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Tc17 cells are a pro-inflammatory, plastic lineage of pathogenic CD8$^+$ T-cells that induce GVHD without anti-leukemic effects

Running Title: Tc17 deletion to separate GVHD and GVL

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Key points:

- Donor derived Tc17 cells differentiate early after allogeneic transplant in response to IL-6 and alloantigen presentation by host-DC.
- Tc17 are a highly pro-inflammatory and pathogenic post-transplant, but exert limited or no graft-versus-leukemia activity.

Abstract:

IL-17-producing cells are important mediators of graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (SCT). Here we demonstrate that a distinct $CD8^+$ Tc17 population develops rapidly after SCT but fails to maintain lineage fidelity such that they are unrecognizable in the absence of a fate reporter. Tc17 differentiation is dependent on alloantigen presentation by host-DC together with IL-6. Tc17 cells express high levels of multiple prototypic lineage-defining transcription factors (e.g. RORγt, T-bet) and cytokines (e.g. IL-17A, IL-22, IFNγ, GM-CSF, IL-13). Targeted depletion of Tc17 early after transplant protects from lethal acute GVHD, however Tc17 cells are non-cytolytic and fail to mediate graft–versus–leukemia (GVL) effects. Thus, the Tc17 differentiation program during GVHD culminates in a highly plastic, hyper-inflammatory, poorly-cytolytic effector population which we term inflammatory Tc17 (iTc17). Since iTc17 mediate GVHD without contributing to GVL, therapeutic inhibition of iTc17 development in a clinical setting represents an attractive approach for separating GVHD and GVL.

Introduction

Bone marrow/stem cell transplantation (BMT/SCT) remains an important curative therapy for hematological malignancies and its success is primarily due to the generation of donor T-cell–mediated graft-versus-leukemia (GVL) effects. The complicating factor for BMT/SCT recipients is the risk of developing graft-versus-host disease (GVHD) within the gastrointestinal tract, liver and skin.[1] GVHD requires the differentiation of donor T-cells and is mediated by both the cytolytic pathways of donor T-cells and pro-inflammatory cytokines. GVHD can be either acute (aGVHD) or chronic (cGVHD), both of which have a significant impact on patient morbidity and mortality and despite advances in immunosuppressive therapies the incidence remains high.[2,3] Therefore a major goal in BMT/SCT remains to prevent GVHD whilst maintaining GVL effects.

GVHD pathology is initiated by conditioning-induced DAMP/PAMP (damage/pathogen-associated molecular patterns) and inflammatory cytokine release, which promotes host APC activation and alloantigen-specific donor T-cell differentiation. Whilst it has been widely recognized that $CD4^+$ T-cell derived IFNγ is central to this process,[4] the role of IL-17 has been more recently established.[5] IL-17 is expressed by both innate and adaptive immune cells including conventional T-cells, mucosal-associated invariant T-cells (MAIT), NK, iNKT and $\gamma\delta$ T-cells,[6-11] and is known to contribute to the pathogenesis of many autoimmune diseases as well as to play a role in host defense from infection and malignancy.[9,12-14] IL-17 producing $CD4^+$ T-cells (Th17) have been extensively characterized in a variety of disease settings; however, IL-17 producing $CD8^+$ T-cells (Tc17) have received comparatively little attention and as a result are relatively poorly understood.

We have previously reported that the mobilization of stem cells in donors with G-CSF promotes Th17 and Tc17 differentiation and that donor IL-17A, which is predominantly derived from Tc17, drives sclerodermatous GVHD following allogeneic SCT (allo-SCT).[15] However, little is known regarding the nature of Tc17 that develop post-transplant, their developmental requirements or functional capabilities. We utilized an IL-17 ‘fate mapping’ reporter model to characterize Tc17 development, plasticity and function in the context of GVHD and to investigate potential routes of therapeutic intervention.

Methods

Mice

Female C57Bl/6 (WT.B6 (H-2Db, CD45.2^{+})), B6D2F1 (H-2Db/d, CD45.2^{+}) and Balb/c (H-2Dd, CD45.2^{+}) mice were purchased from the ARC (Animal Resources Center, WA, Australia). Bm1 (B6.CBy H-2K^{bm1}), IL-17^{Cre} (B6, H-2Db, CD45.2^{+}), Rosa26^{eYFP} (B6, H-2Db, CD45.2^{+}), Rosa26^{iDTR} (B6, H-2Db, CD45.2^{+}), CD11c.DOG.F1 (B6.CD11c- DTR–OVA–eGFP x DBA/2 F1, H-2Db/d, CD45.2^{+}) and IL-6$^{-/-}$ (B6, H-2Db, CD45.2^{+}) mice were bred and housed at QIMR Berghofer. Rosa26^{iDTR} mice were purchased from JAX (stock number 007900), IL-17^{Cre} and Rosa26^{eYFP} mice were provided by Dr B Stockinger (Medical Research Council NIMR, Mill Hill, UK) and crossed to generate IL-17^{cre}Rosa26^{eYFP} and IL-17^{cre}Rosa26$^{eYFP/iDTR}$ heterozygous mice.[16] Mice were housed in sterilized micro isolator cages, and received acidified autoclaved water (pH 2.5) after transplantation. All animal experiments were approved by and performed in accordance with, the QIMR Berghofer Animal Ethics Committee.

Stem cell/bone marrow transplantation

Recipient mice received 900 (Balb/c), 1000 (bm1) or 1100 (B6D2F1, CD11c.DOG.F1) cGy total-body irradiation (^{137}Cs source at 84.6cGy/min) split over 2 doses (d-1), then 10x10^{6} G-CSF treated splenocytes injected (d0). Recombinant human Granulocyte Colony Stimulating Factor was given sc to donors at 10µg/dose/animal (4-6 days).[15] T-cell depletion (TCD) was performed by anti-CD4 (RL172.4), anti-CD8 (TIB211), and anti-Thy1.2 (HO-13-4) treatment, followed by rabbit complement.[17] Cell suspensions contained less than 1% viable CD3^{+} T-cells. Leukemic cells B6D2F1.BCR/ABL-NUP98/HOXA9 (GFP^{+}, H-2Dd/b, CD45.2^{+}) were generated as described.[18] Mice that developed clinical scores ≥ 6 and/or high leukemia burdens (WBC>100x10^{6}) were sacrificed. In CD8^{+} T-cell mixing transplants, G-CSF treated donor mice were given anti-CD8β depleting mAbs (53.5.8) on d-5 and -2. CD8^{+} T-cells were isolated from donor mice treated with G-CSF in parallel. For *in vivo* depletion of inducible diphtheria toxin receptor (iDTR) expressing cells, Diphtheria toxin from *Corynebacterium diphtheriae* was administered ip at 250ng/dose on d4, d5 and d6. In long term experiments, mice were additionally treated with Diphtheria toxin at 100ng/dose on d10, d13, d17 and d20 post-transplant (Sigma-Aldrich, St. Louis, MO, USA). Non-GVHD control groups were injected with TCD grafts. Mice were monitored daily and systemic GVHD assessed weekly using a cumulative

scoring system.[19] Those with GVHD scores of ≥6 were culled and the date of death registered as the next day. For histological analysis, tissue was parafomaldehyde fixed and paraffin embedded prior to tissue sectioning and H&E staining.

Monoclonal Antibodies

A list of antibodies used is provided in Table S1. Rat anti-mouse IL-6R mAb (MR16-1, provided by Chugai Pharmaceutical Co, Japan) or Rat IgG (Sigma-Aldrich) was given ip at 500μg/dose on d-1 and d3 post SCT as described.[20] Mouse anti-TGFβ mAb (1D11) and matched IgG isotype 13C4 (provided by Genzyme Corporation, MA, USA) was administered ip at 500μg/dose on d0 and 100μg/dose on d2, d4 and d6 post-transplant. Anti–IL-12/23p40 (C17.8) and matched control IgG (Anti-AGP3 (cIg, 4D2)) were administered ip at 500μg/dose on d-2, d0, d2, d4 and d6 pre/post-transplant (Amgen Inc. CA, USA).

Cell preparation, culture, and cytokine analysis

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) (Sigma-Aldrich) for 4h with Brefeldin A (BioLegend, CA, USA) included in the last 3 hours of incubation. Cells were surface-labeled and processed for intracellular staining, cytokines were assessed via cytofix/cytoperm kit (BD Biosciences, NJ, USA) and nuclear staining performed via fixation and permeablization (EBioscience, CA, USA). All samples were acquired on BD LSR Fortessa (BD Biosciences) using BD FACSDiva (v7.0) and analyzed with FlowJo software (v9.7, OR, USA).

Gene expression analysis

Total RNA was extracted with the RNeasy Micro kit (Qiagen, Netherlands) from sort-purified (>95% purity) cells and gene expression determined using TaqMan GE assays (Applied Biosystems, MA, USA). All measurements were run in parallel with the housekeeping gene *Hprt*. mRNA was amplified and biotinylated with the Illumina® TotalPrep RNA Amplification Kit (Life Technologies, MA, USA) before hybridization to Mouse Ref-8 v2.0 Expression Bead Chip arrays. Chips were read via iScan Microarray Scanner and analyzed using GenomeStudio (Illumina) and GeneSpring GX v12.5 (Agilent Technologies, CA, USA). Pathway analyses were performed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Qiagen). All microarray analyses have been deposited into the GEO public database under accession number GSE 70931.

