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The Role of Purinergic Signalling in Inflammatory Disorders

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University of Wollongong

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UNIVERSITY
OF WOLLONGONG
AUSTRALIA

Faculty of Science, Medicine and Health
School of Biological Sciences

The Role of Purinergic Signalling in Inflammatory Disorders

Nicholas John Geraghty

B. Med. Biotech. (Hons)

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Final Thesis

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Abstract

The purinergic signalling system comprises extracellular nucleotides such as adenosine triphosphate (ATP), which signals through the two P2 receptor subfamilies; P2X (P2X1-7) and P2Y (P2Y_{1, 2, 4, 6, 11, 12, 13, and 14}). Breakdown of ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP), by the ecto-nucleotidase ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39), and finally AMP to adenosine, by ecto-5'-nucleotidase (CD73) allows activation of adenosine receptors (A₁, A_{2A}, A_{2B} and A₃). Purinergic signalling is important in inflammation and immunity, and has been implicated in transplantation, including hematopoietic stem cell transplantation (HSCT) and a major complication of allogeneic HSCT; graft-versus-host disease (GVHD). In allogeneic mouse models of GVHD, activation of P2X7, or blockade of CD73 or A_{2A}, worsen disease, while A_{2A} activation reduces GVHD. Purinergic signalling is also implicated in skin disorders including psoriasis. Prior to this thesis, the role of purinergic signalling in humanised models of GVHD and the imiquimod (IMQ)-induced psoriasis-like inflammatory mouse model remained unexplored. The overarching aim of this thesis was to establish a humanised non obese diabetic (NOD)-severe combined immunodeficient (SCID)-IL-2 receptor γ common chain null (IL-2R γ^{null}) (NSG) mouse model of GVHD in our laboratory and utilise this model to investigate the role of purinergic signalling in this disease. Additionally, this thesis aimed to establish the IMQ-induced psoriasis-like inflammatory mouse model in our laboratory and to investigate the role of P2X7 in this model.

Chapter 2 details the establishment of the humanised NSG mouse model of GVHD, where NSG mice injected with human (h) peripheral blood mononuclear cells (PBMCs), subsequently develop GVHD. During this study, 10 mice developed clinical GVHD and three mice developed subclinical GVHD only. A comparison of these mice highlighted that clinical GVHD correlated with increased splenic hCD4⁺:hCD8⁺ T cell ratios, serum human interferon

(IFN)- γ concentrations and intestinal interleukin (IL)-17 expression. Comparison of healthy mice with those with subclinical or clinical GVHD allowed characterisation of histological GVHD. This revealed leukocyte infiltration and histological damage in the liver, small intestine and skin, target organs of GVHD.

Chapters 3 and 4 investigated the role of P2X7 in the humanised NSG mouse model of GVHD. A short-term regime of P2X7 blockade using Brilliant Blue G (BBG) (Chapter 3) did not impact survival, but reduced serum hIFN- γ concentrations and leukocyte infiltration and damage to the liver, small intestine and skin. A long-term regime of BBG (Chapter 4) did not impact serum hIFN- γ concentrations but reduced leukocyte infiltration and apoptosis to the livers of humanised NSG mice. Therefore, activation of P2X7 is an important signalling pathway involved in GVHD development in this model.

Chapters 5 and 6 investigated the role of the CD39/CD73/A_{2A} signalling axis in the humanised NSG mouse model of GVHD. CD39 and CD73 blockade using $\alpha\beta$ -methylene-ADP (APCP) (Chapter 5) worsened disease through increased weight loss, leukocyte infiltration and damage to livers, and serum hIL-2 concentrations. However unlike allogeneic mouse models, adenosine receptor blockade with the broad-spectrum antagonist caffeine had no impact on disease. This suggests the accumulation of ATP rather than prevention of adenosine production is promoting GVHD. Conversely, A_{2A} activation using CGS 21680 (Chapter 6) had differing effects on disease development. CGS 21680 caused beneficial effects through reduced leukocyte infiltration and damage in livers, and serum human tumour necrosis factor alpha (hTNF- α) concentrations. CGS 21680 also caused detrimental effects through reduced weight gain and regulatory T cell frequencies, and increased serum hIL-6 concentrations.

Finally, Chapter 7 investigated the role of P2X7 in IMQ-induced psoriasis-like inflammation. IMQ induced ATP release from keratinocytes *in vitro* and up-regulated P2X7 expression in the skin of these mice. However, pharmacological blockade of P2X7 with the antagonists BBG or A804598, or P2X7 deficiency, did not impact IMQ-induced psoriasis-like inflammation.

In conclusion, this thesis demonstrated for the first time that purinergic signalling pathways, predominantly the accumulation of ATP and subsequent activation of P2X7, is important in disease development in the humanised NSG mouse model of GVHD. Additionally, P2X7 is up-regulated in involved skin, but is not essential for the development of IMQ-induced psoriasis-like inflammation.

Statement Indicating Thesis Style

In accordance with the University of Wollongong “Guidelines for preparation and submission of thesis” (2017) and “Higher Degree Research (HDR) Thesis by Compilation Rules” (2017), this PhD is presented in ‘Journal Article Pre-print Compilation Style’. This thesis therefore comprises of a series of articles published in the following journals;

Transplant Immunology (Chapter 2), *Clinical and Experimental Immunology* (Chapter 3), *Cellular Immunology* (Chapter 4), *Immunology and Cell Biology* (Chapter 5), *International Immunopharmacology* (Chapter 6) and *Purinergic Signalling* (Chapter 7).

Chapters are composed of pre-print versions of the articles except for the following changes:

- All language was updated to English (Australian).
- Some abbreviations were altered for uniformity and clarity.
- All references were updated to reflect Harvard style, and listed in one bibliography at the end of this thesis.

I am the first author of all publications. I hereby declare that I was involved in the conceptualisation of these studies, I performed the experiments and data analysis except where indicated, and I prepared and authored the manuscripts.



Nicholas John Geraghty
2018

I consent to the presentation of the PhD in ‘Journal Article Pre-print Compilation Style’ and as Primary Supervisor I acknowledge the above statement pertaining to student contribution to be correct.



Associate Professor Ronald Sluyter
2018

Publications Comprising This Thesis

Published Articles

Geraghty, N. J., Adhikary, S. R., Watson, D. and Sluyter, R. (2019) The A2A receptor agonist CGS 21680 has beneficial and adverse effects on disease development in a humanised mouse model of graft-versus-host disease. *International Immunopharmacology*, 72: 479-486. doi: 10.1016/j.intimp.2019.04.037 .

Geraghty, N. J., Watson, D. and Sluyter, R. (2019) Pharmacological blockade of the CD39/CD73 pathway but not adenosine receptors augments disease in a humanised mouse model of graft-versus-host disease. *Immunology and Cell Biology*, Available Online 7th April, doi: 10.1111/imcb.12251 .

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Publications in Addition to This Thesis

Published Articles

Adhikary, S. R., **Geraghty, N.J.**, Cuthbertson, P., Sluyter, R. and Watson, D. (2019), Altered donor P2X7 activity in human leukocytes correlates with *P2RX7* genotype but does not affect the development of graft-versus-host disease in humanised mice, *Purinergic Signalling*, Available Online 18th April. doi: 10.1007/s11302-019-09651-8.

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Presentations Arising from This Thesis

Oral Presentations

Geraghty, N. J., Adhikary, S. R., Watson, D., Sluyter, R. (2019) Long-term P2X7 blockade reduces liver graft-versus-host disease in humanised mice, Transplantation Society of Australia and New Zealand, Sydney, NSW, Australia

Geraghty, N. J., Adhikary, S. R., Watson, D., Sluyter, R. (2018) The role of the CD73/A2A signalling axis in a humanised mouse model of graft-versus-host disease, Transplantation Society of Australia and New Zealand, Melbourne, Victoria, Australia (*Transplantation Direct*, 4(8S):S1–S50, doi: 10.1097/TXD.0000000000000806)

Geraghty, N. J., Adhikary, S. R., Watson, D., Sluyter, R. (2017) The role of the CD73/A2A signalling axis in a humanised mouse model of graft-versus-host disease: an update, University of Wollongong Post-Graduate Conference, Kioloa, NSW, Australia

Geraghty, N. J. (2017) The humanised mouse model of GVHD, University of Wollongong Animal Ethics Committee, Wollongong, NSW, Australia, (**Invited**)

Geraghty, N. J., Adhikary, S. R., Watson, D., Sluyter, R. (2017) The role of the CD73/A2A signalling axis in a humanised mouse model of graft-versus-host disease: an update, Australasian Society of Immunology, NSW/ACT Branch Meeting, Bowral, NSW, Australia

Geraghty, N. J., Adhikary, S. R., Watson, D., Sluyter, R. (2016) The role of the CD73/A2A signalling axis in a humanised mouse model of graft-versus-host disease, Australasian Society of Immunology, NSW/ACT Branch Meeting, Wollongong, NSW, Australia

Geraghty, N. J., Watson, D., Sluyter, R. (2016) The role of purinergic signalling in inflammatory disorders: an update, University of Wollongong Post-Graduate Conference, Kioloa, NSW, Australia

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Geraghty, N. J., Watson, D., Fuller, S., Sluyter, V., Sluyter, R. (2015) The P2X7 receptor is not essential for imiquimod-induced psoriasis-like inflammation in mice, Australasian Society of Immunology, NSW/ACT Branch Meeting, Bowral, NSW, Australia

Geraghty, N. J., Watson, D., Fuller, S., Sluyter, V., Sluyter, R. (2015) The role of P2X7 in psoriasis, Immunology Group Seminar Series, The Children's Hospital Westmead, Westmead, NSW, August 2015, (**Invited Seminar**)

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Poster Presentations

Geraghty, N. J., Adhikary, S. R., Sluyter, R., Watson, D. (2018) The CD73/A2A signalling axis impacts graft-versus-host disease in a humanised mouse model, The European Congress of Immunology, Amsterdam, Netherlands

Geraghty, N. J., Adhikary, S. R., Watson, D., Sluyter, R. (2017) The purinergic CD73/A2A receptor signalling axis plays an important role in a humanised mouse model of graft-versus-host disease, Transplantation Society of Australia and New Zealand, Brisbane, Queensland, Australia

Geraghty, N. J., Watson, D., Fuller, S., Sluyter, V, Sluyter, R. (2016) The role of the P2X7 receptor in an imiquimod-induced psoriasis-like model, International Congress on Immunology, Melbourne, Victoria, Australia (*European Journal of Immunology*; 46 pp. 393, doi: 10.1002/eji.201670200)

Geraghty, N. J., Belfiore, L., Mullany, P., Alexander, S., Sluyter, R., & Watson, D. (2015) P2X7 blockade reduces interferon gamma in a humanised mouse model of graft-versus-host disease, The Transplantation Society International Scientific Meeting, Lorne, Victoria, Australia (*Transplantation*; 99 (11) pp. S5, doi: 10.1097/01.tp.0000473676.99840.00)

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List of Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APC	Antigen presenting cell
APCy	Allophycocyanin
ATP	Adenosine triphosphate
BBG	Brilliant blue G
BzATP	2',3'-(4-benzoyl)-benzoyl-adenosine triphosphate
cDNA	Complementary DNA
chr	Chromosome
CHS	Contact hypersensitivity
DAMP	Danger associated molecular pattern
DC	Dendritic cell
EC ₅₀	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
GVHD	Graft-versus-host disease
GVT	Graft-versus-tumour
h	Human
H&E	Hematoxylin and eosin
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase
HSCT	Haematopoietic stem cell transplantation
i.p.	Intra-peritoneal
i.v.	Intra-venous
IC ₅₀	Half maximal inhibitory concentration
IFN- γ	Interferon gamma
IL	Interleukin
IMQ	Imiquimod
iNKT	Invariant natural killer T
JAK	Janus associated kinase

KO	Knockout
LC	Langerhans Cell
m	Murine
mAb	Monoclonal antibody
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
miHAg	Minor histocompatibility antigen
MLR	Mixed lymphocyte reaction
mRNA	Messenger RNA
MST	Median survival time
NLRP3	NOD-like receptor family, pyrin domain containing-3 protein
NK	Natural killer
NOD	Non-obese diabetic
NSG	Non-obese diabetic severe combined immunodeficiency IL-2 receptor γ common chain null
oATP	Oxidised adenosine triphosphate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	R-Phycoerythrin
Per-CP-Cy5.5	Peridinin chlorophyll protein
PMA	Phorbol 12-myristate 13-acetate
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
qPCR	Quantitative real-time polymerase chain reaction
ROR γ t	RAR-related orphan receptor gamma
RT	Room temperature
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline containing Tween-20
Tc cell	Cytotoxic T cell
TCR	T cell receptor
TGF	Tumour growth factor
Th cell	Helper T cell
TNF	Tumour necrosis factor
Treg	Regulatory T cell

Contribution to Chapters

As noted on page i, this thesis and any corresponding manuscripts were written entirely, and the experiments within largely conducted, by Nicholas Geraghty. Relative contributions of others are outlined below and as declared on pages 77, 113, 135, 155, 175 and 198.

Chapter 1 consists of an unpublished literature review edited by the Primary Supervisor, Ronald Sluyter and the co-supervisor Debbie Watson.

Chapter 2 consists of a manuscript titled “Increased splenic human CD4⁺:CD8⁺ T cell ratios, serum human interferon- γ and intestinal human interleukin-17 are associated with clinical graft-versus-host disease in humanized mice” (*Transplant Immunology, Revisions requested*).

Author	Figure 1	Figure 2	Figure 3	Figure 4	Figure 5	Editing
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Chapter 3 consists of an original research article titled “The P2X7 receptor antagonist Brilliant Blue G reduces serum human interferon- γ in a humanized mouse model of graft-versus-host disease” (*Clinical and Experimental Immunology, 2017, 190:79-95*).

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Chapter 4 consists of a manuscript titled “Long-term treatment with the P2X7 receptor antagonist Brilliant Blue G reduces liver inflammation and damage in a humanized mouse model of graft-versus-host disease” (*Cellular Immunology, Available online 4 December 2018*).

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Chapter 5 consists of a manuscript titled “Pharmacological blockade of the CD39/CD73 pathway but not adenosine receptors augments disease in a humanised mouse model of graft-versus-host disease” (*Immunology and Cell Biology, Revisions requested*).

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Debbie Watson					33%
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Chapter 6 consists of a manuscript titled “Activation of the adenosine A_{2A} receptor with CGS21680 causes weight loss in a humanised mouse model of graft-versus-host disease”, (*International Immunopharmacology, Revisions requested*).

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Chapter 7 consists of a manuscript titled “The P2X7 receptor is not essential for development of imiquimod-induced psoriasis-like inflammation in mice” (*Purinergic Signalling, 2017, 13:405–415*).

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Debbie Watson							30%
Ronald Sluyter	15%				15%	10%	60%

Chapter 8 consists of an unpublished general discussion edited by Ronald Sluyter and Debbie Watson.

Chapter 1: Introduction

1.1. Inflammatory Diseases

The immune system provides host defence against disease, protecting an organism from infection and death. The immune system incorporates the innate immune system, responsible for rapid non-specific responses, and the adaptive immune system, responsible for slower but specific responses to pathogens. The immune system promotes inflammation to remove pathogens (Janeway Jr and Medzhitov, 2002). The adaptive immune cells; B and T cells, are important in developing memory to pathogens and clear them quickly after subsequent infection (Flajnik and Kasahara, 2009). However, an imbalance in this process or abnormal responses to normal healthy tissues can result in inflammatory diseases. In whole organ transplantation, donor organs can be rejected due to the body recognising foreign tissue (Starzl *et al.*, 1993). After allogeneic hematopoietic stem cell transplantation (HSCT), patients can develop graft-versus-host disease (GVHD). GVHD results from donor (graft) T cells mounting a 'normal' immune response upon detection of 'foreign' antigens, resulting in an immune response against an immunocompromised patient (host) (Billingham, 1965). Alternatively, inflammatory diseases can result from an exaggerated autoimmune response due to genetic or environmental factors, leading to damage to tissues such as the pancreas in diabetes (Donath and Shoelson, 2011), or the skin in psoriasis (Chandra *et al.*, 2015).

1.2. HSCT

HSCT involves the transfer of immunocompetent cells into an immunocompromised patient, and is a curative therapy for blood cancers including acute myeloid leukemia (Bortin and Rimm, 1978) and acute lymphoblastic leukemia (Buckner *et al.*, 1973). HSCT is also considered a curative therapy for other haematological disorders such as aplastic anaemia

(which was the first recorded case of bone marrow transplantation (Osgood *et al.*, 1939)), sickle cell anaemia (Vermylen *et al.*, 1988) and Wiskott-Aldrich syndrome (Ozsahin *et al.*, 2008). Currently there are >25,000 HSCTs performed annually worldwide (D'Souza and Fretham, 2017).

HSCT can be performed in one of two ways. Autologous HSCT involves the *in vivo* expansion of healthy cells, or the *ex vivo* expansion and transplantation of healthy host hematopoietic cells into the host after chemo- and/or radio-therapy (McGuire, 1998), leading to reconstitution of the host's immune system. However, due to the nature of some haematological malignancies, autologous HSCT is not possible, and instead these patients receive allogeneic HSCT. Allogeneic HSCT involves the transplantation of immunocompetent cells (graft) from a healthy individual (donor), to another immunocompromised recipient (host). Allogeneic HSCT reduces the risk of complications by matching donors for histocompatibility, either through using related donors or genetic screening (Sheldon and Poulton, 2006). In cancer patients, the aim of allogeneic HSCT is two-fold; to reconstitute a functional immune system, and to benefit from the graft-versus-tumour (GVT) effect, where donor cells recognise and destroy malignant host cells (Weiden *et al.*, 1979). Hereafter, HSCT will refer to allogeneic HSCT unless stated otherwise.

The reconstitution of the immune system provides the patient with protection against subsequent infection, while the recognition of 'foreign' host tissue forms the basis of the GVT effect (Kolb, 2008). However, immunocompetent cells in the graft can also recognise and mount an immune response against healthy 'foreign' host tissue resulting in GVHD. The current, and only, treatment for GVHD is immunosuppression through the use of steroids (Auletta and Cooke, 2009). However, immunosuppression to prevent GVHD can lead to other problems associated with HSCT. Suppressing the patient's immune system to a great

extent leaves the patient susceptible to subsequent infection or cancer relapse, due to immunosuppression leading to a reduced GVT effect (Fefer, 2004).

1.3. GVHD

GVHD remains a major problem following HSCT, with a morbidity rate of 50% and a mortality rate of 15-30% (Jagasia *et al.*, 2012, Storb *et al.*, 2013). Early GVHD research led Billingham (1965) to outline three criteria for GVHD to occur. Firstly, the graft must contain immunocompetent cells. Secondly, the host must be immunologically disparate from the donor. Finally, the host must be incapable of preventing the inflammatory response of transplanted immunocompetent cells against the genetically disparate host.

Due to difficulties in finding genetically similar donors, in HSCT some patients receive mismatched donor cells. In these cases, recognition of ‘foreign’ host tissues and development of GVHD is due to mismatches of the major histocompatibility complexes (MHC). MHCs are important cell surface receptors present on antigen presenting cells (APCs), where MHC class I molecules (MHC-I) interact with CD8 and MHC class II molecules (MHC-II) interact with CD4 on T cells (Mellman and Steinman, 2001). In humans, MHC is more commonly referred to as human leukocyte antigen (HLA). In humans there are three major (HLA-A, -B, and -C) and three minor (HLA-E, -F, and -G) classes of HLA on MHC-I, and three major (HLA-DP, -DQ, and -DR) and two minor (HLA-DM, and -DO) classes of HLA on MHC-II (Marsh *et al.*, 2002). However, even in HLA-matched HSCT, GVHD can still develop due to differences in minor histocompatibility antigens (miHAg). There are a large number of miHAg (for a recent review see Dzierzak-Mietla *et al.* (2012)). Most miHAg are present on the Y chromosome (chr) and as such sex-matching is also important in HSCT (Miklos *et al.*, 2005, Wang *et al.*, 2018).

As GVHD develops, it can manifest as one of two forms; acute or chronic. Originally acute GVHD was classified as occurring within 100 days, and chronic as persisting beyond 100 days, post-transplantation (Martin *et al.*, 1990, Sullivan *et al.*, 1991). However, subsequent research has demonstrated that acute and chronic GVHD have unique aetiologies, and acute GVHD can emerge after 100 days post-transplantation (Ferrara *et al.*, 2009). For a recent review of chronic GVHD see Zeiser and Blazar (2017b). From this point onwards GVHD will refer to acute GVHD.

GVHD can be characterised by a three step process (Ferrara *et al.*, 1999) (Figure 1.1.). The first stage occurs before the transplantation and is characterised by the inflammatory environment caused by the underlying condition or the treatment regime. This step involves release of pro-inflammatory cytokines including tumour necrosis factor (TNF)- α , interferon (IFN)- γ , and interleukin (IL)-6 (Knulst *et al.*, 1994), as well as other signalling molecules including damage associated molecular patterns (DAMPs) from damaged and dying cells (Wilhelm *et al.*, 2010). The second stage involves antigen processing of MHC or miHAg peptides by APCs, and subsequent presentation to T cells, leading to T cell activation. The release of cytokines due to initial damage results in up-regulation of MHC molecule expression, co-stimulatory molecules and adhesion molecules on APCs (Ferrara *et al.*, 1999). APCs interact with T cells through MHC and co-stimulatory molecules to identify “self”. In the final stage, T cells migrate to target organs and release additional cytokines to mediate inflammatory damage. In particular, CD4⁺ helper T (Th) cells can differentiate into different subtypes and release cytokines, such as Th1 cells release of IFN- γ and IL-2, Th2 cells release of IL-4 and IL-13 (Mosmann *et al.*, 1986), or Th17 cells release of IL-17 (Harrington *et al.*, 2005) to promote GVHD. CD4⁺ T cells subsequently recruit CD8⁺ T cells, which can produce cytokines as well as release perforin and granzyme to promote inflammation and exacerbate GVHD (Graubert *et al.*, 1997). The inflammation and damage caused by GVHD

affects one or more organs, with the most commonly affected organ being the skin, which is involved in 81% of cases, followed by the small intestine (54%) and the liver (50%) (Martin *et al.*, 1990). GVHD is a debilitating disease that manifests according to the involved target organs. Skin involvement can manifest as a painful and/or itchy rash that can cover a majority of a patient's body. When the liver is affected the patient will demonstrate jaundice (a yellowing of the skin and eyes), while involvement of the gastrointestinal tract can lead to abdominal pain, nausea and vomiting (Zeiser and Blazar, 2017a).

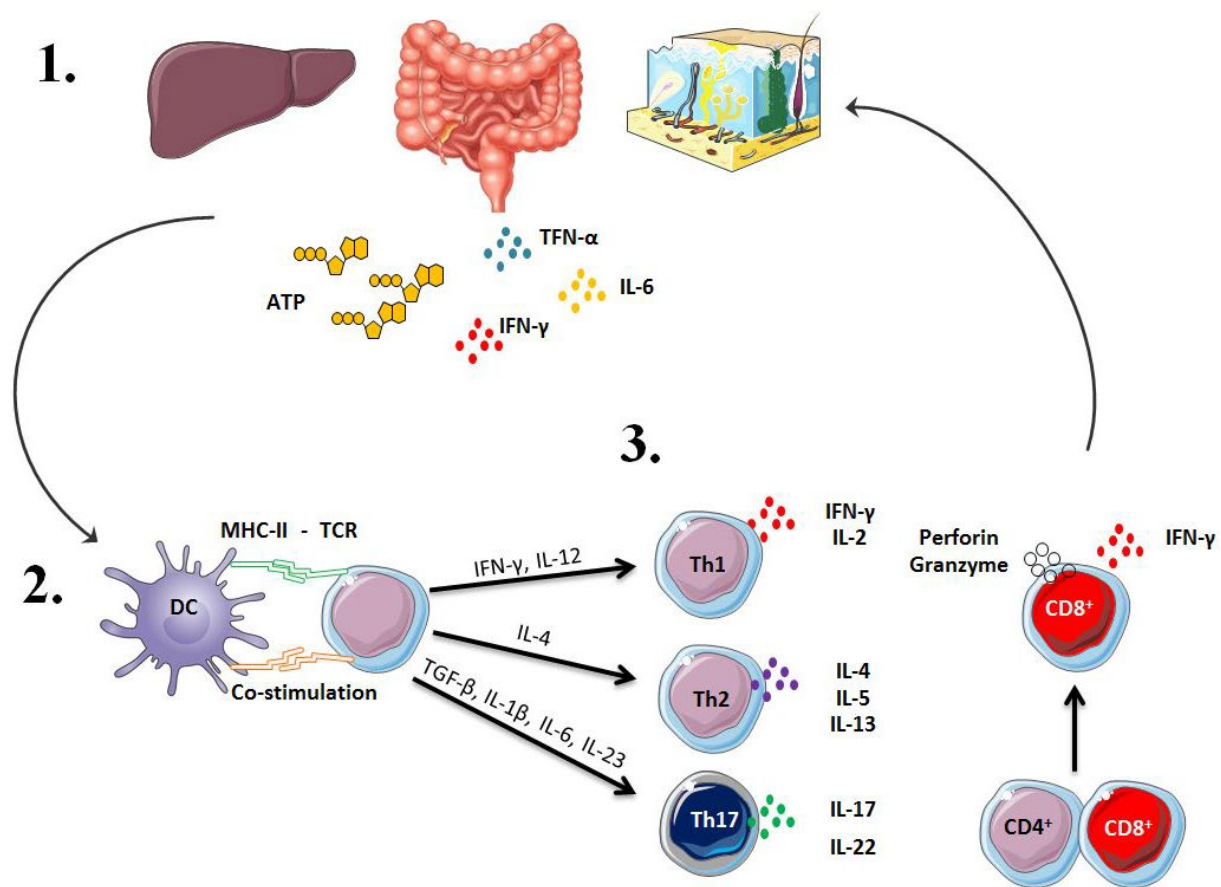


Figure 1.1. The three step process of GVHD. 1. The underlying condition or the treatment regime involves release of pro inflammatory cytokines and DAMPs such as ATP, from damaged and dying cells. 2. DC activation of T cells, leads to T cell differentiation. 3. CD4⁺ and CD8⁺ T cell activation results in cytokine release, leading to a feed forward loop of inflammation. Abbreviations; ATP, adenosine triphosphate, interferon, DC, dendritic cell, IFN, and interleukin , IL tumour necrosis factor, MHC, major histocompatibility complex, TGF, tumour growth factor, TNF- α . Adapted from (Ferrara *et al.*, 1999).

1.4. Cell Types in GVHD

GVHD results in inflammatory damage to target organs, promoted through APC activation of CD4⁺ T cells, CD4⁺ T cell proliferation and subsequent activation of CD8⁺ T cells, and pro-inflammatory cytokine release. Conversely, regulatory T (Treg) cells and natural killer T (NKT) cells have been shown to play an anti-inflammatory role, where higher numbers of these cells inversely correlates with disease severity (Schneidawind *et al.*, 2014). In fact, *ex vivo* expansion of Treg cells is being investigated as a therapeutic strategy to prevent GVHD in HSCT patients (Heinrichs *et al.*, 2016). Additionally, natural killer (NK) cells play a controversial role in GVHD development. Some studies have implicated NK cells in GVHD development, while others have shown NK cells reduce GVHD severity (Simonetta *et al.*, 2017). The role of each different immune cell subset in the development of GVHD is described further below and summarised in Figure 1.2.

1.4.1. APCs

An important part of immunity is the bridging of the innate and adaptive immune systems. Originally, alloantigen expression on host epithelium was thought to be required for initiation of GVHD, however this is not the case (Teshima *et al.*, 2002). Instead, in the development of GVHD, activation of the adaptive immune response is achieved through activation of T and B cells by antigen presentation by APCs (Shlomchik *et al.*, 1999). Notably, non-hematopoietic APCs do have the ability to initiate GVHD (Koyama *et al.*, 2011), and although macrophages can act as professional APCs (Kambayashi and Laufer, 2014), in HSCT dendritic cells (DCs) are the main APC responsible for initiating GVHD development (Markey *et al.*, 2009).

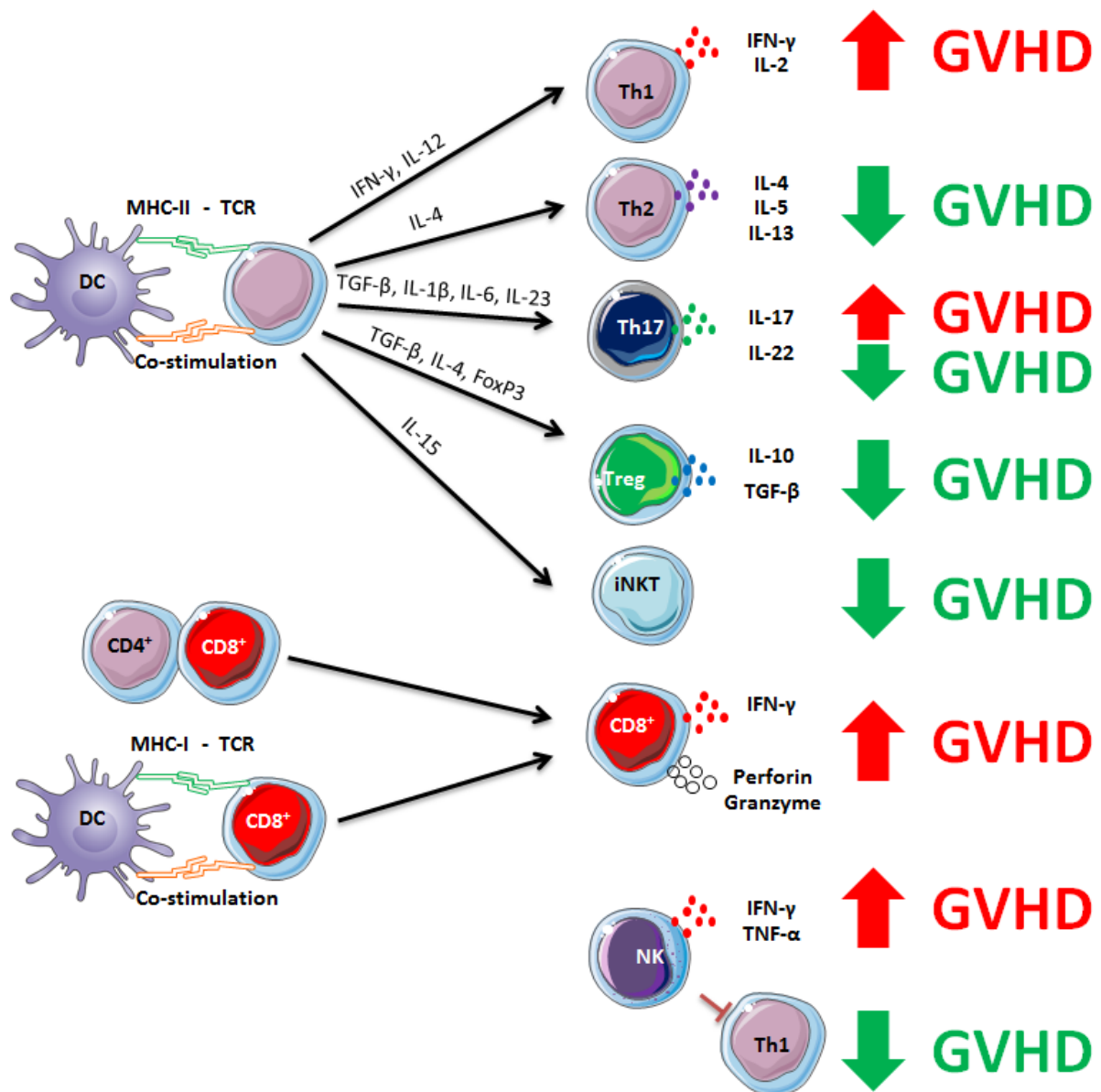


Figure 1.2. The role of different cell types in GVHD development. Activation of naïve T cells by DCs results in T cell differentiation depending on i) the type of DC and the signals it provides, and ii) the cytokines and other factors present. CD4⁺ T cells can differentiate to Th1 cells, which exacerbate GVHD through pro-inflammatory cytokine release, Th2, Treg or iNKT cells which reduce GVHD through anti-inflammatory cytokine release, or Th17 cells which exacerbate or reduce GVHD. CD8⁺ T cells can be activated by DC or CD4⁺ T cells and exacerbate GVHD through IFN- γ and/or perforin/granzyme release. Finally, NK cells can exacerbate GVHD through pro-inflammatory cytokine release, or reduce GVHD through reducing Th1 cell activation. Abbreviations: DC, dendritic cell, FoxP3, forkhead box P3, IFN- γ , interferon gamma, IL, interleukin, iNKT, invariant natural killer T cell, MHC, major histocompatibility complex, NK, natural killer cell, TCR, T cell receptor, TGF- β , tumour growth factor beta, TNF- α , tumour necrosis factor alpha, Treg, regulatory T cell.

1.4.1.1. DCs

Since their initial discovery and classification as an essential mediator of immunity (Banchereau and Steinman, 1998), DCs have been established as professional APCs (Caux *et al.*, 1996). Human DCs can often be identified as CD83 expressing cells (Zhou and Tedder, 1995). DCs express MHC-I and/or MHC-II, and present processed antigen to activate CD8⁺ and/or CD4⁺ T cells, respectively (Banchereau and Steinman, 1998). DCs can be separated into two classes, DC1 cells which activate Th1 cells, and DC2 cells which activate Th2 cells (Banchereau *et al.*, 2000). In GVHD, DC1 cells are deemed pro-inflammatory as Th1 cells mediate GVHD (Mowat, 1989, Garside *et al.*, 1994, Via and Finkelman, 1993), while DC2 are anti-inflammatory as Th2 cells are shown to reduce the incidence and severity of GVHD (Pan *et al.*, 1995, Pan *et al.*, 1999) (discussed in Section 1.4.2. below). DCs have been further characterised and demonstrated to be heterogeneous mix of different subtypes (for a comprehensive review see Mildner and Jung (2014)). DCs can be found in human (h) peripheral blood mononuclear cells (PBMCs). Following HSCT DCs may be of host and/or donor origin, with donor DCs eventually outnumbering host DCs (Auffermann-Gretzinger *et al.*, 2002). Early studies demonstrated that host DCs are sufficient to promote GVHD, even in small numbers, in allogeneic mouse models (Zhang *et al.*, 2002b, Duffner *et al.*, 2004), yet depletion of DCs does not prevent GVHD development (Li *et al.*, 2012). Furthermore, reduced DC numbers also correlates to increased prevalence of GVHD in humans (Reddy *et al.*, 2004). This is due to some DCs being tolerogenic, which can promote Treg cells and reduce GVHD (Sela *et al.*, 2011). Transplantation of *ex vivo* generated tolerogenic DCs can prevent GVHD (Sato *et al.*, 2003). As DCs play major roles in preventing GVHD, manipulation of DCs to promote tolerance represents a potential immunotherapy in HSCT patients.

1.4.2. T Cells

T cells are an important adaptive immune cell subtype and depending on the subset, mediate their effects through release of cytokines and/or perforin and granzymes. T cells are the main immune cell type responsible for mediating the inflammatory damage of GVHD (Sprent *et al.*, 1975, Korngold and Sprent, 1978, Kernan *et al.*, 1986). T cells can be classified by expression of CD3, which along with the T cell receptor (TCR)- α and β chains constitute the TCR complex in the majority of T cells (Yanagi *et al.*, 1984, Hedrick *et al.*, 1984) (with 0.5 – 14% expressing a $\gamma\delta$ TCR instead of an $\alpha\beta$ TCR (Groh *et al.*, 1989)). T cells can be activated by APCs which interact through engagement of the TCR on T cells with MHC molecules present on APCs (Harding and Unanue, 1990). T cells also require co-stimulatory signalling to be activated, which can be through CD28 on the T cell interacting with CD80 or CD86 present on the APC (Lafferty and Cunningham, 1975, June *et al.*, 1987).

All T cells express a TCR, but T cell subsets can be further classified by expression of other markers. Conventional T cells can be classified as CD4⁺ (helper) or CD8⁺ (cytotoxic) T cells. Both CD4⁺ (Korngold and Sprent, 1982) and CD8⁺ (Korngold and Sprent, 1985) T cells play a role in the development of GVHD. CD4⁺ T cells were originally divided into two main subtypes, T helper 1 (Th1) and T helper 2 (Th2) cells depending on the cytokines they produce after activation (Mosmann *et al.*, 1986). Since then other CD4⁺ subtypes have been identified including Th17, Treg, and NKT cells. More information about these cells and the role of each subtype is discussed below.

1.4.2.1. Th1 Cells

Th1 cells are a CD4⁺ T cell subset characterised by expression of the transcription factor Tbet (Szabo *et al.*, 2000) and release of IFN- γ and IL-2 (Mosmann *et al.*, 1986) (Fig 1.2). The role of Th1 cells in GVHD was first characterised by the CD4⁺ T cell production of IFN- γ

(Mowat, 1989, Garside *et al.*, 1994) and IL-2 (Via and Finkelman, 1993) early in GVHD development. IFN- γ and IL-2 blockade confirmed the importance of these cytokines in promoting GVHD (Mowat, 1989, Via and Finkelman, 1993). Th1 cells have also been shown to induce IL-12 production from APCs, which feeds back to further induce Th1 proliferation, and inhibit Th2 differentiation, which collectively exacerbates GVHD (Via *et al.*, 1994). Further, mice with a “quadruple” deficiency in Th2 cytokine production (i.e. mice genetically deficient in IL-4, IL-5, IL-9, and IL-13), exhibit worsened GVHD due to greater Th1 cell differentiation (Tawara *et al.*, 2008). As well as releasing cytokines to promote damage, Th1 cells can damage target tissues through release of Fas ligand (Baker *et al.*, 1996, Kataoka *et al.*, 2001). Additionally, Th1 cells also recruit CD8⁺ T cells to exert damage to target organs, as discussed below (Section 1.3.2.6). In an allogeneic mouse model it was shown that Th1 cells preferentially cause GVHD in the liver and gastrointestinal tract (Yi *et al.*, 2009), whilst in a humanised mouse model of GVHD, human Th1 cells generated *ex vivo* have been shown to drive GVHD development (Amarnath *et al.*, 2010).

Due to the role of Th1 cells and their cytokine production, current treatments for GVHD include the use of cyclosporine and tacrolimus, which target Th1 responses by reducing IL-2 production, and rapamycin, which reduces T cell sensitivity to IL-2 (Khanna, 2000, Antin *et al.*, 2003). However, the role of IFN- γ in GVHD remains controversial (Yang *et al.*, 2005b), as IFN- γ has also shown to have protective roles in GVHD in some models (Brok *et al.*, 1993). Therefore, IFN- γ blockade itself is not considered an efficacious therapy in HSCT recipients.

1.4.2.2. Th2 Cells

Th2 cells are a CD4⁺ T cell subset characterised by the expression of the transcription factor Gata-3 (Zheng and Flavell, 1997), and release of the cytokines IL-4, IL-5 and IL-13 (Mosmann *et al.*, 1986). Originally, DC2 cell activation of Th2 cells was shown to reduce the

incidence and severity of GVHD (Pan *et al.*, 1995, Pan *et al.*, 1999). However, subsequent studies demonstrated genetic deficiency (Murphy *et al.*, 1998) or blockade (Ushiyama *et al.*, 1995) of IL-4 could prevent disease development in allogeneic mouse models of GVHD. Th2 cells have since been shown to exacerbate GVHD, and specifically exert inflammatory damage to the liver and skin, while inflammatory damage to the intestines can be mediated by either Th1 or Th2 cells (Nikolic *et al.*, 2000).

1.4.2.3. Th17 Cells

Th17 cells are a functionally distinct CD4⁺ T cell subset characterised by the expression of the transcription factor RAR-related orphan receptor gamma (ROR γ t) (Ivanov *et al.*, 2006) and release of IL-17A and IL-17F (Harrington *et al.*, 2005), and IL-22 (Chung *et al.*, 2006) (Fig 1.1). Th17 cells have been implicated in GVHD development, where they predominantly exert pro-inflammatory effects; however some studies describe a beneficial effect, so the role of Th17 cells in GVHD remains controversial.

Th17 cells were proposed to exacerbate GVHD, as transfer of IL-17 deficient CD4⁺ T cells delayed, but did not prevent GVHD in an allogeneic mouse model of GVHD (Kappel *et al.*, 2009). Supporting this observed pro-inflammatory role, transplantation of *in vitro* differentiated Th17 cells mediated GVHD in an allogeneic mouse model (Carlson *et al.*, 2009), with inflammatory damage most evident in the skin (Carlson *et al.*, 2009, Hill *et al.*, 2010). Additionally, human Th17 cells exacerbate GVHD in a humanised mouse model due to IL-17 and IFN- γ production (Delens *et al.*, 2018). Blockade of the Janus-associated kinase (JAK)2, signal transducer and activator of transcription (STAT)3 pathway prevents Th17 generation (Betts *et al.*, 2014) and reduces GVHD severity in humanised mice (Betts *et al.*, 2015). Alternatively, Th17 cell-derived IL-22, exacerbates GVHD through a reduction in Treg expansion in target organs and increased CD8⁺ cell infiltration (Couturier *et al.*, 2013). Genetic deficiency of IL-22 in donor T cells (Couturier *et al.*, 2013), or pharmacological

blockade with an anti-IL-22 antibody (Wu *et al.*, 2018) attenuates GVHD through increased Treg cells. As well as reducing Treg cell numbers, in both allogeneic mouse models of GVHD and in HSCT patients Th17 cells exert their pro-inflammatory effects in co-operation with Th1 cells (Carlson *et al.*, 2009). In allogeneic mouse models of GVHD, specific blockade of the transcription factors Tbet and ROR γ t (i.e. simultaneously blocking Th1 and Th17 differentiation), but not blockade of ROR γ t alone, can prevent GVHD development (Yu *et al.*, 2011). Additionally, the use of an anti-IL-12/23p40 antibody can reduce disease severity through decreased Th1 and Th17 cell numbers in target organs in an allogeneic mouse model (Wu *et al.*, 2015) and delay GVHD onset in HSCT patients (Pidala *et al.*, 2017).

The role of Th17 cells in promoting GVHD is confounded by some studies, which describe positive benefits of IL-17 and/or Th17 cells. Absence of Th17 cells leads to increased GVHD severity, due to augmented Th1 cell activation and differentiation (Yi *et al.*, 2008). Additionally, recipient IL-17A was recently shown to be important in preventing intestinal GVHD (Varelias *et al.*, 2017). Finally, IL-22 deficiency in recipients resulted in more severe GVHD (Hanash *et al.*, 2012). A recent study by Cai *et al.* (2018) demonstrated that Th17 cells increase Th1 cell responses, while IL-17 downregulates Th1 cell infiltration of target organs in GVHD, demonstrating individual roles of Th17 cells and IL-17 in an allogeneic mouse model of GVHD. Furthermore, IL-17 deficient donor T cells reduce GVHD, but IL-17 deficiency of all cells in the graft exacerbates GVHD, suggesting Th17 cells exacerbate GVHD, but systemic IL-17 plays a protective role.

Therefore, the role of Th17 cells in allogeneic mouse models can be confounded by the role of IL-17, which notably can also be produced by $\gamma\delta$ T cells (Sutton *et al.*, 2009), NKT cells (Rachitskaya *et al.*, 2008), as well as mast cells and neutrophils (Lin *et al.*, 2011). Thus, the role of Th17 cells and IL-17 requires further elucidation in HSCT patients.

1.4.2.4. Treg Cells

Treg cells are characterised by the expression of the transcription factor Forkhead box protein 3 (FoxP3) (Hori *et al.*, 2003). However, FoxP3 can be expressed by subsets of CD4⁺ T cells other than Treg cells (Morgan *et al.*, 2005). Thus, Treg cells can alternatively be identified by high expression of CD25 and low expression of CD127 (Fazekas de St Groth *et al.*, 2011). Treg cells differ from other CD4⁺ T cells subsets as they predominantly suppress immune responses (Itoh *et al.*, 1999). This suppression is mediated by release of IL-10 and tumour growth factor (TGF)- β (Groux *et al.*, 1997), and/or direct interaction with conventional T cells (Thornton and Shevach, 1998). Treg cells may also exert anti-inflammatory effects through perforin or granzyme release to cause apoptosis of inflammatory T cell subsets (Gondek *et al.*, 2005). However, Treg cells can also exert anti-inflammatory effects by production of adenosine (Deaglio *et al.*, 2007), which can subsequently activate adenosine receptors to promote the release of anti-inflammatory cytokines (Haskó *et al.*, 2008).

Treg cells play important roles in preventing development of GVHD. In allogeneic mouse models, transplantation of donor Treg cells can limit the development of GVHD (Hoffmann *et al.*, 2002) through suppression of alloreactive T cells (Edinger *et al.*, 2003). Moreover, transplantation of *ex vivo* expanded Treg cells can reduce GVHD development in humanised mouse models (Cao *et al.*, 2009, Hannon *et al.*, 2014). In patients, FoxP3 expression on circulating PBMCs inversely correlates with GVHD severity in HSCT patients (Miura *et al.*, 2004), and higher numbers of donor Treg cells present in peripheral blood grafts prior to transplantation reduces the risk of GVHD development in patients (Rezvani *et al.*, 2006). Supporting this, GVHD severity inversely correlates with numbers of Treg cells in the intestine (Rieger *et al.*, 2006) and skin (Fondi *et al.*, 2009) of patients with GVHD. Collectively, these findings have led to the potential use of expanded Treg cells as a preventative therapy for GVHD in HSCT patients (Heinrichs *et al.*, 2016). Unfortunately, the

potential of Treg cell transplantation as a therapy to prevent GVHD is limited by the propensity of Tregs to revert to a pro-inflammatory phenotype in inflammatory environments (Kim *et al.*, 2009, Hanidziar and Koulmanda, 2010).

1.4.2.5. Natural Killer T Cells

NKT cells are a subtype of T cell which were originally characterised by the expression of NK (CD161) and T cell ($\alpha\beta$ -TCR) markers (Makino *et al.*, 1995). Further research has led to the characterisation of NKT cells to be any CD1d-restricted T cell, recognising the non-classical MHC CD1d (Bendelac *et al.*, 1995). NKT cells can be one of three subtypes; Type 1, Type 2, and NKT-like cells (Godfrey *et al.*, 2004). Few studies have investigated the latter two subtypes in GVHD. Donor Type 2 cells (CD1d-restricted cells not expressing the invariant TCR) suppress GVHD development (Kim *et al.*, 2007). Conversely activation of NKT-like cells in *in vitro* cultures suggests they may worsen GVHD (Wang *et al.*, 2008), but this has not been further investigated. However, type 1 NKT, or invariant NKT (iNKT), cells are the most commonly studied CD1d-restricted subset and the role of iNKT cells in GVHD has been elucidated. iNKT cells express an invariant TCR ($V\alpha 14$ - $J\alpha 18$ in mice, and $V\alpha 24$ - $J\alpha 18$ in humans) (Porcelli *et al.*, 1993), and can be further subdivided based on the cytokine production of iNKT1, iNKT2 and iNKT17 cells reflecting Th1, Th2 and Th17 cells, respectively (Chang *et al.*, 2012, Lee *et al.*, 2013). Furthermore, there is recent evidence of another two iNKT cell subsets; a “follicular helper” T cell like-NK cell (iNKT_{FH}) and an IL-10 secreting iNKT cell (iNKT10) (Chang *et al.*, 2012, Lynch *et al.*, 2012, Lynch *et al.*, 2015). However, neither of these cells have been fully characterised, nor has their role in GVHD been investigated.

In general, NKT cells play a role in GVHD, mostly by suppressing GVHD development. In earlier studies, peripheral NKT cells (identified by expression of CD161 and $\alpha\beta$ -TCR) were shown to have a suppressive role in mouse models of HSCT (Zeng *et al.*, 1999, Lan *et al.*,

2001). Mice lacking iNKT1 cells due to a genetic deficiency of the TCR subunit $\text{J}\alpha 18$ demonstrated more severe GVHD (Haraguchi *et al.*, 2005). Co-transplantation of *in vitro* induced (Kuwatani *et al.*, 2006) or *ex vivo* expanded donor (Haraguchi *et al.*, 2005, Yang *et al.*, 2010) or third-party (Schneidawind *et al.*, 2014) iNKT1 cells can suppress disease in allogeneic mouse models of GVHD. The suppressive ability of iNKT cells is through promoting Th2 responses (Hashimoto *et al.*, 2005, Margalit *et al.*, 2005) or up-regulation of Treg cell responses (Pillai *et al.*, 2009, Vela-Ojeda *et al.*, 2010, Schneidawind *et al.*, 2015). In patients, lower numbers of peripheral blood iNKT cells also correlate with worsened GVHD (Crough *et al.*, 2004, Haraguchi *et al.*, 2004).

1.4.2.6. CD8⁺ (Cytotoxic) T Cells

CD8⁺ T cells are characterised by expression of the CD8 molecule, which specifically binds to MHC-I molecules on APCs. Similar to CD4⁺ T cells, CD8⁺ T cells can produce cytokines, predominantly IFN- γ , to exert pro-inflammatory effects (Szabo *et al.*, 2002). CD8⁺ T cells are also known as cytotoxic T cells due to their ability to exert pro-inflammatory effects through the release of perforin and granzymes (Pearce *et al.*, 2003).

In GVHD, CD8⁺ T cells play important roles in causing or exacerbating the inflammatory damage. Whilst, CD4⁺ T cells initiate disease, GVHD is exacerbated by CD8⁺ T cell expansion (Rus *et al.*, 1995). This was supported by demonstrating transplantation of MHC-I deficient donor cells led to reduced GVHD severity in allogeneic (Matte *et al.*, 2004) and humanised (King *et al.*, 2009) mouse models. Infiltration of CD8⁺ T cells into the portal areas of the liver (Murai *et al.*, 1999) and into Peyer's patches in the intestines (Murai *et al.*, 2003) promotes GVHD in these respective organs. Furthermore, CD44^{lo} CD62L^{hi} CD8⁺ T cells, a self-renewing CD8⁺ T cell subset emerging early in GVHD pathogenesis, generates and sustains memory and effector CD8⁺ T cells to further promote GVHD (Zhang *et al.*, 2005). Whilst CD8⁺ T cells are known to exert inflammatory damage in GVHD through perforin and

granzyme release, CD8⁺ T cells also produce IFN- γ to promote inflammation in GVHD (Rus *et al.*, 1995). Alternatively, a role for an IL-17-producing CD8⁺ T cell subset (Tc17) in GVHD has also been identified. Similar to Th17 cells, Tc17 cells exert pro-inflammatory effects through production of IL-17 to promote GVHD in an allogeneic mouse model of GVHD (Gartlan *et al.*, 2015).

1.4.3. Natural Killer Cells

NK cells lack CD3 expression, but express CD56 (Lanier *et al.*, 1989). Similar to CD8⁺ T cells, NK cells can exert their effects through production of cytokines or release of perforin and granzyme (Smyth *et al.*, 2005). Alternatively, NK cells can exert their effects through antibody-dependent cell-mediated cytotoxicity (Katz *et al.*, 1987).

NK cells are another important cell type implicated in GVHD development, but whether these cells play a detrimental or beneficial role is still controversial. Early studies using cell activity assays and immunophenotyping demonstrated donor NK cells were present in GVHD target organs in allogeneic mouse models, suggesting NK cells exacerbate GVHD (Guillen *et al.*, 1986, Ferrara *et al.*, 1989). In allogeneic mouse models of GVHD, transfer of cells with a genetic mutation leading to NK cell deficiency failed to cause GVHD, supporting the idea that NK cells exacerbate GVHD (Ghayur *et al.*, 1987). In a humanised SCID mouse model, transfer of activated NK cells, which produce IFN- γ and TNF- α , exacerbated GVHD (Xun *et al.*, 1993). In patients, higher numbers of NK cells are found in the liver (Dilly and Sloane, 1985), intestines (Roy *et al.*, 1993) and skin (Acevedo *et al.*, 1991) and NK cells correlate with GVHD severity (Dokhelar *et al.*, 1981).

Subsequent research has also identified a beneficial role of NK cells in GVHD. In allogeneic mouse models of GVHD, transfer of NK cells alone does not induce GVHD, whilst co-transfer with splenocytes prevents GVHD (Murphy *et al.*, 1992). NK cells prevent GVHD

development by inhibiting T cell proliferation and depleting APCs (Ruggeri *et al.*, 2002). In patients, higher numbers of alloreactive NK cells inversely correlates with GVHD development (Ruggeri *et al.*, 2007, Tanaka *et al.*, 2012a, Kim *et al.*, 2016b).

1.5. Models of GVHD

Seminal studies of GVHD were conducted in canines. Inflammatory damage to the GVHD target organs; liver, gastrointestinal tract and skin, demonstrated damage was greater in dogs transplanted with tissue that was genetically mismatched for dog leukocyte antigens (Storb *et al.*, 1974, Kolb *et al.*, 1979), which is equivalent to HLA mismatch in humans. Canines also offered useful models for the development of immunosuppression, which was the first therapy for GVHD (Kolb *et al.*, 1973). Although canines offer a good model of GVHD (Markey *et al.*, 2014), the majority of current studies are conducted using mice. Mice afford useful models to study human disease as, like dogs, they are genetically similar to humans, but are much easier and cheaper to maintain, have shorter breeding times and allow the use of transgenic variants and well-established inbred strains. Both allogeneic and humanised mouse models of GVHD have been established and will be discussed below.

1.5.1. Allogeneic Mouse Models of GVHD

Similar to humans, GVHD in allogeneic mouse models can emerge due to differences in either MHC or miHAg, depending on the donor and recipient strains. In mice, MHC antigens are known as H2, with each mouse strain exhibiting a unique haplotype, denoted by a subscript letter. Complete MHC-mismatch models of allogeneic GVHD involve the transfer of bone marrow from one strain of mouse to another with a completely different MHC haplotype (denoted by H2^x where x is the haplotype).

The most common complete MHC-mismatch model of allogeneic GVHD involves the transfer of bone marrow and splenocytes from C57BL/6 mice to BALB/c mice (H2^b to H2^d) (van Leeuwen *et al.*, 2002). Other complete mismatch models include the transfer of bone marrow and splenocytes from C3H/HeJ to C57BL/6 mice (H2^k to H2^b) (Blazar *et al.*, 1991), and C57BL/6 to B10.BR mice (H2^b to H2^k) (Vallera *et al.*, 1981). These complete MHC-mismatch models were used to elucidate that T cells were the major immune cell that drives GVHD inflammation (Sprent *et al.*, 1975, Korngold and Sprent, 1978), and are useful models to study HLA-mismatch transplants in HSCT patients, such as in the cases of unrelated donors.

Other allogeneic mouse models of GVHD include haploidentical models, which involve transplantation where one H2 allele is identical, but the other differs. Some examples of these models include the C57BL/6 to B6C3F1 (H2^b to H2^{k/b}) (Kanamaru *et al.*, 1984), and the C57BL/6 to B6D2F1 (H2^b to H2^{b/d}) (Pickel and Hoffmann, 1977), and are relevant to transplants in HSCT patients when there is a partial HLA-mismatch. Finally, there are allogeneic mouse models of GVHD where the H2 is a complete match, but GVHD arises due to miHAgs. Some examples of these models include the B10.D2 to BALB/c mice (H2^d to H2^d) (Korngold and Sprent, 1987) and C57BL/6 to BALB.b (H2^b to H2^b) mice (Berger *et al.*, 1994). These models are useful to elucidate miHAgs involved in GVHD, and reflect the development of GVHD in HSCT patients who receive an HLA-matched transplant.

Allogeneic mouse models of GVHD have been instrumental in elucidating GVHD pathophysiological mechanisms and derivation of early therapies for GVHD. Therapeutic targets investigated in these models aim to target a specific part of the three-step progression of GVHD. This includes altering the conditioning regime by way of non-myeloablative, or reduced intensity, conditioning to reduce inflammatory cytokines and damage (Aoudjhane *et al.*, 2005). Alternatively therapies target the characteristic pro-inflammatory cytokine storm

such as TNF- α blockade (Hill *et al.*, 1999), or more recently hIL-6 blockade (Tawara *et al.*, 2011). Other therapies aim to prevent the activation and proliferation of T cells, such as the use of post-transplant cyclophosphamide to deplete proliferating cells (Ganguly *et al.*, 2014), and the development of cellular therapies such as transfer of Treg cells to suppress activated cells (Hoffmann *et al.*, 2002, Edinger *et al.*, 2003), which have since been translated to the clinic (Couriel *et al.*, 2009, Kanakry *et al.*, 2014, Di Ianni *et al.*, 2011).

Allogeneic mouse models of GVHD are not without disadvantages. Besides the large differences in immune systems between mice and humans (Mestas and Hughes, 2004, Schroeder and DiPersio, 2011), mice used in allogeneic models of GVHD have low genetic diversity due to inbreeding, and therefore donors have very specific genetic disparities to recipients. Furthermore, mice are usually housed under pathogen-free conditions, unlike HSCT patients (Zeiser and Blazar, 2016). Finally, allogeneic mouse models transfer bone marrow cells supplemented with splenocytes as a source of T cells, whilst in humans grafts often contain peripheral T cells, which will differ in function to those found in the spleen (Zeiser and Blazar, 2016). Therefore, to address these disadvantages and better study human immune responses *in vivo*, numerous humanised mouse models have been developed.

1.5.2. Humanised Mouse Models of GVHD

Humanised mouse models of GVHD utilise mice with genetic abnormalities or deficiencies to allow engraftment of human cells, which subsequently mediate GVHD in these animals. Each model utilises a unique strain of mouse that receives a transfer of human cells, most commonly hPBMCs. The CB17-SCID (*scid/scid*) mouse possesses the severe combined immunodeficiency (SCID) mutation on the protein kinase, DNA-activated, catalytic polypeptide (*Prkdc^{scid}*) which prevents V(D)J recombination leading to impaired B and T cell development (Bosma *et al.*, 1983). This model allows engraftment of human cells, however

after hPBMC injection engraftment of human cells in these mice is low due to spontaneous development of functional murine B and T cells, and constitutive NK cell activity (Greiner *et al.*, 1998). Additionally, the lack of diverse human T cell development in these mice limits the use of this model for investigating potential therapies (Garcia *et al.*, 1997). Subsequently, breeding of CB17-SCID mice with NOD mice allowed the development of the NOD/SCID mouse strain (Shultz *et al.*, 1995), with improved human cell engraftment due to NOD mice possessing a polymorphism in the *Sirpa* gene, which promotes “phagocytic tolerance” (i.e. reduces macrophage clearance) of human leukocytes (Yamauchi *et al.*, 2013). However, human cell engraftment is still low in these mice, due to NK cells, whilst the lifespan of these mice is limited due to lymphoma development (Shultz *et al.*, 1995). Subsequently, the NOD-SCID-IL-2 receptor γ common chain null (IL-2R γ^{null}) (NSG) mouse which lacks IL-2R γ and subsequently lacks functional T, B and NK cells, allowing greater human cell engraftment was developed (Ito *et al.*, 2002, Shultz *et al.*, 2005).

1.5.3. The Humanised NSG Mouse Model of GVHD

Injection of NSG mice with hPBMCs can establish a humanised NSG mouse model of GVHD (King *et al.*, 2009) (Figure 1.3). There are variations of this model, which differ by the number and source of hPBMCs injected and the route of injection (Hogenes *et al.*, 2014, De La Rochere *et al.*, 2018). Whilst these various models exist, the humanised NSG mouse model, established by injection of hPBMCs provides a robust model of GVHD (Ali *et al.*, 2012). Accordingly, from this point forward the humanised NSG mouse model will refer to NSG mice injected with hPBMCs (Figure 1.3).

Within three to eight weeks following injection of hPBMCs, NSG mice develop phenotypical signs of GVHD. These signs include weight loss, hunching, reduced activity, fur ruffling and skin denudation, as first described by Cooke *et al.* (1996) in allogeneic mouse models.

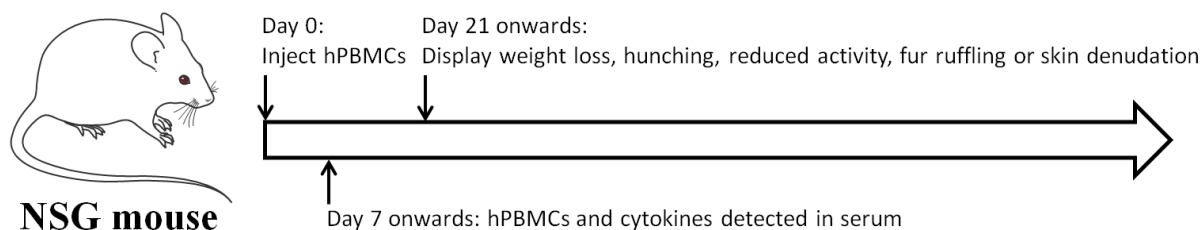


Figure 1.3. The humanised NSG mouse model of GVHD. Immunodeficient NSG mice are injected with freshly isolated hPBMCs and subsequently develop GVHD. GVHD can be assessed by clinical scoring as indicated. Abbreviations: GVHD, graft-versus-host disease, h, human, NSG, non-obese diabetic-severe combined immunodeficient-interleukin-2 receptor γ common chain null, PBMC, peripheral blood mononuclear cell.

King *et al.* (2008) demonstrated that NSG mice rapidly engraft hPBMCs within a matter of weeks following injection with 5×10^6 hPBMCs/mouse, although 100% engraftment is typically observed with injection of $\geq 10 \times 10^6$ hPBMCs/mouse. Indeed, 10×10^6 hPBMCs/mouse is sufficient for 100% engraftment, with no significantly greater engraftment after injection of 20×10^6 or 50×10^6 hPBMCs/mouse (King *et al.*, 2008).

The main GVHD target organs in humans; the liver, gut and skin, are similarly affected in humanised NSG mice, and leukocyte infiltration and inflammation reflects GVHD development as in HSCT patients. The liver demonstrates leukocyte infiltration, concentrated around portal triads (King *et al.*, 2009), with both portal triads and parenchymal tissue damaged (King *et al.*, 2009, Ehx *et al.*, 2017). The gut also demonstrates leukocyte infiltration (King *et al.*, 2009, Nakauchi *et al.*, 2015) as well as apoptosis (Nakauchi *et al.*, 2015). Finally, the skin demonstrates leukocyte infiltration within the dermis and epidermis (King *et al.*, 2009). The original study by King *et al.* (2009) demonstrated that infiltrating leukocytes are of human origin, staining for hCD45⁺. King *et al.* (2009) also demonstrated that the majority of engrafted human leukocytes are T cells, and immunohistochemical analysis of organs using a hCD3 antibody demonstrated human T cells are the main infiltrating leukocyte in the liver (Ehx *et al.*, 2017), and gut (Nakauchi *et al.*, 2015).

Immunohistochemical staining of skin has shown hCD45 cells infiltration (King *et al.*, 2009) but the identity of these human leukocytes is unknown. However, target organ specificity of effector memory T cells that express tissue-specific homing receptors, such as cutaneous lymphocyte antigen have been shown to home to the skin in this model (Ali *et al.*, 2012).

King *et al.* (2009) demonstrated that hCD8⁺ T cells drive GVHD development, as deletion of MHC-I molecules significantly delayed disease, compared to MHC-I deficiency suggesting CD8⁺ T cells are important for promoting more rapid GVHD. However, deletion of MHC-II also delays GVHD (King *et al.*, 2009). Moreover, a subsequent study by Covassin *et al.* (2011) demonstrated CD4⁺ T cells are sufficient to mediate disease as injection of CD4⁺ T cells alone induces GVHD in humanised NSG mice. Activation of human T cells relies on co-stimulatory signals (Lafferty and Cunningham, 1975), and human CD28 can recognise murine B7.2 to act as a co-stimulatory signal (Freeman *et al.*, 1993), which is required for T cell activation in humanised NSG mice (Søndergaard *et al.*, 2013). However, whether murine MHC is responsible for antigen presentation to human T cells, or whether murine MHC is the antigen itself remains to be elucidated. Additionally, human Treg cell numbers inversely correlate to GVHD severity and can prolong survival in humanised NSG mice (Bruck *et al.*, 2013, Achita *et al.*, 2018). This parallels the higher numbers of donor Treg cells correlating to reduced risk of GVHD development in HSCT patients (Rezvani *et al.*, 2006). However, stable long-term engraftment of Treg cells in humanised NSG mice requires expression of human IL-2 (Abraham *et al.*, 2012).

Human T cells migrate to target organs and produce cytokines to exert inflammatory damage, with cytokines implicated in GVHD in HSCT patients also implicated in humanised NSG mouse models. Serum hTNF- α concentrations are increased as soon as 24 hours post-hPBMC injection and pharmacological blockade of TNF- α can prevent GVHD development in humanised NSG mice (King *et al.*, 2009). Similarly, serum human IL-6 is also increased with

GVHD development in humanised mice (Amarnath *et al.*, 2010), and blockade can reduce disease severity (Gu *et al.*, 2016a). Humanised NSG mice demonstrate increased levels of hIL-2 and hIFN- γ (Abraham *et al.*, 2017), and while blockade of these cytokines has not been investigated, successful treatments that reduce and/or prevent GVHD have correlated with reduced serum hIFN- γ (Amarnath *et al.*, 2011, Gregoire-Gauthier *et al.*, 2012) or hIL-2 (Abraham *et al.*, 2012, Gregoire-Gauthier *et al.*, 2012). Furthermore, whilst hIL-17 blockade has not been investigated, reduced serum hIL-17 correlates with successful treatments that reduce and/or prevent GVHD (Gregoire-Gauthier *et al.*, 2012). In contrast, IL-10, which correlates to reduced disease severity in humans (Holler *et al.*, 2000), has been shown to exacerbate GVHD in humanised NSG mice, but can act synergistically with hIL-2 to delay disease (Abraham *et al.*, 2015).

The humanised NSG mouse model of GVHD can be utilised as a preclinical mouse model of GVHD, and therapies that have shown promise in both this model and clinical trials in HSCT patients are discussed briefly below and summarised in Table 1.1. Therapies may aim to prevent inflammation by targeting cytokines or T cells. TNF- α blockade using the humanised monoclonal antibody (mAb) Etanercept delayed GVHD onset, but did not prevent disease development in humanised NSG mice (King *et al.*, 2009) or humans (Choi *et al.*, 2012b, Gatza *et al.*, 2014). Furthermore, although TNF- α is an important pro-inflammatory cytokine in the innate immune system (Serbina *et al.*, 2003), TNF- α blockade may leave HSCT recipients more susceptible to infection (Choi *et al.*, 2012b).

