IMMUNE REGULATION

Eomesodermin promotes the development of type 1 regulatory T (T_R1) cells

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Type 1 regulatory T (T_R1) cells are Foxp3$^-$ interleukin-10 (IL-10)–producing CD4$^+$ T cells with potent immunosuppressive properties, but their requirements for lineage development have remained elusive. We show that T_R1 cells constitute the most abundant regulatory population after allogeneic bone marrow transplantation (BMT), express the transcription factor Eomesodermin (Eomes), and are critical for the prevention of graft-versus-host disease. We demonstrate that Eomes is required for T_R1 cell differentiation, during which it acts in concert with the transcription factor B lymphocyte–induced maturation protein-1 (Blimp-1) by transcriptionally activating IL-10 expression and repressing differentiation into other T helper cell lineages. We further show that Eomes induction in T_R1 cells requires T-bet and donor macrophage–derived IL-27. Thus, we define the cellular and transcriptional control of T_R1 cell differentiation during BMT, opening new avenues to therapeutic manipulation.

INTRODUCTION

Type 1 regulatory T (T_R1) cells are a FoxP3$^-$, interleukin-10 (IL-10)–producing T cell population, which have potent immunosuppressive functions and bear alloantigen specificity (*1*, *2*). IL-10 is the major mediator by which T_R1 cells assert their immunomodulatory role. Direct and bystander-mediated T cell suppression by transforming growth factor–β (TGF-β) and granzyme B (GzmB)–dependent killing of antigen-presenting cells (APCs) have also been described [reviewed in (*3*)]. In addition to IL-10, T_R1 cells show high expression of TGF-β and secrete intermediate amounts of interferon-γ (IFNγ) but not IL-2 or IL-4 (*3*–*5*). Extensive studies have demonstrated the importance of T_R1 cells in maintaining immune tolerance or limiting overt inflammation after transplantation, during autoimmune disease, or after infections (*6*–*9*). IL-27 has been identified as a main driver of T_R1 cell differentiation via the activation of transcription factors that include B lymphocyte–induced maturation protein-1 (Blimp-1), the aryl hydrocarbon receptor (AhR), and c-Maf (*5*–*8*, *10*–*12*). However, the function, phenotype, and lineage development of T_R1 cells in disease states remain poorly understood (*5*, *13*).

Graft-versus-host disease (GVHD) is a common complication of allogeneic bone marrow transplantation (BMT), limiting survival and quality of life (*14*). CD4$^+$FoxP3$^+$ regulatory T (T_{reg}) cells are a well-defined regulatory population important for the generation of tolerance after BMT (*15*). Because of impaired homeostasis of T_{reg} cells after allogenenic BMT (*16*), other suppressive cell populations such as T_R1 cells may be imperative for the prevention and treatment of GVHD. Consistent with this idea, IL-10 deficiency in donor T cells results in more severe GVHD (*17*, *18*). Thus, we developed a mouse model using a dual *Il10*^{GFP}/*Foxp3*^{RFP} reporter mouse strain (*19*, *20*) to delineate T_{reg} cell responses after experimental BMT. Using GVHD as a disease model, we show that T_R1 cells are the most abundant IL-10–producing T_{reg} cell population after experimental BMT. Further analyses demonstrate that T_R1 cells that develop during GVHD express high amounts of Eomesodermin (Eomes), which is required for their development, and its overexpression promotes T_R1 cell development both in vivo and in vitro. Eomes acts in concert with Blimp-1, a known transcriptional regulator of T_R1 cell differentiation (*6*–*8*, *21*), to induce IL-10 expression. We further show that Eomes expression and T_R1 cell development require T-bet and donor macrophage–derived IL-27, resulting in a T-bet^{lo}Eomes^{hi} phenotype. Last, we demonstrate that Eomes$^+$ T_R1 cells are abundant after clinical BMT, indicating the applicability of our findings. Our findings open the way for new therapeutic strategies in transplantation and other clinical settings.

RESULTS

T_R1 cells represent a major T_{reg} cell population in GVHD

We used *Il10*^{GFP} and *Foxp3*^{RFP} dual reporter mice as BMT donors to define CD4$^+$FoxP3$^-$IL-10$^+$ T_R1 cells, CD4$^+$FoxP3$^+$ T_{reg} cells, and CD4$^+$FoxP3$^-$IL-10$^-$ conventional T (T_{con}) cells (Fig. 1A). T cells were the major IL-10 producers after both allogeneic and syngeneic BMT (Fig. 1B), with the highest proportion and intensity of IL-10 produced by T_R1 cells (Fig. 1C). T_R1 cells were present at up to 10-fold higher frequency and number than T_{reg} cells after allogeneic BMT in GVHD target tissues (liver and, to a lesser extent, small intestine), mesenteric lymph nodes (Fig. 1D), and spleen (Fig. 1, D to F). T_R1 cells induced under these conditions had suppressive properties in vitro equivalent to

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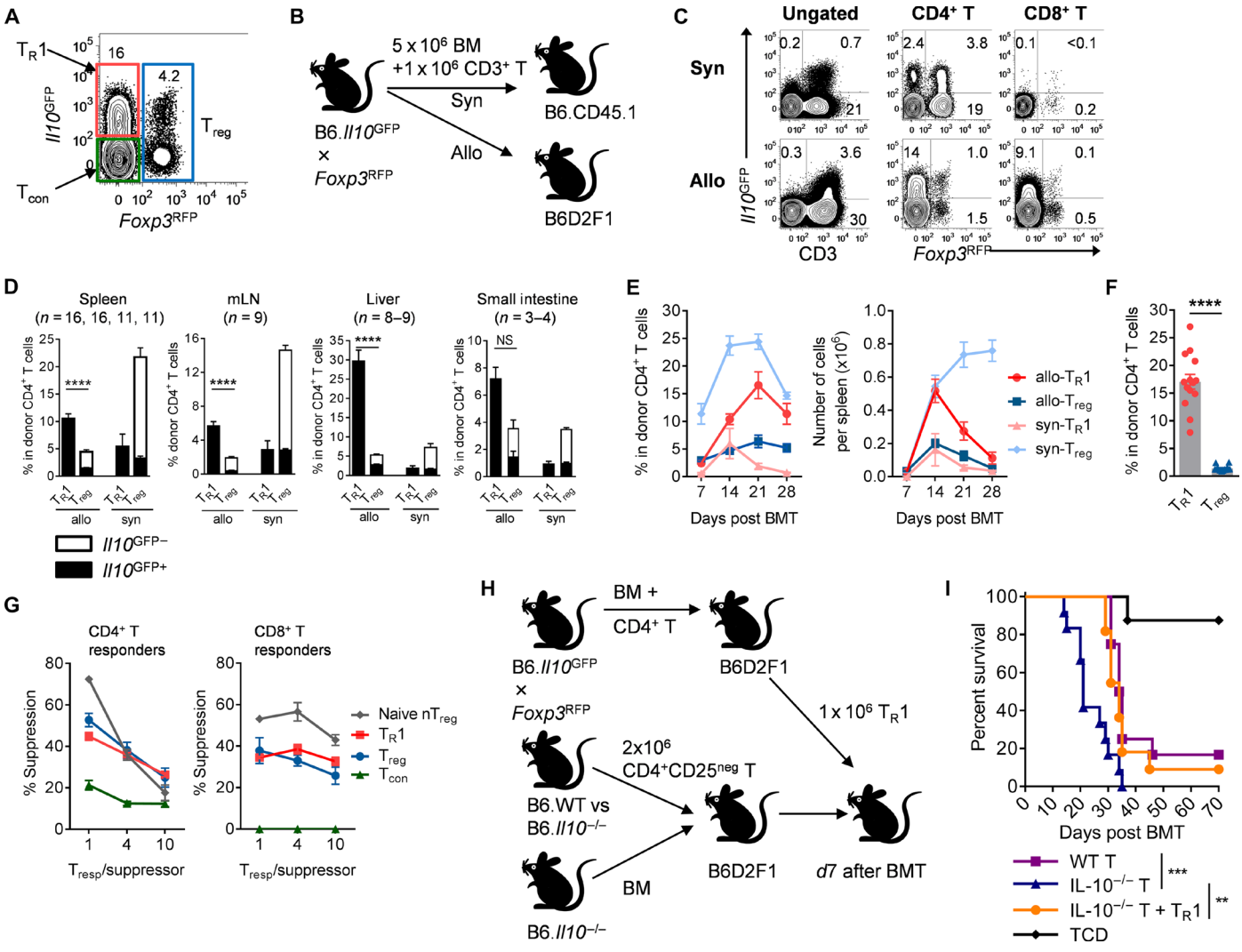

Fig. 1. T_R1 cells constitute the major T_{reg} cells after allogeneic BMT. (**A** to **E**) B6 (Syn) and B6D2F1 (Allo) mice were transplanted with B6 CD3$^+$ T cells (*Il10*^{GFP}/*Foxp3*^{RFP}). (A) Gating strategy after BMT for analysis and fluorescence-activated cell sorting (FACS) of T_R1 (red), T_{reg} (blue), and T_{con} (green) cells. (B) Schema of BMT. (C) Expression of IL-10 and FoxP3 in the spleen at day 14 (representative of more than three experiments). (D) Frequencies of T_R1 and T_{reg} cells at day 14 (solid bar, *Il10*$^{GFP+}$; open bar, *Il10*$^{GFP–}$). (E) CD4$^+$ T cell subsets in spleen after BMT (n = 8 to 9 per group each time point). (**F**) B6D2F1 mice were transplanted with B6 CD4$^+$ T cells (*Il10*^{GFP} and *Foxp3*^{RFP}) and frequencies of T_R1 and T_{reg} cells in the spleen at day 14 (n = 14). (**G**) Suppression of proliferation of carboxyfluorescein diacetate succinimidyl ester–labeled B6 CD4$^+$ and CD8$^+$ responder T cells in vitro by naïve T_{reg} cells versus T_R1, T_{reg}, and T_{con} cell "suppressors" sorted from 10 transplant recipients at day 14 (data combined from two experiments). (**H**) Experimental BMT schema showing adoptive transfer of sorted T_R1 cells to treat established acute GVHD and (**I**) survival of recipients are shown [n = 8 in T cell deplete (TCD) group, others n = 11 to 12]. Data are means ± SEM.

posttransplant T_{reg} cells on a per-cell basis (Fig. 1G and fig. S1). To confirm their suppressive function in vivo, we induced GVHD with wild-type (WT) or *Il10*$^{-/-}$ CD4$^+$CD25$^-$ T cells that cannot develop into functional T_R1 cells. As expected, we observed enhanced GVHD in the absence of IL-10; however, adoptive transfer of limited numbers of T_R1 cells at day 7 after BMT (Fig. 1H), when acute GVHD was established, prolonged survival significantly (Fig. 1I), consistent with potent regulatory function. Thus, T_R1 cells represent the major T_{reg} cell population in GVHD induced by allogeneic BMT and contribute significantly to transplant survival.

