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Eomesodermin driven IL-10 production in effector CD8$^+$ T cells promotes a memory phenotype

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ABSTRACT

CD8$^+$ T cell differentiation is controlled by the transcription factors T-bet and Eomesodermin, in concert with the cytokines IL-2, IL-10 and IL-12. Among these pathways, the mechanisms by which T-box proteins and IL-10 interact to promote a memory T cell fate remain poorly understood. Here, we show that Eomes and IL-10 drive a central memory phenotype in murine CD8$^+$ T cells. Eomes expression led to increased IL-10 expression by the effector CD8$^+$ T cells themselves as well as an increase in the level of the lymph node homing selectin CD62L. Furthermore, exposure of effector CD8$^+$ T cells to IL-10 maintained CD62L expression levels in culture. Thus, Eomes promotes a step-wise transition of effector T cells towards a memory phenotype, synergizing with IL-10 to enhance the expression of CD62L. The early augmentation of lymph node homing markers by Eomes may facilitate the retention of effector T cells in the relatively low inflammatory milieu of the secondary lymphoid organs that promotes central memory development.

1. Introduction

Following the resolution of an immune response, subsequent protection against the same pathogen is largely dependent on the ability of the host to develop immunological memory. The potential to acquire a memory phenotype is limited to 5–10% of antigen-specific CD8$^+$ effector T cells [1,2]. This memory-precursor population is phenotypically distinct from terminally differentiated effector cells, as evidenced by distinct surface marker expression, transcription factor profiles and epigenetic signatures [1–6].

The differentiation of activated CD8$^+$ T cells to effector and memory fates is controlled by the T-box transcription factors T-bet and Eomesodermin (Eomes) [7–12]. Strong antigen-signaling through the T cell receptor (TCR) as well as cytokine signals, including signals downstream of IL-12, results in the upregulation of T-bet. This in turn promotes the development of an effector T cell phenotype, characterized by the expression IFNγ, TNFα, perforin and granzyme B [1,10,11,13]. Conversely, low levels of TCR signaling in the presence of IL-2, but the absence of inflammation, promotes Eomes expression. It is well established that higher Eomes levels favor the maintenance of a functional CD8$^+$ memory T cell population [1,7,11,13,14]. Though these studies provide strong evidence for the roles of T-bet and Eomes expression in CD8$^+$ T cell effector function and memory differentiation, the individual, synergistic and antagonistic contributions that these related T-box factors make to these processes remain poorly understood. T-bet and Eomes share 75% amino acid homology in the T-box domain and their functions are partly redundant [15]. Both induce the expression of IFNγ, perforin, granzyme B, CD122, and CXCR3, albeit to different extents [1,9,16,17]. T-bet deficiency enhances the central memory compartment in lieu of terminal effector cells while conversely, Eomes deficiency severely reduces the population of central memory CD8$^+$ T cells [7,8,10,17,18]. Though the canonical model of memory CD8$^+$ T cell differentiation implies that increasing Eomes expression correlates with decreasing T-bet levels, expression of these transcription factors fluctuates and is not restricted to one distinct cell fate [12,19]. Therefore, it remains incompletely understood how the balance between T-bet and Eomes controls CD8$^+$ T cell memory differentiation.

In addition to the role of T-box transcription factors and cytokine signaling in influencing CD8$^+$ T cell differentiation (e.g. IL-2, IL-7, IL-12, IL-15 and IL-18), recent studies have highlighted the critical role of IL-10 in promoting CD8$^+$ T cell memory maturation [20–24]. This role

Abbreviations: T_n, naïve T cells; T_{eff}, effector T cells; T_{cm}, central-memory T cells; Bcl6, B cell lymphoma 6; WT, wildtype; TKO, T-bet knockout; EKO, Eomes-knockout; DKO, double-knockout

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for IL-10 may be consistent with its role as a cytokine that is essential in resolving inflammation [25]. As low levels of inflammation support memory $CD8^+$ T cell differentiation (and not terminal effector differentiation) [1]. It follows that IL-10 may contribute to memory maturation indirectly by controlling the inflammatory environment. Recent studies show that IL-10 functions at another level in support of memory T cell differentiation. Infection with either *L. monocytogenes* or lymphocytic choriomeningitis virus (LCMV) in IL-10-knockout (KO) mice markedly reduced the function and frequency of memory $CD8^+$ T cells. In the latter study, $CD4^+$ T regulatory cell (T_{reg})-derived IL-10 was shown to rescue the defect in the $CD8^+$ memory T cell population observed in LCMV-infected IL-10-deficient mice [26,27]. A separate study suggested that $CD11c^+$ dendritic cell (DC)-derived IL-10 enhances IL-15-driven homeostatic proliferation of $CD8^+$ memory T cells [28]. Finally, an elegant study by Cui et al. demonstrated that IL-10 signaling acts directly on $CD8^+$ T cells via STAT3 activation to promote memory $CD8^+$ T cell maturation. Accordingly, STAT3 deficiency markedly reduced levels of the memory-associated markers Eomes, Bcl6, B lymphocyte-induced maturation protein-1 (Blimp-1) and suppressor of cytokine signaling 3 (SOCS3) [29]. Taken together, these two parallel streams of studies raise the important question of whether the two pathways converge; i.e. whether T-box transcription factors and IL-10 cooperate to influence $CD8^+$ T cell memory differentiation.

In order to address these questions, we decided to examine the role of T-bet and Eomes in $CD8^+$ T cell memory differentiation, using mice lacking either one or both of these transcription factors. We identified unique subsets of the $CD8^+$ T cell differentiation program controlled individually, redundantly or antagonistically by T-bet and Eomes. This analysis supports a role for Eomes, but not T-bet, in promoting IL-10 production in activated $CD8^+$ T cells. Following up on this observation, we show that both Eomes and IL-10 enhance the expression of markers associated with central memory, including CD62L, Ly6c, and Bcl6 early upon T cell activation. The effects of Eomes and IL-10 on CD62L expression are independent, leading to a $CD44^{hi}/CD62L^{hi}$ central memory phenotype. Our data suggest that Eomes promotes IL-10 expression and cooperates with IL-10 signaling to optimally maintain the central memory phenotype within the $CD8^+$ T cell compartment.

2. Methods

2.1. Mice

Mice were bred, housed and utilized in accordance with University of Maryland School of Medicine Institutional Animal Care and Use Committee Guidelines. $Tbx21^{-/-}$ (TKO) mice, $Eomes^{fl/fl}$CD4–Cre (EKO) and $Tbx21^{-/-}/Eomes^{fl/fl}$CD4–Cre (DKO) mice on a C57BL/6 background were originally obtained from S. Reiner (University of Pennsylvania, Philadelphia, Pennsylvania) and developed as previously described [30]. In order to control for intra-group variance, we housed mice on all experiments under SPF conditions on the same rack. Although different strains were housed in separate cages, they were age and sex matched & subjected to similar husbandry and veterinary care. Mice were weaned for at least one month before use.