Statistical analysis

Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis. An unpaired two-tailed Mann-Whitney test was used to evaluate differences in cytokine and mRNA studies. Data are mean ±SEM and $p<0.05$ considered significant. Gene expression by microarray was assessed after Benjamini-Hochberg false discovery rate (FDR) correction by unpaired t-test ($p<0.05$ cut-off) via GeneSpring GX v12.5 software and then supervised hierarchical clustering performed. 702 differentially expressed probes with >1.2-fold change were identified by unpaired t-test ($p<0.05$ cut-off) and this dataset used in IPA analysis.

Results

Tc17 development after allo-SCT is an early and transient phenomenon

To track Tc17 induction and differentiation we utilized an IL-17A 'fate-mapping' reporter donor IL-17^{Cre}Rosa26^{eYFP}, which permanently induces YFP expression in IL-17A producing cells regardless of ongoing IL-17A gene expression.[16] In time course experiments, CD8$^+$YFP$^+$ Tc17 cells were rapidly induced in all lymphoid tissues and GVHD target organs examined (Figure 1A-B). CD8$^+$YFP$^+$ expansion peaked d7 post-transplant and contracted significantly over the following weeks, with up to 100-fold fewer CD8$^+$YFP$^+$ cells present at d21 relative to d7. Tc17 development was clearly an alloantigen-induced phenomenon since a CD8$^+$YFP$^+$ population did not develop in syngeneic recipients (Figure 1C). Given the relative enrichment of CD8$^+$YFP$^+$ T-cells in the liver, which is a known site of apoptotic clearance,[21] we examined CD8$^+$ T cell apoptosis in both liver and spleen via Annexin V staining. We observed high levels of Annexin V binding in all CD8$^+$ T-cells (~60%), which is likely to explain the overall decrease in organ cellularity normally observed between d7 and d21 in GVHD (data not shown). However, we did not detect any significant differences in apoptosis between CD8$^+$YFP$^+$ and CD8$^+$YFP^{neg} counterparts in either spleen or liver T-cells (Figure 1D). Since CD8$^+$YFP$^+$ T-cell numbers appear to preferentially decline between d7 and d14, these data suggest that differential levels of expansion may contribute to the observed shift in CD8$^+$YFP$^+$ frequencies after d7.

Donor Tc17 are highly inflammatory and plastic in their cytokine profile

CD8$^+$YFP$^+$ Tc17 cells were the dominant IL-17A producing T-cells (Figure 2). At d7 after allo-SCT approximately 35% of CD8$^+$YFP$^+$ cells co-expressed IL-17 and IFNγ following re-stimulation, while the remaining CD8$^+$YFP$^+$ cells no longer expressed IL-17A but maintained IFNγ production. By d21 only 5% of remaining CD8$^+$YFP$^+$ cells continued to produce IL-17A, whereas IFNγ production was largely maintained, demonstrating significant plasticity within Tc17 (Figure 2A-D). Thus Tc17 differentiation is an early and transient phenomenon with most remaining cells ultimately gaining a Tc1-like cytokine profile. To elaborate on the cytokine profile and plasticity observed in IL-17A production within the CD8$^+$YFP$^+$ population, we examined expression of a range of cytokines at d7 and d21 post-transplant. CD8$^+$YFP$^+$ T-cells displayed considerable cytokine heterogeneity at d7, regardless of ongoing IL-17A expression. In addition to IL-17A, a significantly higher frequency of splenic CD8$^+$YFP$^+$ T-cells also expressed the pro-inflammatory cytokines IFNγ, IL-22, IL-13, TNF and GM-CSF relative to

$CD8^+YFP^{neg}$ T-cells and YFP expression was inversely correlated with the immunoregulatory cytokine IL-10 (Figure 2A-C). A similar pattern was also observed in GVHD target tissue, whereby liver $CD8^+YFP^+$ T-cells displayed significantly higher frequencies of IL-22, IL-13, TNF, GM-CSF and reduced IL-10 relative to CD8+YFP^{neg} T-cells (Figure 2D). By d21, in contrast to the relatively consistent cytokine profile observed in $CD8^+YFP^{neg}$ T-cells, $CD8^+YFP^+$ T-cells had transitioned towards a more restricted IFNγ, TNF and GM-CSF cytokine profile (Figure 2B-D). However, the proportion of $CD8^+YFP^+$ T-cells expressing these cytokines remained significantly higher compared to $CD8^+YFP^{neg}$ counterparts, thus the $CD8^+YFP^+$ T-cells maintained a pro-inflammatory profile (Figure 2C-D). Considerable plasticity was also observed in cytokine production within the $CD8^+YFP^+$ compartment, whereby IL-17A, IFNγ, IL-22, IL-13, and GM-CSF production significantly decreased over time in both spleen and liver (Figure 2C-D).

Alloantigen induced Tc17 cells express a unique pattern of genes associated with inflammation and leukocyte, recruitment and migration

Given the observed cytokine heterogeneity and plasticity, we next investigated the expression of lineage defining transcription factors to address differences in T-cell polarization between the $CD8^+YFP^+$ and YFP^{neg} populations. qPCR of $CD8^+$ T-cells sorted from allograft recipients revealed that $CD8^+YFP^+$ T-cells express high levels of both *Rorc* and *Tbet* mRNA, the transcription factors associated with Th17/Tc17 and Th1/Tc1 differentiation respectively (Figure 3A). In addition to increased expression of *Rorc* and *Tbet* in the $CD8^+YFP^+$ population, a general trend toward increased expression of *Bcl6 and Foxp3* was observed. Notably however, at d7 when these cells were most abundant, the *Tbet*–related transcription factor Eomesodermin *(Eomes*) was significantly reduced in $CD8^+YFP^+$ cells. The differential expression of both RORγt and Eomes proteins were confirmed via intracellular staining of sort-purified YFP^+ and YFP^{neg} $CD8^+$ T-cells (Figure 3B–D) and demonstrated an inverse correlation between the expression of RORγt and Eomes. Interestingly, although the $CD8^+YFP^+$ T-cell pro-inflammatory cytokine profile was significantly altered over time, the transcription factor profile remained strikingly diverse (Figure 3A, D). Despite significantly reduced IL-17A expression in $CD8^+YFP^+$ T-cells d21 post-transplant, RORγt protein expression increased over time. Similarly, Eomes protein expression also increased over this same period, but remained low in comparison to $CD8^+YFP^{neg}$ T-cells. Taken together, cytokine and transcription factor profiling highlight distinct differentiation programs in the $CD8^+YFP^+$ and YFP^{neg} populations, with $CD8^+YFP^{neg}$ cells

exhibiting classical and fixed Tc1 polarization in contrast to YFP^+ T-cells which exhibited a unique Tc17 differentiation profile characterized by secretion of multiple inflammatory cytokines.

To further investigate Tc1 and Tc17, we performed a genome-wide microarray study of sorted $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ T-cells. This analysis demonstrated that the two populations differed by ~300 genes (Figure 3E-H), including increased *Rorc* and decreased *Eomes* transcription factor expression in $CD8^+YFP^+$ T-cells as observed in both qPCR and FACS analysis. In confirmation of our earlier findings, multiple genes associated with inflammatory pathways were significantly up-regulated in the $CD8^+YFP^+$ population, including leukocyte adhesion/migration (*Itga3*; Integrin α3, *Itgb4*; Integrin β4), chemotactic factor/cytokines (*Csf2*; GM-CSF, *Csf1*; CSF-1) and chemokine/cytokine receptors (*Ltb4r1*; Leukotriene B4 receptor, *Il1r2*; IL-1R, *Ccr6*) (Figure 3H). The most highly up-regulated gene in the $CD8^+YFP^+$ population was *Klrb1b*, the murine homologue of CD161, whose expression in humans is a marker of IL-17 producing T-cells.[22] In addition, we observed the expression of a number of genes associated with cytolytic function were down-regulated in $CD8^+YFP^+$ cells, including those encoding the degranulation marker CD107a (Lamp1) and the CTL effector molecules FasL and Granzyme K. In agreement with intracellular cytokine staining, gene expression of the immunoregulatory cytokine IL-10 was also down-regulated in the $CD8^+YFP^+$ population as was the IL-10 receptor (*IL10ra*) (Figure 3H), suggesting that not only are Tc17 poor immune-regulators themselves, they may also be less responsive to IL-10 from other sources. Ingenuity Pathway Analysis identified canonical pathways associated with differential gene expression between $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ populations (Table S2-3). These data confirmed these genes were significantly associated with inflammatory pathways and CTL activity and highlighted a number of potential upstream regulators including IL-10Ra, Eomes, FASL and SOCS3.