Other therapies aim to reduce GVHD by preventing T cells activation, proliferation or migration to target organs. The use of post-transplant cyclophosphamide depletes alloreactive T cells, and reduces GVHD severity in humanised NSG mouse models (Kanakry *et al.*, 2013a). This therapy is currently being investigated in HSCT patients, but can cause infection-independent fever (O'Donnell *et al.*, 2015). Alternatively, injection of

immunosuppressive cell types such as Treg cells at the time of hPBMC-injection reduces GVHD in humanised NSG mice (Hannon *et al.*, 2014, Achita *et al.*, 2018) and is being investigated as a potential therapy in HSCT patients. However, Treg cells revert to a pro-inflammatory phenotype in inflammatory environments (Kim *et al.*, 2009, Hanidziar and Koulmanda, 2010), which may limit their use as a therapy for GVHD in HSCT patients. The addition of mesenchymal stem cells, as a suppressive cell type, reduces inflammatory damage of GVHD in humanised NSG mice (Bruck *et al.*, 2013, Huang *et al.*, 2017), but fails to reduce GVHD in HSCT patients (Galipeau, 2013). Finally, CCR5 blockade prevents T cells tracking to target organs in humanised NSG mice (Burger *et al.*, 2017a), but may be limited in humans as CCR5 antagonists can cause hepatotoxicity (Kim *et al.*, 2016a). Therefore, whilst therapies for GVHD are currently being tested in the clinic, these therapies each have limitations, and there is a need for alternative strategies and targets.

The purinergic signalling system plays a role in immunity and has been implicated in numerous inflammatory disorders (see Section 1.10.). Whilst purinergic signalling has been implicated in allogeneic mouse models of GVHD (see Section 1.10.1.), the role of purinergic signalling has not been investigated in humanised mouse models of GVHD and is limited in humans. Due to the importance of purinergic signalling in immunity and inflammation, this signalling system has also been implicated in other inflammatory diseases such as psoriasis. Therefore a brief overview of psoriasis will be presented.

Table 1.1. Therapeutic strategies investigated in the humanised NSG mouse model of GVHD currently in clinical trials. Abbreviations; CCR5, C-C chemokine receptor, IL, interleukin, JAK, Janus associated kinase, mAb, monoclonal antibody, NSG, non-obese diabetic-severe combined immunodeficient-interleukin-2 receptor γ common chain null, Treg, regulatory T cell

Therapeutic strategy in humanised NSG model	Reference(s)	Therapeutic strategy in clinical trial(s)	Current status of clinical trial(s)*	Reference(s)
TNF- α blockade (Etanercept anti-TNF- α)	(King <i>et al.</i> , 2009)	Etanercept (anti TNF- α) Infliximab (anti TNF- α)	Completed Completed	(Choi <i>et al.</i> , 2012b, Gatzka <i>et al.</i> , 2014)
Regulatory T cell co-transplantation	(Hannon <i>et al.</i> , 2014, Achita <i>et al.</i> , 2018)	Donor Treg cell infusion <i>Ex vivo</i> expansion	Recruiting, Suspended, Unknown Recruiting	(Trzonkowski <i>et al.</i> , 2009, Brunstein <i>et al.</i> , 2011)
Post-transplant cyclophosphamide	(Kanakry <i>et al.</i> , 2013a)	Post-transplant cyclophosphamide	Active, Completed, Recruiting	(O'Donnell <i>et al.</i> , 2015)
CCR5 blockade	(Burger <i>et al.</i> , 2017b)	PRO 140 (anti-CCR5 mAb)	Recruiting	
Mesenchymal stem cells	(Bruck <i>et al.</i> , 2013, Huang <i>et al.</i> , 2017)	<i>Ex vivo</i> expansion	Active, Recruiting, Suspended, Unknown	(Galipeau, 2013)
IL-6 blockade (Tocilizumab (anti-IL-6R))	(Gu <i>et al.</i> , 2016b)	Tocilizumab (anti-IL-6R)	Recruiting, Terminated, Withdrawn	Results not published
JAK1/2 blockade	(Betts <i>et al.</i> , 2017, Betts <i>et al.</i> , 2018)	<i>Multiple mAb</i> ; Baricitinib (JAK1/2) Itacitinib (JAK1) Ruxolitinib (JAK1/2)	Recruiting Recruiting Recruiting	

*According to clinicaltrials.gov (last accessed 16/11/2018)

1.6. Psoriasis

Psoriasis affects 1-3% of the general population of Western countries (Lebwohl *et al.*, 2014), and is estimated to cost the United States of America healthcare system alone \$112 billion per year (Brezinski *et al.*, 2015). Psoriasis is an autoimmune disease of the skin, which manifests as red or pink areas of skin covered in white plaque-like lesions of dry flaky skin, present on areas of the body including the scalp, elbows, knees and lower back (Schön and Boehncke, 2005). Whilst the cause of psoriasis is still unknown, the plaque-like lesions form due to an autoimmune response leading to inflammation mediated by T cell activation by APCs, leading to hyper-proliferation of keratinocytes (Schön *et al.*, 2006) and recruitment of neutrophils (Schön *et al.*, 2017).

The typical plaque-like lesions as described above are indicative of psoriasis vulgaris, which is the predominant form of psoriasis constituting ~90% of all cases (Griffiths and Barker, 2007). Less common is pustular psoriasis, where plaques also swell with pus, as opposed to the dry flaky skin of psoriasis vulgaris (Schön and Boehncke, 2005, Nestle *et al.*, 2009b), or guttate psoriasis, which emerges after an infection such as tonsillitis and resolves within a few months of onset (Griffiths and Barker, 2007). However, a third of guttate psoriasis patients subsequently develop psoriasis vulgaris (Martin *et al.*, 1996). If any of these forms are allowed to progress, they can form psoriasis erythroderma, which covers the entire body (Nestle *et al.*, 2009b).

The exact cause of psoriasis is still unknown, but genetic susceptibility is implicated in psoriasis development (Liang *et al.*, 2017). A susceptibility locus, termed *PSORS1* is in the MHC region on chr 6 (6p21) (Balendran *et al.*, 1999) and is associated with up to 50% of psoriasis cases (Asumalahti *et al.*, 2002). Within this gene, the MHC-I molecule HLA-C, and the specific allele *HLA-Cw*0602* have been implicated as causative factors, with patients

homozygous for this allele at a greater risk of developing psoriasis (Gudjonsson *et al.*, 2003). However there are also a number of other susceptibility loci including *PSORS2* (chr 17q) (Tomfohrde *et al.*, 1994), *PSORS3* (chr 4p) (Matthews *et al.*, 1996), *PSORS4* (chr 4q) (de Cid *et al.*, 2009), *PSORS5* (chr 3q) (Vasilopoulos *et al.*, 2008), *PSORS6* (chr 19p) (Lee *et al.*, 2000), *PSORS7* (chr 1p) (Veal *et al.*, 2001), *PSORS8* (chr 16q) (Nair *et al.*, 1997), *PSORS9* (chr 4q) (Zhang *et al.*, 2002a) and *PSORS10* (chr 18p) (Asumalahti *et al.*, 2003).

1.7. Cell Types in Psoriasis

The characteristic plaque-like lesions of psoriasis emerge due to the pro-inflammatory environment mediated by different T cell subsets, predominantly Th1, Th17, and CD8⁺ T cells. Activation of T cells by APCs causes hyper-proliferation of keratinocytes and recruitment of neutrophils to further promote inflammation.

The CD4⁺ subsets Th1 and Th17, and CD8⁺ T cells are all important mediators of psoriasis. The majority of T cells in the serum and psoriatic lesions are Th1 and CD8⁺ T cells, and the high levels of TNF- α and IFN- γ in psoriatic lesions and serum of psoriasis patients suggests psoriasis is a Th1 and CD8⁺ T cell mediated disease (Schlaak *et al.*, 1994, Austin *et al.*, 1999). IFN- γ is an important pro-inflammatory cytokine in psoriasis (Wei *et al.*, 1999, Haider *et al.*, 2008) which can cause hyperproliferation of keratinocytes (Bata-Csorgo *et al.*, 1995). Further, IFN- γ production by Th1 cells can lead to a feedback loop to activate APCs and promote Th17 development (Kryczek *et al.*, 2008). After the discovery and characterisation of Th17 cells (Harrington *et al.*, 2005), these cells were implicated in the pathogenesis of psoriasis due to their activation by the pro-inflammatory cytokine IL-23 (Piskin *et al.*, 2006). IL-23 is upregulated in psoriatic lesions (Piskin *et al.*, 2006) and is essential for activation of Th17 cells (McGeachy *et al.*, 2009), which release IL-17 to promote inflammation in this

disease (Tonel *et al.*, 2010, Rizzo *et al.*, 2011, Guenova *et al.*, 2015). IL-17, as well as TNF- α , IL-21 and IL-22, produced by Th17 cells are present in psoriatic lesions and in higher levels in serum from psoriasis patients compared to healthy controls (Boniface *et al.*, 2007, Lowes *et al.*, 2008, Caruso *et al.*, 2009). These cytokines induce proliferation of, and antimicrobial peptide release from, keratinocytes (Ivanov *et al.*, 2006, Shi *et al.*, 2011).

T cells require antigen presentation by APCs for their activation and therefore APCs play an important role in psoriasis. The classical APCs of the skin are Langerhans cells (LCs) (Streilein and Bergstresser, 1984), which can present antigen to T cells (Romani *et al.*, 1989), but their role in psoriasis remains unknown (Perera *et al.*, 2012). LC numbers are lower in skin from psoriasis patients (Haftek *et al.*, 1983), and their migration is hindered in psoriatic skin compared to healthy skin (Cumberbatch *et al.*, 2006). Furthermore, a seminal paper from (Mizumoto *et al.*, 2002), demonstrates CD39 is the predominant ecto-nucleotidase present on LCs and is essential for degradation of ATP, a danger signal implicated in skin inflammation (Killeen *et al.*, 2013). This suggests LCs play a preventative role in psoriasis, consistent with the tolerogenic nature of these cells (Kitashima *et al.*, 2018).

Plasmacytoid and myeloid DCs are other APCs in the skin and play a major role in psoriasis pathogenesis. Plasmacytoid DCs are normally absent from the skin of healthy people, but are present in both lesional and healthy skin of psoriasis patients (Nestle *et al.*, 2005). These cells can become activated by antimicrobial peptides such as LL-37 (Lande *et al.*, 2007) to infiltrate the skin and release IFN- α (Wollenberg *et al.*, 2002, Nestle *et al.*, 2005). This in turn promotes further release of LL-37 as well as additional antimicrobial peptides; S100 proteins and defensins from keratinocytes (Bando *et al.*, 2007) to promote psoriasis, whilst Plasmacytoid DC production of IFN- α , along with IL-6 and TNF- α can activate myeloid DC (Nestle *et al.*, 2005). Furthermore, LL-37 binds to RNA released by dying cells to activate myeloid DCs (Ganguly *et al.*, 2009), which promote inflammation by release of IL-23 (Tonel

et al., 2010), which promotes Th17 cell responses. Macrophages also play a role in psoriasis, and are found in increased numbers in psoriatic skin of patients (van den Oord and de Wolf-Peeters, 1994). These cells are essential for development of psoriasis-like inflammation in mouse models through production of TNF- α (Wang *et al.*, 2006) and IFN- γ (Stratis *et al.*, 2006).

T cell activation by APCs and cytokine production from both of these cell types causes keratinocyte proliferation which results in the characteristic psoriatic lesions. Keratinocytes constitute up to 90% of the cells of the skin (Nestle *et al.*, 2009a) and are an innate immune cell type involved in psoriasis pathogenesis (Barker *et al.*, 1991). Hyperproliferation of immature keratinocytes results in these cells migrating from the basal layer into the cornified layer in a matter of 1-3 days rather than the usual 28-30 days (Schön and Boehncke, 2005), which results in the thickening of the epidermis (acanthosis) characteristic of psoriatic lesions. As mentioned above, keratinocytes promote inflammation and psoriasis through the release of LL-37, defensins and S100 proteins (Bando *et al.*, 2007), and release of these antimicrobial peptides, as well as chemo-attractants such as IL-8 recruits neutrophils (Bruch-Gerharz *et al.*, 1996), which release cytokines resulting in a feed-forward loop of inflammation (Terui *et al.*, 2000). Neutrophils are found in large numbers in psoriatic skin and there is renewed interest in their role in psoriasis (Schön *et al.*, 2017). Finally, keratinocytes can also release chemo-attractants such as CCL27 (Homey *et al.*, 2002) and IL-8 to recruit T cells into the skin (Homey *et al.*, 2002, Albanesi *et al.*, 2001), as well as produce IL-23 (Piskin *et al.*, 2006), further promoting the feed-forward loop of inflammation.

Although psoriasis is not directly lethal, the disease can greatly reduce the quality of life of an individual, and increase their chance of cardiovascular disease (Neimann *et al.*, 2006), arthritis (Tey *et al.*, 2010), and some cancers (Pouplard *et al.*, 2013). There is currently no cure for psoriasis, and current treatments range from topical treatments to phototherapy, but

satisfaction with these treatments is low (Korman *et al.*, 2015, Schaarschmidt *et al.*, 2015). New therapies for psoriasis aim to suppress the immune response through inhibition of pro-inflammatory cytokines such as TNF- α , IL-12/23 (Rustin, 2012) and IL-17A (Hueber *et al.*, 2010). However, these drugs have off-target effects, and targeting cell receptors represents a better therapy. To achieve this pre-clinical mouse models are required.

1.8. Models of Psoriasis

Psoriasis is a uniquely human disease (Gudjonsson *et al.*, 2007), and due to the complex nature of psoriasis studies are often limited to *in vitro* experiments or inferred from other murine models of skin diseases such as croton oil-induced irritant contact dermatitis (ICD) (Mizumoto *et al.*, 2002) or hapten- or DNFB-induced contact hypersensitivity (CHS) models of atopic dermatitis (AD) (Röse *et al.*, 2012, Gaspari *et al.*, 2016). However, neither ICD nor CHS encapsulate the multifaceted innate and adaptive immune responses of psoriasis, and subsequent models that better reflects psoriasis have been developed. Moreover, current murine models of psoriasis only recapitulate some, but not every aspect of human psoriasis, with each model offering unique advantages and disadvantages. Mouse models of psoriasis can be characterised as belonging to one of four categories; spontaneous, transgenic, xenogeneic, or inducible, as outlined below.

Spontaneous mouse models demonstrate skin pathologies that resemble psoriasis in some ways, but lack some distinct features. The flaky skin ($Ttc7^{fsn}/Ttc7^{fsn}$) mouse model demonstrates neutrophil infiltration into involved skin (Sundberg, 1994). Whilst the chronic proliferative dermatitis mutation ($cpdm/cpdm$) (HogenEsch *et al.*, 1993) and the homozygous asebia ($Scd1^{ab}/Scd1^{ab}$) (Gates and Karasek, 1965) mouse models demonstrate macrophage

and mast cell infiltration. The latter model also demonstrates epidermal thickening but each of these models lack other features of psoriasis, and thus have fallen out of use.

Transgenic mouse models involve the genetic over-expression or knock-out of specific genes to imitate psoriasis. A transgenic mouse model causing a mutation in *Stat3* in basal keratinocytes causes spontaneous psoriatic-like skin lesions, which demonstrates keratinocyte hyperproliferation, and infiltration of neutrophils and T cells (Sano *et al.*, 2005). A transgenic mouse model in which expression of IL-20 is targeted to basal keratinocytes results in altered keratinocyte differentiation through Stat3 signalling (Blumberg *et al.*, 2001). Alternatively, deletion of a gene, such as in the *Ilrn*^{-/-} mouse results in epidermal thickening and formation of psoriatic plaques, with characteristic neutrophil and T cell infiltration (Shepherd *et al.*, 2004). These models are still being used, but not as often as xenogeneic or inducible models which offer more advantages.

Xenogeneic mouse models involve the transplantation of human psoriatic lesions onto one of three immunodeficient strains. Athymic “nude mice”, which lack functional T cells (Krueger *et al.*, 1981), maintain skin for months (Fraki *et al.*, 1982). SCID mice, which lack functional T and B cells, maintain skin grafts but engrafted leukocytes quickly become anergic (Meyerrose *et al.*, 2003). Finally, AGR129 mice, which lack functional T and B cells and have immature NK cells, develop psoriatic lesions spontaneously when engrafted with psoriatic skin due to activation and proliferation of T cells within the graft (Boyman *et al.*, 2004). However, these models are technically difficult and therefore require extensive expertise, involve transplantation of human psoriatic tissue which is hard to obtain, require concurrent transfer of human leukocytes and have low success rates (Mizutani *et al.*, 2003, Hawkes *et al.*, 2018). As a result of the above limitations and difficulties, most *in vivo* psoriasis models use inducible mouse models.

Inducible mouse models involve the injection or application of cells or molecules to promote inflammation and psoriasis development. A T cell-inducible model involves the reconstitution of SCID mice with naïve CD4⁺ T cells, which subsequently develop psoriatic lesions with epidermal thickening due to keratinocyte hyperproliferation, and infiltration of immune cells (Schön *et al.*, 1997). Alternatively, injection of cytokines such as IL-23 promotes keratinocyte hyperproliferation resulting in epidermal thickening and infiltration of immune cells (Chan *et al.*, 2006, Zheng *et al.*, 2007). However, an emerging model is imiquimod (IMQ)-induced psoriasis-like inflammation in mice.

1.8.1. The IMQ-Induced Psoriasis-Like Inflammation Mouse Model

The IMQ-induced psoriasis-like inflammation mouse model is established by application of the Toll-like receptor (TLR)7/8 agonist IMQ onto mouse skin, as outlined in the landmark study by van der Fits *et al.* (2009). IMQ is a commercially available topical treatment for genital warts (Beutner *et al.*, 1998) and basal cell carcinoma (Beutner *et al.*, 1999), but treatment leads to the development of psoriatic-like lesions on healthy skin after IMQ application (Wu *et al.*, 2004). In humans IMQ induces hyperproliferation of keratinocytes (Ha *et al.*, 2014), pDC infiltration and IL-23 production, which promotes activation and proliferation of Th17 cells (Garzorz-Stark *et al.*, 2018) promoting psoriatic-like lesions.

The original study by van der Fits *et al.* (2009) demonstrates that application of IMQ to the ears or shaved backs of mice, induces redness, scaling and thickening of skin resulting in lesions, with this skin thickening being due to altered differentiation and the hyperproliferation of keratinocytes, similar to that observed in humans. Furthermore, this study demonstrated the immune infiltrates consisted of neutrophils, T cells and professional APCs, also characteristic of psoriatic lesions in humans. Further studies have validated the ability of IMQ to mimic the characteristics and mechanisms of human psoriasis. As

mentioned above (Section 1.6.), IL-23-induced Th17 activation and proliferation has been shown to be important in human psoriasis, and IMQ induces psoriasis-like inflammation in mice via the same mechanism (Yoshiki *et al.*, 2014). Furthermore, the presence of cellular infiltrates including T cells, and APCs including pDCs and LCs have been confirmed in IMQ-induced psoriasis-like lesions (Yoshiki *et al.*, 2014, Ha *et al.*, 2014, Vinter *et al.*, 2015, Garzorz-Stark *et al.*, 2018), while eosinophils promote neutrophil infiltration to further promote inflammation in IMQ-induced lesions (Kim *et al.*, 2018a).

The IMQ-induced psoriasis-like inflammation model offers advantages over other models of psoriasis. Unlike spontaneous or transgenic models, the IMQ-induced psoriasis-like inflammation model better recapitulates human psoriasis *in vivo*, and is easy to establish. Additionally, compared to other inducible models, the IMQ-induced psoriasis-like inflammation model does not require expensive reagents (such as recombinant cytokines), and instead can be established relatively cheaply due to commercially available IMQ in the forms of topical cream, such as Aldara™ (iNova Pharmaceuticals, Chatswood, Australia). Finally, mice are not subjected to invasive procedures such as injections, with cream applied topically with minimal animal handling. The IMQ-induced psoriasis-like inflammation model is currently the most commonly used mouse model of psoriasis, with over 200 published studies noted up to 2016 (Hawkes *et al.*, 2017), and an additional 200 studies published in 2017 and 2018 (<https://www.scopus.com>, last accessed 24/10/2018).

1.9. Purinergic Signalling

As outlined above, therapies for both GVHD and psoriasis are limited in humans, highlighting the need to investigate alternative pathways for these inflammatory disorders.

Purinergic signalling plays an important role in numerous inflammatory disorders, and may

play a role in these diseases. The concept of purinergic signalling was first coined, when the purine-based nucleotide adenosine triphosphate (ATP) was suggested to be an important co-transmitter in the nervous system (Burnstock, 1972). Purinergic signalling is a network involving extracellular nucleotides and nucleosides, plasma membrane receptors and ecto-enzymes (Volonté and D'Ambrosi, 2008) (Figure 1.4). Adenine-based nucleotides and nucleosides, and their respective receptors and ecto-enzymes, are the most widely studied purinergic signalling molecules.

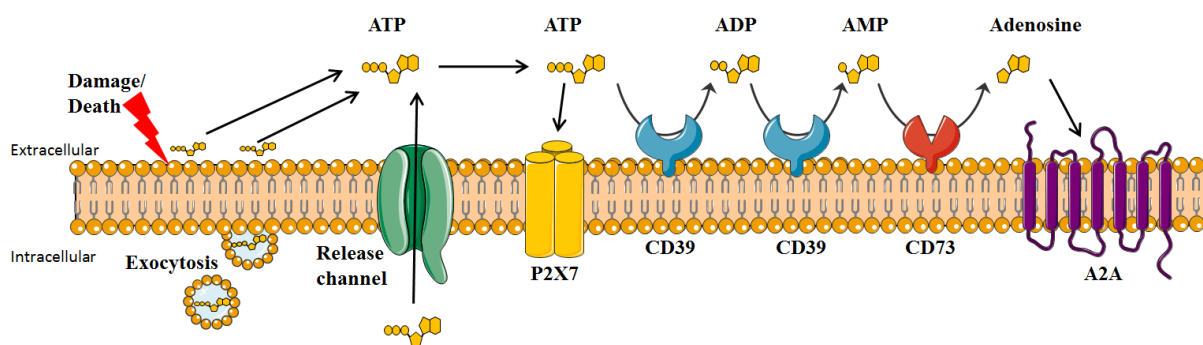


Figure 1.4. The purinergic signalling pathway. ATP is released from damaged or dying cells, or by exocytosis or channels (such as connexin-43 or pannexin-1). Extracellular ATP can activate P2X receptors, including P2X7. ATP is broken down to ADP and AMP by CD39, and AMP is broken down to adenosine by CD73. Adenosine can activate adenosine receptors, including A_{2A} . Finally adenosine is removed by conversion to inosine by adenosine deaminase (not shown). Abbreviations; ADP, adenosine diphosphate, AMP, adenosine monophosphate, ATP, adenosine triphosphate. Figure partly adapted from Junger (2011).

ATP when found extracellularly can act as a DAMP and activate P2 receptors to cause numerous downstream pro-inflammatory effects (Di Virgilio and Vuerich, 2015). ATP can be released into the extracellular space by damaged and/or dying cells via channels such as connexin-43 (Csóka *et al.*, 2015, Qin *et al.*, 2016, Wang *et al.*, 2017) and pannexin-1 (Yang *et al.*, 2015, Lohman *et al.*, 2015, Zhang *et al.*, 2017, Parzych *et al.*, 2017, Saez *et al.*, 2017) or by exocytosis (Sakaki *et al.*, 2013, Ren *et al.*, 2014, Zhang *et al.*, 2017, Kato *et al.*, 2017). This ATP is commonly degraded to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by the cell surface ecto-nucleotidase ecto-nucleoside triphosphate

diphosphohydrolase-1 (CD39), and AMP is subsequently degraded to adenosine by ecto-5'-nucleotidase (CD73) (Zimmermann, 2000). Adenosine can then activate purinergic P1 (adenosine) receptors, which commonly results in anti-inflammatory effects (Antonioli *et al.*, 2013) (Figure 1.4). Purinergic pathway is involved in numerous systems, including the central nervous, cardiovascular, reproductive and immune systems (Burnstock, 2017).

1.9.1. P2 Receptors

1.9.1.1. P2Y Receptors

P2Y receptors are a collection of eight G protein-coupled receptors (GPCRs) (P2Y_{1, 2, 4, 6, 11, 12, 13, and 14}). P2Y receptor stimulation involves the opening or closing of voltage-gated K⁺ (Hille, 1994), Na²⁺ (Cantrell and Catterall, 2001) or Ca²⁺ (Dolphin, 2003) channels and second messenger cascades to exert their effect (Soltoff *et al.*, 1998, Huwiler *et al.*, 2000). P2Y receptors are present on numerous neurological and immune cells (Burnstock and Knight, 2004, Abbracchio *et al.*, 2006). P2Y receptors will not be discussed further, except for a small section on P2Y₂ in GVHD below (Section 1.10.1.1.). For a comprehensive review on P2Y receptors consult Abbracchio *et al.* (2006).

1.9.1.2. P2X Receptors

P2X receptors are a family of seven ligand gated ion channels (P2X₁₋₇) which, upon stimulation by ATP, allow the flux of cations including Na⁺, K⁺ and Ca²⁺ (Khakh *et al.*, 2001). P2X receptors are important in immunity, are present on a number of immune cells and result in numerous downstream signalling effects. For a comprehensive review on P2X receptors see Burnstock and Boeynaems, (2014). In the P2X family of receptors, P2X₄ demonstrates the shortest amino acid sequence and P2X₇ the longest (Valera *et al.*, 1994). Although both subtypes are important in inflammation and immunity, P2X₇ is the most widely studied in this field (Adinolfi *et al.*, 2017).

1.9.1.3. P2X7

P2X7 is encoded by the *P2RX7* gene (Rassendren *et al.*, 1997) located on chr 12 (12q24) in humans (Buell *et al.*, 1998). The *P2RX7* gene is highly polymorphic and contains a number of single nucleotide polymorphisms (SNP) (Sluyter, 2017). P2X7 was originally classified as the “P2Z” receptor, however following the cloning of this receptor was renamed P2X7, becoming the seventh member of the P2X receptor family (P2X1-7) (Surprenant *et al.*, 1996). Each full-length P2X7 subunit has a total molecular weight of 68 kDa (Nicke, 2008) or 75-82 kDa after N-glycosylation (Young *et al.*, 2007, Adinolfi *et al.*, 2010). The original computational structure of P2X7 (Jiang *et al.*, 2013) was based on the crystal structure of the zebrafish P2X4 receptor (Kawate *et al.*, 2009), while the structure of the first native (panda) P2X7 receptor has since been characterised (Karasawa and Kawate, 2016). Overall, recombinant P2X7 has been characterised in 12 different species (seven mammalian and five non-mammalian) (Sluyter, 2017). Each P2X7 subunit is comprised of two transmembrane domains, with the three ATP binding sites present on the extracellular loop, while the carboxyl and amino termini are found intracellularly (Jiang *et al.*, 2013, Karasawa and Kawate, 2016). The structure of the P2X7 receptor is analogous to a “dolphin” with the head, and three fins (dorsal fin, and left and right flippers) representing structurally distinct sections of β -sheets connected to the “body” constituting the extracellular domain, and the “tail” representing the transmembrane proteins anchoring P2X7 to the plasma membrane (Karasawa *et al.*, 2017). P2X7 is expressed on many immune cell types including DCs (Saez *et al.*, 2017), macrophages (De Torre-Minguela *et al.*, 2016), T cells (Sluyter and Wiley, 2014), B cells (Pupovac *et al.*, 2015), NK cells (Gu *et al.*, 2000), neutrophils (Karmakar *et al.*, 2016) and basophils (Tsai *et al.*, 2015) but absent on eosinophils (Mohanty *et al.*, 2001). Expression on mast cells is tissue-dependent (Kurashima *et al.*, 2012).

Due to the expression of P2X7 on a wide range of immune cell subsets, P2X7 plays both a role in innate and adaptive immune responses. In the innate immune system, P2X7 activation is important against bacterial infections, including *Mycobacterium*, *Plasmodium spp.* (Salles *et al.*, 2017) and *Leishmania* (Thorstenberg *et al.*, 2018). However, P2X7 is also important in adaptive immunity. ATP activation of P2X7 can inhibit generation and function of Treg cells (Schenk *et al.*, 2011), and induce apoptosis of Treg and NKT cells (Rissiek *et al.*, 2014). Conversely, P2X7 activation promotes immune homeostasis in follicular Th cells in Peyer's patches (Proietti *et al.*, 2014) and P2X7 is important for survival of memory CD8⁺ T cells (Savio *et al.*, 2018).

P2X7 exerts its effects through a variety of downstream effects including reactive oxygen species formation (Bartlett *et al.*, 2013, Moreira-Souza *et al.*, 2017), shedding of surface molecules such as CD23 (Pupovac *et al.*, 2015), CD62L (Gu *et al.*, 1998, Sluyter and Wiley, 2002) and TNF- α (Barberà-Cremades *et al.*, 2017), and up-regulation of CD80 and CD86 (Wilhelm *et al.*, 2010, Lioi *et al.*, 2015). P2X7 activation also causes NLRP3 inflammasome activation and release of IL-1 β and IL-18 (Buell *et al.*, 1998, Piccini *et al.*, 2008), IL-2 (Yip *et al.*, 2009), IL-6 (Solini *et al.*, 1999, Caporali *et al.*, 2008), IL-17 (Ghiringhelli *et al.*, 2009), IL-18 (Piccini *et al.*, 2008), and prostaglandin E2 (Barberà-Cremades *et al.*, 2012). P2X7 activation can also promote cell death, through apoptosis (Zheng *et al.*, 1991), necrosis (Dagvadorj *et al.*, 2015), or pyroptosis (Vanderpuye-Orgle *et al.*, 2015). The polymorphic nature of *P2RX7* also impacts the downstream effects of P2X7. For example loss-of-function SNPs such as G496A impair ATP-induced IL-1 β and IL-18 release from monocytes (Sluyter *et al.*, 2004b, Sluyter *et al.*, 2004a). In contrast, gain-of-function SNPs such as A348T, increase ATP-induced IL-1 β release from monocytes (Stokes *et al.*, 2010). *P2RX7* SNPs also play a role in some diseases where P2X7 is implicated as discussed below (Section 1.8.1.).

Similar to other P2X receptors, P2X7 is a trimeric channel stimulated by binding of ATP to its extracellular domain to promote subunit rearrangement and opening of a cation channel (Di Virgilio *et al.*, 2018). However, P2X7 is less sensitive to ATP compared to other P2X receptors, requiring $>100 \mu\text{M}$ for activation (Khakh and North, 2006) and therefore can detect large concentrations of extracellular ATP released during cellular stress and damage. Activation of P2X7 results in rapid influx of Na^+ and Ca^{2+} and efflux of K^+ (Surprenant *et al.*, 1996, Rassendren *et al.*, 1997, Egan and Khakh, 2004). However, pro-longed activation of P2X7 also causes the formation of a pore, which can be observed by measuring the uptake of dyes such as 6-carboxyfluorescein (6-FAM), lucifer yellow, fura-2 (Steinberg *et al.*, 1987), ethidium²⁺ (Wiley *et al.*, 1993) and YO-PRO-1²⁺ (Surprenant *et al.*, 1996), but not dyes >900 kDa (Steinberg *et al.*, 1987, Surprenant *et al.*, 1996). P2X7 is believed to be the pore forming molecule (Di Virgilio *et al.*, 2018), however other molecules such as pannexin-1 may also be involved (Pelegrin and Surprenant, 2006) but their relative contribution remains to be resolved.

Despite past and current debates of P2X7 pore formation, ATP-induced dye uptake remains an efficient way to measure P2X7 activity *in vitro* (Jursik *et al.*, 2007, Korpi-Steiner *et al.*, 2008). ATP is the physiological agonist of P2X7, with a half maximal excitatory concentration (EC_{50}) of $80\text{-}100 \mu\text{M}$ at human P2X7 (Wiley *et al.*, 2011). However, the ATP analogue, 2',3'-(4-benzoyl)-benzoyl-adenosine triphosphate (BzATP) is a more potent agonist with an EC_{50} of $7\text{-}20 \mu\text{M}$ at human P2X7 (Carroll *et al.*, 2009, Wiley *et al.*, 2011). Notably, P2X7 can also be activated by antimicrobial peptides such as LL-37, which directly activates P2X7 to cause Ca^{2+} influx, pore formation, and cell proliferation *in vitro* in transfected HEK-293 cells (Tomasinsig *et al.*, 2008).

A number of P2X7 antagonists are commercially available (Bartlett *et al.*, 2014), including Brilliant Blue G (BBG), a non-toxic analogue of the US food and drug administration (FDA)

approved food dye Brilliant Blue FCF (Apolloni and Volonte, 2013), as well as the nucleoside reverse transcriptase inhibitor stavudine (Fowler *et al.*, 2014). The antagonists most relevant to this thesis and the half maximal inhibitory concentrations (IC₅₀) of each are summarised in Table 1.2.

Table 1.2. P2X7 antagonists, and the IC₅₀ exhibited at human P2X7. Abbreviations: IC₅₀, half maximal inhibitory concentration, N.D., not determined.

Antagonist	IC ₅₀ (nM)	Reference
Brilliant Blue G (BBG)	300	(Jiang <i>et al.</i> , 2000)
Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS)	1200	(Donnelly-Roberts <i>et al.</i> , 2009a)
KN-62	12.7	(Gargett and Wiley, 1997)
Stavudine	N.D.	(Fowler <i>et al.</i> , 2014)
A804598	11	(Donnelly-Roberts <i>et al.</i> , 2009b)

1.9.2. Ecto-nucleotidases

Whilst ATP plays a role in the activation of P2 receptors including P2X7 activation in inflammation (Adinolfi *et al.*, 2017), this nucleotide is quickly degraded by ecto-nucleotidases to adenosine in a step-wise manner to limit inflammation. Ecto-nucleotidases are a family of cell surface enzymes that hydrolyse nucleotides and are an important part of the purinergic signalling system throughout the body. They consist mainly of four families; the ecto-nucleoside triphosphate di-phosphohydrolases (E-NTPDases); ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs); alkaline phosphatases (APPs); and ecto-5'-nucleotidases (Zimmermann, 2000). As noted above, the ecto-nucleotidases, CD39

and CD73 mediate the stepwise conversion of ATP to adenosine. Together these ectoenzymes play major roles in inflammation and immunity as outlined below.

1.9.2.1. CD39

CD39 is encoded by the *ENTPDI* gene located on human chr 10 (10q24.1) (Maliszewski *et al.*, 1994). CD39 is composed of two transmembrane domains, with the carboxyl and amino domains found intracellularly, and a large hydrophobic domain found extracellularly (Heine *et al.*, 2001). CD39 has a molecular mass of 70-100 kDa, depending on the extent of glycosylation (Kaczmarek *et al.*, 1996). Originally thought to be a marker of B cell activation (Ling *et al.*, 1989), CD39 has since been found to be present on T cells (Kansas *et al.*, 1991), monocytes, DCs (Koziak *et al.*, 1999) and NK cells (Kansas *et al.*, 1991). CD39 is responsible for Ca^{2+} - and Mg^{2+} -dependent degradation of extracellular ATP to ADP and subsequently to AMP (Heine *et al.*, 2001). The crystal structure of CD39 was partially resolved after over expression in Chinese hamster ovary cells (Zhong *et al.*, 2008), however the full structure has since been characterised from a bacterium (*Legionella pneumophila*) (Zebisch *et al.*, 2012, Zebisch *et al.*, 2013). The full structure of CD39 revealed that both ATP and ADP are hydrolysed at the same site, and this can be done sequentially, demonstrating the efficient removal of ATP by CD39 (Zebisch *et al.*, 2012, Zebisch *et al.*, 2013). Notably, effects of CD39 can be mimicked in part by the use of the ATPDase apyrase (Handa and Guidotti, 1996).

Antagonists of CD39 include nucleotide-based compounds modelled from the structure of ATP, including ARL67156, which is a weak inhibitor of human CD39 with an IC_{50} of 24 μM (Crack *et al.*, 1994) and 8-BuS-ATP with an IC_{50} of $5 \pm 3 \mu\text{M}$ (Lecka *et al.*, 2013). Non-nucleotide-based inhibitors of human CD39 such as PSB-069, with an IC_{50} of 1.6 μM (Longhi *et al.*, 2017) and suramin, with an IC_{50} of 0.3 mM (Leal *et al.*, 2005) are non-specific (Baqi, 2015). Other non-nucleotide inhibitors belonging to the polyoxometalate (POM) group

of compounds are relatively specific to CD39 (Muller *et al.*, 2006). However, at least one of these compounds (POM-1), which is commonly used as a specific CD39 antagonist, blocks murine P2X7 (Pimenta-Dos-Reis *et al.*, 2017). Thus, further investigation of the specificity of these compounds is warranted.

1.9.2.2. CD73

CD73 is encoded by the *NT5E* gene located on human chr 6 (6q14.3) (Boyle *et al.*, 1988). CD73 is a 71 kDa (Misumi *et al.*, 1990) glycosylphosphatidylinositol-linked protein (Zimmermann, 1992). CD73 is present on DCs (Berchtold *et al.*, 1999) and T cells (Thomson *et al.*, 1990), but CD73 is absent on NK cells (Dalh Christensen and Andersen, 1992) and monocytes (Clifford *et al.*, 1997). CD73 is responsible for the degradation of AMP to adenosine (Pearson *et al.*, 1980), which is achieved by exposure of the active site when CD73 changes from the closed to open state, due to the large flexibility of CD73 homodimers (Knapp *et al.*, 2012).

Reactive blue 2 is an antagonist of human CD73, with a K_i of 20 μM (IC_{50} not determined) (Baqi *et al.*, 2010), but is non-specific exerting antagonistic properties at numerous human P2 receptors (Baqi, 2015). The specific antagonist $\alpha\beta$ -methylene-ADP (APCP), can keep CD73 in the closed state (Knapp *et al.*, 2012) and demonstrates an IC_{50} of 3.6-5.6 μM at human CD73 (Crane *et al.*, 2007, McManus *et al.*, 2018). However, APCP is not specific to CD73 as it has since been shown to also inhibit CD39 (Covarrubias *et al.*, 2016).

1.9.3. P1 Receptors

Adenosine receptors, also known as P1 receptors, consist of four subtypes of GPCRs (A_1 , A_{2A} , A_{2B} and A_3) which, as the name suggests, are all activated by extracellular adenosine. Under physiological conditions, activation of A_2 receptors generally results in release of cytokines that promote an anti-inflammatory effect, while A_1 and A_2 can have either pro- or

anti-inflammatory effects. The expression of different subtypes of adenosine receptors varies between cell types (Haskó *et al.*, 2008). A_{2A} and A_{2B} bind the G-protein G_s, which stimulates adenylyl cyclase to produce cAMP. Conversely, A₁ and A₃ bind G_{i/o} to inhibit adenylyl cyclase (Fredholm *et al.*, 2011a). A_{2A} and A_{2B} share 59% sequence homology (Lucattelli *et al.*, 2011), but A_{2A} is better characterised than A_{2B} and more commonly is the target of therapies for diseases, such as Parkinson's and Huntington's disease, currently in human trials (Borea *et al.*, 2018). All four adenosine receptor subtypes are discussed below.

1.9.3.1. A₁

A₁ is encoded by the *ADORA1* gene located on human chr 1 (1q32.1) (Townsend-Nicholson *et al.*, 1995). Similar to all adenosine receptors, A₁ consists of seven transmembrane proteins (Cheng *et al.*, 2017). A₁ can be expressed as homodimers (Ciruela *et al.*, 1995, Namba *et al.*, 2010), however A₁ has also been shown to form heterodimers with β-adrenergic receptors (Komatsu *et al.*, 2012, Chandrasekera *et al.*, 2013), and dopamine receptors (Gines *et al.*, 2000). Furthermore, A₁ can also form heterodimers with other purinergic receptors including A_{2A} (Ciruela *et al.*, 2006) and P2Y₁ (Yoshioka *et al.*, 2001, Yoshioka *et al.*, 2002).

A₁ predominantly plays a role in the nervous system (Chen *et al.*, 2013), but is found on immune cells including DCs (Panther *et al.*, 2001, Schnurr *et al.*, 2004), monocytes and macrophages (Eppell *et al.*, 1989, Thiele *et al.*, 2004), T and B cells (Takahashi *et al.*, 2007), NK cells (Priebe *et al.*, 1990), as well as neutrophils (Cronstein *et al.*, 1990) and eosinophils (Kohno *et al.*, 1996). In innate immunity, A₁ activation on neutrophils promotes a pro-inflammatory response, promoting chemotaxis of neutrophils and their adhesion to endothelial cells (Cronstein *et al.*, 1990, Cronstein *et al.*, 1992). Furthermore, A₁ activation and promotes superoxide release from neutrophils (Cronstein *et al.*, 1990) and eosinophils (Ezeamuzie and Philips, 1999). In adaptive immunity, A₁ activation results in chemotaxis of

DCs and stimulation of T cells (Panther *et al.*, 2001), T and B cell proliferation (Takahashi *et al.*, 2007) and NK cell activation (Priebe *et al.*, 1990).

Adenosine is the physiological agonist of A₁, however a number of selective agonists and antagonists exist. The agonist N⁶-cyclopentyladenosine (CPA) demonstrates a K_i of 2.25 ± 0.18 nM (Cappellacci *et al.*, 2008), 2-chloro-N⁶-cyclopentyladenosine (CCPA) demonstrates a K_i of 0.8 ± 0.06 nM (Cappellacci *et al.*, 2008) and N⁶-cyclohexyladenosine demonstrates a K_i of 7.7 ± 2.2 nM (Kollias-Baker *et al.*, 1997). Conversely, caffeine is commonly used as a general adenosine receptor antagonist, due to its low cost and wide availability (Sattin and Rall, 1970). However, the specific A₁ antagonist DPCPX exhibits a K_i of 3.9 nM, but no IC₅₀ has been published (Klotz, 2000).

1.9.3.2. A_{2A}

A_{2A} is encoded by the *ADORA2A* gene located on human chr 22 (22q11.23) (MacCollin *et al.*, 1994). The crystal structure demonstrates A_{2A} consists of seven transmembrane proteins, and exhibits a unique binding site consisting of four disulphide bonds in the extracellular domain (Jaakola *et al.*, 2008, Lebon *et al.*, 2011b, Weinert *et al.*, 2017), but unlike the other three adenosine receptors, A_{2A} is the only adenosine receptor lacking a site for palmitoylation at the carboxyl terminus (Linden, 2001).

A_{2A} is constitutively expressed as homodimers (Canals *et al.*, 2004), but can interact with other proteins at the cell surface, forming heterodimers with cannabinoid receptors (Carriba *et al.*, 2007, Navarro *et al.*, 2008) and dopamine receptors (Hillion *et al.*, 2002). Association of A_{2A} and dopamine receptors can also lead to the formation of heterotrimers with metabolic glutamate type 5 receptors (Cabello *et al.*, 2009). Furthermore, a seminal paper by Moriyama and Sitkovsky (2010), demonstrated that A_{2A} is required for transport of A_{2B} to the cell surface, as the majority of A_{2B} is degraded by the proteasome in A_{2A} KO cells.

Similar to A_1 , A_{2A} plays a role in the nervous system, and therefore is found in the striatum and other brain regions (Rosin *et al.*, 1998, Lopes *et al.*, 2002, Rebola *et al.*, 2005). However, due to the anti-inflammatory role in immunity, A_{2A} is present on numerous immune subtypes including granulocytes (Sullivan and Linden, 1998), APCs including DCs (Panther *et al.*, 2001), monocytes and macrophages (Eppell *et al.*, 1989, Thiele *et al.*, 2004), T and B cells (Huang *et al.*, 1997), NK cells (Priebe *et al.*, 1990) and mast cells (Ryzhov *et al.*, 2008). A_{2A} plays a role in both adaptive and innate immunity. Activation of A_{2A} on neutrophils inhibits their adhesion to endothelial cells (Eltzschig *et al.*, 2004), reduces reactive oxygen species production (Cronstein *et al.*, 1990), and downregulates L-selectin and β_2 -integrin (Thiel *et al.*, 1996). A_{2A} activation prevents phagocytosis in monocytes (Salmon *et al.*, 1993) and macrophages (Eppell *et al.*, 1989). Upon activation DCs up-regulate A_{2A} expression (Panther *et al.*, 2001, Fossetta *et al.*, 2003) and concomitantly decrease production of pro-inflammatory IL-12, and increase production of anti-inflammatory IL-10 (Banchereau and Steinman, 1998, Panther *et al.*, 2001, Panther *et al.*, 2003). A_{2A} activation on T cells inhibits IFN- γ , TNF- α and IL-4 production (Lappas *et al.*, 2005, Odashima *et al.*, 2005). Furthermore, A_{2A} activation inhibits T cell activation, leading to reduced inflammatory activity (Lappas *et al.*, 2005) and proliferation (Takahashi *et al.*, 2007), while A_{2A} activation on NK cells inhibits their activity (Priebe *et al.*, 1990).

The determination of the crystal structure of A_{2A} in complex with the antagonist ZM241385 identified the ability of this antagonist to keep the receptor in an inactive state, potentially due to the interaction with a tryptophan residue important for its activation (Jaakola *et al.*, 2008). Further characterisation of A_{2A} in complex with the antagonist ZM241385 also identified an allosteric pocket (Sun *et al.*, 2017), whilst A_{2A} in complex with other antagonists (xanthines) (Cheng *et al.*, 2017) may allow development of alternative agonists and antagonists. Characterisation of the structure with an engineered G protein demonstrates how A_{2A} is

activated, and A_{2A} is used as an example to allow elucidation of information about other GPCRs (Carpenter *et al.*, 2016). A_{2A} activation on immune cells stimulates adenylyl cyclase to promote cyclic AMP (cAMP) production, exerting an anti-inflammatory effect by preventing T cell activation (Huang *et al.*, 1997) and reducing pro-inflammatory cytokine release (Ohta and Sitkovsky, 2001).

Adenosine is the physiological agonist of A_{2A} , however a number of potent A_{2A} agonists have been developed. CGS-21680 demonstrates a K_i of 27 nM (Klotz *et al.*, 1998) and an EC_{50} of 24-33 nM at human A_{2A} (Himer *et al.*, 2010). Similarly, ATL-146e demonstrates a K_i value of 11 nM (but no EC_{50} determined) at human A_{2A} (Lebon *et al.*, 2011a), but is not commercially available due to its current use in clinical trials as a therapy for heart conditions (Gao and Jacobson, 2011). Specific antagonists of A_{2A} include ZM241385 (but no IC_{50} determined for human A_{2A}) and SCH-58261, which demonstrates an IC_{50} of 15 ± 3 nM at human A_{2A} (Varani *et al.*, 1996).

1.9.3.3. A_{2B}

A_{2B} is encoded by the *ADORA2B* gene located on human chr 17 (17p12) (Jacobson *et al.*, 1995). Similar to other adenosine receptors A_{2B} consists of seven transmembrane proteins, but also possesses a unique binding site for hypoxia-inducible factor, important for cellular responses during hypoxia and inflammation (Kong *et al.*, 2006, Gessi *et al.*, 2010). Furthermore, A_{2B} is unique from other adenosine receptors requiring higher (micromolar) concentrations for activation (Fredholm *et al.*, 2011b). A_{2B} can be expressed as homodimers (Moriyama and Sitkovsky, 2010), but as mentioned above (section 1.9.5.) A_{2B} can also form heterodimers with A_{2A} (Moriyama and Sitkovsky, 2010), as well as with other molecules including adenosine deaminase (Herrera *et al.*, 2001, Pacheco *et al.*, 2005), SNARE proteins (Wang *et al.*, 2004) and netrin-1 (Corset *et al.*, 2000).

A_{2B} is found on immune cells including monocytes and macrophages (Eppell *et al.*, 1989, Thiele *et al.*, 2004), T and B cells (Gessi *et al.*, 2005), neutrophils (Eckle *et al.*, 2008) and mast cells (Feoktistov and Biaggioni, 1995). A_{2B} was originally thought to be absent on DCs (Panther *et al.*, 2001), but has since been shown to be expressed (Addi *et al.*, 2008, Novitskiy *et al.*, 2008) on these cells. Expression of A_{2B} on neutrophils is controversial. Using Western blotting, one study showed A_{2B} is not present on neutrophils (Fortin *et al.*, 2006), while others have demonstrated the presence of functional A_{2B} on these cells (Wakai *et al.*, 2001, Thiel and Chouker, 1995). A_{2B} plays roles in both innate and adaptive immunity. Activation of A_{2B} on neutrophils inhibits their migration and adhesion to endothelial cells (Wakai *et al.*, 2001) and prevents TNF- α production (Thiel and Chouker, 1995), whilst A_{2B} activation on monocytes promotes production of the anti-inflammatory cytokine IL-10 (Nemeth *et al.*, 2005). In adaptive immunity, A_{2B} activation on T cells reduces IL-2 production (Mirabet *et al.*, 1999).

Similar to the other adenosine receptors, adenosine is the physiological antagonist of A_{2B}, however unlike the other receptors, no specific agonists for A_{2B} exist (Fredholm *et al.*, 2011b). Nevertheless, MRS1754 is a specific A_{2B} antagonist, which exhibits a K_i value of 403 ± 194 nM but no published IC₅₀ value (Kim *et al.*, 2000).

1.9.3.4. A₃

A₃ is encoded by the *ADORA3* gene located on human chr 1 (1p13.2) (Monitto *et al.*, 1995). Similar to all adenosine receptors, A₃ consists of seven transmembrane proteins, and is rapidly desensitised upon activation (Palmer and Stiles, 2000). A₃ is expressed as a homodimer and has been shown to form heterodimers (May *et al.*, 2011), but the specific binding partners have not been identified.

A₃ is expressed on immune cells including DCs (Fossetta *et al.*, 2003, Panther *et al.*, 2001), monocytes and macrophages (Eppell *et al.*, 1989, Thiele *et al.*, 2004), T and B cells (Gessi *et al.*, 2004), neutrophils (Bouma *et al.*, 1997, Walker *et al.*, 1997b), eosinophils (Walker *et al.*, 1997a) and mast cells (Salvatore *et al.*, 2000, Zhong *et al.*, 2003). A₃ is important in both innate and adaptive immunity. A₃ activation on neutrophils results in their migration in response to chemoattractant release by microbes (Chen *et al.*, 2006), whilst activation on monocytes reduces IL-12 production (Ia Sala *et al.*, 2005). In adaptive immunity, A₃ activation on lymphocytes promotes their proliferation (Takahashi *et al.*, 2007).

Adenosine is the physiological agonist of A₃, whilst a selective agonist Cl-IB-MECA demonstrates a K_i of 3.16 ± 0.007 nM at human A₃ (Xia *et al.*, 2018). Specific A₃ antagonists are derived from non-purine based classes, including flavonoids and dihydropyridines (Jacobson *et al.*, 1997). Two examples include MRS-1292 with a K_i of 29 nM (Gao *et al.*, 2002) and an IC₅₀ of 41 ± 11 nM (Yang *et al.*, 2005a), and MRS-3558 with a K_i of 0.3 nM (Joshi and Jacobson, 2005) and an IC₅₀ of 0.4 nM (Zhang *et al.*, 2010).

1.10. Purinergic Signalling in Inflammatory Disorders

Extracellular ATP exerts pro-inflammatory effects in numerous diseases and ATP activation of P2X7 is implicated in a number of these diseases (Adinolfi *et al.*, 2017). Breakdown of ATP to adenosine by CD39 and CD73 on numerous cell types typically results in beneficial anti-inflammatory effects (Bours *et al.*, 2006) and is important in reducing inflammation in numerous inflammatory diseases (Antonioli *et al.*, 2013). CD39 is important for adenosine generation and Treg cell-mediated immune suppression (Deaglio *et al.*, 2007). The immunosuppressive function of Treg cells may in fact rely on the conversion of ATP to adenosine (Deaglio *et al.*, 2007, Ernst *et al.*, 2010).

In tissue transplantation, genetic differences in tissues or cells transplanted from one individual to another is inevitable, and as mentioned above these differences can result in immune responses and inflammation, potentially in the form of GVHD. Due to the importance of purinergic signalling in immunity and inflammation, it is therefore likely purinergic signalling plays a role in transplantation and GVHD. Alternatively, the skin is highly immunogenic and in the case of skin diseases such as psoriasis, where chronic inflammation is a characteristic of pathogenesis, it is likely purinergic signalling also plays a role. The roles of purinergic signalling in transplantation and GVHD, as well as skin biology and psoriasis are summarised below.

1.10.1. Purinergic Signalling in Transplantation

Purinergic signalling has been implicated in transplantation, predominantly studied in the context of whole organ transplantation. Increased ATP concentrations and activation of the P2X7 receptor can increase the risk of whole-organ rejection in heart (Vergani *et al.*, 2013b), lung (Liu *et al.*, 2014), islet (Vergani *et al.*, 2013a), and liver and skin (Amores-Iniesta *et al.*, 2017) transplants. Similarly, genetic deficiency of CD39 or CD73 causes increased ischemia in heart (Enjyoji *et al.*, 1999, Dwyer *et al.*, 2004, Eckle *et al.*, 2007), liver (Yoshida *et al.*, 2015) and renal (Lu *et al.*, 2008) transplants, however genetic deficiency of CD73 was found to be protective in one study of renal transplantation (Rajakumar *et al.*, 2010). Conversely, increased CD39 and CD73 expression, correlates to reduced ischemia in heart (Dwyer *et al.*, 2004), renal (Grenz *et al.*, 2007b, Grenz *et al.*, 2007a, Crikis *et al.*, 2010) and liver (Yoshida *et al.*, 2013) transplantation. The degradation of ATP to adenosine by CD39 and CD73 is important as activation of A_{2A} by adenosine reduces damage and rejection of lung (Sharma *et al.*, 2009, Gazoni *et al.*, 2010), islet (Chhabra *et al.*, 2010), renal (Lee and Emala, 2001, Crikis *et al.*, 2010), and skin (Sevigny *et al.*, 2007) transplants.

Whilst purinergic signalling plays a role in whole organ transplantation, it is also implicated in HSCT. A study by (Lee *et al.*, 2007) demonstrated that *P2RX7* genotypes influence overall survival in HSCT patients. HSCT recipients demonstrated significantly reduced survival when their *P2RX7* genotype, or the *P2RX7* genotype of the donor, was homozygous for the SNP encoding the mutation E496A compared to donors wild-type or heterozygous for this allele. However, this finding was not replicated in a larger study (Karaesmen *et al.*, 2017). A recent abstract by Koldej *et al.* (2018) demonstrates that homozygosity for gain-of-function *P2RX7* SNPs (T375S, G150R, Q460R and A348T) leads to a reduced relapse-free survival and overall survival in HSCT patients, however this data is yet to be published in full. Moreover, both pharmacological and genetic evidence, mainly in allogeneic mouse models, suggests a role for purinergic signalling in GVHD, as discussed below.

1.10.1.1. P2Y2 in GVHD

P2Y2 has been implicated in GVHD, but findings are limited and will only be discussed briefly. P2Y2 is present on neutrophils (Zhang *et al.*, 1996), eosinophils (Mohanty *et al.*, 2001), mast cells (Gao *et al.*, 2013), monocytes (Aga *et al.*, 2002), macrophages (Coutinho-Silva *et al.*, 2005), DCs (Berchtold *et al.*, 1999), as well as T and B cells (Koshiba *et al.*, 1997, Trabanelli *et al.*, 2012). Notably, P2Y2 deficient mice demonstrate reduced mortality, serum IL-6, and histological damage in an allogeneic mouse model of GVHD (Klambt *et al.*, 2015). Specific genetic deletion of P2Y2 in hematopoietic tissues and more specifically on inflammatory myeloid cells reduces GVHD severity (Klambt *et al.*, 2015), while a lack of P2Y2 in Treg cells worsens GVHD (Dürr *et al.*, 2012). Collectively, this suggests both pro- and anti-inflammatory roles of P2Y2 in GVHD potentially limiting its therapeutic potential.

1.10.1.2. P2X7 in GVHD

Evidence for the role of P2X7 in GVHD comes indirectly from studies of downstream effects of P2X7 activation, as well as studies of pharmacological blockade and/or genetic deficiency

of P2X7 in allogeneic mouse models of GVHD. In allogeneic mouse models of GVHD, indirect evidence of the role of P2X7 in GVHD is supported by genetic deletion or pharmacological blockade of IL-1 (the super family of cytokines, including IL-1 β), with the recombinant IL-1 receptor antagonist “anakinra” (10 mg/kg injected i.p. on days -1, 0, 1, 2, 4, 6, and 8) or a neutralising anti-IL-1 β antibody (10 mg/kg injected i.p. on day -1), which delayed GVHD and significantly increased survival (Jankovic *et al.*, 2013). Additionally, genetic deletion of the NLRP3 inflammasome in the host, but not the donor, also reduced GVHD-associated weight loss, histological damage and mortality (Jankovic *et al.*, 2013). Furthermore, micro-RNA (miR)-155, which regulates inflammasome activation and IL-1 β production, as well as IL-6 and TNF- α production (Ceppi *et al.*, 2009), is upregulated in activated T cells in mice with acute GVHD (Ranganathan *et al.*, 2012). Genetic deletion of miR-155 results in reduced damage to target organs and an increased survival due to a reduced number of activated T cells (Ranganathan *et al.*, 2012) as well as reduced DC migration, inflammasome activation and IL-1 β production (Chen *et al.*, 2015).

The role of P2X7 activation by ATP in promoting GVHD pathogenesis has been demonstrated by pharmacological blockade and genetic deletion of P2X7 in allogeneic mouse models of GVHD. First, ATP was shown to be released at the sites of tissue damage in HSCT, where activation of P2X7 causes upregulation of the co-stimulatory molecules, CD80 and CD86 on DCs to promote T cell responses (Wilhelm *et al.*, 2010). Second, apoptosis and inflammation of target organs, as well as serum IFN- γ , was reduced in the presence of either apyrase (4U injected i.p. days 0–2 and 6–8), or PPADS (10 μ mol injected i.p. daily, days 0-10) or KN-62 (1 μ mol injected i.p. daily, days 0-10). Finally, genetic deletion of P2X7 in the host, but not the donor cells, reduces serum concentrations of the pro-inflammatory cytokines IFN- γ , TNF- α , and IL-6, and prolonged survival (Wilhelm *et al.*, 2010). Fowler *et al.* (2014) validated these results by demonstrating that injection of stavudine (25 mg/kg,

twice daily days 0-10) reduces weight loss and serum IFN- γ , TNF- α , and IL-6 concentrations, and prolonged survival. Finally, P2X7 blockade using BBG (50 mg/kg or 75 mg/kg, injected i.p. twice weekly for up to four weeks) reduces liver damage, as well as hepatic expression of P2X7, and the pro-inflammatory cytokines IL-1 β and IL-18 (Zhong *et al.*, 2016).

Clinical studies in humans also support a potential role for P2X7 in GVHD. In HSCT, the presence of loss-of-function SNPs, E496A or I568N in the recipient reduced the incidence of GVHD (Koldej *et al.*, 2018), however this data is yet to be published in full. Furthermore, P2X7 is increased on circulating PBMCs and higher numbers of P2X7 expressing cells are found in the colons of HSCT patients with GVHD (Wilhelm *et al.*, 2010). The role of P2X7 in this disease has not been examined in a humanised mice mouse model of GVHD and is limited in humans.

1.10.1.3. CD39 and CD73 in GVHD

ATP is released at the sites of damage in HSCT recipients to promote inflammation but its degradation by apyrase can prevent GVHD (Wilhelm *et al.*, 2010). Moreover, CD39 and CD73 have been shown to be important in preventing GVHD, in both *in vitro* and *in vivo* allogeneic mouse models of GVHD.

In mixed lymphocyte reactions (MLRs), mesenchymal stem cells express increased CD39 and T cells express increased CD73, to increase adenosine concentrations and reduce activation of conventional T cells (Saldanha-Araujo *et al.*, 2011). Additionally, in co-cultures of Treg and conventional T cells, up-regulation of CD39 and conversion of ATP to adenosine by Treg cells reduces T cell stimulation and inflammation through a reduction in NOTCH1 signalling (Del Papa *et al.*, 2017). Pharmacological blockade of CD73 in allogeneic MLRs using the antagonist APCP (100 μ M) reduces suppression of T cell proliferation by Treg cells and increases IFN- γ concentrations (Wang *et al.*, 2013). Similarly, an anti-human CD73 mAb

(clone 2C5) increases TNF- α , IL-1 β and IFN- γ concentrations in allogeneic MLRs (Young *et al.*, 2016).

Studies have also implicated roles for CD39 and CD73 in allogeneic mouse models of GVHD. Inhibition of aurora kinase A and JAK2 with the dual antagonist AJI-214, increases CD39 expression on the cell surface of Treg cells to promote degradation of extracellular ATP and reduce GVHD (Betts *et al.*, 2017). Furthermore, injection of another dual aurora kinase A/JAK2 antagonist AJI-100 (50 mg/kg, injected i.p. daily, days 0-14) suppressed expansion of activated human CD4⁺ T cells, Th17 cells, CD8⁺ T cells, and improved survival in the humanised NSG mouse model of GVHD (Betts *et al.*, 2017). This effect was shown to be mediated through human CD39^{hi} cells (Gu *et al.*, 2016b). In an allogeneic mouse model of GVHD, Tsukamoto *et al.* (2012) demonstrated that pharmacological blockade of CD73 using APCP (50 mg/kg, injected i.p. daily, days 0-6), increases disease-related mortality. Similarly, Wang *et al.* (2013) demonstrated APCP injection (20 mg/kg injected i.v. twice weekly) also increases GVHD mortality, and increases splenic CD4⁺ and CD8⁺ T cell numbers and serum IFN- γ and IL-6 concentrations. Although both these studies used APCP as a CD73 inhibitor, this compound also blocks CD39 (Covarrubias *et al.*, 2016), so a role for CD39 in this process cannot be excluded. However, genetic deficiency of CD73 also increases histological damage of target organs, splenic CD4⁺ and CD8⁺ T cell numbers and serum IFN- γ and IL-6 concentrations and worsens mortality in allogeneic mouse models of GVHD (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013).

In human allogeneic MLRs, human gingiva-derived mesenchymal stem cells fail to reduce T cell suppression in the presence of APCP (200-500 μ M) or POM-1(100 μ M) (Huang *et al.*, 2017). Whilst, transfer of human gingiva-derived mesenchymal stem cells into humanised NOD/SCID mice reduced histological damage, IL-4, IL-17, IFN- γ , IL-2 and TNF- α production by T cells, and mortality, mediated through adenosine production. Notably, these

effects are lost if these cells were incubated with POM-1 (100 μ M) prior to transplantation (Huang *et al.*, 2017).

In conclusion, whilst CD39 and CD73 have been shown to be important in reducing disease severity in allogeneic mouse models, the role of these ecto-enzymes in disease development in a humanised NSG mouse model of GVHD has not been investigated and studies in humans are limited.

1.10.1.4. A_{2A} in GVHD

The role of A_{2A} has also been implicated in GVHD in allogeneic mouse models of this disease. The non-selective adenosine receptor antagonist caffeine (10 mg/kg injected i.p. day 0-6, and 3-5 times a week onwards) worsens mortality (Tsukamoto *et al.*, 2012). Moreover, activation of A_{2A} was identified as the adenosine receptor subtype responsible for exerting anti-inflammatory effects in allogeneic mouse models of GVHD, as blockade with the A_{2A} antagonist SCH58261, but not the A_{2B} antagonist MRS1754 (both 2 mg/kg, injected i.p. daily day -2-12) increased serum TNF- α , IFN- γ and IL-6, and CD4⁺ and CD8⁺ T cell numbers, and worsened mortality (Wang *et al.*, 2013). Additionally, genetic deficiency of A_{2A} increased CD4⁺ and CD8⁺ T cell numbers (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013), but the effect on mortality was not reported in either study.

Supporting the role of A_{2A} in GVHD, Lappas *et al.* (2010) demonstrated that treatment with the A_{2A} receptor agonist ATL-146e increased serum IL-10, and reduced serum IFN- γ and IL-6. Furthermore, ATL-146e reduced activated splenic CD4⁺ and CD8⁺ T cell numbers, decreased T cell infiltration into target organs and reduced weight loss and mortality in an allogeneic mouse model of GVHD. ATL-146e, as well as the other agonists ATL-370 and ATL-1223, also increased donor derived Treg cells in both the skin and colon in this same model (Han *et al.*, 2013). Finally, Amarnath *et al.* (2015) demonstrated that bone derived

mesenchymal stromal cells reduced IFN- γ - and TNF- α -producing leukocytes, and reversed disease. This beneficial effect is abrogated by the A_{2A} antagonist ZM241385 (1.5 mg/kg injected i.p. daily days 20-35), which prevented T cell suppression, worsened disease and reduced survival in a humanised mouse model of GVHD (Amarnath *et al.*, 2015).

1.10.1.5. Summary

Combined, the above shows that purinergic signalling plays an important role in disease development in allogeneic mouse models of GVHD. Extracellular ATP, released at sites of damage during transplantation can activate P2X7 to promote T cell activation, pro-inflammatory cytokine release and damage to target organs, leading to further ATP release and a feedforward loop of inflammation promoting GVHD. Degradation of extracellular ATP to adenosine by the ecto-enzymes CD39 and CD73, can then promote A_{2A} activation and reduce GVHD (Figure 1.5.). However, the role of this signalling system in humanised mouse models of GVHD remains unknown. Whether ATP release activates P2X7 to promote GVHD, and whether degradation of ATP to adenosine by CD39 and CD73 and activation of adenosine receptors reduces GVHD remains to be fully elucidated in humanised mouse models.

1.10.2. Purinergic Signalling in Skin Biology and Disease

There is a well-established role for purinergic signalling in the skin. The roles of P2 and adenosine receptors, as well as ecto-enzymes on skin cells and in a number of skin diseases, is comprehensively reviewed by Burnstock *et al.* (2012). The role of P2X7 in skin biology and psoriasis is discussed below.

