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

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Abstract

Graft-versus-host disease (GVHD) is a life-threatening consequence of allogeneic haematopoietic stem cell transplantation, a curative therapy for haematological malignancies. The ATP-gated P2X7 receptor channel is implicated in the development of GVHD. P2X7 activity on human leukocytes can be influenced by gain-of-function (GOF) and loss-of-function (LOF) single nucleotide polymorphisms (SNPs) in the *P2RX7* gene. In this study, the *P2RX7* gene was sequenced in 25 human donors and the P2X7 activity on subsets of peripheral blood T cells, natural killer (NK) cells and monocytes was measured using an ATP-induced dye uptake assay. GOF and LOF SNPs representing 10 of the 17 known *P2RX7* haplotypes were identified, and correlated with P2X7 activity on all leukocyte subsets investigated. Notably, invariant (i) NK T cells displayed the highest P2X7 activity amongst all cell types studied. To determine if donor P2X7 activity influenced the development of GVHD, immunodeficient NOD-SCID-IL2R γ^{null} (NSG) mice were injected with human peripheral blood mononuclear cells isolated from donors of either GOF (hP2X7^{GOF} mice) or LOF (hP2X7^{LOF} mice) *P2RX7* genotype. Both hP2X7^{GOF} and hP2X7^{LOF} mice demonstrated similar human leukocyte engraftment, and showed comparable weight loss, GVHD clinical score and overall survival. Donor P2X7 activity did not affect human leukocyte infiltration or GVHD-mediated tissue damage, or the relative expression of human P2X7 or human interferon- γ (hIFN γ) in tissues. Finally, hP2X7^{GOF} and hP2X7^{LOF} mice demonstrated similar concentrations of serum hIFN γ . This study demonstrates that P2X7 activity correlates with donor *P2RX7* genotype on human leukocyte subsets important in GVHD development, but does not affect GVHD development in a humanised mouse model of this disease.

Keywords P2X7 receptor · *P2RX7* gene single nucleotide polymorphism · Purinergic receptor · Xenogeneic graft-versus-host disease · Bone marrow transplantation · Leukocyte · Humanised mice

Introduction

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is one of the few established curative therapies for life-threatening haematological malignancies, which aims to eliminate host tumour cells using myeloablative therapy and transplanted donor leukocytes [1]. However, donor leukocytes can also trigger inflammatory damage against healthy host tissues, resulting in graft-versus-host disease (GVHD) [2]. The initial source of inflammatory damage that promotes GVHD is often the myeloablative regime used to treat the haematological malignancy [3]. This promotes activation of GVHD effector leukocytes which release pro-inflammatory cytokines, such as interferon- γ (IFN γ), that enhance GVHD development [4]. GVHD remains a problem in about half of

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allo-HSCT patients due to ineffective therapies to treat or prevent this disease [5], and the absence of validated biomarkers to predict GVHD onset [6]. Recently, donor single nucleotide polymorphisms (SNPs) in genes important in inflammation have been recognised as possible risk factors for GVHD [7, 8]. Donor SNPs may have potential as future GVHD biomarkers, but these SNPs are not currently screened in allo-HSCT donors. Combined, this highlights a need for new GVHD biomarkers and therapeutics.

The purinergic signalling cascade is a key mediator of the inflammatory immune response, and has an emerging role in the development of GVHD through activation of the P2X7 receptor, an ATP-gated cation channel [9]. P2X7 activation by extracellular ATP, which can be released during tissue damage, initiates the release of pro-inflammatory cytokines which mediate cell death and proliferation, and can trigger inflammatory tissue damage [10]. P2X7 blockade can reduce GVHD severity in allogeneic mouse models [11–13], and reduce concentrations of serum human IFN γ in humanised mice with GVHD [14]. This indicates an important role for P2X7 in mouse models of GVHD, but there are limited studies investigating its effects on GVHD development in humans.

In humans, P2X7 is predominantly expressed on leukocytes, with the highest expression on monocytes followed by natural killer (NK) cells, B cells and T cells [15, 16]. P2X7 activation causes formation of a pore that allows the flux of organic cations including fluorescent dyes such as YO-PRO-1²⁺, providing a method to reliably quantify P2X7 activity on human leukocytes with minimal variation [17, 18]. P2X7 activation on these cells can drive their differentiation into many effector subsets, which can have important roles in GVHD. For example, P2X7 activation on naive CD4⁺ T cells can increase expression of IFN γ , interleukin (IL)-17 and IL-6, which collectively promote T helper (Th) 17 cell responses [19] that are key in GVHD progression [20]. Moreover, P2X7 activation also impacts regulatory T cells (Tregs), which limit GVHD development [21], by potentially regulating Treg numbers [22] and their suppressive functions [23].

One of the biggest influences on human P2X7 activity is SNPs in the human *P2RX7* gene. To date, 16 missense *P2RX7* SNPs have been identified which can cause either a gain-of-function (GOF) or loss-of-function (LOF) in human P2X7 [24]. Notably, four of these SNPs originally defined five haplotype variants (denoted P2X7–1 to 5) which give rise to altered P2X7 activity [25, 26]. Subsequently, analysis of a further seven SNPs revealed that these five haplotypes can be segregated into 17 haplotypes (H1 to H17) [27]. Notably, some *P2RX7* SNPs are associated with various inflammatory diseases such as multiple sclerosis [28] and rheumatoid arthritis [29]. Moreover, the LOF SNP rs3751143 (E496A) in either donors or recipients is associated with poorer clinical outcomes in HSCT [30], although this finding was not supported by a larger study [31]. Therefore, further investigation into the

role of *P2RX7* SNPs in GVHD is warranted. The current study investigated the effect of human donor *P2RX7* genotype on P2X7 activity on leukocyte subsets important in GVHD, and whether altered P2X7 activity affects the development of GVHD in a pre-clinical humanised mouse model of this disease.

Materials and methods

Antibodies

R-Phycoerythrin (PE) conjugated mouse anti-human (h) CD4 (clone RPA-T4) and mouse anti-hCD25 (clone M-A251) monoclonal antibodies (mAb); PE-cyanine 7 (PE-Cy7) conjugated mouse anti-hCD8 (clone RPA-T8), mouse anti-hCD56 (clone B159) and mouse anti-hCD3 (clone SK7) mAb; peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5) conjugated mouse anti-hCD4 (clone SK3), mouse anti-hCD16 (clone 3G8) and rat anti-mouse (m) CD45 (clone 30-F11) mAb; allophycocyanin (APC) conjugated mouse anti-hCD3 (clone UCHT1), mouse anti-hCD45 (clone HI30) and mouse anti-hCD19 (clone HIB19) mAb; Brilliant Violet 711 (BV711) conjugated mouse anti-hCD3 (clone UCHT1) mAb; Brilliant Violet 421 (BV421) conjugated mouse anti-hCD127 (clone HL-7R-M21) and mouse anti-hCD14 (clone MΦPg) mAb were obtained from BD Biosciences (San Jose, USA). PE-Cy7 conjugated mouse anti-hV α 24-J α 18 (clone 6B11) mAb was obtained from BioLegend (San Diego, CA, USA).

