THE ROLE OF THE CD28-CD80 AXIS AND MOLECULAR INHIBITORS OF T CELL CO-STIMULATION IN THE POTENTIAL TREATMENT OF AUTOIMMUNE CHOLESTATIC LIVER DISEASES

by

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Abstract

Primary biliary cholangitis (PBC) is an autoimmune liver disease characterised by bile duct damage, increased portal inflammation and elevated inflammatory activity. In this work we studied the novel CD80 antagonist called RhuDex. Our aim was to block the CD28-CD80 pathway *in vitro* using liver-infiltrating mononuclear cells to study the effect of the drug in the liver of patients with PBC. RhuDex inhibited T cell proliferation in a co-stimulation system using autologous T cells co-cultured with transgenic CHO-CD80 cells. By phenotyping intrahepatic B cells, monocytes, and dendritic cells from patients with end-stage liver diseases we found that B cells and monocytes express the highest levels of CD80 and CD86, however no differences were detected across diseases. Co-culture of intrahepatic T cells with autologous B cells or monocytes in the presence of RhuDex inhibited T cell proliferation irrespective of the presence of CD80 on the surface of the antigen-presenting cells. Similar effects were seen in the absence of APC or upon strong T cell activation. These results demonstrate that RhuDex is a potent inhibitor with the capacity to modulate immune responses in the liver of PBC patients.

Dedication

To my grandfather, Dionysios “Sfiridas” Chionis,

and to my grandmother, Dionysia Leftaki Chioni.

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Abbreviations

aCD28	Anti-CD28
aCD3	Anti-CD3
ADPKD	Autosomal dominant polycystic kidney disease
ADPLD	Autosomal dominant polycystic liver disease
AICD	Activation-induced cell death
AIH	Autoimmune hepatitis
AINR	Activation-induced non-responsiveness
ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine aminotransferate
AMA	Automitochondial antibodies
AML	Acute myeloid leukemia
ANCA	Antineutrophil cytoplasmic antibodies
APC	Antigen-presenting cell
AST	Aspartate aminotransferase
BAFF	B-cell activating factor
BCR	B cell receptor
BEC	Biliary epithelial cell
Breg	Regulatroty B cell
CDAA	coline deficient and amino acid defined diet
cDC1	Conventional dendritic cells 1
cDC2	Conventional dendritic cells 2
CHO	Chinese Hamster Ovary cell
CIA	Collagen type II-induced arthritis
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMP	Damage -associated molecular pattern
DC	Dendritic cell
DN	Double negative
DP	Double positive

EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EMA	European medical agency
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and drug administration
FL	Follicular lymphoma
FXR	Farsenoid X receptor
GC	Germinal centre
GGT	Gamma-glutamyl transferase
hBD-3	Human β defensine-3
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HepA	Hepatitis A virus
HepE	Hepatitis E virus
HFE	Hereditary hemochromatosis
HL	Hodgkin's lymphoma
HSC	Hepatic stellate cell
HTLV	Human T-lymphotropic virus
IBD	Inflammatory bowel disease
ICOS	Inducible co-stimulator
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iNKT	Invariant natural killer T cell
ITC	Isothermal titration calorimetry
LIMC	Liver-infiltrating mononuclear cells
LPL	Lamina propria leukocytes
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cell
MAdCAM-1	Mucosal addressing cell adhesion molecule
MCD	Methionine/choline deficient diet
mDC	Myeloid dendritic cells

mdDC	Monocyte-derived dendritic cells
MFI	Median fluorescence intensity
MLR	Mixed lymphocytic-reaction
MMF	Mycofenolate mofetil
MMPs	Matrix metalloproteinase
MS	Multiple Sclerosis
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NHL	non-Hodgkin's lymphoma
NK	Natural killer cell
NOD	Non-obese diabetic
norUDCA	24-norursodeoxycholic acid
OCA	Obeticholic acid
PB	Plasmablasts
PBC	Primary biliary cholangitis
PBL	Peripheral blood lymphocytes
PBS	phosphate- buffered saline
PBMC	Peripheral blood mononuclear cells
PC	Plasmacells
PC1	Plasma cell alloantigen 1
PD-1	Programmed cell death 1
pDC	Plasmacytoid dendritic cells
PDC-E2	Pyruvate dehydrogenase subunit E2
PLD	Polycystic liver disease
PPD	Purified protein derivative
PsA	Psoriatic arthritis
PSC	Primary sclerosing cholangitis
RA	Rheumatoid Arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640
RT-PCR	Real-time polymerase chain reaction
S1PR1	Sphingosine-1-phosphate receptor-1

SLE	Systemic lupus erythematosus
SLP1	Sphingosine-1-phosphate lyase-1
SPR	Surface plasmon resonance
TBB-5	β-tubulin isotype 5
TCR	T cell receptor
Tfh	T follicular helper cells
Th1	T helper cell 1
Th2	T helper cell 2
Th17	T helper cell 17
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TT	Tetanus Toxoid
UC	Ulcerative colitis
UDCA	Ursodeoxycholic acid

Chapter 1 - Introduction

1.1. The liver architecture, function, and microenvironment

1.1.1. The importance of studying liver diseases

Liver disease refers to more than 100 different conditions all of which affect the liver function leading to liver failure and eventually death. More than 90% of all liver disease is due to high alcohol consumption, viral hepatitis, and obesity (1). 7 out of 10 people are unaware of some liver damage (2). Deaths from major diseases such as cancers, hearth disease, and diabetes are decreasing yearly, however deaths from liver diseases are increasing. Deaths from liver diseases have increased by more than 400% since 1970 (3). In 2018, liver diseases and liver cancer together were responsible for 2.5% of deaths in England, with patients being between the ages of 18-65 (4). In 2018 it was estimated that liver diseases will overtake the deaths by coronary heath diseases by 2020 (5). In the UK alone over 600,000 people have developed liver disease and over 60,000 have developed cirrhosis (3). In 2016-2017 in England, more than 68,000 admissions to hospitals were due to liver disease; £2.1 billion per year is spent to treat the patients (6, 7). Rate of admissions is also increasing; in 2012/2013 admissions to hospitals reached 56,000 patients (7). Liver transplantation is often recommended to patients with extensive liver damage. In 2019, 432 patients were on the active transplant list (8). Unfortunately, the most common reason for not using donor livers for transplantation is due to the high fat content in those livers, which accounts for 39% of all donor livers (3). It is evident from the above data that there is a great need for enhancing our understanding of liver diseases.

1.1.2. The liver as an immune organ

The human liver is the largest internal organ of the body making up 2-3% of the average body weight (9). The liver consists of two lobes and is located in the upper right quadrant of the abdominal cavity. Up to 500 separate functions have been attributed to the liver, including nutrient absorption and storage, bile production, detoxification, digestion, and immune homeostasis. The liver has a dual blood supply, the portal vein where blood is transferred from the intestines, pancreas, spleen, and gallbladder, as well as the hepatic artery which supplies the liver with arterial blood. The majority of the blood enters the liver through the portal vein. Apart from nutrients, pathogen-derived molecules can also enter through the portal vein (10, 11). When the epithelium of the gut becomes damaged, whole pathogens can be translocated into the liver (12). The pathogens avoid tissues where immune system activation could take place such as the lymph nodes and the spleen, and instead immune responses are mounted in the liver (13). This type of pathogen entry does not result in systemic infection, but instead the liver is able to clear the pathogens successfully by mounting its own immune response.

1.1.3. The liver microenvironment

The human liver is comprised of a number of different cells. Around 60-80% of the cells are parenchymal cells, known as hepatocytes (14, 15). They are large cells and are responsible for the main metabolic functions of the liver (9, 16–19). Hepatocytes form links between each other using tight junctions, allowing them to make contact with other non-parenchymal cells (20). Hepatocytes secrete bile into canaliculi which merge to form the bile ducts (21). The canaliculi are present on the apical surface of the hepatocytes whereas on the basolateral surface, hepatocytes come into contact

with venous blood (22–24).

The rest of the cells are called non-parenchymal cells. They comprise of the Kuppfer cells, natural killer cells, invariant natural killer T cells, liver sinusoidal endothelial cells, the biliary cells, stellate cells, T cells, B cells, monocytes and the dendritic cells. Below we will cover their different roles (including hepatocytes) with an emphasis in immune responses, however, T cells, B cells, monocytes, and dendritic cells will be covered later in the introduction chapter.

1.1.3.1. Kupffer cells

The Kupffer cells are resident macrophages in the liver. They do not extravasate to other tissues and make up to 90% of all tissue macrophages (25). They reside in the liver sinusoids and are able to recognise foreign antigens through complement, Fc, Toll-like receptors (TLR), and scavenger receptors (26). Upon recognition, the Kupffer cells phagocytose foreign pathogens and destroy them in the lysosomes (27). Kupffer cells are also able to clear neutrophils, secrete pro- or anti- inflammatory cytokines, and can also present antigen through MHC class I and II to T cells, although they are poor stimulators (28, 29).

1.1.3.2. Natural killer cells

Natural killer cells (NK) make up for one-third to one-half of all lymphocytes in the liver (30, 31). Their role is to screen the liver for possible infections (32). The way NK cells recognise foreign bodies is by testing targets for the absence of self. Upon recognition of cells lacking the expression of self-antigens, NK cells become activated and destroy the cell through cytokine production such as IFN-γ (30). Pathogens or tumours can down regulate the expression of self molecules to avoid detection by the

immune system. Recognition of self takes place through activatory and inhibitory receptors on the surface of the NK cell. If self is not recognised by the NK cell, then the balance between the receptors is lost and the cell releases perforin and granzyme to eliminate the pathogen (26).

1.1.3.3. Invariant natural killer T cells

Invariant natural killer T cells (iNKT) are characterised by their invariant TCR which can recognise glycolipid antigens when bound on the CD1d receptor, an MHC class I type molecule (33, 34). The number of iNKTs in humans is smaller compared to those in mice suggesting that they contribute in greater extent in mice compared to humans (35). INKTs can be detected through the recognition of α-GalCer-CD1d tetramer, which their TCR can bind to (36). Two different subsets of these cells have been identified, the type I which can recognise the tetramer, and the type II which recognises the self-lipid sufatide and cannot bind to α-GalCer or other antigens (37, 38). INKT cells are considered to be part of the innate immune system for a number of different reasons. They express an invariant TCR and a number of different surface receptors which are part of the natural killer cell lineage, they are activated early during immune responses, and are also able to activate a number of different cell types (39, 40). Different iNKT type I subsets have been identified and are categorised by their cytokine expression. They include the IFN-γ, the IL-4, and the Il-17 subset (41–43). In the liver both the IFN-γ subset and the IL-4 subset have been described, with the latter supporting the regrowth the tissue (44). INKT cells are mainly present in the liver and patrol the sinusoids of the liver (26). Upon activation, these cells adhere to the vasculature (45, 46). Cessation of movement is a result of binding to α-GalCer and detection of cytokines such as IL-12 and IL-18 (47).

1.1.3.4. Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSEC) are responsible for the formation of liver sinusoids, and make up for 15-20% of the total liver cells (48). LSEC recognise waste through scavenger receptors on their surface and can remove them from the blood through pinocytosis (49, 50). LSECs can act as APCs, presenting antigen through either MHC class I or class II molecules (51–53). They can also suppress T cell immune responses by expressing the programmed cell death 1 (PD-1) on their surface (51, 52, 54). As a result, these cells can both induce T cell tolerance but also mount immune responses fast in the presence of foreign particles entering the body through the gut.

1.1.3.5. Hepatocytes

Hepatocytes are not only responsible for the various liver functions but also possess immune properties. They express pattern-recognition receptors for the detection of foreign particles and can act as APCs by expressing stimulatory molecules such as MHC class I and class II, CD80 (also known as B7-1), and CD86 (B7-2) (55, 56). Hepatocytes have the capacity to present antigen to naive T cells and mount immune responses (57–60). T cells adhere to hepatocytes through the expression of ICAM-1 (61). CD8 T cells were shown to crawl across the vasculature and patrol the surrounding tissues by protruding their cell membrane through sinusoidal fenestrations searching for antigen presented by the hepatocytes (62). Upon antigen recognition, they adhere and kill the hepatocyte. In the absence of hepatocyte contact during viral infections, infected hepatocytes cross-present antigen to the LSEC which in turn present the antigen to $CD8^+$ T cells through their MHC class I molecule and activate the T cells to secrete TNF, killing the hepatocyte (51).

1.1.3.6. Biliary epithelial cells

Biliary epithelial cells (BEC) make up for 3-5% of the total liver cells and come in many different sizes (63). BEC all come together to form the biliary tree, with is a network of cannaliculae creating intrahepatic ducts of different diameters (64). Hepatocytes are responsible for bile secretion which is then transported through the liver inside the bile canaliculi (65, 66). BEC also play a role in acid transportation and production while also regulating the acidification of the bile through secretion of $HCO3^-$ (63). BEC also show immunogenic features. TLR-2 and TLR-4 are present on the surface of the BEC and ligation of the receptors activates the transcription factor NF-κB (67). Upon isolation BEC express MHC class I and II, and can upregulate their CD80 and CD86 expression in culture (68). However, in this study the researchers found no APC activity from the BEC. In PBC, BEC express MHC class II molecules (69, 70).

1.1.3.7. Hepatic stellate cells

Hepatic stellate cells (HSCs) make up for 8-10% of the total liver cells and reside in the space of Disse, the space between the hepatocytes and the LSEC. HSCs store vitamin A and are responsible for controlling the blood flow in the sinusoids (18, 71). They can also secrete matrix metalloproteinases and deposit collagen leading to liver fibrosis (18). HSCs can present antigens to $CD4^+$, $CD8^+$ T cells, and iNKT cells by expressing MHC class I and class II molecules as well as CD1b and CD1c for lipid presentation (72). They also express CD86 and CD40 (a co-stimulatory molecule expressed by B cells and other APCs which binds to the T cell-expressed ligand CD40L providing activation signals to the T cell) indicating that these cells can fully stimulate T cells and initiate immune responses (73–75). In the presence of IFN-γ,

HSCs upregulate CD80 (72). However, these cells are not as capable as hepatic DCs to mount immune responses (76).

1.1.4. The architecture of the liver

The liver is organised in a way which allows for the maximal interactions of the immune system and the pathogens. The sinusoids are vascular channels which allow for blood flow inside the liver (77). T cells, Kupffer cells, iNKT cells, and DCs are present in that area. The hepatocytes are located around the sinusoids. The sinusoids, composed by the LSEC, allow for molecules and cells to be transferred to the hepatocytes due to their fenestrations. The space between the endothelial cells and the hepatocytes is called the space of Disse which contains the hepatic stellate cells, secreting collagen and fibronectin (21, 78, 79). BEC make up the biliary track and can be the first point of contact with pathogens, which can enter through the biliary tract (80, 81) (**Fig. 1**).

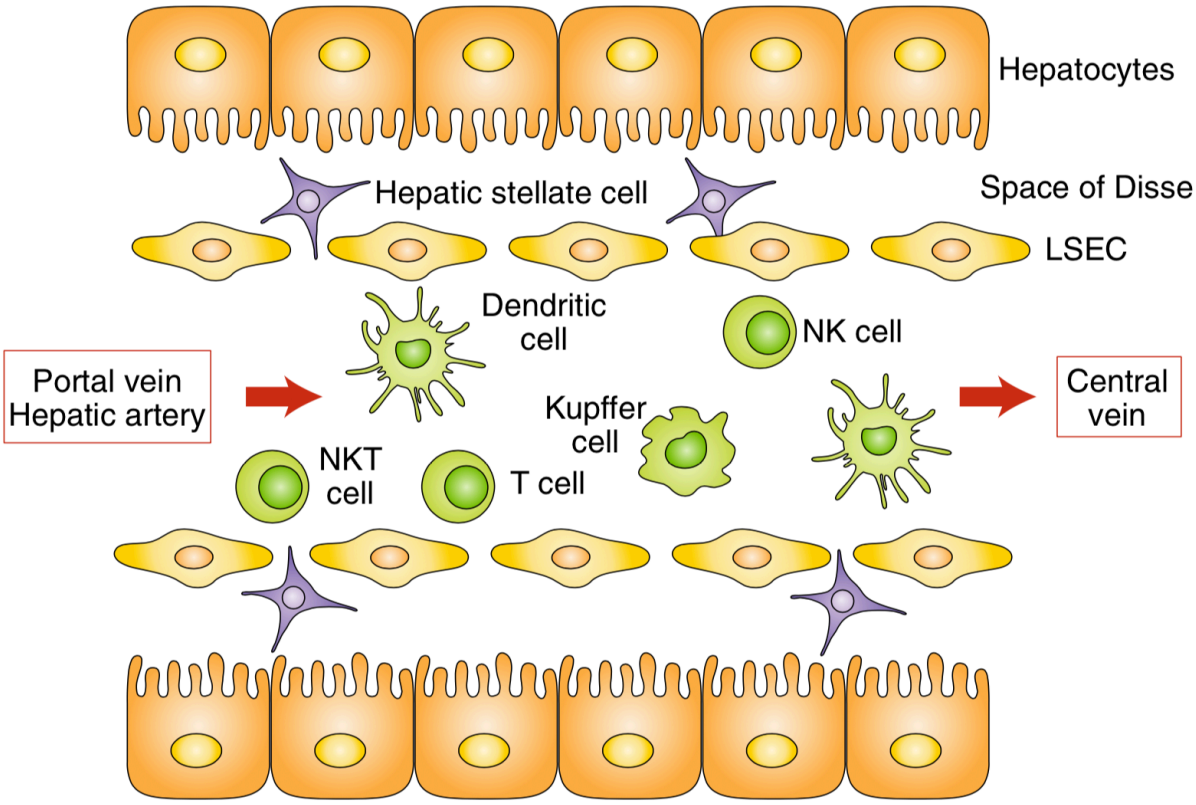

Figure 1: The architecture of the human liver. Blood enters from the portal vein, and traverses through the sinusoids where dendritic, NKT, T, Kupffer, and NK cells are present. The blood then exits the liver through the central vein. The sinusoids are composed of the liver sinusoid endothelial cells (LSEC). The hepatocytes surround the sinusoids and the hepatic stellate cells are found in the space of Disse. From Doherty et al. (77).

1.2. B cell subsets

B cells are part of the adaptive immune system. They compose of 5-10% of the total lymphocyte population and are considered professional antigen presenting cells, similarly to dendritic cells and macrophages (82, 83). They are able to internalise antigens through the B cell receptor (BCR) and present it to T cells via MHC class II surface receptors. Together with the MHC:peptide complex, B cells also present T cells the co-stimulatory molecules CD80 and CD86. Presentation of antigen through MHC class II results in B cell activation and differentiation into either

antibody-secreting B cells or memory B cells (83, 84). Several different B cell subsets exist upon naive B cell activation.

Germinal centre B cells (GC, also known as follicular B cells) differentiate in response to activation and are present in secondary lymphoid organs such as the lymph nodes and the spleen (85). These B cells interact with T cells in the GC and activate them by presenting antigen and co-stimulatory molecules (85, 86).

B cells which have received signals to exit the GC differentiate into memory B cells or plasmablasts (87). Maturation of plasma blasts differentiate them into plasma cells which secrete high amounts of antibodies. Plasma blasts are characterised by rapid proliferation and migration from the blood circulation into the bone marrow from which they secrete antibodies.

Memory B cells are characterised by their big size, increased proliferative, and increased antigen presentation capacity (88–90). Up-regulation of anti-apoptotic transcription factors such as BCL-2 results in the generation of long lasting B cells whereas somatic hypermutation of their BCR is responsible for the generation of several different subsets (91, 92).

Regulatory B cells (Breg) create an anti-inflammatory environment by secretion of IL-10 and TGF-β (93). The importance of these cells is highlighted in mouse studies where mice with IL-10-deficient B cells develop experimental autoimmune encephalomyelitis (EAE) characterised by Th1 responses, whereas adoptive transfer of IL-10-producing B cells suppressed EAE (94).

Transitional B cells represent a B cell subset which arise after immature B cells egress from the bone marrow to mature to other subsets (95, 96). Developmentally, transitional B cells are in a state between the immature B cells and the naive B cells (97). These cells are present in the bone marrow, peripheral blood, and secondary lymphoid organs (98). The development of transitional B cells takes place in two

different steps: the first one is achieved in the bone marrow where autoreactive B cells are eliminated (99). The second checkpoint selection is in the spleen where transitional B cells receive survival signals before they differentiate into other subsets like naive or marginal zone B cells (99, 100).

$CD24^{-}CD38^{-}$ and $CD24^{-}CD38^{int}$ B cells are two novel populations of B cells described by our lab (101). These cells are increased in the livers of end-stage liver disease patients. $CD24^{-}CD38^{int}$ B cells were shown to secrete both pro-inflammatory cytokines (IL-6, INF-γ) and anti-inflammatory cytokines (IL-10). Their role in liver diseases is still under investigation.

1.3. Monocyte subsets

The human monocytes are immune cells generated in the bone marrow and have phagocytic properties (102, 103). They initially circulate the blood and are then recruited into tissues. Monocytes make up for 10% of the total leukocyte population in the human peripheral blood (104). Monocytes are part of the innate immune system, and can mount immune responses against bacteria, viruses, fungi, or parasites (105, 106). They mediate killing of microbial pathogens through phagocytosis, production of reactive oxygen species (ROS), nitric oxide, or through secretion of type I interferons such as IFN-α and IFN-β (106).

Human monocytes are classified into three different subsets based on the expression of two markers, CD14 and CD16. CD14 is a glycoprotein and acts as an accessory protein to TLR-4 which can bind to lipopolysaccharide (LPS) (107). CD16 is also known as Fcγ receptor III and is an immunoglobulin with a role in antibody-dependent T cell cytotoxicity (108). Three different monocyte subsets are the classical ($CD14^{++}CD16^{-}$), the intermediate ($CD14^{+}CD16^{+}$), and the non-classical ($CD14^{+}CD16^{++}$)

monocytes (104, 109–111). The classical monocytes represent 90% of the total monocytes whereas intermediate and non-classical only 10% (102, 112). Classical monocytes derive from the bone marrow and give rise to intermediate monocytes in the blood stream, which will further differentiate into non-classical monocytes (103).

Classical monocytes show greater phagocytic activities compared to the other two subsets (109). They are better at constraining the fungus *Aspergillus fumigatus* and are more efficient than intermediate monocytes in secreting ROS (113–115). Similarly, this subset secretes pro-inflammatory cytokines such as IL-6, IL-8, and CCL2, and is able to migrate to inflamed tissues through expression of chemokine receptors such as CCR2 and CXCR5 (114, 116, 117).

Intermediate monocytes express the highest level of MHC class II molecules compared to the other subsets and secrete TNF-α, IL-1β, IL-6, and IL-10 upon TLR stimulation (116–120). They were increased in the blood of patients undergoing systemic infections however their full role is still under investigation (121, 122).

Non-classical monocytes display a different vasculature crawling pattern compared to the other subsets and are considered as patrolling monocytes (123) They utilise the respiratory chain metabolism compared carbohydrate metabolism used by classical monocytes (124). Furthermore, this subset has the capacity for antigen processing however its transcriptomic profile shows greater capacity for wound healing. They are also able to clear dying endothelial cells through TLR-7 expression (125, 126). Finally, these cells secrete high levels of TNF-α for the recruitment of neutrophils (127, 128).

1.4. Dendritic cell subsets

Dendritic cells (DCs) are part of the adaptive immune system and are responsible for activating T cells in response to antigen uptake through the expression of MHC class I and II on their surface (87). Three DC subsets have been identified, the plasmacytoid DCs (pDC), the myeloid DCs (mDC), and the monocyte-derived DC (mdDC).

Plasmacytoid DCs respond strongly to viral infections by secreting type 1 interferons (129, 130). Human pDCs have antigen-presenting abilities however the are worse APCs compared to mDCs. They present antigen to $CD8^+$ T cells through antigen processing and presentation by MHC class I receptors on their surface, however, $CD4^+$ T cell activation is weaker compared to $CD8^+$ T cell activation (130–132). Two different subsets of pDCs have been identified, pDC1 and pDC2. The first subset is characterised by low expression of MHC class II whereas pDC2 express high levels of MHC class II and CD86 (130). It is worth considering that the two subsets are not part of strong linages and can differentiate between the two subsets depending on the environment.

MDCs, are known for their strong antigen-processing and presentation abilities (129). MDCs are very mobile and are able to traverse between tissues and secondary lymphatic organs (133). Upon maturation, this type of DC can secrete IL-12 which induces naive T cell differentiation into a Th1 phenotype that secretes IFN-γ (134). MDCs can also differentiate into two different subsets, the $CD141^+$ (conventional DC1) and the $CD1c^+$ (conventional DC2). $CD141^+$ DCs are present in tissues like the lungs, skin, and peripheral blood (135). They are strong T cell stimulators by expressing co-stimulatory molecules at high levels while also producing large amounts of type III interferons such as IFN-λ upon antigen recognition (136–138).

Human $CD1c^+$ DCs are the richest type of DC in the peripheral blood (139). They are present in lymphoid and non-lymphoid tissues and can also express CD80 and CD86 upon antigen uptake (140). $CD1c^+$ DCs are able to differentiate naive T cells into Th1, Th2, Th17, and Treg, unlike $CD141^+$ DCs which can only generate Th1 cells (135). Indeed, $CD1c^+$ DCs have been characterised as the main Th2 and Th17 producers upon antigen stimulation (141, 142).

MDDCs are derived from monocytes and resemble the $CD1c^+$ DCs by expressing similar transcriptional signatures (143). Under physiological conditions, generation of mdDCs is not a priority however, upon infection, murine classical monocytes differentiated into inflammatory mdDCs and were recruited to the tissue (144). Indeed, many studies have shown that upon infection (viral or with the use of adjuvant), inflammatory monocytes were recruited to the site by up regulation of tissue-resident chemokine markers (CCR7 for lymph node homing or CCR6 for skin extravasation) and were differentiated into pro-inflammatory mdDCs which were able to mount $CD4^+$ and $CD8^+$ T cell responses (145–147).

1.5. T cell development, stimulation, activation, and inhibition in humans and mice

1.5.1. Development of T cells

The cells responsible for the orchestration and maintenance of immune responses are the T cells. They originate from hematopoietic stem cells which are present in the

bone marrow and are responsible for the production of all the immune cells present in the human blood (148). T cells migrate from the bone marrow to the thymus for development. Only T cells develop in the thymus whereas all other immune cells develop in the bone marrow. At the stage of migration to the thymus, T cells are called early thymocyte progenitors, which home to the thymus by expression of chemokine receptors CCR9 and CCR7 (149, 150). At the thymus, Notch ligand initiates and sustains the development of T cells until they become mature T cells and exit the thymus (151, 152).

Initially, the thymocyte progenitors are negative for both CD8 and CD4 expression, commonly referred to as double negative (DN) $CD4^-CD8^-$ T cells. Upon entering the double negative stage, the fate of these cells to become T cells is locked by genome recombination which results in the formation of the pre-T cell receptor (TCR) (153). Then through pre-TCR signalling, in the presence or absence of antigen, T cells express both *cd4* and *cd8* and enter the second stage of their development, the double positive (DP) stage which is characterised by the double expression of both $CD4^+$ and $CD8^+$ markers (154, 155). The pre-TCR, which consists of the α and β chains, is now supported by CD4 and CD8 molecules, allowing for recognition of peptides presented on MHC class I and class II molecules (156). Upon recognition of self-antigens by the CD8CD4TCR complex, DP T cells undergo positive and negative selection. During this process only a minority of T cells survive which do not recognise self-antigens strongly (157, 158). These cells will eventually mature into functional T cells. The DP T cells which interacted with the MHC class II molecule at the thymus retain their CD4 expression and those who interacted with the MHC class I molecule retain their CD8 expression (159, 160). Eventually these cells become the single positive $CD4^+$ and $CD8^+$ T cells, exit the thymus and become mature naive T cells.

1.5.2. Priming of T cells through signal one

T cell activation requires the ligation of the MHC (class I or II) bearing a peptide (pMHC) to the TCR (156, 161). The TCR comprises of a single α kai β chain (162). In addition, TCR can also consist of γ and δ chains but only in a small number of $\gamma\delta$ T cells are present in humans (163). The constant region of the TCR is bound onto the cell membrane whereas the variable region is exposed extracellularly to allow binding to the pMHC complex. TCR alone is unable to initiate immune responses. Other proteins are needed for activation, like the proteins belonging to the CD3 family, CD3γ, CD3δ, and CD3ε (164). Binding of TCR to the pMHC complex induces conformational changes in the CD3 proteins leading to their phosphorylation and eventually activation of the inflammatory transcription factor NF-κB (165, 166). The role of CD4 and CD8 molecules is to stabilise interactions between the TCR and the MHC class II and MHC class I molecules, respectively (167–169). Furthermore, these two co-receptors participate in signal transduction upon TCR engagement (170, 171).

1.5.3. Activation and expansion of T cells through signal two

1.5.3.1. The two-signal theory

Activation of T cells through the TCR alone is not enough to initiate immune responses. TCR stimulation alone drives the T cell into a state called anergy (172–175). Anergy is defined as a state during which a T cell upon TCR engagement is unable to respond to IL-2 stimulation and thus it does not proliferate. In order for a T cell to become a professional T cell with inflammatory functions, it needs to overcome the activation threshold. This can happen through secondary stimulation

by another T cell receptor called CD28. CD80 and CD86 are the ligands for CD28 and are expressed on APCs. The signal transmitted through the CD28 is referred to as signal two. T cell activation is known as the two-signal theory which states that for a naive T cell to proliferate, two signals are necessary. The first is received through the TCR and signal two through the CD28 (176). In order to study CD80 and CD86, we need to look at them in the context of the CD28-CD80/CD86 pathway, which we will be discussing below **(Fig. 2)**.

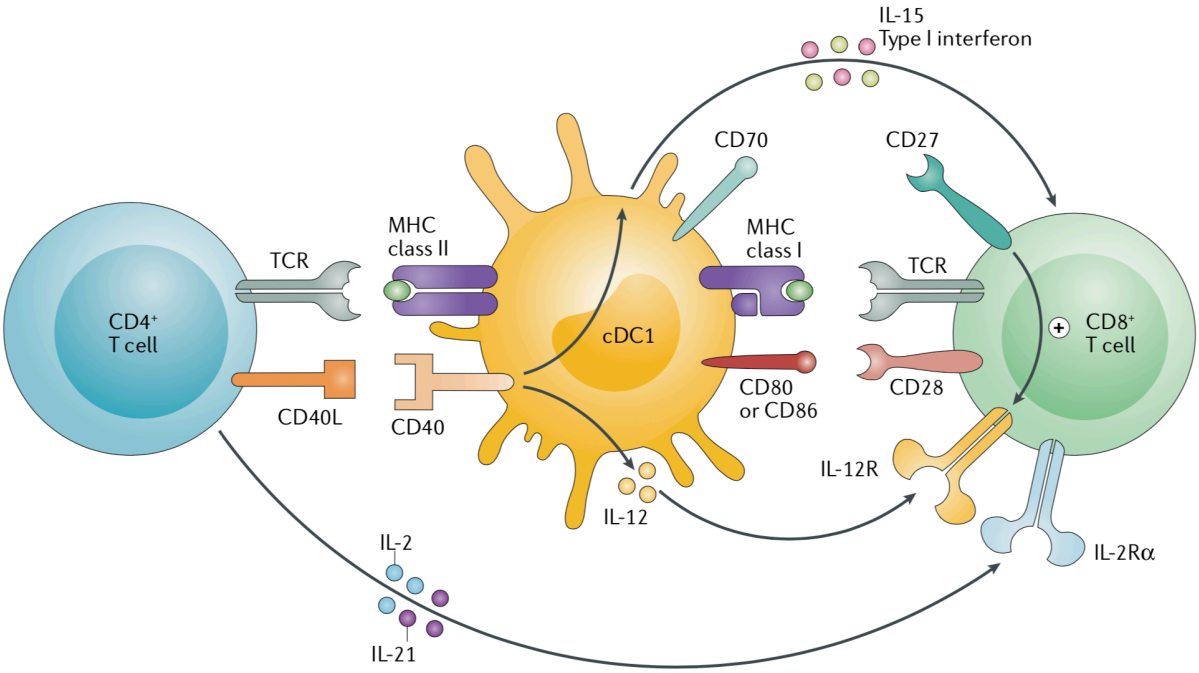

Figure 2: The two-signal model. The two signal model dictates that for a T cell to become primed for activation, two signals are required. The first signal is received through the TCR upon engagement with the MHC:peptide complex, and the second signal is received through the co-stimulatory receptor CD28 as a result of binding to the ligands CD80 and CD86. A third signal can also be provided by the APC, in the form of cytokines, which decides the fate of the T cell. From Borst et al (177).

1.5.3.2. The CD28 receptor

1.5.3.2.1. Structure of CD28

The *CD28* responsible for the generation of the T cell receptor consists of four

exons which encode 220 amino acids (178). CD28 is a homodimer bound together by disulphide bonds. The extracellular domains of CD28 are a paired set of immunoglobulin superfamily domains which attach to a transmembrane domain and a cytoplasmic domain, the latter containing signalling motifs (179).

CD28 has two ligands, the CD80 and the CD86. CD80 is mainly present as a dimer expressed on the cell surface of an APC whereas CD86 exists as a monomer (180, 181). CD28 binds to its two ligands through the two immunoglobulin domains (182). CD80 and CD86 also bind to another receptor called CTLA-4 (CD152), expressed on TCR-activated T cells (183–186). Engagement of CTLA-4 with either CD80 or CD86 results in abrogation of T cell responses (187–190). CD28 and CTLA-4 compete with each other for the binding of the two ligands. The conformation of CD80 binding to CD28 is different compared to CD80 binding with CTLA-4 (191, 192). CD28 does not bind strongly to CD80 despite the presence of an active binding site for the ligand (193). However, CTLA-4 receptor provided a better degree of complementarity for the CD80. This is supported by studies showing that CD80 binds more strongly to CTLA-4 compared to CD28 in a monomeric or dimeric form (183, 194, 195). Interestingly, CD86 shows faster association and dissociation rates with CTLA-4 compared to CD80. The kinetics of binding CD80 or CD86 with CD28 is relatively comparable (193). CD86 is less likely to make stable dimers compared to CD80 (196, 197). Furthermore, the constant region of the ligands as well as the intracellular domains are important for providing proper T cell stimulation (181). Deletion of either resulted in poor secretion of IL-2 by Jurkat cells. These results indicate that CD28 shows preference to bind with monomers whereas CTLA-4 prefers to bind with dimers.

1.5.3.2.2. Functions of CD28 on genetic and epigenetic levels

Engagement of TCR and CD28 with monoclonal antibodies showed that T cells are able to secrete high amounts of IL-2, prolonging their survival (198, 199). Furthermore, CD28 engagement upregulates the expression of Bcl-xL, an anti-apoptotic protein, further prolonging their survival. In addition, CD28 stimulation enhanced the stability of the mRNA of IL-2, IFN-γ, TNF-α, and GM-CSF (200). TCR stimulation alone has little effect in initiating the transcriptional mechanism of naive T cells, however TCR and CD28 stimulation were able initiate the transcription on a large number of genes (201). This effect is enhanced at 24 hours after stimulation (202). IL-2 stimulation can substitute for CD28 engagement. Similarly, blocking the IL-2 receptor α chain known as CD25 did not inhibit the transcription of genes of CD28-stimulated T cells after 24 hours.

CD28 stimulation epigenetically reprograms the genome of a T cell. CD28 promotes extensive chromatin remodelling, increased histone acetylation, and loss of cytosine methylation at the *IL-2* locus (203, 204). *C-Rel*, a proto-oncogene which is part of the Rel/NF-κB transcription factor family, is required for the expression of IL-2 (205, 206). C-Rel is also responsible for the remodelling the promoter region of *IL-2* (207). T cells from mice deficient for c-Rel did not show activity for micrococcal nuclease in the promoter of *IL-2*, an enzyme used to measure chromatin remodelling.

1.5.3.3. The CTLA-4 receptor

1.5.3.3.1. Structure of CTLA-4

CTLA-4 belongs to the immunoglobulin superfamily. The protein is 223 amino acids long and consists of one V-like domain, surrounded by two hydrophobic regions (208). The gene consists of four exons; exon 1 is a signal peptide, exon 2

encodes for the V-like domains which is responsible for binding to CD80 and CD86, exon 3 encodes for the transmembrane domain, and exon 4 encodes the cytoplasm tail. Different splicing variants have been detected for CTLA-4. The protein responsible for the binding to CD80 and CD86 is the full variant which encodes all four exons whereas the soluble form of CTLA-4 lacks exon 3 (209, 210). The full variant is transcribed after T cell activation (211). The soluble variant is present in the cytoplasm of naive T cells however upon activation the expression was suppressed (212). The soluble CTLA-4 was also found in the serum of healthy subjects.

1.5.3.3.2. Expression of CTLA-4

CTLA-4 expression on T cells is strictly regulated. CTLA-4 is expressed 24 hours after T cell stimulation (213). In Treg it constitutively expressed allowing for T cell suppression irrespective of the presence or absence of CD28 on the Tregs (214). CTLA-4 is also expressed on the immune and non immune cells like DCs, monocytes, B cells, and $CD34^{+}$ stem cells however its role is under investigation (215). The transcription factor NFAT is responsible for the transcription of *CTLA-4* in humans, which binds to the proximal promoter of the gene (216). Cyclosporin A, which inhibits NFAT activation, also inhibits the expression of CTLA-4 (217). Furthermore, NFAT interacts closely with the master regulatory T cell gene Foxp3 to modulate the expression of CTLA-4 (218, 219). Mutations of Foxp3 repressed the ability of Treg to secrete IL-2 and upregulated the expression of CTLA-4 and CD25.

CTLA-4 is expressed on the cell surface of resting T cells but only in small amounts and is quickly internalised by clathrin and dynamin (220). In the cytoplasm, CTLA-4 is localised inside the endosomes and can be degraded inside lysosomes. The clathrin adaptor complex AP-1 is responsible for the binding of CTLA-4 intracellularly at the Golgi apparatus through CTLA-4's cytoplasmic tail (221). When

CTLA-4 is on the surface, AP-2 binds to the protein for internalisation. Binding of AP-1 to CTLA-4 in the Golgi compartment prevents transportation of the protein to the lysosomes for degradation, regulating the amount of CTLA-4 present in the Golgi compartment. Upon T cell activation, calcium levels are increased intracellularly and CTLA-4 is transported to the surface, towards the sites of TCR activation (186).

1.5.3.3.3. Functions of CTLA-4

CTLA-4 regulates T cell activation through binding to CD80 and CD86. CTLA-4 in mice blocked IL-2 production, CD25 expression, and inhibited T cell proliferation (222). When CD28 co-stimulation was not present, the inhibitory effects of CTLA-4 were minimal compared to CD28-activated T cells. In addition, IL-2 and CD25 inhibition peaked at 72 hours after activation, and exogenous IL-2 was able to revert the effects of CTLA-4. Moreover, inhibition of the cell cycle progression from the G1 phase to the S phase has been previously described (223). Furthermore, CTLA-4 limits the interaction between T cells and APCs by increasing T cell motility, resulting in reduced antigen-presenting capacity by the APCs (224). CTLA-4 can negatively modulate CD80 and CD86 through transendocytosis (225). The ligands CD80 and CD86 are then degraded in lysosomes of the CTLA-4-expressing cell. Mice lacking for CTLA-4 expression on Treg, develop systemic lymphoproliferation with reduced Treg capacity to suppress DC- and anti-CD3 antibody-stimulated T cells (226). Finally, DCs produce 2,3-dioxygenase, which catalyses tryptophan degradation as a result of Treg-CTLA-4 binding on CD80 or CD86 on DCs (227, 228).

1.5.3.4. The CD80 and CD86 ligands

CD80 and CD86 are the ligands for CD28 and CTLA-4. CD80 was first identified in

1991 by creating a hybrid protein CD80-Ig which bound to CHO-CD28 transfected cells (195). Shortly after that CD86 was also described (184, 185). CD80 is also a ligand partner to PD-L1 (also known as B7-H1) (229). PD-L1 is the binding partner of PD-1 (230). Interaction between PD-1 and PD-L1 inhibited T cell proliferation and cytokine secretion (230, 231). Recently it was shown that PD-L1 can dimerise with CD80 and weaken the interaction between either PD-L1:PD-1 or CD80-CTLA-4 (232). This interaction also prevented the transendocytosis of CD80 from the APC.

CD80 and CD86 are expressed on a number of different APCs including B cells, monocytes, and DCs (233–236). CD86 is constitutively expressed on cells whereas CD80 is expressed upon stimulation (236–241). CD86 is expressed faster after TLR ligation or cytokine stimulation compared to CD80 (242, 243). CD80 is a type I transmembrane glycoprotein with two extracellular domains, one distal variable-like and one proximal constant-like to the membrane Ig domains (244). CD86 shares a similar structure however they only share ~30% homology (197).

To elucidate the differences in the role of CD80 and CD86, deficient transgenic mice were generated for CD80, CD86, or both. Mice deficient for both ligands lacked germinal centre formation and did not generate class-switched immunoglobulins in the presence of adjuvant (245). When CD80 or CD86 was absent in mice stimulated with antigen, immune responses were mounted as normal indicating that CD80 and CD86 have overlapping functions. However, mice deficient for CD86, when immunised without Freund's adjuvant also failed to undergo antibody-switching or form germinal centres whereas CD80-deficient mice acted similarly to wild type mice under stimulation. The above evidence shows that CD86 is important in initiating antibody responses, however CD80 can compensate for the absence of CD86 when antigen is present. Despite these differences, neither ligand is required for the differentiation of naive T cells into a Th1 or Th2 phenotype (246). IL-4 production by

either these molecules is dependant on the activation state of the T cell. Naive T cells require the co-stimulatory molecules CD80 or CD86 to initiate production of IL-4 whereas already activated T cells do not (247). The cytoplasmic tails of CD80 and CD86 are important for initiation of T cell stimulation. Mutations on CD80 or CD86 cytoplasmic tails inhibit T cell stimulation (181, 248, 249).

1.5.3.5. Role of co-stimulation in helper T cell generation

T cell co-stimulation is not only necessary for the initiation of immune responses but also for the differentiation of naive T cells into T helper cells. The strength of TCR engagement is responsible for differential T cell fate. Weak TCR engagement resulted in the generation of Th2 cells able to secrete IL-4, however strong TCR signal did not have the same effect (250). When CD28/B7 interaction was disrupted, T cells were unable to skew into a Th2 phenotype however, cross-linking with an anti-CD28 antibody reversed the effect of the blockade. Different co-stimulatory molecules also promote different T cell fates. CD4, CD28, and OX-40 interactions differentiated T cells into a Th2 phenotype whereas LFA-1 suppressed Th2 responses and supported Th1 responses (251). However, CD28 and OX-40 can also induce Th1 phenotype, suggesting that in order for a naive T cell to follow one fate, multiple signals are necessary (251). The amount of time of TCR engagement also affects the fate of the T cell. When the extracellular signal-regulated kinase (Erk) was transiently expressed, T cells differentiated into Th1 phenotype, whereas when Erk activity was reduced, the levels of IL-4 were increased, indicating that T cells differentiated into Th2 (252). Upon strong antigenic signals such as IL-12 secreted by CD40-primed DCs *in vitro*, T cells differentiate into Th1 and secrete IFN-γ (253, 254). Similar results were obtained from *in vivo* studies in mice. Anti-CD40L antibody differentiated T cells into Th1 phenotype (255). In addition, mice which had a knockout mutation for CD40L were

unable to mount Th1 responses against *Leishmania major*, however administration of IL-12 protected the mice from infection (256). CD28 ligation also promotes differentiation into Th2 phenotype in wild type mice but not in CD28 deficient mice (257). Furthermore, T cells from mice deficient for CD28 expression were able to secrete IL-4 when exogenous IL-4 was added to the culture, whereas wild type T cells did not secrete more IL-4 in the presence of exogenous IL-4 and CD28 stimulation, indicating that IL-4 production is regulated by CD28 (258). Addition of exogenous IL-4 on Th2 cell culture did not increase the production of IL-4 (259).

Generation of Th17 cells, a pro inflammatory subset of T cells responsible for recruiting neutrophils and macrophages to infected tissues, is also mediated through CD28 signals but in an indirect way. CD28 stimulation is responsible for the up-regulation of the inducible co-stimulator (ICOS), a receptor involved in T cell proliferation, cytokine, and antibody secretion by the B cells (260–262). T cells stimulated through ICOS differentiate into a Th17 cell and secrete IL-17, IL-21, and IFN-γ (263). CD28 stimulation overrode the ICOS stimulation which reduced the levels of transcription factor RORC2 as well as the levels of IL-17 secreted by the T cells.

1.5.3.6. Role of co-stimulation in the generation of regulatory T cells

CD28 stimulation is essential for the survival of Tregs. In mice deficient for CD28, 80% of Tregs were depleted (264). Addition of exogenous IL-2 in CD28-deficient mice restored Treg homeostasis indicating that the survival of Tregs depends on IL-2 secretion by CD28 stimulation. Adoptive transfer of Treg from CD28-deficient mice into wild type mice and blockade with anti-B7 antibodies also promoted the deletion

of Tregs, which means that IL-2 is not solely responsible for the survival of Tregs. CD28 stimulation also enhances the expression of *foxp3* as well as *CTLA-4* on double positive thymocytes (265, 266). The differentiation of thymocytes into Tregs is a result of proximal IL-2 secretion as a result of CD28 stimulation. NOD mice deficient for CD28 or CD80/CD86 have reduced numbers of peripheral Tregs and develop spontaneous diabetes (267). Lack of Tregs in these mice is possibly due to the lack of thymic production of Tregs. Adoptive transfer of wild type Tregs into either of the two deficient mice prevents the development of diabetes.

CTLA-4 is also constitutively expressed on Tregs and can be induced on conventional T cells. Mice deficient for CTLA-4 developed extensive lymphoproliferation and tissue destruction, and developed severe myocarditis, which resulted in death by week 4 (268). Absence of CTLA-4 expression in knockout mice does not lead to the development of autoimmune diseases unless the cytoplasmic tail of CD28 is devoid of mutations, since any mutation prevents the development of autoimmunity (269). Similarly, mice lacking CTLA-4/CD80 or CTLA-4/CD86 expression developed severe lymphoproliferative disease whereas mice deficient for CTLA-4/CD80/CD86 did not, further suggesting that co-stimulation is mandatory for the development of a lymphoproliferative phenotype (270). Conditional knockout of CTLA-4 from adult mice also resulted in spontaneous lymphoproliferation, pneumonitis, gastritis, and development of autoantibodies, however the symptoms are not fatal unlike in previous studies (271). Interestingly, CTLA-4 deficient mice were resistant against EAE.

