Immunity, Vol. 13, 771–782, December, 2000, Copyright ©2000 by Cell Press

Selective Activation and Expansion of High-Affinity CD4+ T Cells in Resistant Mice upon Infection with *Leishmania major*

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Summary

Using multimers of MHC class II molecules linked to a peptide derived from the *Leishmania* LACK antigen, we have compared the fate of parasite-specific CD4+ T cells in resistant and susceptible mice transgenic for the β chain of a LACK-specific TCR. Activated T cells were readily detected in the draining lymph nodes of infected animals. Although the kinetics of activation and expansion were similar in both strains, T cells from susceptible and resistant mice expressed low- and high-affinity TCR, respectively. As T cells from resistant mice produced more IFN-γ and less IL-4 than those from susceptible animals, our results suggest that differences in TCR usage between MHC-matched animals may influence the development of the antiparasite immune response.

Introduction

In contrast to most inbred mouse strains that are resistant to infection with the intracellular parasite *Leishmania major*, BALB/c mice are unable to control infection and develop progressive lesions (Reiner and Locksley, 1995). Several studies have shown that CD4+ T cells are critical in determining the outcome of the infection, while B cells (Brown and Reiner, 1999) and CD8+ T cells (Huber et al., 1998) play only a minor role. Moreover, resistance and susceptibility correlate with the development of Th1- and Th2-dominated responses, respectively. Thus, while CD4+ T cells from resistant mice secrete large amounts of IFN-γ and low levels of IL-4 and IL-5, those from susceptible animals produce mainly IL-4. The functional importance of these different cytokine responses was further demonstrated by experiments in which the transfer of *L. major*–specific Th1 and Th2 cell lines to naive hosts led to resistance or susceptibility, respectively (Scott et al., 1990; Titus et al., 1991).

Although CD4+ T cells play a critical role in determining the disease outcome, almost nothing is known about their kinetics of activation, expansion, and death. In early studies, limiting dilution assay (LDA) was used to show that infection of susceptible BALB/c and resistant C57BL/6 mice resulted in a 4-fold increase in the frequency of LN CD4+ cells that formed clones when cultured with soluble Leishmania antigens (SLA) (Morris et al., 1992). While the frequencies of IL-4- and IFN-γ-secreting clones were similar in BALB/c and C57BL/6 mice 1 week after infection, healing of C57BL/6 mice was associated with a decrease in the frequency of IL-4-secreting clones and an increase in those that secreted IFN-γ. More recently, LN cells from infected mice were stimulated in vitro with SLA, or phorbol myristate acetate (PMA) and ionomycin, and the frequency of cytokine-secreting cells was assessed by intracellular staining for IL-4 or IFN-γ (Sommer et al., 1998). One week after infection, a strong but comparable increase in the frequency of IFN-γ- and IL-4-secreting CD4+ T cells (up to 7% of all CD4+ cells) was observed in both C57BL/6 and BALB/c mice. Four weeks later, the frequency of IL-4-secreting cells had dropped to 1% in C57BL/6 mice but not in BALB/c animals.

Although these studies have provided information on the number of cytokine-secreting CD4+ T cells in infected mice, measuring the actual frequency of parasite-specific T cells was not possible due to the lack of suitable reagents. Likewise, it has not been possible to monitor the surface phenotype of these cells during the course of the disease or to compare the kinetics of T cell responses between resistant and susceptible animals. To investigate these issues, we have produced multivalent peptide/MHC molecules that bind selectively to CD4+ T cells reacting to the immunodominant *Leishmania* LACK antigen (Mougneau et al., 1995). We have used these molecules as staining reagents to follow the fate of LACK-specific CD4+ T cells in MHC-matched resistant and susceptible mice upon infection with *L. major*.

Results

Protein A–Bound I-Ad/LACK Dimers Stain LACK-Specific CD4+ T Cells

To visualize parasite-specific CD4+ T cells, we made dimers of soluble peptide/MHC molecules in which an immunodominant peptide from the *Leishmania* LACK antigen was covalently attached to the N-terminal end of the class II I-Ad β chain. I-Ad α and β chains were truncated to eliminate their transmembrane regions and modified by the addition of leucine zipper peptide dimers at their COOH terminus as described (Scott et al., 1996). The α chain was further modified by the addition of the murine IgG2a Fc domain allowing for the production of divalent molecules as previously described for a

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human MHC class II molecule (Appel et al., 2000). The constructs were cloned into the vector pRMHa3 and cotransfected into *Drosophila* S2 cells along with a neomycin resistance gene to produce stable transfectants. I-A^d/LACK divalent molecules were purified to homogeneity by affinity chromatography and subsequent ion exchange chromatography.

We next investigated whether I-A^d/LACK dimers could stain LACK-specific CD4$^+$ T cells. While flow cytometry analysis did not show any binding of I-A^d/LACK dimers to LACK-specific LMR8.2 hybridoma (data not shown), stable and specific staining was achieved when I-A^d/LACK dimers were preincubated with Alexa 488-coupled protein A (Figure 1A). While both LACK-specific LMR8.2 and HEL-specific B04H9.1 hybridomas were stained with control anti-Cβ mAbs, only LMR8.2 hybridoma was stained with protein A–bound I-A^d/LACK dimers. In contrast, LMR8.2 hybridoma was not stained with protein A–bound I-A^d molecules attached to a peptide derived from the *Leishmania* GP63 protein. Specific binding of I-A^d/LACK multimers to LACK-specific T cells was independently demonstrated by staining thymocytes from ABLE $\alpha\beta$ TCR transgenic mice that express a LACK-specific TCR (Reiner et al., 1998). In these mice, I-A^d/LACK multimers stained 70% of CD4$^+$CD8$^-$ thymocytes as compared to only 0.2%–0.3% in wild-type (wt) BALB/c mice (Figure 1B).

As an attempt to track LACK-specific T cells, we injected BALB/c and B10.D2 wt mice with 2×10^6 *L. major* promastigotes. Mice were sacrified at day 6, 10, and 21 days postinfection, and LN T cells were stained with I-A^d/LACK multimers and anti-CD4 mAbs. No selective expansion of I-A^d/LACK$^+$ cells was observed in three independent experiments (data not shown). This was surprising, as infection with *L. major* was reported to induce the expansion of a restricted population of CD4$^+$ cells that expressed Vα8 and Vβ4 (Reiner et al., 1993), two TCR variable regions that are preferentially used by LACK-specific T hybridomas. To rule out the possibility that only a small proportion of LACK-specific T cells were stained by I-A^d/LACK multimers, we have immunized BALB/c mice with LACK, purified LN cells 6 days later, and sorted I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells by flow cytometry. In contrast to I-A^d/LACK$^+$ cells, I-A^d/LACK$^-$ cells did not proliferate when incubated with LACK peptide and syngenic APCs (data not shown). Thus, our inability to detect expansion of I-A^d/LACK$^+$ cells in infected mice was not likely due to the sensitivity of our staining procedure. In this respect, it is noteworthy that other investigators have failed to detect expansion of Vα8$^+$Vβ4$^+$ T cells in infected BALB/c mice (Lohoff et al., 1994; our unpublished data).