Mice

Experiments involving mice were approved by the University of Wollongong Animal Ethics Committee (Wollongong, Australia). Female NOD-SCID-IL2R γ null (NSG) mice (aged 6–8 weeks) were obtained from Australian BioResources (Moss Vale, Australia) and housed in individually ventilated cages (Techniplast; Buguggiate, Italy) with a 12-h light/12-h dark cycle. Mice were provided with autoclaved food and water, ad libitum.

Isolation of genomic DNA

Experiments involving human blood were approved by the University of Wollongong Human Ethics Committee (Wollongong, Australia). Whole blood was collected from consenting donors, by venepuncture, into VACUETTE® lithium heparin tubes (Greiner Bio-One; Frickenhausen, Germany). Genomic DNA was isolated from peripheral blood using a Wizard® Genomic DNA Isolation Kit (Promega; Madison, USA) as per the manufacturer's instructions. DNA was stored at –20 °C. Blood was collected from a total of 25

healthy adult donors (range 20–44 years, mean 28 years; 12 males and 13 females; 24 Caucasian and 1 Asian).

***P2RX7* gene sequencing**

Exons 1–13 of the human *P2RX7* gene were amplified and sequenced by PCR [32] using 12 primer pairs (Invitrogen; Carlsbad, USA) as originally described [33]. PCR was conducted using Mango *Taq* DNA polymerase (Bioline; London, UK) with an initial denaturation step of 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s (for exons 1, 2 and exons 4–13) or 55 °C for 30 s (for exon 3) and extension at 72 °C for 1 min, before a final extension for 5 min at 72 °C using a GeneAmp PCR System 9700 (Applied Bio Systems; Carlsbad, USA). Amplicons were visualised using a 1.5% v/v Tris acetate EDTA (TAE) (20 mM acetic acid, 1 mM EDTA, 40 mM Tris, pH 8) agarose gel stained with GelRed™ (Biotium; Hayward, USA). Amplicons for exons 1–12 for 23 donors were purified using ExoSAP-IT enzyme (Invitrogen) before being sequenced with the above primers and the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as per the manufacturer's instructions, using an Applied Biosystems 2130xl Genetic Analyzer. For two donors, amplicons for exons 1–12 were purified using the QIAquick® Purification Kit (Qiagen; Venlo, Netherlands) as per the manufacturer's instructions, and sequenced with the above primers by the Australian Genome Research Facility (AGRF) (Melbourne, Australia). For all donors, exon 13 was sequenced by AGRF using samples excised from TAE agarose gels using a QIAquick® Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. Sequences were analysed using Chromas Pro software (Technelysium; Brisbane, Australia) by comparing to the NCBI wild-type reference sequence for the human *P2RX7* gene (NG_011471.2).

Isolation of human peripheral blood mononuclear cells

Donor peripheral blood was diluted in an equal volume of sterile Dulbecco's phosphate buffered-saline (PBS) (Thermo Fisher Scientific; Waltham, USA), and human peripheral blood mononuclear cells (hPBMCs) isolated by density-gradient centrifugation (560×g for 30 min) using Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden). hPBMCs were washed twice in two volumes of PBS (300×g for 10 min), resuspended in PBS and counted using an improved bright line haemocytometer (Boeco Neubauer; Hamburg, Germany).

ATP-induced YO-PRO-1²⁺ uptake assay

P2X7 activity on human leukocyte subsets was quantified using an ATP-induced YO-PRO-1²⁺ uptake assay, adapted from Spildreorde et al. [32]. hPBMCs were resuspended at 1×10^6 cells/mL in NaCl medium (145 mM NaCl, 5 mM KCl, 5 mM glucose and 10 mM HEPES, pH 7.5). Cells were pre-incubated at 37 °C for 5 min before being incubated with 1 µM YO-PRO-1 iodide (Molecular Probes; Eugene, USA) in the absence or presence of 1 mM ATP (Sigma-Aldrich; St. Louis, USA) for 5 min. Reactions were stopped with 1 mL ice-cold NaCl medium containing 20 mM MgCl₂ and centrifugation (300×g for 3 min). Cells were washed in PBS (300×g for 3 min), incubated with fluorochrome-conjugated mAb at RT for 15 min and washed in PBS (300×g for 3 min). In some experiments, hPBMCs were incubated in the absence or presence of 60 µM 2-(phenylthio)-*N*-[[tetrahydro-4-(4-phenyl-1-piperazinyl)-2*H*-pyran-4-yl]methyl-3 pyridinecarboxamide (JNJ-47965567) (Tocris Bioscience, Bristol, UK) at 37 °C 15 min prior to the addition of YO-PRO-1²⁺. Data was collected using calibration beads (Spherotech; Lake Forest, USA) and a BD Bioscience LSR Fortessa X-20 flow cytometer (using the band pass filters 450/50 for BV421, 710/50 for BV711, 515/20 for YO-PRO-1²⁺, 586/15 for PE, 780/60 for PE-Cy7, 670/30 for APC and 695/40 for PerCP) and BD Biosciences FACSDiva software v8.0. Geometric mean fluorescence intensity of YO-PRO-1²⁺ uptake was analysed with FlowJo software v8.7.1 (TreeStar Inc.; Ashland, USA). ATP-induced YO-PRO-1²⁺ uptake (P2X7 activity) was determined as the difference in YO-PRO-1²⁺ in the presence and absence of ATP from duplicate samples. P2X7 activity was determined prior to *P2RX7* genotyping. Donor numbers used to determine P2X7 activity in different immune cell subsets varied due to availability of mAb at the time of experiments.

