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HIGHLIGHTS

Basic

REVIEW

Eomesodermin-expressing type 1 regulatory (EOMES$^+$Tr1)-like T cells: Basic biology and role in immune-mediated diseases

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Type 1 regulatory (Tr1) T cells are currently defined all T cells with regulatory functions that lack FOXP3 expression and produce IL-10. Tr1 cells are heterogeneous, and the different reported properties of Tr1-cell populations have caused some confusion in the field. Moreover, understanding the role of Tr1 cells in immune-mediated diseases has been hampered by the lack of a lineage-defining transcription factor. Several independent studies indicated recently that the transcription factor Eomesodermin (EOMES) could act as a lineage-defining transcription factor in a population of IL-10 and IFN-γ co-producing Tr1-like cells, since EOMES directly induces IFN-γ and cytotoxicity, enhances IL-10, and antagonizes alternative T-cell fates. Here, we review the known properties of EOMES$^+$Tr1-like cells. They share several key characteristics with other Tr1 cells (i.e., "Tr1-*like*"), namely high IL-10 production, cytotoxicity, and suppressive capabilities. Notably, they also share some features with FOXP3$^+$Tregs, like downregulation of IL-7R and CD40L. In addition, they possess several unique, EOMES-dependent features, that is, expression of GzmK and IFN-γ, and downregulation of type-17 cytokines. Published evidence indicates that EOMES$^+$Tr1-like cells play key roles in graft-versus-host disease, colitis, systemic autoimmunity and in tumors. Thus, EOMES$^+$Tr1-like cells are key players of the adaptive immune system that are involved in several different immune-mediated diseases.

Keywords: Cytotoxicity · Eomesodermin · IL-10 · Regulatory T cells · Type 1 regulatory T cells

Introduction

It is well accepted that regulatory T cells inhibit excessive immune responses and are required to prevent autoimmunity. Besides FOXP3$^+$ regulatory T cells (FOXP3$^+$ Tregs [1]), different

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Table 1. Characteristics of Eomes+ Tr1-like cells and other regulatory T-cell subsets

	FOXP3+Tregs	Tr1	EOMES$^+$ Tr1-like
Transcription factors:			
- lineage definining	FOXP3$^+$	FOXP3$^-$ (Egr2$^+$)	EOMES$^+$
- IL10 production		AHR, c-Maf, Blimp-1	Eomes, Blimp-1
Generation	Thymus or Periphery	Periphery	Periphery
Inducing cytokines	TGFβ	IL10 (in vitro), IL27	IL4 (+ IL12, in vitro), IL27
Cytokine profile	IL10$^{lo\text{-}hi}$, TGFβ (GARP) IFNγ$^{lo/-}$, IL4$^-$, IL2$^-$, CD40L$^-$	IL10^{hi}, TGFβ, IL21 IFNγ$^{lo/-}$ IL4$^-$ IL2^{lo}	IL10^{hi}, IFNγ^{hi}, IL21 IL4^{lo}, IL2^{lo}, CD40L$^-$
Surface Markers	CD25$^+$IL-7R^{lo} (CTLA-4)	LAG3, CD49b (TIM3, TIGIT, PD1, CTLA4)	CCR5, PD1, CD27, IL-7R^{lo} (TIM3, TIGIT, CTLA4)
Cytotoxicity	+/- (GzmB+)	+ (GzmB+)	+ (GzmK$^+$GzmA$^+$)
Suppression of	CD4, CD8, B, DC	CD4, B, DC	CD4, CD8, B, DC

populations of regulatory T cells that lack FOXP3 expression have been described and they critically contribute to immune regulation. In particular, CD4$^+$ T cells that produce high amounts of the anti- inflammatory cytokine IL-10 appear to be involved in several immune-mediated diseases. Based on a seminal publication by Roncarolo and colleagues, they are often called type 1 regulatory (Tr1) cells [2]. However, while the molecular features of FOXP3$^+$ Tregs have been defined in molecular detail, the molecular properties of Tr1 cells are not well understood. The current definition of Tr1 cells is very broad [3] and comprises potentially all CD4$^+$ T cells that produce IL-10 and that inhibit immune responses. Thus, the term Tr1 is currently used for several different populations, and this has created inevitably confusion in the field. Indeed, a unique lineage-defining transcription factor for Tr1 cells, similar to FOXP3 in Tregs [1], has not been identified. Moreover, the production of effector T-cell cytokines like IFN-γ and IL-17 by Tr1-like cells, as well as the polarizing conditions that promote Tr1 differentiation, is highly variable in different studies. Notably, some authors refer to IL-10 and IFN-γ or IL-10 and IL-17 co-producing CD4$^+$ T cells with regulatory properties not as Tr1 cells, but as "IL-10 producing Th1-cells" [4] or "regulatory Th17-cells" [5], respectively, indicating that a general consensus on the nomenclature of IL-10 producing regulatory T cells has still to be reached. Recently, we and others showed that the transcription factor Eomesodermin ("EOMES") is highly expressed in a population of IL-10 and IFN-γ co-producing FOXP3$^-$ regulatory T cells, that is, CD4$^+$ T cells that possess Tr1-like properties [6–10]. In this review, we summarize the basic features of these EOMES$^+$Tr1-like cells, with a focus on shared and unique properties as compared to other described regulatory T-cell populations (Table 1), and discuss their critical roles in different immune-mediated diseases.

