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Thymic Function Is Maintained during Salmonella-Induced Atrophy and Recovery


Thymic atrophy is a frequent consequence of infection with bacteria, viruses, and parasites and is considered a common virulence trait between pathogens. Multiple reasons have been proposed to explain this atrophy, including premature egress of immature thymocytes, increased apoptosis, or thymic shutdown to prevent tolerance to the pathogen from developing. The severe loss in thymic cell number can reflect an equally dramatic reduction in thymic output, potentially reducing peripheral T cell numbers. In this study, we examine the relationship between systemic Salmonella infection and thymic function. During infection, naive T cell numbers in peripheral lymphoid organs increase. Nevertheless, this occurs despite a pronounced thymic atrophy caused by viable bacteria, with a peak 50-fold reduction in thymocyte numbers. Thymic atrophy is not dependent upon homeostatic feedback from peripheral T cells or on regulation of endogenous glucocorticoids, as demonstrated by infection of genetically altered mice. Once bacterial numbers fall, thymocyte numbers recover, and this is associated with increases in the proportion and proliferation of early thymic progenitors. During atrophy, thymic T cell maturation is maintained, and single-joint TCR rearrangement excision circle analysis reveals there is only a modest fall in recent CD4+ thymic emigrants in secondary lymphoid tissues. Thus, thymic atrophy does not necessarily result in a matching dysfunctional T cell output, and thymic homeostasis can constantly adjust to systemic infection to ensure that naive T cell output is maintained.

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Abbreviations used in this article: DN, double-negative; DP, double-positive; ETP, early thymic progenitor; HSA, heat stable Ag; LN, lymph node; RTE, recent thymic emigrant; sTREC, single-joint TCR rearrangement excision circle; SP, single-positive; STm, Salmonella enterica serovar Typhimurium; TREC, TCR rearrangement excision circle; WT, wild-type.

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The ability to respond to a diverse spectrum of pathogens throughout life requires the constant production of T cells in the thymus and their recruitment to secondary lymphoid tissues. Mature, naive T cells develop within the thymus from immature thymocytes, which themselves derive from early thymic progenitors (ETPs) previously recruited from the bone marrow (BM) (1–3). Thymocyte maturation requires trafficking of immature cells through the distinct anatomical cortical and medullary regions that contain specialized epithelial cell beds (4). This allows thymocytes to undergo sequential positive and negative selection to generate mature cells with diverse TCRs, which have limited self-recognition potential. Deleterious effects on the thymus that compromise its ability to generate naive T cells may have implications for responding to infections. This can be seen as the thymus ages, in which loss of thymic epithelial cells leads to reduced thymic output (5). During aging, thymic atrophy is commonly observed, as it is during immune responses to infectious diseases, including mycobacteria, HIV, parasites, and fungal infections (6–12). Although it is not fully clear why atrophy occurs so frequently during infection, a number of hypotheses have been proposed. These include that thymic atrophy may help reduce thymus activity and prevent the development of tolerance to the pathogen, or that atrophy is a common virulence mechanism shared by multiple pathogens impairing the capacity to respond to the organism. Infection-mediated thymic atrophy can often be linked to increases in apoptosis and subsequent cell loss in the double-positive (DP) thymocyte population, which make up ~80% of the total cell number in the thymus (8, 9, 11, 13). Thus, thymic atrophy is a consequence of many infections in humans and animal models.

Although it is clear that infection induces thymic atrophy, it is unclear what the consequences of this are for the host, and whether the thymus rebounds as infection resolves and if there is a consequence on the regulation of peripheral T cell numbers. For instance, our work has previously shown that there is rapid T cell priming resulting in an accumulation of naive CD4 T cells in the spleen during infection with Salmonella enterica serovar Typhimurium (STm) despite there being similar levels of cell death in naive CD4 T cells before infection and postinfection (14–16). Furthermore, in intact or chimeric CD31-deficient or CD30/OX40 double-deficient animals, in which T cell activation results in T cell death, there appeared to be some continual recruitment of naive CD4 T cells to secondary lymphoid tissues (14, 15). This appears to be paradoxical, with the concept of infection-induced thymic atrophy resulting in a loss of T cell development.
This apparent incongruity led us to examine the effect of STm infection on the thymus and mature T cell production. We demonstrate that systemic, but not local, STm infection results in rapid thymic atrophy. Loss of cellularity is primarily, but not exclusively, in the DP population, and thymocyte numbers recover as bacterial infection resolves. Surprisingly, during both thymic atrophy and recovery, recent thymic emigrant (RTE) numbers appear largely unperturbed. Moreover, ETP show enhanced intrathymic proliferation that precedes the recovery in thymic cellularity. In summary, our data defines the dynamic changes in thymocyte development, thymic recruitment, and output during a systemic bacterial infection and show that, despite a 95% fall in thymocyte numbers, thymic atrophy in response to STm infection does not comparably impede the capacity of the thymus to generate and export newly selected CD4+ T cells.

