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# Helminth antigens modulate human PBMCs, attenuating disease progression in a humanised mouse model of graft versus host disease☆

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ABSTRACT

*Fasciola hepatica* is a trematode worm that causes fascioliasis, a neglected tropical disease in humans and livestock. To gain insight into the host-parasite interactions that facilitate infection, we have investigated the immunomodulatory properties of the parasite's tegumental coat (FhTeg), a major antigen source that is sloughed off and renewed every 2–3 h as the worm migrates through host tissue. Using mouse models of infection, we have previously shown that FhTeg induces a novel phenotype of dendritic cells that induce anergic $CD4^+$ T-cells. We proposed that this induced state of hyporesponsiveness characterised by suppression of cell proliferation and cytokine secretion was one mechanism by which *F. hepatica* prevented host protective immunity to support the parasite survival. To determine if the same mechanisms are utilised during human infections, we have now examined the interaction of FhTeg with human PBMCs. FhTeg binds to and modulates cytokine production in human PBMCs, in particular targeting the $CD4^+$ population resulting in reduced levels of TNF, IL-2 and IFNγ and increased markers of anergy. Furthermore, the adoptive transfer of FhTeg stimulated PBMCs to a humanised model of acute graft versus host disease (GvHD) attenuated disease progression by increasing survival and reducing pathological scores. These mice also displayed a significant decrease in the total number of human $CD4^+$ cells expressing TNF, IL-2 and IFNγ in the spleen, liver and lung. This study therefore concurs with evidence from ruminant and murine models of infection suggesting that anergic $CD4^+$ T cells are associated with successful *Fasciola hepatica* infection and highlights an important role for FhTeg in contributing to the overall immunosuppressive effects of this parasite.

## 1. Introduction

*Fasciola hepatica*, commonly known as liver fluke, is a parasitic worm that causes chronic infection in the liver of livestock and humans (Dalton et al., 2013; Mas-Coma et al., 2019). The life cycle of the parasite alternates between the intermediate snail host and the definitive host where much of the tissue damage in the early stages of infection occurs as juvenile flukes migrate from the duodenum across the peritoneal cavity to penetrate the liver capsule. Flukes mature once reaching the bile ducts residing here for many years triggering the chronic stages of infection (Mas-Coma et al., 2005). Although generally not fatal, infection can cause significant morbidity. In cattle, infection is typically associated with weight loss, anaemia and increased susceptibility to bystander infections such as Bovine salmonellosis and tuberculosis; costing $1 billion losses to the agribusiness annually (Aitken et al., 1978; Kelly et al., 2018). Over one million people are at risk of infection, which can cause significant DALYs (daily adjusted living years) in human populations (Hotez et al., 2008; Mas-Coma et al., 2005). There is widespread resistance to the only chemotherapy available. To date no commercial vaccines exists, partially due to the lack of understanding of host-parasite interactions and also due to the potent immune modulatory effects of the parasite that supress the protective Th1 immune

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responses induced by candidate vaccines (Molina-Hernández et al., 2015).

*Fasciola hepatica* infection is initially associated with mixed Th1/Th2 immune response followed by biased Th2 and then the induction of T-regulatory (Treg) or tolergenic/anergenic T-cells (Fu et al., 2017; Molina and Skerratt, 2005; O'Neill et al., 2000; Ruiz-Campillo et al., 2018; Walsh et al., 2009). While Th1 immune responses are associated with immune protection the strong regulatory responses induced by *F. hepatica* subvert these responses (Dalton et al., 2013). One means of dissecting the impact that the parasite has on the host immune response is to examine the bystander effects of helminth infection to concurrent microbial infections or non-communicable co-morbidities such as autoimmune diseases. In both cattle and mice experimentally co-infected with *F. hepatica* and bacterial infection, exacerbation of bacterial infection coincides with enhanced regulatory immune responses and the suppression of protective anti-bacterial Th1 immune responses (Brady et al., 1999; Flynn et al., 2009). It has been shown that adult flukes release immune modulatory proteins into local tissues, bile, and the general circulation and that these molecules contribute to the regulation of host immune responses by *F. hepatica* (Cwiklinski et al., 2016; El-Bahi et al., 1992).

One major source of antigen is the *F. hepatica* tegumental coat (FhTeg), which is a metabolically active layer that is continuously sloughed off and replaced every 2–3 h during infection. We have shown that FhTeg in a mouse model of sepsis suppresses pro-inflammatory cytokines TNF, IL-12p70 and IFNγ (Hamilton et al., 2009). This is likely mediated by the induction of a novel subset of innate immune cells including dendritic cells (Clare M. Hamilton et al., 2009; K. V. Vukman et al., 2013), mast cells (Krisztina V. Vukman et al., 2013) and macrophages (Adams et al., 2014) that are ultimately unresponsive to a range of Th1 TLR ligands (Clare M. Hamilton et al., 2009; K. V. Vukman et al., 2013). Furthermore, FhTeg prevents the development of antigen specific responses by inhibiting cell to cell communication between innate immune cells and T-cells (Adams et al., 2014; Aldridge and O'Neill, 2016) and FhTeg-treated dendritic cells induce anergic $CD4^+$ T cells *in vitro* (Aldridge and O'Neill, 2016). No studies to date have examined the effect of FhTeg on human PBMCs. Here, we demonstrate that FhTeg binds to and modulates cytokine production in human PBMCs, in particular targeting the $CD4^+$ population. FhTeg stimulated $CD4^+$ cells express reduced levels of IL-2 and increased markers of anergy. The adoptive transfer of these FhTeg stimulated PBMCs to a humanised mouse model of graft versus host disease, attenuates disease progression demonstrating their potential modulatory properties giving some insight into the potential immune suppressive properties of FhTeg in human populations.

## 2. Materials and methods

### 2.1. Animals and ethics

All procedures involving animals or human material were performed by licenced personnel. NOD.Cg-Prkdc$^{scid}$IL2$^{tmlWjl}$/Szj (NOD-Scid IL-2rγ$^{null}$) (NSG) mice were purchased from Jackson Laboratories (distributors Charles River UK Ltd, Kent, UK). Mice were kept under specific pathogen free conditions at the Bioresources unit. All mice were housed following guidelines from the Health Products Regulatory Authority and following protocols approved by the institutional Animal Welfare Body (Maynooth University). Ethical permission for the use of animals was approved by the Department of Health or Health Products Regulatory Authority and Maynooth University Ethics committee.

### 2.2. FhTeg preparation

*Fasciola hepatica* tegumental antigen (FhTeg) was prepared by adapting a previously published method (Hamilton et al., 2009). Live adult flukes were collected from the livers of infected sheep at a local abbatoir and transported to the laboratory in warm sterile RPMI media supplemented with 10% fetal calf serum (Biosciences, Dun Laoghaire, Ireland) and 100u/ml penicillin/streptomycin (Sigma-Aldrich, Arklow, Ireland). Flukes were washed three times in warm sterile PBS and placed in 1% Nonidet P40 for 30 min at 37 °C. The supernatant was collected and the NP-40 was removed using Bio-Rad Bio-Beads SM-2 Adsorbent (Fannin Ltd, Dublin, Ireland). Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Leicestershire, UK), and endotoxin levels were assessed using the Pyrogene endotoxin detection system with levels detected less than 0.01 EU/ml considered acceptable for use (Lonza, Walkersville, USA). FhTeg was used in all cell culture stimulations at 10 μg/ml unless otherwise stated. This concentration was selected because previous dose response studies performed showed this to have an optimum effect both *in vitro* and *in vivo* (Clare M Hamilton et al., 2009; Krisztina V. Vukman et al., 2013).