EOMES determines the cytotoxic fate of lymphocytes

EOMES is a T-box transcription factor that is closely related to T-bet, the lineage-defining transcription factor of Th1 cells [11]. EOMES is highly expressed in cytotoxic lymphocyte lineages, including CD8$^+$ T cells, γ/δ-T cells, and NK cells. The consensus DNA binding sites of T-bet and EOMES are very similar, and, in fact, both transcription factors bind to the IFN-γ promoter and can thus directly induce its transcription [12]. EOMES and T-bet have however also several relevant nonredundant functions [13–15]. Seminal work by S. Reiner and co-workers showed that EOMES controls cytotoxicity in lymphocytes, since it directly induced cytotoxic effector molecules like granzymes (Gzm) and perforin [12]. Moreover, EOMES antagonizes alternative differentiation fates [6, 15–18], and could thus be regarded as a lineage-defining transcription factor of all cytotoxic lymphocytes. CD4$^+$ T cells exert predominantly helper rather than cytotoxic functions, but also CD4$^+$ T cells can upregulate cytotoxic effector molecules upon in vitro TCR stimulation [18, 19]. Moreover, a small fraction of in vivo occurring CD4$^+$ T cells expresses EOMES and cytotoxic effector molecules and possess consequently cytotoxic functions [20]. Notably, there is a rather broad consensus in the field that Tr1 cells possess cytotoxic functions [6, 19, 21]. Three groups reported independently that EOMES is highly expressed in populations of IFN-γ and IL-10 co-producing Tr1-like cells in mice and humans, and acted as a lineage-defining transcription factor [6–10]. EOMES$^+$Tr1-like cells have a very characteristic gene expression profile, which is conserved in humans and mice and that is distantly related to conventional cytotoxic CD4$^+$ T cells [6–10]. However, while FOXP3 expression is sufficient to identify CD25$^+$ Tregs, EOMES is not uniquely expressed in EOMES$^+$ Tr1-like cells [6], but also in conventional cytotoxic CD4$^+$ and CD4$^-$ lymphocytes (CTL) and in some Th1-cells. Moreover, EOMES programs cytotoxicity, but regulatory functions, namely, IL-10 production, may also be regulated by external cues. Therefore, additional markers besides Eomes are necessary to identify Eomes$^+$Tr1-like cells (Table 1).

Features of EOMES$^+$Tr1-like cells

Cytokine production

Tr1-cells were originally defined by their cytokine profile in humans and mice (Table 1). They produced high levels of IL-10, as well as TGF-β, but not of effector cytokines that are characteris-

tic for Th1 and Th2 cells, that is, IFN-γ and IL-4 [2, 3]. However, in particular the production of IFN-γ in Tr1 cells turned out to be highly variable [22, 23]. Notably, since the original concept proposed that Tr1 cells produce little IFN-γ, $CD4^+$ T cells that co-produced IL-10 and IFN-γ were often not called Tr1 cells, even if they possessed potent regulatory functions [4, 24-27]. In 2009, we identified a subset of IL-10 and IFN-γ co-producing T cells with IL-10-dependent regulatory functions in human peripheral blood [22]. They had characteristics of activated effector T cells and responded selectively to persistent self- and foreign antigens, suggesting that they were generated upon chronic TCR stimulation. Notably, the latter appears to be a general feature of Tr1 cells, because Tr1 cells that lack IFN-γ production in allergic patients reacted with persistent or recurrent antigens (i.e., allergens) [23]. Later, we showed that the IL-10 and IFN-γ co-producing Tr1-like cells expressed high levels of EOMES, which directly induced IFN-γ production and blocked type 17 cytokines [6, 9, 10, 16, 18]. Circulating human $EOMES^+$ Tr1 cells produce high levels of IL-21, but only low levels of IL-2, which is entirely consistent with the cytokine profiles of other Tr1 cells (Table 1). $EOMES^+$ Tr1-like cells were also identified in mice [9, 10]. They can produce some IL-4, which is incompatible with the original Tr1 concept [2]. However, it might be explained by the finding that EOMES is induced by IL-4 in T cells in humans and mice [6, 28–30], which induces its own production. Finally, $EOMES^+$ Tr1-like cells largely lack the capacities to produce IL-17, GM-CSF, and IL-22 [6, 9, 10]. Thus, $EOMES^+$Tr1-like cells have a peculiar cytokine profile that is distinct from the originally described Tr1 cells that lack IFN-γ and IL-4 production, and also from IL-10 and IL-17 co-producing T cells with regulatory functions [5]. In addition, IL-22 was recently reported to be produced by Tr1 cells, defined in this study as all $FOXP3^-CD4^+$ T cells that secreted IL-10, and Tr1-cell derived IL-22 promoted tissue repair in the gut [31]. EOMES expression was not analyzed in these IL-22 and IL-10 co-producing T cells, but since EOMES blocks IL-22 production in $CD4^+$ T cells [6, 10], it seems unlikely that they were $EOMES^+$. They might however be closely related to IL-10 producing memory T cells that express the Th17-associated chemokine receptor CCR6, which can produce high levels of IL-2 and IL-22 [32]. Depending on the context, these IL-10 producing memory T cells can possess regulatory or helper functions. In particular, they can exert IL-10-dependent B helper functions and promote autoantibody generation [33]. It could thus be argued that they do not represent professional regulatory T cells. Notably, also in vitro-induced Tr1 cells were reported to possess B helper functions [34], but in vivo differentiated Tr1 cells were in contrast found to suppress B-cell responses in two independent studies [35, 36]. In the case of $EOMES^+$Tr1-like cells, the capacity to inhibit B-cell responses critically depends on the inability to upregulate CD40L [35], a feature that they share with $FOXP3^+$ Tregs [37], but not with IL-10-producing helper T cells [33, 38]. CD40L expression in Tr1 cells that lack Eomes expression is largely unknown, but human IL-10-producing T cells that lack FOXP3 and EOMES express high levels of CD40L. Thus, loss of CD40L may be restricted to $FOXP3^+$ Tregs and $EOMES^+$ Tr1-like cells. In summary, the cytokine profile of $EOMES^+$Tr1-like cells is consistent with the view that they represent Tr1-like cells, but shows also some unique features, in particular the high EOMES-dependent production of IFN-γ and the downregulation of CD40L and type 17 cytokines (Table 1).

