European Journal of Immunology

Adaptive immunity

Research Article

Eomesodermin controls a unique differentiation program in human IL-10 and IFN-γ coproducing regulatory T cells

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Whether human IL-10-producing regulatory T cells ("Tr1") represent a distinct differentiation lineage or an unstable activation stage remains a key unsolved issue. Here, we report that Eomesodermin (Eomes) acted as a lineage-defining transcription factor in human IFN-γ/IL-10 coproducing Tr1-like cells. In vivo occurring Tr1-like cells expressed Eomes, and were clearly distinct from all other CD4$^+$ T-cell subsets, including conventional cytotoxic CD4$^+$ T cells. They expressed Granzyme (Gzm) K, but had lost CD40L and IL-7R expression. Eomes antagonized the Th17 fate, and directly controlled IFN-γ and GzmK expression. However, Eomes binding to the IL-10 promoter was not detectable in human CD4$^+$ T cells, presumably because critical Tbox binding sites of the mouse were not conserved. A precommitment to a Tr1-like fate, i.e. concominant induction of Eomes, GzmK, and IFN-γ, was promoted by IL-4 and IL-12-secreting myeloid dendritic cells. Consistently, Th1 effector memory cells contained precommitted Eomes$^+$GzmK$^+$ T cells. Stimulation with T-cell receptor (TCR) agonists and IL-27 promoted the generation of Tr1-like effector cells by inducing switching from CD40L to IL-10. Importantly, CD4$^+$Eomes$^+$ T-cell subsets were present in lymphoid and nonlymphoid tissues, and their frequencies varied systemically in patients with inflammatory bowel disease and graft-versus-host disease. We propose that Eomes$^+$ Tr1-like cells are effector cells of a unique GzmK-expressing CD4$^+$ T-cell subset.

Keywords: Differentiation · EOMES · Granzyme K · Regulatory T cells · Th17

See accompanying Commentary by Dejean et al.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Immune responses have to be tightly regulated to prevent autoimmunity or overactive immune responses against commensals or pathogens that could lead to immunopathology. Regulatory T cells are specialized cells that fulfill this task, as evidenced by the devastating autoimmune phenotype of mice and patients with genetic disorders with depleted numbers of regulatory T cells [1]. Prototypical regulatory T-cells express CD25 and the transcription factor FOXP3, which regulates several key features of Tregs and distinguishes them from conventional $CD4^+$ T cells [2]. However, it is also well-established that some $Foxp3^-$ T cells possess regulatory functions and might play a nonredundant role in several immune-mediated diseases. In particular, T cells that secrete the anti-inflammatory cytokine IL-10, so-called type 1 regulatory T cells (Tr1), have also been identified [3], and it is becoming increasingly clear that they are a second principal regulatory T-cell subset of the immune system [4, 5]. However, in contrast to $FOXP3^+$ Tregs, and other defined $CD4^+$ T-cell subsets, such as Th1, Th2, and Th17 cells, the molecular identity of Tr1 cells is still enigmatic, in particular, in humans. Features of Tr1 cells are IL-10 production and several researchers also reported GzmB expression, which endows them with suppressive and cytotoxic functions, respectively [6, 7]. Moreover, Tr1 cells express several checkpoint receptors, such as programed cell death protein 1 (PD1), T-cell immunoglobulin and mucin-domain containing-3 (TIM3), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), and Lymphocyte-activation gene 3 (LAG3) [8, 9]. However, neither IL-10 production [10] nor GzmB or checkpoint receptor expression are unique properties of Tr1 cells [11]. Rather, IL-10 is also produced by helper T cells, including T_{FH} cells that promote B-cell responses [10]. Similarly, GzmB is expressed by the large majority of cytotoxic T cells [12], which are abundant among $CD8^+$ T cells, but are also present at low frequencies in the CD4 compartment [13, 14]. Finally, checkpoint receptors are also expressed on other $CD4^+$ T-cell subset, namely $FOXP3^+$Tregs and follicular helper T (T_{FH}) cells that express high levels of CTLA-4 and PD1, respectively. Several transcription factors that regulate IL-10 production in helper and Tr1 cells have been described [7, 15–18], including c-Maf, AHR, and Blimp-1, yet they are not unique to Tr1 cells and are, therefore, insufficient to define Tr1-cells. Indeed, it is still debated if Tr1 cells are only a transient and unstable activation stage of conventional $CD4^+$ T cells [19], or if they represent a unique differentiation stage similar to $Foxp3^+$Tregs [4]. Moreover, while it was originally assumed that Tr1 cells express little or no IFN-γ, it is now clear that IL-10 and IFN-γ coproducing regulatory T cells exist [19, 20], consistent with the findings that IFN-γ can have anti-inflammatory functions under certain conditions. Several protocols to induce Tr1 cells in vitro have been published, including cytokines, such as IL-10 and IFN-α [21] or IL-27 [15] and APCs such as immature DC, IL-10 secreting monocyte-derived DC [22], or plasmacytoid DC (pDC) [23, 24]. In mice, Tr1 cells can be easily identified in IL-10 and Foxp3 reporter mice, or by CD49b and/or LAG-3 expression [8]. LAG3 was also proposed to be a useful surface marker for Tr1 cells in humans [8, 9], but in vivo occurring humans Tr1 cells are still poorly characterized. We have published an alternative strategy to identify human IL-10 and IFN-γ coproducing Tr1-like cells directly ex vivo. These Tr1-like cells are strongly enriched among $CD4^+CD25^-IL\text{-}7R^-$ effector T cells [25, 26], and coexpress CCR5 and PD-1 in lymphoid and nonlymphoid human tissues [26]. They showed selective functional defects in systemic lupus erythematosus [26] and inflammatory bowel diseases (IBDs) [27]. Here, we performed a genome-wide gene expression analysis to provide a molecular blueprint of these in vivo occurring Tr1-like cells. We identified the Tbox transcription factor Eomes as a key regulator, which induced a unique cytotoxic differentiation program that unequivocally distinguishes them from all other $CD4^+$ T cells.

Results

Human IL-10 and IFN-γ coproducing Tr1-like cells express the transcription factor Eomes

We previously showed that IL-10 and IFN-γ coproducing effector cells with Tr1-like regulatory function are present among $CD4^+IL\text{-}7R^-CD25^-$ T-cells ("$IL\text{-}7R^-$") in human peripheral blood of healthy individuals [25]. To provide a molecular blueprint of these in vivo occurring Tr1-like cells, we performed a genome-wide gene expression analysis of $IL\text{-}7R^-$ T cells that secreted IL-10 following brief polyclonal stimulation ex vivo (Supporting Information Fig. 1A). As control, we purified the abundant $CD4^+IL\text{-}7R^+CD25^{lo}$helper T cells ("$IL\text{-}7R^+$") according to IL-10 secretion (Supporting Information Fig. 1A and B). A total of 83 genes were selectively and significantly upregualted and 81 downregulated in IL-10-secreting $IL\text{-}7R^-$ T cells as compared to IL-10-secreting $IL\text{-}7R^+$ T cells and to control populations that failed to produce IL-10 (Supporting Information Table 1). Strikingly, the strongest upregulated gene was Eomesodermin (Eomes, Fig. 1A and Supporting Information Fig. 1C), a Tbox transcription factor that controls cytotoxic functions of $CD8^+$ T cells and NK cells. In addition, among the most strongly upregulated genes there were two cytotoxic molecules, Granzyme (Gzm) A and GzmK, and several surface receptors, including CD27, LAG3, TIM3 (HAVCR2), and 4-1BB (TNFRSF9, Fig. 1A and Supporting Information Table 1). Among the most downregulated genes, we identified several proinflammatory cytokines and chemokines, including IL-22, GM-CSF (CSF2), IL-17F, IL-8, as well as the transcription factors RAR-related orphan nuclear receptor (ROR)α (HS_560343, Fig. 1A and Supporting Information Table 1). Of note, only a low number of genes were specifically up- or downregulated in IL-10 producing $IL\text{-}7R^+$ control T cells (Supporting Information Fig. 1B). The selective expression of Eomes in Tr1-like cells was confirmed at the protein level by intracellular staining. Thus, the majority of IL-10-producing $IL\text{-}7R^-$ T cells expressed Eomes, whereas $IL\text{-}10^+IL\text{-}7R^+$ control cells expressed only low levels (Fig. 1B). Moreover, among $IL\text{-}7R^-$ T cells, Eomes was expressed in the large majority of IL-10 and IFN-γ coproducing cells, but was largely undetectable in cells that lacked IL-10 and IFN-γ producing capacities (Fig. 1C

