Dissemination of Mycobacteria to the Thymus Renders Newly Generated T Cells Tolerant to the Invading Pathogen

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The ability of the thymus to generate a population of T cells that is, for the most part, self-restricted and self-tolerant depends to a great extent on the Ags encountered during differentiation. We recently showed that mycobacteria disseminate to the thymus, which raised the questions of how mycobacteria within the thymus influence T cell differentiation and whether such an effect impacts host–pathogen interactions. Athymic nude mice were reconstituted with thymic grafts from *Mycobacterium avium*-infected or control noninfected donors. T cells generated from thymi of infected donors seemed generally normal, because they retained the ability to reconstitute the periphery and to respond to unspecific stimuli in vitro as well as to antigenic stimulation with third-party Ags, such as OVA, upon in vivo immunization. However, these cells were unable to mount a protective immune response against a challenge with *M. avium*. The observation that thymic infection interferes with T cell differentiation, generating T cells that are tolerant to pathogen-specific Ags, is of relevance to understand the immune response during chronic persistent infections. In addition, it has potential implications for the repertoire of T cells generated in patients with a mycobacterial infection recovering from severe lymphopenia, such as patients coinfected with HIV and receiving antiretroviral therapy. *The Journal of Immunology*, 2010, 184: 351–358.

Several mycobacterial species are important human pathogens, causing a diverse set of diseases that are usually chronic and progressive (1). The host immune response against these pathogens depends, to a great extent, on the activation of infected cells, mostly macrophages and dendritic cells (DCs), by mycobacteria-specific CD4+ T cells. IFN-γ produced by mycobacteria-specific T cells plays a central role in this activation and, consequently, in the control of mycobacterial infections (2). The increase in *Mycobacterium avium* and *Mycobacterium tuberculosis* infections observed in patients with HIV and low CD4+ T cell counts further highlights the relevance of this cell population (3). Unlike *M. tuberculosis*, a primary human pathogen, *M. avium* is a common opportunistic bacteria responsible for localized or disseminated infections, mainly in immune-suppressed individuals (4).

In addition to causing pathology at the site of entry (lung, gut, or skin), mycobacteria are able to disseminate to several other tissues and organs, such as pleura, meninges, and, to a lesser extent, bones and joints (1). A few recent case reports showed the thymus to be an additional site for mycobacterial dissemination (5–9). Using the mouse as a model, we previously showed that the thymus is a target for mycobacterial dissemination, regardless of whether the route of infection used is i.v. or aerogenic (10). In both cases, the bacterial load is initially undetectable or extremely low and increases progressively for several weeks postinfection (10).

Because infection of the thymus with several pathogens is accompanied by premature thymic atrophy, associated or not with the progressive loss of peripheral T cells, research on the topic has focused mostly on thymic cellularity (11). However, although disseminated infection with a highly virulent strain of *M. avium* is accompanied by an intense premature thymic atrophy, this does not seem to be the case when a less virulent strain of *M. avium* is used (12). The possibility that T cell differentiation is maintained, despite the progressive colonization of the thymus with a given pathogen, prompted us to investigate to what extent T cell differentiation is preserved and whether the newly generated T cells, whose differentiation occurred within infected thymi, differ from those generated in noninfected thymi. We observed that, although the production of new T lymphocytes is maintained in infected thymi, these T cells do not mount a protective immune response against the same pathogen in peripheral organs.

Materials and Methods

Mice and infection

C57BL/6 (wild-type [WT]), nude (B6.Cg-*Foxn1^nu*/J), and TCRα^−/− (B6.129S2-*Tcra^tm1Mom*/J) mice were purchased from Charles River Laboratories (Barcelona, Spain), Taconic Farms (Germantown, NY), and The Jackson Laboratory (Bar Harbor, ME), respectively. 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.

Eight- to 10-wk-old female mice were infected i.v. with 10^6 CFU *M. avium* strain 2447 (provided by Dr. F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium). Mice were sacrificed with isoflurane. The bacterial load in the organs was determined as previously described (10).

Thymic transplant

Thymi were aseptically removed from TCRα^−/− mice and maintained in DMEM (supplemented with 10% heat inactivated FCS, 10 mM HEPES,

1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin) for 10–20 min until being transplanted under the kidney capsule of nude mice (anesthetized with xylazine hydrochloride, 200 μg, and ketamine hydrochloride, 200 μg, administered i.v.).

Immunization

Mice were immunized subcutaneously on the back three times, at 1-wk intervals, with 10 μg OVA (Sigma-Aldrich, St. Louis, MO), emulsified in 250 μg dimethyldioctadecyl ammonium chloride (Sigma-Aldrich) and 25 μg monophosphoryl lipid A (Sigma-Aldrich), prepared as specified elsewhere (13). Mice were sacrificed 1 wk after the last immunization.

Immunofluorescence, histopathology, and image analysis

Sections (7 μm) of cryopreserved thymi were fixed in cold acetone, washed, and blocked with 4% BSA in PBS 0.05% Tween. Tissues were incubated overnight at 4°C with purified Abs (rabbit anti-*Mycobacterium* spp. polyclonal Ab [6398-0006, AbD, Serotec, Oxford, U.K.]; an Ab raised with extract from *M. tuberculosis* that reacts mainly with lipoara-binomannan]; anti-keratin 5 [K5] [Abcam, Cambridge, U.K.]; anti-keratin 8 [K8] [Troma1, developed by P. Brulet and R. Kemler and obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biology, Iowa City, IA]; and anti-CD11c [N418] or anti-F4/80 [A3-1] [Serotec]). Secondary Abs used were anti-rat IgG Alexa Fluor 594 or Alexa Fluor 488; anti-rabbit IgG Alexa Fluor 488 (Molecular Probes, Eugene, OR); and biotinylated anti-hamster IgG (Serotec) plus Pacific Blue-conjugated streptavidin (Molecular Probes). In addition, mycobacteria were also detected by auramine-rhodamine staining (AlphaTec Systems, Vancouver, WA) at the end of the immunofluorescence protocol. Inducible NO synthase (iNOS; clone M-19, Santa Cruz Biotechnology, Santa Cruz, CA) detection was performed in paraffin-embedded liver sections, as described elsewhere (14).