Tc17 are a functionally distinct subset with reduced levels of CTL effector protein expression

To confirm differences observed at the gene level, we assessed phenotypic markers associated with T-cell activation/memory, specialized T-cell subsets, chemokine/cytokine receptors and CTL effector function after allo-SCT. Irrespective of YFP expression, the activation markers CD25, CD69, CD44 and co-stimulatory molecules CD27 and GITR were similarly expressed in all $CD8^+$ T-cells 7 days after transplant (Figure 4A), however reduced frequencies of $CD62L^+CD44^{neg}$ (naïve) and $CD62L^+CD44^+$ (central memory/T_{CM}) populations were detected in $CD8^+YFP^+$ T-cells in spleen and liver both early and late post-transplant (Figure 4B, Supplementary Figure 1). Similarly, expression of

the T_{CM}-associated protein Ly6C[23] was significantly lower in $CD8^+YFP^+$ T-cells in concordance with microarray analyses (Figure 4B, E). Given the reduced Eomes and Ly6C expression observed in Tc17 cells, we examined other molecules associated with T-cell differentiation and memory. $CD8^+YFP^+$ T-cells displayed significantly reduced expression of the short-lived effector cell (SLEC) associated protein KLRG1 and increased expression of the memory-precursor effector cell (MPEC) marker CD127 (IL-7Rα).[24] Furthermore, differences were observed in the development of tissue resident memory T-cells (T_{RM}) in lung d21 post-transplant (Supplementary Figure 1). No preferential expression was observed in $CD8^+YFP^+$ T-cells of markers associated with known IL-17 producing, functionally distinct T-cell populations including γδ T-cells (γδ TCR), NKT (NK1.1, CD49b, murine CD1d-α-gal tetramer), MAIT (murine MR1 tetramer), $CD8^+$ 'helper' cells (CD40L) and Th17 (α4β7, CCR6) (Figure 4C).[25-31] IL-12, IL-23, IL-17 and IL-10 have each have been reported to contribute to the regulation of IL-17A production[32-34] and whilst IL-12Rβ1 (involved in both IL-12 and IL23 recognition) and IL-17R were expressed at equal levels in YFP^+ and YFP^{neg} $CD8^+$ T-cell subsets (Figure 4C), IL-10R expression was significantly lower in $CD8^+YFP^+$ T-cells as suggested by microarray analysis (Figure 4C, E). We next assessed cytolytic molecule expression (Figure 4D-E) and noted reduced expression of the cell death-inducing effector molecule FasL and the degranulation marker CD107a (Lamp1). Furthermore we observed strikingly limited granzyme B expression in $CD8^+YFP^+$ T-cells, which was independent of ongoing IL-17A expression within the Tc17 population. Given this inflammatory, non-cytolytic phenotype, we have defined this cell population as inflammatory Tc17 (iTc17) cells.

iTc17 development is promoted by IL-6, recipient dendritic cells and regulated by IFNγ

We next investigated the cytokine and antigen presentation requirements for iTc17 differentiation following allogeneic transplantation. Since both IL-6 and TGFβ are known to drive Th17 polarization, we examined the effect of genetic ablation/antibody neutralization of these cytokines on iTc17 development. The absence of IL-6 had the most prominent effect, whereby mice that received IL-6 receptor blocking mAb after allo-SCT displayed significantly reduced $CD8^+YFP^+$ frequencies and cell numbers in multiple tissues post-transplant (Figure 5A). Similarly, a significant reduction in $CD8^+YFP^+$ numbers was also observed when IL-$6^{-/-}$ recipient mice were transplanted (data not shown) demonstrating that the source of IL-6 required for iTc17 induction is mainly host–derived. In contrast,

administration of TGFβ neutralizing mAb had little effect on iTc17 development in this system, with only the spleen exhibiting a significant reduction in $CD8^+YFP^+$ numbers after TGFβ inhibition (Figure 5B). Neutralization of the shared p40 subunit of IL-12 and IL-23 resulted in a partial enhancement of iTc17 development with increases in $CD8^+YFP^+$ numbers in mesenteric and peripheral LNs (Figure 5C). IFNγ is the dominant cytokine downstream of IL-12 and its neutralization also resulted in a significant increase in $CD8^+YFP^+$ frequencies in multiple tissues post-transplant (Figure 5D). $CD8^+YFP^+$ development was robust in the B6→bm1 model of CD8–dependent GVHD induced by an isolated MHC class I mismatch (Figure 5E). This confirms that iTc17 can develop in a class-I restricted manner in the absence of an alloreactive $CD4^+$ T-cell response and therefore $CD4^+$ T-cell help. Finally, $CD8^+YFP^+$ iTc17 development was partially dependent upon the presence of recipient DC since $CD8^+YFP^+$ frequencies were significantly reduced when host DC were depleted using diphtheria toxin (DT) treated CD11c-DTR (CD11c.DOG.F1) recipients (Figure 5F). Given that iTc17 appear within the first week of BMT, before the reconstitution of donor DC,[35] it would seem less likely that these cells initiate iTc17 development. These data demonstrate that primarily host-derived IL-6 and DC drive development of iTc17 cells after allo-SCT and this process is inhibited by the presence of IFNγ.

Targeted deletion of iTc17 protects against lethal GVHD

Given the pro-inflammatory phenotype exhibited by iTc17 (Figures 2 – 3), we hypothesized that by depleting these cells post-transplant, the severity of GVHD may be reduced. To test this, we utilized a second 'deletable' fate-mapping system ($IL\text{-}17^{Cre}Rosa26^{iDTR}$), in which the diphtheria toxin receptor is induced on IL-17 producing cells regardless of ongoing IL-17 gene expression.[36] By transplanting mice with $CD8^+$ T-cell–depleted G-CSF mobilized allografts supplemented with either WT or $IL\text{-}17^{Cre}Rosa26^{iDTR}$ derived $CD8^+$ T-cells, iTc17 cells could be targeted for depletion whilst other IL-17 producing cells, such as Th17, remained intact. Therefore, by treating mice early post–transplant with diphtheria toxin (DT) and providing ongoing treatment over a period of 3 weeks, we depleted iTc17 cells independent of their continued IL-17 production (Figure 6A). Successful depletion was confirmed in $IL\text{-}17^{Cre}Rosa26^{iDTR/YFP}$ 'reporter-deleter' mice at d7 post–transplant where we detected significantly reduced $CD8^+YFP^+$ T-cells in all organs examined (Figure 6B–C). In line with their pro-inflammatory phenotype, blinded histological analysis demonstrated significantly reduced GVHD pathology in colon tissue d7 post-transplant. Furthermore, we observed significant protection from lethal GVHD after

iTc17 depletion in B6D2F1 recipients (Figure 6D-E), similar to that of Tc17 deficient grafts (Tc17 ko) where IL-17A$^{-/-}$ CD8 T-cells were transplanted. In addition, we also demonstrated significant protection in an alternate major MHC-mismatch model of allo-SCT using Balb/c recipients (Figure 6F) and in a CD8$^+$ T-cell dependent model of GVHD (Figure 6G). These data confirm that iTc17 cells are a pathogenic population and represent a potential target for the prevention of GVHD.

iTc17 cells do not contribute to graft versus leukemia effects

Since CD8$^+$ T-cells are critical mediators of both GVHD and GVL post-transplant, and the quality of anti-tumor responses positively correlate with the incidence of GVHD[37,38] we assessed the contribution of iTc17 cells to GVL using a primary myeloid blast-crisis CML leukemia model. Initial transplants were performed using B6.IL-17^{Cre}Rosa26^{YFP} donors to generate alloantigen induced CD8$^+$YFP$^+$ or CD8$^+$YFP^{neg} cells, which were isolated by FACS sorting on d7 following transplantation. Subsequently, WT.B6 T-cell-depleted BM was supplemented with the purified Tc1 or iTc17 populations and transplanted along with primary leukemia cells[18] into B6D2F1 recipients (Figure 7A). Leukemia burdens and CD8$^+$YFP$^+$ T-cell expansion were monitored in peripheral blood via leukemia expression of GFP and YFP expression in CD3$^+$ T-cells, respectively (Figure 7B).

CD8$^+$YFP$^+$ T-cell numbers increased significantly between days 7 and 14 demonstrating the viability of iTc17 cells and their expansion after secondary transplantation, however this expansion was not maintained during week 3 when tumor burdens were very high (Figure 7C). Importantly, in mice receiving CD8$^+$YFP$^+$ T-cells, leukemia was uncontrolled and both survival and tumor growth curves directly overlapped with that of the recipients receiving no donor T-cells. In contrast, the majority of recipients of CD8$^+$YFP^{neg} donor T-cells cleared leukemia within 3 weeks of transplantation and anti–leukemic immunity was maintained long term (Figure 7D-E). Taken together, the data demonstrate that iTc17 which develop during GVHD represent a unique, highly plastic, hyper-inflammatory, non-cytolytic CD8$^+$ T effector population.

Discussion

Anti-tumor immunity and GVHD after allotransplantation are directly linked to donor T-cell differentiation. Whilst the role of T-cell derived IL-17 in GVHD is increasingly recognized, the focus has been predominantly on IL-17 production by $CD4^+$ T-cells, whereas donor $CD8^+$ T-cell polarization in this context has been largely unappreciated. We have previously shown that in a murine model of allo-SCT, G-CSF mobilization promotes donor Th17/Tc17 differentiation and that IL-17A deficient allografts protect recipients from $CD8^+$ T-cell mediated sclerodermatous GVHD.[15] Recent studies in other disease models have demonstrated significant plasticity within both Th17 and Tc17 lineages,[12,32,34,39-41] however plasticity studies of Tc17 have been limited to the use of *in vitro* polarized $CD8^+$ T-cells. To circumvent this limitation, we used a murine IL-17A 'fate-mapping' reporter model to characterize an inflammatory Tc17 lineage that develops during GVHD, and to our knowledge are the first to do so. We found that iTc17 development is both an early and transient phenomenon after allotransplantation dependent upon host IL-6 and DC; which is regulated by the presence of IL-12 and IFNγ. The iTc17 subset displays considerable lineage promiscuity in transcriptional profile and cytokine production and targeted depletion of iTc17 cells early after transplant was protective in lethal models of acute GVHD. In contrast, iTc17 are significantly impaired in cytolytic and GVL activity and provide little or no direct anti-tumor immunity *in vivo*.

