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The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice

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Summary

CD4 T cells, and especially T follicular helper cells, are critical for the generation of a robust humoral response to an infection or vaccination. Importantly, immunosenescence affects CD4 T cell function and the accumulation of intrinsic defects decreases the cognate helper functions of these cells. However, much less is known about the contribution of the aged microenvironment to this impaired CD4 T cell response. In the current study, we have employed a preclinical model to determine whether the aged environment contributes to the defects in CD4 T cell functions with aging. Using an adoptive transfer model in mice, we demonstrate for the first time that the aged microenvironment negatively impacts at least three steps of the CD4 T cell response to antigenic stimulation. First, the recruitment of CD4 T cells to the spleen is reduced in aged compared to young hosts, which correlates with dysregulated chemokine expression in the aged organ. Second, the priming of CD4 T cells by DCs is reduced in aged compared to young mice. Finally, naïve CD4 T cells show a reduced transition to a T follicular helper cell phenotype in the aged environment, which impairs the subsequent generation of germinal centers. These studies have provided new insights into how aging impacts the immune system and how these changes influence the development of immunity to infections or vaccinations.

Keywords

CD4 T cells; dendritic cells; homeostatic chemokines; secondary lymphoid organs; immune defects; T follicular helper cells

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Supporting information

The detailed experimental procedure for the histology section is available online.

Author contributions

JSL and ACM wrote the manuscript, designed and performed experiments and analyzed data. SME, PAL and MT performed experiments and analyzed data. LH wrote the manuscript, designed experiments and analyzed data.

The authors declare no conflict of interest.

Introduction

Aging negatively impacts most biological processes, including the immune system in both human and murine models. Older individuals are more susceptible to infections and respond less efficiently to new immunization. CD4 T cells, with their helper functions, play a central role in the immune response. These cells are particularly affected by aging and show a decreased response to antigenic stimulation, impaired cytokine production, defective intracellular signaling and a switch in the naïve to memory ratio (Reviewed in (Lefebvre & Haynes, (2012))). In order to understand the mechanisms involved in age-related changes in the immune system, we have developed a preclinical adoptive transfer model in which we can study the impact of aging specifically on CD4 T cells or the microenvironment. We demonstrated that intrinsic defects in aged naïve CD4 T cells contribute to the impaired cognate helper functions of these cells, and lead to a reduced humoral response in aged mice (Maue et al., (2009); Eaton et al., (2004))). While the impact of age-associated intrinsic defects on CD4 T cell functions has been the focus of multiple studies, much less is known regarding the contribution of the aged microenvironment on the impaired CD4 T cell response with aging. This aspect is critical considering that CD4 T cells need to interact with their environment on multiple occasions during the establishment of an immune response.

Naïve CD4 T cells migrate to secondary lymphoid organs in a process partly regulated by the homeostatic chemokines CCL19 and CCL21 (Gunn et al., (1999))). Following immunization or infection, the naïve CD4 T cells will then become activated by dendritic cells (DCs) bearing their cognate antigen. Once activated, some of the CD4 T cells acquire a T follicular helper (Tfh) cell phenotype characterized by a high expression of various surface markers such as programmed cell death-1 (PD-1), inducible costimulator (ICOS) and the chemokine receptor CXCR5 (Crotty, (2011))). Tfh cells are required for optimal B cell activation and germinal center formation that lead to the generation of plasma cells producing high-affinity antibodies (Schaerli et al., (2000); Breitfeld et al., (2000))). Failure of the aged environment to support the recruitment of CD4 T cells to secondary lymphoid organs, their activation by dendritic cells and/or their interaction with B cells could all contribute to the impaired CD4 T cell responses in older individuals.

In the current studies, we aimed to determine the impact of the aged environment on CD4 T cell functions. In order to assess the contribution of the aged environment independently of the intrinsic defects in aged CD4 T cells, we used an adoptive transfer model in which we transferred young congenic (thy1.1) CFSE-labeled OTII cells into young (~2 mo-old) and aged (~20 mo-old) C57BL/6 (thy1.2) hosts. The hosts were then immunized intraperitoneally with ovalbumin, using alum as adjuvant. In this model, the OTII cells isolated from young mice do not exhibit age-associated defects and any functional defects in the antigen-specific CD4 T cell response following immunization would result from environmental defects in the aged hosts. Using this approach, we found that environmental defects play an important role in the functional defects of CD4 T cells in aged individuals. Indeed, naïve CD4 T cells transferred into aged hosts presented delayed priming and proliferation, reduced recruitment to secondary lymphoid organs, and impaired cognate helper functions when compared to cells transferred into young hosts. The incapacity of the aged environment to support proper CD4 T cell activation most certainly contributes to the impaired immune response observed in aged individuals.

