The A$_{2A}$ receptor agonist CGS 21680 has beneficial and adverse effects on disease development in a humanised mouse model of graft-versus-host disease

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A B S T R A C T

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

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative method for numerous haematological malignancies and other blood disorders; however, HSCT is limited by the development of graft-versus-host disease (GVHD) [1]. GVHD develops in up to 60% of HSCT recipients [2], due to donor T cells recognising 'foreign' host cells [3]. GVHD occurs when damage caused by the conditioning regime or the underlying disease promotes the release of inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6. Subsequently, activation of CD4$^+$ T cells by dendritic cells (DCs) results in the further release of TNF-α as well as interferon (IFN)-γ, IL-2 and IL-6 to promote inflammation, and subsequent activation of CD8$^+$ T cells to exacerbate this inflammation. Conversely, T regulatory (Treg) cells and invariant

natural killer T (iNKT) cells can reduce pro-inflammatory effects in GVHD to limit disease development or progression [4].

Adenosine receptors (A$_1$, A$_{2A}$, A$_{2B}$ and A$_3$) are cell-surface G-protein coupled receptors activated by extracellular adenosine [5]. Extracellular adenosine is often produced as a result of ATP hydrolysis mediated by the sequential action of ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39), and ecto-5′-nucleotidase (CD73) [6,7]. The A$_{2A}$ receptor is expressed on numerous immune cell subsets including DCs and T cells [8]. Notably, CD39/CD73-mediated production of adenosine and subsequent activation of A$_{2A}$ is an important anti-inflammatory mechanism [9]. In allogeneic mouse models of transplantation, adenosine production by the CD39/CD73 pathway and subsequent activation of adenosine receptors prevents tissue damage and reduces graft rejection [10]. In allogeneic mouse models of GVHD,

genetic deficiency or pharmacological blockade of CD73 with αβ-methylene ADP (APCP) [11], which results in reduced extracellular adenosine, worsens disease. Similarly, genetic deficiency [11,12] or pharmacological blockade of A_{2A} with SCH58261 [12] also worsens GVHD severity in these models. Conversely, activation of A_{2A} with ATL-146e can ameliorate GVHD in allogeneic mouse models [13,14]. However, the action of A_{2A} activation in humanised mouse models or HSCT patients remains to be explored.

Allogeneic mouse models are often used to investigate potential therapeutics for GVHD, yet therapies investigated in these models often do not translate to the clinic. This lack of translation is possibly due to species differences. In an attempt to address this, preclinical "humanised" mouse models have been developed [15]. A commonly used humanised mouse model involves the intraperitoneal (i.p.) or intravenous (i.v.) injection of human peripheral blood mononuclear cells (hPBMCs) into immunodeficient non-obese diabetic severe-combined immunodeficiency-IL-2 receptor γ^{null} (NSG) mice. Due to defective T and B cells, and a lack of natural killer (NK) cells, these mice readily engraft hPBMCs [16], and subsequently develop GVHD due to the ability of human T cells to recognise the major histocompatibility complex (MHC) I and II of NSG mice [17]. Previous studies have shown that i.p. or i.v. injection of hPBMCs into these mice results in similar splenic engraftment of human leukocytes [17] and progression of clinical GVHD [18].

Using the A_{2A} agonist CGS 21680 [19], this study aimed to investigate the effect of A_{2A} activation on GVHD development in a humanised mouse model. CGS 21680 did not impact clinical score or survival of mice. However, CGS 21680 reduced leukocyte infiltration into livers, and reduced serum hTNF-α concentrations indicative of reduced GVHD severity. Conversely, CGS 21680 worsened weight loss, reduced Treg cell frequency and increased serum hIL-6 concentrations indicating worsened GVHD. Notably, CGS 21680 also prevented weight gain in NSG mice not engrafted with hPBMCs. This suggests that appetite or metabolism may be negatively impacted by CGS 21680. Therefore, the adverse impact on weight, Treg cells and IL-6 caused by CGS 21680 may confound the potential benefits of A_{2A} activation in reducing GVHD in HSCT recipients.

2. Materials and methods

2.1. Humanised mouse model of GVHD

Experiments involving human blood and mice were approved by the respective Human and Animal Ethics Committees of the University of Wollongong (Wollongong, Australia). A humanised mouse model of GVHD was used as described [20]. Briefly, female NSG mice aged 6–8 weeks (Australian BioResources, Moss Vale, Australia) were injected i.p. daily (days −2 to day 11) with saline/0.2% DMSO (Sigma-Aldrich, St Louis, MO, USA) (vehicle) or vehicle containing CGS 21680 (Tocris Bioscience, Bristol, UK) (0.1 mg/kg). This injection schedule was based on that previously used for an A_{2A} agonist in an allogeneic mouse model of GVHD [13]. hPBMCs, isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden) and resuspended in Dulbecco's phosphate-buffer saline (ThermoFisher, Waltham, MA, USA), were injected i.p. (day 0) (10×10^6 hPBMCs/mouse). At 3 weeks post-hPBMC injection, mice were checked for engraftment by immunophenotyping of tail vein blood. Mice were monitored for signs of GVHD using a scoring system, giving a total clinical score out of 10, as described [21]. Mice were euthanized at 10 weeks post-injection of hPBMCs, or earlier if exhibiting a clinical score of ≥8 or a weight loss of ≥10%, according to the approved animal ethics protocol.

2.2. Immunophenotyping by flow cytometry

Tail vein blood (week 3) and spleen cells (end-point) were obtained

Table 1
Monoclonal antibodies used for flow cytometry.

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

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

from mice and lysed with ammonium chloride potassium buffer and immunophenotyped as described [21] using the antibodies listed in Table 1. Data was collected using a BD Fortessa X-20 Flow Cytometer (using band pass filters 450/50 for BV421, 710/50 for BV711, 525/50 for FITC, 586/15 for PE, 695/40 for PerCP-Cy5.5, 780/60 for PE-Cy7 and 670/30 for APC) and FACSDiva software version 8.0. The relative percentages of cells were analysed using FlowJo software v8.7.1 (TreeStar Inc.; Ashland, OR, USA).

2.3. Histological analysis

Formalin-fixed tissue sections (5 μm) were stained with haematoxylin and eosin (POCD; Artarmon, Australia), with histology assessed using FIJI Is Just ImageJ (FIJI) [22] as described [23,24].

2.4. Cytokine analysis by a flow cytometric multiplex assay

Serum was obtained from mice at end-point as described [21] and cytokine concentrations were measured using a Th1 LEGENDPlex kit (BioLegend, San Diego, CA, USA) as per the manufacturer's instructions.

2.5. In vitro T cell activation assay

Freshly isolated hPBMCs (1×10^6 cells/mL) were incubated for 24 h at 37 °C 95% air/5% CO_2 in RPMI-1640 medium containing 2 mM L-glutamine, 1% non-essential amino acids, 55 μM mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin (all Thermo Fisher Scientific) and 10% FBS (Bovogen, East Keller, Australia), in the absence or presence of 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich), and in the absence or presence of 1 μM CGS 21680. Cells were then centrifuged (300 g for 5 min) and washed once with PBS (300 g for 5 min) and immunophenotyped as above. Data were collected using an LSRFortessa X-20 flow cytometer (band-pass filter 450/50 for BV421, 515/20 for FITC, 586/25 for PE, and 780/30 for Zombie NIR (BioLegend)). The relative percentages of Treg cells at 24 h were determined using FlowJo software v8.7.1.

