Investigation of the role of monocytes/macrophages in allogeneic immune response

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Abstract:

Hematopoietic stem cell transplantation (HSCT) is a therapy for many diseases, however, development of graft versus host disease (GVHD) severely limits the successful use of HSCT. Pre-transplant conditioning including total body irradiation (TBI) causes tissue damage and cytokine production that activates antigen presenting cells (APCs). This change triggers monocyte differentiation to macrophages that present alloantigen to T cells during early GVHD. A better understanding of monocytes/macrophages activation during the early phase of alloresponse is needed. This study evaluated monocyte/macrophage function during the early phase alloresponse by measuring surface marker expression in whole blood mixed leukocytes reaction (MLR). To simulate GVHD inflammatory conditions, IFN-γ or IL-4 were added. In response to the allogeneic cells in MLR culture (day 1 and 2), monocytes/macrophages showed significant up-regulation of CD86, HLA-DR, CD64, C3aR and CD204 demonstrating strong monocyte/macrophages activation, accompanied by secretion of TNF-α, IFN-γ, IL-2 and IL-6 (day 2), and T cell activation (day 3). Unexpectedly, IFN-γ addition to MLR culture did not affect co-stimulatory potential or antigen presentation, it instead induced significant up-regulation of CD64 expression, suggesting stimulation of phagocytosis and pathogen defence. Whereas, IL-4 addition significantly up-regulated co-simulation and Ag presentation (day 2), reducing T cell alloreactivity on day 3. Spleen tyrosine kinase (SYK) phosphorylation regulates monocytes/macrophages activation, thus the SYK pathway is a potential therapeutic target for MLR and GVHD. Data in this study showed that the highly selective SYK inhibitor, PRT0603, modulated monocyte/macrophage activation in MLR culture by significantly reducing CD64 (FcγRI) expression, antigen presentation and T cell alloreactivity. In MLR IFN-γ and MLR IL-4 the anti-SYK effects on monocyte/macrophages function were modest. However, a subsequent decrease in T cells alloreactivity was observed in MLR IFN-γ culture due to PRT0603 treatment. More importantly, a significant reduction in cytokine production was observed in all treated cell cultures. This reduction could stop the inflammatory cycle and prevent the development of allogeneic response in GVHD.

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I am dedicating this thesis to the memory of my grandfather Suleiman Alkhuraiji, for being my first English teacher.

Author’s declaration:

I hereby declare that this thesis is my own original work, this work has not been submitted to any institution for basis of any degree or professional qualification before.

I have acknowledged all sources used and have cited these in the references.

Deema Sabir

Abbreviations:

Ab	Antibody
Ag	Antigen
aGVHD	Acute Graft versus host disease
AIRE	Autoimmune regulator
APCs	Antigen presenting cells
AKT	Protein kinase B
BA1	β-Arrestin 1
BCR	B cells receptor
BM	Human bone marrow
BMT	Bone marrow transplant
C3aR	Complement receptor 3a
CB	Cord Blood
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
cDC	Conventional dendritic cells
cGVHD	Chronic Graft versus host disease
CRP	C-Reactive Protein
CSF-1	Colony stimulating factor-1
CTLA-4	Cytotoxic T lymphocyte-associated protein-4
CXCR1	C-X-C motif chemokine receptor 1

D	Day
DAMP	Damage-associated molecules
DC	Dendritic Cells
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F4/801	EGF-like module-containing mucin-like hormone receptor-like
FceR1	High-affinity IgE receptor
FcγR	Fc gamma receptor
FcR	Fc receptor
FSC	Forward scatter light
Fas L	Fas ligand
G-CSF	Granulocyte stimulating factor
GM-CSF	Granulocyte monocyte colony stimulating factor
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
GVT	Graft vs. tumour
HBsAg	Hepatitis B infection
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HLA-DR	Human leukocyte antigen – DR isotype

HSCT	Haematopoietic stem cell transplantation
Hsp70	Heat shock protein 70
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon γ
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-4R	Membrane IL-4 receptor
IL-6R	Membrane IL-6 receptor
iNOS	Inducible nitric oxide synthase
IRF-1	Interferon regulatory factor-1
ITAM	Immunoreceptor-activating motif
JAK	Janus kinase
JNK2	Jun N-terminal protein kinase 2
LDL	Low-density lipoprotein
LIMP-2	Lysosomal integral membrane protein–2
LOX	Lectin-like oxidized low-density lipoprotein receptor
LPS	Lipopolysaccharide
Lyn	Lck/Yes novel tyrosine kinase

LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK	Mitogen-activated protein kinases
MARCO	Macrophage receptor with collagenous structure
M-CSF	Monocyte colony stimulating factor
MDSC	Myeloid derived suppressor cells
MerTK	MER Proto-Oncogene, Tyrosine Kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility
miHA	Minor histocompatibility antigen
MLR	Mixed leukocyte reaction
moDCs	Monocyte-derived dendritic cells
MSC	Mesenchymal stromal cell
mTEC	Medullary thymic epithelial
NF-κB	Nuclear factor κB
NK	Natural killer cells
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen associated molecular patterns
PAMPS	Pathogen molecular pattern
PBMCs	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PD-L1	Program cell death ligand 1
PGJ2	Prostaglandin J2
PPARγ	Peroxisome proliferator–activated receptor γ
PRRs	Pattern-recognition receptors
PS	Phosphatidy serine
RBCs	Red blood cells
ROI	Reactive oxygen intermediates
RT	Room temperature
sIL-6R	Soluble IL-6 receptor
SIRPA	Signal regulatory protein alpha
SR-A	Scavenger receptor
SRB-1	Scavenger receptor B-1
SSC	Side scatter light
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
T reg	Regulatory T cells
TAP	Transporter associated with antigen processing
TBI	Total body irradiation
Tc	cytotoxic T cells

TCR	T cell receptor
TGFB	Transforming growth factor beta
Th	T helper
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
TLRs	Toll-like receptor
TNF-α	Tumour necrosis factor-α
WBC	White Blood Cells

1 Introduction:

1.1 Haematopoietic stem cell transplantation:

One of the key life-saving therapies for malignant and haematological diseases is allogeneic hematopoietic stem cell transplantation (HSCT) (Shlomchik et al., 1999, Markey et al., 2014, Blazar et al., 2013, Koehn et al., 2015, Hill et al., 2018). Essentially, hematopoietic stem cells (HSC) are collected from a health donor and transplanted into the host, to allow them to produce healthy blood cells (Welniak et al., 2007, Aveni et al., 2015). There are three different sources of HSCs, bone marrow (BM), cord blood (CB) and harvesting cells from the peripheral blood (Smith & Wagner, 2009). The latter is feasible after administration of granulocyte stimulating factor (G-CSF) to the donor, which induces mobilisation of HSCs from BM into the blood stream (Welniak et al., 2007, Aveni et al., 2015). One of the main limitations of using HSCT as a therapy is that there is a high risk of developing graft versus host disease (GVHD), which causes high mortality rates in patients after their transplant (Ferrar et al., 1991, Welniak et al., 2007, Markey et al., 2014, Blazar et al., 2013, Koehn et al., 2015, Hiller et al., 2018, Nguyen et al., 2018, Drobyski et al., 2018). In GVHD, the immune cells from the graft target the host patient's tissue (Ferrar et al., 1991). In order to decrease the incidence of GVHD and to maintain graft survival, the patients should undertake chemotherapy and irradiation before transplantation, which is known as a conditioning regimen, and after the HCST they should take immunosuppressive drugs for an extended period of time to control the immune response (Ferrar et al., 1991, Welniak et al., 2007, Hiller et al., 2018, Ganetsky et al., 2019).

1.2 Graft-versus-host-disease:

Although Mathe and his colleagues (1963) succeeded in performing the first BM transplantation, the patient developed a chronic immune reaction, which was characterised by weight loss and cutaneous inflammation (Mathe et al., 1963). Subsequently, Billingham (1966) discovered that there are three elements necessary for the development of the detrimental immune response after transplantation known as GVHD. Firstly, the graft contains immune cells, the host is immune-compromised, so they will not reject the transplant, and the lastly the donor and host tissue express different human leukocyte antigens (HLA), also known as major histocompatibility complex (MHC) molecules (Billingham et al.,

1966, Atkinson et al., 1990). The HSCT patients preparation protocol involves a conditioning regimen, that removes the patient's stem cells and maintains space for the donor graft cells for engraftment (Blazar et al., 2013). However, this causes severe inflammation that results in T cells-interaction with antigen presenting cells (APCs) through MHC molecules to generate a cytokine storm that results in GVHD (Ferrar et al., 1991, Blazar et al., 2013). Interestingly, patients who received matched MHC HSCT grafts can also develop GVHD, which highlights the importance of there being no differences between the host and donor minor histocompatibility (miHA) antigens, as multiple miHA antigens may stimulate a strong immune response, leading to development of GVHD (Ferrar et al., 1991, Shlomchik et al., 1999 , Shlomchik, 2007). GVHD is an allo-immune disease which develops after HSCT; donor T cells recognise mismatched polymorphic human leukocyte antigen complexes and/or miHA antigens on both donor or host APCs (Shlomchik et al., 1999, Shlomchik, 2007). Based on the time of the disease onset after transplantation, GVHD can be classified as either acute or chronic, if the symptoms appear within 100 days after HSCT, it is considered as acute GVHD (aGVHD), and the typical symptoms include skin rash and gastrointestinal disease (Ferrar et al., 1991, Shlomchik, 2007, Blazar et al., 2013, Zhang et al., 2017). However, in chronic GVHD (cGVHD), the disease onset can appear within a year after HSCT, and the immune cells will target all body tissues (Shlomchik, 2007, Blazar et al., 2013). It is widely accepted that donor T cells, host and donor APCs play a critical role in developing GVHD, however, other studies have suggested that T cells are also essential in fighting against leukaemia, which known as the graft versus leukaemia (GVL) effect, which suggests the immune response is complicated and needs further study (Dazzi and Goldman, 1999, Nguyen et al., 2015).

aGVHD is a lethal complication after HSCT and is the most common cause of mortality and morbidity post-transplant (Ferrar et al., 1991, Blazar et al., 2013, Zhang et al., 2017). Patients who survive in the first 100 days are then at a high risk of developed cGVHD (Ferrar et al., 1991). This highlights the need to better understand the complicated mechanism of this disease to maintain effective GVL, prevent GVHD, and to have a strong therapeutic effect of allogeneic HSCT (Lee et al.,2005, Nguyen et al., 2018, Ganetsky et al., 2019).

1.3 Human monocytes:

In 1960 van Furth and Cohn described monocytes as circulatory white blood cells (WBCs) and classified them as mononuclear cells (Furth and Cohn., 1960). Monocytes are mainly generated in the BM from myeloid precursor cells and represent around 4 % of the total leukocyte cell count (Furth and Cohn., 1960, Tacke & Randolph, 2006, Castano et al., 2011, Lee et al., 2017). The half-life of monocytes is relatively short, about 3 days in human circulation (Tacke & Randolph, 2006). Monocytes can be regarded as precursor cells that give rise to macrophages, dendritic cells (DC) and osteoclast. Macrophages and DCs maintain body homeostasis and have critical role in the innate immune response (Tacke & Randolph, 2006, Sprangers et al., 2016). The monocytes in circulation are a heterogeneous, functionally diverse population of cells that can be divided into three sets in (Figure 1.1) based on expression of the cluster of differentiation 14 (CD14) a lipopolysaccharide receptor (LPS) and CD16 the IgG receptor (FcγRIII). The classical monocytes ($CD14^{++}CD16^{-}$), non-classical monocytes ($CD14^{+}CD16^{++}$) and intermediate monocytes ($CD14^{++}CD16^{+}$) (Belhareth and Meg 2015, Boyettel et al., 2016, Sprangers et al., 2016, Lee et al., 2017, Collin et al., 2018, Zhong et al., 2019). Monocytes are further classified based upon their adhesion molecules and chemokine receptors: each subpopulation has a particular function; the classical monocytes ($CD14^{++}CD16^{-}$) mainly count 90-95 % of monocytes count, express C-C chemokine receptor type 2 (CCR2), CD62L (L-selectin) and FcγRI (CD64) and are rapidly recruited to injured tissues during inflammation to phagocytose dead cells and remove microorganisms (Tacke & Randolph, 2006, Sprangers et al., 2016, Lee et al., 2017). Prolong response of classical monocytes result in tissue damage due to inflammation (Sprangers et al., 2016). The intermediate monocytes ($CD14^{++}CD16^{+}$) are 10 % of total monocytes which lack of CCR2, highly express MHC-II and FcγRII (CD32) and recruited in the later stages of inflammation to enhance antigen presentation, secretion of anti- inflammatory cytokines like IL-10 and transforming growth factor TGF-β to initiate wound healing and tissue repair (Tacke & Randolph, 2006, Sprangers et al., 2016, Lee et al., 2017). The non-classical monocytes ($CD14^{-}CD16^{++}$) lack of CCR2 make up 10 % of monocytes surveying the endothelium as part of the innate immune response (Tacke & Randolph, 2006). Classical, intermediate, and non-classical monocytes make important contributions to

innate immune response with different functions (Sprangers et al., 2016). As presented in (Table 1.1) the classical phenotype migrates to the site of infection to start antimicrobial response through phagocytosis (Sprangers et al., 2016). The intermediate monocytes maintain Ag presentation and regulate apoptosis. Whereas the nonclassical monocytes lose the expression of CD14, and mainly initiate wound healing and tissue repair (Sprangers et al., 2016). It has been reported that in GVHD patients the percentage of classical monocytes decreases, while the numbers of intermediate and non-classical monocytes increased (Heller et al., 2017). Interestingly, it has been reported that the presence of monocyte-derived cells in the recipient blood after organ transplantation will suppress immunological rejection and improve graft survival (Ochando et al., 2015). This tolerance could be induced by blocking of co-stimulatory molecules on monocytes, which prevents interaction with T cells and may cause defects in T cell proliferation (Ochando et al., 2015). Thus, although monocytes have a crucial role in inflammation and the initiation of the alloresponse, they could regulate inflammation and mediate tolerance (Wu et al., 2019). This study investigated the role of activated monocytes in an allogenic culture and evaluate the therapeutic strategy by targeting the spleen tyrosine kinase (SYK) pathway in monocytes to induce immune tolerance.

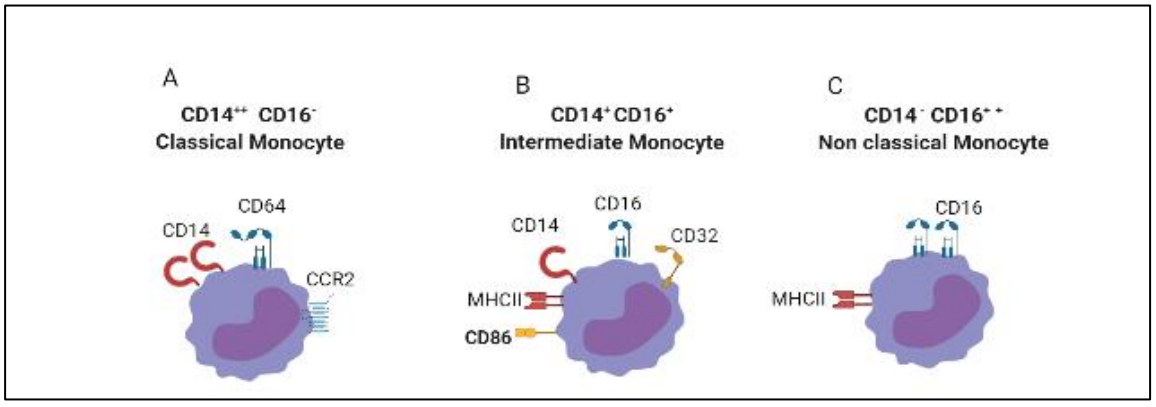

Figure 1. 1: Monocyte's classification based on CD14, CD16 and CCR2 expression.

A) classical monocytes that express $CD14^{++}$ $CD16^{-}$ $CCR2^{+}$ $CD64^{+}$ B) Intermediate monocytes express $CD14^{++}$ $CD16^{+}$ $CCR2^{-}$ $CD32^{+}$ $MHCII^{+}CD86^{+}$ and C) Non-classical -monocytes phenotype express $CD14^{dim}$ $CD16^{++}$ $CCR2^{-}$ $MHCII^{+}$. Created with BioRender.com

Table 1. 1: Human-monocyte subtypes and their function**:**

Monocytes subset	Markers	Chemokine receptors	Main function
Classical	$CD14^{++}CD16^{-}$	$CCR2^{high}$ $CX3CR1^{low}$	Antimicrobial response through phagocytosis
Intermediate	$CD14^{+}$ $CD16^{+}$	CCR2 ^{low} $CX3CR1^{high}$	Ag presentation Regulate apoptosis
Non-classical	$CD14^{-}$ $CD16^{++}$	CCR2 ^{low} $CX3CR1^{high}$	Wound healing Tissue repair

1.4 Macrophages:

Macrophages are myeloid cells, they are classified as mononuclear phagocytic cells and considered as professional APCs. However, they are unlike monocytes in some ways, as they are non-migratory cells that stay in the tissues and carry out their effector and phagocytic functions only in the surrounding area, meaning the activities of inflammatory macrophages are tissue specific (Shechter et al., 2009, Murray & Wynn., 2012, Varol et al., 2015). There are two origins of macrophages; the first type comprises the majority of the tissue macrophage populations, which develop prenatally and are known as embryonic derived macrophages (Belhareth, 2015, Varol et al., 2015, Sprangers et al., 2016). These cells are characterised as independent cells and different from the other hematopoietic cells; they are in charge of cleaning tissue debris which results from tissue development and remodelling (Shechter et al., 2009, Varol et al., 2015, Sprangers et al., 2016). The second type of macrophages originate from adult monocytes that infiltrate to inflamed tissues and are termed adult tissue macrophages or monocyte-derived macrophages (Varol et al., 2015, Sprangers et al., 2016). Phenotypical variations between macrophages from the two different origins have been reported: the tissue resident macrophages highly express CD64, MER Proto-Oncogene, C mer Tyrosine Kinase (MerTK), CD14 and EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80) with a long life span (Belhareth, 2015, Sprangers et al., 2016). In contrast, monocyte-derived macrophages show little expression of CD64, MerTK, CD14 and F4/80 and have a short life span (Belhareth, 2015, Tacke & Ranndolph, 2006). Macrophages play an essential role in inflammation by expressing chemokine receptors and secreting cytokines to activate neutrophils, monocytes and to recruit T cells (Varol et al., 2015). Macrophages are different from monocytes, as they are 5 to 10 times

bigger in size, with increased complexity and have a greater phagocytic capacity (Duque & Descoteaux, 2014). Macrophages are tissue specific cells, which mean that these cells can acquire organ-specific functions based on their location, which can help to maintain tissue homeostasis (Varol et al., 2015). The growth factor granulocyte monocyte colony stimulating factor (GM-CSF) and IL-34 are essential to regulate the development, differentiation, function, and proliferation of macrophages (Sprangers et al., 2016, Belhareth, 2015). The growth factors provided to the macrophages by the tissues they reside in, can enhance tissue-specific functions of the macrophages, meaning that the local environment can influence macrophages function (Sprangers et al., 2016). There are eight macrophage phenotypes based on the site of residence, for example, Kupffer cells are macrophages located in the liver, microglia are macrophages found in the brain tissue, alveolar macrophage-based in the alveolar of the lungs, osteoclast reside in the bone, peritoneal macrophage are found in the peritoneal cavity, mammary gland macrophages in the breast, and gut macrophages are located in the abdomen (Varol et al., 2015, Sprangers et al., 2016). Inflammatory macrophages are highly phagocytic cells that are capable of capturing the pathogen and presenting it to T cells; they can also release lytic factors and proteinases to the extracellular environment to help with the breakdown of invading pathogens (Martinez and Gordon., 2014). Studies have shown that transforming growth factor beta (TGFβ) production by inflammatory macrophages is involved in tissue fibrosis production in cGVHD (Alexander et al., 2014).

1.4.1 Macrophage polarisation:

Monocytes and macrophages are essential components of the innate immune response (Belhareth, 2015, Murray, 2017). In response to pathological infection many monocytes produced from the BM travel to the site of infection and these monocytes will proliferate to form monocyte-derived macrophages (Belhareth, 2015, Murray, 2017). Based on the type, number of cytokines present in the microenvironment, and the time of exposure, macrophages undergothe process of phenotypical polarisation into classical M1 and alternative M2 (Wang et al., 2014, Belhareth, 2015, Obrine et al., 2019). The two subsets have opposite functions (Figure 1.2), M1 mainly have a protection role from pathogens such as viruses, bacteria and have anti-tumour activity (Murray & Wynn, 2012, Belhareth, 2015).

In contrast, M2 maintain tissue repair and anti-inflammatory reaction by releasing IL-10 (Murray & Wynn, 2012, Belhareth, 2015). M1 macrophages are characterised by high antigen (Ag) presentation, increased production of IL-12, IL-23, nitric oxide (NO) and reactive oxygen intermediates (ROI) (Murray, 2017). On the other hand, M2 macrophages perform the protection function by upregulation of mannose receptors, scavenger receptors A, CCR2, C-X-C motif chemokine receptor 1 (CXCR1) and CXCR2, M2 also release ornithineand polyamines (Wang et al., 2014). Interestingly, M1 and M2 polarisation can bereversed during normal physiological and pathological conditions (Wang et al., 2014). It is thought that the classical monocytes give rise to M1 macrophages, whereas $CD16^{+}$ monocytes give rise to M2 macrophages (Shechter et al., 2009).

There are three elements on cellular level that control macrophage polarisation: the extrinsic pathway, intrinsic pathway, and tissue microenvironment (Obrine et al., 2019). *In vivo* extrinsic polarisation of M1 phenotype is stimulated by interferon γ (IFN-γ) released from the type 1 T helper cells (Th1) cells also ligands that bind to the toll-like receptors (TLR) at the surface of macrophages (Obrine et al., 2019). However, type 2 helper T (Th2) cells release IL4 and IL-13, which stimulates M2 polarisation which is presented in (Figure 1.2) (Obrine et al., 2019).

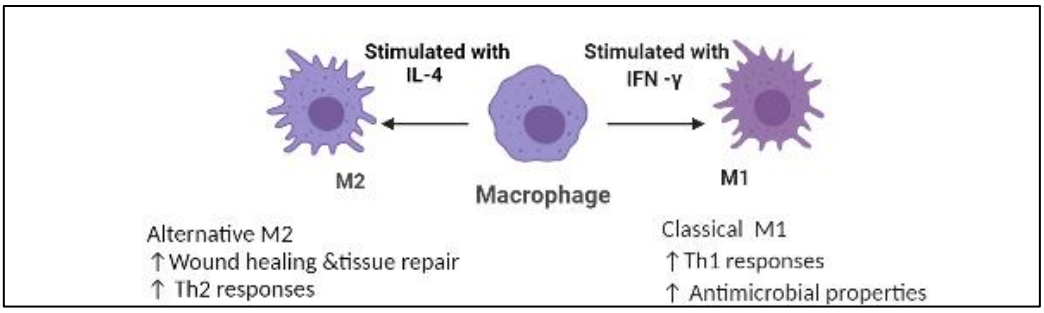

Figure 1. 2: Macrophage polarisation based on IL-4 and IFN-γ stimulus**:**

Polarisation of macrophages with IFN-γ results in the classical M1 subtype that mainly control antimicrobial response and activates the Th1 response. However polarising macrophages with IL-4 results in alternative activated M2 that controls tissue repair, The Th2 response and immunosuppression. Created with BioRender.com

During inflammation, the important factor that enhances monocytes differentiation to macrophages and their further polarization is the alteration of surface marker expression which make cells more sensitive and responsive to cytokines (Murray,

2017). In contrast, *in vitro* extrinsic polarization is maintained by culturing the macrophages with polarizing cytokines to generate a M1 or M2 phenotype to mimic what happens *in vivo*. Studies *in vitro* of monocytes cultured with GM-CSF and LPS, IFN-γ and tumour necrosis factor-α (TNF-α) have shown that they will acquired M1 morphology, however, if monocytes are cultured with monocyte colony stimulating factor (M-CSF), IL-4, IL-10, IL-13, and vitamin D, then the monocytes will give rise to the M2 macrophages (Martinez and Gordon, 2014, Obrine et al., 2019). In addition, hypoxia and lactate production within a tumour are non-cytokine extrinsic pathways that may control macrophage polarization (Murray, 2017). It has been reported that macrophages adapt to the surrounding environment; hypoxia which is one of the characteristics of the inflamed tissue will stimulate M1 activation by increasing gene expression of pro-inflammatory cytokines and enhanced anti-microbial or anti-tumour functions (Wang et al., 2014, Murray, 2017).The second pathway that controls macrophage polarization is known as the intrinsic pathway and is focused on the developmental origin of the macrophages (Murray, 2017). Macrophages originate from two sources and each cell type has a different role in the tissue they are found in, for example microglia originate in the embryo, they maintain tissue homeostasis, remodelling, and removal of dead cells, microglia mainly are located in the central nervous system (Belhareth, 2015). After birth most of the embryonic macrophages are replaced by monocyte-derived macrophages except for microglia cells, which are monocyte independent (Murray, 2017). This replacement occurs depending on time and the type of tissue microenvironment; in gut the transformation occurs within few days after birth, whereas alveolar macrophages need months to years for the full replacement to occur (Murray, 2017).

Interestingly, in a healthy person most tissues will have a mix of embryonic and monocyte-derived macrophages, however in a transplant patient all tissue resident macrophages are replaced by monocyte-derived macrophages due to the effect of irradiation or chemotherapy (Murray, 2017). As macrophage subtypes have opposite functions, the switching from one phenotype to another is controlled by strict pathway signals at the molecular level, such as the interferon regulatory factors (IRF) and signal transducer and activator of transcription (STAT) signalling pathway that controls M1 and M2 switching mainly by IFN-γ and by TLR signalling

(Wang et al., 2014). IFN-γ type 1 which activates STAT1 and enhances production of inducible nitric oxide synthase (iNOS) will skew macrophages towards M1 type (Obrine et al., 2019). However, stimulation of the IRF/STAT pathway with anti-inflammatory or Th2 cytokines, such as IL-4 and IL-13 via STAT6, will skew macrophages toward M2 phenotype (Murray, 2017, Obrine et al., 2019). Indeed, impaired M1 switching may result in chronic inflammatory (Obrine et al., 2019) and autoimmune disease (Murray & Wynn, 2012). Human macrophages and mouse macrophages are polarised by using different signalling pathways (Murray, 2017). This highlights the importance of developing *in vitro* models from human monocytes to assess the effects of blocking specific pathways in the GVHD response (Wang et al., 2014). The aim ofthis study was to optimise a human *in vitro* model of GVHD, using *in vitro* extrinsicpathway activation by (IFN-γ and IL-4), evaluate monocytes/macrophage function (co-stimulation, Ag presentation, activation, capturing apoptotic cells, adhesion, and differentiation) by measuring the expression of surface markers.

1.4.1.1 Pathogen recognition:

Viruses and bacterial products are the standard stimulators for monocytes and macrophages (Neefjes et al., 2011, Wu et al., 2019). Microorganisms display pathogen associated molecular patterns (PAMPs), which are recognised by pattern-recognition receptor (PRRs) present on myeloid cells and some epithelial cells (Neefjes et al., 2011, Wu et al., 2019). The cellular PRRs include CD14, mannose receptors, nucleotide-binding oligomerization domain (NOD)- like receptors, scavenger receptors and Toll like receptor (TLRs), which are all expressed by monocytes and macrophages (Jiménez-Dalmaroni et al., 2016). This recognition is the first line of defence against microorganisms, dead cells and is also essential for initiation of the immune response (Murray & Wynn, 2012, Duque & Descoteaux, 2014, Jiménez-Dalmaroni et al., 2016, Wu et al., 2019). CD14 is a human monocyte marker, known to be the first identified PRR (Wu et al., 2019). CD14 is a phosphoinositolglycan-linked cell surface receptor expressed on monocytes and macrophages that binds to LPS present on gram negative cell walls, which helps to activate the innate immune response (Lin et al., 2004, Neefjes et al., 2011, Wu et al., 2019). The TLR are type I glycoprotein, include 10 members; they are surface or cytoplasmic receptors which can recognise virus,

bacteria, or fungi components (Jiménez-Dalmaroni et al., 2016). TLR1, 2, 4, 5, 6 and 10 are present on the cell surface and recognise PAMPs, whereas TLR 3, 7, 8 and 9 are found intracellularly and maintain detection of the presence of foreign RNA and DNA within the infected cell (Jiménez-Dalmaroni et al., 2016). CD14 helped to transfer LPS to TLR4 to form a complex and allowed internalisation of the TLR4 complex (Wu et al., 2019). The CD14/TLR4 complex activated spleen tyrosine kinase (SYK) through ITAMs that activated the downstream effector phospholipase Cγ 2 (PLCγ2), which resulted in generation of inositol triphosphate (IP3) release, influx of Ca^{2+} from intracellular store result in internalisation of CD14/ TLR4 complex (Wu et al., 2019). Studies have shown the importance of LPS as a key molecule that activates the GVHD response, the presence of this molecule can result in monocyte and macrophage activation (Deeg, 2001). Ag recognition, phagocytosis, Ag processing and peptides presentation via MHCI and MHCII on the surface of monocytes/macrophages, important in the initiation of GVHD as they can lead to recognition of antigens by the T cells, and this can trigger the adaptive immune response (Deeg, 2001, Blazer et al., 2013, Boyette et al., 2016).

1.4.2 Antigen processing and presentation:

In order to stimulate immune response, professional APCs need to recognise, process non-self-antigens, and present digested material (amino acids) via MHC class I and class II to $CD8^+$ and $CD4^+$ T cells respectively, which results in the development of the adaptive immune response (Duque & Descoteaux, 2014, Rock et al., 2016, Wieczorek et al., 2017). MHCI and MHCII are polymorphic proteins because they are expressed from three different gene regions MHCI (HLA-A, HLA-B and HLA-C) MHCII (HLA-DR, HLA-DP and HLA-DQ) (Wieczorek et al., 2017). There are two antigen processing pathways based on the origin of the pathogen: the endogenous and exogenous pathway (Rock et al., 2016, Wieczorek et al., 2017, Junker et al., 2020).In the endogenous pathway, the virus antigen or cancer cells with mutant sequence are presented within the host-nucleated cells and the antigen is broken down to peptides in the proteosome to form peptides of 8-9 amino acids (Rock et al., 2016, Wieczoreket al., 2017, Junker et al., 2020). These peptides are transferred to the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP) channel. After that, the

MHCI molecules will bind to the peptide and transport it to the cell surface of APCs to present the peptide to $CD8^+$ T cells (Neefjes et al., 2011, Rock et al., 2016, Wieczorek et al., 2017, Junker et al., 2020).In contrast, the exogenous pathway occurs when pathogens are endocytosed from the surrounding environment by PRRs or Fc receptors to form endocytic vesicles (Neefjes et al., 2011, Rock et al., 2016). These vesicles are then acidified, which leads to pathogen degradation and produces peptides of 10-20 amino acids long, and at the same time the MHCII molecules are generated in the ER and vesicles are formed (Neefjes et al., 2011, Rock et al., 2016, Junkeret al., 2020). The two types of membrane vesicles will fuse together to facilitate the antigen peptides loading to the MHCII (Neefjes et al., 2011, Rock et al., 2016). Ultimately, the vesicle will fuse with the cell surface membrane to express the MHC-peptide complex and display it to $CD4^+$ T cells (Neefjes et al., 2011, Rock et al., 2016). Monocytes and macrophages can process both endogenous and exogenous antigens and present the antigen-derived peptide fragments either to $CD4^+$ and $CD8^+$ T cells (Rock et al., 2016).

1.4.2.1 FcγRI (CD64):

Fc receptors mediate important links between innate and adaptive immune response, as Fcγ R mainly recognise opsonised Ag coated with IgG or immune complex (IC), the crosslinking results in IC internalisation, processing of the Ag, presenting via MHCII to activate T cells (Junker et al., 2020). The FcγR family include both activation receptors like FcγR I, FcγRIIa, FcγRIIc, FcγRIIIa and the inhibitory receptor like FcγRIIb (Brandsma et al., 2018).

FcγRI known as CD64 which is a transmembrane glycoprotein found on monocytes, macrophages, and neutrophils, with a high binding affinity for the IgG complex (Hepburn et al., 2004, Akinrinmade et al., 2017). As CD64 mainly binds to IgG1, IgG3 and IgG4 isotypes (Junker et al., 2020). Many studies have reported elevated CD64 on activated macrophages and monocytes during chronic inflammation (Hepburn et al., 2004, Delneste et al., 2003, Akinrinmade et al., 2017, Lau et al., 2018). Interestingly, CD64 is involved in autoimmune diseases (Brandsma et al., 2018). For example, CD64 expression was elevated in circulated monocytes in rheumatoid arthritis patients (Hepburn et al., 2004). More

importantly ligation of CD64 with IgG-IC complex result in internalisation, processing of the Ag proteins, leads to Ag presentation via MHCII (Brandsma et al., 2018, Junker et al., 2020). CD64 intracellular signalling is involved in ITAM phosphorylation and spleen tyrosine kinase (SYK family) recruited to the ITAM domain, this intracellular signalling affects cellular function, such as enhancing degranulation, phagocytosis, cytokine, and inflammatory mediator release (Junker et al., 2020). CD64 has gained attention in recent years as a potential target for reducing inflammation (Akinrinmade et al., 2017). Blockage of CD64 with anti-CD64 reduced Ag presentation to T cells (Junker et al., 2020). Targeting CD64 with anti-CD64 showed promising results in treating acute myeloid leukaemia by chemotherapy (Young et al., 2019). It has been reported that monocytes cultured with IFN-γ, IL-4 and GM-CSF upregulate CD64 and shift monocyte differentiation from DCs to macrophages (Delneste et al., 2003). Treating peripheral blood mononuclear cell (PBMC) with IFN-γ polarises macrophages to the M1 type and upregulates CD64 expression. Whereas IL-4 treatment down regulates CD64 expression (Akinrinmade et al., 2017). Targeting of CD64 by anti-CD64 is a potential therapeutic approach to block M1 formation, maintain the M1/M2 balance that allows tissue healing after chronic inflammation(Akinrinmade et al., 2017). A study reported CD64 as an activation marker found in APCs in a skin biopsy from GVHD patients (Kerkhof et al., 2011). There is limited data that links expression of CD64 on $CD14^{+}$ monocytes/macrophages and aGVHD (Kerkhof et al., 2011). Thus, in this study the level of CD64 expression was measured in two-way mixed leukocyte reaction (MLR) as activation marker, and further targeting the SYK pathway through FCγRI tested by highly specific SYK inhibitor to evaluate the therapeutic approach in the histo-incompatible model.

1.4.3 FcγRIIB (CD32B):

CD32 is expressed on DCs, monocytes and monocytes derived macrophages, it is a Fcγ cell membrane receptor protein (FcγRII) that has a medium-low affinity for IgG and has been shown to play a vital role in antibody mediated immune regulation (Bhatnagar et al., 2014, Anania et al., 2019, Zhao et al., 2019). There are three different CD32 isoforms expressed on human cells, such as monocytes and natural killer (NK) cells; CD32A and CD32C which are activating receptors and CD32B which is an inhibitory receptor (Bhatnagar et al., 2014). CD32A is the

most prevalent and abundant activating CD32 on macrophages and monocytes, whereas the inhibitory CD32B is expressed only in relatively low levels on neutrophils, monocytes, and macrophages, and has higher levels of expression on B cells (Zhao et al., 2019, Anania et al., 2019). In human monocytes both cytokines (IL-4 and IL-10) induce the expression of CD32B, and down regulate CD32A expression that mainly linked to macrophage deactivation (Zhao et al., 2019, Anania et al., 2019).CD32 can aggregate IgGs by cross-linking them and this activates a range of effector responses, which is dependent on crosstalk between TLR and FcRs with local cytokine environment that control CD32 expression (Anania et al., 2019).

Studies of asthma and rheumatoid arthritis patients have shown that CD32, and especially CD32B, may be crucial in modulating the allergic immune response by regulating monocyte activity in response to allergic inflammation mediated by IL-4/IL-4Rα signalling (Anania et al., 2019 and Zhao et al., 2019). An imbalance between activation and inhibition FcγR functions can predispose patients to pro-inflammatory, autoimmune diseases (Anania et al., 2019). Patients with GVHD have increase expression of inflammatory cytokines IL-6, TGF-β, TNF-α, IFN-γ and IL-1β, with decreased expression of anti-inflammatory cytokines including IL-10, and upregulation of CD32A expression on B cells, granulocytes, monocytes, and subpopulation of macrophages (Zdziarski et al., 2018). Limited data available regarding expression of inhibitory CD32B and allogeneic response. As such, in this study CD32B was selected as inhibitory marker to investigate the expression of CD32B in the early allogeneic response.

1.4.4 Co-stimulatory molecule (CD80/CD86):

The B7 family, CD80 and CD86, are co-stimulatory molecules for T cells expressed on the surface of APCs including monocytes and macrophages (Deneys et al., 2001, Zheng et al., 2004, Ke et al., 2016, Zeiser et al., 2016). Generally, CD80 and CD86 are ligands for CD28 and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) on T cell surfaces and are involved in T cell activation or tolerance (Ke et al., 2016). Interestingly, CD80 and CD86 are not expressed on the surface of APCs at the same level; their crystal structures show CD80 is present as a dimeric structure that can bind to three ligands: CD28, CTLA-

4 and program cell death ligand 1 (PD-L1), whereas CD86 molecules are present as monomers and they can only bind to CD28 or CTLA-4 (Ke et al., 2016). It has been reported that CD80 binding with CTLA-4 is more efficient than binding with CD86, meaning CD80 has high affinity for CTLA-4, which mainly induces immune tolerance by production of IL-10 and TGF-β from the APCs or upregulation of Treg $CD4^{+}CD25^{+}$ Foxp cells, whereas CD86 preferentially binds to CD28 activating immunereactions (Zhang et al., 2006, Zeiser et al., 2016). Consequently, APCs that expressed high levels of CD86 could impair Treg function, and the expression of CD86 on the surface of APCs is mainly upregulated by danger signals like LPS or IFN-γ signals (Deneys et al., 2001, Zheng et al., 2004, Zeiser et al., 2016). This study focused on upregulation of CD86 expression on $CD14^{+}$, as critical step in T cells co-stimulation, in MLR with Th1 or Th2 cytokines.

1.4.5 Recognition of apoptotic cells by macrophages:

Programmed cell death, which is known as apoptosis, is a vital process to maintain tissue haemostasis (Maderna & Godson, 2003, Gregory & Devitt 2004, Nagata, 2018). Everyday millions of cells generated in human body and millions die based on the cells type and life span (Nagata, 2018). Apoptotic cells are rapidly cleared by macrophages and DCs to avoid release of intracellular component and maintain tissue haemostasis (Maderna & Godson, 2003, Gregory & Devitt 2004, Martinez et al., 2020). The clearance of apoptotic cells mainly occurs in BM, spleen, and liver (Hufford & Ravichandran, 2013, Nagata, 2018). The efficient clearance of apoptotic cells by phagocytic cells reduced proinflammatory response (Gorden & Pluddemann et al., 2018, Nagata et al., 2018). Whereas defects in apoptotic cell clearance and accumulation, results in auto-Ag that drive autoimmune diseases (Maderna & Godson, 2003).

The clearance process is divided in to three stages known as “find me, eat me, and don’t eat me” signals. find me signals like: Fractalkine, ATP, Uridine, and sphingosine I phosphate that mainly chemoattract phagocyte. Don’t eat me signal include: CD31, CD47 which expressed in all healthy cells (Maderna & Godson, 2003, Gorden & Pluddemann, 2018). Whereas phosphatidyl serin (PS) exposer and loss of phospholipid rearrangements on the cell surface play a significant role in apoptotic cell recognition (Maderna & Godson, 2003, Gorden & Pluddemann,

2018). PS exposure in the outer cell surface is considered as “eat me” signal; β-arrestin 1 (BA1)-1 receptors on the macrophage cell surface bind to PS on the apoptotic cell surface, and carbohydrate changes on the apoptotic cells can be recognise by scavenger receptors expressed on macrophage cell surfaces (Gorden & Pluddemann, 2018). Interestingly, a few days after inflammation responses,monocytes and activated macrophages undergo apoptosis (Gorden & Pluddemann, 2018). In this study, to detect the apoptotic cells, all samples were stained with Annexin V that mainly binds to PS, and all Annexin V positive cells were considered as apoptotic cells and excluded from the rest of the experiment. This thesis evaluated monocyte/macrophage recognition of apoptotic cells during the allogeneic response by measuring the level of expression of scavenger receptors (CD36 and CD204) and measure the expression of C3aR and Fc receptors to evaluate ability of monocytes to opsonize Ag within histo-incompatible model.

1.4.6 Scavenger receptor A (CD204):

Scavenger receptors (SRs) are members of the PRRs family, there are 10 classes of SR from A-J (Pombinho et al., 2018). SRs are expressed on neutrophils, macrophages, DCs and B cells (Pombinho et al., 2018). SR-A contain five members they name as A because of structural similarity all have trimeric transmembrane, intracellular N terminus, short cytoplasmic tail including: SR-A1, macrophage receptor with collagenous structure (MARCO), cellular stress response (CSR), SR with C-type lection (SRCL) and SR-A member 5 (SCARA5, Kelley et al., 2014). This report focused on SRA-1, which is known as CD204, and is expressed on macrophages and DCs involve in the innate immune response (Seimon et al., 2006, Murray & Wynn, 2012, Martinez et al., 2020). CD204 can bind to different varieties of ligands, they recognise bacterial cell wall components like LPS, and lipoteichoic acid viral component (Pombinho et al., 2018). The ligation of CD204 results in phagocytosis, inflammatory responses, and secretion of cytokines, like TNF-α (Pombinho et al., 2018). More importantly, CD204 is involved in the uptake of apoptotic cells (Maderna & Godson, 2003, Kelley et al., 2014). Neonatal microglia promote apoptotic clearance through ligation of PSwith SRA (Maderna & Godson, 2003). The CD204 receptor shows a dual function as they contribute to the inflammatory response against pathogens and could be

involve in protecting the host (Kelley et al., 2014). Binding to oxidised low-density lipoproteins (oxoLDL) and PS expressed on apoptotic cells (Gorden & Pluddemann, 2018). Monocytes/macrophages infiltrate and accumulate in different organs causing tissue specific fibrosis and resulting in cGVHD in mice and patients (Konmua et al., 2018). The host alveolar macrophages were replaced with donor non-classical and intermediate monocytes that highly expressed CD204 and were significantly increased in patients with severe lung fibrosis in cGVHD (Knouma et al., 2018). Thus, controlling the expression of CD204 on surface of monocytes/macrophages could prevent tissue-specific pathology in cGVHD (Konmua et al., 2018). The high levels of CD204 expression on monocytes/macrophages is linked to a poor prognosis of cGVHD in HSCT patients (Knouma et al., 2018). Furthermore, aGVHD is characterised by the strong inflammatory response and cells death, this study has investigated the expression of CD204 to evaluate the ability of monocytes to capture apoptotic cells in allogeneic culture.

1.4.7 Scavenger receptor B (CD36):

SRB-1, also known as CD36 is a membrane glycoprotein expressed on mononuclear phagocytes including DCs, macrophages, microglia, cardiac myocytes, hepatocytes, platelets and adipocytes and some epithelia (including gut, kidney, and breast) (Silverstein & Febbraio, 2009, Wang & Li, 2019). On phagocytes, CD36 functions to recognizes specific oxidised lipoproteins and phospholipids and plays a key role in internalisation of modified LDLs (via activation of peroxisome proliferator–activated receptor γ (PPARγ) the nuclear hormone receptor), certain bacterial and fungal pathogens, and apoptotic cells, which contributes to atherothrombotic diseases and inflammatory responses (Silverstein & Febbraio, 2009, Wang & Li, 2019). CD36 has been shown to recognise PS to regulate clearance of apoptotic cells (Perry et al., 2018). CD36 expression in macrophages is reduced by viral infection however, IL-4 can induce CD36 expression in macrophages by activating the PPARγ ligand prostaglandin J2 (PGJ2) and intracellular lipoxygenase result in anti-inflammatory response (Cooper et al., 2016, Wang & Li, 2019). Treatment with IFN-γ has been shown to inhibit CD36 and CD14 expression, but increase expression of CX3CR1,CD16 and HLA-DR on monocytes (Zhong et al., 2019). Phagocytosis is particularly important

in cardiac wound healing to remove apoptotic neutrophils and necrotic cardiomyocytes; the process involves CD36 upregulation, and targeting CD36 reduces macrophage phagocytosis (Daseke et al., 2020). CD36 works by recognising specific lipoprotein and lipid components of bacterial cell walls (especially mycobacterial and staphylococcal organisms), erythrocytes infected with malaria, β-glucans on fungal species, which then triggers opsonin-independent pathogen internalisation(Silverstein & Febbraio, 2009). Interestingly, CD36 showed a protective role in skin infections by generating a host anti-inflammatory response (Pombinh et al., 2018).In GVHD, patients are often found to have skin rashes (Kanuma et al., 2018). CD36 expressed on DCs from mice have been shown to be involved in allo- tolerance, and reduction of CD36 expression was associated with GVHD (Perry et al., 2018).

There is an association between the severity of GVHD, increase production of pro-inflammatory cytokines and activation immunoregulatory intracellular signalling cascades through CD36. As CD36 upregulation initiating allo-tolerance that limits the pathogenesis of as GVHD (Juric et al., 2017). The data about the exact role of $CD14^{+}CD36^{+}$ in early allogeneic response were limited.As such CD36 expression was selected to evaluate the clearance of apoptotic cells by monocytes/macrophages in *in vitro* model of GVHD.

1.4.8 Complement receptors:

Complement receptors play an essential role in pathogen recognition and immune complex clearance (Eredi et al., 1991, Nguyen et al., 2015). Complement activated products and their receptors develop during the innate and adaptive immune responses (Nguyen et al., 2015, Mathern and Heeger, 2015). C3a and C5a are anaphylatoxins that mainly bind to complement receptor 3a (C3aR) and complement receptor 5a (C5aR), respectively (Nguyen et al., 2015). C3aR and C5aR are seven transmembrane spanning G protein coupled receptors (Mathern and Heeger, 2015). C3aR is mainly expressed on the surface of T cells, monocytes, macrophages, neutrophils, and basophils, whereas C5aR is expressed on T cells neutrophils and platelets (Eredi et al., 1991, Cravedi et al., 2013). C3aR and C5aR provide essential signals for APCs survival, maturation, and differentiation, they are also involved in signals that promote T cells priming,

which results in T cells differentiation and proliferation (Nguyen et al., 2015). C3aR and C5aR on APCs promote APC activation via up-regulation of CD86 expression and control cytokine production, as such C3aR and C5aR considered as APC activation markers (Cravedi et al., 2013).C3a, C5a ligation with C3aR and C5aR favour the inflammatory response by increasing vasodilation, chemokines, cytokines, promotes macrophage activation, engulfing of the organism, and intracellular killing to present Ag to T cells (Mathern & Heeger, 2015). Interestingly, APCs that express high levels of C3aR and C5aR activate T cells to differentiate in to Th1 and type 17 T helper (Th17) cells that suppress Treg formation (Nguyen et al., 2018). Also ligation of C3aR and C5aR results in monocyte derived DC activation (Li et al., 2012), elevation of CD86 and HLA-DR levels, secretion of inflammatory cytokines including IL-6 and TNF-α that initiate aGVHD development (Li et al., 2012, Nguyen et al., 2018). More importantly ligation of C3aR, C5aR can result in kidney transplant rejection due to allo-reactiveT cells response (Mathern & Heeger, 2015). Studies has revealed that C3aR and C5aR deficiency on APCs reduces T cell priming and decreases the onset of aGVHD (Nguyen et al., 2015, Mathern & Heeger, 2015). Blocking of C3aR andC5aR inhibit T cell activation and proliferation (Cravedi et al., 2013). Targeting C3aR, C5aR on mice DCs lowers the Ag presentation, with reduced HLA-DR expression, APCs activities, and decreased IFN-γ (Nguyen et al., 2015). Currently, there are limited data on the expression of C3aR, on human monocytes and their role in aGVHD, therefore more study is required. Thus, in this thesis the levels of $C3aR^{+}CD14^{+}$ expression measured to evaluate the role of C3aR during early allogeneic response in two-way MLR.

1.4.9 CD11b:

CD11b is expressed on granulocytes, lymphocytes, monocytes, monocyte-derived cells including DCs, and some resident macrophages (Ochando et al., 2016, Fendl et al., 2018). Human tissue-resident macrophages express MHCII, CD68, CD33 and CD11b under homeostatic conditions (Ochando et al., 2016). CD11b is an important marker for differentiating between different subsets of macrophages; $CD11b^{low}F4/80^{+}$ macrophages express greater numbers of pro-inflammatory M1 markers (including CD11c, CD86 and MHC-II), whereas $CD11b^{high}F4/80^{+}$ express greater numbers of M2 markers with a high phagocytic

activity (Ma et al., 2019). CD11b is crucial for cell adhesion, phagocytosis of pathogens, phagocytosis of apoptotic cells, and complement-opsonised cells by monocytes, but this process does not always involve inflammation (Lukácsi et al., 2017, Gavin et al., 2019).

During cell migration, CD11b, along with CD11c, becomes concentrated in the podosome adhesion ring in human phagocytes, which confirms the important role of CD11b in migration (Lukácsi et al., 2017). Monocytes and macrophages in circulation can be mobilised and activated by the interaction of CD11b and IL-4 (Baba et al., 2020). CD11b selected in this study to evaluate monocytes/ macrophages differentiation state in two-way MLR.

1.5 T cell response:

Naïve T cells are produced from the thymus, after positive and negative selection. Consequently, they can recognise non-self Ag which is presented by APCs to generate an immune response against the pathogen (Neefjes et al., 2011, Rock et al., 2016, Wieczoret et al., 2017). Most APCs are in the secondary lymphoid organs; all pathogen-infected cells in the peripheral sites are transported to the lymph nodes or the spleen. APCs will engulf the pathogen, then process and present the peptides to the naïve T cells via MHC molecules (Murray & Wang 2012, Deretic et al., 2013, Rock et al., 2016, Wieczoret et al., 2017). The activation of naïve T cells usually involves three essential signals; the initial signal comes from the interaction between the T cell receptor (TCR) on both $CD4^+$ and $CD8^+$ and the peptide antigen present via MHCII and I on the surface of APCs(Neefjes et al., 2011, Ka et al., 2016). The later signal after TCR activation is co- stimulation as the absence of co-stimulation leads to T cells apoptosis; naïve T cells express CD28, which is a receptor for CD80, and CD86 expressed on the APCs (Neefjes et al., 2011, Ka et al., 2016). Importantly, binding of CD28 to CD86 enhances T cell proliferation (Ka et al., 2016). CD28 and B7(CD80/CD86) binding up-regulates CTLA-4 on T cell surfaces, which later competes with CD28 to bind with B7 resulting in T cell inhibition, leading to immune tolerance (Blazar et al., 1994, Ka et al., 2016). There are other costimulatory molecules upregulated on T cells during activation, including T cell co-stimulator (ICOS/CD278), members of the TNF ligand/TNF receptor family, such as CD40 ligand (CD154; TNFSF5), CD30

(TNFRSF8), CD137 (TNFRSF9) and OX40 (CD134; TNFRSF4) (Deretic et al., 2013, Zeiser et al., 2016). It has been reported that activation of $CD8^+$ T cells requires co-stimulation signals from CD70 and CD137, unlike $CD4^+$ T cells, which rely on CD28 and B7 co-stimulatory signals (Deretic et al., 2013, Zeiser et al., 2016). The third crucial step for activating T cells are interactions with cytokines, including IL-12 and other cytokines essential for T cell survival, proliferation, and differentiation. This includes cytokines that share a common gamma chain (CD132), which forms a complex receptor for six cytokines, IL2, IL-4, IL-7, IL-9, IL-15, and IL-21 that are present on all lymphocytes, including T cells (Hechinger et al., 2015, Zeiser et al., 2016).

Interestingly, $CD4^+$ cells are further classified into Th1, Th2, Th9, Th17, Th22 and Treg cells based on their cytokine profile and the type of infectious agent (Ouyang et al., 2012, Rahimi et al., 2019). Naïve T cells will differentiate into Th1 if they recognize intracellular infection, whereas T cells will differentiate into Th2 after extracellular pathogen recognition (Romagnani, 1999, Rahimi et al., 2019). Th1 cells secrete IFN-γ, IL-2 and TNF-α, which activate cell mediated immunity and macrophage phagocytic activity, whereas, Th2 cells mainly produce IL-4, IL-5, IL-6, IL-9 IL-10, IL-13, and IL-25, which down regulate macrophages functions, increase antibody production and eosinophil recruitment (Romagnani, 1999, Rahimi et al., 2019). IL-23 prime Th17 cells to secrete the cytokines IL-17, IL-21, and IL-22, which play a crucial role in the defence against extracellular pathogens (Ouyang et al., 2012). Balance in cytokine secretion from Th is essential as an unbalanced Th1/Th2 secretion is associated with many diseases, including inflammatory bowel disease, allergic and ectopic diseases (Rahimi et al., 2019).

