THE ROLES AND CONTROL OF CD4+ EFFECTOR AND REGULATORY T CELLS IN BILIARY AUTOIMMUNITY

by

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Abstract

Autoimmunity represents a misdirection of the adaptive immune response against the self. The prevalence of human autoimmunity is increasing, including an increasing prevalence of autoimmune liver disease. Novel therapeutic options are required and failure of the regulation of CD4+ T cells has been implicated in pathogenesis. OX40, ligated by OX40L, is a secondary co-stimulatory molecule which promotes activation and survival of CD4+ T cells and has been proposed as a therapeutic target in autoimmunity and regulatory failure.

Complete deficiency in FOXP3-positive regulatory T cells results in a lymphocytic hepatitis associated with autoantibodies and loss of effector CD4+ T cell control. This thesis confirms this finding and demonstrates similar pathology in mice deficient in the regulatory molecule CTLA4 and in Roquin sanroque mutant mice. Each of these mouse models is shown to hyperexpress OX40 on liver-infiltrating CD4+ T cells. In FOXP3 deficiency, blockade of OX40-OX40L interactions abrogates hepatitis; whereas in CTLA4 deficiency and the Roquin sanroque mutant mouse, OX40-OX40L blockade is ineffective.

Complementary to these findings, increased expression of OX40 is demonstrated in human autoimmune liver disease, and a negative regulatory mechanism on the expression of OX40L by T cells by the action of gamma-chain cytokines including interleukin-2 is described.

Dedication

This thesis is dedicated to Michelle & Macsen

…grant me the serenity

to accept the things I cannot change;

the courage to change the things I can;

and the wisdom to know the difference.

after K.P. Reinhold Niebühr (1892-1971)

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Abbreviations

AIH autoimmune hepatitis

AILD autoimmune liver disease

AIRE autoimmune regulator

ALP alkaline phosphatase

ALT alanine aminotransferase

AMA anti-mitochondrial antibody

ANA anti-nuclear antibody

APC antigen-presenting cell

APECED autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy

AST aspartate aminotransferase

BEC biliary epithelial cell

BMSU Biomedical Services Unit

CCl_4 carbon tetrachloride

CFA Complete Freud's adjuvant

CFSE 5-carboxyfluorescein diacetate succinimidyl ester

CHO Chinese Hamster Ovary

Con A concanavalin A

CTLA4 cytotoxic T-lymphocyte associated protein 4

CTLA4Ig CTLA4 immunoglobulin

DAB 3,3'-diaminobenzidine

DAPI 4',6-diamidino-2-phenylindole

DC dendritic cell

dH_2O distilled water

DMSO dimethyl sulfoxide

EAE experimental allergic encephalomyelitis

EDTA ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

F_c fragment crystallisable region of antibody

F_{ab} antigen-binding region of antibody

FitC fluorescein isothiocyanate

FOXP3 forkhead box P3

FSC forward scatter

GFP green fluorescent protein

H&E haematoxylin and eosin

HCV hepatitis C virus

HLA human leucocyte antigen

HRP horseradish peroxidase

HTLV human T-lymphotropic virus

ICOS inducible T-cell costimulator

IDO indoleamine 2,3-dioxygenase

IFNγ interferon-gamma

IgA immunoglobulin A

IgE immunoglobulin E

IgG immunoglobulin G

IgM immunoglobulin M

IL1β interleukin-1 beta

IP intraperitoneal

IPEX immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

IV intravenous

LAG3 (the protein encoded by) lymphocyte-activation gene 3

LLN lower limit of normal

MHC major histocompatibility complex

MPA mycophenolic acid

mRNA messenger RNA

mTOR mechanistic target of rapamycin

NAFLD non-alcoholic fatty liver disease

NHS National Health Service

NK natural killer

NKT natural killer T

PBC primary biliary cholangitis (previously primary biliary cirrhosis)

PBS phosphate-buffered saline

PD-1 programmed death protein 1 (CD279)

PDC-E2 E2 subunit of the pyruvate dehydrogenase complex

PDL-1 programmed death protein ligand 1 (CD274)

PMA phorbol 12-myristate-13-acetate

PRR pattern recognition receptors

PSC primary sclerosing cholangitis

RAG recombination activating genes

Roquin$^{M/M}$ mice homozygous for the *sanroque* mutation

RORγ retinoic acid receptor-related orphan receptor gamma

RPMI Roswell Park Memorial Institute medium

SSC side scatter

STAT5 signal transducer and activator of transcription 5

STAT6 signal transducer and activator of transcription 6

TFH T follicular helper cell

TH2 T helper cell type 2

TH1 T helper cell type 1

TCR T-cell receptor

TCRα T-cell receptor alpha chain

TGFβ transforming growth factor-beta

TL1a TNF-like protein 1A

TLR Toll-like receptor

TMB 3,3',5,5'-tetramethylbenzidine

TNF tumour necrosis factor

TNFα tumour necrosis factor-alpha

Treg regulatory T cell

TSLP thymic stromal lymphopoietin

ULN upper limit of normal

WT wildtype

ZAP70 zeta-chain-associated protein kinase 70

1 Introduction

1.1 Immunity and autoimmunity

The immune system represents an interplay of multiple cell types and organ structures that serves to resist a variety of potential insults to the host organism. These insults are primarily microbial, and the immune system serves to prevent its host from being over-run by such invaders.[81] In addition, the immune system has functions in removing cancerous and pre-cancerous cells and in clearing dead and senescent matter from the host. Broadly, the immune system's response to a challenge may be split in two: those components that are, and those that are-not, affected by prior contact with the challenge concerned. These responses are termed *innate* and *adaptive* immunity respectively.

The innate component of the immune system represents a generic response to pathogens. The innate system recognises its triggers through a number of receptors that are pre-encoded in the germline and not subject to the random recombination of receptors and with positive- and negative-selection that characterise the receptors of cells of the adaptive immune response.[274] The cells of the immune system include phagocytes such as macrophages, dendritic cells and neutrophils, as well as natural killer cells, eosinophils, basophils and mast cells. Each of these express a variety of pattern-recognition receptors which respond to a specific component of a pathogen. The family of Toll-like receptors (TLRs) recognise various molecules including microbial glycoproteins, lipopolysaccharide and free double-stranded nucleic acids, whilst other distinct ligand-recognition domains, including leucine-rich repeats, C-type lectin domains and various nucleic acid binding domains triggering other innate responses.[165]

Activation of the initiating receptors of the innate immune response promotes a variety of mechanisms of host defence including complement activation, which targets pathogens for phagocytosis or lysis, release of directly anti-microbial cytokines, the extrusion of neutrophil extracellular traps, release of oxidative respiratory burst, the release of vasoactive peptides like histamine, promotes accumulation of other immune cells via chemokines and through induction of cell adhesion molecules; a further key component of the innate system is the expression of interferons and down-regulation

of surface major histocompatibility complex (MHC) protein by cells infected by viruses.[274, 188] Importantly, the activation of the innate immune system in turn influences the activation of the adaptive response: innate cells present processed antigen to T cells on MHC molecules, cytokines produced by the innate immune system focus the adaptive response by polarising and differentially activating T- and B cells, and by promoting the production of different antibody classes by B cells.[165]

Adaptive immunity

The adaptive immune response encompasses responses that vary depending on an organism's previous exposure to the pathogen or other antigen concerned. The adaptive response primarily concerns the functions of the two major groups of lymphocytes: T cells and B cells. There is induction of quantities of specific antibody by B cells which agglutinate pathogens, promote killing of invading cells by fixing complement or promote antibody-dependent cell-mediated cytotoxicity, promote phagocytosis, and neutralise some toxic chemicals. Equally, activation of T cells includes promotion of CD8+ or cytotoxic T cell killing of other cells, whilst CD4+ T cells control ongoing activation of CD8+ T cells, B cells, phagocytes including macrophages and the production of several systemically active cytokines.[296]

This chapter considers autoimmunity and autoimmune disease in general. It then considers the role of the liver as an important immune organ and then how the liver may be affected by autoimmunity. In two major autoimmune liver diseases, a role for dysregulated CD4+ T lymphocytes appears important. Different mechanisms of regulation of CD4+ T cells are discussed, especially with relation to the effects of disruption of such regulatory mechanisms with a specific focus on regulatory T cells. The manipulation of a tumour necrosis factor (TNF) receptor-ligand pair known as the OX40-OX40L pathway is then considered in relation to the regulation of T cell function, including as potential therapeutic target. Finally, consideration is given to mouse models with disturbances

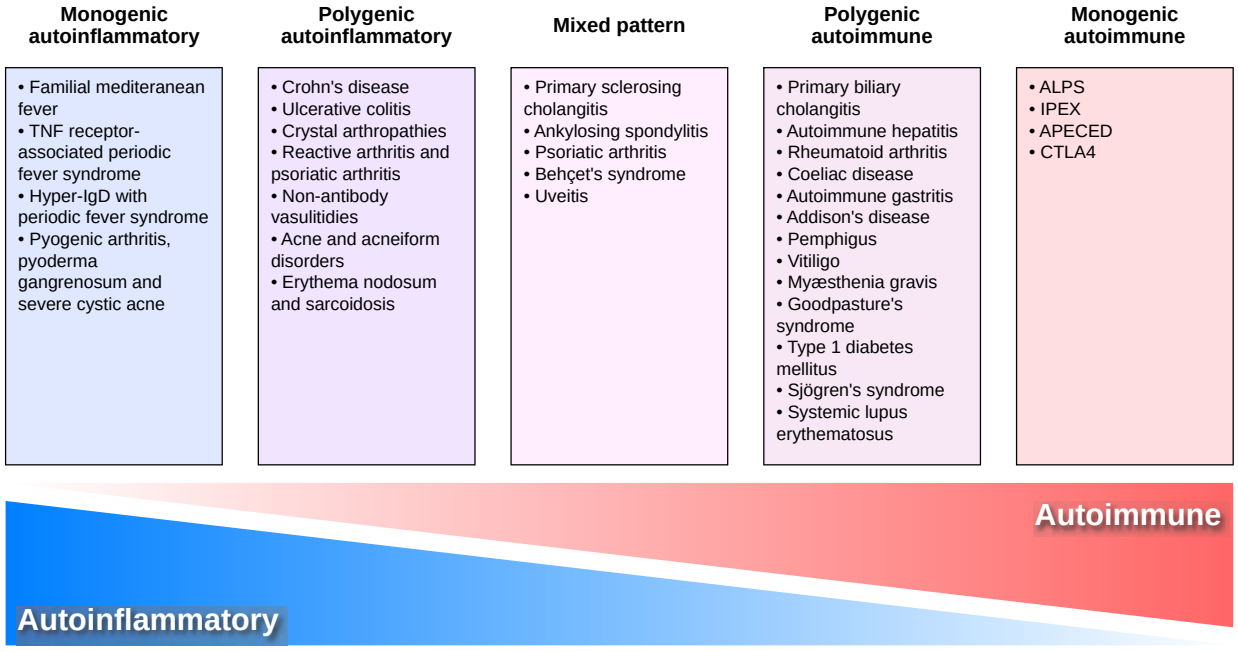

Figure 1.1: Autoimmunity and autoinflammation. A proposed classification of immune-mediated diseases into classical autoimmune conditions and atypical autoinflammatory conditions. Classical autoimmune conditions typically demonstrate autoantibodies, female preponderence, co-incidence with other autoimmune conditions, MHC class II variant risk associations and evidence of an adaptive immune response to self-antigens. Figure adapted from McGonagle & McDermott (2006).[272]

of immune control and that develop hepatitis. These serve as useful systems for exploration of the effects of OX40-OX40L blockade.

Autoimmunity

Autoimmunity may be described as the combination of an adaptive response directed against self tissue with tissue damage or disruption of other physiological processes.[272, 247] More specifically, there is typically a clinical syndrome of single or multi-organ damage or dysfunction caused by the activation of T or B lymphocytes, or both, in the absence of an ongoing infection or other ongoing discernible cause.[79] Typically, this autoimmune adaptive response is defined by the development of autoantibodies: where an antibody's specificity is directed against a self-antigen. Although individuals may develop inflammation that is damaging to self tissues, it is the specificity of the response

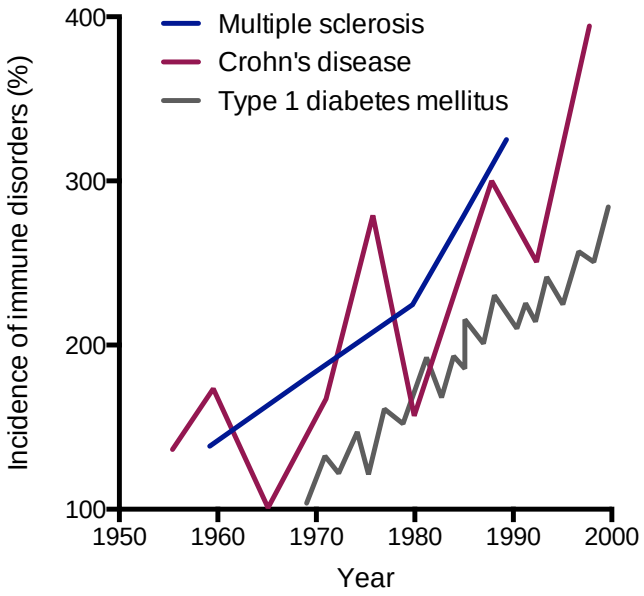

Figure 1.2: Increasing prevalence of autoimmunity worldwide. Figure redrawn from Bach (2002).[12]

that differentiates autoimmunity from simple autoinflammation. Whilst autoinflammation may induce the presence of autoantibodies as a side effect of an uncontrolled innate-like response, in true autoimmune disease there is typically autoantibody production prior to disease development, a female preponderance, genetic association with specific variants in MHCII molecules, and specific increased occurences in the development of autoimmunity in other organ systems (Figure 1.1). The spectrum of severity of autoimmunity ranges from the development of the harmless autoantibodies that may occur in up to 25% of the healthy population to lethal multi-organ disease.[316]

Worldwide, the prevalence of autoimmune conditions is increasing with current estimates suggesting that up to 10% of individuals are affected by an autoimmune disease.[65, 64] Where there are longer-term data available, the prevalence of many autoimmune conditions has increased markedly over the last half-century (Figure 1.2). In addition, a pre-disposition to autoimmunity appears to represent a systemic, rather than organ-specific, deficit with the development of one autoimmune disease predisposing to the development of a second or more.[390, 391] The examination of autoimmunity affecting the liver as part of more generalised autoimmunity is the focus of this thesis.

The liver

The liver functions as both a metabolic and lymphoid organ and is the largest internal organ of the human body. It typically accounts for around 2% of body weight, with this proportion being consistent across mammalian species.[37] Derived from endoderm in early embryogenesis and occupying the superior-right portion of the intra-peritoneal space, the liver receives three-quarters of its blood supply as venous blood from the gastrointestinal tract and spleen via the portal vein. The remainder of its blood supply is arterial from the abdominal aorta via the hepatic artery, and together these two components account for 25% of cardiac output. The portal vein and hepatic artery enter the liver together at the porta hepatis where they branch into smaller venules and arterioles before draining into fenestrated and ultimately discontinuous sinusoidal capillaries. Here blood flow is slow and in direct contact with the major functional cell type of the liver, the hepatocyte. Such slow-flowing direct contact facilitates several of the myriad functions of the liver. The sinusoidal capillaries then drain back into venules that coalesce to form the hepatic vein which in turn drains back into the inferior vena cava (Figure 1.3).[230, 45, 259]

The liver has critical homeostatic functions in glucose metabolism, protein synthesis, maintenance of coagulation function, lipid metabolism, the breakdown and excretion of both endogenous waste and drugs, endocrine control of blood pressure, and, in early life, it is the major haematopoietic organ.[458, 70, 45]. Most of these metabolic functions are performed by hepatocytes with biliary epithelial cells (BECs) providing some excretory function. Liver failure represents the loss of these and other functions and presents with jaundice through the disordered metabolism of bilirubin, coagulopathy through alterations in the production of clotting factors, hypoglycaemia through impaired glucagon metabolism, and encephalopathy through impaired metabolism of nitrogenous compounds originating in the gut. Uncorrected, liver failure is lethal and necessitates transplantation to avoid death.[501]

In addition to its major metabolic functions, the liver represents a key component of the immune

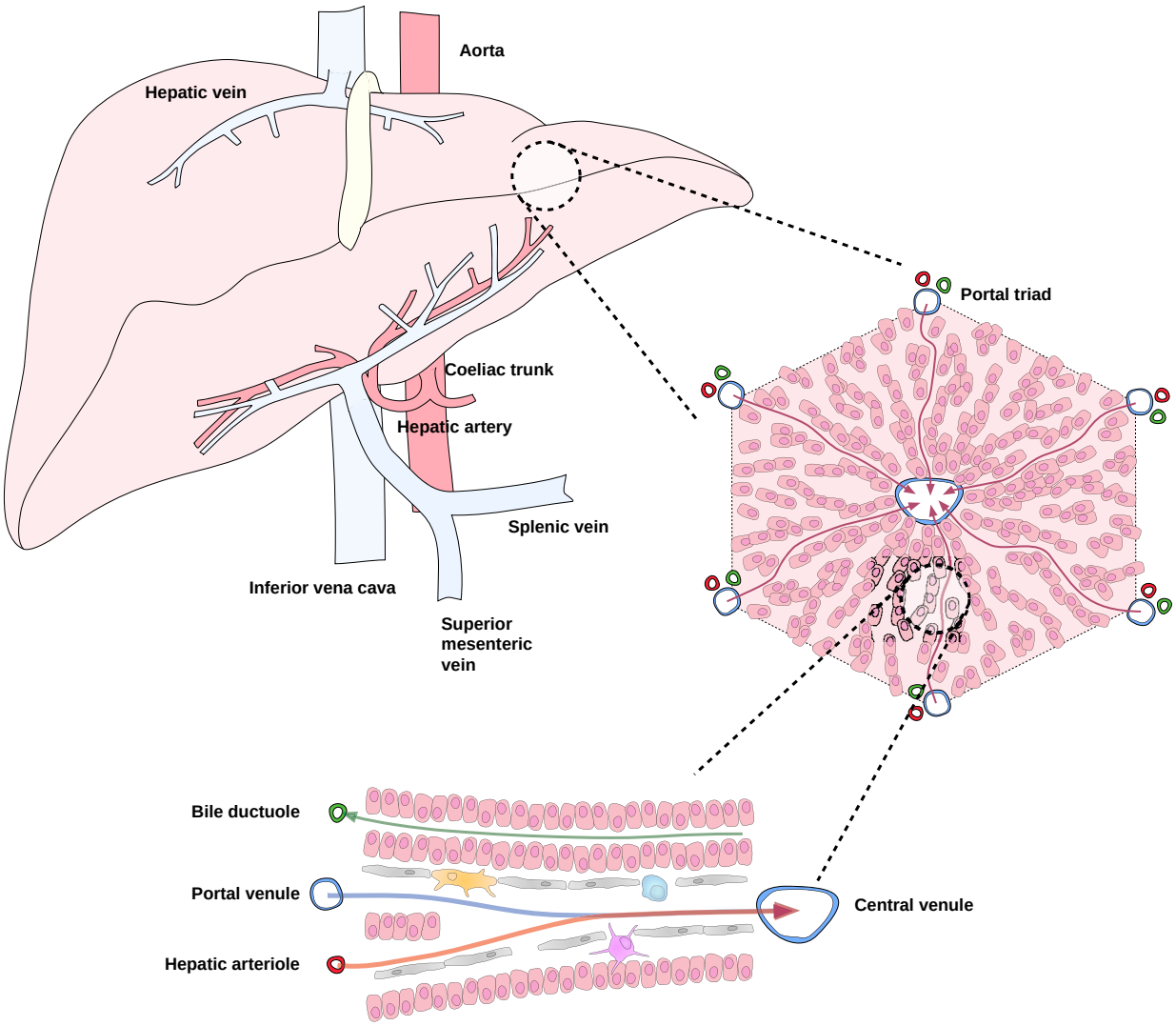

Figure 1.3: Schematic diagram of the functional anatomy of the liver. The liver receives the majority of its blood supply from the portal vein which is formed by the confluence of the superior mesenteric and splenic veins. The superior mesenteric vein receives blood from much of the gastrointestinal tract and the blood it contains is rich in antigen. The liver also receives arterial blood via the hepatic artery. Blood from both sources feeds the liver through progressively smaller venules and arterioles. Together with bile ductuoles, these vessels form the portal triad and area arranged in a roughly hexagonal pattern around central hepatic venules. Blood travels from the portal triad vessels to the central vein by way of fenestrated capillaries. These fenestrations permit exposure of liver resident immune cells and hepatocyte to bloodborne antigen and permits interaction of circulating immune cells with other cells through fenestrations.

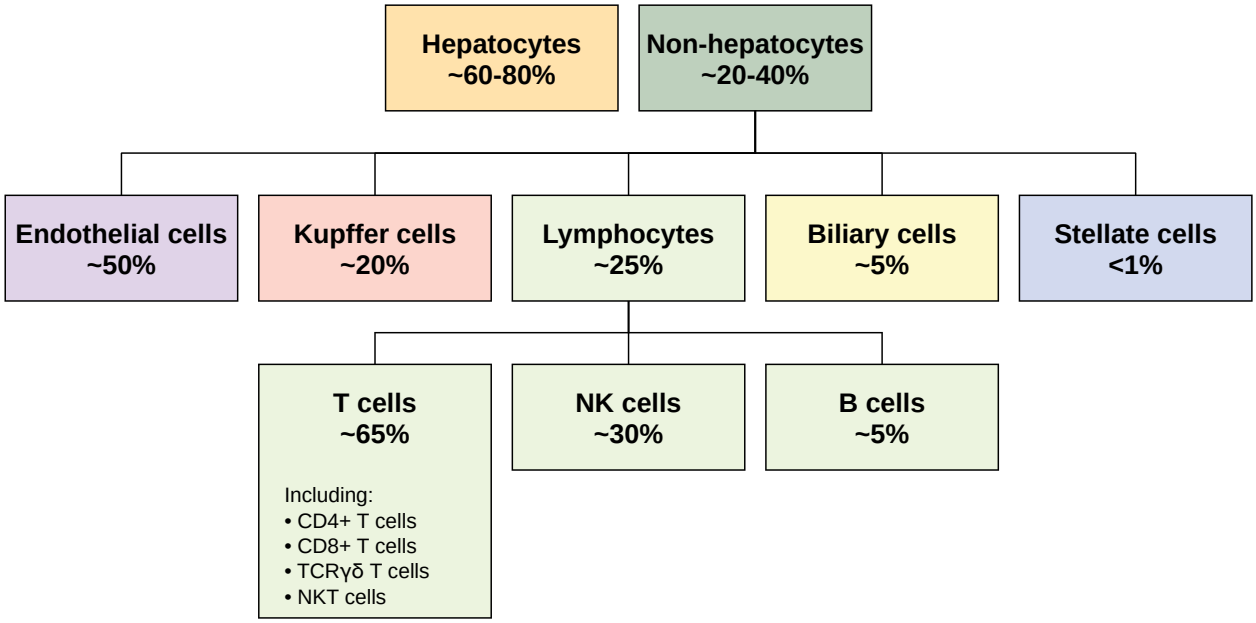

Figure 1.4: Approximate immune cell composition of the human liver. Adapted from Racanelli and Rehermann 2006.[347]

system. The liver's unique vasculature interfaces slow-flowing blood with a large cell surface area. Whilst this facilitates metabolic exchange, it also permits interaction with antigen by the large liver-resident population of immune cells (Figure 1.4); blood in the portal vein, by nature of its contact with the gastrointestinal tract, is particularly antigen-rich.[347] The liver contains large numbers of intravascular macrophages – known as Kupffer cells – and these are seen lining the sinusoids. Lymphocytes are also frequent in the sinusoidal lumen.[206]

Relative to other immune-cell rich structures, the function of the liver is tolerogenic.[173] A number of elegant experiments have highlighted this tolerogenic effect: in early transplant studies performed in pigs, co-transplantation of livers reduce immune-mediated rejection of kidney grafts[48] and such work correlates with the reduced immunosuppressive requirements of human combined liver-kidney recipients as compared to those who receive livers alone, especially those who receive both organs from the same donor.[382] In addition, the liver is unique among solid organs in that it can be transplanted across major MHC mismatches[303] and in that a significant proportion of individuals immunosuppression may successfully be completely withdrawn.[84] Animal studies have

demonstrated an ability to induce tolerance through oral or portal venous introduction of antigen, but not through the liver-bypassing systemic venous route.[49] Some of this may be through the induction of antigen specific regulatory T cells (Tregs).[51] In addition, hepatic Kupffer cells are known to constitutively express molecules with immunoregulatory properties such as programmed death protein ligand 1 (CD274) (PDL-1) at high levels and also to tend to release immunosuppressive IL-10 in response to the usually pro-inflammatory TLR ligands such as lipopolysaccharide ligating TLR4.[140]. A similar phenomenon is seen with hepatocytes and sinusoidal epithelial cells, which also express multiple TLRs. The liver is also a location where chronic infections such as schistosomiasis, malaria, and viral hepatitis may persist [486, 56, 71].

Despite its tolerogenic potential, adaptive immune responses are well documented within the liver. For example, specific antibodies are generated to viral antigens in hepatitis and to bile-salt exporter pump proteins in patients transplanted because of insufficiency of the functional protein.[168, 220, 56] Elegant experiments exploiting T-cell receptor (TCR)-transgenic mice and liver transplants between mice have shown that CD8+ T cells may be primed and activated entirely within the liver (i.e. without the requirement for migration through secondary lymphoid tissue)[202, 83] and that the same is true of CD4+ T cells for at least some antigens.[413]

Liver autoimmunity and its clinical features

In addition to the ability of the immune system to generate adaptive responses to exogenous antigens within the liver, several autoimmune conditions may affect the liver. The three most frequent autoimmune diseases affecting the liver comprise PBC, AIH and primary sclerosing cholangitis (PSC). There is some overlap in clinical and pathological features between the three diseases (Figure 1.5).

Both PBC and AIH have been described as 'archetypal' autoimmune diseases.[143, 435, 319]. Both demonstrate a female preponderance, frequent autoantibodies, and strong associations with a vari-

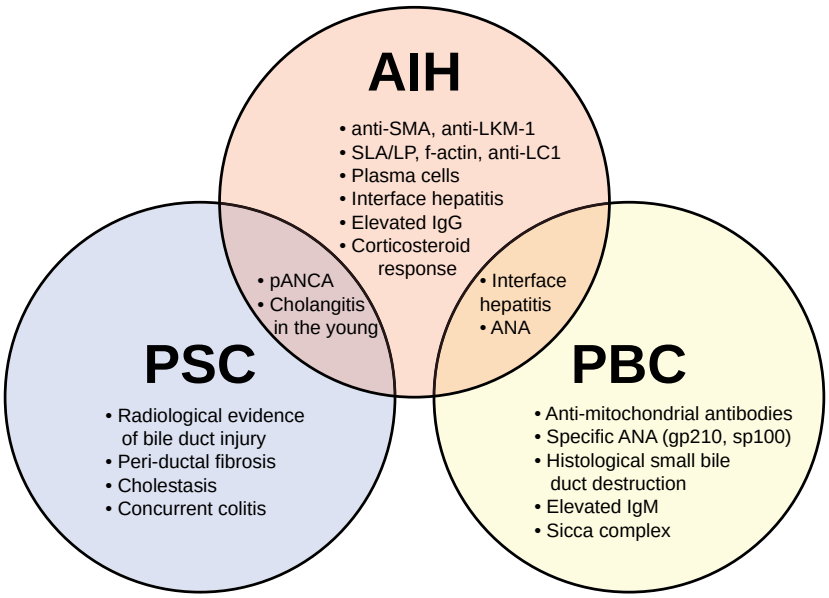

Figure 1.5: Overlapping features in autoimmune liver diseases. Some features of autoimmune liver disease are shared between subtypes. autoimmune hepatitis (AIH) and primary biliary cholangitis (previously primary biliary cirrhosis) (PBC) share the production of anti-nuclear antibody (ANA), polyclonal elevations in immunoglobulins, and the development of histological hepatitis. Figure adapted from Webb et al.[470]

ety of other autoimmune conditions. There is however less consensus that PSC is as typical – it is male-predominant, its associations with autoantibodies are less strong, it is associated with a markedly increased risk of malignancy, and no defined target antigen has been found. It is also strongly associated with inflammatory bowel disease, the ongoing presence of which may affect its development.[432] This last has led some to speculate that PSC represents a response to dysbiosis or another autoinflammatory process rather than a true autoimmune phenomenon.[359] However, a shared genetic architecture with other autoimmune conditions and increased risk of concurrent autoimmunity in other organ systems have led others to support its classification as an autoimmune condition.[176] The remainder of this work considers PBC and AIH, both of which have markedly rising prevalences.

Clinical features of primary biliary cholangitis

PBC represents a chronic immune-mediated destruction of biliary epithelial cells of the small intrahepatic bile ducts.[145] Over time, there is a variable inflammatory infiltrate, the loss of bile ductules, and cholestasis leading to progressive fibrosis over years and ultimately cirrhosis with liver failure and complications of portal hypertension.[53] During this process, cholestasis eventually causes clinically-evident obstructive jaundice, with resultant malabsorption. PBC has a peak incidence at the age of approximately 55 years, a 5:1 to 10:1 female preponderance and a current UK prevalence of approximately 35 per 100,000 persons/year.[52, 466] The disease may present with pruritus, fatigue, pain in the upper abdomen, symptoms and signs of liver failure, or, in a significant proportion of cases, be asymptomatic. Patients may have xanthelasma and/or xanthomata from the hyperlipidaemia that is often present in PBC, there may be hepatomegaly resulting from cholestasis. The cause of the condition is unknown although there are environmental associations with smoking, nail varnish and hair dye exposure, and deprivation.[154, 110, 66, 342, 273] These is marked concordance between monozygotic twins and a number of disease risk-modifying genetic variants have been identified.[473]

PBC is strongly associated with the development of autoantibodies. In over 90% of cases, antibodies directed against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) are detectable and are commonly referred to as anti-mitochondrial antibody (AMA). The majority of patients that do not develop AMAs develop specific ANA.[144] In common with other autoimmune diseases, autoantibodies develop before the onset of clinical disease and predict disease development in unaffected patients.[255]

Other laboratory analyses in PBC typically record an elevation in serum alkaline phosphatase (ALP) activity. In humans, this enzyme is particularly concentrated in the biliary system and is released into the serum by biliary damage. Similarly, the transaminase enzyme alanine aminotransferase (ALT) is concentrated in hepatocytes and its serum activity is typically elevated when there is damage to

hepatocytes. In PBC, elevations in ALT activity are frequent. In addition, for unknown reasons, serum total IgM levels are often increased in patients with PBC.

PBC is generally not responsive to immunosuppressants, but its course is slowed by the drug ursodeoxycholic acid. A proportion of patients require liver transplantation to avoid death from liver failure or to treat intractable pruritus. Recurrence of PBC is well-recognised post-transplantation, and may require repeat transplantation[306, 57]; high titre AMAs typically persist after transplantation highlighting that they are more than an epiphenomenon.

Clinical features of autoimmune hepatitis

AIH represents an adaptive immune response directed against hepatocytes. If uncontrolled, it results in an insufficient number of surviving hepatocytes to provide homeostatic functions and clinically evident liver failure results. AIH may be rapid in onset or more indolent: acute cases may result in liver failure in days, whilst chronic cases progress through increasing fibrosis before established cirrhosis and chronic liver failure. Although most cases respond to treatment with immunosuppressive medications – predominantly corticosteroids, thiopurine antimetabolites, calcineurin inhibitors and mycophenolic acid – a minority of cases are progressive despite therapy and ultimately require liver transplantation.[238, 67]

AIH has a female preponderance of some 3:1 to 9:1 women to men affected.[67] It has a bimodal age of incidence with peaks in the second and sixth decades of life.[466] Autoantibodies are frequent in AIH. A minority of patients have the same AMA as seen in PBC but most develop ANAs, antibodies to components of smooth muscle such as F-actin, elements of the cytochrome P450 system including P450 2D6 , and the Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase that is known as soluble antigen of liver and pancreas cytoplasm.[263, 480, 256, 434] AIH has associations with a number of drug triggers including minocycline, nitrofurantoin, hydralazine and tienilic acid.[36, 231, 118, 7]

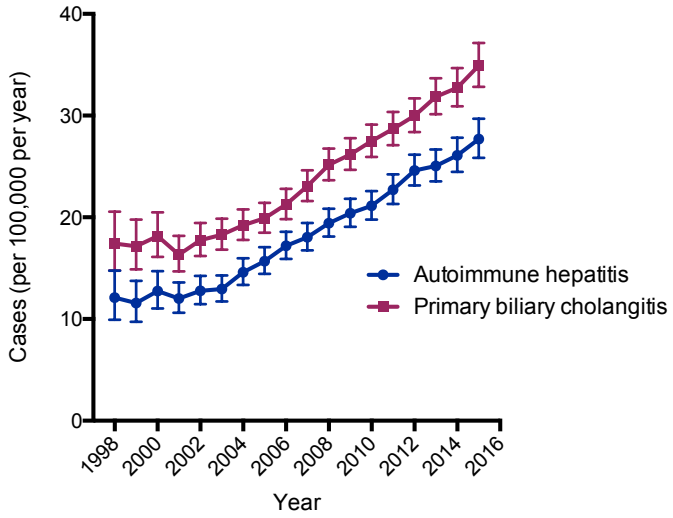

Figure 1.6: Increasing prevalence of autoimmune hepatitis and primary biliary cholangitis. Data are based on an interrogation of The Health Improvement Network of nationwide UK pseudonymised general practice records.[467]

As with PBC, elevations in serum ALT activity are common in PBC and reflect ongoing damage to hepatocytes. In addition, serum total immunoglobulin G (IgG) is often increased. Both of these laboratory indices act as proxies of ongoing inflammation. Similarly to PBC, recurrent disease after transplantation is recognised although difficult to distinguish clinically from rejection.[86]

Overlapping disease

The prevalence of both AIH and PBC is rising in the UK population (Figure 1.6) and also more generally.[31, 125, 430] As noted above, a number of features are shared between the diseases: their frequent co-incidence with other autoimmune phenomena, the presence of autoantibodies, elevations in immunoglobulins, female preponderances, peak ages of incidence, and the fact that a proportion of cases do not respond to standard immunosuppression. A minority of cases of both diseases with shared features are described as overlap syndromes. Typically, these cases are more aggressive and less responsive to standard therapy.

In addition, both diseases clearly represent a problem that is primarily immune rather than a primary hepatic problem as demonstrated by the fact that recurrence is well-reported in both.[306, 290]

CD4+ T cells are implicated in the pathogenesis of autoimmune liver disease

A number of findings have suggested a central role for a subtype of T lymphocyte: the CD4+ T cell in autoimmune liver disease. Most simply, there is a great increase in the number of CD4+ T cells seen on both biopsy and explant studies of AIH and PBC livers (Figure 1.7).[248, 371, 133, 249] CD4+ T cells respond to antigen presented by antigen-presenting cells. Antigen is recognised by specific T cell receptors on the surface but only when presented by the MHC class II molecule, which is in turn encoded by the human leucocyte antigen (HLA)-D locus on the short arm of chromosome six.[128] In many autoimmune conditions, associations with specific variants in HLA-D have been associated with an altered risk of developing multiple autoimmune conditions. In keeping with their being typical autoimmune diseases, such associations have been described both for AIH and PBC in candidate gene studies and also in all genome-wide association studies to date (Figure 1.8). [147, 146, 246, 300, 80, 345]

Supporting observations that antigen presentation to CD4+ T cells is important in autoimmune liver

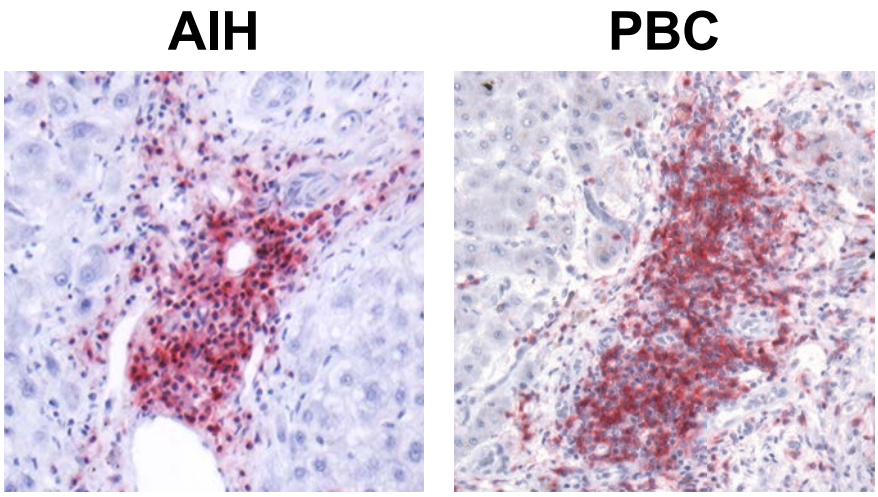

Figure 1.7: CD4+ T cells are prominent in autoimmune liver disease. Representative sections of inflammatory infiltrate from explant livers affected by AIH and PBC. Stained with a rabbit anti-CD4 primary antibody and visualised using an anti-rabbit ALP-conjugated secondary antibody with a red chromogen. Tissue obtained via the University Hospitals Birmingham liver transplantation programme and stained by the author.

disease (AILD) is the fact that MHCII (HLA-DR) is strongly expressed on both hepatocytes in AIH and on BEC in PBC.[429, 16] Such expression is not seen in health. This pattern of expression is also seen on the target tissues of others autoimmune diseases: for example, HLA-DR is expressed on joint synovium in rheumatoid arthritis but not in health.[284]

Both AIH and PBC have defined target antigens: for a proportion of AIH patients the target antigen is LKM-2 and in PBC it is the PDC-E2 subunit that is the target of AMAs.[143, 435, 319] In both, AIH and PBC an IL-2 'cytokine signature' is present: there are increases in expression in the diseased tissue.

T-cell receptor oligoclonality suggestive of an antigen-specific T cell response has been reported for both CD4+ and CD8+ cells in both diseases.[8, 496, 286] Evidence of T cell activation is also supported by evidence of both antibody class switching and production of interferon-gamma (IFNγ) in both diseases [470]. In PBC, where larger numbers of individuals have been studied with genome-wide association techniques, risk conferring variants cluster around genes involved in the T helper cell type 1 (Th1)-IL-12 pathway with its integral role for CD4+ T cells (Figure 1.9). Further evid-

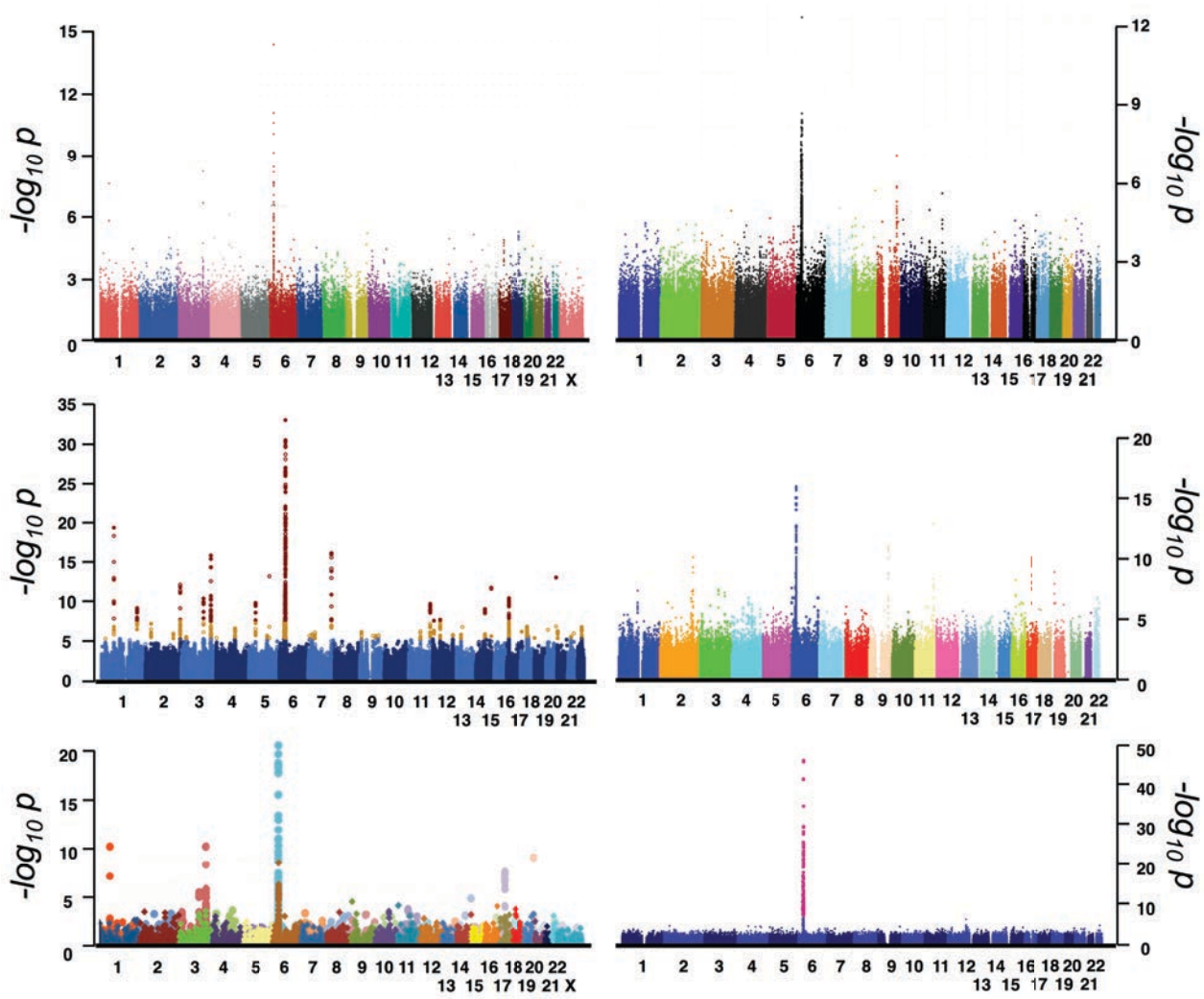

Figure 1.8: HLA-D variants are associated with autoimmune liver disease. Genome-wide association studies of PBC and AIH have demonstrated significant associations with the carriage of multiple single nucleotide variants. In all five major studies of patients with PBC and the single AIH study to date, the strongest association is with variants in HLA-D which encodes MHC class II. *Top-left*: PBC patients from North America[147]; *top-right*: PBC patients from Japan[300]; *centre-left*: British PBC patients[277]; *centre-right*: Chinese PBC patients[345]; *bottom-left*: Italian PBC patients[246]; *bottom-right*: white European AIH patients[80]. Figures adapted from publications referenced.

ence for a central role for CD4+ T cells is the provocation of syndromes similar to AIH after the administration checkpoint inhibitors for cancer immunotherapy, which primarily influence T cell activity[441]. Similarly, a hepatitis indistinguishable from classical type-1 AIH has been reported in immune reconstitution – the recovery of the CD4+ T cell count – during anti-retroviral treatment of the acquired immunodeficiency syndrome.[315]

In a minority of cases, AIH is associated with genetic syndromes associated with multi-system autoimmunity: in each of these cases, disorders of CD4+ T cell control are central to the condition. For example, in the syndrome autoimmune polyendocrinopathy candidiasis ectodermal dystrophy there is dysfunction in autoimmune regulator (AIRE) and resultant impaired presentation of otherwise tissue-restricted antigen to developing T cells and so aberration of both positive and negative selection. Alongside autoimmunity affecting other organs, around 20% of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients develop AIH.[437] In addition, AIH is also reported in patients with heterozygous dysfunction of cytotoxic T-lymphocyte associated protein 4 (CTLA4)[222, 366] and in some cases of the autoimmune lymphoproliferative syndrome where there are aberrations in the T cell apoptosis signalling molecule Fas and its ligand FasL.[331, 388, 484]

Th1 cells are particularly prominent in both PBC and AIH, and genetic variants in molecules implicated in the IL-12-Th1 pathway are associated with PBC (Figure 1.10).[473] This and the other observations above suggest a rationale for further considering CD4+ T cells in the pathogenesis of AIH and PBC, and as a potential therapeutic target.

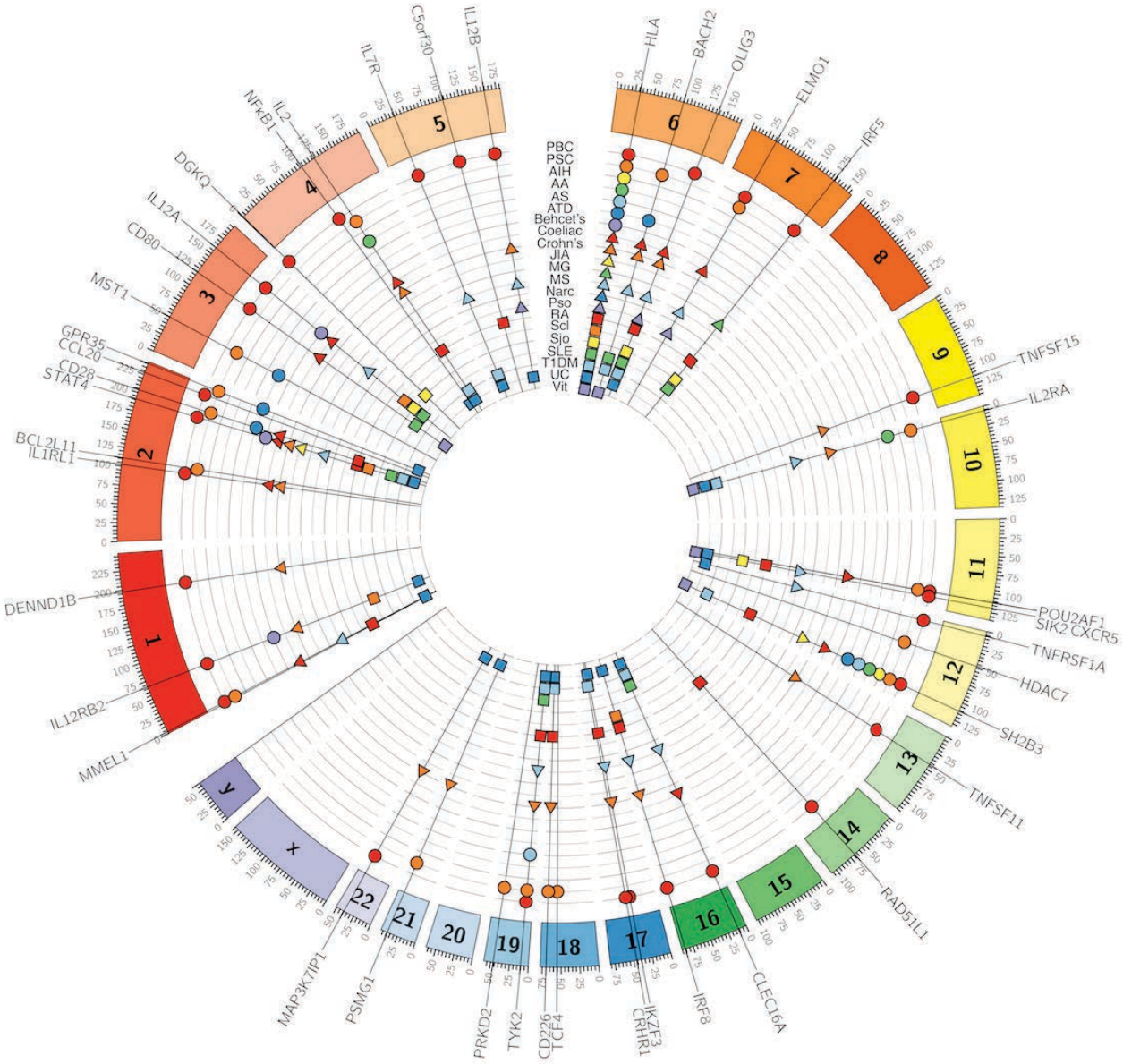

Figure 1.9: Both primary biliary cholangitis and autoimmune hepatitis share risk-conferring single nucleotide variants in loci shared with other autoimmune diseases. Numbers 1-22, X and Y denote the human genome split by chromosomes in descending size. Lines represent the genomic position of loci in which variants have been associated with altered risk of one or more major autoimmune diseases. Symbols on inner circles denote an association between variants in the gene concerned and risk of autoimmune disease. Image produced using Circos[218] and modified from Webb et al.[468] AA = autoimmune alopecia; AS = ankylosing spondylitis; ATD = autoimmune thyroid disease; JIA = juvenile idiopathic arthritis; MG = myaesthenia gravis; MS = multiple sclerosis; Narc = narcolepsy; Pso = psoriasis; RA = rheumatoid arthritis; Scl = scleroderma; Sjo = Sjögren syndrome; SLE = systemic lupus erythematosus; T1DM = type 1 diabetes mellitus; UC = ulcerative colitis; Vit = vitiligo.

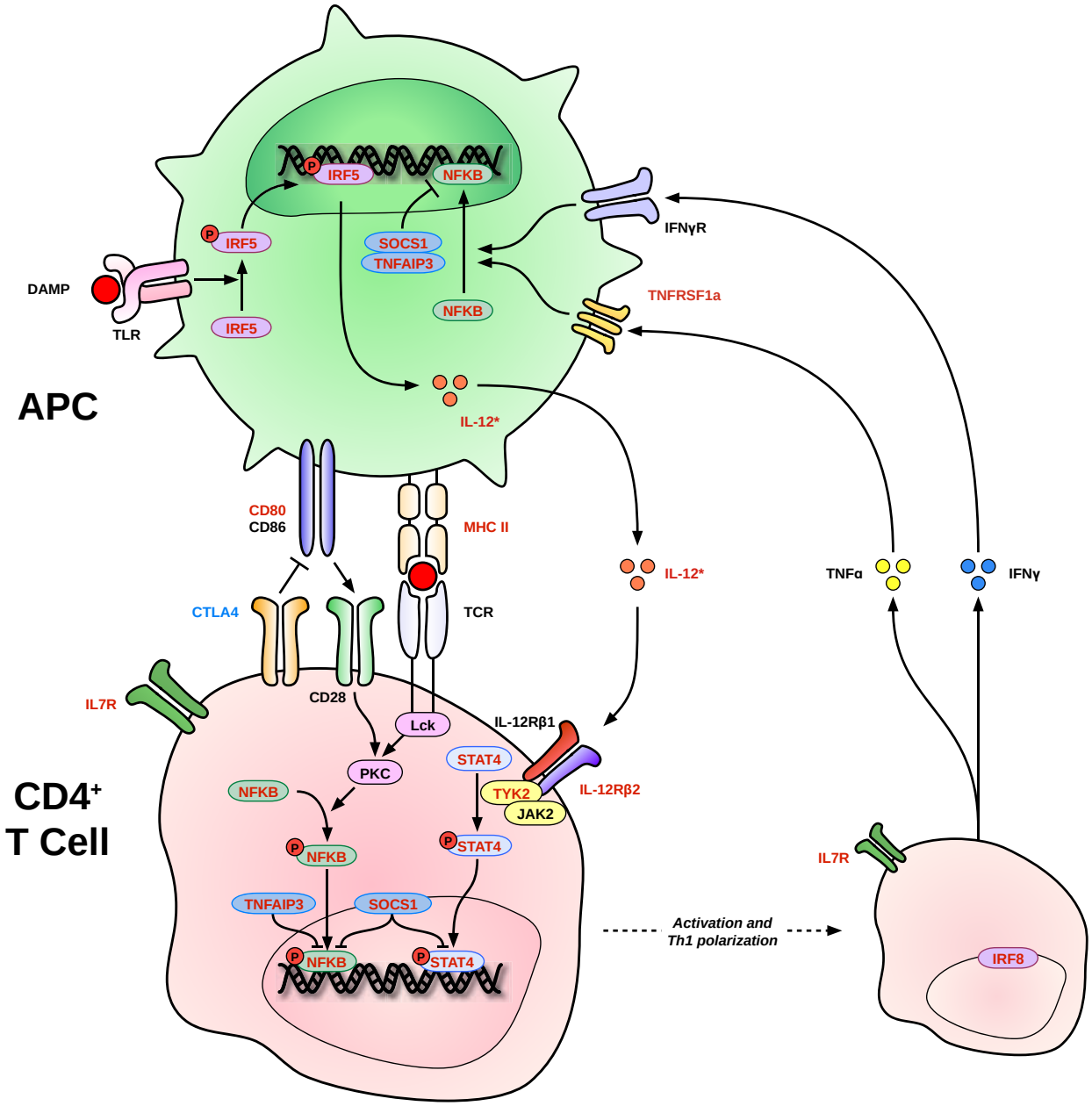

Figure 1.10: Multiple genetic variants associated with PBC are involved in the IL-12-Th1 pathway. Diagram represents the interaction between antigen-presenting cell and CD4+ T cell shown with primary stimulation through presentation of antigen on MHC II to the TCR with secondary stimulation via CD28 from CD80 and CD86. The antigen presenting cell is activated by recognition of danger-associated molecular patterns exemplified by TLR ligation. Genetic association studies have identified a number of signalling molecules key to the IL-12/Th1 pathway. Key elements of that pathway are illustrated with factors with significant genetic associations with PBC highlighted in red and likely associations highlighed in blue. Figure adapted from Webb et al.[473]

1.2 CD4+ T cells

The development of CD4+ T cells

CD4+ T cells represent a subset of lymphocytes. Lymphocytes derive their name from lymphatic vessels and represent the most frequent cell types within lymphatic fluid. They include B cells, T cells, natural killer cells, and natural killer T cells amongst other groups. Haematopoietic stem cells produce committed lymphoid progenitors that migrate to the thymus.[395] Here the lymphoid progenitor cells go through a series of maturation steps before entering a process of negative and positive selection. Initially, there is development of a pre-TCR where there is rearrangement of the beta chain. If the beta chain successfully pairs with an alpha chain, cells proliferate and express both CD4 and CD8: so-called double-positive cells.[109] At this point recombination activating genes (RAG) are suppressed preventing further TCR variation. Double positive cells then continue to mature in the outer and then inner cortex. However, if they receive insufficient signalling through TCRs and co-stimulation, which is the case in around two thirds of cells, there is death by neglect.[364] Depending on whether they receive stronger signalling through CD4 or CD8 associated TCRs, cells become single positive (Figure 1.11).

After selection in the cortex, cells migrate to the thymic medulla. A further sequence of selection ensues, with negative selection now prominent. Here, cells are exposed to self-antigens expressed on medullary thymic epithelial cells. Those that react strongly are selected for deletion, those that react more weakly take on effector phenotypes whilst those of intermediate reactivity – in the presence of sufficient – take on a regulatory phenotype. Within the thymic medulla there is also expression of antigens that are otherwise restricted to non-immune tissues. This allows deletion of potentially autoreactive T cells and selection of self-antigen specific Tregs. Expression of tissue-restricted antigens is mediated by two proteins specific to the thymic medulla: AIRE and Fezf2. Dysfunction of these proteins results in specific and differing patterns of autoimmunity, including in humans with

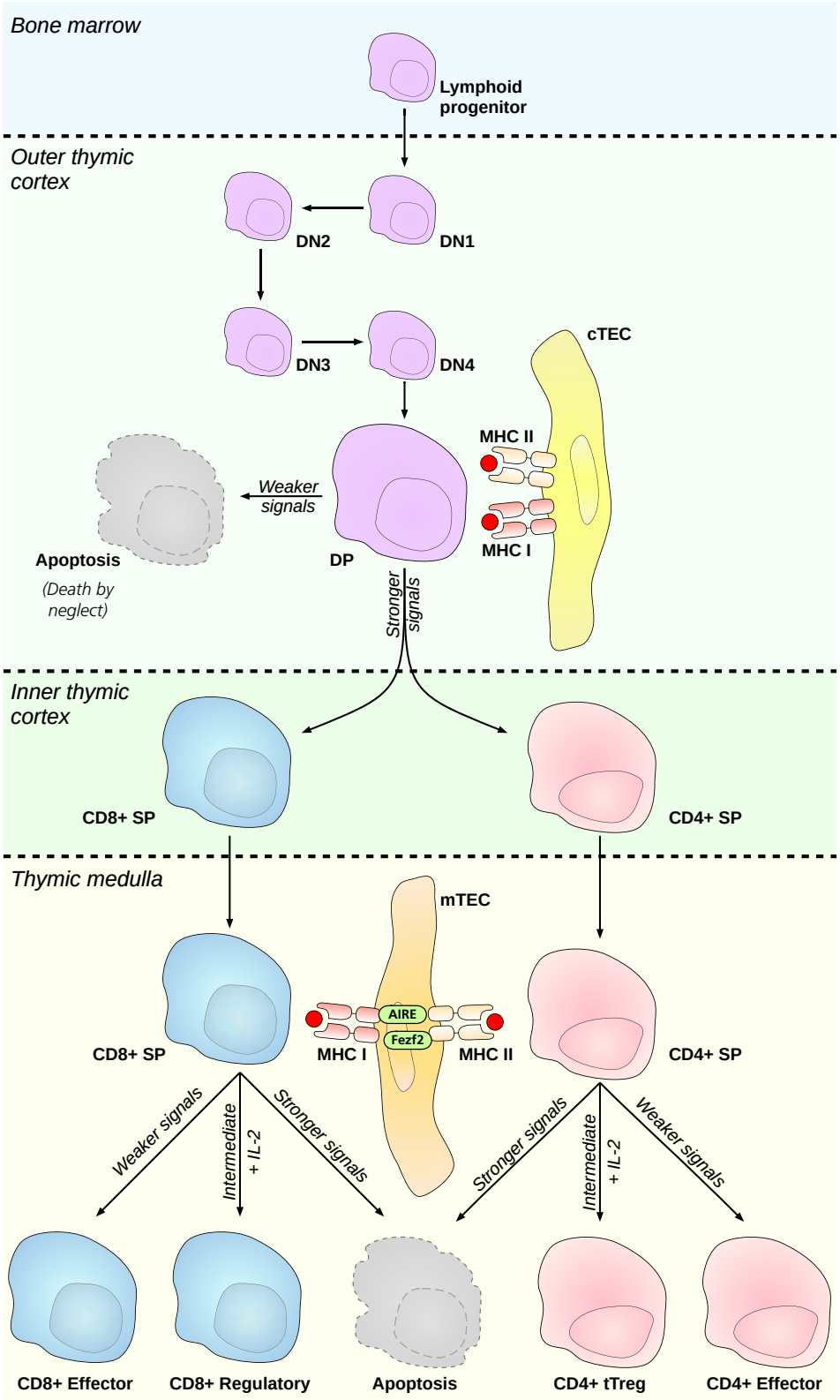

Figure 1.11: (Caption next page.)

Figure 1.11: *(Previous page.)* **Thymic T cell selection**. Lymphoid progenitors migrate from the bone marrow to the thymic cortex. Here they develop through a number of intermediate stages termed double-negative (DN) 1 to 4 where the cells remain negative for both the CD4 and CD8 co-receptors. During this process, the cells lose expression of CD44 and transiently express the IL2 receptor. There is rearrangement of the T cell receptor beta chain, and then if the beta chain successfully pairs with the pre-alpha chain, the alpha chain is rearranged, the cells proliferate and then enter a double-positive (DP) stage expressing the TCR, CD4 and CD8. Here, if the cells receive adequate signals through their TCR in response to antigenic sequences expressed by cortical thymic epithelial cells, they survive and are polarised towards persistent expression of either CD4 or CD8 depending on their specificity. If cells do not receive sufficient stimulation, they enter apoptosis: so-called death by neglect. Next, single-positive (SP) cells enter the thymic medulla and interact with self-antigens expressed on medullary thymic epithelial cells. The expression of otherwise tissue-restricted self antigens is mediated by AIRE and Fezf2. Cells that receive weaker signals adopt an effector phenotype, cells that receive intermediate signals adopt a regulatory phenotype whilst cells that receive strong signals from self antigen enter apoptosis. Figure adapted from Germain (2002), Nature Reviews in Immunology.[109]

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some types of dysfunction, autoimmune hepatitis.[406, 200]

CD4+ T cell effector functions

The overall roles of activated effector CD4+ T cells include the production of effector cytokines, promoting the recruitment of eosinophils, neutrophils, and basophils to sites of activation by supporting the production of chemokines such as CCL5/RANTES, supporting B cell affinity maturation and activation, supporting persistence of memory in CD8+ lymphocytes, promoting increased phagocytic activity in macrophages and regulating the innate immune system by modulating expression of pattern recognition receptors. As such they have been described as "orchestrating the full panoply of immune responses".[510]

The functional role of CD4+ T cells overall can be demonstrated by their deficiency. In mice, this is best achieved by knocking out MHC class II rather than CD4 – if CD4 is knocked out, CD4- and CD8- double negative T cells fulfil some of the roles of CD4+ T cells.[27] Mice lacking MHC class II have minimal CD4+ cells and do not develop germinal centres, show reduced production of immunoglobulin alongside an absence of T cell specific antigen response.[69] Such mice also demonstrate a lack of induction of a cytotoxic CD8+ T cell response[23], marked susceptibility to the

intracellular infection mycobacterium tuberculosis[54], susceptibility to leishmaniasis and protozoa, and exhibit reduced allograft rejection.[127] In humans, the phenotype of patients with low CD4+ counts is visible in the context of human immunodeficiency virus infection. Such individuals show susceptibility to infection with tuberculosis and encapsulated bacteria and fungi, failure to control several viral infections, loss of some specific antibody responses and a tendency to malignancy.[287] A similar phenotype is seen in the rare disease idiopathic CD4+ lymphocytopaenia.[88]

CD4+ effector T cell subtypes

For some time, it has been recognised that CD4+ T cells do not represent a homogeneous group, but that they have unique functions. Primarily, they are subdivided on the cytokines that they produce on stimulation: initially TH1 and T helper cell type 2 (TH2) cells were identified based on their respective polarisation towards either production of IFNγ or IL-4 upon stimulation.[510] Subsequent to this, a number of differently differentiated T cell subsets have been described (Figure 1.12).[349]

The longest recognised effector T cell subset is the TH1 cell. These cells are characterised by their expression of IFNγ on stimulation and their expression of the transcription factor Tbet. Polarisation towards a TH1 phenotype primarily occurs through the action of IL-12. The function of Th1 cells is primarily defined by the effects of their deficiency: susceptibility to intracellular infections such as mycobacteria.[401] The functional importance of IFNγ in this regard is further demonstrated by the susceptibility of humans with genetic aberrations in the IFNγ pathway.[180] TH1 cells have been identified as the major component of target antigen specific T cells in autoimmune diseases such as multiple sclerosis.[317] In several animal models of of autoimmune disease, blocking IFNγ is protective whilst exogenous additional IFNγ worsens pathology.[122]

TH2 cells produce high levels of IL-4 and IL-10 after polarisation by the action of IL-4 and IL-35 and show significant interdependency with innate lymphoid cells such as ILC2.[240] They typically

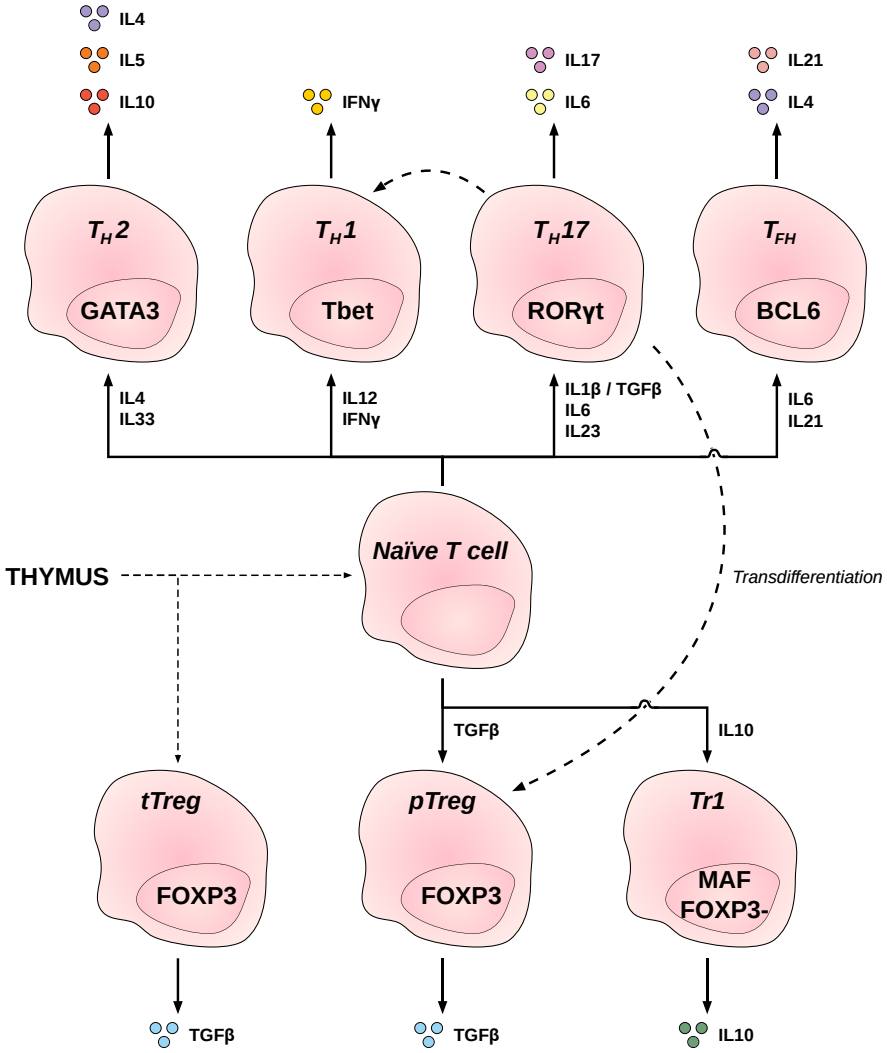


Figure 1.12: CD4+ T cell subsets. T cells leave the thymus in a naïve, antigen non-experiened state. After contact with antigen, T cells may take on one of numerous subsets. These subsets are defined by their cytokine secretion profiles and the transcription factors necessary for their development. Dashed line shows reported transdifferentiation between subsets. Effector subsets are shown at the top of the diagram whilst regulatory subsets are shown at the bottom.

express the transcription factor GATA3. Their role is characterised as being in promoting allergic and anti-parasitic responses: although deficiency in GATA3 is embryonically lethal, their role can be demonstrated by animals deficient in IL-4 which are resistant to allergic responses.[43] Deficiencies in their function predispose to various parasitic infections and cause reduced production of immunoglobulin E (IgE).[301]

TH17 cells develop in response to signals from IL-6, IL-23, transforming growth factor-beta (TGFβ) and tend to express the retinoic acid receptor-related orphan receptor gamma (RORγ). They are characterised by their expression of IL-17 and, as below, they appear to be a particularly plastic subtype of T cell. Their absence is characterised by susceptibility to bacterial and fungal pathogens, and to tumourigenesis.[77, 44] TH17 cells become increasingly frequent in end-stage PBC.[490, 320]

T follicular helper cell (TFH) cells are characterised by their expression of the transcription factor Bcl6. They also tend to express CXCR5, which aids in their positioning in lymphoid tissue, and variable levels of programmed death protein 1 (CD279) (PD-1) and inducible T-cell costimulator (ICOS). They promote germinal centre formation, antibody affinity maturation and the formation of memory B cells. In their absence, specific antibody responses are absent or severely blunted.[75]

Both TH9 and TH22 effector T cells, which produce IL-9 and IL-22 respectively have been identified but are less well described. TH9 T cells are involved in the regulation of mast cell migration and Treg survival and there are reports of their involvement in allergy, autoimmunity and anti-tumour immunity.[184] TH22 cells have major roles in epidermal immunity but equally have been proposed to influence the development of autoimmunity and anti-tumour immunity.[175]

Importantly, T cells are not permanently commited to a specific subset. This process of transdifferentiation has been described using cells that display fate-mapping markers after expressing IL-17: for example, TH17 type cells in inflammatory environments may take on a TH1, IFNγ secreting, phenotype.[103] TH17 also appear to be able to take on a regulatory phenotype during resolution of inflammation.[142]

1.3 Regulatory T cells

Tregs are a subset of CD4+ T cells. The role of Tregs is primarily to control the activation of effector T cells and their importance is keenly demonstrated by the drastic effect of their absence. Mice lacking functional Tregs develop multi-organ infiltrates of activated T lymphocytes, with marked increases in effector cytokine production and a wasting disease that is typically lethal by four weeks of life.[113] Transfer of Tregs prevents the development of disease.[100] The requirement for the action of Tregs is ongoing through life: in mice in which a deficiency in Tregs is inducible by the administration of diphtheria toxin, such depletion rapidly results in lethal multi-system autoimmunity.[194, 224] This same model is also sufficient to demonstrate that the autommunity is due to the excess activation of non-regulatory CD4+ T cells – simultaneous depletion of CD4+ T cells prevents disease.

Tregs may be identified by their expression of the master transcription factor forkhead box P3 (FOXP3). Functional FOXP3 is required both for normal development and activity of Tregs and its transfection alone into naïve T cells promotes the development of a regulatory pheonotype.[153] In mice, the expression of FOXP3 is sufficient to identify Tregs, but in humans the situation is more complicated and activated lymphocytes may also briefly express FOXP3. Tregs also demonstrate constitutive high expression of the IL-2 receptor alpha subunit, relatively low expression of the IL-7 receptor alpha subunit, and constitutive expression of CTLA4 and (the protein encoded by) lymphocyte-activation gene 3 (LAG3). The adoption of a Treg phenotype is not a permanent status and cells may transdifferentiate from other CD4 T cell subsets into FOXP3 expressing cells [103] and that FOXP3 expression may be lost by a proportion of Tregs in inflammatory environments.[283]

Subtypes of regulatory T cell

A number of subtypes of Treg have been proposed and the nomenclature has varied over time. Broadly, there are three types functional *in vivo*: the directly thymic-derived tTreg, the peripherally

derived pTreg which differentiate from naïve T cells and from effector T cells, and Tr1 cells.[1, 379] The former two require FOXP3 for their development, which Tr1 cells do not. The existence of peripherally derived FOXP3-negative cells expressing TGFβ or IL-35 and termed as Th3 and TR35 has also been proposed.

Regulatory T cell effector mechanisms

How the FOXP3-dependent pTregs and tTregs exert their suppressive effects on other T cells is incompletely defined but several important mechanisms have been identified (Figure 1.13).[436, 178] Their respective importance and their functional redundancy are not fully assessed.

First, Tregs produce several cytokines with inhibitory actions on effector T cells. These include IL-10[6, 396], IL-35[63], and TGFβ.[350] A probable role for Treg membrane-bound TGFβ has also been suggested by the demonstration that is it strongly expressed on Treg membranes and that cell-impermeable membranes prevent the anti-TGFβ sensitive suppression of effector T cells in co-culture experiments.[299] The role of IL-10 has also been made less clear by the suggestion that Tregs in fact influence other cells to express immunosuppressive IL-10 via an unknown mechanism.[189, 4]

Second, Tregs have been demonstrated to produce some of their immunosuppressive effects via granzyme B mediated cytolysis, with reduced suppressive capability with Tregs isolated from granzyme B deficient animals.[116]

Third, Tregs constitutively express high levels of IL-2 receptors and upregulate these further when activated. It has been proposed that uptake of IL-2 by Tregs prevents sufficient uptake by other activated T cells to avoid apoptosis.[323]

Fourth, reverse signalling through an uncertain mechanism induces increased Treg expression of

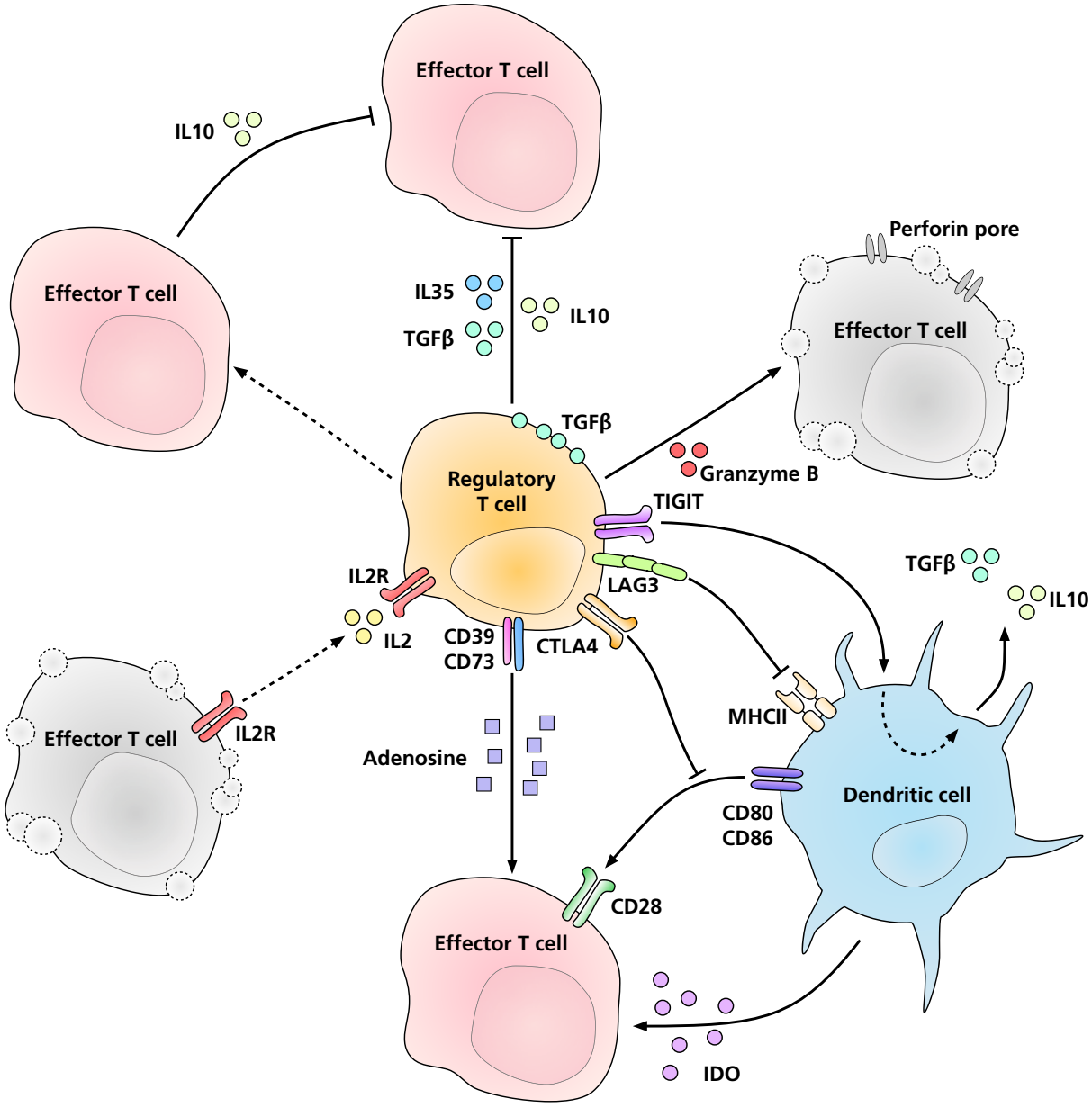


Figure 1.13: Mechanisms of immunosuppression by regulatory T cells. Several mechanisms of suppression of the activity of other cells by Tregs have been proposed. These include the production of immunosuppressive cytokines including TGFβ (which can be membrane-bound), IL-10 and IL-35. In addition, it is reported that Tregs are able to induce other T cells to express IL-10. Expression of cytotoxic granzyme B may permit Tregs to cause the death of other cells. Various interactions with antigen-presenting dendritic cells including induction of TGFβ and IL-10 via TIGIT and interactions between LAG and MHC encourages productions of immunosuppresive TGFβ and IL-10. In addition, because Tregs express high levels of high affinity IL-2 receptors, they appear to have some activity by depriving other T cells of sustaining IL-2.

the immunoregulatory enzyme indoleamine 2,3-dioxygenase and that this in turn has immunsuppressive effects on effector T cells.[343]

Fifth, the two ectoenzymes CD39 and CD73 are highly expressed on Tregs. Recent work has suggested that they convert extracellular 5'-adenosine mono- and triphosphate to adenosine, which in turn has immunosuppressive functions.[208, 34]

Sixth, molecules expressed on the cell surface of Treg surface mediate immunosuppressive effects. Two examples include LAG3, which binds avidly to MHCII and is reported to reduce dendritic cell maturation and co-stimulatory capacity [155], and the immune receptor TIGIT, which decreases production of the effector cytokine IL-12 by dendritic cells.[498]

Finally, and likely to account for a major proportion of the immunosuppressive efficacy of Tregs, the molecule CTLA4 prevents signalling through CD28 on effector cells and so reduces co-stimulation.[449, 450]

Regulatory T cells in autoimmune liver disease

In addition to evidence for uncontrolled action of effector CD4 T cells, other factors support consideration of failures of T cell regulation in the aetiology of AILD. Initial reports suggested that numbers of Tregs were decreased in the inflammatory infiltrate of AIH as compared with other liver diseases.[96] Others have described a decrease in peripheral Treg numbers in AIH.[251, 250, 239, 237] More recently however, reassessments of both peripheral numbers and liver-infiltrating numbers of Tregs with improved molecular phenotyping with specific staining for the transcription factor FOXP3 of both histological samples and cell suspensions prepared from explant livers have demonstrated that both are similar or increased when compared to other liver diseases and, in the case of the peripheral blood, to health.[321, 330, 412, 22]

The function of peripheral Tregs isolated from autoimmune hepatitis patients has been suggested to be impaired in their regulation of activation and proliferation of conventional T cells[250]. Again however, more recent examinations have suggested that this is not the case for Tregs isolated from inflamed liver tissue, highlighting that hepatic and peripheral leucocyte populations may differ in autoimmune hepatitis, and possibly suggesting that more activated Tregs migrate to inflamed tissue.[330, 60, 170] In PBC there is a similar story with regards to Tregs: early reports were made of reduced numbers of Tregs circulating peripherally and in liver tissue[227, 459], but this has been challenged in more recent studies.[321]

In addition, evidence that those treated with drugs that interfere with or deplete Tregs may develop AIH suggests a key role for T cell regulation. The Hirschfield group has also recently reported the first direct association between a complete absence of Treg and AIH in a patient with germline mutation of GATA2.[465]

Complete deficiency in regulatory T cells.

The key role of Tregs can be demonstrated by examining mice with loss of function mutations in the master transcription factor controlling the development of regulatory T cells, FOXP3.[113] In mice the gene for FOXP3 is carried on the X chromosome and a spontaneous mutation affecting FOXP3 in a colony of mice in the Oak Ridge National Laboratory in Tennessee lead to the development of male mice that developed multi-system autoimmunity including lymphadenopathy, autoimmune haemolytic anaemia and marked lymphocytic infiltrates of the liver, skin and bowel.[113] Numbers of CD4+ and CD8+ T cells are greatly expanded, demonstrate increased proliferation, and are hyperresponsive to stimulation.[436] Mice generally die at 3-5 weeks of age. It has been noted that the generalised loss of control of lymphocytes seen in FOXP3 dysfunction is analogous to loss of function of other key regulatory molecules like CTLA4 and to graft-versus-host-disease.[416, 462, 512]

These observations were made before the discovery of FOXP3, but it was later established that mutations in the X-chromosome gene later named FOXP3 were the cause of the syndrome.[42] Formal identification of and cloning of FOXP3 occurred soon after this identification with the demonstration that FOXP3 expression identified a subset of CD4+ T cells with the ability to suppress the activity of other effectors T cells.[100, 153, 191] It was clarified that allografting normal bone marrow was curative[387] and that the disease was primarily driven by CD4+ T cells through the demonstration of ameliorating effects of their depletion. This contrasted with the ineffectiveness of depletion of CD8+ T cells.[28]

In humans, the rare condition immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) bears many phenotypic similarities to mice with deficiencies in FOXP3: there is lymphadenopathy, autoimmune haemolytic anaemia and marked lymphocytic infiltrates of the liver, skin and bowel accompanied by multiple endocrinopathies including diabetes mellitus.[482] The disease requires aggressive immunosuppression, with subsequent protection from infection, and bone marrow allografting for long-term cure.[18] Notably, human IPEX typically represents a spontaneous mutation and some mutations may result in the persistence of FOXP3-positive cells but with a relative loss of function.[85]

Strong corollaries between human and mouse conditions are further highlighted by the fact that transfer of total peripheral T cells from IPEX patients into humanised mice recapitulates the phenotype of mouse FOXP3 dysfunction[115] and that IPEX patients' T cells can be induced to display a regulatory phenotype by FOXP3 gene transfer.[82]

As a result of their combination of peribiliary hepatitis and the development of AMAs in the context of multi-system autoimmunity, mice lacking FOXP3+ Tregs have been described as a potential model of inflammatory PBC.[504] By analogy, AMAs are also reported in a proportion of human cases of IPEX.[422]

Attenuating autoimmune disease in regulatory T cell deficiency

As a condition representative of loss of regulatory control, numerous interventions have been tried to ameliorate the autoimmunity of FOXP3 dysfunction. A number of key manipulations of the $FOXP3^{KO}$ mouse have highlighted several key aspects of the autoimmunity that occurs when there is a loss of regulatory control (Table 1.1). Firstly, signalling through the TCR is required: when mice transgenic for a TCR on a RAG deficient background so that all T cells are specific for a non-self antigen are crossed with mice with FOXP3 dysfunction, no disease is apparent. Secondly, CD4s drive pathology: although both CD4+ and CD8+ cells are greatly increased in number and activation state in FOXP3 dysfunction, co-deficiency in CD8+ T cells does not protect against disease whilst disease is slowed by co-deficiency in CD4+ T cells. Thirdly, TH1 type disease is the mechanism that drives lethality: co-deficiency in the transcription factor Tbet or in IFNγ increase lifespan whereas deficiency in signal transducer and activator of transcription 6 (STAT6) or IL-4 do not.[377, 402] Fourth, co-stimulation is important to the generation of pathology: mice co-deficient in CD28 have a greatly ameliorated phenotype, as do those co-deficient in OX40.[105] Fifth, non αβT cells have a regulatory role: both B cells and γδT cells are reported to ameliorate disease to some extent.[9, 426] Interestingly, some interventions have divergent effects on different organ systems. For example, with co-deficiency in IL-2, skin inflammation is reduced but colitis and hepatitis persist; CD8 depletion is relatively protective for hepatitis but fails to extend lifespan.[507, 377]

1.4 CD28 co-stimulation and CTLA4 control

Co-stimulation of T cells through CD28

Recognition of antigen by a TCR alone is insufficient for full activation of the T cell: cells stimulated in culture without non-T-cells may become anergic or poorly responsive with reduced proliferation

Table 1.1: Preventing autoimmunity in FOXP3 deficiency

Co-deficiency	Change in phenotype	Lifespan	Reference(s)
None	N/A	4-5 weeks	[113]
$CD28^{-/-}$	Reduction in expression of activation markers on lymphocytes; reduced circulating cytokines	25-30 weeks	[385]
$IL\text{-}2^{-/-}$	Skin inflammation reduced	9-10 weeks	[507, 377]
$Fas^{lpr/lpr}$	Delay in onset but unchanged phenotype	16-18 weeks	[507]
$AIRE^{-/-}$	Endocrine organs protected	4 weeks	[61]
NOD	More severe	3-4 weeks	[61]
$CD4^{-/-}$	No CD4+ T cells; minimal change to eventual phenotype. Note that CD4-negative T cells are proposed to fill some of the role of the missing CD4+ cells.	>12 weeks	[28, 271]
$\beta 2M^{-/-}$	No CD8+ T cells; some reduction in hepatitis but otherwise no change	4-5 weeks	[28]
$IFN\gamma^{-/-}$	Lymphocyte expansion delayed	7-8 weeks	[377, 402]
$Tbx^{-/-}$	Lymphocyte expansion delayed	7-8 weeks	[402]
$IL\text{-}4^{-/-}$	IgE and IL-4, 5 & 13 production decreased	4-5 weeks	[377]
$STAT6^{-/-}$	IgE and IL-4, 5 & 13 production decreased	4-5 weeks	[402]
$IL\text{-}10^{-/-}$	No change	4-5 weeks	[377]
Tg.TCR×RAG	All T cells express a T cell receptor specific to a non-self antigen; disease is abolished	Normalised	[499]
$\mu MT^{-/-}$	No B cells. Reduced autoantibody titres; reduced T cell activation	10-12 weeks	[9]
$OX40^{-/-}$	Long delay in onset of otherwise similar phenotype	30 weeks	[105]
$CD30^{-/-}$	Short delay in otherwise similar phenotype	6-7 weeks	[105]
γδ T cells	Reduced organ inflammation including reduced hepatitis; reduced T cell expansion	Not reported	[426]

Table adapted and expanded from Ju et al.[182]

and reduced cytokine production.[171] This observation has been demonstrated to relate to a lack of co-stimulation through the CD28 molecule, with ligating antibodies to the CD28 molecule able to restore responsiveness to pure T cell cultures.[265] The reverse – administration of an antibody that prevented signalling through CD28 – was able to prevent a specific immune response to transplanted pancreatic islet cells.[234]

CD28 is the initial member of a subfamily of co-stimulatory molecules characterised by a variable extracellular immunoglobulin-like domain. Other related molecules in the family include ICOS, CTLA4 and PD-1.[94] CD28 is constitutively expressed on mouse T cells and expressed by a majority of human T cells.[126, 94]

The ligands for CD28 are CD80 and CD86, together known as B7. The expression of both CD80 and CD86 are variable and is upregulated by the activation of antigen-presenting cells. Primarily this occurs through the ligation of pattern recognition receptors (PRRs) such as TLRs and represents a link between the adaptive and innate immune systems where greater antigen-presenting cell (APC) activation will in turn provide stronger co-stimulatory signals to the T cell recognising the antigen.[165] Activation of APCs also increases antigen presentation.

Cellular effects of CD28 ligation

The cytoplasmic domain of CD28 has no intrinsic enzymatic activity but contains highly conserved tyrosine motifs that become phosphorylated after both CD28 ligation and TCR engagement.[94] Once phosphorylated, these motifs complex with a number of kinases and adaptor proteins which initiate signalling cascades that end in altered transcription, primarily through the NFκB, NFAT and AP1 pathways.

Ligation of CD28 has numerous cellular effects on T cells. These include augmentation of TCR signalling[30], promotion of formation of the immunological synapse (or supramolecular activa-

tion cluster) of signalling molecules that forms around ligated TCRs[493], promotion of cellular actin remodelling[493], upregulation of anti-apoptotic proteins such as BCL_{XL}[192, 464], increased IL-2 transcription through NFAT, AP-1 and NFκB[101, 464], mRNA stabilisation resulting in increased production of cytokines including IL-2 and other effector cytokines too[242, 340], increased vesicular transport, enhanced DNA replication and repair[94], and upregulation of secondary co-stimulatory molecules such as CD40L[201] and OX40[448]. Additional effects on Tregs include promoting the thymic development of thymic-derived cells[405] and peripheral differentiation into pTreg development of iTreg[130], maintenance of Treg survival separate from IL-2[503] and promotion of Treg CTLA4 and PD1 expression[503].

T cells stimulated in the absence of CD28 co-stimulation lacking the above effects are typically described as anergic, where there is a general reduction in proliferation and effector function that is partially reversible by exposure to IL-2 or OX40.[367]

CTLA4

The molecule CTLA4 has close homology to CD28 and binds CD80 and CD86. In both mice and humans, the gene for CTLA4 is located near to that for CD28 – on chromosome 1 in mice and on chromosome 2 in humans. CTLA4 is constitutively expressed by FOXP3+ Tregs and is expressed on conventional T cells after activation through the TCR and especially after co-stimulation through CD28.[357]

Mice deficient in CTLA4 demonstrate multi-organ lymphocytic inflammation with activated T cells and succumb to their generalised autoimmune disease at around five weeks of age.[416, 462] The phenotype of these mice is similar to that seen in FOXP3 dysfunction, but differs in that there is typically myocarditis and pancreatitis, but less overt skin involvement.

As exemplified by the effects of its deficiency, CTLA4 has an immunosuppressive role. A num-

ber of mechanisms for this have been proposed and include outcompeting CD28 for its ligands CD80 and CD86, to which CTLA4 binds with higher affinity.[451] It has also been suggested that CTLA4 may have phosphatase activity and so negatively regulate the phosphorylation that is induced by CD28 signalling.[232] Additionally, the ripping, or transendocytosis, of CD80 and CD86 from antigen-presenting cells, so preventing their further co-stimulatory action.[346] Controversially, reverse signalling through CTLA4's ligands CD80 and CD86 and induction of immunosuppressive indoleamine 2,3-dioxygenase (IDO) by dendritic cells has been proposed as one mechanism of immunsuppression.[276] However, CTLA4 blockade remains immunogenic in IDO-deficient mice.[151] Finally, direct signalling through CTLA4 has also been proposed to regulate cell function, but this is not consistent and is disputed by some authors.[207, 450]

Recently two cohorts of humans *heterozygous* for loss of function mutations in CTLA4 have been described.[222, 366] These have varying patterns of autoimmunity with lymphocytic infiltration of a variety of organs alongside autoantibodies. The pattern of autoimmunity is similar to that seen with administration of the blocking anti-CTLA4 antibody ipilimumab[475] and is alleviated by treatment with CTLA4 immunoglobulin (CTLA4Ig).[233]

Manipulations of CTLA4-deficient mice

Similar to the situation in FOXP3 dysfunction, there is an ongoing requirement for CTLA4 through life. One model demonstrating this is a transgenic mouse in which a LoxP flanked CTLA4 gene may be selectively deleted in adulthood by inducing transcription of a Cre recombinase using a tamoxifen-sensitive promoter.[205] These mice develop multi-system lymphocytic inflammation in a similar pattern to constitutively deficient animals. It has been demonstrated that the lethal autoimmunity of CTLA4 deficiency is dependent on interactions between the co-stimulatory receptor CD28 and its major ligands CD80 and CD86: deficiency in either prevents disease.[261, 262]

A number of key experiments have clarified the *in vivo* role of CTLA4 (Table 1.2): First, given

that it has been proposed that both CD28 and CTLA4 interact with ligands other than CD80 and CD86[58], two experiments have clarified that it is in fact the interaction with CD80 and CD86 that is dominant. Firstly, mice deficient in CD28 and CTLA4 do not develop significant autoimmunity[262] and in a complementary experiment, mice deficient in CTLA4, CD80, and CD86 are also protected.[261] Secondly, the creation of a mouse chimeric for wildtype and CTLA4-deficient bone marrow resulted in complete protection from CTLA4-deficiency disease. This included the demonstration that neither subset of cells developed the upregulation of activation markers typically associated with CTLA4 deficiency disease.[13] Thirdly, experiments employing CTLA4-deficient regulatory T cells have demonstrated that deficiency on this subset is sufficient to allow unchecked autoimmunity.[483] Fourthly, similarly to the requirement for FOXP3, there is a peristent requirement for CTLA4 expression through life and inducible deletion precipitates lethal autoimmunity.[205] Fifthly, interactions with self-antigens are key: T cell transfer from CTLA4 deficient mouse peripheral organs are specifically reactive with the same organs in other mice.[162] Manipulations of mice deficient in CTLA4 are discussed in (Table 1.2).

