

# Characterization of eomesodermin and T-bet expression by allostimulated CD8<sup>+</sup> T cells of healthy volunteers and kidney transplant patients in relation to graft outcome

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## Summary

Memory T cell (T<sub>mem</sub>) responses play a critical role in the outcome of allo-transplantation. While the role of the T-box transcription factor Eomesodermin (Eomes) in the maintenance of antigen-specific T<sub>mem</sub> is well studied, little is known about Eomes<sup>+</sup>CD8<sup>+</sup>T cell responses after transplantation. We evaluated the phenotype and function of allo-reactive Eomes<sup>+</sup>CD8<sup>+</sup>T cells in healthy volunteers and kidney transplant patients and their relation to transplant outcome. High Eomes expression by steady-state CD8<sup>+</sup>T cells correlated with effector and memory phenotype. Following allo-stimulation, the expression of both the T-box proteins Eomes and T-bet by proliferating cells increased significantly, where high expression of Eomes and T-bet correlated with higher incidence of allo-stimulated IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> CD8<sup>+</sup>T cells. In patients with no subsequent rejection, Eomes but not T-bet expression by donor-stimulated CD8<sup>+</sup>T cells, increased significantly after transplantation. This was characterized by increased Eomes<sup>hi</sup>T-bet<sup>lo</sup> and decreased Eomes<sup>lo</sup>T-bet<sup>hi</sup> CD8<sup>+</sup>T cell subsets, with no significant changes in the Eomes<sup>hi</sup>T-bet<sup>hi</sup> CD8<sup>+</sup>T cell subset. No upregulation of exhaustion markers programmed-death-1 (PD-1) and cytotoxic-T-lymphocyte-associated-antigen-4 (CTLA4) by donor-stimulated Eomes<sup>+</sup>CD8<sup>+</sup>T cells was observed. Before transplantation, in patients without rejection, there were higher incidences of Eomes<sup>hi</sup>T-bet<sup>lo</sup>, and lower incidences of Eomes<sup>hi</sup>T-bet<sup>hi</sup> and Eomes<sup>lo</sup>T-bet<sup>hi</sup> donor-stimulated CD8<sup>+</sup>T cell subsets, compared to those with subsequent rejection. Overall, our findings indicate that high Eomes expression by allo-stimulated T-bet<sup>+</sup>CD8<sup>+</sup>T cells is associated with enhanced effector function, and that an elevated incidence of donor-stimulated CD8<sup>+</sup>T cells co-expressing high levels of Eomes and T-bet before transplantation, may correlate with an increased incidence of acute cellular rejection.

**Keywords:** Eomesodermin, human, kidney transplantation, memory T cells, rejection, T-bet

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## Introduction

Pro-inflammatory and cytotoxic memory T cells (T<sub>mem</sub>) with enhanced proliferative and functional capacities [1], compared with naïve T cells, are activated by antigen (Ag)-presenting cells in diverse tissues, including organ allografts [2,3]. Additionally, allo-reactive effector CD4<sup>+</sup> and CD8<sup>+</sup>

T<sub>mem</sub> are less susceptible to suppression by regulatory T cells in vivo, than their naïve T cell counterparts [4]. Hence, T<sub>mem</sub> are considered a major barrier to improved long-term allograft survival and tolerance induction [5,6].

T<sub>mem</sub> are immunosuppression-resistant [4], where allo-reactive CD8<sup>+</sup> T<sub>mem</sub> are known to be more resistant than

CD4<sup>+</sup> Tmem [7,8]. In rodents, multiple viral infections result in the development of CD8<sup>+</sup> Tmem that cross-react with allo-antigen (Ag) [9], and mediate resistance to tolerance induction. Unlike naïve CD8<sup>+</sup> T cells, primed CD8<sup>+</sup> Tmem are capable of mediating allograft rejection independent of CD154 co-stimulation [10], whereas treatment with CD154 blockade does not prevent allograft rejection in sensitized allograft recipients [11]. Accordingly, tolerance induction has not been successful in sensitized recipients exhibiting high levels of primed Tmem at the time of transplantation [12,13]. Furthermore, while deletion of allo-reactive T cells is known to be critical for tolerance induction, homeostatic proliferation following lymphodepletion results in the development of Tmem that are immunosuppression-resistant and can promote allograft rejection [14].

Eomesodermin (Eomes), a paralogue of the hallmark T-helper (Th)1 cell transcription factor T-bet, plays a key role in CD8<sup>+</sup>Tmem differentiation, survival and function [15–17]. It is essential for long-term survival of Ag-specific effector CD8<sup>+</sup> Tmem [17]. In rodents, both Eomes and T-bet orchestrate the induction and long-term maintenance of effector CD8<sup>+</sup>T cell function. T-bet is essential for early programming of cytokine production [18], whereas Eomes is induced during CD8<sup>+</sup>T cell differentiation and required to maintain effector function [19]. Additionally, rodent studies have demonstrated critical roles of both T-bet and Eomes in maintaining long-term anti-viral immunity and in preventing chronic viral infection [20–23].

While Th1 responses and T-bet expression have been linked to allograft rejection [24,25], there is little information concerning the role of Eomes in the regulation of allo-immunity. Furthermore, the relative expression of Eomes by allo-reactive CD8<sup>+</sup>T cells and its relation to graft outcome in human organ transplant (tx) recipients is not known. Here, we evaluated Eomes and T-bet expression by allo-stimulated CD8<sup>+</sup>T cells of healthy volunteers and by donor-stimulated CD8<sup>+</sup>T cells, before and after tx, in kidney tx patients given lymphodepletion induction therapy.

## Materials and methods

### Healthy subjects

Healthy adult volunteers of both sexes were recruited following informed consent under an IRB-approved protocol at the University of Pittsburgh. In addition, leukocyte concentrates from healthy volunteers were purchased from the Pittsburgh Blood Bank, Pittsburgh, PA.

### Kidney transplant patients

Cryopreserved peripheral blood mononuclear cell (PBMC) samples from consenting kidney tx patients were obtained

from the human transplant biorepository at the Thomas E. Starzl Transplantation Institute. Demographics and general information concerning these patients are shown in Table 1. Three groups of patients were analyzed: (i) patients with no T cell-mediated rejection within the first year post-tx, i.e. stable (n = 11), (ii) patients with subclinical rejection (SCR), i.e. diagnosis of T cell-mediated rejection in protocol biopsy with stable graft function (n = 5), and (iii) patients with acute cellular rejection (ACR), defined as those patients that had histological findings of cellular rejection and clinical graft dysfunction (n = 5). All patients were recipients of living-donor kidney tx. All patients received T cell-depleting polyclonal Ab (Thymoglobulin) as induction therapy and standard-of-care immunosuppression based on tacrolimus, mycophenolate mofetil (MMF) and methylprednisolone. In 2 patients (with no rejection), Azathioprine was administered instead of MMF.

### Peripheral blood mononuclear cell isolation

PBMC obtained from healthy volunteers and patients were isolated using Ficoll-Paque Plus (GE Healthcare Bio-Sciences; Piscataway, NJ) and cryopreserved with RPMI-1640 (Invitrogen, Carlsbad, CA), 20% v/v fetal bovine serum (FBS) (Atlanta Biologicals; Flowery Branch, GA) and 10% dimethyl sulfoxide (Thermo Fisher Scientific, Pittsburgh, PA). For tx patients, samples were collected before, 1-month, 3-months and 1-year after tx, when available, then processed and stored as above.











