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Eomesodermin Promotes the Development of Type-1 Regulatory T (T_R1) Cells

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Abstract

Type-1 regulatory T (T_R1) cells are Foxp3-negative IL-10-producing $CD4^+$ T cells with potent immune suppressive properties but their requirements for lineage development have remained elusive. Here we show that T_R1 cells constitute the most abundant regulatory population after

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allogeneic bone marrow transplantation (BMT), express the transcription factor Eomesodermin (Eomes) and are critical for the prevention of graft-versus-host disease (GVHD). 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 Th lineages. We further show that Eomes induction in T_R1 cells requires T-bet and donor macrophage-derived IL-27. We thus define the cellular and transcriptional control of T_R1 cell differentiation during bone marrow transplantation, opening new avenues to therapeutic manipulation.

Introduction

Type-1 regulatory T (T_R1) cells are a FoxP3 negative, IL-10 producing T cell population, which have potent immune suppressive 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 TGFβ and granzyme B-dependent killing of antigen presenting cells (APC) have also been described (reviewed in (3)). In addition to IL-10, T_R1 cells show high expression of TGF-β, secrete intermdiate amounts of IFNγ but no 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 remains 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). Due to 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). We thus developed a mouse model using a dual *Il10*^{GFP} / *Foxp3*^{RFP} reporter mouse strain (19, 20) to delineate regulatory T cell responses after experimental BMT. Using GVHD as a disease model, we show that T_R1 cells are the most abundant IL-10 producing regulatory T cell population after experimental BMT. Further analyses demonstrate that T_R1 cells that develop during GVHD express high amounts of Eomes, which is required for their development and its over-expression 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. Finally, 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 regulatory T cell population in GVHD

We used *Il10*^{GFP} and *Foxp3*^{RFP} dual reporter mice as BMT donors to define CD4$^+$ FoxP3^{neg} IL-10$^+$ type-1 regulatory T (T_R1) cells, CD4$^+$ FoxP3$^+$ regulatory T (T_{reg}) cells and CD4$^+$ FoxP3^{neg} IL-10^{neg} conventional T (T_{con}) cells (Fig. 1*A*). T cells were the major IL-10 producers after both allogeneic and syngeneic BMT (Fig. 1*B*), with the highest proportion and intensity of IL-10 produced by T_R1 cells (Fig. 1*C*). Importantly, 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 lesser extent small intestine), mesenteric lymph nodes (Fig. 1*D*) and spleen (Fig. 1*D – F*). T_R1 cells induced under these conditions had suppressive properties *in vitro* equivalent to post-transplant T_{reg} cells on a per cell basis (Figs. 1*G*, S1). To confirm their suppressive function *in vivo*, we induced GVHD with WT or *Il10*$^{-/-}$ CD4$^+$CD25^{neg} 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 *d7* after BMT (Fig. 1*H*) when acute GVHD was established, prolonged survival significantly (Fig. 1*I*), consistent with potent regulatory function. Thus, T_R1 cells represent the major regulatory T 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 co-expression 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. S2*A*). We therefore used *Foxp3*^{RFP}^{neg} 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. S2*B*). Consistent with the T_R1 cell phenotype (3, 5, 9), *Foxp3*^{RFPneg}*Il10*$^{GFP+}$ T_R1 cells expressed high amounts of IL-10 and IFNγ but little T_H2 cytokines such as IL-4, IL-13 and IL-5, or T_H17 cytokines such as IL-17, IL-6 or GM-CSF (Figs. 2*A*, S2*C*).

T_R1 cells have often been considered a terminally differentiated T_H1 cell subset programmed to limit aberrant inflammation (5, 13, 22). Indeed, T_R1 cells expressed high amounts of T-bet, the T_H1 determining transcription factor, but low amounts of GATA-3, BCL-6 and ROR-γt. Strikingly, when we analysed the expression of other transcription factors related to T cell differentiation, we observed high Eomes expression, which was largely restricted to T_R1 cells (Figs. 2*B*, S2*D*). Eomes expression tightly correlated with high expression of IL-10, IFNγ and granzyme B (GzmB) (Fig. 2*C*). In contrast, Eomes$^+$ T_R1 cells expressed low levels of IL-2, IL-17A and GM-CSF (Fig. 2*C*). 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*$^{fl/fl}$x*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 (Figs. 3*A*, S3*A,B*). Critically, the loss of Eomes did not impair the

development of IL-10^{neg}IFNγ^{+} or T-bet^{+} $T_{con,}$ IFNγ^{+}TNF^{+} T_H1, IL-17A^{+} T_H17 cells or IL-10 expression by T_{reg} cells but instead favoured the expression of IL-4 and FoxP3 (Figs. 3*A*, S3*B–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. 3*B*). In addition, over-expression of Eomes promoted the expression of Gzmb whilst suppressing FoxP3, IL-4 and IL-17A expression (Fig. S3*G*). Furthermore, over-expression of Eomes upregulated 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 related factors *Ahr* and *Il21* (10, 23, 24)(Fig. 3*C*).

Notably, 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, iT_{reg} cells (Fig. S4*A*), 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 than other T cell lineages in these cultures (Fig. S4*B*). Consistent with this observation, we did not observe a defect in T_R1 differentiation in the absence of Eomes in these conditions (Fig. S5*A*). However, transduction of Eomes into CD4^{+} T cells and subsequent re-stimulation in culture dramatically promoted the differentiation of IL-10^{+}IFNγ^{+} T_R1 cells and the expression of granzyme B, while suppressing the expression of IL-4 and FoxP3 (Fig. S5*B*). Over-expression of Eomes also suppressed mRNA expression of transcription factors defining other T_H lineages, including *Tbx21, Gata3, Rorc* and *Bcl6* and other T_R1/T_H17 related factors, like *Ahr, Maf* and *Il21*(Fig. S5*C*). Collectively, we show that Eomes is required for T_R1 differentiation and 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 2kb upstream of the transcription start site (TSS) of the *Il10* gene (Fig. 3*D*). The binding of Eomes to the *Il10* promoter was similar to that observed in the *Ifnγ* promoter, suggesting that Eomes regulates expression of both *Il10* and *Ifnγ* directly. Consistent with this concept, the recruitment of RNA polymerase II to the *Il10* promoter, an indicator of transcriptional activity, was reduced in Eomes-deficient CD4^{+} T cells (Fig. 3*D*).

Eomes^{+} T_R1 cells are dependent on Blimp-1, IL-27 and IL-10

Blimp-1 is a well-defined transcriptional promoter of IL-10 in CD4^{+} conventional T 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. S6*A*). 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_R1 cells (Fig. 4*A*) while the expression of T-bet was not impaired (Fig. S6*B*). 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 allogenic BMT recipients. Consistent with a critical role of Eomes in the differentiation of T_R1 cells, over-expression 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 (Figs. 4*B*, S6*C,D*). Furthermore, Eomes transduction enhanced the recruitment of Eomes to the *Il10* promoter regions both in WT and *Blimp1*$^{-/-}$ CD4 T cells (Fig. 4*C*).