1.5 Immune checkpoints as therapeutic targets

Recent years have seen a large increase in the use of immune checkpoint inhibitors for the therapy of human diseases, primarily in use in the treatment of cancers (Figure 1.14). These drugs inhibit the action of immunoregulatory molecules to provoke an increased immune response to cancers. Their marked efficacy first became apparent in the treatment of metastatic melanoma, an immunogenic cancer with poor survival[149], but their use is broadening to multiple different cancer types[325], with new applications reported frequently.[376, 281, 104]

At present two major classes of immune checkpoint inhibitors are in clinical use: those directed against CTLA4 and those directed against either PD-1 or its ligand PDL-1.

Table 1.2: Preventing autoimmunity in CTLA4 deficiency

Intervention	Change in phenotype	Reference(s)
CTLA4 deficiency alone	Massive lymphocyte activation and expansion, multi-organ infiltration and early death	[462, 416]
CTLA4Ig	Reversal of disease	[190]
Deplete CD4+ T cells	Disease prevented	[55]
Deplete CD8+ T cells	Little change to organ infiltration	[55]
$CD80^{-/-}CD86^{-/-}$	Disease prevented	[262]
$CD28^{-/-}$	Disease prevented	[261]
$CTLA4^{-/-}$ and WT bone marrow chimera	Disease prevented	[13]
Depletion of CTLA4 in adulthood	Multi-organ infiltration, lymphoid organ enlargement and early death	[205]
Depletion of CTLA4 on Tregs only†	T cell activation and expansion, slowed multi-organ infiltrate and much delayed death	[166]
Depletion of CTLA4 on Tregs only†	Rapid autoimmunity, T cell activation, organ infiltration and death	[483]
Depletion of Treg CTLA4 in adulthood	Some cell activation, no multisystem autoimmunity, relative resistance to induction of experimental allergic encephalomyelitis (EAE)	[327]
†Note: different mouse backgrounds used		

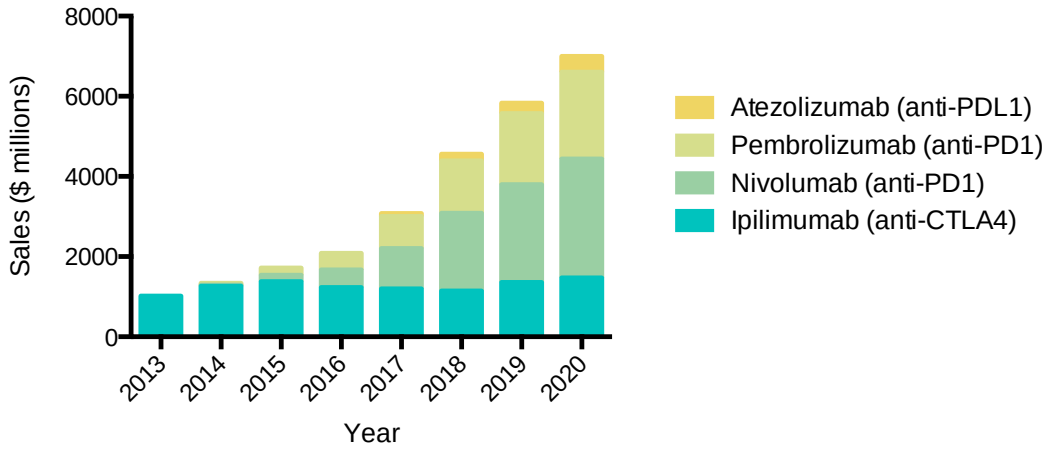


Figure 1.14: Use of immune checkpoint inhibitors is increasing. Graph shows sales to date and estimated future sales of major immune checkpoint inhibitors in the United States, France, Germany, Italy, Spain, United Kingdom and Japan. Sales shown in US$ million. Figure redrawn from Webster, 2014.[476]

CTLA4 blockade as immunotherapy

As discussed above, the major function of CTLA4 is to negatively regulate effector T cell activation largely through modulation of signalling through CD28. Preventing the action of CTLA4 with drugs such as ipilimumab (IgG1) and tremelimumab (IgG2) may therefore increase signalling through CD28 and promote an adaptive immune response. The primary mechanism of anti-CTLA4 medications has been however been the source of some debate. Suspicions as to whether anti-CTLA4 antibodies could penetrate the immune synapse lead to a search for other mechanisms: an alternative major mechanism is the suggestion that the primary clinical activity is through depletion of Tregs, and it has been suggested that reductions in Treg numbers correlate with clinical response.[384, 354] Conversely however, mice made to express human CTLA4 on effector T cells and murine CTLA4 on Tregs through combinatorial adoptive transfer from transgenic hosts develop more significant immune activation with selective blockade of the CTLA4 on effector cells but not with blockade of CTLA4 on Tregs.[329]

Consistent with their role as inhibitors of immunomodulatory function, inhibitors of CTLA4 are associated with the induction of autoimmunity. Most commonly this includes colitis[475, 21], but

may also include hepatitis[195, 119, 177] and a variety of other organs.[441] Such immune-related adverse events are frequent and affect up to 68% of those treated with ipilimumab, the most widely used anti-CTLA4 agent.[474]

PD1/PDL1 blockade as immunotherapy

The second major class of immune checkpoint inhibitors in current use is those that inhibit interactions between the receptor PD-1 and its ligands, which include PDL-1.[325] Ligation of PD-1 decreases cytokine production by T cells and promotes apoptosis and it has been proposed that the system therefore represents a negative feedback mechanism to prevent damage to self tissues during an immune response. Whilst the human anti-PD-1 agents nivolumab and pembrolizumab and the anti-PDL-1 agent atezolizumab are effective as cancer immunotherapies, similar to anti-CTLA4, they too have a high rate of immune-related adverse events, which include hepatitis.[266, 108, 513] Similarly to anti-CTLA4 therapy there has also been speculation that nivolumab also depletes Tregs.[409]

As the use of immune checkpoint inhibitors continues to grow for the therapy of cancer, the incidence of immune-related adverse events is also likely to grow and this new frontier of autoimmunity represents a key area for future control. The frequent hepatitic reactions in response to these drugs may be considered a subtype of autoimmunity.

1.6 OX40, OX40L and the TNF-receptor ligand family

The tumour necrosis factor receptor superfamily represents a related group of at least 17 transmembrane cytokine receptors primarily expressed on leucocytes.[257] The family receives its name from a shared ability to bind tumour necrosis factors through cysteine-rich extracellular domains.[135] The receptors may respond to both soluble and membrane bound ligands and the receptors are chiefly expressed on immune cells.[463] Ligation of TNF-receptors tends to have stimulatory effects on

T cells, but may also be inhibitory. Because the expression of many of the TNFR receptors and their ligands is altered, and typically increased, after leucocyte activation, the TNFRs have been referred to as secondary co-stimulatory molecules.[72]

There is marked heterogeneity in sequence between the receptors of the superfamily with little homology in either extra- or intracellular domains consistent with ligand specificity amongst family members and a number of diverse signalling effects.[158] The tumour necrosis factor receptor superfamily has numerous functional roles.[460] These include altering the functional adjuvant qualities of antigen-presenting cells including dendritic cells[179, 294], in the development of lymphoid structures[199], in mediated cell death, in modulating the innate immune response[11], and in mediating apoptotic stimuli.[444] In T cells, TNF receptor signalling modulates survival, proliferation, cytokine production, and cytokine polarisation.[72, 73] As such, compounds altering receptor-ligand interactions have been proposed as potential therapeutic targets for the management of autoimmunity.[99]

OX40 and OX40L

OX40 (*TNFSFR4*) and its ligand OX40L (*TNFSF4*) represent a cognate tumour necrosis factor receptor and ligand pair. OX40 has also been referred to as CD134 and OX40L has also been referred to as CD134L, CD252 and gp34: the names OX40 and OX40L will be used hereafter.

OX40 and OX40L are relatively typical of the TNFR and TNF superfamilies: OX40 has a cysteine-rich extracellular domain, a single transmembrane domain and trimerises when acting to form a receptor. OX40L is a type II transmembrane protein expressed in trimeric form. One trimeric OX40L molecule binds to three OX40 molecules with high affinity and slow dissociation.[471]

Expression of OX40 in health

Since its identification, the expression profile of OX40 has been confirmed as being predominantly expressed on activated lymphocytes, and, amongst these, predominantly CD4+ T cells; recently however there have been reports of expression on intrahepatic iNKT cells.[226] On human CD4+ T cells, there is no expression of OX40 at rest, although in Tregs constitutive expression is reported.[408] Expression is seen on activated, memory and regulatory CD4+ T cells[47, 389, 74], at lower levels on activated CD8+ cells[293] but not on naïve cells. OX40 expression is also a marker of thymic T cells receiving positive selection signals[204]. Further, lower-level OX40 expression is reported on NKT cells[500, 226], NK cells [275] and neutrophils[20].

TCR ligation alone is sufficient to drive OX40 expression on CD4+ T cells, but co-stimulatory ligation of CD28 augments expression, as does CD40-CD40L ligation.[448, 355] IL-2 may induce OX40 on T cells and IL-1 and TNF also contribute.[353, 72] Further, the proteins Roquin 1 and 2 act as posttranscriptional regulators of protein expression and appears to act to degrade OX40 mRNAs: deficiency in functional Roquin results in increased expression.[439]

Reports on the time-course of OX40 expression vary, but typically expression on previously unstimulated CD4+ T cells reaches maximal 48 hours after TCR stimulation in both mouse [121] and human.[360] Murine memory T cells will re-express OX40 within 4 hours of restimulation.[121] Such rapid re-expression of OX40 also appears to be partly regulated by Sp1/Sp3, YY1 and NFκB. NFκB histone acetylation has been demonstrated in memory T cells, which express OX40 in a few hours on stimulation.[418]

Consistent with observations that it is restricted to activated T cells, OX40 expression is often confined to sites of inflammation and immune activation in human disease.[398] In myelin-immunised rats, which go on to develop EAE, OX40 denoted those T cells that were specific for myelin[478] and that OX40 demarcates antigen specificity is also true after Th1-type response promoting Listeria

infection.[264] In humans, OX40 expression on T cells has been reported to identify autoreactive cells in various autoimmune conditions including type 1 diabetes mellitus.[92]

Expression of OX40 ligand

OX40L expression is upregulated after antigen-presentation on multiple antigen-presenting cells: these include B cells [245], macrophages[185] and dendritic cells,[172] The repertoire of cells that can be induced to express OX40L is wider than for OX40 and reports exist of expression on mast cells[186, 298], bronchial smooth muscle[216], malignancies [46], vascular endothelial cells [159], and Langerhans cells[363]. There is constitutive expression on lymphoid tissue inducer cells.[198]

Factors promoting OX40L expression other than antigen presentation and accompanying co-stimulation include IFNγ, in an IFNγ-receptor dependent mechanism[456, 223], prostaglandin E2 [215], thymic stromal lymphopoietin (TSLP)[164] and IL-18[270]. Finally, human serum soluble OX40L increases with age.[453]

Expression of OX40L on T cells

The original description of OX40L was as an unidentified glycoprotein on human CD4+ T cells following human T-lymphotropic virus (HTLV) infection of human CD4+ T cells.[420, 282] This was later identified as OX40L.[19, 407] Subsequently, there have been reports of OX40L expression on both human[211, 210] and murine T cells[197] without viral infection, including marked expression on gut-derived CD8+ intraepithelial lymphocytes.[452]

On antigen-presenting cells, OX40L is minimally expressed on quiescent cells, however expression is upregulated by activation.[352] In addition the cytokines IL-18, tumour necrosis factor-alpha (TNFα), and TSLP are all reported to upregulate OX40L, as is signalling through CD40.[313, 97, 148,

164, 270] Expression is also reported to be increased by may be enhanced by IL-12 exposure, with CD4+ cells showing greater expression than CD8+.[278, 393]. On neonatal CD4+ CD3- lymphoid tissue inducer cells, TNF-like protein 1A (TL1a) induces expression of OX40L.[198]

In contrast to the situation for OX40, factors controlling expression of OX40L on T cells are poorly described. However, similar to OX40, T-cell receptor ligation is required for expression.[452]. These and other factors that appear to influence expression of OX40L on T cells are summarised as Table 1.3.

Similarly, the function of OX40L expression on T cells is uncertain, although one analysis suggested that a reduction in T cell:T cell interaction reduced proliferation and reduced survival both *in vitro* and *in vivo*.[393] Much other work in this area has involved animals with a generalised deficiency or over-expression of OX40L. Effects cannot therefore be attributed to changes in T cell OX40L expression alone.[294, 295]

Functions of OX40-OX40 ligand interactions

Ligation of OX40 produces a range of effects (Figure 1.15). Experiments with a soluble form of OX40L have shown that its engagement amplifies T cell proliferative responses to a range of stimuli.[114] Activation does not affect early proliferation and activation, but controls late proliferation and activation states (Figure 1.15).[355, 393]

In vivo work has shown that OX40 ligation preferentially expands the antigen-specific T cell pool.[120, 269] Correspondingly, there is reduced CD4+ expansion in OX40 or OX40L deficient mice and constitutive OX40L expression by either DCs[40] or T cells[295] results in a greater numbers of activated CD4+ T cells, and transfecting dendritic cell (DC)s with OX40L messenger RNA (mRNA) increases their CD4+ T cell stimulatory potency and increases T cell polarisation.[78]

OX40 ligation results in augmentation of effector cytokine production[121] and prolongation of activation, and this is partially mediated through stabilisation of mRNA.[280]. An important mech-

Table 1.3: Factors reported to influence the expression of OX40L on T cells

Reported to increase OX40L expression on T cells	**Reported to decrease OX40L expression on T cells**
Mouse	
Signalling through TCR.[452]	IL-4.[196]
TH0 conditions i.e. anti-IL-4 and anti-IL-12.[196]	IFNγ.[278]
IL-12.[278]	
STAT4 activation.[278]	
Lower concentrations of antigen or stimulatory antibody.[278]	
Human	
Signalling through the T cell receptor.[211]	IL-12.[211]
DNA damage.[210]	TGFβ.[211]
Co-culture with OX40L expressing Chinese Hamster Ovary (CHO) cell line.[39]	

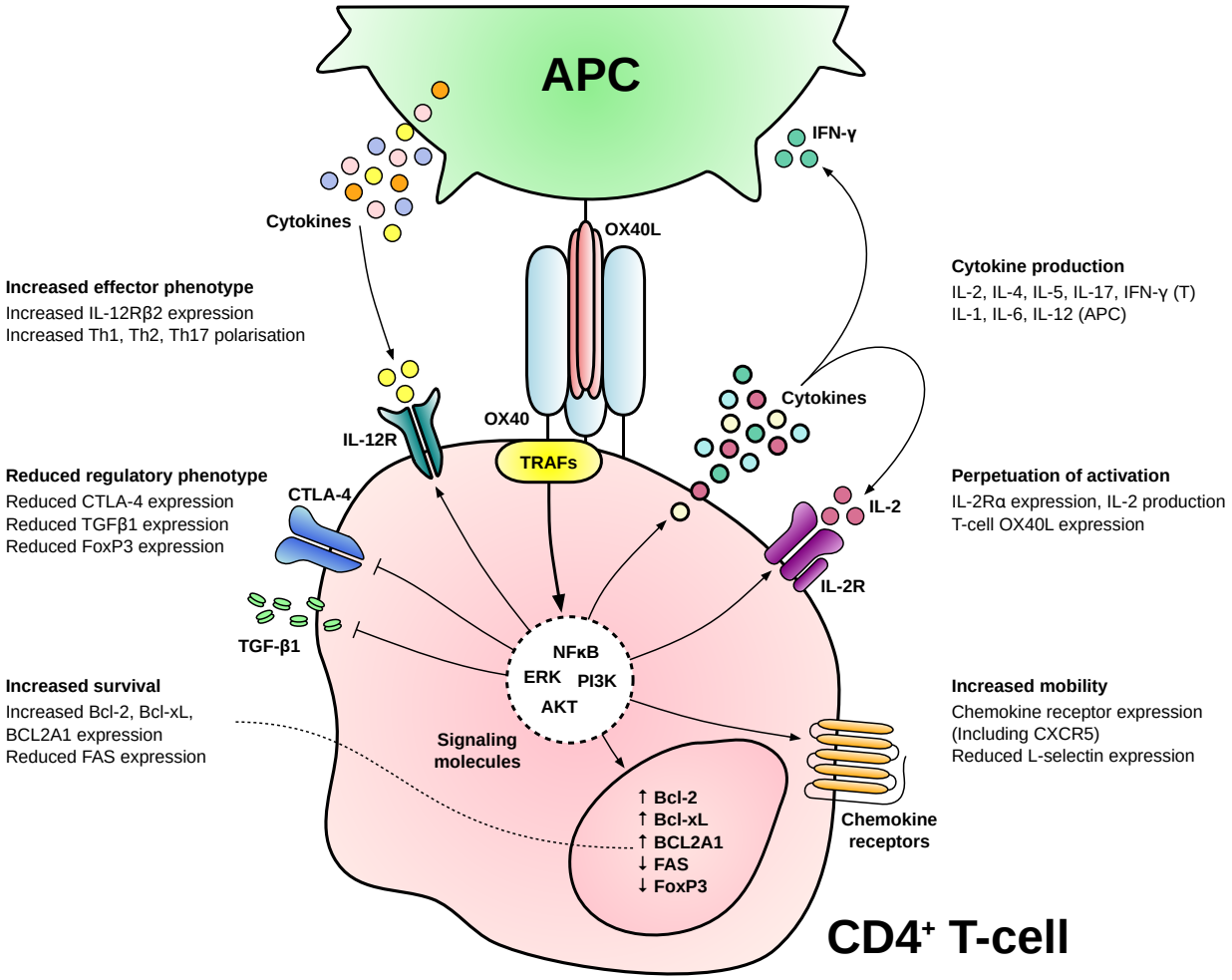


Figure 1.15: Effects of OX40-OX40L interaction. Summary diagram of the effects of OX40-OX40L interaction. Major effects of OX40 ligation are highlighted in bold with effector mechanisms below. Arrows denote positive effects; barred lines denote negative effects; dashed lines show relations. Adapted from Webb et al.[471]

anism by which T cells may prolong activation is through T cell:T cell interactions. By expression of both OX40L and OX40 on activation, T cells may stimulate other T cells, so sustaining activation.[393] Thus, proliferation is reduced in stimulated pure T cell cultures by OX40L blockade. An intriguing observation with relevance to autoimmunity is that T cells activated through OX40 become resistant to subsequent regulation by Treg.[408]

The direction of T cell polarisation is dependent on the cytokine milieu and may favour TH1, TH2 [164, 172], TH9 [487] or TH17 [506] type cytokine profiles depending on circumstances; naïve T cells predominantly produce IL-4.[314] OX40 ligation's general net effect of promoting immune activation was demonstrated in a landmark experiment where a single dose of agonistic OX40 antibody was demonstrated to break tolerance that had been induced to an exogenous peptide.[15]

OX40 and T cell regulation

Both mice and humans lacking OX40 have reduced numbers of natural Tregs alongside a reduction in other non-naïve T cell subtypes.[408, 46] Correspondingly, mice that constitutively express OX40L on T cells have increased numbers of Tregs in their spleens alongside autoimmunity.[295] OX40, together with the other TNFR superfamily members, appears to couple the signal strength of TCR signals and modulate sensitivity to IL-2.[258] Given that both TCR signal strength and IL-2 receptor signalling contribute to thymic Treg selection, this is consistent with the observation that OX40 marks thymic T cells receiving signals of positive selection.[204]

OX40 agonists can drive Treg expansion in TGFβ-treated cultures, although again the cytokine milieu is key. If IFNγ and IL-4 are present, there is preferential expansion of effector CD4+; with blockade of IFNγ and IL-4, there is Treg expansion.[356]. However, OX40 stimulation without IL-2 produces weakly proliferating, poorly suppressive Treg whilst exogenous IL-2 is sufficient to correct this.[487]. Further experiments in lymphocyte cultures treated with the combination of anti-CD3

and anti-CD28 antibodies with exogenous IL-2 have suggested a Treg inhibiting role of OX40: *in vitro* TGFβ-driven conversion to Treg is reduced by OX40 ligation in both mouse and human.[389, 440] In a variety of carcinoma models, agonistic anti-OX40 injected into tumours causes Treg de-activation and depletion, and also mediated tumour regression.[335] Similarly, the expansion and regulatory capability of established 'ICOS+IL-10+' or Tr1 Tregs is inhibited by OX40.[163, 440]

OX40-deficient Treg appear to have reduced suppressive function.

In a study of transfer colitis, Tregs lacking OX40 were ineffective at correcting disease whereas their replete counterparts were effective [124], and in a skin allograft model, OX40 treated Tregs were less able to suppress rejection, effector T cell proliferation or IFNγ production.[442]

OX40 interactions facilitate adhesion and migration

OX40-OX40L interactions facilitate the adhesion of activated T cells to endothelia and their subsequent transmigration. Blockade of OX40L has been demonstrated to reduce T cell adhesion to cultured vascular endothelial cells.[159] In mice with constitutive OX40L expression on dendritic cells, there is greater accumulation of CD4+ T cells in stimulated lymphoid tissue and this has been interpreted as evidence of increased migration, although increased proliferation cannot be excluded.[40] OX40 deficient T cells proliferate faster *in vitro* than OX40-sufficient T cells but survive less well. OX40-OX40L interactions upregulate a number of molecules implicated in migration: CXCR5, which is associated with trafficking to germinal follicles [448] but also sites on inflammation[310]; CXCR4[181]; and RANTES/CCL5[214]. In animals, there is evidence that OX40 deficient T cells may be impaired from reaching sites of inflammation in addition to their reduced effector function.[141, 310, 124]

OX40 and OX40 ligand aberrations in transgenic mice

A key study highlighting OX40-OX40L interactions' role in autoimmunity was performed in 2002 when Murata and co-workers generated mice transgenic for *TNFSF4* with the transgene under the control of the lck promoter.[295] This resulted in the constitutive expression of OX40L on all T cells. Phenotypically, these mice had greatly elevated numbers of CD4+ T cells of which an increased proportion were of a memory phenotype, greatly enlarged lymphoid organs, enhanced antigen-specific T cell responses as measured by proliferation and cytokine production, increased serum antibody concentrations and TH2-type cytokines prior to stimulation and – perhaps most interestingly – multi-lineage infiltrates of both lung and colon; these changes were prevented by the administration of a blocking OX40L antibody. A further interesting observation from the study was that autoimmunity was only induced in C57BL/6 mice and not BALB/c, perhaps relating to the former's greater tendency to produce TH1-type immune responses. Of note, autoimmunity may be similarly induced with constitutive expression of other TNFR ligands such as LIGHT.[374]

If OX40L is constitutively expressed on dendritic cells, increased numbers of CD4+ T cells are seen in B-follicles and these cells are of a more activated phenotype after immunisation with an antigenic nitrophenol conjugate but not at rest or after lipopolysaccharide alone. In contrast to mice with over-expression of OX40L on T cells, there is not overt autoimmunity.[40]

Mice deficient in OX40 were generated in the late 1990s. Such mice breed normally and appear able to generate both immunoglobulin M (IgM) and IgG subclass responses to pathogens such as vesicular stomatitis virus and also to haptenised proteins with maintained germinal centre formation.[213, 336] CD8+ cytotoxic lymphocyte responses are maintained, but stimulated CD4+ T cells show reduced proliferation and IFNγ responses to viruses. However, viral response in OX40 deficiency appears variable and numbers of infiltrating cells on bronchoalveolar lavage in response to influenza virus were reduced in OX40 deficient animals.[213] Similarly, the generation of CD4 memory is greatly impaired in total OX40 deficiency, although haplosufficiency of OX40 appears sufficient

for a phenotypically normal CD4+ response.[120]

Rationale for targeting OX40-OX40L interactions in autoimmunity

OX40-OX40 ligand interactions form part of pathogenic pathway in a number of human diseases and animal models. Thus, whether aberrations in such interactions form part of aetiopathogenesis and initiation of autoimmunity themselves as in constitutively OX40L expressing mice or whether they are resultant on other pathways, preventing their activation may ameliorate disease.[295]

Firstly, blockade of OX40-OX40L interactions ameliorates autoimmunity in a number of models of human autoimmunity (Table 1.4). As identified above, in certain models the effect of OX40-OX40L blockade is superior to T cell depleting therapies alone.[105, 131] This supports exploration of interruption of OX40-OX40L signals in human diseases characterised by loss of T cell regulation.

Secondly, OX40-L should provide inherent targeting to areas of immune activity: expression is largely confined to activated cells and especially autoantigen-specific cells.[478, 264] Certainly increased expression of both OX40 and OX40L is reported in a number of human autoimmune conditions (Table 1.5). Such site-specificity has been demonstrated clinically by OX40 upregulation on the T cells of inflamed tissue but not in peripheral blood in both human colitis and rheumatoid arthritis.[111, 454].

Thirdly, the ideal therapy in autoimmunity would provoke the re-establishment of immune tolerance. The observation that activation through OX40L may render T cells resistant to regulatory signals makes this a logical target.[408, 454] Further, in systems such as CD40L-deficient islet cell allograft recipients, OX40 agonism or blockade alone is enough to determine graft tolerance.[59]

Fourthly, migration of activated T cells across endothelia appears to be at least partly dependent on OX40-OX40L.[159, 160] This observation, coupled with apparent selective tissue expression in

certain autoimmune disease states suggests that inhibition of the interaction might reduce migration into inflamed areas.[10] Work in EAE mice suggests that pathogenic T cells may persist after OX40 blockade, but that they are no longer able to migrate to target sites.[307]

Fifthly, OX40-L blockade appears to be effective after disease onset – e.g. in a diabetes model.[322] Although several animal studies have demonstrated that OX40-L inhibition may ameliorate autoimmunity, many have used OX40/L blockade at, or before, disease onset: something that differs from the clinical situation in which a patient will present for treatment after symptom onset making the observation in diabetic mice particular important.

Sixthly, topical therapy is feasible and effective in mouse models e.g. OX40-agonists intra-tumour[335] or intravitreal OX40L blockade in a uveitis model have powerful disease-modifying effects from topical application alone.[502]

Seventhly, and of particular importance, is the fact that OX40 and OX40L have been recorded as being upregulated in a variety of human autoimmune disease (Table 1.5). To date, no assessment of expression in human liver tissue affected by autoimmune disease or other pathologies has been reported.

A final and particularly promising aspect of targeting OX40-OX40L for the therapy of autoimmunity is the lack of severe side effects in the animal studies reported above and the long-term relative health of OX40 deficient mice and the single OX40 deficient human reported to date (with the caveat of the development of treatment-resistant Kaposi's sarcoma) suggests a low side-effect burden.[213, 46] The one trial of humanised anti-OX40L did not cause significant adverse effects.[107]

1.7 Animal models of autoimmune liver disease

In order to make preliminary assessments of the efficacy of therapeutics and their immunological effects taking into account the multiple immunological interactions within an organism, *in vivo*

experiments are necessary. This thesis aims to address whether liver autoimmunity may be reduced by interruption of OX40-OX40L interactions and therefore animal models that recapitulate features of human AILD are required.

Numerous investigators have sought to develop models of autoimmune liver disease. For PBC the major requirements of an animal model have been interpreted as peri-biliary inflammation in the context of the development of AMAs. In AIH, T lymphocyte-mediated liver damage – as demonstrated by elevations in serum transaminase activities – has been interpreted as sufficient to describe a model as reminiscent of human autoimmune hepatitis[139], but in other models the presence of a lymphocytic infiltrate, hypergammaglobulinaemia and the development of fibrosis have improved representativeness.[470]

There is a convergence between described mouse models of PBC and of AIH towards models in which T cell regulation is derranged.[470, 235, 338] A multitude of different interventions in this pathway produce lymphocytic peri-biliary hepatitis alongside autoimmunity in other organs, with the expression of AMAs alongside other autoantibodies in many models (where they are reported). Thus parallels may be drawn between mice lacking functional FOXP3[504], IL-2, the IL-2 receptor alpha subunit[446], those expressing a dominant negative TGFβRII subunit on T cells (which impairs the generation of regulatory T cells), AE (which is key for Treg survival) [361], thymectomised mice lacking PD-1, AIRE-deficient mice, mice with medullary thymic epithelial TRAF6 dysfunction, and mice with global TGFβ dysfunction (Tables 1.6 & 1.7).

Notably, similar to observations in human diseases, the various mouse models of PBC and AIH demonstrate upregulation of cytokines associated with all T cell subtypes[504] and in numbers of T cells with these phenotypes[491], but with the predominant subtype being Th1. In addition, many of these mouse strains have documented evidence of extra-hepatic autoimmunity consistent with their reported hepatic inflammation being a product of a systemic tendency to autoimmunity.

Of relevance to considering the role of OX40 in autoimmunity involves considering its hyper-expression.

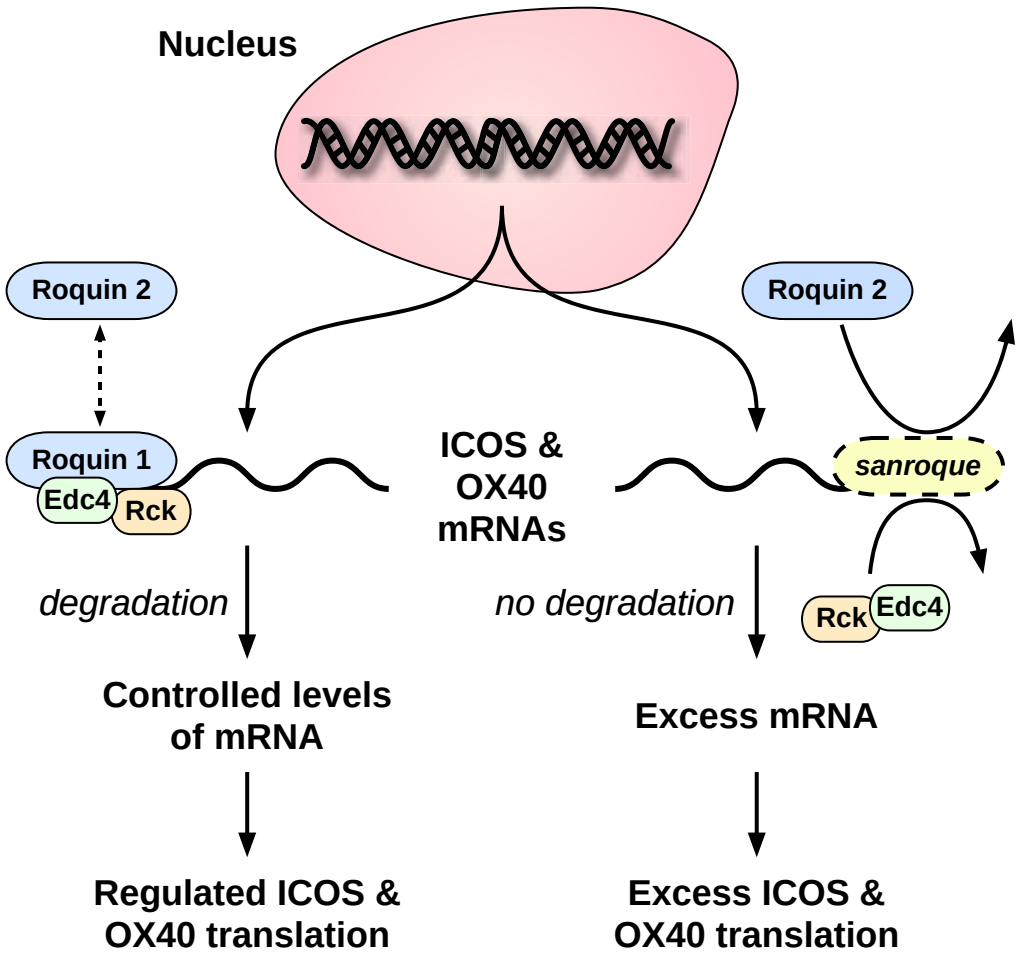


Figure 1.16: Schematic depiction of post-transcriptional regulation of ICOS and OX40 mRNA by Roquin. mRNA encoding both ICOS and OX40 is transcribed in the nucleus. Usually, a proportion of this is degraded before translation into functional protein in a process of post-transcriptional regulation. Either Roquin 1 or Roquin 2 binds the 3-prime untranslated regions of ICOS or OX40 mRNA where they stabilise the binding of two RNA decay proteins known as Edc4 and Rck and collectively facilitate degradation. Reducing the amount of mRNA to be translated into protein regulates the amount of ICOS and OX40 ultimately produced. Because the actions of Roquin 1 and 2 in this regard are redundant (although Roquin 1 binding is preferential), if one or the other is dysfunctional, the other may take its place. In the *sanroque* mutation, there is a mutation in Roquin 1. The mutant Roquin 1 protein retains the ability to bind mRNA but does not stabilise binding of Edc4 and Rck and so does not degrade mRNA. As such, more mRNA is available for translation into functional protein and hyper-expression with resultant autoimmunity follows.[24, 439, 341, 167] (Image adapted from [24])

An additional model of loss of immune control is the Roquin *sanroque* mutant mouse. The RNA-binding proteins Roquin 1 and Roquin 2 usually function to stabilise proteins that act to degrade messenger RNA for the co-stimulatory molecules OX40 and ICOS acting as a limiting factor on their expression (Figure 1.16).[497, 439] However, in mice with a single amino acid substitution from methionine to arginine (M199R) in the ROQ domain of the regulatory protein Roquin 1 – so-called Roquin mutant or *sanroque* mice – there is reduced degradation of both OX40 and ICOS with resultant autoimmunity. These mice homozygous for the *sanroque* mutation ($Roquin^{M/M}$) develop splenomegaly, lymphadenopathy, autoantibodies to nuclear proteins, glomerulonephritis and lymphocytic hepatitis.[438, 497] In addition, the wide expression of ICOS ligand means that signals through ICOS are widely available and are able to replace some of the requirement for co-stimulation through CD28.[244] Notably, the function of mutant Roquin is to actively stabilise mRNA and is more than just a loss of pro-degradation function of the intact wildtype protein: total deficiency in Roquin does not result in similar autoimmunity.[25]

Of additional interest when considering the $Roquin^{M/M}$ mice as potential models of liver autoimmunity is that as well as hyper-expression of OX40 and ICOS, these mice over-express IFNγ. As mentioned above, it has recently been shown that hyperexpression of IFNγ alone is sufficient to cause lymphocytic hepatitis and the development of AMAs.[14]

1.8 Summary

The adaptive immune response may be misdirected against self-antigens. The result is autoimmunity. Autoimmunity may cause organ damage and major clinical disease and its prevalence is increasing. Autoimmune liver disease is also becoming more prevalent, with a proportion of patients resistant to standard therapies and going on to require transplantation to avoid death.

Of the autoimmune liver diseases, the two most frequent – AIH and PBC – display multiple characteristics that suggest a key role for CD4+ T cells in their pathogenesis. The activation of CD4+ T cells may be controlled by the actions of regulatory T cells and by modulating signals through co-stimulatory molecules expressed on CD4+ T cells such as CD28 and the tumour necrosis factor receptor OX40. Many existing mouse models of human autoimmune liver disease use manipulations of CD4+ T cell regulation to produce their phenotype.

OX40-OX40L interactions have important pro-effector activity for T cells and modulation of signals through the receptor-ligand pair has been shown to ameliorate autoimmunity in a number of mouse models of human autoimmune diseases. In addition, their expression is abnormally upregulated in a variety of human non-liver autoimmune diseases. A potential role for OX40 in autoimmune liver disease in either mouse models of autoimmune liver disease or human patients has not been investigated to date. Using mouse models of defective T cell regulation provides an experimental platform in which a potential role for blocking OX40-OX40L for therapeutic effect may be investigated.

1.9 Aims

The aims of this thesis relate to the control of CD4+ T cells by Tregs and by the interactions of OX40 and OX40L.

1. To establish a Treg deficient model of liver injury that re-capitulates findings reported elsewhere, to demonstrate that the disease of Treg deficiency could be transferred between animals, and to explore requirements for its generation.

2. To assess whether prevention of OX40-OX40L signals could control autoimmune liver disease in the context of Treg deficiency.

3. To assess whether prevention of OX40-OX40L signals could control disease in a second mouse model of regulatory failure resulting in generalised and hepatic autoimmunity: the CTLA4-deficient mouse.

4. To assess whether prevention of OX40-OX40L signals could control disease in a mouse model of regulatory failure resulting from excessive co-stimulation and IFNγ production: the Roquin$^{M/M}$ mouse.

5. To assess factors controlling OX40L expression on CD4+ T cells.

Table 1.4: Effects of blocking OX40-OX40L on models of autoimmune disease

Disease and model	Intervention	Effect
Systemic lupus erythematosus		
BXSB mouse	Combined OX40 and CTLA-4 blockade of splenocytes *in vitro*	Reduced proliferation with combined blockade but not either agent alone.[509]
NZB×NZW F1 mouse	Agonist OX40 exacerbated renal disease and increased inflammatory cytokines; inhibitory anti-OX40 fusion protein ameliorated disease.[386]	
Colitis		
Dextran sulfate sodium-induced colitis in mice	Blocking OX40-IgG fusion protein	Reduced clinical score, T cell migration to lamina propria and T-bet mRNA transcription[310]
Transfer colitis in mice	Neutralising OX40L Ab	Reduced histological score, T cell infiltrates and weight loss[260, 419]
	Transfer of OX40-deficient Tregs	Unable to control colitis in contrast to intact Tregs[124]
IL-2$^{-/-}$ mice	Antagonistic OX40-IgG fusion protein	Amelioration of histology, reduced T cell infiltrate and reduced pathogenic cytokines including IL-12, IFNγ and TNFα[141]
Hapten-induced (intrarectal trinitrobenzene sulfonic acid)	Antagonistic OX40-IgG fusion protein	Amelioration of histology, reduced T cell infiltrate and reduced pathogenic cytokines including IL-12, IFNγ and TNFα[141]
Rheumatoid arthritis		

Disease and model	Intervention	Effect
Collagen-induced arthritis	OX40L blocking mAb	Amelioration of clinical score, less production and collagen-specific IgG2a when Ab administered at day -1[495]
	OX40L:Ig fusion protein	Abolished evidence of disease[131]
	Pegylated OX40 blocking Fab fragment	Reduction in joint inflammation and degradation of bone and cartilage[131]
Uveitis		
EAU in rats	OX40 agonistic antibody	Prolonged inflammation, increased Th1 and Th17 cells; increased IFNγ in cell cultures exposed to antigen[485]
EAU in mice	OX40L blocking antibody	Worsening of clinical score and IFNγ production with early administration; when given late had no effect[428]
	OX40L deficiency	Worsening of clinical score and IFNγ production with early administration; when given late had no effect[428]
Intravitreal ovalbumin in OTI mice	OX40L blocking antibody	Amelioration of clinical score[502, 506]
	OX40 agonistic antibody	Worsened clinical score, extent of lymphocytic infiltrate and of Th17 cytokines[502, 506]
Type 1 Diabetes Mellitus		
NOD mouse	Genetic co-deficiency for *TNFSF4*	Prevention of the development of diabetes[267]
	OX40L blocking antibody at 12 weeks of age	Reduction in incidence of diabetes[322]

Disease and model	Intervention	Effect
Multiple sclerosis		
EAE in rats	Soluble OX40 receptor	Increased survival and ameliorated clinical score[479]
EAE in mice	OX40L deficiency	Reduced clinical score, reduced IL-2 and IL-6[305]
	OX40L Ab blockade	Reduced clinical score, reduced spinal cord T cell infiltration[307, 91]
Anti-GBM disease (Goodpasture syndrome)		
Mice given ovine anti-GBM antibody.	OX40L antibody blockade.	Exacerbation in intact mice and $CD86^{-/-}$ mice; amelioration in those also co-deficient in CD80 and CD86.[311]
IPEX		
FOXP3 deficient mouse	OX40 co-deficiency	Prolonged survival, reduced organ T cell infiltration, reduced T cell activation, reduced autoantibody titres, reduced clinical manifestations.[105]
	OX40L Ab blockade	Similar to effects of OX40 co-deficiency.[105]

Table 1.5: OX40 and OX40L disturbances in human autoimmunity

OX40	OX40L
Systemic lupus erythematosus	
Increased OX40 positivity on peripheral T cells with correlation to clinical severity markers[10, 328, 2, 508, 95, 219, 279]	Increased endothelial expression in kidney biopsies from lupus nephritis patients with the appearance of glomerular positivity[10]
OX40 on infiltrating perivascular leucocytes in lupus nephritis[10]	Increased peripheral sOX40L, with further elevations in those with nephritis[95]
More activated phenotype with increased PI3/Akt activation[219]	
Colitis	
Expression increased on lamina propria lymphocytes in inflamed colon	Increased expression on vascular endothelium from inflamed –but not uninflamed – colon[394]
Increased proportion of colonic T cells OX40 positive in UC; peripheral counts unchanged[455]	
Increased biopsy staining of active colitis and unchanged by corticosteroid therapy.[398]	
Coeliac disease	
OX40-positive lymphocytes present in disease but not control duodenal biopsies[398]; peripheral OX40 expression not affected[332]	
Rheumatoid arthritis	
Increased OX40-positivity on lymphocytes in synovial fluid from inflamed joints[41, 495, 111, 326, 229]	OX40L expression on sublining layer of sinovium from inflamed joints[495, 212]
Reduced sOX40[229]	Increased sOX40L with correlation with autoantibody status[229]
Inflammatory myositis	

OX40	OX40L
OX40 expression present in inflammatory myopathies but not controls [411, 324]	OX40L seen on T cells, B-cells, macrophages and myeloid dendritic cells in inflammatory myopathies but not controls[324]
Uveitis	
Multiple OX40-positive infiltrating lymphocytes from ciliary bodies of eyes enucleated for uveitis[485]	
Type 1 Diabetes Mellitus	
Increased proportion of CD4+ cells positive in peripheral blood of newly diagnosed paediatric cases[403]	
Systemic sclerosis	
Elevated serum sOX40 concentrations in comparison with SLE and healthy controls[209]	
Graves' Thyroiditis	
Increased OX40-positivity of circulating CD4+ T cells in patients with anti-TSHR antibodies[35, 454]	Increased OX40L-positivity of circulating CD4+ T cells in patients with anti-TSHR antibodies[454] Elevations of sOX40L[453]
Multiple sclerosis	
Reduction in OX40+CD26+ peripheral CD4+ T cells correlating with clinical response after treatment with natalizumab[32]	OX40 expressed in MS brain and cord sections[337]
Neuromyelitis optica	
Increased OX40 positivity of pathological brain specimens[337]	
Sjögren syndrome	
Increased OX40 expression on peripheral T cells[511]	Increased OX40L expression on peripheral B-cells and monocytes[511]
Myaesthenia gravis	

OX40	OX40L
Increased thymic OX40 staining[318]	OX40L present in myasthenia gravis thymus but not control[318]
Increased OX40-staining on peripheral CD4+ T cells[488]	
Granulomatosis with polyangiitis	
Increased OX40 expression on CD8+ peripheral T cells[112] and on CD4+ T cells[481]	
Henoch-Schönlein purpura	
Increased OX40 positivity on circulating CD4+ T cells[344]	Increased sOX40L[344]

Table 1.6: Mouse models of primary biliary cholangitis

Model	Phenotype	Ref(s)
T cell regulation mediated		
FOXP3 dysfunction	Affected male mice develop multi-system autoimmunity with CD8+ T cell rich peri-biliary lymphocytic infiltrates, serum AMAs, elevations in IFNγ, IL-12, and TNFα with death aged 3-4 weeks	[504]
Dominant negative TGFβ receptor II under CD4 promotor	At 24 weeks, mice had developed T cell dependent peri-biliary lymphocytic infiltrates with a CD8 T cell bias; AMAs, sp100 and gp210 ANA autoantibodies present; increases in serum IFNg, TNFα, and IL-6	[312, 156]
Non-obese diabetic crossed with c3c4 mice	Intra- and extrahepatic bile duct injury associated with dense CD8+ T cell rich infiltrates and the development of AMAs	[161]
AE2 exchanger dysfunction	CD8+ T cell rich but Treg poor peri-biliary lymphocytic infiltrate with BEC damage, AMAs, elevations in serum IgG and death aged 3-4 weeks; notably the AE2 exchanger is important to regulatory lymphocyte survival	[361]
IL-2Rα deficiency	Variable age of onset multi-system autoimmunity with peri-biliary CD8+ T cell rich lymphocytic infiltrate, AMAs, increased serum IFNγ, TNFα, IL-2 and IL-12; 25% mortality at 20 weeks	[446]
IFNγ over-expression through deletion of 3' regulatory region	Female-predominant periportal lymphocytic (subtypes not reported) inflammation at 20 weeks with AMAs; extra-hepatic autoimmunity not detailed; transferable by adoptive transfer of CD4 T cells	[14].

Model	Phenotype	Ref(s)
MRL/lpr mice homozygous for dysfunctional Fas/CD95	Defective T cell apoptosis. At 24 weeks, CD8+ and CD4+ T cell rich peri-portal lymphocytic infiltrates, AMAs alongside ultimately lethal multi-system autoimmunity including vasculitis, glomerulonephritis, arthritis and sialadenitis	[423]
MHC mismatch adoptive transfer into sublethally irradiated hosts	Although described as model of PSC, mice develop dense periportal lymphocytic infiltrates 12 weeks following cell transfer	[308]
Xenobiotic and adjuvant mediated		
Immunisation with 2-octynoic acid and Complete Freud's adjuvant (CFA)	12 weeks post immunisation, peri-biliary CD8+, and activated CD44+ lymphocyte dominant infiltrates with TH1 cytokine elevations in serum, AMA production; no extra-hepatic autoimmunity	[447, 445]
Novosphingobium aromaticivorans injection of NOD 1101 mice	NKT cell dependent hepatic peri-portal lymphocytic infiltrates and bile duct damage with serum AMA development; hepatosplenomegaly	[268]

Table 1.7: Mouse models of autoimmune hepatitis

Model	Phenotype	Ref(s)
Cytokine release		
Concanavalin A injection	Rapid (hours), dose-dependent T cell and NKT cell depenedent necrotic injury of periportal hepatocytes not associated with autoantibodies; blockade of IFNγ and TNFα reduces phenotype	[415, 285]
Lipopolysaccharide injection	Rapid (hours), dose-dependent macrophage derived TNFa-driven hepatocyte toxicity without autoantibodies	[297]
Hepatic antigen manipulation		
Neoantigen expression with specific T cell transfer	T cell dependent injury with fulminant hepatocyte necrosis, lymphocytic infiltrate, elevations in serum IFNγ, TNFα; autoantibody profile not reported; reported with hepatitis B virus surface antigen and ovalbumin and subsequent adoptive transfer of appropriately specific T cells	[5, 83]
Disruption of T cell regulation		
Constitutive over-expression of CD28 ligands	CD4+ and CD8+ T cell dense lymphocytic hepatic infiltrate with necrosis and fibrosis, elevated IFNγ production, elevations in serum transaminase activities	[68]
Neonatal thymectomy with or without PD-1 deficiency	Autoantibodies to liver-specific membrane lipoprotein and mild lymphocytic infiltrate; with concurrent PD-1 dysfunction, fatal necrotic hepatitis with dense CD4+ and CD8+ hepatic infiltrate that could be transferred by transfer of total lymphocytes and controlled by co-transfer of Tregs	[461, 193]
AIRE dysfunction	CD8+ T cell predominant lymphoplasmacytic hepatic infiltrates, increased Tregs, elevated serum TNFα and IL-2, autoantibodies to multiple intrahepatic antigens; reversible by transfer of intact Tregs.	[348, 132]

Model	Phenotype	Ref(s)
TGFβ deficiency	CD4+ T cell and IFNγ dependent hepatitis with CD4+ predominant lymphocytic infiltrate, serum transaminase activity elevations; autoantibodies not reported	[117, 358]
Targetted TRAF6 disruption on medullary thymic epithelium	CD4+-predominant hepatic lymphocytic infiltrate, elevated serum IgG, ANA, fibrosis. Preserved liver Treg numbers. Corticosteroid responsive.	[29]

2 Materials and Methods

2.1 Animals

Animal care

All animals were housed and maintained at the Biomedical Services Unit (BMSU), University of Birmingham. Animals were kept and bred in accordance with UK Home Office Guidelines. Experimental procedures were performed in accordance with pre-specified UK Home Office project licences.

Adult mice were used aged 6-12 weeks. For Roquin mutant mice, mice were aged to 16-20 weeks to permit development of the Roquin mutant phenotype. For $CTLA4^{-/-}$ and $FOXP3^{KO}$ phenotypes, mice were used at 3-4 weeks of age to prevent full development of their otherwise deleterious phenotypes.

Preparation

Where blood or serum was not required for analysis, mice were killed by cervical dislocation; where blood or serum was required for analysis, mice were killed by cardiac puncture under terminal isoflurane anaesthesia. In some instances, blood was collected from the saphenous vein from live mice into heparinised containers to permit flow cytometry.

Serum was prepared by allowing blood to clot on ice. Samples were then centrifuged at 8000 × g for 5 minutes, and the supernatant then centrifuged for a further 5 minutes at 8000 × g. The supernatant sera were then frozen on dry ice before being kept at −20 °C until further analysis.

Survival analysis

Where survival was used as an outcome measure, the condition of mice was assessed by the staff of the University of Birmingham Biomedical Services Unit who were unaware of the treatment groups

to which individual animals belonged. Mice were observed for weight loss of 20% initial weight or greater, continued piloerection, intermittent hunched posture, subdued behaviour for up to 48 hours, intermittent changes to respiration, or deterioration in the condition of fur or eyes. If any of these signs of deteriorating condition were present, the mice were culled and analysed.

Tissues

Mice were dissected to obtain liver, spleen, thymus and lymph node tissues. Mice were sprayed with 70% *v/v* ethanol/distilled water (dH_2O) solution to prevent fur contaminating the abdomen, laid on their back and a midline laparotomy incision made.

The spleen was isolated by blunt dissection and removed *in toto*. The intestine was then unpacked and the mesenteric lymph node chain removed *in toto.* The liver was then retroflected, the gallbladder ligament cut, the stomach dissected free and the liver removed *in toto*.

The liver was washed extensively in Roswell Park Memorial Institute medium (RPMI) before being divided into lobes (Figure 2.1). The left lobe was fixed in formaldehyde solution for use in immunohistochemical analysis, the median lobe frozen, and the caudate lobe used for preparations of cell suspensions.

The incision was then continued to a midline thoracotomy and the two lobes of the thymus exposed by blunt dissection and removed individually.

Induction of chemical hepatitis

For the induction of concanavalin A hepatitis, concanavalin A (Sigma) was dissolved in sterile phosphate-buffered saline (PBS) and administered intravenously at a dose of 10 mg kg^{-1}. Mice were observed for 24 hours and then sacrificed by cardiac puncture under teminal anaesthesia.[285]

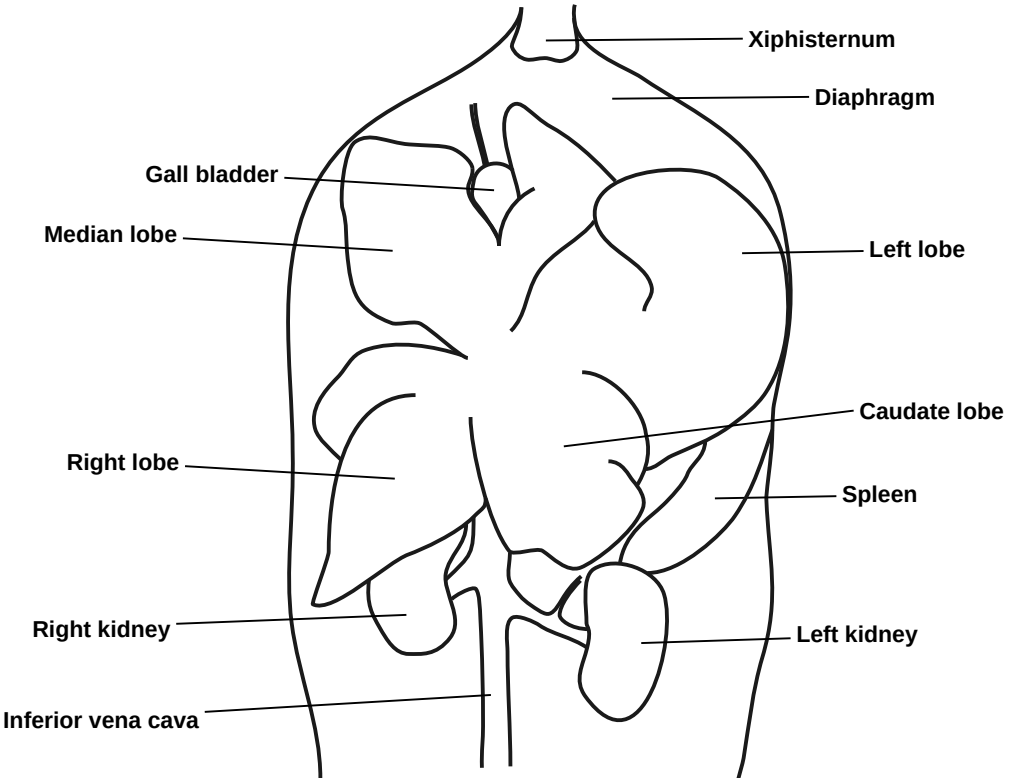


Figure 2.1: Basic mouse liver anatomy after laparotomy, removal of the gastrointestinal tract, and retroflexion of the liver.

For the induction of carbon tetrachloride hepatitis, a solution of 1:3 mineral oil:carbon tetrachloride (CCl_4) (both Sigma-Aldrich) was prepared. Mice were administered the resulting solution at 1 mg kg^{-1} by intraperitoneal injection. Mice were observed for 48 hours and then sacrificed by cardiac puncture under isoflurane terminal anaesthesia.[365]

Genotyping

Genotyping was performed on ear punch specimens by Dr F McConnell using the commercial Transnetyx® platform.

Mouse strains used

All mice were used on an inbred C57BL/6 genetic background. Details are recorded in Table 2.1.

2.2 Human samples

Liver

Samples of explant livers removed at the time of transplantation for various indications were obtained from the tissue bank of the Centre for Liver Research, University of Birmingham. Control samples of normal liver were obtained from either disease-free tissue removed during cancer resection surgeries or from potential graft organs that were not ultimately used for transplantation.

Liver biopsy samples were obtained as part of the Comprehensive Assessment of the Liver with Magnetic Resonance Imaging (CALM) study run by the University of Birmingham. Those taking part gave written informed consent prior to particpation.

Table 2.1: Mouse strains used

Strain	Gross phenotype	Reference(s)
$RAG1^{-/-}$ (RAG^{KO})	Complete deficiency in T cells and B cells; appear well	[289]
$ZAP70^{-/-}$ ($ZAP70^{KO}$)	Lack CD4+ and CD8+ T cells; appear well	[183]
$OX40^{-/-}$ ($OX40^{KO}$)	Mice appear well	[213]
$CTLA4^{-/-}$ ($CTLA4^{KO}$)	Lethal multi-organ autoimmunity associated with hepatitis and T cell activation at a few weeks of age.	[416]
$FOXP3^{y/-}$ ($FOXP3^{KO}$)	Lethal multi-organ autoimmunity associated with hepatitis and T cell activation at a few weeks of age.	[62]
$AIRE^{-/-}$ ($AIRE^{KO}$)	Mice appear well; eventual development of autoimmunity including hepatitis in some	[348]
$CD80^{-/-}CD86^{-/-}$	Mice appear well	[33]
$CTLA4^{-/-}CD80^{-/-}CD86^{-/-}$	Mice appear well; they were crossed in-house but phenotypically reflect those reported elsewhere.	[261]
$FOXP3^{-/-}OX40^{-/-}CD30^{-/-}$	Mice appear well	[105]
FOXP3-GFP	All Tregs labelled with green fluorescent protein	[26]
BoyJ	Mice appear well; all leucocytes allotype marked as CD45.1; used as wildtype controls	[378]
C57BL/6	Mice appear well; CD45.2 allotype; used as wildtype controls	Charles River or in-house
$CTLA4^{-/-}OX40^{-/-}CD30^{-/-}$	not reported	In-house
$OTII.RAG^{-/-}$	Mice appear well	[288, 17]
Roquin *sanroque* mutant	Splenomegaly, lymphadenopathy, multi-system autoimmunity	[438]
$Roquin^{M/M}OX40^{-/-}CD30^{-/-}$	not reported	In-house
VAV1-Cre $OX40L^{(}fl$)	Mice appear well	In-house via Dr D Withers

Blood and serum

Blood was obtained from patients as part of the Cellular Trafficking and Immune Responses in the Human Liver project hosted at the University of Birmingham (approval: 06/q2708/11). Healthy volunteers were used for control samples and gave informed consent.

Serum was obtained by taking blood into a serum-separator tube and allowing it to clot at room temperature. The tube was subsequently centrifuged at 400 × g for 5 minutes, the supernatant pipetted off, re-centrifuged and stored at −80 °C until required.

Clinical characterisation

Blood results and results of other clinical investigations were obtained including values for upper limit of normal (ULN) and lower limit of normal (LLN) as appropriate either directly from clinical records (with written consent) or from a pseudonymised clinical database for biopsy specimens.

2.3 Enzyme-linked immuno-absorbance assays

For analysis of murine serum TNFα concentrations, IgG concentrations and IgE concentrations, eBioscience ready-set-go enzyme-linked immunosorbent assay (ELISA) kits were used. ELISA for IgE was performed by the laboratory of Dr Jessica Strid at Imperial College London.

Briefly, 96-well flat-bottomed plates were coated overnight with capture antibody in PBS. Plates were washed thoroughly with PBS 0.5% Tween 20. Plates were then blocked with a protein-containing solution from the kits. Serum samples were then incubated for ≥60 minutes and washed thoroughly. A standard curve was constructed by creating serial two-fold dilutions. A secondary detection antibody was then added for a further 1 hour and plate again washed thoroughly. Wells were then

filled with the provided 3,3',5,5'-tetramethylbenzidine (TMB) containing solution and the reaction observed. At either 15 minutes or the onset of strong colour change in a large number of wells, sulphuric acid was added to the well to prevent further reaction. Plates were then read on a plate-reader at 450 nm.

For analysis of murine AMAs, a modification of a clinical anti-M2-3E ELISA kit (EurImmun) was used. The secondary antibody was replaced with anti-IgG, anti-IgM and anti-immunoglobulin A (IgA) horse-radish peroxidase conjugated antibodies (all Abcam) to permit measurement of the three respective isotypes. The kit contains a 96-well plate pre-coated with recombinant PDC-E2. Sera were diluted in phosphate-buffered saline and the plate incubated for 1 hour.

For analysis of human soluble OX40L an ELISA kit from Cusabio was used according to the manufacturer's instructions – a similar protocol as that described above.

Where quantification was performed, the values of two-fold dilutions of solutions of known initial concentration were plotted and a standard curve fitted using Prism (GraphPad). Values for other wells were then interpolated against this curve.

2.4 Histology

Frozen tissue sections

Tissues were frozen after dissection by freezing on metal foil placed over dry ice before subsequent storage at −80 °C. Tissues for staining were sectioned by cryostat to 5 µm, mounted on glass slides, fixed in acetone and air-dried overnight. Sections were then stored at −20 °C until required. For staining, sections were rehydrated with phosphate-buffered saline. For some tissues, non-specific streptavidin-biotin binding was blocked with a Streptavidin/Biotin Blocking kit (Vector Labs). Tissues were then blocked with 10% horse serum for 10 minutes before sequential 30-60 minute staining

with primary and subsequent antibodies in 1% bovine serum albumin PBS solution. Secondary and subsequent antibodies were pre-absorbed in 10% *v/v* mouse serum/PBS on ice. Nuclear staining visible after ultraviolet excitation was performed using 4',6-diamidino-2-phenylindole (DAPI). Images were taken using a Zeiss 780 confocal microscope (Carl Zeiss).

Formaldehyde-fixed paraffin-embedded tissue sections

Samples for examination were dissected and placed in 10% formalin-saline solution (4% formaldehyde; Ferndale) for at least five days. Sections were then paraffin embedded either by the staff of the Department of Histology, Royal Orthopædic Hospital National Health Service (NHS) Foundation Trust or the staff of the Centre for Liver Research, University of Birmingham. Samples were then sectioned to 4 μm with a PFM Rotary 3003 microtome onto charged slides before oven-drying for 24 hours at 60 °C.

Samples were sequentially de-waxed by bathing in Clearene® (Leica), then the Clearene® displaced with absolute ethanol, and then rehydrated in tap water. Endogenous peroxidase activity was blocked by bathing for 10 minutes in 0.3% *v/v* hydrogen peroxide / methanol solution (Sigma). Antigen retrieval was performed either in a high-pH ethylenediaminetetraacetic acid (EDTA)-based buffer or a low-pH citrate-based buffer (both Vector Labs). Antigen retrieval buffers were pre-heated for 5 minutes in an 800 W microwave, slides added, and the solution then heated for a further 20 minutes. Non-specific staining was performed with a 2.5% goat serum / casein solution (Vector Labs). Primary antibodies were applied in PBS for 30-60 minutes. Slides were then washed thoroughly in phosphate-buffered saline. Secondary antibodies conjugated to either horseradish peroxidase (HRP) or ALP were then applied in phosphate-buffered saline for 30-60 minutes. Either 3,3'-diaminobenzidine (DAB) or Vector Red ALP substrate (both Vector Labs) were used as chromogens; DAB substrate was applied for 5 minutes and Vector Red for 15 minutes. In some circumstances, both chromogens were used sequentially. For human liver biopsy sections, an automated staining

system using the same parameters was used (Dako Autostainer Link, Dako; operated by Dr Gary Reynolds). Slides were counter-stained with Mayer's haemotylin solution and developed in a bath of tap water at 37 °C. Where haematoxylin and eosin staining was employed, slides were prepared similarly and Harris haematoxylin was used prior to staining with eosin solution.

Where histology was graded according to extent of OX40 expression, this was performed according the greatest number of OX40 positive cells in any one inflammatory aggregate. For explant samples these were categorised as a score of 0 for no positive cells, 1 for 1-5 as a single focus, 2 for 5-10 and 3 for >10 by a single pathologist who was unaware of the diagnosis. For liver biopsy specimens, the total number of cells present was counted by an independent pathologist unaware of other case characteristics. Samples were also graded subjectively as minimal, mild or moderate inflammation. For presentation purposes these counts were logarithmically transformed.

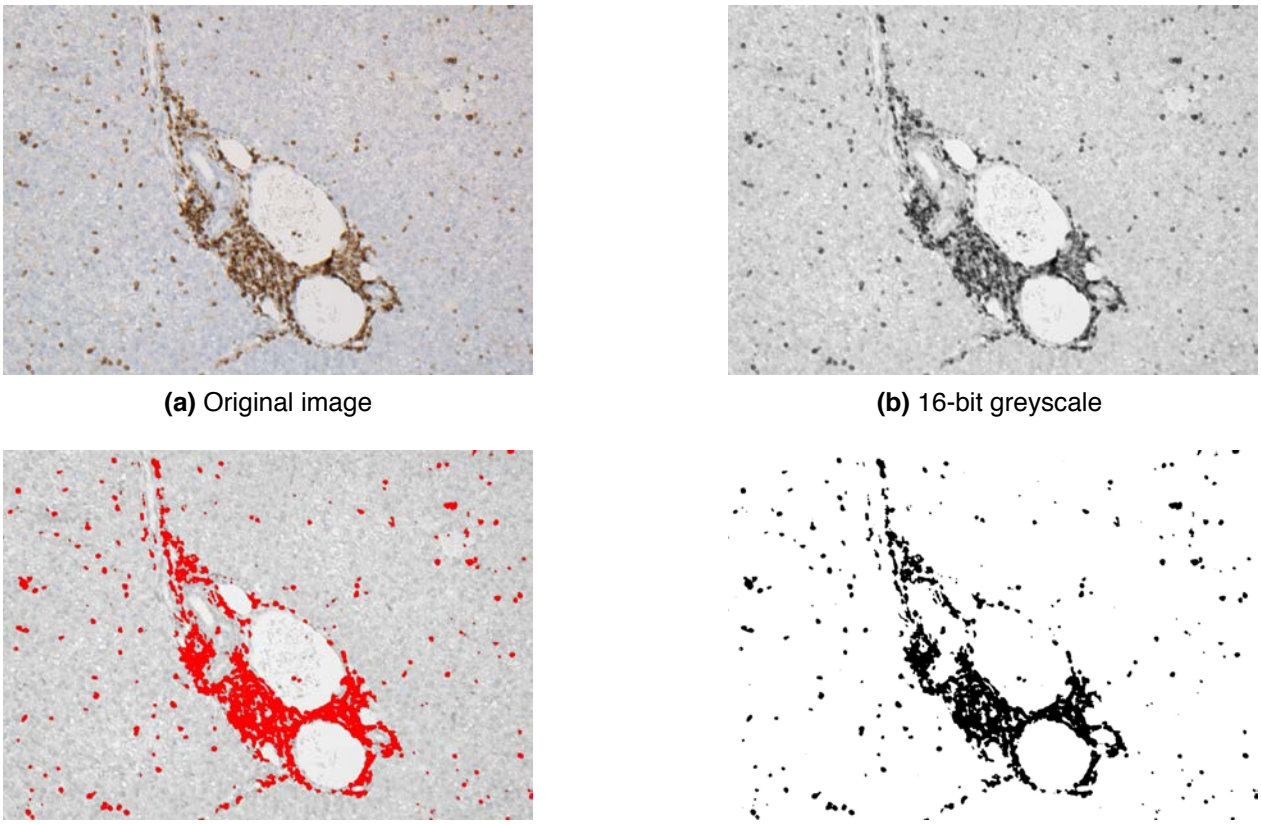
Imaging and quantification

Images for representative purposes were acquired with a Leica DM6000 microscope using a DFC310FX digital camera. Images were individually white-balanced.

For quantification of sections stained with chromogens to demonstrate the presence of markers of interest, five randomly positioned white-balanced images were taken from each tissue section at 10× magnification. Images were then batched through FIJI according to the following workflow:

```
run("Subtract Background...", "rolling=50 light");
run("16-bit");
setAutoThreshold("Huang");
setThreshold(42, 172); // These values were set individually per batch
setOption("BlackBackground", false);
run("Convert to Mask");
run("Analyze Particles...", "size=0-Infinity circularity=0.00-1.00 show=Nothing
display clear summarize");
```

The workflow removes variations in illumination from the microscope using the 'rolling ball method', converts the image into a 16-bit greyscale, thresholds a level of darkness that represents positive staining (adjusted manually on a per batch basis), converts the image into a 1-bit black and white bitmap, and then calculates the proportion of the image that is black (Figure 2.2). This value was calculated for each image taken of each tissue section and used for statistical comparisons.



(a) Original image

(b) 16-bit greyscale

(c) Thresholded image

(d) 2-bit image

Figure 2.2: Digital processing of histological images. An example of image processing to permit quantification of positive staining. Here a section of FOXP3 deficient mouse liver tissue has been stained with an anti-CD45 antibody and brown chromogen.

2.5 Biochemical analysis of serum

Liver biochemical values

For the analysis of liver biochemical values, automated Beckman Coulter AU400 analysers were used. Samples were processed with the assistance of the Department of Biochemistry, Birmingham Women's Hospital. Samples were assessed for ALT and ALP activity using clinical analysers. In some cases, samples were pre-diluted with sterile PBS prior to processing and subsequent readings corrected by the appropriate dilution factor.

Capillary blood glucose

Capillary glucose was measured using an Accu-Chek Nano blood glucose meter and single use glucose check sticks (both Roche) using blood obtained at cardiac puncture.

2.6 Cell suspension preparation and transfer

For the preparation of cell solutions for the analysis or further use of tissue leucocytes, tissues were dissected as above, washed thoroughly with RPMI and then weighed. Tissues were then placed in 3 ml RPMI and mechanically dissociated by repeated pressure with the reverse of a syringe plunger. The resulting cell suspension was then passed through 70 μm pore NYTAL® mesh. The filtered cell suspension was placed into BD Falcon 5 ml round-bottomed polypropylene tubes and centrifuged at 20 × g for 5 minutes. The pelleted débris was discarded and the supernatant was then transferred to a clean tube, which was centrifuged at 400 × g for 5 minutes and the pelleted cells retained. These cells were suspended in Gey's Balanced Salt Solution to cause lysis of red cells. After 5 minutes, an excess of RPMI was added to prevent further haemolysis and the cells washed before further use as described below.

Adoptive cell transfer

For the preparation of cell solutions for cell transfer, the steps above were performed in sterile conditions using sterile solutions. Suspensions were passed through 70 µm sterile filters (Falcon, BD Biosciences) before resuspension at a concentration appropriate for injection in sterile PBS. Cell counting was performed using a haemocytometer. Cell transfers were performed in volumes of 100 µl by either intravenous (IV) injection into one of the tail veins or intraperitoneal (IP) injection to the iliac fossa. For the production of bone marrow chimeras, adult recipient mice were irradiated with two exposures of 4.5 Gy immediately prior to intravenous cell transfer.

For transfer of FOXP3 deficiency disease, 5×10^6 total thymocytes were transferred IP; for all other transfers, preparations of 5×10^6 cells were transferred IV. For adoptive transfer of sorted Tregs, 1×10^5-2×10^5 cells were transferred IV

In some experiments, survival curves were generated. To do this, animals were monitored until they developed signs of autoimmune disease. At this point, they were analysed individually. Those mice that did not develop overt autoimmunity were analysed at the end of the planned experiment; in general this was at 6 weeks. In other experiments, mice were analysed simultaneously at a predetermined time point.

Cell culture suspensions

For the preparation of lymphocyte suspensions for cell culture, spleens were cut into approximately 2 mm pieces and placed into 3 ml RPMI supplemented with 75 µg DNAse and 750 µg collagenase/dispase (Roche diagnostics) for 20 minutes. After 20 minutes, 120 µl of 0.5 M concentration EDTA (Sigma-Aldrich) was added to prevent further enzymatic digestion. Cells were then washed in RPMI and further used as described below.

Purified mouse CD4+ T cells

For the generation of purified cultures of CD4+ T cells, a CD4+ EasySep® negative selection kit (Stemcell Technologies) was used. First a cell suspension of total splenocytes was prepared as above. Cells were resuspended in 500 µl of staining buffer. 25 µl rat serum was added and the sample left to incubate for 10 minutes at room temperature. 25 µl of negative selection cocktail was then added and the sample incubated for a further 10 minutes. Then 37.5 µl of RapidSpheres magnetic beads were added and the suspension resuspended with a pipette. The sample was incubated for 5 minutes and then placed in an EasySep® magnet for a further 5 minutes before a suspension of of CD4+ T cells >90% purity was poured off the magnet.

Human peripheral blood mononuclear cells

For the preparation of suspensions of human peripheral blood mononuclear cells, whole blood was obtained and anticoagulated with EDTA. Whole blood was layered over Lympholyte® solution (Cedarlane Labs) and then centrifuged at 800 × g for 20 minutes. The peripheral blood mononuclear cells containing interface between plasma and Lympholyte® was then aspirated and washed in PBS. Haemolysis was performed with Gey's Buffered Salt Solution. Cell suspensions were then depleted of platelets by centrifugation at 200 × g and discarding the supernatant.

2.7 Flow cytometric analysis

Surface staining

Cell suspensions were prepared as described elsewhere. Cells were washed by centrifugation at 400 × g for 2 minutes before resuspension. Staining was performed in a 96-well U-bottomed plate.

To permit exclusion of dead cells, cells were washed twice in phosphate-buffered saline before resuspension in 50 µl PBS containing a 1:2000 dilution of Zombie NIR™ Live-Dead exclusion dye (Biolegend), where they were left for 10 minutes at room temperature before being washed with PBS. For primary antibody staining, cells were resuspended in 50 µl staining buffer containing primary antibodies at pre-titrated concentrations. Cells were left to stain for 30 minutes at 4 °C. Cells were then washed in staining buffer. In some instances secondary antibodies were then applied and left for a further 30 minutes at 4 °C. After staining, cells were fixed in 4% formaldehyde solution for 30 minutes before resuspension in staining buffer.

Intracellular staining

For intracellular staining, primary antibodies were applied as above. Cells were then fixed for 30 minutes in the fixatives supplied in either the BD Biosciences cytokine kit or eBiosciences transcription factor staining kit (Thermo Fisher Scientific). Cells were then washed twice in 100 µl reconstituted permeabilisation buffer from the kit used before being resuspended in 50 µl permeabilisation buffer with added intracellular antibodies. Cells were then stained for 30 minutes at room temperature before a final 100 µl wash in permeabilisation buffer and then resuspension in staining buffer.

For intracellular staining of cytokines, cells were resuspended in a flat-bottomed 24-well plate in 1 ml culture media supplemented with either 1 µg agonistic anti-CD3 antibody (clone 145-2C11) and 1 µg agonistic anti-CD28 antibody (clone 37.51) or with phorbol 12-myristate-13-acetate (PMA) and ionomycin. Cells were then incubated for 1 hour at 37 °C and 5% CO_2. After 1 hour, cultures were supplemented with either monoensin (BD Biosciences) or brefeldin A (Sigma) before being returned to the incubator for 3 hours. Cells were then fixed, permiabilised and stained for cytokines of interest with appropriate antibodies as described above.

Cell number quantification

Where cell numbers were quantified, AccuCount® counting beads (Spherotech) were added to samples. The number of cells per gram of tissue was then calculated according to the following formula:

$$C_{total} = C_{counted}.F_{dilution}.B_{added}/B_{counted}.W_{tissue}$$

Where C_{total} is the total cells per gram; $C_{counted}$ is the number of cells of interest counted by flow cytometry; $F_{dilution}$ is the dilution factor used in preparing samples; B_{added} is the number of counting beads added; $B_{counted}$ is the number of beads counted during flow cytometry; W_{tissue} is the weight of tissue used in preparation

Acquisition

Acquisition was performed on an LSR Fortessa X-20 flow cytometer using FACS Diva software v8.0.1 (both BD Biosciences). Cell suspensions with cells prepared so as to have populations positive and negative for the fluorochrome of interest were prepared to allow compensation for spectral overlap. Where necessary, control samples stained with a non-specific antibody of the same isotype but with all other antibodies as normal (i.e. fluoresence minus one controls) were used to allow accurate gating. Representative gating strategies are shown below (Figure 2.3 & 2.4).

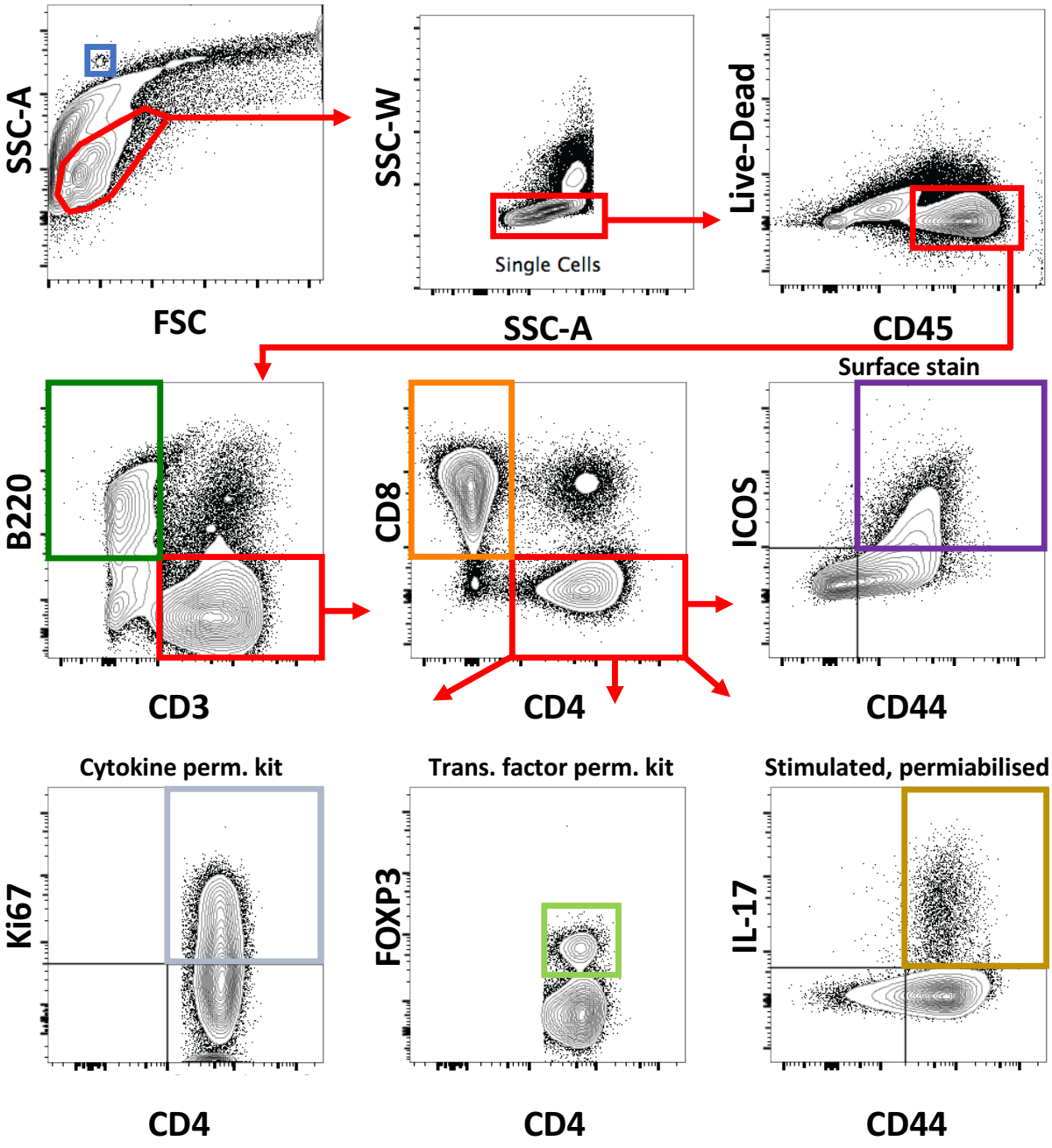


Figure 2.3: (Caption next page.)

Figure 2.3: (Previous page.) **General flow cytometry gating strategy**. Representative plots from cells isolated from liver samples with various staining protocols are shown. First, likely lymphocytes were gated based on their forward scatter (FSC) and side scatter (SSC). To permit counting of cells, counting beads were also selected based on their high SSC values (**blue gate**). Next, aggregations of two or more cells were excluded by plotting SSC-width against SSC-area. Next, cells negative for a fixation-permanent live-dead dye but positive for the pan-leucocyte marker CD45 were then selected. Next, plots of B220 against CD3 were created: B220+CD3- cells were defined as B cells (**dark green gate**), CD3+B220- cells were further examined. CD3+B220- cells were sub-divided by plotting CD4 against CD8. CD8+CD4- negative cells were defined as CD8+ T cells (**orange gate**), whilst CD4+CD8- cells were defined as CD4+ T cells and further examined. Simple surface staining was used to examine T cell markers such as ICOS (**purple gate**). A BD cytokine permiabilisation kit was used to permit staining for some intracellular proteins including CTLA4 and Ki67 (**grey gate**). For transcription factor staining, an eBioscience transcription factor permiabilisation kit was used (**pale green gate**). To reveal cytokine secretion profiles, cells were stimulated with PMA and ionomycin prior to staining with the BD cytokine kit for IFNγ, IL-4 and IL-17 (**ochre**).

2.8 Cell sorting and cell purification

Cell sorting was performed by with the assistance of Drs Matthew Mackenzie and Andrew Owen, University of Birmingham. For cell sorting, cell suspensions were prepared as for cell transfer. Cells were then stained with antibodies as for flow cytometry. Cells were then sorted on an MoFlo XDP Cell Sorter (Beckman Coulter). Purity checks were performed by passing a proportion of the sorted cells back through the cell sorter to confirm that the purity of the desired population was >95%.

2.9 Cell culture

Cells were prepared as described above. For the assessment of cell proliferation or to label cells to allow later discrimination, some cells were loaded with 5-carboxyfluorescein diacetate succinimidyl ester (CFSE): Cells were washed free of extracellular protein in RPMI and resuspended at between 1×10^6-5×10^6 cells/ml. CFSE in dimethyl sulfoxide (DMSO) was then added to a final CFSE concentration of $1\,\mu\text{mol}\,\text{L}^{-1}$ and the cells allowed to stain for 10 minutes in a 37 °C water bath with intermittent agitation. After 10 minutes, an excess of 4 °C culture media was added to absorb unbound CFSE and the cells washed. CFSE dilution through cell proliferation was assessed via flow

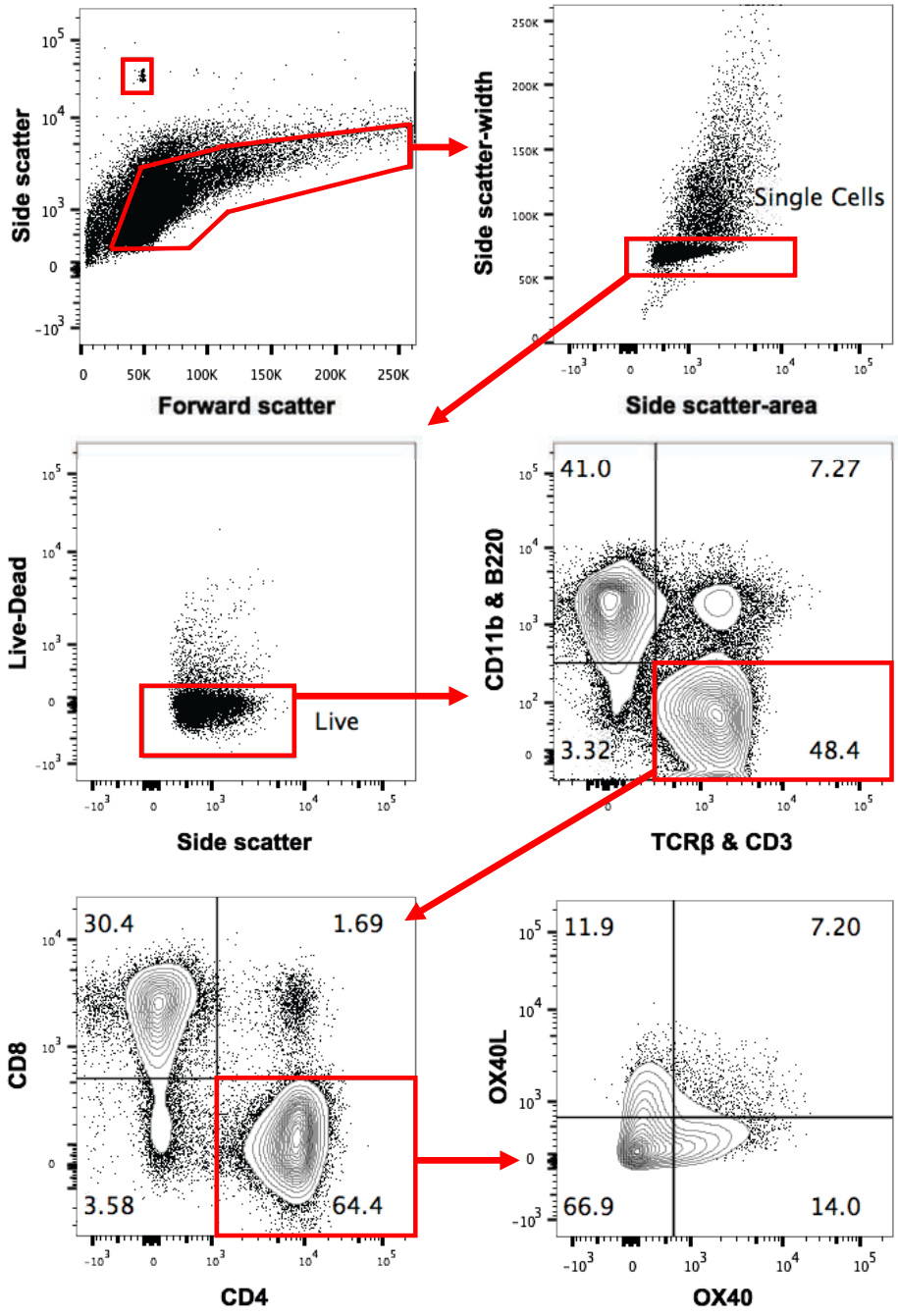


Figure 2.4: Example gating strategy for cell culture experiments. Forward and side scatter were first used to identify counting beads and likely cell populations; doublets were then excluded on side scatter-width; cells positive for live-dead stain were excluded; CD11b and B220-negative, TCRβ or CD3-positive cells were then selected. CD4+ T cells were identified as CD4+ CD8- cells from this population, and were then amenable to further analysis, in this example by comparing OX40 to OX40L expression.

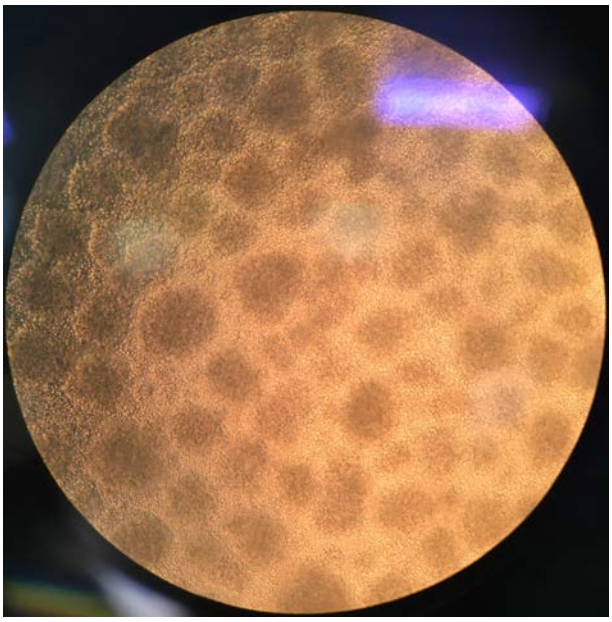


Figure 2.5: Proliferating murine splenocytes. Murine total splenocytes proliferating after being cultured for 72 hours in the presence of agonistic anti-CD3 and anti-CD28 antibodies.

cytometry using the fluorescein isothiocyanate (FitC)/Alexa fluor 488 channel.

For culture, cells were plated in 24-well flat-bottomed plates at 1×10^6 cells in 1 ml of culture medium. In general, cultures were continued for 72 hours at 5% CO_2 and proliferation was confirmed by visual inspection (Figure 2.5). For murine cells, wells were generally supplemented with combinations of agonistic anti-CD3 antibody with or without agonistic anti-CD28; for human peripheral blood mononuclear cells cultures, the same procedure was followed but with antibodies of appropriate specificities substituted.

In some experiments, ovalbumin (323-339; ISQAVHAAHAEINEAGR) peptide or concanavalin A (Con A) (both Sigma-Aldrich) was used to stimulate T cells in place of agonistic anti-CD3 antibody.

In some experiments, blocking antibodies or immunomodulatory drugs were added to culture media. These were added at the beginning of culture in all cases. These are detailed in Table 2.7.

In some experiments, cell cultures were separated by a 0.4 µm Transwell® membrane (BD Biosciences) to permit circulation of soluble molecules across the membrane but exclude movement

of cells or direct contact between cells.

2.10 Statistics

For the comparison of two unpaired groups, the Mann-Whitney U test was used; for comparison of distributed paired samples, the Kolmogorov-Smirnov test was used; for comparison of three or more groups of non-normally distributed data, the Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons was used; for assessment of trend, the *nptrend* function in Stata (Statacorp) was used; for comparison of two survival curves, log-rank analysis was used; for linear regression, a least squares method was used. Values of $p<0.05$ were taken as representing statistical significance; where p values for statistical tests were significant, they are depicted on graphs as horizontal lines between the two groups being compared or across all groups being compared. Graphs show medians unless otherwise stated.

2.11 Software, reagents and suppliers

Software

Text was set in Minion Pro, Helvetica and Arial with LaTeX and XeLaTeX using LyX v2.2.3. Figures were constructed with TouchDraw v1.11.9 (Elevenworks software, Kansas City, MO, USA) and Acorn v5.65 (Flying Meat Inc., Seattle, WA, USA). References were prepared with BibDesk v1.6.11. Image analysis of photographs of immunohistochemical sections was performed with FIJI/ImageJ v2.0.0. Calculations of statistical tests and the generation of graphs was performed with Prism v6.0 (Graphpad, La Jolla, CA, USA) and StataMP v13.0 (StataCorp, College Station, TX, USA). Flow cytometry analysis was performed using FlowJo v10.0.7r2 (Tree Star software, Ashland, OR, USA).

Reagents and suppliers

Solutions (Table 2.2), immunohistochemistry primary antibodies (Table 2.3), immunohistochemistry secondary antibodies (Table 2.4), flow cytometry antibodies (Table 2.5), *in vivo* antibodies (Table 2.6), and cell culture additives (Table 2.7) used, together with a table of suppliers (Table 2.8) are presented in table format below.