Results

The priming of young naïve OTII cells is delayed in aged hosts compared to young hosts

Within the first days following immunization or infection, antigen-specific CD4 T cells migrate to the secondary lymphoid organs (spleen and LNs) where they interact with

antigen-bearing antigen-presenting cells (APCs). CD4 T cells then become activated and, amongst other things, up-regulate the expression of CD69 on the cell surface and begin to proliferate. To examine the impact of the aged microenvironment on these initial steps, young donor cells (congenic OTII TCR Tg) were transferred into intact young and aged hosts. Initially, we evaluated the impact of the aged environment on the kinetic of activation of the OTII cells. Every 12 hours post-immunization, the transferred OTII cells were harvested from the spleen and peripheral lymph nodes (pLNs) of young and aged hosts, and their proliferation and CD69 expression were assessed by flow cytometry.

CFSE dilution profiles show that the proliferation of young OTII cells recovered from the spleen and pLNs of aged hosts was delayed when compared to the OTII cells recovered from young hosts (Fig. 1A). Indeed, whereas almost all of the donor cells recovered from young hosts had undergone over three rounds of division by day 3 post-immunization, a significant proportion of donor cells retrieved from aged hosts had been through fewer than three rounds of division, and many remained undivided. This delay in proliferation correlated with a delay in CD69 expression (Fig. 1B). While over 80% of the OTII cells transferred into young hosts express CD69 on their surface 24h post-immunization, 36-48h were necessary to reach similar proportions of CD69 expression on the donor cells transferred into aged hosts. Moreover, since fewer donor cells were recovered from the spleen of aged compared to young hosts (Fig. 1C), the absolute number of donor cells proliferating and expressing CD69 was also lower in the aged hosts than in the young hosts.

The OTII recruitment to the spleen is impaired in the aged hosts

The privileged site of CD4 T cell priming is the T cell zone found within the B cell follicles of secondary lymphoid organs such as the spleen. A defect in OTII recruitment to the T cell zones could therefore explain the reduced and delayed activation observed in aged mice. In support of this hypothesis, fewer OTII cells accumulated in the spleen of aged hosts than in the spleen of young hosts ($13,943 \pm 3,935$ vs $36,143 \pm 5,679$ cells/spleen, respectively) as early as 12 hours post-immunization (Fig. 1C) even though identical numbers of OTII cells (10^6 OTII cells/mouse) were transferred into both young and aged hosts. At that time point, the delay in cell proliferation in the aged hosts could not account for this difference since the OTII cells did not start proliferating before day 2 post-immunization (Fig. 1A).

To determine whether the OTII cells were indeed reaching the splenic T-cell zones, we next examined frozen spleen sections of both young and aged hosts by immunofluorescence staining 18 hours post-immunization. As expected, the majority of OTII cells (Thy1.1⁺ in red) were found within the T cell zone of the B cell follicles (B220⁺ in green) in the spleen of both young and aged hosts (Fig. 2). However, while a large number of OTII cells accumulated in the T cell zone of young hosts (Fig. 2A i and ii), very few OTII cells were found in the T cell zones of aged hosts (Fig. 2A iii and iv). These findings were highly reproducible with average counts of 0.38 ± 0.05 cells/1000 μm^2 in the T cell zone of aged hosts and 1.28 ± 0.08 cells/1000 μm^2 in the T cell zone of young hosts (Fig. 2B). Interestingly, instead of migrating to the secondary lymphoid organs of the aged hosts, the transferred OTII cells remained in the bloodstream (Fig. 2C). Indeed, aged hosts have a higher proportion ($0.306 \pm 0.022\%$ of total PBLs) and number ($1.23 \pm 0.28 \times 10^6$ OTII cells/ml blood) of circulating OTII cells than young hosts ($0.048 \pm 0.013\%$ of total PBLs and $0.52 \pm 0.13 \times 10^6$ OTII cells/ml blood, respectively) 18 hours post-immunization (Fig. 2C).

In addition, the activation of the transferred cells that did reach the T cell zone in aged hosts was not as efficient as in young hosts. Figure 2D shows the expression of the proliferation marker Ki-67 (blue) by OTII cells (red) in the T cell zone of young (middle panel) and aged (bottom panel) hosts. In naïve mice (top panel), all OTII cells found in the spleen retained the CFSE dye (green, arrows) and do not express Ki-67. Three days post-immunization,

almost all OTII cells within the T cell zone of young hosts express Ki-67 and very few still contained the CFSE dye demonstrating active proliferation (Fig. 2D, middle panel). On the contrary, many OTII cells within the T cell zone of aged hosts did not express Ki-67 and were still bright for CFSE (arrows) suggesting that even the few cells that get to the T cell zone in aged hosts were not optimally activated (Fig. 2D, bottom panel). These data strongly suggest that the recruitment of naïve CD4 T cells to, and activation within, the secondary lymphoid organs are impaired in aged compared to young mice.

The impaired activation of young OTII cells in aged hosts is independent of endogenous CD4 T cells

With increasing age and a lifetime of antigenic exposure, the composition of the T cell compartment gradually shifts from one in which CD4 T cells mainly express a naïve phenotype to one containing CD4 T cells mainly expressing memory markers. Moreover, it has also been reported that aged individuals (both mice and humans) have an increased proportion of regulatory T cells that could negatively influence the activation of CD4 T cells (Sharma et al., (2006); Rosenkranz et al., (2007)). Thus, we next examined the putative impact of the endogenous CD4 T cell pool on the OTII cells activation and splenic recruitment in aged hosts.

