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# The A2A 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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**Abstract**

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative method for blood cancers and other blood disorders, but is limited by the development of graft-versus-host disease (GVHD). GVHD results in inflammatory damage to the host liver, gastrointestinal tract and skin, resulting in high rates of morbidity and mortality in HSCT recipients. Activation of the $A_{2A}$ receptor has been previously demonstrated to reduce disease in allogeneic mouse models of GVHD. 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 $\gamma^{null}$ (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.

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# 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

## Short title: $A_{2A}$ receptor activation in humanised mice

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**KEY WORDS:** Xenogeneic graft-versus-host disease, $A_{2A}$ receptor, CGS 21680, humanised mice, T regulatory cell, cytokine

## ABSTRACT:

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

# 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 when damage caused by the conditioning regime or the underlying disease promotes the release of inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6. Subsequently, activation of T cells by dendritic cells (DCs) results in the further release of TNF-α as well as interferon (IFN)-γ, IL-2 and IL-6 to promote inflammation, and subsequent activation of $CD8^+$ T cells to exacerbate this inflammation. Conversely, T regulatory (Treg) cells and invariant natural killer T (iNKT) cells can reduce pro-inflammatory effects in GVHD to limit disease development or progression [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 $\gamma^{null}$ (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.

96

97 # MATERIALS AND METHODS

98 ## Humanised mouse model of GVHD

99 Experiments involving human blood and mice were approved by the respective Human and
100 Animal Ethics Committees of the University of Wollongong (Wollongong, Australia). A
101 humanised mouse model of GVHD was used as described [20]. Briefly, female NSG mice
102 aged 6-8 weeks (Australian BioResources, Moss Vale, Australia) were injected i.p. daily
103 (days -2 to day 11) with saline/0.2% DMSO (Sigma-Aldrich, St Louis, MO, USA) (vehicle)
104 or vehicle containing CGS 21680 (Tocris Bioscience, Bristol, UK) (0.1 mg/kg). This
105 injection schedule was based on that previously used for an $A_{2A}$ agonist in an allogeneic
106 mouse model of GVHD [13]. hPBMCs, isolated by density centrifugation using Ficoll-Paque
107 PLUS (GE Healthcare; Uppsala, Sweden) and resuspended in Dulbecco's phosphate-buffer
108 saline (ThermoFisher, Waltham, MA, USA), were injected i.p. (day 0) ($10 \times 10^6$
109 hPBMCs/mouse). At 3 weeks post-hPBMC injection, mice were checked for engraftment by
110 immunophenotyping of tail vein blood. Mice were monitored for signs of GVHD using a
111 scoring system, giving a total clinical score out of 10, as described [21]. Mice were
112 euthanized at 10 weeks post-injection of hPBMCs, or earlier if exhibiting a clinical score of ≥
113 8 or a weight loss of ≥ 10%, according to the approved animal ethics protocol.

114 ## Immunophenotyping by flow cytometry

115 Tail vein blood (week 3) and spleen cells (end-point) were obtained from mice and lysed with
116 ammonium chloride potassium buffer and immunophenotyped as described [21] using the
117 antibodies listed in Table 1. Data was collected using a BD Fortessa-X20 Flow Cytometer
118 (using band pass filters 450/50 for BV421, 710/50 for BV711, 525/50 for FITC, 586/15 for
119 PE, 695/40 for PerCP-Cy5.5, 780/60 for PE-Cy7 and 670/30 for APC). The relative

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

**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].

**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.

***In vitro* T cell activation assay**

Freshly isolated hPBMCs (1 x $10^6$ 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), 10% FBS (Bovogen, East Keller, Australia), 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence or absence 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)) and FACSDiva software version 8.0. The relative percentages of Treg cells at 24 h were determined using FlowJo software v8.7.1

### 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.

## RESULTS

### 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 ± 2.8% and 22.8 ± 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 ± 2.8% and 71.1 ± 3.4%, respectively, $P = 0.8065$; not shown). The majority of

the human leukocytes were T cells (95.7 ± 0.8% and 97.3 ± 0.6%, respectively, $P = 0.1086$) (Fig 1b), and a small frequency were non-B/T cells (3.8 ± 0.9 % and 3.4 ± 0.6%, respectively, $P = 0.7502$) (Fig 1c).