### 2.3. Isolation of human PBMCs

Buffy packs (obtained from the Irish Blood Transfusion Service) was transferred to a sterile T75 flask and mixed with 60 ml of sterile PBS. Diluted blood was carefully layered on to 15 ml of Lymphoprep (Stem Cell Technologies, Cambridge, UK) in 50 ml tubes. Samples were centrifuged at 800×*g* for 25 min at room temperature (brake off). The PBMC layer was then transferred to a fresh 50 ml tube using a sterile 3 ml transfer pipette. Cells were centrifuged at 300×*g* for 10 min at 4 °C (brake on). The supernatant was carefully discarded and the cells washed three times in sterile PBS by centrifuging at 300×*g* for 5 min at 4 °C. Red blood cells were then removed from each sample by resuspending the pellet in 5 ml of RBC lysis buffer (Biolegend) for 5 min at room temperature in the dark. RPMI containing 10% FCS was added to neutralise the lysis buffer and cells were then centrifuged at 300×*g* for 10 min at 4 °C. Isolated PBMCs were then resuspended in 25 ml of media and counted. PBMCs were seeded at $2 \times 10^6$/ml for stimulation with LPS (100 ng/ml; Alexis, Enzo Life Sciences, Exeter, UK) or PMA (20 ng/ml; Sigma-Aldrich) and ionomycin (1 μM; Sigma-Aldrich), in the presence or absence of FhTeg (10 μg/ml). After 24 h, supernatant was removed to measure release of cytokines by commercial ELISA. PBMCs were also harvested and counted for use in GvHD model. $CD14^+$ cells were isolated from PBMCs using a positive selection $CD14^+$ isolation kit (Miltenyi, Surrey, UK) and seeded at $1 \times 10^6$/ml for stimulation with LPS for 24 h as optimum cytokine secretion can be measured at this experimental time point. $CD4^+$ cells were isolated from PBMCs using a positive selection $CD4^+$ isolation kit (Miltenyi) and seeded at $1 \times 10^6$/ml for stimulation with PMA and ionomycin for 72 h as optimum cytokine secretion is measured at this experimental time point. The cell population was confirmed using flow cytometry using fluorescently tagged antibodies to CD14/CD4 cells and the percentage of cells was greater than ninety percent.

### 2.4. Cell binding assay

Cellular adhesion assay was performed as previously described (Aldridge and O'Neill, 2016). Briefly, FhTeg and BSA were fluorescently labelled with a FITC-488 label using the Promofluor labelling kit according to the manufacturer's recommendations (Promokine, Heidelberg, Germany). Cells (50,000/well) were seeded in a 96 well plate. Cells were incubated with 1, 5 and 10 μg per ml of 488 labelled FhTeg at 37 °C for 45 min and washed in ice cold PBS before analysis using flow cytometry. As control for non-specific binding, cells were incubated with 10 μg per ml of FITC-488 labelled BSA.

### 2.5. RNA isolation, qPCR and qPCR gene array

RNA from stimulated cells was isolated using a high pure RNA isolation kit (Roche Diagnostics, Dublin, Ireland). The quantity and

quality of the RNA was measured using a nanodrop. RNA was reverse transcribed to cDNA using random primers and a reverse transcription kit (Roche, Dublin, Ireland). Primer probes (Roche) were used to quantify gene expression of the genes of interest, as listed in Table 1. Three housekeeping genes were used as internal standards, *β-actin* (NM_007393.3), *Gapdh* (NM_008084.2) and *Gusb* (β-glucuronidase) (NM_010368.1). These housekeeping genes were chosen based on endogenous control panel genes recommended by Applied Biosystems (http://www.appliedbiosystems.com/). The Cqmean expression of housekeeping genes were stable within each group although slight differences were observed between groups. However, these difference do not account for the enhanced gene expression observed for targeted genes (Supplemental Fig. 1). Gene expression was analysed using a Light Cycler 96 (Roche) and software version 1.1. For qPCR gene array, RNA from stimulated cells was isolated using a Qiagen kit (Qiagen, Manchester, UK), then reverse transcribed to cDNA. Samples were added to wells of a 96 well plate containing specific genes related to T cell anergy induction/maintenance, house-keeping genes and reverse transcription controls. The data was analysed using Qiagen's online data analysis tool.

### 2.6. Flow cytometry (in vitro, extracellular antibody staining)

Cells were harvested after stimulation, washed twice in FACS buffer (PBS, 2% FCS, 1 mM EDTA) and then incubated with the following antibodies: CD4 (clone GK4.5), PD1 (clone J43), CTLA4 (clone UC10-4B9), CD206 (clone C068C2) or the relative isotype control (eBioscienes, Hatfield, UK) for 15–30 min at 4 °C in the dark. The cells were then washed twice with FACS buffer and analysed on a BD FACS Aria. The data was analysed using Flow Jo software (Treestar, Ashland, USA). In all experiments unstained and single stained controls were used for gating and compensation.

### 2.7. Humanised mouse model of acute graft versus host disease

NOD.Cg-Prkdc$^{scid}$IL2$^{tmlWjl}$/Szj (NOD-Scid IL-2rγ$^{null}$) (NSG) mice (Jackson Laboratories) were exposed to a conditioning dose of 2.4 Gy whole body gamma irradiation, then 4 h later injected intravenously (via tail vein) with human PBMCs ($1 \times 10^6$ cells/g) that had been pre-stimulated for 24 h with FhTeg or PBS, in accordance with a humanised mouse model of acute GvHD previously developed in the laboratory (Tobin et al., 2013). Animals were returned to their cages and closely monitored for the first hour and at regular intervals for signs of distress or ill health. Mice were weighed every 2 days until day 7 and every day thereafter. Animals which displayed greater than 15% weight loss on successive days were deemed to have acute GvHD and were sacrificed humanely. Development of disease progression was also assessed using a previously described scoring system to record a series of pathological features, namely posture (hunching), activity, grooming and diarrhoea (Tobin et al., 2013). Any animal which obtained a cumulative score of 7 or higher was considered to have acute GvHD and was subsequently sacrificed humanely. Serum was harvested from mice on Day 12 for measurement of human TNF levels by commercial ELISA (Peprotech, London, UK).

**Table 1**
Primers used for gene expression analysis by qPCR (human).

| Gene | Sense | Anti-sense |
|---|---|---|
| *β-ACTIN* | TTCCTCCCTGGAGAAGAGCTA | CGTGGATGCCACAGGACT |
| *GAPDH* | AGCCACATCGCTCAGACAC | GCCCAATACGACCAAATCC |
| *GUSB* | TCGCCATCAACAACACACTC | TCTGGACAAACTAACCCTTGG |
| *CTLA4* | TCACAGCTGTTTCTTTGAGCA | AGGCTGAAATTGCTTTTCACA |
| *GRAIL* | GGAGGGCAAAAGGAGATACC | CAGAAAAGCCGTTTTGGAGA |

### 2.8. Histology

The lungs, liver, spleen and small intestine were harvested from mice at day 12 and fixed in 10% (v/v) neutral buffered formalin for 24 h, then transferred to 70% ethanol for 24 h. Samples were processed using an automated processor (Thermo Fisher Scientific) which immersed the tissues in fixatives and sequential dehydration solutions including ethanol (70%, 80%, 95% x2, 100% x3) and xylene (×2) (Sigma-Aldrich). After processing, tissues were embedded in paraffin wax using a Shandon Histocentre 2 and allowed to set at 4 °C overnight. A Shandon Finesse 325 microtome was used to cut 5 μm sections of each tissue. Sections were placed in cold water before being transferred to a hot water bath (60 °C) to remove any folding of the sections. Tissue sections were placed onto microscope slides (Thermo Fisher Scientific) and air dried overnight.