Table 2.2: Solutions used

Solution	Composition	Supplier
RPMI	Complete Roswell Park Memorial Institute 1640 Medium with L-glutamine and phenol red	Gibco
Gey's solution	Used for osmotic haemolysis of red blood cells	In house, per [292]
PBS	Dulbecco's PBS with $MgCl_2$and $CaCl_2$.	Gibco
Culture medium	RPMI + 10% fœtal bovine serum (Invitrogen), 2 mM L-glutamine (Sigma) and 1% w/v penicillin & streptomycin (Gibco)	Prepared in house
Staining buffer	PBS + 2% foetal bovine serum with 2.5 mM EDTA (Sigma)	Prepared in house
Staining solution	PBS + 1% w/v bovine serum albumin (Sigma)	Prepared in house

Table 2.3: Immunohistochemical primary antibodies

Target	Clone	Reference	Manufacturer
Mouse OX40	OX86	14-1341-82	eBioscience
IgG_1 control	Polyclonal	13-4301-81	eBioscience
Mouse CD4	4SM95	14-9766-82	eBioscience
Mouse CD45	30-F11	14-0451-85	eBioscience
Mouse OX40	OX86	in house	Dr Margaret Goodall, University of Birmingham, UK
Mouse Ki67	Rabbit polyclonal	none	Dr Johannes Gerdes, Borstel, Germany
Mouse OX40	Rabbit polyclonal	ab203220	Abcam
Human CD4	Rabbit polyclonal	ab133616	Abcam
Human OX40	Ber-ACT35	350002	BioLegend

Table 2.4: Immunohistochemical secondary antibodies

Target	Clone	Conjugate	Reference	Manufacturer
Rat	Polyclonal	HRP	MP-7404	Vector
Mouse	Polyclonal	HRP	MP-7452	Vector
Rabbit	Polyclonal	HRP	MP-7401	Vector
Rabbit	Polyclonal	ALP	MP-5401	Vector

ALP = alkaline phosphatase; HRP = horseradish peroxidase

Table 2.5: Mouse flow cytometry antibodies

Manufacturer	Code	Description
		Intracellular
eBioscience	53-7021-82	IL-2 Antibody, Alexa Fluor® 488 (Monoclonal, JES6-5H4)
eBioscience	16-0281-85	CD28 Antibody, Functional grade (Monoclonal, 37.51)
eBioscience	13-5773-82	FOXP3 Antibody, Biotin (Monoclonal, FJK-16s)
eBioscience	12-7321-82	TNF alpha Antibody, PE (Monoclonal, MP6-XT22)
eBioscience	16-0031-85	CD3e Antibody, Functional grade (Monoclonal, 145-2C11)
eBioscience	25-5698-82	Ki-67 Antibody, PE-Cyanine7 (Monoclonal, SolA15)
eBioscience	25-7311-82	IFN gamma Antibody, PE-Cyanine7 (Monoclonal, XMG1.2)
eBioscience	48-5773-82	FOXP3 Antibody, eFluor® 450 (Monoclonal, FJK-16s)
eBioscience	11-5773-82	FOXP3 Antibody, FITC (Monoclonal, FJK-16s)
Biolegend	106314	PE/Cy7 anti-mouse CD152 Antibody anti-CD152 - UC10-4B9
BD	554436	APC, Rat Anti-Mouse anti IL-4 11B11
		BV421 / AF450
eBioscience	48-0452-82	CD45R (B220) Antibody, eFluor® 450 (Monoclonal, RA3-6B2)
eBioscience	48-0081-82	CD8a Antibody, eFluor® 450 (Monoclonal, 53-6.7)
eBioscience	48-5931-82	Ly-6G (Gr-1) Antibody, eFluor® 450 (Monoclonal, RB6-8C5)
Biolegend	100336	Brilliant Violet 421 anti-mouse CD3epsilon Antibody anti-CD3e - 145-2C11
Biolegend	127628	Brilliant Violet 421 anti-mouse Ly-6G Antibody anti-Ly-6G - 1A8
Biolegend	400429	Brilliant Violet 421 Rat IgG1, kappa Isotype Ctrl
Biolegend	110732	Brilliant Violet 421 anti-mouse CD45.1 Antibody anti-CD45.1 - A20

Manufacturer	Code	Description
Biolegend	109831	Brilliant Violet 421 anti-mouse CD45.2 Antibody anti-CD45.2 - 104
Biolegend	119411	Brilliant Violet 421 anti-mouse CD134 OX-40 Antibody anti-CD134 - OX-86
		AF700
Biolegend	100216	Alexa Fluor 700 anti-mouse CD3 Antibody anti-CD3 - 17A2
Biolegend	109224	Alexa Fluor 700 anti-mouse TCR beta chain Antibody anti-TCR beta chain - H57-597
Biolegend	103232	Alexa Fluor 700 anti-mouse/human CD45R/B220 Antibody anti-CD45R - RA3-6B2
eBioscience	56-5321-82	MHC Class II (I-A/I-E) Antibody, Alexa Fluor® 700 (Monoclonal, M5/114.15.2)
eBioscience	56-0042-82	CD4 Antibody, Alexa Fluor® 700 (Monoclonal, RM4-5)
eBioscience	56-0251-82	CD25 Antibody, Alexa Fluor® 700 (Monoclonal, PC61.5)
		Phycoerythrin (PE)
eBioscience	12-0452-83	CD45R (B220) Antibody, PE (Monoclonal, RA3-6B2)
eBioscience	12-0031-83	CD3e Antibody, PE (Monoclonal, 145-2C11)
eBioscience	12-0081-82	CD8a Antibody, PE (Monoclonal, 53-6.7)
eBioscience	12-0251-83	CD25 Antibody, PE (Monoclonal, PC61.5)
eBioscience	12-5905-83	CD252 (OX40 Ligand) Antibody, PE (Monoclonal, RM134L)
eBioscience	12-0621-83	CD62L (L-Selectin) Antibody, PE (Monoclonal, MEL-14)
eBioscience	12-4031-82	Rat IgG2b kappa, PE Isotype Control
		PE-Cy7
eBioscience	25-0452-82	CD45R (B220) Antibody, PE-Cyanine7 (Monoclonal, RA3-6B2)
eBioscience	25-0441-81	CD44 Antibody, PE-Cyanine7 (Monoclonal, IM7)
eBioscience	25-3351-82	CD335 (NKp46) Antibody, PE-Cyanine7 (Monoclonal, 29A1.4)
eBioscience	25-0041-82	CD4 Antibody, PE-Cyanine7 (Monoclonal, GK1.5)

Manufacturer	Code	Description
eBioscience	25-0112-81	CD11b Antibody, PE-Cyanine7 (Monoclonal, M1/70)
Biolegend	100722	PE/Cy7 anti-mouse CD8a Antibody anti-CD8a - 53-6.7
		Allophycocyanin (APC)
eBioscience	17-0081-83	CD8a Antibody, APC (Monoclonal, 53-6.7)
eBioscience	17-1341-82	CD134 (OX40) Antibody, APC (Monoclonal, OX-86)
eBioscience	50-4801-82	F4/80 Antibody, eFluor® 660 (Monoclonal, BM8)
eBioscience	17-0042-82	CD4 Antibody, APC (Monoclonal, RM4-5)
eBioscience	17-4321-81	Rat IgG2a kappa, APC Isotype Control
eBioscience	17-4301-81	Rat IgG1 kappa, APC Isotype Control
		PerCP5.5
eBioscience	45-0453-82	CD45.1 Antibody, PerCP-Cyanine5.5 (Monoclonal, A20)
eBioscience	45-0454-82	CD45.2 Antibody, PerCP-Cyanine5.5 (Monoclonal, 104)
eBioscience	45-0042-82	CD4 Antibody, PerCP-Cyanine5.5 (Monoclonal, RM4-5)
		Alexa Fluor 647
Biolegend	313516	Alexa Fluor 647 anti-human/mouse/rat CD278 ICOS Antibody anti-CD278 - C398.4A
		Brilliant Violet 510
Biolegend	100559	Brilliant Violet 510 anti-mouse CD4 Antibody anti-CD4 - RM4-5
Biolegend	107635	Brilliant Violet 510 anti-mouse I-A/I-E Antibody anti-I-A/I-E - M5/114.15.2
Biolegend	117337	Brilliant Violet 510 anti-mouse CD11c Antibody anti-CD11c - N418
Biolegend	313525	Brilliant Violet 510 anti-human/mouse/rat CD278 ICOS Antibody anti-CD278 - C398.4A
		Brilliant Violet 711

Manufacturer	Code	Description
Biolegend	100747	Brilliant Violet 711 anti-mouse CD8a Antibody anti-CD8a - 53-6.7
Biolegend	100447	Brilliant Violet 711 anti-mouse CD4 Antibody anti-CD4 - GK1.5
Biolegend	128037	Brilliant Violet 711 anti-mouse Ly-6C Antibody anti-Ly-6C - HK1.4
		Brilliant Violet 785
Biolegend	109839	Brilliant Violet 785 anti-mouse CD45.2 Antibody anti-CD45.2 - 104
Biolegend	100552	Brilliant Violet 785 anti-mouse CD4 Antibody anti-CD4 - RM4-5
Biolegend	103059	Brilliant Violet 785 anti-mouse/human CD44 Antibody anti-CD44 - IM7
BD	563329	BV786, Mouse, Anti-Human, CD335 (NKp46), 9E2/NKp46
		Brilliant Violet 650
Biolegend	100229	Brilliant Violet 650 anti-mouse CD3 Antibody anti-CD3 - 17A2
Biolegend	102038	Brilliant Violet 650 anti-mouse CD25 Antibody anti-CD25 - PC61

Table 2.6: *In vivo* antibodies

Clone	Target	Dose	Source
RM134L	Murine OX40L	250 µg twice weekly	Dr Hideo Yagita, Juntendo University, Japan
OX40 FAb	Murine OX40	250 µg twice weekly	UCB pharma
XMG1.2	Murine IFNγ	500 µg twice weekly	BioXCell

Table 2.7: Cell culture additives

Agent	Source	Concentration(s) used
CTLA4Ig (Abatacept®)	Bristol-Myers-Squibb	100 µg ml^{-1}
Mycophenolate mofetil	Roche	0.2-20 µg ml^{-1}
Interleukin-2	PeproTech	10-40 ng ml^{-1}
Interleukin-4	PeproTech	10-40 ng ml^{-1}
Interleukin-7	PeproTech	10-40 ng ml^{-1}
Interleukin-12	PeproTech	10 ng ml^{-1}
Interferon-γ	PeproTech	10 ng ml^{-1}
Pimozide	Sigma-Aldrich	1 µM
Rapamycin	Sigma-Aldrich	1 µM
Cyclosporin A	Sigma-Aldrich	0.25 µg ml^{-1}
Dexamethasone	Sigma-Aldrich	1 µM
Anti-CD40L (Clone: MR1)	BioLegend	5 µg ml^{-1}
Anti-CD40 (Clone: 1C10)	BioLegend	5 µg ml^{-1}
Anti-CTLA4 (Clone: UC10-4B9)	BioLegend	5 µg ml^{-1}
Anti-IL2 (Clone: JES6-1A12)	BioLegend	5 µg ml^{-1}

Table 2.8: Suppliers

Supplier	Location
Abcam	Cambridge, UK
Beckman Coulter	High Wycombe, UK
BD Biosciences	Oxford, UK
BioLegend	London, UK
BioXCell	West Lebanon, NH, USA
Cedarlane Labs	Burlington, NC, USA
Cusabio Ltd.	College Park, MD, USA
Dako UK Ltd.	Cambridge, UK
EurImmun	Lübeck, Germany
Ferndale	Leeds, UK
Gibco	via Thermo Fisher
Invitrogen	via Thermo Fisher
Leica Microsystems	Milton Keynes, UK
PeproTech	London, UK
Roche	Burgess Hill, Sussex, UK
Sigma-Aldrich	Gillingham, Dorset, UK
Stemcell Technologies	Cambridge, UK
Spherotech Inc.	Lake Forest, IL, USA
Thermo Fisher Scientific	Paisley, UK
UCB Pharma	Slough, UK
Vector Labs	Peterborough, UK

3 Autoimmunity and hepatitis in regulatory T cell deficiency

3.1 Introduction

A complete loss of Tregs causes widespread activation of effector T cells with resultant autoimmunity in multiple organs including the liver.[113] Because of the presence of AMAs, a T cell dominant peri-biliary infiltrate and up-regulation of mRNAs suggestive of TH1 type response, mice deficient in Tregs have been suggested to develop a phenotype partly reminiscent of PBC. This description of FOXP3-deficient mice has however only taken place in a single laboratory.[504] Similar findings have also been described in other models of disturbed Treg function and these have variously been described as models of PBC[312, 446] and AIH.[348, 193, 117, 358, 29]

The protein AIRE is involved in the presentation of otherwise tissue-restricted antigens to developing T cells. Its absence causes deficits in $AIRE^{-/-}$ mice which develop a hepatitis with autoantibodies.[348] A small proportion of human autoimmune hepatitis is related to an analogous mutation in the human AIRE gene. In a mouse model of hepatitis in AIRE dysfunction, the observation has been made that transfer of wildtype, and therefore polyspecific, regulatory T cells corrects the deficit and presents hepatitis.[132] The complimentary experiment of investigating whether AIRE-deficient Tregs are sufficient to prevent multi-system autoimmunity has not been reported. Notably the combination of AIRE deficiency with FOXP3 makes little difference to the phenotype of FOXP3-deficient mice.[61]

This series of experiments aimed to recapitulate findings described elsewhere with respect to the hepatic autoimmunity present in the $FOXP3^{KO}$ mouse and to confirm that $FOXP3^{KO}$ disease could be transferred to other mice as a basis for later experiments. In addition, to confirm that the disease was entirely due to a lack of FOXP3-positive Tregs by replacing them, I assessed whether replacement of polyspecific Tregs would abolish liver autoimmunity, whether transfer of Tregs alone was sufficient to prevent disease and whether Treg self-antigen specificity was necessary to control disease.

3.2 Results

3.2.1 Characteristics of FOXP3^{KO} mice

Male mice deficient in FOXP3 (FOXP3^{KO}) were bred and compared to their FOXP3 heterozygous female littermates. After analysis at 3-4 weeks of age, FOXP3-deficient mice were phenotypically smaller, demonstrated dermatitis affecting the tail and ears and developed marked splenomegaly and generalised lymphadenopathy (Figure 3.1A). To quantify splenomegaly, whole spleens were dissected and weighed. Those from FOXP3^{KO} mice weighed significantly more (Figure 3.1B) whilst total mouse weight was reduced (Figure 3.1C).

FOXP3^{KO} mice develop lymphocytic hepatitis

To evaluate whether FOXP3^{KO} mice developed lymphocytic hepatitis as reported elsewhere, livers were dissected and examined after haematoxylin and eosin (H&E) staining. This demonstrated no significant infiltrate in FOXP3-sufficient mice but marked lymphocytic infiltration surrounding portal triads in FOXP3-deficient mice (Figure 3.2A and B). To better demonstrate leucocytes within liver tissue, sections were stained for the pan-leucocyte marker CD45 and this demonstrated dense aggregates of CD45-positive cells surrounding the bile ducts of FOXP3^{KO} mice with only scattered positive cells in sections from FOXP3-sufficient littermates (Figure 3.2C and D). Subsequent computer quantification demonstrated that there was a significant increase in the proportion of the liver that was CD45-positive in FOXP3^{KO} mice (Figure 3.2E).

To further characterise the hepatitis seen in FOXP3^{KO} animals, frozen sections were stained with markers for three different cell types: anti-CD4 to identify likely CD4+ T cells, anti-CD8 to identify CD8+ T cells and anti-CD11b to demonstrate macrophages and other myeloid cells. Such staining confirmed the presence of multiple CD4-positive and CD8-positive cells with fewer CD11b-positive cells centered around portal triads (Figure 3.3). To further characterise the hepatic infiltrate

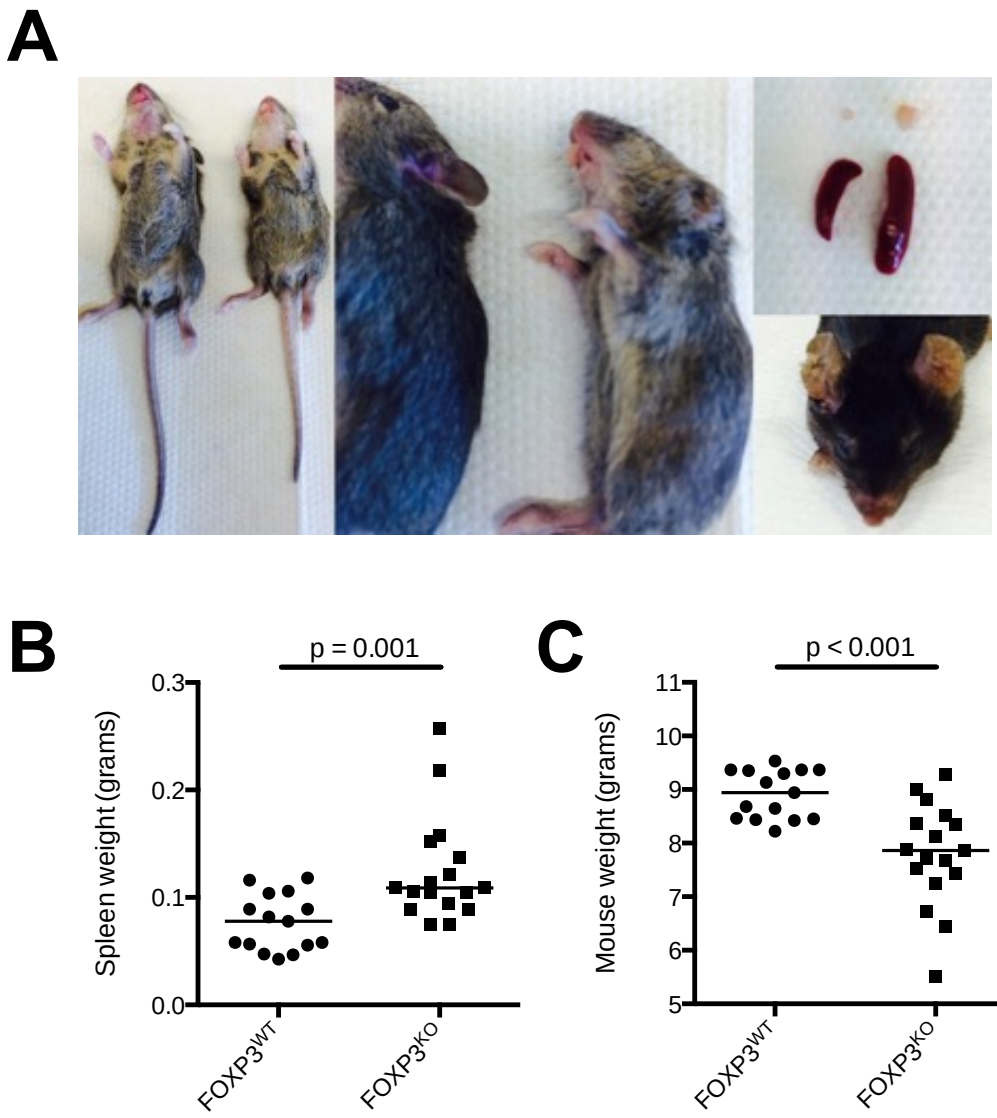


Figure 3.1: The FOXP3^{KO} phenotype. (**A**) FOXP3^{KO} mice (to the right of each panel) are smaller than FOXP3^{het} littermates at 3-4 weeks of age and develop dermatitis affecting the ears and tail with splenomegaly and lymphadenopathy (right inguinal lymph node pictured). (**B**) Splenomegaly and (**C**) reduced growth were consistent findings. Comparisons with Mann-Whitney U test.

associated with FOXP3 deficiency, samples of freshly dissected liver were mechanically digested, cell suspensions prepared, and fluorochrome-conjugated antibodies used to permit flow cytometric analysis. Flow cytometry demonstrated modest increases in intrahepatic B cells numbers and major increases in numbers of CD4+ T cells and CD8+ T cells (Figure 3.4A). Further analysis of CD3+CD4+ T cells demonstrated a small population that were OX40-positive in FOXP3-sufficient mouse liver. The majority of these cells were FOXP3-positive indicating that they were Treg. In contrast, a significantly greater number of cells were positive for OX40 in FOXP3-deficient animals with an expected absence of FOXP3-positive cells. Cells from an OX40KO animals was used to confirm staining specificity.(Figure 3.4B). Further assessment of liver infiltrating cells demonstrated that a higher ratio of CD8+:CD4+ T cells in FOXP3-deficiency (Figure 3.5A), that a higher proportion of CD4+ T cells expressed the activation markers ICOS and CD44 (Figure 3.5B), and that a greater proportion of CD4+ T cells expressed the proliferation marker Ki67 (Figure 3.5C).

FOXP3^{KO} mice express OX40 on infiltrating cells

Having demonstrated flow cytometric evidence of CD4+ rich peri-biliary infiltrates with upregulation of CD4+ T cell expression of OX40 and Ki67 in the livers of FOXP3-deficient mice, immunohistochemistry was used to asssess for supportive evidence and to localise where these cells were located within tissue. Staining demonstrated that CD4+ cells, OX40+ cells and Ki67+ cells were all localised around portal triads and in peri-venular areas (Figure 3.6).

FOXP3^{KO} mice display elevated ALT activity but reduced ALP activity

Serum obtained from FOXP3-deficient mice at cardiac puncture was analysed for serum ALT and ALP activities. Mice deficient in FOXP3 demonstrated significantly increased serum ALT activity when compared to controls, but demonstrated significant reductions in serum ALP activity (Figure 3.7A and B). Some samples were frozen and thawed prior to analysis. To ensure that this was

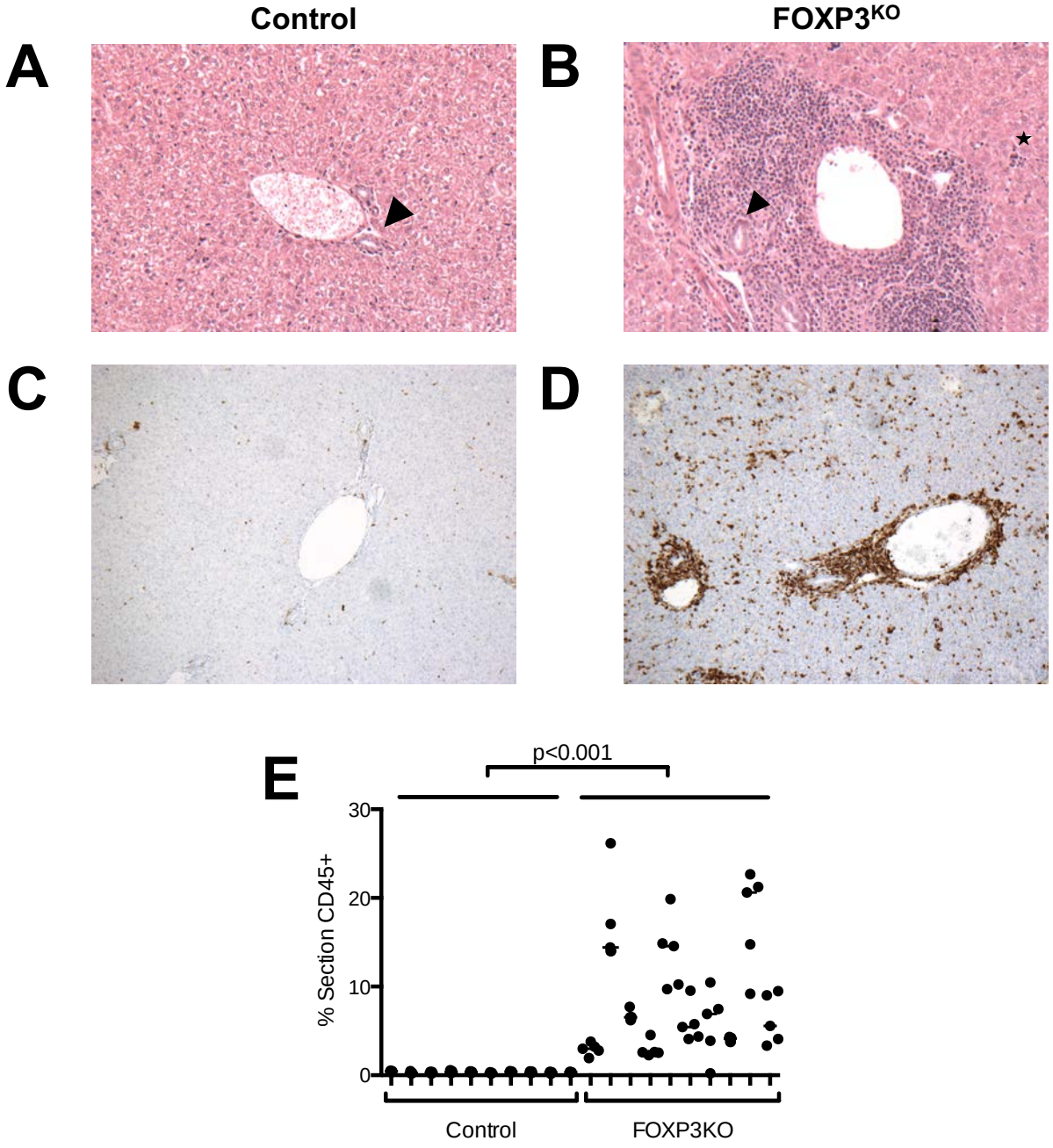


Figure 3.2: FOXP3^{KO} liver histology. FOXP3 sufficient mice (**A**) do not demonstrate hepatitis, whereas FOXP3 deficient mice (**B**) demonstrate dense peri-biliary / peri-venular lymphocytic infiltrates on examination of liver histology. **Arrowheads** denote bile ducts; **star** denotes extramedullary haematopoiesis. Images taken at ×20 magnification, H&E. CD45 staining confirmed marked differences in the number of hepatic leucocytes (**C** and **D**). When quantified, a significantly greater proportion of liver sections from FOXP3KO mice was CD45-positive (**E**). Comparisons with Mann-Whitney U test through across median values; n=20.

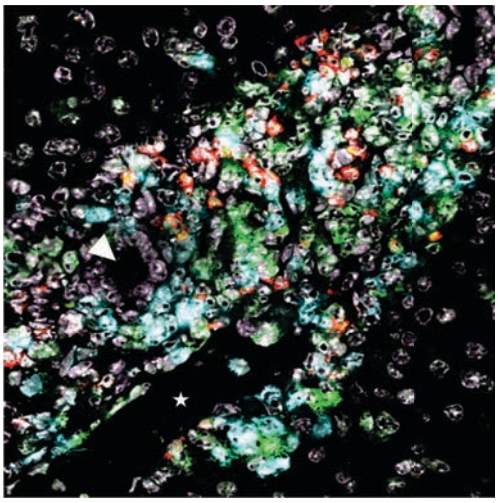


Figure 3.3: Confocal micrograph of inflammatory infiltrate in the liver of a FOXP3^{KO} mouse. Red = CD11b, Green = CD8, Cyan = CD4; Grey = DAPI nuclear stain; **arrowhead** denotes bile duct; ×50 magnification.

not affecting results, a selection of samples were split and analysed either without freezing or after being frozen and thawed. No evidence of alteration in enzymatic acitivity was seen after freeze-thaw for either ALT or ALP (Figure 3.7C and D). In an attempt to determine which of the two major isoforms of ALP was responsible for the readings, a technique used in clinical practice was employed. The bone isoform of ALP is heat labile while the visceral isoform is heat stable. Samples were therefore measured before and after heating at 56 °C for 15 minutes to denature bone-derived ALP.[292] Using this method, nearly all enzyme activity disappeared from samples (Figure 3.7E).

FOXP3^{KO} mice demonstrate elevations in serum immunoglobulins

In human AILD elevations in serum immunoglobulins are present. In order to assess whether serum immunoglobulins were elevated in FOXP3-deficiency, ELISAs for total serum IgG and IgE were performed. In both instances, FOXP3-deficient mice had significantly concentrations of serum immunoglobulin (Figure 3.8A and B).

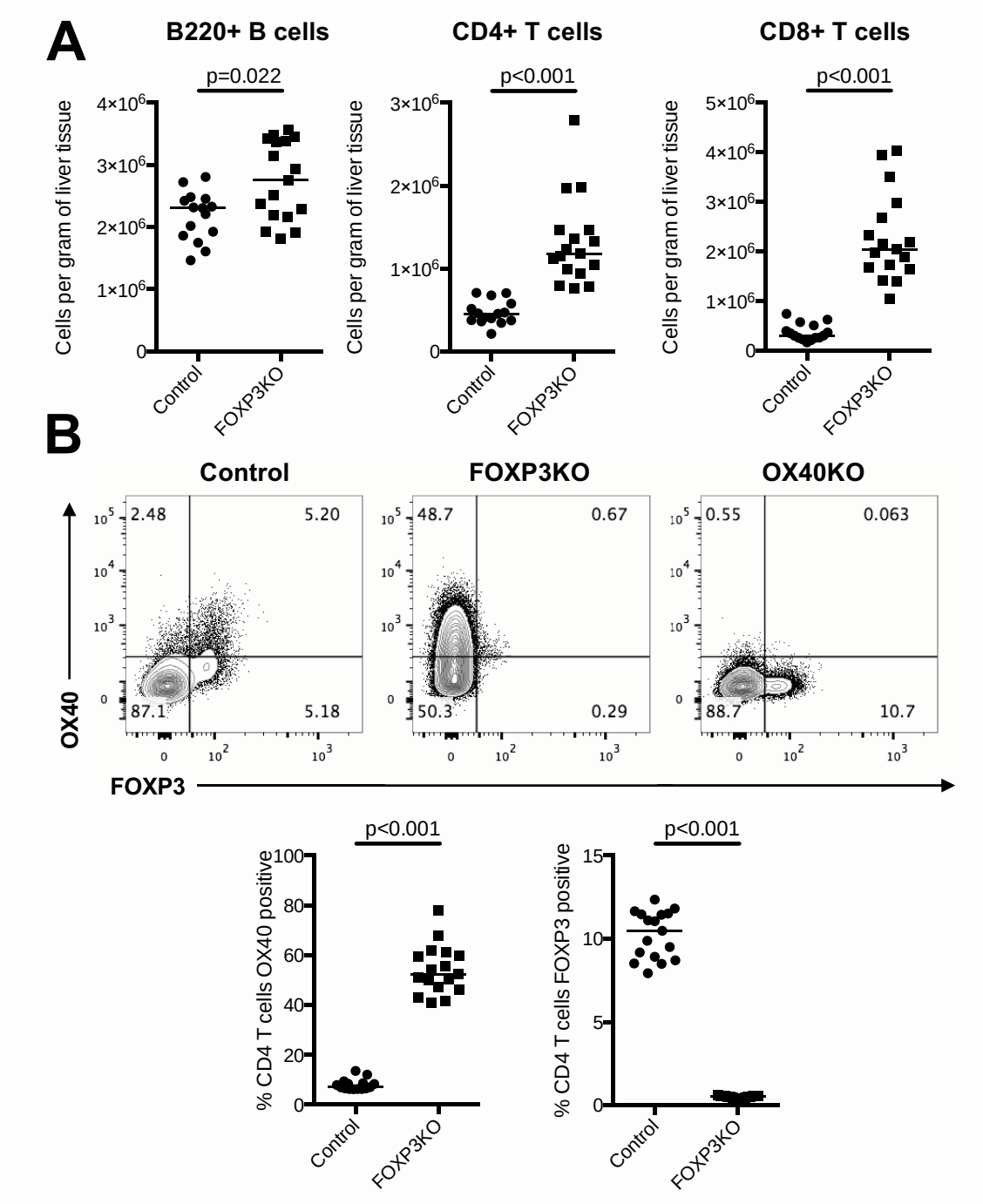


Figure 3.4: Lymphocyte contents of FOXP3^{KO} mouse liver. FOXP3^{KO} and control FOXP3 sufficient mouse livers were assessed at 3-4 weeks of age. (**A**) Numbers of B, CD4+ T cells and CD8+ T cells were increased in FOXP3^{KO} livers. (**B**) OX40 was expressed on a greater proportion of CD4+ T cells in FOXP3^{KO} liver and FOXP3-positive Treg were absent. Representative plots gated on CD4+ T cells. Comparisons with Mann-Whitney U test. n=30

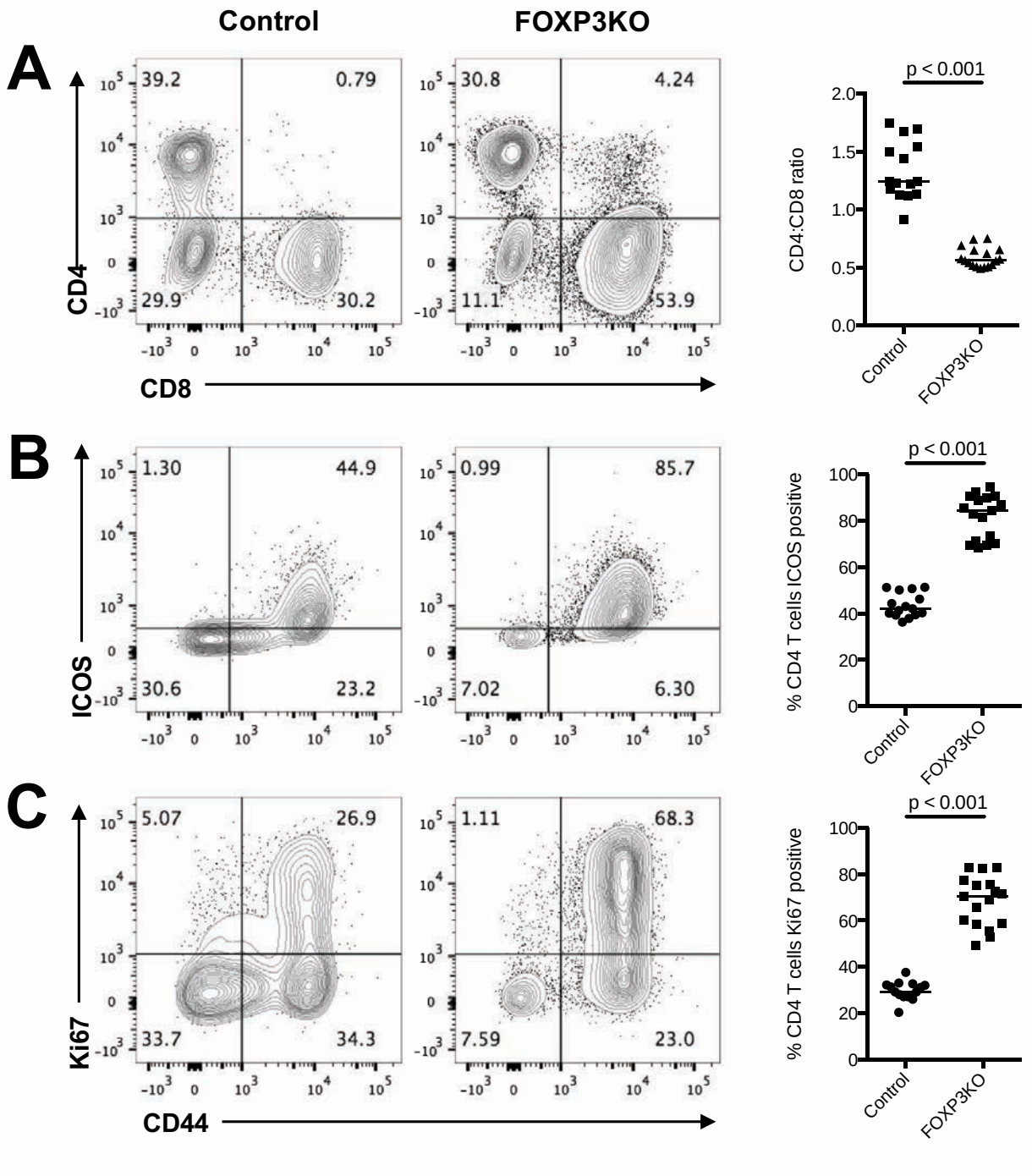
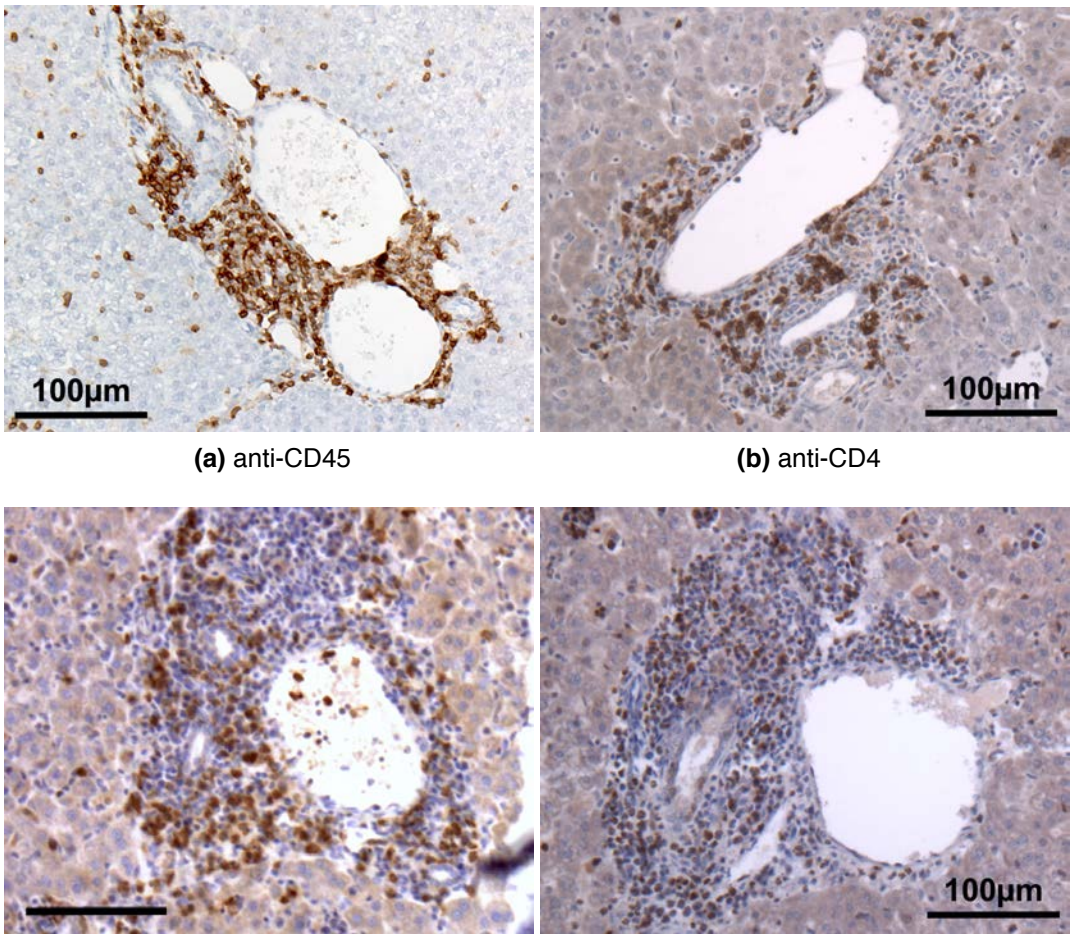


Figure 3.5: Lymphocyte characteristics of FOXP3^{KO} and FOXP3-sufficient mouse livers. FOXP3^{KO} and FOXP3-sufficient control mouse liver was assessed at 3-4 weeks of age. In FOXP3^{KO} mice, the CD4:CD8 ratio was reduced (**A**), the proportion of CD4+ T cells that were ICOS positive was increased (**B**), and the proportion of of CD4+ T cells that were Ki67 positive was increased. Comparisons using Mann-Whitney U test. n=30



(a) anti-CD45 **(b)** anti-CD4

(c) anti-OX40 **(d)** anti-Ki67

Figure 3.6: Histology of the FOXP3^{KO} mouse liver. Numerous (**A**) CD45-positive, (**B**) CD4-positive, (**C**) OX40-positive, and (**D**) Ki67 positive cells were apparently within periportal, perivenular inflammatory infiltrates.

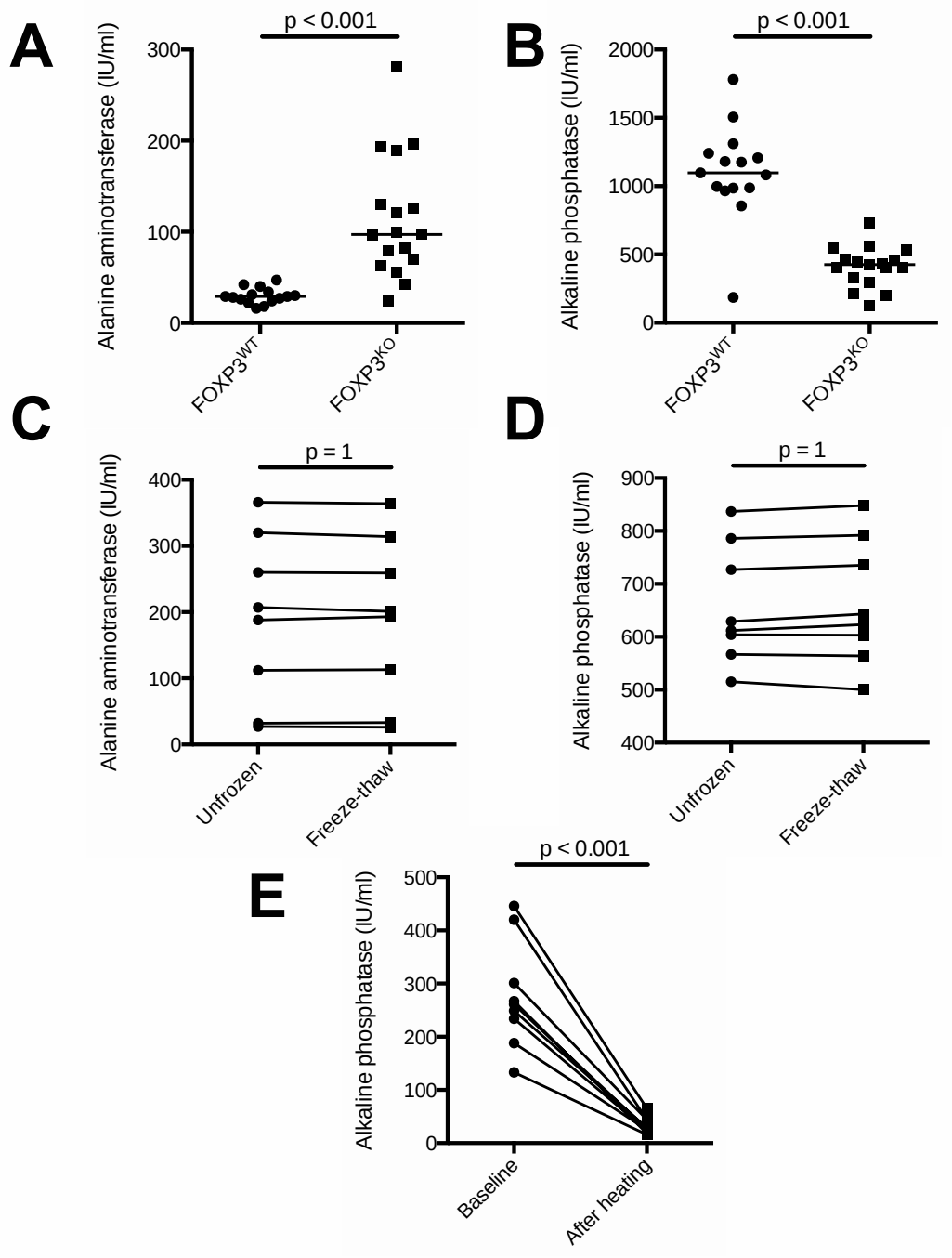


Figure 3.7: FOXP3 ALT and ALP activities. When assessed on clinical analysers, FOXP3^{KO} mice demonstrated elevations in serum ALT activity (**A**) but unexpectedly reductions in serum ALP (**B**); n=30. Because some samples were frozen prior to analysis, some (n=8) samples were split, frozen and then thawed before being compared against the other half of the sample. This procedure made no difference to readings for either ALT (**C**) or for ALP (**D**). Finally, to investigate whether mouse ALP was heat-labile liver derived or heat-stable bone-derived, samples were heated at 56 °C for 15 minutes (**E**). This significantly reduced ALP activity in all instances. Note: all values corrected for any prior dilution, typically 1:3. Comparisons with either Mann-Whitney U test (**A**-**D**) or Kolmogorov-Smirnov test (**E**)

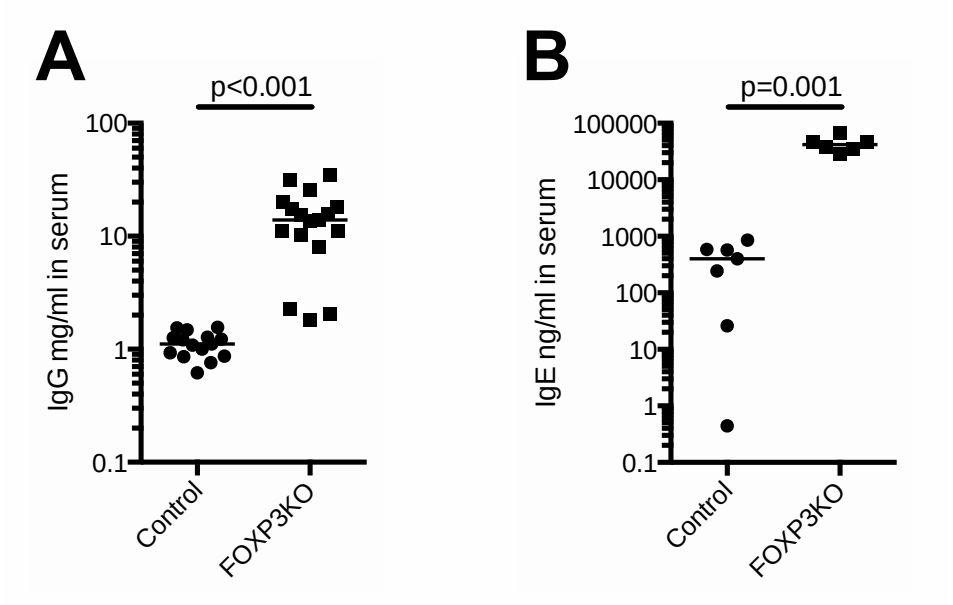


Figure 3.8: FOXP3 deficient mice have elevated serum IgG and IgE. Serum was taken from 3-4 week old FOXP3 sufficient and FOXP3^{KO} mice and assessed for (**A**) IgG and (**B**) IgE by ELISA. The concentrations of both antibodies were significantly increased in FOXP3^{KO} mice. Comparisons by Mann-Whitney U test; n=30.

FOXP3^{KO} mice demonstrate anti-mitochondrial antibodies

It has been reported elsewhere that mice deficient in FOXP3 develop AMA.[504] To confirm this, either control FOXP3-sufficient or FOXP3 deficient serum was applied to composite sections of rat liver, kidney and stomach followed by an anti-IgG FitC-conjugated secondary antibody. This demonstrated diffuse cytoplasmic staining in proximal renal tubule, hepatocyte and gastric parietal cell cytoplasm consistent with the presence of AMA (Figure 3.9A). Similarly, imaging of the similar composite slides stained with serum and then stained with an anti-IgG or anti-IgM antibody demonstrated positive staining in FOXP3-deficient but not FOXP3-sufficient animals (Figure 3.9B).

Having demonstrated evidence of AMA in the sera of FOXP3-deficient animals by microscopy, sera were further assessed by ELISA (Figure 3.10). ELISA demonstrated signal for IgG AMA at up to 1 in 640 titre in pooled sera from FOXP3-deficient animals but not in sera from control animals (**A**). When ELISAs were repeated across multiple animals, there were significant elevations in IgG, IgM, and IgA (**B**).

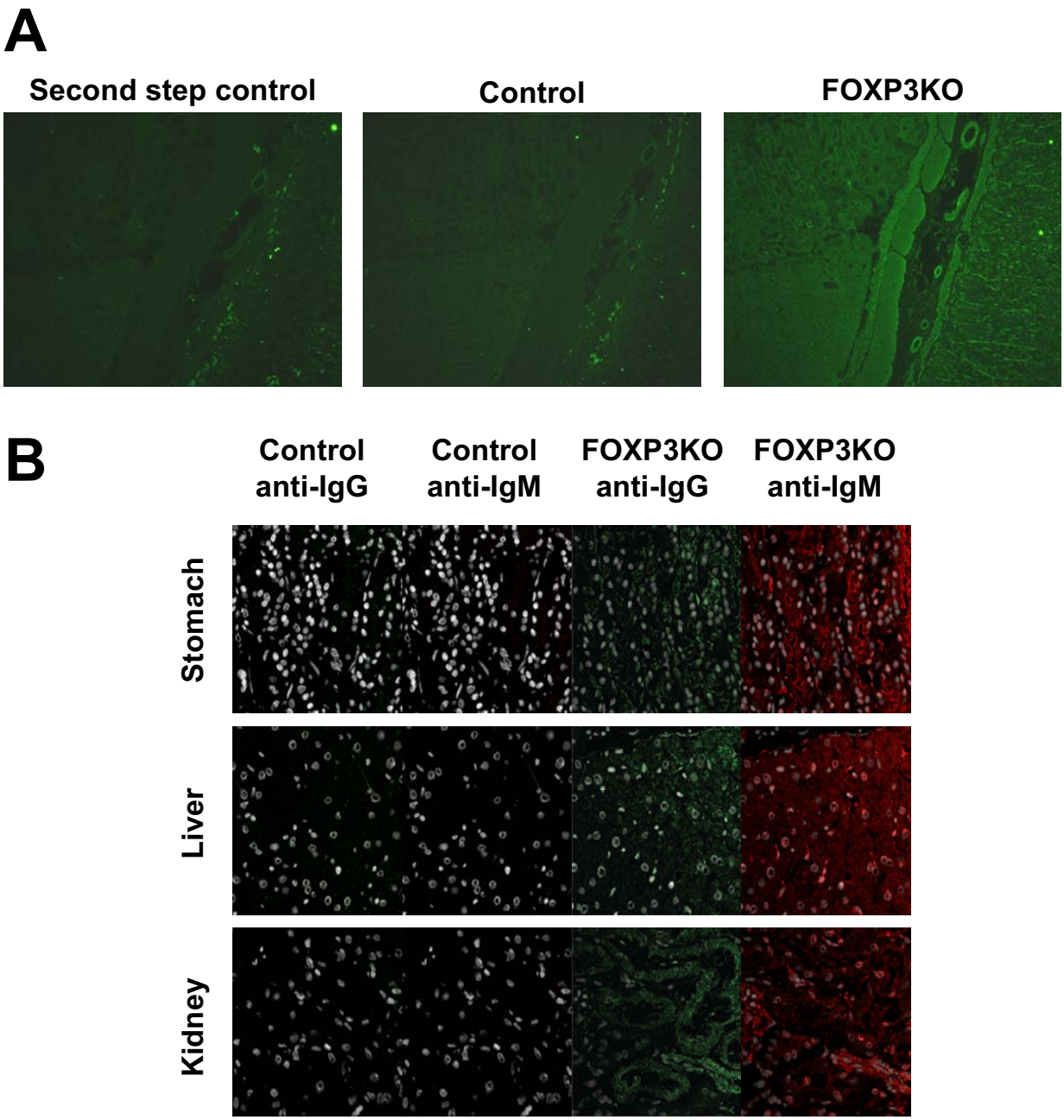


Figure 3.9: Indirect immunofluoresence demonstrates AMAs in FOXP3^{KO} serum. (**A**) Serum applied to composite section of rat liver, kidney and stomach demonstrated diffuse positive staining of cytoplasm in FOXP3-deficiency but not in FOXP3 sufficient animals when assessed using an anti-IgG FitC-conjugated secondary antibody (green). (**B**) Similarly, confocal imaging of composite liver, kidney and stomach sections demonstrated positive staining for IgG (green; FitC) and IgM (red; 555 phalloidin) when analysed by confocal microscopy.

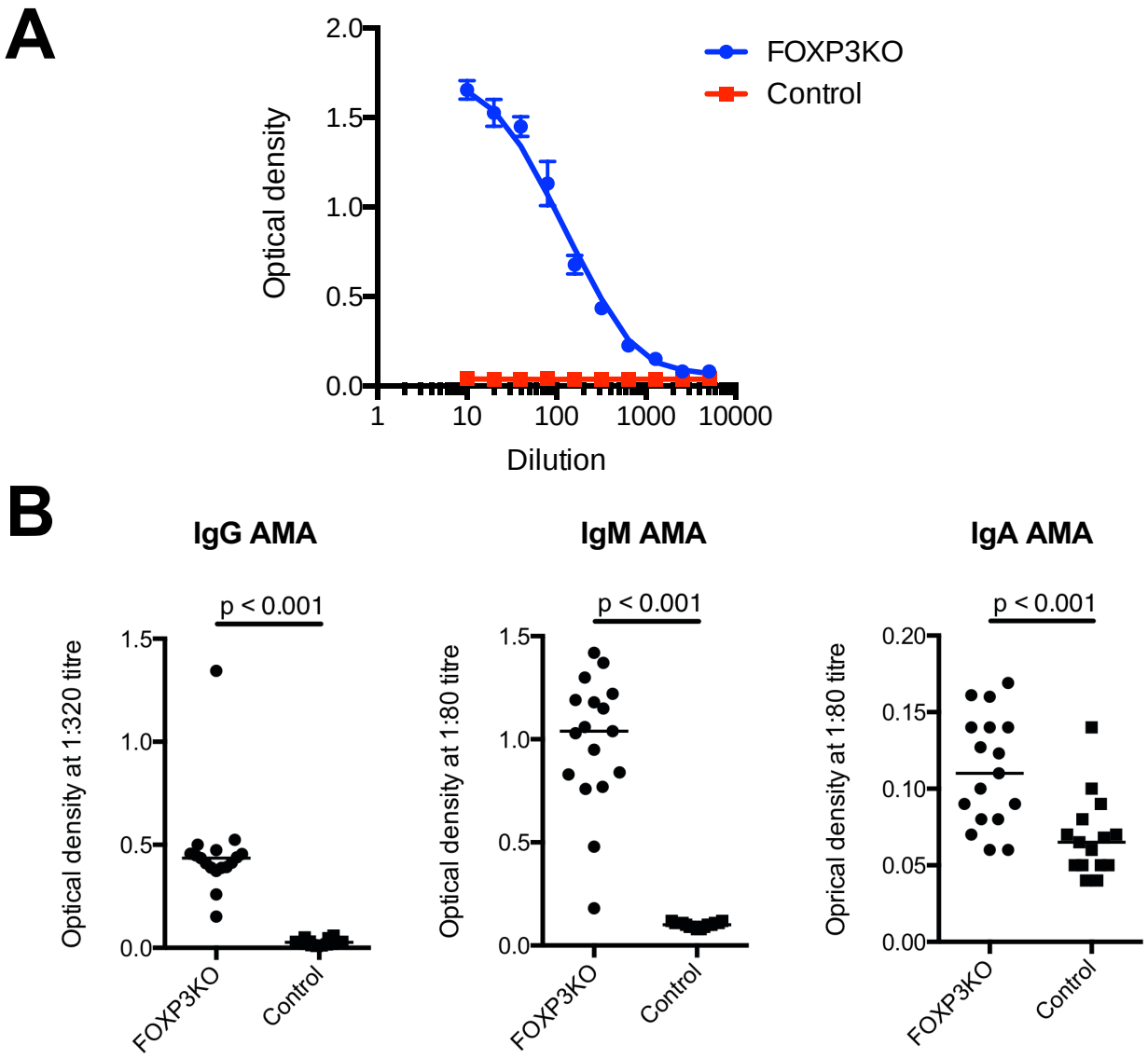


Figure 3.10: AMAs are present in FOXP3^{KO} mouse serum. (**A**) Serial dilutions of FOXP3^{KO} mouse serum revealed high titre reactivity with recombinbant PDC-E2 demonstrating the presence of AMA. (**B**) In contrast to FOXP3 sufficient littermates, FOXP3^{KO} mouse serum contained significantly more IgG, IgM and IgA AMA. Comparisons made with Mann-Whitney U test; n=30.

3.2.2 Comparison of FOXP3^{KO} and FOXP3^{KO}OX40^{het}CD30^{het} mice

Because of breeding limitations, the generation of mice deficient in FOXP3 in the experiments described above was performed by crossing wildtype male mice homozygous for the CD45.1 allele with females from an inbred colony of FOXP3$^{-/-}$OX40$^{-/-}$CD30$^{-/-}$ mice homozygous for CD45.2. This had the result that female offspring had the genotype FOXP3$^{+/-}$OX40$^{+/-}$CD30$^{+/-}$ whilst male offspring had the genotype FOXP3$^{y/-}$OX40$^{+/-}$CD30$^{+/-}$. Phenotypically, the female pups appeared normal as previously reported whilst the male pups developed features consistent with FOXP3-deficiency.[105] Although this breeding was more predictable and sustainable in terms of producing FOXP3-deficient animals for experimentation, it raised the question as to whether these animals heterozygous for functional OX40 and CD30 behaved similarly to animals purely deficient in FOXP3. To assess this, a breeding programme beginning with females from the above cross was initiated to re-derive pups purely deficient in FOXP3. Once female mice that were genotypically identical to standard C57Bl/6 except for being FOXP3$^{+/-}$ were derived these were bred with normal males so that half of male offspring were FOXP3-deficient. These FOXP3$^{y/-}$ pups were then compared to FOXP3$^{y/-}$OX40$^{+/-}$CD30$^{+/-}$ from the original programme. There were no significant differences between pups heterozygous and sufficient for functional OX40 and CD30 (Figure 3.11).

3.2.3 FOXP3^{KO} disease transfers to ZAP70^{KO} mice but is worse in RAG^{KO} mice

In preparation for cohort experiments comparing groups of animals deficient in Treg, adoptive transfer experiments were established. When considering which transfer recipient host mouse strain to use, head-to-head assessment of RAG$^{-/-}$ and ZAP70$^{-/-}$ was performed (Figure 3.12). Animals were simultaneously repleted with 5×10^6 total thymocytes from FOXP3-deficient donors. Mice were monitored for three weeks before sacrifice and analysis. Results demonstrated no difference in spleen weight but significant reductions in serum ALT activity, serum IgG, AMA concentration as assessed by ELISA, and numbers of liver-infiltrating CD8+ T cells in ZAP70$^{-/-}$ as compared to

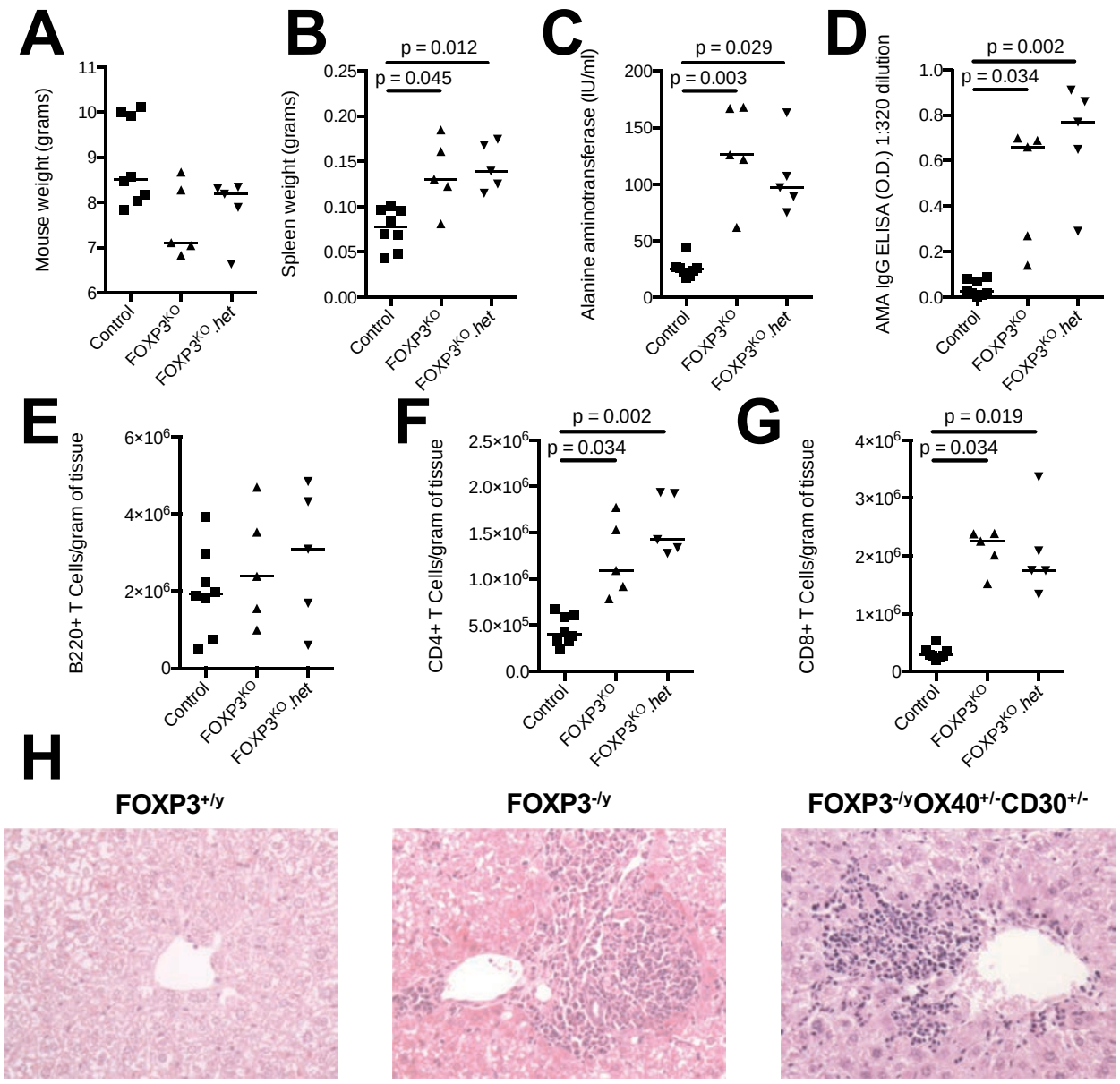


Figure 3.11: FOXP3^{KO}OX40^{het}CD30^{het} mice are similar to pure FOXP3^{KO} mice. Comparisons were made between mice wildtype for FOXP3 (control), OX40 and CD30, mice deficient in FOXP3, and mice deficient in FOXP3 and also heterozygous for OX40 and CD30 (FOXP3^{KO}.het; n=8*v*5*v*5). Mice were analaysed at 3-4 weeks of age. (**A**) There was no significant difference in spleen size but FOXP3-deficient mice demonstrated increased spleen weight (**B**), increased serum ALT acitivity (**C**) and increased titres of serum AMA (**D**) as compared with control animals; there was no difference between FOXP3-deficient animals replete or heterozygous for OX40 and CD30. FOXP3-deficient animals did not show any increase in hepatic B cells (**E**) but demonstrated increases in CD4+ T cells (**F**) and CD8+ T cells (**G**). (**H**) Histological assessment after staining with H&E is shown demonstrating the presence of similar peri-biliary lymphocytic infiltrates in both genotypes of FOXP3 deficient mice. Comparisons made with Kruskal-Wallis test with Dunn's post-hoc test; n=7v5v5.

$RAG^{-/-}$ recipients. Representative sections of liver after H&E staining showed reduced extent of hepatic lymphocytic infiltrate in $ZAP70^{-/-}$ recipients as compared to $RAG^{-/-}$ recipients.

3.2.4 $FOXP3^{KO}$ disease can be prevented by co-transfer of wildtype cells

To investigate whether transfer of intact Tregs could protect mice deficient in FOXP3 from multisystem and hepatic autoimmunity, a co-transfer experiment was performed: zeta-chain-associated protein kinase 70 (ZAP70)-deficient mice were repleted with 5×10^6 total thymocytes from FOXP3-deficient mice. 5 days after initial transfer, hosts received a second transfer of total wildtype lymphocytes sufficient to provide 100 000 Tregs. Mice were then observed for evidence of deterioration by animal house staff unaware of their treatment group, or until 9 weeks. Mice that received wildtype Tregs survived significantly longer than control animals, showed less splenomegaly and reduced hepatic accumulation of CD4+ T cells and CD8+ T cells (Figure 3.13).

Similarly, flow cytometric assessment revealed that whilst co-transfer of intact Tregs did not alter intrahepatic B cell numbers, it prevented intrahepatic accumulation of CD4+ and CD8+ T cells, and reduced numbers of CD44+ CD4+ T cells, IFNγ-secreting CD4+ T cells, and ICOS-positive T cells (Figure 3.15).

3.2.5 $FOXP3^{KO}$ disease can be prevented by co-transfer of regulatory T cells

To confirm that the liver inflammation of FOXP3 deficiency disease could be controlled by transfer of Tregs alone, adoptive transfer of $FOXP3^{KO}$ thymocytes into $ZAP70^{-/-}$ hosts was performed. Then, to test the potential for purified regulatory T cells alone to correct disease, some mice then received a further transfer of 100 000-200 000 purified FOXP3-positive cells. Cells were purified to over 95% purity by sorting on GFP-positive cells from cell suspensions prepared from FOXP3-GFP mice (Figure 3.16). Co-transfer of Tregs abrogated $FOXP3^{KO}$ disease (Figure 3.17 & 3.18)

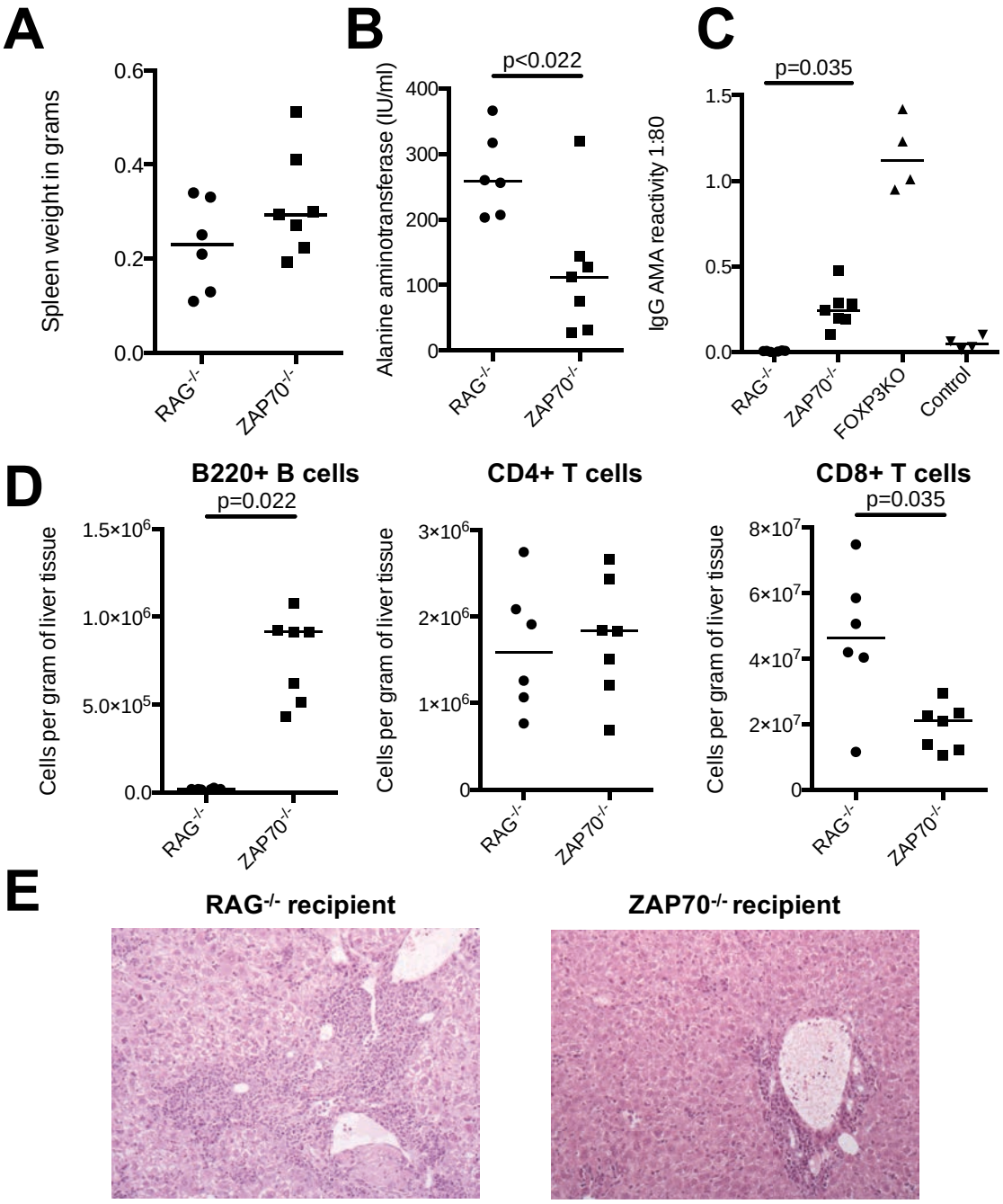


Figure 3.12: FOXP3^{KO} transfer hepatitis is more marked in RAG$^{-/-}$ than ZAP70$^{-/-}$ recipients. Total thymocytes (5×10^6) were transferred into either RAG$^{-/-}$ (n=6) or ZAP70$^{-/-}$ (n=7) recipients. These mice were monitored for three weeks before analysis. There was no difference in spleen weight (**A**), but serum ALT was higher in RAG$^{-/-}$ recipients. AMA were present in ZAP70$^{-/-}$ recipients but not RAG$^{-/-}$ recipients (**C**). **D**. Numbers of B cells were greater in the livers of ZAP70$^{-/-}$ recipients, there was no difference in numbers of CD4+ T cells, but numbers of CD8+ T cells were greater in RAG$^{-/-}$ recipients. H&E staining demonstrated more marked lymphocytic infiltration in RAG$^{-/-}$ than ZAP70$^{-/-}$ recipients (representative histology; 10×magnification). Comparisons with Mann-Whitney U test; n=6v7

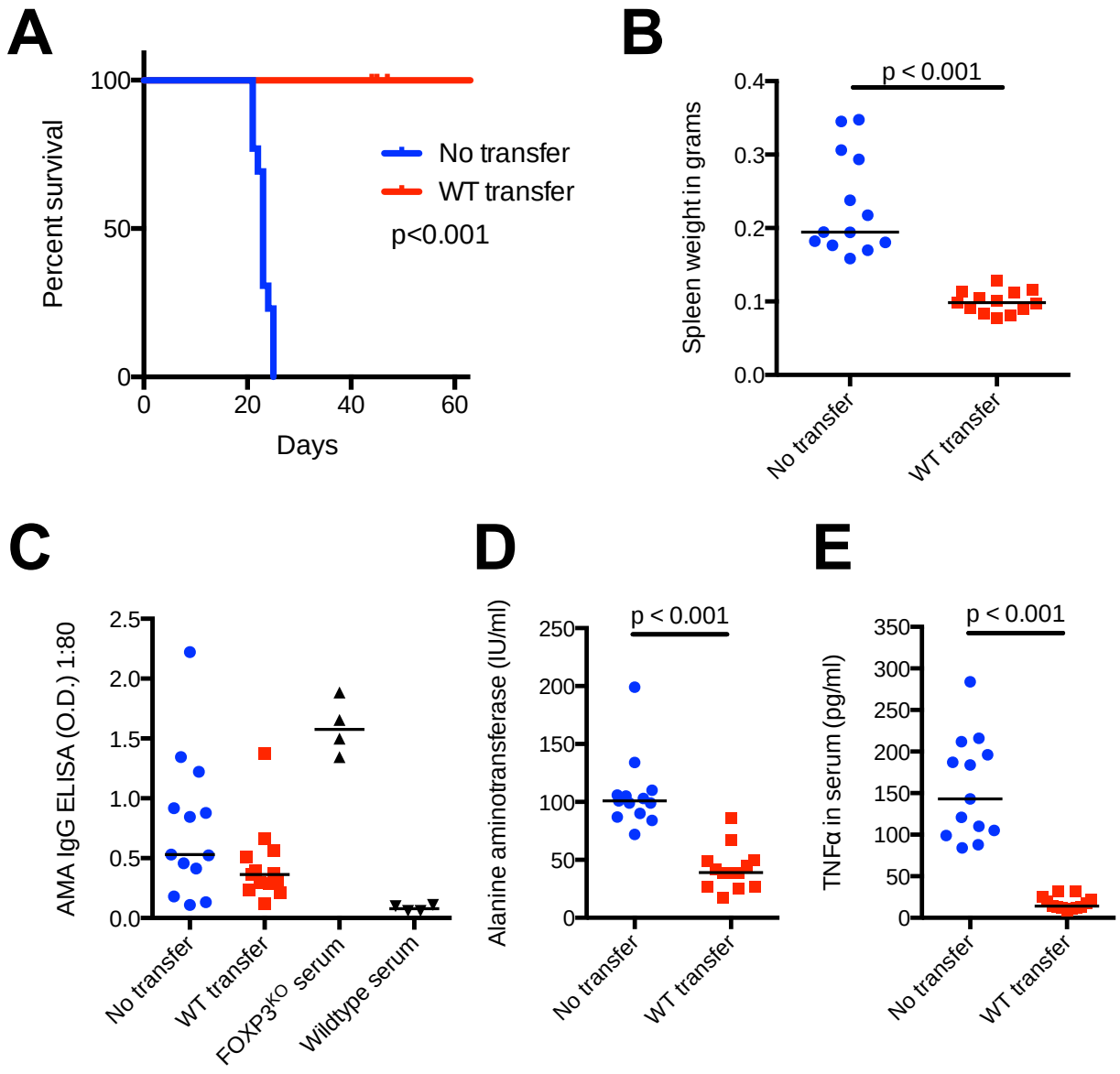


Figure 3.13: Co-transfer of total wildtype cells rescues FOXP3^{KO} transfer disease. ZAP70$^{-/-}$ mice received adoptive transfer of total thymocytes from FOXP3-deficient animals. 5 days later mice received a second transfer of either total wildtype lymphocytes or vehicle alone. Mice that received wildtype lymphocytes showed increased survival (**A**), reduced splenomegaly (**B**), no change in AMA titre (**C**), a reduction in serum ALT activity (**D**), and a reduction in serum TNFα (**E**). Comparisons with Log-Rank or Mann-Whitney U test; n=13v13.

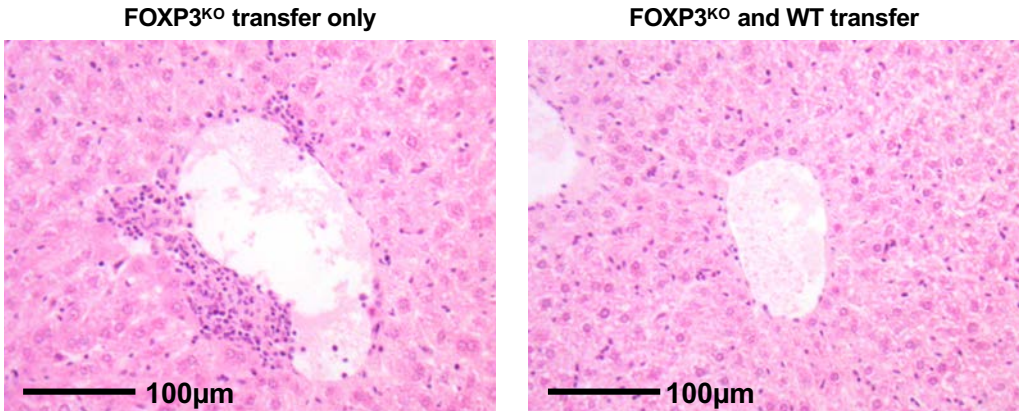


Figure 3.14: Co-transfer of total wildtype cells rescues FOXP3^{KO} transfer disease. ZAP70$^{-/-}$ mice received adoptive transfer of total thymocytes from FOXP3-deficient animals. 5 days later mice received a second transfer of either total wildtype lymphocytes or vehicle alone. Mice that received wildtype lymphocytes showed did not manifest lymphocytic peri-venular infiltrates. Representative H&E images.

3.2.6 FOXP3^{KO} disease can be prevented by transfer of AIRE^{KO} regulatory T cells

Having demonstrated that repletion with exogenous Tregs was sufficient to prevent the development of multi-system autoimmunity and lymphocyte hepatitis in Treg deficiency, the issue of antigen-specificity was considered. Mice bred with loss of function mutations in the AIRE gene were used to prepare suspensions of total lymphocytes. These were assessed by flow cytometry with staining for CD3, CD4 and CD25. CD3+CD4+CD25^{high} cells were assumed to represent Tregs.

ZAP70$^{-/-}$ mice were repleted with 5×10^6 FOXP3-deficient total thymocytes. 5 days later some mice received sufficient total peripheral AIRE-deficient or wildtype lymphocytes to provide 200 000 Tregs or vehicle alone. Mice were then observed for up to 6 weeks or the point at which they were adjudged to be unwell by animal technicians unaware of the treatment arm. At the point of analysis, those mice that had not received an additional cell transfer demonstrated significantly reduced survival (Figure 3.19A), increased serum TNFα (Figure 3.19B), increased splenomegaly (Figure 3.19C), increased ALT activity (Figure 3.19D), but no significant difference in serum IgG AMA concentration as assessed by ELISA (Figure 3.19E). There were no significant differences between animals that

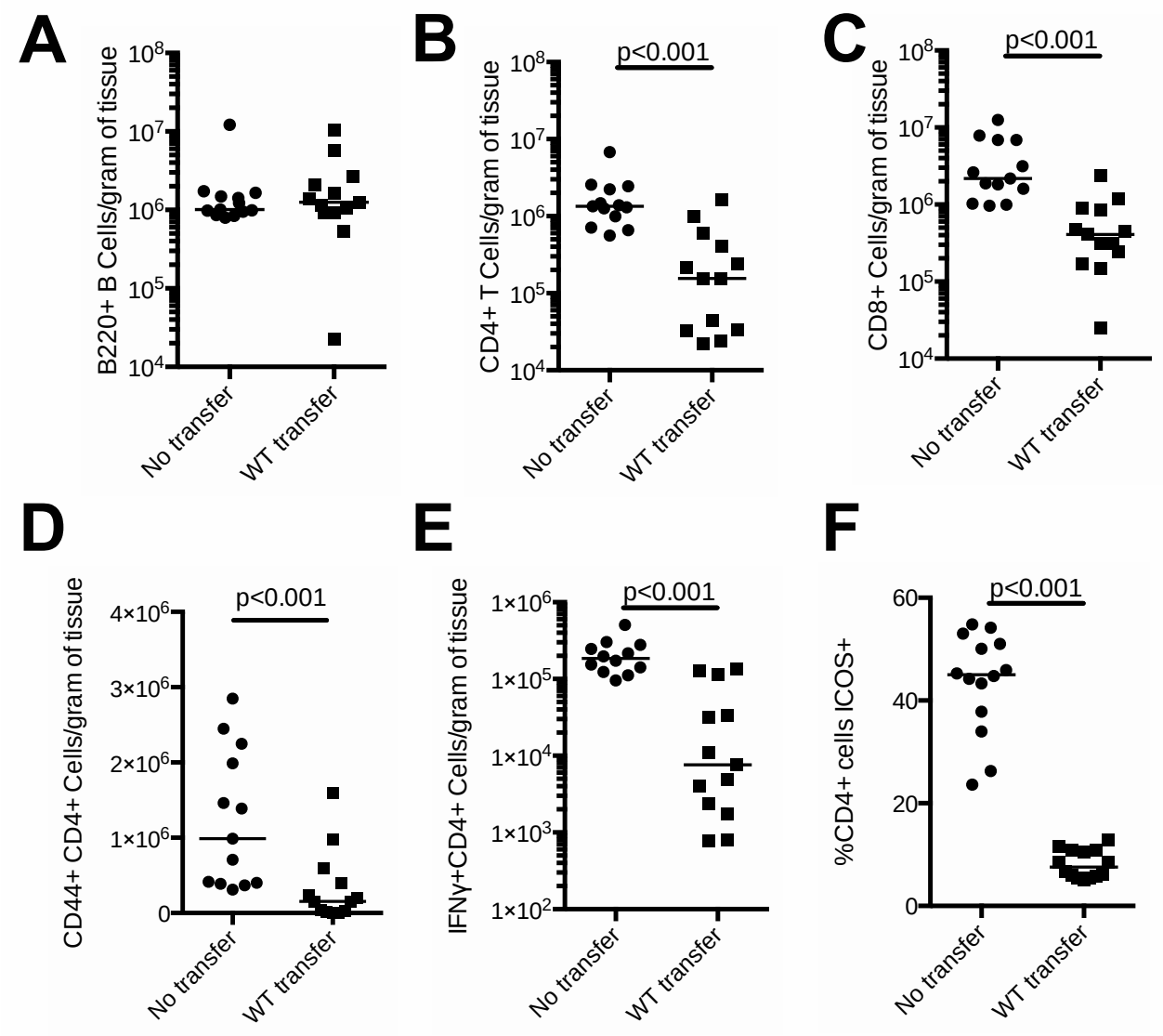


Figure 3.15: Co-transfer of total wildtype cells rescues FOXP3 transfer disease. ZAP70$^{-/-}$ mice received adoptive transfer of total thymocytes from FOXP3-deficient animals. 5 days later mice received a second transfer of either total wildtype lymphocytes or vehicle alone. Mice that received wildtype lymphocytes showed no difference in numbers of intrahepatic B cells (**A**), a reduction in intrahepatic CD4+ T cells, CD8+ T cells, CD44+, IFNγ-producing and ICOS-positive CD4+ T cells (**B-F**). Comparisons with Mann-Whitney U test; n=13v13.

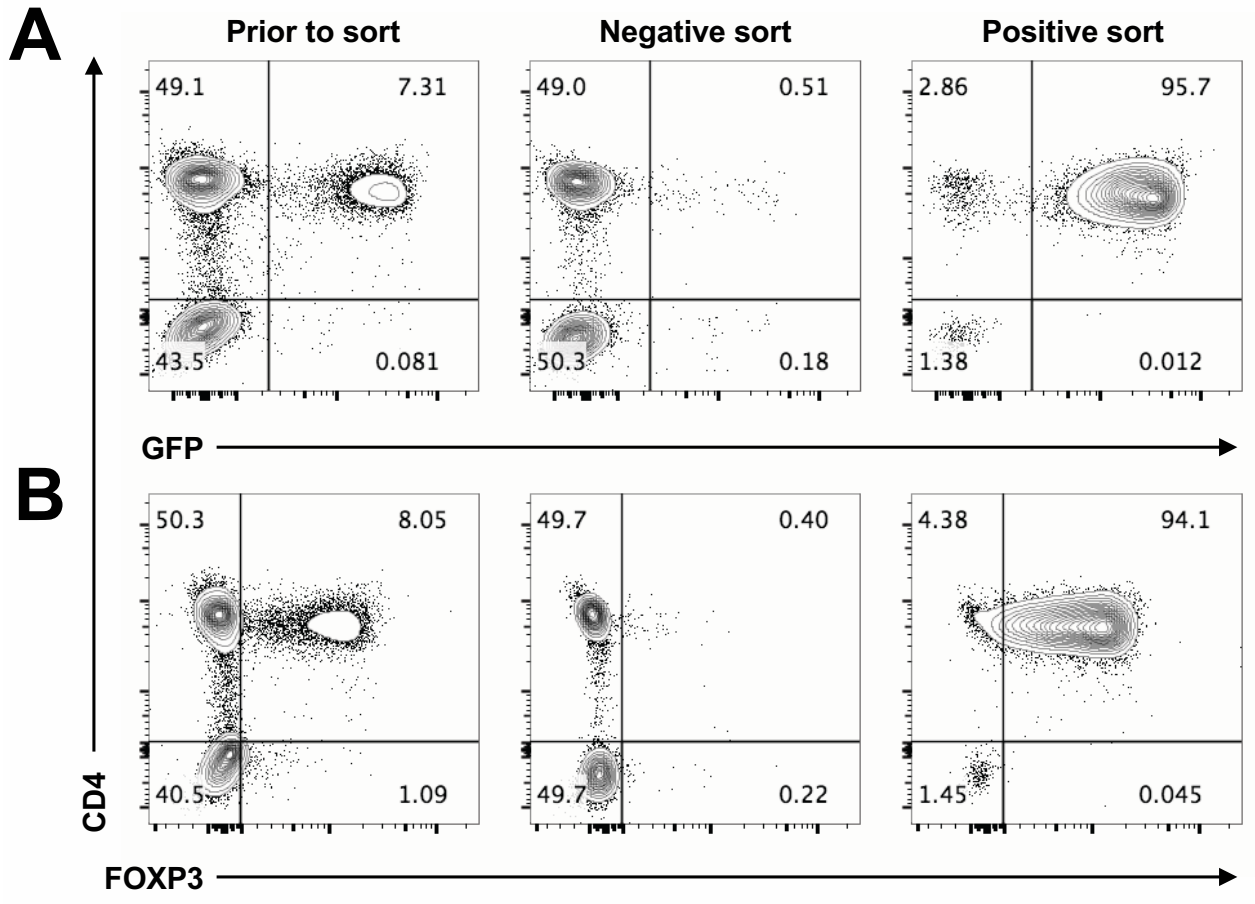


Figure 3.16: Sorting of green fluorescent protein (GFP)-positive regulatory T cells. Prior to transfer experiments, purified suspensions of regulatory T cells were prepared from FOXP3-GFP mice. Representative plots gated on CD3-positive cells derived from the spleens of FOXP3-GFP mice. Approximately 95% purity was obtained after sorting as demonstrated by flow cytometry examining GFP in cells without intracellular staining (**A**) and by using an anti-FOXP3 antibody (**B**).

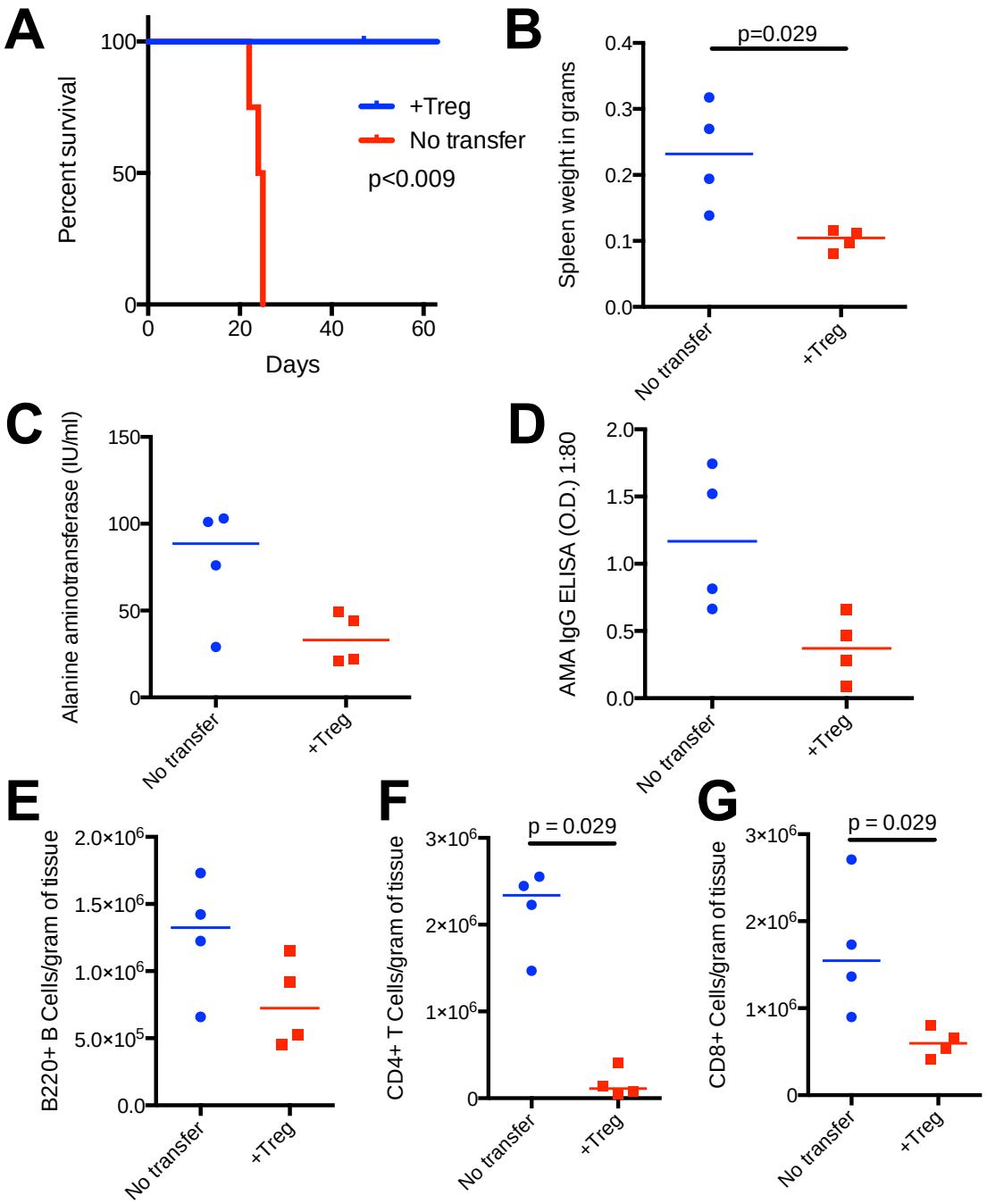


Figure 3.17: Transfer of purified regulatory T cells prevents FOXP3 transfer disease. ZAP70$^{-/-}$ T cell deficient mice received adoptive transfer of FOXP3^{KO} cells. Some mice also received 200 000 purified Tregs generated from FOXP3-GFP mouse spleen. Transfer of purified Tregs prevented overt development of autoimmune disease and increases survival (**A**), reduced the onset of splenomegaly (**B**), non-significantly reduced serum ALT activity (**C**), non-significantly reduced serum AMA titre (**D**), did not significantly affect numbers of hepatic B cells (**E**), reduced intrahepatic CD4+ T cell accumulation (**F**), and reduced CD8+ T cell accumulation (**G**). Comparisons by Mann-Whitney U test; n=8.