To test the role of IL-27 in the induction of Eomes^{+} T_R1 cells after BMT, we transplanted *Il27r*$^{-/-}$ CD4^{+} T cells. Consistent with an important role for IL-27 in T_R1 induction, we found substantially decreased expression of Eomes in *Il27r*$^{-/-}$ CD4^{+} T cells, and T_R1 cells were reduced by >80% (Figs. 4*D*, S6*E*). In contrast, T-bet expression was increased in the absence of IL-27 signalling (Fig. 4*D*), and the development of CD4^{+}IL-10^{neg}IFNγ^{+} conventional T_H1 cells or IL-10 production capabilities of T_{reg} cells were not impaired (Fig. S6*E*).

We next tested whether the differentiation of Eomes^{+} T_R1 cells was dependent on IL-10 itself. The expression of Eomes, T_R1 cells as well as T-bet was not reduced in *Il10r*–deficient CD4^{+} T cells (*Il10r*$^{fl/fl}$ × *Lck*-cre) after BMT (Figs. 4*E*, S6*F*), indicating that IL-10 signalling in T cells was not required for T_R1 cell differentiation. However, when we transplanted *Il10*$^{-/-}$ CD4^{+}CD25^{neg} T cells, Eomes^{+} cells were reduced (Fig. 4*F*), in line with the notion that IL-10 promotes T_R1 cell differentiation indirectly (22, 25). In summary, Eomes expression in T_R1 cells is downstream of IL-27 and Blimp-1 but does not depend on T cell intrinsic IL-10 signalling.

Eomes^{+} T_R1 cells are critical for the prevention of GVHD

We next examined whether *Blimp-1*-deficient and *Il27r*–deficient CD4^{+} T cells would exacerbate GVHD due to impaired expression of Eomes and T_R1 cells. Whilst Blimp1 deletion exacerbated GVHD (Fig. 5*A*), IL-27R deletion did not (Fig. 5*B*). Of note, T_{reg} cells were increased and their IL-10 production intact in recipients of *Il27r*$^{-/-}$ CD4^{+} T cells (Fig. S6*E*, S7*A*), consistent with compensatory regulatory pathways in the absence of T_R1. In contrast, *Il10*$^{-/-}$ CD4^{+} T cells sustain comparable expression of Eomes in conventional T cells and T_{reg} cells (Figs. 4*F*, S7*B*) after BMT and thus reflect a more relevant model to define the regulatory function of T_R1 cells *in vivo*. Consistent with the reduced frequency of T_R1 cells, we observed enhanced GVHD in the skin and liver in recipients of *Il10*$^{-/-}$ CD4^{+}CD25^{neg} T cells (Fig. 5*C*). These findings were confirmed by transplanting *Il10*$^{fl/fl}$ × *Lck*-cre CD4^{+}CD25^{neg} T cells, which also led to exacerbated GVHD in the absence of IL-10 producing T_R1 cells (Fig. 5*D*). Lastly, *Eomes*$^{-/-}$ CD4^{+}CD25^{neg} T cells also resulted in increased GVHD, further confirming the important regulatory role of Eomes^{+} T_R1 cells after BMT (Fig. 5*E*).

Eomes and T-bet cooperate to generate T_R1 cells

As we had observed co-expression of T-bet (encoded by *Tbx21*) and Eomes in T_R1 cells after BMT, we wished to test the role of IFNγ signalling and T-bet in T_R1 cell development. Transplanting *Ifngr*$^{-/-}$ donor T cells or neutralizing IFNγ resulted in reduced expression of

T-bet and Eomes (Fig. 6*A*) with reduced expression of Eomes$^+$ T_R1 cells and expanded T_{reg} cell populations (Fig. 6*B, C*). When we transplanted *Tbx21*$^{-/-}$ CD4$^+$ T cells during BMT, we found that Eomes$^+$ T_R1 cells were dramatically reduced (Figs. 6*D*, S8*A*). Although overall frequencies of IL-10$^+$CD4$^+$ T cells were unaffected, the absolute numbers were reduced (Fig. 6*D*). Importantly, however, the majority 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_H2 or T_H17 cells, respectively (Figs. 6*D*, S8*B*). Gene expression analysis confirmed polarization of donor CD4$^+$ T cells to T_H2 (*Gata3, Il4, Il13*) and T_H17 (*Rorc, Ahr, Il21*) lineages in the absence of T-bet. The transcription of *Il10* (from Th2 cells) was also increased (Fig. S8*C*). Notably, the residual Eomes$^+$ population in *Tbx21*$^{-/-}$ CD4$^+$IL-10$^+$ cells expressed IFNγ but did not express IL-4 (Fig. S8*D*). Thus, T-bet and IFNγ promote Eomes expression within the T_R1 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_R1 cells, we retrovirally transduced *Tbx21*$^{-/-}$ CD4$^+$ T cells with Eomes. The over-expression of Eomes fully rescued the expression of IL-10, IFNγ, and IL-10$^+$IFNγ$^+$ T_R1 cells and correspondingly suppressed the expression of GATA-3$^+$IL-4$^+$ T_H2 and IL-17A$^+$ T_H17 cells (Figs. 6*E*, S8*E,F*).

We next investigated whether there is a temporal and/or spatial collaboration between T-bet and Eomes during T_R1 cell development. Firstly, Eomes expression in T_R1 cells was profoundly time-dependent after BMT (Fig. S8*G*), and CD4$^+$ T cells transited from a T-bet^{hi}Eomes^{lo} to a T-bet^{lo}Eomes^{hi} state over time (Fig. 6*F*), correlating with the increasing frequency of T_R1 cells (Fig. 1*E*). Furthermore, after repeated exposure to high levels of alloantigen *in vivo*, the majority of donor CD4$^+$ T cells had acquired Eomes (>95%) and converted to T_R1 cells (>70%) within four weeks of transfer into secondary BMT recipients (Fig. S8*H*). Consistently, over-expression of Eomes suppressed the expression of T-bet while promoting T_R1 cell differentiation (Figs. 3*B*, S8*I*). T_R1 cells (*Foxp3*^{RFPneg}*Il10*$^{GFP+}$), found in low frequencies in naïve mice, also exhibited higher Eomes expression. This was specific to T_R1 cells as IL-10 producing T_{reg} cells (*Foxp3*$^{RFP+}$*Il10*$^{GFP+}$) expressed some T-bet but not Eomes (Fig. 6*G*). 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 antigen presenting cells (APC) and is influenced by the intensity of conditioning, i.e. total body radiation (TBI) and chemotherapy dose-intensity, in part through inflammatory cytokine dysregulation (26, 27). We thus 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 indeed correlated with the frequency of residual recipient conventional dendritic cells (DC) (Fig. 7*A*) and reduced intensity of TBI that favour the persistence of recipient DC (Fig. S9*A*). Blocking DC function by CD40L inhibition reduced T_R1 cells whilst favouring T_{reg} cell development (Fig. 7*B*). In line with this observation, depletion of both donor and recipient DC dramatically reduced the development of T_R1 cells early after BMT (Fig. 7*C*). While the proportions of T_{reg} cells were unaffected, absolute