Phenotypic markers

Surface markers are critical to track T-cell subsets and isolate viable cells ex vivo for functional studies, in particular in the human system. The most widely used surface marker for Tr1 cells is the co-inhibitory receptor LAG3, alone [39] or in combination with CD49b [40] (Table 1). However, a combination of surface markers appears to be better suited to track Tr1 cells [41]. Human blood IL-10 and IFN-γ co-producing Tr1-like cells were originally identified according to IL-7R downregulation [22], a phenotype that is shared with $FOXP3^+$ Tregs [42] (Table 1) and promoted by chronic TCR stimulation [43]. However, in contrast to $FOXP3^+$Tregs, $EOMES^+IL\text{-}7R^-$Tr1-like cells largely lack CD25 [1]. Moreover, absence of IL-7R and CCR6 distinguishes $EOMES^+$ Tr1-like cells also from other IL-10 producing T cells, such as regulatory Th17 cells in mice [5] and the above-discussed context-dependent regulatory/helper T cells in humans [32, 33]. Notably, CCR6 expression is inhibited by EOMES in human CD4+ T cells in vitro, whereas IL-7R downregulation is promoted by IL-27, but not by EOMES [6]. However, EOMES and T-bet promote IL-7R downregulation in murine NK cells in vivo [15]. A major limitation of this strategy is that human $CD4^+IL\text{-}7R^-CD25^-CCR6^-$ T cells contain effector T cells that lack regulatory functions. However, $EOMES^+$Tr1-like cells co-express the chemokine receptor CCR5 and the co-inhibitory receptor PD1 [8, 35, 44]. Notably, CCR5 expression is promoted by EOMES and IL-27 [6]. PD1 expression was not increased by forced Eomes expression in human $CD4^+$ T cells [6], but Eomes-deficient mouse $CD4^+$ T cells expressed lower levels of PD1 upon transfer into lymphopenic mice. Thus, Eomes may indirectly promote PD1 expression in vivo [10]. In humans, CCR5 and PD1 co-expression allows to distinguish $EOMES^+$Tr1-like cells from most conventional effector cells [35, 41, 44], with the noticeable exception of $CD4^+$CTL, which also express EOMES. However, $CD4^+$CTL can be easily distinguished from $EOMES^+$Tr1 cells, because the former lack CD27 and CD28 expression [45], suggesting that they are terminally differentiated effector cells. Interestingly, T-bet, but not EOMES, inhibits CD27 expression in NK cells in mice [15], suggesting that also in $CD4^+$CTL the loss of CD27 may be mediated by T-bet. CCR5 and PD1 may be co-expressed with LAG3 and CD49b on Tr1 cells, namely in experimental colitis, and in human T cells producing IL-10 upon sustained stimulation with super-antigens [41, 44]). Moreover, several other co-inhibitory receptors, like Tim-3, TIGIT, and CTLA-4, have been consistently identified in different Tr1-cell populations [46], including $Eomes^+$Tr1-like cells [6, 10, 22]. A caveat of the co-inhibitory receptor LAG3 is that it is also expressed on pro-inflammatory Th1/17 cells in ulcerative colitis [47], and that it can be cleaved from the cell surface