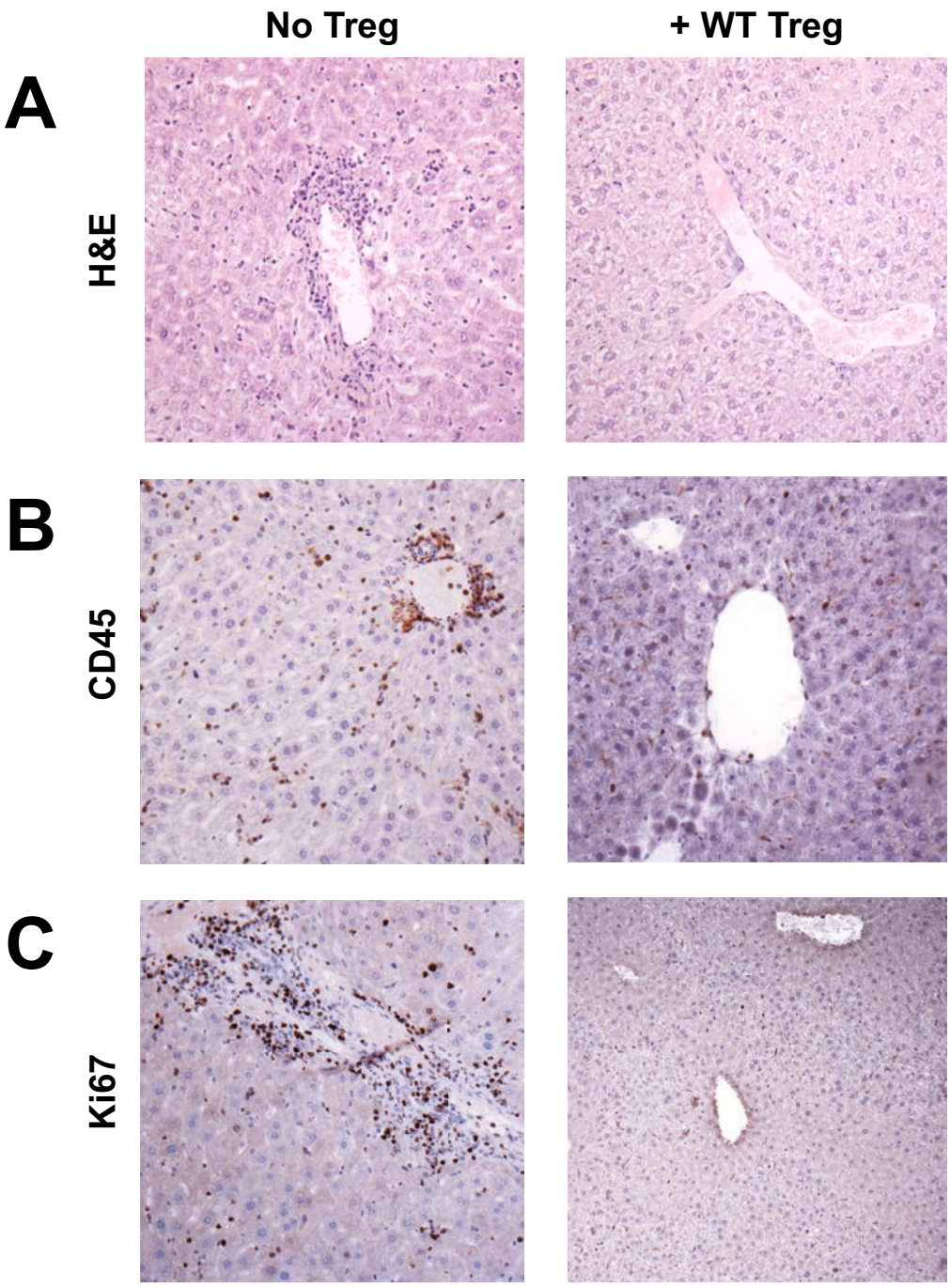


Figure 3.18: Transfer of purified Tregs prevents FOXP3^{KO} transfer disease: histology. ZAP70$^{-/-}$ T cell deficient mice received adoptive transfer of FOXP3^{KO} cells. Some mice also received 200 000 purified Tregs generated from FOXP3-GFP mouse spleen. Transfer of purified Tregs prevented the development of perivenular lymphocytic infiltrates (**A**), reduced numbers of intrahepatic cells staining positive for both CD45 (**B**), and Ki67 (**C**). Representative images shown.

received cells from wildtype and AIRE-deficient animals.

Similarly, flow cytometric analysis of liver-infiltrating cells demonstrated that, similar to mice that received wildtype Tregs, mice that received AIRE-deficient Tregs showed a significant reduction in the numbers of infiltrating T cells but with no differences between mice receiving the two different types of Tregs (Figure 3.20). Consistent with flow cytometric findings, mice that received either wildtype or AIRE-deficient Tregs did not develop the highly Ki67-positive CD4-rich lymphocytic infiltrate seen in those mice that received only $FOXP3^{KO}$ cells (Figure 3.21).

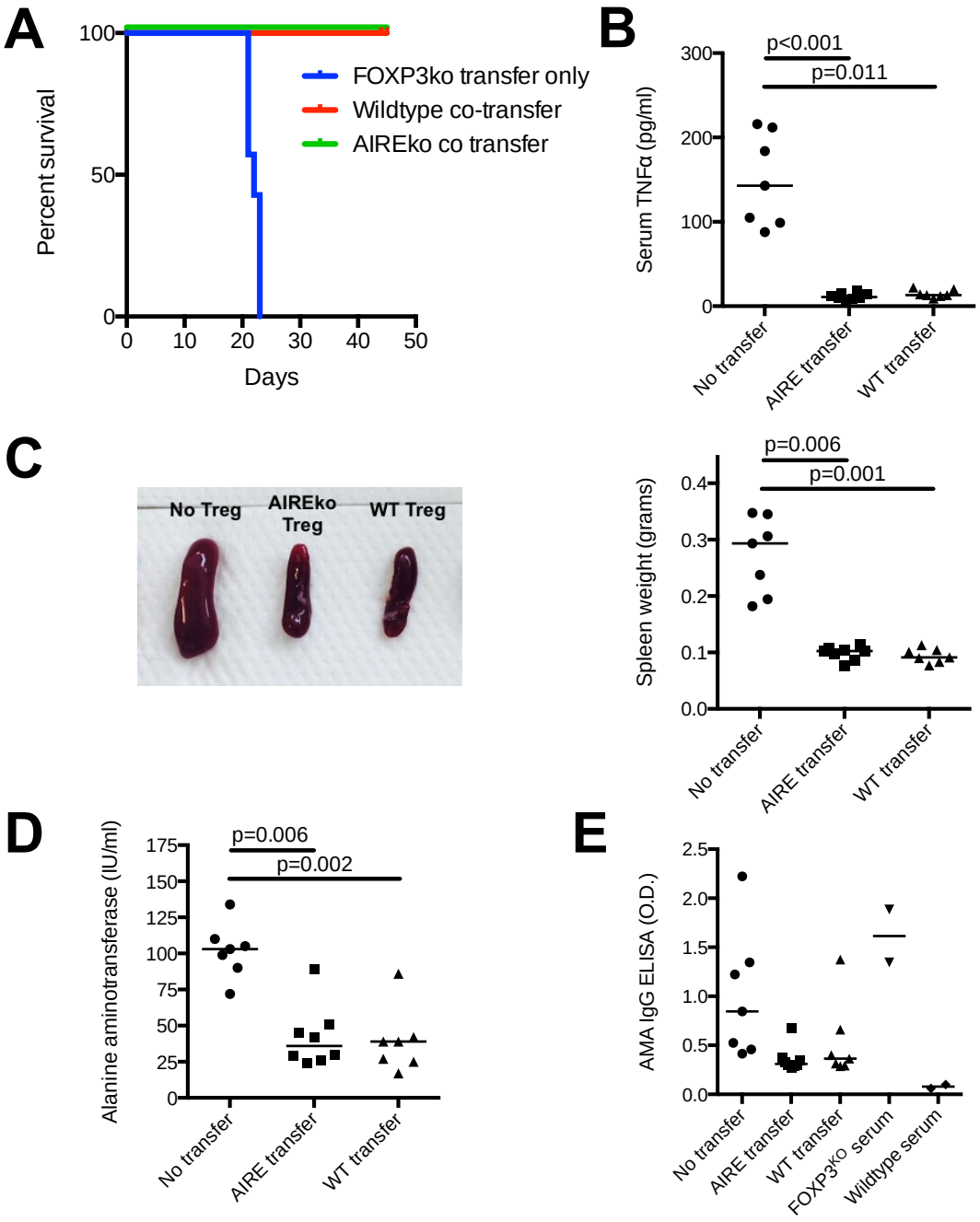


Figure 3.19: AIRE$^{-/-}$ cells protect against FOXP3^{KO} disease: phenotype. ZAP70 T cell deficient mice received adoptive transfer of FOXP3^{KO} cells. Some mice also received either total wildtype splenocytes or total AIRE$^{-/-}$ splenocytes. Receipt of either FOXP3^{KO} or AIRE$^{-/-}$ cells increased survival (**A**), reduced serum TNFα (**B**), reduced splenomegaly (**C**), reduced serum ALT (**D**), and showed a trend towards a reduction in AMA titre; n=7v8v7.

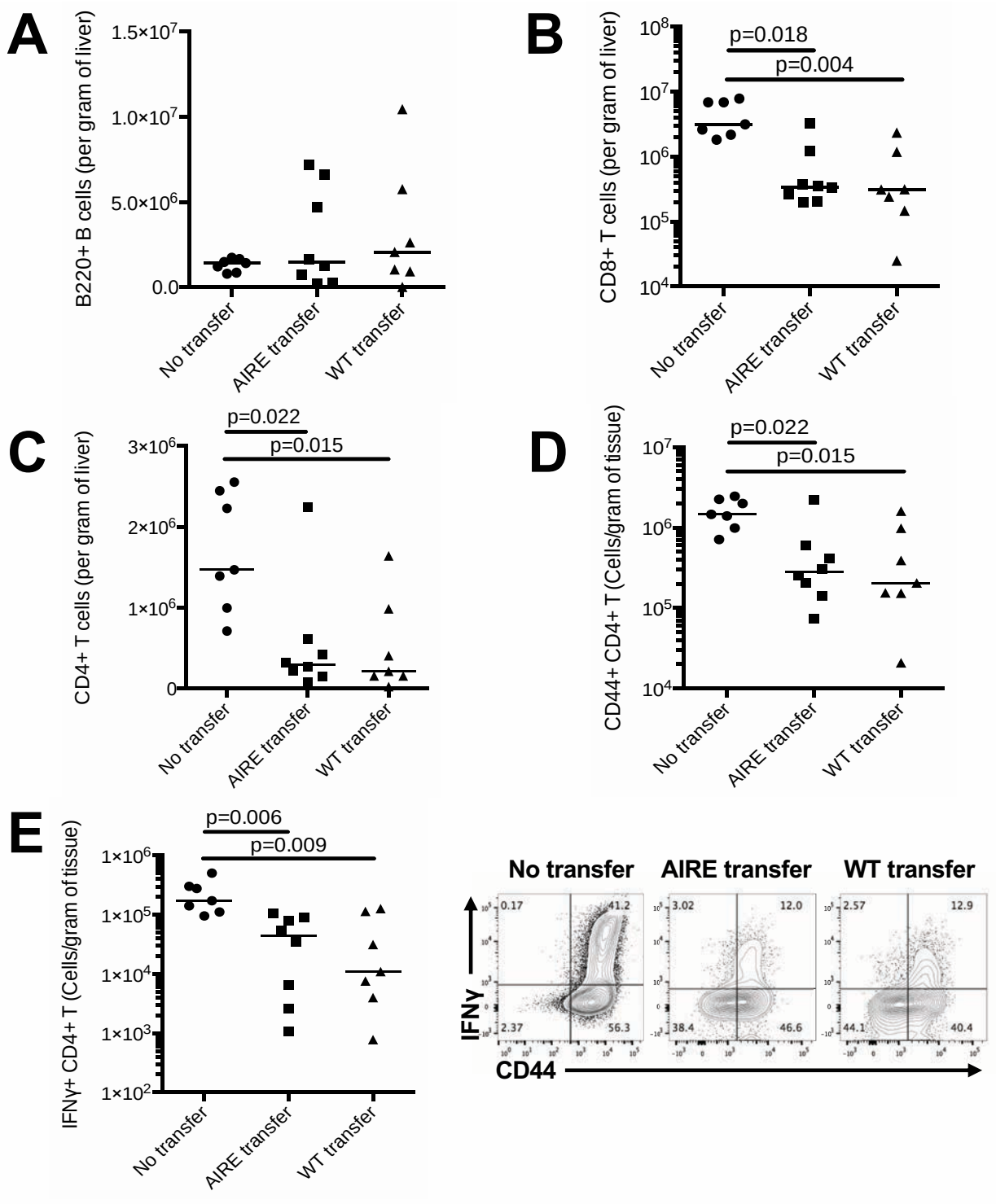


Figure 3.20: AIRE$^{-/-}$ cells protect against FOXP3^{KO} disease: flow cytometry. Plots demonstrating that although co-transfer of either wildtype or AIRE$^{-/-}$ cells into FOXP3^{KO} recipients (**A**) makes no significant difference in numbers of B220+ B cells, (**B** and **C**) it reduces the extent of both CD8+ and CD4+ infiltrate, (**D**) reduces numbers of CD44+ and (**E**) IFNγ-secreting CD4+ T cells. Comparisons via Kruskal-Wallis test; n=7v8v7.

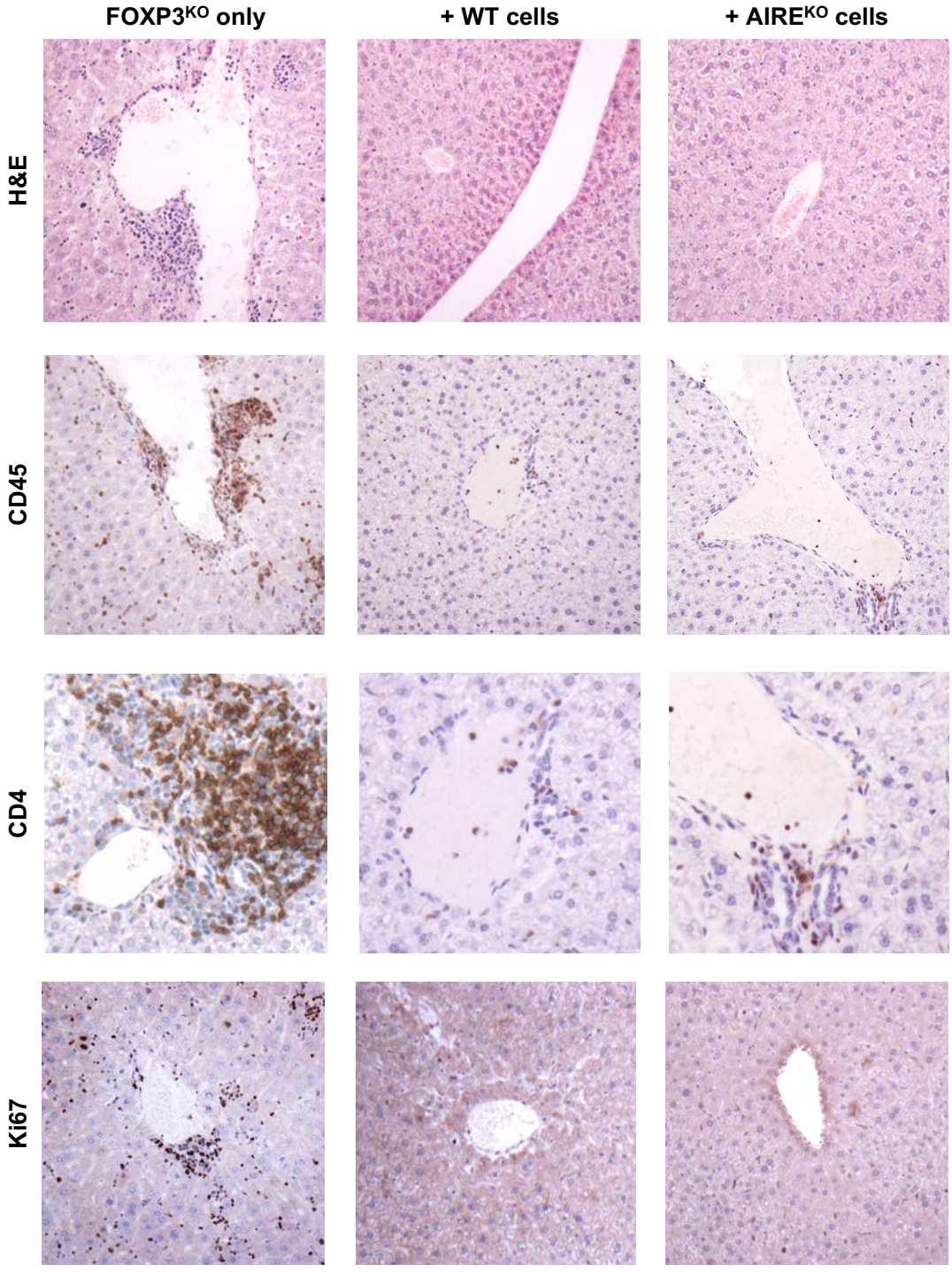


Figure 3.21: AIRE$^{-/-}$ cells protect against FOXP3^{KO} disease: histology. Images show representative liver histology from ZAP70$^{-/-}$ mice that received FOXP3^{KO} cells. Some mice also received total wildtype (WT) splenocytes or total AIRE$^{-/-}$ splenocytes. In mice that received only FOXP3^{KO} cells, a dense lymphocyte hepatitis with numerous CD45-, CD4-, and Ki67-positive peri-venular / peri-biliary cells developed. In mice that also received either WT or AIRE$^{-/-}$ cells, this infiltrate was abolished.

3.3 Discussion

The primary aim of this chapter was to recapitulate the striking findings of hepatic pathology reported by the Gershwin group: dense, T cell rich peri-biliary lymphocytic infiltrates with an increased CD8+:CD4+ ratio, elevations in serum effector cytokines including TNFα, and the development of AMA.[504] The results presented here indeed recapitulate those reported by the Gershwin group, but also add some further findings that represent useful measures to be used in subsequent experiments.

First, I record that both serum total IgG and total IgE concentrations are increased in mice with FOXP3 deficiency: the former a typical finding in human AIH and common in PBC.[203] Second, I demonstrate elevation in serum ALT activity: again a common finding in human AILD; notably however, elevations in ALT activity were not as marked as in some other models of AIH.

A third outcome measured here was serum ALP activity. Unexpectedly, in comparison with others' work suggesting a correlation between biliary injury and ALP in C57Bl/6 mouse models e.g. [373], I record a consistent inverse correlation and subsequently via differential heat stability demonstrate that the enzyme activity that was recorded was likely to be of extra-hepatic origin. This is consistent with a recent analysis of the tissue origins of murine ALP.[241] This finding led to the discontinuation of ALP measurements for the remainder of this project, and questions its suitability as an outcome measure when considering liver disease, at least in the C57Bl/6 strain.

The work presented in this chapter was designed to assist in the design of future experiments. Specifically, because of the unpredictability of breeding, the short natural lifespan, and small size of FOXP3 deficient mice, a transfer system into larger host animals that would permit multiple simultaneous experimental animals to be generated at once was desirable. Thus, the establishment of an adoptive transfer model described represents a useful basis for future experiments.

In choosing potential hosts for these experiments, both RAG deficient and ZAP70 deficient hosts

were used. Interestingly, in head-to-head comparison, the liver injury seen was worsened in RAG-deficient animals. This observation corresponds to a contemporaneous report that γδ T cells have some protective role in the autoimmunity resultant in T cell deficient mice repleted with FOXP3 deficient cells.[426] Notably, ZAP70-deficient mice have γδ T cells, but RAG-deficient mice do not.[183]

A second difference between ZAP70 and RAG deficient mice is that the latter lack B cells. The absence of B cells is reported as important for the generation of pathology in FOXP3 deficient mice, and the findings here contrast with this in that pathology was worse in the relatively B cell deficient $RAG^{-/-}$ recipients.[9] Notably however, the method for cell transfer used here did not remove B cells and so animals were likely to be partially repleted with donor B cells. Given the lesser baseline immunological deficit of ZAP70-deficient mice, this strain was used for subsequent experiments.

As a method of confirmation of the causative deficit in these animals being deficiency in Tregs, co-transfer experiments were performed. First, as expected, co-transfer of total wildtype lymphocytes prevented FOXP3-deficiency transfer disease. To demonstrate absolutely that Tregs alone were sufficient to prevent the manifestations of FOXP3-deficiency, Tregs from mice expressing GFP alongside FOXP3 were isolated using cell sorting and co-transferred into mice. This too was effective and is consistent with previous reports of generally reduced autoimmunity with transfer of Tregs.[100] Such approaches have relevance to IPEX patients in the context of gene therapy: autologous cells might be removed, transfected with functional FOXP3, expanded and re-infused without risk of graft versus host type reactions and little risk of rejection.

Having read with interest that transfer of intact Tregs into mice with an AIH-like phenotype lacking functional AIRE reversed hepatitis[132], and in the context of a direct human disease correlate to these mice,[348, 437] I used a co-transfer model to explore whether AIRE-deficient Tregs could prevent hepatitis in FOXP3-deficiency. In contrast to what might be expected from the reported ability of wildtype Tregs to abolish inflammation in AIRE deficiency, AIRE-deficient Tregs were able to abolish hepatic inflammation in this model.

Weaknesses in this approach

Possible criticisms of the work presented in this chapter include the relatively short time course of the experiments. Human AILDs typically result in chronic fibrosis, an outcome that was not assessed here. Further, arguably to draw greater analogies to human disease, a wider variety of autoantibodies might have been assessed, although testing kits were less available. Arguably, cell populations used for transfer experiments might have been purer: e.g. rendered devoid of B cells and other non T cells, but there was concern that this might alter the transferred T cell phenotype.

In addition, only a small number of potential cell transfer recipients were explored. It may have been preferable to use a strain such as the T-cell receptor alpha chain (TCRα)-deficient mouse which lacks only αβ T cells so as to minimise deviation from wildtype. With respect to experiments assessing the role for AIRE, a determination as to whether intermittent AIRE-negative donor mice had developed hepatitis was not performed.

Future experiments

Future work based on the work presented in this chapter largely revolves around manipulation of the transfer models presented e.g. by the administration of potentially therapeutic compounds or the co-transfer of other cell types such as Tregs deficient in specific compounds. An ideal comparison material would be liver samples from human individuals with the IPEX syndrome or individuals with dysfunction in AIRE, but such material is not readily available.

4 OX40-OX40L blockade in regulatory T cell deficiency

4.1 Introduction

The TNF receptor OX40 is primarily expressed on CD4+ T cells after their activation. It is ligated by its cognate ligand OX40L, which is expressed on activated antigen presenting cells, on some innate lymphoid cells, on natural killer T (NKT) cells, and on T cells. Ligation of OX40 promotes cell survival, enhances effector function and tends to decrease regulatory activity.[72] Blockade of OX40-OX40L interactions has been demonstrated to be effective in many animal models of autoimmunity, but not universally.[471]

The autoimmunity of FOXP3 deficiency results from a complete absence of Tregs. There is unrestrained activation of effector T cells and several organ systems are damaged by infiltration with activated lymphocytes in what is an ultimately fatal systemic disease. Because mice with FOXP3 dysfunction develop hepatitis and the AMA that are characteristic of the human autoimmune liver disease PBC, FOXP3 dysfunction has been described as approximating aspects of the human disease. Previous work has shown marked increases in overall survival of mice with FOXP3 dysfunction given blocking OX40L antibody or co-deficient in OX40, but the livers of these mice were not examined in detail, nor were parameters such as ALT, serum IgG or autoantibodies assessed.[105] One reason for examining the liver specifically is that divergent effects on autoimmunity in different organ systems have been described in other mouse models of regulatory failure.[492, 505, 291]

This chapter details experiments investigating whether OX40 deficient mice have any major disturbance of the intrahepatic lymphocyte population before assessing whether blocking antibody to OX40L prevents the hepatitis seen in $FOXP3^{KO}$ transfer disease. It then proceeds to consider a different blocking mechanism. An anti-OX40 antigen-binding region of antibody (F_{ab}) fragment is then considered in the same model before investigating whether inhibition of OX40-OX40L interaction alters the T-cell dependent Con A model of hepatitis or the T cell independent hepatitis seen after administration of CCl_4. Finally, histological evidence of OX40 expression in human liver disease is sought.

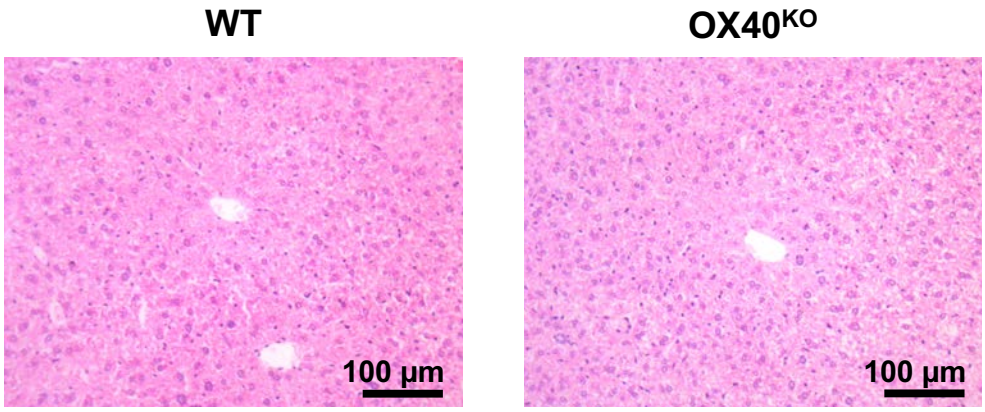


Figure 4.1: Basic histology of OX40^{KO} mouse liver. Representative sections stained with H&E are shown from wildtype and OX40^{KO} mice. Unmanipulated OX40 deficient mice have normal appearances to their livers without visible infiltrate.

4.2 Results

4.2.1 OX40 deficient and wildtype mice have similar intrahepatic lymphocyte populations

Prior to using antibody preparations to block interactions of OX40 and OX40L, the livers of unmanipulated mice both sufficient and deficient (OX40^{KO}) in OX40 were examined as a reference baseline for further experiments. These mice appeared healthy and microscopic examination of their liver structure was normal without obvious infiltrate (Figure 4.1).

Flow cytometric analysis of intrahepatic lymphoyctes was undertaken and although this did not demonstrate alterations in total numbers of B cells, total CD4+ T cells or total CD8+ T cells, proportions and absolute numbers of FOXP3+ Tregs were reduced, and the proportion of cells negative for CD44 was decreased (Figure 4.2).

4.2.2 Blocking anti-OX40L prevents FOXP3^{KO} transfer hepatitis

To investigate the effects of blocking OX40-OX40L interactions on FOXP3^{KO} hepatitis, a transfer model was used. ZAP70$^{-/-}$ T cell deficient mice were injected with 5×10^6 total FOXP3^{KO} thymo-

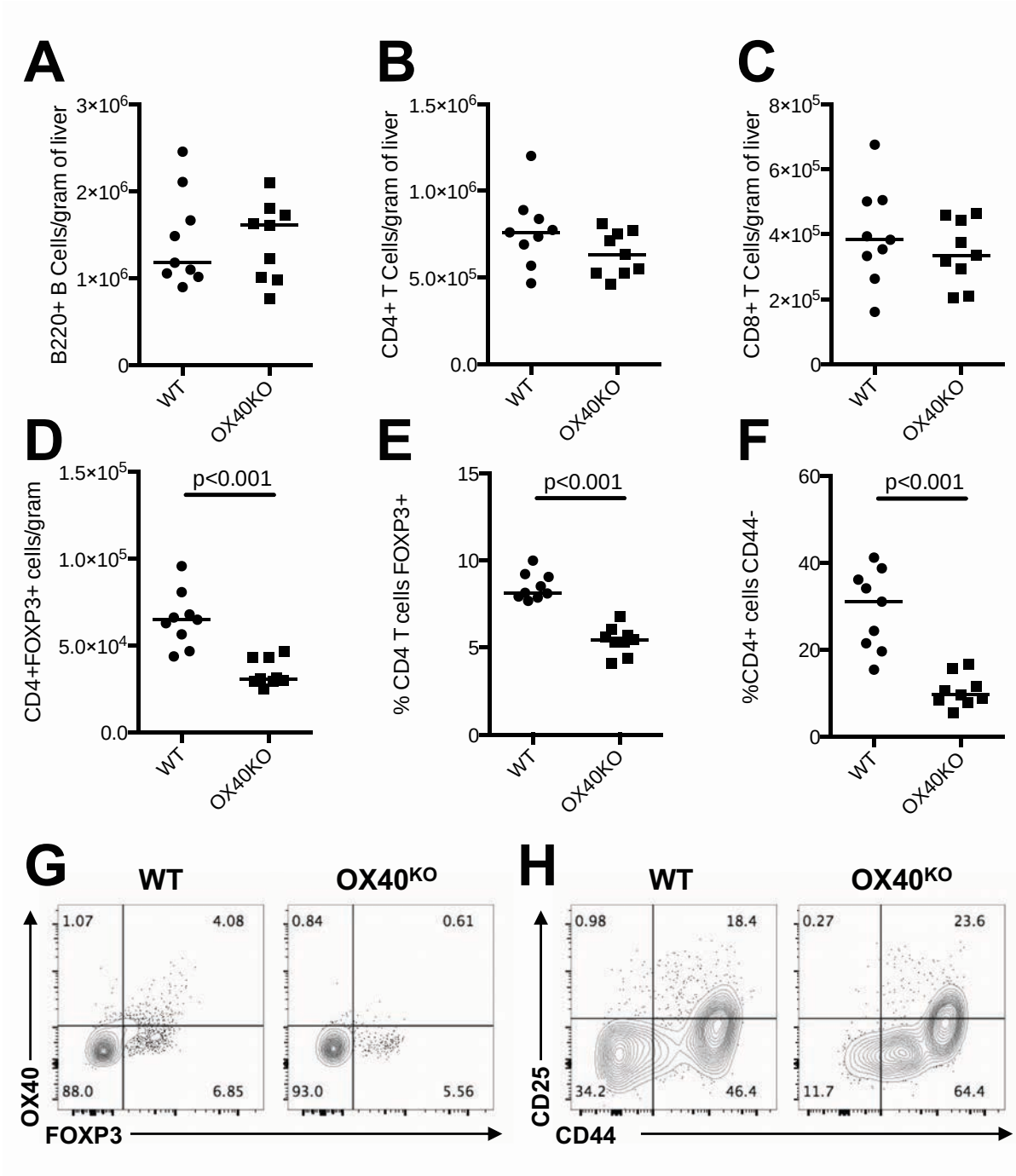


Figure 4.2: OX40^{KO} mouse hepatic lymphocyte populations. The lymphocyte populations of OX40-deficient mice were examined by flow cytometry and compared to samples from age-matched wildtype mice. No significant differences in total numbers of (**A**) B cells, (**B**) CD4+ T cells, or (**C**) CD8+ T cells were evident. However, both numbers (**D**) and the proportion of CD4 T cells that were FOXP3 positive (**E**) were reduced; a higher proportion of CD4+ T cells also expressed CD44 (**F**). (**G** and **H**) flow cytometry panels representative of results presented in panels E and F. Comparisons with Mann-Whitney U test; n=9v9.

cytes IP. Mice were then split into two groups and either treated with 250 μg of rat antagonistic anti-OX40L IgG twice weekly or with an identical dose of non-specific total rat IgG. Mice were then observed for deterioration for six weeks and either analysed at the point of deterioration or the end of the experiment.

Overall, mice treated with anti-OX40L showed markedly increased survival with all individuals surviving to the end of the experiment (Figure 4.3A). Similarly, other markers of evidence of systemic immune activation were decreased by the administration of anti-OX40L including spleen size and serum IgG and IgE concentrations (Figure 4.3B, E & F). Further, both serum ALT and AMA titres were reduced by the administration of anti-OX40L.

When the livers of mice treated with anti-OX40L IgG after FOXP3^{KO} thymocyte transfer were examined, the liver appearances had normalised with complete abolition of lymphocytic infiltrate when examined microscopically (Figure 4.4A&B). This was confirmed by a significant decrease in the proportion of liver sections that stained positive for the pan-leucocyte marker CD45 (Figure 4.4C-E).

Flow cytometric analysis of FOXP3^{KO} thymocyte recipients that had received anti-OX40L showed no significant differences in numbers of hepatic B cells but showed reductions in both CD4+ and CD8+ T cells (Figure 4.5A-C). Further examination of hepatic CD4+ T cells demonstrated that nearly all remained CD44-positive but that expression of the activation marker ICOS was markedly reduced (Figure 4.5D-F).

4.2.3 Blocking anti-OX40 Fab prevents FOXP3^{KO} transfer hepatitis

Having demonstrated that administration of blocking anti-OX40L antibody reduced generalised and liver autoimmunity in deficiency of Tregs, it was considered that the antibody might have been directly causing depletion of T cells through fixation of complement. Therefore to both investigate

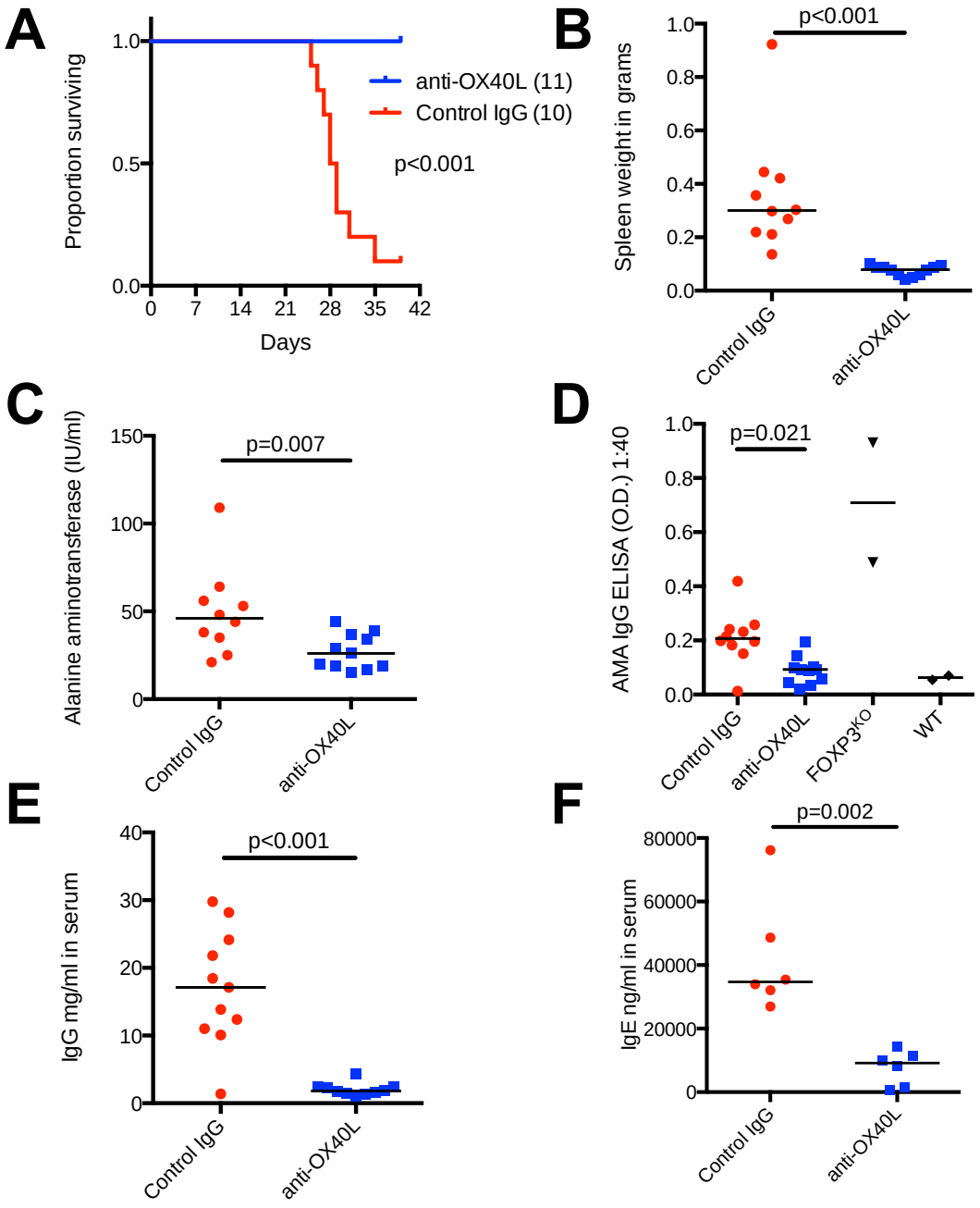


Figure 4.3: FOXP3^{KO} transfer hepatitis and anti-OX40L blocking antibody. ZAP70$^{-/-}$ mice received FOXP3^{KO} total thymocytes and were assessed for the development of generalised autoimmunity and hepatitis. In comparison to administering control rat total IgG, administration of 250 μg of blocking anti-OX40L twice weekly from day 5 after cell transfer, prevented all deaths in the treated group (**A**), prevented the development of splenomegaly (**B**), reduced biochemical evidence of hepatitis in the form of serum ALT activity, reduced AMA titre (**D**), and reduced serum IgG (**E**) and serum IgE (**F**). Results represent the combination of two separate experiments of 21 mice total. p values represent either the log-rank test for survival analysis or the Mann-Whitney U test. Not all samples were analysed for IgE. Comparisons with Mann-Whitney U test; n=10v11.

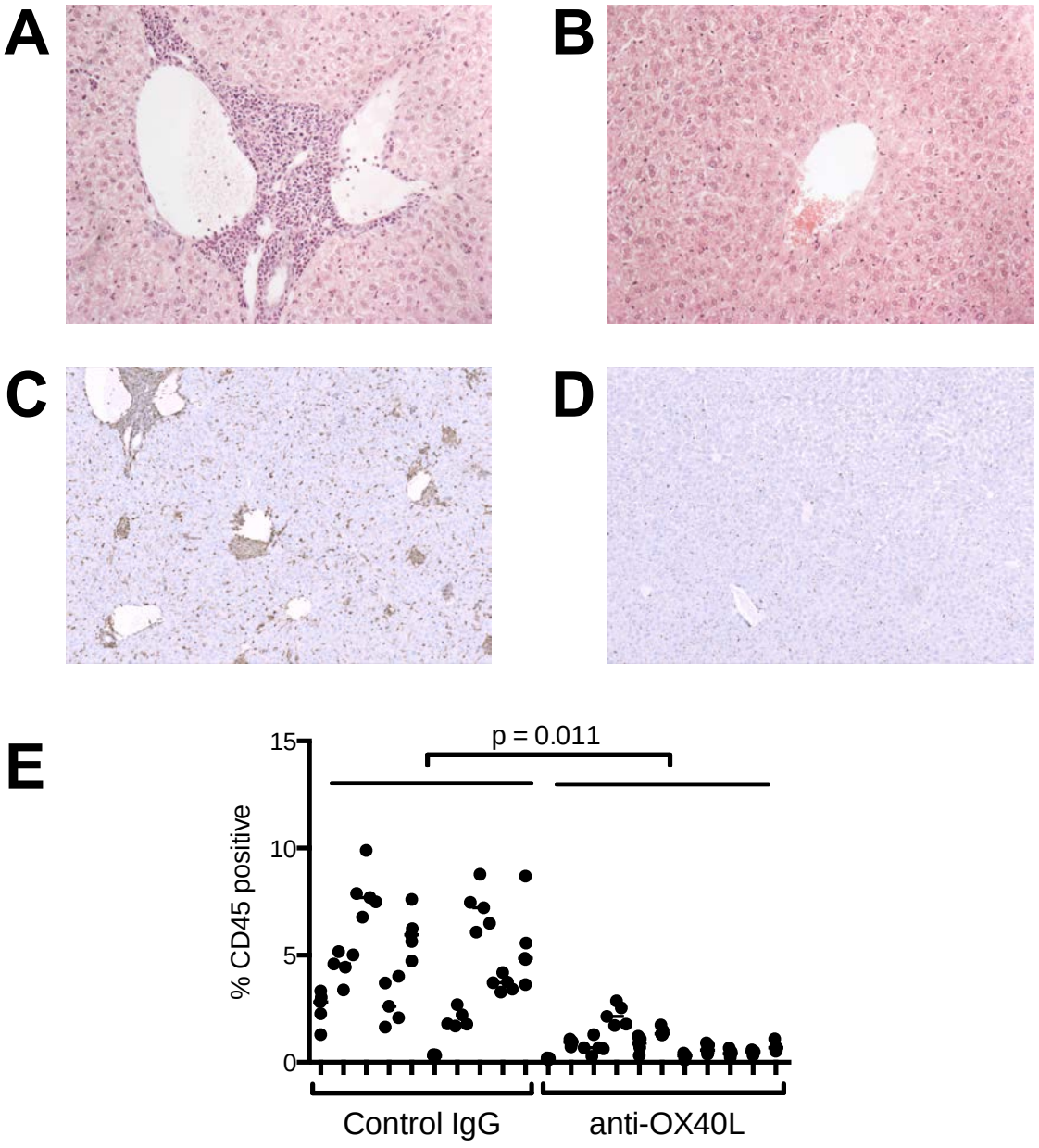


Figure 4.4: FOXP3^{KO} transfer hepatitis and anti-OX40L blocking antibody. ZAP70$^{-/-}$ mice received FOXP3^{KO} total thymocytes and were assessed for generalised autoimmunity and hepatitis. Those mice that were given twice weekly control total rat IgG developed marked lymphocytic peri-venular hepatitis (**A**). However, those mice that were treated with twice weekly from day 5 after cell transfer with anti-OX40L antibody did not develop hepatitis (**B**). Liver sections were stained for the pan-leucocyte marker CD45 highlighting the lymphocytic infiltration in control animals (**C**) and its abolition in those treated (**D**). (**E**) When CD45 infiltrates were digitally quantified, they were significantly reduced in those mice treated with anti-OX40L antibody. Results represent the combination of two separate experiments. Comparisons with Mann-Whitney U test; n=10v11; images A and B taken at × 20 magnification, C and D at × 10.

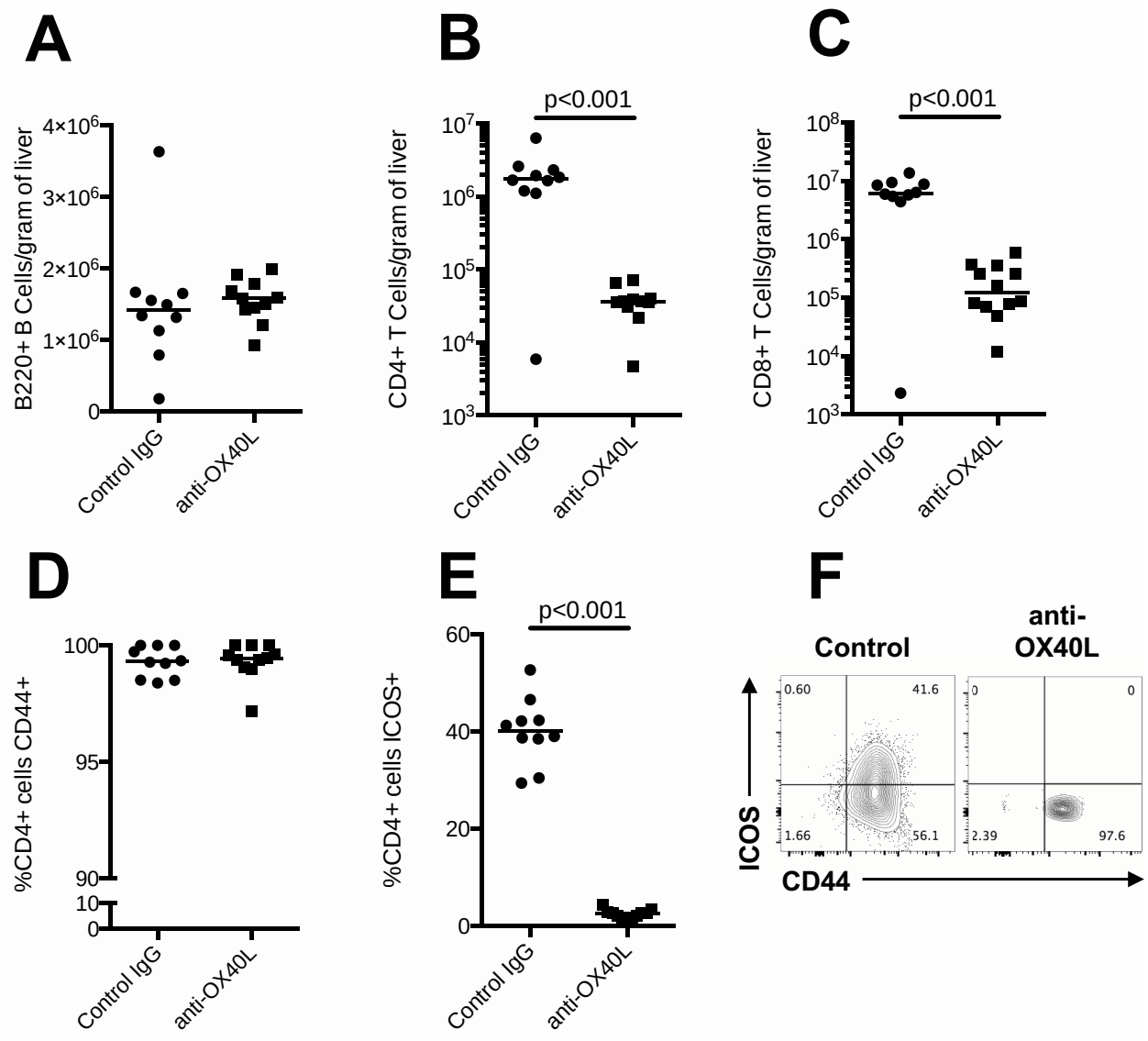


Figure 4.5: FOXP3^{KO} transfer hepatitis and anti-OX40L blocking antibody. ZAP70$^{-/-}$ mice received FOXP3^{KO} total thymocytes and were assessed for generalised autoimmunity and hepatitis. The administration of twice weekly blocking anti-OX40L antibody did not alter numbers of liver B220+ B cells (**A**), but caused significant reductions in numbers of CD4+ T cells (**B**), and CD8+ T cells (**C**). The proportion of CD4+ T cells expressing CD44 was not altered by anti-OX40L (**D**), but the expression of ICOS was markedly reduced (**E**). Representative plots gated on liver CD4+ T cells are shown (**F**). p values represent the Mann-Whitney U test; combination of two experiments with 21 mice in total.

whether blockade of OX40 would have similar results to blockade of OX40L and also to see whether a reagent unlikely to fix complement was effective, the experiment was repeated using an antagonistic anti-OX40 F_{ab} fragment.

Results of blockade of OX40-OX40L interactions with anti-OX40 F_{ab} were similar to those with anti-OX40L antibody. First, mice given anti-OX40 F_{ab} had markedly increased survival (Figure 4.6A), but also decreased markers of immune activation including reduced splenomegaly and levels of serum IgG, but in contrast to treatment with anti-OX40L, no reduction in serum IgE was observed. (Figure 4.6B, E & F). Similarly, reductions in serum ALT and in AMA titres were seen after blocking anti-OX40 (Figure 4.6C&D).

Histological examination of livers from mice that had received anti-OX40 F_{ab} demonstrated elimination of the lymphocytic peri-portal, peri-venular infiltrate seen in those treated with control antibody alone. (Figure 4.7A&B). Consistent with this observation was the significant decrease in the proportion of liver sections that stained positive for the pan-leucocyte marker CD45 (Figure 4.8C-E).

Flow cytometric analysis of $FOXP3^{KO}$ thymocyte recipients that had received anti-OX40 F_{ab} showed no significant differences in numbers of hepatic B cells but showed reductions in both CD4+ and CD8+ T cells (Figure 4.8A-C). Further examination of hepatic CD4+ T cells demonstrated that nearly all remained CD44-positive but that expression of the activation marker ICOS was markedly reduced (Figure 4.8D-E). Further, the proportion of liver CD4+ T cells that expressed IFNγ after stimulation was reduced after adminstration of anti-OX40 F_{ab}.

4.2.4 Concanavalin A induced hepatitis is not affected by OX40 deficency

To investigate whether deficiency in OX40 rendered T cells less able to cause liver damage, the Con A model of hepatitis was used. Mice were given a single dose of IV Con A before analysis 24 hours

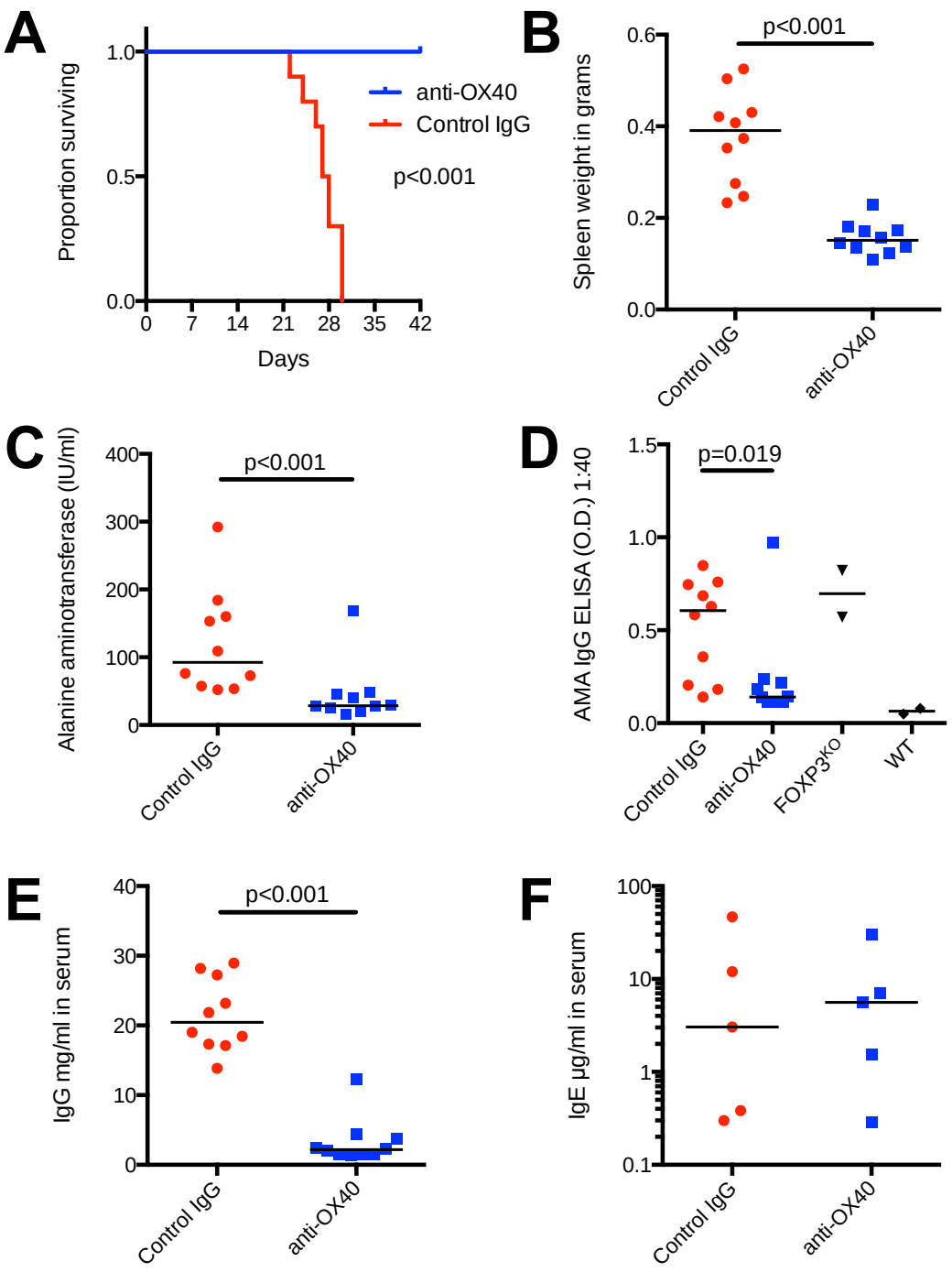


Figure 4.6: FOXP3KO transfer hepatitis and anti-OX40 F_{ab} fragment: phenotype. ZAP70$^{-/-}$ mice received FOXP3^{KO} total thymocytes and were assessed for the development of generalised autoimmunity and hepatitis. In comparison to administering control rat total IgG, administration of 250 μg of blocking anti-OX40 Fab twice weekly from day 5 after cell transfer, increased survival in the treated group (**A**), prevented the development of splenomegaly (**B**), reduced biochemical evidence of hepatitis in the form of serum ALT activity (**C**), reduced AMA titre (**D**), reduced serum IgG (**E**), and reduced serum IgE (**F**). Results represent the combination of two separate experiments. Comparisons with Log-Rank or Mann-Whitney U test; n=10v10.

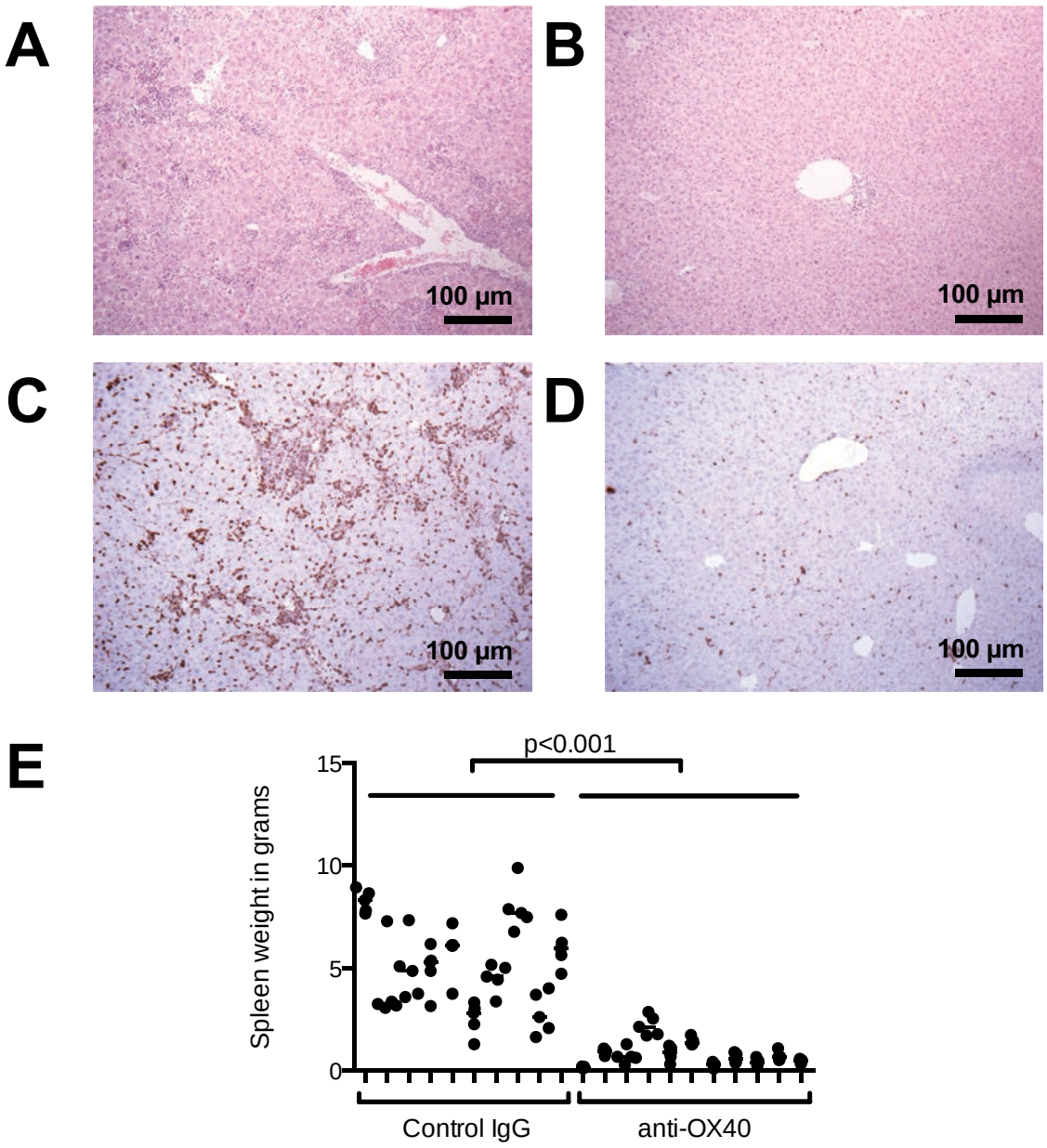


Figure 4.7: FOXP3^{KO} transfer hepatitis and anti-OX40 F_{ab} fragment: histology. ZAP70$^{-/-}$ mice received FOXP3^{KO} total thymocytes and were assessed for the development of generalised autoimmunity and hepatitis. Those mice that were given twice weekly control total rat IgG developed marked lymphocytic peri-venular hepatitis (**A**). However, those mice that were treated with twice weekly with anti-OX40 F_{ab} did not develop hepatitis (given from day 5 after cell transfer; (**B**). Liver sections were stained for the pan-leucocyte marker CD45 highlighting the lymphocytic infiltration in control animals (**C**) and its abolition in those treated (**D**). When CD45 infiltrates were digitally quantified, they were significantly reduced in those mice treated with anti-OX40 F_{ab} (**E**). Results represent the combination of two separate experiments. Comparisons with Mann-Whitney U test; n=10v10.

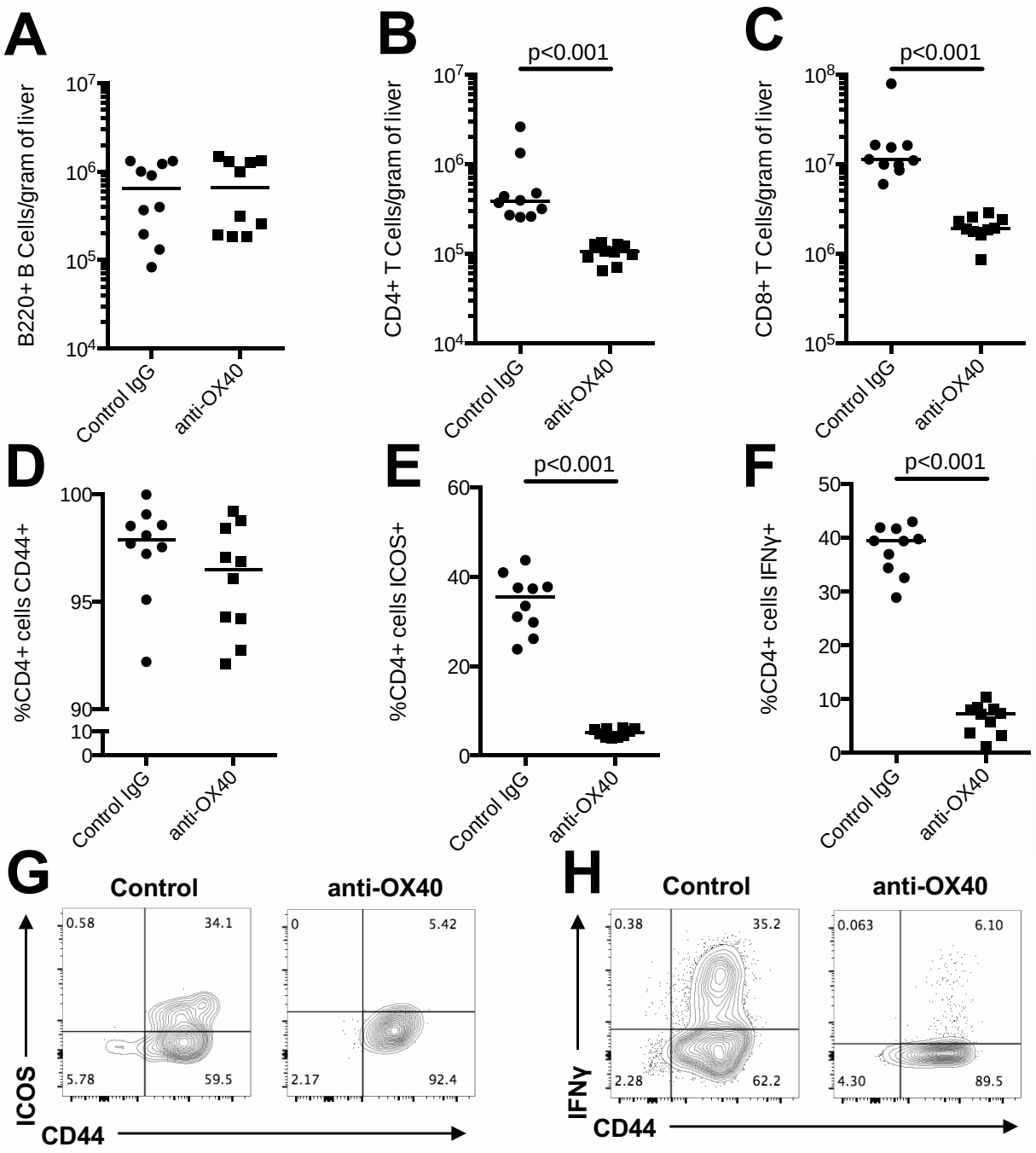


Figure 4.8: FOXP3^{KO} transfer hepatitis and anti-OX40 Fab fragment: flow cytometry. ZAP70$^{-/-}$ mice received FOXP3^{KO} total thymocytes and were assessed for generalised autoimmunity and hepatitis. The administration of twice weekly blocking anti-OX40 F_{ab} did not alter numbers of liver B220+ B cells (**A**), but caused significant reductions in numbers of CD4+ T cells (**B**), and CD8+ T cells (**C**). The proportion of CD4+ T cells expressing CD44 was not altered by anti-OX40 F_{ab} (**D**), but the expression of ICOS was markedly reduced (**E**) as were the proportion that produced IFNγ after stimulation (**F**). Representative plots gated on liver CD4+ T cells are shown (**G**) and (**H**). Comparisons with Mann-Whitney U test; n=10v10.

later.

Con A was given to wildtype, $OX40^{KO}$ and $ZAP70^{-/-}$ mice. When serum ALT was measured, there was no significant difference in serum ALT elevation between wildtype and $OX40^{KO}$ mice, although elevations were entirely absent in $ZAP70^{-/-}$ individuals (Figure 4.9A). A similar pattern was observed with serum glucose: there was no difference in the reductions of capillary glucose seen between wildtype and $OX40^{KO}$ animals (Figure 4.9B). Consistent with these observations, the degree of peri-portal hepatocyte necrosis was similar between wildtype and $OX40^{KO}$ animals but no necrosis was evident in $ZAP70^{-/-}$ animals (or wildtype animals treated with PBS (Figure 4.9C-F)).

Flow cytometric analysis of intrahepatic lymphocytes in animals treated with Con A revealed no significant changes in numbers of B- or CD4+ T cells (Figure 4.10A&B) but did demonstrate an increase in CD8+ T cells that was not altered by deficiency in OX40 (Figure 4.10C). The proportion of CD4+ T cells expressing CD44 and ICOS was increased by Con A administration but not altered by deficiency in OX40, whereas, although adminstration of Con A resulted in the expression of OX40 on CD4+ T cells, this was not seen on $OX40^{KO}$ cells as expected (Figure 4.10D-G)

4.2.5 Carbon tetrachloride induced hepatitis is not affected by anti-OX40L pre-treatment

To investigate whether blocking OX40-OX40L interactions alters the response to chemical liver injury, wildtype mice were pre-treated either with anti-OX40L antibody or control IgG before being injected with CCl_4 IV. The injection of CCl_4 caused the development of peri-portal necrosis as described elsewhere, but this was not altered significantly by pre-treatment with blocking anti-OX40L antibody (Figure 4.11).

Pre-treatment with anti-OX40L did not alter the rise in serum ALT seen with CCl_4 injection (Figure 4.12A). Similarly, flow cytometric analysis of intrahepatic lymphocytes after adminstration of CCl_4 with and without pre-treatment with anti-OX40L antibody revealed no significant differences

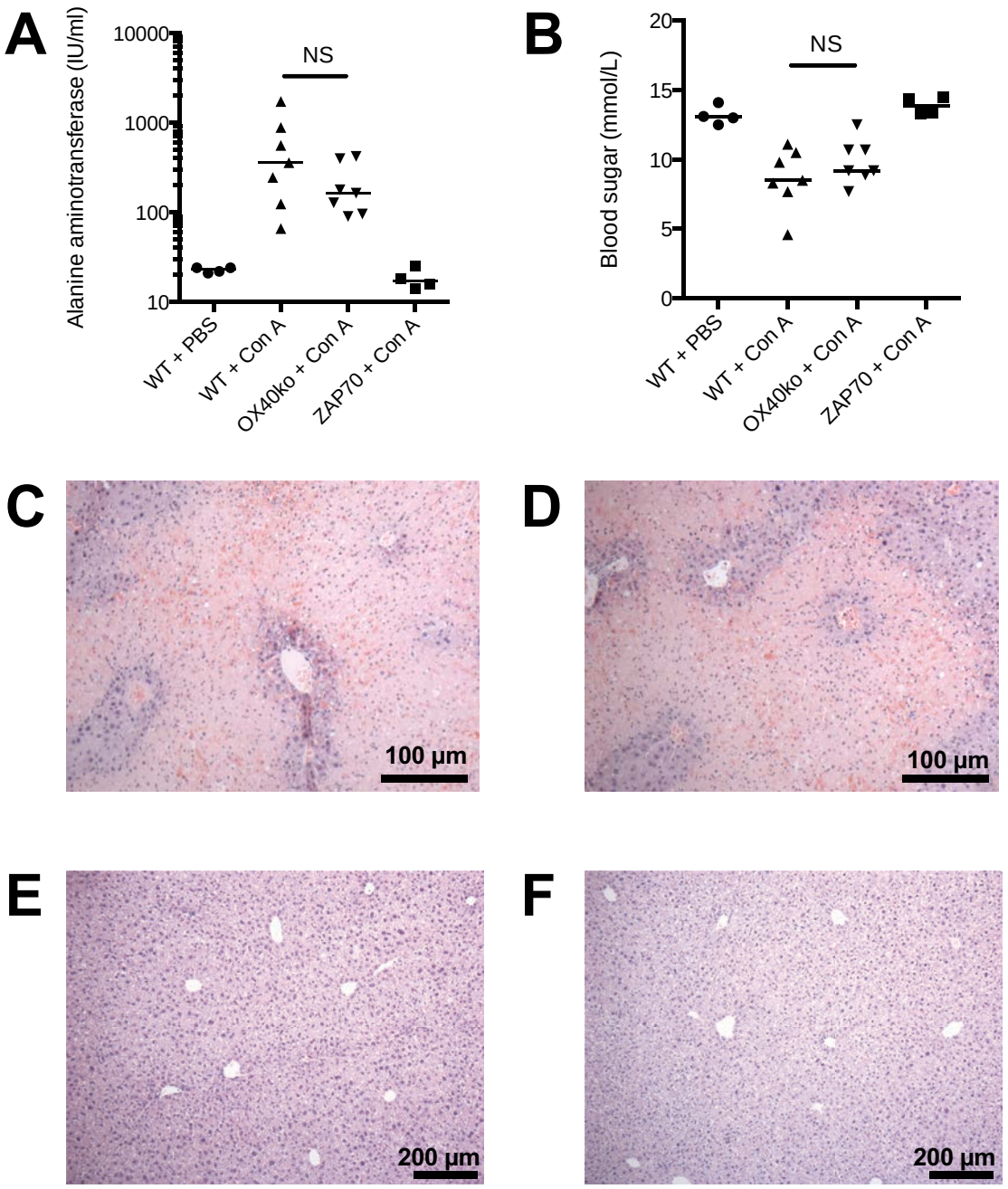


Figure 4.9: OX40 deficiency does not ameliorate concanavalin A hepatitis. Wildtype, OX40-deficient and T cell deficient ZAP70$^{-/-}$ mice were given Con A IV to induce hepatitis with some wildtype animals given PBS as a control. As previously reported, Con A caused an elevation in serum ALT from which T cell deficient animals were protected (**A**). Similarly, Con A caused a reduction in serum glucose from which T cell deficient animals were protected (**B**). No difference was seen between OX40^{KO} and wildtype animals (**A** and **B**). When livers were examined similar confluent peri-portal necrosis was apparent in both wildtype animals (**C**) and OX40^{KO} animals given Con A (**D**), but absent from wildtype animals treated with PBS (**E**) and from ZAP70$^{-/-}$ animals (**F**). Comparisons with Mann-Whitney U test; n=4v7v7v4.

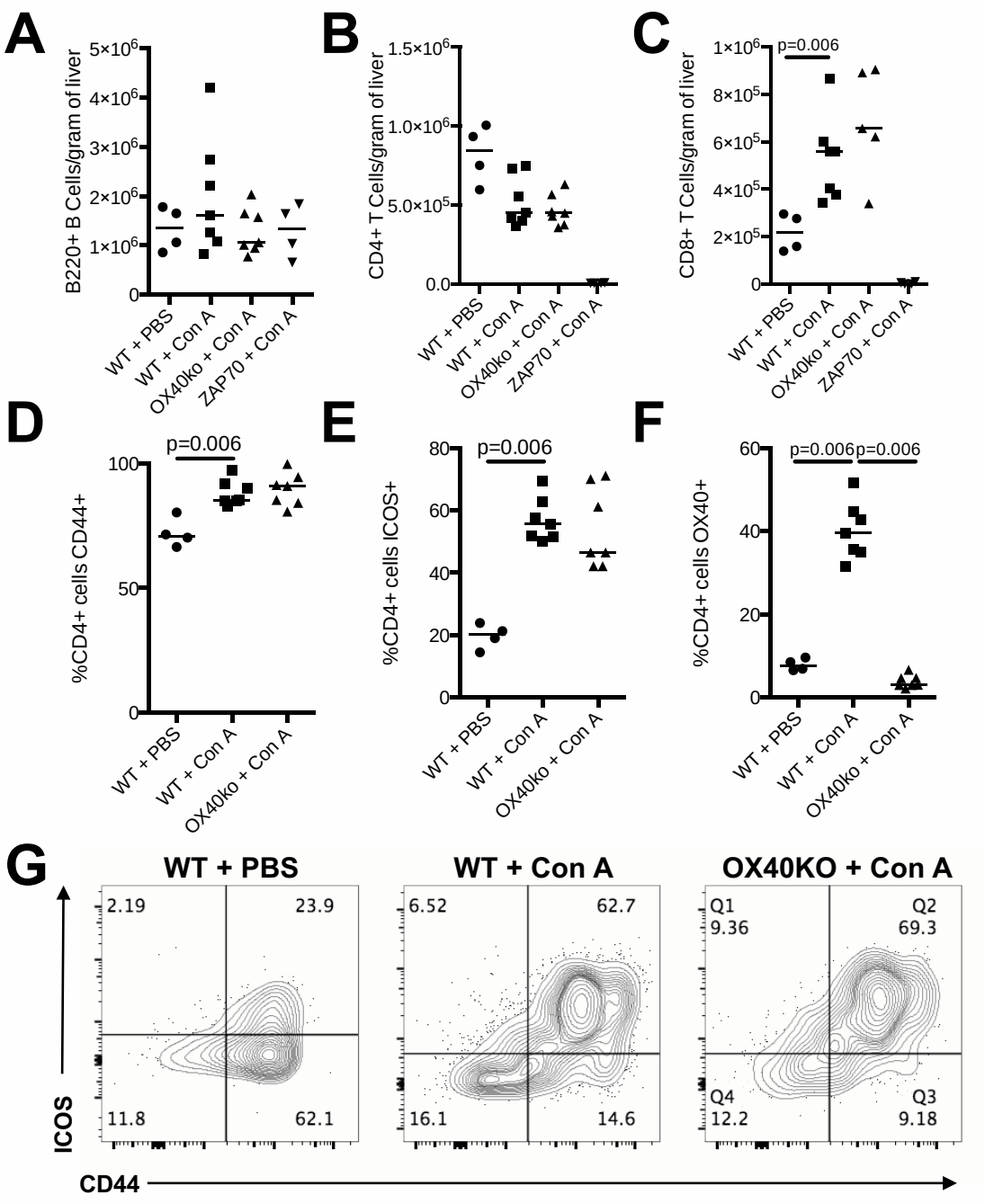


Figure 4.10: OX40 deficiency does not ameliorate concanavalin A hepatitis. Wildtype, OX40-deficient and T cell deficient ZAP70$^{-/-}$ mice were given Con A IV to induce hepatitis with some wildtype animals given PBS as a control. No differences were seen in numbers of B- or CD4+ T cells as a result of administration of (**A**) and (**B**), whilst numbers of CD8+ T cells were increased **C**. No differences in numbers of B cells, CD4+ T cells or CD8+ T cells were seen in OX40^{KO} deficient mice. Similarly, although Con A increased the proportion of CD4+ T cells expressing CD44 (**D**), ICOS (**E**), and OX40 (**F**), deficiency in OX40 did not alter activation as measured by CD44 or ICOS upregulation; (**G**) CD4+ T cells became activated incuded in OX40^{KO} animals as expected. p values denote Mann-Whitney U tests. Values presented represent a combination of three separate experiments; n=4v7v7v4.

in the increases in B and CD8+ T cells nor in the diminution of CD4+ seen with treatment (Figure 4.12B-D).

4.2.6 OX40 and OX40L in human liver disease

Having demonstrated OX40 expression in a mouse model of human autoimmune liver disease and that blocking interactions between OX40-OX40L ameliorated disease in that model, evidence of upregulation of OX40 in human autoimmune liver disease was sought.

OX40 expression in human explant liver

Samples of human liver taken from explants removed at the liver transplant program based at University Hospitals Birmingham were assessed by immunohistochemistry. First it was demonstrated that OX40 was expressed in a variety of liver pathologies including AIH and PBC, but was generally absent in samples from liver explants from patients without known liver disease (Figure 4.13).

Having demonstrated staining of OX40 in sections of liver tissue, dual staining was used to investigate whether OX40 was predominantly expressed on CD4 T cells as reported in other inflammatory conditions. This was indeed the case with dual-positive CD4+ OX40+ cells typically being evident in areas of dense CD4+ T cell accumulation (Figure 4.14).

Having demonstrated that OX40+ cells were present in several liver diseases, a semi-quantitative scale for their assessment was designed to permit basic comparison between diseases (Figure 4.15A). This was then applied to a total of 87 explant cases from livers from invididuals without a known liver diagnosis, those with a history of alcohol-induced liver disease, those with PBC, and those with AIH. Minimal OX40 expression was seen in both the group without known liver disease and those with alcohol-induced disease; in contrast, significantly greater amounts of OX40 staining was seen in the PBC and AIH groups (Figure 4.15B).

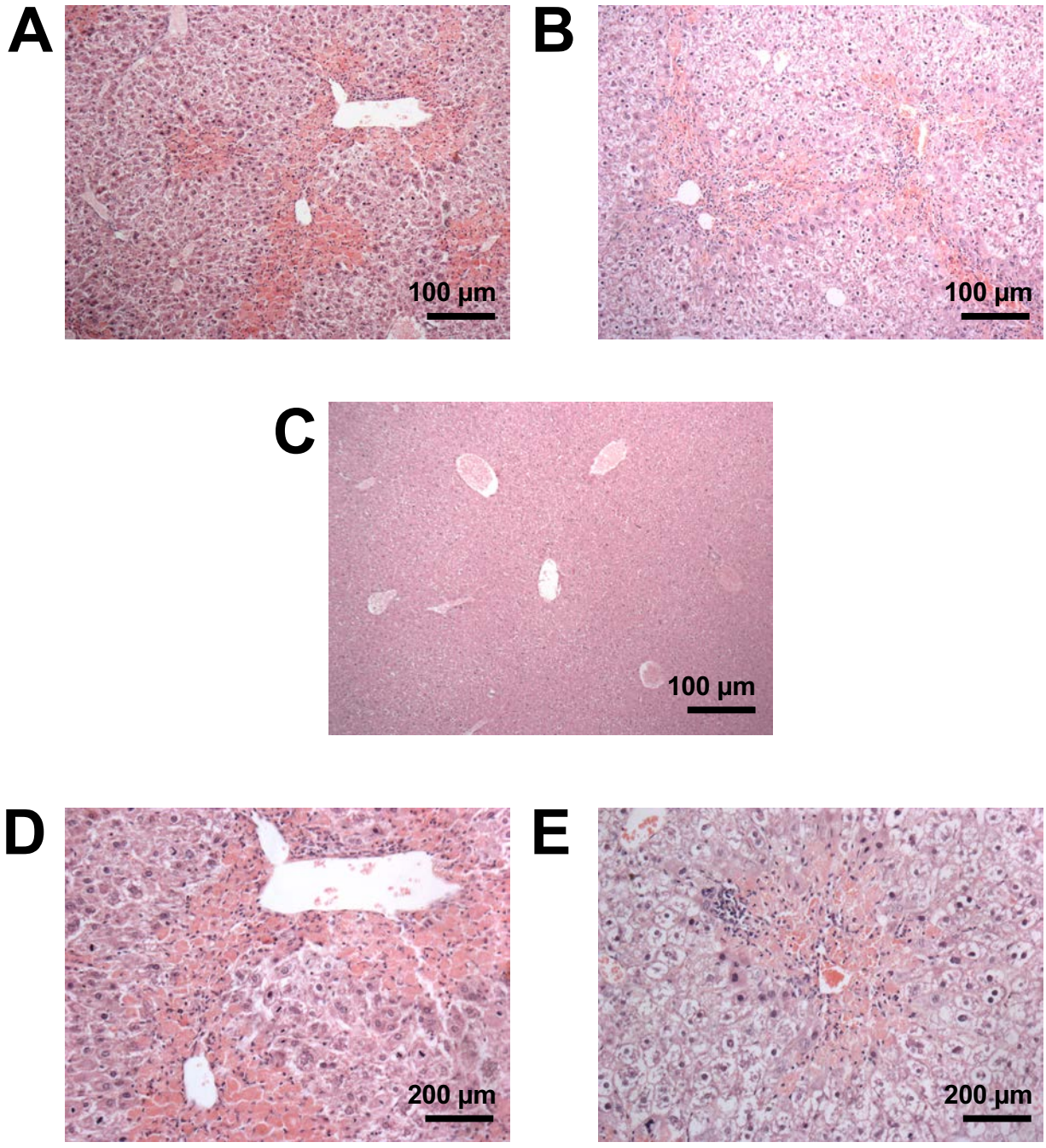


Figure 4.11: Carbon tetrachloride hepatitis in anti-OX40L treated mice: histology. Panels depict representative histology after H&E staining. After administration of CCl_4, confluent necrosis was visible around portal venules with sparing around hepatic venules in both mice administered control IgG (**A**) and in mice pre-treated with anti-OX40L blocking antibody (**B**). Mice treated with vehicle only (mineral oil without CCl_4) did not develop necrosis (**C**). Images labelled (**D**) and (**E**) represent higher magnification images of peri-venular necrosis from mice given control IgG and anti-OX40L antibody respectively. Representative images are shown.

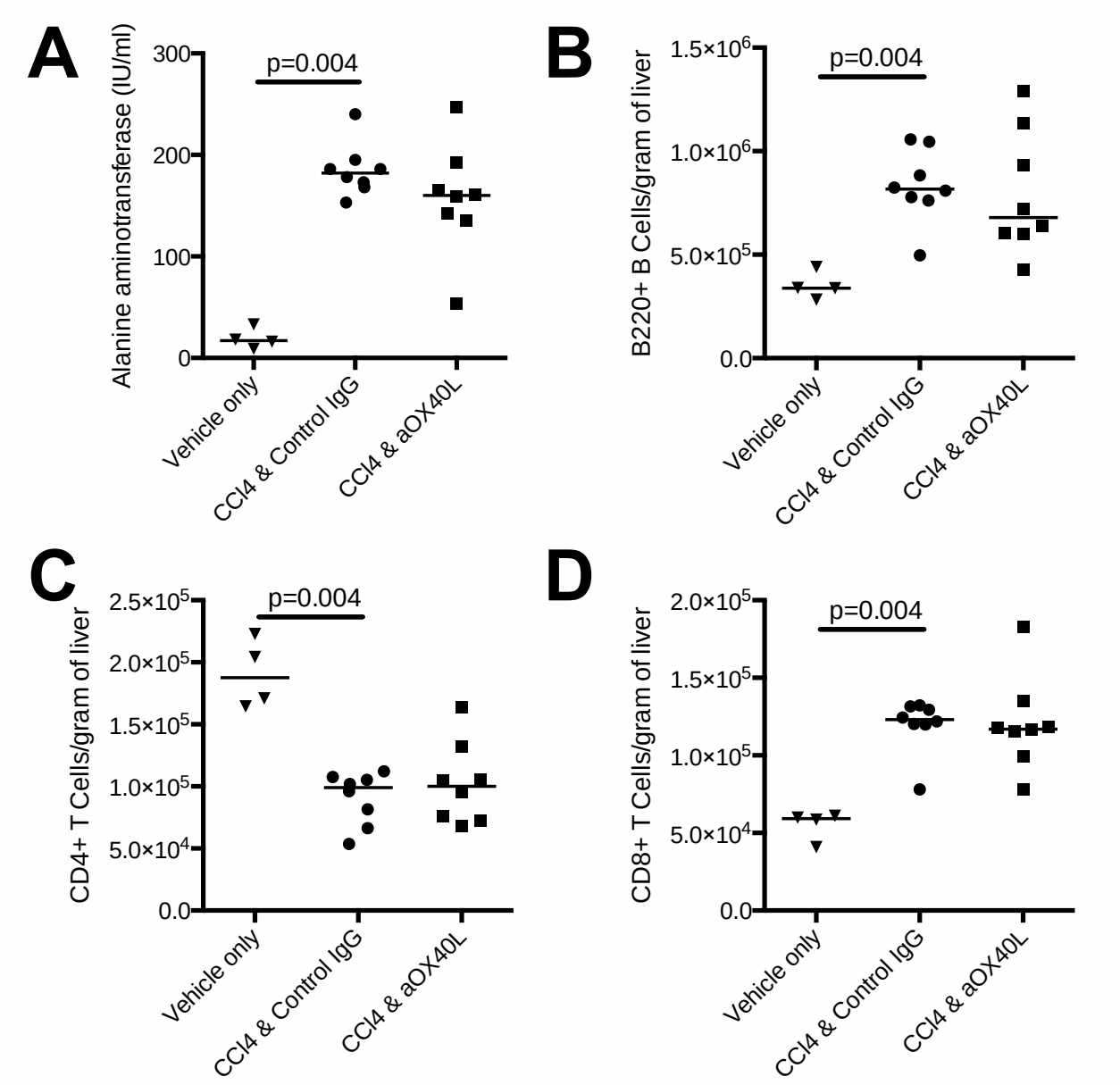


Figure 4.12: Carbon Tetrachloride hepatitis in anti-OX40L treated mice. Wildtype mice were given either mineral oil (vehicle) or carbon tetrachloride:mineral oil as a single IP injection. Some mice were pre-treated with blocking anti-OX40L antibody. 48 hours later mice were analysed for biochemical evidence of hepatitis and numbers of B220+ B cells, CD4+ T cells and CD8+ T cells in the liver by flow cytometry. (**A**) Serum ALT values were significantly increased in those mice that received CCl_4 but were not altered by the administration of anti-OX40L. (**B**) Numbers of liver B cells were increased by the administration of CCl_4 but not altered by pre-treatment with anti-OX40L. (**C**) Numbers of liver CD4+ T cells were reduced by the administration of CCl_4 but were not affected by pre-treatment with anti-OX40L antibody. (**D**) Numbers of CD8+ T cells were increased by CCl_4 but not altered by pre-treatment with anti-OX40L antibody. Plots are combinations of two experiments. Comparisons with Mann-Whitney U tests between 'vehicle only' and other conditions; n=4v8v8.

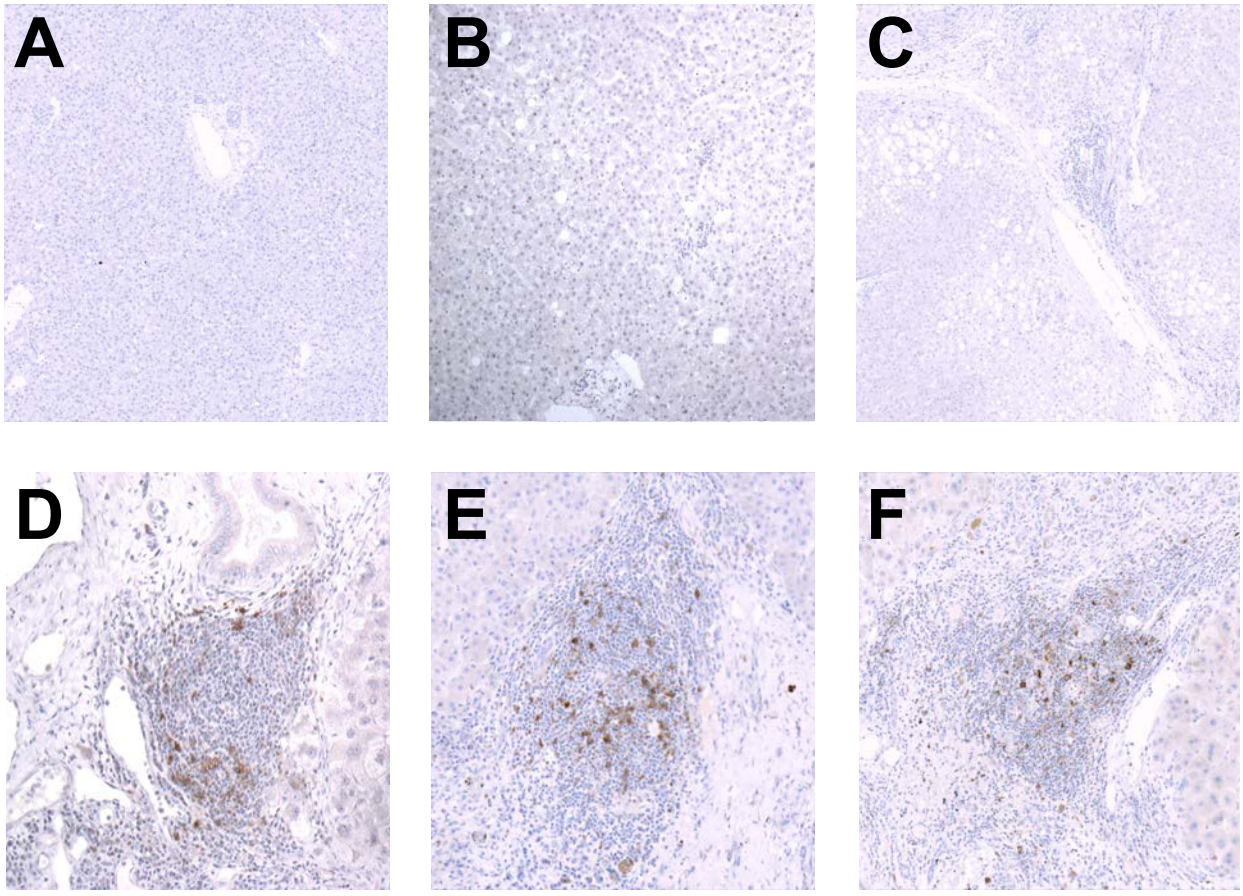


Figure 4.13: OX40 is expressed in diseased human liver tissue. Representative sections of human liver tissue stained for OX40 and counter-stained with haematoxylin. Panels (**A**) to (**C**) represent liver from patients without known liver disease but with varying degrees of steatosis, none of which showed significant positive staining for OX40. The following three panels represent positive staining in three inflammatory liver conditions: the autoimmune conditions PBC (**D**) and AIH (**E**), and the non-autoimmune condition non-alcoholic steatohepatitis (**F**).

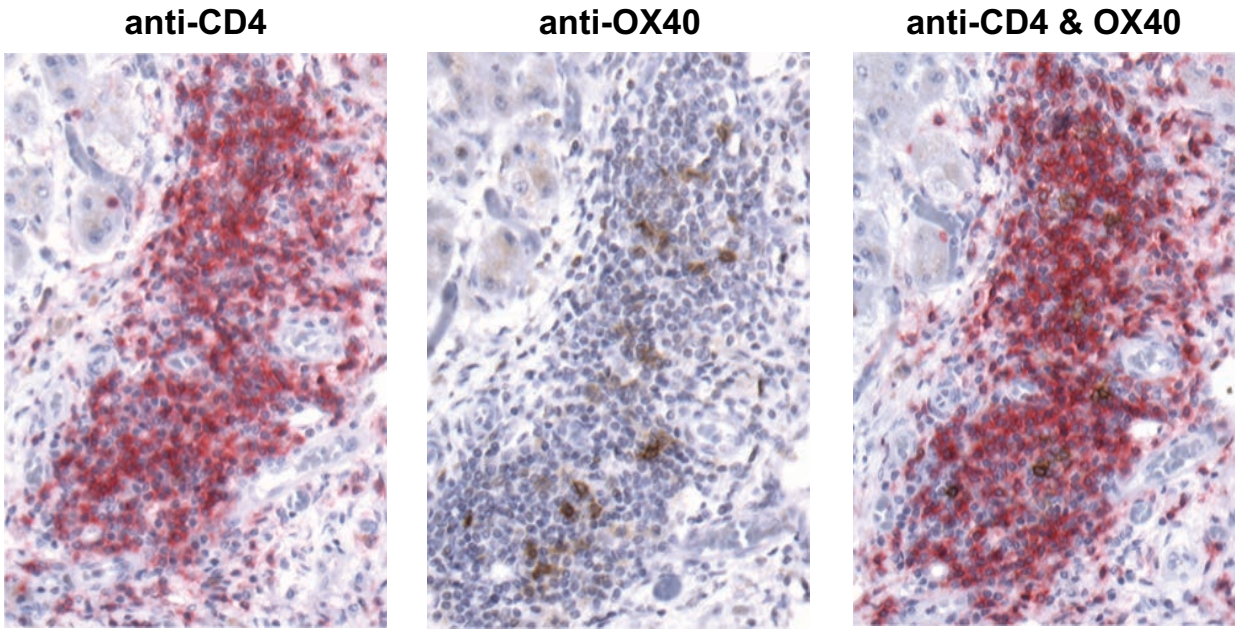


Figure 4.14: Dual staining of OX40 and CD4 in human autoimmune liver disease. Selected liver explant sections were stained for OX40 and CD4, both separately and simultaneously. Representative section of explant liver from a patient with PBC presented. The pattern of OX40 expression being within areas rich in CD4+ T cells was consistent through multiple specimens.