iTc17 numbers declined dramatically in weeks 2-3 following transplant although there was some evidence for their persistence in the liver. This Tc17 contraction is concordant with a human study in which CD161 was used as a surrogate marker of IL-17 producing T-cells,[42] whereby a decline in $CD8^+CD161^+$ T-cell frequencies in peripheral blood was observed over time following allo-SCT. Whilst previous attempts have been made to address the persistence of Tc17 cells *in vivo*, the significant level of plasticity observed in this population has hampered these questions. There are some data to suggest that *in vitro* polarized Tc17 may be more persistent after adoptive transfer than Tc1 polarized $CD8^+$ T-cells,[41,43] however this was not the case in our *in vivo* murine system, where we observed a preferential decline in $CD8^+YFP^+$ frequencies over time following transplant.

iTc17 plasticity was particularly striking early after transplant whereby the majority of $CD8^+YFP^+$ T-cells had already down regulated IL-17 production as early as d7, highlighting the importance of a fate-mapping model to monitor this population. In addition, iTc17 also displayed considerable

heterogeneity in cytokine expression. Notably, co-expression of both IL-17A and IFNγ was observed early post-transplant, which appears to be a general feature of Tc17 cells.[15,32,34] This dual expression has been attributed to epigenetic suppression of the regulatory protein SOCS3,[32] whose absence in donor T-cells we have shown to also result in polyfunctional T-cell responses and exacerbated GVHD.[44] In this study, we found *Socs3* gene expression was down-regulated in $CD8^+YFP^+$ T-cells post-transplant (fold-change -1.306, p=0.001, unpaired moderated t-test with FDR correction), and was highlighted by microarray analysis as a potential upstream regulator of $CD8^+YFP^+$ cells (Table S3). These data suggest differential expression of *Socs3* may also drive the hyper-pro-inflammatory T-cell phenotype observed in iTc17. Multiple studies have looked at the mechanisms behind *in vitro* Tc17 plasticity, finding IL-12 signaling via Stat4 is required for IFNγ induction in both $CD4^+$ and $CD8^+$ populations during inflammation.[40,45] This is also likely to be the case in the context of allotransplantation, since IL-12 is up-regulated early post-transplant and is a well described mediator of acute GVHD.[46]

Our comprehensive characterization of the $CD8^+YFP^+$ T-cell population post-transplant revealed a distinct subset of pro-inflammatory $CD8^+$ T-cells, with diminished capacity for 'classical' effector T-cell function regardless of ongoing IL-17A expression. This unique phenotype appears driven by dual expression of the transcription factors Tbet and RORγt, previously associated with an inflammatory profile likely to be protective during host-pathogen interactions in innate lymphoid cells (ILCs) and $CD4^+$ T-cells.[47] However, the $Tbet^+ROR\gamma t^+$ T-cell differentiation pathway may carry an increased risk of autoimmune disease pathologies,[47-49] which share many of the clinical and histological features of graft-versus-host disease (GVHD). Similarly, expression of the transcription factor Eomes is negatively correlated with IL-17 expression[8,50,51] and has been reported to be necessary for full CTL effector function and is therefore likely to explain the poor cytotoxicity observed in the iTc17 population.[52] Since these iTc17 are difficult to identify after BMT based on surface marker and cytokine profiles (given their promiscuity), it may be that transcription factor expression instead represents a more useful approach to their identification. Given that these cells are in fact highly functional with regard to pro-inflammatory cytokine function, we propose that the iTc17 population represents a unique population of non-classical effector $CD8^+$ T-cells whose primary function is to contribute to inflammation and recruitment of both lymphoid and myeloid effector cells, resulting in GVHD pathology. In agreement with this we observed significantly elevated *Csf1* gene expression by

$CD8^+YFP^+$ T-cells, which we have recently demonstrated in IL-17-dependent models of lung and sclerodermatous GVHD, is a critical factor driving macrophage tissue infiltration and pathology.[53]

The signaling pathways that contribute to type-17 differentiation are relatively well characterized, with IL-6, TGF-β, IL-21 & IL-23 promoting IL-17A production and both IFNγ and IL-12 acting as regulators.[39] We have previously demonstrated that type-17 differentiation in G-CSF mobilized donor T-cells is highly IL-21 dependent[15] and we now show that IL-6R signaling is also required for iTc17 differentiation, coinciding with the presence of elevated IL-6 levels in plasma early post-transplant.[20] These data are in line with our previously published observations that IL-6R blockade significantly protects from lethal GVHD in murine models of GVHD.[20,54] Furthermore, we have recently demonstrated in phase I/II clinical trials that IL-6R inhibition after allotransplantation is a promising therapeutic approach for GVHD intervention in humans.[55] In contrast, TGFβ and IL-12/IL-23p40 were minor contributors to iTc17 development in our systems, with little to no effect observed on iTc17 differentiation when neutralized *in vivo*. IFNγ neutralization significantly enhanced type-17 differentiation, consistent with the known cross-regulation of lineage differentiation by IFNγ and IL-17A. Importantly, iTc17 cells continue to produce high levels of IFNγ in the absence of IL-17 production, thus without a fate reporter iTc17 are indistinguishable from Tc1 cells based on IFNγ and IL-17 expression, and this likely contributes to our early observations regarding the disappearance of $CD8^+IL\text{-}17^+$ T-cells after transplantation.

Since GVHD arises as a complication to therapy designed to provide GVL effects, the identification of factors that contribute to GVHD but not GVL is highly desirable. The pro-inflammatory phenotype and impaired CTL effector function observed in iTc17 post-transplant suggested that this population may represent a suitable target for therapeutic intervention after allotransplantation. There are conflicting reports in the literature regarding the potential for cytolytic activity in Tc17 *in vivo*, particularly with regard to Tc1-shifted cells derived from the Tc17 subset. Reports of Tc17 CTL function range from enhanced in some studies,[34,40,43] and diminished in others.[41,56] In addition, other studies have suggested that Tc17 contribute to host protection via inflammatory mediators (such as IFNγ) as opposed to cytotoxic mechanisms.[12,57] Our studies demonstrate that $CD8^+YFP^+$ iTc17 cells cannot eradicate leukemia *in vivo*, which is concordant with their uniformly poor expression of factors

including Eomes (previously linked with poor anti-tumor immunity)[58] and the cytolytic effector molecule Granzyme B.[59]

This study provides new insights into donor T-cell polarization after allotransplantation, demonstrating that iTc17 differentiation is an early and highly plastic differentiation program culminating in a poorly-cytolytic, hyper-inflammatory donor T-cell. Intervention to prevent this differentiation program, putatively via either IL-6 or RORγt inhibition, would thus be predicted to reduce GVHD whilst maintaining or enhancing GVL effects.

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Authorship Contributions: KHG designed, performed experiments and wrote the manuscript; KAM, AV, MDB, MK & GB performed experiments and provided useful discussion; RDK, NCR, SDO, KEL, MC, BET, ML and IC performed experiments; SWL, MWT, MJS, JM, JR and BS provided essential reagents; ADC performed blinded histological analysis, SRM provided useful discussion and experimental advice; KPAM designed, performed experiments and helped write the manuscript and GRH designed experiments and helped write the manuscript.

Conflict of Interest Disclosures: The authors have declared that no conflict of interest exists.

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Figure Legends:

Figure 1. Tc17 cells develop early post allo-SCT in both central lymphoid and GVHD target organs. (**A – D**) $CD8^+YFP^+$ population development in lethally irradiated allogeneic (B6.IL-$17^{Cre}Rosa26^{eYFP}$→ B6D2F1) or (**C**) syngeneic (B6.IL-$17^{Cre}Rosa26^{eYFP}$→B6) mice transplanted with G-CSF mobilized grafts. (**A**) Time course analysis of $CD8^+YFP^+$ frequencies and absolute numbers within the $CD8^+$ T-cell compartment 3, 7, 14 & 21 days post-transplant (mean ± SEM, *n* = 4 – 15 pooled mice/group from 2-4 independent experiments). (**B**) Representative FACS analyses of $CD8^+YFP^+$ T-cells 7 days after allo-SCT. (**C**) Representative FACS analyses (mLN) and $CD8^+YFP^+$ absolute numbers 7 days after syngeneic or allogeneic SCT (mean ± SEM, *n* = 6 – 10 pooled mice/group from 2 independent experiments, ***p*<0.01, *****p*<0.0001). (**D**) Representative FACS analyses and frequencies of $CD8^+$ T-cells 7 days after allo-SCT co-stained with Annexin V and 7-AAD viability dye (mean ± SEM, *n* = 9 – 10 mice/group).

