Increased P2X7 expression in the gastrointestinal tract and skin in a humanised mouse model of graft-versus-host disease

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Abstract
BACKGROUND: Allogeneic haematopoietic stem cell transplantation (HSCT) is a curative therapy for blood cancers; but results in the development of graft-versus-host disease (GVHD) in up to 70% of recipients. During GVHD, tissue damage results in ATP release into the extracellular compartment activating P2X7 on antigen-presenting cells, leading to the release of pro-inflammatory cytokines and subsequent activation of donor T cells. Therefore, the aim of the present study was to examine murine (m) P2rx7 and human (h) P2RX7 gene expression in GVHD target organs of humanised mice, and further characterise disease impact in these organs. METHODS: NOD-scid IL2Rnull (NSG) mice were injected with human peripheral blood mononuclear cells (hu-PBMC-NSG mice) or phosphate-buffered saline (PBS, control). Leucocytes were assessed by flow cytometry; gene expression was measured by quantitative polymerase chain reaction (qPCR), and tissue sections examined by histology. RESULTS: Compared with control mice, hu-PBMC-NSG mice had increased mP2rx7 and mP2rx4 expression in the duodenum, ileum and skin. hP2RX7 was expressed in all tissues examined. hu-PBMC-NSG mice also displayed increased mReg3g expression in the duodenum and ileum, despite limited histological gut GVHD. hu-PBMC-NSG mice showed histological evidence of GVHD in the skin, liver and lung. Compared with control mice, hu-PBMC-NSG mice displayed increased ear swelling. CONCLUSION: Combined data revealed that P2rx7 is up-regulated in gut and skin GVHD and that P2RX7 is present in target tissues of GVHD, corresponding to human leucocyte infiltration. Data also reveal increased mReg3g expression and ear swelling in hu-PBMC-NSG mice, offering new measurements of early-stage gut GVHD and skin GVHD, respectively.

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Increased P2X7 expression in the gastrointestinal tract and skin in a humanised mouse model of graft-versus-host disease

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Abstract

**Background:** Allogeneic haematopoietic stem cell transplantation (HSCT) is a curative therapy for blood cancers; but results in the development of graft-versus-host disease (GVHD) in up to 70% of recipients. During GVHD, tissue damage results in the release of ATP into the extracellular compartment activating P2X7 on antigen presenting cells, leading to the release of pro-inflammatory cytokines and subsequent activation of donor T cells. Therefore, the aim of this study was to examine murine (m) *P2rx7* and human (h) *P2RX7* gene expression in GVHD target organs of humanised mice, and further characterise disease impact in these organs. **Methods:** NOD-^scid^ IL2Rγnull (NSG) mice were injected with human peripheral blood mononuclear cells (hu-PBMC-NSG mice) or PBS (control). Leukocytes were assessed by flow cytometry; gene expression was measured by qPCR, and tissue sections examined by histology. **Results:** Compared to control mice, hu-PBMC-NSG mice had increased m*P2rx7* and m*P2rx4* expression in the duodenum, ileum and skin. h*P2RX7* was expressed in all tissues examined. hu-PBMC-NSG mice also displayed increased m*Reg3g* expression in the duodenum and ileum, despite limited histological gut GVHD. hu-PBMC-NSG mice showed histological evidence of GVHD in the skin, liver and lung. Compared to control mice, hu-PBMC-NSG mice displayed increased ear swelling. **Conclusion:** Combined this data reveals that *P2rx7* is upregulated in gut and skin GVHD and that *P2RX7* is present in target tissues of GVHD, corresponding to human leukocyte infiltration. Data also reveals increased m*Reg3g* expression and ear swelling in hu-PBMC-NSG mice, offering new measurements of early stage gut GVHD and skin GVHD, respectively.
Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is a common curative therapy for multiple haematological disorders, including blood cancers (1). Acute graft-versus-host disease (GVHD) is a potentially lethal complication that arises following HSCT, damaging the gut, skin, liver and lung (2). Acute GVHD occurs in up to 70% of HSCT recipients with a mortality rate of 20% (3). GVHD is mediated by donor immune cells, which initiate a massive inflammatory response in host organs (4). GVHD develops when host antigen presenting cells activate donor T cells (5). These donor T cells are then directed to target organs by homing molecules including integrin β7 (6) and cutaneous lymphocyte antigen (CLA) (7), which direct inflammatory cells to the gut and skin, respectively. Activated donor T cells release pro-inflammatory cytokines such as interferon gamma (IFNγ) (8), propagating a positive feedback loop leading to further activation of donor T cells, and increased tissue damage (9).

The gut is affected in approximately 62% of GVHD cases and is a major cause of mortality (10). Gut damage arises when donor T cells cause severe inflammation in the intestines, leading to crypt cell degeneration and destruction of the villous epithelium (11). Plasma regenerating islet-derived 3 (REG3)α has emerged as a clinical biomarker of GVHD progression in humans (12). Likewise, mRNA for REG3γ, the murine homologue of REG3α, is upregulated in villous enterocytes of mice with GVHD, corresponding to increased circulating REG3γ in these mice (13).

The skin is affected in approximately 80% of GVHD cases, and is usually the first tissue to show clinical signs of damage (14). Whilst this damage does not typically result in mortality, it causes significant pain to patients (15). The liver is affected in approximately 50% of GVHD cases, and is a major cause of mortality (16). Liver damage occurs when leukocytes infiltrate the area surrounding the portal vein; leading to hepatocyte death and bile duct
destruction (17). In the lung, GVHD presents mainly as an idiopathic pneumonia syndrome, with little to no cell apoptosis (2).