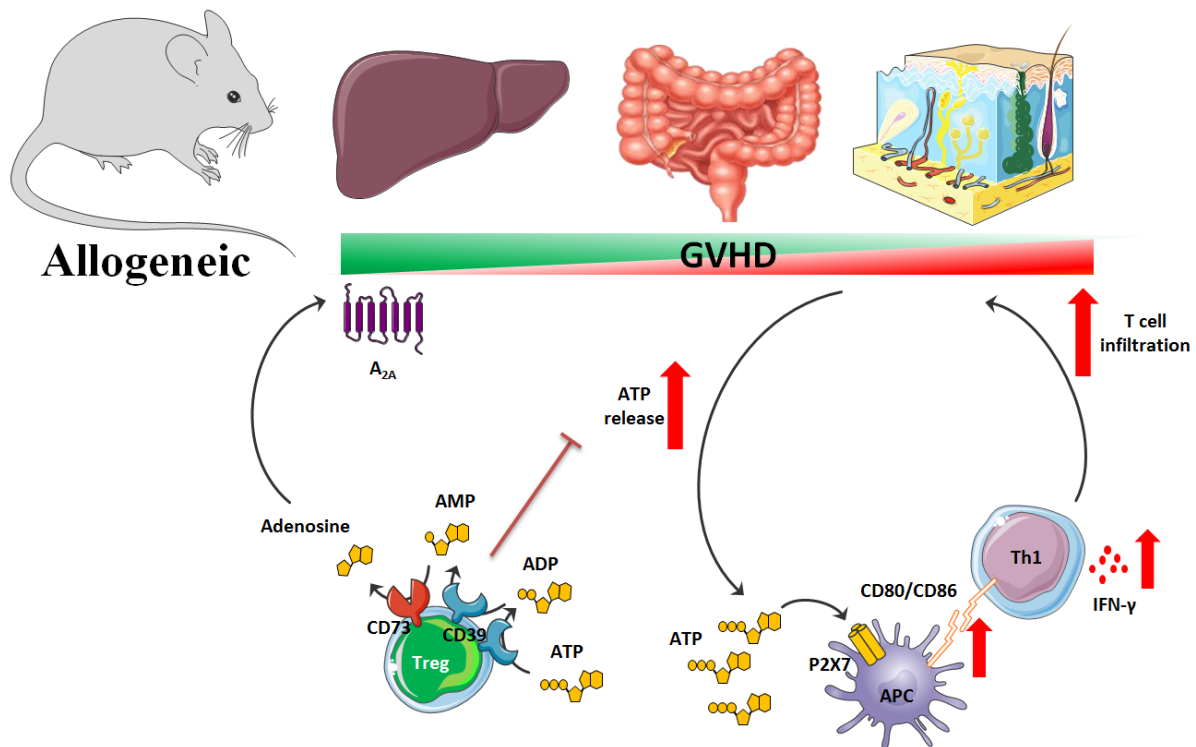


Figure 1.5. Purinergic signalling in allogeneic mouse models of GVHD. ATP is released during the initial stages of inflammatory damage of GVHD, ATP is released. ATP activates P2X7 on APCs, such as DCs, which leads to upregulation of the costimulatory molecules CD80 and CD86, which leads to increased Th1 activation and IFN- γ release. This IFN- γ release up-regulates P2X7 expression on APCs and propagates further tissue damage, resulting in further ATP release and a feed-forward loop of inflammation. However, degradation of ATP to ADP and AMP by CD39, and subsequently to adenosine by CD73, can activate A2A receptors on Treg cells (and mesenchymal stem cells; not shown) to reduce GVHD. Abbreviations; ADP, adenosine diphosphate, AMP, adenosine monophosphate, APC, antigen presenting cell, ATP, adenosine triphosphate, DC, dendritic cell, IFN- γ , interferon gamma.

1.10.2.1. P2X7 in Skin Biology

P2X7 is present on a number of skin cell types including keratinocytes, as well as skin resident DCs including LCs (Georgiou *et al.*, 2005, Tran *et al.*, 2010). Fluorescence based immunostaining of normal skin found P2X7 to be present solely in the stratum corneum of normal skin (Greig *et al.*, 2003), but epidermal P2X7 was not detected by chromogen-based immunohistochemistry (Pastore *et al.*, 2007). P2X7 has been implicated in a number of skin disorders, including AD and ICD. P2X7 is up-regulated in the epidermal basal layer of inflamed skin of AD patients compared to normal human skin (Pastore *et al.*, 2007) and in

murine CHS, a model of AD (Weintraub *et al.*, 2015). Moreover, both pharmacological blockade and genetic deficiency of P2X7 impairs CHS responses in mice due to the absence of P2X7-mediated IL-1 β release from DCs (Weber *et al.*, 2010). Similarly, both pharmacological blockade and genetic deficiency of P2X7 impair oedema, IL-1 β production and neutrophil infiltration in a murine model of croton oil-induced ICD (Da Silva *et al.*, 2013). Additionally, P2X7 on mast cells is involved in retinoid-induced ICD, mediated by aberrant release of ATP within the skin and increased P2X7 expression on skin mast cells (Kurashima *et al.*, 2014). Thus, P2X7 activation is important in promoting AD and ICD but its potential role in psoriasis remains largely unknown but as discussed below a role for P2X7 in this inflammatory disease is emerging.

1.10.2.2. P2X7 in Psoriasis

Although the role of P2X7 has not been investigated in animal models of psoriasis, *in vitro* studies directly, and *in vivo* studies indirectly, support a role for P2X7 in psoriasis pathogenesis. As mentioned above (Section 1.6) antimicrobial release is implicated in psoriasis pathogenesis. LL-37 induces ATP release, caspase-1 activation and IL-1 β release from LPS-primed monocytes, which was inhibited *in vitro* by addition of oxidised ATP (oATP) (100 μ M) or KN-62 (0.1 μ M) (Elssner *et al.*, 2004). LL-37 activation of P2X7 also inhibits skin fibroblast migration, an effect inhibited by addition of BBG (10 μ M) (Kumagai *et al.*, 2013).

Alternatively, β -defensins and S100A proteins can cause ATP release, which activates P2X7 via an autocrine mechanism (Lioi *et al.*, 2015, Wanke *et al.*, 2016, Kim *et al.*, 2018b). Human β -defensin-3 also causes up-regulation of CD86 expression on monocytes, which can be prevented by KN-62 (2 μ M) (Lioi *et al.*, 2015). Additionally, both human β -defensin-2 and -3 induce TNF- α and CXCL8 release from LPS-activated human monocytic THP-1 cells, which can be abrogated by oATP (350 μ M) or A438079 (10 μ M) (Wanke *et al.*, 2016). Finally,

activation of P2X7, and subsequently the inflammasome, by S100A12 (the S100 protein most associated with psoriasis activity (Wilsman-Theis *et al.*, 2015)) can be inhibited by suramin (0.1 μ M) or A804598 (1 μ M) (Kim *et al.*, 2018b).

ATP promotes proliferation of the human HaCaT keratinocyte cell line (Burrell *et al.*, 2008), suggesting a role for P2X7 in the characteristic hyperproliferation in psoriasis. However, this proliferation relied on co-stimulation with parathyroid hormone related protein, which is not associated with psoriasis activity (Sánchez Regaña *et al.*, 2005). Alternatively, IFN- γ , which is important in promoting psoriasis (Bowcock *et al.*, 2001, Haider *et al.*, 2008), can upregulate the expression of P2X7 in primary keratinocytes (Pastore *et al.*, 2007). Furthermore, BzATP injection into normal human skin explants induces increased release of IL-1 β , IL-6 and TNF- α , which is abrogated by KN-62 (1 μ M) (Killeen *et al.*, 2013). P2X7 activation by BzATP also induces the functional maturation, including the up-regulation of CD86 expression, on cutaneous DCs to promote Th17 responses (Killeen *et al.*, 2013). Finally, P2X7 mRNA is up-regulated in non-lesional skin of psoriasis patients compared to healthy controls (Killeen *et al.*, 2013). Combined, the findings above suggest P2X7 activation on DCs by ATP or antimicrobial peptides can promote inflammation through pro-inflammatory cytokine release and/or T cell activation. Furthermore, P2X7 activation on neutrophils or macrophages may also promote pro-inflammatory cytokine release (Barbera-Cremades *et al.*, 2012, Karmakar *et al.*, 2016). These cytokines may up-regulate P2X7 expression in the epidermis, leading to a feed-forward loop of inflammation (Figure 1.6).

1.11. Summary

GVHD remains a major complication following HSCT and current therapies involve general immunosuppression leaving patients susceptible to infection or relapse (Auletta and Cooke, 2009). With >25,000 HSCTs performed annually worldwide (D'Souza and Fretham, 2017),

and the incidence of GVHD approximately 50% (Jagasia *et al.*, 2012), there is a dire need for novel therapeutics. The presence of purinergic signalling molecules on immune cells and the role of purinergic signalling in immunity is well established and represents a potential therapeutic target. In relation to GVHD, P2X7 activation drives disease progression, while the structure of the CD39/CD73/A_{2A} pathway limits this progression. However, the majority of this data is based on allogeneic mouse models, and the role of purinergic signalling in pre-clinical humanised mouse models is completely lacking and is limited in humans with GVHD. Investigation of the role of purinergic signalling in pre-clinical humanised mouse models will provide insight into the role of this signalling system, and possibly identify therapies in a pre-clinical mouse model of GVHD.

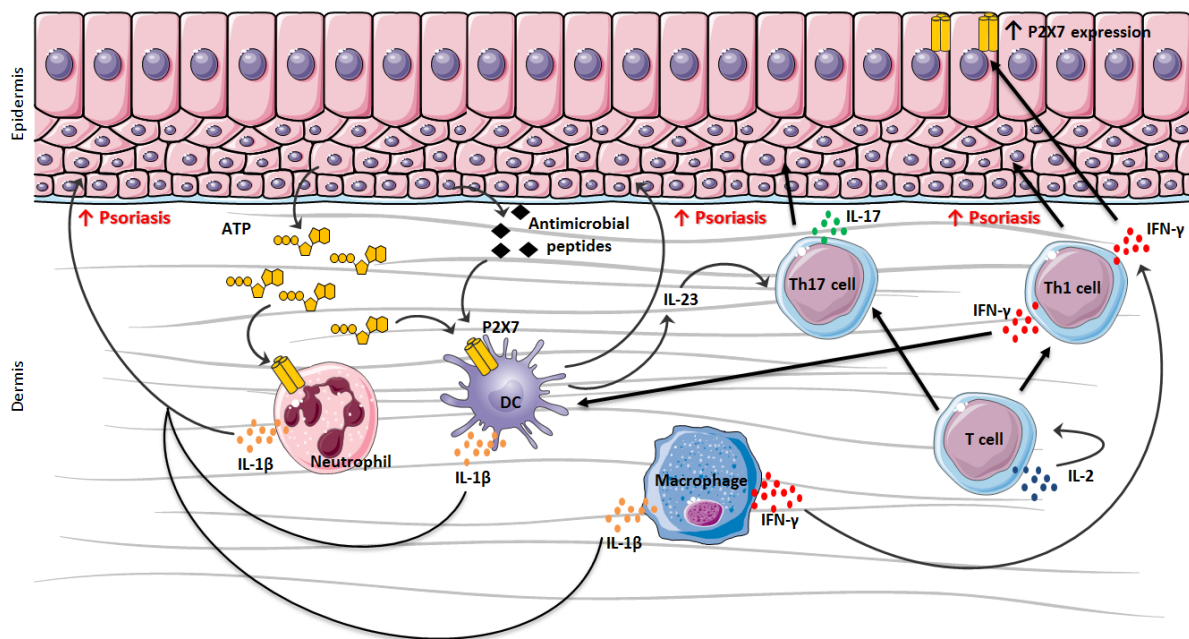


Figure 1.6. The potential role of P2X7 in psoriasis. Initiation of psoriasis could cause the release of DAMPs such as ATP, or antimicrobial peptides such as LL-37. This could act in a paracrine manner and activate, and up-regulate P2X7 in the skin, or alternatively activate P2X7 on a number of cell types. Activation of P2X7 on neutrophils, macrophages and DCs may result in IL-1 β release which can exacerbate psoriasis. LL-37 also inhibits neutrophil and macrophage cell death (not depicted). Alternatively, ATP or LL-37 activation of P2X7 on DCs may result in IL-23 release leading to Th17 activation, proliferation and migration into the skin, where Th17 cells exacerbate psoriasis through IL-17 release. Alternatively, IL-2 release by T cells in psoriasis can also cause Th17 cell differentiation and inflammation, or Th1 cell differentiation which, along with macrophages, promotes inflammation through IFN- γ

release. IFN- γ has also previously been shown to up-regulate P2X7 expression. Abbreviations: ATP, adenosine triphosphate, DAMP, danger associated molecular pattern, DC, dendritic cell, IFN- γ , interferon gamma, IL, interleukin, IMQ, imiquimod.

Psoriasis affects 1-3% of the general population of Western countries (Lebwohl *et al.*, 2014) and remains a significant financial burden. The role of purinergic signalling in skin biology and disease is well characterised, but the role of P2X7 in psoriasis is limited to *in vitro* studies and immunohistodensity studies of patient samples. The IMQ-induced psoriasis-like inflammation mouse model represents a simple and efficient model to study psoriasis pathogenesis and utilising this model may provide insights into the role of P2X7 in psoriasis. Furthermore, the skin is a target organ of GVHD, and thus investigation of the role of purinergic signalling in psoriasis may help elucidate the role of this pathway in other inflammatory skin disorders including cutaneous GVHD.

1.12. Aims

1.12.1. General Aim

This thesis aims to elucidate the role of purinergic signalling, focussing on the roles of P2X7, CD39, CD73, and A_{2A} in a humanised mouse model of GVHD, and the role of P2X7 in IMQ-induced psoriasis-like inflammation in mice.

1.12.2. Specific Aims

The specific aims of this PhD thesis are;

- i. To investigate the role of P2X7 in GVHD in a humanised NSG mouse model using a short-term (Chapter 3) and long-term (Chapter 4) regime with the P2X7 antagonist BBG.

- ii. To investigate the role of the CD39 and CD73 pathway in GVHD in a humanised NSG mouse model using the CD39/CD73 antagonist APCP (Chapter 5).
- iii. To investigate the role of A_{2A} in GVHD in a humanised NSG mouse model using the general adenosine receptor antagonist caffeine (Chapter 5), and the A_{2A} agonist CGS21680 (Chapter 6).
- iv. To investigate the role of P2X7 in an IMQ-induced psoriasis-like disease model using the P2X7 antagonists BBG and A804598 and P2X7 knock-out mice (Chapter 7).

Chapter 2: Increased Splenic Human CD4⁺:CD8⁺ T Cell Ratios, Serum Human Interferon- γ and Intestinal Human Interleukin-17 Are Associated with Clinical Graft-versus-Host Disease in Humanised Mice

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2.1. Statement of Contribution of Authors

I, as one of the authors of this original article, agree with the statement of author contributions stated at the end of this chapter, and originally published in the original journal article.



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2.2. Abstract

Graft-versus-host disease (GVHD) is a frequent complication following allogeneic hematopoietic stem cell transplantation (HSCT) with current therapies limited to general immunosuppression. Humanised mouse models of GVHD are emerging as valuable intermediaries to allow translation of findings from allogeneic mouse models to humans to prevent and treat this disease, but such models require further characterisation. In this study, humanised mice were generated by injecting immunodeficient non-obese diabetic severe combined immunodeficiency interleukin-2 receptor γ common chain null (NSG) mice with human peripheral blood mononuclear cells (hPBMCs). Clinical GVHD development was assessed using established scoring criteria (weight loss, posture, activity, fur texture and skin integrity). Differences between humanised NSG mice that developed clinical or subclinical GVHD were then compared. Both groups of mice demonstrated similar frequencies of human leukocyte engraftment. In contrast, mice that developed clinical GVHD demonstrated increased histological damage compared to mice with subclinical GVHD. Furthermore, mice with clinical GVHD exhibited increases in the splenic human CD4⁺:CD8⁺ T cell ratio, serum human interferon (IFN)- γ and intestinal human interleukin (IL)-17 expression compared to mice with subclinical GVHD. These cellular and molecular changes could be used as potential markers of disease progression in this preclinical model. This study also provides further insights into GVHD development which may be relevant to human HSCT recipients.

2.3. Highlights

- GVHD development in humanised mice is variable with the majority developing clinical disease but a small proportion with subclinical disease.
- Mice with clinical GVHD demonstrate increased splenic human CD4⁺:CD8⁺ T cell ratios compared to mice with subclinical GVHD.

- Mice with clinical GVHD exhibit increased serum human interferon (IFN)- γ and intestinal human interleukin (IL)-17 expression compared to mice with subclinical GVHD.

2.4. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapy for haematological malignancies and other blood disorders. However, graft-versus-host disease (GVHD) occurs in approximately half of the HSCTs conducted annually (Pavletic and Fowler, 2012) and leads to a mortality rate of approximately 20% in these patients (Markey *et al.*, 2014). GVHD emerges when effector donor T cells mount an immune response against host tissues (Billingham, 1965). GVHD is characterised by three stages. The first stage is release of danger signals from cells damaged by the underlying disease and/or conditioning regimes, followed by CD4⁺ T cell activation by antigen presenting cells resulting in cytokine release, and finally CD4⁺ and CD8⁺ T cell-mediated inflammatory damage (Ferrara *et al.*, 1999, Ferrara *et al.*, 2009). This damage can be propagated by the “cytokine storm” in the latter stage, wherein Th1 and Th17 cells release pro-inflammatory cytokines such as interferon (IFN)- γ (Ferrara *et al.*, 1999, Ferrara *et al.*, 2009) and interleukin (IL)-17 (Yi *et al.*, 2009, Gartlan *et al.*, 2015), respectively, perpetuating a feed forward loop of inflammation. Conversely, regulatory T (Treg) cells can modulate effector T cells to reduce T cell-mediated inflammatory damage in GVHD (Edinger *et al.*, 2003). Current therapies for GVHD are limited, with the standard therapy being general immunosuppression achieved through steroids, leaving patients susceptible to subsequent infections and/or disease relapse (Auletta and Cooke, 2009). Therefore, greater understanding of the mechanisms important in GVHD and elucidation of new therapies is essential to improve outcomes in HSCT patients and prevent GVHD.

Current studies into potential therapeutics for GVHD are investigated in allogeneic mouse models before translation to the clinic. However, potentially due to large species differences between humans and mice, therapies that delay or prevent GVHD in allogeneic mouse models often do not translate to the clinic. To address this, previous studies have developed a range of preclinical “humanised” mouse models (Shultz *et al.*, 2007). The most commonly used of these models is the humanised immunodeficient non-obese diabetic severe combined immunodeficiency interleukin-2 receptor γ common chain null (NSG) mouse, developed by Shultz *et al.* (2005) wherein mice readily engraft human peripheral blood mononuclear cells (hPBMCs) due to three defects. First, the *Scid* mutation prevents V(D)J recombination to impair B and T cell development (Bosma *et al.*, 1983). Second, deletion of the *Il2rg* gene results in absence of natural killer (NK) cells. Third, a polymorphism in the *Sirpa* gene, present due to backcrossing onto a NOD background, promotes phagocytic tolerance of xenogeneic leukocytes (Yamauchi *et al.*, 2013). Human T cells recognise major histocompatibility complex (MHC) I and II of NSG mice to cause GVHD in this humanised mouse model (King *et al.*, 2009), demonstrating that human T cell responses can be investigated *in vivo* to better understand this disease in a preclinical setting.

The humanised NSG model of GVHD is emerging as a valuable intermediate to allow translation of findings identified in allogeneic mouse models to human clinical trials. For example, based on an earlier study in an allogeneic mouse model of GVHD (Hill *et al.*, 1999), King *et al.* (2009) demonstrated tumour necrosis factor (TNF)- α blockade could reduce GVHD severity in this humanised mouse model, and Etanercept, an anti-TNF- α monoclonal antibody (mAb) has subsequently shown efficacy in clinical trials (Busca *et al.*, 2007). More recently, Burger *et al.* (Burger *et al.*) demonstrated an anti-CCR5 mAb (PRO 140) could reduce GVHD in humanised NSG mice, with this mAb now being investigated in clinical trials (Green and Dhody). Notably, the discovery of Treg cells, and their therapeutic

potential in GVHD has progressed from allogeneic mouse models (Edinger *et al.*, 2003), to humanised NSG models (Abraham *et al.*, 2012, Hannon *et al.*, 2014), and finally to clinical trials (Heinrichs *et al.*, 2016). Therefore, the humanised NSG model offers a valid preclinical model to test therapies for translation to the clinic. Although this mouse model has offered numerous advancements, the characterization of GVHD in these mice has not been adequately described.

2.5. Objective

The current study aimed to investigate the characteristics of GVHD that were observed in humanised NSG mice injected with human PBMCs that developed clinical disease compared to those mice that displayed subclinical GVHD. This study demonstrated that all humanised NSG mice had similar engraftment of human leukocytes, which were predominantly T cells. In contrast, humanised NSG mice with clinical GVHD demonstrated greater splenic CD4⁺:CD8⁺ T cells ratios, serum human IFN- γ concentrations and intestinal human IL-17 expression than humanised mice with subclinical GVHD. These cellular and molecular changes could potentially be used as biomarkers of disease progression in this preclinical model and provide insights into GVHD development which may be applicable to human HSCT recipients.

2.6. Materials and Methods

2.6.1. Antibodies for Flow Cytometry

Fluorescein isothiocyanate (FITC)-conjugated mouse anti-hCD4 (clone: RPA-T4), and mouse anti-hCD45 (clone: HI30); R-phycoerythrin (PE)-conjugated mouse anti-hCD3 (clone: UCHT1) and mouse anti-hCD8 (clone: RPA-T8); peridinin chlorophyll protein (PerCP)-Cy5.5 conjugated mouse anti-hCD4 (clone: L200) and rat anti-mCD45 (clone: 30-

F11); and allophycocyanin (APCy)-conjugated mouse anti-hCD3 (clone: UCHT1) and mouse anti-hCD19 (clone: HIB19) mAb were obtained from BD (San Jose, CA, USA).

2.6.2. Mice

All mouse experiments were conducted in accordance with approval by the Animal Ethics Committee, University of Wollongong (Wollongong, Australia). Female NSG mice, originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA), were bred at the Westmead Animal Research Facility (Westmead, Australia) or Australian BioResources (Moss Vale, Australia). NSG mice, obtained at 6-10 weeks of age, were housed in filter top cages in Tecniplast (Buggugiate, Italy) isolation cabinets, and provided with autoclaved food and water, *ad libitum*. Mice were allowed to acclimatise for one week prior to injection.

2.6.3. Isolation of Human PBMCs

All experiments with human blood were conducted in accordance with approval by the Human Ethics Committee, University of Wollongong. Peripheral blood was collected by venepuncture into VACUETTE® lithium heparin tubes (Greiner Bio-One; Frickenhausen, Germany). Whole blood, diluted with an equal volume of sterile phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, USA), was underlaid with Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden) and centrifuged (560 x g for 30 min). hPBMCs were collected and washed with PBS (430 x g for 5 min) and resuspended in PBS.

2.6.4. Humanised NSG Mouse Model of GVHD

NSG mice were injected intra-peritoneally (i.p) with 10×10^6 hPBMCs in 100 μ L sterile PBS (hPBMC group) or with 100 μ L sterile PBS alone (control group) (day 0). Mice were checked for engraftment of hPBMCs at 3 weeks post-injection by immunophenotyping tail blood. Mice were monitored up to week 8 for signs of GVHD using a scoring system (Chapter 3) giving a total clinical score out of 10 (Table 1). Mice were euthanised at 8 weeks post-injection, or earlier if exhibiting a clinical score of ≥ 8 or a weight loss of $\geq 10\%$,

according to the approved animal ethics protocol. Mice are assigned a score out of 2 for each of the criterion outlined to give a total score of 10 (as previously described (Chapter 3)). Humanised mice with clinical score < 3 were defined as subclinical GVHD, and mice with a clinical score ≥ 3 were defined as clinical GVHD.

Table 2.1. Scoring system for clinical GVHD.

GRADE	0	1	2
Weight loss ^a	< 5%	5% to 10%	> 10%
Posture	Normal	Hunching noted only at rest	Severe hunching
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling
Skin Integrity	Normal	Scaling of paws/tail	Obvious areas of involved skin

^a Percent of starting weight (day 0)

2.6.5. Immunophenotyping by Flow Cytometry

Tail blood (20-30 μL) was collected into 200 μL of citrate solution (Sigma-Aldrich), diluted with PBS and centrifuged (500 x g for 5 min). Spleens from euthanised mice were mechanically dissociated, filtered through 70 μm nylon filters (Falcon Biosciences, New York, NY, USA) and centrifuged (300 x g for 5 min). Blood and spleen cells were incubated with ammonium chloride potassium (ACK) lysis buffer (150 mM NH_4Cl , 1 mM KHCO_3 , 0.1 mM Na_2CO_3 , pH 7.3) for 5 min and washed in PBS (300 x g for 5 min). Cells were then washed in PBS containing 2% foetal bovine serum (300 x g for 3 min), and incubated for 30 min with fluorochrome-conjugated mAb, including respective isotype controls. Cells were washed with PBS (300 x g for 3 min) and data was collected using a BD LSRII Flow Cytometer (using band pass filters 515/20 for FITC, 575/26 for PE, 675/40 for PerCP-Cy5.5, and 660/20 for APCy). The relative percentages of cells were analysed using FlowJo software v8.7.1 (TreeStar Inc., Ashland, OR, USA).

2.6.6. Histological Analysis

Tissues from euthanised mice were incubated overnight in neutral buffered (10%) formalin (Sigma-Aldrich). Fixed tissues were removed, coated in paraffin, sectioned (5 μ m) and stained with haematoxylin and eosin (POCD; Artarmon, Australia). Histological changes were assessed using a Leica (Wetzlar, Germany) DMRB microscope and Leica Application Software version 4.3.

2.6.7. RNA Isolation and cDNA Synthesis

Tissues from euthanised mice or freshly isolated hPBMCs were stored in RNAlater (Sigma-Aldrich) at -20°C. RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) as per the manufacturer's instructions. Isolated RNA was immediately converted to complementary DNA (cDNA), using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) as per the manufacturer's instructions, and RNA stored at -80°C. cDNA was checked by PCR amplification of the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (Invitrogen, Carlsbad, CA, USA) for 35 cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) and a holding temperature of 4°C. Purity and size of amplicons were confirmed by 2% agarose gel electrophoresis.

2.6.8. Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) reactions of cDNA samples were using TaqMan Universal Master Mix II (Thermo Fisher Scientific) according to the manufacturer's instructions, with VIC-labelled human hypoxanthine phosphoribosyl transferase 1 (Hs99999909_m1) and FAM-labelled hIFN- γ (Hs00989291_m1), hIL-17 (Hs00936345_m1) and hFoxP3 (Hs01085834_m1) primers (Thermo Fisher Scientific). qPCR cycles consisted of two initial steps of 50°C for 2 min, and 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Reactions were conducted in triplicate using a Roche Diagnostics (Indianapolis, IN, USA) LightCycler 480, and analysis was conducted using LightCycler480

software v1.5.1. Human gene expression is shown relative to expression in cDNA from freshly isolated donor hPBMCs.

2.6.9. ELISA

Blood, collected via cardiac puncture from euthanised mice, was incubated for 1 h at RT and centrifuged (1,700 x g for 10 min). Supernatants were re-centrifuged (1,700 x g for 10 min) and sera stored at -80°C. Serum cytokine concentrations were measured using a hIFN- γ Ready-Set-Go! ELISA Kit (eBioscience, San Diego, CA, USA) as per the manufacturer's instructions. Absorbances (450 and 570 nm) were measured using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA).

2.6.10. Statistical Analysis

Data is given as mean \pm standard error of the mean (SEM). Statistical differences were calculated using Student's t-test for single comparisons or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Weight and clinical score were analysed using a repeated measures two-way ANOVA with Tukey's post-hoc test for multiple comparisons. Median survival times (MST) were compared using the log-rank (Mantel-Cox) test. Proportions of mice sacrificed prior to week 8 (mortality rates) were compared using Fisher's Exact test. All statistical analyses and graphs were generated using GraphPad Prism 5 for PC (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

2.7. Results

2.7.1. Clinical GVHD Development Varies in Humanised NSG Mice

NSG mice, injected i.p. with either saline (control) or hPBMCs, were monitored for physical manifestations of GVHD over 8 weeks. After 4 weeks, humanised NSG mice with GVHD started to exhibit clinical signs of disease (Figure 2.1.A.). By end-point it became apparent

that hPBMC-injected mice segregated into one of two groups based on clinical score (Table 2.1.); one group with scores ≥ 3 (clinical GVHD) and another group with scores < 3 (subclinical GVHD) (Figure 1A). Mice with clinical scores ≥ 3 exhibited weight loss, reduced activity, postural changes (hunching) and fur ruffling, with skin involvement in some mice, whilst mice with clinical scores < 3 was solely attributed to fur ruffling and/or postural changes (data not shown). The mean clinical score over the course of the experiment in humanised mice with clinical GVHD was greater than both control mice and mice with subclinical GVHD ($P < 0.0001$ and $P = 0.0010$, respectively) (Figure 2.1.B.). Weight loss alone is indicative of GVHD development (Cooke *et al.*, 1996). Notably, control mice and humanised mice with subclinical GVHD continued to gain weight over the entire 8 weeks, however, humanised mice with clinical GVHD started to lose weight after 4 weeks (Figure 1B). Although this difference was obvious after 4 weeks, the weight loss was not statistically significant between groups ($P = 0.2871$). Humanised mice with clinical GVHD had a MST of 50 days, which was significantly worse than control mice ($P < 0.0001$) and humanised mice with subclinical GVHD ($P < 0.0001$) where all mice survived for 8 weeks (Figure 1C). Humanised mice with clinical GVHD exhibited an 80% mortality rate over 8 weeks, which was significantly greater than control mice ($P < 0.0001$) or humanised mice with subclinical GVHD ($P = 0.0350$) (Figure 2.1.C.).

2.7.2. Increased Histological Damage and Leukocyte Infiltration is Associated with Clinical GVHD

GVHD targets the liver, gastro-intestinal tract and skin in humanised NSG mice (King *et al.*, 2009), similar to humans (Ferrara *et al.*, 2009). To compare control NSG mice and humanised NSG mice with clinical or subclinical GVHD, histological analyses of target tissues were conducted, as well as spleens to examine evidence of engraftment. As expected, control mice demonstrated normal architecture and minimal

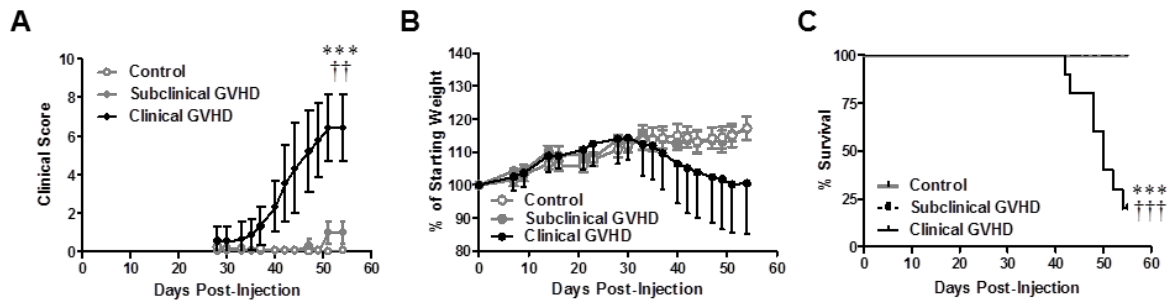


Figure 2.1. Clinical GVHD development varies in humanised mice. (a–c) NSG mice were injected i.p. with PBS (control) (n = 6) or 10 x 10⁶ hPBMCs in PBS (n = 13) and monitored for GVHD over 8 weeks. NSG mice were monitored for (A) clinical score, (B) weight loss and (C) survival. Humanised mice with subclinical GVHD (n = 3) were defined as having a clinical score < 3 and mice with clinical GVHD (n = 10) were defined as having a clinical score ≥ 3. Data represents (a, b) group means ± SEM, or (c) percent survival; *** *P* < 0.0001 compared to control mice, †† *P* < 0.01 and ††† *P* < 0.0001 compared to mice with subclinical GVHD.

presence of leukocytes in spleens, livers, small intestines and skin (Figure2). Both humanised NSG mice with subclinical or clinical GVHD demonstrated leukocyte infiltration in spleens (Figure 2.2.), consistent with engraftment of hPBMCs. However, mice with subclinical GVHD demonstrated leukocyte infiltration and minor apoptosis in the liver, whilst mice with clinical GVHD demonstrated greater leukocyte infiltration and greater apoptosis compared to both control mice and humanised mice with subclinical GVHD (Figure 2.2.). Humanised mice with subclinical GVHD demonstrated leukocyte infiltration and minor damage to enterocytes and occasional crypt epithelial cell apoptosis in the small intestine (Figure 2.2.). Humanised mice with clinical GVHD demonstrated increased leukocyte infiltration and structural damage including rounding of villi, enterocyte loss and crypt epithelial cell apoptosis in the small intestine compared to mice with subclinical GVHD (Figure 2.2.). Humanised mice with subclinical GVHD demonstrated similar intact architecture and leukocyte infiltration to control mice in the skin, except for separation at the dermal subcutaneous boundary in the former group (Figure2.2.). The skin of humanised mice with

clinical GVHD demonstrated leukocyte infiltration, epidermal thickening, basal epithelial cell apoptosis and dermal-subcutaneous boundary separation (Figure 2.2.).

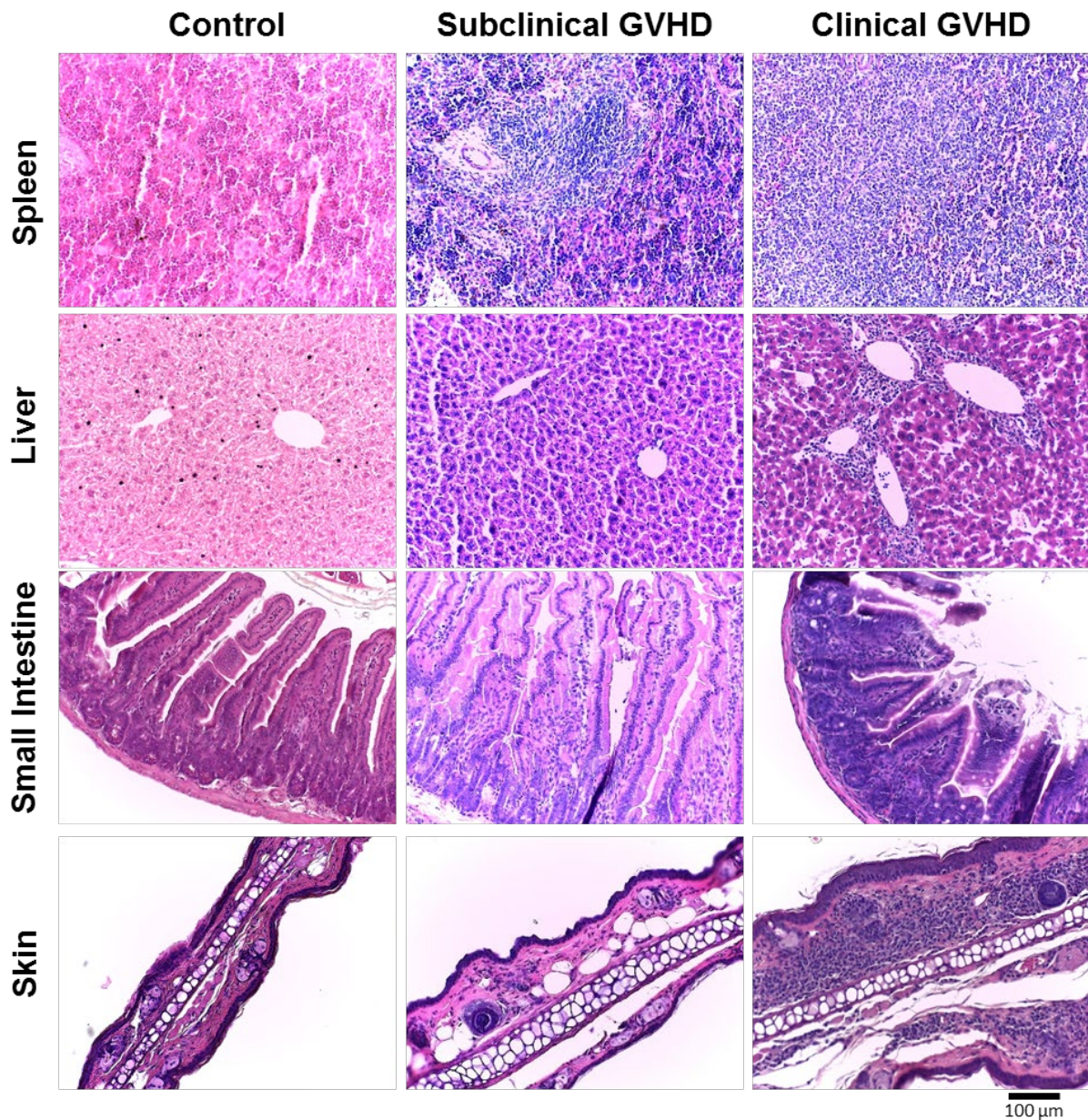


Figure 2.2. Increased histological damage and leukocyte infiltration is associated with clinical GVHD. Tissue sections (spleen, liver, small intestine and skin) from control mice (left panels) or hPBMC-injected mice with subclinical GVHD (middle panels) or clinical GVHD (right panels) at end point were stained with haematoxylin and eosin, and images captured by microscopy. Each image is representative of three mice per group; bar represents 100 μm.

2.7.3. Similar Engraftment of hPBMCs in Mice with Subclinical and Clinical GVHD

To confirm that NSG mice injected with hPBMCs engrafted human leukocytes, blood was collected at 3 weeks post-injection and spleens were collected at end-point and analysed by flow cytometry. Blood (Figure 2.3.A-D.) and spleens (Figure 2.3.E-J.) revealed both murine (m) and human (h) CD45⁺ cells in hPBMC-injected mice (Figure 2.3A, E.). As expected, control mice demonstrated mCD45⁺ but no hCD45⁺ cells in the blood and spleen (Figure 2.1.B, F).

The frequency of hCD45⁺ leukocytes were calculated as a percentage of total hCD45⁺ and mCD45⁺ cells. In the blood, mean hCD45⁺ engraftment was similar between mice with subclinical GVHD ($15.1 \pm 2.2\%$, $n = 3$) and mice with clinical GVHD ($15.3 \pm 3.2\%$, $n = 10$) ($P = 0.9737$) (Figure 2.3.B.). hCD45⁺ cells in the blood of hPBMC-injected mice were then analysed for the presence of hCD3⁺ (human T cells) and hCD19⁺ cells (human B cells) (Figure 3A). NSG mice injected with hPBMCs showed the majority of engrafted human leukocytes were T cells. The mean percentage of T cells in blood did not differ between mice with subclinical GVHD ($99.1 \pm 0.1\%$, $n = 3$) or clinical GVHD ($98.1 \pm 0.4\%$, $n = 10$) ($P = 0.1500$) (Figure 2.3.C.). The remaining cells in the blood were hCD3⁻ and hCD19⁻, the frequencies of which did not differ between mice with subclinical GVHD ($0.8 \pm 0.1\%$, $n = 3$) or clinical GVHD ($1.8 \pm 0.4\%$, $n = 10$) ($P = 0.1869$) (Figure 2.3.D.).

Similar to blood, mean hCD45⁺ engraftment in spleens was similar between mice with subclinical GVHD ($90.9 \pm 1.7\%$, $n = 3$) and clinical GVHD ($87.7 \pm 2.9\%$, $n = 10$, respectively) ($P = 0.5880$) (Figure 2.3.F.). Moreover, NSG mice injected with hPBMCs showed the majority of engrafted human leukocytes in spleens were T cells. The mean percentage of T cells in spleens did not differ between mice with subclinical GVHD ($97.8 \pm 0.5\%$, $n = 3$) or clinical GVHD ($98.4 \pm 0.2\%$, $n = 10$) ($P = 0.1942$) (Figure 2.3.G.). The

remaining cells in spleens were hCD3⁻ and hCD19⁻, the frequencies of which did not differ between mice with subclinical GVHD ($2.2 \pm 0.5\%$, $n = 3$) or clinical GVHD ($1.6 \pm 0.2\%$, $n = 10$) ($P = 0.1942$) (Figure 2.3.H.).

Further analysis of the splenic human T cells (hCD3⁺ hCD19⁻ cells) (Figure 2.3.E.) revealed that mice with clinical GVHD demonstrated significantly greater proportions of hCD4⁺ T cell engraftment ($85.1 \pm 2.0\%$, $n = 10$) than mice with subclinical GVHD ($45.5 \pm 8.4\%$, $n = 3$) ($P < 0.0001$) (Figure 2.3.I.). Conversely, mice with clinical GVHD demonstrated significantly lower proportions of hCD8⁺ T cell engraftment ($12.8 \pm 1.7\%$, $n = 10$) than mice with subclinical GVHD ($50.9 \pm 8.8\%$, $n = 3$) ($P < 0.0001$) (Figure 2.3.I.). Moreover, mice with clinical but not subclinical GVHD demonstrated a significantly greater proportion of hCD4⁺ than hCD8⁺ T cells ($P < 0.0001$), resulting in higher splenic hCD4⁺:hCD8⁺ T cell ratios (8.5 ± 1.7 , $n = 10$) than mice with subclinical GVHD (1.0 ± 0.3 , $n = 3$) ($P = 0.0418$) (Figure 2.3.J.).

2.7.4. Increased Serum Human IFN- γ is Associated with Clinical GVHD

IFN- γ has been implicated in GVHD pathogenesis (Ferrara, 2000) and correlates to worse disease in mice with allogeneic GVHD (Burman *et al.*, 2007). Therefore, to determine if humanised NSG mice with GVHD demonstrate changes in hIFN- γ , mRNA expression and serum concentrations of this cytokine were analysed by qPCR and ELISA, respectively. There was a 3-fold increase in relative hIFN- γ expression in spleens from mice with clinical GVHD (0.7 ± 0.2 , $n = 3$) compared to mice with subclinical GVHD (0.2 ± 0.1 , $n = 3$) ($P = 0.0880$) (Figure 2.4.A.). There was also a 1.5-fold increase in relative hIFN- γ expression in livers from mice with clinical GVHD (0.9 ± 0.1 , $n = 3$) compared to mice with subclinical GVHD (0.6 ± 0.3 , $n = 3$) ($P = 0.2576$) (Figure 2.4.B.). However, small intestines from humanised mice with subclinical GVHD or clinical GVHD demonstrated similar relative hIFN- γ expression (0.7 ± 0.3 vs 0.9 ± 0.1 , $n = 3$) ($P = 0.5907$) (Figure 2.4.C.). Notably, mice

with clinical GVHD demonstrated significantly increased serum human IFN- γ concentrations (42.9 ± 2.7 ng/mL) compared to mice with subclinical GVHD (25.0 ± 5.5 ng/mL) ($P = 0.0155$) (Figure 2.4.D).

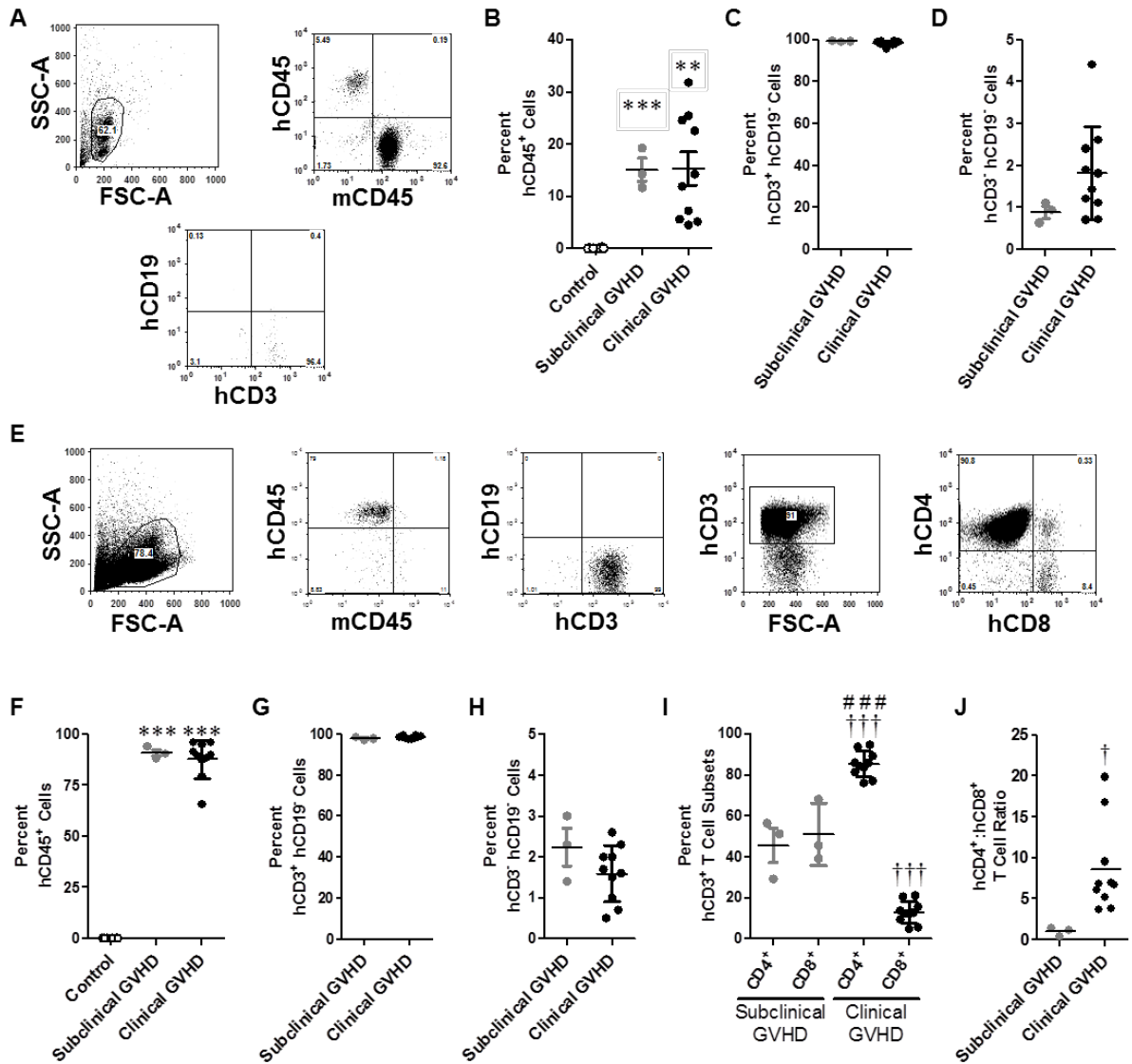


Figure 2.3. Humanised mice with clinical GVHD exhibit increased human CD4⁺:CD8⁺ T cell ratios. (a-d) Blood (3 weeks post-hPBMC injection) and (e-f) spleens (end-point) from mice were analysed by flow cytometry. Representative gates and quadrant regions are shown (a, e). (b - f) hCD45⁺ leukocytes (hCD45⁺ mCD45⁻) are expressed as a percentage of total leukocytes (hCD45⁺ mCD45⁻ + hCD45⁻ mCD45⁺). (c, g) hCD3⁺ hCD19⁻ cells and (d, h) hCD3⁻ hCD19⁻ cells are expressed as a percentage of hCD45⁺ leukocytes. hCD4⁺ and hCD8⁺ T cells are expressed as a percentage of hCD3⁺ T cells and (j) used to determine hCD4⁺:hCD8⁺ T cell ratios. (b-d, f-j) Data represents group means \pm SEM; symbols represent individual mice; ** $P < 0.005$, *** $P < 0.0001$ compared to control mice; † $P < 0.05$, ††† $P < 0.0001$ compared to corresponding cells in mice with subclinical GVHD; ### $P < 0.0001$ compared to corresponding CD8⁺ T cells.

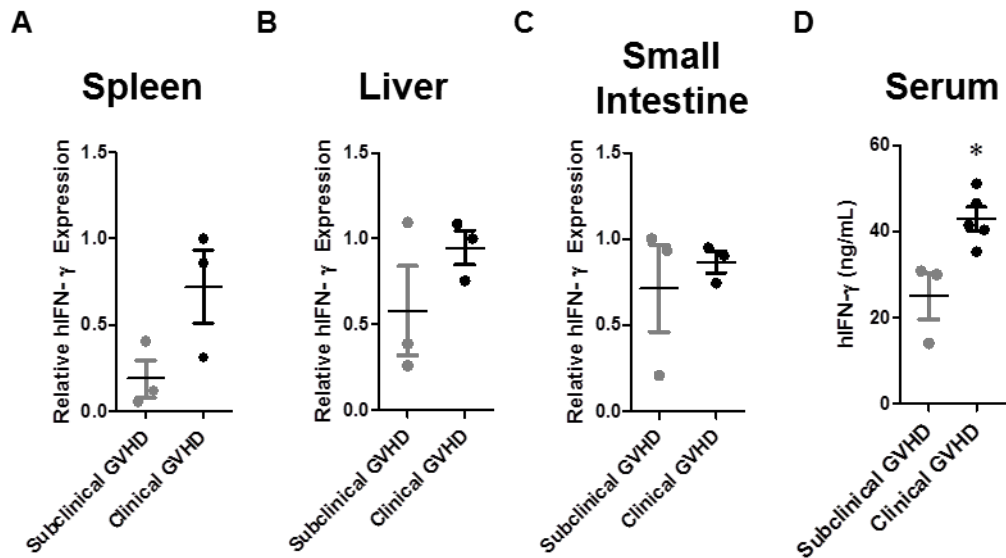


Figure 2.4. Humanised mice with clinical GVHD demonstrate increased serum hIFN- γ concentrations. The relative expression of hIFN- γ in (a) spleens, (b) livers and (c) small intestines from mice with subclinical and clinical GVHD were examined by qPCR. Data represents group means \pm SEM ($n = 3$ per group); symbols represent individual mice. (d) Serum hIFN- γ concentrations were analysed by ELISA. Data represents group means \pm SEM (subclinical GVHD $n = 3$, clinical GVHD $n = 5$); ** $P < 0.005$ compared to mice with subclinical GVHD.

2.7.5. Increased Intestinal hIL-17 is Associated with Clinical GVHD

IL-17 exacerbates GVHD in allogeneic mouse models of this disease (Carlson *et al.*, 2009, Kappel *et al.*, 2009) and in HSCT patients (Dander *et al.*, 2009). Whilst, FoxP3 Treg cells exert anti-inflammatory effects in allogeneic (Edinger *et al.*, 2003) and humanised mouse models (Cao *et al.*, 2009), as well as in humans (Trzonkowski *et al.*, 2009) to prevent GVHD. To further explore potential immunological differences between humanised NSG mice with subclinical or clinical GVHD, relative mRNA expression of hIL-17 and hFoxP3 in the spleen, liver and small intestine was assessed by qPCR. Relative splenic hIL-17 expression in mice with clinical GVHD (0.5 ± 0.2 , $n = 3$) was 40% lower compared to mice with subclinical GVHD (1.3 ± 0.5 , $n = 3$) ($P = 0.2505$) (Figure 2.5.A). However, hepatic hIL-17 expression was similar between mice with clinical GVHD (1.5 ± 0.9 , $n = 3$) and subclinical GVHD (1.2 ± 0.2 , $n = 3$) ($P = 0.7717$) (Figure 2.5.B.). Notably, intestinal hIL-17 expression was evident in mice with clinical GVHD (1.8 ± 0.4 , $n = 3$) but was absent in mice with subclinical GVHD (0.0 ± 0.0 , $n = 3$) (Figure 2.5.C.). Relative splenic hFoxP3 expression was

similar in humanised mice with subclinical GVHD (1.2 ± 1 , $n = 3$) or clinical GVHD (0.6 ± 0.2 , $n = 3$) ($P = 0.5669$) (Figure 2.5.D.). Relative hepatic hFoxP3 expression was also similar in humanised mice with subclinical GVHD (1.3 ± 0.5 , $n = 3$) or clinical GVHD (0.6 ± 0.2 and 2.0 ± 0.8 , $n = 3$) ($P = 0.4838$) (Figure 2.5.E.). However, there was a 17-fold but variable increase in intestinal hFoxP3 expression in mice with clinical GVHD (0.5 ± 0.3 , $n = 3$) compared to mice with subclinical GVHD (0.03 ± 0.01 , $n = 3$) ($P = 0.2020$) (Figure 2.5.F.).

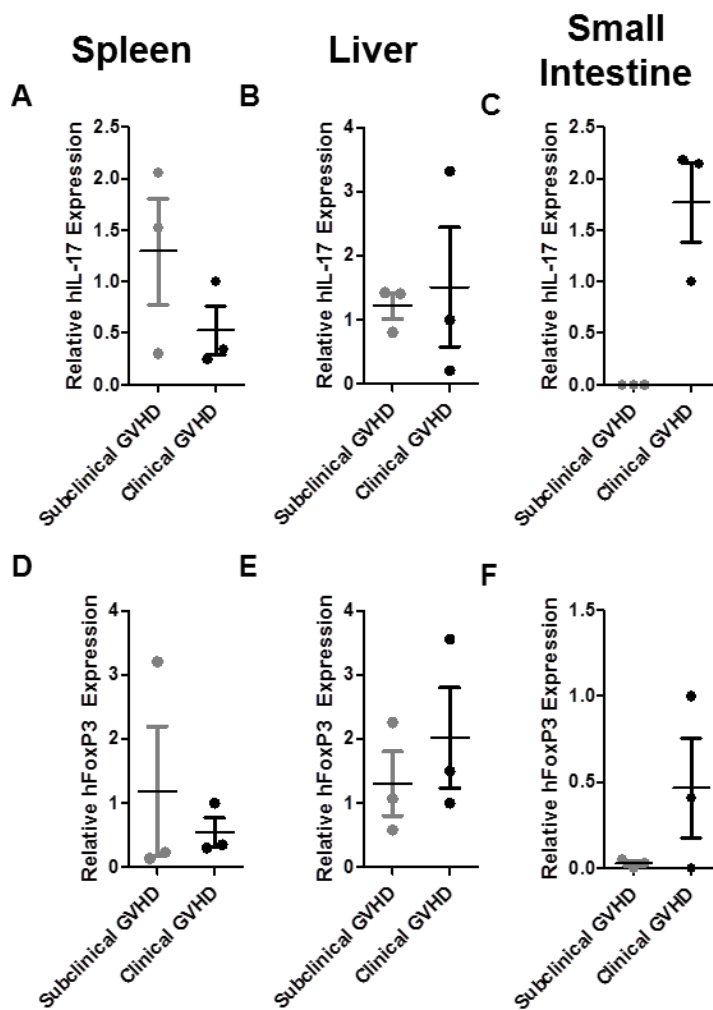


Figure 2.5. Humanised mice with clinical GVHD demonstrate increased intestinal hIL-17.

The relative expression of (a-c) hIL-17 and (d-f) hFoxP3 in (a, d) spleens, (b, e) livers and (c, f) small intestines from mice with subclinical and clinical GVHD were examined by qPCR. Data represents group means \pm SEM ($n = 3$ per group); symbols represent individual mice.

2.8. Discussion

Humanised NSG mouse models of GVHD are used as preclinical models to test potential therapeutics (Shultz *et al.*, 2007), however these models have not been fully characterised. The current study compared humanised NSG mice with subclinical and clinical GVHD, which paralleled the degree of histological disease. Notably, mice with clinical GVHD exhibited greater splenic hCD4⁺:hCD8⁺ T cell ratios, serum hIFN- γ concentrations and intestinal hIL-17 expression than mice with subclinical GVHD.

Humanised mice with a clinical score < 3 were defined as having subclinical GVHD, and only showed signs of mild to moderate fur ruffling and/or hunching only at rest. These two criteria compared to the other three criteria used are relatively subjective (Naserian *et al.*, 2018), and thus may be attributed to events such as being recently awoken rather than GVHD *per se*. Analysis of liver, small intestine and skin revealed that all humanised NSG mice had histological evidence of GVHD (leukocyte infiltration, apoptosis and/or tissue damage). Although the leukocyte infiltrates in these tissues were not assessed, previous studies have confirmed infiltrates comprise hCD45⁺ leukocytes (King *et al.*, 2009) predominantly hCD3⁺ T cells (Vlad *et al.*, 2009, Ehx *et al.*, 2017). Of note, histological evidence of GVHD was more apparent in humanised mice with clinical GVHD compared to those with subclinical GVHD. The reduced histological GVHD in mice with subclinical GVHD is similar to that observed in the livers (Vlad *et al.*, 2009, Abraham *et al.*, 2015, Ehx *et al.*, 2017, Burlion *et al.*, 2017) and small intestine (Vlad *et al.*, 2009, Nakauchi *et al.*, 2015) of humanised mice following various treatments to prevent GVHD. To this end, the current study provided the opportunity to examine differences in humanised mice with subclinical versus clinical GVHD, and to determine factors that correspond with clinical GVHD development. However, it should be noted that this study, like many others (Vlad *et al.*, 2009, Amarnath *et al.*, 2011, Gregoire-Gauthier *et al.*, 2012, Nakauchi *et al.*, 2015), used only female NSG mice.

Whilst other studies (Ali *et al.*, 2012, Kanakry *et al.*, 2013b, Betts *et al.*, 2017 2018) have demonstrated that GVHD can develop in both humanised female and male mice, a comparison of subclinical and clinical disease in humanised male NSG mice remains to be undertaken.

Both humanised NSG mice with subclinical or clinical GVHD were engrafted with similar proportions of human leukocytes as shown indirectly by histology of spleens and directly by flow cytometry of peripheral blood (week 3) and spleens (at end-point). This suggests that a difference in GVHD development between these two groups is unlikely to be due to a dose effect. However, the splenic hCD4⁺:hCD8⁺ T cell ratios were significantly greater in humanised mice with clinical GVHD compared to mice with subclinical GVHD. This finding is consistent with observations that disease development in humanised NSG mice is mediated by hCD4⁺ T cells (King *et al.*, 2009). Furthermore, the high CD4⁺:CD8⁺ T cell ratios in humanised mice with clinical GVHD parallels with studies in humans, in which high CD4⁺:CD8⁺ T cell ratios indicate greater disease severity (Huttunen *et al.*, 2015, Budde *et al.*, 2017). These observations contrast other findings in humanised NSG mice, where increased hCD4⁺:hCD8⁺ T cell ratios correlated with reduced disease severity following IL-21 blockade to prevent GVHD (Hippen *et al.*, 2012). However, this treatment reduced the number of hCD4⁺ (and hCD8⁺) T cells, indicating that the absolute number of hCD4⁺ T cells may also be important for GVHD development.

Serum hIFN- γ concentrations were significantly increased in humanised NSG mice with clinical GVHD compared to those with subclinical GVHD. There was also a trend of increased hIFN- γ expression in the spleens and livers of mice with clinical GVHD. Reduced serum IFN- γ has been used as a marker to demonstrate a reduction in disease severity in allogeneic (Wilhelm *et al.*, 2010) and humanised mouse models (Gregoire-Gauthier *et al.*, 2012) following treatments to prevent GVHD. However, the current study is the first to

demonstrate that increased serum hIFN- γ concentrations in humanised mice are associated with clinical GVHD. It would be of value to determine if blockade or knockdown of hIFN- γ prevents GVHD in humanised NSG mice to directly establish a role for this cytokine in this disease model.

The current study further demonstrated the presence of hFoxP3⁺ Tregs in the spleens, livers and small intestines of humanised NSG mice. This data parallels earlier studies that demonstrated the presence of Treg cells in blood, spleens and livers of humanised NSG mice (Abraham *et al.*, 2012). Collectively, these studies demonstrate that hFoxP3⁺ Tregs can traffic to target organs in this humanised mouse model of GVHD. This is important, as Treg cells need to migrate to target organs to interact with effector T cells to promote anti-inflammatory effects and reduce GVHD severity (Nguyen *et al.*, 2007). In the current study there was a trend of increased FoxP3⁺ Tregs in the small intestines of mice with clinical GVHD compared to subclinical GVHD. Although this result is contrary to expectations, it may reflect a compensatory attempt to suppress the effector T cells mediating intestinal damage in mice with clinical GVHD. Alternatively, the increased intestinal FoxP3 expression may represent FoxP3^{lo} expressing T cells, which can differentiate to Th17 cells (Miyara *et al.*, 2009), consistent with the hIL-17 expression in the small intestines of mice with clinical GVHD compared to mice with subclinical GVHD. This increased intestinal IL-17 expression is consistent with the role of IL-17 in GVHD development (Carlson *et al.*, 2009). Moreover, depletion of donor Th17 cells can delay GVHD (Kappel *et al.*, 2009). However, the role of IL-17 in GVHD is complicated, with IL-17 also showing a protective role in intestinal GVHD (Varelias *et al.*, 2017). Thus, the increased IL-17 expression in the small intestines of mice with clinical GVHD may reflect increased pathology or a compensatory mechanism to protect against intestinal GVHD in these mice.

In conclusion, the current study identified increases in splenic hCD4⁺:hCD8⁺ T cell ratios, serum hIFN- γ concentrations and intestinal hIL-17 in humanised mice with clinical GVHD compared to mice with subclinical GVHD, consistent with observations in human HSCT recipients with GVHD. These cellular and molecular changes could therefore be used as potential biomarkers in this preclinical model. Furthermore, this data provides insight into GVHD development in this preclinical model, which may aid investigation of potential therapeutics due to the ability to target human cells and investigate human immune responses in these humanised mice.

2.9. Acknowledgements

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2.10. Conflict of Interest

The authors declare that they have no conflicts of interest.

2.11. Author Contributions

N. J. G., L. B., S. R. A., S. I. A., R. S. and D. W. designed and performed the experiments. N. J. G., L. B., S. R. A. analysed the data. N. J. G. prepared the figures and wrote the manuscript. L. B. and S. R. A reviewed the manuscript. S. I. A. provided mice and reviewed the manuscript. R. S. and D. W. supervised the project, reviewed the data and edited the manuscript.

Chapter 3: The P2X7 Receptor Antagonist Brilliant Blue G Reduces Serum Human Interferon- γ in a Humanised Mouse Model of Graft-versus-Host Disease

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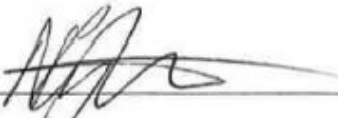
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I, as one of the authors of this original article, agree with the statement of author contributions stated at the end of this chapter, and originally published in the original journal article.



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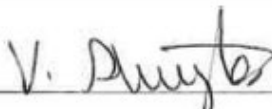
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3.2. Abstract

Graft-versus-host disease (GVHD) remains a major problem after allogeneic haematopoietic stem cell transplantation, a curative therapy for haematological malignancies. Previous studies have demonstrated a role for the adenosine triphosphate (ATP)-gated P2X7 receptor channel in allogeneic mouse models of GVHD. In this study, injection of human peripheral blood mononuclear cells (PBMCs) into immunodeficient NOD-SCID-IL2R γ^{null} (NSG) mice established a humanised mouse model of GVHD. This model was used to study the effect of P2X7 blockade in this disease. From five weeks post-PBMC injection, humanised mice exhibited clinical signs and histopathology characteristic of GVHD. The P2X7 antagonist, Brilliant Blue G (BBG), blocked ATP-induced cation uptake into both murine and human cells *in vitro*. Injection of BBG (50 mg/kg) into NSG mice did not affect engraftment of human leukocytes (predominantly T cells), or the clinical score and survival of mice. In contrast, BBG injection significantly reduced circulating human interferon (IFN)- γ , which was produced by human CD4 $^{+}$ and CD8 $^{+}$ T cells. BBG also reduced human T cell infiltration and apoptosis in target organs of GVHD. In conclusion, the P2X7 antagonist BBG reduced circulating IFN- γ in a humanised mouse model of GVHD supporting a potential role for P2X7 to alter the pathology of this disease in humans.

3.3. Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is a current curative therapy for a range of haematological malignancies, including leukaemia and lymphoma (Markey *et al.*, 2014). A common complication following transplantation is the development of graft-versus-host disease (GVHD), which arises in approximately half of HSCT recipients worldwide (Jagasia *et al.*, 2012). GVHD is mediated by transplanted donor immune cells attacking “foreign” host tissue, resulting in inflammatory damage to healthy host tissue. The initial host tissue damage by pre-conditioning regimes used to treat cancer causes a pro-inflammatory environment, which in turn leads to activation of donor CD4⁺ T cells through host antigen presenting cells (Ferrara *et al.*, 2009). CD4⁺ T cells then release additional pro-inflammatory cytokines including interferon (IFN)- γ and interleukin (IL)-17, directly causing damage to tissue, as well as activating cytotoxic CD8⁺ T cells which further exacerbate disease (Yi *et al.*, 2009, Gartlan *et al.*, 2015). Current therapies aim to prevent GVHD by preventing these T cell responses through general immunosuppression, leaving a patient vulnerable to subsequent infection or relapse, demonstrating a vital need for novel therapeutics (Holtan *et al.*, 2014).

The purinergic system comprises a complex network of extracellular signalling molecules and plasma membrane receptors, and is involved in a range of physiological processes including inflammation and immunity (Cekic and Linden, 2016). Adenosine triphosphate (ATP) is a common signalling molecule in this system, acting as both an autocrine activation molecule, and a danger associated molecular pattern when released by damaged or dying cells (Junger, 2011). In these contexts, extracellular ATP predominantly mediates its effects through activation of the P2X7 receptor (Junger, 2011). P2X7 is an ATP-gated cation channel, whereby activation by ATP allows the flux of Ca²⁺, K⁺ and other cations including fluorescent dyes (De Marchi *et al.*, 2016). P2X7 is present on numerous immune cell types

including antigen presenting cells and T cells (Geraghty *et al.*, 2016). Activation of the P2X7 receptor can result in a variety of downstream signalling events including cytokine release, reactive oxygen species formation and cell proliferation (Wiley *et al.*, 2011).

