T Cells Home to the Thymus and Control Infection

Claudia Nobrega, Cláudio Nunes-Alves, Bruno Cerqueira-Rodrigues, Susana Roque, Palmira Barreira-Silva, Samuel M. Behar and Margarida Correia-Neves

*J Immunol* published online 11 January 2013
http://www.jimmunol.org/content/early/2013/01/11/jimmunol.1202412

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/14/jimmunol.1202412.DC1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
T Cells Home to the Thymus and Control Infection

Claudia Nobrega,*†,1 Cláudio Nunes-Alves,*†,‡,3,1 Bruno Cerqueira-Rodrigues,*† Susana Roque,*† Palmira Barreira-Silva,*† Samuel M. Behar,‡,2 and Margarida Correia-Neves*†,2

The thymus is a target of multiple pathogens. How the immune system responds to thymic infection is largely unknown. Despite being considered an immune-privileged organ, we detect a mycobacteria-specific T cell response in the thymus following dissemination of Mycobacterium avium or Mycobacterium tuberculosis. This response includes proinflammatory cytokine production by mycobacteria-specific CD4+ and CD8+ T cells, which stimulates infected cells and controls bacterial growth in the thymus. Importantly, the responding T cells are mature peripheral T cells that recirculate back to the thymus. The recruitment of these cells is associated with an increased expression of Th1 chemokines and an enrichment of CXCR3+ mycobacteria-specific T cells in the thymus. Finally, we demonstrate it is the mature T cells that home to the thymus that most efficiently control mycobacterial infection. Although the presence of mature T cells in the thymus has been recognized for some time, to our knowledge, these data are the first to show that T cell recirculation from the periphery to the thymus is a mechanism that allows the immune system to respond to thymic infection. Maintaining a functional thymic environment is essential to maintain T cell differentiation and prevent the emergence of central tolerance to the invading pathogens. The Journal of Immunology, 2013, 190: 000–000.

First, not all TCRs generated will recognize self-MHC/peptide complexes. Second, some TCRs will recognize self-MHC/peptide complexes with such a high affinity/avidity that will render them potentially autoreactive. To avoid full differentiation of T cells with these unwanted characteristics, thymocytes go through a complex process of selection during their differentiation in the thymus. T cell precursors emerge from the bone marrow and home to the thymus, a primary lymphoid organ that exists for the purpose of supporting T cell differentiation. Two fundamental selection processes occur during T cell differentiation in the thymus: positive and negative selection (reviewed in Ref. 3). The outcome of thymic selection depends on the organ microenvironment, including which Ags are present within the thymus and the cytokine milieu surrounding the differentiating cells. Positive selection creates a pool of thymocytes that bind self-MHC/peptide complexes whereas T cells expressing TCRs that fail to bind these complexes undergo cell death by apoptosis. T cells expressing TCRs that bind self-MHC/peptide complexes with high affinity/avidity are also eliminated through negative selection. This process of central tolerance is essential to generate a repertoire of T cells that is self-restricted and self-tolerant, and which limits the development of potentially autoreactive T cells. Thus, proper thymic activity is crucial for the development and maintenance of a repertoire of functional T cells. Infants born with genetic mutations that abrogate T cell differentiation are profoundly susceptible to infection, which confirms the essentiality of T cells for cell-mediated immunity (4).

Thymic activity decreases naturally with age, although the adult thymus still supports T cell differentiation and T cells continue to be exported into the peripheral T cell pool (5, 6). Coincident with T cell differentiation and thymic activity, the developing immune system is assaulted by various microbial pathogens. In fact, thymic function is affected during infection by several pathogens, including species of bacteria, virus, fungi, and parasites (7, 8). Both in humans and in animal models the thymus is itself a target of infection (7–19). Infection of the thymus by mycobacterial species has been reported both in humans (16) and in animal models (17–19). The incidence of thymic infection during active tuberculosis...
is unknown, although there are several case reports in the clinical literature (16). However, it is relevant that vertical transmission of tuberculosis (mother to child) frequently occurs during early childhood, at the height of thymic activity (20).

Although the thymus has been formerly considered an immune-privileged site (21), this idea is being reconsidered. In fact, it is clear that protection of the thymus from infection, particularly during childhood, a time of diverse and recurrent infection, should be a function of the immune system. Because negative selection of T cells depends on the Ags encountered in the thymus during differentiation, infection of this organ could theoretically lead to the development of immune tolerance to pathogens. How the immune system prevents or combats thymic infection to maintain the organ’s integrity and function and keep it free from microbial Ags is still unclear. Interestingly, the thymus does contain several populations of mature T cells. These include innate lymphocytes such as invariant NKT cells (22), γδ T cells (23), and MR1-restricted MAIT cells (24), all of which have been implicated in host defense against infection. Additionally, there are recirculating conventional CD4+ and CD8+ T cells (25). Why mature T cells should recirculate back to the thymus is unknown, although others have hypothesized that these T cells play a role in surveying the thymus for infection (reviewed in Refs. 25–27).

Despite the introduction of bacillus Calmette-Guérin vaccination a century ago and the development of multiple pharmacological drugs that are active against mycobacteria, these bacteria are still one of the most prevalent infectious agents worldwide (28). Among these, Mycobacterium tuberculosis alone is estimated to cause ~8.8 million new infections and 1.4 million deaths per year (28). In addition to M. tuberculosis, other members of the Mycobacterium genus, including Mycobacterium marinum, Mycobacterium ulcerans, Mycobacterium leprae, and Mycobacterium avium, cause disease in humans (29). Some of these are opportunistic infections and mainly affect immunocompromised individuals (30). In fact, the spread of HIV dramatically increased the prevalence of active mycobacterial infection, which is the main cause of death in patients with AIDS (31).

We previously showed that experimental infection with M. tuberculosis and M. avium leads to the establishment of thymic infection in chronically infected mice (18, 19). In this study we report that infection of the thymus occurs during differentiation, and that this could theoretically lead to the development of immune tolerance to pathogens. How the immune system prevents or combats thymic infection to maintain the organ’s integrity and function and keep it free from microbial Ags is still unclear. Interestingly, the thymus does contain several populations of mature T cells. These include innate lymphocytes such as invariant NKT cells (22), γδ T cells (23), and MR1-restricted MAIT cells (24), all of which have been implicated in host defense against infection. Additionally, there are recirculating conventional CD4+ and CD8+ T cells (25). Why mature T cells should recirculate back to the thymus is unknown, although others have hypothesized that these T cells play a role in surveying the thymus for infection (reviewed in Refs. 25–27).