For H&E staining, slides were heated to 60 °C for 1 h and then subjected to two xylene treatments (Sigma-Aldrich) for 10 min each, before rehydration by immersion in ethanol at 3 decreasing concentrations (100%, 90% and 80%) for 5 min each. Samples were transferred to $dH_2O$ for 5 min before immersion in Haemotoxylin (Sigma-Aldrich) for 3 min, and then washed in $H_2O$ for 2 min before placing in 1% acid alcohol for 20 s. Samples were then immersed in Eosin Y (Sigma-Aldrich) for 3 min following a wash step with $H_2O$. The dehydration step was repeated and samples air dried and mounted with DPX mounting media (VWR, Dublin, Ireland). Slides were examined under a light microscope and blindly scored by two independent researchers. A semi-quantitative scoring chart was used to assess disease progression in the lungs, liver, spleen and small intestine as previously described (Tobin et al., 2013).

### 2.9. Flow cytometry (Ex vivo, extracellular and intracellular antibody staining)

Mononuclear cells were isolated from lung, liver (by density gradient centrifugation) and spleen of mice as previously described (Tobin et al., 2013). Cells were seeded at $1 \times 10^5$/well in complete RPMI and stimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 5 h in the presence of 1 X Golgi Stop (eBoiscience). After stimulation, cells were transferred to a V-bottom 96 well plate (Lennox Laboratory Supplies, Dublin, Ireland) and following two wash steps with FACS buffer, cells were incubated with cell surface antibodies (CD45 (HI30), CD45RO (UCHL-1), CD27 (0323), CD62L (DREG-56), CD4 (SK3) and CD8 (RPA-T8)) before further wash steps. Fix/Perm buffer (eBioscience) was added to each well and samples were incubated in the dark at 4 °C overnight. Permeabilisation buffer (200 μl) was added to each well and cells were centrifuged at 300×*g* for 5 min at 4 °C. Supernatant was discarded and samples were blocked in 3 μl of 2% rat serum (Sigma-Aldrich) for 20 min at 4 °C. 1 μl of fluorochrome labelled antibody (TNF (Mab11), IFN-γ (4S.B3), IL-2 (MQ1-17H12)) or isotype controls (eBioscience) were added to the cells before incubation in the dark at 4 °C for 1 h. Following two wash steps with FACS buffer, cells were resuspended in counting beads (50 μl) (BD Biosciences, Oxford, UK) and analysed on a BD Accuri C6 flow cytometer. For the gating strategy, live lymphocytes were gated using a FSC SSC plot followed by gating on human CD45$^+$ cells and then human CD4$^+$ or CD8$^+$ T cells. Initial gating for cytokines was selected first on negative expression of the isotype control antibodies followed by positive expression of cytokines in stimulated cells.

### 2.10. Data analysis

All data were analysed for normality prior to statistical testing by Prism® 6.0 (GraphPad Software Inc, La Jolla, CA, USA) software. Where multiple group comparisons were made, data were analysed using one-way or two-way ANOVA. For comparisons between two groups, the Student's *t*-test was used. In all tests, $p < 0.05$ was deemed significant.

## 3. Results

### 3.1. FhTeg suppresses TNF production in human PBMCs and CD4$^+$ cells, but not CD14$^+$ cells

Since FhTeg can bind directly to murine immune cells we investigated if FhTeg could also bind to human PBMCs and isolated subsets of CD14$^+$ (monocytic) and CD4$^+$ cells. PBMCs, CD14$^+$ cells and CD4$^+$ cells were incubated with PBS, FITC labelled FhTeg or FITC labelled BSA for 45 min and then analysed using flow cytometry. FhTeg significantly bound to whole PBMCs, CD14$^+$ and CD4$^+$ cells (Fig. 1A–C).

Since FhTeg can inhibit cytokine production from murine dendritic cells, macrophages and mast cells stimulated with TLR ligands (Clare M Hamilton et al., 2009), and polyclonal stimulated CD4$^+$ cells, we also investigated if FhTeg would have similar effects on human PBMCs. A significant inhibition of TNF production was observed in total PBMCs when stimulated with FhTeg 2.5 h prior to the addition of LPS or PMA and ionomycin (Fig. 4 D, G; $p < 0.01$, $p < 0.0001$). In contrast, pre-treatment of isolated CD14$^+$ cells with FhTeg for 2.5 h before LPS stimulation or without LPS stimulation did not inhibit the production of TNF or IL-1β, but instead significantly enhanced the production of these cytokines (Fig. 1 E, H; $p < 0.001$, $p < 0.0001$). As these are human donors different individuals may respond differently to the antigens *in vitro* while *in vivo* the antigen would interact with multiple cells that in turn with communicate with each other. Interestingly, studies measuring TNF in serum isolated from *F. hepatica* infected individuals demonstrate raised TNF levels (Osman and Abo-El-Nazar, 1999). This does not fit with the observations with see in the mouse model and given the dearth of studies in humans, further investigations need to be carried. Stimulation of isolated CD4$^+$ cells with FhTeg for 2.5 h before PMA and ionomycin stimulation significantly inhibited the production of TNF and IL-2 (Fig. 1 F, I; $p < 0.0001$).

### 3.2. FhTeg enhances the expression of anergy markers in human CD4$^+$ cells and these cells inhibit cytokine production from CD14$^+$ cells

Human CD4$^+$ cells were stimulated with FhTeg for 2 h to determine if FhTeg can induce the anergenic associated markers *CTLA4* and *RNF* 128 by qPCR. The levels of both these genes were significantly enhanced with a fold increase of 1.9 and 2.7 respectively (Fig. 2 A-B; $p < 0.001$, $p < 0.05$). Since our previous findings have shown that mouse dendritic cells stimulated with FhTeg induce anergy in CD4$^+$ cells (Aldridge and O'Neill, 2016), we investigated if FhTeg treated human CD14$^+$ cells had a negative regulatory effect on CD4$^+$ cells. CD14$^+$ cells were stimulated with PBS or FhTeg for 24 h before co-culture with CD4$^+$ cells from both the same and different donors for 72 h. CD14$^+$ cells pre-treated with

