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Critical Synergy of CD30 and OX40 Signals in CD4 T Cell Homeostasis and Th1 Immunity to *Salmonella*¹

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CD30 and OX40 (CD134) are members of the TNFR superfamily expressed on activated CD4 T cells, and mice deficient in both these molecules harbor a striking defect in the capacity to mount CD4 T cell-dependent memory Ab responses. This article shows that these mice also fail to control *Salmonella* infection because both CD30 and OX40 signals are required for the survival but not commitment of CD4 Th1 cells. These signals are also needed for the survival of CD4 T cells activated in a lymphopenic environment. Finally, *Salmonella* and lymphopenia are shown to act synergistically in selectively depleting CD4 T cells deficient in OX40 and CD30. Collectively these findings identify a novel mechanism by which Th1 responses are sustained. *The Journal of Immunology*, 2008, 180: 2824–2829.

Unlike CD8 T cells, CD4 T cells have been shown to depend on survival signals received in secondary lymphoid tissues (1). Both common γ-chain (γc) cytokines (2) and TNF members have been implicated in both the generation and maintenance of memory CD4 T cells (3). With regard to the cellular provision of these maintenance signals, we have been particularly interested in an accessory cell population that constitutively expresses high levels of a number of TNF ligands, including those for OX40 (TNFRSF4; CD134) and CD30 (TNFRSF8), which are expressed on activated CD4 and CD8 T cells. These cells are found throughout the T zone and B cell areas of secondary lymphoid tissues associated with B and T zone stromal cells (4, 5). We have termed these cells adult lymphoid tissue inducer cells (LTI), because of the close genotypic and phenotypic resemblance to LTI present in neonatal rodent life that orchestrate lymph node (LN) development (4, 6–9).

Following exposure to Ag, primed CD4 T cells associate with LTI and in the absence of OX40 and CD30 on CD4 T cells, T cell-dependent memory Ab responses, a hallmark of mammalian adaptive immunity, fail to develop (4, 10). We have published evidence that LTI and the γc cytokine IL-7 act in concert to promote CD4 memory (11); namely, that IL-7 produced by the stromal cells to which LTI are attached up-regulate OX40 on CD4 CD62Llow T cells (10), which then facilitates OX40/CD30-signals from LTI.

In this article, we report studies on the dependence of CD4 Th1 T cells on OX40 and CD30 signals. Double knockout mice (DKO) deficient in both OX40 and CD30 and wild-type (WT) mice were infected with an attenuated strain of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) (12). Control of bacterial replication inside macrophages during primary infection is independent of Ab (13–16), but does depend on CD4 Th1 T cells (17, 18). We report here that, unlike mice deficient in either OX40 or CD30 alone, DKO mice fail to control *S. typhimurium* infection. This is not because DKO CD4 T cells fail to differentiate to Th1 cells as we have found in vitro (10), but because of a failure to sustain activated DKO CD4 CD62Llow T cells. This defective CD4 Th1 survival was confirmed by an impaired ability of splenocytes from DKO but not WT mice infected with *S. typhimurium* to control infection following transfer into Rag1-deficient recipients. Taken together, these data suggest that DKO mice have fundamental defects in sustaining both CD4 T cell help for Ab responses (10) and CD4 Th1 responses. Also these defects are associated with greatly reduced survival of CD4 CD62Llow T cells.

In addition to demonstrating impaired survival of Ag-primed T cells, we found that the survival of the CD4 T cells that down-regulate CD62L following transfer into lymphopenic hosts also depends on OX40 and CD30 signals. Furthermore, the depletion of DKO CD4 T cells was virtually complete when there was concurrent *S. typhimurium* infection. Our data suggest a mechanism whereby infections like *Salmonella* could exacerbate CD4 T cell depletion in HIV infection.

**Materials and Methods**

**Mice, bacteria, and immunization protocols**

The sources and primary reference to each strain of WT, Rag1-deficient, CD154 (CD40L)-deficient, OX40-deficient, CD30-deficient, DKO, and B cell-deficient mice are given in Refs. 4, 10, 13, and 19. Mice were bred and maintained and experiments performed and approved in accordance with Home Office guidelines at the University of Birmingham, Biomedical Services Unit. Mice were age and sex matched and used between the ages of 6 and 12 wk.