1.6. Chronic liver diseases

1.6.1. Autoimmune family liver disorders

1.6.1.1. Autoimmune hepatitis

Autoimmune hepatitis (AIH) is a complex liver disease with not fully understood etiological drivers. AIH was first described in the 1950s (272). The annual prevalence of AIH can range from 4.0 to 24.5 per 100,000 patients depending the geographical location (273, 274). AIH mainly affects females, with a female to male ratio of 4:1 (275). The disease has been split into two subtypes, type 1 and type 2, based on the type of autoantibodies present in the patient. Type 1 is characterised by the presence of anti-nuclear (ANA) and anti-smooth muscle (SMA) antibodies, whereas in type 2 there is evidence of anti-liver kidney microsomal antibody type 1 (LKM1), anti-LKM3, or anti-liver cytosol type 1 (LC1) antibodies (276). Based on the sub-classification of the disease, differences arrise between the two subtypes. Type 1 is more abundant however type 2 is predominantly female with a 9:1 female to male ratio (277).

The pathophysiology of AIH remains incomplete. Genetic, epigenetic, and environmental drivers contribute to disease progression. The *HLA* locus contains many allelic variants for the *DRB1* gene which have been associated with AIH (278). Other genes outside of the *HLA* locus have also been identified as risk factors for AIH, however limited information could be retrieved due to the small patient numbers in these studies. *CTLA-4, STAT-4, IL-13, IL-12A* as well as the common *SH2B3* have been identified as risk loci (279, 280). These associations are weak and without genome-wide significance present ($p \leq 10^{-8}$), however the presence of these loci in other immune-mediated diseases indicates that the findings are probably

genuine (279).

Furthermore, it is suggested that foreign epitopes have been responsible for immune activation through molecular mimicry. They include viruses such as hepatitis A (HepA), hepatitis C (HCV), and hepatitis E (HepE) (281–283). In addition, breaking of tolerance in the microbiome can contribute to increased permeability of external bacteria into the system through the gut, which can trigger immune responses (284). Dysbiosis was shown to result from antibiotics or poor diet. During dysbiosis, the intestinal permeability becomes disrupted resulting in increased introduction of pathogen-associated molecular patterns, damage-associated molecular patterns, and endotoxins, all of which traverse through the hepatic sinusoids, and could lead to initiation of immune responses (285–287).

Immunologically, in the liver of AIH patients tolerance has been broken. This clear by the increased numbers of $CD4^+$ and $CD8^+$ liver-infiltrating T cells (288, 289). Peripheral blood mononuclear cells (PBMCs) from patients with AIH have decreased levels of CD80, CD86, and CTLA-4 compared to healthy controls (290). However, comparison of liver-infiltrating mononuclear cells (LIMCs) to PBMCs from patients with AIH revealed that these markers are elevated in the liver. T cells from patients secreted higher levels of IL-4 and IL-10 compared to patients with viral hepatitis or healthy controls (288). $CD4^+$ T cells localise in portal areas of the liver whereas $CD8^+$ T cells are found in peri-portal areas (291). AIH has been previously described as a result of immune reconstitution of $CD4^+$ T cells after patients received antiretroviral therapy for the treatment of the human immunodeficiency virus (292). Furthermore, in the blood of AIH pattens $CD4^+$ and $CD8^+$ T cells were found to express the apoptotic marker CD95 (Fas/APO-1) whereas healthy controls did not (293). The expression of the marker correlated with the expression of CD45RO on the T cells, an indication of T cells being primed in AIH. It is worth mentioning that high

expression of the Fas ligand has also been detected on $CD4^+$ T cells in ALD and $CD8^+$ T cells in PBC (294). The presence and possible role of $CD8^+$ T cells in the liver of patients with AIH could be highlighted by the presence of MHC class I on damaged hepatocytes (295, 296). This molecule is not present in healthy liver. Furthermore, intrahepatic mRNA levels for perforin and granzyme B were elevated in patients with AIH compared to healthy tissue (297, 298). These molecules are associated with $CD8^+$ T cell cytotoxicity. Furthermore, real-time polymerase chain reaction (RT-PCR) showed that in patients with AIH, $CD8^+$ T cells present features of T cell clonality, indicating a possible antigen-specific response (299). Finally, Treg in AIH were shown to have impaired ability to secrete IL-10, suppress $CD4^+$ T cells and, respond to IL-2 (300).

B cells in AIH are limited (289). They are present in portal tracks, similarly to $CD4^+$ T cells (291). Plasma cells are elevated and are predominantly IgG^+ (301). Mice which were immunised with human liver DNA antigen and then treated with the B cell depleting antibody anti-CD20 had reduced inflammation in the liver, increased number of naive but decreased number of professional $CD4^+$ and $CD8^+$ T cells (302). The levels of secreted IgG however remained the same despite the B cell depletion (302). In another study, B cells were shown to have a protective role in experimental AIH by suppressing $CD4^+$ T cells responses through expression of CD11b (303). This mouse model was generated by immunisation with the S100 antigen which is hypothesised to affect the pathway of the disease. Depletion of B cells exacerbated the disease. Using the anti-CD20 antibody rituximab in humans, the researchers found that it was well tolerated and reduced the levels of IgG in the serum, improving the inflammation score and prevented the increase of Treg in the liver of the patients (304).

Monocytes are rare in AIH compared to healthy subjects (289). AIH-derived

monocytes secrete high levels of TNF-α, express high levels of TLR-4, and are prone to migration in the presence of chemoattractants (305). Co-culture of monocytes with Tregs enhanced the migration of monocytes, upregulated the secretion of TNF-α, and increased the surface expression of TLR-4.

The current treatment options for patients with AIH includes corticosteroids and azathioprine. This line of treatment allows for suppression of the immune system and prevention of liver cirrhosis (306). Second line of treatments are also being explored like the inosine monophospahde dehydrogenase inhibitor Mycofenolate Mofetil (MMF) which inhibits the synthesis of guanosine-5'-monophosphate, or calcineurin inhibitors such as Cyclosporin A and Tacrolimus which inhibit calcineurin, responsible for the production of IL-2 (278). These drugs have shown positive results, however, no data have been collected regarding their efficacy upon disease relapse (307).

1.6.1.2. Primary biliary cholangitis

Primary biliary cholangitis (formerly known as primary biliary cirrhosis, PBC) is a prototypical autoimmune chronic liver disease. It is characterised by the presence of automitochondial antibodies (AMA), small bile duct destruction which results in chronic cholestasis, immune portal infiltration, and fibrosis which may lead to cirrhosis, and eventually liver failure. Patients with PBC can be asymptomatic at the day of diagnosis (308). Most common symptoms of PBC include pruritus and fatigue. In one study, more that 50% of the patients also had another autoimmune condition (309). The most common ones were Sjögren's syndrome, autoimmune thyroid disease, and RA. In the serum of patients, the levels of alkaline phospatase and γ-glutamyl transferase are elevated, however bilirubin levels may vary (310).

The prevalence of PBC in Europe, North America, Asia, and Australia is reported

to be from 19.1 to 402 cases per million inhabitants, whereas the incidence rates range between 0.3 to 6 cases per million inhabitants per year (311). PBC is a female predominant disease. Different studies report different sex ratios, with some reporting 1:10 male to female prevalence whereas male patients have a higher mortality rate (311, 312).

Environmental factors and genetic factors have been suggested to be involved in the development of PBC, however identifying them can be challenging. Smoking (either active or passive), hormone replacement therapy, and frequent use of nail polish have been associated with increased risk of PBC development, which could lead to generation of AMA (313, 314). Molecular mimicry might also play a role in the development of PBC. History of urinary tract infections have been identified as a risk factor (313). T cell clones from PBC patients which react with the auto antigen pyruvate dehydrogenase, the E2 subunit (PDC-E2, more on this later) were able to recognise antigens of *Escherichia coli*, one of the most common bacteria in urinary tract infections (315, 316). Xenobiotics also contribute to disease development through molecular mimicry. Serum of PBC patients reacted with PDC-E2 chemical mimics, including the 2-octynoic acid, resulting in cross reactivity (317). Mice which were immunised 2-octynoic acid developed autoimmune cholangitis, AMA, and increased immune infiltration in the liver (318). 2-octynoic acid and other molecular mimics are present in perfumes, lipstick, and foods (319). Finally, a theory for the presence of a gut-liver axis is currently being investigated. The liver receives blood from the gut through the portal vein. Other products arrive with the blood such as nutrients, bacteria and bacterial components, as well as immune cells (285, 320). Dysbiosis, a process of pathological change and maladaptation of the microbiome in the gut, has been described in cirrhotic patients (321). It is currently unclear whether such process takes place in PBC. However, faecal bacteria have been discovered in

patients with PBC, like *Enterobacteriaceae*, *Pseudomonas*, and *Veillonella*, which are increased in PBC patients whereas *Oscillospira* and *Suterella* are reduced (322).

Multiple genetic risk factors have been identified in PBC, highlighting the important role of genetics in the development of the disease. The HLA and non-HLA variants which have been identified contribute to the disease collectively by modulating different biological processes rather than function individually (323, 324). In large-scale GWAS, HLA loci were shown to contribute greatly in autoimmune diseases (325). However, variability between the the HLA loci of patients with different ethnic groups complicate the understanding of the disease. In caucasian populations the HLA alleles DRB1*11 and DRB1*13 are protective against PBC whereas in Japanese populations HLA-DQB1*0604 and HLA-DQB1*0301 are protective (326–330). In all populations studied however, HLA-DRB1*0803 was highly concentrated in PBC patients.

Non-HLA loci have also been identified for PBC, which highlight the importance of antigen presentation, T cell, and B cell function. The loci, similarly to other autoimmune conditions, are not exclusive to PBC (331–333). *CD80* was identified as a risk locus for PBC by three different GWA studies, studying a UK cohort, Hans Chinese, and Japanese populations, all presenting different SNPs (334–336). In the Mells et al study, 2 SNPs in the region of CD28/CTLA-4/ICOS were also identified as risk loci for PBC (334). Due to linkage disequilibrium it is difficult to conclude which gene or genes are reaching genome-wide significance. These findings highlight a potential dysregulation in the CD28-CTLA-4/CD80 pathway, responsible for the activation and inhibition of T cell responses. However, no functional studies have been conducted to date to elucidate the role of the SNPs in these patients.

PBC is characterised by loss of tolerance to PDC-E2 which results in adaptive immune activation. PDC is a large multi enzyme complex which catalyses the

oxidative decarboxylation of pyruvate to acetyl coenzyme A (337). The complex is located in the inner mitochondrial membrane and consists of multiple copies of the subunits E1, E2, and E3. The presence of the auto-antigen PDC-E2 in the serum of patients however is the result of apoptosis of the biliary epithelial cells (BEC). Bile secretion by BEC is responsible for 25-40% of the total bile flow in humans (338). Under physiological conditions, BEC flush $HCO3^-$ as a protective mechanism to reduce the toxic compounds in the bile through alkalinization. When there is favourable Cl^- gradient across the plasma membrane of the BEC, the Cl^-/HCO_3^- exchanger (known as AE2) secretes the bicarbonate into the bile (339–342). In PBC patients the AE2 exchanger is dysregulated. AE2 expression and activity is decreased in their livers compared to normal controls or PSC patients (343, 344). This leads to cholestasis due to the defective biliary bicarbonate umbrella on the outer membrane of the cholangiocytes (345, 346). Mice deficient for *Ae2* show PBC-like pathology, with features like $CD8^+$ and $CD4^+$ T cell liver infiltration and localisation around bile ducts, and the presence of AMA (347). Similarly in humans, cholestasis leads to hepatocyte injury, chronic inflammation, and bile duct destruction.

Apoptosis is a physiological process which allows for the clearance of apoptotic cells in a manner highly regulated by avoiding secretion of intracellular components and eliciting immune responses against self-antigens (348, 349). In PBC apoptosis of BEC is , resulting in the development of autoimmunity. Apoptosis is increased in PBC compared to PSC (350–353). During apoptosis, PBC-derived BEC do not tag PDC-E2 for cleavage by caspases, neither was the peptide concentrated into apoptotic blebs (354). As a result PDC-E2 leaks out of the mitochondrion, where it is recognised by the immune system resulting in the generation of AMA (355). Sera of PBC patients reacted with the PDC-E2 component (356). Currently, diagnosis of PBC includes identification of AMA in the serum of the patients, where almost 95% of the

patients are positive for the presence of AMA (80). The presence of AMA can precede the clinical onset by a decade (357–359).

The adaptive immune system is believed to be a major driver of the disease. An increased number of $CD4^+$ and $CD8^+$ T cells are present in the portal tracks of PBC patients (360, 361). PBC patients exhibit an ~100-150-fold increase of $CD4^+$ T cells in their liver and lymph nodes compared to the number of T cells isolated from their blood (362). PBC T cells reacted with both a partial PDC-E2 peptide and with the complete molecule. Autoantibodies also reacted with the partial PDC-E2 antigen recognised by the T cells. Similarly, $CD8^+$ T cells were also MHC class I-restricted to identify another epitope of the PDC-E2 component, albeit a different one from the ones recognised by the $CD4^+$ T cells (363). PDC-E2-pulsed DCs produced more autoreactive cytotoxic T cells. B cells are also increased in the livers of PBC patients (101). Furthermore, patients with PBC have reduced number of Tregs in their livers compared to healthy controls and no Treg disfunction was identified (364–366). However, other studies suggest that there is no difference in the frequency of Treg (367, 368). Similarly, Treg defects have been identified by other groups. Treg from PBC patients also upregulated IFN-γ upon IL-12 stimulation through rapid phosphorylation of STAT4 (369). *IL-12A*, *IL-12Rβ2*, and *STAT4* have been identified as risk loci for PBC (332, 370). Tregs were unable to secrete IL-10 (366). When they were co-cultured with $CD4^+$ T cells, they were less immunosuppressive compared to Treg from healthy controls. Furthermore, CD80 expression was similar in the livers of PBC and healthy controls using immunohistochemistry, however, CD86 expression was localised around the bile ducts. In a suppression assay, DCs pulsed with *E. coli* were co-cultured with Tregs from PBC and healthy controls to measure the suppression of CD86 on the surface of the DCs. Only the healthy Treg and not the PBC-derived ones were able to suppress the expression of CD86. These studies highlight the disfunction

of the adaptive immune system in PBC patients. Moreover, double negative T cells ($CD8^-CD4^-$ T cells) are reduced in PBC both in the blood and the liver of patients (371). These cells also showed signs of impaired function, by being unable to suppress $CD8^+$ and $CD4^+$ T cell proliferation and also by having reduced levels of IFN-γ, perforin, and granzyme B.

The first line of treatment for PBC is a drug called ursodeoxycholic acid (UDCA). It is a 7-β epimer of the main human bile acid chenodeoxycholic acid, accounting for up to 40% of the total bile acids (372). UDCA gets absorbed by the small intestine and gets transported into the liver through portal circulation. UDCA converts cholesterol to bile acids, which reduces the amount of cholesterol in the biliary lipids (373). Since its introduction, UDCA improved the outcome of the disease by lowering the mortality rate (374, 375). It also reduced histological features of the PBC patients, it improved the blood makers of cholestasis and reduced the levels of AMA in the blood (376–379). Despite the positive effect it has on PBC patients, only up to 60% of patients respond to UCDA (380). For that reason other lines of treatment are currently being investigated. Obeticholic acid (OCA) is a farsenoid X receptor (FXR) agonist (381). FXR is a bile acid receptor belonging to the superfamily of nuclear receptors and is responsible for the synthesis, secretion and transport of bile acids (381). OCA has shown promise in clinical trials where it was administered in UDCA unresponsive patients. Results showed reduction in blood markers of cholestatis such as alanine aminotransferate (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) (382, 383). Rituximab, the humanised anti-CD20 monoclonal antibody which is widely used for the depletion of B cells in RA, has been on clinical trials for non-UDCA responders. The results showed improvements of alkaline phosphatase (ALP) levels, improvement in pruritus, and depletion of B cells however the biochemical efficacy of the drug was not impressive and as a result

the drug was not recommended for administration (384, 385). Ustekinumab, a monoclonal antibody against IL-12 and IL-23 showed moderate results when administered to PBC patients which responded inadequately to UDCA, with modest reduction of ALP (386). Recently, PBC patients with an incomplete response to UDCA were administered with abatacept. Although there was a reduction in $CD4^+$ T cells, there was no effect on the $CD8^+$ T cells, ALP, ALT, AMA, or liver stiffness (387).

1.6.1.3. Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is another chronic liver disease of unknown aetiology. The hallmark of PSC is cholestasis in the large bile ducts, resulting in strictures in the biliary tree. This leads to liver cirrhosis, portal hypertension, and end-stage liver disease. Patients with PSC usually develop cholangiocarinoma within 2 years of diagnosis (388). Patients with PSC can be asymptomatic at day of diagnosis, or show symptoms of pruritus, fatigue, upper right quadrant abdominal pain, fever, or weight loss.

PSC is a rare disease with fewer than 200,000 reported cases in the US and fewer than 5 per 10,000 cases in Europe (389). The prevalence of the disease is around 1 per 10,000 people in northern Europe and 0.45 - 2.07 per 100,000 in the US (390, 391). The cases of PSC appear to increase in the north American and European countries. Unlike PBC, PSC is more common in men (66-70%) and the median age of diagnosis is around the ages of 30 to 40 years old (392, 393). In a Netherlands study the median survival between diagnosis to either liver transplant or PSC-related death was 21.3 years (393). In the same study, the survival of patients in combined transplant centres was 13.2 years. Inflammatory bowel disease (IBD), which is an umbrella term used to describe chronic inflammation diseases of the digestive track like ulcerative colitis (UC) and Crohn's disease, has been closely associated with PSC, with IBD being

present in the majority of patients (394, 395). 25% of the patients with PSC and IBD usually present with another autoimmune condition which is not part of the liver or the colon, in contrast to 9% of PSC patients without IBD (396). There have also been described cases of PSC patients presenting with IBD before PSC developing post-transplantation despite the patients receiving immunosuppression (397).

Similarly to the other immune-driven liver diseases, PSC is also a complex disease with multiple factors contributing to the development of the disease. Genetics is one of the key drivers. The majority of the risk loci for PSC are found in chromosome 6p21, with weaker links present in other areas of the genome (398). Similarly to the other two liver diseases, the risk loci are grouped as risk loci of the HLA complex and non HLA complex. In the HLA complex, many genetic variants have been described to contribute to disease development. Genetic variations in this area have previously been linked with autoimmunity, immune-related conditions, and infections (399). It is thus hypothesised that the adaptive immune system is one of the key drivers of the disease development. To date, no antigenic peptide has been identified.

Non-HLA genetic variations have also been described. Some of these risk loci overlap with the IBD risk loci, whereas others are involved in autoimmune conditions such as RA or T1DM (400). Of importance in this study is the association of PSC with the SNP rs7426056 of the gene *CD28* (401). As we mentioned earlier, CD28 is a molecule which is directly involved in the priming and activation of T cells. Indeed, it has been previously shown that patients have altered mRNA levels for CD28 if they are carriers of the CD28 variants (402). PSC patients also exhibit loss of CD28 from their surface (403). In addition, *CD28* is at linkage disequilibrium with the genes *CTLA-4* and *ICOS* sugesting that the CD28/CTLA-4 - CD80/CD86 pathway could be dysregulated in patients bearing the allele variants (404, 405). Another interesting susceptibility gene which has been linked with PSC is *FUT2*. This

gene encodes the α-1,2-fucosyltranfersase which is responsible for the presence of ABO blood group antigens on the gastrointestinal tract (406). FUT 2 is also responsible for encoding the H antigen which allows intestinal bacteria to bind to the gastrointestinal tract and use it as a carbon source (407). *FUT2* is also a risk locus for Crohn's disease (408, 409). Patients with IBD experience dysregulation of the gut microbiota (410).

The relationship between the liver and the gut is currently being studied extensively although the theory was established in the mid-late 1960s (394, 395). The gut of patients with PSC is composed of fewer bacteria, with less diversity compared to patients with UC without biliary disease and healthy controls (411–415). Screening of the faecal microbiota is currently being tested as a diagnostic tool for PSC (415, 416). It is unclear whether the decrease in microbiota diversity is a cause or a consequence of the disease.

Immune cells also play a role in PSC. Antibodies against cholangiocytes are present in patients with PSC (417). Biliary cells participate in the development of PSC, either as a victim of the immune system or as an APC, due to their expression of the T cell co-stimulatory molecules HLA class I, class II, and CD86 (418–420). $CD28^{+}$ lymphocytes were also present around bile ducts (420). Furthermore, BEC stimulated with IgG from PSC patients induced the expression of TLR4 and TLR9, as well as phosphorylated the kinase ERK1/2, the transcription factor ELK-1, and NF-κB (421). Furthermore, LPS or CpG stimulation of BEC induced the production of IL-1β, IL-8, IFN-γ, TNF-α, GM-CSF, and TGF-β. Anti-neutrophil cytoplasmic antibodies (ANCA) were also present in patients with PSC and IBD (422). The ANCA were localised perinuclearly of the neutrophils. The antibodies react with the auto antigen β-tubulin isotype 5 (TBB-5) which has a high structural homology with the microbial cell division protein FtsZ (423). ANCAs were also present in the serum of mice deficient

for IL-10, which serves as a mouse model for IBD. Similarly, the serum reacted to recombinant FtsZ as well as human tubulin. This evidence further supports the hypothesis of the liver-gut axis aetiology in PSC. Furthermore, the peripheral $CD4^+$/ $CD8^+$ T cells are increased in PSC compared to inactive UC or healthy controls (424, 425). Other studies have shown that T cells from the liver of PSC patients are reduced and have a low proliferative capacity compared to blood lymphocytes (426). The researchers were also unable to detect cytotoxic activity of either T cells or NK cells. Cytokines are also involved in disease pathogenesis. Gut-activated T cells express the gut homing receptor CCR9 (427). Livers of PSC patients express CCL25, the ligand of CCR9, which was exclusive to PSC and not any other liver diseases, including PBC. Similarly, mucosal addressing cell adhesion molecule (MAdCAM-1), which is responsible for lymphocyte trafficking to the gut, was expressed in the hepatic endothelium of PSC patients (428). Gut recirculating T cells ($\alpha 4\beta 7$ T cells) were present in the blood and liver of patients with PSC and were able to bind to MAdCAM-1.

PSC patients are receiving bile acid-based therapies like UDCA and 24-norursodeoxycholic acid (norUDCA). Unfortunately, results from UDCA treatment have been inconsistent regarding the efficacy of the drug, and as a result, inconsistent prescriptions has been given to patients (429, 430). Due to the lack of understanding of disease pathogenesis, development of reliable treatment for the disease has been difficult. Several clinical trials are currently in place testing different drugs which show promise for the treatment of the disease. They are categorised into three major groups, the bile acid modulants, immunomodulants, and drugs acting on the microbiome. Bile acid modulants include drugs like norUDCA. This drug undergoes absorption by the biliary cells which allows for the cells to become stimulated and flush out their contents (431). Data in mice and PSC patients show that the drug is

well tolerated and can improve cholestasis (431, 432). Immunomodulatory drugs such as cenicriviroc are also being tested. This is a novel antagonist for the chemokine receptors CCR2 and CCR5. This drug has shown promise in mouse models of PSC (*Mdr2*$^{-/-}$ or bile duct ligated mice) by reducing the bile acid pool, the levels of the enzyme bilirubin in the blood, liver necrosis, and fibrosis (433). Furthermore, T cells and neutrophils were also reduced as a result of the cenicriviroc treatment. This drug provides better results in combination with all-trans retinoic acid treatment. This suggests that PSC might require a multimodal approach for treatment. Finally, the easiest way to modulate the gut microbiome is through administration of antibiotics. Some antibiotics have been administered to PSC patients in clinical trials like metronidazole, vancomycin, minocycline, and rifaximin (434–437). These small studies have shown promise for vancomycin, metronidazole, and minocycline, however rifaximin has not shown any positive effects on the patients. It is not clear why that is.

1.6.2. Metabolic liver diseases

1.6.2.1. Alcoholic liver disease

Alcoholic liver disease (ALD) is a condition of the liver which is the result of excessive alcohol consumption. Because the liver is the primary site of ethanol metabolism, it sustains the greatest amount of injury compared to other tissues (438). Development of ALD leads to steatosis (fatty liver), hepatitis, and fibrosis/cirrhosis. These symptoms can appear either simultaneously or sequentially.

According to the Global Burden of Diseases, Injuries and Risk Factors Study, in 2016 alcohol was responsible for 2.2% female and 6.8% male deaths globally, ranking it the seventh leading risk factor causing death (439). ALD develops when the two

drinks per day limit is exceeded (440). Steatosis can induce hepatomegaly which can be reversed after ~7 weeks of abstinence. In 40% of the cases, ALD leads to cirrhosis (441–443). Others report that steatohepatitis only occurs in 25-35% of the consumers and fibrosis/cirrhosis at 8-20% (440). Culture also affects the rate of disease development. Geographical differences indicate different rates of liver cirrhosis, with Islamic countries reporting rates of 10% whereas in France it reaches 90% (440). Furthermore, consumption of alcohol with food reduces the harmful effects of alcohol (444). Factors such as age, gender, ethnicity, and obesity affect the incidence of ALD.

Genetic factors also contribute to ALD pathogenesis. ALD is considered a familial disorder. Familial history of alcohol consumption increased the risk of alcohol-dependence to ~50% for men and ~23% for women (445). Genetic studies have also identified genetic variants associated with ALD. *IL-10* was described as a risk locus for ALD (446–450). This variant is responsible for the production of low levels of IL-10, which is in line with the pro-inflammatory environment present in the inflamed liver of these patients. A later meta-analysis showed that *IL-10* variants were not risk alleles for ALD but for alcoholism (451). *TNF-α* was also identified as a risk locus for ALD (452, 453). The studies mentioned should be carefully considered due to the small number of participants and thus prevents associations from reaching "genome-wide" significance ($p \leq 5 \times 10^{-8}$) which would allow them to draw safer conclusions.

The immune system is also involved in the development of ALD. A gut-liver axis appears to be present in ALD and contributes to the development of the disease. In alcohol-fed mice as well as in humans with ALD, LPS levels were elevated in their serum however LPS was not present in either non-alcohol fed mice or healthy controls (454–458). LPS in the serum correlated with disease severity (457, 458). TNF-

α levels were also increased in alcohol-fed mice but not in ALD patients (454, 457). Lesions in the liver were present in patients and animals fed with alcohol, however, treatment with antibiotics or probiotics reduced the levels of LPS in the serum as well as the severity of the inflammatory lesions (454, 459–461). Translocation of LPS from the gut to the liver results in its uptake by TLRs which release ROS, adhesion molecules (such as ICAM-1 and VCAM-1), and pro-inflammatory cytokines like TNF-α, IL-1, and IL-6, all leading to leukocyte recruitment (462). Also, the phenotype of T cells is altered in ALD patients. The patients have elevated levels of $CD3^+$ T cells with high numbers of both $CD4^+$ and $CD8^+$ T cells in portal areas (463). Hepatocytes as well as bile ducts expressed high levels of MHC class I. Alcohol-fed mice contain T cells which are more prone to secrete IFN-γ and IL-4 (464). Chronic alcohol-fed Rhesus macaques with viral diseases had reduced levels of $CD8^+$ T cells expressing CD28 compared to non-alcohol-drinking macaques (465). Finally, chronic alcohol consumption by mice showed that upon concanavalin A stimulation, T cells upregulated the hepatic NF-κB, STAT1, and STAT3 transcription factors (466). B cells are also affected by chronic alcohol consumption. Melanoma-bearing mice which were fed alcohol in a chronic manner had fewer B cells in their blood. This was a result of downregulation of sphingosine-1-phosphate receptor-1 (S1PR1) and sphingosine-1-phosphate lyase-1 (SLP1) in the spleen, preventing B cells from egressing from the spleen (467). Alcoholic patients with ALD experience severe loss of B cells in their blood (468, 469). Furthermore, low doses of alcohol inhibit antigen-stimulated B cell proliferation and antibody production (470). Finally, the function of DCs was also impaired. Mixed lymphocyte reaction (MLR) using $CD4^+$ T cells with DCs from patients who drink moderately showed that DCs had a reduced capacity to stimulate the T cells (471). This was mediated through decreased secretion levels of IL-12 but elevated production of IL-10, as well as decreased expression of CD80 and

CD86. Monocytes from alcoholic patients, when stimulated to differentiate into DCs had reduced levels of CD86 expression but stronger HLA-DR expression (472). DCs from patients with chronic alcohol consumption but with no liver disease had reduced surface levels of HLA-DR (473).

There are currently several different ways to manage ALD. Long-term abstinence can eliminate mild liver damage from high alcohol consumption (474). Furthermore, a more balanced diet with high protein and low-fat macronutrients can help patients suffering from malnutrition (475). Liver transplantation is the standard of care for patients with advanced ALD, which has a better prognosis for patients with alcoholic hepatitis (476). More targeted approaches are also being developed. Pentoxifylline is a TNF-α transcription inhibitor which reduced mortality in patients with severe ALD (477). TNF-α is released by Kupffer cells as a response to chronic alcohol consumption (478). Another treatment is a course of the antibiotic rifaximin which has shown to alter the gut microbiota and provide an effective treatment of hepatic encephalopathy (479).

1.6.2.2. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease is a spectrum of chronic conditions characterised by hepatic steatosis with no signs of secondary hepatic fat accumulation (for example, excessive alcohol consumption) (480). Non-alcoholic steatohepatitis (NASH) is a type of NAFLD where there is evidence of hepatic steatosis and inflammation in the liver with or without fibrosis. NAFLD is characterised by fat accumulation in hepatocytes as a result of a high fat diet. The prevalence of NAFLD in the US is ~23.5% with NASH being between 1.5-6.5% (481, 482). In Europe the prevalence of NAFLD is ~25% of the general population, however this varies between regions with Romania reporting prevalence rate of 8% and Greece ~45%

(483–486). Risk factors for NAFLD include obesity, type 2 diabetes mellitus, dyslipidaemia, and insulin resistance (487).

Evidence shows that the environment plays a role in the development of the disease. One study showed that areas with increased options for food source (restaurant, grocery stores) accumulate more NAFLD patients (488). Similarly, these patients exhibited unhealthier eating patterns and reported eating at restaurants more often compared to the healthy population. Indeed, dietary habits of patients with NAFLD were increased in high-sodium, high-fat foods, and consumed reduced amounts of fresh fruits (489, 490). Finally, individuals lead a less active life with reduced physical activity and increased sitting times compared to healthy population (491–493).

Genetic predisposition might also play a role in the development of the disease. *PNPLA3* encodes the patatin-like phospholipase domain-containing protein 3 and various GWAS studies have identified variants of this gene to be linked with NAFLD (494–498). The I148M variant has been associated with intracellular lipid accumulation, steatohepatitis, and hepatocellular carcinoma (494, 499, 500). Furthermore, this allele is responsible for invalidating the protective effects of statins and ω-3 fish oils in NAFLD patients who are carriers for the allele (501, 502).

The immune system also contributes to disease progression. The liver of NASH patients is characterised by portal infiltration of lymphocytes and macrophages (503, 504). The level of immune infiltration in the liver of NAFLD patients correlated with disease severity, serum IgG, and IFN-γ levels (503, 505). Lymphocytes formed aggregates in the livers of NAFLD patients and in mouse models of NAFLD (coline deficient and amino acid defined diet (CDAA) and methionine/choline deficient diet (MCD)) aggregates of B cells and T cells were also formed, with B cells preceding T cell accumulation (505). The aggregates resembled ectopic lymphoid structures (506).

Furthermore, the presence of these aggregates correlated with fibrosis and lobular inflammation. $CD4^+$ T cells from NASH mice were polarised into a Th1 phenotype, secreting IFN-γ and TNF-α, and expressing the transcription factor T-bet (507, 508). Mice deficient for IFN-γ, when given methionine- and choline-deficient high-fat diet to develop steatohepatitis, showed fewer signs of disease progression (509). Compared to wild-type mice which were on the same diet, IFN-γ-deficient mice expressed lower levels of TNF-α, TGF-β, and IL-4. Furthermore, their livers were less fibrotic compared to wild type mice. Patients with NASH showed elevated numbers of memory $CD4^+$ and $CD8^+$ T cells and secreted high levels of IFN-γ compared to healthy controls, whereas the frequency of naive $CD4^+$ and $CD8^+$ T cells was decreased (510, 511). In another mouse model for NASH (high fat high carbohydrate), when $CD8^+$ T cells were depleted, the mice had lower triglyceride content, lower ALT levels, and lower infiltrating and resident macrophages compared to wild type mice (512). In the MCD NASH mouse model, steatohepatitis took place at the same time as B cells matured into plasma cells (505). Furthermore, T cell activation ensued after B cell activation. B-cell activating factor (BAFF) was upregulated in those B cells. Depletion of BAFF using the BAFF neutralising antibody Sandy-2 reduced the number of circulating B cells, prevented their maturation and improved the histological scores and inflammation in the MCD mice. Finally, Treg physiology was also disrupted in NAFLD patients, indicating further inadequate immune responses. Tregs were reduced in the circulation of patients with NASH compared to healthy controls (513). Similarly, mice which were fed a high fat diet to develop steatohepatosis had reduced levels of Tregs in their liver (514). Introduction of exogenous LPS in the mice initiated immune response against the antigen, whereas adoptive transfer of Tregs decreased the levels of inflammation.

Many different treatments exist for the management of NAFLD with various

degrees of success. The first step usually recommended is for the patients to focus on healthier lifestyle, by improving their eating habits and by introducing regular exercise into their lives (515). Vitamin E has shown to improve steatosis and inflammation in various clinical trials however it has shown to have no effect in the resolution of NASH or in reducing fibrosis (516–518). Other treatments also exist which aim to treat co-morbidities in NAFLD patients. For example, statins given to NASH patients not only reduced dyslipidaemia but also post-hoc analyses have shown that they might reduce cardiovascular morbidity in the patients (519, 520).

1.6.3. Other conditions

In this section, are focusing on other conditions used as controls for our experiments in chapters 3, 4, and 5.

1.6.3.1. Polycystic liver disease

Polycystic liver disease (PLD) is a rare autosomal dominant genetic disease of the liver which is characterised by more than 20 cysts on the epithelium of the liver, filled with fluid (521). PLD can be subclassified into PDL (autosomal dominant PLD, ADPLD) in isolation or PDL in conjunction with autosomal dominant polycystic kidney disease (ADPKD). To date, no involvement of the immune system has been described for either of the two diseases.

The presence of the cysts is a result of mutations in the genes *PRKCSH* and *SEC63*. These genes encode the hepatocystin and sec-63 proteins, which are involved in fluid transportation and growth of epithelial cells (522). In ADPLD with ADPKD, two of the main genes that have been extensively studied and have contributed to the disease are the *PKD1* and *PKD2* (523, 524). The protein products of these genes are

responsible for regulating intracellular levels of calcium (521).

Current pharmaceutical treatments for patients with PLD are limited. Somatostatin analogues have been used extensively, being the most successful treatment option. Somatostatin is a hormone found naturally in humans and is responsible for the inhibition of production of cAMP (521). In PLD, cAMP was shown to increase the proliferation of biliary cells and the formation of cysts. Furthermore, the options of aspiration of the cysts, resection, or liver transplantation can be considered depending on the severity of the disease, the size, and location of the cysts.

1.6.3.2. Hereditary Hemochromatosis

Hereditary hemochromatosis (HFE) is a hereditary disease during which iron which gets ingested is not excreted from the body but instead accumulates due to ineffective excretory mechanism (525). Over-accumulation of iron in the body leads to damage to many organs including the liver. If the disease remains untreated, it can be fatal.

The mutations responsible for the development of HFE are genes responsible for the activation of hepcidin (such as *HFE, HJV, TRF2*) or hepcidin itself (526). Hepcidin is a negative iron-regulating hormone. When the iron stores are high, it suppresses the protein ferropotin, which blocks the export of iron from macrophages and prevents the internalisation of iron from the duodenum (525). When *HFE* is mutated however, the levels of hepcidin are decreased and the excess iron becomes absorbed (527).

Patients with HFE are more susceptible to infections compared to the general population. *Vibrio vulnificus* and *Yersinia enterocolitica* are two siderophilic bacteria which under physiological conditions are not considered highly pathogenic,

however, in patients with iron overload the mortality rate can reach up to 50% (528, 529).

Not a lot of information exists regarding the role of the immune system in HFE. Patients with HFE had elevated levels of $CD8^+CD28^-$ T cells compared to healthy controls however no difference was found in the $CD4^+$ T cell compartment (530, 531). Furthermore, $CD8^+$ T cell numbers correlated with liver damage in patients with HCV (531). Iron also down regulated the expression of both CD4 and the adhesion molecule CD2 from T cells (532).

Early detection of HFE improves the outcome of the disease. Phlebotomy can decrease the levels of iron in the blood of patients since 400ml of blood contain ~250mg of iron (527). Furthermore, iron chelating drugs have also been used. This type of treatment allows for the excretion of the iron from the body by binding the iron to the drug. These methods as well as early detection of HFE before the development of clinical symptoms, can significantly contribute to a normal life expectancy for the patients.

1.7. RhuDex, a new inhibitor of T cell co-stimulation that targets CD80

It is well established that T cell co-stimulation is critical for the activation of T cells (533, 534). One of the simplest approaches to investigate the effect of co-stimulation is by using inhibitors that will block the CD28-CD80/CD86 pathway either on T cells (CD28 inhibitors) or on the APC (CD80 or CD86 inhibitors). RhuDex is a novel, small molecule with the ability to bind antagonistically on CD80 but not on CD86 (**Fig. 3**) (535). When $CD4^+$ T cells from rhesus monkeys were co-cultured with two rhesus

monkey B cell lines which express high levels of CD80 but not CD86 on their surface, and treated with RhuDex, T cell proliferation was inhibited in a dose-dependent manner (536). RhuDex also suppressed T cell proliferation when human lamina propria leukocytes (LPL) and peripheral blood lymphocytes (PBL) were co-cultured with lamina propria myeloid cells or activated peripheral blood monocytes (537). The blood monocytes expressed both CD80 and CD86 (537), however surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC) data that shows RhuDex being able to bind to CD80 but not CD86 (535).

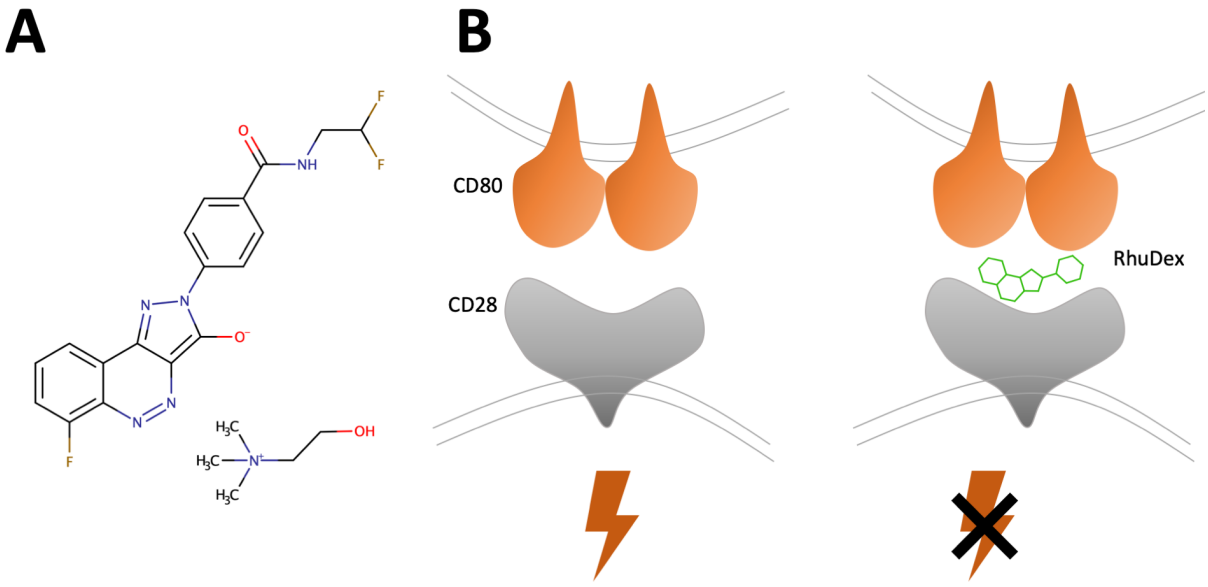

Figure 3: RhuDex is a CD80 inhibitor. (**A**) Structure of active ingredient of RhuDex. (**B**) Mode of action of RhuDex. When CD80 ligand, found on APC, binds to CD28 receptor of a T cell, the cell can proliferate. When RhuDex binds to CD80, the CD28 receptor is unable to bind to CD80 and thus the T cell does not undergo proliferation.

RhuDex also inhibited the release of pro-inflammatory cytokines IFN-γ, and TNF-α as well as IL-2, *in vitro* by T cells of rhesus monkeys (536). Conversely, Heninger et al. did not detect an effect on human T cell IL-2 secretion after treatment with RhuDex *in vitro* (537). RhuDex decreased the mRNA levels of the pro-inflammatory

cytokines *TNF-α* and *IFN-γ* when T cells were co-cultured with activated monocytes derived from atherosclerotic plaques (538). Moreover, RhuDex decreased the mRNA levels of *TNF-α*, *IFN-γ*, and *IL-10* for both LPS-activated monocytes and macrophages (538). Although RhuDex shows potential for inhibiting Th1 responses on both the T cell and APC level, inhibition of *IL-10* levels as well as other off-target effects means that RhuDex has a broader inhibitory effect on cells. Overall, RhuDex shows strong potential for immune modulation.

1.8. Project Aims

The role of the CD28-CD80/CD86 pathway in PBC and other chronic liver diseases has not been established. In addition, the expression of CD80 and CD86 on liver-infiltrating immune cells is currently uncharted. We hypothesised that we can interfere in the CD28-CD80/CD86 pathway using the novel CD80 inhibitor RhuDex and inhibit the development and/or progression of PBC and possibly other immune-driven chronic liver diseases.

Our first aim was to verify that RhuDex can block T cell proliferation through binding to CD80 with a better efficacy compared to abatacept or other CD80 and CD86 inhibitors.

Our second aim was to establish a phenotypic map of CD80 and CD86 on intrahepatic B cells, monocytes, and DCs in PBC and other liver diseases. This allowed us to study the expression pattern of CD80 and CD86 and establish possible relationships between the expression levels of these markers and the diseases we studied. In addition, characterising the expression of these molecules gave us an indication about the possible targets of RhuDex.

Finally, we aimed to co-culture intrahepatic T cells and autologous B cells and

monocytes in the presence or absence of B7 inhibitors including RhuDex. These experiments would elucidate the capacity of liver-infiltrating mononuclear cells (LIMCS) to initiate immune responses through T cell co-stimulation and to study the effect of the drug *in vitro* while assessing its effect before it is administered into patients in clinical trials.

Chapter 2 - Materials and Methods

2.1. Cell isolation

2.1.1. Isolation of peripheral blood mononuclear cells

Venous blood samples were collected from the Liver Unit at the Queen Elizabeth Hospital, Birmingham, UK after acquiring written informed consent from patients with PBC, PSC, or HFE (South Birmingham ethical approval reference 06/Q2708/11). All samples were processed on the day of collection to avoid discrepancies in phenotyping. Blood from HFE patients used as a non-cirrhotic control for this study.

When more than 50 ml of blood were used, leukocytes and plasma was separated from erythrocytes using centrifugation for 20 minutes at 800g (acceleration 5, brake 3). Then, the buffy coat was collected and was resuspended into phosphate- buffered saline (PBS - Gibco, UK) in a 3:2 ratio of buffy coat:PBS. The mixture was then layered on top of a density gradient (1 lympholyte:1.5 blood) (Lympholyte-H media, Cederlane, Canada) and was centrifuged for 20 minutes at 800g (acceleration 5, brake 3). The buffy coat was harvested and washed with PBS twice (5 minutes at 800g, full acceleration and brake). The pellet was then collected, resuspended in PBS. A small portion of the cells were then mixed with Trypan Blue (Sigma-Aldrich, UK) and the live cells were counted using a haemocytometer. For volume of 50 ml of blood or less, the first centrifugation step was skipped.

2.1.2. Isolation of liver-infiltrating mononuclear cells

Liver tissue samples were isolated from liver explants and were collected from the Queen Elizabeth Hospital, Birmingham, UK after acquiring written informed consent from patients undergoing transplantation suffering from PBC, PSC, ALD, and NAFLD (South Birmingham ethical approval reference 06/Q2708/11 and 06/

Q2702/61). Tissue processing was always completed within 12 hours to avoid phenotypic changes and minimise cell death.

Tissue was cut into small pieces (~0.25 cm - 0.5 cm) and was rinsed using ice-cold PBS. Supernatant was discarded until it was relatively clear. The tissue was then transferred in to a Stomacher 400 circulation bag (Seward Limited, UK) with 200-300 ml of warm (~37°C) Roswell Park Memorial Institute 1640 medium (RPMI - Gibco Life Technologies, UK) and was placed into a Stomacher 400 circulator machine (Seward Limited, UK) for 4-7 minutes (depending on the level of cirrhosis and steatosis of the liver) at 260 g to allow for homogenisation of the tissue. The homogenate was then poured through a fine mesh whilst being washed with PBS. The filtrate was then transferred into 50 ml falcon tubes. The samples were centrifuged multiple times, discarding the supernatant and mixing the pellet with PBS, until the supernatant after centrifugation was clear (centrifugation at 800 g for 5 minutes, full brake). The pellet was then resuspended in PBS and layered on Lymphocyte-H-media, and centrifuged at 800 g for 20 minutes, acceleration 5, brake 3. The buffy coat was then harvested, washed twice and the live cells were counted using Trypan Blue and a haemocytometer.

2.1.3. $CD3^+$ T cell isolation

To isolate $CD3^+$ T cells from HFE blood (chapter 3), the negative isolation EasySep™ Human T cell Enrichment Kit (Stemcell Technologies, UK) was used according to manufacturer's instructions. PBMCs were resuspended in a concentration of $5x10^7$ cells/ml in T cell isolation media (PBS, 2% fetal calf serum (FCS - Sigma Aldrich, UK), 1mM ethylenediaminetetraacetic acid (EDTA - ThermoFisher Scientific, UK)). Then, the enrichment cocktail was added at 50 µl/ml to the sample and was incubated for 10 minutes. Following that, already-vortexed 50

μl/ml of sample magnetic particles were added and left to incubate for 5 minutes. The sample was topped up to either 2.5 ml, 5 ml, or 10 ml (depending on the original sample volume) and was placed into an EasySep™ magnet (Stemcell Technologies, UK) for 5 minutes. Finally, the sample was poured into a fresh tube was washed with PBS.