Humanised mouse model of GVHD

A humanised mouse model of GVHD was used as described by Geraghty et al. [14]. NSG mice were injected intraperitoneally (i.p.) with 10×10^6 hPBMCs, isolated from either GOF or LOF *P2RX7* genotype donors (matched for age and sex), in 200 µL sterile PBS. These mice were subsequently classified as hP2X7^{GOF} or hP2X7^{LOF} mice, respectively. Mice were checked for the engraftment of human cells at 3 weeks post-injection by immunophenotyping tail blood. Mice were assessed in a blinded fashion for clinical signs of GVHD for 10 weeks post-injection using a monitoring system that scored 5 criteria (weight loss, hunched posture, decreased activity, fur ruffling and skin scaling) out of 3 (total score out of 15) (Table 1). Mice were euthanised if they demonstrated a score of 3 in any category (excluding fur ruffling), exhibited a total score ≥ 10 , displayed $\geq 10\%$ weight loss over a 7-day period or survived 10 weeks post-injection, according to the

Table 1 Clinical scoring criteria for the assessment of GVHD in humanised NSG mice. NSG mice, injected (i.p.) with 10×10^6 hPBMCs, were monitored for GVHD development by scoring for weight loss, posture, activity, fur loss and skin integrity for up to 10 weeks post-PBMC injection

Score ¹	0	1	2	3
Weight loss ^{2, 3}	< 5%	5 to 9.9%	10 to 15%	> 15%
Hunched posture	Normal	Hunching noted at rest only	Hunching noted at rest and movement	Severe hunching
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated	Permanently stationary
Fur loss	Normal	Mild to moderate ruffling	Severe ruffling and hair loss (thinning)	Areas of hair loss (balding)
Skin integrity	Normal	Scaling of paws and/or tail	Scaling at additional areas	Areas of denuded skin (loss of surface layers)

¹ Mice were euthanised if they scored 3 in any category (excluding fur) or at a total score ≥ 10

² Weight loss determined as percentage loss from starting weight (weight on day 0)

³ Acute weight loss determined as > 10% weight loss over any 7-day period and assigned a score of 3 for weight

approved animal ethics protocol. At euthanasia, samples of spleens were collected for immunophenotyping. Samples of spleen, liver, skin and small intestine were stored in either RNAlater (Sigma-Aldrich) at -20°C for quantitative real-time PCR (qPCR) analysis, or fixed overnight in neutral buffered (10%) formalin (Sigma-Aldrich) and embedded in paraffin for histological analysis. Serum was isolated from mouse blood collected by cardiac puncture, following incubation at RT for 1 h and centrifugation ($1700 \times g$ for 10 min). Sera were stored at -80°C .

Immunophenotyping

Immunophenotyping was performed on cells isolated from mouse tail blood at 3 weeks post-injection and on splenocytes from euthanised mice. Tail blood was collected via tail snip into 200 μL of citrate solution (Sigma-Aldrich). Blood was diluted with PBS and centrifuged ($300 \times g$ for 5 min). Mouse spleens were dissociated in PBS, passed through a 70- μm nylon filter (Falcon Biosciences; New York, USA) and centrifuged ($300 \times g$ for 5 min). Cells were incubated in 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 at RT, washed twice in PBS containing foetal bovine serum (Bovogen Biologicals; Keilor East, Australia) ($300 \times g$ for 5 min) and incubated with fluorochrome conjugated mAb for 15 min at RT. Cells were washed in PBS ($300 \times g$ for 3 min) and data were collected using a BD LSR Fortessa X-20 flow cytometer (using the band pass filters 450/50 for BV421, 710/50 for BV711, 586/15 for PE, 780/60 for PE-Cy7, 670/30 for APC and 695/40 for PerCP) and FACSDiva software v8.0. Cell populations were analysed using FlowJo software v8.7.1.

Histological analysis

Embedded tissue was sectioned (5 μm) and stained with haematoxylin and eosin (POCD; Artarmon, Australia) as previously described [14]. Histology was assessed using a Leica (Wetzlar, Germany) DM750 inverted light microscope at $\times 10$

objective and images captured using a Leica ICC50 HD camera and processed using Leica application suite software version 4.7.

RNA isolation

RNA was isolated, from tissue stored in RNAlater, using TRIzol reagent (Thermo Fisher Scientific) as per the manufacturer's instructions. RNA was converted to complementary DNA (cDNA) using a qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, USA) as per the manufacturer's instructions and stored at -80°C . cDNA was checked by PCR using primers (Invitrogen) to the housekeeping gene *GAPDH* (forward primer 5'-TGCACCACCAACTGCTTAGC-3'; reverse primer 5'-GGAAGGCCATGCCAGTGA-3'). PCR was performed with an initial cycle of 95°C followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension of 72°C for 10 min. Amplicons were visualised with a 1.5% TAE agarose gel stained with GelRed^{TM}.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on cDNA using TaqMan Universal Master Mix II (Thermo Fisher Scientific) according to the manufacturer's instructions. qPCR was conducted with FAM^{TM}-labelled human *IFNG* (Hs00989291_m1) or human *P2X7B* (AIOIXC2) primers, with VIC®-labelled human *HPRT-1* (Hs99999909_m1) primers used for this housekeeping gene (Thermo Fisher Scientific). qPCR cycles consisted of an initial step of 50°C for 2 min, a subsequent 50°C step for 10 min and 50 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were conducted in triplicate using a Roche Diagnostics (Indianapolis, USA) LightCycler 480 with LightCycler480 software v1.5.1. Gene expression was quantified using the $\Delta\Delta\text{Ct}$ method and was calculated relative to the gene expression in cDNA generated from freshly isolated hPBMCs from one randomly selected individual.

Table 2 *P2RX7* genotype and predicted P2X7 activity of healthy human donors. Genomic DNA was isolated from whole blood of 25 human donors. Donor *P2RX7* genotype was determined by sequencing the 13 exons in the human *P2RX7* gene using exon-specific primers.

Donors were classed into *P2RX7* haplotypes based on the presence or absence of different *P2RX7* SNPs according to Stokes et al. [25] (P2X7–1 to 5) and Jørgensen et al. [27] (H1–H17). Predicted P2X7 activity is based on the *P2RX7* SNPs found in each donor

