Cutting Edge: Lymphoid Tissue Inducer Cells Maintain Memory CD4 T Cells within Secondary Lymphoid Tissue

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Phylogeny shows that CD4 T cell memory and lymph nodes coevolved in placental mammals. In ontogeny, retinoic acid orphan receptor (ROR)γ-dependent lymphoid tissue inducer (LTi) cells program the development of mammalian lymph nodes. In this study, we show that although primary CD4 T cell expansion is normal in RORγ-deficient mice, the persistence of memory CD4 T cells is RORγ-dependent. Furthermore, using bone marrow chimeric mice we demonstrate that LTi cells are the key RORγ-expressing cell type sufficient for memory CD4 T cell survival in the absence of persistent Ag. This effect was specific for CD4 T cells, as memory CD8 T cells survived equally well in the presence or absence of LTi cells. These data demonstrate a novel role for LTi cells, archetypal members of the innate lymphoid cell family, in supporting memory CD4 T cell survival in vivo. The Journal of Immunology, 2012, 189: 2094–2098.

The hallmark of mammalian immunity is the capacity to make CD4 T cell-dependent memory immune responses, and this underpins the success of vaccination strategies. Phylogeny shows that both lymph nodes (LNs) and CD4 memory Ab responses evolved in placental animals, whereas monstrepals have evidence of memory (1) and LNs (2, 3), whereas monotremes have neither (4, 5). The formation of LNs is dependent on retinoic acid orphan receptor (ROR)γ-expressing lymphoid tissue inducer (LTi) cells, key members of the recently described innate lymphoid cell family (6). Although the function of LTi cells in the developing embryo is clear, their potential roles within mature secondary lymphoid tissue are currently being elucidated.

Recent studies have found them to be important in the repair of lymphoid tissues after pathogen-related injury (7), the production of IL-22 (8), and T cell-independent production of IgA in the gut (9). We previously found that in mature mice, but not in embryonic mice, LTi cells express high levels of the TNF family members OX40-ligand (L) and CD30L (10, 11), and we have linked signaling through the receptors for these molecules with the capacity to mount CD4 memory Ab responses (12, 13). Unlike APCs such as dendritic cells and B cells that can also express OX40L and CD30L, LTi cells completely lack expression of CD80 and CD86 and do not present Ag (13). Because LNs and CD4 memory Ab responses arose in the same evolutionary window, we speculated that LTi cells might provide survival signals required for the maintenance of memory CD4 T cells in the absence of antigenic stimulation. To test this we have now analyzed CD4 memory responses in mice lacking LTi cells. In this study, we provide direct evidence that LTi cells maintain memory CD4 T cells in vivo, demonstrating a further crucial role for these innate lymphoid cells in supporting adaptive immune responses.

Materials and Methods

Mice

Animals were bred in accordance with Home Office guidelines at the University of Birmingham, Biomedical Services Unit. Mice used were BoyJ, CD3ε<sup>tg26</sup>, CD3ε<sup>tg26RORγ<sup>−/−</sup></sup>, RORγ<sup>−/−</sup>, Rag<sup>−/−</sup>, Rag<sup>−/−</sup> × OTII, and Rag<sup>−/−</sup> × SM1. The following mouse was obtained through the National Institute of Allergy and Infectious Diseases Exchange Program, National Institutes of Health: C57BL/6-Tg(OT-I)-RAG1<sup>−/−</sup>Rag<sup>−/−</sup>(14, 15). Experimental and control CD3ε<sup>tg26RORγ<sup>−/−</sup></sup> mice were sublethally irradiated (1 × 450 rads), given bone marrow (BM) from CD3ε<sup>tg26</sup> or CD3ε<sup>tg26RORγ<sup>−/−</sup></sup> mice i.v., and used 4–5 wk after reconstitution.

Immunization and cell transfer

To track Ag-specific CD4 T cells, mice were infected i.v. with 10<sup>7</sup> ActA mutant Listeria monocytogenes expressing 2W1S peptide, as described (16). To generate memory T cells, 5 × 10<sup>5</sup> SM1, OTII, or OTI T cells were transferred into Rag<sup>−/−</sup> mice, which were then immunized and memory cells harvested 3–4 wk later. To stimulate SM1 cells, recipient mice were immunized i.v. with 10<sup>6</sup> attenuated L. monocytogenes-expressing FlII<sup>−</sup> peptide (target Ag of SM1 T cells), a gift from Dr. Sing Sing Way. To stimulate OTII and OTI cells, recipient mice were immunized i.p. with 100 µg aluminum hydroxide-precipitated OVA.