2.1.4. Cell sorting

We performed fluorescence-activated cell sorting (FACS) to sort for total $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, $CD24^-CD38^-$ B cells, $CD24^-CD38^{int}$ B cells, and $CD24^+CD38^-$/intIgD$^+$ B cells. Cells were stained using fluorescent labeled antibodies (table 1). Two separate sorts took place, the first one was to sort $CD3^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes (CD3-BV510, CD19-PE/Cy5, and CD14-PE/Cy7). The gating strategy is described in **Fig. 22 A**. In the second one we sorted for $CD3^+$ T cells, $CD24^-CD38^-$ B cells, $CD24^-CD38^{int}$ B cells, and $CD24^+CD38^-$/intIgD$^+$ B cells (CD19 PE/Cy5, CD24 APC, CD38 PE/Cy7, IgD BV510). The gating strategy is described in **Fig. 28 A**. The process was the same for both types of sorts with the only difference being the antibodies used.

PBMCs or LIMCs were washed with PBS twice and were stained with fluorescent antibodies for 20 minutes at 4°C in a final volume of 500 μl. The cells were then resuspended in T cell isolation media and were sorted. Upon collection, cells were washed in T cell medium (RPMI, 10% FCS, 100 U/ml Penicillin, 100 μg/ml streptomycin, 1% L-Glutamine). BD FACSAria™ Fusion (Becton Dickinson, UK) was used for sorting. The sorting process was conducted with the help of Dr. Matthew MacKenzie and Dr. Paola Pietroni.

2.2. T cell proliferation assay

To measure T cell proliferation, CellTrace Violet proliferation dye was used (ThermoFisher Scientific, UK) according to manufacturer's instructions. Prior to culturing T cells, they were washed with PBC twice, and were then resuspended at a concentration of 1x106 cells/ml. Then CellTrace Violet was added at a concentration of 1 μg/1x10^6 cells, and samples were incubated for 20 minutes at 37°C, protected from light. Five times the original volume was added to the tube and cells were washed with T cell medium. Then fresh T cell medium was added to the tube and cells were incubated at 37°C in the dark for 10 minutes. Cells were washed again with fresh T cell medium and were counted using Trypan Blue and a hemocytometer.

2.3. Flow cytometric staining and analysis

Cells were washed twice with PBS to remove media and were stained with a mixture of human TruStain FcX (Biolegend, UK) with the Zombie NIR Fixable Viability Dye (1:2000 dilution, Biolegend, UK). The samples were incubated in room temperature for 20 minutes, protected from light. They were then washed with FACS buffer (PBS, 1.5% FCS) twice. Fluorescent antibodies were added onto the samples using the desired concentration in a final volume of 50 μl per 1 million cells. Isotype matched control antibodies were added at the same concentration as the antibodies of interest. The samples were incubated for 20 minutes at 4°C, protected from light. Finally, cells were washed twice using FACS buffer and were then resuspended into PBS and 37% formaldehyde for fixing. Samples were stored at 4°C, protected from light for up to 7 days until running them through the flow cytometer. The final concentration for antibodies were calculated individually after titration experiments.

The list of antibodies used for flow cytometry and other assays are found in table 1.

UltraComp eBeads (ThermoFisher Scientific, UK) were used to calculate the compensation matrix during the analysis of the multicolour experiments. One drop of beads was added per tube, which contained 100 µl of FACS buffer. Then, based on the number of fluorochromes in the experiment, one matched antibody/ fluorochrome was added into the tube for all fluorochromes. Samples were incubated for 20 minutes at 4°C in the dark and were then washed with PBS.

In experiments were Zombie dye was used, ArC Amine Reactive Compensation Beads (ThermoFisher Scientific, UK) were used for compensation according to manufacturer's instructions. The negative ArC beads were added in a tube together with 50 µl of PBS and 1 µl of Zombie NIR dye. The sample was incubated at 4°C in the dark and was then washed with PBS. Finally, ArC positive beads were added in the tube.

Samples were acquired using the ADP Cyan™ flow cytometer (Beckmann Coulter, UK). The acquisition software was Summit v4.3 (Beckmann Coulter, UK). FlowJo (Becton, Dickinson, USA) v10.6.1 was used to analyse the samples.

Table 1: List of antibodies used in flow cytometry.

Target Molecule	Fluorochrome	Clone	Isotype	Final Concentration	Company	Catalogue No
CD80	APC	2D10	Mouse IgG1, κ	0.5 µg/ml	Biolegend	305220
CD24	APC	32D12	Mouse IgG1, κ	1 µg/ml	Miltenyi	130-095-954
CD8	APC	HIT8a	Mouse IgG1, κ	0.48 µg/ml	Biolegend	300912
CD86	BV421	IT2.2	Mouse IgG2b, κ	0.5 µg/ml	Biolegend	305426

Target Molecule	Fluorochrome	Clone	Isotype	Final Concentration	Company	Catalogue No
CD16	BV510	3G8	Mouse IgG1, κ	3 μg/ml	BD Biosciences	563830
CD3	BV510	OKT3	Mouse IgG2a, κ	0.3 μg/ml	Biolegend	317332
CD11c	BV510	S-HCL-3	Mouse IgG2b, κ	1 μg/ml	Biolegend	371514
IgD	BV510	IA6-2	Mouse IgG2a, κ	3 μg/ml	Biolegend	348220
HLA-DR	FITC	G46-6	Mouse IgG2a, κ	1 μg/ml	BD Biosciences	555811
CD4	FITC	OKT4	Mouse IgG2b, κ	1 μg/ml	Biolegend	317408
CD56	PE	NCAM 16.2	Mouse IgG2b, κ	0.0625 μg/ml	BD Biosciences	345812
CD66b	PE	G10F5	Mouse IgM, κ	2 μg/ml	Biolegend	305106
CD3	PE	UCHT1	Mouse IgG1, κ	0.125 μg/ml	BD Biosciences	555333
CD19	PE	HIB19	Mouse IgG1, k	0.5 μg/ml	Biolegend	302208
CD80	PE	2D10	Mouse IgG1, k	2 μg/ml	Biolegend	305208
CD56	PE-Dazzle 594	5.1H11	Mouse IgG1, κ	0.5 μg/ml	Biolegend	362544
CD141	PE-Dazzle 594	M80	Mouse IgG1, k	0.25 μg/ml	Biolegend	344120
CD19	PE-Cy5	HIB19	Mouse IgG1, κ	0.25 μg/ml	Biolegend	302210
CD123	PE-Cy5	6H6	Mouse IgG1, k	0.5 μg/ml	Biolegend	306008
CD14	PE-Cy7	61D3	Mouse IgG1, κ	2 μg/ml	eBioscience	25-0149-42

Target Molecule	Fluorochrome	Clone	Isotype	Final Concentration	Company	Catalogue No
CD8a	PE-Cy7	HIT8a	Mouse IgG1, κ	2 μg/ml	Biolegend	300914
CD38	PE-Cy7	HB-7	Mouse IgG1, κ	0.5 μg/ml	Biolegend	356608

2.4. Cell culture assays

2.4.1. Cell line cultures

The cell lines we used for this project were the Raji, L3055-BCL2, DG-75, THP-1, Jurkat. CHO-GFP, CHO-CD80-GFP, and CHO-CD86-GFP cells were a kind donation by Prof. David Sansom (225). All cell lines apart from the Chinese hamster ovary cell line (CHO) cells were cultured in T cell medium. The CHO cell lines were cultured in DMEM, 10% FCS, 100U/ml Penicillin, 100μg/ml streptomycin, 1% L-Glutamine. Cells were harvested at ~70% confluence.

CHO and THP-1 cell lines required trypsinisation to be removed from the flask due to adherence. Cells were washed with PBS when they were still adherent to the flask. 0.05% Trypsin-EDTA solution (ThermoFisher Scientific, UK) was used and the flask was placed in the incubator at 37°C, 5% CO_2 for 3 minutes. Cells were then washed with T cell medium at 800 g for 5 minutes, full acceleration and brake. Finally, the supernatant was discarded and cells were resuspended in fresh T cell medium.

2.4.2. Co-stimulation assay

Upon collection of the sorted cells ($CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, $CD24^-CD38^-$ B cells, $CD24^-CD38^{int}$ B cells, and $CD24^+CD38^{-/int}IgD^+$ B cells), $CD3^+$ T

cells were stained using CellTrace Violet™ proliferation dye (ThermoFisher Scientific, UK) as described previously. The APCs ($CD19^+$ B cells, $CD14^+$ monocytes, $CD24^-CD38^-$ B cells, $CD24^-CD38^{int}$ B cells, and $CD24^+CD38^{-/int}IgD^+$ B cells) were kept in T cell medium in the incubator at 37°C, 5% CO_2 for the duration of the T cell staining. Upon staining with CellTrace Violet, Trypan Blue was used to measure the viable cells using a haemocytometer. Then, $CD3^+$ T cells were co-cultured with one of each APC at a 2:1 ratio T cells : APC in a U-bottom 96-well plate containing T cell medium. For co-cultures of $CD3^+$ T cells with $CD19^+$ B cells or $CD14^+$ monocytes, we used 100,000 T cells : 50,000 APCs. Due to the small number of B cell subsets collected after the FACS, we were unable to maintain the same number of cells for that ratio. Each experiment contained a different number of total cells however we kept the ratio of 2:1 consistent for all experiments. In the 96-well plate, 0.5 μg/ml of anti-CD3 (OKT3 clone), 0.5 μg/ml anti-CD28 (CD28.2 clone, in control samples) (both ThermoFisher Scientific, UK), and 250 U/ml IL-2 (R&D Systems, UK) were added to the appropriate wells. Finally, RhuDex (Dr. Falk Pharma, DE), Orencia (kind donation of Dr. Gwilym Webb), LEAF Purified anti-CD80, anti-CD86, IgG1, and IgG2b (all Biolegend, UK) were used at the appropriate wells in concentrations indicated in the chapters 3 and 5. The cells were placed in an incubator at 37°C, 5% CO_2 for 5 days.

On day 5, cells were harvested, washed with PBS, and stained with fluorescent antibodies. All samples were stained with CD4-FITC, CD8-PE/Cy7, IgG2b-FITC, and IgG1-PE/Cy7. In all co-stimulation assays apart from the B cell subset co-stimulation assays Zombie dye was used to assess viability of the cells. CD14-PE/Cy7 and CD19-PE/Cy5 were used only where noted.

CHO cells were also used in co-culture experiments. In these experiments $CD3^+$ T cells were not sorted but were isolated using the EasySep™ Human T cell Enrichment Kit. Furthermore, manipulation of CHO cells was different compared to

the primary APC used. CHO cells were harvested from culture using 0.05% Trypsin-EDTA solution (ThermoFisher Scientific, UK). Cells were washed with T cell media. Then CHO cells were fixed using 0.025% of glutaraldehyde for 5 minutes. Cells were further washed three times with PBS and were then added to the co-culture.

2.5. Immunohistochemistry

Immunohistochemistry (IHC) was carried out using formalin-fixed paraffin embedded slices of end-stage PBC livers of 3 μm thick sections. Sections were washed through 3 rounds of xylene and ethanol, and 2 rounds of water for 3 minutes each. Slides were then immersed into a pre-microwaved high pH antigen unmasking solution (Vector Labs, UK) and were microwaved for 15 minutes, followed by an incubation of 10 minutes at room temperature to cool. The samples were then transferred into a dehumidifier box and a hydrophobic DAKO pen (DAKO, UK) was used to draw around the slides. 2 drops of Bloxall (VectorLabs, UK) was used and cells were incubated for 10 minutes while placed on a rocker. Samples were washed twice for 5 minutes (PBS, 0.1% Tween-20 (SigmaAldrich, UK)), followed by an incubation with 2x casein (VectorLabs, UK) for 20 minutes on a rocker. The solution was tipped of and the primary antibody (20 μg/ml of anti-CD80, catalogue no. ab86473, Abcam, UK) was added diluted in PBS. Samples were placed on a rocker and were incubated for 1 hour. They were next washed twice and then 1 drop of the secondary antibody ImmPRESS HRP Universal kit (VectorLabs, UK) was added to the slides and were incubated for 30 minutes, while on a rocker. Slides were washed twice. ImmPACT DAB chromogen dye (VectorLabs, UK) was used according to manufacturer's instructions (30 μl of ImmPACT DAB reagent 1 mixed in 1 ml ImmPACT DAB diluent) and samples were left to be exposed for up to 10 minutes.

Chromogenesis was stopped with water. Finally, the nuclei were stained with the Vector Hematoxylin QS (VertorLabs, UK) for 30 seconds and was washed off using water. Slides were then washed by 2 rounds of water for 3 minutes each, 3 rounds of ethanol, and 3 rounds of xylene for 3 minutes each. Finally, the slides were mounted using the resin-based mounting medium DPX (CellPath, UK). The cells were left to dry overnight and were then imaged using the Zeiss Axio Scanner using the Zeiss Zen software (both Zeiss, UK). Analysis was done using QuPath v0.2 and Fiji software v2.0 (539, 540).

2.6. Statistical analysis

Statistical analyses were conducted using GraphPad Prism v8.1 software (GraphPad, US). The assumption of data not following normal distribution was taken into consideration when choosing which statistical test to follow due to low sample numbers ($n \leq 30$) (541). For comparisons between multiple groups with matched data we used the Friedman test with the ad hoc Dunn's test for multiple comparisons. When the data of the groups were not matched, we used the Kruskal-Wallis test and Dunn's test for multiple comparisons. Statistical tests for each experiment are described in text and figure legend. To produce **figure 8**, outliers were excluded using using the ROUT method with Q=0.1%s. Holm-Šídák test was then used on the dataset to perform multiple t test comparisons for α=0.05 without assuming a consistent standard diviation (542). For all experiments, the statistical error was set at α=5%. I would also like to thank Rehana Rahman for providing help with the samples for figures 13, 14, 15.

Chapter 3 - RhuDex inhibits T cell proliferation

3.1. Introduction

3.1.1. Abatacept, an established inhibitor of T cell co-stimulation that targets CD80 and CD86

Abatacept (commercial name Orencia, Bristol-Myers Squibb) is an established inhibitor of T cell co-stimulation. Abatacept is a CTLA-4-Ig fusion protein, where the human extracellular domain of CTLA-4 is linked with the Fc portion of the human IgG1 immunoglobulin (183, 543). The drug's mode of action lies on the ability of CTLA-4 to bind to either CD80 or CD86 with higher affinity than that of CD28 and inhibit T cell proliferation both *in vitro* and *in vivo* (**Fig. 4**) (183, 543, 544). In 2005, abatacept received US Food and Drug Administration (FDA) approval to be administered to patients suffering from rheumatoid arthritis (RA) and a year later it was also approved in Europe. RA is a chronic, inflammatory joint condition during which T cells, B cells, and monocytes infiltrate the synovial membrane of the joints and promote destruction of the joints (545). The infiltrating immune cells secrete pro-inflammatory cytokines like TNF and IL-6 which result in tissue damage and development of symptoms such as pain, swelling, cartilage degradation, and hyperplasia (546). Immunohistological analysis of the synovial membranes of patients with RA showed increased numbers of $CD4^+$ T cells (547). These pro-inflammatory T cells upregulate the expression of CTLA-4 on their surface after activation (544, 548). However Treg cells, which inhibit T cell responses through surface expression of CTLA-4, were significantly decreased in RA patients compared to Th1 cells, indicating an imbalance between pro- and anti-inflammatory cells in the synovium (549). Naive and memory B cells express higher levels of CD86 on their surface in patients with RA (550). Furthermore, mice with CD80/CD86 deletion from

the B cells failed to develop arthritis (551). In the collagen-induced arthritis (CIA) mouse model for RA, abatacept prevented the development of the disease (552). Furthermore, concomitant administration of anti-CD80 and anti-CD86 resulted in reduced clinical scores for the CIA mice (552). Finally, when abatacept or a combination of anti-CD80 and anti-CD86 were administered to CIA mice, the disease was eliminated (552). Abatacept is showing great potential as a co-stimulatory blockade drug, with many critical trials taking place investigating its potential role in other immune-driven diseases.

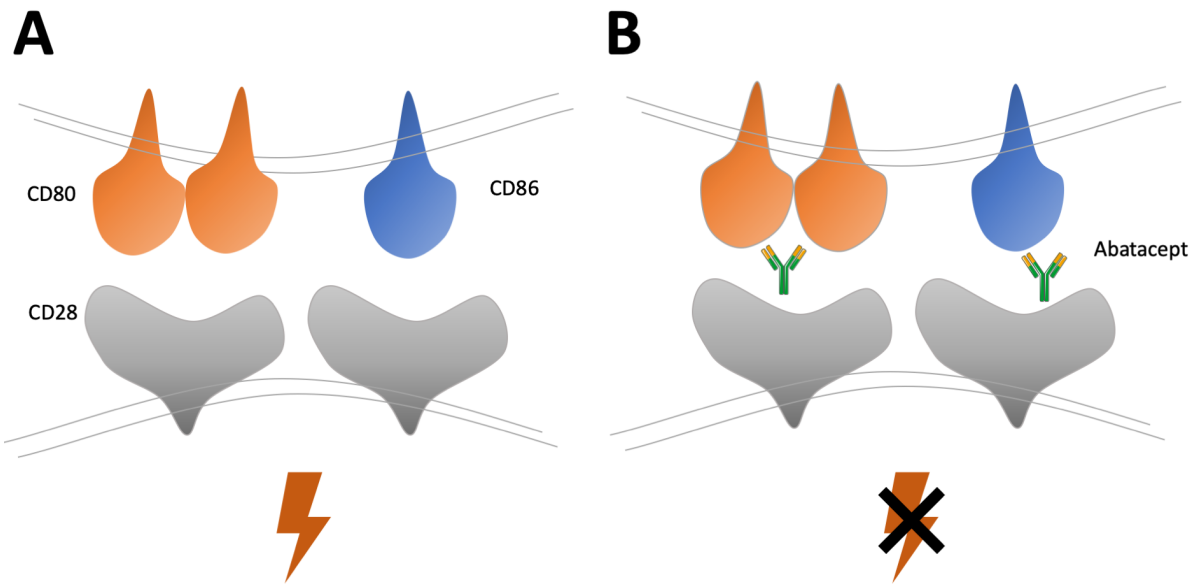

Figure 4: The CD28-CD80/CD86 pathway. (**A**) The CD80 dimers and CD86 monomers both interact with the CD28 receptor found on the T cell surface. The result of this interaction is proliferation of the T cells. (**B**) Mode of action of Abatacept. Abatacept binds specifically to both CD80 and CD86 ligands, disrupting their binding on the CD28 receptor, resulting in inhibition of proliferation and activation of the T cell.

3.1.2. Other CD80 inhibitors

Anti-CD80-specific inhibitors have also been investigated for their effect in CD80 blockade. Galiximab (alternatively known as IDEC-114, Biogen Idec, US) is a primatized monoclonal anti-CD80 antibody. Its constant regions are human derived (IgG1) and the variable regions are from cynomologous monkeys (553, 554). Galiximab has no differences from human antibodies and displays a high affinity with CD80 (554). Unlike CTLA-4, galiximab has no effect on the CTLA-4-CD80 interaction, however it can block the CD28-CD80 pathway (554). As a result, galiximab was developed to treat autoimmune and inflammatory diseases, driven by the CD28-CD80 pathway (553). When galiximab was administered to SCID mice that had received Raji human lymphoma xenografts (a mouse model for the group of lymphoproliferative malignancies called non-Hodgkin's lymphomas (NHLs)), the drug could reduce the size of the tumour *in vivo* (555). When administered with other chemotherapeutic drugs, it prolonged the survival of mice suffering from Raji-induced tumours (555). In a clinical study where the drug was administered to patients with recurrent Hodgkin's lymphoma (HL), the drug had a very limited effect, although its toxicity was low with only a few grade 3 and 4 side effects, with similar results reported in other phase I studies (554, 556). In a doublet phase II study, galiximab was administered in combination with rituximab (anti-CD20 monoclonal antibody) to test its efficacy in follicular lymphomas (FL), a type of NHL. Although the drug did not produce any severe adverse effects and had a favourable outcome (by increasing the survival of patients with FL), the result was not deemed significant enough to continue the study (557). To our knowledge, galiximab is not currently being tested in clinical trial settings to treat any condition.

3.1.3. Hypotheses and aims

We hypothesised that RhuDex inhibits T cell proliferation by blocking CD80/CD28 interactions specifically and thus preventing T cell co-stimulation. We also hypothesised that RhuDex is at least equivalent in efficacy to the well-established drug abatacept. To test these hypotheses, we sought to create the basis for a co-stimulation model which we would use to test the specificity and efficacy of RhuDex and other inhibitors on T cell co-stimulation in the human liver. To isolate the effects of RhuDex on CD80 from other co-stimulatory molecules in human APC, we co-cultured blood-derived human $CD3^+$ T cells with CHO cells as surrogate APC model that expresses CD80, CD86, or lacks any co-stimulatory molecules. The aims for this chapter were:

1. 1. To develop a co-stimulation assay using blood-derived $CD3^+$ T cells in co-culture with either CHO, CHO-CD80, or CHO-CD86 for 5 days and measure proliferation and cell death.
2. 2. To apply this assay to compare the effective dose range of RhuDex, abatacept, anti-CD80, or anti-CD86 inhibitors on T cell co-stimulation.

3.2. Development of in vitro assay for T cell co-stimulation via CD80 and CD86

To develop the co-culture assay, $CD3^+$ T cells were isolated and co-cultured with either CHO, CHO-CD80 or CHO-CD86 for 5 days in the presence of soluble anti-CD3 antibody. Soluble anti-CD3 simulated signal 1 and signals from CHO-CD80 or CHO-CD86 provided signal 2, activating the T cells. A positive control ($CD3^+$ T cells + anti-CD3 + anti-CD28 + IL-2) and a negative control ($CD3^+$ T cells + anti-CD3) were used. At day 5, we measured the percentage of proliferation and cell death of $CD8^+$ and $CD4^+$ cells using CellTrace Violet stain with flow cytometry (**Fig. 5 A, B**). At day 5, $CD8^+$ T cells proliferated after co-culture with CHO-CD80 but not when co-cultured with CHO cells ($p=0.0019$, 63.67% (32.87)) (**Fig. 5 C**). $CD4^+$ T cell proliferation was also measured in the CHO-CD80 but not in the CHO co-culture ($p= 0.0019$, 47.84% (26.37)) (**Fig. 5 D**). Similarly, CHO-CD86 co-culture promoted proliferation of T cells compared to the CHO co-cultures ($CD8^+$ T cells, $p=0.0104$, 63.96% (23.87), $CD4^+$ T cells, $p<0.0001$, 57.77% (21.21)) (**Fig. 5 C, D**). As expected, $CD8^+$ and $CD4^+$ T cells showed a significant increase in proliferation when co-cultured with CHO-CD80 ($CD8^+$ $p=0.0035$, $CD4^+$ $p=0.0462$, **Fig. 5 C, D**) or CHO-CD86 ($CD8^+$ $p=0.0175$, $CD4^+$ $p=0.0006$, **Fig. 5 C, D**) compared to the negative control. We found no differences between the CHO-CD80 and CHO-CD86 co-cultures and the positive control (anti-CD3/anti-CD28/IL-2), however some donors' T cells did not proliferate as well with CHO-CD80 compared to CHO-CD86. These results show that the co-stimulation assay using CHO cells can measure the ability of T cells to proliferate in the presence of signal 2 delivered by CD80 or CD86 alone.

We next measured the percentage of live T cells in the co-culture at day 5 to ensure

that cells remained alive during the co-stimulation assay. It also allowed us to study the effects of the inhibitors on cell death by apoptosis. No differences in apoptosis were observed for either $CD8^+$ or $CD4^+$ T cells at day 5 for all co-cultures (**Fig. 5 E, F**). Of note, the viability of $CD4^+$ T cells was not as consistent as $CD8^+$ T cells. These experiments established the baseline for measuring the effect of the various inhibitors on proliferation and cell death for $CD8^+$ and $CD4^+$ T cells.

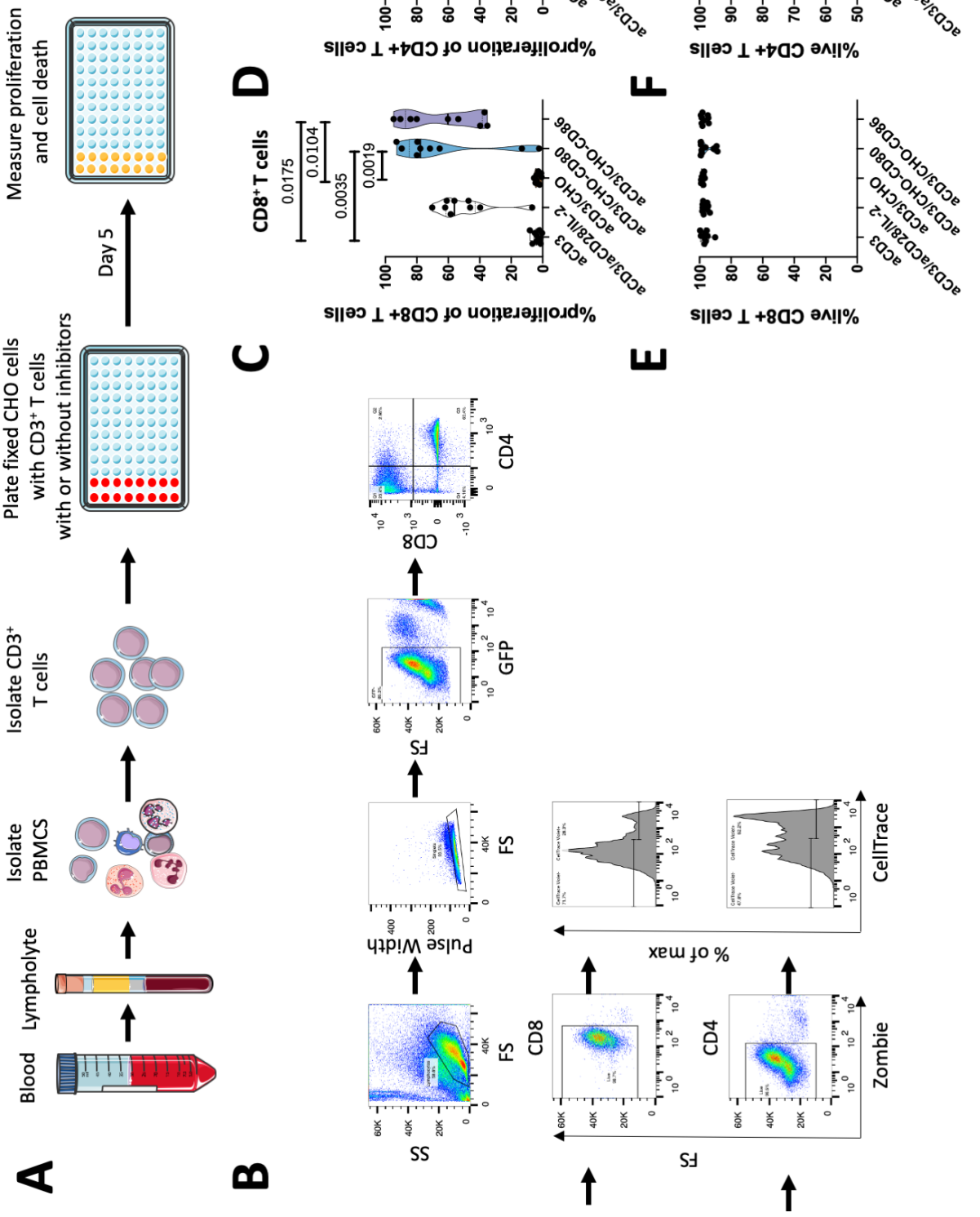

Figure 5: Transfected CHO-CD80 and CHO-CD86 promote $CD8^+$ and $CD4^+$ T cell proliferation. (**A**)

$CD3^+$ T cells were isolated from blood of HFE patients using lympholyte gradient and co-cultured for 5 days in the presence of anti-CD3, anti-CD3/anti-CD28, or they were co-cultured with anti-CD3/CHO, anti-CD3/CHO-CD80, or anti-CD3/CHO-CD86. At day 5 we measured the percentage of proliferation and cell death of $CD8^+$ and $CD4^+$ cells. (**B**) Gating strategy of flow cytometry analysis. Percentage of proliferation of (**C**) $CD8^+$ and (**D**) $CD4^+$ T cells and the percentage of (**E**) live $CD8^+$ and (**F**) live $CD4^+$ T cells. Results are shown as median (solid line) with interquartile range (dotted line). Friedman test was performed with $p < 0.0001$ for (**C**) and (**D**). Statistical analysis was performed using Friedman test with Dunn's post hoc multiple comparisons. N=9 for all samples.

3.3. RhuDex inhibits T cell proliferation in dose dependent manner

To assess the efficacy of RhuDex, we used flow cytometry to analyse the CHO cell/T cell co-culture system while treating the cells with a range of RhuDex concentrations (30 μg/ml, 15 μg/ml, 7.5 μg/ml, 3.75 μg/ml). Treatment with RhuDex inhibited proliferation in a dose-dependent manner for T cell subsets co-cultured with CHO-CD80 ($CD8^+$ and $CD4^+$ T cells when treated with 30 μg/ml: $p<0.0001$, $CD8^+$ and $CD4^+$ T cells when treated with 15 μg/ml: $p=0.0005$ and $p=0.00043$, respectively) (**Fig. 6 B**, **E**) and CHO-CD86 ($CD8^+$ and $CD4^+$ T cells when treated with 30 μg/ml or 15: $p<0.0001$, $CD8^+$ T cells when treated with 7.5 μg/ml: $p=0.0159$) (**Fig. 6 C**, **F**). There was negligible spontaneous proliferation in the CHO co-culture compared to the CHO-CD80 or CHO-CD86 (**Fig. 6 A**, **D**), with the treated cells proliferating at even lower levels than the untreated cells.

Due to the sharp decline in proliferation caused by treatment with RhuDex, we hypothesised that RhuDex could promote cell death in the co-cultures. RhuDex had no effect on the viability of any of the different cell co-cultures at all concentrations

(**Fig. 6 G, H, I**). These data offer concrete evidence that RhuDex can reduce T cell proliferation without promoting cell death; however, this effect does not seem to be limited to the CD28-CD80 axis.

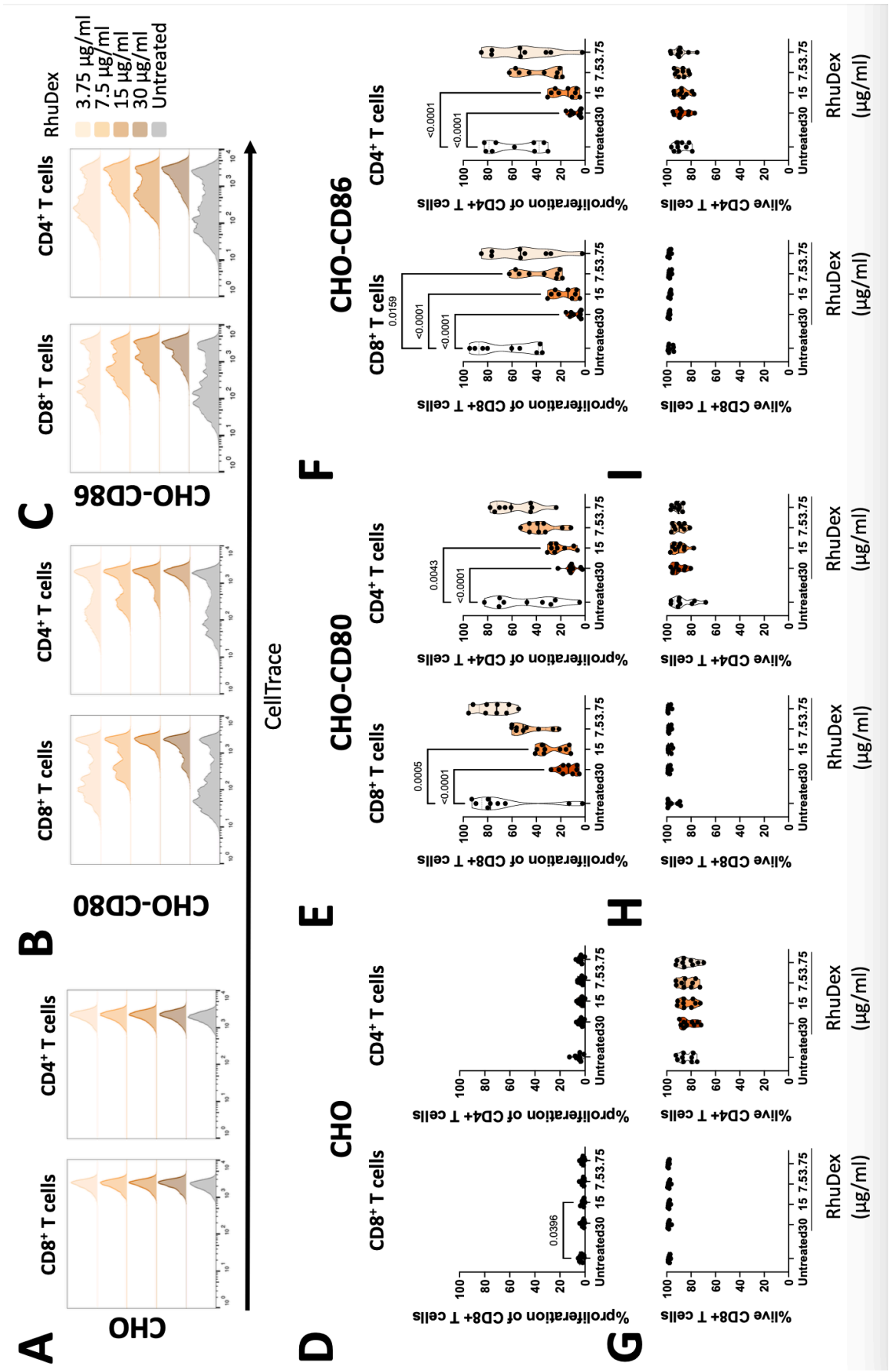

Figure 6: RhuDex inhibits T cell proliferation in a dose-dependent manner. $CD3^+$ T cells were

isolated from blood of HFE patients and co-cultured for 5 days with either anti-CD3/CHO, anti-CD3/CHO-CD80, or anti-CD3/CHO-CD86 in the presence or absence of 30 μg/ml, 15 μg/ml, 7.5 μg/ml, or 3.75 μg/ml of RhuDex. At day 5 we measured percentage of proliferation for $CD8^+$ and $CD4^+$ T cells as well as percentage of live $CD8^+$ and live $CD4^+$ T cells. (**A-C**) Representative flow cytometry histograms of CellTrace stain of $CD8^+$ and $CD4^+$ T cells in the presence or absence of RhuDex co-cultured with (**A**) CHO, (**B**) CHO-CD80, or (**C**) CHO-CD86. Grey histograms denote untreated samples and different hues of orange depict different concentrations of RhuDex. (**D-F**) Accumulative data of the % of proliferation of both $CD8^+$ and $CD4^+$ T cells for (**D**) CHO, (**E**) CHO-CD80, or (**F**) CHO-CD86. (**G-I**) % of live $CD8^+$ and $CD4^+$ T cells when co-cultured with (**G**) CHO, (**H**) CHO-CD80, or (**I**) CHO-CD86. Statistical analysis performed is one-way ANOVA with Dunnett's multiple comparisons test. N=9 for all samples.

3.4. Abatacept inhibits T cell proliferation in co-culture model

We next treated T cells with Abatacept using the co-culture system. Flow cytometry results revealed that the effective range for the drug we used was similar, albeit broader, to what has been used previously (100 μg/ml, 50 μg/ml, 25 μg/ml, and 12.5 μg/ml) (558, 559). Abatacept inhibited proliferation of $CD8^+$ T cells (50 μg/ml: $p=0.0287$, 25 μg/ml: $p=0.0104$, 12.5 μg/ml: $p=0.0287$,) and $CD4^+$ T cells (50 μg/ml: $p=0.0462$, 25 μg/ml: $p=0.0019$) when co-cultured with CHO-CD80 cells (**Fig. 7 B**, **E**). Interestingly, Abatacept suppressed proliferation of $CD4^+$ T cells co-cultured with CHO-CD86 (100 μg/ml: $p<0.0001$, 50 μg/ml: $p=0.0002$), but not of $CD8^+$ T cells (**Fig. 7 C**, **F**). Furthermore, abatacept had no effect on $CD4^+$ T cells co-cultured with CHO cells but it reduced the CHO - $CD8^+$ T co-culture which likely reflects spontaneous proliferation (25 μg/ml:$p=0.0287$) (**Fig. 7 A**, **D**).

Abatacept did not affect the percentage of live T cells in the CHO (**Fig. 7 G**), or

CHO-CD86 (**Fig. 7 I**) co-cultures, however a small effect was present in the CHO-CD80 $CD8^+$ T cell co-culture (50 μg/ml: p=0.0462, 25 μg/ml: p=0.0462) (**Fig. 7 H**), These results show that Abatacept can inhibit both CD80- and CD86-driven T cell proliferation in our assay.

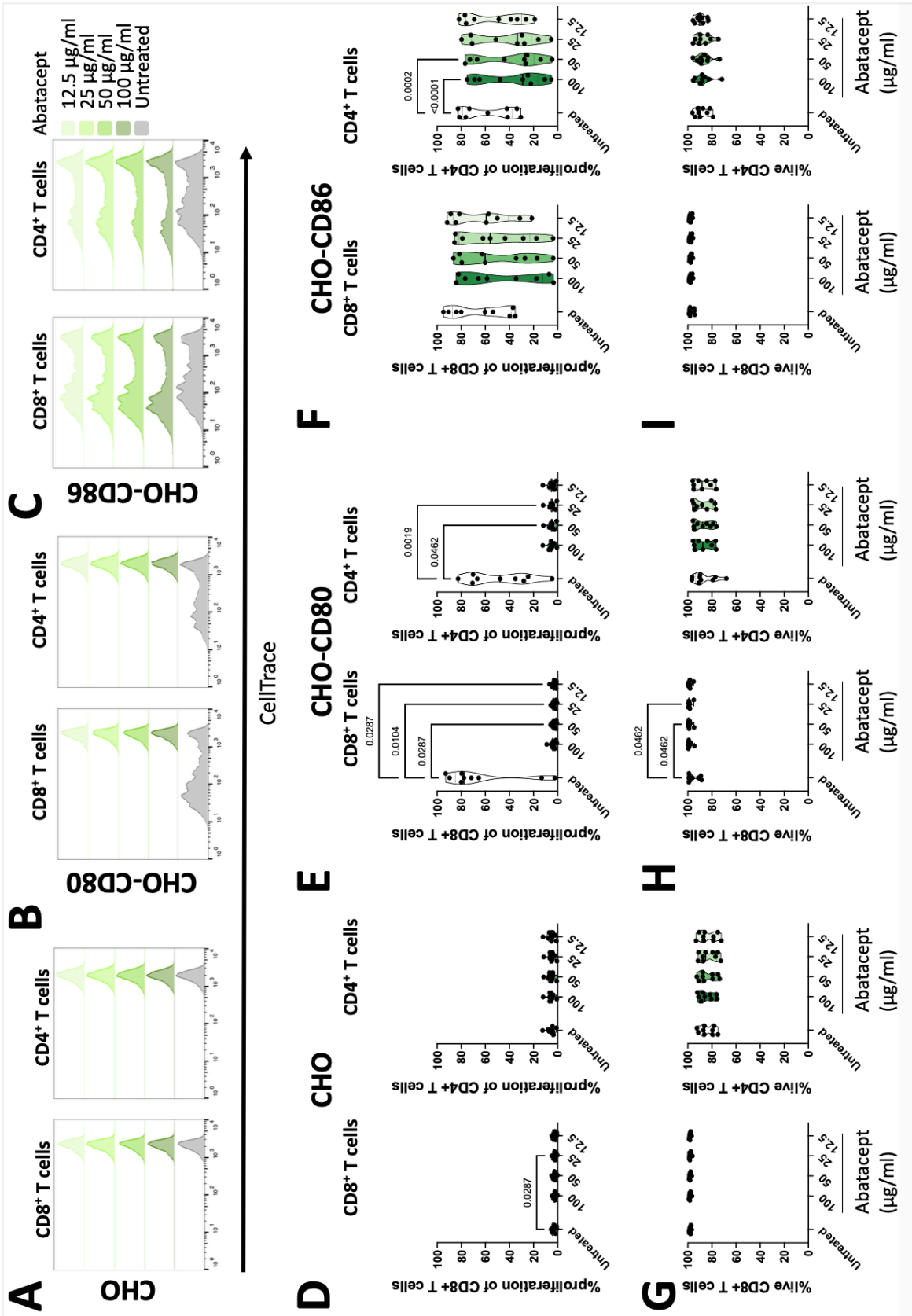

Figure 7: Abatacept inhibits T cell proliferation in both CHO-CD80 and CHO-CD86 co-cultures. $CD3^+$ T cells were isolated from blood of HFE patients and co-cultured for 5 days with either anti-CD3/CHO, anti-CD3/CHO-CD80, or anti-CD3/CHO-CD86 in the presence or absence of 100 µg/ml,

50 µg/ml, 25 µg/ml, or 12.5 µg/ml of RhuDex. At day 5 we measured percentage of proliferation for $CD8^+$ and $CD4^+$ T cells as well as percentage of live $CD8^+$ and live $CD4^+$ T cells. (**A-C**) Representative flow cytometry histograms of CellTrace stain of $CD8^+$ and $CD4^+$ T cells in the presence or absence of Abatacept co-cultured with (**A**) CHO, (**B**) CHO-CD80, or (**C**) CHO-CD86. Grey histograms denote untreated samples and different hues of green depict different concentrations of RhuDex. (**D-F**) cumulative data of the % of proliferation of both $CD8^+$ and $CD4^+$ T cells for (**D**) CHO, (**E**) CHO-CD80, or (**F**) CHO-CD86. (**G-I**) % of live $CD8^+$ and $CD4^+$ T cells when co-cultured with (**G**) CHO, (**H**) CHO-CD80, or (**I**) CHO-CD86. Statistical analysis performed is one-way ANOVA with Dunnett's multiple comparisons test. N=9 for all samples.

When we compared the effectiveness of abatacept to inhibit proliferation at different concentrations we found that abatacept is more potent at inhibiting T cell proliferation for both $CD8^+$ T cells (**Fig. 8 A**) and $CD4^+$ T cells (**Fig. 8 B**) at all doses tested ($p<0.0001$, multiple comparisons with Holm-Sidak). These data suggest that abatacept can work well as a CD80 inhibitor but not as a CD86 inhibitor when it is used in a co-culture with CHO-CD86 cells.

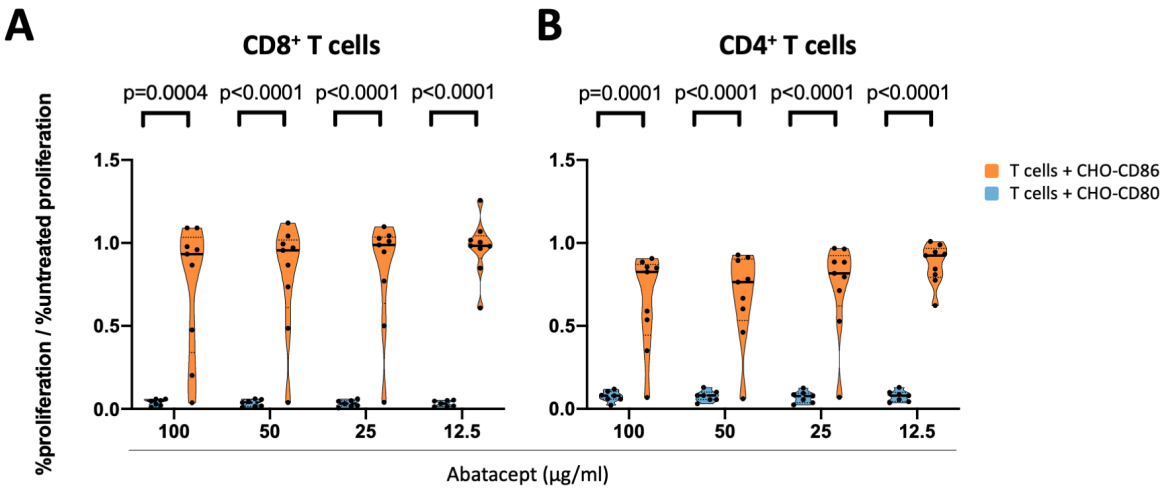

Figure 8: Abatacept is more potent at inhibiting proliferation when blocking the CD80 pathway. $CD8^+$ and $CD4^+$ T cells where co-cultured with Abatacept at different concentrations in the presence of anti-CD3 antibody and either CHO-CD80 or CHO-CD86 cells for (**A**) $CD8^+$ T cells and (**B**) $CD4^+$ T cells. The results were plotted as a ratio of the percentage of proliferation of the treated cells to the untreated

cells. For statistical analysis multiple comparisons using the Holm-Sidak method was used, α=0.05. For CHO-CD80 co-cultured T cells n=7, for CHO-CD86 T cells n=8.

3.5. Anti-CD80 inhibits T cell proliferation in co-culture model

Following abatacept, we used flow cytometry to examine the effect of CD80 blockade using an anti-CD80 antibody in the co-stimulation system at a wide range of concentrations (100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml). Anti-CD80 could only block proliferation at all doses tested only when T cells were co-cultured with CHO-CD80 ($p<0.0001$ for $CD8^+$ and $p=0.0002$ for $CD4^+$ T cells compared to IgG1 control) (**Fig. 9 B, E**) and not with CHO or CHO-CD86 (**Fig. 9 A, D, C, F**).

Toxicity was not promoted when T cells were co-cultured with the anti-CD80 antibody for any of the CHO co-cultures (**Fig. 9 G, I, H**). In conclusion, anti-CD80 antibody worked as expected in our system, by specifically inhibiting CD80-driven T cell proliferation.