Despite the introduction of bacillus Calmette-Guérin vaccination a century ago and the development of multiple pharmacological drugs that are active against mycobacteria, these bacteria are still one of the most prevalent infectious agents worldwide (28). Among these, Mycobacterium tuberculosis alone is estimated to cause ~8.8 million new infections and 1.4 million deaths per year (28). In addition to M. tuberculosis, other members of the Mycobacterium genus, including Mycobacterium marinum, Mycobacterium ulcerans, Mycobacterium leprae, and Mycobacterium avium, cause disease in humans (29). Some of these are opportunistic infections and mainly affect immunocompromised individuals (30). In fact, the spread of HIV dramatically increased the prevalence of active mycobacterial infection, which is the main cause of death in patients with AIDS (31).

We previously showed that experimental infection with M. tuberculosis and M. avium leads to the establishment of thymic infection in chronically infected mice (18, 19). In this study we report that infection of the thymus is followed by the establishment of protective immunity within the thymus, characterized by the appearance of both CD4+ and CD8+ T cells specific for mycobacterial Ags. These Ag-specific T cells do not originate from the pool of differentiating T cells in the thymus, but are instead T cells that recirculate from peripheral organs back to the infected thymus to control infection. Their recruitment to the thymus correlates with the expression of the chemokine receptor CXCR3 and the production of CXCL9 and CXCL10 by the infected thymus. To our knowledge, these data are the first to show that T cell recirculation to the thymus is a mechanism used by the immune system to survey and protect the thymus from infection and maintain thymic integrity.

Materials and Methods

Mice and infection

C57BL/6 wild-type (WT) mice were purchased from Charles River Laboratories (Barcelona, Spain) or from The Jackson Laboratory (Bar Harbor, ME), and CD45.1 mice (B6.SJL-Piprca PepcbBoyJ) (32) and TCRα knockout (KO) (B6.129S2-Tcrd+Mmuc3<sup>−/−</sup>) mice were from The Jackson Laboratory (33). RAG-GFP mice (34) were provided by Dr. Antonino Bandeira (Pasteur Institute, Paris, France). Both TCRα KO and RAG-GFP mice were bred in our facilities. Mice were 7 to 10 wk old at the start of the experiments. All animal experiments were performed in accordance with National and European Commission guidelines for the care and handling of laboratory animals and were approved by the National Veterinary Directorate and by the local Animal Ethical Committee or by the Dana Farber Cancer Institute Animal Care and Use Committee (Animal Welfare Assurance no. A3023-01), under Public Health Service assurance of Office of Laboratory Animal Welfare guidelines. Mice infected with M. tuberculosis were housed in a biosafety level 3 facility under specific pathogen-free conditions at the Animal Biohazard Containment Suite (Dana Farber Cancer Institute, Boston, MA).

Experimental infection

M. avium (strain 2447, provided by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium) infection was performed i.v. through the lateral tail vein, delivering 10<sup>6</sup> CFU per mouse. For each M. tuberculosis (Erdman strain) infection, a bacterial aliquot was thawed, sonicated twice for 10 s in a cup horn sonicator, and then diluted in 0.9% NaCl/0.02% Tween 80. A 15-ml suspension of M. tuberculosis was loaded into a nebulizer (MiniHEART nebulizer; Vortran Medical Technology) and mice were infected via the aerosol route using a mini-one-use particle (Infected Products) and received 100–200 CFU/mouse. At different times postinfection, mice were euthanized by carbon dioxide inhalation or by decapitation and organs were aseptically removed, individually homogenized, and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto 7H10 or 7H11 agar plates for M. avium and M. tuberculosis, respectively. Plates were incubated at 37°C and M. avium and M. tuberculosis colonies were counted after 7 and 21 d, respectively.

Gene expression analysis

Total RNA was isolated from thymi, spleens, and lungs using TRIzol reagent or TRIzol Plus RNA purification system (Invitrogen, Carlsbad, CA). Five hundred nanograms total RNA was amplified using the SuperScript RNA amplification system (Invitrogen) according to the manufacturer’s instructions. mRNA transcripts were assayed by quantitative real-time PCR (qPCR) using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) in a Bio-Rad CFX96 real-time system with a C1000 thermal cycler or a Stratagene Mx3005P thermal cycler. The hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as reference gene. Specific oligonucleotides were used for Hprt (sense, 5’-GCT GAA GAC GCT TTC-3’, antisense, 5’-CAC AGG ACT AGA ACA CCT GC-3’), Ifnγ (sense, 5’-CAG CAA GAC GAA AAA AGG-3’, antisense, 5’-GGG CCA CTC GGA TGA GCT CA-3’), Tgfβ (sense, 5’-TGC GTA TGT CTC AGC CCT TTC-3’, antisense, 5’-GAG GAC ATT TGG GAA CTT CT-3’), Cxcl9 (sense, 5’-CTT TTC TCT TTT GGC GTC AT-3’, antisense, 5’-GAC TGG TGC ATT CCT TAT CA-3’), Cxcl10 (sense, 5’-GCT GCC GTC ATT TTC TGC-3’, antisense, 5’-TCT CAC TGG CCC GTC ATC-3’), Cxcl4 (sense, 5’-AGC ACC AAT GGT CTC TGA-3’, antisense, 5’-TTT GGT M. avium GAA TAC CAC AGC-3’), and inducible NO synthase (iNOS; sense, 5’-CTT GGG AGG GGT TGG CTC CAC TGT-3’, antisense, 5’-GCT GGA AGC CAC TGA CTC TT-3’). The cDNA was denatured for 1 min at 95°C, followed by 40 cycles of 95°C for 15 s, incubation at the optimized melting temperature for 20 s, and 72°C for 20 s. Optimized melting temperatures were 57°C for Cxcl9 and Cxcl4, 58°C for Hprt, Ifnγ, and Cxcl10, and 59°C for iNOS. The expression level of each gene was determined using the ΔΔCt method taking into account the efficiency of the PCR reaction (35). Data are presented as the ratio of the expression level of the gene of interest in infected mice over the mean expression level of the gene in uninfected mice.