2.6. Statistical analysis

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

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

3. Results

3.1. CGS 21680 does not impact initial hPBMC engraftment in NSG mice

NSG mice injected with hPBMCs and either control vehicle ($n = 25$) or the A_{2A} agonist CGS 21680 [19] ($n = 25$) daily (days −2 to 11) were monitored (from day 0) for up to 10 weeks. To determine whether A_{2A} activation affected initial hPBMC engraftment, blood was collected 3 weeks post-hPBMC injection and cells immunophenotyped by flow cytometry. Three mice from each group did not demonstrate human leukocytes ($hCD45^+mCD45^-$) in their blood at 3 weeks (results not shown). In the remaining mice, human leukocytes were observed in the blood with frequency of these cells calculated as a percentage of total leukocytes [$hCD45^+mCD45^-/(hCD45^+mCD45^- + hCD45^-mCD45^+)$]. CGS 21680- and vehicle-injected mice demonstrated similar frequencies of human leukocytes ($21.8 \pm 2.8\%$ and $22.8 \pm 3.2\%$, respectively, $P = 0.8221$) (Fig. 1a). The proportion of human leukocyte engraftment was the same in CGS 21680- and vehicle-injected mice (both 90%, $P = 1.000$). Likewise, the proportion of murine leukocytes was the same between CGS 21680- and vehicle-injected mice ($72.3 \pm 2.8\%$ and $71.1 \pm 3.4\%$, respectively, $P = 0.8065$; not shown). The majority of the human leukocytes were T cells ($95.7 \pm 0.8\%$ and $97.3 \pm 0.6\%$, respectively, $P = 0.1086$) (Fig. 1b), and a small frequency were non-B/T cells ($3.8 \pm 0.9\%$ and $3.4 \pm 0.6\%$, respectively, $P = 0.7502$) (Fig. 1c).

3.2. CGS 21680 reduces human Treg cells in humanised NSG mice

To determine if CGS 21680 impacted hPBMC engraftment at end-point, splenocytes from CGS 21680- ($n = 20$) and vehicle-injected mice ($n = 20$) were analysed by flow cytometry. Human leukocytes were absent in the spleens of the same three mice from each group that failed to show hPBMC engraftment at 3 weeks (results not shown). Frequencies of human leukocytes in mice engrafted at end-point were similar in CGS 21680- and vehicle-injected mice ($70.5 \pm 3.2\%$ and $65.1 \pm 6.0\%$, respectively, $P = 0.4348$) (Fig. 1d). Likewise, the proportion of murine leukocytes was the same between CGS 21680- and vehicle-injected mice ($26.2 \pm 2.9\%$ and $26.7 \pm 4.3\%$, respectively, $P = 0.9278$; not shown).

Similar to blood at 3 weeks post-hPBMC injection, the majority of engrafted human leukocytes in both groups of mice were T cells, which did not differ between CGS 21680- ($93.4 \pm 1.8\%$) and vehicle-injected mice ($93.6 \pm 1.5\%$) ($P = 0.9546$) (Fig. 1e). Further analysis of human T cells demonstrated that CGS 21680- and vehicle-injected mice demonstrated similar engraftment of $hCD4^+$ T cells ($67.2 \pm 3.0\%$ and $58.3 \pm 3.7\%$, respectively, $P = 0.0713$) and $hCD8^+$ T cells ($18.0 \pm 2.3\%$ and $24.4 \pm 3.3\%$, respectively, $P = 0.1260$). Both CGS 21680- and vehicle-injected mice demonstrated greater engraftment of $hCD4^+$ than $hCD8^+$ cells ($P < 0.0001$ and $P < 0.0001$, respectively) (Fig. 1f).

To investigate whether CGS 21680 affected CD39 and/or CD73 on human T cells, these ecto-nucleotidases on $hCD4^+$ and $hCD8^+$ T cell subsets from the spleens of mice were examined. CGS 21680-injected mice demonstrated a trend of increased $hCD39^+hCD73^-hCD4^+$ T cells compared to vehicle-injected mice ($15.6 \pm 3.0\%$ and $9.1 \pm 2.0\%$, respectively, $P = 0.0796$). Conversely, CGS 21680-injected mice demonstrated significantly reduced frequencies of $hCD39^-hCD73^+hCD4^+$ T cells ($2.5 \pm 0.7\%$ and 8.9 ± 2.6, respectively, $P = 0.0227$), and a reduced trend of $hCD39^+hCD73^+hCD4^+$ T cells ($1.3 \pm 0.3\%$ and $3.1 \pm 1.1\%$, respectively, $P = 0.1137$) compared to vehicle-injected mice. However, CGS 21680- and vehicle-injected mice demonstrated

similar frequencies of $hCD39^-hCD73^-hCD4^+$ T cells ($80.6 \pm 3.0\%$ and $78.8 \pm 4.1\%$, respectively, $P = 0.7358$) (Fig. 1g). CGS 21680- and vehicle-injected mice demonstrated similar frequencies of $hCD39^+hCD73^-hCD8^+$ T cells ($40.6 \pm 5.9\%$ and $39.7 \pm 5.0\%$, respectively, $P = 0.9036$), $hCD39^-hCD73^+hCD8^+$ T cells ($3.0 \pm 1.0\%$ and $4.2 \pm 1.2\%$, respectively, $P = 0.4508$), $hCD39^+hCD73^+hCD8^+$ T cells ($4.0 \pm 1.1\%$ and $7.3 \pm 2.2\%$, respectively, $P = 0.1877$) and $hCD39^-hCD73^-hCD8^+$ T cells ($52.3 \pm 4.7\%$ and $51.4 \pm 5.9\%$, respectively, $P = 0.9137$) (Fig. 1h).

In allogeneic mouse models increased frequencies of iNKT and Treg cells correlates with reduced GVHD [4]. Therefore, the frequency of human iNKT cells ($hCD45^+hCD3^+hCD19^-hV\alpha24-J\alpha18^+$) and human Treg cells ($hCD45^+hCD3^+hCD4^+hCD25^+hCD127^{lo}$) in spleens from mice were examined. CGS 21680-injected mice, compared to vehicle-injected mice, demonstrated a reduced trend of iNKT cells ($2.7 \pm 0.6\%$ and $4.7 \pm 1.1\%$, respectively, $P = 0.1223$) (Fig. 3i) and significantly reduced Treg cells ($0.4 \pm 0.1\%$ and $0.9 \pm 0.2\%$, respectively, $P = 0.0130$) (Fig. 1j).

Our group has previously shown that at end-point the spleens of humanised mice do not contain human B cells [21]. Similarly, in the current study the remaining human leukocytes in the spleens of mice were negative for CD19. CGS 21680- and vehicle-injected mice demonstrated small but similar frequencies of $CD3^-CD19^-$ cells present in both groups of mice ($3.2 \pm 0.8\%$ and $4.7 \pm 0.8\%$, respectively, $P = 0.1783$) (Fig. 1k). To determine if human monocytes ($hCD14^+hCD83^-$) or DCs ($hCD14^-hCD83^+$) were present, the remaining non-B/T cell population ($hCD45^+hCD3^-hCD19^-$) was analysed. CGS 21680- and vehicle-injected mice demonstrated similar but low frequencies of monocytes ($0.4 \pm 0.2\%$ and $0.6 \pm 0.2\%$, respectively, $P = 0.6127$) (Fig. 1l) and DCs ($0.2 \pm 0.1\%$ and $0.7 \pm 0.3\%$, respectively, $P = 0.1160$) (Fig. 1m).

