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FoxO3 is a negative regulator of primary CD8+ T cell expansion but not of memory formation

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Abstract

The generation of CD8+ T cells by vaccination represents an important goal for protective immunity to infectious pathogens. It is thus of utmost importance to understand the mechanisms involved in the generation of optimal CD8+ T cell responses. The forkhead box O (FoxO) family of transcription factors plays a crucial role in cellular responses to environmental change. Among them, FoxO3 is critically involved in the regulation of cellular proliferation, apoptosis, metabolism, and stress resistance to withdrawal of nutrients or cytokine growth factors. Since the role of FoxO3 has been poorly studied in the immune system, here we have evaluated its involvement in the CD8+ T cell response. We observe that CD8+ T cells deficient for FoxO3 undergo a significantly greater primary expansion than their wild-type counterparts in response to both infectious (vaccinia virus) or non-infectious (non replicating cellular vaccine) immunogens, resulting in a larger cohort of cells following contraction. These survivors, however, do not undergo a greater secondary response than wild type. Taken together, our data show that FoxO3 is a negative regulator of the CD8+ T cells response, specifically during the primary expansion.

Introduction

Understanding the mechanism(s), which promote effective CD8+ T cell responses, is essential to the design of new vaccines against infectious diseases and cancer. CD8+ T cells play an essential role in the clearance of either infected or abnormal cells through a variety of effector mechanisms[1, 2, 3]. This is preceded by a robust primary expansion in which rare precursors expand up to 10,000 fold[4]. After infection is brought under control, the majority of the cells will die[5] (90–95%), with the remaining cells forming a long-lived memory pool, which can self-renew and rapidly produce new effector cells upon antigen re-encounter.

In the recent years the role of cellular metabolism in regulating CD8+ T cell function and memory has come to the forefront. Recent studies have shown that metabolism is important

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to regulate $CD8^+$ T cell fate, survival and death[6, 7, 8, 9]. Several molecules have been implicated in T cell metabolism. The phosphatidyl-inositol-3-OH kinase (PI(3)K) pathway and subsequently Akt are activated after TCR triggering or cytokine stimuli such as IL-2 or IL-15. Akt activation is due to its phosphorylation status, and mTORC2 is involved in the phosphorylation of one of the Akt serine, whereas Akt activates mTORC1. Akt has been shown to negatively regulate FoxO molecules[6, 10, 11], preventing their entry into the nucleus and their function as transcription factors.

The FoxO transcription factors are mammalian orthologs of the Caenorhabditis elegans longevity protein Daf-16 that are widely conserved through evolution and have been shown to play critical roles in cellular responses to environmental changes[12, 13]. Three of the four known FoxO orthologs (FoxO1, 3, and 4) have overlapping targets of transcriptional regulation and appear to be widely expressed and similarly regulated[14]. FoxO1 and FoxO3 are the main isoforms expressed in immune cells, but their expression levels differ between organs of the immune system and between lymphoid and myeloid cell types: FoxO1 expression is higher in spleen and lymph nodes as compared to FoxO3, which is the main transcript detected in the thymus and bone marrow[15]. FoxO3 plays a crucial role in regulating cellular proliferation, apoptosis, metabolism, and stress resistance to withdrawal of nutrients or cytokine growth factors (reviewed in[10]). Like FoxO1 and −4, the functions of FoxO3 are regulated post-transcriptionally, largely through phosphorylation[16].

Although a role for FoxO1 in the $CD8^+$ T cell memory formation has been established,[17, 18, 19, 20] little is known about the function of FoxO3 in the $CD8^+$ T cell response.