Mice were immunized by i.p. injection with 10⁵ live attenuated *S. typhimurium* strain SL3261 (12) in sterile PBS (200 μl/mouse). *S. typhimurium* was prepared by taking bacteria in log phase and washing twice in PBS as described before (20). At the indicated time points, individual spleens were taken, weighed, and analyzed by flow cytometry. Spleens were also assessed for the level of bacterial load by plating out spleen.
compared with WT mice, DKO bacterial numbers were infection independent of any effects on Ab production. This indicates that there is a T cell defect in DKO mice that impairs their ability to resolve infection (13). During the first week, control of infection, which was assessed by flow cytometry. For intracellular cytokine staining, splenocytes were restimulated for 4 h at 37°C in the presence of GolgiStop (as per the manufacturer’s protocol). The intracellular cytokine staining was performed by stained the cells with IFN-γ-allophycocyanin (BD Pharmingen).

In vivo T cell survival assays
Splenoctyes from either naïve or infected (38 days previously) WT and OX40−/−, CD30−/−, or DKO mice were mixed so that the ratio of WT (CD45.1) to gene-deficient (CD45.2) CD4 T cells was 1:1 and were transferred i.p. either alone or together into Rag1−/− mice (approximately total 10^7 cells/mouse). In some experiments, recipient mice were immunized 24 h later with 10^5 live attenuated S. typhimurium. At the indicated time points, lymphocytes from blood or spleen were stained for CD3, CD4, CD62L, and CD45.1 and the ratio of WT:gene-deficient CD4 T cells was assessed by flow cytometry.

Relative gene expression quantification
CD4 T cells were FACS sorted and RNA and cDNA were prepared as previously described (21). Briefly, RNA was purified from 10^6 sorted cells using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. The RNA pellet was reverse transcribed by standard methods using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). The primer and probe sequences and reaction conditions for β-actin, IFN-γ, and T-bet mRNA and its quantification have been published elsewhere (21, 22). To assess the contribution of each cell population, the relative values were corrected by the absolute number of CD4 CD62Llow T cells at each time point.

Statistics
Statistics were calculated using the nonparametric Mann-Whitney sum rank test. The p values were calculated using the Analyse-It program (Analyse-It).

Results
Mice deficient in CD30 and OX40 fail to control S. typhimurium infection
WT and DKO mice were infected i.p. with 10^7 live attenuated S. typhimurium. During the first week, control of infection, which depends on the innate system (23, 24), was comparable between DKO and WT mice, spleens of which contained equivalent numbers of bacteria and developed comparable degrees of splenomegaly (Fig. 1A, top left panel). In contrast by day 21 of infection, when compared with WT mice, DKO bacterial numbers were >3-fold higher (p < 0.05) and, by day 38, there was a >10-fold difference (Fig. 1 A, top right panel). Both CD30 and OX40 signals contributed to disease susceptibility, because mice deficient in either CD30 or OX40 alone exhibited no defect in bacterial clearance compared with WT mice and were significantly more competent than DKO mice (CD30, p < 0.05; OX40, p < 0.001; Fig. 1C). The defect in clearance was not because of impaired Ab production (10) as neither B cell-deficient (IgH−/−) nor CD154-deficient mice, which do not develop germinal centers and have impaired T-dependent Ig class switching, infected in the same experiment cleared infection comparably to WT mice (Fig. 1C and reported previously (13)). This indicates that there is a T cell defect in DKO mice that impairs their ability to resolve S. typhimurium infection independent of any effects on Ab production.

Following infection DKO mice have reduced CD4 T cell numbers associated with a selective reduction in CD4 CD62Llow T cells
In noninfected WT and DKO mice, the proportions of CD4 T cells in the spleen are similar (Fig. 2, A, top left panel), with a ratio of CD62Lhigh/CD62Llow of 1:1 (naive phenotype):CD62Llow (activated/memory) of 5–10/1 (Fig. 2, A, top right panel). Following infection with S. typhimurium in WT mice, there is a reversal in the CD4 CD62Lhigh/CD62Llow ratio as has been reported by others (Fig. 2A and Ref. 25). In contrast, in DKO mice the proportion of DKO CD4 T cells is reduced at days 18 and 38 (but not day 0 or day 49) and the change in the ratio of DKO CD4 CD62Lhigh/CD62Llow cells is less marked (Fig. 2A). The changes seen in the proportions of the DKO CD4 T cell populations were due to selective loss of CD62Llow cells at all time points postinfection (p < 0.05), whereas the proportions of CD62Lhigh/CD62Llow cells remained similar to WT mice (Fig. 2A). Both OX40 and CD30 contributed to the defect in CD4 CD62Llow cells as the proportions of this population on day 49 of infection were not lower in mice singly deficient in either OX40 or CD30 (Fig. 2B).