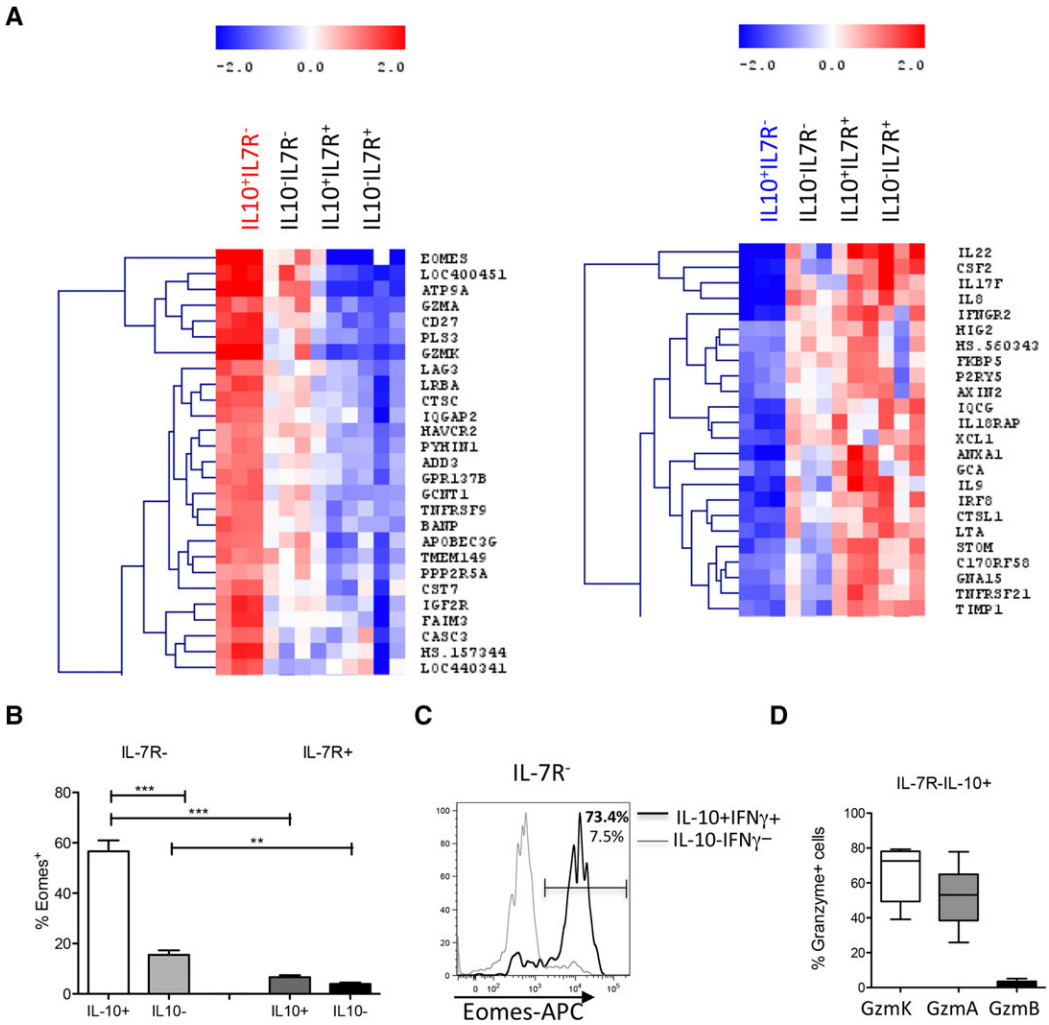

Figure 1. Eomes is highly expressed in human IL-10/IFN-γ coproducing Tr1-like cells. (A) Gene expression analysis of presorted CD4$^+$IL-7R$^+$ and IL-7R$^-$CD25$^-$ T-cell subsets purified from human peripheral blood according to IL-10 secretion, following brief stimulation with PdBu and Ionomycin (Supporting Information Fig. 1A). Heat Map of selectively up- (red, left panel) and downregulated (blue, right panel) genes in IL-7R-IL-10$^+$ Tr1-like cells from peripheral blood as compared to the indicated control populations. Data are from the three donors who were analyzed in the same experiment. (B) Percentages of Eomes$^+$ cells among CD4$^+$IL-7R$^+$ and IL-7R$^-$ T-cell subsets that did or did not produce IL-10 (cells from 16 different donors analyzed in eight experiments) were analyzed by flow cytometry. (C) Eomes protein expression in gated IL-10$^-$IFN-γ$^-$ (dotted line) and IL-10$^+$IFN-γ$^+$ (bold line) cells in purified CD4$^+$IL-7R$^-$ T cells. Numbers indicate the percentage of Eomes$^+$ cells. (D) Intracellular GzmK, A, and B expression in gated IL-10$^+$cells among purified CD4$^+$IL-7R$^-$ T cells (cells from five (GzmB), six (GzmA), or eight (GzmK) donors analyzed in three (GzmB/A) or four (GzmK) experiments). Data from different experiments were pooled and shown as mean + SEM, **/***$p < 0.005/0.00005$ according to statistical analysis with One-way ANOVA.

and Supporting Information Fig. 1D). IL-10$^+$ IL-7R$^-$ Tr1-like cells expressed also high levels of GzmK and GzmA proteins, while GzmB was hardly detectable (Fig. 1D).

In summary, the gene expression analysis revealed that IL-10 and IFN-γ coproducing Tr1-like cells had a unique gene signature, and expressed in particular high levels of the transcription factor Eomes and selected cytotoxic proteins.

Eomes$^+$ Tr1-like cells are distinct from conventional helper and regulatory T cells

Genes of proinflammatory Th17 cells were strongly downregulated in IL-7R$^-$ Tr1 cells (Fig. 1A), suggesting that they were distinct from Th17 cells. Consistently, Eomes$^+$ CD4$^+$ T cells produced high levels of IFN-γ upon stimulation, while IL-17A protein was hardly detectable (Fig. 2A). This was also true for IL-7R$^-$CD4$^+$Eomes$^+$ T cells, which were also negative for the Th17-associated chemokine receptor CCR6 (Supporting Information Fig. 2A) and failed to produce IL-17F, GM-CSF, and IL-22 (data not shown). We then asked how Eomes$^+$ Tr1-like cells were related to conventional regulatory T cells, i.e. CD25$^+$IL-7R^{lo}Tregs ("CD25$^+$"), which express FOXP3. Among total CD4$^+$ T cells, there were two distinct populations of FOXP3$^+$ and Eomes$^+$ cells, while Eomes and FOXP3 co-expressing cells were hardly detectable (Supporting Information Fig. 2B). Furthermore, CD25$^+$Tregs expressed, as expected, high levels of FOXP3, but

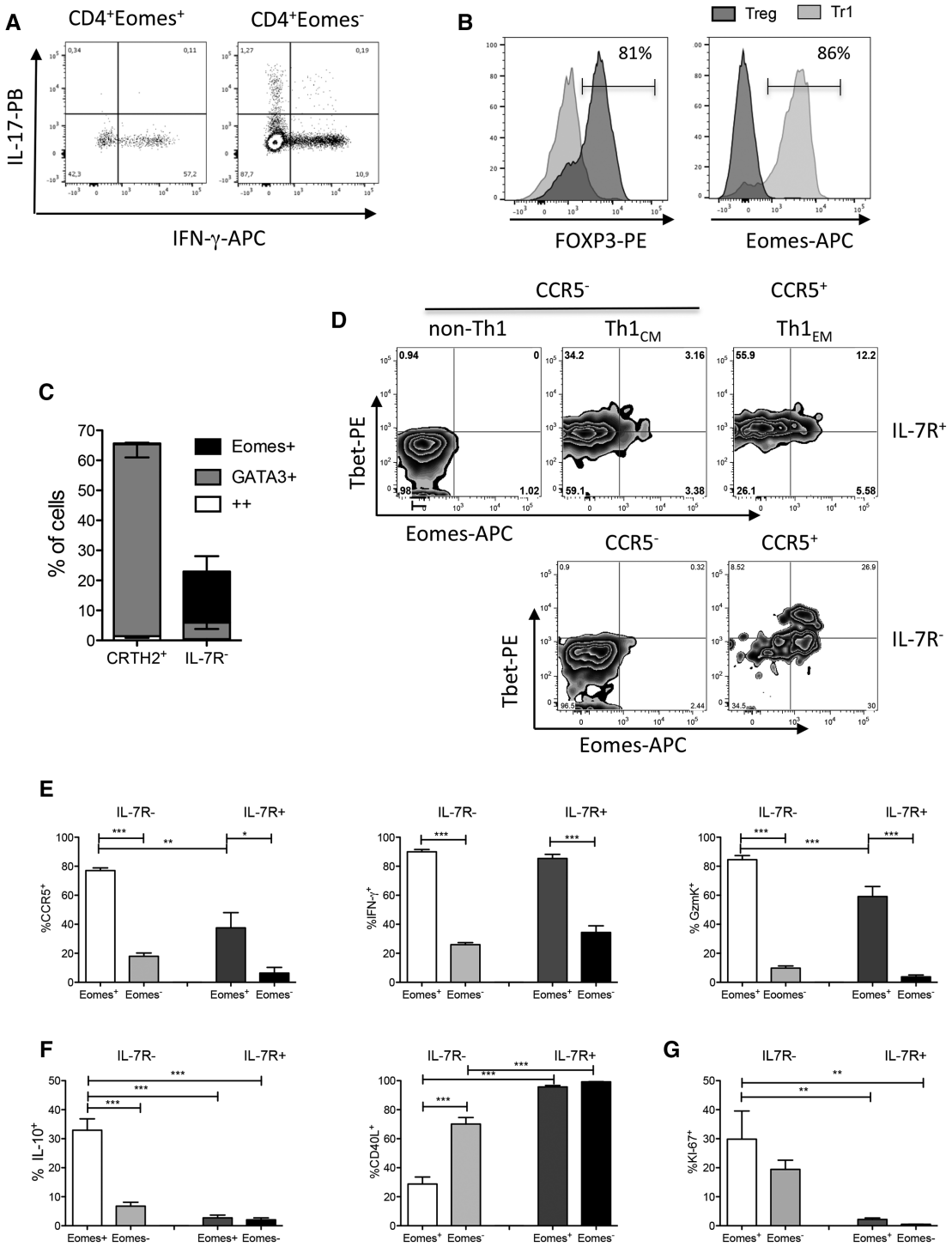

Figure 2. Eomes$^+$Tr1-like cells in human blood are distinct from established CD4$^+$ T-cell subsets. CD4$^+$ T cells from human PBMC were analyzed by flow cytometry. (A) IFN-γ versus IL-17 production in gated CD4$^+$Eomes$^+$ (upper panel) and CD4$^+$Eomes$^-$ T cells (lower panel) was analyzed by flow cytometry. (B) Histogram overlays showing Foxp3 (left) and Eomes (right) expression in CD25$^+$IL-7R^{lo}Tregs (dark grey) and CCR5$^+$CCR6$^-$PD1$^+$IL-7R$^-$Tr1-like cells (light grey). Percentages indicate Foxp3 expression in Tregs and Eomes expression in Tr1 cells. (C) Intracellular GATA3 or Eomes protein expression in gated CRTH2$^+$Th2-cells and Tr1-containing IL-7R$^-$ T cells (four experiments with four different donors). (D) Ex vivo Eomes versus T-bet protein expression in CXCR3$^-$IL-7R$^+$ non-Th1" cells, in CXCR3$^+$IL-7R$^+$Th1 subsets (CCR5$^-$:Th1$_{CM}$/CCR5$^+$:Th1$_{EM}$), and in IL-7R$^-$ cells gated according to CCR5 expression. (E–G) Analysis of CD4$^+$CD25$^-$ T-cells according to Eomes and IL-7R expression. (E) CCR5 expression (cells from four different donors analyzed in three experiments), IFN-γ production (middle, cells from 17 different donors analyzed in nine experiments), and GzmK expression (right, cells from eight different donors analyzed in four experiments). (F) IL-10 production (left, cells from 15 donors analyzed in nine experiments) and CD40L upregulation (right, cells from seven donors analyzed in four experiments) (G) Ex vivo Ki-67 expression (cells from six donors analyzed in two experiments). Data from different experiments were pooled and shown as mean + SEM, */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with One-way ANOVA.

not Eomes (Supporting Information Fig. 2C and D). Importantly, when all surface receptors that are associated with IL-10 and IFN-γ coproduction [25–27] were combined, a very efficient enrichment for Eomes-expressing cells was achieved, and Foxp3$^+$ cells were excluded (Fig. 2B and Supporting Information Fig. 2C and D). Tr1-like cells were also distinct from Th2 cells, since circulating CRTH2$^+$ Th2 cells [28] expressed high levels of the lineage-defining transcription factor GATA3, but little or no Eomes (Fig. 2C). Moreover, although some Eomes$^+$IL-7R$^-$ Tr1-like cells coproduced IL-4 and IFN-γ ([25] and data not shown), there were virtually no IL-7R$^-$ T cells that coexpressed Eomes and GATA-3 (Fig. 2C). We then analyzed how Eomes$^+$ Tr1-like cells were related to Th1 cells, which also secrete IFN-γ, but express the related Tbox transcription factor T-bet and the chemokine receptor CXCR3 [29]. Of note, CCR5 is expressed on both Th1 effector memory cells ("Th1$_{EM}$") and by Tr1-like cells [26], but these two subsets can be distinguished by IL-7R expression [13]. CXCR3$^-$ "non-Th1" cells and CXCR3$^+$CCR5$^-$Th1 central memory cells ("Th1$_{CM}$") expressed respectively no and low levels of T-bet and Eomes (Fig. 2D). Conversely, CCR5$^+$Th1$_{EM}$ cells expressed intermediate levels of T-bet, and a minor fraction was Eomes$^+$. Furthermore, among IL-7R$^-$T-cells, the Tr1-containing CCR5$^+$ subset expressed high levels of Eomes, while CCR5$^-$ cells did not. Notably, among Eomes$^+$ IL-7R$^-$ T cells two distinct populations with high and low expression of T-bet could be distinguished (Fig. 2D), suggesting cellular heterogeneity (see below).