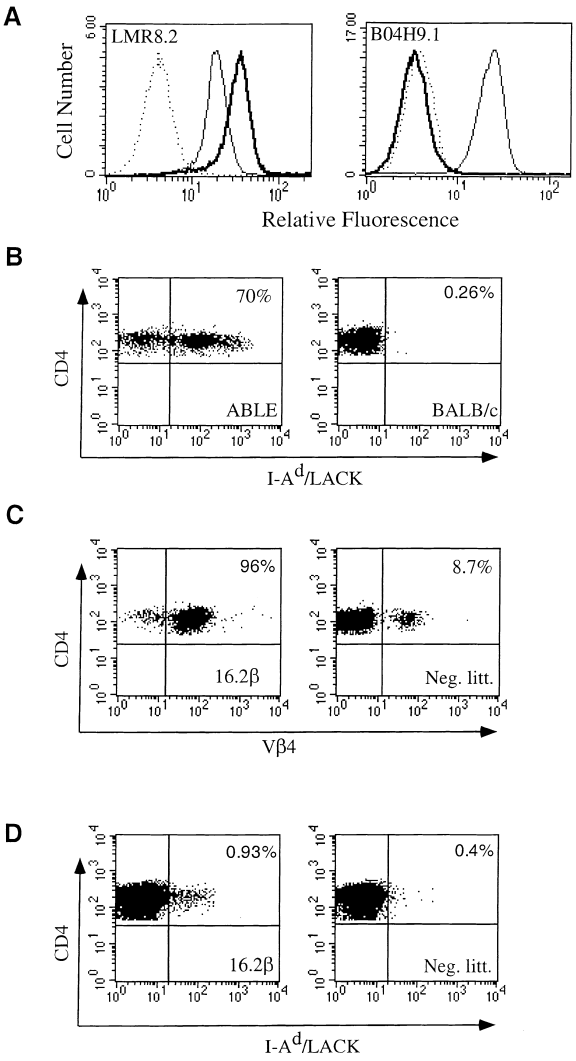

Figure 1. Protein A–Bound I-A^d/LACK Dimers Stain LACK-Specific T Cells

(A) I-A^d/LACK multimer staining of T cell hybridomas. LACK-specific LMR8.2 (left panel) and HEL-specific B04H9.1 (right panel) T cell hybridomas were stained with protein A–bound I-A^d/LACK dimers (thick solid line), anti-Cβ mAbs (thin solid line), or protein A–bound I-A^d/GP63 dimers (dashed line). Data show representative FACS analysis after gating on viable cells.

(B) I-A^d/LACK multimer staining of TCR transgenic thymocytes. Thymocytes from ABLE TCR $\alpha\beta$ transgenic mice (left panel) and BALB/c wt mice (right panel) were stained with I-A^d/LACK multimers and anti-CD4 and anti-CD8 mAbs. Plots display I-A^d/LACK multimer staining after gating on CD4$^+$CD8$^-$ T cells. The percentage of I-A^d/LACK$^+$ cells in the gated population is indicated.

(C and D) LN CD4$^+$ T cells from BALB/c 16.2β transgenic mice (left panels) and from their negative littermates (right panels) were stained with anti-CD4 and (C) anti-Vβ4 mAbs or (D) I-A^d/LACK multimers. Data show representative FACS analysis after gating on CD4$^+$ viable cells. The percentage of (C) Vβ4$^+$ and (D) I-A^d/LACK$^+$ cells in the gated population is indicated.

Fate of LACK-Specific T Cells in Mice Transgenic for the β Chain of a LACK-Specific TCR

To facilitate the detection of LACK-specific T cells, we constructed transgenic mice carrying the rearranged TCR β chain gene of the LACK-specific LMR16.2 T cell hybridoma (Mougneau et al., 1995). These mice, designated 16.2β, were crossed to BALB/c and B10.D2 mice for 16 and 6 generations, respectively. In both transgenic strains, flow cytometry analysis showed that >96% of CD4$^+$ T cells expressed Vβ4 as compared to only 8.7% in their negative littermates (Figure 1C). In addition, the expression of the transgene resulted in an elevated frequency of I-A^d/LACK$^+$ cells (Figure 1D). As observed in wt animals, the outcome of the disease was different in BALB/c and B10.D2 transgenic animals. Thus, while

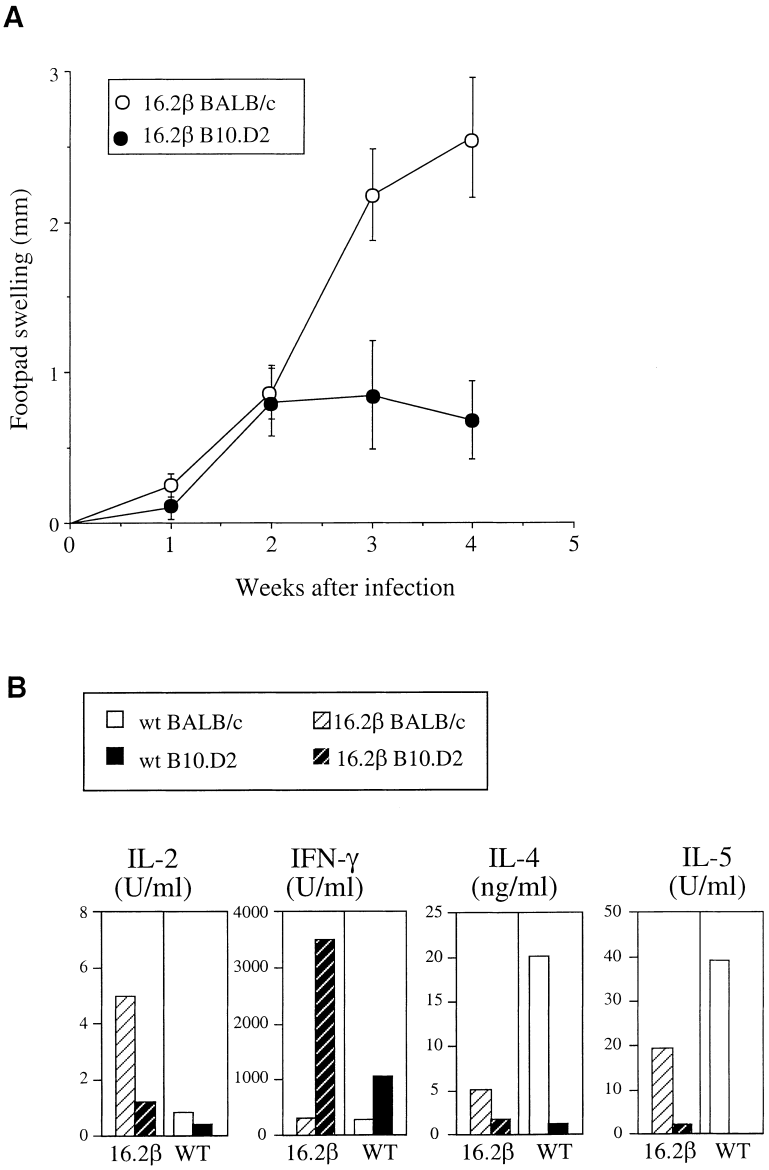

Figure 2. Disease Progression and T Cell Responses in 16.2β Transgenic Mice

(A) BALB/c (open circles) and B10.D2 (filled circles) 16.2β transgenic mice were injected with 2×10^6 *L. major* promastigotes. The increase in footpad thickness was measured at the indicated times after infection. Bars represent the arithmetic mean of six individual mice.

(B) Wt and 16.2β transgenic BALB/c and B10.D2 mice were infected with *L. major* and killed 4 weeks later. CD4+ LN cells were purified and incubated with syngeneic APCs and 30 μg/ml of SLA. Supernatants were harvested 72 hr later and IL-2, IFN-γ, IL-4, and IL-5 contents were measured by ELISA. Data show the results of a representative (out of three) experiments.

BALB/c transgenic mice developed progressive footpad swelling, B10.D2 transgenic animals showed only transient lesions (Figure 2A). Likewise, when analyzed 3 weeks after infection, the draining LN of BALB/c transgenic mice contained 100- to 1000-fold more parasites than those from B10.D2 transgenic animals (data not shown). Despite this latter result, 16.2β BALB/c transgenic mice did not develop necrotic lesions, and parasite loads in these mice reached a plateau 5 to 6 weeks after infection.