Materials and Methods

**Mice, bacteria, and immunization protocols**

Experiments were performed with local and Home Office approval. Wild-type (WT) mice were obtained from Harlan. Mice were infected s.c., i.p., or i.v. with 5 × 10^5 live attenuated STm strain SL3261, as described previously (14, 15, 17). As indicated, at defined time points, spleen, thymuses, and peripheral lymph nodes (LN) were taken and analyzed by flow cytometry and for bacterial infection. Where required, bacteria were killed by heating at 70°C for 1 h. Killing was confirmed by lack of growth of bacteria on agar plates, as described (18).

**Flow cytometry**

Single-cell suspensions were prepared, as previously described (15), and containing erythrocytes were lysed using ammonium chloride buffer, where appropriate. Thymocytes were stained with CD8α-PE, FITC, CD44-PE, TCRβ-PerCP-Cy5.5, CD25-PE-Cy7, CD4-allophycocyanin, CD45-allophycocyanin-eFluor780 (all eBioscience), and the dead cell marker Sytox Blue (Molecular Probes). CD4 single-positive (SP) thymocytes were phenotyped using CD4-eFluor450 and CD8α-allophycocyanin in combination with Q2-FITC, CD24-PE, CD62L-biotin, CD69-PE-Cy7, and CD127-allophycocyanin-eCy5, or appropriate isotype controls (all eBioscience). Biotin Abs were visualized with streptavidin PE-Texas Red (BD Biosciences). ETP were identified using a lineage dump gate (CD8α, CD8β, TCRα, TCRβ, NK1.1, CD11b, Gr-1, and B220 all on FITC) with CD44-PE, CD62L-biotin, CD25-PE-Cy7, CD127-Allexa Fluor 647, and CD117-allophycocyanin-eFluor780 (all eBioscience). All cell populations were assessed using a CyAn ADP flow cytometer (Beckman Coulter).

**Measurement of BrdU uptake by ETP after STm infection**

Mice were pulsed with 1 μg BrdU (BD Biosciences) i.p. 2 h before sacrifice. Thymuses were disaggregated, and immature thymocytes were enriched by depleting cell suspensions using anti-CD4 magnetic beads (Miltenyi Biotec). The percentage of ETP incorporating BrdU was quantified by flow cytometry using the BrdU Flow Kit (BD Biosciences) in combination with surface staining for antilineage mixture-FITC, CD44-PE, CD117-allophycocyanin-eFluor780, and CD25-PE-Cy7 (all eBioscience).

**Immunohistochemistry and confocal microscopy**

Cryofrozen sections of thymic lobes were stained with H&E using standard methodologies. Immunofluorescence staining was performed on 6-μm-thick sections. Mature thymocyte populations were visualized using anti-CD8α biotin and anti-CD4 Alexa Fluor 647 (both eBioscience). Stromal populations were detected using anti-mouse BST (MBL International) amplified with anti-rabbit biotin (Dako) and anti-mouse ERTR5 (gift of W. van Ewijk, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) visualized with anti-rat IgM Alexa Fluor 647 (Molecular Probes, Invitrogen). Streptavidin-Allexa Fluor 555 (Molecular Probes, Invitrogen) was used to visualize biotinylated Abs. Images were captured using either a Leica DM6000 microscope (10× objective [Leica Microsystems], image analysis performed using QCapture software and ImageJ software) or a LSM 510 Meta microscope (10× objective; image analysis performed using Zeiss LSM software [Carl Zeiss]).