We next compared Eomes$^+$ IL-7R$^+$ T cells to Eomes$^+$IL-7R$^-$ Tr1-like cells. Both Eomes$^+$ subsets expressed elevated levels of CCR5, IFN-γ, and GzmK (Fig. 2E). Conversely, Eomes$^-$ subsets, including CD25$^+$Tregs (data not shown), expressed only low levels of these proteins and other cytotoxic molecules (Supporting Information Fig. 2E). Notably, Eomes$^+$ Th1 cells expressed low amounts of IL-10 and high levels of CD40L (Fig. 2F), similar to conventional CD4$^+$Eomes$^-$ T cells. In contrast, Eomes$^+$ Tr1-like cells produced high amounts of IL-10 and low levels of CD40L (Fig. 2F). Furthermore, IL-7R$^-$Eomes$^+$ Tr1-like cells expressed the proliferation marker Ki67, indicating that they had recently divided in vivo, while Eomes$^+$ Th1 cells were largely Ki67$^-$ and, thus, in a resting state (Fig. 2G).

In conclusion, Eomes$^+$ Tr1-like cells are distinct from other established CD4$^+$ T-cell subsets in peripheral blood, including Th17 cells, Th2 cells, and Foxp3$^+$Tregs. Intriguingly, a fraction of Th1$_{EM}$ cells also expressed Eomes and GzmK. They lacked, however, some key characteristics of Tr1-like cells, but might represent precommitted Tr1-like precursors.

Eomes$^+$ Tr1-like cells are distinct from conventional CTL

Eomes controls cytotoxic functions of CD8$^+$ T cells [30], but there are also rare cytotoxic T cells in the CD4 compartment [13, 14]. We, therefore, asked how Eomes$^+$ Tr1-like cells were related to these CD4$^+$ CTL. As CD27 was among the most upregulated genes in Tr1-like cells (Fig. 1A), and is absent on CTL [12], we subdivided IL-7R$^-$CCR5$^+$ T cells into putative CD27$^+$ Tr1-like cells and CD27$^-$CTL (Supporting Information Fig. 3A). Both subsets expressed high levels of Eomes (Fig. 3A). However, GzmK was selectively expressed in CD27$^+$Eomes$^+$ Tr1-like cells, while GzmB was preferentially expressed by CD27$^-$CTL (Fig. 3B and Supporting Information Fig. 3B). GzmA and perforin were expressed in both subsets, but CTL expressed higher levels of perforin (data not shown). Both Tr1-like cells and CTL degranulated upon TCR stimulation by CD1c$^+$DC and induced cell death of monocytes ex vivo, (Fig. 3C). However, Tr1 cells required higher effector–target ratios to kill efficiently, suggesting that conventional CTL had superior cytotoxic capabilities. Of note, CTL expressed high levels of T-bet, whereas Tr1-like cells expressed only low levels (Fig. 3D and Supporting Information Fig. 3A), explaining the observed heterogeneity among total Eomes$^+$ IL-7R$^-$ T cells (Fig. 2D). Moreover, while conventional Eomes$^-$ Th1 cells rapidly upregulated T-bet expression following TCR stimulation, Eomes$^+$ Tr1-like cells failed to do so and remained T-bet^{lo}. Eomes$^+$ CTL, which expressed already high levels of T-bet ex vivo, did not further upregulate T-bet expression upon stimulation (Fig. 3D). Importantly, CD27$^+$ Tr1-like cells produced high levels of IL-10 together with IFN-γ, while CD27$^-$CTL did not (Fig. 3E). Intriguingly, also CD8$^+$Eomes$^+$ T cells with a IL-7R$^-$CCR5$^+$CD27$^+$ Tr1-like phenotype produced some IL-10 and expressed high levels of GzmK, while the corresponding CD27$^-$ subset failed to produce IL-10 and expressed mainly GzmB (Supporting Information Fig. 3C). Finally, while Tr1-like cells and CD25$^+$Tregs consistently suppressed DC-induced naïve CD4$^+$ T-cell proliferation, CD4$^+$CTL had no consistent suppressive effects (Fig. 3F). Suppression by Tr1-like cells was, as expected, dose dependent (Supporting Information Fig. 3D) and decreased upon IL-10 neutralization (Fig. 3G).

We conclude that CD27 expression distinguishes Eomes$^+$ Tr1-like cells from conventional CTL. These two Eomes$^+$ T-cell subsets differ in IL-10 production, suppressive capacities, T-bet expression levels, and their GzmK/B expression profiles, and populations with similar characteristics are present in the CD8$^+$ T-cell pool.

Myeloid DC, IL-12, and IL-4 promote a precommitment to a Tr1-like fate

In other T-cells, IL-12 and, paradoxically, IL-4 were shown to regulate Eomes expression [31–34], but the factors that induce Eomes in human CD4$^+$ T cells are unknown. We screened, thus, for cytokines that could upregulate Eomes expression in anti-CD3 stimulated naïve CD4$^+$ T cells. IL-4 efficiently induced Eomes expression, while all other tested cytokines, including IL-12, IL-27, with or without TGF-β, IL-10, IFN-γ, and IL-13, were inefficient (Fig. 4A and data not shown). Of note, IL-4 induced Eomes also in naïve CD8$^+$ T cells and in CD4$^+$ memory T cells (Supporting Information Fig. 4A). Naïve CD4$^+$ T cells that were stimulated with anti-CD3 stimulation and IL-4 expressed some GzmK (Supporting Information Fig. 4B), but failed to produce IFN-γ (data not shown). We therefore assessed if naïve T cells that upregulated Eomes upon more physiological priming by DCs coexpressed