Allogenic mouse models are commonly used to study GVHD and have provided significant insight into the understanding of this disease (18), but they do not fully replicate GVHD in humans. As such, humanised mouse models of GVHD have been developed to further elucidate the role of human immune cells in this disease. Common examples of these models involve the injection of human peripheral blood mononuclear cells (hPBMCs) into immune deficient mice, such as NOD-scid IL-2Rγnull (NSG) (19) or NOD-scid IL-2Rγmutant (NOG) (20) mice. Humanised mouse studies of GVHD often report disease involvement in the liver, but involvement of the gut, skin or lungs are not always reported (19, 21-23). Homing molecules on donor T-cells have shown importance in both humans receiving allogeneic HSCT (6, 7) and allogeneic mouse models (24), however they have not been extensively examined in humanised NSG or NOG mice.

Purinergic receptors, including the ATP-gated P2X7 receptor, play an important roles in controlling T cell-mediated inflammation following allogeneic transplantation (25, 26). P2X7 blockade delays allograft rejection in both islet (27) and cardiac (28) transplantation in mice, which corresponded with reduced Th1 and Th17 cells and inflammation in both transplanted tissues. Whilst a naturally occurring mutation in human P2X7 leads to excessive Th17 cell generation and poorer outcomes in patients following cardiac transplantation (29). Moreover, P2X7 blockade can also reduce allograft rejection in both lung (30) and skin (31) transplantation in mice, again corresponding with reduced inflammation in both transplanted tissues.

The ATP/P2X7 signalling axis also plays important roles following HSCT. In allogeneic mouse models of GVHD, resulting tissue damage leads to the release of danger associated molecular patterns such as ATP (32). Subsequently, this extracellular ATP activates P2X7 on
antigen presenting cells to promote donor T cell activation (32). Human (h) P2RX7 and mouse (m) P2rx7 gene expression has been shown to be respectively increased in human patients with GVHD and in mouse models of this disease (32), but analysis of P2RX7 expression in GVHD remains limited. The importance of P2X7 activation in mediating GVHD progression has been shown in allogeneic models of this disease (32, 33) and more recently by us in a humanised NSG model of GVHD (34-36). Collectively, this highlights the need for a more thorough analysis of P2RX7 expression in GVHD target organs.

The current study aimed to examine mP2rx7 and hP2RX7 expression in GVHD target organs of humanised NSG mice and to further characterise disease impact in these organs. Humanised NSG mice (hu-PBMC-NSG) had increased mP2rx7 expression in the duodenum, ileum and skin, but not the liver, lung, spleen or other areas of the gut. hP2RX7 was expressed in all tissues examined. Human leukocytes expressing integrin β7 were present in the blood and spleens of hu-PBMC-NSG mice, while mReg3g gene expression was increased in the duodenum and ileum of hu-PBMC-NSG mice despite limited histological gut GVHD. CLA expressing human leukocytes were present in hu-PBMC-NSG mice, with mice displaying histological evidence of cutaneous GVHD and increased ear swelling. Both the liver and lungs showed histological evidence of GVHD with abundant immune cell infiltration.
Methods

Human peripheral blood mononuclear cell isolation

Human blood was acquired and used as approved by the University of Wollongong Human Ethics Committee (Wollongong, Australia). Whole blood was collected from healthy Caucasian donors (one female and six males, aged 21-40 years) into VACUETTE® lithium heparin tubes (Greiner Bio-One, Frickenhausen, Germany) with informed written consent from all donors. Whole blood was mixed with an equal volume of Dulbecco’s modified phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA), under laid with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden), and centrifuged at 560xg for 30 min. PBMCs were collected, washed twice with PBS and resuspended at the indicated concentrations in PBS.

Humanised murine model of graft-versus-host disease

All animal procedures were carried out as approved by the University of Wollongong Animal Ethics Committee (Wollongong, Australia) within the Rodent Research Facility of the University of Wollongong. Female NOD-scid IL-2Rγnull (NSG) mice (aged 5-6 weeks) were obtained from Australian BioResources (Moss Vale, Australia) and housed in ventilated cages (Techniplast, Buggugiate, Italy) with a 12h light/12h dark cycle, and provided with autoclaved food and water provided ad libitum. Mice were acclimitised for 1 week before GVHD was initiated as described (23). Briefly, 10 x 10⁶ human PBMCs (from two male and two female healthy Caucasian donors, aged 21-40 years) were injected intra-peritoneally (i.p.) into NSG mice (hu-PBMC-NSG mice). Control mice were injected i.p. with an equal volume of PBS. Mice were monitored for signs of GVHD three times a week from day 0 until endpoint using a clinical scoring system as described (37). Ear thickness was measured three times a week using Interapid spring-loaded callipers (Rolle, Switzerland). Engraftment was assessed via immunophenotyping of tail vein blood at 3 weeks post-injection. At endpoint all
mice were euthanased by slow-fill carbon dioxide, and blood, spleen, duodenum, jejunum, ileum, colon, liver, lung, skin and ear were removed from mice. The gastrointestinal tract (stomach to rectum) was removed whole and measured to determine total gut, small intestine and large intestine length.

**Antibodies**

Peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse CD45 (clone 30-F11; 1:20 dilution), fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 (clone HI130; 1:20 dilution), allophycocyanin (APC)-conjugated anti-human CD3 (clone UCHT1; 1:20), R-phycoerythrin (PE)-conjugated anti-human CD4 (clone SK3; 1:20 dilution), PE-cyanine 7 (PE-Cy7)-conjugated anti-human CD8 (clone RPA-T8; 1:50 dilution), Brilliant Violet (BV) 421-conjugated anti-human β7 (clone FIB504; 1:20 dilution), and BV605-conjugated anti-human CLA (clone FIB504; 1:20 dilution) monoclonal antibodies (mAb) were obtained from BD (San Jose, CA, USA).