numbers were reduced, albeit much less dramatically than T_R1 cells (Fig. 7*C*). In contrast, the depletion of donor DC or inactivation of donor APC function in isolation did not impair T_R1 cell development (Figs. 7*D*, S9*B,C*), indicating that recipient DC 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 signalling 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. 7*E*). As IL-27R and IL-6R share and compete for the same signalling component, gp130 (28), we hypothesized that blocking IL-6R may favour IL-27R function. As expected IL-6R inhibition blocked STAT3 phosphorylation in response to IL-6 but not IL-27 (Fig. 7*F*). In contrast, IL-6R inhibition enhanced STAT1 phosphorylation in response to IL-27 early after BMT (Fig. 7*F*) and resulted in increased expression of T_R1 cells and a small increase in the frequencies of T_{reg} cells (Fig. 7*G*, S9*D*). The enhanced STAT1 phosphorylation in response to IL-27 following 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. 7*H*). We next sought to identify the cellular sources of IL-27 after BMT. The majority of IL-27 (70–80%) 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$^+$, MCH-II$^+$) (Fig. 7*I*). More than 80% of all Ly6C^{hi} donor macrophages were secreting IL-27 after BMT (Fig. 7*J*). Depletion of donor DC did not impair the overall frequencies or numbers of IL-27$^+$ cells (Fig. S9*B*), consistent with the lack of contribution by donor DC to T_R1 cell development. Lastly we demonstrated that recipient DC did not produce IL-27 early after BMT (Fig. 7*K*) 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 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 immune pathology. To this end we used *Foxp3*$^{GFP\text{-}DTR}$ mice to temporarily deplete T_{reg} cells, thereby causing autoimmunity (29–31). Indeed, depletion of T_{reg} cells from adult mice resulted in a dramatic increase in IL-27 producing Ly6C^{hi} macrophages (Fig. 7*L*) and critically, induced large numbers of Eomes$^+$ T_R1 cells (Fig. 7*M*). 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 as well as BMT recipients were CD25^{lo}, FOXP3^{neg}, IFNγ^{hi}, IL-4^{lo} and IL-17A^{neg} and a proportion secreted IL-10 (Fig. 8*A*). Thus, human Eomes$^+$IL-10$^+$ cells show the characteristics of T_R1 cells. Of note, compared to currently utilized IL-10$^+$IFNγ^+

staining methods, the use of Eomes in defining IL-10 positive T_R1 cells (Eomes$^+$IL-10$^+$) provides better discrimination of T_R1 cells between healthy donors and BMT recipients (Fig. 8*B*). Furthermore, the use of T-bet and Eomes expression defines populations with increasing proportions of IL-10$^+$IFNγ^+ T_R1 cells (Fig. 8*C* – *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^{neg}CCR7^{neg}) phenotype (Figs. 8*C* – *E*, 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. Based on our data and published results (8, 32), we propose a model for the differentiation of T_R1 cells after BMT as illustrated in Figure S11. In this model, antigen presentation by recipient DC and macrophages-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 whilst 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 utilized predominantly a single transplant model, although clinical data were congruent. In addition, while T_{reg} depletion results in T_R1 generation in vivo, it is not yet clear how important T_R1 cells are in other disease settings. Finally, the relative in vivo suppressive activity of T_R1 versus T_{reg} remain to be fully 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 lineage. Eomes is a T-box transcription factor which is more often than not coupled with T-bet in the biology of CD8$^+$ T cells and NK cells (34, 35). Its role in regulating functions of CD4$^+$ T cells (36, 37) and suppressing T_{reg} and T_H17 cells 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 *Ifn*γ promoters. Similarly, it has been shown that Eomes also binds to the promoter of *Gzmb* (35), expression of which is another feature of T_R1 cells. Eomes over-expression 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 has 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 later (12, 41–43). Multiple groups have independently shown IL-27 opposed the functions of IL-6/IL-23 in T_H17 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 lineages including T_H17 cells and its over-expression 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} reconstitution after BMT has also recently been reported (45), consistent with the counter-balanced T_R1 expansion seen here. There appears to be significant 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 signalling 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 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 CTL 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. Notably, both Blimp-1 (6) and Eomes bind to the *Il10* locus, and the activity of both is required to promote efficient T_R1 differentiation and *Il10* expression. Interestingly, similar to Eomes, Blimp-1 is not only required for IL-10 expression but also for granzyme B (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 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 immune pathology.

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, WA, Australia). B6 *Il27r*$^{-/-}$ and *Tbx21*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). B6 *Blimp-1*^{GFP}(52), *Il10*^{GFP} × *Foxp3*^{RFP}(19, 20), *Foxp3*^{GFP},

Foxp3$^{GFP-DTR}$, *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 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'-AAGGGGAAGGGTGGTTAGAA-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 week 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 in order to ensure appropriate power. As stated in 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 processed to CD3$^+$ or CD4$^+$ T cells as described previously (56). On day −1, recipient mice received 1100 cGy (B6D2F1), 1000 cGy (B6), 900 cGy (CD11c-DOG × DBA/2 F1) or otherwise specified doses of total body irradiation ([137Cs] source at 108 cGy/min), split into two doses separated by 3 h. On day 0, recipients were transplanted with 5–10×10^6 BM cells with or without 1–2×10^6 T cells (CD3$^+$ or CD4$^+$). Intraperitoneal injections of rat-anti-mouse IFNγ (XMG1.2, produced in house, 1mg/dose, 3 times per week), hamster-anti-mouse CD40L (MR1, BioXcell, 500ug/dose, *d*0, +2, +4, +6), rat-anti-mouse IL-6R (MR16-1, Chugai Pharmaceutical Co, Japan, 500ug/dose, *d*-1, +3, +7) and control 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 (160ng/dose, 3 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 *d*-3, −1, 0, +1, +3, +5, +7.

T_{reg} depletion

For depletion of T_{reg}, age-matched recipients (B6.WT or B6.*Foxp3*$^{GFP-DTR}$) were given intraperitoneal injections of DT (160ng/dose, 3 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 H&E-stained. The sections

were examined in a blinded fashion using a semi-quantitative scoring system and images acquired as previously described (56, 57).

Flow Cytometry

Single cell suspensions were processed and stained, cells were analyzed on a LSR Fortessa cytometer (Beckman Dickinson) and data were processed using FlowJo Version 9.0 (TreeStar). Cell sorting was performed using a FACSAria or Moflo.

Clinical analysis

Peripheral blood was collected from healthy donors ($n = 27$) or patients ($d60$ after BMT) of an observational study ($n = 18$) and a phase III clinical trial (ACTRN12614000266662) ($n = 25$). All studies were approved by the institutional ethics committee and all subjects signed informed consent. Peripheral blood mononuclear cells (PBMC) 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, Netherlands) and gene expression determined using TaqMan GE assays (Applied Biosystems, MA, USA). All measurements were run in parallel with the housekeeping gene *Hprt*. All primers/probe mixtures were purchased from Applied Biosystems.

Statistics

Results from mouse experiments are presented as mean ± SEM and the Mann–Whitney U test used for comparisons. Results from clinical samples are presented as median ± interquartile range and Mann–Whitney U test 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 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 Material

Refer to Web version on PubMed Central for supplementary material.

