Recruitment and positioning of Regulatory T cells

and

Th17/Tc17 in Inflamed Human Liver

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Abstract

The liver is a unique tolerogenic organ with dual blood supply. Both regulatory lymphocytes and effector lymphocytes are present in the normal and inflamed liver along with innate immune cells. The balance between these two subsets of lymphocyte is crucial in maintaining immune homeostasis by adjusting either hepatic tolerance or mounting immunity against invading pathogens. Thus, it is important to understand the intrahepatic regulatory T cells phenotype and role played by distinct chemokine receptors expressed on regulatory T cells as they are major player in controlling hepatic tolerance. At the same time, it would be crucial to explore the role of new subset of Th17/Tc17 effector lymphocytes characteristic and their positioning in inflamed liver.

This thesis demonstrates the crucial role of chemokine receptors in recruitment and positioning of both intrahepatic regulatory T lymphocytes and IL-17 secreting Th17/Tc17 effector lymphocyte in both normal and inflamed human liver.

This thesis is dedicated to my parents back home that always encourage me to achieve in academic career, and my beloved wife, Myat, whose continued love, encouragement and understanding has helped all my dreams to come true.

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CHAPTER 1 INTRODUCTION

1.1 The human immune system

The mammalian immune system protects the host from a broad range of pathogenic microorganisms while avoiding misguided or excessive immune reactions that would be deleterious to the host. The innate and adaptive immune systems function together to recognize the invading pathogens and coordinate to mount a successful and efficient immune response.

1.1.1 Innate immunity

The innate immune response, which is the frontline of host defence, consists of cellular and biochemical defence mechanisms, which can recognise and neutralize invading pathogens within hours. The diversity of immune recognition for innate system is pre-programmed, limited and it does not possess immunological memory. The cells of the innate immune system include phagocytes (macrophages and neutrophils), dendritic cells, mast cells and natural killer cells. The innate immune system does not require previous exposure to pathogens to mount effective immune response because it is activated by conserved molecular patterns that indicate the presence of pathogens, Pathogen Associated Molecular Patterns (PAMPs) such as bacterial cell wall components or viral RNA or injury cellular stress, e.g. heat shock proteins. Pattern recognition receptors such as toll-like receptors, scavenger receptor and C type lectins (Aderem and Ulevitch, 2000) allow innate immune cells to recognise and respond to PAMPs. The innate immune response also incorporates secreted cytokines particularly those such as type 1 interferon that are secreted early in response to infection, the complement system and acute phase proteins and it's response predominates in tissues at the interface with environment such as skin, the epithelial mucosa of the gut and lungs where the tissues are continuously exposed to antigens(Hoffmann et al., 1999).

1.1.2 Adaptive immunity

The cardinal features of the adaptive immune system are specificity, diversity and immunological memory. The adaptive immune response generates antigen specific lymphocytes that recognise a specific antigenic epitope. It has immunological memory in that a subsequent exposure to the same antigen leads to a more rapid, sustained and potent immune response. Lymphocytes with immunological memory i.e. cells that have been primed to recognise specific antigens are called memory lymphocytes and are widely distributed throughout the body via the blood to the secondary lymphoid tissues and peripheral tissues where they contribute to the process of immune surveillance. These lymphocytes must be rapidly recruited to the site of an infection to allow recognition of foreign antigen and the mounting of an immune response.

The immune system comprises primary, secondary and tertiary structures comprising thymus and bone marrow (primary or central tissue) and lymph nodes, spleen, and mucosa-associated lymphoid tissue such as Peyer's patches (peripheral or secondary lymphoid tissue). Professional antigen presenting cells, such as dendritic cells play a central role in orchestrating complex immune responses and help to determine the nature of an immune response e.g. whether it drives the effector or regulatory arm of the immune system. Antigens from invading pathogens are taken up by dendritic cells which patrol the peripheral tissues before being carried via lymph to secondary lymphoid tissues where antigens are presented to naive lymphocytes (Banchereau et al., 2000). Antigen exposed dendritic cells express high levels of MHC Class II molecules, required to present the peptides and co-stimulatory molecules CD80/CD86 which are required for activation of naive T cells allowing them to fully activate naive T cells which can then mediate effector responses directly or provide T cell help for antibody production. This leads to cellular immunity by proliferation of T effector cells as well as humoral immunity by proliferation of antibody producing B cells.

Lymphocytes have two distinct classes: B and T lymphocytes. B lymphocytes are characterised by the production of antibodies raised against specific antigens and participate in humoral immunity. T lymphocytes provide cell-mediated immunity and consist of cytotoxic T lymphocytes (Tc) and T helper lymphocytes (Th) based on their expression of the cell surface markers CD8 and CD4. Cytotoxic T lymphocytes act on target cells presenting viral or tumour antigens in association MHC class-I molecules. T helper lymphocytes consist of Th1, Th2, Th9, Th17 and T regulatory cells (Bettelli et al., 2006;Mangan et al., 2006;Mosmann et al., 1986;Sakaguchi et al., 1995;Veldhoen et al., 2006;Veldhoen et al., 2008b). They respond to antigen presented in association with MHC class II via APC, and subsequently help B cell production of antibody or differentiation of cytotoxic T cell precursors (Ashwell 1988).

Immune system achieved the homeostasis by distinguishing between self and nonself and maintaining unresponsiveness to self or harmless antigens (immunological tolerance) whilst being able to rapidly recognize foreign antigen to eradicate invading pathogens. Breakdown in immunological self tolerance leads to autoimmunity (Sakaguchi et al., 1995;Sakaguchi, 2004). Self tolerance is maintained by central and peripheral mechanisms (Walker and Abbas, 2002).

1.1.3 Central Tolerance

Billingham, Brent and Medawar first described acquired tolerance by injecting allogenic tissues into foetal mice *in utero* and observing an impairment in their ability to reject skin grafts from the same allogeneic mouse strain when the mice reached maturity (Billingham et al., 1953). Subsequently, studies using TCR transgenic mice (Kisielow et al., 1988) that respond to mouse mammary tumour virus (Kappler et al., 1987) demonstrated that self reactive T lymphocytes can be clonally deleted in the thymus by an apoptotic process (Surh and Sprent, 1994). Central tolerance results from intrathymic selection and clonal deletion of T cells with high avidity for

thymically expressed self-antigen(Kappler et al., 1987) and central tolerance to auto-antigens is programmed in the thymus during lymphocyte development(Anderson et al., 2001).

Thymic selection results in the release of high-affinity T cells specific for non-self-antigens, low-affinity T cells specific for self-antigens, and natural T_{reg} with an intermediate affinity to both self- and non-self-antigens into the circulation. Medullary thymic epithelial cells present self-antigens on MHC class I and class II molecules to thymocytes (developing lymphocytes) and those that respond strongly to these self-antigens are deleted(Anderson et al., 2006). The effective presentation of tissue specific self-antigens by medullary thymic epithelial cells is regulated by the autoimmune regulator gene known as AIRE. The AIRE protein is important for the maintenance of self tolerance (Nagamine, 1997) as demonstrated by the development of autoimmune diseases in both mice and humans with functional mutations in AIRE (Su et al., 2008;Su and Anderson, 2004). AIRE mutations lead to a breakdown of central tolerance associated with decreased expression of self antigens in the thymus. It humans AIRE mutations result in a rare autosomal recessive human autoimmune polyglandular syndrome type 1 (APS type 1) also known as autoimmune polyendocrinopathy, candidosis, ectodermal dystrophy (APECED)(Nagamine, 1997).

The role of the thymus in immune tolerance was demonstrated in mice in which neonatal thymectomy between day 2 and 4 after birth resulted in autoimmune destruction of ovaries (Nishizuka and Sakakura, 1969). Moreover, adult thymectomy of normal rats followed by sub lethal X-irradiation also results in autoimmune thyroiditis with anti-thyroglobulin autoantibody production (Penhale et al., 1973).

1.1.4 Peripheral Tolerance

Self- reactive T cells which escape from thymic deletion are controlled in the periphery to prevent autoimmunity. Many mechanisms contribute to peripheral tolerance. They include ignorance of self-antigens, the apparent absence of antigen recognition, deletion by activation-induced cell death or apoptosis, and the induction of T cell anergy. In addition to these passive mechanisms, active suppression of self-reactive T cells is mediated by immunosuppressive cells the best described of which are regulatory T cells (T_{reg}) which express CD4 $CD25^{high}$ (Walker & Abbas, 2002).

1.1.5 Tolerogenic environment of the liver

The liver is a complex immune organ and its intrahepatic immune environment favour immune tolerance, yet it maintains the capacity to sustain effective immune responses against pathogens (Crispe, 2009). The first evidence of the tolerogenic properties of the liver came from experiments showing acceptance of pig liver allograft transplanted across MHC barriers by Calne and colleagues(Calne RY, 1969). The mechanisms of the effect are in part a consequence of the unique hepatic environment which is shaped by anatomy and by the continuous exposure of the liver to nutrients and microbial products derived from the intestinal bacteria (Benseler et al., 2007). This exposure promotes the expression of cytokines, such as IL-10 and TGF-β that lead to the differentiation of antigen-presenting cells including not only dendritic cells (DCs) but also resident liver cells that tend to generate tolerance or anergy when they activate T cells within the liver. However, full activation of T cells by DCs in draining lymph nodes results in a vigorous and full effector response (Bowen et al., 2004;Bowen et al., 2005;Crispe, 2003).

The liver contains resident lymphocytes, among which $CD8^+$ T cells outnumber $CD4^+$T cells (Winnock et al., 1995). It also contains a high frequency of $CD56^+$ classical natural killer (NK) cells and a population of NK-like T cells expressing both a TCR αβ and NK cell marker

NK1.1 which are found at low frequencies in the blood and most other tissues. The liver also includes myeloid lineage cells including resident tissue macrophages, kupffer cells, and myeloid and plasmacytoid dendritic cells. Antigen presenting DCs and kupffer cells play a key role in regulating the tolerogenic environment (Klein et al., 2007) along with local regulatory T cells (Crispe, 2003;Klein et al., 2007). Hepatic regulatory T cells include naturally occurring, thymic derived T_{reg} and possibly induced T_{reg} in the TGF-β rich intra-hepatic cytokine milieu. In addition to DC, other cells can act as antigen presenting cells in the liver including kupffer cells, stellate cells, and sinusoidal endothelial cells and under some circumstances hepatocytes. The unique architecture of the liver allows T cells to interact directly with several resident liver cells types including hepatocytes which can access naive T cells in sinusoids by extending microprocesses through the fenestrations in sinusoidal endothelium. (Adams et al., 1989;Bowen et al., 2004;Bowen et al., 2005;Goddard et al., 2004a). The liver resident lymphocytes as well as circulating naïve $CD8^+$ T cells make direct contact with hepatocytes through cytoplasmic extensions penetrating the endothelial fenestrations thereby interactions occur between lymphocytes and liver parenchyma cells(Warren, 2006).

The fate of an immune response against intrahepatically expressed antigens depends on the dominant site of antigen expression. Where antigen is overwhelmingly presented to naive T cells within the liver, $CD8^+$ T-cell tolerance ensues; however, if there is significant early antigen expression within the local draining lymph nodes, an effective CTL response is generated, resulting in hepatocellular injury (Bowen et al., 2005). The level of antigen expression is also crucial as the presence of high-level antigens within the liver lead to deletion of $CD8^+$ T cell(Mehal, 2003). There are several mechanisms which contribute to the tolerogenic capacity of the liver.

1.1.6 Intrahepatic lymphocytes in hepatic tolerance

Populations of intrahepatic lymphocytes secrete immuno-regulatory cytokines interleukin-10 and TGF-β and chemokines that maintain tolerance. These may be derived not only from T cells but also from kupffer cells, and even stroma cells (Crispe, 2003). Furthermore, the liver could act as a graveyard or a killing field for activated T cells (Crispe et al., 2000;Crispe and Mehal, 1996). Specific activation induced by systemic administration of peptide results in selective accumulation and apoptosis of $CD8^+$ T cells within the liver of T-cell receptor (TCR) transgenic mice (Huang et al., 1994). Again in transgenic mouse models, the liver is capable of supporting the primary antigen-specific activation of $CD8^+$ T cells(Bertolino et al., 2001;Bowen et al., 2002) however, such intrahepatic activation is ineffectual and leads to impaired effector function and reduced $CD8^+$ T-cell survival (Bowen et al., 2004).

1.1.7 Hepatic dendritic cells and Kupffer cells

Our group and others have demonstrated that intra-hepatic DC tend to act as tolerogenic cells and preferentially express IL-10 compared with for instance skin DC (Goddard et al., 2004a;Thomson et al., 1995;Thomson and Lu, 1999a;Thomson and Lu, 1999b). The Liver is constantly exposed to bacterial LPS via portal blood and this may be responsible for down regulation of its receptor TLR4 on liver DC limiting their response to danger signals and resulting in reduced or altered activation of the hepatic adaptive immune responses (de et al., 2005a). DC also has the capacity to expand functional $CD4^+CD25^+$ T cells (Yamazaki et al., 2006a;Yamazaki et al., 2006b). Recent work in mice suggested that $CCR9^+$ plasmacytoid DC (pDC) are more efficient than $CCR9^-$ pDCs in inducing regulatory T cells and inhibit antigen-specific immune responses both *in vitro* and *in vivo* (Hadeiba et al., 2008). TLR9 stimulation can also promote pDC-mediated generation of $CD4^+CD25^+$ T_{reg} cells and pDC play an important role in the maintenance of immunological tolerance in human peripheral blood (Moseman et al., 2004).

Activation of indolamine 2, 3-dioxygenase (IDO) in DCs inhibit T cell proliferation during antigen presentation by deprivation of tryptophan (Munn et al., 2004) and kupffer cells, resident macrophages of reticuloendothelial system, also contribute to generation of T_{reg} via IDO.(Alabraba E, et al, Manuscript in preparation)

1.1.8 Hepatic sinusoidal endothelial cells

Hepatic sinusoidal endothelial cells (HSEC) tolerize antigens from gut-derived bacterial degradation products such as lipopolysaccharide (LPS) carried by portal venous blood without inducing a local inflammatory reaction (Lalor et al., 2006;Lalor and Adams, 1999). HSEC react to LPS directly via its constitutive Toll-like receptor 4 (TLR4) expressions but gain a LPS-refractory state upon repetitive stimulation without loss of scavenger activity (Uhrig et al., 2005). *In vitro* evidence indicates that HSECs might activate naïve $CD4^+$ T cells but fail to elicit a Th1 type response (Knolle et al., 1999). Thus, HSEC, non-professional APCs which express MHC II influence the outcome of intrahepatic $CD4^+$ T-cell responses.

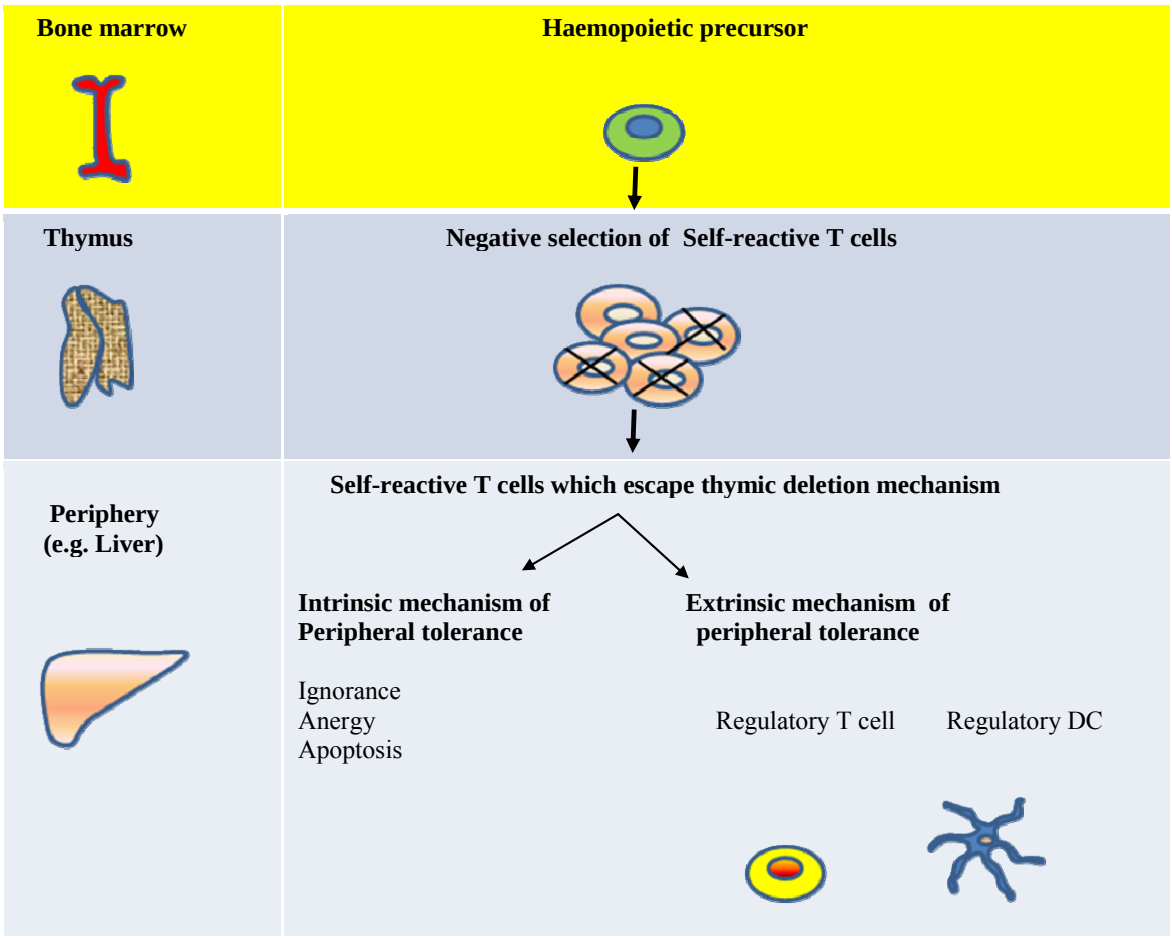

Figure 1-1 Central and peripheral tolerance.

Bone marrow derived haematopoietic precursors migrate to the thymus where they undergo positive and negative selection according to the nature of their TCR interactions with peptide/major histocompatibility complex. Some self-reactive T cells are deleted at this stage (central tolerance), but others escape to the periphery. They are controlled by multiple peripheral tolerance mechanisms acting either passively on the self-reactive T cell (T-cell intrinsic) or actively via regulatory T cells (T-cell extrinsic) or regulatory dendritic cells.

1.2 Lymphocyte lineage

CD4 T helper lymphocytes (Th) are regulators of immune responses to infection and inflammation. They help B cells to make antibodies, secrete cytokines that polarise the immune response and contribute to the recruitment of other effector cells to site of infection or inflammation in an orchestrated immune responses. At least 7 different CD4 subsets have been shown to exist currently namely Th1, Th2, Th17, Th9, naturally occurring thymic derived $T_{reg,}$ peripherally induced T_{reg} and follicular helper T cells.

In 1986, Tim Mosmann & Robert Coffman first demonstrated that effector T cells can be categorized into two distinct subsets, T helper type 1 (Th1) and T helper type2 (Th2), based on their cytokine production profiles(Mosmann et al., 1986). Th1 cells produce large quantities of interferon-γ (IFN-γ), whereas Th2 cells produce interleukin 4 (IL-4), IL-5 and IL-13(Mosmann et al., 1986). Th1 cells elicit delayed-type hypersensitivity (DTH) responses, activate macrophages and are highly effective in clearing intracellular pathogens. DTH reactions were originally defined as cell-mediated immune reactions manifest by swelling, induration and redness appearing 24 to 72 hours after intradermal injection of a challenge antigen equivalent to the tuberculin skin test to the purified protein derivative of *Mycobacterium tuberculosis*. Injection of Th1 clones result in footpad swelling (DTH) in naive mice after 24 hours whereas injection of Th2 clones do not cause swelling (Cher and Mosmann, 1987). Th2 cells, in contrast, are important for the production of immunoglobulin E and eosinophilic inflammation and induce humoral immune responses against helminths and allergic responses (Abbas et al., 1996).

Naive $CD4^+$ T cells proliferate and differentiate into various effector subsets after antigenic stimulation by professional APC such as dendritic cells. This is characterized by the production of distinct cytokines and by their distinct effector functions. DCs are located throughout the body to capture and internalize invading pathogens, subsequently process and present antigen on

MHC class I and class II molecules to $CD8^+$ and $CD4^+$ T cells respectively (Steinman and Banchereau, 2007;Steinman and Young, 1991). Naive T cell activation normally requires two signals: T cell receptor (TCR) signals, and co stimulation through several accessory molecules. The main costimulatory molecule on T cells is CD28, which interacts with CD80 (B7-1) and CD86 (B7-2) expressed on mature DCs. The inducible costimulator (ICOS) is a member of the CD28 superfamily that also regulates naive CD4 T cell activation and effector differentiation. DCs then translate information of the invading pathogen into a cytokine gene expression profile directing the correct T helper cell differentiation pathway. Thus, achievement of effective host defence against invading pathogens involves coordinating complex networks that link innate and adaptive immune systems. Initial T effector response is normally followed by a shift to T regulatory response to limit the collateral damage.

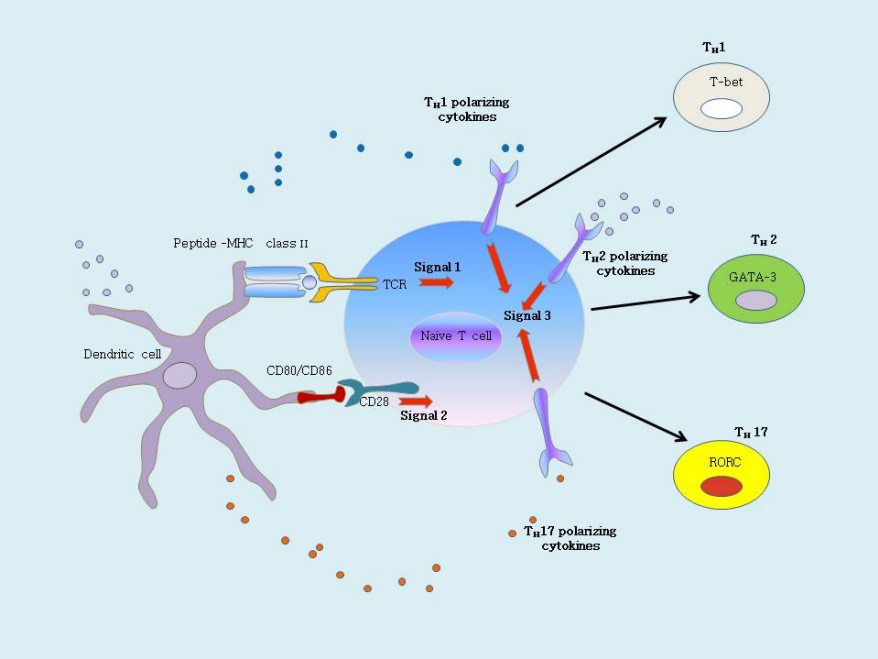

Figure 1-2 T cell stimulation by antigen presenting dendritic cells with polarizing cytokines and co-stimulatory molecules.

T-cell receptor (TCR) ligate to MHC class-II-associated peptides processed from pathogens (signal 1) and co-stimulatory molecule CD28 on lymphocyte bind to CD80 and CD86 expressed by dendritic cells (signal.2). Signal 3 is the polarizing signal that is mediated by various polarizing cytokines that promote the development of Th1, Th2, Th17 lymphocytes lineages.

The cytokine milieu in the local microenvironment plays important role in the differentiation of different T cell subsets. IL-12 produced by innate immune cells and IFN-γ from NK and NKT cells polarize naive T cells towards the IFN-γ secreting Th1 cell differentiation via signal transduction and activator of transcription (STAT)-4 signalling and T box transcription factor T-bet and these Th1 cells are involved in cellular immunity against intracellular organisms(Mosmann et al., 1986). IL-4 signalling through STAT-6 and transcription factor GATA3 will transform naive T cells towards Th2 cells, which are required for humoral immunity to control helminths and other extracellular pathogens(Mosmann et al., 1990). The differentiation pathway also depends to some extent on the strength of the interaction of the T cell antigen receptor with antigen (Boyton and Altmann, 2002;Maldonado et al., 2004).

IL-17-secreting T helper cells (Th17) are identified on the basis of the ability to produce IL-17A, IL-17F and IL-22 (Aggarwal et al., 2003;Harrington et al., 2005;Steinman, 2007). TGF-β stimulation in the presence of IL-6 facilitates naive T cell differentiation towards Th17 in mice (Veldhoen et al., 2006;Veldhoen and Stockinger, 2006a). The signature transcription factor for Th17 is RORγt (Ivanov et al., 2006), which in turn induces transcription of the genes encoding IL-17 and the related cytokine IL-17F in naïve CD4 T helper cells and is also required for their expression in response to IL-6 and TGF-β. IL-1β in combination with IL-6 (costa-Rodriguez et al., 2007a) and IL-23 is required for human Th17 differentiation (Wilson et al., 2007). TGF-β is now accepted as essential cytokine for the differentiation of human Th17 (Manel et al., 2008;Volpe et al., 2008;Yang et al., 2008a). All these Th17 polarizing cytokines activate STAT3 phosphorylation (Chen et al., 2007). Importantly, Th17 cells play crucial roles in the clearance of extracellular bacteria and fungi, especially at mucosal surfaces (Mangan et al., 2006).

Regulation of effector T lymphocytes is necessary for avoidance of autoimmune disease and immunopathology. Effector T cells require control by regulatory T cells to maintain immune homeostasis and prevent collateral tissue damage once the pathogens has been removed. Regulatory T cells which are also crucial for the maintenance of the tolerogenic environment of the liver (Crispe et al., 2006). There are two main types of T_{reg}, thymic derived naturally occurring T_{reg} and TGF-β induced T_{reg}. FoxP3 transcription factor is the master regulator of T_{reg} development and function of both types of T_{reg} (Fontenot et al., 2003a;Hori et al., 2003;Khattri et al., 2003). Naturally occurring, thymic derived T_{reg} are defined by expression of CD4, $CD25^{high}$, $FoxP3^{+}$ (Sakaguchi, 2004), and low expression of the IL-7 receptor/$CD127^{low}$ (Liu et al., 2006;Sakaguchi, 2005;Seddiki et al., 2006). *In vitro* antigenic stimulation of naive T cells in the presence of TGF-β in the periphery results in transient FoxP3 expression and T_{reg} function and these cells are known as induced T_{reg} (Chen et al., 2003). TGF-β is also important for natural T_{reg}

development (Liu et al., 2008). TGF-β activates Smad3 while TCR stimulation induces NFAT activation and both collaborate in remodelling the *Foxp3* enhancer region to promote FoxP3 expression (Tone et al., 2008). IL-2 mediated STAT5 activation is also essential for the induction of FoxP3 expression (Burchill et al., 2007).

A subset of T helper cells which secrete IL-9 known as Th9 was described in mice recently. IL-9 is a T-cell and mast cell growth factor and it is a multifunctional cytokine secreted by many cell types including T lymphocytes, oeosinophils, mast cells and neutrophils (Hultner et al., 1990;Uyttenhove et al., 1988;van et al., 1989). Th9 cells are reprogrammed from Th2 cells under influence of TGF-β and IL-4. Thus, TGF-β constitutes a regulatory 'switch' that in combination with other cytokines can 'reprogram' effector T cell differentiation along different pathways (Dardalhon et al., 2008;Veldhoen et al., 2008b). IL-9 is involved in variety of allergic diseases including human airway inflammation and asthma (Gounni et al., 2004;Longphre et al., 1999;Vermeer et al., 2003). IL-9 affects differentiation of Th17 cells and enhances the suppressive functions of $FoxP3^+$ $CD4^+$ T_{reg} cells *in vitro*. Thus absence of IL-9 signalling weakens the suppressive activity of natural T_{reg} *in vivo*, leading to an increase in effector cells and worsening of experimental autoimmune encephalomyelitis (Elyaman et al., 2009)

Follicular helper T cells (Tfh) are a subset of helper T cells that regulate the maturation of B cells and specialize in the cognate control of antigen-specific B cell immunity. IL-21 cytokine is produced by Tfh cells and it stimulates the differentiation of B cells into antibody-forming cells through IL-21R. Deployment of $CXCR5^+$ Tfh cells to B cell zones of lymph nodes and subsequent stable cognate interactions with B cells are central to the delivery of antigen-specific Tfh cell to generate high affinity antibody production (Fazilleau et al., 2009b). Consequently, dysregulation of Tfh cell function, and over or under-expression of Tfh cell associated molecules such as ICOS (Bauquet et al., 2009) or IL-21, contributes to the pathogenesis of certain

autoimmune diseases or immunodeficiencies (King et al., 2008). Differentiation of these cells requires the cytokine IL-21 (Nurieva et al., 2008;Vogelzang et al., 2008) and may be dependent on the transcription repressor Bcl-6 (Fazilleau et al., 2009a;Nurieva et al., 2009).

Thus, naive $CD4^+$ T cells differentiation to different lineage programs depends on nature of antigen, types of antigen presenting cells, cell surface signals and polarizing cytokines produced by innate immune cells.

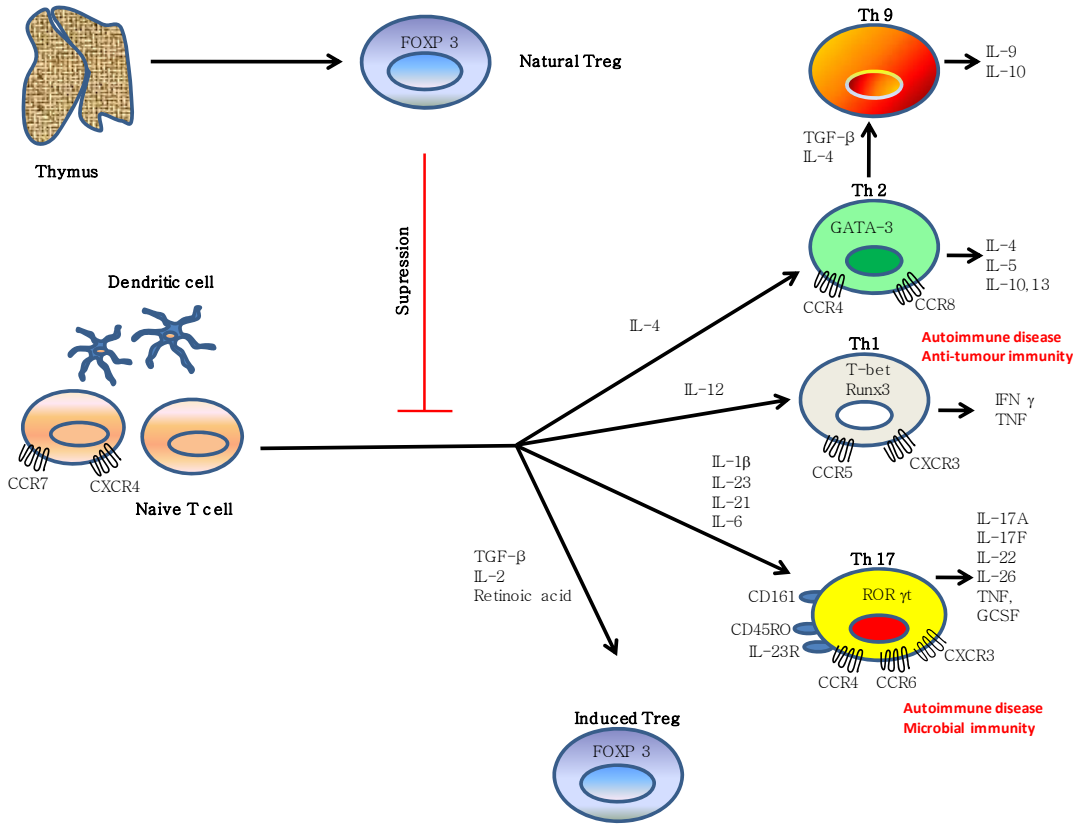

Figure 1-3 T lymphocyte lineage.

Cytokine-mediated amplification of effector $CD4^+$ Th1, Th2, Th-17 and Th-9 cells. Once activated, naive T cells differentiate into distinct $CD4^+$ helper T cell subsets depending on the cytokine milieu which drives a program of differentiation controlled by the induction of key transcription factors. Part of this differentiation programme imprints the subsets with specific combinations of chemokine receptors. Th1 cells express inflammatory type chemokine receptors, CCR5 and CXCR3; Th2 cells express CCR4 and CCR8; recently discovered Th17 cells express CCR4, CCR6, and CXCR3. Th9 subset differentiates from Th2 cells when they are stimulated by IL-4 and TGF-β. Naturally occurring, thymic derived regulatory T cells maintain the peripheral immune tolerance and prevent the excessive damage during infection and inflammation by controlling these T effectors cells.

1.3 Regulatory T cell

The efficient functioning of mammalian immune system requires not only the ability to mount effector responses against pathogens but also to dampen down the system to maintain homeostasis. It protects the body from invading foreign pathogens whilst maintains the self tolerance. Breakdown in self-tolerance leads to auto-immunity (Sakaguchi, 2005). Immunological self-tolerance is achieved by two major mechanisms; central and peripheral tolerance. Central tolerance involves thymic deletion of self reactive T cells, however some of these auto reactive cells escaped to periphery. Peripheral tolerance is maintained by passive and active mechanism. Passive mechanism including ignorance, anergy and apoptosis are not sufficient, and active immunosuppression is required for auto reactive T cells to prevent auto-immunity. Regulatory T cells play an essential role in maintaining the peripheral tolerance (Walker & Abbas, 2002) by actively suppressing the auto-reactive T cells (Sakaguchi et al., 1995).

Regulatory T cells were previously known as T suppressor cells. A proposed determinant of 'suppressor' T-cell function named I-J however, could not be found within the MHC (Steinmetz et al., 1982) which led to the concept of T suppressor cell being discredited until its re-emergence in the late 90s, under the new name of T regulatory cell (T_{reg}) (Sakaguchi et al., 1995;Sakaguchi, 2005).

T-cell–mediated immunosuppression by naturally occurring regulatory T cells is crucial in regulating the immune system. They are key controller in the immunologic tolerance to alloantigen in organ transplantation (Wood and Sakaguchi, 2003). They also prevent excessive inflammation and collateral damage during chronic infection (Belkaid and Rouse, 2005;Sakaguchi, 2005;Wood & Sakaguchi, 2003). There are different types of regulatory T cells which are described individually below.

1.3.1 Naturally occurring regulatory T cell

T_{reg} constitute approximately 5–10% of peripheral lymphocytes. The role of naturally occurring thymic derived $CD4^+CD25^+$ T_{reg} in immune homeostasis and peripheral tolerance was first described by Sakaguchi and colleagues. Transfer of lymphocytes depleted of $CD4^+CD25^+$ into athymic mice cause the development of various autoimmune diseases in the recipient whereas co-transfer of a small number of $CD25^+CD4^+$ T cells with the $CD25^-$ T cells inhibit the development of autoimmunity (Sakaguchi et al., 1995). Removal of $CD25^+CD4^+$ T cells not only provoked the development of autoimmune disease but also enhanced immune responses to non-self antigens including soluble xenogeneic proteins and allograft, and reconstitution with $CD25^+CD4^+$ T cells normalized the responses (Sakaguchi et al., 1995).

Naturally occurring T_{reg} develop in the thymus under strong TCR engagement with self peptides and maintain peripheral self-tolerance and consequently immune homeostasis. They constitutively express CD25, the receptor for the IL-2α chain. IL-2, a 15 kDa α-helical cytokine, is produced almost exclusively by activated T cells and promotes proliferation of lymphocytes, macrophages, and NK cells (Walker et al., 1988). IL-2 which is a key growth and survival factor and functional cytokine for natural T_{reg} and it acts on its receptor, CD25 (Almeida et al., 2002;Schorle et al., 1991;Setoguchi et al., 2005;Suri-Payer et al., 1998;Willerford et al., 1995). The hallmark of $CD4^+CD25^+$ T_{reg} is their functional hyporesponsiveness, low IL2 production and the ability to potently suppress proliferation of other $CD4^+$ and $CD8^+$ T cells in co-cultures stimulated with specific antigen or polyclonal TCR activation (Thornton and Shevach, 1998). CD25 is expressed not only on T_{reg} but also on activated T cells which hampers the phenotypic assessment of T_{reg}. Low level of expression of CD127, the IL-7 receptor α chain on T_{reg}, allows discrimination between T_{reg} and effector populations which express high levels of CD127.

Expression of FoxP3 and the $CD127^{low}$ phenotype is thus highly correlated with $CD4^+CD25^+$ T_{reg} and manifest suppressive activity *in vitro* (Liu et al., 2006;Seddiki et al., 2006).

Fork head box P3 (FoxP3) is the master regulator transcription factor expressed by natural T_{reg} which controls the development and function (Fontenot et al., 2003b;Hori et al., 2003;Khattri et al., 2003). The *Foxp3* gene was identified as the gene mutated in Scurfy mice, which have an X-linked recessive mutation that is lethal in homozygous male mice from severe autoimmunity/inflammation (Brunkow et al., 2001). Mutations of the human *FOXP3* gene, the ortholog of murine *Foxp3*, lead to a similar human disease known as IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is characterized by autoimmune disease in multiple endocrine organs (Type 1 diabetes and Thyroiditis), inflammatory bowel disease, and severe allergy in male children (Sakaguchi et al., 2006).

Retroviral transduction of FoxP3 to normal $CD4^+CD25^-$ T cells converted them into functional T_{reg} with suppressive activity (Fontenot et al., 2003a;Hori et al., 2003). FoxP3 controls T_{reg} functional activity by binding to other transcription factors such as NFAT (nuclear factor of activated T cells) and AML1(acute leukaemia-1), Runx1(runt-related transcription factor-1) and also repress the IL-2 gene in T_{reg} rendering them highly dependent on exogenous IL-2 produced by activated non-T_{reg} cells. FoxP3 also activates the genes encoding other molecules associated with T_{reg} including CD25, CTLA-4 and GITR by binding to their respective promotors (Marson et al., 2007;Ono et al., 2007;Wu et al., 2006;Ziegler, 2005) and these molecules in turn directly suppress non-T_{reg} cells or modulate the function of APC to activate non-T_{reg} cells.

IL-2, the major cytokine for T cells proliferation and cell growth is critical for T_{reg} differentiation and function. Mice lacking IL-2 or its receptor CD25 (Malek et al., 1984), or a deficiency of STAT5 which mediate signalling from IL2 receptor to FoxP3 succumbs to lethal autoimmune diseases (Burchill et al., 2003;Burchill et al., 2007;Yao et al., 2007). Neutralization of

IL-2 with anti-IL2 (Setoguchi et al., 2005) also reduces T_{reg} numbers resulting in autoimmune disease.

CTLA-4 is a potent negative regulator of T-cell immune response (Walunas et al., 1994;Waterhouse et al., 1995) that has been implicated in T_{reg} suppression mechanism. Both murine and human T_{reg} express CTLA-4 constitutively (Takahashi et al.). CTLA-4 knockout (KO) mice die prematurely from multi-organ inflammation (Waterhouse et al., 1995) and blockade of CTLA-4 with antibody results in autoimmunity, inflammatory bowel disease, enhance anti-tumour immunity (Sansom and Walker, 2006). It also prevents regulation of allogenic effectors by alloantigen-specific $CD4^+CD25^+$ T_{reg} (Kingsley et al., 2002). Signalling through CTLA-4 play a role in the induction of peripheral tolerance (Chen et al., 1998) by stimulating secretion of the immunosuppressive cytokine TGF-β (Perez et al., 1997). T_{reg} also express the glucocorticoid-induced TNF receptor family related protein (GITR). Stimulation of GITR abrogates T_{reg} mediated suppression and removal of GITR-expressing T cells or administration of antibody to GITR produced organ-specific autoimmune disease (Shimizu et al., 2002).

The outcome of immune response depends on the balance between T_{reg} and T effector cells. In the context of liver disease, chronicity of viral hepatitis and failure to mount effective immune responses against hepatocellular carcinoma may be due to dominance in the regulatory arm whereas in autoimmune hepatitis and graft rejection the effector arm may outweigh the regulatory arm (Fig.1.4).

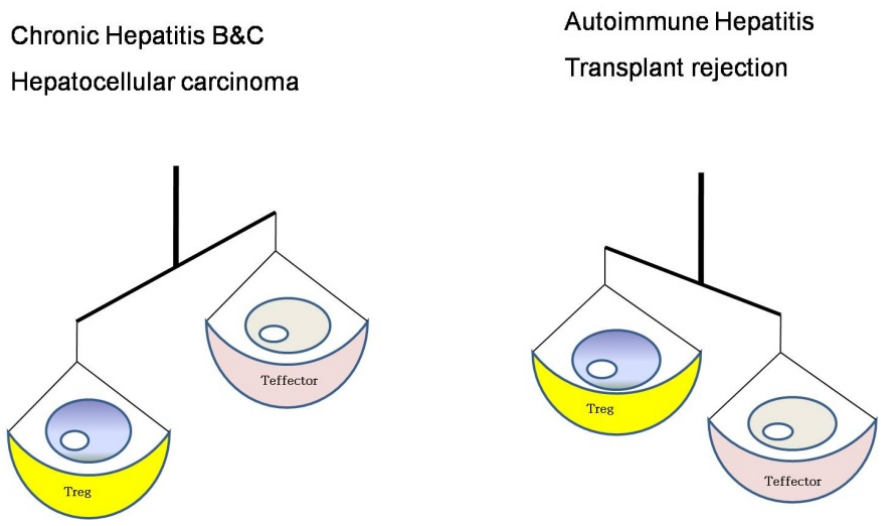

Figure 1-4 Effector arm and regulatory arm balance in the liver.

The balance between effector lymphocytes and regulatory lymphocytes generally decide the final outcome of immune response. In hepatocellular carcinoma and chronic viral hepatitis, the T regulatory response is dominant and T effector arm outweigh the regulatory arm in post-transplant rejection and autoimmune hepatitis.

T_{reg} can also develop outside the thymus and such cells are developmentally, phenotypically and functionally different from natural T_{reg}. They are named as induced or adaptive T_{reg}. Adaptive T_{reg} are induced from naïve T cells by antigenic stimulation in a specific cytokine milieu. They include IL-10 secreting type1 T regulatory cells (Tr1) and TGF-β secreting T helper Th3 cells, and $CD8^+$ $CD28^-$ T cells (Table.1). They all play a pivotal role in the control of autoimmunity and organ transplantation tolerance in rodents and human.

1.3.2 Different subsets of Regulatory T cells

1.3.2.1 Induced T_{reg}

In peripheral tissues, antigenic stimulation of naive T cells in the presence of TGF-β leads to the acquisition of FoxP3 expression and consequently T_{reg} function (Chen et al., 2003). Human naïve T cells transiently express FoxP3 upon TCR stimulation (Gavin et al., 2006) although the expression is generally much lower and more transient than natural T_{reg} (Yagi et al., 2004). In mice, the *Foxp3* gene is more widely demethylated in natural T_{reg} compared with TGF-β induced T_{reg} suggesting that natural T_{reg} are more stable than the induced cells (Floess et al., 2007). Induced T_{reg} can combine with natural T_{reg} to augment the peripheral pool of $FoxP3^+$ T_{reg}.

1.3.2.2 Tr1 cells

Type 1 T regulatory cells (Tr1) differentiate from both human and murine naïve antigen specific $CD4^+$ T cell when they are stimulated *in vitro* and *in vivo* in the presence of IL-10 and APC. They have a low proliferative capacity due to autocrine production of IL-10. Tr1 cells have a distinct cytokine profile that is different from Th1 or Th2 cells as they produce high levels of IL-10 and TGF-β but low amounts of IL-2, IFN-γ and no IL-4 (Groux et al., 1997). These antigen-specific T-cell clones suppress the proliferation of $CD4^+$ T cells in response to antigen,

and prevent colitis induced in SCID mice by pathogenic $CD4^+CD45RB^{high}$ splenic T cells (Groux et al., 1997).

Tr1 cells can be generated in a variety of different tolerogenic microenvironments. The presence of vitamin D and dexamethasone facilitates the *in-vitro* induction of Tr1 cells from naïve $CD4^+$ T cells by IL-10 (Barrat et al., 2002;Levings et al., 2001). Naïve $CD4^+$ T cells stimulated by CD3 and the human complement regulator CD46 in the presence of IL-2 also differentiate into Tr1 cells *in vitro* (Kemper et al., 2003). Again, cord blood derived $CD4^+$ T cells stimulated by allogeneic immature DCs give rise to Tr1 cells (Jonuleit et al.). Furthermore, *in-vivo* generation of tolerogenic Tr1 cells was achieved in diabetic mice by administering IL-10 and rapamycin after pancreatic islet transplantation and antigen-specific tolerance could be transferred to naïve mice by adoptive transfer of Tr1 cells from the mice with tolerance (Battaglia et al., 2006).

Tr1 cells do not express FoxP3, however their properties in vitro are similar to those of FoxP3 T_{reg}. They exhibit diminished proliferation in response to antigen, exert cell contact-dependent suppression, produce very little IL-2, display an activated cell-surface phenotype (Vieira et al., 2004) and suppress both naïve and memory T cell responses. They are inducible, antigen specific, and require activation via TCR to exert their suppressive functions. However, once activated, Tr1 cells exert not only antigen-specific suppression but bystander suppression as well. This bystander suppression is mediated by the local release of IL-10 and TGF-β (Roncarolo et al., 2006).

1.3.2.3 Th3 cells

TGF-β–producing Th3 cells have been identified as regulators in oral tolerance (oral administration of antigens to induce tolerance) both *in vivo* and *in vitro* (Faria and Weiner, 2005). Oral administration of myelin basic protein leads to the generation of $CD4^+$ clones in the mesenteric lymph nodes which produce TGF-β, IL-4 and IL-10 (Chen et al., 1994). Thus,

mucosal derived Th2-like clones induced by oral antigen can actively regulate immune responses *in vivo*.

There are a number of differences between naturally occurring T_{reg} , induced T_{reg}, Tr1 and Th3 in their origin, mode of suppression and these are described in Table 1.1.

Types	Origin	Phenotypic characteristic	Mechanism of suppression	Homing behaviour	Typical features
Naturally occurring T_{reg}	Thymus	$CD4^+CD25^{high}$ $CD127^{low}$ $FoxP3^+$	Cell to cell contact *in vitro* Down regulation of IL-2 transcription in Target T cells	Secondary lymphoid organ or site of inflammation	Anergic *in vitro*
Tr1	Periphery	IL-4^- IL-5^+ 1L-10^+ IFN-γ^{low} TGF-β^+	IL-10, TGF-β mediated	Site of inflammation	Anergic *in vitro* Differentiate with exogenous IL-10 with IFN-α and APCs
Th3	Periphery Oral antigen	$CD4^+$ $FoxP3^+$	TGF-β	Mucosal surface, site of inflammation	Oral antigen
Inducible T_{reg}	Periphery Plasmacytoid DC TGF-β	$CD4^+CD25^{high}$ $Foxp3^+$			?contact dependent

Table 1-1 Types of T_{reg}, origin, phenotype and mode of suppression.

Different types of regulatory T cells, their origin, phenotype and mechanism of suppression are shown in the table. Both naturally occurring T_{reg} and periphery derived T_{reg} maintain the immune homeostasis by controlling the T effectors cells

Abbreviation: Tr1- T regulatory cell type1; Th3- T helper cells type3; IL- interleukin, TGF- Transforming growth factor; FoxP3- fork-head, winged helix box P3.

1.3.3 Suppressive function of regulatory T cells and mechanism of suppression

Inadequate immune suppression results in autoimmunity and allergy on the other hand, too much suppression leads host susceptible to infection. During microbial infection in particular, T_{reg} suppression needs to be attenuated to allow effective anti-microbial immune responses whilst suppressing unwanted immunopathological tissue damage. This involves a cascade of suppressive interactions orchestrated by T_{reg} to control the effector activities of differentiated $CD4^+$ and $CD8^+$ T cells, natural killer cells, natural killer T cells (Miyara and Sakaguchi, 2007a). Once activated by a particular antigen, T_{reg} can suppress responder T cells irrespective of whether they share antigen specificity with the T_{reg} i.e. they can mediate bystander suppression.

1.3.3.1 Contact dependent suppression

Naturally occurring $CD4^+CD25^+$ T_{reg} suppress the proliferation of CD4 and CD8 T effectors *in vitro* via cell-cell contact-dependent mechanisms (Jonuleit et al., 2001;Ng et al., 2001). T_{reg} suppression is abolished when T_{reg} and responder cells are separated by a semi-permeable membrane (Takahashi et al., 1998;Thornton & Shevach, 1998). Several accessory molecules, such as CTLA-4 (CD152) and lymphocyte-activation gene 3 (LAG3), expressed by T_{reg}, and CD80 and CD86 co-stimulatory molecules expressed by APCs contribute to contact-dependent suppression (Huang et al., 2004;Sakaguchi, 2004). T_{reg} are more mobile than naïve T cells *in vitro* therefore, when T_{reg} and $CD4^+CD25^-$ naïve T cells are co cultured with TCR stimulation, the T_{reg} out-compete the $CD4^+CD25^-$ in aggregating around DCs. T_{reg} can then down-regulate the expression of CD80/CD86 on DCs before naive T cells can interact with them thereby preventing the activation of T effector cells by DC (Onishi et al., 2008).

1.3.3.2 LAG-3

LAG-3 (CD4 related lymphocyte activation gene 3, CD223) is a CD4 associated adhesion molecule that binds to major histocompatibility complex(MHC) class 2 molecules. LAG3 is expressed on T_{reg} upon activation and mediates suppression of APC via MHC class II molecules expressed on DCs, B cells, monocots, macrophages (Huang et al., 2004).

1.3.3.3 CTLA-4 and IDO

CTLA-4 and CD28 expressed by T lymphocytes interact with two ligands (CD80 and CD86) on antigen-presenting cells. Although they are structurally similar, the functions of CD28 and CTLA-4 are opposite, with CD28 playing an important role in promoting T-lymphocyte responses, whilst CTLA-4 acts as an essential inhibitor (Sansom, 2006). $CD25^+CD4^+$ natural T_{reg} constitutively express CTLA-4 (CD152), whereas naïve T cells express this molecule only after activation (Sakaguchi, 2005). The expression of CTLA-4 is augmented by T_{reg} stimulation (Sakaguchi, 2005). FoxP3 controls the expression of CTLA-4 and CTLA-4 blockade abrogates suppression(Sansom, 2006). CTLA-4 on $CD4^+CD25^+$ T_{reg} is able to up-regulate indolamine 2,3-dioxygenase (IDO) expression in dendritic cells, through interaction with CD80 and CD86 (Fallarino et al., 2006). IDO catabolise the essential amino acid tryptophan into kynurenine and other metabolite, which are toxic to T cells (Fallarino et al., 2006).

In vivo studies demonstrate that antigen-activated T_{reg} with high expression of LFA-1 are recruited around dendritic cells and out-compete antigen-specific naive T cells thereby hampering activation of other T cells by dendritic cells. T_{reg} then modulate dendritic cell function by down regulating CD80 and CD86 on DC by CTLA-4-dependent mechanism (Misra, 2004;Waterhouse et al., 1995;Wing et al., 2008). *In vitro*, T_{reg} suppress the proliferation and cytokine production of responder T cells when the two populations are co cultured and stimulated by antigen in the presence of APC (Takahashi et al., 1998;Waterhouse et al., 1995). Alternatively, CTLA-4 on T_{reg}

ligate CD80 and, to a lesser extent, CD86 expressed by activated responder T cells and directly transduce a negative signal to the responder T cells (Paust et al., 2004). In addition, activation of Toll like receptors (TLRs) on DCs can also modulate the suppressive function of $CD4^+CD25^+$ T_{reg}(Pasare and Medzhitov, 2003).

1.3.3.4 Cytotoxicity by Perforin and granzyme B

Up-regulation of granzyme B on $CD4^+CD25^+$ T_{reg} correlates with their suppressive activity, and $CD4^+CD25^+$ T_{reg} from granzyme B knockout mice have less suppressive capacity compared with T_{reg} from wild type mice, suggesting granzyme B is of functional importance allowing the T_{reg} to kill target cells (Gondek et al., 2005). In humans, $CD4^+CD25^+$ T_{reg} can also use a perforin-dependent cytotoxic pathway to induce target cell death (Grossman et al., 2004).

1.3.3.5 Immunosuppressive cytokines

The immunosuppressive cytokines TGF-β and IL-10 play an important role in suppression by $CD4^+CD25^+$ T_{reg}. Neutralizing antibodies against TGF-β and IL-10 abrogate the suppression in experimental settings (Sakaguchi, 2005). $Foxp3^+$ natural T_{reg} also predominantly produce the immunosuppressive IL-35, a new member of the IL-12 family(Collison et al., 2007). Alternatively, absorption of cytokine such as IL-2 by T_{reg} induces cytokine-deprived apoptosis in responder T cells (Pandiyan et al., 2007).**Adenosine nucleosides**

T_{reg} express the cell surface ectoenzymes CD39 (ectonucleoside triphosphate diphosphohydrolase1) and CD73 (ecto-5'-nucleotidase) and this distinguishes them from other T cells. CD39 and CD73 generate pericellular adenosine by catalysing extracellular nucleotides and adenosine activate the adenosine A2A receptor on activated T effector cells to increase intracellular cyclic AMP (cAMP) leading to inhibition of T cell proliferation and IL-2 production (Deaglio et al., 2007;Novak and Rothenberg, 1990). This is mediated through protein kinase blockade of nuclear factor κB activity or the activation of the transcriptional repressor ICER(

inducible cyclic AMP repressor)(Bodor et al., 2006). T_{reg} can also increase cAMP levels in the target responder cells by directly delivering them via gap junction (Bopp et al., 2007).

Thus, several mechanisms are involved in T_{reg} mediated suppression and more than one mechanism of suppression may operate for the control of a particular immune response in a synergistic and sequential manner.

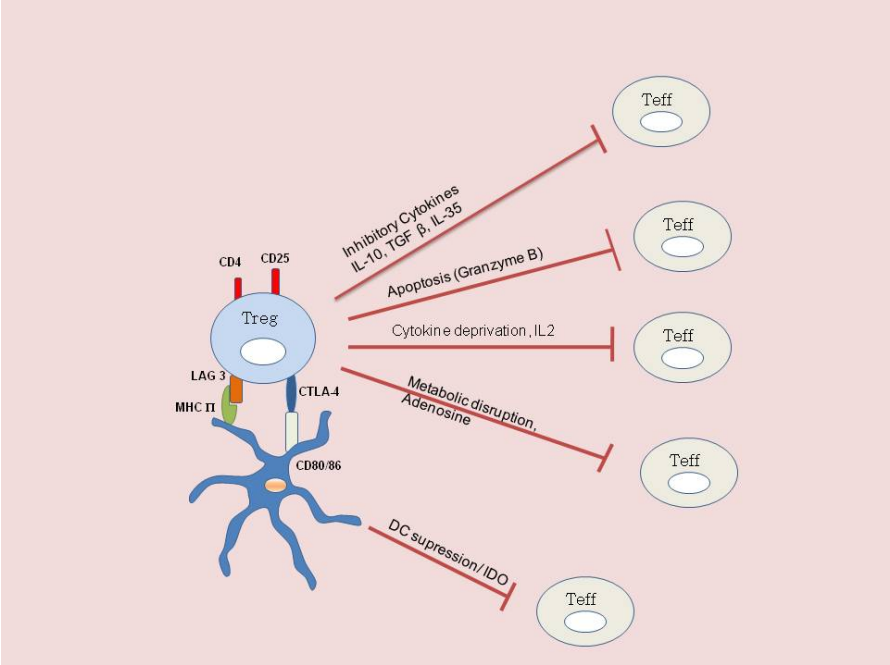

Figure 1-5 Mechanism of suppression of regulatory T cells.

Multiple mechanisms of T_{reg} suppression include both contact dependent interactions with T effector cells and also via dendritic cells. T_{reg} also secrete inhibitory cytokines including interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and interleukin-35 (IL-35). In addition, they can lyse T effector cells via granzyme-B and perforin-dependent killing. Metabolic disruption includes creating cyclic AMP in the vicinity of T effector by ectoenzymes CD39 and CD73. T_{reg} also acts via dendritic cells to modulate DC maturation and function through lymphocyte-activation gene 3 (LAG3/CD223)-MHC-class II mediated suppression of DC maturation. T_{reg} will also act via cytotoxic T-lymphocyte antigen-4 (CTLA-4) and CD80/CD86 mediated induction of indolamine 2,3-dioxygenase(IDO) which leads to tryptophan deprivation to T effector cells.

1.3.4 Regulatory T cell trafficking

Expression of distinct homing chemokine receptors and adhesion molecules (Huehn et al., 2005) by T_{reg} is critical for their recruitment and localization either to inflamed tissue or regional lymphoid tissue. Antigen-specific T_{reg} migrate to regional lymph nodes (Samy et al., 2005) where they become activated by tissue-specific self-antigens or microbial antigens presented by dendritic cells (Scheinecker et al., 2002). FoxP3$^+$ T_{reg} in lymph nodes express high levels of the lymph node homing receptors CCR7 and L-selectin. Once they are activated, they migrate into inflamed tissues, infectious sites, and tumours (Belkaid & Rouse, 2005).

The chemokine receptors expressed by T_{reg} depend upon the site of the tissue, and the cytokine milieu in which they are activated. T_{reg} can express patterns of homing receptors that overlap with T effector cells; Th1 cells (CXCR3, CCR5 and CXCR6), Th2 cells (CCR4 and CCR8) and Th-17 cells (CCR4, CCR6, CCR2 and CXCR3) and Tc17 cells (CXCR6) allowing T_{reg} cells to co-localize with different effector T cells to suppress a wide range of inflammatory conditions(Lee et al., 2007). Our group reported that effector T lymphocytes (Curbishley et al., 2005) and a population of T_{reg} (Eksteen et al., 2006) in the inflamed human liver express CXCR3 which they use to enter the liver via hepatic sinusoids whereas CCR10 expressed by the T_{reg} is important for interactions with biliary epithelium where its ligand CCL28 is expressed (Eksteen et al., 2006). Many other groups also reported that CCR4$^+$ T_{reg} and effector T cells accumulate in the inflamed skin (Sather et al., 2007), ovarian tumours (Curiel et al., 2004) and allograft (Lee et al., 2005a), CCR5$^+$ T_{reg} preferentially migrate to cutaneous lesions of *Leishmania major infection* (Yurchenko et al., 2006) and GVHD target organs (Wysocki et al., 2005), CCR6$^+$ T_{reg} and Th17 are elevated in inflamed joints of murine model of rheumatoid arthritis (Hirota et al., 2007), CCR7$^+$ CD62L^{high} T_{reg} migrate to regional lymph nodes (Szanya et al., 2002). Moreover, CD103$^+$ /integrin α_E β_7 T_{reg} interacts with E-cadherin expressed by epithelial cells and T_{reg} expressing

CD103 are able to control colitis in murine model (Uhlig et al., 2006). They also efficiently migrate into inflammatory sites to suppress peripheral inflammation (Huehn et al., 2004;Lehmann et al., 2002) such as *Leishmania major* infection(Suffia et al., 2005). Thus, appropriate migration to and retention within specific tissues is required for the effective T_{reg} mediated suppression in the microenvironment where regulation is required.

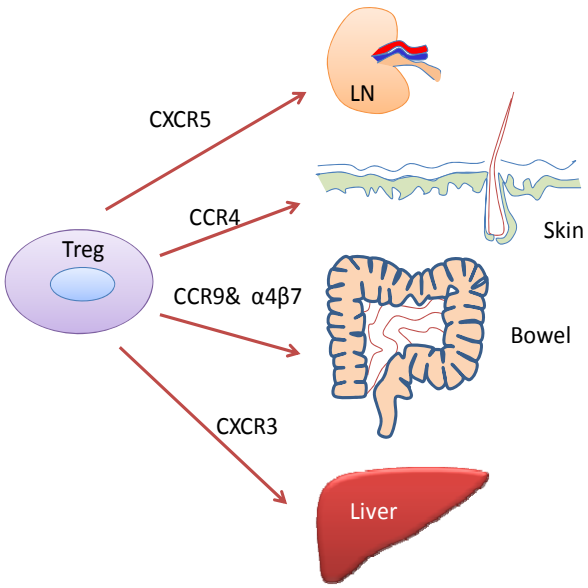

Figure 1-6 Human regulatory T cell trafficking.

Homing of T_{reg} depends on their expression of chemokine receptors that recognise tissue specific chemokines secreted at the site of migration by the resident and infiltrating cells and also by the injury or inflammation of the tissue. T_{reg} could have more than one chemokine receptors and individual receptor may have a defined role in different steps of T_{reg} recruitment, retention and positioning at site of hepatic inflammation.

1.4 Reciprocal relationship between T_{reg} and Th17

There is reciprocity between the Th17 and T_{reg} developmental programs in which IL-6 plays a pivotal role in dictating whether the immune response is dominated by proinflammatory Th17 cells or protective T_{reg}(Bettelli et al., 2006;Mucida et al., 2007). TGF-β is a cytokine produced by natural T_{reg} and cells of the innate immune system. It has broad inhibitory effects on the entire immune system (Li et al., 2006). Naive T cell expresses FoxP3 and becomes T_{reg} on TCR stimulation in the presence of TGF-β alone (Gavin et al., 2006) on the other hand, TGF-β and IL-6 together favour Th17 development and T_{reg} developmental pathway is inhibited. Thus, IL-6 hampers the differentiation of naive T cells to $FoxP3^+$ cells (Bettelli et al., 2006;Veldhoen et al., 2006). IL-6 KO mice have severe defect in the generation of Th17 cells and also have increase numbers of T_{reg} in the periphery (Bettelli et al., 2006;Korn et al., 2007;Korn et al., 2008). In addition to IL6, IL-2, retinoic acids and ligands for the aryl hydrocarbon receptor (AHR) all play crucial roles in Th17 or T_{reg} differentiation pathway.

IL-2, an essential cytokine for T_{reg}(Sakaguchi et al., 1995) and it inhibits the generation of Th17 cells and promotes the generation of T_{reg} in the peripheral immune compartment in vivo (Laurence et al., 2007a;Laurence et al., 2007b). In the tumour microenvironment, IL-2 reduces Th17 differentiation in favour of enhanced T_{reg} development (Kryczek et al., 2007). IL-2 and TGF-β also down-regulate IL-6 receptor expression and IL-6 signalling in induced T_{reg} thus promoting induced T_{reg} resistant to Th17 conversion in an inflammatory milieu (Zheng et al., 2008).

Retinoic acid, a vitamin A metabolite also regulates the balance between proinflammatory Th17 and anti-inflammatory T_{reg}. All trans retinoic acid is generated by a subset of dendritic cells in gut-associated lymphoid tissue. In the presence of TGF-β, retinoic acid facilitates the differentiation of naive T cells to $FoxP3^+$ T_{reg} (Mucida et al., 2007;Yagi et al., 2004). In an

inflammatory setting, retinoic acid inhibits the generation of Th17 whilst enhancing the *de novo* generation of T_{reg}(Mucida et al., 2007;Xiao et al., 2008). Retinoic acid acts directly on naive T cells to enhance TGF-β signalling whilst inhibiting IL-6 signalling and it also suppresses the up regulation of IRF4 and IL-23R, resulting in decreased generation of Th17 cells (Xiao et al., 2008). Furthermore, binding of retinoic acid by its nuclear receptor RARα also increases FoxP3 promoter activity leading to T_{reg} differentiation (Kang et al., 2007)

The aryl hydrocarbon receptor (AHR) was found to be highly expressed in both Th17 cells (Veldhoen et al., 2008a) and T_{reg} (Hill et al., 2007). AHR engagement by its ligand controls the generation of Th17 *in vitro* and *in vivo*. Th17 generation is modulated *in vitro* by endogenous AHR agonists in culture medium such as Iscove's modified Dulbecco's medium which is rich in aromatic amino acids that bind AHR thus yields more Th17 expansion in both mouse and humans (Veldhoen et al., 2009). A nonmetabolizable ligand of AHR, 2,3,7,8-tetrachlorodibenzo-p-dioxin, induce the expression of FoxP3, resulting in the generation of functional T_{reg} ,whereas 6-formylindolo[3,2-b] carbazole, another ligand of AHR, promote the expression of Th17 cells (Quintana et al., 2008;Veldhoen et al., 2008a).

Differentiation of both Th17 and T_{reg} requires TGF-β but depends on distinct transcription factors: RORγt for Th17 cells and FoxP3 for T_{reg} cells(Chen et al., 2003;Ivanov et al., 2006;Veldhoen et al., 2006). At low concentrations, TGF-β synergizes with IL-6 and IL-21(Korn et al., 2007;Nurieva et al., 2007;Zhou et al., 2007) to promote IL-23 receptor expression, favouring Th17 differentiation, however high concentrations of TGF-β repress IL23 receptor expression and favour $FoxP3^+$ T_{reg} cells(Zhou et al., 2008). RORγt and FoxP3 are co-expressed in naive $CD4^+$ T cells exposed to TGF-β (Zhou et al., 2008). Moreover, Runx1 influences Th17 differentiation by inducing RORγt expression and by binding to and acting together with RORγt

during IL-17 transcription. However, Runx1 also interacts with FoxP3, and this interaction is necessary for the negative effect of FoxP3 on Th17 differentiation(Zhang et al., 2008).

Thus, inflammatory cytokine milieu, retinoic acids, AHR ligands, types of antigen presenting cells and transcription factors all in combination influence the reciprocal relation between this two types of cells.

1.5 Interleukin 17 secreting lymphocytes (Th17&Tc17) in the immune system

The $CD4^+$ mediated immune response is highly heterogeneous, depending on the development of distinct subsets which are characterized by cytokine production. After antigen stimulation, $CD4^+$ T cells differentiate into either Th1 or Th2 effector subsets in both mice and humans (Mossman et al., 1988). Th1 cells differentiate from naive T cells in response to IL-12, IFN-γ and they are characterized by IFN-γ production. They protect against intracellular microbes and mediate cellular immunity. In contrast, Th2 cells originate from naive T cells in the presence of IL-4, secrete IL-4, IL-5, IL-9 and IL-13 and drive humoral immunity and responses against parasites and also implicated in allergic disorders (Mossman et al., 1988). Th17 are a separate lineage of $CD4^+$ T helper cells that selectively produce proinflammatory cytokines interleukin-17A and 17F and have been linked to the pathogenesis of autoimmune disease, a function previously attributed to Th1 cells and IFN-γ (Bettelli et al., 2007).

1.5.1 Murine Th17 cells

The Th17 lineage was initially discovered in mice and implicated in experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (Cua et al., 2003;Langrish et al., 2005;Murphy et al., 2003). EAE and CIA were previously believed to be associated with unchecked Th1 response. However, IFN-γ- and IFN-γ receptor deficient mice, as well as mice that lack other molecules involved in Th1 differentiation such as IL-12p35 (IL-12 receptor-β2) were not protected from EAE, but rather developed more severe disease (Gran et al., 2002;Krakowski and Owens, 1996;Tran et al., 2000;Zhang et al., 2003). In addition, IFN-γ deficient or IFN-γ receptor deficient mice are still susceptible to EAE and CIA (Ferber et al., 1996). This led to the discovery of Th17 cells and their role in EAE, CIA and other organ-

specific autoimmune diseases. IFN-γ inhibits the development of Th17 cells(Weaver et al., 2006) suggests that Th1 responses can in some circumstances be protective rather than pro-inflammatory. EAE, driven by IL-23 and IL-17 worsens with administration of neutralizing antibody to IFN-γ as Th1 cells act as an anti-inflammatory brake(Cua et al., 2003).

IL-23 is crucial for Th17 growth, survival and expansion of Th17 rather than for lineage commitment. It is a new member of the IL-12 family which is a heterodimer that shares the p40 subunit with IL-12 associated with p19 rather than p35 (Oppmann et al.). The IL-12 heterodimer is composed of p40 and p35 subunits, and IL-23 is composed of p40 and p19. EAE and CIA do not develop in mice lacking IL-23 p19 subunit, whereas both diseases develop in IL-12 p35 subunit deficient mice (Cua et al., 2003;Murphy et al., 2003). EAE severity is greatly reduced, upon treatment with a monoclonal antibody to IL-17 (Langrish et al., 2005) and mice treated with antibodies to IL-23 also failed to develop EAE(Chen et al., 2006).

The differentiation of naive T cells into Th17 cells depend on the interaction with professional APC which express TCR ligands, costimulatory molecules and secrete specific cytokines. DC secretes IL-23 when stimulated with hyphae from *Candida albicans* and its derivatives zymosan (a TLR2 and Dectin-1 agonist) and β-glucan, a selective Dectin-1 agonist (costa-Rodriguez et al., 2007b;Gerosa et al., 2008). Monocyte derived dendritic cells also produce IL-23 on exposure to *Mycobacterium tuberculosis* H37Rv or a combination of nucleotide-binding oligodimerization domain and Toll-like receptor 2 ligands which mimics activation by *M. Tuberculoisis* (Gerosa et al., 2008).

Th17 differentiation in mice is driven by TGF-β in association with IL-6 and this induces a distinct transcriptional program (Bettelli et al., 2006;Mangan et al., 2006;Veldhoen and Stockinger, 2006b). IL-6 is produced by the innate immune cells including DCs, monocytes, macrophages, B cells, activated T cells, fibroblasts, epithelial cells and tumour cells (van, 1990).

Naive T cells have functional IL-6 receptors composed of IL-6α and the signalling subunit gp130 (Taga and Kishimoto, 1997). Engagement of the IL-6R by IL-6 causes activation of gp130 and activation of STAT3 (Harris et al., 2007) which is necessary for the induction of transcription factors RORγt and RORα (Yang et al., 2008c).

IL-21 is a cytokine produced by NKT and activated T cells (Parrish-Novak et al., 2000). It is secreted by Th17 cells and act as an autocrine amplification loop to regulate its differentiation (Korn et al., 2007;Korn et al., 2009;Nurieva et al., 2007;Nurieva and Dong, 2008;Zhou et al., 2007). Differentiation of murine Th17 cells from naive T cells is directed by two lineage specific transcription factors, retinoic acid-related orphan receptor, RORγt and RORα. RORγt is the transcription factor which acts as the Th17 master regulator (Ivanov et al., 2006) and RORα which is induced by TGF-β and IL-6 (Yang et al., 2008c). RORγt and RORα co expression synergise to drive greater Th17 differentiation and double deficiencies in RORγt and RORα globally impair Th17 generation and completely protect mice against EAE (Yang et al., 2008c).

STAT3 is necessary for Th17 differentiation by regulating RORγt and RORα (Yang et al., 2008b) on the other hand Th17 generation is constrained by IL-2 signalling via STAT5 (Laurence et al., 2007a). Regulatory T cells also control Th17 in a STAT3 dependent manner (Chaudhry et al., 2009). Figure 1.7 shows the cytokines involved in murine Th17 differentiation from naive T cells and cytokines secreted by the Th17 cells.

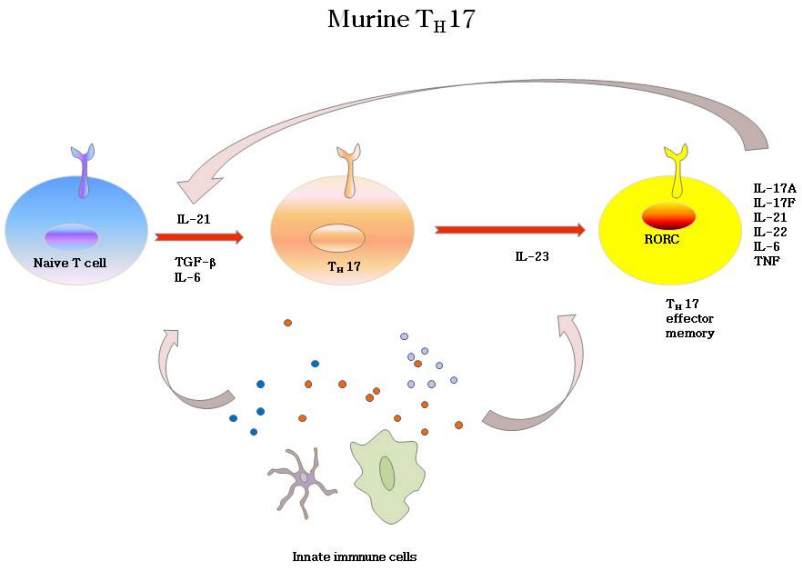

Figure 1-7 Murine Th17 cells differentiation.

IL-6 and TGF-β induce Th17 differentiation and IL-23 is required for the maintenance of Th17. IL-21 which is secreted by Th17 cells act as an autocrine loop in its lineage differentiation. Th17 cells secrete IL-17A, IL-17F, IL21, IL22, IL-6, TNF-α, GMCSF and CCL20 to recruit both innate and adaptive immune cells.

1.5.2 Human Th17 cells

The differentiation of Th17 cells in humans is similar to mice. IL-1β in combination with IL-6 and/or IL-23 is essential for the differentiation of human naive T cells to Th17 (costa-Rodriguez et al., 2007a;Wilson et al., 2007). TGF-β is also essential for the differentiation of human Th17 cells from naive T cells in serum free culture conditions (Manel et al., 2008;Volpe et al., 2008;Yang et al., 2008a) and required to induce RORC (the human homolog of RORγt). However, TGF-β in high doses in both humans and mice inhibits Th17 differentiation by antagonizing RORγt function (Zhou et al., 2008) and an absence of TGF-β induce a shift from a Th17 to a Th1 profile. (Volpe et al., 2008). Thus, TGF-β, IL-23, IL-1β, and IL-6 are all essential

cytokines for human Th17 differentiation. Fig. 1.8 showed the cytokines involved in human Th17 differentiation.

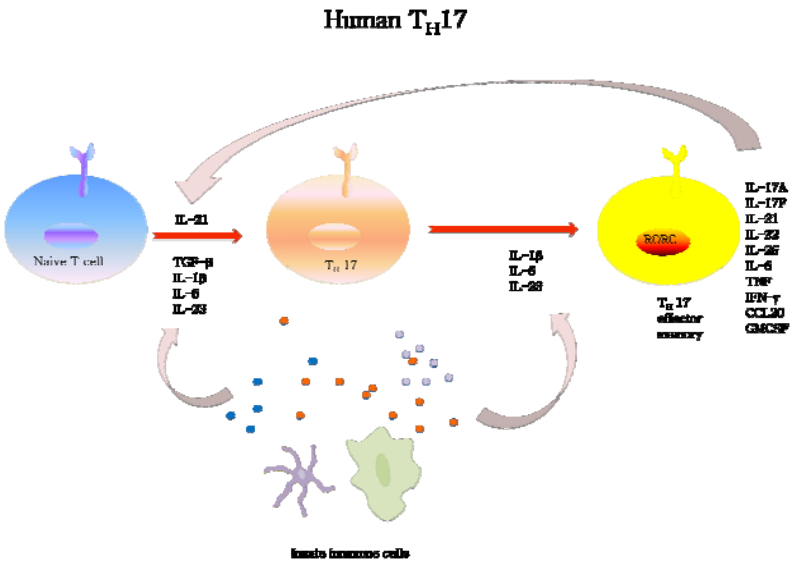

Figure 1-8 Differentiation of Human Th17 cells.

Human Th17 lineage differentiation is similar to mice except for the involvement of IL-1β. In general, IL-1β, IL-6 and TGF-β induce human Th17 differentiation and IL-23 is required for the maintenance of Th17. IL-21 which is secreted by Th17 cells acts as an autocrine loop in its lineage differentiation similar to mice. Human Th17 cells secret IL-17A, IL-17F, IL21, IL22, IL-6, TNF-α, GMCSF and CCL20 to recruit both innate and adaptive immune cells in inflammatory and autoimmune diseases.

STAT3 is required for Th17 differentiation and mutations of STAT3 lead to an inability to produce Th17 cells *in vivo* and *in vitro*. This leads to autosomal dominant hyper IgE syndrome (HIES, 'Job's syndrome') in humans characterized by recurrent severe pulmonary infections, pneumatoceles, eczema, staphylococcal abscesses, mucocutaneous candidiasis, and abnormalities of bone and connective tissue (Holland et al., 2007;Ma et al., 2008;Milner et al., 2008).