Information on the role of FoxO3 in immune functions has emerged from the study of genetically deficient (knockout) mice[21]. This study did not find evidence of immunological abnormalities in unmanipulated FoxO-deficient mice, either by histology or enumeration of T and B cells[21]. However, acute infection of $FoxO3^{-/-}$ mice with lymphocytic choriomeningitis virus (LCMV) or Vesicular Stomatitis Virus (VSV) revealed a more than 3 fold increase in the number of antigen-specific $CD4^+$ and $CD8^+$ T cells. The increased expansion of the primary responder lymphocytes coincided with dysregulated cytokine production by dendritic cells (DC)[21], and highlights a key role for FoxO3 in the regulation of antigen presenting cell (APC) function, which was confirmed by subsequent studies[22, 23, 24]. More recently, a cell-intrinsic role for FoxO3 in regulating the $CD8^+$ T cell response to infectious pathogens such as LCMV[25, 26] or listeria[27] was identified. This was based on the observation that $CD8^+$ T cells lacking FoxO3, mounted proportionally larger responses, which was attributed to decreased apoptosis either during the primary expansion phase[25, 26] or during the contraction phase[27]. Of critical relevance to memory function, however, none of these studies assessed whether secondary responses were influenced by a cell-intrinsic role of FoxO3. We have now examined whether FoxO3 regulates both primary and memory (recall) responses. We find that, in response to both inflammatory and non-inflammatory immunogens, FoxO3-deficient $CD8^+$ T cells undergo a greater primary but comparable secondary response to wild-type (WT) controls. Therefore FoxO3 regulates primary but not memory $CD8^+$ T cell responses.

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Results

FoxO3 regulates the expansion of primary CD8+ effector T cells

To evaluate the intrinsic role of FoxO3 in the CD8$^+$ T cell response, we co-transferred a small number of WT and FoXO3$^{-/-}$ CD8$^+$ T cells, both expressing a transgenic TCR (OT-I) specific for the Chicken Ovalbumin (OVA), into WT recipient mice. Hosts were then immunized with either a non-replicating cellular vaccine (Actm-OVA K$^{b-/-}$ splenocytes)[28] or replicating recombinant vaccinia virus containing OVA (vaccinia-OVA). Seven days after the immunization, at the peak of the primary response, we evaluated the expansion of both populations in lymphoid and non-lymphoid organs and observed that the CD8$^+$ T cells lacking FoxO3 expanded significantly more than their WT counterparts (Figures 1A, B and C). However, the percentages of so called memory precursor effector cells defined by the expression of CD127 and lack of KLRG1 expression and short-lived effector cells (SLEC, CD127$^-$KLRG1$^+$)[29] were comparable between the WT and FoxO3$^{-/-}$ CD8$^+$ OT-I primary responder cells 7 days post-immunization (Figure 2A). This was confirmed by the fact that we did not see, any difference in the expression of T-bet (expressed by effector cells) or Eomes (expressed by memory cells) (Figure 2B). These data demonstrate that FoxO3 negatively regulates the overall expansion of primary CD8$^+$ effector T cells.

FoxO3 does not influence the functional differentiation of primary CD8+ effector T cells

To investigate if in addition to the quantity, FoxO3 also negatively regulates the quality of the primary response, cytokine production was measured 7days post immunization. There was no difference noted however, in the proportion of cells able to produce IFNγ or IFNγ and TNF-α, between the WT and the FoxO3$^{-/-}$ CD8 OT-I T cells (Figure 3A). Moreover there was no difference in the amount of IL-2 production (Figure 3B), indicating that FoxO3 does not control the cytokine production of primary CD8$^+$ effector T cells.

FoxO3 does not control the initial contraction or expansion phase of secondary responder CD8+ T cells

To investigate if FoxO3 controls the magnitude of the memory response through enhanced cell death, we analyzed the contraction phase that follows the primary expansion. In contrast to the enhanced expansion of the FoxO3$^{-/-}$ primary responder OT-I CD8$^+$ T cells, we did not observe any significant change in the proportion between the WT and FoxO3 responder cells during the contraction phase, indicating that FoxO3 does not promote cell death or counteract survival of the primary responder cells (Figure 4A). To investigate if, similar to the primary expansion, FoxO3 also negatively regulates the magnitude of the secondary response; recipient mice were rechallenged with Listeria-OVA 43 days after the initial priming and analyzed 5 days later (Figures 4A and B). WT and FoxO3$^{-/-}$ OT-I responder cells expanded at an equal rate during the secondary response, indicating that in contrast to the primary response, FoxO3 does not control the magnitude of the secondary expansion.

Discussion

In this study we have investigated the impact of FoxO3 during the CD8$^+$ T cell response. Using co-transfer of antigen-specific WT and FoxO3$^{-/-}$ CD8$^+$ T cells we were able to show

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that FoxO3 plays a cell intrinsic role in the primary expansion of the effector cells that follows the first encounter with their specific antigen. On the contrary, we did not find any involvement of FoxO3 in the functional differentiation of the primary effector cells nor did we find an effect of FoxO3 during the contraction phase or memory formation.

