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Suspension of thymic emigration promotes the maintenance of antigen-specific memory T cells and the recall responses

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ABSTRACT

Thymic involution is evolutionarily conserved and occurs early in life. However, the physiological significance remains elusive of this seemingly detrimental process. The present study investigated the potential impact of altered thymic output on T cell memory using ovalbumin (OVA) expressed by *Listeria monocytogenes* as a model antigen. Suspension of thymic emigration by thymectomy was shown to lead to a marked increase in the frequency and total number of OVA-specific memory T cells. In contrast, oversupply of thymic emigrants through thymic grafting caused a significant decrease of such cells. When rechallenged with *L. monocytogenes* expressing OVA, the thymectomized mice mounted a more potent recall response as evidenced by the enlarged population of OVA-specific tetramer$^+$ cells and the accelerated clearance of the bacteria. Notably, the memory-enhancing effect of thymectomy was abrogated following weekly adoptive transfer of naive T cells. Together, data from the present study indicate that reduced thymic output favors the maintenance of the memory T cell pool.

1. Introduction

The thymus is the primary site for T-lymphopoiesis. With the support of signals derived from thymic epithelial cells (TEC), hematopoietic progenitors unfold an intrinsic developmental program, leading to the generation of a T cell repertoire capable of responding to a diverse array of foreign antigens but tolerant to self antigens [1–3]. Recent thymic emigrants (RTE) which survive positive and negative selection undergo a process of post-thymic maturation [4], and are incorporated into the existing T cell pool by replacing resident T cells [5,6].

The continuous supply of new naive T cells by the thymus is believed to be important for the maintenance of a diverse T cell repertoire. However, the thymus starts to undergo progressive regression since childhood [7,8]. This process – known as thymic involution – is characterized by reduced tissue mass, decreased cellularity, disorganized epithelial structure, expanded perivascular space and lessen production and exportation of naive T cells. Despite the reduced input from the thymus, the peripheral T cell pool is maintained at a relatively constant level [9,10]. This is primarily achieved through homeostatic proliferation and enhanced survival of peripheral T cells [10–12]. However, an increasing dependence on homeostatic mechanisms in the absence of new thymic emigrants for maintenance of the peripheral pool leads to a reduced diversity of the TCR repertoire and the accumulation of functionally impaired T cells in the periphery [12–14]. The elderly individuals are thereby rendered more susceptible to infections, autoimmune diseases and cancers [15–17].

As a major contributor to the age-related decline in immune function [18], thymic involution is generally viewed as an entirely undesired process. Paradoxically, it occurs in most, if not all, vertebrates that possess a thymus, indicating that this is an evolutionarily conserved event [19]. Moreover, although the thymus reaches its maximum size around puberty, its productive volume begins to shrink from as early as the first year of human life, which is apparently much earlier than most acknowledged features of aging [20,21].

It is interesting to note the increased presence of T cells of an activated or memory phenotype in the periphery during aging or following thymectomy [22,23]. Among many other possibilities, we speculate that this could represent a genuine increase of antigen-specific memory T cells as the result of reduced input from the thymus. This hypothesis was tested by monitoring the impact of altered thymic output on T cell memory to a model antigen – ovalbumin (OVA). The thymectomized mice showed an elevated level of antigen-specific memory T cells and mounted a more

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vigorous recall response when rechallenged with the same antigen, implying that thymic involution is physiologically relevant for the maintenance of T cell memory.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from the Vital River Laboratories (Beijing, China) and maintained in the animal breeding facility at Peking University Health Science Center under specific pathogen-free conditions. Timed pregnancies were established to obtain fetal thymi at day 15.5–16.5 of gestation. The experimental procedures on use and care of animals had been approved by the ethics committee of Peking University Health Science Center.

2.2. Infection with Listeria monocytogenes

L. monocytogenes expressing the full length ovalbumin protein (LM-ova) was a kind gift from Dr. Qibin Leng, Institute Pasteur of Shanghai. A single colony was inoculated into brain–heart infusion broth (BD Biosciences) and cultured overnight at 37 °C. The culture was diluted at 1:20 into fresh medium and allowed to grow for another 4–5 h to an optical density of 0.8–1.1 at 600 nm. Mice received intravenous injection of $2 * 10^4$ CFU for primary infections and $5 * 10^5$ CFU for secondary infections.