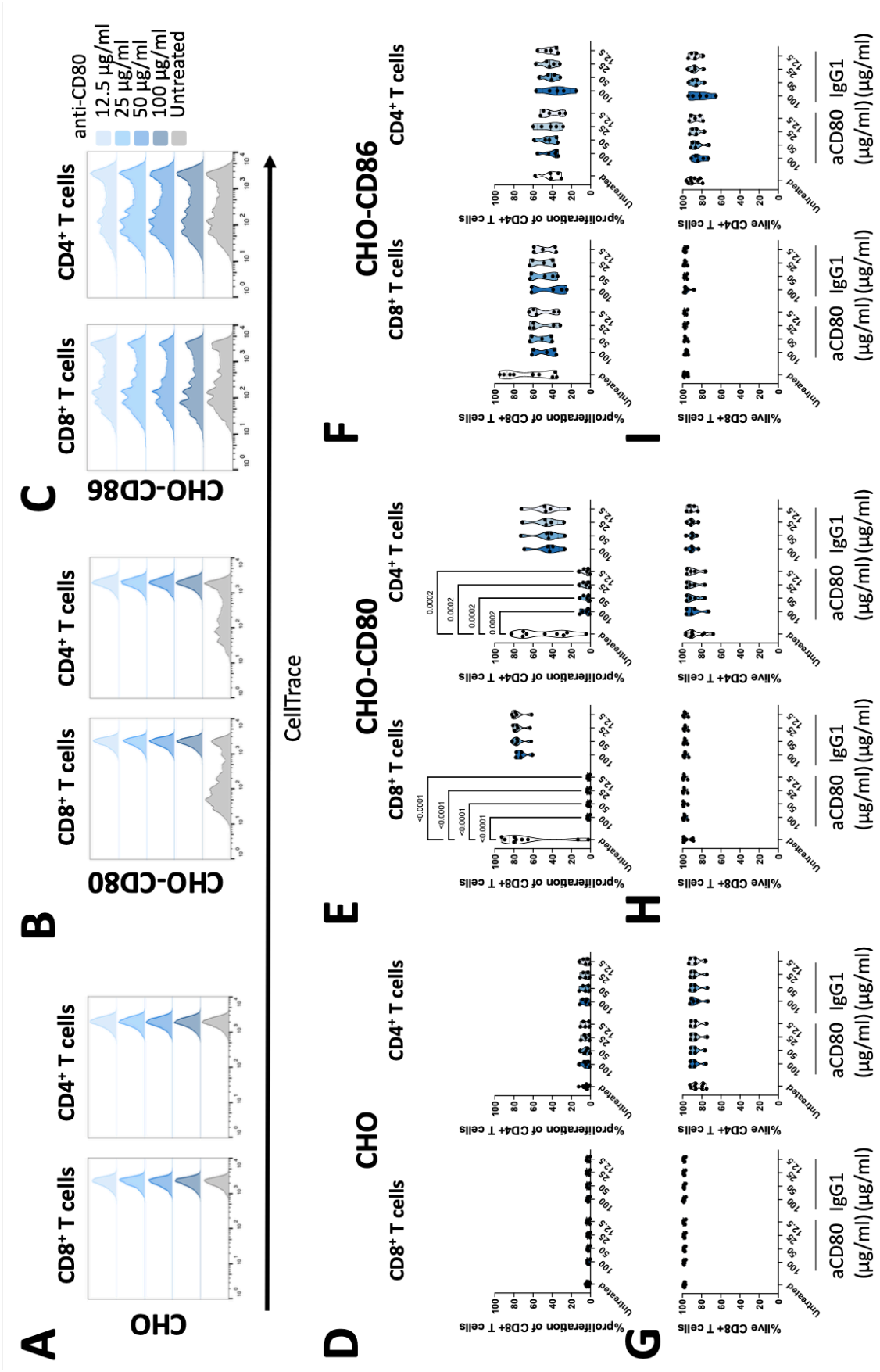

Figure 9: Anti-CD80 inhibits T cell proliferation in CHO-CD80 co-cultures. $CD3^+$ T cells were

isolated from blood of HFE patients and co-cultured for 5 days with either anti-CD3/CHO, anti-CD3/CHO-CD80, or anti-CD3/CHO-CD86 in the presence or absence of 100 μg/ml, 50 μg/ml, 25 μg/ml, or 12.5 μg/ml of of anti-CD80 (aCD80) or IgG1. At day 5 we measured percentage of proliferation for $CD8^+$ and $CD4^+$ T cells as well as percentage of live $CD8^+$ and live $CD4^+$ T cells. (**A-C**) Representative flow cytometry histograms of CellTrace stain of $CD8^+$ and $CD4^+$ T cells in the presence or absence of anti-CD80 co-cultured with (**A**) CHO, (**B**) CHO-CD80, or (**C**) CHO-CD86. Grey histograms denote untreated samples and different hues of blue depict different concentrations of anti-CD80. (**D-F**) cumulative data of the % of proliferation of both $CD8^+$ and $CD4^+$ T cells for (**D**) CHO, (**E**) CHO-CD80, or (**F**) CHO-CD86. (**G-I**) % of live $CD8^+$ and $CD4^+$ T cells when co-cultured with (**G**) CHO, (**H**) CHO-CD80, or (**I**) CHO-CD86. Statistical analysis performed is one-way ANOVA with Dunnett's multiple comparisons test. N=9 for untreated sample, n=5 for all anti-CD80 and IgG1-treated samples.

3.6. Anti-CD86 inhibits T cell proliferation in co-culture model

Finally, we used an anti-CD86 antibody to block signal received from CD86, using the CHO co-stimulation system, in a wide range of doses (100 μg/ml, 50 μg/ml, 25 μg/ml, and 12.5 μg/ml) using flow cytometry. Anti-CD86 had no effect on T cells in CHO or the CHO-CD80 co-cultures (**Fig. 10 A, B, D**, **E**). Anti-CD86 strongly inhibited $CD8^+$ ($p<0.0001$ at all concentrations) and $CD4^+$ T cell proliferation ($p<0.0001$, at all concentrations) when co-cultured with CHO-CD86 (**Fig**. **10 C, F**). A small effect on T cell proliferation was noted in the $CD4^+$ T cell IgG2b control group at 100 μg/ml concentration (**Fig. 10 F)**

Similarly to previous observations, no effect was present on the viability of these cells in all co-cultures when treated with the anti-CD86 antibody (**Fig. 10 G, H, I**).

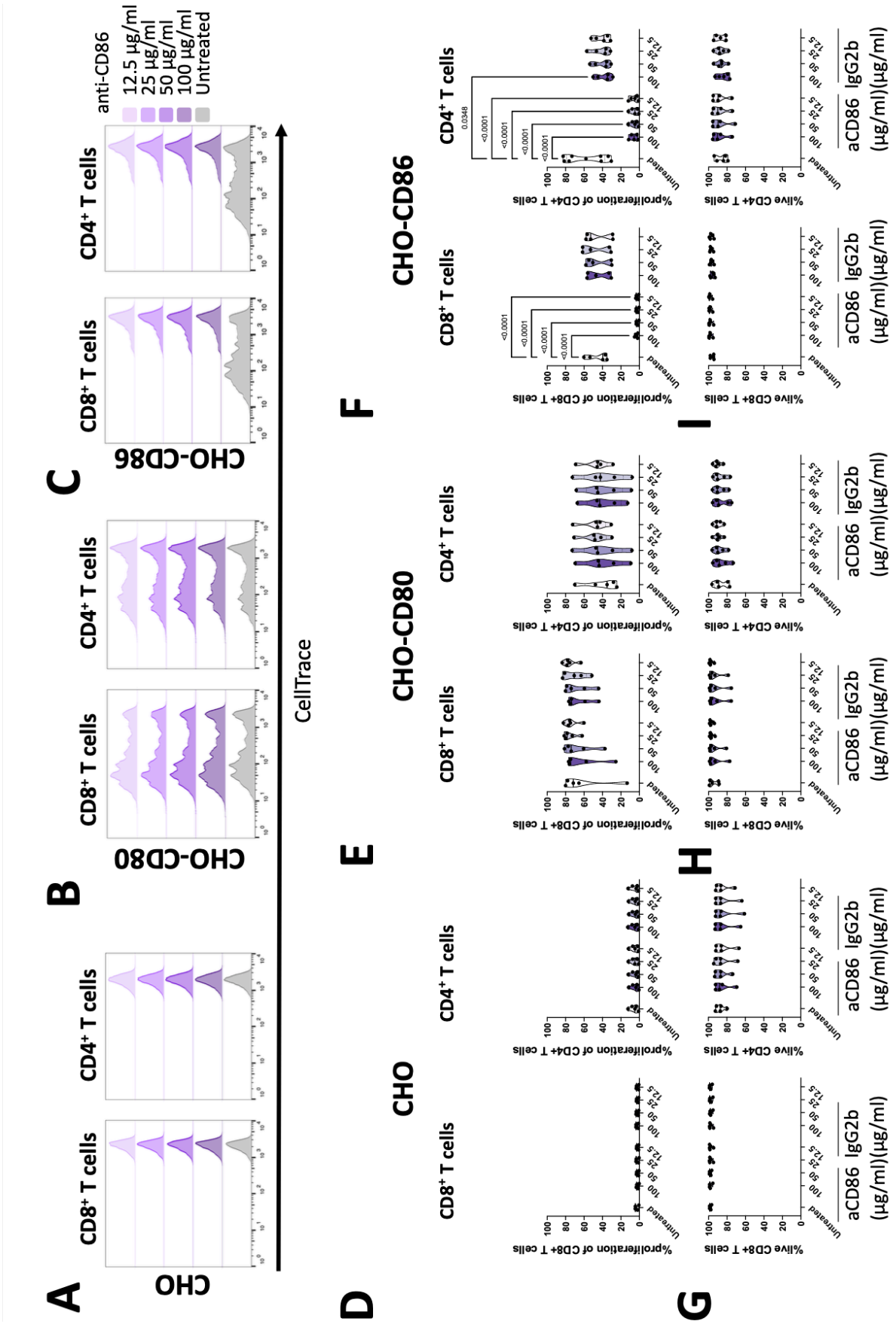

Figure 10: Anti-CD86 inhibits T cell proliferation in CHO-CD86 co-cultures. CD3+ T cells were

isolated from blood of HFE patients and co-cultured for 5 days with either anti-CD3/CHO, anti-CD3/CHO-CD80, or anti-CD3/CHO-CD86 in the presence or absence of 100 μg/ml, 50 μg/ml, 25 μg/ml, or 12.5 μg/ml of of anti-CD86 (aCD86) or IgG2b. At day 5 we measured percentage of proliferation for CD8+ and CD4+ T cells as well as percentage of live CD8+ and live CD4+ T cells. (**A**-**C**) Representative flow cytometry histograms of CellTrace stain of CD8+ and CD4+ T cells in the presence or absence of anti-CD86 co-cultured with (**A**) CHO, (**B**) CHO-CD80, or (**C**) CHO-CD86. Grey histograms denote untreated samples and different hues of purple depict different concentrations of anti-CD80. (**D-F**) cumulative data of the % of proliferation of both CD8+ and CD4+ T cells for (**D**) CHO, (**E**) CHO-CD80, or (**F**) CHO-CD86. (**G-I**) % of live CD8+ and CD4+ T cells when co-cultured with (**G**) CHO, (**H**) CHO-CD80, or (**I**) CHO-CD86. Statistical analysis performed is one-way ANOVA with Dunnett's multiple comparisons test. N=9 for untreated sample, n=5 for all anti-CD80 and IgG1-treated samples.

3.7. Discussion

There are several co-stimulatory molecules that can lead to T cell activation. The CD28-CD80/CD86 pathway is the most thoroughly characterised pathway that contributes to T cell priming, cell growth, survival, and memory formation. Studying this pathway in primary cells, however, is difficult due to a number of co-stimulatory molecules expressed on the surface of APCs, all of which contribute to T cell responses. For that reason, we used a system of transgenic CHO cells which express CD80 or CD86 on their surface. This allows to investigate the effects of CD80 and CD86 on T cells without interference by other co-stimulatory molecules. Indeed, in our assays T cells co-cultured in the presence of CHO not expressing co-stimulatory molecules showed negligible increase in proliferation (**Fig. 5 C, D**). This is likely due to the lack of conserved co-stimulatory proteins between human and Chinese hamster ovary cells, which made this model ideal for the study of human co-stimulatory molecules in isolation.

Co-stimulation studies have been employed successfully in the past to study RhuDex as well as study the CD28-CD80/CD86 pathway itself (225, 536–538). As current literature suggests, only T cells that were co-cultured with CHO-CD80 or CHO-CD86 proliferated, with background levels of proliferation for the T cells co-cultured with CHO cells (**Fig. 5 C, D**). Furthermore, both populations of $CD4^+$ and $CD8^+$ T cells remained alive when co-cultured with either CHO-CD80 or CHO-CD86 for 5 days (**Fig. 5 E, F**). Interestingly, a greater percentage of $CD8^+$ T cells were alive when co-cultured with either CHO-CD80 or CHO-CD86 compared to $CD4^+$ T cells (**Fig. 5 E, F**). T cells require IL-2 for survival in culture (560, 561). Lack of exogenous IL-2 results in limited proliferation response and higher death rates as the proliferation cycles increase (561). Due to absence of exogenous IL-2 in our system, we hypothesise that T cell survival resulted from IL-2 secretion from $CD4^+$ T cells in

an autocrine fashion. However, IL-2 secreted by the cells is typically not enough to promote extended cell survival, with the peak of IL-2 secretion at 24h post-stimulation and a two-fold decrease after 48h (562–564). $CD8^+$ T cells can also secrete IL-2, although evidence points to only $CD8^+$ T cells that either have a memory phenotype or are effector $CD8^+$ T cells with a capacity to become memory cells can secrete IL-2 (565, 566). Moreover, $CD8^+$ T cells become nonresponsive to the autocrine IL-2, and anergic in a process called activation-induced non-responsiveness (AINR) after 3-4 days (567). Exogenous IL-2 can reverse this process and resume their antigen-driven proliferation after 1-2 days (567). $CD4^+$ T cells do not undergo AINR, but instead after activation they can undergo activation-induced cell death (AICD) (568). One study hypothesises that exogenous IL-2 secreted by $CD4^+$ T cells can reverse this process (568). We isolated total $CD3^+$ T cells so our co-cultures contained both $CD4^+$ and $CD8^+$ T cells, which explains their survival resulting from double secretion of IL-2 by both types of cells. However, it does not explain the decrease in survival of $CD4^+$ T cells compared to $CD8^+$ T cells, something that we noticed on all cell death experiments in this chapter. Possibly $CD8^+$ T cells uptake most of the IL-2 in the culture and the remaining IL-2 is consumed by $CD4^+$ T cells, the rest of which undergo AICD. There are no studies which compare the time of IL-2 uptake by $CD4^+$ and $CD8^+$ T cells to our knowledge. In conclusion, we believe that the system we adopted was sufficient to measure the effects of co-stimulation blockade on T cell proliferation as well as measure the survival of cells after being treated with inhibitors.

Using the established co-stimulation system, we treated the three different CHO cell lines with T cells in the presence and absence of RhuDex, according to doses used in the investigator's brochure (535). Treatment resulted in a dose-dependent inhibition of proliferation for both $CD4^+$ and $CD8^+$ T cells co-cultured with CHO-

CD80 cells (**Fig. 6 E**). RhuDex's ability to bind onto CD80 but not CD86 allows for suppression of activation of $CD4^+$ T cells (535). Upon activation, $CD4^+$ T cells can either become pro- or anti-inflammatory cells though signal 3, usually in the form of cytokines, however, evidence that suggests activation through CD80 or CD86 affects the end fate of the cells. In a mouse model for multiple sclerosis, EAE, researchers discovered that *in vitro* and *in vivo* T cells secrete the pro-inflammatory cytokine IFN-γ when anti-CD86 was present in the co-culture (569). When they were treated with anti-CD80, the cells secreted the anti-inflammatory cytokine IL-4 (569). $CD8^+$ T cells derived from non-obese diabetic (NOD) mice which constitutively express CD86 were less activated compared to NOD-CD80 mice (570). *In vitro* studies on the same model showed that $CD8^+$ T cells when co-cultured with β-islets expressing CD80 had an enhanced capacity for cytotoxicity, cytokine secretion, and proliferation (570). There is also evidence that intracellular signals transmitted to the T cells differ if they received signal 2 through CD80 or CD86 (571). Furthermore, blocking of CD80 in an inflammatory bowel disease (IBD) mouse model prevented the development of colitis and it decreased the levels of Th1 cytokines in the colon of the mice (572). Finally, Treg cells enhance their suppressive abilities when co-cultured with allogeneic DCs in the presence of anti-CD86 (243). Even though the evidence points to the role of CD80-activation enhancing the pro-inflammatory responses, Galiximab, which blocks CD80, did not have an effect in clinical trials for lymphoproliferative disorders (554–557). Whether RhuDex has an effect on patients with PBC remains to be established.

RhuDex also inhibited proliferation of T cells treated with CHO-CD86 (**Fig. 6 F**). SRP and ITC data show that RhuDex binds to CD80 with high affinity but not with CD86 (535). Soskic et al. noted that RhuDex inhibits T cell proliferation when co-cultured with CHO-CD86 as well or when incubated with anti-CD3/anti-CD28

antibodies (unpublished data). Furthermore, RhuDex inhibited T cell proliferation even at the CHO co-culture, where the levels of proliferation for those T cells were minimal due to the lack of signal 2 (measuring spontaneous T cell proliferation from *in vivo* activated T cells). There is also evidence that RhuDex can bind to nuclear hormone receptors antagonistically (535). The above results indicate that RhuDex, although it binds specifically to CD80, has potential off-target effects on T cells that have not been yet fully characterised.

The inhibition of proliferation induced by RhuDex did not affect the viability of cells for all $CD8^+$ and $CD4^+$ T cells T cells co-cultured with CHO, CHO-CD80, or CHO-CD86 (**Fig. 6 G, H, I**). Heninger et al. also did not find any negative effects of Rhudex on T cell viability (537). These results are encouraging, since RhuDex allows for T cell modulation without promoting any severe negative effects on T cells *in vitro*.

The next inhibitor we tested was abatacept. The CTLA-4-Ig binds specifically to both CD80 and CD86 and allows for targeted co-stimulation blockade (183, 543). This process is believed to take place through trans-endocytosis of the CD80 or CD86 receptor by CTLA-4 or through sequestration of the cytoskeletal elements of the APC (225, 573). Human B cells can also internalise abatacept after binding to surface CD80 or CD86 through dynamin-dependent internalisation (574). When originally CTLA-4-Ig was used *in vitro* and *in vivo*, the antibody suppressed T cell activation by interfering with T cell co-stimulation (183, 543, 575). Our results also indicate that abatacept blocked T cell proliferation strongly at all doses used when present with the CHO-CD80 cells (**Fig. 7 E**). Abatacept also suppressed T cell proliferation for $CD4^+$ T cells co-cultured with CHO-CD86 cells (**Fig. 7 F**). Inhibition of proliferation for cells cultured with CHO-CD86 was not as strong as it was for the CHO-CD80 co-cultured T cells (**Fig. 8 A, B**). We found no differences in the co-culture of $CD8^+$ T

cells with CHO-CD86 in the presence of abatacept, however a small inhibitory effect was present at the highest concentrations of Abatacept in the $CD4^+$ T cell co-cultures (**Fig. 7 F**). A possible explanation for the selective ability of abatacept to inhibit proliferation of T cells co-cultured with CHO-CD80 versus those with CHO-CD86 could lie in the monomeric binding affinities of CTLA-4. When binding to CD80 or CD86, the equilibrium dissociation constant is ~211 nM and ~2200 nM respectively (194). However, we cannot explain why abatacept has a proliferative effect for $CD4^+$ T cells co-cultured with CHO-CD86 but not for $CD8^+$ T cells co-cultured with the same cells. In contrast to our results, Heninger et al. did not find abatacept to have an anti-proliferative effect on T cells at 10 μg/ml when they were co-cultured with LPS-activated peripheral blood monocytes or non-adherent peripheral blood lymphocytes (537).

In vivo, abatacept has shown promising results for the treatment of PBC. Mice administered with abatacept after developing PBC had reversal of the disease by inhibiting the production of anti-PDC-E2 antibodies, fewer CD4 and CD8 T cell infiltration in the liver, and less bile duct damage (576). Abatacept is currently being used in a clinical trial to assess the efficacy of the drug on PBC patients that are unresponsive to UDCA, the results of which are pending (study number NCT02078882). Abatacept's inhibitory ability can be improved further. Belatacept, another CTLA-4-Ig drug that differs only in two amino acids to abatacept, allows for two times smaller dissociation constant from CD80 and four times smaller dissociation from CD86 compared to abatacept (577). Belatacept has already been approved by the FDA and the European Medicines Agency (EMA) for kidney transplant recipients to kidney rejection.

Abatacept did not promote significant toxicity in any of the co-cultures for either $CD8^+$ T or $CD4^+$ T cells, in line with current literature (**Fig. 7 G, H, I**)(558). The only

difference we found was an increased percentage of live $CD8^+$ T cells when co-cultured with CHO-CD80 cells, however this effect is a possible statistical error (**Fig. 7 H**). Lack of cell death has also been noted before for cells treated with abatacept (537, 558). Furthermore, abatacept could reduce CD95-mediated T cell death for both $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells (578). In conclusion, abatacept does not carry the capacity to induce cell death.

Following the above, we tested two commercially available anti-CD80 and anti-CD86 inhibitor antibodies. The effective concentration for CD80 or CD86 inhibitors is not well documented in the literature (536, 537, 579–582). For that reason, we tested a wide range of concentrations to block the CD28-CD80 pathway effectively. When $CD3^+$ T cells were co-cultured with CHO-CD80 cells in the presence of anti-CD80 inhibitor, proliferation was inhibited at all tested concentrations (**Fig. 9 E**). As expected, cells proliferated when co-cultured with the CHO-CD86 cell lines despite the presence of anti-CD80 (**Fig. 9 F**). Similarly, the antibody did not promote cell death in any of the co-stimulation co-cultures (**Fig. 9 G, H, I**).

Anti-CD86 antibody also inhibited T cell proliferation when T cells were co-cultured with CHO-CD86 (**Fig. 10 F**). However, we found a small suppressive effect of IgG2b at the highest concentration in comparison to the untreated group (**Fig. 10 F**). Similar to previous results, anti-CD86 had no effect on the viability of any of the CHO co-cultures (**Fig. 10 G, H, I**).

In conclusion, we developed a robust system which allows to measure proliferation and cell death under strict conditions, which is in line with our first aim. Then, we sought to evaluate and confirm that the inhibitors we planned to use were having the effects expected from the current literature. Although abatacept, anti-CD80, and anti-CD86 performed as expected, RhuDex exhibited off-target effects and caused consistent, dose-dependent T cell suppression of proliferation without toxic

effects. RhuDex's ability to bypass CD80 blockade and directly effect T cells, regardless of CD80 or CD86 in the culture, brings up more questions regarding its role in the human cells from the liver, which we examined in chapter 5.

Chapter 4 - CD80 and CD86 expression in the human livers

4.1. Introduction

4.1.1. Expression of CD80 and CD86 on B cells

The expression of CD80 and CD86 on B cells has been identified early on (583–585). In 1989, Freeman et al. identified 4 different mRNA transcripts of the B7 molecule on B cells, which were maximally expressed between 4 to 12 hours (586). CD86 is expressed at higher levels on B cells compared to CD80, with a peak expression between 6 to 12 hours (587–591). IL-4 treatment induced the expression of CD80 and CD86 on the surface of B cells (588, 592). Upon IL-4 stimulation CD86 was expressed within 6 hours with a maximal expression at 12 hours and CD80 was expressed after 48 hours with a peak expression at 72 hours. CD80 is also expressed on B cells after 48 - 72 hours upon stimulation with anti-Ig, EBV, HLA-DR, or IL-2 (583–585, 593, 594). Stimulation of antigen-specific B cells with antigen upregulated the expression of CD86 (590). When B cells were stimulated with Con A, CD86 but not CD80 was upregulated (585). In the liver, mouse hepatic B cells have been shown to upregulate their CD80 and CD86 molecules upon LPS stimulation (595).These B cells also secrete elevated levels of pro-inflammatory cytokines such as IFN-γ, IL-6, and TNF-α, while secreting decreased levels of IL-10, compared to splenic B cells. In humans, B cells also upregulated their CD80 and CD86 expression upon LPS stimulation (184). Expression of CD80 and CD86 on the surface of B cells is also dependent on cell contact or CD40 stimulation (596, 597). Expression of CD80 and CD86 declines after 16 days on B cells that have been cultured and grown *in vitro*, however, when B cells are cultured post-isolation with no external stimulus the expression of CD80 and CD86 declines overnight (237, 589, 594). Furthermore, CD80 knockout mice had elevated levels of apoptotic germinal centre B cells and fewer Tfh cells (598). The remaining Tfh cells had lower *IL-21* levels, a cytokine responsible for

sustaining the development of B cells at the germinal centre.

B cells at different activation statuses have a different expression of CD80 and CD86. Naive B cells express CD80 and CD86 at low levels whereas memory B cells as well as germinal centre B cells express CD80 and CD86 constitutively (90, 599–604). Bar-Or et al. suggests that a large portion of circulating B cells are memory B cells which express high levels of CD80 but not CD86 (600). When they compared the activation status of the memory and the naive B cells, they found no significant differences, suggesting that expression of CD80 on the surface of the B cells should not be treated as an activation marker. They also suggest that a novel population of a quiescent memory B cell population exists in the blood that expresses high levels of CD80. These B cells can secrete large amounts of class-switched Igs, have fast proliferative responses, and are efficient T cell activators compared to naive B cells. Good and colleagues showed that mature B cells express CD80 and CD86 on their surface, however the *CD80* transcripts were different for the different subsets (90). Class-switched memory B cells expressed CD80 on their surface in higher amounts compared to IgM memory B cells. Upon CD40 stimulation, upregulation of CD80 and CD86 was faster on memory than on naive B cells (599). Upregulation of CD80 and CD86 upon stimulation from CD40 on naive cells was much lower compared to memory or GC B cells. Transitional B cells were also shown to express low levels of CD86 similar to naive B cells (605). These cells were unable to upregulate their CD86 expression after BCR stimulation (606). Finally, Breg which arise from human transitional B cells and are able to secrete IL-10 were able to downregulate CD86 expression in an autocrine effect through IL-10 secretion (607). This led to decreased $CD4^+$ T cell proliferation and TNF-α. production.

CD80 is also disregulated in certain diseases. Human T-lymphotropic virus (HTLV)-infected patients have increased $CD80^+$ B cells compared to non-infected

patients (608, 609). In SLE, B cells isolated from the blood of patients had increased levels of CD80 and CD86 compared to normal controls (610). Upon culture with T cells, the B cells upregulated their CD86 expression and was shown that this resulted from CD40L expression on the surface of the T cells. SLE patients who had received rituximab had a decreased expression of CD40 and CD80 on their surface after 1 week of the initial injection, while patients saw clinical improvement (611). Memory B cells express similar levels of CD80 and CD86 in chronic HCV infection compared to normal controls, however, upon CD40L or BCR stimulation the memory B cells upregulated their CD80 and CD86 expression (612). Another study also suggested that B cells from HCV but not hepatitis B virus (HBV) have a higher frequency of $CD86^+$ B cells (613). Stimulation through CD40 also upregulated the expression of CD80 on normal B cells and B cells from chronic lymphocytic leukaemia (CLL) (597, 614, 615). Although B-CLL cells don't express CD80 or CD86, upon CD40 stimulation they upregulated the expression of the ligands (614, 615). Following stimulation, the B-CLL cells were able to induce allogeneic T cell proliferation, CD25 and HLA-DR T cell expression. In the proteoglycan-induced arthritis mouse model, a deficiency in the CD80 and CD86 expression of the B cells prevented the development of arthritis in the mice (551). In conclusion, B cells can express the co-stimulatory molecules CD80 and CD86, with evidence that these molecules might play a role in disease development.

4.1.2. Expression of CD80 and CD86 on monocytes

The expression of CD80 and CD86 on monocytes has also been extensively studied. Freshly isolated monocytes do not express CD80 but express CD86 (600, 616). After 6-10 hours of purified protein derivative (PPD) stimulation, CD80 and CD86 expression also increases. Other reports have shown that IFN-α, IFN-γ, and

GM-CSF upregulate the levels of CD80 and CD86 on monocytes (184, 233, 596, 597, 617–619). Recently, TNF-α was also shown to increase the percentage of CD80-expressing peripheral blood monocytes as well as rheumatoid synovial monocytes in patients with RA (620). Stimulation with IFN-γ provided the strongest up regulation of CD80 and CD86. In accordance to the previous results, freshly isolated monocytes express no *CD80* mRNA but the signal appears after 4-6 hours of culture (616, 619). Monocytes upregulated their expression of CD80 in the presence of $CD4^+$ T cells (621). Similarly, the percentage of monocytes that express CD80 and CD86 increased after co-culture with the parasite *T. gondii* (622). The upregulation of the ligands was proportional to the number of tachyzoites used in the co-culture. Furthermore, dead tachyzoites had no effect on the expression levels of the molecules. In another study, the antimicrobial peptide human β defensine-3 (hBD-3) could induce the upregulation of CD80 and CD86 on monocytes (623). Apyrase, a scavenger of extracellular ATP - which can be considered as a damage-associated molecular pattern (DAMP) - was able to induce upregulation of CD86 after incubation with monocytes. Finally, stimulation of the P2X7 receptor with exogenous ATP, a receptor found on the surface of cells including monocytes which becomes activated after receiving ATP, resulted in monocytes expressing CD86, similarly to hBD-3. Blockade of P2X7R resulted in CD80 upregulation.

The different monocyte subsets exhibit different expression patterns of CD80 and CD86. Classical monocytes have the lowest expression levels of CD86 compared to the other two monocyte subsets (624). Intermediate and non-classical monocytes express high levels of CD80, CD86, and HLA-DR on their surface, providing evidence that these subsets may exhibit co-stimulatory capacity (625). In another study, all three subsets were shown to express very low levels of CD80, with intermediate monocytes expressing the ligand at a slightly higher level compared to

the other two subsets (128). Finally, a third study shows that there is no difference in the expression of CD80 and CD86 among the different subsets (116).

CD80 and CD86 expression has been shown to be dysregulated in disease settings. Monocytes from patients suffering from Chagas disease express different levels of CD80 and CD86 compared to normal controls (626). When cultured with antigens from the *Trypanosoma cruzi*, the protozoan responsible for the development of Chagas disease increased the expression of CD80 on classical monocytes compared to untreated controls (627). In chronic HBV infection, monocytes/macrophages from the liver (characterised by CD68 expression using IHC) showed an increased percentage of $CD86^+$ cells present in lobular areas, whereas $CD80^+$ cells showed no differences to controls (628). Correlation was also observed between the $CD68^+CD80^+$ cells and fibrosis score. In fulminant hepatic failure, numerous cells were detected as positive for CD80 and CD86 expression, most of which were characterised as $CD68^+$ liver-infiltrating macrophages (629). In chronic liver diseases, $CD80^+$- and $CD86^+$-macrophages were reduced, and an increased number of lymphocytes were present (629). CD14-expressing monocytes isolated from the synovium of RA patients had increased expression of CD80 (620). A previous study shows that monocytes isolated from the blood of patients with RA had no differences in the expression levels of CD80 and CD86 compared to healthy controls (630). Furthermore, recent evidence suggests that the high mortality in pregnant women during hepatitis E infection could be attributed to increased levels of monocytes in these women, as well as increased expression of CD80 but no CD86, since the levels of the ligand was reduced compared to healthy controls (631). In SLE, monocytes expressed similar levels of CD80 and CD86 to controls (632). Upon isolation, monocytes from both groups were expressing CD86 but only a few monocytes expressed CD80. IL-10 in both groups downregulated CD86 but upregulated CD80. IFN-γ and GM-CSF upregulated both

markers. The expression of CD80 on monocytes has been described to change depending on the cardiac technique used during heart surgery (633).

In disease conditions, the different monocytic subsets also behave differently compared to healthy controls. Patients who are HIV positive and are undergoing antiretroviral therapy were shown to have decreased levels of CD86 on their non-classical monocytes (634). Monocytes from patients with indeterminate, an asymptomatic form of Chagas disease, expressed higher levels of CD86 compared to normal controls or monocytes treated with *T. cruzi* (627). In the same study, intermediate monocytes from non-infected patients expressed the highest level of CD80. CD86 was expressed on similar levels on all the subsets for the non-infected group. During the disease progression, CD80 was reduced from the intermediate and non-classical monocytes and was increased in the classical subset as well as the indeterminate group. Unlike CD80, CD86 was increased on all subsets of the indeterminate group. Intermediate monocytes in type 1 diabetes mellitus also had increased levels of CD86 and HLA-DR compared to patients with Graves disease or healthy controls (635). In another study, intermediate and non-classical monocytes were grouped together and exhibited increased levels of CD80, CD86, and HLA-DR compared to classical monocytes (636). The three monocytic subsets were shown to have no differences in the levels of CD80 and CD86 expression between healthy controls and patients with SLE. Finally, classical and intermediate monocytes from patients with TB expressed higher levels of CD80 and CD86 compared to healthy controls (637). Non-classical monocytes expressed decreased levels of CD80 from patients with TB.

4.1.3. Expression of CD80 and CD86 on dendritic cells

DCs have long been considered as professional APCs and primary T cell stimulators. They can activate and strongly prime immune T cell responses (638, 639). DCs have been shown to be able to induce a stronger T cell stimulus compared to B cells, even in the presence of CD80 and CD86 blockade (640). Freshly isolated DCs do not express CD80 and express very little CD86 (235). Upon *in vitro* culture, the ligands are expressed within 8 hours. CD86 is expressed before CD80, with CD86 being present within 8 hours of the culture and CD80 within 24 hours. The expression levels of the ligands continued to increase during a 48-hour period. *CD80* mRNA was not present in fleshly isolated DCs however *CD86* was. Similarly to the other APCs covered in this chapter, cross-linking of CD40 on DCs resulted in a stark increase in the expression of CD80 and CD86 (641). CD40 cross-linking also increased the CD86 expression at levels higher than those achieved by IFN-γ, GM-CSF, TNF-α, or IL-3 stimulation, all of which contributed to CD80 and CD86 upregulation. Langerhans cells, which are DCs present in the skin, expressed very low levels of CD80 and in some cases CD80 was not present (642). CD86 expression was weak to moderate. CD80 and CD86 mRNAs were detectable for both freshly isolated and cultured Langerhans cells. The induction of CD86 on DCs has been shown to take place through the activation of NF-κB by the transmembrane protein LIGHT (643). Furthermore, the transcription factor PU.1 was also shown to bind to the promoters of CD80 and CD86 of murine DCs and could transactivate the CD80 and CD86 (644). During viral infections in the liver of mice, resident DCs have been shown to upregulate CD80, CD86, and MHC class II molecules, in contrast to a previous study which showed that DCs from murine livers are phenotypically less mature compared to splenic ones (645, 646). These murine liver-resident DCs are $CD103^+$ and are responsible for the generation and priming of $CD8^+$ T cells. In another study, the

effect of CD80/CD86 pathway was studied to assess its role in transplantation. For that, CD80 and CD86 were silenced from DCs using the RNAi technique, and the DCs were transfused into mice. Following, a heart transplant took place. The results showed that the heart of mice with silenced DCs showed signs of only a slight edema without obvious necrosis (647). In the control group, the hearts presented with lymphocyte infiltration, myocardial swelling, and necrosis. In another study, CD86-silenced-DCs suppressed the expansion of T cells (648). The silenced DCs also created an anti-inflammatory environment by producing minimal levels of the pro-inflammatory cytokines IL-2 and IFN-γ. In addition, the expression of IL-10 and TGF-β was increased in the silenced group compared to control groups. Furthermore, DCs from the CD86-silenced group prolonged the survival of xeno-islet grafts.

Myeloid and plasmacytoid DCs also show a difference in the expression levels of CD80 and CD86. Myeloid DCs were able to induce stronger T cell stimulation compared to plasmacytoid DCs, neutrophils, $CD14^+$, or $CD16^+$ monocytes (649). $CD141^+$ myeloid DCs expressed CD80 and CD86 after *in vitro* culture as well as after TLR stimulation (650). $CD1c^+$ DCs expressed only CD86 after *in vitro* culture. Immature DCs expressed both ligands at high levels and were further increased upon TLR stimulation. cDCs isolated from hepatic lymph nodes showed that they express higher levels of CD86 on their surface compared to DCs isolated from liver perfusate, spleen, or inguinal lymph nodes (651). cDCs were also found to have a more activated phenotype compared to DCs from the other tissues. pDCs from liver lymph nodes did not express CD86 on their surface. When liver perfusate was isolated and human DCs were phenotyped, it was discovered that donor myeloid DCs were in an immature phenotype and expressed low levels of CD80 (652). The expression levels of CD80 on mDCs were similar between the liver perfusate and the intrahepatic mDCs, and significantly lower on blood mDCs. Cells isolated from the

hepatic LN contained the highest percentage of CD80-expressing DCs compared to blood or liver perfusate. mDCs from hepatic LN also expressed the highest levels of CD86 compared to liver perfusate but similar levels to those from the blood.

In disease settings, DC CD80 and CD86 expression differs to that in physiological conditions. In early RA, pDCs saw a decrease in CD86 and HLA-DR expression in a longitudinal study which lasted for 12 months, whereas both cDCs, the $CD141^+$ and the $CD1c^+$, had decreased HLA-DR expression but comparable levels of CD86 (653). Compared to healthy controls, early RA patients had significantly increased levels of CD86 expression on their cDCs. $CD141^+$ DCs also had an increased expression of HLA-DR compared to healthy controls. In another study, DCs isolated from the synovial fluid of patients with RA showed that they expressed no CD80 and very little CD86, similar to the expression pattern described earlier in physiological conditions (654). mRNA levels of CD80 was either not present or very low and CD86 mRNA was present in all samples analysed. Finally, upon culturing the DCs in either fresh culture medium or in the presence of IFN-γ or GM-CSF, the expression pattern was mixed, with some DCs from patients not upregulating CD80 or CD86 and in others CD80 was induced variably, implying that in the RA setting DCs may be defective. In a mouse model of allergic asthma, splenic DCs upregulated their CD80 expression compared to control mice, whereas no difference was found regarding their CD86 expression (655). The CD80-expressing DCs were shown to promote Th2 T cell responses, by prompting naive T cells to secrete IL-4 and IL-5 but not IFN-γ (655). Murine DCs which were $CD80/CD86^{-/-}$ were unable to prime immune responses against antigen (656). WT DCs however could induce both T cell proliferation and mount Th2 T cell responses by secretion of IL-4, IL-13, IL-10, and IFN-γ, whereas deficient DCs could not produce cytokines completely or secreted them at low levels.

4.1.4. Hypothesis and aims

Although a lot of phenotypic characterisation has been conducted for the different APCs regarding their CD80 and CD86 expression, there hasn't been a systematic characterisation of professional APCs found in the liver. In this chapter we sought to characterise the expression patterns of CD80 and CD86 on professional APCs found in the liver. Furthermore, since RhuDex's mode of action is to inhibit T cell responses through binding to CD80, it is important to establish an understanding regarding the expression level of CD80 and CD86. We hypothesised that CD80- and CD86-expression might be different in various liver diseases and could contribute to the disease development or progression. Our aims for this chapter were:

1. To phenotypically characterise the expression levels of CD80 and CD86 on B cells, monocytes, and DCs.
2. To assess the expression levels of CD80 and CD86 on B cells and monocytes from the blood and liver of patients with liver diseases.
3. To spatially characterise the expression of CD80 in the human liver.

4.2. CD80 and CD86 expression on total B cells

The first APC we phenotyped for its expression of CD80 and CD86 was the B cells. Using flow cytometry, we gated for the B cells as live, $CD3^{-}CD19^{+}$ cells (**Fig. 11 A**). We initially phenotyped B cells from the blood of patients with PBC or PSC. Samples from the blood of patients with HFE were also collected and were used as healthy controls since patients with HFE do not develop liver diseases with current medications. Comparison between diseases showed that B cells from all three groups had no difference in the percentage of CD80-expressing B cells or the expression levels of the ligand (**Fig. 11 B**). When we looked at the CD86 expression, we found a decreased percentage of B cells expressing CD86 in the PBC patients compared to the HFE group (2.577% (3.757) vs 10.63% (7.161), ANOVA with Dunn's test, $p<0.0001$) (**Fig. 11 B**). The CD86 median fluorescent intensity (MFI) levels however were increased in the PBC group compared to the HFE (85.73 (60.3) vs 36.01 (29.63), ANOVA with Dunn's test, $p=0.0366$) (**Fig. 11 B**).

We next studied the expression of CD80 and CD86 on total B cells in the liver of patients with PBC, PSC, or with a metabolic condition using flow cytometry. In the final group we collected data from patients with ALD, NASH, and NAFLD. Phenotyping the intrahepatic total B cells showed that there was no difference in the percentage of cells expressing CD80 or CD86 or their expression levels across diseases (**Fig. 11 C**). Collectively, these results show that B cells from the blood but not the liver of PBC patients might be more primed for T cell activation with elevated CD86 levels.

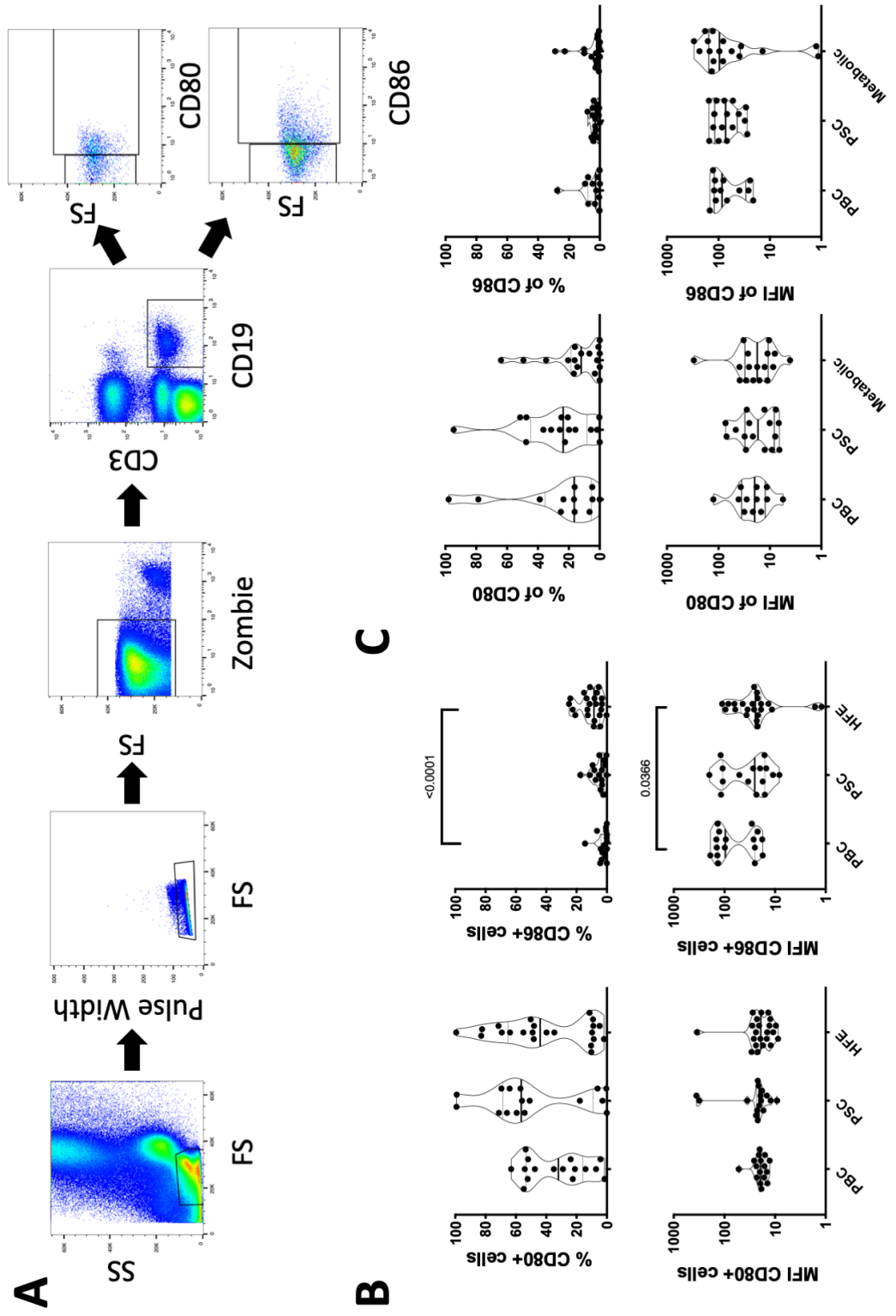

Figure 11: Phenotypic characterisation of CD80 and CD86 on total blood and liver B cells. B cells were isolated from the blood (PBC, PSC, HFE) or liver (PBC, PSC, or metabolic (ALD, NASH/NAFLD) of patients and cells were stained with fluorescent antibodies for their surface expression of CD80 and CD86. (**A**) Gating strategy used to characterise B cells from both blood and liver. (**B**) Percentage and

MFI levels of CD80 and CD86 on $CD19^+$ B cells isolated from blood. N=16-22 (**C**) Percentage and MFI levels of CD80 and CD86 on $CD19^+$ B cells isolated from the liver of patients with PBC, PSC, or metabolic conditions. N=16 for PBC and PSC, n=22 for HFE. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

4.3. CD80 and CD86 expression on B cell subsets

We next looked at the expression of CD80 and CD86 on different B cell subsets derived from the blood of HFE patients or the liver of patients with end-stage liver diseases using flow cytometry. The B cells subsets were gated using the gating strategy used by Buffa et al. and Purswani (101, 657). Live B cells were gated as $CD19^+$ and were then broken down into $CD24^-CD38^-$, $CD24^-CD38^{int}$, $CD24^-$/low$CD38^+$ plasma cells/plasmablasts (PC/PB), $CD24^+CD38^-$ memory cells (which were then further gated as IgD^-), $CD24^+CD38^{int}$ naive cells (which were then further gated as IgD^+), and finally $CD24^+CD38^+$ transitional B cells/Breg (**Fig. 12**).