OX40 expression on liver biopsies

In an attempt to assess whether OX40 expression in human liver disease was limited to end-stage liver disease or present at the point of diagnosis, a number of liver biopsy specimens were obtained and stained for OX40. These were then scored according the maximum number of OX40 positive cells in a given focus. Staining was variable between sections and tended to centre on portal areas with OX40+ cells located within foci of lymphocytic infiltration in a similar manner to the pattern seen in explant samples (Figure 4.16A-C). When the number of OX40 positive cells was correlated with the an independent pathologist's assessment of inflammatory activity, there was a significant but weak correlation between increased OX40 expression and increased inflammation although many or the most inflamed samples did not show OX40 staining (Figure 4.16D). When specimens were divided according to the patient's final diagnosis of either normal, non-alcoholic fatty liver disease (NAFLD), AIH or hepatitis C virus (HCV), there was significantly greater OX40 staining amongst

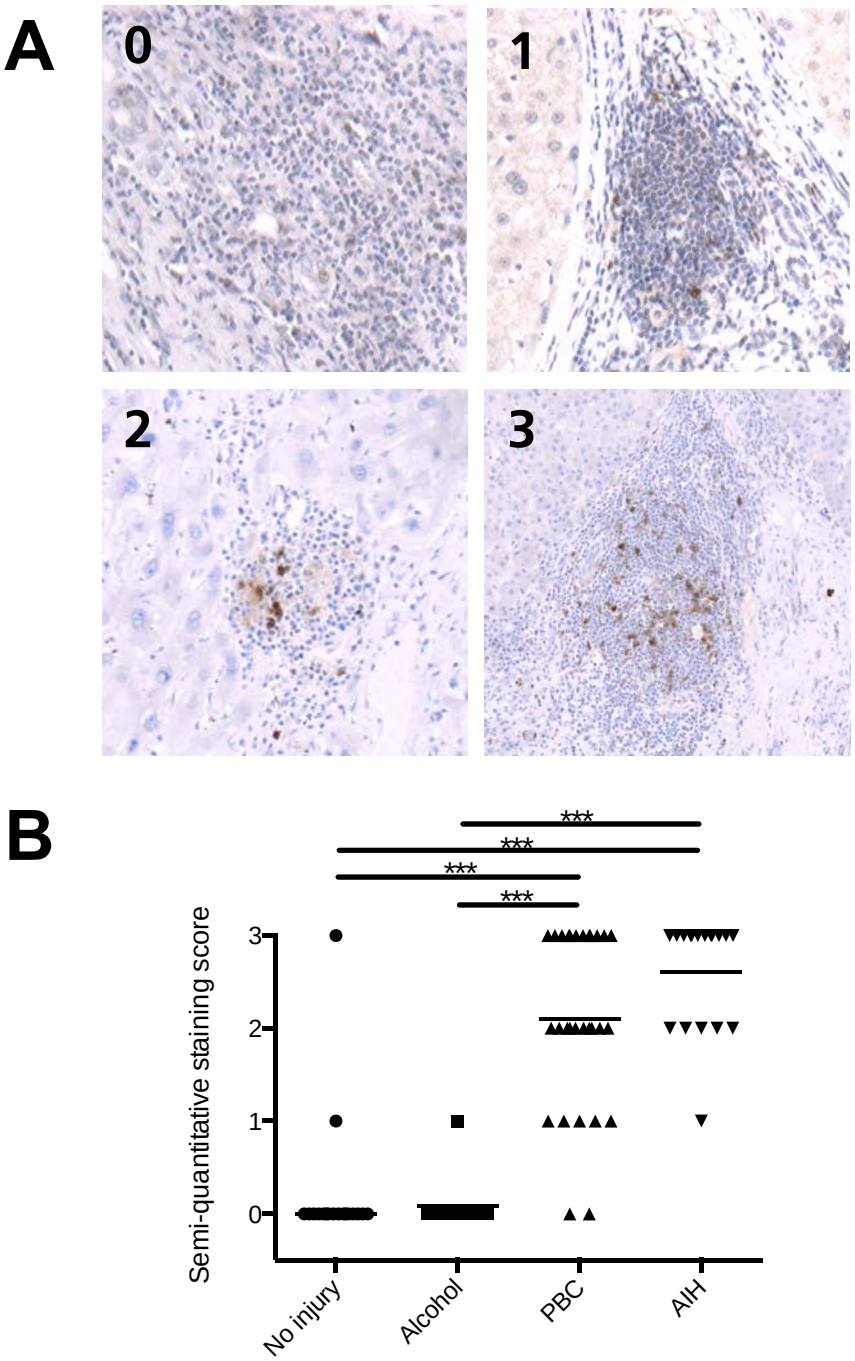


Figure 4.15: Semi-quantitative staining of OX40 expression in human liver disease. To allow assessment of the extent of positive OX40 staining in human liver tissue, a semi-quantitative staining system was devised. This was performed according the greatest number of OX40 positive cells in any one inflammatory aggregate. For explant samples these were categorised as a score of 0 for no positive cells, 1 for 1-5 as a single focus, 2 for 5-10 and 3 for >10 by the author whilst unaware of the pathologist-issued diagnosis (**A**). A total of 77 cases were examined and graded and demonstrated that explant livers from patients affected by either AIH or PBC contained significantly more OX40-positive cells than those from patients with no known injury or injury from alcohol (**B**). Comparisons with Kruskal-Wallis test with Dunn's post-hoc test.

both AIH and HCV cases when compared to normal. (Figure 4.16E).

Given the observation that OX40 positive staining appeared to correlate with inflammation, attempts were made to correlate the extent of OX40 positive staining in biopsy samples with a variety of clinical variables including those associated with hepatic inflammation. 88 samples were analysed. There was a significant but weak positive correlation between both the transaminases ALT and aspartate aminotransferase (AST) and OX40 positivity and a near-significant correlation with serum IgG concentrations (Figure 4.17A-C). In contrast, no significant correlation was seen with hæmoglobin concentrations, serum bilirubin, serum creatinine, age or Fibroscan resistivity – a measure of the severity of liver fibrosis (Figure 4.17D-H).

Serum OX40L in primary biliary cholangitis

Having demonstrated expression of OX40 in human liver tissue both at explant to permit orthotopic liver transplantation and on percutaneous biopsy specimens, evidence of systemic upregulation of the OX40-OX40L axis was sought. To permit this an ELISA was used to measure serum levels of soluble OX40L. However no difference was apparent between patients with PBC and healthy control patients (Figure~4.18).

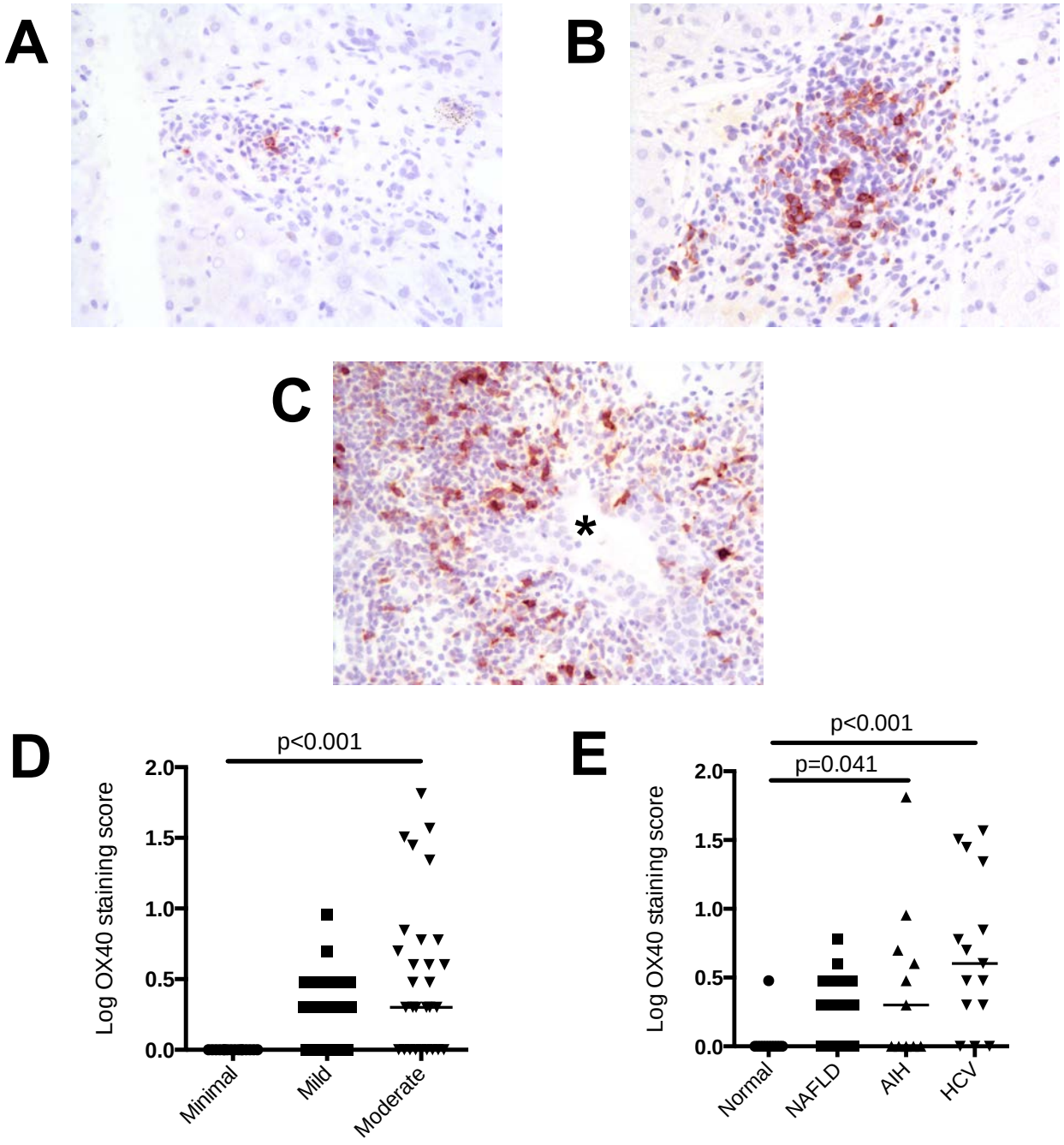


Figure 4.16: Liver biopsies of inflammed livers demonstrate cells positive for OX40. Multiple human liver biopsies were obtained from patients being investigated for liver disease and stained for OX40. Not all biopsies showed positive staining and where there was positive staining this was seen within foci of inflammation on lymphocytes (**A** and **B**). The most dense staining was seen surrounding bile ducts (**C**; bile duct starred). There was a significant but weak correlation between pathologist-assessed extent of inflammation and OX40 staining (**D**). Significantly more OX40 staining was seen in those ultimately diagnosed with AIH or HCV than those who were not diagnosed with any liver disease (**E**). Assessment for trend between inflammation grades was performed with the nptrend test; comparisons between diseases were made with the Kruskal-Wallis test with Dunn's post-hoc comparisons. 88 samples were analysed in total.

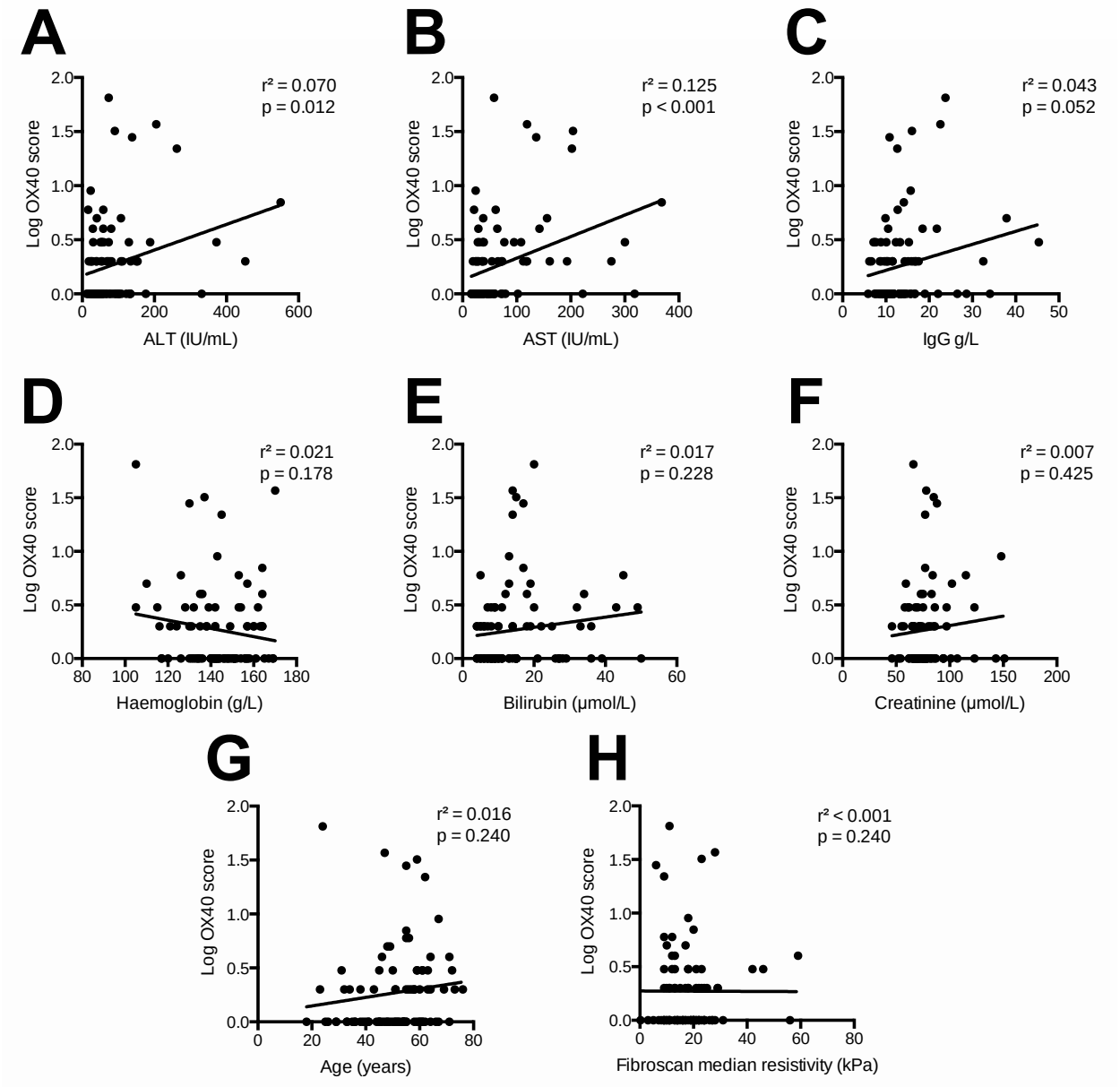


Figure 4.17: OX40 positive staining on liver biopsies correlates with markers of inflammation. Multiple human liver biopsies were obtained from patients being investigated for liver disease, stained for OX40 and the extent of OX40 positive staining correlated with various clinical variables. There was a significant positive correlation as assessed by least squares linear regression with two biochemical indices of liver inflammation: serum ALT and AST activity (**A**&**B**) and a near-significant positive correlation with serum IgG concentration, an indirect marker of inflammation (**C**). There was however no significant correlation with hæmoglobin concentration, serum bilirubin, serum creatinine, age or fibrosis as measured by Fibroscan resistivity (**D-H**).

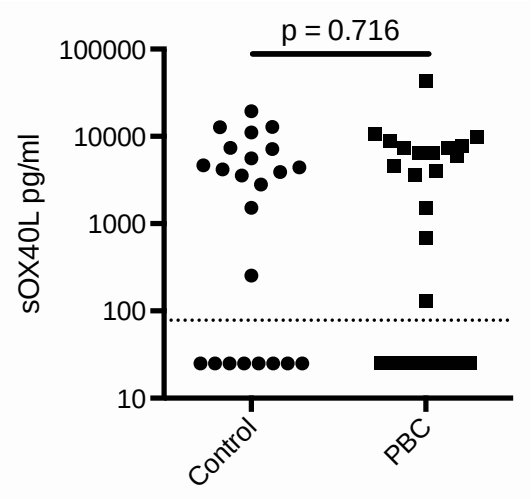


Figure 4.18: Serum soluble OX40L in PBC and in health. Serum from 28 PBC patients and 23 healthy controls was analysed for its soluble OX40L content. 12 PBC samples and 8 control samples were below the assay sensitivity of 28pg/ml. Comparison with Mann-Whitney U-test.

4.3 Discussion

This chapter has presented work suggesting that overall numbers of lymphocytes in OX40-deficient mouse liver are not different from matched wildtypes although there are fewer Tregs and cells tended towards a more activated phenotype. It was then demonstrated that a both a blocking anti-OX40L antibody and a blocking anti-OX40 antibody were sufficient to prevent the development of FOXP3^{KO} transfer hepatitis and also to reduce markers of generalised autoimmunity. Interfering with OX40-OX40L signalling however had no effect on either the T cell dependent hepatitis induced by Con A injection or the chemical hepatitis induced by CCl_4 injection. Finally, evidence of OX40 expression in human liver disease was sought. OX40 was found to be upregulated in both AIH and PBC explanted liver in comparison to undiseased liver and that affected by alcohol; biopsy studies also demonstrated significantly greater OX40 expression in AIH liver than normal liver but also demonstrated postivitiy in HCV, and to a lesser extent, NAFLD affected liver. OX40 positive staining correlated with clinical markers of liver inflammation although systemic serum soluble OX40L was not elevated in PBC.

The observation that OX40^{KO} mice have similar numbers of intrahepatic lymphocytes to wildtype animals represents a baseline with regards to other experiments presented in this thesis and makes it unlikely that the blocking antibodies used will have major effects on intrahepatic T cell numbers. The apparent reductions in intrahepatic Treg numbers in OX40^{KO} animals when compared to wildtype animals differ from a previous report that numbers were similar between wildtype animals and OX40^{KO} animals.[487] However, Xiao et al. only reported that around 1% of total hepatic CD4+ T cells were FOXP3 positive in both wildtype and OX40 deficient animal livers compared with wildtype readings of around 3% [397], around 8% [38] and around 10% [477] in other studies, and around 10% in wildtype mouse liver in this work. This may have masked changes.

In addition, it is reported that stimulation through OX40 by the agonistic OX86 antibody or by the creation of mice transgenic so that they over-express OX40L causes increases in peripheral and hepatic numbers of Treg, and that OX40 is important for Treg survival.[217, 487] These observations support the notion that interrupting OX40 signalling may alter Treg numbers. More experiments, ideally in mice of different backgrounds and in combination with long-term administration of agonistic and blocking antibodies are needed to further investigate this. As well as altering survival or genesis of Tregs, alterations in their migration induced by OX40 blockade may also be important in regulatory homeostasis.

The finding that blocking anti-OX40L prevented the development of hepatitis in T cell deficient animals repleted with FOXP3^{KO} deficient cells is consistent with previous work by the Lane group but extends the observation to specific assessment of the liver.[105] This work also extends observations to specific measurement of autoantibodies. As noted above, this was important because therapeutic manipulations in other mice with analogous deficiencies in T cell regulation have shown divergent effects on inflammation in different organ systems.[492, 505, 291] Importantly when considering administration of OX40-OX40L for potential human therapeutic use, blocking antibody injections were started well before the onset of evidence of FOXP3^{KO} transfer disease at day 5 following cell

transfer. Our animal licence prevented permitting the development of overt autoimmunity before commencing therapy but this would be a logical extension of the study. In addition, divergent effects of OX40-OX40L blockade on autoimmunity have been reported in relation to the initiating insult.[322] Given that OX40 and OX40L are understood to be a cognate receptor-ligand pairing with no significant interactions with other ligands, it could be argued that blocking OX40 with an anti-OX40 F_{ab} fragment preparation was functionally equivalent to using a complete antagonistic anti-OX40L antibody. However, the result that blocking OX40 F_{ab} prevented autoimmunity is important for two reasons: first, because by lacking the ability to fix complement the reagent is unlikely to cause depletion of cells expressing its target antigen and second, because the manufacturer (UCB Pharma) has a directly analogous anti-human compound in production. The effect of blocking anti-OX40 F_{ab} was generally similar to that of blocking OX40L except for the lack of a reduction in serum IgE. This may reflect the fact that total IgE in control animals in this experiment was not as elevated as otherwise so presenting less opportunity for a significant reduction after therapy to be observed. In addition, because IgE measurements could only be completed on a subset of animals, statistical power is reduced.

Administration of Con A causes a massive release of cytokines through the non-specific cross-linkage of TCRs and the subsequent release of cytokines – including IFNγ and TNFα which then induce hepatocyte necrosis and apoptosis.[415, 129] The fact that OX40 deficient mice still suffer this cytokine-mediated hepatitis is consistent with observations that OX40-deficient T cells still retain the ability to secrete effector cytokines.[471] However, it is possible that in less acute insults that were more dependent on co-stimulatory signals differences may have been more apparent.[157] The unchanged ability of CCl_4 to cause hepatitis in the absence of OX40 is consistent with the direct chemical injury caused by that compound , which has otherwise been noted to persist in entirely T cell deficient mice.[309]

In an attempt to demonstrate parallels between the upregulation of OX40 in mouse $FOXP3^{KO}$ trans-

fer hepatitis shown in the previous chapter, the efficacy of blocking OX40-OX40L signals in this model of autoimmunity in this chapter, and human liver disease, evidence of expression of OX40 in human disease was sought. Staining was uncommon in normal liver but was present in a variety of liver diseases including both AIH and PBC. This is consistent with a number of studies of human autoimmunity affecting other organs. However, because differences in the extent of OX40 were not observed between two non-autoimmune diseases, NAFLD and HCV infection, that are associated with lymphocytic infiltrate and because OX40 staining was positively correlated with clinical indices of liver inflammation when multiple diagnoses were assessed together, it may be that the presence of OX40 in human liver disease simply represents T cell activation rather than a process specific to autoimmunity per se. In further investigating OX40 expression in human liver disease, other methods of OX40 quantification including qPCR, flow cytometric analysis and a wider variety of liver diagnoses would be valuable. In addition, when considering analogies between the work presented in this chapter and human disease, samples from patients with IPEX would be valuable because of their pathogenic equivalence.[482]

Weaknesses in this approach

One criticism of this work is that it was not possible to allow overt disease to develop in the mice that received FOXP3^{KO} deficient cells before commencing treatment with either blocking anti-OX40L or anti-OX40. This related to licensing requirements within the animal facility where these experiments took place. A second potential criticism is the subjective methodology with regards to survival analyses: animals were not allowed to die, only to deteriorate to the point where one of the animal technicians became concerned. Although the technicians were blinded as to the treatment groups, this represents an area of potential subjectivity.

Whilst the experiments presented here using cells from mice deficient in AIRE derived from animals that had been genotyped as such and were from colonies known to develop lymphocytic hepatitis,

one criticism of this work is that autoimmune liver disease was not confirmed in these animals prior to their use as donors. Ideally these experiments would be repeated with animals with manifest autoimmune liver disease.

The human work presented here does not account for the other multiple parameters that may affect including gender, age, concurrent pharmacological therapies. Further, the immunohistochemistry based methodology does not subphenotype the cells assessed nor quantify them absolutely.

Future experiments

This work has confirmed that either OX40 or OX40L blockade is effective in reducing autoimmunity in the absence of Tregs. A logical extension of this work is to extend assessment of the efficacy of blockade of OX40 and OX40L interactions in other forms of autoimmunity. For example, AIRE deficient mice where autoimmunity appears to relate to deficiencies in certain subpopulations of antigen-specific Tregs or equally to other similar models such as the recently reported mice with dysfunction of Fzef2 or TRAF6 in which there are failures of thymic selection.[406, 29]

Further, one of the weaknesses of the current assessment is that antibody blockade was administered prior to the onset of clinically apparent disease in contrast to the likely use of these agents in any therapy for human disease. A useful further experiment would be delaying treatment until the onset of observable features of disease and then measuring for efficacy.

An exciting potential application of these findings would be to apply these insights to the human disease IPEX. There is potentially very great utility in limiting autoimmunity in the lead up to stem cell allografting in an attempt to limit use of non-specific immunosuppressive agents. Again, those with AIRE dysfunction and resultant type 2 AIH would be intriguing cases to assess.

To extend the work in human phenotyping presented here, assessment of cell numbers per unit weight of explant liver could be assessed by flow cytometry. This would also allow concurrent assess-

ment of other indicators of cell activation, determination of the proportion that expressed markers of a regulatory phenotype, and comparison with numbers of other cell types present within the liver. It would be particularly interesting to assess whether OX40 expression delineates those cells specific for an antigen of interest as has been suggested in some animal models of autoimmune disease and in human type 1 diabetes mellitus.[478, 264, 92]

5 OX40 blockade in CTLA4-deficient mice

5.1 Introduction

Having demonstrated that blockade of OX40 is effective in preventing the autoimmunity of transfer disease, a related question was whether other models of T cell regulatory failure associated with liver disease may also be ameliorated by blocking OX40-OX40L interactions. Mice deficient in CTLA4, a key molecule involved in negatively regulating co-stimulation through the CD28 pathway, develop multisystem autoimmunity with associated lymphocytic infiltrates.[462, 416] Similar to FOXP3 deficiency[194], there is an ongoing requirement for CTLA4 throughout life with inducible knockouts developing multisystem autoimmunity including hepatitis.[205]

Liver disease in CTLA4 deficient mice as assessed by simple histological examination is described but has not been further characterised.[462, 205] By analogy, both mice and humans treated with blocking antibodies to CTLA4, typically as part of cancer immunotherapy, may develop hepatic autoimmunity.[443, 195, 119, 177] and case series of multi-system autoimmunity in individuals heterozygous for dysfunctional CTLA4 exist, some of whom developed hepatic autoimmunity.[366, 222]

In this chapter, evidence of the production of AMA was sought in a variety of mice that develop liver injury. CTLA4-deficient mice were shown to spontaneously develop lymphocytic hepatitis alongside AMAs, and this was explored further by assessing the efficacy of blocking OX40-OX40L interactions in ameliorating this hepatic and generalised autoimmunity.

5.2 Results

5.2.1 Antimitochondrial antibodies are present in CTLA4-deficient mice

In order to examine whether AMA developed in CTLA4-deficient as well as a selection of other available mice with either induced or spontaneous liver damage, or with multi-system autoimmunity fol-

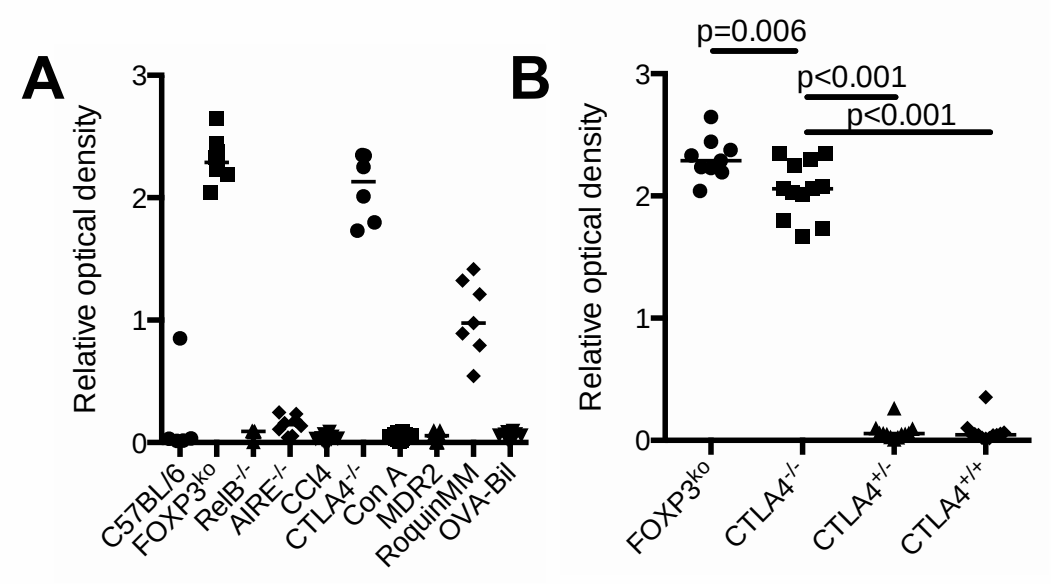


Figure 5.1: AMAs in various mice. (**A**) Sera from a number of mice with immune-dysregulation or liver injury were screened for the presence of AMA using the ELISA described previously. Having identified the presence of AMAs in (**B**) CTLA4$^{-/-}$ mice, these mice were compared against FOXP3^{KO} mice as positive control and against littermates lacking the genotype of interest. C57Bl/6 = wildtype mice; CCl_4 = 8-week carbon tetrachloride treated; Con A = recovered Con A treated mice; MDR2 = multidrug resistance protein-2 deficient (*Abcb4-/-*) mice; OVA-Bil = mice expressing ovalbumin on biliary epithelium injected with OT1 ovalbumin-specific T cells. Comparisons with Kruskal-Wallis test with Dunn's post-hoc test.

lowing aberrations in CD4+ T cell regulation, sera were collected and assessed according to the same AMA ELISA described previously (Figure 5.1A). Here, although AMA were consistently present in CTLA4-deficient mice (Figure 5.1B), they did not develop in other models of generalised autoimmunity associated with disturbances in Treg function (AIRE deficiency and RelB deficiency), in exogenous chemical injury (CCl_4), in T cell cytokine mediated injury (Con A), direct ovalbumin-targeted T cell mediated biliary injury (OVA-Bil), or in endogenous chemical injury following disruption of a key biliary transporter (MDR2$^{-/-}$).

5.2.2 Characterisation of CTLA4 deficiency

Having identified AMA in CTLA4 deficient mice, further assessments of the general and hepatic phenotype of these mice were made. CTLA4-deficient mice demonstrated runting with splenomegaly and generalised lymphadenopathy (Figure 5.2A-C). In addition, and consistent with some analogy to human AILD, CTLA4-deficient mice demonstrated elevations in serum transaminase activity (Figure 5.2D), and elevations in serum IgG (Figure 5.2E).

Flow cytometric analysis confirmed elevations in hepatic lymphocyte numbers in CTLA4-deficient animals with these lymphocytes displaying a more activated phenotype including greater expression of OX40 (Figure 5.3). In addition, proportions of cells expressing FOXP3 consistent with Tregs were identified and a greater proportion of CD4+ T cells showed evidence of recent proliferation through Ki67 staining and produced IFNγ after stimulation (Figure 5.4).

Histological examination of liver from CTLA4-deficient animals confirmed dense lymphocytic infiltrates with numerous cells in these infiltrates staining positive for CD4, OX40, and Ki67 (Figure 5.5).

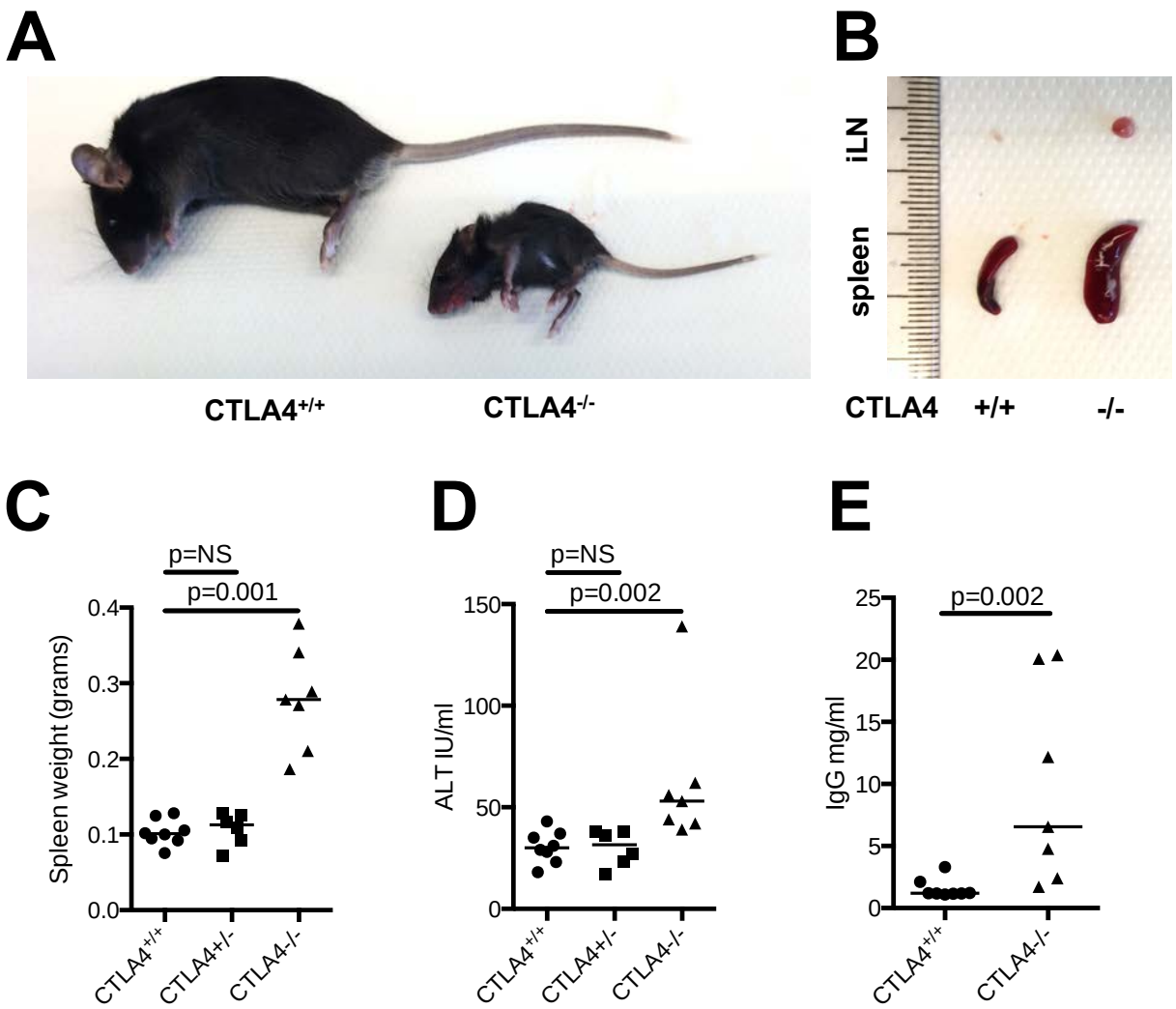


Figure 5.2: Phenotype of CTLA4 deficient mice. Mice either homozygous for dysfunctional CTLA4, heterozygous for functional CTLA4 or with two wildtype CTLA4 genes were analysed at 3-4 weeks of age. (**A**) Mice lacking CTLA4 were runted and (**B**) demonstrated splenomegaly and lymphadenopathy (inguinal lymph node shown; centimetre scale). (**C**) The finding of splenomegaly was consistent between groups. (**D**) CTLA4 deficient mice also demonstrated mild elevations in serum transaminase activities suggestive of liver injury and (**E**) demonstrated elevations in IgG. Comparisons with Mann-Whitney U test; n=8v8v7. Centimetre scale shown in photograph.

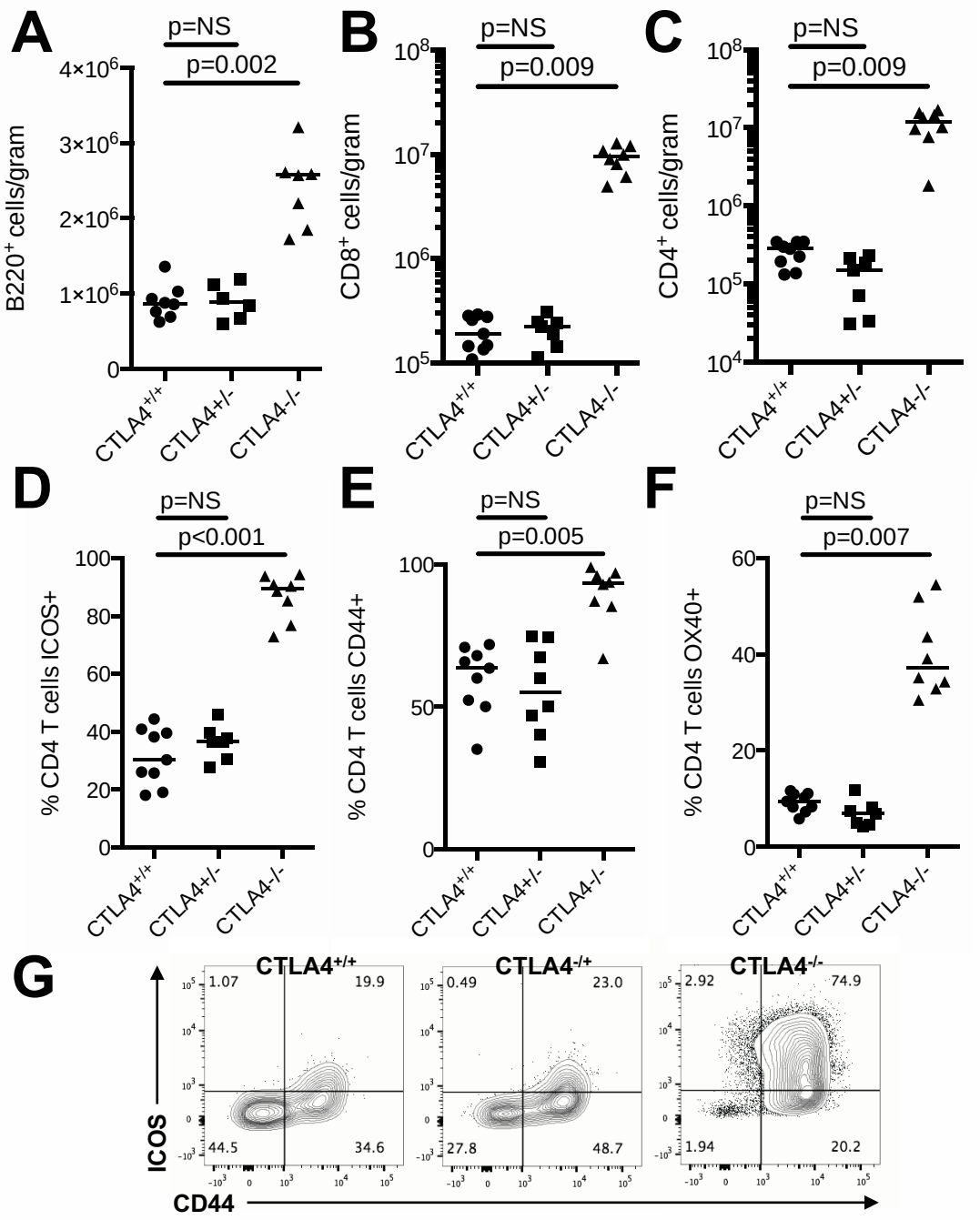


Figure 5.3: Flow cytometric analysis of hepatic lymphocytes in CTLA4 deficiency. Livers from 3-4 week old CTLA4$^{+/+}$, CTLA4$^{+/-}$ or CTLA4$^{-/-}$ pups from several litters were assessed for lymphocyte content. (**A**) B cells (**B**) CD8+ T cells and **C.** CD4+ cells were increased and the proportion of CD4+ T cells positive for (**D**) ICOS (**E**) CD44, and (**F**) OX40 were increased in CTLA4$^{-/-}$ mice as compared with CTLA4$^{+/+}$ controls; heterozygous CTLA4$^{+/-}$ did not differ from CTLA4$^{+/+}$. (**G**) representative plots gated on CD4+ T cells. Comparisons with Kruskal-Wallis test with Dunn's post-hoc test; n=8v8v7.

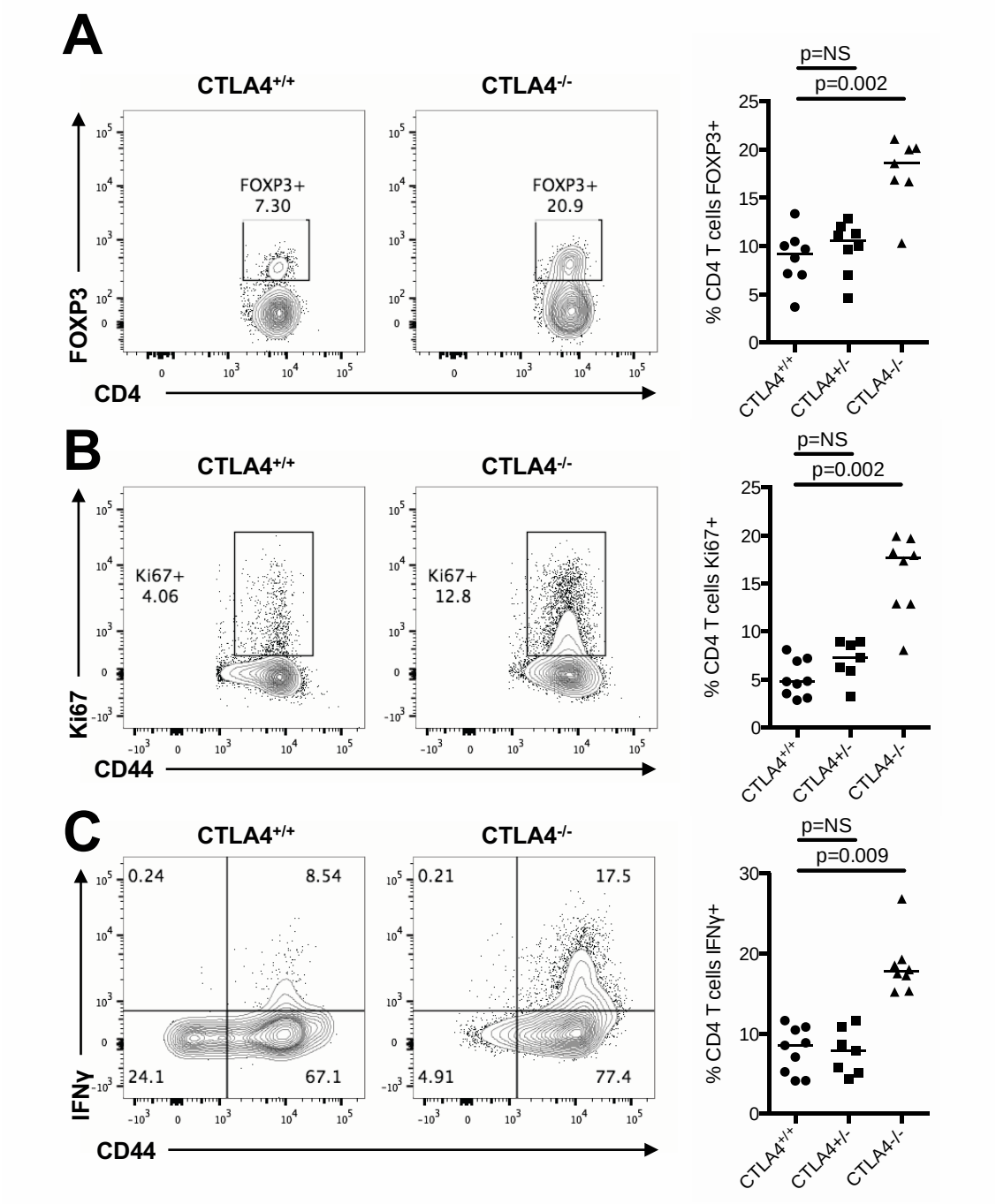


Figure 5.4: CTLA4$^{-/-}$ mouse liver flow cytometric analysis (continued). CTLA4$^{-/-}$ pups have more hepatic regulatory T cells, more IFNγ producing cells and more proliferating cells. Livers from 3-4 week old CTLA4$^{+/+}$, CTLA4$^{+/-}$ or CTLA4$^{-/-}$ pups from several litters were assessed for lymphocyte content. A greater proportion of CD4+ T cells were (**A**) FOXP3+ (**B**) Ki67+ and (**C**) IFNγ positive (after being stimulated for 4 hours with PMA/Ionomycin) in CTLA4$^{-/-}$ animals compared with CTLA4$^{+/+}$ or CTLA4$^{+/-}$. Representative plots gated on CD4+ T cells. Comparisons with Kruskal-Wallis test with Dunn's post-hoc test; n=8v8v7.

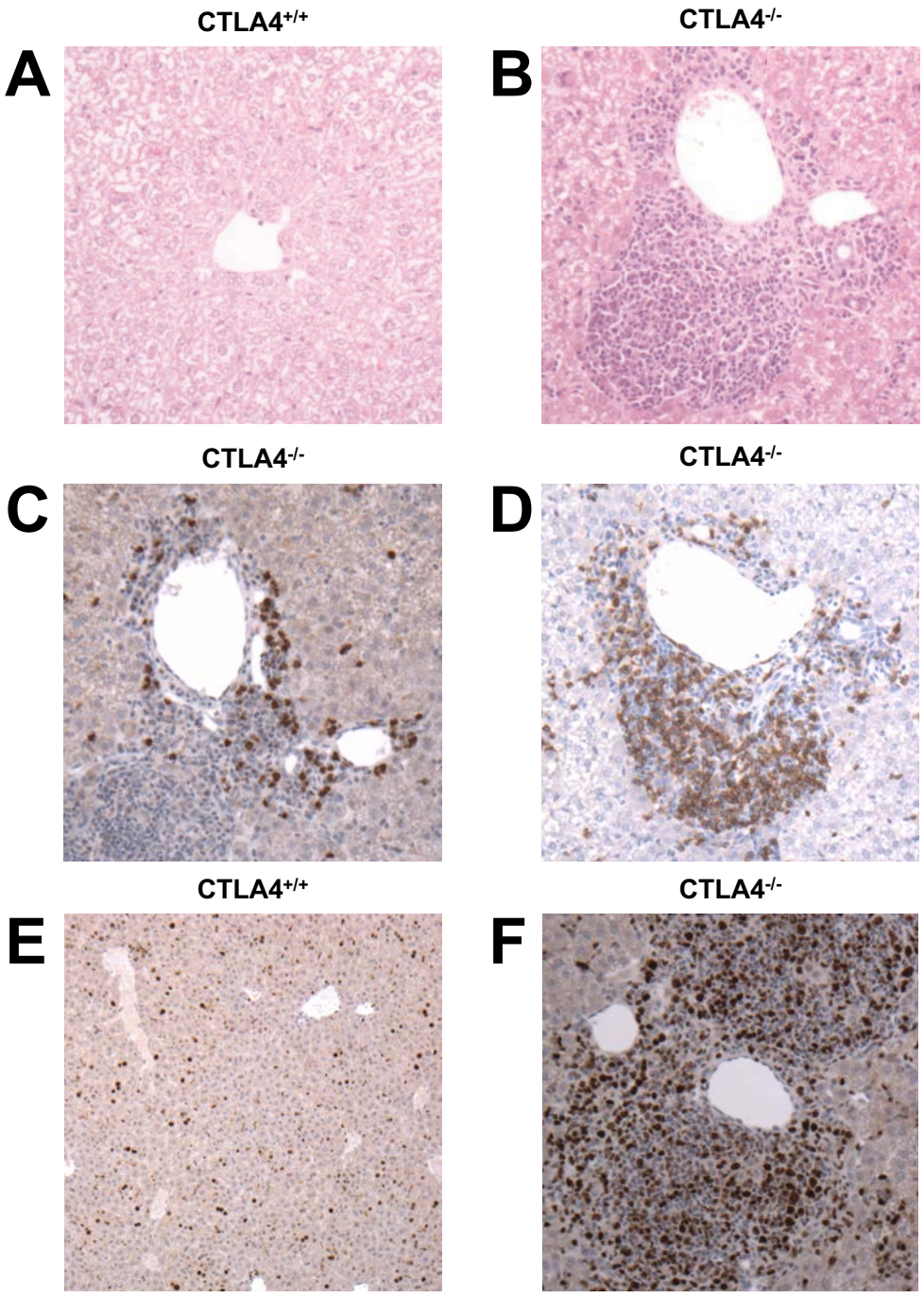


Figure 5.5: Histological analysis of CTLA4 deficient mouse liver. (**A**) and (**B**) CTLA4$^{+/+}$ and CTLA4$^{-/-}$ mouse liver sections stained with H&E. (**C**) CTLA4$^{-/-}$ liver section stained for OX40. (**D**) CTLA4$^{-/-}$ liver section stained for CD4. (**E**) and (**F**) CTLA4$^{+/+}$ and CTLA4$^{-/-}$ mouse liver sections stained for Ki67. All images representative and taken at ×10 and ×20 magnification.

5.2.3 Blockade of OX40 signalling in CTLA4 deficiency

Having demonstrated similarity between the hepatic and general phenotype of CTLA4-deficient and FOXP3-deficient animals, the ability of blockade of OX40-OX40L signalling to ameliorate the autoimmunity of CTLA4 deficiency was assessed. T-cell deficient ZAP70$^{-/-}$ hosts were repleted with total peripheral lymphocytes from CTLA4-deficient donor animals. Animals were then treated with blocking anti-OX40 antibody or total IgG control. In contrast to the significant amelioration of disease in FOXP3 deficiency, OX40 blockade did not increase survival, nor reduce splenomegaly or lymphadenopathy (Figure 5.6). Further, there were no significant differences in the hepatic lymphocyte populations as assessed by flow cytometry (Figure 5.7) or by examination of liver histology (Figure 5.8).

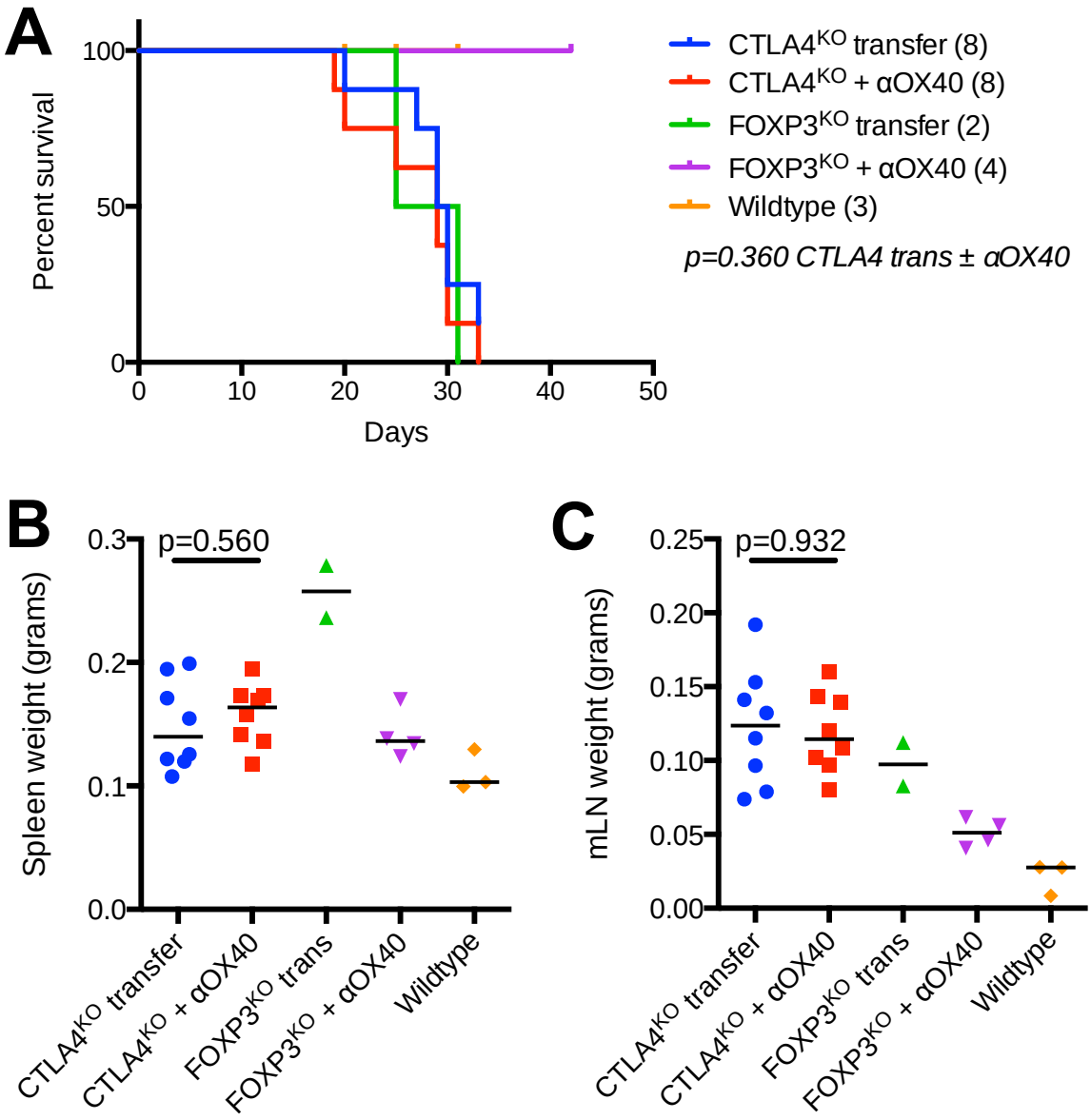


Figure 5.6: Blocking OX40 antibody does not ameliorate disease after transfer of CTLA4$^{-/-}$ cells. ZAP70$^{-/-}$ mice deficient in T cells were repleted with total peripheral lymphocytes from CTLA4$^{-/-}$ animals. Some animals were given blocking anti-OX40 antibody. A smaller number of ZAP70$^{-/-}$ mice were transferred with FOXP3^{KO} cells to confirm the continued efficacy of the antibody in treating that disease. (**A**) Survival of mice that received CTLA4$^{-/-}$ cells was not altered by anti-OX40 antibody; (**B**) Spleen weight was not altered by blocking anti-OX40; (**C**) Mesenteric lymph node weight was not altered by blocking anti-OX40. Comparisons with Log-Rank or Mann-Whitney U test; n=25.

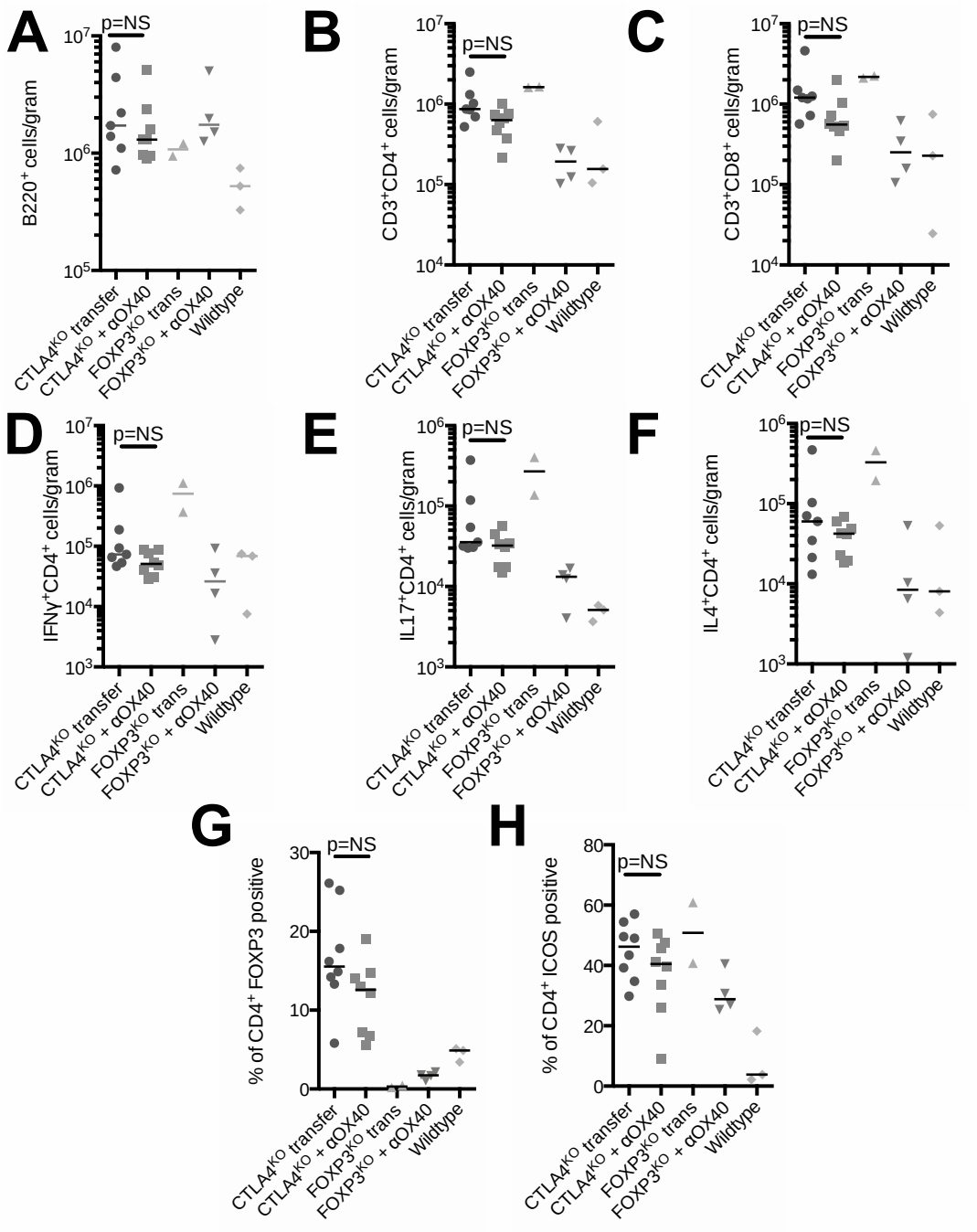


Figure 5.7: Blocking OX40 antibody does not ameliorate disease after transfer of CTLA4$^{-/-}$ cells. ZAP70$^{-/-}$ mice deficient in T cells were repleted with total peripheral lymphocytes from CTLA4$^{-/-}$ animals. Some animals were given blocking anti-OX40 antibody. A smaller number of ZAP70$^{-/-}$ mice were transferred with FOXP3^{KO} cells to confirm the continued efficacy of the antibody in treating that disease. No significant difference were seen in hepatic (**A**) B220+ B cells numbers, (**B**) CD4+ T cell numbers, (**C**) CD8+ T cell numbers, (**D-F**) numbers of CD4+ T cells producing IFNγ, IL-4 or IL-17 after stimulation. There were no differences in the proportions of CD4+ positive T cells positive for FOXP3 (**G**) or ICOS (**H**). Comparisons with Mann-Whitney U test between treated and untreated groups; n=25.

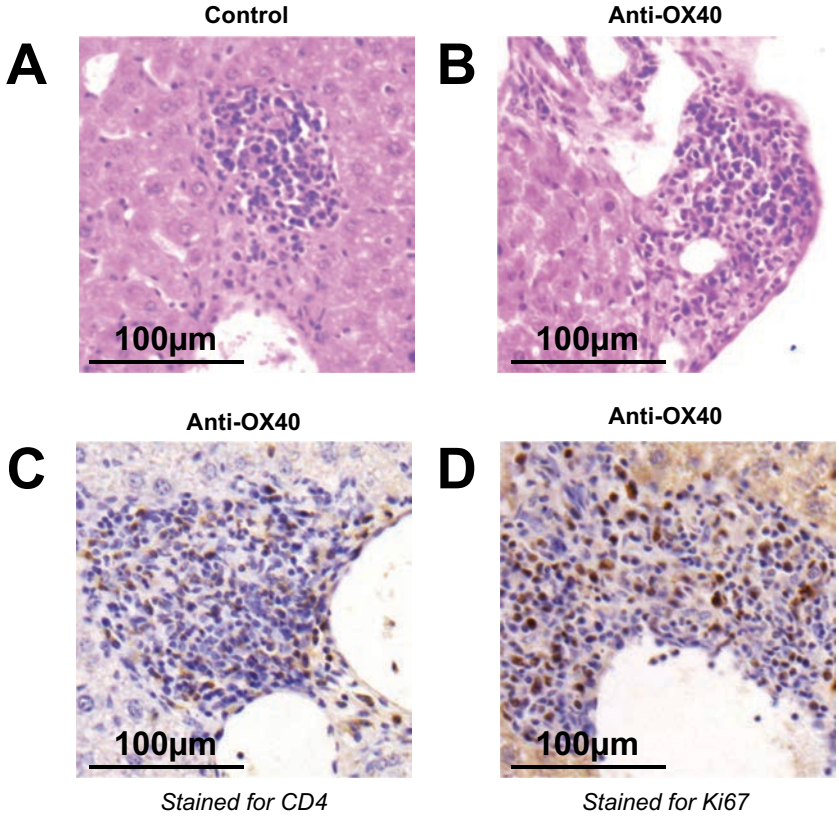


Figure 5.8: Blocking OX40 antibody does not ameliorate disease after transfer of CTLA4$^{-/-}$ cells. ZAP70$^{-/-}$ mice deficient in T cells were repleted with total peripheral lymphocytes from CTLA4$^{-/-}$ animals. Some animals were given blocking anti-OX40 antibody. A smaller number of ZAP70$^{-/-}$ mice were transferred with FOXP3^{KO} cells to confirm the continued efficacy of the antibody in treating that disease. Animals treated with blocking anti-OX40 F_{ab} showed similar lymphocytic infiltrates to control animals (**A**) and (**B**) Infiltrates in treated animals remained rich in (**C**) CD4+ T cells and (**D**) Ki67-positive cells.

5.2.4 Co-deficiency of CTLA4 and OX40

Having demonstrated a lack of efficacy of antibody blockade in ameliorating CTLA4 deficiency disease, to both examine whether the presence of OX40-OX40L interactions during development altered the phenotype of CTLA4 deficiency and to exclude an insufficient dosage of blocking anti-OX40 F_{ab} in the previous experiment, animals co-deficient in CTLA4 and OX40 were produced. Of note, because of limitations in the laboratory's mouse breeding programme, these animals were co-deficient in CD30.

When assessed by degree of splenomegaly or the presence of AMA, there was no significant difference in the phenotype of animals co-deficient in OX40 and CD30 when assessed at 3-4 weeks of age.(Figure 5.9).

When intrahepatic lymphocytes were assessed by flow cytometry, there were increases in the numbers of CD4+ T cells seen with co-deficiency of OX40 and CD30, but no other differences when compared with purely CTLA4-deficient animals (Figure 5.10). Correspondingly, histological examination revealed no significant differences in the lymphocytic, CD4 T cell rich, Ki67-positive cell rich periportal hepatic infiltrate seen in CTLA4-deficient animals (Figure 5.11).

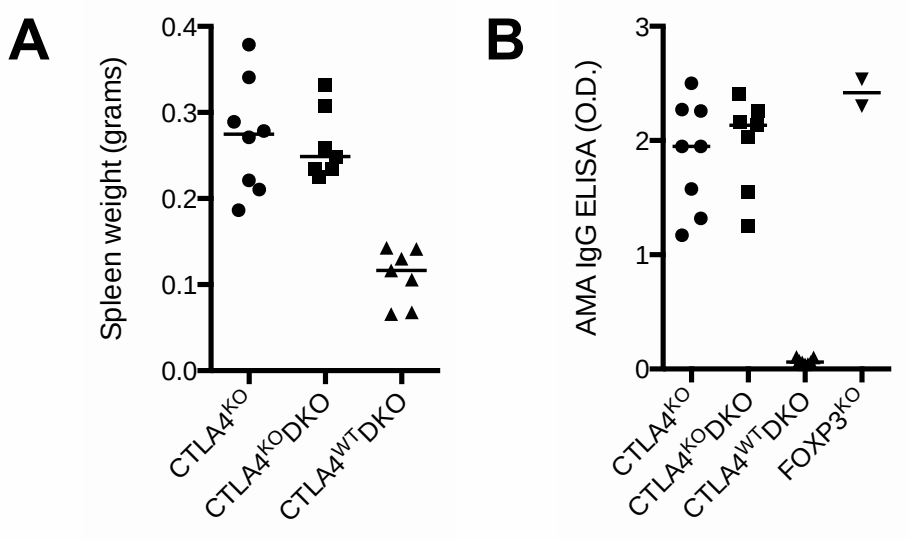


Figure 5.9: Co-deficiency in OX40 and CD30 does not protect against CTLA4$^{-/-}$ disease. 3-4 week old mice that were either deficient in CTLA4 (CTLA4$^{-/-}$), deficient in CTLA4, OX40 and CD30 (CTLA4^{KO}DKO) or sufficient in CTLA4 but deficient in OX40 and CD30 (CTLA4^{WT}DKO)were killed. Co-deficiency in OX40 and CD30 did not significantly alter either (**A**)the splenomegaly associated with CTLA4 deficiency or (**B**) reduce the titre of AMA that developed. Comparisons with Mann-Whitney U test; n=8v8v7.

5.3 Discussion

The two major findings presented in this chapter are, first, that the liver phenotype of mice with dysfunctional CTLA4 is similar to that seen in mice with dysfunctional FOXP3 and, second, that in contrast to FOXP3 dysfunction, CTLA4 dysfunction is not ameliorated by blockade of OX40-OX40L interactions.

The finding that CTLA4-deficient mice develop liver autoimmunity with AMAs and hyper-expression of OX40 in a similar way to those with FOXP3 dysfunction is not surprising: uncharacterised liver infiltrates have been demonstrated in multiple other models of regulatory failure.

By screening a number of mice with different mechanisms of systemic autoimmunity with both autoimmune and otherwise induced liver injury, our assay showed that the development of AMAs was not universal with liver inflammation in contrast to the uncertainty regarding AMAs expressed by others.[150] One reason for this may be that Hohenester et al., used much older mice than those

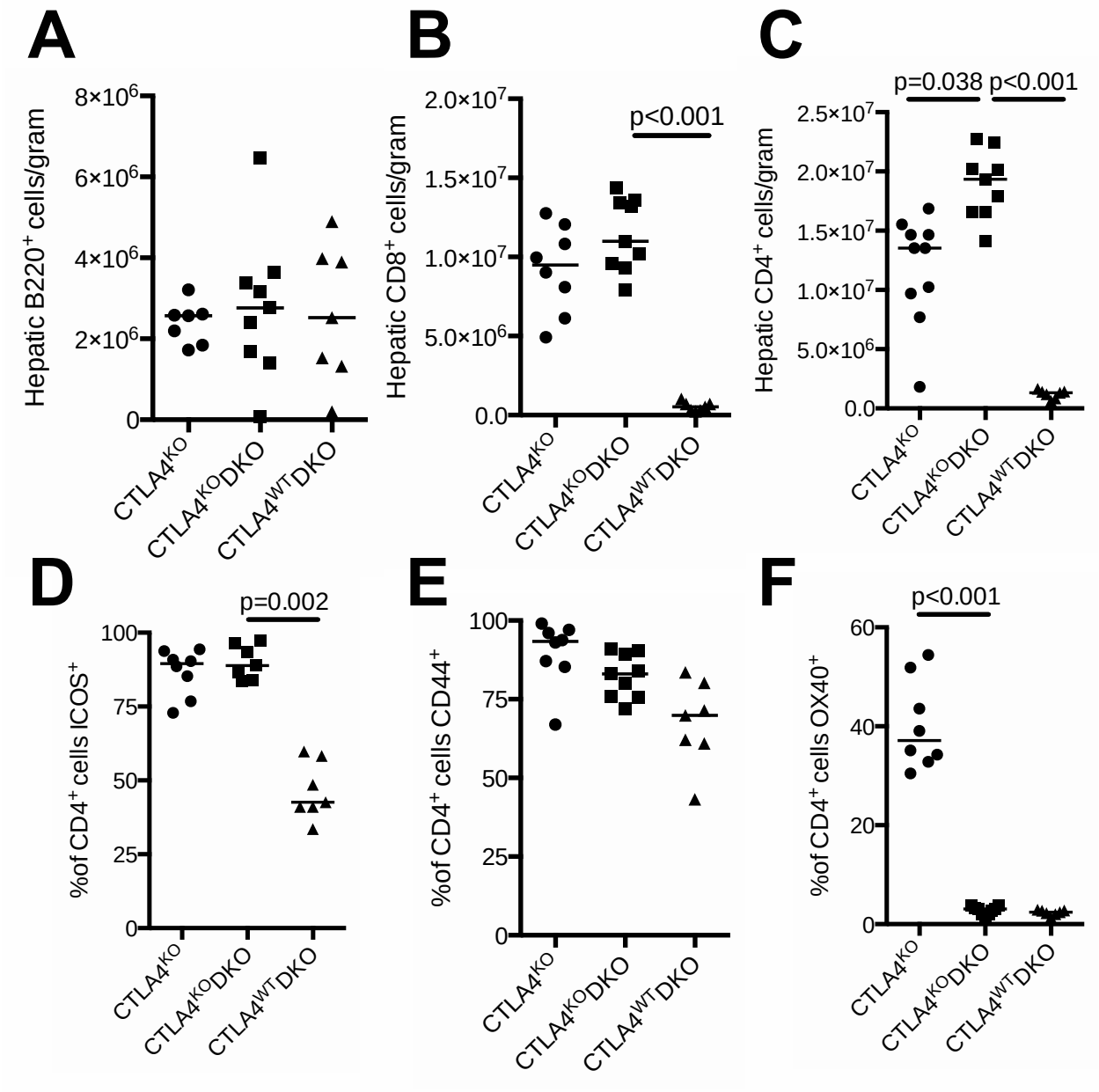


Figure 5.10: Co-deficiency in OX40 and CD30 does not protect against CTLA4$^{-/-}$ disease: flow cytometry. 3-4 week old mice that were either deficient in CTLA4 (CTLA4$^{-/-}$), deficient in CTLA4, OX40 and CD30 (CTLA4^{KO}DKO) or sufficient in CTLA4 but deficient in OX40 and CD30 were killed and their livers analysed. (**A**) There were no differences in numbers of B cells between groups; (**B**) There were markedly greater numbers of CD8+ T cells in both strains deficient in CTLA4; (**C**) There were greater numbers of intrahepatic CD4+ T cells in CTLA4 deficient animals, with slightly more CD4+ T cells in CTLA4^{KO}DKO mice than those purely deficient in CTLA4. (**D**) Fewer CD4+ T cells were ICOS-positive in mice sufficient in CTLA4 whilst (**E**) no significant differences were seen in the proportion of cells that expressed CD44 between groups. (**F**) OX40 expression was consistent with genotype. Comparisons with Mann-Whitney U test; n=8v8v7.

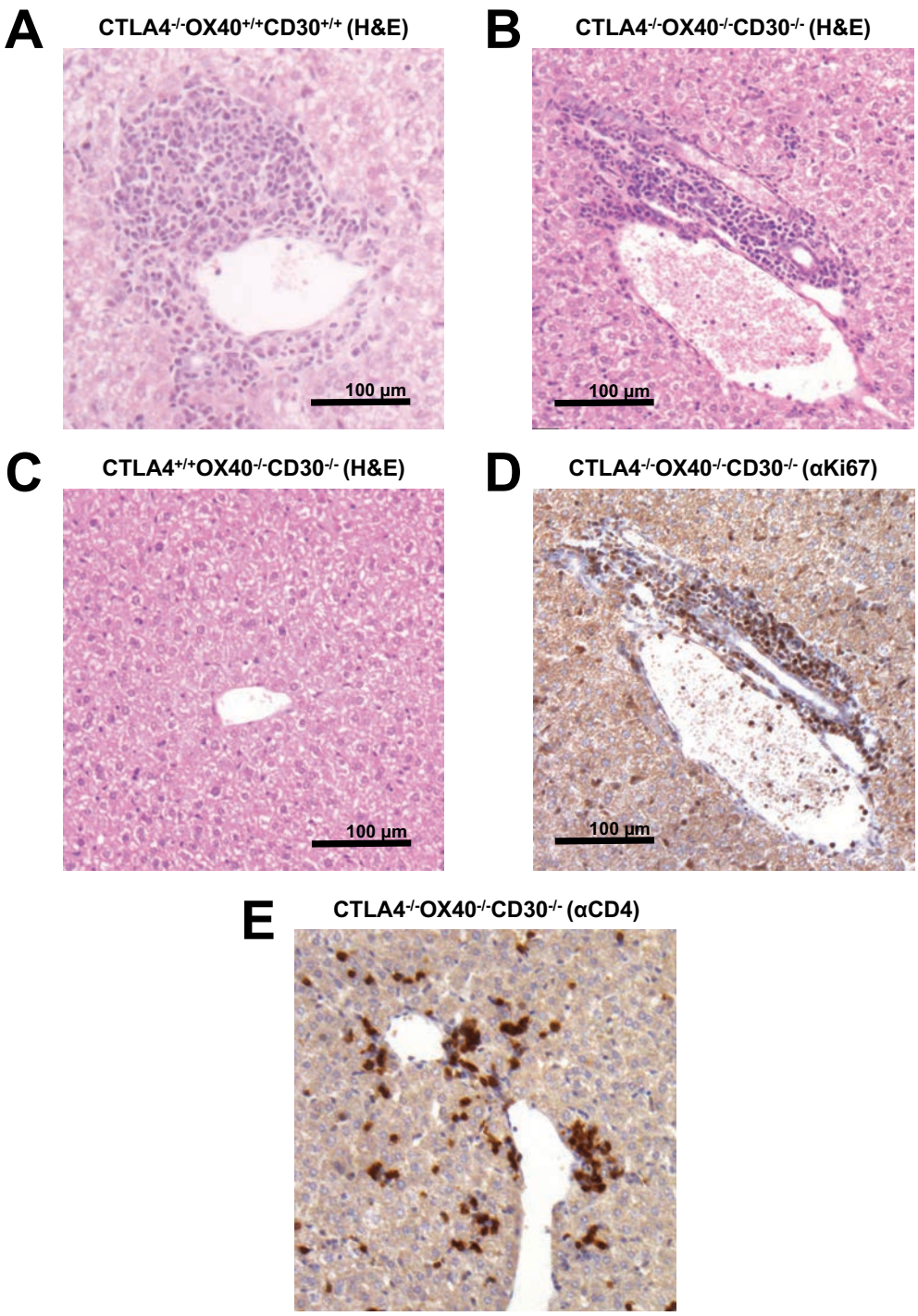


Figure 5.11: Co-deficiency in OX40 and CD30 does not protect against CTLA4$^{-/-}$ disease. 3-4 week old mice that were either deficient in CTLA4 (CTLA4$^{-/-}$), deficient in CTLA4, OX40 and CD30 (CTLA4^{KO}DKO) or sufficient in CTLA4 but deficient in OX40 and CD30 were killed. When stained with H&E, lymphocytic infiltrates were similar between CTLA4$^{-/-}$ (**A**) and CTLA4KO.DKO animals (**B**) but absent in CTLA4$^{-/-}$ sufficient DKO animals **C**. Infiltrates in CTLA4^{KO}DKO animals were rich in Ki67 (**D**) and CD4 (**E**) positive cells.

assessed here, and generalised autoimmunity in aged C57Bl/6 mice is well described.[134]

CTLA4 is nearly exclusively expressed by Treg and by activated and memory effector T cells.[137, 333] As such, the failure of interruptions of OX40-OX40L might reasonably be ascribed to an inability to compensate for the loss of immune regulation caused by a loss of non-Tregs CTLA4. Consistent with an important immune regulatory role for non-Treg CTLA4, some groups have reported being able to selectively delete CTLA4 on Treg without provoking the massive autoimmunity seen in complete CTLA4 deficiency.[166, 327] In these models, loss of Treg CTLA4 results in both expansion in numbers and increased activation of conventional T cells, but not organ infiltration. This phenotype contrasts with the lethal multi-system autoimmunity seen in mice with induced deficiency in CTLA4 across all cells.[205] Supporting a key role for non-Treg CTLA4, one that is more important than that for Treg CTLA4 is the observation that bone marrow chimeras of CD4 deficient mice with CTLA4 deficient mice are able to control multi-system autoimmunity whereas chimeras with mice deficient in all T cells are not.[102]

Such a failure of OX40 blockade to regulate autoimmunity in CTLA4 deficiency despite its apparent efficacy in FOXP3 dysfunction makes it challenging to predict whether OX40-OX40L blockade might be of therapeutic utility in human CTLA4-related disease. In both therapeutic CTLA4 blockade related autoimmunity and constitutive abnormalities in CTLA4, it remains unclear as to whether the dominant driver of pathology is interruption of Tregs or of the action of CTLA4 on conventional T cells. If the former, OX40-OX40L blockade may be effective; if the latter, it seems less likely to work.

Weaknesses in this approach

One criticism of these experiments is the lack of a positive control in the antibody OX40-OX40L blockade experiments. It is possible that the antibody used was not effective. However, the antibody used was drawn from the same batch as that used in successful therapy of animals with FOXP3

dysfunction, and lack of efficacy of co-deficiency in OX40 is consistent with the results of the antibody blockade experiments and excludes an effect from insufficient dosing.

An additional criticism of the co-deficiency experiments presented here is that rather than being purely co-deficient in OX40 and CTLA4, the mice bred here were triply deficient: in OX40, CD30, and CTLA4. This breeding strategy was a constraint of the mouse colonies available. Previous work has shown that dually OX40- and CD30 deficient mice appear healthy but have impaired memory responses.[106] In co-deficiency experiments involving FOXP3 and CD30 and OX40, the expression of OX40 was more important with CD30-deficient FOXP3-deficient animals dying only shortly after those purely deficient in FOXP3.[105]

Future experiments

To further assess whether OX40-OX40L blockade is sufficient to control the loss of immune regulation induced by the loss of Treg CTLA4, a logical extension of this work would be to assess whether OX40-OX40L blockade suppressed the activation and expansion seen in the specific inducible deficiency models.[166, 327] Supportive *in vitro* experiments examining the effect of OX40-OX40L blockade on proliferation and activation of combinations of Tregs and conventional T cells with and without CTLA4 would also be feasible. An additional question is whether the autoimmunity produced by generalised deficiency of CTLA4 induced during adulthood is controllable by OX40-OX40L blockade, and indeed this has been tried and shown to be ineffective (Unpublished observation, Kajsa Wing, Karolinska Institutet; communication to Lane Group).

The uncertainty as to whether the anti-cancer efficacy of CTLA4 blockade was related to activity on Tregs or on effector T cells led one group to develop a mouse which expressed the human form of CTLA4. By co-transfer experiments of Tregs and conventional T cells and the use of anti-human and anti-murine blocking anti-CTLA4 antibodies, it could be demonstrated that the anti-tumour

effect required blockade of effector T cell CTLA4.[329] A similar model would permit delineation of the defect controlled by OX40 blockade.

A further method of investigating the question of the role of non-Treg CTLA4 would be to repeat the CTLA4 deficient transfer experiments reported here, but into a third group of animals also transfer CTLA4-sufficient cells depleted of Tregs and OX40; such experiments might however be complicated by transdifferentiation of conventional T cells into Tregs.

Finally, combining work from the preceding chapter and this, if Tregs from CTLA4-deficient animals could be purified, it would be interesting to see if they could control autoimmunity when transferred into the FOXP3-deficient transfer model reported in the preceding chapter. If the results were consistent with those of selective CTLA4 depletion on Treg reported elsewhere[166, 327], then some resolution might be expected. However, this contrasts with a report that CTLA4Ig is able to rescue FOXP3 deficient mice.[385]

6 OX40 blockade in Roquin *sanroque* mutant mice

6.1 Introduction

Mice homozygous for the *sanroque* mutant Roquin 1 gene have impaired post-transcriptional regulation of mRNA and have greatly increased expression of T cell co-stimulatory molecules alongside the development of multi-organ lymphocytic infiltrates.[438, 497] Key amongst these co-stimulatory molecules are ICOS and OX40.[439] Liver disease is described but its assessment has not been published other than as images of plain histology. No human equivalent mutation is recognised, although the RoquinMM phenotype has been described as analogous to human systemic lupus erythematosus, in which there may be hepatitis.[383] A further aspect of Roquin dysfunction is a loss of control of IFNγ production and subsequent increased expression.[341] Given that others have reported the development of intrahepatic lymphocytic infiltrates and AMA in increased IFNγ production, an association between Roquin$^{M/M}$ and AMA was considered.[14]

Having demonstrated that Roquin *sanroque* mutant mice develop AMA and lymphocytic hepatitis, further experiments to assess whether blockade of OX40-OX40L interactions ameliorated this phenotype were performed. In addition, given the elevations in IFNγ production seen in Roquin mutant mice and their association with liver disease elsewhere, the efficacy of antibody blockade of anti-IFNγ was assessed.

6.2 Results

6.2.1 Roquin *sanroque* mutant mice develop antimitochondrial antibodies

As demonstrated in Figure 5.1, Roquin$^{M/M}$ mice were seen to develop AMA in a screening of sera from a number of different mouse models of liver injury. To confirm this, a larger sample of Roquin$^{M/M}$ mice were compared to both FOXP3^{KO} mice and wildtype littermates of Roquin$^{M/M}$ mice. Roquin$^{M/M}$ mice consistently developed AMA (Figure 6.1).

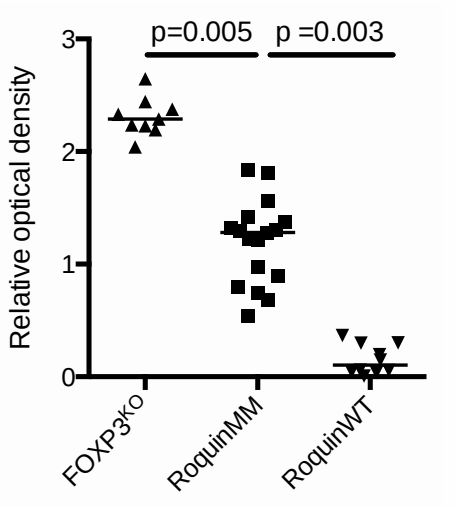


Figure 6.1: AMAs in various mice including Roquin$^{M/M}$. The sera of Roquin$^{M/M}$ mice mice were compared against FOXP3^{KO} mice as positive control and against littermates lacking the Roquin genotype of interest using the AMA ELISA described above. Comparisons with Kruskal-Wallis test with Dunn's post-hoc test.

6.2.2 The phenotype of the Roquin *sanroque* mutant mouse

Having demonstrated AMA in Roquin$^{M/M}$ mice, further analysis of the phenotype of Roquin$^{M/M}$ liver and the intrahepatic lymphocyte population was performed. In comparison with wildtype Roquin controls, Roquin$^{M/M}$ mice demonstrated splenomegaly, lymphadenopathy, elevations in serum ALT activity, elevations in serum IgE and IgG concentrations, and also the presence of high titre AMA (Figure 6.2).

Flow cytometric analysis of Roquin$^{M/M}$ intrahepatic lymphocytes revealed greater numbers of B, CD4+ T, and CD8+ T cells (Figure 6.3A-C). In addition, a greater proportion of CD4+ T cells expressed ICOS, OX40 and FOXP3 (Figure 6.3D-F).

Histological examination of Roquin$^{M/M}$ mouse liver demonstrated peri-portal lymphocytic infiltrates that were not present in age-matched wildtype animals (Figure 6.4), with significant proportions demonstrating positive staining for OX40, Ki67, and CD4 by immunohistochemistry.

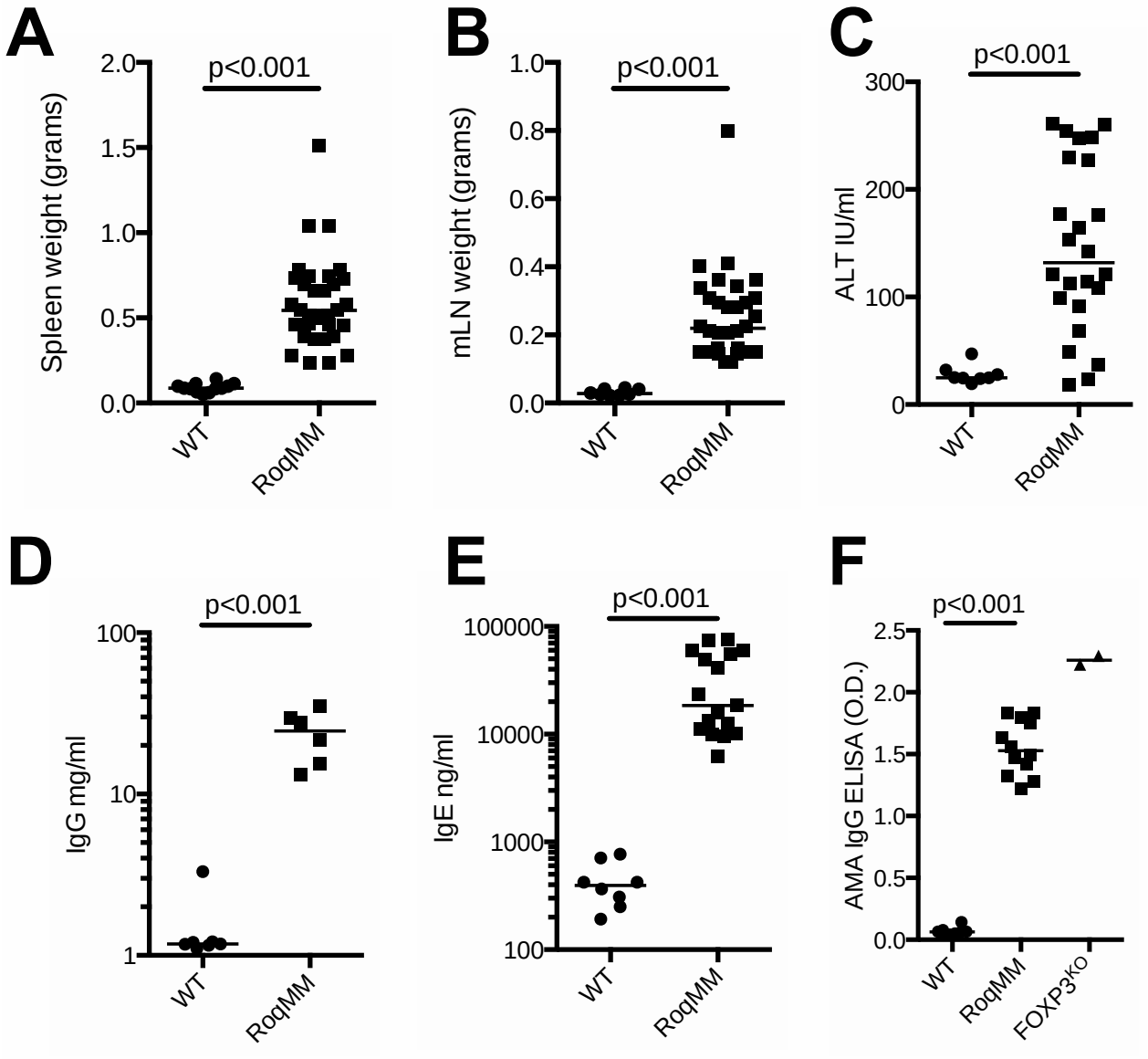


Figure 6.2: Roquin$^{M/M}$ mice demonstrate evidence of systemic autoimmunity. Roquin$^{M/M}$ mice aged 16-20 weeks were compared with wildtype mice. When compared to wildtype mice, Roquin$^{M/M}$ mice showed marked splenomegaly (**A**), lymphadenopathy as assessed by weighing the mesenteric lymph node chain (**B**), elevations in serum ALT (**C**), elevations in serum IgE (**D**), elevations in serum IgG (**E**), and AMA (**F**). Comparisons with Mann-Whitney U test; n=9v26.

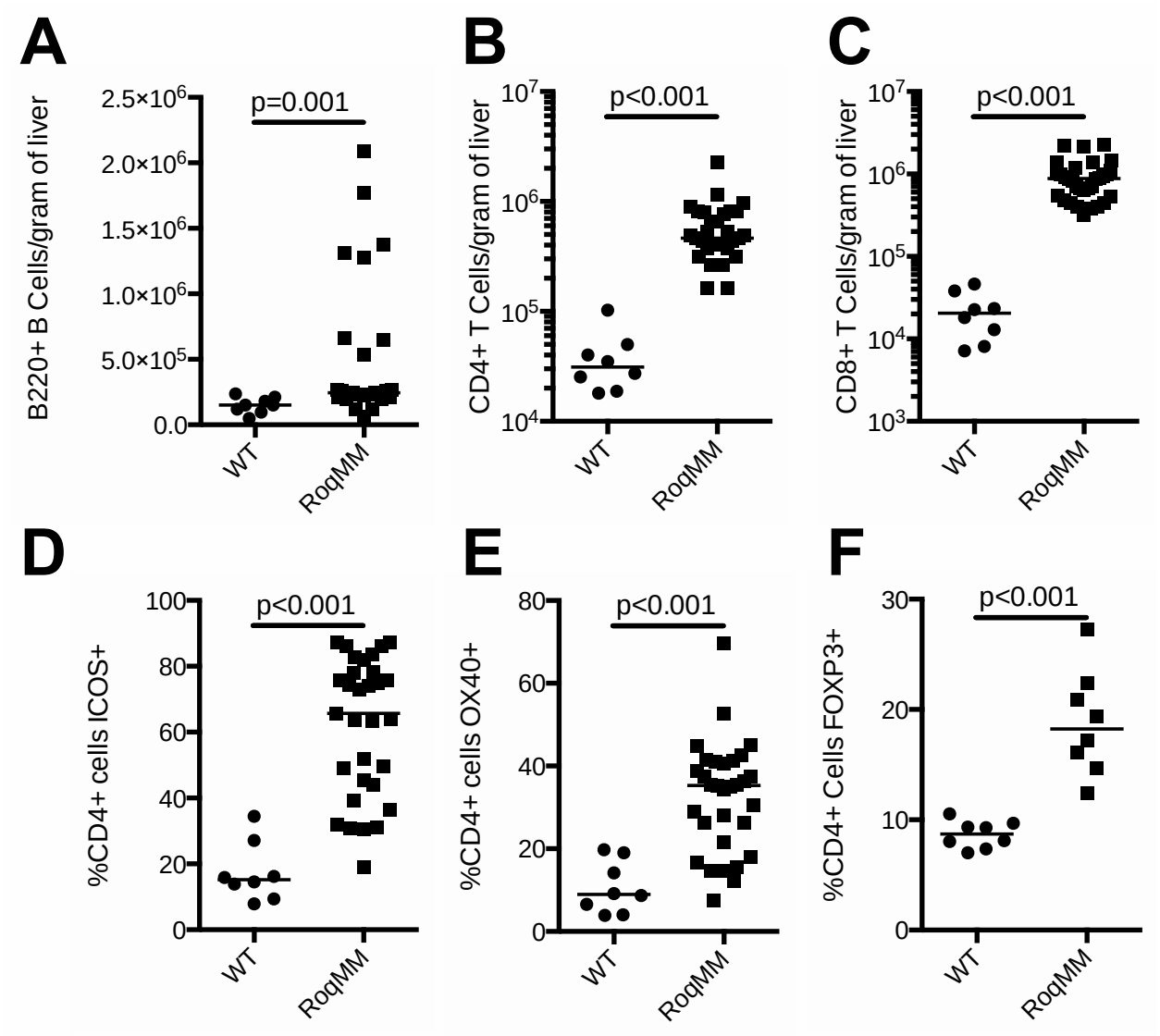


Figure 6.3: Roquin$^{M/M}$ mice demonstrate evidence of elevations in hepatic lymphocyte numbers and activation. Roquin$^{M/M}$ mice aged 16-20 weeks were compared with wildtype mice. When compared to wild-type mice, Roquin$^{M/M}$ mouse liver contained more B cells (**A**), more CD4+ T cells (**B**), and more CD8+ T cells (**C**). Of the hepatic CD4+ T cell population, significantly more were ICOS positive (**D**), OX40 positive (**E**) and FOXP3 (**F**). Statistical comparisons were performed using the Mann-Whitney U test; n=9v26.

