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A Critical Role for Donor-Derived IL-22 in Cutaneous Chronic GVHD

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Abstract

Graft-versus-host disease (GVHD) is the major cause of non-relapse morbidity and mortality after allogeneic stem cell transplant (allo-SCT). Prevention and treatment of GVHD remains inadequate and commonly leads to end-organ dysfunction and opportunistic infection. The role of IL-17 and IL-22 in GVHD remains uncertain, due to an apparent lack of lineage fidelity and variable and contextually determined protective and pathogenic effects. We demonstrate that donor T-cell-derived IL-22 significantly exacerbates cutaneous chronic GVHD, and that IL-22 is produced by highly inflammatory donor $CD4^+$ T-cells post-transplant. IL-22 and IL-17A derive from both

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independent and overlapping lineages, defined as Th22 and IL-22^{+}Th17 cells. Donor Th22 and IL-22^{+}Th17 share a similar IL-6-dependent developmental pathway and whilst Th22 arise independently of the IL-22^{+}Th17 lineage, IL-17 signaling to donor Th22 directly promotes their development in allo-SCT. Importantly, while both IL-22 and IL-17 mediate skin GVHD, Th17-induced chronic GVHD can be attenuated by IL-22 inhibition in preclinical systems. In the clinic, high levels of both IL-17A and IL-22 expression are present in the skin of GVHD patients after allo-SCT. Together, these data demonstrate a key role for donor-derived IL-22 in chronic skin GVHD and confirm parallel but symbiotic developmental pathways of Th22 and Th17 differentiation.

Introduction

A significant and increasing proportion (10–15%) of patients with hematological cancers undergo stem cell transplantation (SCT), meaning it remains an important curative therapeutic option. The success of allogeneic SCT (allo-SCT) is limited by the development of graft-versus-host disease (GVHD) whereby donor T-cells mount an immune response to disparate recipient alloantigens expressed within host tissues. GVHD can manifest acutely or chronically, resulting in significant and widespread tissue damage occurring primarily within the gastrointestinal tract, skin, lung and liver.[1] Despite advances in immunosuppressive therapies, the incidence of GVHD remains high (>50%).[2,3] Cutaneous manifestations including lichenoid and sclerodermatous GVHD are common late after allo-SCT with limited treatment options beyond protracted steroid therapy.[3–5] Chronic GVHD thus has a significant detrimental impact on patient quality-of-life,[3] meaning new therapeutic strategies are urgently needed.

Donor T-cells play a central role in GVHD; depletion of T-cells from the donor graft prevents GVHD, but is associated with increased rates of relapse and infective complications which negate any beneficial effect.[6] Cytokines are critical mediators of inflammatory processes after allo-SCT and interleukin-22 (IL-22) has been shown to play a particularly complex role in GVHD.[7] IL-22 is a member of the IL-10 cytokine superfamily expressed by both the adaptive (CD4^{+} and CD8^{+} T-cells) and innate immune system ($\gamma\delta$ T, NKT and innate lymphoid cells (ILCs) including NKs, ILC3 and lymphoid tissue inducer (LTi) cells).[8–10] The IL-22 receptor shares one subunit with the IL-10 receptor (IL-10RB2), which forms a heterodimer with the IL-22Rα subunit, expressed almost exclusively by non-hematopoietic tissue.[9,10] The IL-22Rα is expressed in epithelia, keratinocytes and fibroblasts such that IL-22 provides an important conduit between the immune system and organ surfaces during infection and tissue injury.[8,9] In adaptive immunity, IL-22 is often linked with IL-17 expression and indeed is commonly considered as a “Th17” cytokine. However, more recent studies have demonstrated IL-22 expression independent of IL-17 in multiple contexts and Th22 are increasingly recognized as a distinct T-cell differentiation program.[11–14]

Recipient-derived IL-22, produced by ILCs, promotes the survival and persistence of intestinal stem cells and helps maintain gut epithelial integrity.[15] In contrast, others have demonstrated a pathogenic role for donor T-cell-derived IL-22 in murine models of acute GVHD and have shown that donor IL-22 does not contribute to graft-versus-leukemia

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effects.[16,17] Given that clinical trials using IL-22-Fc are underway to treat acute gastrointestinal GVHD (clinical trials.gov, NCT02406651),[18] the potential pathogenic effects of IL-22 suggests a better understanding of the cell lineages involved and their clinical relevance is urgently required. Here, we have evaluated the function of IL-22 in the pathophysiology of classic and inflammatory chronic GVHD using pre-clinical models and patient samples.

Methods

Mice

Female C57Bl/6 (WT.B6, $H2^b$), B10.BR (B10.BR, $H2^{k2}$), BALB/c (WT.BALB/c, $H2^d$) and B6D2F1 ($H2^{b/d}$) mice were purchased from the Animal Resources Center (Perth, Australia) or The Jackson Laboratory (Bar Harbor, ME, USA). IL-17^{Cre} (B6, $H2^b$), Rosa26^{eYFP} (B6, $H2^b$), IL-17RC$^{-/-}$ (B6, $H2^b$), IL-22$^{-/-}$ (BALB/c, $H2^d$) and IL-6$^{-/-}$ (B6, $H2^b$) mice were bred and housed at QIMRB. CCR6$^{-/-}$ mice (B6, $H2^b$) were bred and housed at the University of North Carolina DLAM facility. IL-22$^{-/-}$ (B6, $H2^b$) were bred and housed at the University of Minnesota animal facility. IL-17RC$^{-/-}$ mice were provided by Amgen Inc, B6 and BALB/c IL-22$^{-/-}$ mice were provided by Genentech and IL-17^{Cre}, Rosa26^{eYFP} and Rosa26^{iDTR} mice were provided by Dr B Stockinger (Medical Research Council NIMR, UK) and crossed to generate IL-17^{cre}Rosa26^{eYFP} and IL-17^{cre}Rosa26$^{eYFP/iDTR}$ heterozygous mice.[19] B6.IL-17$^{eGFP/+}$IL-22$^{dTom/+}$ (B6, H-2D^b, CD45.2$^+$) were generated by crossing Il17a^{eGFP} (C57BL/6-*Il17atm1*Bcgen/J) and IL-22^{dTom} (C57BL/6-*IL22promTdtomato,* generated by Dr. Scott K. Durum) reporter mice. Mice were housed in sterilized micro isolator cages and received acidified autoclaved water (pH 2.5). Animal experiments were performed in accordance with protocols approved by the local animal ethics committees.