### Flow cytometric analysis

For cell surface staining, the following conjugated Abs (and clones) were used: PerCP-Cy5.5 CD3 (SP34-2), APC-H7 CD4 (L200), PECy7 CCR7 (3D12) and V450 CD45RA (5H9) from Becton Dickinson (BD; Franklin Lakes, NJ) and AF700 or PECy7 CD8 (RPA-T8) from Biolegend (San Diego, CA). For intracellular staining, cell fixation and permeabilization were performed using Fix and Perm reagent (eBioscience, San Diego, CA) and the following conjugated Abs used: eFluor660 Eomes (WD1928) and PE or VB420 T-bet (eBio4B10) from eBioscience; PE IFN $\gamma$  (4SB3), Pacific Blue or AF700 TNF $\alpha$  (MAb11) and V450 Granzyme-B (GrB) (GB11) from Biolegend. For cytokine staining, cells were stimulated with phorbol myristate acetate (PMA; 1ng/ $\mu$ l) and ionomycin (100ng/ $\mu$ l) for 3–4 hr at 37°C. Data were acquired using a Fortessa flow cytometer (BD) and analysis performed using FlowJo software (Tree Star, San Carlos, CA).

### CD3/CD28 activation

After thawing, PBMC were stained with 4 $\mu$ M carboxy-fluorescein succinimidyl ester (CFSE; Invitrogen) and plated at a density of 2 x 10<sup>5</sup> cells per well (round-bottom

**Table 1.** Demographic and clinical characteristics of living donor kidney transplant patients with or without rejection.

Groups	Age	Sex	EBV	CMV	BKV	Rejection (Type)	Time of rejection (days post-tx)	Type of rejection (Banff grade)	Color code			
Group 1	45	M	+	-	+	None	NA	NA				
	28	F	+	-	-							
	33	M	+	-	-							
	41	M	+	-	-							
	19	M	+	NA	-							
	22	F	+	NA	-							
	28	F	+	-	+							
	50	F	+	+	-							
	49	M	+	-	+							
	48	M	+	NA	+							
Group 2	39	M	+	NA	-	Yes (subclinical rejection)						
	23	M	+	+	-					393	IB	
	28	F	+	-	+					373	IB	
	33	F	+	NA	-					362	IA	
	62	M	+	-	-					372	IA	
	56	F	+	+	-					109	IA	
	59	M	+	+	-					756	IA/IB	
Group 3	36	F	+	-	-	Yes (acute cellular rejection)						
	60	M	+	+	-					210	IA	
	56	F	+	-	-					29	IA	
	28	F	+	-	-					23	IA	
												

NA = not available.

96-well plates) with RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2mM L-glutamine (Mediatech Inc., Herndon, VA), 100 U/ml penicillin-streptomycin (BioWhittaker, Walkersville MD), 10 mM HEPES (Mediatech) and 55  $\mu$ M  $\beta$ -2 mercaptoethanol (Invitrogen). Cells were activated with CD3/CD28 beads (T cell activation/expansion kit, Miltenyi Biotec), and cultured at 37°C for 4 days.

#### CFSE-mixed leukocyte reaction (MLR)

In experiments using samples obtained from healthy volunteers, T lymphocytes were purified by magnetic selection (Pan T isolation kit; Cat#130-091-113, Miltenyi Biotec, Auburn, CA) from cryopreserved PBMC, followed by CFSE staining (Invitrogen). T cell-depleted allogeneic PBMC were used as stimulators. Responders were co-cultured with irradiated (3000 cGy) stimulators in complete RPMI-1640 supplemented with 10% v/v FBS, 2mM L-glutamine (Mediatech), 100 U/ml penicillin-streptomycin (BioWhittaker), 10 mM HEPES (Mediatech Inc) and 55  $\mu$ M  $\beta$ -2 mercaptoethanol (Invitrogen), at a ratio of 1:1, in 96-well, U-bottom plates, and at a final concentration of  $4 \times 10^5$  cells/well at 37°C in 5% CO<sub>2</sub> in air. Cultures were harvested on days 3, 5 or 7. Responder cells without stimulation served as controls.

In kidney tx patients, because the number of cells obtained after tx was limited, MLR were set up without sorting responder (recipient) or stimulator (donor) cells. CFSE-stained responder cells were co-cultured with

irradiated (3000 cGy) stimulator PBMC stained with cell tracker violet BMQC (Life Technologies C10094, Carlsbad, CA). Cultures were harvested on day 7. Proliferation was determined by CFSE dilution, after gating out the stimulators (cell tracker violet positive cells).

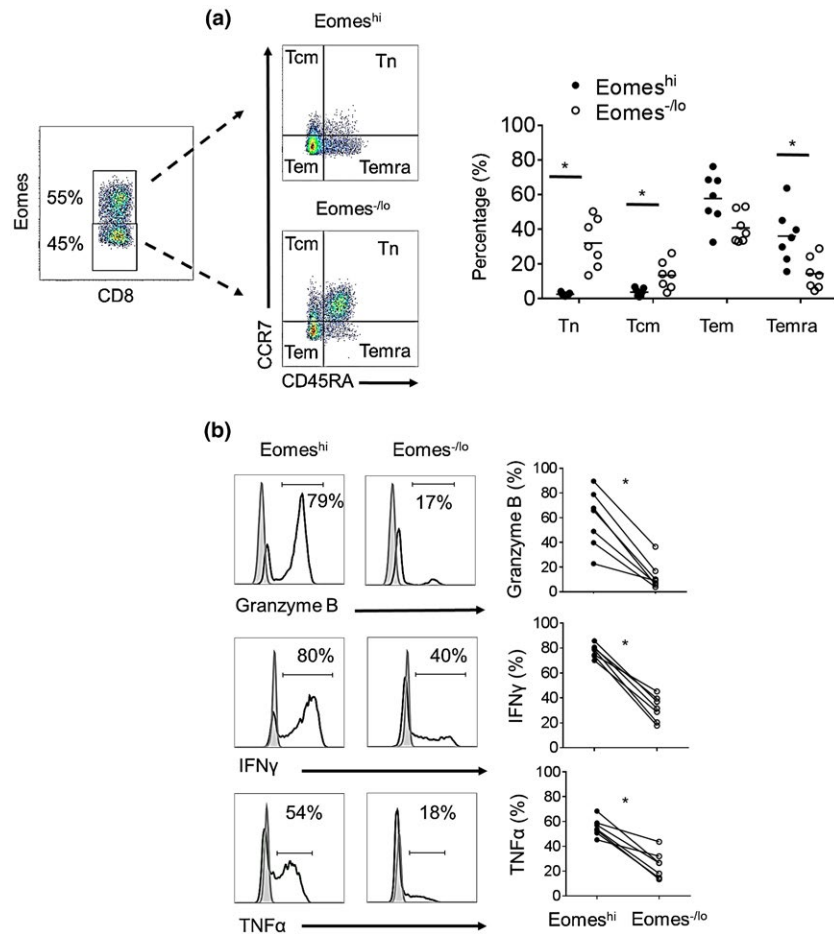
#### Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.07 software (GraphPad). The Wilcoxon test (for paired variables) or Mann-Whitney test was used for analysis.  $P < 0.05$  was considered statistically significant.