**Quantification of TCR rearrangement excision circles containing RTEs**

Purified population of CD4 T cells was isolated by sorting CD3- and CD4-labeled cells using a MoFlo high speed cell sorter (Beckman Coulter) and sorted to >99% purity. Cells were then prepared for PCR by digestion with proteinase K, as previously described (19). A real-time quantitative PCR assay to detect signal-joint TCR rearrangement excision circle (sjTREC) sequences was developed based on methodology from Broers et al. (20). The PCR was performed in 25 μl vol (5 μl cell digest [50,000 cells per reaction] with 2X PCR Master Mix [Applied Biosystems], 900 nM forward and reverse primer, and 200 nM probe). All reactions were performed in duplicate, run on a MX300P quantitative PCR machine, and analyzed using MxPro software (both Stratagene). PCR conditions and primer and probe sequences (all Eurofins, MWG-Biotech) used are described in Supplemental Fig. 2. To calculate the number of sjTREC sequences present in each sample, a murine TCR rearrangement excision circle (TREC) standard was generated by cloning a 262-bp fragment of TREC DNA from the signal-joint region of murine thymus CD4SP cells, as previously described (21). Stock dilutions of 10^-2-10^-1 molecules of TREC in 5 μl were prepared and stored in aliquots at −80°C containing yeast RNA at 30 ng/ml. Each dilution was run in duplicate to generate a standard curve for each individual real-time PCR run, and curve fits had to have a R^2 ≥ 0.995 to be acceptable.

**OP9 stromal ETP cocultures**

To assess the impact of infection on the proliferation potential of ETP, cells were sorted from STm-infected thymuses from CD45.2 mice or PBS controls from CD45.1 controls using a MoFlo high speed cell sorter (Beckman Coulter) using the gating strategy defined (22). ETP were collected in media, and cocultures of 10 cells/well were seeded on OP9-DL1 cell monolayers in 48-well plates, as previously described (23). In some wells, 10 ETP from infected and control congenic donors were mixed at 1:1 ratio before seeding onto stromal cells. All cultures were analyzed by flow cytometry after 14 d.

**Statistics**

Statistics were calculated using the nonparametric Mann-Whitney sum of ranks test. The p values were calculated using Prism5 (GraphPad Software), and the p values ≤0.05 were accepted as significant.

**Results**

Salmonella infection induces thymic atrophy

To assess the impact of *Salmonella* on the thymus, mice were infected i.p. with 5 × 10^5 STm, and bacterial burdens were assessed in the spleens and thymus at intervals postinfection (Fig. 1A). Bacterial numbers peaked in the spleen before gradually falling over the following weeks in a T cell-dependent manner. Surprisingly, bacteria were also found in the thymus from 48 h postinfection, and persisted at a low level between 3 and 4 wk (Fig. 1A and data not shown). Concurrently, STm infection induced splenomegaly, with increases in total cell and CD4 and CD8 T cell numbers (Fig. 1B). Furthermore, this increase in T cell numbers was not only due to an increase in effector T cells (CD62Llow), but also due to an increase in naive CD4 and CD8 T cells (CD62Lhigh). We have previously shown that CD62L expression on naive T cells is a robust marker to discriminate between naive and effector T cells (14, 15, 18). In contrast, infection-induced thymic atrophy occurred from 48 h, and resulted in >90% loss of thymocytes by day 21 (Fig. 1C). After 21 d, when bacterial clearance is well established, thymus size and cellularity start to recover, reaching preinfection levels by day 35 when splenic bacterial levels are <1% of peak levels (Fig. 1B, 1C and data not shown). This loss of cellularity reflected a marked increase in apoptosis of total CD4^+ thymocytes (Fig. 1D) and is in stark contrast to the splenomegaly observed during this infection (Fig. 1B).