2.2. Antibodies

Cells were stained with fluorochrome-labeled antibodies to perforin, granzyme B, CXCR3, Bcl6, Eomes, fixable viability dye (eBiosciences, San Diego, CA), CD44, CD62L, Ly6C, IL-10 (Biolegend, San Diego, CA), Tcf1 (Cell Signaling Technology, Danvers, MA), S1PR1 (R&D, Minneapolis, MN), IFNγ, TCRαβ and CD8 (BD Biosciences, San Jose, CA). Flow data were acquired on an Accuri C6 or LSRII (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.3. Cell staining and flow cytometry

Cells were stained with fluorochrome-labeled antibodies to cell surface molecules for 30 min at 4 °C prior to fixation and permeabilization (FoxP3/Transcription Factor Staining Buffer Set, eBioscience) and stained with fluorochrome-labeled antibodies to intracellular antigens according to the manufacturer's instructions. For analysis of cytokine production, cells were re-stimulated with PMA (50 ng/ml) and ionomycin (1ug/ml) (Sigma-Aldrich, St. Louis, MO) for 6 h in the presence of Brefeldin A (10 μg/mL, Life Technologies, Carlsbad, CA) to inhibit protein secretion. Cells were fixed with 4% PFA/PBS and permeabilized in saponin buffer (1% BSA and 0.1% Saponin in PBS) prior to staining with fluorochrome-labeled anti-IFNγ and anti-IL-10. Fluorescence-activated cell sorting (FACS) was performed on an Accuri C6 or LSRII (BD Biosciences) flow cytometer. Cells were gated on FSC/SSC to identify the lymphocyte population and subsequently on live/$CD8^+$ cells. FACS analysis was performed using FlowJo software (Tree Star Inc.).

2.4. $CD8^+$ T cell activation and in vitro culture

Single cell suspensions were prepared from the spleen, inguinal lymph nodes and axillary lymph nodes of C57BL/6 donor mice. $CD8^+$ T cells were magnetically isolated by negative selection (EasySep Mouse $CD8^+$ T Cell Isolation Kit, STEMCELL Technologies, Vancouver, Canada or Dynabeads Magentic Separation Technology, Thermo Fisher Scientific, Waltham, MA). Isolated $CD8^+$ T cells were activated in 24-well culture plates (2×10^6/well) with immobilized anti-CD3 (1ug/ml) and anti-CD28 (1ug/ml) antibodies and cultured in complete T cell media (IMDM + 10% FBS + 1% Penicillin/Streptomycin + 2 mM β-mercaptoethanol) supplemented with 10 u/mL of IL-2 (herein referred to as Tc0 conditions) with or without IL-10 (20 ng/mL). Cells were split 1:2 every 2 days and supplemented with fresh media and cytokines.

2.5. Retroviral constructs

The MSCV-IRES-Thy1.1 retroviral vector (MiT) was a gift from Dr. Philippa Marrack (Addgene Plasmid #17442) [31]. The MSCV-Puro-IRES-GFP (PiG) was a gift from Dr. Scott Lowe (Addgene plasmid #18751). Eomes cDNA was subcloned into the MiT and PiG backbones to generate the Eomes-MiT and Eomes-PiG constructs, respectively. Empty-vector backbones were used as controls in retroviral transduction experiments, as indicated.

2.6. Retroviral transduction

MiT or PiG retroviral genomes were packaged into retrovirus by co-transfecting 293T cells with MiT or PiG vector plasmid and helper plasmids and viral supernatants were harvested at 48 h. For MiT-RV transductions, $CD8^+$ T cells were transduced one day following T cell activation in complete T cell media in 24-well, RetroNectin-coated plates (Clontech, Mountain View, CA). Cell culture plates containing cells and virus were centrifuged at 2500 rpm for 2 h. After the centrifugation, viral supernatants were replaced with fresh media with IL-2 and transduced cells were cultured for another three days. Successfully transduced T cells were identified by expression of Thy1.1. For PiG-RV transductions, $CD8^+$ T cells were transduced as above and selected with puromycin for an additional 3 days (2ug/ml, Thermo Fisher Scientific, Waltham, MA). Successfully transduced cells were identified by expression of GFP. The cultures were split 1:2 every 2 days and supplemented with fresh media and cytokines. On day 5, cells were stained with the indicated antibodies and analyzed by flow cytometry.

2.7. Microarray and pathway analysis

Single cell suspensions were prepared from spleens of wildtype

(WT), TKO, EKO and DKO mice. $CD8^+$ T cells were isolated via magnetic separation, as above. Isolated $CD8^+$ T cells were activated in 24-well culture plates (2×10^6/well) with immobilized anti-CD3 and anti-CD28 antibodies and cultured in complete T cell media (IMDM + 10% FBS + 1% Penicillin/Streptomycin + 2 mM β-mercaptoethanol) supplemented with IL-2 (10u/mL). Cells were cultured for 5 days and split 1:2 every 2 days and supplemented with fresh media plus IL-2. On day 5, cells lysates were prepared and RNA was harvested per the Qiagen RNeasy protocol (Qiagen, Hilden, Germany). RNA concentration and purity was analyzed via spectrophotometric analysis and submitted to Affymetrix for microarray analysis. Microarray analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team and pathway analysis was conducted using the Ingenuity Pathway Analysis platform (Qiagen). For data filtering, a permutation p-value of < 0.001 was used. Subsequent genes were filtered out at a ± 1.5-fold cut-off ($p < 0.01$).

2.8. qRT-PCR

Total RNA prepared from WT, TKO, EKO and DKO $CD8^+$ T cells (as above) was reverse transcribed into cDNA (SuperScript First-Strand Synthesis, Thermo Fisher Scientific, Waltham, MA). TaqMan probes for Eomes, IL-10, IL-21, Tcf1 and S1PR1 were purchased from Thermo Fisher Scientific. Primers for Bcl6, CD62L, Ly6C and IL-10Rα were designed using Primer3 software. Bcl6: CCTGAGGGAAGGCAATATCA (forward), CGGCTGTTCAGGAACTCTTC (reverse); CD62L: ACCCACTC TCTTGGAGCTGA (forward), CAGGTTGGGCAAGTTAAGGA (reverse); Ly6C: TGTGCAACCACTCTTTCCTG (forward), ATGCCTCTAGGGCCAA GAAT (reverse); IL-10Rα: TCTCCAGGGCAGCCTAAGTA (forward), CTGCAGGTGTACCCCAAGTT (reverse). B-actin or GAPDH was amplified as an internal control. Quantitative PCR was performed in a total reaction volume of 20 µl in 96-well reaction plates using TaqMan or SYBR Green-based detection (Applied Biosystems). Reactions were conducted at 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Plates were run using the 7900HT Fast Real-Time PCR system (Applied Biosystems), and data processing was performed using SDS v2.1 software (Applied Biosystems). The delta–delta Ct method was used as described by Perkin-Elmer Applied Biosystems to determine the relative levels of mRNA expression between experimental samples and controls.

2.9. Statistical analysis

Statistical analyses were performed as indicated using the 2-tailed Student *t* test. A *p*-value of less than 0.05 was considered significant. All graphs show average mean values ± S.E.M., unless otherwise indicated.