**Flow cytometry**

Freshly isolated hPBMCs were obtained as outlined above. At 3 weeks post-injection, tail vein blood from mice was collected into citrate solution (Sigma-Aldrich, St Louis, MO, USA), diluted with PBS and centrifuged (300xg, 5 min). Spleens were manually homogenised in PBS, passed through a 70 µm nylon filter (Falcon Biosciences, New York, NY, USA) and centrifuged (300xg, 5 min). Livers were diced then digested in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) containing 4 mg/mL DNase I (Roche, Basel, Switzerland) and 7 mg/mL collagenase (Sigma-Aldrich) (37°C, 45 min), filtered through a 70 µm nylon filter and centrifuged (300xg, 5 min). Blood, spleen and liver samples were treated with ammonium chloride potassium lysis buffer (150 mM NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂CO₃) for 5 min, to remove red blood cells, and washed once with PBS.
hPBMCs or mouse samples (blood, spleen, and liver) were resuspended at 1 x 10⁶ cells/mL in PBS containing 2% foetal calf serum (Bovogen, Keilor East, Australia) and centrifuged (300xg, 5 min). Samples (1 x 10⁶ cells per tube) were incubated with fluorochrome-conjugated mAb in the dark (ice, 15 min). Cells were washed once with PBS (300xg, 3 min), resuspended in PBS and events were acquired using a BD LSR Fortessa X-20 flow cytometer (BD). Proportions of leukocyte subsets were determined using FlowJo software version 8.7.1 (BD). Single cells were first gated by forward scatter-height and -width, and second by forward and side scatter-height as described (37), and then as indicated in figures.

**Histological analysis**

Tissues from mice were treated with neutral buffered (10%) formalin (Sigma-Aldrich) for 24-48 hours, before being processed and set in paraffin wax. Samples were sectioned (3-5 µm) and stained with haematoxylin and eosin (POCD, Artarmon, Australia). Samples were reviewed in consultation with a histopathologist. All samples were then examined for histological damage, in a blinded fashion, using a Leica DMRB microscope (Leica, Wetzlar, Germany). All tissues were observed for immune cell infiltrates. Intestinal sections were examined for signs of crypt apoptosis and crypt degeneration. Livers were examined for bile duct degradation. Skin and ear were examined to identify apoptotic cells, epidermal thickening and separation at the dermal-epidermal junction.

**RNA isolation and cDNA synthesis**

Tissues from mice were stored in RNAlater (Sigma-Aldrich) at -20°C until required. RNA was isolated from duodenum, jejunum, ileum, colon, liver, spleen and lung using TRIzol reagent (Thermo Fisher Scientific) as per manufacturer's instructions. Flank skin and whole ear samples were homogenised with a Precellys 24 tissue homogeniser (Bertin Instruments,
Montigny-le-Bretonneux, France) as described (38) and RNA was isolated using the ISOLATE II RNA Mini Kit (Bioline, London, UK) as per manufacturer’s instructions. cDNA was synthesised from RNA using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) as per manufacturer’s instructions.

**Quantitative polymerase chain reactions (qPCR)**

Relative gene expression was determined using TaqMan Universal Master Mix II (Thermo Fisher Scientific) as per the manufacturer’s instructions. qPCR was conducted with FAM-labelled- mP2rx7 (Mm01199503_m1), hP2RX7 (Hs00175721_m1), hP2RX7B (custom, For: ‘5GGAAATGGTTTGGAGAAGGAAGTG3’, Rev: 5'CGATGAGGAAGTCGATGAACACA3’) mP2rx1 (Mm01251971_g1), mP2rx4 (Mm00501790_g1), mReg3g (Mm01181783_g1), mIl22 (Mm01226722_g1) or hIL22 (Hs01574154_m1) (Thermo Fisher Scientific), using either VIC-labelled mGapdh (Mm99999915_g1) or hHPRT1 (Hs99999909_m1) as the housekeeping gene (Thermo Fisher Scientific). Amplifications were conducted on a QuantStudio 5 (Thermo Fisher Scientific) with triplicate reactions for each sample and gene. Data was analysed using QuantStudio Design and Analysis Software 1.4.0 (Thermo Fisher Scientific). Gene expression was quantified using the \( \Delta \Delta Ct \) method and made relative to expression in either the duodenum (mP2rx1, mP2rx4 and mReg3g) or spleen (mP2rx7) of a control mouse, or a hu-PBMC-NSG mouse spleen (hP2RX7 and hP2RX7B). Primer efficiency was determined by the generation of a standard curve using serial dilutions of cDNA.

**Enzyme-linked immunosorbent assays (ELISA)**

Whole blood was collected via cardiac puncture, incubated (room temperature, 30 min) and centrifuged (1700xg, 5 min). Serum was collected and stored at -80°C until required. Serum REG3\( \gamma \) was measured with a Murine REG3\( \gamma \) ELISA kit (MyBioSource San Diego, CA,
USA) as per the manufacturer’s instructions. Serum hIFNγ was measured with a Human IFNγ ELISA Kit (Life Technologies) as per the manufacturer’s instructions.

