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*J Immunol* 2009; 183:5079-5084; Prepublished online 28 September 2009;
doi: 10.4049/jimmunol.0901514
http://www.jimmunol.org/content/183/8/5079

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The Survival of Memory CD4\(^+\) T Cells within the Gut Lamina Propria Requires OX40 and CD30 Signals\(^1\)

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Although CD4\(^+\) memory T cells reside within secondary lymphoid tissue, the major reservoir of these cells is in the lamina propria of the intestine. In this study, we demonstrate that, in the absence of signals through both OX40 and CD30, CD4\(^+\) T cells are comprehensively depleted from the lamina propria. Deficiency in either CD30 or OX40 alone reduced CD4\(^+\) T cell numbers, however, in mice deficient in both CD30 and CD40, CD4\(^+\) T cell loss was greatly exacerbated. This loss of CD4\(^+\) T cells was not due to a homing defect because CD30 \(\times\) OX40-deficient OTII cells were not impaired in their ability to express CCR9 and \(\alpha_\beta_2\), or traffic to the small intestine. There was also no difference in the priming of wild-type (WT) and CD30 \(\times\) OX40-deficient OTII cells in the mesenteric lymph node after oral immunization. However, following oral immunization, CD30 \(\times\) OX40-deficient OTII cells trafficked to the lamina propria but failed to persist compared with WT OTII cells. This was not due to reduced levels of Bcl-2 or Bcl-XL, because expression of these was comparable between WT and double knockout OTII cells. Collectively, these data demonstrate that signals through CD30 and OX40 are required for the survival of CD4\(^+\) T cells within the small intestine lamina propria. The Journal of Immunology, 2009, 183: 5079–5084.

The small intestinal mucosa, including the lamina propria (LP),\(^4\) contains the largest reservoir of CD4\(^+\) effector/memory cells in the body (1, 2). The CD4\(^+\) T cells residing in the LP are of essential importance for protection against infections as well as maintaining tolerance (3–6). Survival of effector and memory cells at the LP site is therefore of crucial importance for the maintenance of mucosal immunity (6–8).

Within secondary lymphoid tissue, signals from common cytokine-receptor \(\gamma\)-chain family cytokines such as IL-2, IL-7, and IL-15 promote T cell proliferation, differentiation, and survival during an immune response (9). It has also become increasingly evident that the survival of activated T cells is controlled through their expression of TNF receptor (TNFR) family members such as OX40, CD30, and 4-1BB (10, 11). CD40, CD30, and 4-1BB are type I transmembrane molecules with transient expression, that provide costimulatory signals during T cell activation as well as generating signals enabling the survival of effector T cells (3). Although signals through 4-1BB are important for CD8\(^+\) memory T cells, CD4 responses in 4-1BB- knockout (KO) mice were only slightly impaired (12). Secondary CD8\(^+\) T cell responses were not impaired in OX40KO mice (12), however, signals through OX40 were critical in promoting the survival of CD4\(^+\) T cells (13–16). Further, signals through CD30 also contribute to CD4\(^+\) T cell survival (17, 18). To confer survival, the TNFR family members, including OX40 and CD30, signal through TNFR-associated factors, which link into different signaling pathways resulting in expression of anti-apoptotic proteins such as Bcl-x\(_L\) and Bcl-2 (15, 19). Because different TNFRs use the same TNFR-associated factors, redundancy in the signals generated through these receptors is likely (20).

The severe loss of CD4\(^+\) memory T cells from the spleen of mice lacking both OX40 and CD30 signals (17, 18) led us to investigate whether memory CD4\(^+\) T cells outside secondary lymphoid tissue were similarly dependent on signaling through these pathways. Here we report the selective loss of the CD4\(^+\) T cell compartment from the LP of CD30 and OX40 double knockout (dKO) mice. Although deficiency of either CD30 or OX40 significantly reduced the number of CD4\(^+\) T cells in the LP, the loss of both genes resulted in the almost complete loss of LP CD4\(^+\) T cells. This was not attributable to a defect in either proliferation or the ability of dKO CD4\(^+\) T cells to traffic to the small intestine in vivo. Rather, dKO CD4\(^+\) T cells failed to survive having reached the LP, despite comparable levels of Bcl-2 and Bcl-x\(_L\) expression in wild-type (WT) LP CD4\(^+\) T cells. Materials and Methods

Mice

All experiments were performed in accordance with U.K. laws and with the approval of the University of Birmingham or the Lund/Malmö animal ethics committees. WT (C57BL6 and BoyJ background), CD30KO (C57BL6), OX40KO (C57BL6), CD30KO \(\times\) OX40KO (dKO, C57BL6), WT \(\times\) OTII (BoyJ), dKOxOTII (C57Bl6), and CD3xTg26 mice (BoyJ) (21) were bred and maintained in the animal facilities of the University of Birmingham, U.K., or the Lund University Biomedical Center, Sweden.