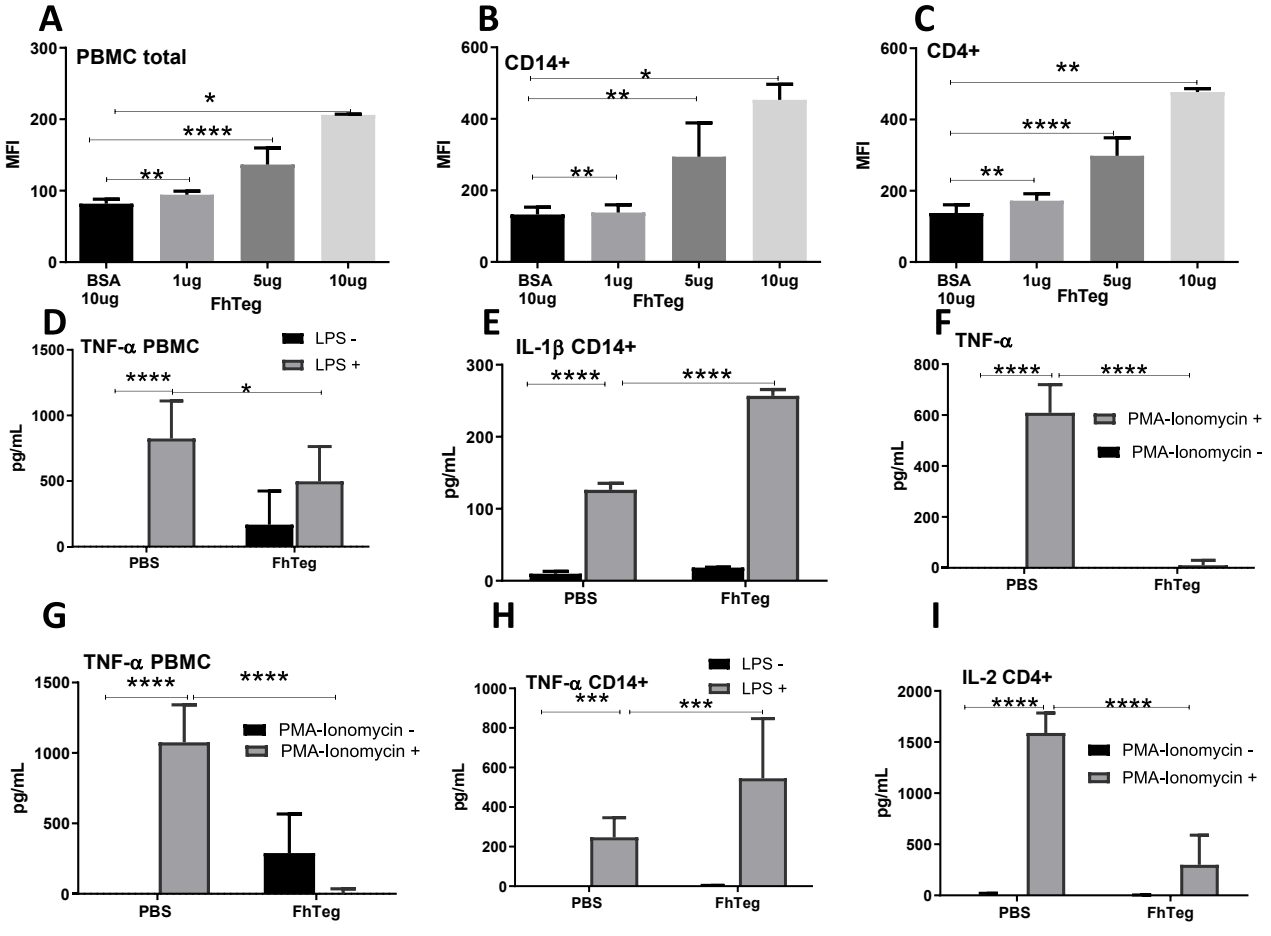


**Fig. 1. FhTeg binds to human PBMCS and inhibits the production of cytokines in PBMCs and isolated CD4$^+$ cells but not isolated CD14$^+$ cells.** (A) PBMCs, (B) CD14$^+$ and (C) CD4$^+$ cells were incubated with PBS, FITC-labelled FhTeg (1, 5 and 10 μg/ml) or FITC-labelled BSA (10 μg/ml) for 45 min and then analysed using flow cytometry. (D, G) PBMCs were stimulated with FhTeg (10 μg/ml) for 2.5 h prior to stimulation with LPS or PMA + ionomycin for a further 24 h. Secreted TNF was quantified by ELISA. (E, H) CD14$^+$ cells were stimulated with FhTeg for 2.5 h prior to stimulation with LPS for a further 24 h. Secreted TNF and IL-1β were quantified by ELISA. (F, I) CD4$^+$ cells were stimulated with FhTeg for 2.5 h prior to stimulation with PMA+ionomycin for a further 72 h. Secreted TNF and IL-2 were quantified by ELISA. The data shown are the mean ± SD of four independent experiments (donor number n = 4) (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

