Systemic toxoplasma infection triggers a long-term defect in the generation and function of naive T lymphocytes

David G. Kugler,1 Francis A. Flomerfelt,4 Diego L. Costa,1 Karen Laky,2 Olena Kamenyeva,3 Paul R. Mittelstadt,5 Ronald E. Gress,4 Stephan P. Rosshart,6 Barbara Rehermann,5 Jonathan D. Ashwell,3 Alan Sher,1 and Dragana Jankovic1

The thymus plays a critical role in adaptive immunity as the site where hematopoietic progenitors give rise to naive T cells ready to respond to antigen stimulation and undergo effector differentiation. Each T lymphocyte, educated by the highly specialized microenvironment of the thymic epithelium, expresses a unique self MHC–restricted TCR selected to recognize an antigenic peptide (Klein et al., 2014). The T cell repertoire is estimated to comprise at least 108 different TCR specificities, enabling the host to defend itself against a wide range of pathogens and malignancies. The thymus grows rapidly during prenatal life and infancy, but then enters an involution phase, leading to decreased production of naive T cells, resulting in impaired immune function during aging (Ventevogel and Sempowski, 2013; Nikolich-Zugich, 2014).

Under steady-state conditions, the peripheral T cell compartment consists of a major population of naive lymphocytes with diverse TCR specificities and a minor population of activated/memory T cells with specificities determined by previous exposure to foreign antigen (Jenkins et al., 2010). In the case of the CD4+ T cell response to infection, clones normally undetectable under steady-state conditions rapidly proliferate and differentiate into different subsets of Th effectors. This vigorous expansion alters the ratio between naive and activated CD4+ T cells. However, fully differentiated CD4+ T effectors are short-lived and contract upon successful control of infection, thereby normalizing the ratio of naive versus antigen-experienced T cells. This type of infection-induced oscillation in the activated T lymphocyte pool has been well documented for a variety of different pathogens (Homann et al., 2001; Schieman et al., 2003; McKinstry et al., 2010; Pepper et al., 2010). In contrast, the population of naive circulating T cells, essential for maintaining immune homeostasis, is considered to be relatively static because the rate of thymic output is independent of fluctuations in the peripheral T lymphocyte compartment (Gabor et al., 1997; Fink, 2013).

Although this homeostatic restoration is the norm, an imbalance in the frequency of activated versus naive T cells has been observed in certain diseases, such as rheumatoid arthritis and multiple sclerosis (Koetz et al., 2000; Hug et al., 2003; Haegert et al., 2011), and after acute control of hepatitis C, HTLV-1, and HIV viral infections (Yasunaga Ji et al., 2003; Haegert et al., 2011), and after acute control of hepatitis C, HTLV-1, and HIV viral infections (Yasunaga Ji et al., 2003; Haegert et al., 2011). In contrast, the population of naive circulating T cells, essential for maintaining immune homeostasis, is considered to be relatively static because the rate of thymic output is independent of fluctuations in the peripheral T lymphocyte compartment (Gabor et al., 1997; Fink, 2013).

This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License Attribution-Noncommercial-Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/.
Perez et al., 2015). In this regard, it is of interest that certain acute infections can lead to decreased numbers of lymphoid progenitors in the bone marrow and/or major reductions in immature T cells in thymus. Indeed, thymic atrophy is frequently observed in infections with viruses, bacteria, fungi, and parasites, and has been speculated to contribute to pathogen virulence (de Meis et al., 2012; Nunes-Alves et al., 2013).

Toxoplasma gondii is an intracellular protozoan parasite that triggers a potent IL-12–dependent Th1 response, which results in production of high levels of IFN-γ and TNF that efficiently control parasite replication in both hematopoietic and nonhematopoietic cells (Yap and Sher, 1999). Chronic infection is maintained by small numbers of parasite cysts localized in the CNS and contained by the residual T cell response (Suzuki et al., 1988). Regulation of the acute CD4 T lymphocyte response is an important aspect of the host–pathogen interaction, as it prevents clearance of the parasite while simultaneously protecting the host against T cell–mediated immune pathology (Gazzinelli et al., 1996; Villarino et al., 2003; Jankovic et al., 2007; Hall et al., 2012; Kugler et al., 2013). Interestingly, T. gondii is also known to induce thymic atrophy and does so in a variety of experimental animal models (Huldt et al., 1973), although the impact of this phenomenon on the host response to the endogenous infection or to resistance to heterologous pathogen challenge has not been addressed.

Here, we demonstrate that T. gondii infection rapidly triggers a profound and persistent reduction in the size of the peripheral naive CD4+ T cell pool. We further show that the resulting perturbation in T cell homeostasis is mechanistically associated with parasite-induced thymic atrophy and, more specifically, with a loss in the architectural integrity of the thymic epithelium. Moreover, this structural degeneration is accompanied by impaired TCR affinity maturation, as indicated by decreased CD5 expression on the few recent thymic emigrants (RTEs) that reach the periphery. Finally, we demonstrate that these alterations in the naive CD4+ T cell compartment lead to decreased host resistance to heterologous pathogen challenge and contribute to the maintenance of chronic T. gondii infection. Interestingly, the changes in thymic structure and function induced by toxoplasma closely resemble those associated with the thymic involution that occurs during aging, suggesting that infection-induced alterations in the thymus could be a factor promoting immunological senescence.

RESULTS

T. gondii triggers a rapid and persistent loss in naive T lymphocytes in the periphery

It has been established in numerous prior studies that acute T. gondii infection triggers activation of large numbers of CD4+ T cells, which rapidly acquire a Th1 phenotype. Using the AS15 tetramer, we found that the parasite-specific CD4 response peaks at day 7, greatly contracts as the acute infection is controlled, and persists at low levels into the chronic phase (Fig. 1, A and B). We further showed that the initial CD4 T cell expansion is the result of extensive expansion of activated Th1 effectors and is accompanied by apoptosis of the same cells (Fig. 1 D). In direct contrast, naive CD62L+CD44− CD4+ T lymphocytes examined in the same animals during the same period failed to display markers of either proliferation or death (Fig. 1 D). Nevertheless, when the absolute number of these cells was determined, a profound reduction in CD62L+CD44− CD4+ T cells was observed from day 9 onward, despite the contraction of the parasite-specific Th1 cell response during the same period (Fig. 1 C). The naive CD62L+CD44− CD8+ T cell population was also reduced in these infected animals (Fig. 1 C).