The above data (Fig. 1j) demonstrates that CGS 21680 significantly reduced Treg cells in humanised mice. To determine if CGS 21680 had a negative impact on Treg cells, hPBMCs were stimulated in vitro for 24 h with PMA and ionomycin in the absence or presence of CGS 21680, and the proportion of Treg cells determined by flow cytometry. CGS 21680 was used at 1 μM, a concentration with known efficacy in vitro [25,26]. As previously reported [27], stimulation with PMA and ionomycin increased the proportion of Treg cells in hPBMC cultures; however this increase did not differ significantly in the absence and presence of CGS 21680 (respective fold-increase of 14.9 ± 7.4 and 11.3 ± 6.3, $P = 0.7294$) (Fig. 1n). The proportion of Treg cells in non-stimulated hPBMC cultures in the absence or presence of CGS 21680 at 24 h was similar (data not shown). This data indicates that CGS 21680 does not have a negative impact on Treg cell differentiation.

3.3. CGS 21680 worsens weight loss in humanised NSG mice

To investigate whether A_{2A} activation impacts GVHD, the above mice were monitored for weight loss and other signs of GVHD for up to 10 weeks. One engrafted vehicle-injected mouse died unexpectedly overnight from unknown causes and was excluded from the following analyses. In those mice which had engrafted hPBMCs, CGS 21680-injected mice ($n = 22$) demonstrated significantly greater weight loss over the 10 weeks than vehicle-injected mice ($n = 21$) ($P = 0.0020$) (Fig. 2a). However, both CGS 21680- and vehicle-injected mice demonstrated signs of GVHD (classified as a score clinical score > 3) from 35 days onwards with similar scores ($P = 0.8008$) (Fig. 2b), survival (MST; 41 days, and 43 days, respectively, $P = 0.6730$) and mortality rates (90% and 82%, respectively, $P = 0.6640$) (Fig. 2c) over the 10 weeks.