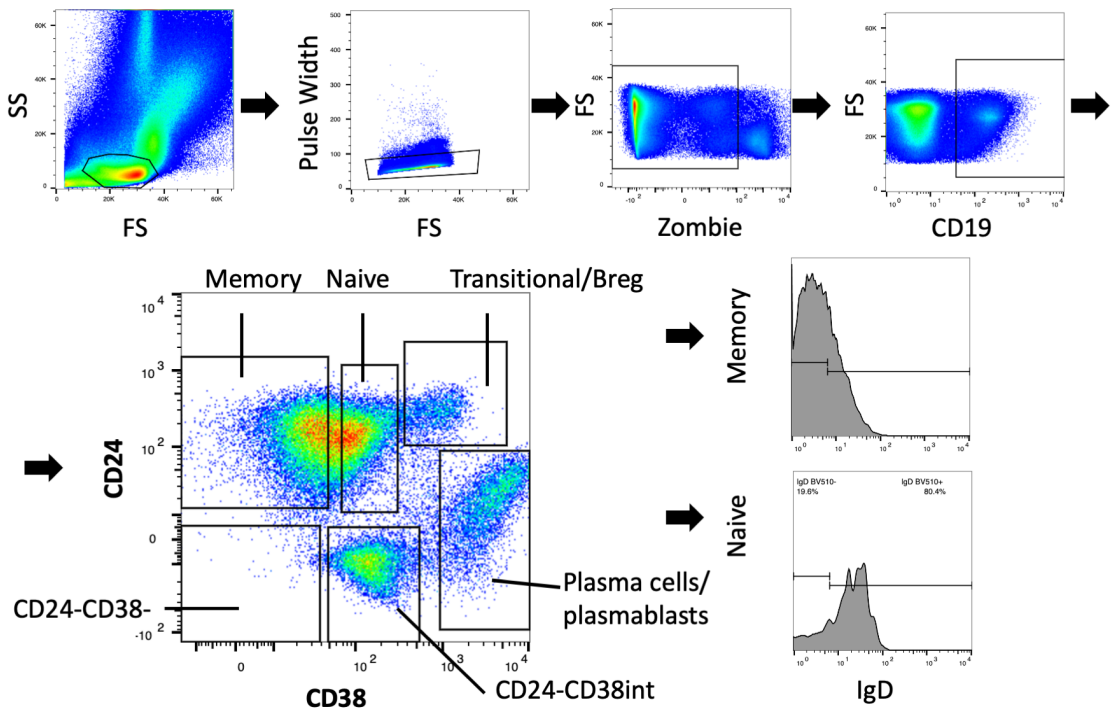

Figure 12: Gating strategy used for characterising B cell subsets isolated from the liver of patients with liver diseases. This is a representative sample from a PSC patient.

Using the established gating strategy, we compared the percentage and expression levels of CD80 and CD86 on blood B cell subsets isolated from patients with HFE. Transitional/Breg cells expressing CD80 (44.59% (34.32)) were enriched in the blood compared to naive (15.34% (21.63), ANOVA with Dunn's test, p=0.0338) and memory B cells (5.959% (6.257), ANOVA with Dunn's test, p=0.0159) (**Fig. 13 A**). PC/PB (64.29% (30.4)) were also increased compared to naive (ANOVA with Dunn's test, p=0.0001) and memory B cells (ANOVA with Dunn's test, p<0.0001). The percentage of PC/PB expressing CD86 (55.9% (27.17)) was increased compared to naive (4.667% (10.59), ANOVA with Dunn's test, p<0.0001), memory (8.314% (15.91), ANOVA with Dunn's test, p=0.0001), transitional/Breg (10.84% (12.15), ANOVA with Dunn's test, p=0.0025), and $CD24^{-}CD38^{int}$ cells (11.43% (18.68), ANOVA with Dunn's test,

p=0.0483) (**Fig. 13 B**). Furthermore, a higher percentage of $CD24^{-}CD38^{-}$ B cells (16.83% (28.27)) expressed CD86 on its surface compared to naive B cells (ANOVA with Dunn's test, p=0.0281). The MFI levels for CD80 were increased in transitional/ Breg cells (239.7 (324.5)) compared to naive (51.56 (39.35), ANOVA with Dunn's test, p=0.0072), memory (40.62 (21.47), ANOVA with Dunn's test, p<0.0001), and $CD24^{-}$ $CD38^{-}$ B cells (48.30 (29.19), ANOVA with Dunn's test, p=0.0048) (**Fig. 13 C**). Similarly, PC/PB (304.8 (314.4)) had elevated levels of CD80 compared to naive, memory, and $CD24^{-}CD38^{-}$ B cells (48.3 (29.19), for all samples, ANOVA with Dunn's test, p<0.0001). Finally, only PC/PB (116 (87.78)) had increased CD86 levels compared to naive (50.82 (25.44), ANOVA with Dunn's test, p=0.0278) and memory B cells (47.85 (22.76), ANOVA with Dunn's test, p=0.0118) (**Fig. 13 D**).

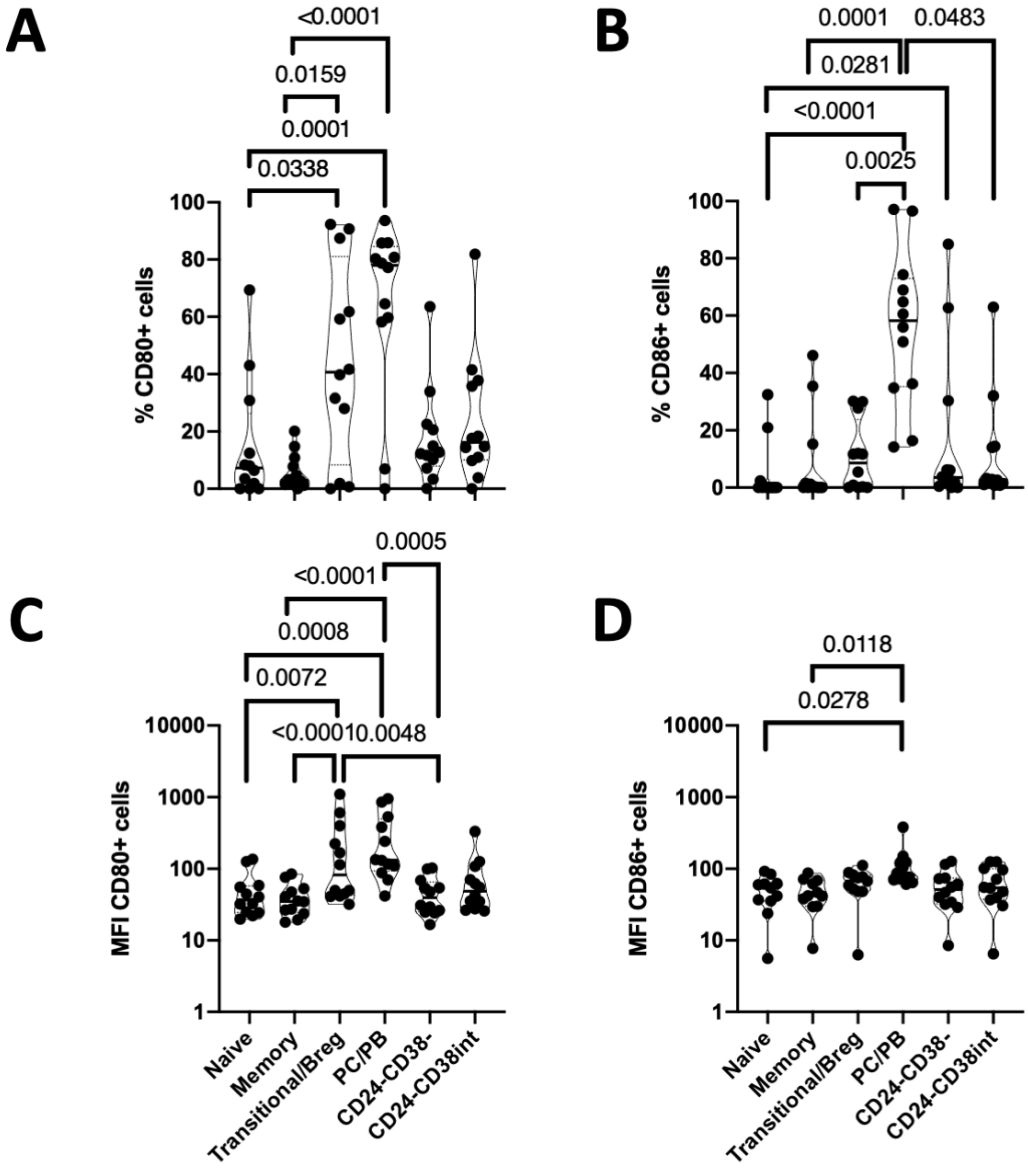

Figure 13: Phenotypic characterisation of CD80 and CD86 on blood B cell subsets. B cell subsets were isolated from the blood of HFE patients and were phenotyped for their percentage expression levels of CD80 and CD86 on the surface using fluorescent antibodies. (**A**, **B**) Percentage of CD80 and CD86 expression on different B cell subsets. (**C**, **D**) MFI levels of CD80 and CD86 on the surface of blood B cells. N=12 for all samples. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

We next measured the percentage of live liver-infiltrating B cell subsets in relation

to the total leukocytes we isolated from liver explants in order to assess the effect these cells might have in the liver. The cells were isolated from PBC, PSC, ALD, and NASH explants. Naive B cells were the most abundant B cell subset in the liver of patients (median 1.09%, interquartile range 0.55%-2.43%), followed by memory B cells (0.45%, 0.11%-1.21%), $CD24^{-}CD38^{int}$ (0.26%, 0.13%-1.6%), transitional/Breg (0.24%, 0.1%-0.33%), PC/PB (0.16%, 0.07%-0.24%) and finally $CD24^{-}CD28^{-}$ (0.023%, 0.014%-0.16%) (**Fig. 14 A**). These results indicate that B cells are only a small fraction of the total leukocytes present in the liver.

The expression of CD80, CD86, and HLA-DR was then measured on intrahepatic B cells to assess the capacity of these cells to stimulate T cells. The samples were isolated from patients with PBC, PSC, ALD, NASH, and NAFLD. CD80-expressing transitional/Breg cells (61.78% (35.01)) were increased compared to naive (38.64% (35.88), ANOVA with Dunn's test, $p=0.0251$), memory (20.62% (29.7), ANOVA with Dunn's test, $p<0.0001$), and $CD24^{-}CD38^{-}$ cells (33.42% (31.45), ANOVA with Dunn's test, $p=0.0035$) (**Fig. 14 B**). Naive cells were also increased compared to memory B cells (ANOVA with Dunn's test, $p=0.0455$). $CD80^{+}$-PC/PB (76.05% (30.06)) were elevated in comparison to naive (ANOVA with Dunn's test, $p=0.0002$), memory (ANOVA with Dunn's test, $p<0.0001$), and $CD24^{-}CD38^{int}$ (57.2% (28.03), ANOVA with Dunn's test, $p<0.0001$). Finally, $CD24^{-}CD38^{int}$ were increased compared to $CD24^{-}CD38^{-}$ (ANOVA with Dunn's test, $p=0.0222$) and memory B cells (ANOVA with Dunn's test, $p<0.0001$). The percentage of CD86-expressing cells was also increased for transitional/Breg cells (9.598% (12.32)) compared to memory B cells (2.901% (7.124), ANOVA with Dunn's test, $p=0.0044$) and PC/PB (43.53% (10.26), ANOVA with Dunn's test, $p<0.0001$) (**Fig. 14 C**). The percentage of PC/PB $CD86^{+}$- expressing cells (6.958% (11.6)) was increased in relation to naive (6.301% (10.59) ANOVA with Dunn's test, $p<0.0001$), memory (ANOVA with Dunn's test, $p<0.0001$), and $CD24^{-}$

$CD38^-$ B cells (6.958% (11.6), ANOVA with Dunn's test, $p<0.0001$). Furthermore, CD86-expressing $CD24^-CD38^{int}$ B cells were also increased compared to naive (ANOVA with Dunn's test, $p=0.0002$), memory (ANOVA with Dunn's test, $p<0.0001$) and $CD24^-CD38^-$ B cells (ANOVA with Dunn's test, $p=0.0095$). We finally studied the percentage of B cell subsets expressing HLA-DR. The results show that more than 60% of all B cell subsets expressed HLA-DR on their surface (**Fig. 14 D**). Naive B cells (89.47% (24.53)) had a higher percentage of HLA-DR-expressing cells compared to transitional/Breg (76.93% (25.26) ANOVA with Dunn's test, $p=0.0001$), PC/PB (60.08% (20.59), ANOVA with Dunn's test, $p<0.0001$), and $CD24^-CD38^-$ cells (78.9% (24.45), ANOVA with Dunn's test, $p=0.0028$). Memory B cells (82.53% (24.79)) also contained more HLA-DR-expressing cells compared to PC/PB (ANOVA with Dunn's test, $p=0.01$). Finally, liver $CD24^-CD38intHLA\text{-}DR^+$ B cells (86.26% (23.28)) were increased compared to PC/PB (ANOVA with Dunn's test, $p=0.0013$). CD80 MFI levels for PC/PB (564 (449.2)) were increased compared to naive (96.91 (107.5), ANOVA with Dunn's test, $p<0.0001$), memory (60.73 (53.61), ANOVA with Dunn's test, $p<0.0001$), $CD24^-CD38^-$ (75.93 (66.54), ANOVA with Dunn's test, $p<0.0001$), and $CD24^-CD38^{int}$ B cells (89.39 (68.7), ANOVA with Dunn's test, $p=0.0001$)(**Fig. 14 E**). Transitional/Breg cells (267.5 (260.8)) cells also expressed high levels of CD80 compared to naive (ANOVA with Dunn's test, $p=0.0229$), memory (ANOVA with Dunn's test, $p=0.0003$) and $CD24^-CD38^-$ B cells (ANOVA with Dunn's test, $p=0.006$). Interestingly, the expression levels for CD86 was similar for all subsets despite their differences in the percentage of cells expressing the ligand (**Fig. 14 F**). Furthermore, HLA-DR expression was decreased in PC/PB (169.7 (57.76)) compared to naive (368.2 (182.5), ANOVA with Dunn's test, $p=0.0005$)) and $CD24^-CD38^{int}$ B cells (206.7 9142), ANOVA with Dunn's test, $p=0.0192$)(**Fig. 14 G**). naive B cells had elevated levels of HLA-DR compared to memory B cells (217.4 (90.88), ANOVA with Dunn's

test, p=0.0445).

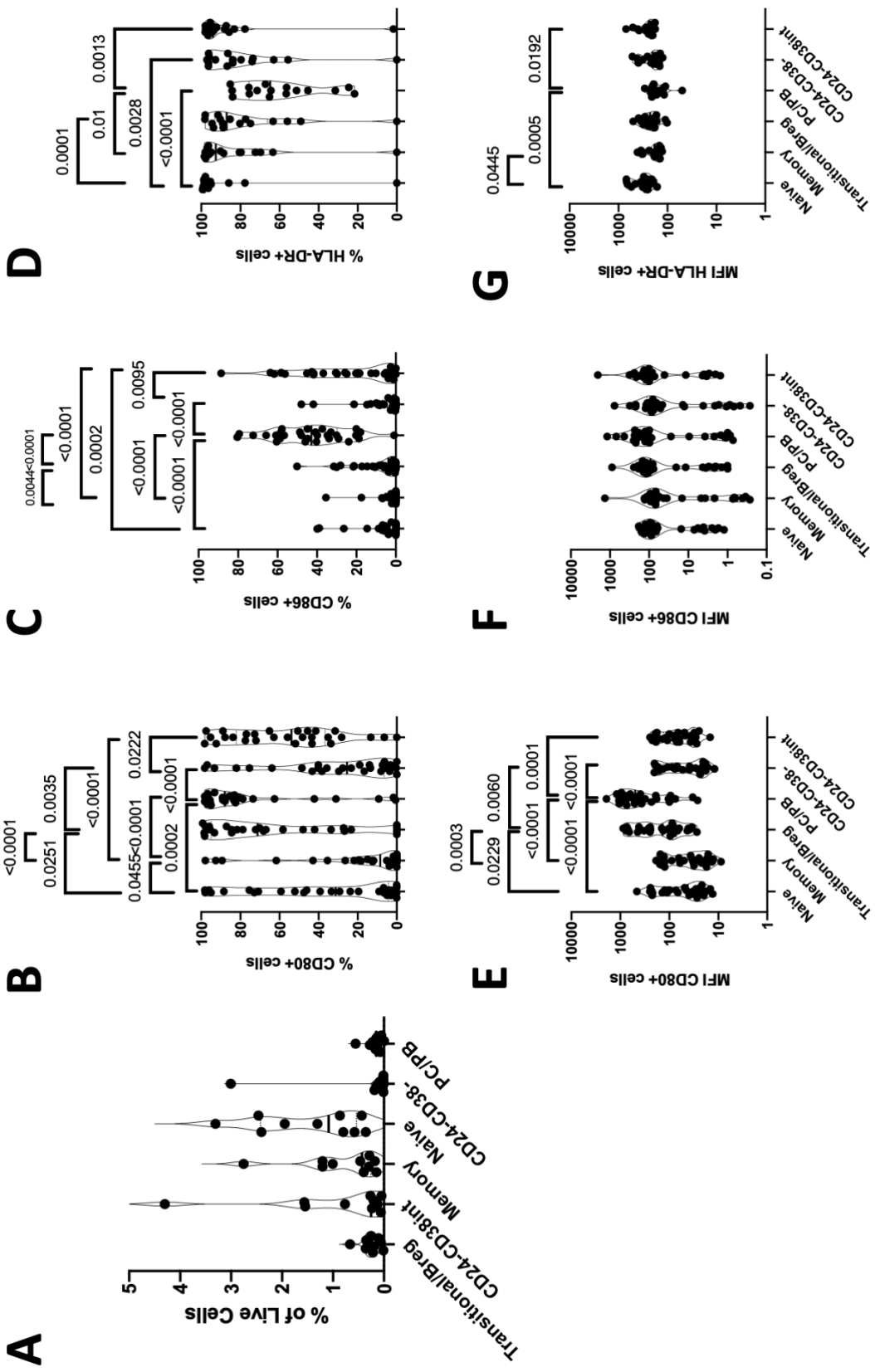

Figure 14: Phenotypic characterisation of CD80, CD86, and HLA-DR on liver B cell subsets. B cell

subsets were isolated from the liver of patients with PBC, PSC, ALD, and NASH/NAFLD and were phenotyped for their percentage and MFI levels of CD80 and CD86 on the surface using fluorescent antibodies. (**A**) Percentage of live B cell subsets in relation to the total number of isolated liver-infiltrating leukocytes (**B**, **C**, **D**) Percentage of CD80, CD86, and HLA-DR expression on different B cell subsets. (**E**, **F**, **G**) MFI levels of CD80, CD86, and HLA-DR on different B cell subsets. N=28 for CD80 samples, n= 29 for CD86 samples, and n=16 for HLA-DR samples. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

Finally, we were interested in assessing the expression levels of CD80, CD86, and HLA-DR in different liver diseases. For that reason we compared the B cell subsets between PBC, PSC, and metabolic group (which comprises of ALD, NASH, and NAFLD patient samples). Our results show that none of the B cell subsets differed in the three groups for either the percentage or MFI levels of cells expressing CD80 (**Fig. 15 A**), CD86 (**Fig. 15 B**), or HLA-DR (**Fig. 15 C**). To sum up, these results suggest that despite the difference in expression of CD80, CD86, and HLA-DR among the different B cell subsets, their differences were not reflected in the diseases we studied.

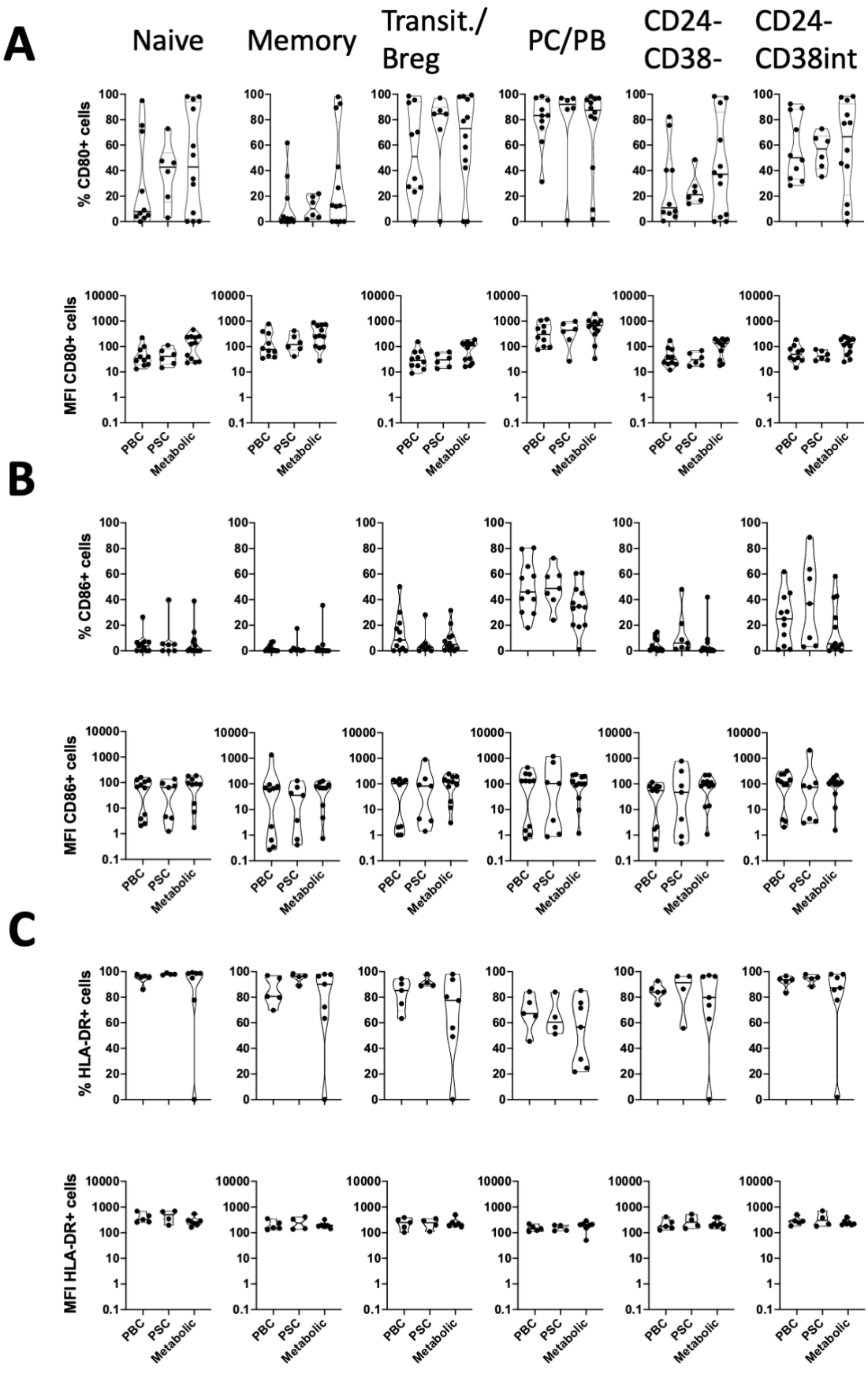

Figure 15: Comparison of CD80, CD86, and HLA-DR expression on B cell subsets in different liver

diseases. B cell subsets were isolated from patients with PBC, PSC, and metabolic (ALD, NASH/ NAFLD) conditions and stained with fluorescent antibodies to measure their percentage and expression levels of CD80, CD86, and HLA-DR. (**A**) Percentage and MFI levels of CD80 on the surface of different B cell subsets. N=10 for PBC, n=6 for PSC, n=12 for metabolic disease. (**B**) Percentage and MFI levels of CD86 on the surface of different B cell subsets. N=11 for PBC, n=7 for PSC, n=12 for metabolic disease. (**C**) Percentage and MFI levels of HLA-DR on the surface of different B cell subsets. N=5 for PBC, n=4 for PSC, n=7 for metabolic diseases. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

4.4. CD80 and CD86 expression on monocytes

We also assessed the expression of CD80 and CD86 on human monocytes. Using flow cytometry, monocytes were gated using the conventional live/$CD14^{-}CD16^{-}$ exclusion gate/$HLA\text{-}DR^{+}CD16$/CD14-CD16 gate (**Fig. 16**). The final gate allows for identification of the classical ($CD14^{++}CD16^{-}$), the intermediate ($CD14^{+}CD16^{+}$), and non-classical monocytes ($CD14^{+}CD16^{++}$). To confirm the right gating for the separation of the monocyte subsets, we used an FMO for CD16.

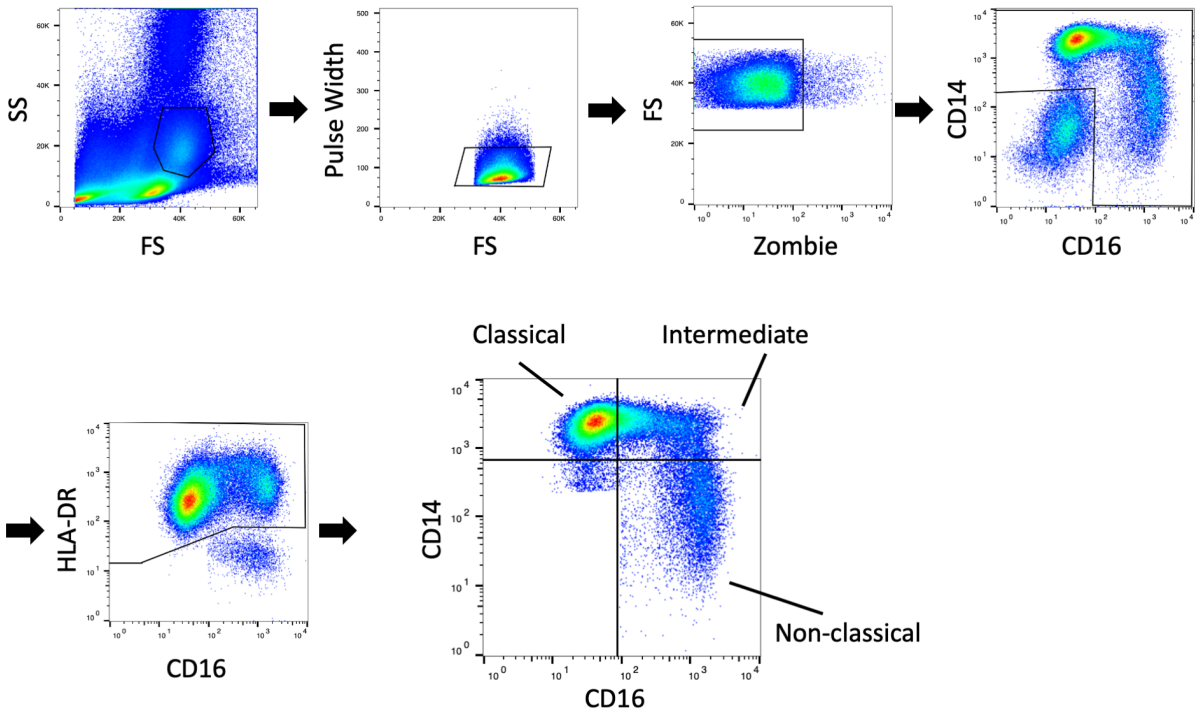

Figure 16: Gating strategy for the characterisation of classical, intermediate, and non-classical monocytes. This is a representative sample from an HFE patient.

In blood, CD80 and CD86 expression was assessed for the three monocytic subsets, isolated from patients with PBC, PSC, or HFE. The percentage of classical

monocytes expressing CD80 (5.065% (9.087)) from PBC patients was higher compared to classical monocytes from HFE patients (3.26% (14.09), ANOVA with Dunn's test, p=0.0472) (**Fig. 17 A**). Intermediate and non-classical monocytes showed no difference in their percentage of cells expressing CD80. The expression levels of CD80 was similar in all three groups across subsets (**Fig. 17 B**). Similarly, the percentage and MFI levels of CD86-expressing cells showed no difference in the three conditions for all subsets (**Fig. 17 C, D**).

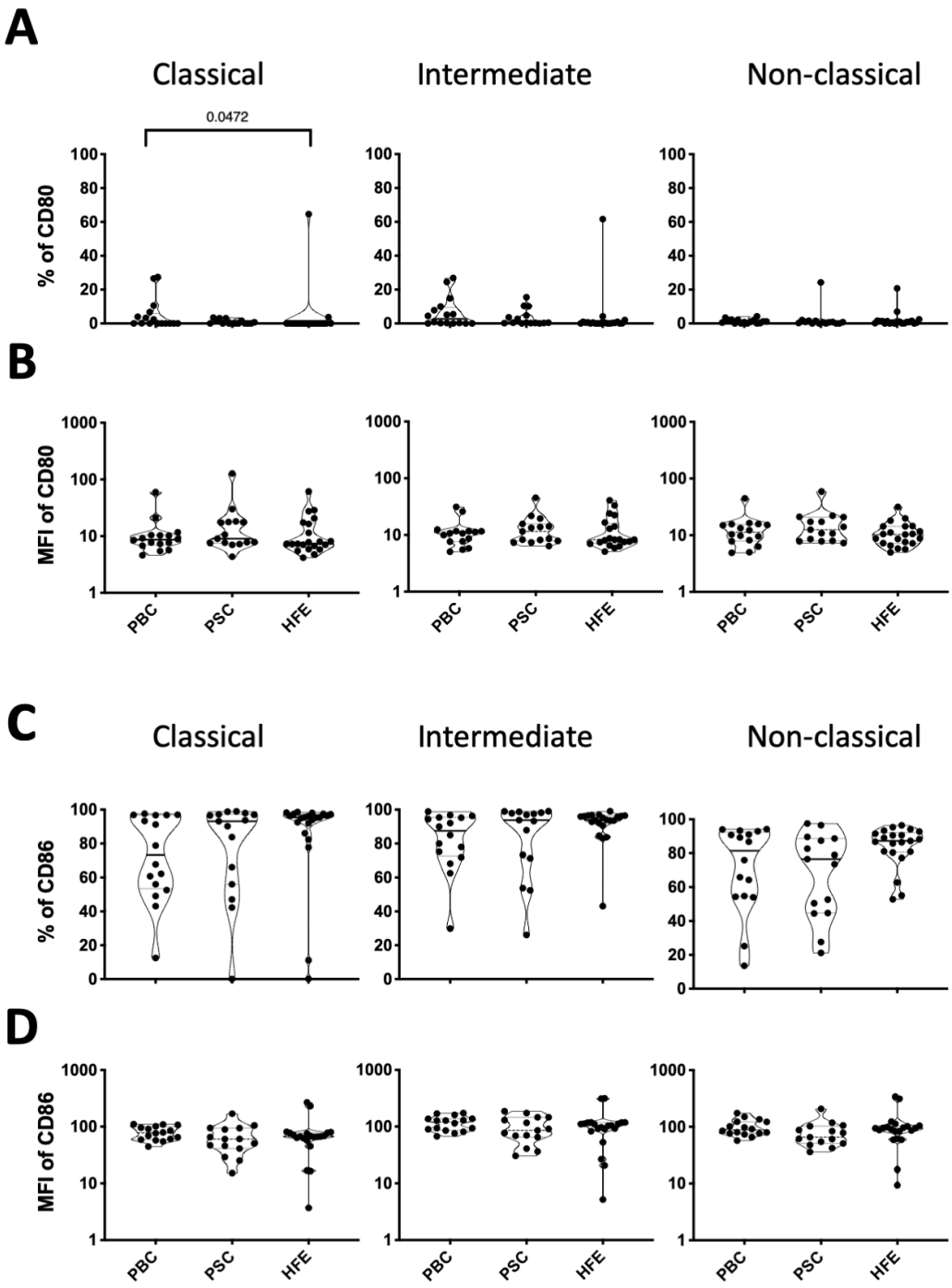

Figure 17: Phenotypic characterisation of blood-derived classical, intermediate and non-classical monocytes. Monocytes isolated from blood of patients with PBC, PSC, or HFE were characterised for their percentage and expression levels of CD80 and CD86 using flow cytometry. (**A**) Percentage of CD80-expressing monocytes. (**B**) MFI levels of CD80-expressing monocytes. (**C**) Percentage of CD86-

expressing monocytes. (**D**) MFI levels of CD86 on monoyctes. N=18 for PBC, n=15 for PSC, and n=21 for HFE. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

Similarly to B cell subsets, we sought to measure the percentage of live liver-infiltrating monocyte subsets in relation to the total number of leukocytes. Our results showed that classical monocytes were abundant in the liver (7.46%, 4.18%-11.3%) followed by intermediate monocytes (0.44%, 0.2%-1.06%), and finally non-classical monocytes (0.13%, 0.05%-0.45%) (**Fig. 18 A**). These results show that monocytes have a relatively strong presence in the liver.

We then studied the expression of CD80 and CD86 on the intrahepatic monocytes from patients with PBC, PSC, or metabolic condition (grouped together like before, containing samples from patients with ALD, NASH, and NAFLD diseases). The three subsets did not show a difference in the percentage of $CD80^+$ monocytes (**Fig. 18 B**). Classical, intermediate and non-classical monocytes from PSC patients expressed higher levels of CD80 compared to the metabolic group (classical PSC 92.51 (158.5) vs metabolic 23.08 (12.44), ANOVA with Dunn's test, p=0.0074, intermediate PSC 116.7 (218.2) vs metabolic 32.19 (16.34), ANOVA with Dunn's test, p=0.0131, nonclassical PSC 60.6 (35.95) vs metabolic 27.96 (16.22), ANOVA with Dunn's test, p=0.0065))(**Fig. 18 C**). The percentage of CD86-expressing monocytes showed no difference between diseases for all monocyte subsets (**Fig. 18 D**). Finally, MFI levels of classical PSC monocytes were elevated compared to the metabolic group (219.7 (106.3) vs 150 (169.2), ANOVA with Dunn's test, p=0.0479) (**Fig. 18 E**). Similarly, intermediate monocytes also showed increased levels of CD86 compared to the metabolic group (267 (107.6) vs 144.3 (107.8), ANOVA with Dunn's test, p=0.0131). In conclusion, these results suggest that monocytes from PSC patients might have the ability to constimulate compared to monocytes from patients with metabolic liver conditions.

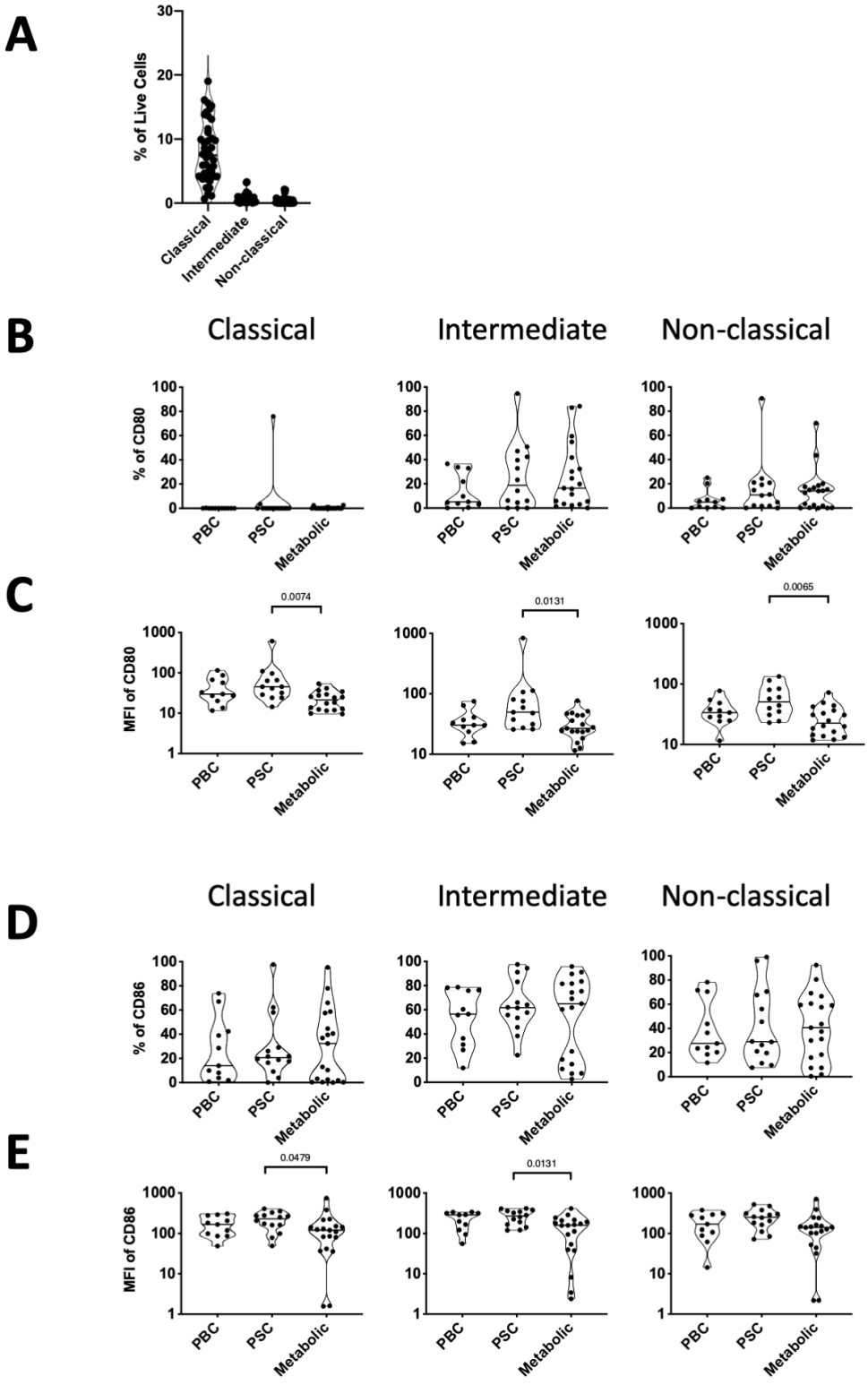

Figure 18: Phenotypic characterisation of liver-infiltrating monocytes. Monocytes were isolated from the liver of patients with PBC, PSC, or metabolic (ALD, NASH/NAFLD) diseases and characterised for their CD80 and CD86 expression using flow cytometry. (**A**) Percentage of live monocyte subsets in relation to the total number of isolated liver-infiltrating leukocytes. (**B**) Percentage of CD80-expressing monocytes. (**C**) MFI levels of CD80+ monocytes. (**D**) Percentage of CD86-expressing monocytes. (**E**)

MFI levels of CD86+ monocytes. N=11 for PBC, n=14 for PSC, n=19 for metabolic conditions. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

4.5. CD80 and CD86 expression on dendritic cells

We also assessed on the expression of CD80 and CD86 on intrahepatic DCs. As mentioned earlier, DCs are considered professional APCs with high stimulatory potency towards T cells. For that reason, we isolated intrahepatic DCs and phenotyped them for their expression of CD80 and CD86 using flow cytometry. We gated on live/$CD14^-$/$HLA\text{-}DR^+$/$CD3^-CD19^-CD56^-$ (as a dump channel). Following that, DCs were split into the conventional myeloid $CD11c^+CD121^-$ and the plasmacytoid $CD11c^-CD121^+$ (**Fig. 19**). With the use of CD141 marker, myeloid cells were then further separated into $CD141^+$ and $CD141^-$, and are most commonly named as conventional DC1 (cDC1) and conventional DC2 (cDC2) respectively.

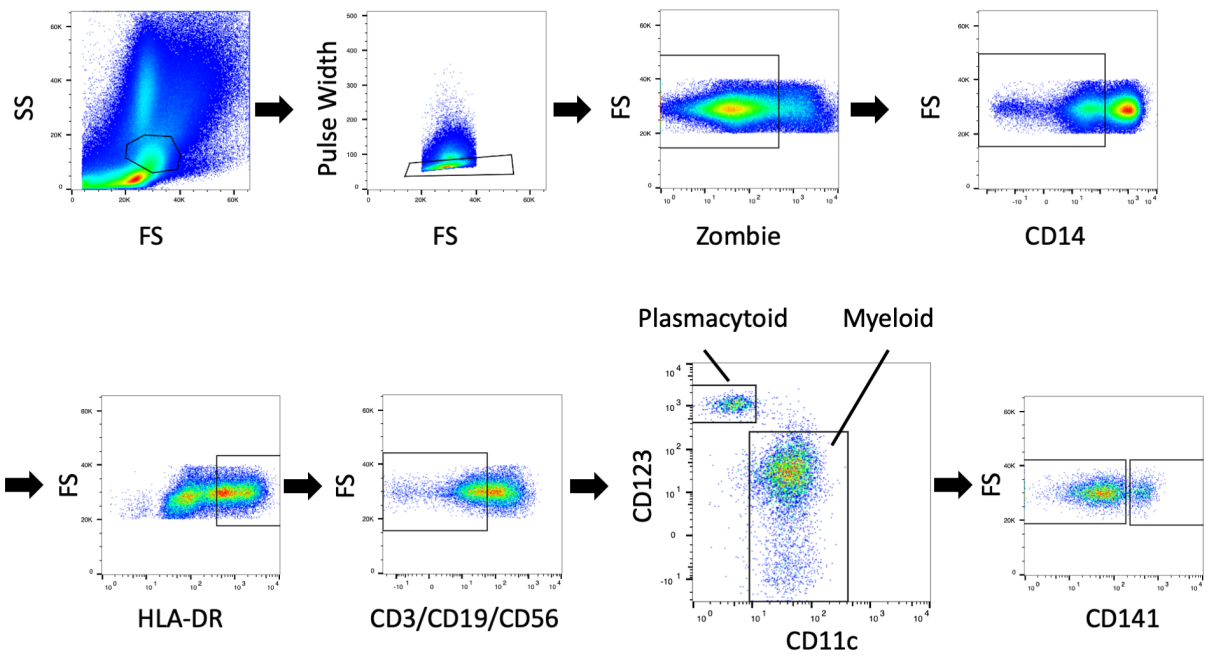

Figure 19: Gating strategy for identification of plasmacytoid, myeloid $CD141^+$, or $CD141^-$ DCs isolated from blood or liver. This is a representative sample of blood-derived DC from a patient with HFE.

Phenotypic characterisation of the percentage of cells expressing CD80 showed no difference among the three DC subsets, isolated from the blood of HFE patients (**Fig. 20 A**). MFI levels for CD80 showed that plasmacytoid DCs (34.71 (3.285)) express higher levels compared to cDC1 (14.86 (6.874), ANOVA with Dunn's test, p=0.0236)) and cDC2 (15.39 (5.239), ANOVA with Dunn's test, p=0.0324). cDC2 (74.16% (17.78)) contained a higher percentage of CD86-expressing cells compared to plasmacytoid DCs (1.607% (2.536), ANOVA with Dunn's test, p=0.0086), however no difference was found regarding their MFI levels (**Fig. 20 B**).

In order to assess the effectiveness of CD80 and CD86 in the liver, we first measured the percentage of live liver-infiltrating DCs from the total number of leukocytes in the liver. We found that $CD141^-$ DCs were the most abundant from the three different subsets (0.35%, 0.17%-0.44%), followed by pDC (0.014%, 0.009%-0.05%), and $CD141^+$ DCs (0.008%, 0.003%-0.019%) (**Fig. 20 C**). This set of data show that DC are extremely rare in the liver explants we studied.

We then isolated intrahepatic DCs from the liver of patients with end-stage liver diseases (PBC, PSC, ALD, NASH, and polycystic livers were used) and phenotyped them for their expression of CD80 and CD86. We found that in the liver a higher percentage of plasmacytoid DCs (57.92% (39.88)) expressing CD80 were present compared to DC1 (4.888% (3.803), ANOVA with Dunn's test, p=0.0127) (**Fig. 20 D**). Similarly, the CD80 MFI levels of plasmacytoid DCs (49.49 (53.04)) were higher compared to both DC1 (11.25 (5.96), ANOVA with Dunn's test, p=0.0011) and DC2 (10.59 (3.817), ANOVA with Dunn's test, p=0.0324). Finally, DC2 (13.32% (9,891)) contained a higher percent of CD86-expressing cells compared to plasmacytoid DCs (0.2656% (0.06881), ANOVA with Dunn's test, p=0.0006), with no difference dated in the expression levels of the marker (**Fig. 20 E**).

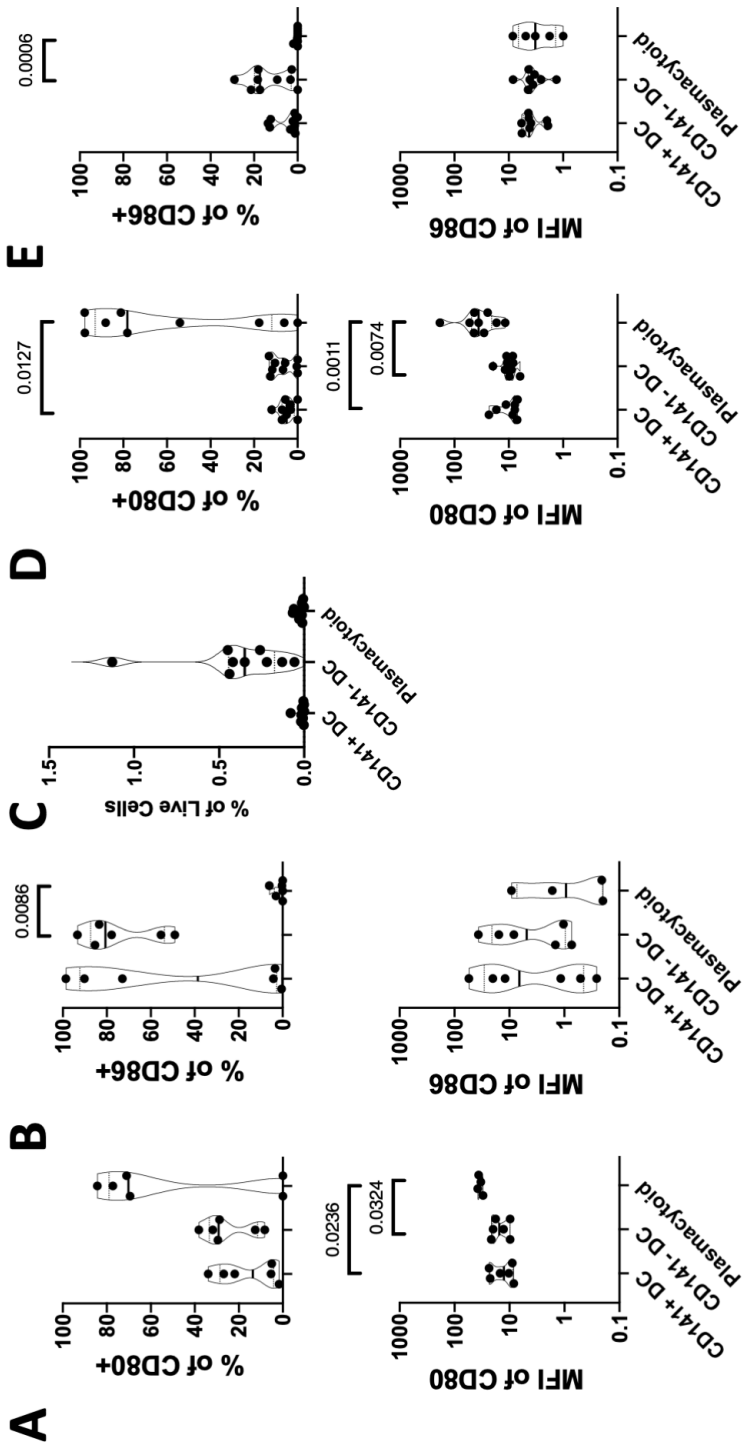

Figure 20: Phenotypic characterisation of DCs isolated from blood and liver. DCs were isolated from the blood of HFE patients or the liver of PBC, PSC, ALD, NASH, and patients with polycystic liver disease. They were then stained with antibodies and characterised for their expression levels of CD80 and CD86 using flow cytometry. (**A**, **B**) Percentage and MFI levels of CD80 and CD86 on the surface of DCs isolated from blood of HFE patients. N=6 (**C**) Percentage of live DC subsets in relation to the total number of isolated liver-infiltrating leukocytes. (**D**, **E**) Percentage and MFI levels of CD80 and CD86

on the surface of DCs isolated from the liver of patients with PBC, PSC, ALD, NASH, and patients with polycystic liver disease. N=8. Kruskal-Wallis was used as a statistical test with Dunn's test as post-hoc analysis.