To analyze parasite-specific cytokine responses, wt and 16.2β transgenic mice were infected with *L. major*. Four weeks later, CD4+ LN T cells were prepared and incubated with syngeneic APCs and SLA. As expected, CD4+ T cells from B10.D2 wt mice secreted more IFN-γ and less IL-4 and IL-5 than those from BALB/c wt mice (Figure 2B). Likewise, B10.D2 transgenic T cells secreted 15-fold more IFN-γ, 3- to 4-fold less IL-2 and IL-4, and 8-fold less IL-5 than BALB/c transgenic T cells. Thus, the expression of the TCR β transgene did not change the tendency of B10.D2 and BALB/c mice to mount Th1- and Th2-dominated responses, respectively. However, transgenic T cells from BALB/c mice secreted less IL-4 and IL-5 than those from their negative littermates, suggesting that 16.2β transgenic animals had an impaired ability to mount a vigorous Th2 response.

To follow the kinetics of activation of LACK-specific T cells, 16.2β BALB/c mice were infected with 2×10^6 promastigotes, and CD4+ cells from their draining LN were stained with I-A^d/LACK multimers. Both the percentage and the absolute number of I-A^d/LACK+ cells increased in the draining LN 1 day after infection and peaked at day 3 (Figure 3A). At this time, the percentage of I-A^d/LACK+ cells dropped but remained higher than those found in uninfected animals up to 28 days after infection. While the absolute number of I-A^d/LACK+ cells per LN also started to decrease after day 3, it increased again after day 14 (Figure 3B).

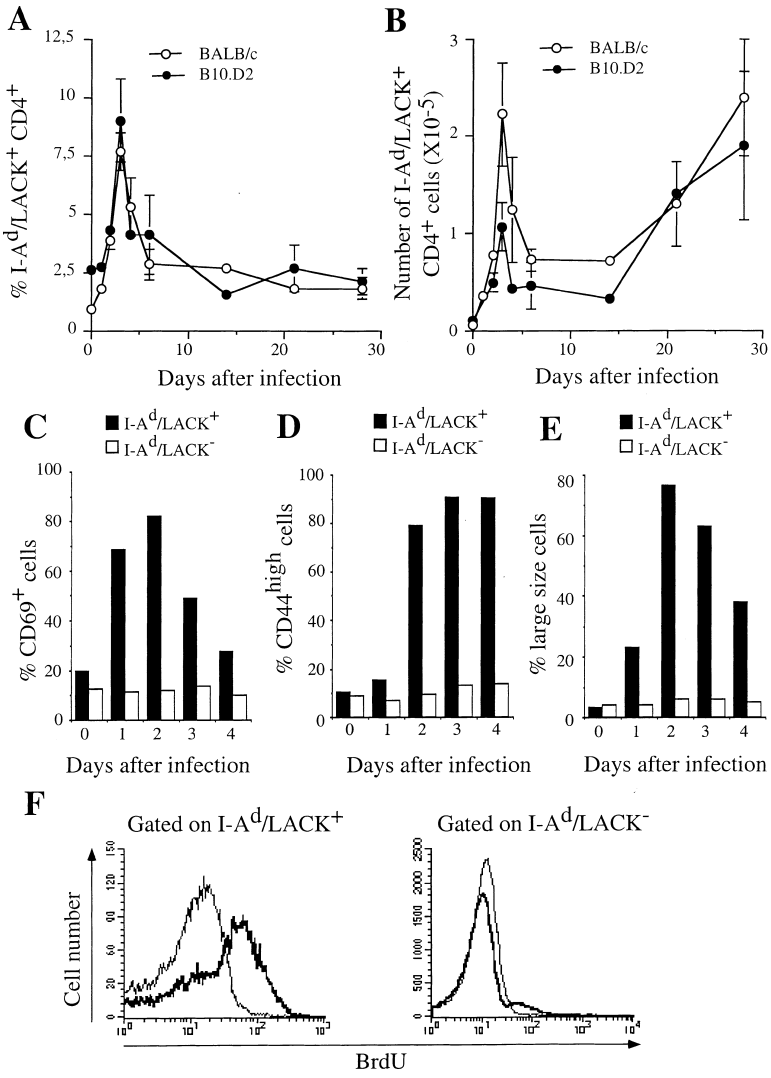

Figure 3. *L. major* Induces the Activation and the Expansion of LACK-Specific T Cells in BALB/c 16.2β Transgenic Mice

(A, B, C, D, and E) BALB/c and (A and B) B10.D2 16.2β transgenic mice were infected with 2×10^6 *L. major* promastigotes. At the indicated times after infection, LN CD4$^+$ T cells were purified and stained with (A, B, C, D, and E) I-A^d/LACK multimers, anti-CD4 and (C) anti-CD69 mAbs, or (D) anti-CD44 mAbs. (A) The percentage and (B) absolute number of I-A^d/LACK$^+$ cells per LN in BALB/c (open circles) and B10.D2 (filled circles) animals were calculated after gating on CD4$^+$ viable cells. Data show the arithmetic mean ± SD of five individual mice.

(C, D, and E) The percentage of I-A^d/LACK$^+$ (filled bars) and I-A^d/LACK$^-$ (empty bars) cells expressing (C) CD69, (D) high levels for CD44, or (E) exhibiting a large size was calculated after gating on live CD4$^+$ T cells. Data show the arithmetic mean of three individual mice.

(F) BALB/c 16.2β transgenic mice were given BrdU in the drinking water at the time of the infection and analyzed 3 days later. LN CD4$^+$ T cells were permeabilized and stained with I-A^d/LACK multimers and anti-BrdU (thick line) or control isotypic (thin line) mAbs. Representative FACS analyses are shown after gating on I-A^d/LACK$^+$ (left panel) or I-A^d/LACK$^-$ (right panel) cells.

Infection with *L. major* induced I-A^d/LACK$^+$ cells to undergo rapid phenotypic changes. Indeed, in contrast to I-A^d/LACK$^-$ cells that did not exhibit apparent surface phenotypic changes during the course of the infection, the frequency of I-A^d/LACK$^+$ cells expressing CD69 increased from 15%–20% at day 0 to 65% and 80% at day 1 and 2, respectively (Figure 3C). Likewise, the frequency of CD44^{high} I-A^d/LACK$^+$ cells increased from 10% at day 0 to 78% at day 2 and remained constant up to 4 days after infection (Figure 3D). To determine whether *L. major* induced I-A^d/LACK$^+$ T cells to progress through the cell cycle, 16.2β mice were given BrdU in their drinking water at day 0, and LN cells were analyzed 3 days later for BrdU incorporation. The proportion of BrdU$^+$ cells among I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells was 60% and 10.3%, respectively (Figure 3F). Similarly, the frequency of large I-A^d/LACK$^+$ cells rapidly increased in infected mice as assessed by FSC analysis (Figure 3E). Thus, in contrast to the frequency of large I-A^d/LACK$^-$ cells that remained constant through the infection, the frequency of large I-A^d/LACK$^+$ cells increased from 3%–5% at day 0 to almost 80% at day 2 and eventually dropped to 40% at day 4. Taken together, these results demonstrate that the phenotype BALB/c I-A^d/LACK$^+$ transgenic cells rapidly changed in response to *L. major* and that these cells accumulated in the draining LN as the result of parasite-induced proliferation.