Th17 cells secrete IL17A and IL17F and both have pro-inflammatory properties (Kolls and Linden, 2004) and act on a broad range of cell types which express the IL17 receptor (IL17RA). IL17RA is the cognate receptor for IL-17A and bind s both IL17A and IL17F (Chang

and Dong, 2009;Moseley et al., 2003). IL17RA is expressed at high levels on haematopoietic cells, and at low levels on osteoblasts, fibroblasts, endothelial cells and epithelial cells. Activation of IL-17RA induces expression of various cytokines including TNF-α, IL-1β, IL-6, GM-CSF, G-CSF, the chemokines CXCL1, CXCL8, CXCL10 and metalloproteinases (Awane et al., 1999;Fossiez et al., 1996;Jovanovic et al., 1998;Laan et al., 1999;Martel-Pelletier et al., 1999;Witowski et al., 2000;Yao et al., 1995). Human Th17 cells produce CCL20 themselves (Wilson et al., 2007), the ligand for CCR6 a signature chemokine receptor for Th17 cells (costa-Rodriguez et al., 2007b). IL17A and IL17F are key cytokines for linking innate and adaptive immunity. Activation of IL-17RA on stroma cells results in CXCL8 secretion and recruitment of neutrophils to the site of inflammation (Aggarwal and Gurney, 2002;Gaffen, 2009). IL-22 is another cytokine secreted by Th17 cells (McGeachy et al., 2007;McGeachy et al., 2009) and its receptor, a heterodimer of the specific IL-22R and the IL-10R2 (Kotenko et al., 2001;Xie et al., 2000) is expressed widely on epithelial and endothelial cells. IL-22 is hepato-protective in liver inflammation (Zenewicz et al., 2007) and promote epithelial repair of the gut (Sugimoto et al., 2008a) thus it is important in epithelial and endothelial barrier having a dual function in promoting tissue inflammation and repair (Pickert et al., 2009;Sugimoto et al., 2008b).

1.5.3 Origin and phenotypic characterization of human Th17 cells

Human IL-17 producing cells originate from $CD161^+$ T cell precursors in both umbilical cord blood and thymus (Cosmi et al., 2008). $CD161^-$ $CD4^+$ naive T cells from both umbilical cord blood and thymus could be induced to Th1 or Th2 with polarizing cytokines however they do not differentiate to IL-17 producing cells(Cosmi et al., 2008). CD161 also known as NKR-P1A is the human homologue of murine NK1.1 (Lanier et al., 1994). CD161 is expressed on most NK cells, NKT cells, and also on some T cells and thymocytes. Human $CD161^+$ Th17

cells have a broad TCR repertoire with MHC Class II restriction and are not CD1d- restricted NKT cells(Godfrey et al., 2004).

IL-17 producing cells are found only in the memory pool of $CD4^+$ T cells (Annunziato et al., 2007;Singh et al., 2008). Stimulation of Th17 cells in the presence of IL-12 down-regulates RORγt and the production of IL-17, and induces IFN-γ suggesting the flexibility of Th17 cells towards Th1 cells(Annunziato et al., 2007). Two distinct subsets of human Th17 cells can be identified based on chemokine receptors expression: high expression of CCR4 and CCR6 defines IL-17 and IL-22 secreting cells and co-existence of CCR6 and CXCR3 is the hallmark of Th17/Th1 cells which produce IL-17 and IFN-γ (Annunziato et al., 2007;costa-Rodriguez et al., 2007b). Both clones selectively express IL-23R, CCR6, and the transcription factor ROR γt. They also help B cells to produce IgG, IgM, IgA but not IgE, express poor proliferative capacity, poor cytotoxicity and are less susceptible to regulation by autologous T_{reg}.

1.5.4 Th17 in immunopathology

Th17 have been implicated in human immunopathology particularly autoimmune and chronic inflammatory diseases. IL17RA, the cognate receptor for IL-17A is widely expressed on haemopoietic cells, osteoblast, fibroblast, endothelial and epithelial cells (Kolls & Linden, 2004;Yao et al., 1995). The interaction of IL17 and its receptor result in chemokines, cytokines and metalloproteinase production by tissue cells. These responses promote the recruitment of lymphocytes, granulocytes and activated monocytes and facilitate matrix destruction (Miossec et al., 2009;Park et al., 2005;van den Berg and Miossec, 2009;Yao et al., 1995). Anti-microbial immunity mediated by Th17 cells is particularly important at epithelial and mucosal surfaces (Aujla et al., 2008;Ye et al., 2001).

Human psoriatic skin lesions consist predominantly of Th17 cells recruited by the action of CCL20 on CCR6 (Pene et al., 2008). Th17 cytokines IL-17A, IL-17F, IL-22 and IL-26 are overexpressed in serum and psoriatic skin lesions and there is increase expression of RORC, IL-6, IL-23 and IL-1β in psoriatic skin (Wilson et al., 2007). Th17 promoting cytokine IL-23 is also expressed in monocytes and dendritic cells in psoriatic skin lesions (Chan et al., 2006;Lee et al., 2004;Piskin et al., 2006). An addition, IL-22 secretion from these cells induces epidermal hyperplasia and acanthosis in psoriasis(Zheng et al., 2007). Treatment with ustekinumab, monoclonal antibody targeting p40 (polypeptide common to both IL-12 and IL-23) is extremely efficient in reducing affected psoriatic skin area (Krueger et al., 2007;Papp et al., 2008).

In rheumatoid arthritis patients, IL-17 expression by synovial cells is predictive of joint destruction (Kirkham et al., 2006) and IL-17 receptor is ubiquitously expressed on monocytes, macrophages, dendritic cells, chondrocytes, osteoblasts and fibroblasts. IL-17 acts on these cells leading to production of matrix proteinases, upregulation of proinflammatory cytokines and chemokines resulting in the recruitment of immune cells and pathological bone erosion and cartilage damage. Th17 cells secrete TNF-α, IL-1, and IL-6 which provide positive feedback for the further generation, expansion and recruitment of Th17 cells to the joint microenvironment. L-23p19 deficient mice do not develop collagen induced arthritis, while IL-12p35 deficient mice actually develop more severe disease suggesting the crucial role of Th17 rather than Th1 in CIA (Murphy et al., 2003).

In the pathogenesis of multiple sclerosis (MS) IL-17 and IL-6 genes are among the most highly expressed genes at the area of MS lesions (Lock et al., 2002) and IL-17 is elevated in the serum and cerebrospinal fluid of multiple sclerosis patients(Matusevicius et al., 1999). IL-17 and IL-22 receptors are expressed on endothelium at the blood brain barrier through which Th17 cells transmigrate to infiltrate the CNS parenchyma (Kebir et al., 2007). Monocyte-derived DCs

from multiple sclerosis patients also show increased expression of IL-23p19 (Vaknin-Dembinsky et al., 2006). IL-23 neutralizing antibody in mice also delays the onset and also decrease the severity of EAE, (Chen et al., 2006) human equivalent of multiple sclerosis.

Th17 and IL-23 are also involved in inflammatory bowel disease (IBD). A single nucleotide polymorphism in the IL-23 receptor (Arg381Gln) is significantly associated with Crohn's disease (Duerr et al., 2006). Clinical remission of Crohn's disease is mediated by antibodies against the p40 subunit which is shared by both IL-23 and IL-12 (Mannon et al., 2004). In murine models of IBD, IL-23 not IL-12 is essential for intestinal inflammation (Hue et al., 2006;Izcue et al., 2008;Kullberg et al., 2006;Yen et al., 2006).

Large numbers of Th17 cells are present in human cancers including hepatocellular carcinoma, renal and ovarian carcinoma. Expression of IL-23 is increased in human tumours and IL-23 upregulates matrix metalloprotease 9 and increases angiogenesis whilst reducing CD8 T-cell infiltration (Langowski et al., 2006). In ovarian tumour local polarizing cytokine milieu leads to expansion of Th17cells (Miyahara et al., 2008). Tumour infiltrating Th17 cells are polyfunctional effector cells and though synergistic actions between IL-17 and IFN-γ, they stimulate CXCL9 and CXCL10 production to recruit more effector T cells to the tumour microenvironment (Kryczek et al., 2009). In hepatocellular carcinoma accumulations of intra-tumour IL-17-producing cells promote tumour progression through fostering angiogenesis (Zhang et al., 2009a). Tumour cells and tumour-derived fibroblasts secrete CCL5 and CCL-2 to recruit Th17 cells and also produce a proinflammatory cytokine milieu to facilitate the generation and expansion of Th17cells(Su et al., 2009).

1.5.5 Tc17 cells

IL-17 secreting cells are not restricted to the CD4 subset, but also found in CD8 T cells when they are known as T cytotoxic IL-17 secreting cells (Tc17) (Kondo et al., 2009). Naïve $CD8^+$ T cells differentiate into cytotoxic killers (Tc1) in response predominantly to intracellular pathogens. Human Tc17 cells are a minor population of $CD8^+$ T cells and their frequency is lower than that of CD4 T cells producing IL-17. Tc17 cells share similar developmental characteristics with Th17 cells (Ciric et al., 2009).

Culture of CD8 T cells in polarizing conditions with IL-1β, IL-6 and IL-23 results in IL-17 secretion and Tc17 generation although such cells lack cytolytic function and express low level of cytotoxic T lymphocyte markers (Huber et al., 2009). They express a similar pattern of cell surface proteins to Th17. STAT3 is again important for Tc17 polarization both *in vitro* and *in vivo* (Huber et al., 2009;Yen et al., 2009) and Tc17 cells express other hallmark molecules of the Th17 program including RORγt, RORα, IL-21 and IL-23 receptor (Huber et al., 2009).

Tc17 are predominantly found in the $CD27^{-/+}$ $CD28^+$ $CD45RA^-$ memory subsets which differentiate from the $CD27^+$ $CD28^+$ $CD45RA^+$ naive subset. They express CCR6 and a high level of CCR5. Most Tc17 cells produce IFN-γ and differentiate from the same precursors that differentiate into IFN-γ-producing $CD8^+$ T cells(Kondo et al., 2009). Protection against lethal influenza via IFN-γ dependent mechanisms is mediated by Tc17 cells which lead to neutrophil influx into the lung in mice (Hamada et al., 2009).

There is little data on Tc17 cells in human liver diseases. Recent report by Klenerman and colleagues suggest that Tc17 cells in healthy donors and HCV patients are restricted to the $CD161^{high}$ population similar to Th17 subsets (Billerbeck et al., 2010). $CD161^{high}$ Tc17 express cytokines IL-17, IL-22, transcription factors RORγt, RUNX2, IL-23R, IL-18R, chemokine

receptors CCR6, CXCR6 and CCR2. $CD161^{high}$ Tc17 cells are markedly enriched in tissue. They co-express IL-17 with high levels of IFN-γ, and/or IL-22. These Tc17 cells represent up to 1 in 6 circulating $CD8^+$ T cells in normal humans and a substantial fraction of tissue-infiltrating $CD8^+$ T cells in chronic hepatitis C (Yu-Hoi Khan, 2010).

1.6 Anatomy and functions of the human liver

Embryologically, the liver is derived from the hepatic diverticulum which arises from the endodermal bud of the foregut. It is the largest solid organ in the human body weighing between 1.2 and 1.5 kg (Dawson, 1985). There are two anatomical lobes, the right six times larger than left and separated anteriorly by the falciform ligament and posteriorly by the fissure for ligamentum venosum and ligamentum teres. Functionally, depending on the independent vascular supply and biliary drainage it has eight segments. The caudate lobe is the equivalent of segment 1 and is separate from the other segments and has its own vascular supply.

The liver receives a dual blood supply, around 75% coming from the portal vein which brings venous blood containing digestive products and toxins absorbed from the alimentary canal (Lalor & Adams 2002) and 25% oxygenated blood via hepatic artery which arise from the coeliac axis. The portal vein is about 8 cm in length, and is formed at the level of the second lumbar vertebra by the union of the superior mesenteric vein and splenic vein behind the neck of the pancreas. In the liver it ramifies like an artery and ends in capillary-like vessels termed sinusoids. Biliary drainage from the liver originates from bile canaliculi formed by adjacent hepatocytes which collect and drain the bile towards the portal tracts. Hepatic artery, portal vein and common bile duct travel along the free edge of the lesser omentum, which is the anterior margin of "Foramen of Winslow" (Gray's Anatomy). The portal tracts, also known as portal triad or Glisson's capsule contain three essential structures; branches of bile ducts, portal vein and hepatic artery. Intrahepatic bile ducts converge ultimately to form the right and left hepatic ducts which unite at porta hepatis to form the common hepatic duct, which later in conjunction with the cystic duct from the gall bladder become the common bile duct. The common bile duct along with the portal vein and hepatic artery runs along the free margin of the lesser omentum, in front

of the "foramen of Winslow" to join the pancreatic duct and finally drains into the second part of the duodenum via the ampulla of Varter (Sherlock, 1948)

The venous drainage of the liver is via the three main hepatic veins (right, middle and left) which drain directly into the inferior vena cava. The hepatic nerve supply accompanies the hepatic artery and bile ducts into their portal tracts and hepatic parenchyma and it arise from hepatic nerve plexus which contains fibres from T7-T10 sympathetic ganglia and the right and left vagus nerve for parasympathetic nerve supply and both synapse at the coeliac plexus. The lymphatic vessels draining the liver terminate in the portal hepatic lymph nodes and from there drains to the coeliac nodes.

1.6.1 Hepatic parenchyma

The hepatocytes or liver cells comprise about 60-80% of the liver(Sherlock 1993). They are polygonal with a single nucleus. They are organised into plates which are one cell thick and separated by the sinusoids. The hepatocyte has three surfaces: the basolateral surface facing the hepatic sinusoid and space of Disse, the apical surface facing the canaliculi and the third facing neighbouring hepatocytes.

1.6.2 Hepatic sinusoid

The human hepatic sinusoids are lined by functionally and phenotypically unique hepatic sinusoidal endothelial cells which lack tight junctions and classical basement membrane but contain fenestrations arranged into sieve plates (Adams, 1996b;Adams and Eksteen, 2006;Smedsrod et al., 1994). Associated with sinusoids are kupffer cells, phagocytic cells of the reticulo-endothelial system and resident liver macrophages involved in taking up pathogens and particulate matter in portal blood (Burt et al., 1993). The space of Disse is a subendothelial tissue space between hepatocytes on one side and the sinusoidal lining cells on the other containing a

loose extracellular matrix (Sherlock & Dooley 1993). The hepatic stellate cell or Ito cell resides in the space of Disse. They are characterised by the presence of vitamin A storing cytoplasmic fat droplets (Friedman, 1996) which are lost on activation in response to injury when they transdifferentiate into myofibroblasts which play a key role in fibrogenesis in liver diseases (Iredale 2001; Iredale 2003). Hepatic sinusoidal endothelial cells are the first point of contact for lymphocytes circulating through the sinusoids. The presence of numerous adhesion molecules and chemokines presented on the surface, serves as a mechanism by which leukocytes are recruited to the liver parenchyma, particularly in inflammatory liver diseases (Lalor & Adams, 1999).

1.6.3 Metabolic and detoxification function

The hepatocytes are involved in protein, carbohydrate and fat metabolism. They synthesize albumin which is crucial for plasma oncotic pressure maintenance and transport of hormones and drugs. The liver also synthesize vitamin K dependant clotting factors II, VII, IX , X and fibrinogen thus it is involved in maintaining coagulation which can be assessed by monitoring the intrinsic pathways of the coagulation cascades or prothrombin time. In the clinical setting, these two parameters, albumin and prothrombin time, are used to access hepatocyte synthetic function.

The liver maintains glucose homeostasis by synthesizing, storing and breaking down glycogen. During the fasting state, glycolysis and glucogenolysis release stored glucose and hepatocytes can also synthesize glucose from non- carbohydrate source such as amino acids, glycerol, lactate and pyruvate via gluconeogensis.

The liver also plays a role in lipid metabolism. It secretes bile across the canalicular membrane of the hepatocytes which is then stored in the gall bladder until required for digestion when it is secreted into the small intestine via cholecystokinin dependent contraction of the gall

bladder. Bile is essential for hydrophobic fat absorption by the formation of chylomicrons using bile acids which are synthesised from cholesterol by hepatocytes. The liver synthesizes VLDL, HDL, cholesterol and phospholipids. In the liver, triacylglycerol is packaged with cholesterol, cholesteryl esters, phospholipid, and protein (apoB-100) to form VLDL which when secreted into blood deliver the newly synthesized lipids to the peripheral tissues. Hepatocytes also oxidize triglycerides to produce energy and synthesize fatty acids and triglycerides from carbohydrate and amino acids. It plays a central role in the regulation of cholesterol by *denovo* synthesis, receiving dietary cholesterol via chylomicrons from the gut and from extra hepatic tissues. It eliminates cholesterol in the bile or as a component of plasma protein or as bile salts secreted into the intestinal lumen. Impaired liver function results in steatorrhoea due to fat malabsorption.

Hepatocytes are also crucial in detoxifying xenobiotics via p450 cytochromes in the endoplasmic reticulum which are crucial for the biotransformation of drugs by glucoronidation and sulphation. Those drugs metabolites which are destined for urinary elimination are transported back to the sinusoids and blood whereas organic lipophilic drugs undergo biliary excretion. Hepatocytes also detoxify ammonia by the synthesis of urea through the Krebs-Henseleit cycle. Thus patients with acute liver failure (massive hepatic necrosis) and chronic decompensated liver diseases(associated with porto-systemic shunting) have high levels of serum ammonia and manifest clinical features of hepatic encephalopathy due to lack of hepatic detoxification of ammonia products from gut.

Bilirubin secreted in bile is the end product of haem metabolism by kupffer cells and it is one of the AHR ligands. UDP-glucuronosyltransferase1A1 (UGT1A1) conjugates bilirubin to make it water soluble. Activation of the AHR induces the reporter gene UGT1A1 in HepG2 cells. High bilirubin in neonates is removed after UGT1A1 induction to prevent jaundice(Togawa et al., 2008).

1.6.4 Immunological aspects of the liver

The liver is the site of T cell tolerance to harmless food antigens, yet maintains the capacity to sustain effective immune response against pathogens. The liver acts as a 'sieve' for the bacterial and other antigens, carried to it via the portal supply from the gastrointestinal tract. The mechanism driving hepatic tolerance is likely to be the continuous exposure of diverse liver cell types to endotoxin, derived from the intestinal bacteria. This exposure promotes the expression of a set of cytokines, antigen-presenting molecules, and costimulatory signals that impose T-cell inactivation, partly via effects on liver antigen-presenting cells. The first report of immunological tolerance in the liver was made by Sir Roy Calne who described liver allograft acceptance across MHC mismatch in the pig in the late 1960s (Calne RY, 1969;Calne and Davies, 1994).

The liver contains an unusual population of resident lymphocytes, which constitute upto 25% of the non-parenchyma cell. $CD8^+$ T cells usually outnumber $CD4^+$T cells, and the blood ratio of CD4:CD8 T cells are reversed(Warnock et al., 1998). Intrahepatic T cells are nearly all memory cells and are scattered throughout parenchyma and also found in portal tracts. The average human liver contains approximately 10^{10} lymphocytes, which include conventional ($CD8^+$ and $CD4^+$ T cells) and unconventional lymphocyte subpopulations of the innate (NKT and NK cells) and adaptive immune systems (T and B cells). Conventional T cells display a diverse repertoire of T cells receptors. About 30% of the total blood circulate through the liver every minute (Sheth and Bankey, 2001) carrying approximately 10^8 peripheral blood lymphocytes in 24 hours (Wick et al., 2002) thus the immune system is continually interacting with the liver.

Regulatory T cells play a key role in the induction and maintenance of hepatic tolerance (Bluestone, 2005;Oo and Adams, 2009;Sakaguchi, 2008;Walker & Abbas, 2002). They interact with resident tolerogenic dendritic cells which may maintain them within the liver or induce their local differentiation. Both hepatic DCs and intrahepatic lymphocytes secrete cytokines that

promote tolerance particularly interleukin-10 (Abel et al., 2006;Bliss et al., 2003;Nelson et al., 2003).

Hepatic antigen presenting cells (APC) include both professional antigen presenting cells (myeloid and plasmacytoid dendritic cells) and non-professional APCs of epithelial and endothelial lineage (hepatocytes, HSEC and stellate cells). Kupffer cells, which accounts for 20% of non parenchyma cells (Gale et al., 1978) are resident hepatic macrophages. Many are long lived although there is a slow turnover and replacement derived from circulating monocytes (Gale et al., 1978;Klein et al., 2007). They reside within the sinusoid predominantly concentrate around the periportal area although they migrate actively throughout the sinusoids. They clear endotoxins and phagocytose debris and microorganisms. They express low levels of costimulatory molecules and are poor antigen presenters thus their major role being the phagocytosis of particulates and pathogens and the secretion of cytokines. The unique architecture of the liver and low flow rates within the sinusoids allows T lymphocytes to interact with kupffer cells, sinusoidal endothelial cells and even hepatocytes which can extend microvilli through the endothelial fenestrations to interact directly with lymphocytes at the sinusoidal endothelial surface (Bowen et al., 2004;Bowen et al., 2005).

Hepatic dendritic cells have an immature phenotype expressing lower levels of costimulatory molecules when compared with langerhands cells in skin. They also preferentially secrete IL-10 (Goddard et al., 2004b). Both of these properties mean that they create a tolerogenic environment in the liver and skew T cell differentiation towards tolerance and regulatory T cell differentiation. The liver lies directly downstream from the gut, and is thus constantly exposed to bacterial LPS, a TLR4 ligand. Low expression of TLR4 by kupffer cells and liver DC and relative tolerance to TLR4 stimulation limit their response to LPS resulting in reduced hepatic adaptive immune responses (Bamboat et al., 2009;de et al., 2005b). Tolerogenic

dendritic cells include the $CCR9^+$ plasmacytoid DC, which has an immature phenotype reside in secondary lymphoid tissue and are potent inducers of regulatory T cells (Hadeiba et al., 2008). In the context of hepatic inflammation, the site of tolerance induction could be within the liver, particularly the portal tracts or within draining lymph nodes. Interactions with immature DCs within the liver inhibit the proliferation and cytokine production of interacting lymphocytes through CTLA-4 and PD-1(Probst et al., 2005).

Hepatic sinusoidal endothelial cells (HSEC) constitute 50% of the nonparenchymal cells in the liver. HSEC express molecules that promote antigen uptake, such as mannose receptor and the scavenger receptor, and molecules that promote antigen presentation like MHC class I and II and co-stimulatory molecules CD40, CD80 and CD86 (Knolle et al., 1999;Lohse et al., 1996). Moreover they can take up and present antigens to CD4 T cells and by cross presentation to CD8 T cells. Although HSEC express constitutive Toll-like receptor 4 (TLR4)/CD14 they gain a LPS-refractory state as a consequence of repetitive stimulation without loss of scavenger activity (Uhrig et al., 2005). When naive CD4 T cells are activated by HSEC they fail to elicit a Th1 type response (Knolle et al., 1999). Thus, MHC II-expressing intrahepatic APCs, such as HSECs and DCs skew local immune activation towards tolerance.

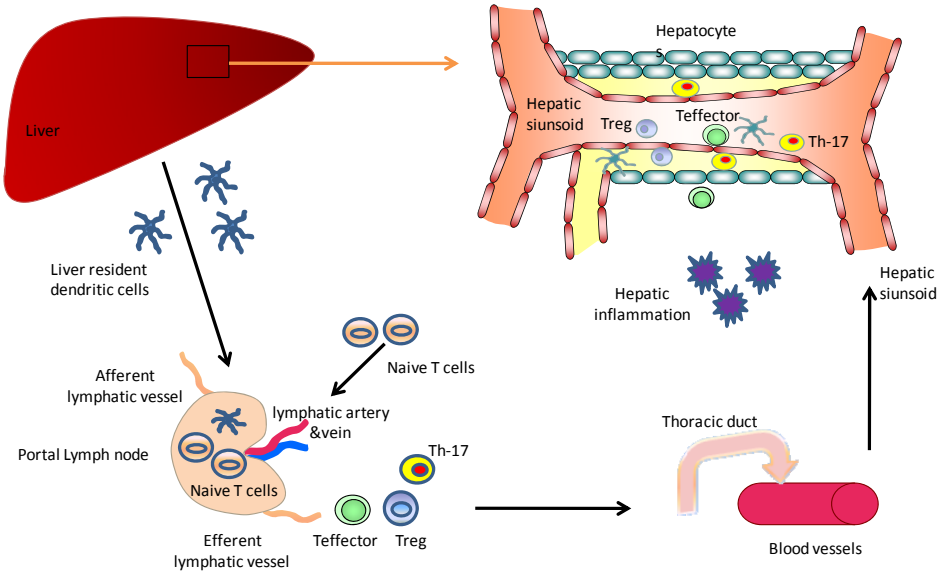

Figure 1-9 Lymphocytes circulation and transmigration across HSEC.

The liver is continuous exposed to gut-derived microbial and foreign antigen. During hepatic inflammation, liver resident dendritic cells sample the foreign antigens and carry them to local portal lymph nodes where antigens are presented to naive T cells recruited via high endothelial venules of lymph nodes. Following antigen presentation, different types of T effector and T regulatory cells leave the regional lymph nodes and drain back to systemic circulation. These antigen experienced lymphocytes are finally recruited towards the site of hepatic inflammation via the low flow hepatic sinusoidal endothelial vascular bed.

1.7 Leukocyte recruitment

Lymphocytes are recruited from the circulation to inflamed or normal tissues to respond to infection and to perform homeostatic immunosurveillance (Imhof & Dunon 1995). The recruitment process involves adhesion molecules on vascular endothelium that support recruitment through endothelium and interactions with extracellular matrix components within tissues (Hauzenberger et al. 1995). The recruitment of leukocytes to the liver occurs via several sites: a) vascular endothelial cells lining the portal vein terminal branches or b) central vein branches or c) through hepatic sinusoidal endothelium which lines the liver parenchyma. Elegant studies in rats suggest that the route of recruitment is through the sinusoidal endothelium with cells either migrating directly into the parenchyma or along the sinusoids to portal tracts (Xu, 2008).

In general, the recruitment of leukocytes through vascular endothelium into the underlying tissue follows a defined four stage process. Initially, free flowing leukocytes are captured and tethered to the endothelium by transient interactions mediated usually by selectins and glycosylated ligands (Rosen, 1985;Rosen, 2004). This dramatically reduces leukocyte velocity inducing them to roll on the vessel wall and thereby to scan the surface of the endothelium for the presence of activation factor such as chemokines (Mackay, 2001) suspended on proteoglycans (Tanaka et al., 1993a). This leads to the clustering and conformational activation of leucocytes integrins allowing them to support firm adhesion and arrest mediated by immunoglobulin superfamily members on the endothelium. The next step is intravascular crawling mediated by chemokines and integrins followed by diapedesis or trans-endothelial migration through endothelium. In most vascular beds the leucocytes then has to cross the basement membrane although in the liver this is missing and they cross the loose matricellular network of the space of

Disse before migrating to the site of inflammation along chemokine concentration gradients within tissue (Ley, 2007).

Hepatic sinusoidal endothelium lacks selectins and they play a minimal role in leukocyte capture in the liver (Wong et al., 1997). Rolling on hepatic sinusoids is greatly attenuated, as a consequence of the low shear stress in hepatic sinusoids (Adams et al., 1996;Wong et al., 1997). Other molecules may mediate capture on sinusoids including vascular adhesion protein-1 (McNab, 1996) and VCAM-1 which is expressed on inflamed sinusoids and can support rolling adhesion by interacting with VLA-4 on the lymphocyte (Alon et al., 1995;Lalor, 1997;Steinhoff et al., 1993).

Chemokines are crucial for leukocyte recruitment via endothelium. They activate cognate G protein coupled receptors resulting in not only activation of leukocyte integrins but also the induction of a migratory response and a motile phenotype. Chemokines secreted by other cells in the microenvironment can be taken up and undergo transcytosis across endothelial cells for presentation on the endothelial glycocalyx thereby localising chemokines to specific sites in the vasculature (Middleton et al., 1997). Chemokines are immobilized and presented via interactions with heparin binding domains on proteoglycans in the glycocalyx (Campbell et al., 1996;Luster, 1998;Springer, 1994;Tanaka et al., 1993a;Tanaka et al., 1993b). A group of promiscuous non-signalling chemokine receptors termed interceptors act as a sump to remove chemokines or in the case of D6 promote their disposal via lymphatics (Nibbs et al., 2003). Thus one such receptor, DARC, when expressed on endothelium promotes chemokine transcytosis and retention whereas on red cells in retains chemokines in the circulation thereby preventing random activation of circulating leukocytes (Colditz et al., 2007).

Stable adhesion is mediated by the activated leukocyte integrins VLA-4 and LFA-1 binding to their respective ligands from immunoglobulin superfamily, VCAM-1 for VLA-4 and

ICAM-1 or ICAM-2 for LFA-1 on the endothelium resulting in arrest of the lymphocyte on the sinusoidal endothelium (Springer, 1990). Both ICAM1 & ICAM-2 have been demonstrated on resting sinusoidal endothelial cells and ICAM-1 substantially upregulated in inflammation whilst VCAM-1 is only expressed on activated sinusoids (Adams et al., 1993;Steinhoff et al., 1993). Stable arrest depends on rapid increases in integrin affinity in response to chemokines displayed on proteoglycans on the endothelial glycocalyx (Tanaka et al., 1993a).

The control of hepatic transendothelial migration is poorly understood. Chemotatic signals remain important in transmigration into tissue (Muller and Randolph, 1999). VAP-1, a homodimeric transmembrane protein of the amine oxidase family plays a role in recruitment to the liver (Lalor et al., 2002a). It is expressed on human hepatic sinusoidal endothelium and supports T cell adhesion to human hepatic endothelium in tissue sections (McNab, 1996;Yoong et al., 1998). Moreover, VAP-1 is also involved in transmigration (Lalor et al., 2002a).

During diapedesis leukocytes migrate across the endothelium and basement membrane to enter tissue (Ley, 2007;Yadav et al., 2003). In most tissues diapedesis is regulated by adhesion receptors that localize to endothelial junctions through which the majority of migrating leukocytes cross. However, hepatic sinusoids lack conventional tight junctions and the signals required for transmigration may differ in the sinusoids (Lee and Kubes, 2008) and how cells traverse the space of Disse is poorly understood. Once they have transmigrated into tissue, the subsequent migration, and retention of effector and regulatory lymphocytes to site of inflammation depends on stroma tissue (Adams and Shaw, 1994;Buckley et al., 2001;Edwards et al., 2005) and microenvironment signals which will also influence the survival of different subsets of recruited lymphocytes (Oo & Adams, 2009).

A multi-stage paradigm (Springer 1994) has been described (Figure 1.10) to elucidate the processes involved in the recruitment of a fast moving blood borne leukocyte into tissue.

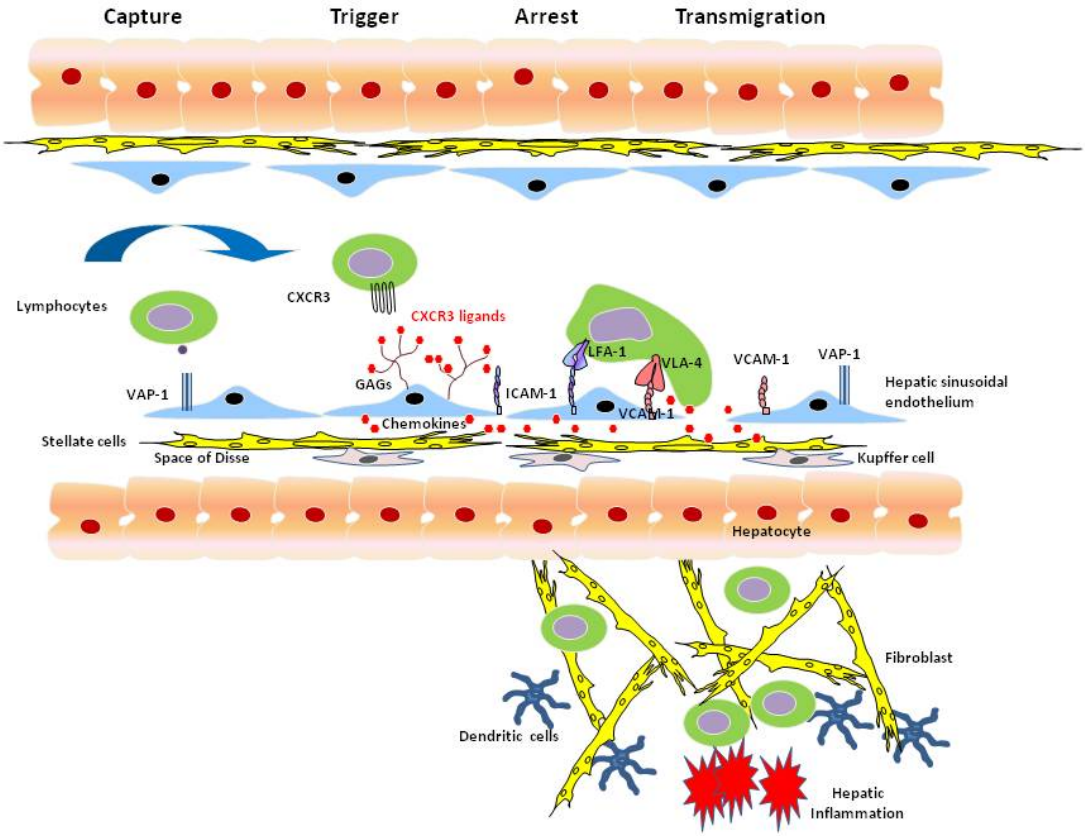

Figure 1-10 The multi-step model of leukocyte recruitment in the liver.

Leukocyte extravasation into tissue involves multiple steps. First of all, lymphocytes are captured by carbohydrate-dependent tethering (which in the liver may be mediated by VAP-1 rather than selectins which are absent from the hepatic sinusoids) allowing the flowing cells to contact the vessel wall where arrest can be triggered by chemokines immobilized on endothelial proteoglycans that activate lymphocyte integrins and binding to endothelial ligands such as ICAM-1 and VCAM-1. During this stage, the leukocyte arrests on the vessel wall and undergo intravascular crawling to sites of transendothelial migration. Once in the tissue, the cell follows chemokine gradients to sites of hepatic inflammation, using chemokine-mediated changes in the actin cytoskeleton, and adhesion molecules to propel migration.

Abbreviations: CXCR, chemokine (C-X-C motif) receptor; ICAM-1, intercellular cell adhesion molecule 1; IFN-γ, interferon-γ; TNF-α, tumour necrosis factor α; VCAM-1, vascular cell adhesion molecule 1. VAP-1, vascular adhesion protein-1; CXCR3 ligands- MIG, IP-10, I-TAC. HSEC= hepatic sinusoidal endothelial cells.

1.8 Lymphocyte Adhesion molecules.

1.8.1 The selectins

In most vascular beds the initial interactions between circulating lymphocytes and endothelium, the rolling or tethering phase is mediated by selectins. The selectins family contains three member named according to the cell type on which they were discovered: L-selectin (Leukocyte selectin, CD62L) (Rosen, 1985), E-selectin (Endothelial selectin, CD62E) which are detected on endothelial surface (Bevilacqua et al., 1987;Bevilacqua et al., 1989) and P-selectin (platelet selectin, CD62P) (Hsu-Lin et al., 1984;Springer, 1995).

Each selectin molecule is a single chain transmembrane glycoprotein with a similar modular structure. The N terminal amino acid, expressed extracellularly, is related to the family of mammalian carbohydrate binding protein known as C-type lectins as ligand binding by selectins is calcium dependent. The lectin domain is followed by a domain homologous to part of the epidermal growth factor (EGF), which in turn is followed by a number of consensus repeats (CR) with homology to structures previously identified as complement regulatory protein. The EGF domain and CR elements mediates adhesion by allowing ligands binding (Jutila et al., 1992;Jutila, 1992;Siegelman et al., 1990). The short CR is followed by a hydrophobic transmembrane region and a short cytoplasmic carboxyl terminal tail which is required for signalling and subsequent cytoskeletal rearrangement.

Selectin	Size	Distribution	Selectin ligands
L- selectin (CD62-L)	90-110 KD (varies depending on glycosylation)	Microvilli of naive lymphocytes (High) Effector & memory lymphocyte (low)	GlyCAM-1 CD34 PSGL-1 MadCAM-1
E-selectin (CD62E)	110 KD	Activated endothelium	CLA-1
P-selectin (CD62P)	140 KD	Storage granules and surface of endothelium and platelets	CD24 PSGL-1

CD34 and GlyCAM-1 which possess frucosylated, sulphated and Lewis^x structure is also known as PNAd or peripheral node addressin which is the ligands on high endothelial venules in lymph node

Table 1-2 Selectins and selectin ligands

L-selectin assist the entry of lymphocytes to secondary lymphoid tissues via high endothelial venules with their ligands, namely; GlyCAM-1, CD34 or MAdCAM-1(Rosen, 2004). L-selectin is located on the microvilli of lymphocyte (Ala et al., 2003), for optimal rolling effect and its shedding occurs once it is activated (Hafezi-Moghadam et al., 2001;Hafezi-Moghadam and Ley, 1999) by proteolytic cleavages mediated by zinc dependant metalloproteinases (Chen et al., 1995). The importance of L-selectin for lymphocyte recruitment to secondary lymph node was demonstrated when L-selectin deficient mice were shown to have impair lymphocyte homing to lymph nodes (Arbones et al., 1994;Xu et al., 1996).

Endothelial expression of E -selectin is activated by inflammatory stimuli (Lasky, 1995) and it is constitutively expressed in the skin in dermal endothelium (Picker et al., 1991). E-selectin is not detected on resting hepatic sinusoidal endothelium *in vivo* or *in vitro*, but is upregulated with inflammation (Adams et al., 1994a;Adams & Shaw, 1994). P-selectin is stored in specialised granules (Weibel-Palade bodies) in endothelium which are absent from sinusoidal endothelium and in α granules in platelets (McEver, 2001;McEver et al., 1989). In mice E-selectin and P-selectin deficiency leads to defective leukocyte recruitment to most sites of inflammation but not the liver (Jung and Ley, 1999). Table.1.2

1.8.2 Integrins and their ligands

Integrins are transmembrane heterodimeric proteins that mediate interactions between adhesion molecules on cells to adjacent cells and/or the extracellular matrix (Tamkun et al., 1986). They are involved in haematopoiesis, haemostasis, immune regulation, inflammatory response and also necessary for cell motility & migration across endothelium and to the site of infection/inflammation. Additionally, they are crucial for embryonic development, influencing cellular survival, proliferation, and differentiation (Danen and Sonnenberg, 2003). Integrin were first described in the late 1980s by Tamkun and colleagues as a protein complex which was integral to the transmembrane connection between the extracellular matrix and the cytoskeleton (Tamkun et al., 1986). Deficiency of many integrins is embryonically lethal (Rosenkranz and Mayadas, 1999).

The integrins are membrane bound α β heterodimers and are divided into families according to their β-chain (Springer and Wang, 2004). Both α subunit and β subunit are non-covalently bonded. Most β-subunits interact with more than one α-subunits and there are at least 18α subunits and 8β subunits in human thus pair to forms 24 different combinations (Hynes, 1992;Shaw and Adams, 1993). The β1 integrins chains can associate with α1 to α9 and they are named VLA or very late integrins; the β2 integrins consist of $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$ and $\alpha_D\beta_2$ and the β7 family consists of α4β7 and αEβ7. Integrins are expressed by many subgroups and shape their migratory responses. VLA-4 of β1 integrins family, LFA-1 of β2 integrins family and LPAM-1 of β7 family are major players involved in lymphocyte homing.

Integrins which are important for leukocyte recruitment via endothelium includes $\alpha_L\beta_2$ (LFA-1, CD11a/CD18) & $\alpha_M\beta_2$ which react its ligands ICAM-1 & ICAM-2 on endothelium, and α4β1 (VLA-4, CD49d/CD29), α4β7 (LPAM-1, CD49d/cd104) via its ligands VCAM-1 and MadCAM-1 (Butcher and Picker, 1996). Some integrins are limited to one cell lineage; for

example αEβ7 is expressed on mucosal lymphocytes (Kilshaw, 1999;Kilshaw and Murant, 1990) and this selective expression promote tissue specific homing (Butcher et al., 1999). An autosomal recessive inherited deficiency in LFA-1, Mac-1, and p150.95 proteins, known as type 1 leukocyte adhesion deficiency (LAD-1) leads to profound defects in adherence-dependent leukocytes function and susceptibility to sepsis (Anderson and Springer, 1987).

The large extracellular domains of α and β subunits combine to form a globular head which contains the ligand binding site and a short cytoplasmic tail (Hynes, 1992). Integrins exist in inactive conformation with low affinity for ligand until activated. Chemokine binding to its appropriate receptor leads to activation of lymphocyte integrins (Campbell and Butcher, 2000), which cluster on the cells surface and in turn leads to a intracellular signalling and conformational activation of the integrin and fully activation of ligand bindin (Dransfield and Hogg, 1989a;Dransfield and Hogg, 1989b) known as inside out signalling. This is followed by increase in the avidity leading to strong endothelial adhesion against sheer stress from blood flow and resulting in successful migration of cells across endothelium.

Integrins	Name	Ligands	Function
α4β1 (CD49d/CD29)	VLA-4,	VCAM-1, Fibronectin	Cell- matrix adhesion; lymphocyte homing
$\alpha_L\beta_2$ (CD11a/CD18)	LFA-1	ICAM-1-4	Leukocyte adhesion to endothelium; T cell-APC adhesion
$\alpha_M\beta_2$ (CD11b/CD18)	MAC-1	Fibrinogen, FactorX, ICAM-1, iC3b	Leukocyte adhesion Cell-matrix adhesion
α4β7 (CD49d/cd104)	LPAM-1	Fibronectin, VCAM-1, MadCAM-1	Lymphocyte homing to mucosal tissue
αEβ7 (CD103)	HML-1	E-cadherin	Retention of intraepithelial cells

Abbreviations: APC, Antigen presenting cell; iC3b, inactivated Complement 3b; ICAM, intracellular adhesion molecules; VCAM-1, vascular adhesion molecules-1; LFA-1, leukocyte function-associated antigen; MadCAM-1, mucosal addressin cell adhesion molecules-1; LPAM-1, lymphocyte Peyer's patch adhesion molecules-1

Table 1-3 Human lymphocyte integrins and their ligands

Integrins binds to ligands that are either cell surface immunoglobulin superfamily members or matrix components. IgSF include ICAM-1, ICAM-2, VCAM-1, CD31 and MAdCAM-1 and they are constitutively expressed or induced on the surface of endothelial cells following stimulation with proinflammatory cytokines (Ala et al., 2003). ICAM-1 is expressed not only on leucocytes also on the endothelium at inflamed sites but ICAM-2 is constitutively expressed on endothelium. Both ICAM-1 & ICAM-2 bind to LFA-1. VCAM-1 is normally expressed at low levels on endothelium and its expression is upregulated during inflammation and also expressed on stroma and epithelial cells which are crucial for post endothelial migration and retention of lymphocytes at site of infection or inflammation. MAdCAM-1 is constitutively expressed in vessels in the gastrointestinal tract and also on HEV in small intestine Peyer's patches and mesenteric lymph nodes (Adams & Eksteen, 2006;Nakache et al., 1989).

Th1 cells, by secreting IFN-γ, may make the vascular endothelium at the site of inflammation more adherent to intravascular lymphocytes. IFN-γ and TNF-α secreted by Th1 cells play a key role in the induction of VCAM-1. VCAM-1 binds lymphocytes with α4 integrin, and this step is a critical in pathophysiology of experimental autoimmune diseases, including EAE, type 1 diabetes mellitus in NOD mouse, and collagen induced arthritis (Yang et al., 1993;Yang et al., 1994;Yednock et al., 1992). Blockade of α4 integrin has led to the most effective therapy to date for MS (Steinman, 2005;Yednock et al., 1992) and has been shown to be effective in the treatment of rheumatoid arthritis and Crohn's disease.

The major functions of lymphocyte integrins are to mediate adhesion to endothelial cells, antigen presenting cell, target cells during cytolysis and extracellular matrix. The expression of integrins on lymphocytes is increased after activation and their ability to bind to matrix molecules is responsible for the retention of antigen primed T cells in lymphoid organs and at peripheral sites of infection.

1.9 Chemokines

Chemokines are 8–12 kDa heparin binding cytokines with the ability to attract leucocytes subsets to specific sites and thereby to shape the outcome of immune responses including intrahepatic inflammation. An efficient immune reaction requires leukocytes to be at the right place at the right time and thus needs a system to regulate the migration and positioning in lymphoid and non-lymphoid tissues (von Andrian and Mackay, 2000). The chemokine system provides cues for the recruitment of effector and regulatory subsets and is central to the pathogenesis of inflammatory diseases.

Chemokines are generally classified into two functional groups, inflammatory and homeostatic/constitutive based on whether they are induced by inflammation or constitutively expressed and involved in homeostatic immune regulation (Table 1.4). Inflammatory chemokines are expressed in inflamed tissues by resident and infiltrating cells on stimulation by pro-inflammatory cytokines or during contact with pathogenic agents (Rot, 2004). These chemokines are secreted early after infection in response to activation of pattern recognition receptors on epithelial, stroma, and immune cells. They recruit the initial wave of innate immune cells which express inflammatory chemokine receptors and immature DCs that provide the link between innate and adaptive immunity (Luster, 2005). After antigen-specific activation of lymphocytes by activated DCs inflammatory chemokines then attract antigen-specific effector T cells to the inflammatory site (Heydtmann and Adams, 2002). At the same time, regulatory cells are also recruited and the balance between effector and regulatory cell recruitment determines the outcome of the local inflammation.

In contrast, homeostatic chemokines are produced in discrete microenvironments within lymphoid (bone marrow, thymus, and secondary lymphoid organs) or non-lymphoid tissues such as the skin and mucosa. These constitutively produced chemokines are involved in physiological

trafficking and positioning of cells, antigen sampling in secondary lymphoid tissue and immune surveillance.

1.9.1 The human chemokine system

The human chemokine system includes more than 50 chemokines and 20 chemokine receptors (Table1.4) that can be divided into structural subsets based on the presence of NH_2-terminal cysteine-motifs (Moser and Loetscher, 2001;Zlotnik and Yoshie). The large CC chemokine family consists of chemokines in which the first two cysteine residues are adjacent, whereas in CXC chemokines they are separated by a single amino acid residue. Fractalkine (CX3CL1) is the only member of the CX3C chemokine family, in which three amino acid residues separate the first two cysteines. Finally, two related chemokines, XCL1 and XCL2 both of which bind the XCR1 receptor, lack two adjacent cysteine residues. Chemokines and their receptors also undergo post-translational modifications which alter their function allowing them to provide almost limitless potential receptor ligand pairs to bring exquisite specificity to the control of leukocyte homing and positioning in tissues (Rot, 2004). Dysregulated expression of chemokines and their receptors is involved in the development of many human diseases, including autoimmune and chronic inflammatory diseases as well as immunodeficiency and cancer.

Chemokine Receptors	Chemokines systematic names	Functions	Distributions of Chemokine receptors
CXC subgroup			
CXCR1	CXCL6, CXCL7,CXCL8	Neutrophil migration; innate immunity; acute inflammation	PMN, Monocytes,Mast cells
CXCR2	CXCL1-3; CXCL5-8	Neutrophil migration; innate immunity; acute inflammation; angiogenesis	PMN, Monocytes, Mast cells
CXCR3	CXCL9, CXCL10, CXCL11	T cell recruitment; adaptive immunity; Th1,Th2,Th17,Treg inflammation	Memory T cells,Th1,Th2,Th17,Treg, NKT
CXCR4	CXCL12	Stem cell migration;B cell lymphopoiesis; bone marrow myelopoiesis; Embryogenesis; HIV infection	T cells, B cells, Macrohpages, Monocytes, Stem cells,NKT
CXCR5	CXCL13	B cell homing in lymphoid organ	B cellls
CXCR6	CXCL16	T cell migration	Memory T cells, Th1,NK, NKT
CXCR7			
CC subgroup			
CCR1	CCL3, CCL5, CCL7, CCL8, CCL13-16, CCL23	T cell and monocyte migration; Hypersenstivity; innate and adaptive immunity; inflammation	Monocytes, Memory T cells,Th1, NK
CCR2	CCL2, CCL7, CCL8, CCL13	T cell and monocyte migration; innate and adaptive immunity; Th1 inflammation	Monocytes, Memory T cells, Basophils, pDC
CCR3	CCL5, CCL7; CCL11, CCL15,16; CCL24, CCL26	Eosinophil, basophil, migration; allergic inflammation;Th2 response	Eosinophils, Basophils
CCR4	CCL17, CCL22	T cell and monocyte migration; allergic inflammation; Regularoty T cells retention; Skin homing; expressed on CD4 Th2 cells	Th2 cells, Treg Eosinophils,Basophils, DC Regulatory T cells
CCR5	CCL3; CCL4; CCL5; CCL8	Th1 response, adaptive immunity; inflammation, HIV infection	Monocytes, Th1 cells, Treg, DC, NK
CCR6	CCL20	Dendritic cell migration, memory T cells, Th17 cells at site of inflammation	Memory T cells, B cells, Th17, immature mDC
CCR7	CCL19, CCL21	T cell and dendritic cells homing to secondary lymphoid tissue; lymphoid development	Naive T, B, mature mDC, Th1,Th2, Treg
CCR8	CCL1	T cell trafficking; Th2 response	Monocytes, Th2, Treg, NK
CCR9	CCL25	T cell homing to gut and Thymus Tolerogenic Dendritic cells	DC, Memory T cells, Thymocytes
CCR10	CCL27, CCL28	T cell homing to skin and bowel	Memory Tcells,Treg
CX_3C and XC family			
CX_3CR1	CX3CL1	T cell and NK cell trafficking and adhesion; innate and adaptive immunity; Th1 inflammation	Monocytes, Th1, NK
XCR1	XCL1-2	NK cell recruitment	NK

Table 1-4 Chemokine nomenclature

Chemokine receptors can be divided into subfamilies on the basis of the position of conserved cysteine residues within a conserved tetracysteine motif. In CC chemokines, the first two consensus cysteines are next to each other; in CXC chemokines, they are separated by a nonconserved amino acid. These two subfamilies account for all but three of the known chemokines, the others being CX3CL1 (three intervening amino acids between the first cysteines) and XCL1 and XCL2, which lack two of four canonical cysteines.

Human chemokines and chemokine receptors could also be classified according to their function; inflammatory (pale blue), homeostatic (yellow). Chemokines and its receptors belongs to both subfamilies are shown in green.

Abbreviations: CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; CX3CL, chemokine (C-X3-C motif) ligand; CX3CR, chemokine (C-X3-C motif) receptor; imm, immature; mat, mature; mDC, myeloid dendritic cell; Mo, monocyte; NK, natural killer; NKT, natural killer T; pDC, plasmacytoid dendritic cell; PMN, polymorphs; T mem, memory T cell; T naïve, naïve T cell; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; T reg, regulatory T cell.

Chemokines exert their chemotactic functions by binding to specific G protein–coupled receptors (GPCRs), seven-transmembrane-spanning proteins coupled to heterotrimeric G proteins (Figure.1.11). Chemokine binding to chemokine receptors dissociates $G\alpha_I$, and $G\beta\gamma$ subunits of the heterotrimeric G proteins, leading to calcium flux and activation of the phosphatidylinositol 3-kinase (PI3K) and the small Rho GTPases signalling pathways (Mellado et al., 2001). Consistent with G_i association, the majority of chemokine responses are inhibited by treatment with pertussis toxin (PTx) (Goldman et al., 1985)which blocks the global G protein receptor.

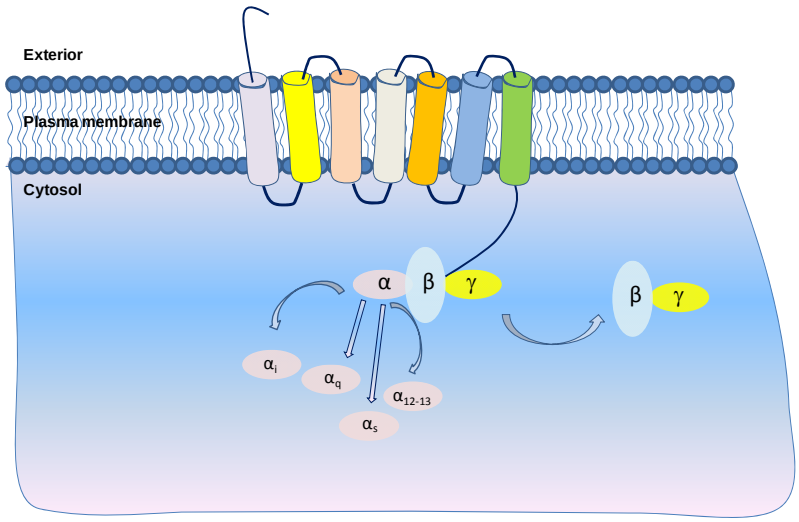

Figure 1-11 Schematic diagram of G- protein coupled receptor (GPCR).

Chemokine receptors are seven-transmembrane molecules coupled to heterotrimeric G proteins. When the chemokine interacts with its cognate receptors, the β- and γ-subunits are assembled into βγ dimers that act as functional units. The α-subunits bind guanine nucleotides, being active when GTP is bound. The G proteins are usually classified into four classes. α_i, α_s, α_q, and $\alpha_{12/13}$ by the nature of their α-subunit. Chemokine receptors can signal through different Gα-protein families, leading to distinct transduction pathways and downstream biological effects.

1.9.2 Roles of chemokines in the context of hepatic inflammation

Immune surveillance in the liver is provided by both resident and blood-borne lymphocytes and macrophages (Lalor & Adams, 1999). During hepatic inflammation the rate of lymphocyte recruitment via sinusoidal endothelium increases. They are retained within the liver by localisation at sites of inflammation or at epithelial surfaces resulting in chronic hepatitis and persistent inflammation (Valiante et al., 2000). Chronic HCV infection and primary biliary cirrhosis are both dominated by Th1 immune responses (Bertoletti et al., 1997;Napoli et al., 1996;Shields et al., 1999) whereas there is some evidence for Th2 involvement in primary sclerosing cholangitis. Recent studies suggest that alcoholic hepatitis involve Th17 responses which may explain the characteristic neutrophil rich infiltrate (Lemmers, 2009). However, unlike some murine models human chronic inflammatory diseases cannot so easily be assigned to Th1 vs. Th2 vs. Th17 dominated responses and it is likely that overlapping mechanism are involved in most situations. Most effector T cell infiltrating the chronically inflamed human liver express high levels of CXCR3, CXCR6, CCR1 and CCR5 consistent with a Th1 predominance and a tissue-infiltrating phenotype (Boisvert et al., 2003;Heydtmann et al., 2006;Kunkel et al., 2002;Leroy et al., 2003;Shields et al., 1999). In addition to recruiting different subsets of lymphocytes there is evidence for compartmentalization of recruitment as well. CCR5 may have a particular role in recruitment to portal tracts whereas CXCR3 appears to be essential for recruitment into the parenchyma (Ajuebor et al., 2004;Curbishley et al., 2005;Harvey et al., 2003;Murai et al., 1999).

Expression of CXCR3 on T cells is closely linked to Th1 function and its ligands CXCL9, CXCL10 and CXCL11 are induced by the Th1 cytokines IFN-γ and TNF-α. Studies reported increase levels of CXCR3 ligands in chronic hepatitis and increased CXCR3 on CD4 and CD8 effector cells within the liver (Apolinario et al., 2002;Arai et al., 2002;Diago et al., 2006;Dumoulin et al., 1997). Increased levels of hepatic CXCR3 ligands are characteristic of many chronic

inflammatory liver diseases suggesting they play a generic role in effector cell recruitment to the inflamed liver (Butera et al., 2005;Curbishley et al., 2005;Nishioji et al., 2001). The sources of CXCR3 ligands in liver inflammation include hepatocytes, stellate cells, sinusoidal endothelial cells and infiltrating leukocytes. The expression of CXCR3 ligands requires stimulation with IFN-γ and TNF-α which are released by activated hepatic macrophages and kupffer cells and the initial wave of infiltrating innate immune cells. An additional contribution comes from activated $CD4^+$ T cells themselves which release CXCR3 ligands after activation within the liver (Cruise et al., 2006) thereby providing a feedback loop in which antigen-specific cells maintain the expression of the chemokines required for effector cell recruitment. CXCR3 ligands on the sinusoidal wall can originate from neighbouring and then be taken up and transcytosed to the endothelial surface (Middleton et al., 2002). In addition, chemokines secreted upstream by other cell types including cholangiocytes in portal tracts can be captured from the slow-flowing sinusoidal blood by proteoglycans within the endothelial cell glycocalyx and presented at the endothelial surface (Curbishley et al., 2005). Thus local presentation of chemokines on the sinusoids will be the result of cross-talk between sinusoidal endothelial cells and stellate cells, kupffer cells, cholangiocytes and hepatocytes, all of which secrete chemokines on appropriate stimulation (Maher, 1995;Morland et al., 1997b;Shiratori et al., 1993). Hepatocytes not only secrete chemokines but also sensitize the endothelium to respond to low levels of TNF-α by increasing chemokine secretion and adhesion molecule expression (Edwards et al., 2005).

In chronic hepatitis many of the infiltrating cells are probably not antigen-specific but rather bystander lymphocytes recruited as part of a broad immune response. Both antigen-dependent and independent infiltrating effector cells express high levels of CD154 whose ligand, CD40, is expressed on hepatocytes, cholangiocytes and kupffer cells. Activation of CD40 on these liver cells triggers NFκB-dependent secretion of CXCR3 ligands(Ahmed-Choudhury et al.,

2003;Alabraba et al., 2007) demonstrating another mechanism by which local chemokine gradients can be amplified during the evolution of the anti-viral immune response. T_{reg} also use CXCR3 to enter liver tissue (Santodomingo-Garzon, 2008), although other signals may determine where they migrate to within the inflamed liver and hence where they mediate their anti-inflammatory effects (Eksteen et al., 2006).

In human blood CXCR6 is expressed on Th1 cells and effector CD8 T cells and our group and others have reported high levels of CXCR6 on CD4 and CD8 T cells within the inflamed human liver (Heydtmann et al., 2005). The CXCR6 ligand CXCL16 is one of only two chemokines that exists in a transmembrane form (Heydtmann et al., 2005;Matloubian et al., 2000;Wilbanks et al., 2001). CXCL16 is upregulated on inflamed bile ducts and hepatocytes and is also expressed by sinusoidal endothelium. The engagement of CXCR6 on T cells by CXCL16 on epithelial cells promotes β1 integrin-dependent adhesion which we believe is important for the positioning, retention and survival of effector cells in the inflamed liver (Germanov et al., 2008). Klenerman and colleagues have recently reported a unique subset of HCV-specific $CXCR6^{+}$ liver-infiltrating CD8 T cells which express the C-type lectin CD161 secrete Il-17 and IFN-γ (Yu Hoi Khan et al, PNAS, 2010) and these cells may represent an important liver-specific subsets of effector cells (Northfield et al., 2008).

Other chemokines involved in retaining T cells within the liver include CXCL12 (Terada et al., 2003;Wald et al., 2004) and the other transmembrane chemokine CX3CL1 (fractalkine) both of which are expressed on inflamed bile ducts (Efsen et al., 2002;Isse et al., 2005). The fractalkine receptor CX3CR1 is expressed by Th1 cells and help to retain these cells at sites of epithelial inflammation. Increased expression of CCL28 on cholangiocytes in a variety of liver diseases and increased numbers of T cells expressing the CCL28 receptor, CCR10, was also reported in the inflamed human liver (Eksteen et al., 2006). A high proportion of these cells were

FoxP3$^+$CD4 cells that secrete IL-10 and suppress T cell activation. Thus CCL28 appears to recruit regulatory rather than effector cells to bile ducts. When compared with T_{reg} in blood the CCR10$^+$ liver-derived T_{reg} express high levels of CXCR3 and low levels of CCR7 consistent with a tissue-infiltrating phenotype. CCL28 was originally isolated from the gut but is widely expressed at mucosal surfaces throughout the body (Kunkel et al., 2003;Pan et al., 2000).

CCR1, CCR2 and CCR5 have all been implicated in hepatic inflammation and their expression is characteristic of memory T cells and CCR5 is also expressed on Th1 cells (Qin et al., 1998;Viola and Luster, 2008). CCR2 and CCR5 expressing CD8 T cells are enriched in the inflamed human liver and CCR1 is important in the regulation of hepatic inflammation in murine models (Boisvert et al., 2003;Shields et al., 1999). The three receptors share chemokine ligands; CCR5 interacts with CCL3, CCL4, CCL5 and CCL8; CCR2 with CCL2, CCL13, CCL7 and CCL8 and CCR1 with CCL3, CCL5, CCL7, CCL23 and CCL14-16 (Rot, 2004;Viola & Luster, 2008). All of these chemokines have been detected in the liver (Leroy et al., 2003;Lichterfeld et al., 2002;Nattermann et al., 2008;Nischalke et al., 2004;Shields et al., 1999).

CCR5 ligands are strongly expressed on portal and vascular endothelium (Goddard et al., 2001) and in murine models of graft versus host disease CCR5 and CCL3 support effector cell recruitment to portal tracts (Murai et al., 1999;Murai et al., 2003). Although other murine models of liver inflammation are characterized by intrahepatic CCR5$^+$ lymphocytes (Ajuebor et al., 2005;Moreno et al., 2005) mice that lack CCR5 are more susceptible to Con A-induced hepatitis and exhibit extensive inflammation mediated by CCR1$^+$ effectors (Ajuebor et al., 2004;Ajuebor et al., 2005). This emphasizes the complexity of chemokine networks and suggests that under some conditions CCR5 recruits anti-inflammatory as well as effector cells (Moreno et al., 2005).

1.9.3 Homeostatic chemokines and lymphocyte egress from the liver

Homeostatic chemokines can be upregulated at sites of inflammation where they play important roles in regulating leukocyte trafficking particularly through the formation of tertiary neolymphoid structures (Grant et al., 2002;Hjelmstrom et al.). Neolymphoid follicles that express CCL19 and CCL21 are a feature of many chronic inflammatory liver diseases particularly PBC, PSC and HCV infection (Heydtmann et al., 2006). They may be sites for ongoing lymphocyte recruitment and provide survival signals to maintain the chronic inflammatory infiltrate within the liver (Bonacchi et al., 2003;Mondelli, 2003;Terada et al., 2003;Wald et al., 2004). The CCR7 receptor is expressed by naive T cells and a subset of central memory cells to promote their recirculation through secondary lymphoid tissues. $CCR7^+$ T cells are detected in livers from patients with chronic autoimmune liver disease and chronic HCV infection (Bonacchi et al., 2003). However, most of these cells were memory rather than naïve cells (Faint et al., 2001;Heydtmann et al., 2006) thus $CCR7^+$ CD8 T cells in the HCV infected liver were $CD62L^{low}$ and LFA-1^{high} characteristic of memory cells. Because CCL19 and CCL21 are expressed on sinusoids and lymphatic vessels in portal tracts (Grant et al., 2002;Heydtmann et al., 2006). These cells use CCR7 to migrate out of the liver via afferent lymphatics to draining lymph nodes where they are either removed during the resolution of infection or restimulated to maintain chronic hepatitis. This pathway may be defective in HCV infection because the numbers of intrahepatic $CCR7^+$ memory T cells are reduced. Others groups have reported an important role for CCR7 in promoting resolution of inflammation and a lack of CCR7 in mice leads to enhanced inflammation (Bromley et al., 2005;Forster et al., 2008;Schneider et al., 2007).

1.9.4 Interstitial migration and position of recruited lymphocytes

Once lymphocytes have crossed the endothelial barrier, these infiltrating cells find cues to position themselves in tissue and chemokine can provide such coordinated directed migration signals (Cyster, 2005) particularly when acting as a surface-bound gradient in extracellular matrix (ECM) and stroma cells(Miyasaka and Tanaka, 2004;von Andrian and Mempel, 2003). The stroma can also modulate the function of the overlying endothelium, emphasizing the importance of the tissue microenvironment in shaping recruitment (Mantovani et al., 2004). Leukocyte migration within interstitial tissue *in vitro* and *in vivo* is only partially integrin-dependent, being directed instead by chemokine dependent migration along the confining ECM scaffold. In the setting of hepatic inflammation, once the lymphocytes are recruited into the inflamed tissue the liver stroma cell network (stellate cells and activated liver myofibroblast) direct the post-endothelial migration (Adams et al., 1994b;Hathaway et al., 1993) which is partly chemokine dependent but also involves chemokine-independent mechanisms (Holt et al., 2009).

Thus, chemokines have the ability to induce migration of both effectors and regulatory cells and are critical regulators of immunity and inflammation in human liver diseases. They play a central role in the generation of cellular inflammation, both in the protective responses to invading pathogens and in the pathological processes associated with infection and immune-mediated diseases.

1.10 Aims of the thesis

Hypothesis: Specific chemokine receptors will regulate the recruitment and positioning of regulatory T cells in the inflamed human liver.

To address this hypothesis I have

1) Carried out a detailed phenotyping of regulatory T cells infiltrating human liver
2) Investigated the molecular basis for the recruitment of T_{reg} across primary human hepatic sinusoidal endothelium under conditions of physiological flow
3) Investigated signals responsible for positioning T_{reg} in tissue and the role of dendritic cells in this process
4) During the thesis the close relationship between Th17 cells and T_{reg} became apparent and I thus extended my work to phenotype liver infiltrating Th17CD4 cells and Tc17CD8 cells in different inflammatory liver diseases and to determine signals involved in the recruitment and positioning of these cells within the inflamed liver

CHAPTER 2 MATERIALS AND METHODS

2.1 Human tissue samples

All liver tissue and matched pre transplant peripheral blood samples were obtained from patients attending the Queen Elizabeth Hospital, Birmingham, UK. Blood samples were also obtained from haemochromatosis patients who underwent regular venesection in liver outpatient clinics or from healthy controls. Diseased liver tissue was collected from explanted livers from patients undergoing orthotopic liver transplantation for a variety of inflammatory conditions. Non-diseased normal liver tissue was from donor material surplus to surgical requirements or from normal areas of liver removed following partial hepatectomy for hepatic tumour or benign focal disease. Lymph nodes were obtained from unused donor/ diseased hepatic nodes. All human samples were collected in accordance with local ethics committee approval and with the consent of patients.

2.2 Isolation of primary human cells

2.2.1 Isolation of peripheral blood lymphocytes & peripheral blood regulatory T cells

Peripheral venous blood was collected from healthy volunteers or haemochromatosis patients into EDTA (ethylenediamine tetra-acetic acid; BD Biosciences, Oxford, UK). The mononuclear fraction was purified by density gradient centrifugation over Lympholyte (VH Bio) for 30 minutes at 550g for 25 minutes. The band at the interface between plasma and gradient material (mononuclear fraction) was removed, resuspended in RPMI /10 % foetal calf serum (FCS) and sedimented by centrifugation at 550Xg for 5 minutes. Harvested lymphocytes were washed and resuspended in RPMI 1640 /10% FCS (Invitrogen) and incubated on plastic for 20 minutes to allow adherence of monocytes before aspiration of the lymphocyte enriched

supernatant. Following one further wash, lymphocytes were resuspended in media containing RPMI-1640/10% FCS. The viability of isolated cells (usually>95%) was determined by assessment of trypan blue exclusion for each sample.

Regulatory T cells were isolated using a CD4 CD25 isolation kit (Invitrogen). Briefly, $1X10^8$ freshly isolated lymphocytes were resuspended in 500µl of isolation buffer and 200 µl of FCS and 200 µl of human CD4 antibody mix (mouse IgG antibodies against CD14, CD16, CD56, CD8 & CD19) were added. The cells were incubated for 20 min at 4ºC, washed with cold isolation buffer (PBS with 0.1%BSA and 2mM EDTA) for 8 minutes at 350Xg and the supernatant was discarded. Then 1ml of Depletion Myone Dynabeads from the Treg isolation kit was added and the cells were incubated for 15 minutes at room temperature with constant agitation. Next, the bead bound cells were resuspended vigorously and bead-free $CD4^+$ fraction was removed by immunomagnetic selection and the $CD4^+$ cells were counted with a haemocytometer. 200µl of CD25 Dynabeads were added to every $15x10^6$ negatively selected CD4 cells and cells were incubated for 30 min at 4ºC with constant rolling. Positive magnetic selection was performed to isolate the bead-bound $CD4^+$ $CD25^+$ fraction. This fraction was used for suppression assay and FoxP3 expression on this $CD4^+$ $CD25^+$ cells population was phenotyped on flow cytometry and more than 90% of these cells are FoxP3 positive regulatory T cells. The $CD4^+$ $CD25^-$ effector cells fraction was kept aside for future functional suppression experiments.

2.2.2 Isolation of monocyte-derived dendritic cells

Monocyte derived dendritic cells (MoDC) from peripheral blood were isolated from buffy coats prepared from whole blood by centrifugation at 900Xg for 20 minutes. 4ml of OptiPrep was added to 10ml of leukocyte rich plasma; LRP (harvested buffy coat in plasma

supernatant) resulting in a density of approximately 1.1g/ml. Two solutions of 1.068g/ml and 1.084g/ml of Optiprep were prepared by mixing OptiPrep and RPMI/10%FCS at one in five and one in four ratios. Next the mixed LRP/OptiPrep was over layered with 7.5ml of the 1.084g/ml solution and 20 ml of the 1.068g/ml solution in 50ml tube and with a final 2ml of RPMI/ 10% FCS layered on top. The 50ml tube was centrifuged at 750Xg for 20minutes without a brake, and the monocytes that floated into the 1.068g/ml layer were collected. These monocytes were cultured for 5 days in dendritic cells (DC) media containing RPMI, 5%FCS and IL-4 with GMCSF. These immature DC were then stimulated with TNF (Peperotec) for 24 hours on day 5 to obtain mature DC. To obtain highly pure dendritic cells, these DC were further selected by using CD11c positive magnetic selection by using Dynal beads (Invitrogen) according to manufacturer instructions. Some of these cells were stimulated with lipopolysaccharide (LPS/1μg/ml; Sigma) and cells were stored in RNA-later (Sigma) for future PCR experiments. Supernatants collected from stimulated and unstimulated DC were stored at -20ºC for ELISA experiments.

2.2.3 Isolation of primary human liver derived cells

This whole project is based on primary human liver derived cells. Therefore, the liver infiltrating lymphocytes (LIL), Liver infiltrating dendritic cells (LIDC), Hepatic Sinusoidal Endothelial Cells (HSEC), and Biliary Epithelial Cells (BEC) were isolated from explanted human liver tissue using a variety of isolation methods (mechanical, enzymatic, immunomagnetic selection) to select for different cell types.

2.2.3.1 Isolation of liver infiltrating lymphocytes & liver infiltrating regulatory T cells

Explanted human liver tissue was chopped into small pieces (5 mm x 5 mm) washed in PBS/10% (v/v) 2%FCS to remove any peripheral blood and resuspended in 200 ml RPMI-1640/10% (v/v) FCS. This suspension was then homogenized for 6 minutes at 260 rpm in a Stomacher® 400 circulator (Seward, Norfolk, UK) before being filtered through a fine (60 μm) nylon mesh. The resulting heterogeneous cell population was reduced to a volume of 80 ml by successive wash steps in RPMI-1640/10% FCS, layered onto Lympholyte (VH Bio) in a 50ml tube (20 ml of cell suspension/30ml of Lympholyte) and centrifuged at 720 g for 25 minutes. Separated liver derived lymphocytes at the interface were aspirated, washed twice more in RPMI-1640/10% FCS and resuspend in culture media containing RPMI-1640/10% FCS supplemented with 2 mM L-glutamine, 60 μg ml-1 benzylpenicillin, 100 μg/ml streptomycin, and 50 ng/ml amphotericin. Liver infiltrating regulatory T cells were then isolated by negative selection of CD4 cells followed by a positive selection of CD25 as described above. Isolated liver infiltrating T_{reg} were cultured in round-bottom 96 well plates with T_{reg} media (see later) and CD3 CD28 beads (2.5μl/10^5 cells) until use in suppression assays. In contrast, chemotaxis assays were performed with freshly isolated liver infiltrating regulatory T cells.

2.2.3.2 Liver derived dendritic cells (LDDC) isolation

Liver derived dendritic cells were isolated from diseased liver using methodology previously established in our laboratory (Lai et al., 2007). Briefly after isolation of liver derived lymphocytes, a further 13.5% Optiprep gradient (Axis Shield) was used to obtain the liver infiltrating dendritic cells and then CD11c positive selection with magnetic beads (Invitrogen) was used as described above to generate a highly purified dendritic cells population.

2.2.3.3 Hepatic sinusoidal endothelial cells isolation

HSEC were isolated from explanted human liver tissue using an established method available in our laboratory (Lalor et al., 2002a). Approximately 150 g of liver tissue was cut into fine pieces and enzymatically digested with Type-1A collagenase (2 mg/ml, Sigma Aldrich, UK) for 30 minutes at 37ºC. Undigested tissue was removed by filtering the suspension through a sterile fine mesh with excess PBS and the cell suspension was then layered onto a 33/77% Percoll gradient (Amersham Biosciences) and centrifuged at 450 g for 25 minutes. The cells layer at the interface were collected, washed and resuspended in PBS. Biliary epithelial cells were removed by indirect immuno-magnetic separation using an antibody raised against the epithelial specific cell-surface glycoprotein HEA-125 (50μg/ml, Progen Biotechnic, Germany) for 30 minutes at 37º followed by incubation with a secondary antibody conjugated to magnetic beads (10μl/ prep, sheep anti-mouse Dynabeads®, Dynal, UK) for 30 min at 4ºC. The remaining suspension was sedimented by centrifugation and sinusoidal endothelial cells isolated by using positive immuno-magnetic selection by incubation with primary antibody raised against CD31 (10 μg/ml, Dynal, UK) for 30 min at 4ºC with occasional agitation to resuspend the beads followed by a positive magnetic selection to select the bead coated sinusoidal cells. The final cells population was resuspended in endothelial media (see later) and cells were cultured in collagen coated T25 flask until a confluent monolayer was obtained.

2.2.3.4 Isolation of biliary epithelial cells

Biliary epithelial cells (BEC) were isolated with the help of Gill Muirhead in our laboratory according to previously described methods used by our group (Afford, 2001). Briefly, liver tissue was finely chopped and digested enzymatically using Collagenase type 1-A (Sigma-Aldrich, UK) for one hour at 37ºC. The digested sample was passed through a sterile metal sieve and the

effluent was centrifuged and the cells pellet was resuspended in PBS with 10 %(v/v) FCS (Sigma) and purified by density gradient centrifugation over 25% metrizamide (Nycomed, UK) at 450Xg for 20 minutes. The cells at the interface between PBS and metrizamide were harvested, washed in PBS and centrifuged 650Xg with brake for 10 minutes to remove the residual metrizamide. BEC were then isolated by positive magnetic selection using antibody raised against the epithelial specific cell-surface glycoprotein HEA-125(10µg/ml, Progen, Germany) followed by adding secondary antibody conjugated to magnetic beads (sheep anti-mouse Dynabeads, Dynal, UK). Subsequently, isolated BEC were plated in rat tail collagen coated 25cm^2 tissue culture flasks (Corning, UK).

2.3 Preparation of media and culture of primary human liver cells

All cell types were cultured on collagen-coated plastic in a humidified incubator at 37ºC with a 5 % CO_2 atmosphere. Cells were maintained in tissue culture grade filtered flasks (Corning Costar, UK)

2.3.1 Preparation of rat tail collagen

Frozen rat tails were washed in PBS and collagen fibres were stripped out mechanically, washed in PBS and weighed. They were then sterilised for 10 minutes in 70 % ethanol (Fisher Scientific, UK) and collagen fibres were dissolved in 4 % acetic acid (1 g of wet collagen per 100 ml 4% acetic acid; BDH, Poole, UK) at 4ºC for 2 days with constant stirring. The resulting solution was first passed through a fine nylon mesh followed by high speed centrifugation (45,000 g for 30 minutes at 4ºC) to remove debris. This purified rat tail collagen (RTC) solution was stored at 4ºC till use for future coating of tissue culture flasks to grow HSEC, BEC and for coating microslides for flow based adhesion assays. Flasks were allowed to air dry in a sterile environment and stored for up to 4 weeks before use and microslides were left air dry for 2 hours.