T_R1 cells express Eomes and display a distinct phenotypic profile

CD49b and LAG-3 coexpression can be used to identify T_R1 cells in models of colitis (*9*); however, their expression is insufficient to identify T_R1 cells after BMT (fig. S2A). Therefore, we used *Foxp3*$^{RFP–}$ and *Il10*$^{GFP+}$ as T_R1 cell markers. Thus defined T_R1 cells demonstrated high expression of CD122, α4β7, LAG-3, Ly6C, and TIGIT and low expression of CD25 and CD69 relative to other CD4$^+$ T cell subsets (fig. S2B). Consistent with the T_R1 cell phenotype (*3*, *5*, *9*), *Foxp3*$^{RFP–}$*Il10*$^{GFP+}$ T_R1 cells expressed high amounts of IL-10 and IFNγ but little T helper 2 (T_H2) cytokines, such as IL-4, IL-13, and IL-5, or T_H17 cytokines, such as IL-17, IL-6, or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 2A and fig. S2C).

T_R1 cells have often been considered a terminally differentiated T_H1 cell subset programmed to limit aberrant inflammation (*5*, *13*, *22*). T_R1 cells expressed high amounts of T-bet, the T_H1 cell–determining transcription factor, but low amounts of GATA-3, BCL-6, and RORγt. When we analyzed the expression of other transcription factors related

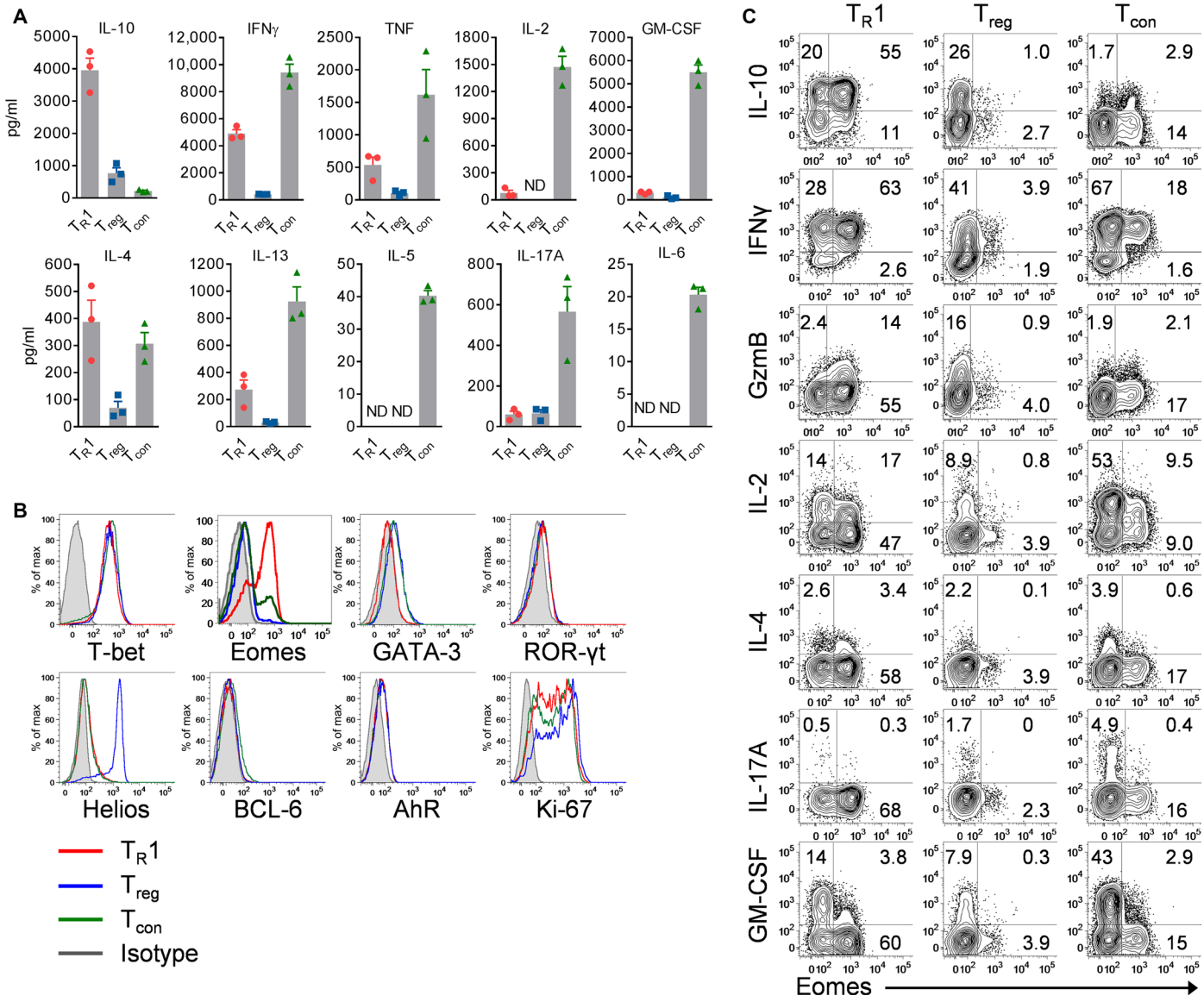

Fig. 2. T_R1 cells express Eomes and display a distinct phenotypic profile. (**A** to **C**) B6D2F1 mice were transplanted with *Il10*^{GFP}*Foxp3*^{RFP} B6 CD3$^+$ T cells. CD4$^+$ T cells from spleen were FACS-sorted into T_R1, T_{reg}, and T_{con} cells at day 14 as in Fig.1A (data from three experiments; ND, not detectable). (A) Cytokine production in culture supernatant of T cell subsets. (B) Expression of transcription factors in T cell subsets (red, T_R1; blue, T_{reg}; green, T_{con}; gray, isotype). (C) Expression of cytokines and Eomes in T cell subsets. Data are means ± SEM.

to T cell differentiation, we observed high Eomes expression, which was largely restricted to T_R1 cells (Fig. 2B and fig. S2D). Eomes expression tightly correlated with high expression of IL-10, IFNγ, and GzmB (Fig. 2C). In contrast, Eomes$^+$ T_R1 cells expressed low levels of IL-2, IL-17A, and GM-CSF (Fig. 2C). Thus, T_R1 cells that develop during allogeneic BMT specifically express Eomes.

Eomes is required for T_R1 cell differentiation

To test the role of Eomes in T_R1 cell development in vivo, we used CD4$^+$ T cells isolated from *Eomes*$^{fl/fl}$×*Cd4*-cre donor mice in allogeneic BMT. Strikingly, T_R1 cell generation was significantly reduced (by >70%) with decreased GzmB expression in recipients of Eomes-deficient CD4$^+$ T cells (Fig. 3A and fig. S3, A and B). Critically, the loss of Eomes did not impair the development of IL-10$^-$IFNγ$^+$ or T-bet$^+$ T_{con}, IFNγ$^+$TNF$^+$ T_H1, and IL-17A$^+$ T_H17 cells or IL-10 expression by T_{reg} cells but instead favored the expression of IL-4 and FoxP3 (Fig. 3A and fig. S3, B to F). To further elucidate the role of Eomes in the differentiation of T_R1 cells and transactivation of *Il10*, we transplanted donor WT or *Eomes*$^{-/-}$ CD4$^+$ T cells, which constitutively expressed Eomes after retroviral transduction. Strikingly, enforced expression of Eomes rescued the development of T_R1 cells from *Eomes*$^{-/-}$ CD4$^+$ T cells after BMT and also promoted their development in WT cells (Fig. 3B). In addition, overexpression of Eomes promoted the expression of GzmB while suppressing FoxP3, IL-4, and IL-17A expression (fig. S3G). Furthermore, overexpression of Eomes up-regulated the transcription of *Il10* but suppressed that of other lineage-defining transcription factors including *Tbx21*, *Gata3*, *Rorc*, *Bcl6*, and *Foxp3*, in addition to the T_R1/T_H17 cell–related factors *Ahr* and *Il21* (Fig. 3C) (*10*, *23*, *24*).

T_R1 cells generated in vitro in the presence of IL-27, a cytokine-promoting T_R1 cell development (*8*, *11*, *12*), did not express Eomes protein, nor did T_H1, T_H2, T_H17, and induced T_{reg} cells (fig. S4A), indicating that short-term in vitro cultures do not replicate the conditions inducing T_R1 cells after BMT. Nevertheless, Eomes mRNA was higher in T_R1 cell than in other T cell lineages in these cultures (fig. S4B). Consistent with this observation, we did not observe a defect in T_R1 cell differentiation in the absence of Eomes in these conditions (fig. S5A). However, transduction of Eomes into $CD4^+$ T cells and subsequent restimulation in culture markedly promoted the differentiation of IL-10^+IFNγ^+ T_R1 cells and the expression of GzmB while suppressing the expression of IL-4 and FoxP3 (fig. S5B). Overexpression of Eomes also suppressed mRNA expression of transcription factors defining other T_H cell lineages, including *Tbx21*, *Gata3*, *Rorc*, and *Bcl6*, and other T_R1/T_H17 cell–related factors, such as *Ahr*, *Maf*, and *Il21* (fig. S5C). Collectively, we show that Eomes is required for T_R1 cell differentiation, IL-10 secretion, and repression of alternative fate differentiation.