Cell preparation for transplantation

For murine stem cell mobilized grafts, recombinant human Granulocyte Colony Stimulating Factor (G-CSF) was given subcutaneously to donors at 10μg/dose/animal (4–6 days)[20] and splenocytes isolated prior to transplant. T-cell depletion was performed by anti-CD4 (RL172.4), anti-CD8 (TIB211), and anti-Thy1.2 (HO-13-4) treatment, followed by rabbit complement.[21] Cell suspensions contained less than 1% viable CD3$^+$ T-cells. For bone marrow (BM) transplantation, donor T-cell depleted (TCD) BM cells were isolated as described previously.[22,23] Total T-cells were isolated using T-cell columns (Cedarlane Laboratories). Following column purification, the sample was antibody depleted using anti-mouse B220 and anti-mouse CD25 conjugated with PE (eBioscience), and magnetic bead isolation was performed using anti-PE beads (Miltenyi Biotec).[23]

In vitro Th17 differentiation: Naïve CD4$^+$ T-cells were isolated from WT.B6 spleens using CD4$^+$ columns (Cedarlane Laboratories) followed by sort purification (CD4$^+$CD62L^{hi}CD25$^-$). Purified T-cells were stimulated with plate-bound anti-CD3 (3μg/ml, eBioscience) and anti-CD28 (5μg/ml, eBioscience) in the presence of TGF-β (2ng/mL, Peprotech), IL-6 (30ng/mL, Peprotech), TNF-α (20ng/mL, Peprotech), IL-1β (10ng/mL, Peprotech), anti-IL-2 antibody (10μg/mL, BioXCell), and anti-IFN-γ (10μg/mL,

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BioXCell) for 2 days. On day 3, cells were replenished with the same cytokines and antibodies and cultured for another 2 days. On day 5, cells were replated into low cluster 24-well plates in the presence of TGF-β1: 2ng/mL, IL-6: 25ng/mL, anti-IL-2: 10μg/mL, anti-IFN-γ: 10μg/mL for 2 days. Cells were re-stimulated on d7 with plate-bound anti-CD3 and anti-CD28 mAbs with TGF-β: 2ng/mL, IL-6: 30ng/mL, TNF-α: 20ng/mL, IL-1β: 10ng/mL, anti-IL-2: 10μg/mL, anti-IFN-γ: 10μg/mL, IL-23 (R&D Systems): 15ng/mL. On d9, cells were again removed from CD3 and CD28 stimulation and cultured in low cluster 24-well plates in the presence of 2ng/mL TGF-β1, 30ng/mL IL-6, 20ng/mL TNF-α, 10ng/mL IL-1β, 10μg/mL anti-IL-2, 10μg/mL anti-IFN-γ, 15ng/mL IL-23. On d10 cells were re-fed with: TGF-β1: 2ng/mL, IL-6: 25ng/mL, IL-23: 15ng/mL, IL-1β: 10ng/mL, anti-IL-2: 10μg/mL, anti-IFN-γ: 10μg/mL. On d12, IL-17 cytokine was assessed by stimulating 1×10^7 cells with 50ng/mL Phorbol 12-myristate 13-acetate (PMA), 500ng/mL ionomycin and 3μg/mL Brefeldin A (Sigma-Aldrich) for 6h.

Stem cell transplantation

For murine stem cell mobilized grafts, B6D2F1 recipients received 10×10^6 G-CSF-treated B6-derived splenocytes (d0) after 1100 cGy total-body irradiation (TBI) split over 2 doses (d-1). B6 recipients received 25×10^6 G-CSF-treated WT (BALB/c) or BALB/c.IL-22$^{-/-}$ derived splenocytes (d0) after 1000 cGy TBI. Non-GVHD control groups were injected with TCD grafts. For iDTR-based cell depletions, mice were injected with 250ng diphtheria toxin (DT) on d4–6 post-transplant. B10.BR recipients received 10×10^6 B6-derived bone marrow (BM) after 2 ip doses of 120mg/kg Cyclophosphamide (d-3 & d-2) and 830 cGy TBI. Grafts were supplemented with or without 8×10^4 T-cells from either WT (B6) or B6.IL-22$^{-/-}$ mice where indicated. B6D2F1 recipients of grafts containing *in vitro* polarized T-cells received 3×10^6 TCD BM and 4×10^6 Th17 cells or total T-cells (d0) after 950 cGy TBI (d-1).

GVHD monitoring and pulmonary function tests

In all *in vivo* transplant systems, mice were regularly monitored and weights recorded each week. For pulmonary function assessment, anesthetized mice were weighed, un-blinded animals were intubated, and lung function assessed by whole body plethysmography using the Flexivent system (SCIREQ) and analyzed using the Flexivent software (version 7.3). B6 and B6D2F1 recipients of G-CSF mobilized grafts were monitored daily and systemic overall GVHD assessed weekly using an established cumulative scoring system.[24] Cutaneous GVHD component: 0: normal; 0.5: scaling of paws/tail/ears, 1: areas of alopecia, skin thickening, 2: obvious ulceration, areas of denuded skin. B6D2F1 recipients of *in vitro* polarized T-cells were scored at least twice a week for GVHD symptoms using an established cumulative scoring system.[25] Cutaneous GVHD Score: 0: No ulcers or alopecia; 1: Skin ulcers with alopecia less than $1cm^2$ in area; 2: 1–2 cm^2 in area; 3: Greater than $2cm^2$ in area; an additional score of 0.3 was added if mice had ulcers or scaling on paws, tail, or ears.

Patients

Patients were enrolled as part of an observational study tracking hematopoietic reconstitution after allo-SCT to treat hematological malignancies. Recipients received either a myeloablative conditioning regimen consisting of cyclophosphamide at 60mg/kg/day for

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two days and 12Gy of fractionated TBI given over three days or a reduced intensity conditioning regimen consisting of fludarabine at 25mg/m^2/day for 5 days (d-7 to d-3) and melphalan at 120mg/m^2 on d-2. All patients received a T-cell-replete G-CSF-mobilized peripheral blood stem cell graft from HLA (A, B, C, DR, DQ) matched donors. GVHD prophylaxis consisted of Cyclosporine A (5mg/kg d-1 to d1 and thereafter 3mg/kg daily with subsequent adjustment to target a therapeutic range of 140–250 ng/mL) and methotrexate on d1 (15mg/m^2), 3, 6 and 11 (10mg/m^2). Ethics approval was obtained from both QIMR Berghofer and the Royal Brisbane and Women's Hospital Human Ethics Committees with written informed consent obtained from participating patients.