Slides were visualized using an epifluorescence microscope (BX61 microscope with an Olympus DP70 camera), and images were analyzed using Image J software (National Institutes of Health, Bethesda, MD). No significant signal was observed in the negative controls (no Abs, no primary Abs, and isotype controls); in the case of antimycobacteria staining, no signal was observed in thymi from noninfected mice.

Analysis of lesions was performed in liver sections stained with hematoxylin and eosin. Using the ×20 objective, five random fields (in a total area of 2.9 mm^2) were photographed from one liver section per mouse. Classification of the lesions was performed blindly.

Quantification of TCR rearrangement excision circles

Genomic DNA was isolated from thymi using TRIzol reagent. Quantification of signal-joint TCR rearrangement excision circles (TRECs) was performed by quantitative RT-PCR using the TCRA constant gene as an endogenous reference [specific primers used were described elsewhere (15)]. Quantitative RT-PCR reactions were performed on a CFX96 Real Time System (Bio-Rad, Hercules, CA) using a QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Hamburg, Germany). All melting curves exhibited a single sharp peak.

Cell preparation, in vitro stimulation, and ELISPOT

Cell suspensions were prepared by gentle disruption of the organs between two notched slide glasses. Es were lysed using a hemolytic solution (155 mM NH$_4$Cl, 10 mM KHCO$_3$, pH 7.2), and cells were resuspended in supplemented DMEM.

Spleen cells were stimulated in vitro as described elsewhere (16). Stimuli used were Con A (4 μg/ml), *M. avium* total extract proteins (4 μg/ml), or a panel of m.w. fractions from *M. avium* short-term culture filtrate (2 μg/ml) (details on the preparation can be found in Supplemental Fig. 1) (16). IFN-γ was quantified by ELISA (R4-6A2 and biotinylated AN18 were used as capture and detection Abs, respectively; BD Biosciences, San Jose, CA). Assay sensitivity was 20 pg/ml.

ELISPOT was performed as described previously (14). The stimuli used were 10 μg/ml mycobacteria epitope Ag85A$_{241-260}$ (Metabion, Martinsried, Germany) (17) or OVA (Sigma-Aldrich).

Flow cytometry

Cells were labeled with Abs specific for CD3 (145-2C11), CD4 (RM4-5), and CD8 (53-6.7, BioLegend, San Diego, CA). Cell acquisition was performed on a FACSCalibur flow cytometer or on a FACSAria cell sorter using Cell Quest software (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Differences among the means of experimental groups were analyzed with the two-tailed Student t test. Differences with a p value ≤0.05 were considered significant.

Results

Infected cells within the thymus are CD11c$^+$

To evaluate the potential impact of mycobacterial dissemination to the thymus on T cell differentiation, the identification of the infected cells, as well as their localization, is essential. Upon i.v. infection with 10^6 CFU of *M. avium*, the thymus became progressively colonized (Fig. 1A), with almost undetectable bacterial loads on day one, to ~100 bacilli at 4 wk, and ~10^5 viable bacteria at 24 wk postinfection. We previously reported that during the first weeks of infection the few bacteria that could be detected within the thymus were mostly at the cortico-medullary region (10). As infection progressed, bacteria were typically present within clumps of large cells, mostly at the cortico-medullary region and within the medulla (10). Using Abs specific for K8 (largely restricted to cortical epithelial cells, with a small subset of K8$^+$ cells within the medulla) and K5 (largely restricted to the medulla, with a small subset of K5$^+$K8$^+$ cells at the cortico-medullary region and scattered in the cortex) (18), we now confirm that the clumps of infected cells are typically at the cortico-medullary and medullary areas and less frequently are within the cortex (Supplemental Fig. 2A–D).

All infected cells stained for CD11c (Fig. 1B–E) and consisted of two populations. The majority were CD11c$^+$F4/80$^+$ (Fig. 1B), a phenotype consistent with foamy macrophages (19, 20). To a lesser extent, bacteria were detected within CD11c$^+$F4/80$^-$ cells (Fig. 1C), a phenotype compatible with DCs. As described previously (21), a weak staining was observed throughout the cytoplasm of infected cells when antimycobacteria Abs were used (Fig. 1B, 1C). This has been attributed to mycobacterial envelope material present in the phagosomes of infected cells (21).

Epithelial cells do not seem to be a target for *M. avium* infection, because no infected cells expressed K8 (Fig. 1D), K5 (Fig. 1E), or epithelial cell adhesion molecule (Supplemental Fig. 1E).

Because DCs within the thymus are known to play an important role in thymocyte selection processes (22–26), the observation that *M. avium*-containing DCs are present in these areas suggests that thymic infection might influence T cell differentiation.