3.4. CGS 21680 prevents healthy weight gain in NSG mice not engrafted with hPBMCs

As noted above, three NSG mice from each treatment group did not engraft hPBMCs. Nevertheless, these mice were also monitored for
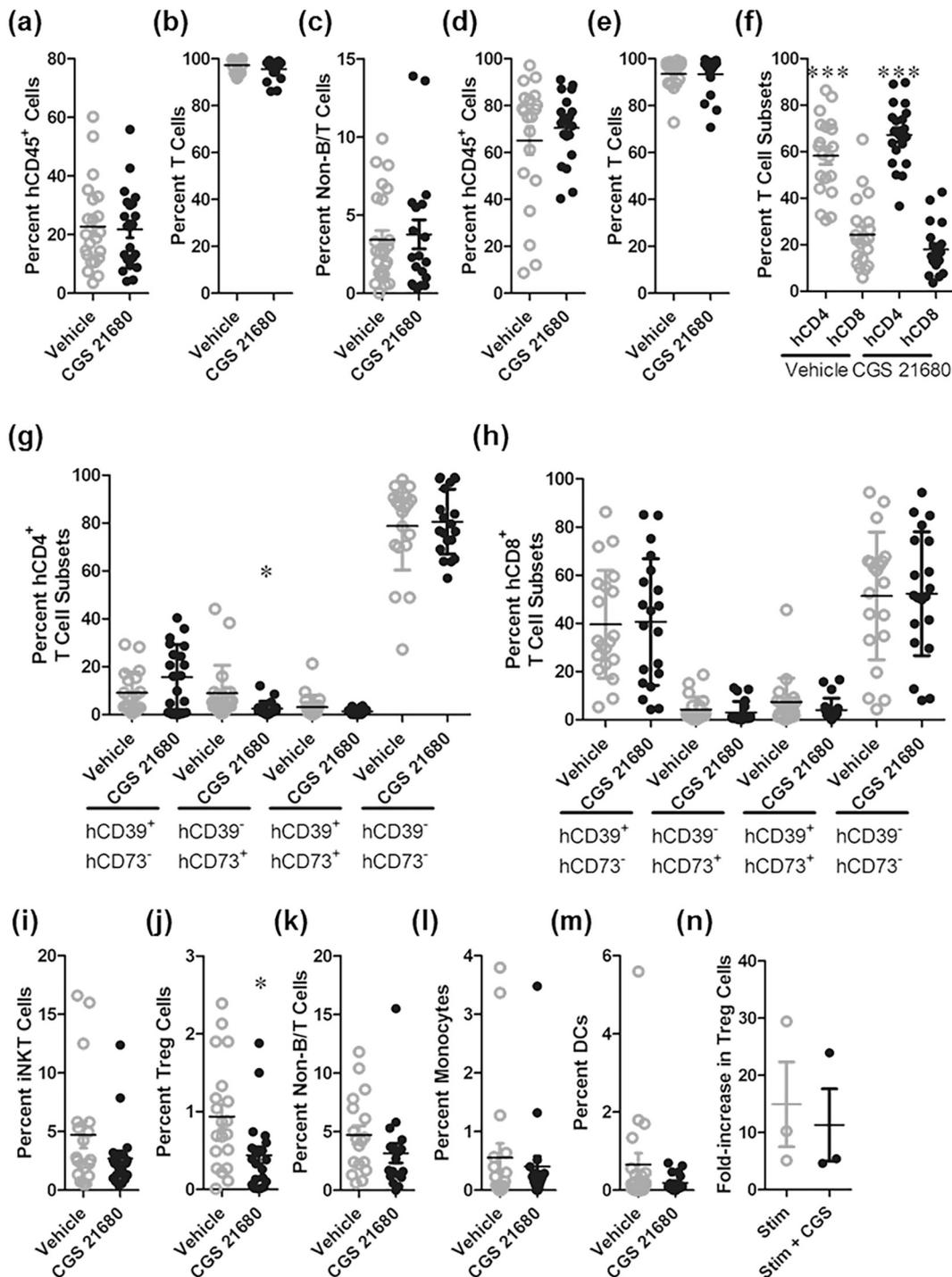
Fig. 1. CGS 21680 reduces human T regulatory cells in humanised NSG mice.
(a–m) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day −2 to day 11), and with 10×10^6 hPBMCs (day 0). The percentages of human (h) leukocytes and subsets in (a–c) blood at 3 weeks post-hPBMC injection and (d–m) spleens at end-point were determined by flow cytometry. (a, d) Human leukocytes (hCD45$^+$mCD45$^-$) are expressed as a percentage of total mCD45$^+$ and hCD45$^+$ leukocytes. Three mice from each group did not engraft hCD45$^+$ leukocytes (not shown). (b, e) hCD3$^+$hCD19$^-$ cells and (c, k) hCD3$^-$hCD19$^-$ cells are expressed as a percentage of total hCD45$^+$ leukocytes. (f) hCD4$^+$ and hCD8$^+$ T cell subsets are expressed as a percentage of total hCD3$^+$ leukocytes. ***$P < 0.0001$ compared to hCD8$^+$ T cells. (g–h) hCD39 and hCD73 expression was analysed on (g) hCD4$^+$ and (h) hCD8$^+$ T cell subsets. *$P < 0.05$ compared to vehicle. (i) Invariant natural killer T (iNKT) cells (hCD45$^+$hCD3$^+$hCD19$^-$hVα24-Jα18$^+$) are expressed as a percentage of hCD3$^+$hCD19$^-$ T cells and (j) T regulatory (Treg) cells (hCD45$^+$hCD3$^+$hCD4$^+$hCD25$^+$hCD127^{lo}) are expressed as a percentage of hCD3$^+$hCD4$^+$ T cells. *$P < 0.05$ compared to vehicle. (l) Monocytes (hCD14$^+$hCD83$^-$) and (m) dendritic cells (DCs) (hCD14$^-$hCD83$^+$) are expressed as a percentage of hCD45$^+$hCD3$^-$hCD19$^-$ cells. (n) Freshly isolated hPBMCs were incubated for 24 h in the absence or presence of 50 ng/mL PMA and 1 µg/mL ionomycin, and in the absence or presence of 1 µM CGS 21680 (CGS), and the percentage of Treg cells (hCD4$^+$hCD25$^+$hCD127^{lo}Zombie NIR$^-$) amongst hCD4$^+$Zombie NIR$^-$ T cells determined by flow cytometry. Data is presented as fold-increase in Treg cells in stimulated PBMCs (Stim) compared to non-stimulated PBMCs. (a–m) Data represents group means ± SEM (vehicle $n = 20$–22, CGS 21680 $n = 20$–22); symbols represent individual mice. (n) Data represents group means ± SEM ($n = 3$); symbols represent individual human donors.
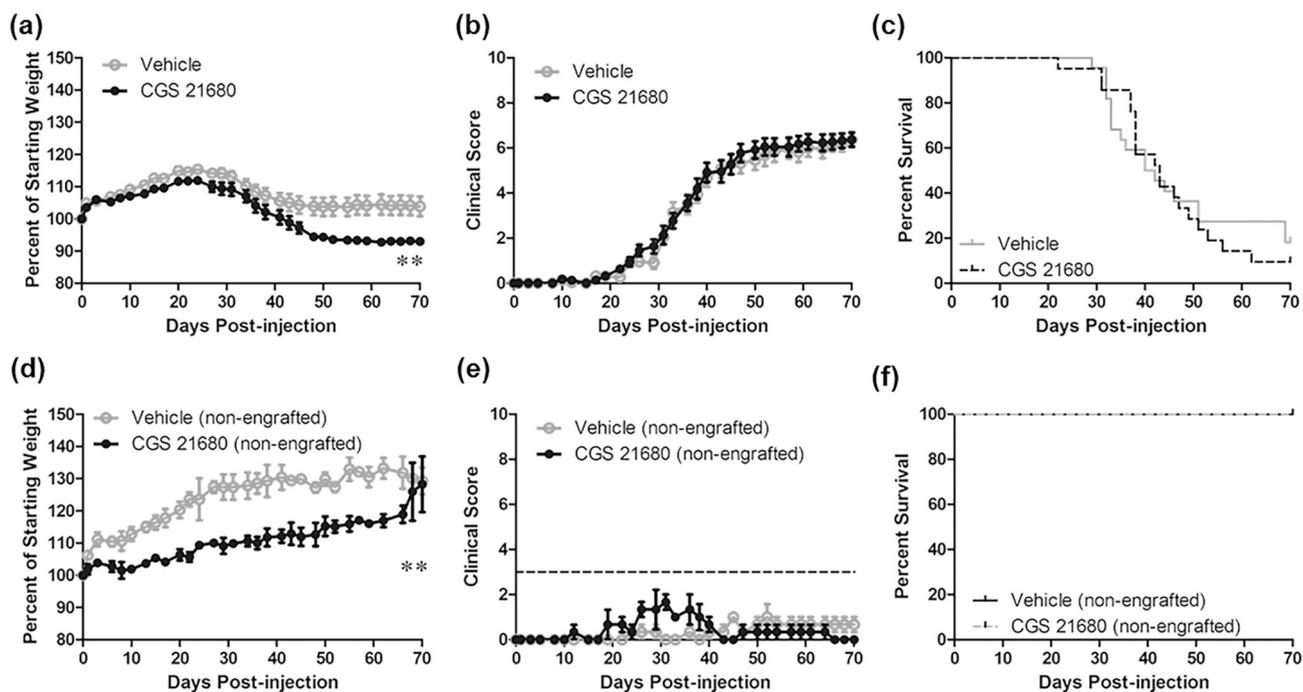
Fig. 2. CGS 21680 worsens weight loss in NSG and humanised NSG mice.
(a–f) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day −2 to day 11), and with 10×10^6 hPBMCs (day 0). NSG mice engrafted with hPBMCs were monitored for (a) weight loss, (b) clinical score, and (c) survival over 10 weeks. Data represents (a, b) group means ± SEM or (c) percent survival (vehicle $n = 22$, CGS 21680 $n = 21$). **$P < 0.01$ compared to vehicle-injected mice. (d–f) Mice which were not engrafted with hCD45$^+$ leukocytes at 3 weeks (blood) and at 10 weeks (spleen) (results not shown) were monitored for (d) weight loss, (e) clinical score, and (f) survival. Data represents (d, e) group means ± SEM or (f) percent survival (vehicle $n = 3$, CGS 21680 $n = 3$). **$P < 0.01$ compared to vehicle-injected mice.

weight loss and signs of GVHD for 10 weeks as per the engrafted mice above. CGS 21680-injected mice gained significantly less weight over the 10 weeks compared to vehicle-injected mice ($P = 0.0029$) (Fig. 2d). Both CGS 21680- and vehicle-injected mice demonstrated similar but minimal clinical signs of GVHD over the 10 weeks (mean clinical scores of 0.4 ± 0.2 and 0.3 ± 0.2) ($P = 0.4557$) (Fig. 2e) consistent with the lack of hPBMC engraftment. Moreover, all CGS 21680- and vehicle-injected mice not engrafted with hPBMCs survived the entire 10 weeks (Fig. 2f).

3.5. CGS 21680 reduces liver infiltrates in humanised NSG mice

Tissues from mice which had engrafted hPBMCs ($n = 20$ per group) were examined by histology. Livers from CGS 21680-injected mice demonstrated reduced leukocyte infiltration (1548.0 ± 121.4 cells per field of view, $n = 9$) compared to vehicle-injected mice (2054.0 ± 103.3 cells per field of view, $n = 9$) ($P = 0.0059$) but all mice demonstrated similar structural damage (Fig. 3a, b). CGS 21680- and vehicle-injected mice demonstrated similar histology and leukocyte infiltration into small intestines (841.6 ± 37.4 cells per field of view, $n = 9$ and 821.1 ± 107.7 cells per field of view, $n = 9$, $P = 0.8530$) and skin (1128.0 ± 97.3 cells per field of view, $n = 9$ and 1139 ± 196.1 cells per field of view, $n = 9$, $P = 0.9593$) (Fig. 3a, b). CGS 21680- and vehicle-injected mice also demonstrated similar epidermal thickening of the skin (88.0 ± 6.9 μm, $n = 14$ and 82.2 ± 5.5 μm, $n = 14$, $P = 0.5235$) (Fig. 3a, c).