4–6 mm punch biopsies of the skin were obtained from patients with cutaneous manifestations of GVHD >100 days post allogeneic stem cell transplant. Half of the biopsied tissue was evaluated for GVHD by a clinical pathologist. The other half of the biopsy was flash frozen in liquid nitrogen and stored at −80°C. mRNA control skin tissue was obtained from the posterior iliac crest from patients undergoing autologous stem cell transplant at the time of bone marrow evaluation. The Ct method was used to calculate fold induction relative to levels found in control samples. All patients provided informed written consent and studies were approved by the University of North Carolina Institutional Review Board and Office of Human Research Ethics.

Monoclonal Antibodies and flow cytometry

Antibodies were purchased from Biolegend: anti-mouse CD3 (145-2C11), CD4 (GK1.5), CD8α (53–6.7), CD90.2 (30-H12), IL-17A (TC11-18H10), IFNγ (XMG1.2), TNF (MP6-XT22), IL-22 (poly5164), anti-human CD3 (UCHT1), CD4 (RPA-T4), CD8α (RPA-T8), TNF (MAb11), IL-17A (BL168), IFNγ (45.B3), or eBioscience: anti-mouse GM-CSF (MP1-22E9). Rat anti-mouse IL-6R mAb (MR16-1, Chugai Pharmaceutical Co) or Rat IgG (Sigma-Aldrich) was given intraperitoneally at 500μg/dose on d-1 and d3 post SCT.[26] In IL-22 *in vivo* blocking studies, mice were injected intraperitoneally with either 16mg/kg of control IgG or anti-IL-22 antibody (provided by Pfizer) once a week for four weeks (wk0 – wk3). After transplant, cells were isolated by mechanical disruption and treated with lysis buffer to remove contaminating erythrocytes. For intracellular cytokine staining, cells were cultured with PMA (5μg/mL) and Ionomycin (50μg/mL) for 4h with Brefeldin A (BioLegend). Cells were surface-labeled and processed for intracellular staining, cytokines were assessed via cytofix/cytoperm kit (BD Biosciences). All samples were acquired on BD LSR Fortessa (BD Biosciences) using BD FACSDiva (v7.0) and analyzed with FlowJo (v9.7).

Gene expression analysis

4mm skin bunch biopsies were taken from affected areas of mice or 4–6 mm punch biopsies from affected areas from patients and homogenized using lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA) and FastPrep Homogenizer (MP Biomedicals). RNA was isolated from skin homogenates using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. RNA was run through the Qiagen clean-up kit and cDNA synthesized using the Superscript III cDNA Synthesis Kit (Invitrogen). Equal amounts of cDNA were analyzed in triplicate for each transcript by real-time quantitative PCR using

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Taqman universal PCR mastermix (Applied Biosystems, Foster City, CA) and the Applied Biosystems' ABI QuantStudio 6 real-time PCR system. The expression level of each gene was standardized to the housekeeping gene, *Gapdh* for mouse studies and 18S for human studies, and the Ct method was used to calculate fold induction relative to levels found in control samples.

Statistical analysis

Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis. Unpaired two-tailed Mann-Whitney tests were used to evaluate differences in cytokine expression and cell frequencies, ANOVA was used when comparisons were made between three or more groups. One-sample t tests were used to compare cytokine expression by qPCR in clinical samples. Data are mean ±SEM unless otherwise stated and $p<0.05$ considered significant.

Results

Donor derived IL-22 promotes cutaneous chronic GVHD in allo-SCT recipients

Given the contrasting roles reported for IL-22 in inflammation and acute GVHD,[8,15–17] we utilized allo-SCT models whereby chronic GVHD was directed to an MHC disparity in WT and IL-22$^{-/-}$ recipients. Since IL-22 is known to contribute to inflammatory skin disease pathology,[11,27–29] we utilized a murine model in which cutaneous chronic GVHD is induced by WT T-cells,[20] and examined the effect of IL-22 deficiency in the donor graft. Here we observed a significant reduction in overall GVHD clinical scores and a striking reduction specifically in cutaneous GVHD scores in the absence of donor IL-22 (Figure 1A–B). Semi-quantitative histopathology of skin 35 days after transplant also demonstrated significantly reduced cellular infiltration and inflammation equating to a reduction in cutaneous GVHD pathology in these mice (Figure 1 C–D). IL-22 is also reported to have a dual role in airway inflammation, whereby IL-22 expression in the lung suppresses antigen-induced eosinophilic infiltration and airway inflammation[30–32], however IL-22 can also exacerbate asthma development in response to subcutaneous antigen sensitization.[33,34] Therefore we utilized a model of chronic GVHD that closely models the features of bronchiolitis obliterans (BO), resulting in alloantigen induced epithelial injury, fibroproliferation, impaired pulmonary function and lung pathology (Figure 1E–F).[35] Lung function studies in mice 60 days after transplant demonstrated clear impairment in lung compliance, and increased elastance and resistance in the presence of donor WT T-cells (Figure 1E), however IL-22 deficiency in donor T-cells did not significantly alter these functional manifestations of BO. Chronic liver and lung GVHD were evident in semi-quantitative histopathology assessments of tissue derived from recipients of T-cell replete grafts, however no significant differences were observed between recipients of either WT or IL-22$^{-/-}$ T-cells (Figure 1F). These data suggest that donor-derived IL-22 plays a relatively specific role in promoting chronic GVHD in the skin late after allo-SCT.