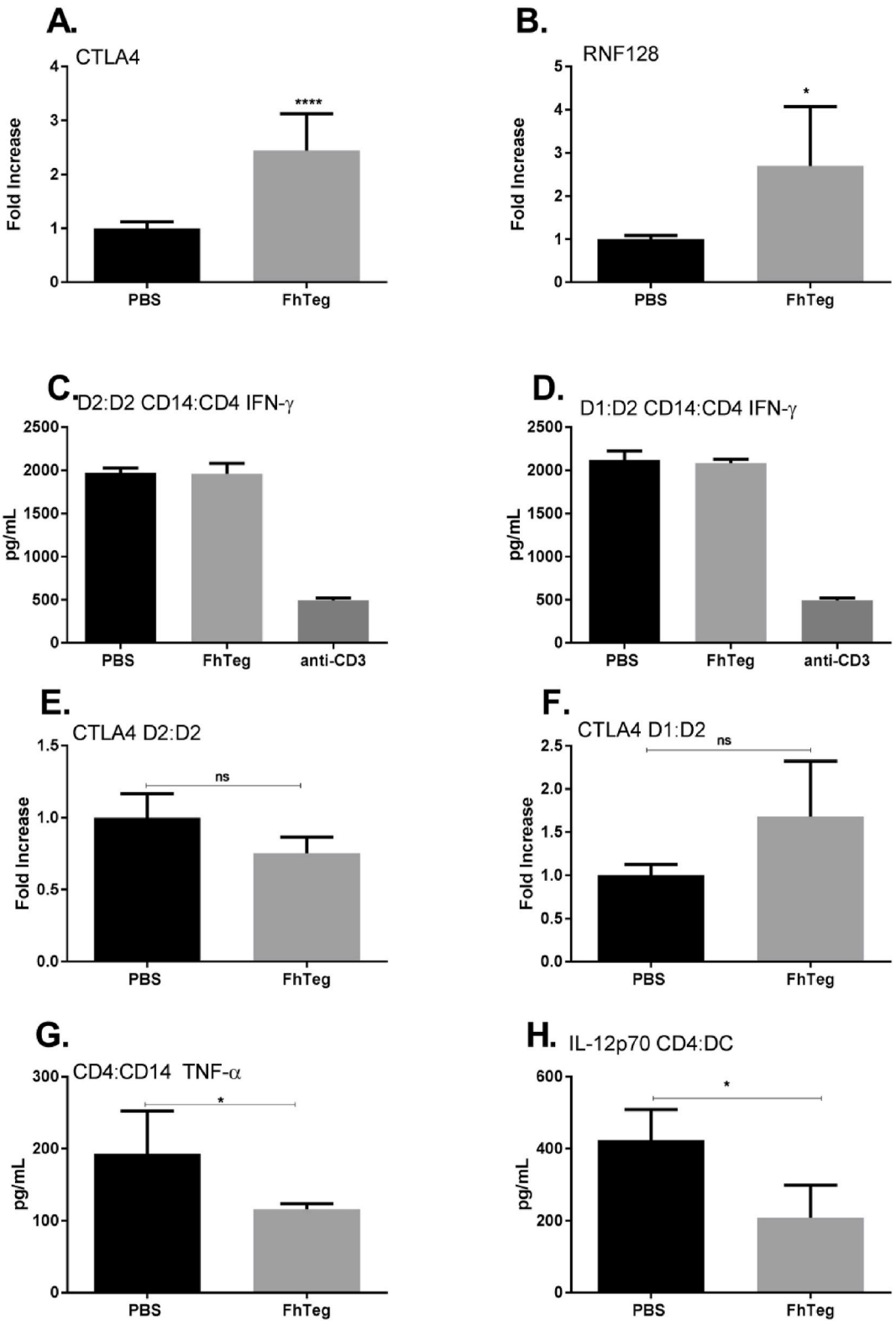


**Fig. 2.** FhTeg enhances expression of anergic genes in human $CD4^+$ cells, and these cells suppress the production of cytokines from $CD14^+$ cells. (A-B) Human $CD4^+$ cells isolated from PBMCs were stimulated with FhTeg (10 μg/ml) for 2 h. *CTLA4* and *GRAIL* gene expression were measured by qPCR. (C-F) Human $CD14^+$ cells were stimulated with FhTeg for 24 h before co-culture with $CD4^+$ cells from the same or different donor in a 1:10 ratio with plate bound anti-CD3 for a further 72 h. Secreted IFN-γ was measured by ELISA and *CTLA4* gene expression analysed by qPCR. (G) Human $CD4^+$ cells were stimulated with PBS or FhTeg for 24 h before co-culture with $CD14^+$ cells in a 1:1 ratio for a further 24 h. $CD14^+$ cells were then re-isolated and rested for 24 h in fresh media before LPS challenge (100 ng/ml) for 24 h. Secreted TNF was measured by ELISA. (H) Mouse $CD4^+$ cells were stimulated with PBS or FhTeg for 24 h before co-culture with bone marrow derived dendritic cells in a 1:1 ratio for a further 24 h. DCs were then re-isolated and rested for 24 h in fresh media before LPS challenge for 24 h. Secreted IL-12p70 was measured by ELISA. The data shown are the mean ± SD of three independent experiments (donor number n = 3), $*p < 0.05$, $****p < 0.0001$. D2:D2 = same donor; D1:D2 = different donor.

FhTeg did not inhibit the production of IFN-γ from $CD4^+$ cells or induce *CTLA4* gene expression (Fig. 2C–F). In contrast, the ability of human $CD4^+$ cells to modulate $CD14^+$ cells was also investigated by stimulation of $CD4^+$ cells with FhTeg for 24 h before co-culture with $CD14^+$ cells for a further 24 h. $CD14^+$ cells were then re-isolated and rested before stimulation with LPS. FhTeg treated $CD4^+$ cells significantly inhibited the production of TNF from $CD14^+$ cells stimulated with LPS after co-culture (Fig. 2 G; $p < 0.05$). Furthermore, the ability of mouse $CD4^+$ cells to modulate dendritic cells was evaluated by a similar co-culture technique, and FhTeg treated $CD4^+$ cells were observed to significantly inhibit the production of IL-12p70 from dendritic cells stimulated with LPS following co-culture (Fig. 2 H; $p < 0.05$).

### 3.3. FhTeg pre-treatment of human PBMCs significantly delayed the development of acute GvHD in a humanised mouse model

Given that FhTeg suppressed human $CD4^+$ T cell cytokine production and induced anergy *in vitro,* we examined the effect of FhTeg stimulated human PBMCs *in vivo* in a humanised mouse model of acute GvHD. Mice that received FhTeg-stimulated PBMCs survived significantly longer (up to 22 days; $p < 0.001$) and had reduced pathological scores ($p < 0.001$) and percentage weight loss ($p < 0.001$) relative to mice injected with unstimulated PBMCs (Fig. 3A–C). The acute GvHD target organs- lung, liver and small intestine appeared normal in control mice (PBS injected), whilst mice that received unstimulated PMBCs displayed typical disease characteristics, including significant mononuclear cell infiltration in the liver, lamina propria, intestinal crypts and the lungs. Sloughing of the lamina propria was a common feature, as was endothelialitis in the liver. In contrast, mice that received FhTeg-treated PBMCs showed reduced mononuclear cell infiltration in the liver, lamina propria, intestinal crypts and lungs. There was also reduced sloughing of the lamina propria and reduced endothelialitis in the liver (Supplementary Fig. 2). When we analysed these mice further, we found a striking reduction in the engraftment of human $CD45^+$ immune cells in mice which received FhTeg-treated PBMC. There was a significant reduction in the total number of tissue infiltrating human $CD45^+$ immune cells in the lung,