In the first approach, the endogenous CD4 T cells from both the young and aged hosts were depleted using a CD4-depleting antibody (GK1.5). We have previously shown that the reconstituted CD4 T cells following antibody depletion are functionally similar whether they were generated from young or aged mice (Haynes et al., (2005)). By using this approach, we eliminate any difference between the endogenous CD4 T cell pool in the young and aged hosts by equalizing them. Depleting endogenous CD4 T cells did not improve the priming of the donor cells in aged hosts (Fig. 3A). Three days post-immunization, fewer donor cells were recovered from the spleen of aged hosts treated with GK1.5 than from the spleen of young hosts similarly treated ($219,300 \pm 30,793$ vs $597,750 \pm 192,447$ OTII cells/spleen) which were not different from their respective isotype-control groups (Fig. 3A). Accordingly, the proliferation of the donor cells transferred into aged hosts was also delayed compared to young hosts in both groups (Fig. 3B). In order to definitely exclude the involvement of endogenous CD4 T cells in the impaired donor cell priming in the aged hosts, we repeated the adoptive transfer experiments using young and aged CD4KO mice as hosts. Again, fewer OTII cells were recovered from the spleen of aged CD4KO hosts ($84,920 \pm 11,390$ OTII/spleen) than from the spleen of young CD4KO hosts ($276,300 \pm 109,500$ OTII/spleen, Fig. 3C). The cells recovered from the aged hosts also showed a delayed proliferation compared to the cells retrieved from the young hosts (Fig. 3D). Taken together, these results lead us to exclude that age-associated changes in the endogenous CD4 T cell compartment, including regulatory T cells, were responsible for the impaired response of the donor cells transferred into the aged hosts.

Impaired DC functions contribute to the delayed T cell response, but not the reduced T cell recruitment to secondary lymphoid organs, in aged mice

One of the first steps of CD4 T cell activation is the interaction with activated APCs bearing cognate antigen. It has been reported that DCs acquire various phenotypic and functional changes with aging in both human and mice (Ciaramella et al., (2011); Qian et al., (2011); Pereira et al., (2011); Wong et al., (2010)). Interestingly, the co-transfer of young DCs was shown to significantly improve the functional defects of young transgenic CD8 T cell response in aged hosts (Jiang et al., (2010)). To determine if young DCs could also restore the defects in the CD4 T cell response, we transferred young LPS-activated BMDCs pulsed with OTII peptide into young and aged hosts 2h prior to the OTII cell transfer. Three days post-transfer, fewer OTII CD4 T cells were recovered from the spleen of aged hosts ($0.598 \pm$

0.152×10^6 OTII cells/spleen) than from the spleen of young hosts ($1.133 \pm 0.132 \times 10^6$ OTII cells/spleen; Fig. 3E). No difference was observed in the CFSE-dilution profile of the donor cells transferred together with activated BMDCs in either young or aged hosts (Fig. 3F). Similar data were also obtained using BMDCs generated from the bone marrow of aged mice (Fig. 3G and 3H), suggesting that defects in the recruitment rather than intrinsic functions of old DCs are responsible for the impaired activation of the donor OTII cells. Further experiments are however required to definitely determine the nature of the defects in DC functions in aged mice. Our data suggests that impaired DC functions contribute to the delay in cell activation and proliferation in aged hosts, but not to the impaired recruitment of the donor cells to the spleen.

The homeostatic chemokines CCL21 and CXCL13 are dysregulated in aged hosts and may contribute to the impaired recruitment of the young OTII donor cells

Since neither the differences in the endogenous CD4 T cells or DCs could explain the impaired splenic recruitment of the donor cells transferred into the aged hosts, we next sought to determine whether the chemotactic signals within the aged host spleen were different than the signals present in the young host spleen. The homeostatic chemokines CCL19, CCL21 and CXCL13 play a critical role in T cell recruitment to secondary lymphoid organs and in orchestrating T-B cell interactions within the B cell follicles (Legler et al., (1998); Ohl et al., (2003)).

Quantitative PCR analyses of RNA purified from the spleen of young and aged hosts showed similar levels of mRNA for CCL19, CCL21 and CXCL13 (Fig. 4A). On the contrary, when measuring CCL19 and CCL21 protein content by ELISA, the spleen of young hosts contains a significantly higher amount of chemokines than the spleen of aged hosts for both CCL21 ($4.3 \pm 0.5 \mu\text{g}$ vs $1.3 \pm 0.1 \mu\text{g/mg}$ of total proteins, respectively) and CCL19 ($0.168 \pm 0.012 \mu\text{g}$ vs $0.085 \pm 0.008 \mu\text{g/mg}$ of total protein, respectively; Fig 4B).