To determine whether the defect in DKO CD4 CD62Llow T cells was due to a failure of cells to traffic normally to the spleen T cell populations in the liver, blood, and mesenteric LN were assessed
18 days after infection. In each case, the proportion of CD4 CD62L<sup>low</sup> T cells was reduced in DKO mice compared with WT mice (Fig. 2C), indicating that there was a widespread loss of these cells from other sites.

*The reduced numbers of DKO CD4 CD62L<sup>low</sup> cells in S. typhimurium infection express normal levels of T-bet and IFN-γ mRNA*

The above data indicate there is selective loss of CD4 CD62L<sup>low</sup> cells in DKO mice. Protection against *S. typhimurium* is dependent upon CD4 Th1 polarization mediated by the transcription factor T-bet (18, 26) and >95% of the mRNA signal for T-bet is found in CD4 CD62L<sup>low</sup> T cells (A. F. Cunningham, unpublished observations). To investigate whether CD4 Th1 polarization was normal in DKO mice, we FACS-sorted CD4 CD3 CD62L<sup>low</sup> cells from infected DKO and WT mice 18 and 49 days after infection, time points when CD4 Th1 development is necessary to control infection (Fig. 1A and Ref. 18). mRNA from purified cells was quantified for expression of T-bet and the key Th1 effector molecule IFN-γ. After correction for the housekeeping gene β-actin, expression of T-bet in WT and DKO CD4 CD62L<sup>low</sup> cells was shown to be similar on day 18, but slightly reduced on day 49 (Fig. 3A; p < 0.05). IFN-γ expression levels in the DKO CD4 CD62L<sup>low</sup> cells were slightly greater on day 18 and comparable on day 49 (Fig. 3A). When the different proportions of CD4 CD62L<sup>low</sup> cells were taken into account, the total amount of both T-bet and IFN-γ mRNA contributed by CD4 CD62L<sup>low</sup> T cells in the spleen was significantly reduced (Fig. 3B; p < 0.05). The difference in total IFN-γ production by WT and DKO CD4 CD62L<sup>low</sup> T cell populations was confirmed by intracellular FACS staining for IFN-γ protein (Fig. 3C; p < 0.01). These data indicate that DKO mice have no intrinsic defect in their capacity to up-regulate the Th1 transcriptional regulator T-bet nor do they have impaired capacity to switch on expression of IFN-γ, but that defective clearance of bacteria in DKO mice is due to the failure to sustain CD4 CD62L<sup>low</sup> Th1 cells.

*CD4 lymphocytes from S. typhimurium primed DKO donors fail to persist after transfer into Rag1-deficient mice and fail to control *S. typhimurium* infection*

To test directly whether DKO splenocytes were defective in their capacity to protect against infection, we transferred splenocytes from DKO and WT animals, primed 38 days previously, into Rag1-deficient mice. Control experiments and previous data showed that at 24 h CD4 posttransfer DKO splenocytes could traffic to the spleen and proliferate normally (data not shown and Ref. 10). At this 24-h point, chimeras were infected with 10<sup>8</sup> *S. typhimurium* (Fig. 4). Mice were sacrificed after 7 days and the numbers...
protection to reinfection. In brief, 10⁷ splenocytes from WT or DKO mice infected 38 days previously with 10⁵ S. typhimurium were injected i.p. into Rag1-deficient mice 24 h before challenge with 10⁷ S. typhimurium. After 1 wk, mice were sacrificed. A, Splenic bacterial counts in Rag1-deficient mice (■) or Rag1-deficient mice that received 10⁷ WT splenocytes (○) or Rag1-deficient mice that received 10⁷ DKO splenocytes (▲) infected with 10⁷ S. typhimurium. B, Relative survival of WT and DKO CD4 T cells. Symbols represent results from individual mice. * p < 0.05. Data are representative of two experiments.