We therefore focused on the population of RTEs by using mice carrying a GFP transgene driven by the RAG2 promoter, in which RTE can be identified as GFP-RAG+ T cells (Berkley et al., 2013). Unexpectedly, the reduction in the naive CD4+ T lymphocyte pool size correlated with a decrease in the frequency of GFP-RAG+ RTE in T. gondii–infected reporter mice (Fig. 2 A). Initially, this decrease occurred preferentially within the brightest GFP-RAG+ CD4+ subpopulation, whereas in chronic phase, the entire population of RTE was affected both locally in spleen (Fig. 2, A–C) and lymph nodes (not depicted), as well as systemically in peripheral blood (Fig. 2 C). A similar decrease in the frequency of GFP-RAG+ CD8+ RTE was observed in the same tissue sites (Fig. 2, B and C; and not depicted).

T. gondii infection induces a persistent thymic atrophy that is largely glucocorticoid (GC) independent

The observed decrease in RTE in T. gondii–infected mice suggested the involvement of the thymic atrophy previously described in these animals (Huldt et al., 1973). Indeed, when examined on day 10, infected mice displayed a marked (>95%) reduction in the number of thymocytes regardless of the route of parasite inoculation (Fig. 3 A), host genetic background (Fig. 3 B), or age of animals (8–12 wk vs. 5 mo vs. 7–8 mo; not depicted) at the time of infection.

We have previously shown that GCs are induced during acute T. gondii infection (Kugler et al., 2013), and this type of stress response is known to lead to thymic atrophy caused by loss of CD4+CD8+ (double-positive; DP) thymocytes. Nevertheless, in T. gondii–infected mice, the loss in thymocytes was more general and not restricted to this DP population (Fig. 3, C and D). To directly evaluate the role of GC in toxoplasma-induced thymic atrophy, we selectively infected targeted knockout mice lacking the GC receptor (GR) in T cells. Although, as expected, the preferential loss in DP thymocytes was less pronounced in these animals (Fig. 3, E and F), they displayed a profound reduction in the number of these cells (Fig. 3, C and D) at the time of infection or death (Fig. 1 D). Nevertheless, when the absolute number of these cells was determined, a profound reduction in CD62L+CD44− CD4+ T cells was observed from day 9 onward, despite the contraction of the parasite-specific Th1 cell response during the same period (Fig. 1 C). The naive CD62L+CD44− CD8+ T cell population was also reduced in these infected animals (Fig. 1 C).
mice treated simultaneously with neutralizing mAb against IL-6, TNF, and IFN-γ (Fig. 4 B). Similarly, T. gondii infection triggered a reduction in thymic cellularity in RAG−/−, IFN-γ−/−RAG−/−, and p40 IL-12−/−IL-10−/−RAG−/− mice comparable to that displayed by WT animals (Fig. 4 C). Thus, the loss of thymic function in toxoplasma infection does not appear to be dependent on the host cytokine response and is only partially dependent on GC signaling.

The thymic atrophy occurring during T. gondii infection is associated with a loss in architectural integrity of the thymic epithelium

During the acute phase, T. gondii disseminates via the blood stream to multiple tissue sites. Because the thymus is a heavily vascularized organ, we hypothesized that the observed damage to the thymic function might be related to the presence of the parasite. Indeed, the dissemination of live and replicating parasites was found to be required for T. gondii–induced thymic atrophy, as challenge with irradiated or temperature-sensitive mutant of the parasite that cannot establish active infection do not trigger thymic atrophy (Fig. 5 A). In addition, loss of thymocytes was not observed in previously vaccinated hosts challenged with wild-type parasites (Fig. 5 B). To identify the cell populations infected within the thymus, we exposed mice to mCherry labeled parasites and performed FACS analyses on total thymic cell preparations. Toxoplasma was detected at similar levels (MFI) in CD11b+ myeloid cells, thymocytes, and thymic epithelial cells (TECs). Although most of the infected cells were myeloid cells (73 ± 7%) and thymocyte (18 ± 5%), the TEC population was the most heavily infected on cell per cell basis (Fig. 5 C, left). Within the TEC population, both, cortical TEC (cTEC) and medullar TEC (mTEC) were found to harbor the parasite (Fig. 5 C, right) although at different ratios depending on the experiment.

Normal thymic function is known to be influenced by interaction of thymocytes with thymic epithelium. To explore the possible role of this process in the decreased thymopoiesis occurring in T. gondii infection, we enumerated the
Infection-induced thymic atrophy dampens immunity | Kugler et al.

Infection-induced thymic atrophy dampens immunity | Kugler et al.

major cellular constituents of the thymic epithelium in mice exposed to the parasite 8 d previously. We observed major decreases (>50%) in the total numbers of TECs, as well as the individual Ly51+ cortical and UEA+ medullar populations when compared with uninfected control animals (Fig. 5 D). As expected the same alterations in TECs were observed in infected GR-/- mice.

Histological examination on day 8 after infection demonstrated that T. gondii infection leads to dramatic changes not only in the size but also in the architecture of the thymus (Fig. 5 E). In addition, staining with anti-keratin 5 and anti-keratin 8 mAbs revealed major alterations in the organization of the cortical and medullary areas of the thymus as indicated by decreased cortical surface area and increased convolution of the corticomedullary border (Fig. 5, F and G).

Persistent loss of thymic function during chronic T. gondii infection

In most cases involving GC-dependent thymic atrophy, recovery of normal thymic function is restored once GC levels return to baseline. However, despite the previously documented transience of the acute GC response induced by T. gondii (Kugler et al., 2013), only limited recovery in thymocyte numbers in WT mice was observed during the chronic phase of infection (Fig. 6 A), although overall thymocyte composition appeared to normalize (Fig. 6 B), and parasite mRNA could no longer be detected in thymus (Fig. 6 D).

To examine the status of thymic epithelium, we analyzed the expression of transcription factor Tbata (Flomertfelt et al., 2010) and the cytokine IL-22 (Dudakov et al., 2012), which are both known to be involved in the regulation of TEC expansion. When mRNA levels for these two proteins were measured by qRT-PCR in thymi on day 8 after infection low Tbata and high IL-22 levels were observed (Fig. 6 C). This suggested that the remaining TECs are responding to damage and attempting to undergo a regenerative process because Tbata inhibits, whereas IL-22 promotes, TEC growth. Interestingly, however, levels of Tbata and IL-22 were found to normalize when measured again on day 60 after infection despite the persistent atrophy (Fig. 6 C) indicating that the new equilibrium achieved in the chronic phase is stable. The observed changes in the expression of Tbata and IL-22 mRNA were not caused by differences in the total number of TEC because similar levels of FoxN1 mRNA were de-
tected at both time points (Fig. 6 C). Nevertheless, the profound changes in the organization of the thymic epithelium as indicated by decreased cortical surface area and increased convolution of the corticomedullary border were found to persist into the chronic phase of infection (Fig. 6 E), as well as the significantly decreased cTEC and mTEC absolute numbers (Fig. 6 F).