Protein quantification in tissue homogenates

Protein was extracted from spleen, lung, and thymus using the Bio-Plex cell lysis kit (Bio-Rad) and the concentrations of IFN-γ, TNF, and CCL4 were measured using a mouse Bio-Plex cytokine assay (Bio-Rad). In the case of M. tuberculosis–infected tissues, the concentrations of CXCL9 and CXCL10 were measured using the mouse CXCL9/MIG Quantikine ELISA kit or mouse CXCL10/IP-10/CRG-2 quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Immunohistochemistry

Detection of iNOS was performed by immunohistochemistry in paraffin-embedded tissues. Briefly, 5-μm thymic sections were dehydrated and Ags were “unmasked” by incubation at 96°C for 30 min in 1 mM EDTA with 0.05% Tween 20 (pH 8). Nonspecific binding was blocked using 4% BSA in PBS with 0.05% Tween 20, and endogenous peroxidases were blocked by incubation with 3% hydrogen peroxide for 30 min. Tissues were incubated overnight, at 4°C, with purified rabbit anti-mouse iNOS (clone M-19; Santa Cruz Biotechnology, Santa Cruz, CA), and detection was performed using a peroxidase goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) followed by incubation with
diaminobenzidine until color development. Mycobacteria were detected by Ziehl-Neelsen, using standard procedures, after the iNOS staining. Slides were visualized using a BX61 microscope with an Olympus DP70 camera. No significant signal was observed when iNOS stain was performed in iNOS KO mice.

In vitro stimulation and IFN-γ measurement by ELISA

Cell suspensions from thymus and spleen were prepared by gentle dissection of the organs between two notched glass slides or by forcing organs through a 70-μm nylon strainer (Fisher Scientific). For lung preparations, tissue was digested for 1 h at 37°C in 1 mg/ml collagenase (Sigma-Aldrich) prior to staining. Erythrocytes were lysed using a hemolytic solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM sodium EDTA [pH 7.2]) and, after washing, cells were resuspended in supplemented DMEM or RPMI 1640 (10% heat-inactivated FCS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 mg/ml streptomycin, and 50 U/ml penicillin, all from Invitrogen). Cells were enumerated in 4% trypan blue on a hemocytometer or on a Countess automated cell counter (Life Technologies). Cells (5 × 10⁵) were plated in each well of a 96-well plate and incubated, in triplicate, in the absence of stimuli was used as positive and negative controls, respectively. Supernatants were collected after 72 h culture and the concentration of IFN-γ was determined by ELISA (R4-6A2 and biotinylated AN18 were used as capture and detection Abs, respectively [eBioscience] or using the mouse IFN-γ ELISA MAX standard kit [BioLegend]).

Flow cytometry

Surface staining was performed with Abs specific for mouse CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD24 (M1/69), CD44 (IM7), and CXCR3 (CXCR3-173) (from BioLegend or from BD Pharmingen). The tetrarmers Ag85280–294-loaded I-Aβ and TB10.44–11-loaded H-2Kb were obtained from the National Institutes of Health Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA); staining was performed for 30 min at 37°C before incubation with the Abs mix for the Ag85280–294-loaded I-Aβ tetramer or for 20 min on ice during the incubation with the surface Abs for the TB10.44–11-loaded H-2Kb tetramer. All stainings, except the one with the Ag85280–294-loaded I-Aβ tetramer, were fixed before acquisition with 2% formaldehyde in PBS for 30 min.

Cell analysis was performed on an LSRII flow cytometer or on a FACSCanto using FACSDiva software (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). Single-lymphocyte events were gated by forward scatter versus height and side scatter for size and granularity.

Thymic transplant

Thymic lobes were aseptically removed from WT mice and kept in cold supplemented DMEM until being transplanted under the kidney capsule of anesthetized TCRα KO mice (200 μg xylazine hydrochloride and 200 μg ketamine hydrochloride, administered i.v.).

T cell chimeras

Single-cell suspensions of pools of spleen and thymus from M. avium–infected RAG-GFP mice (20–22 wk postinfection [wpi]) were prepared. CD4+ T cells were purified from each suspension using the CD4+ T cell isolation kit for splenocytes or CD8α (Ly-2) microbeads (both from Miltenyi Biotec, Bergisch Gladbach, Germany) for thymocytes. Magnetic separation was performed with an autoMACS separator (Miltenyi Biotec). After purification, cells were counted and stained with Abs for CD3, CD4, and CD8 for sorting. Splenic CD4+CD8−CD45Rα− cells, thymic CD4+CD8−CD3+ RAG2−CD45Rα−, and thymic CD4−CD8+CD3+RAG2−RAGα− cells were sorted using a FACSARia cell sortet (Becton Dickinson) with a purity ranging from 98% to 100%, depending on the experiment and on the population. Cells (4 to 4.2 × 10⁵) were transferred i.v. to each TCRα KO mouse.

Adoptive transfer of CXCR3-expressing T cells

Single-cell suspensions of pools of spleens from M. tuberculosis–infected B6 mice (20–26 wpi) were prepared. Total T cells were purified from each suspension using the T cell isolation kit II (Miltenyi Biotec). Magnetic separation was performed with an autoMACS separator (Miltenyi Biotec). After purification, cells were counted and transferred i.v. to each recipient. Recipient mice (CD45.1) had been infected for 12 wks and were analyzed 24 h following transfer. After sacrifice, magnetic bead-based enrichment was performed on single-cell suspensions of thymocytes, as previously described (39). Briefly, cells were stained with a PE-labeled Ab specific for mouse CD45.2 (104) (BioLegend), followed by incubation with anti-PE beads (from Miltenyi Biotec). Magnetic separation was performed with an autoMACS separator. The bound fraction, enriched for donor cells, was analyzed by FACS (as described above).

Statistical analysis

All data are represented as means ± SEM. Student t test was used for normally distributed data; otherwise a Mann–Whitney U test was performed to compare two groups. To compare more than two groups, one-way ANOVA, followed by Bonferroni post hoc test, was performed. A p value of <0.05 was considered statistically significant.