Analysis of murine small intestine using immunofluorescence

Gut tissue was flushed clean with RPMI and frozen sections were prepared and stained as described previously (22). Primary Abs used were anti-B220...
FITC (RA3–6B2, ebioscience), anti-CD3ε FITC (145-2C11, BD Pharmingen), anti-CD4 Alexa Fluor 647 (GXK1.5, ebioscience), and anti-CD11c biotin (N418, ebioscience). Biotinylated Abs were detected with streptavidin Alexa Fluor 555 (Invitrogen). FITC-conjugated Abs were detected using rabbit anti-FITC Abs (Sigma-Aldrich), then goat anti-rabbit-FITC Abs (Southern Biological). Sections were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and mounted using DABCO (Sigma-Aldrich). Confocal images were obtained using a LSM 510 Meta microscope using the Zeiss LSM software. For T cell quantitation, villi were divided into LP and epithelium areas and individual T cells (CD3ε⁺ CD4⁺ and CD3ε⁺ CD4⁻) counted manually.

Cell preparations
Small intestine. Cells were prepared from small intestine as described previously (23). Intestinal tissue was rinsed with cold HBSS/HEPES and Peyer’s patches (PP) and fat carefully removed. The tissue was cut longitudinally and then into smaller pieces of ~5 mm. The tissue pieces were then transferred to HBSS/HEPES/2 mM EDTA, incubated at 37°C for 15 min then shaken vigorously and the resulting mixture was filtered with the tissue remaining in solution. This process was repeated a total of three times and the resulting tissue was washed in cold RPMI/HEPES/10% FCS and transferred to urine beakers which contained 20 ml RPMI 1640/HEPES/10% FCS supplemented with 250 μg/ml collagenase VIII and 50 mM CaCl₂. The urine beakers were incubated for 1 h at 37°C under magnetic stirring. The digested tissue was then filtered and the resulting cell suspension centrifuged and washed with cold RPMI. Lymphocytes were enriched using a 40-70% Percoll gradient.

Flow cytometry
Flow cytometric analysis was performed on cell suspensions as previously described (22). Primary Abs used were: anti-mouse Bcl-2 FITC (3B12, BD Pharmingen); Bcl-XL (7B2.5, Abcam); B220 FITC (RA3–6B2, ebioscience); CD3 ε FITC (145-2C11, ebioscience); CD4 allophycocyanin (GK1.5, ebioscience); CD4 Pacific blue (RM-5, ebioscience); CD11c FITC (N418, ebioscience); anti-αβ γ (DATK32, BD Pharmingen); anti-CCR9 Ab (7B7 purified from hybridomas, gift from Oliver Pabst) (24) then anti-rat IgG2b biotin (G15–337, BD Pharmingen); CD30L biotin (RM153, ebioscience); CD45.1 PE (A20, BD Pharmingen); CD45.2 PE (104, ebioscience); CD45.2 allophycocyanin-A750 (104, ebioscience), IL-7Rα biotin (A7R34, ebioscience); OX40L biotin (RM134L, BD Pharmingen); Thy-1.2 biotin (53–2.1, BD Pharmingen). Biotinylated Abs were detected with streptavidin-PE Cy5.5, streptavidin-QD605 (Invitrogen) or streptavidin-allophycocyanin (BD Pharmingen). DAPI (final concentration 0.25 μg/ml) or propidium iodide were added to samples before acquisition. Data were acquired using a CYAN ADP flow cytometer or a FACSaria (BD Biosciences) and analyzed using Summit software (Beckman Coulter) or FlowJo software (Tree Star).