	P2RX7 Genotype									
Donor ¹	H155Y rs208294	R270H rs7958311	R307Q rs28360457	A348T rs1718119	T357S rs2230911	Q460R rs2230912	E496A rs3751143	Haplotype (P2X7-1 to 5)	Haplotype (H1to H17)	Predicted activity ²
	GOF	LOF	LOF	GOF	LOF	Neutral	LOF			
☒	Y/Y	WT	WT	T/T	WT	R/R	WT	P2X7-4/4	H14/H14	GOF
◐	Y/Y	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H10/H10	GOF
◑	WT	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H2/H8	GOF
○	WT	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H8/H8	GOF
◒	WT	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H8/H8	GOF
▲	WT	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H2/H8	GOF
◐	H/Y	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H2/H5/H8/H10	GOF
◑	Y/Y	WT	WT	T/T	WT	R/R	WT	P2X7-4/4	H14/H14	GOF
●	WT	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H8/H8	GOF
◑	WT	WT	WT	T/T	WT	Q/R	WT	P2X7-2/4	H8/H15	GOF
■	H/Y	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H2/H8/H5/H10	GOF
▼	Y/Y	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H10/H10	GOF
▲	Y/Y	WT	WT	T/T	WT	R/R	WT	P2X7-4/4	H14/H14	GOF
◐	Y/Y	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H5/H10	GOF
⊗	Y/Y	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H5/H10	GOF
◐	WT	WT	WT	T/T	WT	WT	WT	P2X7-1/2	H8/H8	GOF
△	Y/Y	WT	WT	T/T	WT	Q/R	WT	P2X7-2/4	H10/H14	GOF
⊕	WT	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H2/H8	GOF
◆	Y/Y	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H10/H10	GOF
◻	Y/Y	R/H	WT	WT	WT	WT	E/A	P2X7-1/3	H3/H5/H11	LOF
◆	WT	WT	WT	WT	T/S	WT	WT	P2X7-1/5	H2/H16	LOF
◻	Y/Y	WT	WT	WT	WT	WT	E/A	P2X7-1/3	H5/H11	LOF
●	WT	H/H	R/Q	WT	WT	WT	WT	P2X7-1/1	H1/H7	LOF
◻	WT	WT	WT	WT	WT	WT	E/A	P2X7-1/3	H2/H12	LOF
◻	WT	WT	WT	WT	WT	WT	E/A	P2X7-1/3	H2/H12	LOF

¹ Symbols represent individual donors in Figs. 1 and 2

² Predicted activity based on the presence of *P2RX7* SNPs in each donor

GOF, gain-of-function; LOF, loss-of-function

Colours represent P2X7 activity: green, GOF; red, LOF

All donors were wild type for unlisted non-synonymous *P2RX7* SNPs

Fig. 1 Donor P2X7 activity correlates with *P2RX7* genotype on human T cell subsets. **a–k** hPBMCs, from donors of either GOF or LOF *P2RX7* genotype, resuspended in NaCl medium were incubated with 1 μ M YO-PRO-1²⁺ in the absence or presence of 1 mM ATP for 5 min at 37 °C. YO-PRO-1²⁺ uptake into human T cell subsets was analysed by flow cytometry using the gating strategy shown. **a** Single cells were gated by forward scatter area (FSC-A) and height (FSC-H). **b** Viable single lymphocytes were gated using FSC-A and side scatter (SSC-A). Single viable cells were then used to identify **c** CD3⁺ T cells, **d** CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells and **e** CD3⁺CD4⁺CD25⁺CD127^{lo} Tregs. Single, viable cells were also used to gate **f** CD3⁺CD19⁺ cells to subsequently identify **g** CD3⁺Vα24⁺Jα18⁺ iNKT cells. **h** P2X7 activity was determined by comparing YO-PRO-1²⁺ uptake in the absence (blue histograms) and presence (orange histogram) of ATP; histograms for CD3⁺ T cells are shown as examples. P2X7 activity in **i** CD3⁺, CD3⁺CD4⁺ and

CD3⁺CD8⁺ T cells and Tregs was determined in multiple donors and P2X7 activity in **j** iNKT cells and corresponding CD3⁺ T cells was determined in some donors. **k** hPBMCs from three donors were pre-incubated with 60 μ M JNJ-47965567 (JNJ) at 37 °C for 15 min to block P2X7 before incubation in the absence or presence of 1 mM ATP. Symbols correlate to symbols in Table 2 and represent **i–j** mean values of duplicate ATP-induced YO-PRO-1²⁺ uptake measurements into CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺ (GOF, $n = 19$; LOF, $n = 6$), Tregs (GOF, $n = 11$; LOF, $n = 3$) and iNKT cells (GOF, $n = 5$; LOF, $n = 3$); * $P < 0.05$, *** $P < 0.001$ compared to corresponding LOF donors; †† $P < 0.01$, ††† $P < 0.001$, compared to corresponding CD3⁺ T cells. **k** Data represents group means $\pm$ SEM ($n = 3$), **** $P < 0.0001$ compared to hPBMCs incubated with ATP and JNJ. GOF, gain-of-function; iNKT cell, invariant natural killer T cell; LOF, loss-of-function; Tregs, regulatory T cells

ELISA

The concentration of serum hIFN γ was measured using an IFN γ Human Uncoated ELISA Kit (Thermo Fisher Scientific) as per the manufacturer's instructions. Absorbance (570 nm and 450 nm) was measured using a SpectraMax Plus 384 (Molecular Devices; Sunnyvale, USA).

Statistical analysis

All data is given as mean $\pm$ standard error of the mean (SEM). Statistical differences between means were calculated using a Student's *t* test for single comparisons or a one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple comparisons. Comparisons of mouse weight and clinical score over time were completed using a two-way ANOVA. Mouse survival was analysed using a log-rank (Mantel-Cox) test. All statistical analyses and graphs were generated using GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, USA). For all analysis, differences were considered significant if $P < 0.05$.

Results

P2RX7 genotype varies between donors

The *P2RX7* genotype and haplotype, as originally described by Stokes et al. [25] (P2X7–1 to 5) and Jørgensen et al. [27] (H1 to H17), as well as the predicted P2X7 activity of 25 randomly selected human donors, are listed in Table 2. As expected, *P2RX7* haplotype varied between donors. In relation to haplotypes H1 to H17, four donors were homozygous for H8 and another three homozygous for H14. Exact haplotypes could be assigned to 22 of the 25 donors, but exact haplotypes could not be assigned in the remaining three donors. Donors with one wild-type allele were defined as GOF or LOF based on their mutant allele. No donors demonstrated mixed GOF or LOF haplotypes. The presence of different GOF and LOF *P2RX7* SNPs in each donor allowed their P2X7 activity to be predicted. Nineteen donors were predicted to have GOF P2X7 activity and six predicted to have LOF P2X7 activity.