Figure 2. Post-transplant Tc17 cells are highly pro-inflammatory and plastic in cytokine profile. (**A – D**) Donor $CD8^+$ T-cell cytokine expression was examined after allogeneic (B6.IL-$17^{Cre}Rosa26^{eYFP}$→B6D2F1) transplant. Representative FACS analysis showing splenic IL-17A, IFNγ, IL-22, IL-13, TNF, IL-10 and GM-CSF cytokine expression by $CD8^+YFP^{neg}$ or $CD8^+YFP^+$ populations after short-term *in vitro* re-stimulation (**A**) d7 or (**B**) d21 post-transplant. (**C-D**) Frequencies of cytokine expressing $CD8^+$ T-cells isolated from (**C**) spleen or (**D**) liver and assessed 1-3 weeks post-SCT as indicated (mean ± SEM, *n* = ≥ 5 mice/group, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Figure 3. Post-transplant Tc17 cells express a distinct transcription factor and gene expression profile. (**A – C**) $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ T-cell transcription factor expression after allogeneic (B6.IL-$17^{Cre}Rosa26^{eYFP}$→B6D2F1) transplant. (**A**) YFP^+ and YFP^{neg} $CD8^+$ T-cells isolated from spleen, mLNs and liver by FACS on d7 and d21 followed by qPCR (mean ± SEM, n= 3–4 independent experiments each consisting of 10 pooled mice/group, **p*<0.05). (**B**) Representative flow cytometry data from transcription factor staining of $CD8^+$ T-cell populations isolated from pooled spleen, mLNs and liver d7 post–transplant. (**C**) Frequencies of transcription factor expressing $CD8^+$ T-cells gated on YFP expression 7 days post-transplant (mean ± SEM, *n* = 4 independent experiments

each consisting of 10 pooled mice/group, $^{**}p<0.01$). (**D**) Eomes and RORγt expression 21 days post–transplant in YFP^+ and YFP^{neg} $CD8^+$ T-cells isolated from pooled spleen, mLNs and liver. (**E-H**) Microarray analysis of $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ T-cell gene expression after allogeneic (B6.IL-17^{Cre}Rosa26^{eYFP}→ B6D2F1) transplant. (**E**) Gene expression heat map d7 post-transplant showing 299 gene probes differentially expressed between $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ T-cells sorted from 4 independent experiments consisting of 10 mice pooled/group. Genes were identified by unpaired t-test ($p<0.05$) after false discovery rate (Benjamini-Hochberg) *p*-value correction. (**F**) Volcano plot showing the distribution of expression in all genes expressed by either $CD8^+YFP^+$ or $CD8^+YFP^{neg}$ T-cells. Blue data points represent genes with > 1.2 fold differential expression with an uncorrected *p*-value <0.001. (**G**) Scatter plot highlighting the 299 gene probes shown in (**D**). Red scatter data points highlight differentially expressed genes of interest. (**H**) 25 selected immunologically relevant genes differentially regulated in $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ T-cells (FC = fold change, see also Tables S3-S4).

Figure 4. Early post-transplant Tc17 cells express altered T-cell memory markers and reduced CTL effector molecules. Phenotypic analysis of splenic $CD8^+YFP^+$ and $CD8^+YFP^{neg}$ T-cells d7 post-transplant (B6.IL-17^{Cre}Rosa26^{eYFP}→B6D2F1). (**A – D**) Representative flow cytometry analysis of markers associated with (**A**) T-cell activation, (**B**) T-cell memory, (**C**) specialized T-cell functions and chemokine/cytokine receptor expression, and (**D**) CTL effector function. $CD8^+YFP^+$ (filled dark grey), $CD8^+YFP^{neg}$ (thick black), isotype (thin dashed), positive control cells (filled light grey), $CD8^+YFP^+$IL-17^+ (thick grey) and $CD8^+YFP^+$IL-17^{neg} (black dotted). (**E**) Quantitative analysis of Granzyme B, Ly6C, IL-10R, FasL and CD107a expression in $CD8^+$ T-cells (mean ± SEM, *n* = 5-6 mice/group, $^{**}p<0.01$). (**C**) Positive controls used: γδ TCR^+ $CD4^-CD8^-$ naïve T-cells, $NK1.1^+$ naïve NK cells, NKT $tetramer^+CD4^+$ naïve NKT-cells, MR1 $tetramer^+CD4^-CD8^-$ naïve T-cells, $CD40L^+CD4^+$ naïve T-cells.

Figure 5. iTc17 development post transplant is primarily driven by IL-6 and regulated by IFNγ and IL-12p40. (**A-D**) iTc17 development was assessed by enumeration of $CD8^+YFP^+$ T-cells 7 days post-allotransplantation (B6.IL-17^{Cre}Rosa26^{eYFP}→B6D2F1). Mice were treated with either isotype control mAbs or blocking mAbs targeting (**A**) IL-6R (**B**) TGFβ (**C**) IL-12/IL-23p40 and (**D**) IFNγ. (**E-F**)

$CD8^+YFP^+$ T-cell frequencies 7 days post-transplant in (**E**) a B6.IL-17^{Cre}Rosa26^{eYFP}→bm1 model wherein only donor $CD8^+$ T-cells react to alloantigen or (**F**) a B6.IL-17^{Cre}Rosa26^{eYFP}→CD11c.DOG-F1 model wherein recipient mice were treated with DT to deplete DC prior to transplant. Data is pooled from 2 independent experiments (3 – 10 mice/group total ± SEM *p<0.05, **p<0.01, ***p<0.001).

Figure 6. iTc17 depletion protects mice from lethal acute GVHD. Lethally irradiated (**A-E**) B6D2F1, (**F**) Balb/c or (**G**) Bm1 mice received $CD8^+$ T-cell depleted, G-CSF mobilized WT.B6 allografts supplemented with purified $CD8^+$ T-cells derived from either WT.B6 (Tc17 intact), IL-17^{Cre}Rosa26^{iDTR}, IL-17^{Cre}Rosa26^{iDTR} (Tc17 depleted) or IL-17A deficient (Tc17 ko) mice as indicated. WT.B6 G-CSF mobilized WT allografts depleted of both $CD4^+$ and $CD8^+$ T-cells were used as no GVHD controls (TCD). All groups received continued DT treatment during weeks 1 – 3 as described in materials and methods. (**B-C**) $CD8^+YFP^+$ T-cell depletion was assessed d7 post-transplant in B6D2F1 recipients of grafts containing IL-17^{Cre}Rosa$26^{eYFP/iDTR}$ heterozygous $CD8^+$ T-cells. (**B-C**) $CD8^+YFP^+$ T-cell frequencies after Tc17 depletion were enumerated by flow cytometry (mean ± SEM, n = 5 mice/group, * p<0.05, ** p<0.01). (**D**) Quantitative GVHD histopathology analysis was performed on colon tissue isolated d7 post-transplant from B6D2F1 recipients of allografts as described in (**A**), (Tc17 intact; 6 mice/group, Tc17 depleted; 8 mice/group, TCD; 4 mice/group, * p<0.05). Representative d7 colon histology images are shown (scale bar = 0.1mm). (**E-G**) Survival indices by Kaplan-Meier analyses are shown for (**E**) B6D2F1, (**F**) Balb/c and (**G**) Bm1 recipients of allo-SCT as described above. (**E**) Data is pooled from 4 independent experiments (Tc17 intact, Tc17 depleted; 36 mice/group, Tc17 ko; 20 mice/group, TCD; 16 mice/group, **p<0.01). (**F**) Data is pooled from 2 independent experiments (Tc17 intact, Tc17 depleted; 10 mice/group, TCD; 6 mice/group, *p<0.05). (**G**) Data is derived from 1 experiment (Tc17 intact, Tc17 depleted; 9 mice/group, TCD; 3 mice/group, *p<0.05).

Figure 7. iTc17 cells do not directly contribute to post-transplant tumor clearance. (**A**) $CD8^+YFP^+$ or $CD8^+YFP^{neg}$ T-cells were isolated 7 days post-transplant (B6.IL-17^{Cre}Rosa26^{eYFP}→B6D2F1) and combined with WT.B6 T-cell depleted (TCD) BM for secondary transplant into lethally irradiated B6D2F1 recipients. Mice receiving TCD BM only were included as

controls and all grafts supplemented with 1 x 10^5 B6D2F1-derived BCR/ABL-NUP98/HOXA9 GFP^+ CML leukemic cells prior to transplantation. (**B**) Representative flow cytometry analysis of leukemia progression and $CD8^+YFP^+$ T-cell expansion 14 days post-transplant. (**C**) $CD8^+YFP^+$ T-cells were enumerated on d7, d14 and d21 post-transplant (± SEM, n = 10 mice/group, **** p<0.0001 ** p<0.01). (**D**) Survival indices by Kaplan-Meier analysis and tumor growth post-transplant are shown. Data is pooled from 2 independent experiments (10 mice/group total, *** p<0.001). (**E**) Representative images of blood smears collected 3 weeks post-transplant (Wright Giemsa stain, original magnification ×200).

Figure 1.