*T. gondii*-induced thymic atrophy is directly dependent on parasite-induced alteration of the thymic epithelium

*T. gondii* infection triggers mobilization of BM progenitors, as well as BM hypoplasia (Petakov et al., 2002; Glatman-Zaretzky et al., 2014). Indeed, we observed a partial and transient reduction in the number of BM cells in infected animals (unpublished data). To determine whether the persistent thymic atrophy observed during *T. gondii* infection is a consequence of an effect on the thymic epithelium or on the thymic precursors in BM, we performed experiments in which irradiated chronically infected mice were reconstituted with BM from chronically infected animals. After allowing reconstitution for 8 wk, we assessed thymic, BM, and splenic cellularity in both groups (Fig. 7 A). We found that the uninfected mice reconstituted with BM from infected donors displayed the same cellularity in all organs as intact uninfected C57BL/6 animals. In direct contrast, infected mice reconstituted with naive BM displayed normal levels of cells in BM and spleen, but a profound cellular deficiency in the thymus.

Because irradiation is known to induce thymocyte and TEC depletion, we performed an additional set of experiments using RAG−/− mice reconstituted with BM cells in the absence of irradiation. Once again, transfer of naive WT BM failed to reconstitute thymic cellularity in infected recipient, whereas fully reconstituting uninfected animals (Fig. 7 B).

The aforementioned experiments suggested that thymic atrophy in *T. gondii*-infected mice is caused by an effect on a nonhematopoietic component of that organ. To test whether thymic epithelium is the targeted cellular component, we implanted neonatal thymi from RAG−/− mice into naive or...
Infection-induced thymic atrophy dampens immunity | Kugler et al.

chronically WT infected animals. When examined 6 wk later, both the cellularity and histology of the thymic implants (unpublished data) were indistinguishable in the two sets of recipients, despite the expected significant difference in the size of the endogenous orthotopic thymi (Fig. 7 C).

Together these findings argued that the primary defect responsible for the persistent thymic atrophy observed during toxoplasma infection is a loss in epithelial integrity.

T cells display decreased CD5 levels during chronic T. gondii infection

Because TEC provide a critical microenvironment for T cell selection, we questioned whether the thinning of the cortex, and loss of corticomedullary demarcation occurring in thymi of toxoplasma infected mice influences not only the number of thymocytes but also the immunocompetence of naive T cells. CD5 expression on naive T lymphocytes has proven to be a reliable read-out for the strength of the positively selecting TCR–peptide–MHC interaction in thymic cortex (Azzam et al., 1998; Klein et al., 2014). When assessed in our model, we found that the level of CD5 expression on single-positive CD4+ or CD8+ thymocytes was decreased in chronically infected mice when compared with same populations in uninfected mice (Fig. 8, A and B). Importantly, this reduction in CD5 expression was also evident in peripheral GFP-RAG+ RTE, as well as in the total population of naive CD4 and CD8 T cells in T. gondii chronically infected mice (Fig. 8, C and D). These results suggested that the thymic epithelial alterations seen in T. gondii-infected mice may not only affect the number of naive T cells generated (Fig. 1 C), but also result in the development/selection of T cells with lower TCR avidity for self-peptide–MHC.

Consistent with the latter hypothesis, we observed a significantly decreased ratio of CD4/CD8 expression among mature TCRhigh thymocytes in chronically infected when compared with uninfected mice (2.29 ± 0.17 vs. 3.36 ± 0.24).

T lymphocytes expressing low levels of CD5 are known to be poorly responsive to foreign antigens (Mandl et al., 2013; Persaud et al., 2014). Indeed, an attenuated response was observed when naive splenic CD4+ T lymphocytes from 60 d T. gondii–exposed mice were stimulated in an allogeneic mixed lymphocyte reaction using irradiated BALB/c splenocytes (Fig. 9 A). In contrast, we demonstrated that the same cell population displayed normal responsiveness when polyclonally activated with anti-CD3 mAb (Fig. 9 B).

The contraction of the naive T cell pool in T. gondii infection is associated with decreased immunity to heterologous microbial challenge

To assess the in vivo responsiveness of naive CD4 T cells in mice chronically infected with T. gondii, we used a model of heterologous microbial challenge. Bacillus Calmette–Guérin (BCG) was chosen for this purpose because it produces a self-limiting infection that, similar to T. gondii, stimulates a Th1 immune response without triggering thymic atrophy. In addition, in this model, mycobacteria–specific CD4+ T cell responses can be readily tracked using an I–Aβ–Ag85b tetramer.
When assessed 25 d after BCG challenge, mice chronically infected with toxoplasma displayed fourfold higher bacterial CFU in both liver and spleen (Fig. 9 C). This increase in bacterial load was accompanied by a dramatic reduction in the number of Ag85b-specific CD4+ T cells in both organs. The impaired BCG response did not appear to be the result of defective APC function, as irradiated splenocytes from T. gondii–infected mice were able to efficiently prime TCR
transgenic P25 CD4+ T cells specific for Ag85b (unpublished data). In addition, no differences in parasite cyst burden were observed between unchallenged and BCG-challenged T.gondii-infected mice (Fig. 9D), excluding an indirect effect of secondary mycobacterial exposure on the primary protozoan infection as an explanation for the altered response to BCG.

**Restoration of thymic function enhances control of chronic T. gondii infection**
The aforementioned findings raised the interesting possibility that thymic atrophy contributes to parasite persistence itself. In this scenario, acute infection with T. gondii would lead to suppression of the response to stage-specific surface antigens expressed by the parasite during the chronic phase (Kim and Boothroyd, 2005). To test this hypothesis, we examined the effect of thymic grafting on parasite burdens in chronically infected mice. We found that 60-d T. gondii exposed animals implanted with thymi from naive RAG−/− mice displayed a significant threefold reduction in brain cyst loads compared with nonimplanted controls (Fig. 9E). The latter finding supports the concept that parasite-induced thymic involution promotes chronic infection.