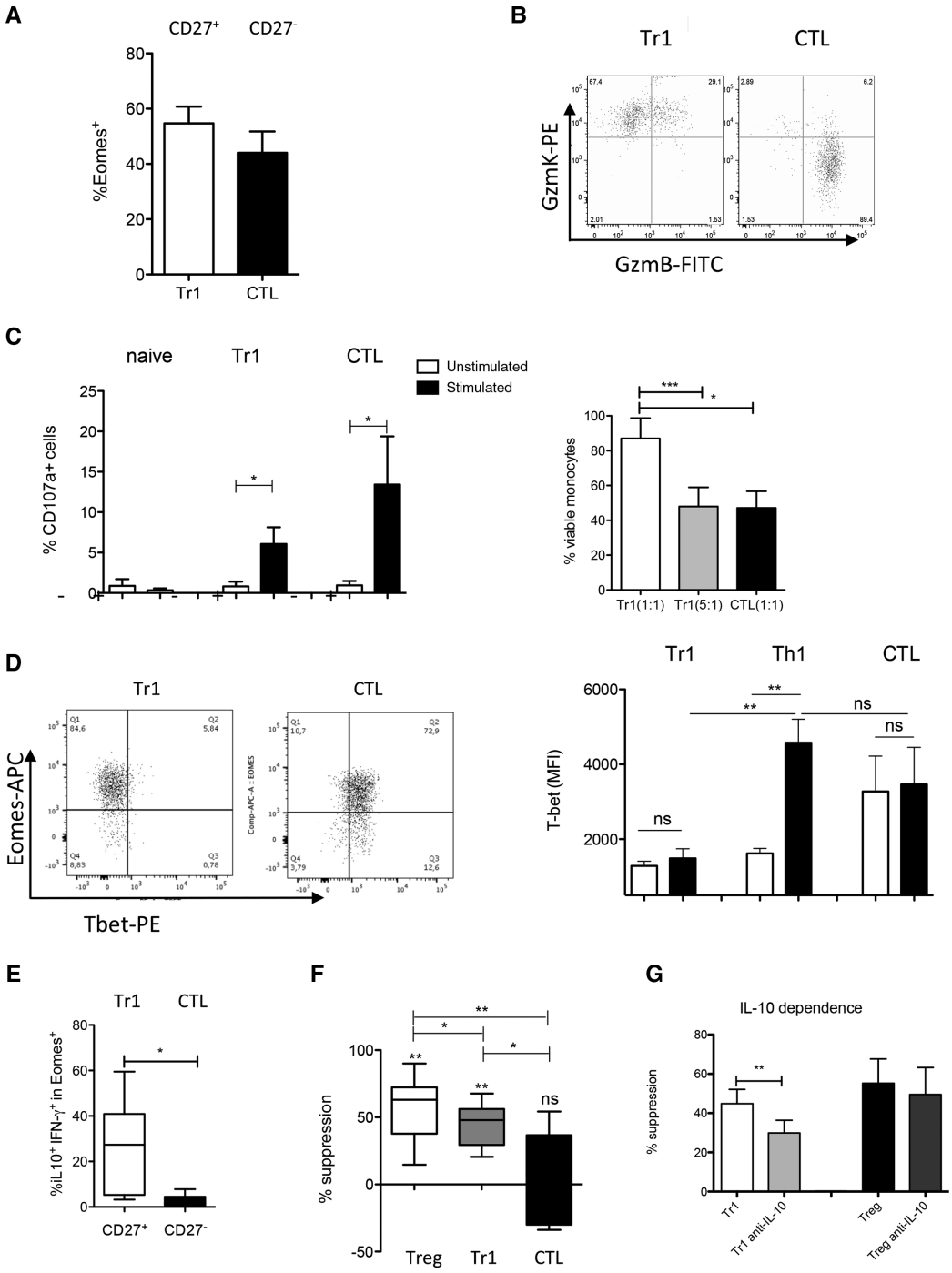

Figure 3. Tr1-like cells and conventional CTL are two distinct Eomes$^+$ T-cell subsets. Shown is an analysis of CD4$^+$ T-cells among mononuclear cells of human peripheral blood mononuclear cells (PBMC). (A) Eomes expression in gated CD27$^+$(Tr1) and CD27$^-$(CTL) subsets among CD4$^+$CCR5$^+$IL-7R$^-$T cells (six experiments with cells from different donors). (B) Intracellular GzmK versus GzmB protein expression in gated Eomes$^+$CD27$^+$Tr1-like cells and Eomes$^+$CD27$^-$CTL. (C) Left: degranulation of Tr1-like cells and CTL following TCR stimulation with SEB (+) presented by CD1c$^+$ DC as compared to naïve control cells was measured by CD107a surface exposure (five experiments with FACS-purified DC and T cells from five different donors). Right: Ex vivo killing of purified monocytes by Tr1 cells and CTL at a Effector–Target ratio of 1.1 or 5:1 as indicated. Percentages are calculated on viable monocytes in the absence of killer cells (100%, three experiments analyzing cells from five different donors). (D) Left: Eomes versus T-bet expression in gated Tr1-like cells and CTL. Right: T-bet expression by Eomes$^-$Th1 cells, Eomes$^+$Tr1-like cells, or CTL before (white bars, "–") or after (black bars, "+") TCR stimulation with anti-CD3 and anti-CD28 antibodies for 24 h (five experiments with cells from five different donors). (E) Percentages of IL-10$^+$IFN-γ$^+$ cells in gated Eomes$^+$ cells in purified IL-7R$^-$CCR5$^+$T-cells were sorted according to CD27 expression. (F) Suppression of DC-induced naïve CD4$^+$ T-cell proliferation by FACS-purified Tregs, Tr1-like cells, and CTL. Proliferation of responder cells in the presence of unlabeled naïve control cells was set to 0% suppression. (G) Suppression by Tregs and Tr1-like cells in the absence or presence of neutralizing anti-IL-10 antibodies. (F/G) (six experiments with cells from six different donors). Data were pooled from different experiments and the mean + SEM is shown; */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with paired student's *t*-test.

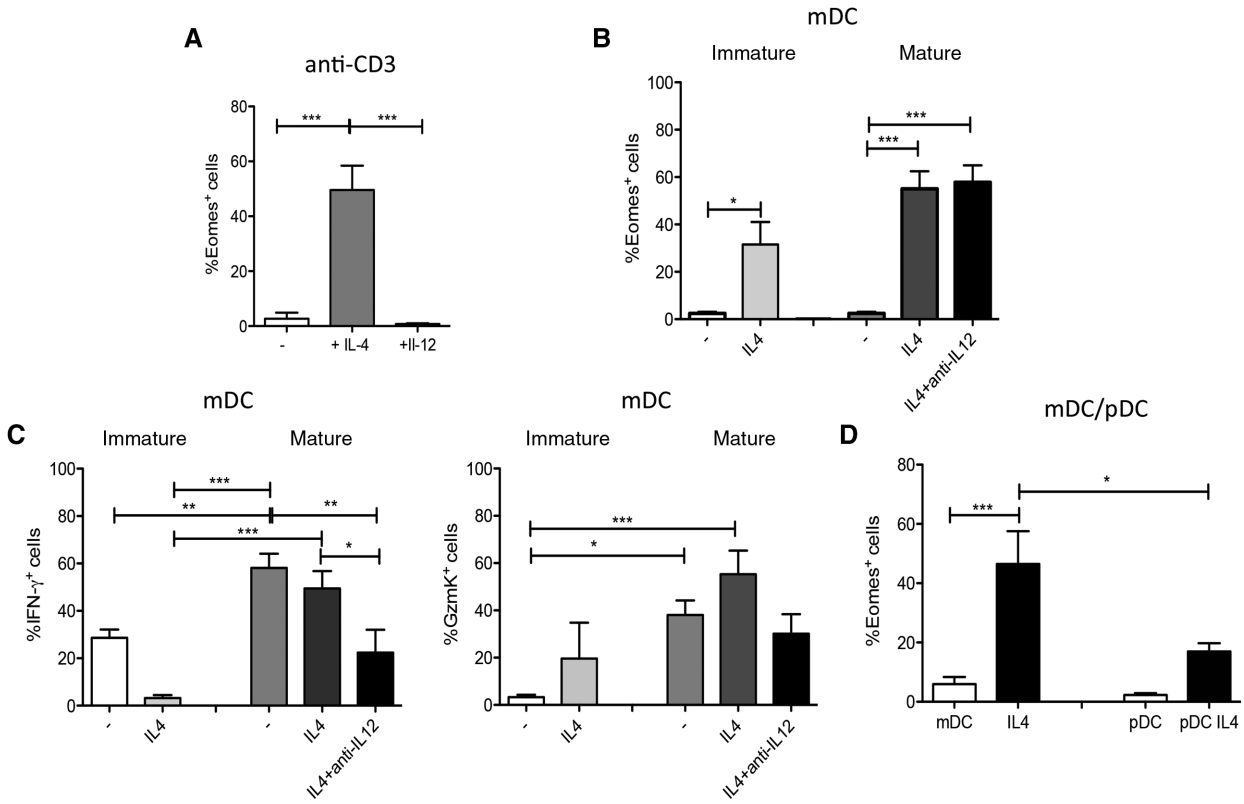

Figure 4. Tr1-like commitment is induced by IL-12-secreting myeloid DC and IL-4. (A) Purified naïve human CD4$^+$ T cells were stimulated with anti-CD3 antibodies in the absence or presence of IL-4 or IL-12. Shown is the mean percentage of Eomes$^+$ cells (nine donors analyzed in nine experiments). (B/C) Naive human CD4$^+$ T cells were stimulated with allogenic CD1c$^+$ DC that were either left unstimulated (immature) or stimulated with LPS and R848 to induce IL-12 (mature). Recombinant IL-4 or neutralizing anti-IL-12 antibodies were added as indicated. Induction of Eomes (B), IFN-γ (C, left), and GzmK (C, right) were assessed (six donors analyzed in six experiments). (D) Eomes induction by CpG-matured pDC or LPS/R848-matured CD1c$^+$ DC in the absence and presence of IL-4 (cells from five donors analyzed in five experiments). Data from different experiments were pooled and is shown as mean + SEM, */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with One-way ANOVA.

IFN-γ and GzmK, as do in vivo occurring Eomes$^+$ CD4$^+$ T cells. CD1c$^+$ myeloid DCs were matured with a combination of LPS and R848, which induces high levels of IL-12 [35]. IL-4 consistently induced Eomes also in DC-primed CD4$^+$ (Fig. 4B) and CD8$^+$ T cells (data not shown). It inhibited IFN-γ production as expected, but DC maturation abrogated this inhibitory effect in an IL-12-ependent manner (Fig. 4C). IL-4 also inhibited GzmB and T-bet induction (data not shown). Conversely, IL-4 increased GzmK expression, and the highest levels of GzmK were induced when both IL-4 and IL-12 were available (Fig. 4C). However, IL-4 failed to downregulate CD40L (Supporting Information Fig. 4C), and CD1c$^+$DC-primed T cells also produced low levels of IL-10 (Supporting Information Fig. 4D). CpG-matured pDC induced higher levels of IL-10 (Supporting Information Fig. 4D), as expected [24, 36, 37]. However, T cells primed with pDC and IL-4 expressed only low amounts of Eomes (Fig. 4D), as well as of GzmK and IFN-γ (Supporting Information Fig. 4D). This was true for pDC matured either with CpG-B or -C (data not shown), which differ in their capacity to induce type 1 interferon.

In summary, IL-4 induces Eomes expression in human T cells, and IL-4 and IL-12 surprisingly cooperated to induce T cells that expressed Eomes, GzmK, and IFN-γ upon priming with myeloid DC. These in vitro-generated Eomes$^+$ T cells lacked, however, some key properties of Tr1-like cells, but had the same characteristics as the in vivo occurring Eomes$^+$ Th1 cells.

Eomes regulates Tr1 effector molecules jointly with IL-27

To understand if Eomes could regulate effector molecules of Tr1-like cells, we forced the expression of Eomes in in vitro-stimulated naïve CD4$^+$ T cells with lentiviral vectors. The cultures were either supplemented with IL-12 or IL-27, which did not induces Eomes (Fig. 3A and data not shown), but promoted CTL and Tr1 differentiation, respectively. Eomes was induced in the majority of cells by a lentiviral vector encoding full-length human Eomes, but was undetectable in cells transduced with a control vector (Fig. 5A). Of note, T-bet was only transiently upregulated by IL-12, and was undetectable at the analyzed late time points (>d6, data not shown).

Eomes induced a moderate but significant increase of IL-10 production, and cooperated with IL-27, but not with IL-12, to induce high levels of IL-10 (Fig. 5A). Eomes had, however, no