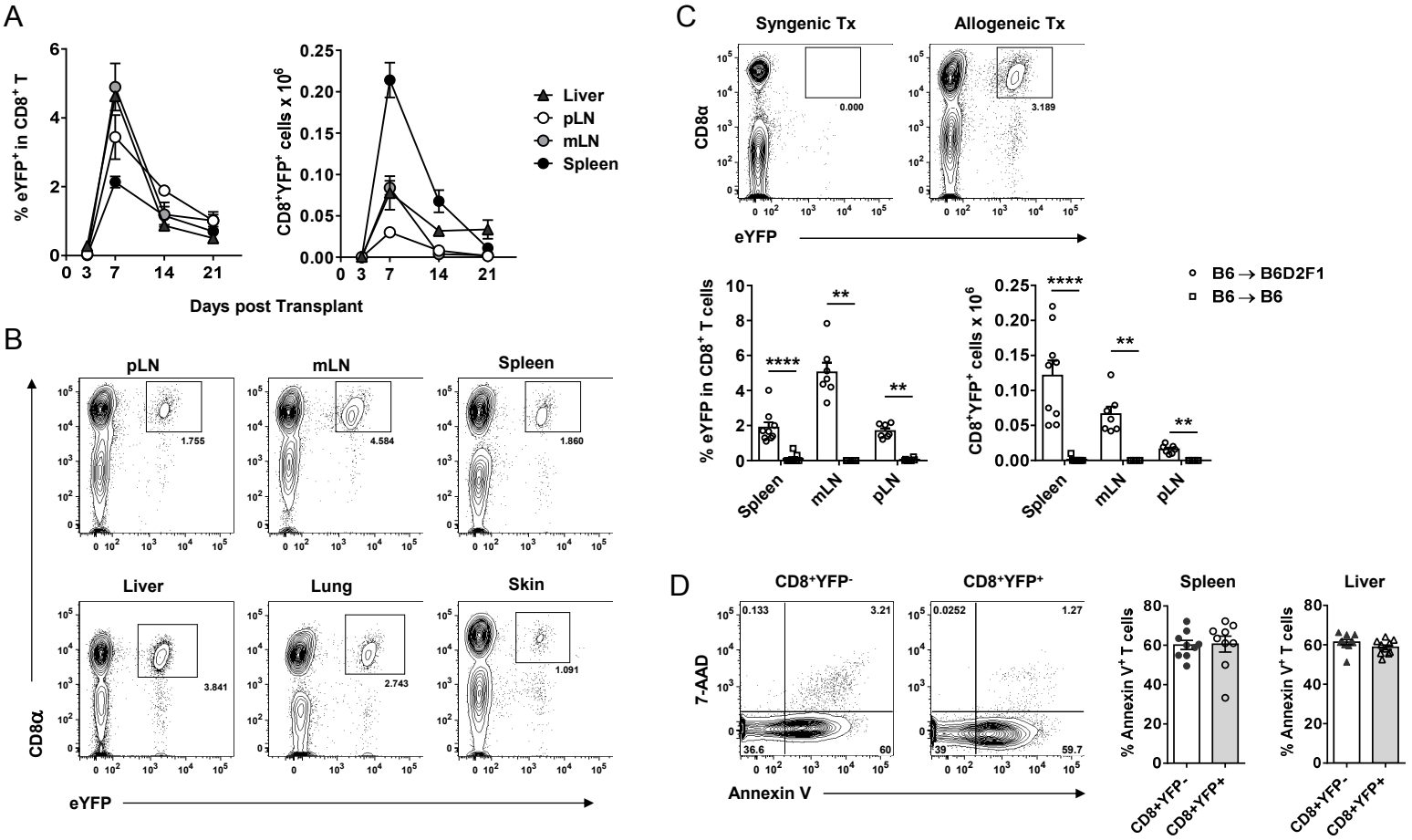
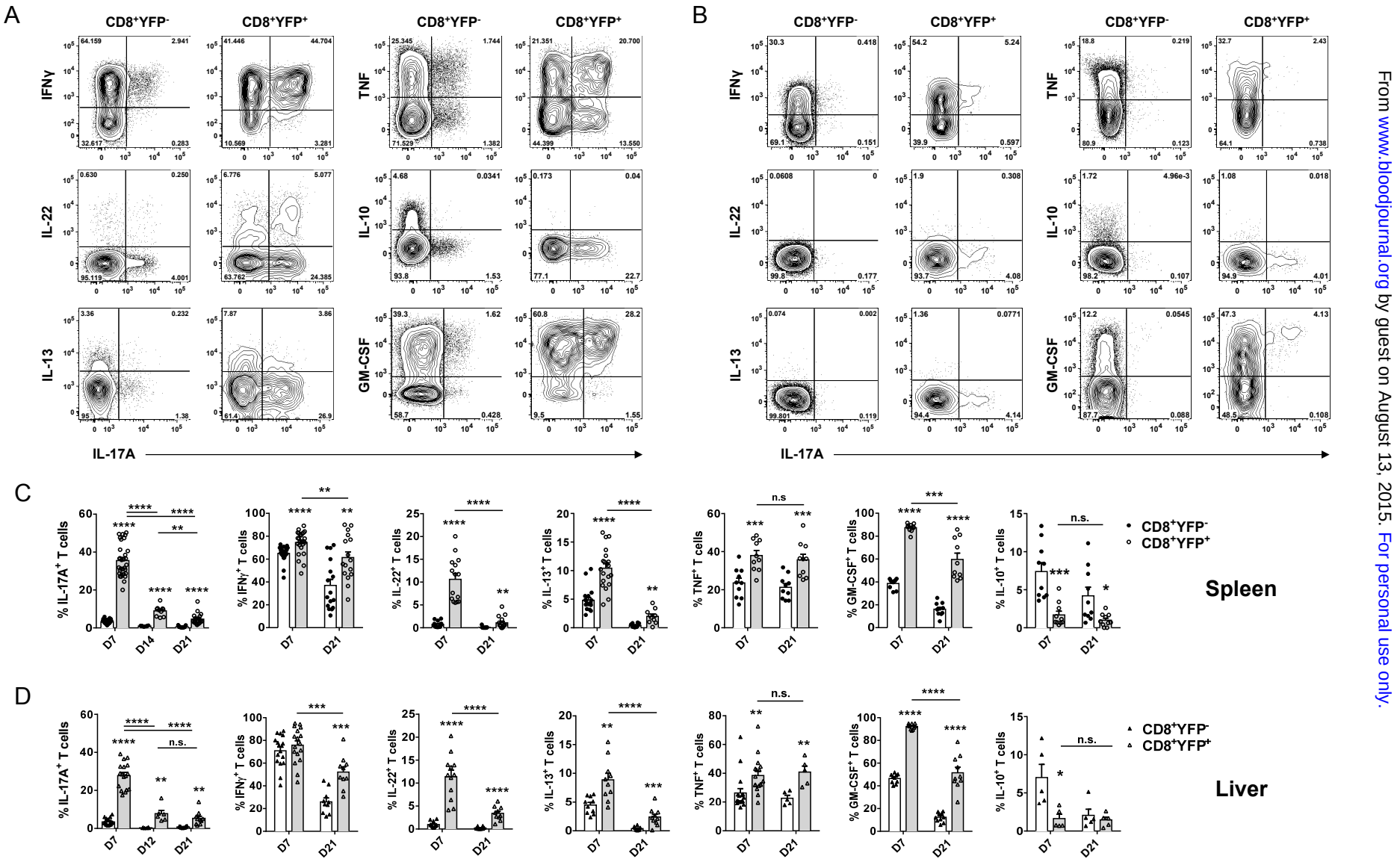


Figure 2.



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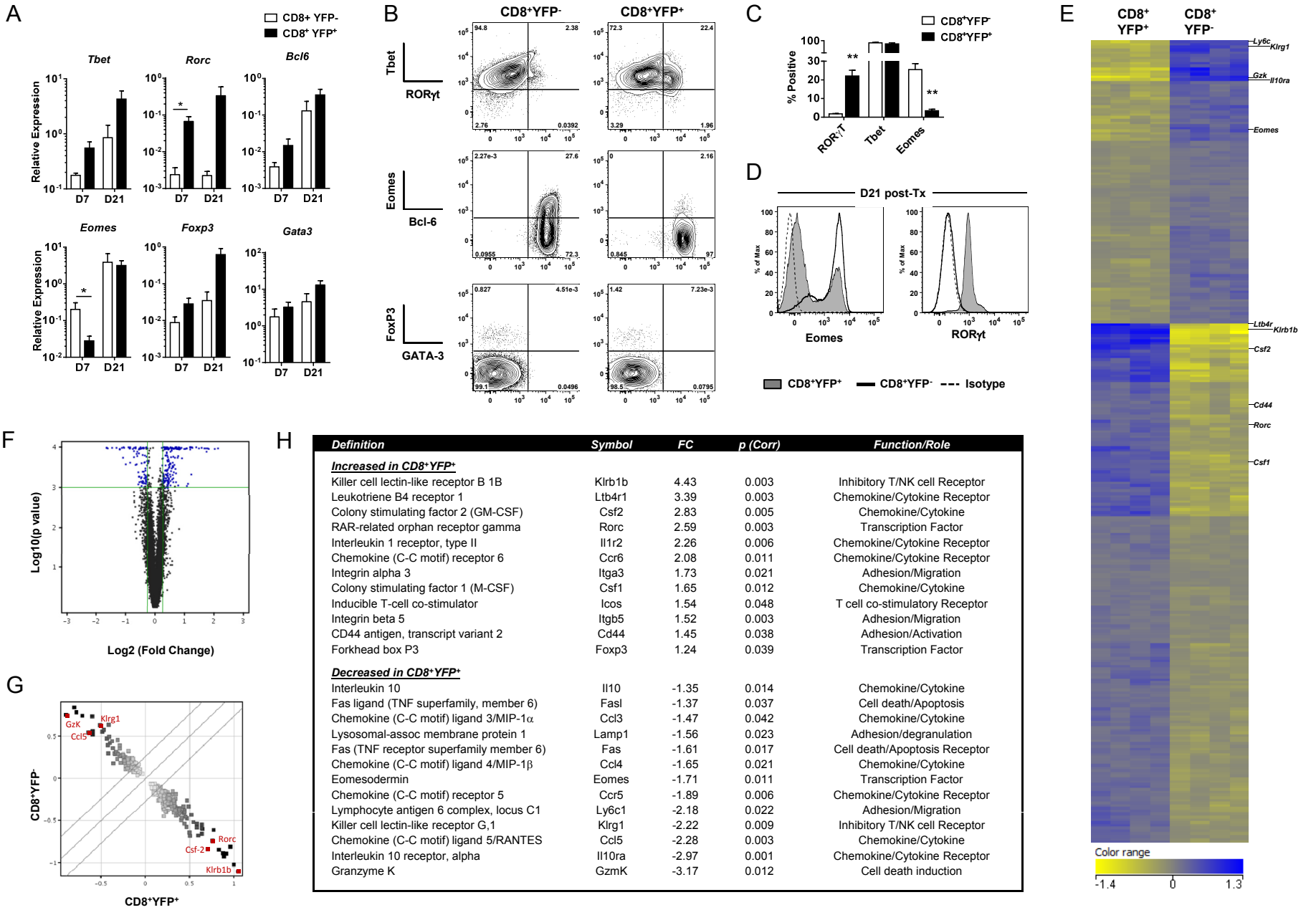