**DISCUSSION**
The thymus plays a major role in the immune system by providing a continuous source of naive T lymphocytes with
a wide variety of TCR specificities that recognize a broad range of foreign antigens (Miller, 2011). As the body reaches adulthood, the gradual involution of the thymus accelerates likely reflecting the increasing need to prevent tolerance to foreign antigen and, conversely, the decreasing need to delete self-antigen–specific T cells (Dowling and Hodgkin, 2009; Shanley et al., 2009). In an ideal situation, host exposure to infections and other antigenic challenges should not affect the normal homeostatic function of the thymus in maintaining T cell availability and diversity. Nevertheless, a wide variety of pathogens trigger an atrophic state in which the thymus undergoes an accelerated decrease in cellularity that in some (Ross et al., 2012), but not all, situations (Douek et al., 1998; Bonzon and Fan, 1999; Borges et al., 2012) rebounds to steady-state levels after acute infection. This infection-induced thymic atrophy has been postulated to be a mechanism promoting pathogen virulence. However, the impact of infectious exposures on the normal age-dependent process of thymic involution is poorly understood.

In this study, we describe a long-term effect of T. gondii infection on the ability of the host to generate naive T cells. This immune alteration is associated with a profound and persistent thymic atrophy that reduces not only the size of the naive T lymphocyte pool but also the functional competence of those cells that successfully emerge. The resulting immunocompromised state manifests in decreased resistance to both heterologous pathogen challenge and established T. gondii infection itself.

Previous studies on thymic atrophy have identified GR–mediated apoptosis of DP thymocytes as an important mechanism underlying this process (Savino, 2006; Pérez et al., 2007). In contrast, the data presented here demonstrate that
Infection-induced thymic atrophy dampens immunity | Kugler et al.

T cell–intrinsic GR signaling is not the major trigger of T. gondii–induced thymic atrophy. Instead, thymic atrophy was closely associated with dramatic changes in the architecture of the thymic epithelium. Interestingly, several clinical reports describe similarly altered thymic architecture in autopsied T. gondii–infected humans (Frenkel and Friedlander, 1951; Yermakov et al., 1982).

The thymus is a highly vascularized organ, but is deficient in immune effector cells relative to secondary lymphoid tissue. This may favor the accumulation of pathogen-infected CD11b cells, which unchecked by the immune response can serve as Trojan horses delivering intracellular microbes to thymocytes and TEC. T. gondii promiscuously invades multiple cell types and, as described here, can infect thymic epithelium during its acute blood-borne dissemination. Nevertheless, the subsequent permanent changes in TEC do not appear to depend on the continued physical presence of the parasite in the tissue. Moreover, our attempts to regenerate the thymus in T. gondii–exposed mice by administering keratin growth factor, and IL-22 were unsuccessful (unpublished data). Based on these observations, we speculate that the persistent thymic atrophy occurring during toxoplasma infection may stem from parasite invasion and the permanent destruction of TEC progenitor cells.

Although not well studied, the thymic atrophy occurring in other infections may also involve related GC–independent mechanisms of TEC impairment. For example, the GC–independent thymic involution seen in mice exposed to Mycobacterium avium is associated with the presence of infected macrophages which accumulate in that organ during the chronic phase (Morrison et al., 1982; Borges et al., 2012). Similarly, in murine influenza (PR8) the tropism of the virus for epithelium may contribute to the thymic atrophy seen in this infection because damage of thymic epithelium was observed even in adrenalectomized mice (Garaci et al., 1974). Likewise, measles infection has been shown to induce terminal differ-
entiation of cortical TEC in vitro (Numazaki et al., 1989) and, importantly, apoptosis of human TEC in a SCID-hu mouse model (Auwaerter et al., 1996). In addition, during chronic LCMV infection, infectious virus has been detected in both thymocytes and cortical stromal cells (King et al., 1992). Consistent with the latter findings, we have observed significant reductions in the numbers of splenic CD44−CD62+ CD4+ and CD8+ T lymphocytes in LCMV-infected mice on day 40 after infection (unpublished data).

Regardless of the mechanism of its induction, infection-triggered thymic atrophy has been postulated to play a role in promoting pathogen persistence. For example, infection of the thymus with M. avium has been proposed to induce a state of central tolerance to antigens of the pathogen, thereby facilitating its long-term survival in the host (Nobrega et al., 2010, 2013). Alternatively, by causing a wholesale decrease in thymic output, atrophy may result in a generalized immunosuppression affecting both pathogen-specific and irrelevant effector T cell responses. Our findings are consistent with the latter outcome.

T lymphocyte expression of CD5 in the periphery is known to correlate with the affinity of TCR for self-antigen and indirectly for foreign antigen, a property imprinted during T cell differentiation in the thymus. It was therefore of interest that both CD4 and CD8 thymocytes, the initial RTE appearing after thymic involution during acute infection (unpublished data) and the subsequent pool of naive T cells present during the chronic phase, express decreased levels of CD5. These observations suggest that the damage to the thymic epithelium induced by T. gondii results in impaired positive selection of the thymocyte repertoire that lessens the ability of the host to mount an adequate immune response. Indeed, chronically infected mice display an increased susceptibility to co-infection with a heterologous pathogen, as well as improved survival of the primary infection. In addition, we have observed a highly significant negative correlation between cyst burden and thymocyte numbers in mice chronically infected with T. gondii (unpublished data), an observation also consistent with a role for thymic involution in parasite persistence.

An interesting aspect of the alterations in thymic architecture and function described here in murine T. gondii infection is that they resemble the changes in this organ observed during natural aging. Thus, senescence of the immune system.

Figure 9. The antigen-dependent responsiveness of CD4+ T cells is compromised during chronic T. gondii infection. (A and B) Decreased antigen responsiveness of naive T cells in chronically infected mice. [H]-thymidine incorporation of CD62+CD44− CD4+ T lymphocytes isolated from mice on day 0 and 60 after infection and cultured for 60 h with indicated number of irradiated BALB/c splenocytes (A) or stimulated with increasing concentrations of anti-CD3 mAb (B). Data are shown as mean ± SD cpm from duplicate cultures from one representative out of two (A) and three (B) experiments performed. (C) Number of BCG CFU (left) and Ag85−CD4+ T cells (right) in liver and spleen of control and 50 d T. gondii-infected mice challenged in parallel with BCG for 25 d. Bars represent the mean ± SEM of values for individual mice (n = 6–10) pooled from two independent experiments. (D) BCG challenge does not affect the parasite burden in chronically infected mice. Data are shown as mean ± SEM of values for individual mice (n = 5) from one representative out of two experiments performed. (E) Increased host resistance of T. gondii chronically infected mice after RAG−/− thymic implant. Bars represent the mean ± SEM of values for individual mice (n = 4–12) from two independent experiments.