BALB/c and B10.D2 Transgenic T Cells Exhibit the Same Kinetics of Activation and Expansion but Secrete Different Cytokines

To compare the kinetics of T cell expansion in B10.D2 and BALB/c mice, transgenic mice from these two strains were injected with *L. major* and cells from their draining LN were analyzed at different times after infection. As observed in BALB/c mice, the frequency of I-A^d/LACK$^+$ B10.D2 transgenic T cells peaked to 8%–10% at day 3 and dropped to 2%–2.5% 4 weeks later (Figure 3A). Although B10.D2 mice exhibited less CD4$^+$ T cells per LN than BALB/c mice, the absolute number of I-A^d/LACK$^+$ cells per LN increased in both strains peaking 3 days after infection, dropping thereafter, and increasing again after day 14 (Figure 3B).

We next analyzed the surface phenotype of I-A^d/LACK$^+$ cells from BALB/c and B10.D2 16.2β transgenic mice 3 weeks after infection. CD4$^+$ LN cells were pre-

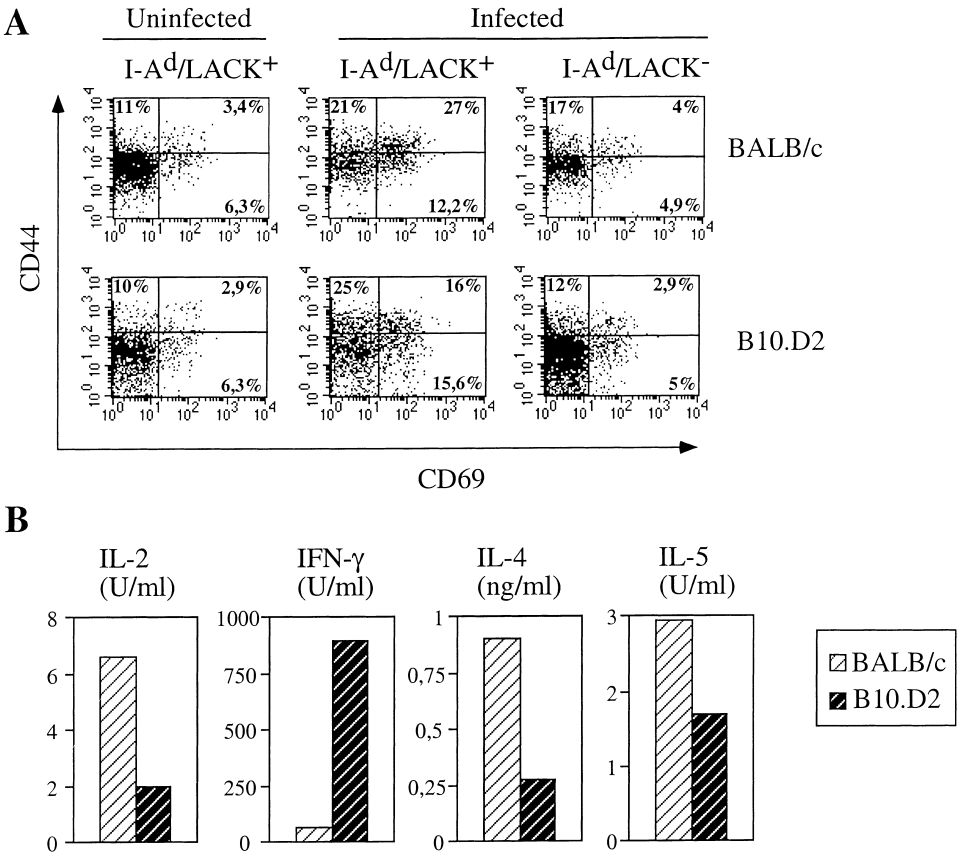

Figure 4. Surface Phenotype and Cytokine Secretion Profiles of LACK-Specific T Cells in BALB/c and B10.D2 16.2β Transgenic Mice

Mice were infected or not with 2×10^6 *L. major* promastigotes, and CD4+ LN T cells were prepared 3 weeks later.

(A) Cells were stained with I-Ad/LACK multimers, anti-CD44 and anti-CD69 mAbs. Data show representative FACS profiles of cells from uninfected (left panel) or infected (right panels) mice. The percentages of CD44high, CD69+, and CD44highCD69+ cells are indicated after gating on the indicated population.

(B) Cells were incubated with syngeneic APCs and 10 μM of LACK peptide. Supernatants were harvested 72 hr later and IL-2, IFN-γ, IL-4, and IL-5 contents were measured by ELISA. Data show the amount of cytokine normalized to 10^3 I-Ad/LACK+ cells. Data show the results of a representative (out of three) experiments.

pared and stained with I-Ad/LACK multimers and anti-CD44 and anti-CD69 mAbs. In both strains, the surface phenotype of I-Ad/LACK− cells did not change during the course of the infection. In striking contrast, the proportion of CD44high I-Ad/LACK+ cells in BALB/c and B10.D2 animals increased from 10%–15% at day 0 to 40%–50% at day 22 (Figure 4A). Similarly, the proportion of I-Ad/LACK+ cells expressing CD69 increased from 8%–10% before infection to 30%–40% 3 weeks later (Figure 4A). At that time, 16% and 27% of I-Ad/LACK+ cells expressed both CD69 and high levels of CD44 in B10.D2 and BALB/c mice, respectively.

To compare the ability of BALB/c and B10.D2 LACK-specific T cells to secrete cytokines, CD4+ LN cells were purified from infected mice 4 weeks after infection and incubated with syngeneic APCs and LACK peptide. After normalizing for each sample, the amount of cytokine to the number of I-Ad/LACK+ T cells, we found that transgenic T cells from B10.D2 mice secreted 12- to 15-fold more IFN-γ, 3-fold less IL-2 and IL-4, and 2-fold less IL-5 than those from BALB/c animals (Figure 4B). Thus, while BALB/c and B10.D2 transgenic T cells exhibited the same kinetics of activation and expansion, they did not secrete the same cytokines.

BALB/c and B10.D2 Transgenic T Cells Express Low- and High-Affinity TCR, Respectively

During the course of these studies, we noticed that I-Ad/LACK+ cells from B10.D2 transgenic mice stained more brightly with I-Ad/LACK multimers than those from BALB/c animals. This phenomenon was observed as early as day 3 after infection, but not at day 0, 1, or 2 (Figure 5A). Differences in staining profiles could result from differences in the level of TCR expression or in the affinity of the TCR for their common peptide/MHC ligand. Indeed, previous studies have demonstrated that there is a direct correlation between the extent of peptide/MHC multimer binding and receptor affinity (Crawford et al., 1998; Savage et al., 1999).

To address this issue, we have used the previously described multimer dissociation assay to compare the half-lives of I-Ad/LACK multimer binding to BALB/c and B10.D2 LACK-specific transgenic T cells (Savage et al., 1999). CD4+ LN cells were purified from mice 3 days