1.5.1 T cells activation markers CD69 and CD25:

T cells express many surface markers based on their activation stage (Bajnok et al., 2017). An early activation marker is the glycoprotein receptor (CD69), that is induced soon after TCR/CD3 ligation with MHC molecules and activating cytokines, CD69 levels remain elevated on the surface of T cells from 3 hours up to 72 hours post activation (Sancho et al., 2005, Han et al., 2009, Bajnok et al., 2017, Cibrian et al., 2017). Interestingly, resting T cells did not express CD69 (Sancho et al., 2005, Han et al., 2009, Cibrian et al., 2017). CD69 have short

cytoplasmic domains binds to JAK3, a ligand to specific carbohydrate binding protein known as Galectin-1 (Gal-1) which is expressed by DCs and macrophages (Cibrian et al., 2017). Activation of CD69 plays an essential role in T cells proliferation and survival during the proinflammatory response (Han et al., 2009, Bajnok et al., 2017).CD69 could control T cells migration to the lymph node and inflamed tissue, as such T cells expressing CD69 are mainly not found in the circulation (Cibrian et al., 2017). CD69 could negatively regulate T cell activity, as ligation of CD69 to calprotectin activate STAT3 result in transcription of TGF-β gen, TGF-β later secreted by activated T cells reduced Ag presentation by APCs which impairs T cells polarisation more importantly maintain Treg differentiation (Sanchoet al., 2005). $CD4^{+}CD69^{-}$ failed to develop into Treg (Sancho et al., 2005, Cibrianet al., 2017). The late activation marker of T cells is the alpha chain trimeric IL-2 receptor prominent surface receptor expressed on lymphocytes (CD25), which is induced 24 hours post TCR activation in response to IL-2, and results in T cells activation and releasing more IL-2 (Yarkoni et al., 2014, Bajnok et al., 2017). Interestingly, CD25 remain on the surface of T cells for a few days (Bajnok et al., 2017). CD25 is up-regulated due to cytokines released by monocytes/macrophages or in responds to substances such as oxoLDL (Bajnok et al., 2017). Many lymphocytes subsets express CD25 on their surface, including regulatory T cells, effector, and memory T cells (Bajnok et al., 2017). It has been reported that incomplete depletion of T cells with CD25 and CD69 produced a good clinical picture of GVHD (Yarkoni et al., 2014). It has been demonstrated that T cells after activation with MHC will go through apoptosis by ligation of the Fas/Fas ligand. Defects of Fas expression will result in accumulation of activated T cells that caused autoimmune damage (O'Flaherty et al., 2000). This study aimed to measure MFI value of CD25 and proportion of % CD69 expression on $CD3^{+}$ and $CD4^{+}$, to evaluate T cells allo-activity in MLR. Based on the well-established characteristics of this molecules, CD69 is regarded as an early activation marker for T cells that is rapidly up-regulated on the cell surface (Han et al., 2009). However, it is difficult to detect difference in the levels of CD69 expression (MFI values), and there is no clear correlation between the level of CD69 expression and the strength of T cell activation (Han et al., 2009). In contrast, after activation CD25 becomes up-regulated on the surface of T cells, and the level of expression

of this marker (detected by changing MFI values-brightness) corresponded to the level of activation. Thus, measuring CD25 level of expression (MFI values) and the proportion of CD69 positive cells (CD69%) were informative and meaningful markers that reflect T cells activation for this study. Indeed, numerous studies have analysed T cell activity based on the proportion (%) of CD69 positive cells and changes in MFI levels for CD25 expression to show the key features of T cells activation (Han et al., 2009, Bajnok et al., 2017, Via et al., 2017).

1.5.2 Th1/ Th2 cells in acute GVHD:

It is thought that during HSCT, donor T cells are primed by APCs, leading to activation and differentiation toward Th and cytotoxic T (Tc) cells, and both T cell subtypes controlled developing of a GVHD (Wyscoki et al., 2005, Blazar et al., 2013, Avin et al., 2015, Koehn et al., 2015, Zeiser et al., 2016, Heller et al., 2017, Hong et al., 2020). More importantly, T cells migrate to lymphoid organs and tissue, T cells can be primed by different APCs at both sites (Yarkoni et al., 2014). It has been reported that the incomplete removal of activated T cells that expressed CD25 and CD69 affect GVHD development (Yarkoni et al., 2014). As previously mentioned, three signals are essential to activate donor T cells to undergo the different stages of activation (naïve, effector and memory) and to initiate aGVHD, starting with activation of TCR, followed by co-stimulatory/ co-inhibitory signals and interaction with cytokines (Zeiser et al., 2016). T cell activation is controlled by the location and the type of APCs, the kind of antigen or peptide presented via the MHC complex, and the capacity for direct peptide recognition (Zeiser et al., 2016). The classical concept was that donor Th cells are primed by host APCs which can further differentiate into Th1, Th2, and Th17 cells that migrate towards target organs such as skin, liver, and intestine (Avin et al., 2015, Zeiser et al., 2016, Hong et al., 2020). Th1 cells-controlled cell mediate immunity like cytokine secretion IFN-γ, IL-2 and TNF-α (Raphael et al., 2014), and these cytokines are essential to induce antigen presentation by APCs and recruit effector T cells during the activation phase of aGVHD (Aveni et al., 2015, Hong et al., 2020). However, Th2 controlled humeral response has been reported to be involved in tissue repair (Raphael et al., 2014). Th2 released cytokines such as IL-4, IL-5 and IL-13 which may lower the risk of GVHD (Aveni et al., 2015). Excessive secretion of Th1 cytokine results in tissue damage whereas excessively

production of Th2 result in hypersensitivity (Raphael et al., 2014). It has been reported that T cells mediated tissue damage controlled by monocytes or macrophages and their microenvironment (Hashimoto et al., 2011).

1.6 Pathophysiology of acute GVHD:

During transplantation, mononuclear phagocytes from the donor organ can migrate into the recipient's secondary lymphoid organs, and when activated initiate the acute inflammatory cascade, which causes secondary tissue injury, for example, activated DCs alter their cytokine, adhesion molecule and co-stimulatory receptor expression to allow them to migrate into lymphoid tissues and stimulate T cells, and this can contribute to initiation of allograft rejection (Ochando, 2016). It has been demonstrated that there are three phases involved in aGVHD: firstly, the conditioning regimen, then T cell activation, and finally the effector phase (Ferrara et al., 1991, Deeg, 2001, Markey et al., 2014).

1.6.1 The conditioning regimen phase:

Before the allogeneic HSCT procedure, patients will take immune suppressor drugs and undergo total body irradiation (TBI), which can result in gastrointestinal tissue damage (Ferrara et al., 1991, Deeg, 2001, Wysocki et al., 2005, Blazer et al., 2013, Hong et al., 2020).

1.6.1.1 Allogeneic reaction in conditioning regimen:

After TBI, the patient's T cells, NK cells and neutrophils are quickly destroyed, and the number of DC and B cells are also reduced in the lymph nodes 48 hours after TBI (Hashimoto et al., 2011). However,the patient's macrophages can persist for many weeks, which may explain the importance of the host macrophages in the pathogenesis of GVHD (Hashimoto etal., 2011). The conditioning regimen mainly leads to exposure in the body to normal bacterial flora products, such as LPS, and many studies have highlightedthe importance of releasing LPS to circulation and the initiation of aGVHD (Deeg,H 2001, Wysocki et al., 2005, Blazer et al., 2013). LPS released activates APCs from haematopoietic sources including B cells, DCs and monocytes/macrophages, and non-haematopoietic sources such as endothelial cells (Chakraverty & Sykes 2007, Zeiser et al., 2016). Activated APCs show upregulation of TLR4, the LPS receptor, on their surface followed by a

cytokine storm in aGVHD (Cooke et al., 2001, Blazer et al., 2016). As such the serum levels of pro-inflammatory cytokines, such as, IL-1β, IL-6 and TNF-α become elevated (Deeg,H 2001, Wysocki et al., 2005, Hong et al., 2020). Monocytes/macrophages can infiltrate tissue in response to the inflammatory response and many studies confirmed the role of monocytes in aGVHD occurrence (Markey et al., 2014, Honget al., 2020). Activated Monocytes/macrophages up-regulate the expression of adhesion molecules, MHC, co-stimulatory molecules in their surfaceready to prime T cells (Deeg,H 2001, Wysocki et al., 2005, Markey et al., 2014, Duque & Descoteaux, 2014).

Koehn et al. (2015) reported that after conditioning the dead cells would release ATP which binds to APCs via endogenous damage- associated molecules (DAMPs) leading to inflammasome activation (Koehn et al., 2015). When the inflammasome is activated the numbers of myeloid-derived suppressor cells will be reduced, which may increase the chance of developing GVHD (Koehn et al., 2015).

1.6.2 The activation phase of alloresponse:

In this phase, alloantigen recognition stimulates donor T cell activation, proliferation, differentiation, and migration of effector T cells from the lymphoid tissues to target tissues, such as the skin, gastrointestinal tract, and the liver (Markey et al., 2014). Post-HSCT, both donor and host APCs process and present peptides to the donor T cells; several studies have confirmed that donor APCs have a role in allogeneic T cell activation (Chakraverty & Sykes, 2007, Markey et al., 2014). Direct presentation occurs when the donor T cells interact with APCs from the host, leading to a proliferation of Th1 $CD4^+$ and Tc $CD8^+$ cells (Chakraverty & Sykes, 2007). The Th1 cells then secrete cytokines, including IL-2 and IFN-γ, which are important in inducing antigen presentation and recruitment of effector T cells, and this phase is crucial for the progression of GVHD (Chakraverty & Sykes, 2007, Blazer et al., 2013). In addition, IL-2 is considered as Tcells factor because it induces T cells to differentiate and to form $CD8^+$ T cells (Chakraverty & Sykes, 2007, Markey et al., 2014). However, cross-presentation, develops when the donor T cells recognize host allogeneic antigens presented indirectly by donor APCs (Matte et al., 2004, Koyama et al., 2015). Interestingly,

antigen presentation may occur via host non-hematopoietic cells like myofibroblast to activate donor T cells (Zeiser et al., 2016,Hechinger et al., 2015). As there is evidence that the activation phase by macrophages is important in the initiation of GVHD, this study focused on evaluation of monocyte-derived macrophages activation, cytokine production and their interaction with T cells using an *in vitro* model of GVHD.

1.6.3 The effector phase of allorespons:

This last phase, occurs when the allo-reactive T cells ($CD4^+$ and $CD8^+$) in response to chemokines and adhesion molecules target the patient's organs, including the skin, liver, and intestine, causing tissue damage, which is the main characteristic of aGVHD (Wysocki et al., 2005, Blaze et al., 2013, Markey et al., 2014, Zeiser etal., 2016, Hill et al., 2018).

1.6.4 Cytokines involved in GVHD pathophysiology:

Cytokines are small, soluble proteins that regulate cellular communication (Duque & Descoteaux, 2014). The main function of cytokines is to control the immune response in health and disease; each cytokine binds to a specific surface receptor to mediate cellular signalling and influence cell function (Duque & Descoteaux, 2014). More importantly, GVHD pathophysiology is characterised by the presence of a cytokine storm (Ferrar et al., 1991, Blazer et al., 2013, Zhang et al., 2017, Drobyski et al., 2018).

Due to patient tissue damaged after irradiation, activated T cells, APCs and NK cells will release pro-inflammatory cytokines, such as IFN-γ, which up-regulate MHCI and MHCII expression on the surface of professional APCs, including monocytes/macrophages. These respond to the initial inflammatory stimulation by releasing significant amounts of TNF-α and IL-1β, which leads to APCs maturation, expression of co-stimulatory molecules, and further cytokine release. This contributes to T cell activation targeting organs and induces GVHD symptoms (Deeg, 2001, Chakraverty & Sykes 2007, Markey et al., 2013, Zhang et al., 2017).Clinical symptoms of aGVHD like weight loss, diarrhoea, and skin rashes, are mainly due to Th1 and pro-inflammatory cytokines like IFN-γ, TNFα, IL-2, IL-1β and nitric oxide (Blazer et al., 2013, Zhang et al., 2017, Hong et al., 2020).

TNF-α is pro-inflammatory cytokine released from macrophages and activated Th1 and is involved in acute inflammation (Deeg, 2001, Duque & Descoteaux, 2014). Releasing of TNF-α in the liver caused vasodilation, stimulated T cells, neutrophil recruitment, and monocytes infiltration (Duque & Descoteaux, 2014). In aGVHD, TNF-α upregulated co-stimulation by monocytes/macrophage followed by priming of T cells (Chakraverty & Sykes 2007, Markey et al., 2013, Zhang et al., 2017). In addition, TNF-α caused cell apoptosis and necrosis, which was associated with tissue damage and resulted in a GVHD symptoms such as liver and intestinal tissue damage (Kumar et al., 2017). Low concentrations of TNF-α followed HST used to lower the risk of aGVHD (Deeg, 2001). Priming of TNF-α could induce Treg functions and lower incidence of aGVHD (Kumar et al., 2017).

IL-2 is one of the key secretory cytokines that act as T cells growth factor, that controls activation and proliferation. IL-2 may control the resistance to apoptosis by up-regulation of anti-apoptotic markers Bcl-2 or Bcxl-x_L on T cells (Blazar et al., 2013, Bajnok et al., 2017, O'Flaherty et al., 2000). During GVHD, IL-2 levels are increased early post transplantation (Deeg, H 2001). IL-2 is secreted from Th1 cells and NK cells to stimulate T cell activation, differentiation and proliferation, secretion of TNF-α from macrophages, and enhanced APCs recruitment to mediate skin and gut damage (Chakraverty & Sykes 2007, Kumar et al., 2017). It has been reported that IL-2 controlled Treg formation is associated with immunotolerance (Blazar et al., 2013, Bajnok et al., 2017). Anti-IL-2 therapy successfully lowers the incidence of GVHD (Deeg,H 2001). Interestingly, low concentrations of IL-2 were associated with Treg proliferation and low GVHD development (Blazar et al., 2013, Kumar et al., 2017).

IL-1 β pro-inflammatory cytokines are produced by several cell types, including monocytes, macrophages, Th1 cells, B cells and NK cells (Chakraverty & Sykes 2007,Ferrara et al., 2009). IL-1β stimulated histamine release that resulted in vasodilation and enhance APCs recruitment to the inflamed tissue to promote Th differentiation (Duque & Descoteaux, 2014). It has been reported that patients with severe forms of aGVHD have higher levels of IL-1β when compared with patients with mild symptoms of aGVHD, which confirms the association between IL-1β, inflammation and GVHD pathology (Ferrara et al., 2009).

In contrast, there are many other cytokines that could lower the GVHD development such as, IL-10 and IL-4 that are mainly produced by Th2 cells (Deeg, H 2001, Kumar et al., 2017). IL-10 and IL-4 could control GVHD formation by inhibiting Th1 cytokine production cytokines like IL-2, IL-6 and TNF-α (Deeg, H 2001, Kumar et al., 2017).

Nevertheless, the clinical pathology of GVHD relies on the alloreactive T cells activities after recognising polymorphic Ag on monocytes/macrophages, which causes T cells activation and differentiation due to local cytokines (Kumar et al., 2017). This study stimulated monocytes/macrophages in alloresponse with two different modulating cytokines (IFN-γ and IL-γ4) to evaluate monocytes/macrophages activation and their interaction with T cells followed by measuring TNF-α, IFN-γ, γ and IL-6 concentrations as an indication of allogeneic inflammatory response.

1.6.4.1 Interferon gamma (IFN-γ):

Interferons (IFNs) are known as viral interfering agents classified as type I and type II, based on their binding receptor, IFN-γ belongs to the type II IFN family (Lee & Ashkar, 2018). The IFN-γ receptor (IFN γ -R) consists of two heterodimer chains, IFNγ-R1 and IFNγ-R2; notably, IFNγ-R1 is expressed on all haematopoietic cells, whereas IFNγ-R2 is highly expressed on myeloid and B cells with variable expression on T cells, which is upregulated during T cells activation (Lee & Ashkar, 2018). Importantly, IFN-γ activate cells via Janus kinase (JAK1 and JAK2) /phosphorylation of STAT1 signalling pathway (Gregory et al., 2000, Lee & Ashkar, 2018). Based on the relatively high level of IFN-γR expression on the cell surface, IFN-γ/JAK/STAT1 may be able to control cellular proliferation or apoptosis activity (Gregory et al., 2000, Lee & Ashkar, 2018).

IFN-γ is mainly produced by $CD4^+$ T helper cells and NK cells, and plays crucial role in activating immune cells, including $CD8^+$ Tc cells, B cells and APCs (Wang & Yang., 2014, Lee & Ashkar, 2018, Rahimi et al., 2019). However, this cytokine is also produced locally by APCs in the early phase of innate immune response, resulting in cell self-activation and nearby cells activation, meaning IFN-γ can control both innate and adaptive immune reactions (Schroder et al., 2004, Wang & Yang., 2014, Lee & Ashkar, 2018). IFN-γ is also known as macrophage

activation factor as IFN-γ is produced when the cells are activated by antigens, mitogens, and allo-antigens (Lee & Ashkar, 2018). IFN-γ stimulated macrophages to up-regulate the expression of complement receptors to mediate phagocytosis (Lee & Ashkar, 2018). It has been reported that IFN-γ stimulates polarisation of macrophages towards the high phagocytic, high Ag presentation M1 subtype via TLR binding and activation of the STAT1 signalling pathway to produce pro-inflammatory cytokines like: IL-12, IL-1β and TNF-α (Wang et al., 2014, Lee et al., 2017, Obrine et al., 2019). Most immune cells express IFN-γ receptors,and they up-regulate the MHCI and II molecules, TLR, IgG class switching, and induced chemokines in response to activation by IFN-γ (Wang & Yang, 2014, Raphael et al., 2014). Interestingly, IFN-γ plays important role in GVHD; the TBI and conditioning regimen results in high amount of IFN-γ, as mentioned earlier it is secreted by activated donor Th1 cells (Chakraverty & Sykes, 2007, Rahimi et al., 2019). This enhances apoptosis of intestinal epithelial cells leading to damagein the gut and increased production of proinflammatory cytokines such as TNF-α from APCs during innate immune reaction in the early phase of aGVHD (Wang &Yang, 2014). Interestingly, IFN-γ showed protective role by controlling T cells trafficking to the lymph node, increased T cells apoptosis and controlling differentiation of T cells (Raphael et al., 2014). Therefore, IFN-γ concentrations were measured in MLR culture, also IFN-γ was selectedas a stimulus to activate monocytes/ macrophages in the *in vitro* model of histo- incompatibility in this thesis.

1.6.4.2 Interleukin-6 (IL-6):

IL-6 is pleiotropic cytokine that has proinflammatory and anti-inflammatory functions (Duque & Descoteaux, 2014). IL-6 is produced by several cells including monocytes, macrophages and DCs during acute inflammation, however,T cells are the main source of IL-6 during chronic inflammation (Ferrara et al., 2001, Tvedt et al., 2017). IL-6 is involved in acute and chronic inflammation and autoimmune diseases (Tvedt et al., 2017). IL-6 receptors exist in two forms, the membrane IL-6 receptor (IL-6R) and soluble IL-6 receptor (sIL-6R) and both receptors rely on glycoprotein (gp)130 (John, 2012, Drobyski et al., 2018). IL-6R is mainly found on hepatocytes, neutrophils, naïve T cells, monocytes, andmacrophages (Drobyski et al., 2018). Notably cells that do not express the previous receptor essentially

bind to IL-6 via sIL-6R, which is known as trans- signalling (Tvedt et al., 2017). Binding of IL-6 to sIL-6R with gp130 leads to pro- inflammatory reactions, whereas complexes of IL-6 with IL-6R and gp130 result in an anti-inflammatory response and tissue repair (John, 2012), which confirms the role of IL-6 in activating monocytes in the pro-inflammatory phase (Duque & Descoteaux, 2014). Interestingly IL-6 is released by classical monocytes in response to M-CSF (Boyette et al., 2016). Several studies have confirmed the elevation of IL-6 in early aGVHD (Tvedt et al., 2017, Drobyski et al., 2018). IL-6 mainly controls the severity of GVHD and blocking the IL-6R lowers the severity of aGVHD and saves the graft vs. tumour (GVT) after BMT (Drobyski et al., 2018). Interestingly, in aGVHD, IL-6 mainly mediated GI tissue damage (Drobyski et al., 2018). IL-6 in GVHD is produced by damaged cells after chemotherapy and irradiation, or donor T cells, and the IL-6 stimulates the production of other cytokines like IL-1β and TNF-□ (Deeg, 2001, Tvedt et al., 2017, Drobyski et al.,2018). IL-6 is essential in GVHD as it controls maturation and proliferation of T and B cells by up-regulating production of Th2, Th17 and down regulating Tregs (Tvedt et al., 2017). M1 activated macrophages mainly release IL-6, TNFα, IL-12,IL-1β and nitric oxide (Obrine et al., 2019, Hong et al., 2020), therefore in this study IL-6 concentration was measured in MLR.

1.6.4.3 Interleukin-4 (IL-4):

IL-4 is an anti-inflammatory cytokine that is produced by $CD4^+$ Th2 cells, basophils, mast cells and NK cells (Lee et al., 2017, Harms et al., 2019). It controls monocytes/macrophages maturation and polarization, B cell activation and IgG IgE class switching (Lee et al., 2017, Harms et al., 2019). IL4 is produced during asthma and the inflammation results in down regulation of the pro-inflammatory cytokines produced by monocytes and macrophages (Bonder et al., 1999, Deeg, 2001). IL-4 can bind to two subunit receptors, the type I receptor (IL- 4Rα/γc) and the type II receptor (IL-4Rα) (Junttila, 2018). Interestingly the type II receptor IL-4Rα is shared by the cytokine IL-13 (Junttila, 2018). IL-4 ligation with the type I receptor activates downstream signal through JAK1, JAK3 and phosphorylation of STAT6 resulting in Th2 and monocytes activation (Junttila, 2018). IL-4 induces Ag presentation by expression of HLA-DR on the monocytes surface, but that activation results in reduced secretion of TNF-α by human monocytes (Rousset et

al., 1988, Velde et al., 1990). Interestingly, IL-4 induced HLA-DR, CD86 expression in both classical and intermediate monocytes, HLA- DR expression was found more in the intermediate subtype with anti-inflammatoryresponse, which resulted in CD4 Th2 cells proliferation (Lee et al., 2017). More importantly IL-4 polarise macrophages towards M2 with tissue repair function (Lee et al., 2017, Junttila, 2018, Obrien et al., 2019). Several studies have confirmed the role of high concentrations of IL-4 in aGVHD tolerance (Deeg, 2001, Via et al., 2017, Tvedt et al., 2017). Study by Via and his colleagues showed that treating un-irradiated mice with 3-10 µg IL-4 reduced donor CD4, CD8 T cells allogeneic response. The study showed that early administration of high dose (10 µg) of IL-4 reduce acute GVHD without inducing chronic GVHD (Via et al., 2017). Notably, the high, non-physiological levels of IL-4 were needed to achieve pharmacological effects in GVHD setting.

It has been reported that IL-4 antagonise the inflammatory cytokines that are essential in aGVHD, including IFN-γ, and reduce T cells alloresponse (Blazar et al., 2013, Via et al., 2017). There are limited data about the effects of IL-4 on monocytes/macrophages function in the early stage of allogeneic culture. Thus, IL-4 was selected as stimulus, to evaluate the anti- inflammatory response of monocytes/macrophages in two-way MLR model.

1.7 Current strategies to control GVHD:

Over the last decades of HSCT, the primary goal was to minimise the risk of GVHD and maintain the graft survival. The patients age, condition regimen and source of HSCT are factors that could control GVHD induction (Markey et al., 2014, Blazar et al., 2013, Ganetsky et al., 2019). The stronger condition regimen caused more tissue injury which resulted in inflammation that favoured GVHD (Blazar et al., 2013, Drobyski et al., 2018). Currently, the first line of treatment to prevent GVHD is immunosuppressive drugs, however the main limitation is that patients will be at high risk of infection with high incidence of relapse (Ferrara et al., 2009, Blazar et al., 2013, Hill et al., 2018). The immunosuppressive regimen aimed to down-regulate TCR signalling pathway and that can be achieved by using calcineurin inhibitors like cyclosporin A, which was introduced in 1980s and became the prominent drug to control T cell alloresponse and in GVHD prophylaxis (Storb et

al., 1989, Zieser et al., 2016). Furthermore, in the 1990s tacrolimus (FK-506) was developed to control GVHD and transplant rejection by reducing IL-2 and maintaining Treg formation in response to TGFβ (Zieser et al., 2016). Steroids were used to treat inflammatory disease including aGVHD however some patients develop steroid resistance (Reinhardt et al., 2017, Hill et al., 2018). Interestingly, both steroid and calcineurin inhibitors reduced IL-2 which is essential for T cells activation and proliferation (Markey et al., 2014). Targeting IL-2 receptor induced Treg formation ($CD4^+CD25^+$ $FoxP3^+$), proven to be beneficial in murine models and human clinical trials. Tregs can prevent GVHD, as they could deactivate allo-reactive T cells after HSCT during activation phase (Wysocki et al., 2005, Markey et al., 2014, Blazar et al., 2013). More importantly, Sirolimus is rapamycininhibitor used as front-line therapy in older aGVHD patients who cannot tolerate steroid treatment or for patients who develop steroid resistance (Zieser et al., 2016, Hill et al., 2018).

Furthermore, Belatacept and Abatacept are nontoxic reagents available in clinical trial provided for patients who intolerance of calcineurin, to block co-stimulation like CD80, CD86, CTLA-4 and CD28 (Markey et al., 2014). It has been reported that the T cell response in aGVHD is mainly controlled by proinflammatory cytokines through JAK2 signalling, it has been demonstrated that kinase inhibitors that target JAK2 could control the T cells alloresponse (Hill et al., 2020). Ruxolitinib is a JAK1/JAK2 inhibitor used in mouse models showed promising results in controlling aGVHD by reducing proinflammatory cytokines and formation of Treg (Hill et al., 2018). Preclinical studies for Tocilizumab (which is humanized anti-IL-6 receptor have shown promise, as IL-6 is essential cytokine involved tissue damage in gastrointestinal track (GI) in GVHD (Drobyski et al., 2018, Ganetsky et al., 2019). IL-6 controls T cell proliferation, trafficking to GI and anti-apoptosis (Drobyski et al., 2018, Ganetsky et al., 2019). As such, a combination of Tocilizumab with a GVHD standard immunosuppressor show therapeutic effects in controlling GVHD (Drobyski et al., 2018, Ganetsky et al., 2019). Fostamatinib is SYK inhibitor (discuss in detail 2.11 and 2.12) that has been used in murine model and showed reduction of GVHD (Hill et al., 2018). It has been demonstrated that activation of innate response through TLR4 caused cytokine storm in aGVHD, and that mutation of the TLR4 gene showed promisingresults in controlling aGVHD in

a mouse model (Blazar et al., 2013). Furthermore,TLR9 signalling through MyD88 induced aGVHD, whereas MyD88 inhibitor reduced the innate immune response and could result in a lower risk of aGVHD (Blazar et al., 2013). Interestingly, T cells could be activated in GVHD even after controlling TLR (Blazar et al., 2013). As mentioned earlier, APCs control Ag presentation in GVHD, targeting or depletion of a single type of APCs could control GVHD (Blazar et al., 2013). Most of the cellular therapies in the clinical trial phase demonstrated promising results by suppressing alloresponse in GVHD targeted lymphocytes (Ferrara et al., 2009, Blazar et al., 2013). However, there are limited studies that targeted monocytes/macrophages function to control GVHD.

From this perspective, this thesis hypothesised that targeting adult monocytes derived macrophages by inhibiting SYK signalling through FcγRI may lower antigen presentation, and this could produce promising results in respect of the down-regulation of allo-reactive T cells after HSCT, as this would reduce the incidence of aGVHD.

1.8 Mixed leukocytes reaction MLR:

Mixed leukocyte reaction (MLR) is mainly used to detect the level of mismatched before organ and stem cells transplants to predict the rejections (O'Flaherty et al., 2000, Bromelow et al., 2000, Reinsomen, 2010). MLR used mainly to allow measuring of T cell activation and proliferation (Bromelow et al., 2000). There are two types of culture for MLR, in one-way MLR stimulatory cells are irradiated and treated with drug or inhibitor, then the responder cells are added so in this model only one population (responder cells) are allowed to proliferate (Reinsomen, 2010). In two-way MLR, which was established by Bain et al. (1964), two populations are mixed with all immune component cells, which results in an allogeneic reaction, interestingly both cellular populations could proliferate (Reinsomen, 2010). Two-way MLR is used to detect mismatch among individuals, one population will disappear after 3 weeks, however the second population will proliferate and survive strongly (Reinsomen, 2010). The allogeneic reaction in two-way MLR is more complex (Sato et al., 1999). Post-transplant the graft will contain immune components that could leave the graft and migrate in the recipient's circulation (Smith & Wang 2009, Reinsomen, 2010). Some protocols

used murine splenocytes as stimulatory cells (Han et al., 2009, Highfill et al., 2010, Ke et al., 2016). Other studies used PBMC as a stimulator population (Bromelow et al., 2000). It has been reported that whole blood MLR will most likely mimic the human immune response better than using PBMC (Bromelow et al., 2000). In this report, two random mismatched samples collected from healthy donors were mixed as two-way MLR. Unlike other protocols that mainly use two-way MLR to measure T cells proliferation, or isolate monocytes (Han et al., 2009, Highfill et al., 2010), this thesis analysed monocytes/macrophages function and T cell activities in two-way MLR saving cellular interaction that could reflect the allogeneic response.

1.9 Limitations of using animal models:

Mouse models are regularly used to test drug efficacy, therapeutic options, or to study immunological reactions during transplantation (Blazar et al., 2013). Although many drugs succeed in mice models, they fail in human systems due to immunological reaction differences between these two species. Basically, human and mice differ in terms of life span, size, behaviour, and innate and adaptive immune reactions (Murray & Wyn, 2011, Heitbrock. L, 2014). One of the important variations in the innate immune system between human and mice is the WBC count; in mice species the lymphocyte count is 75-90 % of the total WBCs count, neutrophils are about 10-20 % and monocytes only make up 2-4 %. However, in humans the ratio of neutrophil is higher, with a count of around 50-70 % of the total WBCs, lymphocytes are 20-40 % and monocytes make up 1-10 % (Zschaler et al., 2014). In addition, there is a recognised variation between the two species especially in respect of the source of monocytes and macrophages (Murray, 2017). For instance, monocytes are the major source of macrophages in humans, however, BM-derived, or peritoneal cavity macrophages are the main source of macrophages in mice, which is due to different polarisation agents (Murray, 2017). There are also differences in expression of cell surface markers on human and mouse monocytes (Zschaler et al., 2014). As mentioned above, there are three functional human monocyte subsets identified based on cell surface marker CD14/CD16 expression, whereas monocytes in mice are classified into two different populations based on expression of Ly6C, CCR2 and CX3CR1 (Sprangers et al., 2016). The $Ly6C^{+}$, $CCR2^{high}$, $CX3CR1^{low}$ monocytes

can be treated as classical and intermediate monocytes in humans, whereas the $Ly6C^{-}$, $CCR2^{low}$, $CX3CR1^{high}$ monocytes can be treated as non-classical monocytes in humans (Sprangers et al., 2016). Although $CD16^{+}$ human monocytes and $Ly6C^{+}$ share some similarities they are not identical (Heitbrock, 2014). These differences may contribute to the variation in immunological reactions between humans and mice for specific stimuli or treatments (Blazar et al., 2013, Kenney et al., 2016).

Humanised mouse models have been developed to overcome the differences in immunological reactions between mice and humans; the most commonly used model is immune-deficient mouse with a mutation in the IL-2 receptor common gamma chain (IL-2γ), xenografted with human innate or adaptive cells (Zschaler et al., 2014, Kenney et al., 2016). IL-2γ receptor is the main receptor that binds to cytokines IL2, IL4, IL7, IL9, IL15 and IL21, and use of this model will eliminate the adaptive immune cells from host and enhance the human grafted cells (Brehm and Shultz., 2012).Although humanised mouse models have been used to help test many drugs in preclinical settings and they also allow for a better understanding of the mechanisms of allogeneic rejection (Brehm and Shultz., 2012). The current humanised model did not show human markers on the surface of monocytes and macrophages, and these could have a crucial role in graft rejection (Brehm and Shultz., 2012). This highlights the need to develop an *in vitro* model of GVHD to allow the study of the functional potential of human monocytes and macrophages in an allogeneic reaction.

1.10 Spleen tyrosine kinase:

Spleen tyrosine kinase (SYK) is an intracellular 72 kDa, non-receptor cytoplasmic tyrosine kinase involved in several signalling pathways within immune cells, including T cells, B cells, monocytes, macrophages and DCs (Wysocki et al., 2005, Yi et al., 2014, Coffy et al., 2017, Hill et al., 2018). SYK is involved in processing downstream signals from cell surface receptors such as TLR, FcR, B cells receptors (BCR) and chemokine receptors (Leonhardt et al., 2012, Flynn et al., 2015). Importantly, activation of these receptors is thought to be involved in the development of GVHD pathology (Leonhardt et al., 2012, Yi et al., 2014, Flynn et al., 2015, Hill et al., 2018). Notably, CD14 and FcR on monocytes/macrophages

are involved in activation of SYK downstream signals that are essential to mediate inflammatory responses by monocytes/macrophages (Yi et al., 2014). As presented in (Figure 1.3) CD14 helps in binding LPS to TLR4, this interaction activated SYK downstream signalling in monocytes/macrophages resulting in CD14, TLR4 endocytosis and internalisation (Yi et al., 2014, Wu et al., 2019). Binding of CD14 to LPS promotes endocytosis of CD14/TLR4 complex, phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM). Then activated tyrosine kinase SYK will activate phospholipase Cγ (PLCγ) leading to generation of phosphoinositide 3-kinase (P13K), resulting in influx of extracellular Ca^{2+} and causing CD14 TLR4 receptors internalisation by IRF phosphorylation (Wu et al., 2019).

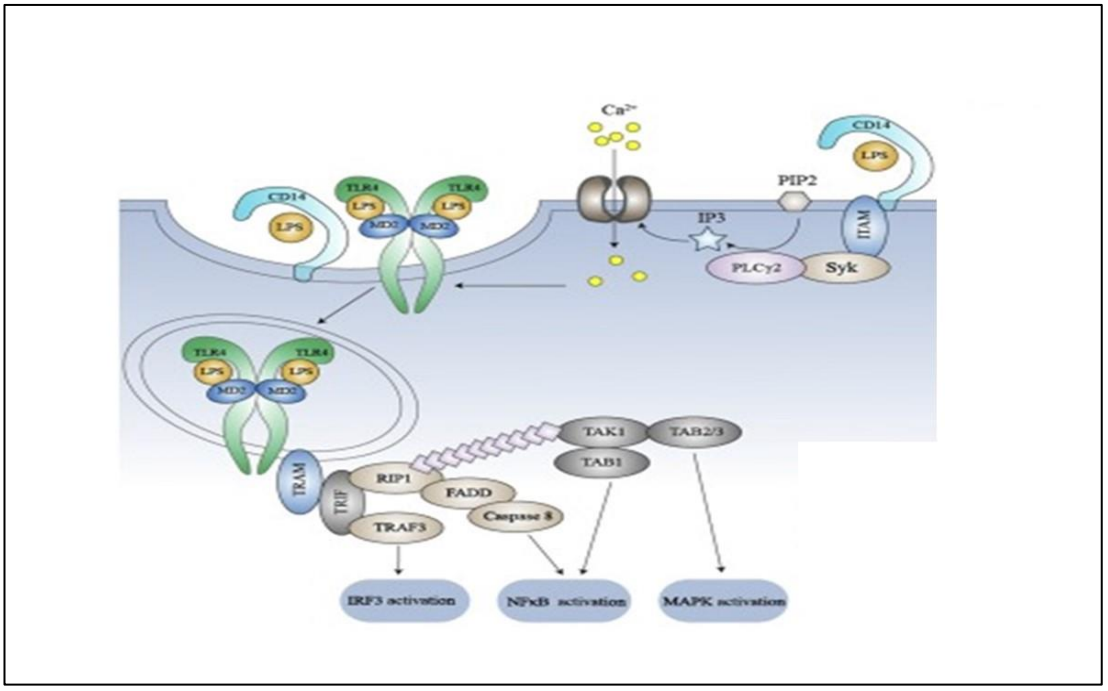

Figure 1. 3: CD14 activation of tyrosine kinase SYK and its downstream effector PLCγ2 through an ITAM-containing receptor.

PLCγ2 promotes the influx of extracellular Ca^{2+} and transports the TLR4 dimer to the endosome. In the endosome TLR4 induces TRIF activation following RIP-1 and TRAF3 recruitment. RIP-1 activates the TAK1 complex and Caspase 8 and the connected MAPK and NFκB. TRAF3 recruits downstream adaptors for IRF3 phosphorylation. (Note : this figure is published by Wu et al., 2019 CD14: Biology and role in the pathogenesis of disease - ScienceDirect).

Similarly, the cross-linking of the FcγR to their ligands will generate signal through SYK as presented in (Figure 1.4). The ligation of the FcR triggers phosphorylation of the cytoplasmic domain through ITAM. Afterwards, the SRC family kinase (Lyn, Src, Fyn, Feg and Hck) starts tyrosine phosphorylation of the ITAMs in the

cytoplasmic domains of these receptors. This is followed by recruitment of SYK-family kinases, resulting in activation of PLCγ. Activation of PLCγ causes an increased in intracellular calcium. This leads to phagosomes formation by membrane remodelling and actin cytoskeleton rearrangements. Then phagosomes fuse with lysosomes, releasing reactive oxygen species which is crucial for digestion of engulfed particles in phagosomes. Thus, activated monocytes/macrophages, up-regulate phagocytosis, Ag uptake, and internalisation of pathogens during inflammation that involves SYK signalling (Ghazizadeh et al., 1995, Poe et al., 2018). This is followed by up-regulation of co-stimulatory molecules and enhanced Ag presentation (Flynn et al., 2015). In addition, SYK activation controls the migration of myeloid cells to the site of infection to perform antigen uptake in response to the chemotactic CX3CL1, which is expressed in inflamed tissues (Gevery et al., 2005, Flynn et al., 2015). More importantly, SYK activation in monocytes/macrophages induced proinflammatory cytokines production including TNF-α, IL-6, IL-12 and the proinflammatory mediator NO (Yi et al., 2014, Hill et al., 2018). Thus, this study aims to control monocytes/macrophages allogenic response by inhibiting SYK signalling.

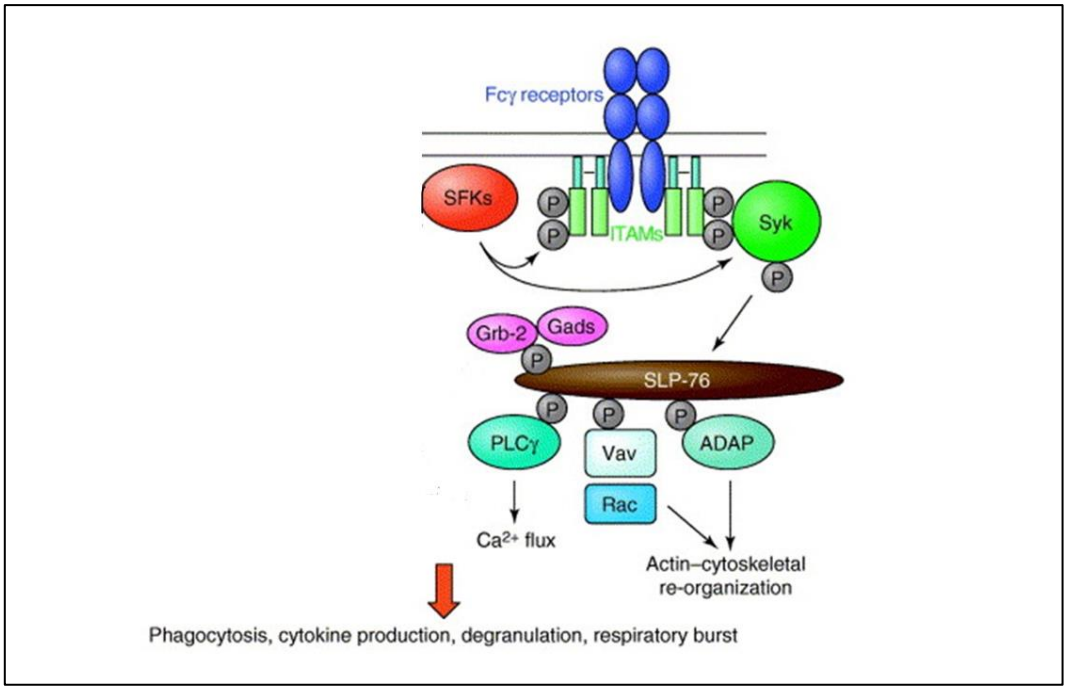

Figure 1. 4: The Fcγ receptors downstream signalling pathways: Signalling -mediated by ITAM involves sequential action of SFKs (red) and SYK kinases (green). Antibody Fc domains are used to aggregate the receptors, which causes SFKs Phosphorylation of the ITAM residues (green) on the receptor-associated γ-chains. The ITAM tyrosine phosphorylation allows SYK SH2 domains

association, which result in activation and unfolding of the SYK enzyme. Additionally, phosphorylation of sites between the kinase domain of SYK and the C-terminal SH2 domain by SFKs also causes SYK enzymatic activation. The activated SYK can then phosphorylates other substrates, including adaptor-type proteins, for example SLP-76 (brown), which then recruits additional molecules, including Grb-2 (pink), phospholipase Cγ (PLCγ) (turquoise), Vav (pale blue), Gads (pink) and ADAP (pale turquoise) (adhesion and degranulation promoting adaptor protein), this result in stimulation of downstream pathways, including mitogen-activated protein kinase (MAPK) activation and actin polymerisation. This can result in cytokine production, phagocytosis and degranulation respiratory burst (Not: this figure is published by Berton et al., 2005 Src and Syk kinases: key regulators of phagocytic cell activation - ScienceDirect).

1.10.1 SYK inhibition:

SYK inhibitors, tested in murine models, showed promising results in controlling autoimmune diseases such as rheumatoid arthritis and systemic lupus (Braselmann et al., 2006, Leonhardt et al., 2011, Hill et al., 2018). Fostamatinib, and Entospletinib are orally available SYK inhibitors in early phase II clinical trials for human (Reilly et al., 2011), mainly their therapeutic approach is in targeting the SYK pathway through BCR on B cells malignancy, by inhibiting ATP phosphorylation in SYK and Y352 regions (Hill et al., 2018).

SYK activation is a fundamental element for B cells migration and survival (Yi et al., 2014), and in murine cGVHD (Flynn et al., 2015). B cells collected from cGVHD patients showed activated SYK signalling (Poe et al., 2018). Studies have shown that Fostamatinib (non-specific SYK inhibitor) can effectively reduce B cell activation in cGVHD (Flynn et al., 2015). Interestingly, 4 µM of Fostamatinib in a murine model showed promising results by deactivation of DCs and successfully reduced T cells alloresponse, with reduction of IFN-γ, IL-6 concentration, which resulted in lowered GVHD and GVL (Leonhardt et al., 2012). It has been reported that daily treatment of Fostamatinib interfered with the expression of co-stimulatory molecules, such as CD80, CD86,MHCI and MHCII in DCs, in a mouse model of cGVHD (Leonhardt et al., 2012, Flynn et al., 2015). Another study used Entospletinib anti-SYK treatment early after HSCT in a murine model, which showed a reduction of the skin manifestations in GVHD (Poe et al., 2018). It has been reported that PRT062607, an oral small SYK inhibitor, selectively inhibits BCR and Fc γ RI on APCs; 1 nM of PRT062607 successfully inhibited SYK phosphorylation after BCR activation in chronic lymphoid leukaemia, which proves

its effectiveness and induce cellular apoptosis (Spurgeon et al., 2013). It has been reported that targeting SYK on macrophages may show a therapeutic effect in cGVHD (Flynn et al., 2015). In autoimmune diseases, the IgG mediated response involved SYK activation which control myeloid cells Ag presentation, blocking of SYK defect Ag recognition, Ag presentation and suppression of T cells activation (Kato et al., 2017).

Although Fc receptors require the presence of antibodies for efficient antigen capture, in GVHD it is feasible that both allogeneic and self-antigens could trigger the generation of specific antibodies and cause this reaction (Claude et al., 2005). Furthermore, it is becoming increasingly evident that autoimmunity, including auto-antibodies may play a significant role in GVHD development (Claude et al., 2005). As FcR are expected to contribute to the efficient capture and presentation of auto-antigens in GVHD (Im et al., 2017, Hong et al., 2020). Meaning targeting FcR signalling on monocytes/macrophages using small molecule SYK inhibitors may show therapeutic approach (Hong et al., 2020). SYK inhibition can inhibit cytoskeletal rearrangement, which is essential for monocytes/macrophages phagocytosis (Braselmann et al., 2006). The therapeutic effect of anti SYK has been extensively study in B cells as a treatment option for cGVHD, with little data about targeting SYK on monocytes/macrophages through FcγR in aGVHD. Monocytes/macrophages are an interesting target due to their role in the early phase of priming T cells, which is essential for the development of aGVHD. PRT062067 is known as P505-15 (Hoellenriegel et al., 2012), a highly selective SYK inhibitor, that completely suppresses BCR, FcR in blood samples collected from heathy donors and rheumatoid arthritis patients (Coffey et al., 2017). In this study, PRT0603 was selected as parent compound of PRT062607 suitable for *in vitro* model testing. PRT0603 also known as PRT318, is a highly selective SYK inhibitor that successfully targeted FcγR SYK downstream signals in platelets to treat Heparin induced thrombocytopenia using mice model (Reilly et al., 2011). PRT0603 inhibits SYK after BCR cross-linked causing CLL cells apoptosis and inhibiting chemotaxis (Hoellenriegel et al., 2012). Although Fostamatinib, and Entospletinib are widely used SYK inhibitors, they are both non-selective and have significant off target effects, including inhibition of FMS-related tyrosine kinase 3 (FLT-3), Lck, Janus kinase 1 and 3, and c-kit (Spurgeon et al., 2012, Coffey et al.,

2017). The nonspecific binding could be the cause of several side effects seen with Fostamatinib treatment in rheumatoid arthritis clinical trials, including hypertension, diarrhoea, nausea, headache, dizziness vomiting, dyspepsia, and arthralgia (Kang et al., 2019). This highlights the need for long term follow up study, to evaluate the efficacy of Fostamatinib (Kang et al., 2019). In contrast, PRT0603 is a highly selective SYK inhibitor that shows therapeutic potential in rheumatoid arthritis and thrombocytopenia patients by inhibiting SYK signalling through BCR on B cells and FcRs on platelets. Although there is broader reactivity of non-selective SYK inhibitors such as Fostamatinib and Entospletinib which could produce therapeutic effects in cancer and autoimmunity, it is important to determine exact contribution of SYK inhibition to the pathological process in a particular disease such as GVHD. This would provide an insight into the disease mechanisms and also define the therapeutic effects of the selective targeting of the single pathway. Indeed, the use of Fostamatinib, or Entospletinib would produce questions about the exact mode of their action and the role of inhibition of individual signalling pathways that are affected as these are non-selective inhibitors.

The downstream signalling via CD14 and FcRs on monocytes/macrophages is controlled by SYK activation was described in section 1.10. This study evaluates the therapeutic potential of PRT0603 to control the early allogeneic response by monocytes/macrophages. This could be achieved by inhibition of the phosphorylation of SYK in the SYK-dependent FcγRs mediated activity in monocytes/macrophages in mismatched MLR. This could modulate early co-stimulation and Ag-presentation by monocytes/macrophages, leading to diminished alloresponse in the histo-incompatible model. Although the data collected in this report may not reflect the physiological condition in MHC matched patients, the data could demonstrate the potential of highly selective SYK inhibitor to control early allogeneic response. Further study will be needed to assess the toxicity and side effects of PRT0603.

1.11 Hypothesis:

This study hypothesised that regulation of the early phase of the allogeneic reaction could be achieved by controlling monocytes/macrophages functions, including their co-stimulation and Ag presentation capacity. Furthermore, the study aimed to define the effects of the highly selective SYK inhibitor, PRT0603, on monocytes/macrophages activation, cytokine production and the allogeneic response.

1.12 Aims:

The early activation of APCs is a crucial event during the initiation of GVHD. Thus, monocytes/macrophages are involved triggering inflammation by presenting the allo-Ag to T cells, which results in T cells activation and proliferation, as well as the secretion of cytokines. The study aims to investigate monocyte derived macrophage function in the allogeneic culture (mixed leukocyte's reaction - MLR). Furthermore, the allogeneic culture was modulated with cytokines. This was achieved by administration of Th1 (IFN-γ) and Th2 (IL-4) cytokines, and their effects on monocytes/macrophages in the early allogeneic response were evaluated. Finally, the therapeutic effect of using SYK inhibitor (PRT0603) in controlling monocytes/macrophages during early phase of allogeneic response was investigated.

1.13 Objectives:

- Monocyte/macrophage function was evaluated by measuring the expression of the surface markers, including co-stimulatory molecule (CD86), Ag presentation function (HLA-DR), marker for classical, intermediate monocyte subtype (CD64 and CD32B), recognition of apoptotic cells (CD36 and CD204), complement receptor (C3aR) and adhesion molecule expression (CD11b) using flow cytometry analysis.
- CD3 and CD4 T cell activation was investigated by measuring median fluorescence intensity (MFI) expression of CD25 and proportion (%) of the CD69 surface marker.
- The level of inflammation was evaluated by measuring the concentrations of the cytokines (TNF-α, IFN-γ, IL-2 and IL-6) using ELISA.

2 Material and Methods:

2.1 Materials:

2.1.1 Reagents, chemicals, and supplier information:

Table 2. 1: Details of the chemicals and reagents used and which supplier they were purchased from.

Reagent	Manufacturer
Annexin V -FITC	BD Biosciences (USA).
Dimethyl sulfoxide (DMSO)	Thermo fisher (USA).
Dulbecco's Phosphate Buffered Saline (DPBS)	Thermo fisher (USA).
Ethanol (100%)	Thermo fisher (USA).
Fatal Calf Serum (FCS)	Thermo fisher (USA).
Fc Blocking	BD Biosciences (USA).
Gluta Max	Thermo fisher (USA).
Fixation buffer	BD Biosciences (USA).
Human IFN-γ DuoSet ELISA Development system	Thermo fisher (USA).
Human IL-2 DuoSet ELISA Development system	Thermo fisher (USA).
Human IL-6 DuoSet ELISA Development system	Thermo fisher (USA).
Human TNF-α DuoSet ELISA Development system	Thermo fisher (USA).
Heparin tube	Thermo fisher (USA).
Hyclone	Thermo fisher (USA).
APC Mouse anti-human CD3 Antibody	BD Biosciences (USA).
APC Mouse anti-human CD4 Antibody	BD Biosciences (USA).
APC Mouse anti-human CD14 Antibody	BD Biosciences (USA).
PE Mouse anti-human CD11b Antibody	BD Biosciences (USA).
PE Mouse anti-human CD25 Antibody	BD Biosciences (USA).
PE Mouse anti-human CD32 Antibody	BD Biosciences (USA).
PE Mouse anti-human CD36 Antibody	BD Biosciences (USA).

PE Mouse anti-human CD64 Antibody	BD Biosciences (USA).
PE Mouse anti-human CD69 Antibody	BD Biosciences (USA).
PE Mouse anti-human CD86 Antibody	BD Biosciences (USA).
PE Mouse anti-human CD204 Antibody	BD Biosciences (USA).
PE Mouse anti-human C3R Antibody	BD Biosciences (USA).
PE Mouse anti-human HLA-DR Antibody	BD Biosciences (USA).
Mouse IgG_1, κ Isotype Ctrl Antibody	BD Biosciences (USA).
Mouse IgG_2b, κ Isotype Ctrl Antibody	BD Biosciences (USA).
Penicillin/Streptomycin	Thermo fisher (USA).
Recombinant Human Interleukin 4 (rhIL-4) (molecularweight 15 kDa)	R&D systems (USA).
Recombinant Human Interleukin 6 (rhIL-6) (molecularweight 20.3 kDa)	R&D systems (USA).
Recombinant Human Interferon gamma (IFN-γ) (molecularweight 16.9 kDa)	R&D systems (USA).
RBCs lysing buffer	BD Biosciences (USA).
Rosewell Park Memorial Institute media (RPMI)	Thermo fisher (USA).
Staining reagent	BD Biosciences (USA).
PRT0603 (anti- SYK inhibitor molecular weight 340.43 kDa)	Synkinase (USA).

2.1.2 Flow cytometer antibodies:

All antibodies used were purchased from BD Biosciences (USA).

Table 2. 2: Details of antibodies used for flow cytometry. Fluorochrome and conjugation antibodies used per 1×10^4 cells.

Antigen	Clone	Conjugation
CD3	UCHT1	APC
CD4	RPA-T4	APC
CD11b	ICRF44	PE

CD14	M5E2	APC
CD25	M-A251	PE
CD32	FLI8.26	PE
CD36	CB38	PE
CD64	10.1	PE
CD69	FN50	PE
CD86	2331	PE
CD204	U23-56	PE
HLA-DR	TU36	PE
C3aR	Hc3Arz8	PE

2.1.3 Cell culture complete media:

Cell culture media, RPMI Gluta MAX (Thermo Fisher Scientific, MA, USA), was supplemented with 10 % foetal bovine serum (R&D Systems, Abingdon, UK) and 1 % penicillin/streptomycin (Sigma-Aldrich, Poole, UK).

2.1.4 Consumables for tissue culture:

15 ml and 50 ml sterile polypropylene centrifuge tubes, falcon 5 ml round bottom polystyrene tubes, cryovials, 24 well plates, pipettes, and sterile tips were purchased from Thermo Fisher Scientific (MA, USA).

2.1.5 Stimulus/inhibitor reconstitution:

2.1.5.1 Recombinant human IFN-γ protein:

IFN-γ (200 µg) powder, was obtained from R&D Systems, Abingdon UK, reconstituted using 1000 µl of sterile PBS and stored at -20 ^{o}C. This generates the stock solution of 200 µg/ml). The working solution was prepared by diluting the stock solution 1:20 with sterile PBS on the day of experiment. This generates diluted solution of 10 µg/ml. The final concentration of 100 ng/ml (0.1 µg/ml) was achieved by further 100x dilution of the 10 µg/ml working solution in tissue culture well. Typically, this was done by adding 10 µl of working solution (IFN-γ 10 µg/ml) into 1ml of the cell culture medium in tissue culture well.