**Systemic STm infection is required to induce thymic atrophy**

To assess whether atrophy was a response to exposure to the multiple innate ligands that bacteria express, we immunized mice with live bacteria and killed bacteria for 7 d. Atrophy was entirely dependent on bacterial viability, as there was no atrophy after immunization with heat-killed bacteria (Fig. 2A). Next, we assessed the impact of bacterial virulence on thymic cellularity and apo-
ptosis by immunizing with STm of differing virulence for 24 h. Both attenuated and virulent STm induced atrophy, although it was more pronounced in mice infected with the more virulent SL1344 strain (Fig. 2B). Thymic atrophy was not dependent upon IFN-γ production by any cell type, Th1 differentiation, or even the presence of peripheral T cells because mice lacking IFN-γ, T-bet, or ZAP70 were equally susceptible to thymic atrophy (see Table I). Indeed, thymic cellularity was reduced in Rag1-deficient mice, indicating that involution occurs in the absence of mature thymocyte populations. Lastly, elevated levels of glucocorticoids have been previously demonstrated to induce thymic atrophy (24, 25). Selective loss of hexose-6-phosphate dehydrogenase and 11β-hydroxysteroid dehydrogenase type 1-converting enzymes, which results in elevated glucocorticoid levels (26, 27), did not prevent STm-mediated thymic atrophy, as found in other systems (28). Thus, the induction of thymic atrophy after STm infection may not be dependent on these enzymes, possibly because of enzyme redundancy or other reasons.

We then examined whether thymic atrophy was dependent upon the infection being systemic or local. Mice were infected s.c. with \(5 \times 10^5\) STm or PBS locally in the foot. In this case, the bacterial infection is largely restricted to the draining popliteal LN with bacterial colonization of the spleen occurring at levels 100- to 1000-fold lower than after i.p. immunization with the same number of bacteria and absent colonization of the thymus (data not shown). In this case, infection did not result in thymic atrophy, demonstrating systemic dissemination and/or colonization of the thymus by the pathogen are required (Fig. 2C). Immunization through the i.v. route showed similar levels of thymic atrophy as i.p. infection and similar levels of colonization by bacteria (representative colonization levels shown in Fig. 1A, and thymus mass and cellularity in Fig. 2D). Therefore, thymic atrophy is a consequence of systemic infection rather than necessarily reflecting the bacterial burden in the host.

**Infection-induced atrophy affects all mature thymocyte subsets**

Thymocytes develop from immature CD4 and CD8 double-negative (DN) cells to mature CD4SP and CD8SP via a CD4 and CD8 DP intermediate. Assessment of mature thymocytes in the four main populations (DN, CD45CD4-CD8-TCRβ-; DP, CD45CD4+CD8-TCRβlow; CD4SP, CD45CD4+CD8-TCRβhigh; CD8SP, CD45CD4-CD8-TCRβhigh) was enumerated using flow cytometry (gating strategy outlined in Supplemental Fig. 1A). Up to day 21 postinfection, when thymic atrophy is most pronounced (Fig. 1C), all mature CD45+ thymocyte populations are reduced (Fig. 3A). The greatest impact was upon the DP population, which accounts for ~85% of all thymocytes in noninfected mice, in which ~95% of these DP cells were lost, accounting for the majority of the fall in thymus cellularity. In contrast to other pathogens (29, 30), STm also impacted upon DN and the mature CD4SP and CD8SP numbers in the thymus. Furthermore, analysis of the DN subsets (DN1–4) (31, 32) (gating strategy outlined in Supplemental Fig. 1A) showed a significant reduction in all of the CD44/CD25 DN subsets (Fig. 3B). Therefore, thymic atrophy impacts upon all CD45+ thymocyte subsets in the thymus. This loss of thymocyte numbers reflects increased apoptosis levels in the most immature DN and DP populations, whereas in the SP populations levels of apoptosis are only marginally increased (Fig. 3C).
FIGURE 2. Thymic atrophy requires systemic dissemination of live bacteria. (A) Mice were immunized for 7 d with $5 \times 10^8$ heat-killed (H.K. STm) or live STm, and thymus cellularity and mass were assessed. (B) Mice were infected i.p. for 24 h with either PBS, $5 \times 10^8$ attenuated STm strain SL3261 (as used in all other experiments), or $5 \times 10^8$, virulent SL1344 STm. Induction of thymic atrophy was assessed by measuring thymic mass, cellularity, and levels of apoptosis in thymocytes (as described in Fig. 1D). (C) Mice were infected s.c. in the footpad with $5 \times 10^5$ STm and contralateral foot with PBS, and popliteal LN and thymic mass were recorded as well as thymic cellularity. (D) Thymic mass and cellularity in mice infected with STm i.p. or i.v. for 7 d. Graphs show mean ± SD of four mice per group and are representative of data from at least two independent experiments; *p ≤ 0.05 or NS compared with PBS or noninfected (n.i.) controls.