3. Results

3.1. Eomes regulates the expression of CD62L in the $CD8^+$ T cell central memory compartment

Differentiation of $CD8^+$ T cells to effector and memory states is typically tracked by the expression of surface markers, including CD62L (*Sell*) and CD44. CD62L is expressed highly on naïve $CD8^+$ T cells but is downregulated in effector T cells following TCR stimulation whereas CD44 levels increase following antigen-specific activation and stay high in memory T cells [1,2]. CD62L is re-expressed in a subset of memory $CD8^+$ T cells known as central memory cells (T_{CM}) and their frequencies correlate with increasing levels of Eomes [32,33]. We first used mice deficient in Eomes (EKO) or T-bet (TKO) to validate the previously reported differences in memory differentiation in the absence of these transcription factors [7] (Fig. 1). Inbred laboratory mice found in most SPF colonies contain ~10–20% $CD44^{hi}CD62L^{hi}$ central memory cells, 70–80% $CD44^{lo}CD62L^{hi}$ naïve cells, and 1–10% $CD44^{hi}CD62L^{lo}$ effector cells [34–38]. Although these frequencies were not significantly altered in TKO mice, in the absence of Eomes, as previously reported, the T_{cm} population was significantly decreased (Fig. 1A, 1B) [7]. Eomes is important in maintaining a central memory phenotype, yet the precise effects of Eomes on features that make up a memory T cell phenotype are not well understood. Indeed we found that EKO mice exhibited a decrease in the percentage of $CD44^{hi}$ cells compared to WT controls, as previously reported (Fig. 1A) [17]. Interestingly, though there was no difference in the frequency of $CD62L^{hi}$ cells between WT and EKO mice, we observed that EKO cells expressed a significantly lower level of CD62L specifically in the central memory population, when compared to WT controls (Fig. 1C, D). This trend in an altered phenotype was also found with the expression of Ly6c, in which EKO cells had a markedly reduced population of $Ly6c^{hi}$ cells compared to WT cells (Fig. S1). Consistent with its role in promoting a T_{cm} phenotype, analysis of Eomes expression in WT $CD8^+$ T cells revealed that memory $CD8^+$ T cells express higher levels of Eomes than naïve cells whereas effector T cells exhibit a bimodal expression of Eomes (Fig. 1E). Taken together, these results suggest that Eomes has a previously unappreciated role in regulating the levels of CD62L in central memory $CD8^+$ T cells.

3.2. Distinct subsets of $CD8^+$ T cell genes are uniquely and redundantly regulated by T-bet and Eomes

The observation that CD62L levels may be regulated transcriptionally by Eomes prompted us to examine the global transcriptome in effector $CD8^+$ T cells that are differentially regulated by T-bet and Eomes. To this end, we analyzed gene expression in WT, TKO, EKO, and mice doubly-deficient in T-bet and Eomes (double-knockout – DKO) $CD8^+$ T cells after activation *in vitro* (since naïve T cells do not typically express T-bet or Eomes to significant levels). Purified $CD8^+$ T cells were activated *in vitro* (see Methods) and their RNA analyzed after 5 days using Affymetrix arrays. Since this study was focused on understanding the role of T-bet vs Eomes in $CD8^+$ T cell activation, we chose a time point for mRNA isolation based on the known timeframe of T-bet and Eomes expression. Typically Eomes is expressed later than T-bet; but by day 5 after initial activation both are expressed at reasonably high levels [11,39]. Therefore we adopted a protocol wherein T cells were purified from mice and stimulated for 5 days (as described in the methods) before analysis. Approximately 695 genes were differentially expressed ± 1.5-fold between the four genotypes ($p < 0.01$). (Fig. 2A). Of these 695 genes, 437 (63%) were affected only in the DKO – suggesting that either T-bet or Eomes were sufficient for maintaining their expression to WT levels. In contrast to these "T-box redundant" target genes, 50 (7.1%) required T-bet for expression while 117 (16.8%) required Eomes. Only 3 genes (0.4%) required both T-bet and Eomes to be doubly-present. This overall transcriptomic picture illustrates the level of redundancy and synergy between these two transcription factors in regulating gene expression in activated $CD8^+$ T cells (Fig. 2B). All genes from each subset in the Venn diagram are listed in Table S1. Genes were differentially expressed (± 1.5-fold) in one, two, or all three genotypes (Table S1). Broadly, genes that are regulated by Eomes (and totally independent of T-bet) are expected to be different in both the EKO and DKO mice (since the latter also lacks Eomes). These genes are listed in column 2 (Table S1). Conversely genes that require only T-bet are in column 4. In addition to these straightforward criteria, and because T-bet and Eomes are closely related, we expected some redundant regulation where certain genes require either T-bet or Eomes. This subset would be unchanged in the single knockouts, but enriched in the DKO cells, which lack both *T*-box proteins (column 1). Interestingly, our analysis also reveals some intriguing crosstalk between these two transcription factors which is not intuitively predictable. For instance, a set of 72 genes were only enriched in the EKO – suggesting that they required Eomes for regulation. However since these were not found in the DKO list, they clearly require basal T-bet

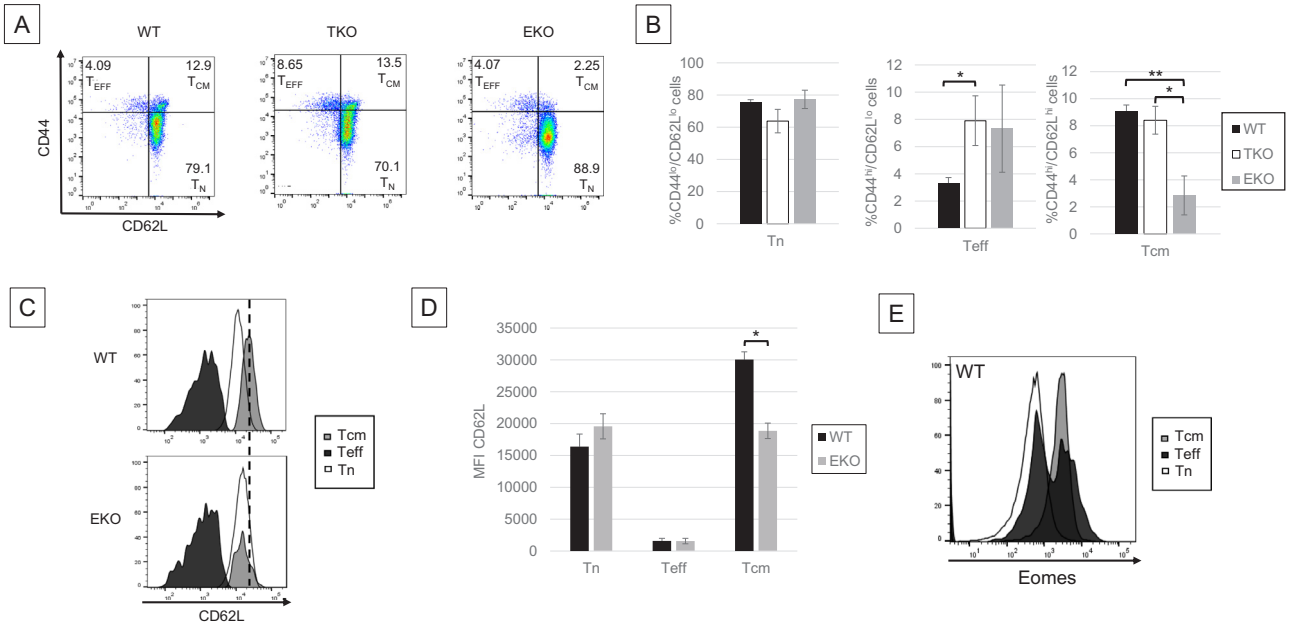

Fig. 1. Eomes regulates the expression of CD62L in the CD8$^+$ T cell central memory compartment. Splenocytes were harvested from uninfected WT BL/6 mice and analyzed for expression of the indicated markers by FACS. Plots represent live/CD8$^+$ T cell populations. Teff = effector, Tcm = central memory, Tn = naïve (A) Representative FACS plots of CD8$^+$ T cell populations in WT, TKO and EKO mice. (B) Frequency of CD8$^+$ T cell populations in WT, TKO and EKO mice based on CD44 and CD62L expression. Data are representative of at least 3 mice per group. (C) Representative histograms of CD62L expression in WT and EKO CD8$^+$ T cell subsets. (D) Comparison of the mean fluorescence intensity (MFI) of CD62L in central memory, effector and naïve subsets of WT and EKO CD8$^+$ T cells from (C). Data are representative of at least 3 mice per group. (E) Expression of Eomes in CD8$^+$ T cell populations as analyzed by FACS in a WT mouse.

expression in order for Eomes to regulate them (column 3). This trend was similarly observed for 11 genes that were differentially regulated in TKO cells but neither of the other three genotypes (column 5). We further identified two genes that were enriched only in TKO and EKO cells but were consistently expressed in the WT and DKO genotypes. This subset represents potentially antagonistic genes (column 6).