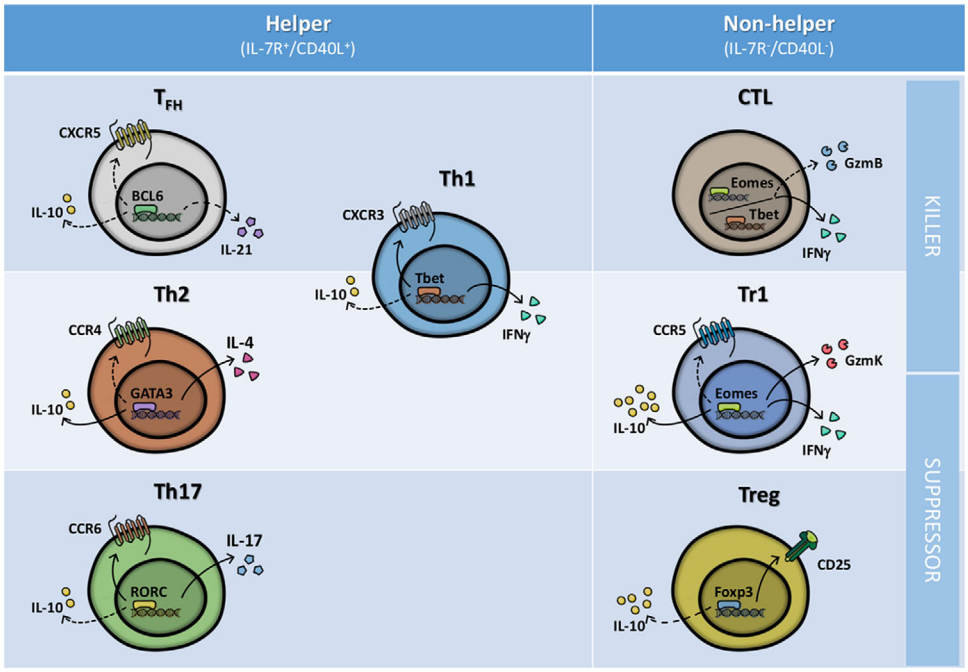

Figure 1. Helper, regulatory, and cytotoxic CD4$^+$ T-cell subsets. CD4$^+$ helper T cells are classified as Th1, Th2, Th17, or TFH cells according to the expression of lineage-defining transcription factors and effector cytokine production. T helper subsets also express characteristic chemokine receptors, but all can produce some IL-10 under permissive conditions. In addition, the CD4$^+$ T-cell compartment also contains regulatory and cytotoxic subsets, namely FOXP3$^+$Tregs and "terminally differentiated" CD4$^+$CTL that co-express T-bet and EOMES. EOMES$^+$Tr1-like cells possess both regulatory and cytotoxic functions, lack T-bet and produce very high levels of IFN-γ and IL-10.

upon T-cell activation [48]. Presumably for the latter reason, ex vivo analyzed human EOMES$^+$Tr1-like cells express in most cases only low levels of LAG3 protein on the cell surface, in spite of the fact that they express high levels of LAG3 m-RNA [6, 44]. Consequently, LAG3 surface expression is not a reliable strategy to identify human EOMES$^+$Tr1-like cells. Furthermore, although CD49b is expressed on the majority of human Eomes$^+$Tr1-like cells in peripheral blood, it is expressed at lower levels in lymphoid and nonlymphoid tissues [45]. CD49b and/or LAG-3 co-expression may however be used to track Eomes$^+$Tr1-like cells in mice [10, 41, 44], although not in all experimental systems [9].

Inductive cues: Cytokines

IL-10 production by CD4$^+$ Tcells is regulated by cytokines and considered to be the key hallmark of Tr1 cells (Table 1). However, IL-10 can be produced by most CD4$^+$ T-cell differentiation lineages (Fig. 1). Consistently, the canonical polarizing cytokines that induce the differentiation of Th1, Th2 and Th17-cells, that is, IL-12 [49], IL-4 [50], and IL-6 plus TGF-β [51], also induce IL-10. Moreover, IL-10 production by human CD4$^+$ T cells is largely unstable in vitro [25], and IL-1β and IL-23 can downregulate T-cell IL-10 production [44, 52]. Altogether, these findings suggest that IL- 10 production is a tunable T-cell effector function, but not a reliable lineage-defining marker of Tr1 cells [25].

IL-10 itself was originally proposed to induce the differentiation of human Tr1-cells in vitro [53]. Later studies showed, however, that IL-10 was dispensable in mice in vivo [54], but was required to maintain IL-10 producing capacities by FOXP3$^+$ and FOXP3$^-$ regulatory T cells [55]. IL-10R signaling in CD4$^+$ T cells was also dispensable for the in vivo generation of Eomes$^+$Tr1-like cells following hematopoetic stem cell transplantation (HSCT). Nevertheless, IL-10 produced by CD4$^+$ T cells promoted Eomes expression in CD4$^+$ T cells in this model, suggesting that IL-10R signaling in other immune cells was instead critical [9]. In lymphopenic mice with leukemia, IL-10R signaling in transferred CD4$^+$ T cells inhibited the accumulation of EOMES$^+$CD4$^+$ T cells, but promoted their cytotoxic functions [10]. A caveat of the latter study is that CD4$^+$ T cells were deficient for the IL- 10Rβ chain, which is not unique for IL-10 but is also used by IFN-λ.