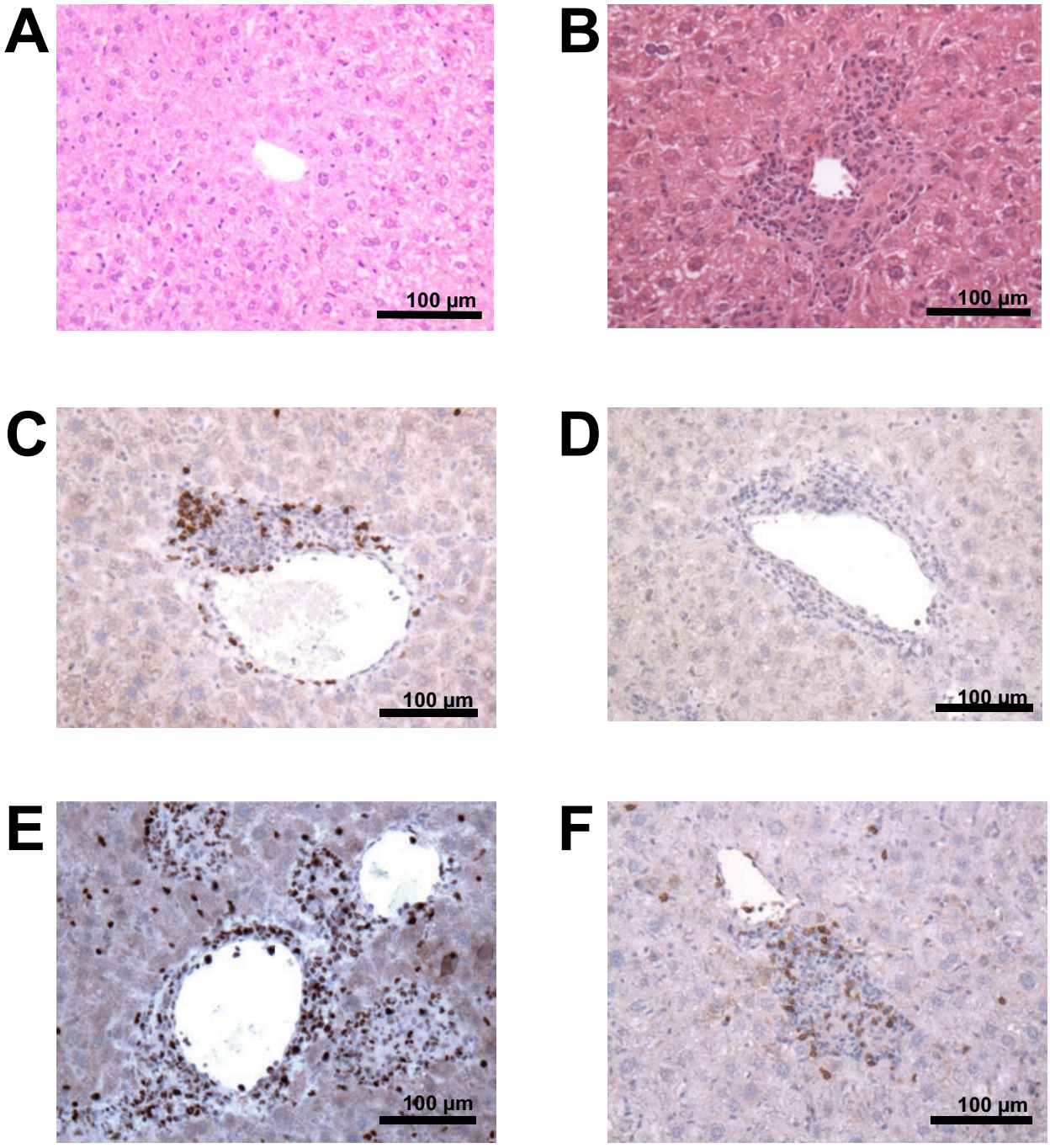


Figure 6.4: Roquin$^{M/M}$ mice develop hepatic inflammation. Roquin$^{M/M}$ mice aged 16-20s week were compared with wildtype mice. When compared to wildtype mice (**A**), liver from Roquin$^{M/M}$ mice demonstrated peri-portal lymphocytic infiltration (**B**). These infiltrating cells were frequently OX40+ (**C**; **D**=isotype control), frequently Ki67 positive (**E**) and contained numerous CD4+ cells (**F**). Representative images shown.

6.2.3 Anti-OX40 antibody blockade in Roquin *sanroque* mutant mice

The presence of lymphocytic peri-portal infiltrates, evidence of liver damage and AMA, and hyper-expression of OX40 led to an attempt to ameliorate Roquin$^{M/M}$ disease by blockade of OX40-OX40L interactions through an OX40 F_{ab}.

A comparison of Roquin$^{M/M}$ treated with either blocking anti-OX40 F_{ab} or total rat IgG control over four weeks demonstrated no significant difference to the gross autoimmune phenotype of Roquin$^{M/M}$ mice in terms of spleen weight and lymphadenopathy (Figure 6.5). Further, no significant differences were apparent in serum AMA titre, ALT activity, serum IgG, or serum IgE (Figure 6.6).

Histological examination of animals treated with and without blocking anti-OX40 F_{ab} revealed a persistence of lymphocyte-rich peri-portal infiltrates in treated animals, and no variation in the extent of OX40 positivity (Figure 6.7). Flow cytometry of intrahepatic lymphocyte populations demonstrated no alteration in the increased numbers of B and T lymphocytes present after blockade of OX40 signalling, and no reduction in the proportion of CD4+ T lymphocytes showing evidence of activation (Figure 6.8). Similarly, stimulation of CD4+ T lymphocytes and analysis of cytokine secretion patterns showed no difference in the number of IFNγ, IL-4, or IL-17 secreting CD4+ T cells present (Figure 6.9).

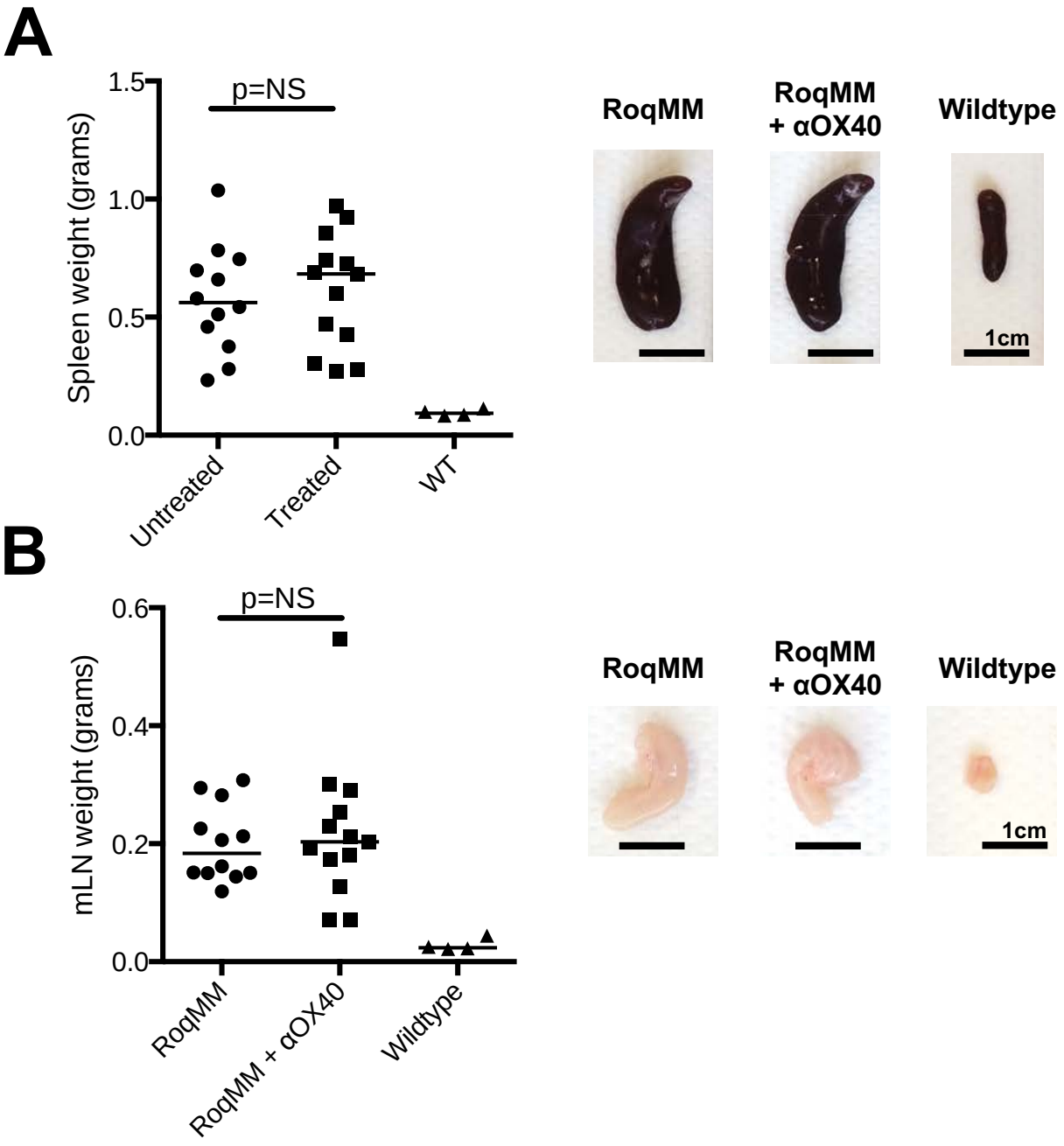


Figure 6.5: Antagonistic anti-OX40 F_{ab} does not reverse splenomegaly and lymphadenopathy in Roquin$^{M/M}$ disease To investigate whether blocking OX40-OX40L interactions could ameliorate autoimmunity in Roquin$^{M/M}$ mice, mice were treated with twice weekly anti-OX40 F_{ab} 250μg for four weeks. Animals were then killed and compared to littermates that had received total rat IgG as a control. Treatment with anti-OX40 F_{ab} made no difference to either (**A**) splenomegaly or (**B**) lymphadenopathy of the mesenteric chain. Comparisons with Mann-Whitney U test; n=12v13v4.

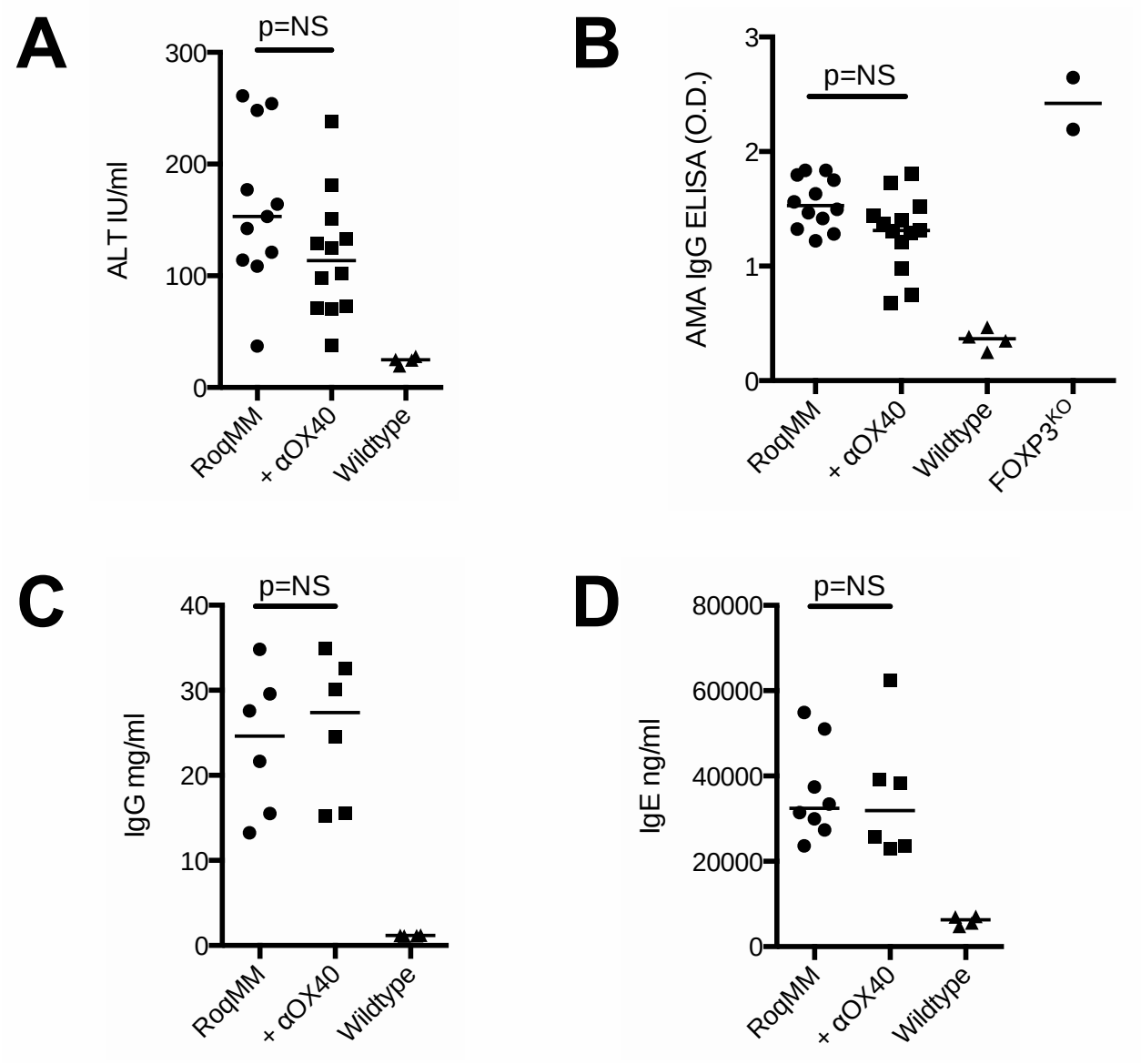


Figure 6.6: Antagonistic anti-OX40 F_{ab} does not reverse markers of autoimmunity in Roquin$^{M/M}$ disease. To investigate whether blocking OX40-OX40L interactions could ameliorate autoimmunity in Roquin$^{M/M}$ mice, mice were treated with twice weekly anti-OX40 F_{ab} 250μg for four weeks. Animals were then killed and compared to littermates that had received total rat IgG as a control. Treatment with anti-OX40 made no difference to the elevations in serum ALT (**A**), AMA titre (**B**), IgG (**C**) or IgE (**D**); n=12v13v4.

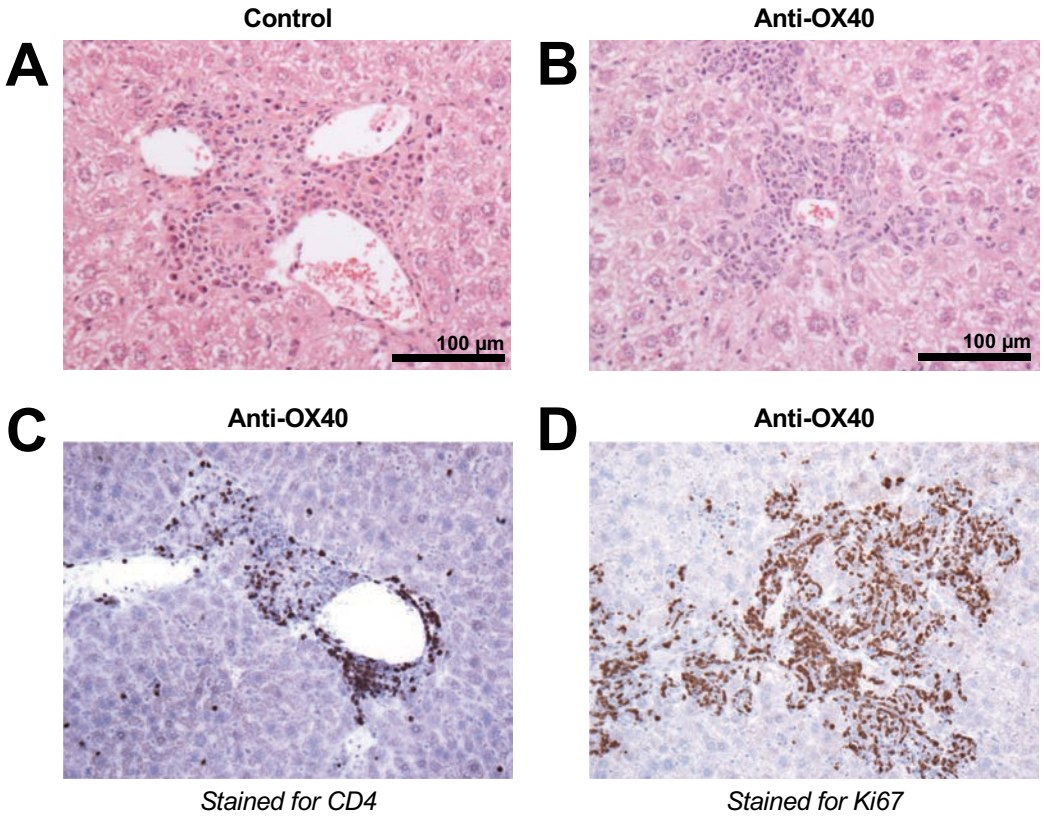


Figure 6.7: Antagonistic anti-OX40 F_{ab} does not reverse liver inflammation in Roquin$^{M/M}$ disease. To investigate whether blocking OX40-OX40L interactions could ameliorate autoimmunity in Roquin$^{M/M}$ mice, mice were treated with twice weekly anti-OX40 F_{ab} 250μg or isotype control for four weeks. Examination after staining with H&E demonstrated peristing infiltrates in both untreated (**A**) and treated mice (**B**). Heavy staining for OX40 was also evident in the inflammatory infiltrate of both untreated (**C**) and treated (**D**) mice.

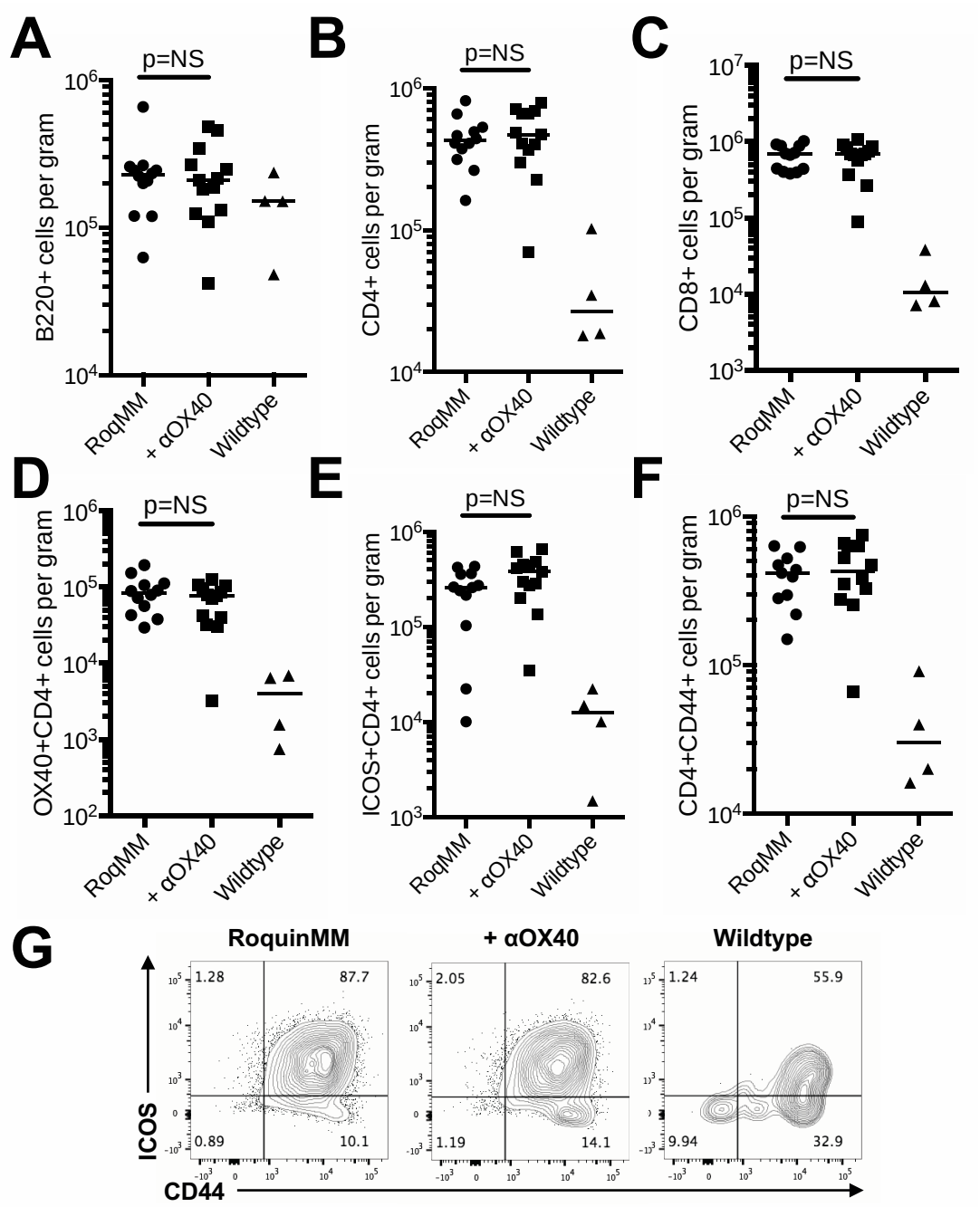


Figure 6.8: Blocking OX40 antibody does not affect Roquin$^{M/M}$ liver infiltration. To investigate whether blocking OX40-OX40L interactions could ameliorate autoimmunity in Roquin$^{M/M}$ mice, mice were treated with twice weekly anti-OX40 F_{ab} 250μg for four weeks. Flow cytometric analysis of hepatic lymphocytes demonstrated persisting numbers of B cells (**A**), CD4+ T cells (**B**), and CD8+ T cells (**C**) after treatment with anti-OX40 F_{ab}. Similarly, anti-OX40 F_{ab} did not reduce the elevations in numbers of OX40+ (**D**), ICOS+ (**E**), and CD44+ (**F**) in Roquin$^{M/M}$ disease. Comparisons with Mann-Whitney U test; n=12v13v4. Representative plots gated on CD4+ T cells are shown (**G**).

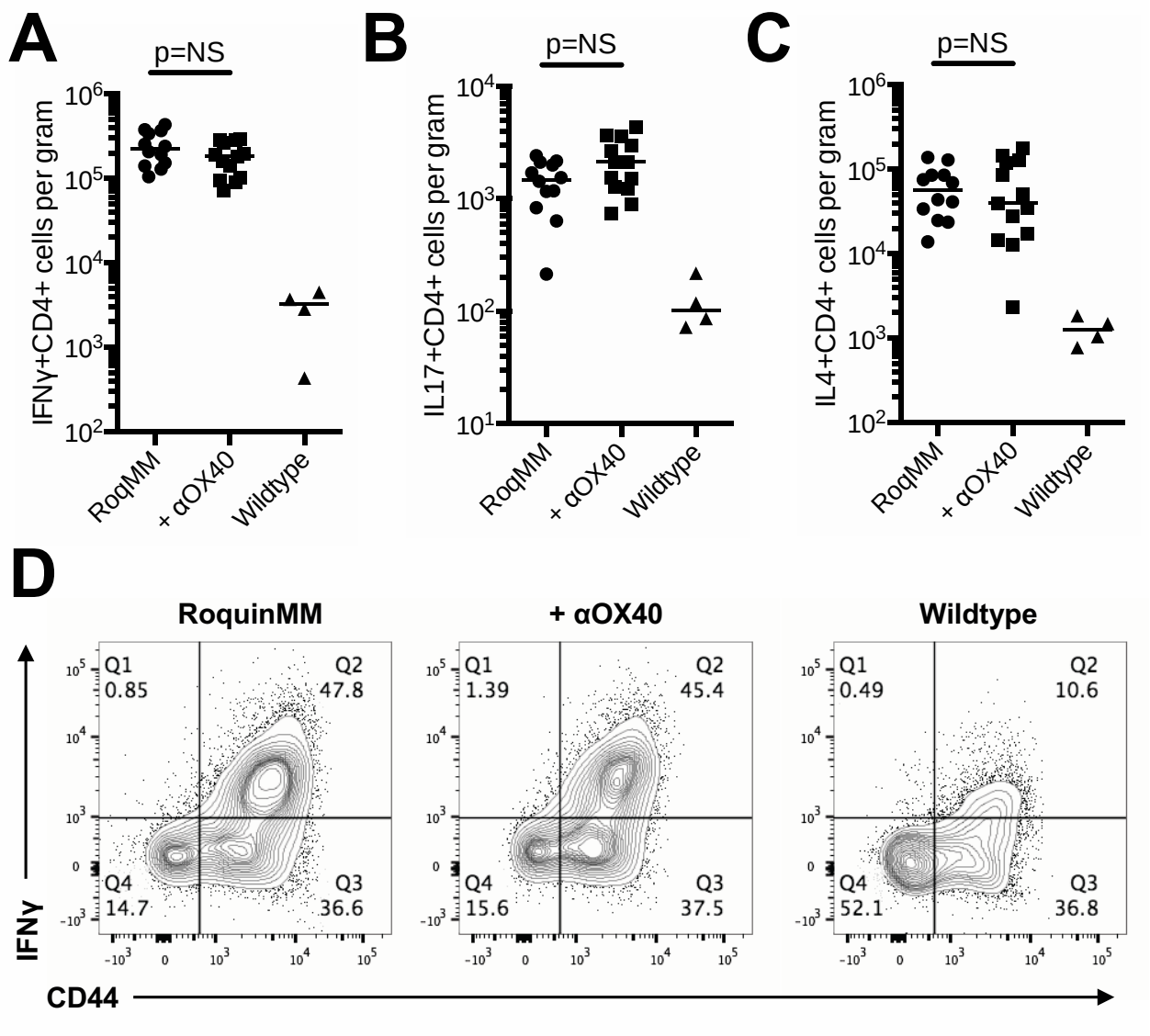


Figure 6.9: Blocking antiOX40 F_{ab} does not affect IFNγ production in Roquin$^{M/M}$ mice. To investigate whether blocking OX40-OX40L interactions could ameliorate autoimmunity in Roquin$^{M/M}$ mice, mice were treated with twice weekly anti-OX40 F_{ab} 250μg for four weeks. Blocking OX40 made no difference to number of IFNγ (**A**), IL-17 (**B**) or IL-4 (**C**) secreting CD4+ T cells assessed after *in vitro* stimulation. Comparisons with Mann-Whitney U test; n=12v13v4.

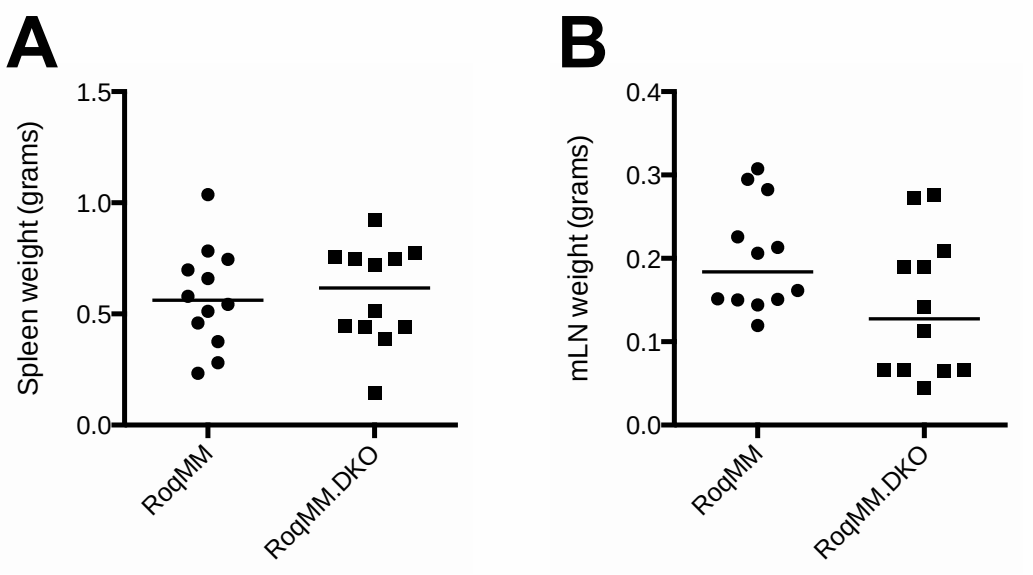


Figure 6.10: Co-deficiency in OX40 does not ameliorate Roquin$^{M/M}$ disease. To investigate whether co-deficiency in OX40 and CD30 ameliorated the Roquin$^{M/M}$ phenotype, Roquin$^{M/M}$ mice were crossed and back-crossed with CD30,OX40 deficient mice to generate Roquin$^{M/M}$.CD30$^{-/-}$OX40$^{-/-}$ mice (Roquin$^{M/M}$.DKO). These mice were then compared with pure Roquin$^{M/M}$ mice. Co-deficiency in OX40 and CD30 made no significant difference to the splenomegaly (**A**) or lymphadenopathy (**B**) in Roquin$^{M/M}$ mice (p=0.831 and p=0.05 respectively). Comparisons with Mann-Whitney U test; n=12v12.

6.2.4 Co-deficiency of OX40 in Roquin *sanroque* mutant mice

To further assess whether developmental OX40-OX40L signalling contributed to the Roquin$^{M/M}$ phenotype, and to exclude an insufficient dose of blocking OX40 in earlier experiments, Roquin$^{M/M}$ mice with and without concurrent deficiency in OX40 and CD30 were compared. The use of mice concurrently deficient in CD30 was mandated by breeding considerations.

Consistent with findings from antibody blockade experiments, no significant difference was seen in the degree of splenomegaly or lymphadenopathy in Roquin$^{M/M}$ mice with and without concurrent OX40 and CD30 deficiency (Figure 6.10). Similarly, histological examination of liver revealed no significant differences between groups (Figure 6.11), and no difference in intrahepatic lymphocyte populations including the proportion producing IFNγ (Figures 6.12 and 6.13).

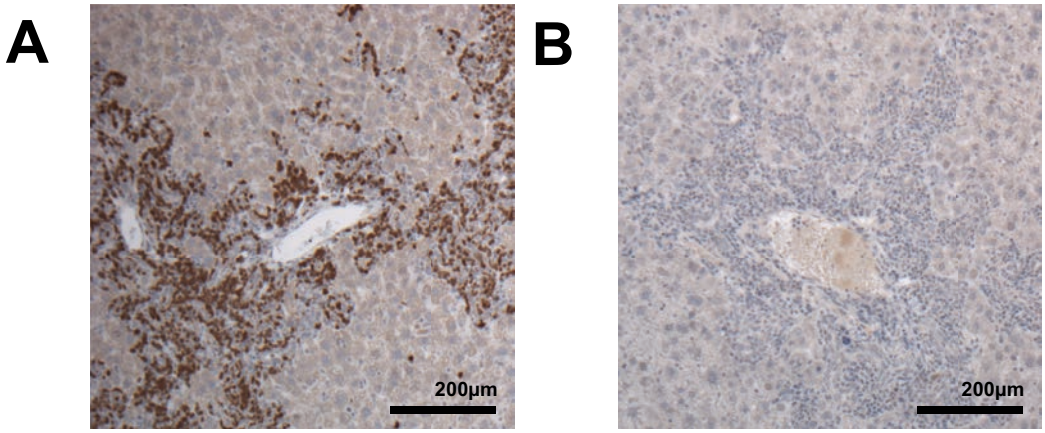


Figure 6.11: Co-deficiency in OX40 does not ameliorate Roquin$^{M/M}$ liver infiltration: histology. To investigate whether co-deficiency in OX40 and CD30 ameliorated the Roquin$^{M/M}$ phenotype, Roquin$^{M/M}$ mice were crossed and back-crossed with CD30,OX40 deficient mice to generate Roquin$^{M/M}$.CD30$^{-/-}$OX40$^{-/-}$ mice (Roquin$^{M/M}$.DKO). These mice were then compared with pure Roquin$^{M/M}$ mice. The extent of lymphocytic infiltration was similar in Roquin$^{M/M}$ (**A**) and Roquin$^{M/M}$.DKO (**B**) mice, despite deficiency in OX40 (representative images stained for OX40).

6.2.5 Treatment of Roquin *sanroque* mutant mice with anti-interferon-γ

Having demonstrated that Roquin$^{M/M}$ mice have increased numbers of IFNγ-producing intrahepatic lymphocytes, are reported to generally hyper-express IFNγ and that generalised hyperexpression of IFNγ in a different model is reported as causing mice to generate AMA and develop intrahepatic lymphocytic infiltrates, an attempt was made to see whether blockade of IFNγ signalling in Roquin$^{M/M}$ mice was effective in ameliorating their autoimmune phenotype.

Administration of blocking anti-IFNγ over four weeks produced a decreased in splenomegaly (Figure 6.14A), but no significant decrease in lymphadenopathy (Figure 6.14B). Similarly, flow cytometric analysis of intrahepatic lymphocytes revealed that blocking anti-IFNγ reduced numbers of infiltrating CD8+ T cells and reduced the proportions of CD4+ T cells expressing OX40, but did not alter other flow cytometric parameters (Figure 6.15). When infiltrating T cells were stimulated and their cytokine expression profiles examined, the proportions producing IFNγ and IL-17 were not altered, but the proportion producing IL-4 was increased (Figure 6.16).

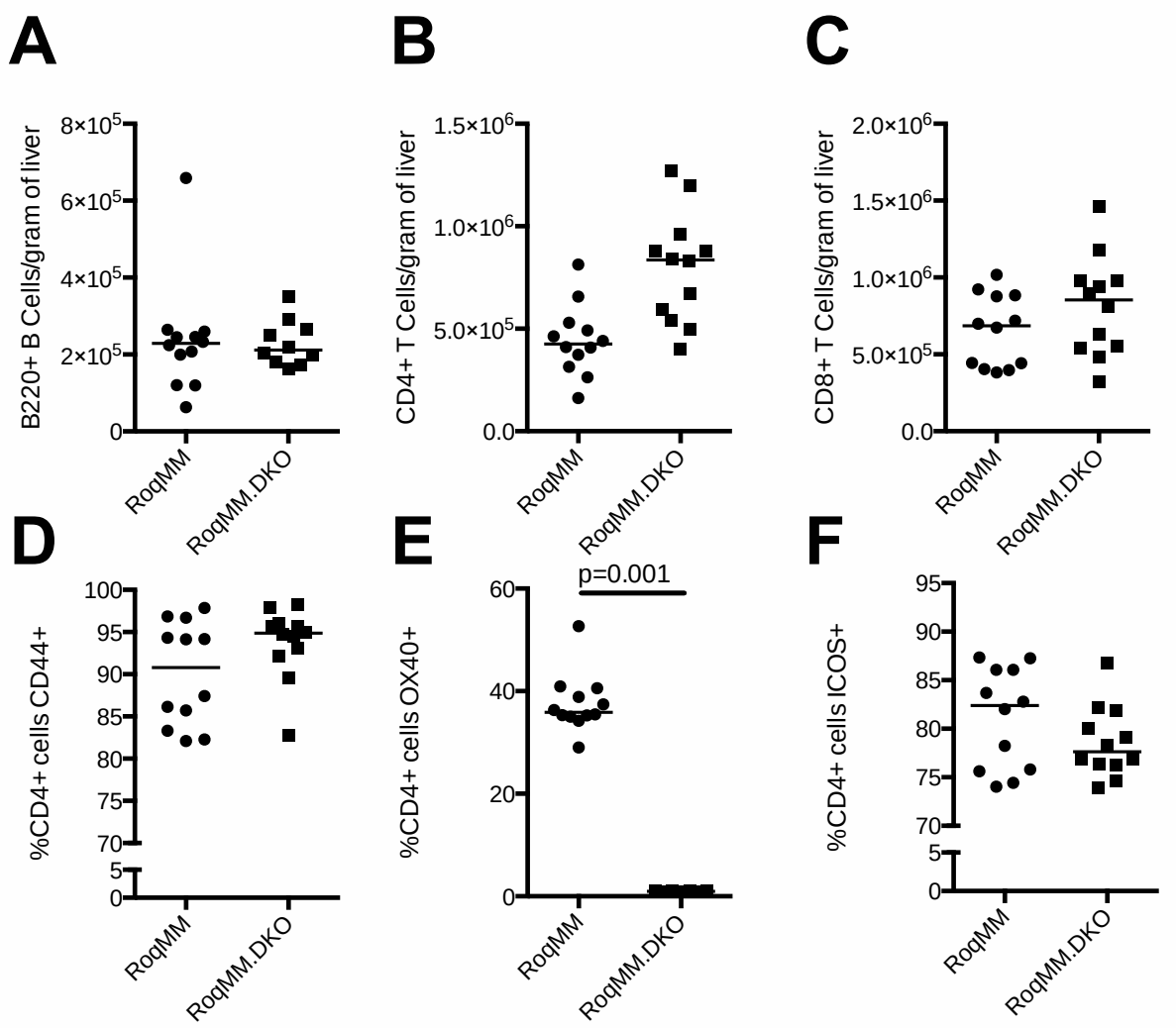


Figure 6.12: Co-deficiency in OX40 does not ameliorate Roquin$^{M/M}$ liver infiltration: flow cytometry. To investigate whether co-deficiency in OX40 and CD30 ameliorated the Roquin$^{M/M}$ phenotype, Roquin$^{M/M}$ mice were crossed and back-crossed with CD30,OX40 deficient mice to generate Roquin$^{M/M}$.CD30$^{-/-}$OX40$^{-/-}$ mice (Roquin$^{M/M}$.DKO). These mice were then compared with pure Roquin$^{M/M}$ mice. Flow cytometric analysis of hepatic lymphocytes demonstrated no differences in numbers of B cells (**A**), CD4+ T cells (**B**), and CD8+ T cells (**C**). When CD4+ T cells were further examined, there were no significant differences in the proportion that were CD44+ (**D**) or ICOS+ (**F**); OX40 was not expressed on Roquin$^{M/M}$.DKO CD4+ T cells (**E**). Comparisons with Mann-Whitney U test; n=12v12.

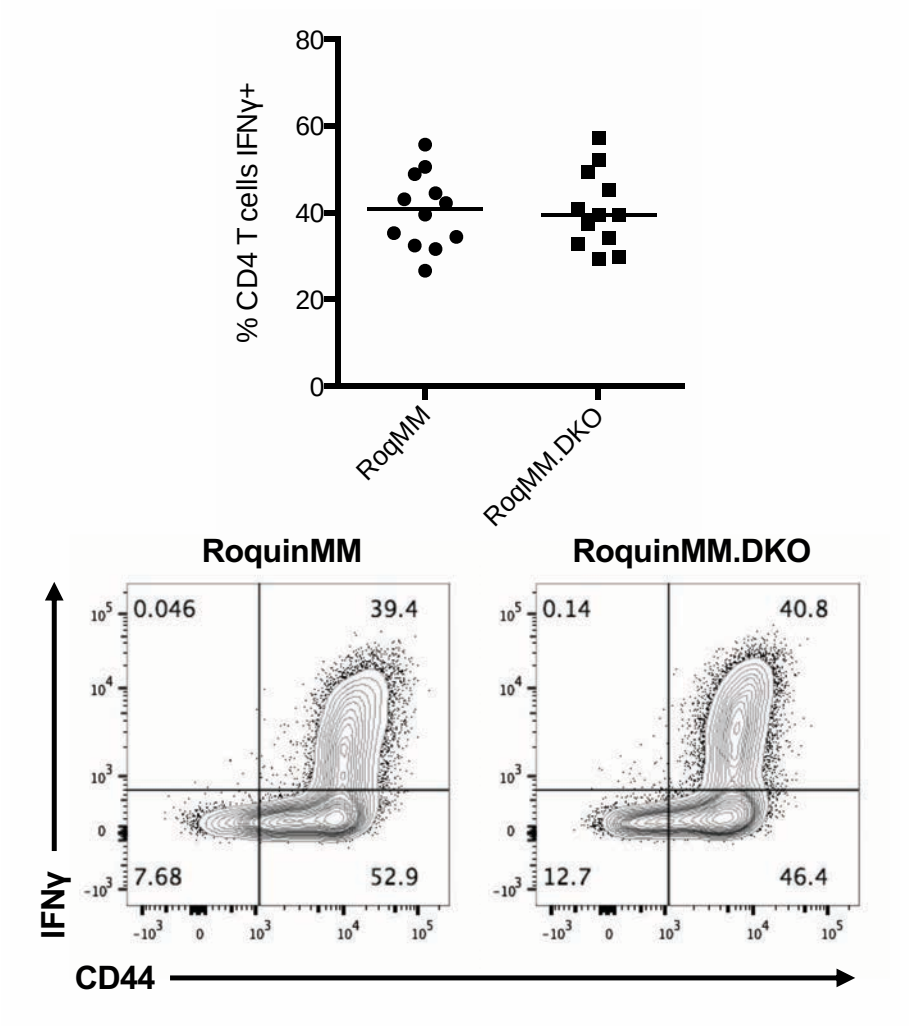


Figure 6.13: Co-deficiency in OX40 does not ameliorate Roquin$^{M/M}$: IFNγ production. To investigate whether co-deficiency in OX40 and CD30 ameliorated the Roquin$^{M/M}$ phenotype, Roquin$^{M/M}$ mice were crossed and back-crossed with CD30,OX40 deficient mice to generate Roquin$^{M/M}$.CD30$^{-/-}$OX40$^{-/-}$ mice (Roquin$^{M/M}$.DKO). These mice were then compared with pure Roquin$^{M/M}$ mice. After *in vitro* stimulation, no significant difference (p=0.962) in the number of hepatic IFNγ-producing CD4+ T cells was present. Comparison with Mann-Whitney U test; n=12v12.

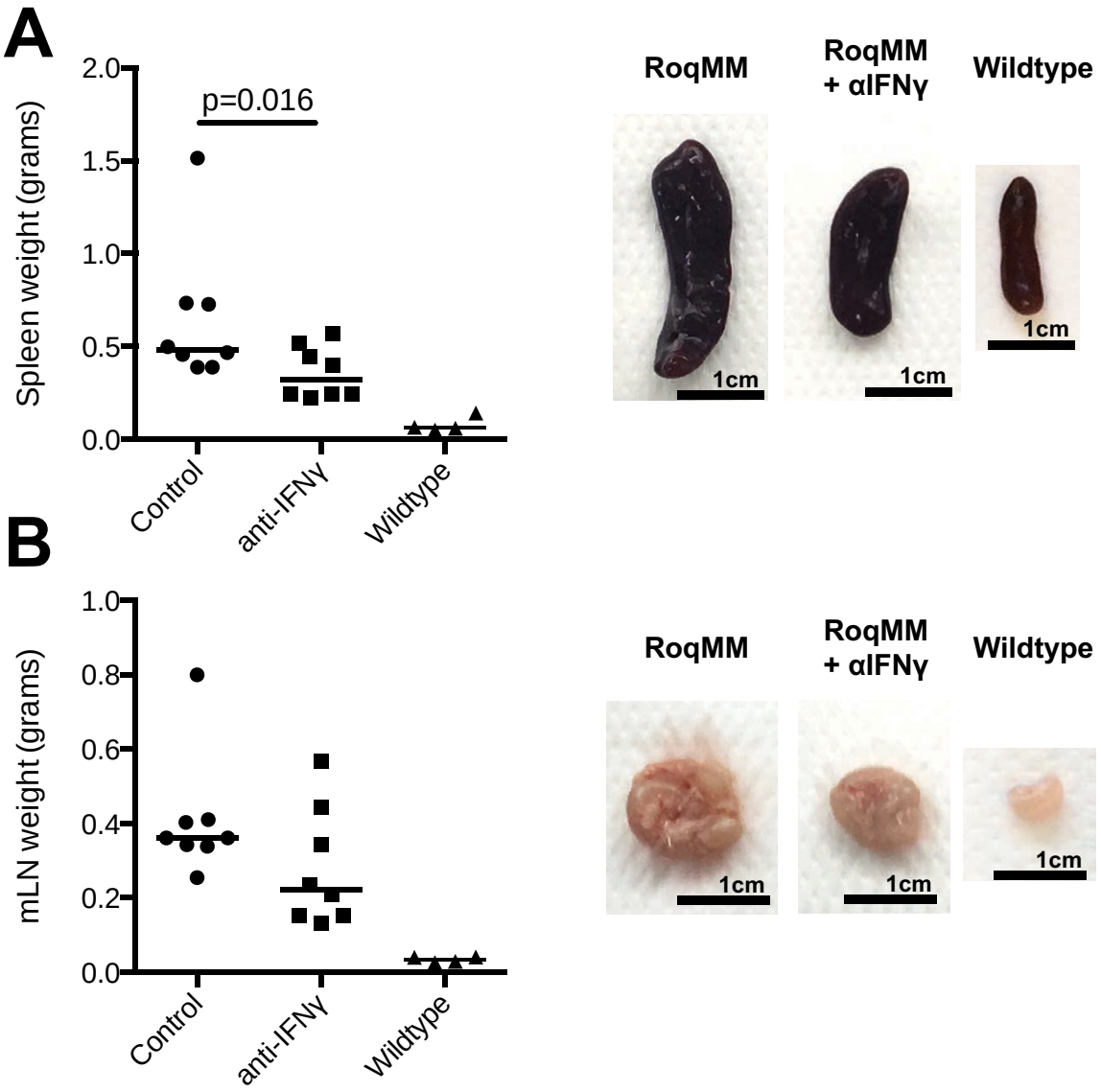


Figure 6.14: Anti-IFNγ antibody partially ameliorates Roquin$^{M/M}$ disease. An anti-IFNγ blocking antibody was administered to Roquin$^{M/M}$ mice aged between 16-20 weeks 500μg twice weekly for four weeks. (**A**) Administration of blocking anti-IFNγ significantly reduced spleen weight in Roquin$^{M/M}$ mice, but (**B**) did not significantly affect mesenteric lymph node weight (p=0.250). Comparisons with Mann-Whitney U test; n=8v8v4.

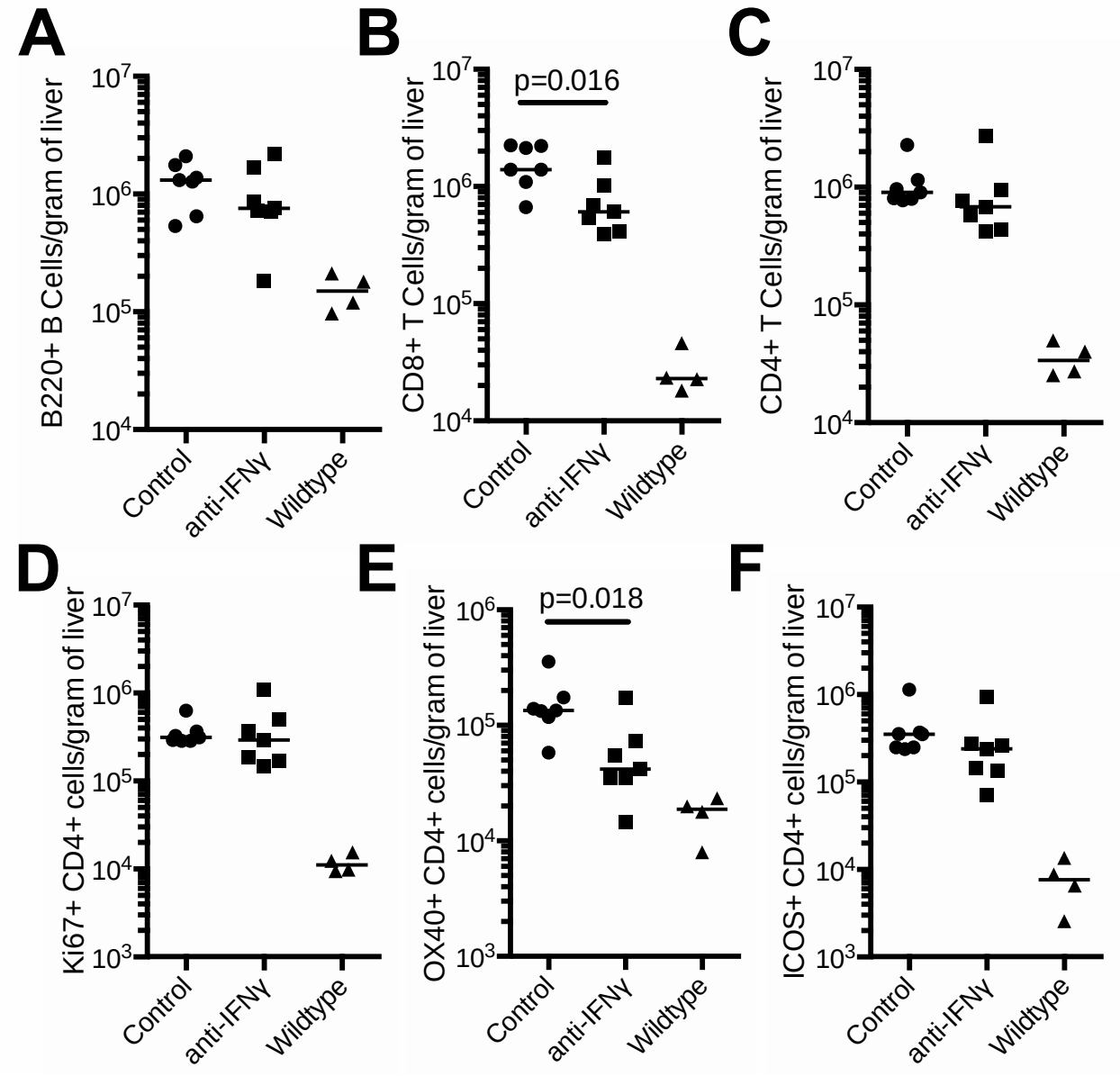


Figure 6.15: Anti-IFNγ antibody partially ameliorates Roquin$^{M/M}$ disease. An anti-IFNγ blocking antibody was administered to Roquin$^{M/M}$ mice aged between 16-20 weeks at a dose of 500μg twice weekly for four weeks. This treatment did not affect the number of hepatic B cells (**A**), did significantly reduce the number of CD8+ T cells (**B**), and did not affect the number of CD4+ T cells (**C**). Blocking anti-IFNγ did not affect numbers of Ki67 CD4+ T cells (**D**), did reduce numbers of OX40+ CD4+ T cells (**E**), and did not alter numbers of ICOS+ CD4+ T cells (**F**). Comparisons between Roquin$^{M/M}$ groups with Mann-Whitney U test; n=8v8v4.

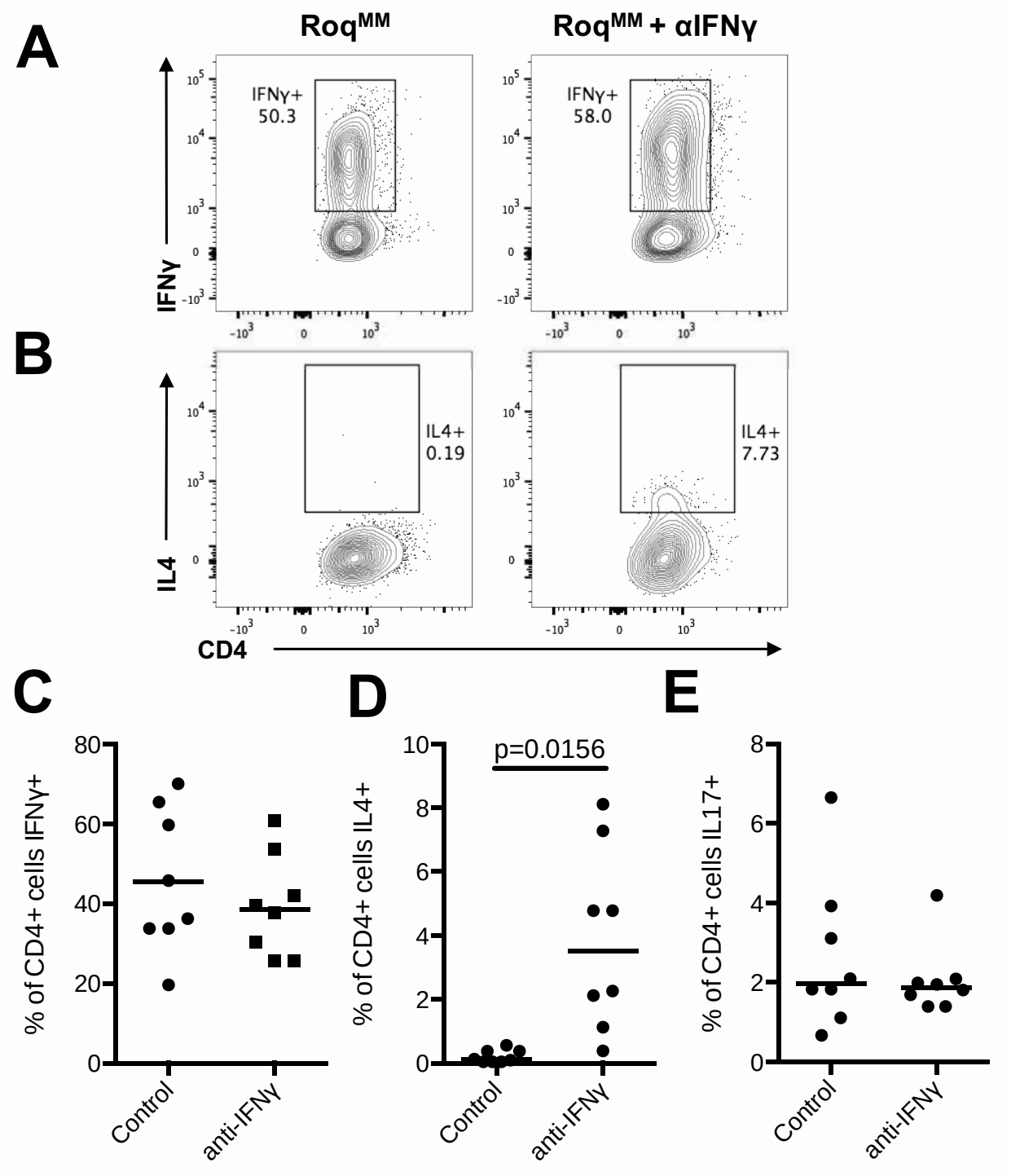


Figure 6.16: Anti-IFNγ antibody partially ameliorates Roquin$^{M/M}$ disease. Treating Roquin$^{M/M}$ mice with blocking anti-IFNγ did not alter the proportion of CD4+ T cells that were positive for IFNγ after stimulation (**A**) and (**C**), nor the proportion that were positive for IL-17 (**D**). However, treatment with anti-IFNγ increased the proportion of cells that were positive for IL-4 after stimulation (**B**) and (**E**); n=8v8.

6.3 Discussion

This set of experiments has shown that Roquin$^{M/M}$ mice demonstrate lymphocytic hepatitis, AMA, hyper-expression of OX40, and some features analagous to human AILD. In contrast to mice deficient in FOXP3 however, there appears to be no change in their phenotype when OX40-OX40L interactions are blocked with either antibody or constitutively. The administration of a blocking IFNγ antibody appears to slightly ameliorate disease.

That Roquin$^{M/M}$ disease greatly increases IFNγ production and results in lymphocytic infiltration and AMA is consistent with the findings of another group who generated a mouse with a different deficit in IFNγ regulation and subsequent over-expression.[14] It is unclear however how much of the Roquin$^{M/M}$ mouse's phenotype is due to IFNγ and how much can be explained by over-expression of ICOS and OX40, and how much relates to other dysregulated processes. For example, animals transgenic for ICOS-ligand generate lymphoid hyperplasia, although specific aspects of autoimmunity were unreported.[494] Mice constitutively expressing ICOS are not yet reported. Similarly, constitutive expression of OX40L can drive autoimmunity.[40, 295]

These experiments serve to highlight that the autoimmune phenotype of Roquin$^{M/M}$ mice is multifactorial consistent with multiple downstream effects of disruption of the Roquin regulatory protein.

Weaknesses in this approach

One criticism of this work is the lack of a positive control in the OX40 and IFNγ antibody blockade experiments. Nevertheless, in the OX40 blockade experiment, antibody from the same batch was efficacious in blocking autoimmunity in FOXP3 deficiency, and results were consistent with the lack of effect of constitutive OX40 blockade.

In the IFNγ antibody blockade experiment, differences were seen between groups including the induction of a larger population of IL-4-secreting cells in the IFNγ-treated groups. These changes

suggest biological activity of the antibody used, although do not exclude the possibility of underdosage

Breeding constraints meant that Roquin$^{M/M}$ mice bred to also be deficient in OX40 were also deficient in CD30: separation of the two variants is relatively uncommon because they are located close together on chromosome 4. Given the lack of differences in groups, this is unlikely to have had a great effect, but it would be preferable to compare groups of animals sufficient in CD30.

Future experiments

To expand on this work, it would be revealing to investigate whether blockade of signalling through ICOS together with blockade of signalling through OX40 would be sufficient to ameliorate the Roquin$^{M/M}$ phenotype. Notably, co-deficiency in ICOS has been reported to ameliorate but not abolish the Roquin$^{M/M}$ phenotype.[497] Blocking ICOS antibodies would also be a consideration.

The experiments with comparing Roquin$^{M/M}$ mice treated with blocking anti-IFNγ antibodies could usefully be extended either by increasing the duration of administration of blocking anti-IFNγ antibody or, with the advantage of preventing concerns about sufficient dosing, by breeding animals deficient in IFNγ with and without the Roquin$^{M/M}$ for comparison.

7 Factors affecting expression of OX40L by T cells

7.1 Introduction

OX40 and OX40L represent an exclusive receptor-ligand pair. OX40 expression and its regulating factors are well established. Briefly, there is constitutive expression on murine regulatory T cells with expression after TCR ligation on multiple subsets of both CD4+ and CD8+ T cells. The situation appears to be similar in humans, although human Tregs require stimulation prior to expression. Lower levels of inducible expression are reported on NKT cells, natural killer (NK) cells and neutrophils.[74]

The expression of OX40 on non-Tregs requires T cell receptor ligation.[121, 433] In addition, the upregulation of OX40 on T cells is slower, reduced and more transient in the absence of CD28 signals.[121, 448, 355] There is a relationship with IL-2, with exogenous IL-2 leading to increased expression of OX40[74] but increased signalling through OX40 also leading to greater T cell production of IL-2.[351] Further, a similar feedback mechanism seems to exist with a related gamma chain cytokine: IL-4 augments and sustains OX40 expression [417], and the expression of IL-4 is augmented by ligation of OX40.[314] The effects of OX40 ligation are numerous but may be summarised as reducing Treg function [442, 335], promoting T cell survival through the induction of anti-apoptotic regulatory proteins[355], promoting the formation of memory[120], and in influencing proliferation: with conflicting suggesting positive[197] and negative effects.[213]

The availability of OX40L, as the only known ligand of OX40, will influence activity through OX40. OX40L is more widely expressed than OX40 with constitutive expression reported on the lymphoid tissue inducer subset of innate lymphoid cells.[196, 228] In addition, induced expression is reported on dendritic cells[172, 215], B cells [399, 400], macrophages[479], mast cells [186, 123], various malignancies[253, 489, 380], endothelia[159], microglia[456], cardiac myocytes[368, 369] and airway smooth muscle in asthma.[381]

The original description of OX40L was however on human CD4+ T cells following HTLV infec-

tion of human CD4+ T cells.[420, 282] This was later identified as OX40L.[19, 407] Subsequently, there have been reports of OX40L expression on both human[211, 210] and murine T cells[197, 278, 393] without viral infection, including marked expression on gut-derived CD8+ intraepithelial lymphocytes.[452]

On antigen-presenting cells, OX40L is minimally expressed on quiescent cells, however expression is upregulated by activation.[352] In addition, the cytokines IL-18, TNFα, and TSLP are all reported to upregulate OX40L, as is signalling through CD40.[313, 97, 148, 164, 270] On neonatal CD4+CD3- lymphoid tissue inducer cells, TL1a induces increased expression of OX40L.[198]

In contrast to the situation for OX40, factors controlling expression of OX40L on T cells are poorly described. However, similar to OX40, T-cell receptor ligation is required for expression.[452] Observations from several groups working on both murine and human T cells have revealed other factors that appear to influence expression, and these are summarised as Table 1.3. The function of OX40L expression on T cells is uncertain, although one analysis suggested that a reduction in T cell:T cell interaction reduced proliferation and reduced survival both *in vitro* and *in vivo*.[393] Much other work in this area has involved animals completely deficient in OX40L in all cells or over-expressing OX40L more widely and so effects cannot be attributed to T cells alone.[294, 295]

During experiments using spleen cell preparations from CTLA4 deficient mice, variable expression of CD4+ T cell OX40L between mice lacking CTLA4 and their CTLA4-sufficient littermates was noted. This suggested a role for the CD80/CD86/CD28/CTLA4 axis in the expression of OX40L on CD4+ T cells, something which has not previously been explored but which complements observations regarding a role of CD28 in T-cell expression of OX40.

The following series of experiments sought to examine factors affecting the expression of OX40L on CD4+ T cells and to consider the physiological role of OX40L expression. These assessments were made in relation to factors already known to affect OX40 expression, factors known to affect OX40L

expression on non-T cells, and with specific reference to the CD80/CD86-CD28-CTLA4 axis which has a major role in OX40 regulation.

7.2 Results

7.2.1 Signals through the T-cell receptor and CD28 control OX40L expression by CD4+ T cells

During experiments assessing splenic and liver-infiltrating lymphocytes in CTLA4$^{-/-}$ mice and their wildtype littermate controls, cultures of lymphocytes were prepared and examined for OX40 and OX40L expression (Figure 7.1). Initially, this occurred during efforts to investigate variations in other lymphocyte markers. It was noticed that OX40L is typically expressed on a minority of CD4+ T cells from wildtype mice after stimulation with soluble agonistic anti-CD3 antibody (Figure 7.1A). In contrast, expression of OX40L on CD4+ T cells from CTLA4$^{-/-}$ animals was noted to be consistently reduced compared with wildtype littermate controls. Interestingly, there was reciprocal expression of OX40L with markers of cell activation including OX40 (Figure 7.1B), ICOS (Figure 7.1C), CD44 (Figure 7.1D), CD25 (Figure 7.2E), and CD69 (Figure 7.2F). These findings were consistent across multiple animals with the proportion of CD4+ T cells expressing OX40 3-4× higher in CTLA4$^{-/-}$ animals and OX40L 3-4× higher in wildtype animals (Figure 7.2G).

Given that a major function of CTLA4 is to negatively regulate signalling through CD28, we proceeded to investigate the effects of manipulating CD28 signals. Stimulation through CD28 was manipulated using either an agonistic antibody or by blocking endogenous ligands by adding CTLA4Ig to cultures. Others have demonstrated that widely available human pharmaceutical CTLA4Ig is effective in binding murine CD80 and CD86.[152] Using this model, it became clear that increased signalling through CD28 is associated with decreased expression of OX40L on CD4+ T cells. Preventing signalling through CD28 by endogenous ligands with CTLA4Ig augmented OX40L expression (Figure 7.3A); this finding was consistent across multiple wildtype mice (Figure 7.3B).

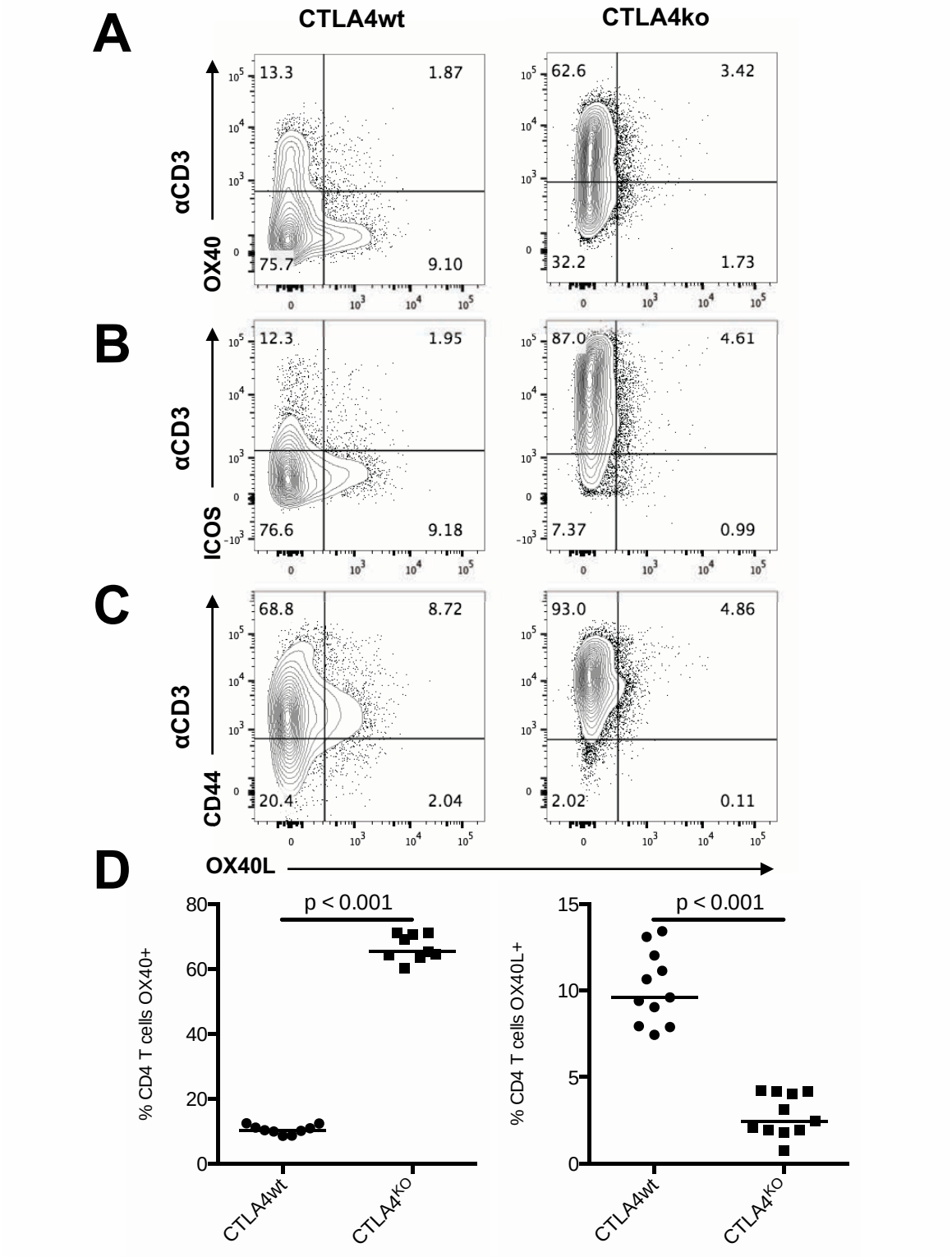


Figure 7.1: OX40L and activation markers are reciprocally expressed on CD4+ T cells. Continues into Figure 7.2

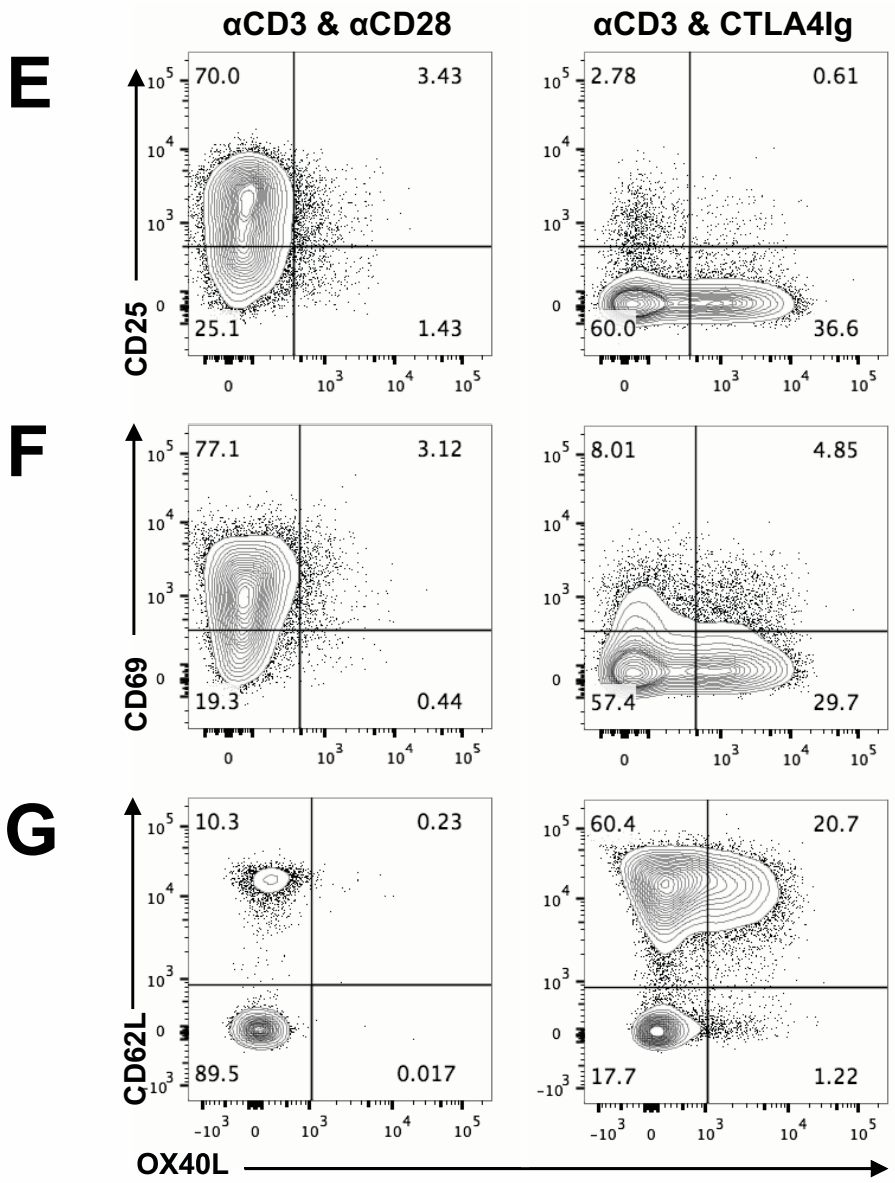


Figure 7.2: OX40L and activation markers are reciprocally expressed on CD4+ T cells. When CTLA4$^{+/+}$ and CTLA4$^{-/-}$ cells were stimulated in the presence of anti-CD3 antibody, there was reciprocal expression of OX40 and OX40L (**A**), of ICOS and OX40L (**B**), and CD44 and OX40L (**C**). These findings were consistent over multiple experiments (**D**). The same pattern was seen for CD25 and OX40L (**E**) expression and CD69 and OX40L expression (**F**) on wildtype cells under different conditions. (**G**) Conversely, loss of CD62L, which is associated with activation related to loss of OX40L expression (All plots are representative of >8 experiments and gated on live CD4 T cells)

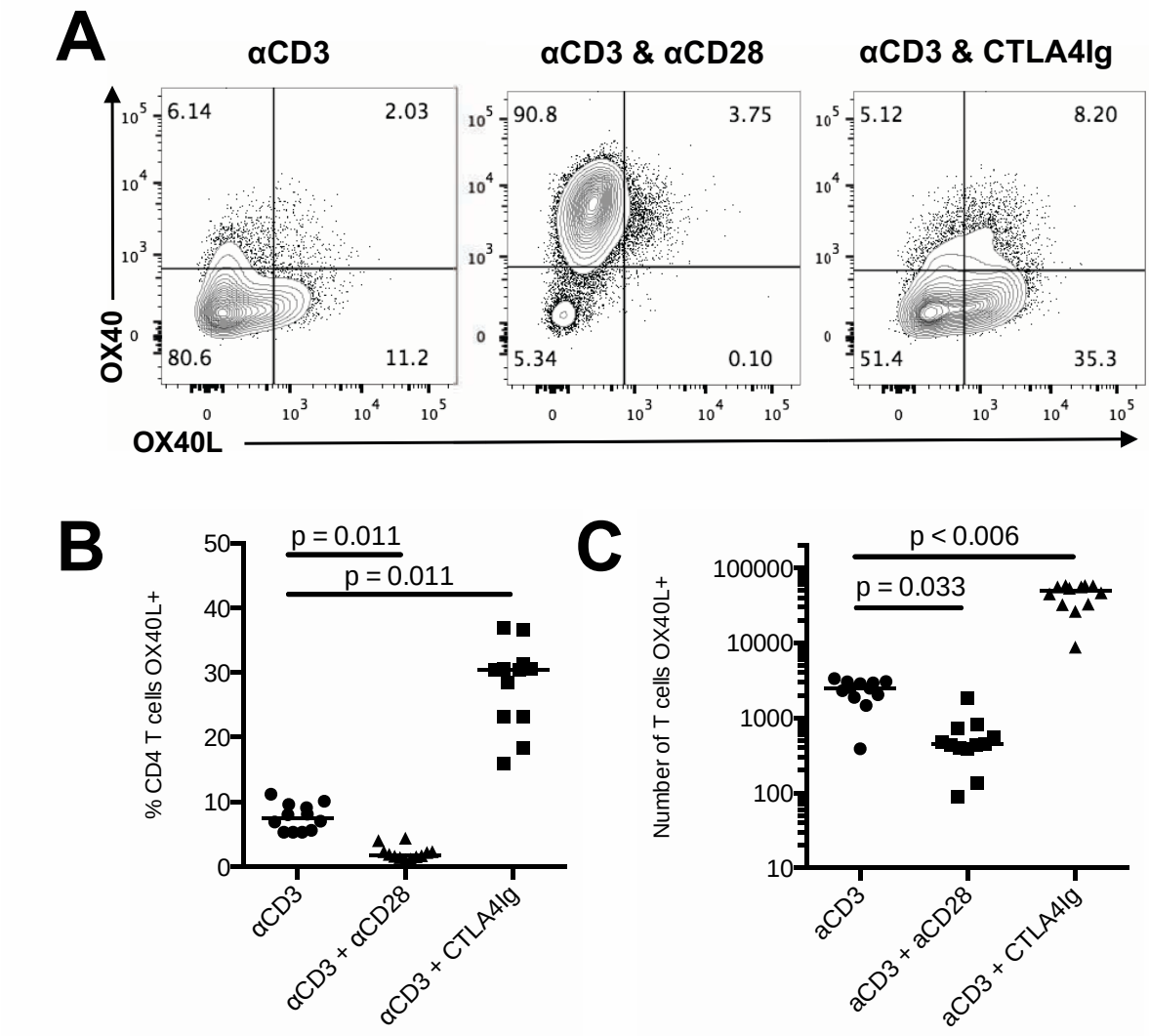


Figure 7.3: The expression of OX40L on CD4+ T cells is negatively regulated by CD28 ligation. (**A**) When total wildtype splenocytes are stimulated in culture with agonistic anti-CD3 antibody, there is reciprocal expression of OX40 and OX40L; if the same culture is repeated with anti-CD3 and anti-CD28, there is increased expression of OX40 and OX40L expression is suppressed; if the culture is repeated with CD28 signalling prevented by CTLA4Ig, OX40 expression is suppressed and OX40L expression increased (plots representative of >10 experiments; gated on CD4+ T cells). (**B**) The findings of CD28 signalling negatively regulating CD4+ T cell OX40L expression are consistent. (**C**) Findings are not due to altered cell proliferation or survival because absolute cell counts demonstrate the same pattern. Kruskal-Wallis test with Dunn's post-hoc test; n=12v12v12.

One potential explanation for the variation in the proportion of CD4+ T cells expressing OX40L is that differential proliferation of subpopulations might alter the proportion remaining positive. To address this possibility, counting beads were used to quantify the number of cells expressing positive for OX40L from multiple cultures from the same stock cell suspension. Again, there was a decrease with CD28 ligation and an increase with the addition of CTLA4Ig (Figure 7.3C).

Having demonstrated that the addition of CTLA4Ig to cultures greatly increased staining for OX40L but accepting that the quality of flow cytometry staining for OX40L in the literature is variable, attempts were made to confirm the specificity of the staining seen in these experiments. First, splenocytes from mice with expressing Cre-recombinase under the control of the *VAV1* promoter, which is transcribed in all haematopoeitic cells including T cells, with and without Lox sequence flanked OX40L genes were isolated. These CD45.1- cells were then cultured with anti-CD3 and CTLA4Ig. CD45.1+ wildtype cells were added to cultures to act as internal controls for OX40L expression. Consistent with results from commercial genotyping, those mice that were OX40L$^{(}$fl) did not have detectable OX40L signal by flow cytometry (Figure 7.4A-B).

To further investigate the role of ligation of CD28 in controlling expression on OX40L on CD4+ T cells, cells isolated from mice deficient in CD28's two endogenous ligands, CD80 and CD86, were used. In cultures of CD80^{KO}CD86^{KO}CTLA4^{KO} splenocytes, CD4+ T cells upregulated OX40L in a manner similar to that seen in wildtype cells stimulated in the presence of CTLA4Ig. The addition of CTLA4Ig to CD80^{KO}CD86^{KO}CTLA4^{KO} did not further increase expression of OX40L, whilst the addition of anti-CD28 agonistic antibody prevented significant expression (Figure 7.5A).

By combining splenocytes from CD45.1 allotype-marked wildtype mice and CD45.2 allotype-marked CD80^{KO}CD86^{KO} mice, the requirement for the addition of CTLA4Ig to induce strong OX40L expression on both cell types was restored (Figure 7.5B). This suggested that interaction with CD80 and CD86 on CD45.1+ cells was sufficient to down-regulate OX40L expression on CD45.2+ CD80^{KO}CD86^{KO} cells i.e. down-regulation could occur *in trans*.

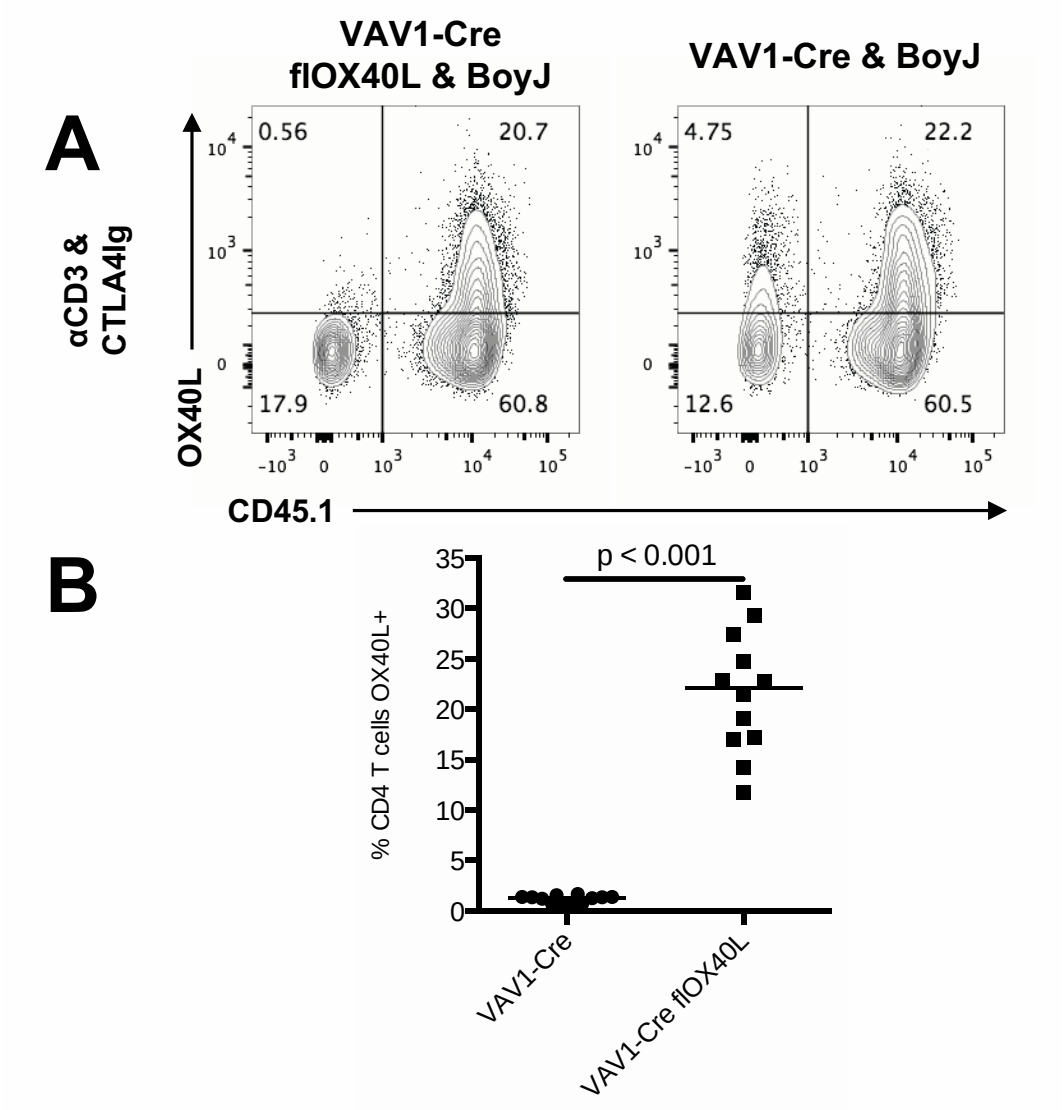


Figure 7.4: Flow cytometric staining of OX40L staining is consistent with genotype. (**A**) Culture of cells from VAV1-Cre flOX40L mice with anti-CD3 and CTLA4Ig demonstrated consistent absence of OX40L expression in mice genotyped as VAV-Cre flOX40L; co-culture with wildtype CD45.1-positive BoyJ cells provided a positive control. (**B**) This was a consistent finding. Comparisons with Mann-Whitney U test; n=12v12.

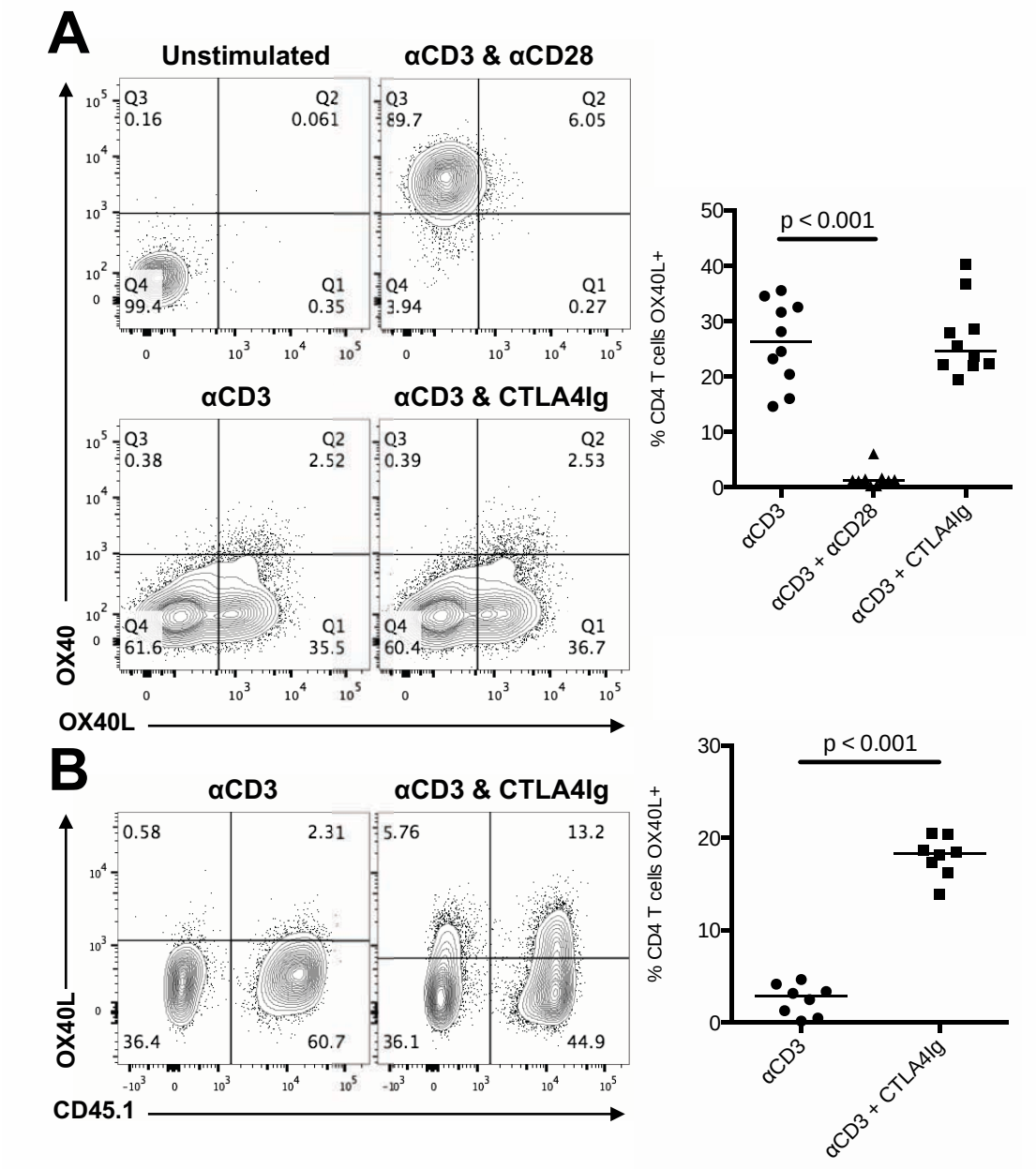


Figure 7.5: CD80 and CD86 negatively regulate OX40L through interaction with CD28. (**A**) Unstimulated, $CD80^{KO}CD86^{KO}$ cells do not express OX40L. When stimulated with anti-CD3 and anti-CD28, these cells behave as wildtype cells and upregulate OX40 without OX40L. However, in contrast to wildtype cells there is a marked upregulation of OX40L in response to anti-CD3 alone that is not augmented by the addition of CTLA4Ig. (**B**) When co-cultured with wildtype CD45.1+ cells, $CD80^{KO}CD86^{KO}$ CD4+ T cells have their anti-CD3-induced expression of OX40L prevented by the presence of wildtype cells; this can be prevented by the addition of CTLA4Ig (All plots gated on live CD4+ T cells). Comparisons with Mann-Whitney U test; n=10v10v10 and 8v8.

Having observed that manipulating the strength of CD28 signals could alter the expression of OX40L on CD4+ T cells in the context of agonistic signalling through the TCR, it was not clear whether TCR signalling was necessary for OX40L expression or whether it was simply a negative response to CD28. To investigate this possibility, cells were stimulated with combinations of CD3 and CTLA4Ig. CTLA4Ig alone was inadequate to cause expression of OX40L and unstimulated cells expressed minimal OX40L (Figure 7.6A).

To establish whether the requirement for agonistic CD3 antibody was specific to that antibody or a more general function of a requirement for activation via the TCR(Figure 7.7A), two other methods of TCR ligation were investigated. First, cells from RAG-OT2 mice, in which all CD4+ T cells express a TCR specific to a sequence in ovalbumin, were stimulated in the presence of CTLA4Ig with the OTII-specific ovalbumin peptide ISQAVHAAHAEINEAGR. Consistent with results from stimulation with agonistic anti-CD3, OX40L was upregulated (Figure 7.7B). Second, similar results were obtained when the lectin Con A, which non-specifically cross-links and activates TCRs, was used to stimulate wildtype cells (Figure 7.7C).

It has been noted that OX40L expression on CD4 T cells is inversely related to the strength of TCR signal[278]; others have previously considered the fact that CD28 signalling strength can be related to TCR signal strength.[3] Combining these two observations, cells were cultured with varying concentrations of agonistic anti-CD3 antibody alone or in the presence of CTLA4Ig to block variations in CD28 signalling. Consistent with the work of others, the strength of CD3 stimulation was inversely correlated to OX40L expression (Figure 7.8A). However, in the presence of CTLA4Ig, this relationship was not apparent and more OX40L was expressed with 0.1 $\mu g\,ml^{-1}$ and 1 $\mu g\,ml^{-1}$ of anti-CD3 than with 0.01 $\mu g\,ml^{-1}$ (Figure 7.8B). A similar reversal in the effect of strength of ligation on OX40L was apparent in the presence of a neutralising antibody to IL-2.

Next, to determine whether our results were related to movement of OX40L between intra- and extracellular compartments, we repeated flow cytometry with and without fixation and permeabil-

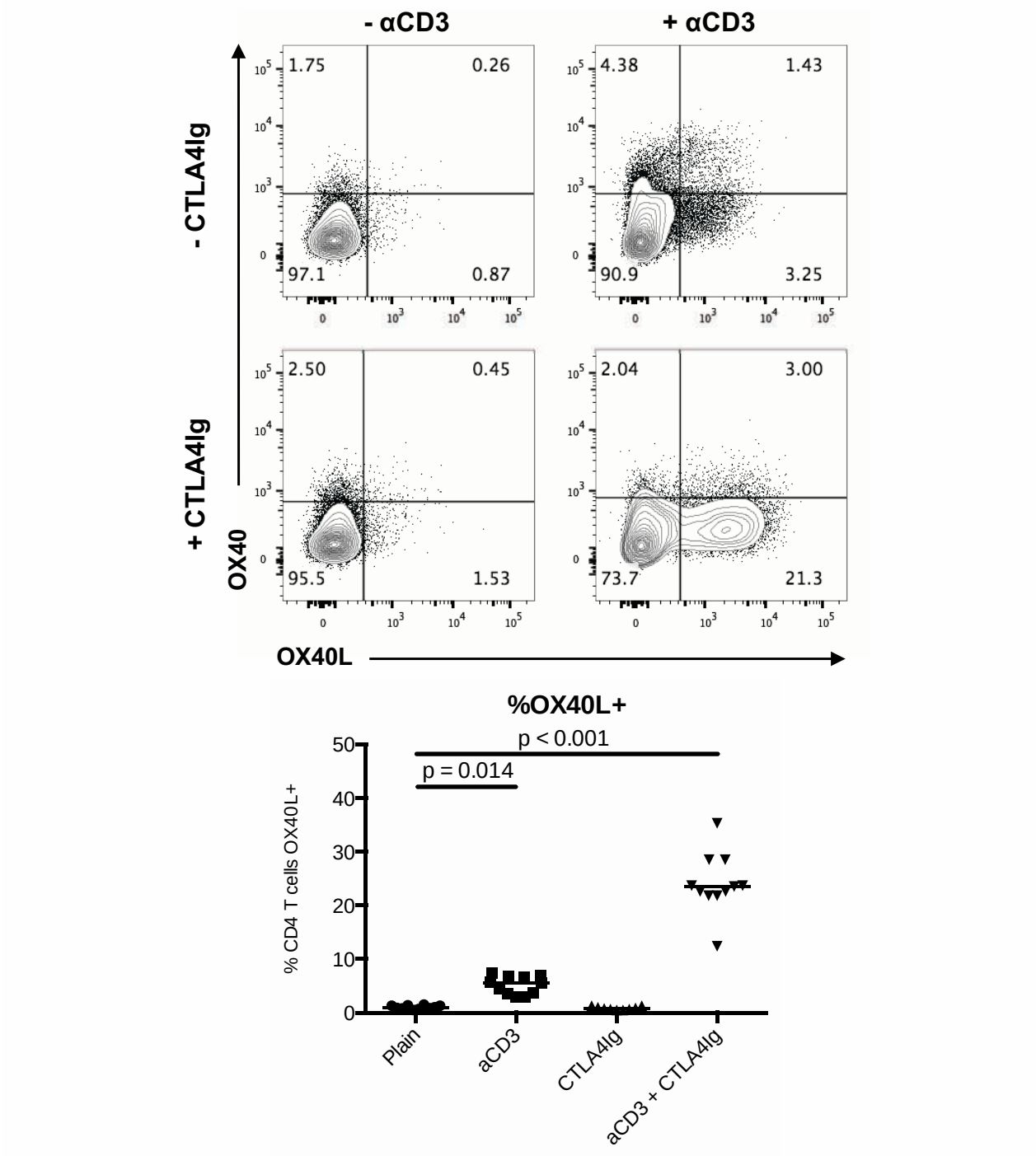


Figure 7.6: Signals through the T cell receptor are required for OX40L expression. Total splenocyte preparations from wildtype mice were cultured in with and without anti-CD3 antibody and with and without CTLA4Ig. Without anti-CD3 there was minimal expression of OX40L with and without CTLA4Ig. However, after the addition of anti-CD3 antibody, OX40L expression was increased; this increase was augmented by the addition of CTLA4Ig (All plots gated on CD4+ T cells). Kruskal-Wallis test with Dunn's post-hoc test; n=12 per group.