GVHD results in pro-inflammatory donor Th22 and IL-22^{+}Th17 differentiation

We next assessed T-cell differentiation after transplant by cytokine expression within donor T-cells in multiple models of GVHD. In both B6 → B6D2F1 and BALB/c → B6 systems,

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we observed IL-22 expression almost exclusively by $CD4^+$ T-cells, a minority of which co-expressed IL-17A (Figure 2A–B). In addition, a large proportion of IL-22^+ cells also co-expressed the pro-inflammatory cytokines TNF, IFNγ and GM-CSF after transplant, all known hallmarks of pathogenic T-cell differentiation.[36–39] Comparisons between $CD4^+$IL-22^+ and $CD4^+$IL-22^- T-cells demonstrated significantly higher frequencies of IFNγ, IL-17A and GM-CSF expression in IL-22 expressing $CD4^+$ T-cells (Figure 2C–D). To explore the relationship between IL-17A and IL-22 further, and to exclude potential artefacts that may arise from *in vitro* re-stimulation, we utilized donor grafts derived from B6.IL-$17^{eGFP/+}$IL-$22^{dTom/+}$ reporter mice. Here we found that unlike IL-17A, IL-22 production is indeed restricted to $CD4^+$ T-cells after allo-SCT (Figure 2E–F). In all transplant systems tested, three distinct populations were clearly apparent in $CD4^+$ T-cells including IL-22^+IL-$17A^-$, IL-22^-IL-$17A^+$ and IL-22^+IL-$17A^+$ subsets, which we defined as Th22, IL-22^+Th17 and Th17 respectively (Figure 2G).

Th22 arise independently of the Th17 lineage but IL-17 promotes Th22 differentiation

Cytokine plasticity is a well-characterized feature of IL-17 producing T-cells.[19,40] We therefore utilized an IL-17 ‘fate-mapping’ reporter system (B6.IL-17^{cre}Rosa26^{eYFP}) to determine whether the Th22 subset identified post-transplant arose independently of the Th17 differentiation pathway. In this system, YFP is permanently expressed in IL-17A producing cells regardless of ongoing IL-17A gene expression.[19] Using G-CSF mobilized stem cell grafts from B6.IL-17^{cre}rosa26^{eYFP} mice, we examined IL-22, IL-17A and IL-17A-induced YFP expression after short-term re-stimulation by PMA/Ionomycin and intracellular cytokine staining (Figure 3A).[40] Again, a distinct IL-$22^+$$YFP^-$ $CD4^+$ T-cell population was clearly observed in these analyses, in parallel to that seen in direct cytokine reporter mice (Figure 2E). Whilst Th22 and Th17 are known to have distinct differentiation pathways (e.g. differential requirements for TGFβ), both populations are reported to be dependent upon IL-6.[8,10,41] We have previously demonstrated systemic IL-6 dysregulation in response to transplant conditioning,[26,42] which we have shown drives ‘type-17’ polarization in mice. Like IL-17A, IL-22 induction was highly dependent upon IL-6 (Figure 3A–B). Whilst the IL-22R is exclusively expressed on non-hematopoietic cells, T-cells commonly express the receptors required for IL-17A recognition (IL-17RA/IL-17RC heterodimer). To assess the influence of IL-17A cytokine on Th22 development we utilized an IL-17 ‘fate-mapping’ reporter-deleter model (B6.IL-17^{cre}Rosa$26^{eYFP/iDTR}$), which enables specific deletion of IL-$17A^+$ cells as they arise, following diphtheria toxin administration (Figure 3C–D).[40] When both donor Th17 and $CD8^+$IL-17^+ T-cells (Tc17) were depleted after allo-SCT, Th22 frequencies were significantly reduced (Figure 3E). Similarly, donor Th22 frequencies were also diminished in the absence of IL-17A signaling within T-cells, demonstrating IL-17A directly acts to promote and/or maintain Th22 differentiation after allo-SCT. This effect was specific to Th22, since Th17 frequencies remained unchanged in the absence of IL-17 signaling (Figure 3F). Together these data demonstrate there are multiple donor T-cell sources of IL-22 (Th22 and Th17), and both IL-6 and IL-17 are key cytokines in Th22 development after transplant.

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Donor Th17 derived IL-22 drives skin GVHD late post-transplant

We have previously demonstrated that *in vitro* polarized Th17 cells can significantly exacerbate skin GVHD[22] and mRNA-seq data indicates that these cells share a similar transcriptome profile with Th17 cells in the skin of patients with inflammatory chronic GVHD (Serody and Coghill unpublished). Thus, we revisited this system to examine the role of IL-22 in this process. To assess Th17 cytokine profiles post-transplant, we compared *Il22*, *Il17* and *Ifng* gene expression by quantitative PCR in murine skin 17 and 21 days after transplantation of grafts containing Th17 cells (Figure 4A). Here we detected high mRNA expression of all three cytokines in comparison to unpolarized T-cells in which only IFNγ encoding mRNA was detected. Since CCR6 expression is important in both IL-17^{+}CD4^{+} and IL-22^{+}CD4^{+} T-cell migration and is reported to be required in IL-22 dependent models of psoriasis,[13,14,43] we examined the capacity of CCR6 deficient Th17 polarized cells to induce GVHD. Here we found that whilst no differences were observed in mortality (data not shown), CCR6$^{-/-}$ Th17 induced significantly less cutaneous GVHD than WT counterparts following allotransplantation (>d37 $p<0.01$; Figure 4B–C). This cutaneous GVHD manifested last after transplant (beyond day 21) with lichenoid features and alopecia, most consistent with chronic disease. To confirm that this pathology was indeed IL-22 dependent, we next assessed the specific capacity of Th17-derived IL-22 to induce GVHD by comparing the effects of IL-22 neutralization after allo-SCT. Mice transplanted with Th17 cells were treated for three weeks with IL-22 blocking mAb, which resulted in a significant reduction in skin GVHD (>d17 $p<0.01$; Figure 4D–E). However, anti-IL-22 mAb did not improve the systemic manifestations of late inflammatory chronic GVHD with no difference in overall survival between control and anti-IL-22 mAb treated animals (data not shown).

IL-22 and IL-17 are elevated in patients with cutaneous GVHD

Immunosuppression during the first 3–4 months after transplant is effective in controlling most donor T-cell inflammatory cytokine production, however during the onset phase of chronic GVHD (>100 days), systemic IL-17A and IFNγ levels increase over time.[42] In one study of pediatric allo-SCT recipients, a significant correlation was observed between increased *Il22* gene expression in peripheral blood mononuclear cells and active cGVHD.[44] Given this and our observations in murine models of chronic GVHD, we assessed plasma IL-22 levels in a clinical cohort after allo-SCT. We found that unlike IL-17A,[42] systemic IL-22 protein levels remained consistently low after transplant (data not shown). To gain a better understanding of cytokine responses in GVHD target tissue, we evaluated cutaneous gene expression in patients >100 days post allo-SCT in parallel with biopsy confirmation of GVHD (Figure 5A). These data were compared to skin biopsies taken from patients after autologous transplant that do not develop GVHD (Figure 5B). We again observed a clear correlation between GVHD and elevated tissue cytokine transcript levels, particularly dysregulated IL-22, IL-17A and IFNγ. These data show striking similarities with that observed in our murine models of skin GVHD and highlight IL-22 as a potential therapeutic target for the treatment of chronic GVHD.