Eomes directly regulates IL-10 expression in T_R1 cells

To understand the mechanism by which Eomes regulates T_R1 cell differentiation, we performed chromatin immunoprecipitation (ChIP) assays on sort-purified T_R1 cells or $CD4^+$ T cells 14 days after BMT. This demonstrated that Eomes is bound to multiple sites within 2 kb upstream of the transcription start site (TSS) of the *Il10* gene (Fig. 3D). The binding of Eomes to the *Il10* promoter was similar to that observed in the *Ifn*γ promoter, suggesting that Eomes regulates expression of both *Il10* and *Ifn*γ directly. Consistent with this concept, the

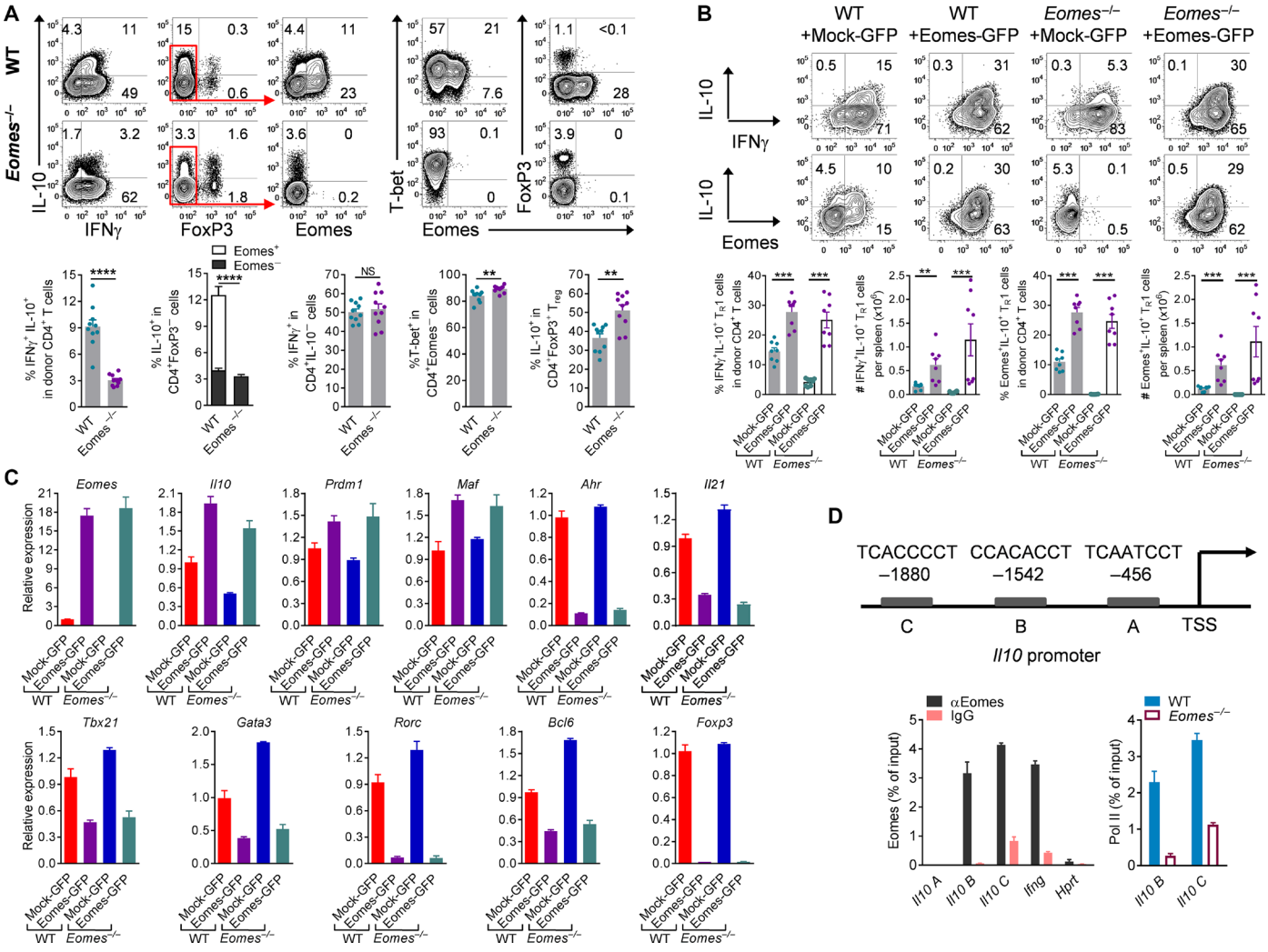

Fig. 3. Eomes is required for T_R1 cell differentiation. (**A** to **D**) B6D2F1 mice were transplanted with primary or retrovirally transduced (Mock-GFP or Eomes-GFP) $CD4^+$ T cells. (A) Expression of IL-10, IFNγ, FoxP3, Eomes, and T-bet (open bar, Eomes$^+$IL-10$^+$; solid bar, Eomes$^-$IL-10$^+$; n = 10 per group) in recipients of WT or Eomes$^{-/-}$ $CD4^+$ T cells at day 14 (n = 10 per group). (B) Expression of IL-10, IFNγ, and Eomes in transduced WT or Eomes$^{-/-}$ $CD4^+$ T cells at day 7 (n = 8 per group) and (C) transcription of *Il10* and related genes (data are from four to five pooled animals in triplicate reactions, representative of two independent experiments). (D) $CD4^+$ T cells or *Foxp3*$^{RFP-}$*Il10*$^{GFP+}$ T_R1 cells were FACS-sorted from the spleen and liver at day 14 (representative of three experiments). A schematic diagram of the mouse IL-10 promoter indicates Eomes binding sites upstream of the TSS, with each sequence shown. Recruitment of Eomes to the *Il10* promoter and control regions in $CD4^+$ T cells from T_R1 cells (data are from 30 pooled animals in triplicate reactions) and recruitment of RNA polymerase II (Pol II) to the *Il10* promoter in WT or *Eomes*$^{-/-}$ $CD4^+$ T cells (data are from 10 pooled animals in triplicate reactions). Data are means ± SEM.