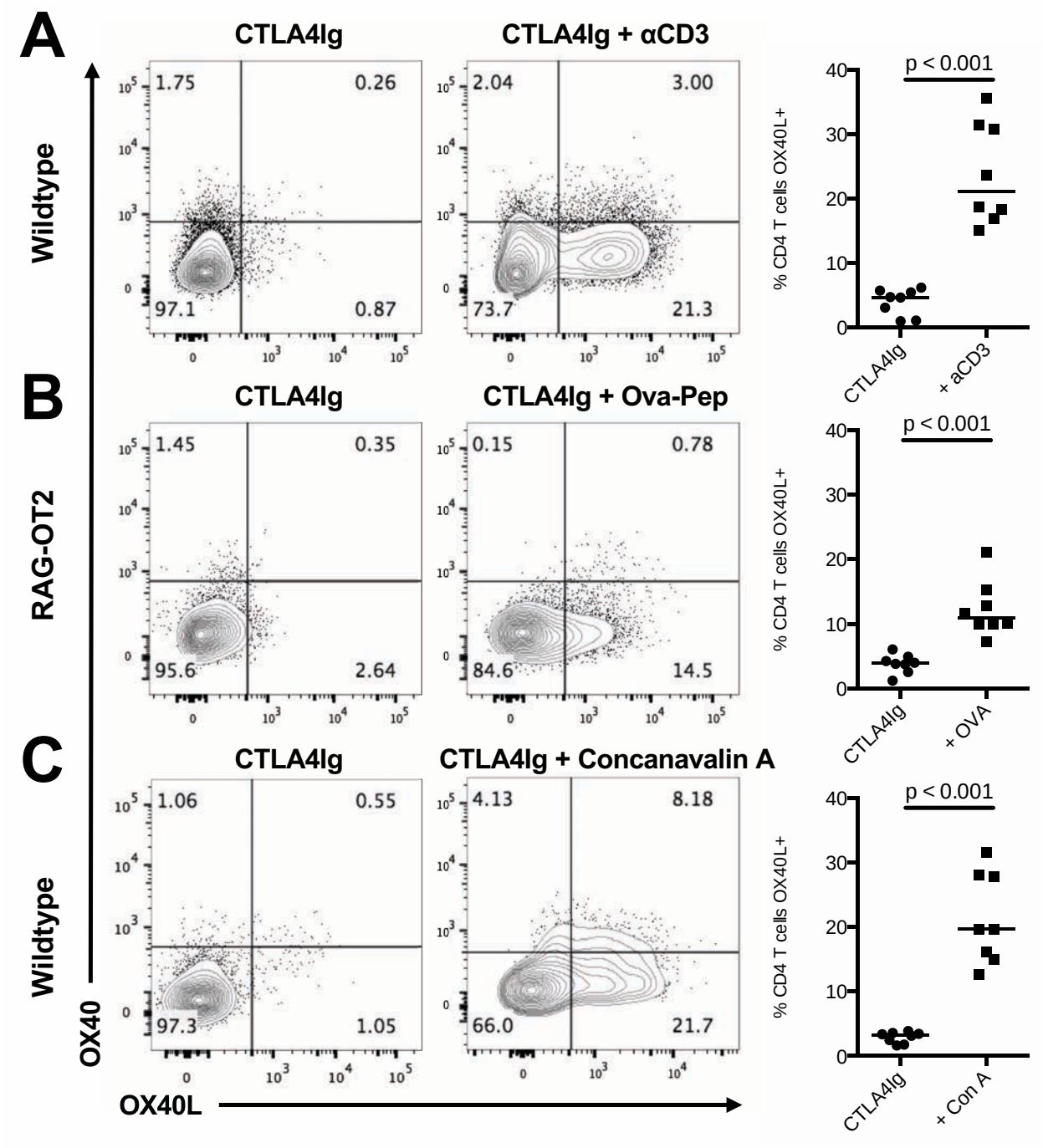


Figure 7.7: Various methods of ligating the T cell receptor induce OX40L expression. (**A**) As demonstrated previously, culturing wildtype cells in the presence of CTLA4Ig alone resulted in no or low expression of OX40L but the addition of anti-CD3 antibody resulted in an increase in OX40L expression. (**B**) Similarly, cultures of RAG-OT2 cells expressing a T cell receptor specific to an ovalbumin peptide expressed minimal OX40L when cultured with CTLA4Ig alone but increased expression when ovalbumin peptide was added. (**C**) Similarly, the lectin concanavalin A was a sufficient to provoke CD4+ T cell expression of OX40L. Comparisons with Mann-Whitney U test; n=8 per group.

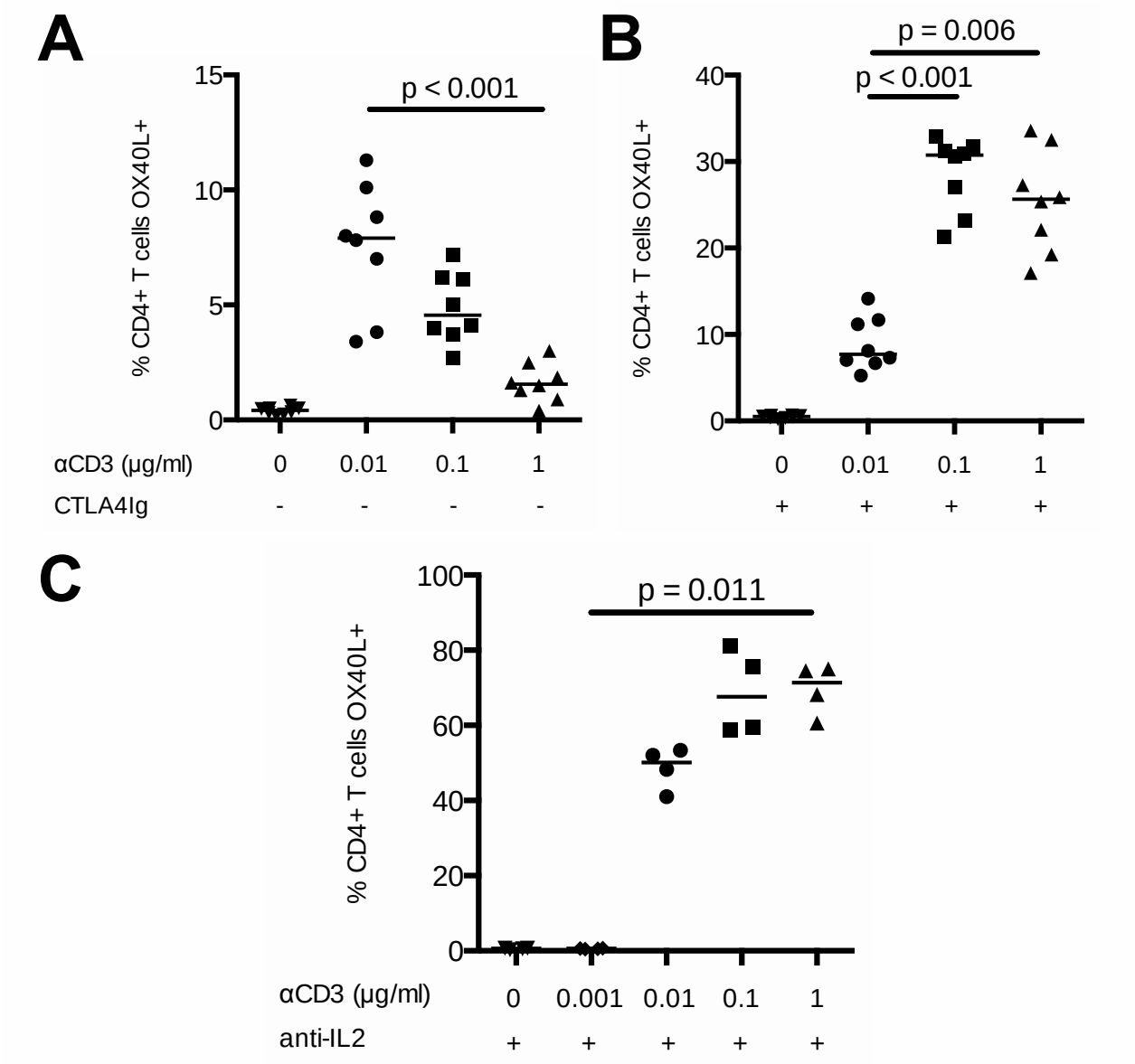


Figure 7.8: Signal strength through the TCR affects OX40L expression. (**A**) Wildtype total splenocytes were cultured in the presence of different concentrations of agonistic anti-CD3 antibody. There was minimal expression without agonistic antibody but a reverse relationship between the concentration of antibody and expression (n = 8 per condition; nptrend test across three samples with anti-CD3). (**B**) The experiment above was repeated with the addition of CTLA4Ig. Here there was a greater expression of OX40L with concentrations of anti-CD3 above 0.01 μg/ml but no difference between 0.1 μg ml^{-1} and 1 μg ml^{-1}. (n=8 per condition; Kruskal-Wallis tests with Dunn's post-hoc analysis across three samples with anti-CD3). (**C**) A further repeat with blocking anti-IL-2 antibody in place of CTLA4Ig was performed. Here, greater concentrations of anti-CD3 were associated with greater expression of OX40L (n=4 per condition; nptrend across all samples treated with anti-CD3).

isation prior to application of anti-OX40 and anti-OX40L antibodies. Notably, detection of CTLA4 is greatly increased by intracellular staining.[243] No difference in OX40L signal was apparent, but fixation and permeabilisation reduced the signal for OX40 (Figure 7.9).

7.2.2 CTLA4 controls OX40L expression as a negative regulator of CD28 signalling

Having demonstrated that deficiency of CTLA4 was associated with an absence of expression of OX40L on CD4+ T cells, it remained uncertain as to whether this effect was through uncontrolled signalling through CD28 or a mechanism directly mediated by CTLA4 itself. Possibilities include signalling through a ligand of CTLA4 induced by CTLA4-binding or by signalling through CTLA4 itself as is reported to be involved in other T cell pathways.[414] To further investigate this, $CTLA4^{-/-}$ cells were cultured in the presence of anti-CD3 and CTLA4Ig. In contrast to the effect of CTLA4Ig on wildtype cells, the addition of CTLA4Ig to cultures of $CTLA4^{-/-}$ cells did not induce OX40L (Figure 7.10). $CTLA4^{-/-}$ cells were then cultured alone or mixed with CD45.1 marked $CTLA4^{+/+}$ cells as a source of CTLA4. When cultured alone, wildtype cells expressed OX40L and $CTLA4^{-/-}$ cells did not express OX40L consistent with previous experiments. However, on mixing the two cell types together, the ability of $CTLA4^{+/+}$ cells to express OX40L was lost (Figure 7.11). This suggested that factors other than a cell's ability to express CTLA4 or signal through CTLA4 were controlling OX40L expression on $CTLA4^{+/+}$ cells.

To further investigate the possibility of a role of signalling through CTLA4, an agonistic anti-CTLA4 antibody was added to cultures of wildtype and $CD80^{KO}CD86^{KO}$ cells. The latter cell type was chosen to prevent the activity of endogenous ligands. Here the addition of varying concentrations of anti-CTLA4 antibody made no difference to the OX40L expression of either cell type (Figure 7.12).

Cells gathered from mice deficient in $CTLA4^{-/-}$ typically display an activated phenotype at harvesting and are likely to have already received signalling through CD28. To create mice with $CTLA4^{-/-}$

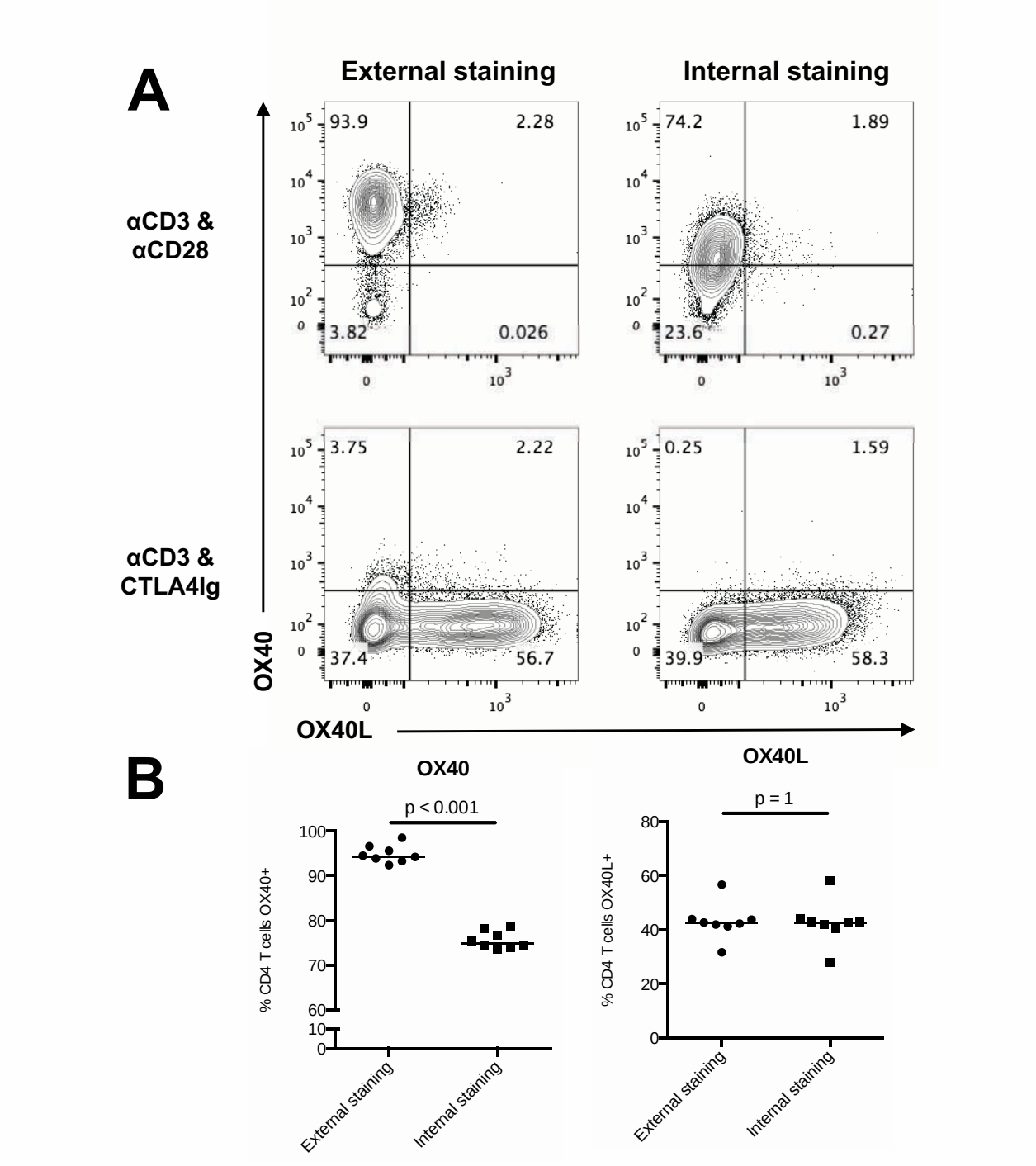


Figure 7.9: Comparison of extra- and intracellular staining for OX40 and OX40L. (**A**) To assess whether intracellular staining would alter signal for OX40 and OX40L, fluorescent antibodies to the two antigens were applied before and after fixation and permeabilisation. There was no apparent difference in staining for OX40L but there was a reduction in the OX40 signal when staining was performed after fixation and permeabilisation. (**B**) The findings in A were consistent across multiple samples (n = 8 vs 8; All plots relate to live CD4+ T cells; Mann-Whitney U-test)

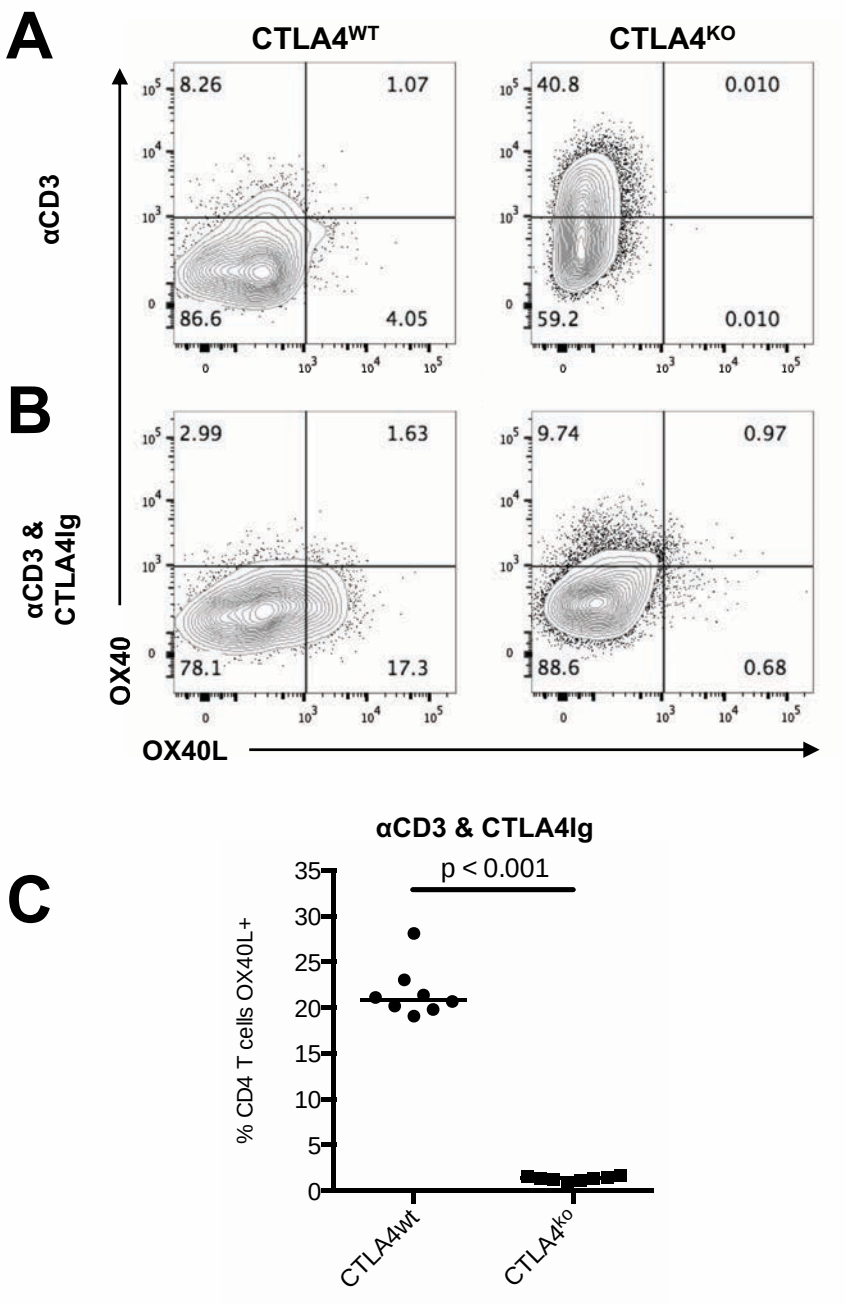


Figure 7.10: CTLA4$^{-/-}$ cells do not express OX40L in the presence of CTLA4Ig. (**A**) Wildtype and CTLA4$^{-/-}$ splenocyte cultures were stimulated with anti-CD3 agonistic antibody. In CTLA4$^{+/+}$ but not CTLA4$^{-/-}$ cultures there was some expression of OX40L. (**B**) With the addition of CTLA4Ig, there was an increase in the expression of OX40L in CTLA4$^{+/+}$ but not CTLA4$^{-/-}$ cells. (**C**) The finding that the addition of CTLA4Ig did not induce expression of OX40L of CTLA4$^{-/-}$ CD4 T cells was reproducible over multiple experiments. (Plots are representative and gated on CD4+ T cells). Comparisons with Mann-Whitney U test; n=8 per group.

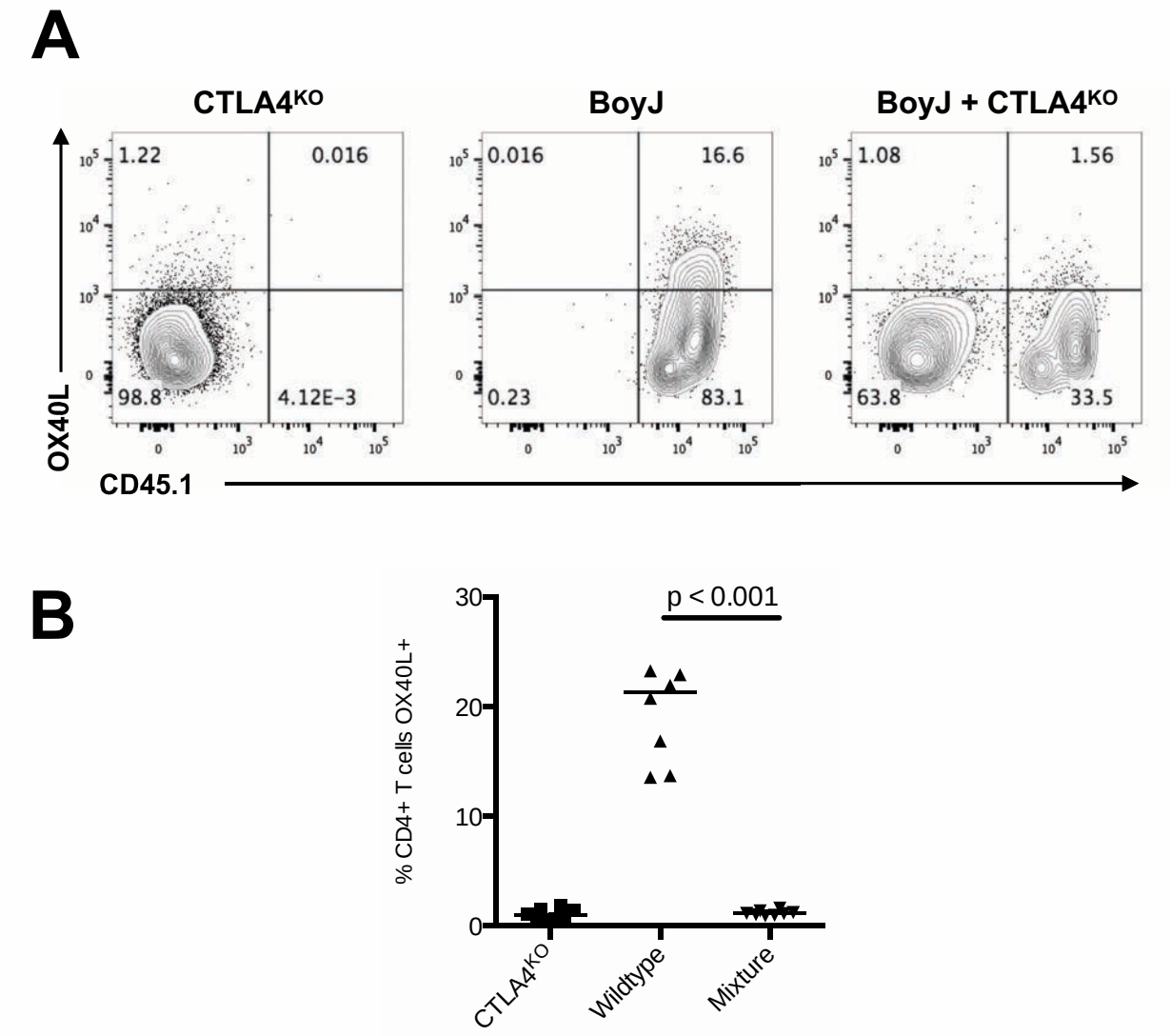


Figure 7.11: CTLA4$^{-/-}$ cells prevent expression of OX40L on wildtype cells. (**A**) As previously, CTLA4$^{-/-}$ deficient splenocyte cultures in the presence of agonistic anti-CD3 antibody and CTLA4Ig did not produce OX40L on T cells whilst this combination produced strong expression of OX40L on wildtype (CD45.1+) cells. Co-culture of the two cells types prevented expression of OX40L on wildtype cells. (**B**) This was a consistent finding (n = 8 per condition; plots are representative and gated on CD4+ T cells). Comparisons with Mann-Whitney U test.

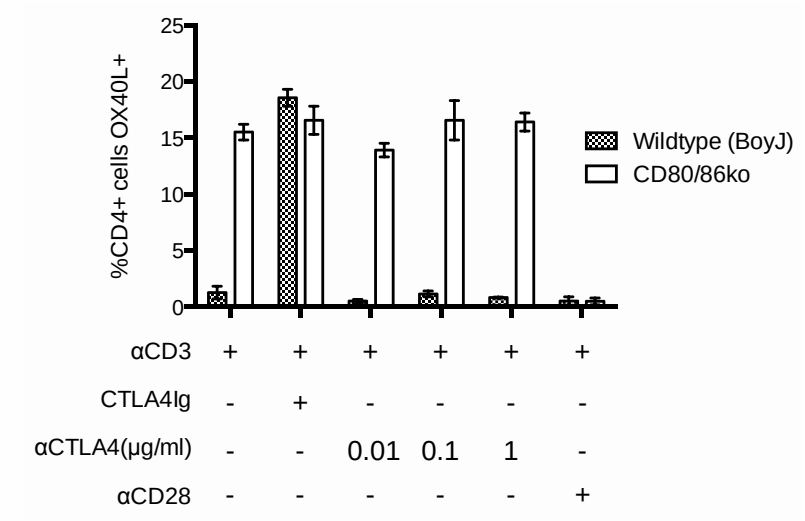


Figure 7.12: An agonistic CTLA4 antibody does not affect OX40L expression. Total splenocytes were isolated from either wildtype mice or mice deficient in CD80 and CD86 and cultured in the presence of agonistic anti-CD3 antibody. As previously, $CD80^{KO}CD86^{KO}$ cells showed upregulation of OX40L that was recapitulated on wildtype cells with the addition of CTLA4. Addition of varying concentrations of agonistic anti-CTLA4 antibody did not alter OX40L expression. As previously, the addition of anti-CD28 decreased OX40L expression (n = 4 per condition; bars shown as median ± range).

cells that were not activated, bone marrow chimeras of $CTLA4^{-/-}$ and CD45.1 marked wildtype cells were created. As described elsewhere, and in contrast to $CTLA4^{-/-}$ mice, which die by 4-5 weeks of age, these mice remained healthy for weeks after chimerisation was confirmed and at sacrifice cells were not abnormally activated.[13] When cells from these mice were cultured, both $CTLA4^{-/-}$ and $CTLA4^{+/+}$ cells behaved as previously: OX40L expression was suppressed by stimulation with anti-CD3 and anti-CD28 whilst OX40L was strongly expressed when stimulated with anti-CD3 in the presence of CTLA4Ig (Figure 7.13). These findings were identical between CTLA4-sufficient (CD45.1+) and –deficient (CD45.2+) cells.

As a second approach to preventing in vivo ligation of CD28 in $CTLA4^{-/-}$ mice, mice deficient in CD80 and CD86 were crossed and then backcrossed with mice haplosufficient for CTLA4 over generations to produce mice triply deficient in CD80, CD86 and CTLA4. The genotype of these mice was confirmed by commercial genotyping. Again as described elsewhere, these mice remained healthy.[261] On stimulation, these triple knockout mouse cells were confirmed to be deficient in

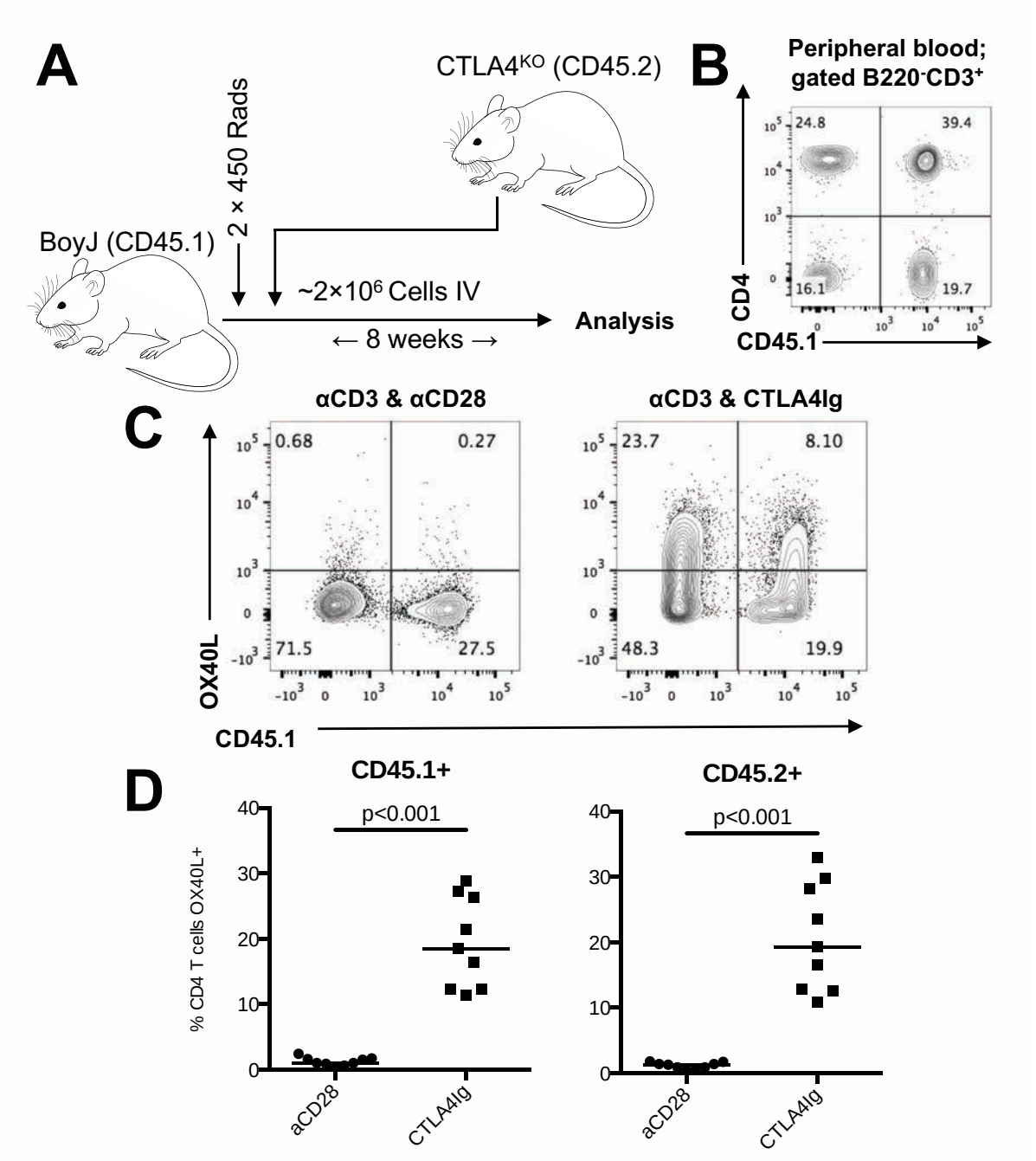


Figure 7.13: Bone marrow chimeras from CTLA4$^{-/-}$ and wildtype mice express OX40L normally. (**A**) Bone marrow chimeras were created by irradiating CD45.1+ wildtype mice and injecting approximately 2 000 000 CTLA4$^{-/-}$ cells intravenously. (**B**) Chimerism was confirmed on peripheral lymphocytes several weeks after irradiation (representative plot gated on live T cells). (**C**) Cell preparations produced from chimeric mice demonstrated suppressed OX40L expression when cultured with anti-CD3 and anti-CD28 and increased OX40L expression when cultured with CTLA4Ig. (**D**) These findings were consistent across samples (9 cell preparations from 3 mice in each group). This experiment performed with the assistance of Dr F Gaspal. Comparisons with Mann-Whitney U test.

CTLA4 (Figure 7.14A) and demonstrated equal CD4+ T cell expression of OX40L after stimulation with anti-CD3 as compared to both $CD80^{KO}CD86^{KO}CTLA4^{WT}$ and $CD80^{KO}CD86^{KO}CTLA4^{KO}$ littermates (Figure 7.14B).

7.2.3 CD4+ T cells require direct interaction with non-T cells to upregulate OX40L

During the course of the experiments described here, cultures of pure CD4+ T cells were generated (typically to >95% purity); Figure 7.15A). It was observed that these cells did not express OX40L after culture with anti-CD3 and CTLA4Ig (Figure 7.15B). It was considered that a soluble factor from non CD4+ T cells might be necessary to drive expression of OX40L. To test this, purified CD4+ T cells were cultured in wells across 0.4μm-pore Transwell® membrane with a total splenocyte preparation on the other side of the membrane thus permitting permeation of soluble molecules but not cells. Here, purified CD4+ T cells did not express OX40L whilst total cells did (Figure 7.15C). However, when CD45.2+ purified CD4+ T cells were added to cultures of CD45.1+ total splenocytes, OX40L expression was again seen on CD45.2+ cells suggesting that direct contact with non CD4+ T cells was necessary for expression (Figure 7.15D-E).

It was not clear as to with which cell type direct contact was required to promote the expression of OX40L. In order to investigate this, mixed cultures of purified CD4+ T cells and purified B220+ T cells were prepared. Here B220+ preparations were made to over 95% purity by magnetic bead purification (Figure 7.16A). The addition of B220+ cells to pure CD4+ T cell cultures stimulated with anti-CD3 and CTLA4Ig restored OX40L expression on T cells (Figure 7.16B). Because CD45 allotype marked B220 and CD4+ T cells were used, it was possible to demonstrate that there was no significant T cell contamination (Figure 7.16C) and that expressed OX40L was all on cells derived from T cell donor mice (Figure 7.16D).

The experimental procedure above was then repeated with the addition of bead-purified CD11b+ cells. Here CD11b preparations were less pure than B220+ preparations with a typical purity of 70-

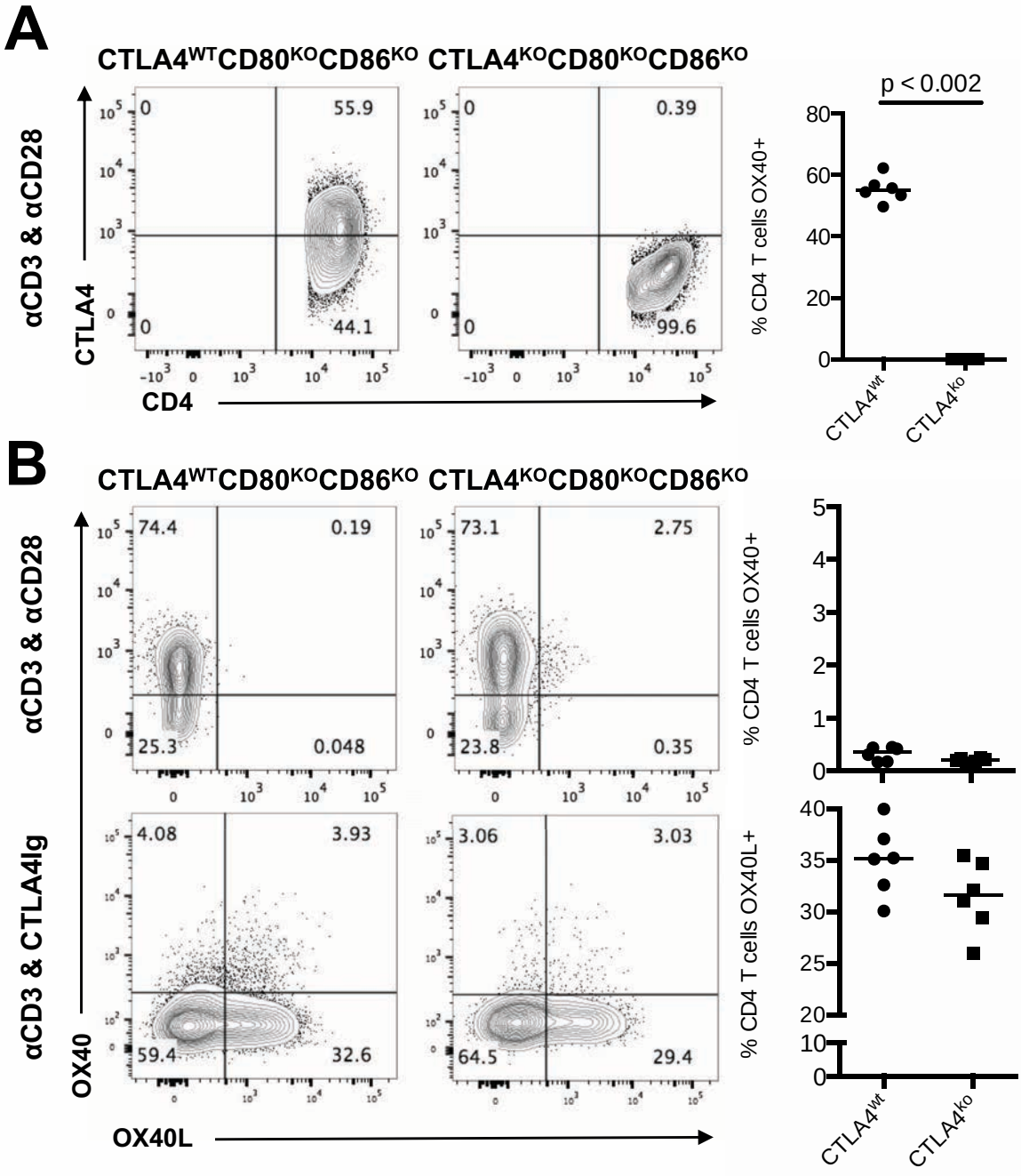


Figure 7.14: CD80^{KO}CD86^{KO}CTLA4^{KO} mice express OX40L normally. (**A**) Mice co-deficient for each of CD80, CD86 and CTLA4 were bred. Cells were compared with those from CTLA4$^{+/+}$ littermates after stimulation with anti-CD3 and anti-CD28 antibodies to confirm deficiency of CTLA4. Findings were consistent with those from commercial genotyping. (**B**) There was no difference in OX40L expression between CD80^{KO}CD86^{KO}CTLA4^{KO} and CD80^{KO}CD86^{KO}CTLA4^{WT} cells: both similarly upregulated OX40L after culture with CTLA4Ig. (All plots relate to live CD4+ T cells). Comparisons with Mann-Whitney U test; n=6v6.

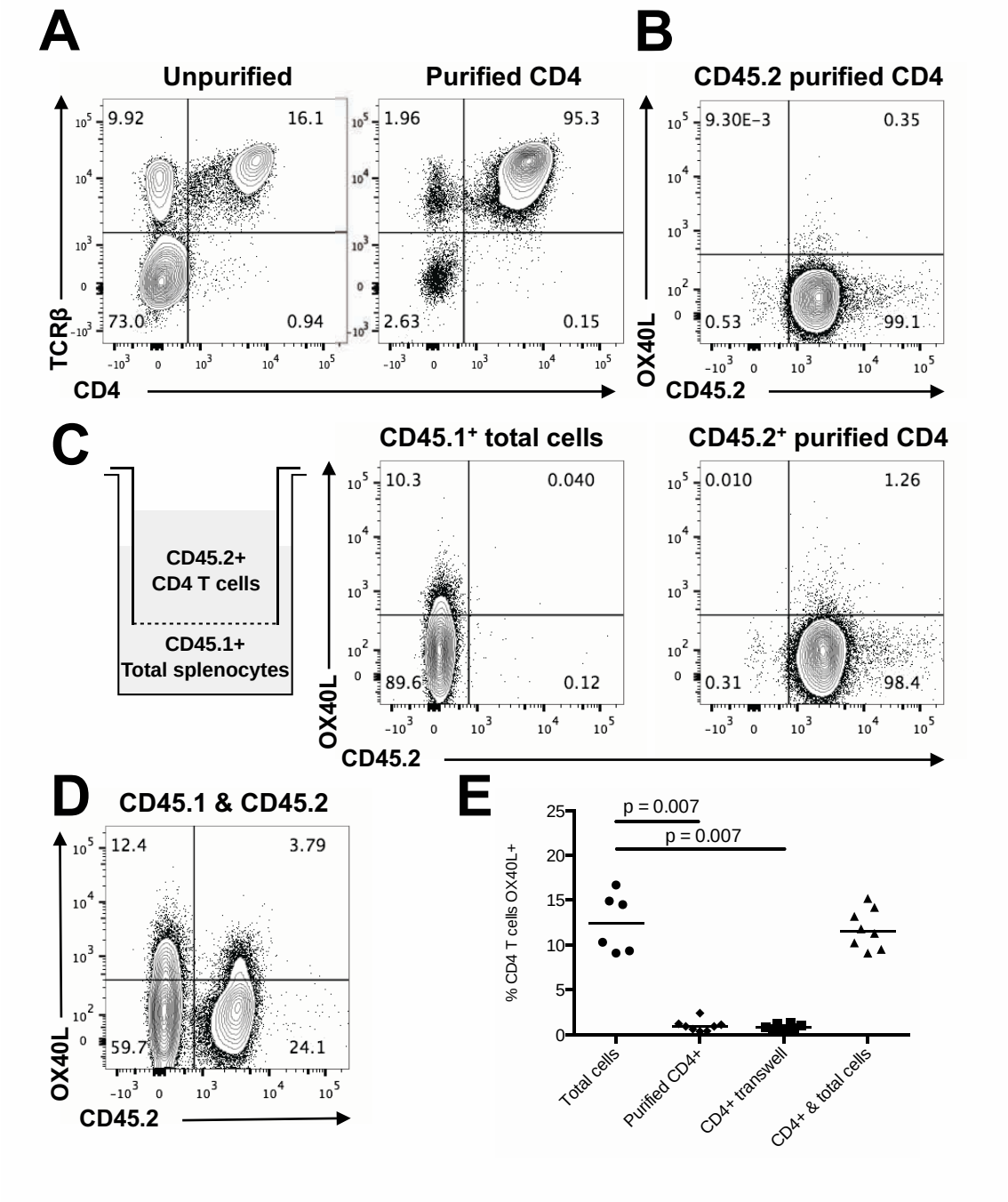


Figure 7.15: CD4+ T cells require direct interactions with APCs to express OX40L. (**A**) CD4+ T cells were purified to $\geq$95% purity (representative plot). (**B**) When cultured with anti-CD3 and CTLA4Ig, purified CD4 T cells did not upregulate OX40L. (**C**) Similarly, purified CD4 T cells did not express OX40L when cultured across a transwell membrane from a total cell population that did express OX40L. (**D**) However, when purified CD45.1+ CD4s were mixed with a total CD45.2+ splenocyte population, they regained the ability to express OX40L. (**E**) These findings were consistent between multiple cultures; n=6v8v8v8.

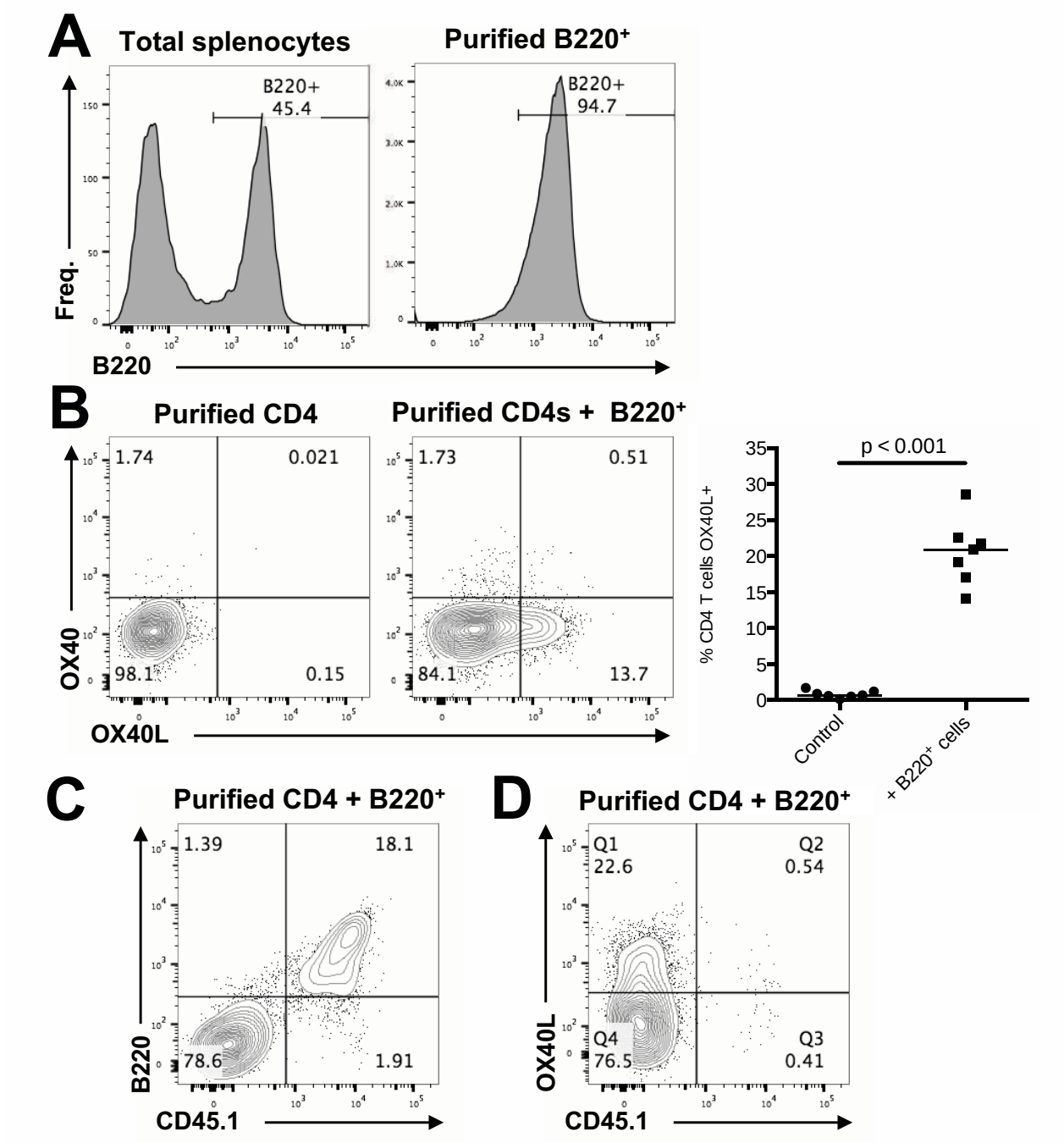


Figure 7.16: B220+ B cells are sufficient to allow CD4+ T cells to express OX40L. (**A**) CD45.1+ wildtype mouse splenocytes were enriched to approximately 95% purity using anti-B220+ antibodies and magnetic beads. (**B**) Purified CD4+ T cells from wildtype CD45.2+ animals were cultured with and without enriched B220+ cells for 72 hours in the presence of anti-CD3 and CTLA4Ig.. There was significantly increased expression of OX40L on CD4+ T cells in the presence of B220+ cells. (n = 7 per sample; Mann-Whitney U-test). (**C**) Representative plot gated on live cells showing that B220+ cells were CD45.1+. (**D**) Representative plot gated on CD4+ T cells showing that OX40L expression was on CD45.1 negative (CD45.2+) cells.

75% (Figure 7.17A). However, again CD4 T cell expression of OX40L was restored by co-culture with CD11b+ cells (Figure 7.17B). In these experiments, some contaminating CD45.2+ non-CD11b+ cells could be seen (Figure 7.17C-D).

Given that both B cells and many CD11b positive cells directly interact with T cells through the CD40-CD40L pathway, total splenocyte cultures were repeated in the presence of agonistic anti-CD3 with either CTLA4Ig or IL-2. These cultures were performed with or without blocking antibodies to CD40 or CD40L (Figure 7.18).

7.2.4 OX40-OX40L interactions do not modulate CD4 T cell OX40L expression

Having demonstrated reciprocal expression of OX40 and OX40L on CD4+ T cells, I considered whether an interaction between the two might either affect OX40L expression or affect its detection by flow cytometry. To investigate this possibility, I used splenocytes from $OX40^{KO}$ mice as a source of CD4+ T cells. To confirm that these cells were deficient in OX40, they were stimulated in the presence of agonistic anti-CD28 antibody. Here, CD4+ T cell expression of the molecule ICOS, which is upregulated in response to CD28 co-stimulation and reciprocally expressed with OX40L, was similar between control and $OX40^{KO}$ cells (Figure 7.19A). However, a complete lack of expression of OX40 in response to CD28 ligation was demonstrated on $OX40^{KO}$ cells as expected (Figure 7.19B). When $OX40^{KO}$ CD4+ T cells were cultured with anti-CD3 antibody alone, a small amount of OX40L was detectable. However, when CTLA4Ig was added to block endogenous signalling through CD28, there was a significant increase in OX40L expression consistent with findings on wildtype cells. This increase could be prevented by the addition of agonistic anti-CD28 antibody (Figure 7.19C).

It has been suggested for human CD4+ T cells that OX40 may be able to transendocytose or 'rip' OX40L from non-T-cells to T cells in a manner analogous to that described for interactions between CD28 and CTLA4.[346, 39] To further investigate the relevance of ripping of ligand to mouse CD4

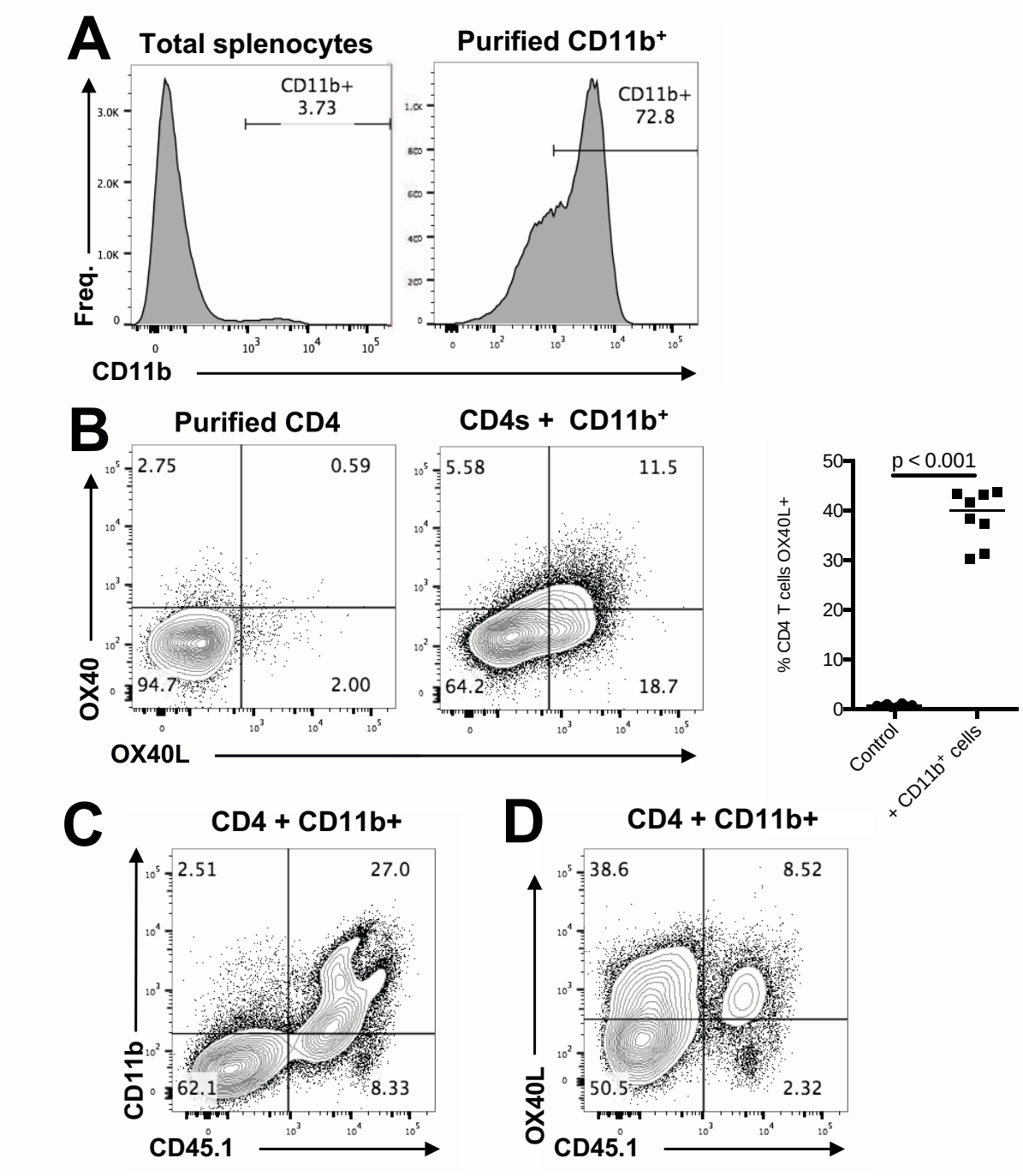


Figure 7.17: CD11b+ cells are sufficient to allow CD4+ T cells to express OX40L. (**A**) CD45.1+ wildtype mouse splenocytes were enriched to approximately 75% purity using anti-CD11b+ antibodies and magnetic beads. (**B**) Purified CD4+ T cells from wildtype CD45.2+ animals were cultured with and without enriched CD11b+ cells for 72 hours in the presence of anti-CD3 and CTLA4Ig.. There was significantly increased expression of OX40L on CD4+ T cells in the presence of CD11b+ cells. (n = 8 per sample; Mann-Whitney U-test). (**C**) Representative plot gated on live cells showing that CD11b+ cells were CD45.1+. (**D**) Representative plot gated on CD4+ T cells showing that OX40L expression was primarily present on CD45.1 negative (CD45.2+) cells.

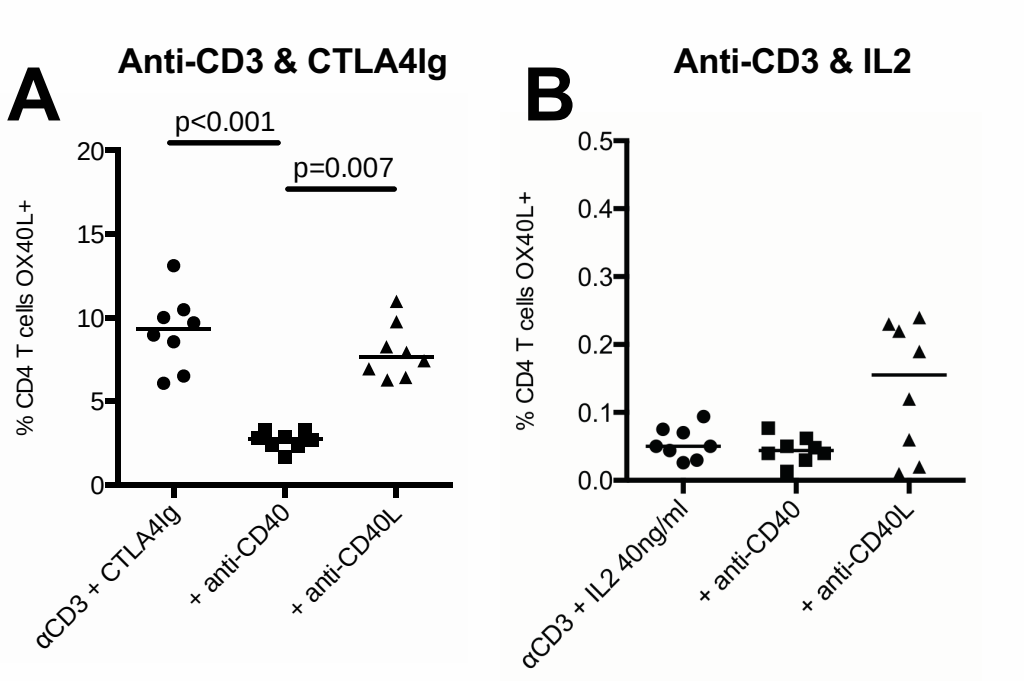


Figure 7.18: CD40 and CD40L ligand blockade and OX40L expression. Total wildtype splenocytes were stimulated with agonistic anti-CD3 antibody and CTLA4Ig and with either anti-CD40 antibody or anti-CD40L antibody (**A**) or with anti-CD3 and IL-2 40ng/ml (**B**) with either anti-CD40 antibody or anti-CD40L antibody. In the presence of anti-CD3 and CTLA4Ig, the addition of anti-CD40 antibody significantly decreased OX40L expression, whilst it was not significantly changed by the addition of anti-CD40L. No significant changes were seen in the presence of IL-2 where OX40L expression was not seen (p=0.191 across conditions). Comparisons with Kruskal-Wallis test and Dunn's post-hoc test; n=8 per group.

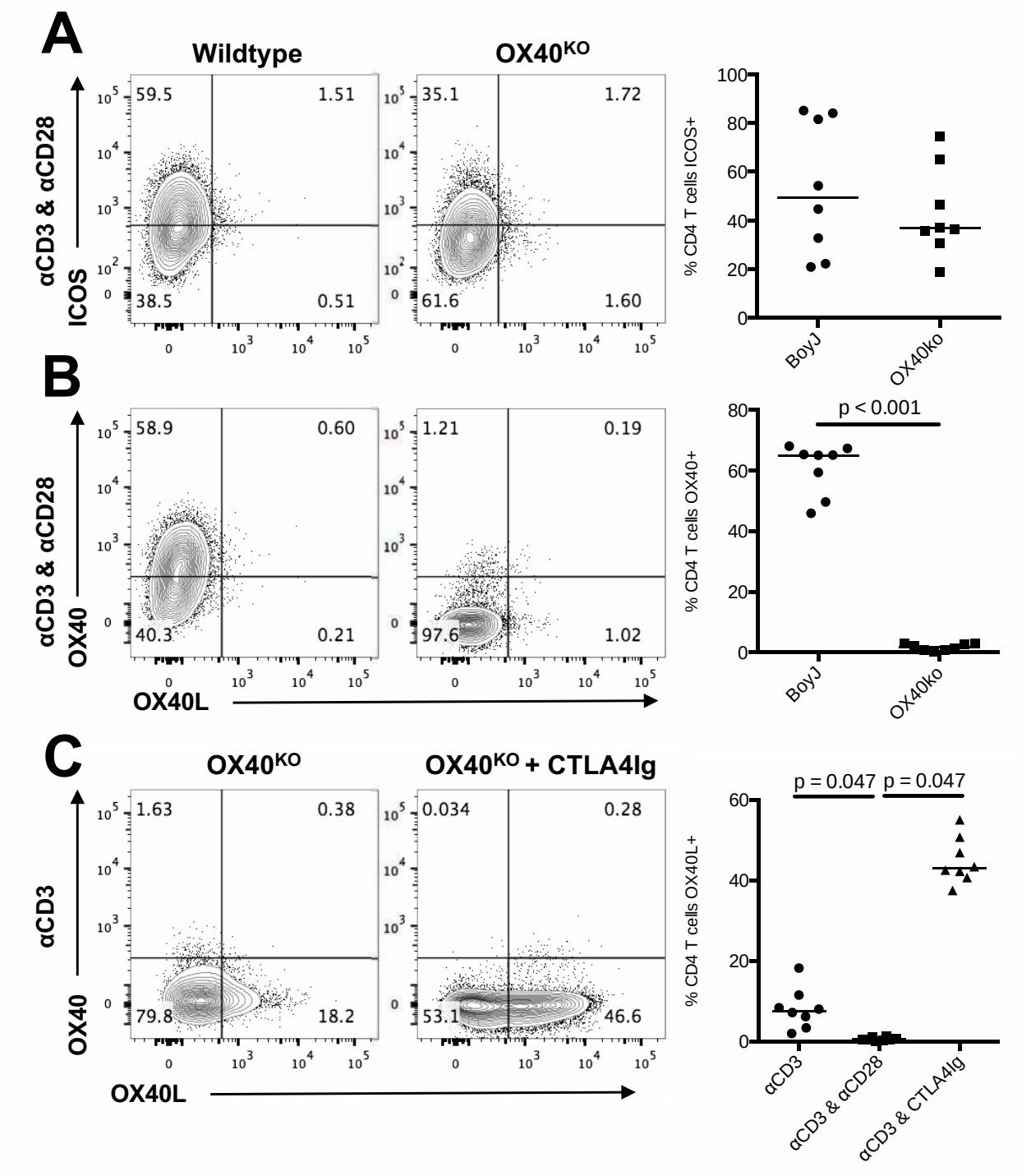


Figure 7.19: OX40^{KO} CD4+ T cells express OX40L normally. Splenocyte cell suspensions were prepared from OX40^{KO} mice and cultured in the presence of: (**A**) and anti-CD3 and (**B**) both anti-CD3 and anti-CD28 to demonstrate absence of OX40 expression (p=0.495) but a preserved ability to express ICOS and (**C**) anti-CD3 alone or with either anti-CD28 or CTLA4Ig. (All plots relate to live CD4+ T cells; comparisons either with Mann-Whitney U-test or Kruskal-Wallis test with Dunn's post-hoc analysis; n=8 per group)

T cells cultures, and also to control for potential inconsistencies in culture conditions between $OX40^{KO}$ and wildtype cells, CD45.2+ $OX40^{KO}$ cells were mixed in approximately 1:1 ratios with CD45.1+ BoyJ wildtype splenocytes. Here, it could be demonstrated that both $OX40^{KO}$ and wildtype cells upregulated OX40L to similar levels when cultured in the same conditions (Figure 7.20). Further, the fact that there were no differences between co-cultured $OX40^{KO}$ and OX40-sufficient cells suggested that OX40-OX40L interactions such as ripping were not important for CD4+ T cell expression of OX40L in this system.

To further investigate the possibility of a need for direct contact with OX40L expression on non-T-cells to permit expression on CD4+ T cells, we used our earlier observation that purified CD4 T cells in culture did not upregulate OX40L. Co-cultures of purified wildtype CD4 T-cells and OX40L-deficient cells were made to see whether this permitted expression of OX40L. By using mice with lox sites around their *TNFSF4* (OX40L) genes and expressing Cre-recombinase under the PGK-1 promoter, OX40L-deficient splenocytes were generated.[225] First, we confirmed that the combination of PGK-Cre with a floxed OX40L locus prevented the expression of OX40L when total cells were cultured in the presence of agonistic anti-CD3 antibody and CTLA4Ig. In contrast, cells from PKG-Cre only mice were able to upregulate OX40L in a manner similar to wildtype cells (Figure 7.21A-B). As previously, wildtype CD4+ T cells lost the ability to upregulate OX40L after purification (Figure 7.21C-D), but when mixed with total splenocytes either sufficient or insufficient in OX40L, the ability of the purified CD4 T cells to express OX40L was restored (Figure 7.21E-F). This finding was consistent over multiple experiments (Figure 7.21G).

7.2.5 Gamma chain cytokines downregulate OX40L expression

Others have reported that cytokines may modulate OX40L expression, both on CD4+ T cells and on antigen-presenting cells. The finding that preventing CD28 signalling greatly increases OX40L expression provides a useful platform to investigate the modulating capacity of cytokine signalling

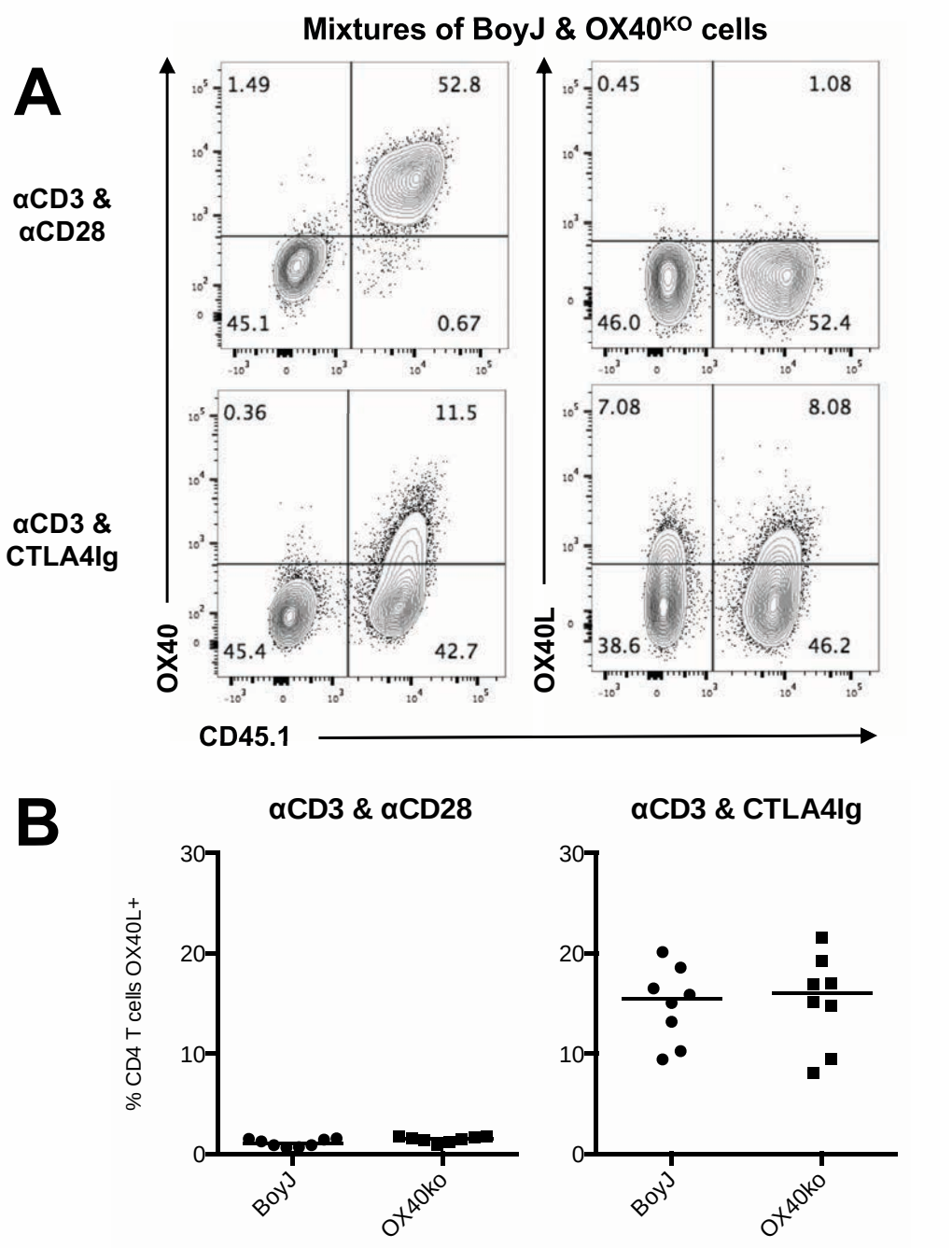


Figure 7.20: OX40-sufficient and -deficient cells express OX40L similarly in co-culture. (**A**) When cultured as approximately 1:1 mixtures, splenocytes from CD45.1-negative OX40-negative mice and CD45.1-positive wildtype mice expressed OX40L in a similar manner and (**B**) this was consistent between cultures ($p=0.089$ and $p=0.854$ respectively)(plots gated on live CD4+ T cells; 8 samples per group; Mann-Whitney U-test)

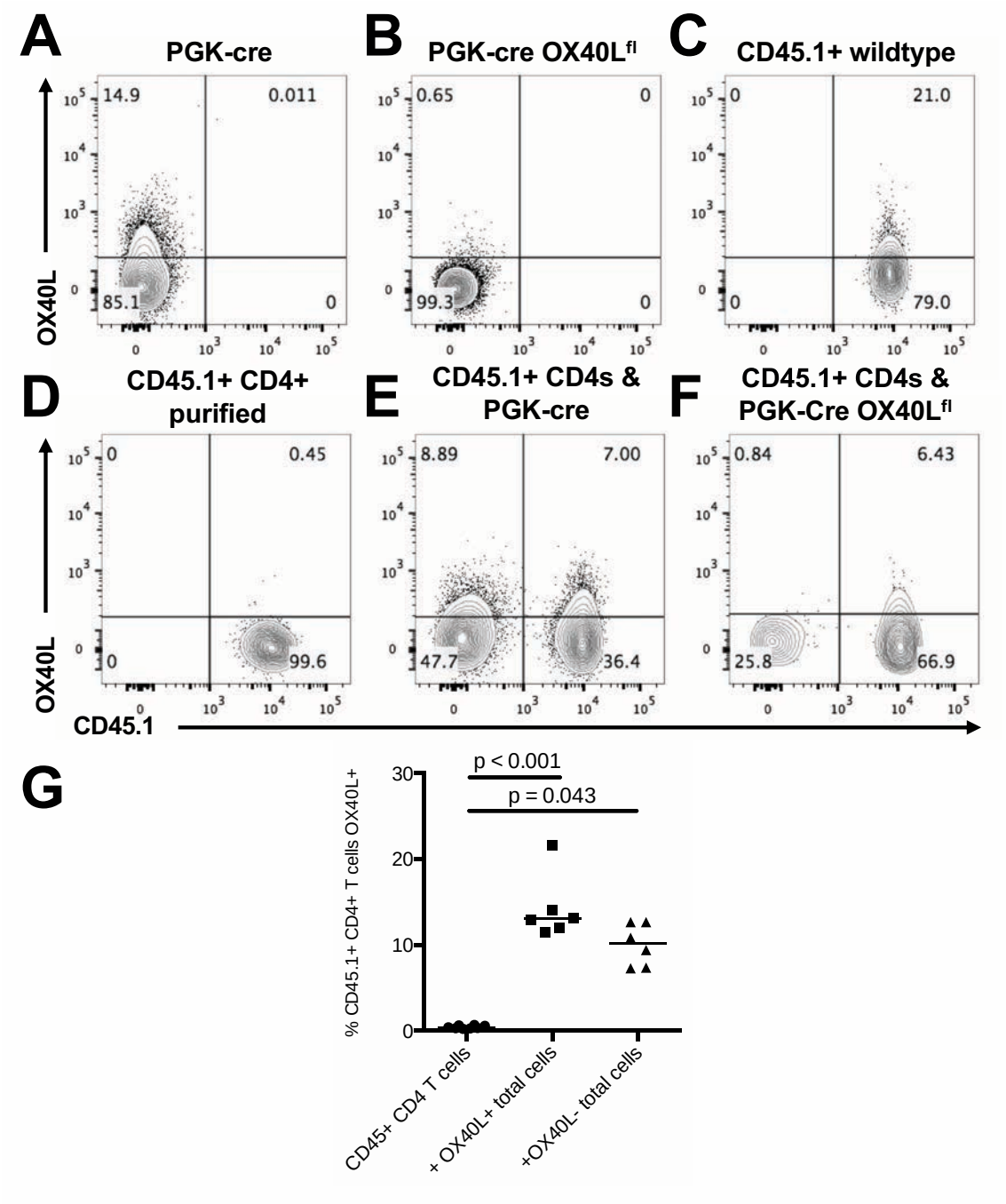


Figure 7.21: Transfer of OX40L from other cells is not important for its expression by CD4+ T cells. Total splenocytes from various mice were cultured with anti-CD3 and CTLA4Ig for 72 hours. (**A**) PGK-Cre cells expressed OX40L on CD4+ T cells; (**B**) PGK-Cre OX40L^{fl} cells did not; (**C**) total CD45.1+ wildtype splenocyte cultures expressed OX40L+ cells; (**D**) purified wildtype CD4+ cells did not; (**E**) and (**F**) purified CD4+ T cells expressed OX40L when mixed with either PGK-Cre or PGK-Cre OX40L^{fl} cells. (**G**) Graphical representation of (**D**-**F**). All plots are gated on CD4 T cells; 6 samples per group; Kruskal-Wallis test with Dunn's post-hoc test.

on OX40L expression. My earlier observations that CTLA4$^{-/-}$ cells could downregulate OX40L on wildtype T cells *in trans* also raised the possibility of a soluble mediator.

The ability of exogenous IL-2 to alter the expression of CD4+ T cell OX40L was investigated. The addition of IL-2 to cultures of wildtype splenocytes stimulated with agonistic anti-CD3 in the presence of CTLA4Ig caused a dose-dependent decrease in OX40L expression (Figure 7.22A). When cultured with 40 ng ml^{-1} of IL-2, OX40L expression was entirely abrogated and when cultured with lower concentrations, there was a dose-dependent reduction in OX40L expression.

As with culture of CD4+ T cells with agonistic anti-CD28 antibody, the addition of IL-2 simultaneously upregulated expression of OX40. To confirm that OX40 expression was not significant in the downregulation of OX40L expression, the experiment above was repeated with OX40 deficient cells. Again, OX40L expression was greatly reduced by the addition of 40 ng ml^{-1} of IL-2 suggesting that OX40 expression did not directly influence OX40L expression (Figure 7.22B).

Combining the observations that CD28 and IL-2 both downregulate OX40L expression with others' demonstration that IL-2 production is CD28-ligation dependent[427], it was hypothesised that the downregulation of OX40L expression seen with CD28 ligation was partially IL-2 induced. To test this, wildtype cells were cultured in the presence of agonistic anti-CD3 and CD28 antibodies with and without a neutralising antibody to IL-2. As previously, culture with anti-CD28 prevented the expression of OX40L. However, the addition of neutralising anti-IL-2 antibody partially restored OX40L expression (Figure 7.22C). This suggested that, at least in part, the action of ligating anti-CD28 antibody was mediated through increasing IL-2 production.

A major mediator of the effects of IL-2 is signalling through signal transducer and activator of transcription 5 (STAT5).[136] As a method of testing whether this pathway was important in the control of T cell OX40L expression, the specific inhibitor pimozide was employed.[138] Consistent with findings from experiments with exogenous IL-2 and with anti-IL-2 blocking antibody, pimozide upregulated OX40L in the presence and absence of IL-2 (Figure 7.23).

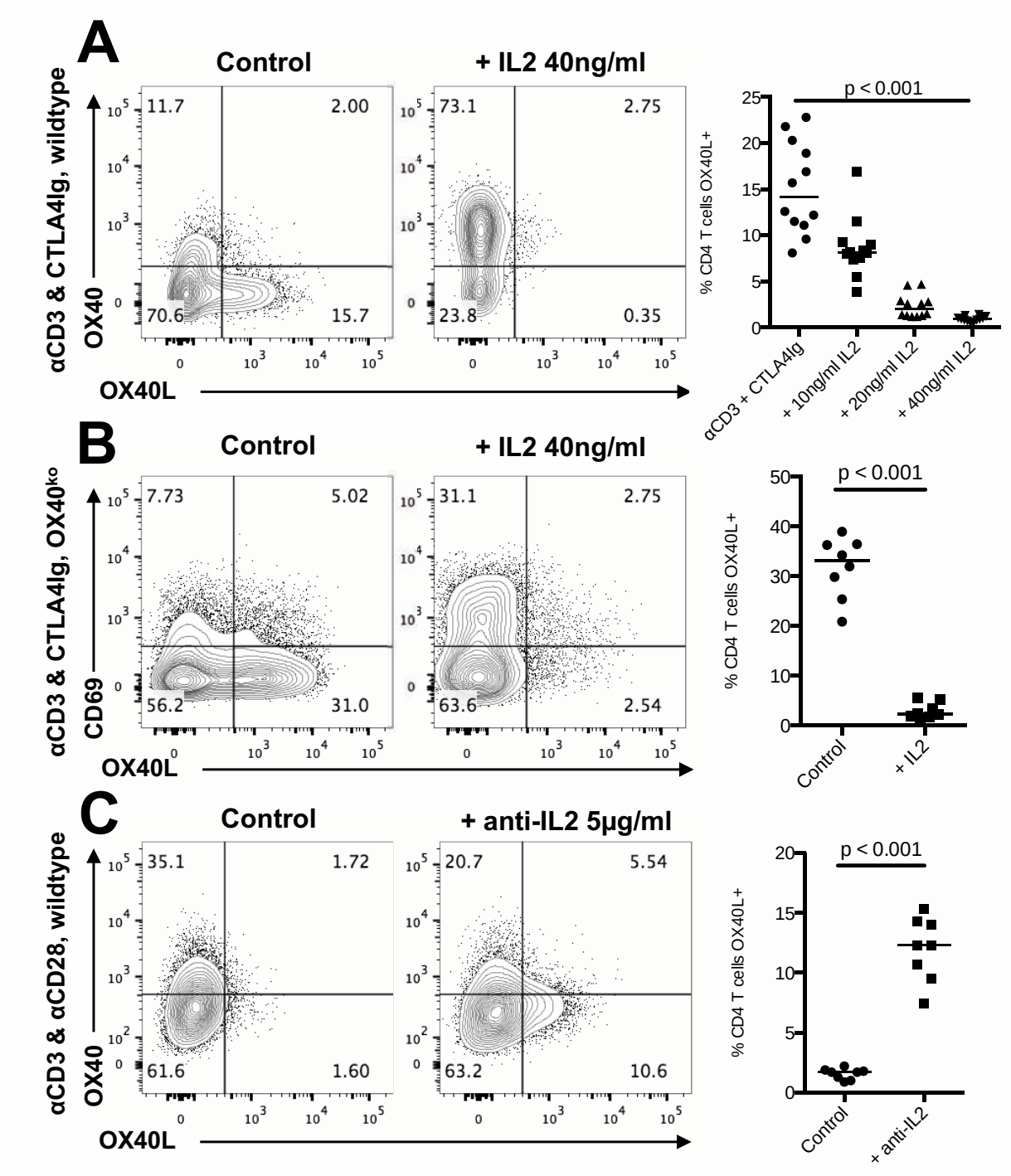


Figure 7.22: IL2 downregulates OX40L expression. (**A**) Wildtype splenocytes were cultured in the presence of anti-CD3 and CTLA4Ig with and without varying concentrations of IL-2. There was a dose-dependent decrease in OX40L expression with increasing concentrations of IL-2 (12 samples per group; nptrend test). (**B**) The effect of IL-2 was similar in OX40ko splenocytes. (**C**) The addition of anti-IL-2 antibody increased OX40L expression when splenocytes were cultured with anti-CD3 and anti-CD28. (all plots representative and gated on live CD4+ T cells). Mann-Whitney U test; n=8 per group.

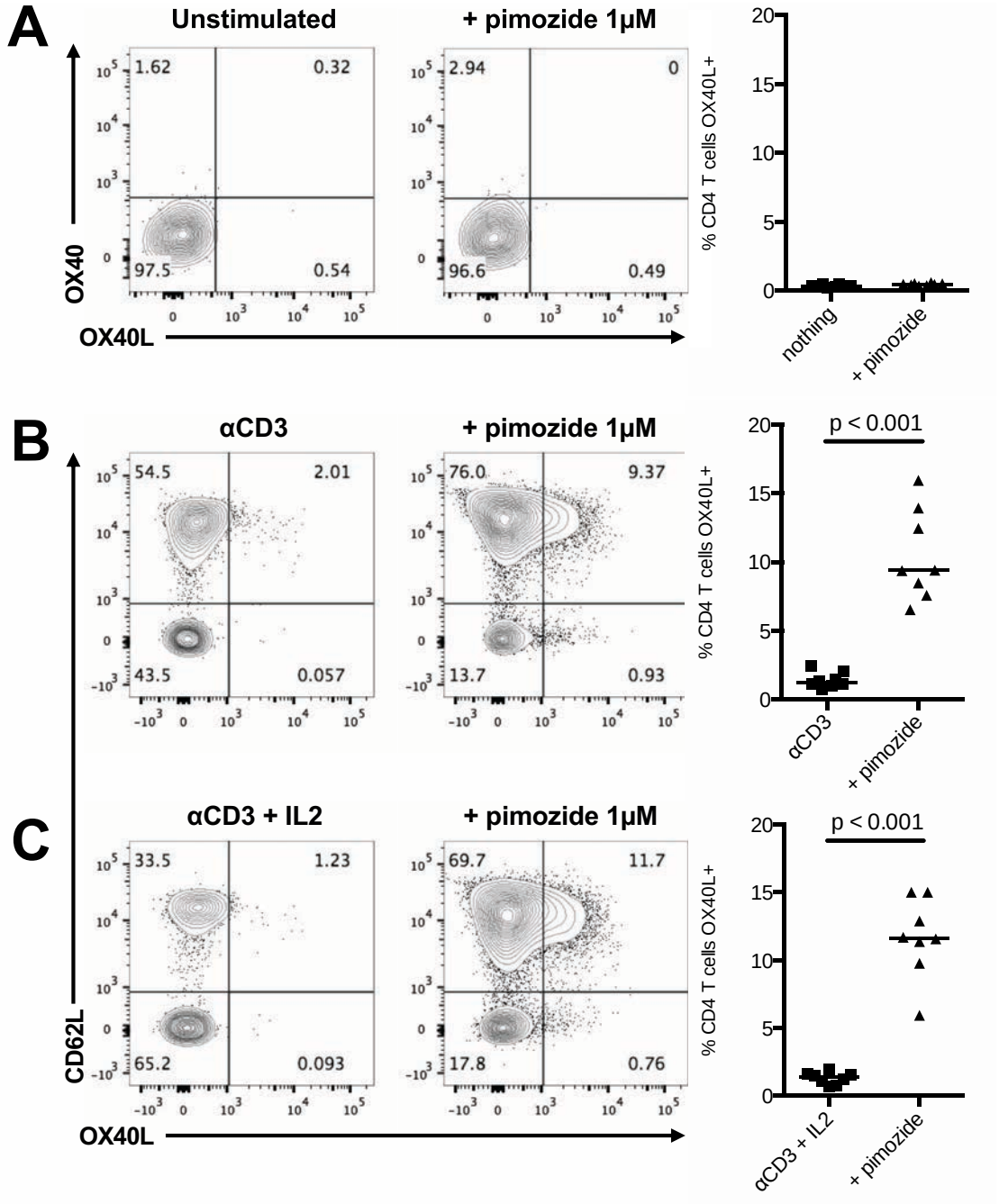


Figure 7.23: STAT5 blockade permits expression of OX40L. The addition of the STAT5-inhibitor pimozide did not cause any change in expression of OX40L on unactivated CD4+ T cells (**A**; p=0.159), but in contrast, pimozide caused upregulation of OX40L on CD4+ T cells stimulated in both the absence (**B**) and the presence (**C**) of exogenous IL-2. Mann-Whitney U test; n=8 per group.

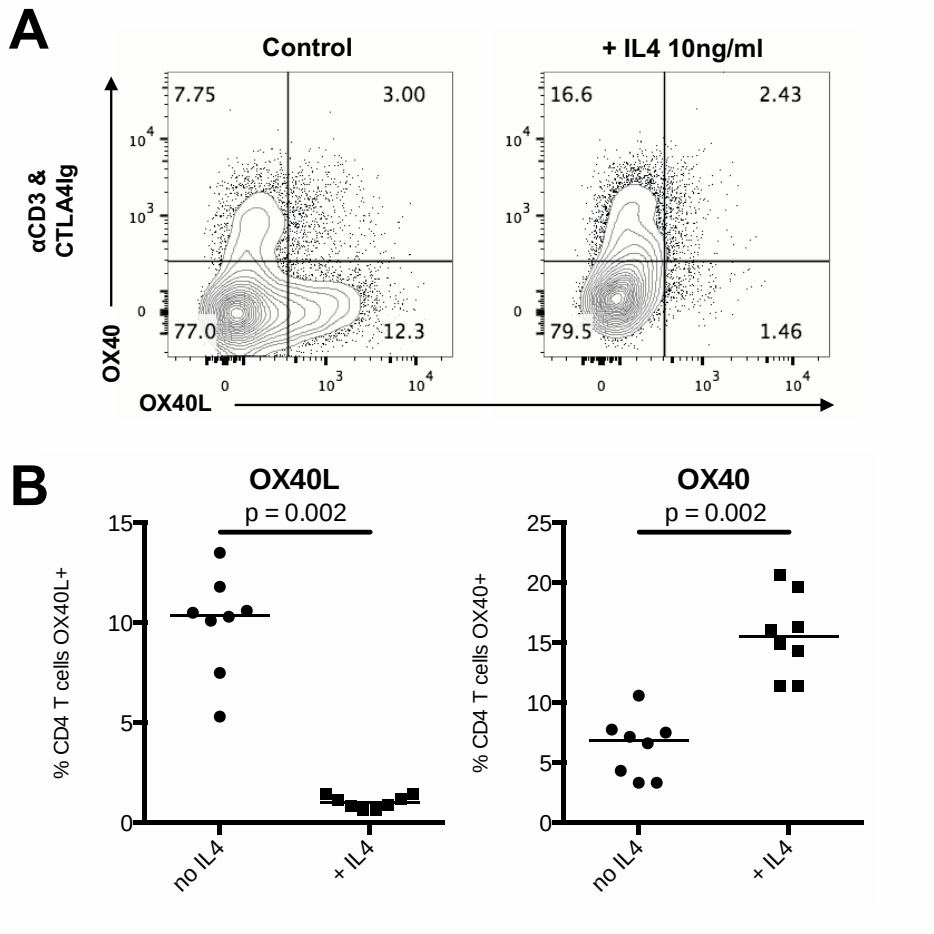


Figure 7.24: IL-4 downregulates OX40L expression. (**A**) The addition of IL-4 to cultures of wildtype splenocytes stimulated with anti-CD3 antibody in the presence of CTLA4Ig reduced OX40L expression and increased OX40 expression (representative plots; gated on liver CD4+ T cells). (**B**) The effects were consistent over multiple samples. Mann-Whitney U test; n=8 per group.

It is reported that IL-4 can downregulate OX40L expression, although it is not clear whether this finding would persist in conditions of controlled CD28 ligation. Splenocytes were therefore cultured with and without exogenous IL-4 in the presence of anti-CD3 and CTLA4Ig. Here, the addition of IL-4 entirely prevented the expression of OX40L (Figure 7.24). Similar to findings with IL-2, there was a concurrent upregulation of OX40.

IL-2 and IL-4 both interact with receptors containing the IL-2 receptor gamma subunit. It was therefore considered whether other cytokines interacting with similar receptors might have similar ef-

fects. IL-7 is one such example and when added to cultures down-regulated OX40L and upregulated OX40 in a dose-dependent manner (Figure 7.25).

It has been suggested elsewhere that the TH1 cytokines IFNγ and IL-12 modulate OX40L expression on T cells. No difference was seen in CD4+ T cell OX40L expression in response to exogenous IL-12 (Figure 7.26A), but an increase was observed in expression with the addition of IFNγ (Figure 7.26B). A number of other cytokines suggested as affecting OX40L expression either on T cells or on other cells types were also assessed, but found no significant difference in OX40L expression was demonstrated on cells treated with exogenous TGFβ, IL-18, interleukin-1 beta (IL1β) or TL1a (Figure 7.27).

7.2.6 CD8+ T cells regulate OX40L similarly to CD4+ T cells

Having demonstrated that CD4+ T cells express OX40L in response to TCR signals without CD28 signals, several of the experiments reported here were repeated with CD8+ T cells in total splenocyte cultures. Consistent with results for CD4+ T cells, CD8+ cells upregulated OX40L when stimulated in the presence of CTLA4Ig but down-regulated OX40L when stimulated with agonistic anti-CD28 antibody. (Figure 7.28A-B). Again, consistent with findings from CD4+ T cells, each of the gamma-chain cytokines IL-2, IL-4 and IL-7 down-regulated OX40L expression (Figure 7.28C-D).

7.2.7 Mycophenolic acid and rapamycin upregulate OX40L by T cells

A number of methods of inducing damage to T cell DNA including mitomycin C, 5-fluorouracil, and X-ray irradiation have been described as potent inducers of OX40L on CD4 T cells.[210] I examined whether the lymphocyte-specific inhibitor of guanine nucleotide synthesis mycophenolic acid could alter OX40L expression on T cells. The addition of mycophenolic acid to cultures increased expression of OX40L when added in addition to agonistic anti-CD3 (Figure 7.29). Assessment of two other

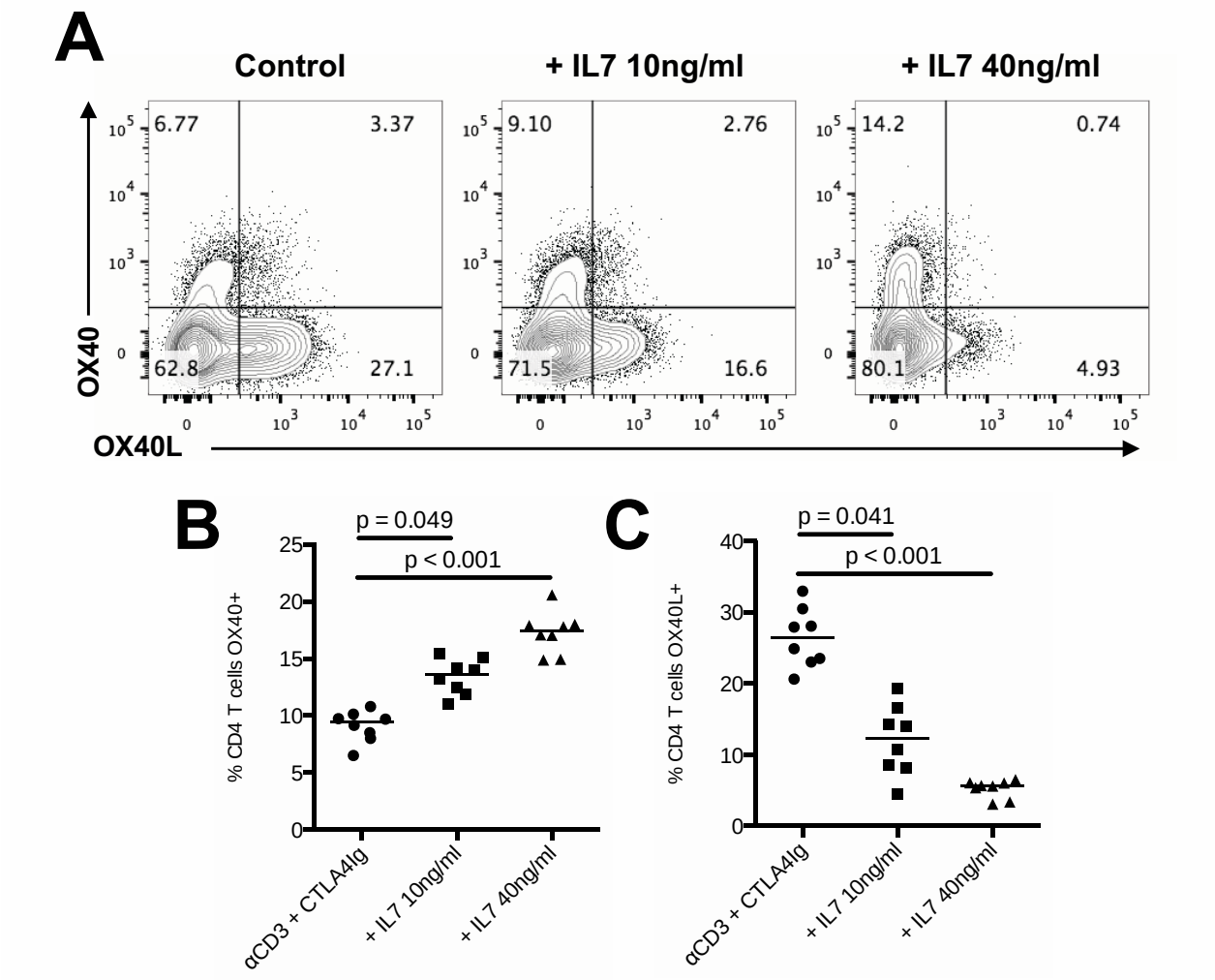


Figure 7.25: IL-7 downregulates OX40L expression. (**A**) The addition of IL-7 to cultures of wildtype splenocytes stimulated with anti-CD3 antibody in the presence of CTLA4Ig reduced OX40L expression and increased OX40 expression (representative plots; gated on liver CD4+ T cells). (**B**) and (**C**) The effects were consistent over several samples. Comparisons with Kruskal-Wallis test with Dunn's post-hoc test; n=8 per group.