Firstly we found that the FoxO3 deficient CD8 primary responder cells expanded significantly more than the cells expressing FoxO3, indicating a negative regulatory function of FoxO3 in the primary expansion of the $CD8^+$ T cell. These results together with previous findings showing that FoxO3 induces the expression of the pro-apoptotic molecules Bim and Puma in $CD8^+$ T cells (27), suggest that FoxO3 promotes cell death during the initial primary response. We did however, not observe any difference in the quality of the effector response and both WT and $FoxO3^{-/-}$ $CD8^+$ T cells were able to produce IL-2 and IFNγ and TNF-α to the same extent, implying that FoxO3 does not affect the quality of the effector $CD8^+$ T cells generated following infectious or non-infectious immunization.

Surprisingly and in contradiction to two previously published studies[25, 26, 27], we did not find any effect of FoxO3 during the contraction phase or the generation of the secondary response. The differences could be due first to the fact that we are using a FoxO3 deficient strain of different origin compared to the other studies, where in the Sullivan et al. and the Tzelepis et al., studies a FoxO3a-trap was used whereas we used the $FoxO3^{Kca}$ [21]($FoxO3^{-/-}$), but since in both cases the FoxO3 protein is absent, it should not explain the differences in our results. In addition, different immunization strategies were used in the published studies compared to our approach here, which might contribute to the discrepancy. To control for this, however, we included 2 types of immunization strategies, a cellular vaccine and an infectious pathogen. Since both approaches rendered the same results, we concluded that the different immunization strategies are likely not the cause of the different outcome of the studies. Another possibility is the difference in the number of cells that was transferred which was much larger in the published studies as compared to our study here. It is well established that the precursor frequency has an impact on the efficiency and the nature of the memory generation[28, 30]. In fact most facets of the $CD8^+$ T cell response, including kinetics, proliferation, surface molecule expression, effector function and the efficiency of memory generation are substantially altered when the initial number of TCR transgenic T cells is sufficiently high to inhibit the endogenous $CD8^+$ T cell response to the same Ag. Those data suggest that the use of TCR transgenic T cells to model the endogenous $CD8^+$ T cell response may only be reliable under conditions where these cells represent only a fraction of the endogenous repertoire. In our case using a low precursor frequency, we noted no difference in the ratio of WT compared to FoxO3 deficient cells during the contraction or upon a secondary challenge, implying that FoxO3 did not affect those phases. Altogether, our results indicate that FoxO3 is not essential for the generation of $CD8^+$ memory T cells. This is in contrast to FoxO1, which was shown to promote $CD8^+$ central memory formation[17, 18, 19, 20] by repressing T-bet and the effector differentiation. Our results are also in line with the notion that there is no compensation by FoxO3 when FoxO1 is absent or conversely. Thus it seems that FoxO transcription factors have differential roles in the $CD8^+$ T cell response where FoxO3 regulates the expansion during priming whereas FoxO1 repress the effector function and participates in the central memory formation.

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Material and methods

Mice

C57BL/6, were purchased from The Jackson Laboratory (Bar Harbor, Maine). OT-I $CD45.1^{+}$ and Act-mOVA/$K^{b-/-}$ mice on a C57BL/6J background have been previously described[31]. The OT-I $FoxO3^{-/-}$ $CD45.1^{+}$ strain was generated by intercross between $FoxO3^{Kca}$ and OT-I $CD45.1^{+}$ mice. Mice were maintained by in-house breeding at the La Jolla Institute for Allergy and Immunology under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International.

T cell preparations

$CD8^{+}$ T cell transfer—OT-I $CD45.2^{+}$ and OT-I $FoxO3^{-/-}$ $CD45.1^{+}$, were harvested from blood and the number of OT-I cells was determined by counting and by FACS staining for $V2^{+}V5^{+}$. WT mice CD45.1/2 were co-transferred with 500 OT-I $CD45.2^{+}$ and 500 OT-I $FoxO3^{-/-}$ $CD45.1^{+}$cells by retro-orbital (RO) injection.