Extracellular ATP and P2X7 have emerging roles in GVHD. In allogeneic mouse models, extracellular ATP accumulates at sites of inflammatory damage, whilst P2X7 is upregulated on dendritic cells (DCs) in lymphoid tissues and livers of mice with severe GVHD (Wilhelm *et al.*, 2010). Notably, pharmacological blockade or genetic deletion of P2X7 reduces disease severity and improves survival in allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016). Studies investigating the role of P2X7 in human GVHD are limited, but it has been shown that P2X7 expression is higher in colon biopsies from HSCT patients (Wilhelm *et al.*, 2010). Additionally, the presence of a loss-of-function *P2RX7* single nucleotide polymorphism (E496A) in either the donor or recipient cells correlates to reduced survival after allogeneic HSCT (Lee *et al.*, 2007).

Therefore, the current study aimed to investigate the effect of the P2X7 antagonist, Brilliant Blue G (BBG), in a pre-clinical humanised mouse model of GVHD. BBG prevented ATP-induced dye uptake into splenic DCs from NOD-SCID-IL2R γ^{null} (NSG) mice and peripheral blood T cells from humans. NSG mice engrafted with human (h) peripheral blood mononuclear cells (PBMCs) demonstrated increased P2X7 expression in the spleen and small intestine. Injection of BBG did not alter the engraftment of human leukocytes or the clinical signs of GVHD in these mice. In contrast, injection of BBG caused a significant reduction in serum hIFN- γ concentrations, and reduced human T cell infiltration and apoptosis in target organs, suggesting a potential role for P2X7 in GVHD pathogenesis in humans.

3.4. Methods

3.4.1. Antibodies for Flow Cytometry

Fluorescein isothiocyanate (FITC) conjugated mouse anti-hCD4 (clone: RPA-T4), mouse anti-hCD8 (clone: RPA-T8) and mouse anti-hCD45 (clone: HI30) monoclonal antibodies (mAb); R-phycoerythrin (PE) conjugated mouse anti-hCD3 (clone: UCHT1) and mouse anti-hCD8 (clone: RPA-T8) mAb; peridinin chlorophyll protein (PerCP-Cy5.5) conjugated mouse anti-hCD4 (clone: L200) and rat anti-mCD45 (clone: 30-F11) mAb; allophycocyanin (APCy) conjugated mouse anti-hCD3 (clone: UCHT1) and mouse anti-hCD19 (clone: HIB19) mAb were obtained from BD Pharmingen (San Jose, CA, USA). PE-conjugated hamster anti-murine (m) CD11c mAb (clone: N418) was from BioLegend (San Diego, CA, USA).

3.4.2. Cells

Human multiple myeloma RPMI8226 cells were obtained from the European Collection of Cell Cultures (Wiltshire, UK). Murine macrophage RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in RPMI 1640 medium containing 2 mM GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA) and 10% heat-inactivated foetal bovine serum (FBS) (Bovogen Biologicals; East Keller, Australia) at 37°C/5% CO₂. Cell lines were checked every two months for *Mycoplasma spp.* infections, using a MycoAlert™ Mycoplasma detection Kit (Lonza; Basel, Switzerland) as per the manufacturer's instructions. Cells were routinely negative for *Mycoplasma spp.*

3.4.3. Mice

Mouse experiments were approved by the Animal Ethics Committee, University of Wollongong (Wollongong, Australia). C57BL/6 mice were obtained from Australian BioResources (Moss Vale, Australia). P2X7 knock-out (KO) mice (Solle *et al.*, 2001), backcrossed onto a C57BL/6 background (Tran *et al.*, 2010) were bred at the University of

Wollongong. Deletion of the *P2RX7* gene was periodically confirmed as described (Solle *et al.*, 2001). Female NSG mice, originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA), were bred at the Westmead Animal Research Facility (Westmead, Australia). NSG mice were housed in filter top cages in Tecniplast (Buggugiate, Italy) isolation cabinets, and provided with autoclaved food and water, *ad libitum*.

3.4.4. Isolation of Human PBMCs

Experiments with human blood were approved by the Human Ethics Committee, University of Wollongong. Blood was collected by venepuncture into VACUETTE® lithium heparin tubes (Greiner Bio-One; Frickenhausen, Germany). Whole blood was diluted with an equal volume of sterile phosphate buffered saline (PBS) (Thermo Fisher Scientific), underlaid with Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden) and centrifuged (560 x g for 30 min). hPBMCs were collected and washed with two volumes of PBS by centrifugation (430 x g for 5 min) and resuspended in PBS.

3.4.5. Humanised Mouse Model of GVHD

NSG mice were injected intra-peritoneally (i.p) with 10×10^6 hPBMCs in 100 μ L sterile PBS (hPBMC group), or with 100 μ L sterile PBS alone (control group) (day 0). At 3 weeks post-hPBMC injection, mice were checked for engraftment by immunophenotyping of tail vein blood. Mice were monitored up to week 8 or 10 for signs of GVHD using a scoring system (involving a score out of 2, for each of the following five criteria; weight loss, hunching, decreased activity, fur ruffling, and skin involvement) giving a total clinical score out of 10. Mice were euthanised at 8 or 10 weeks post-injection of hPBMCs, or earlier if exhibiting a clinical score of ≥ 8 or a weight loss of $\geq 10\%$, according to the approved animal ethics protocol. For BBG blockade, mice were injected with 10×10^6 hPBMCs as above, and with either 200 μ L sterile saline (saline group), or 200 μ L sterile saline containing BBG (50 mg/kg) (BBG group) (Sigma-Aldrich; St Louis, MO, USA) on days 0 (1 h post-hPBMC

injection), 2, 4, 6 and 8. This regime was based on our previous study in which BBG (46 mg/kg) injected i.p. every 2-3 days was shown to have a therapeutic effect in ALS mice (Bartlett *et al.*, 2017), and another study in which i.p. injection of P2X7 antagonists from days 0-10 was shown to have a therapeutic effect in an allogeneic mouse model of GVHD (Wilhelm *et al.*, 2010).

3.4.6. Immunophenotyping by Flow Cytometry

Tail vein blood (50 μ L) was collected into 200 μ L of citrate solution (Sigma-Aldrich), diluted with PBS and centrifuged (500 x g for 5 min). Spleens from euthanised mice were homogenised and filtered through 70 μ m nylon filters (Falcon Biosciences, New York, NY, USA) and centrifuged (300 x g for 5 min). Blood and spleen cells were incubated with ammonium chloride potassium lysis buffer (150 mM NH_4Cl , 1 mM KHCO_3 , 0.1 mM Na_2CO_3 , pH 7.3) for 5 min and washed in PBS (300 x g for 5 min). Cells were washed in PBS containing 2% FBS (300 x g for 3 min), and incubated for 30 min with fluorochrome conjugated mAb, including respective isotype controls. Cells were washed with PBS (300 x g for 3 min) and data was collected using a BD Biosciences LSR II Flow Cytometer (using band pass filters 515/20 for FITC, 575/26 for PE, 675/40 for PerCP-Cy5.5, and 660/20 for APCy). The relative percentages of cells were analysed using FlowJo software v8.7.1 (TreeStar Inc.; Ashland, OR, USA) (Figure S3.1. and Figure S3.2.).

3.4.7. Histological Analysis

Tissues from euthanised mice were incubated overnight in neutral buffered (10%) formalin (Sigma-Aldrich). Fixed tissues were removed, coated in paraffin, sectioned (5 μ m) and stained with haematoxylin and eosin (POCD; Artarmon, Australia). Histological changes were assessed using a Leica (Wetzlar, Germany) DMIL inverted light microscope at 4x objective and images captured using a Motic (Causeway Bay, Hong Kong) Moticam 2 microscope camera, and using Motic Images Plus software v2.0.

3.4.8. Isolation of RNA

Tissues removed from euthanised mice were stored in RNAlater (Sigma-Aldrich) at -20°C until required. RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) as per the manufacturer's instructions. Isolated RNA was immediately converted to complementary DNA (cDNA), using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) as per the manufacturer's instructions, and stored at -80°C. cDNA was checked by PCR amplification of the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (Invitrogen, Carlsbad, CA, USA) for 35 cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) and a holding temperature of 4°C. Purity and size of amplicons were confirmed by 2% agarose gel electrophoresis.

3.4.9. Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) reactions were performed using TaqMan Universal Master Mix II (Thermo Fisher Scientific) according to the manufacturer's instructions, with primers for FAM-labelled murine glyceraldehyde 3-phosphate dehydrogenase (Mm99999915_g1) and mIL-1 β (Mm00434228_m1), and VIC-labelled mP2X7 (Mm01199503_m1), and primers for FAM-labelled human hypoxanthine phosphoribosyl transferase 1 (Hs99999909_m1) and hIL-1 β (Hs01555410_m1), and VIC-labelled hIFN- γ (Hs00989291_m1), hIL-17 (Hs00936345_m1), and hP2X7(B) (AIOIXC2) (Thermo Fisher Scientific), as indicated. qPCR cycles consisted of two initial steps of 50°C for 2 min, and 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. qPCR reactions were conducted in triplicate, and were performed on a Roche Diagnostics (Indianapolis, IN, USA) LightCycler 480, and analysis was conducted using LightCycler480 software v1.5.1.

3.4.10. Immunoblotting

Cell lysates were prepared and immunoblotting was performed as described (Bartlett *et al.*, 2013). Briefly, cell lysates (15 μ g protein per lane) were loaded into a Mini-PROTEAN

TGXTM Precast Gel (Bio-Rad, Hercules, CA, USA) and electrophoresed. Proteins were transferred to a nitrocellulose membrane (Bio-Rad) using a Bio-Rad Trans-Blot Turbo Blotting System. Membranes were washed with Tris-buffered saline (250 mM NaCl and 50 mM Tris, pH 7.5) containing 0.2% Tween-20 (TBST) and blocked overnight at 4°C with blocking solution (TBST containing 5% milk powder). Membranes were incubated with rabbit anti-mP2X7 (extracellular epitope) antibody (Ab) (1:500) (Alomone Labs; Jerusalem, Israel) for 2 h at room temperature (RT), washed with TBST and incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1000) (Rockland Immunochemicals; Limerick, PA, USA) for 1 h at RT. Membranes were washed with TBST and incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and images collected using an Amersham Imager600 (GE Healthcare).

3.4.11. ATP-induced YO-PRO-1²⁺ Uptake Assay

P2X7 pore formation was quantified by measuring ATP-induced YO-PRO-1²⁺ uptake as previously described (Spildreorde *et al.*, 2014). Briefly, cells in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose and 10 mM HEPES, pH 7.4) at 37°C were incubated in the absence or presence of BBG for 15 min, and then incubated with 1 μM YO-PRO-1 iodide (Molecular Probes, Eugene, OR, USA) in the absence or presence of 1 mM ATP (Sigma-Aldrich) for 10 min. Incubations were stopped by addition of ice-cold NaCl medium containing 20 mM MgCl₂ (MgCl₂ medium) and centrifugation (300 x g for 3 min). Cells were incubated with PE-conjugated anti-mCD11c or APCy-conjugated anti-hCD3 mAb, respectively where indicated, for 15 min and washed with NaCl medium. Data was collected using an LSRII Flow Cytometer (band-pass filter 515/20 for YO-PRO-1²⁺, 575/25 for PE and 660/20 for APCy) and FACSDiva software version 8.0. Geometric mean fluorescence intensity of YO-PRO-1²⁺ uptake was analysed using FlowJo software (Figure S3.3.).

3.4.12. Immunohistochemistry

Paraffin-embedded formalin fixed tissues were sectioned (5 μm) and mounted on Snowcoat slides (Leica) coated with porcine gelatin (Sigma-Aldrich). To identify T cells, tissue sections were deparaffinised and heat induced epitope retrieval was performed by incubating in sodium citrate buffer (10 mM trisodium citrate dehydrate (Sigma-Aldrich) and 0.05% Tween-20) for 20 min at 95°C. Before all subsequent steps, tissue sections were washed with Tris-buffered saline (138 mM NaCl, 2.7 mM KCl and 50 mM Tris, pH 7.5) containing 0.05% Tween-20 (TBST2). Tissue sections were blocked with PBS containing 20% goat serum (Thermo Fisher Scientific) and 3% bovine serum albumin (BSA) (Amresco, Solon, OH, USA) for 30 min at RT, and subsequently incubated with rabbit anti-hCD3 mAb (clone: EP449E) (1:50) (Abcam, Cambridge, UK) in TBST2 containing 1% BSA overnight at 4°C. Endogenous peroxidase was quenched by incubating with 3% hydrogen peroxide in PBS containing 1% BSA for 20 min at RT. Tissue sections were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:250) (Invitrogen) in TBST containing 1% BSA for 15 min at RT. Finally, tissue sections were incubated with 3,3 diaminobenzidine tetrachloride (Sigma-Aldrich) for 5 min at RT, counterstained with haematoxylin and dehydrated. To identify apoptotic cells, an *In situ* Apoptosis Detection Kit (Abcam) was used as per the manufacturer's instructions. Immunohistochemistry images were captured using a Leica DMRB microscope and Leica Application Software version 4.3.

3.4.13. ELISA

Blood, collected via cardiac puncture from euthanised mice, was incubated for 1 h at RT and centrifuged (1,700 x g for 10 min). Supernatants were re-centrifuged (1,700 x g for 10 min) and sera stored at -80°C. Serum cytokine concentrations were measured using hIFN- γ , hIL-17, hIL-4 and mIL-1 β Ready-Set-Go! ELISPOT Kits (eBioscience, San Diego, CA, USA) as

per the manufacturer's instructions. Absorbance (450 and 570 nm) was measured using a SpectraMax Plus 384 (Molecular Devices, Sunnyvale, CA, USA).

3.4.14. Intracellular Staining

Splenocytes, following ammonium chloride potassium buffer lysis, were incubated for 4 h in RPMI 1640 medium containing 2 mM L-glutamine (Thermo Fisher Scientific), 10% FBS, 1% non-essential amino acids (Thermo Fisher Scientific), 55 μ M mercaptoethanol (Thermo Fisher Scientific), 100 U/mL penicillin/100 μ g/mL streptomycin (Thermo Fisher Scientific), 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich), 1 μ g/mL ionomycin (Sigma-Aldrich), and 1 μ g/mL GolgiStopTM (BD Biosciences). Cells were centrifuged (300 x g for 5 min) and washed once with PBS (300 x g for 5 min). Staining was performed using the BD Biosciences Intracellular Stain Kit as per the manufacturer's instructions, mAb added and incubated for 30 min at RT. Cells were washed with PBS and data was collected using an LSRII Flow Cytometer (band-pass filter 515/20 for FITC, 575/25 for PE, 675/40 for PerCP-Cy5.5 and 660/20 for APCy) and FACSDiva software version 8.0. The percentage of hIFN- γ ⁺ and hIL-17⁺ cells was analysed using FlowJo software (Figure S3.4.).

3.4.15. Statistical Analysis

Data is given as mean \pm standard error of the mean (SEM). Statistical differences were calculated using Student's t-test for single comparisons or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Weight and clinical score were analysed using a repeated measures two-way ANOVA. Survival was compared using the log-rank (Mantel-Cox) test. Correlations between cytokines were assessed using Spearman correlation. All statistical analyses and graphs were generated using GraphPad Prism 5 for PC (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

3.5. Results

3.5.1. NSG Mice Injected with hPBMCs Engraft Predominantly Human CD3⁺ T Cells and Exhibit Clinical Signs of GVHD

NSG mice injected with hPBMCs develop GVHD from 4 weeks (King *et al.*, 2008). In the current study, NSG mice were injected i.p. with 10×10^6 freshly isolated hPBMCs (hPBMC group) or saline (control group), and observed for signs of GVHD development for up to 8 weeks. At 3 weeks post-hPBMC injection, mice were checked for engraftment of human cells. hPBMC-injected mice demonstrated engraftment of human leukocytes ($14.4 \pm 2.7\%$ hCD45⁺ mCD45⁻ cells, $n = 9$), whilst as expected, no human leukocytes were observed in three randomly selected control mice (Figure 3.1.a.). In hPBMC-injected mice the majority of hCD45⁺ cells were T cells ($97.5 \pm 0.3\%$ hCD3⁺ hCD19⁻ cells, $n = 9$) (Figure 3.1.b.). NSG mice injected with hPBMCs demonstrated no human B cell (hCD3⁻ hCD19⁺) engraftment (data not shown), and the remaining human leukocytes were negative for both hCD3 and hCD19 ($2.5 \pm 0.3\%$, $n = 9$) (Figure 3.1.c.).

Analysis of spleens from mice euthanised at end point confirmed the absence of human leukocytes in control mice ($n = 9$) (Figure 1d). hPBMC-injected mice engrafted large amounts of human leukocytes ($86.4 \pm 2.0\%$ hCD45⁺ mCD45⁻ cells, $n = 9$), whilst as expected, no human leukocytes were observed in control mice ($n = 9$) (Figure 3.1.d.). The majority of human leukocytes in the spleen were T cells ($98.3 \pm 0.2\%$ hCD3⁺ hCD19⁻ cells, $n = 9$) (Figure 3.1.e.). No human B cells (hCD3⁻ hCD19⁺) were found in the spleen (data not shown), and the remaining cells were negative for both hCD3 and hCD19 ($1.7 \pm 0.3\%$, $n = 9$) (Figure 3.1.f.). Analysis of human T cells (hCD3⁺) revealed that hPBMC-injected mice demonstrated a significantly higher percentage of hCD4⁺ T cells ($69.6 \pm 6.6\%$, $n = 9$) compared to hCD8⁺ T cells ($27.6 \pm 6.4\%$, $n = 9$) ($P = 0.0121$) (Figure 3.1.g.).

As stated above, mice were observed for signs of GVHD for up to 8 weeks. Weight changes were similar between hPBMC-injected and control mice from weeks 1-4, with both groups demonstrating weight gain. However, hPBMC-injected mice steadily began to lose weight from day 30, while saline-injected control mice maintained a weight of over 110% of their starting weight throughout the 8 week observation period ($P = 0.4154$) (Figure 3.1.h.). Saline-injected control mice did not show any signs of GVHD, and maintained a clinical score of 0 over the observation period ($n = 9$). In contrast, hPBMC-injected mice displayed clinical signs of GVHD from day 30, first evident as fur ruffling, followed by hunching, reduced activity, weight loss, and/or areas of scaly skin patches. hPBMC-injected mice had a mean clinical score of 4.3 ± 1.1 at end point. The clinical scores between the two groups were significantly different over 8 weeks ($n = 9$) ($P = 0.0038$) (Figure 3.1.i.). hPBMC-injected mice succumbed to disease from day 40, with a median survival time (MST) of 54 days, and exhibited a 55% mortality rate by end point, compared to 100% survival of control mice ($P = 0.0101$) (Figure 3.1.j.).

3.5.2. NSG Mice Injected with hPBMCs Show Histological Evidence of GVHD and Increased Murine P2X7 Expression

Histological analysis of the target organs; liver, small intestine and skin, revealed that mice injected with hPBMCs displayed greater leukocyte infiltration and tissue damage compared to tissues from control mice (Figure 3.2.a.). Compared to control mice, hPBMC-injected mice demonstrated mildly increased leukocyte infiltration, loss of structural integrity and apoptosis of cells in the liver (Figure 3.2.a.), mild damage in the small intestine with increased apoptotic cells and rounded villi (Figure 3.2.a.), and lastly mild leukocyte infiltration and epidermal thickening in the skin compared to control mice (Figure 3.2.a.).

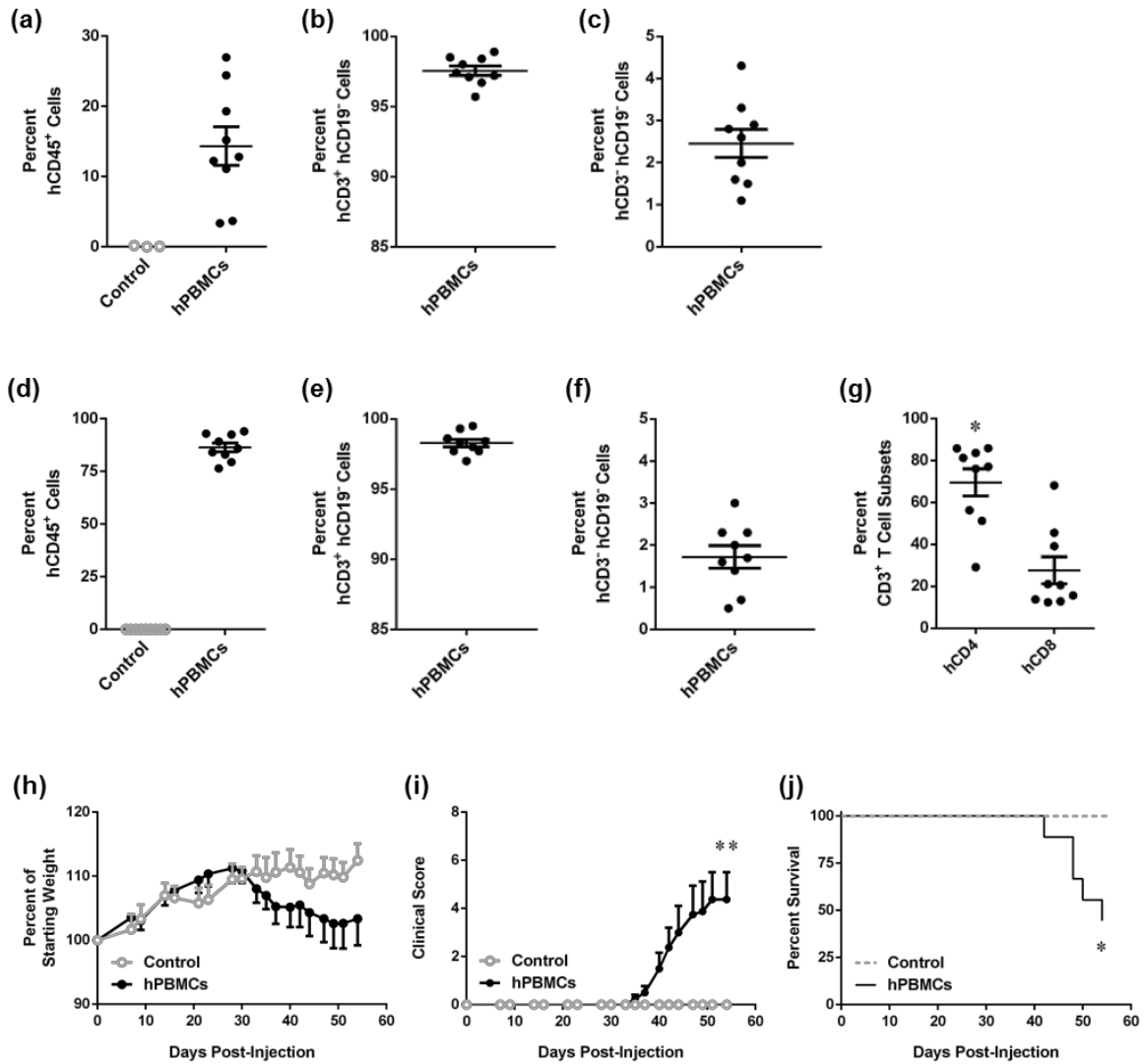


Figure 3.1. Engraftment of human leukocytes and development of GVHD in NOD-SCID-IL2R γ^{null} (NSG) mice. (a-g) NSG mice were injected intra-peritoneally (i.p.) with either 10×10^6 human (h) peripheral blood mononuclear cells (PBMCs) ($n = 9$) or an equal volume of saline (control) ($n = 9$), and monitored for clinical signs of graft-versus-host disease (GVHD) over 8 weeks. (a-g) The percentage of human leukocytes in (a-c) blood at 3 weeks post-hPBMC injection and (d-f) spleens at end point were determined by flow cytometry. (a, d) hCD45⁺ leukocytes are expressed as a percentage of total mCD45⁺ and hCD45⁺ leukocytes. (b, e) hCD3⁺ hCD19⁻ cells and (c, f) hCD3⁺ hCD19⁻ cells are expressed as a percentage of total hCD45⁺ leukocytes. (g) hCD4⁺ and hCD8⁺ T cell subsets are expressed as a percentage of total hCD3⁺ leukocytes. Data represents group means \pm SEM; symbols represent individual mice; * $P < 0.05$ compared to hCD8⁺ T cells. (h-j) NSG mice were monitored for (h) weight loss, (i) clinical score and (j) survival. Data represents (h, i) group means \pm SEM, or (j) percent survival (control, $n = 9$, hPBMC $n = 9$); * $P < 0.05$ and ** $P < 0.005$ compared to control mice.

P2X7 expression is up-regulated in host tissues in allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010, Zhong *et al.*, 2016). Therefore, mP2X7 expression in the spleen, liver and small intestine was analysed by qPCR. mP2X7 expression was increased two-fold in the spleens from hPBMC-injected mice (2.6 ± 0.6 , $n = 7$) compared to spleens from control mice (1.2 ± 0.6 , $n = 4$), but this difference did not reach statistical significance ($P = 0.1656$) (Figure 3.2.b.). Hepatic mP2X7 expression was similar in hPBMC-injected (5.5 ± 0.6 , $n = 6$) and control mice (6.9 ± 1.4 , $n = 3$) ($P = 0.4160$) (Figure 3.2.c.). mP2X7 expression was approximately two-fold greater in the small intestines from hPBMC-injected mice (16.9 ± 2.7 , $n = 6$) compared to control mice (9.9 ± 6.2 , $n = 4$), but this did not reach statistical significance ($P = 0.2668$) (Figure 3.2.d.).

3.5.3. NSG Mice Express Full-Length Functional P2X7 Receptors

The data above (Figure 3.2. b-d) demonstrates that NSG mice express P2X7, but it remained unknown if NSG mice express functional P2X7. Immunoblotting using an antibody against the extracellular domain of P2X7 demonstrated a major band at 81 kDa, corresponding to full-length P2X7 (Masin *et al.*, 2012) in the positive control RAW264.7 macrophages and in NSG splenocytes ($n = 2$) (Figure 3.3.a.).

To determine if NSG mice express functional P2X7, RAW264.7 cells or splenic leukocytes from mice were incubated in the absence or presence of ATP, and YO-PRO-1²⁺ uptake assessed by flow cytometry. ATP induced YO-PRO-1²⁺ uptake into positive control RAW264.7 cells ($n = 4$) and CD11c⁺ splenic DCs from C57BL/6 mice ($n = 4$), but not negative control CD11c⁺ splenic DCs from P2X7 KO mice ($n = 3$) (Figure 3.3.b.). Notably, ATP also induced YO-PRO-1²⁺ uptake into CD11c⁺ splenic DCs from NSG mice ($n = 5$) (Figure 3.3b). This uptake was 50% lower than that of RAW264.7 macrophages ($P < 0.005$), but three-fold greater than that of C57BL/6 CD11c⁺ splenic DCs ($P < 0.005$) (Figure 3.3.b.).

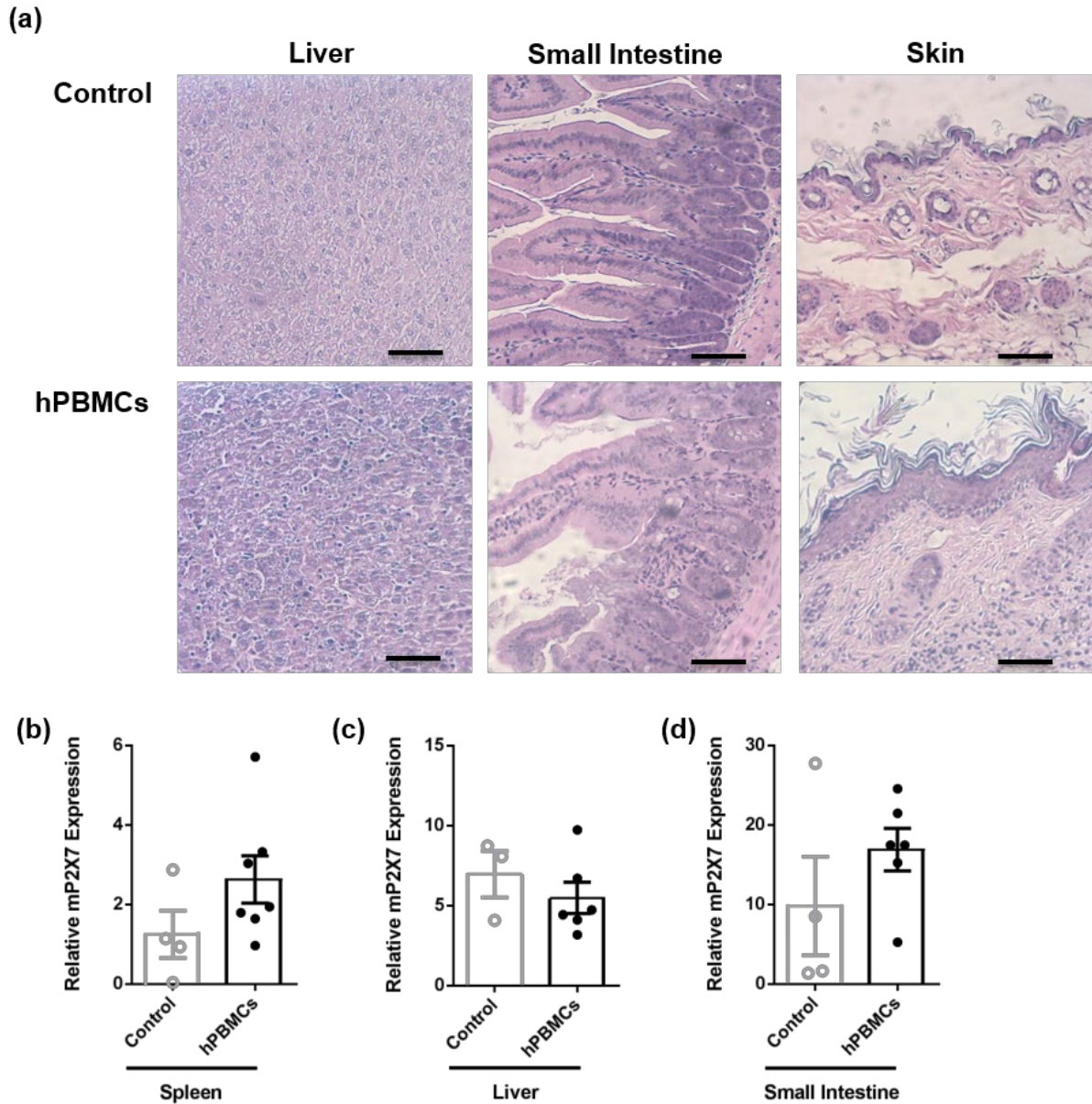


Figure 3.2. NOD-SCID-IL2R γ^{null} (NSG) mice injected with human leukocytes develop graft-versus-host disease (GVHD). (a-d) NSG mice injected intra-peritoneally (i.p.) with either human (h) peripheral blood mononuclear cells (hPBMCs) or saline (control) (day 0) (from Figure 1) were monitored for clinical signs of graft-versus-host disease (GVHD) over 8 weeks. (a) Tissue sections (liver, small intestine and skin) from control (top panel) or hPBMC-injected mice (bottom panel) at end point were stained with haematoxylin and eosin and captured by microscopy. Each image is representative of two mice per group; bar represents 100 μm . (b-d) The relative expression of murine (m) P2X7 in (b) spleen, (c) liver and (d) small intestine from mice at end point was examined by qPCR. Data represents group means \pm SEM ($n = 4-7$); symbols represent individual mice.

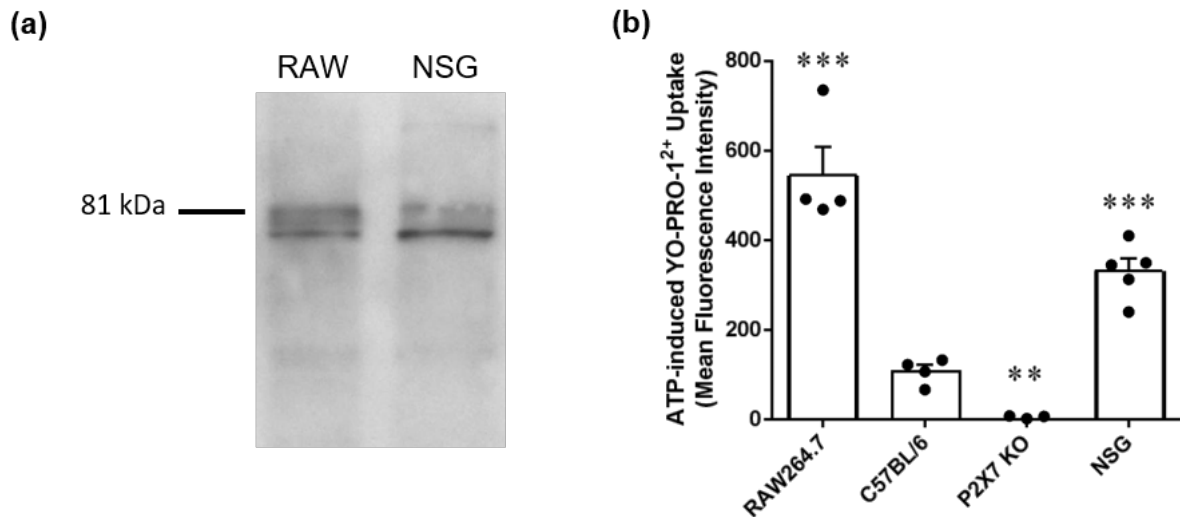


Figure 3.3 NOD-SCID-IL2R γ ^{null} (NSG) mice express full-length functional P2X7. (a) Lysates of RAW264.7 (RAW) macrophages and splenocytes from NSG mice were examined by immunoblotting using an anti-P2X7 antibody. Image is representative of two independent experiments. (b) RAW264.7 cells or splenocytes from C57BL/6, P2X7 KO or NSG mice in NaCl medium containing 1 μ M YO-PRO-1²⁺ were incubated in the absence or presence of 1 mM adenosine triphosphate (ATP) for 10 min at 37°C. The mean fluorescence intensity of YO-PRO-1²⁺ uptake into RAW264.7 cells or CD11c⁺ splenic dendritic cells (DCs) was then assessed by flow cytometry. ATP-induced YO-PRO-1²⁺ uptake was determined as the difference between YO-PRO-1²⁺ uptake in the presence and absence of ATP. Data represents group means \pm SEM ($n = 3 - 5$); symbols represent individual replicates (RAW264.7 cells) or mice (splenic CD11c⁺ DCs); ** $P < 0.005$ and *** $P < 0.0001$ compared to C57BL/6.

3.5.4. BBG Prevents ATP-Induced Cation Uptake into Human and Murine Leukocytes

Since its initial use in rodent models of multiple sclerosis (Matute *et al.*, 2007), spinal cord injury (Peng *et al.*, 2009) and Huntington's disease (Díaz-Hernández *et al.*, 2009), BBG has been used in over 40 studies to establish the role of P2X7 in various disorders (Bartlett *et al.*, 2014) including our group in a murine model of amyotrophic lateral sclerosis (Bartlett *et al.*, 2017). To confirm that BBG can block hP2X7 and mP2X7, human RPMI8226 and murine RAW264.7 cells, both of which express endogenous P2X7 (Farrell *et al.*, 2010, Constantinescu *et al.*, 2010), were pre-incubated with increasing concentrations of BBG and then ATP-induced YO-PRO-1²⁺ uptake was assessed. BBG exhibited a concentration-dependent inhibition of ATP-induced YO-PRO-1²⁺ uptake, with maximal inhibition at 1 μ M

in both cell types, and half maximal inhibitory concentrations (IC_{50}) of 99.0 ± 1.2 nM and 59.7 ± 1.2 nM in RPMI8226 cells and RAW264.7 cells, respectively (Figure 3.4.a.). These IC_{50} values are similar to that known for recombinant hP2X7 and mP2X7 (Bartlett *et al.*, 2014).

To confirm that BBG can block P2X7 in primary leukocytes, hPBMCs and NSG splenocytes were pre-incubated in the absence or presence of BBG, and then ATP-induced YO-PRO-1²⁺ uptake was assessed. ATP induced significant YO-PRO-1²⁺ uptake into hCD3⁺ T cells, which was reduced by 95% by pre-incubation with BBG ($P < 0.0001$, $n = 3$) (Figure 3.4.b.). ATP induced significant YO-PRO-1²⁺ uptake into NSG CD11c⁺ splenic DCs, which was reduced 92% by pre-incubation with BBG (Figure 3.4.c.) ($P < 0.0001$, $n = 3$). Basal uptake of YO-PRO-1²⁺ in the absence or presence of BBG was similar for each respective cell subtype (Figure 3.4.b, c.).

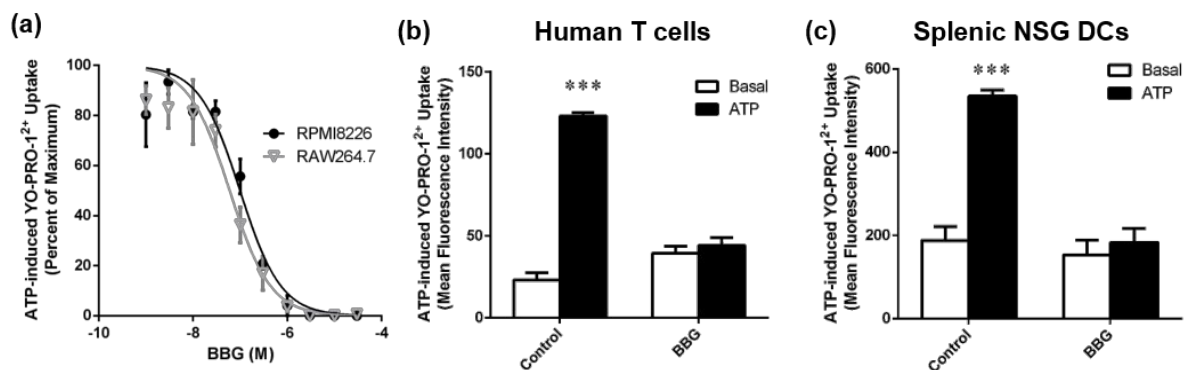


Figure 3.4. Brilliant Blue G (BBG) prevents adenosine triphosphate (ATP)-induced cation uptake into human and murine leukocytes. (a) Human RPMI8226 and murine RAW264.7 cells, (b) human peripheral blood mononuclear cells (PBMCs) or (c) NOD-SCID-IL2R γ^{null} (NSG) splenocytes in NaCl medium were pre-incubated for 15 min at 37°C in the absence or presence of (a) BBG as indicated or (b, c) 1 μ M BBG. Cells were then incubated with 1 μ M YO-PRO-1²⁺ in the absence or presence of 1 mM adenosine triphosphate (ATP) for 10 min at 37°C. The mean fluorescence intensity of YO-PRO-1²⁺ uptake into (a) RPMI8226 or RAW264.7 cells, (b) hCD3⁺ T cells or (b) NSG splenic CD11c⁺ dendritic cells (DCs) was then assessed by flow cytometry. (a) ATP-induced YO-PRO-1²⁺ uptake is represented as a percentage of maximal ATP response in the absence of BBG. (a-c) Data represents group means \pm SEM ($n = 3$); *** $P < 0.0001$ compared to respective basal.

3.5.5. BBG Does Not Affect Engraftment of Human Cells or Prevent GVHD in NSG Mice

To investigate whether P2X7 blockade can prevent disease in a humanised mouse model of GVHD, NSG mice were injected with hPBMCs and the P2X7 antagonist BBG (50 mg/kg) was subsequently injected every two days from days 0 to 8. Mice were monitored for weight loss and signs of GVHD for up to 10 weeks. To determine if BBG affected engraftment of human cells, blood was collected at 3 weeks post-hPBMC injection and cells analysed by flow cytometry. BBG-injected mice demonstrated a similar level of human leukocytes ($10.4 \pm 1.3\%$ hCD45⁺ mCD45⁻ cells, $n = 14$) compared to saline-injected (vehicle control) mice ($10.6 \pm 1.4\%$ hCD45⁺ mCD45⁻ cells, $n = 16$) ($P = 0.9179$) (Figure 3.5.a.). In both groups of mice, the majority of hCD45⁺ cells were T cells, which did not differ between BBG-injected mice ($97.2 \pm 0.4\%$ hCD3⁺ hCD19⁻ cells, $n = 14$) and saline-injected mice ($97.4 \pm 0.4\%$ hCD3⁺ hCD19⁻ cells, $n = 16$) ($P = 0.7014$) (Figure 3.5.b.). Neither group of mice demonstrated engraftment of B cells (hCD3⁻ hCD19⁺) (data not shown). The remaining hCD45⁺ cells were negative for both hCD3 and hCD19, and the percentage of these cells did not differ between BBG-injected mice ($2.7 \pm 0.4\%$, $n = 14$) and saline-injected mice ($2.5 \pm 0.4\%$, $n = 16$) ($P = 0.8291$) (Figure 3.5.c.).

Analysis of spleens from all mice euthanised at end point demonstrated human leukocytes comprised the majority of total murine and human leukocytes. BBG-injected mice engrafted a similar level of human leukocytes ($56.3 \pm 5.0\%$ hCD45⁺ mCD45⁻ cells, $n = 13$) compared to saline-injected mice ($56.2 \pm 6.2\%$ hCD45⁺ mCD45⁻ cells, $n = 15$) ($P = 0.9899$) (Figure 3.5.d.). The majority of human leukocytes in the spleen were T cells, and both BBG-injected mice ($97.1 \pm 0.6\%$ hCD3⁺ hCD19⁻ cells, $n = 13$) and saline-injected mice ($95.4 \pm 0.6\%$ hCD3⁺ hCD19⁻ cells, $n = 15$) demonstrated similar percentages of T cells ($P = 0.2481$) (Figure 3.5.e.). No human B cells (hCD3⁻ hCD19⁺) were found in the spleens (data not

shown). The remaining hCD45⁺ cells were negative for both hCD3 and hCD19, and percentages of these cells did not differ between BBG-injected mice ($2.7 \pm 0.6\%$, $n = 15$) and saline-injected mice ($2.7 \pm 0.6\%$, $n = 15$) ($P = 0.5325$) (Figure 3.5.f.). Analysis of the human T cells (hCD3⁺) revealed that BBG- and saline-injected mice contained similar percentages of hCD4⁺ T cells ($67.0 \pm 4.1\%$, $n = 13$ vs. $72.7 \pm 4.3\%$, $n = 15$, respectively, $P = 0.3515$). BBG- and saline-injected mice also contained similar percentages of hCD8⁺ T cells ($24.0 \pm 3.6\%$, $n = 13$ vs $22.3 \pm 4.1\%$, $n = 15$, respectively, $P = 0.7583$). In both BBG- and saline-injected mice, the percentage of hCD4⁺ T cells was significantly higher than hCD8⁺ T cells ($P < 0.0001$) (Figure 3.5.g.).

As stated above, mice were observed for signs of GVHD for up to 10 weeks. Both BBG- and saline-injected humanised mice began to lose weight from day 30 ($P = 0.8450$) (Figure 3.5.h.). Both BBG- and saline-injected mice began to exhibit signs of mild GVHD at day 35, and both BBG- and saline-injected mice, exhibited similar clinical scores at end point (5.3 ± 0.5 , $n = 16$ vs. 4.9 ± 0.6 , $n = 14$, respectively). The clinical scores between the two groups were not significantly different over 8 weeks ($P = 0.8356$) (Figure 3.5.i.). Survival was also similar in both BBG-injected mice (MST of 64 days, and mortality rate of 57%, $n = 16$) and saline-injected mice (MST of 60 days and mortality rate of 62%, $n = 14$), ($P = 0.9874$ for mortality rate) (Figure 3.5.j.).

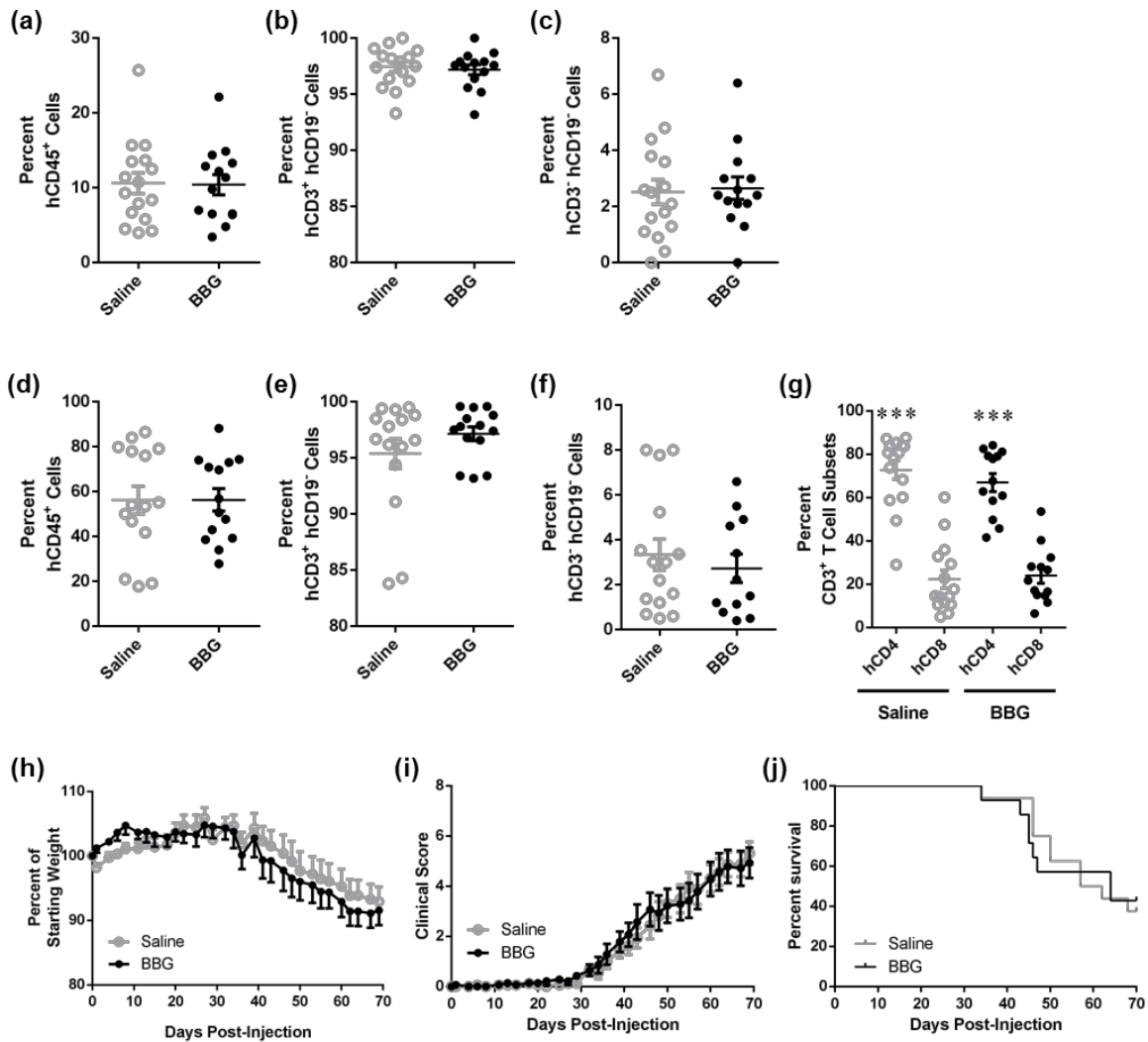


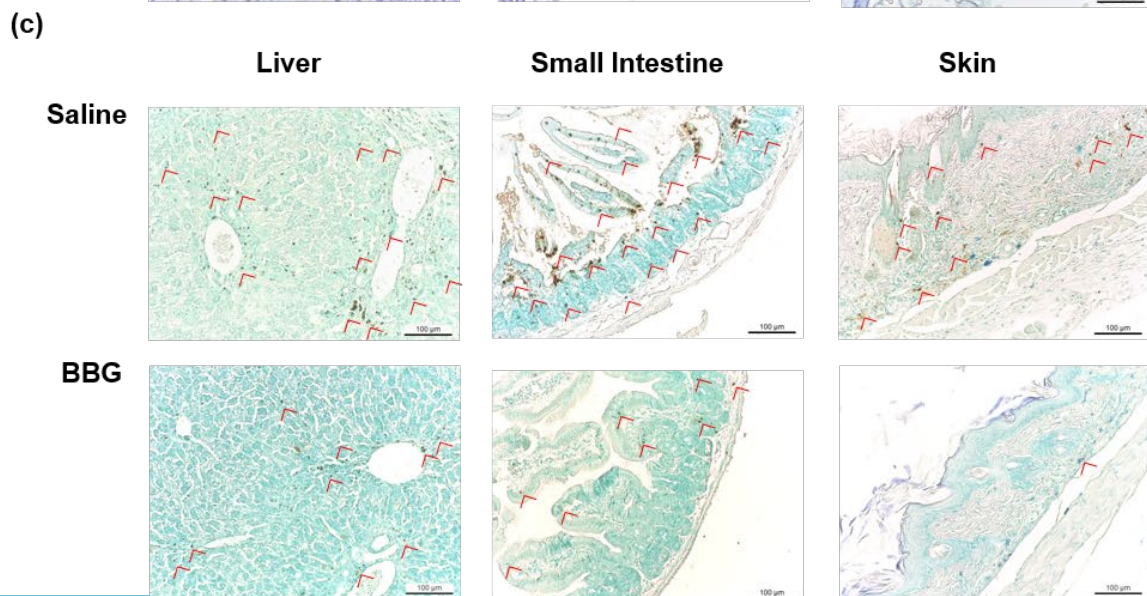
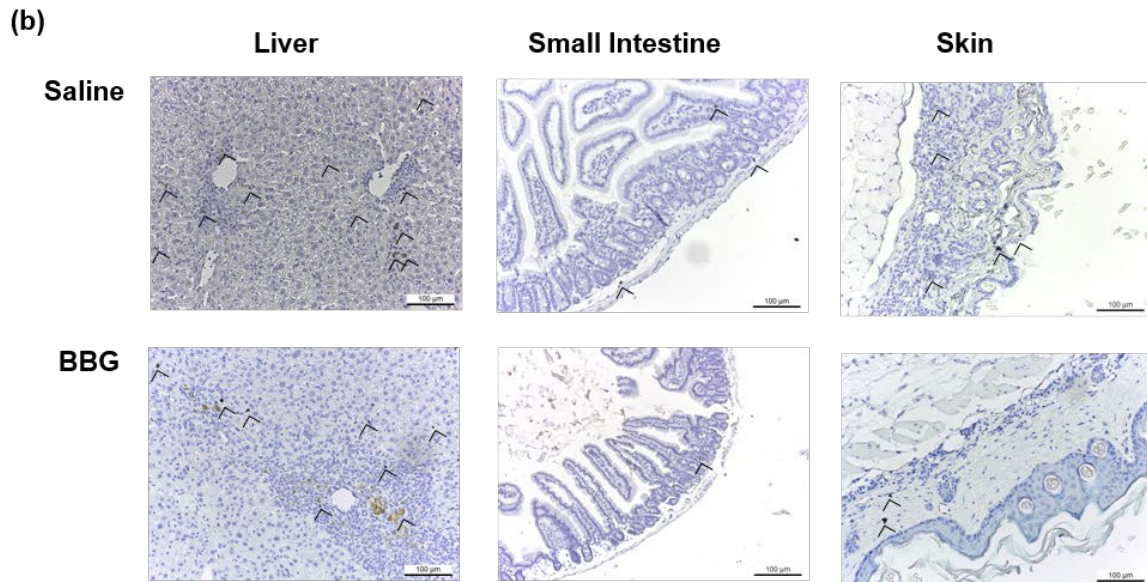
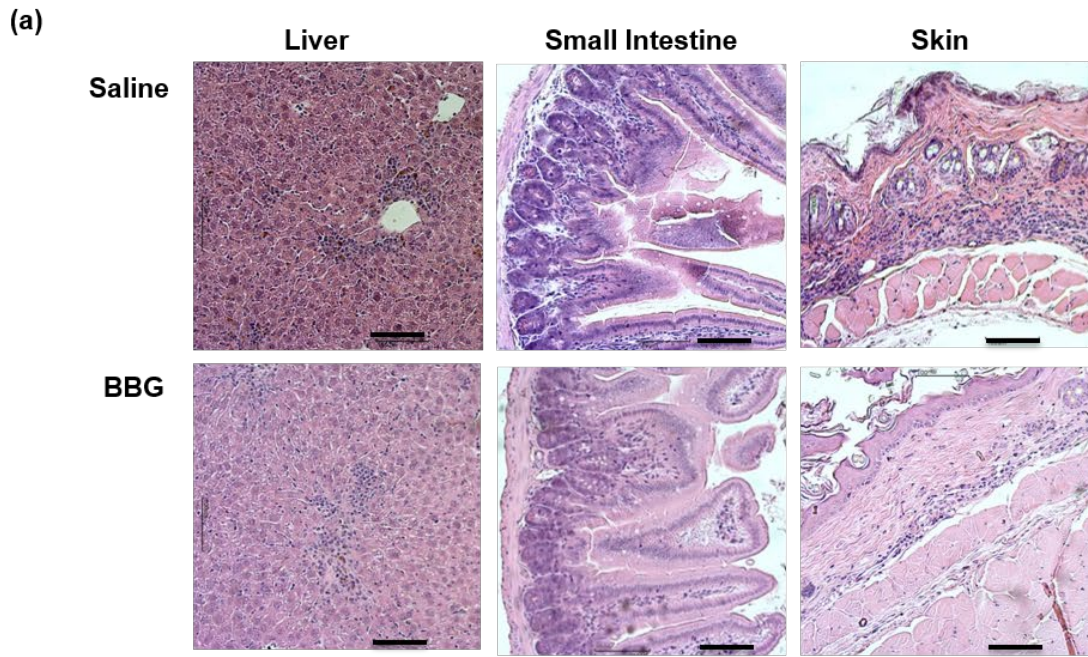
Figure 3.5 Brilliant Blue G (BBG) does not affect engraftment of human cells. (a-j) NOD-SCID-IL2R γ^{null} (NSG) mice were injected intra-peritoneally (i.p.) with 10×10^6 human (h) peripheral blood mononuclear cells (PBMCs), and subsequently with saline (control) ($n = 14$) or saline containing Brilliant Blue G (BBG) (50 mg/kg) ($n = 16$) every two days (from days 0-8), and monitored for clinical signs of graft-versus-host disease (GVHD) over 10 weeks. (a-g) The percentage of human leukocytes in (a-c) blood at 3 weeks post-hPBMC injection and (d-f) spleens at end point were determined by flow cytometry. (a, d) hCD45⁺ leukocytes are expressed as a percentage of total mCD45⁺ and hCD45⁺ leukocytes. (b, e) hCD3⁺ hCD19⁻ cells and (c, f) hCD3⁺ hCD19⁻ cells are expressed as a percentage of total hCD45⁺ leukocytes. (g) hCD4⁺ and hCD8⁺ T cell subsets are expressed as a percentage of total hCD3⁺ leukocytes. Data represents group means \pm SEM; symbols represent individual mice; *** $P < 0.0001$ compared to hCD8⁺ T cells. (h-j) NSG mice were monitored for (a) weight loss, (b) clinical score, and (c) survival. Data represents (h, i) group means \pm SEM or (j) percent survival (saline $n = 14$, BBG $n = 16$).

3.5.6. BBG Reduces Histological Evidence of GVHD in Humanised Mice

BBG- and saline-injected humanised mice demonstrated similar damage to the liver with some leukocyte infiltration, apoptosis of cells and fibrosis (Figure 3.6.a.). Both BBG- and saline-injected humanised mice demonstrated structural damage to the small intestine; however, BBG-injected humanised mice exhibited decreased leukocyte infiltration compared to saline-injected humanised mice (Figure 3.6.a.). BBG-injected humanised mice also exhibited mildly decreased leukocyte infiltrates in the dermis, decreased apoptotic cells and reduced basal vacuolar change of the skin compared to that of saline-injected humanised mice (Figure 3.6.a.).

Immunohistochemistry of the target organs, liver, small intestine and skin, was used to determine if the leukocyte infiltrates observed above included human T cells. T cells (indicated by black arrows) were identified in the liver, and to a lesser extent in the small intestine and skin (Figure 3.6.b.). T cell infiltration in the liver, small intestine and skin appeared to be reduced in BBG-injected mice compared to saline-injected mice (Figure 3.6.b.). To confirm tissue damage in the liver, small intestine and skin, these target organs were analysed for evidence of apoptosis (fragmented DNA) by immunohistochemistry. Apoptotic cells (indicated by red arrows) were evident in the liver and small intestine, and to a lesser extent in the skin (Figure 3.6.c.). Apoptosis in these organs appeared to be reduced in BBG-injected mice compared to saline-injected mice (Figure 3.6.c.).

Figure 3.6. Brilliant Blue G (BBG) does not prevent graft-versus-host disease (GVHD) in humanised mice. (a-c) NOD-SCID-IL2R γ^{null} (NSG) mice injected intra-peritoneally (i.p.) with 10×10^6 human (h) peripheral blood mononuclear cells (PBMCs) (day 0), and with saline (control) or 50 mg/kg Brilliant Blue G (BBG) (from Figure 5) were monitored for clinical signs of GVHD over 10 weeks. Tissue sections (liver, small intestine, and skin) from hPBMC-injected mice injected with saline (control) or BBG at end point were stained with (a) haematoxylin and eosin, (b) anti-hCD3 monoclonal antibody with 3,3'-diaminobenzidine tetrachloride detection system and haematoxylin, or (c) terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end labelling with 3,3'-diaminobenzidine tetrachloride detection system and methyl green. Images were captured by microscopy with each image representative of (a) four, or (b, c) two mice per group; bars represent 100 μm ; (b) black arrow heads indicate T cells, and (c) red arrow heads indicate apoptotic cells. ►



3.5.7. BBG-Injected Mice Demonstrate Similar mP2X7 and hP2X7 Expression in GVHD Target Organs

To determine whether injection of BBG altered expression of murine (host) or human (donor) P2X7 expression in the spleens and target organs of GVHD, tissues from humanised mice were analysed by qPCR. mP2X7 expression was similar in spleens of BBG-injected mice (4.6 ± 1.0 , $n = 6$) and saline-injected mice (5.7 ± 0.5 , $n = 6$) ($P = 0.5446$) (Figure 3.7.a.). mP2X7 expression in the liver was reduced by 58% in BBG-injected mice (1.6 ± 0.3 , $n = 4$) compared to saline-injected mice (3.9 ± 1.2 , $n = 5$), however this difference did not reach statistical significance ($P = 0.1619$) (Figure 3.7.b.). Conversely, mP2X7 expression was increased almost two-fold in the small intestines of BBG-injected mice (2.8 ± 0.7 , $n = 6$) compared to saline-injected mice (1.6 ± 0.3 , $n = 5$), but this did not reach statistical significance ($P = 0.1763$) (Figure 3.7.c.). hP2X7 expression was decreased by 74% in the spleens of BBG-injected mice (3.0 ± 0.9 , $n = 5$) compared to saline-injected mice (11.4 ± 3.5 , $n = 6$) and this difference approached statistical significance ($P = 0.0595$) (Figure 3.7.d.). In contrast, hP2X7 expression was similar in the livers and small intestines of BBG-injected mice (6.3 ± 2.6 , $n = 5$, and 7.6 ± 2.9 , $n = 5$, respectively) compared to saline-injected mice (3.7 ± 1.1 , $n = 4$, and 5.7 ± 1.8 , $n = 6$, respectively) ($P = 0.4296$ and 0.5642 , respectively) (Figure 3.7.e., f.).

3.5.8. BBG Reduces Serum Human IFN- γ in Humanised Mice

IFN- γ and IL-17 are important pro-inflammatory cytokines implicated in the pathogenesis of GVHD (Yi *et al.*, 2009, Gartlan *et al.*, 2015). To determine if BBG treatment altered the amount of these cytokines, serum hIFN- γ and hIL-17 concentrations in humanised mice were assessed by ELISA. hIFN- γ was present in the serum of all mice for which samples were available (Figure 3.8.a.). Notably, treatment with BBG significantly reduced the amount of serum hIFN- γ in BBG-injected mice (10.0 ± 2.4 ng/mL, $n = 12$), which was 54% lower than

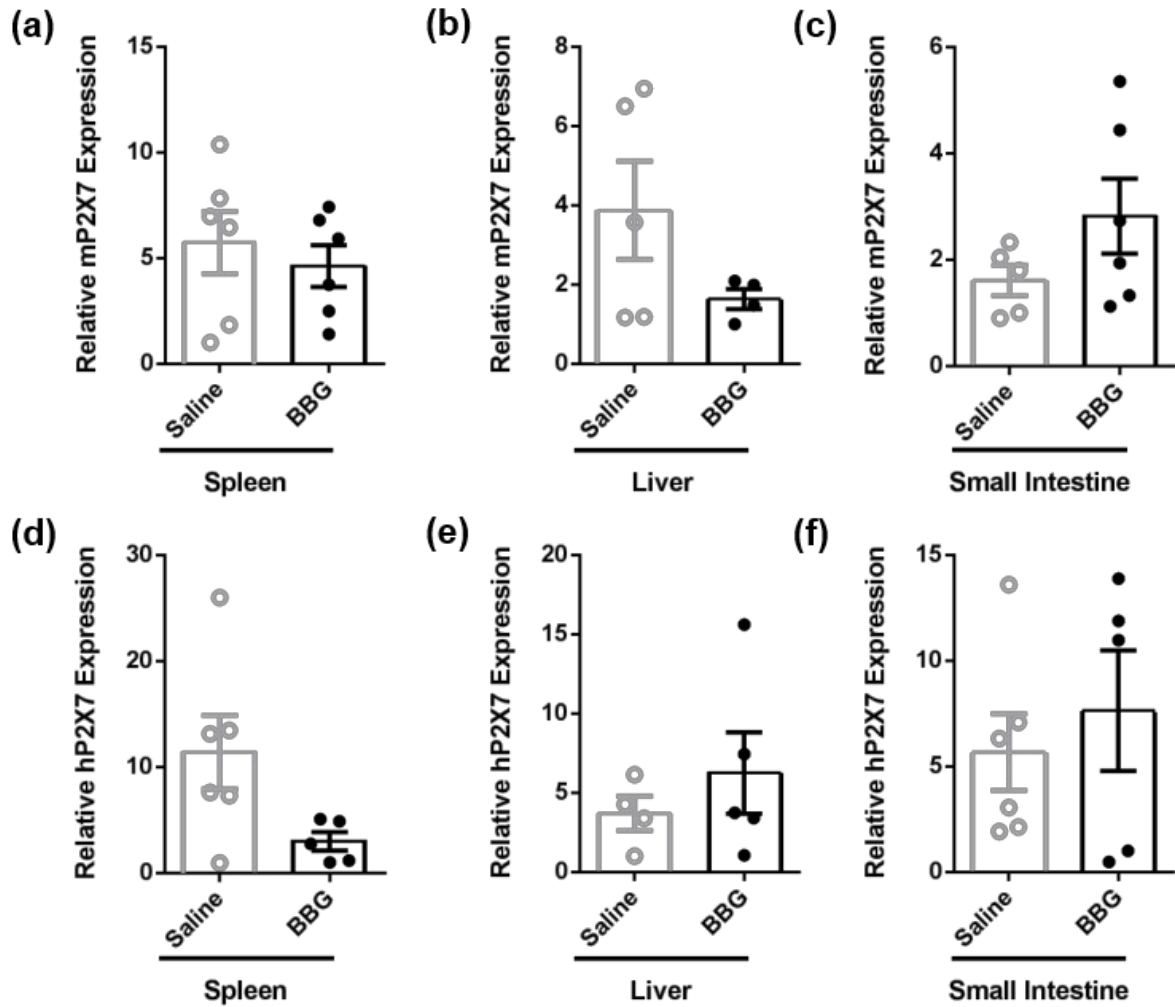


Figure 3.7. Brilliant Blue G (BBG) does not impact expression of mP2X7 or hP2X7 in tissues in humanised mice. (a-f) NOD-SCID-IL2R γ^{null} (NSG) mice injected intra-peritoneally (i.p.) with 10×10^6 human peripheral blood mononuclear cells (day 0), and with saline (control) or 50 mg/kg Brilliant Blue G (BBG) (from Figure 5) were monitored for clinical signs of GVHD over 10 weeks. The relative expression of (a-c) murine (m) P2X7 and (d-f) hP2X7 in (a, d) spleen, (b, e) liver, and (c, f) small intestine from mice at end point were examined by qPCR. Data represents group means \pm SEM ($n = 4-6$); symbols represent individual mice.

that of saline-injected mice (21.8 ± 2.4 ng/mL, $n = 13$) ($P = 0.0023$) (Figure 3.8.a.). In contrast, hIL-17 was only detected in the serum from four BBG-injected mice (33%) and two saline-injected mice (15%) for which samples were available (Figure 3.8.b.). The amount of hIL-17 in the serum of BBG-injected mice (40.8 ± 21.1 pg/mL, $n = 4$) was almost ten-fold

greater than that of saline-injected mice (4.4 ± 4.0 pg/mL, $n = 2$), however this difference did not reach statistical significance ($P = 0.1031$) (Figure 3.8.b.).