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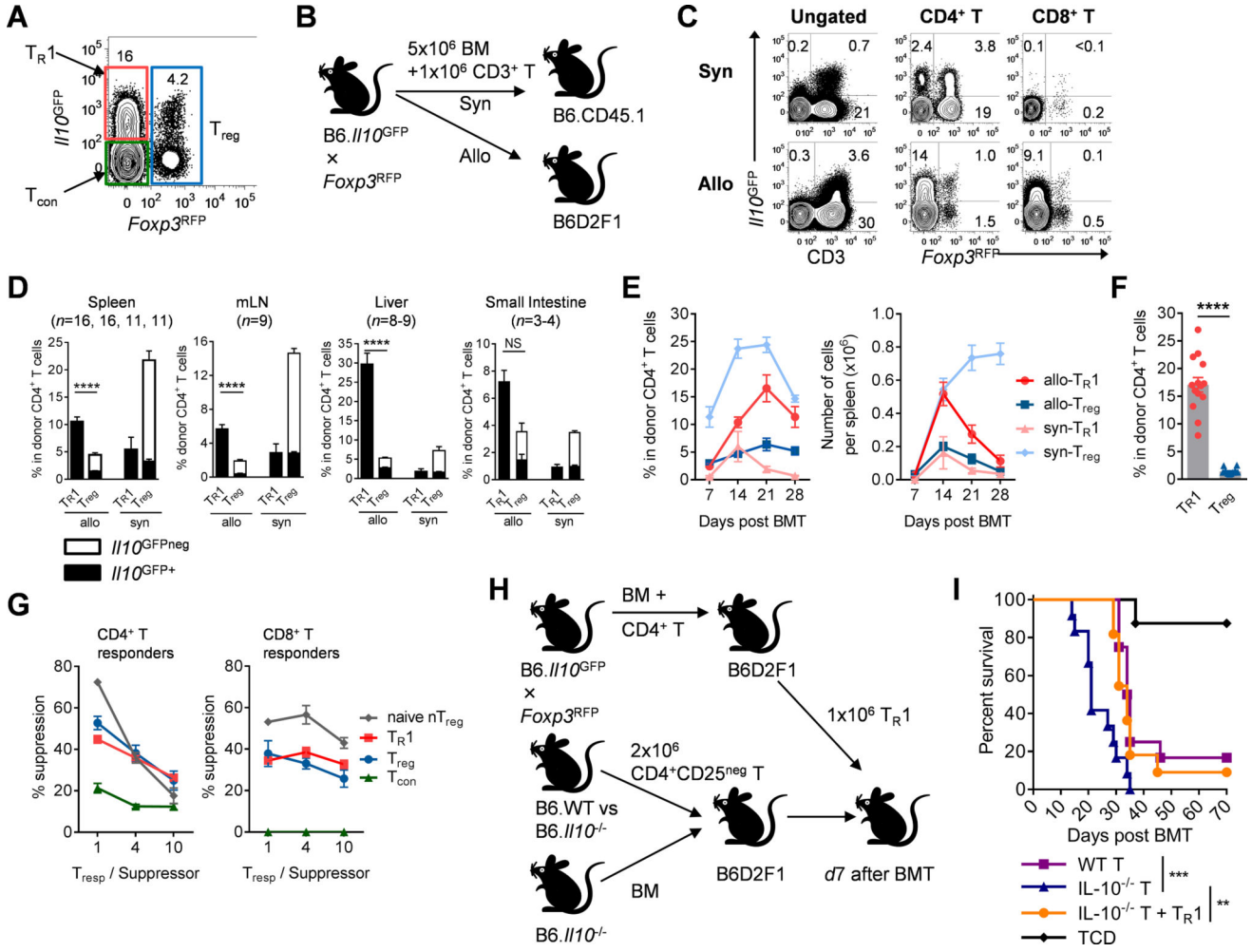

Figure 1. T_R1 cells constitute the major regulatory T cell after allogeneic BMT

(*A – E*) B6 (Syn) and B6D2F1 (Allo) mice were transplanted with B6 CD3$^+$ T (*Il10*^{GFP}/*Foxp3*^{RFP}). (*A*) Gating strategy after BMT for analysis and FACS sorting 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 *d*14 (representative of >3 experiments). (*D*) Frequencies of T_R1 and T_{reg} cells at *d* 14 (*Il10*$^{GFP+}$: solid bar; *Il10*^{GFPneg}: open bar). (*E*) CD4$^+$ T cell subsets in spleen after BMT (n = 8 – 9 per group each time point). (*F*) B6D2F1 mice were transplanted with B6 CD4$^+$ T (*Il10*^{GFP} and *Foxp3*^{RFP}) and frequencies of T_R1 and T_{reg} cells in the spleen at *d*14 (n = 14). (*G*) Suppression of proliferation of CFSE labelled B6 CD4$^+$ and CD8$^+$ responder T cells *in vitro* by naïve T_{reg} cells versus T_R1, T_{reg} and T_{con} "suppressors" sorted from 10 transplant recipients at *d*14 (data combined from 2 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 TCD group, others n = 11 – 12). Data represents mean ± SEM.