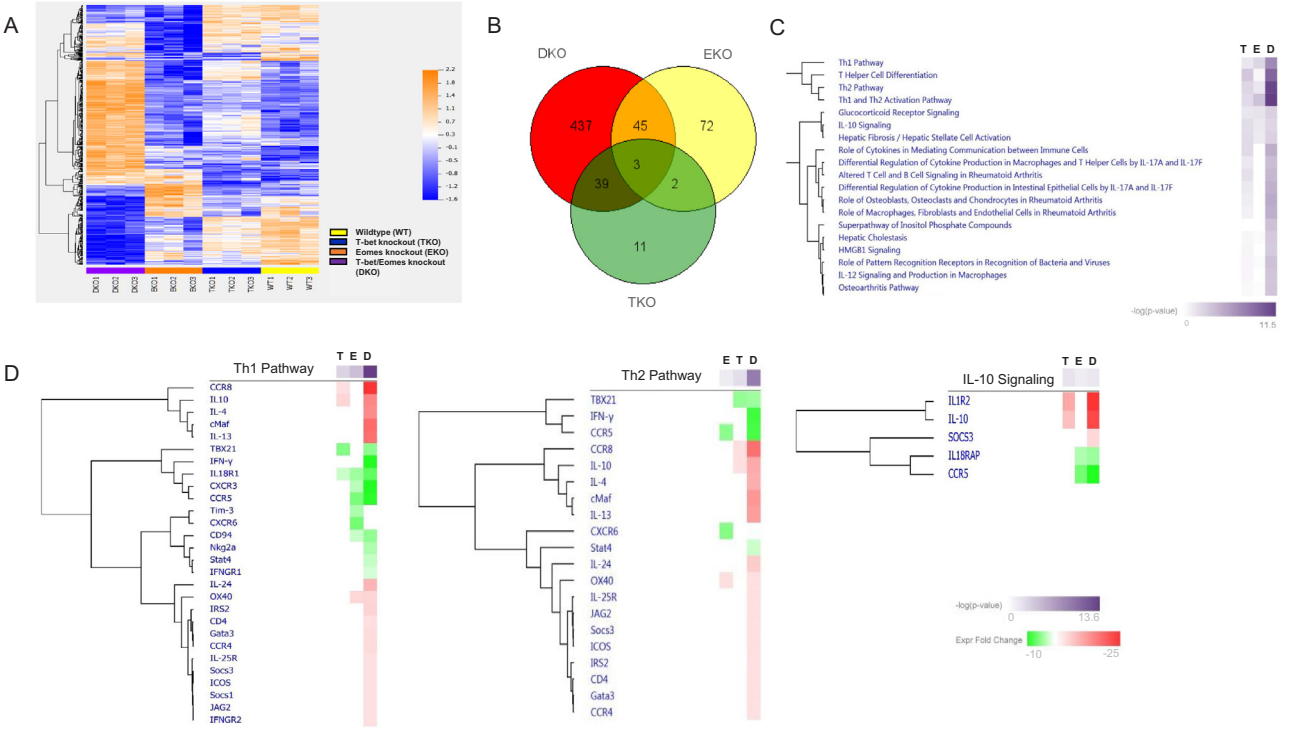

Fig. 2. T-bet and Eomes differentially regulate CD8$^+$ T cell differentiation. (A) Class comparison analysis of genes differentially expressed between WT, TKO, EKO and DKO CD8$^+$ T cells cultured under Tc0 conditions for 5 days. Representative of 695 uniquely expressed genes displaying a difference between at least one of the groups (permutation p-value of < 0.001). (B) Venn diagram comparing differentially expressed genes between each group, relative to WT cells ($\pm$ 1.5-fold, $p < 0.01$). (C) Representative cluster analysis demonstrating highly enriched pathways that are largely dependent on T-bet and/or Eomes. (D) Gene signature profiles from the indicated pathways from (C) demonstrating regulation of individual genes by T-bet and/or Eomes. T (TKO), E (EKO), D (DKO).

Finally, three genes were enriched in the TKO, EKO and DKO genotypes compared to WT cells, suggesting that both T-bet and Eomes are required for expression of these genes (column 7).

From these gene expression data, we focused on pathway analyses to identify major functional clusters that were affected in TKO, EKO and DKO cells (Fig. 2C). The most significant cluster of genes to be affected came off the DKO transcriptome, where the absence of both *T*-box transcription factors led to a pronounced Type 2 gene signature being expressed (Fig. 2D). This was marked by the upregulation of the transcription factors Gata3 and c-Maf as well as the cytokines IL-4 and IL-13. The second cluster (Fig. 2C) that was highlighted in this analysis was one that included the IL-10 family of cytokines. Interestingly, while both TKO and DKO maintained IL-10 expression, the loss of Eomes led to a marked decrease in this cytokine. While the DKO signature is further complicated by the previously discussed Type 2 signature (where the contribution of Gata3 and c-Maf to IL-10 gene regulation cannot be discerned from those attributed to Eomes), we focused further on understanding how Eomes may be involved in regulating IL-10.

3.3. Eomes regulates the early expression of genes involved in CD8$^+$ T cell memory differentiation

Though T-bet and Eomes largely display redundant functions, we observed that of the 695 differentially expressed genes, Eomes alone appeared to control the regulation of more genes than T-bet (16.8% vs 7.1%) (Fig. 2C). Of the genes regulated specifically by Eomes, many were fate-specific transcription factors, cytokines and surface markers implicated in CD8$^+$ T cell memory differentiation. To confirm our microarray data, we performed qRT-PCR analysis to quantitatively compare mRNA levels in WT, TKO, EKO and DKO CD8$^+$ T cells after 5 days of activation. Because CD8$^+$ T cells downregulate CD62L expression upon activation (Fig. 1), we expected a negligible difference in the expression of this marker in activated CD8$^+$ T cells, regardless of Eomes expression. Surprisingly, we found that Eomes deficiency led to a greater than two-fold reduction in the expression of CD62L in activated cells (Fig. 3A). These findings further extend our observations from Fig. 1, and suggest that Eomes is necessary for the maintenance of CD62L expression early upon activation in effector CD8$^+$ T cells, and prior to their differentiation into memory cells. In addition, the memory-associated molecules B cell lymphoma-6 (Bcl6) and Ly6C were also decreased at the transcript level in the absence of Eomes. This was confirmed by flow cytometric analysis on similar cultures (Fig. 3, Fig. S1). Of note, granzyme B and perforin were reduced in EKO cells compared to WT controls, consistent with previous reports (Fig. S2) [9,16]. Bcl6, a transcriptional repressor, was of particular interest, as strong evidence supports its role in CD8$^+$ T cell memory differentiation [1,29,40,41]. Western blot and FACS analysis of Bcl6 expression in WT and EKO CD8$^+$ T cells at day 5 further confirmed the regulation of Bcl6 by Eomes in activated CD8$^+$ T cells, as Bcl6 expression was reduced by an average of 25% in EKO cells (Fig. 3B). Additional replicates corroborated this result, albeit with variable results in the staining intensities between WT and EKO cells (Fig. S2). Together with the gene expression array, these data suggest that Eomes promotes a memory precursor phenotype, quite early in the differentiation of CD8$^+$ T cells through the induction of CD62L and Bcl6.