2.3.2 HSEC culture

Isolated HSEC were cultured in complete medium consisting of basal endothelial medium (EBM; Invitrogen, UK) supplemented with 10% heat-inactivated normal human AB serum (HD Supplies, Bucks, UK), 10ng/ml hepatocyte growth factor (HGF), 10 ng/ml vascular endothelial growth factor (VEGF; both from Peprotec, UK), 2 mM L-glutamine, 60μg/ml benzylpenicillin and 100 μg/ml streptomycin. When confluent, monolayer's were dissociated

with try sin EDTA (0.05% in 0.53 mom EDTA; Nitrogen) and sub-cultured at 1:3 ratios. Briefly. HSEC media was removed and monolayers were washed with PBS before addition of 5 ml trypsin EDTA. Sinusoidal endothelial cells were then incubated for 1–2 minutes to allow dissociation before being collected into PBS/10% FCS to stop any remaining trypsin activity. Following centrifugation at 550Xg for 5 minutes, cells were resuspended in new media and divided between collagen coated T-75 flasks. Cells were used for flow based adhesion assay between passage 2-4 to ensure preservation of integrin ligand and chemokine expression.

2.3.3 Regulatory T cell culture

Regulatory T cells were cultured in U-bottom 96 well plates (Becton&Dickinson, 353077) with T_{reg} media containing RPMI, 10% human serum, L- glutamine, 500 IU/ml of IL-2 and CD3 CD28 beads (Dynal, Invitrogen) were added to culture for T cell receptor (TCR) and co-stimulatory signalling. Cells were fed on alternate days and transferred to 24 well plates after 2 weeks. Purity of cultures (typically more than 97%) was determined by flow cytometry.

Figure 2-1 Culture of isolated Regulatory T cells

Regulatory T cells were cultured in IL-2 containing T_{reg} media (RPMI+10%Human serum+glutamine) in 24 well plate with CD3 CD28 beads for T cell receptor stimulation and co-stimulation. Proliferating T_{reg} clusters attached to CD3 CD28 beads were shown with black arrow. The beads can be observed as black dots.

2.3.4 DC culture

Isolated dendritic cells were cultured in DC media containing RPMI, 5%FCS and IL-4(100U/ml) with GCSF (100U/ml). The immature DCs were stimulated with TNF-α for 24 hours on day 5 of culture to obtain mature DC.

2.3.5 BEC culture

BEC were cultured in Dulbecco's Modified Eagle medium, Hams F12 (50%(v/v); Invitrogen,UK) supplemented with 10% heat-inactivated human AB serum, 10ng/ml epidermal growth factor (EGF; R&D Systems, UK), 10ng/ml HGF, 10ng/ml VEGF, 2mg/ml hydrocortisone, 10ng/ml choleratoxin, 0.124U/ml insulin, 2Nm tri-iodo-thyronine, 2mM L-glutamine, 60μg/ml benzylpenicillin and 100μg/ml streptomycin (all reagents from Sigma). BEC

cell phenotype was confirmed by high level expression of cytokeratin-19. Cells were cultured to confluence and media was changed every other day or as required. Cells between passage 2-4 were used for flow cytometry, ELISA, PCR and chemotaxis experiments.

2.4 Suppression of proliferation assay

RPMI 1640 medium with L-glutamine (Bio Whittaker, Verviers, Belgium) supplemented with 10% human serum (Department of Immunohematology and Blood bank, Leiden University Medical Centre, Leiden, The Netherlands), 100 g/ml penicillin, and 100 g/ml streptomycin (Gibco, Paisley, UK) was used for T regulatory cell culture.

Allogeneic irradiated dendritic cells (1:100 ratios) or 5 μl/ml anti-human CD3/CD28 beads (1:1 ratio, Dynal Biotech) were mixed with autologous $CD4^+CD25^-$ responder T cells in 96-well round-bottom plates (Nune). All cells were cultured in triplicate in a final volume of 200μl of RPMI 1640 medium with L-glutamine, 100U/ml penicillin, 100μl/ml of streptomycin and 10% Human AB serum without IL-2. Suppression of proliferation was determined by adding reducing numbers of liver derived $CD4^+CD25^+$ Treg cells and autologous $CD4^+CD25^-$ responder cells (1:1 to 1:16 ratio). At day 5, cultures were pulsed with [3H] thymidine (Amersham, Little Chalfont, UK) for last 16 hours and plate were harvested. Proliferation was assessed by measuring radioactivity with a liquid scintillation counter (Wallac). Cultures were performed in triplicate and results for radioactive thymidine update are reported in ccpm.

2.5 Cytokines treatment of cultured cells

TNF-α & IFN-γ in combination were used to stimulate cultured HSEC monolayers and other cells to mimic the condition in the inflamed liver environment. All stimulations were

performed in culture media relevant to the cells being treated with the exception that human serum was removed as a possible source of endogenous chemokine contamination. Cells were cultured to confluence as described previously in RTC coated T75 flasks. For test samples all culture medium was removed and replaced with unstimulated media (control) or stimulated media containing TNF-α and IFN-γ. Cytokine concentrations were chosen based upon previous experience in our group and are shown in Table.2.1

Supernatants from BEC and liver derived dendritic cells were collected for sandwich ELISA. BEC were grown to confluence in collagen coated 24 well plates and either left unstimulated (basal conditions) or stimulated with TNF-α, IFNγ, IL-1β and IL-17 prior to collection of supernatant for detection of cytokine and chemokine production. Human liver derived dendritic cells were cultured in 48 well plates and stimulated with LPS 1μg/ml. Following 24 hours stimulation with the above cytokines, supernatant were collected for ELISA and cells were harvested into RNA-Later for future PCR experiments.

Cytokines/ reagents used for stimulation	Final Concentration	Source
TNF-α	**10 ng/ml**	Pepro Tech
IFN-γ	**10 ng/ml**	Pepro Tech
IL-1β	**10 ng/ml**	Pepro Tech
IL-6	**10 ng/ml**	Pepro Tech
LPS	**1 μg/ml**	Sigma Aldrich Ltd.
IL-17	**10 ng/ml**	Pepro Tech

Table 2-1 Reagents used and their concentration for stimulation of cultured HSEC, BEC and DC.

2.6 Histology

Tissue was processed from normal and diseased liver into 1cm^3 blocks and snap- frozen in liquid nitrogen before 5µm cryostat sections were cut using a BRIGHT(model OTF) cryostat for immunohistochemistry and immunofluorescence. These sections were mounted onto poly-lysine coated microscope slides (BDH) and then fixed for 5 minutes in acetone (Fisher Scientific, UK) before storage at 20ºC until staining. Concentrations of primary and secondary antibodies used for staining are detailed in Tables 2.2 and 2.3

2.6.1 Immunohistochemistry

2.6.1.1 Immunohistochemistry on frozen tissue sections

Tissue sections were warmed up to room temperature for 30 minutes and they were re-fixed in acetone for 5 minutes. All staining was carried out in humidified chamber at room temperature to prevent evaporation. Endogenous peroxidase was blocked with 0.3% Hydrogen peroxide in methanol for 10 minutes. Subsequently, the sections were incubated with 10% normal horse serum in Tris buffered saline (TBS, pH 7.6, 0.1% Tween) for 30 minutes. The "Vector Elite" Avidin Biotin staining set was used for amplification and detection steps. Briefly, sections were incubated with an avidin/biotin blocking kit (Vector lab, according to instruction) for 15 min and washed in TBS/ 0.1% Tween buffer. Slides were incubated with mouse anti-human monoclonal primary antibody FoxP3(Abcam) and mouse anti-human monoclonal CD3 (DAKO) in blocking buffer for 60 minutes at 37°C in a humidified container. Control sections were incubated with a relevant isotype-matched control antibody (IgG1, DAKO). Signal amplification was then performed with a biotin- conjugated goat anti-mouse secondary antibody (Vector ABC Elite Kit) in blocking buffer according to manufacturer's instructions. Antibody

localisation was visualised with 3-amino-9-ethylcarbazole (AEC) or ImmPACT DAB (Both from Vector Lab). The sections were washed in distilled water, counterstained with haematoxylin, and then slides were mounted in aqueous mount. Positive staining was identified by the presence of a red colour/brown colour reaction product. All washes were performed with Tris-buffered saline, pH 7.6.

2.6.1.2 Immunohistochemical staining of paraffin-embedded liver tissue

2.6.1.2.1 Single colour immunohistochemical staining

Paraffin-embedded, formalin-fixed liver tissue was cut into 5-μm sections and placed on poly-lysine-coated slides. Slides were initially de-waxed with Xylene for 5 minutes. The sections were then incubated through different strengths of IMS (100%, 96%, 70%, 50%) for 5 minutes each and then distilled water for 3 minutes. Endogenous peroxidase was blocked with 0.3% H_2O_2 for 25 minutes and then slides were washed in TBS/ 0.1%Tween (500ml TBS pH 7.6: 500 μl of Tween) for 5 minutes. Antigen retrieval was achieved via microwave cooking for 20 min in EDTA buffer (EDTA buffer pH 8 was pre-heated on full power in the microwave using 10% EDTA; 70ml EDTA: 630ml distilled water) for 5 minutes. The slides were then placed into a plastic rack, placed in the pre-heated EDTA buffer and microwave for a further 15 minutes at full power. Then the slides were allowed to stand for 10 minutes and cold water was added until room temperature was reached. Slides were then washed in TBS/Tween for 5 minutes and incubated in 2% casein blocking solution (Vector) for 20 minutes. Slides were then incubated with primary antibodies (CD3, FoxP3, IL17, phosphoSTAT-5, HEA-125) or relevant control in blocking buffer for 1 hour in a humidified container. Then slides were then washed in TBS/Tween for 5 minutes and incubated in Vector Immpress secondary antibody reagent for 30 minutes (Vector Goat/Mouse/Rabbit Impress Kit, used according to primary antibody species).

After a further wash, Vector ImmPACT DAB substrate (SK4105, used according to manufacturers recommendations) was applied for 2.5 minutes or VIP substrate (Vector Lab) for 4 minutes or NovaRed substrate (Vector Lab) for 8 minutes was used to visualize the staining. Slides were then washed in tap water for 5 minutes and counter-stained with haematoxylin. Finally slides were dehydrated in dilutions of IMS as before for 5 minutes each and then placed in Xylene for 5 minutes and mounted using DPX. For the enumeration of positive lymphocytes, CD3 lymphocytes were counted in three low-powered fields (lpf; x100). High-powered fields (hpf; x400) were used for counting $FoxP3^{+}$, phosphoSTAT-5 and, IL-17 positive cells because of the lower abundance of these cells in the liver. To investigate frequency estimate of FoxP3 vs CD3 or IL-17 vs CD3, cells were counted five high power fields and average was calculated.

2.6.1.2.2 Dual colour immunohistochemical staining

For double immunostaining, two primary antibodies raised in two different and non-cross-reacting species, or primary antibodies of different isotypes were used. Formalin fixed paraffin embedded tissue sections were dewaxed, rehydrated, endogenous peroxidase blocked, antigen retrieval with EDTA buffer and incubated with 2% casein as described above. Primary mouse monoclonal antibodies, (FoxP3, IL-17 or CD11c) were applied for 30 minutes. Control sections were incubated with same isotype control antibodies at matched concentrations. After a TBS/Tween wash, secondary reagent (ImmPRESS Peroxidase) anti-mouse or anti-goat reagent (Vector Laboratories, UK), was applied for 30 minutes. After 2 further TBS/ 0.1% Tween washes the reaction was visualized with ImmPACT DAB (Vector Laboratories, UK) for 2 minutes. Following a wash in water and TBS/Tween, sections were again incubated with 2%casein for 10 minutes. Then a second round of primary antibodies CD3, HEA-125, CCL22 or MDC, was applied for an hour. After one more wash ImmPRESS universal reagent (Vector), was applied for 30 minutes. After 2 further washes the reaction was visualized with Vector

NovaRed/VIP for 5-8 minutes and, sections were counterstained in heamatoxylin, dehydrated through graded alcohol, placed into xylene and finally mounted in DPX.

Dual staining for IL17 and HEA was done by using Goat ImmPRESS Kit and Mouse ImmPRESS kit; dual staining for CD11c & CCL22 was done by using Universal Mouse & Rabbit ImmPRESS kit. DAB chromogen (2 minutes) was used for detection of first antigen and VIP (10 minutes)/ NovaRed (8 minutes) were used for detection of second antigen. All substrates are purchased from Vector Laboratories.

2.6.2 Immunofluorescent staining

Here, frozen sections were stained as described above but incubated with biotin-conjugated secondary antibody in TBS pH 7.4 for 60 min at 37ºC. Next, strepavidin-FITC mAb (DAKO) was added in TBS pH 7.4 for 60 min at 37ºC. All washing steps in the procedure were performed in TBS containing 2% human serum and 0.1% sodium azide and slides were protected from light throughout by covering the humidified chamber. Slides were mounted in DAKO immunofluorescent mount to retard fading. Immunofluorescent images were captured on a Zeiss Axioscope microscope using AxioVision software (Zeiss, Germany).

2.6.3 Confocal microscopy

2.6.3.1 Detection of regulatory T cells in human liver tissue

Frozen human liver tissue sections were used for this experiments. Tissue sections were deforzed for 30 minutes before staining and rehydrate in Phosphate buffered solution for 10 minutes to rehydrate the slides. PBS 1% BSA was used as a staining buffer. Then the slides were

incubated with blocking buffer (10% horse serum in PBS 1% BSA) for 30 minutes to prevent background staining. Primary antibodies against FoxP3 (IgG1, 1:50), CD3 (IgG2a, 1:100) and CD4 (IgG2b, 1:50) were applied to slides in the staining buffer containing human serum and incubate for an hour. FoxP3 primary antibodies were amplified with goat-anti mouse IgG1 FITC (1:100), followed by rabbit-anti-FITC (1:200) and finally with goat-anti-Rabbit FITC (1:100). All incubation steps were 30 minutes each and all washes in between were done for 10 minutes each in phosphate buffer saline. CD4 antibody was incubated with anti-IgG2b-biotin-conjugated secondary antibody (1:100) in staining buffer containing human serum for 30 min at 37ºC and strepavidin 647(1:400) was applied for amplification. CD3 primary antibody was detected with anti-IgG2a CY-3. All washed were done in a tin foiled covered humidified chamber to protect from light. Then slides were dipped into DAPI (excite at 360 and emit at 460) for 20 seconds followed by 3 wash in PBS and mounted. The edges of slides were cleaned and nail varnish was applied to the edges to secure the cover slip on the slide and dried for 1 hour in light protected box and image taken with confocal microscope.

2.6.3.2 IL-17 secreting lymphocytes detection in human liver tissue

Staining on paraffin embedded human liver tissue sections were used for these experiments. Slides were deparaffinise in Xylene for 5minutes and then rehydrated in Methanol in 5 minutes twice and then washed in distilled water for 3minutes followed by washing in TBS/Tween. Antigen retrieval was done in EDTA buffer for 20 minutes as described above. Slides were then incubated in horse serum for 30 minutes. Without washing, goat polyclonal IL17 (1:100; 1 μg/ml) in staining buffer was incubated for an hour followed by 10 minutes PBS wash twice was done. Then slides were incubated in donkey-anti-goat FITC (1:100) in staining buffer for 30 minutes followed by another wash. Then slides were incubated in rabbit-anti-FITC (1:200)

in staining buffer for 30 minutes and washed twice. Finally slides were incubated in quaternary antibody goat anti-rabbit FITC (1:100) for half an hour followed by two more wash in PBS. Then slides were dipped into DAPI (excite at 360 and emit at 460) for 20 seconds followed by 3 wash in PBS and mounted. The edges of slides were cleaned and nail varnish was applied to keep then in place and dried for 1 hour in light protected box as described above and images were taken with confocal microscope.

2.6.3.3 Detection of T_{reg}, CD4 and CD8 & dendritic cells; Th17 and Tc17; IL-17 cells and Treg in inflamed human liver

Staining protocol was similar to described above. FoxP3 (IgG1) was visualized with anti-IgG1 biotin (1:100) and strepavidin alexa fluor 555(1:1000). Dendritic cells, CD11c (rabbit monoclonal) was visualized with anti-IgG1FITC (1:100) followed by rabbit anti-FITC (1:200) and finally with goat anti rabbit FITC (1:100). Directly conjugated alexa 647 was used for CD8 cells staining.

Staining for Th17/Tc17 was done as follows; IL-17 goat polyclonal antibody was detected by donkey anti-goat FITC (1:100), then with rabbit antiFITC (1:200) and finally with goat anti-rabbit FITC (1:100). CD4 IgG2b antibody was detected with anti-IgG2b biotin-conjugated secondary antibody(1:100) in staining buffer containing human serum for 30 min at 37ºC and strepavidin 555(1:1000) was applied for amplification. IgG1 CD8 alexa 647 was used for CD8 staining.

Staining for IL17 and FoxP3 was done as follow; IL-17 goat polyclonal antibody was detected by donkey anti-goat FITC (1:100), then with rabbit antiFITC (1:200) and finally with goat anti-rabbit FITC (1:100). FoxP3 were visualized with anti-IgG1 biotin (1:100) and strepavidin alexa fluor 555 (1:1000).

Clone	Antigen	Concentration/ working dilution	Source
F 7.2.38	Mouse anti-human CD3 IgG1 (F,P,C)	270 μg /ml	Dako
PS1	Mouse mAb to CD3	1:100 dilution	Abcam
X 0931	Mouse IgG_1	100 μg/ml	Dako
N1698	Universal mouse negative control	200 μl	Dako
AF 317-NA	Goat polyclonal IL-17	0.75 μg/ml	R&D
236A/E7	Mouse anti-human Foxp3 IgG1 (F,P,C)	10 μg/ml(P,F), 20 μg/ml(C)	Abcam
4B12	Mouse anti-human CD4(P) IgG1	0.75 μg/ml	Dako
OKT4	Mouse anti-human CD4 IgG 2b (C)	50 μg/ml	eBioscence
555391	Mouse mAb to CD11c	1:50 dilution	BD
Ab45977	Rabbit monoclonal to MPO	1:50 dilution	Abcam
EP1347Y	Rabbit-antihuman CD11c (F,C)	1:100 dilution	Abcam
9359	Rabbit mAb to Stat5	1:50 dilution	Cell signalling
E208	Rabbit mAb to PhosphoStat5 (Y694)(P)	1:50 dilution	Abcam
144B	Mouse mAb to CD8(P,C)	1:100 dilution	Abcam
OKT3	Mouse anti-human CD3 IgG2a (P,C)	1:50 dilution	eBioscence
MAB 336	Mouse anti-human MDC	1:50 dilution	R&D
SC-12285	Goat polyclonal IgG MDC (C-18)	1:50 dilution	Santa-Cruz
Ab53002	Rabbit polyclonal to MDC	1:50 dilution	Abcam

Table 2-2 Primary Antibodies used for immunohistochemistry

Frozen (F), Paraffin (P), and confocal microscopy(C)

Secondary, Tertiary & Quaternary	Working dilution	Source
Goat anti-mouse IgG1 FITC	1:100 dilution	Southern Biotech (SB)
Rabbit-anti FITC	1:200 dilution	Sigma
Goat-anti Rabbit FITC	1:100 dilution	SB
Anit-IgG2b biotinylated	1:100 dilution	SB
Strepavidin 647	1:400 dilution	Invitrogen
Strepavidin 555	1:1000 dilution	Invitrogen
Anti –IgG2a Cy-3/TRITC	1:50 dilution	SB
Donkey anti-goat FITC	1:100 dilution	SB

Table 2-3 Antibodies used for immunofluorescence and confocal microscopy.

The following staining reagents were used for detection in immunohistochemistry;

Vector Secondary ABC (Avidin-Biotin) & ImmPRESS (Peroxidase)

Vector stain Elite ABC, PK-6102, Vector Laboratories

Avidin/biotin blocking (Vector Laboratories, SP-2001)

ImmPRESS Reagent, Universal Anti-mouse/rabbit Ig, MP – 7500

ImmPRESS Reagent, Anti Goat IgG; MP7405

ImmPRESS Reagent, Anti-Mouse IgG; MP7402

Control:

Dakocytomation; N-Universal; Negative Control; Mouse; Ref: N1698

Goat IgG, Vector Labs

Rabbit IgG, Dako

Substrate

ImmPACT DAB; SK 4105; Vector Labs

VIP; SK-4600; Vector Labs

Nova Red; SK4800; Vector Labs

NICKEL DAB; SK-4100; Vector Labs

2.7 Multi-colour flow cytometry

Flow cytometry provides a mechanism to detect either cell surface or intracellular (cytokine or transcription factors) antigen in individual cells. The flow cytometer passes single cells in suspension through a chamber where they are subjected to a laser light source, the scattering of which is dependent upon the size and granularity of the cells that pass through. Use of fluorochrome-conjugated antibodies result in the laser light being absorbed then emitted at a longer wavelength allowing cells to be identified.

Due to the necessity for staining multiple surface receptors and intra cellular transcription factors to identify Regulatory T cells and Th17 cells, I used the DAKO Cyan 9 colour cytometer for immunophenotyping experiments. The cytometer uses three argon lasers with an emission wavelength of, 405 nm (pacific blue and pacific orange), 488 nm (FITC, PE, PE TxRed, PE Cy5, and PE Cy7) and 635 nm (APC/Alexa fluor 647 and APC Cy7). The use of multi colour cytometer requires careful selection of flurochromes with variable emission spectra, such that interference does not occur. However, some flurochrome overlap is unavoidable and this is compensated for during the cytometer set up. By using Summit software 4.3 any regions of emission spectra that can be superimposed on one another are subtracted from the signal collected by the cytometer by identifying a sub-population of cells labelled with each of the fluorochromes used. I chose to use CD4 antibodies (coupled to different flurochromes) for compensation. Manual compensation was applied using median channel fluorescence intensity of both positive and negative populations to compensate for fluorochrome spill over into other channels. (Figure.2.2)

For the analysis of the percentage of regulatory T cells in freshly isolated LIL and when possible matched PBL, phenotypic markers (CD4, CD25, CD127, FoxP3), chemokine receptors,

integrins, and selectin expression were determined and data were analysed using Summit v4.3 software (Dako Cytomation). The complexity of receptor expression on Regulatory T lymphocytes required the use of four different fluorochromes (three for surface receptors and an intracellular transcription factor) to accurately dissect the phenotype. Antibodies against chemokine receptors were used to detect the receptors expressed by Regulatory T cells, Th17 and Tc17 populations.

Antibodies conjugated to different flurochromes raised against relevant cell surface receptors, chemokine receptors, integrins, intracellular cytokines and transcription factors were used at optimal dilutions. The lymphocyte population was gated using forward and side scatter parameters and then then cells were regated according to expression of CD3 (PE-Cy7)to exclude debris.

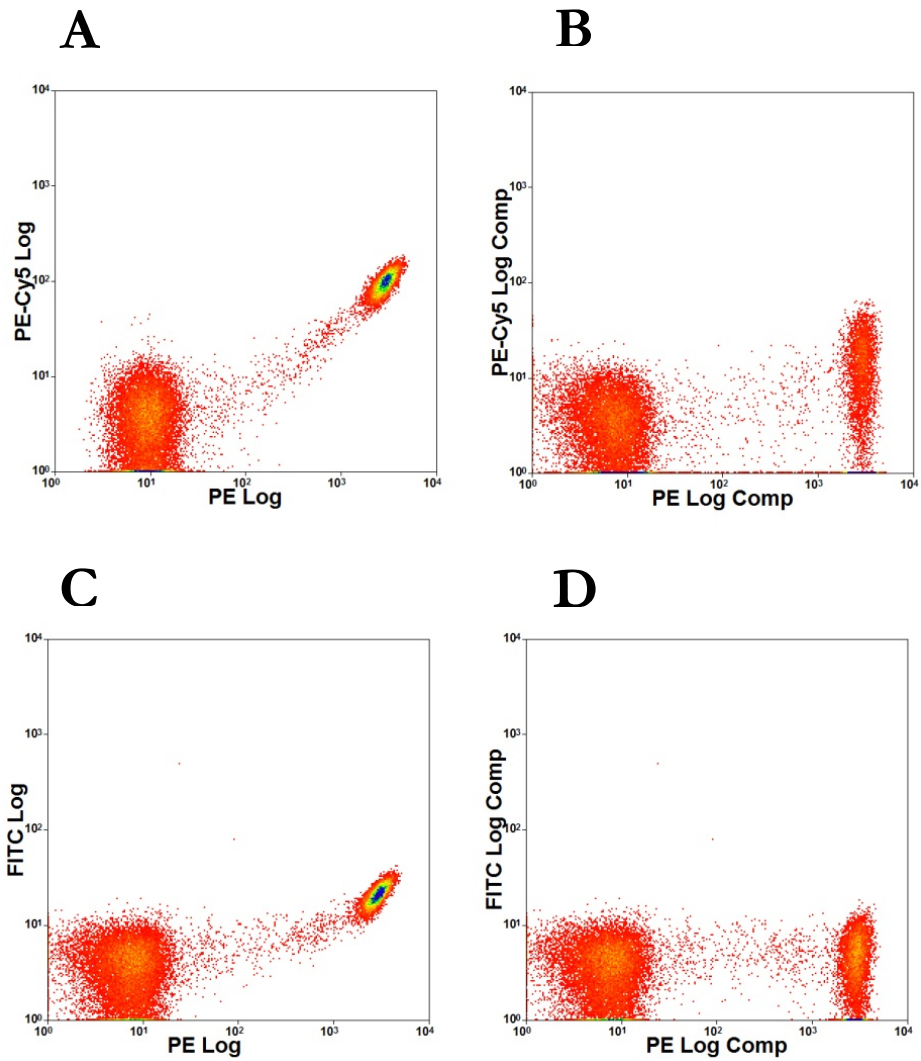

Figure 2-2 Example of a typical compensation for flow cytometry.

Compensation for different flurochromes spillover was performed manually by adjusting the median channel fluorescence intensity in both positive and negative population. Uncompensated samples are shown in A&C and compensated samples are shown in B&D.

Antibodies and chemokine receptors used fot phenotyping of both liver derived and peripheral blood Regulatory T cells an IL-17 secreting cells experiments are described in the Table 2.4, 2.5 and 2.6.

Clone/Name	Antigen/Conjugate	Concentration	Source
RPA-T4	CD4- Pacific Blue	10µg ml^{-1}	BD Pharmingen
MOPC-21	Mouse IgG_1 Pacific Blue	10µg ml^{-1}	BD Pharmingen
IM 2646	CD25 PE Cy-5	1:5 dilution	Beckman Coulter
A 09148	Mouse IgG_{2a} PE-Cy5	1:5 dilution	Beckman Coulter
UCHT1	CD3 PE-Cy7	1:5 dilution	eBioscience
P3	PECy7 IgG1	1:20 dilution	eBioscience
UCHL1	CD45 RO PE-Cy5	1:5 dilution	BD Pharmingen
FAB14001A	IL-23R	1:10 dilution	R&D systems
HCD127	CD127-Alexa 488	1:20 dilution	Biolegend
MOPC-21	Mouse IgG_1 Alexa 488	1:20 dilution	Biolegend
O323	APC/Cy7- CD27	1:5 dilution	Biolegend
MOPC-21	Mouse IgG_1 APC/Cy7	1:5 dilution	Biolegend
BNI3	CD152 PE-Cy5	1:5 dilution	BD Pharmingen
G155-178	Mouse IgG_{2a} PE-Cy5	1:5 dilution	BD Pharmingen
PCH101	FoxP3-PE	1:5 dilution	eBioscience
Ebr2A	Rat IgG_{2a} PE	1:5 dilution	eBioscience
AFKJS-9	ROR -γt	1:100 dilution	eBioscience
eBio4B10	Alexa Fluor 647 T-bet	10µl/ml	eBioscience
51-4714	Alexa Fluor 647 IgG1	10µl/ml	eBioscience
A07748	CD3 PE TexRed	1:500	Beckman Coulter
SK3/ 55852	CD4 PE-Cy7	1:500	BD Pharmingen
PN IM3450	CD161 APC	1:20	Beckman Coulter
130-092-678	CD161 APC	1:500	Miltenyi

Clone/Name	Antigen/Conjugate	Concentration	Source
IC0041A	APC IgG control	1:50	R&D systems
O323	APCCy7 CD27	1:5	Biolegend
MOPC-21	APCCy7 MouseIgG1	1:5	Biolegend
eBioA1	PE conjugated CD39	1:20	eBioscience
559562	CCR6 PE	1:100	BD system
EBioJ105	PE conjugated CD279	1:5	eBiosceicne
556577	FITC conjugated Perforin	1:5	BD
558132	FITC conjugated GranzymeB	1:5	BD
A07795	Mouse IgG1 FITC	1:10	Beckman& Coulter
FAB14001A	IL-23R APC	1:10	R&D
OKT4	Alexa Fluor 647 CD4	1:5	eBioscience
P3	Alexa Fluor 647 IgG1	0.2mg/ml	eBioscience
555347	CD4 PE	1:5	BD
555345	CD4 FITC	1:5	BD
MHCD0818	CD8 PECy5.5	1:400	Invitrogen
MH9D1	IL-9 PE	1:5	eBioscience
550259	CD103 FITC	1:5	BD
550260	CD103PE	1:5	BD
34058	CD56 FITC	1:5	BD

Table 2-4 Antibodies used for phenotyping of T_{reg} and IL-17 secreting cells.

Clone/Catalog#	Antigen/Conjugate	Dilution factors	Source
IC002P	Mouse anti-human IgG1 PE	1:10 dilution	R&D systems
IC003P	Mouse anti-human IgG2a PE	1:10 dilution	R&D systems
IC004P	Mouse anti-human IgG2b PE	1:10 dilution	R&D systems
53504/FAB145P	CCR1	1:10 dilution	R&D systems
48607 /FAB151P	CCR2	1:10 dilution	R&D systems
61828 /FAB 155P	CCR3	1:10 dilution	R&D systems
205410 /FAB1567P	CCR4	1:10 dilution	R&D systems
CTC5 /FAB1802P	CCR5	1:10 dilution	R&D systems
53103 /FAB 195P	CCR6	1:10 dilution	R&D systems
150503 /FAB197P	CCR7	1:10 dilution	R&D systems
191704 /FAB1429P	CCR8	1:10 dilution	R&D systems
112509 /FAB179P	CCR9	1:10 dilution	R&D systems
FAB3478P	CCR10	1:10 dilution	R&D systems
42705 /FAB330P	CXCR1	1:10 dilution	R&D systems
48311 /FAB 331P	CXCR2	1:10 dilution	R&D systems
49801 /FAB160P	CXCR3	1:10 dilution	R&D systems
12G5 /FAB170P	CXCR4	1:10 dilution	R&D systems
51505 /FAB190P	CXCR5	1:10 dilution	R&D systems
56811 /FAB699P	CXCR6	1:10 dilution	R&D systems

Table 2-5 PE conjugated chemokine receptor antibodies used for flow cytometry.

Clone/Catalog#	Antigen/Conjugate	Dilution factors	Source
IC002F	Mouse anti-human IgG1 FITC	1:10 dilution	R&D systems
IC003F	Mouse anti-human IgG2a FITC	1:10 dilution	R&D systems
IC004F	Mouse anti-human IgG2b FITC	1:10 dilution	R&D systems
FAB155F	CCR3	1:10 dilution	R&D systems
FAB1567F	CCR4	1:10 dilution	R&D systems
FAB1802F	CCR5	1:10 dilution	R&D systems
FAB195F	CCR6	1:10 dilution	R&D systems
FAB197F	CCR7	1:10 dilution	R&D systems
FAB1429F	CCR8	1:10 dilution	R&D systems
FAB179F	CCR9	1:10 dilution	R&D systems
FAB330P	CXCR1	1:10 dilution	R&D systems
FAB 331F	CXCR2	1:10 dilution	R&D systems
FAB160F	CXCR3	1:10 dilution	R&D systems
FAB170F	CXCR4	1:10 dilution	R&D systems
FAB190F	CXCR5	1:10 dilution	R&D systems

Table 2-6 FITC conjugated chemokine receptor antibodies used for flow cytometry.

2.7.1 Staining for cell surface markers

Staining for T_{reg} surface markers such as CD4, CD25, CD127, homing receptors such as chemokine receptors, integrins and selectin and isotype control antibodies were done as normal surface staining protocol. Briefly, cells were resuspended to a count of 1 x 10^{6}/ml in staining buffer (500ml PBS-0.1% BSA (v/v), 0.5 mM MgCl2, 1mM CaCl2) and divided into 100 µl aliquots. Antibodies conjugated to FITC (fluorescein-isothiocyante), PE (R-pyhcoerythrin), PE-Cy5 (R-phycoerythrin: Cyanine-5), PE-Cy7 (phycoerythrin: Cyanine-7), PB (pacific blue), APC (allophycocyanin), APC-Cy7 (Allophycocyanin Cyanine-7) raised against relevant cell surface receptors were used at optimal dilutions (Table 2.4). Cells were incubated for 30 minutes with individual conjugated primary mouse anti-human antibodies at 4ºC in the dark before being washed twice with flow cytometry staining buffer (PBS + 5% FCS). Supernatant was discarded and stained cells pellet was vortexed gently and these cells are ready for phenotypic analysis or to proceed to intra-cellular cytokine or transcription factor staining.

2.7.2 Staining for intracellular cytokine and transcription factor for cytometric analysis

Following surface staining, in order to stain intra-cellular cytokines like TNF-α or IL-17 or transcription factors such as FoxP3 and T-bet, cells were treated using fixation/ permeablization buffer (eBioscience Cat.00-5523). Briefly, 1ml of 1% paraformaldehyde (eBioscience 1:4 ratio of fixation concentrate and diluents from the kit) was added to each FACS tube and cells were incubated at room temperature for 20 minutes followed by centrifugation at 400Xg for 5 minutes. Cells were then permeabilized with freshly prepared 0.1% saponin in permeabilization buffer (10X permeablization buffer from the kit) and incubated for 20 min followed by centrifugation. Cells were then blocked with 2% rat or mouse serum (dependant on

antibody used) for 10 minute before application of flurochrome conjugated antibodies or isotype controls.

Prior to detection of intra-cellular cytokines such as IFN-γ, TNF-α, IL-22 and IL-17 in cultured lymphocytes and liver infiltrating lymphocytes, cells were re-stimulated with Ionomycin (500ng/ml) and PdBu (phorbol-12-13-dibutyrate; Sigma-Aldrich, 50nM) or PMA (Phorbol 12-Myristate 13-Acetate, 50ng/ml) for 5 hours at 37ºC. Intracellular cytokine secretion from the Golgi complex was blocked by using a "Golgi block" such as Monensin or Brefeldin (Brefeldin A, 1000ng/ml or Monensin 1- 3 μM) for the last 2 hours of cell stimulation. Cells were washed, incubated with appropriate intracellular cytokine antibodies or control antibodies. Then permeablization buffer was added and cells were incubated in 4ºC for 20 min in the dark before a final wash. Table 2.7 showed the intra-cellular cytokines used for the experiments.

Clone	Antigen/Conjugate	Concentration	Source
340449	IFN- FITC	1:10	BD
MHCIFG05	IFN- APC	1:5	Caltag
64CAP/DEC	IL-17 PE	1:5	eBioscience
51-7229-42	IL-22 Alexa Fluor 647	1:5	eBioscience
IC 7821 P	IL-22 PE	1:5	R&D
554512	TNF-α FITC	1:50	BD

Table 2-7 Intracellular cytokine antibodies used for flow cytometry.

2.8 Western blotting

Fresh normal and disease liver tissue samples were mechanically homogenized in lysis RIPA buffer containing protease inhibitor cocktail (Sigma). The protein concentration of tissue lysates was determined using Biuret reaction. Dilution of BSA (sigma) was used as protein standard. The cell lysates were agitated at 4ºC for 2 to 3 hours and stored at - 20ºC for future use. The lysates were diluted with PBS and SDS-PAGE buffer to protein concentrations prior to loading onto the gels and signals were normalized using beta actin expression. The composition of the stacking and resolving gels are shown in the Table 2.8.

Percent Gel	**dd H_2O**	**30% Degassed Acrylamide/Bis**	**Gel buffer**	**10% SDS**
5%	5.7ml	1.7ml	2.5ml (Stacking)	0.1ml
15%	2.4ml	5ml	2.5ml (Resolving)	0.1ml

Table 2-8 Reagents used to generate SDS-PAGE gels.

2.8.1 SDS-PAGE (SDS Poly Acrylamide Gel Electrophoresis)

Proteins samples from diseased and normal livers were separated by electrophoresis in acrylamide gels made up of a 5% loading gel/ stacking gel over a 15% resolving gel cast in the BioRad Mini Trans Blot cell system. Lysates were diluted in buffer with β-mercaptoethanol, boiled at 100ºC for 10 minutes and centrifuged before loading into wells The lysates were loaded at (20µl/lane) and a pre-stained rainbow molecular weight ladder (Amersham, GE Healthcare) was used as a molecular weight marker (CCL17=8kDa). Protein electrophoresis was performed at 200 V for 30 minutes in 1 x electrophoresis buffer to separate the proteins in the lysates.

2.8.2 Western Blot Transfer

Protein products in the gel were then transferred onto Hybond-ECL nitrocellulose membranes (Amersham) in 1x transfer buffer running at 100 V for 60min with constant stirring. Appearance of the rainbows markers on the membrane confirmed that protein transfer was complete and then the membrane was stained with Ponceau S solution (Sigma) for 5 min followed by rinsing in water to visualise the transfer.

2.8.3 Western Blot Development

Once the transfer was complete, the nitrocellulose membrane were blocked with 5% skimmed milk in PBS + 0.02% Tween-20 for an hour at room temperature and subsequently incubated with goat anti-human CCL17 antibody (R&D systems, Table 2.9) for 1 hour. The blots were then washed in PBS Tween solution for an hour and incubated with a rabbit anti-goat HRP-conjugated secondary antibody (1 in 2500, Dako) for an hour at RT to detect the primary antibody. The membranes were then repeatedly washed in 1 x PBS Tween and immunoreactive bands were detected using the ECL detection system (Amersham) for a minute followed by exposure to a chemosensitive film (Amersham Biosciences) and development using a Kodak X-Omat 1000 processor.

Buffer	Concentration&Volume	Composition
Lysis Buffer in dH2O		Glycerol 1M TrisHCL pH 6.8 Sodium dodecyl sulphate (SDS) Bromophenol Blue β-mercaptoethanol
Resolving gel buffer (pH8.8)	1.5M	Tris based
Stacking gel buffer (pH 6.8)	0.5M	Tris based
10x Electrophoresis Buffer (per 1L)		Glycine (144g); Tris (30.3g) Sodium dodecyl sulphate (SDS) 10g
Transfer Buffer (per 2 Litre)		Glycine (28.8g); Tris(6.0g) Sodium dodecyl sulphate (SDS)1g 400ml MeOH
Blocking buffer (25 ml per membrane)		5% non-fat milk (Marvel) PBS +0.02%Tween-20 (PBS-T)
5X SDS-PAGE sample buffer	200 mM	Tris pH 6.8 20% Glycerol 10% SDS 0.05% bromophenol blue 10 mM β-ME
15 % SDS PAGE Resolving/ Separating gel	2.4ml 2.5ml 5.0ml 100µl	ddH2O Resolving gel buffer Acrylamide/bis-acrylamide 30% solution 10% w/v SDS
5% SDS PAGE Stacking/ Loading gel	5.7ml 2.5ml 1.7ml 100µl	ddH2O Stacking gel buffer Acrylamide/bis-acrylamide 30% solution 10% w/v SDS
Human CCL17/TARC Polyclonal Ab, Goat IgG (R&D systems; AF364) (MW 8kDa)	0.2 µg/ml	

Table 2-9 Buffers, components of SDS-PAGE gels and antibodies used for Western blotting.

(All chemicals from Sigma-Aldrich)

2.9 RT-PCR

Gene expression of the chemokines CCL17, CCL22, CCL20 and IL17RA gene expression was analysed by RT-PCR.

2.9.1 RNA Extraction

Total RNA was extracted from cells using RNeasy kit (Qiagen) according to manufacturer's instructions.

For CCL17 and CCL22 detection, amplification of RNA was carried out from lipopolysaccharide stimulated and unstimulated liver infiltrating dendritic cells from diseased liver tissue, monocyte derived dendritic cells from peripheral blood and TNF-α and IFN-γ stimulated and unstimulated hepatic sinusoidal endothelial cells. For detection of CCL20 and IL-17RA gene expression, RNA was extracted from biliary epithelial cells stimulated with IL1β, IL-17, TNF-α, IFN-γ and TNF-α+IFN-γ. RNA concentration was determined by measuring optical density of a dilution of the sample in the plate reader at 260nm and 280nm.

2.9.2 RT protocol

Individual PCR tubes for each sample were loaded with 1μg RNA and heated at 65º C for 5 min and then placed on ice. A master mix for 1st strand cDNA synthesis was made up as described in Table 2.10. Master mix was added to the RNA samples which were left at 25ºC for 10min then incubated at 42ºC for 1 hour. After a final incubation at 95ºC for 5min, cDNA were stored at -20ºC or -80º C until use. Each reaction was performed with a non-template control, RT-control were included in each PCR detection assays to exclude non-specific detection. Reverse transcription was carried out for the chemokines, IL-17RA and GAPDH was used as an endogenous control.

Master mix for 1st strand cDNA was synthesized as follow

Reagents	Amount	Final concentration	Source
5 X PCR First Strand Buffer(Green GoTaq)	6 µl	X1	Promega,UK
DNTPs	2.5 µl	10Mm	Roche
$MgCl_2$	6 µl	25 Mm	Promega,UK
Random Hexamers	1 µl		Promega,UK
Superscript II reverse Transcriptase	0.5 µl		Invitrogen
PCR water	14 µl		Sigma

Total volume of master mix for each sample is 30µl.

Table 2-10 Reagents in 1st strand cDNA synthesis

2.9.3 PCR Protocol

The PCR reaction was conducted using 1µg/ml of cDNA per reaction (6µl of volume). The reagents used in the PCR protocol are shown in the table below.

Reagents	**Amount**	**Final concentration**	**Source**
5 X Flixi Taq PCR buffer	10 µl	X1	Promega,UK
DNTPs	1µl	10Mm	Roche
$MgCl_2$	3 µl	25 mM	Promega,UK
Reverse Primer	2 µl	0.4µM(stock 10µM)	Alta bioscience
Forward Primer	2 µl	0.4µM(stock 10µM)	Alta bioscience
Flexi Taq polymerse	0.25 µl		Invitrogen
PCR water	25.75 µl		Sigma

Total volume 44µl + 6µl of cDNA = 50 µl per reaction

Table 2-11 Components of PCR reaction

The reaction conditions for CCL17 and CCL22 PCR were hot start at 95°C for 5 min, denaturing at 95°C for 1 min, annealing at 66°C for 1 min and, extension at 72°C for 1 min. 35 cycles were performed for CCL17 &CCL22 chemokines and 27 cycles for GAPDH. A final extension at 72°C for 5 min was performed and samples were stored at 4°C until analysis. For CCL20 and 1L-17RA, initial denaturing was at 94ºC for 3 minutes followed by 35 cycles of 30s of denaturing at 94ºC, 30s of annealing of primers at 55ºC and 60s of extension at 72ºC and final extension at 72°C for 5 min.

All primers were designed based upon GenBank sequences and are detailed in the table below.

Primers	Sequence	Source
CCL17 forward	5'-ACT GCT CCA GGG ATG CCA TCG TTT TT-3'	Alta Bioscience
CCL17 reverse	5'-ACA AGG GGA TGG GAT CTC CCT CAC TG-3'	
CCL22 forward	5'-AGG ACA GAG CAT GGA TCG CCT ACA GA-3'	
CCL22 reverse	5'-TAA TGG CAG GGA GGT AGG GCT CCT GA-3'	
CCL20 forward	5'-GCG CAA ATC CAA AAC AGA CT-3'	
CCL20 reverse	5'- CAA GTC CAG TGA GGC ACA AA-3'	
IL-17RA forward	5'-CCA GAT CCC AGC TTT GAG AG-3'	
IL-17RA reverse	5'-AAA TGC CCG CCA CAT AGT AG-3'	
GAPDH forward	5'-GGC CTC CAA GGA GTA AGA CC-3'	
GAPDH reverse	5'-AGG GGT CTA CAT GGC AAC TG-3'	

Table 2-12 Primers used for RT-PCR

Product sizes for each set of primers were: CCL17, 270 bp; CCL22, 363 bp; CCL20, 200 bp; IL-17RA, 285 bp. Each PCR experiments included negative controls in which RNA was omitted from the RT mixture and cDNA was omitted from the PCR reactions. RNA from human peripheral blood monocyte-derived dendritic cells was used as positive control for CCL17 and CCL22 chemokines.

After the PCR, 15μl of amplified products was run on a 2% agarose gel containing ethidium bromide (Sigma) using 1 x TAE buffer.

2.10 Sandwich ELISA for CCL17, CCL22 and CCL20

The concentration of CCL17 and CCL22 in stimulated liver derived dendritic cells supernatant samples or CCL20 in cytokines stimulated biliary epithelial cells supernatant samples were determined by sandwich ELISA. Human liver derived dendritic cells from different diseased livers were stimulated with LPS (1μg/ml) for 24 hours and supernatant were collected. Similarly biliary epithelial cells were stimulated with TNF-α, IFNγ, IL-1β, IL-17 or combination of these cytokines for 24 hours. Quantikine sandwich ELISA kits for CCL17 (DDN00), CCL22 (DMD00) and CCL20 (DM3A00) were purchased from R&D Systems and used according to manufacturer's instructions. Monoclonal antibodies specific for CCL17, CCL22, and CCL20 were pre-coated in microplates. All the reagents and samples were kept at room temperature before use and wash buffer and protein standards of known concentration was prepared in serial dilutions. Test supernatant samples (from different diseased livers and normal liver) or protein standards were then added to relevant wells and incubated at RT for two hours before washing the plates 4 times with wash buffer. Horseradish peroxidise conjugated antibody directed against the corresponding chemokines were then added to each well, incubated for two more hours and the wells were washed again 4 times. The ELISA was developed by adding tetramethylbenzidine substrate (TMB) in hydrogen peroxide and the enzymatic reaction was stopped by using 2 N sulphuric acid. Colorimetric analysis was performed by measuring absorbance values at 450nm using a Dynatech Laboratories MRX plate reader. All the measurements were performed in triplicates for each experiment.

2.11 Transwell chemotatic migration assay

The migratory properties of T_{reg} or Th17 cells isolated from inflamed livers and peripheral blood were assessed using fibronectin-coated (Sigma-Aldrich) 6.5-mm diameter, 5-μm pore 24 well plate Transwell inserts (Costar Corning). The lower Transwell chambers were filled with 600μL of assay media (RPMI medium with 0.1% BSA; Sigma-Aldrich), BEC media or media supplemented with different chemokines (100 ng/ml recombinant human CCL22, CCL17, CCL20 or BEC supernatant stimulated with different cytokines. LIT_{reg} or peripheral blood Th17 resuspended in RPMI was added to the upper chambers (some after 30 minuntes of Pertussis or CXCR3 block). Anti CXCL9-11 block or Anti CCL-20 block was applied to BEC supernatant for Th17 chemotaxis assay. Then cells were collected from the top and bottom chambers after 4 hours incubation at 37°C. Transmigrated cells were collected from the lower chamber and measured by fixed volume counting (using Caltag counting beads: PCB-100) and also phenotyped for T_{reg} and Th17 markers and chemokine receptors expression by Cyan flow cytometry. Migration assays were conducted in duplicate and migration in test wells was compared with control wells contained medium with 0.1% BSA or BEC media alone. On occasion to inhibit chemokine-mediated signalling, T_{reg} and Th17 were incubated with pertussis toxin (100 ng/ml; Sigma Aldrich Ltd., Aldrich) or CXCR3 blocking antibody or blocking antibody to CCL20 or CXCL9-11 was applied in the BEC supernatant prior to chemotaxis.

To calculate the specific migration, the number of cells in each subpopulation which migrated in the absence of chemokine was subtracted from the number of the corresponding cell subpopulation which migrated in the presence of chemokine. Basal and chemokine directed migration were also evaluated by determining a chemotatic index.

Clone	Antigen	Concentration	Source
300-30	CCL 17	100 ng/ml	R & D Systems
300-36A	CCL22	100 ng/ml	R & D Systems
Clone 87328 MAB 672	Anti I-TAC	5μg/ml	R & D Systems
Clone 33036	Anti-CXCL10	5μg/ml	R & D Systems
Clone 49106 MAB392	Anti-CXCL9	50μg/ml	R & D Systems
MAB 160/49801	Anti-CXCR3	10μg/ml	R & D Systems
Recombinant CCL20 300-29A	CCL-20	100ng/ml	Peprotec
Clone 67310 MAB 360	mAb anti-human CCL20Ab	100ng/ml	R & D Systems
Pertussis Toxin	Anti-GCPR	100ng/ml	Sigma-Aldrich Ltd

Table 2-13 Recombinant chemokines, blocking antibodies and Pertussis toxin used for chemotaxis assay.

2.12 Flow based adhesion assay

Recruitment of T_{reg} to the liver during inflammation is mediated by specific homing receptors. The flow based adhesion assay allows one to study adhesive interactions between T_{reg} and cultured endothelium under inflammatory conditions and physiological fluid flow. This has allowed us to characterise the functional significance of Regulatory T cell homing receptors (chemokine receptors and integrins) and their interaction with cell surface and secreted ligands expressed by hepatic sinusoidal endothelium.

2.12.1 Preparation of microslides for flow based adhesion assay

Glass microslides (Camlab Ltd, Cambridge, UK) were initially acid-washed overnight (70% v/v nitric acid; Sigma Aldrich Ltd.) before being rinsed in an excess of tap water with a final wash in distilled water. They were then blotted dry and dehydrated in anhydrous acetone to remove all traces of water. Microslides were then coated for 10 minutes at room temperature with a 4% (v/v) solution of 3-aminopropyltriethoxysilane (APES; Sigma Aldrich Ltd.) prepared in anhydrous acetone. They were then washed in anhydrous acetone 3 times, and rinsed in distilled water to remove any residual acetone before being blotted and dried in a glass oven overnight at 60ºC. To facilitate connection of microslides to a custom designed multi-port culture dish (Glassblower, School of Chemistry, and University of Birmingham, UK) silicone tubing adaptors (Portex Ltd., Kent, UK) were attached at one end of the slides which were then autoclaved prior to use.

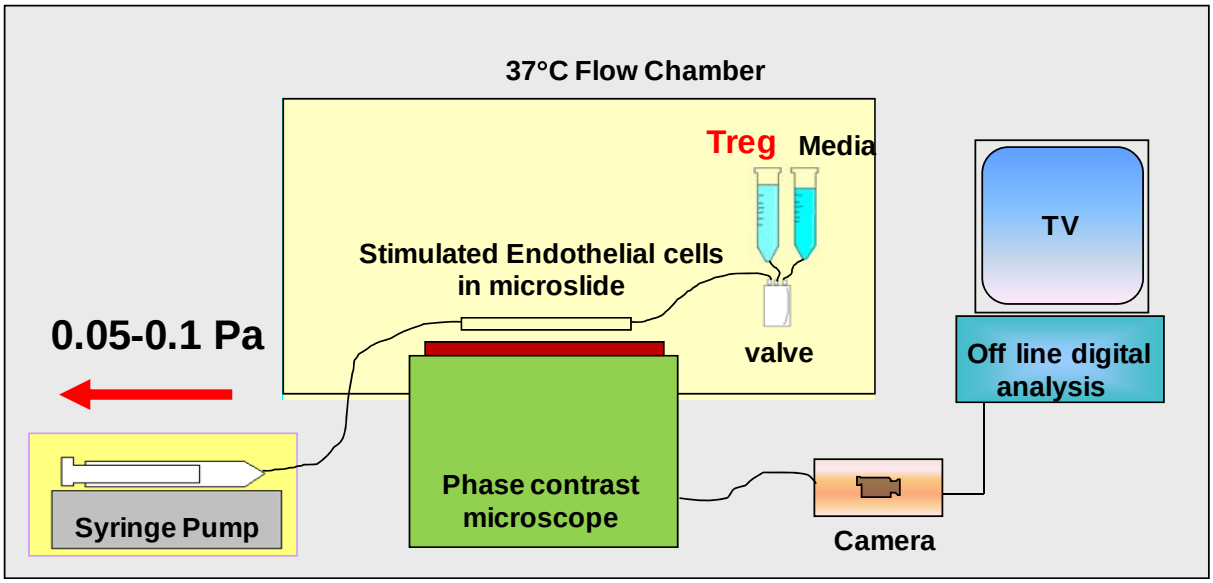

Figure 2-3 Schematic diagram of flow chamber and flow based adhesion assay

Capillary tube glass microslides are attached to silicone adaptor tubing. Hepatic sinusoidal endothelial cells were grown in microslides and stimulated with TNF-α and IFN-γ for 24 hours. One end of microslide was attached to syringe pump and the other end to electronic valve which can switch between cells and wash buffer. Freshly isolated peripheral blood regulatory T cells were perfused over the microslides at a constant shear stress of 0.05Pa which is equivalent to hepatic sinusoidal blood flow. Adhesion cascades were recorded with video monitor and experiments were analysed offline for rolling adhesion and transmigration.

2.12.2 Culture of hepatic sinusoidal endothelial cells in microslides

Sterile microslides were coated with RTC (in house preparation) for 1 hour at 37ºC and washed thoroughly with PBS. Cultured HSEC were trypsinized, washed in PBS and resuspended to a count of 2 x 10^6 /ml in complete medium for seeding of six microslides. Microslides were then attached to a 1 ml pipette and 50 μl of the cell suspension was aspirated into each slide before being incubated at 37ºC for 2 hours to allow cells to settle and adhere. Individual microslides were then attached to one of the six ports within the culture dish via the silicone tubing and the culture dish was filled with 40 ml of complete medium. The culture dish was placed in a humidified incubator at 37ºC and attached via silicone tubing to an external pump, timed to change media within the microslides at 2 hourly intervals. HSEC cultured within microslides were stimulated with TNF-α and IFN-γ, both 10 ng/ml for 24 hours before the flow assay.

The microslides containing stimulated confluent HSEC monolayers were then fixed to a glass slides using cyanoacrylate glue and the two ends of the microslide were coated in double-sided adhesive tape to allow an airtight seal. One end of the microslide was then attached via silicone tubing to a Harvard syringe pump (Harvard Apparatus, South Natic, USA) and the other to an electronic valve (Lee Products Ltd., Gerrards Cross, UK) which permitted alternation between a cell free wash buffer (BEM/0.1% BSA) and Treg cell suspension (1 x 10^6/ml in BEM/0.1% BSA). The withdrawal rate of the syringe pump was altered such that the flow rate within the microslide was at a pre-determined value. Regulatory T cells were perfused over cultured endothelial sinusoidal cells at a constant wall shear stress of 0.05Pa, which approximates the flow rate within the post-capillary venules/sinusoids in vivo. On occasion, HSEC monolayers were pre-treated with blocking antibodies against ICAM-1, and VCAM-1 (or control) to selectively inhibit these adhesion molecules. Alternatively, T_{reg} were incubated with a blocking

antibody against CXCR3 or pertussis toxin (PTX) to selectively inhibit Gi protein linked receptor signalling. Antibodies and blocking agents were prepared in flow assay media which is made up of BEM/0.1% BSA and 50 μl was aspirated into relevant microslides or applied to T_{reg} suspensions for 30 minutes prior to assay. All blocking incubations were at 37°C and details of the blocking antibodies used are described in Table 2.14. In experiments to block individual CXCR3 ligands, CXCL9, CXCL10 and CXCL11 were individually applied to stimulated HSEC in each microslide for 30 minutes before the flow experiments.

T_{reg} captured from flow were visualised (X100 magnification) by phase-contrast microscopy using an Olympus IX50 inverted microscope (Olympus, Southall, Middlesex, UK) and video-microscopic recordings were made along the length of the microslide against the flow to prevent double counting of cells. Both the inflow and wash out phases of the adhesion experiment were recorded on video. Offline analysis allowed the pattern of adhesion and transmigration to be assessed. T_{reg} adherent to the upper surface of the HSEC monolayer appeared phase-bright whilst those that had migrated through the monolayer appeared phase-dark. Adherent cells were classified as exhibiting static adhesion (non-migrated), or migrated and individual categories were expressed as a percentage of total adhesion. Total adhesion was also normalised for adhesive surface area and number of T_{reg} perfused i.e. adherent cells/mm $^2/10^6$ T_{reg} perfused. Total migrated cells were expressed as migrated cells / mm $^2/10^6$ T_{reg} perfused or as percentage of adherent cells which had transmigrated.

Clone	Antigen	Concentration	Company
DAK-G01	Mouse IgG	20 μg/ml	Dako
MAB160/49801	CXCR3	10 μg/ml	R&D Systems
11C81	ICAM-1	10 μg/ml	R&D Systems
4B2	VCAM-1	10 μg/ml	R&D Systems
Pertussis Toxin	-	100 ng/ml	Sigma Aldrich Ltd
49106	Anti- CXCL9	50μg/ml	R&D Systems
33036	Anti-CXCL10	5μg/ml	R&D Systems
87328	Anti-CXCL11	5μg/ml	R&D Systems

Table 2-14 Blocking antibodies used in flow based studies

2.13 Statistical analysis

Data normally distributed were analysed using Student's T-Tests (paired or independent) whilst non-normally distributed data were compared using Wilcoxon signed ranks test (for related samples) or Mann-Whitney U tests (for unrelated samples comparing normal and diseased livers). A value of $p \leq 0.05$ was considered statistically significant.

CHAPTER 3 PHENOTYPE AND FUNCTION OF REGULATORY T CELLS IN HUMAN LIVER

3.1 INTRODUCTION

The liver maintains tolerance to harmless food antigens load from the gastrointestinal tract whilst maintaining the capacity to sustain effective immune response against pathogens. It has been suggested that the fate of an immune response against intrahepatic antigens depends on the dominant site of antigen presentation. Where antigen is overwhelmingly presented within the liver, $CD8^+$ T-cell tolerance is likely to ensue; however, if there is significant early antigen presentation within the lymph nodes, an effective immune response is generated that can clear pathogens from the liver but which may also generate hepatocellular injury (Bowen et al., 2005). The level of antigen expression is also crucial as the presence of high-level antigen within the liver will lead to deletion of $CD8^+$ T cell (Mehal, 2003).

Major players actively involved in maintaining hepatic tolerance include antigen presenting cells (APC) such as tolerogenic dendritic cells (Hadeiba et al., 2008;Thomson, 2010) and regulatory T cells (Eksteen et al., 2007;Lan, 2006). Hepatic antigen presenting cells also include diverse non-professional antigen presenting cells including kupffer cells, hepatocytes, and endothelial cells all of which can present antigen under particular condition. The unique architecture of the liver allows lymphocytes flowing through the low flow sinusoids to interact with resident liver APC, allowing them to direct immune response towards immunity or tolerance (Adams et al., 1989;Goddard et al., 2004a). The normal liver contains a large population of resident lymphocytes that differ from peripheral blood (Crispe, 2003). $CD8^+$ T cells usually outnumber $CD4^+$T cells in the liver (Winnock et al., 1995) in a reversal of the usual ratio in blood.

In chronic hepatitis, both effector subsets and regulatory lymphocytes are found at the site of inflammation and the balance of the cells recruited will determine the nature of the hepatitis and liver disease. The naive CD4 T cell is a multipotential precursor with defined antigen

specificity but substantial plasticity for development of downstream distinct effector or regulatory lineages, contingent upon signals from cells of the innate immune system. The most important lineages are Th1, Th2, Th17 and Th9 effector cells and anti-inflammatory regulatory Tcells.

The maintenance of tolerance to self-antigens and the resolution of inflammation involve central and peripheral mechanism (Walker & Abbas, 2002). Regulatory T cells (T_{reg}) are a subset of CD4 T lymphocytes that play a crucial role in maintaining peripheral tolerance by suppressing self-reactive T cells which escape from central thymic deletion. Thus, T_{reg} prevent collateral autoimmune damage as a consequence of immune responses to infections and inflammation (Sakaguchi and Powrie, 2007). Surviving an infection requires the generation of an effective immune response against the invading pathogen whilst limiting collateral damage to self tissues that may result from an over exuberant immune response. T_{reg} limit excessive inflammation towards the microorganism which could otherwise lead to persistent injury and inflammation (Belkaid & Rouse, 2005)

T_{reg} are crucial in the tolerogenic properties of the liver and help to suppress responses to harmless food antigens and bacterial components (Crispe et al., 2006). Naturally occurring, thymic derived T_{reg} are defined by expression of CD4, $CD25^{high}$, and $FoxP3^{+}$ (Sakaguchi, 2004), and low expression of the IL-7 receptor/$CD127^{low}$ (Liu et al., 2006;Sakaguchi, 2005;Seddiki et al., 2006). FoxP3 is the master regulator of T_{reg} development and function (Fontenot et al., 2003a;Hori et al., 2003;Khattri et al., 2003). The FoxP3 gene was first identified as the defective gene in the mouse strain Scurfy which is an X-linked recessive mutant lethal in hemizygous males exhibiting hyperactivation of $CD4^{+}$ T cells and overproduction of proinflammatory cytokines(Brunkow et al., 2001). Mutations of the human gene FOXP3 are the cause of the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is the human counterpart of Scurfy.

Natural T_{reg} suppress effector cell proliferation and cytokine production in vitro in a cell-contact dependent manner and protect against autoimmunity following adoptive transfer in vivo (Sakaguchi et al., 1995). They can also suppress effector activities of differentiated $CD4^+$ and $CD8^+$ T cells and the function of natural killer cells, natural killer T cells, B cells, macrophages, and dendritic cells (Miyara and Sakaguchi, 2007b;Shevach, 2006;Tang and Bluestone, 2008;von, 2005). Multiple mechanisms of T_{reg} mediated suppression function in a synergistic or sequential manner to suppress inflammation. Antigen-activated T_{reg} are recruited to antigen-presenting dendritic cells as a consequence of their high expression of LFA-1 and out-compete antigen-specific naive T cells (Onishi et al., 2008). T_{reg} then modulate dendritic cell function by downregulation of dendritic cell CD80 and CD86 by a CTLA-4-dependent mechanism (Wing et al., 2008). They also act to absorb IL-2 thereby inducing apoptosis in responder T cells(Pandiyan et al., 2007). Some T_{reg} secrete perforin and actively kill effector cells whereas other secretes immunosuppressive cytokines including IL-10 or IL-35 (Collison et al., 2007). Other mechanisms of suppression involve enzyme activity. Thus, T_{reg} can stimulate dendritic cells to express the enzyme indoleamine 2, 3-dioxygenase, which catabolizes the essential amino acid tryptophan to kynurenines that are toxic to T cells (Munn et al., 2002). T_{reg} also generate pericellular adenosine catalyzed by their cell surface expression of CD39, ectonucleoside triphosphate diphosphohydrolase 1 (Borsellino et al., 2007)and CD73, ecto-5'-nucleotidase (Deaglio et al., 2007).

Interleukin 2 (IL-2) is critical for the function of T_{reg}(Itoh et al., 1999). CD25, a component of the high-affinity IL-2 heterotrimeric receptor complex (IL-2R) is expressed in high levels on T_{reg} and is functionally essential for T_{reg} development. T_{reg} are refractory to TCR-induced proliferation (Itoh et al., 1999;Takahashi et al., 1998) and they depend on IL-2 for their survival (de la Rosa et al., 2004;Furtado et al., 2002). Signalling via the IL-2R complex, in combination with TCR engagement is essential for their proliferation and suppressive function.

The number of FoxP3$^+$ T_{reg} is reduced in mice lacking either CD25 or IL-2(Antony et al., 2006), and autoimmunity in CD25-deficient mice can be prevented by adoptive transfer with wild-type CD25$^+$CD4$^+$ T cells (Malek et al., 1984). Stimulation of T_{reg} with IL-2 leads to phosphorylation and activation of STAT5 with consequent binding to the FoxP3 promotor and enhance FoxP3 expression. T cell-specific deficiency of STAT5a and b, which mediate signalling from the IL-2R β chain to the nucleus, abrogates the development of Foxp3$^+$ T_{reg}, producing autoimmune/inflammatory diseases (Burchill et al., 2007;Yao et al., 2007).

The high levels of CD25 on T_{reg} may absorb IL-2 from other effector cells, the main source of IL-2 in inflamed environment. IL-2 produced by activated non-regulatory T cells contributes to the maintenance, expansion, and activation of natural T_{reg}, which in turn limits the expansion of non-regulatory T cells providing negative feedback control of the immune response. Disruption of this feedback loop promotes the development of autoimmune/inflammatory disease (Sakaguchi, 2008). The poor expansion and impaired function of intrahepatic T_{reg} despite accumulation at sites of chronic viral hepatitis has been attributed to downregulation of STAT5 phosphorylation and upregulation of programmed death-1 expression (Franceschini et al., 2009).

Hepatic inflammation occurs in response to a variety of injuries including hepatitis B and hepatitis C viruses, responses to altered-self antigens in autoimmune diseases. An increased frequency of T_{reg} has been reported in chronic viral hepatitis (Xu et al., 2006) where a failed immune response leads to viral persistence (Belkaid & Rouse, 2005) and also in hepatocellular carcinoma where protective anti-tumour CD8 T cell responses may be suppressed by their presence in the peri-tumor region (Rushbrook et al., 2007;Unitt et al., 2005) promoting tumour progression and poor survival (Fu et al., 2007). Decreased T_{reg} number and impairment in function has been implicated in autoimmune hepatitis (Longhi et al., 2004;Longhi et al., 2005b),

transplant rejection (Demirkiran et al., 2006;Demirkiran et al., 2007). Recently, monocyte overactivation and inability of T_{reg} to restrain them has been reported to contribute the perpetuation of the autoimmune attack (Longhi et al., 2009).

Previous studies indicate that antigen-specific T_{reg} migrate to and become activated in regional lymph nodes where tissue-specific self-antigens or microbial antigens are presented. The concentration of peptide required to activate the peptide-specific T_{reg} to exert suppression in vitro has been estimated to be 10- to 100-fold lower than the concentration needed for activating naive T cells with the same antigen specificity (Takahashi et al., 1998). They also migrate into inflamed tissues, infectious sites, and tumours (Belkaid & Rouse, 2005) . In peripheral lymph nodes, the majority of FoxP3$^+$ T_{reg} express high levels of CCR7 (Huehn et al., 2005) and expression of chemokine receptors and adhesion molecules will control their trafficking and positioning in response to inflammation. Very little is known about T_{reg} recruitment into tissues and thus I undertook a project to investigate the presence of T_{reg} and their positioning in relation to effector and dendritic cells in both liver inflammation and lymph node.

The frequency, distribution, surface phenotype, transcription factor expression and functional capacity of liver infiltrating regulatory T cells were analysed using immunohistochemistry and analysis of fresh human liver infiltrating lymphocytes. I paid particular attention to the proximity of $T_{reg,}$ effector cells and dendritic cells in inflamed liver.

3.2 RESULTS

3.2.1 Presence of FoxP3 regulatory T cells in human liver

Presence of $FoxP3^+$ regulatory T cells in hepatic inflammation was investigated by immunohistochemistry on paraffin embedded liver tissue sections. Few T_{regs} were detected in normal liver tissue by immunohistochemistry (Fig.3.1) although they could be readily detected in the chronically inflamed livers (Figure 3.1 and Figure.3.2).

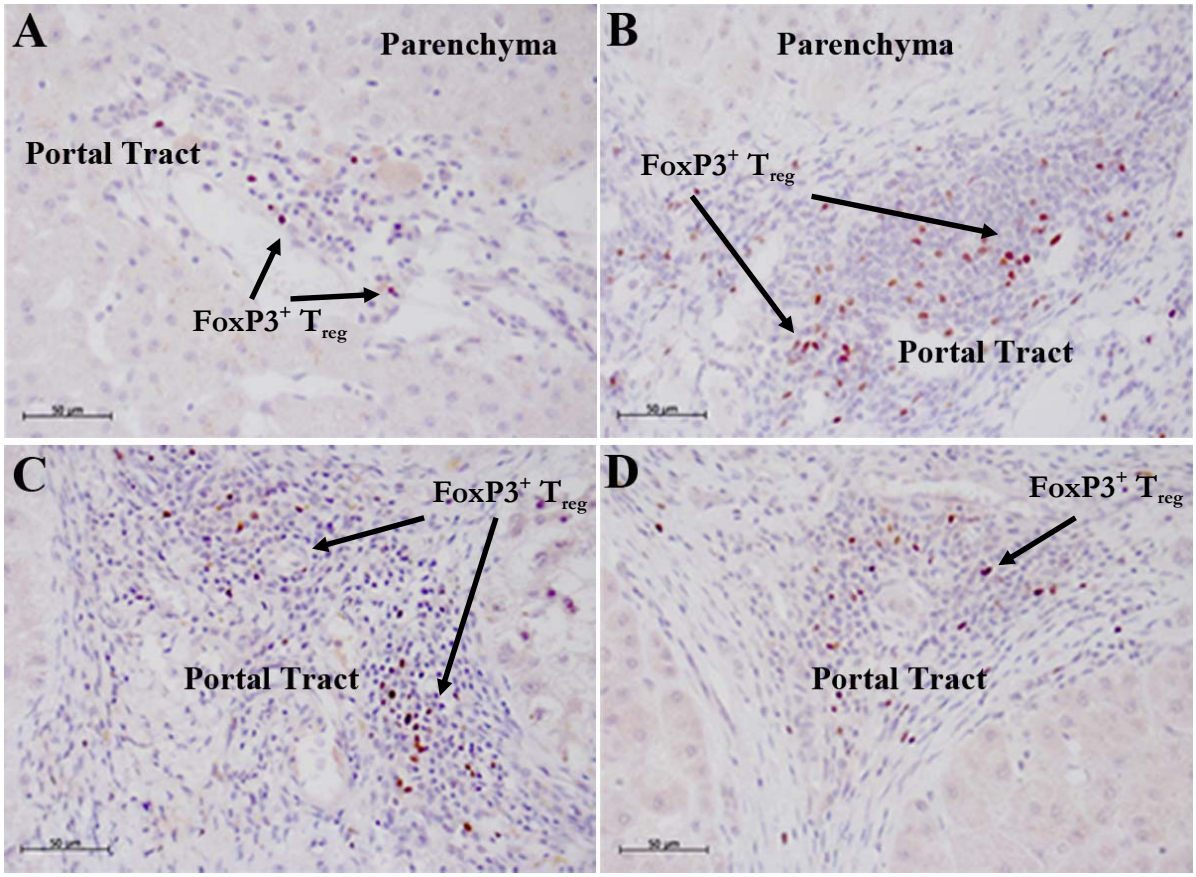

Figure 3-1 Expression of FoxP3$^+$ Regulatory T cells in human liver samples.

Immunohistochemical staining of paraffin embedded liver sections demonstrating expression of FoxP3$^+$ T_{reg} cells (shown with arrows) in (A) Normal Liver (B) Primary Biliary Cirrhosis (C) Autoimmune Hepatitis (D) Hepatitis C. (Positive staining cells are shown as brown stain). Fields were captured using 20x objectives.

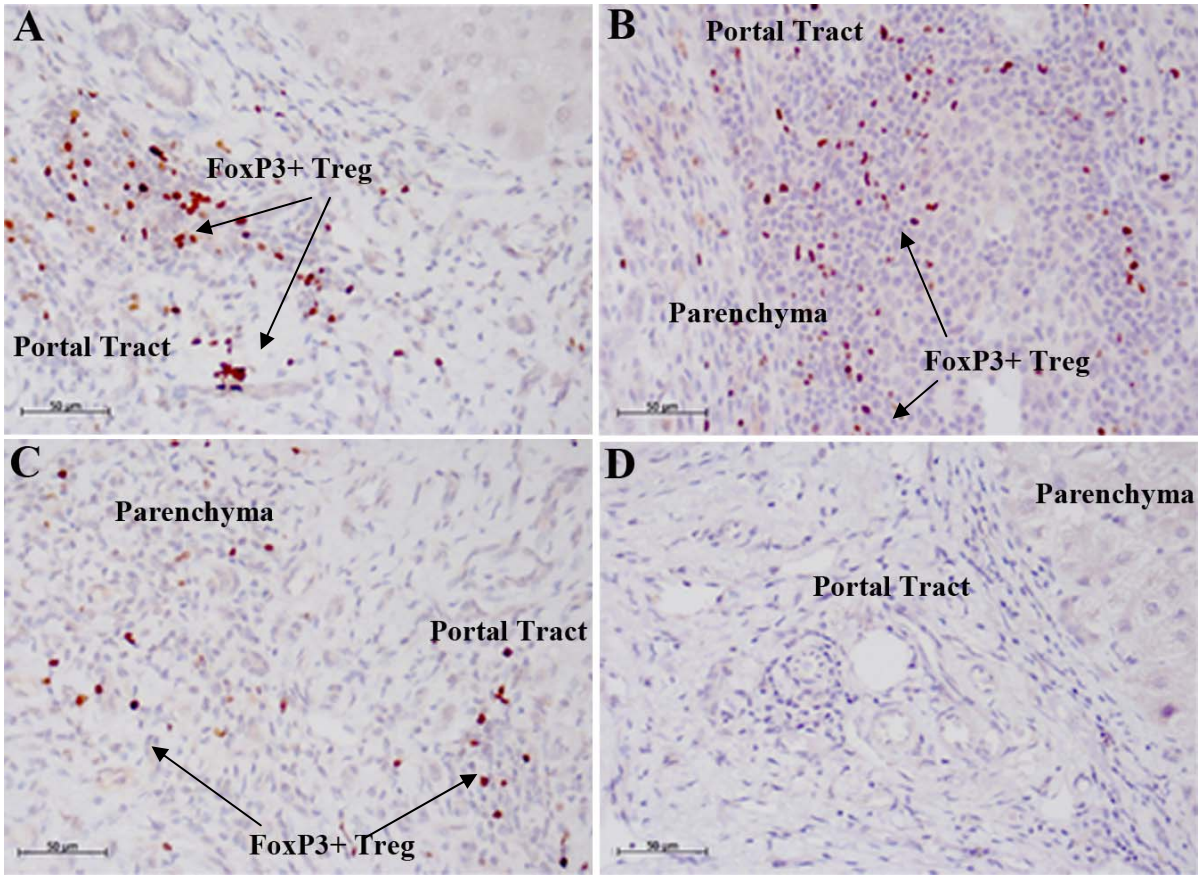

Figure 3-2 Expression of FoxP3$^+$ Regulatory T cells in human liver samples.

Immunohistochemical staining of paraffin embedded liver tissue sections demonstrating the presence of FoxP3$^+$ T_{reg}cells (shown with arrows) in (A) Chronic Hepatitis B (B) Seronegative hepatitis (C) Alcoholic liver disease. (Positive staining FoxP3 cells are shown as brown stain with arrows). Control antibody staining (D) was negative. Fields were captured using 20x objectives.

To confirm the FoxP3$^+$ cells were T_{reg} I carried out costaining with T_{reg} surface markers. Using confocal microscopy I was able to detect cells that were CD4$^+$, CD3$^+$, FoxP3$^+$ in the different diseased liver tissue (Figure 3.3).