T cells generated in mycobacteria-infected thymi are unable to control bacteria proliferation in the periphery

Accelerated thymic atrophy, which is accompanied by reduced or abrogated T cell differentiation, has been frequently reported during systemic infections (11). This is well known in humans infected with HIV (27) and has been shown in several animal models of infection (11, 12). The mycobacterial infection used in this study did not result in altered total thymic cell number up to 22 wk postinfection (Fig. 2A). Only minor differences were detected, at late time points, in the proportion of the four main thymocyte populations (double negative CD4$^-$CD8$^-$CD3$^-$, double positive CD4$^+$CD8$^+$CD3$^{low/-}$, CD4 single positive CD4$^+$CD8$^-$CD3$^+$, and CD8 single positive CD4$^-$CD8$^+$CD3$^+$) (Fig. 2B). Moreover, no differences were detected in the amount of TRECs up to 30 wk postinfection (Fig. 2C). These results suggest that thymocyte differentiation is maintained throughout chronic mycobacterial infection, despite the presence of a considerable number of mycobacteria-infected cells within the thymus.

We next asked to what extent the newly generated T cells, whose differentiation occurred within infected thymi, were able to mount a protective immune response against mycobacterial infections. To
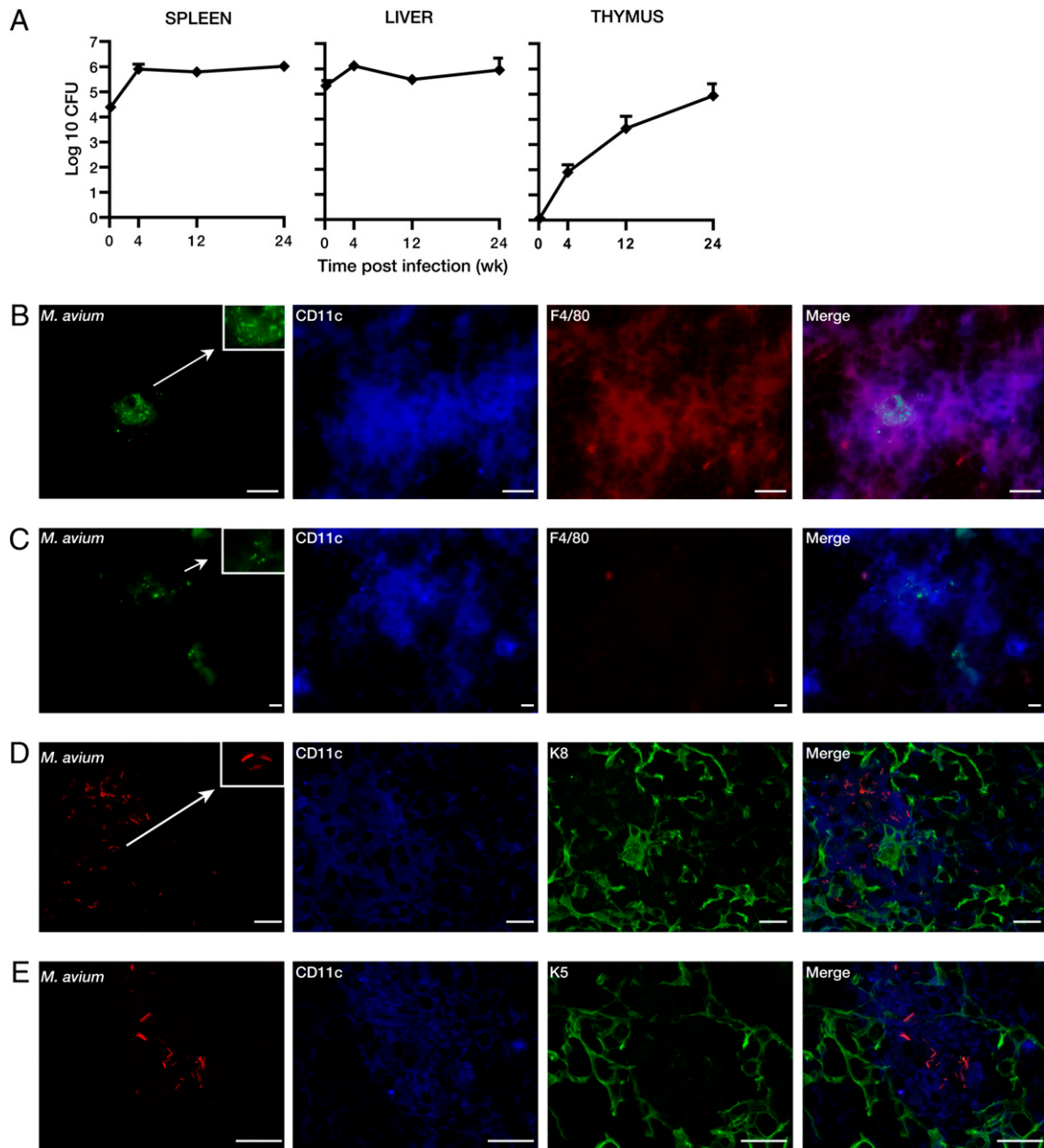
FIGURE 1. Identification of *M. avium*-infected cells in the thymus. *A*, Representative kinetics of *M. avium* bacterial load in the spleen, liver, and thymus. Each point represents the mean ± SD CFU from six mice/group for one of four experiments. *B–E*, Representative thymic sections from *M. avium*-infected WT mice (20–24 wk postinfection) stained with Abs specific for CD11c (*B–E*), F4/80 (*B* and *C*), K5 (*D*), and K8 (*E*). Bacilli were detected with anti-mycobacteria Ab (*B* and *C*) or by auramine-rhodamine staining (*D* and *E*). *B*, Bacteria (green) were mainly found within CD11c⁺ (blue) F4/80⁺ (red) cells. *C*, Bacteria also were detected within CD11c⁺F4/80⁻ cells. *D* and *E*, CD11c⁺ cells (blue) containing bacteria (red) surrounded by K5⁺ (*D*) and K8⁺ (*E*) cells (green). Bar = 20 μm.