2.1.5.2 Recombinant human IL-4 proteins:

10 µg of IL-4 protein was reconstituted in 1000 µl of sterile phosphate buffered saline (PBS) with 0.1 % serum and stored at -20 ^{o}C (stock solution 10 µg/ml). A 100 ng/ml for IL-4 final concentration was prepared by diluting the stock solution 100x or 200x in tissue culture wells on the day of the experiment.

2.1.5.3 PRT0603 SYK inhibitor:

The SYK inhibitor PRT0603 (Mw = 340.4) is soluble in DMSO, and the stock of 5 mg was aliquoted by weighing 0.6808 mg of the substance and dissolving it into 1ml of DMSO. This provides 2 mM solution, which is further diluted 1:10 in RPMI to obtain 0.2 mM (200 µM) working solution. This is followed by pipetting 10 µl of this solution into 1 ml of cell culture to achieve final inhibitor concentration of 2 µM. Notably, the reported IC50% values for SYK inhibitors, including PRT0603, depend on the experimental model and vary from nM to µM concentrations. As this study uses whole blood culture, higher range of inhibitor concentrations is necessary, in contrast to experimental models that use isolated cells or cell lines. In a study by Nani et al., PRT0603 was used at 1 µM (Nani et al., 2014) which is comparable to concentration used in this study.

2.1.6 ELISA:

2.1.6.1 Reagent diluent and washing buffer:

R&D ELISA kit provided x10 reagent diluent and x10 washing buffer, these were diluted freshly on the day of experiment 1:10 with distilled water.

2.1.7 Samples:

Buffy coat samples were provided from the National Health Service, blood, and transplantation service (NHSBT, London, UK).

2.2 Methods:

2.2.1 Buffy coat sample:

Two samples of Buffy coat were received weekly and used in this study. The blood provided from NHSBT. All samples were labelled as non-clinical use and tested negative for human immunodeficiency virus (HIV), hepatitis C virus (HCV), current hepatitis B infection (HBsAg) and syphilis. All blood donors had signed informed

consent, which was provided by NHS, regarding the use of the donors' blood for training and education purposes, to ensure that the samples were ethically approved (NHSBT, 2015). Institutional approval was obtained (ETH1617-0362), All samples preparation was carried out in sterile conditions in a class II safety cabinet.

2.2.2 Samples preparation:

86 samples used in this study were fresh, and 14 samples were excluded as they collected one day before they were received. As 2 Buffy coat samples were received each week, a randomly selected bag was chosen to be the responder cells and the second sample was selected as the stimulatory cells.

2.2.3 Cell count:

Trypan blue (Sigma, UK) was used to assess cell viability and carry out the cell count. 20 µl of cell suspension was mixed with trypan blue dye in a 1:1 dilution. Cell counting was performed using a Neubaur chamber. Typically, >85 % of cells were viable when counted in the four large squares of the chamber. Cell viability and cell counts were calculated using the formulas:

Calculate cells viability = count average live cells / total number of cells x 100

Calculate cell density (cell/ml) = average of live cells x dilution factor/ volume of square

2.2.4 Preparation of responder cells (Res) and stimulatory cells:

The Buffy coat bag was swabbed with 70 % ethanol prior to some being transferred to a 6 ml heparin tube, which was gently inverted 6-8 times to allow mixing of the sample with the anticoagulant. Lysis of the RBCs was carried out with lysis buffer (BD Biosciences, USA). Cell number was analysed using a cell count, then in a 15 ml falcon tube, the sample was diluted 1/2 or 1/5 with complete RPMI to achieve a cell count of around $10x10^6$ cells/ml (Figure 2.1).

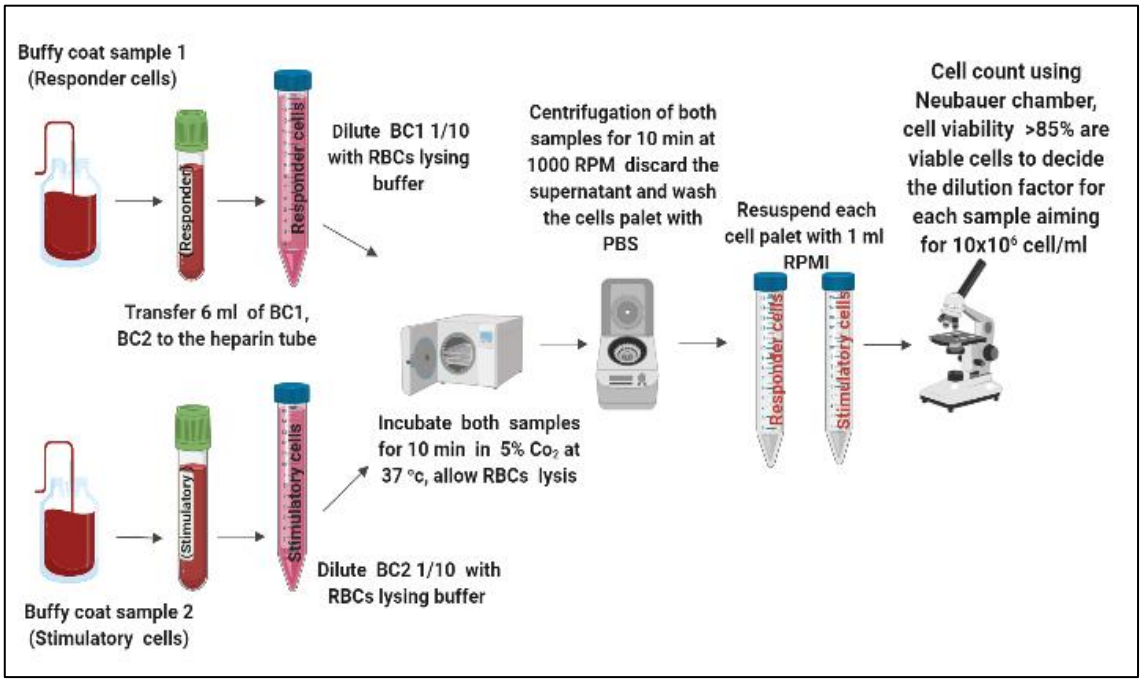

Figure 2. 1: Responder and stimulatory cell preparation and cell counting.

2 Buffy coat samples were randomly selected to prepare Responder cells and Stimulatory cells, samples were transferred to 6 ml heparin tubes followed by RBCs lysis, the cell pellet was resuspended in RPMI and cells were counted to decide the dilution factor for the stimulatory and responder required. Created with BioRender.com

2.2.5 Cell culture preparation:

2.2.5.1 Culture the responder cells (Res):

Buffy coat samples were diluted 1/2 or 1/5 with complete RPMI to achieve a cell count of $10x10^6$ cells/ml. 500 µl of the responder cells sample was added to each well of the 24 well plate and incubated for D1, D2 and D3 at 37 ^oC with 5 % CO_2. Samples were analysed on D1 and D2 for monocyte activity and on D3 for T cell activity (Figure 2.2). Res without any additional cytokines was the -ve allogenic control.

2.2.5.2 Mixed leukocyte reaction (MLR) preparation:

450 µl of responder cells were added to 50 µl of stimulatory cells in a 10/1 ratio with 500,000 cells/well in total. 500 µl of the cellular mix was placed in each well of the 24 well plate and incubated for D1, D2 and D3 at 37 ^{o}C with 5 % CO_2. Samples were analysed by flow cytometry on D1 and D2 for monocytes activity and D3 forT cells activity (Figure 2.2). MLR without any additional cytokines was the optimal +ve allogeneic control.

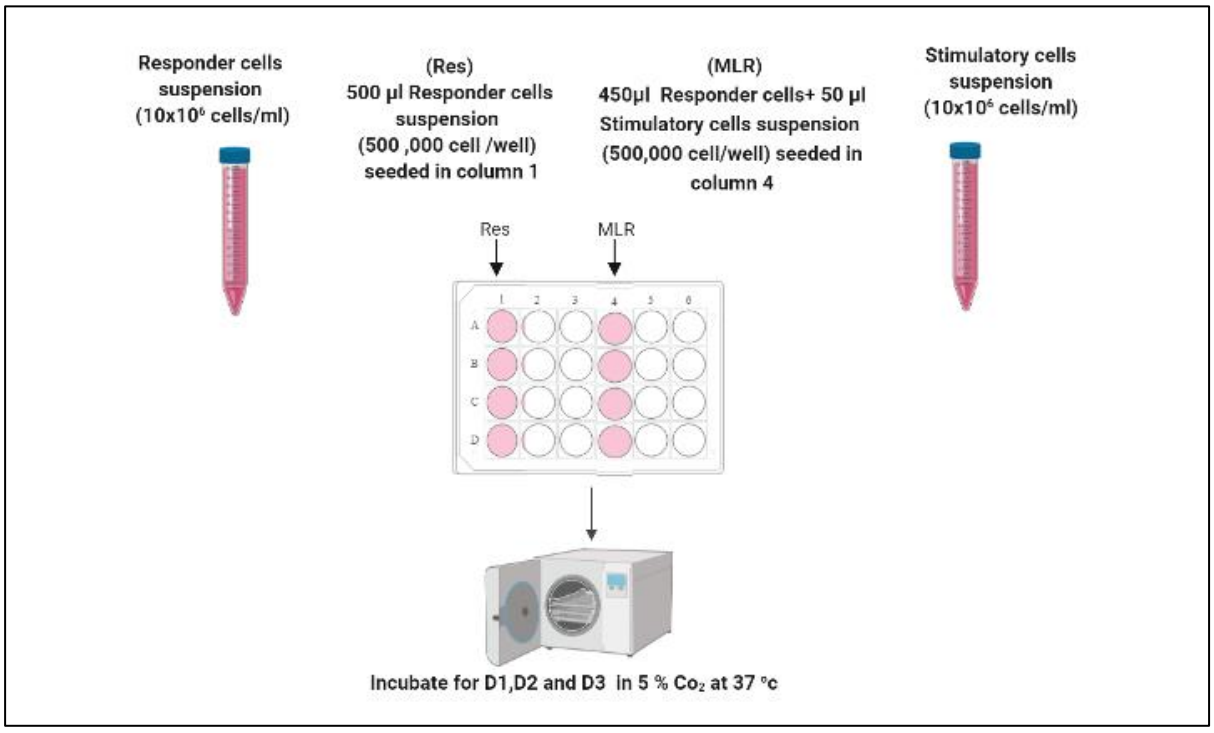

Figure 2. 2: Res and MLR preparation and incubation:

Responder and stimulatory cells were resuspended in complete RPMI at 10x10^6 cells/ml. In column 1 Res cells were seeded (500,000 cells/well) and column 4 MLR, which is mix of responder cells and stimulatory cells diluted 10/1 to have 500,000 cells/well were seeded. Created with BioRender.com

2.2.6 Stimulating cells culture:

2.2.6.1 Optimising IFN-γ concentration:

MLR culture was prepared (Section 2.2.5.2) to optimise the IFN-γ concentration. In MLR, three different concentrations were added (10 ng/ml, 50 ng/ml, and 100 ng/ml) and samples were incubated and analysed by flow cytometry after D1 and D2 of incubation.

2.2.6.2 Optimising IL-4 concentration:

MLR culture was prepared as explained earlier (Section 2.2.5.2). To optimise IL-4 concentration in MLR, three different IL-4 concentrations were tested by adding 10 ng/ml, 50 ng/ml, and 100 ng/ml to 3 MLR samples which were incubated and analysed by a flow cytometer after D1 and D2 of incubation.

2.2.6.3 MLR stimulated with IFN-γ and IL-4:

MLR culture was prepared as explained earlier (Section 2.2.5.2) by mixing two diluted Buffy coat samples in 24 well plate, cells count was around 500,000 cells/well (Figure 2.3). The optimised 100 ng/ml IFN-γ or 100 ng/ml IL-4 were added to MLR during culture preparation, and the samples were incubated and analysed by flow cytometry after D1, D2 and D3 of incubation.

2.2.6.4 Responder cells stimulated with IFN-γ and IL-4:

500,000 cells/well responder cells (Res) added to 24 well plate supplemented with 100 ng/ml of recombined IFN-γ protein or 100 ng/ml IL-4 (R&D systems, USA), on day of tissue culture preparation (Figure 2.3). Res IFN-γ which was used as -ve allogeneic control treated with stimulus to be compared with MLR treated with the same stimulant.

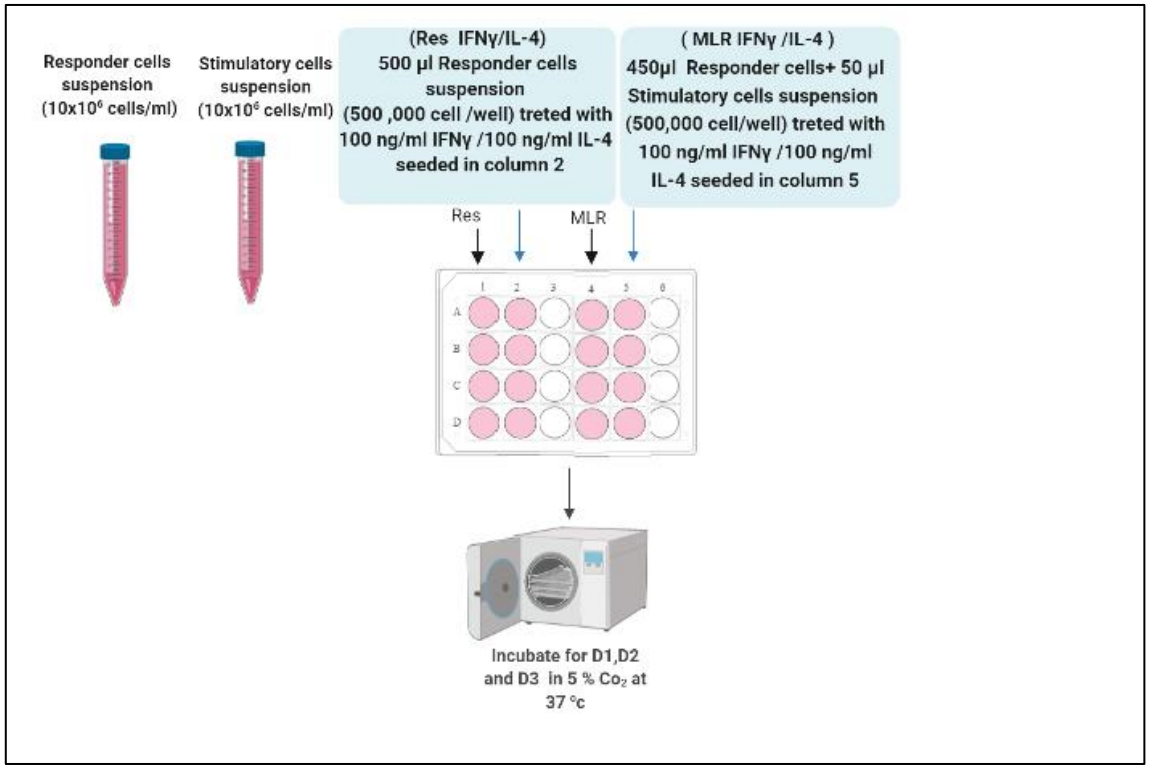

Figure 2. 3: Res IFN-γ/IL-4 and MLR IFN-γ /IL-4 preparation diagram:

Responder and stimulatory cells were resuspended in complete RPMI to have $10x10^6$ cells/ml. In column 1 and 2 Res were seeded (500,000 cells/well) and in column 4 and 5 500,000 cells/well of MLR, which is mix of responder cells and stimulatory were seeded. The additional treatment of 100 ng/mlIFN-γ or IL-4 was added to Res in column 2 to make Res IFN-γ or Res IL-4 similarly IFN-γ or IL-4 added to MLR in column 5 to make MLR IFN-γ or MLR IL-4. Created with BioRender.com

2.2.7 Inhibiting the stimulated cells cultures:

2.2.7.1 Inhibition of MLR IFN-γ and MLR IL-4 with anti-SYK:

MLR IFN-γ and MLR IL-4 cultures was prepared (Section 2.2.6.3) with the addition of 2µM of PRT0603 daily on D0, D1, and D2 (Figure 2.4). The MLR IFN-γ SYK, or MLR IL-4 SYK culture was incubated for three days at 37 ^oC with 5 % CO_2. Analysis performed on day D1, D2 and D3 using flow cytometry. The MLR IFN-γ SYK or MLR IL-4 SYK results were compared to the corresponding MLR IFN-γ, or IL-4, Res IFN-γ or lL-4 and Res IFN-γ SYK or Res IL-4 SYK to evaluate the inhibitory effect of the anti-SYK treatment.

2.2.7.2 Inhibition of Res IFN-γ and Res IL-4 with anti-SYK:

Res IFN-γ and IL-4 cultures were prepared (Section 2.2.6.4) with the addition of 2 µM of anti-SYK on D0, D1, and D2 to make Res IFN-γ SYK or Res IL-4 SYK. The culture incubated for three days at 37 ^oC with 5 % CO_2 (Figure 2.4). Analysis was carried out on D1, D2 and D3 using flow cytometry. The Res IFN-γ SYK or Res IL-4 SYK results were compared to the corresponding Res IFN-γ or Res IL-4, MLR IFN-γ or MLR IL-4 and MLR IFN-γ SYK or IL-4 SYK to evaluate the inhibitory effect of the anti-SYK treatment.

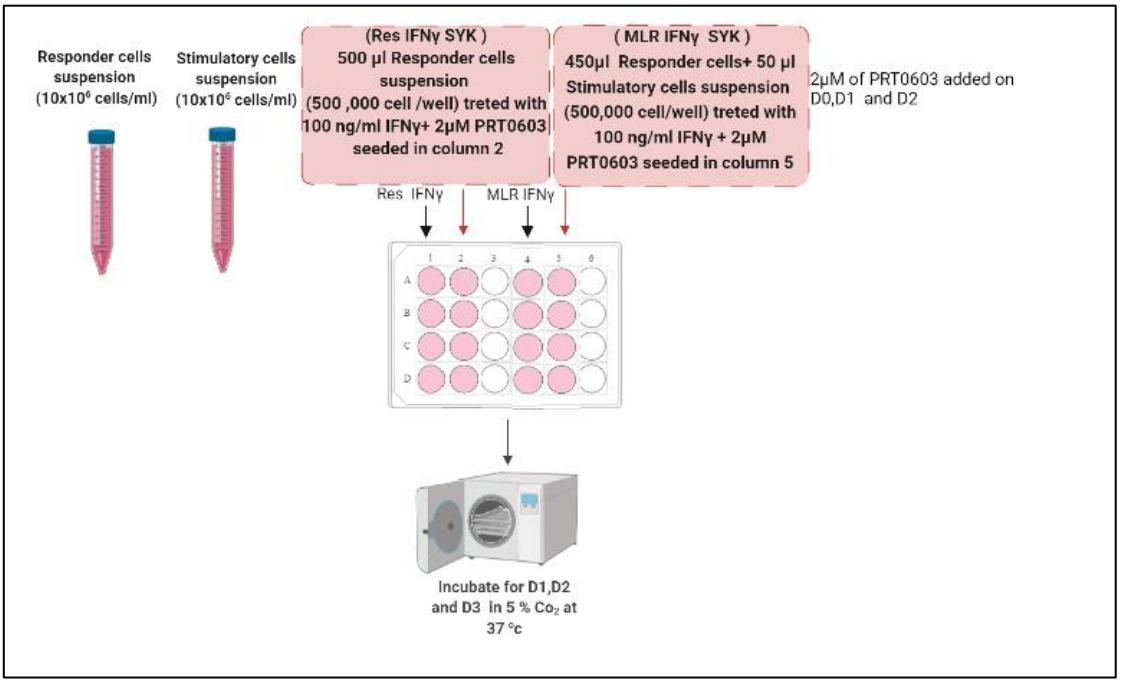

Figure 2. 4: Figure: inhibiting Res IFN-γ and MLR IFN-γ by anti-SYK diagram:

Responder and stimulatory cells resuspend in complete RPMI to have 10x10^6 cells/ml. In column 1 and 2 Res IFN-γ (500,000 cells/well) was seeded with 100 ng/ml IFN-γ. In column 4 and 5 MLR IFN-γ (500,000 cells/well), a mixture of responder cells and stimulatory was treated with 100 ng/ml IFN-γ. Cells in columns 2 and 5 were supplemented with 2 µM of PRT0603 to make Res IFN-γ SYK and MLR IFN-γ SYK, respectively. PRT0603 treatment was applied on D0, D1and D2. Created with BioRender.com

2.2.8 Flow cytometer:

The sample analysis was performed using a CyAn ADP flow cytometer (Beckman Coulter, USA) which has two excitation lasers: a blue laser 488 nm and a red laser 640 nm. Minimum of 5,000 events were acquired for monocytes/T cells. The data were analysed by using the Summit software (version 4.3).

2.2.9 Cell surface phenotyping:

The procedure of cell surface phenotyping was performed in capped, round bottom polystyrene flow cytometer tubes (FACS tubes) after one, two and three days of MLR incubation. Four FACS tubes were used as set-up tubes and a minimum of four tubes were used as sample tubes (depending on number of MLR conditions). 20 µl of the selected antibodies (Table 2.3) conjugated anti-human CD14 APC, conjugated anti-human CD86-PE, conjugated anti-human HLA-DR PE, conjugated anti-human CD64-PE, conjugated anti-human CD32-PE (BD

Biosciences, USA), conjugated anti-human CD11b-PE (BD Biosciences, USA), conjugated anti-human CD36-PE (BD Biosciences, USA) conjugated anti-human C3R-PE and conjugated anti-human CD204-PE (BD Biosciences, USA) were added to identify monocytes/macrophages.

For T cell immunophenotyping (Table 2.3), 20 µl of conjugated anti-human CD3-APC (BD Biosciences, USA), conjugated anti-human CD4-APC (BD Biosciences, USA), conjugated anti-human CD69-PE (BD Biosciences, USA), conjugated anti-human CD25-PE (BD Biosciences, USA), and conjugated anti-human annexin V FITC (BD Biosciences, USA) were used to detect the different markers.

The experimental sample tubes for monocytes/macrophages were all treated with Fc blocking (Section 2.2.12) before the addition of the conjugated antibodies. The conjugated antibodies were mixed in the FACS tubes with 100 µl of sample (100,000 cells/tube) collected from the 24-well plates and incubated for 20 minutes in the dark at room temperature (RT). All samples were washed with 2 ml of PBS, vortexed and centrifuged (1500 RPM for 5 minutes at RT), and this was repeated twice. The supernatant was removed and finally cells were resuspended in 200 µl of fix buffer (BD Biosciences, USA).

2.2.10 Controls:

Negative control: also known as unstained control, was used to identify the negative population, to set the negative gate and to eliminate the autofluorescence cells in flow cytometry.

Setup tube: single stain sample run before the experimental run to adjust the voltage of each channel, APC, PE and FITC.

Isotype control: used to remove any background or non-specific antibody (Ab) binding of the expressed monocyte and macrophage cell surface antigens; the isotype was used in this study to match the host species, fluorophore, and Ig subclass for both IgM, k to match CD36 (BD Biosciences, USA), PE IgG2ak (BD Biosciences, USA), and HLA-DR (BD Biosciences, USA). PE IgG1, k(BD Biosciences, USA) was used to match CD86, CD32, CD69 and C3R.

Table 2. 3: Table of the fluorochrome conjugate panel.

	Antibodies	Fluorochrome
Monocytes/macrophages	CD11b	PE
	CD14	APC
	CD32	PE
	CD36	PE
	CD64	PE
	CD86	PE
	CD204	PE
	C3R	PE
	HLADR	PE
T cells	CD3	APC
	CD4	APC
	CD25	PE
	CD69	PE

2.2.11 Fc blocking:

Following the manufacture's protocol, 100 µl (100,000 cells) was suspended in 100 µl of staining buffer (BD Biosciences, USA) with 2.5 µl of Fc blocking buffer (BD Biosciences, USA), and incubated for 10 minutes at room temperature before immunophenotyping.

2.2.12 Cell viability test:

Annexin V conjugated to FITC used to evaluate cell viability, Annexin V binds to PS, the early marker for apoptotic cells. It was essential to check cell viability as this study aimed to activate monocytes and T cells. All Annexin +ve cells were exclude from the monocytes and T cells gated population.

2.2.13 Cytokine quantification:

2.2.13.1 Sample collection:

Cytokines produced in the cell culture supernatant were collected in cryo-vials. 200 µl was collected from each experimental condition supernatant: responder cells (Res), Res IFN-γ, Res IFN-γ SYK, Res IL-4, Res IL-4 SYK, MLR, MLR SYK, MLR IFN-γ, MLR IFN-γ SYK, MLR IL-4 and MLR IL-4 SYK after D2 of incubation.

All samples were stored at -20 ^{o}C until the day of analysis.

2.2.13.2 Cytokine quantification by enzyme-linked immunosorbentassay (ELISA):

All samples thawed at room temperature. Standards and all samples were diluted 1:2 with reagent diluent (50 µl of the sample + 50 µl of reagent diluent) then added to the 96 well plate in duplicate. TNF-α, IFN-γ, IL-2 and IL-6 sandwich ELISA protocol was provided by the manufacturer (R&D system, USA). 69 well plate coated with capture Ab then the plate blocked with reagent diluent before adding the samples. After adding samples detection Ab added to 69 well incubate at RT. Then streptavidin-HRP added to 69 well plate avoiding direct light, followed by adding substrate solution and finally stop solution to stop the reaction. (Each well aspirated and three time washing with wash buffer between each step). 96 well ELISA plates were analysed using the ELISA reader (SPECTRO star Nano) from BMG Labtech, the reader wavelength set to 450nm, wavelength correction 570.

2.2.14 Statistical analysis:

Statistical analysis was performed using GraphPad Prism version 8.0 (Graph Pad software, CA, USA). The data in this report were analysed by parametric tests. The first assumption in the data analysis by parametric tests, is normality (Maxwell and Delaney, 2004), which was verified using the Shapiro-Wilk test. The Shapiro-Wilk test was selected due to the low number of repeats in this study. Namely, the experiments were repeated 6 times or less, and the Shapiro test requires 3 or more values to assess the normality, also it requires that every value is unique. In contrast, the D'Agostino test requires 8 or more values to perform the normality test, thus the D'Agostino test was not used in the data analysis for this thesis. Importantly, the normality tests used in this study have reported that all the values were sampled from a population that follows a Gaussian distribution. If the data did not follow Gaussian distribution, Dunn's multiple comparisons tests would be used.

Quantile-Quantile plot (QQ plot) is a scatter plot generated by plotting two sets of quantiles. For the QQ plot the X axis is the actual values, and the Y axis is the predicted values. QQ plot was used as a visual normality tool to show if the data were sampled from a Gaussian distribution (Maxwell and Delaney, 2004). The

CD86 expression on monocytes/macrophages, (data collected from Res and MLR cultures after Day 1 (D1) of incubation) data presented on (Figure 2 .1. A) showed normality: The data points fall in line which gave a visual indication that the samples were collected from a normally distributed population. Similarly, the QQ plot (Figure 2.5.B) showed normality of TNF-α concentration, (data collected on D2 from culture supernatant for Res and MLR). The points for MLR and Res fall in line which gave a visual indication that the TNF-α data were normally distributed. The QQ plot (Figure 2 .5.C) illustrated that CD25 expression on T cells, (data collected on D3 of incubation from Res and MLR). Formed a straight line which shows that the CD25 data were collected from Gaussian distribution.

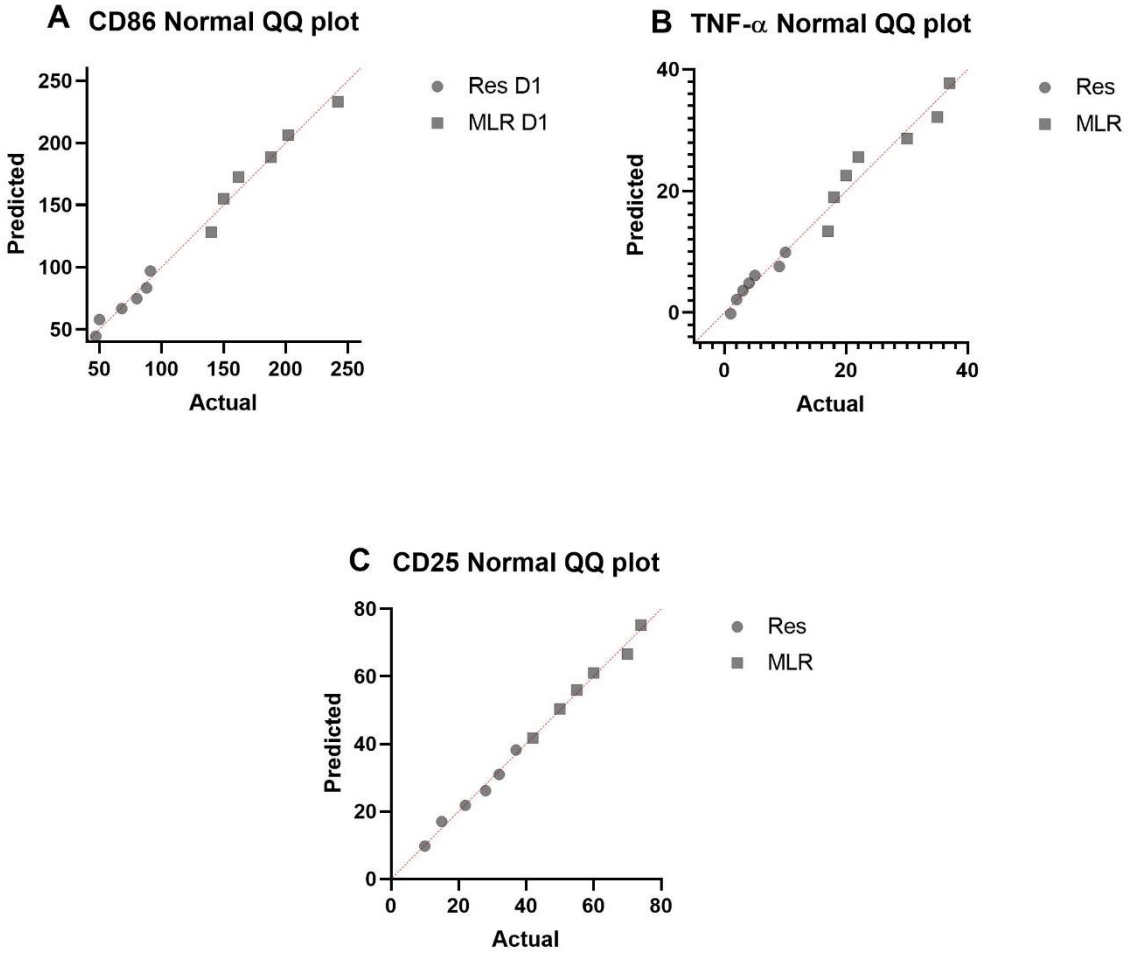

Figure 2. 5: Quantile-Quantile plot **(**QQ plot) shows A) MFI data for CD86 expression, B) TNF-α concentration and C) MFI data for CD25 expression measured from MLR culture and Responder cells controls.

The data were analysed on Day 1 (D1) in graph A, on D2 of incubation in graph B, and analyses performed on D3 in graph C. The X-axis is the actual values, and the Y-axis is the predicted values (assume Gaussian distribution). The data collected from responder cell culture are present as grey dots and data collected from MLR as grey squares. The distribution of data points was following the line, which gave a visual indication that the data collected from MLR, and Responder cells were derived from normally distributed populations.

The second assumption required for parametric tests is homogeneity of variance, and in this thesis, sphericity was selected, as the data were analysed by repeated measures ANOVA. The assumption of sphericity means that the sample was collected from a population with identical standard deviation (variance is square SD, if the SDs are equal, so are the variances). In other words, sphericity assume the differences between treatment groups are equal (Maxwell and Delaney, 2004). Deviations from sphericity in repeated measures ANOVA can be quantified by Mauchly`s test to calculate the value known as epsilon. However, Maxwell and Delaney reported that for small sample sizes repeated measure data sphericity, is usually violated (the *F*-statistic is positively biased and raised the risk of a Type I error). Thus, it is recommended to use Geisser Greenhouse correction to calculate epsilon by correcting the degrees of freedom for the *F*-distribution, to correct the bias that occur in the *F*-table (Maxwell and Delaney, 2004). When more than one independent categorical data was compared to one dependent variable, the data were processed by repeated measures one-way ANOVA. This included T cell activation analysis and cytokine levels comparison. However, when more than two independent categorical data were compared to two dependent variables, the data were processed by repeated measures two-way ANOVA. This included monocytes/macrophages phenotype analysis as the experiments were repeated on day 1 (D1) and day 2 (D2) of incubations with several treatments performed in parallel. Thus, both factors (time and treatment) were repeated measures, as the initial blood samples were cultured continuously throughout the experiment. The analysis of experiments with several cell cultures conditions, and treatment options included post hoc analysis test, such as Tukey or Sidak methods, where comparisons of multiple groups were addressed. Tukey or Sidak tests used compares every mean with every other mean and is used when the n number was identical in all the experimental groups (Kim, H 2015). When comparing means of two independent variables, Welch's test was used (Ruxton D., 2006, Wilcox.,2012). Welch's test was used to analyse T cells activities and cytokine

concentrations in the first result chapter. Welch's test requires the data to be normally distributed, but do not expect the homogeneity of variance. Notably Welch's test controls type I errors when the assumption of the homogeneity is violated and sample sizes are small comparing to student t-test (Zimmerman, D 2010, Delacer et al., 2017). One of limitations in this study is the small sample size, therefore Welch's test was used for analysis. All measured variables were presented as mean, and error bars represent standard deviation (SD). P values below 0.05 were considered significant. In this thesis, mismatched blood samples were used, thus fluctuation of the level of HLA and miHA mismatch resulted in different strengths of immune response in each experiment with consequently high error bars.

3 Results (Part 1):

3.1 Introduction:

Monocytes are myeloid precursors cells, that occur in the circulation of healthy people for up to 3 days. Notably, once these cells move to tissues, they differentiate to macrophages or dendritic cells (DCs) based on the tissue microenvironment (Duque & Descoteaux, 2014, Tacke & Randolph 2006, Sprangers et al., 2016). The differentiation from monocytes to monocyte derived macrophages (monocytes/macrophages) involves an increasein cellular size and more complex organelles, with more phagocytic capacity (Duque & Descoteaux, 2014). Monocytes and macrophages are part of innateimmune response and act as alarm generators to activate the immune system (Visentainer et al., 2003). Monocytes and macrophages cell surfaces are covered with many receptors to support their function such as FcR, scavenger receptors, TLRs and complement receptors (Hong et al., 2020, Martinez et al., 2020). Macrophages are one of the professional APCs, with functions including Ag recognition, Ag internalisation, phagocytosis, Ag processing, loading of MHCI/ MHCII with peptides to present self or non-self Ag to T cells (Chakraverty & Sykes 2007, Hong et al., 2020). Furthermore, following TBI and tissue injury, monocytes/macrophages activation results in a cytokine storm (Chakraverty & Sykes 2007). Naive donor T cells interact with both donor and host macrophages to recognise polymorphic alloantigens, that result in T cells alloactivation (Chakraverty & Sykes 2007, Blazar et al., 2013). In order to activate T cells three signals are required, the first signal involves co-stimulation, followed by Ag presentation through MHCI, MHCII and finally cytokines that are predominantly secretedby APCs (Blazar et al., 2013, Junker et al., 2020).

This chapter addresses the early phase of monocytes/macrophages role in the allo-reactive T cell response using 2-way MLR. This involved optimising the two-way MLR by evaluating the monocytes/macrophages function, T cell activation and production of cytokines. The effects of the presence of allogeneic cells, using the MLR model, was assessed by flow cytometry. All samples were analysed after D1and D2 of incubation, then compared to the control responder cell culture. This allowed differences in CD86, HLA-DR, CD64, CD32, CD36, CD204, C3aR and CD11b expression on $CD14^+$ cells in the MLR culture to be determined using the

expression on $CD14^+$ cells in the responder cells culture as the negative control in the absence of allogeneic cells. The expression of CD86 was measured as it is an essential co-stimulatory molecule that plays a key role in T cell activation (Ke et al., 2016, Bajnok et al., 2017). The expression of HLA-DR was also measured to assess the Ag presentation capacity of the selected monocytes/macrophages (Zheng et al., 2004, Blazar et al., 2013, Duque & Descoteaux, 2014). Several studies confirmed that high expression of CD86 and HLA-DR enhanced T cell activation and proliferation (Zheng et al., 2004, Blazar et al., 2013, Duque & Descoteaux, 2014). CD64 (FCγRI) expression was selected to evaluate the phagocytic capacity of monocytes/macrophages and their activation state. Also, CD64 could be used as an activation marker for classical type of monocytes (Duque and Descoteaux, 2014, Boyette, 2016). CD32B (FCγRIIB) was selected for analysis in this study, as an inhibitory receptor that is expressed on monocytes/macrophages, and plays a role in the regulation of cytokine production and differentiation into dendritic cells (Junker et al., 2020).

Clearance of apoptotic cells during inflammation is an important step to maintain tissue homeostasis, and this is mainly mediated by phagocytic cells (Madrena & Godson, 2003, Gorden & Pluddemann, 2018). The functionality of monocytes/ macrophages to clear apoptotic cells was assessed by measuring the expression of the scavenger receptors CD204 and CD36 (Hufford & Ravichandran, 2013).The complement receptor C3aR as a macrophage activation marker and CD11b was selected as a monocyte/macrophage adhesion molecule and maturation marker (Mathern & Heeger, 2015).CD11b is molecule linked with macrophages differentiation (Ma et al., 2019).

T cells are the key players in the generation of alloresponse (Blazar et al., 2013, Markey et al., 2014, Sprangers et al., 2016, Zeiser et al., 2016). The activation of CD3 T cells and Th CD4 cells were analysed by measuring expression level (MFI) of CD25, and a proportion (%) of CD69 positive cells after three days of incubation. Cytokines production in cell cultures, supernatants were collected after two days of where, TNF-α, IFN-γ, IL-6 and IL-2 concentrations were measured using ELISA. After optimising MLR as an *in vitro* model of alloresponse, the second part of this chapter focused on treating MLR culture with highly specific SYK inhibitor (PRT0603), to determine the inhibitory effects of PRT0603 on

monocytes/macrophages function, T cells activation, TNF-α, IFN-γ, IL-6 and IL-2 concentrations.

Therefore, the aims of this chapter were:

- Determine monocytes/macrophages viability and $CD14^+$ expression.
- Investigate the role of the monocyte/macrophage activation in the alloresponse.
- Investigate the ability of the monocytes/macrophages to present Ag by measuring the level of expression of HLA-DR.
- Investigate the development of the classical and intermediate monocyte phenotypes in the MLR.
- Investigate the expression of phagocytic markers on monocytes in the MLR culture.
- Evaluate the levels of cytokines produced in the MLR culture.
- Examine therapeutic potential of PRT0603 by targeting SYK pathway in monocytes/macrophages in the alloresponse.
- Investigate the effect of PRT0603 in MLR culture to determine if SYK inhibitor could reduce T cell activity.
- Evaluate the effects of SYK pathway inhibitor on cytokine production in the MLR culture.

3.2 Results:

3.2.1 Investigating the early phase of allogeneic reaction in two-way MLR:

$CD14^+$ monocyte activation was analysed using triple staining for CD14-APC, Annexin V-FITC and CD86-PE. All samples were treated with Fc blocking and the results were compared to the isotype control to determine background non-specific antibody binding. The monocyte populations were gated (Figure 3.1.A) based on morphology in forward scatter and side scatter (FSC/SSC), which represents the distribution of cells based on size and intracellular complexity. The plot showed that the monocytes have a medium to large size with moderate granularity. The cell viability was checked by Annexin V- FITC staining after D1 and D2 of incubation (Figure 3.1.B); the D1 results showed 85 % of the gated population were negative for Annexin V, thus 85 % of the gated cells were viable. Similar viability levels were also observed after D2 of incubation (data not shown). To detect the proportional of the $CD14^+$ cells population compared to the $CD14^-$ cells CD14-APC stating was used for the monocyte's phenotype determination (Figure 3.1.C). The staining produced a bimodal histogram with 45 % of gated cells (R1) expressing $CD14^+$ on their surface.

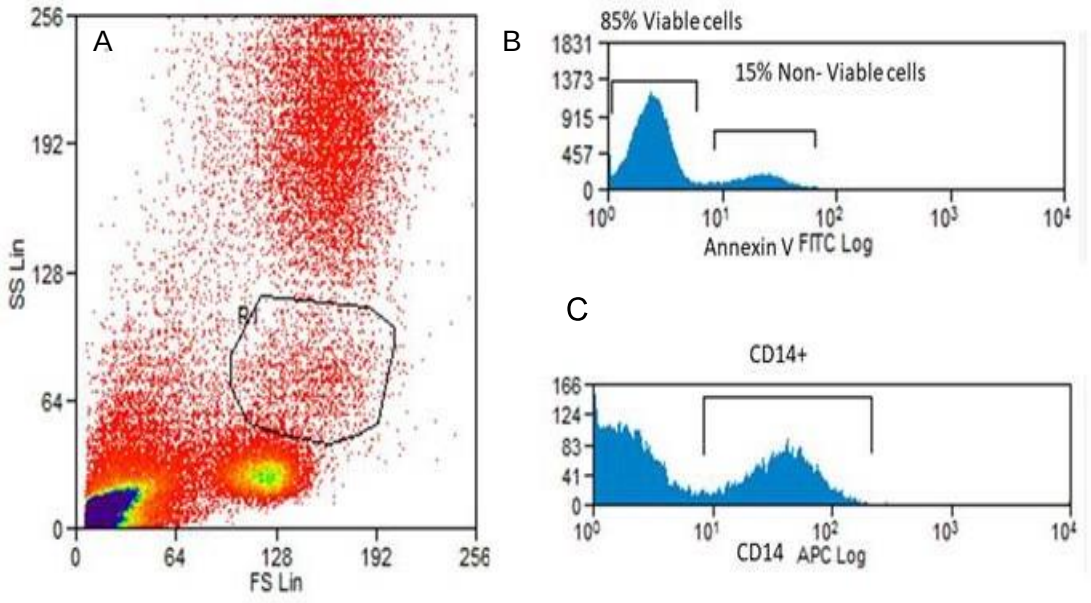

Figure 3. 1: Analysis of monocyte viability and $CD14^+$ expression.

Day 1 of incubation **A)** Monocytes population is gated on (FSC/SSC) in region R1. **B)** The histogram shows Annexin V negative (viable cells) and Annexin V positive, non-viable cells. **C)**

The histogram shows $CD14^-$ and $CD14^+$ cells (45% of cells) populations.

3.2.2 Analysis of monocytes/macrophages co-stimulation capacity:

To measure the co-stimulation capacity of the monocytes, the levels of expression (MFI) of $CD86^+$ on $CD14^+$ cells ($CD14^+$ $CD86^+$) were measured. The results (Figure 3.2.A) suggest that mixing stimulatory cells with responder cells generated an allogeneic response within a 2-way MLR, with enhanced CD86 expression on the surface of $CD14^+$ monocytes when compared to responder cellsculture. There was a significant increase of CD86 expression in the MLR when compared with the responder cells after D1 ($p=0.0319$) and D2 ($p=0.0025$) of incubation, respectively.

3.2.3 Analysis of monocytes/macrophages Ag-presentation capacity:

To determine Ag presentation potential of monocytes, HLA-DR (MHCII) was selected as the marker for this function. Interestingly, the data (Figure 3.2.B) showed significant up-regulation of the MFI values for HLA-DR on the surface of $CD14^+$ in MLR culture when compared to responder cells. The significant changes in the HLA-DR expression levels after D1 and D2 of incubation were ($p=0.0317$ and $p=0.0283$) respectively.

3.2.4 Analysis of the Fc receptor expression on $CD14^+$in the early phase of MLR:

In order to determine the phagocytic function, the expression of $CD64^+$ (FCγRI) was measured, as it is involved phagocytosis result in antigen presentation (Brandsma et al., 2018). The results (Figure 3.2.C) indicate that $CD14^+$ monocytes express higher levels of CD64 in MLR on D1, however, the difference did not reach statistical significance. In contrast, the increase in $CD64^+$ expression was statistically significant in MLR when compared to responder cells on D2 of incubation ($p = 0.0224$). This suggests that up-regulation of FCγRI in the early phase of MLR (D2) could influence key functions, including antigen capture and phagocytic activity.

Furthermore, the expression of $CD32B^+$ (FcγRIIB) on $CD14^+$ monocytes was measured, it is believed that monocytes/macrophages which express CD32B (Fcγ RII-B) are mainly involved in inhibitory response and tissue repair function (Brandsma et al., 2018, Junker et al., 2020). The data (Figure 3.2.D) showed non-

significant up-regulation of CD32B on $CD14^+$ cells in MLR compared to the responder cell control on D1 of incubation. However, on D2 of incubation the $CD14^+$ cells lost the expression of CD32B in MLR.

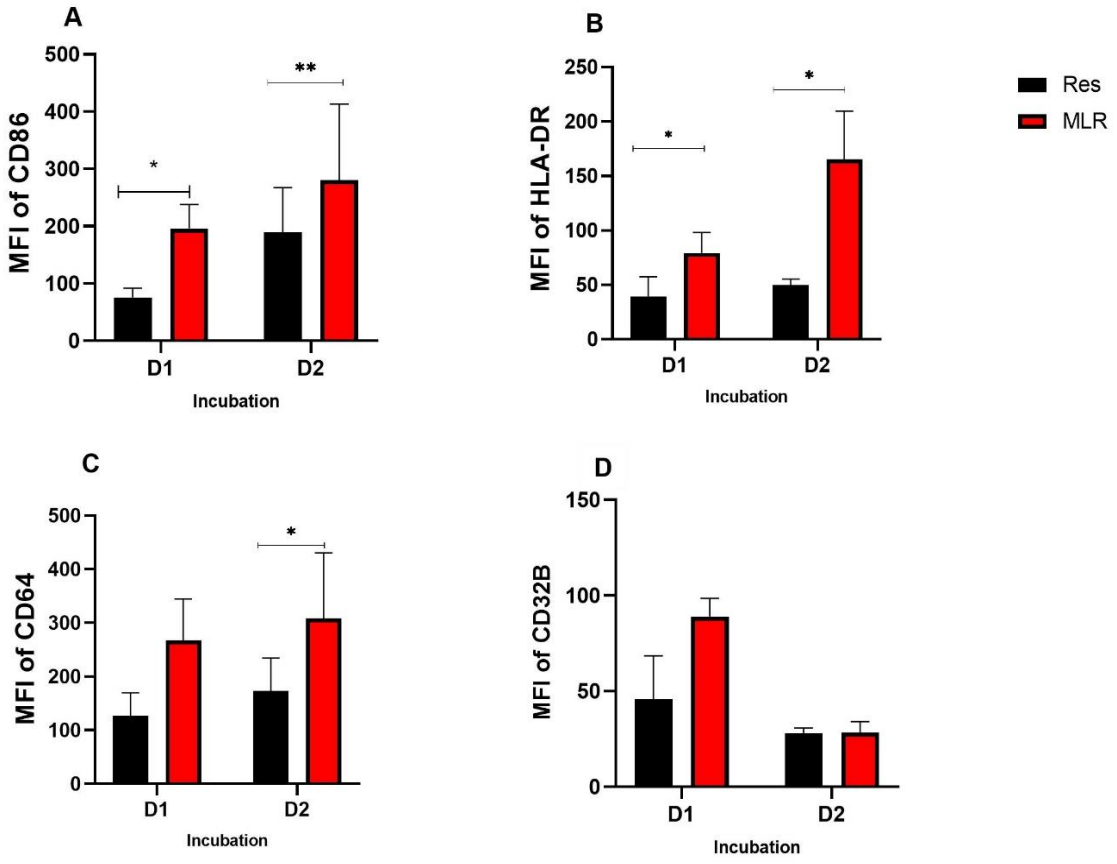

Figure 3. 2: Analysis of **A)** CD86, **B)** HLA-DR, **C)** CD64, **D)** CD32B expression on $CD14^+$ monocytes measure by flow cytometer after one (D1) and two days (D2) of incubation.

The X axis shows incubation days, whereas Y axis present MFI values (levels of expression) for each marker. The data shown represent means and the error bars are SD (n = 6). Black bar represents Res = Responder cells as a negative control, red bar represents MLR = Mixed leukocyte reaction. Statistical significance was analysed using repeated measure (RM) two-way ANOVA with Geisser -Greenhouse correction and Sidak multiple comparisons test, p values < 0.05 were consider significant * =$p < 0.05$, ** =$p < 0.001$**.**

3.2.5 Analysis of scavenger receptors on monocytes/macrophages in early phase MLR:

The expression of CD36 and CD204 scavenger receptors were measured to detect the ability of $CD14^+$ cells to capture apoptotic cells in D1 and D2 culture that included allogeneic cells. Both markers have dual function as they induce inflammation or immunotolerance (Kelley et al., 2014). The data (Figure 3.3**.**A) showed that the levels of $CD36^+$ expression were lower in MLR compared to responder cells on D1 and D2 incubation. However, the differences were not statistically significant.

The results for CD204 expression (Figure 3.3.B) showed that on D1 of incubation, there was a significant increase of expression of this marker in $CD14^+$ in the MLR culture compared to responder control ($p=0.017$). These results suggest that $CD14^+$ monocytes/macrophages in the early phase of allogeneic setting (MLR culture on D1) respond to the presence of allogeneic cells and up-regulate CD204, which could recognise and clear apoptotic cells. In contrast, on D2 of incubation, $CD204^+$ expression on $CD14^+$ monocytes were reduced in MLR and increased in the responder control, but the difference was not statistically significant. This may indicate that the dynamic of the clearance of apoptotic cells differs for allogeneic cells compared to the cell culture of self/responder cells.

3.2.6 Analysis of C3aR expression on $CD14^+$ in the early phase of MLR:

The complement receptor plays an essential role in immune complex binding by monocytes/macrophages. The results (Figure 3.3.C) showed that on D1 of incubation, there was a significant increase in C3aR expression on $CD14^+$ monocytes in the MLR setting ($p < 0.0001$) when compared to the control culture conditions, responder cells only. Notably, C3aR expression was reduced in D2 which is similar to the results for CD204. These data reveal that the presence of allogeneic cells strongly affect C3aR expression in the early phase of MLR culture.

3.2.7 Analysis of CD11b expression on monocytes/macrophages in the early phase of MLR:

The measurements of CD11b expression on $CD14^+$ cells are important, as this adhesion molecule indicates differentiation between different subsets of macrophages. The results (Figure 3.3.D) show that $CD14^+$ cells in the MLR culture express higher levels of $CD11b^+$ on D1 and D2 of incubation when compared to the responder cell control, however, the differences were not statistically significant.

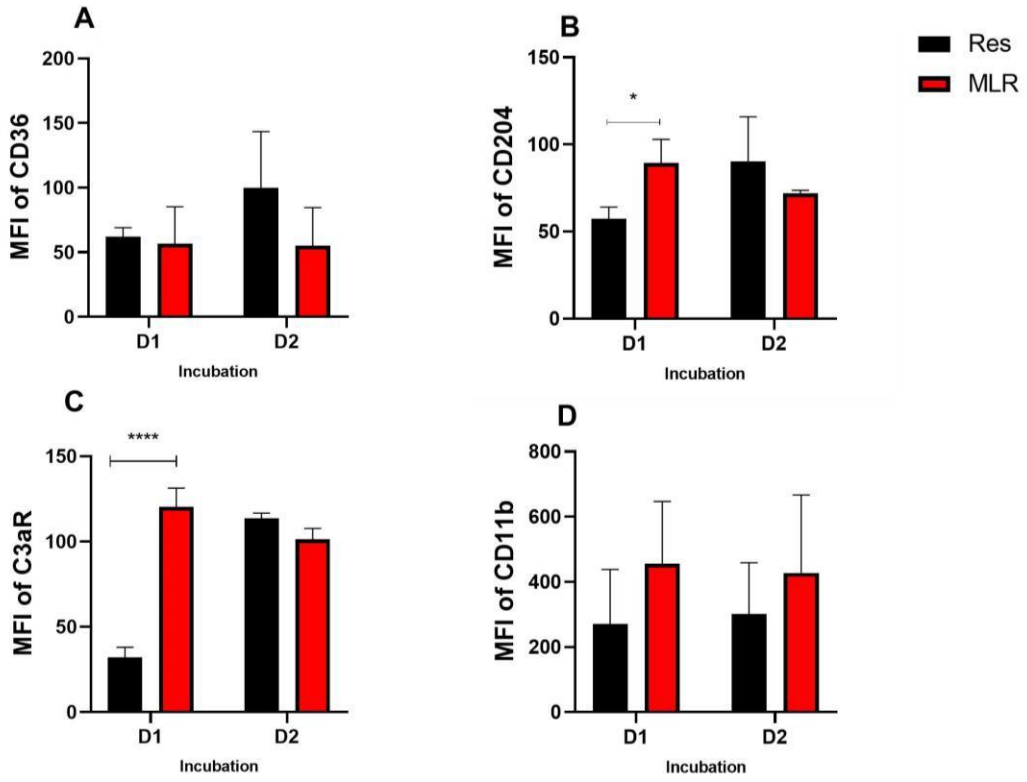

Figure 3. 3: Analysis of **A)** CD36, **B)** CD204, **C)** C3aR and **D)** CD11b expression on $CD14^+$ monocytes measured by flow cytometry after one (D1) and two days (D2) of incubation.