2.3. Thymectomy and thymic grafting

Three weeks after primary infection with LM-ova, mice were thymectomized, sham-operated, or implanted with multiple fetal thymi under sodium pentobarbital anesthesia. For thymectomy, a midline incision of the sternum exposed the thymic lobes, and the thymus was then removed by suction. Sham operation followed the same procedure, but without removal of the thymus. Thymic grafting was performed as previously described [24]. Ten fetal thymic lobes were implanted under the capsule of the left kidney.

2.4. Interferon-γ enzyme-linked immunospot (Elispot) assay

The release of IFN-γ by activated $CD8^+$ or $CD4^+$ T cells was measured by Elispot assay. Plates (Millipore MAHAN4550; Millipore, Bedford, MA) were coated overnight with anti-IFN-γ capture Abs (5 μg/mL, AN/8, Mabtech, Stockholm, Sweden). After blocking with 1640 with 10% FSC for 2 h at 37 °C, total splenocytes or lymph node cells containing $5 * 10^4$ $CD8^+$ or $CD4^+$ T cells were added to each well and pulsed with $OVA_{257–264}$ (SIINFEKL) or $LLO_{190–201}$ (NEKYAQAYPNVS) (1 μg/mL) (Chinese Peptide, Hangzhou, China). After incubation for 20 h at 37 °C, cells were removed and the plates were developed with the biotinylated detecting Ab to IFN-γ (1 μg/mL, R4-6A2-Bidin, Mabtech) and streptavidin-alkaline phosphatase (1 μg/mL, Mebtech). Spots were revealed using BCIP/NBT (Sigma) and counted using a computer-assisted video image analyzer (Sage Creation, Beijing, China). The average number of spots