4.6. Localisation of CD80-expressing cells in the liver

We next identified the location of CD80 in the liver of PBC patients. Using immunohistochemistry, we stained human liver with anti-human CD80 antibody and found that in the liver CD80 localises on cells found on fibrotic tracts (**Fig. 21 A, B**). These cells present characteristics of lymphocytes (small, round cells with varying sizes of cytoplasm). CD80 was also present on immune cells around bile ducts but was not expressed by the biliary cells (**Fig. 21 C**). Furthermore, no expression of CD80 was present on hepatocytes. The IMC showed only minor levels of background staining by the antibody (**Fig. 21 D**). Finally, we also stained human liver with an anti-human CD86 antibody. Unfortunately, the staining did not work after using 2 different batches of the same clone of antibody and 2 different clones (data not shown).

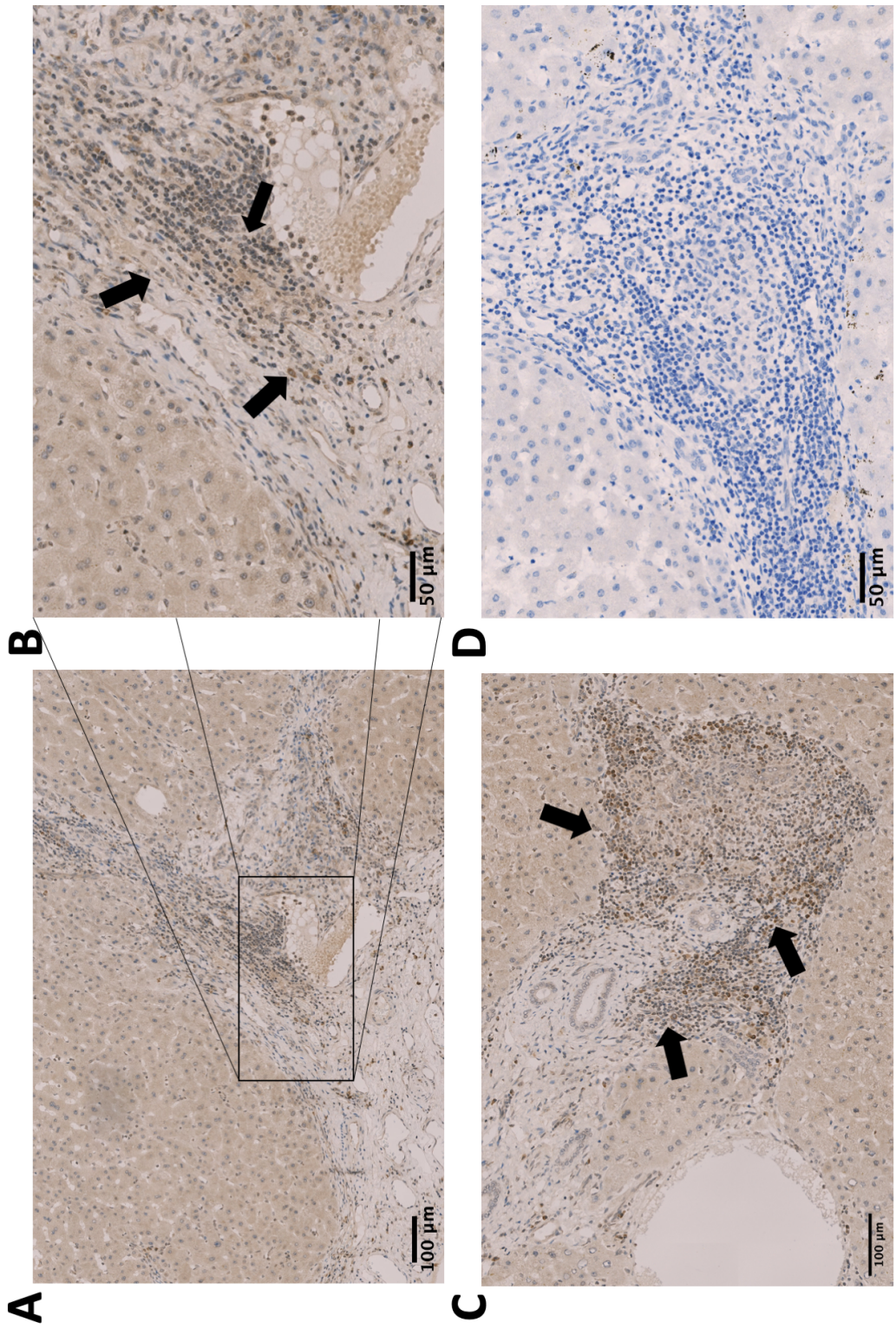

Figure 21: Localisation of CD80 on human liver tissue. FFPE liver tissue from a PBC patient was stained using IHC for the expression of CD80. (**A**) Low power magnification showing the general location of CD80 on the liver. The box denotes the area seen in B. (**B**) High power magnification of the previous section showing cells stained with CD80. (**C**) Alternative area showing cells stained for

CD80. (**D**) FFPE liver tissue stained with the isotype-matched control for the CD80 antibody. Images are representative of 3 different experiments.

4.7. Discussion

The expression of CD80 and CD86 on APCs is well established in many different tissues in both humans and mice, however expression in the liver has not been investigated. Since RhuDex is a CD80 inhibitor and is planned to go into clinical trials for the treatment of patients suffering from PBC, we sought to characterise the expression patterns of CD80 and CD86 in the human blood and liver. This would allow us to predict the possible targets of RhuDex, inhibiting the CD28/CD80 pathway. For this purpose, we concentrated our efforts on phenotyping professional APCs found in the liver. Other liver cells have been shown to have APC functions such as the LSEC, the HSC, or the hepatocytes but these cells are not considered professional APCs and their stimulatory capacity is not as strong as the professional APCs (658).

By phenotyping B cells derived from the blood of PBC, PSC, and HFE patients, we found that total B cells express CD80 on their surface but CD86 expression was limited (**Fig 11 B**). Our data disagree with current literature which states that B cells express CD86 but low levels of CD80 (583, 587). Some studies which looked at the expression of CD80 and CD86 on B cells studied the expression after time in culture and compared the expression to stimulated cells (587). Furthermore, B cells from the blood of PBC patients express higher levels of CD86 compared to HFE B cells, however a smaller percentage of B cells express CD86 on their surface (**Fig 11 B**). We currently cannot explain this observation.

Moreover, our results suggest that CD80 expression was not different across diseases in the liver (**Fig 11 C**). However, the variation in our results for CD80 was large, with some B cells expressing no CD80 and others expressing the marker to almost 100%. The large variation could be due to gating on total B cells and not different B cell subsets, since the different B cell subsets express different levels of

CD80. Another possibility is that B cells from patients with PBC and PSC are already activated. LPS present in the livers of patients could potentially upregulate the expression of CD80. Bacterial LPS has been detected in the livers of PBC and PSC patients, which was derived from the blood and entered through the portal vein (659–662). Injection of LPS into mice has been associated with liver dysfunction (663). The presence of increased levels of LPS could explain the elevated CD80-expressing liver B cells, however it does not explain the lower levels of CD86, since stimulation of B cells with LPS also increases the expression of the ligand (664). IFN-β which is present in the liver of patients with PBC, upregulates CD86 on B cells (608, 665). Similarly, CD86 expression did not differ across diseases in the liver (**Fig. 11 C)**. These result indicate that CD80 and CD86 possibly do not drive the development or progression of the liver diseases we studied through dysregulation of the CD28-CD80/CD86 pathway. The higher percentage of $CD80^+$ B cells in contrast to the lower CD86-expressing B cells could indicate that an ongoing inflammation in the liver is taking place and tolerance has been broken. Evidence has shown before that CD80 and CD80 can differentially modulate Treg responses. Blocking CD80 on Treg stimulated T cells and inhibited Treg function whereas CD86 blockade enhanced the suppressive Treg effects (243). Previous studies showed that blockade of CTLA-4 or CD80 enhanced graft rejection, whereas CD86 blockade enhanced graft survival (666, 667). Furthermore, CD86 co-stimulation was able to greatly increase the cytotoxic effects of $CD8^+$ T cells whereas CD80 only had a small effect (668).Similar effects were detected on the proliferative response of these cells to the two ligands. Although the current consensus is that the two ligands can be used interchangeably, a point can be made for CD80 being the natural ligand for CTLA-4, providing negative immune responses. However, there are studies which have shown that CD80 is not able to regulate immune responses. NOD mice where CD80 was blocked using anti-CD80

developed the disease whereas mice treated with anti-CD86 or CTLA-4-Ig did not (669). Similarly, mice which expressed CD80 constitutively on their B cells had defective B cell responses, however they could be reverted in the presence of anti-CD80 (670). Considering that the liver microenvironment of patients with end-stage liver diseases is pro-inflammatory and we detected a relative absence of CD86 but presence of CD80, the theory of CD80 being a true ligand to CTLA-4 could stand correct. Indeed, Treg are dysregulated in PBC and PSC and have a reduced ability to inhibit co-stimulation in PBC (366, 671). In conclusion, we believe that the pro-inflammatory environment of the liver explants could contribute to the phenotype of the B cells.

Similar expression results were collected after liver B cell subsets were phenotyped for their expression of CD80 and CD86. The liver-infiltrating B cell subsets showed no difference in the percentage of cells expressing CD80, CD86, or HLA-DR across diseases (**Fig. 15 A, B, C**). This further supports the previous hypothesis of B cells possibly not contributing to liver damage in PBC through a clear disregulation of CD80 or CD86.

Focusing on the different B cell subsets we found that naive B cells expressed CD80 and CD86 on their surface, both in the blood and in the liver (**Fig. 13 A, B, C, D, fig. 14 B, C, E, F**). This contradicts previous literature which states that naive B cells do not express CD80 or CD86 on their surface (599, 600, 604). However, CD86 expression was indeed low for blood and liver (**Fig. 13 A, B, D, fig. 14 C, F**).

Memory B cells also expressed low levels of CD80 an CD86 in blood and liver which again does not agree with the majority of published literature but agrees with others (**Fig. 13 A, B, C, D, fig. 14 B, C, E, F**) (90, 599, 600, 602). The two possibilities for this result could be either trans-endocytosis of CD80 and CD86 from the surface of the memory B cells or possibly these cells are similar to the $CD80^-$ memory B cells

described by Bar-Or and colleagues (225, 600).

Transitional/Breg cells showed an increased expression of CD80 compared to naive B cells but not CD86, and were highly expressed across all diseases in the blood and in the liver (**Fig. 13 A, B, C, D, fig. 14 B, C, E, F**). Published data show that transitional B cells indeed express low levels of CD86 (605). The consensus regarding the expression levels of CD80 and CD86 is still not clear for Breg cells. In ascites of cancer patients, Breg cells expressed low CD80/CD86 levels which the researchers believe that to be one of the ways Breg cells exert their suppressive function (672). Upon stimulation with CpG or CD40L, Breg upregulated their CD80 and CD86 expression (673). Non-stimulated Breg cells were unable to inhibit T cell proliferation, whereas stimulated Breg promoted strong T cell inhibition, particularly when co-cultured with autologous T cells rather than allogeneic. In the pro-inflammatory microenvironment of an end-stage liver, Breg cells could be stimulated by the different cytokines present in the liver, as well as other PAMP and DAMP molecules secreted in the area due to the extended liver damage.

Similarly, PC/PB also showed a very high expression of CD80 compared to other subsets in the blood and liver (**Fig. 13 A, B, C, D, fig. 14 B, C, E, F**). Our finding agrees with previous publications proving the existence of CD80 and CD86 on these cells, and showing that CD80 and CD86 are necessary for the survival of long-lived PC (598). Loss of CD80, CD86, or CD28 from T cells resulted in rapid long-lived PC death, and were unable to maintain antibody titres for long-term (674). A recent study found that PC from the bone marrow interact with Treg through CTLA-4 (675). Deletion of CTLA-4 from Treg resulted in increased numbers of PC. However, Treg depletion from mice decreased the number of PC in the periphery and the bone marrow PC. These results show that CD80 and CD86 on PC/PB play a different role for these B cell subsets, which puts an emphasis on B cell survival rather than T cell

stimulation.

$CD24^{-}CD38^{-}$ and $CD24^{-}CD38^{int}$ B cells also contained a population of B cells expressing CD80, with CD86 being lower on $CD24^{-}CD38^{-}$ compared to $CD24^{-}CD38^{int}$ B cells in the liver (**Fig. 14 B, C, E, F**). As we mentioned earlier, studies about these B cell subsets are still in progress. Our data agree with the results previously published by our group showing that intrahepatic $CD24^{-}CD38^{int}$ $CD80^{+}$ B cells are increased compared to memory B cells (101). However, in the present study we did not find any differences in the frequency of CD80-expressing cells between $CD24^{-}CD38^{int}$ and transitional/Breg cells in the liver (**Fig. 14 B, E**). We also found no difference in the percentage of cells expressing CD80 in the blood of HFE patients (**Fig. 13 A, C**). $CD24^{-}CD38^{-}$ cells expressing CD86 were increased in the blood compared to naive and $CD24^{-}CD38^{int}$ were decreased compared to PC/PB (**Fig. 13 B, D**). In the liver, we saw a different picture, with increased $CD24^{-}CD38^{int}CD86^{+}$ B cells compared to $CD24^{-}CD38^{-}$, naive, and memory B cells (**Fig. 14 C, F**).The $CD24^{-}CD38^{-}$ compartment had fewer CD86-expressing cells compared to PC/PB(**Fig. 14 C, F**). None of the two subsets showed any differences across the different diseases for CD80 or CD86 (**Fig. 15 A, B**). The high expression of CD80 and the relatively low expression of CD86 could possibly hint towards a more anti-inflammatory role for these B cells, however this hypothesis depends on the acceptance of CD80 being the natural ligand for CTLA-4. Nevertheless, upon stimulation both subsets secreted both pro- and anti-inflammatory cytokines (IFN-γ, IL-6, TNF-α, IL-10)(101). The relative low expression of CD80 and CD86 on these cells in the blood compared to the liver could indicate that they act in the liver, further considering these cells are present in the liver but not in the blood (101).

HLA-DR expression levels as well as the number of cells expressing the marker was also elevated in all B cell subsets (**Fig. 14 C, D**). PC/PB showed the lowest

expression levels as well as frequency of cells expressing the marker. Indeed, PC/PB lose the expression of MHC class II molecules during the plasmacytic differentiation (676). High expression of HLA-DR together with the expression of CD80 and CD86, suggests that liver-infiltrating B cells have the capacity to prime T cells.

Monocytes also had different expression pattern of CD80 and CD86 among subsets in the blood and liver. In the blood, monocytes expressed extremely low quantities of CD80 and only a marginal difference was present in the classical CD80-expressing monocytes from PBC compared to HFE patients (**Fig. 17 A, B**). A high frequency of CD86-expressing monocytes were present in all diseases in the blood (**Fig. 17 C, D**). The expression pattern for both ligands in the blood-derived monocytes agrees with the published literature (600, 616). Another report described that intermediate monocytes express higher levels of CD80 compared to classical monocytes isolated from blood of patients with ALD (677). No differences were detected in any of the other subsets. In the same study, no differences were found between ALD subsets and healthy controls (677). In the liver, the expression levels of CD80 and CD86 on monocytes from PSC patients were increased when we compared them to monocytes from metabolic conditions (**Fig. 18 B, C, D, E**). This result could be indicative of the pro-inflammatory liver microenvironment in the end-stage livers we studied, resulting in up-regulation of CD80 (184, 233, 596, 597, 617–619). Classical monocytes have been studied extensively for their expression of CD80 since they are the most numerous subset from all three (**Fig. 18 A**). The classical compartment contained a high percentage of CD80-expressing cells (678). In the liver, classical monocytes were shown to contain fewer CD80-expessing classical monocytes compared to intermediate monocytes for both normal and diseased liver (679). Classical monocytes were recruited to the site of inflammation and secreted pro-inflammatory cytokines such as TNF-α and anti-inflammatory cytokines like IL-10

after IFN-γ stimulation (680). After parasitic infection however, it was the intermediate monocytes which expressed high levels of IL-10. The reason for the increased CD80 and CD86 expression on all subsets from PSC patients compared to metabolic diseases could be because PSC is an immune-driven disease whereas ALD and NASH/NAFLD are the result of increased alcohol and fat intake. However, the lack of similar results from PBC patients, another immune-driven disease, raise the question of why similar results were not obtained from that condition as well. Furthermore, a limited number of non-classical monocytes were present in the liver compared to the blood. $CD16^+$ monocytes are less frequentt compared to classical monocytes in the blood and liver (also **fig. 18 A**) (128, 679). Classical monocytes in mice have been shown to transition to non-classical monocytes with a half-life of ~20 hours (681). Human non-classical monocytes have been shown to have a half-life of ~7 days in circulation (103). It is possible for these monocytes to enter the liver in order to be recycled explaining their absence, similarly to erythrocytes getting recycled in the liver, however more experiments are needed to verify this hypothesis (682).

Finally, we focused our efforts on phenotyping DCs in the blood and in the liver. Due to the low number of explants collected and time constraints, we were unable to stratify the livers according to disease, but we were able to compare the expression of CD80 and CD86 in the different subsets. Results from the blood of HFE patients did not show any differences regarding the percentage of CD80-expressing cells among subsets, however, there was a higher percentage of $CD86^+$ cDC2 cells in the blood compared to plasmacytoid DCs (**Fig. 20 A, B**). This is in partial agreement to other reports showing DCs from the blood having few positive cells for CD80 and CD86, however Heidkamp et al. have found similar results to ours regarding the expression of CD86 in the three subsets (235, 652, 683). These reports however focus on total DCs

and myeloid DCs. Plasmacytoid DCs upregulate their CD80 and CD86 expression upon TLR7 or TLR9 stimulation (684). However, we cannot assume that the expression we found on the DCs is due to TLR stimulation because we found a very high percentage of DCs expressing CD80 and almost none expressing CD86. Similarly, expression levels of plasmacytoid cells in the blood were increased for CD80 but not CD86. DCs isolated from the liver showed that plasmacytoid DCs had a higher expression level of CD80 compared to the two myeloid subsets. Similarly, a high percentage of cells were positive for CD80. High expression of CD80, but not CD86, on intrahepatic myeloid and plasmacytoid DCs have been shown before to be a result of HCV infection (685). In mice, intrahepatic DCs are phenotypically immature with low expression of CD80, CD86, and MHC class II on their surface compared to spleen DCs (645, 686, 687). Upon culture with GM-CSF or Flt-3 ligand - the ligand for the cytokine FL which is responsible for the generation of myeloid cells - DCs upregulated their CD80 and CD86 expression (688). Although reports suggest that intrahepatic DCs are immature, the DCs we have isolated were in a pro-inflammatory environment for a very long time so it is possible these DCs are not immature. The presence of CD80 together with the absence of CD86 from plasmacytoid DCs however could further support the hypothesis of CD80 a negative regulator of immune responses. Finally, it is important to acknowledge a limitation in the current study. For the phenotyping we made the assumption that $CD141^+$ DCs are the commonly known cDC1 type and the $CD141^-$ are the cDC2, although separation of the two subsets usually requires more phenotypic markers. The inability to add more markers was due to limitations of the flow cytometer we used, which was able to analyse up to nine different fluorochromes. CD141 alone is not a reliable marker for the separation of cDC1 and cDC2. Evidence shows that cDC2 can express both CD141 and CD1c, the latter being a common marker for identification of

cDC2 (689). Similarly, $CD141^+$ DCs were found to be present in both $CD14^+$ and $CD14^-$ DCs, further complicating the DC phenotyping (137). Both of these cells are present in the human liver. Although we have used CD14 marker and gated at $CD14^-$ cells, other markers should still be used to identify and discriminate cDC1 and cDC2 such as CLEC9A, a receptor expressed in necrotic cells, the adhesion molecule NECL2, the T cell inhibitor BTLA, or 2,3-dioxygenase, a DC activator (690–695). Due to the limited numbers of DCs present in the liver, we focused our efforts on using total B cells, B cell subsets as well as monocytes as intrahepatic APCs to elucidate the role of RhuDex in the human liver using the co-culture model since we would not be able to isolate enough DCs to conduct our experiments (more on this on chapter 5).

To assess the expression of CD80 and CD86 on B cells, monocytes and DCs in the human liver, we measured the frequencies of these cell subsets in relation to the total liver leukocytes. Classical monocytes were present in relatively high frequency compared to intermediate and non-classical monocytes (**Fig. 18 A**). The liver infiltrating B cell subsets however were present in a very small proportion in relation to the rest of the intrahepatic leukocytes (**Fig. 14 A**). Similarly, all DC subsets were present in very small frequencies in the liver explants we studied (**Fig. 20 C**). The small presence of B cells and DCs indicates that these cells are not very likely to co-stimulate a large number of T cells in the liver. It is possible that other areas of the liver possess a higher percentage of these cells. The liver is a very large organ and not all of the areas of the liver possess the same homogeneous function (696, 697).

Lastly, staining PBC livers for CD80 revealed that CD80 expression was localised in fibrotic tracts and surrounding bile ducts (**Fig. 21 A, B, C**). The only other evidence of CD80 staining in human liver came from Said and colleagues, which showed that in livers with HCV, CD80-expressing Kupffer cells were increased in lobular areas compared to controls, with no difference between portal areas (698). This group did

not find any differences regarding CD80 expression in lobular or portal areas in HBV livers compared to controls (628). Our results agree with the published results, showing presence of CD80-expressing cells in periportal areas and along fibrotic tracks. CD80 is localised in the cytoplasm of the cells, and these cells have a wide cytoplasmic area indicating that they are some type of APC. More markers as well as comparison with other conditions will help us understand the type of these cells and their role in the diseased PBC livers.

In conclusion, these results inform us about the frequency of the CD80 and CD86 expression on B cells, monocytes, and DCs in human livers. Although CD80 was differentially expressed in different APC subsets, it was present on all cells that we studied. Because of this, we believe that RhuDex has a chance to bind to CD80 and inhibit the CD28-CD80 pathway. Of course, the question then is whether the CD80-expressing cells contribute to disease pathogenesis, and at what level. Published evidence has shown that CD80 drives T cell differentiation towards a Th1 phenotype and CD86 towards a Th2 profile (699–702). Moreover, patients with fulminant hepatic failure had elevated levels of both CD80 and CD86 in their liver compared to livers with chronic liver diseases and normal controls (629). They also found that the number of $CD80^+$ and $CD86^+$ cells correlated with the number of $CD28^+$ lymphocytes. The final question to be answered is whether the relatively low levels of CD80 expressed on most cells can actually contribute in any way to the disease so that RhuDex can have an effect on those cells. In the next chapter we sought to evaluate the efficacy of RhuDex in inhibiting T cell co-stimulation using intrahepatic cells.

Chapter 5 - RhuDex is a potent immunomodulator

5.1. Introduction

To predict the immunomodulatory value of RhuDex in patients with liver inflammation, it is important to consider its effects on liver antigen presenting cells. Few cells in the liver expressed CD80, however it was most abundant in B cells and monocytes.

5.1.1. The role of B cells as antigen-presenting cells

Using autologous APCs to study T cell responses is often difficult, particularly when focusing on tissue immunity where access to sufficient cells is challenging. In vaccine development, mapping the antigen epitopes is problematic due to the large number of unique peptides a pathogen contains (703). Usage of tetramers carry the limitation of testing for a few antigens (704). As a result, the most efficient way of measuring activation of T cells is by using APCs, isolated from patients which can then be expanded *in vitro*. However, DCs and macrophages are found in low numbers in the blood and are short lived in cultures (705–707). Expansion and differentiation of monocytes to either DCs or macrophages can further complicate the process. B cells are more abundant and if needed, are easier to expand in culture compared to macrophages and DCs (594, 708–710). For that reason, many studies that opt to work with APCs *in vitro* will either use B cells, monocytes, or monocyte-derived DCs.

B cells have been classified as professional APCs together with DCs and macrophages (711, 712). Their function as APCs lies in their capacity to activate naive T cells and prime them for effector functions. Antigen-specific B cells present antigen to T cells and drive their activation, resulting in proliferation and polarisation of the cells (713–716). On the other hand, resting B cells are unable to stimulate T cells (83,

717). Depletion of the murine conventional B cells (also known as B-2 cells) completely inhibited all proliferative responses after adjuvant stimulation (718). Su et al. showed that autologous human B cells do not promote T cell activation, however allogenic B cells could when co-cultured with either $CD4^+$ or $CD8^+$ T cells *in vitro* (594). Cells derived from patients which had been vaccinated against tetanus toxoid were co-cultured with autologous T cells in the presence of microbial antigen and induced T cell proliferation (594).

It is important to note that different B cell subsets differ in their ability as APCs. Naive B cells that were stimulated either through CD40 or by using a TLR9 agonist matured into an activated state allowing them to gain APC functions and prime T cells, however unstimulated naive B cells were tolerogenic in humans and mice (719–722). Memory B cells are more potent than naive B cells for inducing strong proliferative responses in alloreactive T cells (599, 723). Memory CD80-expressing B cells activated T cells stronger than memory $CD80^-$ B cells, even at low B cell:T cell ratio (600). Breg inhibit the production of IFN-γ by $CD8^+$ T cells through low CD80 and CD86 surface expression as well as secretion of IL-10 (672). This was reverted by either CD28 stimulation or IL-10 depletion. A novel B cell subset has been shown to promote strong T cell proliferative responses and increased expression of CD86 (724). This subset is characterised as $CD21^{low}CD86^+$ and is present in small numbers in the human peripheral blood and in higher frequencies in women than men below the age of 50 years. In autologous MLR these cells showed stronger stimulatory capacity compared to $CD21^+CD86^+$ B cells. Prolonged CD40L activation results in downregulation of CD21 (724). As a result, it is theorised that $CD21^{low}CD86^+$ B cells derive from the $CD21^+CD86^+$ B cells which themselves arise from the $CD21^+CD86^-$ resting B cells. Also, these $CD21^{low}CD86^+$ B cells are found in higher frequencies in patients with RA compared to healthy subjects (724).Finally, transitional B cells were

shown to induce $CD4^+$ T cell responses despite the lack of CD86 expression on their surface, however not as strongly as mature B cells (725). This was a result of incomplete co-stimulation and not ineffective antigen presentation by the B cells.

There is increasing evidence that B-1 cells are also involved in T-dependent responses. Murine B-1a cells are innate-like B cells present in the peritoneal cavity with the ability to secrete high levels of IgM but low levels of IgD, and stimulate $CD4^+$ T cells (726, 727). They are responsible for the first line of difference against a number of infections (728, 729). This B cell subset also increased the percentage of IL-10^-, IL-4^-, and IFN-γ-producing $CD4^+$ T cells. Moreover, B1-a.$PC1^{lo}$ and B1-a.$PC1^{hi}$ B cells, two subsets of B1-a type B cells expressing different levels of plasma cell alloantigen 1 (PC1), can also stimulate T cells (730, 731). Interestingly, B-1a cells in mice express high levels of CD80, CD86, and MHC class II (732). CD80 and CD86 in B-1 B cells are expressed in levels higher than that of the conventional B-2 B cells (733). Under inflammatory stimuli such as LPS or bacterial suspension, these cells upregulate their expression of CD80, CD86, and MHC class II (734, 735). Anti-CD86 antibody could prevent T cell stimulation by these cells (736). These B cells can present antigen to T cells and provide stronger stimulation than conventional B cells (737, 738). B-1 cells are present in secondary lymphoid organs, the thymus, kidney, and lung in a mouse model of SLE, indicating that loss of immunological tolerance in SLE could be taking place in those areas (739).

Dysregulation of the APC function of B cells results in disease development. In many cases, B cells have been heavily involved the development or progression of autoimmune diseases. Murine autoreactive B cells were the first type of APCs to present self-antigen to T cells in a systemic lupus erythematosus model (SLE) and break the immune tolerance *in vivo* (740, 741). In humans, B cell depletion resulted in clinical remission in patients with lupus nephritis, with lower levels of $CD4^+$ T cell

activation after 1 month following depletion (742). Involvement of B cells as APCs in RA has also been studied. Depletion of B cells from mice prevented the development of RA (743). When CD80/CD86 was deleted from the surface of B cells, the mice did not develop the disease (551). Similarly, B cells deficient in MHC class II were unable to promote EAE in mice (744). In humans, risk alleles for CD80 and CD86 have been associated with higher or lower risk for developing multiple sclerosis (MS) (745). One allele has been associated with higher expression of CD86 in B cells resulting in increased T cell proliferation (746). MS patients with high neurodegeneration show increased expression of CD80 on naive, unswitched, and class switched B cells (747). CD86 was present only on naive and switched B cells. B cells also play a role in the development of autoimmune diabetes. Depletion of B cells from non-obese diabetic (NOD) mice results in protection from autoimmune diabetes (748–751). Deletion of I-Ag7 from B cells in mice, an MHC class II molecule found in NOD mice, also protected the mice from diabetes (752).

B cells have also been implicated in non-autoimmune conditions. In mouse models of atherosclerosis, B cell depletion prevented the development of atherosclerosis (753). Treating mice with an anti-CD20 antibody depleted B cells and decreased insulin resistance in diet-induced obese mice (754). Similarly, B cells contributed to allograft rejection in mice by supporting the activation of T cells (755). CD40 stimulation from T cells has shown to upregulate CD80 expression on B cells making them even better T cell activators (597, 599, 719, 756). Upregulation of both CD80 and CD86 was observed on B cells isolated from cervical cancer patients after treatment with soluble CD40 and IL-4 (757). CD40-treated B cells from those patients were able to stimulate T cells in an MHC class II dependent manner. Human B cells in chronic lymphocytic leukaemia (CLL) constitutively express intracellular CTLA-4 but with no surface expression (758). A similar observation was noted for freshly

isolated human B cells (215). B cells isolated from the TCL1 transgenic mouse, a mouse model used to study CLL, expressed CTLA-4 on their surface. In contrast, human and mouse CLL cell lines did not express CTLA-4 in either mRNA or protein form. B cells down regulate the expression of CD80 from the surface of CD80-expressing cells by transferring CD80 within the CTLA-4-expressing B cell, similarly to the mode of action of CTLA-4 expressed on T cells (225).

5.1.2. The role of monocytes as antigen-presenting cells

Monocytes are also involved in antigen presentation and priming of T cells (713). However until the 1990s, monocytes were considered as cells which play a role in host defense but do not have APC functions (759). We now know that monocytes are good APCs. $CD16^+$ monocytes promote T cell proliferation in both mice and humans (760, 761). When co-cultured with autologous human T cells in the presence of purified protein derivative of tuberculin (PPD), they promoted T cell proliferation (616, 762). Monocytes which had engulfed bacteria had reduced ability to stimulate T cells and could not be reversed even after stimulation of monocytes with antigen or mitogen (622). However, antigen-pulsed monocytes exhibited strong stimulation to T cells regardless of the presence or absence of phagocytosed bacteria. Although expression of CD80 and CD86 on the surface of monocytes is present, only anti-CD80 and CTLA-4-Ig antibodies inhibited proliferation, with anti-CD86 having no effect on the system (616). A combination of anti-CD80 with anti-CD86 did not enhance inhibition. Depletion of monocytes from mice resulted in impaired $CD8^+$ T cell priming, and adoptive transfer of monocytes into the transgenic mice reversed the effect (145). Tacke et al. found that monocytes can take up antigen through phagocytosis in the bone marrow and present it to B cells long after the monocytes have entered the blood stream (763). This could be due to low proteolytic capacity of

monocytes, similar to DCs (764, 765). DCs and B cells, in contrast to macrophages, carry fewer lysosomal proteases allowing them to breakdown antigens. Although it is widely accepted that when monocytes enter tissues they differentiate into DCs or macrophages, Jakubzick et al. showed that monocytes can enter the skin and draining lymph nodes without differentiation (765, 766). Monocytes surveil peripheral tissues and transfer antigens into the lymph nodes while also upregulating the expression of MHC class II molecule. Monocytes are retained in the vasculature and interact with T cells inducing T cell proliferation (767). MHC class II^+ monocytes are responsible for glomerular inflammation in a mouse model, since depletion of monocytes results in reduced $CD4^+$ T cell dependent inflammation. Moreover, human endothelial cells increased the expression levels of CD80 on the surface of monocytes only when T cells were present (621). Anti-CD28 fragment and anti-CTLA-4 antibodies inhibited the T cell activation in that system. Monocytes also dysregulated their expression of CD80 and CD86 in the presence of the live parasite *Toxoplasma gondii* (622). Dead *T. gondii* or parasitic antigens did not elicit the same response by the monocytes. Co-culture of autologous *T. gondii*-seronegative T cells with *T. gondii*-infected PBMCs resulted in T cell proliferation which was inhibited in the presence of CTLA-4-Ig or a combination of anti-CD80 with anti-CD86 antibodies. It is worth noting that inhibition of proliferation was also detected in the presence of anti-CD80 or anti-CD86, however CD86 blockade induced stronger inhibition compared to CD80 blockade. Finally, IFN-γ was only secreted by T cells after co-culture with CD80- and CD86-expressing monocytes, a result of infection of monocytes with the parasite. Monocytes in mice have also been responsible for mounting immune responses against fungi (768).

In the last few years, we learned more about the different subsets of monocytes and their distinct roles. Human classical and non-classical monocytes isolated from

seropositive donors for cytomegalovirus (CMV) or influenza were able to induce $CD4^+$ T cell proliferation stimulated with either CMV or influenza antigens, albeit at lower levels compared to conventional DCs (649). Classical monocytes also exhibit a higher capacity for T cell stimulation in MLR system compared to the intermediate monocytes (769).

Intermediate monocytes however are more potent stimulators in autologous MLR compared to classical or non-classical monocytes (770). Zawada et al. and others have found that intermediate monocytes upregulated *IFI30*, *CD74*, and other *HLA-DR* paralogues, which are involved in antigen processing and presentation through MHC class II (116, 770). An earlier study supports this finding by showing that intermediate monocytes express higher levels of HLA-DR (771). In contrast to these findings, Lee et al. reported that classical monocytes express the highest amount of MHC class II proteins after cytokine stimulation compared to the other two subsets and all monocyte subsets decreased their expression of HLA-DR after IL-10 was present in the culture (118). In pigs, the $2A10^+$ monocytes, the homologues for the intermediate monocytes in humans, are able to induce strong allogeneic responses to T cells (772).

Non-classical monocytes were worse at autologous T cell co-stimulation compared to the other two subsets but were better than the intermediate monocytes in MLR (769). Non-classical monocytes upregulate genes which are linked to the MHC class I mechanism (*B2M*, *HLA-B*, *HLA-E*, and *PSMB9*) (770). Proliferation of $CD4^+$ T cells was stronger after co-culture with $CD16^+$ monocytes from healthy individuals compared to $CD16^-$ monocytes, however no distinction between the three different subsets were made in this study (636). Similarly, $CD16^+$ monocytes stimulated $CD4^+$ T cells stronger than the $CD16^-$ monocytes isolated from patients suffering from SLE. Interestingly, $CD16^+$ monocytes skewed $CD4^+$ T cells towards a Th1 and Th2

response, whereas $CD16^-$ monocytes only promoted Th1 responses.

Co-stimulation by monocytes was also possible using cells from acute myeloid leukaemia (AML) patients. Human $CD4^+$ T cells proliferated in the presence of AML cells and proliferation was strongly inhibited using either a mixture of anti-CD80 and anti-CD86 antibodies or the CTLA-4 Ig (773). Anti-CD80 or anti-CD86 antibodies did not promote a strong T cell inhibition. IFN-γ led to upregulation of both CD80 and CD86 in both the healthy as well as the leukaemic monocytes but upregulation of these proteins were lower in lymphoma cells (184, 614, 773). CD40 can similarly upregulate CD80 and CD86 in normal monocytes and in lymphomas (773).

5.1.3. Hypothesis and aims

In this chapter we set out to test RhuDex's effect in T cell co-stimulation in the human liver. We expected a strong effect on liver B cells and monocytes since CD80 was most abundantly detected in those subsets. To assess that, we conducted a series of *in vitro* experiments using autologous liver B cells, monocytes, and T cells in the autologous MLR system adapted in chapter 3. The overall aims of this chapter were:

1. To verify the ability of RhuDex to block T cell co-stimulation using primary cells isolated from blood of non-cirrhotic HFE patients.
2. To assess RhuDex's capacity to block T cell co-stimulation using liver-isolated immune cells from patients with end-stage diseases.
3. To further evaluate the mode of action of RhuDex on T cells as well as on the APCs.

5.2. Establishment of co-stimulation assay using blood-derived cells

In order to assess the capacity of RhuDex to inhibit T cell co-stimulation in the liver, we tested the effectiveness of the drug in a more physiological system compared to that we used in chapter 3. We isolated $CD3^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes using fluorescence-activated cell sorting (FACS) from the blood of patients with HFE (**Fig. 22 A**). The gating strategy we followed was to select the $CD3^+$ and $CD3^-$ cells. The $CD3^-$ cluster was further sorted into $CD19^+$ and $CD14^+$ cells. Then the $CD3^+$ T cells were co-cultured with either the autologous $CD19^+$ B cells or the $CD14^+$ monocytes for 5 days in the presence of anti-CD3. After 5 days, we assessed the proliferation of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells using flow cytometry. The purity of all cells was consistently over ~90%.

In order to get the most optimal T cell proliferation in our co-cultures, we assessed T cell : APC ratio, using 100,000 T cells per well together with 30,000, 10,000, 3,000 or 1,000 APC. T cells proliferated stronger at a ratio of 2:1, or 100,000 T cells co-cultured with 50,000 APCs (**Fig. 22 B**). For all future experiments, we used the 2:1 ratio to provide the strongest T cell stimulation.

We next investigated the ability of B cells to present signal 2 to the T cells. To achieve that, we phenotyped the blood-derived B cells at the day of isolation to assess their CD80 and CD86 expression. The percentage of blood-derived B cells expressing CD80 or CD86 was low for both proteins (median=6.195%, IQR= 0.4875-44.06% and 3.885%, 1.023-5.195%, respectively) (**Fig. 22 C**). The MFI for CD80 and CD86 was similarly low (16.27, 13.31-55.2 and 85.17, 77.48-103 respectively). Following that, we co-cultured the autologous $CD19^+$ B cells with the $CD3^+$ T cells for

5 days in the presence of anti-CD3 to assess the ability of B cells to present signal 2 to the T cells. B cells promoted T cell proliferation of $CD4^+$ T cells (p=0.016), however, there was no statistical difference for the $CD8^+$ T cells (p=0.1017) (**Fig. 22 E**). Of note, the baseline of untreated liver $CD8^+$ T cell proliferation was higher than $CD4^+$ T cells (~20% vs 10%).

We then followed the same strategy to evaluate the ability of autologous monocytes isolated from the blood of HFE patients to promote T cell proliferation. The percentage of monocytes expressing CD80 and CD86 was higher than the B cells and had a high variation between patients (33.35%, 1.505-69.37%, and 27.57%, 11.43-76.75% respectively) (**Fig. 22 D**). The MFI levels for CD80 was 7.495 (4.955-39.66) and for CD86 166.8 (126.9-189.7). When the $CD14^+$ monocytes were co-cultured with autologous $CD3^+$ T cells from HFE blood in the presence of anti-CD3, proliferation was strongly induced for both $CD8^+$ and $CD4^+$ T cells (p=0.014 and p=0.024, respectively) (**Fig. 22 F**). In conclusion, both B cells and monocytes provided efficient signal 2 to the T cells to induce proliferation.

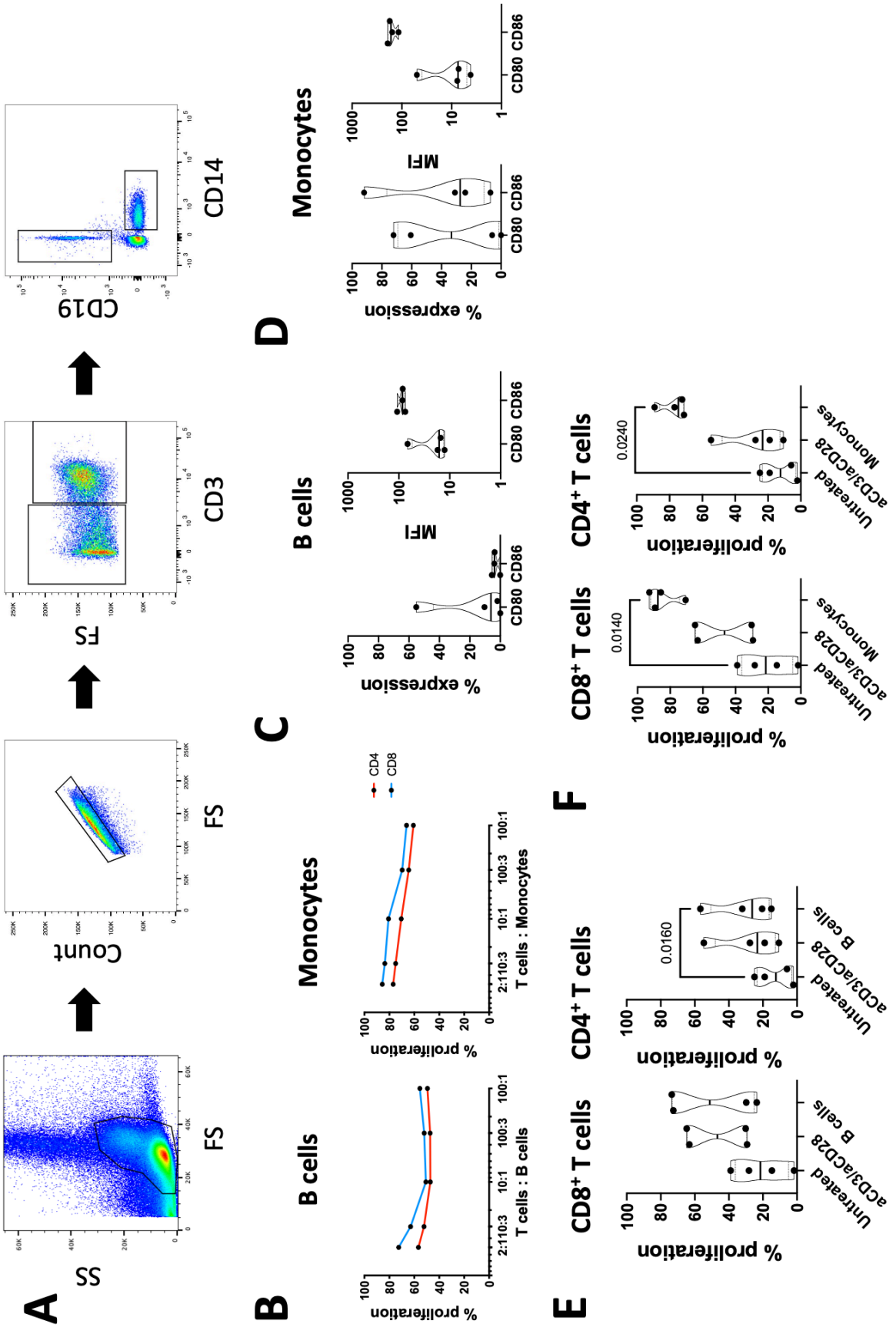

Figure 22: CD19^{+} B cells and CD14^{+} monocytes induce proliferation of autologous CD8^{+} and CD4^{+} T

cells from blood. (**A**) Representative gating strategy for fluorescent-activated cell sorting of lymphocytes isolated from blood of patients with HFE. (**B**) Percentage of proliferation for $CD8^+$ (blue line) and $CD4^+$ (red line) T cells when co-cultured either with $CD19^+$ B cells or $CD14^+$ monocytes at different T cell: APC ratio. N=1 (**C**) Percentage of expression and MFI levels for CD80 and CD86 on $CD19^+$ B cells from patients with HFE. N=4 (**D**) Percentage of expression and MFI levels for CD80 and CD86 on $CD14^+$ monocytes from HFE patients. N=4 (**E**) Percentage of proliferation of FAC sorted $CD3^+$ T cells which were later stained with CD8 and CD4 after co-culture with autologous $CD19^+$ B cells for 5 days. T cells were cultured alone (untreated), cultured with anti-CD3 and anti-CD28 or with anti-CD3 and $CD19^+$ B cells. N=4 (**F**) Percentage of proliferation of FAC sorted $CD3^+$ T cells which were later stained with CD8 and CD4 after co-culture with autologous $CD14^+$ monocytes for 5 days. T cells were cultured alone (untreated), cultured with anti-CD3 and anti-CD28 or anti-CD3 with $CD14^+$ monocytes. N=4. Results in **C**, **D**, **E**, and **F** are shown as median (solid line) with interquartile range (dotted line). Statistical analyses for **E** and **F** were conducted using Friedman test with Dunnett's multiple comparisons.

5.3. RhuDex inhibits T cell proliferation in blood-derived B cell and monocyte co-cultures

We next assessed the ability of RhuDex, abatacept, anti-CD80, anti-CD86, and their isotypes to inhibit T cell proliferation in co-cultures where $CD3^+$ T cells were co-cultured with autologous $CD19^+$ B cells and anti-CD3 for 5 days using flow cytometry. RhuDex inhibited T cell proliferation for both $CD8^+$ (30 μg/ml: p=0.0451), and $CD4^+$ T cells (30 μg/ml: p=0.018, 15 μg/ml: p=0.0393) despite the lack of high expression of CD80 by the B cells (**Fig. 23 A**). Abatacept did not affect T cell proliferation of either $CD8^+$ or $CD4^+$ T cells in any of the concentrations we used. Similarly, proliferation was unaffected for T cells co-cultured with B cells when

treated with either anti-CD80 or anti-CD86.