characterize T cells whose differentiation occurred within infected thymi and their ability to control mycobacteria growth, it was necessary to create a system in which these cells could be analyzed with no interference from T lymphocytes whose differentiation occurred prior to thymic infection. To do so, thymic lobes from *M. avium*-infected TCRα$^{-/-}$ mice (28) were transplanted under the kidney capsule of athymic nude mice. Nude mice have no thymus but have competent T cell precursors (29), and TCRα$^{-/-}$ mice have a thymus, but their T cell precursors are unable to fully differentiate (28). By performing these thymic transplants, we generated mice with T cells derived from nude mice's bone marrow that differentiated exclusively within *M. avium*-infected TCRα$^{-/-}$ thymi (Fig. 3*A*). Nude mice transplanted with non-infected thymi were used as controls. Most bacteria within the

infected transplanted thymi (containing ∼10$^{5-6}$ CFU) seemed to remain within the thymus, because we were only able to detect a low number of bacteria in other organs in a few transplanted animals (three of eight) 4–8 wk posttransplantation (data not shown). However, even if residual, these could contribute to peripheral T cell response when posterior infection is induced. Therefore, to guarantee that a similar bacterial load was present in the peripheral organs of nude mice transplanted with infected or noninfected thymi at the moment of peripheral T cell colonization, all animals were infected 3 d postthymic transplantation (i.v. infection with 10^6 CFU) (Fig. 3*A*). At 5 wk posttransplantation, similar numbers of newly formed CD4⁺CD3⁺ and CD8⁺CD3⁺ T cells were detected in the blood of mice transplanted with infected or noninfected thymi (Fig. 3*B*). At sacrifice (7–9 wk
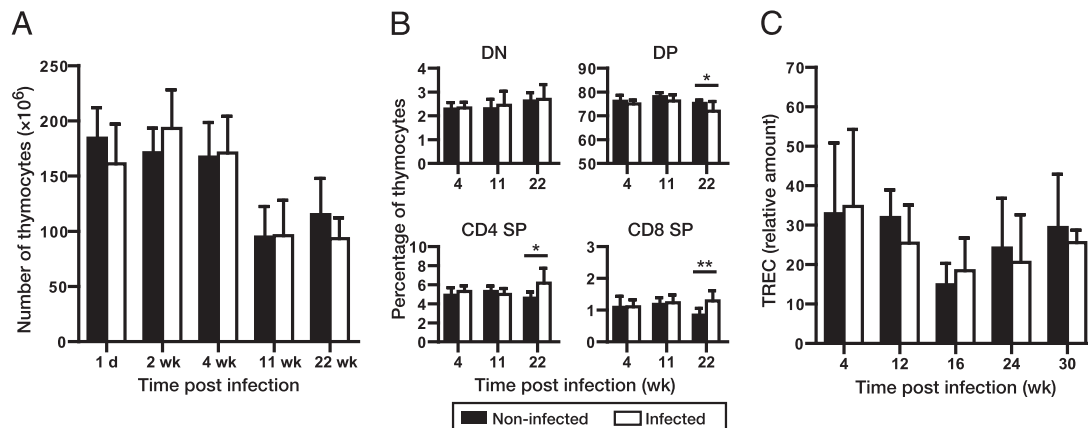
FIGURE 2. *M. avium* infection of the thymus does not lead to premature thymic atrophy but causes minor changes in cell populations at late time points. *A*, Number of thymocytes in infected and age-matched control mice. *B*, The percentage of the four main thymic populations (accordingly to the expression of CD4, CD8, and CD3) in infected and age-matched control animals at different time points postinfection. $*p \leq 0.05$; $**p \leq 0.005$. *C*, TCR rearrangement in thymi assessed by the relative quantification of TREC. Each bar represents the average $\pm$ SD of the number (*A*) and percentage (*B*) of cells or relative amount of TREC (*C*) from three to six mice per group.

postinfection, depending on the experiment), thymic engraftments under the kidney capsule were confirmed macroscopically and microscopically for each animal. The numbers of T cells in the spleen were similar for mice transplanted with infected or non-infected thymi (Fig. 3*C*). Moreover, we found no differences in the activation profile of the T cells or in the proportion of effector/regulatory CD4$^+$ T cells (data not shown). Thus, T cells arising from infected or noninfected thymi were equally able to colonize the periphery of nude recipient mice. However, when the bacterial load was assessed, mice transplanted with noninfected thymi presented almost ten times fewer viable bacteria in the liver and spleen than those transplanted with infected thymi (Fig. 3*D*). Interestingly, the bacterial load in the liver of animals transplanted with infected thymi was not different from that found in nude mice. These observations clearly indicate that T cells that differentiate within infected thymi have an impaired ability to protect against the same pathogen in peripheral organs.