#### Results

##### Memory and effector phenotype of Eomes<sup>hi</sup> versus Eomes<sup>lo</sup> CD8<sup>+</sup>T cells in healthy volunteers, before and after allo-stimulation

Naïve and memory subsets of non-activated Eomes<sup>hi</sup> versus Eomes<sup>lo</sup> CD8<sup>+</sup>T cells in peripheral blood of healthy volunteers were evaluated based on their differential expression of CD45RA and CCR7, i.e. naïve (Tn; CD45RA<sup>+</sup>CCR7<sup>+</sup>), central memory (Tcm; CD45RA<sup>-</sup>CCR7<sup>+</sup>), effector memory (Tem; CD45RA<sup>-</sup>CCR7<sup>-</sup>) and terminally-differentiated effector memory (Temra; CD45RA<sup>+</sup>CCR7<sup>-</sup>). Eomes<sup>hi</sup> CD8<sup>+</sup>T cells were comprised mostly of Tem and Temra. Of note, the percentages of Temra among the Eomes<sup>hi</sup> CD8<sup>+</sup>T cell population were



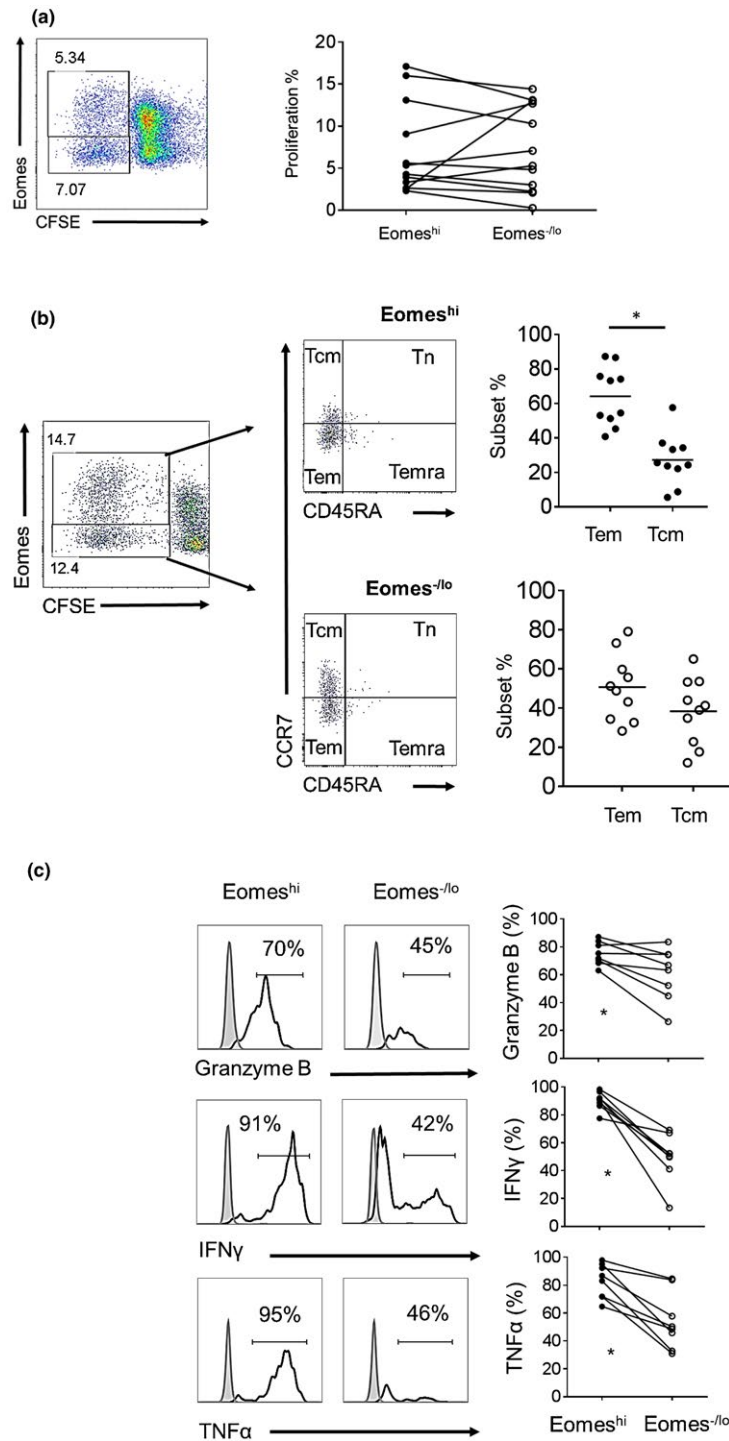
**Fig. 1.** Memory and effector phenotype of *Eomes*<sup>hi</sup> versus *Eomes*<sup>-/lo</sup> non-activated CD8<sup>+</sup>T cells in healthy human volunteers. (a) Memory CD8<sup>+</sup>T cell subsets were defined based on their differential expression of CD45RA and CCR7: naïve T cells (Tn; CD45RA<sup>+</sup>CCR7<sup>+</sup>), central memory T cells (Tcm; CD45RA<sup>-</sup>CCR7<sup>+</sup>), effector memory T cells (Tem; CD45RA<sup>+</sup>CCR7<sup>-</sup>) and terminally-differentiated effector memory T cells (Temra; CD45RA<sup>+</sup>CCR7<sup>-</sup>). Dot plots are from one representative individual. Mean values are indicated by horizontal bars (n = 7 individuals). (b) Expression of TNFα, IFNγ and granzyme-B was assessed after 3-4 hours of PMA/ionomycin stimulation (n = 7 individuals). Histograms (left) are from one representative individual; results are expressed as percent positive cells. Gray histograms indicate isotype controls. Data from all 7 individuals examined are shown on the right. Wilcoxon-Mann-Whitney test; \**P* < 0.05.

significantly higher than among *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells (*P* < 0.05), while naïve and Tcm were significantly higher in *Eomes*<sup>-/lo</sup> compared to *Eomes*<sup>hi</sup> CD8<sup>+</sup> T cells (Fig. 1a). Next, we examined the expression of the effector molecules GrB, IFNγ and TNFα by *Eomes*<sup>hi</sup> versus *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells following their stimulation for 3-4 hr with PMA/ionomycin. *Eomes*<sup>hi</sup> CD8<sup>+</sup>T cells consistently comprised significantly higher percentages of GrB<sup>+</sup>, IFNγ<sup>+</sup> and TNFα<sup>+</sup> cells (*P* < 0.05) than *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells (Fig. 1b).

Following stimulation with allogeneic human T cell-depleted PBMC in CFSE-MLR, two distinct *Eomes*<sup>hi</sup> and *Eomes*<sup>-/lo</sup> proliferating CD8<sup>+</sup>T cell populations were consistently observed (Fig. 2a). The percent proliferation of *Eomes*<sup>hi</sup> versus *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells in response to allo-stimulation was variable between individuals. This variability in proliferation of *Eomes*<sup>hi</sup> versus *Eomes*<sup>-/lo</sup> cells was

also observed when the same responder CD8<sup>+</sup>T cells were stimulated by allogeneic cells from different individuals (*data not shown*). As reported previously [26], *in vitro* allo-stimulated CD8<sup>+</sup>T cells exhibited a memory phenotype (i.e. CD45RO<sup>+</sup>; not shown). We further evaluated the memory phenotype of proliferating *Eomes*<sup>hi</sup> versus *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells following allo-stimulation based on CD45RA and CCR7 expression. As expected, proliferating cells comprised mainly CD45RA negative cells, i.e. either Tcm or Tem. While *Eomes*<sup>hi</sup> CD8<sup>+</sup>T cells comprised significantly higher percentages of Tem than Tcm (*P* < 0.05), the mean percentages of Tem and Tcm were similar for *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells (Fig. 2b).