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Discussion

IL-17 and IL-22 play an important and complex role in allo-SCT; both have been described to have dual pathogenic and protective roles in GVHD that are largely dependent upon their cellular origin. Recipient-derived IL-17 and IL-22 both play early protective roles in the GI tract during GVHD, where IL-22R signaling helps maintain gut epithelial integrity[15] and IL-17R signaling suppresses inflammatory responses and prevents dysbiosis in the gastrointestinal tract.[45] Conversely, we and others have demonstrated that donor T-cell-derived IL-22 and particularly IL-17 are pathogenic and exacerbate GVHD.[16,20,22]

The contrasting roles observed for IL-22 in GVHD mirror reports in other systems that show IL-22 can be both protective and pathogenic. For example, IL-22 promotes skin wound healing and epithelial integrity, however dysregulated IL-22 expression exacerbates psoriatic skin disease.[8,46] Keratinocyte responses to excess IL-22R signaling result in hyperproliferation, abnormal differentiation, epidermal hyperplasia, skin thickening and disruption,[46] which are pathological features commonly observed in cutaneous GVHD. The presence of other pro-inflammatory cytokines has a significant impact on IL-22 responses, particularly IL-17, IFNγ and TNF, which synergize with IL-22 to enhance chemokine production in the skin.[47,48] Indeed, both pathological and protective functions have also been identified for IL-22 in the context of damage-induced airway inflammation and these effects are regulated by the presence of IL-17.[33,34] This is of particular interest since we have demonstrated that donor T-cell polarization towards an IL-17-producing phenotype in both $CD4^+$ (Th17) and $CD8^+$ (Tc17) subsets exacerbates GVHD after allo-SCT.[20,22,40,49,50]

To date, investigation of the effects of donor derived IL-22 in allo-SCT has focused on acute GVHD, with little data regarding the role of IL-22 in chronic GVHD. It is important to note that whilst clear overlap exists between the etiologies of acute and chronic GVHD, it is increasingly recognized that divergent mechanisms are also involved.[51,52] A role for IL-22 in the pathophysiology of acute GVHD cannot be extrapolated to cGVHD. A single murine study has noted a correlation between lower IL-22 secretion and a reduction in cGVHD in the context of IL-12/23 blockade, without establishing any cause and effect.[53] Given the complexities and the potential competing risks of targeting IL-22 early post-transplant, we therefore examined IL-22 deficiency in murine models of chronic GVHD. We observed a specific role for IL-22 in driving cutaneous chronic GVHD, which is in line with the pathogenic role of IL-22 in autoimmune skin disease.[11,27–29] We identified $CD4^+$ T-cells as the major source of donor IL-22 by both direct protein detection and cytokine reporter systems, where we observed co-expression of IL-22 with a range of pro-inflammatory cytokines including IL-17, IFNγ, GM-CSF and TNF. Importantly, we identified both Th17 and Th22 T-cell lineages contributing to IL-22 production and excluded potential cytokine plasticity to identify a definitive Th22 population in allo-SCT. Intriguingly, Bronchiolitis Obliterans (BO), a manifestation of chronic GVHD that mirrors BO after lung transplant, was not IL-22-dependent. Given that we have recently established the importance of IL-17A and Th17 cells in driving BO,[38,54] this would suggest that IL-17A rather than IL-22 is the most appropriate therapeutic target for inhibition of BO after stem cell or solid organ transplant.

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IL-22 induction in $CD4^+$ T-cells is reported to be dependent upon IL-6,[8,10] which is significantly elevated early after allo-SCT.[26,42] Moreover, the magnitude of IL-6 release directly correlates with the intensity of pre-transplant conditioning and the incidence of subsequent acute GVHD.[49,55] To a lesser extent, IL-23 and IL-1β have also been reported to promote IL-22 expression in $CD4^+$ T-cells, however TGFβ suppresses IL-22 production in all T-cell subsets, redirecting T-cell polarization towards Th17/Tc17 development. It has been suggested that IL-17 is required to mediate IL-22 dependent airway inflammation; Th22 differentiation persisted in the absence of IL-17 but pathogenicity was reduced.[34] This is distinct from our observations in allo-SCT, where Th22 frequencies are significantly reduced when donor IL-17-producing cells are depleted or when IL-17A/F signaling responses are abrogated within donor T-cells. This dependency suggests that investigation of Th22 differentiation needs to be undertaken in the context of concurrent Th17 responses, providing the impetus for us to examine the role of IL-22 in an established adoptive transfer model of Th17-induced cutaneous GVHD. Here we observed high levels of both *Il17* and *Il22* gene expression in the skin of recipients of Th17 cells that persisted over time post-transplant. $CCR6^{-/-}$ Th17 cells failed to induce severe skin GVHD, as did Th17 cells in the presence of IL-22 neutralization. Finally, we observed a striking similarity in cytokine gene expression in samples derived from patients who developed cutaneous GVHD, suggesting comparable pathological processes during clinical GVHD.

In sum, we demonstrate that IL-22 is secreted by distinct but interacting Th22 and Th17 lineages that develop in response to IL-6 and IL-17 and mediate late-onset skin GVHD. Given the complex function of IL-22 in GVHD, therapeutic modulation will require a careful and staged approach to ensure that the benefits of IL-22R signaling early post-transplant are maintained. Our novel observations regarding the relationship between IL-22 and IL-17 suggest that an additional strategy may be to target IL-17 directly in the skin using small molecule inhibition.[38] These data provide insight for rational therapeutic strategies to inhibit IL-22 secreting T-cells late after transplant, particularly for the treatment of cutaneous chronic GVHD.