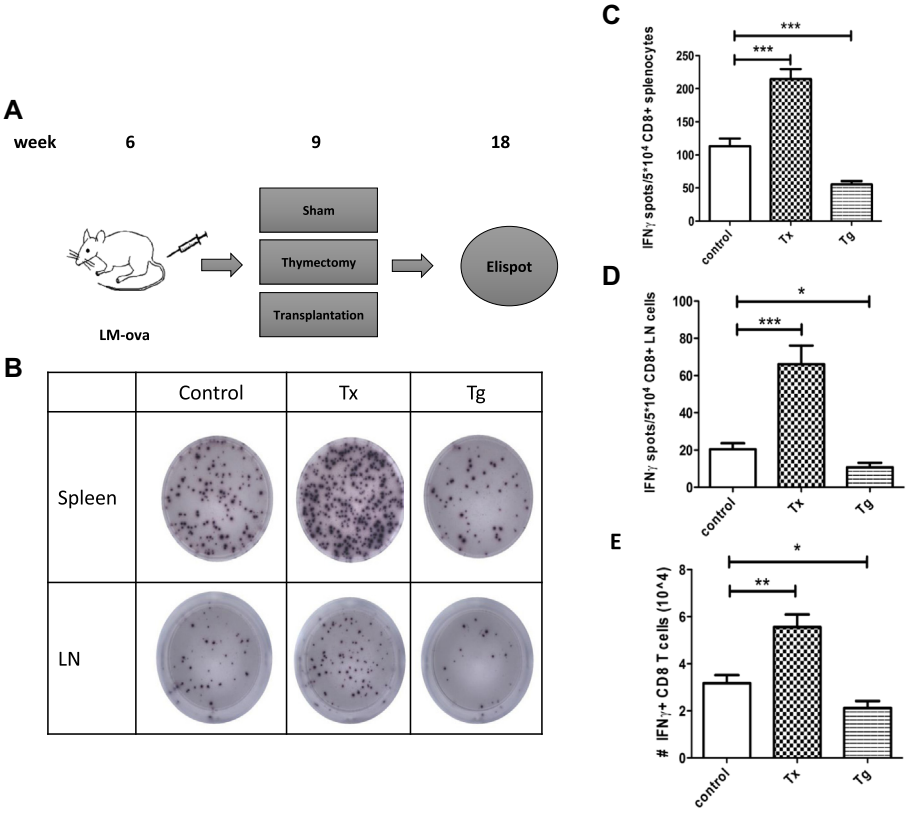

Fig. 1. OVA-specific $CD8^+$ memory T cells following manipulation of thymic output. (A) C57BL/6 mice were infected with LM-ova at week 6, underwent thymectomy (Tx), thymic grafting (Tg) or sham operation (Control) at week 9, and examined for the frequency of OVA-specific $CD8^+$ memory T cells using Elispot assay at week 18. (B) Elispot assay was performed to detect OVA257-264 peptide-specific, IFNγ-producing cells. Representative results are shown for cells obtained from one mouse of each group. (C–E) The frequencies of IFNγ-producing cells per $5 * 10^4$ $CD8^+$ T cells were calculated based on the percentage of $CD8^+$ T cells in spleen (C) and lymph nodes (D). The total number of OVA-specific $CD8^+$ memory T cells were calculated as the number of such cells in the spleen plus twice the number of these cells in the lymph nodes (pooled from mesenteric, inguinal, and axillary nodes) (E). $n = 15, 17, 15$ for the control, thymectomy and thymic grafting groups, respectively.

in duplicate wells was calculated and expressed as the number of spot forming cells/5 * 10^4 $CD8^+$ or $CD4^+$ T cells.

2.5. Flow cytometry and cell sorting

Single cell suspensions was stained for 30 min at 4 °C with FITC-, PE-, PerCP-Cy5.5-, APC-, or APC-Cy7-conjugated mAb specific for mouse CD4, CD8, CD62L (BD Biosciences), CD25, CD44 (eBioscience), and TCRβ (Quantobio, Beijing, China). To detect OVA-specific T cells, cells were also stained with PE-conjugated tetramers, H-2K(b) (SIINFEKL) for $CD8^+$ T cells and I-A(b) (NEKYAQAYPNVS) for $CD4^+$ T cells, both of which were kindly provided by NIH Tetramer Facility. Flow cytometry was performed on a BD FACSCalibur, and the data were analyzed using flowJo software. For cell sorting, the $TCR\beta^+CD62L^{hi}CD25^{low}CD44^{low}$ population was collected on a BDAriaII cytometer with a purity >95%.

2.6. Adoptive transfer

Thymectomized mice received weekly intravenous injections of 5–6 * 10^6 $TCR\beta^+CD62L^{hi}CD25^{low}CD44^{low}$ naive T cells from the second to the ninth week after operation. One week after the last transfer, mice were sacrificed and analyzed for the presence of OVA-specific memory T cells derived from LM-ova infection prior to thymectomy.

2.7. Quantification of L. monocytogenes in the liver of infected mice

The liver was removed 1 day after the secondary infection and homogenized in 10 mL 0.05%Triton-PBS. The homogenate and its dilution (1:10) were plated in a volume of 100 μL onto brain-heart infusion agar with streptomycin and erythromycin. Colonies were counted after incubation at 37 °C for 2 days.

2.8. Statistics

Data are presented as mean ± SD. Statistical significance was evaluated by one way ANOVA test using GraphPad Prism software (GraphPad). Throughout the text, figures, and figure legends, the following terminology is used to denote statistical significance: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Impact of altered thymic output on the frequency and absolute number of antigen-specific memory T cells

In order to elucidate the potential influence of altered thymic output on the antigen-specific memory T cells, we first treated C57/BL6 mice with *L. monocytogenes* expressing the full-length ovalbumin protein (LM-ova) at the age of 6 weeks. Three weeks