To evaluate their effector phenotype, we examined allo-stimulated *Eomes*<sup>hi</sup> versus *Eomes*<sup>-/lo</sup> CD8<sup>+</sup>T cells for GrB, IFNγ and TNFα expression following PMA/ionomycin



**Fig. 2.** Memory and effector phenotype of allo-stimulated Eomes<sup>hi</sup> versus Eomes<sup>lo</sup> CD8<sup>+</sup>T cells. (a) CFSE-labeled purified T cells were co-cultured with allogeneic stimulators for 5 days. Percent responder cell proliferation was determined by CFSE dilution and Eomes expression by proliferating cells was then evaluated by flow cytometry. The dot plot is from one representative individual. Data from all individuals examined are shown on the right (n = 12). (b) Memory CD8<sup>+</sup>T cell subsets of proliferating cells were defined based on their differential expression of CD45RA and CCR7 (as in Fig. 1). The dot plots are from one representative individual. Data from 10 individuals examined are shown. (c) Expression of TNF $\alpha$ , IFN $\gamma$  and granzyme-B assessed 3-4 hours after PMA/ionomycin stimulation following coculture with allogeneic stimulators. Histograms (left) are from a representative individual; results are expressed as percent positive cells, gating on proliferating cells. Gray histograms indicate isotype controls. Data from all individuals examined (n = 8) are shown on the right. Wilcoxon-Mann-Whitney test; \*P < 0.05.

stimulation. Like their non-stimulated counterparts, allo-stimulated Eomes<sup>hi</sup> CD8<sup>+</sup>T cells exhibited significantly higher incidences of GrB<sup>+</sup>, IFN $\gamma$ <sup>+</sup> and TNF $\alpha$ <sup>+</sup> cells ( $P < 0.05$ ) than Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells (Fig. 2c).

Both proliferating and non-proliferating Eomes<sup>hi</sup> CD8<sup>+</sup>T cells comprised a significantly higher incidence of dual IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells than Eomes<sup>-/lo</sup> cells (Supporting Information Fig. 1). The mean percentages of dual IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells among Eomes<sup>hi</sup> proliferating cells were similar to those of Eomes<sup>hi</sup> non-proliferating cells. Furthermore, in the non-proliferating CD8<sup>+</sup>T cell populations, Eomes<sup>hi</sup> cells comprised a significantly higher incidence of dual IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells than Eomes<sup>-/lo</sup> cells. In correlation, non-proliferating Eomes<sup>hi</sup> CD8<sup>+</sup>T cells were also comprised mainly of Tem and Temra (*data not shown*). These observations suggest that higher Eomes expression correlates with enhanced effector function, even in the absence of proliferation after allo-stimulation.

Next, we evaluated the percentages of Eomes<sup>hi</sup> CD8<sup>+</sup>T cells and their intensity of Eomes expression 3, 5 and 7 days following allo-stimulation (Fig. 3). Eomes expression (MFI) increased progressively over time, such that the incidence of proliferating Eomes<sup>hi</sup> CD8<sup>+</sup>T cells was higher than that of Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells after 7 days of culture. This suggests that prolonged allo-stimulation *in vitro* is associated with increased incidence of Eomes<sup>hi</sup> CD8<sup>+</sup>T cells.

#### T-bet expression by Eomes<sup>hi</sup> and Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells of healthy volunteers, before and after allo-stimulation

The T-box protein T-bet is essential for effector CD8<sup>+</sup>T cell development and function [16,21,27]. Furthermore, T-bet drives Th1 differentiation [28,29], which plays a major role in T cell responses to allo-Ag [24,25]. We evaluated Eomes<sup>hi</sup> and Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells for T-bet expression before and after allo-stimulation. Non-stimulated Eomes<sup>hi</sup> CD8<sup>+</sup>T cells exhibited significantly higher T-bet expression than Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells ( $P < 0.001$ ) (Fig. 4a), which parallels the enhanced effector phenotype of Eomes<sup>hi</sup> CD8<sup>+</sup>T cells (Fig. 2c).

Following allo-stimulation, T-bet expression was upregulated by Eomes<sup>hi</sup> and Eomes<sup>-/lo</sup> proliferating cells. However, T-bet expression by proliferating Eomes<sup>hi</sup> CD8<sup>+</sup>T cells remained significantly higher than that by Eomes<sup>-/lo</sup> cells ( $P < 0.05$ ) (Fig. 4a). Meanwhile, T-bet expression was strongly upregulated by Eomes<sup>hi</sup> and Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells after CD3/CD28 activation (Supporting Information Fig. 2). Next, we assessed the effector phenotype of CD8<sup>+</sup>T cells co-expressing T-bet and Eomes following allo-stimulation (Fig. 4b). Proliferating Eomes<sup>hi</sup>T-bet<sup>hi</sup> cells comprised the highest incidence of dual IFN $\gamma$  and TNF $\alpha$  positive (IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup>)

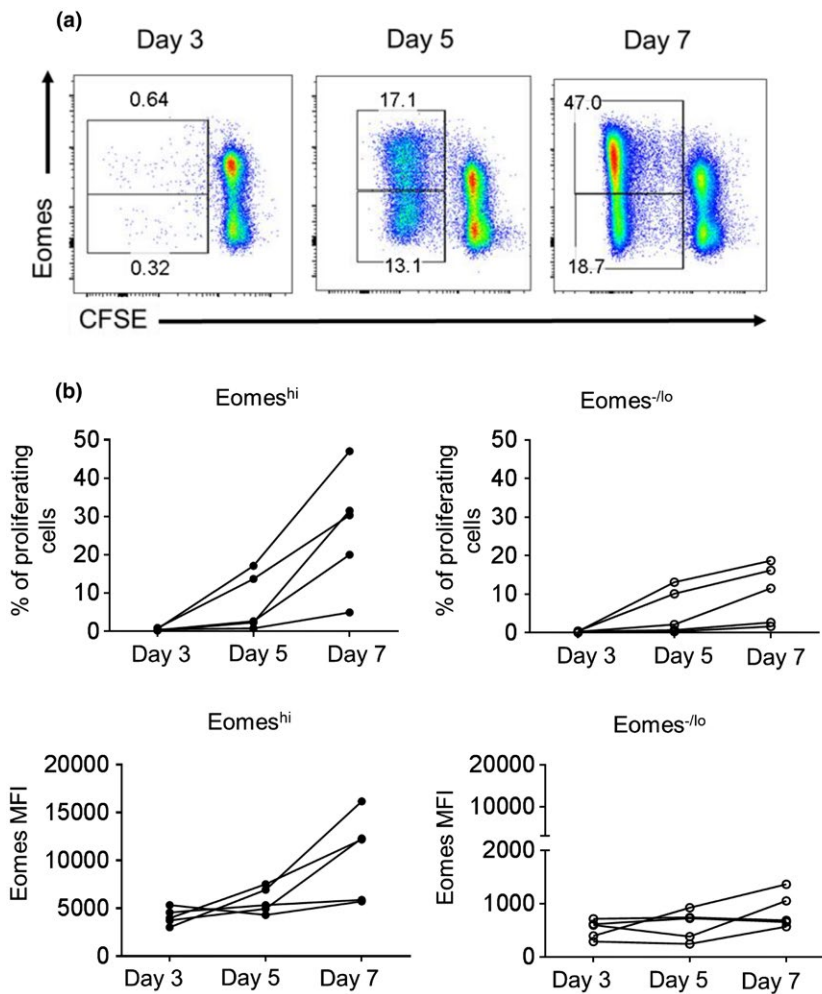
cells, while proliferating Eomes<sup>-/lo</sup>T-bet<sup>-/lo</sup> cells comprised the lowest incidence of these cells. Notably, within the T-bet<sup>hi</sup> or T-bet<sup>-/lo</sup> populations, higher Eomes expression correlated with higher incidences of IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells ( $P < 0.05$ ). When total CD8<sup>+</sup>T cells (both proliferating and non-proliferating) were evaluated, similar incidences of IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells were observed (not shown). These findings suggest that, irrespective of T-bet expression, higher Eomes expression by alloreactive human CD8<sup>+</sup>T cells correlates with an enhanced effector phenotype, even in the absence of proliferation.