In vivo experiments

OT-I cells were transferred respectively into C57BL/6J CD45.1/2 mice one day prior to immunization. The mice were immunized with either 5×10^{6} Act-mOVA/$Kb^{-/-}$ splenocytes by RO injection (prime) and re-challenged by RO injection with 5000 cfu *Listeria monocytogenes*-OVA, or were inoculated ip with 1×10^{6} pfu *vaccinia*-OVA (priming), and boosted by RO injection with 5000 cfu *Listeria monocytogenes*-OVA.

Ex-vivo restimulation and antibody staining

Cytokine production in $CD8^{+}$ T cells was assessed as described: splenocytes from immunized mice were resuspended in IMDM medium (Invitrogen) supplemented with 8% FCS (Omega Scientific), 1% L-glutamine (Invitrogen), 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM 2-ME (Sigma-Aldrich). Cells ($1–2 \times 10^{6}$) were plated in 96-well round bottom plates in case of Act-mOVA/$K^{b-/-}$ and *Vaccinia*-OVA stimulation in 200 μl medium plus $OVA_{257–264}$ peptide (SIINFEKL) at 1 μg/ml in presence of GolgiPlug (BD Biosciences) for 5 h at 37°C. Cells were stained with anti-CD8 (5H10, PE-TR), CD44 (IM7, Alexa-Fluor 700), CD45.1 (A20, Pacific Blue) and CD45.2 (104, Percp-Cy5.5) followed by fixation with Cytofix-Cytoperm (BD Biosciences) for 20 min at 4°C. Fixed cells were subjected to intracellular cytokine staining in Perm/Wash buffer (BD Biosciences) for 30 min at 4°C. Anti-TNF (MP6-XT22, Pe-cy7), IFN- (XMG1.2, APC) and IL-2 (JES6-5H4, PE). The antibodies were purchased from BD Pharmingen, eBiosciences or biolegend. The samples were acquired on a LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo software.

Transcription factor and Flow cytometry analysis

Cells were stained with CD8 (5H10, PE-TR), CD44 (IM7, Alexa-Fluor 700), CD45.1 (A20, Pacific Blue), CD45.2 (104, Percp-Cy5.5 or FITC), CD62L (MEL-14, APC eFluor780), KLRG-1 (2F1, PeCy7), CD127 (A7R34, APC). In the case of transcription factor study the

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staining was followed by fixation with Cytofix-Cytoperm (BD Biosciences) for 20 min at 4°C. Fixed cells were subjected to intracellular staining in Perm/Wash buffer (BD Biosciences) for 30 min at 4°C with Eomes (Dan11mag, PercP-eFluor710) and Tbet (eBio4B10, APC). The antibodies were purchased from BD Pharmingen, eBiosciences or Biolegend. The samples were acquired on an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo software.

Statistical analysis

Data were analyzed using PRISM software (GraphPad, San Diego, CA). Differences between groups were examined for statistical significance using an unpaired two-tailed Student's *t* test. Unless otherwise indicated, data represent the mean ± SEM, with * = $p<0.05$ considered statistically significant.

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Abbreviations

APC	Antigen Presenting Cell
DC	Dendritic Cells
Eomes	Eomesodermin
FoxO	Forkhead box O
−/−	knockout
Listeria	*Listeria monocytogenes*
LCMV	lymphocytic choriomeningitis virus
MPEC	memory precursor effector cells
OVA	chicken Ovalbumine
PI(3)K	phosphatidyl-inositol-3-OH kinase
SLEC	short-lived effector cells
Tbet	T-box expressed in T cells
VSV	Vesicular Stomatitis Virus
WT	wild-type