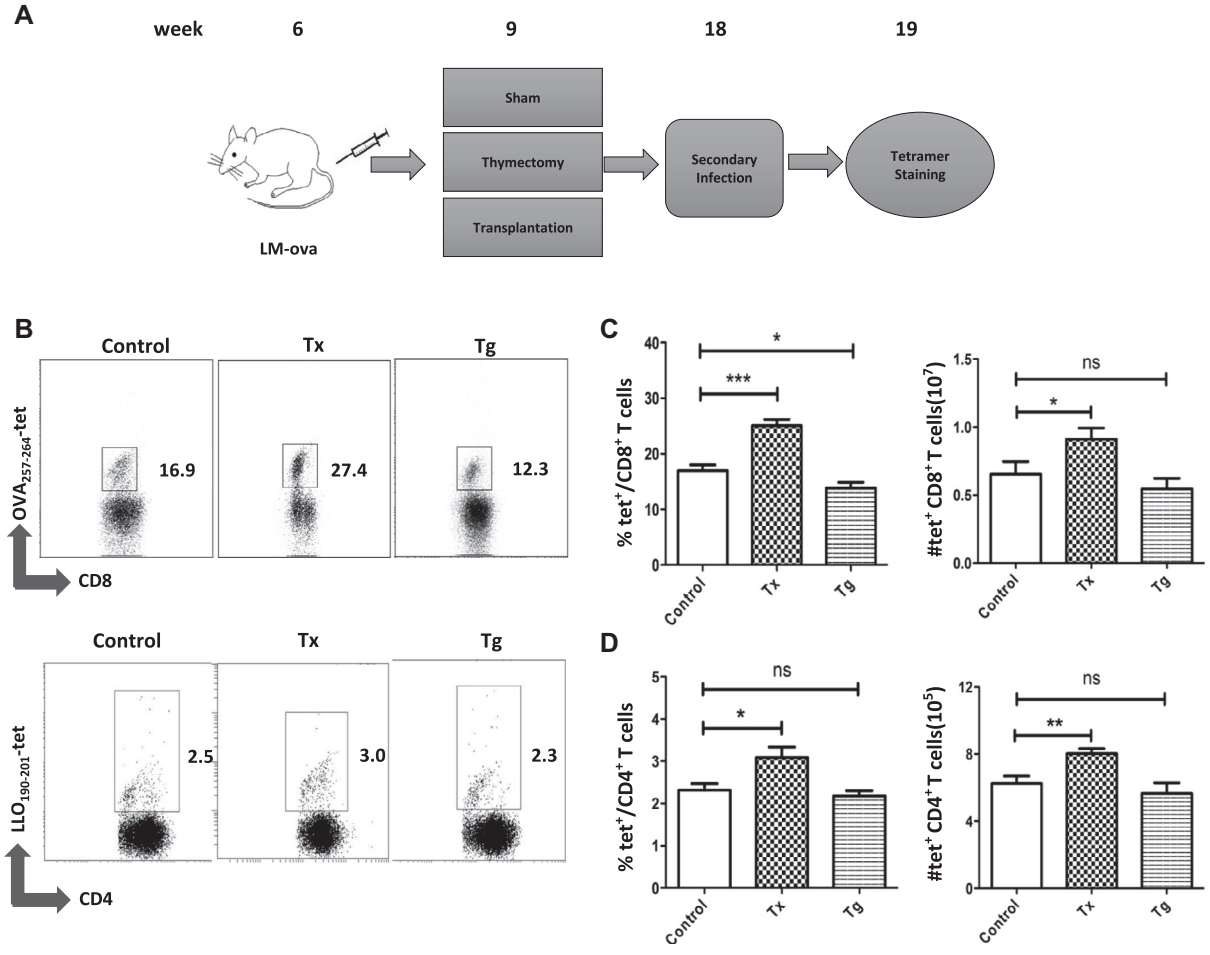

Fig. 2. Enhanced OVA-specific recall responses in thymectomized mice. (A) The same procedure was followed as described in Fig. 1A except that the mice received a secondary challenge with LM-ova at week 18 and were sacrificed for further analysis at week 19. (B) Splenocytes were staining with anti-CD4, anti-CD8, anti-TCRβ, and H-2K(b) (SIINFEKL) or I-A(b) (NEKYAQAYPNVS) tetramer. Representative dot plots are shown for one mouse from each group. The number indicates the percentage of $tetramer^+$ cells within the gate in total $CD8^+$ or $CD4^+$ $TCR\beta^+$ cells. (C) The percentage of $OVA_{257-264}$-$tetramer^+$ cells among $CD8^+$ T cells (*Left*) and the total number of such cells in the spleen (*Right*). (D) The percentage of $LLO_{190-201}$-$tetramer^+$ cells among $CD4^+$ T cells (*Left*) and the total number of such cells in the spleen (*Right*). $n = 14, 16, 15$ for the control, thymectomy and thymic grafting groups, respectively.