The X axis shows incubation days, whereas Y axis presents MFI value (level of expression) for each marker. The data shown represent means and the error bars are SD (n = 6). Black bar represents Res = Responder cells as a negative control, red bar represents MLR = Mixed leukocyte reaction. Statistical significance of data was analysed using repeated measure (RM) two-way ANOVA with Geisser -Greenhouse correction and Sidak multiple comparisons test, p values < 0.05 were consider significant *= $p < 0.05$, ****= $p < 0.0001$**.**

3.2.8 Analysis of T cell activation in the allogeneic setting:

T cell activity was checked on D3 by assessing the activation state of $CD3^+$ and $CD4^+$ T cells and the results were compared to the T cell activity observed in responder cells culture (negative control for allogenic reaction). The proportion of CD69 positive cells (%) and the MFI for CD25 were selected as markers to detect T cells activation in the MLR.

The data for CD25 (Figure 3.4.A), showed that there was a significant increase in the $CD3^+CD25^+$ MFI in MLR when compared to the responder cells (p=0.056).For the analysis of CD69 positive cells (Figure 3.4.B), the $CD3^+$ cells showed significantly elevated CD69 (%) in the MLR culture compared to responder cells culture (p=0.0029).

The investigation of the $CD4^+$ T helper cell activity (Figure 3.4.C) showed $CD4^+CD25^+$ MFI significantly increased in MLR when compared to the respondercells (p=0.0113). The results for $CD4^+$ helper T cells followed the same trend forCD69 expression (Figure 3.4.D) and showed significantly higher proportional of $CD4^+CD69^+$ (%) in MLR than in responder cells (p=0.0060).

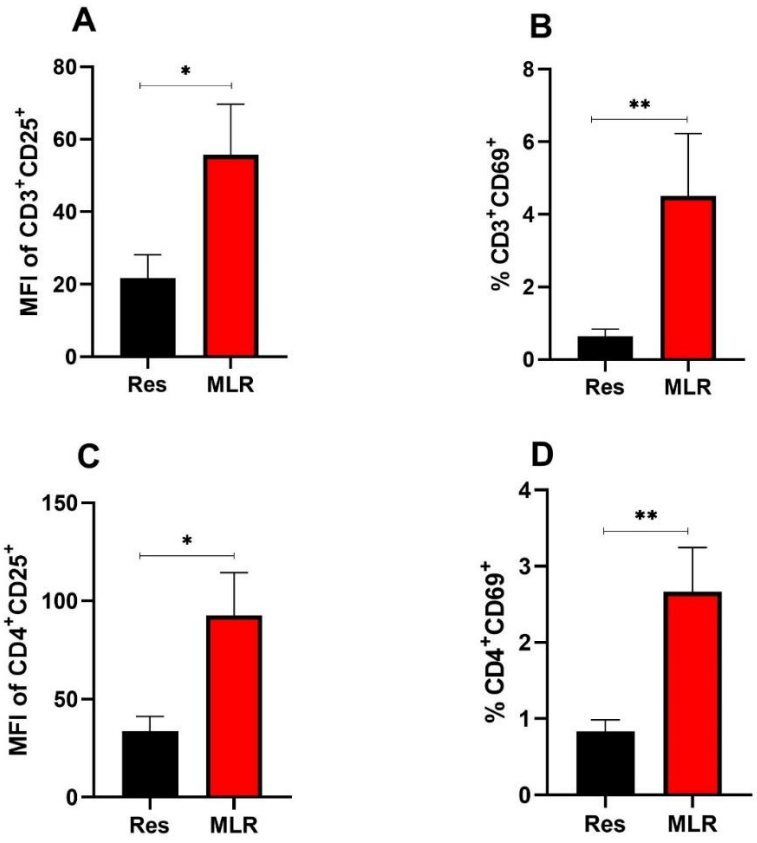

Figure 3. 4: Analysis of T cells activation measured by flow cytometry after three days ofincubation.

A) MFI values for $CD3^+CD25^+$ T cells where Y axis shows MFI values for CD25 expression, **B**) Percent of $CD3^+CD69^+$ T cells where Y axis shows proportion (%) of CD69-positive T cells. **C**) MFI values for $CD4^+CD25^+$ T helper cells, and **D**) percent of $CD4^+CD69^+$ T helper cells, where the Black bar represents Res = Responder cells as a negative control, red bar represents MLR = Mixed leukocyte reaction culture (n=6). Statistical significance of data was analysed using Welch's tests, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$.

3.2.9 Measurement of cytokines in allogeneic media:

To detect inflammatory response in the MLR, the level of TNF-α, IFN-γ, IL-6 and IL-2 cytokines were measured sample supernatant collected on D2 of incubation

from MLR compared to Res, measured by ELISA. The ELISA results for TNF-α (Figure 3.5.A) showed that the concentration of this cytokine on D2 of incubation in the MLR culture were significantly higher than responder cells supernatant ($p=0.0021$). Furthermore, the IFN-γ data (Figure 3.5.B) revealed that the MLR culture generated significantly higher levels of IFN-γ compared to responder cells ($p=0.0026$). The IL-6 data (Figure 3.5.C) showed that in the responder cell supernatant the concentration of IL6 was below the detection limit, whereas the concentration in the MLR culture in D2 was significantly higher ($p=0.0020$). The same trend could be seen for IL-2 (Figure 3.5.D) as the concentration of IL-2 was below the detection limit in the respondercell supernatant and was significantly higher in the MLR cell culture ($p=0.0023$). These data have revealed that the MLR culture resulted in higher levels of production of inflammatory cytokines than the control responder cells culture.

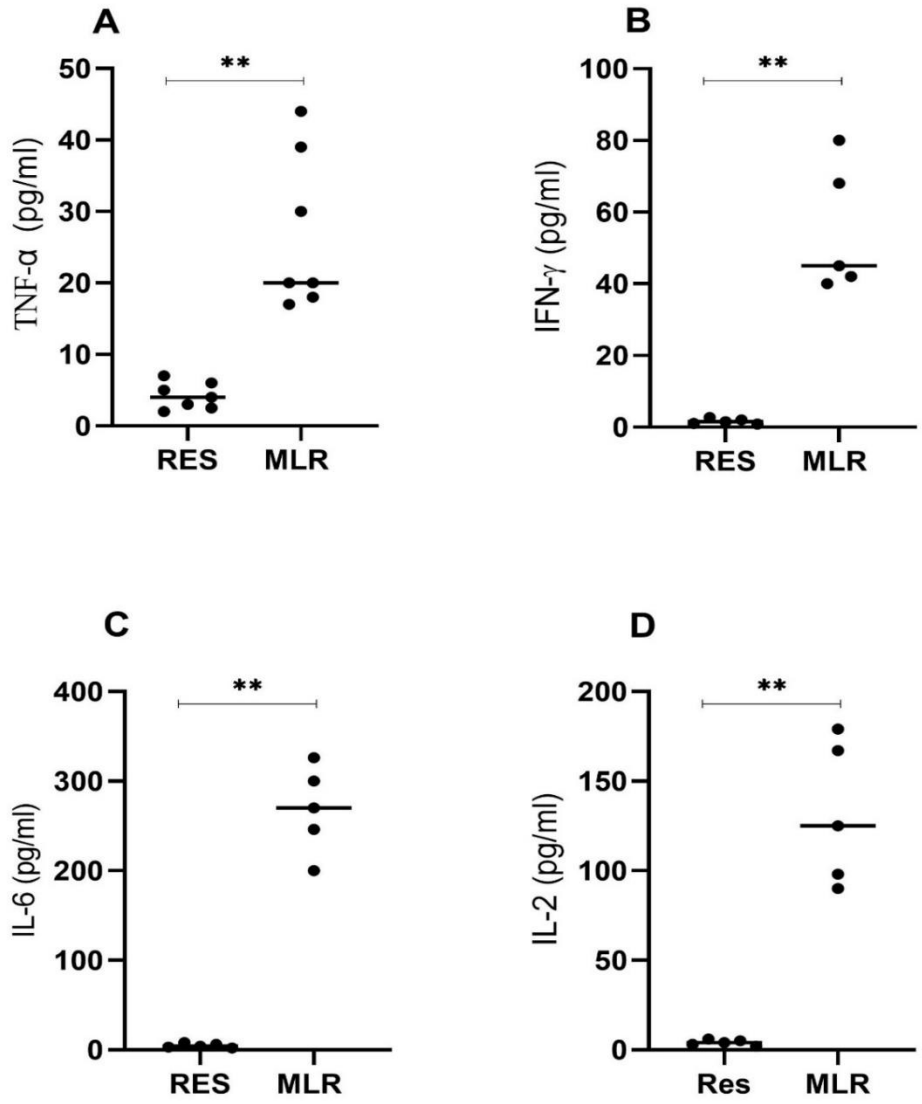

Figure 3. 5: Measuring inflammatory cytokines in MLR cultures after two days of incubation.

A) TNF-α, **B)** IFN-γ, **C)** IL-6 and **D)** IL-2. X axis shows comparison of two conditions: Res= responder cells culture, MLR = Mixed leukocyte reaction culture. Y axis shows the cytokine concentrations in pg/ml. Scatter Dot plot show five repeats (n = 5 except TNF-α n=7). Statistical significance of data was analysed using Welch's test, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$, and ***= $p < 0.001$.

3.2.10 Effects of the inhibition of the SYK pathway in monocytes/ macrophages in MLR culture:

MLR culture versus MLR treated with 2 μM SYK inhibitor (MLR SYK) were analysed for CD86, CD64 and HLA-DR expression on $CD14^+$ monocytes. From the previous section, the data indicates that alloresponse in MLR shows $CD14^+$ monocytes/macrophages up-regulation of co-stimulation (CD86), Ag presentation (HLA-DR level) and FcγRI (CD64) expression. This section focused on the inhibition of monocyte-mediated co-stimulation and Ag presentation in the alloresponse (MLR culture) setting.

The data (Figure 3.6.A) showed that $CD14^+$ monocytes/macrophages down regulated CD86 in MLR SYK compared to MLR however, the result did not reach statistical significance on D1 or D2 of incubation. The only significant differences observed were between MLR and Res (responder cell culture) ($p=0.028$) and Res SYK ($p=0.032$), which supports the idea that the difference in CD86 expression is due to the allogeneic response.

Significant reductions of HLA-DR expression (Figure 3.6.B) on $CD14^+$ were observed in MLR SYK ($p = 0.034$) compared to MLR on D1 of incubation. Whereas the difference did not reach statistical significance on D2. MLR showed significantly higher HLA-DR levels compared to Res ($p=0.0028$) and Res SYK ($p = 0.0004$) on D1, Res ($p=0.0029$) and Res SYK ($p=0.0012$) on D2. Importantly,the data revealed that treating MLR with SYK inhibitor significantly down regulated HLA-DR expression on $CD14^+$ monocytes/macrophages in allogeneic culture with no effect on responder cells.

The data (Figure 3.6.C) demonstrated significant reduction of CD64 expression in MLR SYK compared to MLR on D1 ($p=0.0044$) and on D2 ($p=0.035$). Again,MLR showed significantly higher CD64 expression compared to Res ($p = 0.0012$)and Res SYK ($p=0.0006$) on D1, Res ($p < 0.0001$) and Res SYK ($p < 0.0001$) on D2. Therefore, treating MLR with SYK inhibitor down regulated monocyte/macrophage Ag capture, possibly by targeting the FcγRI (CD64) signalling pathway. The SYK treatment modulating monocytes/macrophages function in allogeneic culture.

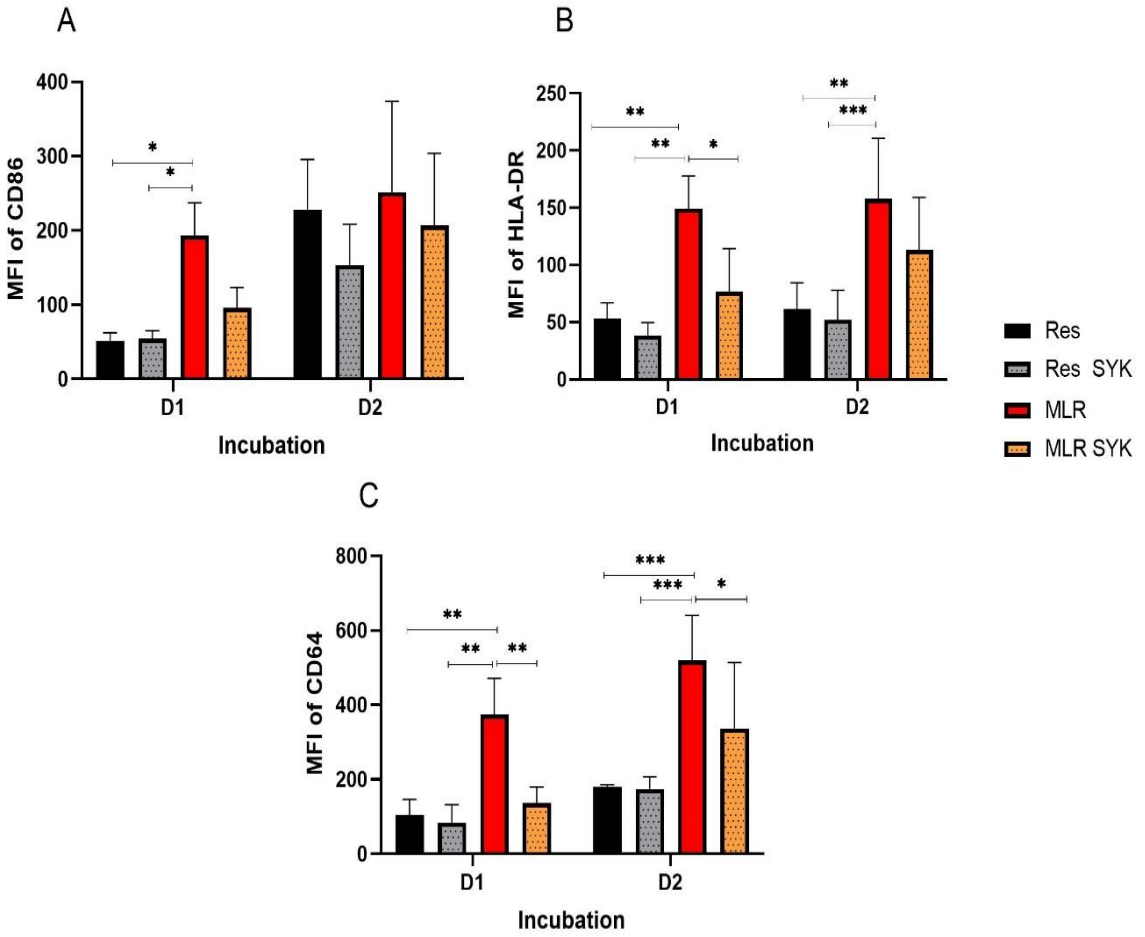

Figure 3. 6: Analysis of A) CD86, B) HLA-DR and C) CD64 expression on $CD14^+$ in MLR culture with/without SYKinhibitor.

Monocytes on D1 and D2 of incubation, co-stimulation, Ag presentation functions and FcγRI expression. The X axis shows the four experimental conditions, black bar represent Res = response cells, dark grey bar with dot pattern represents responder cell treated with 2 μM SYK inhibitor, orange bar represents MLR = Mixed leukocyte culture, and light orange with dots pattern bar represents MLR SYK = MLR treated with 2 μM of SYK inhibitor. The Y axis shows MFI values in arbitrary units. Data shown are mean and the error bars are SD (n = 4). Statistical significance of data was analysed using repeated measure (RM) two-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test, p values < 0.05 were consider significant *=p < 0.05, ** = p < 0.01 and ***=p < 0.001**.**

3.2.11 Analysis of the effects of the SYK inhibitor on $CD3^+$ and $CD4^+$ T cells:

The analysis of T cells was performed after three days of incubation. Based on the monocytes/macrophages data, it was postulated that in the MLR setting, $CD14^+$ cell activity was down regulated by 2 μM SYK inhibitor on D1 and D2 of incubation (Section 3.2.10). Consequently, it would be expected that

monocytes/macrophages were not able to co-stimulate and present antigens to T cells on D1 and D2 of MLR culture. Thus, MLR cultures were incubated for 3 days to analyse changes in T cells activity and the results were compared to those observed in MLR treated with 2 μM SYK inhibitor daily. The double gated $CD3^+CD25^+$ T cells were measured, and the median MFI is presented. Also, the double gated $CD3^+CD69^+$ T cells were measured and presented as the number/proportion of positive cells.

The data (Figure 3.7.A) showed that the MLR culture treated with 2 μM SYK inhibitor significantly down-regulated CD25 (IL-2 receptor) expression on $CD3^+$ T cells (p=0.0403) compared to MLR. Significant down regulation was reported due to allogeneic culture in MLR compared to Res (p=0.033), Res SYK (p=0.032).Also, the proportion (%) of $CD3^+CD69^+$ activated T cells (Figure 3.7.B) showed significant down regulation in the MLR treated with 2 μM SYK inhibitor when compared to the MLR (p=0.031). Significant reductions in T cells activities were observed again due to allogeneic culture in MLR compared to Res (p=0.012), Res SYK (p=0.011).

The results for the CD25 expression on $CD4^+$ helper T cells (Figure 3.7.C) showed that the MLR culture treated with 2 μM SYK inhibitor down-regulated CD25 expression, but the decrease did not reach statistical significance. The only significance difference was the reduction between MLR and Res (p=0.045). The proportion (%) of positive $CD4^+CD69^+$ T cells (Figure 3.7.D) were slightly lower (but not statistically significant) in the MLR treated with SYK inhibitor when compared to MLR. Thus, these results confirmed that treating MLR with 2 μM PRT0603 SYK inhibitor has reduced $CD3^+$ T cells activity.

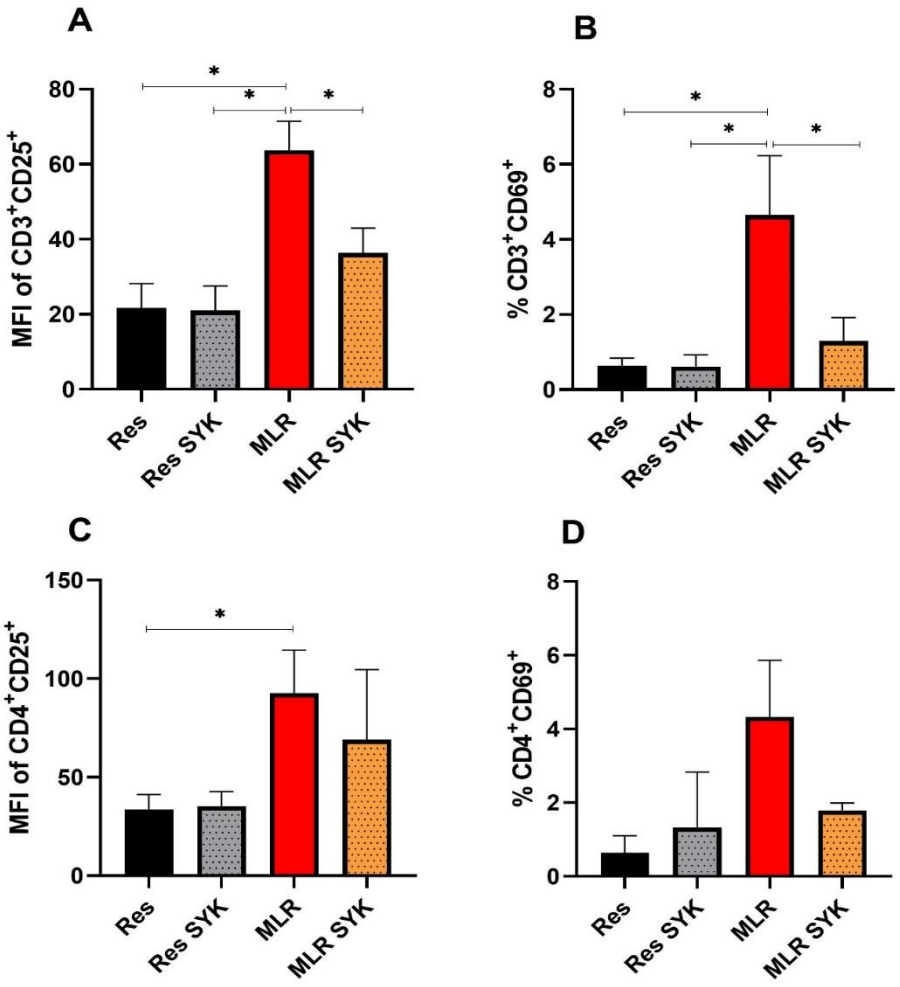

Figure 3. 7: T cells inhibition in the presence of SYK inhibitor on D3of incubation.

A) MFI values for $CD3^{+}CD25^{+}$ T cells where Y axis shows MFI values for CD25 expression, **B)** Percent of $CD3^{+}CD69^{+}$ T cells where Y axis shows proportion (%) of CD69-positive T cells. **C)** MFI for $CD4^{+}CD25^{+}$ and **D)** Percent (%) $CD4^{+}CD69^{+}$ cells, black bar represents Res= responder cell culture, grey bar with dots pattern represents = Res SYK= responder cells treated with SYK, dark orange bar represents MLR = Mixed leukocyte reaction culture and light orange with dots pattern bar represents MLR treated with 2 µM of SYK inhibitor. Data shown are mean values and the error bars are SD (n = 3). Statistical significance of data was analysed using repeated measure (RM) two- way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test, p values < 0.05 were consider significant * =$p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3.2.12 Analysis the inhibitory effect of PRT0603 SYK on the production of inflammatory cytokines:

Cell culture supernatants were collected from MLR and MLR treated with SYK inhibitor (MLR SYK) after two days of incubation and TNF-α, IFN-γ, IL-6 and IL-2 concentrations were measured by ELISA to determine the inhibitory effects of

PRT0603 SYK inhibitor on the production of the inflammatory cytokines.

The data (**Error! Reference source not found.**.A) showed that the TNF-α concentration was significantly reduced in the MLR treated with SYK inhibitor compared to the MLR ($p = 0.011$). As previously observed, significant differences were evident in the allogeneic culture (MLR) compared to responder cells (Res) ($p=0.031$) and Res SYK ($p= 0.031$). The data for IFN-γ concentration (**Error! Reference source not found.**.B) showed a significant drop of IFN-γ levels in the MLR treated with SYK compared to the MLR ($p=0.008$). Also, allogeneic culture (MLR) showed significant differences compared to Res ($p= 0.007$) and Res SYK ($p=0.007$). For IL-6 (**Error! Reference source not found.**.C) there was a significant reduction in concentration in the MLR SYK compared to MLR only ($p=0.047$). Furthermore, modest but significant differences were detected in MLR compared to Res ($p = 0.048$) and Res SYK ($p=0.048$).

The data (**Error! Reference source not found.**.D) for IL-2, showed significantly lower concentrations in the MLR treated with SYK inhibitor in comparison to the MLR ($p=0.082$). Also, significant differences in MLR culture compared to Res ($p=0.002$) and Res SYK ($p=0.002$) were confirmed.

Overall, these results highlight that PRT0603 SYK inhibitor successfully reduced the inflammatory cytokine concentrations in the allogeneic cell cultures. Also, the role of allogeneic culture in triggering an increase in the cytokine production has been confirmed.

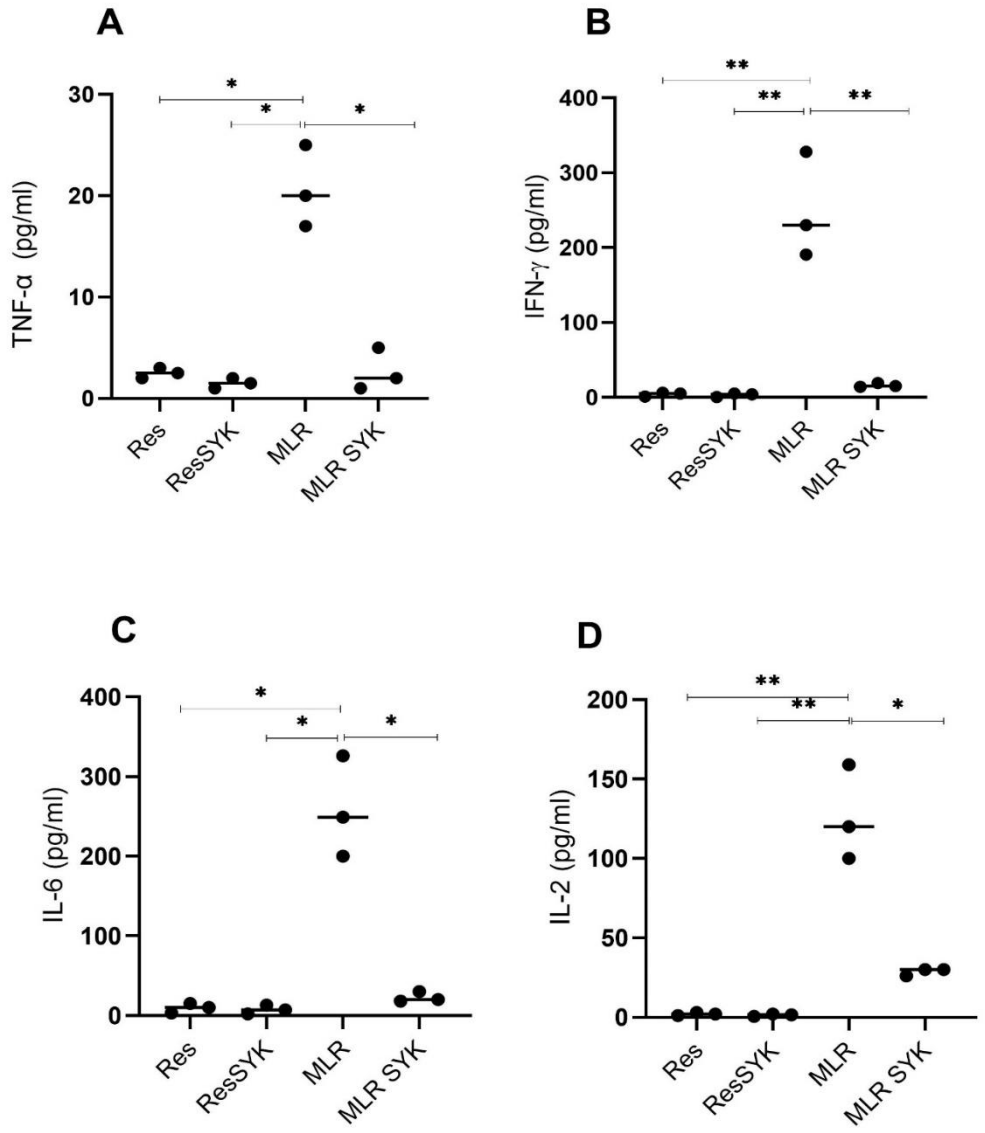

Figure 3. 8: Concentrations of inflammatory cytokines in MLR cultures after two days of incubation in the presence of SYK inhibitor.

A) TNF-α, **B)** IFN-γ, **C)** IL-6 and **D)** IL-2. X axis shows comparison of two conditions, Mixed leukocyte reaction (MLR) and MLR treated with 2 μM of SYK inhibitor (MLR SYK). Y axis shows the cytokine concentrations in pg/ml. Scatter dot plots show mean values of 3 independent repeats (n = 3). Statistical significance of data was analysed using repeated measure (RM) two- way ANOVA with Geisser -Greenhouse correction and Tukey's multiple comparisons test, p values < 0.05 were consider significant * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

3.3 Discussion (Part 1):

Monocytes make up 5-10 % of total WBCs count but they play an important role in innate and adaptive immunity (Tacke & Randolph, 2006, Sprangers et al.,2016). Monocytes have a short half-life in human circulation, after 3 days monocytes based in the microenvironment differentiate to macrophages or DC (Tacke & Randolph, 2006). The aim of this study was to investigate the role of monocytes/macrophages in whole blood allogeneic culture, in contrast to most other studies that isolated monocytes to study their function. In this study whole blood, MLR was used to evaluate monocytes/macrophage function in the early allogeneic reaction. The MLR culture used in this study allows activation, stimulation, and proliferation of both populations stimulatory cells and responder cells. The benefit of using whole blood was to preserve the cellular interactions including neutrophils, monocytes, and T cells. Using MHC mismatched *in vitro* culture could not represent the exact setting in matched GVHD patients. However, the data in this report showed that to activate monocytes *in vitro* strong activation stimulus is required, similar to that present in the complete MHC mismatched blood samples. It has been reported that during the innate immune response neutrophils first arrive at the affected area, recognise the pathogen via PAMPS/DAMPS, neutrophils then release their granular content and generate reactive oxygen species (ROS) (Zeise R, 2019). This enhances the recruitments of monocytes/macrophages to the site of infection, where later monocytes/macrophages will phagocytose the pathogen, the apoptotic neutrophil, and present antigens to T cells (Zeise, R 2019). Therefore, monocytes activation and macrophages polarisation are controlled by neutrophils degranulation. Interestingly, it has been reported that Lactoferrin, which is a neutrophil granule product, controls formation of M1 macrophages with a consequent proinflammatory response. Whereas IL-13 that is released by neutrophils polarises macrophages to M2, which results in anti-inflammatory response (Kumar et al., 2011). Monocytes/macrophages responses that could be either pro-inflammatory or anti-inflammatory are involved in the early phase of GVHD (Zeise R, 2019). Similarly, platelets-monocytes interaction mediates the proinflammatory response and cytokine release by monocytes (Passacquale et al., 2011). Therefore, this study has optimised the histo-incompatible model to activate host (stimulatory) and donor (responder) monocytes, maintaining the cellular

interactions, which is important for a strong immune activation. Then the effects of allogeneic cells on the monocytes/macrophages function, T cell activation and cytokine production in the MLR culture were evaluated. The second part of this study evaluates the efficacy of the SYK inhibitor PRT0603 to control monocytes/macrophages. Indeed, if the inhibitor has a potential to reduce the early allogeneic response in this MHC mismatched model with a strong activation stimulus, this could highlight their potential to control immune response in a challenging GVHD setting. Thus, whole blood, MHC mismatched MLR culture could be used for future studies, as a model that simulates early immune response in GVHD, and this could complement rather than replace the current mouse models.

The alloresponse was investigated using following experimental conditions: MLR (mixed leukocyte reaction) and Res (-ve control, responder cells only). For the flow cytometry immunophenotyping all samples were treated with Fc blocking and the results were compared to the isotype control to eliminate any background of nonspecific antibody binding. As 80 to 85 % of monocytes in the gated population were Annexin V -ve this confirmed cell viability on D1 and D2 of incubation, respectively.

Monocytes/macrophages function was assessed by evaluating the expression level of surface markers. CD86 and HLA-DR were measured, reflecting co-stimulation capacity and Ag presentation, respectively. Based on published studies, high expression of CD86 and HLA-DR are essential to prime T cells, enhance T cell activation and proliferation (Zheng et al., 2004, Blazar et al., 2013, Markey et al., 2014, Duque & Descoteaux, 2014). As FcRs links the innate and adaptive immune response (Brandsma et al., 2018, Junker et al., 2020), the levels of CD64 (FcγRI) and CD32B (FcγRIIB) were measured alongside CD36, CD204, C3aR and CD11b to evaluate the capability of the monocytes, to capture phagocytes and opsonise immune complexes (Gorden & Pluddemann, 2018, Mathern & Heeger, 2015).

The results showed a significant increase of monocytes CD86, HLA-DR, CD64, CD204 and C3aR expression in MLR compared to the responder cells (raw data for CD86 are presented in the appendix Figure S.1.3). Thus, the data in this study

could show that monocytes in MHC mismatched MLR are strongly activated, which supports the concept that the allogeneic cell culture up-regulates phagocytosis, co-stimulatory and Ag presentation capacity of monocytes/macrophages. This agrees with previous reports that during early aGVHD, host and donor APCs up-regulate co-stimulation and Ag presentation markers (Chakraverty & Sykes, 2007, Markey et al., 2014). This observation is discussed in detail in this chapter.

CD64 is an FcγRI receptor expressed on monocytes/macrophages and other cells, CD64 has a well-established role in inflammation and autoimmune diseases (Brandsma et al., 2018). These data could link CD64 up-regulation on monocytes/macrophages with increased HLA-DR expression. It is generally accepted that CD64 is involved in inflammation, as ligation of CD64 to the immune complex or opsonised Ag activates the downstream signalling of CD64. This includes SYK phosphorylation, Ag internalisation, Ag processing and presentation via MHCII (Junker et al., 2020, Brandsma et al., 2018). Intracellular signalling triggered by FcγR results in degranulation and secretion of pro-inflammatory cytokines by macrophages (Brandsma et al., 2018). Furthermore, the research study reported by Tacke and Randolph (2006) proposed that classical monocytes express $CD14^+$ $CD16^-$ $CCR2^+$ $CD64^+$ markers (Tacke & Randolph, 2006). Thus, the findings in this work, with $CD14^+$ $CD64^+$ phenotype in MLR culture, broadly could supports the concept that early alloresponse promoted development of a classical monocyte subtype.

CD32B is a low affinity Fcγ RIIB, that binds to the IgG complex, and is expressed in human circulating monocytes (Hepburn et al., 2004, Brandsma et al., 2018). More importantly, CD32B expression is involved in inhibitory immune response (Hepburn et al., 2004, Brandsma et al., 2018). In MLR culture, the changes in monocyte CD32B expression were modest and cannot be associated with either pro-inflammatory or inhibitory effects. The intermediate monocytes phenotype was described as $CD14^+CD16^+CD32^+$HLA-DR^{++} (Tacke & Randolph 2006, Lee et al., 2017). In this study, the characterisation of monocyte subtypes was incomplete, due to limited 3 colours analyses by flow cytometry. This needs to be considered in future work to allow the analysis of the subtypes of monocytes. However, the current observation of up-regulation of $CD14^+CD64^+$ and modest

down regulation of $CD14^{+}CD32B^{+}$ on D2 suggests that monocytes/macrophages in MLR culture are more likely to develop into the classical subtype.

In MLR culture, scavenger receptors CD36 and CD204 were assessed to evaluate the functional ability of monocytes/macrophages to capture apoptotic cells. Clearance of apoptotic cells by phagocytes is essential to control inflammatory response (Hufford & Ravichandran, 2013). Notably, these two markers, CD36 and CD204, have dual function, as they could induce both inflammation and immune tolerance (Kelley et al., 2014). Therefore, the data reported here indicate that although allogeneic cells trigger inflammatory response in MLR culture, this was not accompanied by changes in CD36 expression on $CD14^{+}$ monocytes/macrophages. Remarkably, a previous study showed that the monocytes/macrophages that did not regulate CD36 could still prime alloreactive T cells in GVHD patients (Perry et al., 2018).

The second scavenger receptor selected in this study is CD204, that can increase macrophages recruitment to the site of injuries (Kelley et al., 2014). The ligation of CD204 results in phagocytosis, inflammatory response, and secretion of cytokines, including TNF-α (Pombinho et al., 2018). Furthermore, CD204 is involved in the uptake of apoptotic cells (Maderna & Godson, 2003, Kelley et al., 2014), which could be particularly relevant for MLR culture and GVHD. A study by Knouma et al. (2018) demonstrated that high levels of CD204 expression on monocytes/macrophages was linked to tissue fibrosis in GVHD patients (Knouma et al., 2018). From the data it could be concluded that clearance of apoptotic cells in allogeneic cell culture on D1 of incubation maybe controlled by CD204 and not by using the CD36 receptor. These data question the role of the different monocyte subtypes in the GVHD pathology, as increased expression of CD204 on non-classical monocytes was reported to induce cGVHD (Kanuma et al., 2018). However, $CD14^{+}$ monocytes in this study showed characteristics of the classical monocyte subtype, and yet reveal up-regulation of CD204 expression in the early phase of allogeneic reaction.

This finding highlights the role of allogeneic cells in inducing C3aR expression in monocytes, which is followed by up-regulation of co-stimulation and antigen presenting capacity as both markers were up-regulated on D1 of MLR culture. It

has been previously reported that C3aR is up-regulated on APCs in response to complement pathway stimulation (Raedler & Heeger 2011). The ligation of C3a to C3aR on APCs results in intracellular signalling that involves phosphorylation of AKT (protein kinase B), which in turn controls expression of co-stimulatory molecules (Raedler & Heeger 2011). Furthermore, C3aR ligation mediates IFN-γ and IL-2 secretion by T cells (Nguyen et al., 2018). Thus, ligation of C3aR appears to be involved in the pathology of GVHD (Nguyen et al., 2015, Nguyen et al., 2018). Although, a previous study showed that ligation of C3aR on APCs controls CD86 and MHCII expression on DCs and this results in T cells activation in GVHD (Nguyen et al., 2018), this could be the first report of such effects in monocytes, and in the early phase of alloresponse.

Surface expression of CD11b on $CD14^{+}$ monocytes was measured in this study, as the CD11b is crucial for cell adhesion and phagocytosis of pathogens (Lukácsi et al., 2017, Gavin et al., 2019). CD11b is also considered as a macrophage differentiation marker with the low levels of expression on pro-inflammatory M1, whereas M2 anti-inflammatory macrophages express $CD11b^{high}$ (Ma et al., 2019). The data in this report showed that CD11b was not-significantly up-regulated in MLR on D1 and D2 of incubation when compared to responder cells. Therefore, it appears that CD11b expression is not involved in inflammation induced by allogeneic cells. To confirm this conclusion, larger number of repeats will be done in future work.

Although the monocytes subtypes were not fully characterised in this study, the phenotype observed in the early phase of allogeneic cell culture, $CD14^{+}CD86^{++}$HLA-$DR^{++}CD64^{++}$, broadly could aligns with a classical subtype. However, the monocyte phenotype in MLR culture appears to be complex and dynamic with rapid changes in marker expression on D1 and D2 of incubation. Furthermore, the monocytes tend to differentiate into macrophages, and the line of transition between the different cell types is not clear. Therefore, it could be argued that monocyte subtypes are artificial, and that observed cell phenotype represents a transitional stage of monocyte development rather than a stable, defined cell type.

The activation levels of $CD3^{+}$ and $CD4^{+}$ T cells were analysed by measuring the

intensity (MFI) for CD25 expression, and the proportion (%) of $CD69^+$ cells on D3 of incubation. It is generally accepted that T cells are the key mediators of alloresponse (Blazar et al., 2013, Markey et al., 2014, Sprangers et al., 2016, Zeiser et al., 2016). Notably, in this study the levels of CD25 expression and the proportion of CD69 positive cells were significantly up regulated in MLR culture on $D3^+$ and $CD4^+$ T helper cells compared to responder cells control. These findings indicate that activation of monocytes/macrophages precedes a strong, measurable T cell alloresponse, including both total $CD3^+$ T cells and helper $CD4^+$ T cells on D3 of MLR culture. This could be supported by the monocyte data described above. Based on the generally accepted concepts and the data from this study, the alloresponse is likely to be initially triggered by the recognition of allogeneic cells by TCR, as TCR is the only molecule that can detect polymorphic alloantigens (Ferrat et al., 1991, Blazar et al., 2013). However, this early event appears difficult to detect. Indeed, this study has shown there is a lack of measurable T cell activation on D1 and D2 in the MLR culture (data not shown). Nevertheless, an activation of monocytes is clearly detectable at this early stage of alloresponse, suggesting that a subtle signal generated by T cells and possibly combined with the presence of cell injury and inflammation results in a strong activation of APCs/monocytes. Subsequently, co-stimulatory signals and cytokines generated by $CD14^+$ monocytes/macrophages could play a critical role in enabling a full T cell activation that is detected on D3 of MLR culture.

To detect inflammatory response in the MLR, the level of TNF-α, IFN-γ, IL-6 and IL-2 cytokines were measured. Activated monocytes/macrophages release cytokines to cause an inflammatory response (Deeg, H 2001, Duque & Descoteaux, 2014). Indeed, it is thought that cytokines storm originates from the innate immune response that is activated in aGVHD (Blazar et al., 2013, Markey et al., 2014, Duque & Descoteaux, 2014). The samples from MLR culture and the control responder cells supernatants were collected on D1, D2 and D3 of incubation. The cytokine concentrations with consistent values above detection limits were observed on D2, which were analysed by ELISA and presented in this report. The results showed that the concentrations of TNF-α and IFN-γ in the MLR culture were significantly higher than the responder cells culture. This could indicate the presence of the inflammatory conditions in the early phase of

alloresponse/MLR culture. The origin of these cytokines (the producing cells) has not been determined, due to lack of intracellular staining, which is considered in future work. Based on the current data, it could be assumed that activated monocytes/macrophages make a substantial contribution to the cytokine generation due to their early activation, observed on D1 and D2 of incubation. In contrast, T cells were not measurably activated on D1 and D2 and are unlikely to play a major role in cytokine production determined on D2, with a possible exception for IL-2. Indeed, it has been reported that both TNFα and IFN-γ were secreted in the early stage of aGVHD by activated APCs (Blazar et al., 2013, Markey et al., 2014, Duque & Descoteaux, 2014, Sprangers et al., 2016). Notably, the high concentrations of TNF-α demonstrated in this study could explain the up-regulation of the CD86, HLA-DR and CD64 on the surface of monocytes/macrophages observed in the early MLR culture. This is supported by the reported role of TNF-α in the control of CD86 expression and subsequent priming of T cells in aGVHD (Markey et al., 2013, Sprangers et al., 2016, Zhang et al., 2017). As discussed above, activated monocytes in the early MLR culture show some features of the classical subtype. The classical monocytes are regarded as the main source of TNF-α cytokines. This is based on the data reported by several studies, where activation of classical monocytes results in the secretion of TNF-α (Deeg, H 2001, Sprangers et al. 2016, Boyette et al., 2016).

It is thought that IFN-γ promotes monocyte differentiation into macrophages, and polarisation towards the M1 phenotype with high Ag presentation capacity (Delneste et al., 2002, Lee et al., 2017, Murray, 2017, Obrien et al., 2019). Furthermore, several studies have shown that exposing naïve monocytes to IFN-γ encourages development of classical $CD14^{+}$ monocytes, which can then secrete pro-inflammatory cytokines, including TNF-α, IL1-β, IL-6, IL-12, and IL-23, and this promotes T cell proliferation (Duque & Descoteaux, 2014, Sprangers et al., 2016, Lee et al., 2017). Whereas stimulating monocytes with TNF-α and IFN-γ increases CD64 expression and allows binding/phagocytosis of the immune complex (Brandsma et al., 2018). A study has suggested that IFN-γ production plays a vital role during the generation of GVHD, and that IFN-γ is mainly produced by T cells and NK cells to control the recruitment of classical monocytes and their differentiation into macrophages (Sprangers et al., 2016). This supports the

findings in this study, where $CD14^+$ monocytes in the MLR culture, and due to the secretion of IFN-γ, showed some of the features of the classical phenotype. Furthermore, it has been proposed that once classical monocytes are stimulated with IFN-γ, they become highly phagocytic M1 macrophages (Galan et al., 2015).

Importantly, IL-6 and IL-2 concentrations were below the detection limit in the responder cells on D2 of incubation, whereas, in the MLR culture, IL-6 and IL-2 had significantly higher concentrations. It has been reported that activated classical monocytes are the main producers of IL-6 (Boyette et al., 2016). The secretion of IL-6 in MLR culture could be linked with the up-regulation of CD64 expression on $CD14^+$ monocytes, as a previous study in rheumatoid arthritis showed that IL-6 cytokine controls CD64 expression in classical and intermediate monocytes (Luo et al. 2018).

IL-2 is Th1 cytokine that controls T cells activation and proliferation in immune response in general and GVHD in particular (Kumar et al., 2017, Nassereddine et al., 2017). The data in this study showed high concentrations of IL-2 in the early phase of MLR culture, thus clearly confirming the role of this cytokine in alloresponse. Indeed, the IL-2 cytokine could control CD25 expression and increase the proportion of $CD69^+$ T cells in MLR culture, as observed in this study on D3 of incubation. This interpretation of the data is supported by a recent study that showed that IL-2 controls early and late activation markers on T cells (Bajnok et al., 2017). Furthermore, the role of IL-2 in controlling the alloresponse could include its effects on TNF-α secretion by macrophages, which may result in the skin and tissue damage in GVHD (Kumar et al., 2017, Nassereddine et al., 2017).

In summary, allogeneic reaction in the early MLR culture resulted in the increased co-stimulation capacity (CD86 expression), enhanced Ag presentation potential (HLA-DR expression), stimulation of the complement pathway (C3aR expression) and a strong phagocytosis function (CD64 and CD204 expression) by monocytes/macrophages. This striking activation of monocytes/macrophages appears to be induced by T cell alloreactivity and cytokine production. These data suggest that the use of whole blood MLR culture as an *in vitro* model for GVHD provides valuable data on the role of monocytes/macrophages in the early phase of allogeneic culture. The second part of this chapter focused on controlling

monocytes/macrophages function, T cells activation by treating MLR culture with a specific SYK inhibitor. As controlling GVHD by immunotherapy has been extensively studied for T cell activation, whereas there is limited data available about controlling monocytes/macrophages co-stimulation and Ag presentation, as well as cytokine production (Leonhardt et al., 2012). Evaluation obtained by observing the expression of CD64 (FcγRI), CD86 and HLA-DR on monocytes/macrophages. It has been reported that SYK activation is involved in monocytes/macrophages inflammatory response such as phagocytosis, Ag presentation, co-stimulation, and cytokines secretion. Thus, SYK inhibition is expected to suppress monocytes/macrophages inflammatory response (Yi et al., 2014, Coffey et al., 2017). PRT0603 is a highly selective SYK inhibitor that has been shown in a murine model to block FcγRII in platelets to prevent heparin induced thrombocytopenia and thrombosis (Reilly et al., 2011). Interestingly, PRT0603 has selectively inhibited SYK signalling in B cells which resulted in deactivation of B cells, in a CLL model (Hoellenriegel et al., 2012). PRT0603 is a parent compound for PRT0626, which is highly selective for SYK molecules and showed complete suppression of BCR and FcRs signalling that resulted in reduced inflammation in rheumatoid arthritis patients (Coffey et al., 2013). To determine the potential effects of SYK inhibitor PRT0603 in controlling monocytes/macrophages during the allogeneic reaction, samples from MLR were compared to MLR with SYK treatment. Furthermore, to detect the potential toxicity of SYK, the control responder cells were treated with 2 µM of PRT0603 and compared to untreated samples of responder cells culture. Optimising SYK concentration extensively studied in this work, the optimal concentration selected showed an inhibitory effect on monocytes function. That was when the MLR culture treated daily with 2 µM. The results indicated that treatment of the MLR cultures with 2 µM SYK inhibitor significantly lowered CD64 and HLA-DR expression on monocytes and reduced the cytokine concentration, followed by decrease of T cells activities. Interestingly, the data showed that the SYK pathway deactivated in MLR SYK as CD64 expression diminished in both days of incubation, because PRT0603 is a highly selective SYK inhibitor that could target FCγRI signalling. It has been reported that CD64 controlled inflammation by up-regulation of MHCII expression to enhance Ag presentation (Brandsma et al., 2018). Interestingly, the results showed SYK inhibitor mediated down regulation

of HLA-DR observed on D1. This may suggest that due to suppression of SYK, downstream signalling $CD14^+$ in monocytes/macrophages the Ag presentation capacity reduced. However, no significant down regulation of CD86 was observed (raw data presented in appendix Figure S.1.3), thus the SYK inhibitor did not control co-stimulation on $CD14^+$ monocytes. To confirm this observation additional experiments are needed to determine the interaction of SYK and other signalling pathways in regulation of the CD86 expression on monocytes. Furthermore, no changes in CD86, HLADR and CD64 expression were observed between Res and Res SYK. This indicates the inhibitory effect of SYK worked only in allogeneic culture.

As stated earlier, three signals are important to activate T cells (Ka et al., 2016, Junker et al., 2020). Targeting SYK by the SYK inhibitor in MLR culture impaired Ag processing that could reduce Ag presentation capacity by monocytes/macrophages. This could affect at least one of the signals necessary for the full activation of T cells in alloresponse.

Notably, the data showed on D3 in the presence of PRT0603 there was a significant reduction of $CD25^+$ expression levels and percent of $CD69^+$ positive cells among $CD3^+$ T cells in MLR SYK compared to the MLR (raw data provided in the appendix Figure S.1.2). These results fit with studies that have shown that SYK phosphorylation plays a role in T cell allo-activation in GVHD (Leonhardt et al., 2014). This has confirmed the potential of the SYK inhibitor to reduce activation of $CD3^+$ T cells, and suggests the possible mechanism: namely, targeting of the monocytes FcγRI, as the main pathway affected by SYK inhibition in the MLR culture. This could result in down regulation of the key monocyte/macrophage functions ultimately leading to a reduced T cell alloresponse. This highlights that early Ag presentation provided by monocytes/macrophages was reduced, as the most likely indirect effect on T cells alloreactivity in D3 of incubation. This is supported by the recent study that showed that targeting FcγR resulted in inhibited Ag presentation, which could affect T cells activation (Junker et al., 2020). As $CD3^+$ T cells represent both T cell helper $CD4^+$ cells and cytotoxic $CD8^+$ cells, the effects of SYK inhibitor are broad and affect the total T cell population. However, the effects of the SYK inhibitor on $CD4^+$ T helper cells were modest and did not reach statistical significance.

Importantly MLR treated with the SYK inhibitor had significantly reduced concentrations of TNF-α, IFN-γ, IL-6 and IL-2 cytokines compared to the untreated MLR. The results are in accordance with studies that showed that Fostamatinib (non-specific SYK inhibitor) lowered the concentration of IFN-γ and IL-6 in GVHD (Leonhardt et al., 2014). However, unlike Fostamatinib, PRT0603 significantly reduced TNF-α. It has been reported that the activation of the SYK pathway is involved in the control of TNF-α production by macrophages (Su Yi et al., 2014). The data in this report highlighted that deactivation of SYK by PRT0603 is the likely mechanism responsible for the reduced TNF-α production by monocytes/macrophages. TNF-α is mainly released by macrophages in GVHD to up-regulate co-stimulatory molecules and to stimulate T cells (Markey et al., 2013, Zhang et al., 2017). The previous data revealed a reduction of CD86 expression on monocytes/macrophages, due to reduction of TNF-α, as a result T cells alloreactivity being reduced after PRT0603 treatment. This explanation is supported by other studies which reported that low TNF-α concentrations are linked with a low risk of GVHD (Kumar et al., 2014). However, in this study the data showed a reduction of TNF-α with non-significant changes on CD86 expression which could be due to the limited number of repeats. Reduction of IFN-γ levels could deactivate monocytes/macrophages (Ashkar et al., 2018, Rahimi et al., 2019). IL-6 is mainly released by classical monocytes (Boyette et al., 2016). In GVHD IL-6 controlled T cell maturation and differentiation (Tawara et al., 2011, Tvedet et al., 2017). The data in this study highlighted that a reduction of IL-6 and IL-2 concentration in MLR after PRT0603 treatment could explain the reduction of T cells activity. This data was supported by studies that showed low IL-2 concentrations are associated with lower GVHD incidences (Blazar et al., 2013, Kumar et al., 2017). In MLR, the results showed that SYK inhibitor was able to control cytokine secretion and that demonstrated its potency to reduce inflammation in alloresponse. Indeed, targeting cytokines is a valid therapeutic approach to control inflammation (Coffey et al., 2013).

In summary, it is difficult to clearly characterise the monocytes subtypes in allogeneic response, the data indicated that early MLR culture activates monocyte/macrophages to show an inflammatory phenotype. The activated $CD14^{+}$ could be the minor secretory cells of TNF-α, IFN-γ and IL-6 cytokines on

D2. Consequently, this early monocytes/macrophage activation could contribute to priming /alloresponse of T cells on D3 of cell culture. Importantly, these findings could highlight that the monocytes/ macrophages in the MHC mismatched MLR could react like monocytes in the matched model. In addition, the data may suggest that targeting SYK by 2 µM PRT0603 in MLR culture modulated monocytes/macrophages function and reduced cytokine secretion which is followed by interference with T cell allo-reactivity. Indeed, reducing the inflammation driven by cytokines that are produced during the allogeneic reaction could be an important therapeutic approach. Thus, the primary data revealed that the SYK inhibitor PRT0603 could be considered as a therapeutic option to control monocytes/macrophages and T cells alloreactivity in the histo-incompatible model of aGVHD.

4 Results (Part 2):

4.1 Introduction:

IFN-γ is a cytokine produced by Th1 and NK cells. IFN-γ promotes monocytes differentiation into macrophages and polarisation towards the M1 phenotype with high Ag presentation capacity (Deeg, 2001, Wang et al., 2014, Lee et al., 2017, Lee & Ashkar, 2018). It is thought that in GVHD, tissue injury that occurs post TBI is accompanied by bacterial product (LPS) release from GIT epithelium, and this triggers macrophages activation and production of inflammatory cytokines such as TNF-α and IFN-γ (Chakraverty & Sykes, 2006). In GVHD, patients' serum contains high concentrations of IFN-γ, however, this does not reflect GVHD severity. Nevertheless, IFN-γ stimulates the secretion of TNF-α and IL-6, which could contribute to pathology (Nakamura et al., 2000). The alloresponse is started by T cells after recognising the foreign Ag presented by APCs (Leonhardt et al., 2012, Heller et al., 2017, Poe et al., 2018). This study investigated the effects of IFN-γ on monocytes function in the *in vitro* model of early allogeneic response. Furthermore, the effects of SYK inhibitor, PRT0603, on MLR in the presence of IFN-γ were assessed. This therapeutic approach could control alloresponse, inhibit monocyte function and T cells activation, leading to a decrease in cytokine production.

Therefore, the aims of this chapter were to:

- Investigate the effect of IFN-γ on monocytes/macrophages function and activation in alloresponse.
- Investigate the effect of IFN-γ on T cell activity.
- Evaluate the effect of IFN-γ on cytokine production in MLR culture.
- Examine the effect of SYK inhibitor, PRT0603 on CD86, HLA-DR and CD64 expression on monocytes/macrophages in MLR culture in the presence of IFN-γ.
- Investigate the effect of PRT0603 on T cells activity in MLR culture in the presence of IFN-γ.
- Evaluate the effect of SYK inhibitor on cytokine production in MLR culture in the presence of IFN-γ.