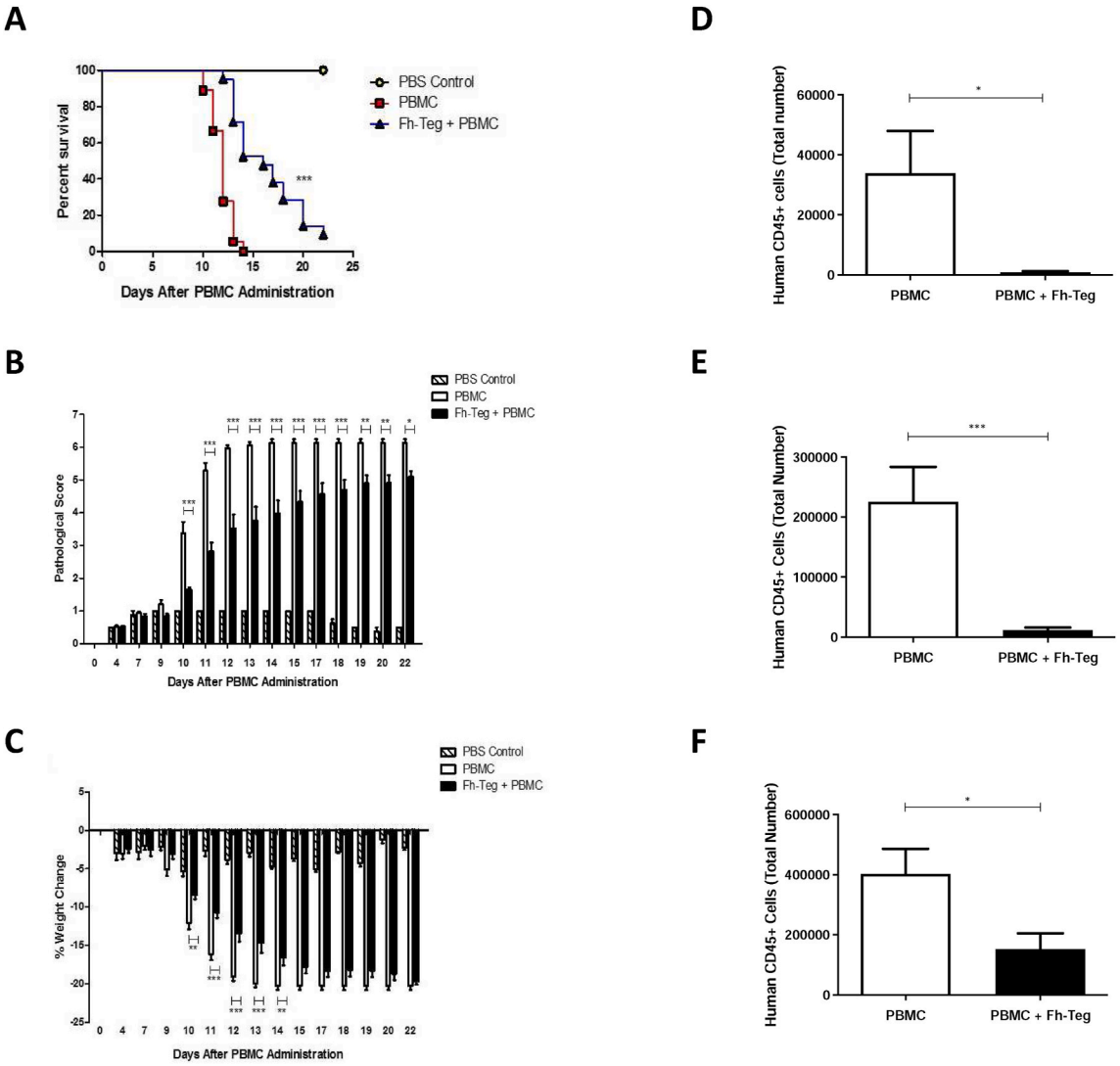


**Fig. 3. FhTeg pre-treatment of human PBMCs delays the development of acute GvHD by significantly impairing human PBMC engraftment in a humanised mouse model.** NSG mice were sub-lethally irradiated (2.4 Gy) and administered PBS, PBMCs or FhTeg-treated PBMCs ($1 \times 10^6$ cells/g) via tail vein injection on day 0. Mice were monitored for (A) survival, (B) pathological score and (C) weight change for the duration of the experiment. PBS control mice are represented by diagonally hatched bars, mice which received PBMCs are represented by white bars and mice which received FhTeg-treated PBMCs are represented by black bars. Statistical significance was determined using Student's *t*-test where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; n = 20. GvHD target organs (lung (D), liver (E) and spleen (F)) were harvested on day 12 and mechanically digested. PBMCs were isolated via density gradient centrifugation and the total number of infiltrating $CD45^+$ human immune cells was determined via flow cytometry; n = 12 per group.

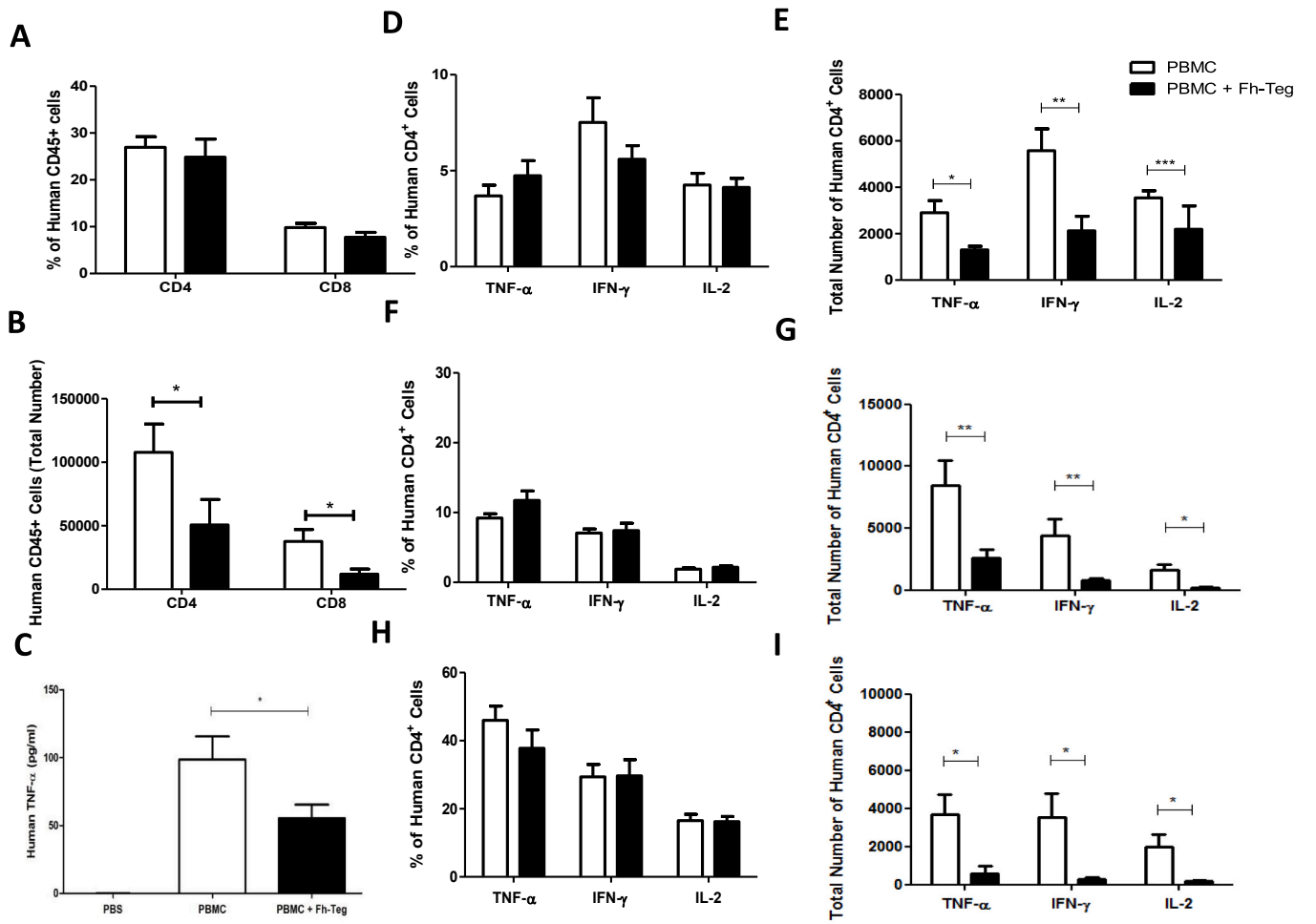


**Fig. 4. FhTeg significantly reduced the number of human TNF, IFN-γ and IL-2 producing CD4$^+$ cells in acute GvHD mice.** NSG mice were sub-lethally irradiated (2.4 Gy) and administered PBMCs or FhTeg-treated PBMCs ($1 \times 10^6$ cells/g) via tail vein injection. GvHD target organs were harvested for analysis on day 12. (A-B) The spleen was examined for engraftment levels of human CD4$^+$ and CD8$^+$ T cells by flow cytometry. (C) Serum levels of human TNF were analysed by ELISA. n = 12 per group (4 PBMC donors, 3 mice per donor). Intracellular flow cytometry was used to examine cytokine levels produced by CD4$^+$ cells in the spleen, liver and lung. Results are presented as both the percentage and total number of TNF, IFN-γ and IL-2 producing CD4$^+$ cells recovered from the spleens (D-E), livers (F-G) and lungs (H-I) of PBMC (white bars) or FhTeg PBMC (black bars) treated mice. Statistical significance was determined using Student's *t*-test where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

liver and spleen of mice which received FhTeg-treated PBMC (Fig. 3D–F).

