UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Eomes in the Maintenance of $CD8^+$ Tissue-Resident Memory T cells

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The thesis of Han Duong is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

The Role of Eomes in the Maintenance of $CD8^+$ Tissue-Resident Memory T cells

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Master of Science in Biology

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Professor John Tzu-yu Chang, Chair
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$CD8^+$ tissue-resident memory T cells play a well-established role in host defense against microbial pathogens but have also been recently implicated in playing a role in the pathogenesis of inflammatory and autoimmune diseases. Previous work demonstrated that the T-box transcription factor Eomes is extinguished during the formation of skin T_{RM} cells owing to TGF-β signaling. However, our lab previously observed high expression of Eomes in a pathogenic $CD8^+$ T_{RM} subset enriched in patients with ulcerative colitis, a form of inflammatory bowel

disease. Thus, we sought to investigate the role of Eomes in the maintenance of T_{RM} in small intestine and colon tissue compartments. We adoptively transferred congenically distinct wild-type and $Eomes^{fl/fl}$ ER-Cre^{+} $CD8^{+}$ P14 cells into recipient mice and infected them with LCMV. We administered tamoxifen injections to inducibly delete Eomes at 30 days post-infection after the formation of T_{RM} cells. Our results suggest that Eomes plays a previously unappreciated function in intestinal T_{RM} maintenance through a potential role in regulating tissue damage and TGF-β responsiveness.

Introduction

$CD8^+$ T cells play an important role in protecting the body against infection and cancer. The advent of new technology such as single-cell RNA sequencing allowed for the discovery and characterization of subsets of $CD8^+$ T cells. The subsets of T cells include effector, central memory, peripheral memory, long-lived effector memory, and tissue-resident memory T cells (1). $CD8^+$ tissue-resident memory T cells (T_{RM}) normally reside in non-lymphoid tissues where they are uniquely positioned as rapid responders to local infections (1). They work to limit the spread of infection, recruit other immune cells, and clear pathogens at the site of infection (1). T_{RM} cells have also been recently implicated in the pathogenesis of inflammatory and autoimmune diseases such as ulcerative colitis (UC) (1,2). In a recent study, our lab utilized single-cell RNA sequencing to characterize immune cell subsets within healthy individuals and patients with UC. We found a pathogenic T_{RM}-like cluster enriched in patients with UC. Eomesodermin (Eomes) was found to be upregulated within this cluster, suggesting that it might function as a potential regulator for this pathogenic T_{RM} subset (2). Eomes is a T-box transcription factor that has been shown to be involved in effector function as well as central memory formation (3,4). The T-box transcription factors, Eomes and its homolog T-bet, were reported to be downregulated via TGF-β signaling to enable formation of $CD8^+CD103^+$ T_{RM} cells in the skin (5). However, the role of Eomes in T_{RM} maintenance, particularly in the intestinal tissue compartments, is largely unknown.

Results

To test whether Eomes has a role in the maintenance of $CD8^+$ tissue-resident memory T cells (T_{RM}), we adoptively transferred congenically distinct wild-type (WT) and $Eomes^{fl/fl}$ ER-Cre (iKO) $CD8^+$ P14 T cells into wild-type recipient mice prior to infection with LCMV. At 30 days post-infection, we inducibly deleted Eomes by administering tamoxifen injections to assess

the role of Eomes in the maintenance of intestinal T_{RM}. In preliminary experiments, we observed that the prototypical T_{RM} markers, CD69 and CD103, which are variably expressed in different tissues, were expressed at different levels in $CD8^+$ T_{RM} in the small intestine and colon intraepithelial (siIEL and cIEL) and lamina propria (siLPL and cLPL) compartments. Therefore, we injected anti-CD8a antibody prior to sacrifice to label $CD8^+$ T cells within the intravascular space (IV^+) as a method of identifying putative T_{RM} (IV^-) within these tissue compartments, as previously published. Upon flow cytometric analysis, we found that the majority of $CD8^+$ P14 cells in each compartment were IV^- putative T_{RM} cells (Fig 1B). When we looked at the distribution of WT and iKO within the T_{RM} cells, iKO cells exhibited a severe numerical defect within the small intestine compared to WT cells; notably, this defect was much less pronounced compared to the colon (Fig 1C), suggesting that Eomes may play a critical role in the maintenance of established small intestinal $CD8^+$ T_{RM} cells. To characterize expression of Eomes at homeostasis, we looked at the relative WT expression of Eomes in each tissue compartment using flow cytometry. We observed that colon $CD8^+$ T_{RM} cells tended to express higher levels of Eomes compared to those in the small intestine. (Fig 1D). The siIEL T_{RM} cells had the lowest percentage of $Eomes^+$ cells while the cIEL T_{RM} cells had the highest. These data suggest an inverse correlation between Eomes expression and importance in T_{RM} cell maintenance, as siIEL and siLPL T_{RM} cells were the most dependent on Eomes for their maintenance despite their apparent low expression.