Regulatory T cells express in general high levels of IL-10Rα, consistent with the concept that IL-10 signaling is important for their function. We showed that IL-10Rα is also highly expressed on human EOMES$^+$Tr1-like cells, and that its expression was inversely associated with miR-125a, which could inhibit IL-10Rα expression [7].

Cytokines that are particularly relevant for IFN-γ and IL-10 co-producing Tr1-cells are IFN-α and IL-27, since they promote both IL-10 and IFN-γ production in CD4$^+$ T cells [53, 56, 57]. IL-27 is however not sufficient to induce Eomes in vitro, neither in humans nor in mice [6, 9]. Conversely, IL-4 was consistently found to induce EOMES expression in different T-cell compartments in humans and mice [6, 28-30], alone or in combination with IL-12 [6, 8, 29]. IL-4 has anti-inflammatory properties [58] and induces IL-10 production in CD4$^+$ T cells [50]. A role for

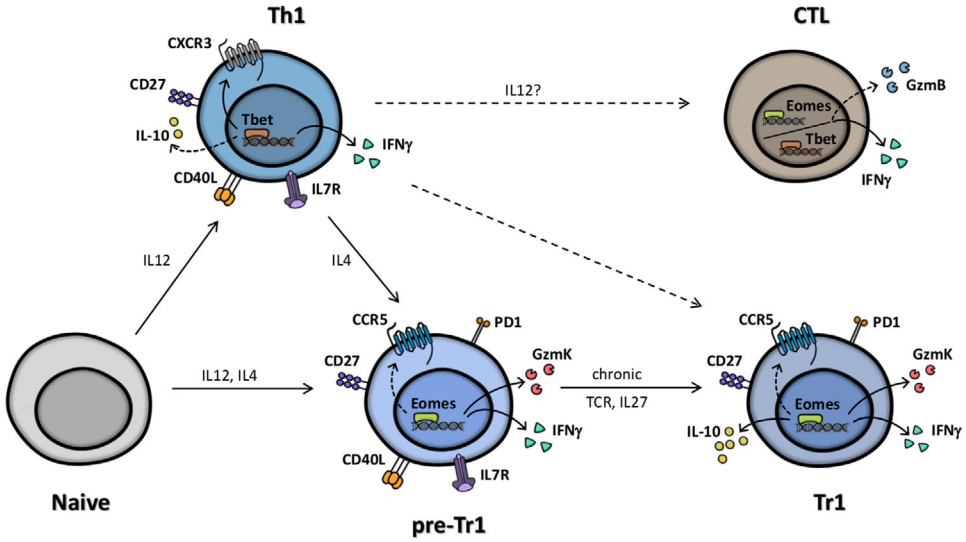

Figure 2. Hypothetical differentiation pathway of EOMES$^+$Tr1-like cells. EOMES$^+$Tr1-like cells could differentiate from T-bet$^+$Th1-cells in response to chronic antigenic stimulation and IL-27. In humans, pre-committed EOMES$^+$GzmK$^+$ "pre-Tr1"-cells are present among Th1 memory cells and could be induced with IL-12 and IL-4 in vitro. EOMES is also expressed in cytotoxic CD4$^+$ T cells that lack IL-10 producing and suppressive capabilities but display a different pattern of cytotoxic molecules and Tbox transcription factors.

IL-4 in the generation of EOMES$^+$Tr1-cells is nevertheless surprising, since it inhibits not only IFN-γ production upon priming, but also GzmB expression and cytotoxicity in in vitro developing human Tr1 cells [59]. IL-4 promotes however a Tr1-like precommitment in established and developing human Th1 cells, since it induces EOMES and GzmK at the dispense of T-bet and GzmB. Of note, the latter are highly expressed in conventional cytotoxic T cells (Fig. 1) [6]. The induction of high levels of IL-10 and other Tr1-relevant features like downregulation of CD40L and IL-7R in human Eomes$^+$CD4$^+$ T cells requires additional factors, such as IL-27 [6]. Indeed, although IL-27 is insufficient to induce EOMES expression in vitro, it is critical for the generation of EOMES$^+$Tr1-like cells in mice in vivo [9].