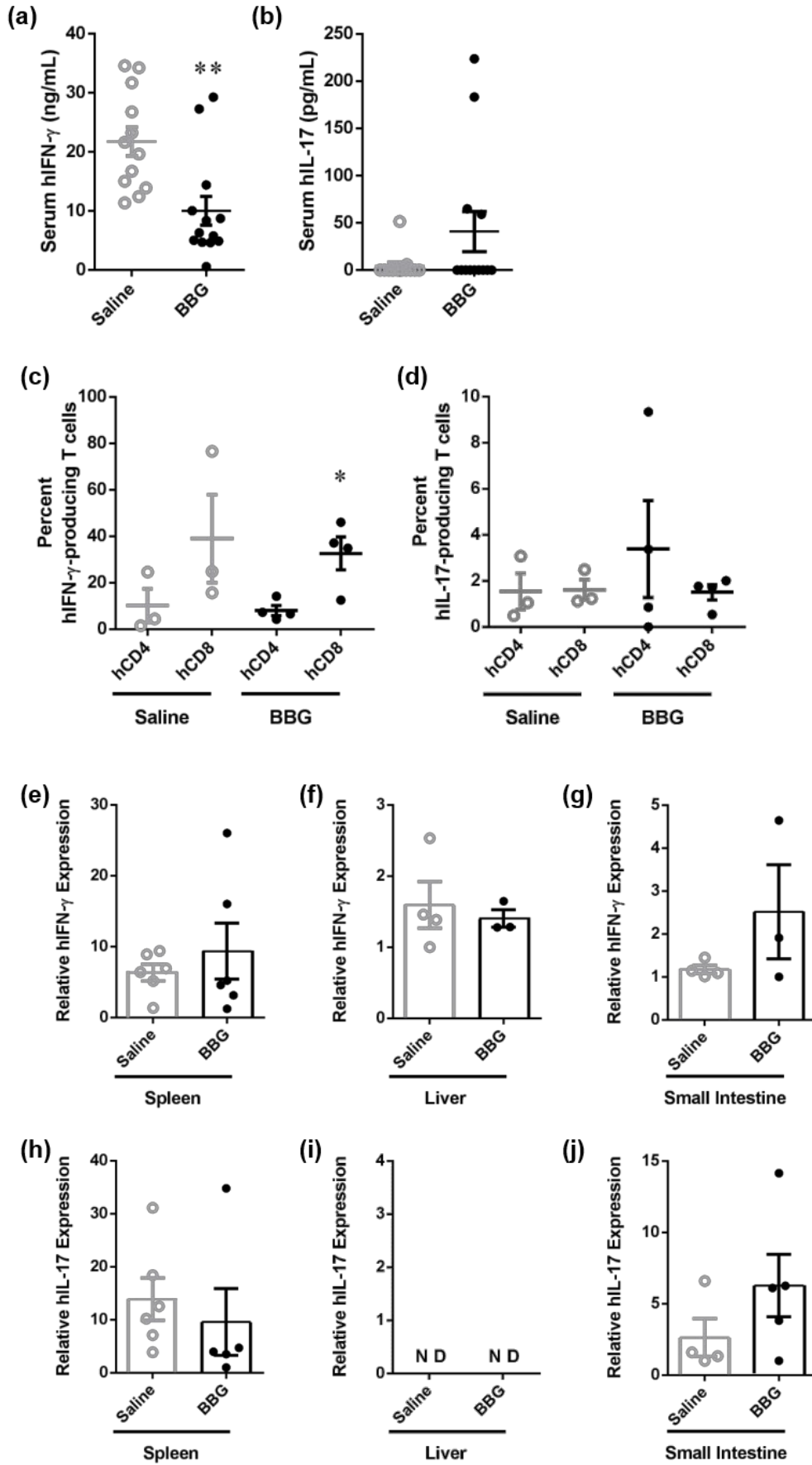
To determine the potential source of hIFN- γ and hIL-17, splenocytes were isolated from humanised mice and the intracellular expression of hIFN- γ and hIL-17 was analysed by flow cytometry. Both hCD4⁺ and hCD8⁺ T cells produced hIFN- γ and to a lesser extent hIL-17 (Figure 3.8.c, d). The percentage of hIFN- γ -producing hCD8⁺ T cells was four-fold greater than hIFN- γ -producing hCD4⁺ T cells from BBG-injected mice ($32.6 \pm 7.1\%$ vs $8.1 \pm 2.1\%$, respectively, $n = 4$) and saline-injected mice ($39.0 \pm 18.9\%$ vs $10.2 \pm 7.3\%$, respectively, $n = 3$), but this only reached statistical significance in the BBG-injected group ($P = 0.0165$) (Figure 3.8.c.). The percentage of hIL-17-producing hCD4⁺ and hCD8⁺ T cells in BBG-injected mice ($3 \pm 4\%$ vs. $2 \pm 1\%$, $n = 4$, respectively) was similar to saline-injected mice ($2 \pm 1\%$ vs. $2 \pm 1\%$, $n = 3$, respectively) ($P = 0.5046$ and 0.8437 , respectively) (Figure 3.8.d.).

To determine if expression of IFN- γ and IL-17 were altered in the spleens, and target organs of GVHD, tissues from humanised mice were analysed by qPCR. hIFN- γ expression was 1.5-fold greater in the spleens of BBG-injected mice (9.4 ± 3.9 , $n = 6$) compared to saline-injected mice (6.4 ± 1.2 , $n = 6$), but this difference did not reach statistical significance ($P = 0.4825$) (Figure 3.8.e.). hIFN- γ expression was similar in livers of BBG-injected mice (1.4 ± 0.1 , $n = 6$) and saline-injected mice (1.6 ± 0.3 , $n = 6$) ($P = 0.6549$) (Figure 3.8.f.). hIFN- γ expression was two-fold greater in the small intestines of BBG-injected mice (2.5 ± 1.1 , $n = 3$) compared to saline-injected mice (1.2 ± 0.1 , $n = 4$) but again this difference did not reach statistical significance ($P = 0.2050$) (Figure 3.8.g.). hIL-17 expression in spleens of BBG-injected mice (9.6 ± 6.3 , $n = 5$) was similar to saline-injected mice (13.9 ± 4.0 , $n = 6$) ($P = 0.5441$) (Figure 3.8.h.). hIL-17 was not detected in the livers of either BBG-injected mice ($n = 2$) or saline-injected mice ($n = 4$) (Figure 3.8.i.). hIL-17 expression was two-fold greater in the small intestines of BBG-injected mice (6.3 ± 2.2 , $n = 5$) compared to saline-injected mice

(2.6 ± 1.3 , $n = 4$), but this difference did not reach statistical significance ($P = 0.2263$) (Figure 3.8.j).

3.5.9. BBG Does Not Alter Murine or Human IL-1 β Expression in Humanised Mice

Activation of P2X7 causes IL-1 β maturation and release from antigen presenting cells (Englezou *et al.*, 2015), a pro-inflammatory cytokine implicated in the pathogenesis of GVHD (McCarthy Jr *et al.*, 1991). Moreover, P2X7-mediated IL-1 β release is involved in the production of IFN- γ (Ghiringhelli *et al.*, 2009) and IL-17 (Zhao *et al.*, 2013). Given the identity of the antigen presenting cells involved in human T cell activation in our humanised model of GVHD remains unknown and since mIL-1 β can stimulate human cells (Huang *et al.*, 1988, Vandenabeele *et al.*, 1990), the expression of both mIL-1 β and hIL-1 β in the spleen, liver and small intestine from these mice was analysed by qPCR. mIL-1 β expression was 3-fold greater in the spleens of BBG-injected mice (6.3 ± 2.8 , $n = 3$) compared to saline-injected mice (2.0 ± 0.9 , $n = 5$), but this difference did not reach statistical significance ($P = 0.1183$) (Figure 3.9.a). mIL-1 β expression was similar in the livers and small intestines of BBG- injected (2.7 ± 1.2 , $n = 5$ and 2.5 ± 0.5 , $n = 6$, respectively) and saline-injected mice (2.7 ± 0.7 , $n = 4$ and 1.9 ± 0.6 , $n = 6$, respectively) ($P = 0.9552$ and 0.4829 , respectively) (Figure 3.9.b, c). In contrast to mIL-1 β , hIL-1 β expression was approximately 50% lower in the spleens of BBG-injected mice (2.2 ± 1.1 , $n = 5$) compared to saline-injected mice (5.0 ± 2.1 , $n = 5$), but this difference did not reach statistical significance ($P = 0.2986$) (Figure 3.9.d). hIL-1 β expression was not detected in livers or small intestines from either BBG- or saline-injected mice (both $n = 5$) (data not shown). Therefore, given the broad expression of mIL-1 β within the humanised mice, serum mIL-1 β was assessed by ELISA. However, mIL-1 β was not detected in the serum from BBG-injected (0.0 ± 0.0 , $n = 12$) or saline-injected mice (0.0 ± 0.0 , $n = 11$) using an ELISA with a reported sensitivity of 8 pg/mL (eBioscience).



◀ **Figure 3.8. Brilliant Blue G (BBG) significantly reduces serum human interferon (IFN)- γ in humanised mice.** (a-j) NOD-SCID-IL2R γ^{null} (NSG) mice were injected intra-peritoneally (i.p.) with 10×10^6 human (h) peripheral blood mononuclear cells (PBMCs) (day 0), and with saline (control) or 50 mg/kg Brilliant Blue G (BBG) (from Figure 5). Concentrations of serum (a) hIFN- γ , and (b) hIL-17 were analysed by ELISA. Data represents group means \pm SEM (saline $n = 12$, BBG $n = 13$); $**P < 0.005$ compared to saline-injected mice. (c, d) Splenocytes were incubated in complete RPMI 1640 medium containing phorbol 12-myristate 13-acetate and ionomycin for 4 h, and the percentage of hCD4 $^+$ and hCD8 $^+$ T cells producing intracellular (c) hIFN- γ or (d) hIL-17 was determined by flow cytometry. Data represents group means \pm SEM (saline $n = 3$, BBG = 4); symbols represent individual mice; $* P < 0.05$ compared to corresponding hCD4 $^+$ T cells. (e-j) The relative expression of (e-g) hIFN- γ and (h-j) hIL-17 in (e, h) spleen, (f, i) liver and (g, j) small intestine from mice at end point were examined by qPCR. Data represents group means \pm SEM ($n = 2-6$); symbols represent individual mice. ND = not detected.

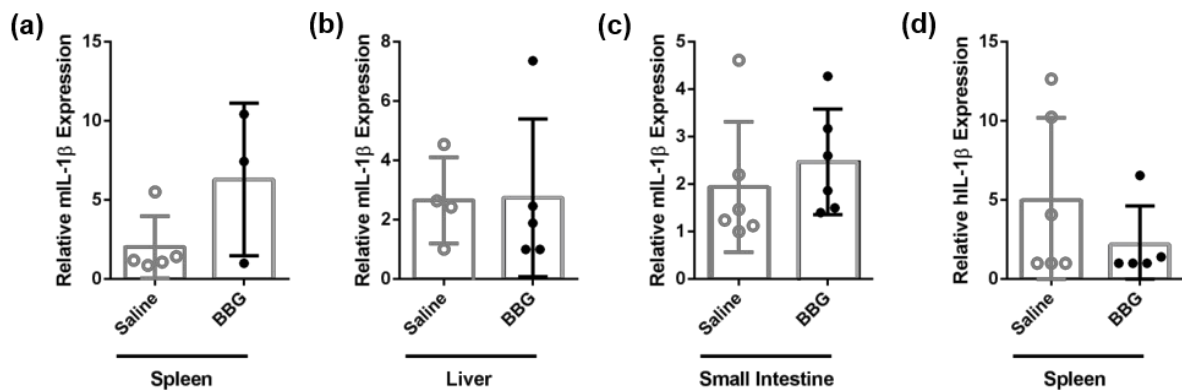


Figure 3.9. Brilliant Blue G (BBG) does not alter relative expression of human or murine IL-1 β in humanised mice. (a-d) NOD-SCID-IL2R γ^{null} (NSG) mice were injected intra-peritoneally (i.p.) with 10×10^6 human (h) peripheral blood mononuclear cells (PBMCs) (day 0), and with saline (control) or 50 mg/kg Brilliant Blue G (BBG) (from Figure 5). The relative expression of (a-c) murine (m) interleukin (IL)-1 β and (d) hIL-1 β in (a, d) spleen, (b) liver and (c) small intestine from mice at end point were examined by qPCR. Data represents group means \pm SEM ($n = 3-6$); symbols represent individual mice.

3.6. Discussion

This study aimed to investigate the effect of the P2X7 antagonist, BBG, in a pre-clinical humanised mouse model of GVHD. Injection of BBG into humanised mice did not alter human leukocyte engraftment or disease progression, as assessed by weight loss, clinical score and survival. However, treatment with BBG significantly reduced serum hIFN- γ .

Intracellular hIFN- γ was detected in both splenic hCD4⁺ and hCD8⁺ T cells of humanised mice, with a larger proportion (four-fold) of hIFN- γ ⁺ hCD8⁺ T cells than hIFN- γ hCD4⁺ T cells. However, splenic hCD4⁺ T cells were more frequent (three-fold) than hCD8⁺ T cells in humanised mice, and therefore the relative contribution of each subset to circulating IFN- γ production needs to be further elucidated. Pharmacological blockade or genetic deficiency of P2X7 has also been shown to reduce serum IFN- γ in allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014). Additionally, P2X7 activation of the NLRP3 inflammasome and subsequent IL-1 β production promotes the generation of IFN- γ ⁺ CD8⁺ T cells in mice (Ghiringhelli *et al.*, 2009). Combined, these findings support a potential role for P2X7 in GVHD pathology in humans, possibly through the regulation of T cell activation and subsequent IFN- γ production. To this end, IFN- γ plays important roles in the up-regulation of CXCR3 and subsequent trafficking of T cells to target tissues in allogeneic mouse models of GVHD (Choi *et al.*, 2012a). Conversely, others have suggested that IFN- γ ⁺ CD8⁺ T cells and IFN- γ ⁺ CD4⁺ T cells are important in the initiation and progression of disease, respectively, in an allogeneic mouse model of GVHD (Zhao *et al.*, 2016). Nevertheless, it remains to be determined if P2X7 functions through these mechanisms in allogeneic or humanised mouse models of GVHD.

The current study also identified the presence of serum hIL-17 in a small proportion of humanised mice (15-30%), and intracellular hIL-17 in an even smaller population of splenic hCD4⁺ and hCD8⁺ T cells (2-3%) from these mice. Due to the relatively small populations, it is not possible to positively determine the role of P2X7 on hIL-17 production in this humanised mouse model of GVHD. However, the mean serum hIL-17 concentration was 10-fold greater in BBG-injected mice compared to saline-injected mice. These results may suggest P2X7 blockade promotes hIL-17 production in humanised mice, which contradicts previous *in vitro* results in which P2X7 activation promoted hIL-17 production in T cells

(Ghiringhelli *et al.*, 2009). Others have shown that a shift from an IFN- γ -producing to an IL-17-producing T cell phenotype exacerbates GVHD in an allogeneic mouse model of GVHD (Pan *et al.*, 2012). Similarly, increased numbers of IL-17-producing T cells were observed in patients with more severe GVHD (Zhao *et al.*, 2011). Investigating IL-17-producing T cells at time points prior to end point or through increased sample sizes may assist in addressing the role of P2X7 blockade in IL-17 production in humanised mice with GVHD.

Due to the variation observed in hIL-17 mRNA expression between humanised mice, and the role of IL-1 β in the differentiation of IL-17-producing T cells (Acosta-Rodriguez *et al.*, 2007) including models of P2X7 activation (Zhao *et al.*, 2013), mIL-1 β and hIL-1 β was also examined in the current study. mIL-1 β , but not hIL-1 β , mRNA expression was detected in the livers and small intestines of humanised mice, with no differences observed between BBG- and saline-treatments. In contrast both mIL-1 β and hIL-1 β mRNA expression was detected in the spleens of humanised mice, with BBG treatment resulting in a 3-fold increase in mIL-1 β mRNA expression and a 50% decrease in hIL-1 β mRNA expression. However, there was no correlation between hIL-17 and hIL-1 β , or between hIL-17 and mIL-1 β mRNA expression (results not shown), although *n* values are relatively small, limiting interpretation of this data. Due to the inability to detect serum mIL-1 β in these mice, serum mIL-1 β could not be compared with serum hIL-17 (hIFN- γ). The inability to detect serum mIL-1 β was not limited to this cytokine, as serum hIL-4 was not detected in initial experiments to establish the humanised mouse model, despite detection of serum hIFN- γ in these mice (results not shown).

As previously mentioned, injection of BBG into humanised mice did not alter disease progression, as assessed by weight loss, clinical score and survival. However, histological examination revealed that BBG reduced the infiltration of leukocytes into the small intestine and skin in this humanised mouse model of GVHD. Moreover, there was also histological

evidence of reduced tissue damage in these tissues from BBG-injected mice compared to saline-injected mice, observations supported by immunohistochemical analysis of T cell infiltrates and apoptosis. In contrast, BBG did not alter the proportion of hCD4⁺ or hCD8⁺ T cells in blood and spleens of humanised mice. Others have reported that humanised NSG mice exhibit leukocyte infiltration and tissue damage in the liver, small intestine and skin, characteristic of GVHD (King *et al.*, 2009). T cells are the main mediators of disease in this humanised mouse model of GVHD (Covassin *et al.*, 2013, Abraham *et al.*, 2015). Consistent with these reports, immunohistochemical analysis with a human CD3 specific mAb detected T cells in the liver, small intestine and skin in humanised mice with GVHD. In support of this observation, hP2X7 mRNA was detected in the livers and small intestines of humanised mice using qPCR probes to the P2X7B splice variant, which is predominantly expressed in human T cells (Adinolfi *et al.*, 2010), with P2X7 present on both hCD4⁺ and hCD8⁺ T cells (Sluyter and Wiley, 2014).

Collectively, the above data investigating the effect of the P2X7 antagonist, BBG, in a humanised mouse model of GVHD both confirms findings from previous studies using P2X7 antagonists in allogeneic mouse models of GVHD, and provides additional novel insights. Similar to allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016), P2X7 antagonism reduced serum IFN- γ , immune infiltrates and tissue damage in humanised mice with GVHD. By contrast, no therapeutic benefit (weight loss, clinical score or survival) was observed in humanised mice with GVHD, but therapeutic benefits were observed in allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016). Besides obvious laboratory, species and mouse strain differences, a number of other notable differences remain between these models of GVHD. First, survival time in these allogeneic mouse models (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014) is at least half that compared to humanised mice. Thus, extended or delayed injection

regimes may be required to show therapeutic benefits in humanised mice. Second, in the allogeneic mouse models, P2X7 antagonists were injected daily for the first 10 days (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014) or twice weekly for 28 days (Zhong *et al.*, 2016) in contrast to five injections over the first 8 days in our humanised mouse model. Thus, more frequent or extended injection regimes may be required to show therapeutic benefits with BBG in humanised mice with GVHD. Third, the two studies that reported improved survival in the allogeneic mouse models (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014) used the P2X7 antagonists, pyridoxal-phosphate-6-azophenyl-2',4-disulphonic acid, KN-62 or stavudine, rather than BBG. Thus, testing of P2X7 antagonists other than BBG in humanised mice with GVHD may be warranted. Although it should be noted that BBG at either 50 or 70 mg/kg (i.p. twice weekly for 28 days) prevented weight loss in one allogeneic mouse model of GVHD (Zhong *et al.*, 2016), indicating BBG can display some therapeutic benefit in this disease. Finally, in each of the allogeneic mouse models, recipient mice were first irradiated (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016), which causes ATP release (Wilhelm *et al.*, 2010). In contrast, the humanised mice in our study were not irradiated, potentially eliminating this initial release of ATP, which may subsequently activate P2X7 to promote disease. Thus, the therapeutic potential of P2X7 antagonists in irradiated humanised mice with GVHD could be investigated.

Finally, this study showed that NSG mice demonstrate full-length functional P2X7. Firstly, qPCR revealed expression of *P2RX7* mRNA in the spleens, livers and small intestines of NSG mice. Secondly, immunoblotting revealed a major band at 81 kDa, correlating to glycosylated, full-length P2X7 (Pupovac *et al.*, 2015). Finally, ATP induced YO-PRO-1²⁺ uptake into splenic DCs from NSG mice, a process impaired by BBG, and which was absent in splenic DCs from P2X7 KO mice. ATP-induced YO-PRO-1²⁺ uptake into splenic DCs from NSG mice was significantly higher than splenic DCs from C57BL/6 mice. This is most

likely due to the absence and presence of a loss-of-function mutation (P451L) (Adriouch *et al.*, 2002) in NSG and C57BL/6 mice, respectively. Although it is yet to be formally demonstrated that NSG mice are wild-type at this allele, NSG mice are derived from NOD mice (Shultz *et al.*, 2007), a strain known to encode a proline residue at amino acid position 451 (Syberg *et al.*, 2012).

In conclusion, the current study demonstrates P2X7 blockade with BBG can significantly reduce serum hIFN- γ , and inflammation and tissue damage in a humanised mouse model of GVHD. The similar clinical course with skewing to Th17 suggests that clinically P2X7 blockade may be of additive benefit if combined with strategies that limit Th17, such as IL-6 receptor blockade (Koenecke *et al.*, 2009). This study suggests a potential benefit for P2X7 blockade, but highlights the need to also address alternative pathways of immune activation.

3.7. Acknowledgements

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3.8. Disclosure

All authors declare that they have no disclosures.

3.9. Author Contributions

N.J.G., L.B., D.L., S.R.A., S.I.A., M.S.S., R.S. and D.W. designed the experiments. N.J.G., L.B., D.L., S.R.A., V.S., R.S. and D.W. performed the experiments. N.J.G., L.B., D.L., S.R.A., R.S. and D.W. analysed the data. S.F. and W.V. provided histological analysis. N.J.G. prepared the figures and wrote the manuscript. R.S. and D.W. edited the manuscript. L.B., D.L., S.R.A, S.F., W.V., M.S.S. and S.I.A. co-edited the manuscript.

Chapter 4: Long-Term Treatment With the P2X7 Receptor Antagonist Brilliant Blue G Reduces Liver Inflammation in a Humanised Mouse Model of Graft-versus-Host Disease

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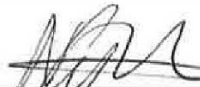
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4.1. Statement of Contribution of Authors

I, as one of the authors of this original article, agree with the statement of author contributions stated at the end of this chapter, and originally published in the original journal article.



Nicholas Geraghty



Debbie Watson



Ronald Sluyter

4.2. Abstract

Allogeneic haematopoietic stem cell transplantation (HSCT) is a frequent curative therapy for numerous haematological malignancies. However, HSCT is limited by the occurrence of graft-versus-host disease (GVHD), with current therapies restricted to general immunosuppression. Activation of the P2X7 receptor by extracellular adenosine triphosphate (ATP) causes tissue damage and subsequent inflammation exacerbates GVHD. Short-term pharmacological blockade of P2X7 has been shown to reduce clinical disease and/or reduce inflammatory markers in allogeneic and humanised mouse models of GVHD. The current study demonstrates that long-term P2X7 blockade by intra-peritoneal injection of Brilliant Blue G (BBG) thrice weekly for up to 10 weeks did not impact human (h) peripheral blood mononuclear cell (PBMC) engraftment, predominantly T cells, in blood at 3 weeks post-hPBMC injection or in spleens at end-point in humanised mice. Histological analysis demonstrated long-term BBG treatment reduced leukocyte infiltration in the livers of humanized mice. Immunohistochemical analysis demonstrated that BBG treatment reduced liver apoptosis. Immunohistochemical analysis confirmed reduced human T cell infiltration and apoptosis in livers from long-term BBG-treated humanised mice. Long-term BBG treatment did not alter mRNA expression of pro-inflammatory markers in tissues or serum human interferon (IFN)- γ concentrations. Therefore, this study demonstrates that P2X7 activation plays a role in GVHD pathogenesis in the livers of humanised mice, supporting a role for this receptor in GVHD development in HSCT recipients.

4.3. Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is a frequent curative method for various haematological malignancies. However, a major side effect of this treatment is graft-versus-host disease (GVHD). GVHD occurs in approximately half of HSCT recipients (Jagasia *et al.*, 2012) and results in damage to the liver, gastrointestinal tract and skin, with the liver affected in approximately 50% of GVHD patients (Martin *et al.*, 1990). GVHD emerges due to transplanted effector T cells mounting an immune response against the host (Billingham, 1965). This immune response is driven by release of cytokines and danger associated molecular patterns (DAMP), which causes antigen presenting cells to release cytokines resulting in activation of T cells (Reddy and Ferrara, 2003, Ferrara and Reddy, 2006, Ferrara *et al.*, 2009). T cell activation leads to proliferation and differentiation of T cell subsets such as Th1 cells which release interferon (IFN)- γ (Reddy and Ferrara, 2003, Ferrara and Reddy, 2006), and Th17 cells which release interleukin (IL)-17, resulting in inflammatory damage (Yi *et al.*, 2009, Gartlan *et al.*, 2015). Current therapies are largely limited to general immunosuppression through the use of steroids (Jagowski and Devine, 2014), highlighting the need for better therapies.

Targeting a pathway in one of the above stages of GVHD represents a potential therapeutic strategy. Purinergic signalling is an important pathway of immune cell signalling (Yip *et al.*, 2009) and plays important roles in transplantation (Vergani *et al.*, 2014, Apostolova and Zeiser, 2016b, Castillo-Leon *et al.*, 2018). Of all the purinergic receptors, the P2X7 receptor is the most widely studied in immune and inflammatory responses (Di Virgilio *et al.*, 2018). Activation of P2X7 induces a number of downstream effects including DC activation and cytokine release (Saez *et al.*, 2017), T cell activation (Schenk *et al.*, 2008) and inhibition of regulatory T cells (Schenk *et al.*, 2011). Thus, given the central role of these immune processes in GVHD, blockade of P2X7 represents a potential therapeutic approach to limit

GVHD development. In allogeneic mouse models the DAMP, adenosine triphosphate (ATP), is released from damaged and dying cells, to promote GVHD (Wilhelm *et al.*, 2010). ATP exacerbates GVHD by activating P2X7 in allogeneic mouse models (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016). Short term pharmacological blockade of P2X7 with either KN-62, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid or stavudine during the first week post-transplantation can extend survival of recipient mice in these models (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014). Whilst, P2X7 blockade with Brilliant Blue G (BBG) (50 or 75 mg/kg) twice per week for 4 weeks reduces weight loss and liver GVHD in an allogeneic mouse model (Zhong *et al.*, 2016). Finally, our group recently demonstrated that short-term blockade of P2X7 with BBG (50 mg/kg, days 0, 2, 4, 6, 8) reduced serum human (h) IFN- γ and tissue inflammation in a humanised non-obese diabetic severe combined immunodeficient IL-2 gamma receptor null (NOD-SCID-IL2R γ^{null} ; NSG) mouse model of GVHD (Chapter 3).

To further extend the above observations ((Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016) and Chapter 3) to humans, the current study investigated the effect of long-term P2X7 blockade with BBG (50 mg/kg; thrice weekly for up to 10 weeks) in a humanised NSG mouse model of GVHD. Similar to our previous study (Chapter 3), injection of BBG did not impact human leukocyte engraftment, weight loss, clinical score or survival. Notably, this regime reduced leukocyte infiltration and apoptosis in the livers of mice, supporting a role for P2X7 activation in GVHD pathogenesis in the liver.

4.4. Materials and Methods

4.4.1. Humanised Mouse Model of GVHD

Experiments with human blood and mice were approved by the Human Animal Ethics Committees, respectively (University of Wollongong, Wollongong, Australia). Humanised NSG mice were established as described (Chapter 3). Briefly, hPBMCs, isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden), were injected intra-peritoneally (i.p) (10×10^6 hPBMCs/mouse) into female NSG mice aged 5-7 weeks (Westmead Animal Research Facility, Westmead, Australia) housed at the University of Wollongong. Mice were injected i.p. 2 hours later with 200 μ L of saline (saline group) or saline containing BBG (Sigma-Aldrich; St Louis, MO, USA) (50 mg/kg; BBG group), then thrice weekly (every second or third day apart) for up to 10 weeks. At 3 weeks post-hPBMC injection, engraftment was examined by immunophenotyping of tail vein blood. Mice were monitored up to 10 weeks for signs of clinical GVHD using a scoring system, giving a total clinical score out of 10 as described (Chapter 3) and euthanised at 10 weeks post-hPBMC injection, or earlier if the clinical score was ≥ 8 or weight loss was $\geq 10\%$, as per the approved ethics protocol.

4.4.2. Immunophenotyping by Flow Cytometry

Tail vein blood and spleen cells were obtained from mice and lysed with ammonium chloride potassium buffer and immunophenotyped as described (Chapter 3) with the addition of an allophycocyanin conjugated mouse anti-hCD56 monoclonal antibody (clone: B159) (BD, San Jose, CA, USA). Data was collected using a BD Fortessa-X20 Flow Cytometer using band pass filters of 525/50 for fluorescein isothiocyanate, 586/15 for R-phycoerythrin, 695/40 for peridinin chlorophyll protein and 670/30 for allophycocyanin. The relative percentages of cells were analysed using FlowJo software v8.7.1 (TreeStar Inc.; Ashland, OR, USA) (Figure S1 and Figure S2).

4.4.3. Histological and Immunohistological Analysis

Formalin-fixed tissue sections (5 µm) were stained with haematoxylin and eosin (POCD; Artarmon, Australia) for histological analysis. Other formalin-fixed tissue sections were also stained with either rabbit anti-hCD3 mAb (clone: EP449E) (Abcam, Cambridge, UK) and haematoxylin as described (Chapter 3) or an *In situ* Apoptosis Detection Kit (Abcam) as per the manufacturer's instructions. Total numbers of leukocyte and hCD3⁺ T cell infiltrates and apoptotic cells in livers were quantified from captured images (Leica DMRB microscope, Wetzlar, Germany) using FIJI is just ImageJ (FIJI) software (Schindelin *et al.*, 2012). Data is represented as the total number of cells measured per field of view. Epidermal thickness of skin sections was measured as described (Chapter 7).

4.4.4. Quantitative Real-Time PCR

RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) and converted to cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) as per the respective manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed as described (Chapter 7) with the addition of a human tumour necrosis factor (TNF)- α (Hs01113624_g1) primers (Thermo Fisher Scientific). Human gene expression was normalised to cDNA from hPBMCs, directly isolated from a human donor. Murine gene expression was normalised to cDNA from a spleen of a BALB/c mouse (Bartlett *et al.*, 2017).

4.4.5. ELISA

Serum was obtained from mice as described (Chapter 3), and hIFN- γ and hIL-17 concentrations determined using respective Ready-Set-Go! ELISA Kits (eBioscience, San Diego, CA, USA) as per the manufacturer's instructions.

4.4.6. Statistical Analysis

Data is given as mean \pm standard deviation (SD). Statistical differences were determined using the Student's t-test for single comparisons or one-way analysis of variance (ANOVA)

with Tukey's post-hoc test for multiple comparisons. Weight and clinical score over time were compared using a repeated measures two-way ANOVA with Tukey's post-hoc test for multiple comparisons. Survival (median survival time; MST) was compared using the log-rank (Mantel-Cox) test. Mortality was compared using Fisher's exact test. All statistical analyses and graphs were generated using GraphPad Prism 5 for PC (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

4.5. Results

4.5.1. BBG Does Not Impact Engraftment of Human Cells in NSG Mice

To determine if long-term P2X7 blockade can ameliorate disease development in a humanised mouse model of GVHD, NSG mice injected with hPBMCs, were subsequently injected with the P2X7 antagonist BBG (Sluyter, 2017) at 50 mg/kg or with saline thrice weekly for up to 10 weeks. To investigate if human cell engraftment was affected by BBG treatment, flow cytometric analysis of blood was conducted at 3 weeks post-hPBMC injection (Supplementary Figure 4.1.). Mice injected with BBG demonstrated a similar frequency of human leukocytes ($13.2 \pm 2.4\%$ hCD45⁺ mCD45⁻ cells, $n = 10$) compared to mice injected with saline (control) ($9.7 \pm 2.8\%$ hCD45⁺ mCD45⁻ cells, $n = 7$) ($P = 0.3561$) (Figure 4.1.a.). T cells comprised the majority of hCD45⁺ cells, and the frequencies of T cells did not vary between mice injected with BBG ($93.2 \pm 1.4\%$ hCD3⁺ hCD56⁻ cells, $n = 10$) or saline ($91.6 \pm 2.1\%$ hCD3⁺ hCD56⁻ cells, $n = 7$) ($P = 0.5188$) (Figure 4.1.b.). In all mice, there was a small population of hCD3⁺ hCD56⁺ cells, potentially natural killer T (NKT) cells (Ortaldo *et al.*, 1991), but the frequency of these cells did not vary between mice injected with BBG ($3.1 \pm 1.1\%$ hCD3⁺ hCD56⁺ cells, $n = 10$) or saline ($3.3 \pm 1.0\%$ hCD3⁺ hCD56⁺ cells, $n = 7$) ($P = 0.8768$) (Figure 4.1.c.). Both groups of mice demonstrated engraftment of a

small proportion of natural killer (NK) cells (hCD3⁻ hCD56⁺), which did not significantly differ between mice injected with BBG (1.0 ± 0.3% hCD3⁻ hCD56⁺ cells, *n* = 10) or saline (2.3 ± 0.8% hCD3⁻ hCD56⁺ cells, *n* = 7) (*P* = 0.1067) (Figure 4.1.d.). Finally, there was a small population of cells that were neither T, NKT, nor NK cells; the frequency of which did not vary between mice injected with BBG (2.7 ± 0.8% hCD3⁻ hCD56⁻ cells, *n* = 10) or saline (2.8 ± 1.1% hCD3⁻ hCD56⁻ cells, *n* = 7) (*P* = 0.9854) (Figure 4.1.e.).

Splenocyte analysis at end point (Supplementary Figure 4.2.) showed human leukocytes comprised the majority of total murine and human leukocytes. Mice injected with BBG exhibited a similar frequency of human leukocytes (76.7 ± 3.2% hCD45⁺ mCD45⁻ cells, *n* = 10) compared to mice injected with saline (71.4 ± 6.1% hCD45⁺ mCD45⁻ cells, *n* = 7) (*P* = 0.4308) (Figure 4.1.f.). Similar to engraftment at 3 weeks post-hPBMC injection, T cells comprised the majority of human leukocytes in the spleen, and both mice injected with BBG (86.4 ± 5.2% hCD3⁺ hCD56⁻ cells, *n* = 10) and mice injected with saline (96.1 ± 1.9% hCD3⁺ hCD56⁻ cells, *n* = 7) demonstrated similar frequencies of T cells (*P* = 0.1390) (Figure 4.1.g.). Similar to blood, there was a small population of hCD3⁺ hCD56⁺ cells, and the percentage of these cells did not vary between mice injected with BBG (1.3 ± 0.6% hCD3⁺ hCD56⁺ cells, *n* = 10) or saline (0.9 ± 0.1% hCD3⁺ hCD56⁺ cells, *n* = 7) (*P* = 0.5154) (Figure 4.1.h.). Few mice demonstrated engraftment of NK cells in spleens at time of euthanasia, which did not significantly differ between mice injected with BBG (0.7 ± 0.5% hCD3⁻ hCD56⁺ cells, *n* = 10) or saline (0.1 ± 0.0% hCD3⁻ hCD56⁺ cells, *n* = 7) (*P* = 0.3064) (Figure 4.1.i.). Finally, there was a remaining population of cells that were neither T nor NK cells; the frequency of which did not vary between mice injected with BBG (10 ± 4.2% hCD3⁻ hCD56⁻ cells, *n* = 10) or saline (3.0 ± 2.0% hCD3⁻ hCD56⁻ cells, *n* = 7) (*P* = 0.1868) (Figure 4.1.j.).

Human T cell analysis (hCD3⁺) in spleens at end point (Supplementary Figure 4.2.) revealed that mice injected with BBG or saline contained similar frequencies of hCD4⁺ T cells (71.3 ±

4.7%, $n = 10$ vs. $75.6 \pm 3.8\%$, $n = 7$, respectively, $P = 0.5345$) and hCD8⁺ T cells ($17.9 \pm 2.9\%$, $n = 10$ vs $14.6 \pm 2.3\%$, $n = 7$, respectively, $P = 0.4500$) (Figure 4.1.k.). In both mice injected with BBG or saline, the percentages of hCD4⁺ T cells were significantly greater than that of hCD8⁺ T cells (both $P < 0.0001$ and $P < 0.0001$, respectively) (Figure 4.1.k.).

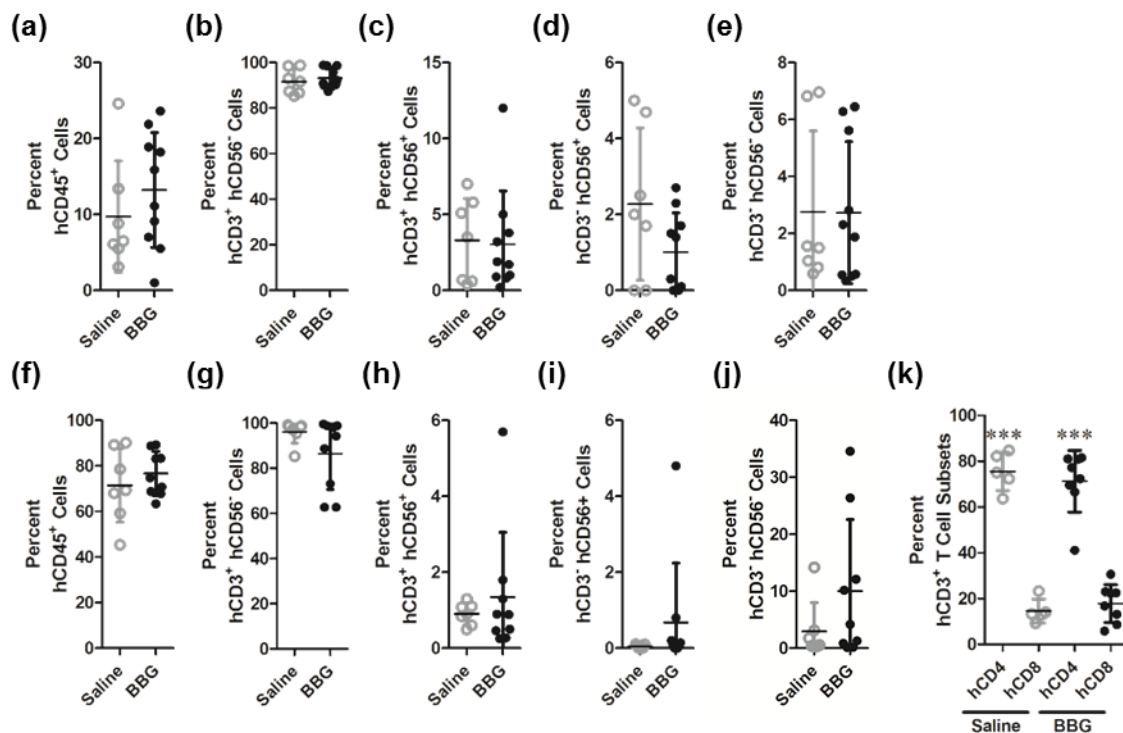


Figure 4.1. Long-term BBG treatment does not affect engraftment of human cells in a humanised mouse model of GVHD (a-k) NOD-SCID-IL2R γ null (NSG) mice were injected intraperitoneally (i.p.) with 10×10^6 human (h) peripheral blood mononuclear cells (hPBMCs), and subsequently with saline ($n = 7$) or saline containing Brilliant Blue G (BBG) (50 mg/kg) ($n = 10$) thrice weekly. The percentages of human leukocytes in (a-e) blood at 3 weeks post-hPBMC injection and (f-k) spleens at end-point were determined by flow cytometry. (a, f) hCD45⁺ leukocytes are expressed as a percentage of total mCD45⁺ and hCD45⁺ leukocytes. (b, g) hCD3⁺ hCD56⁻, (c, h) hCD3⁺ hCD56⁺, (d, i) hCD3⁻ hCD56⁺ and (e, j) hCD3⁻ hCD56⁻ cells are expressed as a percentage of total hCD45⁺ leukocytes. (k) hCD4⁺ and hCD8⁺ T cell subsets are expressed as a percentage of total hCD3⁺ leukocytes. Data represents group means \pm SD; symbols represent individual mice; *** $P < 0.0001$ compared to corresponding hCD8⁺ T cells.

4.5.2. BBG Does Not Prevent Clinical GVHD in NSG Mice

For up to 10 weeks, mice were monitored for weight loss and signs of GVHD. All mice injected with BBG or saline exhibited weight loss from 4 weeks, with similar weight loss between groups over the course of the study ($P = 0.2853$) (Figure 4.2.a.). Both groups of mice began to show signs of mild GVHD from day 35, and both mice injected with BBG or saline had similar clinical scores at end point (5.8 ± 0.5 , $n = 10$ vs. 5.9 ± 0.7 , $n = 7$, respectively; $P = 0.9494$), and mean clinical scores over 10 weeks ($P = 0.1286$) (Figure 4.2.b.). Similar survival was also observed between mice injected with BBG (mortality of 90%, $n = 10$) or saline (mortality of 86.7%, $n = 7$) ($P = 1.000$) (Figure 2c), and mice injected with BBG or saline also demonstrated similar MSTs (45.5 days vs. 52.0 days, respectively) ($P = 0.3014$) (Figure 4.2.c.).

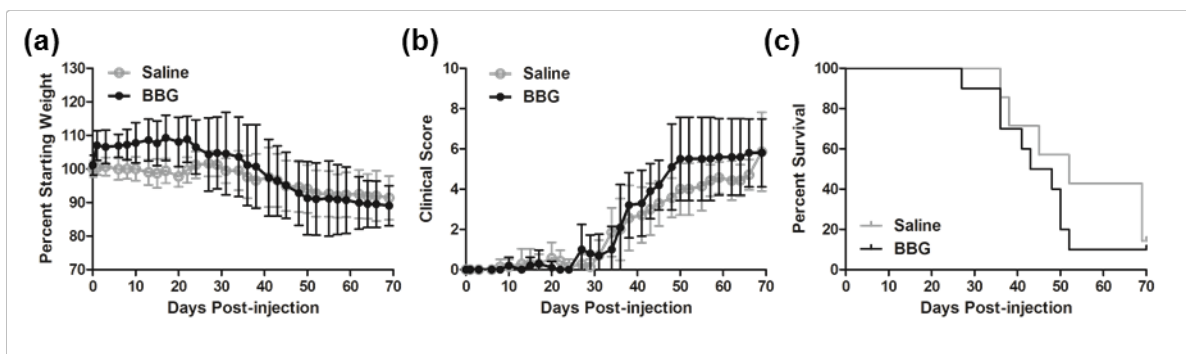


Figure 4.2. Long-term BBG treatment does not affect disease development in a humanised mouse model of GVHD. (a-c) Saline- and BBG-injected humanised NOD-SCID-IL2R γ null (NSG) mice (Figure 1) were monitored for (a) weight loss, (b) clinical score (using a scoring system, giving a total clinical score out of 10), and (c) survival for up to 10 weeks. Data represents (a, b) group means \pm SD or (c) percent survival (saline $n = 7$; BBG $n = 10$).

4.5.3. BBG Reduces Leukocyte Infiltration and Apoptosis in Livers of NSG Mice

Short-term BBG has been shown to partly reduce leukocyte infiltrates, predominantly T cells, and apoptosis in GVHD target organs including liver, small intestine and skin in humanised NSG mice (Chapter 3). Therefore, to assess if long-term BBG treatment could reduce leukocyte infiltration and damage, target tissues from humanised NSG mice were analysed via histology. There were no histological differences in the small intestines or skin, including epidermal thickening, of BBG- and saline-injected mice (Supplementary Figure 4.2.). However, livers from mice injected with BBG demonstrated reduced histological damage with less apoptotic cells (Figure 4.3.a.). Image analysis demonstrated a significant 52% reduction in leukocytes in livers from BBG-injected mice (517 ± 244 cells/field of view; $n = 9$) compared to saline-injected mice (1082 ± 324 cells/field of view; $n = 6$) ($P = 0.0020$) (Figure 4.3.a.).

To determine the identity of the cells above (Figure 4.3.a.), immunohistochemistry using an anti-hCD3 mAb was conducted. Image analysis demonstrated a 55% reduction in CD3⁺ cell infiltration in livers from BBG-injected mice (117 ± 157 cells/field of view; $n = 4$) compared to saline-injected mice (263 ± 404 cells/field of view; $n = 4$), however this difference did not reach statistical significance ($P = 0.5263$) (Figure 4.3.b.). Moreover, the number of hCD3⁺ T cells detected (Figure 4.3.b.) represented only 24% of the total leukocytes detected by histology (Figure 4.3.a.). Finally, an *In Situ* Apoptosis detection kit was used to confirm that there was reduced apoptosis in the liver. Image analysis demonstrated there was a significant 57% decrease in apoptotic cells in livers from BBG-injected mice (65 ± 32 cells/field of view; $n = 4$) compared to saline-injected mice (150 ± 47 cells/field of view; $n = 4$) ($P = 0.0244$) (Figure 4.3.c.).

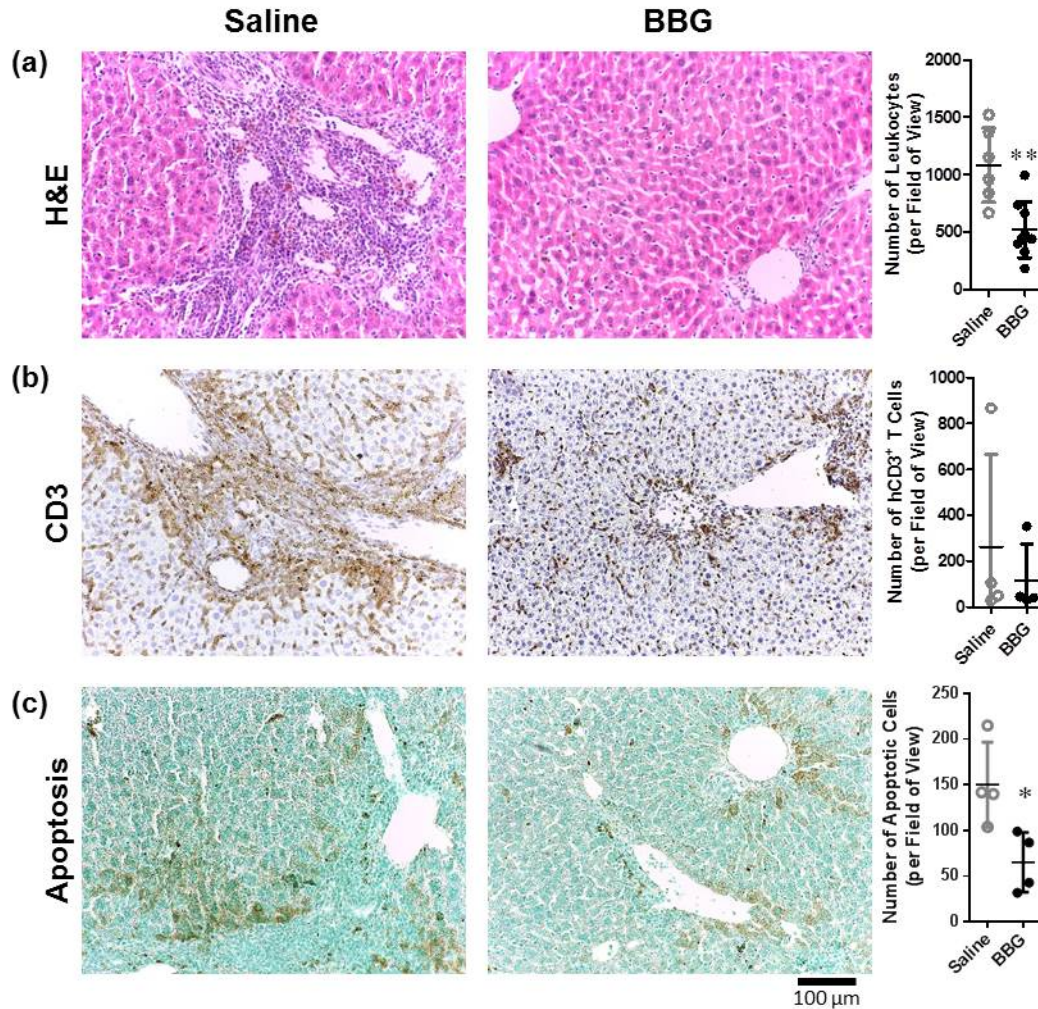


Figure 4.3. Long-term BBG treatment reduces leukocyte infiltration and apoptosis in livers of mice in a humanised mouse model of GVHD. (a-c) Livers from saline- and BBG-injected humanised NOD-SCID-IL2R γ null (NSG) mice (Figure 1) at end-point were stained with (a) haematoxylin and eosin, (b) anti-human (h) CD3 monoclonal antibody and haematoxylin or (c) terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end and methyl green. (a-c) Images were captured by microscopy; representative images from one mouse per group shown; bar represents 100 μ m. Image analysis was used to quantitate the mean number of cells per field of view from three different captured images per mouse. Data represents group means \pm SD (saline n = 4-6; BBG n = 4-9); symbols represent individual mice; * $P < 0.05$ or ** $P < 0.01$ to saline.

4.5.4. BBG Does Not Impact Cytokine Expression in the Liver of NSG Mice

IFN- γ , IL-17 and TNF- α are important pro-inflammatory cytokines implicated in GVHD pathogenesis (Yi *et al.*, 2009, Gartlan *et al.*, 2015). To investigate if long-term BBG treatment alters these cytokines in the liver, hIFN- γ , hIL-17 and hTNF- α expression were analysed by qPCR. hIFN- γ expression was similar in mice injected with BBG (2.8 ± 0.3 , $n = 7$) or saline (3.0 ± 0.6 , $n = 7$) ($P = 0.7347$) (Figure 4.4.a.). There was a two-fold increase in hIL-17 expression in mice injected with BBG (4.0 ± 2.0 , $n = 7$) compared to mice injected with saline (1.8 ± 0.5 , $n = 7$), but this did not reach statistical significance ($P = 0.2940$) (Figure 4.4.b.). hTNF- α expression was low, and similar in mice injected with BBG (0.10 ± 0.03 , $n = 7$) or saline (0.08 ± 0.03 , $n = 7$) ($P = 0.7754$) (Figure 4.4.c.).

Short-term BBG treatment reduces serum IFN- γ in allogeneic (Wilhelm *et al.*, 2010) and humanised (Chapter 3) mouse models of GVHD. To determine if prolonged BBG treatment could alter the amount of these cytokines, serum hIFN- γ and hIL-17 concentrations in humanised NSG mice were assessed by ELISA. hIFN- γ was present in the serum of all mice (Figure 4.4.d.). In contrast to previous data involving short-term BBG treatment (Chapter 3), serum hIFN- γ concentrations were similar in mice injected with BBG (15.8 ± 3.5 ng/mL, $n = 10$) or saline (14.9 ± 3.2 ng/mL, $n = 7$) ($P = 0.8462$) (Figure 4.4.d.). hIL-17 was not detected in the serum of any mice injected with BBG ($n = 10$) or saline ($n = 7$) using an ELISA with a reported sensitivity of 4 pg/mL (eBioscience).

P2X7 expression is significantly increased in livers of mice with allogeneic GVHD (Wilhelm *et al.*, 2010). Therefore, to determine the impact of P2X7 blockade on expression of hP2X7 and mP2X7, livers of humanised NSG mice were analysed by qPCR. There was a four-fold increase in relative hP2X7 expression in mice injected with BBG (8.7 ± 5.6 , $n = 7$) compared to mice injected with saline (2.0 ± 0.6 , $n = 7$), however this did not reach statistical significance ($P = 0.2547$) (Figure 4.4.e.). Relative mP2X7 expression was similar in mice

injected with BBG (0.07 ± 0.02 , $n = 7$) or saline (0.07 ± 0.01 , $n = 7$) ($P = 0.9986$) (Figure 4.4.f).

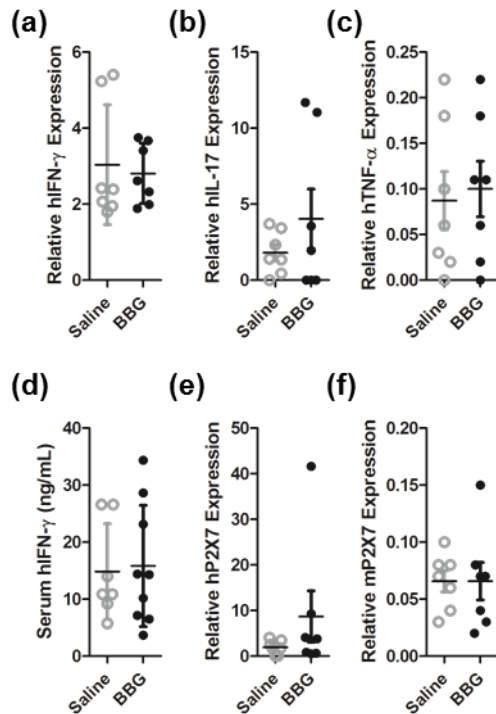


Figure 4.4. Long-term BBG treatment does not affect serum or liver inflammatory markers in a humanised mouse model of GVHD. (a-f) cDNA from livers of saline- and BBG-injected humanised NOD-SCID-IL2R γ null (NSG) mice at end-point (Figure 1) were used to assess the relative expression of human (h) (a) interferon (IFN)- γ , (b) interleukin (IL)-17, (c) tumour necrosis factor (TNF)- α , (e) hP2X7, and (f) murine (m) P2X7 by qPCR. (d) Concentrations of serum hIFN- γ from mice at end-point were analysed by ELISA. (a-f) Data represents group means \pm SD (saline $n = 7$; BBG $n = 7-9$); symbols represent individual mice.

4.6. Discussion

The current study demonstrated that a long-term regime of P2X7 blockade using BBG (50 mg/kg i.p. thrice weekly for up to 10 weeks) can reduce liver inflammation and apoptosis in a humanised mouse model of GVHD. Saline-injected control mice exhibited characteristic leukocyte infiltration and apoptosis in the liver, consistent with previous observations in humanised NSG mice (King *et al.*, 2009, Vlad *et al.*, 2009). The long-term regime of BBG

reduced leukocyte infiltrates, including human T cells, into the livers compared to control mice. This reduction was greater than our previous study using a short-term regime of BBG (50 mg/kg i.p. on days 0, 2, 4, 6, 8) in humanised NSG mice (Chapter 3). Leukocyte infiltrates are also reduced in livers from allogeneic mice following a long-term regime of BBG (50 or 75 mg/kg i.p. twice weekly for four weeks) (Zhong *et al.*, 2016). The long-term BBG regime in the current study also reduced apoptosis in livers, an effect that was greater than the previous short-term BBG regime in humanised NSG mice (Chapter 3). Similarly, the long-term BBG regime in allogeneic mice also reduced inflammatory damage (Zhong *et al.*, 2016). The reduction in leukocyte infiltrates and apoptosis in livers is also similar to other GVHD therapies in humanised mice (Vlad *et al.*, 2009, Abraham *et al.*, 2012, Ehx *et al.*, 2017). Notably, P2X7 blockade is an efficacious therapy in drug-induced inflammatory liver damage (Hoque *et al.*, 2012, Amaral *et al.*, 2013). Thus, the current and past (Zhong *et al.*, 2016) studies indicate that P2X7 is important for liver GVHD development and that P2X7 represents a potential therapeutic target in HSCT to prevent this form of GVHD.

The current study demonstrated that a long-term regime of BBG did not impact clinical disease in humanised mice. This result is similar to that of the short-term BBG regime in this model (Chapter 3) but differs to that of a long-term BBG regime in the allogeneic mouse model of GVHD above (Zhong *et al.*, 2016). In the latter study, BBG prevented weight loss, with a stronger effect observed with 75 mg/kg BBG compared to 50 mg/kg BBG, but clinical score and survival were not reported. Nevertheless, since the larger dose of 75 mg/kg significantly reduced histological GVHD and weight loss compared to the dose of 50 mg/kg in this allogeneic mouse model, investigation of this or larger doses in a humanised mouse model are warranted. Moreover further studies are required to reconcile why clinical GVHD development in humanised mice is not altered by BBG, despite BBG impairing histological liver GVHD in these mice. Finally, it should be noted that administration of BBG into

humanised NSG mice caused rapid weight gain, the reasons for which remain unknown. However, P2X7 blockade with BBG or *P2RX7* gene deficiency also induces weight gain in C57BL/6 mice within one week (Costa-Junior *et al.*, 2011). This previous study hypothesised that a consensus motif similar to Janus kinase 2 within the C-terminus of P2X7 may be a substrate of protein-tyrosine phosphatase 1B, which has known roles in obesity (Rondinone *et al.*, 2002). Thus, blockade of P2X7 may induce an initial weight gain in mice via modulation of this phosphatase.

The long-term regime of BBG in the current study did not detect any differences in the expression of hIFN- γ , hIL-17, and hTNF- α , or mP2X7 in the livers of humanised mice. However, there was a 2-fold increase in hP2X7 in the liver, similar to the 1.7-fold increase in the short-term regime (Chapter 3), but this did not reach statistical significance. This result is somewhat unexpected given the reduced leukocyte infiltration and apoptosis in the livers of these mice. This suggests that other molecules may be controlling T cell migration and T cell-mediated damage in the livers of these mice. Alternatively, the similar expression of these molecules may simply reflect the comparison of livers at end-stage disease, but this does not explain observed differences in leukocyte infiltration and apoptosis. Analysis of liver samples earlier in disease progression may reveal further differences between BBG- and saline-treated mice.

Short-term (Chapter 3) but not long-term BBG treatment (the current study) reduced serum hIFN- γ in humanised mice. The reason for this difference remains unknown, but suggests long-term P2X7 blockade negates any benefits observed with short-term P2X7 blockade in relation to serum hIFN- γ . This suggests that P2X7 blockade shortly after hPBMC engraftment reduces the development of hIFN- γ -producing T cells in humanised mice, similar to P2X7 deficiency reducing IFN- γ production early in disease development in allogeneic mice (Ghiringhelli *et al.*, 2009). However, P2X7 blockade at later stages of

engraftment and GVHD development may promote development of hIFN- γ -producing T cells in humanised NSG mice. Alternatively, comparison of serum hIFN- γ concentrations between the current and previous (Chapter 3) studies indicates that the long-term regime with saline reduced serum hIFN- γ compared to the short-term saline regime. This suggests long-term injections of saline may reduce some disease parameters in this humanised mouse model of GVHD. In support of this, injection of saline is used in other inflammatory disease models to rescue mice from weight loss ((van der Fits *et al.*, 2009) and Chapter 7).

Finally, the current study demonstrates that a long-term BBG regime does not affect engraftment of donor human cells in NSG mice. Although the clinical benefits of this regime were limited, this indicates long-term P2X7 blockade may permit reconstitution of the immune system, an essential aim of HSCT (Paloczi, 2000). In the current study, the majority of engrafted leukocytes were T cells, consistent with previous studies ((King *et al.*, 2008, King *et al.*, 2009) and Chapter 3). Our previous study demonstrated that B cells are not present in the blood or spleens of humanised NSG mice, but the identity of the remaining human leukocytes remained unknown (Chapter 3). In the current study, NK cells constituted a portion of the remaining leukocyte population, consistent with similar frequencies of NK cells in humanised NSG mice observed by others (Gregoire-Gauthier *et al.*, 2012, Tanaka *et al.*, 2012b). NK cells contribute to the graft-versus-leukemia effect but not GVHD in both mice (Hüber *et al.*, 2015) and humans (Ruggeri *et al.*, 2002), suggesting that NSG mice injected with hPBMCs could be potentially used to study the graft-versus-leukemia effect. Importantly, BBG did not affect NK cell engraftment suggesting P2X7 blockade may not impact graft-versus-leukemia immunity, another key aim of HSCT. The current study also demonstrated, for the first time, that humanised NSG mice engraft a small proportion of NKT cells (Koreck *et al.*, 2002), although in the absence of additional markers these cells may represent activated T cells instead or in part (Kelly-Rogers *et al.*, 2006). NKT cells suppress

GVHD in allogeneic HSCT in mice (Lan *et al.*, 2001, Haraguchi *et al.*, 2005, Schneidawind *et al.*, 2015) and higher numbers of NKT cells in humans correlates to reduced GVHD (Rubio *et al.*, 2012, Chaidos *et al.*, 2012). BBG did not alter the percentage of these cells indicating that P2X7 blockade does not affect engraftment of NKT cells. Thus, humanised NSG mice may help elucidate the role of human NK and NKT cells in GVHD. It should be noted that a limitation of the current study was that the flow cytometric analyses did not include gating of singlets or dead cell exclusion using a viability dye. Thereby increasing the likelihood of small errors in determining the proportions of human leukocyte subsets especially rarer subsets such as NK and NKT cells.

The current study demonstrated that long-term P2X7 blockade does not impact human leukocyte engraftment, weight loss, clinical score or mortality associated with GVHD. However, this regime can reduce leukocyte infiltration and apoptosis in the liver. Therefore, this study demonstrates that P2X7 activation plays a role in GVHD pathogenesis in the livers of humanised mice, supporting a role for this receptor in GVHD development in HSCT recipients. Moreover, given that long-term P2X7 blockade does not affect donor human leukocyte engraftment or appear to have any other adverse effects, P2X7 may represent a potential therapeutic target to reduce liver GVHD in HSCT recipients.

4.7. Acknowledgements

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4.8. Disclosures

None.

4.9. Author Contributions

N.J.G., D.W. and R.S. designed the experiments. N.J.G. performed the experiments, analysed the data, prepared the figures and wrote the manuscript. D.W. and R.S. supervised the project, reviewed the data and edited the manuscript.

Chapter 5: Pharmacological Blockade of the CD39/CD73 Pathway But Not Adenosine Receptors Augments Disease in a Humanised Mouse Model of Graft-versus-Host Disease

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5.1. Statement of Contribution of Authors


I, as one of the authors of this original article, agree with the statement of author contributions stated at the end of this chapter, and originally published in the original journal article.



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5.2. Abstract

Allogeneic haematopoietic stem cell transplantation (HSCT) is a curative therapy for a number of haematological malignancies, but is limited by the development of graft-versus-host disease (GVHD). CD39 and CD73 form an ecto-enzymatic pathway that hydrolyses extracellular adenosine 5'-triphosphate (ATP) to adenosine, which respectively exacerbate or alleviate disease in allogeneic mouse models of GVHD. The current study aimed to explore the role of the CD39/CD73 pathway and adenosine receptor (AR) blockade in a humanised mouse model of GVHD. Immunodeficient non-obese diabetic-severe combined immunodeficiency-IL-2 receptor γ^{null} (NSG) mice were injected with human peripheral blood mononuclear cells (PBMCs), and subsequently injected with the CD39/CD73 antagonist $\alpha\beta$ -methylene-ADP (APCP) (50 mg/kg) or saline for 7 days, or the AR antagonist caffeine (10 mg/kg) or saline for 14 days. Mice predominantly engrafted human CD4⁺ and CD8⁺ T cells, with smaller proportions of human regulatory T (Treg) cells, invariant natural killer T (iNKT) cells, monocytes and dendritic cells (DCs). Neither APCP or caffeine altered engraftment of these human leukocyte subsets. APCP (CD39/CD73 blockade) augmented GVHD as shown through increased weight loss and worsened liver histology. This treatment also increased serum human IL-2 concentrations and decreased the frequency of human CD39⁻CD73⁻CD4⁺ T cells. In contrast, caffeine (AR blockade) did not alter GVHD severity or human serum cytokine concentrations (IL-2, IL-6, IL-10 or TNF α). In conclusion, blockade of CD39/CD73 but not ARs augments disease in a humanised mouse model of GVHD. These results indicate that CD39/CD73 blockade maintains sufficient extracellular ATP concentrations to promote GVHD in this model.

5.3. Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is a common curative therapy for numerous haematological malignancies. However, 30-60% of transplant recipients develop graft-versus-host disease (GVHD) as a result of the donor T cells recognising the recipient tissue as 'foreign' (Jagasia *et al.*, 2012). The first stage of GVHD is characterised by the initial release of cytokines, such as TNF- α and IL-6, from damaged tissue caused by the underlying disease or pre-conditioning regimes. Following this, activation of CD4⁺ T cells by antigen presenting cells, such as dendritic cells (DC), can cause release of T helper-1 (Th1) cytokines such as TNF- α , IFN- γ and IL-2. Finally, both CD4⁺ and CD8⁺ T cells migrate to the liver, gastrointestinal tract and skin to cause inflammatory damage of these tissues (Ferrara *et al.*, 2009). Alternatively, IL-10 can suppress immune responses and can potentially reduce GVHD (Blazar *et al.*, 1998). The T cell subsets regulatory T (Treg) cells and invariant natural killer T (iNKT) cells can also reduce GVHD severity (Schneidawind *et al.*, 2013).

Purinergic signalling includes the plasma membrane P1 and P2 receptors. P1 or adenosine receptors (AR; A₁, A_{2A}, A_{2B} and A₃) are activated by extracellular adenosine, while P2 receptors (P2X₁₋₇ and P2Y_{1, 2, 4, 6, 8, 11-14}) are activated by a range of extracellular nucleotides including adenosine 5'-triphosphate (ATP) (Burnstock, 2007). Purinergic receptors are important in inflammation and immunity, and are expressed on immune cells including antigen presenting cells and T cells (Di Virgilio and Vuerich, 2015). In general activation of P2 receptors by extracellular ATP exerts pro-inflammatory effects (Idzko *et al.*, 2014). In contrast, hydrolysis of ATP to adenosine by a pathway involving ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73) commonly results in anti-inflammatory effects via activation of ARs (Antonioli *et al.*, 2013).

ATP, released from activated, damaged or dying cells, can activate P2 receptors and promote inflammatory responses in transplantation (Castillo-Leon *et al.*, 2018). Moreover, P2 receptor activation can promote development of GVHD. Extracellular ATP can be released from damaged cells in GVHD (Wilhelm *et al.*, 2010) and subsequently activate P2X7 to promote disease development in allogeneic (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016) and humanised (Chapter 3) mouse models of GVHD. Likewise, P2Y2 activation by ATP can promote leukocyte infiltration and tissue damage in allogeneic models of GVHD (Klamt *et al.*, 2015). In contrast, activation of ARs can limit GVHD development (Lappas *et al.*, 2010, Han *et al.*, 2013). Moreover, pharmacological blockade of ARs with caffeine (Tsukamoto *et al.*, 2012) or A_{2A} with SCH58261 (Wang *et al.*, 2013) or genetic deletion of A_{2A} (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013) worsens disease in allogeneic mouse models of GVHD. Similarly, pharmacological blockade of CD39 and CD73 with $\alpha\beta$ -methylene-ADP (APCP), which prevents adenosine generation and increases extracellular ATP (Moody *et al.*, 1984, Covarrubias *et al.*, 2016), augments GVHD in allogeneic mouse models (Wang *et al.*, 2013). However, the roles of the CD39/CD73 pathway and ARs in a humanised mouse model of GVHD have not been reported.