T cells that differentiate in an infected thymus have a reduced ability to produce IFN-γ in response to M. avium *Ags*

The impaired ability of T cells arising from infected thymi to mount a protective immune response against *M. avium* could be due to a general defect of these cells to respond to stimuli or due to a specific *M. avium*-induced tolerance. Because the immune response against mycobacterial infections depends greatly on the ability of mycobacteria-specific T cells to produce IFN-γ (16), splenocytes were stimulated in vitro (Fig. 4*A*) with a panel of *M. avium* antigenic fractions (Supplemental Fig. 1). Splenocytes from WT, as well as from nude mice transplanted with noninfected thymi, produced similar amounts of IFN-γ upon stimulation with Con A or with *M. avium* antigenic fractions (Fig. 4*A*). In contrast, splenocytes from mice transplanted with infected thymi generated undetectable levels of IFN-γ in response to most of the *M. avium* antigenic fractions. As expected, splenocytes from non-transplanted nude mice did not produce detectable levels of IFN-γ (data not shown). In our thymic transplant system, an innate immune response to infection is taking place when de novo generated T cells colonize the periphery. Although the results suggest that thymic export from infected and noninfected thymi is similar (as shown by no premature thymic atrophy and no differences on the amounts of TRECs in the thymus of infected versus non-infected mice and by similar peripheral T cell reconstitution in mice transplanted with infected or noninfected thymi), we cannot

exclude the possibility that a difference in the timing of thymic export exists, which could delay the acquired immune response in one of the experimental groups. To clarify this aspect, purified CD4$^+$ T cells from mice transplanted for 9 wk were transferred to new TCRα$^{-/-}$ mice (Fig. 4*B*). These mice were infected 3 d after the adoptive transfer and sacrificed 18 wk later. T cells transferred from both types of mice reconstituted equally well the periphery of TCRα$^{-/-}$ mice (Supplemental Fig. 3). When stimulated in vitro with *M. avium* Ags, splenocytes from mice that received CD4$^+$ T cells from nude mice transplanted with infected thymi produced extremely low levels of IFN-γ compared with mice that received CD4$^+$ T cells from animals transplanted with noninfected thymi (Fig. 4*C*). Further supporting the hypothesis that T cells differentiating in infected thymi are specifically impaired in their ability to respond to mycobacterial Ags was the observation that OVA-immunized mice transplanted with infected thymi mounted an immune response to OVA indistinguishable from that of OVA-immunized mice transplanted with noninfected thymi (Fig. 5). It is important to stress that because a minute amount of bacteria from the infected thymi may disseminate to peripheral organs in some animals, as reported previously, all animals were infected 3 d after thymic transplant; the immunization regimen was initiated at 3 wk posttransplant.

Differentiation within infected thymi generates T cells unable to participate in the formation of organized granulomas

Although Ag-specific T cells are required for the formation of fully mature granulomas, only poorly organized granulomas are observed in T cell-deficient hosts, provided that there is IFN-γ secretion by innate immune cells (30). The liver inflammatory lesions of nude mice transplanted with infected or noninfected thymi, as well as the nontransplanted nude and WT mice, were classified, taking into consideration their organization as nonorganized infiltrates (lymphocytes and/or macrophage-like cells showing no signs of organization), poorly organized granulomas (a central core of macrophage-like cells surrounded by lymphocytes but not forming a clear cuff), and well-organized granulomas (a central core of macrophage-like cells surrounded by a well-defined cuff of lymphocytes) (Fig. 6*A*, *right*, *middle*, and *left panels*, respectively). Although the three types of lesions were present in the liver of WT infected animals, well-organized granulomas, which depend on the existence of Ag-specific T cells, were virtually absent from nude mice, as described previously (30). Mice transplanted with infected
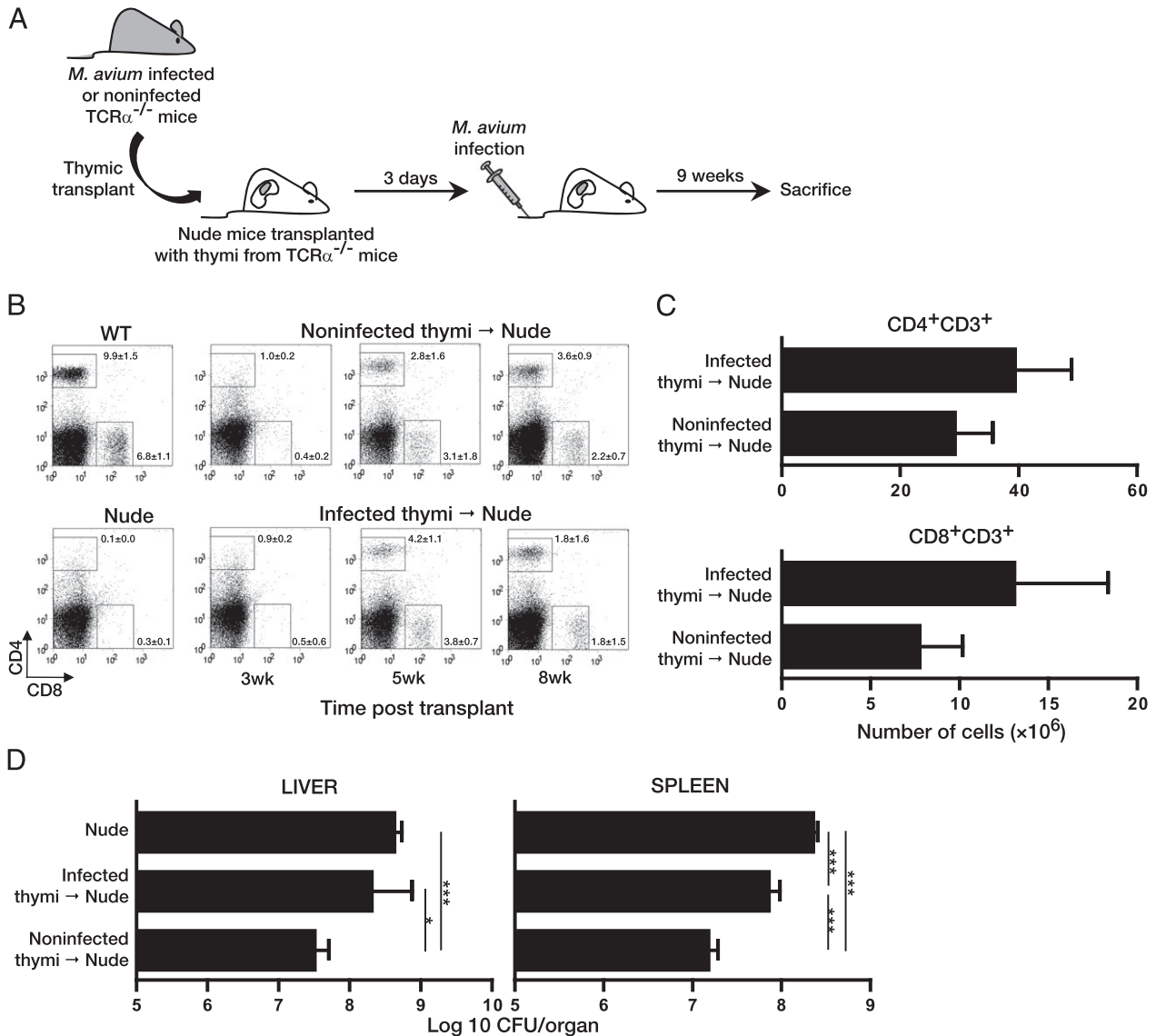
FIGURE 3. T cells whose differentiation takes place within infected thymi present an impaired ability to fight the pathogen in peripheral organs. *A*, Thymic lobes from TCRα$^{-/-}$ mice were transplanted under the kidney capsule of nude mice (thymic donors were animals previously infected with *M. avium* or age-matched noninfected controls). Transplanted mice were infected with *M. avium* 3 d after surgery and sacrificed 9 wk later. *B*, Representative plots of the CD4 and CD8 staining in the blood at different time points after transplantation with noninfected (*upper panels*) or infected (*lower panels*) thymic lobes. WT (*left upper panel*) and nude (*left lower panel*) blood cells are also depicted. *C*, Number of CD4$^+$ T cells and CD8$^+$ T cells in the spleen. *D*, Bacterial loads were assessed in liver and spleen 9 wk after infection. Each column represents the mean ± SD of the numbers of cells (*C*) or the CFU (*D*) from three to seven mice per group from one of three experiments. *$p \leq 0.05$; ***$p \leq 0.001$.