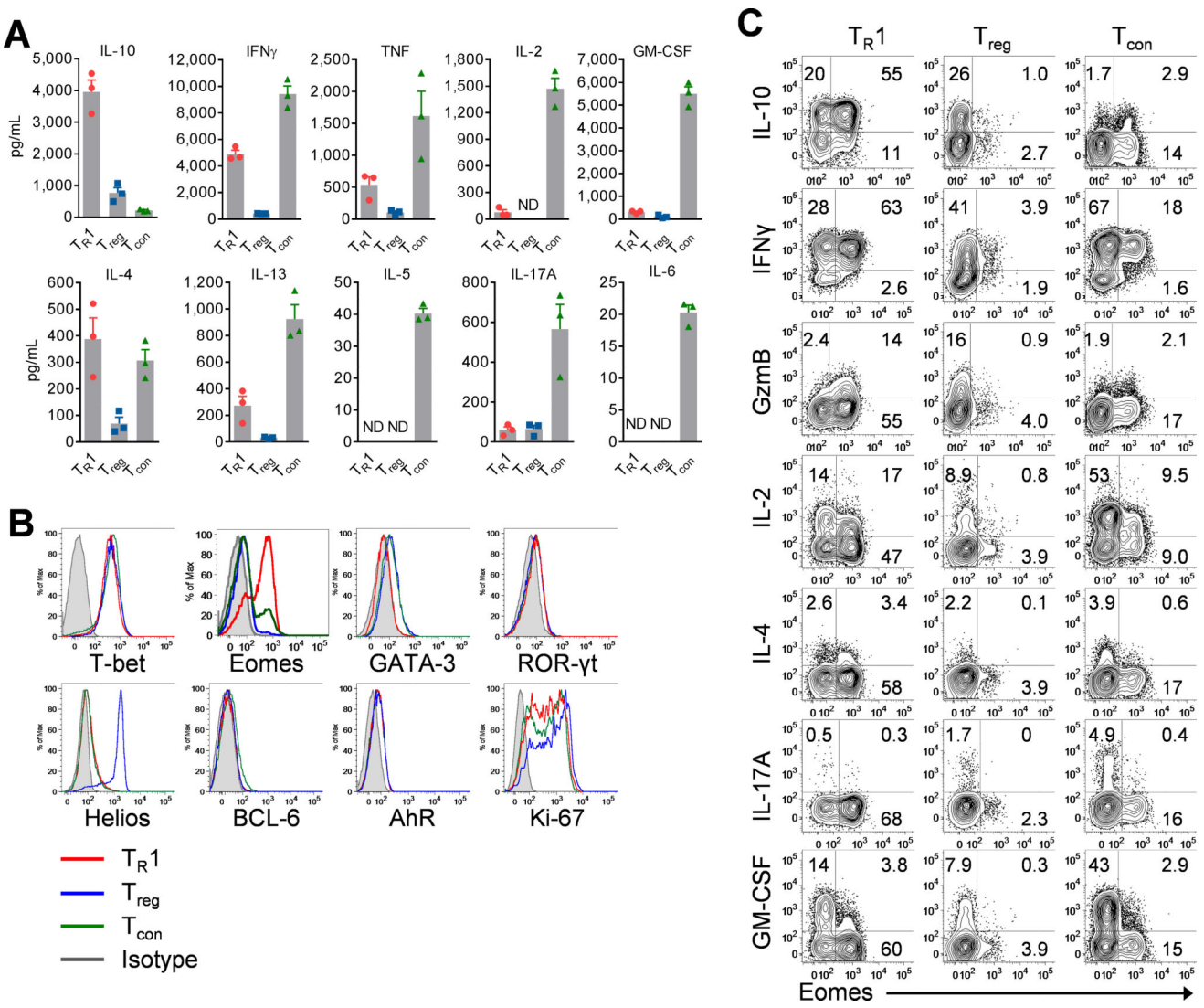

Figure 2. T_R1 cells express Eomes and display a distinct phenotypic profile

(*A* – *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 *d*14 as in Fig 1*A*. (Data from 3 experiments, ND = not detectable) (*A*) Cytokine production in culture supernatant of T cell subsets. (*B*) Expression of transcription factors in T cell subsets (T_R1: red, T_{reg}: blue, T_{con}: green, isotype: gray). (*C*) Expression of cytokines and Eomes in T cell subsets. Data represents mean ± SEM.

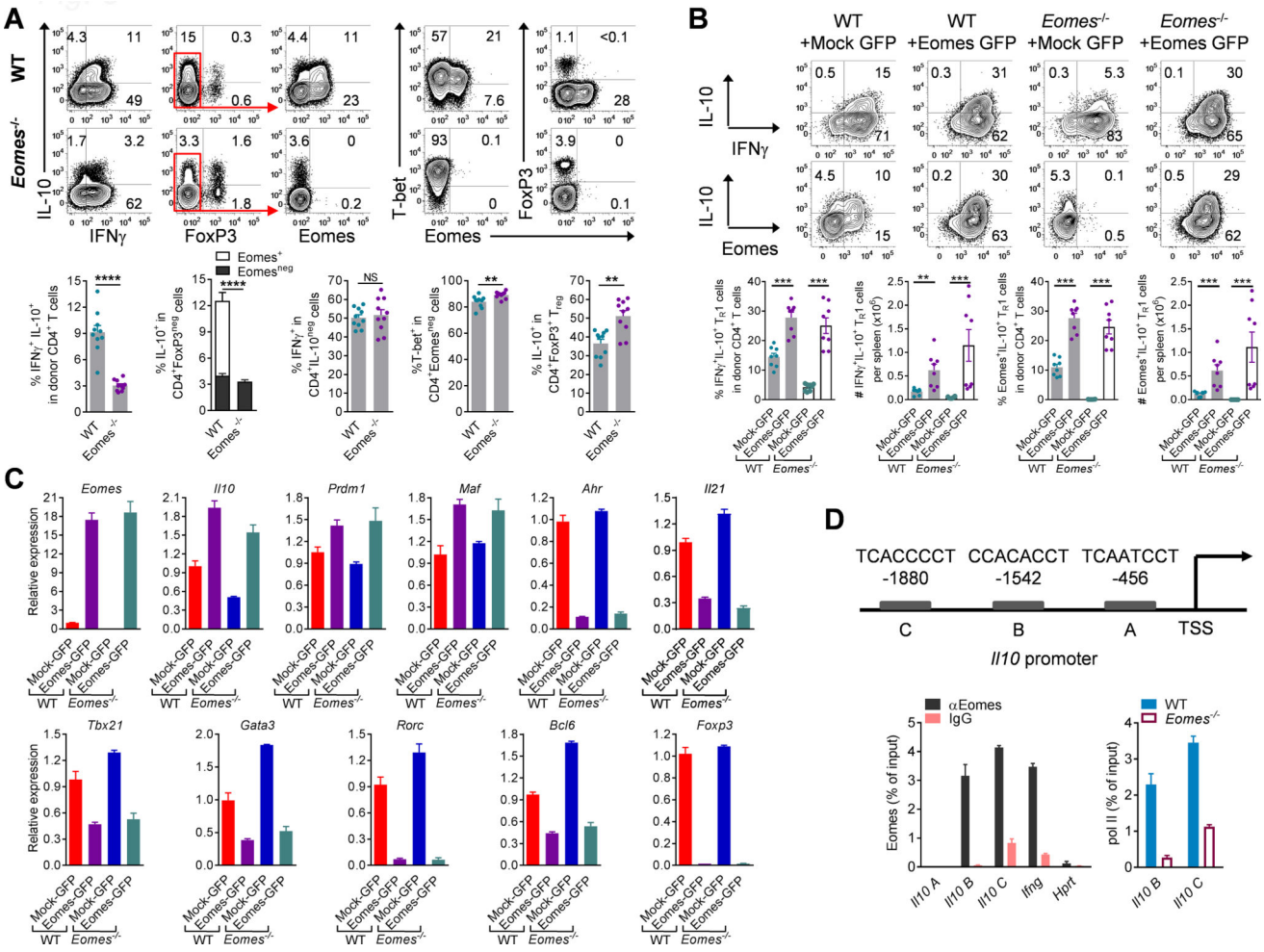

Figure 3. Eomes is required for T_R1 cell differentiation

(*A* – *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 (Eomes$^+$IL-10$^+$: open bar; Eomes^{neg}IL-10$^+$: solid bar, n = 10 per group) in recipients of WT or Eomes$^{-/-}$ CD4$^+$ T cells at d14 (n = 10 per group). (*B*) Expression of IL-10, IFNγ, and Eomes in transduced WT or Eomes$^{-/-}$ CD4$^+$ T cells at d7 (n = 8 per group) and (*C*) transcription of *Il10* and related genes (data are from 4–5 pooled animals in triplicate reactions, representative of 2 independent experiments). (*D*) CD4$^+$ T cells or *Foxp3*^{RFPneg}*Il10*$^{GFP+}$ T_R1 cells were FACS sorted from spleen and liver at d14 (representative of 3 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 Pol II to the *Il10* promoter in WT or *Eomes*$^{-/-}$ CD4$^+$ T cells (data are from 10 pooled animals in triplicate reactions). Data represents mean ± SEM.