Inductive cues: APC subsets and TCR signal strength

Besides cytokines, antigen-presenting cell (APC) subsets and maturation state as well as TCR signal strength [60] are critical factors for Tr1-cell generation. Tolerogenic dendritic cells (DCs), in particular immature monocyte-derived DC in the presence of IL-10 [61] or plasmacytoid (pDCs), could induce high levels of IL-10 in CD4$^+$ T cells [61, 62]. Notably, pDC and pDC-derived IFN-α enhanced IL-10 expression also in our hands but failed to induce EOMES [6, 63]. Conversely, dual co-stimulation with OX40 and 4-1BB was reported to induce EOMES and cytotoxicity in CD4$^+$ T cells in the mouse [63, 64]. IL-10 production and regulatory functions of EOMES$^+$T cells were however not assessed in this study. Human CD4$^+$ T cells that co-expressed EOMES, IFN-γ, and GzmK could be primed by TLR-activated, IL-12 secreting myeloid DC (CD11c$^+$CD1c$^{+\prime\prime}$cDC2$^{\prime\prime}$ [65]) in the presence of IL-4 [6]. Notably, IL-4-dependent EOMES induction in T cells is nevertheless promoted by low TCR signal strength [8, 28, 60]. Finally, myeloid CD11c$^+$DC that presented persistent (allo-)antigens also induced Eomes$^+$Tr1-like cells in mice following HSCT [9]. Interestingly, IL-27, which was required to induce Eomes$^+$Tr1-like cells in this model, was derived from macrophages and not from DC [9]. In conclusion, EOMES and IL-10 induction in CD4$^+$ T cells have partially different requirements, but they may be acquired sequentially upon chronic T-cell stimulation. A sequential induction of EOMES and IL-10 would also explain why EOMES$^+$Th1-cells produce only low levels of IL-10 (Fig. 2).

Transcription factors

The transcription factor Egr-2 was proposed to play a key role to induce LAG3$^+$Tr1 cells [39]. However, a lineage-defining transcription factor expressed in all Tr1 cells has not been identified (Table 1), consistent with the notion that Tr1 cells are heterogeneous. EOMES acts as a lineage-defining transcription factor in a subset of in vivo differentiated IFN-γ and IL-10 co- producing Tr1-like cells. EOMES induces directly the transcription of IFN-γ in CD4$^+$ T cells [66] and programs stable cytotoxic functions [6, 8, 64]. Moreover, it binds to the promoters of the ROR-γt and IL-17A genes in humans and mice, and it consequently blocks Th17 differentiation [9, 18, 67]. In mice, EOMES was also shown to inhibit the generation of FOXP3$^+$Tregs [9, 17]. Moreover, in mouse Th2 effector memory like-cells, EOMES inhibited selectively IL-5 production by blocking the binding of GATA3 to the IL-5 promoter [68]. Finally, EOMES could also inhibit the expression of GATA3 and IL-4 as well as of T-bet and BCL6 [9]. Thus, EOMES induces a cytotoxic differentiation program also in CD4$^+$ T cells and antagonizes alternative cell fates.

Several transcription factors have been identified that directly induce IL-10 production in T cells [24, 69]. EOMES itself binds

a distal enhancer of the IL-10 gene in mouse T cells [9], which is however not conserved in humans [6]. Nevertheless, EOMES enhanced IL-10 production also in human CD4$^+$ T cells. Importantly, in two different mouse models where EOMES$^+$Tr1-like cells were abundant, EOMES-deficient CD4$^+$ T cells were largely unable to produce IL-10 in vivo [9, 10]. Transcription factors that induce IL-10 production in Tr1 cells generated with IL-27 in vitro are c- Maf, AHR, and Blimp-1 [24, 69]. Forced expression of EOMES inhibited AHR but increased the expression of c-Maf and Blimp-1 in vivo. Moreover, Blimp-1 was required for IL-10 production in EOMES$^+$Tr1-like cells in mice in vivo [9].

IL-10 and IFN-γ producing CD4$^+$ T cells may also express T-bet [4, 27]. Notably, the generation of mouse EOMES$^+$Tr1-like cells was T-bet dependent in vivo [9], suggesting that they are derived from Th1-cells. Consistently, EOMES could be induced in human Th1 memory cells with IL-4 in vitro, but not in other memory subsets [8]. Moreover, TCR clonotype sharing between EOMES$^+$Tr1-like cells and Th1-like cells was detected in human tumors [8]. Human EOMES$^+$Tr1-like cells express only low levels of T-bet [6, 9] and failed—in contrast to Th1 cells—to upregulate T-bet upon TCR stimulation [6]. Altogether these findings suggest that EOMES$^+$Tr1-like cells are derived from Th1-cells (Fig. 2), but switch progressively from T-bet to EOMES expression. This T-box expression pattern distinguishes them also from CD4$^+$CTL, which constitutively co-express high levels of T-bet together with EOMES.