3.4. Eomes promotes IL-10 expression in activated CD8$^+$ T cells

In addition to the surface markers discussed above, the absence of Eomes also resulted in differential expression of key cytokines and signaling pathways (Fig. 2D). Of these, the IL-10 pathway was especially intriguing. Recent studies suggest that IL-10 influences the development of CD8$^+$ memory T cells [26–29]. Zhang et al. found that Eomes directly bound to and promoted IL-10 expression in CD4$^+$ Tr1 cells [42]. However, a connection with Eomes as a regulator of IL-10 in CD8$^+$ T cells has not been studied. We therefore sought to determine if Eomes regulates IL-10 expression in CD8$^+$ T cells. We isolated CD8$^+$ T cells from WT, TKO and EKO mice and cultured them *in vitro* under Tc0

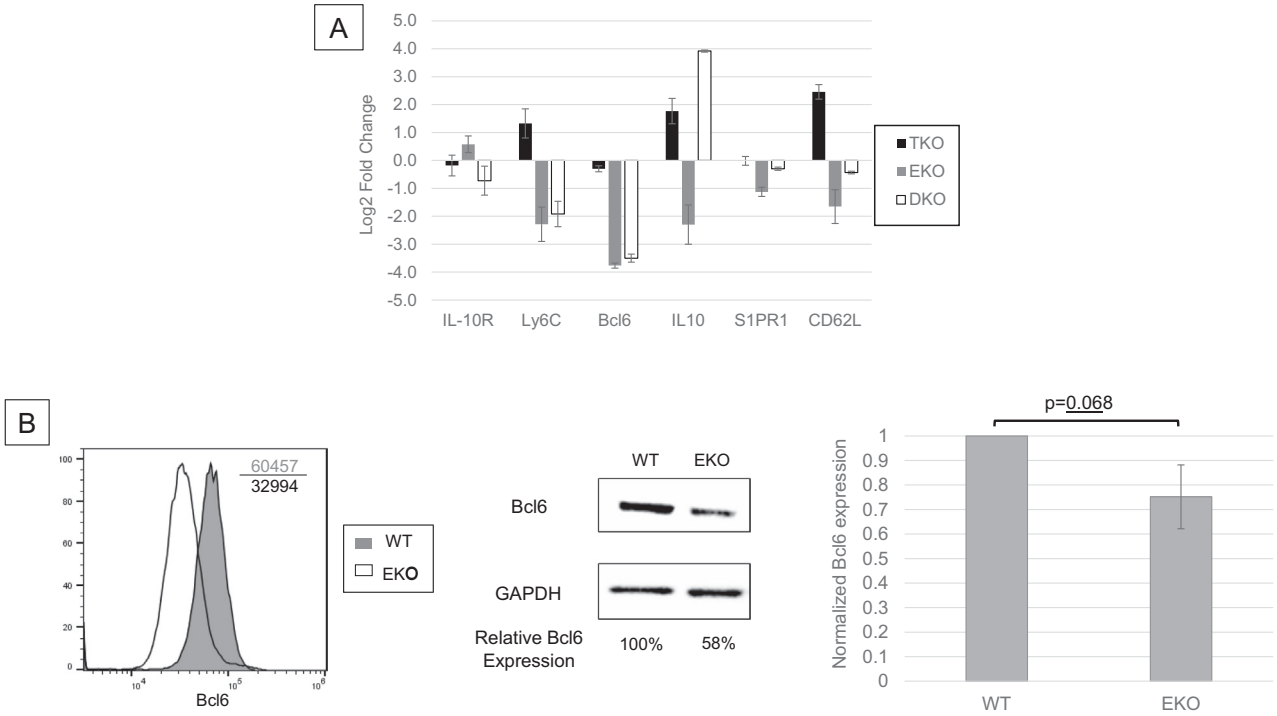

Fig. 3. Eomes regulates the early expression of genes involved in CD8$^+$ T cell memory differentiation. (A) qRT-PCR analysis of the indicated genes was performed with RNA harvested from WT, TKO, EKO and DKO CD8$^+$ T cells cultured *in vitro* for 5 days under Tc0 conditions, as in Fig. 1. Expression levels are relative to WT RNA and normalized to GAPDH or β-actin. Plot is representative of 3 mice per genotype (n = 3). (B) Representative FACS and Western blot analyses comparing Bcl6 expression in WT and EKO CD8$^+$ T cells after 5 days of culture *in vitro*. Western blot data (middle, right) are representative of 3 independent experiments.