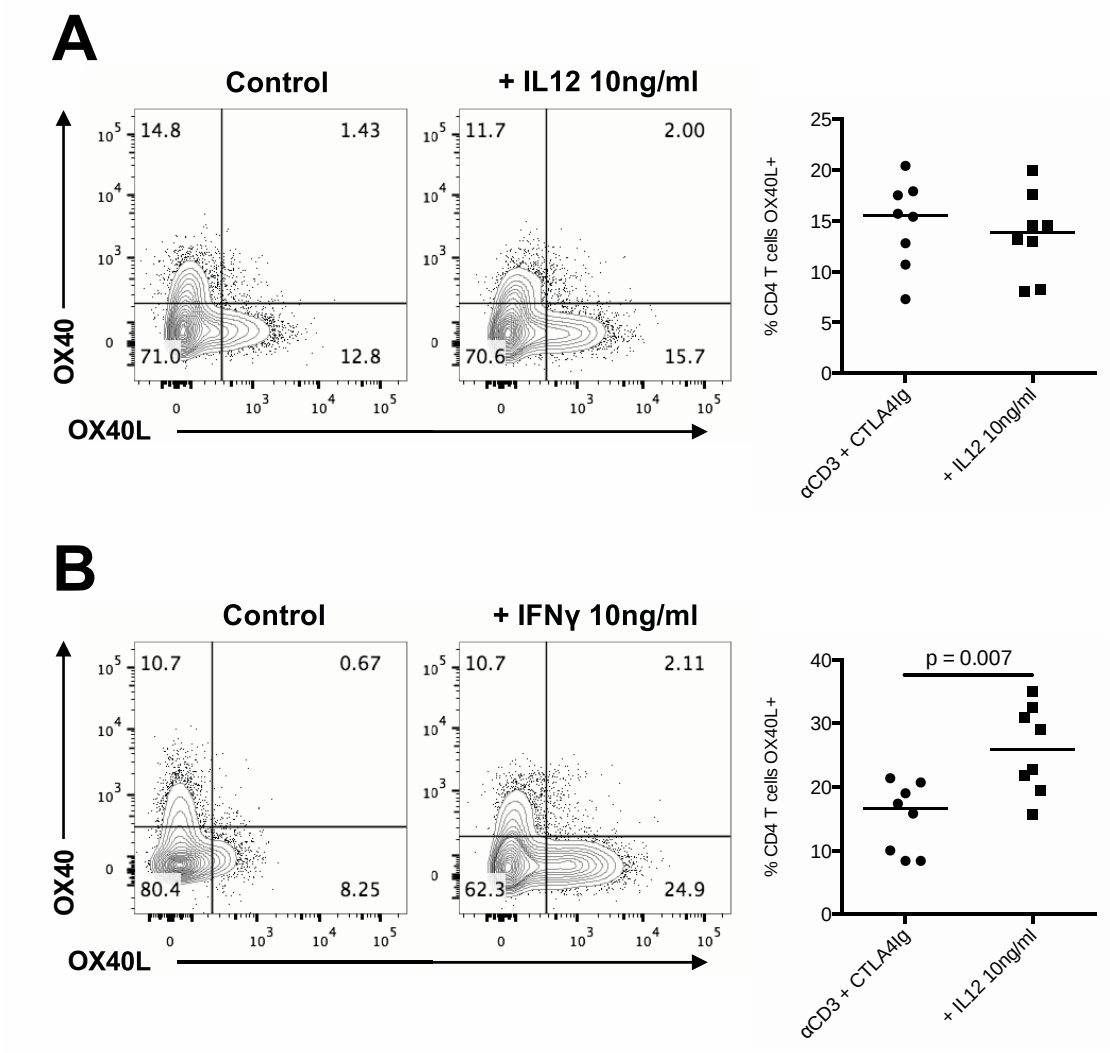


Figure 7.26: IFNγ increases expression of OX40L. (**A**) The addition of IL-12 to mixed wildtype splenocytes cultures did not significantly alter expression of OX40L CD4+ T cells (p=0.629). (**B**) The addition of IFNγ to the same cultures augmented OX40L expression. (n = 8 per group; Mann-Whitney U-test)

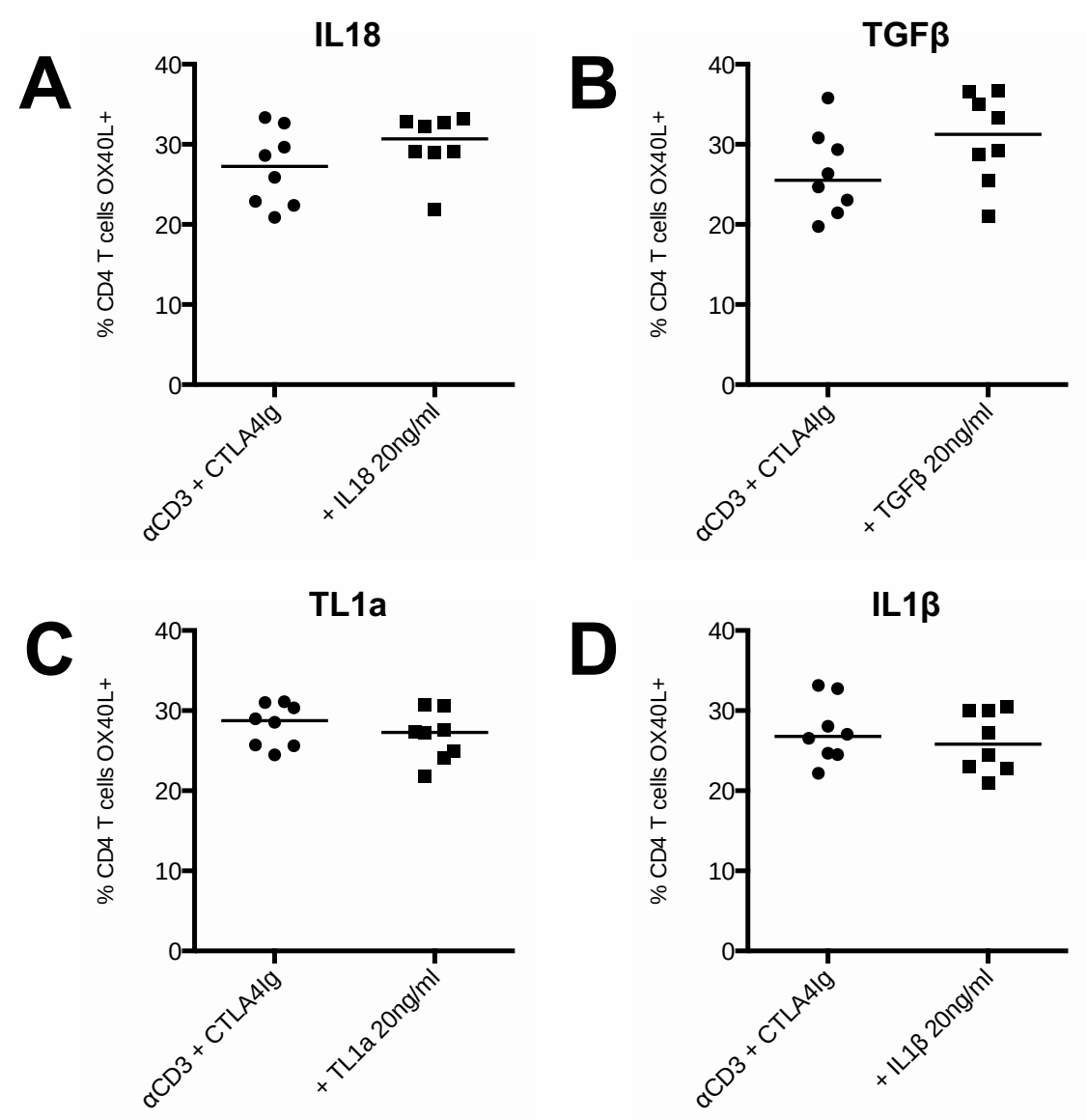


Figure 7.27: Not all cytokines affect OX40L expression. Wildtype total splenocytes were cultured for 72 hours in the presence of $1\,\mu g\,ml^{-1}$ anti-CD3 antibody and CTLA4Ig with and without additional (**A**) IL-18 (p=0.275); (**B**) TGFβ (p=0.193); (**C**) TL1a (p=0.323); and (**D**) IL1β (p=0.560). The addition of these cytokines did not affect expression of OX40L on CD4+ T cells. (n=8 per group; Mann-Whitney U-test)

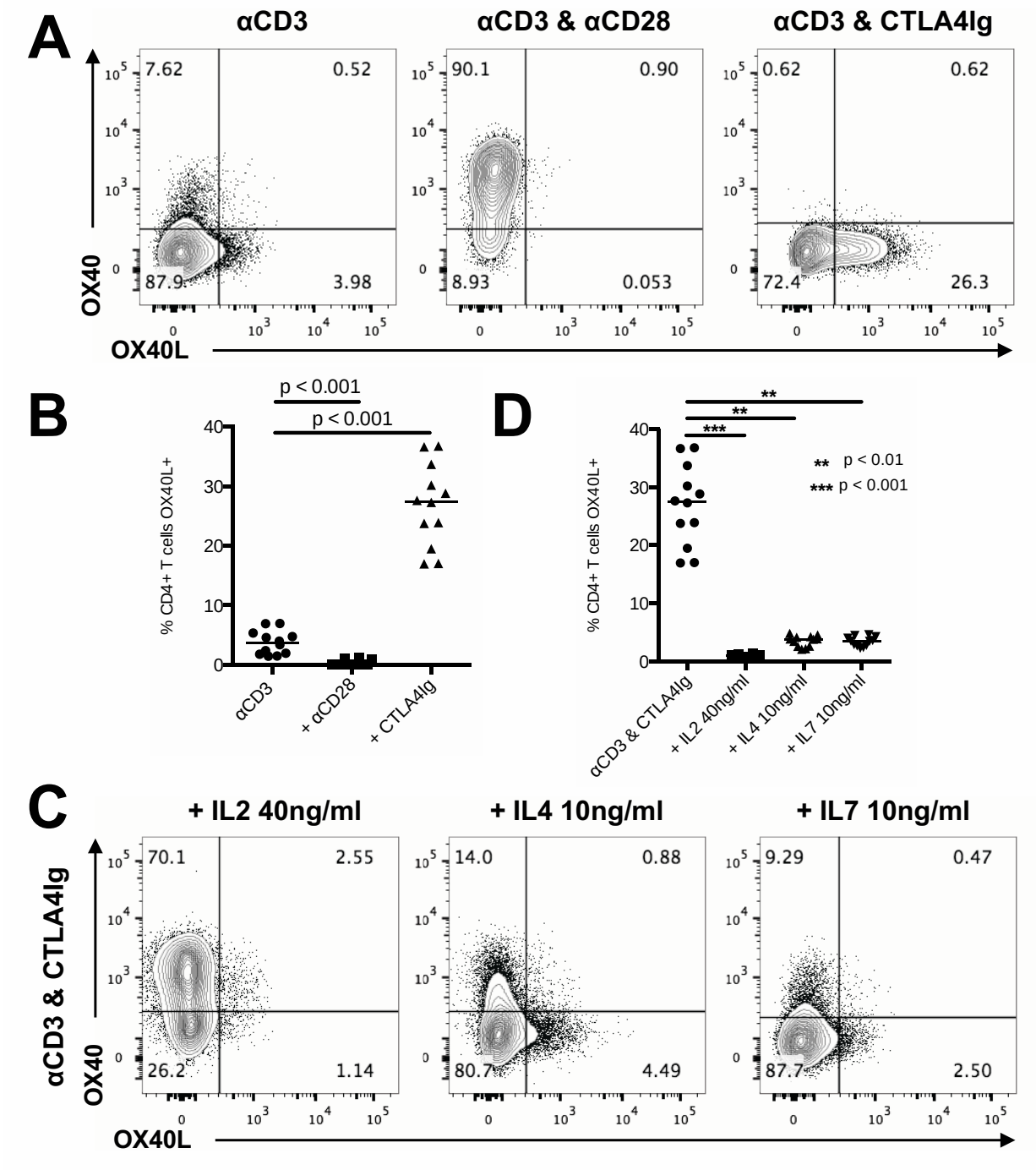


Figure 7.28: CD8+ T cells regulate OX40L similarly to CD4+ T cells. (**A**) When cultured with agonistic anti-CD3 antibody, CD8 T cells express OX40L on a small minority of cells. The addition of anti-CD28 to cultures reduced expression whilst addition of CTLA4Ig greatly increased expression. (**B**) These findings were consistent between cultures. (**C**) The addition of IL-2, IL-4 or IL-7 reduced OX40L expression when added to cultures containing anti-CD3 and CTLA4Ig. (**D**) These findings were consistent between cultures. (n = 12 per condition; Kruskal-Wallis tests with Dunn's post-hoc tests to test significance from leftmost control group; plots gated on live CD8+ T cells)

immunosuppressant agents active on T cells revealed that ciclosporin A – a calcineurin inhibitor – decreased OX40L expression (Figure 7.30A); the addition of dexamethasone made no difference to OX40L expression (Figure 7.30B).

Because CD28 signalling is partly effected by signalling through the mechanistic target of rapamycin (mTOR), rapamycin was added to cultures of wildtype cells stimulated with either anti-CD3 or the combination of anti-CD3 and anti-CD28. In both instances the addition of rapamycin induced an increase in expression of OX40L (Figure 7.31). Interestingly, the addition of rapamycin was sufficient to induce double-positive OX40-OX40L cells whereas in previous experiments expression of OX40 and OX40L was generally reciprocal.

7.2.8 OX40L may be associated with reduced proliferation

To assess the expression of OX40L in relation to whether cells had proliferated, wildtype CD4+ T cells were stained intracellularly for the nuclear protein Ki67, a marker of proliferation. Here, when cells were stimulated with anti-CD28 or deprived of CD28 signals by adding CTLA4Ig, there was reciprocal OX40L and Ki67 staining (Figure 7.32A). To further assess proliferation, cells were loaded with the membrane dye CFSE and stimulated. Again a reciprocal pattern between OX40 and OX40L expression was apparent: with OX40 expression greater on those cells that had diluted CFSE and OX40L expression greater on cells that had not diluted CFSE (Figure 7.32B-C).

In an attempt to assess the functional role of OX40L on T cells with respect to proliferation, experiments to estimate the increase in their number in response to a standardised stimulus was conducted. To control for variations in culture conditions between culture wells and for the fact that my sources of OX40L cells was from Cre-recombinase positive cells, and so relatively hypoproliferative, cells were cultured in approximately 1:1 mixes with CD45.1+ wildtype cells. Prior to culture, and at the end of culture, the ratio of CD45.2:CD45.1 CD4+ T cells was calculated and a further ratio of the

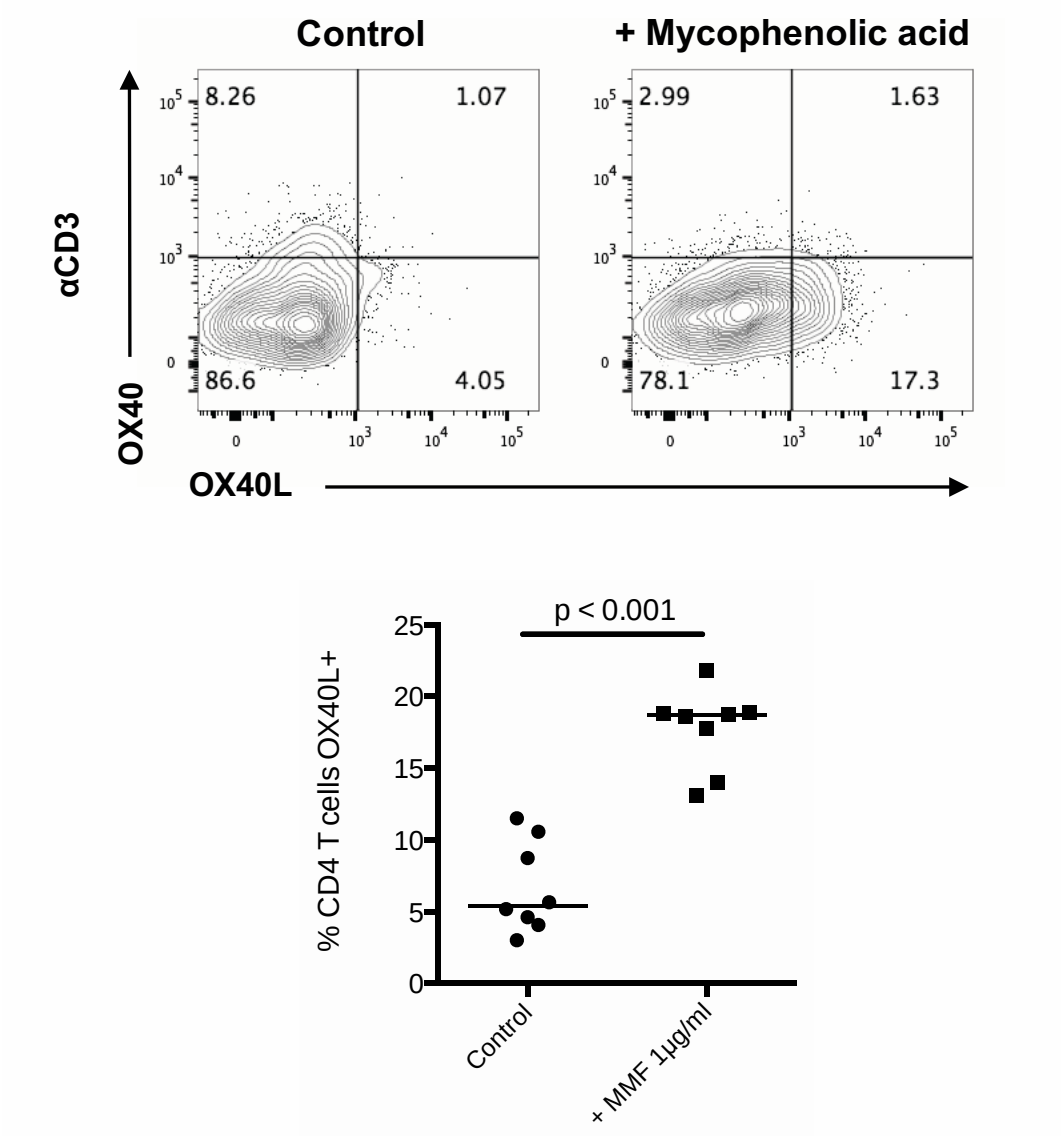


Figure 7.29: Mycophenolic acid upregulates OX40L expression. The addition of mycophenolic acid (1 µg/ml) to cultures of total wildtype splenocytes stimulated with agonistic anti-CD3 increased expression of OX40L on CD4+ T cells (8 samples per group; plots gated on live CD4+ T cells; Mann-Whitney U-test).

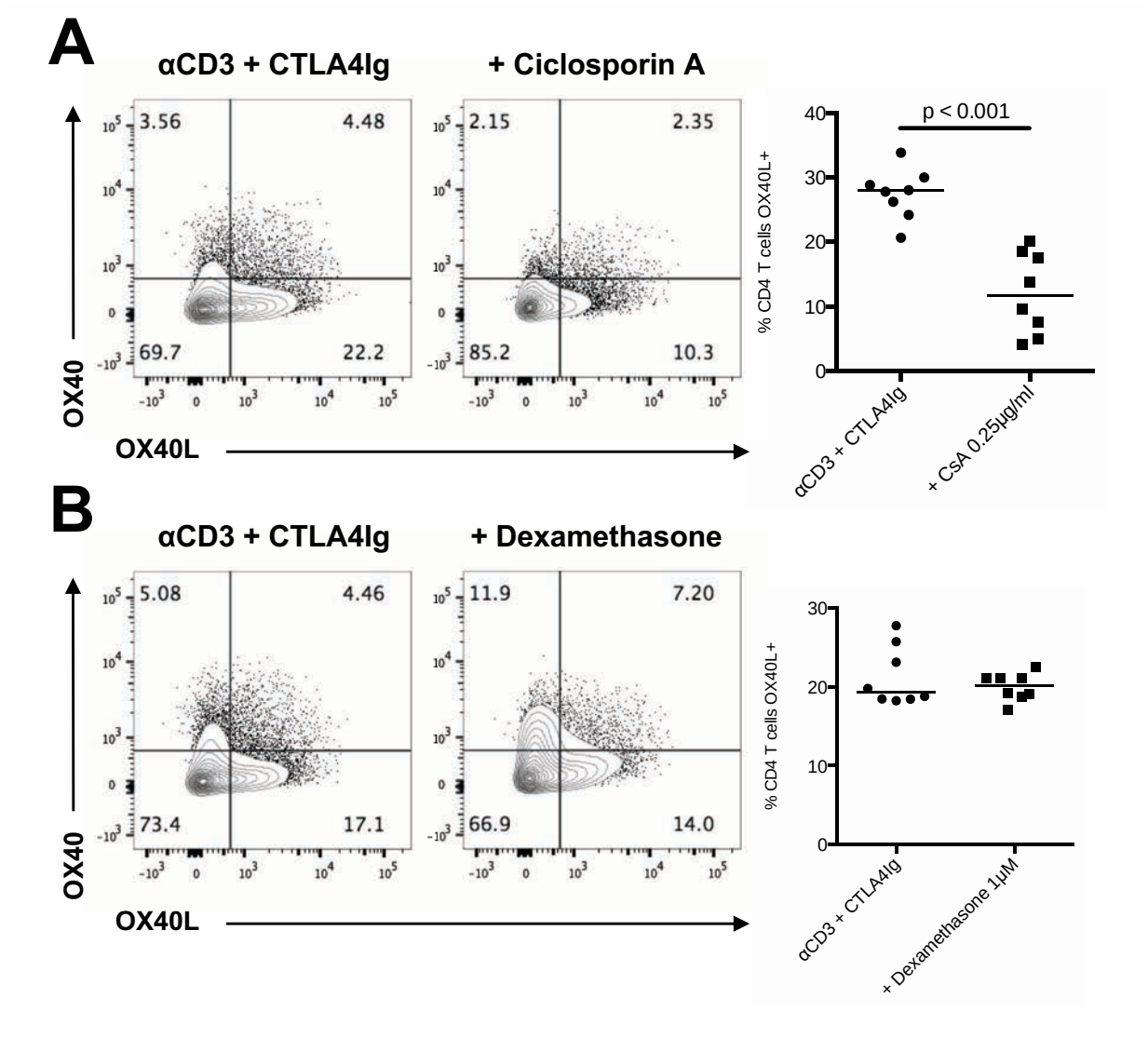


Figure 7.30: Ciclosporin reduces OX40L expression. (**A**) Wildtype total splenocytes were cultured in the presence of anti-CD3 and CTLA4Ig with and without ciclosporin A 0.25 μg ml^{-1} for 72 hours. Those cultures treated with ciclosporin A expressed significantly less OX40L. (**B**) Wildtype total splenocytes were cultured in with anti-CD3 and CTLA4Ig with and without dexamethasone 1 μm for 72 hours. There was no difference in CD4+ T cell expression of OX40L (p=0.932). (8 samples per group; Mann-Whitney U-tests)

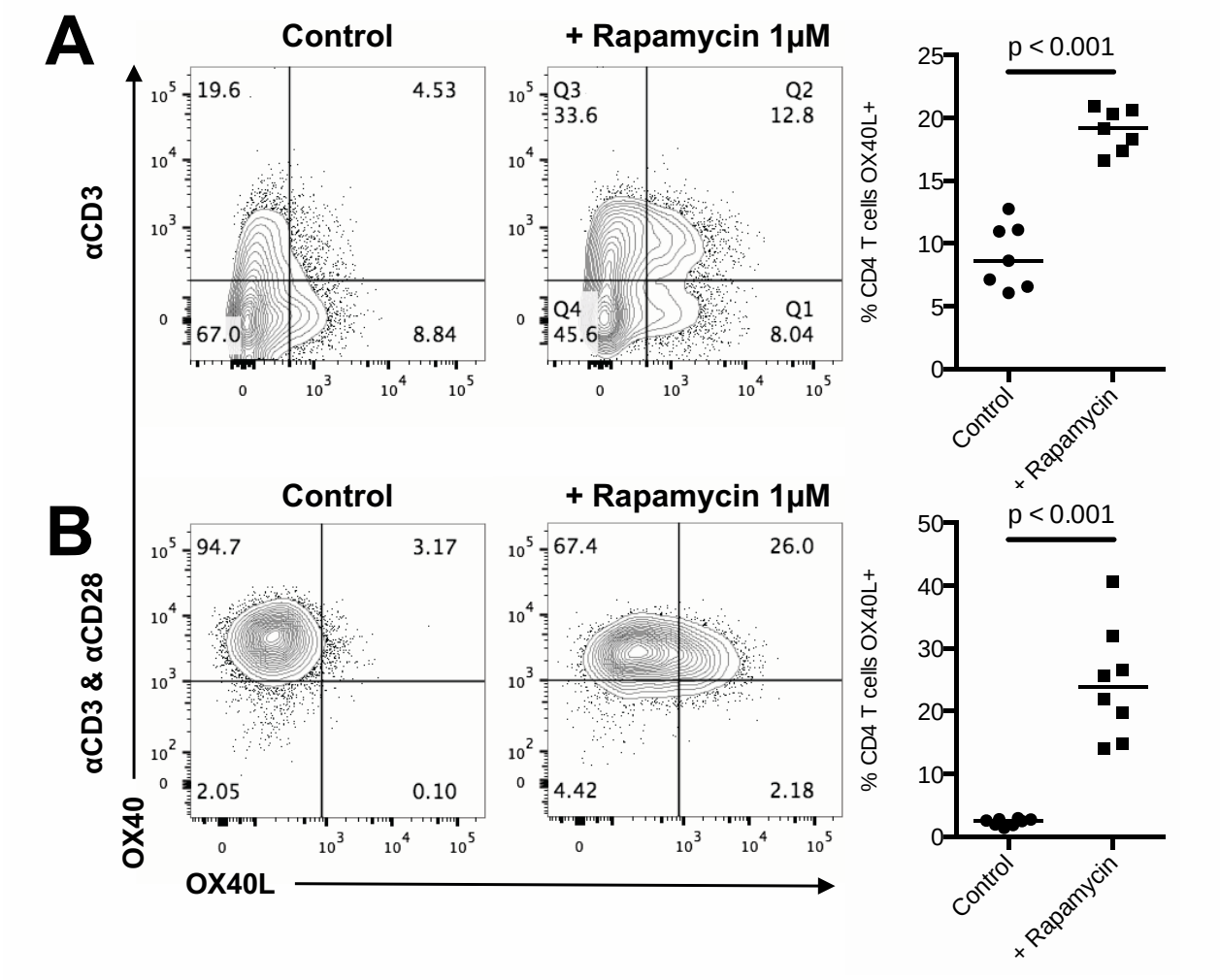


Figure 7.31: Rapamycin induces expression of OX40L. (**A**) Total splenocytes were cultured in the presence of agonistic anti-CD3 antibody with and without rapamycin (1 μm). The addition of rapamycin increased expression of OX40L on CD4+ T cells. (**B**) The addition of rapamycin also increased expression of OX40L on CD4+ T cells cultured with anti-CD3 and anti-CD28 (plots are representative and gated on live CD4+ T cells; 8 samples per condition; Mann-Whitney U-test)

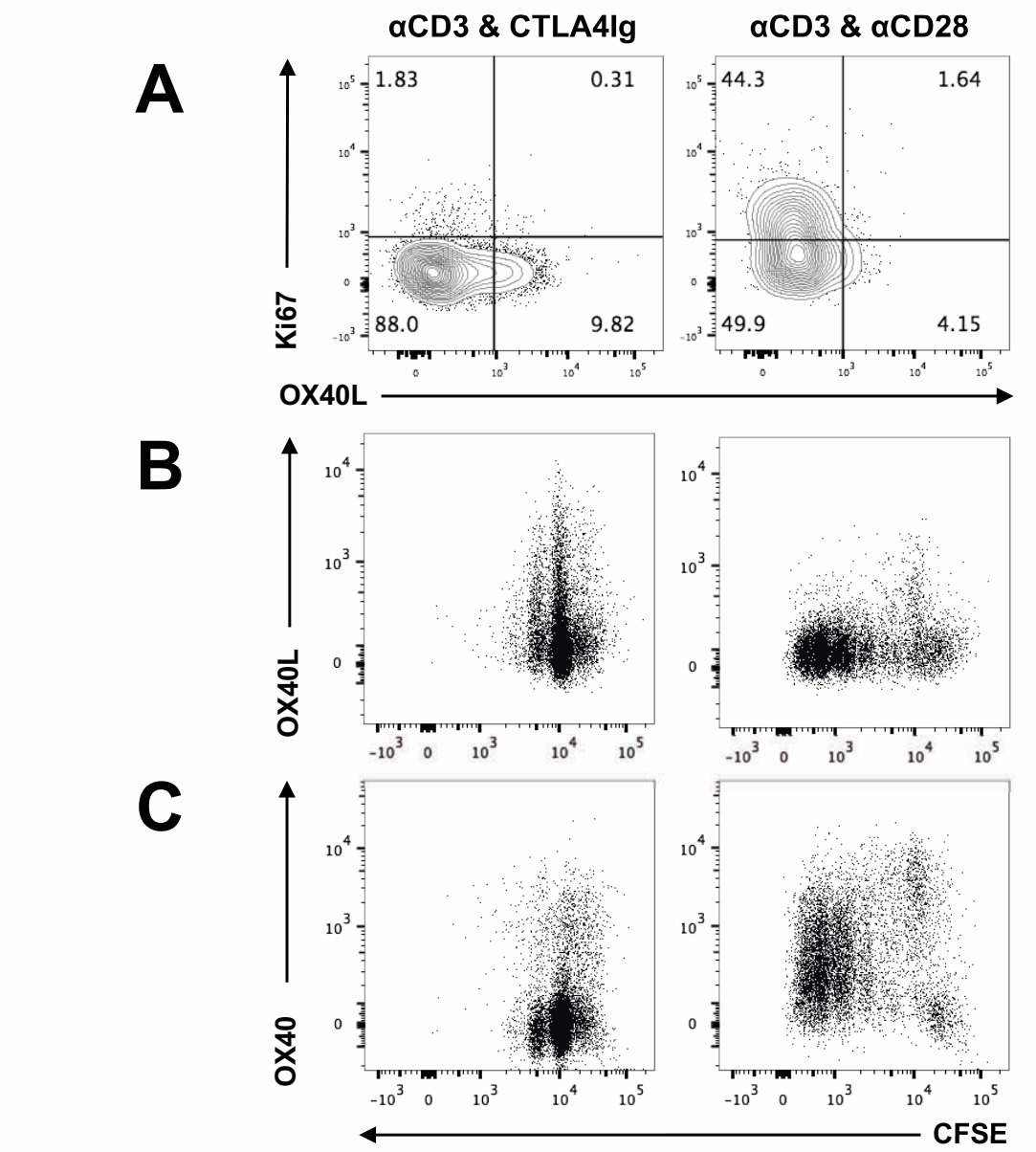


Figure 7.32: OX40L expression on CD4+ T cells is associated with reduced proliferation. Representative plots (from >8 experiments per condition; all panels gated on live CD4+ T cells) demonstrating (**A**) reciprocal expression of Ki67 with OX40L; (**B**) that OX40L expression is predominantly on cells that have not diluted CFSE; (**C**) that OX40 expression, in contrast to OX40L expression, is primarily on cells that have diluted CFSE.

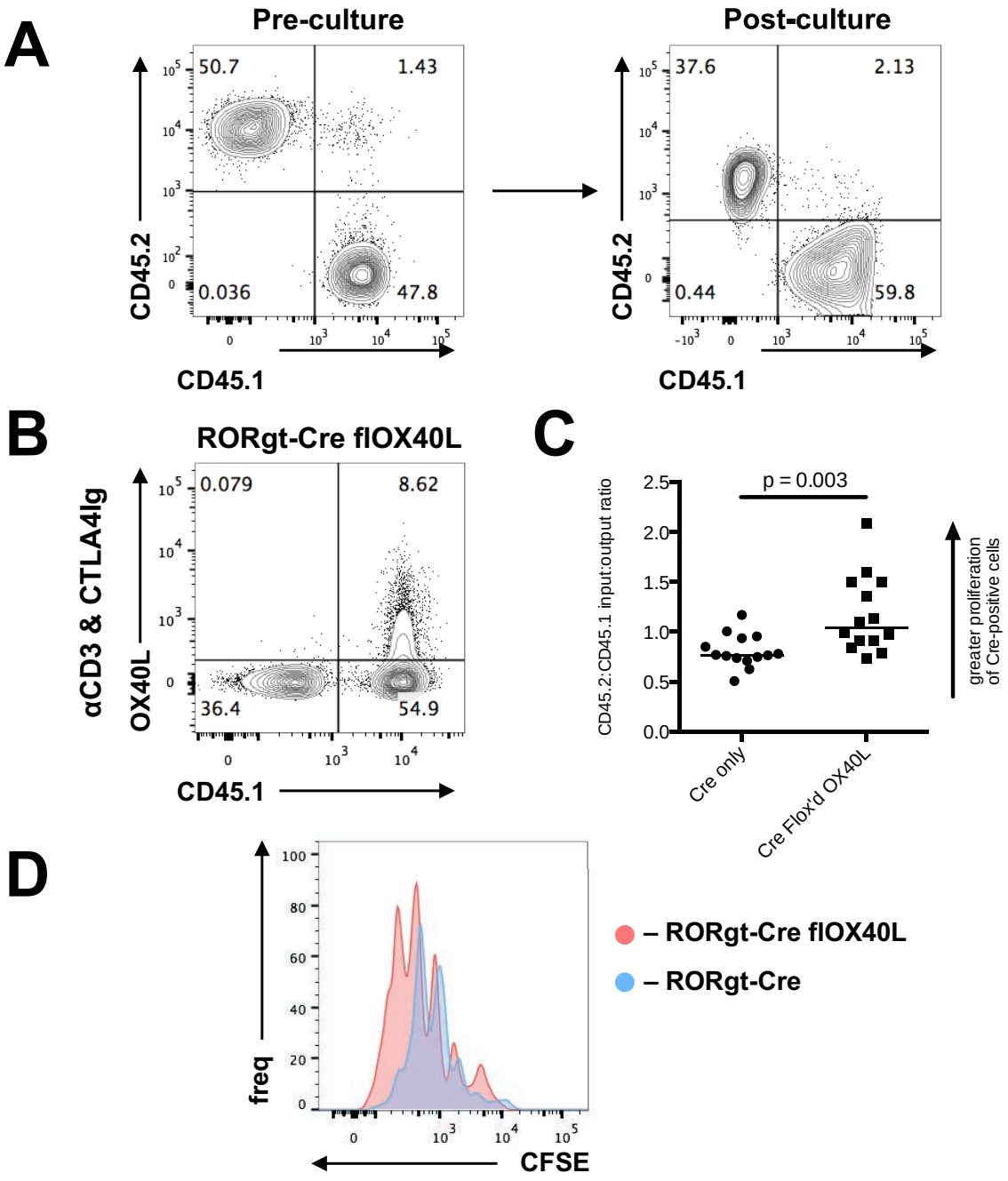


Figure 7.33: OX40L deficient CD4+ T cells are hyperproliferative. Cells from CD45.1+ wildtype and CD45.2+ RORGt-Cre mice with and without flox'd OX40L genes were mixed in approximately 1:1 ratios and cultured for 72 hours in the presence of anti-CD3 antibody. (**A**) Ratios of CD45.2+:CD45.1+ cells were calculated at the beginning and end of culture (representative plots gated on CD4+ T cells). (**B**) The absence of OX40L was confirmed by separate culture with anti-CD3 and CTLA4Ig. (**C**) At the end of the experiment the proportion of Cre-positive OX40L-deficient cells was greater than cells Cre-positive but OX40L-sufficient; Mann-Whitney U test; n=14 per group. (**D**) In some experiments CFSE was added and was diluted more in OX40L deficient cells (representative overlay).

initial and final cell counts calculated (Figure 7.33A). A higher final ratio would therefore suggest a relatively more positive change in the number of CD45.2+ cells and indirectly greater proliferation.

The relative proliferation of Cre-recombinase positive and Cre-flOX40L OX40L-deficient T cells was compared over three days of culture in the presence of anti-CD3. Deficiency in OX40L was checked by co-culture with anti-CD3 and CTLA4Ig. Although Cre-recombinase expressing cells increased in number less than CD45.1+ cells, overall OX40L deficient cells increased in number more than their OX40L sufficient littermates counterparts (Figure 7.33B-C). In some wells, CFSE was added and dilution in representative samples was greater in cultures of cells lacking OX40L than those with OX40L (Figure 7.33D).

7.2.9 Human T cells regulate OX40L similarly to murine T cells

Given that the literature on T cell expression of OX40L describes several similarities between human and murine cells, human peripheral blood mononuclear cells were cultured with either anti-CD3 and CTLA4Ig or anti-CD3 and anti-CD28. Here, human CD4+ T cells behaved similarly to murine T cells by downregulating OX40L expression in response to CD28 signalling and upregulating it in the absence of CD28 signals (Figure 7.34).

Further, similar to murine cells, human cells downregulated OX40L in response to IL-4 (Figure 7.35) and upregulated OX40L in a dose-dependent manner after exposure to mycophenolic acid (Figure 7.36).

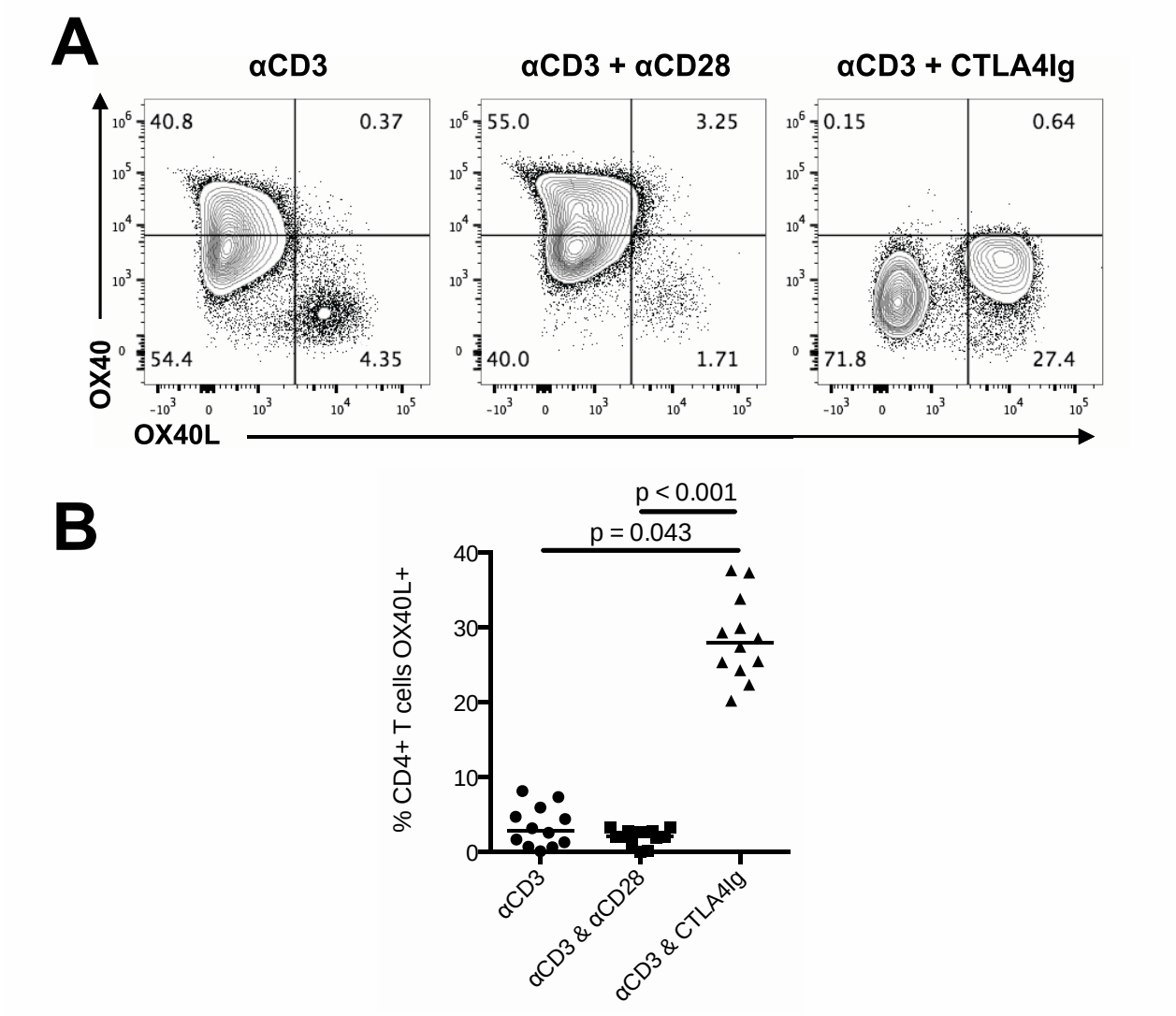


Figure 7.34: Human cells downregulate OX40L in response to signals through CD28. (**A**) Human total peripheral blood mononuclear cells were cultured for 72 hours in the presence of either agonistic anti-CD3 antibody, anti-CD3 and anti-CD28 or anti-CD3 and CTLA4Ig. There was an upregulation in OX40L on CD4+ T cells at the end of culture with the addition of CTLA4Ig and (**B**) this was consistent between experiments.(12 samples per condition; Kruskal-Wallis test with Dunn's post-hoc test; plots are representative and gated on live CD4+ T cells).

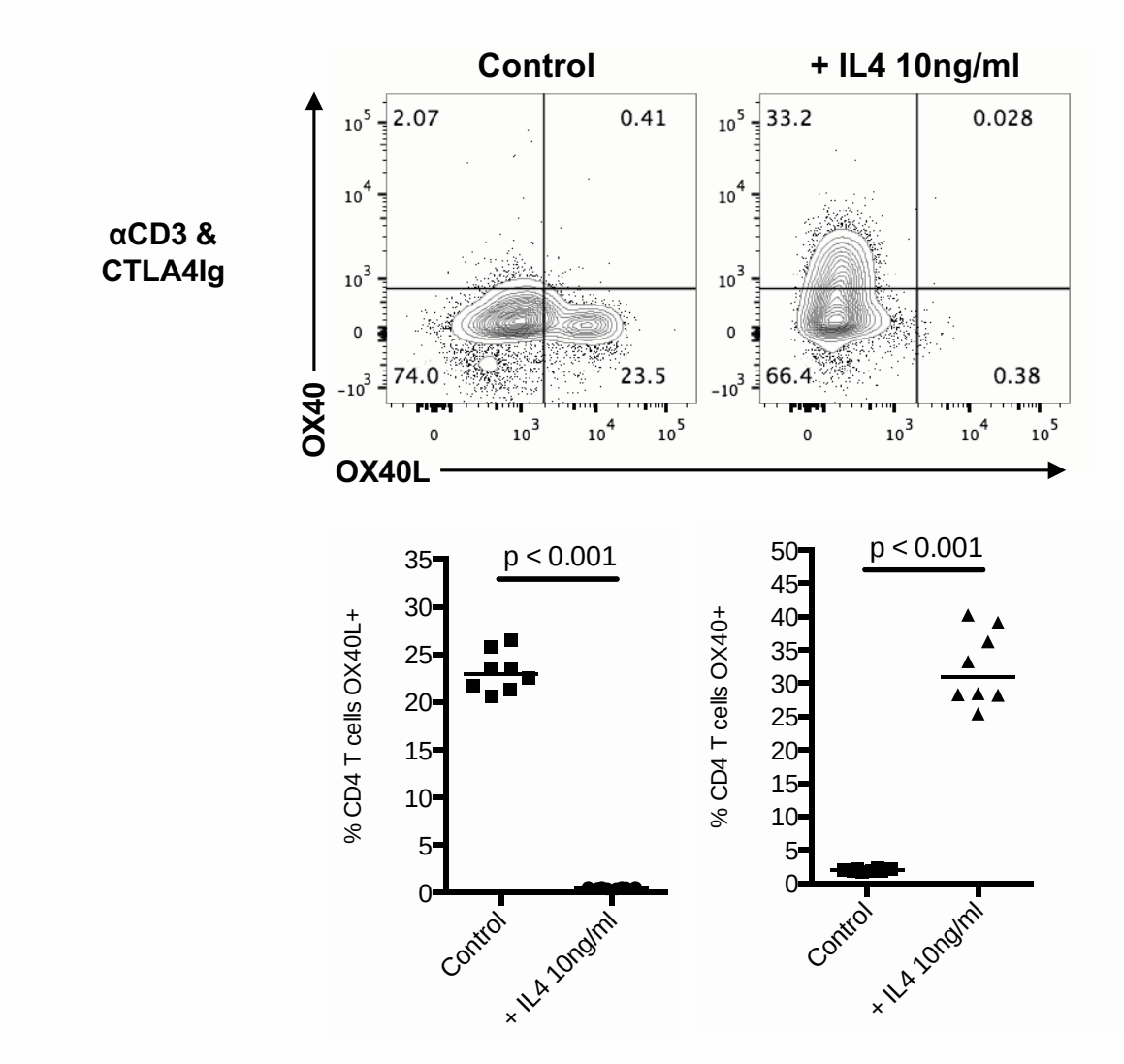


Figure 7.35: Human cells downregulate OX40L in response to IL-4. Human peripheral blood mononuclear cells were cultured for 72 hours in the presence of anti-CD3 and CTLA4Ig. IL-4 was added to some cultures. Cultures with added IL-4 demonstrated greatly reduced OX40L expression on CD4+ T cells (plots are gated on live CD4+ T cells; 8 samples per group; Mann-Whitney U-test).

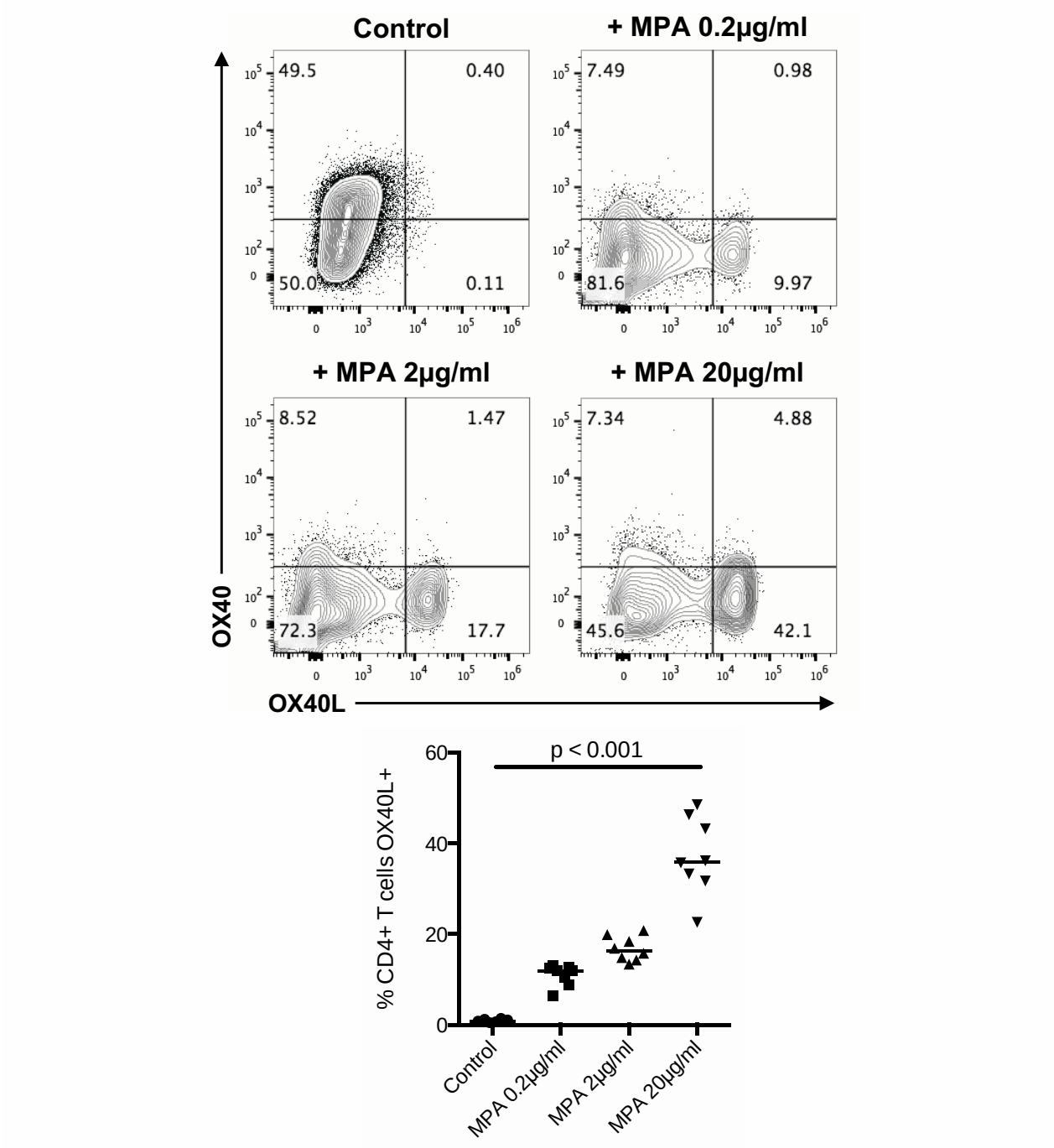


Figure 7.36: Human cells upregulate OX40L in response to mycophenolic acid. Human peripheral blood mononuclear cells were cultured in the presence of agonistic anti-CD3 antibodies for 72 hours in the presence of varying concentrations of mycophenolic acid (MPA). Increasing concentration of mycophenolic acid was associated with increased CD4+ T cell expression of OX40L (plots are gated on live CD4+ T cells; 8 samples per condition; nptrend test).

7.3 Discussion

The experiments presented in this chapter originated from the observation that CTLA4-deficient mice did not express OX40L on CD4+ T cells whilst some expression was evident on T cells from their CTLA4-sufficient littermates.

The phenotype of mice deficient in CTLA4 is of widespread lymphadenopathy, lymphocytic organ infiltration and early death. There is an increase in numbers of circulating and tissue lymphocytes with both CD4 and CD8 numbers increased; cells generally express markers consistent with activation such as CD69, CD25, CD44 and a loss of CD62L.[416, 462] A number of mechanisms of action for CTLA4 have been proposed including regulatory signals originating via ligation of CTLA4 or through CTLA4's ligands after interaction with CTLA4. However, the majority of the effect of CTLA4 appears to be a function of modulating signalling through CD28 by its major ligands CD80 and CD86: this is proposed to be a combination of high affinity regulatory binding by either cell-bound or soluble CTLA4 and by transendocytosis or the 'ripping' and internalising of ligand from the surface of expressing cells.[362, 93, 449, 450]. Mice co-deficient in CTLA4 and either CD28 or CD80 and CD86 are protected from their otherwise lethal phenotype.[261, 262] It therefore appeared that the CD80-CD86-CD28/CTLA4 axis was important in controlling T cell OX40L expression.

These experiments show that it is signals through CD28 that are key in regulating OX40L expression and that CTLA4 is only involved as modulator of signals through CD28. This was evident when agonistic anti-CD28 was used to ensure maximal signalling through CD28 or when CTLA4 was used to prevent the action of endogenous CD28 ligands (CD80 and CD86). Notably, endogenous ligands could act to downregulate OX40L *in trans*: wildtype cells down-regulated expression on $CD80^{KO}CD86^{KO}$ cells. Signals through CD28 have multiple cellular effects including the activation of many signalling kinase cascades and further work will be required to determine which is most important in decreasing OX40L expression.[3] These observations fit well with the observations that signalling through CD28 augments OX40 expression[355], although is not absolutely required for

it, and the observation presented here that OX40-OX40L expression is typically reciprocal.

A series of further experiments was performed with the intention of demonstrating conclusively that it was the modulation of signals through CD28 rather than a direct interaction with CTLA4 that explained changes in OX40L expression. First a clone of an agonistic anti-CTLA4 antibody described as being able to signal through CTLA4 was used in both wildtype and $CD80^{KO}CD86^{KO}$ splenocyte preparations.[414] This made no difference to OX40L expression in either cell type, although this experiment was hindered by the lack of a positive control for signalling through CTLA4; nonetheless this antibody at the concentrations used here is described as having effects on T cells.

To further clarify whether CTLA4 had an important role, mice chimeric for wildtype and CTLA4-deficient T cells were created. Consistent with results reported elsewhere, these mice did not become ill and T cells were not activated.[13] The resulting unactivated, but CTLA4-deficient, cells behaved identically to wildtype cells. Finally, another method of producing non-activated CTLA4-deficient T cells was employed: generating $CD80^{KO}CD86^{KO}CTLA4^{KO}$ triple-knockout mice. These mice lack the major ligands for CD28 and so activation of this system is minimal. As expected, these mice also remained healthy and *ex vivo* T cells were not activated.[261] Cells from these mice also behaved identically to those from $CD80^{KO}CD86^{KO}$ animals. Thus there is firm support for the notion that CTLA4 is not directly required to permit OX40L expression, but that it acts as an important negative regulator of CD28 signalling.

The observation that $CTLA4^{-/-}$ cells could inhibit the expression of OX40L on co-cultured CTLA4-sufficient cells in the presence of CTLA4Ig suggested that a mechanism other than ligation through CD28 was involved. IL-2 is both upregulated as a result of CD28 ligation and positively regulates OX40 expression.[101, 433] IL-2 is one of a family of related cytokines each of which contains the common cytokine receptor gamma-chain and which also includes IL-4, -7, -9, -15, and -21.[236] IL-4 is also noted to upregulate OX40.[417] It was therefore logical to investigate whether these cytokines influenced OX40L expression in addition.

In a series of experiments where T cells were cultured in the presence of CTLA4Ig to prevent endogenous CD28-ligation, I have demonstrated that administration of IL-2, IL-4 or IL-17 prevents OX40L expression on activated CD4+ T cells whilst increasing OX40 expression in a manner similar to that seen with CD28 administration. Importantly, when an anti-IL-2 antibody was added to cultures of anti-CD3 and anti-CD28 treated splenocytes, expression of OX40L could be partially restored without preventing OX40 expression: some double-positive cells were produced. This suggests that the negative regulation of OX40L may be more sensitive to IL2 than to CD28 signals, whilst OX40 expression in response to stimulation with anti-CD28 signals may be maintained.

Although it has pleiotropic effects, inhibition of mTOR signalling by rapamycin reduces IL-2 expression. The observed effect of rapamycin on OX40L expression may therefore potentially be explained through preventing the action of IL-2. Notably, in others' work OX40L expression may take many days or be of relatively low level: one potential explanation is supplementation of cultures with either or both of IL-2 and anti-CD28 antibody[278], while those who used CD3 alone revealed OX40L more quickly.[393]

Others have suggested a direct interaction between OX40 on T cells and OX40L on other cell types in producing T cell expression of OX40L. Work on human T cells cultured with CHO cells engineered to express a fluorescently marked OX40L has suggested transfer analogous to the transendocytosis reported for ligands of CTLA4.[346, 39] Others have suggested that an interaction between OX40 and OX40L limits staining for OX40L. This was in response to the observation that staining OX40L on OX40-deficient cells was reduced when they were co-cultured with OX40L-deficient cells activated so that they expressed OX40.[393]

To address the potential importance of interactions between OX40 and OX40L on OX40L expression, several experiments were performed that suggest that such an interaction is not important. First, OX40-deficient cells upregulate and downregulate OX40L similarly to wildtype cells in response to CD28 signals and exogenous IL-2; second, OX40-deficient cells co-cultured with wild-

type CD45-allotype-marked cells showed no difference in the response to stimulation with either CD28 or CTLA4Ig in otherwise identical culture milieu; third, OX40L-deficient T cells do not acquire OX40L when co-cultured with OX40L sufficient cells; fourth, OX40L-deficient non T cells are sufficient to support the expression of OX40L on purified CD4+ T cells that are otherwise unable to express OX40L when cultured alone. These experiments provide evidence against significant OX40-OX40L interactions with respect to ripping. However, it should be noted that my observations do not currently extend to human T cells. My results showing the importance of IL-2 in negatively regulating OX40L expression raise the possibility that the trans downregulation of OX40L seen in Soroosh et al.'s experiments was due to IL-2 being produced by activated, OX40-expressing cells.[393] This last would be consistent with my findings with regard to the concentration of anti-CD3 agonistic antibody being inversely related to OX40L expression.

The need for direct contact of murine CD4+ T cells with non-T cells to induce OX40L expression was unexpected. The mechanisms also remains unexplained by these experiments other than to demonstrate that B220+ B cells and probably CD11b+ cells are individually sufficient to support OX40L expression in the right circumstances. One possible candidate for this were CD40-CD40L interactions: interactions with APC CD40 are described as regulating OX40L expression and it is plausible that signals through CD40L might influence T cell expression: T cells do not express CD40.[90] However, culture with antibodies intended to block this interaction did not greatly impair expression of OX40L: the small reduction in expression with the anti-CD40 used may be explained by its partially agonistic effects and release of an unknown cytokine. Further possibilities for the identity of this interaction include a soluble molecule not stable over the distances required by our transmembrane system, or another membrane protein.

Weaknesses in this approach

Whilst these results suggest that the loss of OX40L on T cells might cause increased proliferation, they are from *in vitro* experiments only and may be influenced by differential survival. Further, the use of a Cre-recombinase system whilst assessing survival is not ideal because the presence of Cre-recombinase itself has an anti-proliferative effect in T cells.[252] Thus, *in vivo* survival and proliferation studies comparing cells constitutively deficient in OX40L and wildtype cells are necessary. In addition, whether OX40L regulation is upregulated *in vivo*, rather than *in vitro*, in response to TCR stimulation without CD28 co-stimulation is as yet unexplored but could be conducted in $CD80^{KO}CD86^{KO}$ mice. Finally, it has not been demonstrated that the OX40L expression induced in these experiments is functional, either in terms of binding OX40 or by ligating OX40 on other cell types.[393, 211, 210]

Future experiments

These experiments suggest several avenues for further investigation. One is identifying the key pathway that negatively regulates OX40L expression in response to CD28 stimulation or stimulation by IL-2, IL-4 or IL-7. One possibility is STAT5 which is activated in common by all cytokines in the IL-2 family but which does not require ligation of CD28 except as a mediator of IL-2 transcription.[431] Investigations using staining for STAT5-phosphorylation and the use of specific inhibitors represent potential methods of exploring this further. A possible common role for the IL-2 receptor common gamma chain could be further investigated with mice deficient for the gamma chain.[50] Such investigation could be combined with an assessment of the activity of other related cytokines active through the common gamma-chain subunit, for example IL-9 and IL-15.

The negative relationship of variable TCR signal strength of OX40L may be explicable by variable induction of IL-2.[334] This could be confirmed by investigating various concentrations of agonist

antibodies in the presence of blocking IL-2 antibody. Exploration of the mechanisms of regulation with respect to CD28 and IL-2-family cytokines of the other TNF ligands expressed by CD4+ T cells would be instructive; possibilities include: CD30L, LIGHT, 41BBL, GITRL and CD27L. Determining the precise cell types that are able to support the induction of OX40L would be possible by sequential cultures of purified CD4+ T cells with appropriately purified non-T cell populations. The use of flow cytometric cell-sorting should allow cell populations of greater purity. A better understanding of the cell types that were capable of supporting OX40L induction would then inform the use of blocking antibodies or cells from knockout mice in determining the molecular basis of the direct interaction.

Summary

The experiments presented in this chapter investigated the expression of the TNF receptor ligand OX40L on murine CD4+ T cells. Having noted that CTLA4-deficient T cells did not express OX40L when stimulated with anti-CD3 antibody but that CTLA4-sufficient cells did express OX40L, the role of CTLA4-CD28 was explored. It was demonstrated that OX40L required signals through the T cell receptor and that signals through CD28 negatively regulate expression; correspondingly the addition of CTLA4Ig to block signals through CD28 increases expression. Ligation of CD28 causes increased production of IL-2 and, when co-cultured with wildtype cells, CTLA4-deficient cells downregulated OX40L expression. Exogenous IL-2 downregulated OX40L expression, whilst the addition of an anti-IL-2 antibody to cultures promoted the expression of OX40L. The related gamma-chain-containing cytokines IL-4 and IL-7 had similar actions to IL-2. Thus, it appears that IL-2 may be a key mediator of CD28-control of OX40L expression.

Combination cultures of purified CD45-allotype marked T cells with mixed splenocytes, purified B cells and purified myeloid lineage cells in direct contact and across Transwell® membranes demonstrated a requirement for an unidentified direct interaction between CD4+ T cells and non-T cells

to permit expression of OX40L. In general across all experiments, OX40 and OX40L were reciprocally expressed: others have suggested interactions between the two molecules in inducing T cell expression of OX40L. However, OX40-deficient CD4+ T cells upregulated normally and there was no difference in expression between cell types in co-cultures of wildtype and OX40-deficient cells. OX40L-deficient T cells did not acquire OX40L when co-cultured with OX40L-sufficient cells, whilst OX40L-sufficient cells were sufficient to support OX40L expression in purified wildtype T cells in co-culture suggesting that OX40-OX40L interactions were not important. Early experiments suggested that loss of OX40L might be associated with increased proliferative capacity. Analogous CD28/IL-2 dependent regulation of OX40L was seen on CD8+ T cells and on human CD4+ T cells. These findings build on hitherto limited knowledge of the regulation of OX40L expression on T cells.

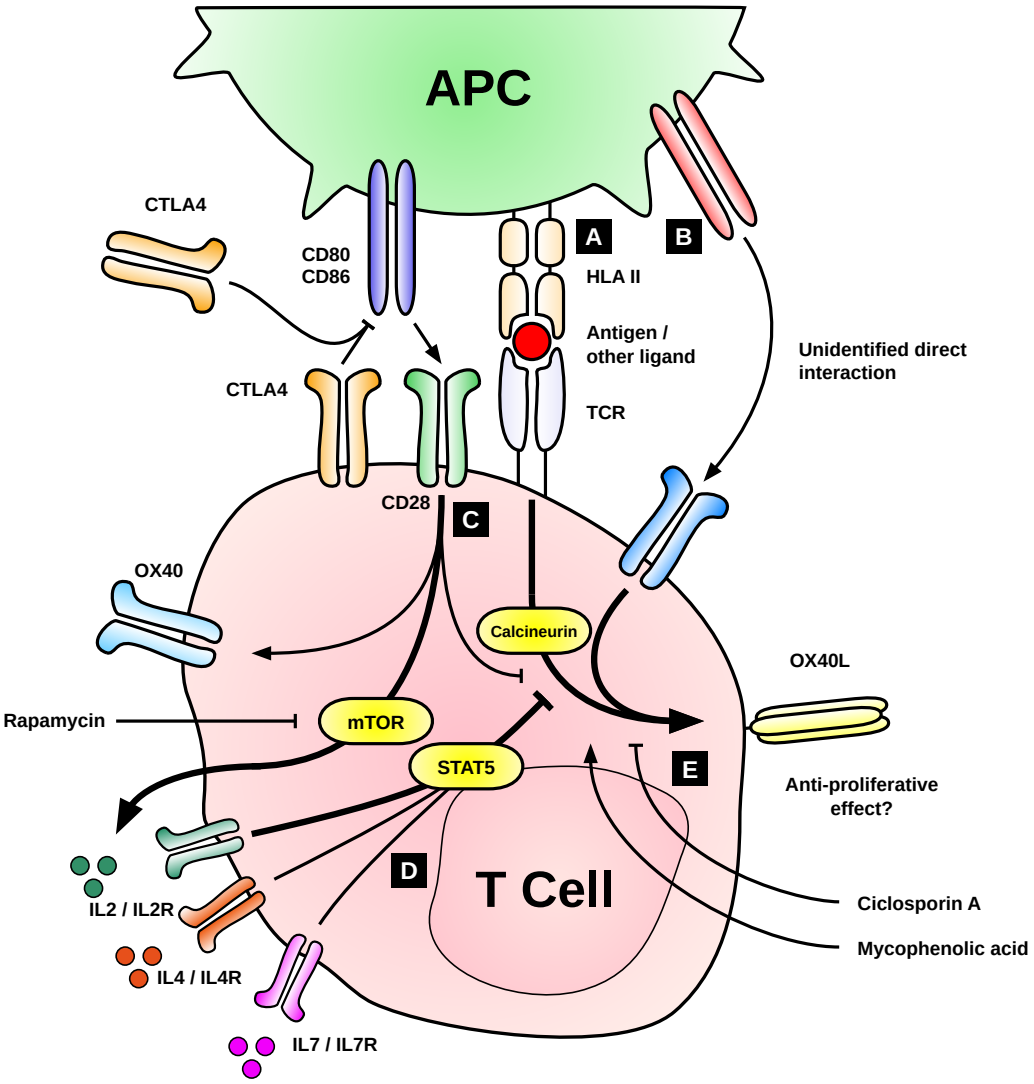


Figure 7.37: Proposed summary diagram of control of OX40L expression on T cells. T cell expression of OX40L requires ligation of the TCR, which in vivo occurs through the presentation of antigen by HLA II (**A**). An unknown second direct signal between APC and the T cell is also necessary for expression of OX40L (**B**). The ligation of CD28 promotes the expression of OX40 (**C**) as well as causing release of IL-2. IL-2 and other gamma-chain cytokines cause a STAT5-mediated reduction in OX40L expression (**D**). Ciclosporin reduces OX40L expression whilst mycophenolate enhances it, the latter through an unknown mechanism (**E**).

8 General discussion

8.1 Aims of this project

The series of experiments presented in this thesis aimed to build on the laboratory's prior work demonstrating that blockade of OX40/OX40L interactions could greatly increase overall survival in mice deficient in Tregs. Following others' observation that FOXP3-deficient mice shared some similarities with important human autoimmune liver diseases, this project aimed to assess whether such OX40/OX40L blockade also ameliorated hepatic autoimmunity in Treg deficient animals. The same approach was then applied to other mouse models of autoimmunity associated with T cell regulatory failure which also developed liver-related autoantibodies and lymphocytic hepatic infiltrates. Finally, variations in OX40L expression by T cells were apparent during experimentation and the factors influencing OX40L expression were further explored.

The aims established for this project as presented at the end of the introductory chapter are repeated below followed by a summary of findings.

1. To establish a Treg deficient model of liver injury that re-capitulates findings reported elsewhere, to demonstrate that the disease of Treg deficiency could be transferred between animals, and to explore requirements for its generation.

2. To assess whether prevention of OX40-OX40L signals could control autoimmune liver disease in the context of Treg.

3. To assess whether prevention of OX40-OX40L signals could control disease in a second mouse model of regulatory failure resulting in generalised and hepatic autoimmunity: the CTLA4-deficient mouse.

4. To assess whether prevention of OX40-OX40L signals could control disease in a mouse model of regulatory failure resulting from excessive co-stimulation and IFNγ production: the Roquin$^{M/M}$ mouse.

5. To assess factors controlling expression of OX40L expression on CD4+ T cells.

8.2 Findings by aim

1. This work has demonstrated that mice deficient in FOXP3 and therefore Treg, develop lymphocytic hepatitis. This work confirms and extends the original description of such hepatitis in these animals[504] by recording elevations in serum ALT activity, IgG, IgE, and serum TNFα as well as providing additional phenotyping of liver-infiltrating T cells. In addition, it is demonstrated that transfer of intact Tregs is sufficient to prevent the FOXP3-deficiency phenotype and that antigen specificity among transferred Tregs, at least to those antigens presented by AIRE, is not essential.

2. This project has demonstrated that blockade of OX40-OX40L interactions are sufficient to prevent the development of autoimmune liver injury in FOXP3 deficiency. In addition, immunohistochemical work has demonstrated an upregulation of OX40 in various human liver diseases including AILDs.

3. Although CTLA4-deficient mice demonstrated CD4 T cell-dense hepatic infiltrates alongside AMAs and multisystem autoimmunity in a similar manner to FOXP3-deficient animals, no significant difference was seen in the extent of this autoimmunity when OX40-OX40L interactions were prevented. This was true for both antibody-mediated blockade and constitutive deficiency in OX40.

4. Roquin$^{M/M}$ mice also demonstrated CD4 T cell-dense hepatic infiltrates alongside AMAs and multisystem autoimmunity with marked increases in ICOS expression and in IFNγ production by CD4+ T cells. Autoimmunity was not altered by OX40 blockade. This was true for both antibody blockade and for constitutive deficiency in OX40.

5. The examination of factors controlling T cell expression of OX40L has resulted in the novel observations that OX40L expression is negatively regulated by signalling through CD28 via IL-2 and other related members of the gamma-chain cytokine family including IL-4 and IL-7, perhaps acting through STAT5. Further, a requirement for direct interaction between CD4+ T cells and non T cells for the expression OX40L was established whilst the lack of transfer of OX40L to OX40L-negative cells in co-culture with OX40L sufficient cells made direct transfer of OX40L unlikely. Finally, a possible association between loss of OX40L expression and increased proliferation was demonstrated. Notably, similar findings were present in murine CD8+ T cells and human CD4+ T cells.

Incidental findings

In addition to exploring its stated aims, this project resulted in several incidental findings. The first of these was the paradoxical observation that, in contrast to human autoimmune and biliary disease, mice appeared to have serum ALP activity that was reduced in association with hepatic inflammation. Further laboratory assessment using heating of the serum suggested that the ALP present in the mouse serum analysed was heat-labile and therefore likely of bone origin. This lead to a search of the literature and the consistent finding that in the C57Bl/6 mouse strain, ALP was absent from the liver.[241] From the perspective of this project, this led to a discontinuation of measurement of serum ALP activity. Serum ALT was assessed instead. This finding of an inverse relationship with liver damage and ALP activity has wider ramifications: serum ALP activity is widely reported in the literature in relation to mouse models of biliary injury including in models on a C57Bl/6 background.[338, 425, 339, 187]. The findings presented here, together with the work of Linder et al., question this approach.[421, 241]

Adoptive transfer of T cells from FOXP3-deficient mice was necessary to permit the generation of multiple similar subjects to permit cohort experimentation. In developing the adoptive transfer

model, it was noted in preliminary experiments that transfer of FOXP3-deficient cells into ZAP70$^{-/-}$ hosts produced less marked disease than transfer into RAG$^{-/-}$ hosts. The major difference between these hosts is the presence of B cells in the former but their absence in the latter. Although in this series of experiments, experiments were continued with ZAP70$^{-/-}$ hosts because they closer to the wildtype phenotype, such findings point to a potential anti-inflammatory role for B cells. This observation contrasts with that reported by Aschermann et al who crossed FOXP3-deficient mice with μMT$^{-/-}$ mice co-deficient in B cells. The group recorded increased survival and reductions in tissue-specific infiltrates in the absence of B cells.[9] Notably my methodology differed by involving adoptive transfer: certainly the effects of the absence of B cells were apparent with the absence of AMA and an absence of B cells on flow cytometry (Figure 3.12). It is possible that the differences in T cell homeostatic proliferation in the two models may account for the variation in liver inflammation. The small numbers of B cells transferred at adoptive transfer may also have been important. A further consideration is the potential residual presence of some γδ T cells in ZAP70$^{-/-}$ animals, and these may regulate the T cell driven autoimmunity of Treg deficiency, and may perhaps explain this difference.[426]

Having established that adoptive transfer of intact Tregs was sufficient to prevent hepatic and multisystem autoimmunity associated with FOXP3 deficiency, the same model was used to investigate whether transfer of Tregs from mice deficient in the protein AIRE; AIRE is involved in the presentation of otherwise antigens that are otherwise restricted to their usual tissue of expression to developing T cells in the thymus. As such, it is important in the generation of antigen-specific Treg. In so doing, I sought an indirect answer to the question of whether antigen specificity was necessary for control of generalised regulatory failure, which is important when considering Treg therapy.[410] The issue is also of specific interest to hepatology: mutations in AIRE underlie a proportion of cases of AIH.[437]

Our demonstration that AIRE-deficient Treg were able to effectively suppress autoimmunity in the

FOXP3-deficient model suggests that antigen-specificity – at least on the spectrum dictated by AIRE – is not important for Treg control. This contrasts with work reported by a German group who transferred intact Tregs into AIRE-deficient mice with hepatitis and prevented the development of hepatitis.[132]

8.3 Application to human disease

This work supports a potential therapeutic role for blockade of OX40 / OX40L interactions in controlling the autoimmunity seen in complete deficiency of Treg in mice. Notably however, the lack of therapeutic effect seen in controlling the multi-system immunity of either CTLA4 deficiency or in Roquin$^{M/M}$ mice suggests against a generalised immunosuppressive effect. It is intriguing therefore to consider whether these observations might be applicable to human diseases associated with a loss of regulatory control.

Compounds with effective blocking activity of OX40 / OX40L interactions in humans are already in existence: for example, after evaluation in non-human primate models of asthma[372], an OX40L-specific blocking antibody has been trialled in patients with atopic asthma.[107] Although this study did not demonstrate efficacy in the clinical endpoints considered – primarily responses to allergen challenges – there were no significant adverse effects with a dosing regime of four doses over three months. Notably, small molecule inhibitors and agonists of OX40 are in development although have not yet entered clinical trials.[392] Caution with regard to increased susceptibility to infection is however warranted when using blockade of OX40-OX40L interactions: details of a single human invididual homozygous for loss of function missense mutations in OX40 has been reported.[46] Although this person was relatively healthy until the age of 19, she had suffered from childhood Kaposi's sarcoma and visceral leishmaniasis, although it is not clear if the latter was related to her immunodeficiency. In mice, susceptibility to leishmaniasis, an infection primarily controlled by the action of TH2 cells, is not increased in deficiency of OX40L.[424]

The contrasting effectiveness of OX40 / OX40L blockade in preventing autoimmunity in mice with total deficiency of FOXP3 and in those with deficiency of CTLA4 suggests that if blockade were to be assessed in human disease, directly analogous models are likely to be important. The rare IPEX syndrome results in loss of Treg function and lethal autoimmunity in affected humans. Current treatment is with non-specific immunosuppression followed by allogeneic stem cell transplant for which mortality may reach 50% and which appears to have a worsened prognosis with increasing age.[221] Thus, given the high mortality of IPEX and the potential value of a temporising therapy, an argument might be made for trials of the agent in IPEX sufferers prior to allografting. One caution if such an approach were to be tried would be careful definition of the precise subtype of IPEX being evaluated: in contrast to FOXP3-deficient mice, some IPEX patients still generate Treg but these are largely non-functional.[85]

A further potential situation where OX40 blockade might be proposed for translation to human therapy is in preventing or ameliorating autoimmunity during checkpoint inhibitor therapy for cancer. Anti-CTLA4 antibodies have become widespread in treating malignancies including melanoma, but are associated with colitis in some 35% at standard doses and hepatitis in 5%.[474] Notably, the mechanism of action of ipilimumab appears to be partially dependent on fragment crystallisable region of antibody (F_c)-mediated depletion of Tregs.[384, 370] In addition, the development of colitis is associated with colonic Treg depletion.[302] As such, it is feasible that OX40 blockade in combination with ipilimumab may represent an autoimmunity-limiting option in cancer immunotherapy, although notably the occurence of Treg depletion in human cancer immunotherapy has recently been challenged.[375]

The Lane group has gone some way towards supporting an approach of OX40 blockade to ameliorate autoimmunity in a mouse melanoma model where the addition of an anti-OX40 antibody to the combination of CTLA4 and PD-1 blockade reduced hepatic autoimmunity.[304]. Such interventions need careful assessment: the timing of OX40 blockade in relation to the autoimmune precipitant has

been shown to alter its effect in other models of autoimmunity [471, 356]; more importantly, it is not clear whether interfering with OX40 may affect anti-tumoral immunity in other models: in the Birmingham mouse model, a significant difference in anti-melanoma responses was not apparent. However, building on the observation that OX40 agonism appears to have anti-tumour effects in cancers including prostate cancer, a potentially negative effect on anti-tumour immunity cannot be discounted.[174]

8.4 Extending this work

Animal work

One of the striking aspects of this work is of the differing efficacy of OX40-OX40L blockade in preventing autoimmunity between mice deficient in FOXP3 and CTLA4 despite these mice having relatively similar OX40 blockade in cells only deficient in CTLA4 on Tregs. Given that CTLA4 is expressed by both Tregs and activated effector T cells, this suggest a role for the absence of non-Treg CTLA4 in provoking autoimmunity in my model.[166] It would be intriguing to study the effect of OX40- OX40L blockade in two alternative mouse models. First, in mice deficient in CTLA4 on Tregs alone, autoimmunity still develops [483]. If OX40 controlled this autoimmunity, it would be further evidence for a major role for non-Treg CTLA4 in driving the autoimmune response. Second, it is unclear as to whether timing of OX40 blockade dictates why it is unsuccessful in germline CTLA4 deficiency. To trial this, one could repeat the experiment in an inducible knockout of CTLA4 which also develops hepatic and multisystem lymphocytic infiltrates.[205] Preliminary data show that OX40 is not effective in this manner (Kajsa Wing, personal communication), and this is consistent with the observation that germline OX40-deficient mice are not protected from marked autoimmunity.

OX40-OX40L blockade was ineffective in ameliorating autoimmunity in Roquin$^{M/M}$ mice. This is is likely because OX40 hyperexpression is either a less important mediator of autoimmunity or there

are redundant mechanisms generating autoimmunity. Although OX40 is upregulated in Roquin$^{M/M}$ mice, greater upregulations are seen in the alternative T cell costimulatory molecule ICOS.[438] It has been demonstrated that reductions in ICOS signalling ameliorate the autoimmune phenotype seen in Roquin$^{M/M}$ disease[497], and that in other situations OX40 and ICOS cooperate to promote the overdevelopment of follicular T cells, a characteristic of Roquin$^{M/M}$ mice.[404] Therefore, combinatorial blockade of ICOS and OX40 signalling would be a logical next experiment.

In addition to considering OX40 and ICOS hyperexpression in the Roquin$^{M/M}$ mouse, it is notable that isolated hyperexpression of IFNγ is sufficient to produce hepatic infiltration with activated T cells and the production of AMA.[14] Although antibody blockade of IFNγ was not sufficient to abrogate autoimmuntiy in the experiments reported here, it might be that combinations of ICOS, OX40 and IFNγ blockade would be more effective.

Two different strains of mice that develop hepatic autoimmunity associated with a failure to generate self-antigen specific Tregs have been reported: AIRE dysfunction and the more recently reported FezF2[406] dysfunction. Given the efficacy of OX40-OX40L blockade in the autoimmunity of complete Treg deficiency, it is likely that OX40-OX40L blockade would be efficacious in these models. This is of particular interest because AIRE dysfunction has a direct human correlate in APECED.[98]

OX40L expression on T cells

With regard to OX40L expression on T cells, several avenues are appropriate for following up on the key findings of this project: that OX40L is negatively regulated by CD28 ligation through the production of IL-2 in a mechanism that appears to involve STAT5; that an uncertain direct interaction between T cells and non T-cells is required to support the expression of OX40L; and the demonstration of an association of OX40L expression with reduced proliferation.

Of the mechanisms above, the first might reasonably be assessed by using cells from animals either

constitutively deficient in STAT5 or by assessing inducible knockouts. These could be assessed alone and in co-culture experiments where STAT5-deficient cells might be expected to express more OX40L than their wildtype counterparts and be resistant to IL-2-induced downregulation.[457]

The obsevation – of the requirement for direct interaction to induce OX40L – should ideally be confirmed by using a different conformation of culture chamber: ideally where cells were closer together but still separated by a cell-impermeable membrane to reduce the likelihood of an unstable soluble mediator causing the effect. Further co-culture experiments with different purified preparations of support cells other than the B220+ and CD11b+ positive populations would permit a search, perhaps in conjunction with databases of molecular expression, for a potential cell-surface molecule permitting OX40L expression.[137] The role of OX40L in affecting cell turnover could valuably be further assessed by increasing the number of experiments presented here. In addition to expanding in vitro work, similar methodology *in vivo* could be used to assess survival[254], as could the creation of bone marrow chimeras of OX40L-sufficient and OX40L-deficient animals.

Human translation

A further characterisation of human OX40+ T cells in health and disease would be enlightening in the context of these results: is upregulation seen only on CD4+ T cells? What state are these cells in and what are their subtypes? Is upregulation seen primarily in autoimmune conditions or other causes of chronic inflammation such as viral hepatitis? Extraction of lymphocytes from human liver is well-reported and consistent and would provide a mechanism to answer these questions.[76] As described above, there is a rationale for extending this work into humans by trialling OX40 / OX40L blockade in either IPEX or AIRE-deficiency. There is a potential role in ipilimumab-associated autoimmunity associated with Treg depletion. Given that we have demonstrated increased expression of OX40 on T cells and within the livers of mice lacking Treg, it would be a logical step to look for upregulation of OX40 in inflamed tissue from IPEX and AIRE-deficient patients and in tissue

samples from patients with ipilimumab-associated colitis or hepatitis. Were widespread upregulation of OX40 to be seen such tissues this might further support a rationale for trialling OX40 / OX40L blockade in that disease, although it is noted that OX40 expression was also upregulated in Roquin$^{M/M}$ and CTLA4-deficient mice where OX40 / OX40L blockade is not effective.

8.5 Overall summary

The interactions of the TNF-receptor OX40 and its ligand OX40L primarily act to sustain activated T cells, especially CD4+ T cells. Mice deficient in Tregs develop lethal multisystem autoimmunity which shares some features with human autoimmune liver disease including production of CD4+ T cell rich hepatic infiltrates and the presence of the autoantibody AMA. Previously the Lane group has demonstrated that interrupting OX40 / OX40L interactions increases survival in Treg deficiency.

In this series of experiments, I recapitulate the presence of selected features of human autoimmune liver disease in mice lacking Tregs including CD4+ T cell rich liver infiltrates and AMA. In addition, this project draws further parallels with human disease by recording evidence of liver damage by elevations in serum ALT activity and demonstrating elevations in serum IgG and IgE concentrations. I go on to demonstrate increased expression of OX40 on T cells in these mice. I then demonstrate that replacing healthy Treg either as a component of total cells from syngeneic mice or sorted Treg alone is sufficient to control disease. Antigen specificity of transferred T cells was unlikely to be important because cells from AIRE-deficient mice were also able to abrogate autoimmunity.

The effects of blocking antibodies to OX40L and a non-depleting blocking anti-OX40 F_{ab} were examined in FOXP3-deficiency. Both treatments totally abrogated autoimmunity but blockade of OX40-OX40L interactions did not prevent hepatic damage in the T cell cytokine mediated model of Con A liver damange nor in chemically mediated CCl_4 liver injury.

Two other models of systemic autoimmunity associated with deficits in T cell regulation – the CTLA4$^{-/-}$

mouse and the Roquin$^{M/M}$ mouse were examined for analogous hepatitis. This was present in both animals but in neither was liver disease ameliorated by either antibody blockade of OX40-OX40L interactions or co-deficiency of OX40.

During these experiments variablility in CD4 T cell expression of OX40L was noted and subsequently explored in a number of culture experiments. I demonstrate that OX40L expression on both CD4+ and CD8+ murine T cells requires TCR ligation but that it is negatively regulated by signals through CD28. The effects of these signals through CD28 appear to limit OX40L expression through the action of IL-2 and other related gamma-chain cytokines partly through the action of STAT5. Further, for CD4 T cells to express OX40L, direct contact with non T cells including either B cells or macrophages is essential. OX40L expression appears to be associated with reduced proliferation. Human CD4+ T cells appear to behave similarly.

These results suggest that interruption of OX40-OX40L signals may powerfully control T cell mediated autoimmunity in some forms of dysregulation. However, the effect is not universal. These results suggest potential value in OX40-OX40L blockade as a therapy in some forms of human autoimmune disease, but the specific situations will need to be carefully chosen.

9 Publications associated with this thesis

9.1 Journal articles

- **Webb, G. J.**, Siminovitch, K. A. & Hirschfield, G. M. (2015). The immunogenetics of primary biliary cirrhosis: A comprehensive review. *Journal of Autoimmunity*, 64, 42–52.[473]

- Dyson, J. K., **Webb, G.**, Hirschfield, G. M., Lohse, A., Beuers, U., Lindor, K., & Jones, D. E. J. (2015). Unmet clinical need in autoimmune liver diseases. *Journal of Hepatology*, 62(1), 208–218.[89]

- **Webb, G. J.** & Hirschfield, G. M. (2016). Using GWAS to identify genetic predisposition in hepatic autoimmunity. *Journal of Autoimmunity*, 66, 25–39.[468]

- **Webb, G.**, Chen, Y.-Y., Li, K.-K., Neil, D., Oo, Y. H., Richter, A., et al. (2016). Single-gene association between GATA-2 and autoimmune hepatitis: A novel genetic insight highlighting immunologic pathways to disease. *Journal of Hepatology*, 64(5), 1190–1193.[465]

- **Webb, G. J.**, Hirschfield, G. M. & Lane, P. J. L. (2016). OX40, OX40L and Autoimmunity: a Comprehensive Review. *Clinical Reviews in Allergy & Immunology*, 50(3), 312–332.[471]

- **Webb, G. J.** & Hirschfield, G. M. (2017). Primary biliary cholangitis in 2016: High-definition PBC: biology, models and therapeutic advances. *Nature Reviews Gastroenterology and Hepatology*, 14(2), 76–78.[469]

- Nawaf, M. G., Ulvmar, M. H., Withers, D. R., McConnell, F. M., Gaspal, F. M., **Webb, G. J.**, Jones, N. D., Yagita, H., Allison, J. P., and Lane, P. J. L. (2017). Concurrent OX40 and CD30 Ligand Blockade Abrogates the CD4-Driven Autoimmunity Associated with CTLA4 and PD1 Blockade while Preserving Excellent Anti-CD8 Tumor Immunity. *The Journal of Immunology*, 199(3), 974–981.[304]

- Jeffery, H. C., Jeffery, L. E., Lutz, P., Corrigan, M., **Webb, G. J.**, Hirschfield, G. M., et al. (2017). Low-dose interleukin-2 promotes STAT-5 phosphorylation, Treg survival and CTLA-

4-dependent function in autoimmune liver diseases. *Clinical & Experimental Immunology*, 188(3), 394–411. http://doi.org/10.1111/cei.12940.[169]

- Dufton, N. P., Peghaire, C. R., and Osuna-Almagro, L., and Raimondi, C., and Kalna, V., and Chuahan, A., **Webb, G.**, et al. (2017) Dynamic regulation of canonical TGFβ signalling by endothelial transcription factor ERG protects from liver fibrogenesis. *Nature Communications*. 8(1)895.[87]

- **Webb, G. J.**, Hirschfield, G.M., Krawitt, E.L., & Gershwin, M.E. (2018) Cellular and Molecular Mechanisms of Autoimmune Hepatitis. *Annual Review of Pathology: Mechanisms of Disease*, 13, 247–292.[470]

- **Webb, G. J.**, Rana, A., Hodson, J., Akhtar, M. Z., Ferguson, J. W., Neuberger, J. M., et al. (2018). Twenty-Year Comparative Analysis of Patients With Autoimmune Liver Diseases on Transplant Waitlists. *Clinical Gastroenterology and Hepatology*, 16(2), 278–287.e7.[472]

9.2 Letter

- Trivedi, P. J., Weston, C. J., **Webb, G. J.**, Newsome, P. N., Hirschfield, G. M., & Adams, D. H. (2015). Serum alkaline phosphatase in multidrug resistance 2 ($Mdr2^{-/-}$) knockout mice is strain specific. *Hepatology*.[421]

9.3 Conference abstracts

- Autoimmune Liver Disease In The UK: A National Primary Care Evaluation Of Disease Geoepidemiology. **Webb, G. J.**, Ryan, RP., Marshall, TPM., and Hirschfield, GM.. **BASL 2017, platform presentation**

- Blockade of OX40-OX40L prevents the development of hepatic autoimmunity in a regulatory T cell deficient model. **Webb, G. J.**, Gaspal, F., Eddowes, P., Reynolds, G., Hirschfield, G. M. and Lane, P. **EASL 2017, platform presentation**

- Blockade of OX40-OX40L prevents the development of hepatic autoimmunity in a regulatory T-cell deficient model. **Webb, G. J.**, Gaspal, F., Reynolds, G.M., Eddowes, P. J., Hirschfield, G. M., and Lane, P. J. L. **AASLD 2016, poster**

- Examining unmet need: international trends in transplantation for autoimmune liver disease. **Webb, G. J.**, Rana, A., Akhtar, M. Z., Ferguson, J. W., Neuberger, J. M., Vierling, J. M., and Hirschfield, G. M. **AASLD 2016, poster**

- AIRE is not required for T regulatory cells to regulate the hepatic autoimmunity of FOXP3 dysfunction. **Webb, G. J.**, Gaspal, F., McCarthy, N., Hirschfield, G. M., and Lane, P. J. L. **AASLD 2016, poster**

- Autoimmune hepatitis with T-regulatory cell deficiency in the context of GATA-2 dysfunction: A novel single gene association. **Webb, G. J.**, Richter, A. G., Blahova, M., Curbishley, S. M., Li, K. -K., Neil, D. A. H., Oo, Y. H. , Adams, D. H., Bigley, V. H., Collin, M. P. and Hirschfield, G. M. **EASL 2015, poster**

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