H

Definition	Symbol	FC	p (Corr)	Function/Role
Increased in CD8+YFP+				
Killer cell lectin-like receptor B 1B	Klrb1b	4.43	0.003	Inhibitory T/NK cell Receptor
Leukotriene B4 receptor 1	Ltb4r1	3.39	0.003	Chemokine/Cytokine Receptor
Colony stimulating factor 2 (GM-CSF)	Csf2	2.83	0.005	Chemokine/Cytokine
RAR-related orphan receptor gamma	Rorc	2.59	0.003	Transcription Factor
Interleukin 1 receptor, type II	Il1r2	2.26	0.006	Chemokine/Cytokine Receptor
Chemokine (C-C motif) receptor 6	Ccr6	2.08	0.011	Chemokine/Cytokine Receptor
Integrin alpha 3	Itga3	1.73	0.021	Adhesion/Migration
Colony stimulating factor 1 (M-CSF)	Csf1	1.65	0.012	Chemokine/Cytokine
Inducible T-cell co-stimulator	Icos	1.54	0.048	T cell co-stimulatory Receptor
Integrin beta 5	Itgb5	1.52	0.003	Adhesion/Migration
CD44 antigen, transcript variant 2	Cd44	1.45	0.038	Adhesion/Activation
Forkhead box P3	Foxp3	1.24	0.039	Transcription Factor
Decreased in CD8+YFP+				
Interleukin 10	Il10	-1.35	0.014	Chemokine/Cytokine
Fas ligand (TNF superfamily, member 6)	Fasl	-1.37	0.037	Cell death/Apoptosis
Chemokine (C-C motif) ligand 3/MIP-1α	Ccl3	-1.47	0.042	Chemokine/Cytokine
Lysosomal-assoc membrane protein 1	Lamp1	-1.56	0.023	Adhesion/degranulation
Fas (TNF receptor superfamily member 6)	Fas	-1.61	0.017	Cell death/Apoptosis Receptor
Chemokine (C-C motif) ligand 4/MIP-1β	Ccl4	-1.65	0.021	Chemokine/Cytokine
Eomesodermin	Eomes	-1.71	0.011	Transcription Factor
Chemokine (C-C motif) receptor 5	Ccr5	-1.89	0.006	Chemokine/Cytokine Receptor
Lymphocyte antigen 6 complex, locus C1	Ly6c1	-2.18	0.022	Adhesion/Migration
Killer cell lectin-like receptor G,1	Klrg1	-2.22	0.009	Inhibitory T/NK cell Receptor
Chemokine (C-C motif) ligand 5/RANTES	Ccl5	-2.28	0.003	Chemokine/Cytokine
Interleukin 10 receptor, alpha	Il10ra	-2.97	0.001	Chemokine/Cytokine Receptor
Granzyme K	GzmK	-3.17	0.012	Cell death induction

Figure 4.

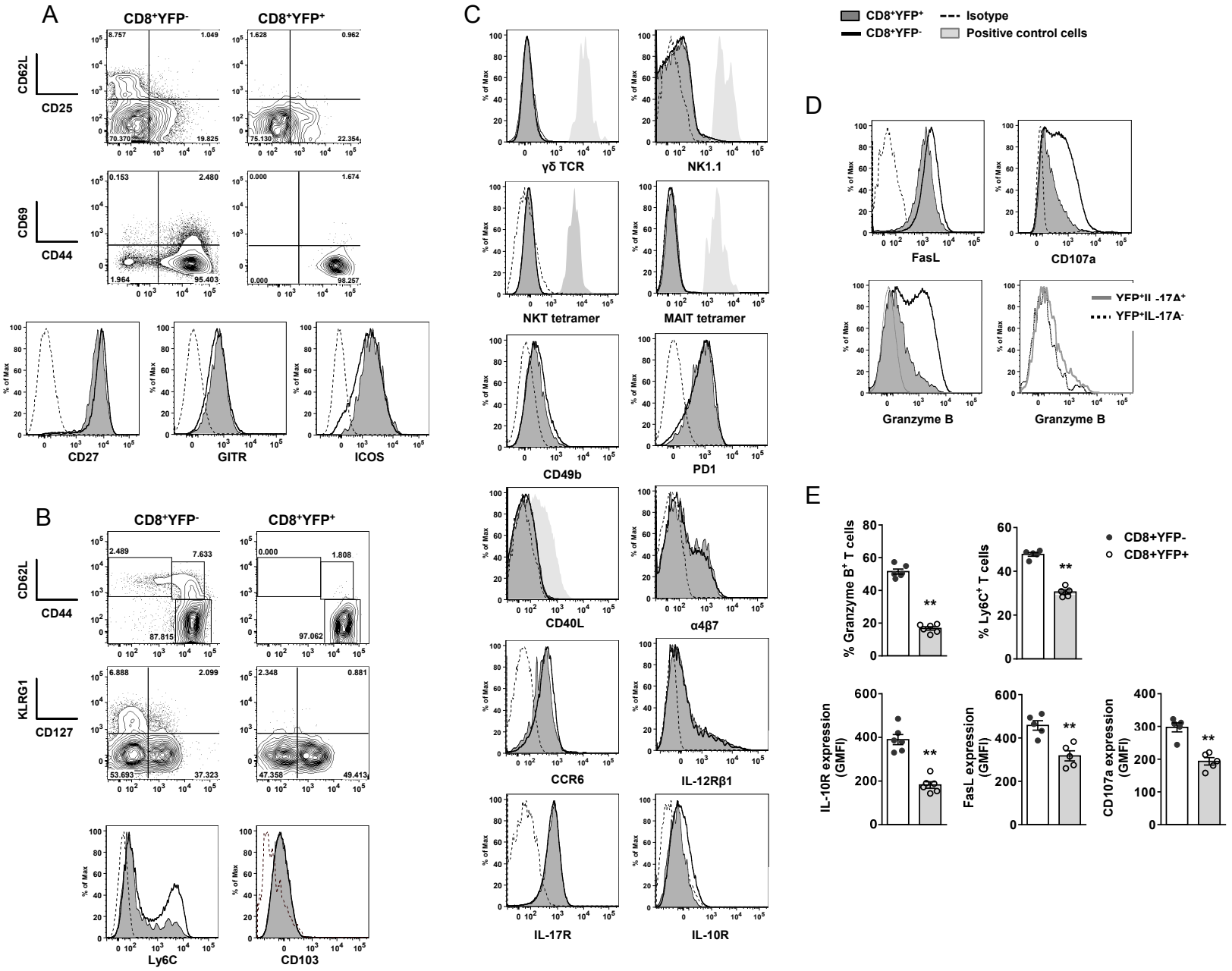


Figure 5.