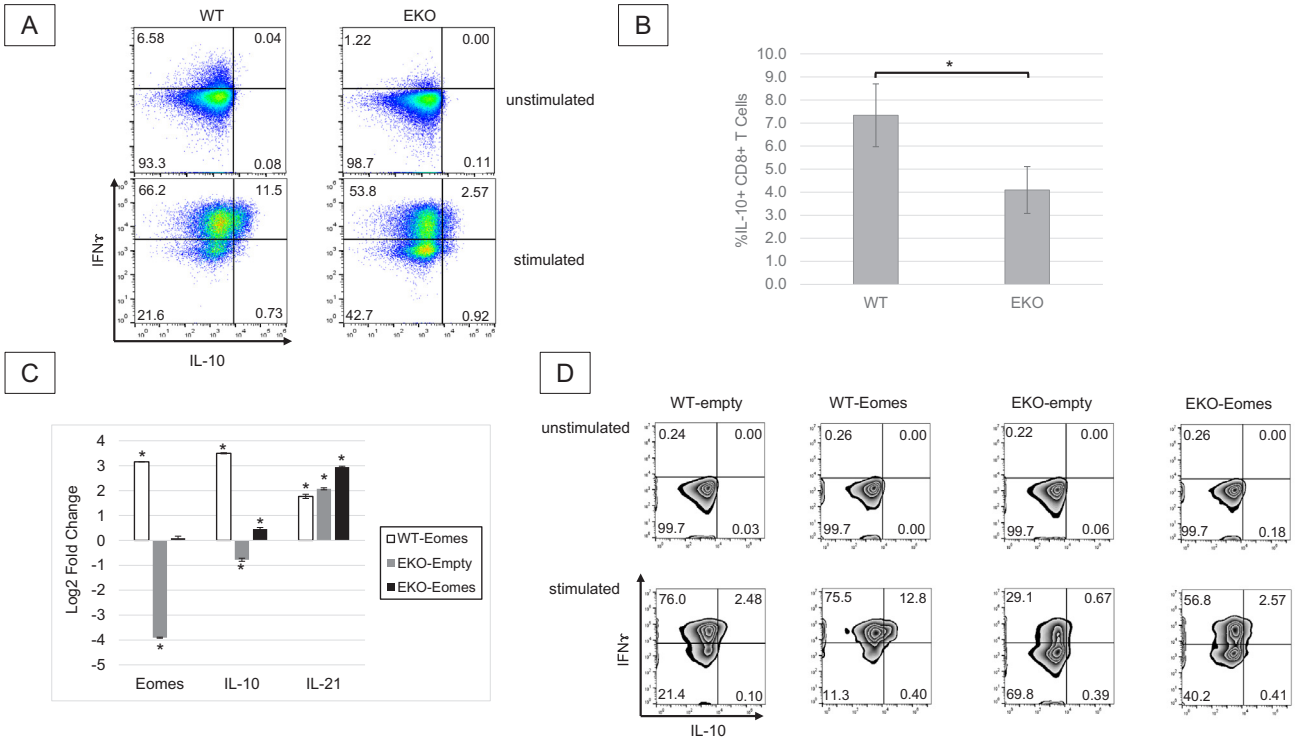

Fig. 4. Eomes promotes the expression of IL-10 in activated CD8$^+$ T cells. (A) CD8$^+$ T cells isolated from WT, TKO and EKO mice were cultured under Tc0 conditions for 5 days. On day 5, cells were restimulated (bottom panel) with PMA and ionomycin and analyzed for IFNγ and IL-10 expression. Cells are gated on live/CD8$^+$ cells. (B) Representative percentages of IL-10-secreting CD8$^+$ T cells in WT and EKO mice from (A). Plots are representative of 5 independent experiments (n = 5). (C) WT or EKO CD8$^+$ T cells were activated *in vitro* and transduced with Eomes or empty-vector retrovirus. On day 5, RNA was harvested from cell cultures and transcript levels were analyzed by RT-qPCR. Expression levels were analyzed relative to empty-vector transduced WT cells and normalized to GAPDH. Error bars represent standard deviation of replicates done in triplicate. Asterisks indicate a p-value < 0.05. (D) WT or EKO CD8$^+$ T cells were activated *in vitro* and transduced with Eomes or empty-vector retrovirus as in (C). Cells were selected with puromycin for 3 days, restimulated with PMA/ionomycin (bottom panel) on day 5 and stained for IFNγ and IL-10. Cells are gated on live/CD8$^+$ cells.

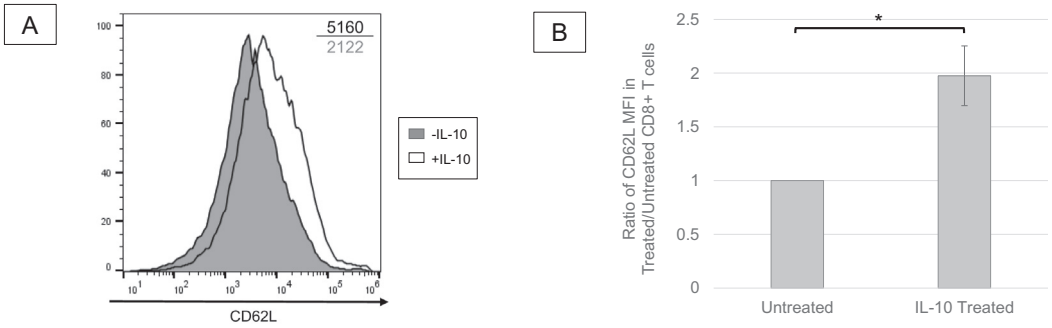

Fig. 5. IL-10 maintains the expression of the T_{cm}-associated molecule CD62L. (A) WT CD8$^+$ T cells were cultured for 5 days under Tc0 conditions with or without the addition of exogenous IL-10 (20 ng/ml). On day 5, cells were analyzed for expression of CD62L by FACS. (B) The ratio of CD62L expression in IL-10-treated vs untreated CD8$^+$ T cells is shown. Data are representative of cell cultures from 7 mice (n = 7) in 3 independent experiments. Median MFI values were used for analysis.

conditions for 5 days before analyzing for IFNγ and IL-10 expression. Consistent with qPCR data from Fig. 3, EKO cells exhibited a roughly 2-fold decrease in IL-10 production as compared to both WT and TKO cells (Fig. 4A, B). Of note, there was no observed difference in expression of IL-10R at either the mRNA or protein levels (Fig. 3A, Fig. S2). To assess whether Eomes alone was sufficient to regulate IL-10 expression, CD8$^+$ T cells were isolated from WT and EKO mice and transduced with Eomes-containing retroviruses. The expression levels of Eomes and IL-10 were analyzed by qRT-PCR and flow cytometry on day 5. Relative to WT cells transduced with empty-retrovirus (control), the lack of Eomes (EKO-empty) decreased levels of Eomes and IL-10 mRNA approximately 2-fold (Fig. 4C). This result correlated with low expression of IL-10 protein in the EKO-empty cells (1.06%) (Fig. 4D, Fig. S3). Overexpression of Eomes in WT CD8$^+$ T cells (Eomes-WT) increased Eomes as expected – but also the IL-10 transcript levels (Fig. 4C). This upregulation of mRNA was similarly evident at the protein level as 13.2% of WT-Eomes cells expressed IL-10 compared to 2.5% in the WT-empty control (Fig. 4D, Fig. S3). Induction of Eomes expression in EKO cells rescued the IL-10 phenotype to a similar extent as observed in the WT-empty control (2.98% and 2.5%, respectively) (Fig. 4C, D). Together, these data suggest that Eomes alone is sufficient to promote IL-10 production in activated CD8$^+$ T cells.

3.5. IL-10 induces the expression of CD62L in activated CD8$^+$ T cells

The precise role of IL-10 in influencing CD8$^+$ T cell memory

differentiation is unclear, and while it has been suggested to have roles in effector function or dampening activation, our data suggests that it might also impact the Eomes-mediated orchestration of the T_{cm} program. To evaluate this further, we added IL-10 to our 5 day cultures of $CD8^+$ T cells. $CD8^+$ T cells cultured with IL-10 for 5 days exhibited increased expression of CD62L (Fig. 5A). In assessing the ratio of IL-10-treated to untreated cells, we consistently observed a ~2-fold increase in CD62L expression at day 5 when cells were cultured in the presence of IL-10 (Fig. 5B). In analyzing cell cultures on days 1–5, there was no observed difference in the percentage of $CD62L^{hi}$ $CD8^+$ T cells regardless of the presence of IL-10 (data not shown), suggesting that IL-10 functions to maintain the expression of CD62L early upon activation, but does not increase the percentage of $CD62L^{hi}$ cells. Overall, these data show that the timing of maximal IL-10 receptivity in activated $CD8^+$ T cells is relatively early – suggesting that this exposure in the activating secondary lymphoid organs (SLOs) may then prime the subsequent differentiation program of T_{cm} cells.