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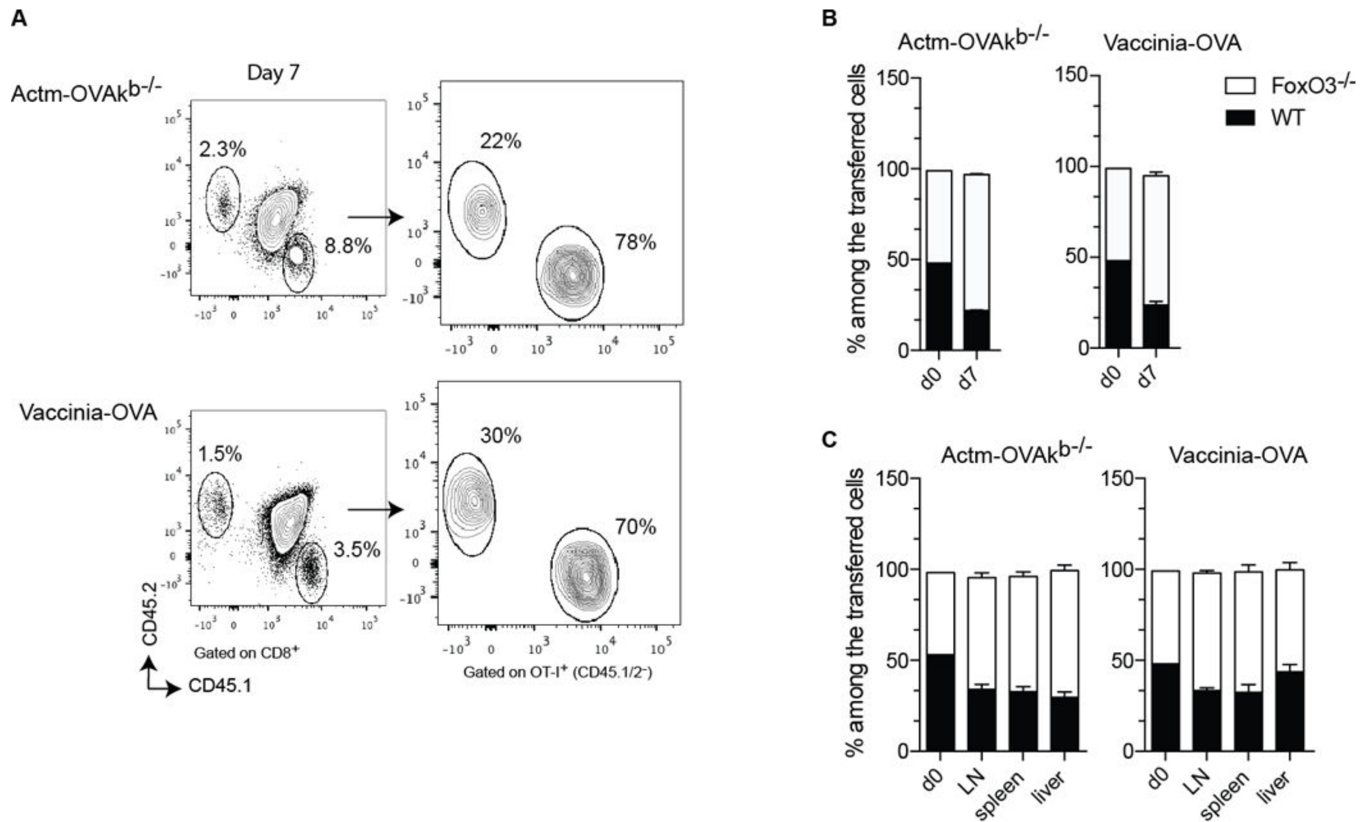

Figure 1.

FoxO3 regulates the expansion of $CD8^+$ T cells. A–C 500 OT-I CD45.1 $FoxO3^{-/-}$ and 500 OT-I CD45.2 cells were co-injected into WT (CD45.1/2) mice one day before immunization. The mice were infected with 1×10^6 vaccinia-OVA or with 5×10^6 Actm-$OVAk^{b-/-}$ splenocytes. In A and B the responses were measured in the blood at day 7. In C the ratio of OT-I was measured at day 7 in different organs. Data are representative of groups of 4 to 5 mice and represents the most representative result of 2 to 3 independent experiments.