* P < 0.05; ** P < 0.01; *** P < 0.001.
system is associated with gradual structural deterioration of the thymic epithelium (Gui et al., 2007; Kim et al., 2015) and a concurrent decline in the frequency of peripheral naïve T lymphocytes (Fagnoni et al., 2000; Naylor et al., 2005; Schulz et al., 2015). These parallel manifestations of infection and senescence raise the intriguing possibility that exposure during childhood to pathogens that, along with T. gondii, target the thymus could accelerate the natural aging process, rendering the host more susceptible to new infections. A proposed link between prior infectious exposure and the kinetics of immune senescence is supported by several observations (Gavazzi and Krause, 2002; Deeks, 2011; Derhovanessian et al., 2014). Perhaps the best studied example is the association of seropositivity for cytomegalovirus (CMV), a pathogen known to infect TEC (Mocarski et al., 1993; Price et al., 1993), and accelerated aging of the immune system (Pawelec et al., 2010). Although this phenomenon has been attributed to memory T cell inflation (Arens et al., 2015), the results presented here raise virus-induced thymic disruption as a possible alternative explanation of the CMV infection data. Future studies that analyze in depth the longitudinal relationship between age, infection history, and thymic output could be used to evaluate the contribution of this proposed mechanism to immune senescence.

**MATERIALS AND METHODS**

**Experimental animals**

C57BL/6 and BALB/c mice were purchased from Taconic Farms, whereas C57BL/6 CD45.2 RAG1−/−, CD45.1 RAG1−/−, IL-10−/−, and IFNAR (IFN-α/βR−/−) mice were provided by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Supply Contract at Taconic Farms. TNF−/−, IFN-γR−/−, and CCR2−/− animals were obtained from The Jackson Laboratory; STAT1−/− animals (Durbin et al., 1996) were provided by D. Woodland (Trudeau Institute, Saranac Lake, NY); MyD88−/−, MyD88−/−TRIF−/− double-deficient (Yamamoto et al., 2003), IL-27R−/− (Batten et al., 2008), GFP-RAG (Yu et al., 1999), and GrkCre (Mittelstadt et al., 2012) mice were bred in our facilities. IFN-γ−/−RAG−/− and p40−/−IL-10−/−RAG−/− animals were generated by intercrossing mice with the respective single gene deficiency. All animals were maintained at an American Association for the Accreditation of Laboratory Animal Care–accredited and specific pathogen–free facility at the NIAID/National Institutes of Health or National Cancer Institute (NCI)/National Institutes of Health. All procedures were performed in accordance with the protocols outlined in the Guide for the Care and Use of Laboratory Animals and described in an animal study proposal approved by the NIAID Animal Care and Use Committee. 8–12-wk-old age- and sex-matched experimental and littermate control mice were used in all experiments, except when noted otherwise.

**T. gondii infection and parasite burden determination**

Type II avirulent strain ME-49 cysts were obtained from the brains of chronically infected C57BL/6 mice. Cyst preparations were pepsin treated to eliminate potential contamination with host cells and mice were inoculated i.p. with an average of 15 cysts. Parasite burden was assessed by enumerating infected cells in cytospin-smeared of PEC (105 cells) during the acute phase of infection or by counting cysts in brain homogenates during the chronic phase. Tachyzoites of the RH-88 type I strain of T. gondii, its temperature sensitive mutant, ts-4 (Pfefferkorn and Pfefferkorn, 1976), and an mCherry expressing recombinant (Koshy et al., 2010), were cultured and harvested from infected monolayers of human foreskin fibroblasts.

**Vaccination experiments**

Groups of mice were vaccinated by two biweekly i.p. injections of 2 × 10⁵ ts-4 tachyzoites in 0.5 ml PBS, while a control group was injected with PBS alone. Both groups were challenged in parallel 2 wk later by subcutaneous injection of 1,000 virulent RH strain tachyzoites.

**BCG cultures, infections, and quantitation**

*M. bovis* BCG (strain Pasteur) were expanded to log phase in Middlebrook 7H9 liquid medium (Difco) supplemented with OADC (Difco), 0.05% glycerol (Invitrogen), and 0.05% Tween-80 (Thermo Fisher Scientific), washed, aliquoted in PBS, and stored at −80°C until further use. Animals were inoculated i.v. with 5 × 10⁶ CFU. Quantification of bacterial stocks and the numbers of viable bacteria in whole-organ homogenates on day 25 after infection were measured by plating serial dilutions supplemented Middle-brook 7H11 nutrient agar (Difco) with OADC and 0.5% glycerol. Plates were incubated for 20 d at 37°C before counting bacterial colony formations.

**In vivo mAb treatment**

To block TNF, IFN-γ, and IL-6 function mice were injected i.p. with the combination of 1 mg each of neutralizing antibody XT3-11, XMG-1.2, and MP5-20F3 (BioXcell) on days −1, 1, 3, 5, and 7 after infection. Control groups of animals received an equivalent amount of rat mAb GL113.

**Cell preparation and flow cytometry analyses**

At the indicated time points, single-cell suspensions were prepared from peritoneal exudate cell (PEC), spleen, liver, thymus, or BM from individual naïve and infected animals, and after counting nucleated cells were used for further analysis (Jankovic et al., 2007). Samples from individual animals were stained with Fixable Viability Dye (eBioscience) and an appropriate combination of mAb specific for CD4 (RM4-5), CD8 (53–6.7), CD44 (IM7), CD62L (MEL14), TCRβ (H57–597), T-bet (O4–46), CD5 (53–7–3), CD24 (M1/69) B220 (RA3–6B2), NK1.1 (PK136), CD90.1 (HS51), CD90.2(53–2.1), CD45.1 (A20), or CD45.2. (104) purchased from eBioscience, BioLegend, or BD. Parasite-specific CD4⁺ T cells were determined using a fluorescently labeled MHC class II tetramer bound to *T. gondii* antigenic peptide AS15.
(Grover et al., 2012), whereas BCG-specific CD4+ lymphocytes were detected with a fluorescently labeled Ag85b260-294-loaded I-A\(^b\) tetramer (Vogelzang et al., 2014), both provided by the National Institutes of Health Tetramer Core Facility (Bethesda, MD).