Next, we evaluated the kinetics of Eomes and T-bet expression by allo-stimulated CD8<sup>+</sup>T cells. Following allo-stimulation, proliferating (dividing) CD8<sup>+</sup>T cells exhibited significant increases in both Eomes and T-bet expression ( $P < 0.01$ ) after the first division, compared to non-proliferating cells (division 0). While a gradual increase in T-bet and Eomes expression was observed initially over the first 3 divisions, expression of both factors declined gradually with subsequent divisions (Fig. 5). These observations indicate that proliferation of CD8<sup>+</sup>T cells in response to allo-stimulation is associated with a concomitant increase in Eomes and T-bet expression.

#### Eomes and T-bet co-expression by donor stimulated CD8<sup>+</sup>T cells of kidney tx patients with no rejection

Next, we aimed to evaluate Eomes and T-bet co-expression in human renal allograft recipients with no rejection (Table 1). First, we evaluated the incidences of Eomes<sup>+</sup>CD8<sup>+</sup>T cells in patients before tx. No significant difference was observed in the intensity of Eomes expression (MFI), when compared to healthy controls (Supporting Information Fig. 3a). Similar to healthy volunteers, Eomes<sup>hi</sup> CD8<sup>+</sup>T cells comprised significantly higher incidences of T-bet<sup>+</sup> cells ( $P < 0.05$ ) than Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells (Supporting Information Fig. 3b). Following allo-stimulation, within the proliferating T-bet<sup>-/lo</sup> or T-bet<sup>hi</sup> populations, higher Eomes expression also correlated with a higher incidence of IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells (Supporting Information Fig. 3c). These data indicate that, in patients with end stage renal disease, the effector phenotype of Eomes<sup>+</sup>CD8<sup>+</sup>T cells is similar to that of healthy controls.

Next, we determined the kinetics of Eomes and T-bet expression by donor (allo)-reactive CD8<sup>+</sup>T cells in renal allograft recipients with no rejection, before and after tx. In these experiments, proliferation of donor-stimulated CD8<sup>+</sup>T cells was only observed in post-tx samples obtained from 6/11 patients. In 5/11 patient samples, no proliferation of donor-stimulated CD8<sup>+</sup>T cells was observed. Both proliferating and non-proliferating Eomes<sup>hi</sup> CD8<sup>+</sup>T cells were similar in regards to their effector phenotype, i.e.

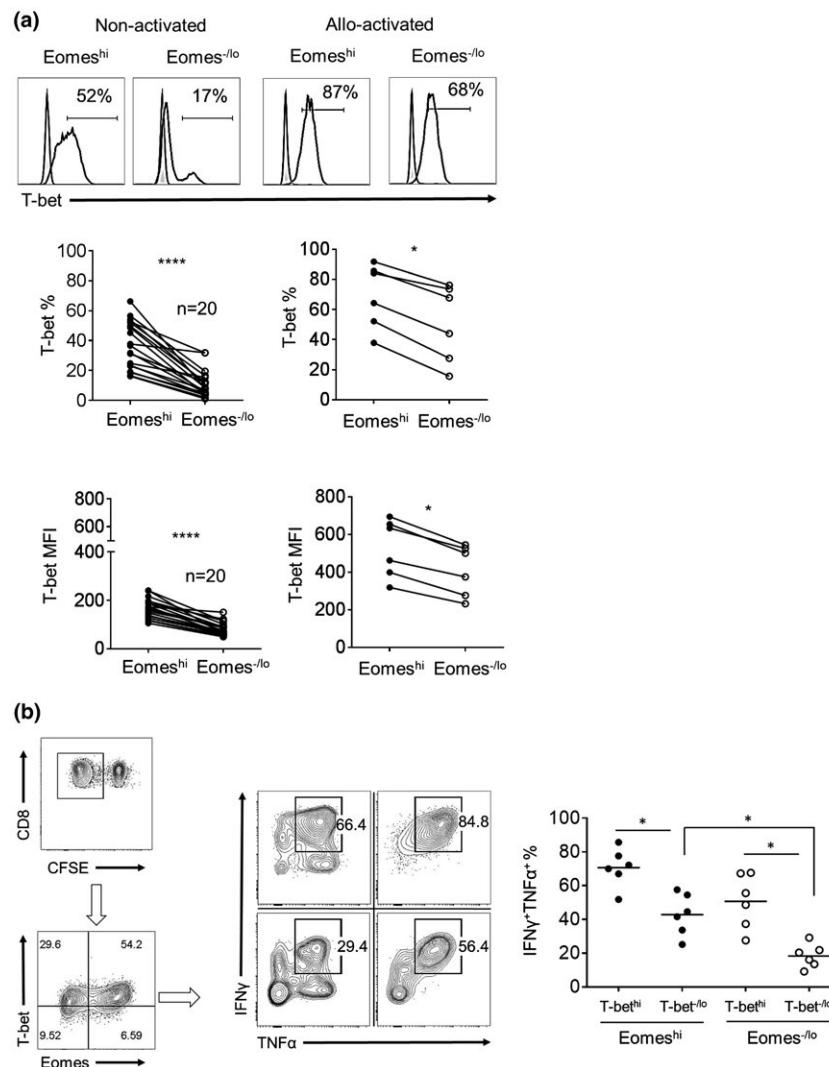


**Fig. 3.** Kinetics of Eomes expression by CD8<sup>+</sup>T cells following allo-stimulation in vitro. CFSE-labeled purified T cells were co-cultured with allogeneic stimulators for 3, 5 and 7 days. Percent responder cell proliferation determined by CFSE dilution and Eomes expression by proliferating cells over time was then evaluated by flow cytometry. (a) The dot plots are from one representative individual. (b) Percent of proliferation of Eomes<sup>hi</sup> and Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells (top) and MFI for Eomes expression (bottom). Combined data from five individuals examined are shown.

the incidence of the IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells (Supporting Information Fig. 1). Hence, total CD8<sup>+</sup>T cells were examined after coculture with donor cells ( $n = 11$ ). Eomes<sup>hi</sup>T-bet<sup>hi</sup>, Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> and Eomes<sup>-/lo</sup>T-bet<sup>hi</sup> CD8<sup>+</sup>T cell subsets were evaluated (Fig. 6a). In donor-stimulated recipient CD8<sup>+</sup>T cells (Fig. 6b), no significant changes were observed in the incidence of the Eomes<sup>hi</sup>T-bet<sup>hi</sup> subset after tx, compared with before tx. The Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> CD8<sup>+</sup>T cell subset was increased significantly 1 month ( $P < 0.05$ ) and 3 months ( $P < 0.05$ ) post-tx, while the Eomes<sup>-/lo</sup>T-bet<sup>hi</sup> subset was reduced significantly ( $P < 0.05$ ) 1 month after tx. In MLR experiments where proliferation was observed (6/11), proliferating CD8<sup>+</sup>T cells exhibited similar incidences of Eomes<sup>hi</sup>T-bet<sup>hi</sup>, Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> and Eomes<sup>-/lo</sup>T-bet<sup>hi</sup> CD8<sup>+</sup>T cell subsets before and after tx (*not shown*).