Cytotoxic molecules

Cytotoxicity is a potent immune effector mechanism to eliminate infected or transformed cells, but it can also promote tolerance when APC are killed [21]. Cytotoxic capabilities are a consistently reported feature of Tr1-cells, which are related to the expression of cytotoxic molecules, in particular of GzmB [19, 21]. Notably, however, GzmB is expressed by most cytotoxic lymphocytes, and even FOXP3$^+$Tregs may express GzmB and exert cytotoxicity in vivo [70]. A caveat in the human system is furthermore that GzmB is efficiently induced in CD4$^+$ T cells by in vitro TCR stimulation with anti-CD3 antibodies [19], a condition used to induce Tr1 cells in most early human studies. In marked contrast, in vivo GzmB expression in human CD4$^+$ T cells is restricted to CD4$^+$ CTL [45], which lack IL-10 producing capacities and regulatory functions [6] (Fig. 1). EOMES was initially proposed to induce GzmB directly in mouse CD8$^+$T-cells [12], but a later study suggested instead that GzmB is induced by RUNX3 [14]. Mouse EOMES$^+$Tr1-like cells express some GzmB [10], but at rather low levels [9]. Human EOMES$^+$Tr1-cells express very high levels of GzmA and GzmK, but not of GzmB [6, 45]. However, while GzmA is also highly expressed by CD4$^+$CTL, GzmK is restricted to EOMES$^+$Tr1-like cells (Table 1). Importantly, EOMES binds to the human GzmK promoter and directly induces its expression [6]. Moreover, GzmK was also among the most upregulated genes in EOMES$^+$Tr1-like cells in leukemia-bearing mice [10]. In human blood, GzmK is also expressed in a subset of EOMES$^+$Th1 memory cells, which produce only little IL-10 and efficiently upregulate CD40L [6]. These EOMES$^+$GzmK$^+$Th1-cells may represent pre-committed precursors of EOMES$^+$Tr1-like cells (Fig. 2). Moreover, "unconventional" EOMES$^+$Th1 cells, which are derived from Th17 cells and express CCR6, were reported to have pro-inflammatory functions [18]. The generation of anti-inflammatory EOMES$^+$Tr1-cells from highly pro-inflammatory EOMES$^+$Th1-cells upon persistent antigenic stimulation might represent an important negative feed-back mechanism to inhibit immunopathology [71] (Fig. 2).

Involvement of EOMES$^+$Tr1-like cells in immune-mediated diseases

Graft versus host disease

Tr1 cells have been originally identified among in vitro cloned T cells from patients with graft- versus-host-disease (GvHD) [72], a life-threatening clinical complication that often develops after HSCT. HSCT is a therapeutic approach to induce a novel, healthy immune system, but in the case of allogenic donors results also in the persistent stimulation of T cells by allo-antigens. Clinical trials with FOXP3$^+$Tregs and with in vitro generated Tr1 cells have been performed to treat GvHD [73]. Intriguingly, also EOMES$^+$Tr1-cells were first described in GvHD in mice [9]. They were shown to represent the most abundant regulatory population after HSCT and to be able to inhibit GvHD [9]. As observed in mice, EOMES$^+$Tr1-like cells and their presumed precursors were also significantly increased in human patients after HSCT [6, 9]. These findings raise the provocative question if exclusively EOMES$^+$Tr1-cells protect from GvHD, or if also other Tr1-cell populations contribute.

Colitis and inflammatory bowel diseases

Tr1-cells are of particular relevance for inflammatory bowel diseases (IBDs) and experimental colitis [2]. Indeed, genetic defects in the IL-10/IL-10R pathway results in early, severe colitis in humans and mice [74]. Studies in mice indicated further that T-cells were the relevant source of protective IL-10, and that colitis required an intact intestinal microflora [75]. Conversely, the main intestinal manifestation in patients with genetic defects in FOXP3 is autoimmune enteropathy [76, 77], which is associated with anti-enterocyte autoantibodies in the small intestine. Nevertheless, both Tr1-cells and FOXP3$^+$Tregs can inhibit experimental colitis in an IL-10-dependent manner in vivo [78]. Importantly, human Tr1-like cells were consistently found to possess reduced IL-10 producing capacities in IBD patients [41, 44, 79]. Intriguingly, we found that selectively IL-10 production by IFN-γ producing CD4$^+$ T cells was reduced, while IL-10 in intestinal FOXP3$^+$Tregs and in conventional CD4$^+$ T cells was unaffected [44]. This selective effect on IL-10 and IFN-γ co-producing Tr1-like cells could be explained with their high responsiveness to IL-

23, a key colitogenic cytokine [80] that could downregulate IL-10 production together with IL-1β [44]. As expected, these intestinal IL-10 and IFN-γ co-producing T cells in IBD patients expressed high levels of EOMES [6]. Overall, these findings suggest that EOMES$^+$Tr1-like cells also play a key role in IBDs.

Autoimmunity

Patients that lack FOXP3 expression develop severe, multi-organ autoimmune manifestations known as Immune dysregulation, poly-endocrinopathy, enteropathy, and X-linked inheritance (IPEX) [77]. Tr1-cells are present in these patients, suggesting a nonredundant role for FOXP3$^+$Tregs to prevent autoimmunity [76, 81]. Nevertheless, also IFN-γ producing Tr1-cells may contribute [27]. In particular, in vivo induction of Tr1-like cells with nanoparticles coated with disease-relevant MHC-peptide complexes in mice suggested that they could be used to treat autoimmune diseases [82]. A peculiar case is systemic lupus erythematosus (SLE), where IL-10 is suspected to play a paradoxical pathogenic role [38], presumably because it stimulates autoreactive B-cells. In vivo differentiated Tr1-cells were consistently found to inhibit B-cell responses, independently of IL-10 production [35, 36], and to be dysfunctional in SLE patients. Conversely, FOXP3$^+$Tregs in the same patients efficiently suppressed B-cell responses [35]. In the case of EOMES-expressing Tr1-like cells, a switch from regulatory to B helper functions may be caused by a defect to shut-down CD40L expression [35]. Notably, the latter is regulated concomitantly by EOMES and IL-27 [6].