later, these mice were subject to different surgical procedures: one group were thymectomized (Tx) to terminate thymic output; another group received thymic grafting (Tg) to maintain a high level of thymic output; and still another group were sham-operated and allowed to undergo natural involution process (Control). T cell compartment was then analyzed at week 18 (Fig. 1A). As expected, the total number of T cells in the periphery was decreased by 35% in thymectomied ($3.96 \pm 0.25 * 10^7$) mice in comparison to control mice ($6.05 \pm 0.35 * 10^7$), whereas mice with thymic grafts showed a 47% increase ($8.92 \pm 0.67 * 10^7$). We subsequently measured the frequencies of OVA-specific $CD8^+$ memory T cells in the peripheral lymphoid tissues following *in vitro* stimulation with the $OVA_{257-264}$ peptide. Elispot assay detected higher frequencies of IFN-γ secreting cells in thymectomized mice than control mice, with 2- and 4-fold increases in the spleen and lymph nodes, respectively (Fig. 1B–D). Despite the overall shrunk T cell pool in thymectomized mice, the total number of OVA-specific $CD8^+$ memory T cells in these mice was almost twice as many as that in control mice (Fig. 1E). Similar changes were also observed with $LLO_{190-201}$-reactive $CD4^+$ memory T cells (Fig. 4D). On the other hand, mice with thymic grafts showed a substantial reduction of OVA-specific $CD8^+$ memory T cells compared to the control mice (Fig. 1B–E). These results suggest that altered thymic output has a substantial influence on the maintenance of existing memory T cells.

3.2. Enhanced memory responses and accelerated pathogen clearance by thymectomized mice

We next examined whether the increased presence of OVA-specific memory T cells would lead to a more potent recall response. Similar procedures were followed as described above. At week 18, the mice were re-challenged with LM-ova. Tetramer staining was then performed to identify T cells recognizing the two dominant epitopes, $OVA_{257-264}$ and $LLO_{190-201}$ (Fig. 2A). As the majority of memory T cells were found in the spleen (Fig. 1C and D), the analysis was focused on splenocytes. As shown in Fig. 2B, the secondary infection induced a massive expansion of memory T cells. In control mice, $16.96 \pm 1.09\%$ of $CD8^+$ T cells and $2.32 \pm 0.15\%$ of $CD4^+$ T cells were stained positive for $OVA_{257-264}$ and $LLO_{190-201}$ tetramer, respectively. They were further increased in thymectomized animals ($25.08 \pm 1.09\%$ for $CD8^+$ and $3.09 \pm 0.25\%$ for $CD4^+$), whereas mice with thymic grafts had significantly lower proportion ($13.86 \pm 1.03\%$) of $OVA_{257-264}$-$tetramer^+$ cells but a comparable $LLO_{190-201}$-$tetramer^+$ subpopulation ($2.18 \pm 0.12\%$) (Fig. 2C and D). We also calculated the absolute number of $tetramer^+$ T cells in the spleen. A significant difference was observed between the thymectomized and control mice. But the difference became less obvious between the control and the thymic grafting group, partly because of the overall increased T cell population in the latter group (Fig. 2C and D).

The potency of the recall response was further evaluated by comparing the efficiency of pathogen clearance among the various groups. One day after re-challenge with LM-ova, the number of live bacteria in the liver tissue was determined through colony formation (Fig. 3A). Compared to the control mice, thymectomized mice contained fewer bacteria in the liver (1.06 ± 0.13 versus $0.57 \pm 0.10 * 10^5$ CFU/liver), indicating a more rapid clearance of the pathogens. The thymic grafting group, on the other hand, were indistinguishable from the control group ($1.12 \pm 0.37 * 10^5$ CFU/liver) (Fig. 3B). Together, these results suggest that reduced thymic output favors the development of a stronger memory responses against antigens previous experienced by the host.

3.3. Reversal of thymectomy-induced effect on T cell memory by adoptive transfer of naïve T cells

In addition to the supply of new naive T cells, thymectomy may affect other less well-defined aspects of the thymic function, which in turn contributes to the enhanced memory responses in these