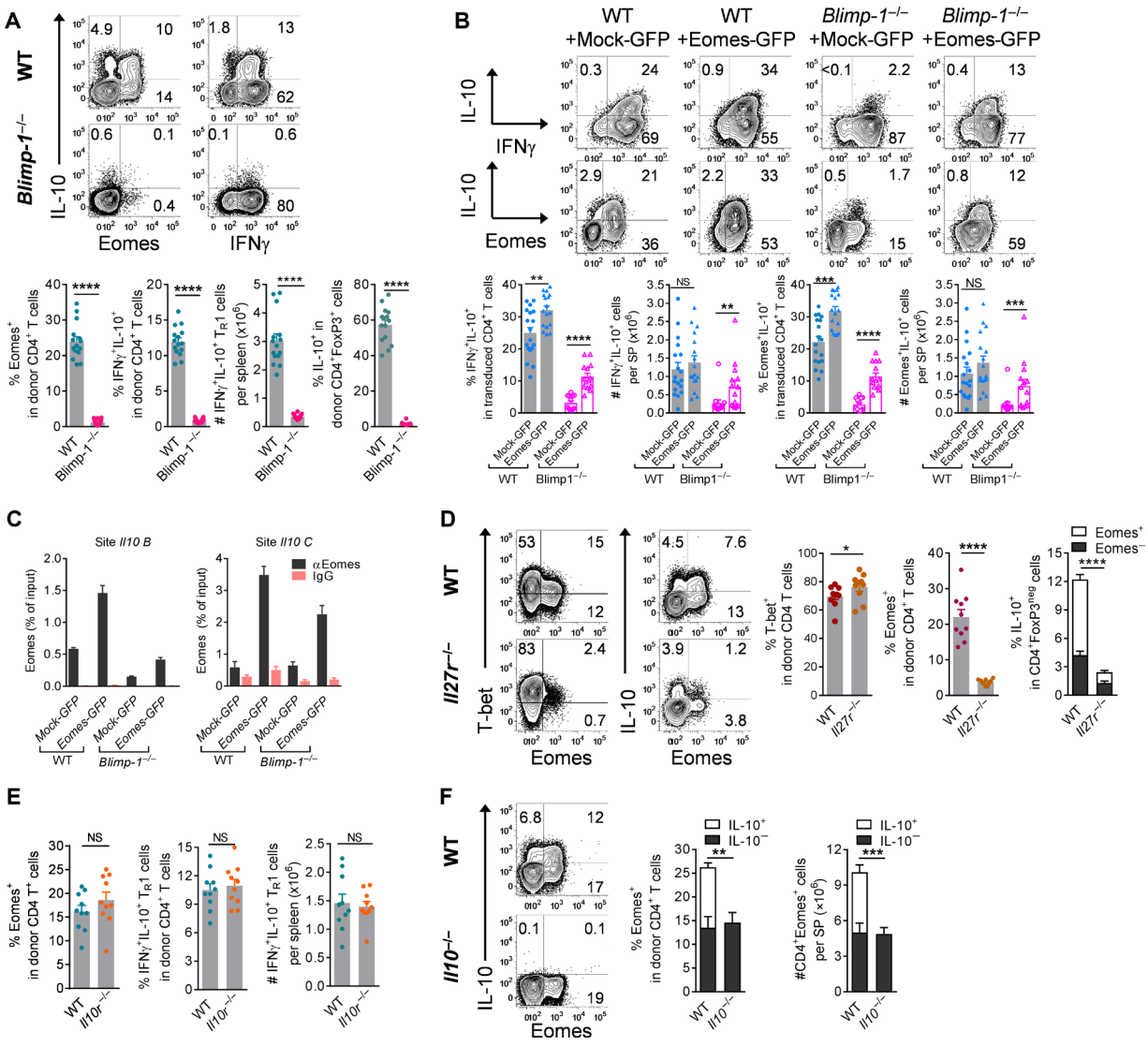

Fig. 4. Eomes$^+$ T$_R$1 cells are dependent on Blimp-1, IL-27, and IL-10. (**A** to **F**) B6D2F1 mice were transplanted with primary or retrovirally transduced (Mock-GFP or Eomes-GFP) CD4$^+$ T cells, and spleen was examined after BMT. (A) Expression of Eomes, IL-10, and IFNγ in WT or *Blimp-1*$^{-/-}$ CD4$^+$ T cells at day 14 (n = 14 to 15 per group). (B) Expression of Eomes, IL-10, and IFNγ (n = 18 and 17 for WT; n = 13 and 14 for *Blimp-1*$^{-/-}$) in transduced CD4$^+$ T cells at days 7 to 10. (C) Recruitment of Eomes to *Il10* promoter in transduced CD4$^+$ T cells (WT or *Blimp-1*$^{-/-}$) at day 10 (data are from four animals in duplicate or triplicate reactions). (D) Expression of T-bet, Eomes, and IL-10 in WT or *Il27r*$^{-/-}$ CD4$^+$ T cells at day 14 (n = 10 per group). (E) Expression of Eomes and IFNγ$^+$IL-10$^+$ T$_R$1 cells in WT or *Il10r*$^{-/-}$ CD4$^+$ T cells at day 14 (n = 10 per group). (F) Expression of Eomes and IL-10 (open bar, Eomes$^+$IL-10$^+$; solid bar, Eomes$^+$IL-10$^-$) in CD4$^+$ T cells in recipients of WT or *Il10*$^{-/-}$ CD4$^+$CD25$^-$ T cells at day 14 (n = 10 to 11 per group). Data are means ± SEM.

recruitment of RNA polymerase II to the *Il10* promoter, an indicator of transcriptional activity, was reduced in Eomes-deficient CD4$^+$ T cells (Fig. 3D).

Eomes$^+$ T$_R$1 cells are dependent on Blimp-1, IL-27, and IL-10

Blimp-1 is a well-defined transcriptional promoter of IL-10 in CD4$^+$ T$_{con}$ and T$_{reg}$ cells (*6*, *11*, *21*). Consistent with this notion, after BMT, IL-10 production in all CD4$^+$ T cells was confined to Blimp-1–expressing cells (fig. S6A). Critically, conditional ablation of Blimp-1 (*Prdm1*$^{fl/fl}$×*Lck*-cre) in donor T cells resulted in a near-complete loss of both IL-10 and Eomes expression in CD4$^+$ T cells, demonstrating a near-complete lack of T$_R$1 cells (Fig. 4A), whereas the expression of T-bet was not impaired (fig. S6B). To elucidate the relative contribution of Eomes and Blimp-1 to the expression of IL-10, we transferred Eomes-transduced WT or *Blimp-1*$^{-/-}$ CD4$^+$ T cells into allogeneic BMT recipients. Consistent with a critical role of Eomes in the differentiation of T$_R$1 cells, overexpression of Eomes in Blimp-1–deficient CD4$^+$ T cells partially rescued their defective expression of IL-10 and GzmB and suppressed the expression of IL-2, IL-4, IL-17A, GM-CSF, and FoxP3 after BMT (Fig. 4B and fig. S6, C and D). Furthermore, Eomes transduction enhanced the recruitment of Eomes to the *Il10* promoter regions in both WT and *Blimp-1*$^{-/-}$ CD4 T cells (Fig. 4C).

To test the role of IL-27 in the induction of Eomes$^+$ T$_R$1 cells after BMT, we transplanted *Il27r*$^{-/-}$ CD4$^+$ T cells. Consistent with an important role for IL-27 in T$_R$1 cell induction, we found substantially decreased expression of Eomes in *Il27r*$^{-/-}$ CD4$^+$ T cells, and T$_R$1 cells were reduced

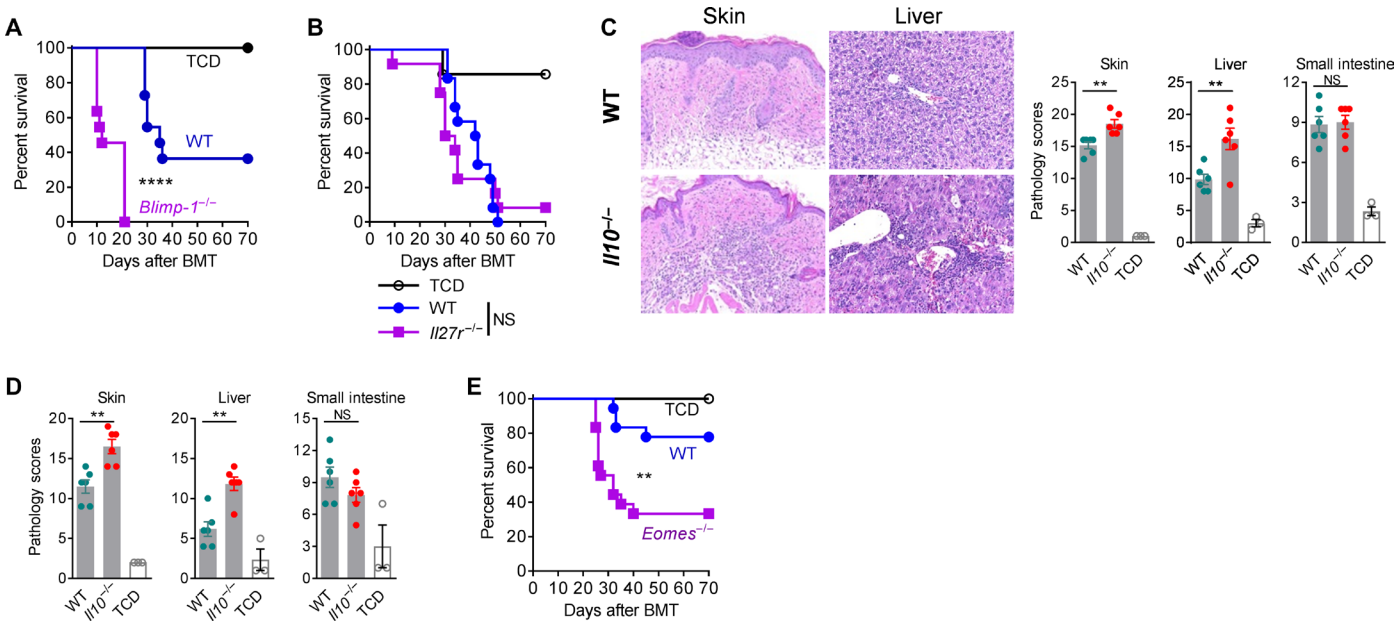

Fig. 5. Attenuation of GVHD by Eomes$^+$ T$_R$1 cells. (**A** to **E**) B6D2F1 recipients were transplanted with CD4$^+$ T cells, and survival or histopathology was examined. (A) Survival of recipients of WT or *Blimp-1*$^{-/-}$ CD4$^+$ T cells (2×10^6 per mouse) ($n = 11$ per T cell group, $n = 7$ in TCD; two experiments). (B) Survival of recipients of WT or *Il27r*$^{-/-}$ CD4$^+$CD25$^-$ T cells (2×10^6 per mouse) ($n = 12$ per T cell group, $n = 7$ in TCD; two experiments). (C and D) Histology in recipients of (C) WT versus *Il10*$^{-/-}$ or (D) WT versus *Il10*$^{fl/fl}$×*Lck*-cre CD4$^+$CD25$^-$ T cells (1×10^6 per mouse) at day 28 ($n = 6$ per T cell group, $n = 3$ in TCD group). (E) Survival of recipients of WT or *Eomes*$^{-/-}$ CD4$^+$CD25$^-$ T cells (1×10^6 per mouse) ($n = 12$ per T cell group, $n = 7$ in TCD; two experiments). Histology represents mean ± SEM.

by >80% (Fig. 4D and fig. S6E). In contrast, T-bet expression was increased in the absence of IL-27 signaling (Fig. 4D), and the development of CD4$^+$IL-10$^-$IFNγ$^+$ conventional T$_H$1 cells or IL-10 production capabilities of T$_{reg}$ cells were not impaired (fig. S6E).

We next tested whether the differentiation of Eomes$^+$ T$_R$1 cells was dependent on IL-10 itself. The expression of Eomes, T$_R$1 cells, and T-bet was not reduced in *Il10r*-deficient CD4$^+$ T cells (*Il10r*$^{fl/fl}$×*Lck*-cre) after BMT (Fig. 4E and fig. S6F), indicating that IL-10 signaling in T cells was not required for T$_R$1 cell differentiation. However, when we transplanted *Il10*$^{-/-}$ CD4$^+$CD25$^-$ T cells, Eomes$^+$ cells were reduced (Fig. 4F), in line with the notion that IL-10 promotes T$_R$1 cell differentiation indirectly (*22*, *25*). In summary, Eomes expression in T$_R$1 cells is downstream of IL-27 and Blimp-1 but does not depend on T cell–intrinsic IL-10 signaling.

Eomes$^+$ T$_R$1 cells are critical for the prevention of GVHD

We next examined whether *Blimp-1*– and *Il27r*-deficient CD4$^+$ T cells would exacerbate GVHD due to impaired expression of Eomes and T$_R$1 cells. Whereas Blimp-1 deletion exacerbated GVHD (Fig. 5A), IL-27 receptor (IL-27R) deletion did not (Fig. 5B). Notably, T$_{reg}$ cells were increased, and their IL-10 production was intact in recipients of *Il27r*$^{-/-}$ CD4$^+$ T cells (figs. S6E and S7A), consistent with compensatory regulatory pathways in the absence of T$_R$1 cells. In contrast, *Il10*$^{-/-}$ CD4$^+$ T cells sustain comparable expression of Eomes in T$_{con}$ and T$_{reg}$ cells (Fig. 4F and fig. S7B) after BMT and thus reflect a more relevant model to define the regulatory function of T$_R$1 cells in vivo. Consistent with the reduced frequency of T$_R$1 cells, we observed enhanced GVHD in the skin and liver in recipients of *Il10*$^{-/-}$ CD4$^+$CD25$^-$ T cells (Fig. 5C). These findings were confirmed by transplanting *Il10*$^{fl/fl}$×*Lck*-cre CD4$^+$CD25$^-$ T cells, which also led to exacerbated GVHD in the absence of IL-10–producing T$_R$1 cells (Fig. 5D). Last, *Eomes*$^{-/-}$ CD4$^+$CD25$^-$ T cells also resulted in increased GVHD, further confirming the important regulatory role of Eomes$^+$ T$_R$1 cells after BMT (Fig. 5E).