**Data presentation and statistical analysis**

All data is given as mean ± standard deviation unless otherwise stated. All statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Statistical differences were calculated using a student’s t test for single comparisons or one-way analysis of variance (ANOVA) with a Tukey’s post-hoc test for multiple comparisons. Survival differences were determined by a log-rank (Mantel-Cox) test.
Results

NSG mice injected with hPBMCs contain circulating human T cells and develop clinical GVHD

NSG mice typically develop GVHD following hPBMC injection and engraftment (19). In the current study NSG mice were injected i.p. with either $10 \times 10^6$ hPBMCs (hu-PBMC-NSG) or PBS (control). Tail vein blood was collected from all mice at 3 weeks post-injection, and analysed by flow cytometry (Figure 1a). As expected, control mice lacked hCD45+ cells, whilst hu-PBMC-NSG mice engrafted hCD45+ cells (Figure 1b), the majority of which were hCD3+ T cells (Figure 1c). Further analysis of the hCD3+ T cell population revealed similar proportions of hCD4+ or hCD8+ subtypes (Figure 1d).

The above mice were monitored for up to 10 weeks. Weight change was similar between hu-PBMC-NSG and control mice until week 3, at which time hu-PBMC-NSG mice steadily lost weight (Figure 1e). hu-PBMC-NSG mice developed significant signs of disease from 3 weeks post-hPBMC injection, with significantly increased clinical score over time compared to control mice (Figure 1f). As a result, hu-PBMC-NSG mice had significantly reduced survival compared to control mice over 10 weeks, with the majority of hu-PBMC-NSG mice succumbing to disease by 10 weeks, whilst all control mice remained alive during this time (Figure 1g). The median survival time of hu-PBMC-NSG mice was 37.5 days. Collectively, these data reveal that following injection of hPBMCs, NSG mice engraft hCD4+ and hCD8+ T cells and develop signs of clinical GVHD from 3 weeks.

hu-PBMC-NSG mice engraft hCD45+ hCD3+ T cells in the spleen and display circulating hIFNγ
Spleens from control and hu-PBMC-NSG mice at endpoint were examined by flow cytometry (Figure 2a). As above, control mice lacked hCD45+ cells, whilst hu-PBMC-NSG mice contained hCD45+ cells (Figure 2b); the majority of which were hCD3+ T cells (Figure 2c). In contrast to the data at 3 weeks, the hCD3+ T cell population contained a significantly greater proportion of hCD4+ T cells than hCD8+ T cells (Figure 2d).

P2X7 activation has an emerging role in GVHD in hu-PBMC-NSG mice (34-36). qPCR analysis of spleens from control and hu-PBMC-NSG mice demonstrated similar expression of mP2rx7 (Figure 2e). Moreover, qPCR revealed the presence of hP2RX7 in the spleens of hu-PBMC-NSG mice, as well as the presence of hP2RX7B (Figure 2f), an isoform associated with lymphocyte proliferation (39). The human housekeeping gene (hHPRT1), hP2RX7 and hP2RX7B were not detected in control mice, despite detection of mGapdh in these same samples (data not shown).

Human IFNγ is produced by human T cells and has been shown to play important roles in GVHD development (8). Thus, hIFNγ concentrations in sera from endpoint mice were assessed by ELISA. hIFNγ was detected in all hu-PBMC-NSG mice, but no control mice tested (Figure 2g). Combined, these data indicate that hu-PBMC-NSG mice display a greater proportion of hCD4+ T cells compared to hCD8+ T cells, and express both mP2rx7 and hP2RX7 in the spleen, and have circulating human IFNγ.

**hu-PBMC-NSG mice engraft gut-homing T cells but do not develop histological signs of gut GVHD**

The gastrointestinal tract is a major target of GVHD (2). Thus, the current study aimed to characterise gut GVHD in hu-PBMC-NSG mice. Integrin β7 is an important homing molecule that directs various T cell subsets to the gut, and has been linked to GVHD progression (6). To first assess the presence of integrin β7 on circulating T cells, PBMCs
were isolated from the blood of seven healthy donors and integrin β7 expression on CD4⁺ or CD8⁺ T cell subsets examined by flow cytometry. Integrin β7 was expressed on the majority of CD4⁺ T cells, but a significantly smaller proportion of CD8⁺ T cells (Figure 3a). Next, integrin β7 expressing T cells were then examined in hu-PBMC-NSG mice. Again, integrin β7 was expressed on the majority of hCD4⁺ T cells and a significantly smaller proportion of hCD8⁺ T cells in the blood of hu-PBMC-NSG mice at 3 weeks post-hPBMC injection (Figure 3b), and in the spleens of these mice at endpoint (Figure 3c).

Tissue from the duodenum, jejunum, ileum and colon were collected from mice at endpoint, stained with haematoxylin and eosin, and examined for signs of histological GVHD. There was limited evidence of histological GVHD throughout the gut of hu-PBMC-NSG mice with mild immune cell infiltration, no crypt degeneration and few apoptotic bodies (Figure 3d). As expected, guts of control mice displayed normal histology (Figure 3d). Of note, there was no evidence of diarrhoea from hu-PBMC-NSG mice (results not shown), as occasionally reported in other mouse models of GVHD (18). Thus, hu-PBMC-NSG mice engraft gut-homing T cells but display limited evidence of histological gut GVHD.

**mP2rx7 and mReg3g expression are upregulated in the duodenum and ileum of hu-PBMC-NSG mice, while gut length is unaltered**

It was hypothesised that shortened gut length may serve as an indicator of gastrointestinal GVHD as evidenced in inflammatory bowel disease in humans and mice (40, 41). Following euthanasia the gastrointestinal tracts were removed from control and hu-PBMC-NSG mice and the lengths of the lower gastrointestinal tract (duodenum to colon inclusive), small intestine and large intestine were measured. There was no significant difference in the lengths of the lower gastrointestinal tract, small intestine or large intestine between hu-PBMC-NSG and control mice (Figure 4a).
Despite limited changes in gut histology or length between mouse groups, qPCR analysis revealed that mP2rx7 was significantly upregulated in the duodenum and ileum, but not jejunum or colon, of hu-PBMC-NSG mice compared to control mice (Figure 4b). In contrast, hP2RX7 and hP2RX7B expression in each of these regions of the gut was limited, with no significant differences between sites or isoforms (Figure 4c).