Additionally, we evaluated Eomes and T-bet expression (MFI) by CD8<sup>+</sup>T cells in renal allograft recipients with no rejection ( $n = 11$ ) before and after stimulation with donor cells (Supporting Information Fig. 4a). In non-stimulated CD8<sup>+</sup>T cells, Eomes expression was significantly higher at 1 month ( $P < 0.01$ ), 3 months ( $P < 0.01$ ) and 1 year ( $P < 0.05$ ) post-tx. By contrast, T-bet expression was increased significantly only at 1 year ( $P < 0.05$ ) post-tx. Following stimulation with donor cells (Supporting Information Fig. 4b), total CD8<sup>+</sup>T cells exhibited significantly increased Eomes expression 1 month ( $P < 0.01$ ), 3 months ( $P < 0.01$ ) and 1 year ( $P < 0.05$ ) post-tx. By contrast, T-bet expression was not increased.

To determine whether upregulation of Eomes expression was associated with exhaustion of donor-stimulated CD8<sup>+</sup>T cells in patients with no rejection, we evaluated



**Fig. 4.** T-bet expression by non-stimulated and allo-stimulated  $Eomes^{hi}$  and  $Eomes^{-/lo}$  CD8<sup>+</sup>T cells. (a) T-bet expression by non-activated CD8<sup>+</sup>T cells (left) and proliferating cells after allo-stimulation (right). Histograms are from one representative individual. Data from multiple normal healthy individuals are shown below (non-activated; n = 20 and allo-stimulated; n = 6). (b) Dual expression of IFN $\gamma$  and TNF $\alpha$  by  $Eomes^{hi}T-bet^{hi}$ ,  $Eomes^{hi}T-bet^{-/lo}$ ,  $Eomes^{-/lo}T-bet^{hi}$  and  $Eomes^{-/lo}T-bet^{-/lo}$  CD8<sup>+</sup>T cell subsets, following coculture with allogeneic stimulators. Dot plots (left and center) are representative of one individual. Data from 6 individuals are shown in the graph (right). Gray histograms indicate isotype controls. Horizontal bar represents the mean. Wilcoxon-Mann-Whitney test; \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ .

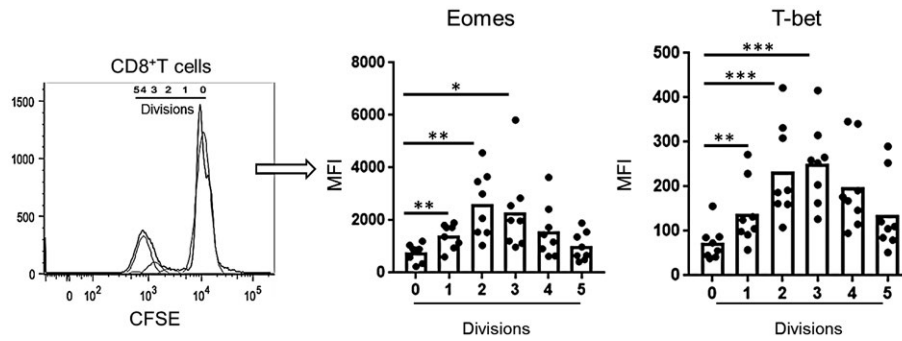
the expression of the exhaustion markers programmed death 1 (PD-1) and cytotoxic T-lymphocyte associated antigen 4 (CTLA4). Compared to before tx, donor-stimulated CD8<sup>+</sup>T cells maintained similar levels of PD-1 after tx. In contrast, there was a significant reduction in CTLA4 expression by donor-stimulated CD8<sup>+</sup>T cells at 1 month ( $P < 0.05$ ), 3 months ( $P < 0.01$ ), and 1 year ( $P < 0.01$ ) after tx. Furthermore, donor-stimulated CD8<sup>+</sup>T cells exhibited significant reduction of the activation marker CD57 expression by donor-stimulated CD8<sup>+</sup>T cells, at 1 month ( $P < 0.05$ ) and 3 months ( $P < 0.01$ ) after tx (Fig. 6c).

### Pre-tx Eomes and T-bet co-expression by donor-stimulated CD8<sup>+</sup>T cells of kidney tx patients with or without subsequent rejection

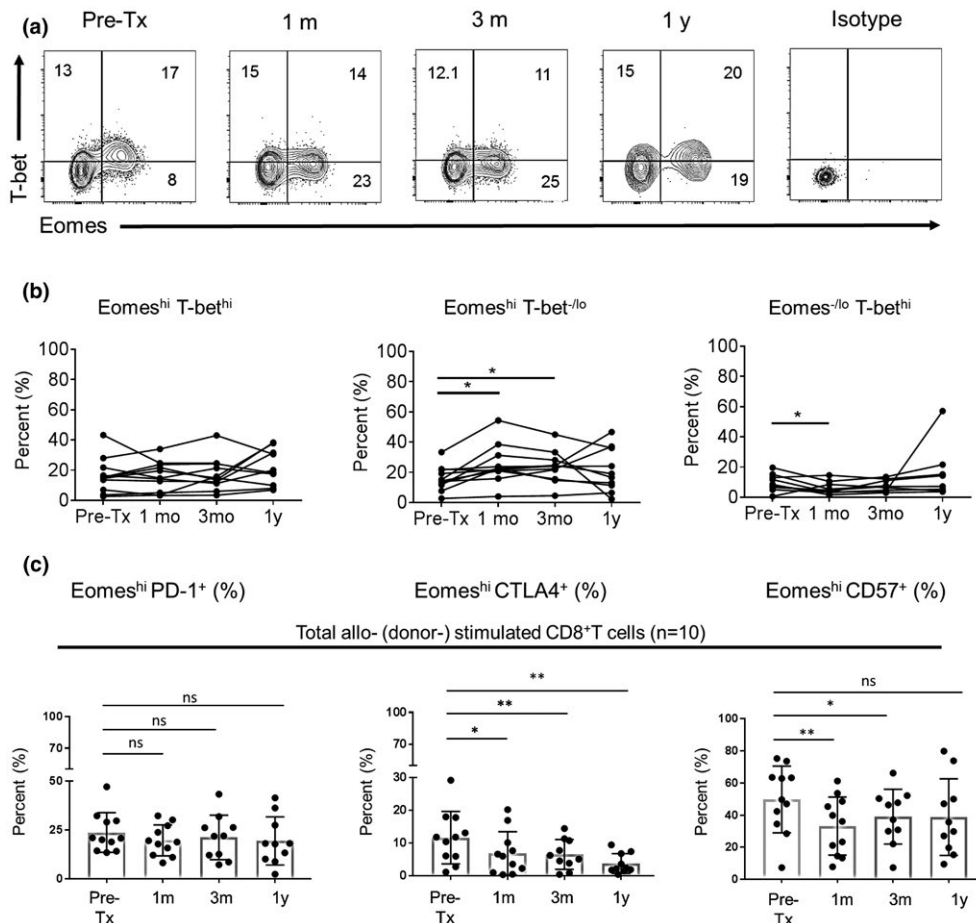
PBMC were collected before tx from renal allograft recipients (Table 1) with either no subsequent rejection (n = 11), subclinical rejection (SCR; n = 5) or acute cellular rejection (ACR; n = 5). Pre-tx recipient CD8<sup>+</sup>T cells were evaluated for  $Eomes^{hi}T-bet^{hi}$ ,  $Eomes^{hi}T-bet^{-/lo}$  and  $Eomes^{-/lo}T-bet^{hi}$  CD8<sup>+</sup>T cell subsets following stimulation with donor cells. Samples from healthy volunteers served as controls (Fig. 7).