From the nadir at 21 d postinfection, thymic regeneration occurs rapidly so that recovery of all mature CD45$^+$ thymocyte subsets is seen by day 35 and detectable by 28 d postinfection (Fig. 3A, 3B). Recovery at day 35 is concomitant with reduced apoptosis in all populations compared with day 21 (Fig. 3C). Thus, STm infection reveals that thymic atrophy is a reversible, dynamic process.

**CD4 T cell maturation is maintained during thymic atrophy**

Naive CD4 T cell numbers in secondary lymphoid tissues, such as the spleen, increase postinfection, leading to an accumulation of RTEs in the periphery (Fig. 5). This bias in mature CD4SP T cells is highlighted when the relative proportion of total CD4SP T cells of immature or mature phenotype in the population is assessed (Fig. 3E). This shows that, before infection, between 60 and 70% of CD4SP T cells are of an immature phenotype and between 20 and 30% are mature. By 21 d postinfection, the situation has reversed, so that now ~60% of CD4SP are mature and 20% immature. This suggests that mature CD4SP thymocyte numbers are less affected by thymic atrophy during infection.

**Medullary and cortical organization is not impaired during infection**

Efficient maturation of T cells in the thymus requires sequential transit of cells through the thymic medulla and cortex. Some parasitic (e.g., Trypanosoma cruzi, Plasmodium berghei) and viral (e.g., HIV, measles) infections can disrupt the organization of these distinct anatomical regions (9, 11–13, 33). In contrast, STm infection does not alter gross thymic architecture, as shown by confocal microscopy using H&E (Fig. 4A) or immunofluorescence staining (Fig. 4B; medullary, anti-ERTR5, blue; cortical, anti-βST, red). In addition, DP, CD4SP, and CD8SP thymocytes were localized in the correct anatomical regions at all time points studied (Fig. 4C; CD4, red; CD8, green). Therefore, thymic atrophy is not a consequence of disrupted organization of the cortical and medullary epithelium.

**Naive CD4 T cell output is maintained during thymic atrophy**

The accumulation of naive CD4 T cells in secondary lymphoid tissues after STm infection and the differential loss of immature and mature CD4SP thymocytes suggested that CD4 T cell release to the periphery may be maintained during infection. To assess this, we examined numbers of RTEs before infection and postinfection. This was done by quantifying the numbers of sjTREC containing peripheral CD4 RTEs in secondary lymphoid tissues (Fig. 5). This found that, despite there being a 50-fold loss in total thymocytes, numbers of sjTREC RTEs in the spleen containing T cells at the nadir of thymic atrophy were only 4-fold lower and had recovered by day 35. In other sites (mesenteric LN and axillary LN), numbers remained similar to noninfected or actually increased during infection. A fall in the proportion of CD4 T cells that were sjTREC was also seen in the spleen, but not in the LN. In the spleen, this fall may have been exaggerated because RTE may be

| Table I. STm-induced thymic atrophy in gene-deficient mice |
|-----------------|-----------------|-----------------|-----------------|
| KO Strain       | Thymus Cellularity ($\times 10^5$ Cells per Tissue) | p Value |
| WT              | PBS             | STm             |                 |
| T-bet           | 8.35 ± 3.87     | 2.14 ± 0.57     | 0.05            |
| IFN-γ           | 18.8 ± 2.77     | 5.88 ± 0.81     | 0.001           |
| ZAP70           | 10.8 ± 1.83     | 2.26 ± 1.09     | 0.01            |
| Rag1            | 0.08 ± 0.001    | 0.0325 ± 0.008  | 0.01            |
| H6P DH/11β-HSD1 | 9.87 ± 1.96     | 4.49 ± 1.67     | 0.001           |

*WT and the genetically altered mouse strains shown were infected i.p. with $5 \times 10^8$ STm for 7 d. Thymic cellularity was measured and compared with PBS controls. Data are shown as mean ± SD (n = 4 mice per group and is representative of at least two independent experiments).