Results

Thymic integrity is maintained following mycobacterial infection

The ability of mycobacteria to disseminate to the thymus has been previously described upon aerosol challenge with M. tuberculosis (Fig. 1A and Ref. 18) and following i.v. infection with M. avium (18, 19). In both cases the bacterial load in the thymus is initially low or undetectable, but increases profoundly until it plateaus, in a pattern similar to the one observed in the lung and spleen, indicative of immunological restriction of bacterial replication. However, bacterial dissemination to the thymus is delayed compared with other organs, and it peaks late during the course of infection, at 16 wpi for M. avium (18, 19) and at 12 wpi for M. tuberculosis (Fig. 1A). Additionally, thymic infection by M. tuberculosis is also more heterogeneous, with a small fraction of mice having undetectable bacterial load within the thymus even at 24 wpi (Fig. 1A). The fact that we did not observe this heterogeneity previously (18) might be a consequence of the greater number of animals studied in the present experiments or of slight differences in the infection protocol (different M. tuberculosis strains and/or different apparatus used for aerosol infection).

As previously observed for M. avium infection with a low virulence strain (19), infection with M. tuberculosis does not induce loss of thymic cellularity even at 24 wpi, as compared with uninfected age-matched controls (Fig. 1B). Percentages of the four main thymic populations, as assessed by CD3, CD4, and CD8 expression (CD4−CD8−CD3− double-negative, CD4+CD8−CD3+ double-positive, CD4+CD8+CD3+ single-positive [SP] CD4+CD4SP, and CD4−CD8−CD3+ SP CD8+CD8SP) are also maintained following infection, although a small increase in the percentages of SP cells, both CD4SP and CD8SP, and a decrease in double-positive cells are observed from 12 wpi on (Fig. 1C). These data indicate that the presence of M. tuberculosis within the thymus does not induce premature thymic atrophy (a common consequence of systemic infection) (8) or major alterations in thymic cell populations, as have been shown previously for M. avium (19).

An immune response develops in the thymus following mycobacterial infection

The stabilization of bacterial growth in the primary infected organ, spleen for M. avium and lung for M. tuberculosis, is associated with the establishment of an effective acquired immune response (40–42). To evaluate whether an immune response is established in the thymus, the expression of key cytokines required for immunity to mycobacteria was measured in whole-organ homogenates of mice infected with M. avium (i.v.) or with M. tuberculosis (by aerosol) and compared with uninfected controls. The selected time points reflect periods when: 1) the peak in the immune response is observed in the primary infected organs upon M. tuberculosis (lung) (43) and M. avium (spleen) infection (40) (4 wpi for both infections); 2) the bacterial burden in the thymus is still
FIGURE 1. M. tuberculosis infection of the thymus. (A) Bacterial burden in the lung, spleen, and thymus after aerosol infection with M. tuberculosis. Data are pooled from 11 independent experiments each, with 3–7 subjects per time point, for a total of 97 subjects. Each dot represents one mouse, the solid line is the mean, and the dashed line is the lower detection limit. Number of thymocytes (B) and percentage of the main thymocyte populations (based on CD3, CD4, and CD8 expression) (C) after M. tuberculosis infection are shown. Both infected (filled bars) and age-matched uninfected controls (open bars) are represented. *p < 0.05 by Mann–Whitney U test. Bars represent the mean ± SEM (n = 5 mice/group). Data are representative of two independent experiments.

increasing (4 wpi for M. tuberculosis, 4 and 12 wpi for M. avium); 3) the bacterial burden plateaus in the thymus (12 wpi for M. tuberculosis, 16 wpi for M. avium); and 4) bacterial growth in the thymus has been sustained for a long period (24 wpi) (Fig. 1) (18, 19).

During both infections, Ifnγ expression is significantly increased as early as 4 wpi in the primary infected organ (spleen for M. avium, Fig. 2A; lung for M. tuberculosis, Fig. 2B). In the thymus, Ifnγ expression is increased only at later time points, consistent with the delayed dissemination and establishment of bacterial control (16 wpi for M. avium, Fig. 2A; 12 wpi for M. tuberculosis, Fig. 2B). The kinetics of Tnf expression is similar to that of Ifnγ in mice infected with M. tuberculosis (Fig. 2B). In contrast, in mice infected with M. avium, Tnf is not upregulated in the thymus and is only transiently upregulated in the spleen (Fig. 2A). In agreement with the observed changes in gene expression, elevated levels of IFN-γ protein are detected in the thymus 16 wpi with M. avium (Fig. 2C), and both IFN-γ and TNF protein are increased in the thymus 12 wpi with M. tuberculosis (Fig. 2D). These data show that the thymus is the site of an ongoing immune response that involves the production of proinflammatory cytokines after infection, and the cytokine levels peak concordantly with the control of bacterial replication.

Mycobacteria-specific T cells are detected in the thymus after infection

To determine whether a mycobacteria-specific T cell response accompanies the inflammatory changes observed in the infected thymus, IFN-γ production by thymus cells obtained from infected mice after stimulation with defined mycobacterial Ags was assessed. IFN-γ production was specifically induced when spleen or thymus cells from M. avium–infected mice were stimulated with M. avium protein extract or with Ag85_280–294 peptide (primarily recognized by CD4+ T cells) (36). These Ags led to maximal IFN-γ production by splenocytes at 4 wpi (Fig. 3A), as previously described (40). In contrast, these Ags did not trigger IFN-γ production by thymic cells until 16 wpi, and even then they elicited much lower amounts of IFN-γ (Fig. 3A). Similarly, large amounts of IFN-γ were produced by lung cells obtained from M. tuberculosis–infected mice after stimulation with the immunodominant epitope ESAT6_1–20 (recognized by CD4+ T cells) (38) or TB10.4_4–11 (recognized by CD8+ T cells) (37) as early as 4 wpi. Although an M. tuberculosis–specific response could be detected in the thymus as early as 4 wpi, a more substantial response was detected 12 wpi (Fig. 3B) and, as in the case of M. avium, in quite lower amounts and associated with control of the infection and a plateau of the bacterial burden in the thymus (Fig. 3B).