### 3.4. FhTeg pre-treatment of human PBMCs significantly reduced the number and function of activated, naïve, Tem and Tcm human CD4$^+$ T cells in aGvHD mice

In order to determine if the reduced disease pathology observed with FhTeg stimulated PBMCs in the mouse model was due to reduced cell engraftment, GvHD target organs (spleen, liver and lung) were harvested from mice and examined for engraftment levels of human CD45$^+$-CD4$^+$ and -CD8$^+$ T cells by flow cytometry. The total number of these cell populations were significantly reduced in the spleens of mice that received FhTeg-treated PBMCs versus mice that received unstimulated PBMCs (Fig. 4 A-B; $p < 0.05$). In addition, serum levels of human TNF were significantly reduced in mice that received FhTeg-treated PBMCs versus unstimulated PBMCs (Fig. 4 C; $p < 0.05$). While there was no difference in the percentage of human CD4$^+$ T cells producing human TNF, IFN-γ and IL-2 in the spleen, liver and lung of mice that received FhTeg-treated PBMCs, there was a significant decrease in the total number (Fig. 4D–I).

Furthermore, we quantified the numbers of activated (CD45RO$^+$), naïve (CD45RO$^-$), effector memory (Tem: CD45RO$^+$CD27$^+$) and central memory (Tcm; CD45RO$^+$CD27$^+$CD62L$^+$) human CD4$^+$ T cells in the spleen, liver and lung of the experimental mice. The total number, but not percentage, of each of these subsets was significantly reduced in the spleen (Fig. 5A–B), liver (Fig. 5C–D) and lung (Fig. 5E–F) of mice that received FhTeg-treated PBMCs relative to untreated PBMCs.

## 4. Discussion

*Fasciola hepatica* can survive for many years within the host because it is constantly secreting and excreting molecules that potently regulate the normal host protective immune response. While significant advances have taken place in understanding host parasite interactions between *F. hepatica* and its natural or experimental hosts, few studies have examined immune responses in human populations (Dalton et al., 2013). Therefore, this study using a humanised murine model of acute GvHD to shed some light on the potential immune modulatory properties of *F. hepatica* antigens when exposed to human cells. FhTeg-treated human PBMCs administered to mice increased survival and reduced pathological scores compared to unstimulated cells and this coincided with the reduction of the total number of CD4$^+$ cells expressing TNF, IL-2 and IFNγ. Examining the effects of the FhTeg on human PBMCs *in*

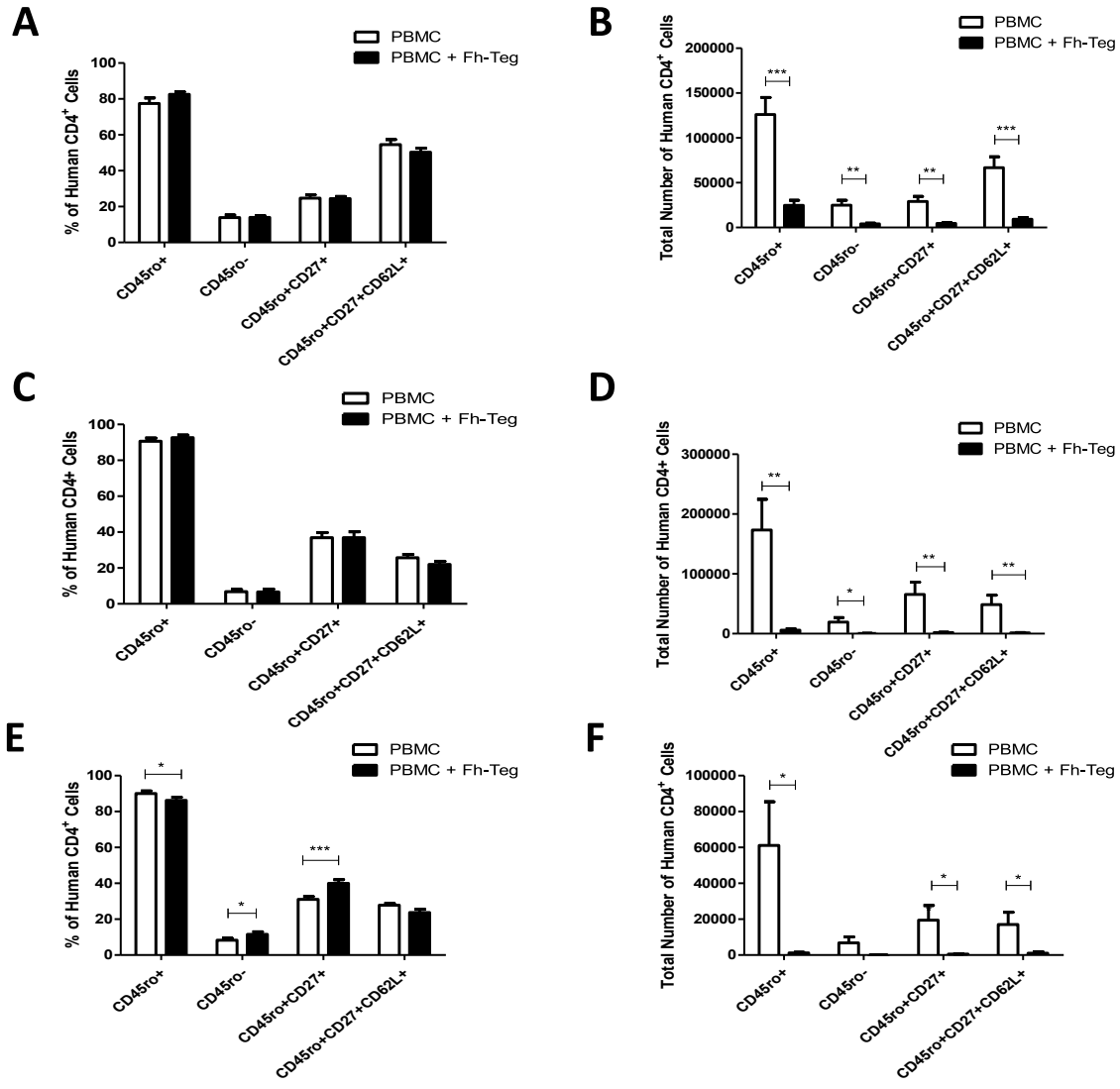


**Fig. 5. FhTeg significantly reduced the number of activated (CD45RO$^+$), naïve (CD45RO$^-$), effector memory (CD45RO$^+$ CD27$^+$) and central memory (CD45RO$^+$ CD27$^+$CD62L$^+$) human CD4$^+$ T cells in acute GvHD mice.** NSG mice were sub-lethally irradiated (2.4 Gy) and administered PBMCs or FhTeg-treated PBMCs ($1 \times 10^6$ cells/g) via tail vein injection and monitored for 12 days. Results are presented as both the percentage and total number of active, naïve, effector memory and central memory CD4$^+$ T cells recovered from the spleens (A-B), livers (C-D) and lungs (E-F) of PBMC (white bar) or FhTeg PBMC (black bar) treated mice. n = 12 per group (4 PBMC donors, 3 mice per donor). Statistical significance was determined using Student's *t*-test where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

*vitro*, revealed that FhTeg binds to the CD4$^+$ population of cells and inhibits TNF, IL-2, and IFNγ secretion while enhancing the expression of anergy makers. While these FhTeg-treated CD4$^+$ cells modulate the activity of CD14$^+$ dendritic cells the FhTeg stimulated CD14$^+$ cells do not reciprocally modulate CD4$^+$ cells which does not support previous studies in bovines or mice, thus highlighting potential differences between different species (Aguayo et al., 2019; Ramos-Benitez et al., 2018).