Spatial regulation of chemokine production is another important aspect that can influence cellular responses to chemokines. To determine whether the homeostatic chemokines were also spatially dysregulated in aged mice compared to young mice, we next looked at CCL19, CCL21 and CXCL13 expression by immunofluorescence staining of frozen spleen sections. Immunofluorescence staining of CCL19 did not provide a strong enough signal to visualize, most certainly because of the very low CCL19 content in our mouse spleens that was more than 10 times lower than CCL21 (Fig. 4B). In accordance with previous findings (Luther et al., (2000); Nolte et al., (2003)), CCL21 was found within the T cell zone of the B cell follicles with very little, if any, within the B cell zone in young hosts (Fig. 4C i-iii). CCL21 expression in the aged hosts showed much more individual variability as represented by the three examples shown in figure 4C iv-vi. Although CCL21 was also mainly found within the T cell zone in aged mice, the signal was much less focused and could be seen scattered in the B cell zone in many sections (Fig. 4C iv and v). CXCL13 is a B cell chemokine mainly found in the B cell zone of the B cell follicles in secondary lymphoid organs (Nolte et al., (2003)). Figure 4D shows CXCL13 expression in young (left panel) and aged (right panel) hosts one day post-immunization. In young spleens, a bright CXCL13 signal is found in the marginal zone surrounding each follicle (arrows). This signal is greatly reduced or absent from the marginal zones of aged spleen B cell follicles as reported previously (Wols et al., (2010)).

The fact that CCL19 and CCL21 production is reduced and dysregulated in aged mice provides circumstantial evidence for the involvement of these T cell homeostatic chemokines in the reduced T cell recruitment and delayed priming in aged hosts. Further supporting this role, and in agreement with the findings of Mori and co-workers (Mori et al., (2001)), a lower number of OTII cells was recovered from *plt* hosts (deficient for both

CCL19 and CCL21) than wild type hosts at all time-point tested (Fig. 4E). The proliferation of the OTII cells was also delayed in *plt* hosts with 48.6% of the donor cells still undivided at day 3 post-immunization compared to only 1.35% in wild type hosts (Fig. 4F). Taken together, these findings strongly support an important contribution of the dysregulated chemokine expression in the spleen of aged hosts in the impaired recruitment and priming of the OTII donor cells.

Young OTII donor cells transferred into aged hosts have impaired helper functions

As noted above, the priming and proliferation of the donor cells transferred into the aged hosts were delayed but not abrogated. The number of OTII cells in the spleen of aged hosts significantly increased starting at day 5 post-immunization (Fig. 1). This could suggest that the aged environment, although delaying the donor cell activation, does not affect later functions of these cells. In the next series of experiments, we therefore evaluated whether the donor cells transferred into young and aged hosts could acquire a Tfh phenotype (defined as CXCR5^{hi} PD-1^{hi} cells) and promote GC B cells generation (defined as CD19⁺ CD38^{lo} PNA^{hi}). Ten days post-immunization, 8.5% of the OTII cells harvested from the spleen of young hosts expressed the Tfh markers CXCR5 and PD-1 (Fig. 5A, left panel). Only 2.9% of the OTII cells recovered from the spleen of aged hosts expressed a similar phenotype (Fig. 5A, right panel).

The total number of OTII cells expressing a Tfh phenotype in the aged hosts was therefore significantly reduced compared to the number of OTII Tfh cells generated in young hosts ($7,685 \pm 2,081$ vs $35,490 \pm 6,561$ OTII Tfh cells/spleen, respectively; Fig. 5B). This impaired Tfh generation resulted in a reduced production of germinal center B cells in the aged hosts compared to young hosts in both percentages (Fig. 5C) and numbers ($124,800 \pm 32,360$ vs $1,004,000 \pm 140,800$ GC B cells/spleen, respectively; Fig. 5D). The reduced GC B cell generation in aged hosts correlated with fewer and smaller germinal centers observed by immunofluorescence staining of GL-7, a marker for germinal center B and T cells (Laszlo et al., (1993); Yusuf et al., (2010)), in frozen spleen sections (Fig. 5E, green).

Discussion

CD4 T cells play a critical role in the establishment of an efficient humoral response by providing help for B cell activation, differentiation and antibody production. Importantly, CD4 T cells accumulate intrinsic defects during the normal course of aging (Reviewed in (Lefebvre & Haynes, (2012))), which contributes to the reduced humoral responses seen in older individuals (Maue et al., (2009); Eaton et al., (2004)). The contribution of the aged environment on the impaired CD4 T cell response in aging, however, remains to be clearly established. The results presented herein provide strong evidence that the aged environment significantly contributes to the impaired response of CD4 T cells, independently of the age-associated intrinsic defects in these cells. While, a role for the aged microenvironment in impaired CD4 T cell responses has been previously suggested (Linton et al., (2005)), the disruption of normal chemokine expression with aging has not been shown to be involved in age-related changes in CD4 T cell responses.