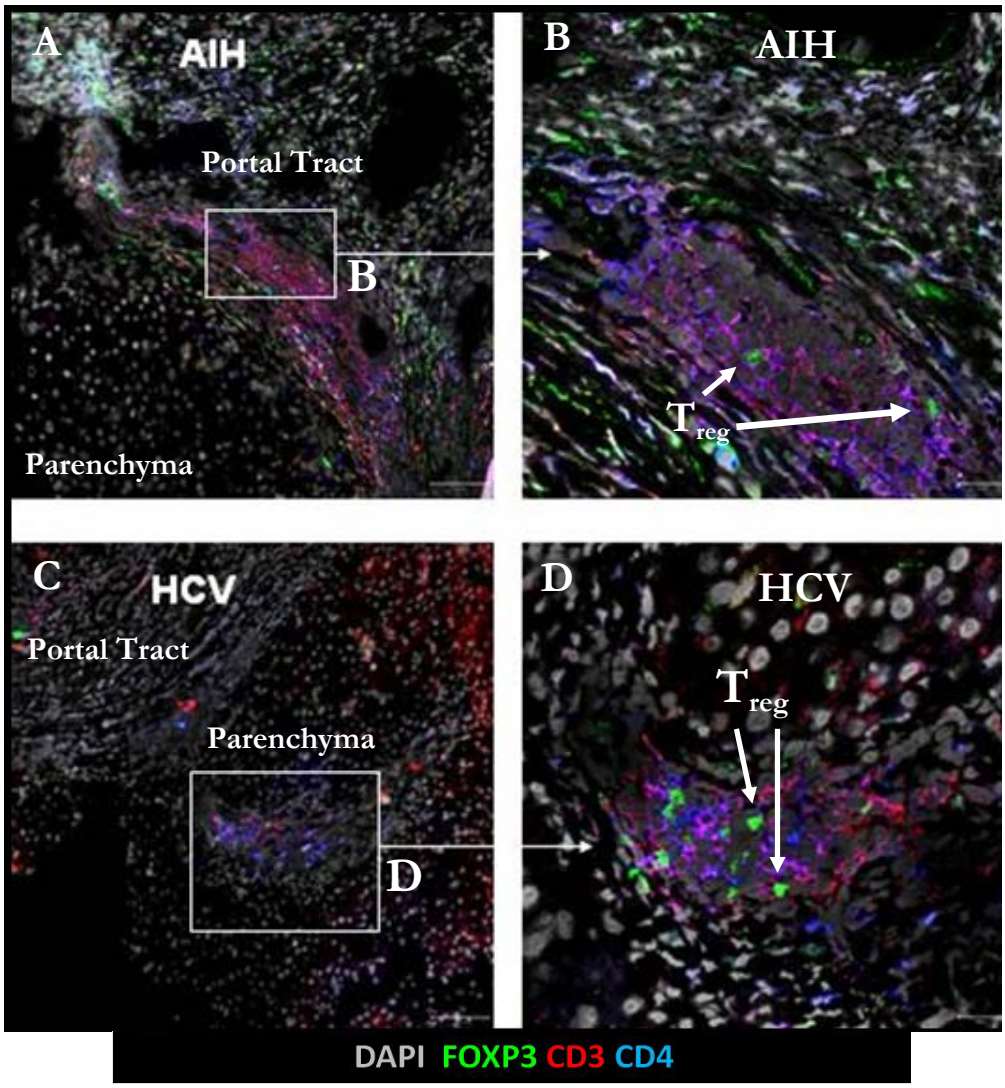

Figure 3-3 Confocal microscopy of FoxP3$^+$ regulatory T cells in inflamed human liver.

Confocal microscopy using CD3, CD4, FoxP3 demonstrating the distribution and presence of liver infiltrating regulatory T cells in inflamed human livers (A&B=AIH; autoimmune hepatitis, C&D=HCV; chronic hepatitis C). FoxP3$^+$ regulatory T cells are shown in portal tract of inflamed livers. Areas of CD3$^+$, CD4$^+$, FoxP3$^+$ costaining were shown with arrows. Pictures were taken at x25 and x40 magnification. CD3=Red; CD4=Blue; FoxP3=Green; Nuclear staining DAPI=Grey. (N=6)

3.2.2 FoxP3$^+$ cells in normal and chronically inflamed livers

I then calculated the proportion of the CD3 infiltrate that was FoxP3$^+$ in a variety of liver diseases. All diseased livers contained a significantly higher proportion of T_{reg} compared with non-diseased livers (Fig 3.4). Paraffin embedded serial liver sections from different liver diseases were stained for CD3 antibody, FoxP3 antibody and control antibody. Numbers of CD3 and FoxP3 cells are counted in same area in five high power fields for each diseased liver. Average values were calculated and the ratio of CD3 lymphocytes which are positive for FoxP3 cells was calculated for each liver diseases. Table 3.1 showed the frequency of CD3 and FoxP3 cells and percentage in each disease livers.

Interestingly greater numbers of FoxP3$^+$ T_{reg} were seen in the patients (explanted liver tissue samples) with the most severe inflammation with the highest frequencies are detected in patients who had undergone emergency liver transplantation for fulminant liver failure as a consequence of severe seronegative (nonA nonB) hepatitis (Fig.3.4).

Disease state	CD3 Portal Tract Mean±SD	CD3 Parenchyma Mean±SD	FoxP3 Portal Tract Mean±SD	FoxP3 Parenchyma Mean±SD	Total % of CD3 which are FoxP3$^+$ Mean±SD
Normal Liver	51 ± 28	11.6 ± 3.8	2.2 ± 1.2	0.21 ± 0.1	3.04±0.76
Chronic hepatitis C	239.5 ± 84.6	40.1 ± 16.6	25.7 ± 5.7	2.5 ± 1.2	8.85±3.6
Chronic hepatitis B	327 ± 118	39 ± 10	28 ± 4.5	2.6 ± 1	8.2±2.8
Seronegative hepatitis	343.8 ± 121.7	60.1 ± 25.7	38.3 ± 8.4	10.1 ± 4.6	15.6±6.9
Autoimmune hepatitis	280 ± 119	53.8 ± 30.6	27.6 ± 5.8	6.7 ± 4.3	11.6±3.5
Primary biliary cirrhosis	226 ± 94	35.5 ± 13	28.6 ± 6.9	2.7 ± 1.2	11.4±5.4
Alcoholic liver disease	258.8 ± 116	24.7 ± 8.5	20 ± 7.4	1.6 ± 1	7.4±3.4

Table 3-1 Total cells number of CD3 and FoxP3 in different liver diseases.

Table shows the total numbers of CD3 and FoxP3 in portal tract and parenchyma of different liver diseases. Immunohistochemistry were done on liver sections with CD3 and FoxP3. CD3 and FoxP3 from five areas of portal tract and parenchyma were counted at high power fields and average was calculated. 7-8 cases of normal and diseased livers were studied. Seronegative hepatitis= non A non B hepatitis.

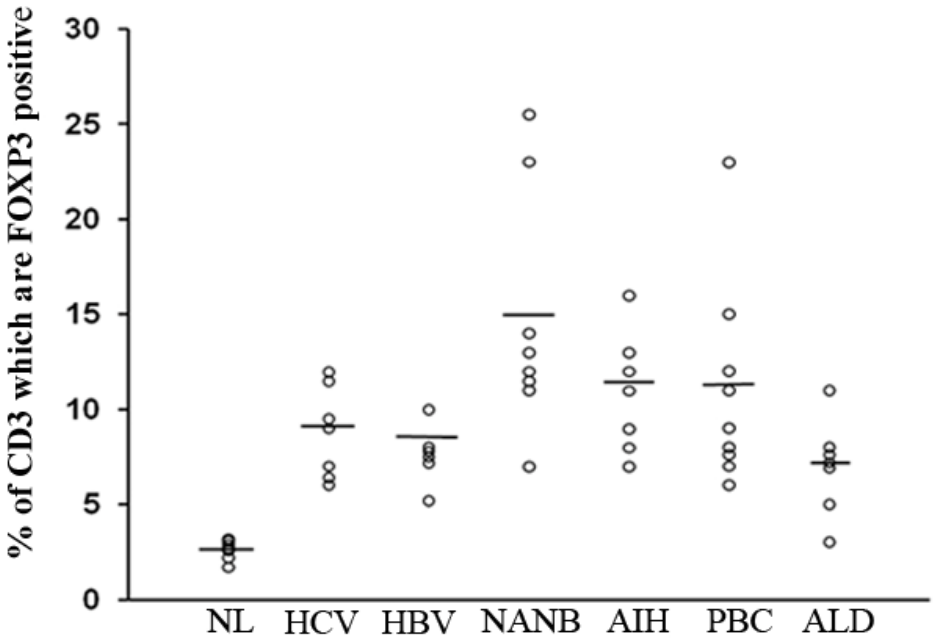

Figure 3-4 Percentage of CD3 T cells which are FoxP3$^+$ in normal and different human liver diseases.

The ratio of FoxP3$^+$ cells to CD3 staining T cells was quantified by immunohistochemistry in normal livers (NL) and different chronic liver diseases. Compared to normal liver, diseased livers has significantly higher infiltration of FoxP3$^+$ cells ($p < 0.05$ comparing normal to different diseased livers, Mann Witney U tests; n= 7-8 for normal and diseased livers) (NL=normal liver, HCV=chronic hepatitis C, HBV=chronic hepatitis B, NANB= nonA nonB or seronegative hepatitis, AIH= autoimmune hepatitis, PBC=primary biliary cirrhosis, ALD=alcoholic liver disease)

Multi-colour flow cytometry was used to phenotype T_{reg} isolated from normal and inflamed liver tissue. Liver infiltrating lymphocytes were isolated from both normal and inflamed liver tissues using mechanical digestion and density gradient centrifugation. Cells were stained for T_{reg} surface markers CD4, CD25, CD127 and transcription factor FoxP3. The total percentage of $CD4^{+}CD25^{high}CD127^{low}$ $FoxP3^{+}$ T_{reg} was greater in diseased than normal liver. In normal liver T_{reg} frequency varied from 0.5-2 % whereas in diseased livers it ranged from 3-7 %.(Figure 3.5).

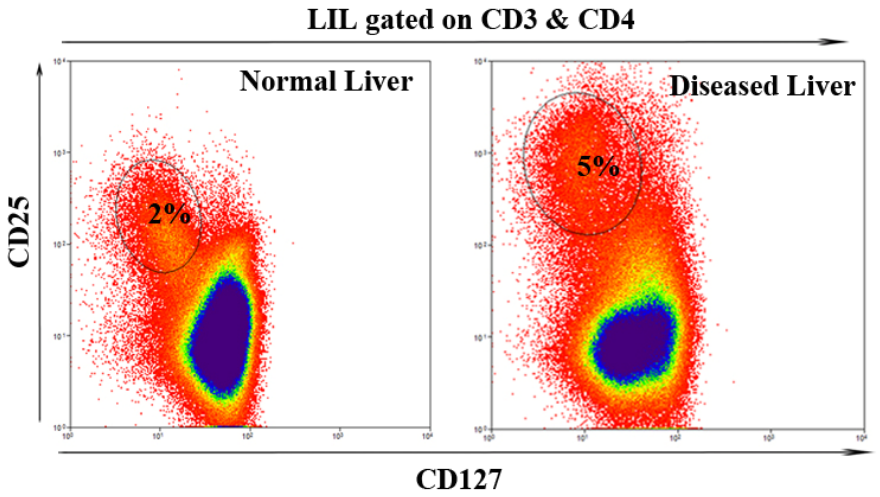

Figure 3-5. Liver infiltrating regulatory T cells in normal liver and inflamed liver.

Flow cytometry dot plot comparing the frequency of liver infiltrating T_{reg} in inflamed diseased liver (one representative plots shown from 8 diseased livers studied and 7 normal livers). Liver infiltrating T_{reg} (LIT_{reg}) were identified by gating on liver infiltrating lymphocytes $CD3^{high}$ $CD4^{high}$ followed by $CD25^{high}$ (Y axis) and $CD127^{low}$ (X axis).

Percentage of $CD4^+CD25^{high}CD127^{low}$ T_{reg} in different liver diseases was shown in Table 3.2. The frequency was much greater in all diseased livers compared to normal liver. Majority, >90% of these $CD4^+CD25^{high}CD127^{low}$ cells are FoxP3 positive by intracellular transcription factor staining.

Disease state	% of $CD25^{high}$ $CD127^{low}$
Normal Liver	1.24± 0.3
Chronic hepatitis C	4.3±1.62
Non alcoholic steatohepatitis	4.3±1.17
NonA nonB/ Seronegative hepatitis	6.1±1.98
Autoimmune hepatitis	5.2±1.2
Primary Biliary cirrhosis	4.8±1.6
Alcoholic liver disease	3.4±1.72

Table 3-2 Percentage expression of $CD4^+CD25^{high}CD127^{low}$cells in normal liver and different diseased livers.

Percentage expression of liver infiltrating regulatory T cells in normal and different diseased livers was shown. Data were analysed from flow cytometry on freshly isolated liver infiltrating lymphocytes using CD4, $CD25^{high}$ and $CD127^{low}$ population. Mean ± SD (N=2 for NonA NonB hepatitis; N=2-4 for each diseased livers and N= 5 for normal livers)

3.2.3 Distribution of FoxP3$^+$ cells in different liver diseases

Immunohistochemical staining suggested that T_{reg} are present in both septal and parenchyma areas. In order to assess the distribution of FoxP3$^+$ T_{reg} in hepatic inflammation, the staining of cells on different liver diseases was quantified in the portal tract and the hepatic parenchyma separately. Numbers of CD3 and FoxP3 cells are counted in same area of portal tract and parenchymal in five high power fields for normal and each diseased livers. Average values were calculated and the ratio of CD3 lymphocytes which are positive for FoxP3 cells was calculated for portal tract and parenchyma.

Percentage of FoxP$^+$ cells in relation to CD3 suggested that FoxP3$^+$ T_{reg} were found at higher frequencies in the parenchyma compared with portal tracts in patients with seronegative hepatitis and autoimmune hepatitis whereas in the other liver diseases and in normal liver, FoxP3$^+$ T_{reg} were detected at higher frequencies in portal tracts (Fig. 3.6)

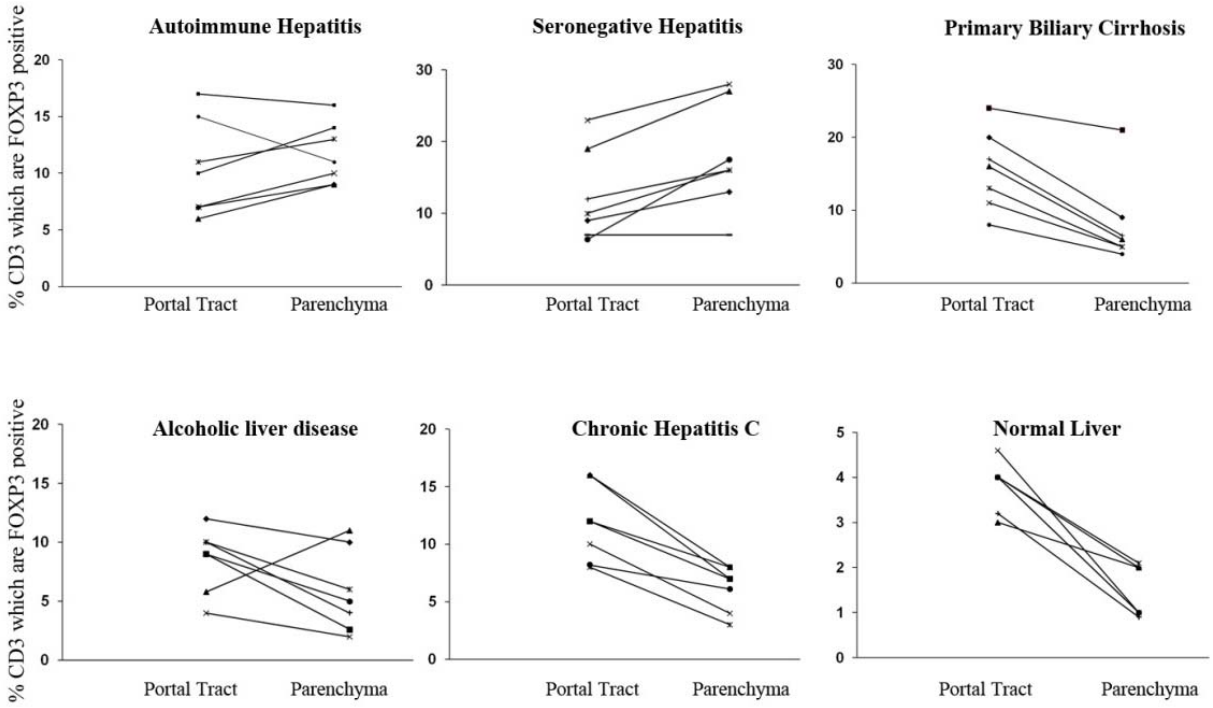

Figure 3-6 FoxP3$^+$ T_{reg} in parenchyma and portal tract of different diseased livers and normal liver.

Percentage of FoxP3$^+$ cells in relation to CD3 in portal tract and parenchyma are shown for normal liver and different chronic liver diseases (autoimmune hepatitis, seronegative hepatitis, primary biliary cirrhosis, alcoholic liver disease, chronic hepatitis C, normal liver). Autoimmune hepatitis, seronegative hepatitis cases have higher preponderance of FoxP3$^+$ cells in parenchyma compared to portal tract. (N=7 for normal liver and diseased livers)

3.2.4 T effector cells in chronically inflamed human livers

Chronic hepatic inflammation is characterised by infiltration by both CD4 and CD8 lymphocytes. Thus, I went on to study the presence of these T effector lymphocytes in the liver. Immunohistochemical and confocal staining suggested that both CD4 and CD8 subsets are present in inflamed liver (Figure 3.7 and Figure 3.8).

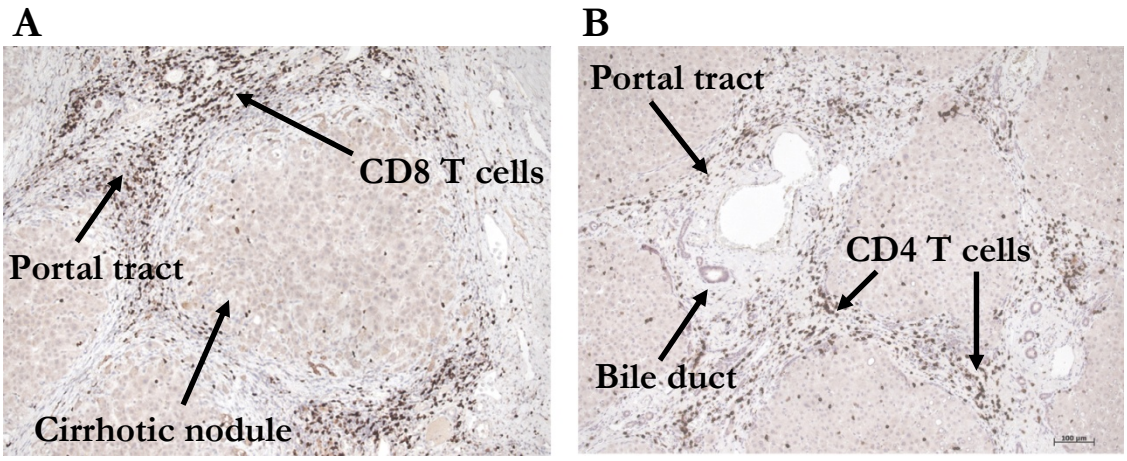

Figure 3-7 CD4 and CD8 effector T lymphocytes in inflamed liver.

Immunohistochemistry of inflamed diseased liver tissue sections illustrating $CD8^+$ Tcells (A) and $CD4^+$ T cells (B) in inflamed liver tissues. Cells were shown with arrows. Image of a patient with HCV was shown. Images were captured with x20 lens (HCV=chronic hepatitis C).

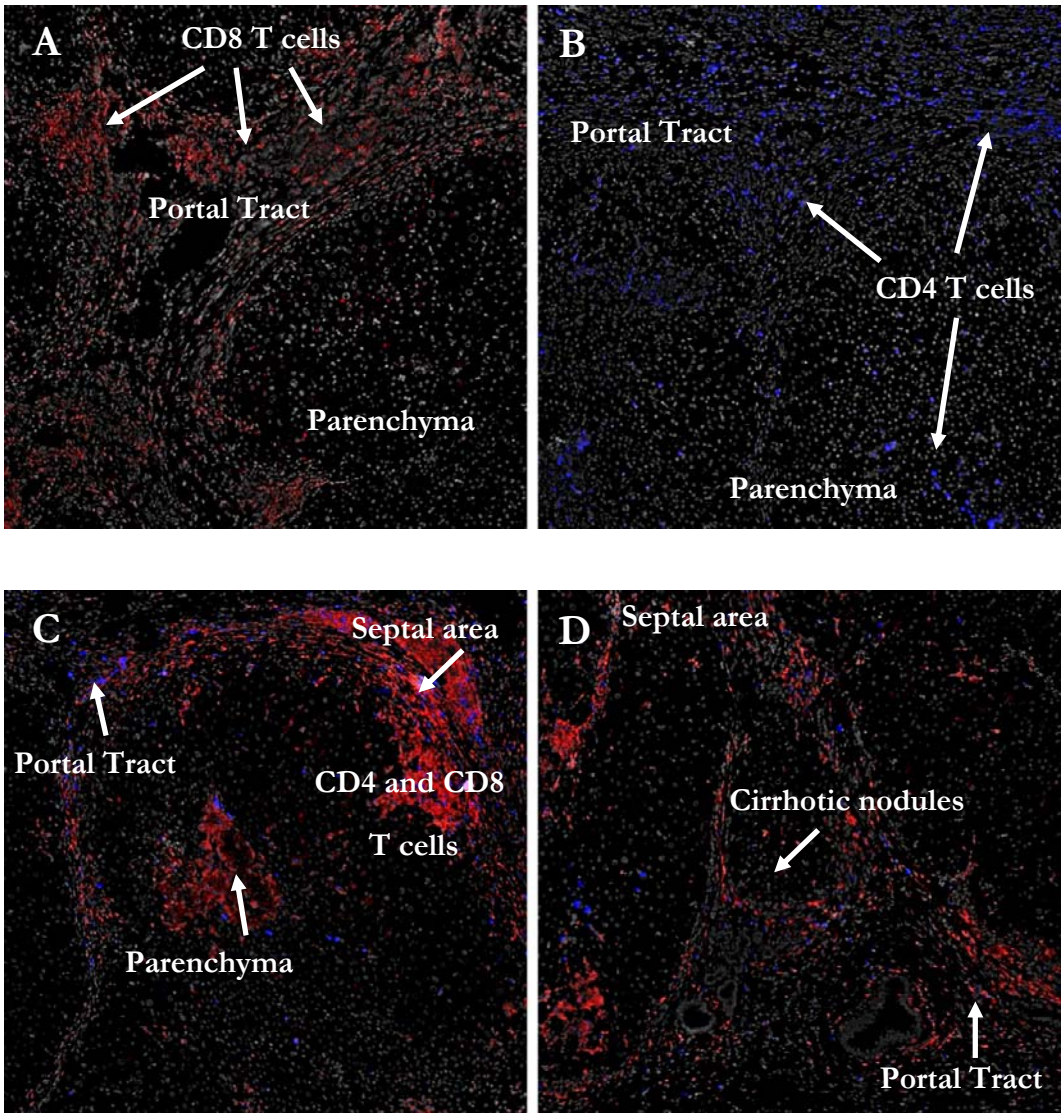

Figure 3-8 Confocal images of CD4 and CD8 T cells in inflamed human liver.

Presence of CD8 (Panel A, red colour) or CD4 (Panel B, blue colour) T lymphocytes in inflamed parenchyma, septal area and portal tract is shown in this three colour confocal images. CD8 and CD4 together (Panel C&D, both red and blue colour) was noted in septal area, portal tract, parenchyma. Nuclear staining was done with DAPI=Grey. (Confocal images of a patient who was transplanted for autoimmune hepatitis was shown)

T effector cells involved in inflammation also include Th1 cells and also the recently described Th17 subset. I detected Th1 CD4 and Th1 CD8 cells defined by their expression of IFN-γ and TNF-α by intracellular cytokine (Fig.3.9). Presence of IFN-γ and TNF-α in Th1 cells in diseased livers were analysed by stimulating freshly isolated liver infiltrating lymphocytes with PMA and ionomycin and then secretion of the cytokines were blocked with BrefeldinA. Figure 3.9 demonstrate the Th1 cytokine expression of liver infiltrating CD4 and CD8 T cells and Table.3.3 showed percentage expression from each donor.

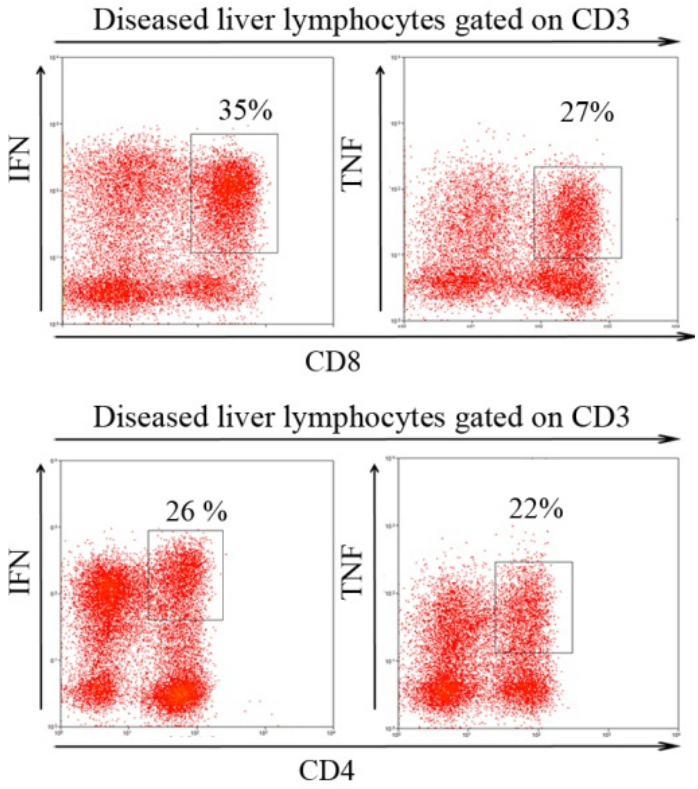

Figure 3-9 Th1 cells in inflamed human liver.

Flow cytometry dot plot analysis of Th1 lymphocytes isolated from a diseased liver (primary biliary cirrhosis). Th1CD8 cells (top panel) and Th1CD4 cells (lower panel) expressing IFN-γ and TNF-α are shown. Cells were gated on freshly isolated liver infiltrating lymphocytes and regated on CD3 lymphocytes. Intracellular cytokine staining was done after stimulating liver infiltrating lymphocytes with PMA and ionomycin for 5 hours. (N=4, diseased livers and N=3, normal livers)

Disease state	CD8 IFN-γ	CD8 TNF-α	CD4 IFN-γ	CD4 TNF-α
Alcoholic liver disease	34%	24%	18%	18%
Autoimmune hepatitis	31%	31%	19%	19%
NonA nonB/ Seronegative hepatitis	51%	44%	19%	20%
Primary Biliary Cirrhosis	35%	27%	26%	22%

Table 3-3 Percentage expression of CD8 IFN-γ, CD8 TNF-α, CD4 IFN-γ and CD4TNF-α in inflamed livers.

Liver infiltrating lymphocytes were freshly isolated and stimulated with PMA and inomycin for 5 hours and then cells were stained for surface markers, CD3, CD4, CD8 and intra cellular cytokines IFN-γ and TNF-α and analysed on flow cytometry for Th1 cytokine expression. Experiments were done on one donor from each different explanted diseased liver.

In addition to Th1 cells, I investigated interleukin-17 expressing CD4 T cells (Th17) (Figure 3.10) and interleukin-17 expressing CD8 T cells (Tc17) cells (Figure 3.11) in chronic liver diseases studied. Intracellular cytokines staining for IL-17 were performed after stimulating the cells with PMA and Ionomycin for 5 hours. Percentage expression of Th17 and Tc17 from normal and diseased livers is shown in Table 3.4.

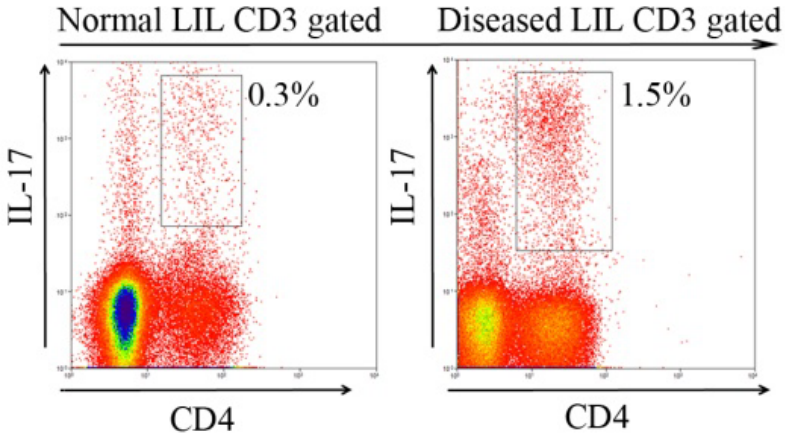

Figure 3-10 Th17 cells in normal and diseased livers

Flow cytometry dot plot showing the presence of Th17 lymphocytes isolated from normal liver and diseased liver (primary biliary cirrhosis as example). Cells were gated on freshly isolated liver infiltrating lymphocytes and then re-gated on CD3 lymphocytes (one representative example of 6 experiments). These cells were stimulated with PMA and ionomycin for 5 hours before labelling.

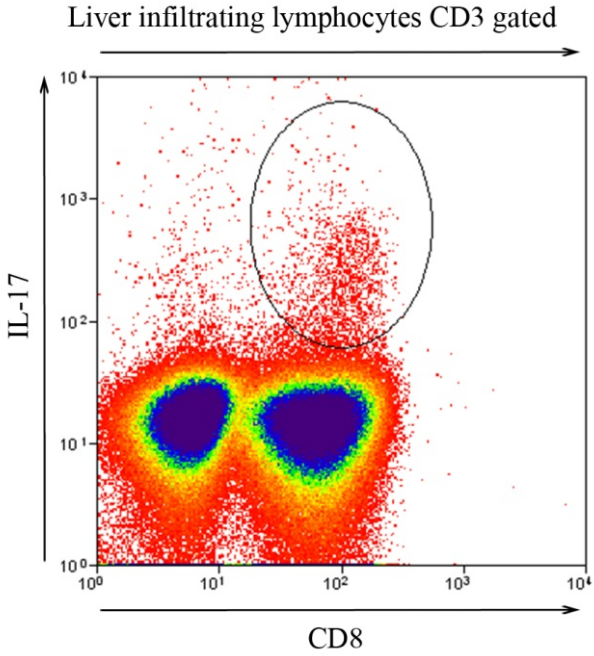

Figure 3-11 Tc17 cells in inflamed liver

Presence of Tc17 in diseased liver is analysed by flow cytometry. Cells were gated on liver infiltrating lymphocytes and re-gated on CD3 lymphocytes. Explanted liver from a patient with autoimmune hepatitis is shown. Cells were stimulated for 5 hours before labelling.

Disease state	CD4 Th17	CD8 Tc17
Normal Liver 1	0.37%	0.1%
Normal Liver 2	0.4%	0.12%
Normal Liver 3	0.52%	0.14%
Autoimmune hepatitis	2.2%	0.6%
Primary Biliary Cirrhosis	1.6%	0.4%
Alcoholic liver disease	1.7%	0.5%
NonA nonB/ Seronegative hepatitis	2.3%	0.6%
Chronic Hepatitis C	1.5%	0.6%

Table 3-4 Percentage expression of Th17 and Tc17 in normal and different diseased livers.

Liver infiltrating lymphocytes from normal liver and diseased livers are freshly isolated from explanted donors. Then cells were stimulated with PMA and ionomycin for 5 hours and intracellular cytokines staining with IL-17 was performed along with surface staining for CD3, CD4 and CD8. Percentage expression of both Th17 and Tc17 was shown for normal and diseased livers.

3.2.5 Defining Regulatory T cells

Both liver and blood-derived T_{reg} were defined by the characteristic phenotype $CD4^{+}CD25^{high}$ $CD127^{low}FoxP3^{+}$ (Liu et al., 2006;Sakaguchi, 2004;Seddiki et al., 2006). Liver-infiltrating lymphocytes are freshly isolated from explanted human liver tissue by mechanical digestion and density gradient centrifugation for each experiment before being stained with antibodies against CD3, CD4, CD25, CD127 and the intracellular transcription factor FoxP3. More than 95% of $CD4^{+}CD25^{high}$ $CD127^{low}$ liver-infiltrating lymphocytes expressed FoxP3 (Fig.3.12).

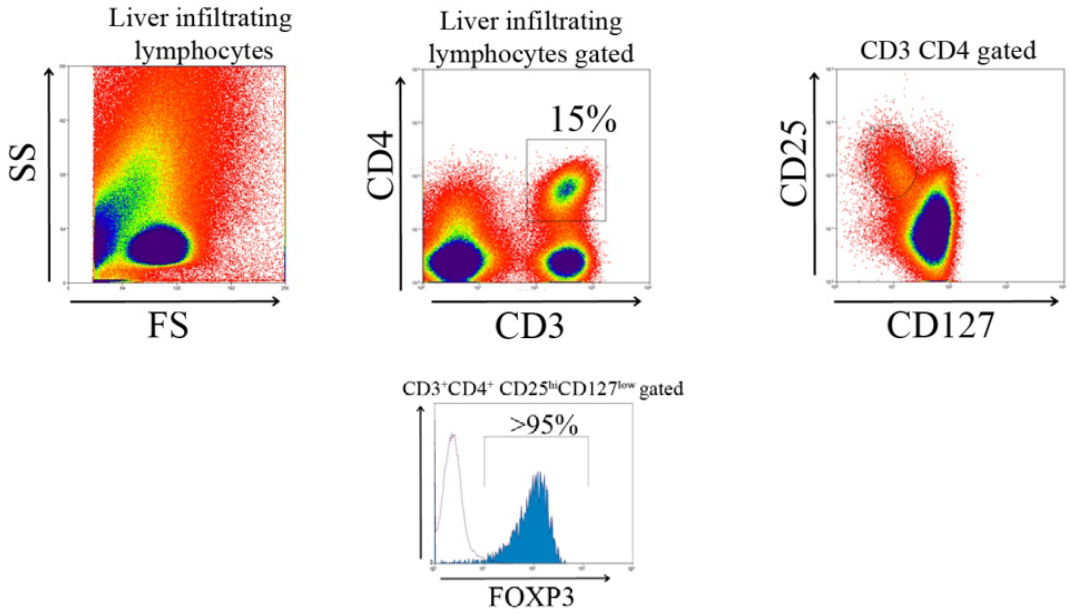

Figure 3-12 Phenotyping of liver infiltrating regulatory T cells by flow cytometry.

Human liver infiltrating lymphocytes were stained for cell surface expression of CD4, CD25, and CD127. The stained cells were fixed, permeabilized and stained intracellularly for FoxP3. For analysis, liver infiltrating lymphocytes were gated on forward scatter and side scatter and liver infiltrating T_{reg} (LIT_{reg}) identified by gating on liver infiltrating $CD3^{high}$ $CD4^{high}$ lymphocytes followed by $CD25^{high}$ (Y axis) and $CD127^{low}$ (X axis). The oval box represents the population of liver infiltrating T_{reg} cells. Histogram shows the expression of transcription factor, FoxP3 which was analysed on liver infiltrating T_{reg} ($CD3^{high}$ $CD4^{high}$ $CD25^{high}$ $CD127^{low}$) by flow cytometry.

3.2.6 Phenotypic characteristic of liver infiltrating regulatory T cells

The vast majority of liver-infiltrating T_{reg} expressed CD45RO and more than 80% expressed high levels of CD27 (Fig.3.13) consistent with a tissue-infiltrating phenotype (Ruprecht et al., 2005a). Furthermore, there is high expression of ectoenzyme CD39 on liver infiltrating T_{reg}. They also express OX-40.

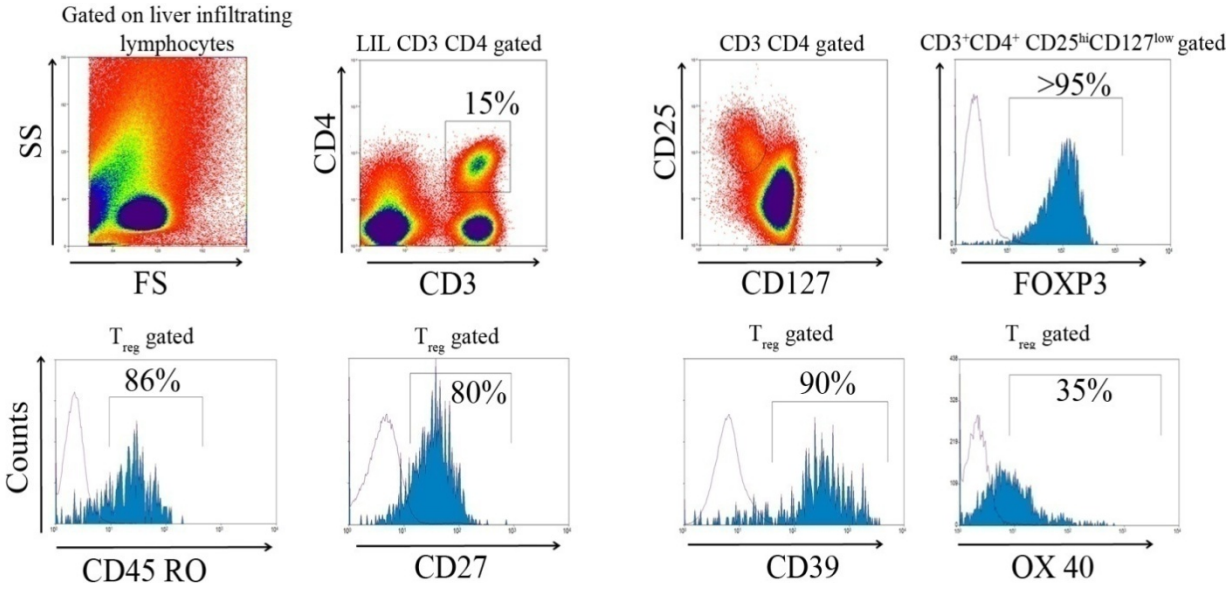

Figure 3-13 Flow cytometry analysis of LITreg phenotypic markers

Flow cytometry overlay histogram shows the percentage positivity of liver-infiltrating T_{reg} for CD27, CD45RO, CD39, and OX-40. The numbers in the histograms indicate the percentage expression of relevant markers. Cells were gated on liver infiltrating $CD3^{high}$ $CD4^{high}$ $CD25^{high}$ $CD127^{low}$ lymphocytes for phenotypic markers analysis. N=6

3.2.7 Positioning of regulatory T cells, dendritic cells and CD8 T cells in the human liver

Multicolour confocal analysis was performed to detect the positioning of liver infiltrating regulatory T cells (LIT_{reg}) with resident CD11c dendritic cells (Figure 3.14); dendritic cells and CD4 and CD8 cells (Figure 3.15). I then went on to perform four colour confocal microscopy to investigate the positioning of these three cell types. LIT_{reg}, dendritic cells and CD8 T cells are in close proximity at sites of inflammation in both inflamed portal tract and parenchyma (Figure 3.16). Quantification of each cell types in relation to anatomical distribution of each diseased liver studied was shown in Table 3.5.

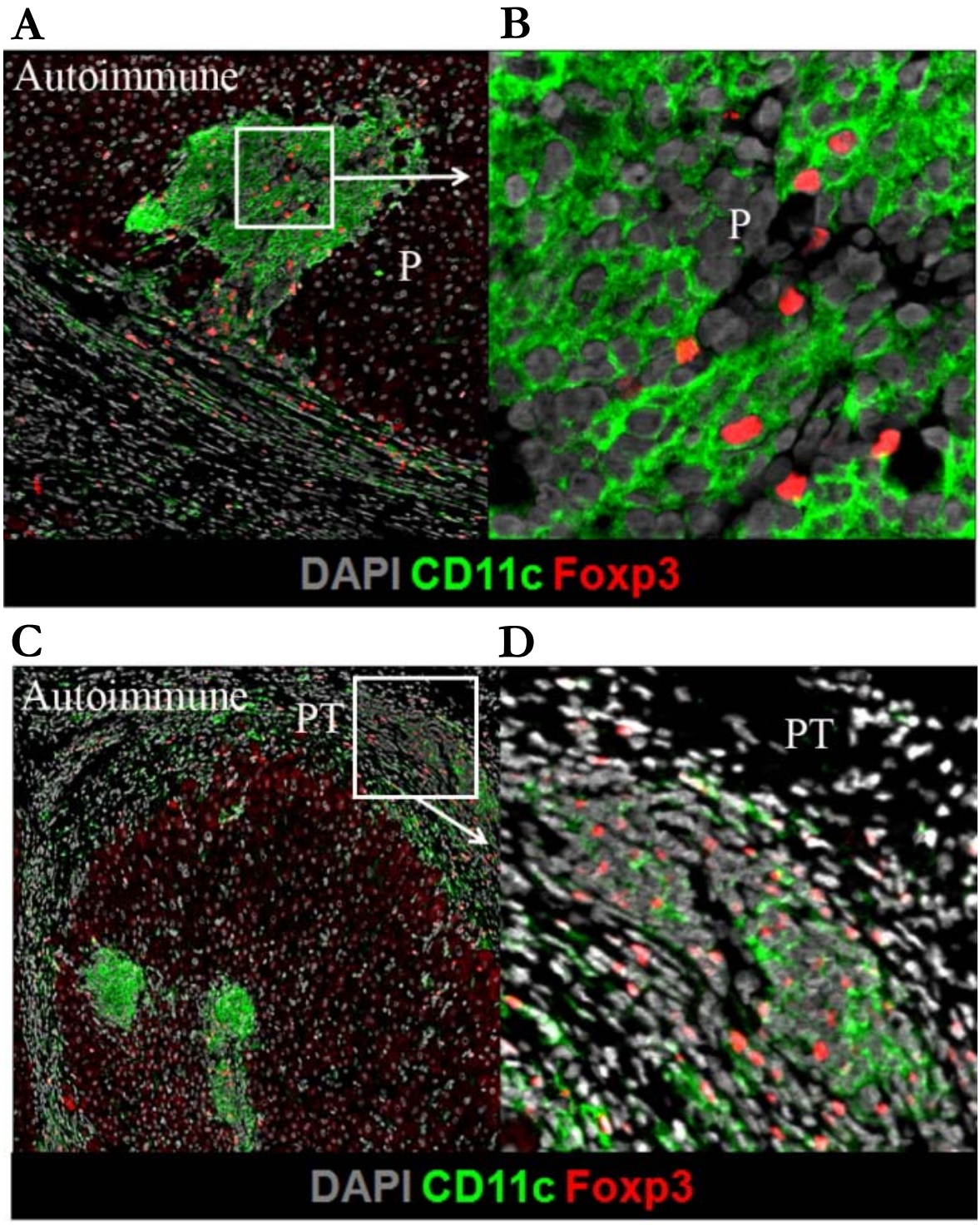

Figure 3-14 Liver infiltrating T_{reg} and liver resident dendritc cells in inflamed human liver portal tract and parenchyma.

Confocal images of the hepatic parenchyma infiltrate, interface hepatitis (A&B) and portal tract (C&D) demonstrating FoxP3 Treg (Red/TRITC) and CD11c dendritic cells (Green/FITC) in close proximity in both the inflamed lobule (B) and portal tract (D). Cell nuclei stained with DAPI (Grey). Staining from a patient explanted liver tissue who had liver transplantation for autoimmune liver disease was shown. P=parenchyma; PT=portal tract.

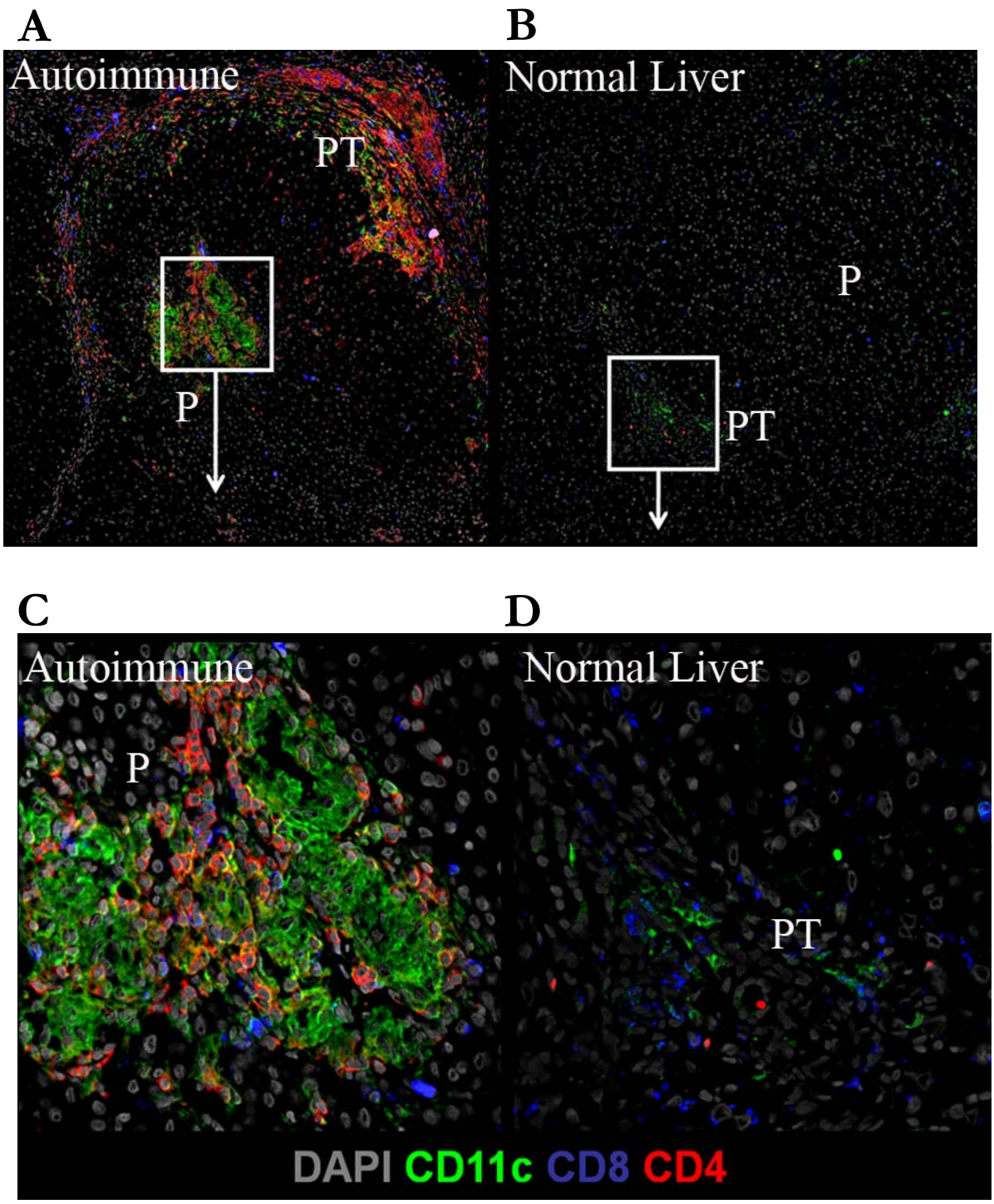

Figure 3-15 Liver infiltrating CD4 and CD8 T cells resides close to resident dendritic cells.

Four colour confocal microscopy of liver infiltrating dendritic cells (Green/FITC), CD4(Red/TRITC), and CD8 (Blue/Cy3) T cells in inflamed portal tract (PT) and parenchyma (P) in a patient with autoimmune liver disease (A&C) and normal liver (B&D). CD8 and CD4 T cells are detected in close proximity with CD11c dendritic cells. P=parenchyma; PT=portal tract. Top panel magnificationx25; lower panel magnification=x40

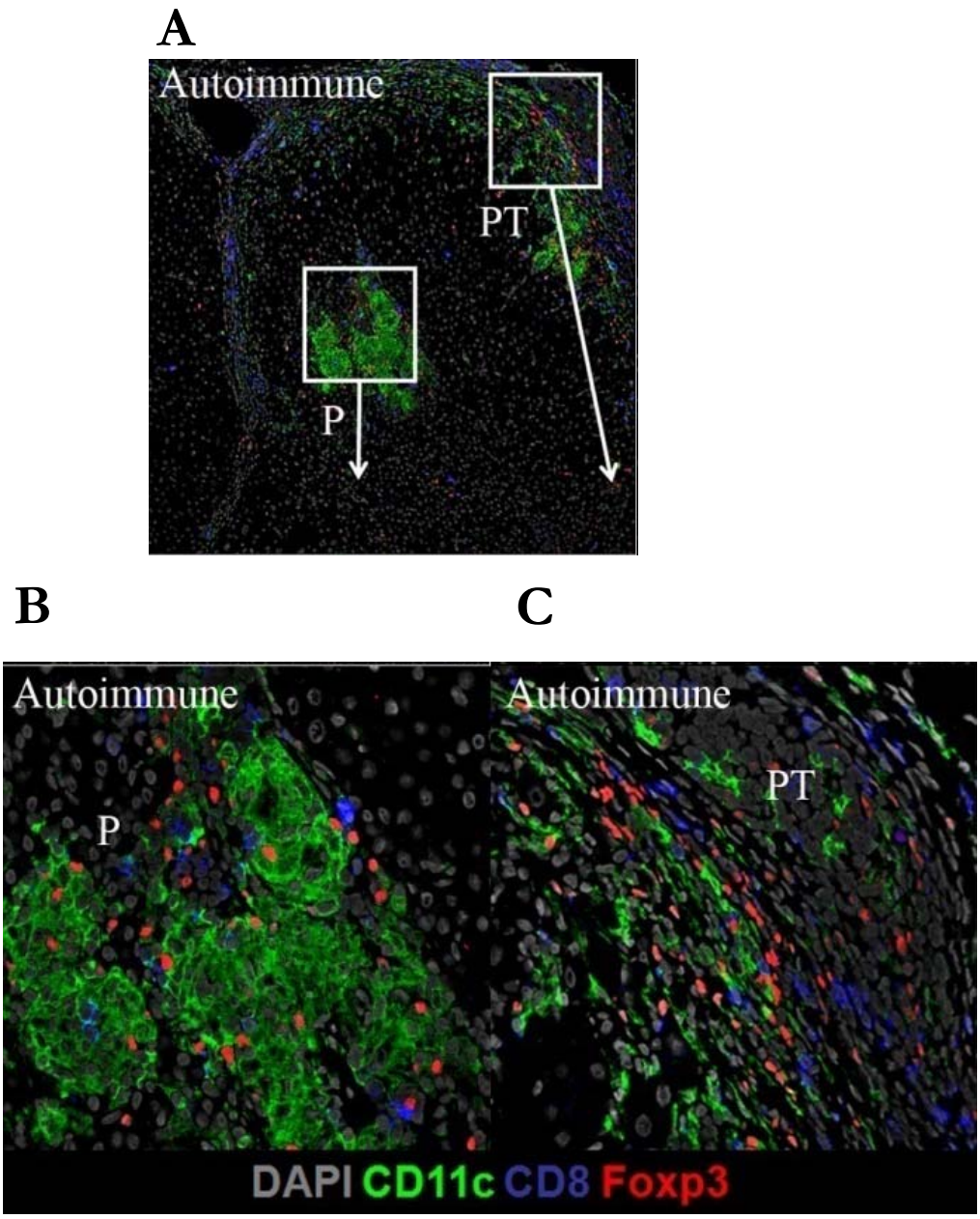

Figure 3-16 CD11c dendritic cells, CD8 T cells and FoxP3 T_{reg} in human liver.

Four colour confocal images of inflamed liver portal tract (PT) and lobular hepatitis in parenchymal area (P) showing LIT_{reg}, CD11c dendritic cells and CD8 T cells (Figure A). Explanted liver tissue from autoimmune hepatitis liver was shown. Figure B is the higher magnification of lobular hepatitis in parenchyma (P) and Figure C is the higher magnification of portal tract (PT). Figure A was captured with x25 and Figure B&C were captured with x40 magnification.

T_{reg} (FoxP3; TRITC, Red), (CD8, Blue, Cy5), (CD11c, Green, FITC); (cell nuclei, DAPI, Grey)

Types of cells	Portal Tract	Parenchyma	Septal area
CD8	31±7	18±5	27±5
HCV1	26	12	28
HCV2	35	16	32
PBC	34	19	28
AIH1	38	25	30
AIH2	22	20	19
FoxP3	21±4	5±1	16±4
HCV1	19	4	14
HCV2	23	6	18
PBC	18	4	12
AIH1	28	5	23
AIH2	18	4	14
Dendritic cells	23±8	17±6	21±8
HCV1	14	9	8
HCV2	22	12	21
PBC	19	20	24
AIH1	35	24	30
AIH2	26	19	23

Table 3-5 Localization and frequency of different cell types in inflamed human liver

Total numbers of individual cells were counted in high power field of confocal image of liver tissues from patients transplanted for chronic hepatitis C, N=2; primary biliary cirrhosis, N=1 and autoimmune hepatitis, N=2.

3.2.8 Liver infiltrating regulatory T cells are functional

3.2.8.1 Liver infiltrating regulatory T cells suppress proliferation of allogenic T responder cells in vitro

To investigate whether liver infiltrating regulatory T cells are functional, allogeneic stimulation/suppression assays were used with ^{3}H thymidine incorporation. These confirmed the ability of liver infiltrating T_{reg} to suppress T responder cell proliferation *in vitro* at ratios of 1 to 4 (Fig 3.17 and Table 3.6).

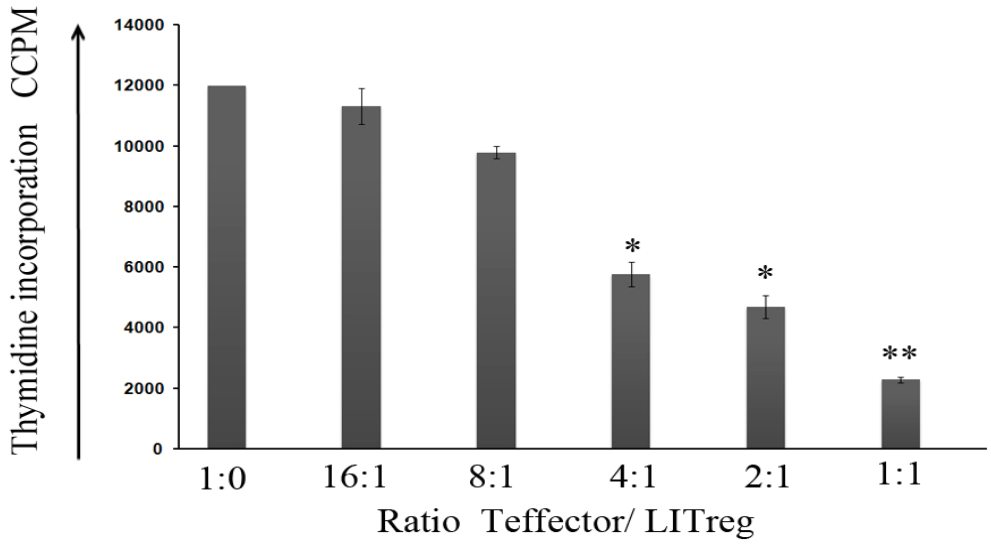

Figure 3-17 LIT_{reg} are suppressor to autologous T responder cells

The suppressive function of freshly isolated human liver-infiltrating T_{reg} was assessed by ^{3}H thymidine incorporation assay. Liver infiltrating T_{regs} from diseased livers were freshly isolated using the Dynal magnetic T_{reg} isolation kit. Suppression of proliferation was done ratios of 1:16 to 1:1 freshly isolated liver infiltrating T_{reg} suppress proliferation of autologous CD4 $CD25^{-}$ lymphocytes activated by allogenic liver-derived DC (p by Student's t test * $p < 0.05$, **$p < 0.005$). Data represent mean± SEM of 3 independent experiments.

Liver Treg	T responder	Treg: Tresponder	CCPM Exp 1	CCPM Exp 2	CCPM Exp 3	Mean	SEM
100000	100000	1:1	1902	2345	2146	2131	130
50000	100000	1:2	4078	4354	5420	4617	416
25000	100000	1:4	4996	5363	6520	5626	467
12500	100000	1:8	9102	9643	10206	9650	324
6250	100000	1:16	11102	10904	13120	11708	721

Table 3-6 Suppression of proliferation assay using liver infiltrating regulatory T cells and autologous responder cells.

Freshly isolated liver infiltrating regulatory T cells were used to assess the suppressive function. Suppression of proliferation was done ratios of 1:16 to 1:1 freshly isolated liver infiltrating T_{reg} vs. autologous T responder cells which were activated by allogenic liver-derived DC. ^{3}H thymidine incorporation was used. Data from three diseased livers was shown. Exp= experiment.

3.2.8.2 STAT5 signalling in liver infiltrating regulatory T cells

IL-2 is critical for the differentiation and survival of T_{reg}. Binding of IL-2 to its receptor, CD25 on T_{reg} leads to STAT5 phosphorylation which is a critical factor in T_{reg} differentiation and expression of FoxP3. Thus, to determine whether T_{reg} within liver tissue are functional, we stained for phosphoSTAT5 on serial liver tissue sections and quantified the percentage of FoxP3 cells which contained phosphoSTAT5. Only 5-15% of the liver infiltrating $FoxP3^+$ cells expressed phosphoSTAT5 (Fig. 3.18).

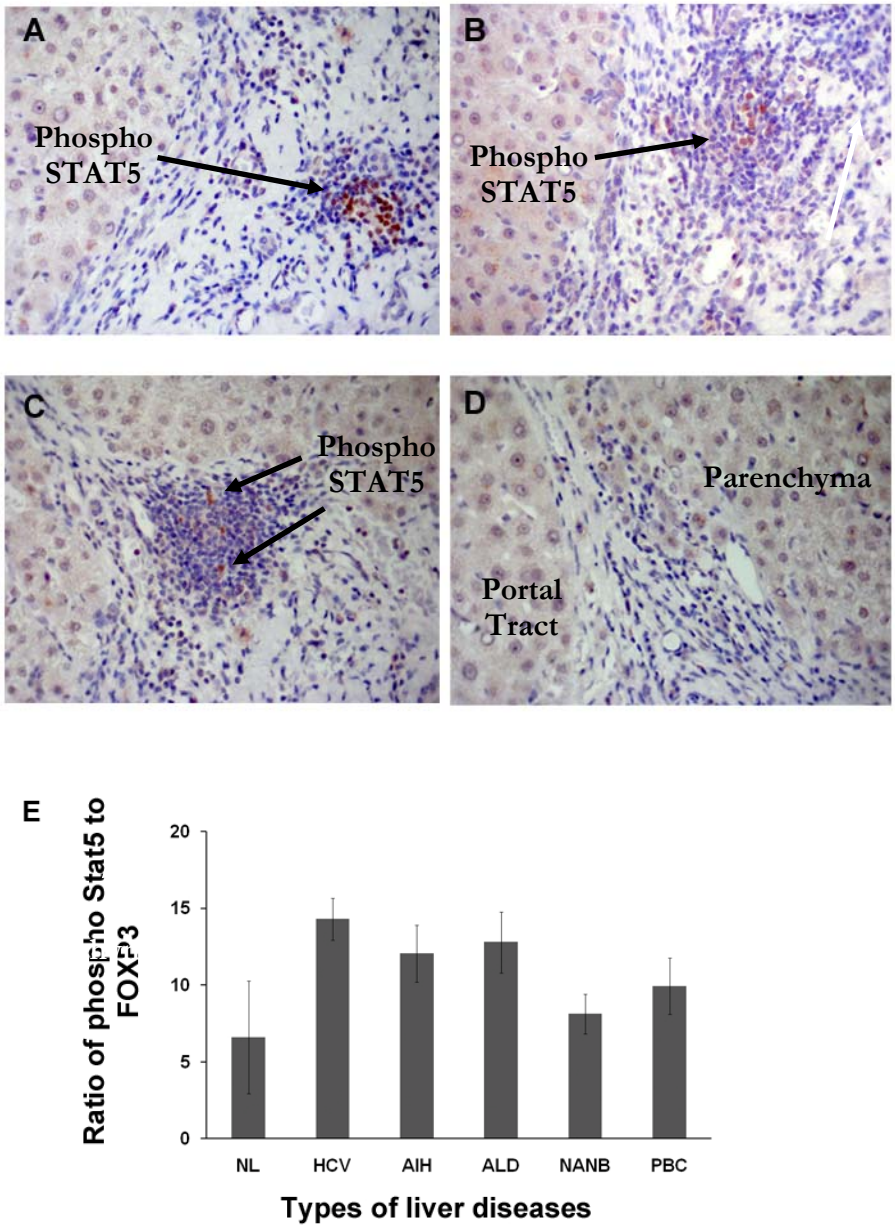

Figure 3-18 Presence of phosphoSTAT5 in inflamed human liver

A-D) Single immunohistochemical staining of formalin-fixed, paraffin-embedded liver sections showed phophoSTAT5 staining using monoclonal antibody (shown with arrows, detected as a brown pigment) in patients with autoimmune hepatitis (A &C) liver and alcoholic liver disease (B). Pictures were taken at x 20 magnification. Control sections are staining was negative (D).

Percentage of FoxP3 cells which are phosphoSTAT5 positive in normal and inflamed liver is shown (E). Serial liver sections were stained with FoxP3 & phosphoSTAT5 antibodies. Numbers of positive staining were counted from the same areas and % positivity of phosphoSTAT5 in relation to FoxP3 was calculated (n=6 for each diseased livers and normal liver).

3.3 DISCUSSION

The liver is one of the most tolerogenic organs in the body despite being constantly exposed to antigens from the gut (Adams & Eksteen, 2006;Adams and HUTCHINSON, 1997;Knolle and Gerken, 2000). The intrahepatic immune environment exhibits dichotomy as it is not only associated with the induction of tolerance but can also mount effective immune responses against invading organisms.

It has been described that both resident tolerogenic dendritic cells (Knolle & Gerken, 2000;Munn et al., 2002;Munn, 2002;Thomson & Lu, 1999a) and regulatory T cells play an important role in maintaining this tolerance (Sakaguchi, 2005). In addition to passive mechanisms of unresponsiveness such as ignorance and anergy, naturally occurring, thymic derived, $CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ T_{reg} are essential in maintaining peripheral tolerance by actively suppressing the cytokine production and proliferation of T effector cells (Sakaguchi et al., 1995;Sakaguchi, 2004). Hence, a thorough examination of the presence, distribution, phenotype, function of these liver infiltrating T_{reg} is necessary to determine whether these cells are involved in intrahepatic tolerance under homeostatic conditions and in the context of established chronic inflammation.

In an attempt to identify and characterize intrahepatic regulatory T cells, I have utilized normal liver tissues from organ donors, normal margins of hepatic resections, and explanted liver tissues from individuals who underwent liver transplantation for end-stage liver cirrhosis. I have confirmed that T_{regs} are present within both normal and inflamed human liver tissue characterised by three surface markers CD4, $CD25^{high}$, $CD127^{low}$ as described (Liu et al., 2006;Sakaguchi et al., 1995;Seddiki et al., 2006). Moreover, > 95% of liver infiltrating CD4, $CD25^{high}$, $CD127^{low}$ express the T_{reg} signature transcription factor $FoxP3^+$, which controls the development and function of these cells, as previously reported (Hori et al, 2003).

By immunohistochemistry, the infiltrate contained a 4 fold greater proportion of $FoxP3^+$ T_{reg} to CD3 cells in chronically inflamed livers compared with non-diseased livers. Indeed, only 3% of CD3 cells co-expressed $FoxP3^+$ in the normal liver, but up to 15% of CD3 cells in the inflamed liver co-expressed $FoxP3^+$. A similar difference in T_{reg} ($CD4^+CD25^+FoxP3^+CD127^{low}$) frequency was noted by FACS analysis of total liver tissues (1.5% vs. 6% of LIL in normal and disease livers, respectively). However, fewer T_{reg} were detected by FACS compared with immunohistochemistry. One explanation would be that $FoxP3^+$ immuno-reactive cells include peripherally induced T_{reg} in TGF-β rich hepatic microenvironment, but this is unlikely as the majority of $FoxP3^+$ liver-derived lymphocytes were $CD4^+CD25^+CD127^{low}$. More likely, the protocol used to isolate liver infiltrating lymphocytes which used a mechanical rather than enzymatic digestion to preserve cell surface receptors may not completely retrieve all liver-infiltrating T_{reg}. Although the proportions of T_{reg} varied between the two methods, the overall results were similar with the highest frequencies detected in patients with fulminant seronegative hepatitis, the most florid and destructive form of hepatitis which leads to liver failure. These findings suggest that T cell-mediated hepatitis and liver destruction is not a consequence of a failure of T_{reg} to infiltrate the liver but rather an inability of the recruited cells to control the effector arm of the response.

$FoxP3^+$ T_{reg} contribute to viral persistence by suppressing immune response in chronic hepatitis B and chronic hepatitis C (Franzese et al., 2005;Stoop et al., 2005) and also inhibit anti-tumour immune responses mediated by effector CD8 T cells in hepatocellular carcinoma (Fu et al., 2007;Unitt et al., 2005). Previous studies have suggested there is an increased frequency of T_{reg} in chronic HCV and HBV. However, I did not see higher frequencies compared with autoimmune diseases in which it has been described that T_{reg} are reduced in frequency and function (Longhi et al., 2004;Longhi et al., 2005b). In fact, I detected higher frequencies of

FoxP3$^+$ T_{reg} in autoimmune disease compared with chronic viral infection. A possible explanation is that the increased numbers of liver infiltrating T_{reg} are part of a failed regulatory arm in the face of mounting effector T cells recruitment and activation but they suggest T_{reg} numbers are not the cause of the differential immune responses in autoimmune versus chronic viral infection.

A higher proportion of the T cells infiltrate in the hepatic parenchyma or inflamed lobules were FoxP3$^+$ T_{reg} in autoimmune hepatitis and seronegative hepatitis. On the other hand, other chronic liver diseases such as alcoholic liver disease, hepatitis C and primary biliary cirrhosis there was high preponderance of FoxP3$^+$ T_{reg} in portal tracts. This likely reflects the more severe lobular inflammation and interface hepatitis in autoimmune hepatitis and seronegative liver diseases will require more suppression by regulatory T cells.

The outcome of hepatic inflammation will be determined by the balance of effectors, and regulatory cells activated and recruited to the liver. Chronically inflamed liver is infiltrated by effector T lymphocytes including CD4 Th1 and CD8 Th1 cells which play a major role in acute and chronic hepatitis (Adams and Hubscher, 2006;Heydtmann et al., 2006;Shields et al., 1999). The recently described Th17 has been implicated in autoimmune and chronic inflammatory diseases (Annunziato et al., 2007;Harada et al., 2009;Veldhoen et al., 2006). Polarised Th1 cells in inflamed liver express distinct chemokine receptors CCR5, CXCR3, CXCR6 (Curbishley et al., 2005;Heydtmann et al., 2005;Shields et al., 1999). I found that effector T cells and regulatory T cells were both recruited at site of hepatic inflammation. CD8 Th1 and CD4 Th1 cells were capable of expressing both TNF-α and IFN-γ in hepatic inflammation. However, this potential was found after stimulation with PMA and ionomycin ex-vivo and it may not represent the actual state of inflamed environment. I also detected both CD4 IL-17 secreting cells (Th17) and CD8 IL-17 secreting cells (Tc17) after stimulation in different inflammatory liver diseases.

Immunophenotyping demonstrated that majority of LIT_{reg} are CD45RO memory cells consistent with previous studies of other subsets (Heydtmann et al., 2006;Volpes et al., 1991). These LIT_{reg} cells are $CD27^{high}$ and CD27 expression has been reported as a characteristic of tissue-infiltrating T_{reg} in inflamed synovial tissue (Mack et al., 2009;Ruprecht et al., 2005a). Moreover, CD39, one of the ectonucleotidases which generate pericellular adenosine from extracellular nucleotides is also highly expressed on $LIT_{reg.}$ Binding of adenosine to the adenosine A2A receptors on activated T cells will increase the intracellular cyclic AMP in the responder cells leading to inhibition of T cell proliferation and IL-2 production (Deaglio et al., 2007;Novak & Rothenberg, 1990).

Analysis of the distribution of T_{reg} in diseased and normal liver tissue demonstrated that they are closely associated with $CD11c^+$ DCs and CD8 T cells at sites of inflammation both in portal tracts and in the hepatic lobules. Thus, T_{reg} are ideally situated to mediate suppression of intrahepatic immune responses. High levels of CD39 expression on $FoxP3^+$ T_{reg} suggest that they may need to be in contact with effector cells to maintain full suppression. We have previously described that liver dendritic cells preferentially express immunosuppressive IL-10 (Goddard et al., 2004b) which may play an important role in T_{reg} mediated suppression. Liver derived dendritic cells (Thomson, 2010) and kupffer cells also express indoleamine 2,3-dioxygenase (Munn et al., 2002;Munn, 2002) which is implicated in T_{reg} mediated suppression.

Tissue infiltrating T_{reg} have been shown to suppress effectors T cells in other tissues (Ruprecht et al., 2005b) and could thus contribute to the local control of ongoing inflammation. To confirm the cells I detected in liver tissue were functional, I showed that $CD4^+CD25^+FoxP3^+CD127^{low}$ cells from liver tissue suppressed allogeneic responder cells in *vitro*. However, this does not address whether T_{regs} in tissue are functional. Thus, although these cells are functional *in vitro* it is possible that their function is suppressed within the liver

microenvironment *in vivo*. In order to investigate this possibility I stained the intrahepatic T_{reg} in situ for phosphorylated STAT5. STAT5 phosphorylation is a consequence of IL-2 binding of its receptor, CD25 and thus provides an indication of whether the intrahepatic T_{reg} are activated (O'Gorman et al., 2009). STAT 5 has been described as a critical mediator of T_{reg} biological activity and STAT5 activation promotes T_{reg} differentiation and function while constraining Th17 generation. We quantified the numbers of pSTAT5 positive cells in the liver and found that only 5-15% of the liver infiltrating $FoxP3^+$ cells expressed pSTAT5. Thus it is possible that these LIT_{reg} are suppressed in situ, possibly by PD-1 dependent mechanisms, as has been proposed for chronic HCV infection (Franceschini et al., 2009), allowing the effector arm to drive persistent inflammation. However, it is also possible that low proportion of cells staining with phosphoSTAT5 may be a reflection of the transient nature of phosphorylation and may not be an ideal to investigate the functional property of regulatory T cells. This is the limitation of human study and *in-vivo* study in future may explain these functional aspects of tissue infiltrating T_{reg}.

Taken it all together, I have shown the presence of functional immunoregulatory T_{reg} cells in human hepatic inflammation which express characteristic receptors associated with T_{reg}. The frequency and distribution in the hepatic lobules compared with portal tract varies depending on the nature of inflammatory liver disease. Surprisingly, I did not find low levels of T_{reg} in aggressive autoimmune hepatitis nor did I detect higher frequencies in chronic hepatitis B and C infection. Intrahepatic T_{reg} may limit liver injury by controlling inflammation in diseases such as autoimmune hepatitis leading to stable chronic inflammation. The most aggressive immune-mediated disease I studies fulminant hepatitis had the highest frequency of both IFN-γ and TNF-α expressing T effectors along with $FoxP3^+$ T_{reg} cells at the site of inflammation suggesting both subsets are recruited. The question remains whether the regulatory arm of intrahepatic

lymphocytes is functioning properly or more dominating effector arms plays a role still needs further investigations.

CHAPTER 4 RECRUITMENT AND POSITIONING OF REGULATORY T CELLS IN INFLAMED HUMAN LIVER

4.1 INTRODUCTION

The hepatic tolerogenic environment is required to suppress immune activation as a consequence of constant antigen exposure from the gut. Several mechanisms maintain this tolerogenic environment including tolerogenic hepatic dendritic cells and the tolerogenic consequences of antigen presentation by other liver cell types including sinusoidal endothelium and stellate cells. It has recently become apparent that regulatory T cells also play an important role in controlling intrahepatic immune responses (Crispe, 2009). Naturally occurring, thymic derived CD4, $CD25^{high}$ $FoxP3^{+}$ (Sakaguchi, 2004), $CD127^{low}$ (Liu et al., 2006;Sakaguchi, 2005;Seddiki et al., 2006) T_{reg} are present in the inflamed liver and suppress effector cell proliferation and cytokine production at sites of intrahepatic inflammation. As mentioned in the previous chapter, T_{reg} are crucial in maintaining peripheral immune tolerance to self antigen and in the context of the liver this includes food antigens from the gut and antigens generated by metabolism by hepatocytes. They also limit immune responses to infection to prevent persistent inflammation and autoimmunity and in this context a defect in T_{reg} function has been associated with autoimmune hepatitis and over active T_{reg} has been implicated in the failed immune response and persistent infection in chronic hepatitis B and C infection.

In the healthy organism T_{reg} suppress effector T cell activation in secondary lymphoid tissues to maintain steady-state self-tolerance. However in chronic inflammatory states such as autoimmune hepatitis, effector T cells are activated in local secondary lymphoid tissues by dendritic cells which take up, process and transport antigen from the site of infection or inflammation. Thus, when steady-state self-tolerance is breached as a consequence of infection or chronic inflammation T_{reg} become further activated to allow their migration into peripheral tissue where they limit local bystander and collateral damage.

T_{reg} are detected at sites of inflammation where they suppress immune responses locally (Belkaid, 2008;Cao et al., 2003;Ruprecht et al., 2005b;Strauss et al., 2007;Tang & Bluestone, 2008;van Amelsfort et al., 2004). As naturally occurring regulatory T cells are generated in the thymus, their presence in the liver implies they are recruited from the blood to the site of hepatic inflammation to limit immune damage.

Lymphocyte subsets including T_{reg} display distinct migratory behaviour which is determined by their ability to respond to chemokine and adhesion molecule which provide a tissue "post code" and this in turn depends on the presence of specific combinations of adhesion receptors and chemokine receptors on the T_{reg}. The ability of activated lymphocytes to enter peripheral tissues such as the liver requires the downregulation of the chemokine receptor CCR7 and adhesion molecule L-selectin that promotes entry to secondary lymphoid tissues and the expression of receptors such as CXCR3 and CCR5 and the integrins LFA-1 and VLA-4 which allow the cell to respond to signals at sites of inflammation. Chemokines secreted by the damaged tissues or local innate immune cells such as dendritic cells at the sites of inflammation are crucial in directing the recruitment and positioning of effector cells and regulatory lymphocytes.

Our group has previously shown that CCR10 expressing T_{reg} home to inflamed bile ducts in response to the chemokine CCL28 in the human liver (Eksteen et al., 2006). Moreover, our previous work demonstrated that CXCR3 and CCR5 are highly expressed on liver infiltrating lymphocytes (Shields et al., 1999;Shields and Adams, 2002) and can promote transendothelial migration through liver sinusoids. In other tissues the CCR4 chemokine receptor has been implicated in directing T_{reg} recruitment in inflamed tissue particularly skin (Cavassani et al., 2006;Iellem et al., 2001a;Iellem et al., 2003) but also the joint, mouth (Cardoso et al., 2008a), breast, inflamed gut(Enarsson et al., 2006;Gobert et al., 2009;Yuan et al., 2007), and cardiac allograft tolerance(Lee et al., 2005b).

Chemokines are critical for directing the recruitment of both innate and adaptive immune cells including T_{reg} to the site of inflammation. They are involved in homeostatic and inflammatory trafficking so that T_{reg} are recruited appropriately to lymphoid tissues and to sites of inflammation. Chemokines modulate migration by activating integrins to allow them to bind their ligands and mediate lymphocyte adhesion, arrest under flow and subsequent transmigration. They also activate small GTPases resulting in cytoskeletal reorganization and the induction of a motile migratory phenotype. Different combinations of chemokines, chemokine receptors, integrins and their ligands provide an address/post code to which T_{reg} expressing the appropriate receptors can respond. In the context of hepatic inflammation we have reported that CXCR3 ligands are expressed and presented on activated human hepatic sinusoidal endothelium at sites of inflammation where they can operate to recruit effector cells expressing the CXCR3 receptor (Curbishley et al., 2005). In support of the importance of this pathway the CXCR3 chemokine receptor is highly expressed on liver infiltrating T cells including the $CCR10^{+}$ T_{reg} population (Eksteen et al., 2006).