In patients with ACR, there was a trend towards higher Eomes and T-bet expression (MFI) by donor-stimulated





**Fig. 5.** Eomes and T-bet expression by proliferating CD8<sup>+</sup> T cells following allo-stimulation in vitro. Divisions of CFSE-labeled proliferating cells measured using flowjo software. Mean fluorescence intensity (MFI) of Eomes and T-bet expression was evaluated for each division. The histogram (left) is from one representative individual coculture. Combined data from 8 individuals examined are shown (left). Wilcoxon-Mann-Whitney test; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Fig. 6.** Pre- and post-tx Eomes and T-bet expression by donor-stimulated CD8<sup>+</sup> T cells of kidney tx recipients with no rejection. (a) After co-culture of recipient PBMC with donor PBMC, total CD8<sup>+</sup> T cells were evaluated for Eomes and T-bet co-expression. Dot plots are representative of one kidney tx recipient. (b) The percentages of Eomes<sup>hi</sup>T-bet<sup>hi</sup>, Eomes<sup>hi</sup>T-bet<sup>lo</sup> and Eomes<sup>lo</sup>T-bet<sup>hi</sup> subsets in total CD8<sup>+</sup> T cells were determined. Results are expressed as percentages of each subset. (c) Percentages of PD-1<sup>+</sup>, CTLA4<sup>+</sup> and CD57<sup>+</sup> Eomes<sup>hi</sup> CD8<sup>+</sup> T cells following stimulation with donor cells. Wilcoxon-Mann-Whitney test; \* $P < 0.05$ , \*\* $P < 0.01$ .

CD8<sup>+</sup>T cells before transplantation compared to healthy controls, patients with no rejection, and patients with SCR (Fig. 7a). Meanwhile, the incidences of the Eomes<sup>hi</sup>T-bet<sup>hi</sup> and Eomes<sup>-/lo</sup>T-bet<sup>hi</sup> subsets were significantly higher ( $P < 0.05$ ), but the incidences of the Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> subset were lower in patients with ACR compared to those with no rejection (Fig. 7b).

Collectively, these data suggest that an increased incidence of donor-reactive CD8<sup>+</sup>T cells co-expressing high levels of Eomes and T-bet before tx, may correlate with an increased incidence of ACR.

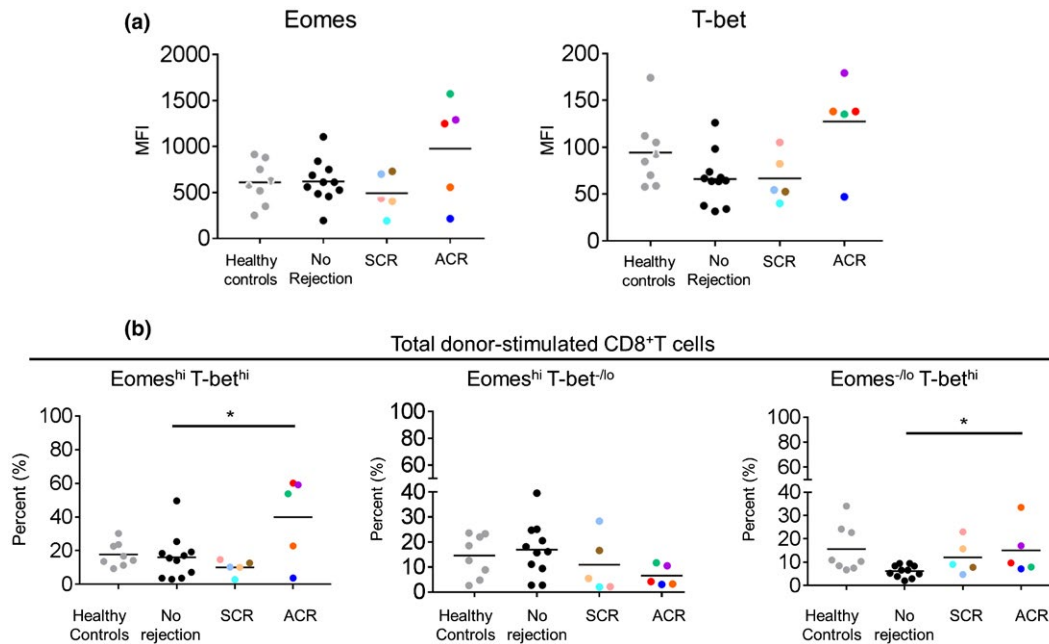
### Discussion

Allo-reactive Tmem constitute a major barrier to improved long-term organ allograft survival [6,30-32], where impairment of Tmem responses is crucial to attain Ag-specific tolerance [9]. T cell depletion is a common induction therapy in tx protocols. However, lymphodepletion leads to the generation of Tmem that are resistant to co-stimulation blockade [14]. Additionally, in rodents [7,33] and nonhuman primate (NHP) [8] allo-tx models, CD8<sup>+</sup>Tmem are known to mediate immunosuppression-resistant allograft rejection.

The T-box transcription factors Eomes and T-bet, are considered master regulators of Ag-specific CD8<sup>+</sup> Tmem

differentiation, function and survival [15,16]. Rodent studies have demonstrated the critical roles of both Eomes and T-bet in long-term survival of effector CD8<sup>+</sup>T cells. Reduced Eomes expression results in deficient long-term Ag-specific Tmem development and homeostatic renewal [17], whereas in chronic viral infection [23] both Eomes and T-bet are essential for the maintenance of anti-viral effector CD8<sup>+</sup> T cell responses. In the latter study, Eomes<sup>hi</sup> virus-specific CD8<sup>+</sup>T cells displayed enhanced cytotoxicity, despite expressing the T cell exhaustion markers B lymphocyte-induced maturation protein-1 (Blimp-1) and PD-1. These virus-specific Eomes<sup>hi</sup> CD8<sup>+</sup> T cells exhibited robust proliferation, but were associated with lower effector function, in contrast to T-bet<sup>hi</sup> CD8<sup>+</sup>T cells. Of importance, genetic deletion of either T-bet<sup>+</sup> or Eomes<sup>+</sup> subsets results in failure of mice to control chronic viral infection [23], suggesting that both transcription factors are required for the maintenance of anti-viral Tmem with effector function.

In humans, Eomes is highly expressed by CD8<sup>+</sup> compared to CD4<sup>+</sup> T cells [34,35]. In kidney tx patients with primary cytomegalovirus (CMV) infection [36], T-bet and Eomes are upregulated in CMV-specific CD8<sup>+</sup>T cells. In lung tx patients, [37], reduced T-bet but not Eomes expression by CMV-specific CD8<sup>+</sup>T cells is associated with reduced viral control and increased incidence of CMV



**Fig. 7.** Pre-tx Eomes and T-bet expression by donor-stimulated CD8<sup>+</sup>T cells of kidney tx recipients with or without subsequent rejection. PBMC were obtained before transplantation from kidney allograft recipients with no subsequent rejection ( $n = 11$ ), subclinical rejection (SCR;  $n = 5$ ) and acute cellular rejection (ACR;  $n = 5$ ). Recipient PBMC were co-cultured with donor PBMC for 7 days. After coculture, total CD8<sup>+</sup>T cells were evaluated for (a) Mean fluorescence intensity (MFI) of Eomes and T-bet expression, and (b) for the incidences of Eomes<sup>hi</sup>T-bet<sup>hi</sup>, Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> and Eomes<sup>-/lo</sup>T-bet<sup>hi</sup> subsets. Color coded dots correspond with the same patients in Table 1. Wilcoxon-Mann-Whitney test; \* $P < 0.05$ .

relapse after tx. Meanwhile, the role of Eomes in the development and maintenance of allo- (donor-) reactive Tmem in tx patients is not known. The present data show that, after CD8<sup>+</sup>T cell allo-stimulation, two distinct populations of proliferating Eomes<sup>hi</sup> and Eomes<sup>-/lo</sup> CD8<sup>+</sup>T cells can be identified. Importantly, irrespective of T-bet expression, both proliferating and non-proliferating CD8<sup>+</sup>T cells expressing high levels of Eomes demonstrated a stronger effector phenotype, compared to low Eomes-expressing cells. Nevertheless, elevated co-expression of Eomes and T-bet by allo-stimulated CD8<sup>+</sup>T cells correlated with the highest incidence of dual IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> cells.