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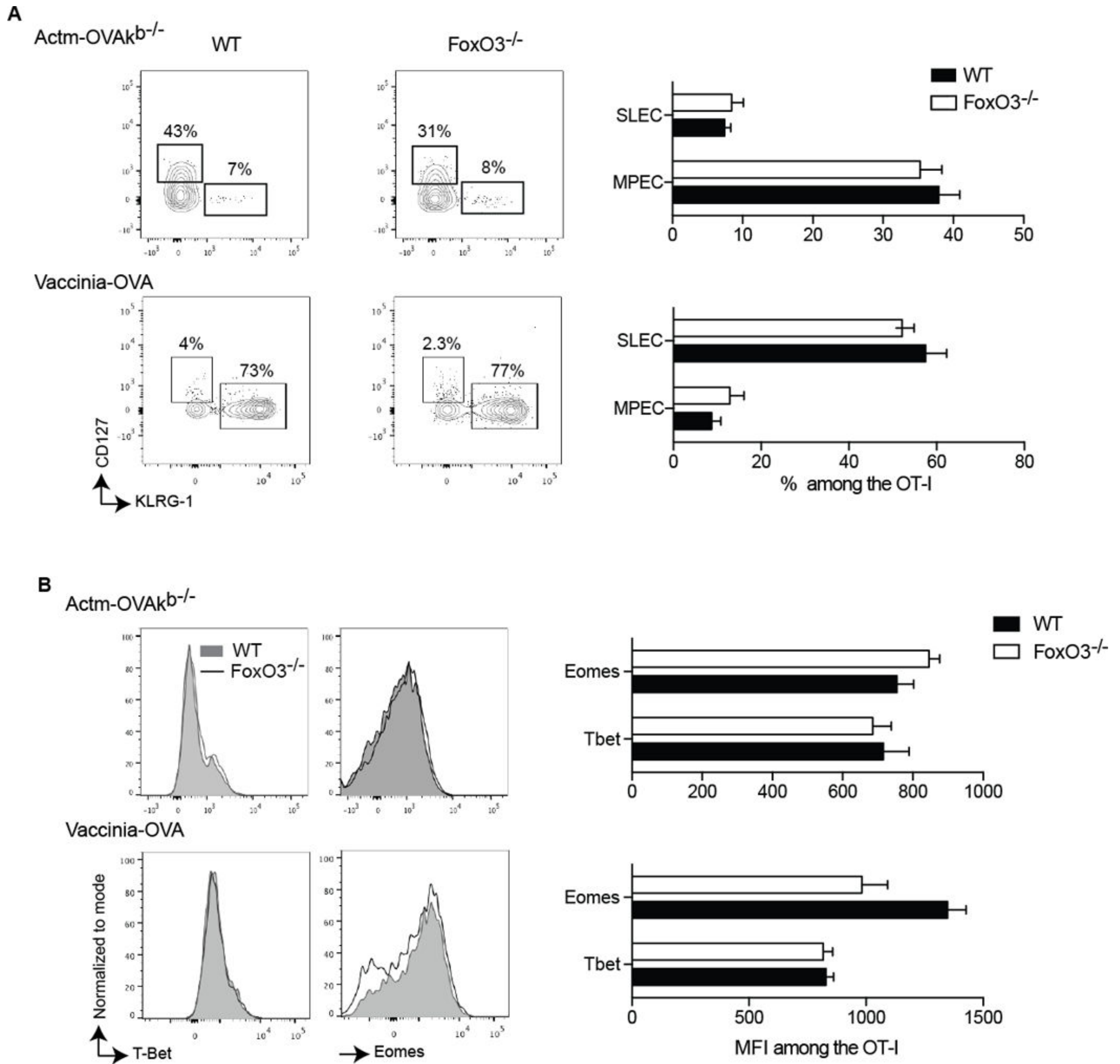

Figure 2.

FoxO3 does not regulate the number of memory precursor effector cells (MPEC). A–B 500 OT-I CD45.1 FoxO3$^{-/-}$ and 500 OT-I CD45.2 were co- injected into WT (CD45.1/2) mice one day before immunization. The mice were infected with 1×10^6 vaccinia-OVA or with 5×10^6 Actm-OVAk$^{b-/-}$ A represents the FACS plots and the percentage of OT-I expressing CD127^{+}KLRG1^{-} (MPEC) or CD127^{-}KLRG1^{+} (SLEC) at day 7 post immunization. C represents the histograms and the MFI for the transcription factors Tbet and Eomes at day 7-post immunization within the OT-I. Data are representative of groups of 4 to 5 mice and represents the most representative result of 2 to 3 independent experiments.