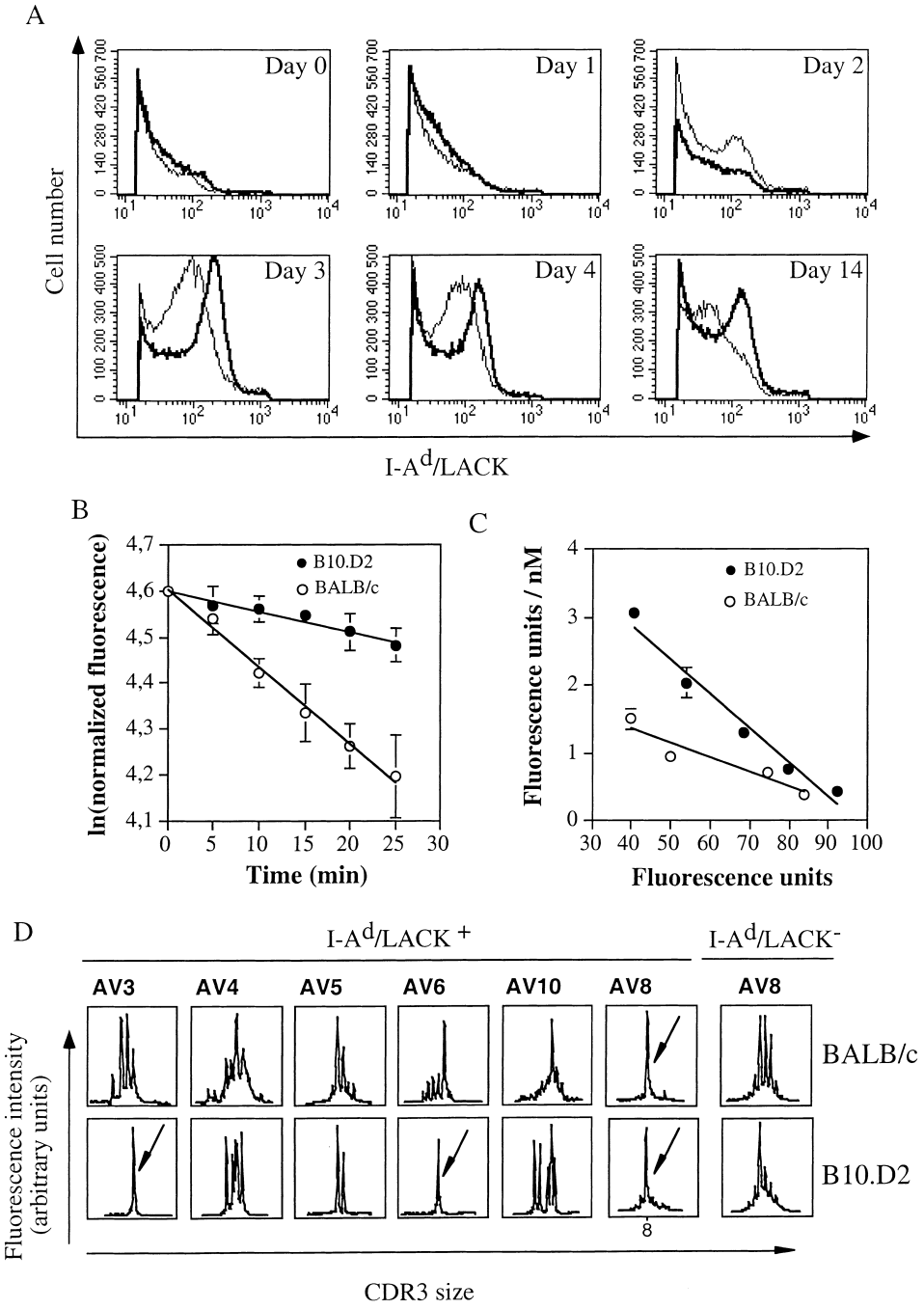

Figure 5. B10.D2 and BALB/c Transgenic T Cells Express TCR of Different Affinity

16.2β transgenic mice were infected with 2×10^6 *L. major* promastigotes. At the indicated times after infection (A) or 3 days after infection (B, C, and D), LN CD4$^+$ T cells were stained with I-A^d/LACK multimers and anti-CD4.

(A) Representative FACS analysis of BALB/c (thin line) and B10.D2 (thick line) transgenic T cells after gating on I-A^d/LACK$^+$ CD4$^+$ viable cells.

(B) Multimer staining decay kinetics for BALB/c and B10.D2 I-A^d/LACK$^+$ cells stained with I-A^d/LACK multimer. The natural logarithm of the normalized fluorescence is plotted versus time after the addition of M5/114 mAbs. Multimer staining was measured at 0, 5, 10, 15, 20, and 25 min after M5/114 addition. Data show I-A^d/LACK multimer dissociation from B10.D2 (filled circles) and BALB/c (open circles) I-A^d/LACK$^+$ cells, respectively.

(C) Scatchard analysis of I-A^d/LACK multimer binding to I-A^d/LACK$^+$ T cells. BALB/c (open circles) and B10.D2 (filled circles) cells were stained with I-A^d/LACK multimer over a range of concentrations. Apparent K_D values were derived from Scatchard plots of bound multimer/free multimer versus bound multimer.

(D) Immunoscope analysis of CDR3α regions. I-A^d/LACK$^+$ and I-A^d/LACK$^-$ CD4$^+$ LN cells from 16.2β BALB/c (upper panel) and B10.D2 (lower panel) mice were sorted by flow cytometry. cDNA were amplified by PCR using AC and the indicated AV-specific primers followed by a run-off with a nested fluorescent AC-specific primer. The run-off products were size fractionated in an automated DNA sequencer, and the CDR3 size distribution was analyzed using the Immunoscope software.

after infection and stained with I-A^d/LACK multimers at 4°C. After washing, the rate of staining decay was measured by flow cytometry in the presence of saturating amounts of the M5/114 monoclonal antibody. While I-A^d/LACK multimers label on B10.D2 LACK-specific T cells decayed with a $t_{1/2}$ of 162 ± 32 min, I-A^d/LACK multimers label on BALB/c LACK-specific T cells decayed with a $t_{1/2}$ of 43 ± 7 min (Figure 5B). To determine apparent K_D values for multimer binding to BALB/c and B10.D2 LACK-specific T cells, multimer binding to these cells was evaluated under equilibrium staining conditions as described (Savage et al., 1999). While I-A^d/LACK multimer binding to B10.D2 trangenic T cells exhibited an apparent K_D of 19.5 ± 0.5 nM, the apparent K_D value for binding to BALB/c trangenic T cells was 46 ± 4 nM (Figure 5C).

To independently confirm that BALB/c and B10.D2 LACK-specific T cells expressed low- and high-affinity TCR, respectively, we generated hybridomas from the LN of BALB/c and B10.D2 infected transgenic mice. These hybridomas were stained separately with I-A^d/LACK multimers and anti-Vβ4 mAbs to normalize the mean fluorescence intensity (MFI) obtained after multimer binding to the level of TCR surface expression. While seven out of eight BALB/c hybridomas exhibited a normalized MFI ranging from 0.2 to 0.68, all B10.D2 hybridomas exhibited MFI ranging from 1.2 to 1.7 (Table 1). In agreement with this latter result, the apparent K_D value for multimer binding to the BALB/c LMR4.1 and LMR4.2 hybridomas were higher than those measured for the B10.D2 LMR7.5 and LMR8.4 hybridomas (53 and 21.5 nM versus 11.9 and 7.5 nM) (Table 1).

If BALB/c and B10.D2 transgenic T cells express TCR of different affinity, they should use different α chains. To confirm this, the TCR α chains expressed by our panel of LACK-specific hybridomas were analyzed by RT-PCR and sequenced. While six different Vα regions were found in the eight BALB/c-derived hybridomas, three out of three B10.D2-derived hybridomas used the same AV3-J25 rearrangement with a conserved 10 amino acid CDR3 loop (Table 1). Because of a possible bias in hybridoma sampling, we used flow cytometry to purify I-A^d/LACK$^+$ and I-A^d/LACK$^-$ cells from *L. major*–infected transgenic animals. We then analyzed the CDR3 size distribution of TCR α chains in the sorted populations using six different AV- and AC-specific primers and the Immunoscope technique (Pannetier et al., 1993) (Figure 5D). For five out of the six rearrangements analyzed (AV3, AV4, AV5, AV6, and AV10), the CDR3 size pattern distribution was different between BALB/c and B10.D2 transgenic T cells. This was striking for AV3-AC and AV6-AV elongation products where a unique CDR3 peak was observed in B10.D2, but not in BALB/c mice. After sequencing, only AV3-AC PCR products gave a readable sequence that was identical to the CDR3 region of the LMR7.5 and LMR8.1 hybridomas (Table 1). In contrast, Immunoscope analysis of rearranged TCR bearing the AV8 gene segment revealed a single peak in both BALB/c and B10.D2 mice. In both strains, direct sequencing of the AV8-AC PCR products gave a readable AV8-AJ8 sequence that was identical to the sequence found in the LMR4.11 hybridoma. As a control, the analysis of the AV8-C rearrangements performed on I-A^d/LACK$^-$ cells showed a Gaussian-like distribution of the peaks (Figure 5D).