4.2 Results:

4.2.1 Optimising the IFN-γ concentration:

10 ng/ml, 50 ng/ml, and 100 ng/ml IFN-γ were added to MLR, and the expression levels (MFI value) of CD86, HLA-DR and CD64 were measured by flow cytometry after D1 and D2, of cell culture. The cell viability was checked by Annexin V. staining. Notably, 80-85% of cells were viable on D1 and D2 (data not shown). All samples were treated with Fc blocking and the results were compared to the isotype controls to eliminate any background of non-specificantibody binding (isotype raw data provided in the appendix figure S.1.3).

The results (Figure 4.1.A and Figure 4.1.C) show that the expression of CD86 and CD64 were increased after treatment with 100 ng/ml IFN-γ when compared to the other concentrations for both days of incubation, but the differences were not significant. The results for HLA-DR (Figure 4.1.B) revealed slight changes in expression on the $CD14^+$ monocytes/macrophages after D1, however, after D2 the $CD14^+$ cells treated with 100 ng/ml and 50 ng/ml IFN-γ maintained the same expression level. Therefore, 100 ng/ml of IFN-γ was selected as the optimal concentration for the rest of the experiments.

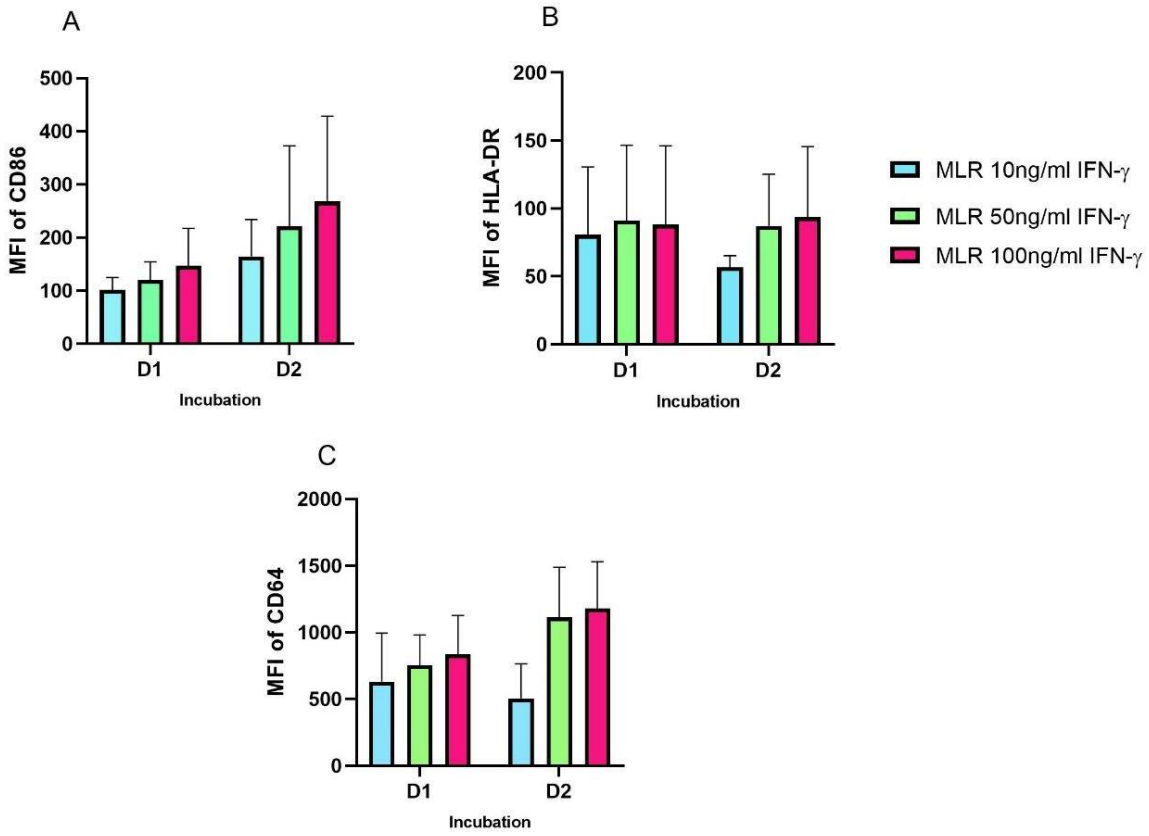

Figure 4. 1: Analysis of the level of expression (MFI) for **A)** CD86, **B)** HLA-DR and **C)** CD64 on $CD14^+$ monocytes measured by flow cytometry after one (D1) and two days (D2) of incubation.

The X axis shows incubation days, Y axis presents MFI expression. Data shown are mean and the error bars are SD (n = 3). Blue bar = MLR treated with 10 ng/ml IFN-γ, green bar = MLR treated with 50 ng/ml IFN-γ and pink bar = MLR treated with 100 ng/ml IFN-γ. Statistical significance of data was analysed using RM two-way ANOVA with Tukey`s multiple comparisons test and Geisser Greenhouse, p values below 0.05 were considered significant.

4.2.2 Monocytes/macrophages co-stimulation capacity in MLR culture with addition of IFN-γ:

To further investigate the effect of 100 ng/ml IFN-γ on monocyte co-stimulation capacity, four conditions were compared: Res (responder cells negative control), MLR (alloresponse control), MLR treated with 100 ng/ml IFN-γ (experimental condition, alloresponse in the presence of cytokine) and Res IFN-γ (responder cells treated with 100 ng/ml IFN-γ control for the effects of the cytokine). The results for D1 (Figure 4.2.A) revealed that the presence of IFN-γ did not affect CD86 expression on $CD14^+$ monocytes in allogeneic culture. Indeed, the only statistically significant difference in CD86 expression on D1 was observed between MLR and responder controls ($p = 0.0071$), MLR IFN-γ Vs Res ($p = 0.042$)

and Res IFN-γ (p = 0.043). Notably, CD86 expression had increased in all cell culture conditions on D2. However, the effects of IFN-γ were modest when added to MLR culture and resulted in a slight, non-significant increase compared to MLR without cytokine. In contrast, addition of IFN-γ to responder cells culture resulted in lower levels of CD86 expression compared to responder cells only culture. Consequently, the statistically significant difference on D2 was observed between MLR IFN-γ culture compared to responder cells treated with IFN-γ (p = 0.0087) and Res control (p = 0.023) on D2 of incubation. These data suggest that IFN-γ has neutral effects on CD86 expression in monocytes. Furthermore, the combined presence of allogeneic cells and IFN-γ did not result in significant changes in monocyte/macrophages CD86 expression. Thus, the presence of IFN-γ did not boost alloreactive response by increasing co-stimulation via CD86 in the early phase of MLR culture.

Conversely, the addition of IFN-γ did not interfere with the MLR-induced CD86 expression, as the CD86 up-regulation on the monocytes was mainly due to the allogeneic response. It could be concluded that effects of IFN-γ on monocyte are not focused on their co-stimulatory capacity and that other monocyte functions are more likely targets of this cytokine.

4.2.3 Monocytes/macrophages Ag presentation capacity in MLR culture with addition of IFN-γ:

The same four experimental conditions were investigated for Ag presentation capacity by measuring HLA-DR expression on monocytes/macrophages. The data (Figure 4.2.B) showed that $CD14^+$ monocytes/macrophages expressed slightly more of HLA-DR in the MLR treated with 100 ng/ml IFN-γ compared to MLR only on D1 and D2, however, the difference was notsignificant. On D1 of cell culture the significant difference was observed between MLR IFN-γ vs. Res IFN-γ (p=0.040) and Res control (p=0.0118). Similarly, on D2 of incubation the HLA-DR expression was significantly increased in MLR IFN-γ culture compared to Res IFN-γ (p=0.0052) and Res control (p=0.0010).

In contrast, it appears that IFN-γ has no effect on monocyte HLA-DR expression in the absence of alloresponse (responder cells culture in the presence of IFN-γ) compared to responder cells only. These findings suggest that the monocytes up-

regulated Ag presentation due to the alloresponse, and IFN-γ presence results in a modest further increasedof HLA-DR$^+$ expression on CD14$^+$ monocytes. Thus, it could be concluded that IFN-γ stimulates HLA-DR expression in monocytes only if monocytes were already activated by alloresponse.

4.2.4 Analysis of CD64 (FCγRI) and CD32B (FCγRIIB) expression on monocytes CD14$^+$ in MLR treated with IFN-γ:

The same four experimental conditions were investigated for CD64$^+$ (FCγRI) and CD32B (FCγRIIB) expression. The data (Figure 4.2.C) showed that CD14$^+$CD64$^+$ expression was significantly increased in MLR IFN-γ compared to the other experimental conditions on D1 ($p = 0.0075$ vs. Res, $p = 0.017$ vs. MLR) and D2 ($p = 0.0004$ vs. Res and $p = 0.0012$ vs. MLR). Notably, on D1 and D2 in Res IFN-γ the expression of CD14$^+$CD64$^+$ was significantly higher than Res ($p = 0.029$ for D1 and $p = 0.013$ for D2) The results highlight the strong stimulatory effects of IFN-γ on monocyte expression of CD64, and the synergy between IFN-γ and early alloresponse leading to further increase in the expression of CD64.

The data for CD32B expression (Figure 4.2.D) showed high levels of variability on D1 of incubation. Thus, there were no clear trends for changes in monocyte CD32B expression in the presence of allogeneic cells, IFN-γ or both stimuli. On D2 of incubation, the expression of CD32B was lower on D1 in all four experimental conditions. Furthermore, there were no effects of IFN-γ or alloresponse on monocyte CD32B expression. Thus, it appears that CD14$^+$CD32$^+$ expression is decreasing during the cell culture regardless of the addition of IFN-γ or the presence of allogeneic cells.

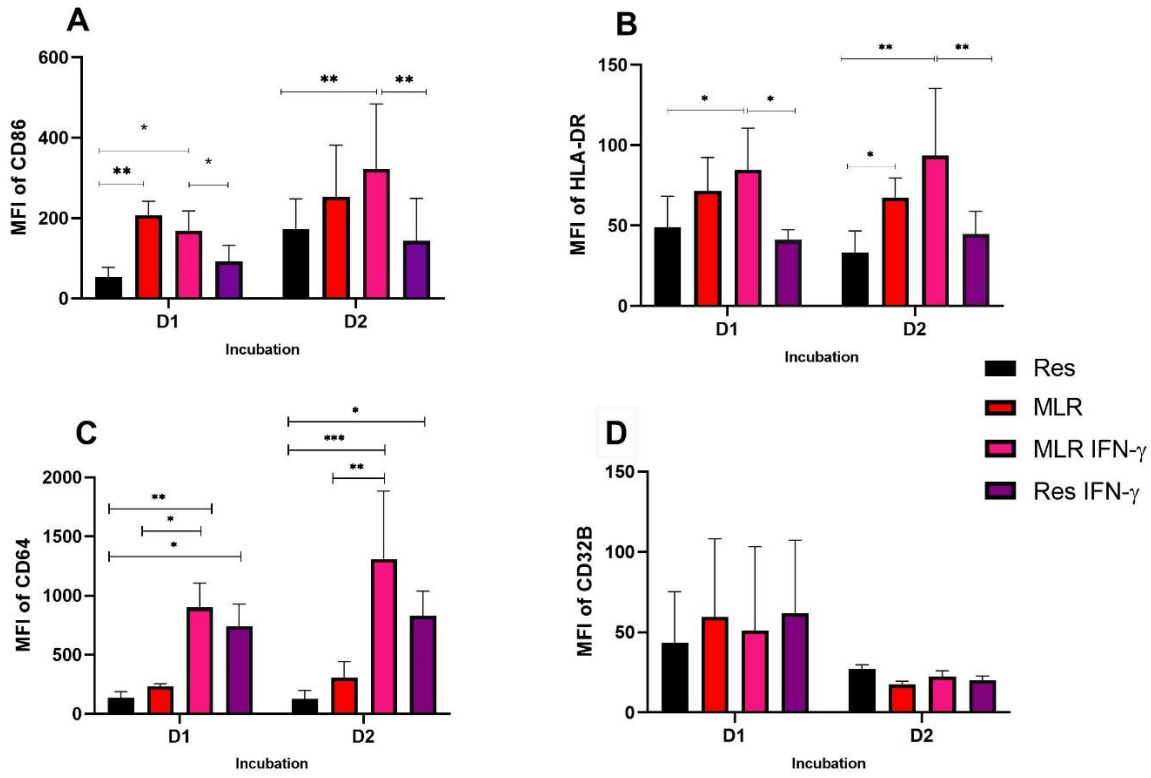

Figure 4. 2: Analysis of expression levels on CD14^{+} monocytes after IFN-γ stimulation after D1 and D2 of incubation. **A)** CD86, **B)** HLA-DR, **C)** CD64, **D)** CD32B.

The X axis shows incubation days, Y axis presents MFI expression level. Data shown are mean and the errorbars are SD (n = 6). Black bar represents Res = Responder cells as a negative control, orange barrepresents MLR = Mixed leukocyte reaction, pink bar represents MLR treated with 100 ng/ml IFN-γ, and purple bar represents Responder cells treated with100 ng/ml IFN-γ. Statistical significanceof data was analysed using RM two-way ANOVA with Tukey`s multiple comparisons test and Geisser-Greenhouse correction, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.001$, and *** = $p < 0.0001$.

4.2.5 Analysis of CD36 and CD204 expression on CD14^{+}in the presence of allogeneic cells and IFN-γ:

The expression of the scavenger receptors CD36 and CD204 were measured in the same four conditions to determine functional changes in monocytes/macrophages and their potential to clear apoptotic cells. The results (Figure 4.3.A) showed that the expression of CD14^{+}CD36^{+} was similar in all four conditions on D1 of incubation. Interestingly, on D2 of incubation, CD14^{+} in the control, responder cells culture had higher expression of CD36 compared to MLR, MLR IFN-γ and Res IFN-γ, although the differences did not reach statistical significance. These data suggest that the expression of CD36 on

monocytes/macrophages is not affected by the presence of allogeneic cells, IFN-γ or both stimuli combined.

The results for CD204 expression on $CD14^+$ cells (Figure 4.3.B) showed that on D1 of incubation, there was a significant increase in expression in the MLR ($p = 0.034$), and in MLR IFN-γ ($p = 0.021$) when compared to responder cells. These results suggest that IFN-γ stimulate $CD14^+$ monocytes to up-regulate $CD204^+$ scavenger receptor. The effects of early D1 alloresponse (MLR culture) on monocyte/macrophages CD204 expression were also significant, compared to responder cells. However, the presence of two stimuli, MLR IFN-γ compared to MLR, resulted in a modest further increase in CD204 expression. Thus, IFN-γ and alloresponse show modest, non-significant synergy in respect to monocyte/macrophages expression of CD204 biomarker. It could be argued that IFN-γ upregulates CD204 on monocytes/macrophages in order to improve recognition and clearing of the apoptotic cells. However, these effects of IFN-γ and alloresponse on CD204 expression appeared to be short lasting, as on D2 of incubation, $CD14^+CD204^+$ expression was reduced in MLR and MLR IFN-γ settings and increased in the responder cell control. Indeed, on D2 there was a statistically significant increase in responder cells expression of CD204 compared to Res IFN-γ ($p=0.018$).

4.2.6 Analysis of Ca3R expression on $CD14^+$ in the presence of allogeneic cells and IFN-γ:

The effect of IFN-γ and alloresponse on the complement receptor C3aR was investigated in the four experimental conditions described above. The results (Figure 4.3.C) showed that MLR culture resulted in striking significant increase in C3aR expression on D1 of incubation compared to responder cells ($p < 0.0001$), MLR IFN-γ ($p < 0.0001$) and Res IFN-γ ($p < 0.0001$). Furthermore, MLR IFN-γ had significantly up-regulated C3aR expression compared to the negative control of responder cells only ($p = 0.021$). These data suggest that an early alloresponse (D1) showed a dramatic effect on monocyte C3aR expression. In contrast, the effects of IFN-γ on monocyte/macrophage C3aR expression were modest. More importantly, IFN-γ appeared to interfere with stimulatory effects of alloresponse, as shown by significant decrease in monocyte/macrophage C3aR expression in early (D1) MLR IFN-γ culture compared to MLR.

Although the prominent feature of D2 data was a large increase in monocyte/macrophage C3aR expression in the control, responder cell culture, the apparent inhibitory effect of IFN-γ on the expression of this complement receptor was still observed.Indeed, D2 results showed that $CD14^+C3aR^+$ expression in MLR was significantlyincreased compared to MLR IFN-γ ($p < 0.0001$) and Res IFN-γ ($p < 0.0001$). Notably, the control responder cells expressed $CD14^+C3aR^+$ at a higher level than MLR IFN-γ ($p < 0.0001$) and Res IFN-γ ($p < 0.0001$). Overall, these data demonstrate a strong stimulatory effect of early alloresponse on monocytes/macrophages C3aR expression, whereas IFN-γ reduced the expression of this complement receptor.

4.2.7 Analysis of CD11b expression on $CD14^+$ monocytes in the presence of allogeneic cells and IFN-γ:

CD11b expression on $CD14^+$ was also analysed using the four experimental conditions, as described above. The results (Figure 4.3.D) revealed that the monocytes/macrophages in MLR and MLR IFN-γ showed a similar level of $CD14^+CD11b^+$ expression with no significant differences. This indicates that neither IFN-γ or early alloresponse play a significant role in controlling CD11b on monocytes/macrophages. Although, CD11b in MLR and MLR IFN-γ was expressed at a slightly higher level than in responder cells, particularly on D1, the difference did not reach statistical significance. As CD11b is a well-established marker of monocyte adhesion and migration, it appears that this important function is not significantly affected by IFN-γ or alloresponse.

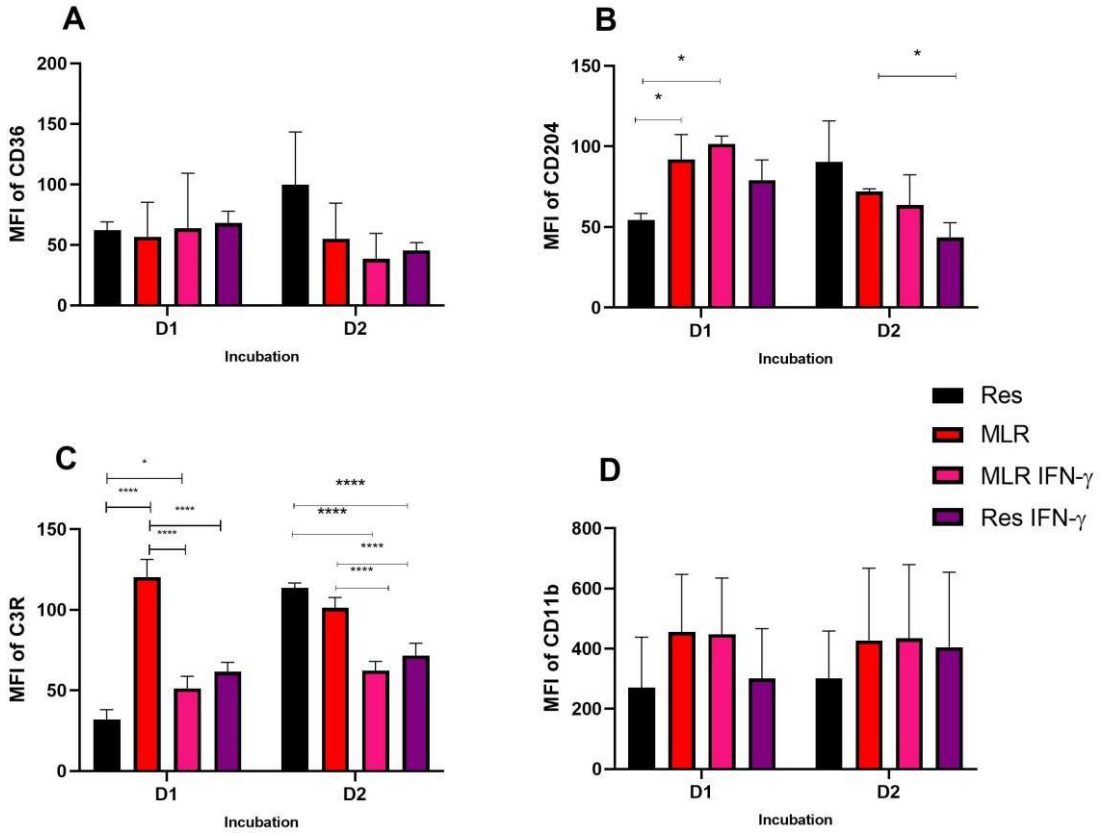

Figure 4. 3: Analysis of the expression CD36, CD204, C3aR and CD204 on $CD14^{+}$ monocytes after IFN-γ stimulation on D1 and D2 of incubation.

A) CD36, **B)** CD204, **C)** C3aR and **D)** CD11b. The X axis shows incubation days, Y axis is MFI expression. Data shown are mean and the error bars are SD (n = 3). Black bar represents Res = Responder cells as a negative control, orange bar represents MLR = Mixed leukocyte reaction, pink bar represents MLR treated with 100 ng/ml IFN-γ, and purple bar represents Responder cells treated with100 ng/ml IFN-γ. Statistical significance of data was analysed using RM two-way ANOVA with Tukey multiple comparisons test and Geisser-Greenhouse correction, p values below 0.05 were considered significant, * = $p < 0.05$** = $p < 0.01$, and *** = $p < 0.001$.

4.2.8 Activation of T cells in MLR treated with 100 ng/ml IFN-γ:

T cell activity was investigated on D3 by assessing the percent (%) of $CD3^{+}CD69^{+}$ and $CD4^{+}CD69^{+}$cells and the level of expression (MFI) of $CD3^{+}CD25^{+}$ and $CD4^{+}CD25^{+}$ T cells was determined in the four conditions described above

The data (Figure 4.4.A) showed a significant increase of MFI values $CD3^{+}CD25^{+}$ in the MLR IFN-γ compared to Res IFN-γ ($p=0.0013$) and Res ($p=0.0024$). The results for $CD3^{+}$ population (Figure 4.4.B) showed a significantly elevated percentage (%) of activated, $CD69^{+}$ T cells in MLR IFN-γ when compared to Res IFN-γ ($p=0.019$) and Res ($p=0.021$). Similar trends for increased $CD69^{+}$ T cells

were observed for MLR culture when compared to Res ($p = 0.014$) and Res IFN-γ ($p = 0.019$). Although proportion of $CD69^+$ T cells in MLR IFN-γ was slightly increased when compared to MLR, the difference was not statistically significant.

Thus, it can be concluded that IFN-γ on its own did not activate $CD3^+$ T cells, as demonstrated by the analysis of the expression of CD69 and CD25 markers on D3 of incubation. Furthermore, adding IFN-γ to MLR culture had modest, non-significant effects on T cell activation.

For the population of $CD4^+$ T helper cells, the level of expression (MFI) of CD25 (Figure 4.4.C) was significantly increased in MLR compared to Res ($p=0.046$) and Res IFN-γ ($p=0.046$). Notably, $CD4^+CD25^+$ expression was slightly lower in MLR IFN-γ than MLR, although difference was not significant. For CD69 expression (Figure 4.4.D), the results showed the high level of variability with a significant increase in the proportion (%) of activated $CD4^+$T cells in MLR IFN-γ compared to Res IFN-γ ($p=0.043$). However, the difference was not significant between MLR and MLR IFN-γ cultures. These findings for $CD4^+$ T helper cells suggest that MLR culture on D3 of incubation results in T cell activation, as demonstrated by increased levels of CD25 expression and a higher proportion of $CD69^+$ T cells. In contrast, the addition of IFN-γ did not boost T cell activation on D3 of incubation and, more importantly, did not act synergistically with MLR.

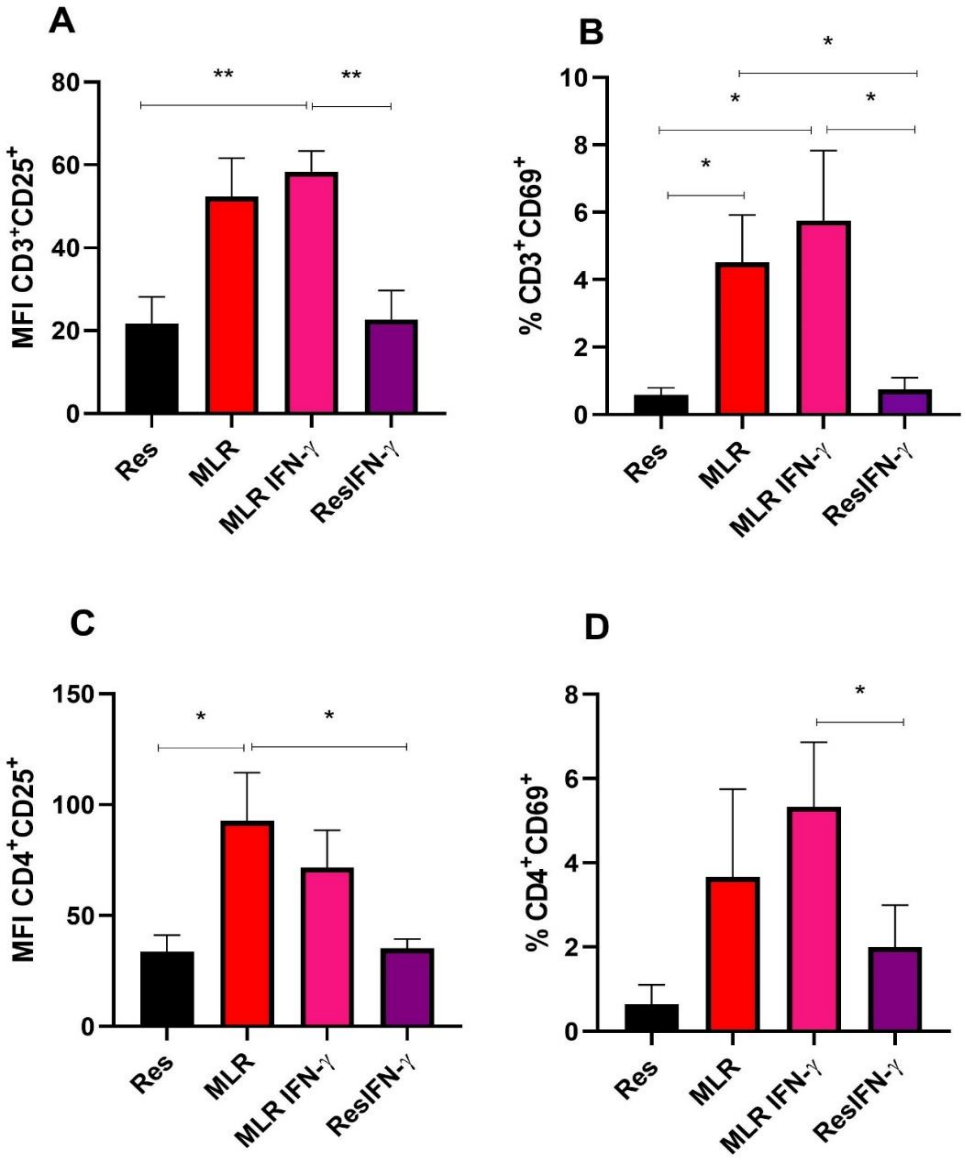

Figure 4. 4: Analysis T cells activation measured by flow cytometry after three days (D3) of incubation.

A) MFI values for $CD3^+CD25^+$ T cells where Y axis shows MFI values for CD25 expression, **B)** Percent of $CD3^+CD69^+$ T cells where Y axis shows proportion (%) of CD69-positive T cells. **C)** MFI values for $CD4^+CD25^+$ T helper cells, **D)** Percent of $CD4^+CD69^+$ T helper cells. Data shown are mean and the error bars are SD (n = 3). Black bar represents Res = Responder cells as negative control, orange bar represents MLR = Mixed leukocyte reaction culture, pink bar represents MLR treated with 100 ng/ml IFN-γ and purple bar represents responder cells treated with 100 ng/ml IFN-γ. Statistical significance of data was analysed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey multiple comparison values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

4.2.9 Measurement of cytokines in MLR treated with IFN-γ:

TNF-α, IL-2 and IL-6 concentration were measured by ELISA after two days of incubation analysis determined in the four conditions described above, to determine the effects of IFN-γ and the features of inflammatory reaction in allogeneic culture. The results for TNF-α (Figure 4.5.A) showed that on D2 of incubation in MLR IFN-γ levels of this cytokine were significantly higher than in the control Res culture (p=0.0069) and Res IFN-γ (p = 0.015). The levels of TNF-α in MLR culture were significantly higher than in the Res (p=0.028) andRes IFN-γ cell cultures (p=0.0105). Furthermore, the concentration of TNF-α in MLR IFN-γ was higher than MLR, but the result was not statistically significant.

The results (Figure 4.5.B) for IL-2 in MLR IFN-γ were clearly measurable and were significantly higher than Res (p=0.043) and Res IFN-γ (p=0.032). Notably, MLR IFN-γ showed slightly higher IL-2 concentrations compared to MLR, however the difference was not significant.

For IL-6 (Figure 4.5.C) the levels of IL-6 cytokine in MLR were detectable and significantly increased compared to Res (p=0.029) and Res IFN-γ (p=0.016). Interestingly, the levels of IL-6 in MLR culture were significantly higher than in MLR IFN-γ cell culture (p=0.016). Also, IL-6 concentrations were significantly higher in MLR IFN-γ than in the Res (p=0.061) and Res IFN-γ (p=0.011). These data indicate that the presence of IFN-γ interferes with the stimulatory effects of alloresponse on IL-6 production.

In summary, the results presented above highlight that the inflammatory cytokines such as TNF-α, IL-2 and IL-6 were induced in early alloresponse (D2 of incubation), as MLR culture showed significantly increased levels of these cytokines. However, addition of IFN-γ to MLR culture resulted in a modest synergy with a trend for a slight increase in the production of TNF-α and IL-2. In contrast, IFN-γ showed strong antagonistic effects on IL-6 production even in the presence of the stimulatory allogeneic response, MLR culture. Regarding the source of cytokines in a complex cell culture, at this early stage of alloresponse (D2), monocytes/macrophages were clearly activated, thus it could be postulated that

TNF-α and IL-6 were likely to be secreted by these cells. On the other hand, IL-2 could be produced by T cells, despite the markers of T cells activation such as CD25 and CD69 being negative at this stage (D2 of incubation) of alloresponse. Thus, intracellular analysis will consider in future work.

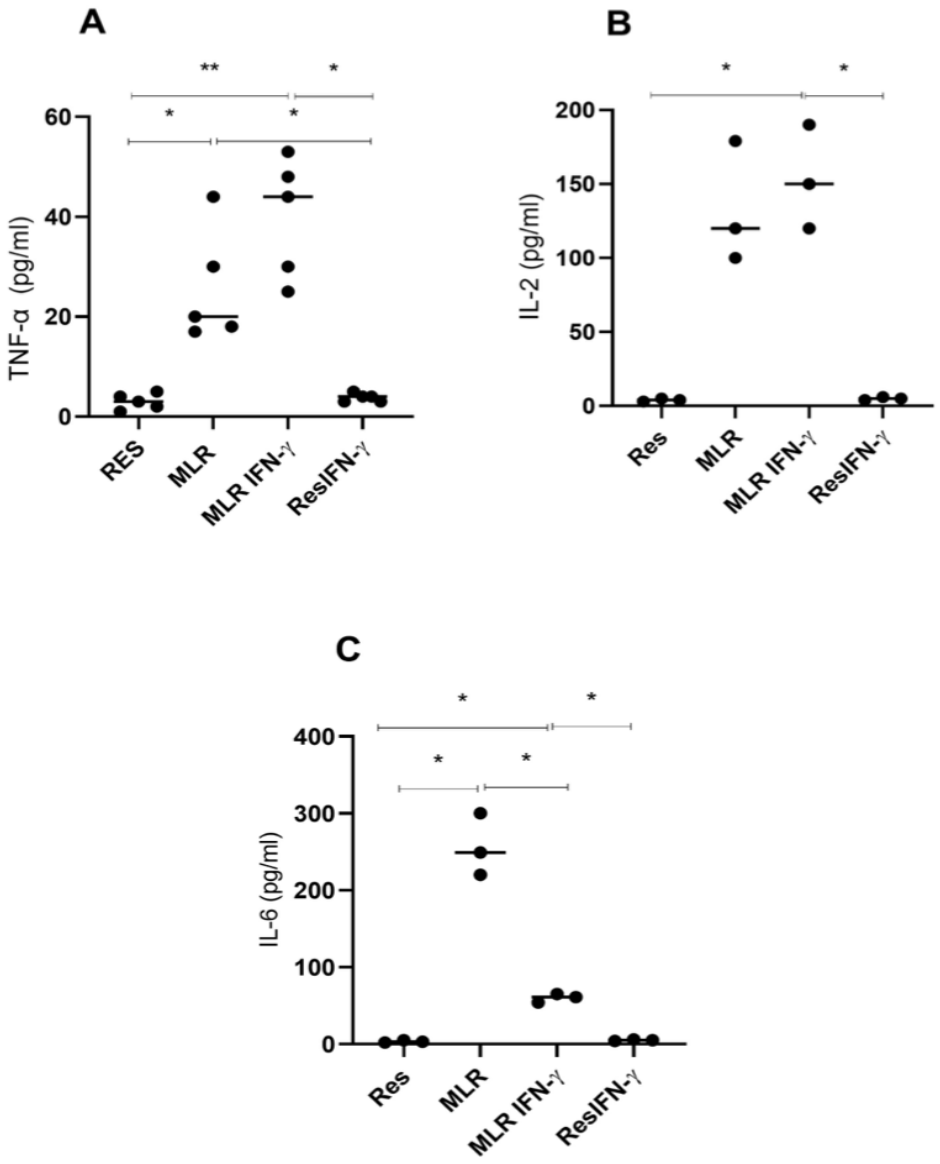

Figure 4. 5: Measuring inflammatory cytokines on D2 of incubation with/without IFN-γ treatment.

A) TNF-α, **B**) IL-2, and **C**) IL-6 concentrations. X axis shows comparison of four conditions: Res = responder cells, MLR = Mixed leukocyte reaction, MLR IFN-γ = MLR treated with 100 ng/ml IFN-γ and Res IFN-γ = Responder cells treated with 100 ng/ml IFN-γ. Y axis shows the cytokine concentrations in pg/ml. Scatter dot plots show repeats (n = 3 and 5 for TNF-α). Statistical significance of data was analysed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey multiple comparison, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

4.2.10 Effects of SYK inhibitor on CD14$^+$ in MLR treated with IFN-γ:

The expression levels (MFI values) of CD86, HLA-DR and CD64 were measured for the four test conditions: MLR IFN-γ (+ve control MLR treated with 100 ng/ml IFN-γ), MLR IFN-γ SYK (experimental condition MLR treated with 100 ng/ml IFN-γ and 2 μM anti-SYK), Res IFN-γ (control responder cells treated with 100 ng/ml IFN-γ) and Res IFN-γ SYK (inhibitor effects on control responder cells treated with 100 ng/ml IFN-γ and 2 μM anti- SYK).

The results (Figure 4.6.A) showed that there was a modest reduction in CD86$^+$ expression on CD14$^+$ monocytes in the MLR IFN-γ treated with SYK inhibitor when compared to the MLR IFN-γ on D1 and D2 of incubation, however the difference was not significant. The data also confirmed stimulatory effects of MLR as demonstrated by significant higher levels of CD14$^+$CD86$^+$ expression in MLR IFN-γ compared to Res IFN-γ ($p=0.0042$) and Res IFN-γ SYK ($p= 0.0046$). following the same trend significant difference of CD86 expression in MLRIFN-γ SYK compared to Res IFN-γ ($p= 0.034$) and Res IFN-γ SYK ($p=0.039$). These data confirm strong effects of MLR on the expression of monocyte/macrophages co-stimulatory molecule CD86, modest effects of IFN-γ and a trend for slight inhibitory effects of anti SYK that did not reach statistical significance.

For HLA-DR expression (Figure 4.6.B), a reduction of MFI was observed for MLR IFN-γ compared to MLR IFN-γ with SYK inhibitor on D1 and D2 of incubation, although difference was not statistically significant. The trend for significant reduction in HLA-DR was seen in Res IFN-γ ($p = 0.040$) and Res IFN-γ with SYK inhibitor ($p=0.030$) compared to MLR IFN-γ on D1. Similarly significant difference in HLA-DR expression was seen in Res IFN-γ ($p=0.045$) and Res IFN-γ with SYK inhibitor ($p=0.032$) compared to MLR IFN-γ on D2. This is a consequence of the lower HLA-DR expression in the presence of IFN-γ in responder cells compared to the combined stimulatory effects of IFN-γ plus MLR. Also, SYK inhibition could contribute to low HLA-DR expression in Res IFN-γ with SYK inhibitor culture, thus SYK pathway could modulate monocytes/macrophages Ag presentation in certain inflammatory settings.

For $CD14^{+}CD64^{+}$ expression (Figure 4.6.C), the data revealed that there was a modest down regulation after treatment of MLR IFN-γ with 2 μM of SYK inhibitor on D2 of incubation, and in Res IFN-γ SYK compared to Res IFN-γ on D1 of incubation. However, the difference did not reach statistical significance.

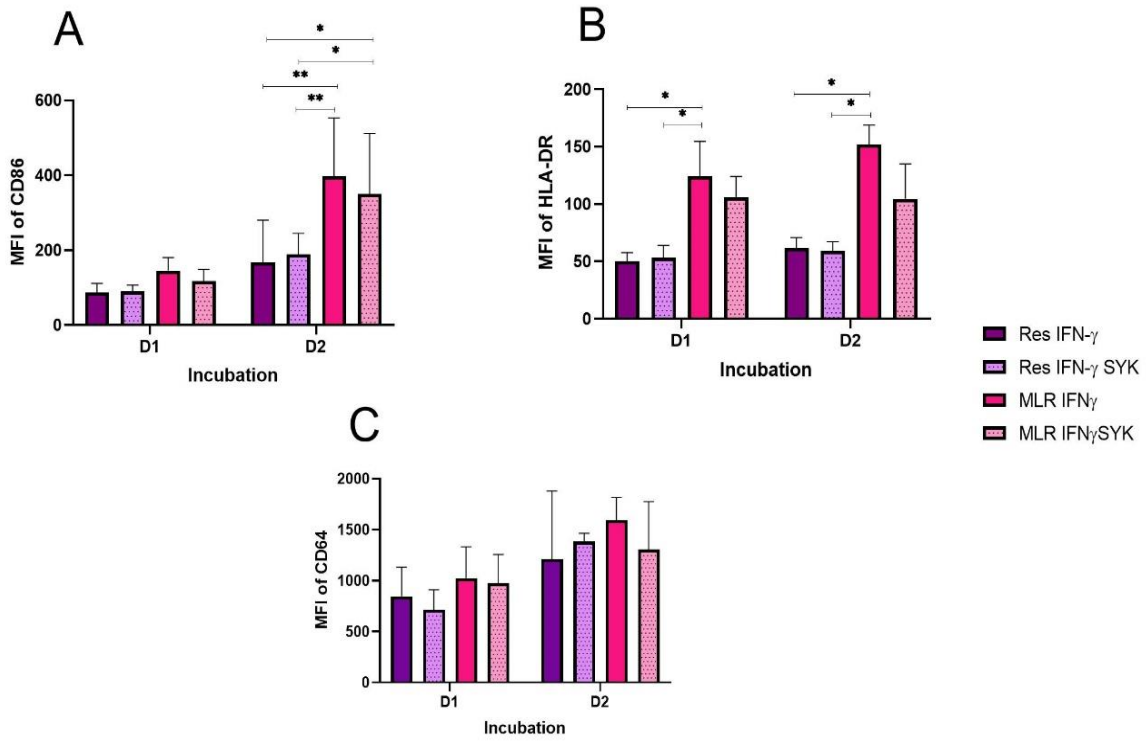

Figure 4. 6: Analysis of **A)** CD86, **B)** HLA-DR and **C)** CD64 expression on $CD14^{+}$ monocytes on D1 and D2 of incubation in the presence of IFN-γ, MLR and SYK inhibitor.

The X axis shows incubation days, Y axis is MFI expression. Data shown are mean and the error bars are SD (n = 5). Dark purple bar represents Res IFN-γ = Responder cells cell treated with 100 ng/ml IFN-γ as a negative control, light purple bar with dot pattern represents responder cell treated with 100 ng/ml IFN-γ and 2 μM SYK inhibitor, pink bar represents MLR IFN-γ = Mixed leukocyte reaction treated with 100 ng/ml IFN-γ, and light pink with dot pattern bar represents MLR treated with 100 ng/ml IFN-γ and 2μM SYK inhibitor. Statistical significance of data was analysed using RM two-way ANOVA with Geisser-Greenhouse correction and Tukey multiple comparison test, p values below 0.05 were considered significant, * = $p < 0.05$, and ** = $p < 0.01$.

4.2.11 Effects of SYK inhibitor on T cell activity in MLR treated with IFN-γ:

$CD3^+$ and $CD4^+$ T cells were analysed after three days of incubation for CD25 expression levels (MFI values), and proportion (%) of CD69 positive cells in four conditions described above. Cell viability was checked using Annexin V FITC and more than 80 % of the cells were Annexin -ve after gating for the T cell population (data not shown).

The data (Figure 4.7.A) revealed that $CD3^+CD25^+$ T cells in MLR IFN-γ showed significantly higher levels of expression than Res IFN-γ (p=0.041) and Res IFN-γ SYK (p=0.043). Importantly, treatment with 2 μM SYK inhibitor (MLR IFN-γ SYK) significantly reduced $CD3^+CD25^+$ expression compared to MLR IFN-γ culture (p = 0.024).

Following the same trend, the results (Figure 4.7.B) for the % of $CD3^+CD69^+$ T cells showed significant reduction in MLR IFN-γ treated with 2 μM SYK inhibitor compared to MLR IFN-γ (p=0.045). Also, MLR IFN-γ cell culture showed significantly higher proportion (%) of $CD69^+$ T cells than Res IFN-γ (p=0.011) and Res IFN-γ SYK (p=0.032).

For $CD4^+$ cells (Figure 4.7.C) there was a slight reduction in $CD4^+CD25^+$ expression for MLR IFN-γ treated with SYK inhibitor when compared to MLR IFN-γ. However, the difference did not reach statistical significance. Interestingly, $CD4^+CD25^+$ expression levels were significantly higher in MLR IFN-γ than in Res IFN-γ SYK (p=0.034).

The results (Figure 4.7.D) for $CD4^+CD69^+$ showed similar proportion of $CD69^+$ T helper cells for MLR IFN-γ compared to cell culture in the presence of inhibitor MLR IFN-γ SYK. Notably, $CD4^+CD69^+$ proportion (%) was higher in MLR IFN-γ and MLR IFN-γ SYK compared to Res IFN-γ, Res IFN-γ SYK.

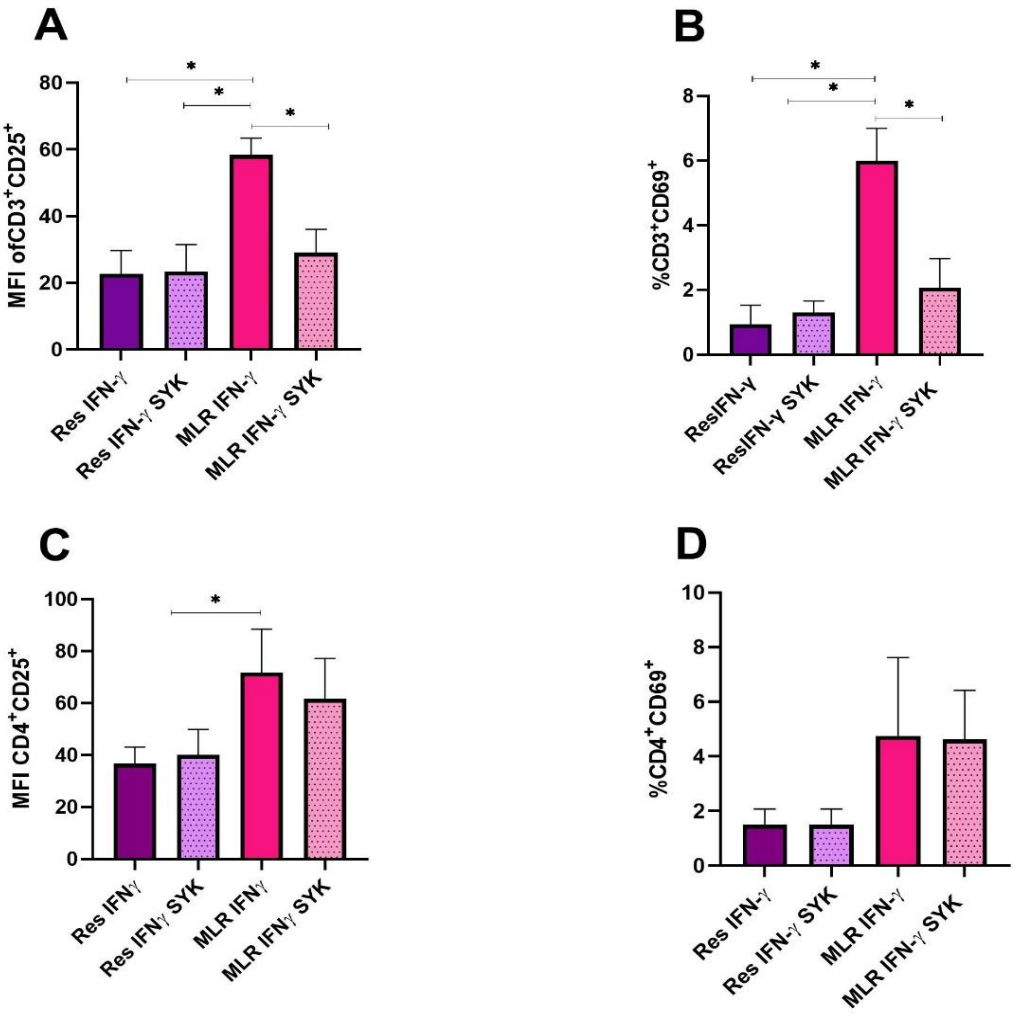

Figure 4. 7: Analysis of T cell activation by flow cytometry on D3 of incubation with IFN-γ and SYK inhibitor.

A) $CD3^+CD25^+$, B) $CD3^+CD69^+$, C) $CD4^+CD25^+$ D) $CD4^+CD69^+$. Y axis is MFI expression in A andC and % in B and D. The X axis shows treatment, dark purple bar represents Res IFN-γ = Responder cells treated with 100 ng/ml IFN-γ, light purple bar with dot pattern representsresponder cells treated with 100 ng/ml IFN-γ and 2 μM SYK inhibitor, pink bar represents MLR IFN-γ = Mixed leukocyte reaction treated with 100 ng/ml IFN-γ and light pink with dot pattern bar represents MLR IFN-γ SYK = MLR treated with 100 ng/ml IFN-γ and 2μM of SYK inhibitor. Data shown are mean and the error bars are SD (n = 4). Statistical significance of data was analysed RM one-way ANOVA with Geisser-Greenhouse correction and Tukey multiple comparison, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$.

4.2.12 Measuring cytokines concentrations in MLR culture treated with IFN-γ and SYK inhibitor:

The samples were collected on D2 of incubation from four conditions were described above. All samples were analysed by sandwich ELISA for TNF-α, IL-2 and IL-6 cytokines concentrations.

The results (Figure 4.8.A) revealed that SYK inhibitor significantly decreased the TNF-α concentration in MLR IFN-γ SYK compared to MLR IFN-γ ($p=0.0017$). Also, significant differences were observed in MLR IFN-γ compared to Res IFN-γ ($p=0.013$) Res IFN-γ SYK ($p=0.045$).

For IL-2 (Figure 4.8.B), there was a significant reduction in IL-2 concentration due to SYK inhibitor, MLR IFN-γ SYK compared to MLR IFN-γ ($p=0.032$). Also, significant differences were observed in MLR IFN-γ compared to Res IFN-γ ($p=0.023$) Res IFN-γ SYK ($p=0.024$).

For IL-6 (Figure 4.8.C) the results showed a significant reduction in the presence of inhibitor in MLR IFN-γ SYK compared to MLR IFN-γ ($p=0.081$). Significant differences in IL-6 concentration were seen in MLR IFN-γ compared to Res IFN-γ ($p=0.021$) Res IFN-γ SYK ($p=0.025$).

Overall, SYK inhibitor significantly reduced concentrations of TNF-α, IL-2 and IL-6 cytokines in allogeneic culture (MLR) in the presence of IFN-γ.

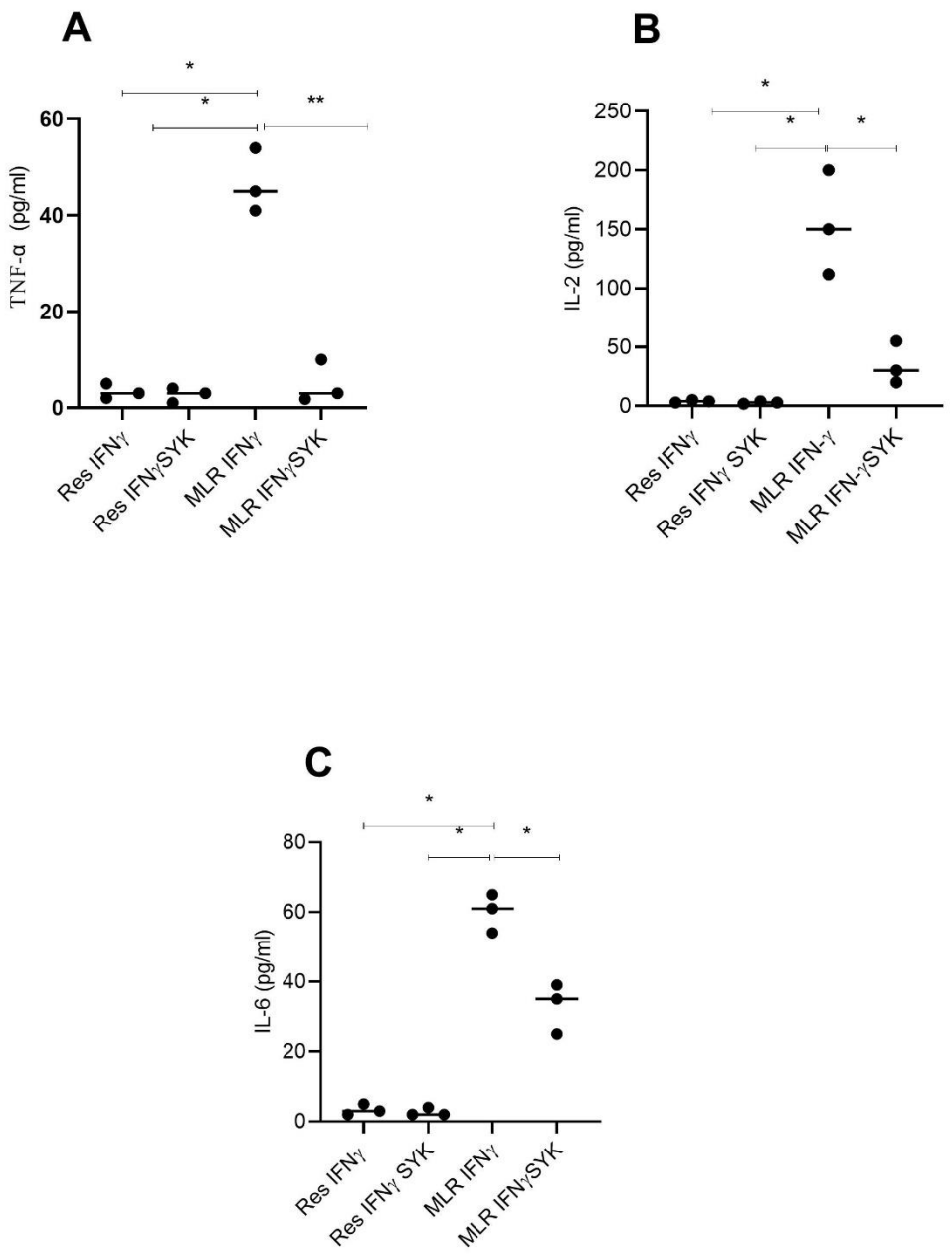

Figure 4. 8: Measuring proinflammatory cytokines on D2 of incubation in the presence of allogeneic cells, IFN-γ and SYK inhibitor.

A) TNF-α, **B)** IL-2 and **C)** IL-6 concentrations. X axis shows comparison of four conditions: Res IFN-γ = Responder cell culture with 100 ng/ml IFN-γ, Res IFN-γ SYK = Responder cell culture with 100 ng/ml IFN-γ and 2 μM of SYK inhibitor, MLR IFN-γ = Mixed leukocyte reaction with 100 ng/ml IFN-γ, and MLR IFN-γ SYK = MLR treated with IFN-γ and 2 μM of SYK inhibitor. Y axis shows the cytokine concentration in pg/ml. Scatter Dot plot shows triplicate repeats (n = 3). Statistical significance of data was analysed using RM one-way ANOVA with Geisser-Greenhouse correction and Tukey multiple comparison, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

4.3 Discussion (Part 2):

In the context of GVHD development, IFN-γ is regarded as a proinflammatory cytokine that is released from activated APCs in response to allo-Ag (Wang et al., 2014, Lee & Ashkar, 2018). IFN-γ is known as a Th1 cytokine, as it is mainly produced by Th1 cells and NK cells, and it control monocyte/macrophage activation (Nakamura et al., 2000, Murray, 2012, Raphael et al., 2014, Rahimi et al., 2019). Binding of IFN-γ to the IFN-γ receptor on the surface of monocytes/macrophages, will activate JAK-STAT signal transduction. This controls gene transcription and many monocyte functions, including Ag capture Ag processing, Ag presentation, anti-microbicidal function and anti-viral response (Schorder et al., 2003). It is thought that IFN-γ promotes differentiation of monocytes towards M1 macrophages with a high phagocytic capacity (Nakamura et al., 2000, Duque & Descoteaux, 2014, Raphael et al., 2014, Lee et al., 2017). However, the exact role of IFN-γ is still debated as it can act as an inhibitor of inflammation (Yong et al., 1998, Raphael et al., 2014, Nassereddin et al., 2017). Also, its association with GVHD pathogenesis remains controversial (Nakamura et al., 2000, Visentainer et al., 2003). In the previous chapter, MLR culture was shown to activate monocytes/macrophages and T cells. This is likely to involve contribution of multiple cytokines produced by the activated immune cells, however, the role of an individual cytokine such as IFN-γ needs to be defined. Therefore, this chapter investigated the role of IFN-γ in the early phase of response to allogeneic cells.