Using pharmacological approaches, the current study aimed to investigate the role of the CD39/CD73 pathway and AR blockade in disease development in a humanised mouse model of GVHD. In this model, immunodeficient non-obese diabetic-severe combined immunodeficiency-IL-2 receptor γ^{null} (NSG) mice are injected with human peripheral blood mononuclear cells (PBMC) and develop clinical signs of GVHD over 10 weeks (Chapter 3). CD39/CD73 blockade with APCP, but not AR blockade with caffeine, augments GVHD in humanised mice. These results support the known roles of extracellular ATP in promoting GVHD, but do not provide evidence that extracellular adenosine can limit GVHD in this humanised model.

5.4. Materials and Methods

5.4.1. Humanised Mouse Model of GVHD

Experiments involving human blood and mice were approved by the respective Human and Animal Ethics Committees (University of Wollongong, Wollongong, Australia). A humanised mouse model of GVHD was used as described (Chapter 3). Briefly, hPBMCs, isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden), were injected intra-peritoneally (i.p.) (10×10^6 hPBMCs/mouse) into female NSG mice aged 5-7 weeks (Australian BioResources; Moss Vale, Australia). For CD73 blockade, humanised mice were injected i.p. daily with APCP (Sigma-Aldrich; St Louis, MO, USA) (50 mg/kg) or saline 2 hours following hPBMC injection and then daily over the next 6 days (Tsukamoto *et al.*, 2012). For AR blockade, mice were injected i.p. daily with caffeine (Sigma-Aldrich) (10 mg/kg) or saline 2 hours following hPBMC injection and then daily for the next 13 days (Tsukamoto *et al.*, 2012). At 3 weeks post-hPBMC injection, mice were checked for engraftment by immunophenotyping of tail vein blood. Mice were monitored for signs of GVHD using a scoring system, giving a total clinical score out of 10, as described (Chapter 3). Mice were euthanised at 10 weeks post-injection of hPBMCs, or earlier if exhibiting a clinical score of ≥ 8 or a weight loss of $\geq 10\%$, according to the approved animal ethics protocol.

5.4.2. Immunophenotyping by Flow Cytometry

Tail vein blood (week 3) and spleen cells (end-point) were obtained from mice and lysed with ammonium chloride potassium buffer and immunophenotyped as described (Chapter 3) using the antibodies listed in Table 5.1 and the gating strategy depicted in the Supplementary Figures S1 and S2. Data was collected using a BD (San Jose, CA, USA) Fortessa-X20 Flow Cytometer (using band pass filters 450/50 for BV421, 710/50 for BV711, 525/50 for FITC, 586/15 for PE, 695/40 for PerCP-Cy5.5, 780/60 for PE-Cy7 and 670/30 for APCy). The

relative percentages of cells were analysed using FlowJo software v8.7.1 (TreeStar Inc.; Ashland, OR, USA) (FigureS1 and S2).

Table 5.1. Monoclonal antibodies used for flow cytometry.

Target	Fluorochrome	Clone*
CD14	BV421	MφP9
CD127	BV421	HIL-7R-M21
CD3	BV711	UCHT1
CD45	FITC	HI30
CD8	FITC	RPA-T8
CD25	PE	M-A251
CD3	PE	UCHT1
CD83	PE	HB15e
CD4	PerCP-Cy5.5	L200
CD45	PerCP-Cy5.5	30-F11
CD73	PE-Cy7	AD2
Vα24-Jα14	PE-Cy7	6B11
CD19	APCy	HIB19
CD39	APCy	TU66

*All antibodies mouse anti-human except clone 30-F11 (rat anti-mouse); all antibodies from BD Biosciences except clone 6B11 (BioLegend).

5.4.3. Histological Analysis

Formalin-fixed tissue sections (5 µm) were stained with haematoxylin and eosin (POCD; Artarmon, Australia) and histology assessed as described (Chapter 3).

5.4.4. Cytokine Analysis by a Flow Cytometric Multiplex Assay

Serum was obtained from mice at end-point as described (Chapter 3) and cytokine concentrations were measured using a human Th1 LEGENDPlex kit (BioLegend; San Diego, CA, USA) as per the manufacturer's instructions.

5.4.5. Statistical Analysis

Data is given as mean ± standard error of the mean (SEM). Statistical differences were calculated using Student's t-test for single comparisons or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Weight and clinical score

were analysed using a repeated measures two-way ANOVA. Survival (MST) was compared using the log-rank (Mantel-Cox) test. Mortality was compared using Fisher's exact test. All statistical analyses and graphs were generated using GraphPad Prism 5 for PC (GraphPad Software; La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

5.5. Results

5.5.1. APCP Does Not Impact hPBMC Engraftment in NSG Mice

To investigate a role for the CD39/CD73 pathway in GVHD development in humanised NSG mice, these mice were injected with the CD39/CD73 inhibitor, APCP, using the same regime efficacious in an allogeneic mouse model of GVHD (Tsukamoto *et al.*, 2012). First, to investigate whether APCP (CD39/CD73 blockade) altered hPBMC engraftment in NSG mice, 3 weeks post-hPBMC injection blood cells from APCP- ($n = 9$) or saline-injected ($n = 9$) mice were analysed by flow cytometry. APCP- and saline-injected mice demonstrated similar frequencies of human leukocytes (hCD45⁺mCD45⁻ cells), calculated as a percentage of total leukocytes (hCD45⁺mCD45⁻/(hCD45⁺mCD45⁻ and hCD45⁻mCD45⁺)) ($22.1 \pm 3.9\%$ and $22.2 \pm 5.4\%$, respectively, $P = 0.9869$) (Figure 5.1.a.). These human leukocytes from APCP- and saline-injected mice predominately comprised T cells (hCD3⁺hCD19⁻) ($99.5 \pm 0.1\%$ and $99.3 \pm 0.3\%$, respectively, $P = 0.4897$) (Figure 5.1.b.), with a small amount of non-B/T cells (hCD3⁻ hCD19⁻) ($0.5 \pm 0.1\%$ and $0.7 \pm 0.3\%$, respectively, $P = 0.4897$) (Figure 5.1.c.).

At end-point, splenocytes from APCP- ($n = 9$) and saline-injected ($n = 8$) mice were analysed by flow cytometry. APCP- and saline-injected mice demonstrated similar frequencies of human leukocytes ($75.1 \pm 9.4\%$ and $90.2 \pm 3.0\%$, respectively, $P = 0.1691$) (Figure 5.1.d.). One APCP-injected mouse revealed a human leukocyte frequency of only 9.7% (Figure 1d) but was included in subsequent analyses. APCP- and saline-injected mice

demonstrated similar frequencies of human T cells ($92.5 \pm 1.9\%$ and $95.1 \pm 1.6\%$, respectively, $P = 0.3128$) (Figure 5.1.e.), which comprised both hCD4⁺ T cells ($58.3 \pm 6.5\%$ and $64.0 \pm 7.0\%$, respectively, $P = 0.5558$) and hCD8⁺ T cells ($29.6 \pm 6.0\%$ and $19.2 \pm 3.3\%$, respectively, $P = 0.1633$) (Figure 5.1.f.). Both APCP- and saline-injected mice demonstrated greater frequencies of hCD4⁺ T cells than hCD8⁺ T cells ($P = 0.0053$ and $P < 0.0001$, respectively) (Figure 5.1.f.).

To investigate whether APCP altered the frequency of CD39^{+/−} and/or CD73^{+/−} human T cells, these markers on hCD4⁺ and hCD8⁺ T cells from the spleens of humanised mice were analysed by flow cytometry. APCP-injected mice, compared to saline-injected mice, demonstrated a trend of increased hCD39⁺hCD73[−]hCD4⁺ T cells ($10.2 \pm 4.1\%$ and $5.5 \pm 2.1\%$, respectively, $P = 0.3441$) and hCD39[−]hCD73⁺hCD4⁺ T cells ($13.1 \pm 5.5\%$ and $3.7 \pm 0.9\%$, respectively, $P = 0.1347$), but a similar frequency of hCD39⁺hCD73⁺hCD4⁺ T cells ($1.3 \pm 0.4\%$ and $0.8 \pm 0.4\%$, respectively, $P = 0.4247$). Conversely, the frequency of hCD39[−]hCD73[−]hCD4⁺ T cells was significantly reduced in APCP-injected mice compared to saline-injected mice ($75.4 \pm 5.8\%$ and $89.9 \pm 2.4\%$, respectively, $P = 0.0441$) (Figure 5.1.g.). In contrast to hCD4⁺ T cells, APCP- and saline injected mice demonstrated similar frequencies of hCD39⁺hCD73[−]hCD8⁺ T cells ($41.4 \pm 3.0\%$ and $36.4 \pm 5.0\%$, respectively, $P = 0.3867$), hCD39[−]hCD73⁺hCD8⁺ T cells ($3.8 \pm 1.0\%$ and 2.8 ± 0.6 , respectively, $P = 0.4342$), hCD39⁺hCD73⁺hCD8⁺ T cells ($5.5 \pm 1.6\%$ and 4.1 ± 1.2 , respectively, $P = 0.5035$) and hCD39[−]hCD73[−]hCD8⁺ T cells ($49.3 \pm 4.6\%$ and 56.7 ± 5.8 , respectively, $P = 0.3247$) (Figure 5.1.h.).

Previous studies have demonstrated that increased proportions of iNKT and Treg cells correlate to reduced GVHD severity in allogeneic mouse models of GVHD (Schneidawind *et al.*, 2013), but the presence of human iNKT cells in humanised NSG mice is unknown. Therefore, the presence and frequency of human iNKT cells (hCD45⁺hCD3⁺hCD19[−]

hV α 24-J α 18⁺), as well as human Treg cells (hCD45⁺hCD3⁺hCD4⁺hCD25⁺hCD127^{lo}), in the spleens of these mice was examined. APCP- and saline-injected mice demonstrated the presence and similar frequencies of iNKT cells ($2.4 \pm 1.0\%$ and $1.4 \pm 0.4\%$, respectively, $P = 0.3674$) (Figure 5.1.i.). APCP- and saline-injected mice also demonstrated similar frequencies of Treg cells (1.4 ± 0.6 and $0.9 \pm 0.2\%$, respectively, $P = 0.4983$) (Figure 5.1.j.).

Previously, our group observed that the spleens of humanised mice at end-point do not contain human B cells (Chapter 3). Consistent with this observation, the remaining human leukocytes in the spleens of APCP- and saline-injected mice were negative for CD19, with similar frequencies of CD3⁻CD19⁻ cells present in both groups of mice ($7.5 \pm 1.9\%$ and $3.2 \pm 1.0\%$, respectively, $P = 0.0703$) (Figure 5.1.k.). Therefore, this remaining non-B/T cell population (hCD45⁺hCD3⁻hCD19⁻) was further analysed to determine if these cells were human monocytes (hCD14⁺hCD83⁻) or DCs (hCD14⁺hCD83⁺). APCP- and saline-injected mice demonstrated small but similar frequencies of human monocytes ($0.03 \pm 0.02\%$ and $0.001 \pm 0.001\%$, respectively, $P = 0.1804$) (Figure 5.1.l.) and human DCs ($0.4 \pm 0.2\%$ and $0.3 \pm 0.1\%$, respectively, $P = 0.6066$) (Figure 5.1.m.).

5.5.2. APCP Augments Clinical and Histological GVHD in Humanised Mice

To investigate whether APCP (CD39/CD73 blockade) impacted GVHD development, the above mice were monitored for disease development for up to 10 weeks. APCP-injected mice ($n = 9$) demonstrated significantly greater weight loss over the 10 weeks than saline-injected mice ($n = 9$) ($P = 0.0350$) (Figure 5.2.a.). APCP-mice demonstrated signs of GVHD (classified as a score clinical score ≥ 3) from 38 days onwards, while saline-injected mice demonstrated signs of GVHD from 45 days, however, clinical scores were similar over 10 weeks ($P = 0.1711$) (Figure 5.2.b.). Moreover, APCP- and saline-injected mice exhibited identical median survival times (MST) (57 days and 57 days, respectively, $P = 0.2634$) and similar mortality over 10 weeks (100% and 78%, respectively, $P = 0.2059$) (Figure 5.2.c.).

GVHD affects the liver, gastrointestinal tract and skin in humans (Glucksberg *et al.*, 1974), and in allogeneic (Schroeder and DiPersio, 2011) and humanised (King *et al.*, 2009) mouse models. APCP-injected mice exhibited greater leukocyte infiltration and histological damage of the liver compared to saline-injected mice (Figure 5.2.d.). In contrast, the small intestines of APCP- and saline-injected mice exhibited similar amounts of leukocyte infiltration, crypt epithelial cell apoptosis and structural damage including rounding of villi (Figure 5.2.d.). Similarly, the skin of APCP- and saline-injected mice displayed similar amounts of leukocyte infiltration, with similar but minimal amounts of basal epithelial cell apoptosis and epidermal thickening (Figure 5.2.d.).

5.5.3. APCP Blockade Increases Serum hIL-2 Concentrations in Humanised Mice

An important part of GVHD development is the pro-inflammatory cytokine storm that drives immune cell proliferation and inflammatory damage (Ferrara *et al.*, 2009). Therefore, to determine if APCP altered cytokine production in humanised mice, serum hIL-2, hIL-6, hIL-10, hTNF- α and hIFN- γ concentrations from APCP- ($n = 9$) and saline-injected ($n = 8$) mice were analysed by a flow cytometric multiplex assay. APCP-injected mice demonstrated a significant increase in serum hIL-2 concentrations (3.2 ± 0.5 pg/mL) compared to saline-injected mice (1.6 ± 0.3 pg/mL) ($P = 0.0080$) (Figure 5.2.e.). In contrast, APCP- and saline-injected mice demonstrated similar serum concentrations of hIL-6 (3.9 ± 1.5 pg/mL, and 1.4 ± 0.7 pg/mL, respectively, $P = 0.1743$) (Figure 5.2.f.), hIL-10 (22.2 ± 4.1 pg/mL, and 19.1 ± 3.1 pg/mL, respectively, $P = 0.7621$) (Figure 5.2.g.), and TNF- α (21.3 ± 6.5 pg/mL, and 16.6 ± 3.4 pg/mL, respectively, $P = 0.7851$) (Figure 5.2.h.). Serum hIFN- γ concentrations in both treatment groups exceeded the highest standard ($>10,000$ pg/mL) and thus could not be compared (data not shown). Attempts to reassess serum hIFN- γ concentrations by ELISA were unsuccessful (results not shown), possibly due to freeze-thawing of serum.

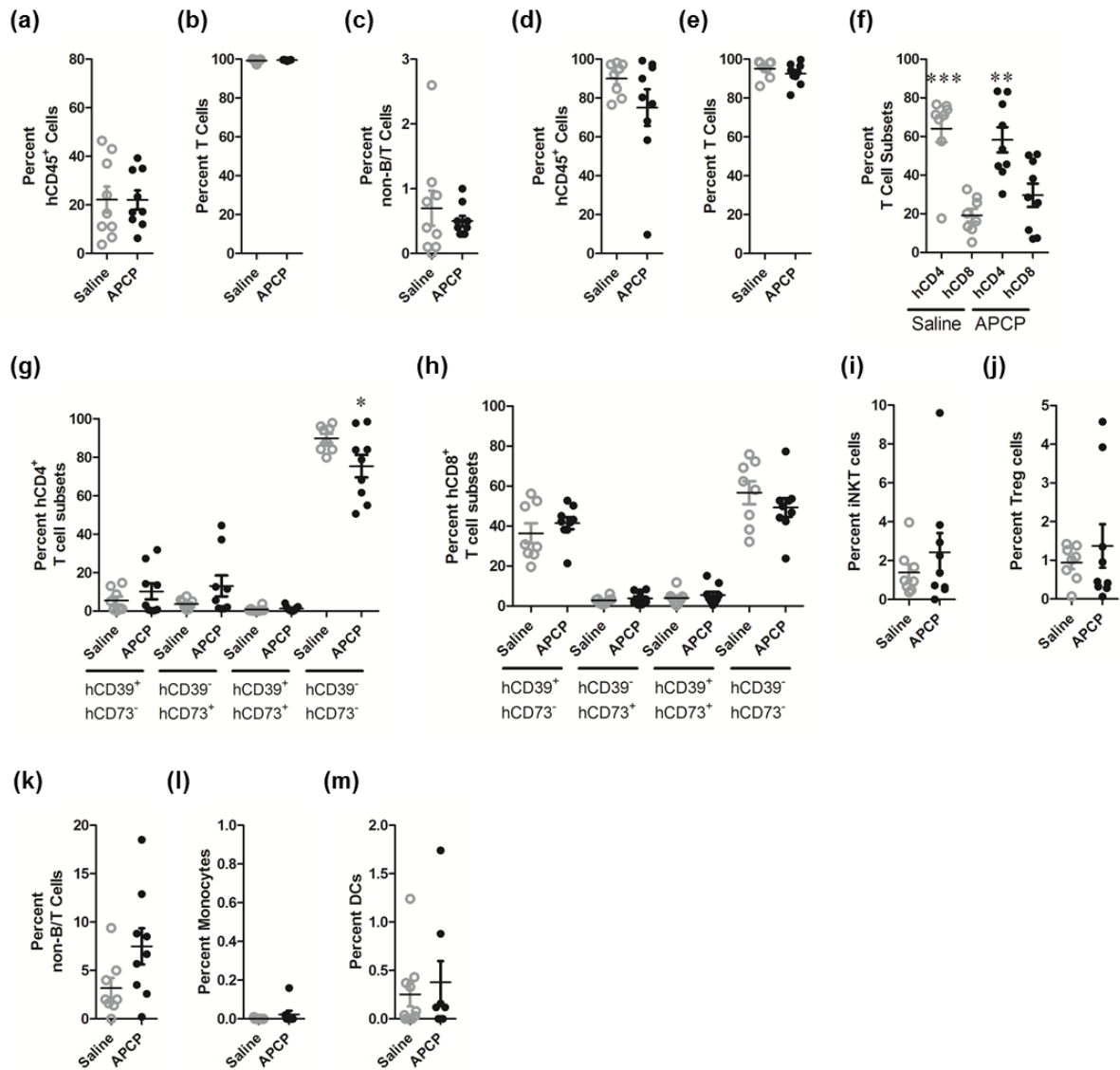


Figure 5.1. APCP reduces human CD4⁺CD39⁺CD73⁻ cells in a humanised mouse model of GVHD (a-m) The percentage of human leukocytes in (a-c) blood at 3 weeks post-hPBMC injection and (d-m) spleens at end-point from humanised NSG mice injected with saline (control) or the CD39/CD73 inhibitor APCP (50 mg/kg) were determined by flow cytometry. (a, d) hCD45⁺ leukocytes are expressed as a percentage of total (mCD45⁺ and hCD45⁺) leukocytes. (b, e) T cells (hCD3⁺hCD19⁻) and (c, k) non-B/T cells (hCD3⁻hCD19⁻) are expressed as a percentage of hCD45⁺ leukocytes. (f) hCD4⁺ and hCD8⁺ T cell subsets are expressed as a percentage of hCD3⁺ leukocytes. ** *P* < 0.005, *** *P* < 0.0001 compared to hCD8⁺ T cells. (g, h) The expression of hCD39 and hCD73 was analysed on (g) hCD4⁺ and (h) hCD8⁺ T cell subsets. * *P* < 0.05 compared to respective saline control. (i) iNKT cells (hVα24-Jα18⁺) are expressed as a percentage of hCD3⁺hCD19⁻ T cells and (j) Treg cells (hCD25⁺hCD127^{lo}) are expressed as a percentage of hCD3⁺ T cells. (l) monocytes (hCD14⁺CD83⁻) and (m) DCs (hCD14⁺CD83⁺) are expressed as a percentage of non-B/T cells. Data represents group means ± SEM; symbols represent individual mice (saline *n* = 9, APCP *n* = 8-9).

5.5.4. Caffeine Does Not Impact hPBMC Engraftment in NSG Mice

The above data suggests that decreased adenosine and/or increased ATP production due to APCP-mediated blockade of CD39/CD73 worsens GVHD in humanised NSG mice. Thus, a role for ARs using the broad-spectrum AR antagonist, caffeine, in this process was examined using the same regime efficacious in an allogeneic mouse model of GVHD (Tsukamoto *et al.*, 2012). First, to determine if caffeine altered hPBMC engraftment, 3 weeks post-hPBMC injection blood cells from caffeine- ($n = 13$) and saline-injected ($n = 15$) mice were analysed by flow cytometry. Caffeine- and saline-injected mice demonstrated similar frequencies of human leukocytes ($23.5 \pm 3.5\%$ and $28.5 \pm 4.7\%$, respectively, $P = 0.4135$) (Figure 5.3.a.), which comprised T cells ($92.5 \pm 1.1\%$ and $94.2 \pm 0.7\%$, respectively, $P = 0.1897$) (Figure 5.3.b.), and non-B/T cells ($7.5 \pm 1.1\%$ and $5.8 \pm 0.7\%$, respectively, $P = 0.1897$) (Figure 5.3.c.).

At end-point, splenocytes from caffeine- ($n = 13$) and saline-injected ($n = 14$) mice were analysed by flow cytometry. Caffeine- and saline-injected mice demonstrated similar frequencies of human leukocytes ($72.2 \pm 6.2\%$ and $75.6 \pm 3.8\%$, respectively, $P = 0.6346$) (Figure 5.3.d.). One caffeine-injected mouse revealed a human leukocyte frequency of only 11.2% (Figure 5.3.d.) but was included in subsequent analyses. Caffeine- and saline-injected mice demonstrated similar frequencies of human T cells ($96.1 \pm 1.0\%$ and $93.7 \pm 1.9\%$, respectively, $P = 0.2934$) (Figure 5.3.e.), which comprised hCD4⁺ T cells ($51.7 \pm 4.4\%$ and $52.7 \pm 3.4\%$, respectively, $P = 0.8658$) and hCD8⁺ T cells ($13.5 \pm 2.3\%$ and $18.5 \pm 4.0\%$, respectively, $P = 0.3011$). Both caffeine- and saline-injected mice demonstrated greater frequencies of hCD4⁺ than hCD8⁺ T cells ($P < 0.0001$ and $P < 0.0001$, respectively) (Figure 5.3.f.).

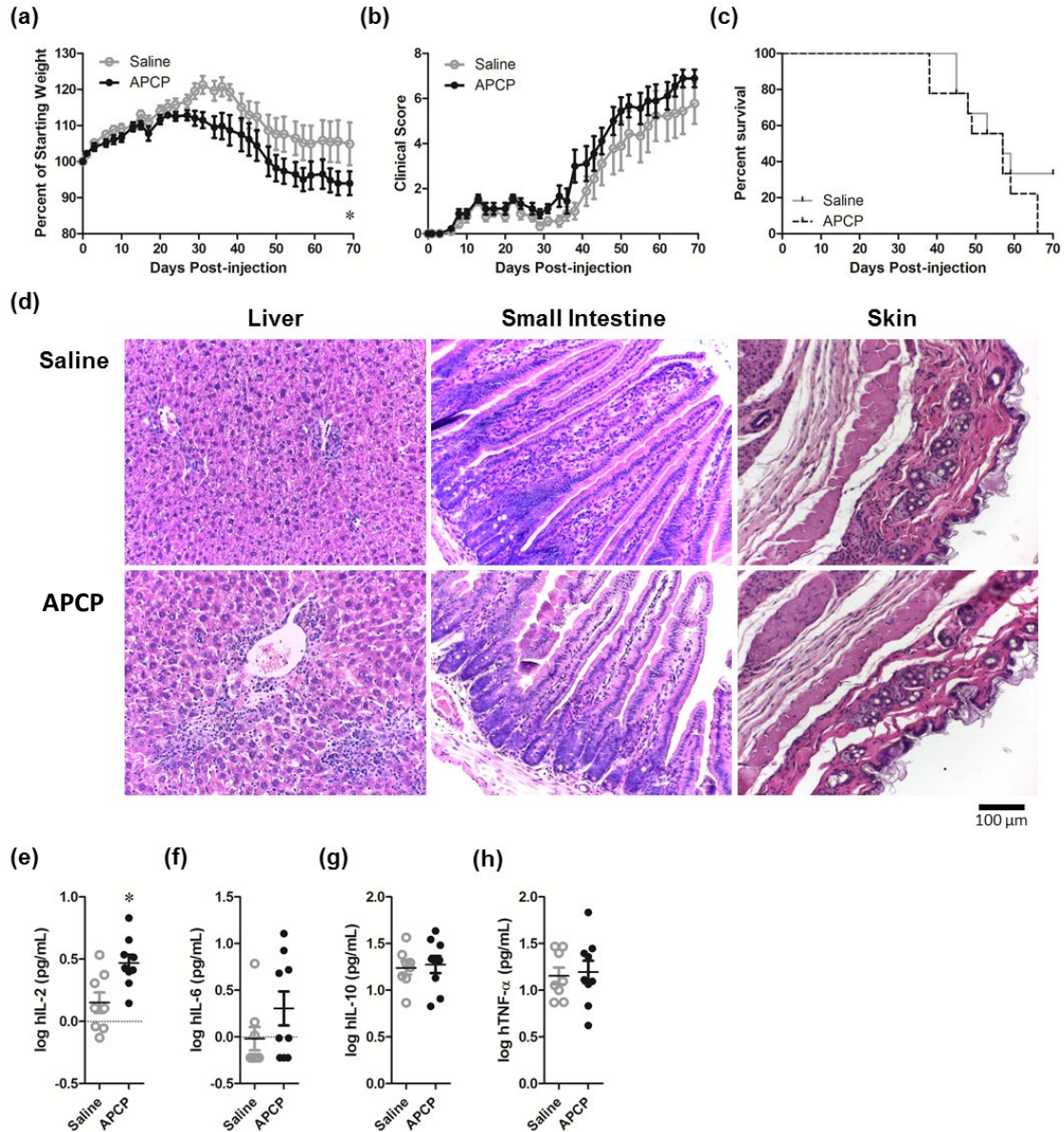


Figure 5.2 CD73 blockade augments disease in a humanised mouse model of GVHD.

(a-i) Humanised NSG mice injected with saline (control) or the CD39/CD73 inhibitor APCP (50 mg/kg) were monitored for (a) weight loss, (b) clinical score and (c) survival over 10 weeks. * $P < 0.05$ compared to saline-injected mice. Data represents (a, b) group means \pm SEM or (c) percent survival (saline $n = 9$, APCP $n = 9$). (d) Tissue sections (liver, small intestine, and skin) were stained with haematoxylin and eosin and images were captured by microscopy, with each image representative of eight mice per group; bar represents 100 μm . (e-h) Concentrations of serum human (e) IL-2, (f) IL-6, (g) IL-10 and (h) TNF- α were analysed using a flow cytometric multiplex assay. * $P < 0.05$ compared to respective saline control. Data represents group means \pm SEM (saline $n = 8$, APCP $n = 9$).

Caffeine- and saline injected mice demonstrated similar frequencies of hCD39⁺hCD73⁺hCD4⁺ T cells ($14.2 \pm 4.6\%$ and $11.0 \pm 3.6\%$, respectively, $P = 0.5926$),

hCD39⁺hCD73⁺hCD4⁺ T cells ($3.2 \pm 1.0\%$ and 5.3 ± 1.9 , respectively, $P = 0.3572$), hCD39⁺hCD73⁺hCD4⁺ T cells ($0.8 \pm 0.2\%$ and 0.7 ± 0.3 , respectively, $P = 0.9430$) and hCD39⁺hCD73⁻hCD4⁺ T cells ($81.8 \pm 4.4\%$ and 83.0 ± 3.5 , respectively, $P = 0.8425$) (Figure 5.3.g.). Caffeine- and saline-injected mice also demonstrated similar frequencies of hCD39⁺hCD73⁻hCD8⁺ T cells ($33.3 \pm 6.2\%$ and $39.5 \pm 5.4\%$, respectively, $P = 0.4544$), hCD39⁺hCD73⁺hCD8⁺ T cells ($3.3 \pm 0.7\%$ and $5.9 \pm 2.8\%$, respectively, $P = 0.3922$), hCD39⁺hCD73⁺hCD8⁺ T cells ($4.9 \pm 1.4\%$ and $5.2 \pm 2.0\%$, respectively, $P = 0.9055$) and hCD39⁺hCD73⁻hCD8⁺ T cells ($57.4 \pm 6.9\%$ and $50.6 \pm 6.0\%$, respectively, $P = 0.4627$) (Figure 5.3.h.).

Caffeine- and saline-injected mice also demonstrated similar frequencies of iNKT cells ($3.1 \pm 0.9\%$ and $1.6 \pm 0.3\%$, respectively, $P = 0.1088$) (Figure 5.3.i.) and Treg cells ($0.8 \pm 0.1\%$ and $0.9 \pm 0.2\%$, respectively, $P = 0.8964$) (Figure 5.3.j.). Caffeine- and saline-injected mice demonstrated similar frequencies of non-B/T cells ($4.1 \pm 1.1\%$ and $5.8 \pm 1.9\%$, respectively, $P = 0.4381$) (Figure 5.3.k.), which included similar frequencies of monocytes ($0.2 \pm 0.1\%$, $n = 11$ and $0.3 \pm 0.1\%$, $n = 10$ respectively, $P = 0.5873$) (Figure 5.3.l.) and DCs ($0.6 \pm 0.2\%$, $n = 11$ and $0.4 \pm 0.2\%$, $n = 10$ respectively, $P = 0.7984$) (Figure 5.3.m.).

5.5.5. Caffeine Does Not Impact Clinical or Histological GVHD in Humanised Mice

To determine if caffeine (AR blockade) impacted GVHD development, the above mice were monitored for disease development for up to 10 weeks. Caffeine- ($n = 13$) and saline-injected ($n = 15$) mice demonstrated similar weight loss ($P = 0.7574$) (Figure 5.4.a.) and clinical scores ($P = 0.0846$) (Figure 5.4.b.) over 10 weeks. Moreover, caffeine- and saline-injected mice exhibited similar MSTs (47 days and 45 days, respectively, $P = 0.8741$) and mortality over 10 weeks (87% and 87%, respectively, $P = 1.000$) (Figure 5.4.c.).

Caffeine- and saline-injected mice demonstrated similar amounts of leukocyte infiltration and histological damage of the liver, small intestine and skin (Figure 5.4.d.). Moreover, the small intestines from mice from either group exhibited similar amounts of structural damage including enterocyte loss, crypt epithelial cell apoptosis and rounding of villi (Figure 5.4.d.). Similarly, skin from both caffeine- and saline-injected mice displayed mild basal epithelial cell apoptosis and epidermal thickening (Figure 5.4.d.).

5.5.6. Caffeine Does Not Impact Serum Cytokine Concentrations in a Humanised Mouse Model of GVHD

Finally, to determine if caffeine altered cytokine production in humanised mice, serum hIL-2, hIL-6, hIL-10, hIFN- γ and hTNF- α concentrations were analysed as above. Caffeine- ($n = 12$) and saline-injected ($n = 11$) mice demonstrated similar concentrations of serum hIL-2 (1.5 ± 0.4 pg/mL and 1.0 ± 0.3 pg/mL, respectively, $P = 0.7556$), hIL-6 (2.9 ± 1.3 pg/mL and 3.7 ± 2.8 pg/mL, respectively, $P = 0.6577$), and hIL-10 (23.0 ± 2.6 pg/mL and 26.8 ± 6.4 pg/mL, respectively, $P = 0.8594$). There was a trend of increased hTNF- α concentration in caffeine-injected mice, which approached but did not achieve statistical significance (18.6 ± 3.2 pg/mL and 13.7 ± 3.3 pg/mL, respectively, $P = 0.0852$). Serum hIFN- γ concentrations in both treatment groups exceeded the highest standard ($>10,000$ pg/mL) and thus could not be compared (data not shown). Attempts to reassess serum hIFN- γ concentrations by ELISA were unsuccessful (results not shown), possibly due to freeze-thawing of serum.

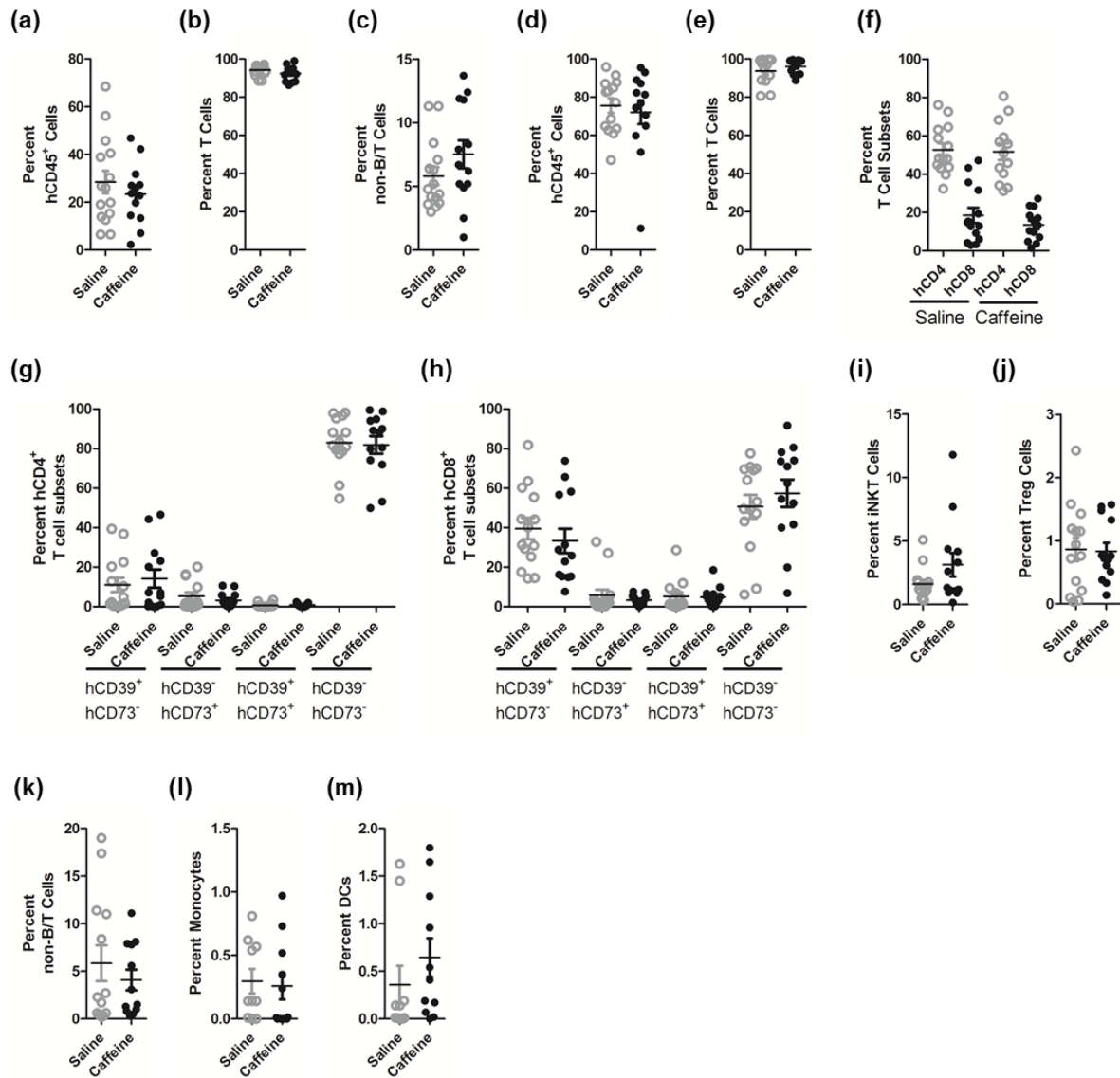


Figure 5.3. Caffeine does not impact human leukocyte engraftment in a humanised mouse model of GVHD. (a-m) The percentage of human leukocytes in (a-c) blood at 3 weeks post-hPBMC injection and (d-m) spleens at end-point from humanised NSG mice injected with saline (control) or the AR antagonist caffeine (10 mg/kg) were determined by flow cytometry. (a, d) hCD45⁺ leukocytes are expressed as a percentage of total (mCD45⁺ and hCD45⁺) leukocytes. (b, e) T cells (hCD3⁺hCD19⁻) and (c, k) non-B/T cells (hCD3⁻hCD19⁻) are expressed as a percentage of hCD45⁺ leukocytes. (f) hCD4⁺ and hCD8⁺ T cell subsets are expressed as a percentage of hCD3⁺ leukocytes. *** $P < 0.0001$ compared to hCD8⁺ T cells. (g, h) The expression of hCD39 and hCD73 was analysed on (g) hCD4⁺ and (h) hCD8⁺ T cell subsets. (i) iNKT cells (hV α 24-J α 18⁺) are expressed as a percentage of hCD3⁺ hCD19⁻ T cells and (j) Treg cells (hCD25⁺hCD127^{lo}) are expressed as a percentage of hCD3⁺ T cells. (l) monocytes (hCD14⁺CD83⁻) and (m) DCs (hCD14⁻CD83⁺) are expressed as a percentage of non-B/T cells. Data represents group means \pm SEM; symbols represent individual mice (saline $n = 15$, caffeine $n = 13$).

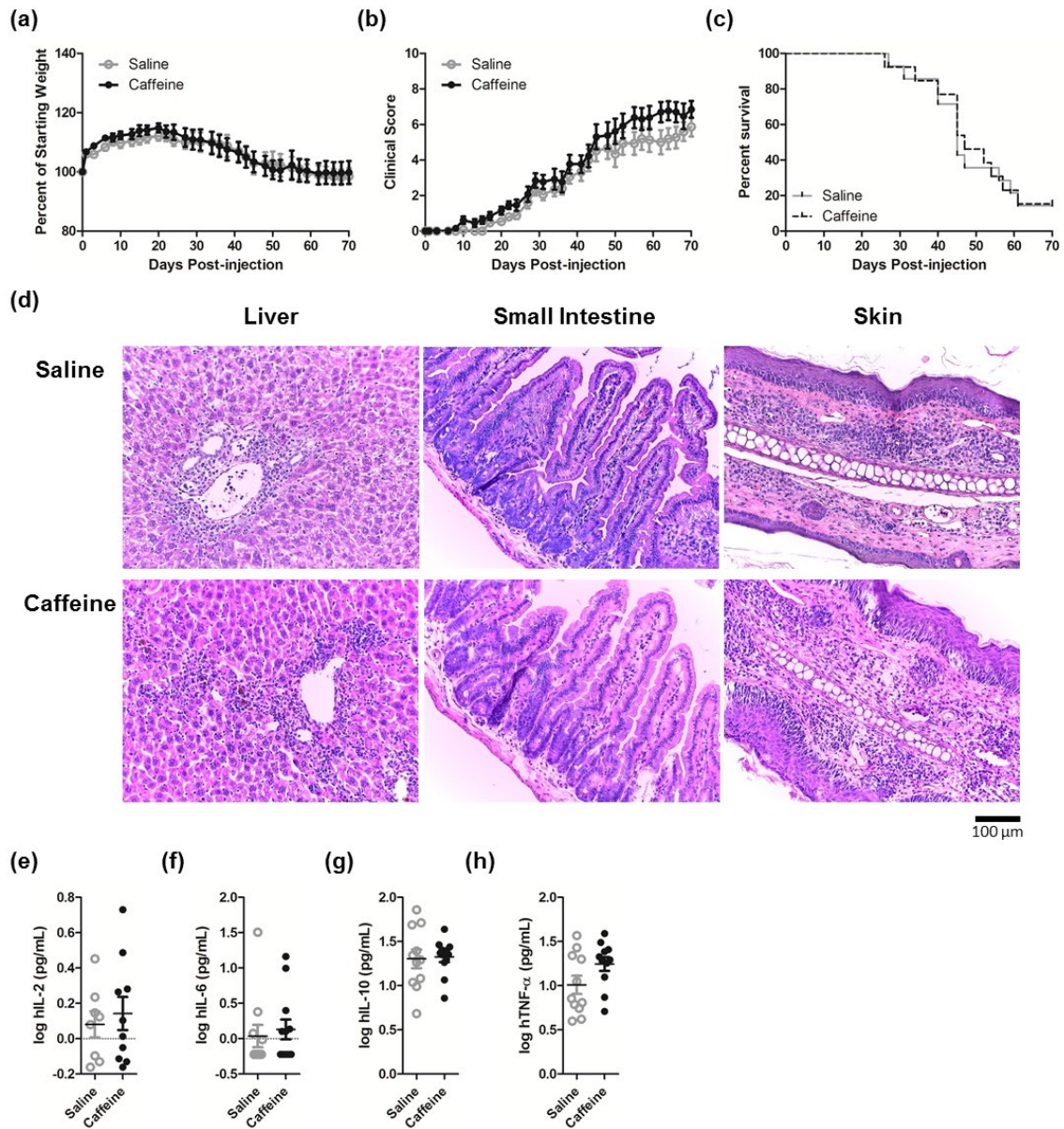


Figure 5.4. Caffeine does not impact disease in a humanised mouse model of GVHD.

(a-i) Humanised NSG mice injected with saline (control) or the AR antagonist caffeine (10 mg/kg) were monitored for (a) weight loss, (b) clinical score, and (c) survival over 10 weeks. Data represents (a, b) group means \pm SEM or (c) percent survival (saline $n = 9$, caffeine $n = 9$). (d) Tissue sections (liver, small intestine, and skin) were stained with haematoxylin and eosin and images were captured by microscopy, with each image representative of 13 mice per group; bar represents 100 μ m. (e-h) Concentrations of serum human (e) IL-2, (f) IL-6, (g) IL-10 and (h) TNF- α were analysed using a flow cytometric multiplex assay. Data represents group means \pm SEM (saline $n = 15$, caffeine $n = 13$).

5.6. Discussion

Accumulation of extracellular adenosine resulting from the CD39/CD73 pathway and subsequent activation of ARs is important in limiting disease in allogeneic mouse models of GVHD (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013). However, the role of this signalling axis in humanised mouse models of GVHD remained unknown. The current study demonstrated that CD39/CD73 blockade with APCP increases weight loss, liver GVHD and serum hIL-2 concentrations, and decreases the frequency of hCD39⁺hCD73⁺hCD4⁺ T cells in humanised mice. In contrast, blockade of ARs with caffeine did not impact clinical or histological GVHD or associated leukocytes or inflammatory markers. It is unlikely that caffeine failed to block ARs in the humanised mice, as this same caffeine regime augmented GVHD in allogeneic mice (Tsukamoto *et al.*, 2012) and caffeine impairs both murine and human ARs with similar efficacy (El Yacoubi *et al.*, 2000, Abo-Salem *et al.*, 2004). Thus, the results of this study do not support a role for AR activation in limiting GVHD in humanised mice. Rather, this study supports a role for extracellular ATP in augmenting GVHD in this model. Blockade of CD39 and/or CD73 with APCP maintains or increases extracellular ATP concentrations (Moody *et al.*, 1984, Covarrubias *et al.*, 2016). This ATP in turn may subsequently act on P2X7 and/or P2Y2 to promote GVHD in humanised mice. These P2 receptors are involved in GVHD progression in humanised (Chapter 3) and/or allogeneic models (Wilhelm *et al.*, 2010, Fowler *et al.*, 2014, Zhong *et al.*, 2016, Klambt *et al.*, 2015) of this disease, although the role of P2Y2 in the former remains to be elucidated.

The current study demonstrates that CD39/CD73 blockade with APCP augments liver GVHD in humanised mice. This finding is similar to the increased liver GVHD in CD73 deficient mice compared to wild-type mice in allogeneic GVHD (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013). The inability of AR blockade with caffeine to augment liver GVHD in humanised mice supports the concept that extracellular ATP rather than adenosine contributes to liver

GVHD in humanised mice. In support of this, ATP activation of P2X7 worsens liver GVHD in an allogeneic mouse model (Wilhelm *et al.*, 2010), and pharmacological blockade of P2X7 reduces liver GVHD in both allogeneic (Wilhelm *et al.*, 2010, Zhong *et al.*, 2016) and humanised (Chapter 3) mouse models of this disease.

The current study also demonstrated the presence of serum hIL-2, hIL-6, hIL-10, hTNF- α and hIFN- γ in humanised mice with GVHD, and implicates a pro-inflammatory role for hIL-2 in GVHD development in this model. Consistent with this, administration of low dose IL-2 in allogeneic and humanised mouse models of GVHD results in the activation of effector T cells (Abraham *et al.*, 2017, Pérol *et al.*, 2014), which circumvent any potential clinical benefits imparted by the expanded Treg cell population in these mice (Pérol *et al.*, 2014). Notably, co-administration of IL-10 with IL-2 can limit the expansion of effector T cells and increase survival in humanised mice with GVHD (Abraham *et al.*, 2017). However in the current study, hIL-10 concentrations were similar in APCP- and saline-injected mice suggesting that any potential effects of hIL-10 on hIL-2 was the same between treatments. Finally, in the current study, serum concentrations of hIL-2, hIL-6, hIL-10 and hTNF- α were in the pg/mL range, which contrasted the 1000-fold greater amounts of serum hIFN- γ . Thus, this suggests hIFN- γ may have a major role in disease development in this humanised mouse model of GVHD. Consistent with this, P2X7 blockade in this model reduced histological GVHD, which coincided with reduced concentrations of serum hIFN- γ (Chapter 3), but the impact of P2X7 blockade on serum hIL-2 remains to be elucidated.

The current study demonstrated that APCP but not caffeine decreases human CD39⁻CD73⁻CD4⁺, but not CD39⁻CD73⁻CD8⁺ T cells. The reduced frequency of human CD39⁻CD73⁻CD4⁺ T cells due to APCP treatment may be due a compensatory mechanism that involves upregulating CD39 and/or CD73 to increase ATP degradation and promote adenosine generation. This is supported by the trend of increased frequencies of human

CD39⁺CD73⁻CD4⁺ and CD39⁻CD73⁺CD4⁺ T cells following APCP treatment. However, these increases were not statistically significant and there was no increased trend in human CD39⁺CD73⁺CD4⁺ T cells in these mice. Thus, the reason for the reduced frequency of human CD39⁻CD73⁻CD4⁺ T cells following APCP treatment requires further elucidation.

The current study confirms that NSG mice injected with hPBMCs engraft hCD4⁺ and hCD8⁺ T cells as well as Tregs, but shows for the first time that humanised NSG mice also engraft human iNKT cells. This engraftment of human iNKT cells suggests that this model could be used to study these cells *in vivo* and allow manipulation of these cell types to alter disease outcomes. In allogeneic mouse models of GVHD, both host (Zeng *et al.*, 1999) and donor (Kim *et al.*, 2007) iNKT cells can attenuate disease, whilst adoptive transfer of these cells can prevent GVHD in such models (Schneidawind *et al.*, 2015). The current study also demonstrates that humanised NSG mice engraft human monocytes and DCs. Previous studies have shown that NSG mice engraft human monocytes and DCs when injected with human cord blood (Audigé *et al.*, 2017), and engraft human monocytes when injected with hPBMCs (Palamides *et al.*, 2016, Jodeleit *et al.*, 2017). Similarly, SCID mice injected with hPBMCs engraft human DCs (Seldon *et al.*, 2015), but the current study is the first to demonstrate DC engraftment in NSG mice injected with hPBMCs. Human T cells recognise murine major histocompatibility complex I and II molecules in NSG mice to cause GVHD (King *et al.*, 2009), but the identity of the APCs involved remains to be determined. However it is known that both host and donor APCs are involved in GVHD development in allogeneic mouse models of this disease (Shlomchik *et al.*, 1999, Markey *et al.*, 2009). The engraftment of human monocytes and DCs in NSG mice suggests that human T cells may at least in part be activated by human antigen presenting cells in this model.

Although these results demonstrate that AR blockade with caffeine has no impact on disease, caffeine is a broad-spectrum adenosine receptor antagonist which may confound

interpretation of its effects in the current study. Furthermore, caffeine is an antagonist of phosphodiesterases (Choi *et al.*, 1988) and γ -aminobutyric acid receptors (Nistri and Berti, 1984, Vigh and Lasater, 2003), and can promote calcium release from intracellular stores (Nistri and Berti, 1984, Vigh and Lasater, 2003) further confounding interpretation of current findings. Therefore, the future use of more selective AR antagonists such as SCH 52861, which worsened GVHD in allogeneic mouse models (Lappas *et al.*, 2010), is warranted in humanised mouse models of GVHD to further explore a role for ARs in this model. Alternatively, deletion of specific ARs (or CD39 or CD73) in NSG mice may provide further insight into ATP and adenosine signalling pathways in the development of GVHD in humanised mice. This was not performed in this study due to issues with dissolving SCH 52861, and the ethically unnecessary use of mice.

In conclusion, CD39/CD73 blockade with APCP, but not AR blockade with caffeine, augmented GVHD in humanised mice. These results support the known roles of extracellular ATP in promoting GVHD but do not support evidence that extracellular adenosine can limit GVHD in this humanised model.

5.7. Acknowledgements

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5.8. Conflict of Interest

All authors declare that they have no conflicts of interest.

5.9. Author Contributions

N.J.G., D.W. and R.S. designed the experiments. N.J.G. performed the experiments, analysed the data, prepared the figures and wrote the manuscript. D.W. and R.S. supervised the project, reviewed the data and edited the manuscript.

Chapter 6: The A2A Receptor Agonist CGS 21680 Has Differential Effects on Disease Development in a Humanised Mouse Model of Graft-versus-Host Disease

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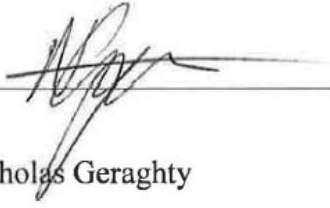
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6.1. Statement of Contribution of Authors

I, as one of the authors of this original article, agree with the statement of author contributions stated at the end of this chapter, and originally published in the original journal article.



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6.2. Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative method for blood cancers and other blood disorders, but is limited by the development of graft-versus-host disease (GVHD). GVHD results in inflammatory damage to the host liver, gastrointestinal tract and skin, resulting in high rates of morbidity and mortality in HSCT recipients. Activation of the A_{2A} receptor has been previously demonstrated to reduce disease in allogeneic mouse models of GVHD using CGS 21680. This study aimed to investigate the effect of A_{2A} activation on disease development in a humanised mouse model of GVHD. Immunodeficient non-obese diabetic-severe combined immunodeficiency-interleukin (IL)-2 receptor γ^{null} (NSG) mice injected with human (h) peripheral blood mononuclear cells (hPBMCs), were treated with either the A_{2A} agonist CGS 21680 or vehicle control. Contrary to the beneficial effect of A_{2A} activation in allogeneic mouse models, CGS 21680 increased weight loss, and failed to reduce the clinical score or increase survival in this humanised mouse model of GVHD. Moreover, CGS 21680 reduced T regulatory cells and increased serum human IL-6 concentrations. Conversely, CGS 21680 reduced serum human tumour necrosis factor (TNF)- α concentrations and leukocyte infiltration into the liver, indicating that A_{2A} activation can, in part, reduce molecular and histological GVHD in this model. Notably, CGS 21680 also prevented healthy weight gain in NSG mice not engrafted with hPBMCs suggesting that this compound may be suppressing appetite or metabolism. Therefore, the potential benefits of CGS 21680 in reducing GVHD in HSCT recipients may be limited and confounded by adverse impacts on weight, decreased T regulatory cell frequency and increased IL-6 production.

6.3. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative method for numerous haematological malignancies and other blood disorders; however, HSCT is limited by the development of graft-versus-host disease (GVHD) (Jaglowski and Devine, 2014). GVHD develops in up to 60% of HSCT recipients (Jagasia *et al.*, 2012), due to donor T cells recognising 'foreign' host cells (Billingham, 1965). GVHD begins when damage caused by the conditioning regime or the underlying disease promotes the release of inflammatory cytokines such as tumour necrosis factor (TNF)- α and interleukin (IL)-6. Subsequently, activation of T cells by dendritic cells (DCs) results in the further release of TNF- α as well as interferon (IFN)- γ , IL-2 and IL-6 to promote inflammation, and subsequent activation of CD8⁺ T cells to exacerbate this inflammation. Conversely, T regulatory (Treg) cells and invariant natural killer T (iNKT) cells can reduce pro-inflammatory effects in GVHD to limit disease development or progression (Schneidawind *et al.*, 2013).

Adenosine receptors (A₁, A_{2A}, A_{2B} and A₃) are cell-surface G-protein coupled receptors activated by extracellular adenosine (Fredholm *et al.*, 2011a). Extracellular adenosine is often produced as a result of ATP hydrolysis mediated by the sequential action of ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39), and ecto-5'-nucleotidase (CD73) (Ferrero *et al.*, 2018, Apostolova and Zeiser, 2016a). The A_{2A} receptor is expressed on numerous immune cell subsets including DCs and T cells (Burnstock and Boeynaems, 2014). Notably, CD39/CD73-mediated production of adenosine and subsequent activation of A_{2A} is an important anti-inflammatory mechanism (Deaglio *et al.*, 2007). In allogeneic mouse models of transplantation, adenosine production by the CD39/CD73 pathway and subsequent activation of adenosine receptors prevents tissue damage and reduces graft rejection (Vergani *et al.*, 2014). In allogeneic mouse models of GVHD, genetic deficiency or pharmacological blockade of CD73 with $\alpha\beta$ -methylene ADP (APCP) (Tsukamoto *et al.*, 2012), which results

in reduced extracellular adenosine, worsens disease. Similarly, genetic deficiency (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013) or pharmacological blockade of A_{2A} with SCH58261 (Wang *et al.*, 2013) also worsens GVHD severity in these models. Conversely, activation of A_{2A} with ATL-146e can ameliorate GVHD in allogeneic mouse models (Lappas *et al.*, 2010, Han *et al.*, 2013). However, the action of A_{2A} activation in humanised mouse models or HSCT patients remains to be explored.

Allogeneic mouse models are often used to investigate potential therapeutics for GVHD, yet therapies investigated in these models often do not translate to the clinic. This lack of translation is possibly due to species differences. In an attempt to address this, preclinical “humanised” mouse models have been developed (Shultz *et al.*, 2007). A commonly used humanised mouse model involves injection of human peripheral blood mononuclear cells (hPBMCs) into immunodeficient non-obese diabetic severe-combined immunodeficiency-IL-2 receptor γ^{null} (NSG) mice. Due to defective T and B cells, and a lack of natural killer (NK) cells, these mice readily engraft hPBMCs (King *et al.*, 2008), and subsequently develop GVHD due to the ability of human T cells to recognise the major histocompatibility complex (MHC) I and II of NSG mice (King *et al.*, 2009, Brehm *et al.*, 2018). As such, humanised NSG mice have been widely used as a model of GVHD (Pino *et al.*, 2010, Hippen *et al.*, 2011, Ali *et al.*, 2012, Bruck *et al.*, 2013, Søndergaard *et al.*, 2013, Abraham *et al.*, 2015, Gregoire-Gauthier *et al.*, 2015, Hilger *et al.*, 2016, Ehx *et al.*, 2017).

Using the A_{2A} agonist CGS 21680 (Klotz *et al.*, 1998), this study aimed to investigate the effect of A_{2A} activation on GVHD development in a humanised mouse model. CGS 21680 did not impact clinical score or survival of mice. However, CGS 21680 reduced leukocyte infiltration into livers, and reduced serum hTNF- α concentrations indicative of reduced GVHD severity. Conversely, CGS 21680 worsened weight loss, reduced Treg cell frequency and increased serum hIL-6 concentrations indicating worsened GVHD. Notably, CGS 21680

also prevented weight gain in NSG mice not engrafted with hPBMCs. This suggests that appetite or metabolism may be negatively impacted by CGS 21680. Therefore, the adverse impact on weight, Treg cells and IL-6 caused by CGS 21680 may confound the potential benefits of this agonist in reducing GVHD in HSCT recipients.

6.4. Materials and Methods

6.4.1. Humanised Mouse Model of GVHD

Experiments involving human blood and mice were approved by the respective Human and Animal Ethics Committees of the University of Wollongong (Wollongong, Australia). A humanised mouse model of GVHD was used as described (Chapter 3). Briefly, female NSG mice aged 6-8 weeks (Australian BioResources, Moss Vale, Australia) were injected intraperitoneally (i.p.) daily (days -2 to day 11) with saline/0.2% DMSO (Sigma-Aldrich, St Louis, MO, USA) (vehicle) or vehicle containing CGS 21680 (Tocris Bioscience, Bristol, UK) (0.1 mg/kg). hPBMCs, isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden) and resuspended in Dulbecco's phosphate-buffer saline (ThermoFisher, Waltham, MA, USA), were injected i.p. (day 0) (10×10^6 hPBMCs/mouse). At 3 weeks post-hPBMC injection, mice were checked for engraftment by immunophenotyping of tail vein blood. Mice were monitored for signs of GVHD using a scoring system, giving a total clinical score out of 10, as described (Chapter 3). Mice were euthanised at 10 weeks post-injection of hPBMCs, or earlier if exhibiting a clinical score of ≥ 8 or a weight loss of $\geq 10\%$, according to the approved animal ethics protocol.

6.4.2. Immunophenotyping by Flow Cytometry

Tail vein blood (week 3) and spleen cells (end-point) were obtained from mice and lysed with ammonium chloride potassium buffer and immunophenotyped as described (Chapter 3) using the antibodies listed in Table 1. Data was collected using a BD Fortessa-X20 Flow Cytometer

(using band pass filters 450/50 for BV421, 710/50 for BV711, 525/50 for FITC, 586/15 for PE, 695/40 for PerCP-Cy5.5, 780/60 for PE-Cy7 and 670/30 for APCy). The relative percentages of cells were analysed using FlowJo software v8.7.1 (TreeStar Inc.; Ashland, OR, USA).

Table 6.1. Monoclonal antibodies used for flow cytometry.

Target	Fluorochrome	Clone*
CD3	BV711	UCHT1
CD4	PerCP-Cy5.5	L200
CD8	FITC	RPA-T8
CD14	BV421	MφP9
CD19	APCy	HIB19
CD25	PE	M-A251
CD39	APCy	TU66
CD45	FITC	HI30
CD45	PerCP-Cy5.5	30-F11
CD73	PE-Cy7	AD2
CD83	PE	HB15e
CD127	BV421	HIL-7R-M21
Vα24-Jα14 TCR	PE-Cy7	6B11

*All antibodies were mouse anti-human except clone 30-F11 (rat anti-mouse); all antibodies were from BD Biosciences except clone 6B11 (BioLegend). Abbreviations: APCy, allophycocyanin; BV, brilliant violet; Cy, cyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; TCR, T cell receptor.

6.4.3. Histological Analysis

Formalin-fixed tissue sections (5 µm) were stained with haematoxylin and eosin (POCD; Artarmon, Australia), and histology assessed, as described (Chapter 3).

6.4.4. Cytokine Analysis by a Flow Cytometric Multiplex Assay

Serum was obtained from mice at end-point as described (Chapter 3) and cytokine concentrations were measured using a Th1 LEGENDPlex kit (BioLegend, San Diego, CA, USA) as per the manufacturer's instructions.

6.4.5. Statistical Analysis

Data is given as mean \pm standard error of the mean (SEM). Statistical differences were calculated using Student's t-test for single comparisons or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Weight and clinical score were analysed using a repeated measures two-way ANOVA. Survival (median survival time; MST) was compared using the log-rank (Mantel-Cox) test. Proportion of engraftment and mortality were compared using Fisher's exact test. All statistical analyses and graphs were generated using GraphPad Prism 5 for PC (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

6.5. Results

6.5.1. CGS 21680 Does Not Impact Initial hPBMC Engraftment in NSG Mice

NSG mice, injected with hPBMCs and either control vehicle ($n = 25$) or the A_{2A} agonist CGS 21680 (Klotz *et al.*, 1998) ($n = 25$) daily (days -2 to 11), were monitored (from day 0) for up to 10 weeks. To determine whether CGS 21680 affected initial hPBMC engraftment, blood was collected 3 weeks post-hPBMC injection and cells immunophenotyped by flow cytometry. Three mice from each group did not demonstrate human leukocytes (hCD45⁺mCD45⁻) in their blood at 3 weeks (results not shown). In the remaining mice, human leukocytes were observed in the blood with frequency of these cells calculated as a percentage of total leukocytes [hCD45⁺mCD45⁻/(hCD45⁺mCD45⁻ + hCD45⁻mCD45⁺)]. CGS 21680- and vehicle-injected mice demonstrated similar frequencies of human leukocytes ($21.8 \pm 2.8\%$ and $22.8 \pm 3.2\%$, respectively, $P = 0.8221$) (Fig 1a). The proportion of human leukocyte engraftment was the same in CGS 21680- and vehicle-injected mice (both 90%, $P = 1.000$). The majority of these human leukocytes were T cells ($95.7 \pm 0.8\%$ and $97.3 \pm$

0.6%, respectively, $P = 0.1086$) (Fig 1b). The remaining cells were non-B/T cells ($3.8 \pm 0.9\%$ and $3.4 \pm 0.6\%$, respectively, $P = 0.7502$) (Fig 1c).

6.5.2. CGS 21680 Reduces Human Treg Cells in Humanised NSG Mice

To determine if CGS 21680 impacted hPBMC engraftment at end-point, splenocytes from CGS 21680- ($n = 20$) and vehicle-injected mice ($n = 20$) were analysed by flow cytometry. Human leukocytes were absent in the spleens of the same three mice from each group that failed to show hPBMC engraftment at 3 weeks (results not shown). Frequencies of human leukocytes in mice engrafted at three weeks were similar in CGS 21680- and vehicle-injected mice ($70.5 \pm 3.2\%$ and $65.1 \pm 6.0\%$, respectively, $P = 0.4348$) at end-point (Figure 6.1.d.).

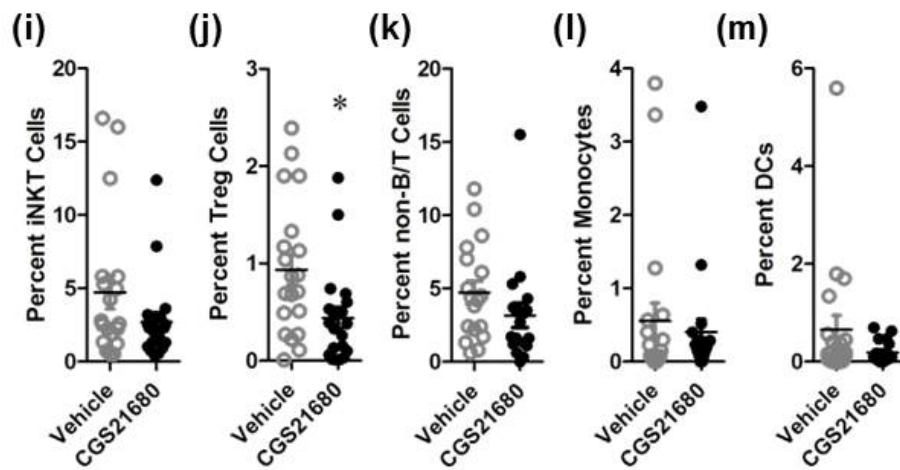
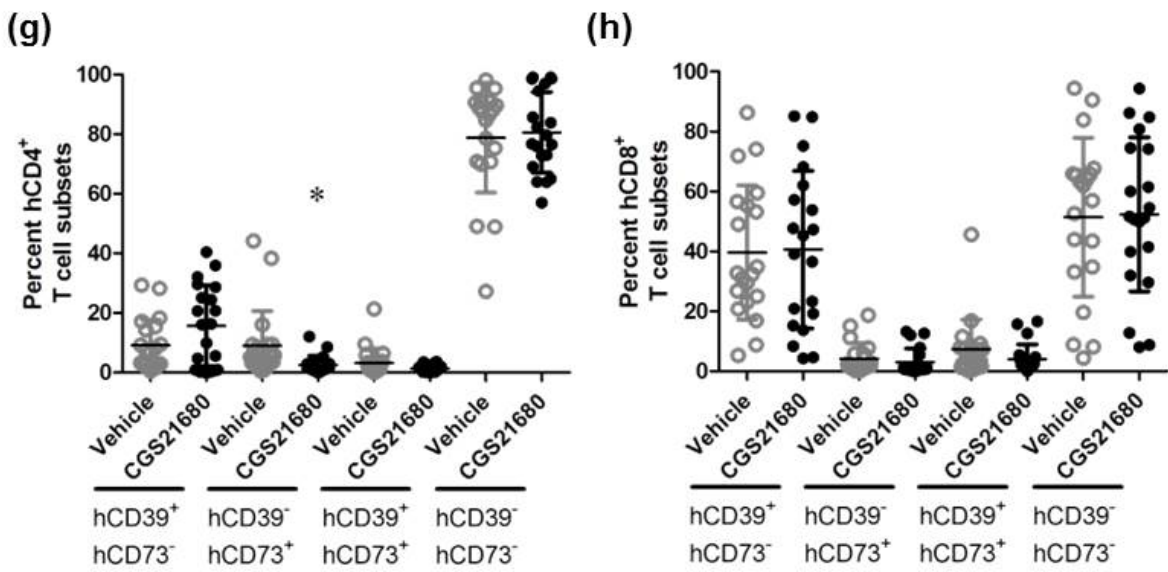
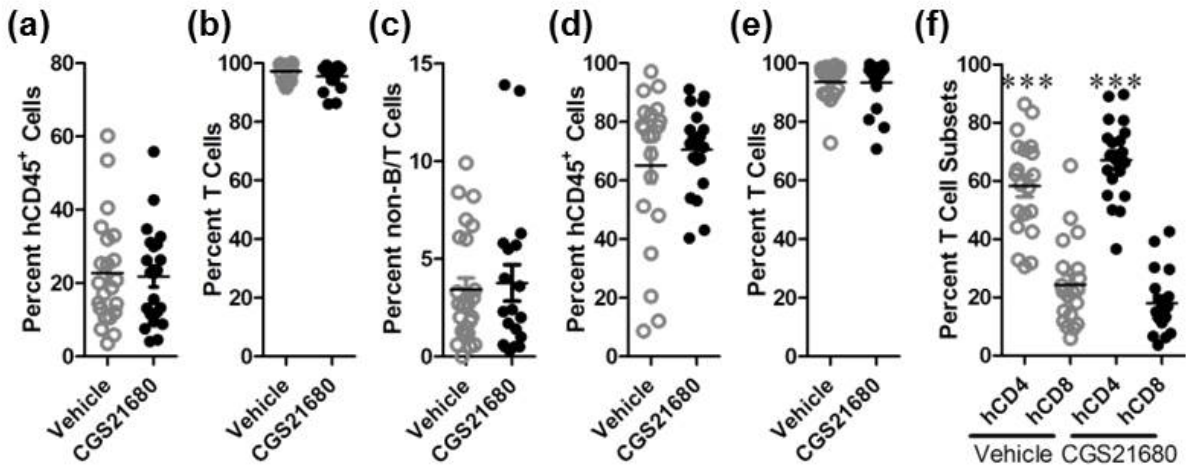
Similar to blood at 3 weeks post-hPBMC injection, the majority of engrafted human leukocytes in both groups of mice were T cells, which did not differ between CGS 21680- ($93.4 \pm 1.8\%$) and vehicle-injected mice ($93.6 \pm 1.5\%$) ($P = 0.9546$) (Figure 6.1.e.). Further analysis of human T cells demonstrated that CGS 21680- and vehicle-injected mice demonstrated similar engraftment of hCD4⁺ T cells ($67.2 \pm 3.0\%$ and $58.3 \pm 3.7\%$, respectively, $P = 0.0713$) and hCD8⁺ T cells ($18.0 \pm 2.3\%$ and $24.4 \pm 3.3\%$, respectively, $P = 0.1260$). Both CGS 21680- and vehicle-injected mice demonstrated greater engraftment of hCD4⁺ than hCD8⁺ cells ($P < 0.0001$ and $P < 0.0001$, respectively) (Figure 6.1.f.).