Flow cytometry

For tetramer staining, cells from secondary lymphoid tissue were pooled and stained for 1 h at room temperature with 2W1S:I-Ab. All cell surface staining
was done at 4°C for 30 min. Samples were run using a Fortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Immunofluorescence and image analysis
Frozen tissue sections were cut and stained as described previously (11).

Statistics
Statistical significance was tested using the Mann–Whitney U test, and a two-tailed p value was calculated.

**Results and Discussion**

**Memory CD4 T cells fail to survive in RORγ−/− mice**

To investigate whether CD4 T cell survival was dependent on LTi cells, we first immunized RORγ−/− and RORγ+/− littermate mice with nitrophenylated chicken γ-globulin. We assessed the primary anti-(4-hydroxy-3-nitrophenyl)acetyl IgM and IgG responses in the serum 7 d after immunization and found no significant difference (Supplemental Fig. 1). To assess a memory response, we analyzed splenic (4-hydroxy-3-nitrophenyl)acetyl–specific IgG plasma cells at 4 d after challenge (Supplemental Fig. 1). Although RORγ−/− mice mounted a characteristic memory response, this was absent in RORγ−/− littermates (Supplemental Fig. 1, p = 0.004). Although consistent with defective memory CD4 T cell survival, these experiments did not discriminate between B or T cell defects in the Ab response. To specifically test memory CD4 T cell survival in RORγ−/− mice, we analyzed endogenous 2W1S+ CD4 T cells (16). Mice were infected with L. monocytogenes–2W1S peptide (16), and the pMHC class II tetramer 2W1S-I-\(\text{κ}\)\(^{\text{A}}\) was used to identify responding cells in secondary lymphoid tissue (spleen and LNs from wild-type [WT] mice, only spleen from RORγ−/− mice). Comparable numbers of 2W1S-I-\(\text{κ}\)\(^{\text{A}}\) CD4 T cells were recovered from WT and RORγ−/− mice 7 d postinfection (dpi) (Fig. 1). No significant differences were seen in the expression of CXCR5, PD-1, Bcl-6, or T-bet by 2W1S+ CD4 T cells at 7 dpi, indicating a normal primary response with formation of follicular T helper cells and other T effector cell subsets (data not shown) (17). Strikingly, by 28 dpi, numbers of 2W1S-I-\(\text{κ}\)\(^{\text{A}}\) cells were significantly reduced (\(p = 0.003\)) in RORγ−/− mice compared with WT controls, and this difference was even greater at 70 dpi (Fig. 1B).

**RORγ-expressing LTi cells mediate memory CD4 T cell survival**

Although these data demonstrated that polyclonal Ag–specific CD4 memory T cells were not maintained in RORγ−/− mice, they did not show directly that LTi cells were responsible. Differences between WT and RORγ−/− mice could have been attributed to the absence of LNs in the latter, and other cell populations are RORγ-dependent (18–20). Therefore, to test directly the requirement for LTi cells we performed the following reductionist experiment, exploiting the CD3e\(^{\text{tg26}}\) mouse (21) that lacks T and NK cells but is LTi cell-sufficient (10). These mice were crossed with RORγ−/− mice to make mice deficient in T, NK, and LTi cells (CD3eRORγ−/−) (Supplemental Fig. 2). To reconstitute LTi cells in the spleen, CD3eRORγ−/− mice were irradiated and reconstituted with BM from CD3e\(^{\text{tg26}}\) mice (Supplemental Fig. 2, mice designated LTi+). CD3eRORγ−/− mice irradiated and reconstituted with BM from CD3eRORγ−/− (mice designated LTi−) were used as controls. Because LNs only develop during an embryonic window, they were absent in both LTi+ and LTi− mice. LTi-like cells expressing NK1.1 and NKP46 (22) were absent in the spleens of CD3e\(^{\text{tg26}}\) mice compared with the spleens of Rag−/− mice (Supplemental Fig. 2). The LTi cells in CD3e\(^{\text{tg26}}\) mice expressed high levels of OX40L, whereas expression of OX40L by dendritic cells was comparable in CD3e\(^{\text{tg26}}\) and CD3eRORγ−/− mice (Supplemental Fig. 2). Therefore, we had generated mice with the same secondary lymphoid tissue but sufficient or deficient in LTi cells to test the role of LTi cells in memory T cell survival.