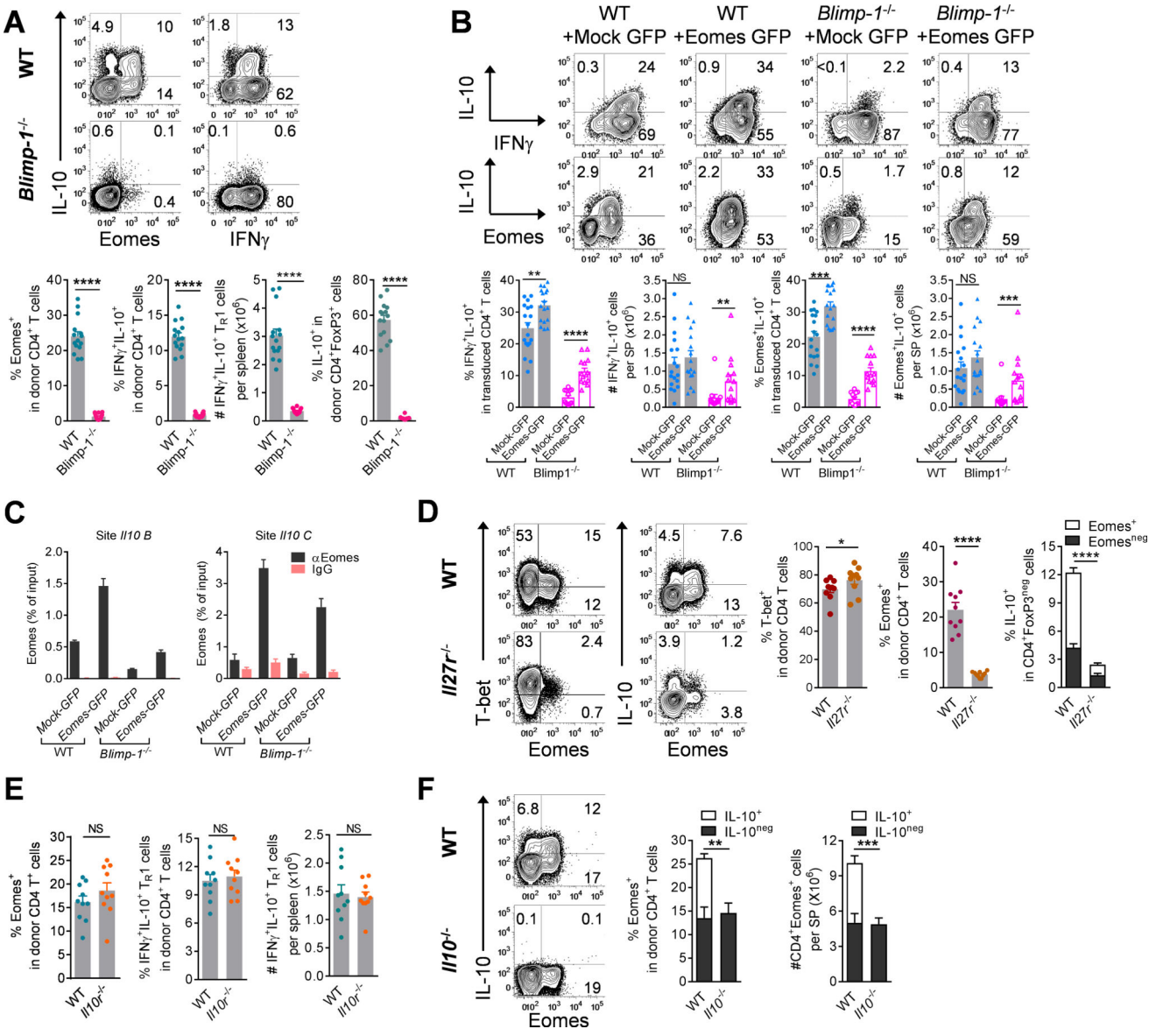

Figure 4. Eomes^{+} T$_{R}$1 cells are dependent on Blimp-1, IL-27 and IL-10

(*A* – *F*) B6D2F1 mice were transplanted with primary or retrovirally transduced (Mock-GFP or Eomes-GFP) CD4^{+} T cells and spleen examined after BMT. (*A*) Expression of Eomes, IL-10 and IFN**γ** in WT or *Blimp-1*$^{-/-}$ CD4^{+} T cells at *d*14 (*n* = 14 – 15 per group). (*B*) Expression of Eomes, IL-10 and IFN**γ** (*n* = 18, 17 for WT; *n* = 13, 14 for *Blimp-1*$^{-/-}$) in transduced CD4^{+} T cells at *d*7–10. (*C*) Recruitment of Eomes to *Il10* promoter in transduced CD4^{+} T cells (WT or *Blimp-1*$^{-/-}$) at *d*10 (data are from 4 animals in duplicate or triplicate reactions). (*D*) Expression of T-bet, Eomes and IL-10 in WT or *Il27r*$^{-/-}$ CD4^{+} T cells at *d*14 (*n* = 10 per group). (*E*) Expression of Eomes and IFN**γ**$^{+}$IL-10^{+} T$_{R}$1 cells in WT or *Il10r*$^{-/-}$ CD4^{+} T cells at *d*14 (*n* = 10 per group). (*F*) Expression of Eomes and IL-10 (Eomes^{+}IL-10^{+}: open bar; Eomes^{+}IL-10^{neg}: solid bar) in CD4^{+} T cells in recipients of WT or *Il10*$^{-/-}$ CD4^{+}CD25^{neg} T cells at *d*14 (*n* = 10 – 11 per group). Data represents mean ± SEM.