Eomes and T-bet cooperate to generate T$_R$1 cells

As we had observed coexpression of T-bet (encoded by *Tbx21*) and Eomes in T$_R$1 cells after BMT, we wished to test the role of IFNγ signaling and T-bet in T$_R$1 cell development. Transplanting *Ifngr*$^{-/-}$ donor T cells or neutralizing IFNγ resulted in reduced expression of T-bet and Eomes (Fig. 6A), with reduced expression of Eomes$^+$ T$_R$1 cells and expanded T$_{reg}$ cell populations (Fig. 6, B and C). When we transplanted *Tbx21*$^{-/-}$ CD4$^+$ T cells during BMT, we found that Eomes$^+$ T$_R$1 cells were markedly reduced (Fig. 6D and fig. S8A). Although overall frequencies of IL-10$^+$CD4$^+$ T cells were unaffected, the absolute numbers were reduced (Fig. 6D). However, most of the *Tbx21*$^{-/-}$ IL-10$^+$ CD4$^+$ T cells did not express IFNγ but rather IL-4 and GATA3 or IL-17A, indicating that these cells had been diverted to T$_H$2 or T$_H$17 cells, respectively (Fig. 6D and fig. S8B). Gene expression analysis confirmed polarization of donor CD4$^+$ T cells to T$_H$2 (*Gata3*, *Il4*, and *Il13*) and T$_H$17 (*Rorc*, *Ahr*, and *Il21*) cell lineages in the absence of T-bet. The transcription of *Il10* (from T$_H$2 cells) was also increased (fig. S8C). The residual Eomes$^+$ population in *Tbx21*$^{-/-}$ CD4$^+$IL-10$^+$ cells expressed IFNγ but did not express IL-4 (fig. S8D). Thus, T-bet and IFNγ promote Eomes expression within the T$_R$1 cell lineage after BMT and, in concert with Eomes, repress alternate cell fates. To further understand the relative function of Eomes and T-bet in the differentiation of T$_R$1 cells, we retrovirally transduced *Tbx21*$^{-/-}$ CD4$^+$ T cells with Eomes. The overexpression of Eomes rescued the expression of IL-10, IFNγ, and IL-10$^+$IFNγ$^+$ T$_R$1 cells and correspondingly suppressed the expression of GATA-3$^+$IL-4$^+$ T$_H$2 and IL-17A$^+$ T$_H$17 cells (Fig. 6E and fig. S8, E and F).

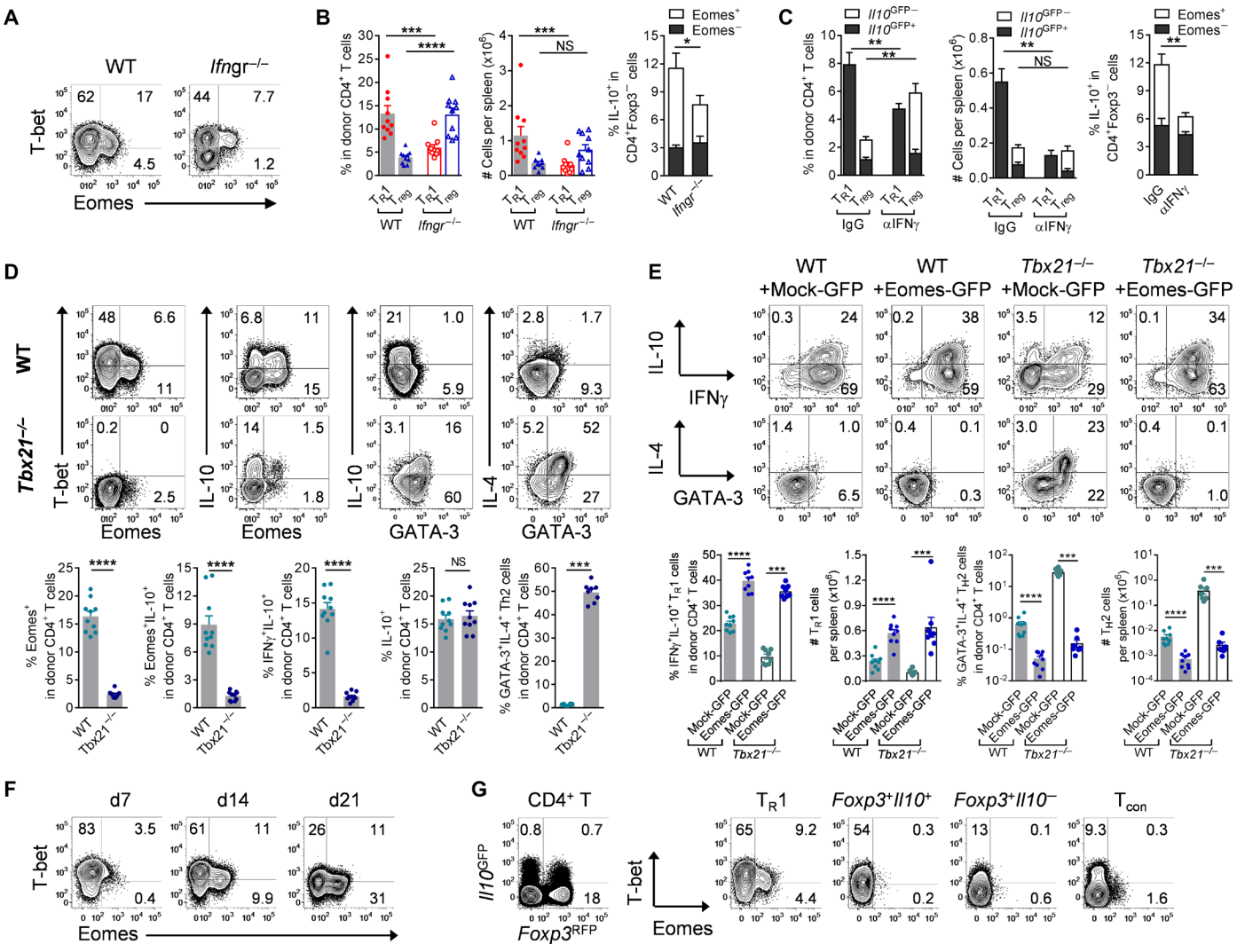

Fig. 6. Eomes and T-bet jointly regulate T_R1 cell development. (**A** and **B**) B6.WT or B6.*Ifngr*$^{-/-}$ $CD3^+$ T cells were transplanted into B6D2F1 mice, and splenic $CD4^+$ T cells were examined at day 14. (A) Representative plots show expression of T-bet and Eomes and (B) frequencies of T_R1 and T_{reg} cells and expression of IL-10 and Eomes (n = 10 per group). (**C**) B6.*Il10*^{GFP}*Foxp3*^{RFP} $CD3^+$ T cells were transplanted into B6D2F1 mice receiving αIFNγ or control mAb, and splenic $CD4^+$ T cells were examined at day 12 (n = 5 per group). Frequencies of T_R1 and T_{reg} cells and expression of Eomes and IL-10 are shown. (**D**) B6D2F1 mice were transplanted with WT or *Tbx21*$^{-/-}$ $CD4^+$ T cells, and expression of transcription factors and cytokines in splenic $CD4^+$ T cells at day 12 is shown (n = 10 per group). (**E**) B6D2F1 mice were transplanted with retrovirally (Mock-GFP or Eomes-GFP) transduced WT or *Tbx21*$^{-/-}$ $CD4^+$ T cells, and expression of IL-10, IFNγ, IL-4, and GATA-3 in splenic $CD4^+$ T cells at day 7 is shown (n = 8 per group). (**F**) Coexpression of T-bet and Eomes in $CD4^+$ T cells over time (representative of at least two experiments). (**G**) Splenic $CD4^+$ T cells from naïve mice FACS-sorted to four subsets based on *Il10*^{GFP} and *Foxp3*^{RFP} expression and T-bet and Eomes were evaluated (representative of two experiments). Data are means ± SEM.

We next investigated whether there is a temporal and/or spatial collaboration between T-bet and Eomes during T_R1 cell development. First, Eomes expression in T_R1 cells was profoundly time-dependent after BMT (fig. S8G), and $CD4^+$ T cells transited from a T-bet^{hi}Eomes^{lo} to a T-bet^{lo}Eomes^{hi} state over time (Fig. 6F), correlating with the increasing frequency of T_R1 cells (Fig. 1E). Furthermore, after repeated exposure to high levels of alloantigen in vivo, most donor $CD4^+$ T cells had acquired Eomes (>95%) and converted to T_R1 cells (>70%) within 4 weeks of transfer into secondary BMT recipients (fig. S8H). Consistently, overexpression of Eomes suppressed the expression of T-bet while promoting T_R1 cell differentiation (Fig. 3B and fig. S8I). T_R1 cells (*Foxp3*$^{RFP-}$*Il10*$^{GFP+}$), found in low frequencies in naïve mice, also exhibited higher Eomes expression. This was specific to T_R1 cells because IL-10–producing T_{reg} cells (*Foxp3*$^{RFP+}$*Il10*$^{GFP+}$) expressed some T-bet but not Eomes (Fig. 6G). Collectively, these data show that both T-bet and Eomes are required for T_R1 cell differentiation, which is characterized by the initial up-regulation of T-bet, the acquisition of Eomes expression, and the subsequent down-regulation of T-bet, resulting in a T-bet^{lo}Eomes^{hi} phenotype.