thymi presented the same pattern of lesions as nude animals (Fig. 6*B*). These observations corroborate the finding that T cells arising from infected thymi are mostly depleted of activated mycobacteria-specific T cells. The inability of T cells from mice transplanted with infected thymi to participate in the formation of structured granulomas is further supported by the lower number of liver lesions expressing iNOS, an enzyme whose expression is upregulated in activated macrophages (12), compared with WT or mice transplanted with noninfected thymi (Fig. 6*C*, 6*D*).

Discussion

This study shows that thymi infected with *M. avium* retain the ability to generate new T cells. However, T cells generated within infected thymi are unable to mount a protective response against *M. avium* in the periphery. This raises the possibility of a defect in the T cells generated within infected thymi. The similarities in the ability of infected and noninfected thymi (or of isolated T cells recovered from transplanted recipients) to repopulate the periph-

ery, as well as their indistinguishable ability to produce IFN-γ in response to Con A, strongly argue against a general defect of the T cells generated within infected thymi. Moreover, the observation that T cells that differentiate within infected thymi mount an immune response to OVA that is similar to that of T cells that differentiate in noninfected thymi shows that, in both cases, T cells are able to mount T cell-specific immune responses. Therefore, the T cell tolerance observed against *M. avium* Ags in animals transplanted with infected thymi seems specific to this pathogen, as evaluated by their inability to control bacterial growth and their reduced aptitude to produce IFN-γ upon stimulation with a set of mycobacterial Ags. These results show that T cells generated in infected thymi are specifically impaired in their ability to respond to *M. avium* Ags.