We also determined the frequency of TB10.4_4–11-specific CD8+ T cells during M. tuberculosis infection using K7/TB10.4_4–11 tetramers, which can identify Ag-specific CD8+ T cells independently of their function (Fig. 3C, Supplemental Fig. 1). Similar to our other data, the frequency of Ag-specific T cells was closely correlated with the production of IFN-γ in the infected organs. Taken together, these data indicate that mycobacteria-specific T cells are present within the thymus following infection. Although the magnitude of the response differs from that observed in the dominant target organs, the kinetics in the thymus resemble other tissue-specific responses to mycobacterial infection.

Mycobacteria-infected cells in the thymus express iNOS

The production of IFN-γ by Ag-specific T cells is a central feature of protective immunity against mycobacterial infection (44, 45). Among the important antibacterial actions of IFN-γ is upregulation of iNOS expression by macrophages. iNOS catalyzes the production of NO, which has a significant role in controlling M. tuberculosis infection (46). Despite the fact that NO plays no role in the protective immunity to M. avium (47, 48), iNOS expression represents...
a suitable marker of macrophage activation in this scenario. The elevated IFN-γ level in the infected primary organs was associated with increased iNos expression by 4 wpi. An increase in iNos expression in the thymus was only observed at later time points: 24 wpi for M. avium (Fig. 4A) and 12 wpi for M. tuberculosis (Fig. 4B). These data are consistent with a protective T cell response in the thymus leading to iNOS induction, NO production, and control of bacterial replication.

In addition to measuring iNos gene expression, we detected iNOS protein by immunohistochemical staining. Although iNOS is expressed in the thymus even in the absence of infection (49), we detected an increase in the number of cells containing M. avium (Fig. 4C) or M. tuberculosis (Fig. 4D) and expressing iNOS throughout the course of infection, with most of the infected cells expressing iNOS at later time points. For M. avium and M. tuberculosis, most infected cells in the thymus were located in the medulla or corticomedullary region, although occasional bacteria were observed in the cortex. M. tuberculosis–infected cells were also infrequently observed in the subcapsular zone. Thus, our data showing augmented iNOS expression and its colocalization with infected cells indicates the establishment of a bona fide protective immune response within the thymus.

Mycobacteria-infected thymi contain cells able to transfer protection against infection

Having shown that the thymus is a site of infection where Ag-specific immune responses are detected, we next asked whether cells within infected thymi could confer protection against subsequent infection. To that end, we transplanted M. avium–infected or uninfected thymic lobes from WT mice under the kidney capsule of TCRα KO mice (33), which lack peripheral αβ T cells (Fig. 5A).

The transplanted mice were challenged with M. avium and sacrificed at 4 or 8 wpi, at which time thymic engraftment was confirmed macroscopically. At both time points, the spleens of mice transplanted with infected or uninfected thymic lobes had similar numbers of CD4+ T cells, indicating that cells leaving the infected or uninfected thymus were equally able to reconstitute the peripheral T cell pool of the recipient TCRα KO mice (Fig. 5B).
groups receiving thymic transplants had lower bacterial burdens than did the untransplanted TCRαKO mice (Fig. 5C). This demonstrates that the cells emerging from the thymic grafts are functional and can mediate protection against microbial pathogens. Importantly, mice receiving infected thymic grafts were significantly more protected than those receiving uninfected grafts, despite similar T cell reconstitution (Fig. 5C). These data support the hypothesis that the greater protection conferred by the infected thymic grafts is mediated by *M. avium*–specific T cells contained within the grafts, which efficiently confer protection against infectious challenge.

**Mycobacteria-specific T cells within the thymus are recirculating cells**

After establishing that Ag-specific T cells within the infected thymus produce protective cytokines, induce NO production, and transfer protection, we next sought to determine the origin of these cells. In particular, we wanted to determine whether differentiating T cells were primed in the thymus, or, alternately, whether mature mycobacteria-specific T cells, primed in the periphery, traffic to the thymus.

Our previous data demonstrated that T cells that differentiate within *M. avium*–infected thymi are tolerant to the invading pathogen (19). Moreover, newly differentiated T cells arising from the thymus are not fully mature (50) and need additional signals within secondary lymphoid organs to achieve full differentiation (51, 52). Therefore, we used a genetic model in which GFP is expressed under the control of the RAG2 promoter (34), for the purpose of identifying newly differentiated T cells in the thymus. The RAG2 enzyme is essential for genetic recombination of B and T cell Ag receptors and is downregulated afterward. Even though RAG2 is downregulated after T cell differentiation in the thymus, cells remain GFP+ for ∼2 wk (50). As such, it is highly expressed in differentiating T cells and its expression is a useful marker to distinguish between newly differentiated T cells (high to intermediate GFP expression) and mature peripheral T cells (no GFP expression).

When RAG-GFP mice were infected with *M. avium* there was no difference in the percentage of GFP	extsuperscript{+} among CD4SP cells, suggesting that there is no difference in the amount of thymic recirculating T cells (Fig. 6A, Supplemental Fig. 1). However, upon infection, most of the Ag85280–294–specific CD4SP cells in the thymus were GFP	extsuperscript{+}, showing a modification in the repertoire of recirculating T cells (Fig. 6A). In contrast with what was observed for *M. avium*, thymi from *M. tuberculosis*–infected mice showed an increase in the percentage of GFP	extsuperscript{+} within CD8SP cells, suggesting an increase in T cell trafficking from the periphery back to the thymus (Fig. 6B). A similar increase was detected when analyzing mycobacteria-specific T cells (TB10.44–11–specific CD8SP T cells) during *M. tuberculosis* infection, in agreement with what was observed for Ag-specific cells in the thymus of *M. avium*–infected mice (Fig. 6B). In both cases, no tetramer	extsuperscript{+} cells were found in the thymus of control uninfected mice, which is in agreement with the small precursor frequency we observed for T cells reactive against these mycobacterial Ags in naive mice (data not shown). In parallel, we show that most TB10.44–11–specific CD8SP T cells within the thymus of WT mice infected with *M. tuberculosis* are CD44	extsuperscript{hi}CD24	extsuperscript{lo} (Fig. 6C), a phenotype typical of recirculating CD8	extsuperscript{+} T cells (25, 53).