Similarly to chapter 3, we were interested in studying the capacity of RhuDex to induce cell death to $CD8^+$ and $CD4^+$ T cells in the culture after 5 days. RhuDex did not promote apoptosis to the T cells (**Fig. 23 B**). Abatacept, anti-CD80, and anti-CD86 also did not affect the viability of both T cell subsets. Finally, we looked at the capacity of the inhibitors of promoting apoptosis to the autologous B cells. RhuDex and the other inhibitors we tested did not promote apoptosis towards the co-cultured B cells (**Fig. 23 C**).

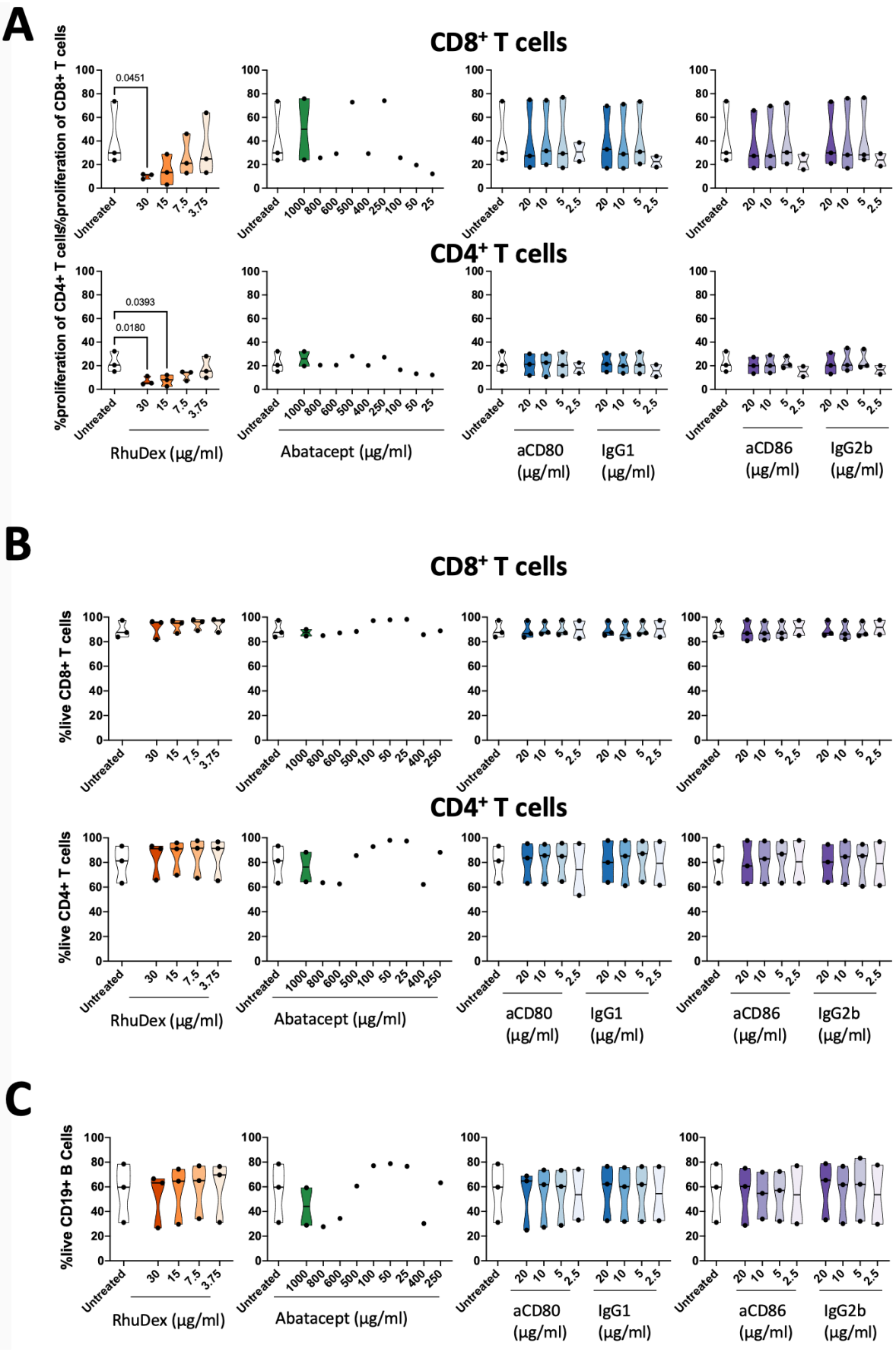

Figure 23: RhuDex is the most potent inhibitor of blood-derived T cell proliferation compared to

abatacept, anti-CD80 and anti-CD86 when T cells are co-cultured with $CD19^+$ B cells. Autologous $CD3^+$ T cells were co-cultured with anti-CD3 and blood-derived $CD19^+$ B cells alone (untreated) or in the presence of RhuDex (30, 15, 7.5, and 3.75 ug/ml), abatacept (1000, 800, 600, 500, 400, 250,100, 50, and 25 ug/ml), anti-CD80 (20, 10, 5, 2.5 ug/ml), IgG1 (20, 10, 5, 2.5 ug/ml), anti-CD86 (20, 10, 5, 2.5 ug/ml), and IgG2b (20, 10, 5, 2.5 ug/ml) for 5 days. (**A**) Percentage of proliferation of $CD8^+$ and $CD4^+$ T cells in the presence of RhuDex, abatacept, anti-CD80, IgG1, anti-CD86, and IgG2b. (**B**) Percentage of live $CD8^+$ and $CD4^+$ T cells in the presence of RhuDex, abatacept, anti-CD80, IgG1, anti-CD86, and IgG2b. (**C**) Percentage of live $CD19^+$ B cells after co-culture with autologous $CD3^+$ T cells and anti-CD3 at day 5 in the presence of RhuDex, abatacept, anti-CD80, IgG1, anti-CD86, and IgG2b. RhuDex n=3, abatacept n=1-2. Results are shown as median (solid line) with interquartile range (dotted line). Statistical analysis for samples treated with RhuDex were conducted with the Friedman testt with Dunnett's multiple comparisons. The rest of the samples were analysed with the Kruskal-Wallis test with Dunn's multiple comparisons.

Following that, we used the same system to assess the ability of RhuDex to inhibit T cell proliferation using autologous blood-derived $CD14^+$ monocytes instead of $CD19^+$ B cells. RhuDex strongly suppressed the proliferation of $CD8^+$ and $CD4^+$ T cells (p=0.0195 for both) when co-cultured with anti-CD3 and monocytes (**Fig. 24 A**). Abatacept, anti-CD80, and anti-CD86 did not inhibit T cell proliferation.

Furthermore, RhuDex did not have toxic effects on $CD4^+$ or $CD8^+$ T cells (**Fig. 24 B**). Moreover, abatacept, anti-CD80, and anti-CD86 did not affect the viability of either $CD8^+$ or $CD4^+$ T cells. Finally, we assesed the effect of the inhibitors on the viability of the monocytes. Untreated monocytes had a low viability (median below 20%), and treatment with the inhibitors did not induce further reduction in their viability (**Fig. 24 C**). The above results enhance the hypothesis that RhuDex is a potent inhibitor compared to commercially available blockers of the CD28-CD80 pathway.

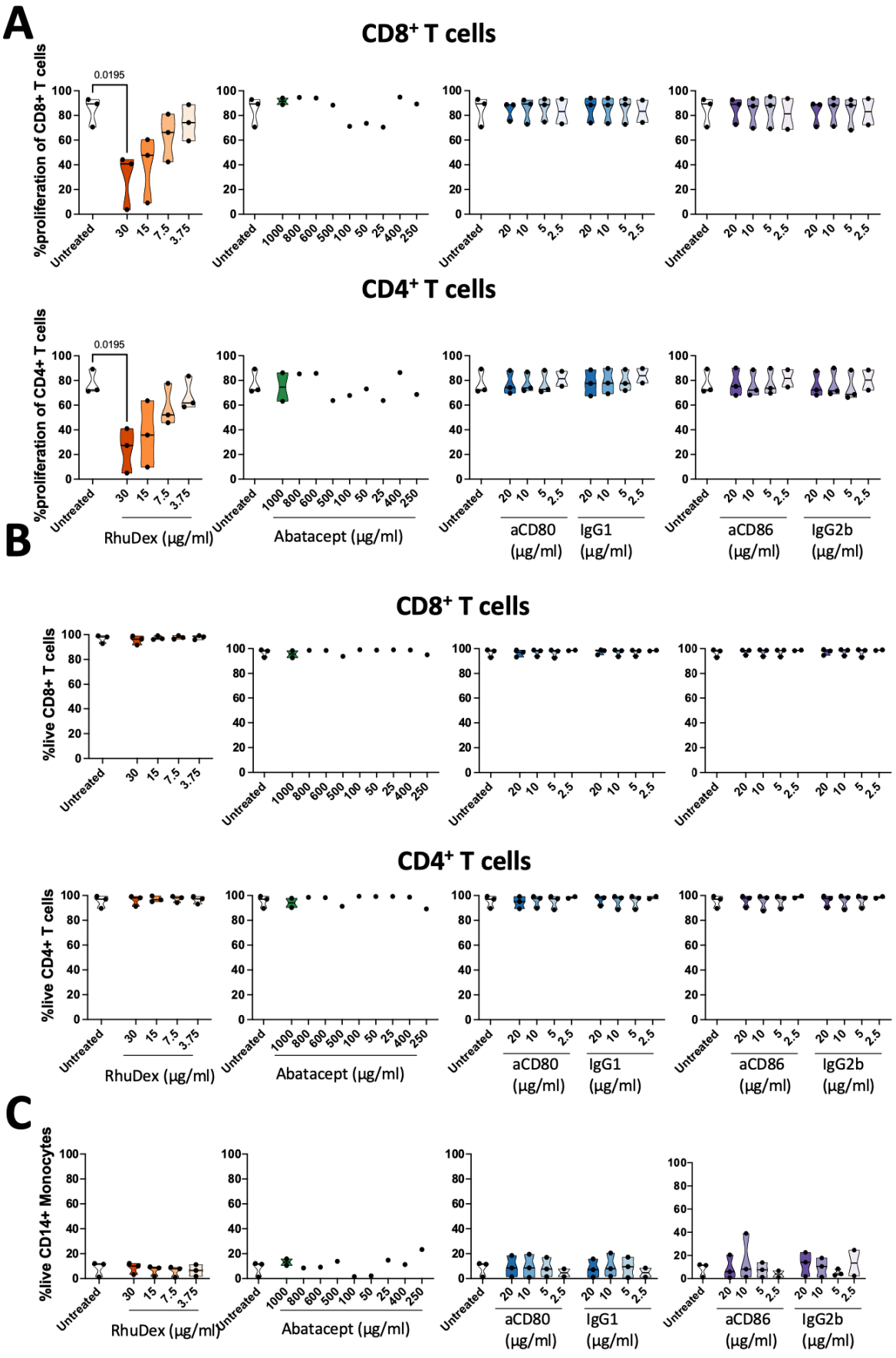

Figure 24: RhuDex is the most potent inhibitor of blood-derived T cell proliferation compared to

abatacept, anti-CD80 and anti-CD86 when T cells are co-cultured with $CD14^+$ monocytes. Autologous $CD3^+$ T cells were co-cultured with anti-CD3 and blood-derived $CD14^+$ monocytes alone (untreated) or in the presence of RhuDex (30, 15, 7.5, and 3.75 ug/ml), abatacept (1000, 800, 600, 500, 400, 250,100, 50, and 25 ug/ml), anti-CD80 (20, 10, 5, 2.5 ug/ml), IgG1 (20, 10, 5, 2.5 ug/ml), anti-CD86 (20, 10, 5, 2.5 ug/ml), and IgG2b (20, 10, 5, 2.5 ug/ml) for 5 days. (**A**) Percentage of proliferation of $CD8^+$ and $CD4^+$ T cells in the presence of RhuDex, abatacept, anti-CD80, IgG1, anti-CD86, and IgG2b. (**B**) Percentage of live $CD8^+$ and $CD4^+$ T cells in the presence of RhuDex, abatacept, anti-CD80, IgG1, anti-CD86, and IgG2b. (**C**) Percentage of live $CD14^+$ monocytes after coculture with autologous $CD3^+$ T cells and anti-CD3 at day 5 in the presence of RhuDex, abatacept, anti-CD80, IgG1, anti-CD86, and IgG2b. RhuDex n=3, abatacept n=1-2. Results are shown as median (solid line) with interquartile range (dotted line). Statistical analysis for samples treated with RhuDex were conducted with Friedman test with Dunnett's multiple comparisons. The rest of the samples were analysed with the Kruskal-Wallis test.

5.4. Establishment of co-stimulation assay using liver-infiltrating mononuclear cells

Based on the above data, we wanted to study the effects of RhuDex on liver-derived cells. For that reason, we isolated total LIMCs and used FACS following the same gating strategy as described in chapter 5.2 to isolate $CD3^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes. The purity of the isolated cells was consistently over ~90%. When we co-cultured $CD3^+$ T cells from a NASH patient with either autologous B cells or monocytes together with anti-CD3 we found that a 2:1 ratio of T cells:APCs (or 100,000 $CD3^+$ T cells : 50,000 $CD19^+$ B cells/$CD14^+$ monocytes) resulted in a maximal amount of T cell proliferation (**Fig. 25 A**).

We next phenotyped $CD19^+$ B cells and $CD14^+$ monocytes to measure the percentage and expression levels of CD80 and CD86 (1 PBC patient, 2 PSC patients, 2 NASH patients, 1 ALD patient) using flow cytometry. A small percentage of B cells expressed CD80 (4.22%, 1.7-13.63%) and CD86 (20%, 6.77-47.73%) (**Fig. 25 B**). The MFI for CD80 and CD86 was 8.645 (5.7-9.3) and 244.7 (135.4-308.2) respectively. We then co-cultured the liver-isolated $CD19^+$ B cells with autologous $CD3^+$ T cells for 5 days in the presence of soluble anti-CD3 antibody. The results indicate that $CD4^+$ T cells had received a strong signal 2 which promoted strong proliferation to the T cells ($p=0.0187$) (**Fig. 25 D**). $CD8^+$ T cells also proliferated in the presence of B cells ($p=0.0418$).

Following that, we phenotyped the $CD14^+$ monocytes at day of isolation to assess their capacity to induce T cell proliferation. Flow cytometry results revealed that the majority of the monocytes did not express CD80 (0.335%, 0-1.05%), however, they favoured CD86 expression (42.9%, 8.16-59%) (**Fig. 25 C**). The expression levels of CD80 and CD86 on the surface of monocytes were 8.4 (4.2-51.75) and 168.4 (127.5-203.8) respectively. When we co-cultured $CD3^+$ T cells with $CD14^+$ monocytes and anti-CD3, we saw that monocytes promoted strong proliferative responses to the T cells ($CD8^+$ $p=0.0078$, $CD4^+$ $p=0.0078$) (**Fig. 25 E**). These results indicate that the co-culture system we have set up is a valid tool for measuring T cell responses, in both blood and liver.

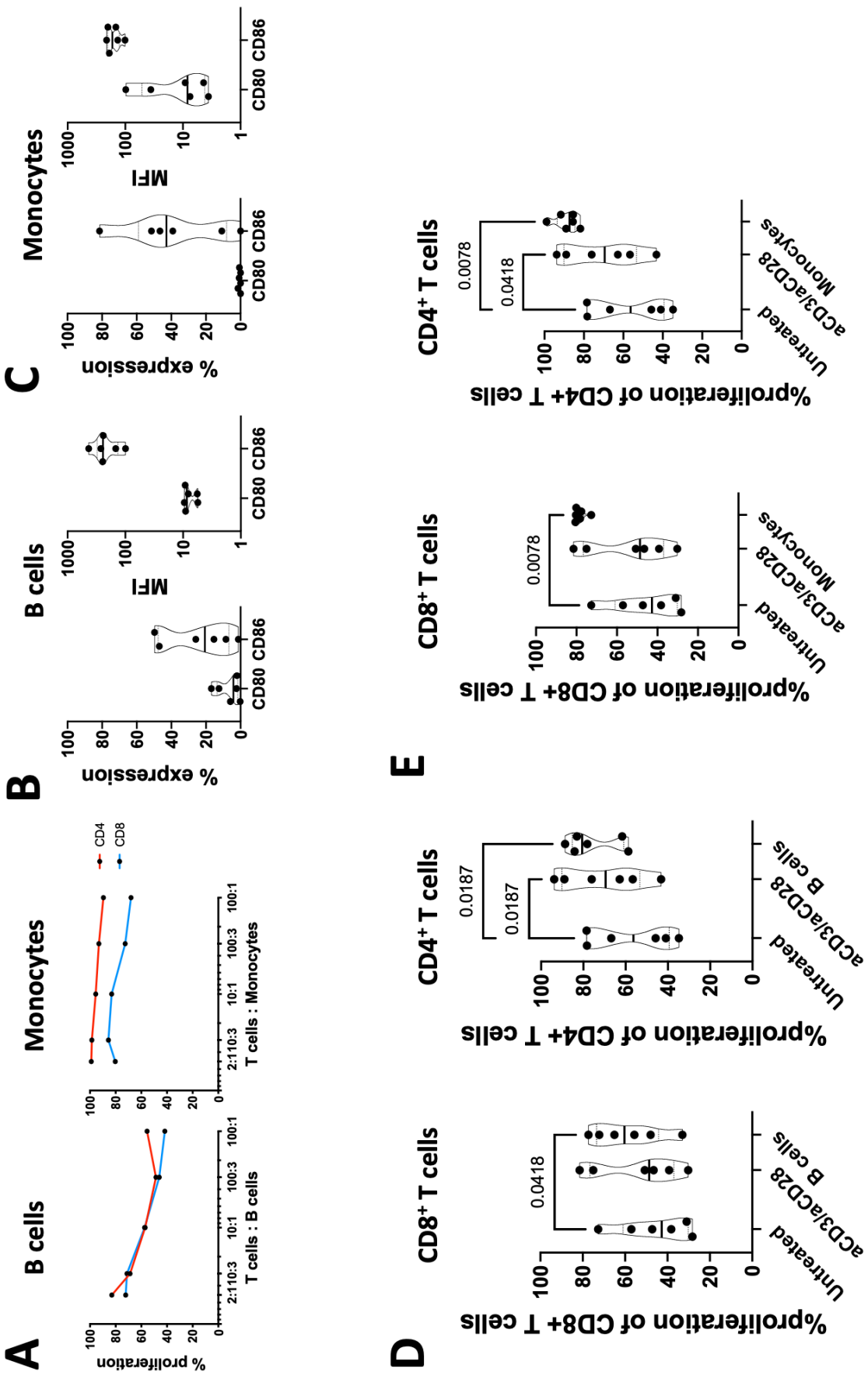

Figure 25: CD19$^+$ B cells and CD14$^+$ monocytes induce proliferation of autologous CD8$^+$ and CD4$^+$ T

cells isolated from liver of patients with autoimmune and metabolic diseases. Liver-infiltrating $CD19^+$ B cells and $CD14^+$ monocytes were isolated and FAC sorted with autologous liver-infiltrating $CD3^+$ T cells and co-cultured for 5 days either alone, in the presence of anti-CD3 and anti-CD28, or with anti-CD3 and either $CD19^+$ B cells or $CD14^+$ monocytes. (**A**) Percentage of proliferation for $CD8^+$ (blue line) and $CD4^+$ (red line) T cells when co-cultured either with $CD19^+$ B cells or $CD14^+$ monocytes at different T cell: APC ratio. N=1 (**B**) Percentage of expression and MFI levels for CD80 and CD86 on $CD19^+$ B cells. N=6 (**C**) Percentage of expression and MFI levels for CD80 and CD86 on $CD14^+$ monocytes. N=6 (**D**) Percentage of proliferation of FAC sorted $CD3^+$ T cells which were later stained with CD8 and CD4 after co-culture with autologous $CD19^+$ B cells for 5 days. T cells were cultured alone (untreated), cultured with anti-CD3 and anti-CD28 or with anti-CD3 and $CD19^+$ B cells. N=6 (**E**) Percentage of proliferation of FAC sorted $CD3^+$ T cells which were later stained with CD8 and CD4 after co-culture with autologous $CD14^+$ monocytes for 5 days. T cells were cultured alone (untreated), cultured with anti-CD3 and anti-CD28 or with anti-CD3 and $CD14^+$ monocytes. N=6. Results in **B**, **C**, **D**, and **E** are shown as median (solid line) with interquartile range (dotted line). Statistical analyses for **D** and **E** were conducted with the Friedman test with Dunnett's multiple comparisons.

5.5. RhuDex blocks T cell co-stimulation in liver-derived B cell and monocyte co-cultures

Our next aim was to study the effect of RhuDex on liver cells and verify whether RhuDex could work with cells isolated from the liver. For that reason, we co-cultured autologous $CD3^+$ T cells with $CD19^+$ B cells and anti-CD3 in the presence and absence of RhuDex or abatacept (1 PBC patient, 2 PSC patients, 2 NASH patients, 1 ALD patient). RhuDex strongly inhibited T cell responses for $CD8^+$ and $CD4^+$ T cells (30 μg/ml: p=0.0165 and p=0.0356 respectively) (**Fig. 26 A**). Abatacept did not inhibit responses of either T cell subset at the tested concentrations (**Fig. 26 A**).

We next examined the toxicity levels of RhuDex and abatacept to the T cells in the co-culture. Neither RhuDex nor abatacept promoted cell death to $CD8^+$ or $CD4^+$ T cells (**Fig. 26 B**). Furthermore, the drugs had no effect on the viability of the autologous liver-infiltrating B cells which were co-cultured with the T cells (**Fig. 26 C**).

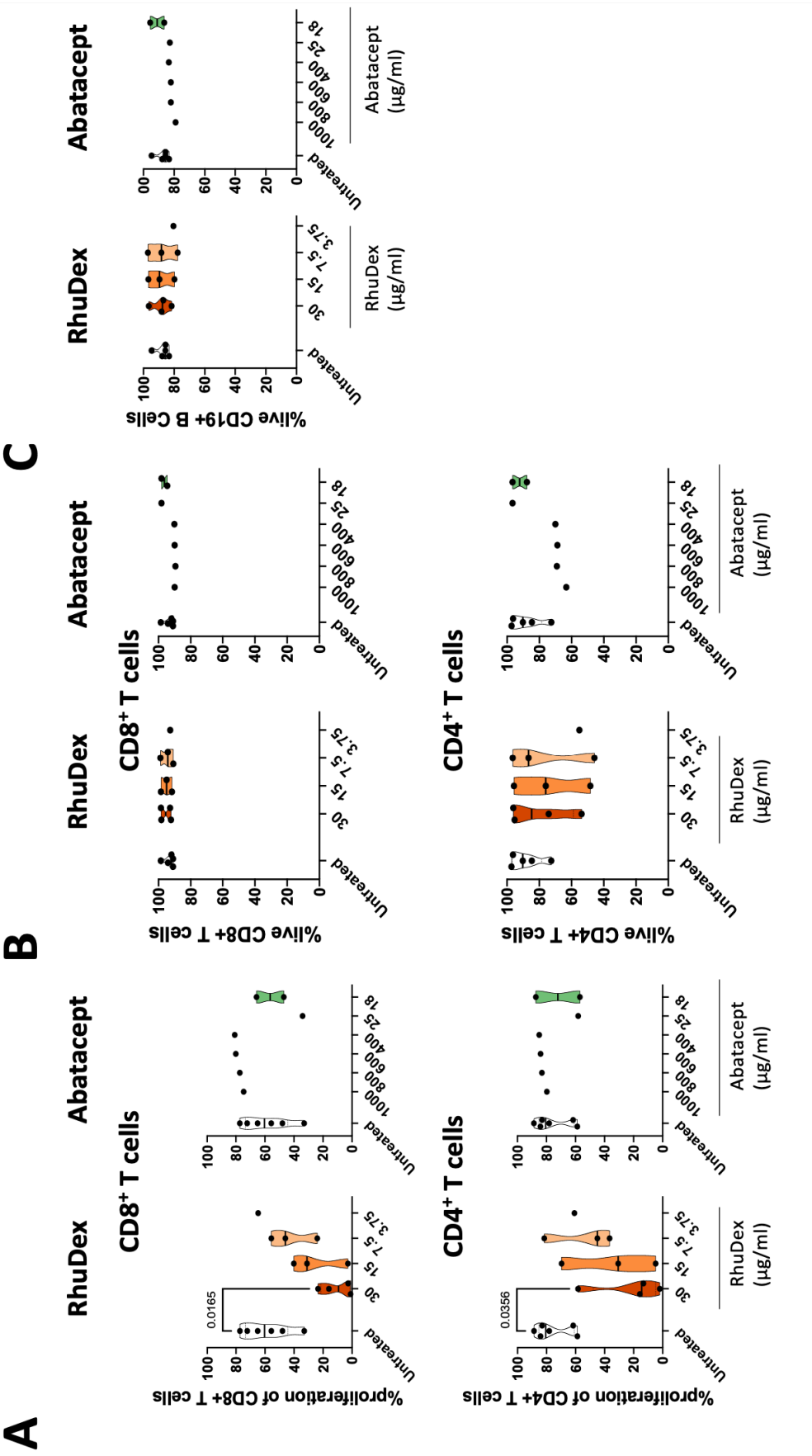

Figure 26: RhuDex is a potent inhibitor of liver-infiltrating T cell proliferation when co-cultured with

$CD19^+$ B cells. Autologous $CD3^+$ T cells were co-cultured with anti-CD3 and liver-infiltrating $CD19^+$ B cells alone (untreated) or in the presence of RhuDex (30, 15, 7.5, and 3.75 ug/ml) or abatacept (1000, 800, 600, 400, 25, and 18 ug/ml) for 5 days. (**A**) Percentage of proliferation of $CD8^+$ and $CD4^+$ T cells (first and second row respectively) in the presence of RhuDex or abatacept. (**B**) Percentage of live $CD8^+$ and $CD4^+$ T cells in the presence of RhuDex or abatacept. (**C**) Percentage of live $CD19^+$ B cells after coculture with autologous $CD3^+$ T cells and anti-CD3 at day 5 in the presence of RhuDex or abatacept. RhuDex n=1-4, abatacept n=1-2. Results are shown as median (solid line) with interquartile range (dotted line). Kruskal-Wallis with Dunn's multiple comparisons statistical analysis was performed for all graphs.

Following that, we assessed the ability of RhuDex to inhibit T cell responses when co-cultured with autologous monocytes in the presence of anti-CD3. RhuDex was able to inhibit $CD8^+$ (30 μg/ml: p=0.032) but $CD4^+$ T cells (p=0.0573) in the presence of signal 2 (**Fig. 27 A**). This is possibly due to the small sample size. Abatacept did not block the CD28-CD80/CD86 pathway (**Fig. 27 A**).

RhuDex and abatacept did not see have an effect on the viability of the T cells (**Fig. 27 B**). The viability of monocytes after treatment with RhuDex or abatacept in co-culture with T cells after 5 days was unaffected by the presence of the drugs (**Fig. 27 C**). To conclude, RhuDex but not abatacept inhibited liver-isolated T cell proliferation without affecting the viability of cells.

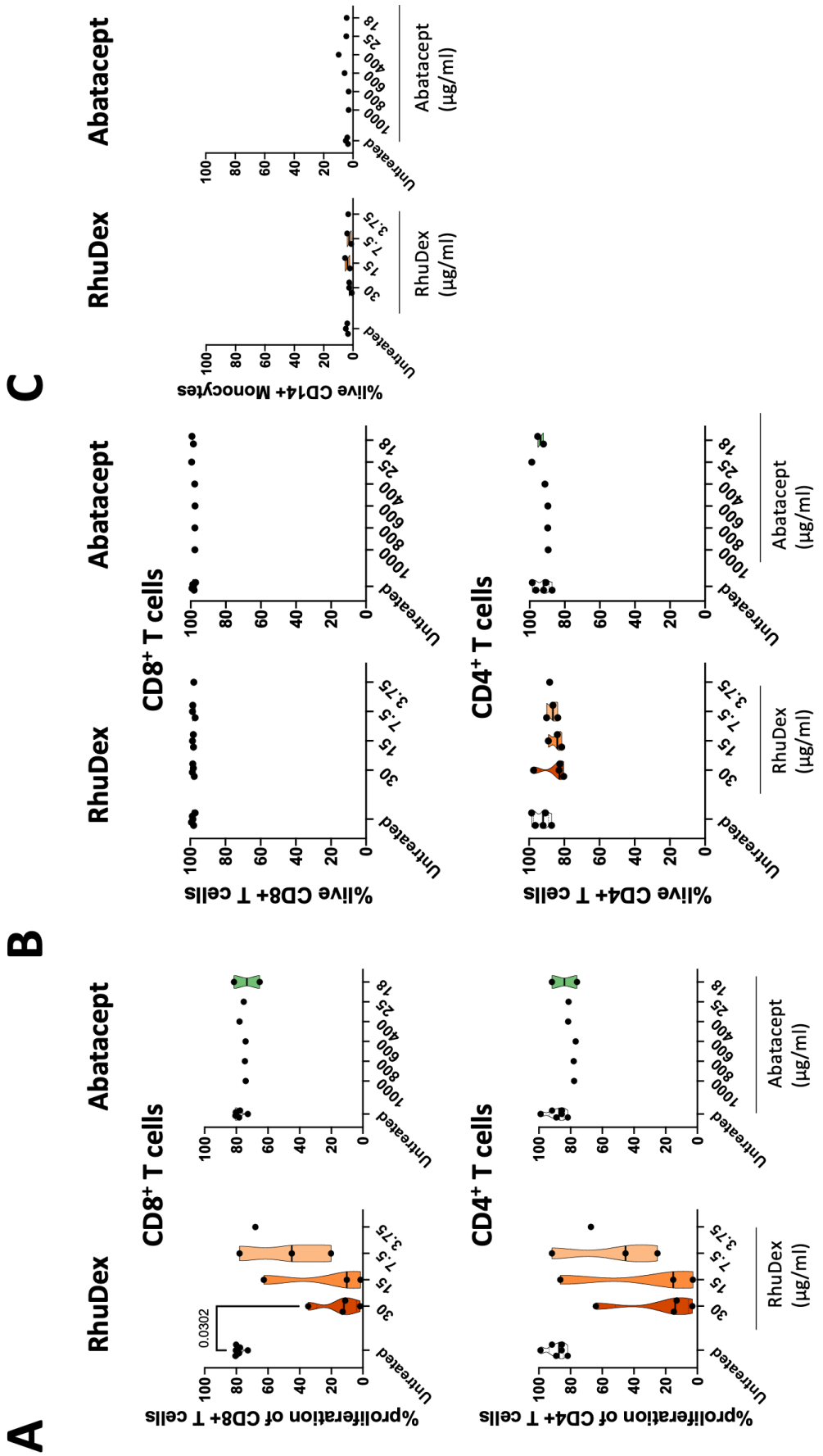

Figure 27: RhuDex is a potent inhibitor of liver-infiltrating T cell proliferation when co-cultured with

$CD14^+$ monocytes. Autologous $CD3^+$ T cells were co-cultured with anti-CD3 and liver-infiltrating $CD14^+$ monocytes alone (untreated) or in the presence of RhuDex (30, 15, 7.5, and 3.75 ug/ml) or abatacept (1000, 800, 600, 400, 25, and 18 ug/ml) for 5 days. (**A**) Percentage of proliferation of $CD8^+$ and $CD4^+$ T cells (first and second row respectively) in the presence of RhuDex or abatacept. (**B**) Percentage of live $CD8^+$ and $CD4^+$ T cells in the presence of RhuDex or abatacept. (**C**) Percentage of live $CD14^+$ monocytes after coculture with autologous $CD3^+$ T cells and anti-CD3 at day 5 in the presence of RhuDex or abatacept. RhuDex n=1-4, abatacept n=1-2. Results are shown as median (solid line) with interquartile range (dotted line). Kruskal-Wallis with Dunn's multiple comparisonsstatistical analysis was performed for all graphs.

5.6. Liver-infiltrating B cell subsets are unable to induce T cell co-stimulation *in vitro*

Based on the above data, we wanted to investigate whether the two novel liver-enriched B cell populations discovered by our lab, $CD24^-CD38^-$ and $CD24^-CD38^{int}$, could induce T cell activation though the CD28-CD80/CD86 axis and if RhuDex and abatacept had the ability to inhibit such signal. Since these B cells are enriched in the liver (101), we isolated autologous cells from explant livers with end-stage disease.

To test the above hypotheses, we used flow cytometry to sort the LIMCs isolated from patients with autoimmune or metabolic liver diseases (2 PBC patients, 1 NAFLD patient). The gating strategy we followed was to identify first $CD3^+$ cells and $CD19^+$ B cells. Then we gated on the $CD24^-CD38^-$, $CD24^-CD38^{int}$, and the $CD24^+CD38^{-/int}$. From the final cluster of cells, we gated on the IgD^- cells to sort for the memory B cells (**Fig. 28 A**). The purity of the B cell subsets was ~80% and for the $CD3^+$ T cells

was ~90%.

The different B cell subsets expressed different levels of CD80 and CD86. Flow cytometry showed that only a small percentage of memory and $CD24^{-}CD38^{-}$ B cells expressed CD80 on their surface (3.91%, 0-26.5% and 8.14%, 0.4-29.7%, respectively), however $CD24^{-}CD38^{int}$ had a larger percentage of CD80 expressing cells (51.96%, 48.26-83.77%) (**Fig. 28 B**). The CD80 MFI levels for the three subsets were 18.24 (17.51-24.86) for the memory B cells, 20.54 (18.11-24.06) for the $CD24^{-}CD38^{-}$ B cells, and finally 37.14 (36.46-101.3) for the $CD24^{-}CD38^{int}$ B cells. Similarly to CD80, a small percentage of memory and $CD24^{-}CD38^{-}$ B cells expressed CD86 on their surface (2.46%, 1.49-7.12% and 9.04%, 1.92-12.85%, respectively) while a great percentage of $CD24^{-}CD38^{int}$ B cells expressed the marker (42.74%, 41.79-61.75%). The MFI levels for all three markers were lower than those for the CD80 (memory- 0.74, 0.27-2.16, $CD24^{-}CD38^{-}$- 1.07, 0.27-2.1, $CD24^{-}CD38^{int}$ - 3.44, 1.54-4.05).

Next, we assessed the ability of the three B cell subsets to induce T cell co-stimulation upon anti-CD3 stimulation. None of the three B cell subsets provided a signal strong enough to promote proliferation in the $CD8^{+}$ and $CD4^{+}$ T cells (**Fig. 28 C**). Treating the co-stimulation co-cultures with either a high dose of RhuDex or abatacept did not have an effect in the systems in either of the T cell subsets (**Fig. 28 D, E, F**). In conclusion, although these B cell subsets are present in the liver, *in vitro* they were not capable of promoting T cell co-stimulation.

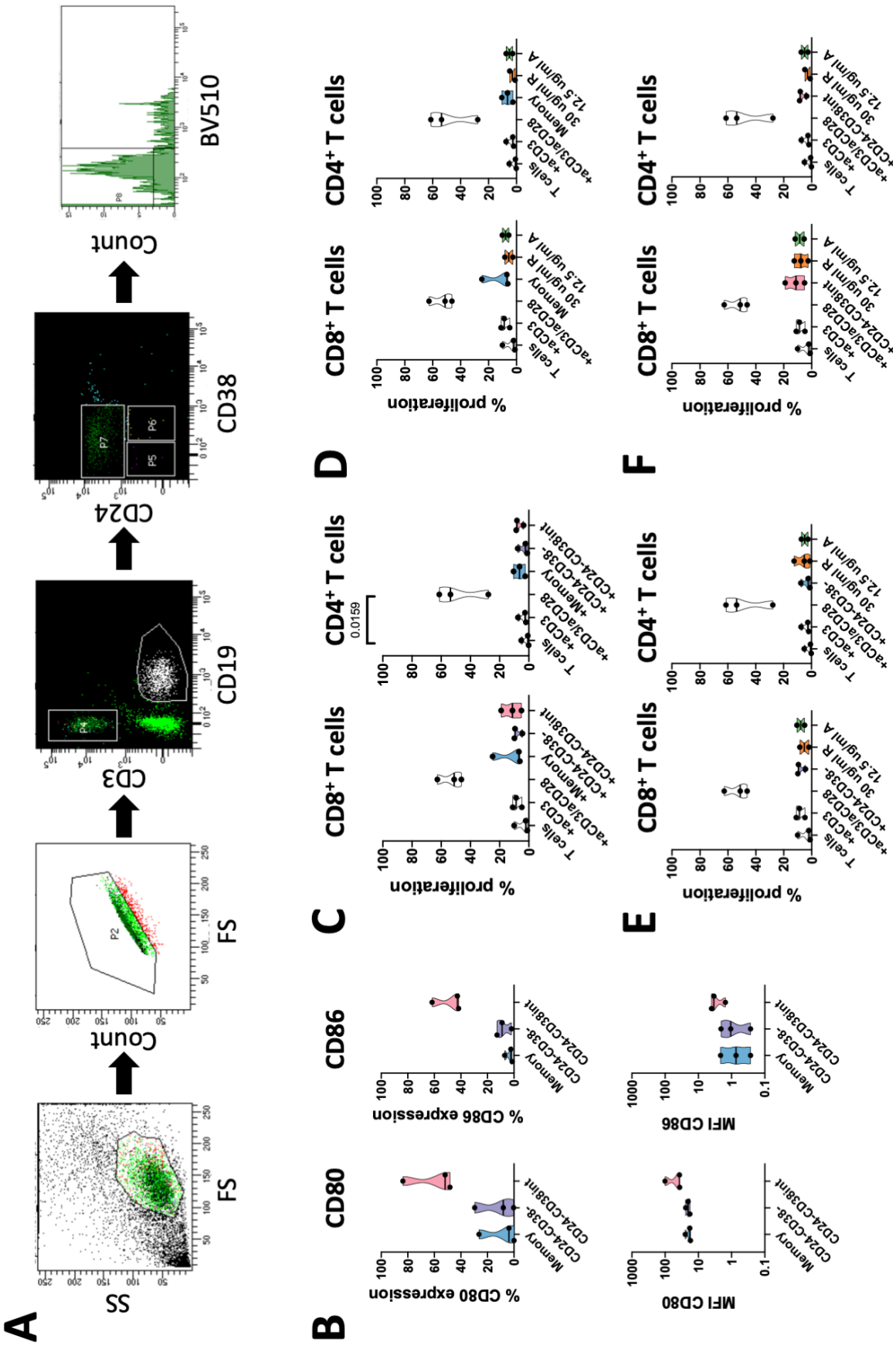

Figure 28: B cell subsets do not provide an efficient T cell co-stimulation signal. (**A**) Representative

gating strategy for fluorescent-activated cell sorting of lymphocytes isolated from the liver of patients with autoimmune and metabolic conditions. (**B**) Percentage of expression (top row) and MFI (bottom row) of CD80, CD86, and HLA-DR on $CD24^{-}CD38^{-}$, $CD24^{-}CD38^{int}$, and memory B cells. N=3 (**C**) $CD3^{+}$ T cells were FAC sorted from liver of patients with autoimmune or metabolic diseases and cultured alone, with anti-CD3, anti-CD3 and anti-CD28, or co-cultured with $CD24^{-}CD38^{-}$, $CD24^{-}CD38^{int}$, or memory B cells in the presence of anti-CD3 for 5 days. Graphs show the percentage of proliferation of $CD8^{+}$ and $CD4^{+}$ T cells. N=3, Friedman test was used for statistical analysis. (**D**) Percentage of proliferating $CD8^{+}$ and $CD4^{+}$ FAC sorted $CD3^{+}$ T cells when co-cultured with anti-CD3 and memory B cells in the presence of 30 ug/ml RhuDex (R) or 12.5 ug/ml abatacept (A). N=2-3, Kruskal-Wallis test was used. (**E**) Percentage of proliferating $CD8^{+}$ and $CD4^{+}$ FAC sorted $CD3^{+}$ T cells when co-cultured with anti-CD3 and $CD24^{-}CD38^{-}$ B cells in the presence of 30 ug/ml RhuDex (R) or 12.5 ug/ml abatacept (A). N=2-3, Kruskal-Wallis test was used. (**F**) Percentage of proliferating $CD8^{+}$ and $CD4^{+}$ FAC sorted $CD3^{+}$ T cells when co-cultured with anti-CD3 and $CD24^{-}CD38^{int}$ B cells in the presence of 30 ug/ml RhuDex (R) or 12.5 ug/ml abatacept (A). N=2-3, Kruskal-Wallis test with Dunn's mutliple comparisons. Results are shown as median (solid line) with interquartile range (dotted line).

5.7. RhuDex inhibits T cell proliferation in the absence of APCs

Evidence from above as well as from chapter 3 indicate that RhuDex could potentially act outside of the CD28-CD80 pathway. In order to further elucidate the mechanism of action of RhuDex and its possible off-target effects, we cultured $CD3^{+}$ T cells isolated from the blood of HFE patients alone or in the presence of anti-CD3, anti-CD3 and anti-CD28, or 30 μg/ml of RhuDex for 5 days. Flow cytometry results showed that RhuDex had a strong inhibitory effect on both $CD8^{+}$ and $CD4^{+}$ T cell proliferation (3.085%, 0-6.17% and 1.055%, 0-2.11%, respectively) compared to T cells

stimulated with anti-CD3 and anti-CD28 (38.05%, 11.2-64.9% and 13.85%, 0-27.7%, respectively) (**Fig. 29 A**). RhuDex had no toxic effects on the viability of the blood-derived T cells (**Fig. 29 B**).

We next repeated these experiments using LIMCs from explanted livers of patients with autoimmune and metabolic diseases (1 PBC, 2 NASH/NAFLD patients). Treatment of T cell cultures with 30 μg/ml of RhuDex resulted in inhibition of $CD8^+$ and $CD4^+$ proliferation (10.7%, 3.12-20.8% and 13.1%, 2.44-28.1%, respectively) compared to proliferating T cells after treatment with only anti-CD3 and anti-CD28 (62.7%, 51.2-86.5% and 61.4%, 53.7-88.4%, respectively) (**Fig. 29 C**).

Finally, we looked at the effect of RhuDex on the proliferating T cell line, Jurkat, in a culture without any external stimuli but in the presence of RhuDex or abatacept for 2 and 5 days. This preliminary experiment showed that Jurkat cells had not proliferated by day 2 but on day 5 proliferation levels reached 50.8% (**Fig. 29 D**). RhuDex inhibited proliferation in a dose dependent manner, however in this sample abatacept has shown no strong effects at the tested doses. Cells were alive at both day 2 and day 5 in the presence of RhuDex and abatacept. The above results indicate that one of RhuDex's off-target effects is to affect the activation of T cells without requiring the presence of CD80 on APCs to exert its function.

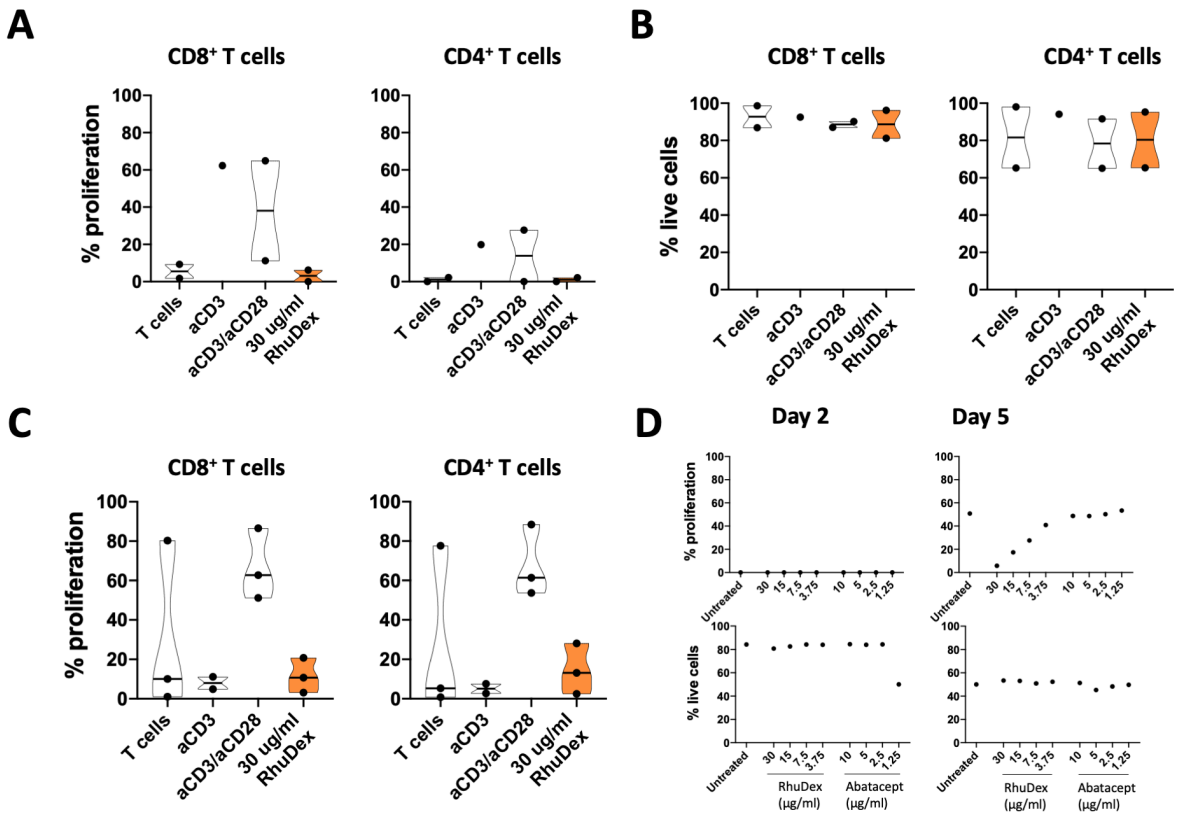

Figure 29: RhuDex inhibits T cell proliferation independent of the presence of APCs. $CD3^+$ T cells were FAC sorted and were cultured alone, in the presence of anti-CD3, anti-CD3 and anti-CD28, or RhuDex (30, 15, 7.5, 3.75 ug/ml) for 5 days and stained with CD4 and CD8. (**A**) Percentage of proliferating $CD8^+$ and $CD4^+$ T cells at day 5, isolated from blood of HFE patients. N=1-2. (**B**) Percentage of live $CD8^+$ and $CD4^+$ T cells at day 5, isolated from blood of HFE patients. N=1-2. (**C**) Percentage of proliferation of liver-infiltrating $CD8^+$ and $CD4^+$ T cells at day 5, isolated from patients with autoimmune and metabolic liver diseases. N=2-3. Kruskal-Wallis was used for the statistical test. (**D**) Percentage proliferation of the T cell line Jurkat (top row) and percentage of live Jurkat cells at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=1. Results are shown as median (solid line) with interquartile range (dotted line).