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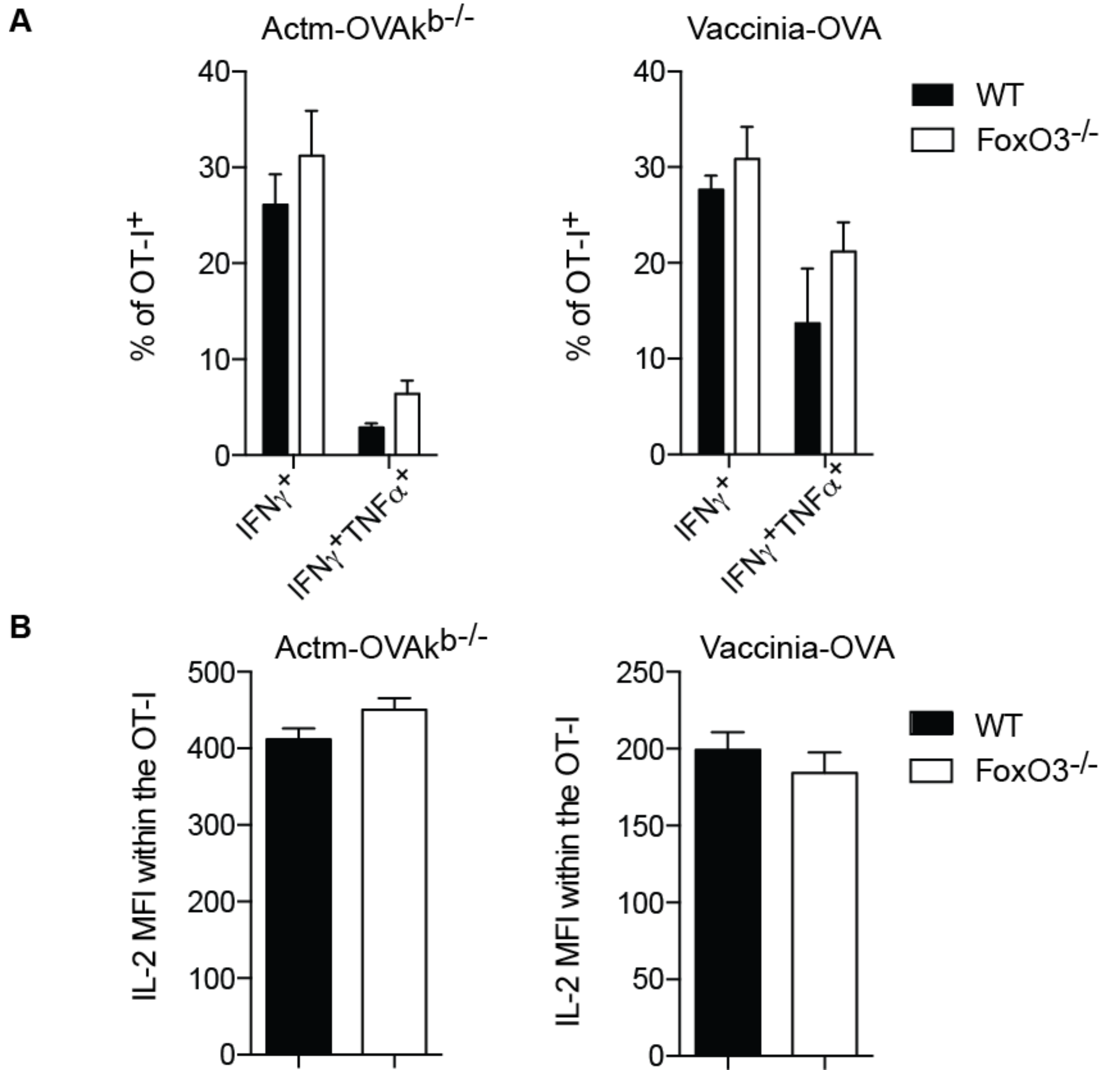

Figure 3.

FoxO3 does not influence cytokine production. A–B 500 OT-I CD45.1 $FoxO3^{-/-}$ and 500 OT-I CD45. 2 were coinjected into WT (CD45.1/2) mice one day before immunization. The mice were infected with 1×10^6 vaccinia-OVA or with 5×10^6 Actm-$OVAk^{b-/-}$. The cytokine response was measured in the spleen at day 7. A represents the percentage of OT-I producing IFNγ or IFNγTNF. B represents the MFI of IL-2 within the OT-I. Data are representative of groups of 4 to 5 mice and represents the most representative result of 2 to 3 independent experiments.