To determine if the increased expression of mP2rx7 in the duodenum and ileum observed above corresponded to changes in other P2X receptors commonly expressed on murine leukocytes (42, 43) associated with GVHD (44, 45), mP2rx1 and mP2rx4 in these tissues was assessed by qPCR. There was no difference in mP2rx1 expression in either the duodenum or ileum between hu-PBMC-NSG and control mice (Figure 4d). In contrast, mP2rx4 was significantly increased in the duodenum and partly increased in the ileum (albeit not significantly) of hu-PBMC-NSG mice compared to control mice (Figure 4e).

With limited histological evidence of GVHD in the gut, the current study sought to determine if molecular signs of gut GVHD were present. REG3α is a clinical biomarker of human gut GVHD (12), whilst the murine homologue, REG3γ, is increased in the gut and serum of mice with allogeneic GVHD (13). Therefore, relative mReg3g expression was measured in control and hu-PBMC-NSG mice by qPCR. mReg3g expression in the duodenum and ileum, but not jejunum and colon, was significantly increased in hu-PBMC-NSG mice compared to control mice (Figure 4f). ELISA measurements revealed that there was no significant difference in serum REG3γ concentrations between hu-PBMC-NSG mice and control mice (Figure 4g). Notably, seven (47%) hu-PBMC-NSG mice had REG3γ concentrations greater than two standard deviations above the control mean, whilst three (20%) hu-PBMC-NSG mice had REG3γ concentrations two standard deviations below the control mean.

Since IL-22 can regulate REG3γ and is involved in the pathogenesis of various gut disorders (46) including GVHD (47), the expression of mIL22 and hIL22 in duodenum and ileum,
tissues in which mReg3g expression was found to be significantly increased in hu-PBMC-NSG mice above, were assessed by qPCR. mIl22 was not detected in duodenum or ileum from control or hu-PBMC-NSG mice (n = 4 and n = 8, respectively; data not shown). Whilst, hIL22 was only detected in the duodenum of one of 8 hu-PBMC-NSG mice (data not shown). Collectively, these data reveal that mP2rx7 and mReg3g are upregulated in the duodenum and ileum of hu-PBMC-NSG mice, and that these changes correspond to increases in mP2rx4 expression but not mP2rx1, mIl22 or hIL22 expression.

hu-PBMC-NSG mice engraft skin-homing T cells and develop cutaneous GVHD, including ear swelling

The skin is a major target of GVHD and a leading cause of morbidity in HSCT recipients (14). Thus, the current study aimed to characterise cutaneous GVHD in hu-PBMC-NSG mice. Cutaneous lymphocyte antigen (CLA) is an important skin homing molecule for T cells, which has been linked to cutaneous GVHD (7). To assess the presence of CLA on circulating T cells, PBMCs were isolated from the blood of seven healthy donors and CLA expression on CD4+ or CD8+ T cell subsets examined by flow cytometry. CLA was present on approximately half of the CD4+ T cells, but near absent on CD8+ T cells (Figure 5a). Next, CLA expressing T cells were examined in hu-PBMC-NSG mice. Compared to PBMCs directly isolated from human blood, hCD4+ and hCD8+ T cells in the blood of hu-PBMC-NSG mice at 3 weeks post-hPBMC injection displayed high proportions of CLA+ T cells, with significantly higher proportions of CLA+ hCD4+ T cells compared to CLA+ hCD8+ T cells (Figure 5b). In contrast, there was no difference in the proportions of hCD4+ or hCD8+ T cells expressing CLA in the spleens of these mice at endpoint (Figure 5c).

Flank skin and ears from endpoint mice were collected and examined for evidence of histological GVHD. Both the flank skin and ears of hu-PBMC-NSG mice displayed signs of histological GVHD, with the presence of apoptotic bodies, epidermal hyperplasia
(thickening) and separation of the dermal-epidermal junction (Figure 5d). As expected, control mice did not display histological evidence of GVHD (Figure 5d).

Measurements of ear swelling have long been used as an assay of cutaneous inflammation (48), but to our knowledge, this assay has not been used to assess GVHD in mice. As such, ear thickness, as a measure of ear swelling, was assessed thrice weekly in control and hu-PBMC-NSG mice over 10 weeks. Mean ear thickness in control mice remained unchanged over this time (Figure 5e). In contrast, mean ear thickness in hu-PBMC-NSG mice increased from week 3, reaching a maximum plateau by week 6 and was significantly increased compared to that of control mice (Figure 5e).

qPCR analysis revealed mP2rx7 expression was significantly upregulated in the flank skin of hu-PBMC-NSG mice compared to control mice (Figure 5f). In contrast, mP2rx7 expression was decreased in the ears of hu-PBMC-NSG mice compared to control mice, although this difference failed to reach statistical significance due to one outlier in the hu-PBMC-NSG group (Figure 5f). qPCR analysis also revealed that both hP2RX7 and hP2RX7B were expressed in the flank skin and ears of hu-PBMC-NSG mice, with no significant differences between sites or isoforms (Figure 5g).