Recipient DC and donor-derived IL-27 promote T_R1 cell development

GVHD is initiated by recipient APCs and is influenced by the intensity of conditioning, that is, total body irradiation (TBI) and chemotherapy dose intensity, in part through inflammatory cytokine dysregulation (*26*, *27*). Thus, we hypothesized that T_R1 cells may also be generated in an APC- and conditioning-dependent fashion. The frequency of T_R1 cells in donor $CD4^+$ T cells correlated with the frequency of residual

recipient conventional dendritic cells (DCs) (Fig. 7A) and reduced intensity of TBI that favors the persistence of recipient DC (fig. S9A). Blocking DC function by CD40L inhibition reduced T_R1 cells while favoring T_{reg} cell development (Fig. 7B). In line with this observation, depletion of both donor and recipient DC markedly reduced the development of T_R1 cells early after BMT (Fig. 7C). Whereas the proportions of T_{reg} cells were unaffected, absolute numbers were reduced, albeit much less markedly than T_R1 cells (Fig. 7C). In contrast, the depletion of donor DC or inactivation of donor APC function in isolation did not impair T_R1 cell development (Fig. 7D and fig. S9, B and C), indicating that recipient DCs are required for the development of T_R1 cells early after BMT.

Consistent with the notion that Eomes$^+$ T_R1 cells are dependent on IL-27 signaling and further confirming critical role of IL-27 in promoting T_R1 cell development, we found that the number of T_R1 cells significantly correlated with the number of IL-27$^+$ cells in the spleen (Fig. 7E). Because IL-27R and IL-6R share and compete for the same signaling component, gp130 (*28*), we hypothesized that blocking IL-6R may favor IL-27R function. As expected, IL-6R inhibition blocked signal transducer and activator of transcription 3 (STAT3)

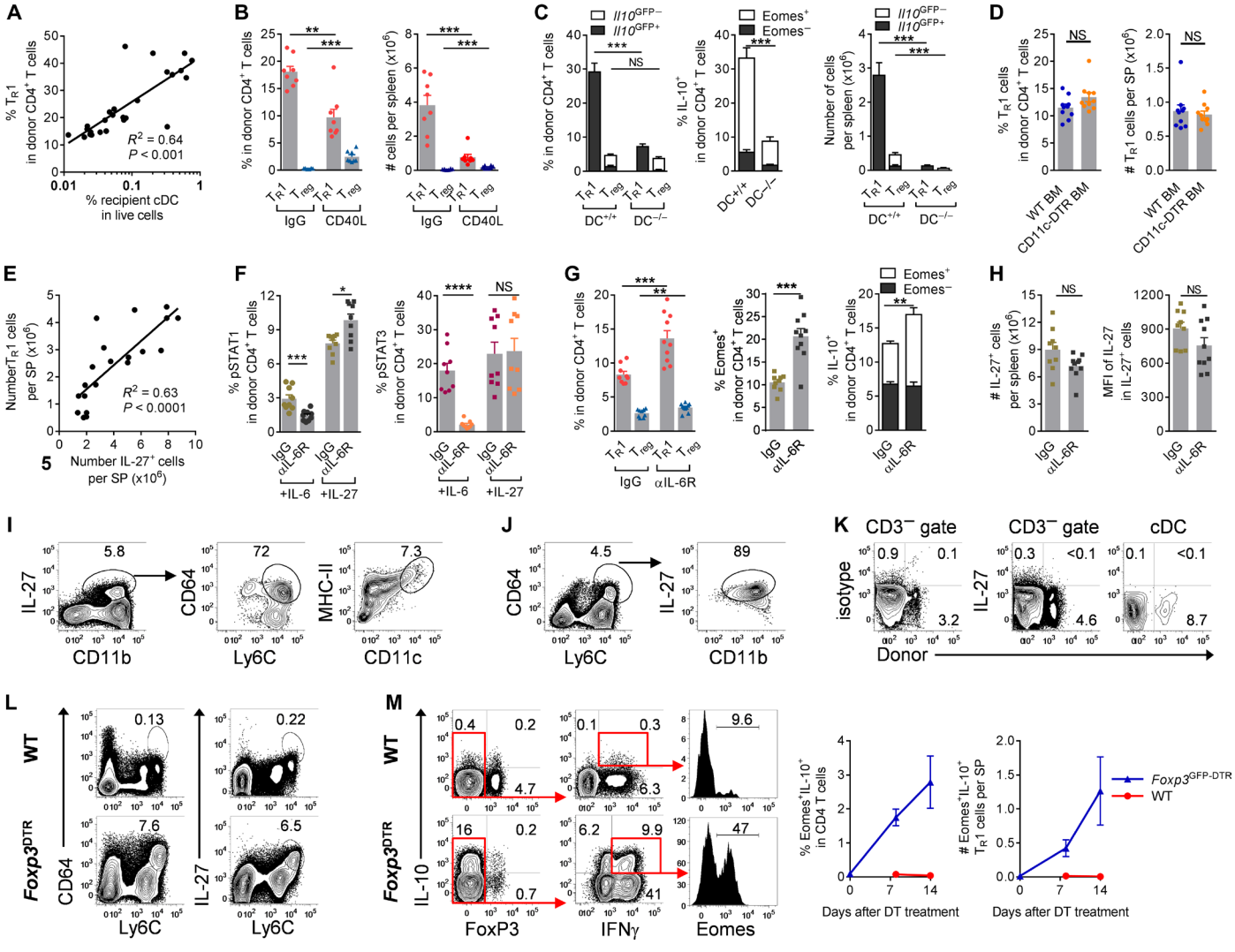

Fig. 7. Recipient DC and macrophage-derived IL-27 promote the development of T_R1 cells. (**A** to **K**) B6D2F1 mice were transplanted with TCD BM and CD4$^+$ T cells, and spleen was examined. (A) Correlation of T_R1 cells (*Il10*$^{GFP+}$*Foxp3*$^{RFP-}$) with proportions of recipient DC at day 14 (n = 26). (B) Frequencies of T_{reg} (*Foxp3*$^{GFP+}$) and T_R1 (IFNγ$^+$IL-10$^+$) cells at day 14 in the presence or absence of CD40L inhibition (n = 8 per group, grafts were CD4$^+$*Foxp3*$^{GFP-}$). (C) WT.B6D2F1 or CD11c-DOG×DBA/2 F1 recipients were treated with DT to deplete recipient conventional DC and received B6.WT or *MHC-II*$^{-/-}$ BM, respectively. Expression of T_R1 cells, T_{reg} cells, Eomes, and IL-10 at day 14 is shown (n = 10 and 7, respectively). (D) Recipients of WT or CD11c-DOG BM were treated with DT to deplete donor conventional DC, with expression of T_R1 and T_{reg} cells at day 10 shown (n = 10 per group). (E) Data from (A) and (B) demonstrate correlation between numbers of T_R1 cells and IL-27$^+$ cells per spleen at day 14 (n = 20). (F) Recipients were treated with IL-6R, and spleens were analyzed at day 5. Phosphorylation of STAT1 and STAT3 in response to IL-6 or IL-27 (n = 10 per group). (G and H) Recipients were treated with IL-6R, and spleens were analyzed at day 10. (G) Expression of *Foxp3*$^{RFP-}$*Il10*$^{GFP+}$ T_R1 cells, *Foxp3*$^{RFP+}$ T_{reg} cells, Eomes, and IL-10 in donor CD4$^+$ T cells and (H) numbers of IL-27$^+$ cells with intensity [mean fluorescence intensity (MFI)] of IL-27 (n = 9 to 10 per group). (I and J) Phenotypes of CD3$^-$ IL-27–secreting cells at day 14 are shown. (K) Expression of IL-27 from recipient DC at day +1 after BMT. (**L** and **M**) B6.WT or B6.*Foxp3*$^{GFP-DTR}$ mice were treated with DT for up to 2 weeks, and spleens were analyzed. (L) Phenotype of IL-27–secreting macrophage in CD3$^-$ splenocytes and (M) expression of Eomes$^+$IL-10$^+$ cells over time with representative plots at day 14. Data are means ± SEM.