**FIGURE 3.** Mycobacteria-specific T cell responses are detected in the thymus after *M. avium* and *M. tuberculosis* infection. Cells from *M. avium* (A)– or *M. tuberculosis* (B)–infected mice were stimulated in vitro in the presence of *M. avium* protein extract or Ag85280–294 peptide (A) and ESAT61–20 or TB10.44–11 peptides (B). Age-matched uninfected mice were used as controls. Unstimulated and Con A or anti-CD3/anti-CD28–stimulated cultures were used as negative and positive controls of the in vitro stimulation, respectively (data not shown). IFN-γ quantification in cell supernatants was performed by ELISA. (C) TB10.4–specific CD8	extsuperscript{+} T cells were detected in lung and thymus of *M. tuberculosis*–infected mice using the K7/TB10.44–11 tetramer. Filled symbols represent infected mice and open symbols uninfected mice. Data points represent the mean ± SEM (n = 4–6 mice/group). Data are representative of two to four independent experiments. *p < 0.05 by Mann–Whitney U test.
**FIGURE 4.** Mycobacterial infection in the thymus is associated with increased Inos expression. iNOS RNA expression levels were quantified by qPCR in tissues of *M. avium* (A)– and *M. tuberculosis* (B)–infected mice. Bars refer to fold increase of infected mice in comparison with the average of uninfected mice and represent the mean ± SEM (n = 4–8 mice/group). Data are representative of two to three independent experiments. *p < 0.05 by Mann–Whitney U test (statistics were performed by comparing uninfected with infected mice, before performing the ratio). (C and D) Representative medullary thymic sections of *M. avium* (C)– and *M. tuberculosis* (D)–infected thymi stained for iNOS (brown). Bacilli were detected by Ziehl-Neelsen staining. Shown are representative images obtained from the analysis of three to five thymi per time point from two to three independent experiments. Scale bars, 10 μm.

**T cell chemokines are increased in the thymus during infection**

The above results indicate that Ag-specific T cells from the periphery respond to infection in the thymus by trafficking back to this organ. To determine whether increased chemokine production is associated with the recruitment of mycobacteria-specific T cells to the thymus, we measured the expression of chemokines that recruit Th1 cells (54), including CXCL9, CXCL10, and CCL4, after *M. avium* and *M. tuberculosis* infection. We found increased mRNA expression of all three chemokines in the primary infected organs during *M. tuberculosis* infection and at least at the initial phase of *M. avium* infection (Fig. 7A, 7B). These chemokines were also detected in the thymus although at lower levels and at later time points, that is, after 16 wpi for *M. avium* (Fig. 7A) and 12 wpi for *M. tuberculosis* (Fig. 7B). At the protein level, increased CCL4 was detected in the spleen but not in the thymus during *M. avium* infection, possibly because the expression levels were low (data not shown). During *M. tuberculosis* infection, the expression of these chemokines was strongly induced in the lungs of mice infected for 4 wk, and the responses appeared maximal for all three chemokines by 12 wpi (Fig. 7C). These chemokines were also highly expressed in the thymus, but their expression was not detected until 12 wpi (Fig. 7C).

The high levels of CXCL9 and CXCL10 detected in the infected thymus led us to investigate whether mycobacteria-specific T cells express CXCR3, the receptor for these chemokines (54). We found most TB10.4L11-specific CD8SP T cells in *M. tuber-
As infected thymi to express high levels of the chemokine receptor CXCR3 (Fig. 7D, 7E).

To support a role for CXCR3 in the recruitment of Ag-specific T cells to the infected thymus, we adoptively transferred purified splenic T cells obtained from M. tuberculosis–infected donors into congenically marked infected recipients (Supplemental Fig. 2). Analysis of the donor cells immediately before transfer found that 30–40% of the CD4+ and CD8+ T cells expressed CXCR3. Twenty-four hours after injection, the proportion of donor CD4+ and CD8+ T cells recruited to the thymus that expressed CXCR3 was increased to 65–85%. The increased CXCR3 expression was limited to the infected thymus and was not observed in the spleen or the lung or the recipient mice (Fig. 7E, Supplemental Fig. 2). These data suggest that CXCR3 participates in the recruitment of Ag-specific cells to the infected thymus. Thus, although the thymus is the key organ for T cell differentiation, mycobacteria-specific T cells from the periphery are recruited to the thymus following infection. Our data are consistent with the chemokines CXCL9 and CXCL10 playing an important role in the recruitment of these Ag-specific T cells from peripheral organs to the thymus to fight infection.

Peripheral recirculating T cells within infected thymi efficiently confer protection against infection

To confirm that peripheral T cells recirculating to infected thymi are the ones that confer protection to infection, RAG-GFP mice were infected with M. avium and 20 wpi sacrificed and their thymi and spleens collected. Highly purified T cell populations were sorted accordingly to the expression of GFP and of surface markers: 1) total CD4+ T cells from the spleen (CD3+CD4+CD8−), 2) thymic recirculating CD4SP cells (CD3+CD4+CD8−GFP+CD24−), and 3) thymic newly differentiated CD4SP cells (CD3+CD4+CD8−GFP+CD45R+B220+). A GFP+ population was sorted to the detriment of the total GFP+ population, as we wanted to exclude the most immature GFP− CD4SP cells (Fig. 8A). Each cell subset was transferred to TCRα KO mice that had been infected the previous day with M. avium. Eight weeks later, the mice were analyzed (Fig. 8B).

Despite receiving the same number of cells, mice that received recirculating thymic CD4SP cells (GFP+) had lower numbers of CD4+ T cells than did animals that received either newly differentiated thymic T cells from the thymus (GFP+) or total splenic T cells (Fig. 8C, left panel). Nevertheless, administration of recirculating thymic CD4SP cells provided significantly more protection from M. avium infection in the spleen and liver compared with animals that received newly differentiated thymic T cells (Fig. 8C, middle and right panels). Additionally, the protection provided by recirculating thymic CD4SP T cells is comparable to that of total splenic CD4+ T cells. Although transfer of newly differentiated T cells from the thymus provided partial protection against M. avium infection in comparison with TCRα KO mice, this was significantly reduced compared with protection conferred by recirculating thymic CD4SP cells. Taken together, these results indicate that T cells recirculating from the periphery into the thymus are capable of protecting mice against infection.