As for gut tissues above, mP2rx1 and mP2rx4 expression in the flank skin of mice was analysed by qPCR. Similar to the duodenum and ileum, there was no difference in mP2rx1 expression in flank skin between the two groups (Figure 5h), but mP2rx4 expression was significantly increased in this tissue from hu-PBMC-NSG mice compared to control mouse (Figure 5i). Thus, hu-PBMC-NSG mice engraft skin homing T cells and develop cutaneous GVHD, corresponding to increases in both mP2rx7 and mP2rx4 but not mP2rx1 expression, or the expression of hP2RX7 in the skin.

hu-PBMC-NSG mice develop hepatic GVHD with human T cells present in the liver
The liver is another major target of GVHD (16). Thus, the current study examined hepatic GVHD in hu-PBMC-NSG mice. Histological analysis revealed that hu-PBMC-NSG mice showed increased immune cell infiltration of the liver compared to control mice, and early signs of bile duct destruction (Figure 6a). King et al (19) demonstrated that hCD45+ cells were present in the livers of humanised mice. The current study confirmed, and expanded upon, this finding by showing the presence of hCD45+ cells in digested livers by flow cytometry, the majority of which were hCD3+ T cells (Figure 6b). qPCR analysis revealed similar mP2rx7 expression in the livers of hu-PBMC-NSG and control mice (Figure 6c). Moreover, both hP2RX7 and hP2RX7B were expressed in the livers of hu-PBMC-NSG mice, with no difference between isoforms (Figure 6d). Thus, hu-PBMC-NSG mice develop hepatic GVHD, corresponding with the presence of human T cells and hP2RX7 expression, but not changes in mP2rx7 expression, in the liver.

**hu-PBMC-NSG mice develop histological evidence of lung GVHD**

Previous studies have reported involvement of the lung in hu-PBMC-NSG mice (19). Therefore, the current study examined pulmonary GVHD in hu-PBMC-NSG mice. Histological analysis revealed increased immune cell infiltration into the lungs of hu-PBMC-NSG mice compared to those of control mice (Figure 7a). qPCR analysis revealed similar mP2rx7 expression in the lungs of both control and hu-PBMC-NSG mice (Figure 7b). Moreover, both hP2RX7 and hP2RX7B were expressed in the lungs of hu-PBMC-NSG mice, with no difference between isoforms (Figure 7c). Thus, hu-PBMC-NSG mice develop pulmonary GVHD, corresponding with the presence of hP2RX7 expression.


Discussion

The current study aimed to examine mP2rx7 and hP2RX7 expression in GVHD target organs of humanised mice, and further characterise disease impact in these organs. In this study, injection of hPBMCs into NSG mice resulted in the development of GVHD with clinical signs increasing over time, and weight loss commencing from week 3. The rate of disease progression was slower in the current study compared to other studies which involved the same injection regime of hPBMCs (19, 49). This difference is presumably due to the absence of irradiation in the present study, compared to pre-transplant irradiation used in these former studies. In contrast, disease progression was similar to previous works from our group in which mice were not irradiated (23, 34-37). The current study also confirmed the engraftment of human leukocytes, predominately CD4+ and CD8+ T cells. Engraftment of human leukocytes was similar to previous studies of irradiated and non-irradiated mice which demonstrated that hCD45+ cells make up between 40-80% of the leukocyte population, and that the majority of these cells are hCD3+ (19, 23, 34-37, 49). Furthermore, the current study further confirmed that the proportion of CD4+ T cells is greater than that of CD8+ T cells at endpoint, similar to previous studies from our group (23, 34-37) and others (19, 49).

The current study examined the expression of mP2rx7, hP2RX7 and hP2RX7B in GVHD target tissues of control and hu-PBMC-NSG mice. mP2rx7 tended to be higher in hu-PBMC-NSG mice compared to control mice but such increases only reached statistical significance in the duodenum, ileum and skin. These increases in mP2rx7 expression in the duodenum and skin, and to a lesser extent the ileum, corresponded to increases in mP2rx4, but not mP2rx1 expression. This suggests that the increases in mP2rx7 expression in these tissues may be due to the infiltration of macrophages, which co-express both P2X7 and P2X4 (43). Notably, mP2rx7 expression in the ear was an exception to the above tissues with gene expression decreased. Whether this reflects a compensatory mechanism or possible changes in ear
cartilage, which also express P2X7 (50), remains unknown. hP2RX7 and hP2RX7B expression was also observed in all examined tissues. Given the expression of hP2X7 on T cells (51) and other human leukocytes (37), this indicates that human leukocytes were present in these tissues from most mice. Finally, it should be noted that relative mP2rx7 expression in tissues from NSG mice closely resembles hP2RX7 expression profiles seen in humans (52), with greatest expression in the skin, followed by the lungs, spleen, liver and gut.

Although flow cytometric analyses in hu-PBMC-NSG mice in former studies (19, 23, 34-37, 49) is extensive, the histological analysis of GVHD tissues in each is limited. The current study provides the broadest tissue analysis of this model to date, including analysis of four regions of the gut; duodenum, jejunum, ileum and colon. Previous studies have reported blunting of villi (53) and lymphocyte infiltration of intestinal crypts (19, 53). In contrast to previous studies (19, 53), and despite the presence of potential gut-homing integrin β7+ human T cells, histological evidence of gut GVHD was limited in hu-PBMC-NSG mice in the current study. Recent evidence suggests that radiation plays a large role in controlling gut-homing T cells in allogeneic models of GVHD (54). Furthermore, NSG mice, and other strains encoding the IL-2Rγnull mutation, show reduced T cell trafficking and infiltration into the gastrointestinal tract (55). Thus, the absence of irradiation in the current model and defects in gut homing in NSG mice provides an explanation for the limited gut GVHD observed in the current study.