**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 ± 3.2% and 65.1 ± 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 ± 2.9% and 26.7 ± 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 ± 1.8%) and vehicle-injected mice (93.6 ± 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 ± 3.0% and 58.3 ± 3.7%, respectively, $P = 0.0713$) and $hCD8^+$ T cells (18.0 ± 2.3% and 24.4 ± 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 demonstrated a trend of increased $hCD39^+hCD73^-hCD4^+$ T cells compared to vehicle-injected mice (15.6 ± 3.0% and 9.1 ± 2.0%, respectively, $P = 0.0796$). Conversely, CGS 21680-injected mice demonstrated significantly reduced

frequencies of $hCD39^{-}hCD73^{+}hCD4^{+}$ T cells (2.5 ± 0.7% and 8.9 ± 2.6, respectively, $P$ = 0.0227), and a reduced trend of $hCD39^{+}hCD73^{+}hCD4^{+}$ T cells (1.3 ± 0.3% and 3.1 ± 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 ± 3.0% and 78.8 ± 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 ± 5.9% and 39.7 ± 5.0%, respectively, $P$ = 0.9036), $hCD39^{-}hCD73^{+}hCD8^{+}$ T cells (3.0 ± 1.0% and 4.2 ± 1.2%, respectively, $P$ = 0.4508), $hCD39^{+}hCD73^{+}hCD8^{+}$ T cells (4.0 ± 1.1% and 7.3 ± 2.2%, respectively, $P$ = 0.1877) and $hCD39^{-}hCD73^{-}hCD8^{+}$ T cells (52.3 ± 4.7% and 51.4 ± 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 ($hCD45^{+}hCD3^{+}hCD19^{-}hV\alpha24\text{-}J\alpha18^{+}$) 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 ± 0.6% and 4.7 ± 1.1%, respectively, $P$ = 0.1223) (Fig 3i) and significantly reduced Treg cells (0.4 ± 0.1% and 0.9 ± 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 ± 0.8% and 4.7 ± 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 ± 0.2% and 0.6 ± 0.2%,

respectively, $P = 0.6127$) (Fig 1l) and DCs (0.2 ± 0.1%, and 0.7 ± 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 (Fig 1n), however the proportion were not significantly up-regulated in the absence (14.9 ± 7.4%) or presence (11.3 ± 6.3%) of CGS 21680 ($P = 0.7294$). This data indicates that CGS 21680 does not have a negative impact on Treg cell differentiation.

**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.

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

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

weeks as per the engrafted mice above. CGS 21680-injected mice gained significantly less weight over the 10 weeks compared to vehicle-injected mice ($P$ = 0.0029) (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).

**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, $n$ = 9) compared to vehicle-injected mice (2054.0 ± 13.3, $n$ = 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, $n$ = 9 and 821.1 ± 107.7, $n$ = 9, $P$ = 0.8530) and skin (1128.0 ± 97.3, $n$ = 9 and 1139 ± 196.1, $n$ = 9, $P$ = 0.9593) (Fig 3a). 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 3c).

**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.

## 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 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 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 these $CD8^+$ 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.

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## 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.

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# FIGURE LEGENDS

**Figure 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 x $10^6$ hPBMCs (day 0). The percentages of human (h) leukocytes and subsets in (a-c) blood at 3 weeks post-hPBMC injection and (d-m) spleens at end-point were determined by flow cytometry. (a, d) Human leukocytes ($hCD45^+mCD45^-$) are expressed as a percentage of total $mCD45^+$ and $hCD45^+$ leukocytes. Three mice from each group did not engraft $hCD45^+$ leukocytes (not shown). (b, e) $hCD3^+hCD19^-$ cells and (c, k) $hCD3^-hCD19^-$ cells are expressed as a percentage of total $hCD45^+$ leukocytes. (f) $hCD4^+$ and $hCD8^+$ T cell subsets are expressed as a percentage of total $hCD3^+$ leukocytes. ** $P < 0.005$, *** $P < 0.0001$ compared to $hCD8^+$ T cells. (g-h) hCD39 and hCD73 expression was analysed on (g) $hCD4^+$ and (h) $hCD8^+$ T cell subsets. * $P$