Discussion

Our understanding of the role of T cells in resistance to microbial infection is generally limited to the effector or memory phases of the immune response, or their role in vaccine-induced immunity. Despite the key role of the thymus in generating such T cells, the consequences of infection of the thymus itself have been rarely studied. Our previous studies have shown that mycobacteria disseminate to the thymus (18, 19) and alter the process of T cell differentiation, leading to tolerance against the invading pathogen (19). In this study, we address whether and how the immune system is able to defend the thymus against infection, as well as to preserve its structure and its ability to generate T cells.

Our results show that similar to other tissues that are the target of mycobacterial infection, mycobacteria-specific T cells appear in the thymus following the dissemination and subsequent growth of bacteria. Furthermore, the appearance of Ag-specific T cells roughly correlates with the host’s ability to control bacterial growth in the thymus. Ag-specific T cells in the thymus secrete IFN-γ and stimulate antimicrobial functions of infected cells, as manifested by the upregulation of iNOS. Thus, T cell responses in the thymus resemble ones occurring in the lung or spleen, although they differ in magnitude. In fact, both the number of Ag-specific

**FIGURE 5.** M. avium–infected thymi contain T cells able to confer protection during infection. (A) Schematic representation of the experiment. Thymic lobes from M. avium–infected mice (24 wpi) or from uninfected WT mice were transplanted under the kidney capsule of TCRα KO receptor mice. Transplanted mice were infected 2–3 d after transplant and sacrificed 4 and 8 wk later. Nontransplanted TCRα KO mice were used as controls. Numbers of CD4+ T cells (B) and bacterial load (C) were assessed in the spleen. Each column represents mean ± SEM (n = 4 mice/group) from one of two experiments. *p < 0.05 by one-way ANOVA.
T cells and related immune molecules detected in the thymus are significantly lower than those found in the lung or the spleen. Although the thymic immune response is less pronounced, its ability to control bacterial growth seems similarly efficient as the one established in other peripheral organs. Additionally, our previous data show that cellular infiltrates and granuloma-like lesions, characteristic of inflammation in other tissues, are not detected in the infected thymus (18). The reasons for this difference are not clear, but we speculate that avoiding a massive proinflammatory state and preventing disruption of the cellular architecture is required for the thymus to remain functional. The smaller magnitude of the thymic-associated immune response might contribute to the maintenance of thymic integrity during the course of infection. It has previously been shown that infection with a highly virulent *M. avium* strain, which is associated with a vigorous immune response, causes robust thymic atrophy (48). Recent results from our laboratory (55) show that this depends on a synergistic effect between IFN-γ-induced iNOS induction and corticosteroid production, as blocking the effect of each one individually prevents thymic atrophy. Of note, infection with the low virulence *M. avium* strain used in the present study is not accompanied by increased serum levels of corticosteroids (55), which may contribute to the maintenance of thymic integrity reported in this study.

An important question that our data address is the origin of Ag-specific T cells in the thymus that mediate protection against mycobacterial infection. In our model, T cells from the periphery recirculate back to the thymus and are responsible for bacterial control. Thus, although the thymus maintains the differentiation of T cells during infection, these do not appear to be the cells that defend the thymus against infection. These data are consistent with our previous results showing that newly differentiated T cells that mature in a thymus infected by *M. avium* are tolerant to mycobacterial Ags, and consequently they are not optimally able to establish protective immunity (19). Interestingly, T cells that dif-
Differentiate in an infected thymus were not completely impaired in their ability to proliferate, to traffic to infected organs, and to confer a small amount of protection against infection, albeit less efficiently than T cells that develop within an uninfected thymus (19). In agreement with those findings, we now report that newly differentiated T cells are much less effective at conferring protection than are peripheral T cells that recirculate back to thymus. Therefore, we conclude that recirculation of peripheral activated T cells back to the thymus is a mechanism to survey and protect this organ from invading pathogens.

These data are also in agreement with other work that finds newly differentiated T cells defective in Th1 commitment (dampened cytokine production and transcriptional factor expression) and biased toward the Th2 lineage (56), as well as a requirement for newly differentiated T cells to exit the thymus and reside in the periphery before they become fully functional (50–52).

Another interesting question arising from this study concerns whether the Ag-specific cells found in the thymus are primed in peripheral organs and traffic back to the thymus because of thymic infection, or are recirculating T cells that traffic back and are then

---

**FIGURE 7.** Recirculation of mycobacteria-specific T cells into infected thymi correlates with increased Th1-recruiting chemokines and their cognate receptors. RNA expression (A, B) and protein concentration (C) were determined in tissues of *M. avium* (A)– and *M. tuberculosis* (B, C)–infected mice. Bars refer to fold increase of infected mice in comparison with the average of uninfected mice and represent mean ± SEM (*n* = 4–8 mice/group). Data are representative of two to three independent experiments. *p < 0.05 by Mann–Whitney *U* test (statistics were performed by comparing uninfected with infected mice, before performing the ratio). (D) FACS plot of CXCR3 expression in thymic CD8SP TB10.4− and CD8SP TB10.4+ cells from *M. tuberculosis*–infected mice. Represented are the concatenated data from three mice analyzed. Gating strategy is depicted in Supplementary Fig. 1. (E) Percentage of CXCR3 expressing cells between CD8SP TB10.4− and CD8SP TB10.4+ cells in *M. tuberculosis*–infected mice. Each bar represents mean ± SEM (*n* = 4–8 mice/group). Data are representative of two to three independent experiments. (F) CXCR3 expression by donor CD4SP and CD8SP in the spleen and thymus. T cells from *M. tuberculosis*–infected CD45.2 mice were transferred to *M. tuberculosis*–infected CD45.1 recipient mice, and their CXCR3 expression was analyzed the next day. See Supplemental Fig. 2 for scheme and gating strategy. Each bar represents mean ± SEM (*n* = 3 mice). Data are representative of three independent experiments. *p < 0.05 by one-way ANOVA.
primed within the infected thymus. Although we have not addressed this question directly, our data on the production of Th1 chemokines within the infected thymus, on the expression of the cognate chemokine receptors by the Ag-specific T cells found within this organ, and on the preferential recruitment of CXCR3+ cells to the infected thymus strongly support the notion that the Ag-specific T cells found in the thymus are primed in other tissues during infection and then traffic to the thymus in response to a chemokine gradient.