Dendritic cells are professional antigen presenting cells with the ability to fully activate naive T cells and thereby to shape the outcome of immune activation. They are involved in immune homeostasis, helping to maintain surveillance without triggering inappropriate activation by self-antigens and also implicated in immunopathology. They take up, process and present sampled antigen from peripheral tissue and present the antigen to both effector and regulatory lymphocyte in draining lymph nodes. Dendritic cells are also targets of T_{reg} mediated suppression. CTLA-4 (CD152) and lymphocyte activation gene 3 (LAG3) which are expressed by T_{reg} allow the T_{reg} to interact with CD80 and CD86 costimulatory molecules expressed by dendritic cells. These interactions result in contact dependent suppression of immune responses (Huang et al., 2004;Sakaguchi, 2005) partly by downregulating CD80 and CD86 on DC (Wing et al., 2008) and hampering activation of other T cells by DC. Antigen-activated T_{reg} also exert suppression by the

formation of LFA-1-dependent aggregates which can prevent the activation of immature DCs thus preventing antigen-reactive naïve T cells from being activated resulting in specific immune suppression (Onishi et al., 2008).

Although the phenotype of blood T_{reg} has been described, little is known about the chemokine receptors involved in recruitment and positioning of T_{reg} in chronically inflamed human tissues. I hypothesised that chemokine receptors regulate regulatory T cells recruitment and positioning in inflamed liver.

4.2 RESULTS

4.2.1 Chemokine receptor expression on liver infiltrating T_{reg}

Chemokine receptor expression on liver infiltrating lymphocytes was investigated by using multi-colour flow cytometry. Liver infiltrating regulatory T cells were defined by surface markers CD4, $CD25^{high}$, CD127low. More than 95% of the cells in this population expressed transcription factor FoxP3. Fresh liver infiltrating lymphocytes from explanted liver were isolated using mechanical digestion instead of collagenase to preserve cell surface chemokine receptors (Shields et al., 1999;Shields & Adams, 2002). This is followed by density gradient separation over lympholyte to obtain the liver infiltrating lymphocytes. A panel of antibodies against chemokine receptors (CCR1-CCR10; CXCR1-CXCR6) along with T_{reg} surface markers and FoxP3 were used to stain fresh liver infiltrating lymphocytes. The results for chemokine receptors expression on freshly isolated liver-derived T_{reg} are shown (Figure 4.1).

4.2.2 Higher expression of CCR4 & CXCR3 in inflamed liver infiltrating T_{reg}

Approximately 20% (20±5% STD) of liver-infiltrating T_{reg} expressed CCR7. The majority of LIT_{reg} expressed CCR4 (74±7% SD) and CXCR3 (67±9%SD) (Fig, 4.1). Furthermore, the levels of these receptors were higher on liver-infiltrating T_{reg} isolated from inflamed liver compared with normal liver tissue; (74±7%SD) % vs (32±7SD) % for CCR4 and (67±9% SD) vs (21± 6%SD) for CXCR3 (both $p \leq 0.05$).

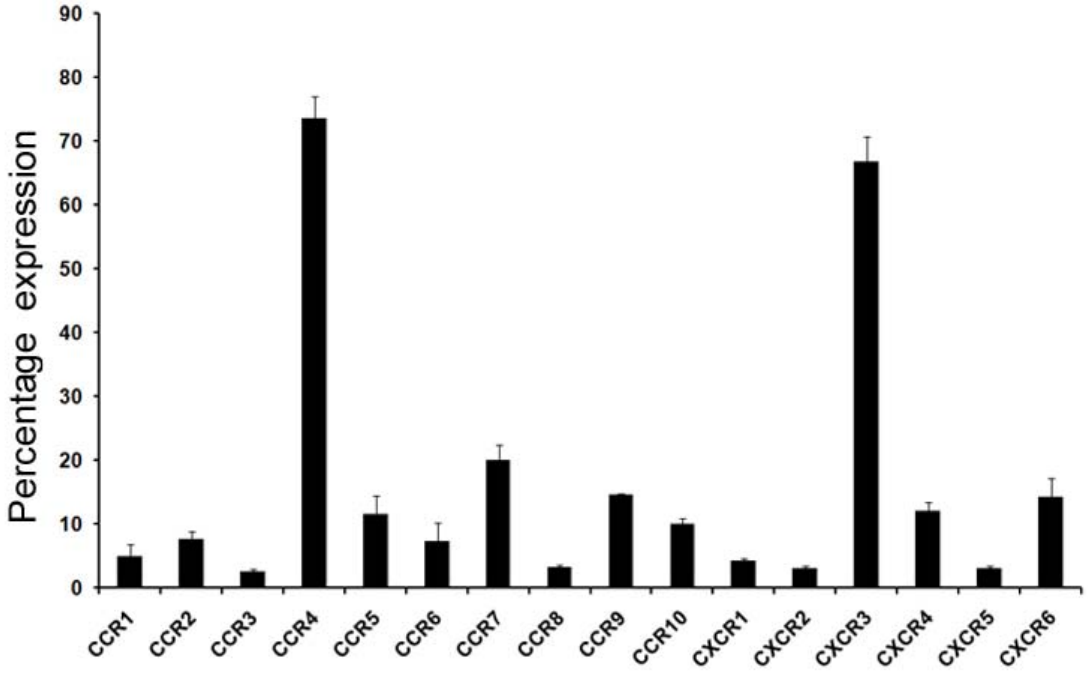

Figure 4-1 Chemokine receptors expression on liver infiltrating regulatory T cells.

Freshly isolated disease liver infiltrating lymphocytes were stained with regulatory T cells marker CD4, CD25, CD127 and FoxP3. $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ T_{reg} were gated for the analysis and this gating was applied to chemokine receptor staining and compared with control. As shown in the figure, the percentage of LIT_{reg} expressing CCR4 and CXCR3 were much higher compared to other chemokine receptors. CCR7 expression was also detected and there is also moderate level expression of CCR5, CCR6, CCR9, CCR10, CXCR4 and CXCR6. Data represents mean±SEM for 6 experiments from patients transplanted for primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, cryptogenic cirrhosis.

4.2.3 CCR4 and CXCR3 expression in human liver

Similar proportion of liver infiltrating T_{reg} or liver-infiltrating $CD4^{+}CD25^{low}$ effector cells expressed CXCR3 (Figure 4.2) but more liver infiltrating T_{reg} expressed CCR4 (74%) compared with liver-infiltrating $CD4^{+}CD25^{low}$ cells from diseased livers (40%) suggesting that CCR4 is preferentially expressed on liver-infiltrating T_{reg} (Figure 4.2).

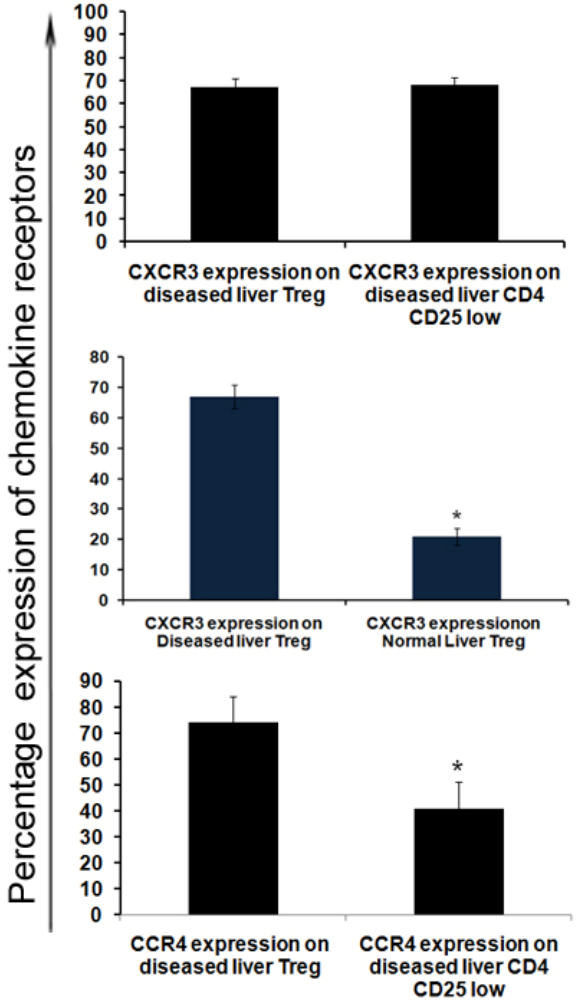

Figure 4-2 High expression of CCR4 and CXCR3 on liver infiltrating regulatory T cells.

CXCR3 expression on LIT_{reg} was analysed with flow cytometry. The expression of CXCR3 on LIT_{reg} was compared with liver infiltrating $CD4^{+}$ $CD25^{low}$ in diseased livers and there was no difference in expression between LIT_{reg} 67% (±4SEM) and $CD4^{+}$ $CD25^{low}$ 68% (±3SEM). However, significantly higher expression of CXCR3 was observed on diseased LIT_{reg} compared to normal liver (67% vs. 21%). Data represent mean ±SEM of 4 experiments.

4.2.4 CXCR3 and CCR4 expression in matched samples.

Circulating and liver-derived T_{reg} of patients from matched blood and liver samples were compared to detect whether CCR4 and CXCR3 have higher expression in inflamed liver (Fig 4.3). 82±7 % of liver-infiltrating T_{reg} stained for CCR4 vs. 67±6 % in matched blood T_{reg} and 66±6% of liver-infiltrating T_{reg} stained for CXCR3 compared with 43±7% of matched blood T_{reg} ($P<0.05$).

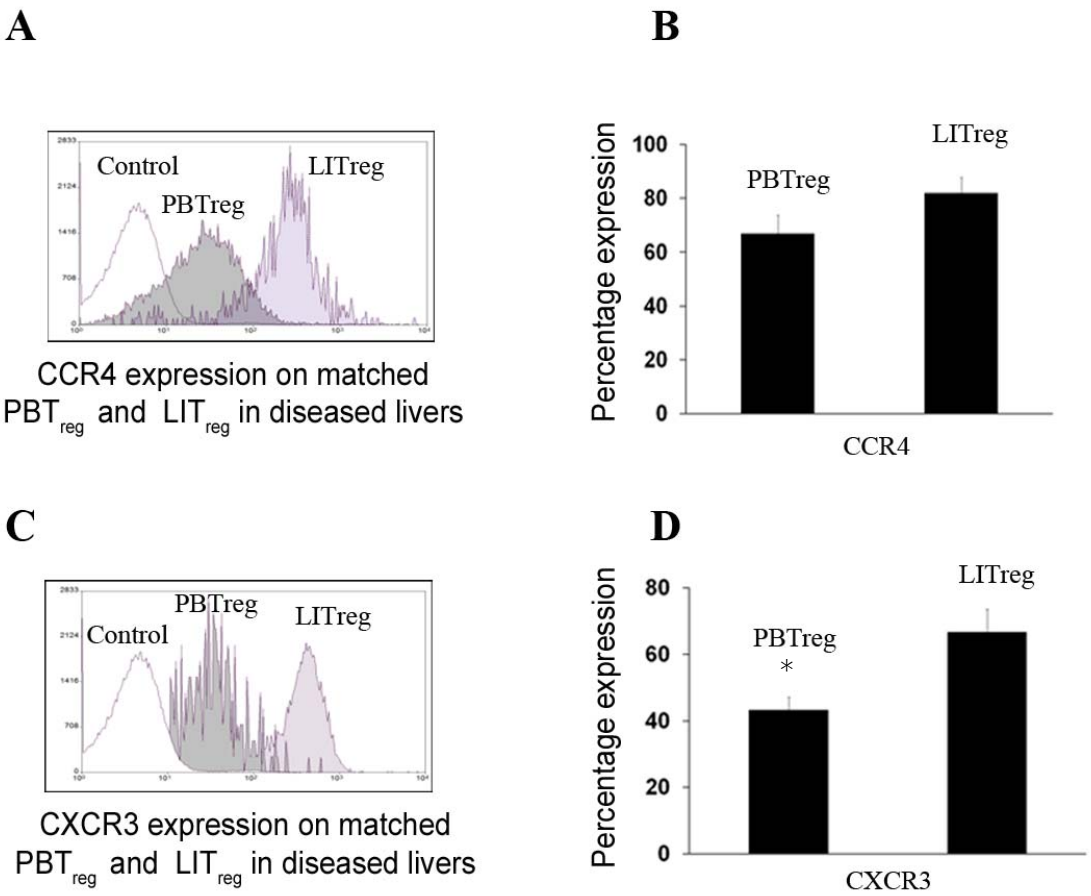

Figure 4-3 Expression of CCR4 and CXCR3 in matched patients.

CCR4 expression overlay histogram in peripheral blood T_{reg} and liver T_{reg} in matched patient who underwent liver transplantations (A) and percentage expression of CCR4 in matched samples (B). Figure (C) showed CXCR3 expression overlay histogram in matched patient and and CXCR3 frequency on peripheral blood T_{reg} and LIT_{reg} from explanted livers in same patients. Cells were gated on $CD4^{high}$, $CD25^{high}$ $CD127^{low}$ $FoxP3^{+}$. 5 matched donors were studied.

4.2.5 Co-expression of CXCR3 and CCR4 chemokine receptor on liver infiltrating regulatory T cells.

Once I have shown that CCR4 and CXCR3 are expressed at higher levels on a high proportion of liver-infiltrating T_{reg}, I then investigated whether they are co-expressed on the same cells or whether there were two sub-populations of liver-infiltrating T_{reg} each expressing individual receptor. More than 75% of liver-infiltrating T_{reg} co-express CCR4 and CXCR3 (Fig.4.4).

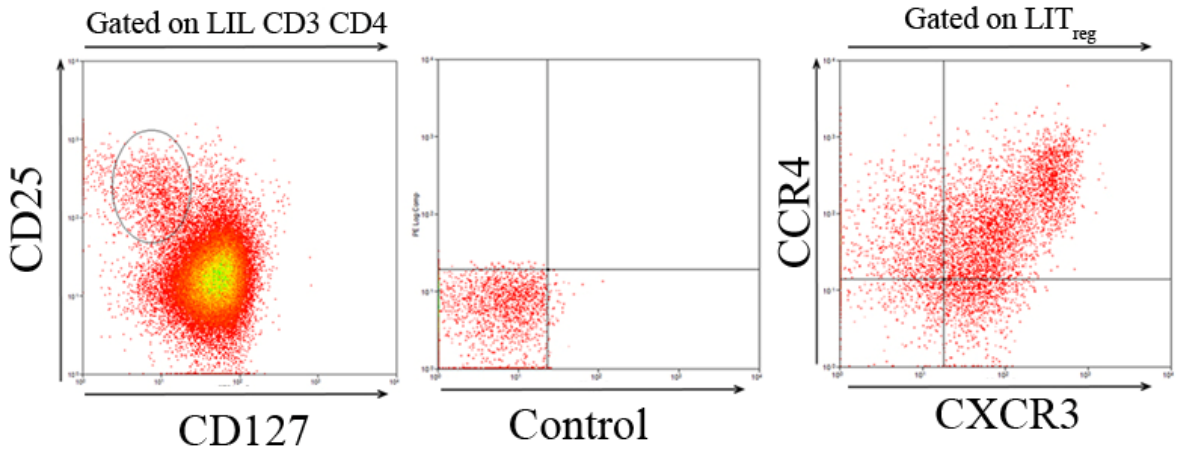

Figure 4-4 Co-expression of CCR4 and CXCR3 in liver infiltrating regulatory T cells.

Flow cytometry dot plot showing co-expression of CXCR3 and CCR4 on liver infiltrating regulatory T cells. Freshly isolated liver infiltrating lymphocytes are labelled with CD4, CD25, CD127, FoxP3 and chemokine receptors CCR4 and CXCR3. Liver-infiltrating lymphocytes were gated on $CD3^{+}CD4^{+}CD127^{low}CD25^{high}$ (left panel) and this gating was applied to CCR4 PE(Y axis) and CXCR3 APC (X axis) confirming co-expression of two chemokine receptors on the same cell in approximately 75% of the liver infiltrating T_{reg} (right panel). One representative experiment of 5 is shown. Control antibody staining is shown in middle panel.

4.2.6 CXCR3 expression on LIT_{reg} does not correlate with the proportion of liver infiltrating Th1 cells.

Because CXCR3 ligands are IFN-γ dependent we looked for a correlation between the expression of CXCR3 on liver infiltrating T_{reg} and infiltrating IFN-γ secreting CD4 and CD8 cells (Figure 4.5). I used intracellular cytokine staining and flow cytometry to analyse TNF-α and IFN-γ expression by CD4 and CD8 cells after PMA and ionomycin stimulation and correlated the frequency of these cells with levels of CXCR3 on LIT_{reg} from the same liver sample. Inflamed liver contained a similar proportion of IFN-γ and TNF-α secreting CD4 and CD8 cells compared with normal liver although as the total infiltrate is far greater in diseased livers (Figure 4.6). CXCR3 expression on T_{reg} from normal liver ranged from 15-28% and from diseased liver between 60-80 % (Figure 4.6). The highest levels of CXCR3 were detected on T_{reg} from severe seronegative hepatitis where CD8 IFN-γ and CD8 TNF-α secretion were highest as well (Figure 4.6). However, there was no overall correlation between CXCR3 expression on T_{reg} and CD4 and CD8 Th1 cytokine secretion (Figure 4.6)

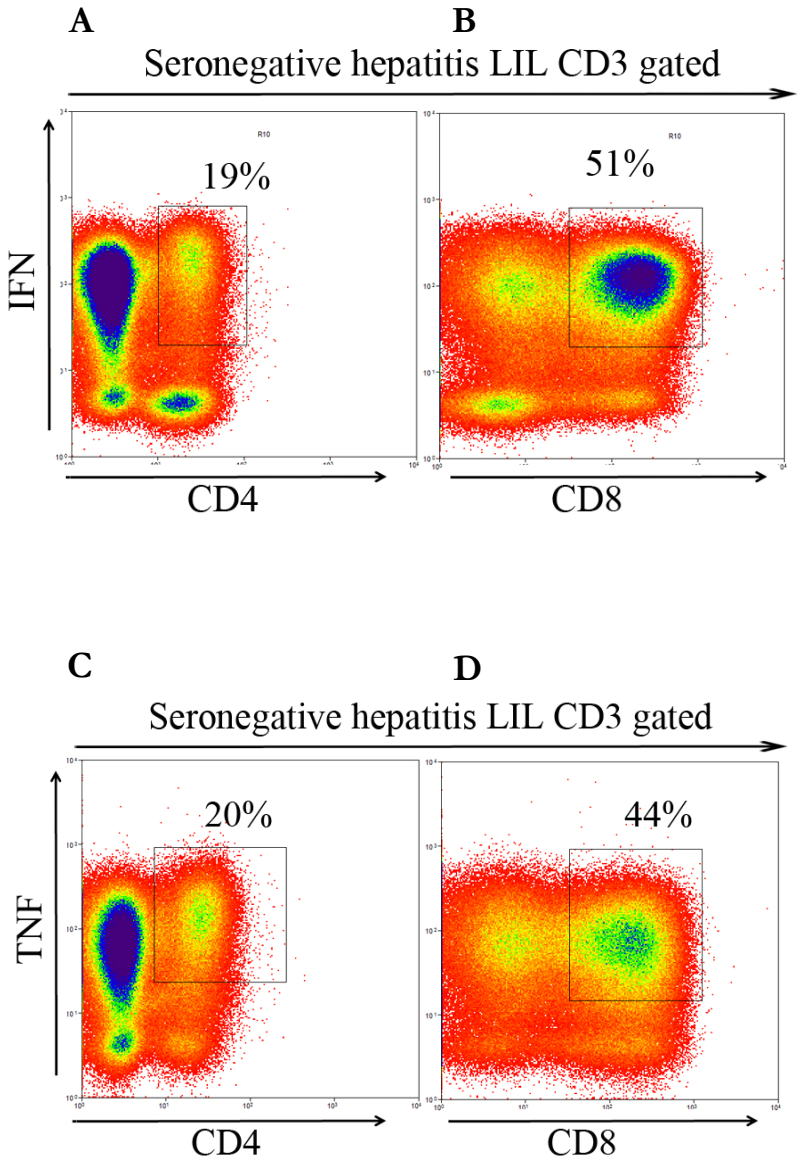

Figure 4-5 CD8Th1 and CD4Th1 cytokine expression in chronically inflamed liver.

Flow cytometry from a diseased liver (seronegative hepatitis) is shown to demonstrate IFN-γ expressing CD4 and CD8 (A&B) and TNF-α expressing CD4 and CD8 (C&D). Cells were gated on liver infiltrating CD3 lymphocytes. (Representative example from a patient with seronegative hepatitis was shown).

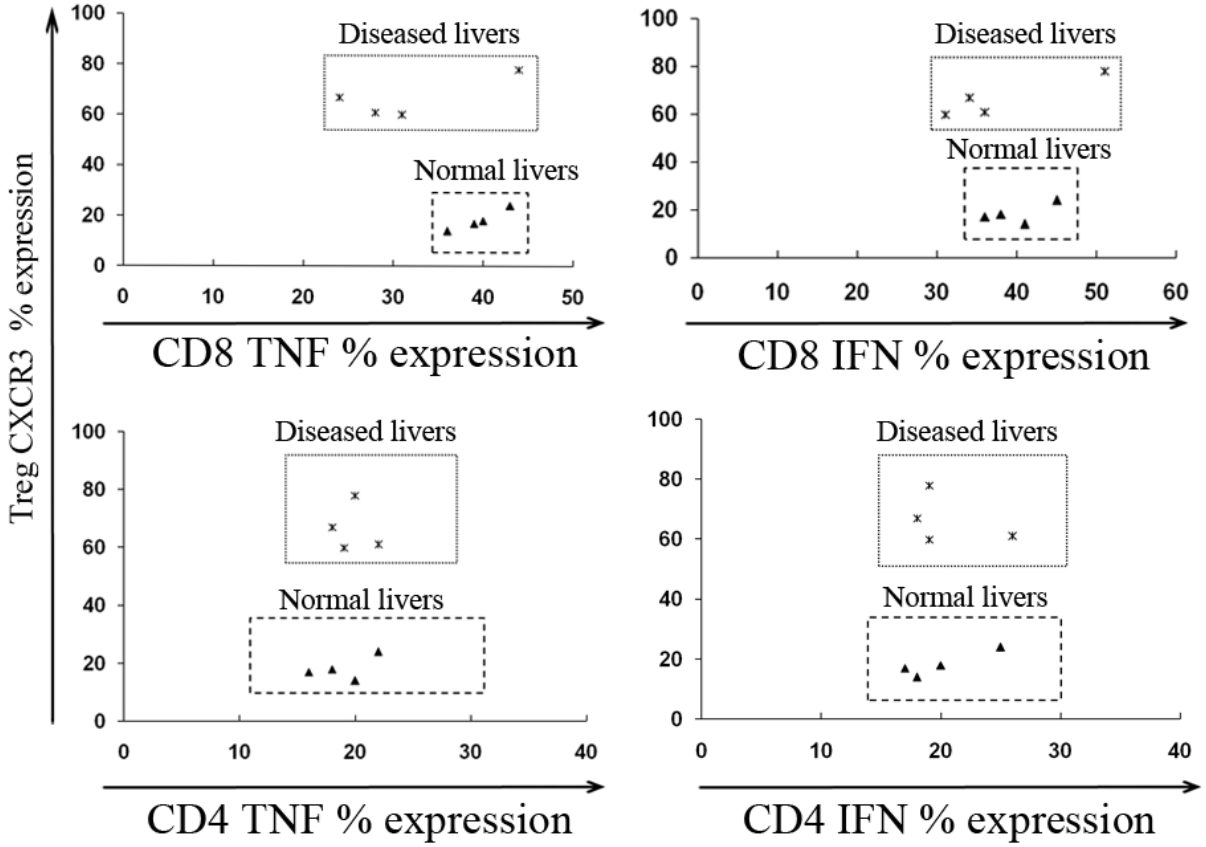

Figure 4-6 No correlation between LIT_{reg} CXCR3 expression and TNF-α and IFN-γ expression on liver infiltrating CD4 and CD8 cells.

Flow cytometry analysis of matched cells from the same liver samples on both diseased and normal liver was performed to determine the correlation between the levels of CXCR3 expression by liver-infiltrating T_{reg} and TNF-α and IFN-γ expression by liver-infiltrating CD4 and CD8 effector cells. The percentage of liver-infiltrating CD8 and CD4 T cells expressing TNF-α and IFN-γ is shown on the x axis and the frequency of CXCR3% T_{reg} ($CD4^{+}CD25^{high}CD127^{low}$) isolated from the same donor liver and analysed without expansion is shown on the y axis. Data from 4 normal livers and 4 diseased livers (autoimmune hepatitis, primary biliary cirrhosis, alcoholic liver disease and nonAnonB hepatitis) are shown. There was no clear correlation between CXCR3 frequencies in the T_{reg} population and cytokine secretion by T effectors. T_{reg} were gated on $CD4,CD25^{high}CD127^{low}$. Intracellular cytokine, TNF-α and IFN-γ staining was performed by incubating liver infiltrating lymphocytes with PMA and ionomycin for 5 hours with Brefeldin A Golgi block in the last 2 hours followed by intracellular cytokines staining.

4.2.7 Recruitment of T_{reg} is dependent on VLA-4 and CXCR3

The immunophenotyping revealed that CXCR3 is expressed at high levels on most liver infiltrating T_{reg} (67%±9 SD). We have previously shown that liver infiltrating lymphocytes also expressed high levels of CXCR3 in the inflamed livers and CXCR3 ligands, CXCL9, CXCL10 and CXCL11 are expressed at high levels on hepatic sinusoids *in vivo* and on TNF-α and IFN-γ stimulated human sinusoidal endothelial cells *in vitro* (Curbishley et al., 2005). The integrin ligands ICAM-1 and VCAM-1 are also expressed on HSEC in response to TNF-α and IFN-γ stimulation (Lalor et al., 2002b). In the following studies I used the flow-based adhesion assays to determine the function of CXCR3 and integrins in peripheral blood derived T_{reg} interactions with endothelium.

I have previously phenotyped both peripheral blood and liver derived regulatory T cells in patients who underwent for liver transplantation and observed that chemokine receptors CXCR3 and CCR4 are highly expressed on the both peripheral blood T_{reg} and liver infiltrating T_{reg} but liver T_{reg} has higher expression of these chemokine receptors. Peripheral blood derived T_{reg} were used for flow based adhesion assay to study the recruitment cascade. PBT_{reg} were the closest to represent hepatic sinusoidal T_{reg} for the recruitment process as lymphocytes including regulatory T cells flow across hepatic sinusoid, adhere and transmigrate across the human sinusoidal endothelium to the site of liver inflammation. Thus, peripheral blood regulatory T cells were freshly isolated with CD4 CD25 magnetic beads for flow based adhesion experiments and kept in IL-2 (50U/ml) before the experiments and resuspended in basal endothelial flow assay media just before the experiment.

The basal levels of T_{reg} adhesion on TNF-α and IFN-γ stimulated HSEC was 157±15 SD cells//$mm^2/10^6$ perfused and this was reduced by about 60% following pre incubation of T_{reg}

with anti CXCR3 mAb for 30minutes ($p \leq 0.001$) (Figure 4.7). A similar level of inhibition was seen when T_{reg} were pre-treated with pertussis toxin ($p \leq 0.004$). This suggests that CXCR3 is the dominant receptor involved in the adhesion of T_{reg} to stimulated HSEC. Adhesion was also significantly reduced to 30% by blocking antibodies to VCAM-1 (the ligand for VLA-4). In contrast, antibody to ICAM-1 on HSEC did not significantly reduce adhesion and blocking both these integrin ligands together did not increase blockade over the use of anti-VCAM-1 alone suggesting that VCAM-1 is the dominant integrin ligand mediating adhesion (Figure 4.7 and Figure 4.8).

I then calculate the proportion of adherent T_{reg} which subsequently underwent transendothelial migration across HSEC under condition of flow. On average, 37% of adherent T_{reg} transmigrated across HSEC within 5 minutes of initial adhesion. This was reduced significantly by incubation with anti-CXCR3 mAb or pertussis toxin applied to T_{reg} for 30 minutes ($p<0.05$ for both). Addition of anti-VCAM-1 mAb to HSEC for 30 minutes also reduced the transmigration of adherent T_{reg} but this did not reach statistical significance (average 31%) and no significant effect was noticed with anti-ICAM-1 blockage (Figure 4.9).

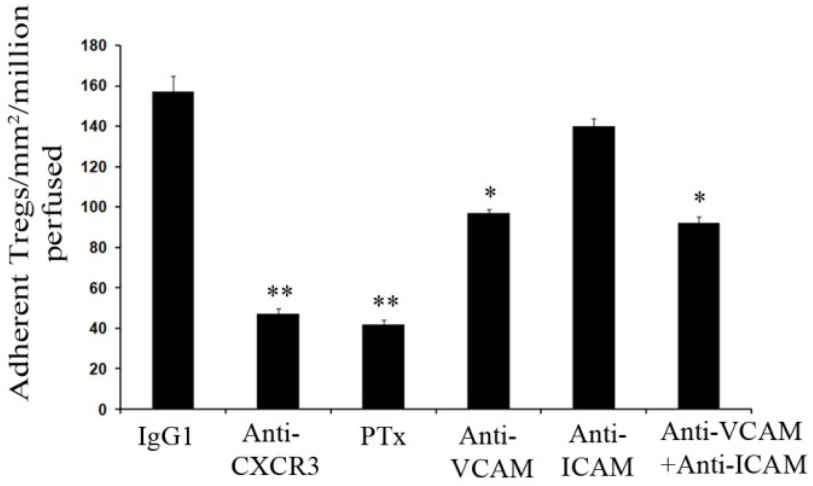

Figure 4-7 The effect of antibodies raised against CXCR3, integrin ligands and pertussis toxin on the adhesion of regulatory T cells.

CXCR3 inhibition with a function blocking antibody or PTX (Gi protein block) significantly reduces the total adhesion of regulatory T cells to 24 hours TNF-α and IFN-γ stimulated HSEC. Adhesion was further reduced by the addition of blocking antibodies against ICAM-1/VCAM-1. Data represent number of adherent $T_{reg}/mm^2/10^6$ perfused and are the mean ±SEM of five experiments. All experiments are performed at a flow rate of 0.182ml/min (0.05Pa), which is equivalent to sheer stress at hepatic sinusoid. Statistical significance was calculated using paired T-tests, comparing treatment with control. (* =p≤0.05; **=p ≤0.005)

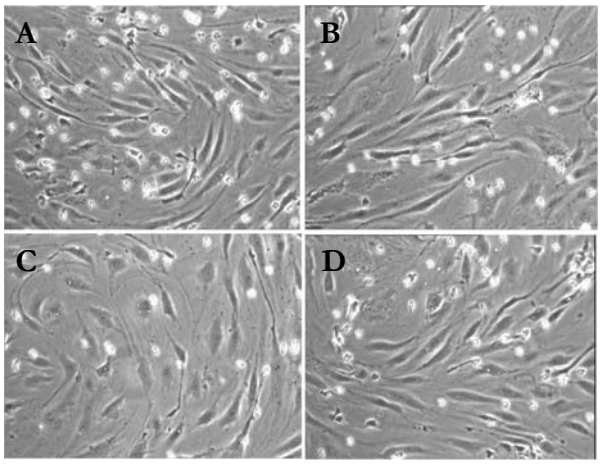

Figure 4-8 Adhesion of T_{reg} on stimulated primary hepatic sinusoidal endothelium

Adherent T_{reg} on control HSEC (A) and reduction of adherent T_{reg} with Pertussis block (B), anti-CXCR3 block (C) and anti-VCAM block (D).

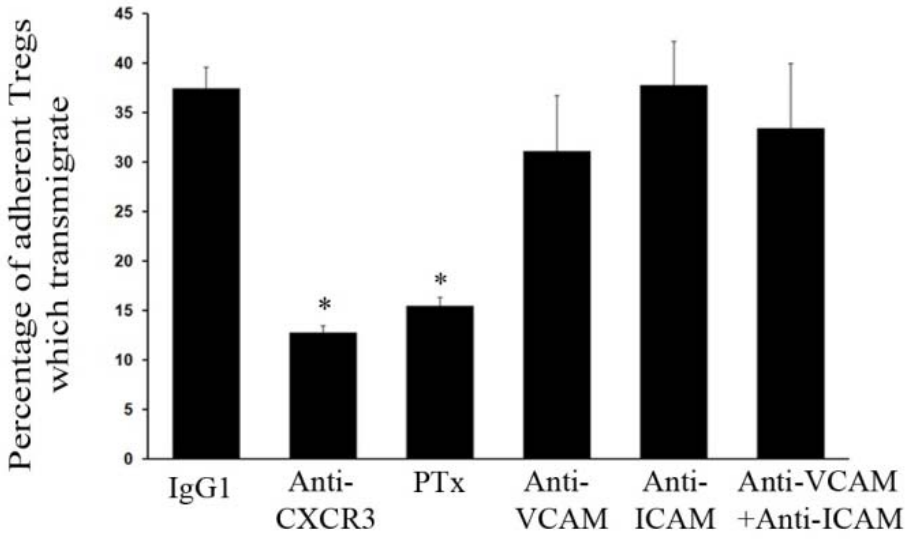

Figure 4-9 The effect of antibodies raised against CXCR3, integrin ligands and Pertussis toxin on the transmigration of regulatory T cells.

CXCR3 inhibition with a function blocking antibody or PTX (Gi protein block) significantly reduces the transmigration of regulatory T cells to 24 hours TNF-α and IFN-γ stimulated HSEC. Transmigration was also reduced by the addition of blocking antibodies against ICAM-1/VCAM-1. Data represent number of adherent $T_{reg}/mm^2/10^6$ perfused and are the mean ±SEM of five experiments. All experiments are performed at a flow rate of 0.182ml/min (0.05Pa), which is equivalent to sheer stress at hepatic sinusoid. Statistical significance was calculated using paired T-tests, comparing treatment with control.(*=$p \leq 0.05$)

4.2.8 Effect of individual CXCR3 chemokines on T_{reg} recruitment

The activation of CXCR3 on T_{reg} could be mediated by the three CXCR3 ligands, namely CXCL9, CXCL10 and CXCL11 all of which are found on sinusoidal endothelium. I therefore studied the effect of blocking antibodies to the individual ligands. Peipheral blood derived regulatory T cells were used for these experiments to mimic the normal physiological process as described before. HSEC are stimulated for 24 hours with TNF-α and IFN-γ and incubated with individual CXCR3 receptor chemokines block, anti-CXCL9, anti-CXCL10 and anti-CXCL11 for 30 minutes before the flow based adhesion assay. The basal adhesion of peripheral T_{reg} was around 150 adherent $T_{reg}/mm^2/10^6$ perfused similar to the previous assay and this was taken as 100%. Inhibiting the individual chemokines reduced both adhesion (Figure 4.10) and transmigration (Figure 4.11) however it did not reached to statistical significance. However, significant inhibition was only achieved when CXCR3 was blocked (Figure 4.7 & 4.9) suggesting there is redundancy in the system and all CXCR3 chemokines contribute in combination or in a serial cascade for T_{reg} recruitment.

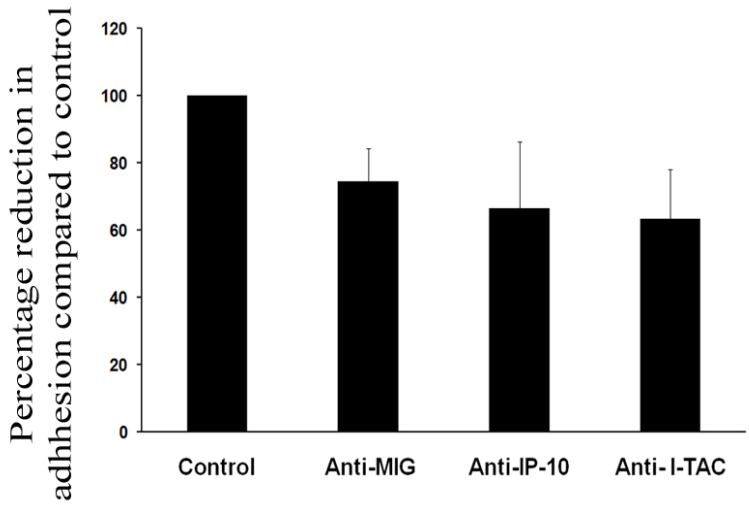

Figure 4-10. Effect of Individual CXCR3 chemokines block on total adhesion of T_{reg}.

To investigate the individual effect of CXCR3 ligands, anti-CXCL9-11 were blocked individually on 24 hours stimulated HSEC. Adhesion was calculated using frame by frame analysis of experiment videos to count the percentage of cells which are adhered to HSEC. Individual chemokine blockade reduce the adhesion of T_{reg} to stimulated endothelium. Data represent mean± SEM of four experiments. All experiments were performed at 0.05Pa.

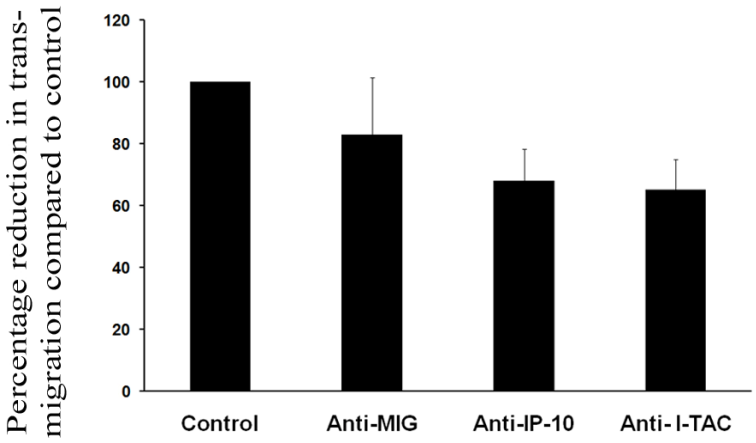

Figure 4-11 Effect of Individual CXCR3 chemokines block on transmigration of T_{reg}.

To investigate the effect of individual CXCR3 ligands on transmigration, anti-CXCL9-11were blocked individually on stimulated HSEC. Transmigration was calculated using frame by frame analysis of experiment videos to count the percentage of adherent cells which transmigrated across the HSEC. Individual chemokine blockade reduce the transmigration of T_{reg} to stimulated endothelium. Data represent mean± SEM of four experiments.

4.2.9 Expression of CCR4 ligands on liver infiltrating dendritic cells

CCR4 is the other chemokine receptor which is highly expressed on liver infiltrating T_{reg} and it is co-expressed with CXCR3 on more than 75% of LIT_{reg}. CCR4 has two ligands, CCL17 and CCL22. Immunohistochemistry was used initially to localise the source of the CCR4 ligands in human liver tissue. Expression of CCL22 could be detected in inflammatory liver diseases although it was not abundant (Figure 4.12) and staining was restricted to dendritic like cells at areas of active inflammation including portal infiltrates, interface hepatitis and lobular hepatitis within the parenchyma. There was very little detectable staining on normal liver tissue and staining with control antibody was negative. I was unable to detect CCL17 and CCL22 in normal liver tissue but CCL17 chemokine were detected in chronically inflamed liver tissue by western blotting (Figure 4.12).

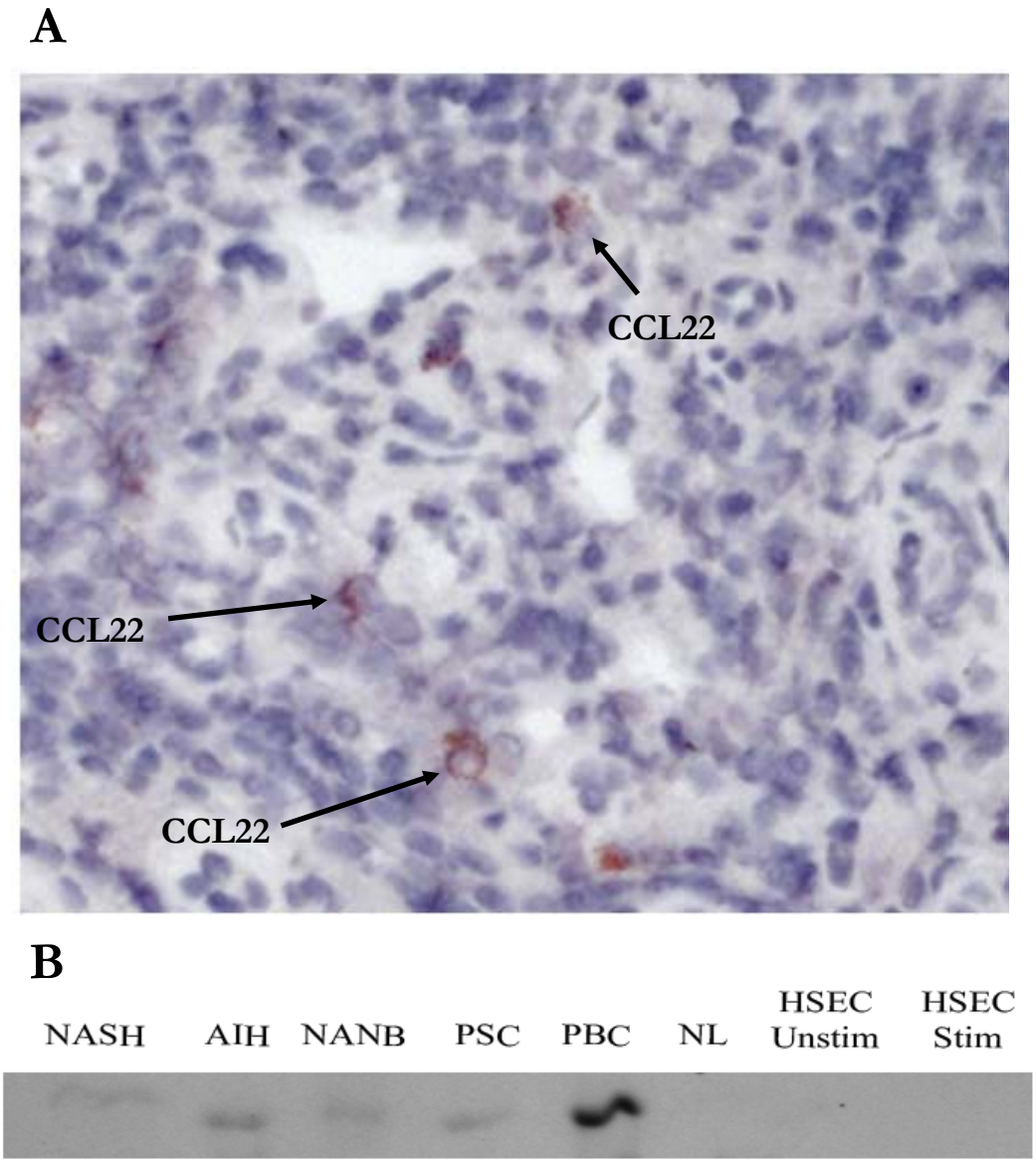

Figure 4-12 Protein expression of CCL22 and CCL17.

(A) Expression of CCL22 in human liver tissue samples by immunohistochemistry. Staining pattern of CCL22 is on liver infiltrating dendritic cells (LIDC) within inflammatory infiltrates (positive staining cells shown with arrows) in a patient with autoimmune hepatitis. Image was taken at x40 magnification.

(B) Detection the presence of CCL17 was observed in whole liver lysates not on HSEC lysates. Western blot analysis of CCL17 in liver tissue lysate from different chronic liver diseases and either unstimulated (Unstim) or TNF-α/IFN-γ stimulated HSEC (Stim) cell lysate. CCL17 is present in chronically inflamed diseased livers but could not be detected in normal liver tissue lysates or in lysates of HSEC. NASH = non-alcoholic steatohepatitis; AIH = autoimmune hepatitis; NANB = nonAnonB seronegative hepatitis; PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; NL = normal liver. HSEC Unstim = HSEC unstimulated and HSEC Stim = HSEC stimulated with TNF-α and IFN-γ.

Co-localisation of CD11c$^+$ dendritic cells and CCL22 chemokine in inflamed liver tissue was confirmed by dual immunofluorescence (Figure 4.13). Moreover, CCL22 staining are present in the same areas with CD11c$^+$ dendritic cells in immunohistochemistry staining in serial liver tissue sections (Figure 4.14).

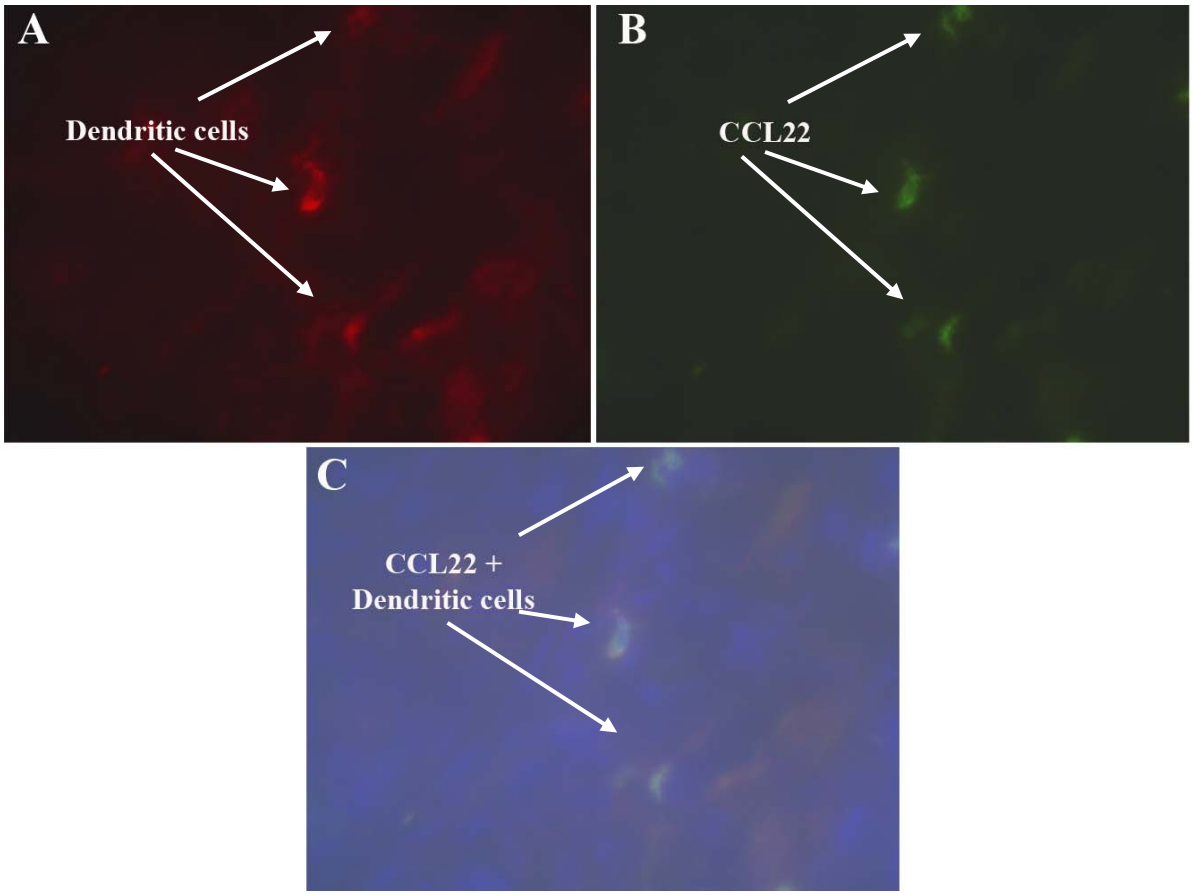

Figure 4-13 Co-expression of CD11c and CCL22 in diseased liver tissues.

Liver infiltrating CD11c$^+$ dendritic cells expression of CCL22 chemokine was confirmed by co-localisation of TexRed-labelled CD11c dendritic cells (A) with FITC labelled CCL22 (B). Areas of co-expression appeared as yellow (C). DAPI=Blue. Staining with control antibody was negative. Pictures were taken at X20 lens. The results are representative of the pattern of immunofluorescence staining seen in tissue sections from donors with PBC, ALD, AIH.

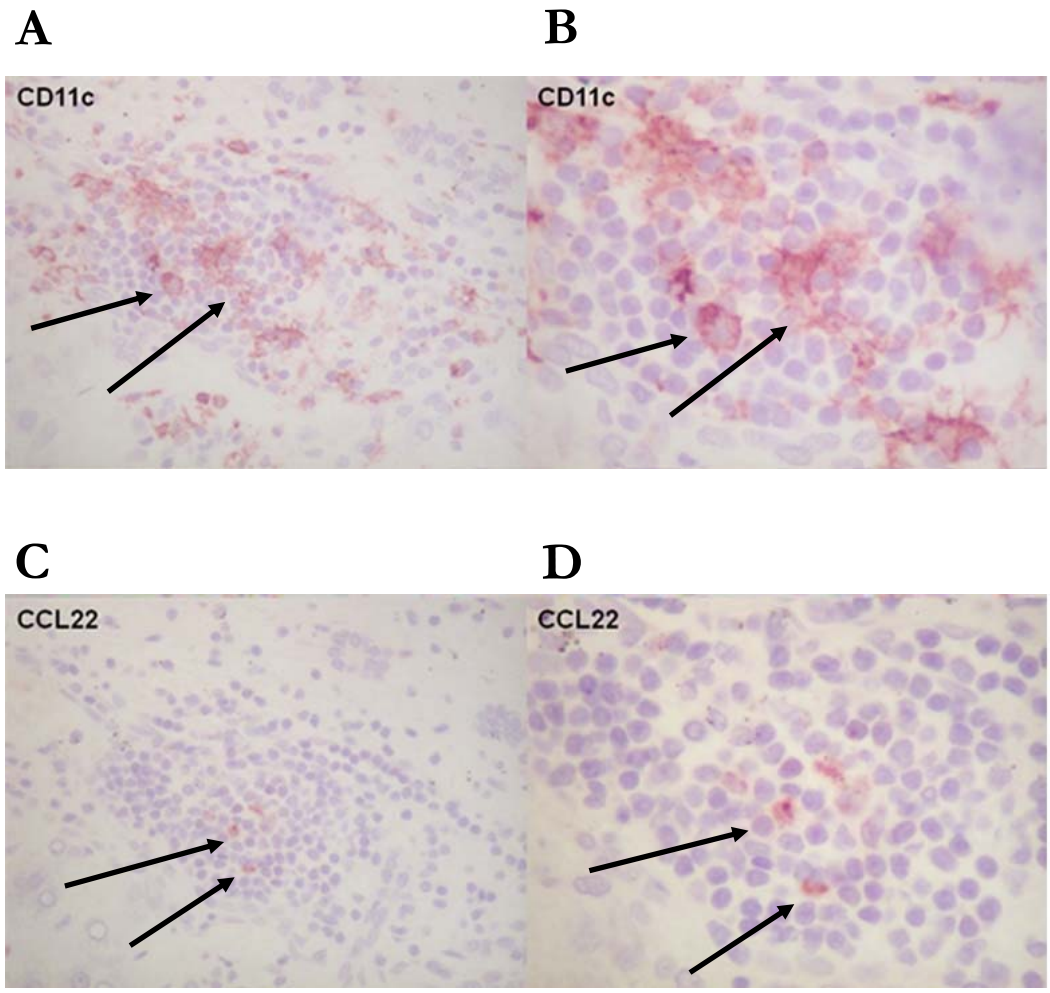

Figure 4-14. Co-localisation of dendritic cells and CCL22 in inflamed liver tissue.

Serial liver sections are stained with CD11c (Figures A&B, B is the higher magnification of A) and CCL22 (Figures C&D, D is the higher magnification of C). CCL22 staining was detected in the same areas as CD11c (B&D). Images were taken at x10 lens (A&C) and x20 lens (B&D). Images were taken from a patient with primary biliary cirrhosis.

4.2.10 *De novo* synthesis and secretion of CCR4 ligands by liver infiltrating dendritic cells.

To confirm the immunohistochemistry finding of CCR4 ligands expression by dendritic cells. I initially used RT-PCR to detect mRNA from stimulated and unstimulated liver infiltrating myeloid dendritic cells isolated from diseased livers. Neither stimulated (TNF-α + IFN-γ) nor unstimulated HSEC low passage (passage1-2) or high passage (passage4-6) showed any detectable staining or mRNA suggesting that CCL17 and CCL22 are absent from HSEC. However, *de novo* synthesis of CCL17 and CCL22 was detected by disease liver infiltrating dendritic cells (LIDC) both unstimulated and stimulated with lipopolysaccharide (Figure 4.15).

To detect the secretion of CCR4 ligands by liver infiltrating dendritic cells, ELISA was performed on supernatants collected from liver infiltrating dendritic cells which were stimulated with LPS 1μg/ml for 24 hours. Compared with normal liver, DC from diseased livers secreted more of both chemokines stimulated and unstimulated ($P \leq 0.05$) (Figure 4.16).

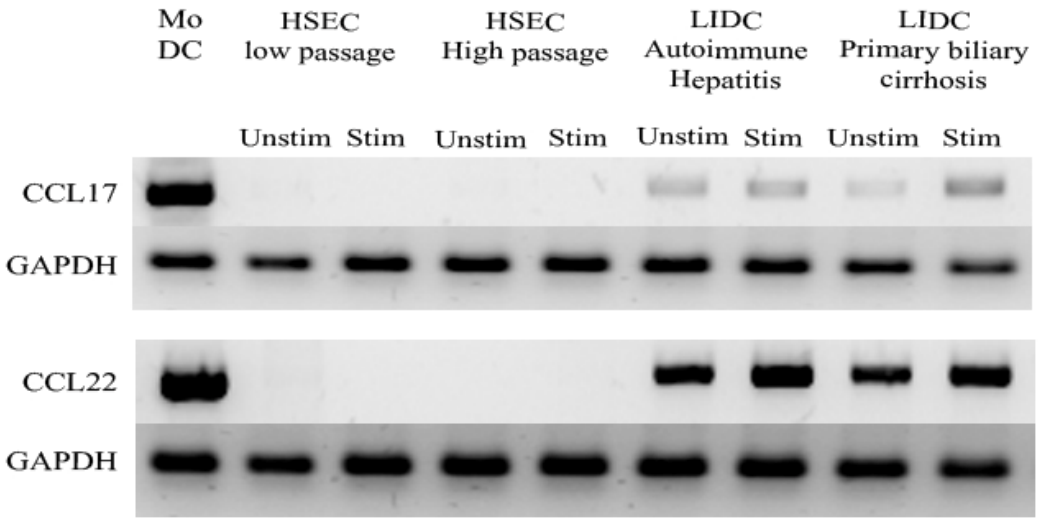

Figure 4-15 Measurement of CCR4 ligand transcript in HSEC and liver infiltrating dendritic cells.

De novo synthesis of CCR4 ligands, CCL17 and CCL22 by liver-derived DC was confirmed by RT-PCR. Liver infiltrating myeloid DCs (LIDC) were isolated from liver tissue from patients with autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) and analysed either unstimulated (U) or after stimulation (S) with lipopolysaccharides (LPS). Monocyte-derived TNF-α activated DCs (MoDC) are shown as a positive control. CCL17 and CCL22 mRNA was detected in diseased liver-infiltrating DCs (LIDC) but not on HSEC. Representative data from one of three experiments is shown (HSEC= Hepatic sinusoidal endothelial cells low passage; HSEC= Hepatic sinusoidal endothelial cells high passage; LIDC= Liver infiltrating dendritic cells) CCL17=270bp; CCL22 363bp.

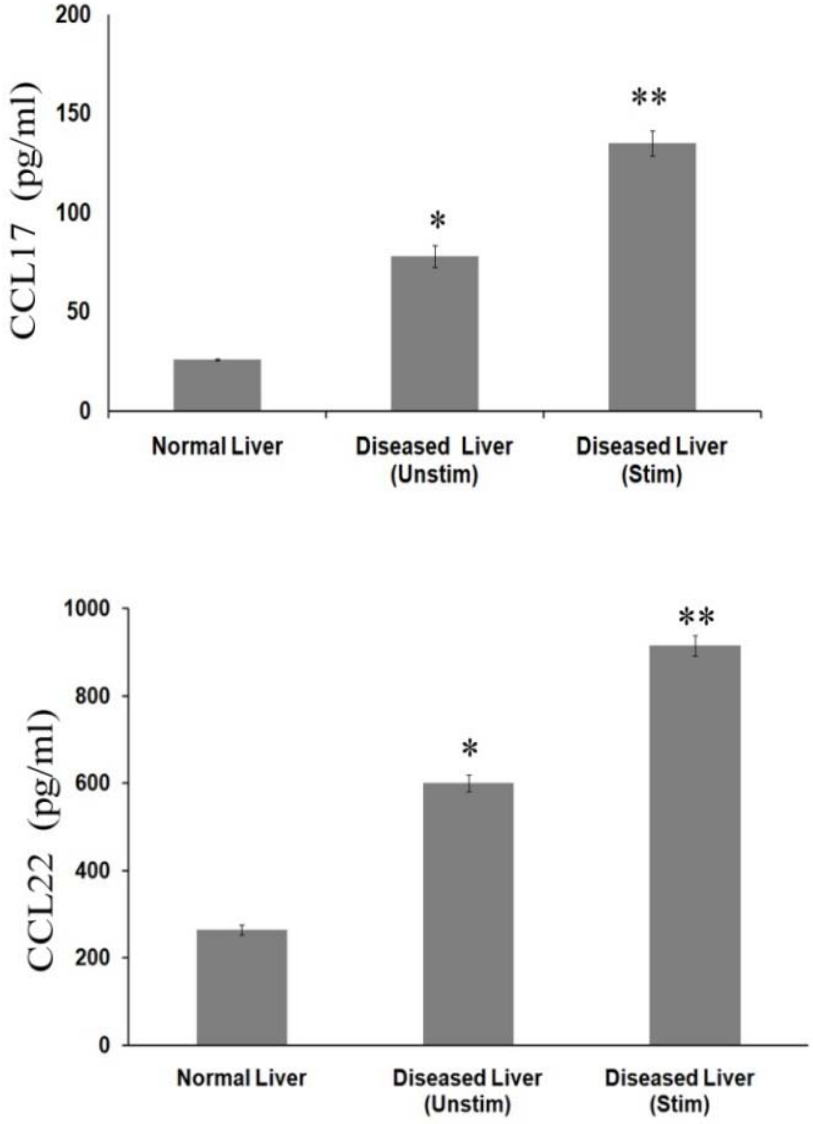

Figure 4-16 Measurement of CCR4 ligands CCL17 & CCL22 production by liver infiltrating dendritic cells following stimulation with LPS.

In order to determine the CCL17 & CCL22 production, primary liver infiltrating dendritic cells are cultured and stimulated them with LPS (1μg/ml) for 24 hours. CCL17 (TARC) and CCL22 (MDC) proteins levels were measured in cultured supernatant by sandwich ELISA. Experiments reflect liver infiltrating dendritic cells isolated from 3 donors (2X AIH; 1PBC) and assays conducted in triplicates. Results were expressed as mean± SEM. * P< 0.05, ** P< 0.005. Statistical significance was determined using Student's t test comparing normal and diseased livers. Stim=stimulated with LPS, Unstim=unstimulated.

4.2.11 CCR4 receptor on liver infiltrating T_{reg} is functional

To confirm that CCR4 on freshly isolated liver-infiltrating T_{reg} is functional, I used a chemotaxis assay to study responses to CCL17 and CCL22 using fresh LIT_{reg} (Fig 4.17). The chemotactic indices for CCL17 and CCL22 were around 3 times compared to control and this increase was inhibited by pertussis toxin. Liver-infiltrating T_{reg} were phenotyped before the chemotaxis assays and confirmed that more than 75% expressed CCR4.

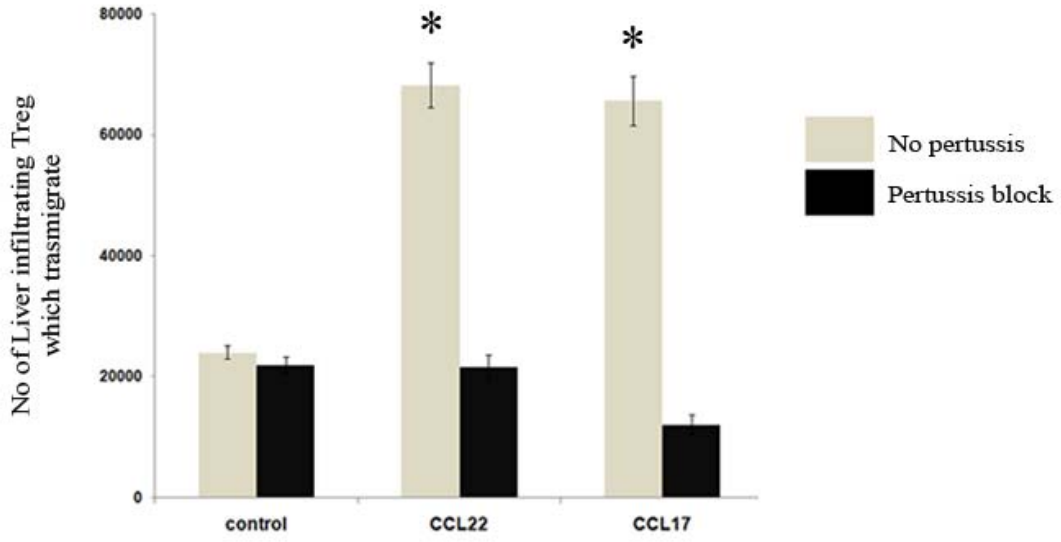

Figure 4-17 Chemotaxis of LIT_{reg} to CCL17 and CCL22 demonstrating CCR4 is functional.

Liver-infiltrating T_{reg} migrate towards CCR4 ligands *in vitro*. Migration of liver-infiltrating T_{reg} to CCR4 ligands, CCL22 and CCL17 was assessed using fibronectin-coated 5μm pore transwell migration chambers. The lower chambers were filled with 600μl assay media (RPMI medium with 0.1% BSA; Sigma-Aldrich) alone or supplemented with chemokines (100 ng/ml recombinant human CCL22 and CCL17 from R&D Systems). 0.1%BSA was used as control. A total of $5x10^5$ T_{reg} isolated from inflamed livers (n=4) were loaded per well in the upper chamber; experiments were done in duplicates. T_{reg} were collected from the top and bottom chambers after 5 hours incubation at 37°C in 5% CO_2. The proportion of transmigrated cells was calculated by fixed volume counting and phenotyped for T_{reg} markers and chemokine receptor expression by flow cytometry. To inhibit chemokine-mediated signalling LIT_{reg} were incubated with pertussis toxin (100 ng/ml; Sigma Aldrich Ltd) prior to the assay. Results are expressed as mean ± SEM. LIT_{reg} showed significant migration to CCL22 and CCL17 which was inhibited by pertussis toxin compared with migration to BSA (*= $p<0.05$). Phenotyping of the migrated LIT_{reg} in the lower chamber revealed they were $CCR4^+$.

4.2.12 Close proximity of liver infiltrating dendritic cells, T effector cells & regulatory T cells.

Regulatory T cells exert their suppressive function on T effectors cells via many mechanisms, contact dependent and also operate through effects on antigen presenting cells such as dendritic cells. Therefore, T_{reg} need to be close to T effector cells and dendritic cells to function efficiently. Confocal analysis in inflamed human liver tissue suggested that liver infiltrating T_{reg} are in close vicinity to effectors and dendritic cells (Figure 4.18). Table 3.5 in Chapter 3 showed the quantitative analysis of these cells types in different liver diseases.

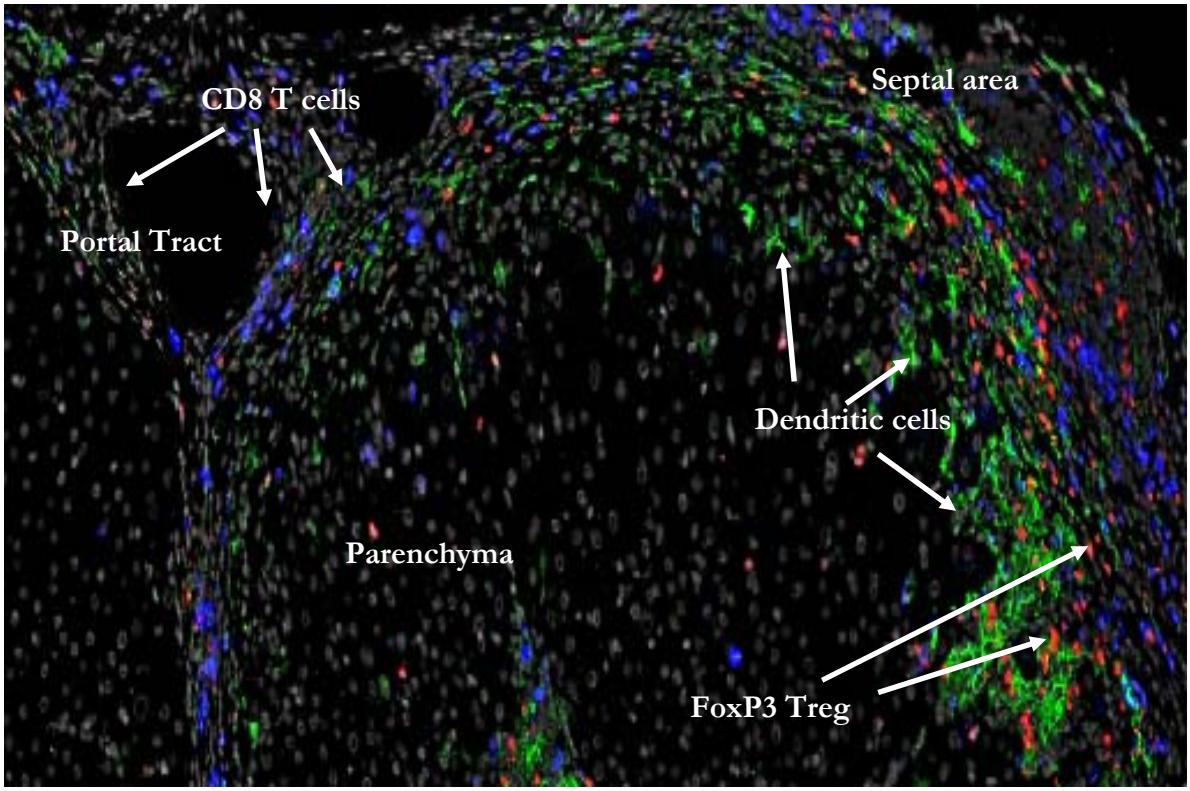

Figure 4-18 CD11c dendritic cells, CD8 T cells and FoxP3 T_{reg} in human liver.

Confocal image of inflamed liver portal tract and parenchyma autoimmune hepatitis liver. T_{reg} (FoxP3; TRITC, Red), (CD8, Blue, Cy5), (CD11c, Green, FITC); (cell nuclei, DAPI, Grey).

4.3 DISCUSSION

Regulatory T cells operate at sites of inflammation in peripheral organs as well as in secondary lymphoid tissues (Tang & Bluestone, 2008;Walker et al., 2003). At inflammatory sites they mediate bystander and antigen-specific suppression of local immune responses to prevent collateral tissue damage in response to injury and infection (Holmen et al., 2006;Ni et al., 2006;O'Connor et al., 2007). The outcome of chronic inflammation is the result of a balance between pro and anti-inflammatory responses in which T_{reg} maintain stable chronic inflammation whilst preventing fulminant destructive inflammation (Westendorf et al., 2005). This is particularly important in liver disease where intrahepatic T_{reg} are detected in chronic hepatitis from a variety of causes (Lan, 2006;Longhi et al., 2005a;Rushbrook et al., 2005). The frequency and function of intrahepatic T_{reg} has been reported to affect the outcome of chronic infection with hepatitis B and C viruses and to be associated with suppression of anti-tumour immune response in hepatocellular carcinoma (Rushbrook et al., 2005;Unitt et al., 2005).

The migration and positioning of leukocytes in tissues during homeostasis and in response to inflammation is controlled by the coordinated expression of adhesion molecules and chemokine receptors that determine a) where and when cells will be recruited via endothelium b) their subsequent migration and positioning in tissue. Little is known about the molecular control of T_{reg} recruitment to inflamed tissues although this will be critical in defining their role in disease and in developing strategies for their therapeutic use. In order to determine the receptors responsible for the recruitment of T_{reg} to human liver we compared chemokine receptor expression on liver-derived T_{reg} (analysed straight from liver tissue without expansion) with blood-derived T_{reg} isolated from the same patient at the time of liver transplantation. Two receptors in particular were over-expressed on liver-infiltrating cells, CXCR3 and CCR4 with the majority of liver-infiltrating T_{reg} in chronic inflammatory liver diseases expressing both receptors.

CXCR3 has been reported on circulating human T_{reg} (Lim et al., 2006;Lim et al., 2008) where its expression is presumed to favour homing to inflamed tissue sites. Our data confirmed that this is the case because the majority of T_{reg} in inflamed human liver expressed high levels of functional CXCR3 which promoted their transmigration across liver endothelium under flow. We found only 15-20% of T_{reg} in normal liver expressed CXCR3 which further supports its particular role in the recruitment of T_{reg} to sites of inflammation. Levels of the CXCR3 ligands are low to undetectable in normal liver but markedly increased in inflammatory liver diseases (Curbishley et al., 2005;Harvey et al., 2003).

In chronic inflammatory liver disease the CXCR3 ligands CXCL9, CXCL10 and CXCL11 are increased as a consequence of secretion by hepatocytes, stroma cells and endothelium. They can be readily detected on the glycocalyx of sinusoidal vessels (Narumi et al., 1997;Shields et al., 1999;Tamaru et al., 2000) where they promote the transendothelial migration of effector T cells (Curbishley et al., 2005). We have developed an *in vitro* flow-based adhesion assay in which primary cultures of human sinusoidal endothelial cells (HSEC) are grown in microslides under physiological levels of shear stress to model the hepatic sinusoid (Curbishley et al., 2005). Treatment of the HSEC with TNF-α and IFN-γ induces expression of CXCL9, CXCL10 and CXCL11 as well as the adhesion molecules ICAM-1 and VCAM-1 thereby recapitulating the phenotype of chronically inflamed sinusoids *in vivo* (Curbishley et al., 2005). In the present study we used this assay to determine the role of CXCR3 on liver-infiltrating T_{reg}. We found that signalling through CXCR3 not only activated integrin-mediated arrest from flow but also promoted the transendothelial migration of T_{reg} across inflamed hepatic sinusoidal endothelium. The fact that a similar degree of inhibition to both processes was seen when either a blocking CXCR3 antibody or the G_{α} protein inhibitor pertussis toxin used suggests that CXCR3 is the dominant chemokine receptor involved. In the *in vitro* flow assays all three CXCR3 ligands were

involved because blocking individual chemokines had a lesser effect than blocking the common receptor CXCR3. These findings are consistent with studies in murine models of inflammatory disease that report the requirement for CXCR3 expression to allow T_{reg} to be recruited to inflamed tissues in GVHD (Hasegawa et al., 2008) and to the brain in EAE (Muller et al., 2007). Recently CXCR3 has been implicated in the recruitment of T_{reg} into the murine liver in response to inflammation and IFN-γ production from NKT cells (Santodomingo-Garzon, 2008). Thus interferon-inducible chemokines CXCL9 and CXCL10 expressed on inflamed hepatic sinusoidal endothelium promote the recruitment of both effector cells and CXCR3 expressing T_{reg} under shear stress. A difference between the behaviour of effector cells and T_{reg} was the involvement of VCAM-1 but not ICAM-1 in adhesion of T_{reg} to HSEC whereas effector cells used ICAM-1.

The other chemokine receptor that was strongly over-expressed on liver-infiltrating T_{reg} was CCR4 which was co-expressed with CXCR3 in nearly 80% of T_{reg} from chronically inflamed livers. Liver-derived T_{reg} migrated to the CCR4 ligands CCL17 and CCL22 *in vitro* demonstrating that the receptor is functional. CCR4 expression has been associated with T_{reg} and shown to control migration and immune suppression in the skin (Iellem et al., 2001b;Sather et al., 2007) and also in immunologically tolerant cardiac allograft (Lee et al., 2005a). Until this report CCR4 was not believed to play a role in recruitment of T cells to the liver. Only few effector cells in the liver express CCR4 (Shields et al., 1999). CCR4 is the receptor for two chemokines, CCL17 and CCL22 both of which are secreted by DCs on maturation and which serve to recruit and retain T_{reg} in contact with DCs in lymph nodes (Cardoso et al., 2008b;Curiel et al., 2004;Iellem et al., 2001b;Katou et al., 2001;Tang and Cyster, 1999;Vulcano et al., 2001). The interaction between T_{reg} and DC inhibits DC maturation and the expression of co-stimulatory molecules required for effector T cell activation (Bayry et al., 2007;Houot et al., 2006;Sansom, 2006;Wing et al., 2008). We were unable to detect either chemokine in normal human liver tissue by western blotting of

whole liver tissue or immunohistochemistry. However both chemokines were detected in inflamed liver tissue and immunohistochemistry showed staining restricted to $CD11c^+$ DCs within inflammatory infiltrates in septal areas and lobules. In order to confirm that liver-infiltrating DCs secrete CCR4 ligands we isolated myeloid DCs from liver tissue and showed they expressed and secreted both CCL17 and CCL22.

Confocal microscopy of liver tissue revealed that in chronic hepatitis liver-infiltrating T_{reg} were closely associated with both DCs and CD8 T cells suggesting that they are ideally positioned to maintain intrahepatic immune suppression. We propose that localisation to these infiltrates occurs in response to sequential chemokine signals. Local proinflammatory cytokines including IFN-γ lead to the secretion of CXCL9 and CXCL10 by sinusoidal and parenchyma cells; these chemokines are presented on the glycocalyx of sinusoidal endothelium where they recruit $CXCR3^{high}$ circulating T_{reg} into the liver. Subsequent migration within the inflamed liver is guided by CCR4 which allows tissue-infiltrating T_{reg} to respond to CCL17 and CCL22 secreted by activated intrahepatic DCs thereby resulting in their accumulation within DC-rich inflammatory infiltrates in liver tissue.

Therefore, our experimental data suggested that in chronic inflammatory human liver disease there is a high frequency of liver-infiltrating $CD4^+CD25^+CD127^{low}FoxP3^+$ T_{reg} which are detected in close association with intrahepatic DCs at sites of inflammation. Moreover, liver-infiltrating T_{reg} use CXCR3 to undergo transendothelial migration across hepatic sinusoidal endothelium and CCR4 to respond to CCL17 and CCL22 secreted by intrahepatic DCs. Thus different chemokine receptors play distinct roles in the recruitment and positioning of T_{reg} at sites of hepatitis in chronic liver disease.

CHAPTER 5

IMMUNOPHENOTYPE OF IL-17 SECRETING LYMPHOCYTES AND THEIR POSITIONING IN INFLAMED HUMAN LIVER

5.1 INTRODUCTION

$CD4^+$ T helper (Th) lymphocytes and $CD8^+$ T effector lymphocytes are essential mediators of successful hepatic immune responses and are also implicated in inflammatory diseases (Adams & Eksteen, 2006;Adams and Lloyd, 1997;Adams & Shaw, 1994). The Th1/Th2 paradigm, first introduced by Mosmann & Coffman (Mosmann et al., 1986) explained many phenomena in adaptive immunity but proved to be an over-simplification. Recently a subset of IL-17 secreting lymphocytes has been described which includes both $CD4^+$ T helper (Th) lymphocytes (Th17) (Harrington et al., 2005;Park et al., 2005) and $CD8^+$ cytotoxic T effector lymphocytes (Tc17) (Kondo et al., 2009). Th17 has been shown to play a role in human chronic inflammatory and autoimmune related diseases such as rheumatoid arthritis (Sato et al., 2006), multiple sclerosis, psoriasis, acanthosis, and inflammatory bowel diseases (Duerr et al., 2006;Lock et al., 2002;Pene et al., 2008;Zheng et al., 2007).

IL-17 secreting cells have been implicated in a variety of human liver diseases. An increased frequency of intrahepatic IL-17 lymphocytes was observed in primary biliary cirrhosis liver compared to healthy liver (Lan et al., 2009) and high concentration of Th17 cells were also reported around inflamed peri-ductal regions in chronic biliary inflammation (Harada et al., 2009). Th17 cells have also been detected in human alcoholic hepatitis and alcoholic liver disease where the ability of IL-17 to recruit neutrophils may link the adaptive and innate immune responses and explain some of the features of alcoholic hepatitis (Lemmers, 2009). Regarding the role of IL-17 cells in viral hepatitis, I collaborated with Paul Klenerman group in Oxford, to define a novel subset of Tc17 cells (IL-17 secreting CD8) cells in the inflamed chronic hepatitis C infected human liver. More recent studies report increased frequencies of Th17 cells in the blood of patients with chronic hepatitis B compared with normal healthy individual. In this study Th17

cells were inversely correlated with Th1 cells and showed a positive correlation with serum transaminases (Ge et al., 2009).