583 < 0.05 compared to vehicle. (i) Invariant natural killer T (iNKT) cells
584 ($hCD45^+hCD3^+hCD19^-hV\alpha24\text{-}J\alpha18^+$) are expressed as a percentage of $hCD3^+hCD19^-$ T cells
585 and (j) T regulatory (Treg) cells ($hCD45^+hCD3^+hCD4^+hCD25^+hCD127^{lo}$) are expressed as a
586 percentage of $hCD3^+hCD4^+$ T cells. * $P < 0.05$ compared to vehicle. (l) Monocytes
587 ($hCD14^+hCD83^-$) and (m) dendritic cells (DCs) ($hCD14^-hCD83^+$) are expressed as a
588 percentage of $hCD45^+hCD3^-hCD19^-$ cells. (n) Freshly isolated hPBMCs were incubated for
589 24 h in the absence (No Stim) or presence (Stim) of 50 ng/ml PMA and 1 μg/ml ionomycin,
590 in the absence or presence of 1 μM CGS 21680 (CGS), and the proportion of Tregs
591 ($hCD4^+hCD25^+hCD127^{lo}$Zombie $NIR^-$) as a percentage of $hCD4^+$Zombie $NIR^-$ T cells
592 determined by flow cytometry. (a-m) Data represents group means ± SEM (vehicle $n$ = 20-
593 22, CGS 21680 $n$ = 20-22); symbols represent individual mice. (n) Data represents group
594 means ± SEM ($n$ = 3); symbols represent individual human donors.

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

605 **Figure 3. CGS 21680 reduces liver infiltrates in humanised NSG mice**
606 (a-c) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle
607 containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10 x $10^6$ 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 ± SEM; ** $P < 0.005$ compared to vehicle-injected mice.