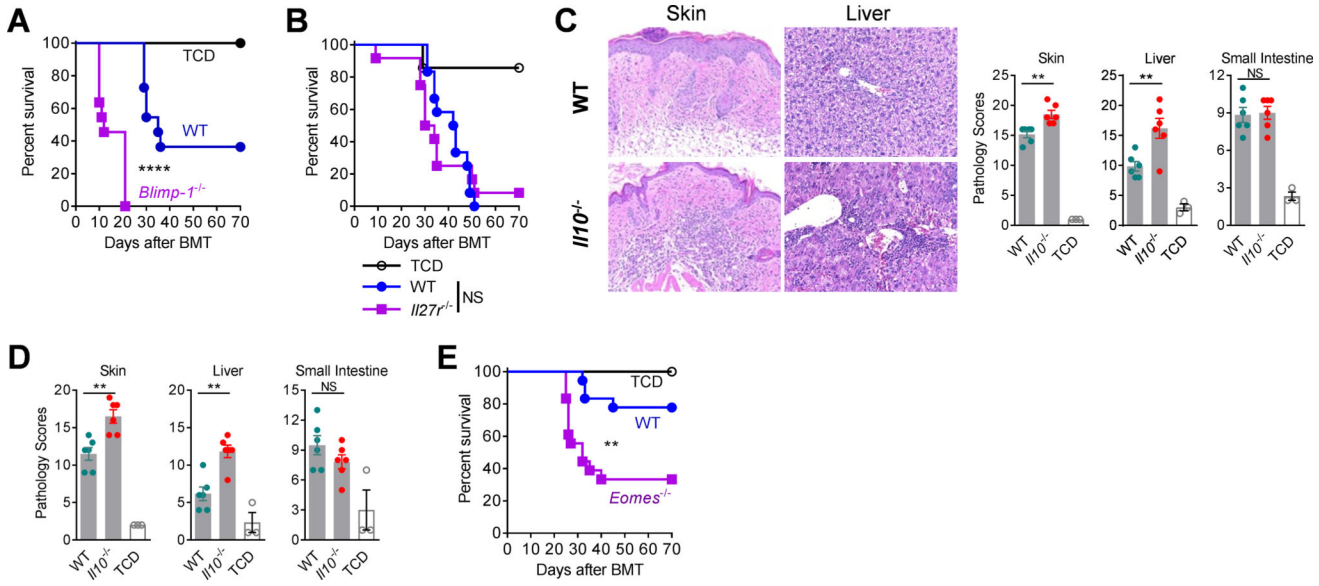

Figure 5. Attenuation of GVHD by Eomes^{+} T$_{R}$1 cells

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

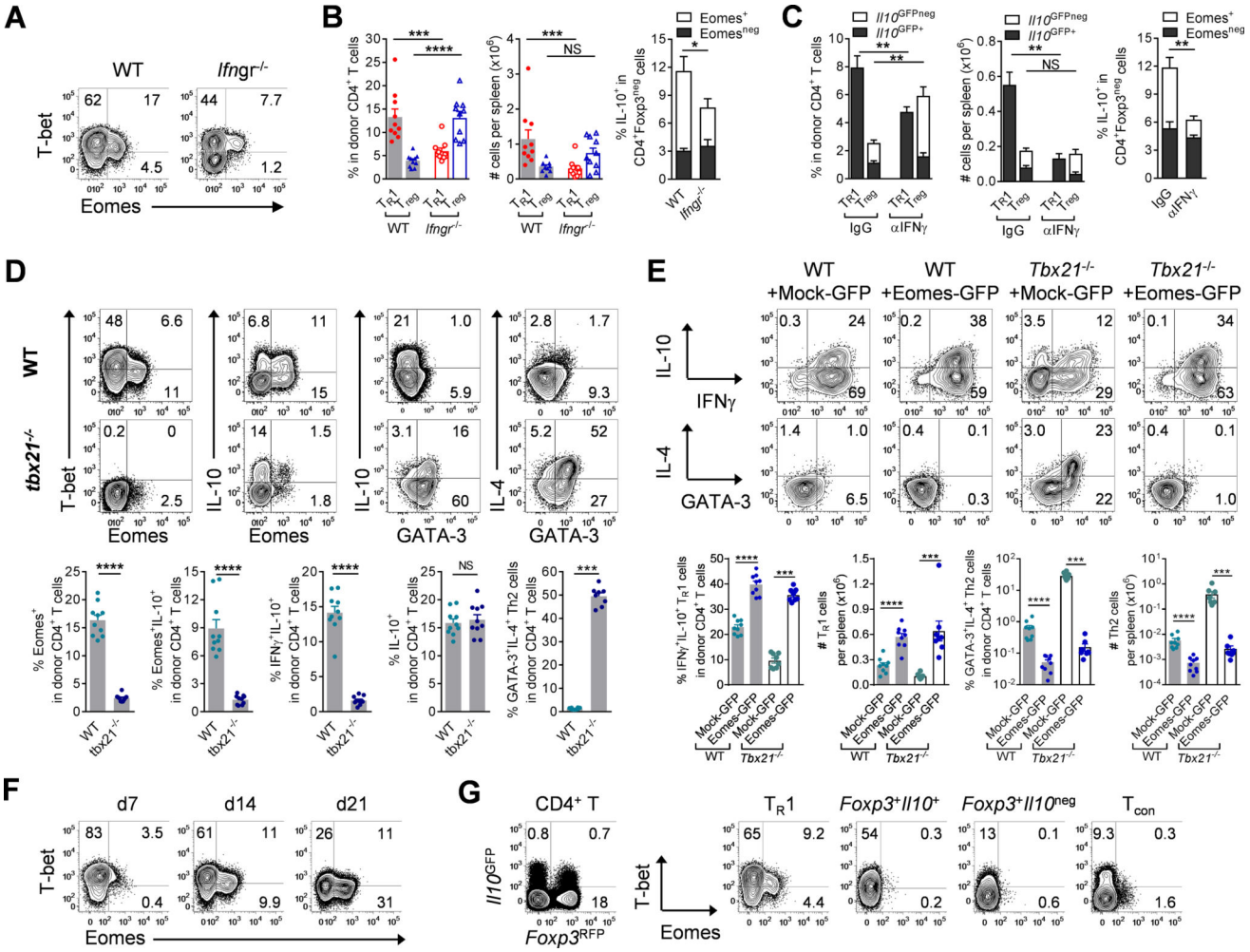

Figure 6. Eomes and T-bet jointly regulate T_R1 cell development

(*A* and *B*) B6.WT or B6.*Ifngr*$^{-/-}$ CD3$^+$ T cells were transplanted into B6D2F1 mice and splenic CD4$^+$ T cells examined at *d*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 examined at *d*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 *d*12 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 *d*7 shown (*n* = 8 per group). (*F*) Co-expression of T-bet and Eomes in CD4$^+$ T cells over time (representative of at least 2 experiments). (*G*) Splenic CD4$^+$ T cells from naïve mice FACS sorted to 4 subsets based on *Il10*^{GFP} and *Foxp3*^{RFP} expression and T-bet and Eomes evaluated (representative of 2 experiments). Data represents mean ± SEM.

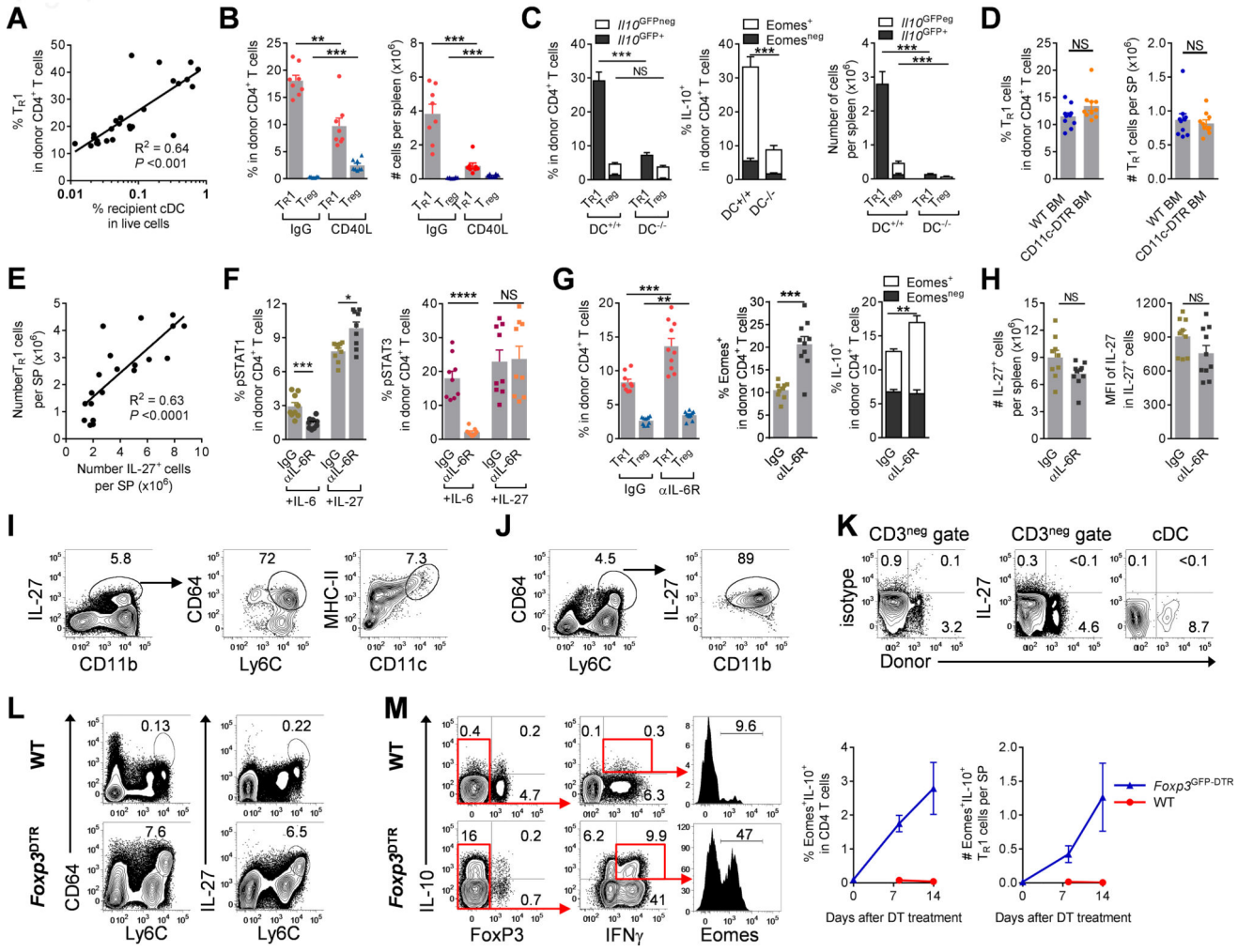

Figure 7. Recipient DC and macrophage-derived IL-27 promote the development of T_R1 cells