3.6. CGS 21680 increases serum hIL-6 but reduces hTNF-α in humanised NSG mice

The pro-inflammatory cytokine storm that preludes immune cell infiltration and inflammatory damage of target organs is an important stage of GVHD pathogenesis [28]. Therefore, to determine if A$_{2A}$ activation impacts human cytokines, a multiplex assay was used to analyse concentrations of serum hIL-2, hIL-6, hIL-10, hTNF-α, and hIFN-γ from CGS 21680-injected mice ($n = 15$) and vehicle-injected ($n = 18$).

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

4. Discussion

Previous studies have shown that the CD73/A$_{2A}$ pathway reduces disease severity in allogeneic mouse models of GVHD [11–14]. However, the effect of A$_{2A}$ activation in humanised mouse models or in HSCT patients has not been reported. Using a humanised NSG mouse model of GVHD, the current study demonstrated that the A$_{2A}$ agonist CGS 21680 had opposing roles in disease development. CGS 21680 did not affect clinical score or mortality in humanised mice but reduced GVHD severity, as indicated by decreased leukocyte infiltration into the liver and serum hTNF-α in these mice. Unexpectedly, CGS 21680 increased weight loss and serum hIL-6, and reduced the frequency of Tregs, indicating this A$_{2A}$ agonist worsens these disease parameters in this humanised mouse model of GVHD.

CGS 21680 reduced leukocyte infiltration into the liver and serum hTNF-α indicating GVHD is reduced, at least in part, in humanised
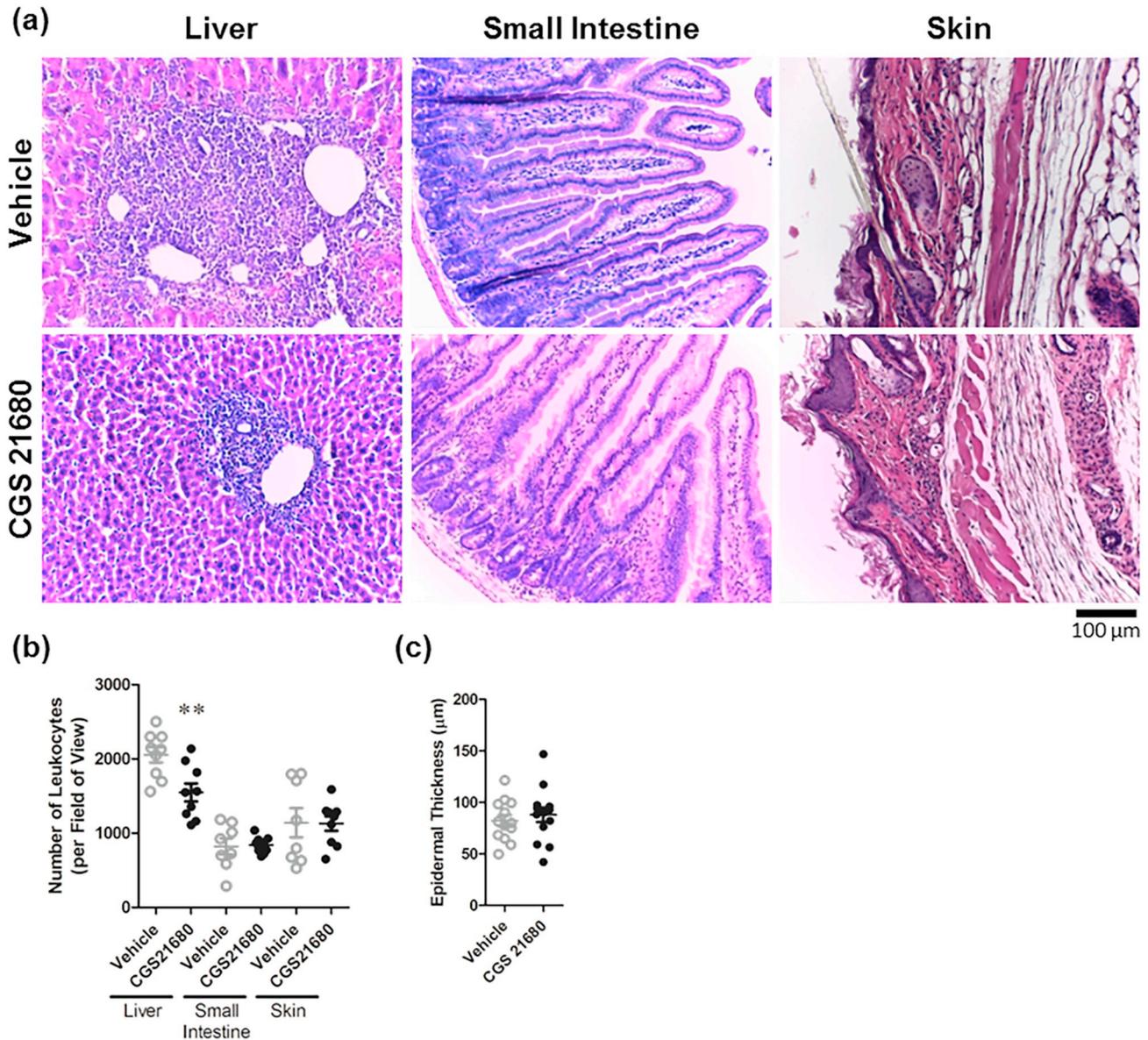
Fig. 3. CGS 21680 reduces liver infiltrates in humanised NSG mice.
(a–c) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10×10^6 hPBMCs (day 0). Tissue sections (liver, small intestine, and skin) from hPBMC-engrafted mice were stained with haematoxylin and eosin. Images were captured by microscopy with each image representative of twenty mice per group; bars represent 100 μm. (b) Leukocyte infiltration (vehicle $n = 9$, CGS 21680 $n = 9$) and (c) epidermal thickness (vehicle $n = 14$, CGS 21680 $n = 14$) were quantified using FIJI Is Just ImageJ (FIJI). Data represents group means $\pm$ SEM; **$P < 0.01$ compared to vehicle-injected mice.

mice. This indicates that A_{2A} activation has beneficial roles in this model. This finding parallels similar observations in allogeneic mouse models of GVHD where the A_{2A} agonist ATL-146e reduces histological damage, leukocyte infiltration in livers and serum TNF-α [13,14]. Conversely, pharmacological blockade or genetic deficiency of CD73, which limits extracellular adenosine [29], worsens liver histology in allogeneic mouse models of GVHD [11,12]. Of note, in allogeneic and humanised mouse models of GVHD, increased TNF-α levels correspond with disease severity [30,31] and blockade of this cytokine impairs GVHD in both of these models [17,32]. Thus, collectively these studies suggest a crucial role for TNF-α in GVHD, and that activation of A_{2A} can limit this in both allogeneic and humanised mouse models of this disease.