Historically, T_{RM} cells have been identified using CD69 and CD103, an early activation and tissue retention marker, respectively (1). However, there has been growing appreciation for phenotypic heterogeneity among T_{RM} cells from the same tissues as well as different tissues (1, 6). To explore this, we examined CD69 and CD103 expression in the siIEL, siLPL, cIEL, and

cLPL tissue compartments. We observed that the siIEL contained the most $CD69^+CD103^+$ T_{RM} cells compared to the other compartments (Fig 2A). The LPL compartments contained fewer $CD103^+$ cells when compared to their IEL counterparts. Additionally, the cIEL was the most phenotypically heterogenous compartment encompassing three different T_{RM} sub-populations ($CD69^-CD103^-$, $CD69^+$, $CD69^+CD103^+$). Eomes, along with its homolog T-bet, has been found to regulate $CD8^+CD103^+$ T_{RM} formation in skin through their coordinate downregulation via TGF-β signaling (5). Upon deletion of Eomes, we saw an increased frequency of $CD69^+CD103^+$ T_{RM} cells compared to WT T_{RM} cells within the siIEL, cIEL, and cLPL compartments. Moreover, the percentage of $CD69^+$ T_{RM} cells increased in only the cIEL and cLPL compartments, while $CD103^+$ cells increased in both cIEL and siIEL compartments. The mean fluorescent intensity (MFI) of CD69 was increased in all compartments while MFI of CD103 expression was increased in only the siIEL and siLPL (data not shown). This data highlights Eome's role in regulating CD103 expression as well as its potential role in regulating CD69 expression.

To investigate the mechanism by which Eomes promotes T_{RM} maintenance, we looked at markers known to be involved in T_{RM} survival and maintenance. IL-15 signaling is required for T_{RM} survival and the β-chain (CD122) of the IL-15 receptor has been reported to be a direct gene target of Eomes (7). We observed that CD122 expression correlated with the relative expression of Eomes in the small intestine and colon at homeostasis (Fig. 2B). In WT $CD8^+$ T_{RM}, CD122 expression was lower in the small intestine compared to the colon. The deletion of Eomes resulted in a decrease of CD122 expression only in the cIEL and cLPL compartments, but a slight increase in the siIEL compartment. Based on these data, Eomes may not be required for T_{RM} maintenance in an IL-15 dependent manner in the small intestine.

P2RX7 is a purinergic receptor reported to have a role in supporting T_{RM} cell longevity by promoting TGF-β sensitivity and metabolic function (10,11). P2RX7 also senses extracellular ATP or NAD^+ which is increased when inflammation or tissue damage occurs (12). When P2RX7 is triggered with NAD^+, it resulted in selective T_{RM} death (12). In WT $CD8^+$ T_{RM}, P2RX7 expression was higher in the small intestine compared to the colon (Fig. 2C). The LPL compartments contained higher percentages of $P2RX7^+$ cells compared to their IEL counterparts. Upon deletion of Eomes, P2RX7 expression was increased in all four compartments in terms of percentage and MFI of $P2RX7^+$ cells (data not shown). Thus, these data indicate the potential role of Eomes in suppressing P2RX7 expression which corresponds to decreased tissue damage sensitivity.

TGF-β is also required for the formation and maintenance of $CD103^+$ T_{RM} in the small intestine (8,9). TGF-β responsiveness is regulated through the expression of TGF-βRII (5). In addition, TGF-β has been shown to downregulate T-box transcription factors, Eomes and T-bet, to promote CD103 expression in the skin (5). Based on our data in WT $CD8^+$ T_{RM}, TGF-βRII expression did not correlate with relative CD103 expression across the four tissue compartments (Fig. 2, A and D). We observed that the highest TGF-βRII expression is in the colon compared to the small intestine at homeostasis. This result suggests intestinal differences in TGF-β responsiveness based on distinct TGF-βRII expression in each intestinal compartment. The deletion of Eomes resulted in the downregulation of TGF-βRII in all tissue compartments, suggesting that Eomes may play a role in promoting TGF-β responsiveness. These results suggest that the requirement of T_{RM} cells in the small intestine for Eomes may result from a greater dependence on TGF-β signaling than that of colon T_{RM} cells.