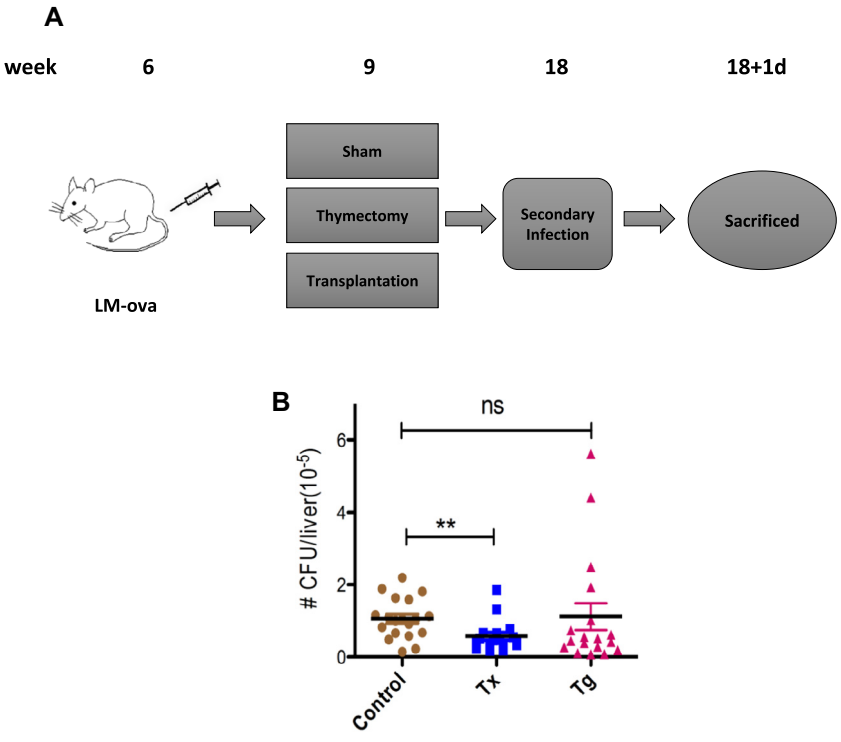

Fig. 3. Accelerated clearance of bacteria in thymectomized mice in the secondary infection with LM-ova. (A) The same procedure was followed as described in Fig. 2A except that the mice were sacrificed 24 h after infection. (B) The number of LM-ova in the liver was measured by inoculating tissue homogenate on the brain–heart infusion agar. Each dot, square or triangle represents the result from individual mice. $n = 19, 18, 18$ for the control, thymectomy and thymic grafting groups, respectively.