3.6. The relative roles of Eomes and IL-10 in the regulation of CD62L expression

Taken together, these data suggested at least two potential mechanisms for the interplay between Eomes and IL-10 in the regulation of CD62L expression. For one, Eomes and IL-10 may independently regulate CD62L, or alternatively, both could be part of the same pathway whereby Eomes regulates IL-10, which in turn regulates CD62L (via STAT3). In order to distinguish between these mechanisms, we analyzed CD62L expression in WT and IL-10 knockout mice. WT $CD8^+$ T cells were transduced with Eomes-containing or empty-vector retrovirus and cultured for 5 days under Tc0 conditions. Consistent with our previous data, constitutive Eomes expression in WT cells led to a greater than 2-fold increase in the percentage of $Eomes^{hi}/CD62L^{hi}$ cells compared to the empty-vector controls (Fig. 6A, left panel). Because IL-10 maintains CD62L expression in $CD8^+$ T cells (Fig. 5), we next investigated whether Eomes requires IL-10 to induce CD62L expression. To this end, we transduced $CD8^+$ T cells from IL-10KO mice with Eomes-containing or empty-vector retrovirus and cultured for 5 days under Tc0 conditions, as above. Constitutive Eomes expression in IL-10KO cells led to a 4-fold increase in the frequency of $Eomes^{hi}/CD62L^{hi}$ cells, indicating that Eomes induces CD62L expression independent of IL-10 (Fig. 6A, right panel). In analyzing the CD62L-expressing population alone, we observed similar percentages of $CD62L^{hi}$ $CD8^+$ T cells in both the WT and IL10KO cells following Eomes induction (Fig. 6B). In this experimental timeframe, Eomes overexpression increased the percentage but did not affect the overall MFI of CD62L in either WT or IL-10KO cells (Fig. 6C). Whereas IL-10 functions to maintain the CD62L MFI (Fig. 5), forced Eomes expression increases the overall frequency of CD62L-expressing $CD8^+$ T cells. Together our data show that Eomes and IL-10 can independently induce CD62L expression in activated $CD8^+$ T cells.

4. Discussion

The differentiation of memory $CD8^+$ T cells is dependent on a balance between T-bet and Eomes [1,19,43]. Both T-bet and Eomes induce the expression of the IL-2/15 receptor beta chain (CD122) early on in effector $CD8^+$ T cells, which supports the eventual formation of T cell memory by conferring responsiveness to the cytokines IL-2 and IL-15 [9,10]. The availability of IL-2 in both the priming and effector phases influences memory $CD8^+$ T cell differentiation, and IL-15 supports the maintenance of differentiated memory T cells [44–47]. Inhibition of IL-2, among other pro-inflammatory signals, or T-bet deficiency, increases the development of $KLRG1^{lo}L\text{-}7R^{hi}$ memory $CD8^+$ T cells in TCR-transgenic models of LCMV infection [10,11,43]. Overall, these and other studies have offered the field a model that incorporates *T*-box transcription factors and gamma-chain-dependent cytokines. Our understanding of the role of IL-10, however, is still in its infancy.

Several recent studies implicate IL-10 in the differentiation of $CD8^+$ memory T cells. IL-10 derived from either $CD4^+$ T regulatory cells (T_{regs}) or $CD11c^+$ dendritic cells promotes the development and

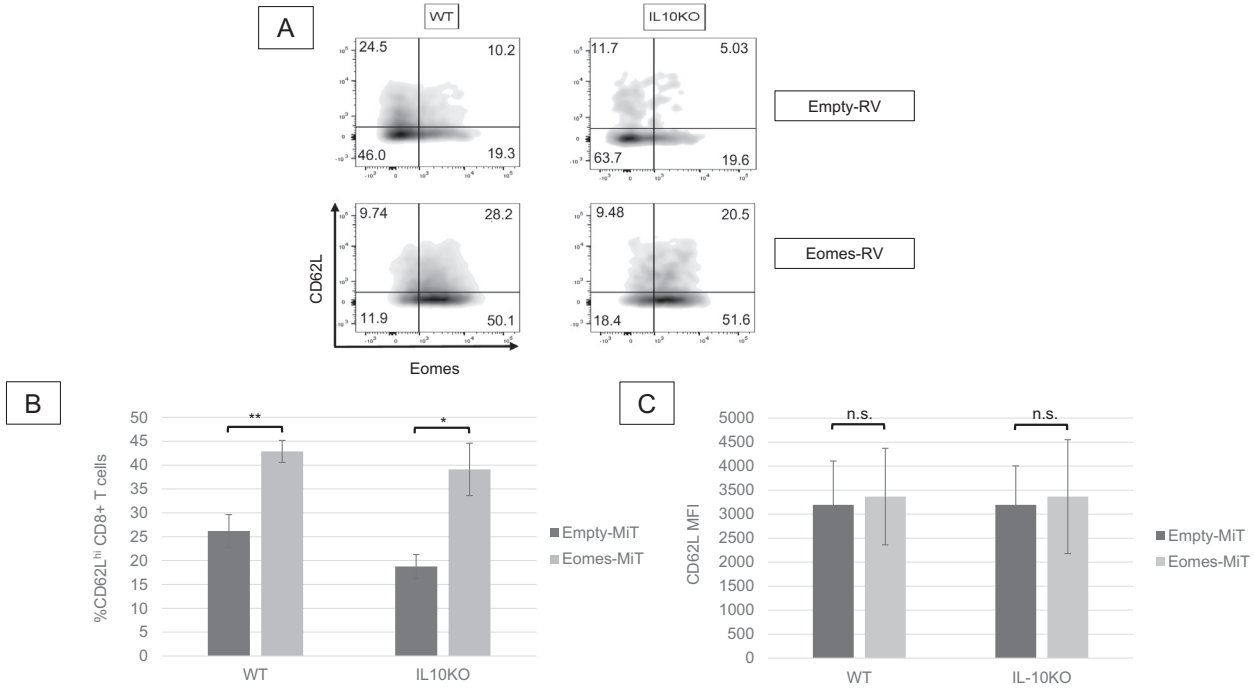

Fig. 6. Eomes does not require IL-10 to induce CD62L expression in $CD8^+$ T cells. (A) $CD8^+$ T cells were isolated from WT or IL-10KO mice and stimulated under Tc0 conditions for 2 days. On day 2, cells were transduced with Eomes-containing or empty-vector retrovirus (RV) and cultured through day 5. Cells were then analyzed for CD62L and Eomes expression by FACS. Cells are gated on live/$CD8^+$/$Thy1.1^+$ populations. (B) Representative frequency of $CD62L^{hi}$ cells from (A). (C) Representative CD62L MFI values from $CD62L^+$ populations in (A). Plots are representative of 2 independent experiments with 2 mice per genotype (n = 4).

maturation of $CD8^+$ memory precursor T cells [27,28]. Cui et al. demonstrated that IL-10 blockade resulted in a significantly reduced percentage of $KLRG1^{lo}L\text{-}7R^{hi}$ memory $CD8^+$ T cells in LCMV infection [29]. The authors further showed that IL-10/21 signaling via STAT3 is critical for the functional maturation of memory $CD8^+$ T cells. Importantly, $STAT3^{-/-}$ memory $CD8^+$ T cells expressed significantly less Eomes, Bcl6, Blimp1 and a lower percentage of $CD62L^{hi}$ cells than WT controls, suggesting that IL-10 and Eomes have critical roles in memory development [29]. Here, we identified IL-10 as an early target of Eomes in activated $CD8^+$ T cells, and show that Eomes and IL-10 act independently to regulate CD62L expression. Our data are in line with a recent study demonstrating that Eomes directly binds the IL-10 promoter in $CD4^+$ Tr1 cells and induces IL-10 expression [42]. Similarly, $CCR6^+$ Th17 cells express high levels of both Eomes and IL-10 in experimental autoimmune encephalitis [48]. In line with this, one report demonstrated that treatment of $CD8^+$ T cells with the mammalian target of rapamycin (mTOR) inhibitor rapamycin increased both Eomes and IL-10 expression in a model of pulmonary aspergillosis [49]. A related group of studies revealed an additional link between Eomes and IL-10. Jiang et al. showed that type I IFNs, IL-2 and IL-27 induced IL-10 expression in $CD8^+$ T cells in a Blimp1- and Irf4-dependent manner [50]. A subsequent study demonstrated that type I IFNs similarly promote Eomes expression in memory $CD8^+$ T cells in an IRF9-dependent manner [51]. The correlation of Eomes expression in effector and memory $CD8^+$ T cells with the ability of these cells to make and respond to IL-10 warrant a modified model of memory T cell differentiation which includes distinct roles for Eomes and IL-10 in a T cell-intrinsic program driving memory formation.