The homeostatic chemokines CCL19, CCL21 and CXCL13, through the activation of their receptor, play a major role in both the micro-organization of the secondary lymphoid organs as well as the recruitment of B cells, T cells and dendritic cells to these organs (Gunn et al., (1999); Legler et al., (1998); Ohl et al., (2003)). Disruption of the balance between these homeostatic chemokines (CCL19/CCL21/CXCL13) or their receptors (CCR7/CXCR5) have a major impact on the efficient migration of immune cells. For example, the recruitment of T cells to the secondary lymphoid organs of *plt* mice (deficient for both CCL19 and CCL21) or in mice deficient for the receptor CCR7 has been shown to be delayed or impaired (Mori

et al., (2001); Forster et al., (1999)). Similarly, CXCR5 is required for the proper interaction of T and B cells in germinal centers (Junt et al., (2005)). In agreement with previously published data (Wols et al., (2010)), we show that the production of CXCL13 in the marginal zone of the B cell follicles is reduced or completely lost in old mice (Fig. 4D). Most importantly, our findings show a significant reduction in the amount of CCL19 and CCL21 produced in the spleen of aged hosts compared to young hosts (Fig. 4B). To our knowledge, a difference in the production of these chemokines between young and aged mice has not been published. In unpublished data, Linton and co-workers observed by *in situ* staining as well as PCR analysis low levels of both CCL19 and CCL21 in naïve young and aged mice that increased after immunization in young but not in aged mice (Linton et al., (2005)). In our experiments, we could not see any difference between the mRNA levels of CCL19 or CCL21 in the spleen of young or aged mice whether naïve (data not shown) or 18 hours post-immunization (Fig. 4A). Moreover, we did not observe any difference between the immunofluorescence staining of CCL21 from spleens of naïve and immunized mice (data not shown), and we have not been able to detect any CCL19 using this technique. Several things could explain these discrepancies such as the use of different mouse strains (Balb/c vs C57BL/6) as well as the immunization strategy (influenza vs OVA/Alum). The use of different immunization strategies is particularly likely to have an important impact on chemokine expression since it has been reported that the modulation of CCL19 and CCL21 expression in secondary lymphoid organs varies according to the infectious agent used (Mueller et al., (2007)).

Given that the balance between the chemotactic signals provided by CCL19, CCL21 and CXCL13 is of critical importance for the proper interactions between activated B and T cells (Reif et al., (2002)), it is likely that the dysregulation of these chemokines contribute to the impaired Tfh cells and germinal center formation observed in aged mice. Moreover, mice deficient for CXCL13 or CXCR5 have impaired germinal center formation (Allen et al., (2004)). Recent advances in Tfh cell biology demonstrated that optimal Tfh cell generation requires the initial interaction with dendritic cells (within the first few days) which induce the expression of surface markers generally thought to be Tfh markers, such as CXCR5, PD-1 and ICOS, on the majority of responding cells (Kerfoot et al., (2011)). The maintenance of those Tfh markers is however dependant on cognate interactions between the activated CD4 T cells and B cells. Indeed, in the absence of cognate B cells, Tfh cells are generated but not maintained (Kerfoot et al., (2011); Choi et al., (2011)). These interactions therefore provide the necessary signals to induce the B cells to become GC B cells and form germinal centers and to maintain Tfh cells in the follicles. Since the co-transfer of activated BMDCs did not restore Tfh or germinal center B cell numbers in aged mice ten days post-immunization (data not shown) despite the improvement of the initial priming, this suggests that the interaction with B cells is impaired in the aged animals. Our immunofluorescence data show that the B cell follicles in aged mice are not as well organized as the B cell follicles in young mice (Fig. 2A and D, Fig. 4C and D, and Fig 5E). Whereas the B cells are tightly arranged in young mice to form well-defined follicles, the B cells in aged mice appear more loosely arranged. This looser structure of the follicles in aged spleens sometimes leads to the loss of a defined T cell zone (Fig. 4C iv and v). The lack of organization of the aged spleen combined with the dysregulation of the homeostatic chemokine signals that direct activated B and T cell movement within the follicles are likely preventing optimal interactions between these two cell types. Our current data, however, do not allow us to establish whether the defect in Tfh cell generation in aged mice is the result of their impaired induction or rather of their impaired long-term maintenance. These questions are currently under investigation.

Altogether, our data support the theory that there are at least three different steps involved in the priming and activation of naïve CD4 T cells that are impaired in the aged environment.

First, the chemokine signals directing CD4 T cell migration to the secondary lymphoid organs are decreased and spatially dysregulated in aged hosts and prevents their efficient recruitment of the cells to their privileged priming site. Second, the antigen presentation by DCs and/or their recruitment to the spleen is not optimal in the aged hosts, which results in the reduced activation of the CD4 T cells. Finally, the aged environment does not support the induction and/or maintenance of Tfh cells and, consequently, of germinal center B cells. This impaired Tfh cell generation is likely caused by inappropriate interactions with cognate B cells.

Experimental procedures

Animals

All mice used in these studies were bred and housed at the Trudeau Institute animal facilities, except some aged C57BL/6 mice (20 months) that were obtained from the National Institute of Aging. Young (8-12 weeks) and aged (20-24 months) C57BL/6, young and aged CD4KO mice (in the C57BL/6 background), and young *plt* mice (deficient for CCL19/CCL21) were used as hosts. CD90.1 (Thy1.1) OTII TCR Tg mice (6-8 weeks) expressing a V α 2V β 5 TCR specific for a peptide fragment of OVA presented in Class II MHC molecules (IA^b) were used as donors (Barnden et al., (1998)). All mice were housed in sterilized, HEPA-filtered, individually ventilated caging at the animal facility of the Trudeau Institute until their use. The Trudeau Institute Institutional Animal Care and Use Committee approved all experimental procedures involving mice.