Transfer of premade memory cells into LTi+ or LTi− mice would exclude possible effects of LTi cell deficiency on the generation of these cells. A pure cohort of memory CD4 SM1 TCR transgenic T cells was generated (see Materials and Methods) and then transferred (\(\sim 2.5 \times 10^5\)) into LTi+ and LTi− hosts. Four weeks after transfer of memory SM1 T cells and without further exposure to Ag, a clear population persisted principally in the spleen of LTi+ mice, and to a lesser extent in the BM and other nonlymphoid tissues such as the lung and liver (Fig. 2A–C). In contrast, this population was almost entirely absent in LTi− mice. To control for complexities arising from chimeric mice, a similar experiment was performed using Rag−/−RORγ−/− and Rag−/−RORγ−/− mice, with similar results (Fig. 2D). We also confirmed these findings with a second TCR transgenic T cell, OTII cells, which again persisted only in LTi+ mice (Fig. 2E).

Finally, we have found that numbers of LTi cells are dependent on RORγ expression, as Rag−/−RORγ−/− mice have fewer LTi cells than do Rag−/−RORγ+/− mice (Supplemental...
Fig. 2). In these mice, survival of CD4 T cells is highly correlated with LTi cell number, consistent with CD4 T cell survival influenced by LTi cells. It is presently unclear how LTi cells maintain memory CD4 T cells. Agonistic anti-OX40 Abs did maintain memory OTII cells in LTi2 mice (Fig. 2F), indicating that provision of this signal could keep memory CD4 T cells alive in vivo. Our previous observations that LTi cells express high levels of OX40L and CD30L, and that signals by these molecules are essential for the survival of memory CD4 T cells (12, 13), would suggest this may be how LTi cells provide survival signals. We have previously identified that memory OTII cells associate with LTi cells much more frequently than do naive OTII cells within the spleen (23), supporting a model where cellular interactions mediate survival.

LTi cells are not required for memory CD8 T cell survival
To investigate whether memory CD8 T cell survival was also LTi cell-dependent, memory OTI cells were generated in Rag−/− mice and transferred into Rag−/−RORγ−/− and Rag−/−RORγ+/− mice, which were then immunized with OVA. Three weeks after immunization the survival of OTI cells in either the spleen or BM was comparable between LTi cell-sufficient and LTi cell-deficient hosts (Fig. 3A, 3B). When memory OTI and SM1 cells were cotransferred into LTi+ and LTi− chimeric mice, memory OTI cells could be recovered from both LTi+ and LTi− mice, but memory SM1 cells were absent specifically in LTi− mice (Fig. 3C).

LTi cells reside at sites of memory cell recirculation
To investigate where LTi cells and memory CD4 T cells might interact in vivo, we analyzed RORγ expression in secondary lymphoid tissue to determine the location of these cells. Within the spleen, LTi cells (defined as RORγ+IL-7Rα+CD3ε+) were located at the marginal sinus and within the bridging channels (24), coinciding closely with the sites of lymphocyte entry and trafficking into the white pulp (24). Similarly, within the LN, LTi cells were found clustered at the marginal sinus and interfollicular spaces (Fig. 4B), the site where recirculating T cells enter from the afferent lymph (25). Thus, LTi cells are ideally placed to encounter recirculating memory CD4 T cells. Additionally, these locations are known to be rich in IL-7 expression (26), and the IL-7 signal is recognized as an essential part of CD4 memory survival (27). We have previously shown that in vitro, culture with IL-7 will increase OX40 expression on memory but not naive CD4 T cells (12). Culture with IL-7 also increased CD30L expression by LTi cells in vitro (10). Based on these data and our previous findings, we propose a simple model whereby memory CD4 T cells upregulate OX40 in response to IL-7 signals received as they re-enter lymphoid tissues, enabling them to engage OX40L expressed by LTi cells in the immediate vicinity.
Therefore, LTi cells can function as regulators of memory CD4 T cells, distinct from OX40- and CD30-controlled effector function driven by APCs (28). In summary, we show that a further function of LTi cells is the support of memory CD4 T cell survival. These data link the evolution of LN and CD4 memory in placental mammals with LTi cell functions.