**Proliferation assay**

Splenic naive Th lymphocytes were isolated as CD62L\(^+\)CD44\(^-\) CD4\(^+\) T cells by sorting total splenocytes on a FACSARia III (BD) and gating out B220\(^+\), NK1.1\(^+\), I-A/I-E\(^b\), and CD8\(^+\) T cells. Naive CD4\(^+\) T lymphocytes (2 × 10\(^5\)/well) were stimulated with indicated number of irradiated BALB/c spleenocytes. Alternatively, naive CD4\(^+\) T cells (7.5 × 10\(^5\)/well) were cultured in 96-well plates coated with 1 µg of anti-CD28 (37–51) and increasing amounts of anti-CD3 mAbs (2C11). After 48 h of incubation, cultures were pulsed with \(^3\)H-thymidine for an additional 18 h before harvesting.

**TEC isolation**

Thymus from uninfected and infected mice were processed individually by mincing in a Mickle Tissue Homogenizer, followed by incubation at 37°C for 15 min and shaking at 50 RPM for 37°C in 0.5 ml RPMI containing 20 µl of Liberase TH (28 U/ml) and DNase1 (5 µg/ml). Phenotypic analysis of thymic epithelial cells was performed on CD45\(^{neg}\) cells stained with anti-EpCAM (G8.8) Ulex Europaeus Agglutinin I (Vector Laboratories), anti-Ly51 antibody (BP-1), anti-CD31 antibody (390), and anti-MHC class II antibody (M5/114). The TEC-gating strategy as described previously (Xing and Hogquist, 2014) is shown in Fig. S1.

**Bone marrow reconstitution**

In one set of experiments, 48-d T. gondii–infected and uninfected age-matched C57BL/6 mice were lethally irradiated with 950 rad and subsequently reconstituted with 2 × 10\(^6\) congenic whole bone marrow cells using mismatched CD45.1/2 markers from either uninfected or 48-d T. gondii–infected donors, respectively. Alternatively, 8-d T. gondii–infected and uninfected age-matched RAG2\(^{-/-}\) mice were injected i.v. with 10\(^7\) syngeneic bone marrow cells from naive WT donors after depletion of T lymphocytes by negative selection on CD90.2 MACS columns (Miltenyi Biotech). All BM recipients were maintained on antibiotic TMS (0.5 mg/ml), and 0.67 mg/ml Sulfamethoxazole (0.67 mg/ml) throughout the duration of the experiment 8 and 6 wk (0.13 mg/ml), and 0.13 mg/ml Trimethoprim (0.13 mg/ml), Sulfadiazine Vitrogen). Gene expression analysis was performed using SYBR Green-based real-time quantitative PCR (RT-qPCR) on an ABI Prism 7900HT analyzer (Applied Biosystems). Arbitary units represent the ratio of tested mRNA levels compared with hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA levels. The following primer pairs were used: Tbata: 5′-TCAGGGACGATGTTCTCTGGT-3′ (forward) and 5′-GGTTCCAGATGGACTGTGA-3′ (reverse); Il22: 5′-CCTGCTGCCCTGCTGCA-3′ (forward) and 5′-TCTCCTGCTGGCGAG-3′ (reverse); Foxn1: 5′-TCAGGGACGATGTTCTCTGGT-3′ (forward) and 5′-GGTTCCAGATGGACTGTGA-3′ (reverse); Mouse K5: 5′-CATCTCCACAGGAAG-3′ (forward) and 5′-AGCAGGCTAGAGCTCCTGTTCAC-3′ (reverse); and 5′-GCAGTCTGGTGTCGCA-3′ (forward) and 5′-GCAGTCTGGTGTCGCA-3′ (reverse) (Burg et al., 1989).

**Histology and immunofluorescent microscopy**

Tissues were fixed in Bouin's fixative, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For immunofluorescence, sections of a tissue embedded in OCT freezing media were prepared on a CM3050s cryostat (Leica), adhered to Superfrost Plus slides (VWR), and subsequently permeabilized and blocked in PBS containing 0.3% Triton X-100 (Sigma-Aldrich) plus 10% normal mouse serum (Jackson ImmunoResearch Laboratories). Staining was performed with rabbit anti-K5 mAb (EP1601Y; Abcam) and rat anti-K8 mAb (TROMA-I; DSHB). Histological sections were evaluated (based on a 1–10 scoring system) for two parameters: cortical area that measures the distance from the thymic capsule to the corticomedulary border and convolution of the corticomedulary border that measures the length of the junction between cortex and medulla.

**Confocal immunofluorescence microscopy**

Isolated mouse thymii were sliced into 8–10-µm sections using a cryostat and stained with fluorescently labeled anti-K5, K8, and CD31 mAbs. Confocal microscopy of immunostained sections was performed using a Leica SP8 inverted five-channel confocal microscope equipped with 2 HyD ultra-sensitive detectors (Leica) and a broad range of visible lasers. The microscope configuration was adjusted for three-dimensional analysis (x, y, z) of cell segregation within tissue sections, and 20-µm z stacks were collected.

**Thymus grafting**

Thymus were recovered from newborn congenitally marked C57BL/6 CD45.1 RAG1\(^{-/-}\) donor mice. Recipient 48-d T. gondii–infected and uninfected age-matched C57BL/6 animals were shaved and scrubbed, and a suitable incision was made in the right shoulder blade in which the donor thymus was inserted. The topical analgesic bupivacaine was applied into the incision for immediate post-surgical analgesia, and the wound was closed with two sterile wound clips.

**Quantitative RT-PCR**

Total RNA was isolated (RNeasy Mini kit; QIAGEN) and reverse-transcribed (SuperScript II Reverse transcription; Invitrogen). Gene expression analysis was performed using SYBR Green-based real-time quantitative PCR (RT-qPCR) on an ABI Prism 7900HT analyzer (Applied Biosystems). Arbitrary units represent the ratio of tested mRNA levels compared with hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA levels. The following primer pairs were used: Tbata: 5′-TCAGGGACGATGTTCTCTGGT-3′ (forward) and 5′-GGTTCCAGATGGACTGTGA-3′ (reverse); Il22: 5′-CCTGCTGCCCTGCTGCA-3′ (forward) and 5′-TCTCCTGCTGGCGAG-3′ (reverse); Foxn1: 5′-CATCTCCACAGGAAG-3′ (forward) and 5′-AGCAGGCTAGAGCTCCTGTTCAC-3′ (reverse); and 5′-GCAGTCTGGTGTCGCA-3′ (forward) and 5′-GCAGTCTGGTGTCGCA-3′ (reverse) (Burg et al., 1989).