H6P DH, Hexose-6-phosphate dehydrogenase; 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1.
recruited into the response and also because total CD4 T cell numbers increase (Fig. 1B), through it being a major site of T cell priming and expansion. By day 21, up to 80% of CD4 T cells have a CD62Llow effector phenotype (15), and together these effects are likely to result in a reduction of sjTREC RTEs in the spleen (34). Nevertheless, in combination with the data from examining RTE numbers and proportions in the LN, it indicates that CD4 T cell RTE migration to secondary lymphoid tissues is maintained during both thymic involution and regeneration.

Thymic atrophy does not ablate ETP numbers during infection
Thymic T cell production requires the recruitment of progenitors from the BM, as the adult thymus does not contain a population of self-renewing T cell progenitors (2, 3). The earliest of these progenitors are ETPs, and the development of mature T cells from these precursors is estimated to take ∼14 d (35). We examined how infection influences the numbers of ETPs in the thymus. By using the gating strategy outlined, numbers of ETPs were quantified (Fig. 6A) and presented as either the total number per thymus or as a proportion of the total DN1 subset. This showed that, although thymocyte numbers fall dramatically postinfection (Fig. 1), numbers of ETPs are affected to a much lesser extent, and do not fall below one-third of ETP numbers found in noninfected mice (Fig. 6A). When ETPs are examined as a proportion of the earliest DN1 thymocyte population, then proportions are either similar to or greater than those of nonimmunized mice. Importantly, between day 14 and 21 postinfection there is a significant increase in this proportion (Fig. 6A). This increase occurs prior to the thymus reconstitution, which is well established by day 28 (see Fig. 1). ETP are induced to proliferate as they enter the thymus (2). Therefore, we assessed levels of proliferation in ETP after a 2-h pulse in PBS-immunized mice and mice infected for 14 d. This showed there was a near doubling in the proportion of ETP that were BrdU+ in the infected mice (Fig. 6B). Thus, ETP numbers are substantially less impacted upon by infection than other thymocyte populations.

To examine whether infection altered the capacity of ETP to proliferate after exposure to appropriate stimuli, ETP were sorted from congenic mice that had been immunized with either PBS (CD45.1) or STm infected for 10 d (CD45.2). After sorting, ETP

FIGURE 3. Effects of STm infection on distinct thymocyte populations. Mice were infected with 5 × 10^5 STm i.p., and responses were evaluated at discrete times postinfection. (A) Total numbers of thymocytes in the four major thymocyte populations (based on the expression of CD4, CD8, and TCRβ) were quantified by flow cytometry (CD8SP were gated on TCRβ^high cells to exclude DN cells maturing to the DP stage). (B) Thymocyte numbers in the four DN subsets (DN1–4) were defined using CD44 and CD25 and quantified by flow cytometry. (C) Apoptosis in the four major thymocyte subsets postinfection was quantified by Sytox Blue uptake and flow cytometry. (D) The effect of STm infection on CD4SP and CD8SP maturation was quantified by flow cytometry using markers to distinguish total numbers of immature (Qa2^lowCD69^highHSA^highCD62L^low) and mature (Qa2^highCD69^lowHSA^low CD62L^high) cells per thymus. (E) The proportion of immature and mature cells in the total CD4SP pool per thymus was assessed. Graphs show mean ± SD of at least four mice per group (*p ≤ 0.05 or NS compared with noninfected [n.i.] controls) and are representative of data from three independent time courses in (A)–(C) and two in (D) and (E).
were seeded onto confluent OP9-DL1 cells in three groups and cultured for 14 d before total numbers of CD45^+ cells were assessed by flow cytometry. The first group contained 10 ETP from PBS-immunized mice, the second group 10 ETP from STm-infected mice, and the third group 10 ETP from both groups together. These experiments (Fig. 6C) showed that ETP from STm-infected mice could expand to similar levels as ETP from PBS-immunized mice when cultured alone or together.

Finally, we examined the role of the ETP migration receptor CCR9 and its ligand CCL25 during thymic involution and recovery, as these molecules can regulate aspects of ETP recruitment and thymopoiesis (36–38). Infection of WT, CCR9, or CCL25-deficient mice revealed that all genotypes atrophied similarly by day 14 (Fig. 6D). During the recovery phase at day 35, all groups had increased thymocyte numbers, although the recovery was slightly lower at this time in the absence of CCR9 or CCL25. Therefore, thymic atrophy and recovery after STm infection are not dependent upon CCR9 or CCL25.