Naïve CD4 T cell isolation and phenotyping

Spleens and peripheral lymph nodes (subiliac, proper axillary, accessory axillary, mandibular, accessory mandibular, superficial parotid and cranial deep cervical lymph nodes ((Van den et al., (2006)); pLNs) of young OTII TCR Tg mice were harvested and homogenized into single cell suspensions in RPMI + 1% FBS (Hyclone, Logan, UT). Cell suspensions were then pelleted (1400 rpm, 7 min, 4°C), resuspended in 2 ml of warm (37°C) RPMI containing 0.18 μ M 2-ME, 4 mM glutamine, Antibiotic-antimycotic solution (Cellgro, Manassas, VA), 10 mM HEPES and 10% FBS (TCM) and placed onto nylon wool columns for 40 min at 37°C to remove adherent cells. Cells were eluted from the column and pelleted. Contaminating RBCs were eliminated by resuspending the cell pellet in RBC lysis buffer (0.15M NH₄Cl, 1mM KHCO₃, 0.1 mM Na₂EDTA; 1 ml/mouse) for 1 min. Lysis was stopped by the addition of 4 volumes of RPMI + 1% FBS; the cells were then pelleted, resuspended in 2 ml of cold (4°C) RPMI + 1% FBS and layered onto a discontinuous Percoll (GE HealthCare, Piscataway, NJ) gradient (90%/62%/53%/40%, 3 ml/fraction in a 15 ml conical tube). After centrifugation (3000 rpm, 20 min, 4°C), small naïve T cells were collected at the interface between the 90% and 62% fractions. Naïve CD4 T cells were next purified by MACS using a CD4 T cell enrichment kit (Miltenyi Biotec, Cambridge, MA) according to manufacturer's instructions. The purity and phenotype of TCR Tg CD4 T cells were determined by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA) using FITC-conjugated anti-V α 2 TCR, PerCP- or APC-conjugated anti-CD4 and PE-conjugated anti-CD25, CD44, CD62L and CD69 or isotype control antibodies (BD Pharmingen, San Diego, CA). The cell suspensions obtained were generally 90% naïve CD4 T cells.

BMDC generation and transfer

Bone marrow was harvested from one young or aged C57BL/6 mouse and cultured in polystyrene Petri dishes (100mm $\times$ 15mm, Fisherbrand, Pittsburgh, PA) containing 10 ml RPMI supplemented with 4 mM glutamine, Antibiotic-antimycotic solution, 10% FBS and 20 ng/ml GM-CSF in a humidified incubator (37°C, 7% CO₂). Ten ml of media were added

on day 3. On day 6 and 8, 10 ml of media were removed from the dishes and replaced with 10 ml of fresh media. On day 9, cells were harvested, counted and plated in non-treated 6-well plates (Nunc, Rochester, NY) at 2×10^6 cells/ml, 4 ml/well and activated with 50 ng/ml LPS (Sigma) for 14-16 hours. BMDCs were next pulsed for 2h with 50 μ g/ml of OTII peptide. The LPS-activated, peptide-pulsed BMDCs were finally harvested, washed in PBS, resuspended at 10^7 cells/ml in PBS and 100 μ l were injected i.v. into the young or aged hosts. OTII CD4 T cells were injected 2 hours later.

Cell labeling, adoptive transfer and immunization

When indicated, OTII TCR Tg CD4 T cells were labeled with CFSE according to manufacturer's instructions (Molecular Probes, Grand Island, NY). In brief, the purified CD4 T cells were resuspended in TCM at 10^7 cell/ml and incubated in a water bath (37°C) with 300 μ M CFSE for 13 min, mixing every 2 min. The labeled cells were then washed 3 times and resuspended in PBS (10^7 cell/ml). Donor Tg CD4 T cells were finally transferred i.v. into adoptive hosts (100 μ l/mouse). For immunization, the vaccine was prepared by mixing 1 volume alum (9%, w/v) with 1 volume of PBS containing 500 μ g/ml OVA. The pH of the solution was then adjusted to 6.5 with NaOH 10N and allowed to precipitate for 30 min at room temperature. The suspension was then washed (2000 rpm, 7 min, 4°C) and resuspended to original volume with PBS. At the time of cell transfer, mice were simultaneously immunized i.p. with 200 μ l of vaccine (50 μ g OVA adsorbed on alum (4.5%, w/v)). For each experiment, three to five hosts were used in each group and each experiment was performed from two to four times.

In vivo Ab depletion

In some experiments, host endogenous CD4 T cells were depleted using the anti-CD4 Ab (GK1.5); isotype control (FLK) antibody-treated group was also included (BioXCell, West Lebanon, NH). Young and aged C57BL/6 mice were administered 250 μ g of Ab in 100 μ l PBS i.p. on days 0 and 3, and allowed to rest for 8 wks. After that resting time, endogenous CD4 T cells have replenished and the Ab is cleared from the circulation (Haynes et al., (2005)). These hosts with newly generated CD4 T cell pool were then used for adoptive transfer experiments as described above.