**Statistical analysis**

The statistical significance of differences between data means was evaluated using an unpaired, two-tailed Student's t test.
and, in the case of qRT-PCR data, by nonparametric ANOVA (Kruskal-Wallis) with multiple-comparisons.

Online supplemental material
Fig. S1 shows the gating strategy for FACS analysis of TEC.

ACKNOWLEDGMENTS
We thank Calvin Eigsti for performing FACS sorting, Sandy White, Sara Hieny, and Deborah Surman Depew for technical help with toxoplasma infections, and Sharon Evans for assistance with thymus grafting. We are also grateful to Dr. Rima McLeod for discussions and input concerning human congenital toxoplasmosis.

This work was supported by the Intramural Research Programs of the National Institute of Allergy and Infectious Diseases and National Institute for Diabetes and Digestive and Kidney Diseases, National Institutes of Health and by an Intramural AIDS Research Fellowship (to D.G. Kugler).

The authors declare no competing financial interests.

Submitted: 14 October 2015
Revised: 5 August 2016
Accepted: 18 October 2016

REFERENCES
Arens, R., E.B. Remmerswaal, J.A. Bosch, and R.A. van Lier. 2015. 5th International Workshop on CMV and Immunosenescence - A shadow of cytomegalovirus infection on immunological memory. *Eur. J. Immunol.* 45:954–957. http://dx.doi.org/10.1002/eji.201570044

Auwaerter, P.G., H. Kaneshima, J.M. McCune, G. Wiegand, and D.E. Griffin. 1996. Measles virus infection of thymic epithelium in the SCID-hu mouse leads to thymocyte apoptosis. *J. Virol.* 70:3734–3740.

Azzam, H.S., A. Grinberg, K. Lui, H. Shen, E.W. Shores, and P.E. Love. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J. Exp. Med.* 188:2301–2311. http://dx.doi.org/10.1084/jem.188.12.2301

Batten, M., N.M. Kljavin, J. Li, M.J. Walter, EJ. de Sauvage, and N. Ghilardi. 2008. Cutting edge: IL-27 is a potent inducer of IL-10 but not Foxp3 in murine T cells. *J. Immunol.* 180:2752–2756. http://dx.doi.org/10.4049/jimmunol.180.5.2752

Berkley, A.M., D.W. Hendricks, K.B. Simmons, and PJ. Fink. 2013. Recent thymic emigrants and mature naive T cells exhibit differential DNA methylation at key cytokine loci. *J. Immunol.* 190:6180–6186. http://dx.doi.org/10.4049/jimmunol.1300181

Bonzon, C., and H. Fan. 1999. Moloney murine leukemia virus-induced pre-leukemic thymic atrophy and enhanced thymocyte apoptosis correlate with disease pathogenicity. *J. Virol.* 73:2434–2441.

Borges, M., P. Barreirasilva, M. F, M.B. Jordan, M. Correia-neves, and R. Appelberg. 2012. Molecular and cellular mechanisms of *Mycobacterium avium*-induced thymic atrophy. *J. Immunol.* 189:3600–3608. http://dx.doi.org/10.4049/jimmunol.1201525

Burg, J.L., C.M. Grover, P. Pouletty, and J.C. Boothroyd. 1989. Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction. *J. Clin. Microbiol.* 27:1787–1792.

Cabrera-Perez, J., S.A. Condotta, B.R. James, S.W. Kashem, E.L. Brincks, D. Rau, T.A. Kucaba, V.P. Badovinac, and T.S. Griffith. 2015. Alterations in antigen-specific naive CD4 T cell precursors after sepsis impairs their responsiveness to pathogen challenge. *J. Immunol.* 194:1609–1620. http://dx.doi.org/10.4049/jimmunol.1401711

Deeks, S.G. 2011. HIV infection, inflammation, immunosenescence, and aging. *Annu. Rev. Med.* 62:141–155. http://dx.doi.org/10.1146/annurev-med-042909-093756

de Men, J., D.Aurélio Farias-de-Oliveira, PH. Nunes Panzenhagen, N. Maran, D.M. Villa-Verde, A. Morrot, and W. Savino. 2012. Thymus atrophy and double-positive escape are common features in infectious diseases. *J. Parasitol.* 2012:574020. http://dx.doi.org/10.1155/2012/574020

Derhovanessian, E., A.B. Maer, K. Hähnel, J.E. McElhaney, E.P. Slagboom, and G. Pawelec. 2014. Latent infection with cytomegalovirus is associated with poor memory CD4 responses to influenza A core proteins in the elderly. *J. Immunol.* 193:3624–3631. http://dx.doi.org/10.4049/jimmunol.1303361

Douck, D.C., R.D. McFarland, PH. Keiser, E.A. Gage, J.M. Massey, B.E. Haynes, M.A. Polis, A.T. Haase, M.B. Feinberg, J.L. Sullivan, et al. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature.* 396:690–695. http://dx.doi.org/10.1038/25374

Dowling, M.R., and P.D. Hodgkin. 2009. Why does the thymus involute? A selection-based hypothesis. *Trends Immunol.* 30:295–300. http://dx.doi.org/10.1016/j.it.2009.04.006

Dudakov, J.A., A.M. Hanash, R.R. Jenq, L.F. Young, A. Ghosh, N.V. Singer, M.L. West, O.M. Smith, A.M. Holland, J.J. Tsai, et al. 2012. Interleukin-22 drives endogenous thymic regeneration in mice. *Science.* 336:91–95. http://dx.doi.org/10.1126/science.1218004

Fagnoni, F.F., R. Vescovini, G. Passeri, G. Bologna, M. Pedrazzoni, G. Lavagetto, A. Casti, C. Franceschi, M. Passeri, and P. Sansoni. 2000. Shortage of circulating naive CD8+ T cells provides new insights on immunodeficiency in aging. *Blood.* 95:2860–2868.

Fink, P.J. 2013. The biology of recent thymic emigrants. *Annu. Rev. Immunol.* 31:31–50. http://dx.doi.org/10.1146/annurev-immunol-032712-100010

Flomerfelt, F.A., N. El Kassar, C. Gurunathan, K.S. Chu, S.C. League, S. Schmitz, T.R. Gershon, V. Kapoor, X.Y. Yan, R.H. Schwartz, and R.E. Gress, 2010. Thata modulates thymic stromal cell proliferation and thymus function. *J. Exp. Med.* 207:2521–2532. http://dx.doi.org/10.1084/jem.20092759

Frenkel, J.K., and S. Friedlander. 1951. Toxoplasmomiasis: pathology of neonatal disease, pathogenesis, diagnosis, and treatment. United States Government Printing Office, Washington. 12-35 pp.