This chapter aimed to analyse two areas, first was to evaluate the effects of IFN-γ on the monocyte/macrophages function, T cell activation and cytokines production in MLR culture. The second area was to investigate the effects of SYK pathway inhibition in MLR treated with IFN-γ, by evaluating changes in the $CD14^{+}$ monocytes phenotype, T cell activation and cytokine production.

The initial, extensive study of the effects of various concentrations of IFN-γ (from 1 to 100 ng/ml) on the early phase of alloresponse/MLR showed that a relatively high concentration, 100 ng/ml of this cytokine ensured a stable response without affecting cell viability. Although this concentration of IFN-γ is much higher than the levels that were observed in GVHD patients (Nakamura et al., 2000, Visentainer

et al., 2003). It is important to note that the reported systemic (serum) concentration of this cytokine does not represent the actual levels in the local, inflamed tissues. Furthermore, the inflamed tissue is likely to provide a continuous supply of newly produced cytokines, which compensates for the physiological, short half-life of these unstable molecules. In contrast, *in vitro* or *ex vivo* experimental conditions need to ensure the optimal presence of a cytokine in a challenging environment where cytokine stability and bioavailability is difficult to monitor. Indeed, the complex experimental conditions such as those based on the whole blood culture used in this model, represent a distinct setting where the cytokines are exposed to enzymes released by neutrophils, platelets, and other blood cells. Thus, the whole blood MLR culture used in this study cannot be directly compared to *in vivo* conditions or experimental models based on the use of isolated peripheral blood mononuclear cells (PBMCs). As the relevant, tissue levels of IFN-γ remain speculative and the *in vitro* stability of cytokine depends on the specific cell culture conditions, it could be argued that using a relatively high concentration of the cytokine ensures its presence during the 1-3 days of incubation. This approach was evaluated by confirming the known stimulatory effects of IFN-γ, such as an increased CD64 expression on monocytes and demonstrated the lack of negative effects on cell viability. Similar to this report, the study by Bayik et al. (2017) on human monocyte maturation has used 500 ng/ml of IFN-γ after optimising the cytokine concentration for cell culture conditions (Bayik et al., 2017). This is 5 times more than the levels of cytokine used in this study. Furthermore, using a mouse model by Brok et al. (1998) reported that high dose of IFN-γ immediately after transplantation was crucial for the prevention of GVHD (Bork et al., 1998). In that study, treatment with 50,000 IU (International Units) of IFN-γ (approximately 10 μg (10,000 ng) twice weekly for a period of 5 weeks was required to prevent GVHD in a fully MHC-mismatched model. Therefore, based on these published reports and the extensive optimisation experiments, it was concluded that the optimal concentration of IFN-γ was 100 ng/ml, and therefore this concentration was used in all subsequent experiment. Nevertheless, a high concentration of cytokines appears to be a limitation for the *in vitro* monocytes studies, as most of the studies required a high pharmacological concentration of cytokines to achieve phenotype changes.

The effects of the addition of IFN-γ to MLR culture were investigated using four experimental conditions: MLR IFN-γ (MLR treated with 100 ng/ml IFN-γ), MLR only (alloresponse control), Res (-ve control, responder cells only) and Res IFN-γ (control for IFN-γ effects). For the flow cytometry immunophenotyping all samples were treated with Fc blocking and the results were compared to the isotype control to eliminate any background of nonspecific antibody binding (raw data shown in the appendix figure S.1.4). Notably, 80 to 85 % of monocytes in the gated population were Annexin V -ve, thus confirming acceptable cell viability on D1 and D2, respectively.

This study has used the MLR model where stimulatory cells were not irradiated or treated by mitomycin c. Consequently, both populations, stimulator and responder cells could respond to the presence of allogeneic cells. Nevertheless, the large numerical advantage of responder cells (10:1 ratio) favours this population. Indeed, the control experiments using carboxy fluorescein diacetate succinimidyl ester (CFSE) labelled stimulator cells have shown minimal contribution of stimulator cells to the overall MLR response (data not shown Sabine Broc, unpublished findings).

Recently, it has been reported that the skin lesion from GVHD patients contain large numbers of activated macrophage infiltrates, derived from the donor circulatory monocytes (Jardine et al., 2020). Thus, donor (responder) APCs are involved in the initiation of GVHD (Jardine et al., 2020). Therefore, this study could argue that the MLR model described in this report might reflects the allogeneic response in the GVHD setting, regardless of the differences in MHC matching between this model and HSCT patients.

Monocyte/macrophage function was assessed by evaluating the expression levels of the surface markers by measuring MFI values for CD86, HLADR, CD64, CD32B, CD36, CD204, C3aR and CD11b. The MLR culture in the presence of 100 ng/ml IFN-γ compared to control conditions. The data revealed that IFN-γ up-regulate the expression of CD64 and downregulated C3aR expression significantly. Interestingly, IFN-γ antagonised IL-6 concentrations in allogeneic culture. Non-significant difference observed in CD86 and HLA-DR expression that could be due to the small sample size. Although MLR IFN-γ culture showed a

significant up-regulation of CD86 and HLA-DR expression on $CD14^+$ monocytes when compared to Res IFN-γ and Res control, this can be attributed to the predominant effects of allogeneic cells/MLR, rather than effects of the IFN-γ cytokine. Therefore, these findings indicate that IFN-γ has a modest effect on CD86 and HLA-DR expression in monocytes in allogenic/MLR culture, and that IFN-γ did not show any synergistic effects with the allogeneic response. Notably, these data question the previously proposed role of IFN-γ in controlling co-stimulation in circulatory monocytes in GVHD (Deneys et al., 2001, Delneste et al., 2003, Zheng et al., 2004, Zeiser et al., 2016). Thus, this study supports the concept that the effects of IFN-γ on monocytes/macrophages in the early allogeneic culture are not focused on their co-stimulatory capacity and the other monocytes/macrophages functions are more likely targets of this cytokine.

The presence of allogeneic cells stimulates HLA-DR and IFN-γ slightly enhanced expression of this maker for Ag presentation capacity. This could suggest that IFN-γ stimulates HLA-DR expression in monocytes/macrophages only if $CD14^+$ monocytes were already activated by the alloresponse. This finding supports the importance of donor derived IFN-γ to control HLA-DR and CD86 expression in the MLR/GVHD. Indeed, it has been reported that donor derived IFN-γ plays a role in controlling GVHD initiation (Young et al., 1998). And the donor derived MHC molecules could be involved as well (Poe et al., 2018). This data could be explained as the following, in the MLR culture both stimulatory and responder cells could involve in the Ag presentation and cytokines secretion.

It has been reported by previous studies that release of IFN-γ in the early phase of GVHD up-regulated MHCII expression (Nakamura et al., 2000, Deeg, H 2001, Markey et al., 2013). This could lead to formation of M1 macrophages, as several studies have reported that IFN-γ induced MHCII on monocytes and controlled M1 formation (Murray & Wang 2012, Raphael et al., 2014, Lee et al., 2017). However, in this report the effect of IFN-γ on HLA-DR expression is weak to moderate. Therefore, it could be argued that the IFN-γ mediated increase in Ag presentation may prepare monocytes for defence against pathogens without prominent inflammatory response. These results are supported by previous studies, which reported elevated level of serum IFN-γ in GVHD patients was linked to viral infection, and not GVHD development (Nakamura et al., 2000, Visentainer et al.,

2003).

The Fc receptor, CD64 is high affinity IgG receptor, that is saturated with IgG monomer (Brandsma et al., 2018). In the previous chapter significant up-regulation of CD64 observed due to allogeneic response. The data in this study showed significantly increased expression of CD64 on $CD14^+$ monocytes in the MLR IFN-γ culture when compared to control conditions (MLR, Res and Res IFN-γ) on D1 and D2 (data presented in the appendix Figure S.1.4). The explanation of these results could be that the binding of the IFN-γ cytokine to the cytokine receptor on the surface of $CD14^+$ monocytes caused the clustering of CD64 on the surface of monocytes/macrophages (Brandsma et al., 2018). More importantly the clustering of CD64 induces downstream signalling leading to ITAM phosphorylation, followed by the recruitment of SYK (Kiefer et al., 1998, Coffey et al., 2016). Similarly, the cross-linking of opsonised Ag led to CD64 internalisation, processing of the Ag and loading of the processed Ag to MHC molecule (Junker et al., 2020). Thus, the up-regulation of CD64 could be linked to the increase of HLA-DR expression in MLR culture. Interestingly, this was only observed during allogeneic culture/MLR not with the responder cell culture. Although, this study cannot confirm SYK activation as intracellular staining was not performed, it could be assumed that the up-regulation of CD64 and increase of HLA-DR expression could be SYK pathway dependent (Coffey et al., 2016). The treatment of $CD14^+$ monocytes in MLR culture with IFN-γ resulted in a strong up-regulation of CD64 expression. This could have an important role in monocytes/macrophages phagocytosis and defence against pathogens (Delneste et al., 2003). Indeed, in this study IFN-γ appears to promote the monocyte response aimed at clearing the pathogens. However, the effects of the early alloresponse on CD64 expression and the synergy with IFN-γ are novel observations, and the mechanisms responsible are not fully understood. It is plausible that early interaction of TCR with allogeneic cells results in a stimulatory T cell-monocyte molecular interaction or the secretion of yet undefined inflammatory mediators by T cells that trigger an increase in CD64 expression. Importantly, as in this study relatively high concentrations of IFN-γ were used, it is unlikely that any additional secretion of IFN-γ by T cells could have an additive/synergistic effect by simply increasing the total concentration of this cytokine.

CD32B (FcγRIIB) is a low affinity IgG receptor, generally considered as an inhibitory FcγR, which is expressed on monocytes/macrophages, mast cells, eosinophils, and B cells (Junker et al., 2020). The results collected from MLR IFN-γ culture showed a modest, non-significant down regulation of CD32B expression. Indeed, it has been previously reported that during inflammation CD32B expression on $CD14^+$ cells was reduced due to the presence of IFN-γ (Anania et al., 2019). Interestingly, the results in this study suggest that monocytes/macrophages in MLR IFN-γ culture up-regulated the expression of stimulatory FcγRI (CD64) but not the inhibitory FcγRII (CD32B), which indicate an inflammatory nature of the response in allogeneic culture treated with IFN-γ.

Further analysis to evaluate the ability of the monocytes/macrophages to capture apoptotic cells and immune complexes was carried out by investigating CD36, and CD204 expression on the $CD14^+$ monocytes. In the previous chapter data suggest that the clearance of apoptotic cells during early alloresponse is mainly conducted by CD204, and not CD36 molecule (D1 of incubation). In this chapter expression of CD36 and CD204 on monocytes is not affected by both stimuli (the presence of allogeneic cells and IFN-γ). This contrasts with the previous study that reported IFN-γ mediated inhibition of CD36 expression on monocytes (Zhong et al., 2019). Interestingly, it has been reported that inhibition of CD36 does not affect phagocytosis of bacteria by human macrophages (Cooper et al., 2016, Wang et al., 2019). Similarly, this result disagrees with previous studies that reported IFN-γ caused down regulation of CD204 expression (Yoichi Ohtaki et al., 2010, Obrien et al., 2019). The role of expression of CD36 or CD204 in early alloresponse combine with IFN-γ is not clear. That could be due to the small sample size, future work will overcome this limitation that may cause difference in the current result.

The effects of IFN-γ and the alloresponse on the complement receptor, C3aR, were also investigated. C3aR is expressed on APCs and T cells (Raedler & Heeger, 2011), and it has been reported that up-regulation of C3aR expression on APCs and T cells is involved in alloreactivity and transplant rejection (Raedler & Heeger, 2011). In this study, the data demonstrated a strong stimulatory effect of the early alloresponse in C3aR expression on monocytes. Notably, it has been demonstrated that APCs up-regulate C3aR, as an early sign of aGVHD (Nguyen et al., 2018). Although monocytes and macrophages highly express C3aR during

innate immune response (Mommert et al., 2018), the current understanding of the molecular pathways that control C3aR expression is limited. Thus, the links between TCR recognition of allogeneic cells and rapid upregulation of C3aR on the surface of monocytes/macrophages remain poorly defined. Interestingly, MLR IFN-γ culture significantly reduced expression of $CD14^{+}C3aR^{+}$ compared to MLR culture. This observation reveals that the early alloresponse and IFN-γ have opposing effects on C3aR expression in monocytes /macrophages. Previous studies reported that FcγR and C3aR are co-expressed on the surface of monocytes to mediate the inflammatory response, and the ligation of FcγR with IgG controlled C3aR either to inhibit or amplify the C3aR response (Mommert et al., 2004). In this study, a dramatic increase of $CD14^{+}CD64^{+}$ expression was observed in MLR IFN-γ culture and that could be responsible for down-regulating C3aR expression. The inhibitory effects of IFN-γ on C3aR expression in monocytes/macrophages may have important functional consequences. A possible scenario could be that binding of C3a to C3aR results in activation of APCs and T cells, followed by IFN-γ release by the Th1 cells. The high local concentration of IFN-γ could result in the reduction of inflammation, down regulation of C3aR and increase in T cell apoptosis due to induction of Fas/FasL expression (Raedler & Heeger, 2011, Mathern & Heeger, 2015). As this study used high, pharmacological IFN-γ concentrations, this may contribute to the reduction of C3aR expression, which could be linked to reduced inflammatory response provided by monocytes/macrophages.

CD11b is regarded as an important differentiation marker, for macrophage subsets. Notably, CD11b was expressed slightly more in the allogeneic culture/MLR when compared to the responder cells. This could indicate that CD14 monocytes in MLR and MLR IFN-γ cultures were in a similar differentiation stage, as monocytes derived macrophages. Interestingly CD11b is crucial for cell adhesion, and phagocytosis of pathogens, apoptotic cells, and complement-opsonised cells by monocytes, but this process does not always involve inflammation (Gavin et al., 2019, Lukácsi et al., 2017). Therefore, it appears that CD11b expression is not involved in inflammation triggered by allogeneic cells.

T cell activations were analysed on D3 of incubation, by evaluating the expression of MFI CD25 and % of $CD69^{+}$. Notably, IFN-γ did not act synergistically with the

allogeneic response to enhance T cells activation. It has been reported that activated monocytes/macrophages control T cells priming (Bajnok et al., 2017, Murray, 2017). The data in this study showed that IFN-γ activated monocytes/macrophages appeared to have enhanced phagocytosis on D1 and D2 of incubation due to higher levels of CD64 expression. This was accompanied by a modest increase of Ag presentation (HLA-DR expression) and co-stimulatory capacity (CD86 expression). Also, the addition of IFN-γ to MLR culture has modest effects on T cell activation and failed to act synergistically with the early phase of alloresponse. It has been reported that IFN-γ could limit alloreactive T cells expansion in allogeneic culture, by up-regulation of apoptotic receptors (Wang & Yang, 2014, Via et al., 2017). Although these studies support the findings reported here, additional investigations are needed to establish if the modest effects of IFN-γ on T cell activation could reach statistical significance with a larger number of experimental repeats.

The levels of cytokines (TNF-α, IL-6 and IL-2) in MLR culture with/without the addition of IFN-γ were measured. The cell culture supernatant samples were collected on D1, D2 and D3. However, the only results with consistent values above detection limits were on D2, which were presented in this report. Notably, this corresponded with the increased levels of expression of the activation markers on monocytes/macrophages that were also at their peaks on D2 of incubation.

The data in this study have shown that MLR culture in the presence of IFN-γ produced only slightly more TNF-α and IL-2 when compared to the MLR. Indeed, a significant difference was only observed in MLR IFN-γ when compared to Res and Res IFN-γ. Based on the expression of the cell surface markers at this early stage of alloresponse/MLR culture, monocytes/macrophages were clearly activated. Thus, it could be postulated that TNF-α is likely to be secreted by these cells on D1 and D2 of incubation. On the other hand, IL-2 is likely to be produced by T cells, however, the source of cytokines remains speculative, which highlights the need for intracellular cytokine staining to determine which cells produce each cytokine.

The data in this report revealed that IFN-γ had modest effects on TNF-α secretion in allogeneic cell culture. This is in contrast with previous studies that reported that

IFN-γ strongly stimulated TNF-α production from APCs (Nakamura et al., 2000), which was attributed to functional characteristics of classical monocytes (Wang et al., 2014, Spragers et al., 2016). Furthermore, the increased production of IL-2 in the early phase of the alloresponse could explain the modest up-regulation of CD69 in both $CD3^+$ and $CD4^+$ T cells. Indeed, a previous study showed that CD69 is an early T cells activation marker was induced by IL-2 cytokine (Bajnok et al., 2017). Interestingly, this study showed a decrease in IL-6 concentration in MLR IFN-γ when compared to the MLR culture. IL-6 is a pleiotropic cytokine that has proinflammatory and anti-inflammatory functions (Duque & Descoteaux, 2014). The data in this study highlights that early administration of IFN-γ negatively regulated IL-6, which is opposite to what has been reported previously and interpreted as IFN-γ induced classical monocytes that differentiate in to M1 macrophages to produce IL-6, IL-2, IL-12, and IL-23 (Nakamura et al., 2000, Wang et al., 2014). The apparent inhibitory effects of IFN-γ on the alloresponse and reduced IL-6 production implied that IFN-γ antagonise IL-4 by inhibiting IL-6 production, which could be linked to suppression of cytokine signalling mediated by STAT6 inhibition (Delneste et al., 2002). These data highlight the link between the effects of IFN-γ, IL-4 and IL-6 in the early allogeneic culture. Thus, IL-4 concentration in the MLR culture could be measured to determine the interaction between this cytokine and IFN-γ, and their effects on the production of IL-6.

Overall, the addition of IFN-γ to the early MLR culture resulted in increased CD64 expression/phagocytosis function with down regulation of C3aR, slightly enhanced co-stimulation capacity and Ag presentation potential. The addition of IFN-γ to the MLR culture resulted in a cytokine production profile that broadly matched the classical subtype, except for IL-6 reduction. Importantly, this study did not focus on classifying the monocytes subtypes. The main concern was the function of monocytes/macrophages and the T cell activation in early MLR culture, which was not significantly boosted by the presence of IFN-γ. These data show that the presence of IFN-γ in the early phase of the alloresponse does not play a pro-inflammatory role (Nakamura et al., 2000, Visentrain et al., 2003). Therefore, the contribution of IFN-γ to GVHD pathology may need to be examined in a different light. Indeed, IFN-γ could drive monocyte differentiation towards macrophages, and defence against pathogens with limited T cell co-stimulation

and inflammatory potential. However, due to the small sample size and limitations of using an *in vitro* model in this study it is difficult to generalise this conclusion.

The second part of this chapter focused on modulating monocytes/macrophage's function and T cells activation by treating MLR IFN-γ culture with a SYK inhibitor. The SYK pathway is involved in transmitting signals from FcRs expressed on monocytes/macrophages that control crucial events in the development of GVHD (Leonhardt et al., 2012, Poe et al., 2018). It has been reported that SYK pathway activation controls monocytes/macrophages phagocytosis and Ag presentation (Su Yi et al., 2014, Coffey et al., 2017). SYK inhibitors that were tested in murine models and clinical trials have shown promising results in the reduction of inflammation in autoimmune diseases (Leonhardt et al., 2012). More importantly, targeting SYK signalling is an attractive concept aiming to reduce GVHD pathology (Leonhardet et al., 2012, Poe et al., 2018). Therefore, this study evaluated the effects of highly selective SYK inhibitor (PRT0603), on modulating monocyte/macrophage activation and production of cytokines in the early phase of allogeneic cell culture with/without the addition of IFN-γ.

The findings in this study showed that $CD14^+$ monocytes expressed high levels of CD64 in the MLR IFN-γ culture. Although this implies SYK pathway activation, this was not confirmed using intracellular staining for phosphorylated SYK protein. Nevertheless, it is well established that clustering of CD64 molecules on monocyte/macrophage surface results in ITAM phosphorylation and SYK pathway activation (Kefer et al., 1998, Coffey et al., 2017). In this part of the study four experimental conditions were compared: MLR IFN-γ (allogeneic culture with addition of IFN-γ), MLR IFN-γ SYK (allogeneic culture with addition of IFN-γ and SYK inhibitor), Res IFN-γ (control for the effect of IFN-γ) and Res IFN-γ SYK (control with responder cells IFN-γ and SYK inhibitor).

Interestingly expression of CD86 HLA-DR and CD64 on $CD14^+$ monocytes were slightly reduced in MLR IFN-γ SYK compared to MLR IFN-γ. However, the reduction was modest and non-significant. This non-significant reduction could be due to the small sample size. It has been reported that PRT0603 is a highly selective SYK inhibitor that targets intracellular signalling through FcγRI (Hoellenriegel et al., 2012), and targeting of this receptor affects Ag presentation

(Junker et al., 2020). The modest effects of PRT0603 SYK inhibitor on monocyte/macrophage functions such as co-stimulation and Ag presentation, suggest that FcγRI signalling represents a single component of the complex interactions between multiple pathways driven by the alloresponse and IFN-γ cytokine. Interestingly, the addition of IFN-γ could reduce the potency of the SYK inhibitor on monocytes, as in the previous chapter the SYK inhibitor significantly down-regulated CD64 expression. In contrast, in this chapter only a modest reduction of CD64 expression was observed. Nevertheless, it is feasible that relatively small changes in monocytes/macrophages function could lead to significant effects on T cells alloreactivity.

The analysis of T cells in the presence of the SYK inhibitor was performed on D3. Notably, the data showed a significant reduction of CD25 expression levels and CD69 on $CD3^+$ T cells in MLR IFN-γ SYK when compared to MLR IFN-γ. This has confirmed the potential of the SYK inhibitor to reduce activation of $CD3^+$ T cells. As $CD3^+$ T cells represent both T cell helper $CD4^+$ cells and cytotoxic $CD8^+$ cells, the effects of the SYK inhibitor are broad and affect the total T cell population. However, the effects of the SYK inhibitor on $CD4^+$ T helper cells were modest and did not reach statistical significance (raw data are provided in the appendix figure S.1.5). Indeed, targeting the SYK pathway could indirectly reduce T cell activation (Coffey et al., 2017). More importantly, the addition of IFN-γ to the MLR culture did not reduce the efficacy of the SYK inhibitor. As described in the previous chapter, SYK inhibitor has significantly reduced T cell activation in MLR, in a similar way to the data presented in this chapter.

To evaluate inflammatory response in the MLR IFN-γ culture with/without SYK, the concentrations of TNF-α, IL-6 and IL-2 cytokines were measured. The supernatant samples were collected on D2 of incubation from MLR IFN-γ, MLR IFN-γ SYK, Res IFN-γ and Res IFN-γ SYK cell cultures. Notably, the TNF-α, IL-2 and IL-6 concentrations were significantly reduced in MLR IFN-γ SYK when compared to MLR IFN-γ. Therefore, T cell activities were reduced on D3 as both IL-2 and IL-6 are essential for T cells activation. Indeed, IL-2 is mainly produced by helper T cells, and it could be regarded as an essential growth factor for both $CD8^+$ and $CD4^+$ T cells (Blazar et al., 2013). It has been previously reported that, in the early period post HSCT, IL-2 concentrations are increased, which results in

stimulation of T cell proliferation and differentiation (Blazar et al., 2013). Therefore, lower concentrations of IL-2 due to PRT0603 treatment could affect T cells alloreactivity, leading to significant reduction of CD25 expression and a decrease in the proportion of CD69 positive $CD3^+$ T cells. Indeed, it has been shown that low IL-2 concentrations are associated with lower rate of GVHD (Blazar et al., 2013, Kumar et al., 2017). Similarly, it is thought that IL-6 is mainly produced by classical monocytes (Boyette et al., 2016). In GVHD setting it was proposed that IL-6 is involved in controlling T cells maturation and differentiation (Tawara et al., 2011, Tvedet et al., 2017), and diminishing T cells apoptosis (Duque & Descoteaux, 2014). However, inhibition of the SYK pathway resulted in reduction of TNF-α concentration in the presence of allogeneic cells and IFN-γ, which did not influence significant effect on monocytes. As TNF-α is mainly produced by macrophages in GVHD, this cytokine could trigger the inflammatory response culminating in up-regulation of co-stimulatory molecules on APCs and activation of T cells (Nakamura et al., 2000, Markey et al., 2013, Zhang et al., 2017). The data in this study demonstrate that treatment of MLR IFN-γ culture with the SYK inhibitor resulted in a modest reduction of monocyte/macrophages expression of CD86 and HLA-DR, which is followed by a significant decrease in T cell activations. This confirms the importance of the SYK pathway in alloresponse (MLR culture) and by implication in GVHD. Although T cells are not directly targeted by the SYK inhibitor, it could be postulated that reduced activation of the B cell receptor and FcRγ in the alloresponse resulted in a significant change in the inflammatory milieu, which indirectly affects T cell response. Indeed, targeting cytokine productions is a well-established therapeutic approach to control inflammation (Coffey et al., 2013).

In summary, these findings suggest that administration of IFN-γ to early MLR has enhanced the phagocytosis function of monocytes. This could promote monocyte differentiation towards a phagocytic phenotype without T cell co-stimulation and limited pro-inflammatory potential. Targeting the SYK signalling pathway on monocytes/macrophages, in the early phase of MLR IFN-γ culture results in the reduction of production of inflammatory cytokines and a subsequent decrease in T cells alloreactivity. Notably, the addition of IFN-γ did not reduce the efficacy of SYK inhibition in the allogeneic culture.

5 Results (Part 3):

5.1 Introduction and aims:

IL-4 is a cytokine secreted by mast cells, basophils and Th2 cells (Mosser & Edward, 2008, Rahimi et al., 2019). Monocytes and macrophages express IL- 4R, and its ligation will activate these cells to become an alternatively activated subtype that supports tissue repair (Mosser & Edward, 2008, Raphael et al., 2014, O'Brien et al., 2019), and anti-inflammatory response (Lee et al., 2017). Interestingly, it has been reported that IL-4 activates macrophages to up-regulate CD86 (Deszo et al., 2004). However, IL-4 activated monocytes fail to present Ag to T cells (Mosser & Edward, 2008). Earlier studies reported that high doses (10 µg) of IL-4 could suppress aGVHD, by antagonisingIFN-γ and reducing donor Th1 cells activities and proliferation in a murine model (Blazer et al., 2013, Via et al., 2017).

In this chapter, the Th2 cytokine IL-4 was selected, with the aim of investigating monocytes/macrophages function in the early phase of MLR culture for D1 and D2 of incubation in response to this cytokine.

Therefore, the aims of this chapter were:

- Investigate the effects of IL-4 on activation ofmonocytes/macrophages in the early phase of alloresponse.
- Investigate the effects of MLR in the presence of IL-4 cytokine on Tcell activity.
- Evaluate the effects of adding IL-4 to early phase MLR culture on the production of cytokines.
- Examine the effects of SYK pathway inhibitor on monocytes/macrophages in the early phase alloresponse in the presence of IL-4 cytokine.
- Investigate the effects of selective SYK inhibitor PRT0603, on T cells activation in MLR culture in the presence of IL-4 cytokine.
- Evaluate the effects of SYK pathway inhibition on cytokineproduction in early phase MLR in the presence of IL-4.

5.2 Results:

5.2.1 Optimising IL-4 concentration:

10 ng/ml, 50 ng/ml and 100 ng/ml IL-4 were added to MLR culture, and the level of expression (MFI) of CD86, HLA-DR and CD64 were measured by flow cytometry on D1 and D2 of incubation. The cell viability was confirmed by Annexin V staining, where 80-85 % of cells were viable on D1 and D2. All samples were treated with Fc blocking and the results were compared to the isotype control to eliminate any background of non-specific antibody binding.

The results (Figure 5.1.A and Figure 5.1.B) show that the expression levels of CD86 and HLA-DR were increased after treatment with 100 ng/ml IL-4 when compared to the other concentrations mainly on D2, thought the differences were not statistically significant. The results for CD64 (Figure 5.1.C) revealed slight changes in expression on the$CD14^+$ monocytes/macrophages on D1 and D2 of incubation. Therefore, 100 ng/ml of IL-4 was selected as the optimal concentration for the rest of the experiments.

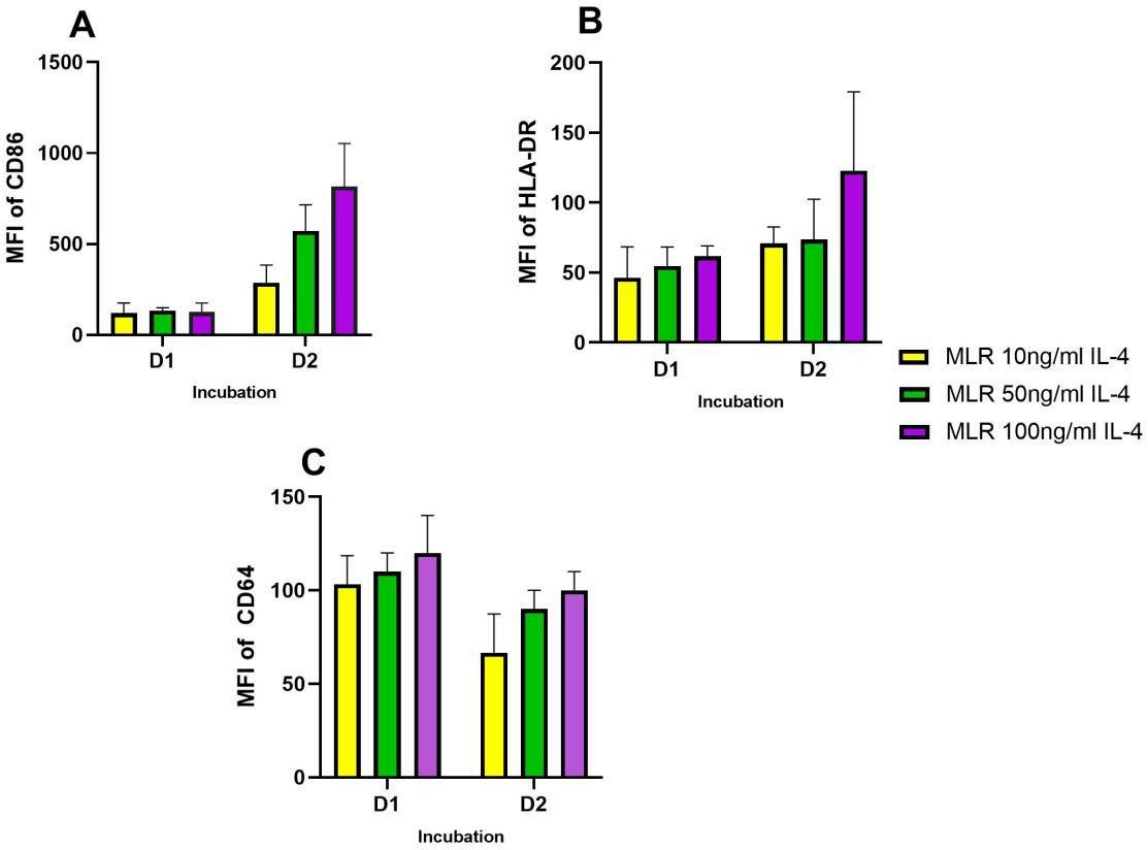

Figure 5. 1: Analysis of the level of (MFI) expression on $CD14^+$ monocytes by flow cytometry after D1 and D2 of incubation in the presence of with IL-4. **A)** CD86, **B)** HLA-DR and **C)** CD64.

The X axis shows incubation days, Y axis is MFI expression. Data shown are mean and the error bars are SD (n = 3). Yellow bar = MLR treated with 10 ng/ml IL-4, green bar = MLR treated with 50 ng/ml IL-4 and purple bar = MLR treated with 100 ng/ml IL-4. Statistical significance of data was analysed using RM two-way ANOVA with Tukey multiple comparisons test and Geiser - Greenhouse correction, p values below 0.05 were considered significant.

5.2.2 Monocyte/macrophage co-stimulation capacity in MLR culture in the presence of IL-4 cytokine:

The expression of the co-stimulatory molecule CD86 was investigated in four conditions: responder cells (negative control), MLR (positive alloresponse control), MLR treated with 100 ng/ml IL-4 (experimental condition, alloresponse in the presence of IL-4) and Res IL-4 (control for IL-4 effects, responder cells treated with 100 ng/ml IL-4).

The results (Figure 5.2.A) showed that MLR culture (alloresponse) in the presence of 100 ng/ml of IL-4 significantly up-regulated $CD14^+CD86^+$ expression compared to the responder (Res) cells ($p=0.005$), MLR ($p=0.0020$) and Res IL-4 ($p=0.0027$) on D2 of incubation. There were no significant differences between the four

conditions on D1. These data indicate that the presence of allogeneic cells and IL-4 cytokine during early phase of response resulted in a strong synergistic effect, leading to significant up-regulation on $CD14^+$ monocytes co- stimulation capacity.

5.2.3 Monocyte/macrophage Ag presentation in early MLR culture with addition of IL-4 cytokine:

To investigate monocytes Ag presentation capacity, HLA-DR expression was determined in the four conditions described above. The results (Figure 5.2.B) showed that $CD14^+$ cells expressed significantly higher levels of HLA-DR in the MLR with addition of 100 ng/ml IL-4 (MLR IL-4) compared to Res ($p=0.044$) and Res IL-4 ($p=0.020$) on D1. Following the same trend, the $CD14^+$ monocytes in the MLR IL-4 culture expressed significantly more HLA-DR when compared to MLR ($p = 0.0015$), Res ($p < 0.0001$) and Res IL-4 ($p=0.0015$) on D2 of incubation. These results highlight that during the early phase of alloresponse monocytes/ macrophages significantly up-regulate Ag presentation capacity due to the combined presence of allogenic cells in MLR and IL-4 cytokine.

5.2.4 Analysis of monocytes/macrophages CD64 and CD32B expression in early MLR with addition of IL-4 cytokine:

It is generally regarded that CD64 and CD32B play important roles in monocyte/macrophage function, as these Fc receptors contribute to phagocytosis of opsonised pathogens and the capture of antibody-antigen complexes. The expression of these two markers of monocyte function was investigated in the four conditions described above. For CD64 (Figure 5.2.C), on D2 of incubation the MLR culture showed higher levels of CD64 expression compared to the other conditions with significant difference when compared to MLR IL-4 ($p=0.0170$), and Res IL-4 ($p=0.001$). Thus, it appears that the presence of IL-4 cytokine in the culture inhibits MLR-induced CD64 expression on monocytes.

For CD32B (Figure 5.2.D) the results showed that $CD14^+CD32B^+$ expression in MLRIL-4 appeared increased compared to the MLR and other control conditions on D1however, the difference was not significant. Interestingly, $CD14^+CD32B^+$ expression down regulated in MLR on D2 compared to the other conditions, although the difference did not reach statistical significance. These results suggest that unlike CD64 marker, CD32B expression was not significantly affected by the

presence of allogeneic cells however, the addition of IL-4 cytokine could up-regulate the inhibitory receptor CD32B.

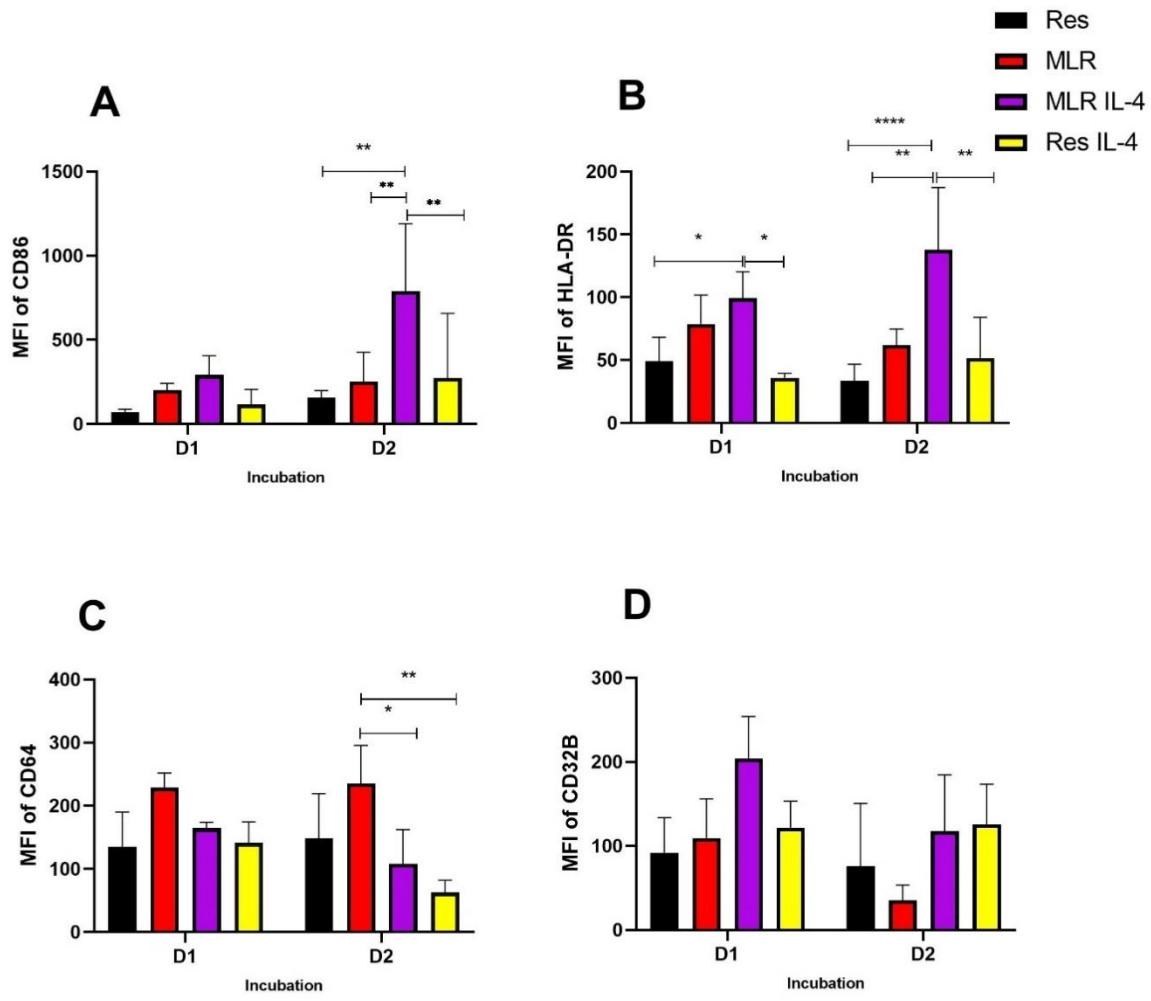

Figure 5. 2: Analysis of expression on $CD14^{+}$ monocytes by flow cytometry on D1 and D2 of incubation in the presence of allogeneic cells (MLR) and cytokine.IL-4.

A) CD86, **B)** HLA-DR, **C)** CD64, **D)** CD32B. The X axis shows incubation days, Y axis presents MFI expression. Data shown are means and the error bars are SD (n = 3). Black bar represents Res = Responder cells as control, orange bar represents MLR = Mixed leukocyte reaction, purple bar represents MLR treated with 100 ng/ml IL-4, and yellow bar represents Responder cells treated with 100 ng/ml IL-4. Statistical significance of data was analysed using RM two-way ANOVA with Tukey multiple comparisons test and Geiser -greenhouse correction, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

5.2.5 Analysis of monocytes/macrophages scavenger receptors in MLR culture treated with IL-4 cytokine:

The monocytes/macrophages expression of scavenger receptors was analysed by measuring the level of CD36, and CD204, that could be linked to the capacity of monocytes to clear apoptotic cells in the four experimental conditions described above. The results for CD36 (Figure 5.3.A) showed that in MLR IL-4 the expression of $CD14^+CD36^+$ was slightly higher than in the other culture conditions (Res, MLR and Res IL-4) on D1, however the difference did not reach statistical significance. Interestingly, on D2 of incubation $CD14^+CD36^+$ expression was reduced in the MLR compared to other experimental conditions, although the differences were not significant. Thus, it appears that effects of IL-4 on CD36 expression in $CD14^+$ are not significant.

The results for CD204 (Figure 5.3.B) showed that on D1 of incubation, there was a significant increase in $CD14^+CD204^+$ expression for the MLR compared to Res IL-4 culture (p=0.040). There was a modest reduction of $CD14^+CD204^+$ expression in the MLR IL-4 compared to the MLR, although differences were not significant on D1 and D2 of incubation. On D2, there was a significant increase of $CD14^+CD204^+$ in Res compared to Res IL-4 (p=0.022). These results suggest that IL-4 cytokine down regulates $CD14^+CD204^+$ expression in monocytes/macrophages during D1 and D2 of cell culture, although the observed effects were modest.

5.2.6 Analysis of C3aR expression on $CD14^+$ monocytes in the presence of allogeneic cells and MLR IL-4:

The monocytes/macrophages expression of complement receptor C3aR was investigated in four experimental conditions described above. The results for C3aR (Figure 5.3.C) showed that on D1 of incubation monocytes/macrophages expression of C3aR was at the highest level in MLR compared to other experimental conditions,including significant difference from MLR IL-4 culture ($p < 0.0001$). This demonstrates a strong stimulatory effect of early alloresponse on monocytes/macrophages C3aR expression and inhibitory effects of IL-4 cytokine. Furthermore, C3aR expression was significantly increased in the MLR IL-4 compared to Res IL-4 ($p < 0.0001$) probably due to dominant effects of MLR over

IL-4 mediated inhibition. Interestingly, on D2 of incubation the highest levels of monocytes/macrophages C3aR expression was observed in the control responder (Res) culture, significantly higher than Res IL-4 ($p < 0.0001$) and MLR IL-4 ($p=0.030$), indicating inhibitory effects of IL-4 cytokine. Although the stimulatory effects of MLR were largely masked on D2 due to the high levels of $C3aR^+$ expression in the control Res culture, inhibitory effects of IL-4 were still evident. Thus, both MLR and MLR IL-4 expressed significantly higher levels of C3aR than Res IL-4 culture ($p=0.007$ and $p=0.0067$, respectively).

5.2.7 Analysis of CD11b expression on $CD14^+$ monocytes in the presence of allogeneic cells and IL-4 cytokine:

The monocytes/macrophages expression of adhesion molecule CD11b was investigated using four experimental conditions described above. The results for $CD14^+CD11b^+$ (Figure 5.3.D) on D1 revealed the strong stimulatory effects of IL-4 as both MLR IL-4 and Res IL-4 expressed significantly higher levels of this marker compared to Res control ($p = 0.0027$ and $p = 0.0477$, respectively) and MLR culture ($p = 0.0149$ for MLR IL-4 vs. MLR).

Following the same trend, on D2 of incubation both MLR IL-4 and Res IL-4 expressed significantly higher levels of $CD11b^+$ than Res controls ($p=0.0043$ and $p = 0.0054$, respectively) and MLR culture ($p=0.0375$ and $p =0.0460$, respectively). This highlights that IL-4 up-regulated CD11 bexpression, whereas the presence of allogeneic cells did not have significant effects on this marker of monocytes/macrophages functions.

Overall, these results demonstrate distinct effects of IL-4 on monocytes/macrophages phenotype and function. Indeed, IL-4 cytokine significantly enhanced monocytes/macrophages levels of expression of CD86 and HLA-DR. Importantly this, effect was synergistic with the stimulatory potential of alloresponse (MLR culture). Furthermore, IL-4 showed inhibitory effects on the expression of CD64 and C3aR during early phase of monocytes/macrophages culture and this could not be reversed by alloresponse. Also, IL-4 enhanced the expression of CD11b adhesion molecule on monocytes.

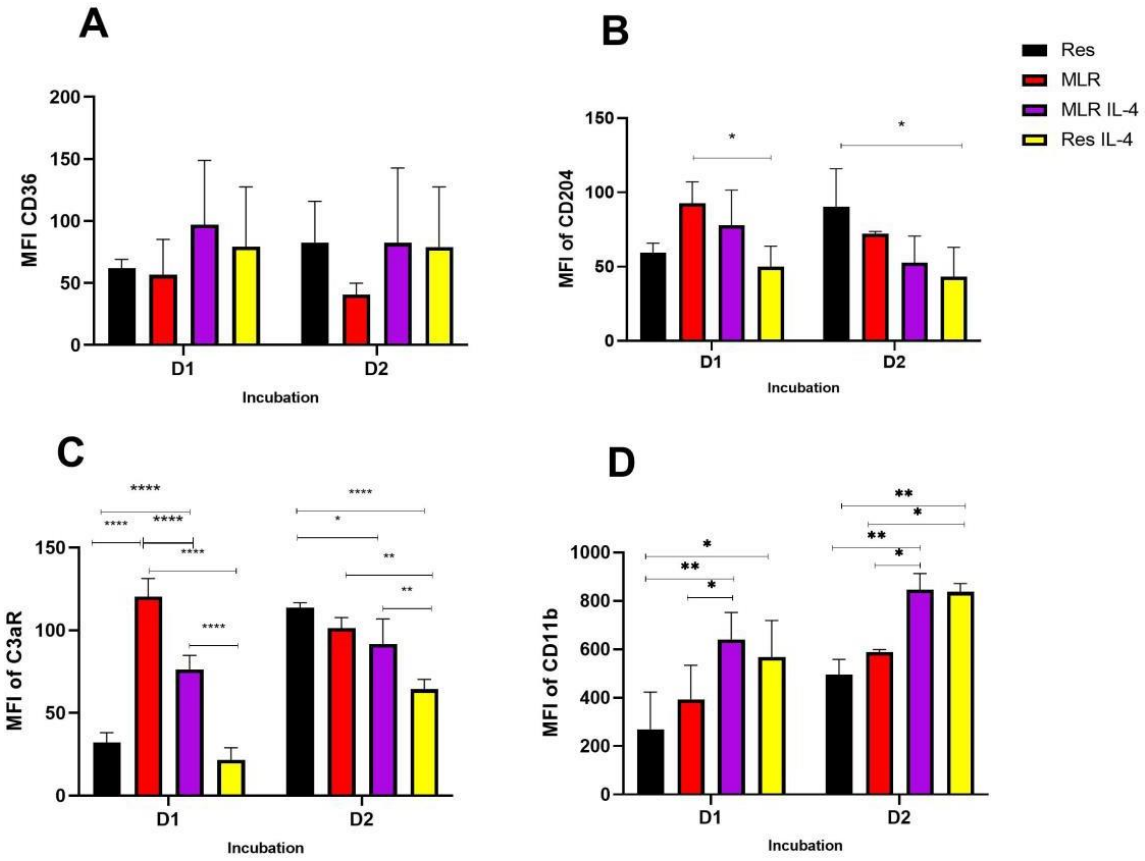

Figure 5. 3: Analysis of expression on $CD14^{+}$ monocytes by flow cytometry after D1 and D2 of incubation in the presence of allogeneic cells/MLR and IL-4 cytokine.

A) CD36, **B)** CD204, **C)** C3aR, **D)** CD11b. The X axis shows incubation days, Y axis is MFI expression. Data shown are mean and the error bars are SD (n = 3). Black bar represents Res = Responder cells as negative control, orange bar represents MLR = Mixed leukocyte reaction, purple bar represents MLR treated with 100 ng/ml IL-4, and yellow bar represents Responder cells treated with 100 ng/ml IL-4. Statistical significance of data was analysed using RM two-way ANOVA with Tukey multiple comparisons test and Geisser -Greenhouse correction, p values below 0.05 were considered significant, * = $p < 0.05$, ** =$p < 0.\ 01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

5.2.8 Analysis of T cell activity in MLR treated with IL-4 cytokine:

After three days of incubation the proportion percent (%) of T cells that express CD69 ($CD3^{+}CD69^{+}$ and $CD4^{+}CD69^{+}$), and the levels of expression (MFI) of CD25 ($CD3^{+}CD25^{+}$ and $CD4^{+}CD25^{+}$) were assessed to investigate T cells in the four experimental conditions: responder cells (-ve control), MLR (+ve allogeneic control), MLR IL-4 (experimental condition, MLR treated with 100 ng/ml IL-4) and Res IL-4 (control for the effects of IL-4).The data (Figure 5.4.A) showed that the MFI values for $CD3^{+}CD25^{+}$ in the MLR IL-4 culture were significantly lower(p =

0.034) than in the MLR.

Notably, $CD3^+CD25^+$ expression level was significantly higher in the MLR compared to Res (p=0.0025), which was seen previously (Figure 3.4.A) and Res IL-4 (p=0.0042). Therefore, a strong stimulatory effect of the presence of allogeneic cells (MLR) on the expression levels of CD25 in $CD3^+$ T cells on D3 of culture was confirmed in this set of experiments.

The results for CD69 expression (Figure 5.4.B) showed a modest reduction of the proportion (%) of $CD3^+CD69^+$ T cells in the MLR IL-4 when compared to the MLR. However, the difference was not statistically significant. Importantly, the percent of $CD3^+CD69^+$ T cells was significantly higher in the MLR compared to Res (p = 0.0001) and Res IL-4 (p=0.0033), which confirms the stimulatory effects of MLR on D3 of culture. Furthermore, the proportion of $CD69^+$ T cells was significantly increased in MLR IL-4 compared to Res (p=0.0062). This indicates that IL-4 hasa modest or neutral effect on CD69 expression, as an inhibitory effect would resultin a much lower percent of $CD69^+$ T cells in MLR IL-4 culture and significant difference from MLR culture.

For the $CD4^+$ T helper cells, the expression levels of CD25 (Figure 5.4.C) were significantly increased in the MLR compared to Res (p=0.0499) and Res IL-4 (p=0.016). Although, $CD4^+CD25^+$ expression was lower in the MLR IL-4 culture than MLR the difference was not statistically significant. This indicates stimulatory effects of MLR on CD25 expression in helper T cells and a modest/neutral effect of the addition of IL-4 to MLR culture.

For the proportion of $CD69^+$ Th cells (Figure 5.4.D), the results showed that there was a modest, non-significant decrease for the MLR IL-4 compared to the MLR on D3 of culture. Although the percent of $CD4^+CD69^+$ cells appeared higher in MLR culture compared to other conditions, the difference did not reach statistical significance.

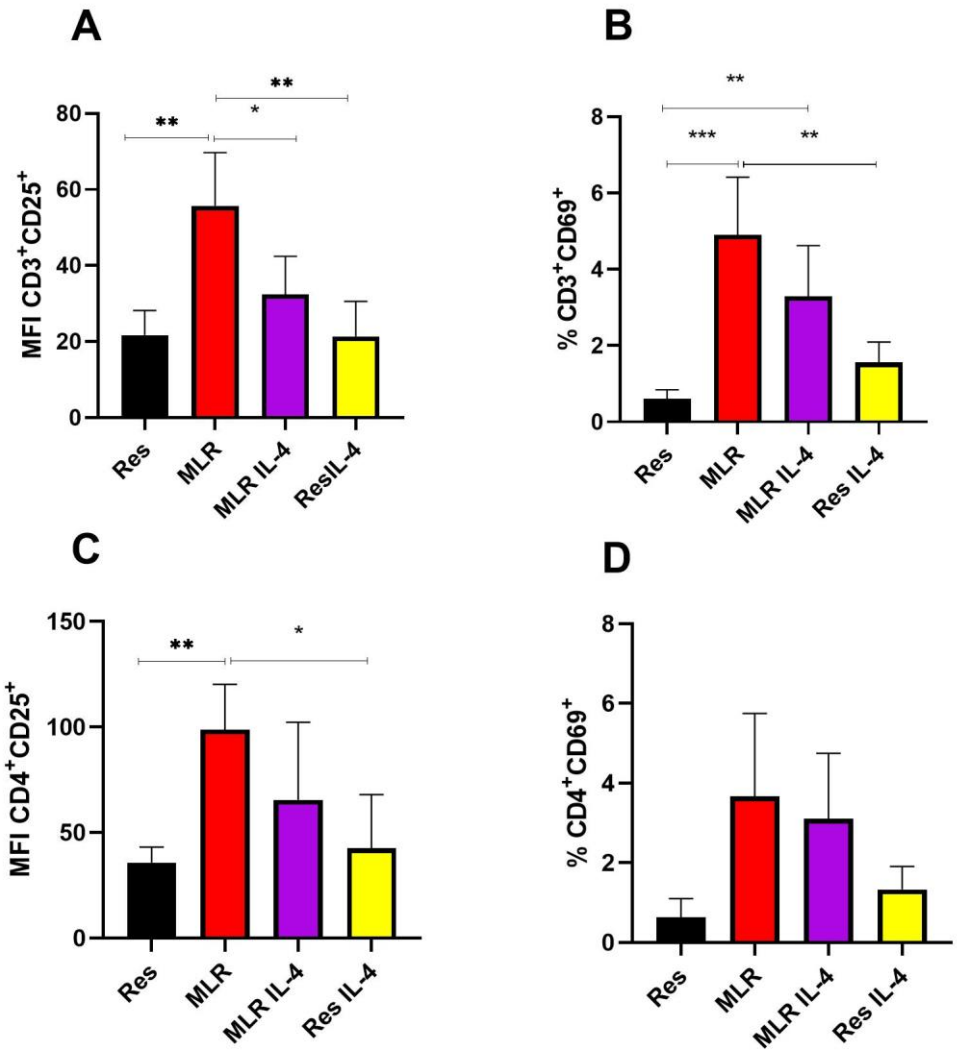

Figure 5. 4: Analysis T cells activation by flow cytometry on D3 of incubation with/without addition of IL-4.