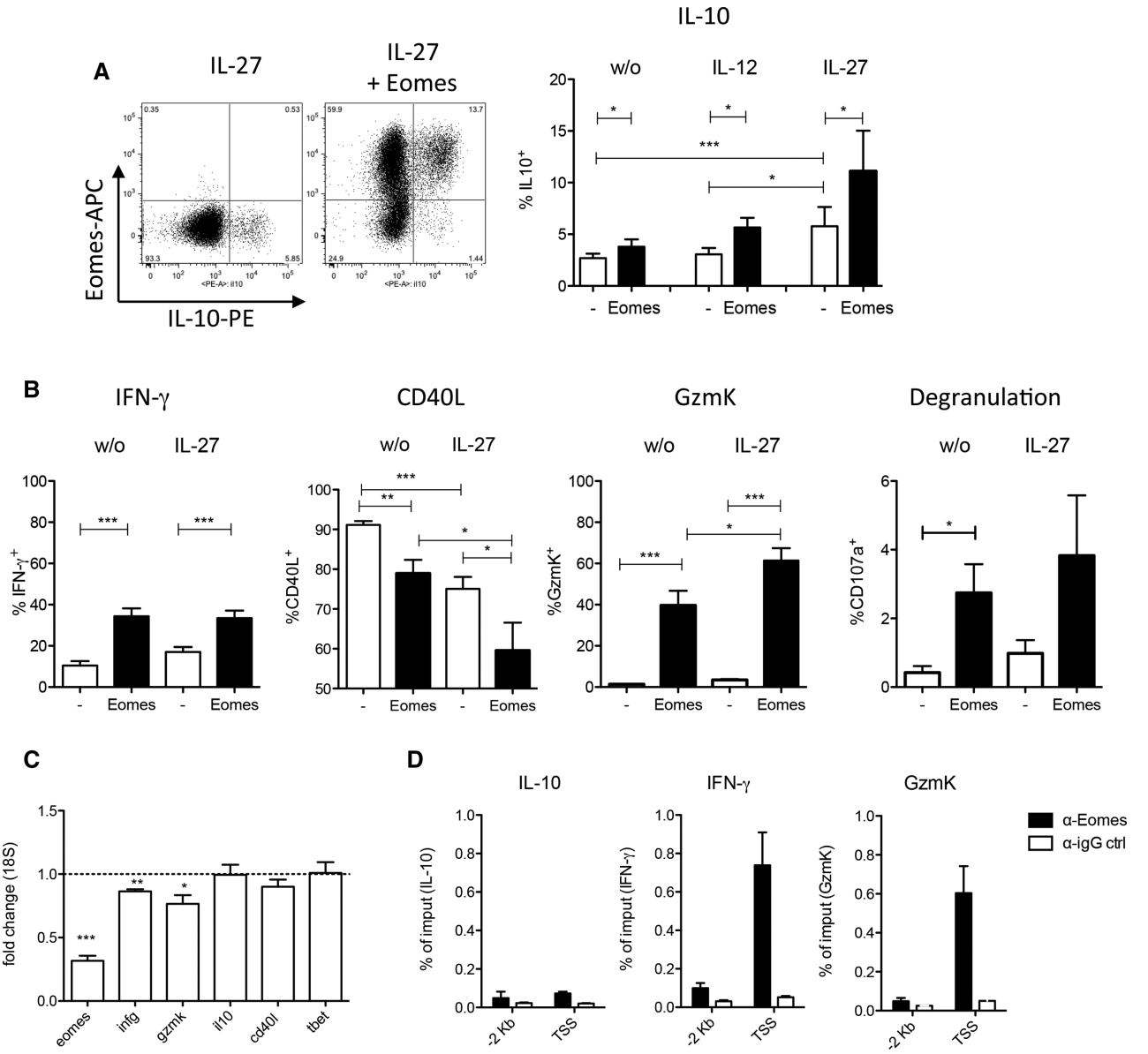

Figure 5. An Eomes controls GzmK and IFN-γ expression, and cooperates with IL-27 to promote Tr1-like properties. (A/B) Naïve human CD4$^+$ T cells were activated with anti-CD3 and -CD28 antibodies with or without IL-12 or IL-27, and viral vectors coding for either GFP and Eomes ("Eomes," black bars) or for GFP only ("–," white bars). Expression of indicated markers was analyzed by flow cytometry (A) Left panel: Eomes versus IL-10 expression in the presence of IL-27. Right panel. Mean percentage of IL-10$^+$ cells in the absence or presence of Eomes and IL-12 or IL-27 (seven experiments with cells from different donors). (B) IFN-γ production, CD40L upregulation, and GzmK expression (six experiments with cells from different donors) or degranulation (CD107a surface exposure; five experiments with cells from different donors) in the absence or presence of Eomes and IL-27 as indicated. (C) Eomes was downregulated with siRNAs in human CCR5$^+$CD4$^+$ T cells and mRNAs for Eomes, IFN-γ, GzmK, IL-10, CD40L, and T-bet measured by RT-PCR (six experiments with cells from different donors). mRNA levels were normalized and the foldchange to scrambled control siRNA was calculated. (D) CHIP analysis of Eomes binding to proximal ("TSS") and distal ("−2kb") regulatory regions (see Supporting Information Fig. 5) of the IL-10, IFN-γ, and GzmK genes in human Tr1 clones (three experiments with the same clone). Data from different experiments were pooled and shown as mean + SEM, */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with One-way ANOVA.

clear effect on the expression of Blimp-1, AHR, and c-Maf mRNAs (Supporting Information Fig. 5A), transcription factors that promote IL-10 production in IL-27-induced Tr1 cells [7, 15, 16]. IFN-γ production was also induced by Eomes (Fig 5B) or by IL-12 (data not shown), and to a lesser degree by IL-27. Importantly, Eomes significantly inhibited CD40L expression and cooperated with IL-27 to efficiently downregulate CD40L (Fig. 5B), while IL-12 was inefficient (data not shown). In addition, Eomes was sufficient to induce high levels of GzmK, while IL-27 (Fig. 5B) and IL-12 (data not shown) alone induced only low levels. Eomes had also a weak positive effect on GzmB, but GzmB expression was low unless IL-12 was added (Supporting Information Fig. 5B). Finally, Eomes-expressing CD4$^+$ T cells also degranulated (Fig. 5B). Overall, these results show that Eomes regulates the expression of

several effector molecules that are characteristic for in vivo occurring Tr1-like cells, and is sufficient to induce IFN-γ and GzmK. In addition, Eomes promoted a switch from CD40L to IL-10 in cooperation with IL-27.

To understand if Eomes was necessary for the expression of Tr1-associated genes, we downregulated Eomes expression with siRNAs in $CD4^+$ $CCR5^+$ T cells, and measured the expression of the most relevant mRNAs by qRT-PCR. Eomes expression was reduced to approximately 30% by specific siRNAs [33], and both IFN-γ and GzmK mRNAs were also significantly reduced (Fig. 5C). In contrast, the levels of CD40L, IL-10, and T-bet control mRNAs were not affected by Eomes downregulation. To understand if Eomes could directly regulate these genes by binding to their promoter regions, we searched for predicted Eomes/Tbox binding sites in the relevant regulatory elements. We identified predicted binding sites in the proximal promoter regions of IFN-γ and GzmK (Supporting Information Fig. 5C). Conversely, the CD40L promoter contained no potential binding sites for Eomes (data not shown). Notably, only the proximal, but not the distal part of the IL-10 promoter was conserved in humans and mice (Supporting Information Fig. 5D). Consequently, two T-box binding sites in the distal part of the murine IL-10 promoter [17] were not conserved in humans. Nevertheless, we also identified a predicted, partially altered Eomes/Tbox binding site in the distal part of the human IL-10 promoter (Supporting Information Fig. 5C). CHIP analysis in cloned Tr1 cells with high IL-10 production (Supporting Information Fig. 5E) revealed that Eomes bound to the proximal promoter regions of IFN-γ and GzmK, but neither to the proximal nor to the distal analyzed promoter regions of IL-10 (Fig. 5D). Similar results were obtained with naïve $CD4^+$ T cells that were forced to express Eomes (data not shown).

Overall these results indicate that IFN-γ and GzmK are directly controlled by Eomes in human $CD4^+$ T cells. Conversely, IL-10 and CD40L are probably not regulated in a direct manner, which could explain why their expression depends more heavily on IL-27.

Eomes regulates chemokine receptor expression and antagonizes the Th17 fate

Since $Eomes^+$ Tr1-like cells had a characteristic IL-$7R^-CCR5^+PD1^+CCR6^-$ phenotype (Fig. 2B), we analyzed whether the expression of these membrane proteins was regulated by Eomes or by IL-27. Eomes induced CCR5 in $CD4^+$ T cells, and collaborated with IL-27 (Fig. 6A) and with IL-12 (data not shown) to induce high levels of CCR5. In contrast, IL-7R expression was not affected by Eomes expression, whereas IL-27 had a significant inhibitory effect (Fig. 6A). Although $Eomes^+$Tr1-like cells expressed high levels of PD1 (Supporting Information Fig. 6A), forced expression of Eomes actually inhibited PD1 expression (Fig. 6B), while IL-12 or IL-27 had no effect (data not shown). Similar results were obtained for CTLA-4 (data not shown), another checkpoint receptor that is highly expressed by Tr1-like cells [25, 26]. Conversely, surface expression of LAG3 was low on $Eomes^+$Tr1-like cells (Supporting Information Fig. 6B), despite of the fact that LAG3 was among the most upregulated genes in Tr1-like cells (Fig. 1A). This was not unexpected, since $IL\text{-}7R^-$ Tr1-like cells express LAG-3 mRNA, but they barely express LAG3 protein on the cell surface [27]. Of note, the large majority of $LAG3^+$ $CD4^+$ T cells were $IL\text{-}7R^+$ (Supporting Information Fig. 6C), and only a small fraction expressed Eomes (data not shown). Thus, $LAG3^+CD4^+$T-cells contain some $Eomes^+$ Th1-cells, but they show virtually no overlap with $Eomes^+IL\text{-}7R^-$ Tr1-like cells.

As Tr1-like cells expressed neither IL-17 nor CCR6, we analyzed if Eomes could antagonize human Th17 differentiation. Since in vitro human Th17 differentiation is inefficient [38] and CCR6 upregulation is unstable [39], we assessed if forced expression of Eomes could suppress Th17 signature genes in ex vivo isolated $CCR6^+IL\text{-}7R^+$T-cells, which contain in vivo differentiated Th17 cells [40]. Indeed, Eomes strongly inhibited CCR6 expression (Fig. 6C), as well as production of IL-17A/F (Fig. 6C) and IL-22 (data not shown). Moreover, Eomes significantly reduced the expression of RORC and RORA mRNAs (Fig. 6D), which code for the lineage-defining transcription factors of Th17 cells. Finally, even under optimal Th17 polarizing conditions [38], Tr1-like failed to acquire IL-17 producing capacities, and maintained Eomes and GzmK expression (data not shown).

In conclusion, Eomes induces CCR5 and inhibits CCR6 expression, consistent with the $CCR5^+CCR6^-$ phenotype of in vivo occurring $Eomes^+$Tr1-like cells. In addition, Eomes blocks the RORA/C transcription factors and the production of Th17 cytokines, explaining why Th17-associated genes are strongly downregulated in $Eomes^+$Tr1-like cells.