Despite limited evidence of histological gut GVHD, the current study provided molecular evidence of gut GVHD with increased mReg3g expression in the duodenum and ileum. These increases did not correspond to an increase in mIl22 or the presence of hIL22, which were largely absent in these tissues, suggesting that up-regulation of mReg3g expression in hu-PBMC-NSG mice may be IL-22-independent. Of note, the increase in mReg3g in the gut regions paralleled increases in mP2rx7, suggesting these regions may be more susceptible to
GVHD. Moreover, there was an increased trend in serum REG3γ consistent with gut GVHD. However, serum REG3γ was increased in only some hu-PBMC-NSG mice and failed to correlate with integrin β7+ human T cells or the limited histological damage (data not shown). Increases in serum REG3α/REG3γ concentrations are thought to be due to damage of the mucosal epithelial cells (12). However, given that some hu-PBMC-NSG mice displayed relatively high concentrations of REG3γ compared to control mice, this may suggest that this antimicrobial peptide may also enter circulation in the absence of epithelial cell damage.

The current study also provides the most thorough analysis of cutaneous GVHD in hu-PBMC-NSG mice. King et al (19) originally reported minimal histological GVHD involvement in the skin of humanised mice; however more recent studies reported lymphocyte infiltration in the skin of such mice (34, 49). Consistent with these previous findings, the current study identified extensive lymphocyte infiltration and signs of apoptosis in flank skin of hu-PBMC-NSG mice. This was further expanded by observing the same histological signs of cutaneous GVHD in the ears of these mice. GVHD damage in the ears was also consistent with increases in ear swelling over time, shown for the first time in any mouse model of GVHD. Changes in ear thickness provide a quantitative method for determining cutaneous GVHD in mouse models and could be used alongside traditional clinical measures such as scaling of skin and fur loss. Although the proportions of CLA+ T cells did not correlate with histological evidence of GVHD or ear swelling (results not shown), others have shown that these cells home to the skin of hu-PBMC-NSG mice (49).

The present study revealed histological evidence of hepatic GVHD in hu-PBMC-NSG mice consistent with previous findings (19, 34). We also demonstrated that a large proportion of leukocytes in the liver were of human origin consistent with King et al (19). This was further expanded with the current study demonstrating that the majority of these human leukocytes were T cells, as similarly seen in the spleens of hu-PBMC-NSG mice. Of note, our previous
studies have revealed that activation of P2X7 plays an important role in hepatic GVHD in hu-PBMC-NSG mice (35, 36), but despite this the expression of mP2rx7 was similar between hu-PBMC-NSG and control mice. This may suggest activation of P2X7 to mediate hepatic GVHD in this model may be regulated by P2X7 present elsewhere in these mice.

Finally, the current study revealed that lungs are also a target in this hu-PBMC-NSG model of GVHD. Lung involvement following HSCT is used to diagnose chronic GVHD (56), however lung involvement has also been reported in patients with acute GVHD (57). Similar to the current study, previous allogeneic (58) and humanised (19, 22) mouse studies have reported immune cell infiltration in the lungs of mice with GVHD. Therefore, even though the lung is involved, hu-PBMC-NSG mice can still be considered a model of acute GVHD.

In conclusion, this study demonstrated that mP2rx7 expression was increased in the duodenum, ileum and skin of hu-PBMC-NSG mice supporting a role for P2X7 in these organs. hP2RX7 is present in target tissues of GVHD, consistent with human leukocyte infiltration into these tissues. The present study also demonstrated that the skin, liver and lung are highly involved in this model, with limited histological gut involvement. Finally, increased mReg3g expression and ear swelling in hu-PBMC-NSG mice offer potential measurements of early stage gut, and later stage skin GVHD, respectively.
Perspectives

- P2X7 plays an important role in GVHD but expression has not been characterised in disease tissues in humanised mouse models of GVHD, and disease impact in these tissues is oftentimes not reported
- Hu-PBMC-NSG mice have increased mP2rx7 and mReg3g expression in areas of the gut, but histological gut GVHD was limited compared to the skin, liver and lungs which displayed severe signs of disease
- Better characterisation of P2X7, and GVHD impact, in the gut, skin, liver and lung in this humanised mouse model will allow for a more thorough analysis when trialling therapeutics to reduce or prevent GVHD

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Conflict of Interest

Peter Cuthbertson declares no conflict of interest
Sam Adhikary declares no conflict of interest
Nicholas Geraghty declares no conflict of interest
Thomas Guy declares no conflict of interest
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Author Contribution Statement

The majority of experiments were performed by P Cuthbertson with assistance from S Adhikary. N Geraghty and T Guy provided assistance with flow cytometry. T Guy, A Hadjiashrafi and S Fuller provided assistance with histology. D Ly collected the majority of human blood samples. P Cuthbertson, D Watson and R Sluyter designed the project. D Ly, D Watson and R Sluyter supervised the project. The manuscript was drafted by P Cuthbertson, and edited by D Watson and R Sluyter. All authors revised the manuscript.
References


Figure Legends

Figure 1: NSG mice injected with hPBMCs contain circulating human T cells and develop clinical GVHD. (a-e) NSG mice were injected i.p. with 10 x 10⁶ hPBMCs or PBS on day 0. (a-d) Blood from all mice (week 3) was examined by flow cytometry for the presence of leukocytes. (a) Cells were gated as shown and the proportion of (b) hCD45+ or (c) hCD3+ T cells of hCD45+ cells, and (d) hCD4+ or hCD8+ cells of hCD3+ T cells determined. All mice were monitored 3 times a week for up to 10 weeks for (e) weight loss, (f) clinical score and (g) survival. (b-f) Data represented as group mean ± standard deviation. (g) Data represented as percent survival. (b-d) Symbols represent individual mice. (b-g) n = 4, control mice; n = 26 hu-PBMC-NSG mice. Significance of hu-PBMC-NSG versus control determined by (b, d) Student’s t-test, (e, f) repeated measures ANOVA or (g) log-rank (Mantel-Cox). *P < 0.05, **P < 0.01, ***P < 0.001. Non-significant values not shown.