The presence of IL-17 secreting lymphocytes in the liver during chronic hepatic inflammation suggests that Th17 and Tc17 cells are either recruited into the liver from blood or they differentiate locally within the liver from memory T lymphocytes under a polarising cytokine microenvironment. It is perhaps more likely that both processes are involved with recruitment of Th17 cells being mediated by specific chemokines and local conditions maintaining their polarisation and survival in tissue. The recruitment and positioning of lymphocyte subsets in inflamed microenvironments in critically dependent on chemokines (Rot, 2004). Our laboratory previously reported that biliary epithelial cells are the source of inflammatory chemokines such as CXCL9, CXCL10, CXCL11 (Curbishley et al., 2005), CXCL16 (Heydtmann et al., 2005) that can attract liver infiltrating effector lymphocytes expressing CXCR3 and CXCR6 and CXCL8 (Morland et al., 1996;Morland et al., 1997a) which can attract neutrophils thereby linking innate and adaptive responses in hepatitis.

CCR6 is the chemokine receptor which best defines IL-17 secreting cells suggesting that together with its ligand CCl20 it might be a critical recruitment signal for Th17 cell recruitment to tissue. CCL20, which is the only CCR6 ligand was originally named as LARC (liver and activation-regulated chemokine) as it was detected in liver cell lines (Hieshima et al., 1997). CCL20 is also alternatively named as macrophage inflammatory protein-3α (MIP-3α) or Exodus-1. CCL20 messenger RNA expression has been reported on human biliary epithelial cells (Harada et al., 2009) and it has been shown to be involved in the local necroinflammatory response in the human liver (Shimizu et al., 2001). Epithelial cells such as human bronchial epithelial cells also secrete CCL20 in response to pro-inflammatory cytokines.

CCR6 is expressed on memory T cells and immature dendritic cells (Liao et al., 1999;Yang et al., 1999). The ligand-receptor pair CCL20-CCR6 has been described to be responsible for the chemoattraction of immature dendritic cells, effector/memory T cells and B cells and reported to play a role at skin and mucosal surfaces under homeostatic and inflammatory condition, as well as in pathology (Schutyser et al., 2000;Schutyser et al., 2003).

I was interested in determining the chemokine receptors that might be responsible for recruiting Th17 and Tc17 cells to the liver. The potential candidates include CXCR3 and its ligands, CXCL9 CXCL10 CXCl11 and CCR6 and its ligand CCL20. Our group have shown that these ligands are expressed by inflamed bile ducts. Importantly, Th17/Th1 cells expressing both CCR6 and CXCR3 have been reported previously (costa-Rodriguez et al., 2007b).

Although Th17 have been described in human liver diseases, little is known about their phenotype, homing receptors and how they are positioned in inflamed liver. Even less is known about Tc17. This lead me to investigate the phenotype of liver infiltrating Th17and Tc17cells, their frequency and distribution and also to address the questions of which chemokine receptor/chemokine pair might be involved in their recruitment to the liver and positioning around bile ducts.

5.2 RESULTS

5.2.1 IL-17 secreting lymphocytes in human liver

IL-17 staining lymphocytes were detected in both normal and diseased human liver tissue. Very few cells were seen in normal liver but in chronically inflamed tissue cells could be detected in inflamed portal tracts, septa and within the lobules in the parenchyma. There was no detectable staining on hepatocytes, epithelial cells or mesenchymal tissue. IL-17 staining in human liver tissues was shown in Figure 5.1.

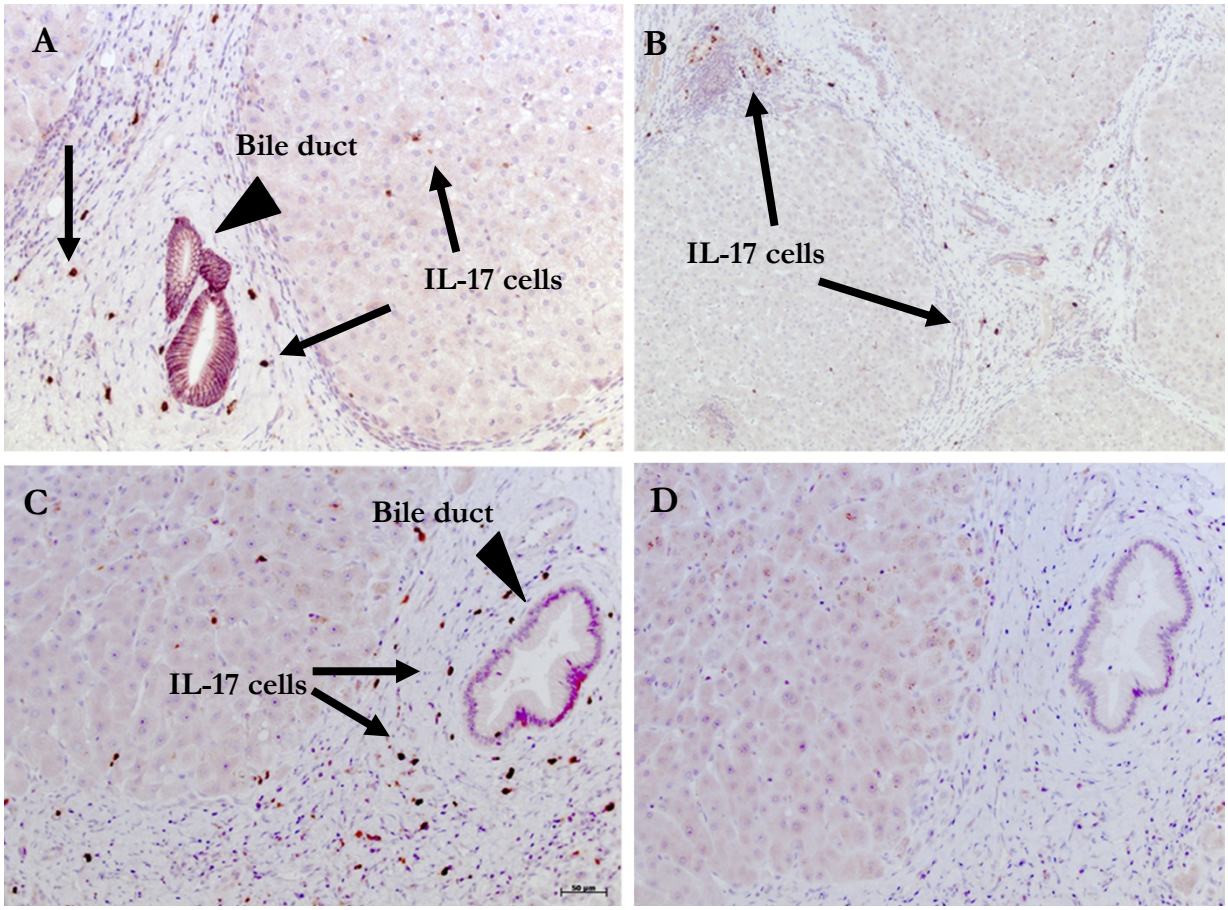

Figure 5-1 Hepatic IL-17 expression in different inflamed diseased livers.

Immunohistochemical staining of IL-17 lymphocytes (shown with arrows) in inflamed human non-alcoholic steatohepatitis liver (A), alcoholic liver disease (B) and primary biliary cirrhosis (C) using goat polyclonal antibody against IL-17. IL-17 cells were noted mainly in portal tract but they are also present in parenchyma. No detectable staining was seen in control tissue (D). Images were taken at magnification X200.

Having identified the presence of IL-17 cells in inflamed liver by immunohistochemistry, I used flow cytometry to analyse freshly isolated liver infiltrating lymphocytes. Il-17 secreted cells were defined by expression of Il-17 after PMA ionomycin treatment. In chronic inflammatory liver diseases the frequency of Il-17^{+} cells defined in this way was 1-2% of the total lymphocyte population whereas Il-22^{+} cells represented 0.3% (Figure 5.2)

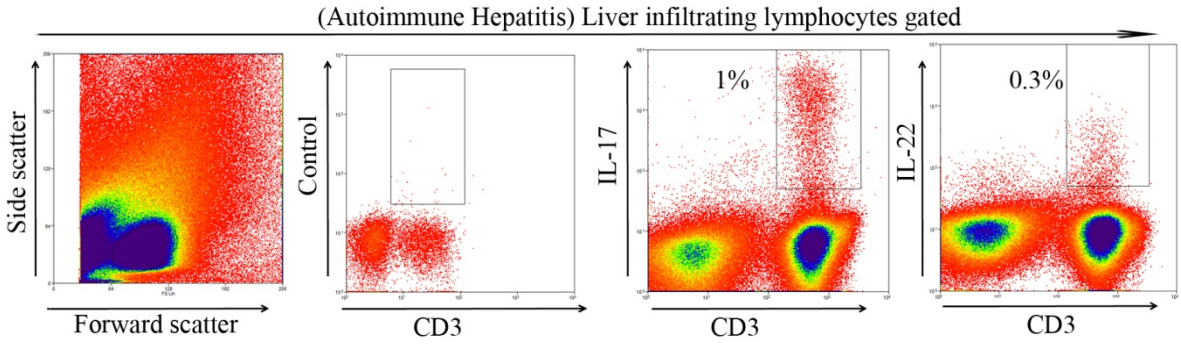

Figure 5-2 IL-17 & IL-22 secreting CD3 and CD4 lymphocytes (Th17) in human liver

Freshly isolated liver infiltrating lymphocytes were stimulated with PMA and ionomycin for 5 hours and Brefeldin A was used to block Golgi apparatus for intracellular cytokines. Then the cells were stained with lymphocyte markers CD3, and CD4. These cells were then fixed with paraformaldehyde and permeabilized with saponin and stained for intracellular IL17 and IL22. Flow cytometry analysis of isolated liver infiltrating lymphocytes showed the presence of IL-17 and IL-22 in total CD4^{+} liver infiltrating lymphocytes compared with isotype control. Cells are gated on total liver derived lymphocytes and the presence of IL-17 and IL-22 in these lymphocytes was shown. Control sample did not have any staining for intracellular cytokines. (Analysisi of explanted liver tissue from autoimmne hepatitis was shown)

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5.2.2 IL-17 expressing cells frequency in normal and diseased liver

Immunohistochemical analysis was performed to investigate the frequency of IL-17 secreting cells and their distribution in inflamed liver tissue and control sections. Histological sections of undiseased liver and end stage explanted primary biliary cirrhosis, autoimmune hepatitis, chronic hepatitis C, nonAnonB/seronegative hepatitis, alcoholic liver disease and non-alcoholic steatohepatitis patients were stained with IL-17 antibody. Diseased livers contained significantly higher numbers of IL-17 positive cells per examined area (portal tract/parenchyma) compared with normal/resection liver tissues. The highest numbers of IL-17 expressing cells were seen in steatohepatitis (alcoholic and non-alcoholic) but they were also seen in other chronic liver diseases including chronic HCV infection, primary biliary cirrhosis and autoimmune hepatitis. Interestingly, patients with fulminant seronegative hepatitis had the highest frequencies of IL-17 secreting cells.

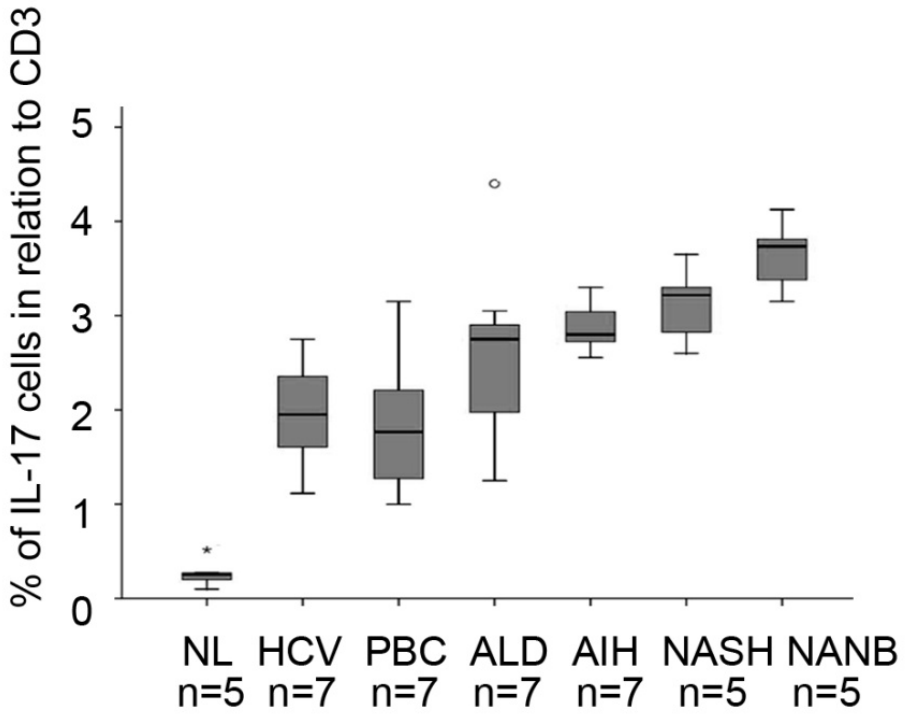
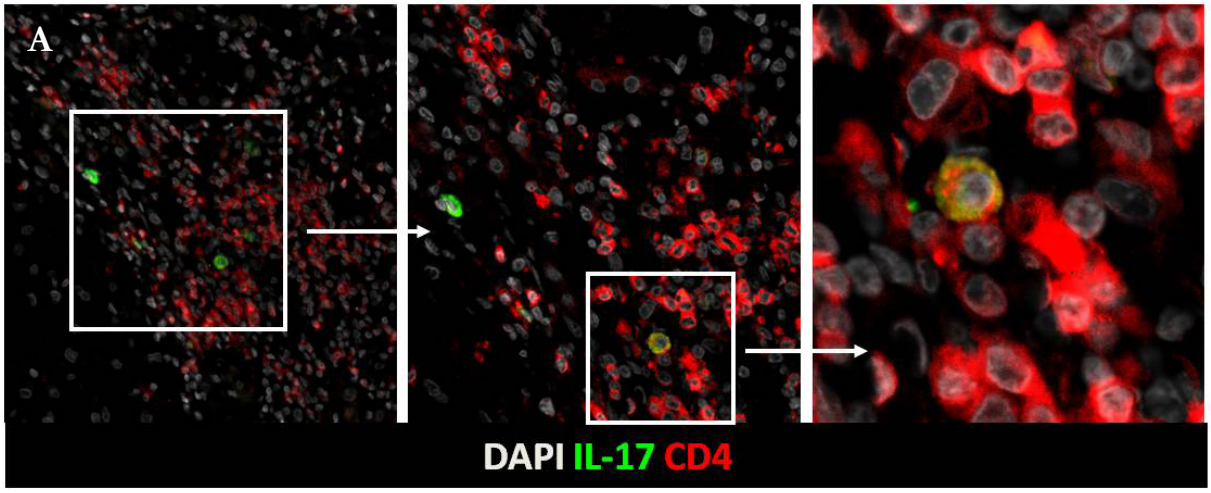


Figure 5-3 IL-17 lymphocytes frequency in different chronic liver diseases and normal liver.

Frequency of IL-17 expressing cells were accessed in normal liver and different types of chronically inflamed liver diseases (normal liver; n=5, diseased livers n=5-7). Five areas were examined and average was taken for each cases. Compared to normal liver, diseased livers have significantly higher percentage of IL-17 expressing cells ($p \leq 0.05$). Y axis showed the percentage of IL-17^+ cells in relation to CD3 and X axis showed the different types of liver diseases ($p \leq 0.05$ comparing normal with different diseased liver diseases using Mann Whitney U test).

5.2.3 Presence of Th17 cells in inflamed human liver

To further define the $IL17^+$ cells in tissue I used confocal microscopy staining CD4 intracellular IL-17 and DAPI as a nuclear stain in paraffin embedded liver tissue after antigen retrieval. The staining detected both $CD4^+$ $IL\text{-}17^+$ dual positive cells in inflamed liver tissues (Figure 5.4A). Th17 staining in hepatic inflammation was confirmed by analysing the profile through a cell from confocal microscopy (Figure 5.4B).



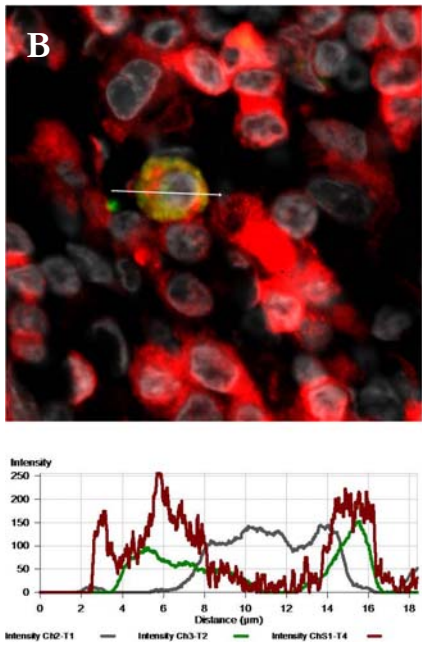


Figure 5-4 Th17 cells in chronic liver disease.

(A) Confocal staining of a patient with autoimmune hepatitis showing $CD4^+$ $IL\text{-}17^+$ dual positive Th17 cells. Middle and right panel showed the magnified image. Original magnification x 25.

(B) Cross section of a Th17 cells shows three different plane of staining in Th17 cell ($CD7^+IL\text{-}17^+$). Red line represents CD4 staining. Green line represents intracellular cytoplasmic IL-17 staining and Gray line represents DAPI nuclear staining at different wavelength. In this cross section of the Th-17 cell, CD4 surface staining was detected in the periphery (red line/Cy5) and IL-17 staining was in the cytoplasm (green line/FITC) and nuclear staining (grey line/DAPI).

5.2.4 IL-17 secreting lymphocytes are not restricted to CD4 subset.

When confocal images were analysed closely, not all IL-17^{+} cell costained with CD4 (Th17 cells), and some IL-17 staining cells are not at CD4 T cells areas. This finding leads me to investigate whether there are subsets of lymphocytes such as CD8 cells which express IL-17.

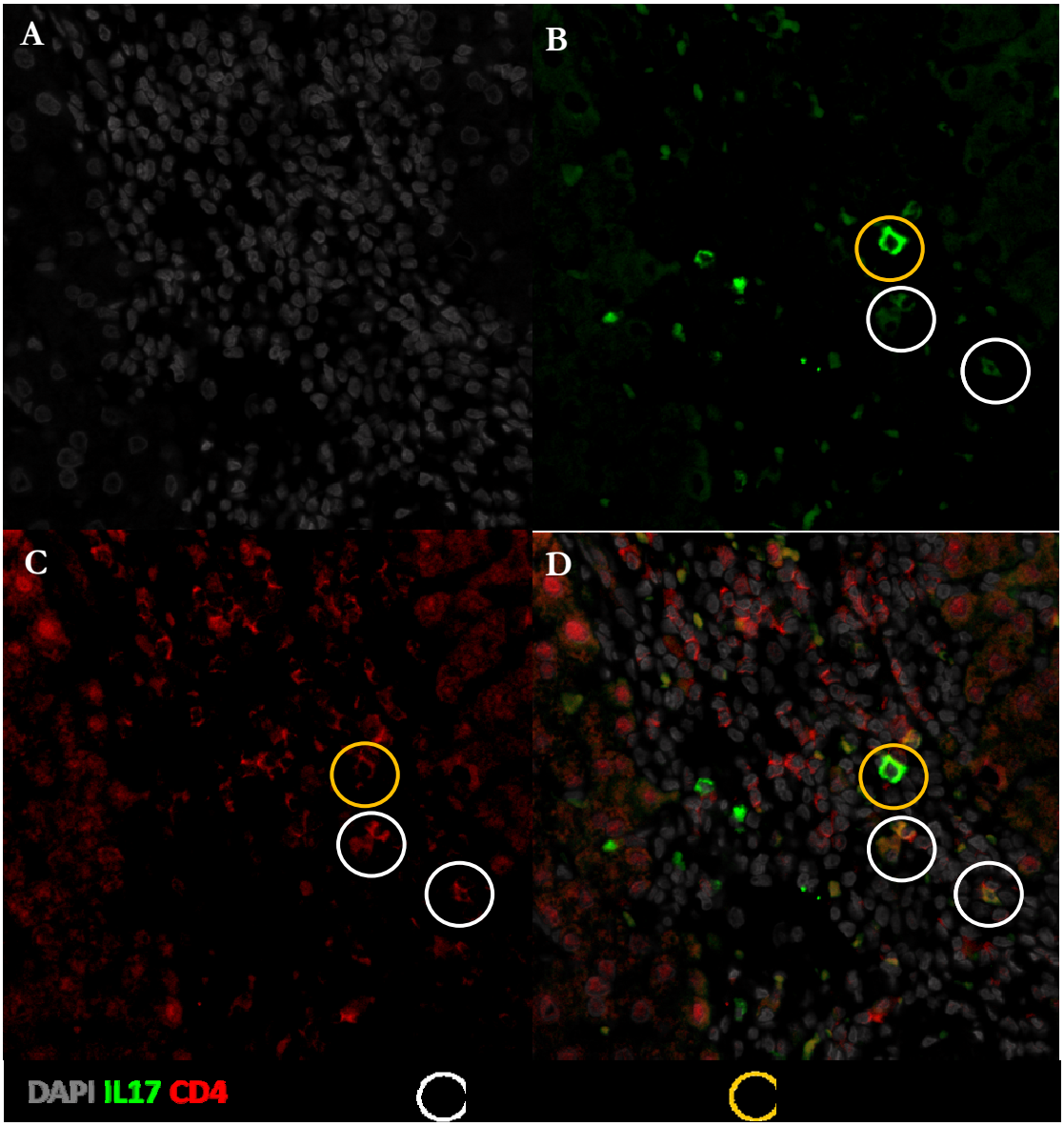


Figure 5-5 IL-17 secreting cells in liver are not restricted to CD4 cells.

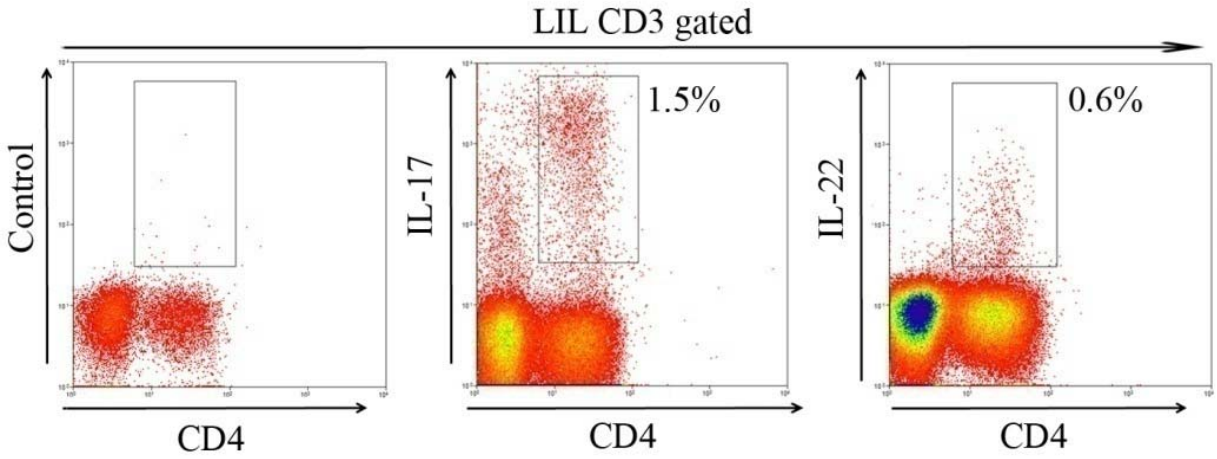
Confocal microscopy image of autoimmune hepatitis stained with DAPI (grey colour; A), IL-17 (green colour; B) and CD4 staining (red colour; C). Images were captured at x25. Dual staining show area of CD4 and IL-17 together shown in white circle (D) suggests presence of Th17 cells. However there are areas of IL-17 staining are without CD4 staining (yellow circle).

Th17 = IL-17 secreting CD4 cells; Tc17= IL-17 secreting CD8 cells.

5.2.5 IL-17 secreting CD4 (Th17) and IL-17 secreting CD8 (Tc17) are present in inflamed human liver

In order to analyse the subsets of IL-17 secreting cells in inflamed liver, I stained for CD4 and CD8 along with intracellular cytokines in flow cytometry analysis. The freshly isolated liver infiltrating lymphocytes were gated using forward and side scatter, then gated again on CD3 to exclude other cell types. These cells were then further analysed for CD4/CD8 against IL-17 and IL-22. This clearly demonstrated that IL-17 and IL-22 secreting cells are both detected in the CD4 Th17 (Figure 5.6A) and CD8 Tc17 (Figure 5.7A). The percentage of Th17 cells was much higher compared with Tc17 cells. Confocal analysis using CD4 and CD8 lymphocyte surface markers and IL-17 also suggested that both Th17 and Tc17 cells are present in the inflamed human livers (Figure 5.6B and Figure 5.7B)

A



B

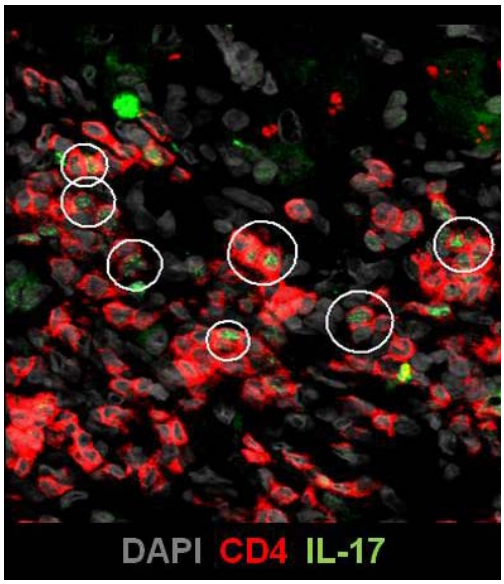


Figure 5-6 (A) Flow cytometry and (B) confocal microscopy showing Th17 in inflamed human liver.

(A) Liver derived lymphocytes were stained with surface marker CD3 and CD4. Then cells were stimulated for 5 hours with PMA + ionomycin and followed by intra-cellular IL-17 & IL-22 cytokine staining. Cells were gated on liver infiltrating CD3 showing that presence of IL-17 secreting CD4 cells (Th17) and IL-22 secreting CD4 cells. Flow cytometry dot plot from a patient with non-alcoholic steatohepatitis was shown.

(B) Staining of non-alcoholic steatohepatitis cirrhosis liver section showing presence of IL-17 secreting CD4 lymphocytes (Th17). CD4 lymphocyte = red; IL-17 = green; Nuclei were stained with DAPI = Grey. Infiltration of Th17 is mainly observed in portal tract area (X40)

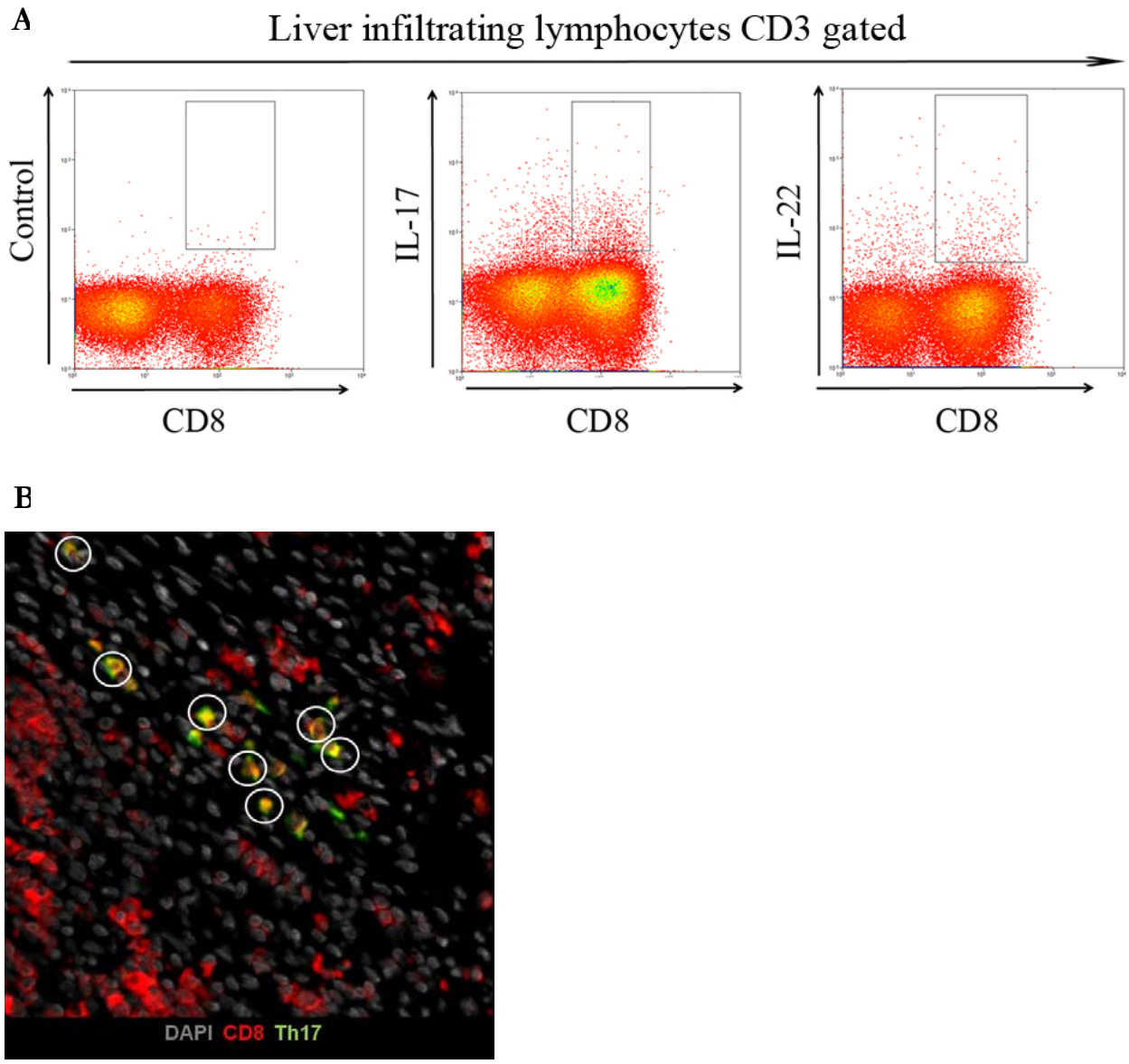


Figure 5-7 (A) Flow cytometry and (B) confocal microscopy demonstrating Tc17 in inflamed human liver.

(A) Liver derived lymphocytes were stained with surface marker CD3, and CD8. Then cells were stimulated for 5 hours with PMA + ionomycin followed by intra-cellular IL-17 & IL-22 cytokine staining. Cells were gated on liver infiltrating CD3 demonstrating the presence of IL-17 secreting CD8 subsets Tc17 in a patient with primary biliary cirrhosis. IL-22 expressing CD8 cells were also noted.

(B) Staining of Primary biliary cirrhosis liver section showing the presence of IL-17 secreting CD8 lymphocytes (Tc17). CD8 lymphocyte = red; IL-17 = green; Nuclei were stained with DAPI = Grey. Infiltration of Tc17 are mainly observed in portal tract area (X40)

5.2.6 Th17 and Tc17 in normal and diseased liver

The frequency of Th17 and Tc17 in normal and diseased liver tissue was investigated by flow cytometry. In order to get the actual frequency, all the experiments were done on freshly isolated explanted human liver and stimulation and staining was done on the same day. In general, Th17 cell were more frequent than Tc17 in diseased livers (Table 5.1).

Th17 percentage was higher in diseased liver compared to normal liver (Figure 5.8A). IL-22 expression on Th17 cells are also much more frequent in diseased liver compared with normal liver (Figure 5.8B).

Diseased aetiology	Th17 positive cells%	Tc17 positive cells%
Hepatitis C	1.5%	0.6%
Primary Biliary Cirrhosis	1.6%	0.4%
Alcoholic liver disease	1.7%	0.5%
Autoimmune Hepatitis	2.2%	0.6%
Non alcoholic steatohepatitis	1.8%	0.5%
Normal Liver	0.3%	0.1%
Mean±SD	1.5±0.64	0.45±0.18

Table 5-1 Frequency of Th17 and Tc17 in normal liver and different diseased livers.

Liver infiltrating lymphocytes were freshly isolated, PMA and ionomycin stimulated for 5 hours and then cytokines secretion were blocked with BrefeldinA and stained for both surface and intracellular IL-17 cytokines.

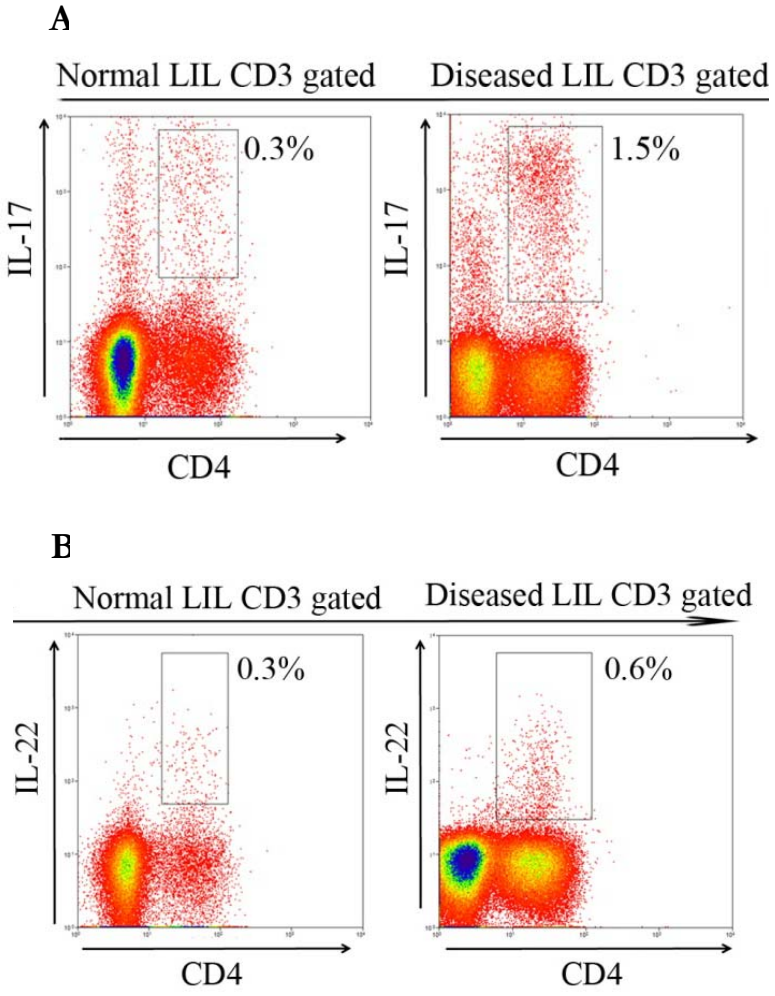


Figure 5-8 Th17 cells in diseased and normal liver

Expression of IL-17 (A) and IL-22 (B) in CD4 subset of lymphocytes analysed by flow cytometry. Frequency of expression was compared between normal and diseased liver. Freshly isolated liver infiltrating lymphocytes were stimulated with PMA and Ionomycin for 5 hours and then stained with CD3, CD4 surface markers. Then cells were fixed with paraformaldehyde and permeabilized with saponin. Intracellular cytokines staining was done with IL-17, IL-22. (N=4 normal; N=5 diseased liver, ALD, PBC, HCV, AIH, NANB).

Tc17 cells and IL-22 expressing CD8 T cells are present in diseased liver tissue (Figure 5.9) however, there is very minimal expression in normal liver to detect by flow cytometry.

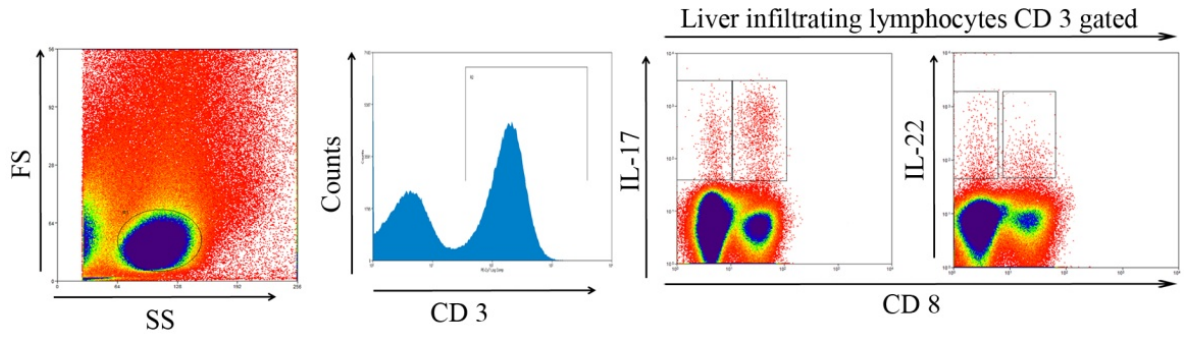


Figure 5-9 Flow cytometry analysis of Tc17 in diseased liver.

Expression of IL-17 and IL-22 in CD8 subset of lymphocytes. Freshly isolated liver infiltrating lymphocytes were stained as described above and analysed on flow cytometry. (N=5 diseased liver, ALD, PBC, HCV, AIH, NANB).

5.2.7 Immunophenotype of human liver infiltrating Th17 and Tc17 in chronic liver diseases

5.2.7.1 Th17 and Tc17 are restricted to CD161^{high} population

It has been reported that human Th17 cells originate from CD161^{+} naive CD4^{+} T cells precursor (Cosmi et al., 2008). Moreover, IL-17-producing cells are contained in the CD161^{+} fraction of CD4^{+} T cells present in the circulation or in inflamed tissues, although they are not CD1-restricted natural killer T cells (Cosmi et al., 2008). In collaboration with Paul Klenerman group in Oxford, I have noted that both liver-infiltrating Th17 and Tc17 cells are mainly restricted to CD161^{high} population as shown in Figure 5.10.

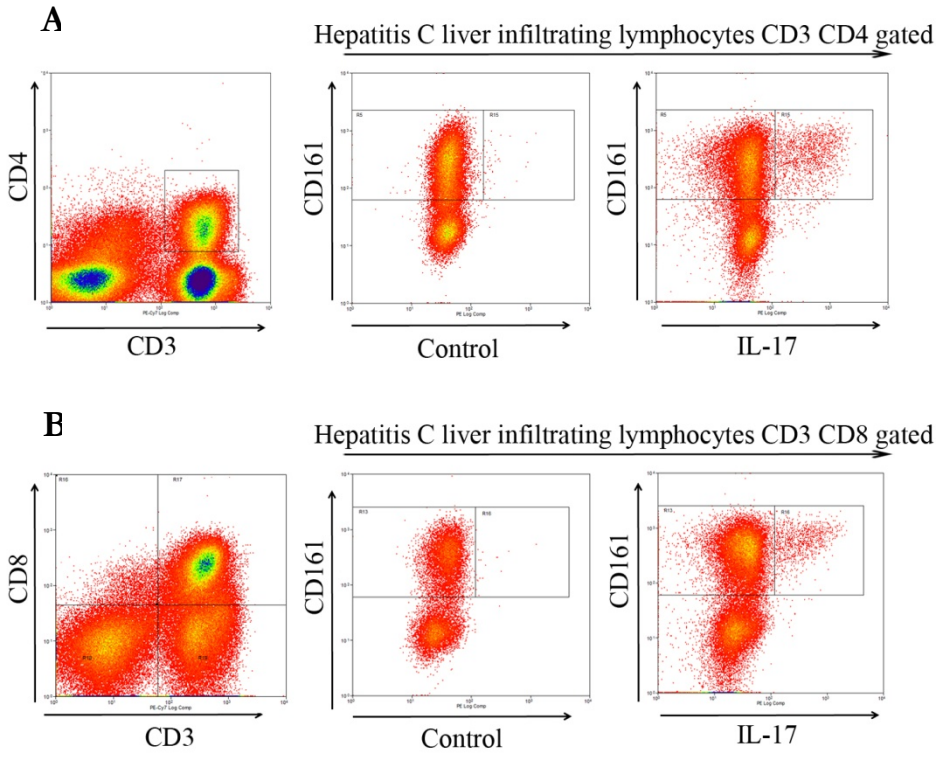


Figure 5-10 Th17 and Tc17 in inflamed human liver are mainly CD161^{high}.

Liver infiltrating lymphocytes are gated on CD4 or CD8 population and this gating was applied to either control or IL-17 vs CD161. The result suggested that both Th17 (A) and Tc17 (B) were restricted to CD161^{high} population. One representative patient (HCV) of six experiments was shown. (HCV, ALD, PBC, NANB, NL).

5.2.7.2 Th17 and Tc17 cells express IL-23 receptor.

IL-23 is a heterodimeric cytokine of the IL-12 family composed of p19 subunits and the p40 subunit which is also shared by IL-12 (Oppmann et al.). Initial Th17 lineage commitment of CD4 cells requires IL-1β, IL-6 and TGF-β and once they are differentiated, IL-23 has been shown to be crucial for survival and stabilization of Th17 cells. IL-23 p19-deficient mice are resistant to the development of EAE, a Th17 driven disease in mice, compared to p-35 deficient mice (Cua et al., 2003). IL-6 and IL-21 induce expression of the IL-23 receptor in a STAT3 dependent manner (Zhou et al., 2007) which also depends on RORγt because RORγt deficient mice have reduced expression of IL-23R (Nurieva et al., 2007). The presence of IL-23 receptor on Th17 cells is thus essential for IL-23 responsiveness. Flow cytometry analysis on fresh liver infiltrating lymphocytes has shown high IL-23 receptor expression on Th17 and Tc17 cells (Figure 5.11)

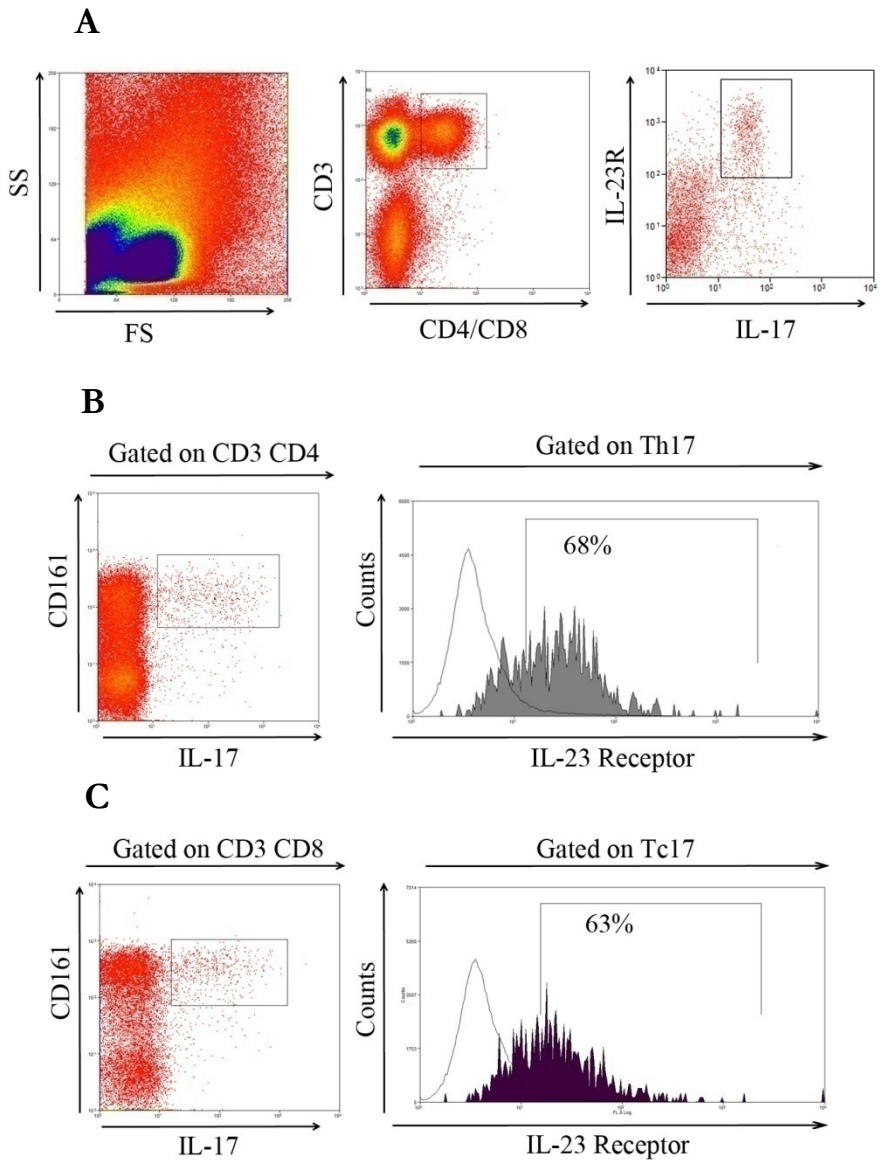


Figure 5-11 IL-23 Receptor expression on liver infiltrating Th17 and Tc17 cells.

Flow cytometry analysis showed the IL-23R expression on LITh17 (B) and LITc17 (C). Liver infiltrating lymphocytes were gated on liver infiltrating CD4 or CD8 cells. (A) dot plot showing the co-expression of IL-17 and IL23R. (B&C) Overlay histogram of percentage IL-23 receptor expression in liver infiltrating Th17 and Tc17 lymphocytes. Representative overlay histogram is of a patient with alcoholic liver disease. N=3.

5.2.7.3 Th17 cells in the liver secrete other cytokines

Th17 cells also secrete other cytokines including IL-22 (Wilson et al., 2007). Thus, freshly isolated liver infiltrating CD4 lymphocytes were gated as above and cytokines expression on Th17 cells analysed. Th17 cells not only expressed IL-17 but also other cytokines including IFN-γ, TNF-α and IL-22. Co-expression with TNF-α was more prominent compared to other cytokines. Thus, Th17 cells are polyfunctional, polysecretor effector cells (Figure 5.12 and Table 5.2)

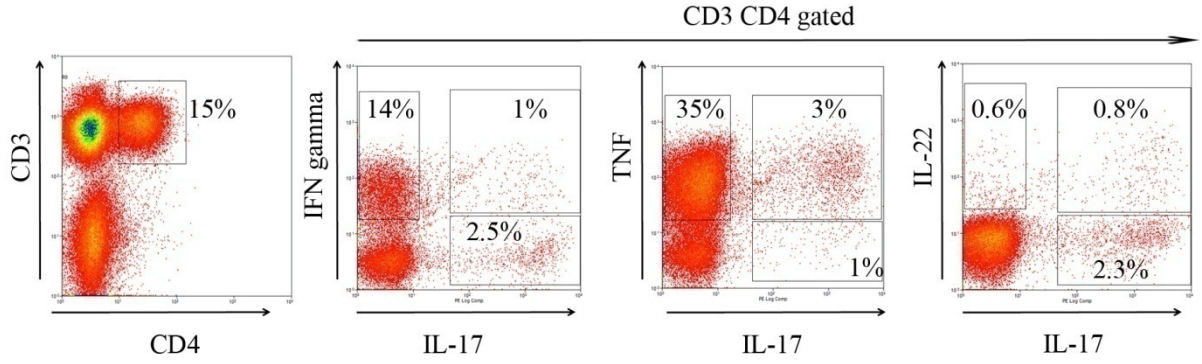


Figure 5-12 Liver infiltrating Th17 cells are polysecretor.

Flow cytometry analysis of liver infiltrating CD4 lymphocytes from a patient with autoimmune hepatitis. There is co- expression of intracellular IL-17 with IFN-γ, TNF-α and IL-22. Cells were gated on $CD3^+$ $CD4^+$ liver infiltrating lymphocytes. (N=4)

Disesese state	IL-17+IFN-γ	IL-17+TNF-α	IL-17+IL-22
Autoimmune hepatitis	1%	3%	0.8%
Primary biliary cirrhosis	1.2%	1.4%	0.7%
Alcoholic liver disease	1.1%	1.2%	0.7%
Autoimmune hepatitis	1.2%	1.3%	1%

Table 5-2 Percentage coexpression of different cytokines on liver infiltrating Th17 cells.

Table showing the percentage coexpression of different cytokines on Th17 cells in diseased livers. Four diseased livers were studied and they all showed co-expression of intracellular cytokines.

5.2.7.4 Expression of transcription factor RORC in Th17 and Tc17 cells.

Retinoid nuclear receptor RORC is the human homologue of murine RORγt and RORα the signature Th17 transcription factor. RORγt is selectively expressed *in vitro* differentiated Th17 cells (Ivanov et al., 2006). RORα is another member of the retinoid receptor family and plays a similar role in the differentiation of Th17 cells (Yang et al., 2008c). The induction of RORγt is dependent on STAT3 (Yang et al., 2008c;Zhou et al., 2007) and IL-23 R expression is dependent on RORγt (Nurieva et al., 2007). My analysis on liver infiltrating Th17 and Tc17 cells suggested that both highly express the signature transcription factor RORC (Figure 5.13)

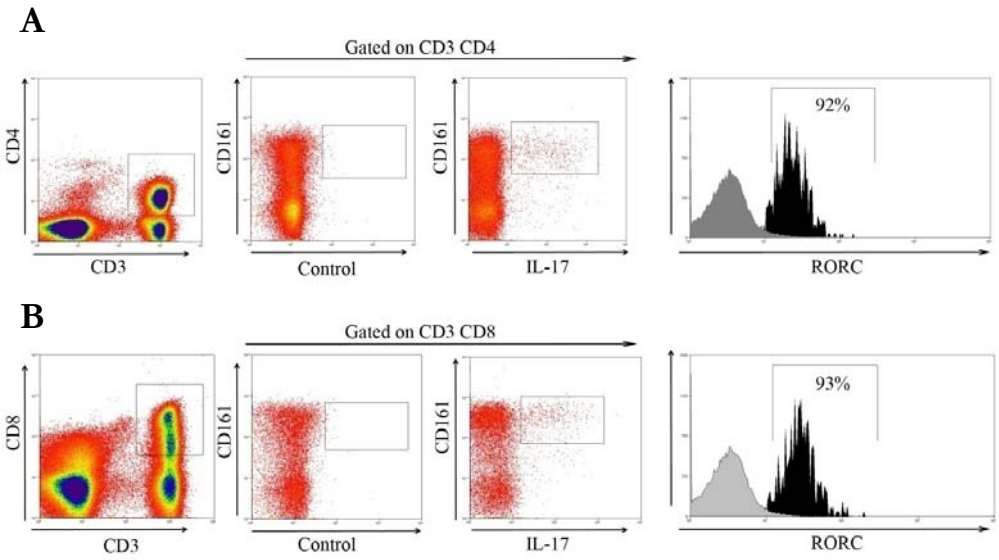


Figure 5-13 Expression of transcription factor RORC in liver infiltrating Th17 and Tc17 cells

Freshly isolated liver infiltrating lymphocytes were stimulated with PMA and ionmycin. Then cells were fixed, permeablized and stained with intracellular cytokine for IL-17 and transcription factor RORC. Liver infiltrating Th17 (A) and Tc17 (B) both has high expression of transcription factor RORC.

5.2.7.5 Chemokine receptors expression on LITh17 & LITc17 cells

The expression of potential homing receptors on liver infiltrating Th17 and Tc17 lymphocytes was assessed by using flow cytometry (Figure 5.14). LITh17 cells express CCR4, CCR6 and CXCR3 (Figure 5.14A). LITc17 cells express CCR6 and CXCR3 (Figure 5.14B). Importantly, CCR6 and CXCR3 were highly expressed in both subsets. Percentage expression of chemokine receptors on both subsets in liver were described in Figure 5.14 and Table 5.3.

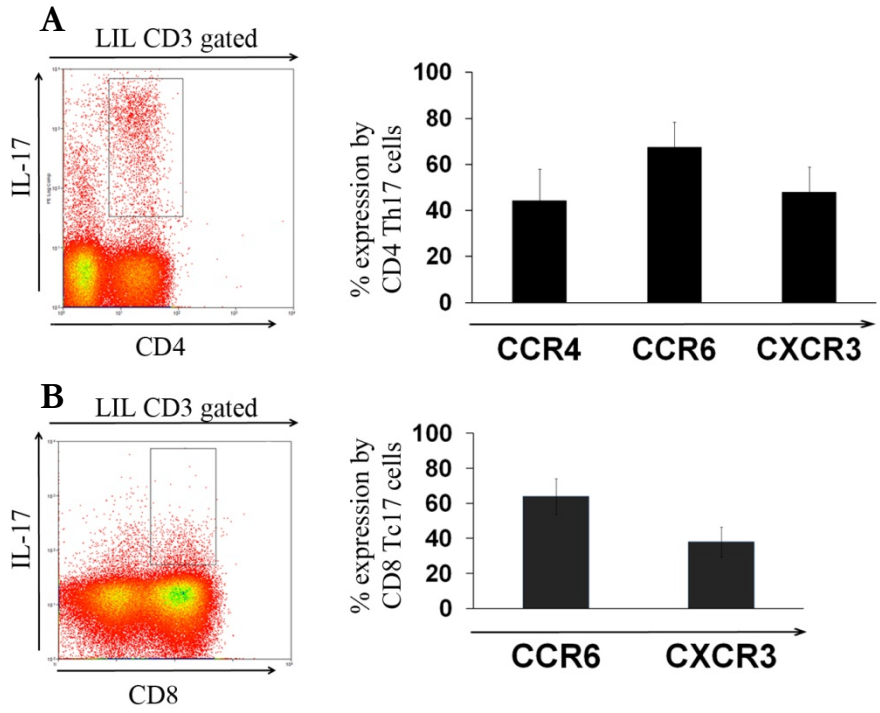


Figure 5-14 Chemokine receptors expression on liver infiltrating Th17 and Tc17 cells.

Liver infiltrating lymphocytes were freshly isolated and stained for surface receptor CD3, CD4 and CD8. Then cells were stimulated with PMA and inomycin for 5 hours with final golgi block for last 2 hours. These cells were then stained for intra-cellular cytokine IL-17 and chemokine receptors and analysed on flow cytometry. Data is from diseased livers (2x autoimmune hepatitis, primary biliary cirrhosis, alcoholic hepatitis) N=4

	CCR4	**CCR6**	**CXCR3**
Th17 % (Mean±SD)	44±13.7	67±9.8	48±11.8
	CCR6	**CXCR3**	
Tc17 % (Mean±SD)	65±10.8	38±8.5	

Table 5-3 Chemokine receptors expression on liver infiltrating Th17 and Tc17 cells.

Liver infiltrating Th17 and Tc17 chemokine receptors expression percentage is shown in Table 5.3.

5.2.7.6 Integrins expression on LITh17 and LITc17

Both β1 and β2 integrins were present at high levels on most LITh17 and LITc17 cells as seen in other lymphocytes subsets. Integrins which are important for lymphocyte recruitment via endothelium include $\alpha_L\beta_2$ (LFA-1, CD11a/CD18) which binds ICAM-1 & ICAM-2 on endothelium, and α4β1 (VLA-4, CD49d/CD29), α4β7 (LPAM-1, CD49d/cd104) via its ligands VCAM-1 and MadCAM-1 (Butcher & Picker, 1996). Flow cytometry analysis suggested that $\alpha_L\beta_2$ is highly expressed on both subsets and there was also high expression of β1 integrins (Figure 5.15).

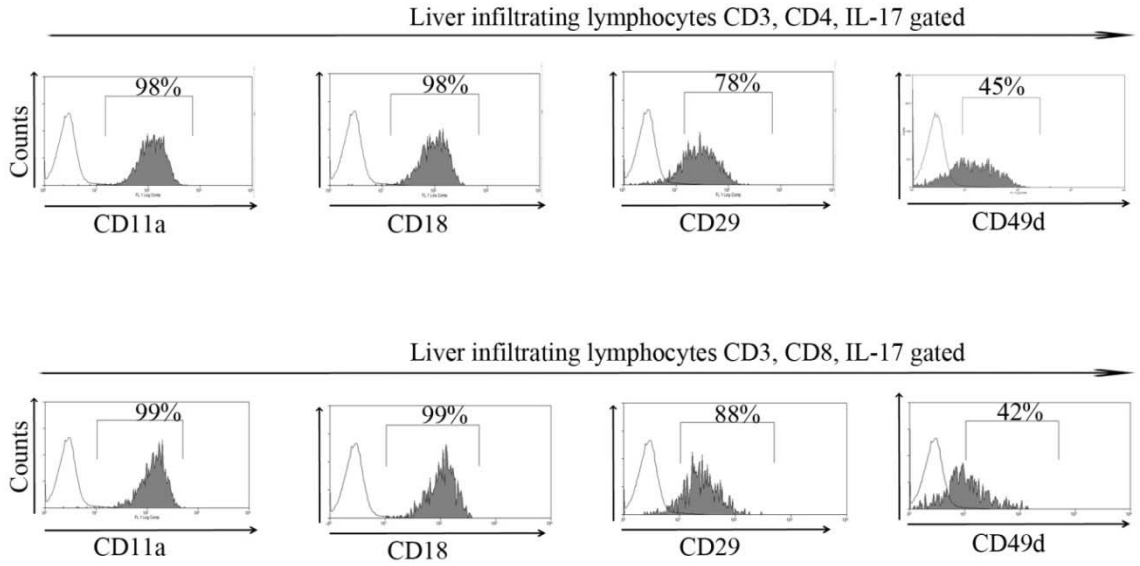


Figure 5-15 Expression of integrins on LITh17 and LITc17 cells.

Flow cytometry analysis on liver infiltrating Th17 and Tc17 stained for surface markers for CD3, CD4, CD8 and intra-cellular cytokines IL-17. Then these Th17 and Tc17 cells were gated on CD11a, CD18, CD29 and CD49d. Both Th17 and Tc17 in inflamed liver express high levels of LFA-1 and VLA-4. Sample from a patient transplated for autoimmune hepatitis was shown. N=4.

5.2.8 IL-17 secreting lymphocytes were located around human biliary epithelial cells

Immunohistochemistry demonstrate many of the IL-17 cells in portal tracts were closely associated with bile ducts (Figure 5.16). Dual staining with the biliary epithelial cells marker (HEA125) and IL-17 also suggest that IL-17 cells are in close proximity to bile ducts (Figure 5.1A&C). This finding was similar to previous studies suggesting that IL-17 cells were observed in peri-ductal region and increase frequency was noted in biliary disease (Harada et al., 2009;Lan et al., 2009).

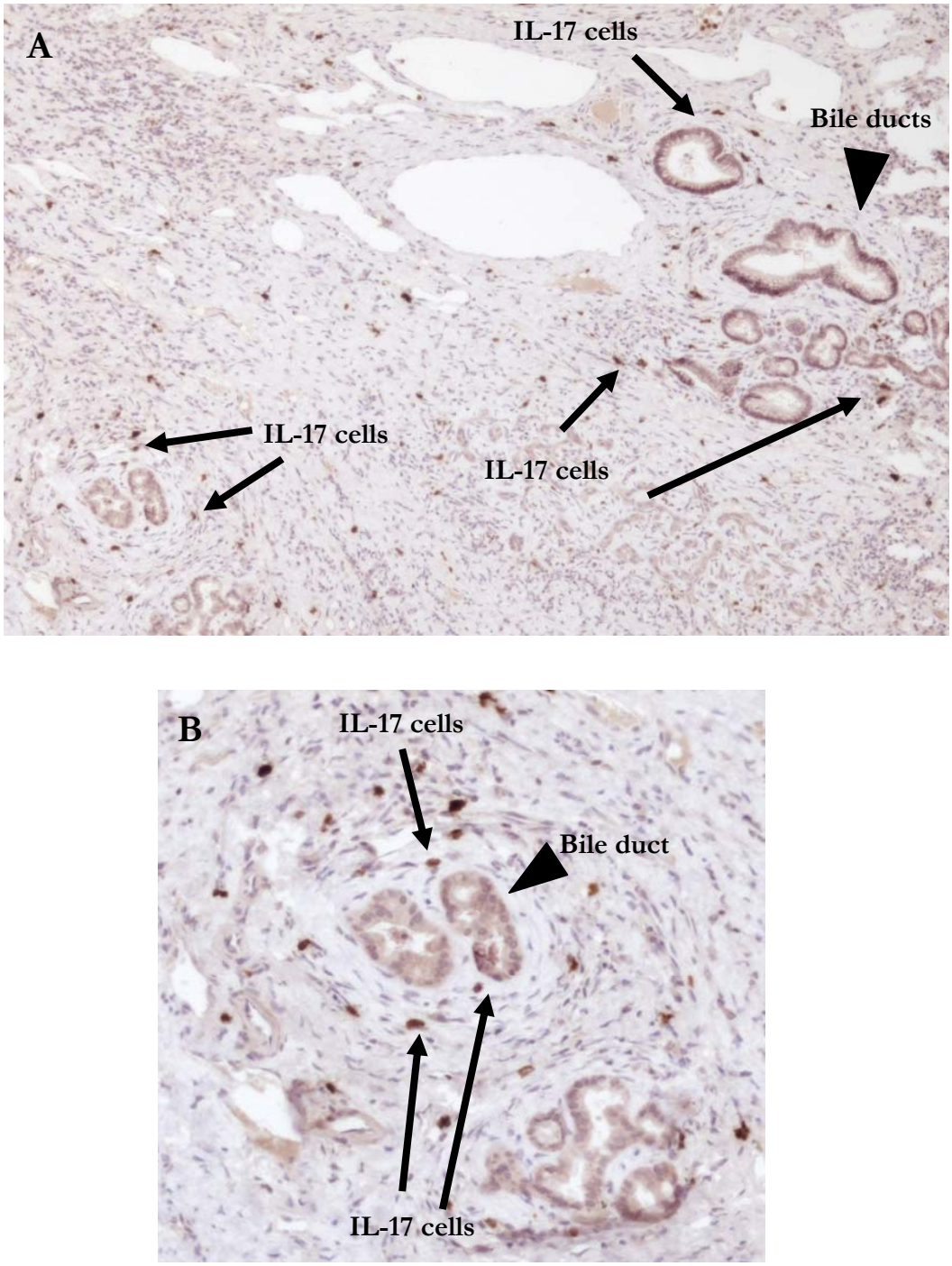


Figure 5-16 Presence of IL-17 cells in close proximity to bile ducts.

Immunohistochemical staining of IL-17 demonstrate that IL-17 cells (arrow) are located around bile ducts (arrow head). IL-17 staining was shown as brown pigment. Picture was taken at x20 lens. Patient with primary biliary cirrhosis sample was shown.

5.2.9 Biliary Epithelial cells express IL-17RA receptor

As IL-17 secreting cells were detected around the bile ducts, I proceed to investigate whether biliary epithelial cells (BEC) express the IL-17 receptor. IL-17 receptor family includes IL-17RA to IL-17RE. In humans, IL-17RA can form a heterodimer with IL-17RC that binds to human IL17A and IL-17F (Toy et al., 2006), two major cytokines secreted from Th17 cells. IL-17RC is the cognate receptor for IL-17F (Kuestner et al., 2007). IL-17 receptor has been shown to be expressed widely on epithelial cells, endothelial cells, haemopoietic cells and fibroblasts.

Human biliary epithelial cells (BEC) from diseased livers were isolated and flow cytometry analysis was performed to detect IL-17 receptor (IL-17RA). Intracellular staining of CK19 marker (normally >97% of gated population) was used after to confirm that the cells were biliary epithelial cells. BEC were stimulated with TNF-α (10ng/ml) and IFN-γ (50ng/ml each) for 24 hours. Biliary epithelial cells express the IL-17RA receptor (Figure 5.17). More than 90% of early low passage cells (P1, P2) express IL-17RA receptor and its expression regress with higher passage (P4- P6).

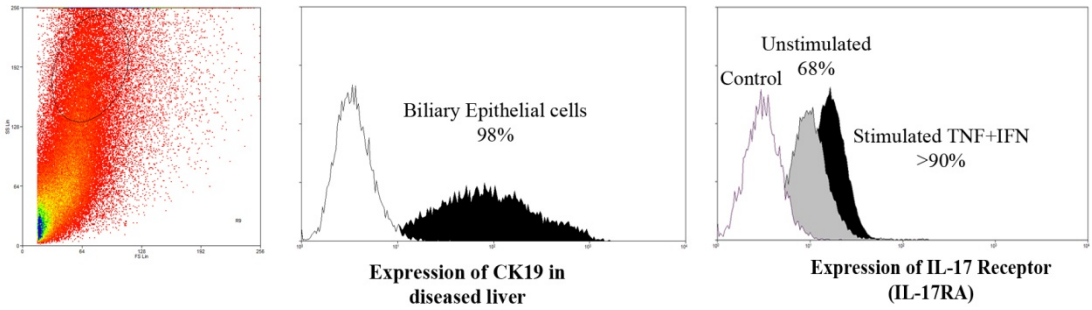


Figure 5-17 Expression of IL-17 Receptor (IL-17RA) on the biliary epithelial cells.

Biliary epithelial cells (BEC) were stimulated with TNF-α (10ng/ml) and IFN-γ (50ng/ml) for 24 hours and expression of IL-17RA receptor was accessed by flow cytometry. Cells were gated on forward and side scatter. CK19 was used as biliary cell marker and stained after fixation and permeabilization of cell. Expression of IL-17RA receptor was higher on stimulated BEC (93%) compared to unstimulated BEC (68%). One representative example of 4 experiments was shown.

5.2.10 Expression of CCL20 on human biliary epithelial cells.

5.2.10.1 Immunohistochemistry for CCL20

Detection of Th17 cells around bile ducts associated with expression of the IL-17 receptor on biliary epithelium led me to investigate whether biliary epithelial cells secrete the chemokine CCL20 (the ligand for CCR6) which could recruit $CCR6^+$ IL-17 cells to the bile ducts. First, I determined the expression and distribution of CCL20 in normal and inflamed liver tissue using immunohistochemistry on paraffin embedded liver tissue sections. The expression of CCL20 was largely restricted to bile ducts and bile ductules in inflammatory liver disease (Figure 5.18A). There were no detectable staining on hepatocytes and staining with the control antibody was negative (Figure 5.18B).

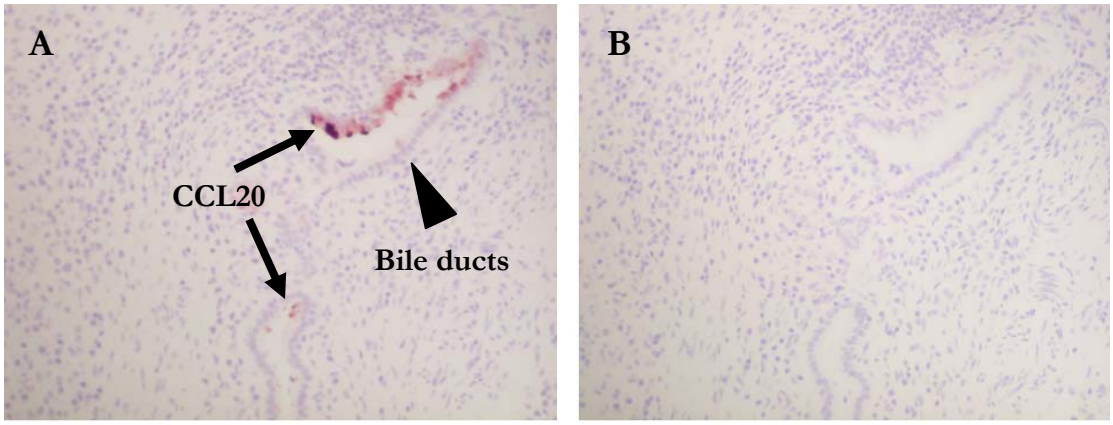


Figure 5-18 Expression of CCL20 on human biliary epithelial cells

Liver tissue sections were stained with CCL20 antibody. CCL20 staining was shown in Figure A (red pigment shown with arrow). Staining was particularly intense on bile ducts (arrow head) around the portal tract area. There was no detectable staining of hepatocytes, or vascular endothelium. Staining of control antibody (B) was negative. Staining on primary biliary cirrhosis and primary sclerosing cholangitis was shown. N=4; Original magnification X 20.

5.2.10.2 ELISA for CCL20

To confirm the immunohistochemical CCL20 staining on bile ducts and to determine whether CCL20 protein is secreted by biliary epithelial cells. I measured CCL20 secretion using a sandwich ELISA in biliary epithelial cells conditioned supernatant. Biliary epithelial cells were stimulated with IL-1β, IL-17, TNF-α, IFN-γ or combination of TNF-α and IFN-γ (10 ng/ml) for 24 hours and the supernatants were collected for Quantikine sandwich ELISA analysis was performed (Figure 5.19).

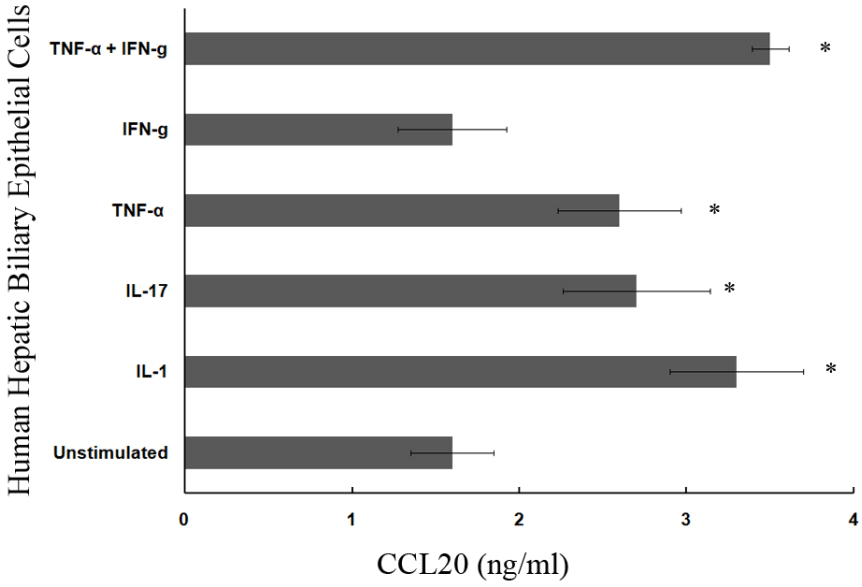


Figure 5-19 CCL20 secretion by biliary epithelial cells.

Sandwich ELISA was used to measure CCL20 in culture supernatants from unstimulated biliary epithelial cells (BEC) and those stimulated with different cytokines. This confirmed the immunohistochemical findings of detection of CCL20 on bile ducts. BEC were stimulated with different cytokines including TNF-α, IFN-γ, combination of both, IL-1β and IL-17(all 10ng/ml) for 24 hours before supernatants were collected for ELISA. Data represent the mean ±SEM of four diseased livers (PBC, ALD, PSC, and NASH). Statistical significance was calculated using student's t test (*p ≤0.05) and compares the unstimulated and different cytokines stimulated biliary epithelial cells.

5.2.10.3 *Transcription of* CCL20 and IL17RA by biliary epithelial cells

To investigate whether biliary epithelial cells has messenger RNA for CCL20 and IL-17RA receptor, I carried out PCR reaction on isolated human BEC cells. PCR was done on both unstimulated and biliary epithelial cells stimulated with different cytokines (Figure 5.20). In all reactions, a negative PCR control was included. The message for CCL20 and IL-17 RA receptor was observed on both unstimulated and different cytokines stimulated biliary epithelial cells. Each reaction was performed with a non-template control and RT-control included in the same run.

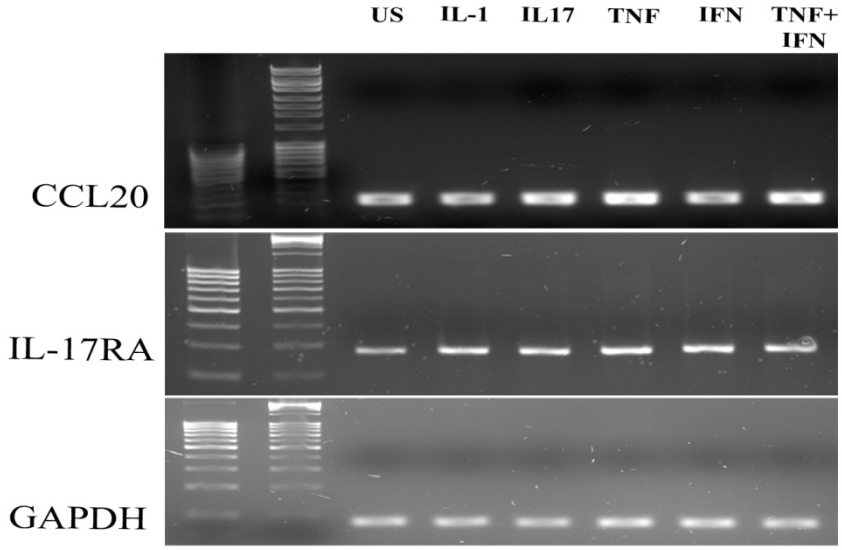


Figure 5-20 Expression of CCL20 and IL-17 receptor transcripts (mRNA) in human biliary epithelial cells.

De novo synthesis of CCL20 and IL-17RA receptor messenger RNA by both cytokine stimulated and unstimulated biliary epithelial cells were shown. RT-PCR was performed and product was developed on 2% Agarose gel. Data from one of 3 replicate experiments was shown. (US=unstimulated); IL-1=interleukin 1β; IL17=interleukin17; TNF=tumour necrosis factor-α; IFN=interferon-γ. Negative PCR control without cDNA was used in each reaction.

5.2.11 CCR6 on Th17 is functional

The chemokine receptor CCR6 and its ligand CCL20 has been reported to mediate Th17 cell migration (Hirota et al., 2007;Singh et al., 2008) and I investigated whether CCR6 Th17 cells was functionally active. Chemotaxis assays was used to detect whether CCR6 chemokine receptor expressing Th17/Tc17 cells migrate towards biliary epithelial cells which express and secrete CCL20. CD4 cells were isolated using a Dynal CD4 isolation from which highly purified Th17 cells were isolated again using Miltenyi IL-17 secretion and capture. These cells were cultured in Th17 polarizing cytokines with IL-1β, IL-6, TGF-β and IL-2 previously described (Bettelli et al., 2006;costa-Rodriguez et al., 2007a;Cua et al., 2003;Korn et al., 2007;Langrish et al., 2005;Mangan et al., 2006;Nurieva et al., 2007;Veldhoen et al., 2006;Zhou et al., 1998).

Th17 cells were cultured in Iscove's modified Dulbecco's medium which is rich in aromatic amino acids and AhR agonists which has been shown to be essential for expansion of both mice and human Th17 cells (Veldhoen et al., 2008a;Veldhoen et al., 2009). These cultured Th17 cells were then used for migration assay. Biliary epithelial cells were stimulated with different cytokines including IL-17 and supernatants were collected. Purely isolated Th17 cells migrate towards the IL-17 stimulated BEC supernatant (chemotactic index was 2.5 times compared to control) and this migration could be blocked significantly by blocking antibodies against CCL20 in the supernatant or blocking the G protein coupled chemokine receptors with PTX. These findings demonstrate that Th17 cells use chemokine receptors which may include CCR6 to migrate towards CCL20 ligand secreted by biliary epithelial cells (Figure 5.21). However, subsequent investigation following this thesis suggested that chemokines CXCL9-11 also contributed to Th17 migration.