$Eomes^+$ Tr1-like subsets are present in human tissues and are modulated in immune-mediated diseases

We previously identified Tr1-like cells also in human lymphoid [26] and nonlymphoid tissues [27], and we, therefore, assessed if these tissue-derived Tr1 cells also expressed Eomes. Indeed, Eomes was highly expressed by IL-10 producing $IL\text{-}7R^-$T cells in human tonsils, but was hardly detectable in tonsillar $IL\text{-}10^-IL\text{-}7R^-$, $IL\text{-}7R^+$, or $CD25^+$ control populations (Fig. 7A). These tonsillar Tr1-like cells also expressed GzmK and IFN-γ (Supporting Information Fig. 7B). A caveat in human tonsils is, however, that they contain activated follicular helper T cells ("T_{FH}"), which have also downregulated IL-7R expression [41] and produce IL-10. However, analyzing T_{FH}- and Tr1-like cells according to their specific surface markers (Supporting Information Fig. 7B) revealed that Eomes was expressed selectively in tonsillar Tr1-like cells, but not in T_{FH} cells (Fig. 7B). Moreover, BCL6 was as expected expressed by T_{FH}-cells, but was hardly detectable in tonsillar Tr1-like cells (Supporting Information Fig. 7C). In the intestinal lamina propria, $CD4^+IL\text{-}7R^-$ T cells expressed high amounts of Eomes (Fig. 7C). As observed in the blood (Fig. 2D), both in the lamina propria (Fig. 7C) and in intestinal lymph nodes (Supporting Information Fig. 7D), two subsets of $Eomes^+$T-bet^{lo}Tr1-like cells and of $Eomes^+Tbet^{hi}$CTL were distinguishable. Intestinal

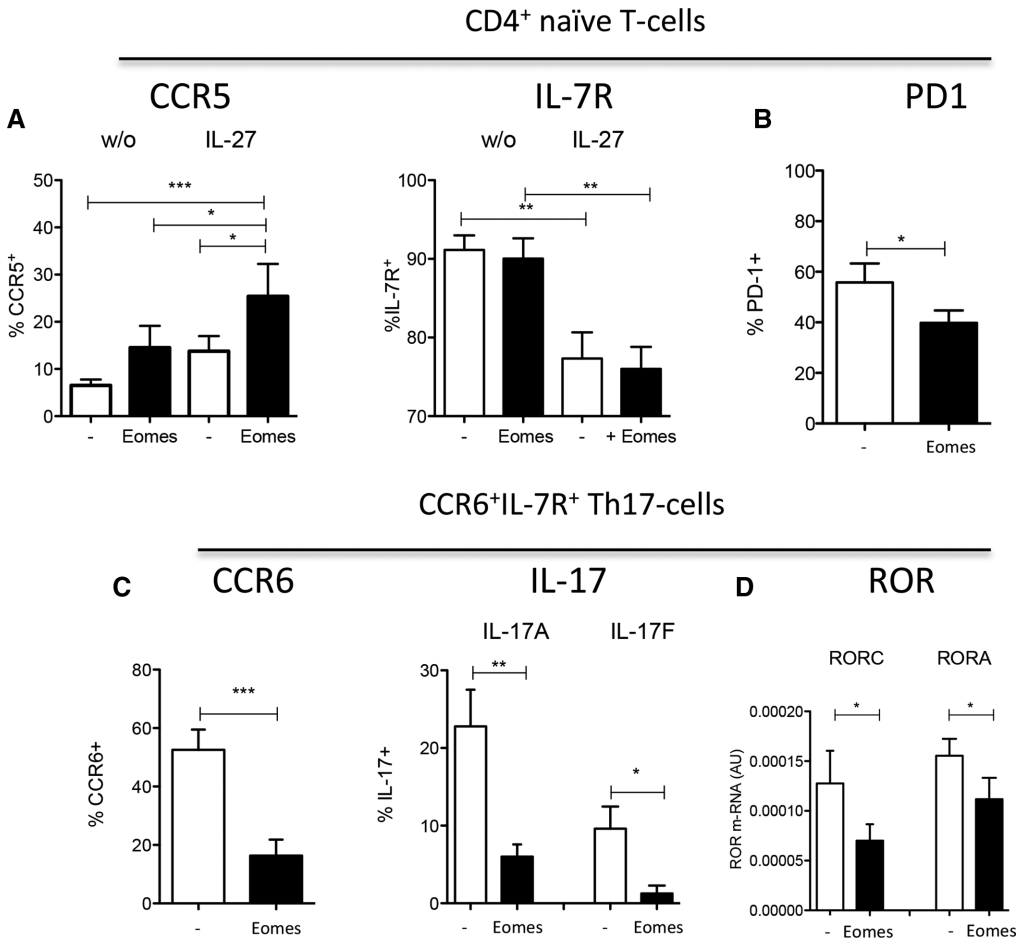

Figure 6. Eomes induces CCR5, but not PD1, and inhibits Th17 signature genes. (A/B) Human naïve CD4$^+$ T cells were transduced with Eomes- and/or GFP- encoding lentiviral vectors and analyzed for phenotypic markers of Tr1-like cells. (A) CCR5 and IL-7R expression in the absence (–) or presence of Eomes or IL-27 (eight for CCR5 and six for IL-7R) experiments with cells from different donors. */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with One-way ANOVA. (B) Effects of forced Eomes expression on PD1 (five experiments with cells from different donors). *$p < 0.05$ according to statistical analysis with paired student's *t*-test. (C/D) Human CCR6$^+$IL-7R$^+$ T cells were stimulated and transduced with lentiviral vectors coding for Eomes and/or GFP and analyzed for features of Th17 cells. Left: CCR6 surface expression and Right: production of IL-17A/F in the absence (–) or presence of Eomes. (D) Expressions of RORC and RORA transcription factors were measured by qRT-PCR. (C/D) Five experiments with cells from different donors. Data from different experiments were pooled and shown as mean + SEM; */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with paired student's *t*-test.

Eomes$^+$Tr1-like cells expressed also high levels of GzmK (Supporting Information Fig. 7E).

Since Eomes$^+$ Tr1-like cells were present in tissues, we analyzed whether the frequencies of Eomes$^+$ subsets in the CD4 compartment were altered locally or systemically in immune-mediated diseases where Tr1 cells are of therapeutic relevance, namely IBDs and graft-versus-host disease (GvHD) [42, 43]. In the inflamed gut of IBD patients, but also in the healthy control mucosa obtained from colorectal cancer patients (CRC), Eomes$^+$Tr1-like cells were strongly enriched as compared to peripheral blood (Fig. 7D). Intriguingly, IBD patients had significantly lower frequencies of Eomes$^+$IL-7R$^+$ T-cells than healthy individuals, suggesting that Tr1-like precommitment might be compromised. FOXP3$^+$Tregs were, in contrast, increased in the blood of CRC patients (Fig. 7D). Finally, we analyzed Eomes$^+$ and FOXP3$^+$T-cell subsets also in the blood of patients that develop a new immune system following allogenic stem cell transplantation. This is a peculiar clinical condition where Tr1 cells were first described, are increased and could potentially inhibit GvHD [43]. In these patients, Eomes$^+$ Tr1-like cells were indeed significantly increased (Fig. 7E). In a smaller cohort of patients, we also observed an increase of GzmK$^+$CD4$^+$ T cells (Supporting Information Fig. 7F). Notably, we detected highly variable frequencies of IL-7R$^+$Eomes$^+$ T cells, which were very abundant in some patients. Foxp3$^+$Tregs showed much less variability and were only moderately increased (Fig. 7E).

We conclude that Eomes$^+$Tr1-like cells are present in human lymphoid and nonlymphoid tissues, and are enriched in the gut. Moreover, the frequencies of Eomes$^+$ Tr1-like cells or their potential precursors were systemically altered in two prototypical clinical conditions where Tr1 cells play a critical role. Finally, the strong variability of Eomes$^+$ Tr1-like subsets in patients suggests that their in vivo homeostasis is highly dynamic, and that they should thus be included in monitoring strategies to assess the equilibrium between regulatory and effector T cells in diseases.