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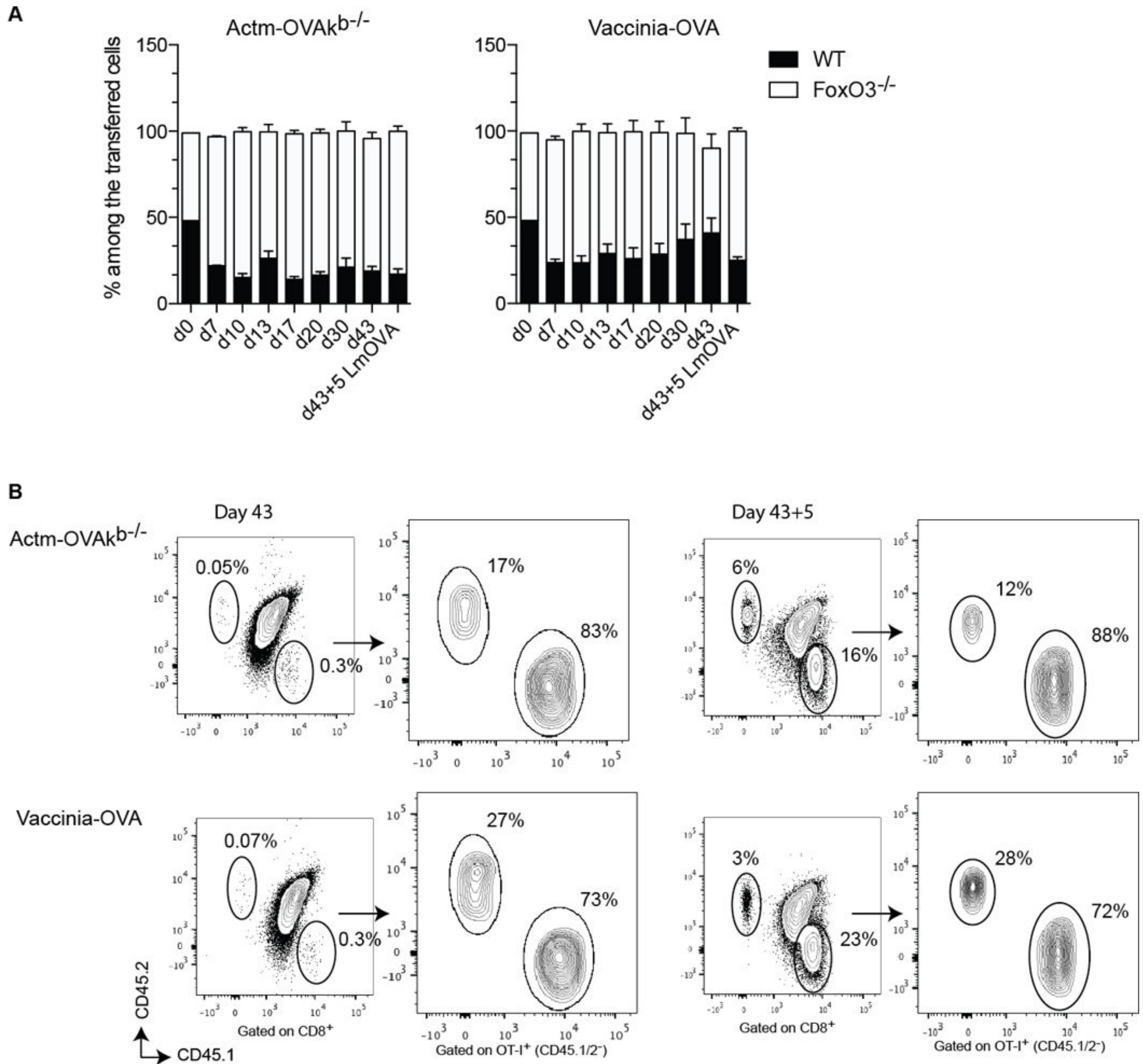

Figure 4.

FoxO3 does not regulate the contraction and secondary expansion. A–B 500 OT-I CD45.1 FoxO3$^{-/-}$ and 500 OT-I CD45.2 were co-injected into WT (CD45.1/2) mice one day before immunization. The mice were infected with 1×10^6 vaccinia-OVA or with 5×10^6 Actm-OVAk$^{b-/-}$. In A the response was measured in the blood at different time points during priming, contraction, and memory. The mice were then rechallenged at day 43 with 5000 Lm-OVA and the response was measured in the blood 5 days later. B represents the FACS plot during memory and secondary response. Data are representative of groups of 4 to 5 mice and represents the most representative result of 2 to 3 independent experiments.