Because mycobacteria were detected within macrophages and DCs in the medulla and at the cortico-medullary region and that DCs are recognized key players in the clonal deletion of specific T cells (22, 26, 31), the clonal deletion of *M. avium*–specific T cells is the most likely
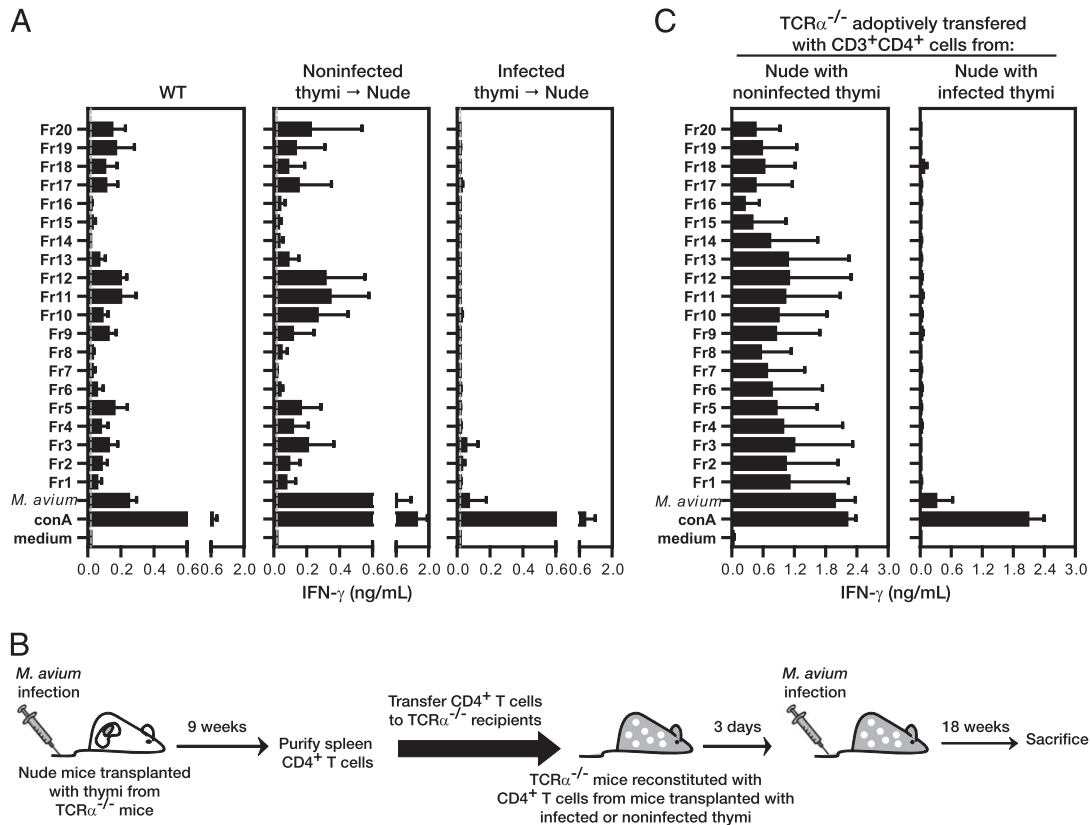
FIGURE 4. T cells differentiated within infected thymi show impaired ability to produce IFN-γ in response to *M. avium* Ags. *A*, Splenocytes from WT and nude mice transplanted with noninfected or infected thymi (*right*, *middle*, and *left panels*, respectively) were stimulated in vitro with Con A, *M. avium* total extract (referred to as *M. avium* in the figure), and a panel of antigenic fractions prepared from the supernatant of the *M. avium* culture (Fr1 to Fr20) (details on the preparation can be found in Supplemental Fig. 1). IFN-γ was quantified in the cell supernatants by ELISA. *B*, Thymic lobes from TCRα$^{-/-}$ infected or noninfected mice were transplanted under the kidney capsule of nude recipient mice. Transplanted mice were infected 3 d later with *M. avium* and sacrificed after 9 wk. Purified CD4$^+$ T cells from these mice were adoptively transferred to TCRα$^{-/-}$ mice that were infected with *M. avium* 3 d later and sacrificed 18 wk afterward. *C*, Splenocytes of TCRα$^{-/-}$ mice adoptively transferred with CD4$^+$ T cells from nude mice transplanted with noninfected (*left panel*) or infected thymi (*right panel*) were stimulated with the same Ags described in *A*. Each bar represents the mean ± SD of the IFN-γ concentration from three to six mice per group from one of three independent experiments.

mechanism responsible for the observed *M. avium* tolerance. It was recently shown that adoptively transferred DCs loaded with a nonself Ag migrate to the thymus, leading to clonal deletion of T cells that
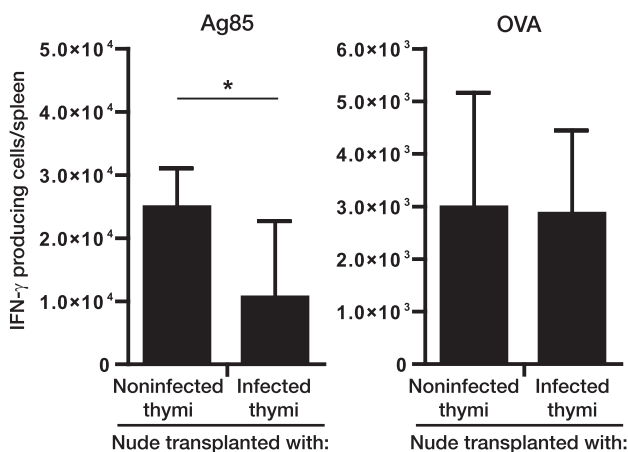

FIGURE 5. Upon immunization, T cells that differentiated within infected thymi respond in vitro to OVA. Nude mice transplanted with infected or non-infected thymic lobes from TCRα$^{-/-}$ mice were infected, immunized with OVA, and sacrificed 1 wk after the last immunization. The number of IFN-γ–producing splenocytes, when stimulated with Ag85 (*left panel*) or with OVA (*right panel*), was assessed by ELISPOT. Each bar represents the mean ± SD of the number of IFN-γ–producing cells from four to five mice per group. *$p \leq 0.05$.

recognize that Ag (23), and that within the thymus, DCs of extrathymic origin are mainly localized at the cortico-medullary region (32).

Because regulatory T cells participate in the immune response against mycobacteria (33), the possibility that differentiation within infected thymi alters the generation of regulatory T cells, leading to the differentiation of a T cell population enriched in regulatory T cells or particularly enriched in mycobacteria-specific regulatory T cells, should also be taken into consideration. This is particularly relevant because of the recent data that associate DCs of extrathymic origin with the generation of regulatory T cells within the thymus (24). Although infected DCs are detected within the thymus, our results do not support the possibility that regulatory T cells are responsible for the decreased protection against *M. avium*. Mice that were transplanted with infected thymi did not present more regulatory T cells in the periphery, either in absolute numbers or in the proportion of regulatory/effector CD4$^+$ T cells. Effector and regulatory Ag-specific T cells located in the infected tissues were shown initially for *Leishmania major* infection (34); more recently, it was reported that regulatory T cells do participate in the formation of mycobacteria-induced granulomas (33). Thus, our observation that the pattern of inflammatory lesions is similar in nude mice that were and were not transplanted with infected thymi strongly suggests the lack of *M. avium*-specific T cells, whether regulatory or effector, in animals transplanted with infected thymi.