**Figure 4. CGS 21680 impacts serum cytokines in humanised NSG mice**

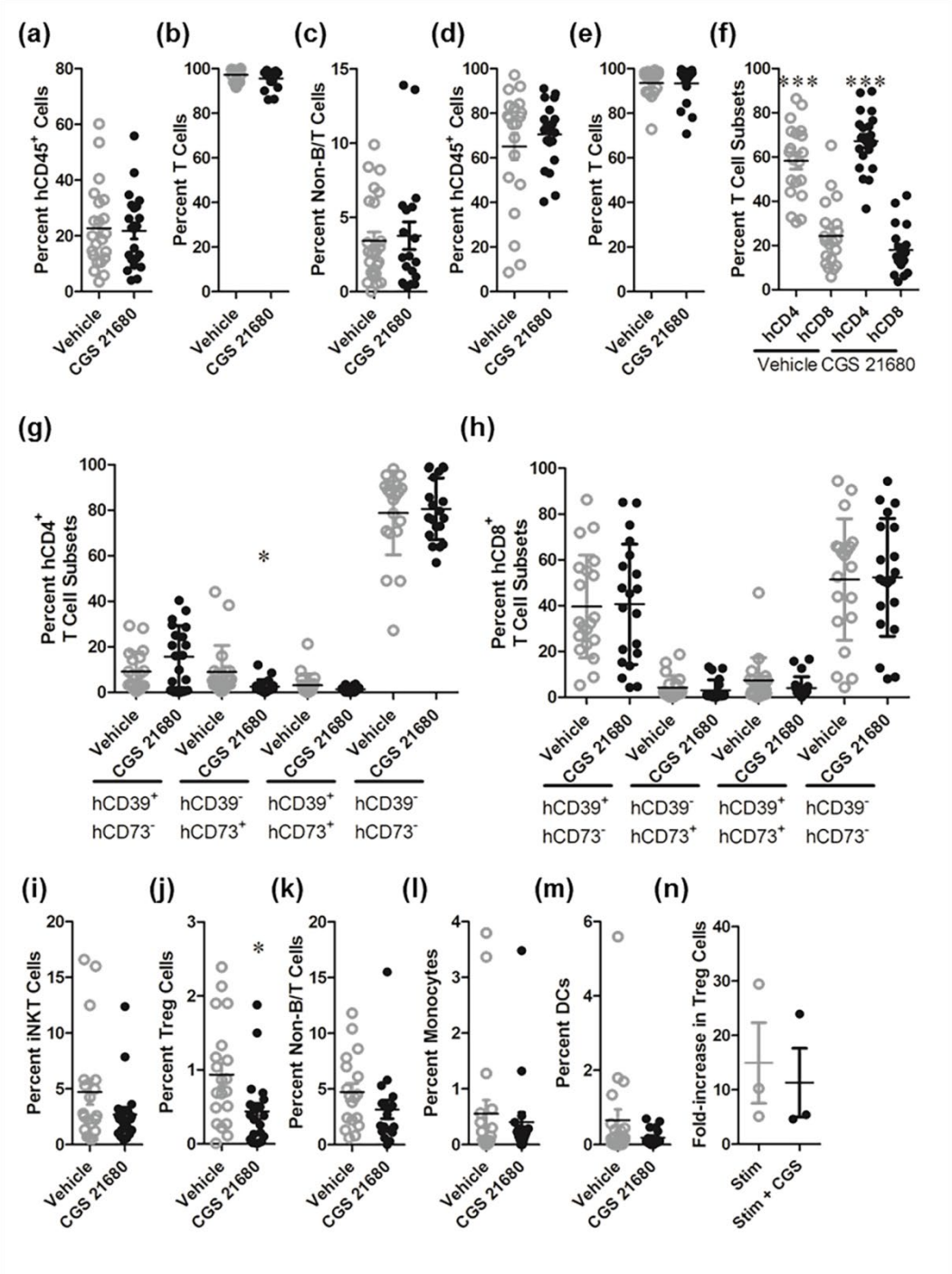
(a-d) NSG mice were injected daily with either saline/0.2% DMSO (vehicle) or vehicle containing CGS 21680 (0.1 mg/kg) (day -2 to day 11), and with 10 x $10^6$ hPBMCs (day 0). Concentrations of serum human (a) interleukin (IL)-2, (b) IL-6, (c) IL-10, and (d) tumour necrosis factor (TNF)-α from hPBMC-engrafted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

## TABLE

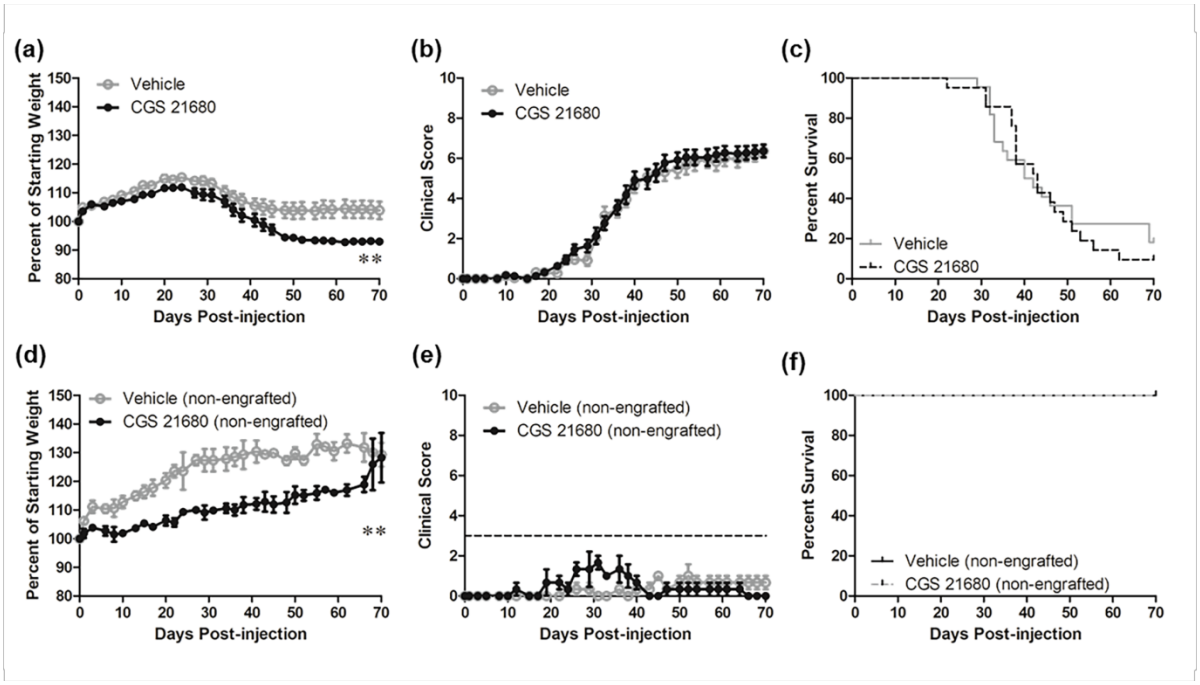
**Table 1. Monoclonal antibodies used for flow cytometry.**