Exploring the role of cytokines and trophic factors that drive memory differentiation has also led to an increasing appreciation for the importance of tissue localization of effector T cells in their eventual differentiation to memory [7,52]. Perhaps the most prevalent concept stems from the expression of CD62L by central memory T cells with stem-like properties that sustain long-term memory [32,53,54]. In this context, the gradual increase in CD62L expression by effector T cells, with the help of Eomes and IL-10, could set the stage for the arrival of memory precursors to niches where they can undergo further differentiation towards central memory cells. Studies to date have reported decreased frequencies of $CD62L^{hi}$ $STAT3^{-/-}$ memory $CD8^+$ T cells and reduced numbers of $CD44^{hi}/CD62L^{hi}$ EKO central memory $CD8^+$ T cells [7,17], but have not addressed the potential for regulation of the expression levels (MFI) of CD62L. We observed that Eomes deficiency reduced the MFI of CD62L to levels comparable to those in $CD8^+$ T_n cells. As T_n do not express Eomes, this suggests that Eomes selectively increases CD62L expression in effector cells that may be destined to become central memory $CD8^+$ T cells. At the resolution of an immune response, the differentiation and survival of memory $CD8^+$ T cells may be viewed as a competitive process. In this model, $CD8^+$ T cells compete for the memory niche in SLOs, where they receive the appropriate survival signals at the expense of competing cells. Thus, the relative expression of CD62L might influence the potential for some of the effector cells to preferentially receive memory-promoting signals. In this way, Eomes and IL-10 may be critical for promoting and maintaining memory $CD8^+$ T cells.

In addition, our study lays out a transcriptional map for the overlapping and unique regulatory effects of T-bet versus Eomes. Previous studies have demonstrated that $CD8^+$ T cell-specific Eomes-deficiency led to reduced numbers, persistence and re-expansion of memory T cells, suggesting both functional and anatomical defects in $CD8^+$ T cells lacking Eomes. Indeed, several molecules that are regulated by Eomes in the memory compartment including CD122, CXCR3, CXCR4 and Bcl-2 are known [7]. Our study extends this list, by focusing on effector stage $CD8^+$ cells, to include Bcl6, CD62L, Ly6C and IL-10, all of which may collectively support $CD8^+$ T cell memory differentiation.

The most prominent paradigm unraveled in our analysis, however, is the key role played by T-box transcription factors in overriding what seems to be a default Type 2 gene signature in effector $CD8^+$ T cells. DKO cells expressed high levels of the Type 2 transcription factors Gata3 and c-Maf as well as many type 2 cytokines. This suggests that T-bet or Eomes is required to redundantly repress the native Gata3/c-Maf-driven program in these cells. A notable consequence is that DKO cells also express higher levels of IL-10 than their WT, TKO and EKO counterparts. Since it is possible that this IL-10 expression may be Gata3-dependent (and not Eomes) as has been observed in $CD4^+$ Th2 cells [55], we excluded these cells in our analyses. This concept has precedent in the literature. One study observed increased Eomes expression in $CD8^+$ T cells cultured under Tc2 conditions but did not address whether they express Gata3 or IL-10 under those conditions [56]. However, IL-10-secreting Type 2 $CD8^+$ T cells have been observed in several pathological conditions and may be quite relevant clinically [57–61]. Earlier reports have also demonstrated that DKO $CD8^+$ T cells display a Type 17 response both *in vitro* and *in vivo*. Under Tc2 or Tc17 polarizing conditions, TKO, EKO and DKO $CD8^+$ T cells expressed IL-4 or IL-17 at higher levels compared to WT controls, respectively [17]. Intlekofer et al. demonstrated a robust Type 17 response in DKO mice infected with LCMV, where DKO cells expressed high amounts of IL-17 but not IL-10 [30]. In contrast with these studies, our study suggests that under non-polarizing Tc0 conditions, $CD8^+$ T cells lacking T-bet and Eomes are primed to revert to a Type 2 phenotype. It is not clear in *in vivo* models, whether the availability of some polarizing cytokine signatures can modulate the fate specificity of DKO $CD8^+$ T cells accordingly. Our pathway analyses revealed several other genes that may be individually linked to the Type 1 or Type 2 $CD8^+$ T cell phenotype and are regulated by T-bet and Eomes. Two genes, namely *Ifi204* and *Ifitm3*, are regulated by T-bet or Eomes but display normal expression levels in both WT and DKO animals. Interestingly, both are interferon-inducible genes that have been implicated in dendritic cell biology and particularly in IFNβ signaling and autophagy [62,63]. Three additional genes, *Il18r1*, *Lum* and *Fam26f*, require both T-bet and Eomes to maintain expression levels relative to WT cells. Apart from the IL-18R, these genes are poorly defined and their individual and collective roles in T cell biology warrant additional insight.

We and others have shown that $CD8^+$ T cells are capable of secreting IL-10, express the IL-10R and effectively respond to distinct cellular sources of IL-10 [26–29,50,64–73]. These findings suggest that IL-10 may support memory $CD8^+$ T cell differentiation via an autocrine or paracrine loop. This then begs the question as to the role of IL-10 secretion by $CD8^+$ T cells in the context of an immune response. Our study shows that Eomes uses IL-10 to induce CD62L expression, which supports homing to and retention in secondary lymphoid organs. In line with this, our data propose a modified model of memory differentiation where Eomes acts as a molecular switch in effector $CD8^+$ T cells in inflamed tissues. As antigen levels and inflammatory stimuli decrease, Eomes expression is favored and induces IL-10. IL-10 then exerts its canonical anti-inflammatory role, harboring T cells from further inflammatory stimuli, while both Eomes and IL-10 cooperate to induce CD62L expression on a subset of $CD8^+$ T cells. In this model, the capacity to become a central memory $CD8^+$ T cell depends on a delicate balance of local inflammatory cues. An anti-inflammatory environment favors Eomes and IL-10 expression, which promotes CD62L expression and confers a competitive advantage to those cells competing for the memory niche.

5. Conclusion

Our aim was to assess the interplay between *T*-box transcription factors and cytokines in regulating $CD8^+$ T cell memory differentiation. Our study highlights an important role for Eomes in maintaining CD62L expression levels in central-memory T cells *in vivo*. Early upon activation, Eomes promotes the expression of the memory-associated molecules CD62L, Bcl6 and IL-10 in effector $CD8^+$ T cells. In turn, Eomes and IL-10 independently regulate CD62L, which enhances trafficking to

and retention in secondary lymph tissues. These findings further elucidate the role of Eomes in supporting memory differentiation and have implications in improving vaccine design as well enhancing memory responses against infection and cancer.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.11.008.

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