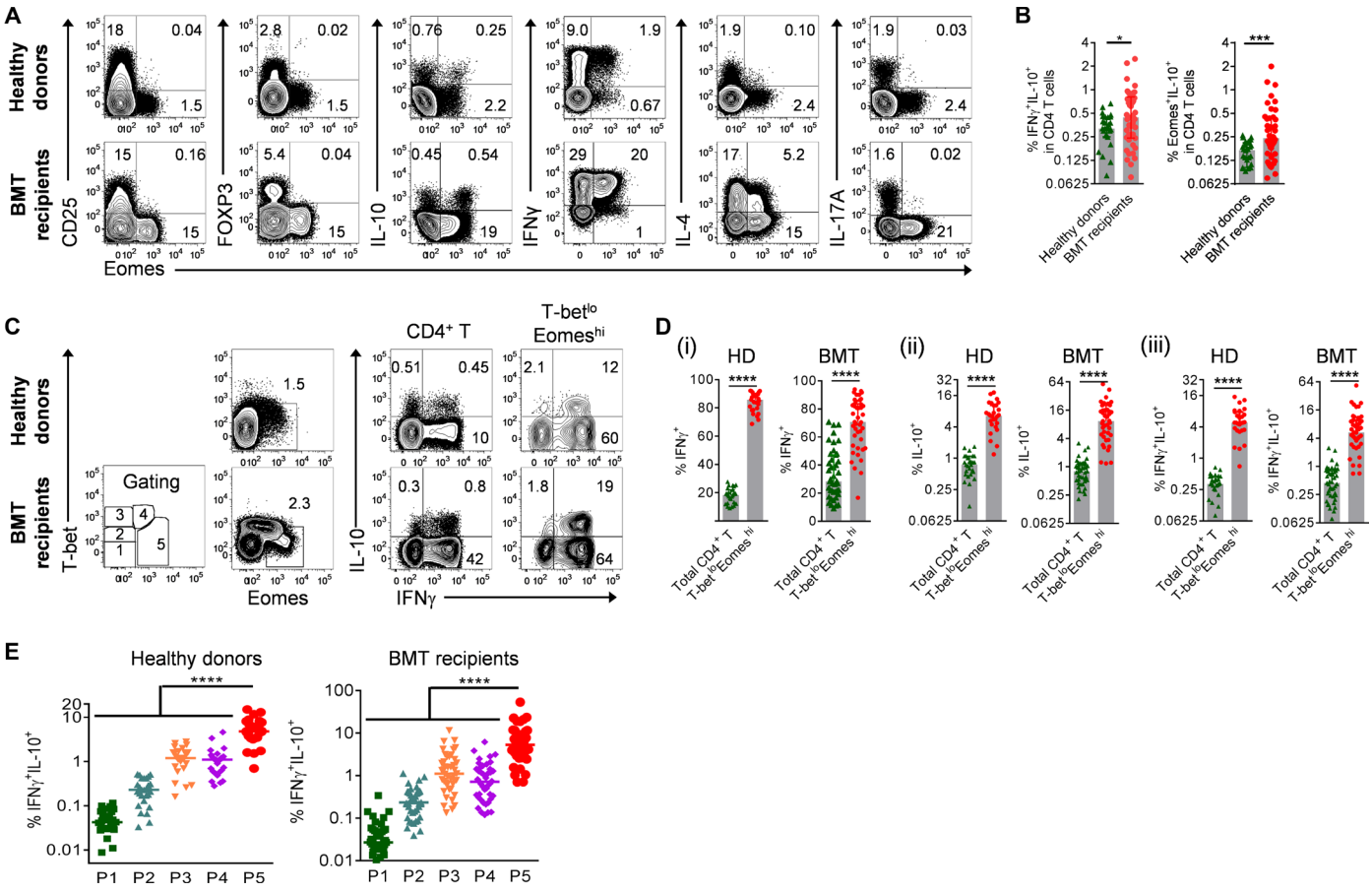

Fig. 8. Coexpression of T-bet and Eomes identifies a T_R1 cell–enriched population in human $CD4^+$ T cells. (**A**) Representative plots show the correlation of Eomes to CD25, FOXP3, and cytokines in $CD4^+$ T cells in healthy individuals and at day 60 after clinical allo-BMT. (**B**) Frequencies of T_R1 cells defined as $IFN\gamma^+IL\text{-}10^+$ or $Eomes^+IL\text{-}10^+$ in $CD4^+$ T cells in healthy donors ($n = 27$) or day 60 after clinical allo-BMT ($n = 43$). (**C** to **E**) Expression of cytokines in the T-$bet^{lo}Eomes^{hi}$ population relative to total $CD4^+$ T cells or subpopulations defined with differential expression of Eomes and T-bet in healthy individuals (HD; $n = 27$) and at day 60 after allo-BMT (BMT; $n = 43$). Data represent median ± interquartile range.

phosphorylation in response to IL-6 but not IL-27 (Fig. 7F). In contrast, IL-6R inhibition enhanced STAT1 phosphorylation in response to IL-27 early after BMT (Fig. 7F) and resulted in increased expression of T_R1 cells and a small increase in the frequencies of T_{reg} cells (Fig. 7G and fig. S9D). The enhanced STAT1 phosphorylation in response to IL-27 after IL-6R inhibition was not a result of an increase in the number of cells producing IL-27 itself or IL-27 production on a per-cell basis (Fig. 7H). We next sought to identify the cellular sources of IL-27 after BMT. Most IL-27 (70 to 80%) was produced by $Ly6C^{hi}$ donor macrophages ($CD11b^+$, MHC-II^+, $Ly6C^{hi}$, $F4/80^{hi}$, $CD64^+$, and $CCR2^+$), with a more limited contribution from donor DC ($CD11c^+$ and MCH-II^+) (Fig. 7I). More than 80% of all $Ly6C^{hi}$ donor macrophages were secreting IL-27 after BMT (Fig. 7J). Depletion of donor DC did not impair the overall frequencies or numbers of $IL\text{-}27^+$ cells (fig. S9B), consistent with the lack of contribution by donor DC to T_R1 cell development. Last, we demonstrated that recipient DC did not produce IL-27 early after BMT (Fig. 7K), suggesting that the requirement of recipient DC to T_R1 cell development relates to their capacity for alloantigen presentation and not IL-27 production. Thus, donor macrophages appear as the main producers of IL-27 and, in concert with the initial stimulation by recipient DC, drive Eomes-dependent T_R1 development after BMT.

To further understand the requirement of Eomes in T_R1 cell development, we investigated the expression of $Eomes^+$ T_R1 cells in other models of immunopathology. To this end, we used *Foxp3*$^{GFP\text{-}DTR}$ mice to temporarily deplete T_{reg} cells, thereby causing autoimmunity (*29*–*31*). Depletion of T_{reg} cells from adult mice resulted in a marked increase in IL-27–producing $Ly6C^{hi}$ macrophages (Fig. 7L) and, critically, induced large numbers of $Eomes^+$ T_R1 cells (Fig. 7M). Thus, our data show that different inflammatory conditions result in the development of $Eomes^+$ T_R1 cells. Furthermore, our results demonstrated that defects in T_{reg} cells are associated with compensatory increases in $Eomes^+$ T_R1 cells.

Identification of T_R1 cells in humans

To validate whether our findings from experimental BMT can be translated into humans, we analyzed the expression of Eomes, IL-10, and other markers in $CD4^+$ T lymphocytes collected from healthy donors and BMT recipients. $Eomes^+CD4^+$ cells from healthy individuals and BMT recipients were $CD25^{lo}$, $FOXP3^-$, $IFN\gamma^{hi}$, $IL\text{-}4^{lo}$, and $IL\text{-}17A^-$, and a proportion secreted IL-10 (Fig. 8A). Thus, human $Eomes^+IL\text{-}10^+$ cells show the characteristics of T_R1 cells. Notably, compared with currently used $IL\text{-}10^+IFN\gamma^+$ staining methods, the use of Eomes in defining $IL\text{-}10^+$ T_R1 cells ($Eomes^+IL\text{-}10^+$) provides better discrimination of T_R1 cells between healthy donors and BMT recipients (Fig. 8B). Furthermore, the

use of T-bet and Eomes expression defines populations with increasing proportions of IL-10$^+$IFNγ$^+$ T_R1 cells (Fig. 8, C to E), consistent with the requirement for these transcription factors at different stages of differentiation both in steady state and after clinical BMT. IL-10$^+$IFNγ$^+$ T_R1 cells were enriched (>10-fold) in the T-bet^{lo}Eomes^{hi} population, which exhibited an effector memory (CD45RA$^-$CCR7$^-$) phenotype (Fig. 8, C to E, and fig. S10). Thus, consistent with the findings in the mouse model, after clinical BMT, high Eomes and low T-bet expression in CD4$^+$ T cells can be used to identify a population that is enriched for T_R1 cells.

DISCUSSION

We demonstrate that Eomes acts together with Blimp-1 and specifically drives the development of T_R1 cells. On the basis of our data and published results (*8*, *32*), we propose a model for the differentiation of T_R1 cells after BMT, as illustrated in fig. S11. In this model, antigen presentation by recipient DC and macrophage-derived IL-27 provide the cellular and molecular cues for the development of T_R1 cells, inducing Blimp-1 expression, which initiates the transcription of *Il10*. Blimp-1 is also required for Eomes expression, and both factors act in concert, enabling stable IL-10 production and T_R1 cell differentiation. Concurrently, T-bet is required to suppress GATA3 and RORγt while driving IFNγ and Eomes expression, ultimately leading to a T-bet^{lo}Eomes^{hi} phenotype, which can reliably identify T_R1 cells after BMT as well as in steady state in mouse and man. There are some limitations to this study. Our preclinical studies used predominantly a single transplant model, although clinical data were congruent. In addition, although T_{reg} cell depletion results in T_R1 cell generation in vivo, it is not yet clear how important T_R1 cells are in other disease settings. Last, the relative in vivo suppressive activity of T_R1 cells versus T_{reg} cells remains to be explored.

There is still debate whether T_R1 cells constitute an independent lineage or simply represent IL-10–producing T_H1 cells. In particular, the lack of a master transcriptional factor for T_R1 cells has made progression of the field difficult (*5*, *13*, *33*). Multiple transcription factors, including Blimp-1, AhR, and c-Maf, are induced by IL-27 and have been shown to be critical for T_R1 cell differentiation (*5*–*8*, *10*); however, none of them appear to be specific to the T_R1 cell lineage. Eomes is a T-box transcription factor that is more often than not coupled with T-bet in the biology of CD8$^+$ T cells and natural killer cells (*34*, *35*). Its role in regulating functions of CD4$^+$ T cells (*36*, *37*) and suppressing T_{reg} and T_H17 cell differentiation have been described recently (*38*, *39*). Here, we demonstrate that IL-10$^+$IFNγ$^+$ T_R1 cells are uniquely dependent on Eomes. We found that Eomes bound to the *Il10* and *Ifnγ* promoters. Similarly, it has been shown that Eomes also binds to the promoter of *Gzmb* (*35*), the expression of which is another feature of T_R1 cells. Eomes overexpression was sufficient to promote IL-10 and GzmB and suppress other lineage-characteristic transcription factors (e.g., FoxP3, GATA-3, RORγt, and BCL-6) and cytokines (e.g., IL-2, IL-4, IL-13, GM-CSF, and IL-17A). Therefore, expression of Eomes and IL-10 within CD4$^+$ T cells defines the T_R1 cell lineage.