Discussion

This study demonstrates, using a systemic, resolving model of STm infection, that infection results in thymic atrophy, with a >95% decrease in total thymocyte numbers (Fig. 1). Thymic atrophy is reversible, with cellularity recovering to levels comparable to those preinfection. Atrophy was most prominent in the immature thymocyte subsets. In the CD4SP population, numbers of mature CD4SP were largely maintained, whereas immature CD4SP numbers were more profoundly affected (Fig. 3D, 3E). Despite such a profound impact on thymocyte numbers, RTE numbers in secondary lymphoid tissues were much less affected (Fig. 5). This contrasts to events observed postinfection with other pathogens, in which loss of predominantly DP thymocytes can lead to reduced thymic output (13, 39, 40), or in which there is premature release of thymocytes at the DP stage (41). Such a release of immature cells is not a feature of STm infection (data not shown). This suggests that thymic atrophy may be a common feature in response to pathogens, but that the consequences on thymic function may differ markedly during different infections.

In the current study, we show that the distinct thymic cortex and medullary anatomical microenvironments were maintained, thus providing the required environment for T cell maturation (Fig. 4). In other infections, such as parasitic (e.g., Trypanosoma cruzi, Plasmodium berghei) and viral (e.g., HIV, measles) infections, this is not the case (9, 11, 13, 33, 42). The current study indicates that persistent thymic atrophy and loss of T cell production is not an inevitable consequence of...
systemic infection, countering the concept that thymic atrophy is a strategy employed to prevent pathogen tolerance from developing. This is important, as it suggests that age-related diminished thymic output may not simply reflect the accumulated effects of multiple systemic infections experienced over a lifetime. Furthermore, the study of how thymic rebound is controlled postinfection may provide significant insights into how thymic output can be improved in humans. Thymic atrophy occurred irrespective of the presence of peripheral T cells, or of the capacity of peripheral CD4 T cells to prime or differentiate to Th1 effector cells in response to STm and occurred normally in the complete absence of IFN-γ (Table I). Therefore, the control of thymic atrophy is not likely to relate to events in the secondary lymphoid tissues, suggesting there may not be feedback mechanisms involved in controlling thymic atrophy. This indicates that atrophy during STm infection is likely to be an intrinsically thymus-regulated event. In other situations, such as after high-dose (250 μg) poly:IC, thymic atrophy can be induced through the induction of IFN-α (43). Although IFN-α is not typically associated with bacterial infection, it can be induced in the spleen after STm and can have marked effects on cellular function (44). Nevertheless, these levels are only modestly greater than nonimmunized mice, and there appears to be some site variability to its induction. Thus, although identifying the factors that drive thymic atrophy was not a key aim of this study, future work will examine whether IFN-α influences thymic atrophy after STm infection. If so, it would be intriguing because it offers a common mechanistic through which bacteria and viruses can induce thymic atrophy.

The event most associated with thymic rebound was the change in ratio of ETP to DN1 thymocytes (Fig. 6A), and this increased ETP proportion was accounted for, at least in part, by increased proliferation in the thymus (Fig. 6A). Indeed, during thymic atrophy, ETP numbers were much less affected than mature thymocyte numbers and ETP retained the capacity to proliferate in vitro in response to coculture with Notch ligand (Fig. 6A). Although thymic atrophy and recovery were largely independent of CCR9 and its ligand CCL25 (Fig. 6D), this may have been because of the existence of alternative receptor/ligand pairing compensating for these gene deletions. This concept is supported by recent reports using mice deficient for both CCR7 and CCR9 (45, 46). The earliest increase in ETP:DN1 ratio in the thymus was after day 14 postinfection, reflecting the detection of increased mature T cells in the thymus seen at day 28 postinfection, because it takes ∼2 wk for ETPs to develop into mature T cells (35). The persistence of ETP numbers in the thymus is surprising considering the impact infection has been reported to have on BM progenitor homeostasis. Postinfection with the malaria parasite *Plasmodium chabaudi* (47) or *Mycobacterium avium* (48), there are significant transient increases in the stem cell compartment, which can result in contraction of their downstream progeny and the emergence of atypical progenitors. In contrast, after viral infection, a prolonged reduction in BM progenitor numbers is observed (49). Interestingly, STm infection can have marked effects on the production of other cell types. For instance, over time infection causes an increase in myeloid cells such as neutrophils and monocyte lineages, which can persist when infection is cleared.