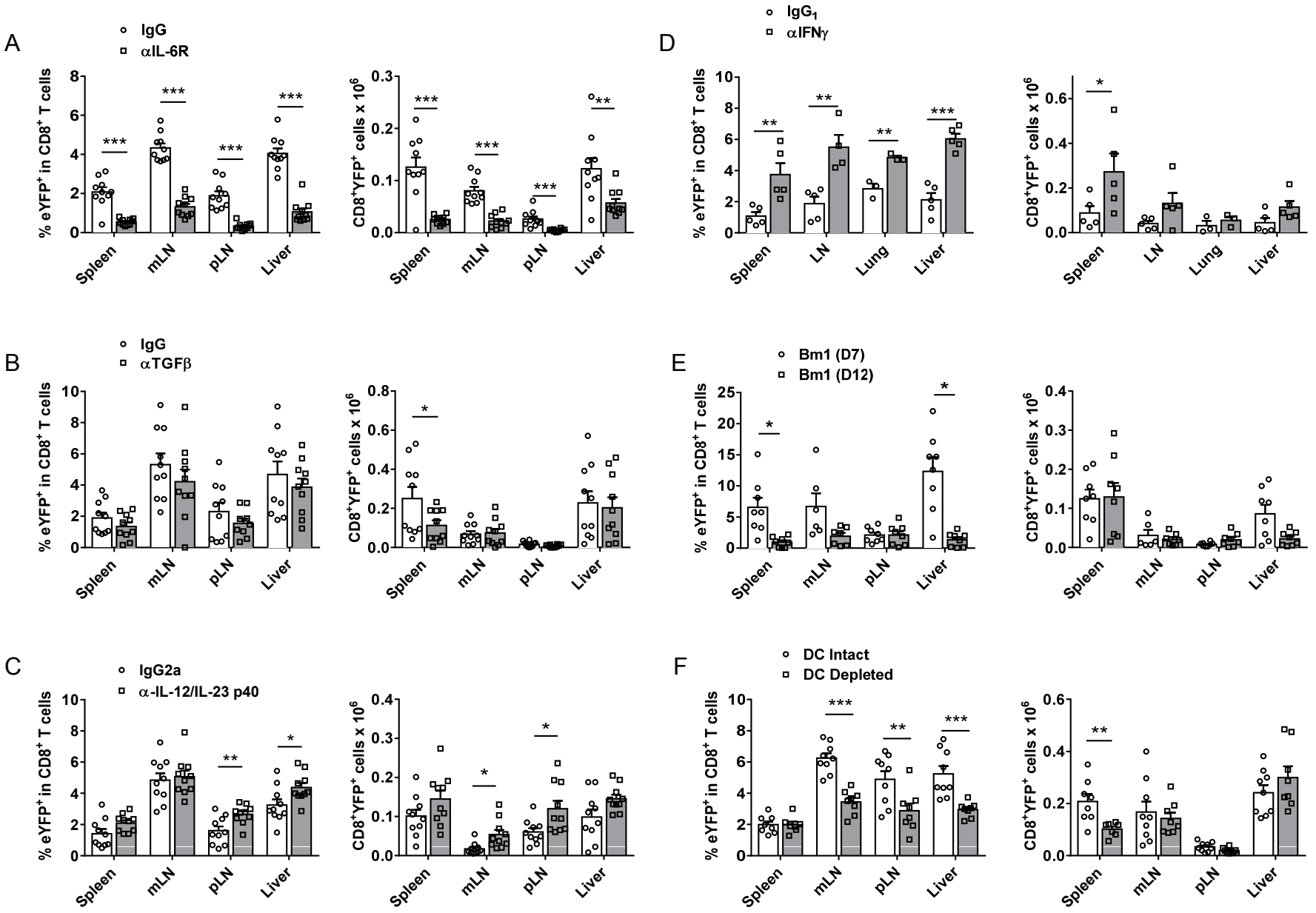


Figure 6.

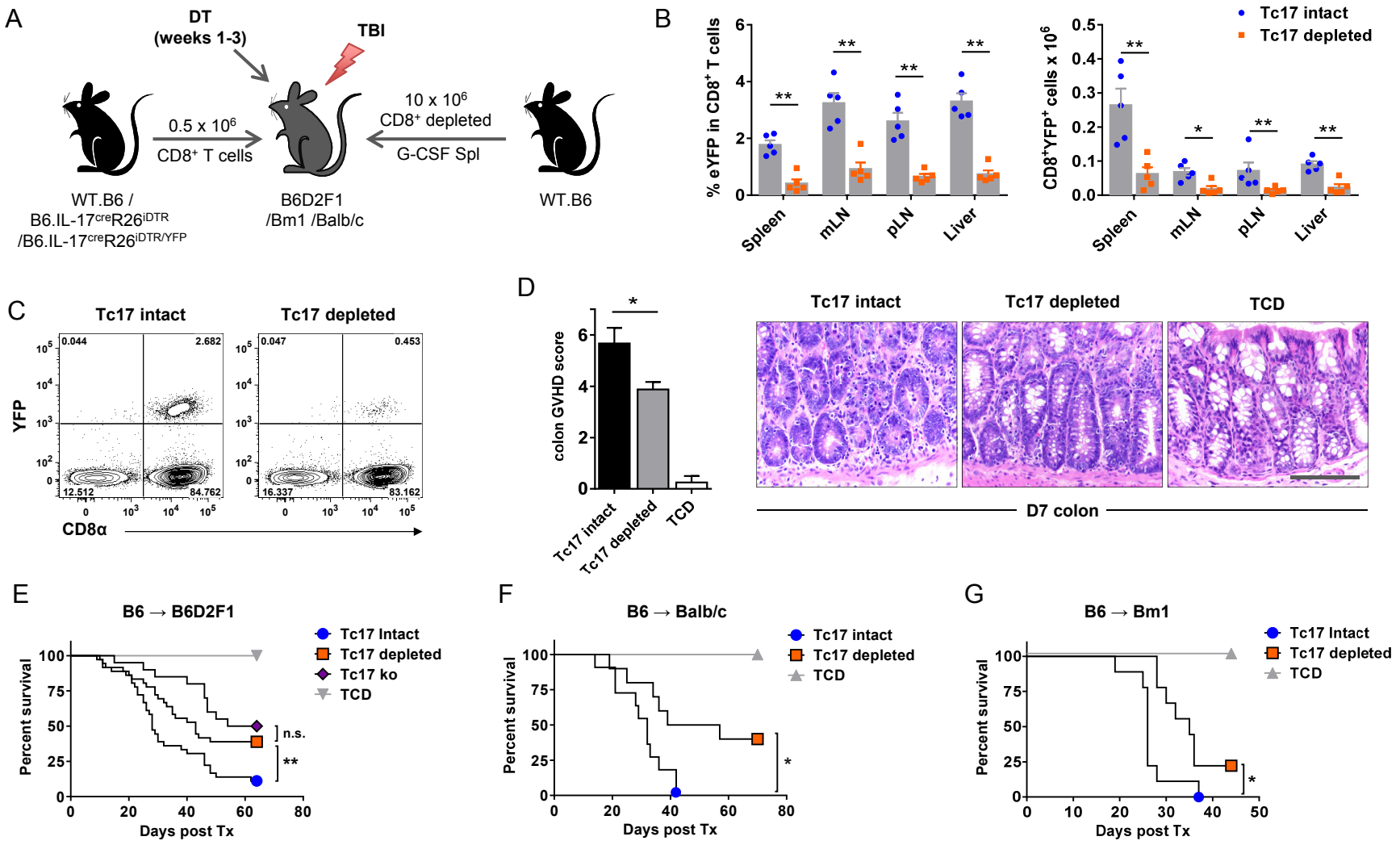
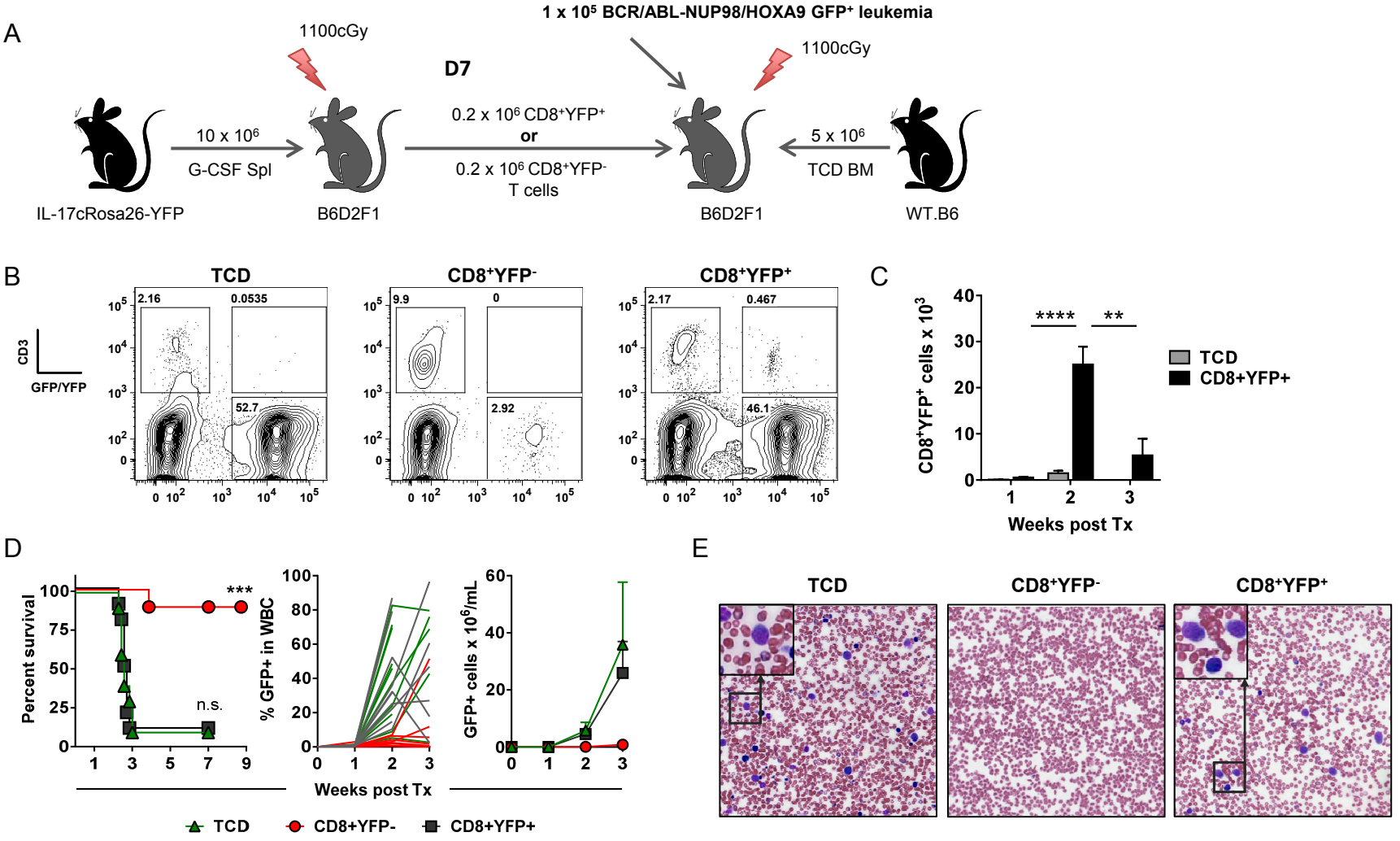


Figure 7.



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Tc17 cells are a pro-inflammatory, plastic lineage of pathogenic CD8$^+$ T-cells that induce GVHD without anti-leukemic effects

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