Increasing data have suggested a close relationship between T_R1 and T_H17 cells linked via AhR, c-Maf, and IL-21 (*10*, *23*, *24*, *40*). However, T_R1 and T_H17 cells require different cytokines for their respective differentiation: IL-27/IL-10 for the former and IL-6/TGF-β/IL-23 for the latter (*12*, *41*–*43*). Multiple groups have independently shown that IL-27 opposed the functions of IL-6/IL-23 in T_H17 cell differentiation (*8*, *28*, *44*). Our data demonstrate that inhibition of IL-6R signaling favors IL-27 function and subsequent development of Eomes$^+$ T_R1 cells. We further show that Eomes distinguishes T_R1 cells from other T_H cell lineages including T_H17 cells, and its overexpression represses polarization to T_H17 cells. This is in line with the notion that Eomes suppresses T_H17 cell differentiation by directly inactivating *Rorc* and *Il17a* promoters (*39*). A role for IL-27 in inhibiting T_{reg} cell reconstitution after BMT has also recently been reported (*45*), consistent with the counterbalanced T_R1 cell expansion seen here. There appears to be substantial interplay between IL-6 and IL-27 (*28*), an effect also seen during GVHD. IL-6 inhibition has an intriguing capacity to enhance IL-27 responses and thereby to promote T_R1 cell differentiation, an effect likely contributing to clinical efficacy (*46*).

Eomes can be regulated by T-bet in a Runx-3–dependent manner, and the differential expression of these two T-box transcription factors is critical for the differentiation of CD8$^+$ T cells (*47*, *48*). In line with this notion, we show that IFNγ signaling and T-bet expression were required for Eomes expression, demonstrating an important role of T-bet in the early phase of T_R1 cell development. Downstream of IL-27, Blimp-1 is critical for the expression of IL-10 in CD4$^+$ T cells in various models (*6*–*8*, *21*, *49*). Here, we show that Eomes$^+$ T_R1 cells were regulated by both Blimp-1 and T-bet, consistent with a recent report that demonstrated close collaboration between Blimp-1 and T-bet in cytotoxic T lymphocyte generation (*50*). In addition, binding of Blimp-1 to the *Eomes* promoter in CD8$^+$ T cells during viral infection has been described (*32*), suggesting that Blimp-1 not only regulates IL-10 expression directly but also contributes to the induction or maintenance of Eomes expression in T_R1 cells. Both Blimp-1 (*6*) and Eomes bind to the *Il10* locus, and the activity of both is required to promote efficient T_R1 cell differentiation and *Il10* expression. Similar to Eomes, Blimp-1 is required not only for IL-10 expression but also for GzmB (*51*). We also confirmed that IL-10 itself contributes to T_R1 cell differentiation, a T cell–extrinsic effect likely via myeloid cells (*22*, *25*). Overall, these data suggest that the functional interactions between Blimp-1, T-bet, and Eomes are important for the differentiation of CD4$^+$ T cells and T_R1 cell lineage in particular. Identification of the bona fide transcriptional and cellular control of T_R1 cell development should allow for therapeutic utilization of T_R1 cells in transplantation and other diseases, where excessive and aberrant immunity results in immunopathology.

MATERIALS AND METHODS

Study design

Female C57BL/6 (B6.WT, H-2b, CD45.2), B6.SJL-Ptprca (PTPrca, H-2b, CD45.1), and B6D2F1 (H-2b/d, CD45.2) mice were purchased from the Animal Resource Center (Perth, Western Australia, Australia). B6 *Il27r*$^{-/-}$ and *Tbx21*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). B6 *Blimp-1*^{GFP} (*52*), *Il10*^{GFP}×*Foxp3*^{RFP} (*19*, *20*), *Foxp3*^{GFP}, *Foxp3*$^{GFP\text{-}DTR}$, *Il10*$^{-/-}$, *Ifnγr*$^{-/-}$, MHC-II$^{-/-}$, *Il10r*$^{fl/fl}$×*Lck*-cre (*53*), *Il10*$^{fl/fl}$×*Lck*-cre (*54*), *Prdm1*$^{fl/fl}$×*Lck*-cre (*Blimp-1*$^{-/-}$) (*51*), CD11cDOG, and DBA2×B6.CD11cDOG mice were bred at the Queensland Institute of Medical Research (QIMR) Berghofer Medical Research Institute animal facility. B6 *Eomes*$^{fl/fl}$ mice were derived from the Eomes$^{floxed/mcherry}$ mice previously generated by GTB and described in (*55*). The Eomes$^{floxed/mcherry}$ mice were crossed to the B6.129S4-*Gt(ROSA)26Sor*$^{tm2(FLP*)Sor}$/J line, which induces FLP-mediated recombination to remove the mCherry/Amp cassette to generate the Eomes^{floxed} line. Removal of the Frt sites (and hence the IRES-Cherry cassette) was detected using primers (a) 5′-ggacttggggagccaaaa-3′ (forward) and (b) 5′-cacatctgtaaccgcagcat-3′ (reverse) (deleted allele, 306 bp). The primers (c) 5′-agtcggtttgagctggtgac-3′ (forward), (d)

5′-tttggaacagcctccaaatc-3′ (reverse) were used to detect the wild-type (339 bp) and floxed allele (421 bp) while primer (e) 5′-AAGGGGAAGG-GTGGTTAGAA-3′ (reverse) was used to detect the floxed allele (1941 bp) and germline deletion (587 bp). This Eomes^{floxed} line was subsequently crossed with *Cd4*-cre or *Lck*-cre mice to generate T cell–restricted *Eomes*$^{-/-}$ offspring. All recipient mice were used between 6 and 10 weeks of age, and age-matched female donor mice were used. Mice were housed in microisolator cages and received acidified autoclaved water (pH 2.5) after BMT. All animal studies were performed in accordance with the QIMR Berghofer Medical Research Institute Animal Ethics Committee. We chose sample sizes based on estimates from initial and previously published results to ensure appropriate power. As stated in the figure legends and wherever possible, *n* values were derived from individual mice from replicated experiments.

Bone marrow transplantation

BM (B6.CD45.1$^+$ or where indicated) was T cell–depleted, and splenocytes were processed to CD3$^+$ or CD4$^+$ T cells, as described previously (*56*). On day −1, recipient mice received 1100 centigray (cGy) (B6D2F1), 1000 cGy (B6), 900 cGy (CD11c-DOG×DBA/2 F1), or otherwise specified doses of TBI (^{137}Cs source at 108 cGy/min), split into two doses separated by 3 hours. On day 0, recipients were transplanted with 5×10^6 to 10×10^6 BM cells with or without 1×10^6 to 2×10^6 T cells (CD3$^+$ or CD4$^+$). Intraperitoneal injections of rat anti-mouse IFNγ (XMG1.2, produced in-house, 1 mg per dose, three times per week), hamster anti-mouse CD40L (MR1, BioXcell; 500 μg per dose, days 0, +2, +4, and +6), rat anti-mouse IL-6R (MR16-1, Chugai Pharmaceutical Co., Japan; 500 μg per dose, days −1, +3, and +7), and control monoclonal antibody (mAb) were administered to recipients. In some experiments, CD11c-DOG mice [in which diphtheria toxin (DT) receptor is driven off the CD11c promoter] were used as BM donors. Recipients were given intraperitoneal injections of DT (160 ng per dose, three times per week) after BMT to deplete donor DC. For depletion of recipient DC, B6.CD11c-DOG×DBA/2 F1 mice were used as recipients and treated with DT on days −3, −1, 0, +1, +3, +5, and +7.

T_{reg} cell depletion

For depletion of T_{reg} cells, age-matched recipients (B6.WT or B6.*Foxp3*$^{GFP\text{-}DTR}$) were given intraperitoneal injections of DT (160 ng per dose, three times per week) for up to 2 weeks.

Histology

GVHD target tissues (skin, liver, and small intestine) were taken, preserved in 10% formalin, embedded in paraffin, processed to 5-mm-thick sections, and stained with hematoxylin and eosin. The sections were examined in a blinded fashion using a semiquantitative scoring system, and images were acquired as previously described (*56*, *57*).

Flow cytometry

Single-cell suspensions were processed and stained, cells were analyzed on an LSRFortessa cytometer (Becton Dickinson), and data were processed using FlowJo version 9.0 (Tree Star). Cell sorting was performed using FACSAria or MoFlo.

Clinical analysis

Peripheral blood was collected from healthy donors ($n = 27$) or patients (day 60 after BMT) of an observational study ($n = 18$) and a phase 3 clinical trial (ACTRN12614000266662) ($n = 25$). All studies were approved by the institutional ethics committee, and all patients signed informed consent. Peripheral blood mononuclear cells were purified from whole blood using Ficoll-Paque centrifugation and stained immediately.

Gene expression analysis

Total RNA was extracted with the RNeasy Micro Kit (Qiagen), and gene expression was determined using TaqMan GE assays (Applied Biosystems). All measurements were run in parallel with the housekeeping gene *Hprt*. All primer/probe mixtures were purchased from Applied Biosystems.

Statistics

Results from mouse experiments are presented as means ± SEM, and the Mann-Whitney *U* test was used for comparisons. Results from clinical samples are presented as median ± interquartile range, and Mann-Whitney *U* test was used for comparisons. Survival is estimated and plotted using Kaplan-Meier methods, and the difference between subgroups is estimated using log-rank methods. Ordinary least-squares method is used in the linear or semi-log regression analysis. A two-sided *P* value of 0.05 is considered statistically significant. Statistical analyses are performed using Prism version 6 software (GraphPad) (NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.)

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. T_R1 cells are suppressive in vitro.
Fig. S2. T_R1 cells display a distinct profile of markers.
Fig. S3. Eomes is required for the development of T_R1 cells after BMT.
Fig. S4. Expression of Eomes during in vitro culture.
Fig. S5. Role of Eomes in the generation of T_R1 cells in vitro.
Fig. S6. Eomes$^+$ T_R1 cells are dependent on Blimp-1, IL-27, and IL-10.
Fig. S7. T_{reg} cell development in *Il27r*$^{-/-}$ and *Il10*$^{-/-}$ T cells after BMT.
Fig. S8. Both T-bet and Eomes are required for T_R1 cell generation.
Fig. S9. Recipient DC and donor IL-27 promote T_R1 cell development after experimental BMT.
Fig. S10. Eomes and T-bet can be used to identify T_R1 cells after clinical BMT.
Fig. S11. Proposed cellular and transcriptional regulation of T_R1 cell development after BMT.
Table S1. Primer sets used for ChIP assays.
Table S2. Tabulated data for figures.
Reference (*58*)

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