To our knowledge this is the first study to examine the interactions of FhTeg with human CD4$^+$ T cells demonstrating reduced IFNγ following co-stimulation with LPS or polyclonal activators. The suppression of IFNγ is a common feature of *F. hepatica* infection that occurs within in the early stages of infection due to its importance in immunity following vaccination (Clery and Mulcahy, 1998). While typically IFNγ is expressed in the early stages of infection albeit in addition to elevated IL-4, IL-5 and IL-10, as the infection progresses the levels of IFNγ decreases significantly (Bossaert et al., 2000; Clery and Mulcahy, 1998; Walsh et al., 2009; Zafra et al., 2010). Helminth released antigens can mimic the immune responses associated with infection and numerous studies have shown that one of the common properties of Fasciola antigens is the suppression of IFNγ. These molecules directly block IFNγ promoting cytokines such as IL-12 in innate immune cells or induces M2 like macrophages that secrete TGF or IL-10 that can suppress IFNγ. As a bystander effect to Fasciola infection, the suppression of IFNγ contributes to susceptibility to co-infection (Brady et al., 1999; Flynn et al., 2009) and confers protection to co-morbidities such as autoimmune diseases. In studies examining the beneficial effects of *F. hepatica* molecules in murine models of arthritis, EAE, and sepsis the suppression of IFNγ is observed (Donnelly et al., 2010; Khan et al., 2015; Lund et al., 2014; Quinn et al., 2019; Ramos-Benitez et al., 2018).

In addition to supressing IFNγ, FhTeg enhanced expression of *CTLA4* and RNF128 in human CD4$^+$ cells and reduced IL-2 which are characteristics of T-cell anergy. Anergic T cells are often found following chronic infection or after multiple exposures to antigens (Taylor et al., 2012). Anergic CD4$^+$ T cells are present during *F. hepatica* infection, and injecting of FhTeg *in vivo* is sufficient to induce this cell population

(Aldridge and O'Neill, 2016). Anergic T-cells during infection could supress' immune responses that associated with the immune pathology in the host induced by flukes migrating from the gut through the peritoneal cavity, and liver parenchyma towards the bile ducts (Stempin et al., 2016). Anergy is an induced state of T cell hyporesponsiveness where cytokine secretion and proliferation are suppressed, reversible by the addition of exogenous or recombinant IL-2 (Schwartz, 2003). Therefore, the characteristics of anergic T cells are a lack of production of IL-2, which in turn leads to a suppression of proliferation and a block in the cell cycle at the G1 phase (Powell et al., 2001). This could explain the reduction of total numbers of $CD4^+$ cells and the decrease in cytokine production in the humanised mouse model of acute GvHD.

In addition to decreasing cytokine secretion from $CD4^+$ cells, the application of FhTeg stimulated human PBMCs in the humanised mouse model of acute GvHD significantly increased survival and reduced pathological score, showing that FhTeg can be functionally relevant *in vivo* in a preclinical model where pathology is mediated by human T cells. Acute aGvHD is a life-threatening condition that can occur in patients following allogenic haematopoietic stem cell transplantation (HSCT) and is mediated by the recognition of host cells and tissues as antigens by donor T cells. Current treatment of aGvHD involves inhibition of donor T cells using glucocorticosteroids and immunosuppressive drugs, which improve survival for many patients but overall the prognosis remains poor (Ferrara et al., 2009; Messina et al., 2008). As TNF is a key effector in the pathology of acute GvHD, and anti-TNF is an important treatment for patients with corticosteroid refractory disease (Lv et al., 2014), these data are consistent with FhTeg having a potential therapeutic function. However, there was a significant reduction in engraftment of human $CD4^+$ cells in GvHD target organs (spleen, liver and lung) of NSG mice receiving FhTeg stimulated PBMCs. Since engraftment of donor T cells is critical for the success of HSCT, it is unlikely that FhTeg pre-treatment would be feasible as a preventative therapy for acute GvHD. Instead, FhTeg might be more suitable in organ transplantation where it could help prevent T cell mediated graft rejection.

We also demonstrate that FhTeg stimulated $CD4^+$ cells enhanced rather than suppressed TNF or IL-1beta in $CD14^+$ cells which supports studies that observed enhanced IL-1beta and/or TNF in the serum of rats (Balog et al., 2010) and humans (Osman and Abo-El-Nazar, 1999) during the chronic stages of *F. hepatica*. The interface of innate and adaptive immunity is often predominantly viewed as APC modulation of T cells, however there are many studies detailing the influence of both Tregs and anergic T cells on innate immune cells (Frasca et al., 2003; Misra et al., 2004; Oderup et al., 2006; Tadokoro et al., 2006). Given that so few studies have measured this parameter during infection, we cannot draw any firm conclusions, although from *in vitro* studies examining *F. hepatica* antigens a suppression of TNF and IL-1B in this population could be proposed (Aguayo et al., 2019; Ramos-Benítez et al., 2017). Indeed, in this study, serum levels of human TNF were significantly reduced which would reflect other studies examining this parasite (Garcia-Campos et al., 2019; Khan et al., 2020; Rojas et al., 2016).

In conclusion, this study deepens our understanding of how helminths circumvent host defence through the release of powerful immune modulatory antigens. It also provides us with some insight into the modulatory properties of *F. hepatica* in human populations in the absence of robust field studies. This model provides a unique advantage as PMBCs migrate to peripheral tissues and organs which facilitates the study of FhTeg effects in local tissues. This is important as studies in bovine and sheep have shown differences in responses between PBMCs compared to local lymph nodes and liver tissue during *F. hepatica* infection. We have also demonstrated a direct interaction of FhTeg with $CD4^+$ cells inducing an anergy like population. Few antigens have been shown to directly interact with $CD4^+$ cells to elicit an immune response; however superantigens produced by pathogenic microbes such as bacteria can cause non-specific activation of T cells. In addition to the 95% of $CD4^+$ T cells, human PBMCs also comprise of B-cells, natural killer cells, monocytes and dendritic cells and numerous studies have implicated their modulatory effects on $CD4^+$ cells population. We therefore, cannot rule out a role for these remaining 5% of cells. We need to further characterise these interactions and expand the characterisation of $CD4^+$ T-cells by measuring other genetic markers that are important for the induction and maintenance of T cell anergy (Doherty et al., 2010; Whiting et al., 2011; Zheng et al., 2012). Such advancement of the preliminary data presented here will cultivate a deeper understanding of the relationship between *F. hepatica* and its human host. Furthermore, the role of anergic T-cells in helminth infection is much less known opening up this new area of study in helminth biology.

## Credit author statement

O'Neill SM, Mahon BP, English K: Conceptualization. Healy M; Aldridge A; Glasgow AMA; Mahon BP; English K; O'Neill SM: Methodology. Healy M; Aldridge A; Glasgow AMA; Mahon BP; English K; O'Neill SM: Data curation. Healy M; Aldridge A; English K; O'Neill SM Writing- Original draft preparation. Mahon BP; English K; O'Neill SM: Supervision. Healy M; Aldridge A; Glasgow AMA; Mahon BP; English K; O'Neill SM: Writing- Reviewing and Editing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2022.108231.

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