Gabor, M.J., R. Scollay, and D.J. Godfrey. 1997. Thymic T cell export is not influenced by the peripheral T cell pool. *J. Immunol.* 157:798–805. http://dx.doi.org/10.4049/jimmunol.157.3.798

Garaci, E., R. Caliò, and W. Djaczenko. 1974. Effect of influenza virus PR8 infection on thymus in intact and adenalecetomized mice. *Experientia.* 30:358–360. http://dx.doi.org/10.1007/BF01921662

Gavazzi, G., and K.H. Krause. 2002. Ageing and infection. *Lancet Infect. Dis.* 2:659–666. http://dx.doi.org/10.1016/S1473-3099(02)00437-1

Gazzinelli, R.T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12. *IFN-γ* and TNF-α. *J. Immunol.* 157:798–805.

Glattman Zaretsky, A.J.B. Engules, and C.A. Hunter. 2014. Infection-induced changes in hematopoietic *J. Immunol.* 192:27–33. http://dx.doi.org/10.4049/jimmunol.1302061

Grover, H.S., N. Blanchard, F. Gonzalez, S. Chan, E.A. Robey, and N. Shastri. 2012. The *Toxoplasma gondii* gondii peptide AS15 elicits CD4 T cells that can control parasite burden. *Infect. Immun.* 80:3279–3288. http://dx.doi.org/10.1128/IAI.00425-12

Gu, J., X. Zhu, J. Dobkan, L. Cheng, P.F. Barnes, and D.M. Su. 2007. The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. *Int. Immunol.* 19:1201–1211. http://dx.doi.org/10.1093/immunol/dxm095
Pfefferkorn, E.R., and L.C. Pfefferkorn. 1976. *Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants. *Exp. Parasitol.* 39:365–376. http://dx.doi.org/10.1016/0014-4894(76)90040-0

Price, P., S.D. Olver, A.E. Gibbons, H.K. Teo, and G.R. Shellam. 1993. Characterization of thymic involution induced by murine cytomegalovirus infection. *Immunol. Cell Biol.* 71:155–165. http://dx.doi.org/10.1038/icb.1993.18

Ross, E.A., R.E. Coughlan, A. Flores-Langarica, S. Lax, J. Nicholson, G.E. Price, P., S.D. Olver, A.E. Gibbons, H.K. Teo, and G.R. Shellam. 2003. Differences in maintenance of CD8+ and CD4+ bacteria-specific effector-memory T cell populations. *Eur. J. Immunol.* 33:2875–2885. http://dx.doi.org/10.1002/eji.200324224

Savino, W. 2006. The thymus is a common target organ in infectious diseases. *PLoS Pathog.* 2:e62. http://dx.doi.org/10.1371/journal.ppat.0020062

Schiemann, M., V. Busch, K. Linkemann, K.M. Huster, and D.H. Busch. 2003. Differences in maintenance of CD8' and CD4' bacteria-specific effector-memory T cell populations. *Eur. J. Immunol.* 33:2875–2885. http://dx.doi.org/10.1002/eji.200324224

Suzuki, Y., M.A. Orellana, R.D. Schreiber, and J.S. Remington. 1988. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science.* 240:516–518. http://dx.doi.org/10.1126/science.3128869

Ventevogel, M.S., and G.D. Sempowski. 2013. Thymic rejuvenation and aging. *Curr. Opin. Immunol.* 25:516–522. http://dx.doi.org/10.1016/j.coi.2013.06.002

Villarino, A., L. Hibbert, L. Lieberman, E. Wilson, T. Mak, H. Yoshida, R.A. Kastelijn, C. Saris, and C.A. Hunter. 2003. The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection. *Immunology.* 19:645–655. http://dx.doi.org/10.1016/S1076-7598(03)00300-5

Vogelzang, A., C. Perdomo, U. Zedler, S. Kuhlmann, R. Hurwitz, M. Gengenbacher, and S.H.E. Kaufmann. 2014. Central memory CD4+ T cells are responsible for the recombiant Bacillus Calmette-Guérin ΔureC:hly vaccine’s superior protection against tuberculosis. *J. Infect. Dis.* 210:1928–1937. http://dx.doi.org/10.1093/infdis/jiu347

Xing, Y., and K.A. Hogquist. 2014. Isolation, identification, and purification of murine thymic epithelial cells. *J. Vi. Exp.* 90:e51780. http://dx.doi.org/10.3791/51780

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science.* 301:640–643. http://dx.doi.org/10.1126/science.1087262

Yap, G.S., and A. Sher. 1999. Effector cells of both nonhemopoietic and hemopoietic origin are required for interferon (IFN)-γ- and tumor necrosis factor (TNF)-α-dependent host resistance to the intracellular pathogen, *Toxoplasma gondii*. *J. Exp. Med.* 189:1083–1092. http://dx.doi.org/10.1084/jem.189.7.1083

Yasunaga Ji, T., K. Sakai, Nosaka, S. Etoh Ki, S. Tamiya, S. Koga, M. Mita, H. Uchino, Matsuya, and M. Matsusaka. 2001. Impaired production of naive T lymphocytes in human T-cell leukemia virus type I-infected individuals: its implications in the immunodeficient state. *Blood.* 97:3177–3183. http://dx.doi.org/10.1182/blood.V97.10.3177

Yermakov, V., R.K. Rashid, J.C. Vuletin, L.P. Pertschuk, and H. Jakobs. 1982. Disseminated toxoplasmosis. Case report and review of the literature. *Arch. Pathol. Lab. Med.* 106:524–528.

Yonkers, N.L., S. Sieg, B. Rodriguez, and D.D. Anthony. 2011. Reduced naive CD4 T cell numbers and impaired induction of CD27 in response to T cell receptor stimulation reflect a state of immune activation in chronic hepatitis C virus infection. *J. Infect. Dis.* 203:635–645. http://dx.doi.org/10.1093/infdis/jiq101

Yu, W., H. Nagaoka, M. Jankovic, Z. Misulovin, H. Suh, A. Rolink, F. Melchers, E. Meffre, and M.C. Nussenzweig. 1999. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. *Nature.* 400:682–687. http://dx.doi.org/10.1038/23287