Flow cytometry

At indicated time points following immunization, spleens, pLNs and blood were harvested from mice to generate single cell suspensions. For detection of donor cells, cells were stained with FITC-conjugated anti-V α 2 TCR, PE-conjugated anti-Thy1.1, APC-conjugated CD69 and PerCP- or Pacific Blue-conjugated CD4 Abs. For the determination of Tfh phenotype cells were stained with PE-conjugated anti-Thy1.1, biotin-conjugated CXCR5 followed by APC-conjugated streptavidin, PECy7-conjugated anti-PD-1, Pacific Blue-conjugated anti-CD4 and dead cells were excluded using propidium iodide. For GC B cells determination, cells were stained with FITC-conjugated PNA, PE-conjugated CD38, PECy7-conjugated CD19 and dead cells were excluded using propidium iodide. Acquisition of samples was performed on a Calibur, Canto II or LSRII (BD biosciences) flow cytometer. For determination of CFSE profiles and phenotypic analysis, at least 2000 donor cells were acquired. Data analysis was performed using FlowJo software (TreeStar, Inc., Ashland, OR).

Histology

The spleen sections were fixed in acetone/ethanol (75/25, v/v) for 10 min, washed 3 times in PBS, and blocked with 10 μ g/ml purified Fc Block Ab (BioXCell) in PBS containing 5% serum for 30 min. The sections were then probed with the primary Abs in a humidified chamber for 30 min, washed 3 times with PBS and incubated with respective secondary Abs

for an additional 30 min. The sections were washed again 3 times with PBS and mounted with AquaMount (PolySciences, Inc., Warrington, PA). In some cases, sections were counterstained with 100 nM Hoechst (Sigma-Aldrich, St-Louis, MO) for 15 min before mounting the slides. Pictures were captured using a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany) or with a Leica TCS SP5II confocal microscope.

Real-time quantitative RT-PCR

Eighteen hours following adoptive transfer and immunization, spleens and pLNs were harvested from young and aged mice. Total RNA was isolated from tissues using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. cDNA was reverse transcribed from 1 µg of total RNA using SuperScriptIII reverse transcriptase and random hexamers according to manufacturer's instructions. RNA samples were reverse transcribed to generate cDNA, which was amplified with Taqman reagents on the ABI Prism 7700 sequence detection system (Applied Biosystems, Carlsbad, CA). Primers and probes were purchased from Applied Biosystems Gene Expression Assays. The genes assayed for each mouse were CCL19, CCL21, CXCL13, TNFα, IFNβ1, RANTES, IL-1, IL-6 or IL-7. Levels of gene expression were normalized to levels of GAPDH for each sample and the fold increase in signal was determined using the $\Delta\Delta C_T$ calculation recommended by Applied Biosystems. Results shown are log fold changes in gene expression compared to uninfected controls.

CCL19 and CCL21 ELISAs

Spleens portions were collected 18h following immunization, snap-frozen on dry ice and kept at -20°C. Samples were then processed as previously described (Wols et al., (2010)), with some modifications. In brief, spleens were homogenized in 1 ml PBS containing Complete protease inhibitor cocktail (Roche Applied Biosciences, Indianapolis, IN), sonicated (2 pulses of 5 sec at power 5) using a 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA), and centrifuged at 2000 rpm for 10 min. Supernatants were next filtered through a 1.2 µm Versapor® membrane Acrodisc® syringe filter (Pall Life Sciences, Ann Arbor, MI). The amount of CCL19 and CCL21 was assessed by ELISA according to the manufacturer's instructions (R&D Systems). The quantity of CCL19 and CCL21 in each sample was normalized to the total protein content determined by Bicinchoninic Acid Assay (Thermo Scientific, Rockford, IL).

Statistical analysis

All experiments were performed using 3-8 animals per group. Experiments were performed 2-4 times. Differences between groups were determined using a two-tailed unpaired Student's t test or two-way ANOVA followed by Bonferroni's post-tests (GraphPad Prism 5.0; GraphPad Software, Inc., San Diego, CA), as specified in figure legends. Differences were considered significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proliferation and priming of OTII donor cells in young and aged hosts. (A) CFSE-dilution profile of OTII donor cells recovered from the spleen and lymph nodes of young and aged hosts 1, 2, 3 and 4 days post-immunization. Histograms represent concatenated data from 4-5 mice per time point. (B) Percentage of OTII donor cells expressing CD69⁺ and (C) total number of OTII donor cells recovered from the spleen of young and aged hosts 12 to 120 hours post-immunization. Each time point represents the mean $\pm$ SEM of 4-5 mice per group of one representative experiment out of 2. Statistical significance was determined by 2-way ANOVA (Age and Time) followed by Bonferroni's posttests *, $p < 0.05$; ***, $p < 0.001$ of young vs aged groups. The interaction between age and time was extremely significant ($p < 0.0001$).

Figure 2.