P2RX7 genotype correlates with P2X7 activity on human immune cell subsets

P2X7 activity on human CD3⁺ T cells [15], CD4⁺ and CD8⁺ T cells [34] and classical, non-classical and intermediate monocytes [35] has been previously assessed; however, there are limited studies on P2X7 activity on human NK cell subsets [15], Tregs [23, 36] or invariant (i) NKT cells. Moreover, correlation between various *P2RX7* genotypes and P2X7

activity on these leukocyte subsets is lacking. Therefore, P2X7 activity on these cells, from randomly selected donors, was determined by flow cytometric measurements of ATP-induced YO-PRO-1²⁺ uptake [32]. P2X7 activity was measured on CD3⁺ T cells, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, CD3⁺CD4⁺CD25⁺CD127^{lo} Tregs and CD3⁺CD19[−]V α 24[−]J α 18⁺ iNKT cells using a defined gating strategy (Fig. 1 a–h) and was found to be similar between CD3⁺ T cells, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells and Tregs in each donor ($P > 0.05$) (Fig. 1 i). Moreover, donor *P2RX7* genotype corresponded with P2X7 activity on these cells as ATP-induced YO-PRO-1²⁺ uptake in GOF donors was significantly greater compared to corresponding LOF donors in each T cell subset ($P < 0.0001$). This was also true in iNKT cells, where GOF donors demonstrated significantly higher P2X7 activity than LOF donors ($P < 0.05$). Notably, P2X7 activity on iNKT cells was significantly higher than on CD3⁺ T cells in each corresponding donor analysed, regardless of *P2RX7* genotype ($P < 0.005$) (Fig. 1 j).

Human T cells and Tregs are the main mediators [37, 38] and regulators of GVHD [39] in humanised NSG mice, respectively. Therefore the role of P2X7 in ATP-induced YO-PRO-1²⁺ uptake was assessed in these cells using the antagonist JNJ-47965567, which blocks P2X7 but not other P2X subtypes [40] present on T cells [41, 42]. P2X7 blockade with JNJ-47965567 caused a significant reduction in ATP-induced YO-PRO-1²⁺ uptake in CD3⁺ T cells (98.7% inhibition), CD4⁺ T cells (99.4% inhibition), CD8⁺ T cells (98.4% inhibition) and Tregs (94.69% inhibition) ($P < 0.0001$ for all T cell subsets) (Fig. 1 k). These results demonstrate that P2X7 is the major P2X subtype mediating ATP-induced YO-PRO-1²⁺ uptake in these T cell subsets.

P2X7 activity was then measured on classical (CD14⁺CD16[−]), non-classical (CD14[−]CD16⁺) and intermediate (CD14⁺CD16⁺) monocytes using a defined gating strategy (Fig. 2 a–d). In each donor, all monocyte subsets demonstrated similar P2X7 activity ($P > 0.05$). This activity corresponded to donor *P2RX7* genotype, with GOF donors demonstrating significantly greater ATP-induced YO-PRO-1²⁺ uptake than corresponding monocyte subsets from LOF donors ($P < 0.0001$) (Fig. 2 h).

Finally, P2X7 activity was measured on CD56⁺CD16[−], CD56[−]CD16⁺ and CD56⁺CD16⁺ NK cell subsets using a defined gating strategy (Fig. 2 a, b, e–g). Again, no difference in P2X7 activity was observed between any subset in each donor ($P > 0.05$) (Fig. 2 i). P2X7 activity also corresponded with donor *P2RX7* genotype on these cells, with GOF donors demonstrating significantly higher ATP-induced YO-PRO-1²⁺ uptake than corresponding NK cell subsets from LOF donors ($P < 0.001$) (Fig. 2 i).

Notably, one LOF donor of the H2/H16 haplotype demonstrated P2X7 activity comparable to GOF donors in all immune cell subsets analysed. This donor was heterozygous for

Fig. 2 Donor P2X7 activity correlates with *P2RX7* genotype on human monocyte subsets and NK cell subsets. **a–i** hPBMCs, from donors of either GOF or LOF *P2RX7* genotype, in NaCl medium were incubated with 1 μ M YO-PRO-1²⁺ in the absence or presence of 1 mM ATP for 5 min at 37 °C. YO-PRO-1²⁺ uptake into human monocyte and NK cell subsets was analysed by flow cytometry using the gating strategy shown. **a** Single cells were gated by forward scatter area (FSC-A) and height (FSC-H) and **b** viable cells gated using FSC-A and side scatter (SSC-A). Single viable cells were then used to identify **c** CD14⁺CD16⁻, CD14⁺CD16⁺ or CD14⁻CD16⁺ monocyte subsets. Single, viable cells were used to gate **e** CD3⁻ cells to subsequently identify **f**

CD16⁺CD56⁻, CD16⁺CD56⁺ or CD16⁻CD56⁺ NK cell subsets. **d, g** P2X7 activity on these cells was determined by comparing YO-PRO-1²⁺ uptake in the absence (blue histograms) and presence (orange histogram) of ATP; histograms for **d** CD14⁺CD16⁻ monocytes and **g** CD16⁺CD56⁺ NK cells are shown as examples. **h, i** P2X7 activity in **h** monocyte subsets and **i** NK cell subsets was determined in multiple donors. Symbols correlate to Table 2 and represent mean values of duplicate ATP-induced YO-PRO-1²⁺ uptake tests from different donors (GOF, $n = 7$; LOF, $n = 3$); *** $P < 0.001$ compared to corresponding LOF donors. GVHD, graft-versus-host disease; GOF, gain-of-function; NK cell, natural killer cell; LOF, loss-of-function

the T357S SNP which is a partial LOF allele [43], and as such may be less dominant than the wild-type allele, thus accounting for their higher than expected P2X7 activity. Alternatively, the effect of this LOF SNP may have been confounded by the presence of a GOF SNP outside of the *P2RX7* coding region.

hP2X7^{GOF} and hP2X7^{LOF} mice demonstrate similar engraftment of human leukocytes

Injection (i.p.) of hPBMCs into NSG mice is a well-established pre-clinical model of GVHD [37]. To determine if human donor *P2RX7* genotype effects the development of GVHD in this model, NSG mice were injected with hPBMCs isolated from either GOF (hP2X7^{GOF} mice) or LOF (hP2X7^{LOF} mice) *P2RX7* genotype donors ($n = 4$ donors per genotype) (Table 3) and monitored for GVHD for up to 10 weeks. These eight donors were part of the original donor cohort (Table 2). Donor *P2RX7* genotype did not affect the engraftment of human leukocytes or T cell subsets in NSG mice (Fig. 3 a–g). At 3 weeks post-hPBMC injection, comparable frequencies of human leukocytes were observed between hP2X7^{GOF} mice ($28.67 \pm 2.30\%$ hCD45⁺ mCD45[−] cells, $n = 30$) and hP2X7^{LOF} mice ($24.49 \pm 1.78\%$ hCD45⁺ mCD45[−] cells, $n = 37$) ($P = 0.1495$) (Fig. 3 a). The majority of these human leukocytes were T cells, the frequency of which was similar between the two groups (hP2X7^{GOF} $92.31 \pm 0.73\%$ hCD3⁺ hCD19[−] cells, $n = 30$; hP2X7^{LOF} $94.6 \pm 0.90\%$ hCD3⁺ hCD19[−] cells, $n = 37$) ($P = 0.0776$) (Fig. 3 b). Both hP2X7^{GOF} mice and hP2X7^{LOF} mice were also engrafted with similar frequencies of hCD4⁺ T cells ($40.71 \pm 2.14\%$, $n = 30$ vs. $41.36 \pm 1.68\%$, $n = 37$, respectively) ($P = 0.8102$) and hCD8⁺ T cells ($43.43 \pm 2.20\%$, $n = 30$ vs. $45.83 \pm 1.67\%$, respectively, $n = 37$) ($P = 0.3792$). There were no significant differences between the percentages of hCD4⁺ and hCD8⁺ T cells in hP2X7^{GOF} mice ($P = 0.3803$) or hP2X7^{LOF} mice ($P = 0.0625$) (Fig. 3 c).