 FIGURE 3. Expression of T-bet and IFN-γ in WT and DKO CD4 CD62Llow T cells. WT and DKO CD4 CD62Llow T cells were isolated from mice by FACS 18 and 49 days after infection with 10⁵ S. typhimurium. Expression of mRNA was assessed by quantitative PCR. Symbols indicate results from individual mice. A, Expression of T-bet and IFN-γ mRNA relative to β-actin. B, Expression of T-bet and IFN-γ mRNA after correction for CD4 CD62Llow cell numbers. C, Intracellular FACS staining for IFN-γ in WT (top panels) and DKO (bottom panels). Left-hand panels, The CD4 T cell gate used; right-hand panels, the IFN-γ and CD62L expression in CD4 T cells. The graph shows the percentage of CD4 CD62Llow T cells expressing IFN-γ for individual WT and DKO mice. *, p < 0.05. Panels are representative of two experiments.

 FIGURE 4. DKO splenocytes from S. typhimurium-primed donors transferred into Rag1-deficient mice fail to survive and confer only partial protection to reinfection. In brief, 10⁷ splenocytes from WT or DKO mice infected 38 days previously with 10⁵ S. typhimurium were injected i.p. into Rag1-deficient mice 24 h before challenge with 10⁷ S. typhimurium. After 1 wk, mice were sacrificed. A, Splenic bacterial counts in Rag1-deficient mice (■) or Rag1-deficient mice that received 10⁷ WT splenocytes (○) or Rag1-deficient mice that received 10⁷ DKO splenocytes (▲) infected with 10⁷ S. typhimurium. B, Relative survival of WT and DKO CD4 T cells. Symbols represent results from individual mice. *, p < 0.05.
Final ratio of WT CD4 cells to deficient CD4 cells was clearly well placed to provide these signals to CD4 T cells which were activated by Ag. Consistent with a global defect in survival of primed CD4 T cells, we found profound depletion of CD4 CD62L\textsuperscript{low} but not CD4 CD62L\textsuperscript{high} cells in DKO mice infected with \textit{S. typhimurium}.

In this study, we report that CD4 Th1-dependent control of \textit{S. typhimurium} infection also depends on both OX40 and CD30 signals. We have published a report (10) showing that commitment to protective CD4 Th1 differentiation in vitro is not affected by the absence of CD30 and OX40, but in the current study we find that both signals were required for their survival. L-selectin (CD62L) down-regulation has been used as a marker for CD4 T cells activated by Ag. Loss of both OX40 and CD30 signals contributed to this reduction in survival rates. The very striking finding, however, was the synergy between \textit{S. typhimurium} infection and lymphopenia, as virtually all DKO CD4 T cells perished under these conditions.

Are these observations relevant to human disease? Because murine adult LTI coexpress CD4 and CXCR4, ligands for CXCR4 tropic strains of HIV, we have speculated that their human equivalent could be targeted and depleted, rendering individuals effectively deficient in CD30 and OX40-mediated CD4 T cell survival signals. This would explain the initial loss of memory CD4 T cells not infected by virus, but would also offer an explanation for the depletion of naive CD4 T cells activated under conditions of lymphopenia (27). Since in intact DKO, but not lymphopenic DKO Rag1-deficient chimeras CD4CD62L\textsuperscript{high} and CD4CD62L\textsuperscript{low} T cells were detectable throughout the infection, it implies that CD4 T cells are likely to be consistently recruited into the response against \textit{S. typhimurium}.

With regard to \textit{Salmonella} infection, recurrent disease commonly occurs in lymphopenic HIV-infected individuals in sub-Saharan Africa (28). Our data suggest that susceptibility to \textit{Salmonella} infection in HIV disease could not only be consequent on CD4 immunodeficiency, but in the context of lymphopenia might also further exacerbate CD4 depletion.

In summary, our data highlight two important points: first, the roles of OX40 and CD30 signals in sustaining CD4 Th1 responses and, second, the synergy between lymphopenia and concurrent \textit{S. typhimurium} infection in rendering CD4 T cells dependent on OX40 and CD30 survival signals. Pinpointing human LTI will establish whether they are deleted in HIV disease, although the promiscuous expression of CD4 on many different cell types in humans makes this task difficult.

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Disclosures
The authors have no financial conflict of interest.

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