Acknowledgments

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Abbreviations

GVHD	graft-versus-host disease
SCT	stem cell transplantation
allo-SCT	allogeneic stem cell transplant
ILC	innate lymphoid cell
NK	natural killer
LTi	lymphoid tissue inducer

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G-CSF	granulocyte colony stimulating factor
TCD	T-cell depleted
BM	bone marrow
TBI	total-body irradiation
BO	Bronchiolitis obliterans
DT	diphtheria toxin

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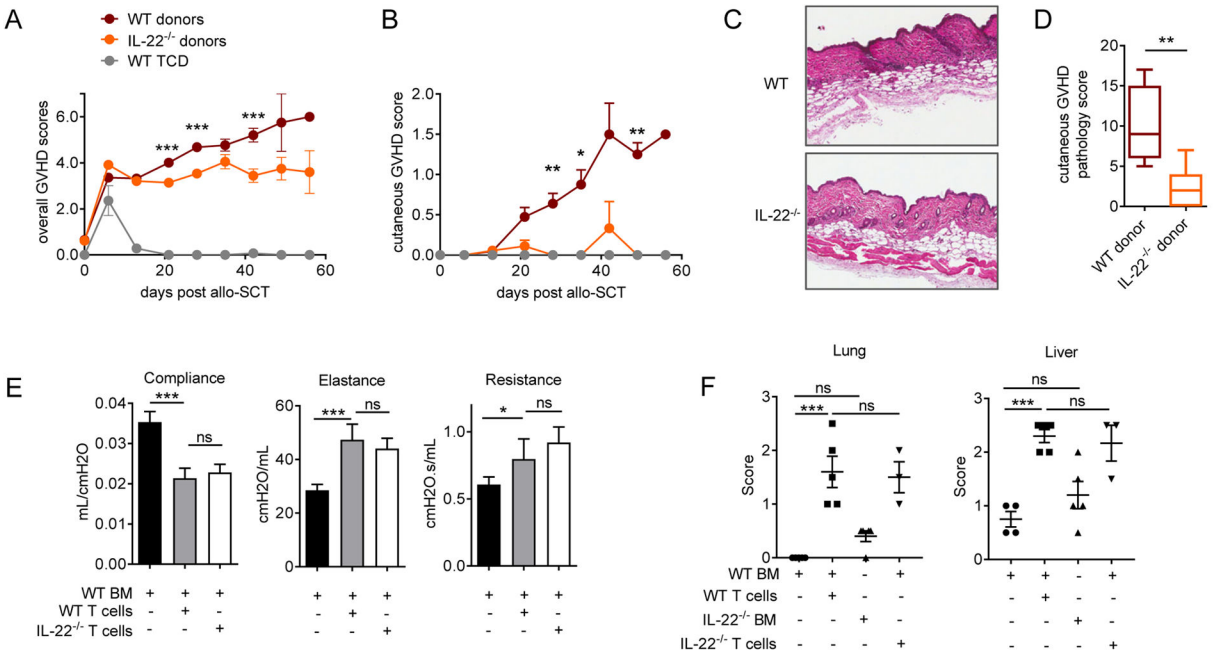

Figure 1. Donor derived IL-22 drives cutaneous GVHD in allo-SCT recipients

(**A–D**) 25×10^6 G-CSF mobilised BALB/c.WT or BALB/c.IL-$22^{-/-}$ splenocytes transplanted into lethally-irradiated allogeneic B6 recipients. (**A**) Overall GVHD clinical scores (WT & IL-$22^{-/-}$ donors, n = 49 mice/group, ***p<0.001) and (**B**) cutaneous GVHD are shown (WT & IL-$22^{-/-}$ donors, n = 24 mice/group, *p< 0.05, **p< 0.01). (**C**) Representative images and (**D**) quantitative histopathology scores of skin GVHD in haemotoxylin and eosin stained sections collected 35 days post-transplant (original magnification ×200, n = 5–7 mice/group, **p< 0.01). (**E–F**) 10×10^6 T-cell depleted BM and 8×10^4 purified T-cells derived from WT.B6 or IL-$22^{-/-}$ B6 mice were transplanted into lethally irradiated allogeneic B10.BR recipients. (**E**) Pulmonary function of recipient mice 60 days post-transplant (n=3–11 mice/group, *p< 0.05, ***p<0.001). (**F**) Quantitative histopathology scores of liver and lung GVHD target organs assessed in haemotoxylin and eosin stained sections collected 60 days post-transplant (n=3–5 mice/group, *p< 0.05, ***p<0.001).

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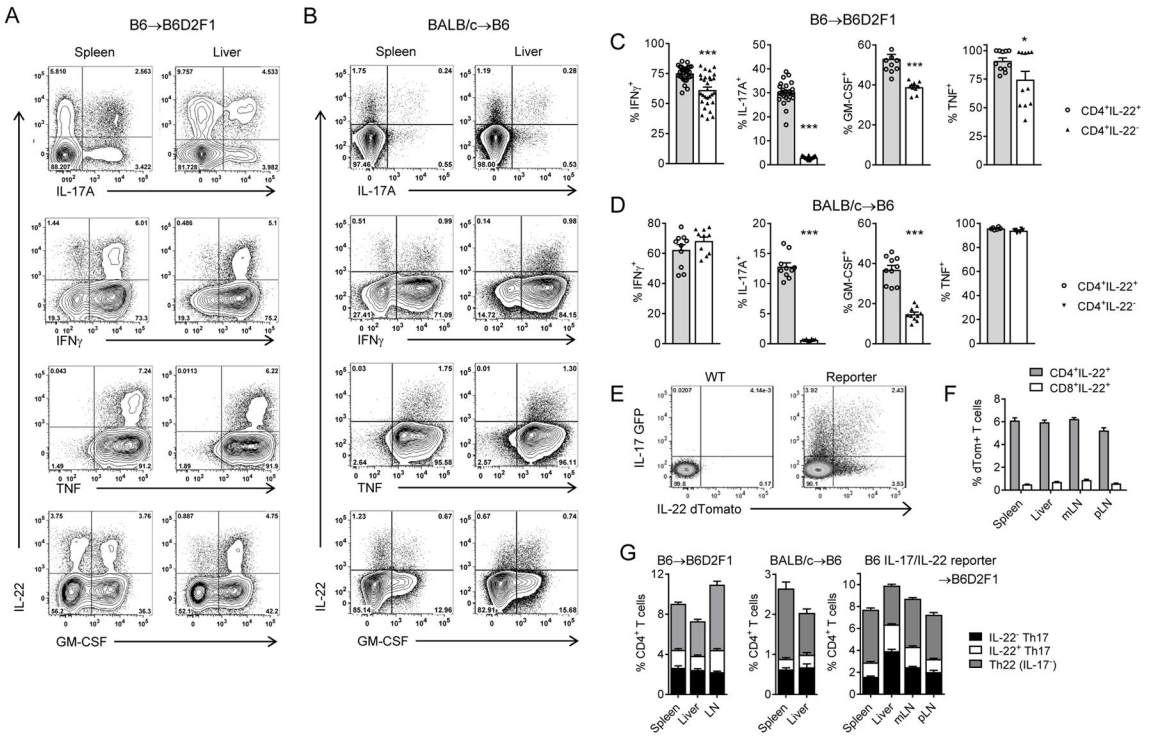