A Possible Link between T Cell Repertoire Selection and Cytokine Secretion

To get further insights into a possible relationship between T cell repertoire and cytokine secretion, (BALB/c × B10.D2) F1 16.2β transgenic mice were generated and intercrossed to obtain F2 transgenic mice. These F2 mice and sex-matched BALB/c and B10.D2 control transgenic animals were infected with *L. major* and killed 3 weeks later. CD4$^+$ LN T cells from individual mice were prepared and analyzed for cytokine production following in vitro stimulation with LACK peptide. In parallel, the apparent K_D values for multimer binding to I-A^d/LACK$^+$ cells were measured under equilibrium staining conditions at 4°C. As previously observed for mice analyzed 3 days after infection, the apparent K_D values for multimer binding to BALB/c T cells were higher than those measured for B10.D2 T cells (22.0 ± 1.1 nM versus 8.9 ± 1.7 nM) (Figure 6). In addition, BALB/c T cells secreted less IFN-γ and more IL-4 (measured as a lower IFN-γ/IL-4 ratio) than B10.D2 T cells. Among 26 F2 mice, 24 exhibited relatively low apparent K_D values (ranging from 6 to 15 nM) and relatively high IFN-γ/IL-4 ratio. Interestingly, the only two mice that exhibited high apparent K_D values (29.0 nM and 31.0 nM) were also characterized by a low IFN-γ/IL-4 ratio.

Discussion

The use of peptide/MHC tetramers to stain T cells has revolutionized our appreciation of the numerology of the CD8$^+$ T cell response to bacterial or viral infections. Here, we have used a similar approach to follow the fate of parasite-specific CD4$^+$ T cells in mice infected with *L. major*. To this aim, we made molecular probes in which a peptide derived from the immunodominant LACK antigen was linked to MHC class II I-A^d molecules. Dimers of I-A^d/LACK molecules were produced in *Drosophila* cells and incubated with fluorochrome-conjugated protein A. Protein A–bound I-A^d/LACK dimers stained LACK-specific hybridoma and CD4$^+$ T cells from TCR transgenic mice that carried the rearranged TCR α and β chain gene of a LACK-specific hybridoma. Our approach to make peptide/MHC multimers was different from the standard strategy in which peptide/MHC monomers were biotinylated and eventually obtained as fluorescent tetramers following incubation with fluorochrome-conjugated streptavidin. Although streptavidin-bound tetramers are now routinely used to stain antigen-specific CD8$^+$ T cells, only a few investigators have successfully used it to stain CD4$^+$ T cells (McMichael and Kelleher, 1999). Our strategy does not require peptide/MHC molecules to be biotinylated in vitro, a procedure that can be technically difficult. Although we have not systematically compared our strategy with others, all protein A–bound MHC/peptide dimers that we have made so far were biologically active, including those in which antigenic peptides were bound to the unstable NOD I-A^{g7} molecules (our unpublished data).

To facilitate the detection of LACK-specific T cells, we have constructed a transgenic mouse strain, 16.2β,

Table 1. CDR3α Regions Used by LACK-Specific T Cell Hybridomas

Strain	Hybridoma	AV-J	CDR3 Size (aa)	V	CDR3 Loop	J	MFI	Apparent K_D (nM)
BALB/c	LMR3.11	AV3-J33	8	TGT GCT GCG C A A	AGG ACT GGG GGA AAC TAC AAA TAC R T G G N Y K Y	GTC TTT GGA V F G	0.2	N.D.
	LMR3.5	AV8-J9	10	TGT GCT TTG C A L	AGG CTC CTA ACT GCC AGT TTG GGG AAA CTG R L L T A S L G K L	CAG TTT GGA Q F G	0.45	N.D.
	LMR4.1	AV8-J41	5	TGT GCT TTG C A L	AAG TAC CAG AAC TTC K Y Q N F	TAT TTT GGG Y F G	0.27	53
	LMR4.11	AV8-J8	8	TGT GCT CTG C A L	AGT GAA GAC ATG GGC TAC AAA CTT S E D M G Y K L	ACC TTC GGG T F G	0.3	N.D.
	LMR5.1	AV10-J47	10	TGT GCT ATG C A M	GAC ATG GCT ACT GGA GGC AGT AAT AAG CTG D M A T G G S N K L	ACT TTT GGT T F G	0.35	N.D.
		AV17-J35	6	TGT GCT ATG C A M	AGA GAC AAC AAT GCC CCA R D N N A P	CGA TTT GGA R F G		
	LMR3.7	AV11-J44	12	TGT GCT GCT C A A	GAG CCG CTT AAC ACT GGA GCT AAC ACT GGA AAG CTC E P L N T G A N T G K L	ACG TTT GGA T F G	0.24	N.D.
	LMR4.9	AV11-J28	6	TGT GCT GCC C A A	GAG CCC GCA AGT GCG CTG E P A S A L	ACA TTT GGA T F G	0.24	N.D.
	LMR4.2	AV18-J41	5	TGT GCT CCG C A P	GGT TAC CAG AAC TTC G Y Q N F	TAT TTT GGG Y F G	0.68	21.5
B10.D2	LMR7.5	AV3-J25	10	TGT GCT CTG C A L	AGC TTC TAT GGG AGC AGT GGC AAC AAG CTC S F Y G S S G N K L	ATC TTT GGA I F G	1.67	7.5
	LMR8.1	AV3-J25	10	TGT GCT CTG C A L	AGC TTT TAT GGG AGC AGT GGC AAC AAG CTC S F Y G S S G N K L	ATC TTT GGA I F G	1.35	N.D.
	LMR8.4	AV3-J25	10	TGT GTT TTG C V L	AGC CAT TAT GGG AGC AGT GGC AAC AAG CTC S H Y G S S G N K L	ATC TTT GGA I F G	1.74	11.9

For each hybridoma, data show the Vα and Jα segments, the size of the CDR3α loop, the nucleotide and predicted amino acid sequences of the CDR3α loop, and the normalized MFI as calculated as the ratio of binding of I-A^d/LACK multimers to binding with anti-Vβ4 mAbs. For some hybridomas, the apparent K_D values of multimer binding to I-A^d/$LACK^+$ cells are indicated. ND, not determined.