At endpoint, human leukocytes constituted the bulk of the total leukocyte population in spleens of both hP2X7^{GOF} mice ($69.73 \pm 2.59\%$, hCD45⁺ mCD45[−] cells, $n = 30$) and hP2X7^{LOF} mice ($71.36 \pm 2.51\%$, hCD45⁺ mCD45[−] cells, $n = 37$) with comparable levels of engraftment ($P = 0.6550$) (Fig. 3 d). T cells comprised the majority of the splenic human leukocyte population in both hP2X7^{GOF} mice ($83.32 \pm 2.22\%$ hCD3⁺ hCD19[−] cells, $n = 30$) and hP2X7^{LOF} mice ($82.82 \pm 1.97\%$ hCD3⁺ hCD19[−] cells, $n = 37$). Both mouse groups demonstrated similar frequencies of human T cells ($P = 0.8654$) (Fig. 3 e). hCD4⁺ and hCD8⁺ T cell subsets were also identified at endpoint, with significantly higher frequencies of hCD4⁺ T cells than hCD8⁺ T cells in both hP2X7^{GOF} mice ($62.77 \pm 3.35\%$ vs. $28.47 \pm 2.33\%$, respectively, $n = 30$) ($P < 0.0001$) and hP2X7^{LOF} mice ($64.98 \pm 2.76\%$ vs. $28.93 \pm 2.22\%$, respectively, $n = 37$) ($P < 0.0001$). However, frequencies of hCD4⁺ and hCD8⁺ T cells were similar between hP2X7^{GOF} and hP2X7^{LOF} mice (hCD4⁺ T cells, $P = 0.6093$; hCD8⁺ T cells, $P = 0.8894$) (Fig. 3 f). Analysis of

hCD4⁺ T cells revealed that hP2X7^{GOF} mice and hP2X7^{LOF} mice contained similar frequencies of hCD4⁺hCD25⁺hCD127^{lo} Tregs ($1.68 \pm 0.27\%$, $n = 30$ vs. $1.395 \pm 0.16\%$, $n = 37$, respectively) ($P = 0.3490$) (Fig. 3 g).

hP2X7^{GOF} and hP2X7^{LOF} mice demonstrate similar development of GVHD

GVHD development in humanised mice was analysed by monitoring weight loss and scoring clinical symptoms. Donor P2X7 activity did not affect GVHD development in humanised mice (Fig. 4 a–c). Weight loss was similar between hP2X7^{GOF} and hP2X7^{LOF} mice ($P = 0.3398$), which was first observed from day 25 in both groups (Fig. 4 a). Both hP2X7^{GOF} and hP2X7^{LOF} mice exhibited clinical symptoms from day 16, with comparable mean clinical scores over 10 weeks ($P = 0.6093$) and at endpoint (7.26 ± 0.35 , $n = 30$ vs. 7.53 ± 0.26 , $n = 37$, respectively) ($P = 0.6512$) (Fig. 4 b). Finally, all mice in both groups succumbed to the ethical endpoint by 10 weeks, with no significant difference in median survival time (MST) between hP2X7^{GOF} mice (MST of 34 days, $n = 30$) and hP2X7^{LOF} mice (MST of 36 days, $n = 37$) ($P = 0.8107$) (Fig. 4 c).

hP2X7^{GOF} and hP2X7^{LOF} mice demonstrate similar histological evidence of GVHD

NSG mice injected with hPBMCs demonstrate histological signs of GVHD damage in the liver, small intestine and skin [14, 37, 44]. In the current study, leukocyte infiltration was observed in the livers of hP2X7^{GOF} and hP2X7^{LOF} mice with similar amounts of tissue fibrosis between the two mouse groups (Fig. 4 d). Both groups also demonstrated similar leukocyte infiltration and overall tissue damage in the small intestine, and also showed mild signs of skin GVHD with comparable amounts of epidermal and dermal thickening alongside detachment of the epidermis from the dermis (Fig. 4 d).

Relative hIFN γ expression is significantly increased in the livers of hP2X7^{GOF} compared to hP2X7^{LOF} mice, but serum hIFN γ concentrations are similar

P2X7 expression is upregulated in allogeneic models of GVHD [11], whilst P2X7 blockade reduces serum IFN γ in both allogeneic [11, 12] and humanised mouse models of GVHD [14]. Therefore, qPCR was used to measure the relative expression of hP2X7 and hIFN γ in spleens and GVHD target organs. hP2X7^{GOF} and hP2X7^{LOF} mice demonstrated similar relative hP2X7 expression in the spleen (4.87 ± 0.38 , $n = 18$ vs. 4.19 ± 0.33 , $n = 18$, respectively) ($P = 0.1912$) (Fig. 5 a), liver (2.16 ± 0.19 , $n = 18$ vs. 2.29 ± 0.16 , $n = 18$, respectively) ($P = 0.6005$) (Fig. 5 b) and small intestine (17.62 ± 0.37 , $n = 18$ vs. 16.77 ± 0.42 , $n = 18$, respectively) ($P = 0.1444$) (Fig. 5 c). Relative

hIFN γ expression was also comparable in the spleens of hP2X7^{GOF} mice (12.46 ± 0.52 , $n = 18$) and hP2X7^{LOF} mice (13.11 ± 0.27 , $n = 18$) ($P = 0.3363$) (Fig. 5 d). In contrast, a significant but small (1.3-fold) increase in relative hIFN γ expression was observed in livers from hP2X7^{GOF} mice (3.82 ± 0.24 , $n = 18$) compared to hP2X7^{LOF} mice (3.12 ± 0.14 , $n = 18$) ($P = 0.0154$) (Fig. 5 e). Similar relative hIFN γ expression was observed in the small intestines of hP2X7^{LOF} mice (3.66 ± 0.15 , $n = 18$) and hP2X7^{GOF} mice (3.25 ± 0.15 , $n = 18$) ($P = 0.0623$) (Fig. 5 f).