Discussion

The previously published observation that Eomes is highly expressed in a pathogenic subset of T_{RM} cells in UC patients prompted us to study this transcription factor in T_{RM}, using a murine infection model. The role of Eomes in early T_{RM} formation and maintenance has been well-characterized, but only in skin T_{RM} cells. It was suggested that the extinguishment of Eomes expression is necessary for T_{RM} formation and maintenance in the skin. However, our results suggest that Eomes plays a previously unappreciated function in intestinal T_{RM} maintenance through a potential role in regulating tissue damage and TGF-β responsiveness. This evidence suggests that the transcriptional regulation of T_{RM} cell maintenance and longevity may be tissue-specific. Additionally, we showed phenotypic heterogeneity across all four intestine tissue compartments based on CD69, CD103, Eomes, CD122, P2RX7, and TGF-βRII expression in $CD8^+$ T_{RM} cells. The question of whether this phenotypic heterogeneity confers functional differences remains unanswered. It has been reported that $CD69^+$ T_{RM} cells have potential for tissue egress while $CD103^+$ cells may be more retained in the tissue (5). $CD103^{hi}$ T_{RM} cells have also been reported to have higher cytotoxic capacity in human lung cancer and their frequency is predictive of improved survival outcomes (13). Thus, modulation of Eomes expression may promote the formation of certain T_{RM} subsets to improve disease prognoses. Emerging research has also implicated T_{RM} cells in playing a role in the pathogenesis of certain diseases. Based on our data, the upregulation of Eomes in T_{RM} cells may confer continued persistence through decreased tissue damage responsiveness and increased TGF-β signaling. The decrease in tissue damage responsiveness may prevent the deletion of T_{RM} subsets upon tissue damage or inflammatory signals and the increase in TGF-β signaling may improve tissue retention, resulting in continued persistence T_{RM} in the intestinal compartments. Based on our data, the downregulation of Eomes may be a potential strategy in targeting these pathogenic T_{RM} cells.

Additional experiments will be required to confirm and explore the mechanisms to which Eomes may promote tissue maintenance.

Materials and Methods

Study design

The purpose of this study was to elucidate the role of Eomes in the T_{RM} maintenance in various intestinal compartments. To do so, we utilized flow cytometry to look at protein expression of selected markers by T_{RM} in the siIEL, siLPL, cIEL, and cLPL compartments at memory timepoints after inducible deletion of Eomes.

Mice

All mice were housed in an American Association of Laboratory Animal Care-approved facility at the University of California, San Diego (UCSD), and all procedures were approved by the UCSD Institutional Animal Care and Use Committee. The recipient mice used were 6-9 weeks of age, male, and purchased from Jackson Laboratories. $Eomes^{fl/fl}$ and ER-Cre mice were purchased from Jackson Laboratories. These mice were bred with P14 mice to obtain distinct $Eomes^{fl/fl}$ ER-Cre^{+} P14 mice.

Adoptive Cell Transfer, Infection, and Deletion of Eomes

Splenocytes were collected from $Eomes^{fl/fl}$ ER-Cre^{-} CD45.1 P14 and $Eomes^{fl/fl}$ ER-Cre^{+} CD45.1.2 P14 mice. $1x10^5$ $CD8\alpha^{+}CD45.1^{+}$ and $1x10^5$ $CD8\alpha^{+}CD45.1.2^{+}$ cells were adoptively transferred to congenically distinct CD45.1 recipient mice 30 minutes prior to infection. To infect, we used $2x10^5$ plaque-forming units (PFU) of LCMV Armstrong intraperitoneally. At 21 days post-infection, we bled the mice to check the percentage of circulating P14 cells. To delete Eomes, we administered tamoxifen injections (1 mg / mouse / day) consecutively for 4 days and allowed the mice to rest for 5 days before sacrifice. The sacrifice occurs at 30 days post-infection.