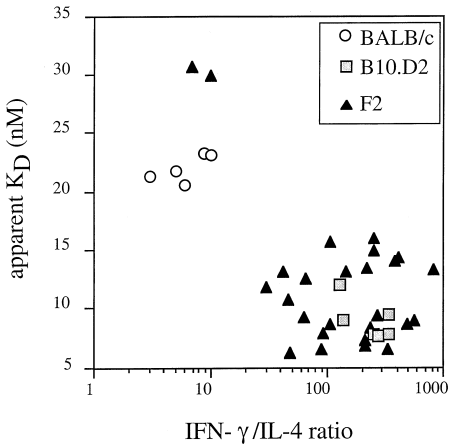

Figure 6. Apparent K_D Values and Cytokine Secretion Profiles of T Cells from (B10.D10 × BALB/c) F2 Transgenic Mice

BALB/c (open circles), B10.D2 (gray squares), and (B10.D10 × BALB/c) F2 (filled triangles) 16.2β transgenic mice were infected with *L. major*, and LN CD4+ cells were purified 3 weeks later. The apparent K_D value for multimer binding to I-A^d/LACK+ cells was determined for individual mice as described in the legend to Figure 5. LN cells were stimulated with LACK for 72 hr, and cellular supernatants were analyzed for IL-4 and IFN-γ content by ELISA. For each mouse, data show the apparent K_D value versus the ratio between IFN-γ and IL-4 production.

which carried the rearranged β gene of a LACK-specific TCR and exhibited an increased frequency of LACK-specific T cells. As observed wt animals, 16.2β transgenic mice that have been crossed to BALB/c mice developed larger lesions and exhibited higher parasite loads than those crossed to B10.D2 mice. In agreement with a critical role of Th1 cells in resistance to infection, B10.D2 transgenic T cells secreted more IFN-γ and less IL-4 and IL-5 when incubated with SLA than BALB/c transgenic T cells. However, while both BALB/c and B10.D2 transgenic T cells secreted more IFN-γ than those from their negative littermates, they secreted less IL-4 and IL-5, suggesting that 16.2β transgenic mice were impaired in their ability to mount a strong Th2 response. At least two hypotheses could account for this latter result. First, the TCR β transgene used to generate 16.2β mice was derived from a Th1 clone, and it could be that T cells expressing this particular β chain may have a predisposition to differentiate into IFN-γ-secreting cells. Alternatively, the efficient allelic exclusion resulting from the expression of the TCR β transgene may have prevented the development of regulatory T cells required for a vigorous Th2 response.

Using I-A^d/LACK multimers as staining reagents, we found that LACK-specific T cells rapidly expanded in the draining LN of infected transgenic mice. Selective expansion was readily detectable 1 day after infection and peaked 2 days later. At that time, the number of I-A^d/LACK+ cells per LN increased by 50- to 100-fold, mainly as the result of antigen-driven proliferation. *L. major* also induced LACK-specific T cells to rapidly express CD69 and to upregulate CD44, a phenomenon that was previously observed in mice immunized with cytochrome C (McHeyzer-Williams and Davis, 1995) or ovalbumin (OVA) (Kearney et al., 1994). In striking contrast, although the number of I-A^d/LACK− cells increased in the draining LN as the result of the infection, these cells did not progress through the cell cycle and their surface phenotype did not change over time, arguing against *L. major*–induced activation of bystander T cells. Altogether, our results suggest that the increase in the number of LN CD4+ T cells induced by *L. major* resulted both from the nonspecific recruitment of cells to the draining LN and from the antigen-driven proliferation of parasite-specific T cells within this lymphoid organ.

At any time after infection, the number of antigen-specific T cells in the draining LN reflects the rates at which T cells proliferate, die, or migrate to other organs. Because of this dynamic process, it is difficult to draw conclusions from variations in the number of antigen-specific cells during the course of the disease. Nevertheless, it is striking that both the frequency and the absolute numbers of LACK-specific T cells in the draining LN dropped 3 days after infection. At that time, the proportion of cells expressing CD69 and/or progressing through the cell cycle also started to decrease, a result that could be explained by the transient disappearance of DCs as the result of their killing by activated T cells (Ingulli et al., 1997). At later times, the frequency of LACK-specific T cells in the LN of both resistant and susceptible mice reached a plateau, but the absolute numbers of these cells increased again. Three weeks after infection, a relatively high proportion of LACK-specific T cells expressed CD69, suggesting that antigen-dependent stimulation was still taking place. Thus, in the late stages of the disease, parasite-specific T cells were continuously activated but their frequency in the draining LN did not increase, possibly because of homeostatic mechanisms. Chronic stimulation of parasite-specific T cells was not unexpected in BALB/c mice as they exhibited high parasite loads. However, although *L. major* amastigotes have been demonstrated to persist in resistant mice for more than 1 year, it was unclear whether chronic infection was associated with persistent activation of parasite-specific T cells (Aebischer et al., 1993). Our results suggest that this is indeed the case.

Using I-A^d/LACK multimers, we found that infection with as little as 2×10^6 promastigotes caused a dramatic expansion of LACK-specific T cells in 16.2β transgenic mice. As *L. major* promastigotes express ~30,000 LACK molecules per cell (Fowell and Locksley, 1999), maximal expansion of LACK-specific T cells was observed with less than 2×10^{-11} moles of LACK antigen, i.e., 1000-fold fewer molecules than needed to activate T cells in BALB/c mice adoptively transferred with OVA-specific T cells and immunized with OVA (Kearney et al., 1994). Epidermal Langerhans cells (LC) can internalize *L. major* amastigotes and transport them from the skin to the draining LN (Moll et al., 1993). In addition, parasite internalization by LC-like dendritic cells induces these cells to upregulate MHC class I and II surface antigens, to express high levels of costimulatory molecules (CD40, CD54, CD80, and CD86), and to release IL-12 p40 (von Stebut et al., 1998). Thus, the ability of *L. major* promastigotes to induce the activation and the expansion of parasite-specific T cells may result from their ability to be captured by DCs and to induce their differentiation.

While the kinetics of expansion and contraction of

LACK-specific T cells were similar in BALB/c and B10.D2 transgenic mice, B10.D2 T cells stained more brightly with I-A^d/LACK multimers than BALB/c T cells. Differences in I-A^d/LACK multimer staining were associated with strain-specific differences in AV gene usage. In BALB/c mice, the TCR AV repertoire was quite diverse, with T cells using different AV gene segments with CDR3 loops of variable size. In contrast, the TCR AV repertoire in B10.D2 mice was characterized by the preferential expansion of T cells expressing the AV3-J25 rearrangement with a conserved 10 amino acids long CDR3 motif. Further experiments showed that the apparent K_D values for multimer binding to BALB/c T cells were higher than those measured for B10.D2 T cells. Likewise, I-A^d/LACK multimers dissociated more rapidly from BALB/c than from B10.D2 T cells. Thus, while I-A^d/LACK$^+$ T cells from infected BALB/c mice expressed a diverse repertoire and low-affinity TCR, those from B10.D2 animals expressed a restricted repertoire and high-affinity TCR.

What are the reasons for this phenomenon? Although we cannot rule out that the preimmune repertoires of BALB/c and B10.D2 16.2β transgenic mice were different, our preliminary data suggest that this is not the case. By analyzing the CDR3 size distribution among rearranged TCR AV3, AV6, and AV8 gene segments on CD4$^+$ T cells from naive BALB/c and B10.D2 transgenic mice, we could not find any significant bias in CDR3 length between these two strains. Thus, the absence of high-affinity T cells in the LN of infected BALB/c transgenic mice was likely to result from an impaired ability of these mice to select high-affinity T cells during the course of the infection. This phenomenon may be related to strain-specific T cell–intrinsic differences (Guler et al., 1996; Bix et al., 1998) or to differences in the non-T cell compartment (Scott, 1991; Laskay et al., 1995).