In vitro culture of OTII cells with all-trans retinoic acid
Splenid dendritic cells (DCs) and OTII CD4⁺ T cells were isolated as described (23). DCs (10⁵ cells) were incubated with OVA (1 mg/ml), 10 nM all-trans retinoic acid, and WT or dKO OT-II cells (2 x 10⁵ cells) in flat-bottom 96-well plates (Nunc) in 200 μl of complete RPMI/HEPES/10% FCS. After 4 days, cells were resuspended in RPMI/HEPES/10% FCS to a total volume of 500 μl supplemented with 10 mg/ml IL-7 and 10 ng/ml IL-15 (R&D Systems) and transferred into 48-well plates. Six days after culture setup, cells were analyzed for their expression of αβγ and CCR9.

Cell isolation, labeling, and adoptive transfers for in vivo activation of OTII cells
CD4⁺ T cells were isolated from WT × OTII and dKO × OTII spleens (23), labeled with 2.5 μM CFSE in PBS for 8 min at RT and washed twice. For adoptive transfers, CD4⁺ T cells (3 x 10⁵) were injected i.v. into recipient mice which 1 day later received oral OVA (50 mg/mouse, Sigma-Aldrich) and RA85 (10 μg/mouse, Alexis Biochemicals). Twenty hours after OVA administration, mice where indicated received FTY720 i.p. (1 mg/kg, Cayman Chemical). Mice were sacrificed at 3, 4, 5, or 7 days after OVA administration.

Statistics
For quantitation of T cell numbers in the LP and epithelium, the Wilcoxon Two Sample Test was applied. For WT and dKO OTII cell survival the t test for paired samples was used.

Results
CD4⁺ T cells in the LP depend on both OX40 and CD30 signals
To investigate whether CD4⁺ T cells in the LP depend on OX40 and CD30 signals, sections of small intestine prepared from WT, CD30KO, OX40KO, and dKO mice were stained for the expression of CD3 and CD4 (Fig. 1A). In WT mice, CD4⁺ T cells were clustered specifically within the LP core of each villus, whereas CD8⁺ T and particularly γδ T cells were mostly associated with gut epithelium. Compared with WT mice (mean = 2315.6 cells per mm², STDEV = 802.8), both CD30KO (mean = 1211.9 cells per mm², STDEV = 507.7) and OX40KO (mean = 766.2 cells per mm², STDEV = 366.8) mice had significantly reduced numbers of CD4⁺ T cells in the LP (Fig. 1B). In mice deficient in both CD30 and CD40, the effects on the LP CD4⁺ T cell compartment were dramatic. Many sections of villi in dKO mice contained no CD4⁺ T cells and this was reflected in a severe reduction in villus CD4⁺ T cell numbers (mean = 156.9 cells per mm², STDEV = 203.9). This represents an ~14.8-fold loss compared with WT mice and a ~7.7- and ~4.9-fold further reduction compared with CD30KO and OX40KO mice, respectively.

The effects of OX40 and CD30 deletion was most evident for CD4⁺ T cells, CD4⁻ T cell numbers in the LP were only slightly reduced in dKO mice compared with WT mice (WT mean = 1250.6 vs dKO mean = 1064.1, Fig 1C). The intraepithelial lymphocyte population was modestly affected by the absence of both CD30 and OX40 genes (Fig. 1D), however, both CD8⁺ T cells and γδ T cells were readily detected in the villi of dKO mice (Fig. 1E). Therefore, combined signals through both CD30 and OX40 appear critical specifically for the presence of the CD4⁺ T cell compartment of the small intestine LP.

dKO CD4⁺ T cells show normal activation and gut homing ability
Our previous studies have shown that dKO CD4⁺ T cells initially proliferate and differentiate normally both in vitro (16) and in vivo (17). To exclude a specific defect related to their gut homing capacity during priming in the mLN, CFSE-labeled CD45.1⁺ WT and CD45.2⁺ dKO transgenic OTII cells (25) were transferred in a 1:1 ratio into CD45.1⁺ CD45.2⁺ recipient mice, allowing host T cells to be discriminated from donor T cell populations. After 24 h, these mice were given 50 μg OVA orally, followed by FTY720 at 20 h after OVA to prevent lymphocyte egress from the mLN (26). Three days later, the ratio of dKO:WT OTII cells in spleen and mLN remained ~1:1 (Fig. 2A). Furthermore, the proliferation of these cells, assessed by dilution of CFSE, was comparable between WT and dKO OTII cells, as was the expression of CCR9 and αβγ (Fig. 2B).