Although infection of the thymus has previously attracted the curiosity of researchers, to the best of our knowledge, this study is
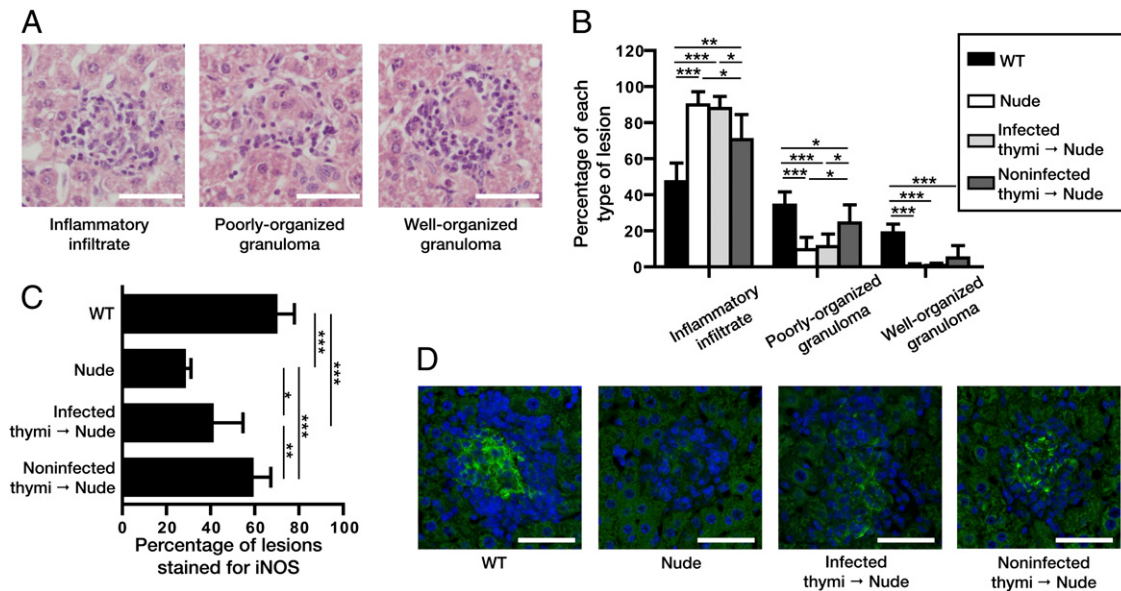
FIGURE 6. T cells differentiated in infected thymi do not participate in the formation of well-organized granulomas. Liver sections from mice transplanted with infected or noninfected thymic lobes (as described in Fig. 2) and control mice were analyzed 9 wk posttransplant. *A*, Representative hematoxylin and eosin staining showing three types of hepatic lesions. *B*, The percentage of each type of lesion was assessed. *C*, Percentage of iNOS$^+$ lesions in the liver. Each bar represents the mean $\pm$ SD from five to nine mice per group. *D*, Representative iNOS staining of liver lesions from WT, nude, and nude mice transplanted with infected or noninfected thymic lobes. Bar = 50 μm (original magnification $\times$200). $*p \leq 0.05$; $**p \leq 0.005$; $***p \leq 0.001$.

the first demonstration that bacteria within the thymus lead to the generation of T cells that are impaired in their ability to mount a protective immune response against the infecting pathogen in the periphery. The fact that the newly generated T cells are, to a great extent, tolerant to *M. avium* raises new questions for understanding the host–parasite interaction during chronic persistent infections. One needs to consider the hypotheses that this induced-tolerance on de novo generated T cells may represent an advantage or a disadvantage for the host. In fact, the process might be part of a broader mechanism that prevents excessive immune response and, consequently, immunopathology. It should be noted that in immune-competent animals, newly generated T cells are not necessary to control a persistent infection, because mycobacterial growth in mice thymectomized 2 wk prior to infection is the same as that of sham-thymectomized mice [data not shown and (12, 35)]. However, this might also be detrimental, especially in situations in which the peripheral T cell population depends to a great extent on thymic export, as we showed in this study. In humans, despite the progressive physiologic thymic atrophy observed with age, it is becoming clear that the thymus maintains its ability to generate new T cells in adulthood (36, 37). This is of relevance in situations of T cell reconstitution upon severe lymphopenia, such as in patients with AIDS (37) and those submitted to cytoreductive transplant (38) or receiving chemotherapy (39). After autologous transplant, thymopoiesis has been shown to be critical to restore the peripheral CD4$^+$ T cell population. This recovery encompasses the reconstitution of CD4$^+$ T cells expressing a diverse TCR repertoire (36). Moreover, for HIV$^+$ patients receiving highly active antiretroviral therapy, thymic volume increase was associated with better CD4$^+$ T cell recovery (37). The data presented in this study suggest that, when the peripheral T cell population reconstitution depends greatly on de novo thymic export, infection of the thymus by mycobacteria may have, in humans, a relevant negative effect on the ability to control chronic slow-progressing or latent infections.

In summary, our observations show that during infection with *M. avium,* infected cells within the thymus induce tolerance specifically to mycobacterial Ags. This is the first report showing induced central tolerance to an infecting pathogen. Knowing that the thymus is also a target organ for mycobacterial infections in humans, it is now important to evaluate to what extent the newly generated T cells from patients infected with *M. avium* or *M. tuberculosis* display an impaired ability to respond to mycobacterial Ags.

Disclosures

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