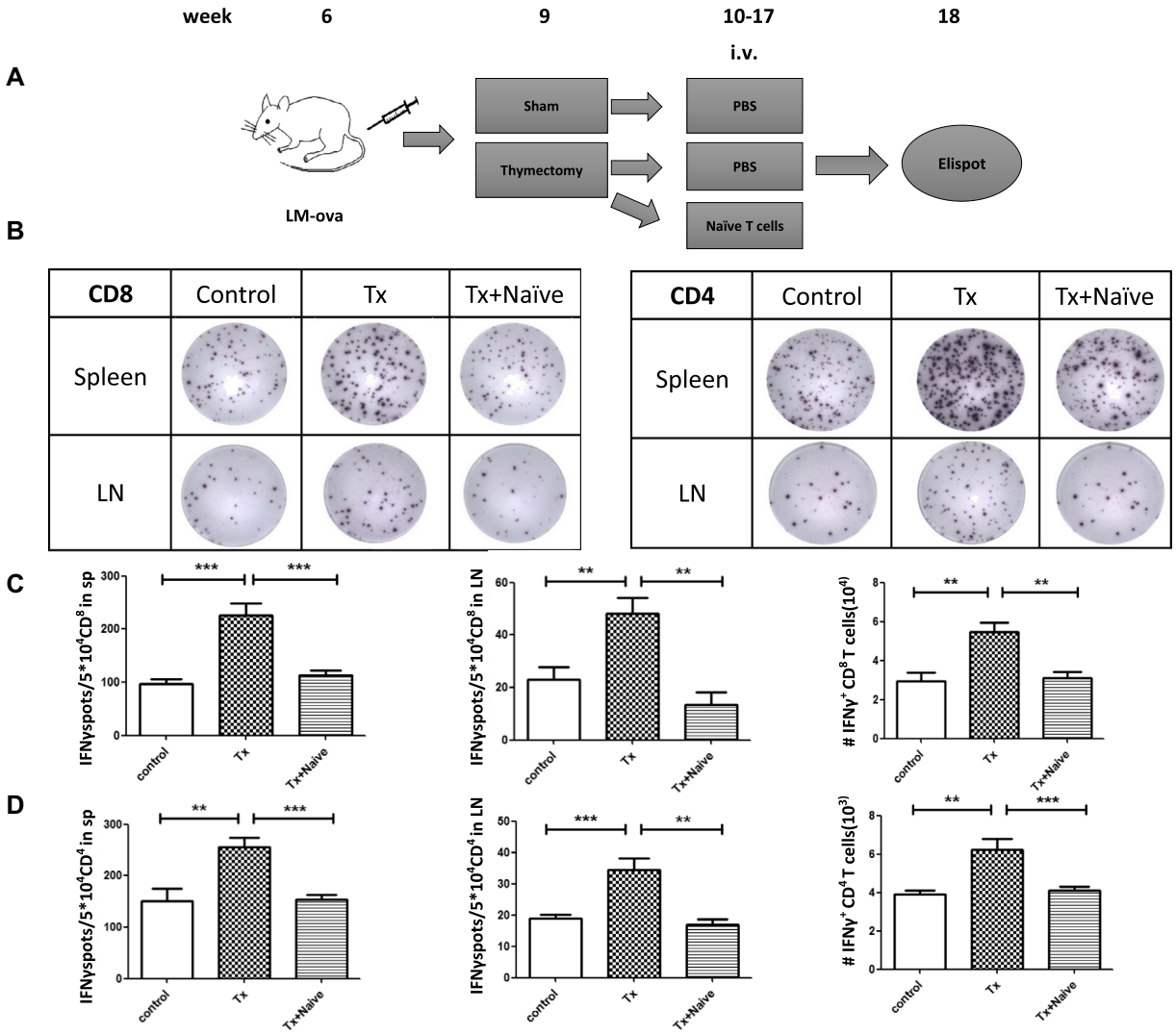

Fig. 4. Reversal of the effect of thymectomy by adoptive transfer of naïve T cells. (A) C57BL/6 mice were infected with LM-ova at week 6, underwent thymectomy (Tx) or sham operation (Control) at week 9, and received weekly intravenous injection of 5–6 * 10^6 naive T cells (Tn) or PBS between week 10 and 17. The frequency of OVA-specific $CD8^+$ and $CD4^+$ memory T cells were analyzed using Elispot one week after the last transfer. (B) Splenocytes and lymph node cells were stimulated with $OVA_{257–264}$ or $LLO_{190–201}$ peptide for 20 h. IFNγ-producing cells were detected by Elispot assay. Representative results are shown for cells obtained from one mouse of each group. (C) The frequencies of $OVA_{257–264}$-responsive memory T cells per 5×10^4 $CD8^+$ T cells in spleen (*Left*) and lymph nodes (*Middle*) and the total number of OVA-specific $CD8^+$ memory T cells in each mouse (*Right*). (D) The frequencies of $LLO_{190–201}$-responsive cells per 5×10^4 $CD4^+$ T cells in spleen (*Left*) and lymph nodes (*Middle*) and the total number of OVA-specific $CD4^+$ memory T cells in each mouse (*Right*). $n = 8$ for each group.

animals. To address this concern, we treated a subgroup of thymectomized mice with weekly intravenous injection of purified naive T cells between week 10 (1 week after operation) and 17 to mimic continual replenishment of the peripheral T cell pool by a functional thymus. At week 18, OVA-specific memory T cells were enumerated in these mice using Elispot assay (Fig. 4A and B). While thymectomy alone caused an increase in the frequency and total number of OVA-specific $CD4^+$ and $CD8^+$ memory T cells, this effect was completely abrogated following weekly adoptive transfer of naive T cells. As such, the thymectomized mice replenished with naive T cells were essentially indistinguishable from the control mice. In fact, the frequency of CD8+ memory cells in the lymph nodes was even found to be lower than that in the control mice (Fig. 4C and D). Therefore, the enhanced memory T cell response observed with thymectomy is most likely the result of premature termination of thymic output rather than any other effect introduced by the removal of the thymus.

4. Discussion

The present study showed that the thymic exportation had a profound impact on the maintenance of T cell memory. Using a mouse model infected with LM-ova, we demonstrated that thymectomy resulted in a marked increase in the frequency and total number of OVA-specific $CD8^+$ and $CD4^+$ memory T cells. Such an effect was primarily ascribed to the disruption of thymic output as it could be reversed by adoptive transfer of naive T cells. In accordance with the elevated levels of memory T cells, the thymectomized mice, when re-challenged with LM-ova, mounted a more vigorous recall response as evidenced by the enlarged population of tetramer$^+$ cells and the accelerated clearance of the pathogen. Sustained high levels of thymic output, on the other hand, caused a reduction of OVA-specific memory T cells, even though this relatively small difference did not translate into an overt change in the secondary responses of thymus grafted mice.