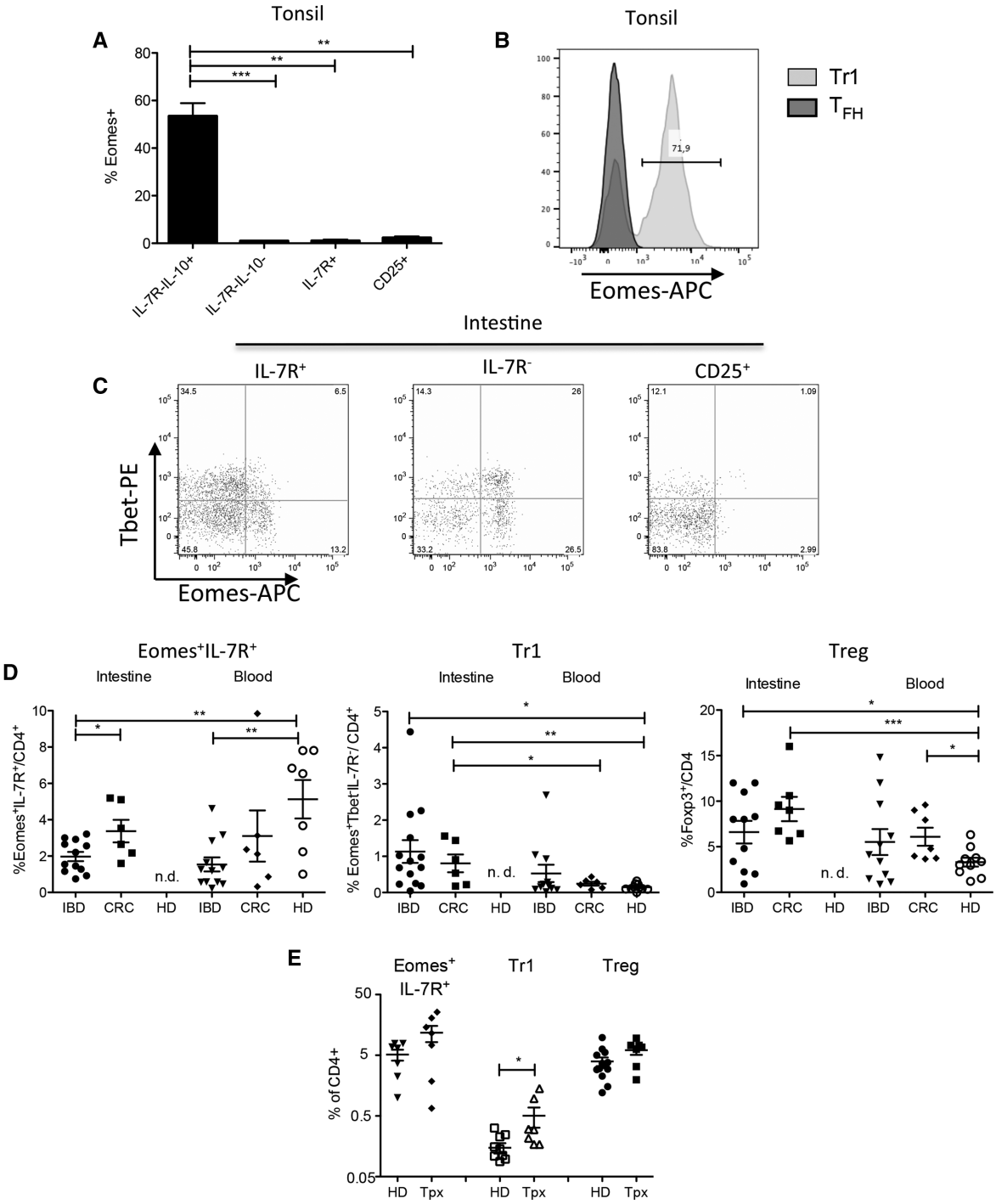

Figure 7. Eomes$^+$ T-cell subsets are present in human solid tissues and are modulated in Tr1-relevant clinical settings. (A) Purified tonsillar CD4$^+$IL-7R$^+$, CD25$^+$IL-7R^{lo}, and IL-7R$^-$CD25$^-$ subsets gated according to IL-10 expression were analyzed for the expression of Eomes (five experiments with cells from different patients). (B) Tonsillar IL-7R$^-$CCR5$^+$CXCR5$^-$ICOS$^-$Tr1-like cells and IL-7R$^-$CCR5$^-$CXCR5$^+$ICOS$^+$T$_{FH}$-cells were analyzed for Eomes expression. (C) CD4$^+$ T-cell subsets gated according to the expression of IL-7R and CD25 from the intestinal lamina propria were analyzed for T-bet and Eomes expression. (D) Frequencies of IL-7R$^+$Eomes$^+$ T-cells (left panel), Tr1-like cells (central panel), and FOXP3$^+$Tregs (right panel) among CD4$^+$ T cells in the inflamed intestinal lamina propria (intestine) of IBD patients ("IBD", 14 (Tr1), 12 (Eomes+IL-7R$^+$), or 11 (FOXP3$^+$Tregs) patients analyzed in different experiments) or the healthy, noninflamed lamina propria of CRC control patients ("CRC", six experiments with cells from different patients). Eomes$^+$ and FOXP3$^+$ T-cell subsets were also analyzed in peripheral blood ("blood") of the same patients and compared to healthy donors (HD, seven experiments with cells from different donors); n.d., not determined. (E) Eomes$^+$ and Foxp3$^+$ subsets among CD4$^+$ T cells in healthy donors ("HD", five experiments analyzing cells from nine donors) and in patients who received stem cell transplantation ("Tpx," seven experiments with cells from seven different patients). Frequencies of Eomes$^+$IL-7R$^+$ cells, of Eomes$^+$T-bet$^-$IL-7R$^-$Tr1-like cells ("Tr1"), and of Foxp3$^+$Tregs ("Treg") are shown. Data from different experiments were pooled and shown as mean + SEM; */**/***$p < 0.05/0.005/0.00005$ according to statistical analysis with One-way ANOVA.

Discussion

Regulatory T-cell subsets are required to prevent overshooting immune responses. It is increasingly recognized that, besides, Foxp3$^+$Tregs, also Tr1-cells play a crucial and sometimes nonredundant role [4, 5]. However, while the molecular identity of Foxp3$^+$Tregs is well established, the biology of in vivo occurring human Tr1 cells is poorly understood. We showed here that Eomes acted as a lineage-defining transcription factor in human Tr1-like cells. Moreover, we identified putative precursor cells and identified the APC and cytokine conditions that could induce Eomes$^+$ precursor and effector Tr1-like cells.

Several transcription factors have been proposed to regulate IL-10 production in murine Tr1 cells, but the transcriptional regulation of in vivo occurring human Tr1-like cells remains an understudied area. We performed here the first detailed molecular characterization of in vivo occurring IFN-γ and IL-10 coproducing Tr1-like cells, allowing their unequivocal identification and their distinction from all other CD4$^+$ T cells. Key to their biology is Eomes, the lineage-defining transcription factor of cytotoxic lymphocytes. Consistent with a recent report in mice [17], our results suggest that Eomes acted as a lineage-defining transcription factor in human Tr1 cells. The induction of cytotoxicity and the suppression of Th17 differentiation by Eomes [44], as well as the expression of Eomes in IL-10 and IFN-γ coproducing regulatory T cells appear to be conserved between humans and mice [17]. Conversely, our results suggest that the regulation of IL-10 by T-box transcription factors differs in humans and mice, and whether murine Tr1 cells also express GzmK remains to be shown. In human Tr1-like cells, Eomes establishes a unique cytotoxic differentiation program, which is characterized by the expression of GzmK, IFN-γ, and CCR5. In the CD4 compartment Eomes, CCR5 and IFN-γ are also expressed by conventional CTL. Conversely, GzmK expression was characteristic for Tr1-like cells and their putative precursor cells in humans, and is, thus, the most powerful single marker to identify in vivo occurring Tr1-like cells. Surprisingly, GzmB was not expressed in human Tr1-like cells in vivo, but was selectively expressed in conventional CD4$^+$CTL. Thus, GzmB is not only not a marker of in vivo occurring Eomes$^+$Tr1-like cells, but is on the contrary useful to distinguish them from conventional CTL.

Besides IL-10 and GzmB, also checkpoint receptors are characteristic for Tr1 cells. Since they are expressed on the cell surface they could be exploited to purify viable Tr1 cells [8, 26]. Checkpoint receptors are, however, also expressed on other activated T-cell subsets, and Eomes neither induce PD1 nor CTLA4. LAG3 was proposed to be more specific for Tr1 cells [8, 9], but a limitation in humans is that LAG3 protein has to be induced on the surface of Eomes$^+$Tr1-like cells by in vitro TCR stimulation [27]. The fact that human LAG3$^+$T-cells possess regulatory functions [8], but are distinct from Eomes$^+$Tr1-like cells, suggests that additional Tr1-like populations might exist. However, since they lack Eomes they are expected to have a different biology. Importantly, the Tr1-associated chemokine receptor pattern CCR5$^+$CCR6$^-$ [26] was determined by Eomes, consistent with the notion that chemokine receptors are powerful in vivo differentiation markers of human T-cells [13, 29]. Together with IL-7R, CD25 [25], and CD27, these chemokine receptors allow to sort unstimulated Eomes$^+$Tr1-like cells with high purity.

In mice, Tr1-cells could be generated in vivo with nanoparticles from precommitted precursors, possibly Th1 cells [45]. Consistently, the potential Tr1 precursor cells that we identified in humans had a $Th1_{EM}$ phenotype, produced IFN-γ and expressed Eomes and GzmK. Unlike Tr1-like cells, however, they were resting and expressed IL-7R and CD40L, but not IL-10. Intriguingly, all these missing features of Tr1-like effector cells could be induced by TCR stimulation in the presence of IL-27, a well-known condition to induce Tr1-cells. However, IL-27 failed to induce Eomes in human T cells, suggesting that it induces Eomes$^+$ Tr1-like cells from Eomes$^+$ Th1 precursors rather than from naive T cells [29]. It seems likely that the putative IL-7R$^+$ Tr1 precursors express not only Eomes and GzmK, but have also the same phenotype as Eomes$^+$ Tr1-like cells, i.e. expression of CCR5, CD27, and PD1, but not of CCR6. Notably, an accompanying article by Mazzoni et al. shows that Th17-derived "nonconventional" Th1 cells that coexpress Eomes and CCR6 possess proinflammatory properties [46], suggesting that CCR6$^+$Eomes$^+$Th1-cells are indeed more distantly related to anti-inflammatory Tr1 cells. Nevertheless, since they already express Eomes, they could transdifferentiate more efficiently to Eomes$^+$ Tr1-like cells upon resolution of inflammation [47]. Cells with characteristics of Eomes$^+$ Tr1-like precursor cells could be induced by IL-4 and mature DC, IL-12 secreting myeloid DC, consistent with the view that Eomes$^+$ Tr1-like cells are generated in immune responses against pathogens [23, 25]. IL-4 and IL-12 are known to act antagonistically to induce Th2 and Th1 differentiation, respectively, and IL-4 blocks, in addition, the induction of conventional CTL. However, IL-4 was previously shown to induce Eomes in other T-cell subsets, including human CD8$^+$ and γ/δ T cells [31–34], suggesting that the induction of Eomes is similarly regulated in all human T-cell subsets. In addition, CD56^{bright} NK cells, which possess regulatory properties, express high levels of GzmK [48] (and data not shown), suggesting that GzmK might be a universal marker of lymphocytes with regulatory functions. IL-4 can be induced upon tissue damage via IL-33, in particular in the gut [49]. Thus, IL-4 and IL-12 could collaborate to induce Tr1-like commitment in response to tissue damage induced by pathogens. Interestingly, IBD patients had reduced frequencies of Eomes$^+$ Th1 cells, suggesting that Tr1 commmittment might be affected. In addition, Tr1-like cells downregulate IL-10 production in the inflamed gut of IBD patients [27], and the Tr1 response in IBD might, thus, be affected at multiple levels.

In conclusion, we provided here a molecular blueprint of human IL-10 and IFN-γ coproducing Tr1-like cells. The identification of Eomes and GzmK as Tr1-associated markers in humans will greatly facilitate their study in immune-mediated diseases in the future.

Materials and methods

Human samples and patients

Buffy-coated blood of healthy donors and tonsils specimens were obtained from the IRCCS Policlinico Ospedale Maggiore, Milan, Italy and the Deutsches Rotes Kreuz (DRK), Germany), as described [26]. The ethical committee approved the use of tonsil specimen for research purposes (permission EA1/107/10). Intestinal specimens were obtained from IBD or CRC patients undergoing therapeutic resection as described [27]. The ethical committee approved the use of specimens for research purposes (permission n. 2476) and informed consent was obtained from patients. The cohort of IBD was composed of 12 severe patients, six with Crohn's Disease and six with ulcerative colitis who were not treated with anti-TNF antibodies. The cohort of transplanted pediatric patients was composed of 7 patients, 3 were transplanted with cord-blood derived stem cells and 4 with mobilized, circulating $CD34^+$ stem cells from a first-degree relative. Five patients developed GvHD, one chronic GvHD and four acute GvHD of grade I or II. Three patients experienced viral reactivations, two with CMV and one with HHV6.

Cell isolation

Mononuclear cells from PBMCs, tonsils, and the intestinal lamina propria molecular cells were isolated as described [26, 27]. $CD4^+$T-cell subsets were purified by cell sorting on a FACSAria (BD Biosciences) based on CD25 and IL-7R marker expression into IL-$7R^+CD25^{-/lo}$ helper T cells, IL-$7R^-CD25^-$ Tr1-containing cells, and IL-$7R^{lo}CD25^+$Tregs. IL-$7R^-$ cells were further subdivided into $CCR5^+CCR6^-CD27^+$Tr1-like cells and $CCR5^+CCR6^-CD27^-$CTL. $CD1c^+$DC and pDC were enriched with magnetic beads (Miltenyi) and purified by cell sorting as described [37].