A) MFI values for $CD3^+CD25^+$ T cells where Y axis shows MFI values for CD25 expression, **B)** Percent of $CD3^+CD69^+$ T cells where Y axis shows proportion (%) of CD69-positive T cells, **C)** MFI values for $CD4^+CD25^+$ T helper cells, and **D)** proportion (%) of $CD4^+CD69^+$ T helper cells. Data shown are mean and the error bars are SD (n = 3). Black bar represents Res = Responder cells as negative control, orange bar represents MLR = Mixed leukocyte reaction culture, purple bar represents MLR treated with 100 ng/ml IL-4, and yellow bar represents responder cells treated with 100 ng/ml IL-4. Statistical significance of data was analysed using RM one-way ANOVA with Tukey multiple comparison with Geisser greenhouse correction. Values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

5.2.9 Measurement of cytokines in MLR culture treated with IL-4:

The cytokines TNF-α, lFN-γ, lL-2 and IL-6 were measured by ELISA on D2 of incubation, using the following four conditions: responder cells (negative control), MLR (positive alloresponse control), MLR IL-4 (experimental condition, MLR treated with 100 ng/ml IL-4) and responder cells treated with 100 ng/ml IL-4 (control for the effect of IL-4).

The results for TNF-α (Figure 5.5.A) showed that the concentration of this cytokine on D2 of incubation in the MLR IL-4 was significantly lower than in the MLR culture (p=0.0042). This indicates an inhibitory effect of IL-4 on MLR- induced TNF-α production. Furthermore TNF-α concentration was significantly higher in the MLR compared to Res (p=0.0041) and Res IL-4 (p=0.0062). Interestingly, the levels of TNF-α in MLR IL-4 were slightly higher than those in Res IL-4. Although the difference did not reach statistical significance, this observation suggests that IL-4 does not completely inhibit the MLR effects on TNF-α production.

The results for IFN-γ (Figure 5.5.B) demonstrated that the concentration of this cytokine in MLR IL-4 culture was significantly reduced compared to the MLR (p = 0.0018), thus indicating a strong inhibitory effect of IL-4. Notably, the concentrations of IFN-γ were significantly higher in the MLR than Res (p=0.0002) and Res IL-4 (p=0.0002), confirming stimulatory effects of MLR on IFN-γ production on D2 of culture.

The data for IL-2 (Figure 5.5.C) showed that MLR IL-4 culture had significantly decreased levels of this cytokine compared to the MLR (p=0.0160). Also, IL-2 concentrations were significantly higher in the MLR culture compared to Res (p = 0.0017) and Res IL-4 (p=0.0015). These data indicate that MLR strongly stimulates IL-2 production on D2 of incubation and that this effect is significantly inhibited by the addition of IL-4 to MLR culture.

The result for IL-6 concentrations (Figure 5.5.D) showed significantly lower levels in the MLR IL-4 compared to MLR culture (p=0.0011), implying a strong inhibitory effect of IL-4 on MLR-induced IL-6 production. Notably, IL-6 concentrations were significantly higher in the MLR compared to Res (p=0.0002) and Res IL-4 (p =0.0002), confirming the stimulatory effects of MLR on IL-6 production on D2 of

cell culture.

Overall, these results highlight that treatment with 100 ng/ml of IL-4 showed a significant inhibitory effect on the production of TNF-α, IFN-γ, IL-2 and IL-6 in the early phase of alloresponse, D2 of cell culture.

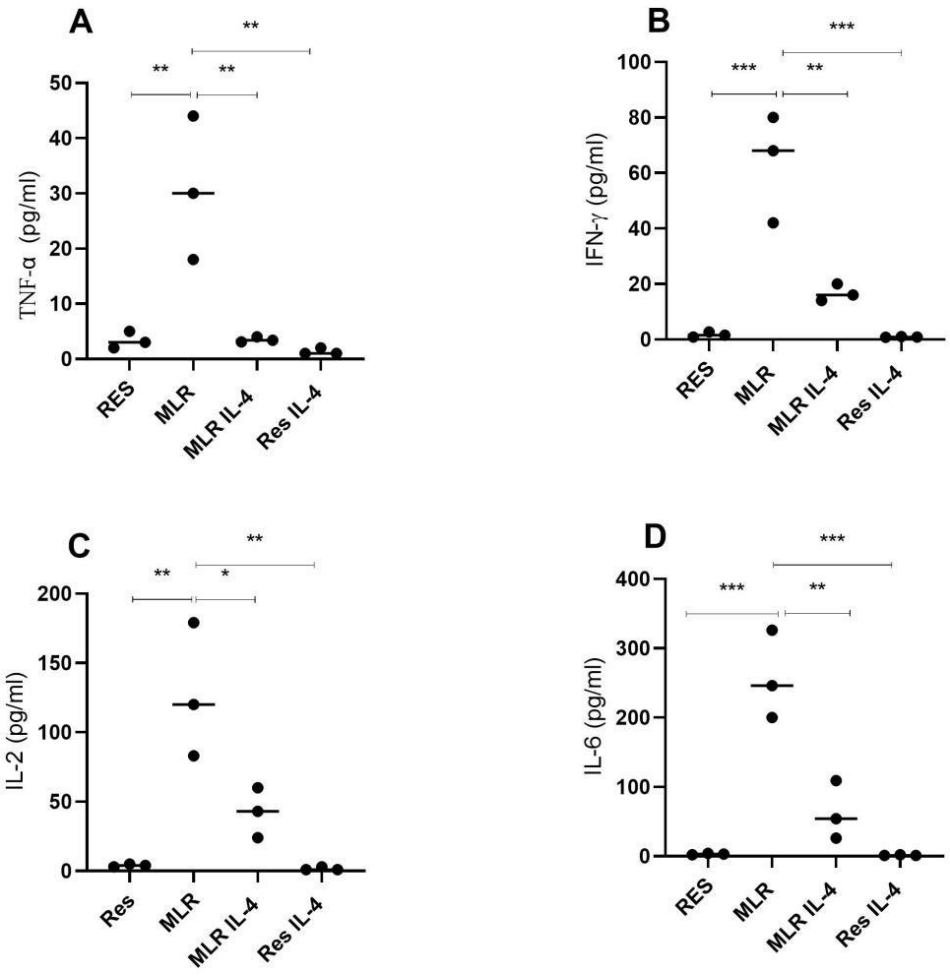

Figure 5. 5: Measuring cytokines by ELISA on D2 of incubation in MLR culture with/without IL-4.

A) TNF-α, **B)** IFN-γ **C)** IL-2 and **D)** IL-6 concentrations. X axis shows comparison of four conditions: Res = responder cells, MLR = Mixed leukocyte reaction, MLR treated with 100 ng/ml IL-4 and Responder cells treated with 100 ng/ml IL-4. Y axis shows the cytokine concentrations in pg/ml. Scatter Dot plots show three repeats (n = 3). Statistical significance of data was analysed using RM one-way ANOVA, Tukey multiple comparison with Geisser Greenhouse correction. p values below 0.05 were considered significant, * = $p < 0.01$, ** = $p < 0.001$ and *** = $p < 0.0001$.

5.2.10 Analysis of the effects of SYK inhibitor on monocytes/macrophages in MLR treated with IL-4:

Previously in this chapter, the addition of IL-4 to MLR culture showed a distinct effect on monocytes/macrophage. Therefore, this section examined the effects of daily treatment with SYK inhibitor, 2 μM PRT0603 on co-stimulation and Ag presentation capacity of monocytes/macrophages. The expression of (MFI) for $CD14^+CD86^+$, $CD14^+CD64^+$ and $CD14^+HLA\text{-}DR^+$ were measured in the following four conditions: MLR IL-4 (MLR treated with 100 ng/ml IL-4), MLR IL-4 SYK (MLR treated with 100 ng/ml IL-4 and 2 μM anti-SYK), Res IL-4 (Responder cells treated with 100 ng/ml IL-4 control for the effects of IL-4) and Res IL-4 SYK (Responder cells treated with 100 ng/ml IL-4 and 2 μM anti-SYK).

For CD86 (Figure 5.6.A), there was a modest reduction in the expression in the MLRIL-4 culture treated with anti-SYK when compared with MLR IL-4 on D1 and D2 of incubation, however the difference was not significant. Furthermore, there were no changes of CD86 expression in Res IL-4 SYK compared to Res IL-4 on D1 and D2. As previously note, the presence of allogeneic cells and IL-4 cytokine during early phase of response resulted in a strong synergistic effect, leading to significant up-regulation of monocytes/macrophages co-stimulation capacity in MLR IL-4 compared to ResIL-4 and Res IL-4 SYK ($p=0.019$, $p=0.026$ respectively). Also, there were significant differences in MLR IL-4 SYK compared to Res IL-4 and Res IL-4 SYK ($p=0.018$, $p=0.024$ respectively) on D2.

The data for HLA-DR (Figure 5.6.B) showed similar expression in the MLR IL-4 SYK culture compared to the MLR IL-4 on D1 and D2 of incubation. Following the same trend, there were no changes in HLA-DR expression in Res IL-4 SYK compared to ResIL-4 on D1 and D2. Thus, it appears that the SYK inhibitor does not affect MLR- induced increases in $HLA\text{-}DR^+$ expression and this is not altered by the presence of IL-4 cytokine. As noted in the previously described experiments, during early phase of the alloresponse monocytes/macrophages have significantly up-regulated Ag presentation capacity due to the combined presence of allogeneic cells in (MLR) and IL-4 cytokine. Consequently, $CD14^+$ monocytes cells expressed significantly higher levels of HLA-DR in MLR IL-4 compared to Res IL-4 ($p=0.02$) and Res IL-4 SYK ($p =0.04$) on D2 of incubation. Following the same trend, the $CD14^+$ in the MLR IL-4 SYK culture expressed

significantly more HLA-DR when compared to Res IL-4 (p = 0.04), Res IL-4 SYK (p=0.049) on D2 of incubation.

For CD64 (Figure 5.6.C), the data revealed the MLR IL-4 with 2 μM of anti-SYK showed similar CD64 expression on the $CD14^+$ monocytes compared to MLR IL-4. Res IL-4 SYK showed same levels of $CD14^+CD64^+$ expression when compared to Res IL-4. Therefore, SYK inhibition did not show the effects on $CD14^+$ $CD64^+$ expression in the presence of allogeneic cells and IL-4 cytokine.

In summary, these results reveal that although MLR and MLR IL-4 have strongly up-regulated CD86 and HLA-DR on $CD14^+$, the daily treatment with SYK inhibitor PRT0603 did not affect these markers. Thus, it appears that the co-stimulation and Ag presentation capacity of monocytes/macrophages in the presence of allogeneic cells and IL-4 are not regulated by SYK pathway.

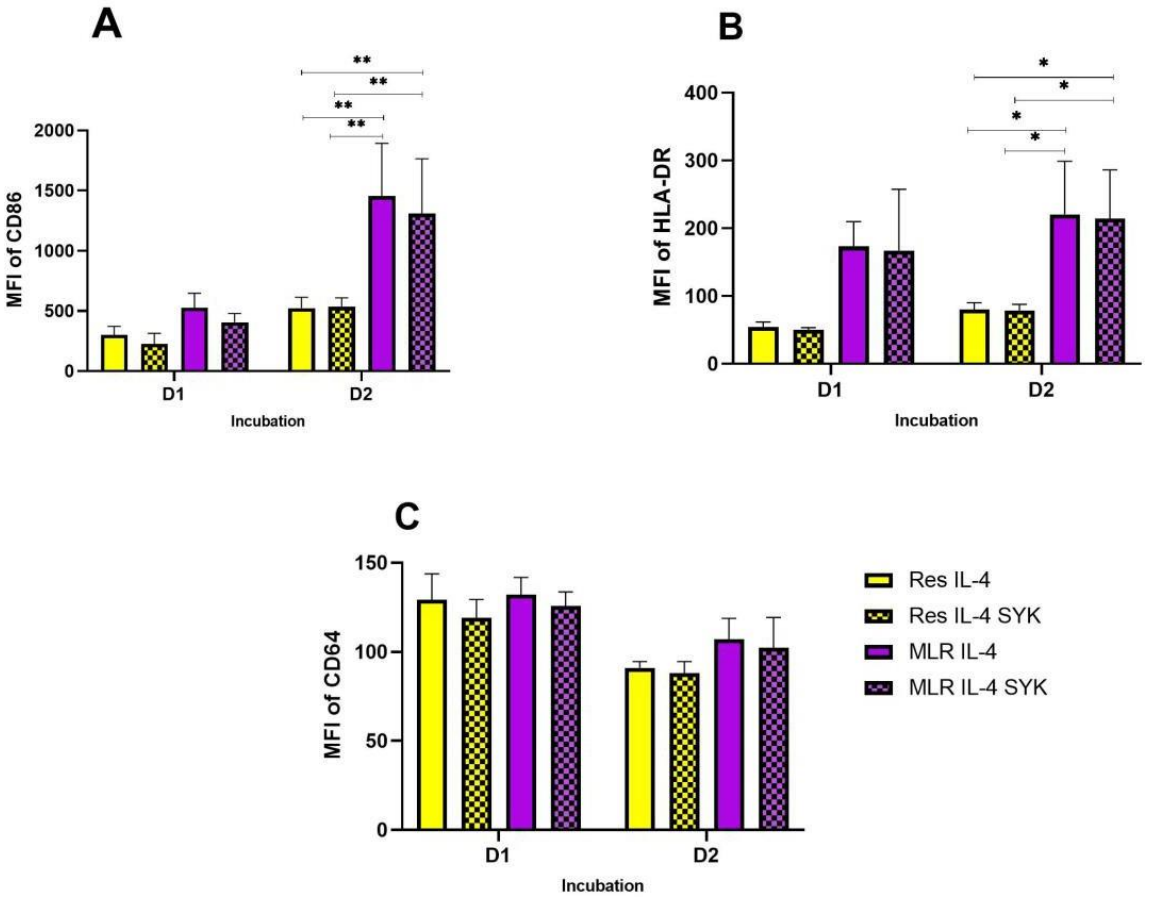

Figure 5. 6: Analysis of the expression CD86, HLA-DR and CD64 on $CD14^+$ monocytes/macrophages by flow cytometry on D1 and D2 of incubation with/without IL-4 and anti-SYK.

A) CD86, **B)** HLA-DR and **C)** CD64. The X axis shows incubation days and Y axis is MFI expression. Data shown are mean and the error bars are SD (n = 3). Yellow bar represents Res IL-4 = Responder cells as negative allogeneic control treated with 100 ng/ml IL-4, dark yellow bar with pattern represents responder cell treated with 100 ng/ml IL-4 and 2 µM anti-SYK, purple bar represents MLR IL-4 = Mixed leukocyte reaction treated with 100 ng/ml IL-4, and light purple bar with pattern bar represents MLR treated with IL-4 and 2 µM of anti-SYK. Statistical significance of data was analysed using RM two-way ANOVA with Tukey multiple comparisons with Geisser Greenhouse correction, p values below 0.05 were considered significant. * = $p < 0.01$, ** = $p < 0.001$ and *** = $p < 0.0001$.

5.2.11 Analysis of the effects of SYK inhibitor on T cell activity in the presence of MLR and IL-4 cytokine:

In this section, analysis of the effects of daily treatment with SYK inhibitor (2 µM PRT0603) on T cells on D3 of incubation was carried out. The $CD3^+$ and $CD4^+$ T cells were analysed for the level of expression of CD25 (MFI) and percent (%) of $CD69^+$ cells using four experimental conditions described above. The cell viability was investigated using Annexin V and showed that more than 90-95 % of cells were Annexin -ve after gating on the T cells population (data not shown).

The data for CD25 expression (Figure 5.7.A) revealed that there was almost no difference in $CD3^+CD25^+$ MFI values in the MLR IL-4 treated with 2 µM anti-SYK inhibitor and the MLR IL-4. Also, no differences were found between Res IL-4 and Res IL-4 SYK.

The data for the $CD3^+CD69^+$ cells (Figure 5.7.B) showed that the proportion (%) of $CD69^+$ T cells was reduced in the MLR IL-4 treated with 2 µM anti-SYK compared to the MLR IL-4, although the difference did not reach statistical significance. Following the same trend, Res IL-4 SYK showed a lower proportion (%) of $CD3^+CD69^+$ T cells compared to Res IL-4, with a modest difference that was not significant.

For the $CD4^+$ cells (Figure 5.7.C), there were a slight reduction in MFI values for $CD4^+CD25^+$ in the IL-4 treated with 2 µM anti-SYK when compared to the MLR IL-4, however the difference was not statistically significant. Furthermore a small

reduction in MFI values for $CD4^+CD25^+$ observed for Res IL-4 SYK compared to Res IL-4, was not significant.

Similarly, the results for the proportion (%) of $CD4^+CD69^+$ cells (Figure 5.7.D) showed a modest reduction in MLR IL-4 with SYK inhibitor compared to MLR IL-4, however, the difference did not reach statistical significance.

Overall, these results highlight that treatment with 2 µM anti-SYK resulted in slightly lower T cell activation levels in the presence of allogeneic cells (MLR) and IL-4 cytokine, although the effects were not statistically significant. Thus, the therapeutic potential of SYK inhibitor in alloresponse in the presence of IL-4 cytokine appears to be rather modest.

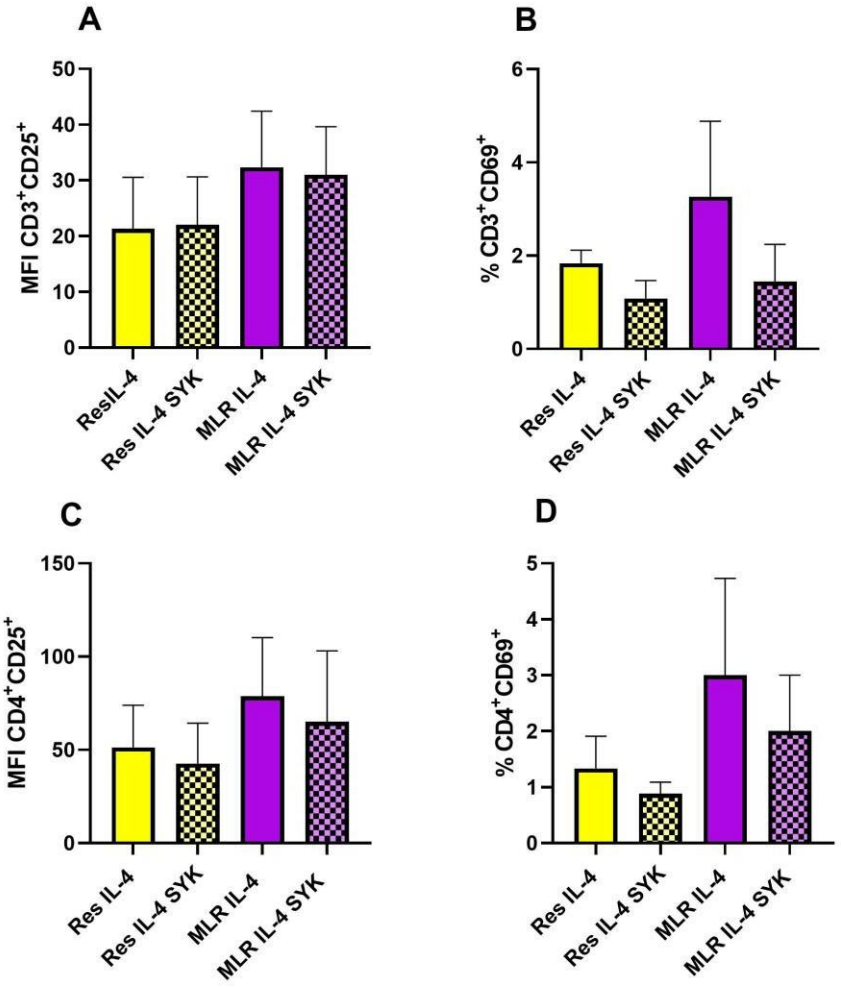

Figure 5. 7: Analysis of T cells activation by flow cytometer on D3 of incubation in the presence of MLR, IL-4 and anti-SYK.

A) $CD3^+CD25^+$, **B)** $CD3^+CD69^+$, **C)** $CD4^+CD25^+$, and **D)** $CD4^+CD69^+$. Y axis presents MFI expression in A and C whereas B and D are present (%) of CD69 positive cells. The X axis shows: yellow bar represents Res IL-4 (responder cells treated with 100 ng/ml IL-4), yellow bar with pattern represents Res IL-4 SYK (responder cells treated with 100 ng/ml IL-4 and 2 µM anti-SYK), purple bar represents MLR IL-4 (Mixed leukocyte reaction treated with 100 ng/ml IL-4), and purple bar with pattern represents MLR IL-4 SYK (MLR treated with 100 ng/ml IL-4 and 2 µM of anti-SYK). Data shown are mean and the error bars are SD (n = 3). Statistical significance of data was analysed using RM one-way ANOVA Tukey multiple comparison with Geisser-greenhouse correction, p values below 0.05 were considered significant.

5.2.12 Measuring cytokine concentrations in MLR treated with IL-4 and SYK inhibitor:

The results presented in (Figure 5.5) showed that the addition of 100 ng/ml of IL-4 to MLR culture resulted in a decrease in the concentrations of TNF-α, IFN-γ, IL-2 and IL-6 cytokines. Nevertheless, the levels of cytokines in MLR IL-4 cultures were higher than in a control, responder cell cultures. Therefore, this section evaluated the effects of combining 100 ng/ml IL-4 and 2 µM anti-SYK daily treatment on the cytokine production. Thus, TNF-α, IFN-γ, IL-2 and IL-6 concentrations were measured by sandwich ELISA and the samples were collected on D2 of incubation from the same four experimental conditions described above.

The results for TNF-α (Figure 5.8. A) revealed that 2 µM of SYK inhibitor treatment significantly decreased TNF-α concentrations in the MLR IL-4 SYK when compared to the MLR IL-4 ($p=0.0173$). For IFN-γ (Figure 5.8.B), the results showed a significant reduction in the MLR IL-4 SYK compared to the MLR IL-4 ($p = 0.0007$). With significant difference in MLR IL-4 compared to Res IL-4 ($p=0.034$) and Res IL-4 SYK ($p = 0.021$).

Also, IL-2 (Figure 5.8.C) data showed that the concentrations of this cytokine were significantly reduced with 2 µM anti-SYK treatment MLR IL-4 SYK comparedto the MLR IL-4 ($p = 0.0035$), significant difference in MLR IL-4 compared to Res IL-4 ($p = 0.001$) and Res IL-4 SYK ($p=0.008$).

Although the result for IL-6 (Figure 5.8.D) showed a small decrease in concentrations of this cytokine in MLR IL-4 SYK compared to MLR IL-4, the difference was not significant. However, significant differences were observed in

MLR IL-4 compared to Res IL-4 (p=0.03) and Res IL-4 SYK (p=0.03).

In summary, these results indicate that SYK inhibitor significantly decreased the production of TNF-α, IFN-γ and IL-2 cytokines in MLR culture in the presence of IL-4. Notably, this further reduction of cytokine production due to SYK pathway inhibition was evident despite the negative influence of IL-4 on the MLR-induced cytokine levels. Therefore, relatively modest effects of SYK inhibition on IL-4 influenced alloresponse may still prove beneficial, as further reduction of Th1 type cytokines to below detection levels could result in the quenching of the initial inflammation.

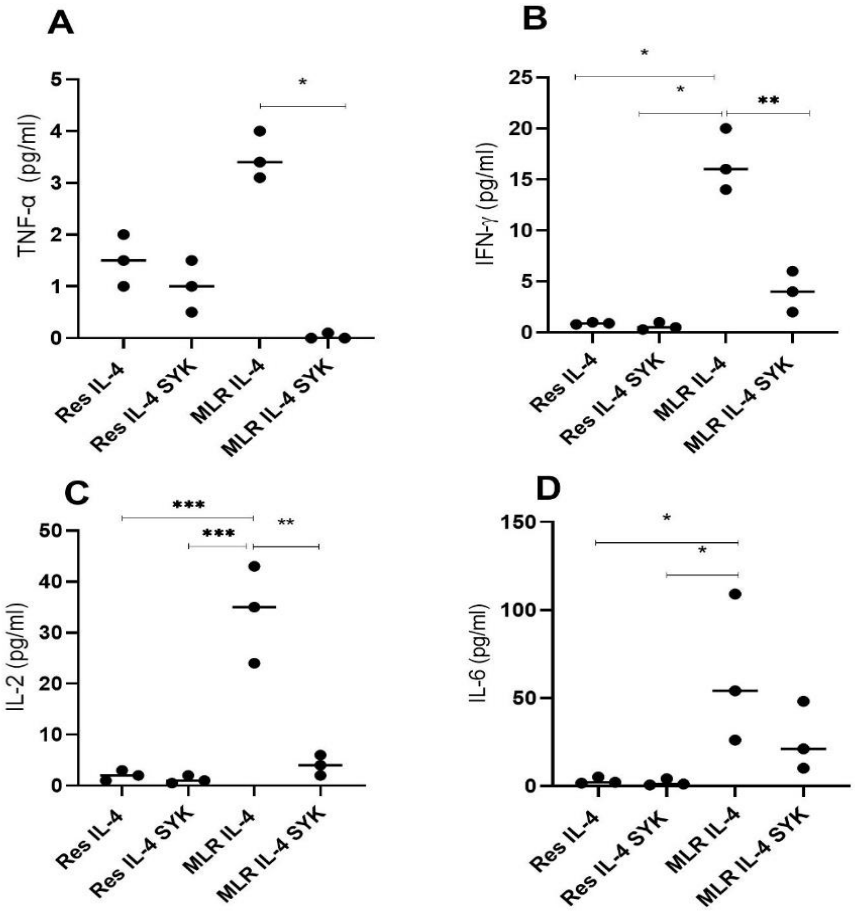

Figure 5. 8: Measuring cytokines by ELISA on D2 of incubation in the presence of MLR, IL-4 and anti-SYK.

A) TNF-α, **B)** IFN-γ, **C)** IL-2 and **D)** IL-6 concentrations. X axis shows comparison of the two conditions: MLR IL-4 = Mixed leukocyte reaction treated 100 ng/ml IL-4 and MLR IL-4 SYK = Mixed leukocyte reaction treated 100 ng/ml IL-4 and 2 µM anti-SYK. Y axis shows the cytokine concentration in pg/ml. Scatter Dot plot presents triplicate repeats (n = 3). Statistical significance

of data was analysed using RM one-way ANOVA Tukey multiple comparison with Geisser-Greenhouse correction, p values below 0.05 were considered significant, * = $p < 0.05$, ** = $p < 0.01$ and ***= $p < 0.001$.

5.3 The effects of SYK inhibitor when MLR is unmodified compared to the MLR culture in presence of IFN-γ and IL-4:

To quantify the effects of the selective SYK inhibitor PRT0603, the percent of inhibition of the expression of monocytes/macrophages markers was calculated. Furthermore, the percent reduction in T cell activity and a decrease in cytokine concentrations were also calculated. The following formula was used: (mean of MFI (or cytokine concentration) with SYK/ mean of MFI (or cytokine concentration) without SYK x 100) -100.

5.3.1 Evaluation of the inhibitory effects of PRT0603 on monocytes/ macrophages:

In Figure 5.9 the data for unmodified MLR culture on D1 of incubation showed that the percent of inhibition by SYK inhibitor treatment was 32 % for CD86 expression (Figure 5. 9.A), 48 % for HLA-DR (Figure 5. 9.B) and 28 % for CD64 (Figure 5. 9.C). Similar trends were shown on D2 of incubation 29 % inhibition of CD86 expression, 34 % of HLA-DR and 32 % inhibition of CD64 expression was observed. Interestingly, in the presence of cytokines, IFN-γ and IL-4, the potency of the SYK inhibitor was reduced compared to unmodified MLR culture. Indeed, the inhibition of CD86, HLA-DR and CD64 expression was 20 % or less on D1 of incubation. Similarly, on D2 of incubation the presence of cytokines interfered with the SYK inhibitor potency. Thus, the expression of CD86 was inhibited by 14 % in MLR IFN-γ and by 27% in MLR IL-4. The expression of HLA-DR reduced by 10 % in MLR IFN-γ and by 15% in MLR IL-4. The expression of CD64 decreased by 5 % in MLR IFN-γ and by 8% in MLR IL-4.

Overall, the presence of IFN-γ or IL-4 strongly reduced the potency of SYK inhibitor in controlling monocytes activities in early allogeneic culture compared to unmodified MLR culture. This has important repercussions for the potential clinical use of SYK inhibitors, as both acute and chronic GVHD are likely to involve production of cytokines, which could interfere with therapeutic efficacy of drugs targeting SYK pathway. (This data will be discussed in the general discussion section 6).

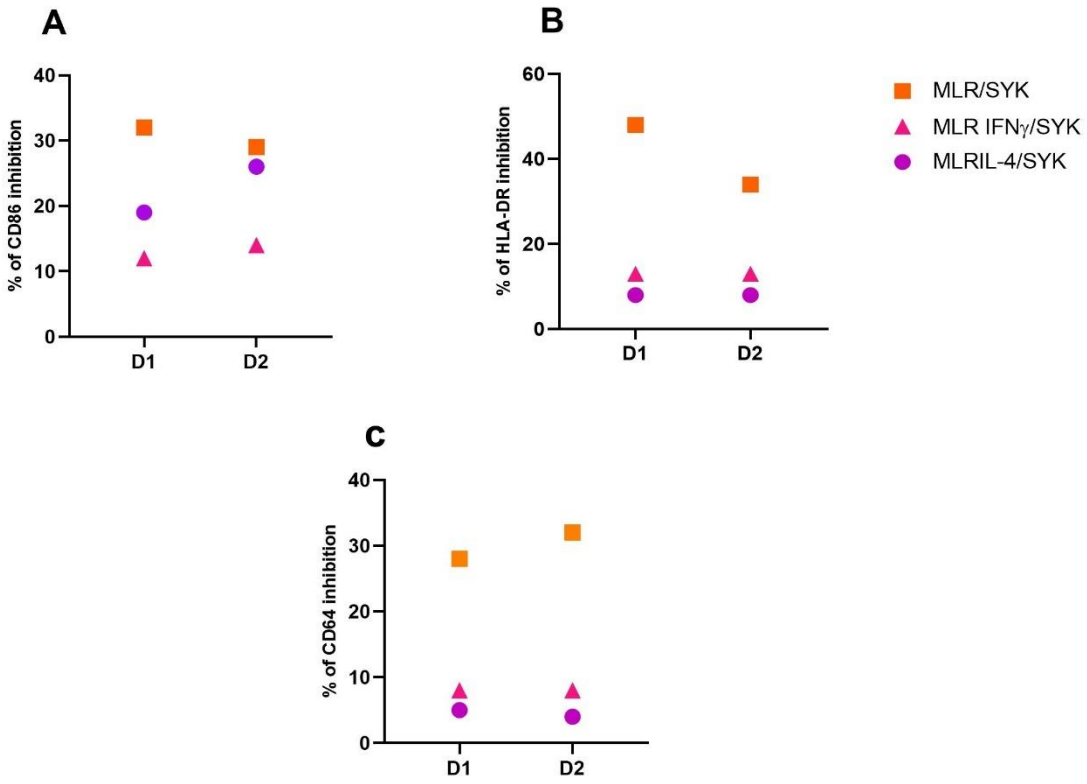

Figure 5. 9: Comparing the precent of inhibition of **A)** CD86, **B)** HLA-DR and **D)** CD64 expression after SYK inhibitor treatment:

Y axis presents the % of inhibition, X axis presents incubation day. Orange square show data calculated from MLR after SYK treatment, pink triangle MLR IFN-γ after SYK treatment and MLR IL-4 purple circle. The data calculated followed this formula (mean of MFI with SYK/ mean of MFI without SYK x 100) -100.

5.3.2 Evaluation of the inhibitory effects of PRT0603 on T cells and cytokine concentrations:

To compare the inhibitory effect of PRT0603 on the levels of CD25 expression (MFI values) on T cells and the proportion of CD69 positive T cells in unmodified MLR culture and in the presence of cytokines (IFN-γ or IL-4), following formula was used. The mean of MFI (or proportion of $CD69^+$ cells) with SYK/ mean of MFI (or proportion of $CD69^+$ cells) without SYK x 100) -100.

The data showed (Figure 5. 10) that the SYK inhibitor reduced $CD3^+CD25^+$ MFI values by 42 % in unmodified MLR and by 44 % in MLR IFN-γ cultures. The reduction was less prominent in the MLR IL4 culture by only 15 %. For early T cell activation marker, CD69 expression change from negative to positive, the

reduction in $CD3^+$ population was pronounced in all conditions. Indeed, the SYK inhibitor resulted in 65 %, 59 %, 54 % reduction in MLR, MLR IFN-γ and MLR IL-4 respectively.

By evaluating the effects of the SYK inhibitor on $CD4^+$ T cell population, the data showed that the SYK inhibitor reduced CD25 expression levels (MFI) by 25 %, 15% and 17% in unmodified MLR, MLR IFN-γ and MLR IL-4 respectively. The proportion of $CD69^+$ cells reduced by 33 % in unmodified MLR culture, 20% in MLR IFN-γ and 33% in MLR IL-4. Overall, reductions of the proportion of $CD69^+$ T cells were more pronounced after SYK inhibitor treatment in both $CD3^+$ and $CD4^+$ T cells. This is in contrast with the less potent inhibition of CD25 expression levels. This is an interesting observation, as CD25 expression is linked with both effector and regulatory T cells. Thus, relative sparing of CD25 expression may allow SYK inhibitors to preferentially target effector T cells. Notably, the presence of cytokines IFN-γ and IL-4 slightly affected the potency of SYK inhibitor effects on T cells compared to unmodified MLR culture. This finding highlights the importance of SYK pathway in T cell activation and suggests that IFN-γ and IL-4 do not provide additional signals to T cells that could override SYK inhibition. This is in sharp contrast with the data on monocytes, where the presence of cytokines interferes with SYK inhibitor effects.

In Figure 5.10.B the data presented the reduction of cytokine concentrations due to the SYK inhibitor treatment. TNF-α concentration were reduced by 87 % in unmodified MLR culture, reduced by 91 % in MLR IFN-γ and the reduction reached 92 % in MLR IL-4. Furthermore, IFN-γ concentrations were reduced due to SYK inhibitor treatment by 88 % in unmodified MLR and 80 % in MLR IL-4 culture. Similarly, IL-2 concentration decreased due to the SYK treatment by 82 %, 79 % and 80 % in unmodified MLR, MLR IFN-γ and MLR IL-4 culture respectively. However, IL-6 concentration decreased by 90 % in unmodified MLR, whereas the presence of IL-4 and IFN-γ have substantially interfere with the effectiveness of SYK inhibitor by 55 % in MLR IFN-γ and 48 % in MLR IL-4.

Similar to effects on T cells, the potency of SYK inhibitor to diminish the cytokine concentrations was not affected by the presence of cytokines IL-4 or IFN-γ added to MLR culture. Indeed, the SYK inhibitor mediated reduction of cytokines was

impressive in all investigated conditions, the unmodified MLR, the MLR IFN-γ and the MLR IL-4 culture. This points out that the SYK inhibitor therapy could show robust effects in challenging, inflammatory environment with a presence of large quantities of cytokines. This data will be discussed in the general discussion section 6.

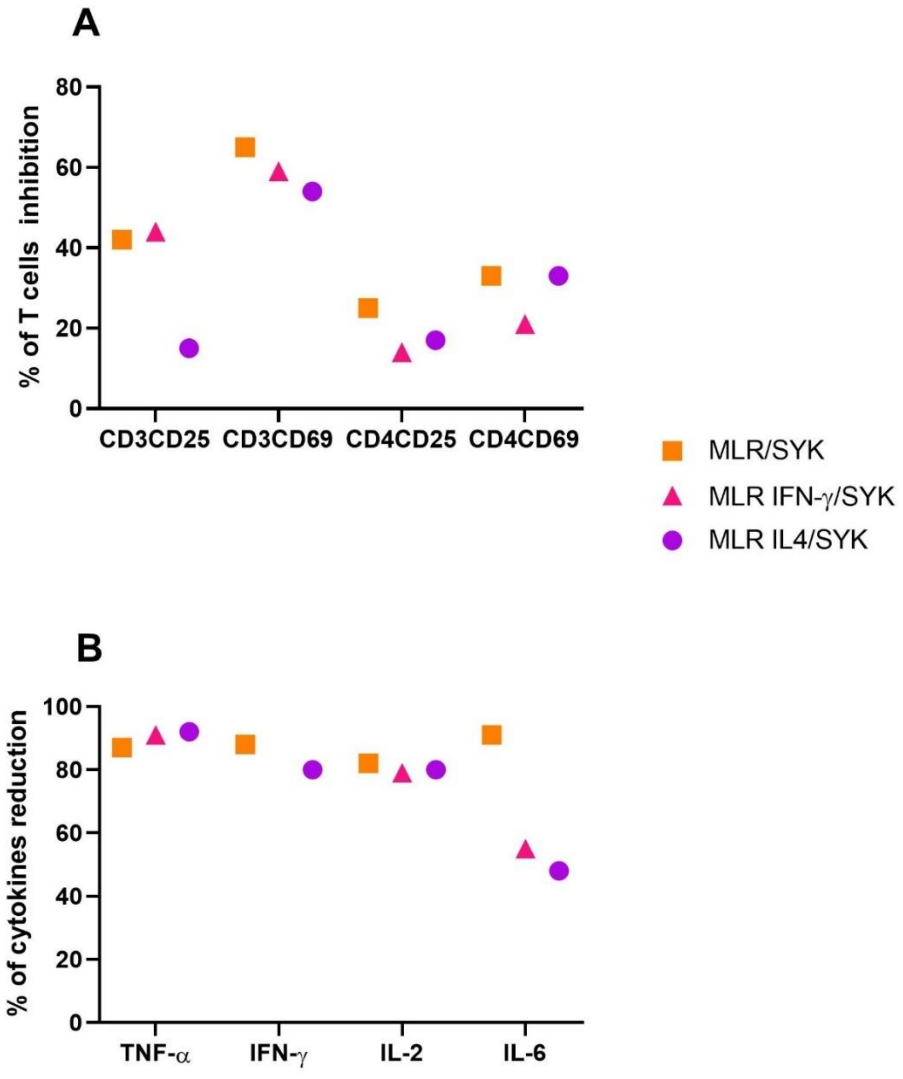

Figure 5. 10: Comparing the potency of inhibition of **A)** T cells activation **B)** cytokine concentration after the SYK inhibitor treatment:

Y axis presents the % of inhibition, X axis A) T cells surface markers, B) Cytokine concentrations (TNF-α, IFN-γ, IL-2 and IL-6). Orange square data calculated from MLR after SYK inhibitor treatment, pink triangle represents MLR IFN-γ after SYK inhibitor treatment and data from MLR IL-4 are presented as purple circle. The data were calculated following this formula: (mean of MFI with SYK/ mean of MFI without SYK x 100) -100.

5.4 Discussion (Part 3):

The discussion for the results presented in chapter 5 (MLR IL-4), can be divided into two parts. The first part evaluated the effects of IL-4 on alloresponse/MLR monocytes/macrophages function and, T cells activation. The second part evaluated the effects of combining IL-4 and treatment with the SYK inhibitor PRT0603 in the MLR setting. Notably, IL-4 is produced by T cells, NK cells basophils and eosinophils (Junttila, 2018, Via et al., 2017). IL-4 was selected in this study as a Th2 cytokine that could modify the inflammatory response and antagonise Th1 cytokines (Via et al., 2017). IL-4 controls lymphocytes function: it causes T cells to differentiate into the Th2 phenotype, and controls immunoglobulin class switching by B cells (Junttila, 2018, Zhao et al., 2018). Many studies have highlighted the therapeutic potential of IL-4 to control T cell activities (Raphael et al., 2014, Kumar et al., 2017, Junttila, 2018). The study by Via and colleagues (2017) showed that early administration of a high dose (10 µg) of IL-4 control T cells alloresponse, reduced aGVHD (Via et al., 2017). Notably, the high doses of IL-4, above the physiological levels were needed to achieve pharmacological effects in GVHD setting. Furthermore, it has been proposed that IL-4 enhances monocyte differentiation into intermediate monocytes and to polarise macrophages toward the alternative macrophage (M2) type, which have anti-inflammatory and tissue repair functions (Duque & Descoteaux, 2014, Raphael et al., 2014, Murray et al., 2017). However, human monocytes or macrophages subsets and their functions are different, as each subset reacts differently, depending on the activator context (Mosser & Edwards, 2008, Boyette et al., 2016). IL-4 binds to two types of IL-4 receptors based on the cellular type, T cells mainly express IL-4RI, whereas epithelial cells expressed IL-4 RII (Junttila, 2018, Zhao et al., 2018). Interestingly myeloid cells including monocytes and macrophages expressed both receptors type (Junttila, 2018, Zhao et al., 2018).

This study evaluated monocytes/macrophage's function and T cell activity after administration of IL-4 to the early allogeneic cell culture (MLR). To optimise the IL-4 concentration in MLR setting, three different concentrations 10, 50 and 100 ng/ml of IL-4 were used and evaluated for the expression of CD86, CD64 and HLA-DR. The higher dose of 100 ng/ml IL-4 was the most consistent in the whole blood 2-way MLR model. Importantly, none of the IL-4 concentrations used in this

study showed cytotoxic effects, thus the viability of cells in the culture was >85 % in all experimental conditions. The study by Delneste et al. (2003) on human monocytes differentiation *in vitro* used IL-4 and IFN-γ, the cytokine concentrations in the range of 25-100 ng/ml to achieve changes in monocytes phenotype (Delneste, 2003). This is remarkably similar to the concentrations used in this study. Furthermore, Delneste et al. (2003) used purified monocytes cell culture, whereas this study was based on whole blood culture. As the bioavailability of cytokines is affected by the presence of the complex whole blood matrix, the effective cytokine concentrations in this study are likely to be lower than that used by Delneste et al. (Delneste, 2003). It has been reported that low doses of IL-4 will bind to IL-4 R type I with high affinity (Junttila, 2018). However, the use of high concentrations of IL-4 in this report did not show any negative effects on cell viability, and changes in the monocyte phenotype showed similar trends across the full range of investigated concentrations. Therefore, it could be argued that a relatively high cytokine concentration could preserve its activity during several days of cell culture without affecting cell viability and their function. Obviously, this may not apply to all conditions and an optimal cytokine concentration needs to be evaluated in a particular experimental model. Notably, several cell cultures studies that activate monocytes used high non-physiological cytokine concentrations (Delneste, 2003, Bayik et al., 2017)

This study evaluated the effects of treating the alloresponse/MLR with IL-4, by analysing the expression of CD86, HLA-DR, CD64, CD32, CD36, CD204, C3aR and CD11b on the $CD14^+$ monocytes. The results observed in MLR IL-4 culture were compared to MLR, Res and Res IL-4. During the flow cytometer staining procedure, all samples were treated with Fc blocking and the results were compared to the isotype control to eliminate background of nonspecific antibody binding. Cell viability was assessed after D1 and D2 of incubation days by Annexin V, and 80 to 85 % of monocytes in the gated population were Annexin V negative, confirming that the cells were viable. However, $CD14^+$ monocytes viability data on D3 showed 45 to 50 % Annexin positive cells, thus the D3 result were excluded from analysis.

Interestingly, the data in this report showed that $CD14^+$ monocytes in MLR IL-4 culture significantly up-regulated CD86, HLA-DR and CD11b expression, with

significant down regulation of CD64 and C3aR expression. The explanation for this observation is based on the following: when IL-4 binds to IL-4R on monocytes, this activates JAK1-JAK3 signalling which results in STAT6 phosphorylation. Subsequently, the STAT6 dimer is translocated to the nucleus, triggering transcriptions of the genes for HLA-DR and CD86 on APCs (Deneys et al., 2001, Zhao et al., 2018). Thus, IL-4 controlled CD86 and HLA-DR expression via STAT6 pathway (Deneys et al., 2001, Deszo et al., 2003, Balce et al., 2011). Although this report did not address the precise mechanism of IL-4 effects on isolated monocytes, it could be postulated that IL-4 could trigger STAT6 phosphorylation, resulting in enhanced co-stimulation and Ag presentation capacity compared to untreated MLR control. Thus, the data from this study helped to clarify the effects of IL-4 in the early phase of immune response to allogeneic cells, with particular focus on the role of $CD14^+$ monocytes/macrophages, highlighting that IL-4 did not control the inhibitory effect through co-stimulation. However, this study did not evaluate the expression of the co- inhibitory CD80 molecule, which needs to be measured in the future work. These results support previous studies that have showed IL-4 mediated induction of HLA-DR expression in monocytes (Roussetf et al., 1988, Anje et al., 1990, Lee et al., 2017). However, the data in this study contradicted the observation of via et al (2017), where IL-4 controlled T cell proliferation indirectly by reducing Ag presentation capacity in an aGVHD mouse model (Via et al., 2017). In this work, CD64 was selected as an activator FcγRI and CD32B (FcγRIIB) as an inhibitory receptor (Zhao et al., 2018). When FcγR binds to the Fc region of the immune complex (antibody-antigen complex), this controls internalisation and phagocytosis of the immune complex, and later Ag presentation (Brandsma et al., 2018, Zhao et al., 2018). The observations described here suggest that the presence of allogeneic cells (MLR) enhances phagocytic and antibody-antigen capture functions of monocytes. Whereas the addition of IL-4 cytokine to the MLR culture inhibited these effects. Furthermore, these results could be interpreted in the light of changes in the monocyte phenotype in the alloresponse/MLR triggering an increase in the classical monocytes, whereas IL-4 showed the opposite effects. The results reported here support previous studies that showed the down regulation of CD64 expression, due to IL-4 treatment which limited classical monocytes differentiation (Akinrinmade et al., 2018, Duque & Descoteaux, 2014). The reduction of CD64

expression could reflect the status of SYK downstream signalling. The role of CD64 clustering and SYK activation was already discussed in Chapter 4 (Kiefer et al., 1998, Coffey et al., 2016). The further analysis of intracellular staining for the SYK phosphorylation could be useful to show the activation state of the SYK pathway in this model. However, previous extensive study of SYK signalling (Coffey et al., 2016) enable the prediction of the effects of FcγR ligation and SYK pathway inhibition, without the need to repeat experiments that were already reported.CD32B was selected as a monocyte inhibitory marker, although its role in human monocytes and particularly in GVHD pathology have been less studied, with most research focused on CD32B expression in DCs (Brandsma et al., 2019, Junker et al., 2020). This study evaluated CD32B expression on monocytes/macrophages in early response to the allogeneic reaction and treatment with IL-4 cytokine. The results in MLR IL-4 cell culture showed up-regulation of CD32B on $CD14^+$ monocytes compared to MLR, Res and Res IL-4 on D1 but the differences were not statistically significant (raw data are presented in the appendix Figure S.1.6). These results may suggest that unlike the CD64 marker, CD32B expression was not significantly affected by the presence of allogeneic cells or the addition of IL-4 cytokine. Regarding the monocyte phenotype, the alloresponse and IL-4 on CD32B expression, which is associated with intermediate monocyte phenotype, did not produce significant effects. That could be due to limited number of experiments, that needs to consider in further investigation. Notably, up-regulation of CD32B is mainly involved in the monocyte inhibitory response (Brandsma et al., 2018). Although this study did not investigate monocytes subtype and CD16 expression was not determined to define $CD14^+CD16^-CD64^+$ and $CD14^+CD16^+CD32B^+$ populations, down-regulation of CD64 and up-regulation of $CD32B^+$ in the MLRIL-4 culture suggest that IL-4 may promote an intermediate monocyte subtype. This could infer that the presence of IL-4 combined with alloresponse resulted in monocyte differentiation towards a subtype that has some anti-inflammatory features. Interestingly, monocytes that express CD32B (inhibitory receptor) can capture and process immune complexes and present the processed Ag to T cells, resulting in weak T cell activation in an experimental model based on murine DCs (Junker et al., 2020). Therefore, the intermediate monocytes highly express MHC-II, FcγRIIB and are recruited in the later stages of inflammation to enhance antigen presentation, secretion of anti-

inflammatory cytokines and chemokines, and to initiate wound healing (Tacke & Randolph, 2006, Belhareth & Mege, 2015).

In this study, phenotypical analysis of CD36 and CD204 expression on $CD14^+$ cells were performed to evaluate clearance of apoptotic cells. Interestingly, CD36 is considered an important phagocytic marker expressed on the surface of M2 macrophages to recognise apoptotic cells (Pennathur et al., 2015). The current data on the $CD14^+$ monocytes expression of CD36 in the early phase of GVHD are limited and it is unclear if this molecule is a marker for immune tolerance or a strong alloresponse. Based on this study, the role of the CD36 molecule appears to be limited, with modest increase in its expression on monocytes observed in the early phase of MLR culture in the presence of IL-4 cytokine.

The expression of CD204 on $CD14^+$ monocytes is slightly down regulated in the MLR IL-4 when compared to the MLR culture. The reduction of $CD14^+CD204^+$ expression was also observed on Res IL-4 compared to Res. These data contradict a previously published study, which reported that CD204 expression was enhanced by IL-4 (Ohtaki et al., 2010). The possible explanation of this observation could be that IL-4 antagonise the effect of TNF-α, which is increased in the allogeneic culture. Hence, the reduction of TNF-α could result in down regulation of CD204. In this study, administration of IL-4 to the early MLR culture may have promoted the capture of apoptotic cells by monocytes via CD36 rather than CD204, which could affect monocyte function and their differentiation into a distinct phenotype. However, this concept needs to be interpreted with caution, as the IL-4 mediated changes in the expression of scavenger receptors were modest and did not reach statistical significance. Nevertheless, the monocytes expression of CD36 and CD204 showed large variations, thus the future work will need to address this by increasing the number of experimental repeats or by improving the methodology.

Monocytes/macrophages expressed C3aR at high concentration (Mommert et al., 2018). The data revealed that the addition of IL-4 to early MLR culture significantly down-regulated the expression of C3aR on monocytes/macrophages when compared to the control MLR culture on D1 of incubation. The monocyte expression of C3aR dropped even further on D2 of incubation, confirming that IL-

4 treatment has negative effect on C3aR levels. The data in this study support the previous observation obtained using a different experimental model, which showed a reduction of C3aR on human M2 macrophages in an allergic reaction in the presence of IL-4 and histamine (Mommert et al., 2018). Notably, it has been reported that targeting C3aR on mice DCs lowered the Ag presentation capacity, APCs activation, reduced HLA-DR expression and decreases IFN-γ production (Nguyen et al., 2015). More importantly, C3aR deficiency on APCs reduced T cell priming and decreased the onset of aGVHD (Nguyen et al., 2015). Notably, the reduction of C3aR could be a potential sign that IL-4 could control monocytes activities in early allogeneic culture. However, in this study CD86 and HLA-DR were highly expressed on $CD14^{+}$ monocytes/macrophages after IL-4 treatment. Thus, the role of IL-4 and the expression of C3aR on human monocytes/macrophages in early allogeneic cell culture requires further study, to confirm IL-4 function. At present, it could be postulated that IL-4 mediated decrease in C3aR expression which reflects the development of M2 macrophages and promotes an anti-inflammatory response. Nevertheless, this effect of IL-4 cytokine needs to be balanced with a striking stimulation of monocyte/macrophages CD86 expression.

CD11b is one of the key markers that are expressed on the surface of monocytes/macrophages particularly the M2 subtype, with an anti-inflammatory function (Ma et al., 2019). CD11b is also considered as a main regulator for monocyte migration and adhesion (Zheng et al., 2015, Lukácsi et al., 2017, Baba et al., 2020). Furthermore, CD11b is considered as a differentiation and activation marker (Sprangers 2016, Baba et al., 2020). Notably, monocytes/macrophages can change their response to inflammation based on the microenvironment milieu (Zheng et al., 2015).

The data in this study showed that CD11b expression is mainly controlled by IL-4 treatment, without a significant contribution by the allogeneic reaction. Thus, IL-4 showed a strong effect on monocytes/macrophage migration and adhesion functions, which was not affected by the presence of allogeneic cells. The data reported here broadly agreed with previous studies that showed IL-4 induced CD11b expression on macrophages due to STAT6 activation after IL-4R ligation (Deszo et al., 2003, Zheng et al., 2015). Therefore, it appears that the MLR culture

represents a robust model of an immune response with a good insight into monocyte function. However, it is difficult to confirm the role of the specific subtypes due to monocytes plasticity in the MLR culture. However, the early allogeneic culture with IL-4 cytokine activated monocytes and they are mainly differentiated to monocytes derived macrophages, as demonstrated by CD11b expression, and they are capable of co-stimulation and presenting Ag to T cells.

Furthermore, T cell activation levels were analysed on D3 of incubation by evaluating the proportion of (%) CD69 and expression (MFI) CD25 on the surface of $CD3^+$ and $CD4^+$ cells. Generally, $CD4^+$T helper cells have been associated with early GVHD development (Hong et al., 2020). Moreover, it has been reported that IL-4 reduces T cell proliferation and migration in aGVHD (Via et al., 2017). The data in this study showed a significant reduction of CD25 expression levels (MFI values) on $CD3^+$ T cells with modest non-significant decrease in $CD3^+CD69^+$, and $CD4^+CD69^+$, $CD4^+CD25^+$ markers in the MLR treated with IL-4 when compared to the control MLR culture. These findings showed that the early phase of the alloresponse/MLR culture, IL-4 stimulates $CD14^+$ cells co-stimulatory and Ag presenting functions, while at the same time this cytokine down regulates T cell activation. This agrees with a previous study that reported activation of macrophages *in vitro* with IL-4, leading to enhanced Ag presentation, but failure to present Ag to T cells (Mosser & Edwards, 2008). Thus, the T cell response is mainly controlled by the cytokine environment (Junttila, 2018).