To investigate whether CGS 21680 effected CD39 and/or CD73 on human T cells, these ecto-nucleotidases on hCD4⁺ and hCD8⁺ T cell subsets from the spleens of mice were examined. CGS 21680-injected demonstrated a trend of increased hCD39⁺hCD73⁻hCD4⁺ T cells compared to vehicle-injected mice ($15.6 \pm 3.0\%$ and $9.1 \pm 2.0\%$, respectively, $P = 0.0796$). Conversely, CGS 21680-injected mice demonstrated significantly reduced frequencies of hCD39⁻hCD73⁺hCD4⁺ T cells ($2.5 \pm 0.7\%$ and $8.9 \pm 2.6\%$, respectively, $P = 0.0227$), and a reduced trend of hCD39⁺hCD73⁺hCD4⁺ T cells ($1.3 \pm 0.3\%$ and $3.1 \pm 1.1\%$,

respectively, $P = 0.1137$) compared to vehicle-injected mice. However, CGS 21680- and vehicle-injected mice demonstrated similar frequencies of hCD39⁻hCD73⁻hCD4⁺ T cells ($80.6 \pm 3.0\%$ and $78.8 \pm 4.1\%$, respectively, $P = 0.7358$) (Figure 6.1.g.). CGS 21680- and vehicle-injected mice demonstrated similar frequencies of hCD39⁺hCD73⁻hCD8⁺ T cells ($40.6 \pm 5.9\%$ and $39.7 \pm 5.0\%$, respectively, $P = 0.9036$), hCD39⁻hCD73⁺hCD8⁺ T cells ($3.0 \pm 1.0\%$ and $4.2 \pm 1.2\%$, respectively, $P = 0.4508$), hCD39⁺hCD73⁺hCD8⁺ T cells ($4.0 \pm 1.1\%$ and $7.3 \pm 2.2\%$, respectively, $P = 0.1877$) and hCD39⁻hCD73⁻hCD8⁺ T cells ($52.3 \pm 4.7\%$ and $51.4 \pm 5.9\%$, respectively, $P = 0.9137$) (Figure 6.1.h.).

In allogeneic mouse models increased frequencies of iNKT and Treg cells correlates with reduced GVHD (Schneidawind *et al.*, 2013). Therefore, the frequency of human iNKT cells (hCD45⁺hCD3⁺hCD19⁻hV α 24-J α 18⁺) and human Treg cells (hCD45⁺hCD3⁺hCD4⁺hCD25⁺hCD127^{lo}) in spleens from mice were examined. CGS 21680-injected mice, compared to vehicle-injected mice, demonstrated a reduced trend of iNKT cells ($2.7 \pm 0.6\%$ and $4.7 \pm 1.1\%$, respectively, $P = 0.1223$) (Figure 6.1.i.) and significantly reduced Treg cells ($0.4 \pm 0.1\%$ and $0.9 \pm 0.2\%$, respectively, $P = 0.0130$) (Figure 6.1.j.).

Our group has previously shown that at end-point the spleens of humanised mice do not contain human B cells (Chapter 3). Similarly, in the current study the remaining human leukocytes in the spleens of mice were negative for CD19 (results not shown). CGS 21680- and vehicle-injected mice demonstrated small but similar frequencies of CD3⁻CD19⁻ cells present in both groups of mice ($3.2 \pm 0.8\%$ and $4.7 \pm 0.8\%$, respectively, $P = 0.1783$) (Figure 1k). To determine if human monocytes (hCD14⁺hCD83⁻) or DCs (hCD14⁻hCD83⁺) were present, the remaining non-B/T cell population (hCD45⁺hCD3⁻hCD19⁻) was analysed. CGS 21680- and vehicle-injected mice demonstrated similar but low frequencies of monocytes ($0.4 \pm 0.2\%$ and $0.6 \pm 0.2\%$, respectively, $P = 0.6127$) (Figure 1l) and DCs ($0.2 \pm 0.1\%$, and $0.7 \pm 0.3\%$, respectively, $P = 0.1160$) (Figure 1m).



◀ **Figure 6.1. CGS 21680 reduces human T regulatory cells in humanised NSG mice.**

(a - m) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10×10^6 hPBMCs (day 0). The percentages of human (h) leukocytes and subsets in (a-c) blood at 3 weeks post-hPBMC injection and (d-m) spleens at end-point were determined by flow cytometry. (a, d) Human leukocytes (hCD45⁺mCD45⁻) are expressed as a percentage of total mCD45⁺ and hCD45⁺ leukocytes. Three mice from each group did not engraft hCD45⁺ leukocytes (not shown). (b, e) hCD3⁺hCD19⁻ cells and (c, k) hCD3⁻hCD19⁻ cells are expressed as a percentage of total hCD45⁺ leukocytes. (f) hCD4⁺ and hCD8⁺ T cell subsets are expressed as a percentage of total hCD3⁺ leukocytes. ** $P < 0.005$, *** $P < 0.0001$ compared to hCD8⁺ T cells. (g-h) hCD39 and hCD73 expression was analysed on (g) hCD4⁺ and (h) hCD8⁺ T cell subsets. * $P < 0.05$ compared to vehicle. (i) Invariant natural killer T (iNKT) cells (hCD45⁺hCD3⁺hCD19⁻hV α 24-J α 18⁺) are expressed as a percentage of hCD3⁺hCD19⁻ T cells and (j) T regulatory (Treg) cells (hCD45⁺hCD3⁺hCD4⁺hCD25⁺hCD127^{lo}) are expressed as a percentage of hCD3⁺ T cells. * $P < 0.05$ compared to vehicle. (l) Monocytes (hCD14⁺hCD83⁻) and (m) dendritic cells (DCs) (hCD14⁺hCD83⁺) are expressed as a percentage of hCD45⁺hCD3⁺hCD19⁻ cells. Data represents group means \pm SEM (vehicle $n = 20-22$, CGS 21680 $n = 20-22$); symbols represent individual mice.

6.5.3. CGS 21680 Worsens Weight Loss in Humanised NSG Mice

To investigate whether CGS 21680 impacts GVHD, the above mice were monitored for weight loss and other signs of GVHD for up to 10 weeks. One engrafted saline-injected mouse died unexpectedly overnight from unknown causes and was excluded from the following analyses. In those mice which had engrafted hPBMCs, CGS 21680-injected mice ($n = 22$) demonstrated significantly greater weight loss over the 10 weeks than vehicle-injected mice ($n = 21$) ($P = 0.0020$) (Figure 6.2.a.). However, both CGS 21680- and vehicle-injected mice demonstrated signs of GVHD (classified as a score clinical score > 3) from 35 days onwards with similar scores ($P = 0.8008$) (Figure 6.2.b.), survival (MST; 41 days, and 43 days, respectively, $P = 0.6730$) and mortality rates (90% and 82%, respectively, $P = 0.6640$) (Figure 6.2.c.) over the 10 weeks.

6.5.4. CGS 21680 Prevents Healthy Weight Gain in NSG Mice Not Engrafted with hPBMCs

As noted above, three NSG mice from each treatment group did not engraft hPBMCs. Nevertheless, these mice were also monitored for weight loss and signs of GVHD for 10

weeks as per the engrafted mice above. CGS 21680-injected mice gained significantly less weight over the 10 weeks compared to vehicle-injected mice ($P = 0.0029$) (Figure 6.2.d.). Both CGS 21680- and vehicle-injected mice demonstrated similar but minimal clinical signs of GVHD over the 10 weeks (mean clinical scores of 0.4 ± 0.2 and 0.3 ± 0.2) ($P = 0.4557$) (Figure 6.2.e.) consistent with the lack of hPBMC engraftment. Moreover, all CGS 21680- and vehicle-injected mice not engrafted with hPBMCs survived the entire 10 weeks (Figure 6.2.f.).

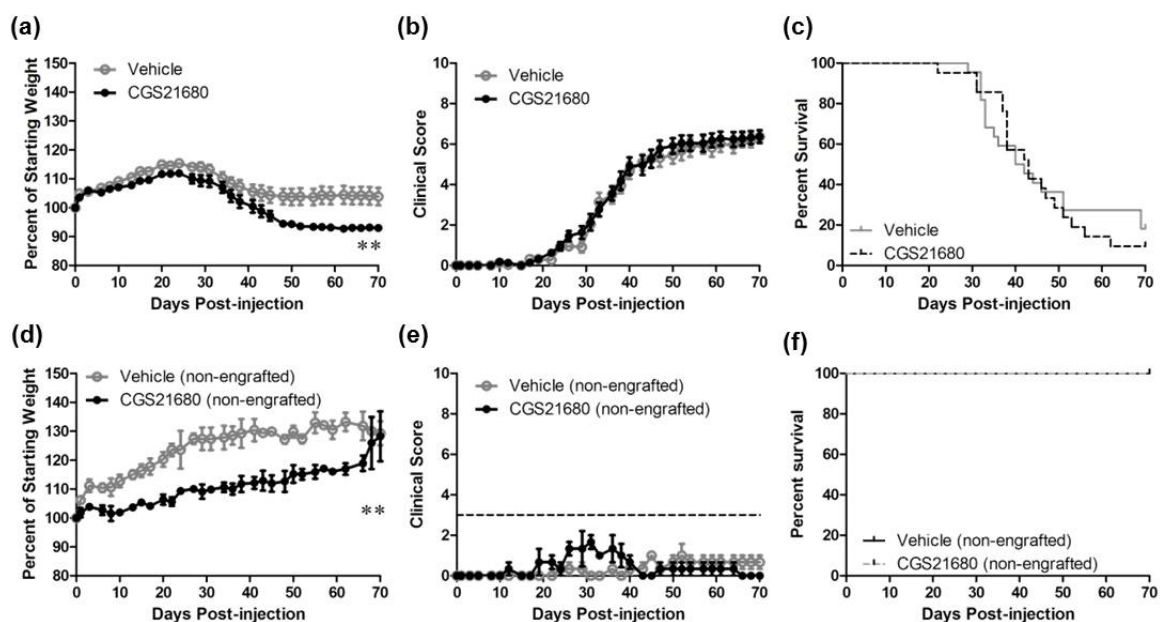


Figure 6.2. CGS 21680 worsens weight loss in NSG and humanised NSG mice. (a - f) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10×10^6 hPBMCs (day 0). NSG mice engrafted with hPBMCs were monitored for (a) weight loss, (b) clinical score, and (c) survival over 10 weeks. Data represents (a, b) group means \pm SEM or (c) percent survival (vehicle $n = 22$, CGS 21680 $n = 21$). ** $P < 0.005$ compared to vehicle-injected mice. (d - f) Mice which were not engrafted with hCD45⁺ leukocytes at 3 weeks (blood) and at 10 weeks (spleen) (results not shown) were monitored for (d) weight loss, (e) clinical score, and (f) survival. Data represents (d, e) group means \pm SEM or (f) percent survival (vehicle $n = 3$, CGS 21680 $n = 3$). ** $P < 0.005$ compared to vehicle-injected mice.

6.5.5. CGS 21680 Reduces Liver Infiltrates in Humanised NSG Mice

Tissues from mice which had engrafted hPBMCs ($n = 20$ per group) were examined by histology. Livers from CGS 21680-injected mice demonstrated reduced leukocyte infiltration

but similar structural damage compared to vehicle-injected mice (Figure 6.3.). CGS 21680-injected mice demonstrated mildly reduced leukocyte infiltration into small intestine of vehicle-injected mice, but there were no structural differences, with all mice demonstrating minimal rounding of villi and crypt cell apoptosis (Figure 6.3.). CGS 21680- and vehicle-injected mice demonstrated similar skin histology, with all mice demonstrating leukocyte infiltration and epidermal thickening (Figure 6.3.).

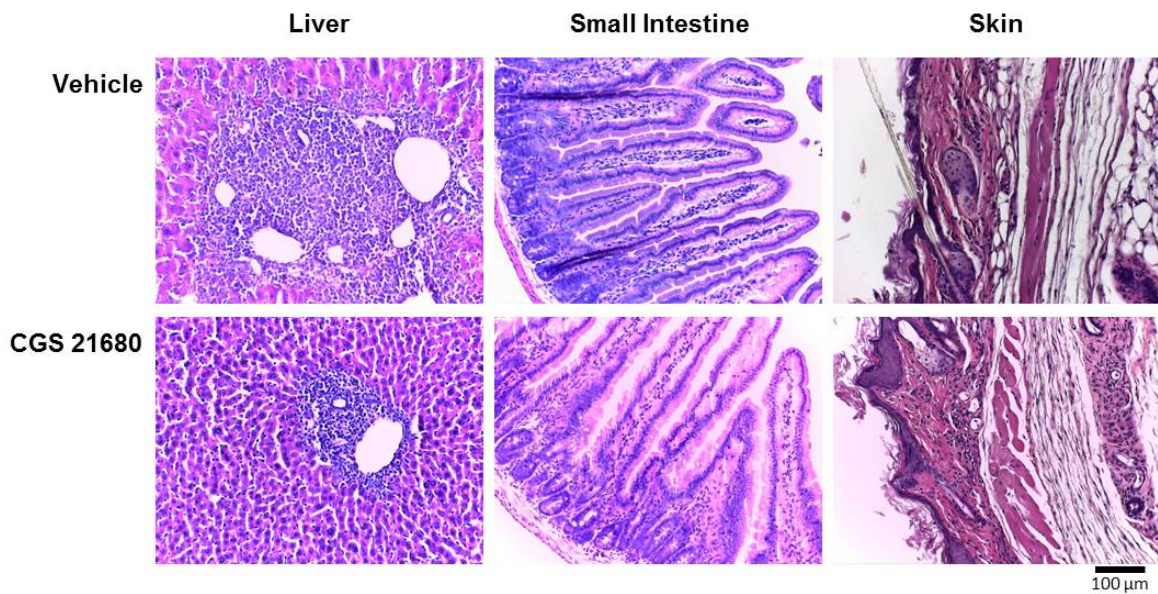


Figure 6.3. CGS 21680 reduces liver infiltrates in humanised NSG mice. NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10×10^6 hPBMCs (day 0). Tissue sections (liver, small intestine, and skin) from hPBMC-engrafted mice were stained with haematoxylin and eosin. Images were captured by microscopy with each image representative of twenty mice per group; bars represent 100 μm .

6.5.6. CGS 21680 Increases Serum hIL-6 but Reduces hTNF- α in Humanised NSG Mice

The pro-inflammatory cytokine storm that preludes immune cell infiltration and inflammatory damage of target organs is an important stage of GVHD pathogenesis (Ferrara *et al.*, 1999). Therefore, to determine if CGS 21680 impacts human cytokines, a multiplex

assay was used to analyse concentrations of serum hIL-2, hIL-6, hIL-10, hTNF- α , and hIFN- γ from CGS 21680-injected mice ($n = 15$) and vehicle-injected ($n = 18$).

Mean serum hIL-2 concentrations were 83% lower in CGS 21680-injected mice compared to vehicle-injected mice but this was not significantly different (7.0 ± 1.2 pg/mL and 41.8 ± 28.1 pg/mL, respectively; $P = 0.2379$) (Fig 3a). CGS 21680-injected mice demonstrated a significant four-fold increase in hIL-6 concentrations compared to vehicle-injected mice (141.0 ± 79.3 pg/mL and 35.4 ± 27.8 pg/mL, respectively; $P < 0.0001$). There was an 89% decrease in serum hIL-10 in CGS 21680-injected mice compared to vehicle-injected mice, but this did not reach statistical significance (18.9 ± 4.8 pg/mL and 170.0 ± 93.1 pg/mL, respectively; $P = 0.0800$) (Fig 4c). There was a significant 75% decrease in hTNF- α concentrations in CGS 21680-injected mice compared to vehicle-injected mice (18.9 ± 4.8 pg/mL, and 77.0 ± 40.1 pg/mL, respectively; $P = 0.0411$) (Fig 4d). hIFN- γ concentrations in both treatment groups exceeded the highest standard ($>10,000$ pg/mL) (data not shown) and could not be compared.

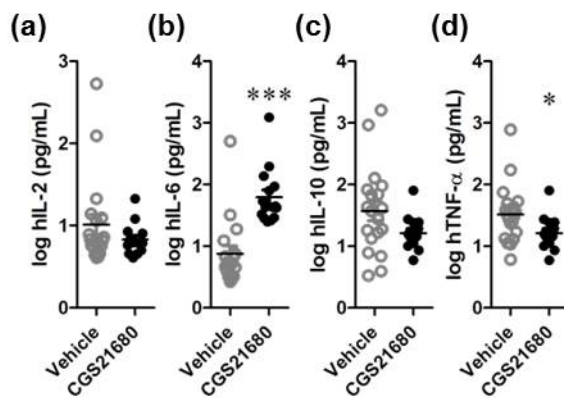


Figure 6.4. CGS 21680 impacts serum cytokines in humanised NSG mice. (a - d) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10×10^6 hPBMCs (day 0). Concentrations of serum human (a) interleukin (IL)-2, (b) IL-6, (c) IL-10, and (d) tumour necrosis factor (TNF)- α from hPBMC-engrafted mice were analysed by a flow cytometric multiplex assay. Data represents group means \pm SEM (vehicle $n = 18$, CGS 21680 $n = 15$); * $P < 0.05$, *** $P < 0.0001$ compared to vehicle-injected mice.

6.6. Discussion

Previous studies have shown that the CD73/A_{2A} pathway reduces disease severity in allogeneic mouse models of GVHD (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013, Lappas *et al.*, 2010, Han *et al.*, 2013). However, the effect of A_{2A} activation in humanised mouse models or in HSCT patients has not been reported. Using a humanised NSG mouse model of GVHD, the current study demonstrated that the A_{2A} agonist CGS 21680 had opposing roles in disease development. CGS 21680 did not affect clinical score or mortality in humanised mice but reduced GVHD severity, as indicated by decreased leukocyte infiltration into the liver and serum hTNF- α in these mice. Unexpectedly, CGS 21680 increased weight loss and serum hIL-6, and reduced the frequency of Tregs, indicating that this A_{2A} agonist worsens these disease parameters in this humanised mouse model of GVHD.

CGS 21680 reduced leukocyte infiltration into the liver and serum hTNF- α indicating GVHD is reduced, at least in part, in humanised mice. This indicates that A_{2A} activation has beneficial roles in this model. This finding parallels similar observations in allogeneic mouse models of GVHD where the A_{2A} agonist ATL-146e reduces histological damage, leukocyte infiltration in livers and serum TNF- α (Lappas *et al.*, 2010, Han *et al.*, 2013). Conversely, pharmacological blockade or genetic deficiency of CD73, which limits extracellular adenosine (Bruns, 1980), worsens liver histology in allogeneic mouse models of GVHD (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013). Of note, in allogeneic and humanised mouse models of GVHD, increased TNF- α levels correspond with disease severity (Kuroiwa *et al.*, 2001, Abraham *et al.*, 2015) and blockade of this cytokine impairs GVHD in both of these models (Hill *et al.*, 1999, King *et al.*, 2009). Thus, collectively these studies suggest a crucial role for TNF- α in GVHD, and that activation of A_{2A} can limit this in both allogeneic and humanised mouse models of this disease.

Contrary to above, the current study suggests that CGS 21680 worsens aspects of GVHD in humanised mice as evidenced through increased weight loss. This indicates that A_{2A} activation by this agonist also has adverse roles in this model. Weight loss is a common indicator of disease severity in mouse models of GVHD (Naserian *et al.*, 2018). The dose of CGS 21680 (0.1 mg/kg) used in this study is sufficient to prevent disease in mouse models of acute lung inflammation (Impellizzeri *et al.*, 2011), pleurisy (da Rocha Lapa *et al.*, 2012) and collagen-induced arthritis (Mazzon *et al.*). These studies reported no effect of CGS 21680 on weight, suggesting weight loss in CGS 21680-injected humanised mice is due in part to worsened GVHD. Supporting this, serum IL-6 was also increased in humanised mice. Although, hIL-6 has not been previously detected in the serum of humanised mice (Gregoire-Gauthier *et al.*, 2012, Abraham *et al.*, 2015, Abraham *et al.*, 2017), and its role in humanised mouse models of GVHD remains to be elucidated, IL-6 is implicated in GVHD progression in allogeneic mouse models (Koenecke *et al.*, 2009, Tawara *et al.*, 2011). In the current study, the increased serum hIL-6 concentrations were unexpected, as previously ATL-146e reduced serum IL-6 concentrations in allogeneic mouse models of GVHD (Lappas *et al.*, 2010, Han *et al.*, 2013). Moreover, CGS 21680 reduces IL-6 release from *ex vivo* anti-CD3/anti-CD28-stimulated murine effector T cells (Romio *et al.*, 2011). Conversely, increased serum IL-6 concentrations correlate to disease severity as a result of A_{2A} blockade in an allogeneic mouse model of GVHD (Wang *et al.*, 2013). The reason for increased serum hIL-6 concentrations in CGS 21680-injected humanised mice remains unknown but supports the concept that A_{2A} activation with this agonist has adverse roles in this model.

In the current study, the frequency of Treg cells was also reduced in CGS 21680-injected humanised mice. This further supports the concept that A_{2A} activation with this agonist has detrimental roles in humanised mice. Treg cells inversely correlate to GVHD progression in allogeneic (Edinger *et al.*, 2003) and humanised mouse models (Cao *et al.*, 2009), as well as

in HSCT recipients (Rieger *et al.*, 2006, Fondi *et al.*, 2009). The observed reduction in Treg cells was contrary to expectations, as in allogeneic mouse models ATL-146e increases Treg cells to ameliorate disease development (Lappas *et al.*, 2010, Han *et al.*, 2013). The mechanism by which CGS 21680 decreases Treg cells in the current study remains to be established. However, CGS 21680 reduced, albeit not significantly, serum hIL-2 and hIL-10 in humanised mice. These cytokines are important for maintenance of Treg cells in humanised mice (Abraham *et al.*, 2012, Abraham *et al.*, 2017). Thus a decrease in one or both of these cytokines may have contributed to the lower frequency of Treg cells in CGS 21680-injected mice. Alternatively, although not mutually exclusive to the above, IL-6 with IL-1 β reduces Treg cell numbers through their conversion to Th1 and/or Th17 cells (Gu *et al.*, 2016b). Thus, the increased IL-6 in CGS 21680-injected mice may have contributed to the reduction in Treg cells. However, it should be noted that the amount of hIL-1 β mRNA (Chapter 3) and serum hIL-1 β (Abraham *et al.*, 2017) in humanised mice is negligible, thereby potentially limiting the ability of hIL-6 to convert Treg cells to Th cells in this model.

Concerning the above comparisons between CGS 21680 and ATL-146e in GVHD, it should be noted that these compounds differ pharmacologically, which may explain their differing effects in this disease. ATL-146e has two-fold greater affinity than CGS 21680 for human A_{2A} (K_i of 45 \pm 15 nM and 82 \pm 18 nM, respectively) (Sullivan *et al.*, 2001) and a three-fold greater affinity for murine A_{2A} (4 \pm 1.9 nM and 13 \pm 3.5 nM, respectively) (Lappas *et al.*, 2005). Thus, these differences may be contributing to the varying effects of these two compounds in GVHD progression. As discussed above, ATL-146e could not be used in humanised NSG mice due to the lack of commercial availability.

The current study also demonstrated that CGS 21680 prevented healthy weight gain in NSG mice not engrafted with hPBMCs. This suggests that this A_{2A} agonist may be suppressing appetite or metabolism in these mice, which may also be contributing to the increased weight

loss in hPBMC-engrafted NSG mice in addition to GVHD. In rats, CGS 21680, at the same dose used in the current study, is sufficient to prevent weight gain by reducing food intake (Mingote *et al.*, 2008, Micioni Di Bonaventura *et al.*, 2012). Moreover, CGS 21680 at this same dose increases energy expenditure in mice to prevent diet-induced obesity (Gnad *et al.*, 2014) and causes hypothermia (Carlin *et al.*, 2018). The reasons for the different effects of CGS 21680 on mouse weight in these and the current studies compared to those discussed above (Impellizzeri *et al.*, 2011, da Rocha Lapa *et al.*, 2012, Mazzon *et al.*, 2011) remains unknown, but may reflect strain differences. CGS 21680-mediated effects on metabolism and weight were observed in C57BL/6 mice (Gnad *et al.*, 2014, Carlin *et al.*, 2018) and NSG mice (this study), whilst CGS 21680 was reported to have no such effects in CD1, Swiss and DBA/1 mice (Impellizzeri *et al.*, 2011, da Rocha Lapa *et al.*, 2012, Mazzon *et al.*, 2011). Finally, it should be noted that IL-6 can potentially impact mouse body weight, as genetic deficiency of IL-6 promotes obesity (Wallenius *et al.*, 2002). Thus, the increased weight loss observed in CGS 21680-injected humanised mice may be mediated via an IL-6-dependent mechanism.

In summary, the A_{2A} agonist CGS 21680 has opposing effects in a humanised mouse model of GVHD. Moreover, CGS 21680 can prevent weight gain in healthy NSG mice. As discussed above, CGS 21680 does not alter weight in all mouse strains, whilst adverse effects on weight have not been reported for the A_{2A} agonist ATL-146e. Therefore, the therapeutic efficacy of A_{2A} activation with certain agonists in HSCT recipients or a subset of these patients may be limited by adverse effects on weight as well as decreased Treg cell frequency and increased IL-6 production.

6.7. Acknowledgements

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6.8. Disclosures

All authors declare that they have no disclosures.

6.9. Author Contributions

N.J.G., D.W. and R.S. designed the experiments. N.J.G. and S. R. A. performed the experiments. N. J. G. analysed the data, prepared the figures and wrote the manuscript. S. R. A. co-edited the manuscript. D.W. and R.S. supervised the project, reviewed the data and edited the manuscript.

Chapter 7: The P2X7 Receptor is Not Essential for Development of Imiquimod-Induced Psoriasis-Like Inflammation in Mice

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7.1. Statement of Contribution of Authors

I, as one of the authors of this original article, agree with the statement of author contributions stated at the end of this chapter, and originally published in the original journal article.



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7.2. Abstract

Psoriasis is a chronic inflammatory skin disorder, characterised by epidermal hyperplasia (acanthosis) and leukocyte infiltration of the skin. Current therapies are inadequate, highlighting the need for new therapeutic targets. The P2X7 receptor is implicated in the pathogenesis of psoriasis. This study investigated the role of P2X7 in imiquimod (IMQ)-induced psoriasis-like inflammation. Topically-applied IMQ caused two-fold greater ear swelling in BALB/c mice compared to C57BL/6 mice, which encode a partial loss-of-function missense mutation in the *P2RX7* gene. However, there was no difference in histological skin pathology (acanthosis and leukocyte infiltration) between the two strains. IMQ treatment up-regulated P2X7 expression in skin from both mouse strains. Additionally, IMQ induced ATP release from cultured human keratinocytes, a process independent of cell death. Injection of the P2X7 antagonist Brilliant Blue G (BBG) but not A-804598 partly reduced ear swelling compared to vehicle-injected control mice. Neither antagonist altered skin pathology. Moreover, no difference in ear swelling or skin pathology was observed between C57BL/6 and P2X7 knock-out (KO) mice. Flow cytometric analysis of IMQ-treated skin from C57BL/6 and P2X7 KO mice demonstrated similar leukocyte infiltration, including neutrophils, macrophages and T cells. In conclusion, this study demonstrates that P2X7 is not essential for development of IMQ-induced psoriasis-like inflammation, but does not exclude a role for this receptor in psoriasis development in humans or other mouse models of this disease.

7.3. Introduction

Psoriasis affects 1-3% of the general population of Western countries, reducing quality of life and posing a significant economic burden (Lebwohl *et al.*, 2014). In the USA alone, psoriasis is estimated to cost \$112 billion per year (Brezinski *et al.*, 2015). Inflammation and hyper-

proliferation of keratinocytes in psoriasis results in epidermal hyperplasia (acanthosis) and plaque- or pustular-like skin lesions (Nestle *et al.*, 2009b). This characteristic inflammation is driven by infiltrating leukocytes, including neutrophils, macrophages and T cells (Nestle *et al.*, 2009a). These leukocytes exert their effects by release of pro-inflammatory cytokines such as interferon gamma (IFN- γ) (Nestle *et al.*, 2009a, Nestle *et al.*, 2009b), which promotes the hyper-proliferation of keratinocytes, with the resulting cell damage exacerbating inflammation and leading to plaque formation (Nestle *et al.*, 2009a).

The imiquimod (IMQ)-induced psoriasis-like inflammation mouse model is a valuable model for studying psoriasis pathogenesis. This model involves application of AldaraTM cream, containing IMQ, which causes epidermal hyperplasia and immune infiltration similar to psoriasis in humans (van der Fits *et al.*, 2009). IMQ treatment results in scaling and thickening of skin as well as infiltration of immune cells 48-72 hours after first application (van der Fits *et al.*, 2009). This model has been well characterised (Flutter and Nestle, 2013) and used in some 200 different studies to date (Hawkes *et al.*, 2017).

The ATP-gated P2X7 receptor channel has been implicated in a number of skin diseases including psoriasis (Geraghty *et al.*, 2016). P2X7 is present on keratinocytes (Greig *et al.*, 2003) and immune cells important in psoriasis including neutrophils (Karmakar *et al.*, 2016), macrophages (De Torre-Minguela *et al.*, 2016) and T cells (MacLeod *et al.*, 2014), where its activation can drive cytokine release and potentially promote inflammation (Geraghty *et al.*, 2016). Notably, IFN- γ can up-regulate the expression of P2X7 in primary keratinocytes (Pastore *et al.*, 2007) and P2X7 expression is increased in human psoriatic lesions (Pastore *et al.*, 2007, Killeen *et al.*, 2013). A functional role for P2X7 in psoriasis has also been suggested. Injection of the P2X7 agonist 3'-O-(4-benzoyl)benzoyl ATP into normal human skin explants induces increased expression of cytokines and other molecules commonly associated with psoriasis, including interleukin (IL)-1 β , IL-6 and tumour necrosis factor alpha

(TNF- α) (Killeen *et al.*, 2013). Importantly, these responses could be prevented through pharmacological blockade of P2X7 (Killeen *et al.*, 2013). P2X7 activation of skin migrating dendritic cells also promoted Th17 responses (Killeen *et al.*, 2013), a T cell subtype known to contribute to psoriasis pathogenesis (Zheng *et al.*, 2007).

This study investigated the role of P2X7 in the pathogenesis of psoriasis using a murine model of IMQ-induced psoriasis-like inflammation. Results indicate that P2X7 is up-regulated in psoriatic skin in both BALB/c and C57BL/6 mice, which encode the wild-type (P451) or partial loss-of function (451L) allele, respectively (Adriouch *et al.*, 2002). IMQ also induced ATP release from human keratinocytes. However, pharmacological blockade or genetic deletion of P2X7 showed a limited role for P2X7 in this murine model of psoriasis.

7.4. Materials and Methods

7.4.1. Cells

Human HaCaT keratinocytes and murine J774 macrophages were obtained and cultured as described (Farrell *et al.*, 2010, Sluyter and Vine, 2016). Cell lines were checked for *Mycoplasma spp.* infections every two months, using a MycoAlertTM Mycoplasma detection kit (Lonza, Basal, Switzerland) as per the manufacturer's instructions. Cells were routinely negative for *Mycoplasma spp.*

7.4.2. Mice

Female and male mice (aged 6-10 weeks) were used in experiments conducted under protocol AE14/10, which was approved by the University of Wollongong Animal Ethics Committee (Wollongong, Australia). BALB/c and C57BL/6 mice were obtained from Australian BioResources (Moss Vale, Australia). P2X7 knock-out (KO) mice (Solle *et al.*, 2001) backcrossed onto a C57BL/6 background (Tran *et al.*, 2010), were maintained at the

University of Wollongong and genotyped as described (Solle *et al.*, 2001). All mice were housed in open top cages and provided with food and water, *ad libitum*. All mice were housed in a temperature-controlled environment with a 12 h light/12 h dark cycle.

7.4.3. Murine Model of IMQ-Induced Psoriasis-Like Inflammation

A total of 25 mg of Aldara™ cream (1.25 mg IMQ) (iNova Pharmaceuticals, Thornleigh, Australia) was applied to both sides of one ear of each mouse and the contralateral ear remained untreated (control) (days 0-5) as described (Riol-Blanco *et al.*, 2014). Prior to treatment mice were weighed and ear measurements were recorded using Interapid (Rolle, Switzerland) spring-loaded calipers (days 0-6). In some experiments, Brilliant Blue G (BBG) or A-804598 (both Sigma-Aldrich, St Louis, MO, USA) (final concentration 50 mg/kg in mice), and their respective diluent controls (saline or dimethyl sulfoxide (DMSO), respectively), were injected every second day (as indicated). Mice were sacrificed on day 6 and ears collected.

7.4.4. Histological Analysis

Ears from euthanised mice were incubated overnight in neutral buffered (10%) formalin (Sigma-Aldrich). Fixed tissues were removed, embedded in paraffin, sectioned (5 µm) and stained with haematoxylin and eosin (POCD, Artarmon, Australia). Histology was observed using a Leica (Wetzlar, Germany) DM500 inverted light microscope, with images captured and processed using Leica application suite software version 4.7. Acanthosis (epidermal thickness) was measured using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA).

7.4.5. Isolation of RNA and cDNA Synthesis

Ears from euthanised mice were stored in RNAlater (Sigma-Aldrich) at -20°C until required. RNA was isolated using the ISOLATE II RNA Mini Kit (Bioline, London, UK) as per the manufacturer's instructions. Briefly, tissue in lysis buffer containing 1% (v/v) β-

mercaptoethanol (Sigma-Aldrich) was homogenised (two 20 second cycles at 5500 rpm) in a Bertin Technologies (Montigny-le-Bretonneux, France) Precellys 24 tissue homogeniser. Isolated RNA was immediately converted to cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) as per the manufacturer's instructions. cDNA was checked by PCR amplification of the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (Invitrogen, Carlsbad, CA, USA) for 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and a holding temperature of 4°C. Purity and size of amplicons were confirmed by a 2% agarose gel electrophoresis.

7.4.6. Quantitative Real-Time Polymerase Chain Reaction

qPCR reactions were performed using TaqMan Universal Master Mix II (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions with primers for FAM-labelled GAPDH (Mm99999915_g1) and VIC-labelled *P2RX7* (Mm01199503_m1) (Thermo Fisher Scientific). qPCR cycles consisted of an initial step of 50°C for 2 min, followed by 50°C for 10 min, and 40 cycles at 95°C for 15 s, and 60°C for 1 min. qPCR reactions were conducted in triplicate on a Roche Diagnostics (Indianapolis, IN, USA) LightCycler 480 and analysis was conducted using LightCycler480 software version 1.5.1.

7.4.7. ATP Release Assay

ATP release assays were performed as described (Mizumoto *et al.*, 2003). Briefly, HaCaT keratinocytes were plated in 12-well plates (Greiner Bio-One, Frickenhausen, Germany) at a concentration of 2×10^5 cells/well in RPMI 1640 medium containing 2 mM L-glutamine, 1% non-essential amino acids (all Thermo Fisher Scientific), and 10% (v/v) heat-inactivated foetal bovine serum (Bovogen Biologicals, Keller East, Australia), and incubated for 24 h at 37°C/5% CO₂. Cells were washed thrice with PBS (Thermo Fisher Scientific), and incubated in 1 mL PBS containing DMSO or 1 µg/mL IMQ (Sigma-Aldrich) in DMSO for 30 min at

37°C/5% CO₂. The final concentration of DMSO in both groups was 0.1%. Cells were then centrifuged through 10% bovine serum albumin (4,000 x g for 3 min) and cell free supernatants were stored in duplicate at -20°C until required. ATP concentrations in supernatants were assessed using a Bioluminescence ATP Assay Kit (Sigma-Aldrich) as described (Mansfield and Hughes, 2014). The amount of lactate dehydrogenase (LDH) in supernatants was determined using a Cytotoxicity Detection Kit (LDH) (Roche Diagnostics) as per the manufacturer's instructions using a Molecular devices (Sunnyvale, CA, USA) Spectramax Plus 384 plate reader.

7.4.8. ATP-Induced Ethidium⁺ Uptake Assay

P2X7 pore formation was quantified by measuring ATP-induced ethidium⁺ uptake as described (Bartlett *et al.*, 2013). Briefly, J774 cells in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose and 10 mM HEPES, pH 7.5) were pre-incubated in the absence or presence of BBG or A-804598 (as indicated) for 15 min at 37°C. Cells were then incubated with 25 µM ethidium bromide (Sigma-Aldrich) in the absence or presence of 1 mM ATP (Sigma-Aldrich) for 10 min. Incubations were stopped by addition of ice-cold NaCl medium containing 20 mM MgCl₂ (MgCl₂ medium) and cells were washed with NaCl medium (300 x g for 3 min). Data was collected using a BD Biosciences (San Jose, CA, USA) LSRFortessa X-20 flow cytometer (using band-pass filter 530/30 for ethidium⁺) and FACSDiva software version 8.0. Mean fluorescence intensity (MFI) of ethidium⁺ uptake was analysed using FlowJo software version 8.7.1. (TreeStar Inc., Ashland, OH, USA).

7.4.9. Immunophenotyping

CD16/32 Fc Block (clone 2-4G2), Brilliant Violet (BV)450-conjugated rat anti-mouse Ly-6G (clone 1A8), peridinin chlorophyll protein (PerCP)-conjugated rat anti-mouse CD4 (clone RM4.5), allophycocyanin (APCy)-conjugated rat anti-mouse CD45 (clone 30-F11) and R-phycoerythrin (PE)-Cy7 conjugated hamster anti-mouse CD3e (clone 145-2C11) were from

BD Biosciences. PE-conjugated rat anti-mouse CD11b (clone M1/70) was from BioLegend (San Diego, CA, USA).

Enzymatic digestion of ears was performed as described (Rana *et al.*, 2008). Briefly, dorsal and ventral sides of ears were separated and incubated in 20 mM EDTA (Sigma-Aldrich) in Tris-buffered saline (pH 7.3) for 2 hours at 37°C. Epidermal and dermal layers were mechanically separated, cut into smaller fragments using a scalpel and homogenised in RPMI 1640 medium containing 1 mg/mL collagenase IV and 15 U/mL DNase I (both Sigma-Aldrich), and 5% heat-inactivated foetal bovine serum at room temperature for 90 min with agitation. Enzymatic digestion was stopped by addition of 100 mM EDTA and suspensions filtered through 70 µm cell strainers (Falcon, Franklin, NJ, USA). Cells were centrifuged (300 x g for 5 min), resuspended in PBS and counted manually using a Boeco (Hamburg, Germany) Neubauer Improved Bright Line haemocytometer. Cells were incubated with CD16/32 Fc Block for 10 min and then with fluorochrome-conjugated monoclonal antibodies in the dark for 10 min. Cells were washed twice with PBS (300 x g for 3 min), resuspended in PBS and data was collected using a LSRFortessa X-20 flow cytometer (using band pass filters 450/50 for BV421, 695/40-A for PerCP, 575/25-A for PE, 780/60 for PE-Cy7 and 670/30 for APCy) and FACSDiva software. The relative percentage of cells was analysed using FlowJo software.

7.4.10. Statistical Analysis

Data is given as mean \pm standard error of the mean (SEM). Statistical differences were calculated using Student's t test for single comparisons or one-way analysis of variance (ANOVA) with Tukeys post-hoc test for multiple comparisons. Weight and ear measurements were analysed using a repeated measures two-way ANOVA. All statistical analyses and graphs were generated using Prism 5 for Windows software (GraphPad Software, La Jolla, CA, USA).

7.5. Results

7.5.1. IMQ Treatment Induces Greater Ear Swelling in BALB/c Mice Compared to C57BL/6 Mice

To investigate the role of P2X7 in psoriasis, IMQ-induced psoriasis-like inflammation was first compared over 6 days in BALB/c and C57BL/6 mice. C57BL/6 mice encode a loss-of-function mutation (P451L) in the *P2RX7* gene, while BALB/c mice are wild-type at this allele (Adriouch *et al.*, 2002). IMQ induced ear swelling in ears relative to control (contralateral untreated) ears from day 3 in both strains (Figure 7.1.a.). However, IMQ-induced ear swelling was greater in BALB/c mice compared to C57BL/6 mice over 6 days ($P < 0.0001$) (Figure 1a). Histological analysis of IMQ-treated ears from BALB/c and C57BL/6 mice demonstrated acanthosis (epidermal thickening) and leukocyte infiltration compared to control ears; however, there was no difference between strains (Figure 7.1.b.). Image analysis of histological sections revealed that epidermal thickness was significantly greater in IMQ-treated ears compared to control ears from both BALB/c mice ($P < 0.0001$) and C57BL/6 mice ($P < 0.0001$). However, IMQ-induced epidermal thickening did not significantly differ between the two strains (Figure 7.1.c.).

7.5.2. P2X7 mRNA is Up-Regulated in IMQ-Induced Psoriatic Lesions and IMQ Induces ATP Release From Keratinocytes

Next, P2X7 mRNA expression in IMQ-treated and control ears was analysed by qPCR. P2X7 was significantly up-regulated almost three-fold and two-fold in IMQ-treated ears compared to control ears from BALB/c mice ($P = 0.0002$) and C57BL/6 mice ($P = 0.0343$), respectively (Figure 7.2.a.). To further determine a potential role for P2X7 activation in IMQ-induced psoriasis-like inflammation, HaCaT keratinocytes were incubated in the presence of 1 $\mu\text{g/mL}$ IMQ or DMSO (vehicle control) for 30 min and the amount of ATP released measured using a bioluminescence assay. IMQ induced significantly greater amounts of ATP

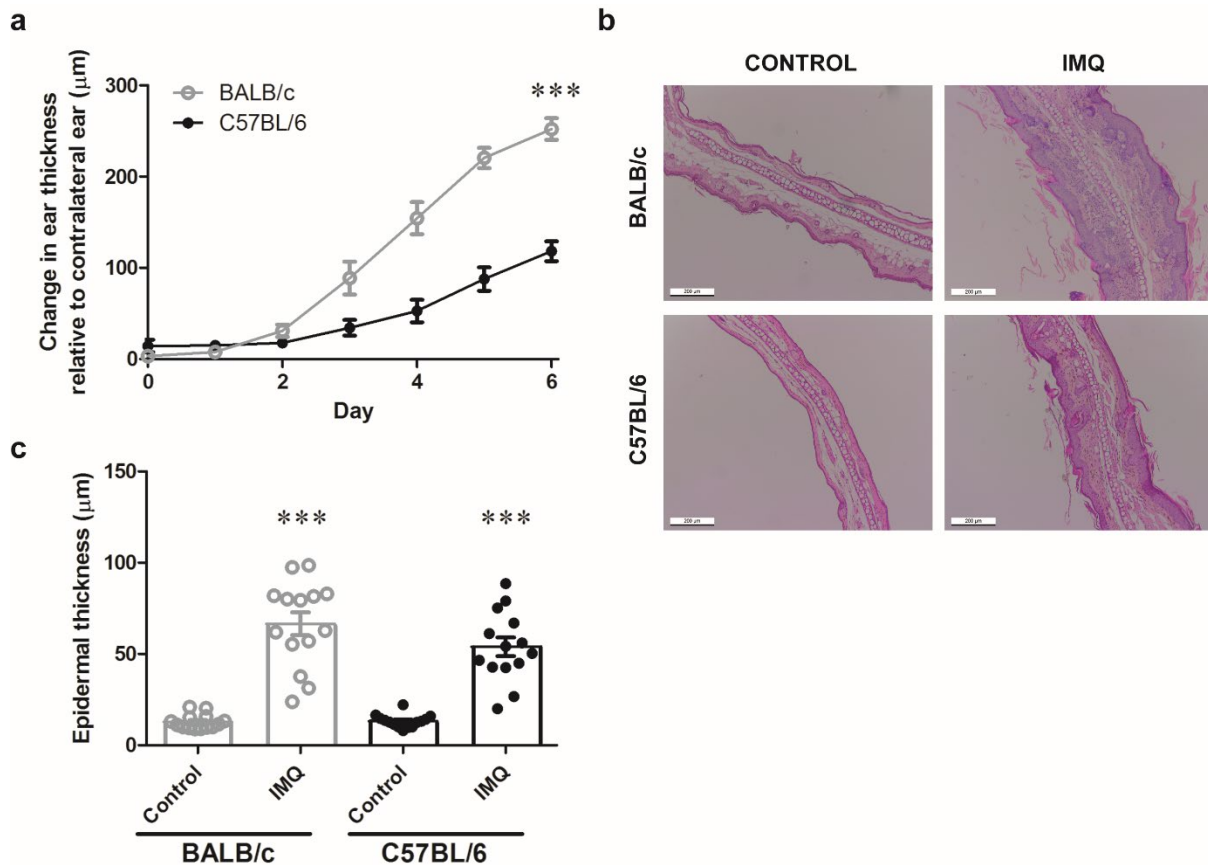


Figure 7.1. IMQ treatment induces greater ear swelling in BALB/c mice compared to C57BL/6 mice. (a-b) AldaraTM cream (containing 5% IMQ) was applied to one ear of BALB/c and C57BL/6 mice from day 0 to 5. Contralateral ears were left untreated (control). (a) Ear swelling was measured over 6 days. Data represents group means \pm SEM ($n = 14$ per strain). *** $P < 0.0001$ compared to C57BL/6. (b) Ears from mice at end-point were stained with haematoxylin and eosin, and viewed by microscopy. Each image is representative of 14 mice per group. Bars represent 200 μm . (c) Epidermal thickness was measured on histological images using ImageJ. Data represents group means \pm SEM ($n = 14$ mice per group); symbols represent individual ears; *** $P < 0.0001$ compared to corresponding control.

release compared to DMSO ($P = 0.0425$) (Figure 7.2.b). To examine if this ATP release was due to cytotoxicity by IMQ, the relative amount of LDH in the above supernatants was determined using a spectrophotometric assay. Although the absorbance in the IMQ cells was reduced the absorbance values were not significantly different between IMQ and control groups (Figure 7.2.c).

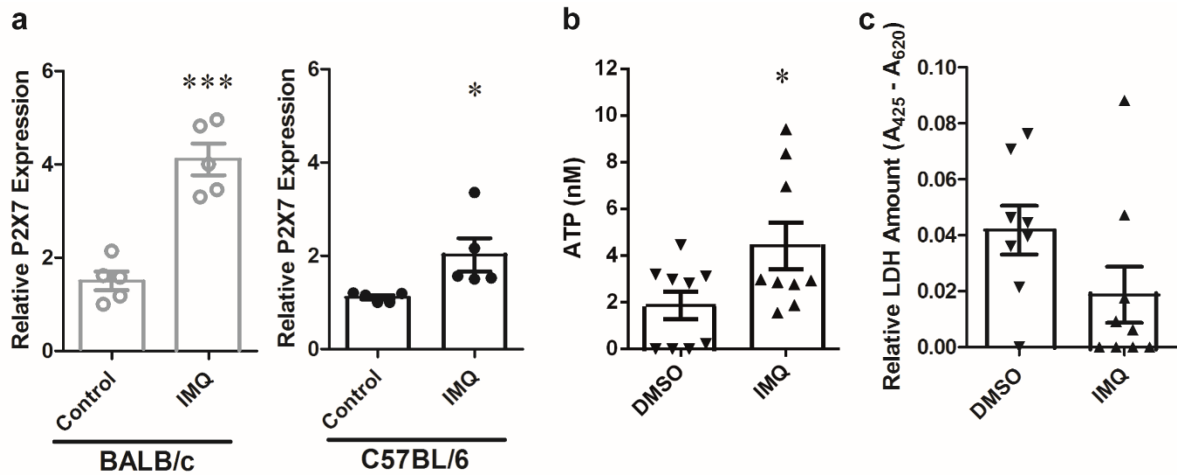


Figure 7.2. P2X7 is up-regulated in IMQ-treated skin and IMQ can induce ATP release from keratinocytes. (a) P2X7 expression in untreated (control) and IMQ-treated ears from BALB/c and C57BL/6 mice at end-point (Figure 1) was assessed by qPCR. Data represents group means \pm SEM ($n = 5$ mice per strain); symbols represent individual ears; * $P < 0.05$, *** $P < 0.0001$ compared to corresponding control. (b and c) Human HaCaT keratinocytes were incubated in DMSO or 1 μ g/mL IMQ for 30 min and (b) ATP release assessed using a bioluminescence assay, and (c) LDH release was assessed using a spectrophotometric assay. Data represents group means \pm SEM ($n = 9$ from three individual experiments); * $P < 0.05$ compared to DMSO.

7.5.3. BBG Does Not Prevent IMQ-Induced Psoriasis-Like Inflammation

To investigate the role of P2X7 in IMQ-induced psoriasis-like inflammation, the P2X7 antagonist BBG (Jiang *et al.*, 2000) was used in this model. First, to confirm that BBG can impair murine P2X7 activation, J774 macrophages, which express functional P2X7 (Coutinho-Silva *et al.*, 2005), were pre-incubated in the absence or presence of 10 μ M BBG and ATP-induced cation dye uptake was measured by flow cytometry. BBG significantly reduced ATP-induced ethidium⁺ uptake by 87% ($P < 0.0001$) (Figure 7.3.a). To determine if BBG could prevent the development of IMQ-induced psoriasis-like inflammation BALB/c mice were treated with IMQ as above and injected with BBG or saline (vehicle control) every second day (days 0, 2 and 4). IMQ induced ear swelling in mice from day 3 regardless of treatment. However, IMQ-induced ear swelling was partially reduced (by 21%) in BBG-injected mice compared to saline-injected mice over 6 days ($P = 0.0068$) (Figure 7.3.b).

Histological analysis of IMQ-treated ears from BBG- and saline-injected mice demonstrated epidermal thickening and leukocyte infiltration compared to control ears; but there were no differences between BBG and saline treatments (Figure 7.3.c.). Image analysis of histological sections revealed that epidermal thickness was greater in IMQ-treated ears compared to control ears from BBG-injected mice ($P < 0.0001$) and saline-injected mice ($P < 0.0001$). Epidermal thickness in IMQ-treated and control ears was similar between BBG and saline treatments (Figure 7.3.d.).

7.5.4. A-804598 Does Not Prevent IMQ-Induced Psoriasis-Like Inflammation

To further examine the potential effects of P2X7 blockade on psoriasis-like inflammation, the P2X7 antagonist A-804598 (Donnelly-Roberts *et al.*, 2009b) was used. Similar to BBG, A-804598 near-completely abrogated ATP-induced ethidium⁺ uptake in J774 macrophages ($P < 0.0001$) (Figure 7.4.a.). BALB/c mice were treated with IMQ as above and injected with A-804598 or DMSO (vehicle control) every second day (days 0 and 2). IMQ induced ear swelling in mice from day 3 regardless of treatment. A-804598- and DMSO-injected mice demonstrated similar ear swelling (Figure 7.4.b.). Histological analysis of IMQ-treated ears from A-804598-injected and DMSO-injected mice demonstrated epidermal thickening and leukocyte infiltration compared to control ears with no difference between A-804598 and control treatments (Figure 7.4.c.). Image analysis of histological sections revealed that epidermal thickness was greater in IMQ-treated ears compared to control ears from A-804598-injected mice ($P < 0.0001$) and DMSO-injected mice ($P < 0.0001$). Epidermal thickness in IMQ-treated and control ears was also similar between A-804598 and control treatments (Figure 7.4.d.).

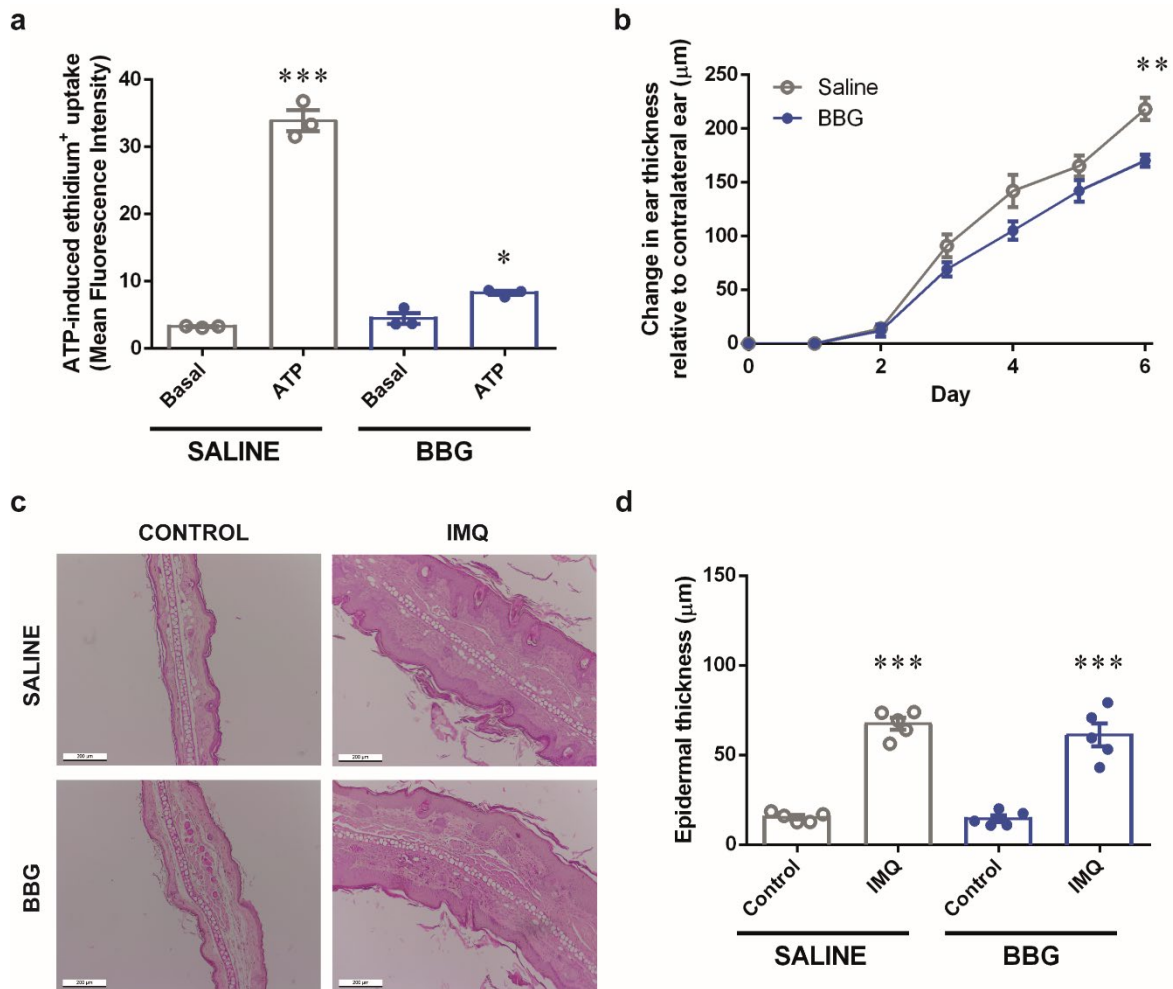


Figure 7.3. BBG does not prevent IMQ-induced psoriasis-like inflammation. (a) Murine J774 macrophages were incubated for 15 min at 37°C in the absence or presence of 10 μM BBG, and then with 25 μM ethidium bromide in the absence or presence of 1 mM ATP for 10 min at 37°C. Assays were stopped by addition of MgCl₂ medium, and ethidium⁺ uptake was then assessed by flow cytometry. Data represents group means ± SEM (*n* = 3); * *P* < 0.05, *** *P* < 0.0001 compared to corresponding basal. (b-d) AldaraTM cream (containing 5% IMQ) was applied to one ear of BALB/c mice from day 0 to 5. Contralateral ears were left untreated (control). Mice were injected i.p. with BBG or saline (control) on days 0, 2, and 4. (b) Ear swelling was measured over 6 days. Data represents group means ± SEM (*n* = 5 mice per treatment); ** *P* < 0.005 compared to BBG. (c) Ears from mice at end-point were stained with haematoxylin and eosin, and viewed by microscopy. Each image is representative of 5 mice per treatment. Bars represent 200 μm. (d) Epidermal thickness was measured on histological images using ImageJ. Data represents group means ± SEM (*n* = 5 mice per treatment); symbols represent individual ears; *** *P* < 0.0001 compared to corresponding control.

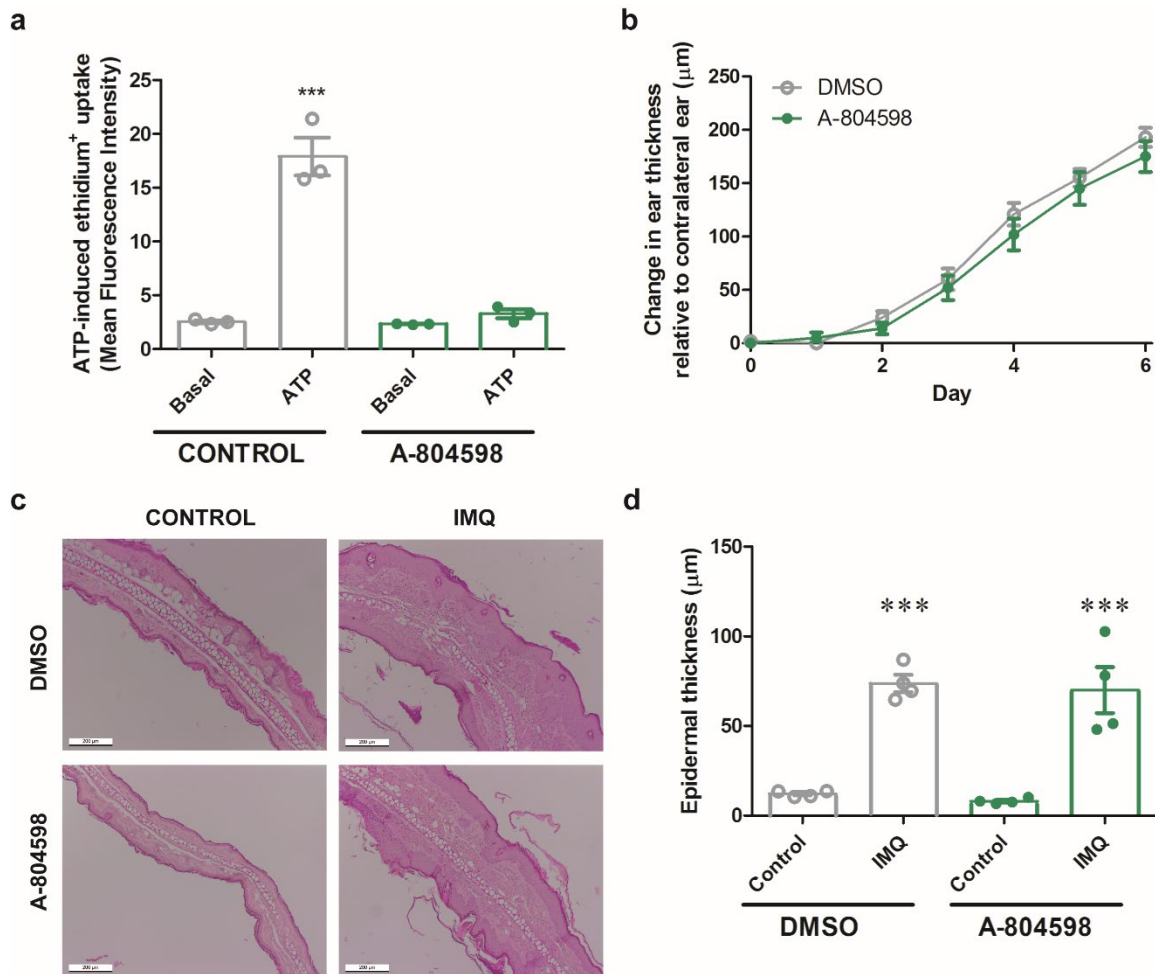


Figure 7.4. A-804598 does not prevent IMQ-induced psoriasis-like inflammation. (a) Murine J774 macrophages were incubated for 15 min at 37°C in the absence or presence of 1 μM A-804598, and then with 25 μM ethidium bromide in the absence or presence of 1 mM ATP for 10 min at 37°C. Assays were stopped by addition of MgCl₂ medium, and ethidium⁺ uptake was then assessed by flow cytometry. Data represents group means ± SEM (*n* = 3); *** *P* < 0.0001 compared to corresponding basal. (b-d) AldaraTM cream (containing 5% IMQ) was applied to one ear of BALB/c mice from day 0 to 5. Contralateral ears were left untreated (control). Mice were injected i.p. with A-804598 or DMSO (control) on days 0 and 2. (b) Ear swelling was measured over 6 days. Data represents group means ± SEM (*n* = 5 mice per treatment). (c) Ears from mice at end-point were stained with haematoxylin and eosin, and viewed by microscopy. Each image is representative of 5 mice per treatment. Bars represent 200 μm. (d) Epidermal thickness was measured on histological images using ImageJ. Data represents group means ± SEM (*n* = 5 mice per treatment); symbols represent individual ears; *** *P* < 0.0001 compared to corresponding control.

7.5.5. Genetic Deletion of P2X7 Does Not Prevent IMQ-Induced Psoriasis-Like Inflammation

Finally, to determine if genetic deficiency of P2X7 could alter IMQ-induced psoriasis-like inflammation, C57BL/6 and P2X7 KO mice were treated with IMQ as above. Both C57BL/6 and P2X7 KO mice demonstrated ear swelling in IMQ-treated ears from day 3, but this was not significantly different between the two strains over 6 days (Figure 7.5.a.). Histological analysis of IMQ-treated ears from C57BL/6 and P2X7 KO mice demonstrated epidermal thickening and leukocyte infiltration compared to control ears, with no difference between strains (Figure 7.5.b.). Image analysis of histology revealed that epidermal thickness was significantly greater in IMQ-treated ears compared to control ears from C57BL/6 mice ($P < 0.0001$) and P2X7 KO mice ($P < 0.0001$). Epidermal thickness in IMQ-treated and control ears was also similar between C57BL/6 and P2X7 KO mice (Figure 7.5.c.).

7.5.6. IMQ-Induced Leukocyte Infiltration is Similar in C57BL/6 and P2X7 KO Mice

The data above indicates that P2X7 deficiency does not prevent IMQ-induced psoriasis-like inflammation in mice. However, the possibility remained that P2X7 deficiency may have altered the number of leukocyte subsets infiltrating the skin in this disease model. Therefore, whole ear cell suspensions of IMQ-treated and control ears from C57BL/6 and P2X7 KO mice were examined by flow cytometry. IMQ induced a significant increase in total skin leukocytes in both C57BL/6 ($P = 0.0002$) and P2X7 KO ($P = 0.0029$) mice, but there was no significant difference between strains (Figure 7.6.a.). Subset analysis also revealed IMQ induced a significant increase in neutrophils and macrophages in both C57BL/6 ($P = 0.0027$ and $P < 0.0001$, respectively) and P2X7 KO ($P = 0.0020$ and $P = 0.0046$, respectively) mice, but there was no significant difference between strains (Figure 7.6.b-c.). IMQ induced a significant increase in T cells in C57BL/6 ($P = 0.0006$) but not P2X7 KO mice; yet there was no significant difference between strains (Figure 7.6.d.).