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Disclosures
The authors have no financial conflicts of interest.

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SUPPLEMENTARY DATA

Supplementary Figure 1. Impaired memory antibody responses in RORγ−/− mice.

To investigate whether memory antibody responses were RORγ-dependent, RORγ−/− and +/+ littermates were immunised then challenged with NP-CγG.

A, Primary IgM (squares) and IgG (triangles) anti-NP response in RORγ+/+ (white) and RORγ−/− (grey) mice at day 7 post immunisation with 200μg alum-precipitated NP-CγG, assessed by ELISA. Results representative of 10 mice of each type. Bars show medians.

B, Sections of spleen from RORγ+/+ and RORγ−/− mice, initially immunised with NP-CγG, then challenged at day 30 post immunisation with 100μg NP-CγG and then analysed at 4 days post challenge; stained for IgG (green), IgM (blue) and anti-NP (red) expression by immunofluorescence. Images acquired at room temperature using Zeiss Metahead microscope with x10 objective and LSM software (Zeiss). Scale bar 100μm. Results representative of 2 independent experiments, 6 mice per group.

C, Quantitation of anti-NP specific IgG+ and IgM+ plasma cells (PCs) in sections of spleen from RORγ+/+ (white) and RORγ−/− (grey) mice 4 days after challenge with NP-CγG. Results representative of 2 independent experiments. Bars show medians.

Supplementary Figure 2. Generation of mice restricting RORγ expression to LTI cells.

To demonstrate that LTI cells were the RORγ expressing cell population required for memory CD4 T cell survival, chimeric mice were generated in which other RORγ-dependent populations were absent.

A, Expression of CD4+ and IL-7Rα+ on CD3−B220−CD11c− splenocytes isolated from CD3ε−ε− and CD3ε−ε− RORγ−/− (LTI) mice. Highlighted box marks LTI.
B, Reconstitution of irradiated LTi− mice with CD45.2+ CD3εtg26 BM restores donor splenic LTi (LTi+ mouse). Highlighted box marks LTi.

C, Expression of NK1.1 and NKp46 on CD3¢B220¢CD11c¢CD4¢IL-7Rα+ splenocytes from CD3εtg26 and Rag−/− mice. Plots representative of 3 mice.

D, Location of LTi in spleen sections from LTi+ versus LTi− mice. Expression of RORγ (green), IL-7Rα (yellow) and CD11c (blue), counterstained with DAPI (grey). Images acquired at room temperature using Zeiss Metahead microscope with x10 and x40 objectives and LSM software (Zeiss). Scale bar 50μM.

E, Expression of OX40L by LTi and DCs (CD11c+) from spleens of CD3εtg26 and CD3εtg26RORγ−/− mice. Plots representative of 3 mice.

F, Numbers of splenic LTi cells recovered from Rag−/−, Rag−/−RORγ−/+ and Rag−/−RORγ−/− mice 7 days post transfer of ~10⁴ SM1 T cell transfer and i.v. immunisation with Lm expressing FliC peptide. LTi cells were defined as non-SM1 B220CD11cCD3− cells expressing CD4 and IL-7Rα. Data points represent individual mice. Graphs show pool of 3 independent experiments.

G, Numbers of splenic SM1 TCR transgenic T cells recovered from Rag−/−, Rag−/−RORγ−/+ and Rag−/−RORγ−/− mice 7 days post transfer of ~10⁴ SM1 T cell and i.v. immunisation with Lm expressing FliC peptide. Data points represent individual mice. Graphs show pool of 3 independent experiments.

H, Numbers of LTi cells versus SM1 T cells recovered from spleens of Rag−/−, Rag−/−RORγ−/+ and Rag−/−RORγ−/− mice 7 days post transfer of ~10⁴ SM1 T cell transfer and i.v. immunisation with Lm expressing FliC peptide, r=0.9605 (Pearson Correlation test).
Supplementary Figure 1