The culture supernatants collected on D2 of incubation were measured for the concentrations of specific inflammatory cytokines: TNF-α, IFN-γ, IL-2 and IL-6. The data in this study showed that 100 ng/ml of IL-4 significantly lowered the concentrations of TNF-α, IFN-γ, IL-2 and IL-6 in MLR-IL-4 when compared to the control MLR culture, which could be a protective effect of IL-4 in controlling the early allogeneic response. These data agree with a previous study in a different model, which reported that the Th2 type response can down regulate the production of Th1 cytokines, resulting in reduced pathogenesis of GVHD (Kumar et al., 2017). In GVHD, TNF-α plays an essential role by controlling tissue damage and enhancing cells apoptosis (Kumar et al., 2017, Via et al. ,2017).

Interestingly, in a mouse model, blockage of TNF-α or absence of IFN-γ showed

therapeutic effects in aGVHD but promoted development of cGVHD (Kumar et al., 2017, Via et al., 2017). These results are in accordance with a study that reported that IL-4 reduced IFN-γ secretion and resulted in suppression of CD8 T cells (Raphael et al., 2014, Via et al., 2017). Whereas, in this report, the effect of IL-4 to antagonise IFN-γ was linked to a significant reduction of CD25 level on $CD3^+$ cells, which comprises $CD8^+$ T cell population.

In this report, the reduction of IL-2 and IL-6 could be linked with the low TNF-α concentration observed in the MLR IL-4 culture. It has been reported that IL-6 in GVHD stimulates the production of TNF-α by APCs (Deeg, H 2001, Tawara et al., 2011, Tvedt et al., 2017, Drobyski et al., 2018). Furthermore, the antagonism between IL-4 and IL-2 could explain the lack of T cell activation in the MLR IL-4 culture (Markey et al., 2014), as low concentrations of IL-2 post HSCT were associated with a decrease in GVHD incidence (Kumar et al., 2017). Indeed, the data in this report support the concept that IL-6 cytokine could be involved in the control of T cells activation (Tawara et al., 2011, Drobyski et al., 2018). In this context, reduction of IL-6 concentrations due to IL-4 treatment could result in the lowered levels of alloresponse, as IL-6 could be secreted by activated monocytes/macrophages, and this cytokine has been associated with the development of aGVHD (Tawara et al., 2011, Tvedt et al., 2017, Drobyski et al., 2018, Hong et al., 2020). Notably, in some experimental models, IL-4 showed a protective role, such as reduced inflammation in GVHD (Kumar et al., 2017, Via et al., 2017). In this report, the addition of the IL-4 cytokine to the MLR culture generates the unique inflammatory conditions that promote monocyte/APC activation, while at the same time antagonised the inflammatory cytokines in MLR culture that result in limiting T cell response. The findings in this study suggests that treating MLR with 100 ng/ml of IL-4 could showed a therapeutic potential in the early allogeneic response. Notably, the data showed that monocytes in the 2-way MLR with non-physiological IL-4 concentration could reacted similarly to monocytes in isolated cell culture.

The second part of this chapter evaluated the effects of combining a treatment with 2 µM PRT0603 (anti-SYK) and 100 ng/ml IL-4 cytokine. The expression levels (MFI values) for CD86, HLA-DR and CD64 were measured by flow cytometry to evaluate the phenotype and function of the monocytes/macrophages in following

conditions: MLR IL-4 SYK (experimental condition, MLR treated with 100 ng/ml IL-4 and 2 µM anti-SYK), MLR IL-4 (allogeneic response/MLR treated with IL-4), Res IL-4 (responder cells treated with IL-4) and Res IL-4 SYK (responder cells treated withIL-4 and anti-SYK). The results showed that with 2 µM of the SYK inhibitor, there were no changes in CD86, HLA-DR and CD64 expression. This suggests that the SYK pathway is involved in the control of CD86 and HLA-DR expression during the alloresponse and not in the IL-4 driven response. This is in contrast to previous observations that showed that Fostamatinib (a non-selective SYK inhibitor) mediated a decrease in CD86 expression on macrophages in a mouse model of GVHD (Flynn et al., 2015).

Considering that CD64 is a Fcγ receptor, which uses the SYK pathway for downstream signal transmission, it could be regarded as unexpected that the SYK inhibitor does not affect expression of this marker. However, it is feasible that IL-4 treatment controls CD64 expression in such a way that any further inhibition by the SYK inhibitor is non-significant.

Importantly, on D3 of incubation $CD3^+$ and $CD4^+$ T cell activation levels were evaluated by measuring MFI values for CD25 and percent (%) of T cells positive for CD69 expression. The data indicate that SYK treatment may not affect T cells activation levels in the MLR IL-4 culture, although the effects did not reach statistical significance (raw data in appendix Figure S.1.7). Thus, the data needs to be interpreted with caution and further study is required. Notably, the effects of SYK inhibitor were observed despite the low levels of TNF-α, IFN-γ IL-2 cytokine production in the presence of IL-4. Thus, SYK inhibition achieves further, profound reduction of cytokine production with values that were often below detection limits. This could be particularly important in GVHD setting, as even a minute increase in cytokine levels could trigger a spiral of inflammation leading to severe pathology. The data in this study support a previous report that showed that SYK pathway inhibition resulted in lower TNF-α secretion (Su Yi et al., 2014). Furthermore, significant reduction in IL-2 secretion could explain the slightly lower levels of T cells activation in the MLR IL-4 SYK culture. Interestingly, despite the reduction in TNF-α and IFN-γ concentrations, the levels of CD86 expression on $CD14^+$ remained relatively high in the MLR IL-4 SYK culture. This is in contrast with previous studies, which reported that TNF-α is the principal factor that

controlled CD86 expression, whereas IFN-γ controlled HLA-DR expression and Ag presentation (Raphael et al., 2014, Lee et al., 2017). However, these results could support the concept that CD86 and HLA-DR expression is regulated by IL-4 ligation to their receptor which does not involve SYK activation. As such, the SYK treatment did not control that high expression.

In summary, addition of IL-4 cytokine to the early allogeneic culture, activated monocytes/macrophages, resulting in cells that highly express CD86 and MHCII with low phagocytosis capacity through FcR, C3aR and CD204. That caused neutral effects on T cells activities and as expected, IL-4 antagonise other cytokines in the allogeneic culture. However, using IL-4 as a treatment is not a safe option to control monocytes/macrophages in the allogeneic response. Indeed, IL-4 combined with the SYK inhibitor did not control monocytes/macrophages or T cells activation, although this treatment did significantly reduce cytokine concentrations.

6 General discussion:

HSCT is a therapeutic option for many diseases (Welniak et al., 2007, Hill and Koyama 2020), however, infections and GVHD severely limit the successful use of HSCT (Deneys et al., 2001, Blazar et al., 2013, Wang and Young, 2014, Hill and Koyama 2020). Despite improvements in MHC matching, the inevitable presence of mismatched miHA antigens may stimulate a strong immune response by donor T cells, leading to the development of GVHD (Shlomchik, 2007, Hill and Koyama 2020). Furthermore, activation of APCs, especially monocytes, in the early phase of GVHD, play a crucial role in the pathology. Monocytes maintain an important role in innate and adaptive immunity (Tack and Randolph 2006, Sprangers et al., 2016). Monocytes have short half-life in human circulation, after 3 days monocytes based in the microenvironment differentiate to macrophages or DCs (Tack and Randolph 2006). Monocyte-derived macrophages mainly adapt to the surrounding environment (Wang et al., 2014, Murray., 2017). Changes in extrinsic pathway, such as cytokine levels, could control macrophage polarisation, therefore, it is possible to evaluate *in vitro* monocyte-derived macrophage surface marker expression maintained by changing cytokines in the extrinsic pathway to control monocytes differentiation and polarisation towards the M1 phenotype, with high Ag presentation capacity (Wang and Yang, 2014, Murray, 2017). Several studies have shown that IFN-γ is essential for activation of monocytes that appear to adapt to the proinflammatory response (Blazar et al., 2013, Deneys et al., 2001, Wang and Young, 2014). It was proposed that after TBI, damaged tissue activated APCs that release IFN-γ and this cytokine control monocytes differentiation into macrophages during the early phase of GVHD (Deeg, 2001, Blazar et al., 2013, Murray, 2017). Furthermore, activated donor T helper cells secret high concentrations of IFN- γ in response to endotoxin (LPS) (Deeg, 2001, Murray, 2017). This in turn activates the innate immune response to produce additional proinflammatory cytokines (Wang and Yange, 2014). Notably, monocytes cultured with IFN-γ and TNF-α will acquired M1 morphology, however, if monocytes are cultured with IL-4, then the monocyte-derived macrophages will give rise to the M2 macrophages (Martinez and Gordon, 2014). This knowledge was obtained using either cell cultures of PBMCs, isolated monocytes or animal models, in contrast, this study avoided experimental manipulation of human monocytes by using whole blood culture and

a short incubation period. Although this study used high, non-physiological concentrations of IFN-γ and IL-4 to activate the monocytes, the dosing of these cytokines was based on extensive concentration optimising experiments. Interestingly, other papers support the use of high cytokine concentrations to activate monocytes *in vitro*. The study by Delneste et al. (2003) on human monocyte differentiation *in vitro* used IL-4 and IFN-γ cytokine concentrations in the range of 25-100 ng/ml to achieve changes in the monocyte phenotype (Delneste, 2003). These are remarkably similar to the concentrations used in this study. Furthermore, Bayik et al. (2017) used 500 ng/ml of IFN-γ to allow human monocyte maturation, which is 5 times more than the levels of cytokine used in this study (Bayik et al., 2017). Importantly, the data in this study showed that in the unmodified, whole blood MLR culture, the monocytes activation pattern shares many features of the monocytes obtained from inflamed tissues of GVHD patients (Jardine et al., 2019). The MLR culture in this study allowed activation, stimulation, and proliferation of both stimulatory and responder cells. Furthermore, the complex nature of the whole blood MLR allows interactions of many cells, including neutrophils, platelets, and the differentiation of monocytes to macrophages, depending on the cytokine milieu. This could be similar to the conditions in GVHD patients, where the graft T cells interact with both host and donor cells, including neutrophils and myeloid cells (Jardine et al., 2019). Indeed, recent studies in GVHD patients have confirmed the critical role of infiltrated donor monocyte-derived macrophages in the affected skin, which activate pathogenic T cells (Jardine et al., 2019, Young et al., 2020). Thus, the data from this report could highlights the similarity between recent clinical observations (Jardine et al., 2019, Young et al., 2020) and the phenotype of activated monocytes in the whole blood MLR culture. Nevertheless, further studies are needed to confirm these findings. The current understanding of how multiple cytokines are integrated in the allogeneic environment remains limited. As the concept of cytokine therapy is well established in GVHD, this study evaluated the effect of IFN-γ and IL-4 on early monocyte activation that contributed to the response of allogeneic T cells in MLR culture.

Signalling for both cytokines IFN-γ and IL-4 is mediated by the JAK/STAT pathway via STAT1/2 and STAT6, respectively (Lin and Leonard, 2019).

JAK/STAT signalling in monocytes/macrophages results in transcriptional enhancement or changes in cellular functions such as phagocytosis, Ag presentation and co-stimulation. A previous study has reported that these two cytokines can share the same STAT protein to enhance or antagonise the effects of each other (Lin and Leonard, 2019). This study used administration of IFN-γ and IL-4 to the MLR culture, followed by evaluation of the levels of expression (MFI) values for 8 surface receptors on monocytes/macrophages by flow cytometry: CD86, HLA-DR, CD64, CD32B, CD36, CD204, C3aR and CD11b, as each marker could be linked to one of the monocytes functions. In addition, this study investigated the therapeutic potential of highly selective SYK inhibitor (PRT0603) that targets monocytes/macrophages FcγR signalling by inhibiting the SYK pathway. This enabled evaluation of the role of FcγR in the early phase of allogeneic cell culture.

The data collected from early MLR culture represented the allogeneic reaction. Notably, TCR is the only molecule that can recognise MHC I or MHC II polymorphisms (Ferrat et al., 1991, Blazar et al., 2013, Zeiser et al., 2016 and Lee et al., 2018). In this study, T cells could recognise the polymorphic Ag on D1 that result in IL-2 secretion to activate monocytes, the activated monocytes released cytokines that also control the expression of monocytes markers. Thus, based on the data available, it could be concluded that this mechanism is likely to operate in all conditions: MLR, MLR IFN-γ and MLR IL-4 cultures. Notably, the data reveal that activated monocytes in unmodified MLR culture expressed several surface markers and produced cytokines that could be linked with the classical subtype. As monocytes share similarity to data reported by previous studies, where activation of classical monocytes (Deeg, H 2001, Tawara et al., 2011, Sprangers et al. 2016, Boyette et al., 2016, Tvedt et al., 2017). Furthermore, based on the preceding (D1 and D2) activation of monocytes/macrophages in the MLR culture, it appears that the early functional response of monocytes plays an important role in subsequent T cells alloresponse (on D3 of incubation), including both total $CD3^+$ T cells and helper $CD4^+$ T cells. Notably, it has been reported that priming of the T helper cells has been associated with early GVHD development (Koehen et al., 2015, Zeiser et al., 2016 and Hong et al., 2020). The presence of inflammatory conditions in the

early phase of alloresponse/MLR culture due to stimulatory signals provided by allogeneic cells is confirmed by significant increases of TNF-α, IFN-γ, IL-6 and IL-2 concentrations in the MLR culture, when compared to the responder cells control. Although this study did not determine the origin of these cytokines, at this early stage of alloresponse, monocytes/macrophages were clearly activated. Thus, it could be postulated that TNF-α, IFN-γ and IL-6 are likely to be secreted by monocytes/macrophages. On the other hand, IL-2 could be produced by T cells. However, intracellular cytokine analysis is needed to address the limitation of current study in the future work.

The results of this study revealed that the allogeneic response in whole blood MLR culture could activates monocytes/macrophages in a similar way to the reactions generated in a clinical aGVHD setting. However, due to limited number of monocyte markers analysed in this study, limited cytokine measurements, and the lack of intracellular cytokines staining, the relevance of this model to the clinical setting remains speculative. Furthermore, whole blood MLR culture was also used to evaluate the effect of administration of IFN-γ and IL-4 cytokines on monocytes/macrophage's function.

These data indicate that the presence of IFN-γ does not play a strong pro-inflammatory role during the early phase of alloresponse. The key contribution of IFN-γ to the alloresponse appears to be to drive $CD14^+$ monocytes differentiation towards a defence against pathogens mode, with limited inflammatory response and modest effects on T cell priming.

In contrast, administration of IL-4 to the MLR culture steers monocytes towards a distinct functional state, characterised by the high levels of CD86, HLA-DR and CD11b expression, and decreased the levels of CD64 and C3aR expression.

However, IL-4 inhibited MLR-induced T cell activation and showed antagonistic effects on Th1 type cytokines. Indeed, this highlight the need of further analysis of Tregs in the future work, as it has been recently reported that combining IL-2 and IL-4 could induce Treg formation (Zhou et al., 2020). Interestingly, in response to allo-Ag, activated APCs released IFN-γ cytokine (Wang et al., 2014, Lee & Ashkar, 2018). HLA-DR expression is thought to be controlled by IFN-γ binding to IFN-γR to activate JAK/STAT signalling, resulting in increased transcription of MHC II genes: HLA-DR, DP, DQ, Li and HLA-DM (Axelrod et al.,

2019). However, in this study, the effects of IFN-γ on monocytes expression of HLA-DR were rather modest. This could be a distinct feature of peripheral blood monocytes that differs from other APCs. Alternatively, the unique environment of the whole blood culture may have influenced the effects of IFN-γ. In contrast, in MLR IL-4 culture a strong increase in HLA-DR expression was observed, which highlights the role of IFN-γ independent pathway responsible for HLA-DR up-regulation that was triggered by the ligation of IL-4 to IL-4R.

CD86 expression is controlled by either receptor cross-linking or cytokine stimulation (Axelrod et al., 2019). The data in this study showed that CD86 expression was up-regulated by different pathways, in MLR and MLR IFN-γ cultures CD86 could be triggered by cross-linking of MHC II to TCR during allogeneic recognition. However, the MLR IL-4 culture showed a strong up-regulation of CD86 expression that could be linked to the well-known phosphorylation of STAT6 protein that follows ligation of IL-4R (Tugal et al., 2013). Indeed, the data revealed that IL-4 stimulation of CD86 expression was much stronger than that observed in MLR and MLR IFN- γ cultures.

The data in this study revealed that stimulating monocytes by allogeneic cells in MLR culture initiates inflammation due to the observed production of cytokines. Furthermore, it is generally accepted that the CD64 molecule is involved in inflammation. CD64 expression is controlled by IFN-γ binding to IFN-γ receptor. Indeed, the results presented here show that CD64 expression is increased in MLR and MLR IFN-γ cultures. Notably, the expression of CD64 was higher after adding IFN-γ to the MLR culture. As expected, IL-4 has opposite effects to IFN-γ, which was confirmed by the observed significant reduction of CD64 expression in the MLR IL-4 culture. The likely explanation could be that IL-4 mediated STAT6 phosphorylation which inhibits activation of a number of genes, including CD64 (Tugal et al., 2013). This finding highlights the importance of CD64 expression on monocytes/macrophages as an early marker in allogeneic reaction that indicates the predominant effects of IFN-γ cytokine. Notably, the data showed that the phenotype of monocytes/macrophages in MLR-IL-4 culture could be defined as M2, which is different from the phenotype in MLR and MLR IFN-γ cultures.

The allogeneic culture (MLR) significantly enhanced C3aR expression on $CD14^{+}$ monocytes. Unexpectedly, both cytokines, IFN-γ and IL-4, appear to negatively control C3aR expression on monocytes, and that could lead to a defect in T cells

priming. Notably, CD64 and C3aR are co-expressed on the surface of monocytes to mediate inflammatory response (Mommert et al., 2004). Therefore, it appears that an increased level of expression of CD64 on monocytes is controlled by the administration of IFN-γ to the cell culture. Conversely, the high concentration of IFN-γ negatively controls C3aR expression. Furthermore, administration of the IL-4 cytokine to the MLR culture significantly down-regulated the expression of C3aR on $CD14^+$ monocytes as monocytes in MLR IL-4 showed some anti-inflammatory response. It has been reported that reducing C3aR could have beneficial effects and may down regulate the inflammatory response. This is supported by the previous report that showed targeting C3aR on mice DCs lowers their Ag presentation capacity and decreases IFN-γ production (Nguyen et al., 2015). More importantly, C3aR deficiency on APCs reduces T cell priming and delays the onset of aGVHD (Nguyen et al., 2015). The data in this study showed that monocyte CD11b expression was not involved in the inflammatory response induced by allogeneic cells in MLR, or allogeneic cells treated with the IFN-γ cytokine in MLR IFN-γ culture. Although additional experiments may be needed to confirm this observation and to determine mechanisms responsible for CD11b expression on monocytes. However, this study indicates that CD11b was controlled by IL-4 signalling. These data support previous studies reporting that IL-4 induces CD11b expression in the cell line with characteristics of alternative activated M2 macrophages (Deszo et al., 2003), and in a mouse model (Zheng et al., 2015).

Furthermore, based on the preceding (D1 and D2) activation of monocytes/macrophages in the MLR culture, it appears that early functional response of the monocytes plays an important role in subsequent T cells alloresponse (on D3 of incubation), including both total $CD3^+$ T cells and helper $CD4^+$ T cells. Moreover, the data in this study has revealed that adding IFN-γ cytokine to the MLR culture caused modest effects on T cell activation. There were no synergistic effects when the IFN-γ cytokine was combined with MLR stimulus. Indeed, the significant increase in T cell activity in MLR IFN-γ culture can be attributed to the role of allogeneic cells but not IFN-γ. Although T cells express IFN-γ receptors on their surface (Boehm et al., 1997), the data in this study showed that IFN-γ did not activate T cells directly, which highlights the role of allogeneic cells and monocytes/macrophages in the priming of T cells.

Recently, it has been reported that IFN-γ induced apoptosis of activated T cells and reduced proliferation of activated allogeneic cells by up-regulation of PD-1 (Lee et al., 2017). Therefore, the findings in this study could be explained by IFN-γ induced apoptosis of T cells, which highlights the importance of analysis of the expression of PD-1 on T cells and PDL-1/PDL-2 on macrophages in the future work. Notably, IL-4 showed opposing effects on monocyte/macrophages and T cells, as IL-4 enhanced monocyte/macrophages capacity to co-stimulate and present antigens, and at the same time it appeared to limit T cell activation. This could be linked to differentiation into intermediate monocytes and the development of M2 macrophages, which were reported to show high Ag capacity and co-stimulation potential, but generate weak T cells activation (Lee et al., 2017 and Junker et al., 2020). Alternatively, it is feasible that monocyte activated by IL-4 release IL-10, and this cytokine could inhibit effector T cells and induce regulatory T cells. This highlights the importance of considering measurements of IL-10 concentrations and T reg analysis in the future work. It has been reported that one of the strategies to control GVHD in early phase is reducing T cells priming by administration of IL-4 (Markey et al., 2014 and Via et al., 2017). However, it is difficult to ensure the therapeutic potential of IL-4, as it strongly activates monocytes/macrophages. Indeed, the finding in this study could explain the recent report of the limited benefits of using IL-4 to control aGVHD, and subsequent development of cGVHD (Via et al., 2017).

Overall, the data in this study demonstrate the plasticity of the monocyte responses to the presence of allogeneic cells and to the addition of specific cytokines. In fact, these findings strongly argue against the oversimplified categorisation of monocytes into pro- and anti-inflammatory subtypes. Instead, based on these novel data, it could be proposed that monocyte function and phenotype is determined by the presence of the dominant cytokine. Thus, two subtypes are proposed in this study, IFN-γ and IL-4 driven monocytes. Notably, these two monocyte subtypes could differentiate into monocyte-derived macrophages and their phenotypes are clearly distinct from previously defined categories. Importantly, these novel, cytokine driven monocyte subtypes were defined using a whole blood culture and early phase of alloresponse. This ensures minimal alteration of monocytes and maintains conditions that could closely mimic the physiological setting.

The second part of this study investigated the effects of the SYK inhibitor, PRT0603, on controlling monocytes/macrophage's function, cytokine secretion and T cells activation in MLR, MLR IFN-γ and MLR IL-4 cultures. SYK is a protein tyrosine kinase expressed on B cells, mast cells, monocytes, and macrophages. It controls outside/in signal transduction, resulting in distinct cellular responses (Leonhardt et al., 2012, Yi et al., 2014, Coffey et al., 2017, Kato et al., 2017). It is well established that monocytes/macrophages functions, including phagocytosis, Ag presentation, oxidative burst and cytokines secretion are controlled by SYK activation. Thus, SYK inhibition suppresses the monocytes/macrophages inflammatory response (Yi et al., 2014, Coffey et al., 2017, Leonhardt et al., 2012). Importantly, SYK activation is involved in transmitting signalling from FcRs expressed on monocytes/macrophages, which controls crucial events in the development of GVHD (Leonhardt et al., 2012, Poe et al., 2018). Consequently, targeting SYK signalling by using an SYK inhibitor is an attractive approach to control GVHD pathology (Leonhardet et al., 2012, Poe et al., 2018). Notably, SYK inhibition appears to control T cells activation indirectly (Leonhardet et al., 2012, Coffey et al., 2017). The results in this study suggest that there was a significant increase in the levels of monocyte CD64 expression (high-affinity human IgG receptor, FcγRI) in allogeneic culture with and without IFN-γ, which could be linked to activation of SYK downstream signalling. An earlier study reported that ligation of FcγR on macrophages directly regulates phosphorylation signalling, even before the peak of phagocytosis (Kiefer et al., 1998). On the other hand, the MLR IL-4 culture showed a reduction of CD64 expression that may indicate antagonism with SYK pathway. However, intracellular staining was not performed in this study to confirm SYK activation status. This study used PRT0603 (a parent compound for PRT0626), which is a highly selective SYK inhibitor that showed complete suppression of BCR and FcRs signalling, resulting in reduced inflammation in rheumatoid arthritis patients (Coffey et al., 2013). This study evaluated the therapeutic potential of targeting the SYK pathway in monocytes/macrophages by using 2 µM PRT0603 and measuring expression of CD64 (FcγRI), CD86 and HLA-DR on $CD14^+$ monocytes/macrophages.

The data revealed that PRT0603 significantly reduced CD64 expression in MLR SYK culture. Furthermore, SYK inhibition resulted in a short term (observed on D1 of incubation) inhibition of HLA-DR expression. This could be explained by

PRT0603 mediated down regulation of CD64, as CD64 was reported to control inflammatory response by up-regulation of HLA-DR expression (Brandsma et al., 2018). However, inhibition of SYK pathway did not significantly control expression of the co-stimulatory molecule CD86 on $CD14^+$ monocytes. This could imply that the co-stimulatory function of monocytes/macrophages is regulated independently from SYK, however, this important observation needs to be confirmed by additional experiments and the precise mechanisms should be determined. Notably, the MLR IFN-γ culture treated with PRT0603 showed a modest, non-significant reduction in monocytes/macrophages functions, FcγRI expression, co-stimulation, and Ag presentation. Thus, it is feasible that the activation of monocytes/macrophages to support phagocytosis function is a component of the innate immune response induced by IFN-γ treatment, which is not affected by SYK inhibitor. Furthermore, the SYK inhibitor did not affect CD86, HLA-DR and CD64 expression on monocytes/macrophages in the MLR IL-4 culture. Thus, it appears that co-stimulation and Ag presentation capacity of monocytes/macrophages in the presence of allogeneic cells and IL-4 are not regulated by the SYK pathway. Alternatively, IL-4 treatment reduces CD64 expression to such an extent that any further inhibition by SYK inhibitor is non-significant. The inhibitory effect of PRT0603 in various experimental conditions was compared by calculating the percent of inhibition for each marker on monocytes/macrophages (Figure 5.9). Overall, SYK inhibition resulted in reduced monocytes marker expression in unmodified MLR, however, both cytokines (IFN-γ and IL-4) reduced the potency of this inhibitor. Thus, SYK treatment showed less inhibitory effect on monocytes/macrophages when compared to unmodified MLR. This is an important observation suggesting a limited efficacy of this SYK inhibitor in controlling monocytes/macrophages activation in inflammatory condition, which are likely to be present in aGVHD.

Notably, PRT0603 treatment resulted in reduced activation of $CD3^+$ T cells in MLR SYK. This could be linked to the down-regulation of Ag presentation capacity provided by monocytes/macrophages, followed by reduction in cytokine concentration. Therefore, T cells alloreactivity appears to be affected indirectly by SYK inhibitor treatment. That agrees with the hypothesis in this study that controlling early monocytes/macrophages response could regulate T cells alloresponse. Whereas in MLR IFN-γ cultures PRT0603 treatment reduced $CD3^+$

T cells without significant changes in monocytes/macrophages activation. Therefore, T cells alloreactivity appears to be affected by the reduction of cytokine concentration. The data in this study indicate that the SYK inhibitor does not significantly affect T cells activation levels in the MLR IL-4 culture. However, as administration of IL-4 to the allogeneic culture has previously shown a reduction in T cells activation, it is feasible that the SYK inhibitor has a limited scope for effects in a condition with a minimal level of T cell activity. This observation was confirmed by calculating the relative inhibitory effect of PRT0603 and comparing the percent of inhibition of T cells in unmodified MLR, MLR IFN-γ and MLR IL-4 (Figure 5.10. A). The presence of IL-4 cytokine in the MLR culture (IL-4 MLR) showed low potency of the SYK inhibitor to prevent activation of $CD3^+$ T cells. That could be due to minimal T cell activities in MLR IL-4. Notably, the reductions of $CD3^+$ $CD25^+$ T cells were more pronounced after SYK inhibitor treatment in both unmodified MLR and MLR IFN-γ. This highlights the efficacy of controlling T cell activation by SYK inhibitor treatment. Furthermore, the results in this study indicate that the SYK inhibitor predominantly controls cytokine secretion in all experimental conditions, MLR, IFN-γ MLR and IL-4 MLR. That probably affected indirectly T cells early activation. Nevertheless, the SYK inhibitor has shown therapeutic potential in a new model of allogeneic response by targeting cytokine production to control inflammation (Coffey et al., 2013). Although SYK treatment did not control monocytes/macrophages functions, it resulted in the reduction of cytokine production, which could be considered as an important tool in controlling inflammation in the alloresponse in an *in vitro* model that could mirrors allogeneic reaction.

To verify this, the precent of cytokine reduction was calculated (Figure 5.10.B). Interestingly, the SYK inhibitor mediated reduction of cytokines was impressive in all investigated conditions, the unmodified MLR, the MLR IFN-γ and the MLR IL-4 culture. This points out that the SYK inhibitor therapy could show robust effects in challenging, inflammatory environment with a presence of large quantities of cytokines.

In conclusion, this study suggests that monocytes/macrophages activation in the early phase of allogeneic response is followed by priming of T cells. The difference between IFN-γ and IL-4 effects on monocytes/macrophages function in the early allogeneic culture appears to originate from their different stimulation

mechanisms: IFN-γ has enhanced phagocytosis function whereas, IL-4 cytokine antagonised this effect and up-regulated the co-stimulation and the Ag presentation on monocytes/macrophages during the early allogeneic culture. This study has shown that blocking of the SYK pathway results in the reduction of monocyte CD64 and HLA-DR expression in the unmodified MLR culture, as well as a profound decrease in the production of inflammatory cytokines. This led to a reduction in T cells activation. PRT0603 SYK inhibitor showed therapeutic potential in reducing cytokine concentrations that lead to T cell inhibition in MLR IFN-γ. When MLR culture is stimulated with IL-4 the SYK inhibitor reduced cytokine concentrations. Thus, PRT0603 could be therapeutic option in controlling the cytokine driven inflammatory response in allogeneic culture and presumably in GVHD as well. That may contribute to successful control of the GVHD pathology.

7 Limitations and future work:

7.1 Limitations:

There are a number of limitations of this study, firstly due to the relatively small number of repeats for each experiment analysing monocytes/macrophages and T cells surface markers expression and cytokine concentrations, it is necessary to interpret the data with caution. Indeed, a small number of repeats could be the reason for loss of statistical significance in some experiments, particularly when this is combined with a large variation in measured values. Furthermore, the data which show significance with this low number of repeats may benefit from increased power of statistical analysis that is associated with a larger number of experiments. Notably, this was due to the pandemics and lockdown, which restricted the time available for experimental work. Furthermore, some of the blood samples received from the NHS were rejected due to their collection time, as only samples delivered within 24 hours showed acceptable level of monocytes viability.

Flow cytometry instrument that was used in this study was CyAn ADP flow cytometer (Beckman Coulter, USA), which has two excitation lasers. Consequently, this instrument allows the analysis of the limited number of markers in a single sample. Conversely, an instrument with 3 or more lasers, such as LSR Fortessa, would allow in depth analysis of the cell phenotype with a large number of markers analysed simultaneously. For example, $CD14^{+}$ monocytes could be analysed for the expression of CD16, CD64, CD86 and HLA-DR markers, and at the same time apoptotic cells could be excluded using Annexin, and the responder population could be distinguished from allogeneic stimulators by using CFSE staining. This approach could be used in the future work by introducing a more powerful flow cytometer instrument.

This study uses high, non-physiological cytokine concentrations, and MHC mismatched blood samples in MLR cell cultures, which are not reflecting the conditions present in GVHD patients. However, in vitro models can rarely mimic in vivo environment. Indeed, MHC matched blood samples that only differ in minor histocompatibility antigens cannot be used for MLR cultures and analysis of monocytes, as the immune response develops slowly and would require

incubation periods of 3-4 weeks (Delaney et al., 2015). Furthermore, this study has used extensive optimisation of the cytokine concentrations to activate monocytes in MHC mismatched MLR model. The preliminary results have indicated that high concentration of cytokine is required to activate monocyte cultured *in vitro* in whole blood MLR model. Notably, similar concentrations of IL-4 and IFN-γ were used by other study (Delneste, et al., 2003) or even 5 times more than the levels of cytokine used in this study by Bayik et al (2017). Thus, in order to differentiate human monocytes *in vitro*, a high concentration of cytokine appears to be needed. Importantly, high cytokine concentrations did not affect monocytes or T cells viability, and they showed strong allogeneic responses, similar to that reported in other studies (Delneste et al., 2003, Bayik et al., 2017).

Furthermore, a limited number of surface markers were evaluated to characterise monocytes/macrophage's phenotype and function. Additional markers would benefit the study and should be considered in the future work. The expression of CD80 was measures in preliminary experiments, however, no difference in the levels were observed between MLR culture and Responder cell control (data not shown). Nevertheless, the analysis of CD80 expression in other conditions such as IFN-γ MLR and IL-4 MLR cultures should be conducted in the future. As this study has only evaluated T cells activation in MLR cultures, it would be valuable to determine T cells proliferation in the future work. Furthermore, there was a lack of cytokine intracellular staining to specify the source of cytokines and measurements of additional cytokines such as IL-5. IL-7, IL-12 and IL-17 could help in a more comprehensive evaluation of immune response in MLR model. Notably, IL-1β was measured, but the data were below detection limits. Thus, the data for IL-1β were not presented in this study.

7.2 Future work:

In the future work, analysing the samples with BD LSR Fortessa flow cytometry instrument should be considered, as this will allow multicolour panel design and analysis of T cells and monocytes within the same sample. Thus, a single experimental run will provide detail characterisation of the phenotypes of the key cell populations and subpopulations. For example, $CD14^+$ monocytes could be analysed for $CD1c^+$ expression to exclude DCs, and monocytes subtypes could be determined based on the expression of CD16, CCR2 and CX3CR1 markers. The multi-colour panel also allows analysis of T cells populations, including Tregs that could be affected by the presence of IL-4 in MLR culture. Thus, Tregs could be analysed in MLR IL-4 and compared to unmodified MLR culture. Furthermore, the effects of SYK inhibitor on Tregs could be evaluated by analysing MLR SYK and MLR IL-4 SYK cultures. It could be valuable to analyse the expression of program death 1 (PD-1) marker on T cells and program death ligand 1 (PDL-1) on monocytes/macrophages, as these molecules are involved in immune tolerance. Thus, the future work could include investigation of the role of PD-1/PDL-1 interaction in the regulation of early allogeneic culture in unmodified MLR and in the presence of IFN-γ, IL-4 and SYK inhibitor.

The use of intracellular analysis with a mixed panel can be used to evaluate monocytes and, T cells cytokines and specify the cells that secrete TNF-α, IFN-γ, IL-2, and IL-6. That will help to conclude which cells are affected by SYK treatment and which reduced the proinflammatory cytokine concentrations. Further cytokine analysis will consider measuring the concentration of IL-12 and IL-17, as both are essential for T cell formation. Measuring the concentration of the anti-inflammatory cytokines IL-10 and IL-4 will evaluate if the anti-inflammatory reaction is enhanced after SYK treatment.

Furthermore, T cells proliferation could be assessed using carboxy fluorescein diacetate succinimidyl ester (CFSE) staining. Thus, T cells proliferation could be determined in unmodified MLR, and MLR culture in the presence of cytokines, IFN-γ and IL-4. The efficacy of SYK inhibition to control T cells proliferation could be also investigated.

The comparison of the highly selective SYK inhibitor, PRT0603, and the less selective first generation SYK inhibitor Fostamatinib could reveal the importance of the “off-target” effects in controlling allogeneic response. Indeed, it has been recently reported that in addition to SYK, Fostamatinib inhibited 117 kinases, 100 of which had half-maximal inhibitory concentration (IC50) values within 3-fold of the IC50 value for SYK (Zarrinet al.,2021). The off targets for Fostamatinib include FLT1, vascular endothelial growth factor receptor 2 (VEGFR2), SRC, FLT3, LYN and LCK. Consequently, it is feasible that Fostamatinib could show more potent effects than PRT0603 in MLR model, however, this would also indicate potential risk of side effects associated with the functional roles of off-target pathways. Conversely, the therapeutic potential of PRT0603 could be similar to that of Fostamatinib, suggesting that SYK pathway plays a prominent role in allogeneic response, and that the use of the second generation, selective SYK inhibitors could represent a promising treatment option. However, the data cannot be confirmed *in vitro* as this model focused on cellular interaction, and this model did not measure the level of toxicity for each treatment. Thus, further study *in vivo* is required to confirm the efficacy of using PRT0603 and Fostamatinib to control the early allogeneic reaction.

8 Appendix:

8.1 T cells gating strategies:

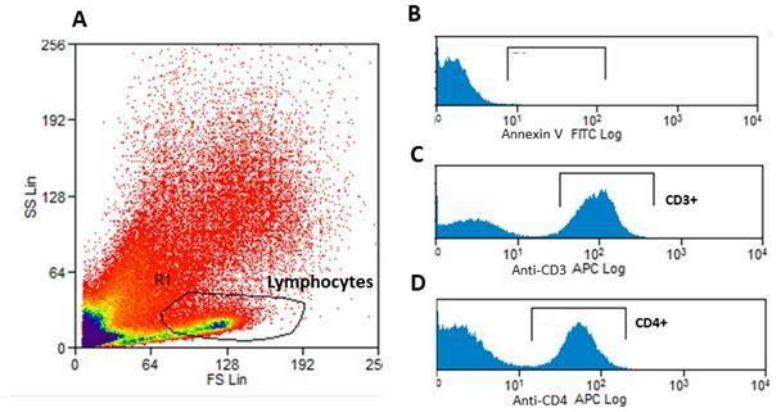

Figure S1. 1: Analysis of lymphocytes viability, CD3 $^{+}$ and CD4^{+} expression.

Day 3 of incubation **A)** Lymphocytes population is gated on (FSC/SSC) in region R1. **B)** The histogram shows Annexin V negative (viable cells) and Annexin V positive, non-viable cells. **C)** The histogram shows CD3^{-} and CD3^{+} cells (65% of cells) populations. **D)** The histogram shows CD4^{-} and CD4^{+} cells (55% of cells) populations.

8.2 Analysis of CD3 T cells activity (in MLR, MLR SYK, Res and Res SYK):

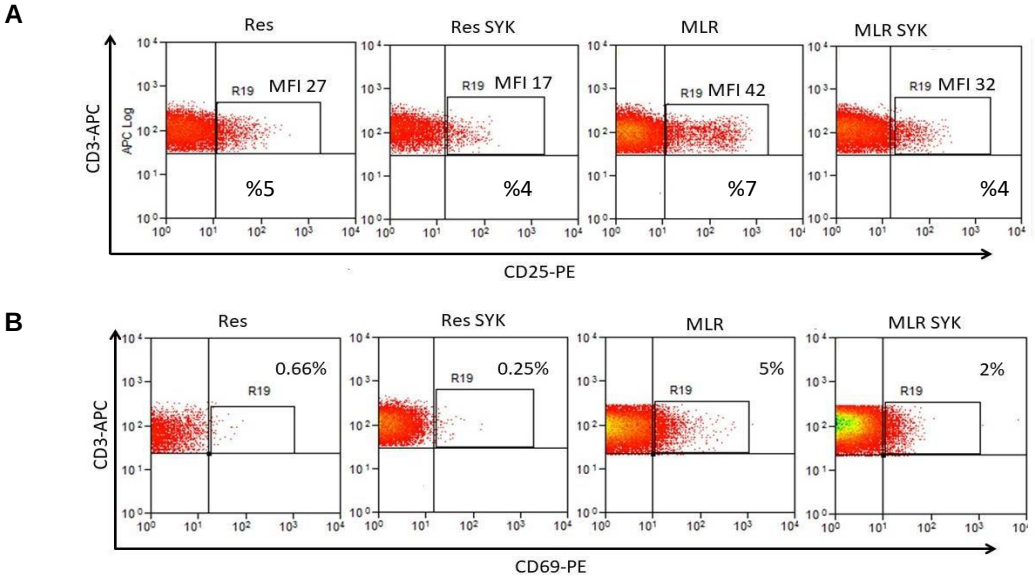

Figure S1. 2: Analysis of CD3 T cells activation by flow cytometer on D3 of incubation.

The dot plots comparing 4 conditions: responder cells as negative allogeneic control (Res), responder cells with 2µM PRT0603 negative control treated with inhibitor to check SYK toxicity (Res SYK), mixed leukocyte reaction (MLR) as +ve allogeneic control and mixed leukocyte reaction

treated with 2µM PRT0603 the experimental conditions (MLR SYK) **A**) analysis of MFI values of $CD3^{+}CD25^{+}$, X axis represents CD25-PE, Y axis presents CD3-APC **B)** analysis % of $CD3^{+}CD69^{+}$, X axis represents CD69-PE, Y axis presents CD3-APC.

8.3 Analysing the expression of CD86 on $CD14^{+}$ monocyte/macrophages (in MLR, MLR SYK, Res and Res SYK):

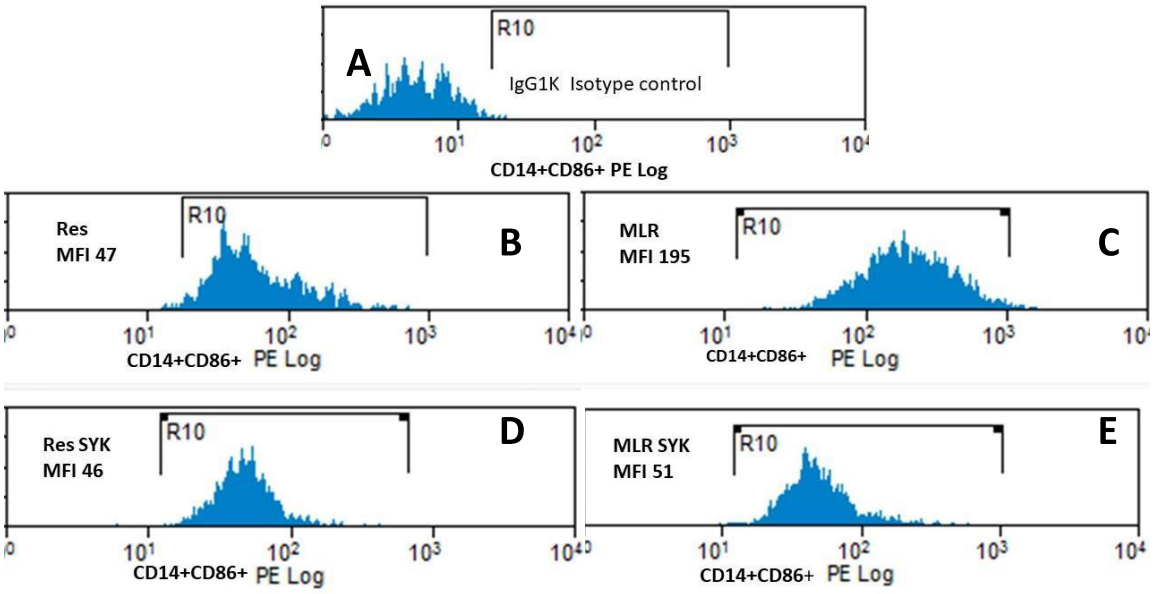

Figure S1. 3: Flow cytometry histograms that present expression of $CD86^{+}$ on $CD14^{+}$ in four experimental conditions on D2 of cell culture.

A) Isotype control, **B)** Res = responder cells as a negative allogeneic control, **C)** MLR = mixed leukocyte reaction as a positive allogeneic control, **D)** Res SYK = responder cells treated with 2 µM PRT0603, **E)** MLR SYK = mixed leukocyte reaction treated with 2 µM PRT0603 γ, the histograms shifted to the right when the MFI values for CD86 expression increased.

8.4 Analysing the expression of CD64 on CD14^{+} monocyte/macrophages (in MLR, MLR IFN-γ, Res and Res IFN-γ):

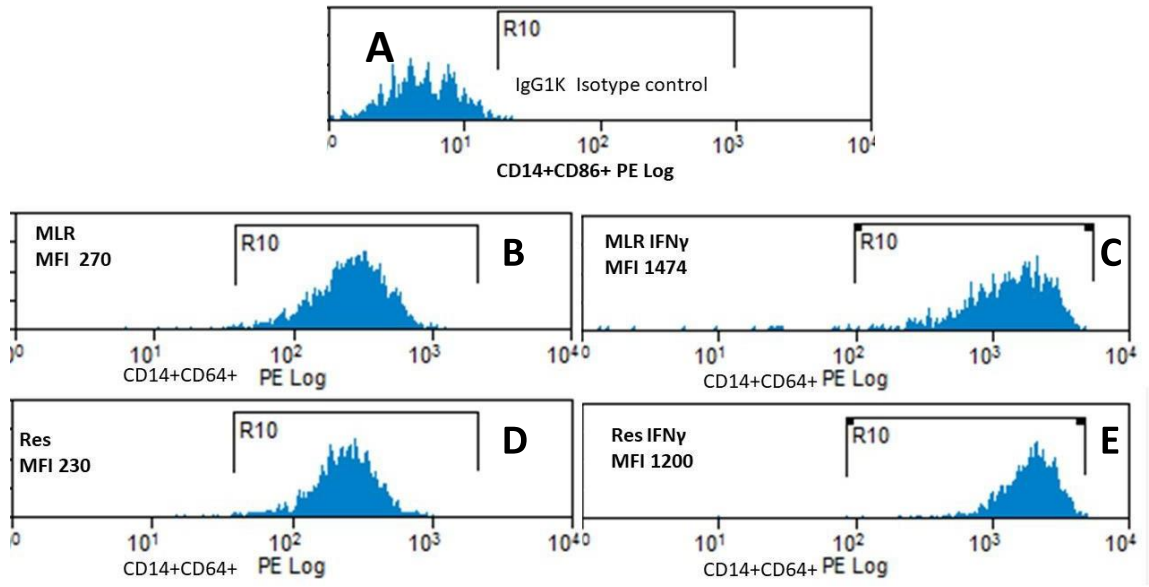

Figure S1. 4: Flow cytometry histograms that present expression of CD64^{+} on CD14^{+} in four experimental conditions on D2 of cell culture.

A) Isotype control, **B)** MLR = mixed leukocyte reaction as a positive allogeneic control, **C)** MLR IFN-γ = mixed leukocyte reaction treated with 100 ng/ml IFN-γ, **D)** Res = responder cells as a negative allogeneic control, **E)** Res IFN-γ = responder cells treated with 100 ng/ml IFN-γ. the histograms shifted to the right when the MFI values for CD64 expression increased.

8.5 CD4^{+}CD25^{+} T cells activation analysis before and after SYK treatment (MLR IFN-γ, Res IFN-γ, MLR IFN-γ SYK and Res IFN-γ SYK):

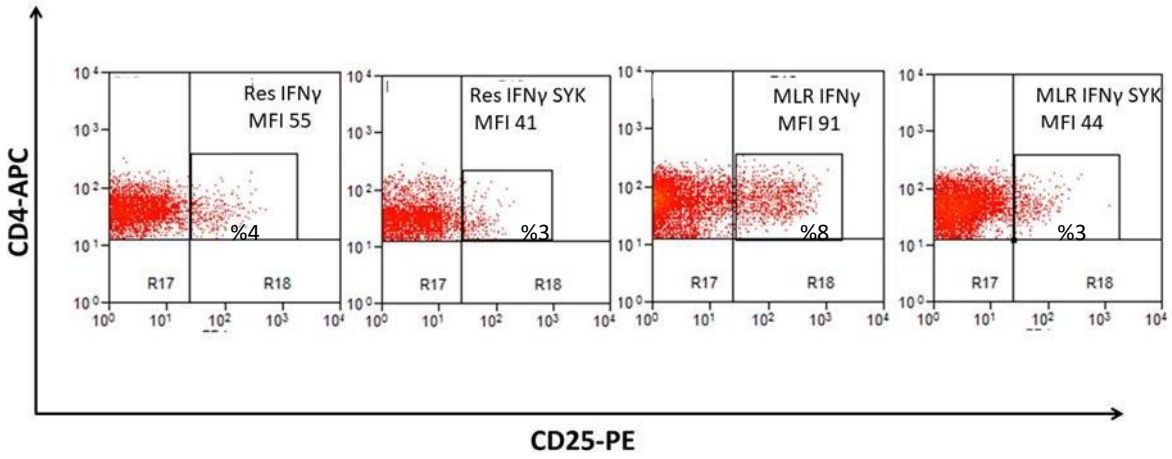

Figure S1. 5: Analysis of CD4 T cells activation by flow cytometer on D3 of incubation.

The dot plots comparing 4 conditions: Res IFN-γ (responder cells as a negative control treated with 100 ng/ml IFN-γ), Res IFN-γ SYK (control for the effect of IFN-γ and PRT0603 SYK inhibitor), MLR IFN-γ (allogeneic control treated with IFN-γ), MLR IFN-γ SYK (mixed leukocyte reaction treated with IFN-γ and PRT0603, the experimental conditions). To determine MFI values for CD25

expression on CD4⁺ helper T cells, X axis presents CD25 -PE and Y axis presents CD4-APC.

8.6 Analysing the expression of CD32 on CD14⁺ monocyte/macrophages (in MLR, MLR IL-4, Res and Res IL-4):

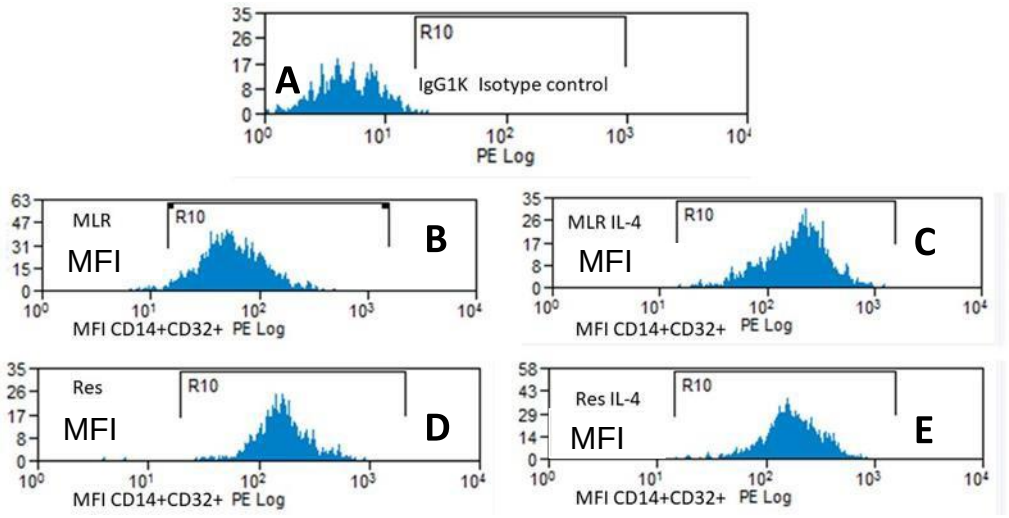

Figure S1. 6: Flow cytometer histograms presenting expression of CD14⁺CD32⁺ in four conditions on D1of incubation.

A) Isotype control **B)** MLR = mixed leukocyte reaction, as allogeneic control **C)** MLR IL-4 = mixed leukocyte reaction treated with 100 ng/ml IL-4 **D)** Res = responder cells as a negative control **E)** Res IL-4 = responder cells as treated with 100 ng/ml IL-4. The histogram shifted to the right due to the increase in MFI values for CD32 expression.

8.7 CD3⁺CD69⁺ T cells activation analysis before and after SYK treatment (MLR IL-4, Res IL-4, MLR IL-4 SYK and Res IL-4 SYK):

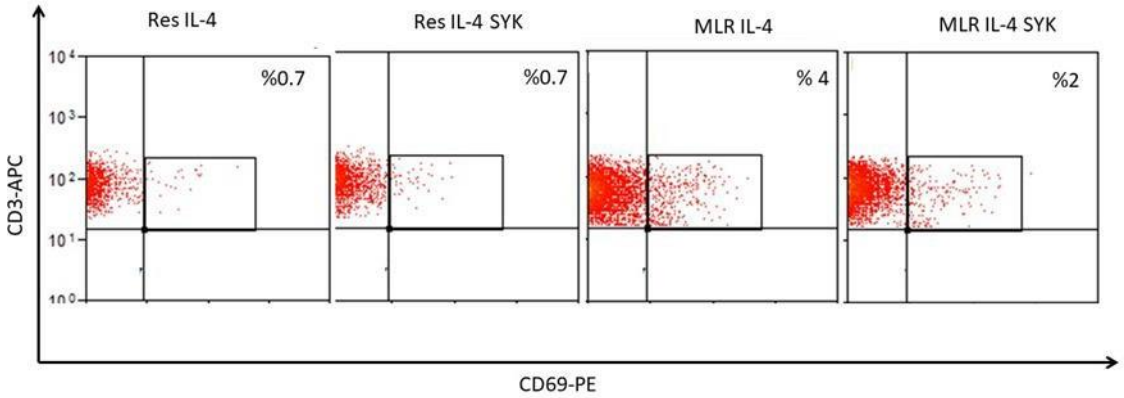

Figure S1. 7: Analysis of CD3⁺ T cells activation by flow cytometer on D3 of incubation.

The dot plot graphs compare 4 conditions: Res IL-4 = responder cells as control treated with 100 ng/ml IL-4, Res IL-4 SYK = responder cells treated with 100 ng/ml IL-4 and 2 µM PRT0603, MLRIL-4 = mixed leukocyte reaction treated with 100 ng/ml IL-4, MLR IL-4 SYK = experimental condition, mixed leukocyte reaction treated with 100 ng/ml IL-4 and 2 µM PRT0603. To evaluate % of CD3⁺CD69⁺ cells, X axis represents CD69-PE and Y axis presents CD3-APC.

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