Figure 2. Allo-SCT induces pro-inflammatory donor Th22 and IL-22+Th17 differentiation

(**A,C**) 10×10^6 B6 G-CSF mobilised, splenocytes transplanted into lethally-irradiated allogeneic B6D2F1 recipients. (**B, D**) 25×10^6 BALB/c G-CSF mobilised, splenocytes transplanted into lethally-irradiated allogeneic WT.B6 recipients. (**A**) Representative flow cytometry analysis of cytokine expression in $CD4^+$ T-cells isolated d7 post-transplant (B6→B6D2F1) from spleen and liver after *in vitro* restimulation. (**B**) Representative flow cytometry analysis of cytokine expression in $CD4^+$ T-cells isolated d7 post-transplant (BALB/c→B6) from spleen and liver after *in vitro* restimulation. (**C**) Frequency of cytokine co-expression by $CD4^+$ T-cells isolated d7 post-transplant after *in vitro* restimulation (B6→B6D2F1, 5–17 mice/group, ***p<0.001). (**D**) Frequency of cytokine co-expression by $CD4^+$ T-cells isolated d7 post-transplant after *in vitro* restimulation (BALB/c→B6, 6–10 mice/group, ***p<0.001). (**E–F**) 10×10^6 G-CSF mobilised, B6.IL-$17^{eGFP/+}$IL-$22^{dTom/+}$ splenocytes transplanted into lethally-irradiated allogeneic B6D2F1 recipients. (**E**) Representative flow cytometry analysis d7 post-transplant and (**F**) frequencies of IL-22^{dTom+} cells within the $CD4^+$ and $CD8^+$ T-cell subsets in spleen, liver, mesenteric lymph nodes (mLN) and peripheral lymph nodes (pLN) (5 mice/group). (**G**) Th22, Th17 and IL-22^+Th17 frequencies d7 post-transplant (B6→B6D2F1: 15 mice/group, BALB/c →B6: 10 mice/group, B6.IL-$17^{eGFP/+}$IL-$22^{dTom/+}$ →B6D2F1: 5 mice/group).

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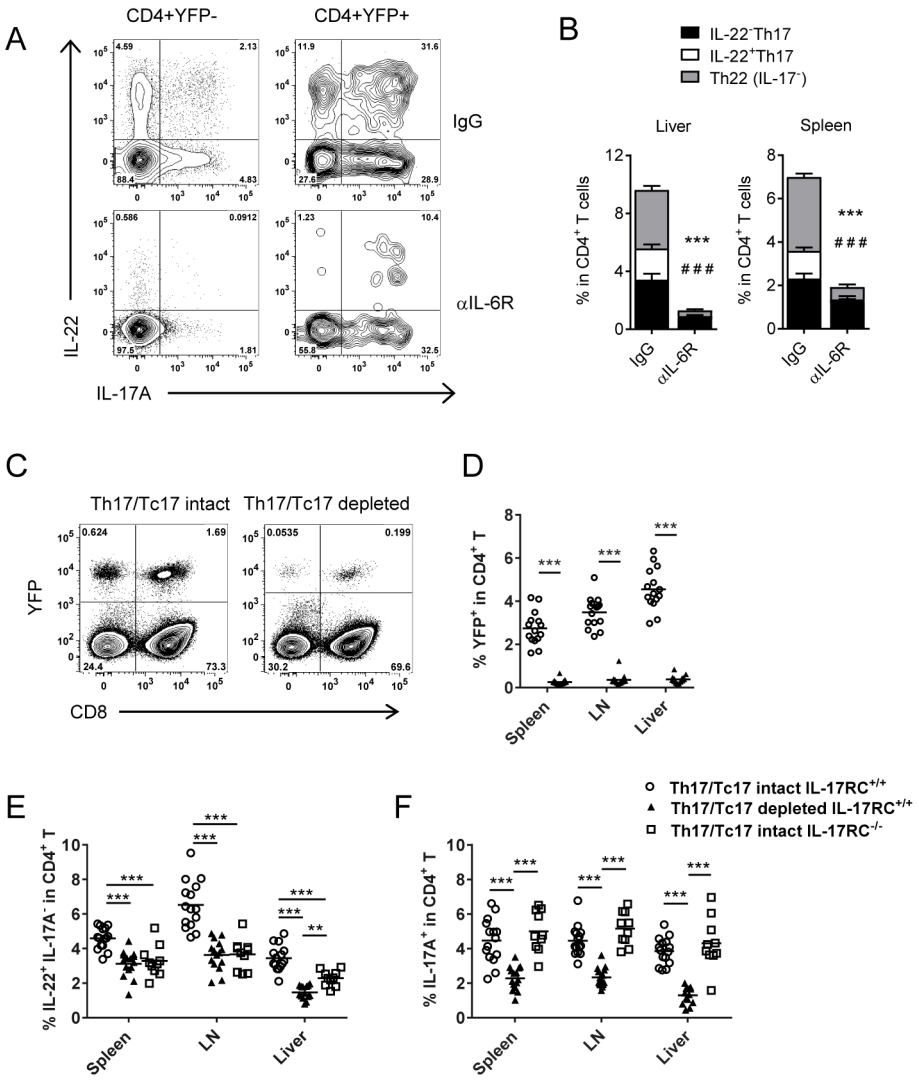