In tx patients with stable graft function, upregulation of Eomes, but not T-bet, by donor-stimulated CD8<sup>+</sup>T cells was observed at 1 and 3 months after tx. This was associated with maintained PD-1 expression by donor-stimulated Eomes<sup>+</sup>CD8<sup>+</sup>T cells after tx, compared to before tx, suggesting an exhausted phenotype as previously reported in patients with human immunodeficiency virus (HIV) infection [38]. In contrast, CTLA4 expression by Eomes<sup>+</sup>CD8<sup>+</sup>T cells after tx was reduced significantly (Fig. 6c). Recently, using a NHP model [39,40], we have shown that prolonged kidney allograft survival is associated with reduced Eomes, but increased CTLA4 expression by recipient donor-reactive CD8<sup>+</sup>Tmem, which can be due to the immunoregulatory influence of regulatory dendritic cell infusion before tx, and/or different immunosuppressive regimen.

Higher incidences of T cells primed to produce high levels of IFN $\gamma$  correlate directly with severity of rejection in human kidney tx [41], and increased incidences of ACR [42]. Recently, use of donor-specific IFN $\gamma$  ELISPOT assays demonstrated that increased anti-donor reactivity before tx, correlated with higher incidences of ACR [43,44], while increased anti-donor reactivity after tx was associated with increased incidences of SCR and subsequent development of anti-donor HLA Abs [45]. This supports earlier observations demonstrating the role of Th1 (T-bet-mediated) T cell responses in renal allograft rejection [24,25]. However, mounting evidence suggests that IFN $\gamma$  can have immunoregulatory properties [46-48]. Prolonged allograft survival using a co-stimulation blockade-based regimen is not achievable in IFN $\gamma$ <sup>-/-</sup> recipients [49,50]. Also, the Th1 transcription factor T-bet is essential for regulatory T cell homeostasis and migration to sites of inflammation [51]. These observations suggest that IFN $\gamma$  expression alone cannot be linked directly to allograft rejection [52,53]. While Eomes and PD-1 co-expression correlates with exhausted virus-specific CD8<sup>+</sup>T cells [38], co-expression of Eomes and T-bet by donor-reactive CD8<sup>+</sup>T cells might prove to be a useful marker to determine the risk of T cell-mediated rejection in kidney tx patients.

Hence, in patients with subsequent ACR, the percentages of donor-stimulated Eomes<sup>hi</sup>T-bet<sup>hi</sup> CD8<sup>+</sup>T cells were higher before tx, compared to those with no subsequent rejection (Fig. 7).

Before tx, patients with subsequent ACR exhibited increased percentages of donor-reactive CD8<sup>+</sup>T cells expressing significantly higher levels of T-bet, i.e. Eomes<sup>hi</sup>T-bet<sup>hi</sup> and Eomes<sup>-/lo</sup>T-bet<sup>hi</sup>. Also, the same group of patients exhibited lower percentages of donor-reactive Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> CD8<sup>+</sup>T cells, although not significantly (Fig. 7). After tx, patients with subsequent stable graft function maintained low percentages of donor-reactive Eomes<sup>hi</sup>T-bet<sup>hi</sup> and Eomes<sup>-/lo</sup>T-bet<sup>hi</sup> CD8<sup>+</sup>T cells up to 1 year after tx (Fig. 6b). In reverse correlation, the percentages of donor-reactive Eomes<sup>hi</sup>T-bet<sup>-/lo</sup> CD8<sup>+</sup>T cells were significantly increased at 1 and 3 months after tx in the same group of patients. These observations suggest upregulation of Eomes expression by donor-reactive CD8<sup>+</sup>T cells, without concomitant upregulation of T-bet expression, may correlate with absence of anti-donor reactivity early after tx and hence reduced incidence of acute cellular rejection. However, considering the potential role of Eomes in effector T cell development and maintenance, particularly in the absence of T-bet-mediated effector mechanisms, it is yet to be determined whether such observations correlate with long-term stable graft function. Indeed, rodent data suggest that Eomes may compensate for the lack of T-bet function to promote Th1-driven allograft rejection [54]. In this rodent model, adoptive transfer of allogeneic CD4<sup>+</sup>T cells deficient in both T-bet and retinoid-related orphan receptor (ROR)  $\gamma$ t (the hallmark Th17 cell transcription factor) is associated with enhanced Eomes expression increased IFN $\gamma$  production by the double-deficient cells and enhanced skin allograft injury. This suggests that, under these conditions, Eomes may compensate for the lack of T-bet function to drive Th1-driven allograft rejection.

Continuous exposure of CD8<sup>+</sup>T cells to Ag results in the downregulation of T-bet expression and upregulation of Eomes expression, associated with reduced cytokine production and increased PD-1 expression, but enhanced cytotoxic function [23]. In our study, patients with no rejection exhibited significantly higher Eomes expression after tx, but no changes in PD-1 expression were observed (Fig. 6c). Additionally, this was associated with significant reduction in CTLA4 expression. Furthermore, CD57 expression by donor-stimulated Eomes<sup>+</sup>CD8<sup>+</sup>T cells was reduced significantly only 1-3 months after tx in patients with stable graft function (Fig. 6c). Of note, in patients with HIV infection, CD57<sup>+</sup>CD8<sup>+</sup>T cells with high Eomes expression correlate with better viral control [55]. Accordingly, increased Eomes expression may not

correlate with long-term exhaustion of antigen-specific CD8<sup>+</sup>T cells after tx [56], which may have implications for transplant outcome, as well as future therapeutic strategies, e.g. potential weaning of immunosuppression.

In conclusion, this is the first report, to our knowledge, that has examined Eomes and T-bet co-expression by allo-reactive CD8<sup>+</sup>T cells of human healthy volunteers and of organ tx patients in response to donor Ag stimulation. Our study has revealed that high Eomes expression correlates with enhanced effector phenotype by allo-stimulated CD8<sup>+</sup>T cells. Importantly, our data suggest that high Eomes and T-bet co-expression by donor-reactive CD8<sup>+</sup>T cells before tx may correlate with cellular rejection. These findings provide new insight into allo-reactive CD8<sup>+</sup>T cells in allograft recipients. It will now be important to conduct a long-term follow-up study involving a larger number of patients to further evaluate the significance of these findings.

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### Disclosure

The authors of this manuscript have no conflict of interest to disclose.

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### Supporting Information

Additional Supporting information may be found in the online version of this article at the publisher's web-site.

**Fig. S1.** Percentage of TNF $\alpha$  and INF $\gamma$  double positive cells in proliferating and non-proliferating human CD8<sup>+</sup>T cell populations after allo-stimulation.

**Fig. S2.** T-bet expression by Eomeshi and Eomes-/lo human CD8<sup>+</sup>T cells following CD3/CD8 activation.

**Fig. S3.** T-bet expression and function of Eomeshi and Eomes-/lo human CD8<sup>+</sup>T cells in patients with end stage renal disease (ESRD) before transplantation.

**Fig. S4.** Eomes and T-bet expression (MFI) by allo-(donor-) stimulated CD8<sup>+</sup>T cells, before and after tx, in kidney tx recipients with no rejection.