Lymphocyte Isolation

Treg-Protector (Biolegend) was administered via intraperitoneal injections15 minutes prior to sacrifice. To identify IV negative T_{RM} CD8+ T cells, 3 μg anti-CD8α antibody was injected intravenously 5 minutes prior to sacrifice. The spleen, small intestine, and colon were harvested. For lymphocyte isolation in the spleen, the splenocytes were mashed and placed in a single cell suspension prior to red blood cell lysis using Red Blood Cell Lysing Buffer Hybri-Max (Sigma). For lymphocyte isolation in the small intestine and colon, the Peyer's patches were removed, and the tissues were cut longitudinally and washed with PBS. The tissues were cut in 1-2 cm pieces and then shaken in DTE buffer (1ug/ml dithioerythritol (Thermo Fisher Scientific) in 10% HBSS and 10% HEPES bicarbonate) at 37°C for 30 minutes. The supernatant (IEL) was collected and spun down while the remaining tissues pieces (LPL) was resuspended in IEL solution (PBS and EDTA) and vortexed for 15 minutes. The IEL cells were placed in 44/67 Percoll gradient solution. After spinning the IEL tubes, the IEL cells were collected from the cloudy lymphocyte layer in the Percoll gradient. The LPL tissue pieces were cut up in small pieces and resuspended in LPL solution (RPMI 1640 with Glutamine (Corning), 10% FBS (Genclone), Collagenase P from Clostridium histolyticum (Sigma), and DNase (Sigma)). The tissue pieces were shaken for 12 minutes at 37°C. The LPL supernatant was collected, washed with cold PBS, and spun down.

Antibodies and Flow Cytometry

The cells were stained for 10 minutes on ice with Fixable Viability Dye eFluor 780 (Invitrogen/Thermo Fisher). The surface markers were stained for 30 minutes at 4°C with the following antibodies: CD45.1 (A20), TGF-βRII (FAB532P), CD122 (TM-β1), CD8b(YTS156.7.7), P2RX7(1F11), CD45.2(104), CD69 (H.2F3), CD127 (LG.3A10), and CD103(2E7). The cells were then fixed and permeabilized using eBioscience

Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher). After fixation, the cells were stained with intracellular marker, Eomes, for 30 minutes in permeabilization buffer at room temperature. For analysis, all the samples were run on a Novocyte cytometer (ACEA Biosciences) and the data analyzed using Flowjo software (BD Biosciences). The statistical analysis was done on Graphpad Prism.

Figure 1. Eomes plays a critical role in maintenance of established small intestine CD8+ T_{RM} cells.

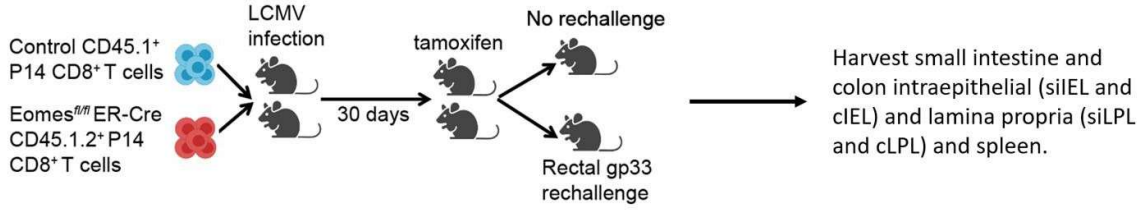
(A) Experimental setup. Wild-type (WT) CD45.1$^+$ P14 CD8$^+$ T cells and Eomes$^{fl/fl}$ ER-Cre (iKO) CD45.1.2$^+$ P14 CD8$^+$ T cells adoptively transferred into CD45.2$^+$ congenic hosts 30 minutes prior to infection LCMV-Armstrong. Tamoxifen was administered 30 days post-infection. Lymphocytes were harvested from the small intestine (siIEL and cIEL) and lamina propria (siLPL and cLPL) and spleen.

(B) Intravascular labelling using anti-CD8α antibody prior to sacrifice was employed to identify circulating T cells (IV$^+$) and putative tissue-resident memory T cells (IV$^-$). Representative flow cytometry plots displaying distribution of IV$^+$ and IV$^-$ populations across all four tissue compartments.