Finally, the concentrations of serum hIFN γ in hP2X7^{GOF} and hP2X7^{LOF} mice were analysed by ELISA (Fig. 5 g). hIFN γ was present in the serum of all mice; however, there

was no significant difference in the concentration of serum hIFN γ between hP2X7^{GOF} mice (8.68 ± 1.26 pg/mL, $n = 30$) and hP2X7^{LOF} mice (9.67 ± 1.20 pg/mL, $n = 37$) ($P = 0.6662$).

Discussion

This study demonstrated that P2X7 activity correlates with GOF and LOF *P2RX7* genotypes on human T cell, NK cell and monocyte subsets, and showed this correlation for the first time on human CD4⁺CD25⁺CD127^{lo} Tregs and iNKT cells. Despite the impact of *P2RX7*

Table 3 *P2RX7* genotype and P2X7 activity of paired human donors for a humanised mouse model of GVHD. Each donor pair with opposing *P2RX7* genotypes were matched for sex and age. Donor *P2RX7* genotype was sequenced from genomic DNA by sequencing 13 exons in the human *P2RX7* gene. Donors were classed into *P2RX7* haplotypes based on the

presence or absence of different *P2RX7* SNPs according to Stokes et al. [25] (P2X7-1 to 5) and Jørgensen et al. [27] (H1-H17). Loss or gain of P2X7 activity in CD3⁺, CD4⁺ and CD8⁺ T cells was confirmed by flow cytometric measurements of ATP-induced YO-PRO-1²⁺ uptake prior to mouse studies

	<i>P2RX7</i> Genotype										P2X7 activity ²
	Donor ¹	H155Y rs2082 94	R270H rs7958 311	R307Q rs2836 0457	A348T rs1718 119	T357S rs2230 911	Q460R rs2230 912	E496A rs3751 143	Haplot ype (P2X7- 1 to 5)	Haplot ype (H1 to H17)	
		GOF	LOF	LOF	GOF	LOF	Neutra l	LOF			
Study 1	☐	Y/Y	WT	WT	T/T	WT	R/R	WT	P2X7-4/4	H14/H14	GOF
	●	WT	H/H	R/Q	WT	WT	WT	WT	P2X7-1/1	H1/H7	LOF
Study 2	○	WT	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H8/H8	GOF
	☐	Y/Y	WT	WT	WT	WT	WT	E/A	P2X7-1/3	H5/H11	LOF
Study 3	◆	Y/Y	WT	WT	T/T	WT	WT	WT	P2X7-2/2	H10/H10	GOF
	☐	WT	WT	WT	WT	WT	WT	E/A	P2X7-1/3	H2/H12	LOF
Study 4	■	H/Y	WT	WT	A/T	WT	WT	WT	P2X7-1/2	H2/H8/H5/H10	GOF
	☐	WT	WT	WT	WT	WT	WT	E/A	P2X7-1/3	H2/H12	LOF

¹ Symbols represent individual donors in Figs. 1 and 2

² P2X7 activity was confirmed in T cells by flow cytometry of ATP-induced YO-PRO-1²⁺ uptake

GOF, gain-of-function; LOF, loss-of-function

Colours represent P2X7 activity: green, GOF; red, LOF

All donors were wild type for unlisted non-synonymous *P2RX7* SNPs

Fig. 3 Engraftment of human leukocytes is similar in hP2X7^{GOF} and hP2X7^{LOF} mice. NSG mice were injected (i.p.) with 10×10^6 hPBMCs isolated from donor of either GOF (hP2X7^{GOF} mice) or LOF (hP2X7^{LOF}) *P2RX7* genotype (four donors per group). hP2X7^{GOF} and hP2X7^{LOF} mice were checked for the engraftment of human leukocytes in **a–c** the blood at 3 weeks post-injection and **d–g** spleen at endpoint. **a, d** hCD45⁺ leukocytes are expressed as a percentage of total mCD45⁺ and hCD45⁺

leukocytes. **b, e** hCD3⁺ cells are expressed as a percentage of total hCD45⁺ leukocytes. **c, f** hCD4⁺ and hCD8⁺ cells are expressed as a percentage of hCD3⁺ cells. **g** hCD4⁺hCD25⁺ hCD127^{lo} Tregs are expressed as a percentage of CD4⁺ T cells. Data is presented as a group mean $\pm$ SEM; symbols represent individual mice; **** $P < 0.0001$ compared to hCD8⁺ T cells. GOF, gain-of-function; LOF, loss-of-function

genotype on P2X7 activity on human immune subsets, altered donor P2X7 did not affect the development of clinical or histological disease in a pre-clinical humanised mouse model of GVHD. Our results in humanised mice are contrary to previous findings in allogeneic mouse models that have shown that genetic ablation of P2X7 [11] or P2X7 blockade with KN-62 [11], stavudine [12] or BBG [13] can reduce GVHD development. However, it must be noted that whilst P2X7 activity is implicated in exacerbating GVHD, it is P2X7 activation on host, not donor, antigen-presenting cells that is believed to worsen disease [11]. Consequently, the current study is one of the few to examine the importance of human donor P2X7 activity on GVHD development. Despite the current results suggesting that donor P2X7 activity does not affect GVHD development in this humanised mouse model, individual *P2RX7* SNPs may still have important effects on GVHD since they can influence survival outcomes in allo-HSCT patients [30]. Additionally, during the course of the current study, a conference abstract was published highlighting the potential role of *P2RX7* genotype in predicting GVHD onset [45]. This preliminary data indicated that the presence of the LOF SNPs rs3751143 (E496A) or rs16536624 (I568N) in recipients reduces GVHD post-HSCT. Lastly, given that *P2RX7* genotypes

are associated with a number of inflammatory diseases such as multiple sclerosis [28] and rheumatoid arthritis [29], further investigation into the role of host *P2RX7* genotype on the development of GVHD in humans is warranted.