Contrary to above, the current study suggests that CGS 21680 worsens aspects of GVHD in humanised mice as evidenced through increased weight loss. This indicates that A_{2A} activation also has

adverse roles in this model. Weight loss is a common indicator of disease severity in mouse models of GVHD [33]. The dose of CGS 21680 (0.1 mg/kg) used in this study is sufficient to prevent disease in mouse models of acute lung inflammation [34], pleurisy [35] and collagen-induced arthritis [36]. These studies reported no effect of CGS 21680 on weight, suggesting weight loss in CGS 21680-injected humanised mice is due in part to worsened GVHD. Supporting this, serum IL-6 was also increased in humanised mice. Although, hIL-6 has not been previously detected in the serum of humanised mice [31,37,38], and its role in humanised mouse models of GVHD remains to be elucidated, IL-6 is implicated in GVHD progression in allogeneic mouse models [39,40]. In the current study, the increased serum hIL-6 concentrations were unexpected, as previously ATL-146e reduced serum IL-6 concentrations in allogeneic mouse models of GVHD [13,14]. Moreover, CGS 21680 reduces IL-6 release from ex vivo anti-CD3/anti-CD28-stimulated murine effector T cells [41]. Conversely, increased serum IL-6 concentrations
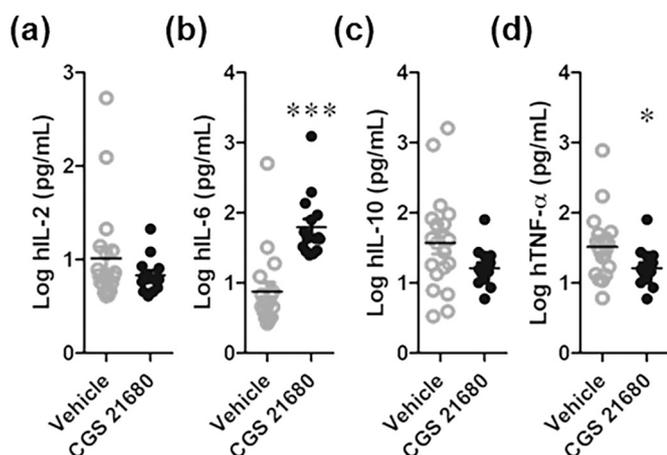
Fig. 4. CGS 21680 impacts serum cytokines in humanised NSG mice.
(a–d) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10×10^6 hPBMCs (day 0). Concentrations of serum human (a) interleukin (IL)-2, (b) IL-6, (c) IL-10, and (d) tumour necrosis factor (TNF)-α from hPBMC-engrafted mice were analysed by a flow cytometric multiplex assay. Data represents group means $\pm$ SEM (vehicle $n = 18$, CGS 21680 $n = 15$); *$P < 0.05$, ***$P < 0.0001$ compared to vehicle-injected mice.

correlate to disease severity as a result of A_{2A} blockade in an allogeneic mouse model of GVHD [12]. The reason for increased serum hIL-6 concentrations in CGS 21680-injected humanised mice remains unknown but supports the concept that A_{2A} activation has adverse roles in this model.

In the current study, the frequency of Treg cells was also reduced. This further supports the concept that A_{2A} activation has detrimental roles in humanised mice. Treg cells inversely correlate to GVHD progression in allogeneic [42] and humanised mouse models [43], as well as in HSCT recipients [44,45]. The observed reduction in Treg cells was contrary to expectations, as in allogeneic mouse models ATL-146e increases Treg cells to ameliorate disease development [13,14]. The mechanism by which CGS 21680 decreases Treg cells in the current study remains to be established. However, CGS 21680 reduced, albeit not significantly, serum hIL-2 and hIL-10 in humanised mice. These cytokines are important for maintenance of Treg cells in humanised mice [38,46]. Thus, a decrease in one or both of these cytokines may have contributed to the lower frequency of Treg cells in CGS 21680-injected mice. Alternatively, although not mutually exclusive to the above, IL-6 with IL-1β reduces Treg cell numbers through their conversion to Th1 and/or Th17 cells [47]. Thus, the increased IL-6 in CGS 21680-injected mice may have contributed to the reduction in Treg cells. However, it should be noted that the amount of hIL-1β mRNA [21] and serum hIL-1β [38] in humanised mice is negligible, thereby potentially limiting the ability of hIL-6 to convert Treg cells to Th cells in this model.

One other point of note is that a high, but similar, proportion of human CD39$^+$CD73$^-$CD8$^+$ T cells were observed at end-point in both CGS 21680- and vehicle-injected humanised mice. These cells may represent exhausted CD8$^+$ T cells, as recently described in a murine model of viral infection [48]. If so, this would be consistent with persistent antigen experience and activation of human CD8$^+$ T cells [49] in humanised mice. Which provides indirect evidence that CD8$^+$ T cells (along with CD4$^+$ T cells) mediate GVHD in this model, as demonstrated by others [17,50,51].

The current study also demonstrated that CGS 21680 prevented healthy weight gain in NSG mice not engrafted with hPBMCs. This suggests that this A_{2A} agonist may be suppressing appetite or metabolism in these mice, which may also be contributing to the increased weight loss in hPBMC-engrafted NSG mice in addition to GVHD. In rats, CGS 21680, at the same dose used in the current study, is sufficient to

prevent weight gain by reducing food intake [52,53]. Moreover, CGS 21680 at this dose increases energy expenditure in mice to prevent diet-induced obesity [54] and causes hypothermia [55]. The reasons for the different effects of CGS 21680 on mouse weight in these and the current studies compared to those discussed above [34–36] remains unknown, but may reflect strain differences. CGS 21680-mediated effects on metabolism and weight were observed in C57BL/6 mice [54,55] and NSG mice (this study), whilst CGS 21680 was reported to have no such effects in CD1, Swiss and DBA/1 mice [34–36]. Finally, it should be noted that IL-6 can potentially impact mouse body weight, as genetic deficiency of IL-6 promotes obesity [56]. Thus, the increased weight loss observed in CGS 21680-injected humanised mice may be mediated via an IL-6-dependent mechanism. Regardless, given the confounding effects of CGS 21680 on GVHD in humanised NSG mice, as well as the effect of this compound on weight loss in non-engrafted NSG mice and other mouse strains, the use of CGS 21680 to activate A_{2A} in allogeneic mouse models of GVHD should be avoided.

In summary, the A_{2A} agonist CGS 21680 has opposing effects in a humanised mouse model of GVHD. Moreover, CGS 21680 can prevent weight gain in healthy NSG mice. Therefore, the therapeutic efficacy of A_{2A} activation with CGS 21680 in HSCT recipients may be limited by adverse effects on weight, decreased Treg cell frequency and increased IL-6 production.

Disclosure

All authors declare that they have no disclosures.

Author contributions

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

References

[1] S.M. Jaglowski, S.M. Devine, Graft-versus-host disease: why have we not made more progress? Curr. Opin. Hematol. 21 (2) (2014) 141–147, https://doi.org/10.1097/Moh.0000000000000026.
[2] M. Jagasia, M. Arora, M.E.D. Flowers, et al., Risk factors for acute GVHD and survival after hematopoietic cell transplantation, Blood 119 (1) (2012) 296–307, https://doi.org/10.1182/blood-2011-06-364265.
[3] R.E. Billingham, The biology of graft-versus-host reactions, Harvey Lect. 62 (1965) 21–78.
[4] D. Schneidawind, A. Pierini, R.S. Negrin, Regulatory T cells and natural killer T cells for modulation of GVHD following allogeneic hematopoietic cell transplantation, Blood 122 (18) (2013) 3116–3121, https://doi.org/10.1182/blood-2013-08-453126.
[5] B.B. Fredholm, A.P. Ijzerman, K.A. Jacobson, J. Linden, C.E. Müller, International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update, Pharmacol. Rev. 63 (1) (2011) 1–34, https://doi.org/10.1124/pr.110.003285.
[6] E. Ferrero, A.C. Faini, F. Malavasi, A phylogenetic view of the leukocyte ectonucleotidases, Immunol. Lett. (2018), https://doi.org/10.1016/j.imlet.2018.06.008.
[7] P. Apostolova, R. Zeiser, The role of danger signals and ectonucleotidases in acute graft-versus-host disease, Hum. Immunol. 77 (11) (2016) 1037–1047, https://doi.org/10.1016/j.humimm.2016.02.005.
[8] G. Burnstock, J.M. Boeynaems, Purinergic signalling and immune cells, Purinergic Signal 10 (4) (2014) 529–564, https://doi.org/10.1007/s11302-014-9427-2.