OTII donor cell accumulation and activation in the splenic T cell zones of young and aged hosts. (A) Immunofluorescence staining of frozen spleen sections 18 hours post-immunization showing B cell follicles using B220 staining (green), donor CD4 T cells using thy1.1 staining (red) and nuclei using Hoechst staining (blue). The figure shows 2 representative follicles of young hosts (i and ii) and aged hosts (iii and iv). (B) Number of donor OTII cells per T cell zone 18 hours post-immunization. (C) Percentage (left panel) and number (right panel) of donor OTII cells in peripheral blood lymphocytes. Results represent the mean $\pm$ SEM of 4-5 mice/group from one representative experiment of 3. Statistical significance was determined using Student's unpaired t test. *, $p < 0.05$; ***, $p < 0.001$. (D) Immunofluorescence staining of frozen spleen sections three days post-immunization showing B cell follicles (white), donor CD4 T cells (red), CFSE (green), the proliferation marker Ki-67 (blue) from naïve (top panels), young (middle panels) or aged (lower panels) mice. The arrows in the inset of the merged images (last column) show some donor T cells still containing CFSE. All pictures were taken at a magnification of 200X. Scale bars represent 100 μm .

Figure 3.

Impact of hosts dendritic cells and CD4 T cells on OTII donor cell proliferation. (A, C, E and G) Numbers and (B, D, F and H) CFSE-dilution profiles of OTII donor cells recovered from the spleen of young or aged hosts three days post-immunization. (A-B) Hosts were previously treated with the CD4-depleting antibody GK1.5 or the isotype control administered i.p. Data are the mean $\pm$ SEM of 7-10 mice/group from 3 experiments. (C-D) CD4 deficient (CD4KO) mice were used as hosts. Data are the mean $\pm$ SEM of 4 young and 15 aged hosts from 1 of 2 experiments. (E-H) 2 hours prior to donor OTII cell transfer, young and aged mice received 10⁶ young (E and F) or aged (G and H) LPS-activated BMDCs pulsed with 50 μ g OTII peptide i.v. Data are the mean $\pm$ SEM of 8-10 mice/group from 2 experiments. Statistical significance was determined by 2-way ANOVA (A) or Student's unpaired t test (B, E and G). ns, not significant; *, $p < 0.05$; **, $p < 0.002$.

Figure 4.

Expression of the homeostatic chemokine CCL19, CCL21 and CXCL13 in the spleen of young and aged mice 18 hours post-immunization. Snap-frozen spleen portions were homogenized and analyzed for CCL19, CCL21 and CXCL13 mRNA content by real-time PCR (A), or for CCL19 and CCL21 protein content by ELISA (B). Data represent the mean $\pm$ SEM of 5-8 mice/group. Statistical significance was determined by unpaired Student's *t* test. ***, $p < 0.0001$. (C) Localization of CCL21 expression by immunofluorescence staining of frozen spleen sections showing the B cell follicles using B220 staining (green) and CCL21 (red). The figure shows 3 representative spleen sections from young (i-iii) and aged (iv-vi) hosts of at least 10. Each panel shows whole spleen sections created from stitched images taken at a magnification of 200X. Scale bars represent 500 μm . (D) Localization of CXCL13 expression by immunofluorescence staining of frozen spleen sections from one representative young (left panel) and aged (right panel) host. The arrows point to the marginal zone expression of CXCL13 in young mice that is absent in aged mice. Pictures were taken at a magnification of 200X. Scale bars represent 100 μm . (E) Number of OTII donor cells recovered from the spleen of *plt* and WT hosts 1, 2, 3 and 4 days post-immunization. (F) CFSE-dilution profile of OTII donor cells recovered from *plt* and WT mice 3 days post-immunization. The gate delimits the undivided population (48.6% for *plt* mice and 1.5% for WT mice). Error bars represent the mean $\pm$ SEM of 2-3 mice/group. Statistical significance was determined by 2-way ANOVA followed by Bonferroni's post-tests. ***, $p < 0.001$ WT vs *plt* mice.

Figure 5.

Donor CD4 T cell helper functions in young and aged hosts ten days post-immunization. (A) Representative flow cytometric dot plots of CXCR5 and PD-1 expression by the donor cells OTII cells. The gate shows the proportion of donor cells showing a typical Tfh phenotype (CXCR5^{hi} PD-1^{hi}). (B) Total number of donor cells expressing a Tfh phenotype per spleen of young and aged hosts using the gating strategy shown in (A). (C) Representative flow cytometric dot plots showing germinal center (GC) B cells (PNA^{hi} CD38^{lo}) from the CD19⁺ population. (D) Total number of GC B cells per spleen of young and aged hosts using the gating strategy shown in (C). Dot plots (A and C) shows concatenated data of 5-7 mice/group from 1 of 2 experiments. Data (B and D) represent the mean $\pm$ SEM of pooled data from 2 experiments with a total of 10-13 mice/group. Statistical significance was determined by Student's unpaired t test. ***, $p < 0.0002$. (E) Immunofluorescence staining of frozen spleen sections showing B cell follicles using B220 staining (blue) and germinal centers using GL-7 staining (green). Each panel shows whole spleen sections created from stitched images taken at a magnification of 200X. Scale bars represent 500 μ m. Shown is 1 representative picture of 5-7 mice/group.