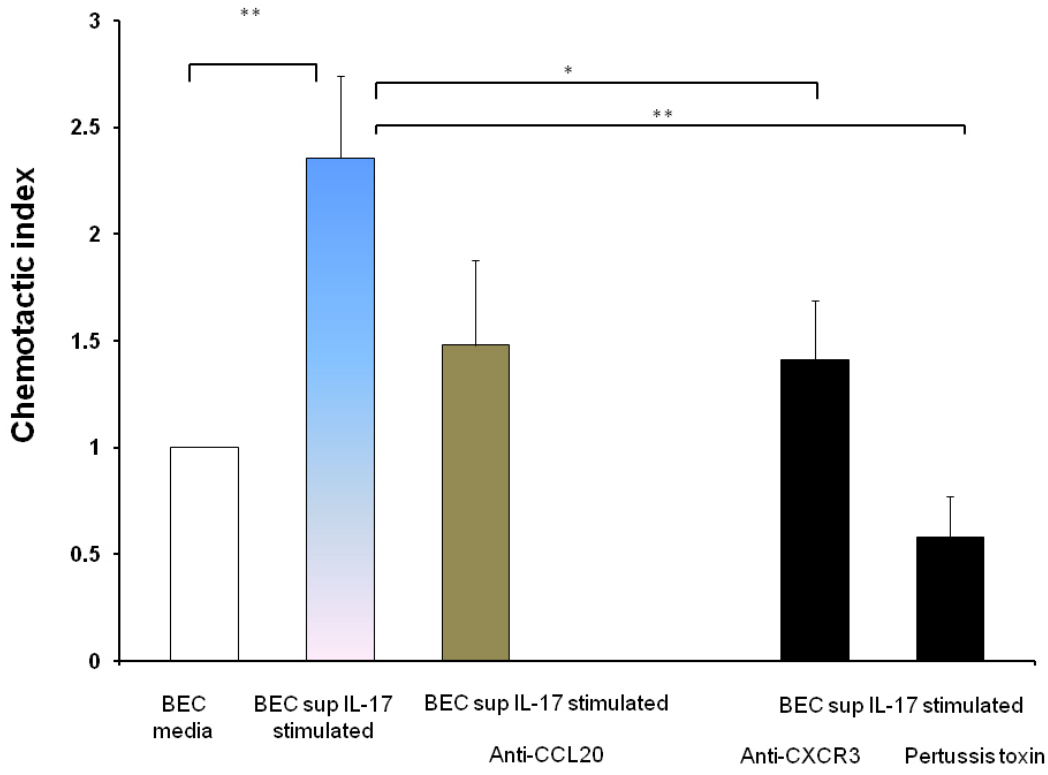


Figure 5-21 Chemotasis of Th17 cells towards BEC supernatant.

Pure Th17 cells were freshly isolated and kept overnight in Th17 polarizing media. BEC culture media was used as negative control. Biliary epithelial cells were stimulated with IL-17 and supernatant were collected after 24 hours. 5μ pore gelatin coated Conning chemotactic migration chambers were used for the assay. The lower chamber was filled with 600μl of BEC media (negative control) or IL-17 stimulated BEC supernatant ± anti CCL20 block. Isolated Th17 cells resuspended in 1ml of RPMI are loaded per well in upper chambers. Assay was done in duplicates. For the chemokine receptors blockade, Th17 cells were incubated with PTX for 30 minutes before the assay and recombinant CCL20 in BEC media was used in the lower chamber.

IL-17 stimulated BEC supernatant induced significantly higher levels of Th17 cells migration compared with unconditioned medium ($p=0.0013$) and this migration could be blocked by adding anti-CCL20 in the stimulated supernatant. The migration could also be significantly reduced by blocking global G-protein with PTX or CXCR3 on Th17. N=4. Student t test was used for statistical analysis.

5.2.12 Positioning of liver infiltrating IL-17 lymphocytes and liver infiltrating T_{reg}

Regulatory T cells and Th17 have a reciprocal relationship in their developmental programs. TGF-β is required for expression of both FoxP3 and RORγt. Naive T cells can be converted to FoxP3 expressing T_{reg} upon TCR stimulation in the presence of TGF-β. However, in the presence of IL-6, TGF-β promotes the differentiation of naive T lymphocytes into proinflammatory IL-17 producing Th17 cells, which promote autoimmunity and inflammation (Mucida et al., 2007). Thus, IL-6 plays a pivotal role in dictating whether proinflammatory Th17 or protective T_{reg} differentiate in inflamed state or steady state of immune system.

I used confocal analysis of FoxP3 and IL-17 staining to assess the positioning of LIT_{reg} and liver infiltrating IL17 cells. Both population of cells are present in inflamed human liver tissue however, there was no co-staining or double positive cells. Furthermore although both subsets were detected in inflammatory infiltrates they were not found in close proximity unlike the T_{reg} and DCs for instance suggesting that at least in the inflamed hepatic environment the reprogramming of T_{reg} to Th17 cells is probably not taking place (Figure 5.22).

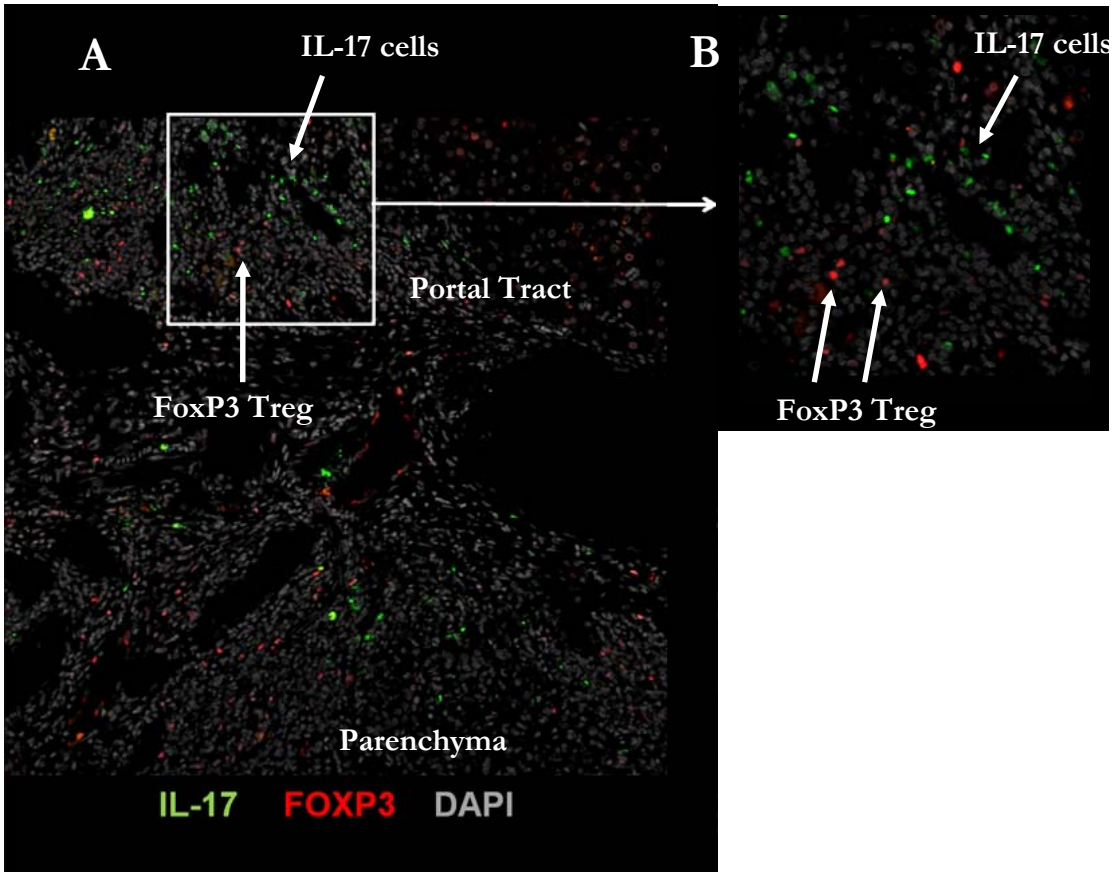


Figure 5-22 Confocal images of liver infiltrating IL-17 cells with LIT_{reg} cells.

Confocal microscopy was done on paraffin embedded inflamed liver tissue sections (primary biliary cirrhosis). IL-17 goat polyclonal and FoxP3 mouse monoclonal antibodies and DAPI (nuclear staining) were used. Confocal images show the presence of both cells however they reside discrete from each other (N=3; AIH, PBC, NASH).

5.3 DISCUSSION

The discovery of IL-23 extended the Th1/Th2 paradigm to more a complex Th1/Th2/Th17 paradigm. IL-23 shares the p40 subunit with IL-12, the heterodimer of IL-12 being composed of p40 and p35, and that of IL-23 being composed of p40 and p19(Cua et al., 2003;Oppmann et al.). Murine model of experimental allergic encephalomyelitis (EAE/a model of human multiple sclerosis) and collagen induced arthritis (CIA/a model of human rheumatoid arthritis) were accepted to have association with unchecked Th1 responses. This is based on studies where disease development was ablated by treatment with neutralizing antibodies specific for IL-12 p40 or gene targeted mice deficient in the p40 subunit of IL-12. However it turns out that these diseases also involve Th17 responses proving the existence of another effector pathway. Subsequently many groups have started to investigate the role of these IL-17 secreting cells in human autoimmune and inflammatory diseases (Harada et al., 2009;Lan et al., 2009;Lemmers, 2009;Zhang et al., 2009b). I hypothesized that the IL-17 pathway may be involved in hepatic inflammation either causing injury or repair of hepatocytes and/or biliary epithelial cells. This led me to investigate the immunophenotype, migration and positioning of human liver infiltrating IL-17 secreting cells.

Initial lineage commitment for human Th17 requires the combined activity of IL-1β, IL-6 and TGF-β, and IL-21 as an autocrine loop. Then IL-23 is required for stabilization and survival (costa-Rodriguez et al., 2007a;Manel et al., 2008;Volpe et al., 2008;Yang et al., 2008a). Thus, following pure Th17 cell isolation I use culture media with all these Th17 polarizing cytokines in IMDM media which is enriched with AhR ligands essential for Th17 generation (Veldhoen et al., 2008a;Veldhoen et al., 2009) to maintain Th17 phenotype.

Initially, I focussed on Th17 (IL-17 secreting CD4 cells) however, during a collaborative investigation with Paul Klenerman group we observed that not only Th17, but Tc17 (IL-17

secreting CD8 cells) are present in the inflamed liver. Tc17 cells have been reported to protect from lethal influenza infection and neutrophil influx into the lung in murine model (Hamada et al., 2009). $CD8^+$ T cells activated in the presence of the cytokines IL-6 or IL-21 plus TGF-β develop into Tc17 which display greatly suppressed cytotoxic function along with low levels of T-box transcription factor Eomesodermin, granzyme B and IFN-γ. Instead, these cells express hallmark molecules of the Th17 program including RORγt, RORα, IL-21 and IL-23R (Huber et al., 2009). Functionally, both Tc17 cells and IL-17/IFN-γ double-positive cells generated under Tc17 conditions lacked lytic activity, expressed similar patterns of cell surface proteins to IL-17-producing cells. STAT3 signalling pathway is necessary for Tc17 polarization, both *in vitro* and *in vivo* (Yen et al., 2009). Moreover, adoptive transfer study by Yen and colleagues also demonstrated functional plasticity of Tc17cells to Tc1 cells. However, our study is the first to report human liver infiltrating Tc17 cells.

Phenotyping of fresh liver infiltrating Th17 and Tc17 demonstrated that they are mainly memory cells expressing CD45RO and, IL-23R. The presence of IL-23 is important because once Th17 cells have differentiated from naive T cells, IL-23 is crucial to maintain Th17 stabilization and survival (Cua et al., 2003;Murphy et al., 2003). The presence of IL23R on Tc17 cells suggest a similar survival pathway operates in these cells.

Studies have shown that human Th17 may have a different origin from murine Th17 cells. While murine Th17 cells originate from naive T cells in response to IL-6 and TGF-β (Bettelli et al., 2006;Bettelli et al., 2007;Mangan et al., 2006), human Th17cells originate from $CD161^+$ naive CD4 precursors present in neonatal blood and thymus (Cosmi et al., 2008). CD161 (NKR-P1A) is the human homologue of the mouse NK1.1 (Lanier et al., 1994) which is expressed not only on NK cells also on NKT cells (Godfrey et al., 2004). During collaborative work to investigate the role of Tc17 in hepatitis C (Yu-Hoi Khan, Klenerman, PNAS, 2010), I studied CD161

expression on human Th17 and Tc17 isolated from liver tissue. Immunophenotyping of liver infiltrating Th17 and Tc17 suggested that both subsets are mainly restricted to $CD161^{high}$ population. Thus, our findings of both Th17 and Tc17 cell express CD161 consistent with results from the other groups.

I next investigated the cytokine profiles secreted by both Th17 and Tc17 isolated from inflamed liver. They expressed not only IL-17, but also IL-22, TNF-α and IFN-γ. Among these cytokines, IL-22 has been shown to have protective effect on liver epithelium (Zenewicz et al., 2007). Cosmi and colleagues previously reported Th17/Th1 cells co-express IL-17 and IFN-γ (Cosmi et al., 2008). RORC is the human homologue to murine RORγt which is the essential transcription factor for Th17 generation (Ivanov et al., 2006;Ivanov et al., 2007) and I found that liver infiltrating Th17 and Tc17 cells highly expressed this signature transcription factor, RORC.

Recent studies have shown that expression of CCR6 and CCR4 together identified human memory $CD4^+$ T cells selectively producing IL-17 and expressing RORC transcription factor, whereas CCR6 and CXCR3 identified T helper cells producing both IFN-γ and IL-17 (costa-Rodriguez et al., 2007b). In the present study, the homing receptor and chemokine receptor phenotype of liver infiltrating Th17 was found to be CCR6 and CXCR3. Liver infiltrating Th17 are most likely Th1/Th17 phenotype (CCR6/CXCR3) as they express high levels of these two receptors. Liver-infiltrating lymphocytes express high levels of CXCR3 and use this receptor to migrate across human hepatic endothelium under flow *in vitro* (Curbishley et al., 2005;Steinmetz et al., 2009). Similar recruitment pattern would be possible for LITh17 cells and this work is currently in process. In a murine lupus model, CXCR3 deficiency was shown to attenuate both the Th1 and Th17 responses (Steinmetz et al., 2009).

The frequency of IL-17 cells in the human liver was also quantified by immunohistochemistry. The findings suggested that the frequency was increased in chronic inflammatory liver diseases including autoimmune, viral, steatohepatitis or seronegative hepatitis. This finding was similar to the studies from other groups (Lan et al., 2009;Lemmers, 2009). Interestingly, the highest frequencies were seen in steatohepatitis and fulminant seronegative hepatitis. Lemmers and colleagues have shown recently that in alcoholic hepatitis, the frequency of Il-17 secreting cells is higher than in alcoholic cirrhosis. My observation of increased IL-17 secreting cells in autoimmune hepatitis is consistent with previous human studies showing increased numbers of Th17 cells in tissues of patients with autoimmune diseases such as rheumatoid arthritis, and juvenile idiopathic arthritis (Hirota et al., 2007;Nistala et al., 2008). In general, IL-17 cells were present in higher frequencies in the portal tract compared to areas of lobular infiltration. However, in steatohepatitis, autoimmune hepatitis and seronegative hepatitis there is an enrichment of IL-17 cells in lobules and at areas of interface hepatitis.

I detected a high concentration of IL-17 secreting cells around intrahepatic bile ducts. Similar finding has been reported in a recent study on Th17 involvement in primary biliary cirrhosis (Harada et al., 2009). Some of these IL-17 cells are even found to be present on the biliary epithelial cells. It has been shown that IL-17 differentiation cytokines IL-1β, IL-6 and stabilization cytokines IL23 p19 mRNA and protein are present on biliary epithelial cells (Harada et al., 2009). Thus it is possible that biliary epithelial cells may provide a local Th17 polarizing cytokine milieu by secreting these cytokines for IL-17 cells generation around bile ducts.

IL17 receptor is widely express on epithelial cells, endothelial cells and mesenchymal tissue. A recent study on alcoholic liver disease suggested that IL-17RA is also expressed on fibroblast (Lemmers, 2009). I found IL-17RA receptor expression on biliary epithelial cells at both mRNA level and protein level by PCR and flow cytometry. Harada and colleagues also

found the presence of mRNA on biliary epithelial cells in human liver (Harada et al., 2009). Thus, I have postulated that Il-17 secreted by Th17 and Tc17 cells act on IL-17 receptor on biliary epithelial cells leading to downstream cytokines and chemokine secretion from BEC cells further attracting IL-17 cells to the portal tracts.

I have focussed on one chemokine of particular interest, CCL20 because its receptor CCR6 is highly expressed on both Th17 and Tc17 cells. Others important chemokines are CXCR3 ligands such as CXCL9, CXCL10 and CXCL11, as there is also high expression of CXCR3 on liver infiltrating IL-17 cells. *De novo* generation of CCL20 and IL-17RA messenger RNA has been shown on biliary epithelial cells (Harada et al., 2009) and in accordance with previous finding, I have confirmed expression of CCL20 protein by immunohistochemical staining around bile ducts and CCL20 chemokine secretion by sandwich ELISA in BEC supernatants. Our group have shown that chemokines CXCL9-11 were secreted from inflamed bile ducts (Curbishley et al., 2005). Chemotaxis studies on freshly isolated Th17 showed that the both CCR6 and CXCR3 receptor on Th17 is functional and allows the cells to migrate towards CCL20 or CXCL-10 secreted by biliary epithelial cells. The migration could be blocked if the chemokine receptor was blocked with global G protein blockade pertussis toxin or if CCL20 was removed by using blocking antibodies.

The balance of effector and regulatory arms determines the outcome of hepatic inflammation. I found T_{reg} in inflamed liver along with IL-17 cells in confocal studies. Reciprocal relation of frequency between these effector and regulatory subset of cells has been described in juvenile chronic arthritis (Nistala et al., 2008). Confocal findings did not suggest that the two subsets are in particularly close proximity. It has been described before that double positive RORγt and FoxP3 cells were noted in human tonsil (Voo et al., 2009) however, I did not notice costaining of T_{reg} markers FoxP3 and IL-17.

The reciprocal relation between T_{reg} and Th17 depends on TGF-β and IL-6 and IL-6 plays a pivotal role in shaping the proinflammatory Th17 dominant or anti-inflammatory T_{reg} dominant environment (Mucida et al., 2007). In normal liver, the ability of stellate cells to secrete large amounts of TGF-β and retinoic acid (Eksteen et al., 2009) may generate T_{reg} dominant microenvironment. However, in the inflamed liver environment where stellate cells differentiate into activated liver myofibroblasts resulting in large amounts of IL-6, TGF-β secretion and switch to Th17 dominant inflamed microenvironment.

Our findings demonstrated that both Th17 and Tc17 cells are present in inflamed human liver. They expressed IL-23R, homing receptors CCR6 & CXCR3, and were restricted to the $CD161^{high}$ population and have signature transcription factor RORC. Both Th17 and Tc17 are polysecretor, polyfunctional cells expressing not only IL-17, but IL-22, TNF-α and IFN-γ. These IL-17 cells are found around bile ducts which possess the IL-17RA receptor and secrete CCL20 which could allow them to recruit more functional $CCR6^{+}$ Th17 cells. Whether IL-17 cells cause biliary damage or help promote regeneration of bile ducts is still unclear. Furthermore, whether these Th17/Tc17 cells are recruited fully differentiated or generated in the local microenvironment by polarising cytokines around the bile ducts will need addressing in future studies.

CHAPTER 6

GENERAL DISCUSSION

In chronic liver disease, the liver is infiltrated by different subsets of lymphocytes that make up the characteristic lesion of chronic hepatitis. The infiltrate includes regulatory T cells (T_{reg}) that are believed to suppress inflammation and effector T cells that include Th1, Th2 and Th17 effector T lymphocytes in addition to innate immune cells. T_{reg} suppress effector T lymphocyte proliferation and cytokine secretion thereby providing an important regulatory control of immune responses (Sakaguchi, 2004). Thus, the balance between effector and regulatory lymphocytes plays a pivotal role in dictating whether an immune response in the liver leads to tolerance or resolution on one hand or immunity and persistent inflammation on the other (Crispe, 2003).

It has been proposed that in human disease a quantitative or qualitative T_{reg} failure leads to autoimmune liver diseases (Lan, 2006;Longhi et al., 2004;Longhi et al., 2005b), and liver allograft rejection (Demirkiran, 2006) whereas too much immune suppression by T_{reg} results in chronicity of viral hepatitis (Chang, 2005;Ward, 2007;Xu et al., 2006) and poor anti-tumour immune responses to hepatocellular carcinoma (Fu et al., 2007). On the effector arm, the recently described IL-17 secreting lymphocytes which include Th17 and Tc17 cells (Veldhoen et al., 2006) has been implicated in many human liver diseases such as autoimmune liver diseases (Lan et al., 2009), hepatocellular carcinoma (Kuang, 2010) and alcoholic liver disease (Lemmers, 2009).

T_{reg} and Th17 have a close reciprocal developmental relationship (Mucida et al., 2007;Veldhoen et al., 2006). The potential for translational cell-based therapy by manipulating these two subsets of lymphocytes is apparent although for this to become a reality requires a greater understanding of their biology in humans and their behaviour in clinical disease. Presently, little is known about the phenotypic characteristics of either liver infiltrating T_{reg} or IL-17 secreting lymphocytes and several questions remain unanswered regarding the recruitment and positioning of these cells in inflamed human tissue. Thus, my studies aimed to address these

questions by characterising the detailed phenotype of liver infiltrating T_{reg}, IL-17 secreting Th17CD4 cells, IL-17 secreting Tc17CD8 cells in different human liver diseases and by investigating the molecular basis of their recruitment and positioning within the inflamed human liver.

6.1 Balance between Th17 effectors and regulatory T cells in inflamed human liver

Both effector and regulatory subsets of lymphocytes will play critical role in the generation and outcome of immune responses in chronic inflammatory liver disease. T_{regs} have been shown to be essential for the maintenance of peripheral tolerance (Sakaguchi, 2004;Walker & Abbas, 2002) and Th17 cells can now be added to Th1 and Th2 cells as important players in inflammatory liver diseases (Kuang, 2009;Lan et al., 2009;Zhang et al., 2009b). To understand the role of T_{reg} and Th17 cells in inflammatory liver diseases, I investigated their frequency, distribution in the normal and inflamed human liver.

I studied the presence and distribution of CD4Th1, CD8Th1, CD4Th17 and CD8Tc17 effector subsets and regulatory T cells in both normal and inflamed human livers. I found that inflamed human liver is infiltrated by TNF-α and IFN-γ secreting CD4Th1 and CD8Th1cells as described previously (Adams & Eksteen, 2006;Crispe, 2003;Knolle & Gerken, 2000) and that the frequency of CD8Th1 is higher than CD4Th1. In addition to classical Th1 cells, I also detected IL-17 secreting cells and found they included IL-22, TNF-α and IFN-γ polysecretor CD4Th17 cells (costa-Rodriguez et al., 2007b;Wilson et al., 2007) as well as polysecretor CD8Tc17 cells (Kondo et al., 2009). I also contributed to work reporting this latter subset of cells in the peripheral blood and livers of patients with chronic HCV infection (Billerbeck & Khan, 2010).

Similar to effector lymphocytes, $CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ regulatory T cells are also present in normal and a variety of diseased livers.

I found a significantly higher frequency of both $FoxP3^+$ T_{reg} and IL-17^+ cells in inflamed liver compared with normal liver. Previous reports suggested that increased frequencies of T_{reg} correlate with chronicity of viral hepatitis (Xu et al., 2006) and the reduce numbers of T_{reg} and impairment in function occurs in autoimmune hepatitis (Longhi et al., 2004;Longhi et al., 2005b). However, my data showed a similar frequency of Th17 and T_{reg} in both chronic HCV infection and autoimmune hepatitis. However, I was studying patients with end-stage disease at transplantation (explanted liver tissue) thus the situation may differ earlier in disease course. It is however interesting that we detected higher frequencies of T_{reg} in autoimmune disease given that the aetiopathogenesis is presumed to be breakdown in self-tolerance. This suggests that either T_{reg} are overwhelmed by the effector response at site of chronic inflammation or that the T_{reg} are defective. This finding implies that not only the number but the functional integrity of intrahepatic T_{reg} determines the outcome. The Th17 frequency was greater in inflammatory liver diseases as previously reported (Lan et al., 2009;Lemmers, 2009). I found the most intense infiltration of IL-17 cells in alcoholic and non alcoholic steatohepatitis suggesting that they are important effector cells in steatohepatitis as the tissue injury is associated with the presence of neutrophils, a characteristic feature of Th17 immune responses(Lemmers, 2009).

The similarly increased frequency of T_{reg} and Th17 cells suggests that more regulatory activity is present in inflamed liver where there is more severe inflammation. The parallel increase in frequency of both populations in inflamed livers suggests that persistent hepatic inflammation is not due to a reduction in the number of T_{reg}. Other factors including functional suppression defect of these cells in the local microenvironment may explain their failure to control ongoing hepatitis. Alternatively the regulatory cells may be fully functional but overwhelmed by the vigour

of the effector response. The balance in the liver between T_{reg} and T effector cells during homeostasis, infection/inflammation and chronic hepatitis is shown in Figure 6.1.

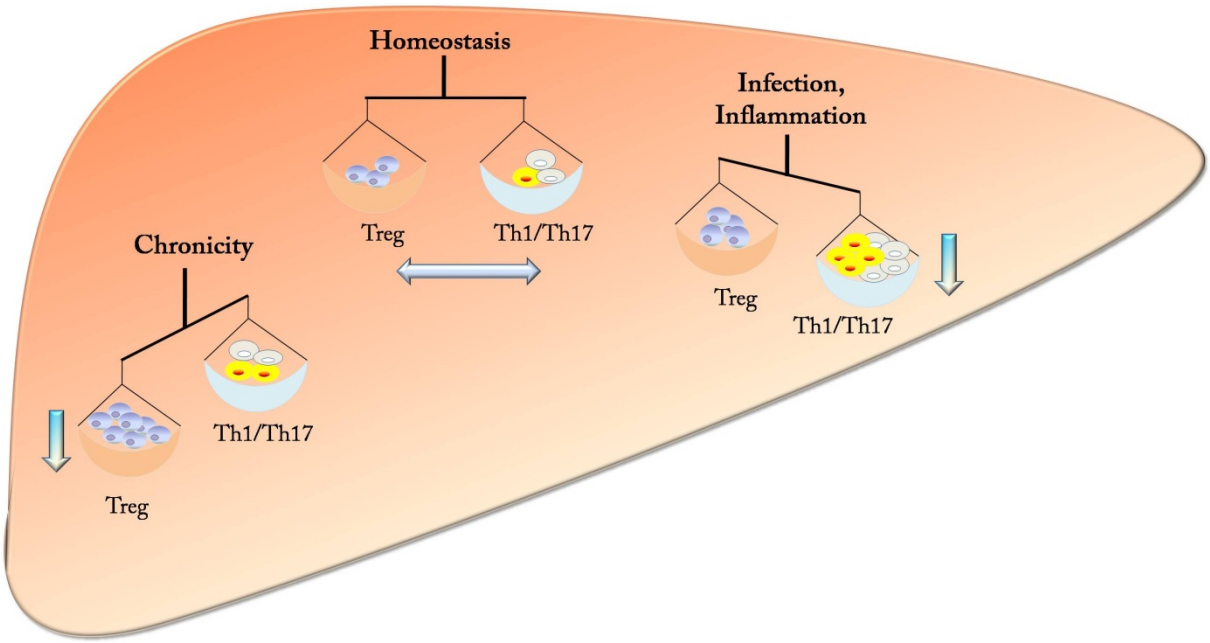


Figure 6-1 The balance (quantity or functional quality) between effector and regulatory lymphocytes.

During the homeostatic state, both regulatory and effector lymphocytes are in balance. However, the effector cells outweigh the regulatory T cells during hepatic infection or inflammation. On the other hand, increase in frequency of T_{reg} may contribute to the chronicity of hepatitis.

Both T_{reg} and IL-17 secreting lymphocytes are detected in portal tracts, inflamed lobules and septa. In most liver diseases, there is a predominant portal tract distribution of both subsets consistent with the pattern of the hepatitis. However, the highest frequencies of IL-17 staining were observed in alcohol and non-alcoholic steatohepatitis and autoimmune hepatitis, all diseases where the injury is targeted at hepatocytes. Initial reports of functional roles for Th17 cells were in autoimmune models such as EAE and CIA in mice (Harrington et al., 2005;Park et al., 2005). More recently Lemmers *et al* described Th17 cells in human alcoholic hepatitis with a similar frequency and distribution to my findings (Lemmers, 2009). Similarly, I detected the highest frequency of $FoxP3^+$ T_{reg} in autoimmune hepatitis and seronegative nonAnonB hepatitis, both diseases which target hepatocytes. Histologically, lobular hepatitis and interface hepatitis are common in both diseases (Hubscher, 1998). Thus it is likely that more T_{regs} will be required at these sites to control effector cell activity.

Although I have focused on naturally occurring $FoxP3^+T_{reg}$, other subsets of regulatory T cells had been reported by other groups including Tr1 and/or Th3 (Bluestone, 2003;Roncarolo et al., 2006;Sakaguchi, 2004) and these cells may also be present in the inflamed liver. Interleukin-10 secreted from liver myeloid dendritic cells (Goddard et al., 2004b) is enriched in the inflamed liver and could promote the generation of Tr1 cells. Furthermore, the liver is TGF-β rich microenvironment (Adams et al., 1991;Bowen et al., 2005;Crispe, 2009) making it potentially ideal milieu for Th3 differentiation. Thus, other subsets of T_{reg} not studied in this thesis would need further investigations to determine whether they are also implicated in chronic liver diseases. Regarding to Th17 project, I have not had time to study the local generation of these cells in the inflamed hepatic microenvironment which is enriched with Th17 polarizing cytokines, TGF-β, IL-1β and IL-6. All these experiments should be done in the future.

6.2 Liver infiltrating T_{reg} and IL-17 secreting cells characteristics

It is crucial to define the detailed phenotype and functional properties of intra-hepatic T_{reg} and IL-17 secreting cells in order to understand their role in disease pathogenesis. Such findings also have translational importance if regulatory T cells are to be manipulated for immunotherapy in the future. Our group and others have reported that liver infiltrating T lymphocytes are mainly activated and of the memory phenotype (Heydtmann et al., 2006;Volpes et al., 1991). I made similar observations with liver infiltrating T_{reg}. Freshly isolated T_{reg} from explanted livers expressed CD45RO and CD27 which has been claimed to be characteristic of tissue-infiltrating T_{reg} in other organ (Mack et al., 2009;Ruprecht et al., 2005a).

T_{reg} can suppress immune responses through antigen specific and antigen independent mechanisms (Tiegs and Lohse, 2009;Walker & Abbas, 2002) and multiple mechanisms of T_{reg} suppression have been reported (Sakaguchi & Powrie, 2007). I noted that the liver infiltrating T_{reg} express high level of CD39, the ectonucleotidases which suggest that adenosine play a role in intrahepatic T_{reg} suppressor function as previously described (Borsellino et al., 2007). However, I did not investigate other potential suppressive mechanisms of intrahepatic T_{reg} including their ability to secrete IL-10, IL-35 or TGF-β; determining the secretion of these cytokines by liver infiltrating T_{reg} and their effect on responder T cells will be important study in the future. Contact dependant molecules such as LAG-3, GITR and IDO and how they promote interactions between T_{reg} and hepatic dendritic cells will also require investigation to elucidate the mechanisms of intrahepatic T_{reg} suppression. I used proliferation assays to demonstrate that LIT_{reg} are suppressor in vitro as has been reported for other tissue infiltrating T_{reg} (Ruprecht et al., 2005b) but whilst this shows the cells are functional in vitro it does not mean they function fully within the inflammatory environment of the liver.

I detected both CD4 and CD8 IL-17 secreting cells in the liver which we designated liver infiltrating CD4Th17 and CD8Tc17 respectively. Both subsets express the IL-23 receptor. IL-23 is a recently described member of the IL-12 cytokine family that is critical for the survival and maintenance of Th17 cells in chronic inflammation (costa-Rodriguez et al., 2007b;Cua et al., 2003;Langrish, 2004;Murphy et al., 2003;Oppmann et al.). The fact that I found similar levels of IL-23 receptor on Th17 and Tc17 cells within the liver suggests that IL-23 is important for both CD4 and CD8 IL-17 secreting cells. Cosmi and colleagues recently reported that peripheral blood Th17 cells are all found within a subset of $CD161^{high}$ T cells (Cosmi et al., 2008). I found that liver infiltrating CD4Th17 comprise around 1.5% of the intrahepatic T cells population and are similarly all $CD161^{high}$. Collaborative work with Paul Klenerman's group has allowed me to define CD8Tc17 from chronically HCV infected liver and other inflammatory liver diseases. These studies confirmed that CD8Tc17 cells are also restricted to the $CD161^{high}$ population (Billerbeck & Khan, 2010). They are less frequent than Th17 cells comprising around 0.5% of the intrahepatic lymphocyte pool. Both Th17 and Tc17 are polysecretor that not only express IL-17 and IL-22 but also secrete TNF-α and IFN-γ. The ability of these cells to secrete a range of proinflammatory cytokines suggests that they might activate other effector pathways leading to the recruitment of other effector cells and broadening of the inflammatory response (Adams, 1996a;Curbishley et al., 2005;Eksteen, 2006;Holt et al., 2009). Of interest, IL-22 has been shown to be hepatoprotective in murine models and in some circumstances inhibiting IL17 can exacerbate liver injury and damage (Radaeva, 2004;Zenewicz et al., 2007).

The activation of specific transcription factors determines the differentiation of the different T cells lineages. For instance, T bet drives Th1 differentiation and GATA-3 leads to Th2 differentiation. Liver infiltrating T_{reg} express high levels of FoxP3 and liver infiltrating Th17 and Tc17 RORC, which are respectively the transcription signature that drive T_{reg} and Th17/Tc17

differentiation. Recently, plasticity between T cells lineages has been reported and this is especially pertinent to my studies because of the capacity of T_{reg} to transform into Th17 when in an inflammatory milieu in both mice (Kitani and Xu, 2008) and human (Voo et al., 2009). These cells coexpress both FoxP3 and RORγt transcription factor, however interestingly they maintain suppressive function (Voo et al., 2009). I could not detect any IL-17 and FoxP3 co-expression in any of the livers studied by confocal microscopy. Furthermore T_{reg} and Th17/Tc17 cells were not found in close contact in the infiltrates both of which suggest that plasticity events might not take place in the inflamed human liver.

The increased frequency of liver infiltrating T_{reg} I detected in diseased livers in the face of ongoing chronic inflammation suggests that T_{reg} are either suppressed in situ in the inflamed liver or overwhelmed by the strength of the effector response. Thus, failure of T_{reg} to function in the inflamed hepatic microenvironment rather than an inability to recruit these cells may be the reason for parenchyma tissue destruction despite the presence T_{reg}. This led me to investigate the function of LIT_{reg}. As discussed above the LIT_{reg} were able to suppress T cell activation *in vitro* but these experiments were of course without the inflammatory milieu in the liver. In order to assess T_{reg} function in situ we exploited the fact that IL-2 signalling is essential for T_{reg} function (Burchill et al., 2007). The effects of IL-2 signalling are mediated via phosphorylation of STAT5 and downstream activation of the transcription factor FoxP3 which controls T_{reg} development and function (Hori et al., 2003). I thus decided to stain liver tissue for FoxP3 and with an antibody that detects a phosphorylated form of STAT5. Thus detection of pSTAT5 implies the cell has recently received a signal via the IL-2 receptor suggesting it may be functional whereas a lack of pSTAT5 suggests a lack of function at that specific time. I found that only a minority of liver infiltrating T_{reg} expressed pSTAT5 suggesting that most of them are not functional at that time. However, dynamic studies are required to see if the cells are receiving cyclical activation

through the IL-2 receptor which could be missed by studying one time point. This may not be the best way of investigating the functional role of LIT_{reg} however there are limitations with *in vitro* studies.

The functional role of IL-17 and Th17 cells in liver disease remain unknown and I was not able to investigate the pathogenesis role of IL-17 in this study. It is possible that Th17 cells may damage biliary epithelium or hepatocytes via IL-17, IL-23, TNF-α, IFN-γ as they secrete all of these cytokines *in vitro*. However, once again the *in vitro* intracellular cytokine analysis does not mean that the cells secrete these cytokines at sites of inflammation *in vivo*. On the other hand, their ability to secrete IL-22 may be protective and support liver regeneration and repair. The precise role of IL-17 in specific liver disease needs to be determined before anti-IL17 therapy is translated to the clinic. However, real caution needs to be taken when interpreting murine studies on the role of IL-17 as attempts to translate findings on Th17 in mice to humans have generated surprising and conflicting results reflecting underlying fundamental differences between the human and murine systems with regard to IL-17 and anti p40 therapy. For these reasons, Th17 mechanistic action will certainly require further investigations in future.

6.3 Role of chemokine receptors and integrins in recruitment

Naturally occurring T_{reg} and Th17 are thymic derived (Cosmi et al., 2008;Sakaguchi, 2005) and T cells that circulate in the blood are recruited to sites of inflammation via endothelium in the liver, in the case of liver disease the specialised hepatic sinusoidal endothelium. The migration of leukocytes from blood stream or from hepatic sinusoids into and through tissues either during homeostasis or in response to inflammation is controlled by the adhesion molecules and chemokine receptors on the leukocytes and their ability to bind to and interact with endothelial

cells, stroma cells and matrix (Adams & Shaw, 1994;Butcher & Picker, 1996;Ley, 2007). Tissues display chemokines and cell adhesion molecules (CAMs) that act like post codes to attract and position specific subsets of leukocytes (Butcher & Picker, 1996;Rot, 2004;Shetty, 2008). Such signals also determine the recruitment of T_{reg} and Th17 cells to tissues (Sallusto, 2000). At sites of inflammation, T_{reg} mediate both bystander and antigen-specific suppression of local immune responses to limit tissue injury and prevent collateral tissue damage (Holmen et al., 2006;Ni et al., 2006;O'Connor et al., 2007;Walker, 2004;Walker & Abbas, 2002). On the other hand, Th17 subset are implicated in inflammatory, viral and autoimmune related liver diseases (Harada et al., 2009;Lan et al., 2009;Zhang et al., 2009b).

I found high levels of chemokine receptors CXCR3 and CCR4 on the majority of liver-infiltrating T_{reg} in this study. Circulating human T_{reg} have previously been reported to express CXCR3 although few studies have examined expression on tissue infiltrating cells (Lim et al., 2006;Lim et al., 2008). Although the majority of T_{reg} in inflamed human liver expressed high levels of CXCR3 it was also detected on 15-20% of T_{reg} from normal liver. Hepatocytes, stroma cells and hepatic sinusoidal endothelium secrete the CXCR3 ligands CXCL9, CXCL10 and CXCL11 in response to IFN-γ (Curbishley et al., 2005;Holt et al., 2009) and these chemokines can be detected on epithelial cells, stroma cells and the glycocalyx of sinusoidal vessels in the inflamed human liver (Narumi et al., 1997;Shields et al., 1999;Tamaru et al., 2000) where they promote the transendothelial migration of effector T cells (Curbishley et al., 2005). CXCR3 ligands are low to undetectable in normal liver consistent with them being inflammation induced (Curbishley et al., 2005;Harvey et al., 2003). The finding of high levels of CXCR3 on tissue-infiltrating T_{reg} suggested that this receptor might be involved in the recruitment of T_{reg} across hepatic sinusoidal endothelium. Peripheral blood derived regulatory T cells were used as they are the closes representation of hepatic sinusoid regularoty T cells. Using flow-based adhesion assays with stimulated human sinusoidal endothelial cells, I had demonstrated that activation of CXCR3

on T_{reg} promotes their adhesion and transmigration across human liver sinusoidal endothelium under condition of flow. These results are similar to those our group previously reported using liver infiltrating effector T cells suggesting that common signals recruit both T_{reg} and T effector to the inflamed liver. In contrast, neither of the CCR4 ligands, CCL17 and CCL22 could be detected on hepatic sinusoidal endothelium at either messenger RNA or protein levels. This finding suggested that CCR4 is not involved in recruitment through endothelium.

I concluded that all three CXCR3 ligands were involved in transendothelial migration because blocking individual chemokines had a lesser effect than blocking the common receptor CXCR3 (Oo et al., 2010). CXCR3 blockage was as potent as inhibiting all chemokine receptors with pertussis toxin suggesting that CXCR3 is the dominant receptor involved in transendothelial migration. The findings in this study are consistent with published *in vivo* studies that report the requirement for CXCR3 expression to allow T_{reg} to be recruited to inflamed tissues in GVHD (Hasegawa et al., 2008) and, to the brain in EAE (Muller et al., 2007). Recent in *vivo* studies in mice support my findings. Santodomingo-Garzon and colleagues reported that IFN-γ production from innate NKT cells promotes homing of $CXCR3^+$ T_{reg} to the murine liver (Santodomingo-Garzon, 2008). Thus, IFN-γ inducible chemokines CXCL9 and CXCL10 expressed on inflamed hepatic sinusoidal endothelium provide a common recruitment signal for both CXCR3 expressing effector cells (Curbishley et al., 2005) and T_{reg} (Oo et al., 2010).

I also studied the adhesion molecules involved in T_{reg} adhesion to hepatic sinusoidal endothelium. I found that VCAM-1 was required for adhesion but not for transendothelial migration and surprisingly I was unable to block either adhesion or migration with anti-ICAM antibodies. This is in contrast to previous studies of effector cells using the same system they used VCAM-1 for adhesion and both CXCR3 and ICAM-1 were necessary for transmigration (Curbishley et al., 2005). This leads to the question of what role other adhesion molecules might

play in T_{reg} transmigration. Two potential candidates are VAP-1 (amine oxidase and adhesion receptor that is expressed by endothelium in the human liver) and CLEVER-1 (common lymphatic endothelial and vascular endothelial receptor-1) both expressed on the hepatic sinusoids. Our group have previously demonstrated that VAP-1 supports adhesion and transmigration of lymphocytes across hepatic sinusoids under physiological shear stress (Lalor et al., 2002a). CLEVER-1 has also been shown to mediate adhesion and transmigration of leukocytes via vascular endothelium (Salmi, 2004). Since completing the work for this thesis I have collaborated with my colleague Dr Shishir Shetty to show that both of these receptors are involved in T_{reg} transendothelial migration through HSEC under flow. The results with CLEVER-1 were particularly interesting because we were unable to found a role for it in the recruitment of CD8 T cells or CD4 effector cells suggesting its function is specific to T_{reg} (Shetty et al, submitted to J Exp Med).

Phenotyping of fresh CD4Th17 and CD8Tc17 from explanted liver tissues demonstrated that they express high levels of CCR6 and also expression of CXCR3. Furthermore, Th17 and Tc17 cells expressed high levels of LFA-1 and VLA-4 integrins. Previous studies have reported expression of CCR6 and CCR4 on circulating CD4Th17 cells whereas CCR6 and CXCR3 were identified on CD4 T cells that secrete both IFN-γ and IL-17 so-called Th17/Th1 cells (costa-Rodriguez et al., 2007b). Based on my findings of both CCR6 and CXCR3 on liver infiltrating Th17 cells, I predicted that they would also be Th17/Th1 phenotype and this was indeed the case as demonstrated by polysecretor type intracellular cytokine staining.

Study in murine lupus have shown that CXCR3 deficiency attenuates both the Th1 and Th17 responses (Steinmetz et al., 2009) suggesting that CXCR3 plays a role in recruitment of Th17 cells. I did not have time to extend my experiments to look into the role of specific

chemokines and CAMs in the recruitment of Th17 cells but this is the subject of ongoing research in our laboratory.

CXCR3 and CXCL-10 are potential targets for anti-inflammatory therapy and blocking antibodies to both have been developed and tested in preclinical studies. However, my findings that not only effector cells (Curbishley et al., 2005) but also regulatory T cells (Oo et al., 2010) require CXCR3 to enter inflamed liver makes the outcome of CXCR3 blockade less predictable and it may depend on the level of expression of CXCR3 on both subsets of lymphocytes, their recovery following therapy and many other factors in the inflamed hepatic microenvironment.

6.4 Hepatic dendritic cells and positioning of liver infiltrating T_{reg}

Different chemokine receptors play distinct roles in recruiting and positioning cells in tissues including the liver. In this study, I found that CCR4 is expressed at high levels on liver-infiltrating T_{reg}. Because ligands for CCR4 are not detected on hepatic sinusoidal endothelium, I investigated the role of CCR4 in positioning of T_{reg} in the inflamed liver once they have undergone transendothelial migration. Similar to a previous report by Shields (Shields et al., 1999), I found that few effector cells expressed CCR4 suggesting that CCR4 is selectively expressed on T_{reg} in tissue consistent with findings showing CCR4 expression on T_{reg} in the circulation. Studies have reported local control of responder T cells by $CCR4^{+}T_{reg}$ in the skin (Iellem et al., 2001b;Sather et al., 2007) and cardiac allograft (Lee et al., 2005a). I demonstrated that CCR4 on liver infiltrating T_{reg} is functional receptor because freshly isolated liver-derived T_{reg} migrated *in vitro* towards the CCR4 ligands CCL17 and CCL22 in chemotaxis assays. CCL17 and CCL22 are reported to be secreted by mature dendritic cells and to recruit and retain T_{reg} in contact with dendritic cells in lymph nodes (Cardoso et al., 2008b;Curiel et al., 2004;Iellem et al.,

2001b;Katou et al., 2001;Tang & Cyster, 1999;Vulcano et al., 2001). I demonstrated both chemokines in inflamed liver tissue but not in normal liver. Furthermore, I showed that myeloid DCs isolated from liver tissue express and secrete both CCL17 and CCL22. Using immunohistochemistry and multi-colour immunofluorescense I was able to show that in tissue CCL17 and CCL22 expression was restricted to $CD11c^{+}$DCs within inflammatory infiltrates in septal areas and lobules. In addition, confocal microscopy revealed that liver-infiltrating $FoxP3^{+}T_{reg}$ are in close proximity to both intrahepatic DCs and CD8 T cells in inflammatory infiltrates. Previous studies have shown that T_{reg} exert their function either by contact dependant interaction with effector cells or via dendritic cells (Miyara & Sakaguchi, 2007b;Sakaguchi, 2008;Wing et al., 2008). Thus, my findings are consistent with a model in which intrahepatic inflammatory DC secrete CCL17 and CCL22 chemokines and thereby attract $CCR4^{+}T_{reg}$ to the infiltrates. The nature of the interactions between intrahepatic DC and T_{reg} are an area of ongoing investigation in our group and it will be important to determine whether DCs in the inflamed liver inhibit or enhance the function or survival of T_{reg}.

This study is the first to provide evidence that CXCR3 and CCR4 play distinct role in T_{reg} recruitment and positioning in inflamed tissues. However, other groups have demonstrated that CCR4 plays a crucial role in T_{reg} positioning either at sites of inflammation or at draining lymph nodes in mice *in vivo* (Haas, 2008;Sather et al., 2007;Yuan, 2007). It would be interesting to see if mice that lack the chemokine receptor CCR4 have defective intrahepatic migration of T_{reg} *in vivo* and such studies could be done using models of liver inflammation. However most of these models support acute inflammation, for instance the concanavalin-A hepatitis model and there are also a few good models that recapitulate the changes of chronic hepatitis model and inflammation seen in human disease.

6.5 Biliary epithelial cells and IL-17 secreting cells positioning

I also investigated the role of IL-17 cells in inflamed human liver in this study. Most of the IL-17 secreting cells are detected around bile ducts and some are seen in close contact with biliary epithelial cells. This is consistent with a previous study on primary biliary cirrhosis (Harada et al., 2009). This study also reported that cytokines involved in Th17 differentiation, IL-1β, IL-6 and IL-23 are present on biliary epithelial cells (Harada et al., 2009). Thus, it is possible that human biliary epithelial cells may provide a local Th17 polarizing cytokine milieu by secreting these cytokines which maintains or even generate IL-17 cells around bile ducts.

The IL-17 receptor is widely expressed on epithelial cells, endothelial cells and mesenchymal tissue (Korn et al., 2009). A recent study on alcoholic liver disease suggested that IL-17RA is also expressed on fibroblasts (Lemmers, 2009). As IL-17 cells are located in the vicinity of bile ducts, I looked for IL-17RA expression on bile ducts and isolated human biliary epithelial cells *in vitro*. I detected IL-17RA receptor expression on human biliary epithelial cells at both mRNA and protein level in accordance with previous report by Harada and colleagues (Harada et al., 2009). Moreover, treatment of biliary epithelial cells with IL-17 lead to secretion of CCL20 suggesting that Th17/BEC interaction might amplify further Th17 cell recruitment thereby perpetuates the local response. Thus, I hypothesize that Il-17, TNF-α, IFN-γ secreted by Th17 and Tc17 cells may act on IL-17 receptor on bile ducts leading to cytokines and chemokine secretion from biliary epithelial cells.

Both Th17 and Tc17 cells express CXCR3 and CCR6. CCL20, also known as LARC (liver activation regulated chemokine) is the ligand for CCR6 which is constitutively expressed on both Th17 and Tc17 cells. *De novo* generation of CCL20 mRNA and IL-17RA mRNA was reported on biliary epithelial cells in recent study (Harada et al., 2009). Similarly, I have detected CCL20 expression by immunohistochemistry around bile ducts and CCL20 secretion by ELISA

in biliary epithelial cells supernatant stimulated with IL-17, TNF-α, IFN-γ or combination of cytokines. CXCL9-11 are ligands for CXCR3 which is expressed at high levels on liver infiltrating Th17 and Tc17 cells. We have described previously that CXCL9-11 secreted from inflamed bile ducts recruit activated T cells (Curbishley et al., 2005). I used chemotaxis assays and freshly isolated Th17 to show that the both CCR6 and CXCR3 receptors on Th17 cells are functional by blocking cells migration with anti-CCL20 in the supernatant of stimulated biliary epithelial cells or blocking CXCR3 receptor on Th17 cells. The migration could be blocked by G protein blockade with pertussis toxin as well suggesting that other chemokine receptors may be involved.

Nevertheless, it still remains unanswered whether Th17/Tc17 cells cause biliary damage or promote regeneration of bile ducts; whether these cells are recruited fully differentiated or generated in the local microenvironment by polarising cytokines around the bile ducts. Both these question need addressing with future studies.

Thus, complex cellular interactions activate networks of chemokine and cytokine secretion within the inflamed liver microenvironment that determine which functional subsets are recruited to the liver and hereby the balance between intrahepatic immunity and tolerance. Understanding these networks may allow us to manipulate the natural anti-inflammatory processes by using adoptive transfer of autologous regulatory T cells to suppress immune and inflammatory responses in autoimmune hepatitis and chronic inflammatory liver disease. This project also provides a better understanding of the distinct roles of specific chemokine receptors in the recruitment of intrahepatic regulatory T cells and effector Th17/Tc17 cells. These findings may be useful in future to develop new therapeutic tools for the treatment of immune mediated liver diseases by for instance blocking the recruitment of damaging Th17 and Tc17 cells whilst promoting anti-inflammatory T_{reg} recruitment.

6.6 Planned future works

6.6.1 Autologous Regulatory T cell Infusion Therapy

Regulatory T cells are physiological regulators of inflammation and immune activation and their adoptive transfer should in theory be able to re-establish immune homeostasis and resolution of inflammation in autoimmune and inflammatory liver disease and following liver transplantation. Regulatory T cell-based therapy has been tested in some clinical settings such as GVHD (Hoffmann et al., 2005;Hoffmann et al., 2006;Hoffmann and Edinger, 2006) with encouraging outcomes.

Generation of robust, sterile, reproducible populations of these cells is essential. Antigen specificity would avoid generalized immunosuppression (Bluestone et al., 2007) but is not practical for most autoimmune inflammatory diseases where the antigens are unknown. Preservation of functional regulatory activity and T_{reg} phenotype after selection and expansion *ex vivo* is an absolute prerequisite for safe and successful cell-based therapy and this requires that a) the cells home to the right sites on transfer and b) survive and maintain functional differentiation at sites of inflammation.

We are currently exploring the use of adoptive T_{reg} therapy in chronic immune-mediated liver disease. We are isolating the T_{reg} from leukapheresis products by using CD19 depletion and CD25 positive selection with clinical grade CliniMACS (under good manufacturing practice/GMP conditions) to yield highly purified T_{reg} in a closed system which we can then expand *ex vivo* with TCR stimulation by clinical grade CD3/CD28 beads and IL-2. Our group has previously reported that $CCR9^+$ effector lymphocytes home to the gut and under some circumstances to the inflamed liver where they can drive injury and chronic hepatitis (Adams & Eksteen, 2006). Retinoic acid from gut dendritic cells has been shown to induce this phenotype

and is also an important factor in driving FoxP3 expression and T_{reg} differentiation (Mora, 2003;Mora, 2004). Thus, our current studies plan to generate $CCR9^+$ T_{reg} using CD3/CD28 clinical grade expander beads as artificial dendritic cells together with adding IL-2 and clinical grade retinoic acid. The addition of 1-20 ng/ml rapamycin to CD25-enriched cultures has been reported to increase the purity and stability of cells with the phenotype and function of T_{reg}(Battaglia, 2005;Battaglia, 2006;Keever-Taylor, 2007;Strauss, 2007) and to suppress differentiation of pathogenic Th17 cells (Kopf, 2007). Thus, we plan to add rapamycin to our isolated T_{reg} culture to ascertain that proliferating cells lineage remain as the regulatory T cells.

The functional integrity, purity and sterility of cultured T_{reg} would need to be assessed before they can be used to treat liver disease patients. As an initial step, we plan to label these cells and track their homing with MRI scanning. Following that, if we are convinced that they home to the inflamed gut and liver, autologous T_{reg} cell immunotherapy would be an option to administer to patients with liver disease and inflammatory bowel disease to see if such therapy can ameliorate disease activity.

6.6.2 Reciprocal relation and local generation of T_{reg} and Th17 cells

Regulatory T cells have a similar developmental pathway to Th17 and the outcome depends on the local cytokine environment. In the liver this will depend on activation of innate immune cells and stroma and epithelial cells at sites of chronic hepatitis. TGF-β is critical factor in both T_{reg} and Th17 differentiation (Veldhoen & Stockinger, 2006a). The liver is a TGF-β rich environment and this cytokine is essential for differentiation of anti-inflammatory T_{reg} on the other hand it is required for proinflammatory Th17 generation (Bettelli et al., 2006;Mangan et al., 2006;Veldhoen et al., 2006). Thus, I plan to co-culture liver derived CD4 cells with resident DC and fibroblasts together with specific cytokines to recapitulate the liver microenvironment and

attempt to model its effect on survival and lineage outcome. Hypothesized mechanisms of homeostasis in the hepatic microenvironment is shown below in Figure 6.2.

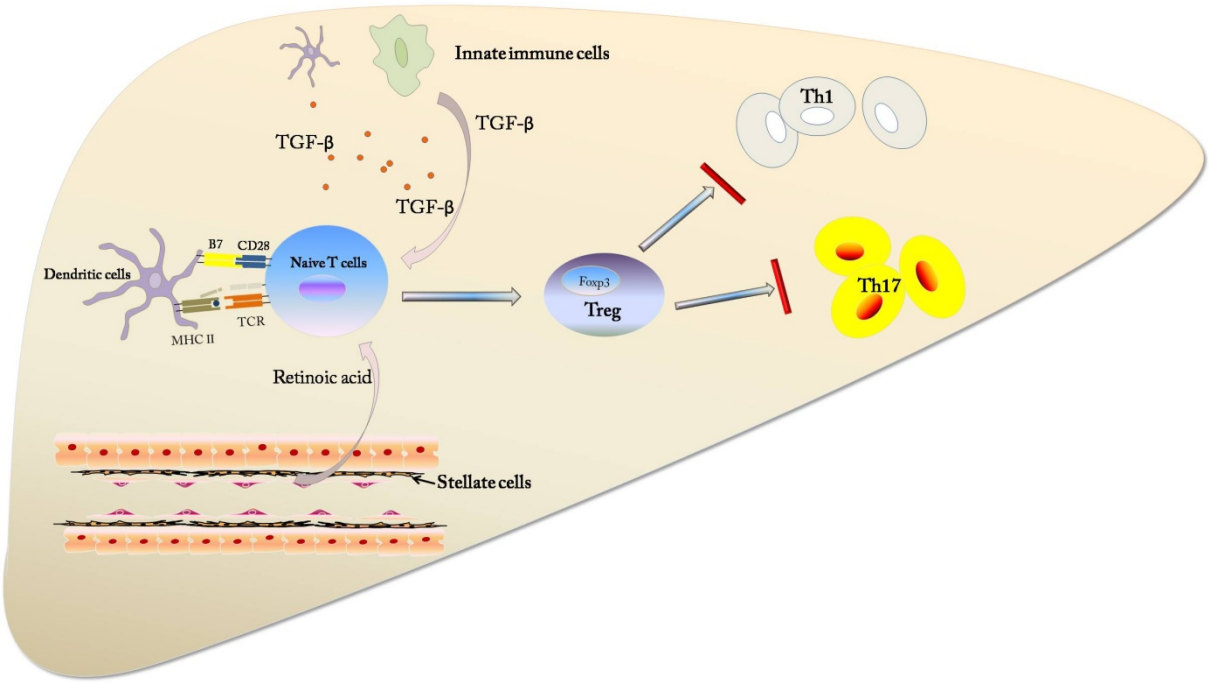


Figure 6-2 Hypothesized mechanisms of immune homeostasis in the hepatic microenvironment.

In normal liver, there are low levels of proinflammatory cytokines but high level of TGF-β which may drive intrahepatic CD4 lymphocytes towards T_{reg} differentiation. Thus, T_{reg} dominate the non-inflamed hepatic microenvironment to control of effector T lymphocytes like Th1 and Th17. Retinoic acid which is stored in hepatic stellate or Ito cells may also favour the T_{reg} differentiation.

6.6.3 Plasticity of intrahepatic lymphocytes lineage

The potential plasticity of lymphocyte lineage is of much interest recently. T_{reg} differentiation towards Th17 in inflamed microenvironment and the ability of Th17 cells to secrete Th1 cytokines may be related to complex interactions between transcription factor genes and cytokines. Thus, I plan to investigate the stability of intrahepatic lymphocytes subsets in future study for the plasticity and lineage outcome.

Abbreviations

Aa	Amino acid
Ab	Antibody
AhR	Aryl hydrocarbon receptor
AIH	Autoimmune hepatitis
ALD	Alcoholic liver diseases
APC	Antigen presenting cell
BEC	Biliary epithelial cells
BFA	Brefeldin A
BM	Basement membrane
BSA	Bovine serum albumin
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CHC	Chronic hepatitis C
CLA	Cutaneous lymphocyte antigen
CLEVER-1	Common lymphatic endothelial and vascular endothelial receptor-1
CNS	Central nervous system
ConA	Concavalin A
CTLA-4	Cytotoxic T lymphocyte antigen-4
CXCL	CXCL-chemokine ligand
CXCR	CXC-chemokine receptor
DC	Dendritic cell
DMSO	Dimethyl sulfoxide

DTH	Delayed type hypersensitivity
EAE	Experimental allergic encephalomyelitis
EDTA	Ethylediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	Florescence activated cell sorter
FCS	Foetal calf serum
FoxP3	Fox head box P3 (master regulator of T_{reg})
GITR	Glucocorticoid-induced TNF receptor family related protein
GlyCAM-1	Glycosylation–dependent cell adhesion molecule-1
HEV	High endothelial venules
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidise
HSEC	Hepatic sinusoidal endothelial cells
ICAM	Intracellular cell adhesion molecule
ICOS	Inducible costimulator
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant natural killer T cell
LFA-1	Lymphocyte function associated antigen-1
LILs	Liver infiltrating lymphocytes
LIT_{reg}	Liver infiltrating regulatory T lymphcytes
LITh17	Liver infiltrating IL-17 secreting CD4 lymphocytes
LITc17	Liver infiltrating IL-17 secreting CD8 lymphocytes
LPS	Lipopolysaccharide

MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MALT	Mucosa associated lymphoid tissue
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MIP	Macrophage inflammatory protein
NK	Natural killer cell
NKT	Natural killer T cell
NL	Normal liver
PAMPs	Pathogen associated molecular patterns
PBC	Primary biliary cirrhosis
PBL	Peripheral blood lymphocytes
PBS	Phosphatase buffer saline
PCR	Polymerase chain reaction
PLN	Peripheral lymph node
PMA	Phorbol 12-myristate 13-acetate
PNAd	Peripheral node addressin
PPs	Peyer's patches
PRRs	Pattern recognition receptor
PSC	Primary sclerosing cholangitis
PSG	Penicillin streptomycin glutamine
PSGL-1	P–selectin glycoprotein ligand-1
RANTES	Regulated activation normal T–cell expressed and secreted
RA	Retinoic acid
ROR	Retinoic acid related orphan receptor
SCID	Severe combined immunodeficiency

TBS	Tris-buffered saline
TCR	T cell receptor
Tc17	Interleukin-17 secreting CD8 T effector cell
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th1	T helper 1 cell
Th2	T helper 2 cell
Th3	TGF-β secreting T helper cells
Th17	Interleukin-17 secreting CD4 T helper cell
Th9	Interleukin-9 secreting CD4 T helper cell
TLRs	Toll like receptors
TNF-α	Tumour necrosis factor alpha
Treg	T regulatory cell
Tr1	IL-10 secreting type1 T regulatory cells
VAP-1	Vasculat adhesion protein-1
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

References

Abbas, A. K., Murphy, K. M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. **Nature,** 383 (6603): 787-793

Abel, M., Sene, D., Pol, S. et al (2006) Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. **Hepatology.,** 44 (6): 1607-1616

Adams, D. H. (1996a) Biliary epithelial cells: innocent victims or active participants in immune-mediated liver disease?

Adams, D. H. (1996b) Lymphocyte-endothelial cell interactions in hepatic inflammation. **Hepatogastroenterology,** 43 (7): 32-43

Adams, D. H., Burra, P., Hubscher, S. G. et al (1994a) Endothelial activation and circulating vascular adhesion molecules in alcoholic liver disease. **Hepatology,** 19 (3): 588-594

Adams, D. H. and Eksteen, B. (2006) Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel disease. **Nat.Rev.Immunol.,** 6 (3): 244-251

Adams, D. H., Harvath, L., Bottaro, D. P. et al (1994b) Hepatocyte growth factor and macrophage inflammatory protein 1 beta: structurally distinct cytokines that induce rapid cytoskeletal changes and subset-preferential migration in T cells. **Proc.Natl.Acad.Sci.U.S.A,** 91 (15): 7144-7148

Adams, D. H., Hathaway, M., Shaw, J. et al (1991) Transforming growth factor-beta induces human T lymphocyte migration in vitro. **J.Immunol.,** 147 (2): 609-612

Adams, D. H. and Hubscher, S. G. (2006) Systemic viral infections and collateral damage in the liver. **Am.J.Pathol.,** 168 (4): 1057-1059

Adams, D. H., Hubscher, S. G., Fisher, N. C. et al (1996) Expression of E-selectin and E-selectin ligands in human liver inflammation. **Hepatology,** 24 (3): 533-538

Adams, D. H. and HUTCHINSON, I. V. (1997) Microchimerism and graft tolerance: cause or effect? **Lancet,** 349 (9062): 1336-1337

Adams, D. H. and Lloyd, A. R. (1997) Chemokines: leucocyte recruitment and activation cytokines. **Lancet,** 349 (9050): 490-495

Adams, D. H. and Shaw, S. (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. **Lancet,** 343 (8901): 831-836

Adams, D. H., Shaw, S., & Van Seventer, G. (1993) "Adhesion molecules: role in cellular adhesion and communication.". In: Snow E. C. (eds.) **Handbook of T and B Lymphocytes**. 1 ed. San Diego: Academic Press, Inc. pp. 3-25.

Adams, D. H., Wang, L., Hubscher, S. G. et al (1989) Hepatic endothelial cells. Targets in liver allograft rejection? **Transplantation,** 47 (3): 479-482

Aderem, A. and Ulevitch, R. J. (2000) Toll-like receptors in the induction of the innate immune response. **Nature.,** 406 (6797): 782-787

Afford, S. C. (2001) CD40 activation-induced, Fas-dependent apoptosis and NF-kappaB/AP-1 signaling in human intrahepatic biliary epithelial cells.

Aggarwal, S., Ghilardi, N., Xie, M. H. et al (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. **J.Biol.Chem.,** 278 (3): 1910-1914

Aggarwal, S. and Gurney, A. L. (2002) IL-17: prototype member of an emerging cytokine family. **J.Leukoc.Biol.,** 71 (1): 1-8

Ahmed-Choudhury, J., Russell, C. L., Randhawa, S. et al (2003) Differential induction of nuclear factor-kappaB and activator protein-1 activity after CD40 ligation is associated with primary human hepatocyte apoptosis or intrahepatic endothelial cell proliferation. **Molecular Biology of the Cell,** 14 (4): 1334-1345

Ajuebor, M. N., Aspinall, A. I., Zhou, F. et al (2005) Lack of chemokine receptor CCR5 promotes murine fulminant liver failure by preventing the apoptosis of activated CD1d-restricted NKT cells. **J.Immunol.,** 174 (12): 8027-8037

Ajuebor, M. N., Hogaboam, C. M., Le, T. et al (2004) CCL3/MIP-1alpha is pro-inflammatory in murine T cell-mediated hepatitis by recruiting CCR1-expressing CD4(+) T cells to the liver. **Eur.J.Immunol.,** 34 (10): 2907-2918

Ala, A., Dhillon, A. P. and Hodgson, H. J. (2003) Role of cell adhesion molecules in leukocyte recruitment in the liver and gut. **Int.J.Exp.Pathol.,** 84 (1): 1-16

Alabraba, E. B., Lai, V., Boon, L. et al (2007) Coculture of human liver macrophages and cholangiocytes leads to CD40-dependent apoptosis and cytokine secretion. **Hepatology,**

Almeida, A. R., Legrand, N., Papiernik, M. et al (2002) Homeostasis of peripheral CD4+ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers. **The Journal of Immunology,** 169 (9): 4850-60

Alon, R., Kassner, P. D., Carr, M. W. et al (1995) The integrin vla-4 supports tethering and rolling in flow on vcam-1. **Journal of Cell Biology,** 128 1243-1253

Anderson, D. C. and Springer, T. A. (1987) Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. **Ann.Rev.Med.,** 38 175-194

Anderson, G., Jenkinson, W. E., Jones, T. et al (2006) Establishment and functioning of intrathymic microenvironments. **Immunol.Rev.,** 209 10-27

Anderson, G., Pongracz, J., Parnell, S. et al (2001) Notch ligand-bearing thymic epithelial cells initiate and sustain Notch signaling in thymocytes independently of T cell receptor signaling. **Eur.J.Immunol.,** 31 (11): 3349-3354

Annunziato, F., Cosmi, L., Santarlasci, V. et al (2007) Phenotypic and functional features of human Th17 cells. **J.Exp.Med.,** 204 (8): 1849-1861

Antony, P. A., Paulos, C. M., Ahmadzadeh, M. et al (2006) Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. **J.Immunol.,** 176 (9): 5255-5266

Apolinario, A., Majano, P. L., Alvarez-Perez, E. et al (2002) Increased expression of T cell chemokines and their receptors in chronic hepatitis C: relationship with the histological activity of liver disease. **American Journal of Gastroenterology,** 97 (11): 2861-2870

Arai, K., Liu, Z. X., Lane, T. et al (2002) IP-10 and Mig facilitate accumulation of T cells in the virus-infected liver. **Cell Immunol,** 219 (1): 48-56

Arbones, M. L., Ord, D. C., Ley, K. et al (1994) Lymphocyte homing and leukocyte rolling and migration are impaired in L- selectin-deficient mice. **Immunity.,** 1 (4): 247-260

Aujla, S. J., Chan, Y. R., Zheng, M. et al (2008) IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. **Nat.Med.,** 14 (3): 275-281

Awane, M., Andres, P. G., Li, D. J. et al (1999) NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. **The Journal of Immunology,** 162 (9): 5337-5344

Bamboat, Z. M., Stableford, J. A., Plitas, G. et al (2009) Human liver dendritic cells promote T cell hyporesponsiveness. **J.Immunol.,** 182 (4): 1901-1911

Banchereau, J., Briere, F., Caux, C. et al (2000) Immunobiology of dendritic cells. **Annu.Rev.Immunol.,** 18 767-811

Barrat, F. J., Cua, D. J., Boonstra, A. et al (2002) In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. **J.Exp.Med.,** 195 (5): 603-616

Battaglia, M. (2005) Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells.

Battaglia, M. (2006) Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients.

Battaglia, M., Stabilini, A., Draghici, E. et al (2006) Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. **Diabetes,** 55 (1): 40-9

Bauquet, A. T., Jin, H., Paterson, A. M. et al (2009) The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. **Nat.Immunol.,** 10 (2): 167-175

Bayry, J., Triebel, F., Kaveri, S. V. et al (2007) Human dendritic cells acquire a semimature phenotype and lymph node homing potential through interaction with CD4+CD25+ regulatory T cells. **J.Immunol.,** 178 (7): 4184-4193

Belkaid, Y. (2008) Role of Foxp3-positive regulatory T cells during infection. **Eur.J.Immunol.,** 38 (4): 918-921

Belkaid, Y. and Rouse, B. T. (2005) Natural regulatory T cells in infectious disease. **Nat.Immunol.,** 6 (4): 353-360

Benseler, V., McCaughan, G. W., SCHLITT, H. J. et al (2007) The liver: a special case in transplantation tolerance. **Semin.Liver Dis.,** 27 (2): 194-213

Bertoletti, A., D'Elios, M. M., Boni, C. et al (1997) Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections [see comments]. **Gastroenterology,** 112 (1): 193-199

Bertolino, P., Bowen, D. G., McCaughan, G. W. et al (2001) Antigen-specific primary activation of CD8+ T cells within the liver. **The Journal of Immunology,** 166 (9): 5430-5438

Bettelli, E., Carrier, Y., Gao, W. et al (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. **Nature.,** 441 (7090): 235-238

Bettelli, E., Korn, T. and Kuchroo, V. K. (2007) Th17: the third member of the effector T cell trilogy. **Current Opinions in Immunology,** 19 (6): 652-657

Bevilacqua, M. P., Pober, J. S., Mendrick, D. L. et al (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. **Proc.Natl.Acad.Sci.USA,** 84 9238-9242

Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr. et al (1989) Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. **Science,** 243 1160-1164

Billerbeck, E., Kang, Y. H., Walker, L. et al (2010) Analysis of CD161 expression on human CD8+ T cells defines a distinct functional subset with tissue-homing properties. **Proc.Natl.Acad.Sci.U.S.A.,** 107 (7): 3006-3011

Billingham, R. E., Brent, L. and MEDAWAR, P. B. (1953) Actively acquired tolerance of foreign cells. **Nature.,** 172 (4379): 603-606

Bliss, S. K., Alcaraz, A. and Appleton, J. A. (2003) IL-10 prevents liver necrosis during murine infection with Trichinella spiralis. **J.Immunol.,** 171 (6): 3142-3147

Bluestone, J. A. (2003) Natural versus adaptive regulatory T cells.

Bluestone, J. A. (2005) Regulatory T-cell therapy: is it ready for the clinic?

Bluestone, J. A., Thomson, A. W., Shevach, E. M. et al (2007) What does the future hold for cell-based tolerogenic therapy? **Nat.Rev.Immunol.,** 7 (8): 650-654

Bodor, J., Fehervari, Z., Diamond, B. et al (2006) Regulatory T cell-mediated suppression: potential role of ICER. **J Leukoc Biol,**

Boisvert, J., Kunkel, E. J., Campbell, J. J. et al (2003) Liver-infiltrating lymphocytes in end-stage hepatitis C virus: subsets, activation status, and chemokine receptor phenotypes. **Journal of Hepatology,** 38 (1): 67-75

Bonacchi, A., Petrai, I., Defranco, R. M. et al (2003) The chemokine CCL21 modulates lymphocyte recruitment and fibrosis in chronic hepatitis C. **Gastroenterology,** 125 (4): 1060-1076

Bopp, T., Becker, C., Klein, M. et al (2007) Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. **J Exp Med,** 204 (6): 1303-1310

Borsellino, G., Kleinewietfeld, M., Di, M. D. et al (2007) Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. **Blood.,** 110 (4): 1225-1232

Bowen, D. G., McCaughan, G. W. and Bertolino, P. (2005) Intrahepatic immunity: a tale of two sites? **Trends Immunol.,** 26 (10): 512-517

Bowen, D. G., Warren, A., Davis, T. et al (2002) Cytokine-dependent bystander hepatitis due to intrahepatic murine CD8 T-cell activation by bone marrow-derived cells. **Gastroenterology,** 123 (4): 1252-1264

Bowen, D. G., Zen, M., Holz, L. et al (2004) The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. **J Clin Invest,** 114 (5): 701-712

Boyton, R. J. and Altmann, D. M. (2002) Is selection for TCR affinity a factor in cytokine polarization? **Trends Immunol.,** 23 (11): 526-529

Bromley, S. K., Thomas, S. Y. and Luster, A. D. (2005) Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. **Nat.Immunol.,** 6 (9): 895-901

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A. et al (2001) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. **Nat Genet,** 27 (1): 68-73

Buckley, C. D., Pilling, D., Lord, J. M. et al (2001) Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. **Trends Immunol.,** 22 (4): 199-204

Burchill, M. A., Goetz, C. A., Prlic, M. et al (2003) Distinct effects of STAT5 activation on CD4+ and CD8+ T cell homeostasis: development of CD4+CD25+ regulatory T cells versus CD8+ memory T cells. **The Journal of Immunology,** 171 (11): 5853-64

Burchill, M. A., Yang, J., Vogtenhuber, C. et al (2007) IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. **J.Immunol.,** 178 (1): 280-290

Burt, A. D., Le Bail, B., Balabaud, C. et al (1993) Morphologic Investigation of Sinusoidal Cells. **Seminars in Liver Disease,** 13 (1): 21-38

Butcher, E. C. and Picker, L. J. (1996) Lymphocyte homing and homeostasis. **Science,** 272 (5258): 60-66

Butcher, E. C., Williams, M., Youngman, K. et al (1999) Lymphocyte trafficking and regional immunity. **Adv Immunol,** 72 209-253

Butera, D., Marukian, S., Iwamaye, A. E. et al (2005) Plasma chemokine levels correlate with the outcome of antiviral therapy in patients with hepatitis C. **Blood,** 106 (4): 1175-1182

Calne RY, S. R. P. J. e. al. Induction of Immunological Tolerance by Porcine Liver Allografts. Nature 223, 472-476. 1969.
Ref Type: Generic

Calne, R. and Davies, H. (1994) Organ graft tolerance: the liver effect. **Lancet,** 343 (8889): 67-68

Campbell, J. J. and Butcher, E. C. (2000) Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. **Current Opinions in Immunology,** 12 (3): 336-341

Campbell, J. J., Qin, S. X., Bacon, K. B. et al (1996) Biology of chemokine and classical chemoattractant receptors - differential requirements for adhesion-triggering versus chemotactic responses in lymphoid-cells. **Journal of Cell Biology,** 134 255-266

Cao, D., Malmstrom, V., Baecher-Allan, C. et al (2003) Isolation and functional characterization of regulatory CD25brightCD4+ T cells from the target organ of patients with rheumatoid arthritis. **Eur.J.Immunol.,** 33 (1): 215-223

Cardoso, C. R., Garlet, G. P., Moreira, A. P. et al (2008a) Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. **J.Leukoc.Biol.,** 84 (1): 311-318

Cardoso, C. R., Garlet, G. P., Moreira, A. P. et al (2008b) Characterization of CD4+CD25+ natural regulatory T cells in the inflammatory infiltrate of human chronic periodontitis. **J.Leukoc.Biol.,** 84 (1): 311-318

Cavassani, K. A., Campanelli, A. P., Moreira, A. P. et al (2006) Systemic and local characterization of regulatory T cells in a chronic fungal infection in humans. **J.Immunol.,** 177 (9): 5811-5818

Chan, J. R., Blumenschein, W., Murphy, E. et al (2006) IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. **J.Exp.Med.,** 203 (12): 2577-2587

Chang, K. M. (2005) Regulatory T cells and the liver: a new piece of the puzzle.