To test the capacity of dKO OTII cells to traffic to the LP, WT and dKO OTII cells were primed in vitro with splenic DCs and OVA in the presence of retinoic acid, conditions that induce a gut homing phenotype (27). Six days later, almost all (~95%) dKO and WT OTII cells expressed αβγ and CCR9 (Fig. 2C). Both primed CD45.1⁺ WT and CD45.2⁺ dKO OTII cells were then transferred (in 1:1 ratio) into CD45.1⁺ CD45.2⁺ recipients. After 24 h, the distribution of WT and dKO OTII cells was analyzed (Fig. 2D), revealing no significant differences between the mouse strains in spleen, mLN, and LP. Combined, these data indicate that
dKO CD4+ T cells display normal activation in mLN in vivo as well as no disadvantage in ability to home to the small intestinal LP after in vitro activation.

Survival of LP OTII cells depends on OX40 and CD30

To analyze whether dKO T cells had a defect during the in vivo generation of gut homing, we adoptively transferred dKO and WT OTII cells into recipient mice, which were subsequently immunized by oral administration of OVA. Three days after immunization, the mLN was analyzed. Comparing immunized and unimmunized mice, we could clearly see an expansion of both dKO OTII (CD45.1+ and CD45.2+) and WT (CD45.1+CD45.2+) cells after OVA administration, while the ratio in immunized and naive mice was similar (Fig. 3, A and B). Furthermore, the CFSE profile of dKO and WT OTII cells as well as expression of CCR9 and WT OTII cells in mLN remained fairly constant, albeit skewed in favor of WT OTII cells. However, the ratio was significantly decreased in the LP compared with the mLN at day 7 (p = 0.03 and p = 0.047 in two separate experiments, Fig. 4B). Together, these results provide evidence that OX40 and CD30 are required for the survival of CD4+ T cells within the LP.

Normal levels of Bcl-2 and Bcl-xL in dKO LP CD4+ T cells

Activation of OX40+/CD4+ T cells in vitro has indicated that in the absence of this receptor, expression of both Bcl-2 and Bcl-XL is reduced (15). To investigate whether the failure of survival of CD4+ T cells in the LP of dKO mice was due to reduced levels of Bcl-2 and Bcl-xL, their expression was analyzed by intracellular flow cytometry. Surprisingly, no differences in the expression of Bcl-2 or Bcl-xL were detected in CD45.1+CD45.2+ T cells isolated from the LP (Fig. 5A), spleen, mLN, or PP of WT and dKO mice (data not shown). Expression of Fas by CD4+ T cells in the mLN was also comparable between WT and dKO mice (data not shown).

To further analyze expression of Bcl-2 or Bcl-xL by responding dKO CD4+ T cells that had been induced to traffic to the LP, WT
OTII cells (CD45.1+ and dKO OTII cells (CD45.2+)) were transferred (1:1 input ratio) into CD3eTg26 mice (which lack T and NK cells). The mice were then orally immunized with OVA and the spleen, mLN, PP, and LP analyzed 4 days after immunization. A clear population of OTII cells was detected in the LP, however, expression of Bcl-2 and Bcl-xL was comparable between WT and dKO-derived OTII cells (Fig. 5B), despite skewing of the WT: dKO OTII cell ratio in the gut (Fig. 5C). Transfer of WT and dKO OTII cells into WT mice with subsequent OVA administration yielded similar results (data not shown). In summary, dKO OTII cells fail to survive in the LP compared with WT OTII cells, however, this is not due to impaired expression of either Bcl-2 or Bcl-xL.

Discussion
In this study, we describe the severe depletion of CD4+ T cells from the LP of the small intestine in the absence of OX40 and CD30. The loss of these cells was not due to an inability to traffic to the LP, neither were dKO CD4+ T cells impaired in their ability to proliferate in the mLN following oral immunization. Rather, these data demonstrate the failure of dKO OTII cells to survive within the LP.