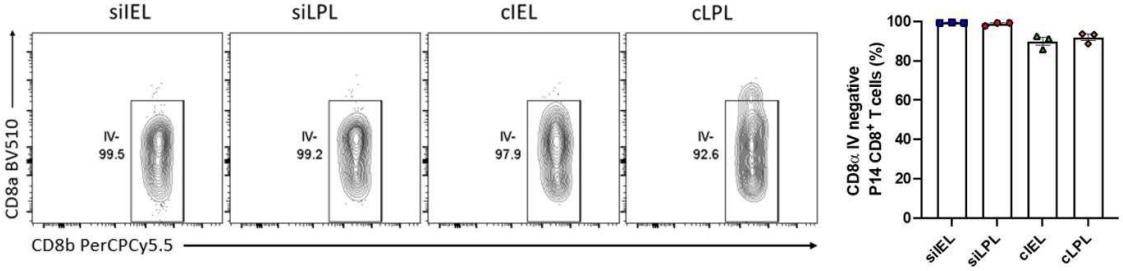
(C) Representative flow cytometry plots of the ratio of WT and iKO in all four tissue compartments. Percentages of WT and iKO gated within IV$^-$ cells were normalized to the percentages of WT (blue dots) and iKO (red dots) at the time of transfer.

(D) Bar graph displaying relative WT Eomes expression from flow cytometry data within tissue-resident memory T cells across all four tissue compartments. *P<0.05, **P<0.01, ***P<0.001 (Paired t-test).

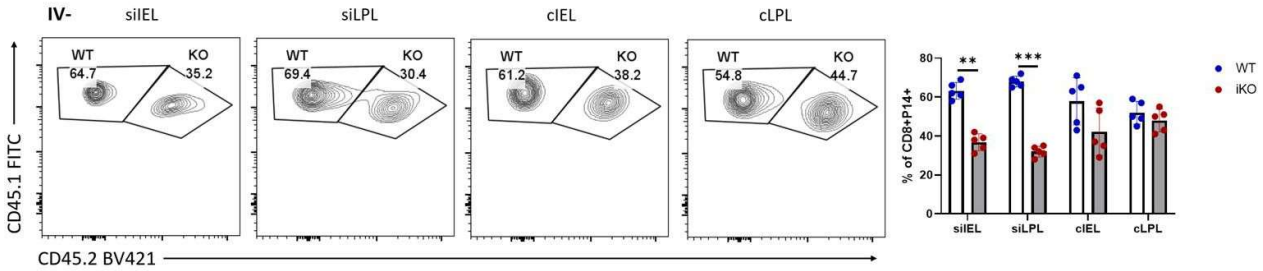
A


Control CD45.1+
P14 CD8+ T cells
Eomesfl/fl ER-Cre
CD45.1.2+ P14
CD8+ T cells
LCMV
infection
30 days
tamoxifen
No rechallenge
Rectal gp33
rechallenge
Harvest small intestine and
colon intraepithelial (siIEL and
cIEL) and lamina propria (siLPL
and cLPL) and spleen.

B


siIEL
siLPL
cIEL
cLPL
CD8a BV510
CD8b PerCPCy5.5
IV-
99.5
IV-
99.2
IV-
97.9
IV-
92.6
CD8α IV negative
P14 CD8+ T cells (%)
100
80
60
40
20
0
siIEL
siLPL
cIEL
cLPL

C


IV-
siIEL
siLPL
cIEL
cLPL
WT
KO
64.7
35.2
69.4
30.4
61.2
38.2
54.8
44.7
CD45.1 FITC
CD45.2 BV421
**
***
% of CD8+P14+
WT
iKO