5.8. Proliferation of B cell and monocytic cell lines is inhibited by RhuDex

The ability of RhuDex to inhibit T cell proliferation in the absence of APC prompted us to inquire about RhuDex's ability to inhibit APC proliferation as well. To answer that question, we first phenotyped the three CHO cell lines we used on chapter 3. As expected, CHO cells expressed neither CD80 nor CD86, 97.72% of CHO-CD80 cells expressed CD80 and no CD86, and finally, 95.05% of CHO-CD86 cells expressed CD86 and no CD80 (**Fig. 30 A**).

We then cultured these cell lines in the presence or absence of a range of doses of RhuDex (abatacept was used as control) for 2 and 5 days to establish a timeframe under which the effect of RhuDex takes place. Our results showed that abatacept had no effect on proliferation on day 2 or day 5 no effect was detected (**Fig. 30 B**). We saw no effects in the proliferation of CHO-CD80 and CHO-CD86 cultures by either RhuDex or abatacept at either day 2 or day 5. We furthermore found that RhuDex caused a small drop in the viability of CHO-CD80 T cells in day 2 but not in day 5, whereas abatacept showed no effect in either of the co-culture for both days (**Fig. 30 C**). These results illustrate that RhuDex's inhibitory capacity has no effect on the proliferation of the cell lines, even when CD80 is present on the surface of the cell. However, there might be a small effect on the viability of cells expressing CD80 early during the treatment.

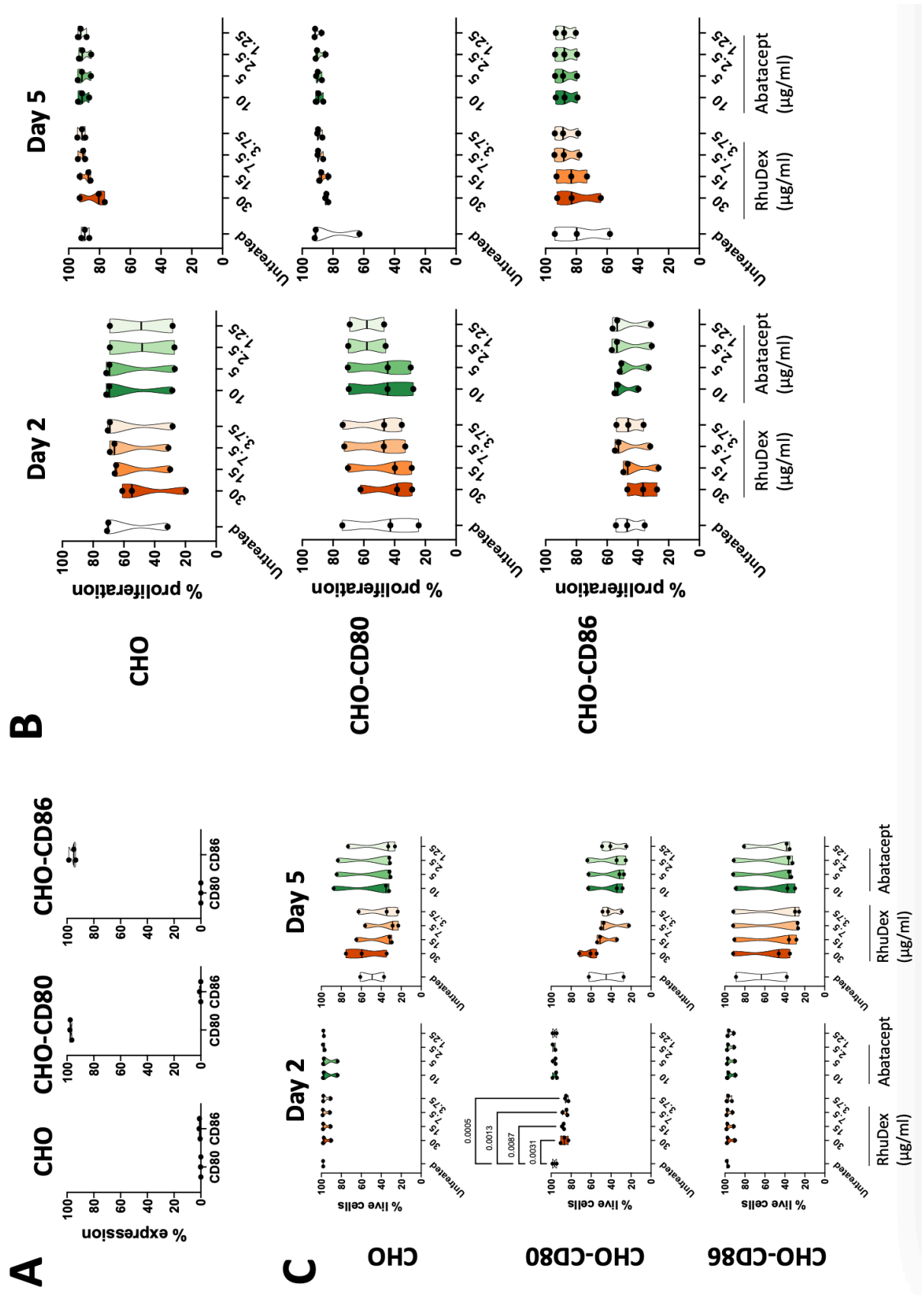

Figure 30: RhuDex does not inhibit proliferation of CHO cells. (**A**) Percentage of expression of CD80 and CD86 on the surface of CHO, CHO-CD80, and CHO-CD86. N=3. (**B**) Percentage of

proliferating cells for CHO, CHO-CD80, and CHO-CD86 at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=2-3. Statistical analysis performed were Friedman and Kruskal-Wallis tests. (**C**) Percentage of live CHO, CHO-CD80, and CHO-CD86 at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=2-3. Statistical analysis performed was one-way ANOVA with Dunn's mutliple comparisons. Results are shown as median (solid line) with interquartile range (dotted line).

Our focus then turned to human cells and for that reason we repeated the above experiments using the human B cell lines, Raji, DG-75, and L3055-BCL2. Phenotypically, these cell lines express different levels of CD80 and CD86. Specifically, 65.03% (with a range of 50.58-79.47%) of Raji express CD80 and 49.01% (with a range of 44.81-53.2%) express CD86 on their surface (**Fig. 31 A**). 15.74% (5.99-25.49%) of DG-75 express CD80 and 96.45% (96.28-96.61%) of CD86. Finally, 0.33% (0-0.66%) of L3055-BCL2 express CD80 and 15.53% (4.91-26.15%) express CD86 on their surface.

Next we cultured the cell lines for 2 and 5 days in the presence and absence of different doses or RhuDex and abatacept. RhuDex did not inhibit the proliferation of Raji cells at day 2 or day 5 despite the increased presence of CD80 on their surface (**Fig. 31 B**). DG-75 cells were not affected by either RhuDex or abatacept at day 2. At day 5, RhuDex inhibited the proliferation of the cells, however no statistical effect was detected for the cells treated with abatacept. Moreover, RhuDex blocked the proliferation of L3055-BCL2 at day 2 but no effect was detected at day 5. Abatacept showed no signs of inhibition of proliferation at either or those days. We found no effect of RhuDex or abatacept on the viability of any of the cell lines at day 2 or 5. (**Fig. 31 C**).

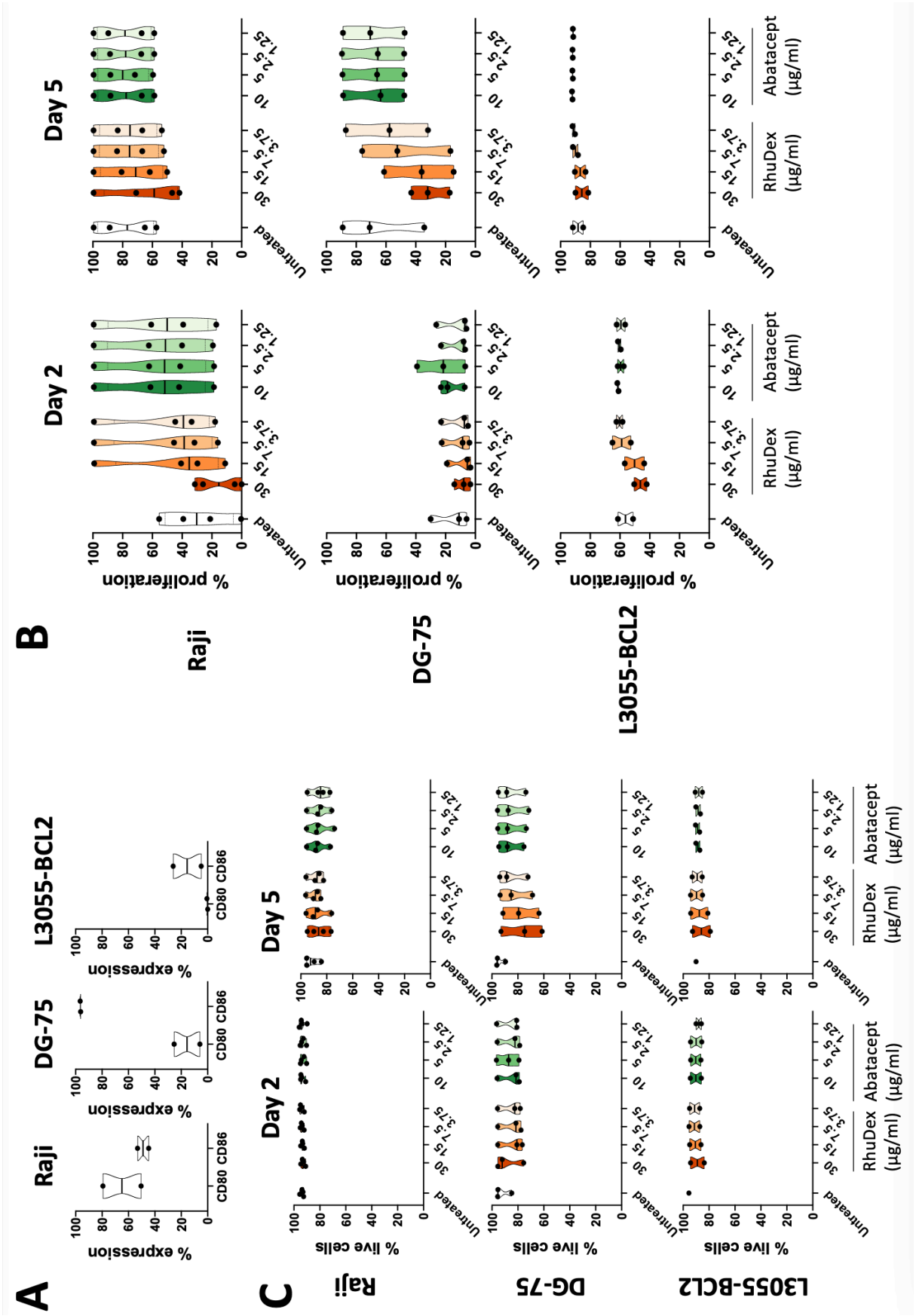

Figure 31: RhuDex does not inhibit proliferation of Raji, DG-75, L3055-BCL2 cells. (**A**) Percentage

of expression of CD80 and CD86 on the surface of the B cell lines Raji, DG-75, and L3055-BCL2 cell lines. N=2. (**B**) Percentage of proliferating cells for Raji, DG-75, and L3055-BCL2 at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=2-4. Statistical analysis performed were Friedman and Kruskal-Wallis tests. (**C**) Percentage of live Raji, DG-75, and L3055-BCL2 at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=2-4. Statistical analysis performed were one-way ANOVA with Dunn's mutliple comparisons. Results are shown as median (solid line) with interquartile range (dotted line).

The final cell line we studied for the effect of RhuDex was the human monocytic cell line THP-1. Only a small percentage of these cells expressed CD80 (0.415%, 0-0.8%) and a relatively small number of cells expressed CD86 on their surface (20.31%, 13.64-26.98%) (**Fig. 32 A**). When we cultured this cell line in the presence of different doses of RhuDex or abatacept we found that neither molecule inhibited proliferation of these cells both at day 2 and day 5 (**Fig. 32 B**). Finally, neither RhuDex nor abatacept promoted cell death at any concentration (**Fig. 32 C**). To conclude, RhuDex inhibited proliferation of the cell lines we tests at various degrees suggesting that surface expression of CD80 does not correlate with RhuDex-induced inhibition.

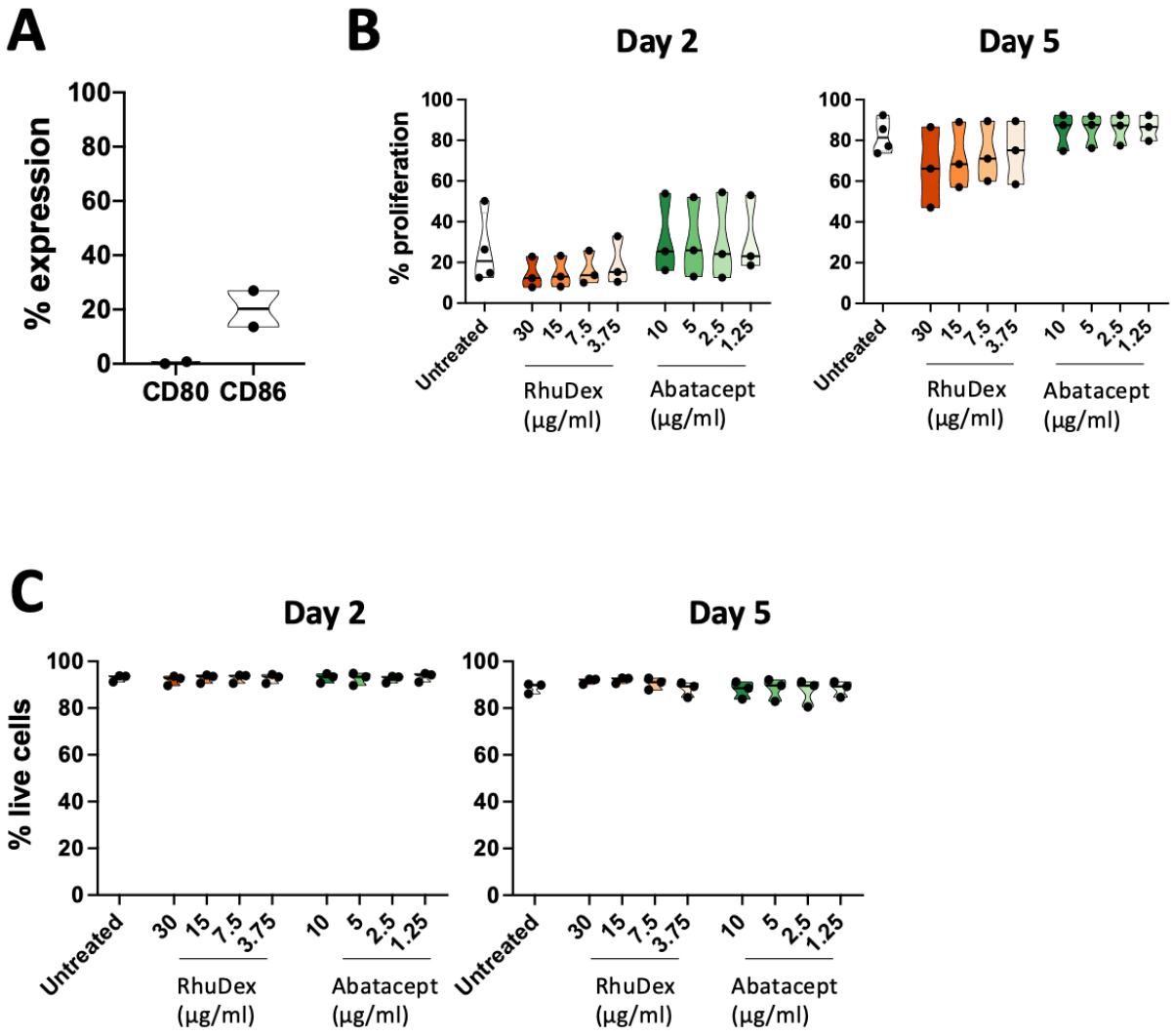

Figure 32: RhuDex does not inhibit proliferation of the monocytic cell line THP-1. (**A**) Percentage of expression of CD80 and CD86 on the surface of THP-1 cell line. N=2. (**B**) Percentage of proliferating THP-1 cells at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=3-4. (**C**) Percentage of live THP-1 cells at day 2 and day 5 in the presence or absence of RhuDex (30, 15, 7.5, 3.75 ug/ml) or abatacept (10, 5, 2.5, 1.25 ug/ml). N=3. Statistical analysis is one-way ANOVA with Dunn's mutliple comparisons.Results are shown as median (solid line) with interquartile range (dotted line).

5.9. Discussion

RhuDex is hypothesised to bind to CD80 with high affinity (535–538). The interaction blocks the CD28-CD80 pathway and could potentially prevent the development or progression of autoimmune diseases. RhuDex could provide an additional treatment option for patients suffering from PBC and PSC. However, no evidence exists regarding the role of RhuDex in the liver of these patients. In this chapter we sought to understand the mode of action of RhuDex in the context of the CD28-CD80 pathway and the effects of the drug on the liver-infiltrating immune cells.

We initially focused on the frequency of B cells and monocytes expressing CD80 and CD86 to assess their ability to offer co-stimulatory molecules to the T cells in the co-culture. The expression levels of CD80 on blood B cells were similar to those we found on chapter 4, however, the percentage of cells expressing CD80 was lower in these experiments (**Fig. 22 B**). CD86 data agree with the results we found on chapter 4 for both the expression levels on B cells and the percentage of cells expressing the marker (**Fig. 22 B**). For B cells isolated from the liver, the percentage of cells expressing the CD80 was lower compared to the results of chapter 4, however the expression levels were similar (**Fig. 25 B**). We also noticed that the percentage of liver B cells expressing CD86 had a broader range compared to the results in chapter 4 (**Fig. 25 B**). The expression levels of CD86 on liver B cells were the same in both chapters (**Fig. 25 B**).

The variation in the percentage of blood and liver monocytes expressing CD80 and CD86 was very broad compared to the results we found on chapter 4 (**Fig. 22 C, 25 C**). This is because when we phenotyped these cells for this chapter, we gated on the total $CD14^+$ cells and did not distinguish between the different subsets since we used total monocytes in the autologous MLRs. The expression levels of blood-

derived monocytes agree with our data from chapter 4 (**Fig. 22 C**). The liver monocyte data also correlate with our data from chapter 4 in both their expression levels, as well as the percentage of CD80 and CD86 expressed on their surface (**Fig. 25 C**).

We next used the autologous MLR model we used in chapter 3 to assess on the ability of T cells to proliferate in the presence of APC. Both blood monocytes and B cells were able to induce T cell proliferation (**Fig. 22 B**). $CD8^+$ T cells had a slightly higher proliferative capacity than the $CD4^+$ T cells, whether they were co-cultured with B cells or monocytes (n=1)(**Fig. 22 B**). Indeed evidence in the literature shows that $CD4^+$ T cells are programmed to restrict their ability to proliferate, in contrast to $CD8^+$ T cells (774, 775). Furthermore, the metabolic programming between $CD4^+$ and $CD8^+$ T cells is completely different. Activated $CD8^+$ T cells use the glycolytic pathway in a higher degree compared to activated $CD4^+$ T cells, which are more oxidative (775). During T cell activation, T cell metabolic programming switches from oxidative phosphorylation to glycolysis (776). The difference in the pathways used by the two T cell subsets suggests that the ability of $CD8^+$ T cells to proliferate is greater compared to $CD4^+$ T cells. Furthermore, activated $CD8^+$ T cells can also proliferate in the presence of low doses of IL-2 quicker than $CD4^+$ T cells, with naive subsets remaining unresponsive to low IL-2 (777). This suggests that the proliferation we are seeing in the co-culture is not from naive but from matured T cells, since no IL-2 was added in our co-cultures. When we used liver-infiltrating $CD8^+$ and $CD4^+$ T cells, we saw a different picture. Both T cell subsets co-cultured with B cells had similar levels of proliferation, whereas when occulted with monocytes, $CD4^+$ T cells proliferated stronger than $CD8^+$ T cells (n=1) (**Fig. 25 A**). $CD4^+$ T cells co-cultured with hepatocytes have been shown to proliferate stronger than $CD8^+$ T cells, however the proliferation was minimal (778). It is worth noting that in this study the T cells

were isolated from blood and not the liver. Another point of consideration is the strength at which liver-infiltrating monocytes could induce proliferation of T cells. More than 90% of $CD4^+$ T cells proliferated whereas $CD8^+$ T cell proliferation was above 75%, with a minimal difference between the different T:APC ratio.

Blood $CD8^+$ T cells proliferated strongly compared to T cells cultured alone, which agrees with current literature (**Fig. 22 E**) (714–716) We found similar results when we used liver T and B cells instead of blood-derived cells (**Fig. 25 D**). When we used blood- or liver-isolated monocytes, proliferation increased for both $CD4^+$ and $CD8^+$ T cells at a striking level of 70% compared to T cells cultured alone (**Fig. 22 F, 25 E**). One reason for the strong T cell co-stimulation could be due to potential cross-linking of the anti-CD3 antibody with the Fc receptor on the surface of the monocytes, CD16. It could also be hypothesised that monocytes are better co-stimulators that B cells, similarly to DCs being more potent T cell stimulators to B cells, but such evidence does not currently exist to our knowledge (640). Another possibility is that monocytes express a higher percentage of CD80 and CD86 compared to B cells. As of this time, there is no research that links percentage co-stimulatory molecule expression with levels of proliferation by T cells. Liver isolated T cells also proliferated strongly when they were co-cultured with autologous monocytes, however the increase in proliferation was less extensive, possibly due to an already increased activation state of the T cells when cultured alone (**Fig. 25 E**). T cells in the liver of PBC patients have been shown to be present at an activated state (779). Furthermore, we know that liver can activate or sequester already activated $CD8^+$ T cells (60, 780, 781). Finally, the patients could have been going or had gone through a viral infection.

In order to assess the ability of RhuDex to inhibit T cell responses we first tested the drug using cells from the blood of patients with HFE. This allowed us to test the

drug with cells which we know how they will react under physiological conditions, unlike liver-isolated cells. Furthermore, we compared the effects of RhuDex on blood-isolated T cell stimulation with that of abatacept, anti-CD80, and anti-CD86 antibodies. RhuDex-treated $CD4^+$ and $CD8^+$ T cells in co-culture with B cells could not proliferate in a dose-dependant manner (**Fig. 23 A**). This is in line with current literature showing RhuDex having inhibitory effects on T cell proliferation (536, 537). The effect of RhuDex was present despite the low percentage of B cells expressing CD80 on their surface. Using a wide range of abatacept concentrations, we found that abatacept did not induce strong inhibition in the co-culture, which has also been reported previously (**Fig. 23 A**) (537). However, this contradicts several other studies as well as the results we saw in chapter 3 when using the transfected CHO cells (183, 543, 575). These studies show that abatacept was effective when used with either transfected CHO cells or primary cells. One reason for the difference in results could be due to a higher expression of other co-stimulatory molecules expressed by the B cells which could promote T cell proliferation such as ICOS or OX40, however this cannot fully justify the poor inhibition in our experiments (782–784). Furthermore, the antibodies anti-CD80 and anti-CD86 showed no effect in inhibiting T cell responses, possibly due to the low percentage of cells expressing the markers (**Fig. 23 A**). Additionally, absence of effect for abatacept and the inhibitors anti-CD80 and anti-CD86 could be due to the very low T cell proliferating responses in our co-cultures in the presence of B cells.

We found similar results in the liver. RhuDex strongly inhibited the T cell responses in both subsets when co-cultured with B cells in a dose-dependant manner (**Fig. 26 A**). This is in contrast to the James et al. study which showed that liver cells have a diminished ability to promote stimulation to autologous liver T cells and showed that cells isolated from PBC patients have a deficiency in autologous MLR

(785). However, the same cells could induce strong T cell proliferative responses in the co-culture. Our result was surprising since we were expecting T cells to proliferate in the presence of CD80- and CD86-expressing B cells. Since RhuDex can only bind to CD80 but not CD86, that means that RhuDex has an off-target effect, something which we also observed in chapter 3. Unpublished results also showed that RhuDex might had off-target effects (535). One of the studies suggested that RhuDex down-regulated CD80 from CD80-expressing cells in a dose- and time-dependent manner. However, this could be due to RhuDex binding to the CD80 ligand and competing with the anti-CD80 for the binding site. Another unpublished evidence showed that RhuDex bound antagonistically (mainly but not always) on nuclear hormone receptors such as FXR, RARα, RARβ, and RXRα among others (535). Furthermore, RhuDex inhibited the transcription of PPAR and NF-κB gene on the THP-1 cell line (535). In carotid artery plaques, RhuDex inhibited the NF-κB and AP-1 expression, as well as the ERK1 and ERK2 (538). These enzymes and transcription factors are heavily involved in the induction of pro-inflammatory signals from immune cells (786–788). We believe that based on the current evidence, RhuDex's off-target effects are not mediated through CD80 binding. Similarly to previous findings, abatacept did not to dampen the stimulatory responses when used with LIMCs (n=1) (**Fig. 26 A**). Unfortunately, because of the low number of cells isolated from the liver and retrieved post-FACS, we were unable to test the anti-CD80 and anti-CD86 antibodies. As expected, RhuDex did not promote T cell death in either the blood or the liver co-cultures (**Fig. 23 B, 26 B**). Indeed, Heninger et al. also did not find induction of apoptosis on RhuDex-treated T cells (537). Finally, RhuDex had no effect on the viability of B cells in the co-culture (**Fig. 23 C, 26 C**). Abatacept had also no effect on either cell, a finding which agrees with results from chapter 3 (**Fig. 23 C, 26 C**)(558).

Similar results were obtained after using monocytes in co-culture with T cells. The strong inhibition induced by RhuDex in co-cultures with CD80- and CD86-expressing monocytes points towards the direction of RhuDex having off-target effects (**Fig. 24 A, 27 A**). Using liver monocytes, the off-target effects become more evident since liver monocytes do not express CD80 on their surface but over 40% express CD86 (**Fig. 22 D, 25 C**). LPS-activated blood or lamina propria monocytes co-cultured with T cells have been previously shown to be unable to induce T cell proliferation in the presence of RhuDex (537). Another study however, showed evidence that monocytes are incapable of promoting autologous MLR from PBC patients (785). Many studies cited in this chapter regarding the efficacy of monocytes as good APCs should be treated cautionary because positive selection has been used to isolate the monocytes with the use of either CD14 or CD16 which could activate the cells. That is also a limitation in our study, since in order to isolate $CD14^+$ monocytes from the LIMCs, we had to use $CD14^+$ antibodies to target them. RhuDex did not promote T cell death in either blood or liver monocytic co-cultures (**Fig. 24 B, 27 B**).

Abatacept did not show an ability to inhibit T cell stimulation despite the presence of CD80- and CD86-expressing monocytes which agrees with prior observations but not with the chapter 3 results (**Fig. 24 B, 27 B**) (537). Similarly to what we saw before, abatacept did not induce cell death in the co-cultures using blood or liver cells (**Fig. 24 B, 27 B**). Interestingly, only a few monocytes from both blood and liver were alive at the day the experiments were stopped (**Fig. 24 C, 27 C**). Under controlled conditions, monocytes can survive in culture for up to 14 days (789). No monocyte stimulant was added in our cultures to promote their survival in order to avoid potential phenotypic differentiation. Other reports suggest that unstimulated PBMCs in culture do not proliferate and undergo apoptosis, however PBMCs were not

separated into different cell types and as a result, stimuli from other leukocytes might have affected the survival of the cells (790–792). Different surfaces have been shown to affect the monocytic cell adhesion and the secretion of cytokines and chemokines (793). Fibronectin-coated plates and fetal bovine serum (FBS)-supplemented serum had no effect on the viability of monocytes. Although abatacept decreased the viability of monocytes in culture by attaching to the Fc receptor of monocytes, we saw no difference in the viability of monocytes among the different treatments (794). Another possibility for the low viability is because monocytes did not detach from the plate.

We also looked at the ability of different liver-infiltrating B cell subsets to induce T cell co-stimulation and RhuDex's ability to inhibit T cell proliferation in that system. We primarily focused on $CD24^{-}CD38^{-}$ and $CD24^{-}CD38^{int}$ B cells since they expressed co-stimulatory molecules and were enriched in the liver compared to blood (101). We used memory B cells as comparators because we expected memory B cells to be good T cell co-stimulators. Looking at the different B cell subsets, we found that the percentage of CD80 and CD86 on the $CD24^{-}CD38^{int}$ B cells was increased compared to the naive and $CD24^{-}CD38^{-}$ B cells (**Fig. 28 B**). This result agrees with the phenotypic characterisation completed by a previous member of our team, where it was shown that CD80-expressing $CD24^{-}CD38^{int}$ B cells were present in the liver (101). This result also agrees with the phenotypic characterisation conducted in chapter 4. Unfortunately, due to the novelty of these B cells, there is no published study to further validate our results. When these cells were used in AMLR co-cultures, memory B cells were unable to induce $CD8^{+}$ and $CD4^{+}$ T cell co-stimulation, possibly due to the low percentage of cells expressing CD80 and CD86 (**Fig. 28 C**). In the literature studies have shown that memory B cells could promote T cell proliferation whilst also expressing CD80 and CD86 on their surface (599, 600). As expected,

RhuDex and abatacept had no effect on the three B cell subsets (**Fig. 28 D, E, F**). It is worth noting that the number of cells we were able to collect per experiment was limited. As a result, only a small number of $CD24^{-}CD38^{-}$ and $CD24^{-}CD38^{int}$ B cells were retrieved after liver isolation and FACS. Distributing the cells in wells for the different conditions left us with less than 10,000 B cells co-cultured with T cells. As stated before, a reduction in the T cell:APC ratio results in poorer T cell activation, an event which could have taken place here despite the expression of CD80 and CD86 on $CD24^{-}CD38^{int}$.

The ability of RhuDex to inhibit T cell proliferation in the absence of CD80 in the co-culture led us to question the necessity of CD80 in RhuDex's mode of action. For that reason we cultured T cells receiving strong signal 1 and signal 2 in the form of antibodies and measured RhuDex's ability to inhibit T cell proliferation. RhuDex-treated T cells could not proliferate even when stimulated with anti-CD3 and anti-CD28, when using either cells isolated from the blood or the liver (**Fig. 29 A, C**). The effect of RhuDex was not due to induction of T cell death (**Fig. 29 B**). RhuDex's effect could be exerted through the expression of CD80 on the surface of T cells. CD80 and CD86 expression has been previously detected on the surface of mouse and human peripheral T cells (237, 795, 796). Furthermore, expression of CD80 on T cells was found to be present in RA and SLE (797–799). However, the presence of CD80 on the surface of mouse and human T cells was a result of CD80 acquisition from CD80-expressing APCs (800, 801). In fact, these CD80-bearing T cells had the ability to stimulate other T cells through the ligand. Furthermore, CD80-expressing $CD8^{+}$ T cells were more prone to apoptosis than $CD80^{-}$ T cells because of the lack of interaction between PD-L1 and CD80 (802). CD86 expression was induced on T cell surface after IL-2 stimulation in the absence of bystander APC (803, 804). CTLA-4 could inhibit IL-2 secretion by $CD4^{+}$ T cells which suggests that CD86 expression on

the T cells is regulated (223, 805). In the liver of patients with hepatitis C, $CD8^+$ T cells expressed CD86 after IL-2 stimulation (806). The absence of natively expressed CD80 suggests that RhuDex might not interact with the T cells through the possible CD80 found on the surface. However, it could indirectly interact with the NF-κB or PPAR transcription factors as described before (535, 538). Finally, Jurkat cells had reduced proliferation only when treated with RhuDex and not abatacept when co-cultured for 5 days (n=1) (**Fig. 29 D**). Heninger et al. did not detect inhibition of proliferation in Jurkat cells after 20 hours of treatment with RhuDex or abatacept (537). Heninger et al. as well as our lab also did not detect apoptosis induced by RhuDex or abatacept in Jurkat cells (**Fig. 29 D**)(537).

The effects of RhuDex on the T cells led us to wonder if the drug has similar off-target effects on the APCs as well. As a starting point, we used the transfected CHO cells because they allow us to study the APCs in an environment where the surface CD80 expression has no role on their function. As expected, we did not see a significant decrease in the proliferation of CHO, CHO-CD80, or CHO-CD86 when treated with either RhuDex or abatacept for 2 or 5 days, in spite of the high percentage of CD80-expressing CHO cells (**Fig. 30 B**). We then focused on the B cell lines Raji, DG-75, and L3055-BCL2. All three cell lines are isolated from patients with Burkitt's lymphoma. A high percentage of Raji cells express CD80 and CD86 (**Fig. 31 A**)(807–810). Almost all of the DG-75 cells expressed CD86 but very few expressed CD80 (**Fig. 31 A**)(811). Finally, the cell line L3055-BCL2, a transfected B cell line which over expresses the protein BCL-2, contained a small population of cells expressing CD86 and almost no CD80 (**Fig. 31 A**)(812). We did not detect a difference in the proliferation of Raji co-cultured in the presence of either RhuDex or abatacept at day 2 or day 5 (**Fig. 31 B**). The median percentage of proliferating DG-75 decreased when we treated them with RhuDex but not abatacept after 5 days however more repeats

are necessary (n=3)(**Fig. 31 B**). Finally, the drugs did not inhibit the proliferation of the cell line L3055-BCL2 at day 5 but we did note a small drop at day 2, however more repeats are necessary (n=2)(**Fig. 31 B**). As described before, neither of the drugs showed any effect on the viability of the three cell lines, however, a small drop on the viable DG-75 was present (n=2)(**Fig. 31 C**).

Finally, we used the monocytic cell line THP-1 to look at the effects of RhuDex on monocytes. This cell line was derived from a patient with acute monocytic leukaemia (813). There were no cells which expressed CD80 and only a few of them expressed CD86 (**Fig. 32 A**). Indeed, in the literature THP-1 express low levels of CD80 and CD86 (814–818). Treatment with RhuDex did not inhibit their proliferation at day 2 or at day 5, similar to abatacept (**Fig. 32 B**). Finally, neither of those drugs affected the viability of the cells at either day 2 or day 5 (**Fig. 31 C**). These results indicate that THP-1 proliferation levels cannot be decreased by RhuDex despite prior evidence of RhuDex acting through a non-CD80 mechanism.

Chapter 6 - Discussion

6.1. General discussion

Autoimmune liver diseases are characterised by elevated immune infiltration and cellular destruction of the liver due to the active inflammation taking place in the liver. Currently there are limited treatment options and their efficacy is poor. Liver transplant is not only a risky and expensive procedure, but the waiting time on transplant list can be unpleasant for the patients who might be waiting for more than a year for a new liver (819). In PBC, increasing evidence shows that the immune system in actively involved in the process of liver damage, with loss of tolerance to self antigens and mounting of immune responses against the self being the primary drivers of the disease. It is evident that new therapies must be discovered which will allow for targeting and disengaging of the immune responses building up in the liver of patients. In the last 10 years, checkpoint inhibitors have gained traction as a cure for cancer. They include antibodies which block pathways which could activate or inactivate the immune system such as the anti-CTLA-4 antibody ipillimumab, the anti-PD-1 antibodies nivolumab and pembrolizumab, or the more recent anti-PD-L1 antibodies nivolumab, pembrolizumab, and avelumab (820). We proposed that checkpoint inhibition could also provide benefits for patients with autoimmune liver diseases by targeting the immune system for inactivation rather than activation, such as the CD28-CD80 pathway. For that reason, we used the novel drug RhuDex, a CD80 inhibitor, to measure its potency in inhibiting immune responses *in vitro* from patients with autoimmune liver diseases.

Initially, we sought to establish that RhuDex can indeed inhibit the CD28-CD80 pathway by binding to CD80, rendering T cells inactive. To achieve that we used a transgenic model of CHO cells which expressed either CD80 or CD86, and co-cultured them with primary T cells. Our results showed that RhuDex was able to

inhibit T cell co-stimulation in a dose-dependant manner in both CD80- and CD86-expressing CHO cell system. However, results from Dr. Falk Pharma indicated that RhuDex was unable to bind to CD86 (535). This was interesting as it was a first hint of RhuDex having an off-target effect.

We also used other inhibitors for comparison to RhuDex such as abatacept, and the commercially available antibodies anti-CD80 and anti-CD86. Anti-CD80 or anti-CD86 antibodies showed complete inhibition of co-stimulation in the cells which expressed either CD80 or CD86 respectively. Abatacept however, showed variation in its inhibitory ability which we didn't expect. Although abatacept completely inhibited T cell co-stimulation in CHO-CD80 cells, similar results were not obtained from $CD8^+$ T cell co-cultured with CHO-CD86. Further analysis showed that abatacept is more potent at inhibiting CD80 than CD86 signals, which is independant of the T cell subset. Indeed, the binding affinity of CTLA-4 to CD80 is much greater compared to CD86, even though the association and dissociation rates for CD86 are much higher (183, 194, 195).

Knowing that RhuDex can inhibit CD80 signals, we then sought to establish the levels of CD80 in T cells, B cells, monocytes, and DCs in the blood and the liver of healthy controls and patients with end-stage liver diseases. Total B cells express high levels of CD80 but not CD86 in the blood in contrast to previous findings (583, 587). Expression of the ligands on different B cell subsets revealed that CD80 expression is mainly a feature of PC/PBs and Bregs, whereas CD86 was favoured only by PC/PB. Expression of CD80 and CD86 on these cells is associated with an activated phenotype and prolonged survival (598, 673, 674).

Despite the variations in the expression levels between the subsets, no differences were found across the different diseases we studied. This suggests that the CD80 and CD86 expression is not dysregulated in the end-stage chronic liver diseases we

studied. We cannot make assumptions regarding association between the risk loci of *CD80* and the expression levels of CD80 because we do not have genetic data from those patients. This type of experiment would require genotyping a large number of patients to avoid underpowered results, taking a significant amount of time to complete. For this reason we chose not follow this question.

Monocytes also showed variable expression levels of CD80 and CD86 depending on the subset and the disease. The overall expression of CD80 and CD86 on all blood monocyte subsets remained the same across diseases. The percentage of liver isolated CD80- and CD86-expressing monocytes was similar across all diseases, however, PSC monocytes had elevated levels of both markers in the classical and intermediate compartments. We cannot explain this result, since in end-stage liver disease, the liver microenvironment is similar for all diseases, especially between PSC and PBC. A recent study has shown that shown that CD16-expressing monocytes were preferentially recruited to the livers of PSC patients compared to other liver diseases including PBC and ALD (821). The authors hypothesise that these monocytes may differentiate into $CD68^{+}CD206^{+}$ macrophages, which were shown to also be increased in PSC. These results indicate that monocytes probably play a unique role in PSC which is yet to be established.

We next phenotypically characterised the liver-infiltrating DCs which showed elevated levels of CD80-expressing pDCs whereas their expression for CD86 was almost non-existant for both blood and liver. pDCs have been described as cells able to express co-stimulatory molecules, however they are not as efficient APCs as cDCs (822). pDCs are involved in viral immunity by secretion of type I interferon (IFN-α) and recognition of viral DNA through TLR-7 or TLR-9 sensing pathway (823, 824). The patients we studied could have been undergoing a viral infection at the time of the liver transplant. One hypothesis states that PBC could result from viral infection

with the betaretrovirus (825). Retroviral antibodies were detected in PBC patients, and the virus has the ability to stimulate biliary cells to express PDC-E2 on their surface (826, 827). Such explanation is inefficient for our work since not all of our samples were from PBC patients. Due to low number of patients, we were unable to stratify the expression of CD80 and CD86 based on diseases. Whether CD80 expression on pDCs or other DC subsets is different between diseases remains to be established.

We next focused our attention on the ability of liver-infiltrating cells to induce co-stimulation on autologous T cells and evaluating the effect of RhuDex in those systems. Because DC are present in low frequencies in humans, we concentrated our efforts on B cells and monocytes due to the large number of cells needed to complete the experiments (828). Our results showed that it is possible to co-stimulate LIMC *in vitro* using total B cells and monocytes. However, three different B cell subsets were unable to induce T cell proliferation. Interestingly, memory and $CD24^{-}CD38^{-}$ B cells did not show expression for either CD80 or CD86 and as a result, we did not expect to induce T cell proliferation. However, $CD24^{-}CD38^{int}$ B cells did show expression for both CD80 and CD86 but proliferation was still not detected. We are unable to explain the reason behind this since in chapter 4 we showed that these cells express adequate levels of HLA-DR. One possible reason is that the B cells underwent apoptosis shortly after the sort. Alternatively, the reason could the small numbers of the cells used in our experiments. Upon sorting, we only retrieved a small number of cells back (<10,000 cells in total). The small number of cells retrieved could have prevented them from being in close proximity and in result strong co-stimulation might have not taken place.

RhuDex was able to inhibit T cell proliferation either in the presence of total B cells or monocytes. Abatacept, anti-CD80, and anti-CD86 were unable to provide co-

stimulatory blockade. A possible reason for this is the presence of other co-stimulatory molecules on the surface T cells such as ICOS. ICOS is able to induce T cell proliferation similarly to CD28 (829). However, expression of ICOS on T cells is dependant on TCR engagement or CD28 stimulation (830, 831). It is worth considering that we sorted for total T cells and not only naive, which implies that some of these cells would be activated T cells and as a result ICOS might be present on their surface.

Still captivated by the off-target effect of RhuDex, we chose to investigate this event even further. Our results indicate that RhuDex can inhibit T cell proliferation in a non-CD80 dependant way. This does not limit the ability of RhuDex to bind to CD80. At this current stage we are unable to establish the level of inhibition derived by the CD80-binding and by the off-target of RhuDex. No inhibition of proliferation was detected on APCs regardless of their expression of CD80 on their surface. Some inhibition was achieved at different levels in all cell lines, however none was statistically significant, possibly due to low sample numbers. As shown before, inhibition of proliferation was not a result of cell death. RhuDex had off-target effects through its interaction with nuclear hormone receptors (535). From these results we can conclude that RhuDex is a potent proliferation inhibitor.

6.2. Future work

Throughout this work we focused on the ability of RhuDex to inhibit T cell co-stimulate through a CD80-dependent and independent way. This work could be further expanded by investigating the precise mechanism which allows RhuDex to inhibit T cell and APC proliferation. Since we know that RhuDex does not promote cell death, it is likely that the drug induces cellular senescence in the cells. Cellular

senescence is a phenomenon during which a cell does not undergo cell division. RhuDex treated and untreated liver tissue could be stained for p21 and p16 to measure for senescence using IHC (832). Elucidating the mode of action of RhuDex is very important since the pathway it uses to inhibit T cell and APC proliferation is one step ahead of its ability to block T cell co-stimulation. As such, the drug could then be tested in other diseases where immune cell proliferation is prominent such as lymphomas. It is also critical to test RhuDex's inhibitory ability in more cell types to study the full effect of RhuDex and assess potential adverse effects in humans. In pre-clinical studies on cynomolgus monkeys RhuDex had a favourable toxicological profile (535).

The effect of RhuDex on the cytokine profile of T cells is also worth studying. Heninger et al. has shown that RhuDex-treated T cells, especially $CD4^+$ T cells, inhibit the secretion of IL-17 and IFN-γ without affecting IL-2 expression (537). Anti-IL-17 monoclonal antibodies are currently being used successfully to treat psoriasis (833). Targeting Th17 responses with RhuDex is an exciting prospect and the drug could be further tested for the treatment of psoriasis. Furthermore, RhuDex was shown to upregulate the secretion of IL-4 in naive and memory T cells when co-cultured with monocytes (535). Understanding RhuDex's ability to modulate T cells towards an anti-inflammatory phenotype will be very desirable for the treatment chronic liver diseases if proven correct.

Future work could also expand on our CD80 and CD86 phenotyping. Cells from matched blood, lymph nodes, and liver from patients would allow to compare the expression of the ligands between systems. In addition, use of healthy discarded donor liver would provide a better control for our experiments. Although we tried to use non-transplantable healthy donor livers in our experiments as controls, the majority of them were high in fat content preventing us from isolating immune cells

from the tissue. Moreover, further work could concentrate on deeper phenotyping of the cells we studied and examine additional characteristics to better understand the state of the isolated cells, for example pro- or anti-inflammatory phenotype or chemokine marker expression. We were unable to analyse more markers due to the 9-colour limitation by our flow cytometer. New technologies are being developed which allow to analyse additional markers. For example, mass cytometry can analyse up to 40 different markers (834). Such results would increase our understanding of the expression patterns of CD80 and CD86 on the APCs we studied.

6.3. Conclusion

PBC is an immune-driven disease characterised by the loss of self-tolerance and aberrant immunoproliferation in the livers of patients. In this study we tested the novel drug RhuDex, a CD80 inhibitor, for its ability to block T cell co-stimulation and dampen the immune responses in the inflamed liver. We initially verified that RhuDex can inhibit T cell proliferation successfully *in vitro* under co-stimulatory conditions, similarly to other co-stimulation inhibitors, abatacept, anti-CD80, and anti-CD86. We also demonstrated that the expression of CD80 and CD86 is highly variable for B cells, monocytes, and DCs in the blood and liver of patients with end-stage liver disease. We further showed that there is no difference in the expression of CD80 and CD86 in PBC compared to other end-stage liver diseases. These findings allowed us to hypothesise which are RhuDex's possible targets in the liver. In a co-stimulation assays using autologous liver-infiltrating T cells with either B cells or monocytes we found that RhuDex was able to inhibit T cell responses by blocking T cell proliferation. We also found that RhuDex was able to inhibit T cell responses in the absence of CD80 co-stimulation or in the presence of strong CD28 co-stimulation.

Similarly, it also inhibited the proliferation of B cell and monocyte cell lines without the induction of cell death. This evidence clearly shows that RhuDex have immunomodulatory functions beyond the ones discovered previously. These findings combined indicate that although the CD80 expression shows no signs of dysregulation in PBC patients, RhuDex can still immunomodulate T cells and APC regardless of their CD80 expression, providing promising results in the management or treatment of PBC and possibly other chronic liver diseases.

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