Intriguing evidence that IL-10 and IFN-γ co-producing Tr1-like cells play a protective role was provided in experimental autoimmune encephalomyelitis (EAE) [83], the most widely used animal model for multiple sclerosis (MS). Consistently, IL-10 production by in vitro induced Tr1 cells was reduced in MS patients [84]. However, EOMES$^+$Th1 cells and EOMES$^+$CD4$^+$CTL may in contrast play a detrimental role in MS [18, 85, 86]. Finally, EOMES-expressing Tr1-like cells are probably also enriched in the synovial fluid of patients with rheumatoid arthritis [87], but the role of EOMES$^+$Tr1-like cells in rheumatoid arthritis and in other organ-specific autoimmune diseases remains to be understood.

Cancer

It is well established that FOXP3$^+$Tregs are expanded and highly suppressive in tumors, and they also inhibit efficient anti-tumor responses of CD8$^+$T cells in cancer [88]. IL-10 plays a complex role in cancer [89], but some early studies suggested that Tr1-cells might also be involved [90]. A recent study showed that EOMES$^+$Tr1-like cells are highly enriched in lymph nodes of leukemia patients [10]. EOMES-deficient CD4$^+$ T cells failed to control leukemia in mice, suggesting an unexpected protective role of cytotoxic EOMES$^+$CD4$^+$ T cells in this model [10]. Furthermore, transfer of IL- 10Rβ-deficient T cells into lymphopenic mice suggested that IL-10 promoted the cytotoxicity of EOMES$^+$CD4$^+$ T cells. Dual effector and regulatory functions of IL-10 and IFN-γ co-producing T cells have been reported previously [4], but it could not be excluded in this study that EOMES-expressing CD4$^+$CTL rather than EOMES$^+$Tr1-like cells were killing the leukemia cells.

Recent evidence unveiled that EOMES$^+$Tr1-like cells also play a key role in solid human tumors [8]. Tumor-infiltrating CD4$^+$ T cells were analyzed by single cell RNA and TCR sequencing and compared to cells from the adjacent normal tissue. Strikingly, only two conserved tumor-associated CD4$^+$ T-cell clusters were identified, which corresponded to EOMES$^+$Tr1-like cells and FOXP3$^+$Tregs [8]. EOMES$^+$Tr1-like cells were strongly enriched in tumors and correlated with the disease stage in non-small cell lung cancer and colorectal cancer. Importantly, they were inversely associated with patient's survival in several different types of cancer, indicating that they play a detrimental role. Intriguingly, they were clonally expanded, and the same clonotypes could be identified in primary intestinal tumors and in liver metastasis. Notably, there was virtually no clonotype sharing with FOXP3$^+$Tregs, indicating that EOMES$^+$Tr1-like cells and FOXP3$^+$Tregs are not derived from the same precursors. Moreover, this lack of clonotype sharing suggests that FOXP3$^+$Tregs EOMES$^+$Tr1-like cells may respond to different antigens and are thus likely to play nonredundant roles. Both FOXP3$^+$Tregs and EOMES$^+$Tr1-like cells suppressed the proliferation of human antigen-experienced CD8$^+$T cells [8]. Notably, suppression of CD8$^+$T cells by other Tr1 cells has to our knowledge not be reported (Table 1). Overall these findings suggest that FOXP3$^+$Tregs and EOMES$^+$Tr1-like cells jointly suppress cytotoxic immune responses against solid tumors. Surprisingly, EOMES$^+$Tr1-like cells were also associated with the survival of melanoma patients treated with anti-PD1 antibodies, suggesting that intratumoral EOMES$^+$Tr1-like cells could predict the responsiveness of patients to immunotherapy. This apparently paradoxical association could be explained by the observed inefficient suppression of CD8$^+$T-cells by EOMES$^+$Tr1-like cells in the presence of blocking anti-PD1 antibodies [8]. Moreover, EOMES$^+$Tr1-like cells in tumors are likely to be generated upon chronic stimulation with tumor antigens, and might thus also indirectly reflect the "hotness" of tumors, that is, the local presentation of immunogenic tumor antigens to T cells.

Conclusions

EOMES$^+$Tr1-like cells are a molecularly well-defined and unique CD4$^+$ T-cell subset (Fig. 1) that can be easily monitored and isolated to assess molecular and functional properties. They are generated from Th1 cells upon persistent antigenic stimulation and have a characteristic cytokine profile, which distinguishes them from other IL-10 producing T cells. EOMES$^+$Tr1-like cells are in general enriched in inflamed tissues and play key roles in GvHD, IBDs, SLE, and in cancer. It seems likely that they are also involved in other pathologies, such as stroke [91] and organ-specific autoimmune diseases [87].

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Abbreviations: **Gzm**: granzyme · **HSCT**: hematopoetic stem cell transplantation · **IBD**: inflammatory bowel disease · **SLE**: systemic lupus erythematosus · **Tr1**: Type 1 regulatory

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