Flow cytometry

Flow cytometry was performed according to the published guidelines [50]. T cells were stained at the cell surface with the following antibodies: CD4 (RPA-T4, BD), CCR5 (27D, BD), PD-1 (MIH4, Biolegend, San Diego, CA), CD25 (M-A251, Biolegend), CD127 (eBioRDR5, eBioscience), LAG3 (REA351, Miltenyi), CXCR3 (1C6, BD), CCR6 (R6H1 Ebioscience), CRTH2 (BM16, Miltenyi), CD27 (L128, BD), and CD45RA (HI100, BD). For the analysis of intracellular proteins, cells were fixed and permeabilized and stained intracellularly for GzmA (Cb9, BD), GzmB (GB11, BD), GzmK (GM6C3, Santa Cruz), Eomes (WD 1928, Ebioscience), FOXP3 (PCH101, Ebioscience), GATA3 (L50-823, BD), T-bet (O4-46, BD), BCL6 (K112-91, BD), or the proliferation marker KI67. Cytokine production was assessed by intracellular staining after stimulation with 0.1 μM phorbole ester (PMA) and 1 μg/mL ionomycin (both from Sigma–Aldrich) in the presence of 10 μg/mL Brefeldin A (Sigma). Cells were fixed, permeabilized, stained, and analyzed by flow cytometry for intracellular IL-17A (BL168, Biolegend), IL-17F (SHLR17, Ebioscience), IL-22 (22 URTI; Ebioscience), IFN-γ (B27, Biolegend), IL-10 (JES-19F1, Biolegend), as well as CD40L (2431, Biolegend) and CTLA-4 (BNI3, BD) expression.

T-cell stimulation

Naïve $CD4^+CD45RA^+$T-cells, $CD4^+CD45RA^-$ memory T-cells, and naive $CD8^+CD45RA^+CD27^+$T-cells were labeled with CellTrace and 10^5 cells per well activated by plate-bound anti-CD3 antibodies (UCHT1, BD; 2 μg/ml) in the absence or presence of 10 ng/mL recombinant IL-12or IL-4 (R&D systems). Cells were analyzed by flow cytometry after 5 days. For priming with DC, $CD1c^+$DC or BDCA-4^+pDC was isolated and either left untreated or matured with a combination of LPS and R848 to induce IL-12 ($CD1c^+$DC) or CpGB/C (pDC) as described [37]. Naïve $CD4^+$ and $CD8^+$ T cells were cocultured with DC at a 1:5 ratio in the absence or presence of 10 ng/mL recombinant IL-4 and 10 μg/mL neutralizing anti-IL-12 antibodies.

Suppression assay

Naïve $CD4^+$ T cells were sorted as $CD4^+CD45RA^+$ and labeled with CellTrace Violet (Life Technologies). A total of 2.5×10^4 naive T cells were cocultured at different ratios with sorted Tregs, Tr1-like cells, or CTL. A total of 5×10^3 allogenic $CD1c^+$DCs were added as stimulators. Unlabeled naive cells were used as a negative control to assess specific suppression. After 4–5 days, cells were stained and analyzed for CellTrace Violet dilution by FACS.

Generation of T-cell lines and clones

Tr1-like cells were isolated according to phenotypic markers (single cells were positioned with a FACSAria in wells containing 10^6/mL PBMC from five different donors and 2×10^5/mL Rosi-EBV cells as feeder cells, 1 μg/mL anti-CD3 antibodies (OKT3) and 200 U/mL IL-2 in RPMI 10% FCS. Fresh medium was added every 3 days. Clones were used after 3–4 weeks and restimulated with the same protocol every 4–6 weeks.

Cytotoxicity assays

Ex vivo isolated or cloned Tr1-like cells were incubated with $CD1c^+$DC as target cells in the absence or presence of Staphylococcus enterotoxin B (SEB) at a 1:1 ratio. Degranulation was assessed by surface exposure of the lysosomal protein CD107a after 16 h. Cytotoxicity of Tr1 clones was assessed by staining of $CD1c^+$DC with AnnexinV and propidium iodide. To assess cytotoxicity of Tr1 cells ex vivo, $CD14^+$ monocytes were isolated with anti-CD14

beads (Miltenyi) from peripheral blood. They were either incubated alone, or with *ex vivo* isolated Tr1-like cells and SEB at a 1:1 and 5:1 Effector–Target ratio for 16 h. UV irradiation was used as a positive control (data not shown). Percentage of viable $CD14^+$monocytes that were negative for DAPI and LIVE/DEAD dye (Invitrogen) in the absence of T cells was set to 100%.

Lentivirus-mediated EOMES gene transfer in primary T cells

Purified $CD4^+$ naive or Th17-enriched $CCR6^+IL\text{-}7R^+$ T-cells were activated at a density of 10^5 cells per well in 96-well MaxiSorp plates (Nunc) coated with anti-CD3 (0.1 μg/mL; UCHT1; BD) and anti-CD28 (6 μg/mL; CD28.2; BD) and IL-2 (20 IU/mL; Novartis). In some cases, 10 ng/mL IL-12 or IL-27 was added. Lentiviral particles were produced according to a standard protocol (System Biosciences User Manual). T cells were simultaneously activated and transduced with either GFP control lentiviral vector or a lentiviral vector encoding whole length wildtype EOMES at a multiplicity of infection of 1×10^7 transducing units/mL. Cells were detached on day 3, and transduction efficiency was assessed by flow cytometry on day 4 as the frequency of GFP^+ cells (normally >50%). Transduced cells were then transferred to uncoated wells and were cultured in complete RPMI medium. After 7–14 days cells were analyzed by flow cytometry for various proteins according to Eomes expression.

Eomes downregulation in primary $CD4^+$T cells

A total of 10^6 FACS-purified $CD4^+CCR5^+$T-cells were transfected with Eomes siRNA [33] or scrambled control siRNA. Cells were activated with PMA and Ionomycin for 5 h, and RT-PCR for Tr1-associated genes was performed.

In silico prediction of Eomes binding sites

Prediction of Tbox binding sites in the regulatory elements of the human IL-10, GzmK, IFN-γ, and CD40L was done with two different softwares, Jaspar and Matinspector. Sequences with a score >0.85 were considered as candidates.

Chromatin immune precipitation analysis

A total of 10^7 cloned Tr1 cells or lentiviral-transduced $CD4^+$ T cells were activated with PMA and Ionomycin, lysed and DNA shredded. Eomes was immune precipitated and bound DNA revealed with primers was located in the proximal or distal part of the promoters of IL-10 (distal fw 5′-CCCTGTTGGGACAGATGAAAA-3′; distal rev 5′-TTGGCCCTGCCACTCTATAGTC-3′; proximal fw 5′-TGAGAACA GCTGCACCCACTT-3′; proximal rev 5′-TCGGAGATCTCGAAGCAT GTTA-3′), GzmK (distal fw 5′-GCATTCTGATGCGTGATCTG-3′; distal rev 5′-GCCAGGCCTTCAATACAAAA-3′; proximal fw 5′-CC TGAAAGTCCCCAAACTGA-3′; proximal rev 5′-CCACAGGTGCTCT AGGGGTA-3′), or IFN-γ (distal fw 5′-TGCCCCATAACTGCAATA CTG-3′; distal rev 5′- CCTCCACTCTTTGGTTCAAACC-3′; proximal fw 5′-CCAACCACAAGCAAATGATCA-3′; proximal rev 5′-TGGCCCTGGTAAAATGTTGA-3′). Primers specifc for the promoter region of GAPDH (fw 5′-ACAGTCAGCCGCATCTTCTT-3′; rev 5′- TGACTCCGACCTTCACCTTC-3′), as well as isotype control antibodies were used to control specificity of binding.

RNA isolation and mRNA expression profiling

Total RNA Integrity Number (RNA) was isolated using mirVana miRNA Isolation Kit (Ambion) following the standard protocol. Briefly, the lysates were extracted once with acid — phenol chloroform and further purified to yield total RNA with specific miRNA retention. Extracted RNA was quantified with RiboGreen Quantitation Kit (Molecular Probes) on an Infinite F200 plate reader (Tecan Trading AG). All extracted RNA samples were quality controlled for integrity with 2100 Bioanalyzer (Agilent Technologies) and samples with RNA Integrity Number (RIN) lower than eight were discarded. The standard Megaplex protocol (Applied Biosystems) was performed starting from 10 ng of total RNA for each sample with preamplification. Gene expression of whole transcriptome was performed on $CD4^+$ T-cells subsets isolated as described in Supporting Information Fig. 1A, with Illumina Direct Hybridization Assays according to the standard protocol (Illumina Inc.).

Gene expression profiling

Gene expression arrays were quantile normalized, with background subtraction, and average signals were calculated on gene-level data for genes whose detection p value was lower than 0.001 in at least one of the cohorts considered. Normalized data were log2 transformed and presented as z-scores. A one-way analysis of variance (ANOVA) (p value < 0.001) was used to select selectively expressed genes. Statistical tests were performed on MeV software version 4.5.

TaqMan gene expression assays

For assessment of gene expression levels, TaqMan gene expression assays (Thermo Fisher Scientific) were used. 200 ng of total RNA was used for reverse transcription with VILO Reverse Transctiptase (Thermo Fisher Scientific). 1 ng of diluted cDNA was then used as input for RT-qPCR to assess the expression of *EOMES* (Hs 00172872_m1), *IL10* (Hs 00961622_m1), *GZMK* (Hs 00157878_m1), *PRDM1* (Hs 01068508_m1), *AHR* (Hs 00169233_m1), *CD40LG* (Hs 00163934_m1), *TBX21* (Hs 00203436_m1), *FOXP3* (Hs 03987537_m1), and *IFNG* (Hs 00989291_m1). Gene expression levels were normalized on 18s rRNA (Hs 99999901_s1) and reported as arbitrary units.

Statistics

Statistical significance for two variables was calculated using paired two-tailed Student's *t*-test. In the case of multiple comparisons, one-way ANOVA with Tukey's post-test was used. $p < 0.05$(*), $p < 0.005$(**), and $p < 0.0005$(***) were regarded as statistically significant. Error bars reflect ±SEM.

Acknowledgments: This work was supported by the Cariplo and AIRC foundations (Grants nos. 2013-0937 and IG 2015 Id17448).

Conflicts of interest: The authors declare no financial or commercial conflicts of interest.

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Abbreviations: **GvHD**: graft-versus-host disease · **IBDs**: inflammatory bowel diseases

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See accompanying Commentary:
https://onlinelibrary.wiley.com/doi/10.1002/eji.201848000

Received: 28/5/2018
Revised: 28/9/2018
Accepted: 9/11/2018
Accepted article online: 15/11/2018