[9] S. Deaglio, K.M. Dwyer, W. Gao, et al., Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression, J. Exp. Med. 204 (6) (2007) 1257–1265, https://doi.org/10.1084/jem.20062512.

[10] A. Vergani, S. Tezza, C. Fotino, G. Visner, A. Pileggi, A. Chandraker, P. Fiorina, The purinergic system in allotransplantation, Am. J. Transplant. 14 (3) (2014) 507–514, https://doi.org/10.1111/ajt.12567.

[11] H. Tsukamoto, P. Chernogorova, K. Ayata, et al., Deficiency of CD73/ecto-5′-nucleotidase in mice enhances acute graft-versus-host disease, Blood 119 (19) (2012) 4554–4564.

[12] L. Wang, J. Fan, S. Chen, Y. Zhang, T.J. Curiel, B. Zhang, Graft-versus-host disease is enhanced by selective CD73 blockade in mice, PLoS ONE 8 (3) (2013) e58397, , https://doi.org/10.1371/journal.pone.0058397.

[13] C.M. Lappas, P.-C. Liu, J. Linden, E.M. Kang, H.L. Malech, Adenosine A2A receptor activation limits graft-versus-host disease after allogenic hematopoietic stem cell transplantation, J. Leukoc. Biol. 87 (2) (2010) 345–354.

[14] K.L. Han, S.V. Thomas, S.M. Koontz, C.M. Changpririoa, S.-K. Ha, H.L. Malech, E.M. Kang, Adenosine A2A receptor agonist–mediated increase in donor-derived regulatory T cells suppresses development of graft-versus-host disease, J. Immunol. 190 (1) (2013) 458–468, https://doi.org/10.4049/jimmunol.1201325.

[15] L.D. Shultz, F. Ishikawa, D.L. Greiner, Humanized mice in translational biomedical research, Nat. Rev. Immunol. 7 (2) (2007) 118–130, https://doi.org/10.1038/nri2017.

[16] M. King, T. Pearson, L.D. Shultz, et al., A new Hu-PBL model for the study of human islet alloreactivity based on NOD-scid mice bearing a targeted mutation in the IL-2 receptor gamma chain gene, Clin. Immunol. 126 (3) (2008) 303–314, https://doi.org/10.1016/j.clim.2007.11.001.

[17] M.A. King, L. Covassin, M.A. Brehm, et al., Human peripheral blood leucocyte non-obese diabetic-severe combined immunodeficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex, Clin. Exp. Immunol. 157 (1) (2009) 104–118, https://doi.org/10.1111/j.1365-2249.2009.03933.x.

[18] Y. Kawasaki, K. Sato, H. Hayakawa, et al., Comprehensive analysis of the activation and proliferation kinetics and effector functions of human lymphocytes, and antigen presentation capacity of antigen-presenting cells in xenogeneic graft-versus-host disease, Biol. Blood Marrow Transplant. 24 (8) (2018) 1563–1574, https://doi.org/10.1016/j.bbmt.2018.04.016.

[19] K.N. Klotz, J. Hessling, J. Hegler, C. Owman, B. Kull, B.B. Fredholm, M.J. Lohse, Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells, Naunyn Schmiedeberg's Arch. Pharmacol. 357 (1) (1998) 1–9, https://doi.org/10.1007/PL00005131.

[20] N.J. Geraghty, L. Belfiore, S.R. Adhikary, S.I. Alexander, R. Sluyter, D. Watson, Increased splenic human CD4$^+$:CD8$^+$ T cell ratios, serum human interferon-γ and intestinal human interleukin-17 are associated with clinical graft-versus-host disease in humanized mice, Transpl. Immunol. (2019), https://doi.org/10.1016/j.trim.2019.02.003.

[21] N.J. Geraghty, L. Belfiore, D. Ly, et al., The P2X7 receptor antagonist Brilliant Blue G reduces serum human interferon-γ in a humanized mouse model of graft-versus-host disease, Clin. Exp. Immunol. 190 (1) (2017) 79–95, https://doi.org/10.1111/cei.13005.

[22] J. Schindelin, I. Arganda-Carreras, E. Frise, et al., Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (7) (2012) 676–682, https://doi.org/10.1038/nmeth.2019.

[23] N.J. Geraghty, K.J. Mansfield, S.J. Fuller, D. Watson, R. Sluyter, The P2X7 receptor is not essential for development of imiquimod-induced psoriasis-like inflammation in mice, Purinergic Signal (2017), https://doi.org/10.1007/s11302-017-9569-0.

[24] N.J. Geraghty, D. Watson, R. Sluyter, Long-term treatment with the P2X7 receptor antagonist Brilliant Blue G reduces liver inflammation in a humanized mouse model of graft-versus-host disease, Cell. Immunol. 336 (2019) 12–19, https://doi.org/10.1016/j.cellimm.2018.12.001.

[25] N. Zhang, D. Yang, H. Dong, Q. Chen, D.I. Dimitrova, T.J. Rogers, M. Sitkovsky, J.J. Oppenheim, Adenosine A2a receptors induce heterologous desensitization of chemokine receptors, Blood 108 (1) (2006) 38–44, https://doi.org/10.1182/blood-2005-06-2599.

[26] A.J. Williams, B.N. Cronstein, The effect of A2A adenosine receptor activation on C-C chemokine receptor 7 expression in human THP1 macrophages during inflammation, Inflammation 35 (2) (2012) 614–622, https://doi.org/10.1007/s10753-011-9353-1.

[27] H. Wang, V. Daniel, M. Sadeghi, G. Opelz, Differences in the induction of induced human CD4$^+$ CD25$^+$ FoxP3$^+$ T-regulatory cells and CD3$^+$ CD8$^+$ CD28$^-$ T-suppressor cells subset phenotypes in vitro: comparison of phorbol 12-myristate 13-acetate/ionomycin and phytohemagglutinin stimulation, Transplant. Proc. 45 (5) (2013) 1822–1831, https://doi.org/10.1016/j.transproceed.2012.10.061.

[28] J.L. Ferrara, R. Levy, N.J. Chao, Pathophysiologic mechanisms of acute graft-vs.-host disease, Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation 5 (6) (1999) 347–356.

[29] R.F. Bruns, Adenosine receptor activation by adenine nucleotides requires conversion of the nucleotides to adenosine, Naunyn Schmiedeberg's Arch. Pharmacol. 315 (1) (1980) 5–13, https://doi.org/10.1007/bf00504224.

[30] T. Kuroiwa, E. Kakishita, T. Hamano, et al., Hepatocyte growth factor ameliorates acute graft-versus-host disease and promotes hematopoietic function, J. Clin. Investig. 107 (11) (2001) 1365–1373, https://doi.org/10.1172/JCI11808.