(*A* – *K*) B6D2F1 mice were transplanted with TCD BM and CD4$^+$ T cells and spleen examined. (*A*) Correlation of T_R1 cells (*Il10*$^{GFP+}$*Foxp3*^{RFPneg}) with proportions of recipient DC at *d*14 (n = 26). (*B*) Frequencies of T_{reg} (*Foxp3*$^{GFP+}$) and T_R1 (IFNγ^+IL-10$^+$) cells at *d*14 in the presence or absence of CD40L inhibition (n = 8 per group, grafts were CD4$^+$*Foxp3*^{GFPneg}). (*C*) WT.B6D2F1 or CD11c-DOG × DBA/2 F1 recipients were treated with DT to deplete recipient cDC and received B6.WT or *MHC-II*$^{-/-}$ BM respectively. Expression of T_R1, T_{reg} cells, Eomes and IL-10 at *d*14 are shown (n = 10 and 7 respectively). (*D*) Recipients of WT or CD11c-DOG BM were treated with DT to deplete donor cDC with expression of T_R1 and T_{reg} cells at *d*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 *d*14 (n = 20). (*F*) Recipients were treated with IL-6R and spleens analyzed at *d*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 analyzed at *d*10. (*G*) Expression of *Foxp3*^{RFPneg}*Il10*$^{GFP+}$ T_R1, *Foxp3*$^{RFP+}$ T_{reg}, Eomes and IL-10 in donor CD4$^+$ T cells and (*H*) numbers of IL-27$^+$ cells with intensity (MFI) of IL-27 (n = 9 – 10 per group). (*I* and *J*) Phenotypes of CD3^{neg} IL-27 secreting cells at *d*14 are shown. (*K*) Expression of IL-27 from

recipient DC at *d*+1 after BMT. (*L* and *M*) B6.WT or B6.*Foxp3*$^{GFP\text{-}DTR}$ mice were treated with DT for up to 2 weeks and spleens analyzed. (*L*) Phenotype of IL-27 secreting macrophage in CD3^{neg} splenocytes and (*M*) expression of Eomes^{+}IL-10^{+} cells over time with representative plots at *d*14. Data represents mean ± SEM.

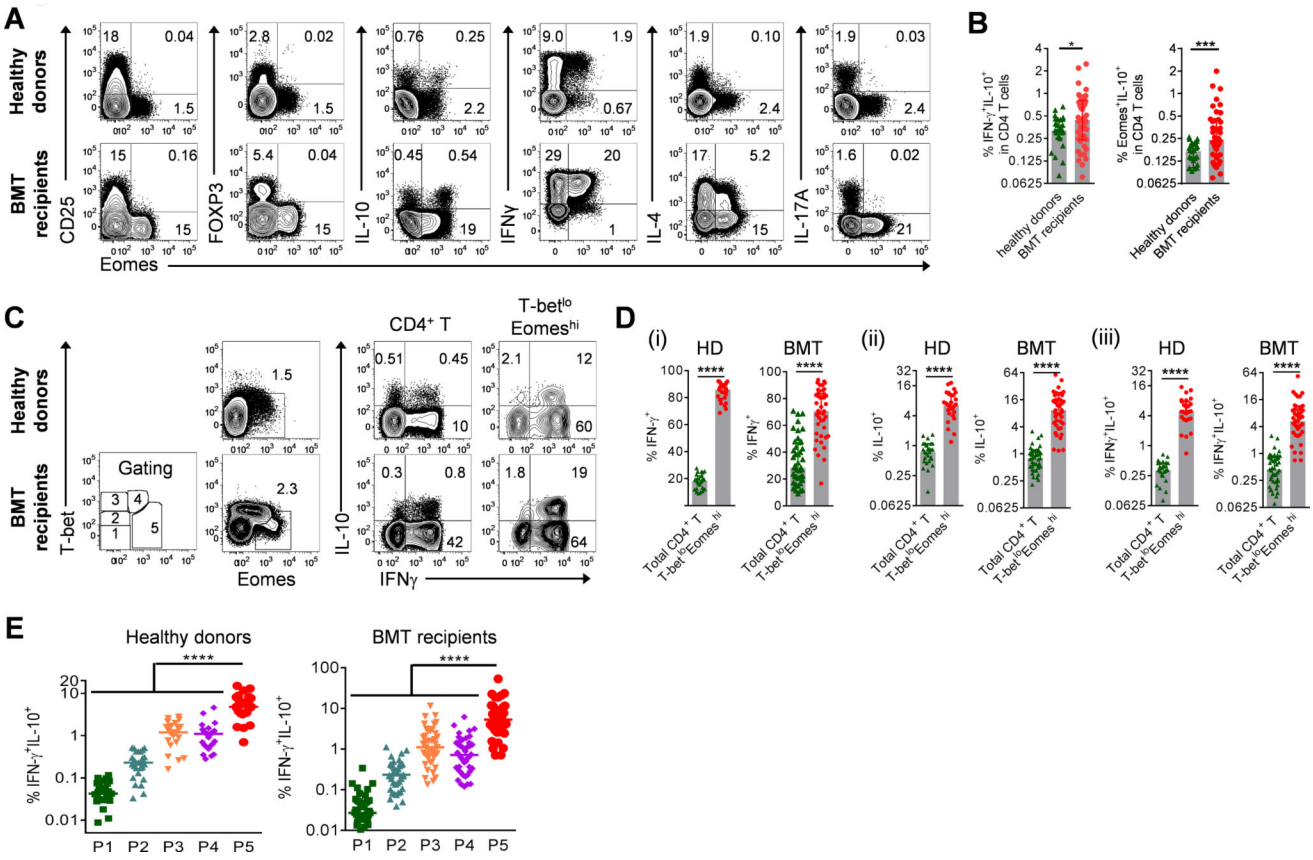

Figure 8. Co-expression of T-bet and Eomes identifies a T_R1 cells enriched population in human $CD4^+$ T cells

(*A*) Representative plots show the correlation of Eomes to CD25, FOXP3 and cytokines in $CD4^+$ T cells in healthy individuals and at *d*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 *d*60 after clinical allo-BMT (n = 43). (*C* – *E*) Expression of cytokines in the $T\text{-}bet^{lo}Eomes^{hi}$ 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 *d*60 after allo-BMT (BMT, n = 43). Data represents median ± interquartile range.