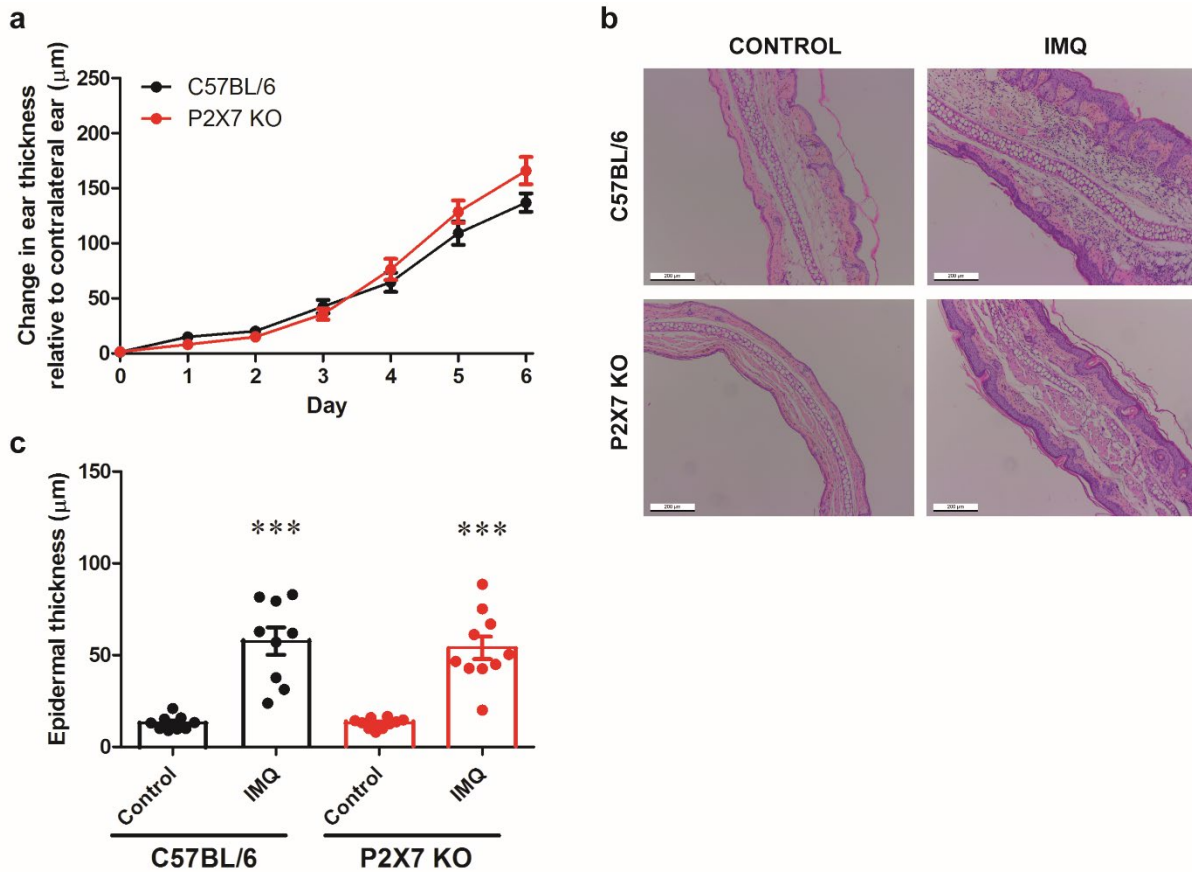


Figure 7.5. Genetic deletion of P2X7 does not prevent IMQ-induced psoriasis-like inflammation. (a-c) Aldara™ cream (containing 5% IMQ) was applied to ears of C57BL/6 and P2X7 KO mice from day 0 to 5. Contralateral ears were left untreated (control). (a) Ear swelling was measured over 6 days. Data represents group means ± SEM ($n = 10$ mice per strain). (b) Ears from mice at end-point were stained with haematoxylin and eosin, and viewed by microscopy. Each image is representative of 10 mice per group. Bars represent 200 μm. (c) Epidermal thickness was measured on histological images using ImageJ. Data represents group means ± SEM ($n = 10$ mice per strain); symbols represent individual ears; *** $P < 0.0001$ compared to corresponding control.

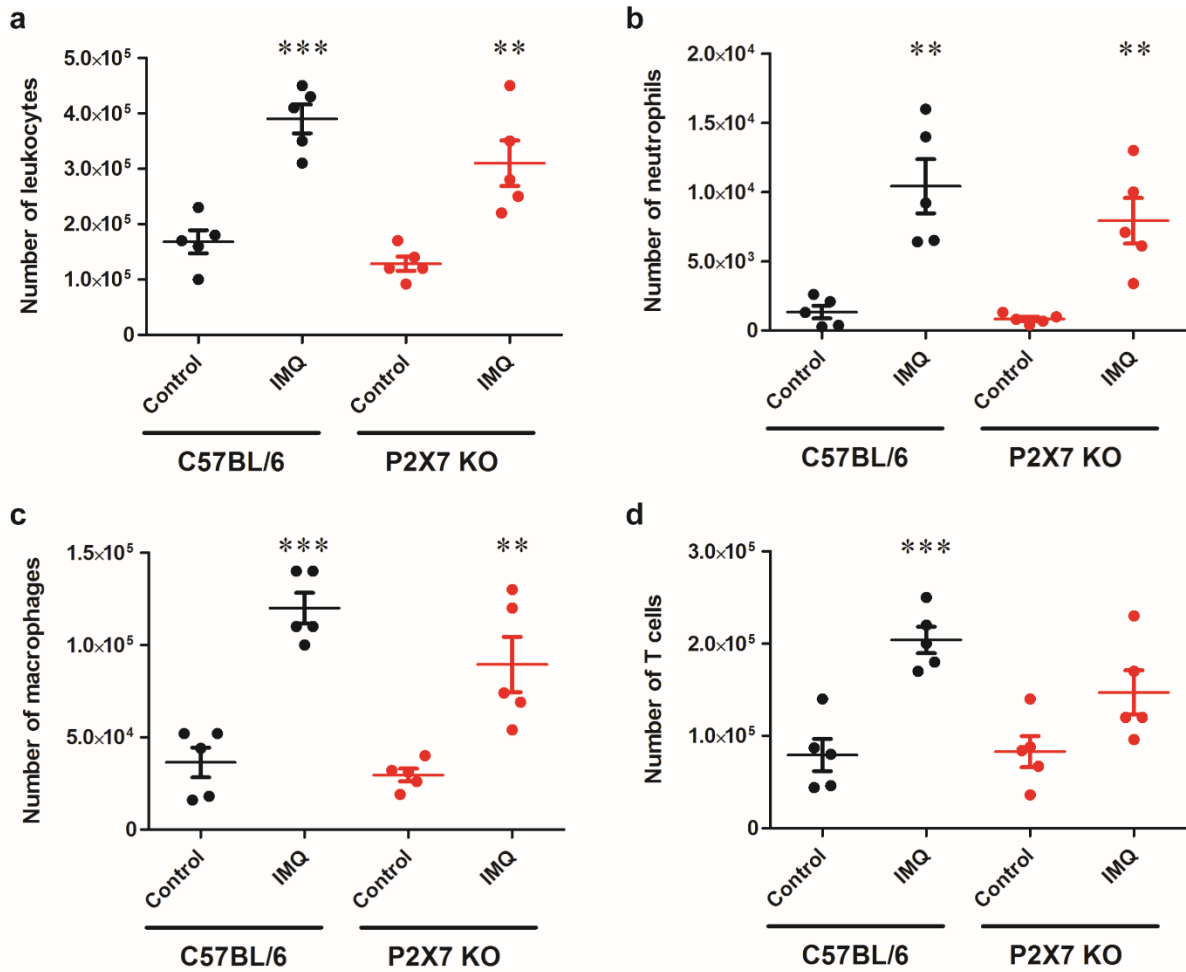


Figure 7.6. IMQ-induced leukocyte infiltration is similar in C57BL/6 and P2X7 KO mice.

(a-d) Single whole ear cell suspensions of ears from untreated (control) and IMQ-treated C57BL/6 and P2X7 KO mice at end-point (Figure 5) were labelled with fluorochrome-conjugated mAb and analysed by four-colour flow cytometry. Data represents total numbers of group means \pm SEM for (a) leukocytes ($CD45^+$), (b) neutrophils ($CD45^+ Ly6G^+ CD11b^+$) (c) macrophages ($CD45^+ Ly6G^- CD11b^+$), and (d) T cells ($CD45^+ CD3^+$) in each cell suspension ($n = 5$ mice per group); symbols represent individual ears; ** $P < 0.005$, *** $P < 0.0001$ compared to respective control ears.

7.6. Discussion

This study demonstrated that P2X7 is not essential for the development of IMQ-induced psoriasis-like inflammation in mice. With the exception of a partial effect on ear swelling by BBG, pharmacological blockade or genetic deletion of P2X7 did not impact development of IMQ-induced psoriasis-like inflammation. The general lack of effect of either P2X7 antagonist was not due to the preparations of BBG or A-804598, as both compounds blocked ATP-induced dye uptake into murine macrophages in this study. Moreover, both compounds were used *in vivo* at concentrations known to inhibit murine P2X7 (Bartlett *et al.*, 2014). In regards to IMQ-induced ear swelling, BBG may be blocking alternate molecules, such as P2X1 (Seyffert *et al.*, 2004), P2X5 (Bo *et al.*, 2003), the ATP channel pannexin-1 (Qiu and Dahl, 2009) or a voltage-gated sodium channel (Jo and Bean, 2011).

Genetic deficiency of P2X7 did not impact leukocyte infiltration in IMQ-induced psoriasis-like inflammation. In contrast, a previous study reported that P2X7 KO mice had reduced neutrophil infiltration into skin in a croton oil model of irritant contact dermatitis (Da Silva *et al.*, 2013). However, in line with our current study, ear swelling was similar between wild-type and P2X7 KO mice in croton oil-induced irritant contact dermatitis (Weber *et al.*, 2010, Da Silva *et al.*, 2013). Conversely, P2X7 KO mice are protected from allergic contact dermatitis, which was also assessed by ear swelling measurements (Weber *et al.*, 2010). This previous study demonstrated that P2X7-mediated IL-1 β release, which requires NLRP3 inflammasome activation (Di Virgilio, 2007), was essential for allergic contact dermatitis (Weber *et al.*, 2010). In contrast, activation of the NLRP3 inflammasome is not required for IMQ-induced psoriasis-like inflammation (Rabeony *et al.*, 2015). Thus, this provides a possible explanation as to why P2X7 is not essential in this model of psoriasis. Collectively, this indicates P2X7 plays differing roles in various inflammatory skin disorders, but given previous findings (Pastore *et al.*, 2007, Killeen *et al.*, 2013) further investigation of P2X7 in

human psoriasis and other mouse models of this disease is warranted. Moreover, it should be noted that C-terminal truncated P2X7 variants are present at low amounts in C57BL/6 and Pfizer P2X7 KO mice (Masin *et al.*, 2012), the same strains used in the current study. Although P2X7-induced pore formation is absent in splenic T and B cells (Pupovac *et al.*, 2015), epidermal Langerhans cells and keratinocytes (Tran *et al.*, 2010) from these P2X7 KO mice, a role for these escape P2X7 variants in IMQ-induced psoriasis-like inflammation in P2X7 KO mice cannot be excluded. P2X7 KO strains in which escape variants have not been reported, such as those from Lexicon Pharmaceuticals (Basso *et al.*, 2009) or conditional humanised P2X7 KO mice (Metzger *et al.*, 2016), provide alternatives to assess the potential role of P2X7 in psoriasis.

Ear swelling, but not histological acanthosis or inflammation, differed between BALB/c and C57BL/6 mice. Thus, this difference in ear swelling most likely reflects differences in oedema. Coincidentally, this difference in ear swelling corresponds to *P2RX7* genotype (P451L mutation), but P2X7 deficiency in mice on a C57BL/6 background does not alter ear swelling compared to wild-type C57BL/6 mice. This suggests that the difference between BALB/c and C57BL/6 mice is not due to reduced P2X7 activity as a result of the P451L mutation in C57BL/6 mice. In this regard, whilst some have reported that the P451L mutation results in a loss of P2X7 activity (Adriouch *et al.*, 2002, Young *et al.*, 2006), others have observed no differences in the activity or pharmacological profiles between recombinant BALB/c and C57BL/6 P2X7 (Donnelly-Roberts *et al.*, 2009a). Moreover, it remains to be established if P2X7 variants differ between BALB/c and C57BL/6 mice, and if P2X7 activity differs between these two mouse strains *in vivo*. BALB/c and C57BL/6 mice have a diverse lineage and display phenotypic and genetic differences (Simpson *et al.*, 1997, Beck *et al.*, 2000). Thus, differences in IMQ-induced ear swelling between these strains could be due to any number of genotypic or phenotypic differences. Notably, IL-22 expression is increased in

IMQ-induced psoriasis-like inflammation in BALB/c, but not C57BL/6, mice (van der Fits *et al.*, 2009). Conversely, IL-6 is detected in C57BL/6 mice, but not BALB/c mice (van der Fits *et al.*, 2009). Thus, a role for these cytokines in oedema during psoriasis warrants further investigation.

Analysis by qPCR revealed P2X7 mRNA expression was up-regulated in IMQ-treated skin in both BALB/c and C57BL/6 mice. Although, it was not determined if P2X7 protein is increased in these mice, P2X7 protein is increased in lesional skin from psoriasis patients (Pastore *et al.*, 2007, Killeen *et al.*, 2013). Notably, P2X7 is up-regulated on human keratinocytes by IFN- γ (Pastore *et al.*, 2007), a cytokine implicated in psoriasis pathogenesis (Johnson-Huang *et al.*, 2012) including IMQ-induced psoriasis-like inflammation (van der Fits *et al.*, 2009). Therefore, P2X7 may be up-regulated on keratinocytes due to IFN- γ present in IMQ-treated skin. Alternatively, since P2X7 is also found on various skin immune cells (Geraghty *et al.*, 2016), increased P2X7 expression in IMQ-treated ears may simply reflect increased numbers of infiltrating leukocytes in IMQ-induced skin as observed in the current study.

Finally, this study demonstrated that HaCaT keratinocytes can constitutively release ATP, as previously observed (Burrell *et al.*, 2005), and that this release could be increased by IMQ. The IMQ-induced ATP release was not due to cytotoxicity, as IMQ did not significantly alter LDH release. Although genetic deletion or pharmacological blockade of P2X7 did not impact IMQ-induced psoriasis-like inflammation in this study, extracellular ATP may be involved in the development of this disease through activation of purinergic receptors other than P2X7. In particular, P2Y1, P2Y2 and P2Y6 have been implicated in psoriasis or other inflammatory skin conditions (Burnstock *et al.*, 2012). Conversely, ATP can be degraded to adenosine by ecto-nucleotidases such as CD39, present on Langerhans cells in the skin (Mizumoto *et al.*, 2002), and CD73, present on dendritic cells (Airas and Jalkanen, 1996), to promote anti-

inflammatory effects through activation of adenosine receptors (Gessi *et al.*, 2014). Therefore, CD39 or CD73 may also play a role in the development of psoriasis-like inflammation in this model.

In conclusion, pharmacological blockade and genetic deletion of P2X7 could not prevent IMQ-induced psoriasis-like inflammation. However, given the potential roles of P2X7 in human psoriasis (Pastore *et al.*, 2007, Killeen *et al.*, 2013) and other skin diseases (Geraghty *et al.*, 2016), a role for this receptor in human psoriasis or other mouse models of this disease cannot be excluded.

7.7. Acknowledgements

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7.8. Conflicts of Interest

The authors wish to declare that there are no conflicts of interest.

7.9. Author Contributions

N.J.G., K. J. M., D.W. and R.S. designed the experiments. N.J.G. and K. J. M. performed the experiments. N. J. G. analysed the data, prepared the figures and wrote the manuscript. S. J. F. provided histological analysis. K. J. M. and S. J. F. co-edited the manuscript. D.W. and R.S. supervised the project, reviewed the data and edited the manuscript.

Chapter 8: General Discussion

8.1. Introduction

The purinergic signalling system is important in immunity, with P2X, P2Y and adenosine (P1) receptors found on a number of immune cell subtypes (Burnstock and Boeynaems, 2014). Extracellular adenosine triphosphate (ATP) can act as a danger associated molecular pattern (DAMP), and exert pro-inflammatory effects through P2X7 activation (Adinolfi *et al.*, 2017). ATP is broken down by ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP), and subsequently by ecto-5'-nucleotidase (CD73) to adenosine, which can activate adenosine receptors to exert anti-inflammatory effects (Antonioli *et al.*, 2013). Purinergic signalling has been studied in transplantation, including hematopoietic stem cell transplantation (HSCT) (Section 1.8.1). Moreover, this signalling pathway has been implicated in graft-versus-host disease (GVHD), a common complication of allogeneic HSCT. In particular, allogeneic mouse models of GVHD have implicated a pro-inflammatory role for the P2X7 receptor in this disease (Wilhelm *et al.*, 2010). Conversely, allogeneic mouse models of GVHD have supported an anti-inflammatory role of the CD73/A_{2A} receptor pathway in this disease (Lappas *et al.*, 2010, Tsukamoto *et al.*, 2012, Han *et al.*, 2013, Wang *et al.*, 2013). However, prior to this thesis the role of purinergic signalling had not been investigated in a humanised mouse model of GVHD.

8.2. Establishment and Characterisation of a Humanised Mouse Model of GVHD

In GVHD, humanised mouse models are valuable preclinical models to investigate cell signalling pathways and to identify potential therapeutics (Hogenes *et al.*, 2014). The most

commonly used model is the humanised non-obese diabetic (NOD)-severe combined immunodeficient (SCID)-interleukin (IL)-2 receptor γ common chain null (IL-2R γ^{null}) (NSG) mouse model, due to the superior engraftment of human leukocytes after injection of human (h) peripheral blood mononuclear cells (PBMCs) (King *et al.*, 2008). Thus, this model was chosen to investigate the role of purinergic signalling in GVHD (Chapters 3-6). However, prior to these studies, the humanised NSG mouse model of GVHD was established at the University of Wollongong (Wollongong, Australia) (Chapter 2). This initial study (Chapter 2) described the successful establishment of this model, with 77% of mice injected with hPBMCs (10 out of 13) developing clinical GVHD. All mice engrafted human leukocytes (hCD45⁺), with the majority of engrafted leukocytes identified as T cells (hCD3⁺), similar to previous studies (King *et al.*, 2009, Ali *et al.*, 2012). Furthermore, histological evidence of GVHD was similar to previous studies including leukocyte infiltration and inflammatory damage of target organs (King *et al.*, 2009). The current study (Chapter 2) also reported that 23% of mice injected with hPBMCs (3 out of 13) developed subclinical GVHD but did not progress to clinical GVHD. This afforded the opportunity to compare differences between humanised NSG mice with clinical or subclinical GVHD. Humanised NSG mice that develop clinical GVHD demonstrate increased splenic hCD4⁺:hCD8⁺ T cell ratios compared to mice with subclinical GVHD. This finding is consistent with human studies, where higher CD4⁺:CD8⁺ T cell ratios indicate greater disease severity (Huttunen *et al.*, 2015, Budde *et al.*, 2017). Serum hIFN- γ was also increased in humanised NSG mice with clinical GVHD compared to those with subclinical GVHD. Whilst reduced hIFN- γ correlates with reduced disease severity in allogeneic (Wilhelm *et al.*, 2010) and humanised mouse models (Gregoire-Gauthier *et al.*, 2012), this thesis is the first to highlight this cytokine as a potential biomarker in this humanised mouse model of GVHD.

Although histology of saline-injected and humanised NSG mice has been compared previously (King *et al.*, 2009, Choi *et al.*, 2011), this study (Chapter 2) also provided a comparison of histology of GVHD target organs between healthy (saline-injected) NSG mice, mice with subclinical GVHD and mice with clinical GVHD. Mice with subclinical GVHD demonstrated some human cell infiltration into spleens, liver, small intestines and skin. Conversely, histological damage and leukocyte infiltration were more evident in mice with clinical GVHD, with greater leukocyte infiltration and inflammatory damage to target organs, similar to previous studies (King *et al.*, 2008). The lesser amount of damage in mice with subclinical GVHD compared to clinical GVHD is similar to that observed in the liver (Vlad *et al.*, 2009, Abraham *et al.*, 2015, Ehx *et al.*, 2017, Burlion *et al.*, 2017), and small intestine (Vlad *et al.*, 2009, Nakauchi *et al.*, 2015) of humanised mice following various treatments to prevent GVHD.

Although our initial study (Chapter 2) provided insight into the humanised NSG mouse model of GVHD, further studies could investigate engraftment of human antigen presenting cells (APCs), including subtype analysis. Murine APC activation of human T cells has been demonstrated, as genetic deficiency of MHC I or II molecules delays GVHD development (King *et al.*, 2009), and deficiency of both prevents GVHD almost completely (Brehm *et al.*, 2018) in humanised mice. Further, murine $\beta 7.2$ on APCs interaction with CD28 on human T cells can provide co-stimulation for human T cell activation (Freeman *et al.*, 1993). Yet, later mouse models (Chapters 5 and 6) highlighted engraftment of a $hCD45^+ hCD3^- hCD19^- hCD83^+$ population, which are likely dendritic cells (DCs), as CD83 is a marker of DCs (Zhou and Tedder, 1995), which could potentially be activating human T cells to promote GVHD in this humanised NSG mouse model. Therefore, whilst murine MHC activation is likely activating T cells in humanised NSG mice in these studies, the possibility of engrafted

human APCs resulting in T cell activation remains to be elucidated, potentially using the MHC I and II deficient NSG mouse model.

Future studies could also further explore leukocyte infiltration into target organs. Neutrophils are recruited into the intestines early during GVHD (Hülsdünker *et al.*, 2018) and are important in disease pathogenesis in HSCT patients with GVHD (Schwab *et al.*, 2014). Whilst human T cells are the predominant human cell type responsible for mediating damage in the humanised NSG mouse model used throughout this thesis, one study (Chapter 4) highlighted that T cells were present in target organs but did not constitute all immune infiltrates. The origin (human or murine) of neutrophils could also be explored, since NOD mice, the background strain of NSG mice, demonstrate increased numbers of murine neutrophils into target organs (Shultz *et al.*, 1995), and it is unknown whether human neutrophils will engraft after injection of hPBMCs.

Nevertheless, this thesis has demonstrated that the humanised NSG mouse model of GVHD is reproducible, having been performed multiple times, with five different human donors. Clinical and histological evidence (characterised in Chapter 2 and demonstrated in Chapters 3-6) highlights the efficacy of this humanised mouse model as a useful preclinical model of GVHD.

8.3. The Role of Purinergic Signalling in a Humanised Mouse Model of GVHD

These studies (Chapters 3 and 4) investigated the previously unexplored role of P2X7 in a humanised NSG mouse model of GVHD. In allogeneic mouse models of GVHD, ATP released from damaged and dying cells accumulates at sites of damage (Wilhelm *et al.*, 2010) and P2X7 blockade using the antagonists pyridoxalphosphate-6-azophenyl-2',4'-disulfonic

acid (PPADS), KN-62 (Wilhelm *et al.*, 2010), stavudine (Fowler *et al.*, 2014), and BBG (Zhong *et al.*, 2016) can reduce disease severity. The first of these studies (Chapter 3) demonstrated that a short-term regime of BBG was able to reduce serum hIFN- γ and histological GVHD in humanised mice, paralleling features of mice with subclinical GVHD (Chapter 2). Moreover, this reduction in serum hIFN- γ and histological GVHD parallels studies of P2X7 blockade or deficiency in allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010). However, the short-term BBG regime did not impact clinical GVHD (Chapter 3), suggesting that other mechanisms are involved in subclinical GVHD (Chapter 2) and in the increased survival observed in allogeneic GVHD following P2X7 blockade or deficiency (Wilhelm *et al.*, 2010).

In contrast to the short-term BBG regimen (Chapter 3), a long-term regimen of BBG (Chapter 4) only showed reduced leukocyte infiltration and histological damage in the liver of humanised mice, and did not reduce serum IFN- γ . The greater reduction in serum hIFN- γ concentrations and reduced histological damage, using the short-term BBG regimen, compared to the long-term regimen may indicate the importance of P2X7 early in GVHD induction, indicating more frequent P2X7 blockade early post-transplantation (days 0, 2, 4, 6, 8) may be more important than prolonged P2X7 blockade (3 times weekly). Alternatively, comparison of humanised mice following short-term or long-term treatment regimens with either BBG or saline suggest that long-term treatment with saline in humanised NSG mice reduces serum hIFN- γ concentrations and negating any benefits of long-term BBG. This is supported by the injection of saline as a means to rescue mice from weight loss in other inflammatory disease models (van der Fits *et al.*, 2009).

Potential limitations in these studies include the use of BBG as the P2X7 antagonist. BBG was chosen as it is cheap, and has shown efficacy in over 40 studies of disease (Bartlett *et al.*, 2014). However, BBG has a relatively high half maximal inhibitory concentration (IC₅₀)

(Jiang, 2012), and has off-target effects (Qiu and Dahl, 2009). Thus, other more potent, selective P2X7 antagonists, including A804598 (Chapter 7), could be used in future studies in the humanised NSG mouse model of GVHD. Otherwise, the use of PPADS or KN-62, the drugs shown to be efficacious in allogeneic mouse models of GVHD (Wilhelm *et al.*, 2010) could be used to further investigate the role of P2X7, and the therapeutic potential of P2X7 blockade to prevent disease in a humanised mouse model of GVHD. Alternatively, biologics such as nanobodies could be tested in this model. Nanobodies against P2X7 can limit the inflammation associated with experimental glomerulonephritis and allergic contact dermatitis (Danquah *et al.*, 2016) and thus represent a potential therapy for GVHD prevention.

Further, this thesis aimed to explore the role of the CD73/A_{2A} signalling axis on the development of GVHD in humanised NSG mice. However, during the course of this thesis Covarrubias *et al.* (2016) revealed that the purported CD73 inhibitor $\alpha\beta$ -methylene ADP (APCP), could also inhibit CD39. Thus, data using APCP (Chapter 5) could not be solely attributed to the prevention of adenosine production but also to the accumulation of ATP.

Administration of APCP or the broad-spectrum adenosine receptor antagonist caffeine (Chapter 5) were hypothesised to worsen GVHD in humanised NSG mice based on previous studies in allogeneic mouse models of GVHD (Tsukamoto *et al.*, 2012, Wang *et al.*, 2013). In contrast to this hypothesis, APCP, but not caffeine, worsened GVHD in humanised NSG mice. APCP worsened disease through increased weight loss, liver GVHD and serum hIL-2 concentrations. Collectively, this suggests that accumulation of ATP rather than adenosine receptor blockade worsened GVHD in humanised NSG mice. Given that P2X7 is involved, in part, in GVHD development in these mice (Chapters 3 and 4), the accumulation of ATP, as a result of CD39 blockade by APCP, may be acting on P2X7 to promote GVHD in this model. However, serum hIFN- γ concentrations from APCP-injected mice exceeded the scale using the flow cytometric cytokine assay whilst subsequent analysis of this cytokine using an

ELISA failed to detect any hIFN- γ , possibly due to freeze-thawing samples. Thus, this serum cytokine could not be compared between BBG- and APCP-injected humanised NSG mice.

The inability of caffeine to alter GVHD in humanised NSG mice contrasts the worsening effect of this compound in allogeneic mouse models of GVHD (Tsukamoto *et al.*, 2012). Notably, the same caffeine regime was used in these two studies suggesting that the observed differences in GVHD progression relates to differences between the two models, and that the CD73/A_{2A} pathway may have a minor role in the humanised NSG mouse model of GVHD.

This may be attributed to species selective differences of caffeine on murine and human A_{2A} or other adenosine receptors and other targets (Fredholm *et al.*, 2011a). However, due to problems dissolving the more selective A_{2A} antagonist SCH 52861 (Varani *et al.*, 1996) and that caffeine did not offer observable effects, A_{2A} blockade was not explored further in humanised NSG mice. Given the role of A_{2A} in allogeneic GVHD (Lappas *et al.*, 2010, Tsukamoto *et al.*, 2012, Han *et al.*, 2013, Wang *et al.*, 2013) investigation with other A_{2A} antagonists or agonists in humanised NSG mice remains warranted.

The A_{2A} agonist CGS 21680 was hypothesised to reduce GVHD progression in humanised NSG mice based on previous studies where another A_{2A} agonist, ATL-146e, reduced disease severity and mortality in allogeneic mouse models of GVHD (Lappas *et al.*, 2010, Han *et al.*, 2013). This study (Chapter 6) demonstrated that activation of A_{2A} with CGS 21680 had both positive and negative effects on disease progression in humanised NSG mice. This was unlikely due to the effectiveness of CGS 21680, as the same dose (0.1 mg/kg) has been efficacious in preventing disease in mouse models of acute lung inflammation (Impellizzeri *et al.*, 2011), pleurisy (da Rocha Lapa *et al.*, 2012) and arthritis (Mazzon *et al.*, 2011). In the allogeneic mouse models of GVHD, the anti-inflammatory effects of adenosine receptor activation was shown to rely on the production of adenosine by regulatory T (Treg) cells

(Han *et al.*, 2013). Human (h) Treg cells were shown to be present in humanised NSG mice (Chapters 5 and 6), but may not be present in sufficient numbers in this model to drive adenosine production to protect GVHD target organs. Abraham *et al.* (2012) demonstrated that functional hTreg cells can only be maintained in humanised NSG mice by providing hIL-2. Thus, the lack of functional hTreg cells in humanised NSG mice may explain why CGS 21680 did not have a significant impact on GVHD development. Although CGS 21680 had confounding effects, and caused weight loss in healthy NSG mice, it is worthwhile investigating the effect of ATL-146e in this humanised mouse model of GVHD, due to the beneficial effects of ATL-146e, and its analogues ATL-370 and ATL-1223, in allogeneic mouse models of GVHD (Lappas *et al.*, 2010, Han *et al.*, 2013). These drugs could not be investigated in the current study, as ATL-146e is currently being investigated in clinical trials (Gao and Jacobson, 2011), and all three are no longer commercially available.

In summary, the above studies support a role for extracellular ATP and P2X7 activation in GVHD progression in humanised NSG mice, whilst A_{2A} activation has both beneficial and detrimental effects on disease progression in this model. Short-term P2X7 blockade with BBG (Chapter 3) led to reduced serum hIFN- γ concentrations, and reduced histological damage in all organs, while a long-term regime (Chapter 4) reduced leukocyte infiltration and damage to a greater extent. Conversely, CD39/CD73 blockade using APCP (Chapter 5) led to increased leukocyte infiltration and histological damage, and an increase in the pro-inflammatory cytokine hIL-2. However, A_{2A} activation had conflicting results (Chapter 6). Therefore, theoretically in this humanised mouse model of GVHD the release and accumulation of ATP leads to activation of P2X7, potentially on APCs such as DCs. This leads to T cell activation and proliferation, and release of pro-inflammatory cytokines such as IFN- γ and IL-2. T cells then migrate to target tissues, predominantly the liver, to promote inflammation (Figure 8.1.).

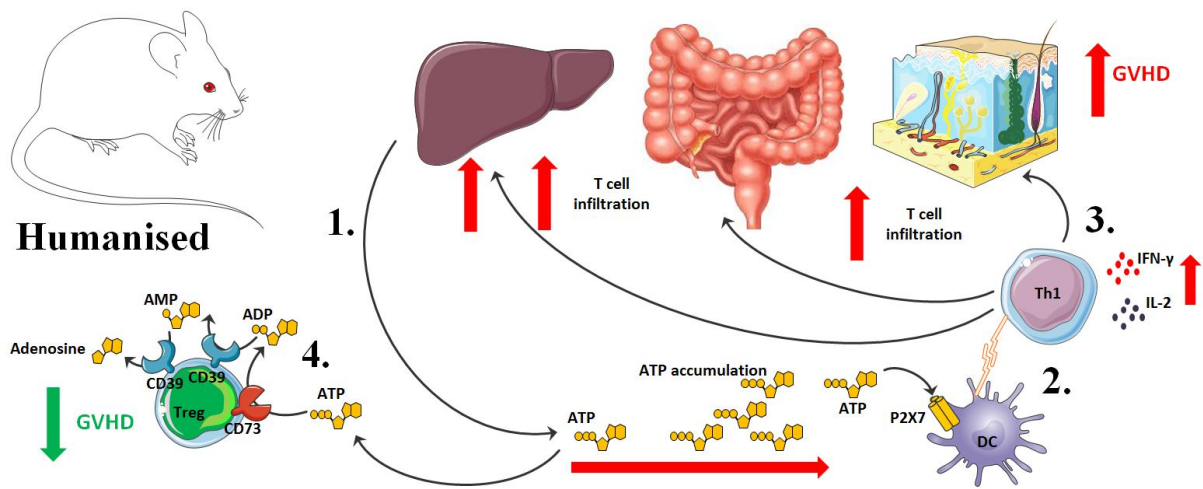


Figure 8.1. The potential role of purinergic signalling in the humanised NSG mouse model of GVHD. 1. Early after transplantation of hPBMCs, ATP is released, and accumulation of ATP leads to P2X7 activation on APCs, such as DCs. 2. APCs subsequently activate Th1 cells which release IL-2 and IFN- γ . 3. These cytokines propagate damage, potentially resulting in further ATP release and P2X7 activation on APCs (1.), resulting in a feed-forward loop of inflammation. A_{2A} activation cannot prevent GVHD development in humanised NSG mice (not shown). 4. Degradation of ATP to ADP, AMP and subsequently adenosine can potentially reduce GVHD. Abbreviations; ADP, adenosine diphosphate, AMP, adenosine monophosphate, APC, antigen presenting cell, APCP, $\alpha\beta$ -methylene-ADP, ATP, adenosine triphosphate, DC, dendritic cell, IFN- γ , interferon gamma, IL-2, interleukin-2, Th, T helper, Treg, regulatory T cell.

8.4. The Role of P2X7 in a Psoriasis-Like Inflammatory Mouse Model of Inflammation

A preliminary study was undertaken to assess the role of P2X7 in psoriasis (Chapter 7), as an alternate inflammatory disease to GVHD. This involved the establishment of the imiquimod (IMQ)-induced psoriasis-like inflammation model (van der Fits *et al.*, 2009) at the University of Wollongong. The landmark study by van der Fits *et al.* (2009) demonstrated IMQ induces redness, scaling and thickening of skin due to altered differentiation and the hyperproliferation of keratinocytes, as well as immune infiltrates consisting of neutrophils, T cells and professional APCs resulting in psoriatic-like lesions. Our study confirmed that BALB/c mice develop greater psoriasis (ear swelling and inflammation) compared to C57BL/6 mice as previously described (van der Fits *et al.*, 2009). IMQ also induced ATP

release from keratinocytes *in vitro* and up-regulated P2X7 expression in the skin of these mice. Nevertheless, pharmacological blockade of P2X7 with the antagonists BBG or A804598 or P2X7 deficiency failed to alter disease progression in this model. During the course of this PhD a study by Diaz-Perez *et al.* (2018) confirmed that P2X7 does not impact IMQ-induced psoriasis-like inflammation. Thus, the IMQ-induced psoriasis-like inflammation model was not explored further, with time and resources directed to exploring the role of purinergic signalling in GVHD. Moreover, it was never intended to use the IMQ-induced psoriasis-like inflammation mouse model of psoriasis to investigate the role of adenosine receptor signalling as it is well known that IMQ can interact as an antagonist of adenosine receptors (Schön *et al.*, 2006).

Notably, Diaz-Perez *et al.* (2018) demonstrated a role for P2X7 in recombinant IL-23 (rIL-23) induced psoriasis-like inflammation, a finding consistent with the up-regulation of P2X7 in human psoriatic skin (Pastore *et al.*, 2007, Killeen *et al.*, 2013). To reconcile these differences in the role for P2X7 in IMQ- and recombinant IL-23-induced psoriasis-like inflammation, Diaz-Perez *et al.* (2018) propose that IMQ bypasses the need to activate the NLRP3 inflammasome/IL-1 β to directly induce IL-17 production, which is a key cytokine in psoriasis (Rizzo *et al.*, 2011). This proposal was based on prior studies (Rabeony *et al.*, 2015) in which IMQ-induced psoriasis was found to be independent of the NLRP3 inflammasome. Thus, although further studies of P2X7 in psoriasis were not continued in this model, future studies could potentially utilise the recombinant IL-23 induced psoriasis-like inflammation model to explore the role of P2X7 in this disease.

8.5. Conclusion

This thesis demonstrated that the purinergic signalling system plays a role in the development of disease in a humanised mouse model of GVHD. Increased serum hIFN- γ was identified as a marker of worsened disease severity in humanised mice. Similar to allogeneic mouse models, short-term P2X7 blockade with BBG reduced GVHD severity through decreased serum hIFN- γ and reduced histological damage, and long-term P2X7 blockade with BBG reduced liver GVHD compared to control mice. Additionally, CD39/CD73 blockade using APCP worsened GVHD in humanised mice, paralleling results in allogeneic mouse models. However, in contrast to allogeneic mouse models, adenosine receptor blockade using the broad spectrum antagonist caffeine did not worsen GVHD severity in humanised mice. Further, adenosine receptor activation with the specific agonist CGS 21680 did not prevent GVHD, but instead exaggerated both positive and negative aspects of GVHD. This suggests that extracellular ATP and P2X7 activation is the main purinergic signalling pathway involved in disease pathogenesis in a humanised mouse model of GVHD.

P2X7 was also investigated in the IMQ-induced psoriasis-like inflammation mouse model of GVHD, where genetic deficiency or pharmacological blockade did not impact disease development in this model. However, the role of this purinergic signalling pathway in psoriasis cannot be excluded based on this one model, as it has been shown to be important in other models of this disease.

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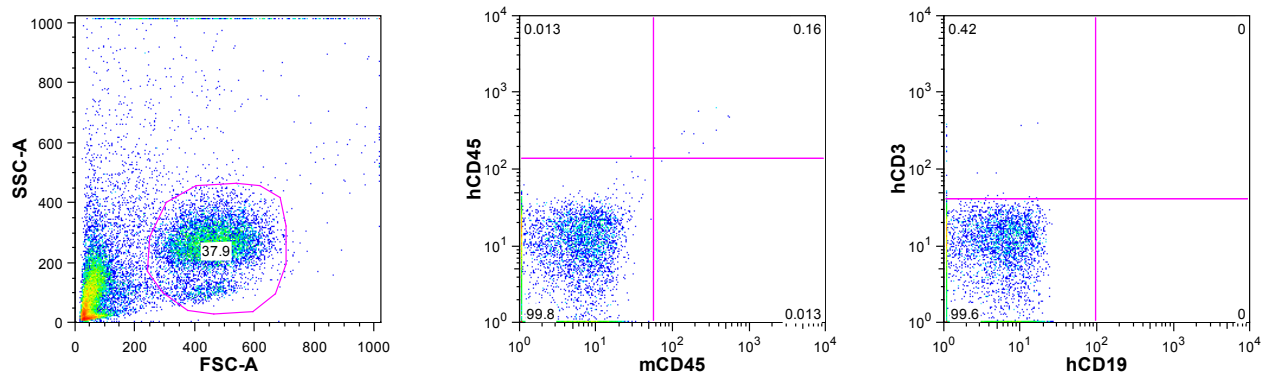
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Appendices: Supplementary Data

Isotype control mAb



CD specific mAb

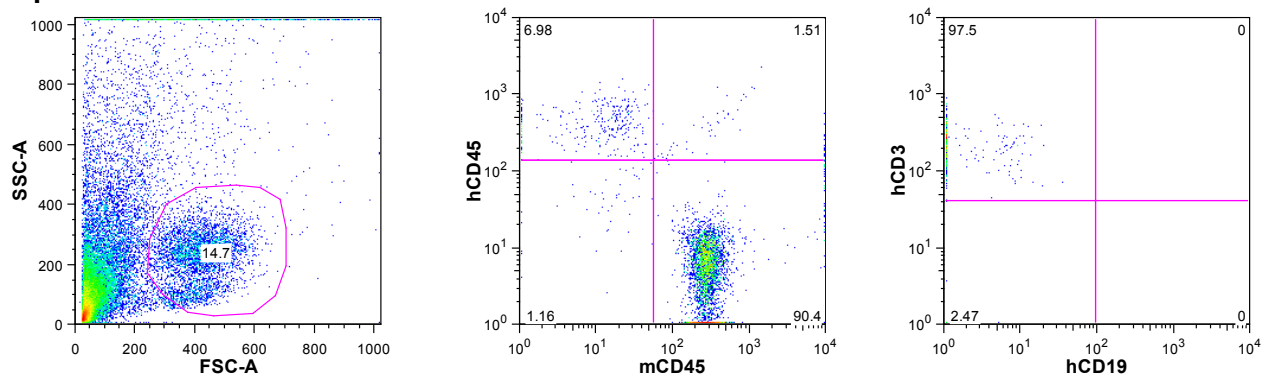
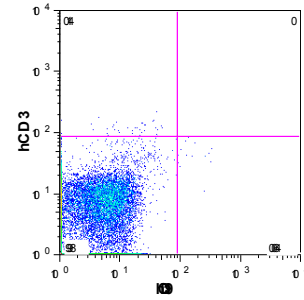
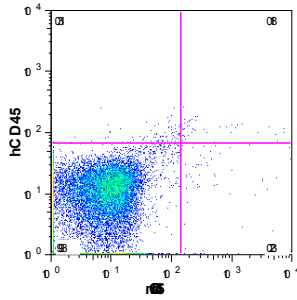
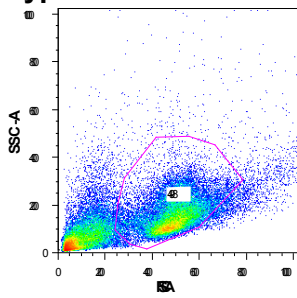


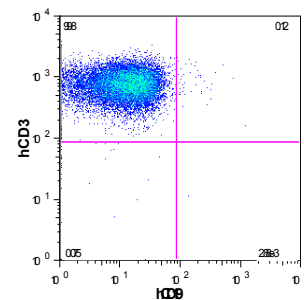
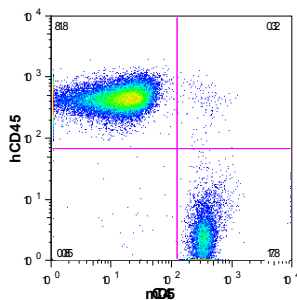
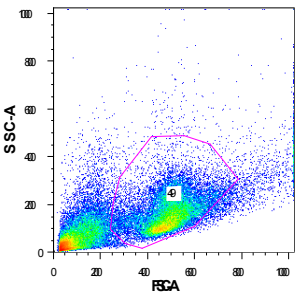
Figure S3.1. Flow cytometric gating of leukocytes in blood from humanized mice.

Blood cells from humanized mice were labelled with isotype control or CD-specific monoclonal antibodies (mAb). Forward and side scatter was used to identify leukocytes, which were subsequently used to identify human leukocytes ($hCD45^+ mCD45^-$), human T cells ($hCD45^+ mCD45^- hCD3^+ hCD19^-$) and human B cells ($hCD45^+ mCD45^- hCD3^- hCD19^+$).

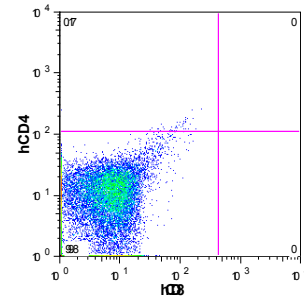
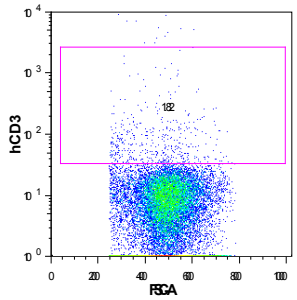
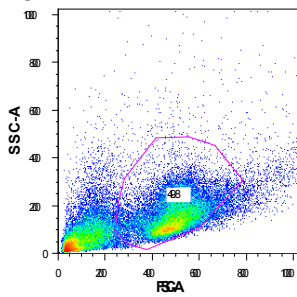
Isotype control mAb



CD specific mAb



Isotype control mAb



CD specific mAb

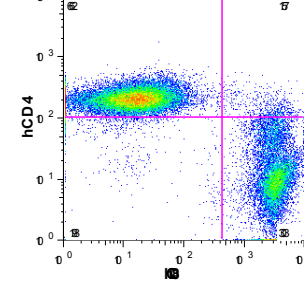
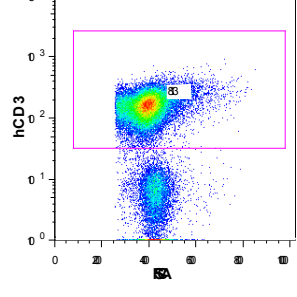
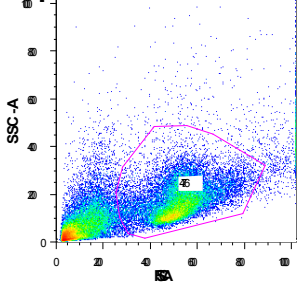
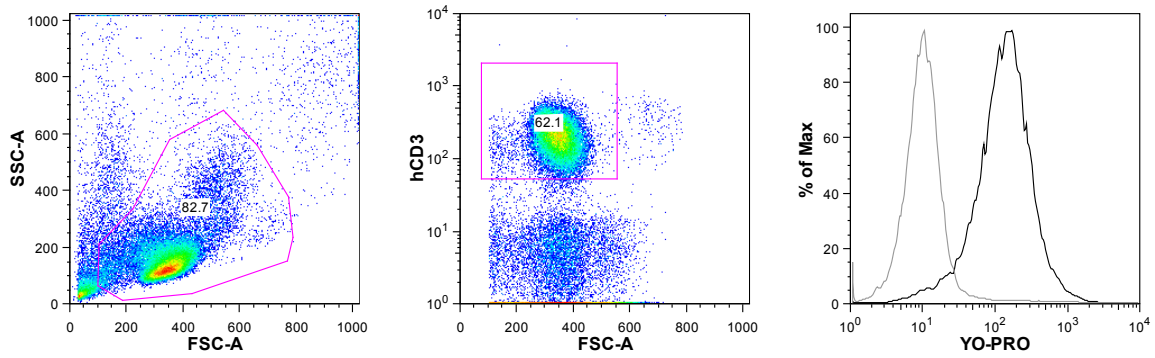


Figure S3.2. Flow cytometric gating of leukocytes in spleens from humanised mice.

Splenocytes from humanised mice were labelled with isotype control or CD-specific monoclonal antibodies (mAb). Forward and side scatter was used to identify leukocytes, which were subsequently used to identify human leukocytes ($hCD45^+ mCD45^-$), human T cells ($hCD45^+ mCD45^- hCD3^+ hCD19^-$) and human B cells ($hCD45^+ mCD45^- hCD3^- hCD19^+$). Forward and side scatter was used to identify leukocytes, which were subsequently used to identify human $CD4^+$ T cell ($hCD3^+ hCD4^+ hCD8^-$), and human $hCD8^+$ T cell ($hCD3^+ hCD4^- hCD8^+$) subsets.

Human T Cells



Splenic NSG DCs

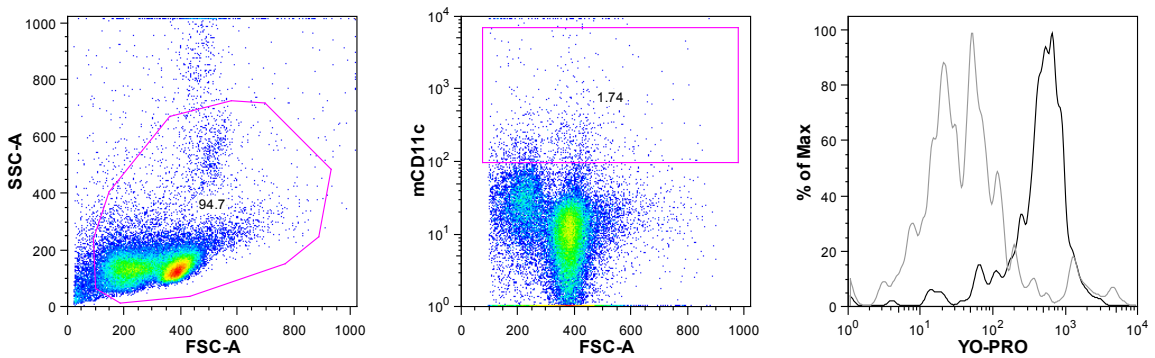


Figure S3.3. Flow cytometric analysis of YO-PRO-1²⁺ uptake into human hCD3⁺ T cells and murine CD11c⁺ splenic dendritic cells. Human PBMCs and splenocytes from NOD-SCID-IL2R γ^{null} mice were incubated with YO-PRO-1²⁺ in the absence (basal) (grey histograms) or presence of adenosine triphosphate (ATP) (black histograms), and subsequently labelled with anti-human (h) CD3 or anti-murine (m) CD11c monoclonal antibody. Forward and side scatter was used to identify leukocytes, which were subsequently used to identify hCD3⁺ T cells or murine CD11c⁺ splenic DCs. YO-PRO-1²⁺ uptake into these cells was then quantified using geometric mean fluorescence of histograms.

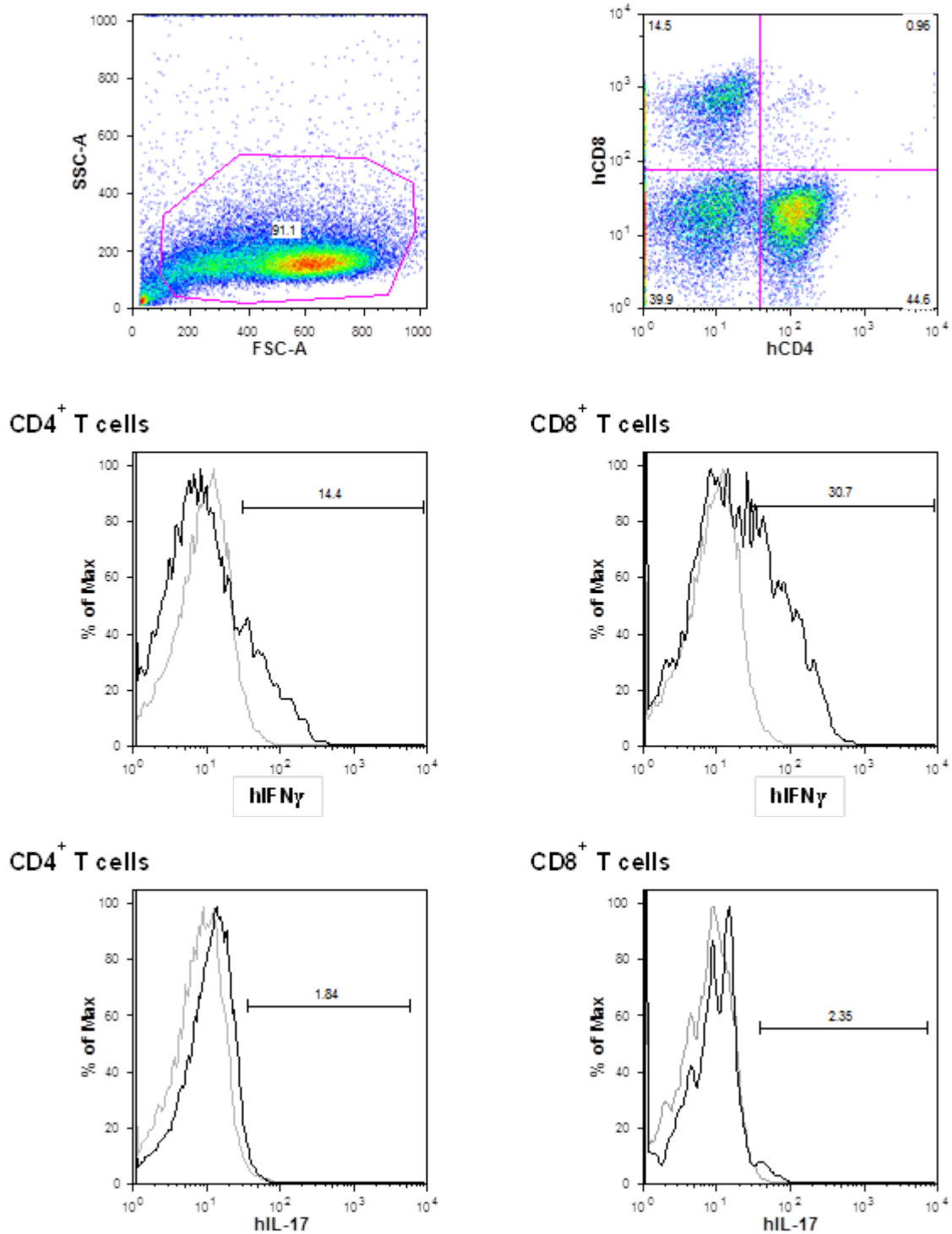


Figure S3.4. Flow cytometric analysis of intracellular human IFN- γ and IL-17 in human CD4⁺ and CD8⁺ T cells. Splenocytes from humanised mice were incubated with phorbol 12-myristate 13-acetate and ionomycin, and labelled with isotype control or specific monoclonal antibodies. Forward and side scatter was used to identify leukocytes, which were subsequently used to identify human CD4⁺ T cell (hCD3⁺ hCD4⁺ hCD8⁻), and human hCD8⁺ T cell (hCD3⁺ hCD4⁻ hCD8⁺) subsets. The percentage of hIFN- γ and hIL-17 was quantified as the difference between cytokine specific (black histograms) and isotype control (grey histograms) mAb labelling (indicated by marker regions as shown).

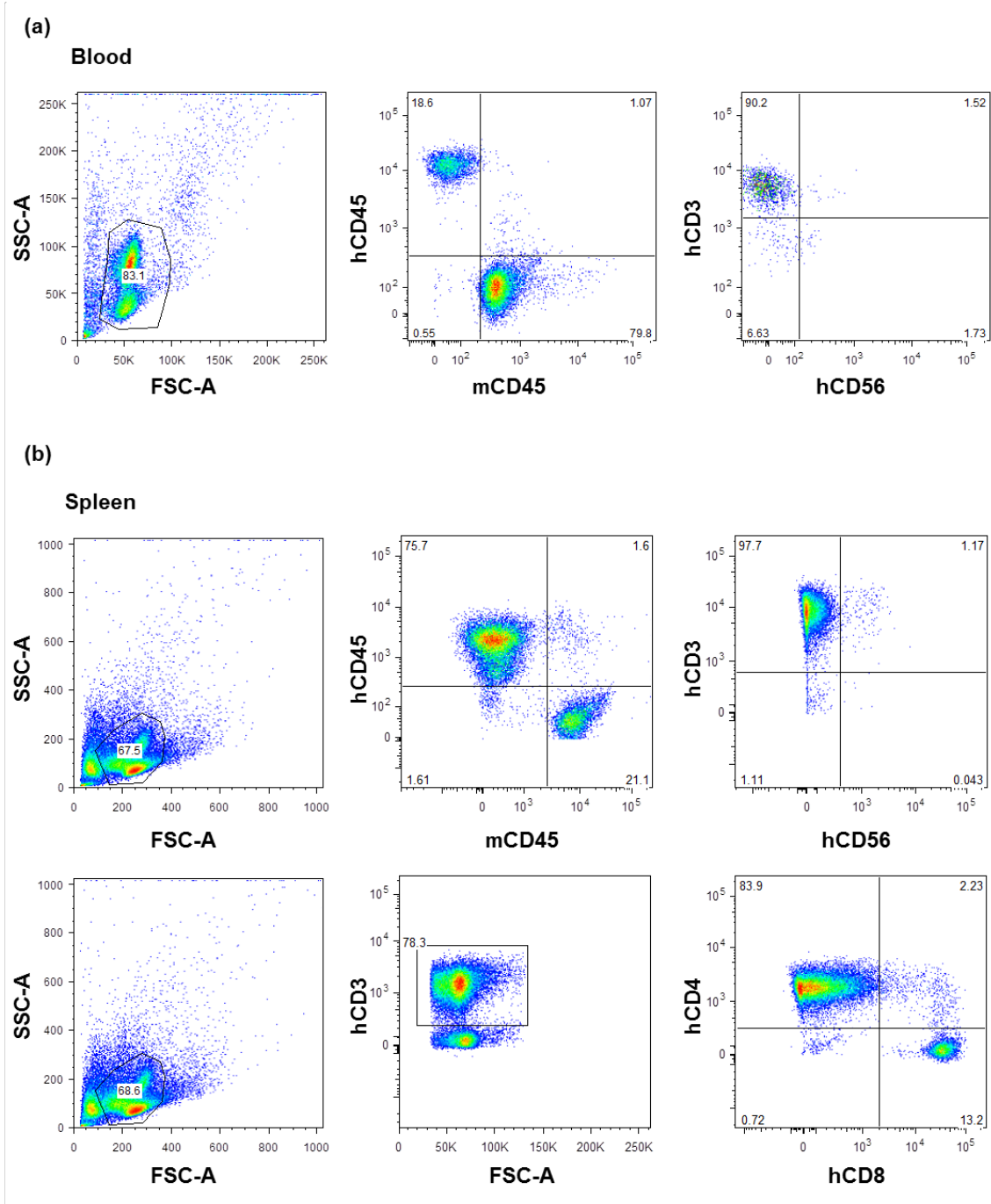


Figure S4.1. Flow cytometric gating of leukocytes in blood and spleens from humanised mice
 . (a-b) NOD-SCID-IL2R γ null (NSG) mice were injected intra-peritoneally with 10×10^6 human (h) peripheral blood mononuclear cells, and subsequently with saline or saline containing Brilliant Blue G (BBG) (50 mg/kg) thrice weekly. (a) Blood cells and (b) splenocytes from humanised NSG mice were labelled with isotype control or CD-specific monoclonal antibodies. (a, b) Leukocytes, initially gated by forward scatter (FSC-A) and side scatter (SSC-A), were analysed to determine the percentages of human leukocytes (hCD45⁺ mCD45⁻), human T cells (hCD45⁺ mCD45⁻ hCD3⁺ hCD56⁻), human NK cells (hCD45⁺ mCD45⁻ hCD3⁻ hCD56⁺), human NKT cells (hCD45⁺ mCD45⁻ hCD3⁺ hCD56⁺), human non-T/NK/NKT cells (hCD45⁺ mCD45⁻ hCD3⁻ hCD56⁻), and (spleen only) human CD4⁺ T cell (hCD3⁺ hCD4⁺ hCD8⁻), and human hCD8⁺ T cell (hCD3⁺ hCD4⁻ hCD8⁺) subsets.

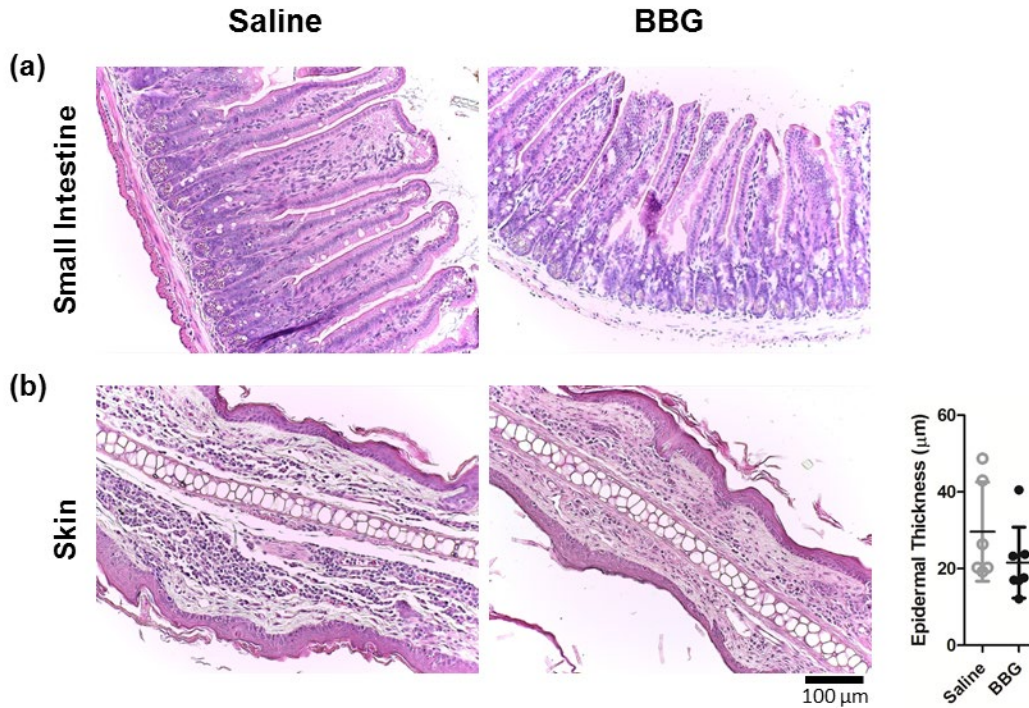


Figure S4.2. Long-term BBG treatment does not affect intestinal or skin damage in a humanised mouse model of GVHD. (a-b) NOD-SCID-IL2R γ null (NSG) mice were injected intra-peritoneally (i.p.) with 10×10^6 human (h) peripheral blood mononuclear cells (hPBMCs), and subsequently with saline or saline containing Brilliant Blue G (BBG) (50 mg/kg) thrice weekly. (a) Small intestines and (b) skin from hPBMC-injected mice injected with saline or BBG at end-point were stained with haematoxylin and eosin. Images were captured by microscopy; representative images from one mouse of seven saline- or 10 BBG-injected mice. (b) Image analysis was used to quantitate the mean epidermal thickness from three different skin images per mouse. Data represents group means \pm SD (saline n = 6; BBG n = 7; P = 0.2174); symbols represent individual mice.

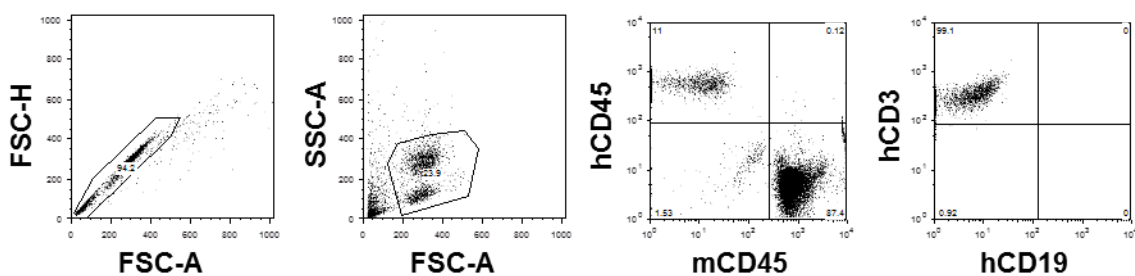


Figure S5.1. Flow cytometric gating of leukocytes in blood from humanised mice.

The percentage of human leukocytes in blood at 3 weeks post-hPBMC injection collected from all humanised NSG mice were determined by flow cytometry. Forward scatter area (FSC-A) and forward scatter height (FSC-H) were used to identify single cells (far left panel). Forward scatter (FSC-A) and side scatter (SSC-A) (middle left panel) were used to identify and subsequently analyse the percentages of human leukocytes (hCD45 $^+$ mCD45 $^-$; middle right panel), which comprised T and non-B/T cells (hCD3 $^+$ hCD19 $^-$ and hCD3 $^-$ hCD19 $^-$ respectively; far right panel).

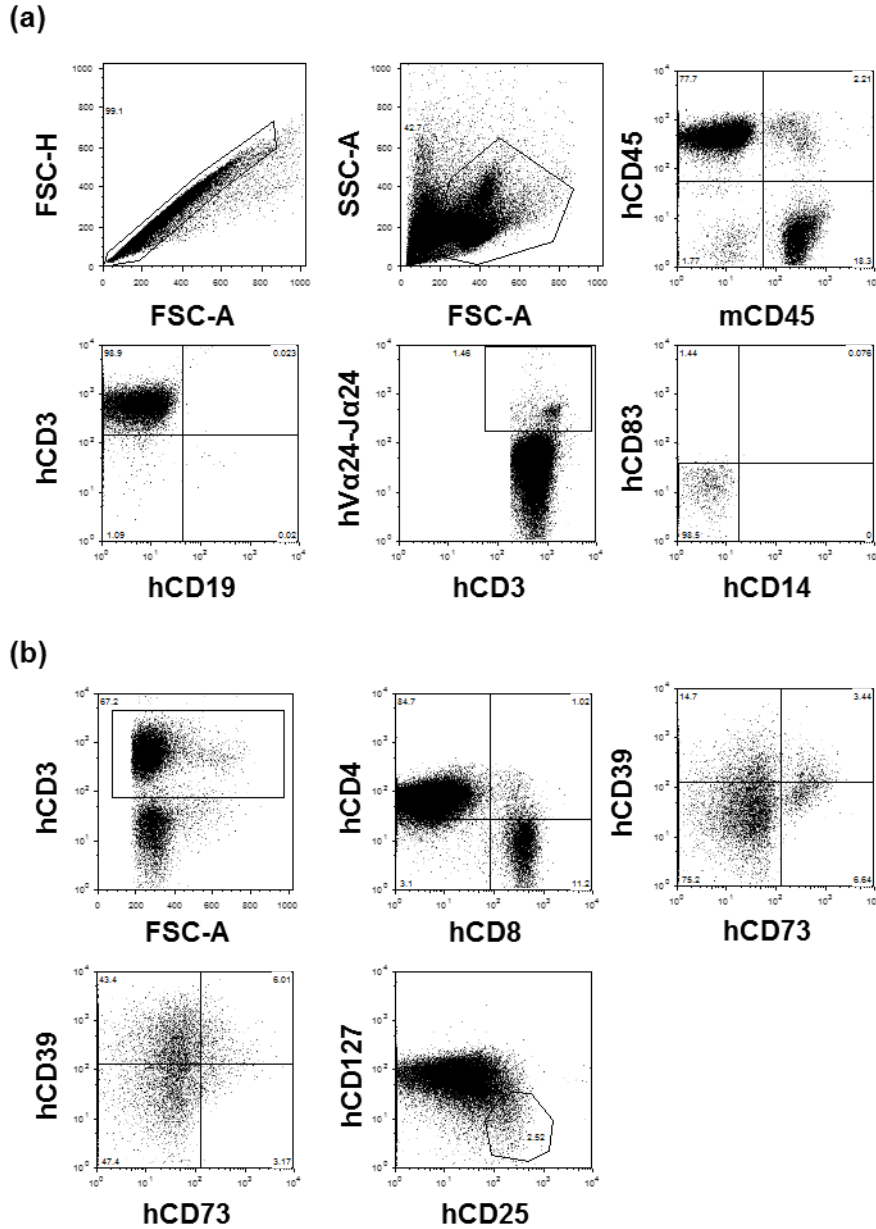


Figure S5.2. Flow cytometric gating of leukocytes in spleens from humanised mice.

(a-b) The percentage of human leukocytes in spleens at end-point collected from all humanised NSG mice were determined by flow cytometry. (a) Forward scatter (FSC-A, FSC-H) and side scatter (SSC-A) were used to identify and subsequently analyse single leukocytes (top left and middle panels). These leukocytes were analysed to determine the percentages of human leukocytes ($hCD45^+mCD45^-$; top right panel), and subsequently used to identify T cells ($hCD3^+hCD19^-$; bottom left panel) and iNKT cells ($hCD3^+hCD19^-hV\alpha24-J\alpha18^+$; bottom middle panel), or non-B/T cells ($hCD3^+hCD19^-$; bottom left panel), which were subsequently used to identify monocytes and DCs ($hCD14^+CD83^-$ and $hCD14^+CD83^+$ respectively; bottom right panel). (b) Single leukocytes were gated as above. T cells were identified ($hCD3^+$; top left panel), and subsequently used to determine $hCD4^+$ and $hCD8^+$ T cell subsets (top middle panel), and $hCD39$ and $hCD73$ expression on $hCD4^+$ (top right panel) and $hCD8^+$ (bottom left panel) T cells was analysed. $hCD4^+$ T cells were also gated to identify Tregs ($hCD25^+hCD127^{lo}$; bottom right panel).