Chang, S. H. and Dong, C. (2009) IL-17F: regulation, signaling and function in inflammation. **Cytokine.,** 46 (1): 7-11

Chaudhry, A., Rudra, D., Treuting, P. et al (2009) CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. **Science.,** 326 (5955): 986-991

Chen, A., Engel, P. and Tedder, T. F. (1995) Structural requirements regulate endoproteolytic release of the L-selectin (CD62L) adhesion receptor from the cell surface of leukocytes. **J.Exp.Med.,** 182 (2): 519-530

Chen, W., Jin, W., Hardegen, N. et al (2003) Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. **J Exp Med,** 198 (12): 1875-86

Chen, W., Jin, W. and Wahl, S. M. (1998) Engagement of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) induces transforming growth factor beta (TGF-beta) production by murine CD4(+) T cells. **J.Exp.Med.,** 188 (10): 1849-1857

Chen, Y., Kuchroo, V. K., Inobe, J. et al (1994) Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. **Science,** 265 (5176): 1237-40

Chen, Y., Langrish, C. L., McKenzie, B. et al (2006) Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. **J.Clin.Invest.,** 116 (5): 1317-1326

Chen, Z., Laurence, A. and O'Shea, J. J. (2007) Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. **Seminars in Immunology,** 19 (6): 400-408

Cher, D. J. and Mosmann, T. R. (1987) Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. **J.Immunol.,** 138 (11): 3688-3694

Ciric, B., El-behi, M., Cabrera, R. et al (2009) IL-23 drives pathogenic IL-17-producing CD8+ T cells. **J.Immunol.,** 182 (9): 5296-5305

Colditz, I. G., Schneider, M. A., Pruenster, M. et al (2007) Chemokines at large: in-vivo mechanisms of their transport, presentation and clearance. **Thromb.Haemost.,** 97 (5): 688-693

Collison, L. W., Workman, C. J., Kuo, T. T. et al (2007) The inhibitory cytokine IL-35 contributes to regulatory T-cell function. **Nature.,** 450 (7169): 566-569

Cosmi, L., De, P. R., Santarlasci, V. et al (2008) Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. **J.Exp.Med.,** 205 (8): 1903-1916

costa-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A. et al (2007a) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. **Nat.Immunol.,** 8 (9): 942-949

costa-Rodriguez, E. V., Rivino, L., Geginat, J. et al (2007b) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. **Nat.Immunol.,** 8 (6): 639-646

Crispe, I. N. (2003) Hepatic T cells and liver tolerance. **Nat.Rev.Immunol.,** 3 (1): 51-62

Crispe, I. N. (2009) The liver as a lymphoid organ. **Annu.Rev.Immunol.,** 27 147-163

Crispe, I. N., Dao, T., Klugewitz, K. et al (2000) The liver as a site of T-cell apoptosis: graveyard, or killing field? **Immunol.Rev.,** 174 47-62

Crispe, I. N., Giannandrea, M., Klein, I. et al (2006) Cellular and molecular mechanisms of liver tolerance. **Immunol.Rev.,** 213 101-118

Crispe, I. N. and Mehal, W. Z. (1996) Strange brew: T cells in the liver. **Immunology Today,** 17 (11): 522-525

Cruise, M. W., Lukens, J. R., Nguyen, A. P. et al (2006) Fas ligand is responsible for CXCR3 chemokine induction in CD4+ T cell-dependent liver damage. **J.Immunol.,** 176 (10): 6235-6244

Cua, D. J., Sherlock, J., Chen, Y. et al (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. **Nature,** 421 (6924): 744-748

Curbishley, S. M., Eksteen, B., Gladue, R. P. et al (2005) CXCR3 Activation Promotes Lymphocyte Transendothelial Migration across Human Hepatic Endothelium under Fluid Flow. **Am J Pathol.,** 167 (3): 887-899

Curiel, T. J., Coukos, G., Zou, L. et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. **Nat Med.,** 10 (9): 942-949

Cyster, J. G. (2005) Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. **Annu.Rev Immunol,** 23 127-159

Danen, E. H. and Sonnenberg, A. (2003) Integrins in regulation of tissue development and function. **J.Pathol.,** 201 (4): 632-641

Dardalhon, V., Awasthi, A., Kwon, H. et al (2008) IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. **Nat.Immunol.,** 9 (12): 1347-1355

Dawson, J. L. (1985) "Normal Anatomy". In: Wright R., Millward-Sadler, G. H., Alberti, K. G. M. M. et al. (eds.) **Liver and Biliary Disease**. Second ed. Eastbourne, United Kingdom: Bailliere Tindall and W.B. Saunders Company Ltd. pp. 3-12.

de la Rosa, M., Rutz, S., Dorninger, H. et al (2004) Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. **Eur J Immunol,** 34 (9): 2480-8

de, C. A., Abe, M., Lau, A. H. et al (2005a) Low TLR4 expression by liver dendritic cells correlates with reduced capacity to activate allogeneic T cells in response to endotoxin. **J.Immunol.,** 174 (4): 2037-2045

de, C. A., Abe, M., Lau, A. H. et al (2005b) Low TLR4 expression by liver dendritic cells correlates with reduced capacity to activate allogeneic T cells in response to endotoxin. **J.Immunol.,** 174 (4): 2037-2045

Deaglio, S., Dwyer, K. M., Gao, W. et al (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. **J Exp Med,** 204 (6): 1257-65

Demirkiran, A. (2006) Low circulating regulatory T-cell levels after acute rejection in liver transplantation.

Demirkiran, A., Baan, C. C., Kok, A. et al (2007) Intrahepatic detection of FOXP3 gene expression after liver transplantation using minimally invasive aspiration biopsy. **Transplantation,** 83 (6): 819-823

Demirkiran, A., Kok, A., Kwekkeboom, J. et al (2006) Low circulating regulatory T-cell levels after acute rejection in liver transplantation. **Liver Transpl,** 12 (2): 277-84

Diago, M., Castellano, G., Garcia-Samaniego, J. et al (2006) Association of pretreatment serum interferon gamma inducible protein 10 levels with sustained virological response to peginterferon plus ribavirin therapy in genotype 1 infected patients with chronic hepatitis C. **Gut,** 55 (3): 374-379

Dransfield, I. and Hogg, N. (1989a) Monoclonal antibody defining a cation-dependent epitope on the beta subunit (CD18) of the leucocyte adhesion molecule family. **Tissue Antigens,** 33 p. 255

Dransfield, I. and Hogg, N. (1989b) Regulated expression of Mg2+ binding epitope on leukocyte integrin alpha subunits. **EMBO J.,** 8 3759-3765

Duerr, R. H., Taylor, K. D., Brant, S. R. et al (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. **Science,** 314 (5804): 1461-1463

Dumoulin, F. L., Bach, A., Leifeld, L. et al (1997) Semiquantitative analysis of intrahepatic cytokines mRNA in chronic hepatitis C. **J.Infect.Dis.,** 175 681-685

Edwards, S., Lalor, P. F., Nash, G. B. et al (2005) Lymphocyte traffic through sinusoidal endothelial cells is regulated by hepatocytes. **Hepatology,** 41 (3): 451-459

Efsen, E., Grappone, C., Defranco, R. M. et al (2002) Up-regulated expression of fractalkine and its receptor CX3CR1 during liver injury in humans. **Journal of Hepatology,** 37 (1): 39-47

Eksteen, B. Expression and plasticity of the gut homing molecules, CCR9 and á4â7 ON T cells IS dependent on retinoic acid. 2006.
Ref Type: Journal (Full)

Eksteen, B., Afford, S. C., Wigmore, S. J. et al (2007) Immune-mediated liver injury. **Semin Liver Dis,** 27 (4): 351-366

Eksteen, B., Miles, A., Curbishley, S. M. et al (2006) Epithelial Inflammation Is Associated with CCL28 Production and the Recruitment of Regulatory T Cells Expressing CCR10. **J.Immunol.,** 177 (1): 593-603

Eksteen, B., Mora, J. R., Haughton, E. L. et al (2009) Gut homing receptors on CD8 T-cells ARE retinoic acid dependent and not maintained by Liver dendritic or stellate cells. **Gastroenterology,**

Elyaman, W., Bradshaw, E. M., Uyttenhove, C. et al (2009) IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. **Proc.Natl.Acad.Sci.U.S.A.,** 106 (31): 12885-12890

Enarsson, K., Lundgren, A., Kindlund, B. et al (2006) Function and recruitment of mucosal regulatory T cells in human chronic Helicobacter pylori infection and gastric adenocarcinoma. **Clin.Immunol.,** 121 (3): 358-368

Faint, J. M., Annels, N. E., Curnow, S. J. et al (2001) Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics. **J.Immunol.,** 167 (1): 212-220

Fallarino, F., Grohmann, U., You, S. et al (2006) The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. **The Journal of Immunology,** 176 (11): 6752-61

Faria, A. M. and Weiner, H. L. (2005) Oral tolerance. **Immunol.Rev.,** 206 232-259

Fazilleau, N., Heyzer-Williams, L. J., Rosen, H. et al (2009a) The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. **Nat.Immunol.,** 10 (4): 375-384

Fazilleau, N., Mark, L., Heyzer-Williams, L. J. et al (2009b) Follicular helper T cells: lineage and location. **Immunity.,** 30 (3): 324-335

Ferber, I. A., Brocke, S., Taylor-Edwards, C. et al (1996) Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). **J.Immunol.,** 156 (1): 5-7

Floess, S., Freyer, J., Siewert, C. et al (2007) Epigenetic control of the foxp3 locus in regulatory T cells. **PLoS.Biol.,** 5 (2): p. e38

Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003a) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. **Nat.Immunol.,** 4 (4): 330-336

Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003b) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. **Nat Immunol,** 4 (4): 330-6

Forster, R., valos-Misslitz, A. C. and Rot, A. (2008) CCR7 and its ligands: balancing immunity and tolerance. **Nat.Rev.Immunol.,** 8 (5): 362-371

Fossiez, F., Djossou, O., Chomarat, P. et al (1996) T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. **J Exp Med,** 183 (6): 2593-2603

Franceschini, D., Paroli, M., Francavilla, V. et al (2009) PD-L1 negatively regulates CD4+CD25+Foxp3+ Tregs by limiting STAT-5 phosphorylation in patients chronically infected with HCV. **J.Clin.Invest,** 119 (3): 551-564

Franzese, O., Kennedy, P. T., Gehring, A. J. et al (2005) Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. **J.Virol.,** 79 (6): 3322-3328

Friedman, S. L. (1996) Hepatic stellate cells. **Prog.Liver Dis.,** 14 101-130

Fu, J., Xu, D., Liu, Z. et al (2007) Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. **Gastroenterology,** 132 (7): 2328-39

Furtado, G. C., Curotto de Lafaille, M. A., Kutchukhidze, N. et al (2002) Interleukin 2 signaling is required for CD4(+) regulatory T cell function. **J Exp Med,** 196 (6): 851-7

Gaffen, S. L. (2009) Structure and signalling in the IL-17 receptor family. **Nat.Rev.Immunol.,** 9 (8): 556-567

Gale, R. P., Sparkes, R. S. and Golde, D. W. (1978) Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. **Science.,** 201 (4359): 937-938

Gavin, M. A., Torgerson, T. R., Houston, E. et al (2006) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. **Proc Natl Acad Sci U S A,** 103 (17): 6659-64

Ge, J., Wang, K., Meng, Q. H. et al (2009) Implication of Th17 and Th1 Cells in Patients with Chronic Active Hepatitis B. **J.Clin.Immunol.,**

Germanov, E., Veinotte, L., Cullen, R. et al (2008) Critical Role for the Chemokine Receptor CXCR6 in Homeostasis and Activation of CD1d-Restricted NKT Cells. **J.Immunol.,** 181 (1): 81-91

Gerosa, F., Baldani-Guerra, B., Lyakh, L. A. et al (2008) Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. **J.Exp.Med.,** 205 (6): 1447-1461

Gobert, M., Treilleux, I., driss-Vermare, N. et al (2009) Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. **Cancer Res.,** 69 (5): 2000-2009

Goddard, S., Williams, A., Morland, C. et al (2001) Differential expression of chemokines and chemokine receptors shapes the inflammatory response in rejecting human liver transplants. **Transplantation,** 72 (12): 1957-1967

Goddard, S., Youster, J., Morgan, E. et al (2004a) Interleukin-10 secretion differentiates dendritic cells from human liver and skin. **Am.J.Pathol.,** 164 (2): 511-519

Goddard, S., Youster, J., Morgan, E. et al (2004b) Interleukin-10 secretion differentiates dendritic cells from human liver and skin. **Am.J.Pathol.,** 164 (2): 511-519

Godfrey, D. I., MacDonald, H. R., Kronenberg, M. et al (2004) NKT cells: what's in a name? **Nat.Rev.Immunol.,** 4 (3): 231-237

Goldman, D. W., Chang, F. H., Gifford, L. A. et al (1985) Pertussis toxin inhibition of chemotactic factor-induced calcium mobilization and function in human polymorphonuclear leukocytes. **J.Exp.Med.,** 162 (1): 145-156

Gondek, D. C., Lu, L. F., Quezada, S. A. et al (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. **J.Immunol.,** 174 (4): 1783-1786

Gounni, A. S., Hamid, Q., Rahman, S. M. et al (2004) IL-9-mediated induction of eotaxin1/CCL11 in human airway smooth muscle cells. **J.Immunol.,** 173 (4): 2771-2779

Gran, B., Zhang, G. X., Yu, S. et al (2002) IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. **J.Immunol.,** 169 (12): 7104-7110

Grant, A. J., Goddard, S., Ahmed-Choudhury, J. et al (2002) Hepatic expression of secondary lymphoid chemokine (CCL21) promotes the development of portal-associated lymphoid tissue in chronic inflammatory liver disease. **Am.J.Pathol.,** 160 (4): 1445-1455

Grossman, W. J., Verbsky, J. W., Barchet, W. et al (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. **Immunity.,** 21 (4): 589-601

Groux, H., O'Garra, A., Bigler, M. et al (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. **Nature,** 389 737-742

Haas, J. (2008) Specific recruitment of regulatory T cells into the CSF in lymphomatous and carcinomatous meningitis.

Hadeiba, H., Sato, T., Habtezion, A. et al (2008) CCR9 expression defines tolerogenic plasmacytoid dendritic cells able to suppress acute graft-versus-host disease. **Nat.Immunol.,** 9 (11): 1253-1260

Hafezi-Moghadam, A. and Ley, K. (1999) Relevance of L-selectin shedding for leukocyte rolling in vivo. **J.Exp.Med.,** 189 (6): 939-948

Hafezi-Moghadam, A., Thomas, K. L., Prorock, A. J. et al (2001) L-selectin shedding regulates leukocyte recruitment. **J.Exp.Med.,** 193 (7): 863-872

Hamada, H., Garcia-Hernandez, M. L., Reome, J. B. et al (2009) Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. **J.Immunol.,** 182 (6): 3469-3481

Harada, K., Shimoda, S., Sato, Y. et al (2009) Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis. **Clin.Exp.Immunol.,** 157 (2): 261-270

Harrington, L. E., Hatton, R. D., Mangan, P. R. et al (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. **Nat.Immunol.,** 6 (11): 1123-1132

Harris, T. J., Grosso, J. F., Yen, H. R. et al (2007) Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. **J.Immunol.,** 179 (7): 4313-4317

Harvey, C. E., Post, J. J., Palladinetti, P. et al (2003) Expression of the chemokine IP-10 (CXCL10) by hepatocytes in chronic hepatitis C virus infection correlates with histological severity and lobular inflammation. **J.Leukoc.Biol.,** 74 (3): 360-369

Hasegawa, H., Inoue, A., Kohno, M. et al (2008) Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. **Gene Ther.,** 15 (3): 171-182

Hathaway, M., Burnett, D., Elias, E. et al (1993) Secretion of soluble chemotactic factors, including interleukin-6: a mechanism for the recruitment of CD8-positive T lymphocytes to human liver allografts during rejection. **Hepatology,** 18 (3): 511-518

Heydtmann, M. and Adams, D. H. (2002) Understanding selective trafficking of lymphocyte subsets. **Gut,** 50 (2): 150-152

Heydtmann, M., Hardie, D., Shields, P. L. et al (2006) Detailed analysis of intrahepatic CD8 T cells in the normal and hepatitis C-infected liver reveals differences in specific populations of memory cells with distinct homing phenotypes. **J.Immunol.,** 177 (1): 729-738

Heydtmann, M., Lalor, P. F., Eksteen, J. A. et al (2005) CXC chemokine ligand 16 promotes integrin-mediated adhesion of liver-infiltrating lymphocytes to cholangiocytes and hepatocytes within the inflamed human liver. **J.Immunol.,** 174 (2): 1055-1062

Hieshima, K., Imai, T., Opdenakker, G. et al (1997) Molecular cloning of a novel human CC chemokine liver and activation- regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2. **J.Biol.Chem.,** 272 (9): 5846-5853

Hill, J. A., Feuerer, M., Tash, K. et al (2007) Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. **Immunity.,** 27 (5): 786-800

Hirota, K., Yoshitomi, H., Hashimoto, M. et al (2007) Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. **J.Exp.Med.,** 204 (12): 2803-2812

Hjelmstrom, P., Fjell, J., Nakagawa, T. et al) Lymphoid tissue homing chemokines are expressed in chronic inflammation. **Am.J.Pathol.2000.Apr.;156.(4.):1133.-8.,** 156 (4): 1133-1138

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. et al (1999) Phylogenetic perspectives in innate immunity. **Science,** 284 (5418): 1313-1318

Hoffmann, P., Boeld, T. J., Eder, R. et al (2006) Isolation of CD4(+)CD25(+) Regulatory T Cells for Clinical Trials. **Biol.Blood Marrow Transplant.,** 12 (3): 267-274

Hoffmann, P. and Edinger, M. (2006) CD4+CD25+ regulatory T cells and graft-versus-host disease. **Semin.Hematol.,** 43 (1): 62-69

Hoffmann, P., Ermann, J. and Edinger, M. (2005) CD4+CD25+ regulatory T cells in hematopoietic stem cell transplantation. **Curr Top Microbiol Immunol,** 293 265-85

Holland, S. M., DeLeo, F. R., Elloumi, H. Z. et al (2007) STAT3 mutations in the hyper-IgE syndrome. **N.Engl.J.Med.,** 357 (16): 1608-1619

Holmen, N., Lundgren, A., Lundin, S. et al (2006) Functional CD4+CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. **Inflamm.Bowel.Dis.,** 12 (6): 447-456

Holt, A. P., Haughton, E. L., Lalor, P. F. et al (2009) Liver myofibroblasts regulate infiltration and positioning of lymphocytes in human liver. **Gastroenterology,** 136 (2): 705-714

Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. **Science,** 299 (5609): 1057-1061

Houot, R., Perrot, I., Garcia, E. et al (2006) Human CD4+CD25high regulatory T cells modulate myeloid but not plasmacytoid dendritic cells activation. **J.Immunol.,** 176 (9): 5293-5298

Hsu-Lin, S., Berman, C. L., Furie, B. C. et al (1984) A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. **J.Biol.Chem.,** 259 (14): 9121-9126

Huang, C. T., Workman, C. J., Flies, D. et al (2004) Role of LAG-3 in regulatory T cells. **Immunity.,** 21 (4): 503-513

Huang, L., Soldevila, G., Leeker, M. et al (1994) The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. **Immunity.,** 1 (9): 741-749

Huber, M., Heink, S., Grothe, H. et al (2009) A Th17-like developmental process leads to CD8(+) Tc17 cells with reduced cytotoxic activity. **Eur.J.Immunol.,** 39 (7): 1716-1725

Hubscher, S. G. (1998) Histological grading and staging in chronic hepatitis: clinical applications and problems.

Hue, S., Ahern, P., Buonocore, S. et al (2006) Interleukin-23 drives innate and T cell-mediated intestinal inflammation. **J.Exp.Med.,** 203 (11): 2473-2483

Huehn, J., Siegmund, K. and Hamann, A. (2005) Migration rules: functional properties of naive and effector/memory-like regulatory T cell subsets. **Curr.Top.Microbiol.Immunol.,** 293 89-114

Huehn, J., Siegmund, K., Lehmann, J. C. et al (2004) Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. **J Exp.Med,** 199 (3): 303-313

Hultner, L., Druez, C., Moeller, J. et al (1990) Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). **Eur.J.Immunol.,** 20 (6): 1413-1416

Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. **Cell,** 69 11-25

Iellem, A., Colantonio, L. and D'Ambrosio, D. (2003) Skin-versus gut-skewed homing receptor expression and intrinsic CCR4 expression on human peripheral blood CD4+CD25+ suppressor T cells. **Eur.J.Immunol.,** 33 (6): 1488-1496

Iellem, A., Mariani, M., Lang, R. et al (2001a) Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. **J.Exp.Med.,** 194 (6): 847-853

Iellem, A., Mariani, M., Lang, R. et al (2001b) Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. **J.Exp.Med.,** 194 (6): 847-853

Isse, K., Harada, K., Zen, Y. et al (2005) Fractalkine and CX3CR1 are involved in the recruitment of intraepithelial lymphocytes of intrahepatic bile ducts. **Hepatology,** 41 (3): 506-516

Itoh, M., Takahashi, T., Sakaguchi, N. et al (1999) Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. **The Journal of Immunology,** 162 (9): 5317-26

Ivanov, I. I., McKenzie, B. S., Zhou, L. et al (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. **Cell,** 126 (6): 1121-1133

Ivanov, I. I., Zhou, L. and Littman, D. R. (2007) Transcriptional regulation of Th17 cell differentiation. **Seminars in Immunology,** 19 (6): 409-417

Izcue, A., Hue, S., Buonocore, S. et al (2008) Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. **Immunity.,** 28 (4): 559-570

Jonuleit, H., Schmitt, E., Schuler, G. et al) Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. **J.Exp.Med.2000.Nov.6.;192.(9.):1213.-22.,** 192 (9): 1213-1222

Jonuleit, H., Schmitt, E., Stassen, M. et al (2001) Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. **J Exp Med,** 193 (11): 1285-94

Jovanovic, D. V., Di Battista, J. A., Martel-Pelletier, J. et al (1998) IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. **The Journal of Immunology,** 160 (7): 3513-3521

Jung, U. and Ley, K. (1999) Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. **The Journal of Immunology,** 162 (11): 6755-6762

Jutila, M. A. (1992) Leukocyte traffic to sites of inflammation. **Apmis,** 100 (3): 191-201

Jutila, M. A., Watts, G., Walcheck, B. et al (1992) Characterization of a functionally important and evolutionarily well-conserved epitope mapped to the short consensus repeats of E-selectin and L-selectin. **J.Exp.Med.,** 175 (6): 1565-1573

Kang, S. G., Lim, H. W., Andrisani, O. M. et al (2007) Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. **J.Immunol.,** 179 (6): 3724-3733

Kappler, J. W., Roehm, N. and Marrack, P. (1987) T cell tolerance by clonal elimination in the thymus. **Cell.,** 49 (2): 273-280

Katou, F., Ohtani, H., Nakayama, T. et al (2001) Macrophage-derived chemokine (MDC/CCL22) and CCR4 are involved in the formation of T lymphocyte-dendritic cell clusters in human inflamed skin and secondary lymphoid tissue. **Am.J.Pathol.,** 158 (4): 1263-1270

Kebir, H., Kreymborg, K., Ifergan, I. et al (2007) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. **Nat.Med.,** 13 (10): 1173-1175

Keever-Taylor, C. A. (2007) Rapamycin enriches for CD4(+) CD25(+) CD27(+) Foxp3(+) regulatory T cells in ex vivo-expanded CD25-enriched products from healthy donors and patients with multiple sclerosis.

Kemper, C., Chan, A. C., Green, J. M. et al (2003) Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. **Nature,** 421 (6921): 388-92

Khattri, R., Cox, T., Yasayko, S. A. et al (2003) An essential role for Scurfin in CD4+CD25+ T regulatory cells. **Nat.Immunol.,** 4 (4): 337-342

Kilshaw, P. J. (1999) Alpha E beta 7. **Mol.Pathol.,** 52 (4): 203-207

Kilshaw, P. J. and Murant, S. J. (1990) A new surface antigen on intraepithelial lymphocytes in the intestine. **Eur.J.Immunol.,** 20 2201-2207

King, C., Tangye, S. G. and Mackay, C. R. (2008) T follicular helper (TFH) cells in normal and dysregulated immune responses. **Annu.Rev.Immunol.,** 26:741-66. 741-766

Kingsley, C. I., Karim, M., Bushell, A. R. et al (2002) CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. **J.Immunol.,** 168 (3): 1080-1086

Kirkham, B. W., Lassere, M. N., Edmonds, J. P. et al (2006) Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). **Arthritis and Rheumatism,** 54 (4): 1122-1131

Kisielow, P., Bluthmann, H., Staerz, U. D. et al (1988) Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. **Nature.,** 333 (6175): 742-746

Kitani, A. and Xu, L. (2008) Regulatory T cells and the induction of IL-17. **Mucosal.Immunol.,** 1 Suppl 1:S43-6. p. S43-S46

Klein, I., Cornejo, J. C., Polakos, N. K. et al (2007) Kupffer cell heterogeneity: functional properties of bone marrow derived and sessile hepatic macrophages. **Blood.,** 110 (12): 4077-4085

Knolle, P. A. and Gerken, G. (2000) Local control of the immune response in the liver. **Immunol.Rev.,** 174 21-34

Knolle, P. A., Schmitt, E., Jin, S. et al (1999) Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells. **Gastroenterology,** 116 (6): 1428-1440

Kolls, J. K. and Linden, A. (2004) Interleukin-17 family members and inflammation. **Immunity.,** 21 (4): 467-476

Kondo, T., Takata, H., Matsuki, F. et al (2009) Cutting edge: Phenotypic characterization and differentiation of human CD8+ T cells producing IL-17. **J.Immunol.,** 182 (4): 1794-1798

Kopf, H. (2007) Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells.

Korn, T., Bettelli, E., Gao, W. et al (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. **Nature.,** 448 (7152): 484-487

Korn, T., Bettelli, E., Oukka, M. et al (2009) IL-17 and Th17 Cells. **Annu.Rev.Immunol.,** 27:485-517. 485-517

Korn, T., Mitsdoerffer, M., Croxford, A. L. et al (2008) IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. **Proc.Natl.Acad.Sci.U.S.A.,** 105 (47): 18460-18465

Kotenko, S. V., Izotova, L. S., Mirochnitchenko, O. V. et al (2001) Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. **J.Biol.Chem.,** 276 (4): 2725-2732

Krakowski, M. and Owens, T. (1996) Interferon-gamma confers resistance to experimental allergic encephalomyelitis. **Eur.J.Immunol.,** 26 (7): 1641-1646

Krueger, G. G., Langley, R. G., Leonardi, C. et al (2007) A human interleukin-12/23 monoclonal antibody for the treatment of psoriasis. **N.Engl.J.Med.,** 356 (6): 580-592

Kryczek, I., Banerjee, M., Cheng, P. et al (2009) Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. **Blood.,** 114 (6): 1141-1149

Kryczek, I., Wei, S., Zou, L. et al (2007) Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. **J.Immunol.,** 178 (11): 6730-6733

Kuang, D. M. (2009) Activated monocytes in peritumoral stroma of hepatocellular carcinoma promote expansion of memory T helper 17 cells.

Kuang, D. M. (2010) Activated monocytes in peritumoral stroma of hepatocellular carcinoma promote expansion of memory T helper 17 cells.

Kuestner, R. E., Taft, D. W., Haran, A. et al (2007) Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. **J.Immunol.,** 179 (8): 5462-5473

Kullberg, M. C., Jankovic, D., Feng, C. G. et al (2006) IL-23 plays a key role in Helicobacter hepaticus-induced T cell-dependent colitis. **J.Exp.Med.,** 203 (11): 2485-2494

Kunkel, E. J., Boisvert, J., Murphy, K. et al (2002) Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. **Am.J.Pathol.,** 160 (1): 347-355

Kunkel, E. J., Campbell, D. J. and Butcher, E. C. (2003) Chemokines in lymphocyte trafficking and intestinal immunity. **Microcirculation.,** 10 (3-4): 313-323

Laan, M., Cui, Z. H., Hoshino, H. et al (1999) Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. **The Journal of Immunology,** 162 (4): 2347-2352

Lai, W. K., Curbishley, S. M., Goddard, S. et al (2007) Hepatitis C is associated with perturbation of intrahepatic myeloid and plasmacytoid dendritic cell function. **Journal of Hepatology,** 47 (3): 338-347

Lalor, P. F. (1997) Association between receptor density, cellular activation, and transformation of adhesive behavior of flowing lymphocytes binding to VCAM-1.

Lalor, P. F. and Adams, D. H. (1999) Adhesion of lymphocytes to hepatic endothelium. **Mol.Pathol.,** 52 (4): 214-219

Lalor, P. F., Edwards, S., McNab, G. et al (2002a) Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. **The Journal of Immunology,** 169 (2): 983-92

Lalor, P. F., Lai, W. K., Curbishley, S. M. et al (2006) Human hepatic sinusoidal endothelial cells can be distinguished by expression of phenotypic markers related to their specialised functions in vivo. **World J.Gastroenterol.,** 12 (34): 5429-5439

Lalor, P. F., Shields, P., Grant, A. et al (2002b) Recruitment of lymphocytes to the human liver. **Immunol Cell Biol,** 80 (1): 52-64

Lan, R. Y. (2006) Liver-targeted and peripheral blood alterations of regulatory T cells in primary biliary cirrhosis.

Lan, R. Y., Salunga, T. L., Tsuneyama, K. et al (2009) Hepatic IL-17 responses in human and murine primary biliary cirrhosis. **J.Autoimmun.,** 32 (1): 43-51

Langowski, J. L., Zhang, X., Wu, L. et al (2006) IL-23 promotes tumour incidence and growth. **Nature.,** 442 (7101): 461-465

Langrish, C. L. (2004) IL-12 and IL-23: master regulators of innate and adaptive immunity.

Langrish, C. L., Chen, Y., Blumenschein, W. M. et al (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. **J.Exp.Med.,** 201 (2): 233-240

Lanier, L. L., Chang, C. and Phillips, J. H. (1994) Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. **J.Immunol.,** 153 (6): 2417-2428

Lasky, L. A. (1995) Selectin-carbohydrate interactions and the initiation of the inflammatory response. **Annu.Rev.Biochem.,** 64:113-39. 113-139

Laurence, A., Tato, C. M., Davidson, T. S. et al (2007a) Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. **Immunity.,** 26 (3): 371-381

Laurence, A., Tato, C. M., Davidson, T. S. et al (2007b) Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. **Immunity.,** 26 (3): 371-381

Lee, E., Trepicchio, W. L., Oestreicher, J. L. et al (2004) Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. **J.Exp.Med.,** 199 (1): 125-130

Lee, I., Wang, L., Wells, A. D. et al (2005a) Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. **J.Exp.Med.,** 201 (7): 1037-1044

Lee, I., Wang, L., Wells, A. D. et al (2005b) Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. **J.Exp.Med.,** 201 (7): 1037-1044

Lee, J. H., Kang, S. G. and Kim, C. H. (2007) FoxP3+ T cells undergo conventional first switch to lymphoid tissue homing receptors in thymus but accelerated second switch to nonlymphoid tissue homing receptors in secondary lymphoid tissues. **J.Immunol.,** 178 (1): 301-311

Lee, W. Y. and Kubes, P. (2008) Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs. **Journal of Hepatology,** 48 (3): 504-512

Lehmann, J., Huehn, J., de la Rosa, M. et al (2002) Expression of the integrin alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. **Proc Natl Acad Sci U S A JID - 7505876,** 99 (20): 13031-13036

Lemmers, A. (2009) The interleukin-17 pathway is involved in human alcoholic liver disease.

Leroy, V., Vigan, I., Mosnier, J. F. et al (2003) Phenotypic and functional characterization of intrahepatic T lymphocytes during chronic hepatitis C. **Hepatology,** 38 (4): 829-841

Levings, M. K., Sangregorio, R., Galbiati, F. et al (2001) IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. **J.Immunol.,** 166 (9): 5530-5539

Ley, K. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated.

Li, M. O., Sanjabi, S. and Flavell, R. A. (2006) Transforming growth factor-beta controls development, homeostasis, and tolerance of T Cells by regulatory T cell-dependent and -independent mechanisms. **Immunity,** 25 (3): 455-71

Liao, F., Rabin, R. L., Smith, C. S. et al (1999) CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. **J.Immunol.,** 162 (1): 186-194

Lichterfeld, M., Leifeld, L., Nischalke, H. D. et al (2002) Reduced CC chemokine receptor (CCR) 1 and CCR5 surface expression on peripheral blood T lymphocytes from patients with chronic hepatitis C infection. **J.Infect.Dis,** 185 (12): 1803-1807

Lim, H. W., Broxmeyer, H. E. and Kim, C. H. (2006) Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. **J.Immunol.,** 177 (2): 840-851

Lim, H. W., Lee, J., Hillsamer, P. et al (2008) Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. **J.Immunol.,** 180 (1): 122-129

Liu, W., Putnam, A. L., Xu-Yu, Z. et al (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. **J Exp Med,** 203 (7): 1701-11

Liu, Y., Zhang, P., Li, J. et al (2008) A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. **Nat.Immunol.,** 9 (6): 632-640

Lock, C., Hermans, G., Pedotti, R. et al (2002) Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. **Nat.Med.,** 8 (5): 500-508

Lohse, A. W., Knolle, P. A., Bilo, K. et al (1996) Antigen-presenting function and b7 expression of murine sinusoidal endothelial-cells and kupffer cells. **Gastroenterology,** 110 1175-1181

Longhi, M. S., Ma, Y., Bogdanos, D. P. et al (2004) Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease. **Journal of Hepatology,** 41 (1): 31-37

Longhi, M. S., Ma, Y., Mitry, R. R. et al (2005a) Effect of CD4+ CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. **J Autoimmun,** 25 (1): 63-71

Longhi, M. S., Ma, Y., Mitry, R. R. et al (2005b) Effect of CD4+CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. **J.Autoimmun.,** 25 (1): 63-71

Longhi, M. S., Mitry, R. R., Samyn, M. et al (2009) Vigorous activation of monocytes in juvenile autoimmune liver disease escapes the control of regulatory T-cells. **Hepatology.,** 50 (1): 130-142

Longphre, M., Li, D., Gallup, M. et al (1999) Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. **J.Clin.Invest.,** 104 (10): 1375-1382

Luster, A. D. (1998) Chemokines--chemotactic cytokines that mediate inflammation. **N.Engl.J.Med.,** 338 (7): 436-445

Luster, A. D. (2005) Immune cell migration in inflammation: present and future therapeutic targets.

Ma, C. S., Chew, G. Y., Simpson, N. et al (2008) Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. **J.Exp.Med.,** 205 (7): 1551-1557

Mack, D. G., Lanham, A. M., Palmer, B. E. et al (2009) CD27 expression on CD4+ T cells differentiates effector from regulatory T cell subsets in the lung. **J.Immunol.,** 182 (11): 7317-7324

Mackay, C. R. (2001) Chemokines: immunology's high impact factors. **Nat Immunol,** 2 (2): 95-101

Maher, J. J. (1995) Rat hepatocytes and kupffer cells interact to produce interleukin-8 (cinc) in the setting of ethanol. **American Journal Of Physiology-Gastrointestinal And Liver Physiology,** 32 p. G 518-G 523

Maldonado, R. A., Irvine, D. J., Schreiber, R. et al (2004) A role for the immunological synapse in lineage commitment of CD4 lymphocytes. **Nature.,** 431 (7008): 527-532

Malek, T. R., Ortega, G., Jakway, J. P. et al (1984) The murine IL-2 receptor. II. Monoclonal anti-IL-2 receptor antibodies as specific inhibitors of T cell function in vitro. **J.Immunol.,** 133 1976-19782

Manel, N., Unutmaz, D. and Littman, D. R. (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. **Nat.Immunol.,** 9 (6): 641-649

Mangan, P. R., Harrington, L. E., O'Quinn, D. B. et al (2006) Transforming growth factor-beta induces development of the T(H)17 lineage. **Nature.,** 441 (7090): 231-234

Mannon, P. J., Fuss, I. J., Mayer, L. et al (2004) Anti-interleukin-12 antibody for active Crohn's disease. **N.Engl.J.Med.,** 351 (20): 2069-2079

Mantovani, A., Sica, A., Sozzani, S. et al (2004) The chemokine system in diverse forms of macrophage activation and polarization. **Trends Immunol.,** 25 (12): 677-686

Marson, A., Kretschmer, K., Frampton, G. M. et al (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. **Nature,** 445 (7130): 931-5

Martel-Pelletier, J., Mineau, F., Jovanovic, D. et al (1999) Mitogen-activated protein kinase and nuclear factor kappaB together regulate interleukin-17-induced nitric oxide production in human osteoarthritic chondrocytes: possible role of transactivating factor mitogen-activated protein kinase-activated proten kinase (MAPKAPK). **Arthritis Rheum,** 42 (11): 2399-2409

Matloubian, M., David, A., Engel, S. et al (2000) A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. **Nat Immunol,** 1 (4): 298-304

Matusevicius, D., Kivisakk, P., He, B. et al (1999) Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. **Mult Scler,** 5 (2): 101-104

McEver, R. P. (2001) Adhesive interactions of leukocytes, platelets and the vessel wall during hemostasis and inflammation. **Thromb Haemost,** 86 746-756

McEver, R. P., Beckstead, J. H., Moore, K. L. et al (1989) GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. **J.Clin.Invest,** 84 (1): 92-99

McGeachy, M. J., Bak-Jensen, K. S., Chen, Y. et al (2007) TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. **Nat.Immunol.,** 8 (12): 1390-1397

McGeachy, M. J., Chen, Y., Tato, C. M. et al (2009) The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. **Nat.Immunol.,** 10 (3): 314-324

McNab, G. (1996) Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium.

Mehal, W. Z. (2003) Intrahepatic T cell survival versus death: which one prevails and why? **Journal of Hepatology,** 39 (6): 1070-1071

Mellado, M., Rodriguez-Frade, J. M., Manes, S. et al (2001) Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. **Annu.Rev.Immunol.,** 19:397-421. 397-421

Middleton, J., Neil, S., Wintle, J. et al (1997) Transcytosis and surface presentation of IL-8 by venular endothelial cells. **Cell,** 91 (3): 385-395

Middleton, J., Patterson, A. M., Gardner, L. et al (2002) Leukocyte extravasation: chemokine transport and presentation by the endothelium. **Blood,** 100 (12): 3853-3860

Milner, J. D., Brenchley, J. M., Laurence, A. et al (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. **Nature.,** 452 (7188): 773-776

Miossec, P., Korn, T. and Kuchroo, V. K. (2009) Interleukin-17 and type 17 helper T cells. **N.Engl.J.Med.,** 361 (9): 888-898

Misra, N. (2004) Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells.

Miyahara, Y., Odunsi, K., Chen, W. et al (2008) Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. **Proc.Natl.Acad.Sci.U.S.A.,** 105 (40): 15505-15510

Miyara, M. and Sakaguchi, S. (2007a) Natural regulatory T cells: mechanisms of suppression. **Trends Mol.Med.,** 13 (3): 108-116

Miyara, M. and Sakaguchi, S. (2007b) Natural regulatory T cells: mechanisms of suppression. **Trends Mol.Med.,** 13 (3): 108-116

Miyasaka, M. and Tanaka, T. (2004) Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. **Nat.Rev.Immunol.,** 4 (5): 360-370

Mondelli, M. U. (2003) Multifaceted functions of B cells in chronic hepatitis C virus infection. **Antiviral Res.,** 60 (2): 111-115

Mora, J. R. (2003) Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells.

Mora, J. R. (2004) Retinoic Acid; an educational "vitamin elixir" for gut-seeking T cells.

Moreno, C., Gustot, T., Nicaise, C. et al (2005) CCR5 deficiency exacerbates T-cell-mediated hepatitis in mice. **Hepatology,** 42 (4): 854-862

Morland, C. M., Fear, J., Joplin, R. et al (1996) Human biliary epithelial-cells express chemokines interleukin-8 and monocyte chemotactic protein-1 in response to inflammatory cytokine stimulation. **Hepatology,** 24 p. 823

Morland, C. M., Fear, J., Joplin, R. et al (1997a) Inflammatory cytokines stimulate human biliary epithelial cells to express interleukin-8 and monocyte chemotactic protein-1. **Biochem.Soc.Trans.,** 25 (2): p. 232S

Morland, C. M., Fear, J., McNab, G. et al (1997b) Promotion of leukocyte transendothelial cell migration by chemokines derived from human biliary epithelial cells in vitro. **Proc.Assoc.Am.Physicians,** 109 (4): 372-382

Moseley, T. A., Haudenschild, D. R., Rose, L. et al (2003) Interleukin-17 family and IL-17 receptors. **Cytokine Growth Factor Rev.,** 14 (2): 155-174

Moseman, E. A., Liang, X., Dawson, A. J. et al (2004) Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. **The Journal of Immunology,** 173 (7): 4433-4442

Moser, B. and Loetscher, P. (2001) Lymphocyte traffic control by chemokines. **Nat Immunol,** 2 (2): 123-128

Mosmann, T. R., Cherwinski, H., Bond, M. W. et al (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. **J.Immunol.,** 136 (7): 2348-2357

Mosmann, T. R., Schumacher, J. H., Fiorentino, D. F. et al (1990) Isolation of monoclonal antibodies specific for IL-4, IL- 5, IL-6, and a new Th2-specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay. **J.Immunol.,** 145 2938-2945

Mossman, T., Cherwinski, H., Bond, M. W. et al (1988) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. **J.Immunol.,** 136 2348-2357

Mucida, D., Park, Y., Kim, G. et al (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. **Science,** 317 (5835): 256-260

Muller, M., Carter, S. L., Hofer, M. J. et al (2007) CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. **J.Immunol.,** 179 (5): 2774-2786

Muller, W. A. and Randolph, G. J. (1999) Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes. **J Leukoc.Biol.,** 66 (5): 698-704

Munn, D. H. (2002) Tolerogenic antigen-presenting cells. **Ann.N.Y.Acad.Sci.,** 961 343-345

Munn, D. H., Sharma, M. D., Hou, D. et al (2004) Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. **J Clin Invest,** 114 (2): 280-290

Munn, D. H., Sharma, M. D., Lee, J. R. et al (2002) Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. **Science,** 297 (5588): 1867-1870

Murai, M., Yoneyama, H., Ezaki, T. et al (2003) Peyer's patch is the essential site in initiating murine acute and lethal graft-versus-host reaction. **Nat.Immunol.,** 4 (2): 154-160

Murai, M., Yoneyama, H., Harada, A. et al (1999) Active participation of CCR5(+)CD8(+) T lymphocytes in the pathogenesis of liver injury in graft-versus-host disease. **J.Clin.Invest,** 104 (1): 49-57

Murphy, C. A., Langrish, C. L., Chen, Y. et al (2003) Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. **J.Exp.Med.,** 198 (12): 1951-1957

Nagamine, K. (1997) Positional cloning of the APECED gene.

Nakache, M., Berg, E. L., Streeter, P. R. et al (1989) The mucosal vascular addressin is a tissue-specific endothelial cell adhesion molecule for circulating lymphocytes. **Nature,** 337 179-181

Napoli, J., Bishop, G. A., McGuinness, P. H. et al (1996) Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. **Hepatology,** 24 (4): 759-765

Narumi, S., Tominaga, Y., Tamaru, M. et al (1997) Expression of IFN-inducible protein-10 in chronic hepatitis. **The Journal of Immunology,** 158 (11): 5536-5544

Nattermann, J., Sherzada, R., Iwan, A. et al (2008) Hepatitis C Virus-Induced Secretion of Inflammatory Chemokines Preferentially Recruits NKG2A(+)CD8(+) T Cells. **J.Infect.Dis,** 198 (2): 213-217

Nelson, D. R., Tu, Z., Soldevila-Pico, C. et al (2003) Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. **Hepatology.,** 38 (4): 859-868

Ng, W. F., Duggan, P. J., Ponchel, F. et al (2001) Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. **Blood,** 98 (9): 2736-2744

Ni, C. N., MacConmara, M., Zang, Y. et al (2006) Enhanced regulatory T cell activity is an element of the host response to injury. **J.Immunol.,** 176 (1): 225-236

Nibbs, R., Graham, G. and Rot, A. (2003) Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6. **Seminars in Immunology,** 15 (5): 287-294

Nischalke, H. D., Nattermann, J., Fischer, H. P. et al (2004) Semiquantitative analysis of intrahepatic CC-chemokine mRNas in chronic hepatitis C. **Mediators.Inflamm.,** 13 (5-6): 357-359

Nishioji, K., Okanoue, T., Itoh, Y. et al (2001) Increase of chemokine interferon-inducible protein-10 (IP-10) in the serum of patients with autoimmune liver diseases and increase of its mRNA expression in hepatocytes. **Clin Exp Immunol,** 123 (2): 271-279

Nishizuka, Y. and Sakakura, T. (1969) Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. **Science,** 166 (906): 753-5

Nistala, K., Moncrieffe, H., Newton, K. R. et al (2008) Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. **Arthritis and Rheumatism,** 58 (3): 875-887

Northfield, J. W., Kasprowicz, V., Lucas, M. et al (2008) CD161 expression on hepatitis C virus-specific CD8+ T cells suggests a distinct pathway of T cell differentiation. **Hepatology,** 47 (2): 396-406

Novak, T. J. and Rothenberg, E. V. (1990) cAMP inhibits induction of interleukin 2 but not of interleukin 4 in T cells. **Proc.Natl.Acad.Sci.USA,** 87 9353-9357

Nurieva, R., Yang, X. O., Martinez, G. et al (2007) Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. **Nature.,** 448 (7152): 480-483

Nurieva, R. I., Chung, Y., Hwang, D. et al (2008) Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. **Immunity.,** 29 (1): 138-149

Nurieva, R. I., Chung, Y., Martinez, G. J. et al (2009) Bcl6 mediates the development of T follicular helper cells. **Science.,** 325 (5943): 1001-1005

Nurieva, R. I. and Dong, C. (2008) Keeping autoimmunity in check: how to control a Th17 cell controller. **Immunity.,** 29 (6): 841-843

O'Connor, R. A., Malpass, K. H. and Anderton, S. M. (2007) The inflamed central nervous system drives the activation and rapid proliferation of Foxp3+ regulatory T cells. **J.Immunol.,** 179 (2): 958-966

O'Gorman, W. E., Dooms, H., Thorne, S. H. et al (2009) The initial phase of an immune response functions to activate regulatory T cells. **J.Immunol.,** 183 (1): 332-339

Onishi, Y., Fehervari, Z., Yamaguchi, T. et al (2008) Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. **Proc.Natl.Acad.Sci.U.S.A.,** 105 (29): 10113-10118

Ono, M., Yaguchi, H., Ohkura, N. et al (2007) Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. **Nature,** 446 (7136): 685-689

Oo, Y. H. and Adams, D. H. (2009) The role of chemokines in the recruitment of lymphocytes to the liver. **J.Autoimmun.,**

Oo, Y. H., Lalor, P. F., Withers, G. D., Hubscher, S. G. and Adams, D. H. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. Journal of Immuology . 2010.
Ref Type: In Press

Oppmann, B., Lesley, R., Blom, B. et al) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. **Immunity.2000.Nov.;13.(5.):715.-25.,** 13 (5): 715-725

Pan, J., Kunkel, E. J., Gosslar, U. et al (2000) A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. **J.Immunol.,** 165 (6): 2943-2949

Pandiyan, P., Zheng, L., Ishihara, S. et al (2007) CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. **Nat.Immunol.,** 8 (12): 1353-1362

Papp, K. A., Langley, R. G., Lebwohl, M. et al (2008) Efficacy and safety of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with psoriasis: 52-week results from a randomised, double-blind, placebo-controlled trial (PHOENIX 2). **Lancet.,** 371 (9625): 1675-1684

Park, H., Li, Z., Yang, X. O. et al (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. **Nat.Immunol.,** 6 (11): 1133-1141

Parrish-Novak, J., Dillon, S. R., Nelson, A. et al (2000) Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. **Nature.,** 408 (6808): 57-63

Pasare, C. and Medzhitov, R. (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. **Science,** 299 (5609): 1033-1036

Paust, S., Lu, L., McCarty, N. et al (2004) Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. **Proc.Natl.Acad.Sci.U.S.A,** 101 (28): 10398-10403

Pene, J., Chevalier, S., Preisser, L. et al (2008) Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. **J.Immunol.,** 180 (11): 7423-7430

Penhale, W. J., Farmer, A., McKenna, R. P. et al (1973) Spontaneous thyroiditis in thymectomized and irradiated Wistar rats. **Clin.Exp.Immunol.,** 15 (2): 225-236

Perez, V. L., Van Parijs, L., Biuckians, A. et al (1997) Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. **Immunity.,** 6 (4): 411-417

Picker, L. J., Kishimoto, T. K., Smith, C. W. et al (1991) ELAM-1 is an adhesion molecule for skin-homing T cells. **Nature,** 349 796-799

Pickert, G., Neufert, C., Leppkes, M. et al (2009) STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. **J.Exp.Med.,** 206 (7): 1465-1472

Piskin, G., Sylva-Steenland, R. M., Bos, J. D. et al (2006) In vitro and in situ expression of IL-23 by keratinocytes in healthy skin and psoriasis lesions: enhanced expression in psoriatic skin. **J.Immunol.,** 176 (3): 1908-1915

Probst, H. C., McCoy, K., Okazaki, T. et al (2005) Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. **Nat.Immunol.,** 6 (3): 280-286

Qin, S., Rottman, J. B., Myers, P. et al (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. **Journal of Clinical Investigation,** 101 746-754

Quintana, F. J., Basso, A. S., Iglesias, A. H. et al (2008) Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. **Nature.,** 453 (7191): 65-71

Radaeva, S. (2004) Interleukin 22 (IL-22) plays a protective role in T cell-mediated murine hepatitis: IL-22 is a survival factor for hepatocytes via STAT3 activation.

Roncarolo, M. G., Gregori, S., Battaglia, M. et al (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. **Immunol Rev,** 212 28-50

Rosen, S. D. (1985) Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation.

Rosen, S. D. (2004) Ligands for L-selectin: homing, inflammation, and beyond.

Rosenkranz, A. R. and Mayadas, T. N. (1999) Leukocyte-Endothelial Cell Interactions - Lessons from Knockout Mice. **Exp Nephrol,** 7 (2): 125-136

Rot, A. (2004) Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells.

Ruprecht, C. R., Gattorno, M., Ferlito, F. et al (2005a) Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. **J Exp Med,** 201 (11): 1793-803

Ruprecht, C. R., Gattorno, M., Ferlito, F. et al (2005b) Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. **J.Exp.Med.,** 201 (11): 1793-1803

Rushbrook, S. M., Hoare, M. and Alexander, G. J. (2007) T-regulatory lymphocytes and chronic viral hepatitis. **Expert.Opin.Biol.Ther.,** 7 (11): 1689-1703

Rushbrook, S. M., Ward, S. M., Unitt, E. et al (2005) Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. **J.Virol.,** 79 (12): 7852-7859

Sakaguchi, S. (2004) Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. **Annu.Rev Immunol,** 22 531-562

Sakaguchi, S. (2005) Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. **Nat.Immunol.,** 6 (4): 345-352

Sakaguchi, S. (2008) Regulatory T cells and immune tolerance.

Sakaguchi, S., Ono, M., Setoguchi, R. et al (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. **Immunol.Rev.,** 212 8-27

Sakaguchi, S. and Powrie, F. (2007) Emerging challenges in regulatory T cell function and biology. **Science,** 317 (5838): 627-9

Sakaguchi, S., Sakaguchi, N., Asano, M. et al (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. **J.Immunol.,** 155 (3): 1151-1164

Sallusto, F. (2000) The role of chemokine receptors in primary, effector, and memory immune responses.

Salmi, M. (2004) CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium.

Samy, E. T., Parker, L. A., Sharp, C. P. et al (2005) Continuous control of autoimmune disease by antigen-dependent polyclonal CD4+CD25+ regulatory T cells in the regional lymph node. **J.Exp.Med.,** 202 (6): 771-781

Sansom, D. M. (2006) The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology.

Sansom, D. M. and Walker, L. S. (2006) The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. **Immunol.Rev.,** 212:131-48. 131-148

Santodomingo-Garzon, T. (2008) Natural killer T cells regulate the homing of chemokine CXC receptor 3-positive regulatory T cells to the liver in mice.

Sather, B. D., Treuting, P., Perdue, N. et al (2007) Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. **J.Exp.Med.,** 204 (6): 1335-1347

Sato, K., Suematsu, A., Okamoto, K. et al (2006) Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. **J.Exp.Med.,** 203 (12): 2673-2682

Scheinecker, C., McHugh, R., Shevach, E. M. et al (2002) Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. **J.Exp.Med.,** 196 (8): 1079-1090

Schneider, M. A., Meingassner, J. G., Lipp, M. et al (2007) CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. **J.Exp.Med.,** 204 (4): 735-745

Schorle, H., Holtschke, T., Hunig, T. et al (1991) Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. **Nature,** 352 621-624

Schutyser, E., Struyf, S., Menten, P. et al (2000) Regulated production and molecular diversity of human liver and activation-regulated Chemokine/Macrophage inflammatory protein-3alpha from normal and transformed cells [In Process Citation]. **The Journal of Immunology,** 165 (8): 4470-4477

Schutyser, E., Struyf, S. and Van, D. J. (2003) The CC chemokine CCL20 and its receptor CCR6. **Cytokine Growth Factor Rev.,** 14 (5): 409-426

Seddiki, N., Santner-Nanan, B., Martinson, J. et al (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. **J.Exp.Med.,**

Setoguchi, R., Hori, S., Takahashi, T. et al (2005) Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. **J.Exp.Med.,** 201 (5): 723-735

Shaw, S. and Adams, D. H. The adhesion cascade of leukocyte binding to endothelium: relevance to transplantation biology. Transplantation Science 3, 166-167. 1993.
Ref Type: Journal (Full)

Sheth, K. and Bankey, P. (2001) The liver as an immune organ. **Curr.Opin.Crit Care.,** 7 (2): 99-104

Shetty, S. (2008) Lymphocyte recruitment to the liver: Molecular insights into the pathogenesis of liver injury and hepatitis.

Shevach, E. M. (2006) From vanilla to 28 flavors: multiple varieties of T regulatory cells. **Immunity,** 25 (2): 195-201

Shields, P. L. & Adams, D. H. (2002) "Chemokines and Chemokine Receptor Interactions and their Functions". In: O'Brien T. R. (eds.) **Chemokine Receptors and AIDS**. New York, United States of America: Marcel Dekker Inc. pp. 1-30.

Shields, P. L., Morland, C. M., Salmon, M. et al (1999) Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver. **J.Immunol.,** 163 (11): 6236-6243

Shimizu, J., Yamazaki, S., Takahashi, T. et al (2002) Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. **Nat.Immunol.,** 3 (2): 135-142

Shimizu, Y., Murata, H., Kashii, Y. et al (2001) CC-chemokine receptor 6 and its ligand macrophage inflammatory protein 3alpha might be involved in the amplification of local necroinflammatory response in the liver. **Hepatology,** 34 (2): 311-319

Shiratori, Y., Takada, H., Hikiba, Y. et al (1993) Production of chemotactic factor, interleukin-8, from hepatocytes exposed to ethanol. *Hepatology* 18, 1477-1482. **Hepatology,** 18 (1477): p. 1482

Siegelman, M. H., Cheng, I. C., Weissman, I. L. et al (1990) The mouse lymph node homing receptor is identical with the lymphocyte cell surface marker Ly-22: Role of the EGF domain in endothelial binding. **Cell,** 61 611-622

Singh, S. P., Zhang, H. H., Foley, J. F. et al (2008) Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. **J.Immunol.,** 180 (1): 214-221

Smedsrod, B., De Bleser, P. J., Braet, F. et al (1994) Cell biology of liver endothelial and Kupffer cells. **Gut,** 35 (11): 1509-1516

Springer, T. A. (1990) Adhesion receptors of the immune system. **Nature,** 346 (6283): 425-434

Springer, T. A. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. **Cell,** 76 (2): 301-314

Springer, T. A. (1995) Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. **Annu.Rev.Physiol.,** 57:827-72 827-872

Springer, T. A. and Wang, J. H. (2004) The three-dimensional structure of integrins and their ligands, and conformational regulation of cell adhesion. **Adv.Protein Chem.,** 68:29-63. 29-63

Steinhoff, G., Behrend, M., Schrader, B. et al (1993) Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia - lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2 and LFA-3. **American Journal of Pathology,** 142 481-488

Steinman, L. (2005) Blocking adhesion molecules as therapy for multiple sclerosis: natalizumab. **Nat.Rev.Drug Discov.,** 4 (6): 510-518

Steinman, L. (2007) A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. **Nat.Med.,** 13 (2): 139-145

Steinman, R. M. and Banchereau, J. (2007) Taking dendritic cells into medicine. **Nature.,** 449 (7161): 419-426

Steinman, R. M. and Young, J. W. (1991) Signals arising from antigen-presenting cells. **Current Opinions in Immunology,** 3 361-372

Steinmetz, M., Minard, K., Horvath, S. et al (1982) A molecular map of the immune response region from the major histocompatibility complex of the mouse. **Nature,** 300 35-42

Steinmetz, O. M., Turner, J. E., Paust, H. J. et al (2009) CXCR3 mediates renal Th1 and Th17 immune response in murine lupus nephritis. **J.Immunol.,** 183 (7): 4693-4704

Stoop, J. N., van der Molen, R. G., Baan, C. C. et al (2005) Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. **Hepatology.,** 41 (4): 771-778

Strauss, L. (2007) Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin.

Strauss, L., Bergmann, C., Szczepanski, M. et al (2007) A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. **Clin.Cancer Res.,** 13 (15 Pt 1): 4345-4354

Su, M. A. and Anderson, M. S. (2004) Aire: an update. **Current Opinions in Immunology,** 16 (6): 746-752

Su, M. A., Giang, K., Zumer, K. et al (2008) Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire. **J.Clin.Invest,** 118 (5): 1712-1726

Su, X., Ye, J., Hsueh, E. C. et al (2009) Tumor Microenvironments Direct the Recruitment and Expansion of Human Th17 Cells. **J.Immunol.,**

Suffia, I., Reckling, S. K., Salay, G. et al (2005) A role for CD103 in the retention of CD4+CD25+ Treg and control of Leishmania major infection. **J.Immunol.,** 174 (9): 5444-5455

Sugimoto, K., Ogawa, A., Mizoguchi, E. et al (2008a) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. **J.Clin.Invest.,** 118 (2): 534-544

Sugimoto, K., Ogawa, A., Mizoguchi, E. et al (2008b) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. **J.Clin.Invest.,** 118 (2): 534-544

Surh, C. D. and Sprent, J. (1994) T-cell apoptosis detected in situ during positive and negative selection in the thymus. **Nature.,** 372 (6501): 100-103

Suri-Payer, E., Amar, A. Z., Thornton, A. M. et al (1998) CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. **The Journal of Immunology,** 160 (3): 1212-1218

Szanya, V., Ermann, J., Taylor, C. et al (2002) The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. **J.Immunol.,** 169 (5): 2461-2465

Taga, T. and Kishimoto, T. (1997) Gp130 and the interleukin-6 family of cytokines. **Annu.Rev.Immunol.,** 15:797-819. 797-819

Takahashi, T., Kuniyasu, Y., Toda, M. et al (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. **Int Immunol,** 10 (12): 1969-80

Takahashi, T., Tagami, T., Yamazaki, S. et al) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. **J.Exp.Med.2000.Jul.17.;192.(2.):303.-10.,** 192 (2): 303-310

Tamaru, M., Nishioji, K., Kobayashi, Y. et al (2000) Liver-infiltrating T lymphocytes are attracted selectively by IFN-inducible protein-10. **Cytokine,** 12 (4): 299-308

Tamkun, J. W., DeSimone, D. W., Fonda, D. et al (1986) Structure of Integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. **Cell,** 46 271-282

Tanaka, Y., Adams, D. H., Hubscher, S. et al (1993a) T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. **Nature,** 361 (6407): 79-82

Tanaka, Y., Adams, D. H. and Shaw, S. (1993b) Regulation of leukocyte recruitment by proadhesive cytokines immobilized on endothelial proteoglycan. **Curr Top Microbiol Immunol,** 184 99-106

Tang, H. L. and Cyster, J. G. (1999) Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. **Science.,** 284 (5415): 819-822

Tang, Q. and Bluestone, J. A. (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. **Nat.Immunol.,** 9 (3): 239-244

Terada, R., Yamamoto, K., Hakoda, T. et al (2003) Stromal cell-derived factor-1 from biliary epithelial cells recruits CXCR4-positive cells: implications for inflammatory liver diseases. **Lab Invest,** 83 (5): 665-672

Thomson, A. W. (2010) Tolerogenic Dendritic Cells: All Present and Correct? **Am.J.Transplant.,**

Thomson, A. W. and Lu, L. (1999a) Are dendritic cells the key to liver transplant tolerance? **Immunology Today,** 20 (1): 27-32

Thomson, A. W. and Lu, L. (1999b) Dendritic cells as regulators of immune reactivity: implications for transplantation. **Transplantation,** 68 (1): 1-8

Thomson, A. W., Lu, L., Wan, Y. et al (1995) Identification of donor-derived dendritic cell progenitors in bone marrow of spontaneously tolerant liver allograft recipients. **Transplantation,** 60 (12): 1555-1559

Thornton, A. M. and Shevach, E. M. (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. **J.Exp.Med.,** 188 (2): 287-296

Tiegs, G. and Lohse, A. W. (2009) Immune tolerance: What is unique about the liver. **J.Autoimmun.,**

Togawa, H., Shinkai, S. and Mizutani, T. (2008) Induction of human UGT1A1 by bilirubin through AhR dependent pathway. **Drug Metab Lett.,** 2 (4): 231-237

Tone, Y., Furuuchi, K., Kojima, Y. et al (2008) Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. **Nat.Immunol.,** 9 (2): 194-202

Toy, D., Kugler, D., Wolfson, M. et al (2006) Cutting edge: interleukin 17 signals through a heteromeric receptor complex. **J.Immunol.,** 177 (1): 36-39

Tran, E. H., Prince, E. N. and Owens, T. (2000) IFN-gamma shapes immune invasion of the central nervous system via regulation of chemokines. **J.Immunol.,** 164 (5): 2759-2768

Uhlig, H. H., Coombes, J., Mottet, C. et al (2006) Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. **J.Immunol.,** 177 (9): 5852-5860

Uhrig, A., Banafsche, R., Kremer, M. et al (2005) Development and functional consequences of LPS tolerance in sinusoidal endothelial cells of the liver. **J.Leukoc.Biol.,** 77 (5): 626-633

Unitt, E., Rushbrook, S. M., Marshall, A. et al (2005) Compromised lymphocytes infiltrate hepatocellular carcinoma: the role of T-regulatory cells. **Hepatology,** 41 (4): 722-730

Uyttenhove, C., Simpson, R. J. and van, S. J. (1988) Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. **Proc.Natl.Acad.Sci.U.S.A.,** 85 (18): 6934-6938

Vaknin-Dembinsky, A., Balashov, K. and Weiner, H. L. (2006) IL-23 is increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. **J.Immunol.,** 176 (12): 7768-7774

Valiante, N. M., D'Andrea, A., Crotta, S. et al (2000) Life, activation and death of intrahepatic lymphocytes in chronic hepatitis C. **Immunol.Rev.,** 174:77-89 77-89

van Amelsfort, J. M., Jacobs, K. M., Bijlsma, J. W. et al (2004) CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. **Arthritis and Rheumatism,** 50 (9): 2775-2785

van den Berg, W. B. and Miossec, P. (2009) IL-17 as a future therapeutic target for rheumatoid arthritis. **Nat.Rev.Rheumatol.,** 5 (10): 549-553

van, S. J. (1990) Interleukin-6: an overview. **Annu.Rev.Immunol.,** 8:253-78. 253-278

van, S. J., Goethals, A., Renauld, J. C. et al (1989) Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). **J.Exp.Med.,** 169 (1): 363-368

Veldhoen, M., Hirota, K., Christensen, J. et al (2009) Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. **J.Exp.Med.,** 206 (1): 43-49

Veldhoen, M., Hirota, K., Westendorf, A. M. et al (2008a) The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. **Nature.,** 453 (7191): 106-109

Veldhoen, M., Hocking, R. J., Atkins, C. J. et al (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. **Immunity.,** 24 (2): 179-189

Veldhoen, M. and Stockinger, B. (2006a) TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. **Trends Immunol.,** 27 (8): 358-361

Veldhoen, M. and Stockinger, B. (2006b) TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. **Trends Immunol.,** 27 (8): 358-361

Veldhoen, M., Uyttenhove, C., van, S. J. et al (2008b) Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. **Nat.Immunol.,** 9 (12): 1341-1346

Vermeer, P. D., Harson, R., Einwalter, L. A. et al (2003) Interleukin-9 induces goblet cell hyperplasia during repair of human airway epithelia. **Am.J.Respir.Cell Mol.Biol.,** 28 (3): 286-295

Vieira, P. L., Christensen, J. R., Minaee, S. et al (2004) IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. **J.Immunol.,** 172 (10): 5986-5993

Viola, A. and Luster, A. D. (2008) Chemokines and their receptors: drug targets in immunity and inflammation. **Annu.Rev.Pharmacol.Toxicol.,** 48 171-197

Vogelzang, A., McGuire, H. M., Yu, D. et al (2008) A fundamental role for interleukin-21 in the generation of T follicular helper cells. **Immunity.,** 29 (1): 127-137

Volpe, E., Servant, N., Zollinger, R. et al (2008) A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. **Nat.Immunol.,** 9 (6): 650-657

Volpes, R., van den Oord, J. J. and Desmet, V. J. (1991) Memory T cells represent the predominant lymphocyte subset in acute and chronic liver inflammation. **Hepatology.,** 13 (5): 826-829

von Andrian, U. H. and Mackay, C. R. (2000) T-cell function and migration. Two sides of the same coin. **N.Engl.J.Med.,** 343 (14): 1020-1034

von Andrian, U. H. and Mempel, T. R. (2003) Homing and cellular traffic in lymph nodes. **Nat.Rev.Immunol.,** 3 (11): 867-878

von, B. H. (2005) Mechanisms of suppression by suppressor T cells. **Nat.Immunol.,** 6 (4): 338-344

Voo, K. S., Wang, Y. H., Santori, F. R. et al (2009) Identification of IL-17-producing FOXP3+ regulatory T cells in humans. **Proc.Natl.Acad.Sci.U.S.A.,** 106 (12): 4793-4798

Vulcano, M., Albanesi, C., Stoppacciaro, A. et al (2001) Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. **Eur.J.Immunol.,** 31 (3): 812-822

Wald, O., Pappo, O., Safadi, R. et al (2004) Involvement of the CXCL12/CXCR4 pathway in the advanced liver disease that is associated with hepatitis C virus or hepatitis B virus. **Eur.J.Immunol.,** 34 (4): 1164-1174

Walker, E., Leemhuis, T. and Roeder, W. (1988) Murine B lymphoma cell lines release functionally active interleukin 2 after stimulation with Staphylococcus aureus. **J.Immunol.,** 140 (3): 859-865

Walker, L. S. (2004) CD4+ CD25+ Treg: divide and rule?

Walker, L. S. and Abbas, A. K. (2002) The enemy within: keeping self-reactive T cells at bay in the periphery. **Nat.Rev.Immunol.,** 2 (1): 11-19

Walker, L. S., Chodos, A., Eggena, M. et al (2003) Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. **J Exp.Med,** 198 (2): 249-258

Walunas, T. L., Lenschow, D. J., Bakker, C. Y. et al (1994) CTLA-4 can function as a negative regulator of T cell activation. **Immunity.,** 1 (5): 405-413

Ward, S. M. (2007) Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection.

Warnock, R. A., Askari, S., Butcher, E. C. et al (1998) Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. **J.Exp.Med.,** 187 (2): 205-216

Warren, A. (2006) T lymphocytes interact with hepatocytes through fenestrations in murine liver sinusoidal endothelial cells.

Waterhouse, P., Penninger, J. M., Timms, E. et al (1995) Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. **Science,** 270 (5238): 985-988

Weaver, C. T., Harrington, L. E., Mangan, P. R. et al (2006) Th17: an effector CD4 T cell lineage with regulatory T cell ties. **Immunity,** 24 (6): 677-88

Westendorf, A. M., Templin, M., Geffers, R. et al (2005) CD4+ T cell mediated intestinal immunity: chronic inflammation versus immune regulation. **Gut,** 54 (1): 60-69

Wick, M. J., Leithauser, F. and Reimann, J. (2002) The hepatic immune system. **Crit Rev.Immunol.,** 22 (1): 47-103

Wilbanks, A., Zondlo, S. C., Murphy, K. et al (2001) Expression cloning of the strl33/bonzo/tymstr ligand reveals elements of cc, cxc, and cx3c chemokines. **The Journal of Immunology,** 166 (8): 5145-5154

Willerford, D. M., Chen, J., Ferry, J. A. et al (1995) Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. **Immunity,** 3 (4): 521-30

Wilson, N. J., Boniface, K., Chan, J. R. et al (2007) Development, cytokine profile and function of human interleukin 17-producing helper T cells. **Nat.Immunol.,** 8 (9): 950-957

Wing, K., Onishi, Y., Prieto-Martin, P. et al (2008) CTLA-4 control over Foxp3+ regulatory T cell function. **Science,** 322 (5899): 271-275

Winnock, M., Garcia, B. M., Lukomska, B. et al (1995) Human liver-associated lymphocytes: an overview. **J.Gastroenterol.Hepatol.,** 10 Suppl 1 p. S43-S46

Witowski, J., Pawlaczyk, K., Breborowicz, A. et al (2000) IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. **The Journal of Immunology,** 165 (10): 5814-5821

Wong, J., Johnston, B., Lee, S. S. et al (1997) A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. **J.Clin Invest,** 99 (11): 2782-2790

Wood, K. J. and Sakaguchi, S. (2003) Regulatory T cells in transplantation tolerance. **Nat.Rev.Immunol.,** 3 (3): 199-210

Wu, Y., Borde, M., Heissmeyer, V. et al (2006) FOXP3 controls regulatory T cell function through cooperation with NFAT. **Cell,** 126 (2): 375-87

Wysocki, C. A., Jiang, Q., Panoskaltsis-Mortari, A. et al (2005) Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. **Blood,** 106 (9): 3300-3307

Xiao, S., Jin, H., Korn, T. et al (2008) Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. **J.Immunol.,** 181 (4): 2277-2284

Xie, M. H., Aggarwal, S., Ho, W. H. et al (2000) Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R. **J.Biol.Chem.,** 275 (40): 31335-31339

Xu, D., Fu, J., Jin, L. et al (2006) Circulating and Liver Resident CD4+CD25+ Regulatory T Cells Actively Influence the Antiviral Immune Response and Disease Progression in Patients with Hepatitis B. **J.Immunol.,** 177 (1): 739-747

Xu, J., Grewal, I. S., Geba, G. P. et al (1996) Impaired primary T cell responses in L-selectin-deficient mice. **J.Exp.Med.,** 183 (2): 589-598

Xu, X. D. (2008) Trafficking of recirculating lymphocytes in the rat liver: rapid transmigration into the portal area and then to the hepatic lymph.

Yadav, R., Larbi, K. Y., Young, R. E. et al (2003) Migration of leukocytes through the vessel wall and beyond. **Thromb.Haemost.,** 90 (4): 598-606

Yagi, H., Nomura, T., NAKAMURA, K. et al (2004) Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. **Int.Immunol.,** 16 (11): 1643-1656

Yamazaki, S., Inaba, K., Tarbell, K. V. et al (2006a) Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. **Immunol Rev,** 212 314-29

Yamazaki, S., Patel, M., Harper, A. et al (2006b) Effective expansion of alloantigen-specific Foxp3+ CD25+ CD4+ regulatory T cells by dendritic cells during the mixed leukocyte reaction. **Proc.Natl.Acad.Sci.U.S.A,**

Yang, D., Howard, O. M., Chen, Q. et al (1999) Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-beta 1 express functional C-C chemokine receptor 6. **The Journal of Immunology,** 163 (4): 1737-1741

Yang, L., Anderson, D. E., Baecher-Allan, C. et al (2008a) IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. **Nature.,** 454 (7202): 350-352

Yang, X. D., Karin, N., Tisch, R. et al (1993) Inhibition of insulitis and prevention of diabetes in nonobese diabetic mice by blocking L-selectin and very late antigen 4 adhesion receptors. **Proc.Natl.Acad.Sci.U.S.A.,** 90 (22): 10494-10498

Yang, X. D., Michie, S. A., Tisch, R. et al (1994) A predominant role of integrin alpha 4 in the spontaneous development of autoimmune diabetes in nonobese diabetic mice. **Proc.Natl.Acad.Sci.U.S.A.,** 91 (26): 12604-12608

Yang, X. O., Nurieva, R., Martinez, G. J. et al (2008b) Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. **Immunity.,** 29 (1): 44-56

Yang, X. O., Pappu, B. P., Nurieva, R. et al (2008c) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. **Immunity.,** 28 (1): 29-39

Yao, Z., Fanslow, W. C., Seldin, M. F. et al (1995) Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. **Immunity,** 3 (6): 811-821

Yao, Z., Kanno, Y., Kerenyi, M. et al (2007) Nonredundant roles for Stat5a/b in directly regulating Foxp3. **Blood.,** 109 (10): 4368-4375

Ye, P., Rodriguez, F. H., Kanaly, S. et al (2001) Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. **J.Exp.Med.,** 194 (4): 519-527

Yednock, T. A., Cannon, C., Fritz, L. C. et al (1992) Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha4beta1 integrin. **Nature,** 356 63-66

Yen, D., Cheung, J., Scheerens, H. et al (2006) IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. **J.Clin.Invest.,** 116 (5): 1310-1316

Yen, H. R., Harris, T. J., Wada, S. et al (2009) Tc17 CD8 T Cells: Functional Plasticity and Subset Diversity. **J.Immunol.,**

Yoong, K. F., McNab, G., Hubscher, S. G. et al (1998) Vascular adhesion protein-1 and ICAM-1 support the adhesion of tumor-infiltrating lymphocytes to tumor endothelium in human hepatocellular carcinoma. **J.Immunol.,** 160 (8): 3978-3988

Yuan, Q. (2007) CCR4-dependent regulatory T cell function in inflammatory bowel disease.

Yuan, Q., Bromley, S. K., Means, T. K. et al (2007) CCR4-dependent regulatory T cell function in inflammatory bowel disease. **J.Exp.Med.,** 204 (6): 1327-1334

Yurchenko, E., Tritt, M., Hay, V. et al (2006) CCR5-dependent homing of naturally occurring CD4+ regulatory T cells to sites of Leishmania major infection favors pathogen persistence. **J Exp Med,** 203 (11): 2451-60

Zenewicz, L. A., Yancopoulos, G. D., Valenzuela, D. M. et al (2007) Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. **Immunity.,** 27 (4): 647-659

Zhang, F., Meng, G. and Strober, W. (2008) Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. **Nat.Immunol.,** 9 (11): 1297-1306

Zhang, G. X., Gran, B., Yu, S. et al (2003) Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. **J.Immunol.,** 170 (4): 2153-2160

Zhang, J. P., Yan, J., Xu, J. et al (2009a) Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. **Journal of Hepatology,** 50 (5): 980-989

Zhang, J. Y., Zhang, Z., Lin, F. et al (2009b) Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B. **Hepatology.,**

Zheng, S. G., Wang, J. and Horwitz, D. A. (2008) Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. **J.Immunol.,** 180 (11): 7112-7116

Zheng, Y., Danilenko, D. M., Valdez, P. et al (2007) Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. **Nature.,** 445 (7128): 648-651

Zhou, L., Ivanov, I. I., Spolski, R. et al (2007) IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. **Nat.Immunol.,** 8 (9): 967-974

Zhou, L., Lopes, J. E., Chong, M. M. et al (2008) TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. **Nature.,** 453 (7192): 236-240

Zhou, L., Peng, S., Duan, J. et al (1998) A human B cell line AF10 expressing HIL-17. **Biochem Mol Biol Int,** 45 (6): 1113-1119

Ziegler, S. F. (2005) FOXP3: Of Mice and Men. **Annu.Rev.Immunol.,**

Zlotnik, A. and Yoshie, O.) Chemokines: a new classification system and their role in immunity. **Immunity.2000.Feb.;12.(2.):121.-7.,** 12 (2): 121-127