| Target | Fluorochrome | Clone* |
|---|---|---|
| CD3 | BV711 | UCHT1 |
| CD4 | PerCP-Cy5.5 | L200 |
| CD8 | FITC | RPA-T8 |
| CD14 | BV421 | MϕP9 |
| CD19 | 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 |

*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.

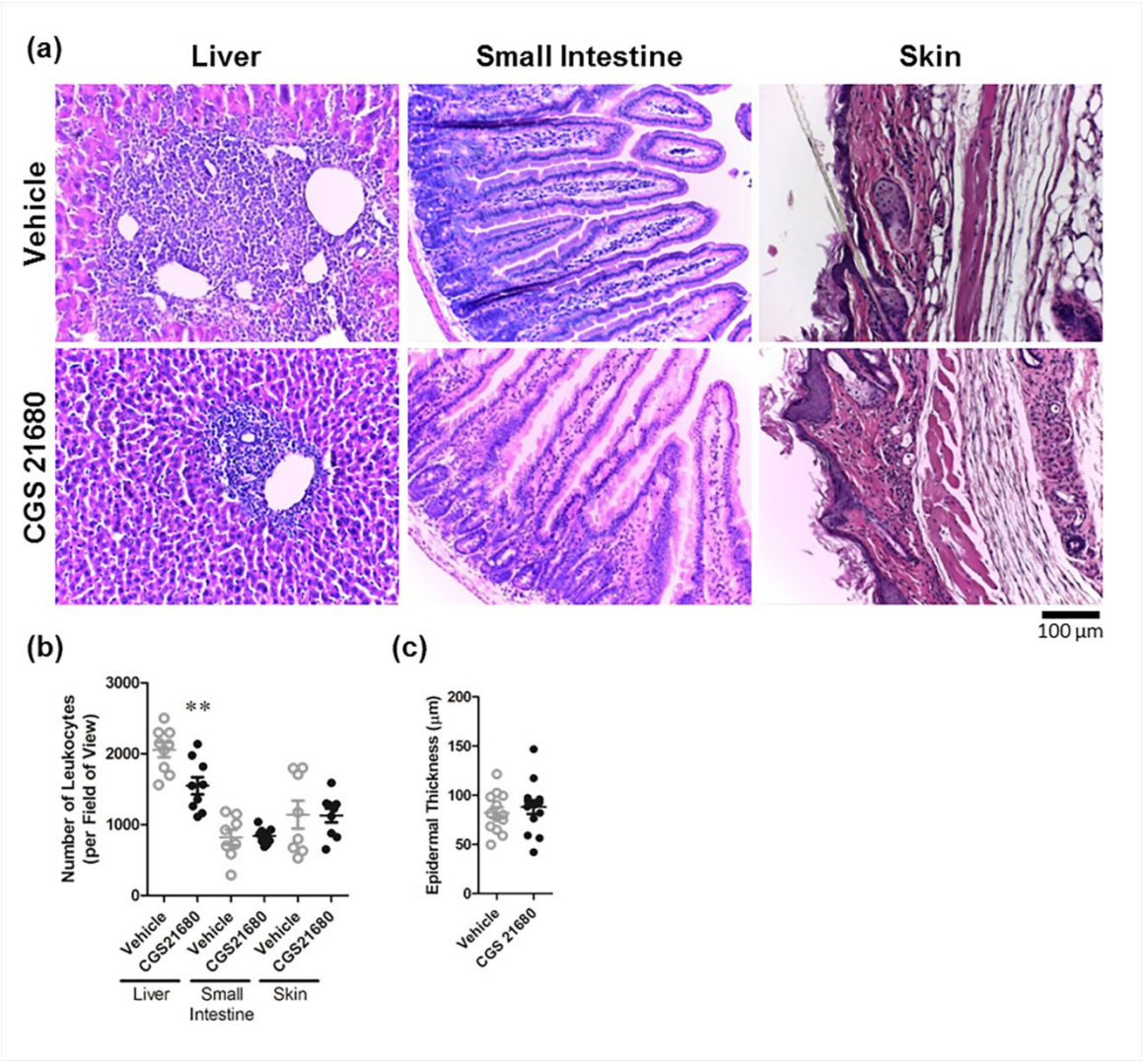

(a)
Percent hCD45+ Cells
Vehicle
CGS 21680
(b)
Percent T Cells
(c)
Percent Non-B/T Cells
(d)
Percent hCD45+ Cells
(e)
Percent T Cells
(f)
Percent T Cell Subsets
***
***
hCD4
hCD8
hCD4
hCD8
Vehicle CGS 21680
(g)
Percent hCD4+ T Cell Subsets
*
hCD39+ hCD73-
hCD39- hCD73+
hCD39+ hCD73+
hCD39- hCD73-
(h)
Percent hCD8+ T Cell Subsets
(i)
Percent iNKT Cells
(j)
Percent Treg Cells
*
(k)
Percent Non-B/T Cells
(l)
Percent Monocytes
(m)
Percent DCs
(n)
Fold-increase in Treg Cells
Stim
Stim + CGS

629
630

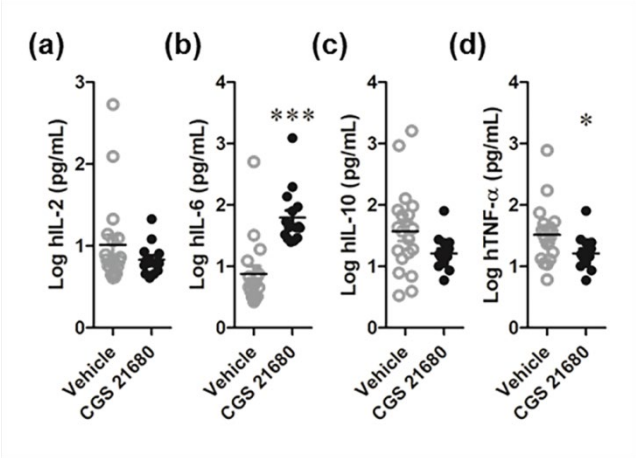

(a)
Vehicle
CGS 21680
Percent of Starting Weight
Days Post-injection
**
(b)
Vehicle
CGS 21680
Clinical Score
Days Post-injection
(c)
Vehicle
CGS 21680
Percent Survival
Days Post-injection
(d)
Vehicle (non-engrafted)
CGS 21680 (non-engrafted)
Percent of Starting Weight
Days Post-injection
**
(e)
Vehicle (non-engrafted)
CGS 21680 (non-engrafted)
Clinical Score
Days Post-injection
(f)
Vehicle (non-engrafted)
CGS 21680 (non-engrafted)
Percent Survival
Days Post-injection

631

632


(a)
Liver
Small Intestine
Skin
Vehicle
CGS 21680
100 µm
(b)
Number of Leukocytes (per Field of View)
3000
2000
1000
0
**
Vehicle
CGS21680
Liver
Small Intestine
Skin
(c)
Epidermal Thickness (µm)
200
150
100
50
0
Vehicle
CGS 21680

633
634


(a)
(b)
(c)
(d)
Log hIL-2 (pg/mL)
Log hIL-6 (pg/mL)
Log hIL-10 (pg/mL)
Log hTNF-α (pg/mL)
***
*
Vehicle
CGS 21680

635