ORIGIN  

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

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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 has 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 (WT) 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 WT. Taken together, our data show that FoxO3 is a negative regulator of the CD8+ T-cell response, specifically during the primary expansion.

*Immunology and Cell Biology* (2015) 93, 120–125; doi:10.1038/icb.2014.78; published online 23 September 2014

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 have an essential role in the clearance of either infected or abnormal cells through a variety of effector mechanisms.1–3 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 (90–95%),5 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 past, 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 to regulate CD8+ T-cell fate, survival and death.6–9 Several molecules have been implicated in T-cell metabolism. The phosphatidylinositol-3-0H kinase pathway and subsequently Akt are activated after T-cell receptor (TCR) triggering or cytokine stimuli such as interleukin 2 (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 forkhead box O (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 with FoxO3, which is the main transcript detected in the thymus and the bone marrow.15 FoxO3 has a crucial role in regulating cellular proliferation, apoptosis, metabolism and stress resistance to withdrawal of nutrients or cytokine growth factors (reviewed in Accili and Arden16). 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,17–20 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 un manipulated FoxO-deficient mice, either by histology or by enumeration of T and B cells.21 However, acute infection of FoxO3 knockout (FoxO3−/−) mice with lymphocytic choriomeningitis virus or Vesicular Stomatitis Virus revealed a more than threefold 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,21 and highlights a key role for FoxO3 in the regulation of antigen presenting cell (APC) function, which was confirmed by subsequent studies.22–24 More recently, a cell-intrinsic role for FoxO3 in regulating the...
CD8+ T-cell response to infectious pathogens such as lymphocytic choriomeningitis virus or listeria 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 or during the contraction phase. 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.

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 Kb−/− splenocytes) or a 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–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 (CD127-KLRG1+) 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 Tbet (expressed by effector cells) or Eomesodermin (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 whether in addition to the quantity, FoxO3 also negatively regulates the quality of the primary response, cytokine production was measured 7 days post immunization. There was no difference noted however, in the proportion of cells able to produce interferon gamma (IFNγ) or IFNγ and tumor necrosis factor alpha (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 whether 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 whether, 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 that FoxO3 has 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.

First, 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, 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 with the other studies, where in the Sullivan et al. and the Tzelepis et al. studies a FoxO3a trap was used whereas we used the FoxO3Kca (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 with our approach here, which might contribute to the discrepancy. To control for this, however, we included two types of immunization strategies, a cellular vaccine and an infectious pathogen. Since both approaches rendered the same results, we concluded that the different

Figure 3 FoxO3 does not influence cytokine production. (a, b) 500 OT-I CD45.1 FoxO3−/− and 500 OT-I CD45.2 were co-injected into WT (CD45.1/2) mice 1 day before immunization. The mice were infected with 1 × 10^6 vaccinia-OVA or with 5 × 10^6 Actm-OVAb−/−. The cytokine response was measured in the spleen at day 7. (a) Represents the percentage of OT-I producing IFNγ or IFNγ+TNF. (b) Represents the mean fluorescence intensity (MFI) of IL-2 within the OT-I. Data are representative of groups of 4–5 mice and represent the most representative result of 2–3 independent experiments.

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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 with 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 with 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 formation17–20 by repressing Tbet 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.

**METHODS**

**Mice**

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). OT-I CD45.1+ and Act-mOVA/Kb−/− mice on a C57BL/6J background have been previously described.31 The OT-I FoxO3−/− CD45.1+ strain was generated by intercross between FoxO3−/− and OT-I CD45.1+ mice. Mice were maintained by in-house breeding at the La Jolla Institute for Allergy and

**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 1 day before immunization. The mice were infected with 1 × 10⁶ vaccinia-OVA or with 5 × 10⁶ Actm-OVA/Kb−/−. 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 4–5 mice and represent the most representative result of 2–3 independent experiments.
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+Vβ5+ WT mice. CD45.1/2 were co-transferred with 500 OT-I CD45.2+ and 500 OT-I FoxO3−/− CD45.1+ cells by retroorbital injection.

**In vivo experiments**

OT-I cells were transferred respectively into C57BL/6J CD45.1/2 mice 1 day before immunization. The mice were immunized with either 5×10^6 Act-mOVA/Kb+− T cells by modulating fatty acid metabolism. Nature 2009; 460: 103–107.

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17. Dejan AS, Hedrick SM, Kerdiles YM, Pe, CD44 (IM7, Alexa-Fluor 700), and CD45.2 (104, Perpc-Cy5.5 or FITC), IL-2 (JES6-5H4, PE), and CD45.1 (A20, Pacific Blue), CD8+ (5H10, PE-TR), and CD45.2 (104, Percp-Cy5.5 or FITC), IL-2 (JES6-5H4, PE). The antibodies were purchased from BD Biosciences, eBiosciences or Biologend (San Diego, CA, USA). The samples were acquired on an LSRII cytometer (Becton Dickinson, Carlsbad, CA, USA) supplemented with 8% FCS (Omega Scientific, Tarzana, CA, USA). L-glutamine (Invitrogen), 100 μM l-glutamine, 100 U ml−1 penicillin, and 50 μg 2-βE (Sigma-Aldrich, St Louis, MO, USA). Cells (1–2×10^6) were plated in 96-well round bottom plates in the presence of Act-mOVA/Kb−/− and Vaccinia-OVA stimulation in 200 μl medium plus OVA326-344 peptide (SINFEKL) at 1 μg ml−1 in the presence of GolgiPlug (BD Biosciences, San Jose, CA, USA) 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, Perpc-Cy5.5 or FITC), IL-2 (JES6-5H4, PE). The antibodies were purchased from BD Biosciences, eBiosciences or Biologend. The samples were acquired on an LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using the Flowjo Software (Tree Star, Ashland, OR, USA).

**Transcription factor and flow cytometry analysis**

Cells were stained with CD8 (5H10, PE-TR), CD44 (IM7, Alexa-Fluor 700), CD45.1 (A20, Pacific Blue) and CD45.2 (104, Perpc-Cy5.5 or FITC), IL-2 (JES6-5H4, PE). The antibodies were purchased from BD Biosciences, eBiosciences or Biologend (San Diego, CA, USA). The samples were acquired on an LSRII flow cytometer (Becton Dickinson) and analyzed using the Flowjo Software.

**Statistical analysis**

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

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

We thank Cheryl Kim and Kurt Van Gunst for assistance with flow cytometry. We thank Dr Stephen M Hedrick for providing the FoxO3KO mice. This work was supported by National Institutes of Health grants AI070010 (Schoenerberger). This is manuscript number 1713 from La Jolla Institute for Allergy and Immunology, La Jolla, CA.

**Author contributions**

ST, AL and SF designed and performed the experiments and analyzed the data. SPS provided the funds and resources to pursue this study, SF wrote the manuscript. ST, AL and SPS provided significant assistance in writing and editing the manuscript.