D

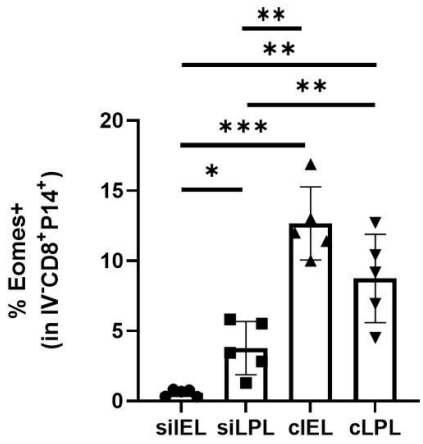
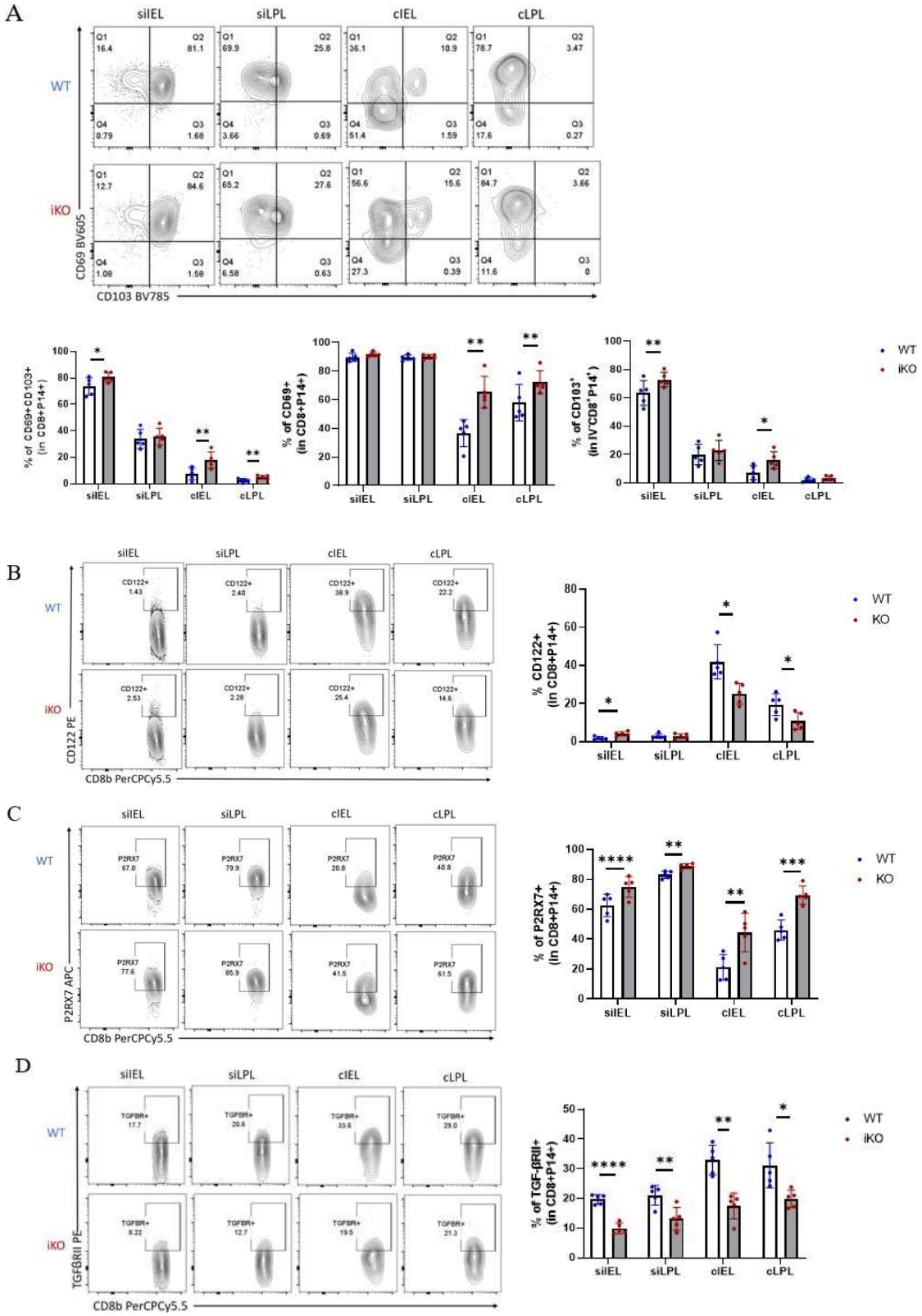

**
**
**
***
*
% Eomes+
(in IV-CD8+P14+)
20
15
10
5
0
siIEL
siLPL
cIEL
cLPL

Figure 2. Eomes may regulate expression of several genes that have been previously shown to play a role in T_{RM} maintenance.

(A to D) Representative flow cytometry plots of various markers in all four tissue compartments and bar graph of percentages of positive expression within T_{RM} population.

(A) CD69 and CD103 expression.
(B) CD122 expression.
(C) P2RX7 expression.
(D) TGF-βRII expression. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Paired t-test).


A
siIEL
siLPL
cIEL
cLPL
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iKO
CD69 BV605
CD103 BV785
% of CD69+CD103+ (in CD8+P14+)
% of CD69+ (in CD8+P14+)
% of CD103+ (in IV-CD8+P14+)
WT
iKO
B
CD122 PE
CD8b PerCPCy5.5
% CD122+ (in CD8+P14+)
WT
KO
C
P2RX7 APC
CD8b PerCPCy5.5
% of P2RX7+ (in CD8+P14+)
WT
KO
D
TGFβRII PE
CD8b PerCPCy5.5
% of TGF-βRII+ (in CD8+P14+)
WT
iKO

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