The current study also demonstrated that human donor *P2RX7* genotype does not affect the engraftment of human leukocytes (predominantly T cells) in this humanised mouse model of GVHD. Moreover, the engraftment of hCD4⁺ and hCD8⁺ T cells in these humanised mice was comparable to the proportions of hCD4⁺ and hCD8⁺ T cell engraftment observed by Geraghty et al. [14]. This is consistent with these T cell subsets being the main effectors of GVHD in this model [37, 46, 47], and the similar engraftment of T cell subsets between hP2X7^{GOF} or hP2X7^{LOF} mice parallels the similarity in GVHD development between the two mouse groups. The current study also demonstrated no difference in the engraftment of Tregs between hP2X7^{GOF} and hP2X7^{LOF} mice, which have protective roles against GVHD in humanised NSG mice [39, 48] and in allo-HSCT patients [49, 50]. Since P2X7 is present on human Tregs [36] and can influence the proliferation and suppressive capabilities of these cells in vitro [51], it is possible that GOF and LOF *P2RX7* genotypes may influence GVHD development by

Fig. 4 Clinical and histological GVHD is similar in hP2X7^{GOF} and hP2X7^{LOF} mice. NSG mice were injected (i.p.) with 10×10^6 hPBMCs isolated from either a GOF (hP2X7^{GOF} mice) or LOF (hP2X7^{LOF}) *P2RX7* genotype donor (four donors per group). **a–c** Mice were monitored for up to 10 weeks post-hPBMC injection for clinical GVHD development including **a** weight loss, **b** GVHD clinical score and **c** survival. Data

represents **a, b** group means $\pm$ SEM or **c** percentage survival (GOF, $n = 30$; LOF, $n = 37$). **d** At endpoint, tissue sections (liver, small intestine and skin) from hP2X7^{GOF} mice (top panel) and hP2X7^{LOF} mice (bottom panel) were stained with haematoxylin and eosin, and viewed by microscopy. Representative images from four mice per group are shown; bars represent 100 μ m. GOF, gain-of-function; LOF, loss-of-function

enhancing and reducing Treg numbers and function, respectively. However, the similar engraftment of Tregs, and severity of GVHD in both groups of mice, indicates that this is not the case in this study.

Donor *P2RX7* genotype did not alter relative expression of hP2X7 in the spleen, liver or small intestine in this humanised mouse model. Although P2X7 is upregulated in allogeneic mice and humans with GVHD [11], in humanised mice, the P2X7 expression observed indirectly reflects human donor cell engraftment and supports human leukocyte engraftment being similar between *P2RX7* genotypes. In contrast, hIFN γ RNA expression was significantly higher in the livers of hP2X7^{GOF} than in hP2X7^{LOF} mice. Although this difference was small, and assuming IFN γ expression correlates with GVHD severity [52], the possibility exists that molecular GVHD is worse in the livers of mice injected with GOF *P2RX7* genotype donors. Nonetheless, such differences were not readily observed by histology, and serum hIFN γ was similar between donor *P2RX7* genotypes. This latter finding contrasts previous data in allogeneic mouse models of GVHD where genetic deficiency of *P2RX7* reduced serum

IFN γ [11, 12], and in this humanised mouse model, where short-term P2X7 blockade with BBG reduced serum hIFN γ and tissue leukocyte infiltration and damage [14]. This suggests that BBG may be acting on host (murine) rather than donor (human) P2X7 to reduce GVHD development in humanised mice.

This study demonstrated for the first time the presence of functional P2X7 on CD4⁺CD25⁺CD127^{lo} Tregs and iNKT cells, as well as on CD16⁺CD56[−], CD16⁺CD56⁺ and CD16⁺CD56[−] NK cell subsets. Furthermore, this study confirms the presence of functional P2X7 on CD4⁺ and CD8⁺ T cells [34] and non-classical (CD14[−]CD16⁺), intermediate (CD14⁺CD16⁺) and classical (CD14⁺CD16[−]) monocytes [35, 53, 54]. Notably, *P2RX7* genotype corresponded to P2X7 activity on each of these subsets, validating the functional predictions of the respective haplotypes made by both Stokes et al. [25] and Jørgensen et al. [27]. The possibility remains that some *P2RX7* haplotypes may reduce or increase cell-surface P2X7 expression; however, previous attempts using an anti-P2X7 mAb have repeatedly been unable to reveal this in primary leukocytes [25, 33, 43, 55, 56]. Whilst mean P2X7 activity was similar

Fig. 5 Relative hIFN γ expression is significantly increased in the livers of hP2X7^{GOF} mice compared to hP2X7^{LOF} mice. **a–g** NSG mice were injected (i.p.) with 10×10^6 hPBMCs isolated from either a GOF or LOF *P2RX7* genotype donor (four donors per group). **a–f** Relative expression of **a–c** hP2X7 and **d–f** hIFN γ in **a, d** spleen, **b, e** liver and **c, f** small intestine at endpoint was examined by qPCR. **g** Concentrations of hIFN γ

in serum of humanised mice were analysed by ELISA. **a–g** Data represents group mean $\pm$ SEM (**a–f**, $n = 12–18$; **g** GOF, $n = 30$; LOF, $n = 36$); symbols represent individual mice; * $P < 0.05$ compared to hP2X7^{LOF} mice. hIFN γ , human interferon- γ ; GOF, gain-of-function; LOF, loss-of-function

between all leukocyte subsets in the current study, iNKT cells demonstrated significantly higher activity than all other cell subsets but whether this reflects differences in cell-surface P2X7 expression remains to be determined. The current results contrast previous studies which have shown higher P2X7 activity in monocytes compared to NK cells, B cells and T cells [15, 16]. The reasons for the differences between the current and past studies remain unclear, but most likely reflect the assessment of dye uptake in fixed-time and real-time measurements, respectively. Although the identity of the P2X7 pore remains elusive

[57], ethidium⁺ is thought to pass through the same pore as YO-PRO-1²⁺ [58]. Thus, differences between studies due to the use of different dyes are unlikely.

In conclusion, the current study demonstrates that donor *P2RX7* genotype affects P2X7 activity on human T cell, monocyte and NK cell subsets. This study also demonstrated that altered donor P2X7 activity does not affect the development of GVHD in this humanised mouse model. This indicates that hPBMCs, regardless of *P2RX7* genotype, can be used to establish GVHD in NSG mice. However, whether certain *P2RX7* genotypes can predict GVHD and other

outcomes in allo-HSCT recipients will need to be confirmed by larger, adequately powered studies.

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Compliance with ethical standards

Conflict of interest Sam R Adhikary declares that he has no conflict of interest.

Nicholas J Geraghty declares that he has no conflict of interest.

Peter Cuthbertson declares that he has no conflict of interest.

Ronald Sluyter declares that he has no conflict of interest.

Debbie Watson declares that she has no conflict of interest.

Ethical approval All animal experiments were approved under protocol AE16/03 by the University of Wollongong Animal Ethics Committee. All human experiments were approved under protocol HE12/290 by the University of Wollongong Human Ethics Committee.

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