**FIGURE 6.** Infection does not ablate numbers of ETPs. (A) ETPs were quantified by flow cytometry using the gating strategy shown, and total numbers of ETPs in the thymus (left) and the proportion (right) of ETPs relative to the DN1 subset during infection are shown. (B) The percentage of ETPs in cycle at 14 d postinfection as assessed by BrdU incorporation and flow cytometry. (C) ETPs were purified by cell sorting from STm-experienced (10 d post-infection) or control (PBS-immunized) mice and cocultured in vitro with OP9-DL1 stromal cells either alone or mixed together at 1:1 ratio. After culturing for 14 d, expansion of CD45+ cells was enumerated by flow cytometry. (D) CCR9KO and CCL25KO mice were infected i.p. with 5 × 10⁵ STm, and thymic mass and cellularity were assessed at 14 and 35 d postinfection. Graphs shown in (A), (B), and (D) are mean ± SD (n = 4 mice per group), and are representative of at least two independent time course experiments. Data shown in (C) are combined from two independent experiments. *p ≤ 0.05 or NS compared with PBS or noninfected controls.
(16, 50). Furthermore, there is a marked shift of erythropoiesis from the BM to the spleen, which accounts for a large part of the splenomegaly, a signature event of primary, systemic murine salmonellosis (49, 51). This reflects an increase in serum concentrations of the CSF erythropoietin postinfection (51). Furthermore, infection results in rapid increases in myeloid cell types, such as macrophages and neutrophils, in the spleen and BM, probably through emergency myelopoiesis, that for some infections can require IFN-γ, but not IFN-α (50, 52–54). Interestingly, the splenomegaly observed in this model of infection is reversible (50, 55), similar to the thymic atrophy, because erythropoiesis reverts to the BM once STm infection has largely resolved. In this study, numbers of myeloid cells in the thymus did not increase radically postinfection, although this does not discount the possibility that there were changes in the subsets present. This may indicate that, although this organ is colonized by the bacterium, it does not result in the marked cellular infiltrate seen elsewhere postinfection-induced myelopoiesis.

The most surprising finding from this work was that T cell production and release from the thymus persisted throughout infection, despite thymic atrophy. Thus, whereas thymocyte numbers were nearly 30-fold lower at their nadir, numbers of RTEs as measured using sjTREC analysis were only reduced 3-fold (Fig. 5). This is also reflected in the reduced impact infection had on numbers of mature CD4SP cells in the thymus postinfection, which were largely stable or only reduced by 2-fold during the course of the infection. This supports our original findings in CD31KO or CD30/OX40 double-deficient mice in which there is defective T cell survival after STm infection, yet there are near normal numbers of naive T cell numbers, suggesting there must be renewal of the peripheral T cell pool from the thymus. Furthermore, as we have shown previously and in this study, there is a rapid accumulation of naive CD4 T cells in WT mice postinfection (15). This presumably functions to allow a greater T cell repertoire to enhance the probability of a productive T cell response being primed. RTEs are the only source of new T cell diversity, and have been shown to proliferate and replenish the peripheral T cell pool during times of stress (56). This suggests the increased naive T cell pool seen postinfection may reflect the increased level of RTE released into the periphery. In this model of STm infection, up to 80% of all CD4 T cells become activated at the peak of the response, as measured using CD44 and CD62L. Moreover, virtually all of the IFN-γ cytokine signal derives from the CD62Llow population, with <1% of CD4 T cells having a CD62LhighCD44high phenotype (15). This suggests that the maintenance of a large, activated CD4 T cell pool is important for the host’s capacity to control STm infection, and the progenitors for this response must be continuously renewed.

Therefore, our work suggests that during our lifetime and constant exposure to pathogens, the thymus may be constantly adapting, but will also give new insights into strategies for promoting infection induces thymic atrophy, followed by recovery. Therefore, investigating how the thymus is altered upon constant exposure to pathogens will be key to not only understanding how the thymus adapts, but will also give new insights into strategies for promoting and extending the functional life span of the thymus and thus our immune surveillance capacity as we age.

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