Although activated and memory CD4+ T cells reside within secondary lymphoid tissue, the main reservoir for these cells is the LP of the small intestine. Because signals through CD30 and OX40 are required for the persistence of memory CD4+ T cells in the spleen (17, 18), we investigated whether these signals were also required for survival in the LP. Immunofluorescence analysis of the small intestine of dKO mice revealed a severe loss of CD4+ T cells from the small intestine LP, significantly more dramatic than observed in either OX40KO or CD30KO mice. To exclude the possibility that dKO CD4+ T cells were unable to traffic to the small intestine oral immunization with OVA, in addition to in vitro culture with retinoic acid, was performed. In all assays, WT and dKO OTII cells expressed comparable levels of both CCR9 and αβ7 and migrated as efficiently as the WT CD4+ T cells to the LP at early time points. Therefore, dKO CD4+ T cells were neither...
are not dependent upon RORγt/H9253 and Bcl-xL/H11001 port activated CD4+/H11001 T cells, lymphoid tissue inducer cells (LTi) are well placed to support the generation of memory T cells in the LP are currently unclear. Within the Tg26 mice which A staining was done. The population is clearly distinct from LTi and although it may be CD30 signals within the LP. T cells consistent with a further requirement for OX40 and post immunization, demonstrated a significant loss of the dKO mLN does indicate that OX40 and CD30 signals are required and dKO OTII cells within the mLN being comparable, the strongly arguing against this. Despite the initial priming of WT and dKO CD4+/H11001 T cells of LP isolated from WT and dKO mice. B, WT (CD45.1) and dKO (CD45.2) OTII cells were transferred into CD3εTg26 mice which were then orally immunized with OVA and analyzed 4 days later. Expression of Bcl-2 and Bcl-xL by WT and dKO OTII cells of LP. C, Ratio of WT:dKO OTII cells in spleen, mLN, PP, and LP at 4 days after oral immunization. Numbers show percentage of cells in gate, data from three mice.

An alternative explanation for the loss of dKO T cells from the LP was the inability of these cells to proliferate within the mLN. However, CFSE-labeled WT and dKO OTII cells showed comparable dilution of the dye 3 days after oral immunization strongly arguing against this. Despite the initial priming of WT and dKO OTII cells within the mLN being comparable, the skewing of the WT:dKO OTII ratio in favor of WT cells in the mLN does indicate that OX40 and CD30 signals are required either during or soon after priming at this site. Because the ratio of WT:dKO OTII cells remained at the input ratio in the spleen after oral immunization, this provides further evidence that OX40 and CD30 signals are required post activation. Comparison of the ratio of WT:dKO OTII in the mLN and LP at 7 days post immunization, demonstrated a significant loss of the dKO T cells consistent with a further requirement for OX40 and CD30 signals within the LP.

The OX40L/CDC30L+-requiring cells that provide signals for the survival of memory T cells in the LP are currently unclear. Within the mLN, lymphoid tissue inducer cells (LTi) are well placed to support activated CD4+ T cells, constitutively expressing high levels of OX40L and CD30L. Within the small intestine, LTi reside within cryptopatches (29) and ILFs where a role in T cell independent switching to IgA was identified (30). Although a CD4+CD3- population of cells resides within the LP, these cells are not dependent upon RORγt (29) and lacked expression of IL-7Rα (D. Withers, unpublished observations). This CD4+CD3- population is clearly distinct from LTi and although it may be involved in mediating T cell survival, other APCs resident within the LP may also provide OX40L and CD30L signals.

To investigate the mechanisms behind the impaired survival of dKO CD4+ T cells, expression of Bcl-2 and Bcl-xL were analyzed, because these have been reported to be reduced in the absence of OX40 signals (15). Surprisingly, we found expression of the anti-apoptotic factors Bcl-2 and Bcl-xL to be identical when WT and dKO CD4+ T cells in the LP were compared. Furthermore, when WT and dKO OTII cells were induced to traffic to the LP, levels of Bcl-2 and Bcl-xL were again comparable, despite the impaired survival of dKO OTII cells. In contrast to our in vivo study, previous studies demonstrated impaired expression of Bcl-2 and Bcl-xL in vitro-stimulated OX40+/H11001 T cells, and these experimental differences may explain this discrepancy. Nevertheless, why dKO CD4+ T cells survive less well than WT CD4+ T cells in the LP remains unclear.

In summary, our data demonstrate that signals through OX40 and CD30 are required for the persistence of CD4+ T cells within the small intestine LP. This shows the relevance of the OX40 and CD30 signaling pathways in the survival of tissue memory CD4+ T cells and has implications for the modulation of these pathways in intestinal inflammatory diseases.

Acknowledgments
We acknowledge the work of all the staff at Biomedical Services Unit, University of Birmingham for animal breeding and welfare.

Disclosures
The authors have no financial conflict of interest.

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