Figure 2: hu-PBMC-NSG mice engraft hCD45+ hCD3+ T cells in the spleen and display circulating hIFNγ. (a-d) Spleens from control and hu-PBMC-NSG mice (endpoint) were examined by flow cytometry. (a) Cells were gated as shown and the proportion of (b) hCD45+ or (c) hCD3+ T cells of hCD45+ cells, and (d) hCD4+ or hCD8+ cells of hCD3+ T cells determined. Relative expression of (e) mP2rx7, and (f) hP2RX7 and hP2RX7B in spleen at endpoint were determined by qPCR. (g) Serum hIFNγ at endpoint was measured by ELISA. (b-g) Data represented as group mean ± standard deviation; symbols represent individual mice (n = 2-4, control mice; n = 8–26, hu-PBMC-NSG mice). Significance of hu-PBMC-NSG versus control determined by Student’s t-test; *P < 0.05, ***P < 0.001. Non-significant values not shown.

Figure 3: hu-PBMC-NSG mice engraft gut-homing T cells but do not develop histological signs of gut GVHD. Human CD4+ or CD8+ T cells from (a) human blood and (b) mouse
blood (week 3) or (c) spleens (endpoint) from hu-PBMC-NSG mice were examined for integrin β7 expression by flow cytometry. (d) Sections of duodenum, jejunum, ileum and colon from mice at endpoint were examined for histological evidence of GVHD. (a-c) Data represented as group mean ± standard deviation; symbols represent individual (a) humans (n = 7) or (b, c) mice (n = 16). Significance determined by Student’s t-test; **p < 0.01, ***p < 0.001. (d) Scale bar represents 100 µm.

**Figure 4:** mP2rx7 and mReg3g expression is upregulated in the duodenum and ileum of hu-PBMC-NSG mice while gut length is unaltered. (a) Lower gastrointestinal tract (duodenum to colon inclusive) (left panel), small intestine (middle panel) and colon (right panel) length was measured at endpoint. (b-f) Relative expression of (b) mP2rx7, and (c) hP2RX7 and hP2RX7B, (d) mP2rx1, (e) mP2rx4 and (f) mReg3g in gut sections (as indicated) at endpoint were determined by qPCR. (g) Serum REG3γ at endpoint was measured by ELISA. (a-g) Data represented as mean ± standard deviation; symbols represent individual mice (n = 4, control mice; n = 7–15, hu-PBMC-NSG mice). (g) Dashed lines represent two SDs from control mean. Significance of hu-PBMC-NSG versus control determined by Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001. Non-significant values not shown.

**Figure 5:** hu-PBMC-NSG mice engraft skin-homing T cells and develop cutaneous GVHD, including ear swelling. Human CD4+ or CD8+ T cells from (a) human blood and (b) blood (week 3) or (c) spleens (endpoint) from hu-PBMC-NSG mice were examined for cutaneous lymphocyte antigen (CLA) expression by flow cytometry. (d) Sections of flank skin and ears at endpoint were examined for histological evidence of GVHD. (e) Ear swelling in all mice was measured using a spring loaded calliper three times a week for 10 weeks or until endpoint. (f-i) Relative expression of (f) mP2rx7, (g) hP2RX7 and hP2RX7B, (h) mP2rx1 and (i) mP2rx4 in (f-i) skin or (f, g) ears at endpoint were determined by qPCR. (a-c, e-i) Data is represented as mean ± standard deviation; (a-c, f-i) symbols represent individual mice (n = 4,
control mice; n = 8–26 hu-PBMC-NSG mice). Significance of hu-PBMC-NSG versus control determined by Student’s t-test; *P < 0.05, **P < 0.01, ***P < 0.001. Non-significant values not shown. (d) Scale bar represents 100 µm, black arrowheads indicate dermal-epidermal junction separation, red arrowheads indicate apoptotic keratinocytes, stars indicate areas with epidermal thickening.

**Figure 6:** hu-PBMC-NSG mice develop hepatic GVHD with human T cells present in the liver. (a) Sections of liver were examined for histological evidence of GVHD. (b) hCD45+ (left) and hCD3+ (right) cells were examined in the liver by flow cytometry. (c, d) Relative expression of (c) mP2rx7, and (d) hP2RX7 and hP2RX7B in the liver at endpoint were determined by qPCR. (b-d) Data is represented as mean ± standard deviation; symbols represent individual mice (n = 4, control mice; n = 6–8, hu-PBM-NSG mice). Significance of hu-PBMC-NSG versus control determined by Student’s t-test. Non-significant values not shown. (a) Scale bar represents 100 µm.

**Figure 7:** hu-PBMC-NSG mice develop histological evidence of lung GVHD. (a) Sections of lung from control and hu-PBMC-NSG mice were examined for histological evidence of GVHD. (b, c) Relative expression of (b) mP2rx7, and (c) hP2RX7 and hP2RX7B in lungs at endpoint were determined by qPCR. (b, c) Data is represented as mean ± standard deviation; symbols represent individual mice (n = 4, control mice; n = 8, hu-PBMC-NSG mice). Significance of hu-PBMC-NSG versus control determined by Student’s t-test. Non-significant values not shown. (a) Scale bar represents 100 µm.
a. Human Blood

b. hu-PBMC-NSG

c. hu-PBMC-NSG

d. Control

Duodenum

Jejunum

Ileum

Colon