Figure 3. Th22 differentiation is promoted by IL-6 and IL-17A in allo-SCT

(**A–B**) Th22 development and lineage fidelity was assessed in lethally irradiated allogeneic mice transplanted with G-CSF mobilized grafts (B6.IL-17^{Cre}Rosa26^{eYFP}→B6D2F1) in the presence (αIL-6R) or absence (IgG) of IL-6 blockade. (**A**) Representative flow cytometry analysis and (**B**) frequencies of IL-17A and IL-22 expressing splenic CD4$^+$ T-cells in the absence of IL-6R signalling d7 post-transplant (n= 8–10 mice/group, ***p<0.001 Th22 (IL-17$^-$) treated vs untreated, $^{###}$p<0.001 IL-22$^+$Th17 treated vs untreated. (**C–F**) 5×10^6 TCD BM and 2×10^6 T-cells isolated from IL-17^{Cre}Rosa26^{eYFP}, IL-17^{Cre}Rosa26$^{eYFP/iDTR}$ or IL-17RC$^{-/-}$ mice were transplanted into lethally-irradiated allogeniec B6D2F1 recipients. (**C**) Representative flow cytometry analyses and (**D**) frequency of YFP$^+$CD4$^+$ T-cells d7 post-transplant after DT treatment. Frequency of (**E**) Th22 (IL-22$^+$IL-17$^-$) and (**F**) IL-17$^+$ cells in CD4$^+$ T-cell compartment d7 post-transplant in the presence and absence of Th17/Tc17 and IL-17RC signalling (n=10 mice/group; **p< 0.01, ***p<0.001).

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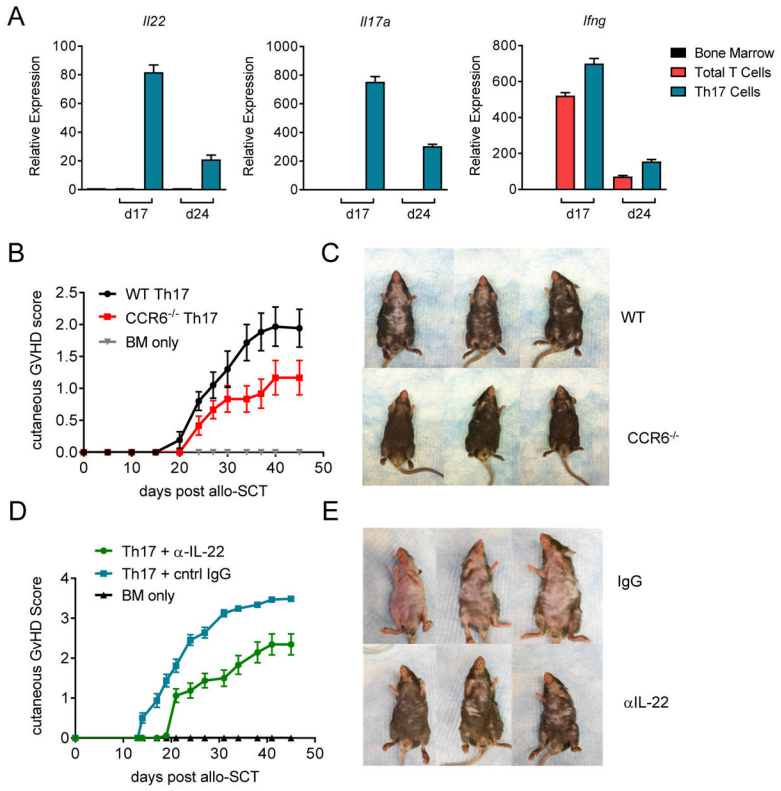

Figure 4. IL-22 derived from Th17 polarised T-cells exacerbates skin GVHD

(**A–H**) 5×10^6 TCD BM and 3×10^6 *in vitro* polarized Th17, or control T $CD4^+$ T-cells, were transplanted into lethally irradiated B6D2F1 recipients. (**A**) *Il22*, *Il17a*, *Ifng* gene expression in murine skin d17 and d24 post transplant (9–12 mice/group/time point). (**B**) cutaneous GVHD scores and (**C**) representative images (d25) from mice transplanted with either WT or $CCR6^{-/-}$ *in vitro* polarized Th17. (**D**) Cutaneous GVHD scores and (**E**) representative images (d35) from mice treated with either control IgG or IL-22 blocking mAbs post-transplant (control IgG & αIL-22: 18–20 mice/group).

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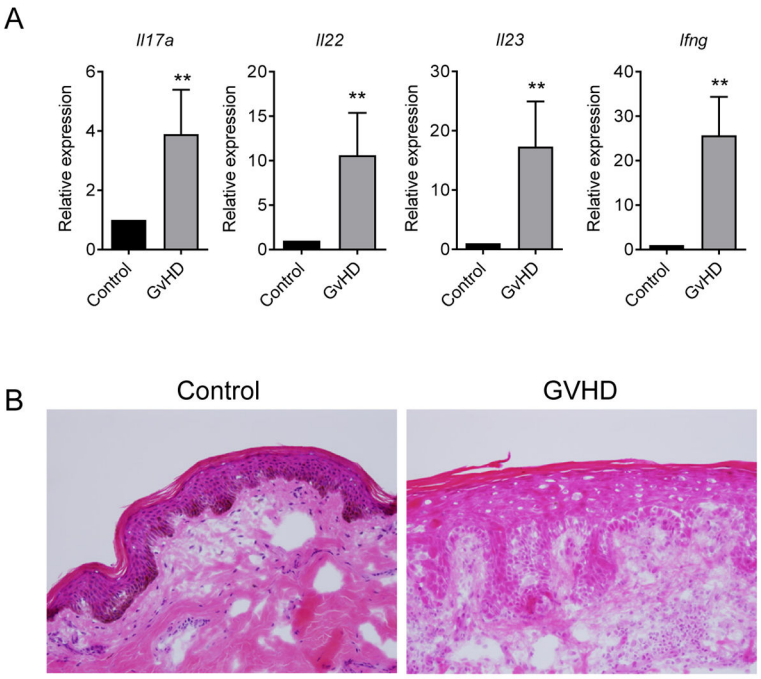

Figure 5. Cutaneous IL-22 cytokine levels are dysregulated in GVHD patients

(**A**) Gene expression in skin explants derived from 12 allo-SCT patients with cutaneous GVHD and 8 autologous-SCT control patients. (**B**) Representative haemotoxylin and eosin stained images from skin explants of cutaneous GVHD and healthy autologous-SCT patient control patients (as in **A**).