[31] S. Abraham, J.G. Choi, C. Ye, N. Manjunath, P. Shankar, IL-10 exacerbates xenogeneic GVHD by inducing massive human T cell expansion, Clin. Immunol. 156 (1) (2015) 58–64, https://doi.org/10.1016/j.clim.2014.11.004.

[32] G.R. Hill, T. Teshima, A. Gerbitz, L. Pan, K.R. Cooke, Y.S. Brinson, J.M. Crawford, J.L.M. Ferrara, Differential roles of IL-1 and TNF-α on graft-versus-host disease and graft versus leukemia, J. Clin. Investig. 104 (4) (1999) 459–467.

[33] S. Naserian, M. Leclerc, A. Thiolat, et al., Simple, reproducible, and efficient clinical grading system for murine models of acute graft-versus-host disease, Front. Immunol. 9 (10) (2018) 10, https://doi.org/10.3389/fimmu.2018.00010.

[34] D. Impellizzeri, R. Di Paola, E. Esposito, et al., CGS 21680, an agonist of the adenosine (A2A) receptor, decreases acute lung inflammation, Eur. J. Pharmacol. 668 (1–2) (2011) 305–316, https://doi.org/10.1016/j.ejphar.2011.06.049.

[35] F. da Rocha Lapa, M.D. da Silva, D. de Almeida Cabrini, A.R.S. Santos, Anti-inflammatory effects of purine nucleosides, adenosine and inosine, in a mouse model of pleurisy: evidence for the role of adenosine A2 receptors, Purinergic Signal 8 (4) (2012) 693–704, https://doi.org/10.1007/s11302-012-9299-2.

[36] E. Mazzon, E. Esposito, D. Impellizzeri, D.I.P. R, A. Melani, P. Bramanti, F. Pedata, S. Cuzzocrea, CGS 21680, an agonist of the adenosine (A2A) receptor, reduces progression of murine type II collagen-induced arthritis, J. Rheumatol. 38 (10) (2011) 2119–2129, https://doi.org/10.3899/jrheum.110111.

[37] J. Gregoire-Gauthier, L. Durrieu, A. Duval, et al., Use of immunoglobulins in the prevention of GvHD in a xenogeneic NOD/SCID/γc- mouse model, Bone Marrow Transplant. 47 (3) (2012) 439–450.

[38] S. Abraham, H. Guo, J.G. Choi, et al., Combination of IL-10 and IL-2 induces oligoclonal human CD4 T cell expansion during xenogeneic and allogeneic GVHD in humanized mice, Heliyon 3 (4) (2017), https://doi.org/10.1016/j.heliyon.2017.e00276.

[39] C. Koenecke, N. Czeloth, A. Bubke, et al., Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD, Eur. J. Immunol. 39 (11) (2009) 3091–3096, https://doi.org/10.1002/eji.200939432.

[40] I. Tawara, M. Koyama, C. Liu, et al., Interleukin-6 modulates graft-versus-host responses after experimental allogeneic bone marrow transplantation, Clin. Cancer Res. 17 (1) (2011) 77–88.

[41] M. Romio, B. Reinbeck, S. Bongardt, S. Hüls, S. Burghoff, J. Schrader, Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells, Am. J. Phys. Cell Phys. 301 (2) (2011) C530–C539, https://doi.org/10.1152/ajpcell.00385.2010.

[42] M. Edinger, P. Hoffmann, J. Ermann, K. Drago, C.G. Fathman, S. Strober, R.S. Negrin, CD4$^+$ CD25$^+$ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation, Nat. Med. 9 (9) (2003) 1144–1150.

[43] T. Cao, A. Soto, W. Zhou, W. Wang, S. Eck, M. Walker, G. Harriman, L. Li, Ex vivo expanded human CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells prevent lethal xenogenic graft versus host disease (GVHD), Cell. Immunol. 258 (1) (2009) 65–71.

[44] K. Rieger, C. Loddenkemper, J. Maul, et al., Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD, Blood 107 (4) (2006) 1717–1723.

[45] C. Fondi, C. Nozzoli, S. Benemei, et al., Increase in FOXP3$^+$ regulatory T cells in GVHD skin biopsies is associated with lower disease severity and treatment response, Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation 15 (8) (2009) 938–947.

[46] S. Abraham, R. Pahwa, C. Ye, et al., Long-term engraftment of human natural T regulatory cells in NOD/SCID IL2rγcnull mice by expression of human IL-2, PLoS ONE 7 (12) (2012) e51832, , https://doi.org/10.1371/journal.pone.0051832.

[47] J. Gu, X. Ni, X. Pan, et al., Human CD39hi regulatory T cells present stronger stability and function under inflammatory conditions, Cell. Mol. Immunol. 14 (6) (2016) 521–528, https://doi.org/10.1038/cmi.2016.30.

[48] P.K. Gupta, J. Godec, D. Wolski, et al., CD39 expression identifies terminally exhausted CD8$^+$ T cells, PLoS Pathog. 11 (10) (2015) e1005177, , https://doi.org/10.1371/journal.ppat.1005177.

[49] E.J. Wherry, M. Kurachi, Molecular and cellular insights into T cell exhaustion, Nat. Rev. Immunol. 15 (2015) 486, https://doi.org/10.1038/nri3862.

[50] M.A. Brehm, L.L. Kenney, M.V. Wiles, et al., Lack of acute xenogeneic graft-versus-host disease, but retention of T-cell function following engraftment of human peripheral blood mononuclear cells in NSG mice deficient in MHC class I and II expression, FASEB J. (2018) fj201800636R, , https://doi.org/10.1096/fj.201800636R.

[51] G. Ehx, J. Somja, H.J. Warnatz, et al., Xenogeneic graft-versus-host disease in humanized NSG and NSG-HLA-A2/HHD mice, Front. Immunol. 9 (1943) (2018) 1943, https://doi.org/10.3389/fimmu.2018.01943.

[52] S. Mingote, M. Pereira, A.M. Farrar, P.J. McLaughlin, J.D. Salamone, Systemic administration of the adenosine A2A agonist CGS 21680 induces sedation at doses that suppress lever pressing and food intake, Pharmacol. Biochem. Behav. 89 (3) (2008) 345–351, https://doi.org/10.1016/j.pbb.2008.01.006.

[53] M.V. Micioni Di Bonaventura, C. Cifani, C. Lambertucci, R. Volpini, G. Cristalli, M. Massi, A2A adenosine receptor agonists reduce both high-palatability and low-palatability food intake in female rats, Behav. Pharmacol. 23 (5–6) (2012) 567–574, https://doi.org/10.1097/FBP.0b013e3283566a60.

[54] T. Gnad, S. Scheibler, I. von Kügelgen, et al., Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors, Nature 516 (7531) (2014) 395–399, https://doi.org/10.1038/nature13816.

[55] J.L. Carlin, S. Jain, R. Duroux, et al., Activation of adenosine A2A or A2B receptors causes hypothermia in mice, Neuropharmacology 139 (2018) 268–278, https://doi.org/10.1016/j.neuropharm.2018.02.035.

[56] V. Wallenius, K. Wallenius, B. Ahrén, M. Rudling, H. Carlsten, S.L. Dickson, C. Ohlsson, J.-O. Jansson, Interleukin-6-deficient mice develop mature-onset obesity, Nat. Med. 8 (1) (2002) 75–79, https://doi.org/10.1038/nm0102-75.