These findings have important implications for our understanding of the physiological significance of thymic involution. As a major contributor of immunosenescence, thymic involution is generally viewed as an undesirable process [16]. However, its evolutionary conservation and early occurrence in life suggests that this process may not be wholly detrimental. The data generated from this study suggest a direct impact on one important aspect of the immune function, i.e., T cell memory. Loss of thymic function actually favors the maintenance of memory T cells, thereby facilitating the secondary immune responses. In a broad sense, this conclusion is in concert with the model forwarded by Hodgkin and Dowling [25], in which a reduced input from the thymus is proposed to be important for post-thymic selection of naive T cells and finetuning of the peripheral T cell repertoire. As an integral component of the repertoire, the memory T cell compartment may also be subject to the influence of thymic output. From such a perspective, thymic involution may serve as an active mechanism to facilitate the establishment of an optimal peripheral repertoire with well balanced receptor diversity and frequency of T cells of defined specificities. A large clone size of memory T cells apparently favors immune responses to antigens to which the immune system has been primed. In adulthood, it may be more important to maintain good memory responses rather than use reserves to meet new challenges that may never come. As such, the decline in immunocompetence seen in thymic involution may simply be an unfortunate side effect, as suggested by Hodgkin and Dowling [25].

In fact, thymic involution has long been known to be accompanied by an increase of $CD62L^{-}CD44^{+}$ activated/memory T cells and a concomitant reduction of $CD62L^{+}CD44^{-}$ naive T cells in the periphery [22]. But the increased representation of the $CD62L^{-}CD44^{+}$ population does not necessarily predict an enhanced T cell memory to specific antigens. In principle, this population could be accumulating as a result of repeated exposures to an increasing number of antigens in aged mice. Moreover, peripheral T cells undergo homeostatic expansion to maintain the peripheral pool [10,11] and this homeostatic expansion frequently induces a conversion from naive to activated/memory phenotype [26–28], making it difficult to identify memory cells purely based on phenotypic characters. By directly analyzing the presence and response of memory T cells against a specific antigen, the present study unambiguously shows that the disruption of continuous supply of new naive cells does have a beneficial effect on T cell memory.

A previous study by Tanchot and Rocha suggests that naive and memory cells occupy different niches and the sizes of the two pools are independently regulated [6]. Results from the present study, however, argue that the homeostatic regulation of naive and memory T cell compartments is somehow interconnected. The reason for the discrepancy is unclear. Apart from the difference of a cloned transgenic versus a diversified TCR repertoire, we are comparing the substitution of resident cells in a full and intact repertoire versus an initially empty and reconstituted repertoire. In addition, although male cells are believed to be efficiently eliminated in the periphery of the chimeras [29], it remains possible that the bone marrow may provide a continuous supply of newly generated male antigen-expressing hematopoietic cells. Therefore, the high levels of activated/memory T cells in these mice could rather be the result of constant TCR stimulation than homeostatic activities.

The mechanisms underlying peripheral T cell homeostasis have been intensively studied [30,31]. The survival of naive T cells requires continuous TCR interaction with MHC molecules as ablation of either TCR or MHC expression in the periphery causes their rapid disappearance [32–34]. Memory T cells, especially $CD4^{+}$ memory T cells show much less stringent dependence on the TCR signal for survival [35,36]. On the other hand, cytokine signals, especially those mediated by the common γ chain receptor, are critically involved in the long term survival of both naive and memory T cells [35–37]. Therefore, it is not totally surprising that they may be in direct competition for limiting resources such as IL-7 and IL-15, and the memory cells may be better positioned in the competition with a decline of thymic exportation.

In summary, the present study reveals a previously unappreciated positive impact of thymic involution on immune responses. As a long-term consequence, the timely reduction in thymic exportation of more virgin cells favors the maintenance of T cell memory established from previous antigen exposures. This finding has shed light on the physiological relevance of thymic involution and may have implications for the design of intervention strategies for immunosenescence.

Acknowledgments

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