Thymic recirculating T cells, the focus of multiple studies, have had numerous functions attributed to them, including thymic surveillance, tolerance induction, and even modulation of both negative and positive selection (25–27). To our knowledge, the results presented in this study are the first evidence that recirculating T cells fight infection in the thymus, a function that has been previously hypothesized (25, 57, 58). The observations that mycobacteria-specific T cell trafficking to the thymus is associated with an increase in the levels of Th1-related chemokines in this organ, as well as the expression of specific chemokine receptors such as CXCR3 by the recirculating T cells, lead us to propose that the immune system has evolved mechanisms to recruit peripheral T cells to the thymus during infection. This might ensure that invading pathogens are unable to disrupt the thymus, the primary lymphoid organ where T cells are generated. Such mechanisms must be particularly relevant for prenatal and early childhood infections. In both scenarios, pathogen dissemination to the thymus could lead to the presentation of microbial Ags in a “self” context and lead to the deletion of developing T cells that recognize pathogen-specific Ags. Exclusion of pathogen-reactive T cells from the peripheral pool could have severe effects during childhood, when the peripheral T cell pool is still developing, but the consequences could persist throughout adulthood.

Childhood infections are a great cause of morbidity and mortality, particularly in the developing world (59). Interestingly, HIV and M. tuberculosis, two of the most important pathogens in infants and young children, are both able to infect the thymus (16, 60), and thymic infection alters the output and selection of T cells in experimental models using these pathogens (9, 19). The evidence that thymic infection can compromise thymic function implies that the recruitment of protective immune responses to the thymus is essential to prevent detrimental alterations in immunological function.

The implications of our study extend beyond childhood infection. Skepticism about the potential immunological consequences of thymic infection would not be surprising if one were to believe the dogma that the adult thymus becomes immunologically useless as it ages and progressively loses function. However, our understanding of the role of the thymus during adulthood is rapidly evolving. The control of persistent infections has been suggested to benefit from the continuous recruitment of naive T cells generated in the thymus (61, 62). In this case, alterations in the development of these T cells could be detrimental to the on-
going immune response. The integrity of the adult thymus is re-
quired for other aspects of ongoing immune responses such as Ab
generation (63). This observation raises the hypothesis that thymic
disruption secondary to infection can impair T cell responses to
infection as well as other components of the immune system.
Additionally, decreased thymic output contributes to the pro-
gression from HIV infection to AIDS (7, 64), indicating that
thymic export is important in the context of chronic infection in
adults, especially in the context of M. tuberculosis/HIV coin-
collection. Collectively, these data show that thymic infection has an
impact on the development of the immune system and ongoing
immune responses to pathogens. Thus, it is not surprising that
mechanisms have evolved to specifically respond to the invasion
of the thymus by microbial pathogens. We propose that the rec-
circulation of T cells from the periphery to the thymus, as well as the
presence of mature T cells in this organ, represents strategies that
evolved to protect the thymus and sustain its activity during and
after infectious episodes.

Acknowledgments
We thank Dr. Antônio Bandeira for the kind gift of the RAG-GFP mice, the National Institutes of Health Tetramer Facility for providing tetrasters, Goriets Pinto for technical assistance, and the Behar Laboratory for helpful
discussions.

Disclosures
The authors have no financial conflicts of interest.

References
4. Aloj, G., G. Giardino, L. Valentino, F. Maio, V. Gallo, T. Esposito, R. Naddei,
E. Cirillo, and C. Pignata. 2012. Severe combined immuno deficiencies: new
5. Lynch, H. E., G. L. Goldberg, A. Chidgey, M. R. Van den Brink, R. Boyd, and
7. Ho Tsong Fang, R., A. D. Colantonio, and C. H. Uittenbogaart. 2008. The role of
8. Savino, W. 2006. The thymus is a common target organ in infectious diseases.
9. King, C. C., B. D. Jamieson, K. Reddy, N. Bali, R. J. Concepcion, and
1990. Neonatal exposure to thymotropic gross murine leukemia virus induces virus-
12. Andrade, C. F., J. Gameiro, P. R. Nagib, B. O. Carvalho, R. L. Talaisy,
F. T. Costa, and L. Verinaud. 2008. Thymic alterations in Plasmodium berghei-
Thymus atrophy and involution induced by Paraechococciosis brasiliensis in
15. Sotomayor, C. E., H. R. Rubinstein, C. M. Riera, and D. T. Masih. 1995. Im-
munosuppression in experimental cryptococcosis: variation of splenic and thy-
16. Sprent, J., and C. D. Suh. 2009. Re-entry of mature T cells to the thymus:
Switzerland.
18. Nobrega, C., P. J. Cardona, S. Roque, P. Pinto do O, R. Appelberg, and
Mycobacterium tuberculosis and Mycobacterium avium among HIV-infected patients
after the introduction of highly active antiretroviral therapy. EuroSIDA Study Group JD.
7363.
20. Kariyone, A., K. Higuchi, S. Yamamoto, A. Nagasaka-Kametaka, M. Harada,
A. Takahashi, N. Harada, K. Ogasawara, and K. Takatsu. 1999. Identification of
amino acid residues of the T-cell epitope of Mycobacterium tuberculosis alpha
E. Meffre, and M. C. Nussenzweig. 1999. Continued RAG expression in late
stages of B cell development and no apparent re-induction after immunization.
4810.
expansion, and control of cellular immunity to tuberculosis. Immunol. Rev. 226:
191–204.
2002. Dissemination of Mycobacterium tuberculosis is influenced by host factors and
B. R. Bloom. 1993. An essential role for interferon gamma in resistance to
27. Appelberg, R. A. G. Castro, J. Pedrosa, R. A. Silva, I. M. Orme, and
P. Minoprio. 1994. Role of γ interferon and tumor necrosis factor α during
T-cell-independent and -dependent phases of Mycobacterium avium infection.
Mycobacterium tuberculosis by reactive nitrogen intermediates produced by
clearance of Mycobacterium avium upon disruption of the inducible nitric oxide

T CELLS HOME TO THE THYMUS AND CONTROL INFECTION

12

Downloaded from http://www.jimmunol.org/ by guest on August 31, 2017

by guest on August 31, 2017 http://www.jimmunol.org/ Downloaded from