It may be of interest that BALB/c and B10.D2 transgenic T cells expressed TCR of different affinities and secreted different amounts of IL-4 and IFN-γ. Although it remains to be established whether there is a causal relationship between TCR affinity and phenotype acquisition, many studies have suggested that the strength of signal through the TCR may influence the generation of Th1 and Th2 effector lymphocytes in vivo (Pfeiffer et al., 1995) and in vitro (Constant et al., 1995; Hosken et al., 1995). As a first attempt to address this issue, we have generated F2 transgenic mice by intercrossing F1 (BALB/c × B10.D2) 16.2β mice. When mice were analyzed 3 weeks after infection, 24 out of 26 F2 mice exhibited apparent K_D values for multimer binding to I-A^d/LACK$^+$ cells that were in the same range as those observed for B10.D2 animals. These 24 F2 mice also exhibited Th1-polarized immune responses similar to those observed in B10.D2 animals. In striking contrast, two F2 mice exhibited relatively high apparent K_D values and mounted Th2-dominated responses comparable to those developed by BALB/c transgenic mice. Although we cannot rule out that repertoire selection and cytokine secretion were governed by different genes that would have segregated together in these two F2 mice, our results strongly suggest that whatever determines T cell differentiation also governs the LACK-specific TCR repertoire.

Although we have not measured parasite loads in F2 mice, it is striking that all these animals, including the two that exhibited relatively high apparent K_D values and relatively low IFN-γ/IL-4 ratios, developed smaller lesions than control BALB/c mice. Previous studies have demonstrated the complexity of the mechanisms that lead to resistance (Shankar and Titus, 1995; Beebe et al., 1997; Roberts et al., 1997). Thus, both T cell and non–T cell compartments were shown to independently determine resistance to *Leishmania major* (Shankar and Titus, 1995). Furthermore, genetic experiments have shown that resistance is under the control of at least six genetic loci. The presence of all loci was not necessary to confer resistance and no single locus was required. Rather, a variety of combinations of these loci were capable of interacting to confer resistance (Beebe et al., 1997). Together with these previous studies, our data suggest that mice may be able to control the infection despite developing a Th2-biased immune response. Thus, while the expansion of high-affinity T cells and the subsequent development of Th1 cells may be sufficient to confer resistance, it may not be necessary.

In earlier studies, Bretsher et al. (1992) have infected BALB/c mice with various numbers of *L. major* promastigotes. While mice infected with high numbers of parasites developed progressive lesions and mounted a Th2-dominated response, those infected with low numbers developed a protective Th1-polarized T cell response. More recently, Rees et al. (1999) have demonstrated that there was an inverse relationship between T cell receptor affinity and antigen dose during CD4$^+$ T cell responses in vivo. Our data provide a link between these two studies by suggesting that infection of BALB/c mice with low number of promastigotes could favor the selective expansion of high-affinity T cells that may eventually differentiate along the Th1 pathway.

Experimental Procedures

Mice and Parasites

BALB/c and B10.D2 mice were purchased from Harlan, UK. All mice were used between 8 and 12 weeks of age. Mice were bred and housed under specific pathogen-free conditions. ABLE TCR αβ transgenic mice have been described (Reiner et al., 1998). 16.2β transgenic mice were produced by microinjection of the rearranged TCR β chain gene of the LACK-specific LMR16.2 T cell hybridoma (Mougneau et al., 1995) into (B10.S × C57BL/6) F1 fertilized eggs and implantation into pseudopregnant foster mothers. One transgene positive founder was identified by Southern analysis of tail DNA, and crossed to BALB/c and B10.D2 for 15 and 5 generations, respectively. Offspring were monitored for expression of the TCR β transgene by flow cytometry of peripheral blood cells using KT4.1 anti-Vβ4 mAbs. For infection, *L. major* (World Health Organization strain WHOM/IR/-/173) promastigotes were grown in M199 containing 20% FCS and used as described (Mougneau et al., 1995).

Reagents and Antibodies

LACK peptide (aa 158 through 173; FSPSLEHPIVVSGSWD) was purchased from Mimotopes (Suresnes, France). BrdU was purchased from Sigma SARL (St. Quentin Fallavier, France). SLA was prepared as described (Scott et al., 1990). The following mAbs were used for stimulation, cell purification, or flow cytometry analysis: GK1.5, anti-CD4; 53-6.7, anti-CD8α; KT4.1, anti-Vβ4; H57-597, anti-Cβ; H1.2F3, anti-CD69; IM7, anti-CD44; M1/70, anti-CD11b; RA3-6B2, anti-B220; 2G9, anti-I-A^d/I-E^d; and 3D4, anti-BrdU. All mAbs were purchased from Becton Dickinson SA (Le Pont de Claix, France).

Production of Soluble I-A^d/LACK Dimers and Multimeric Staining Reagents

Constructs encoding the extracellular domains of I-A^d α and β chains fused to acidic/basic leucine zipper sequences (Scott et al., 1996)

were modified as followed. The α chain construct was modified by the addition of a sequence encoding the Fc region of murine IgG2a antibodies. For the β chain construct, a sequence coding for either a LACK-derived peptide (amino acids 156 through 173) (FSPSLEH PIVVSGSW) or a GP63-derived peptide (amino acids 364 through 378) (GSCTQRASEAHASLL) was covalently attached by a six-residue linker and a thrombin cleavage site to the amino terminus of the I-A^d β chain as described (Kozono et al., 1994). Recombinant cDNA were cloned into the pRMHa3 expression vector and transfected into *Drosophila* S2 cells, and stable transfectants were selected as described (Scott et al., 1996). For large scale culture, transfectants were grown at 24°C in SFM medium (Life Technology SARL, Cergy Pontoise, France) supplemented with 1% FCS. When the cell density reached 5×10^6 cells/ml, expression was induced by addition of copper sulfate (0.5 mM). Supernatants were harvested 5 days later and concentrated using a Prepscale Concentrator (Millipore S.A., St. Quentin Yvelines, France). I-A^d/LACK dimers were purified from supernatants by affinity chromatography using MK-D6 mAbs. Bound molecules were eluted by CAPS 50 mM (pH 11.5), and the collected fractions were neutralized with 200 mM sodium phosphate (pH 6.2). Fractions containing I-A^d/peptide dimers were identified by SDS/PAGE (10%), pooled, and I-A^d/peptide dimers were further purified by anion exchange HPLC using a MonoQ HR5/5 column (Amersham Phamarcia Biotech Europe GmbH, Saclay, France). The column was equilibrated with 20 mM Tris (pH 8.0), and dimers were eluted with a linear gradient of 0%–50% 1 M NaCl. The protein was dialyzed against PBS 1× (pH 7.4), and the final protein concentration was determined by BCA assay (Pierce Chemical Co, Rockford). For preparing staining reagent, 1.4 μl of Alexa 488-coupled protein A (Molecular Probes Inc., Eugene) at the concentration of 0.5 mg/ml in PBS 1× was incubated for 30 min at 20°C with 8 μg of I-A^d/peptide dimers.

Purification of CD4+ T Cells

CD4$^+$ T cells were purified by negative depletion of CD8$^+$, B220$^+$, CD11b$^+$, and I-A^{d+} cells using sheep anti-rat Ig-coated Dynabeads (Dynal S.A., Compiègne, France) as previously described (Julia et al., 1996). The resulting populations were pure to more than 95% as assessed by flow cytometry.

Cytokine Secretion

Purified CD4$^+$ T cells (2×10^5) were incubated with 6×10^5 mitomycin C–treated syngeneic splenocytes with or without SLA (30 μg / ml) or LACK peptide (10 μM) in flat-bottomed 96-well plates. Supernatants were harvested 72 hr later, and IFN-γ, IL-2, IL-4, and IL-5 contents were measured by ELISA as described (Julia et al., 1996).

T Cell Hybridomas

LACK-specific hybridomas were generated by fusing purified LN CD4$^+$ T cells from *L. major*–infected 16.2β transgenic mice with BW5147 $\alpha^-\beta^-$ cells, and hybridomas were selected in HAT medium. To identify LACK-specific hybridomas, 10^5 cells were incubated with 0.5 μg of I-A^d/LACK dimers in the wells of a flat-bottom 96-well plates. Supernatants were harvested 24 hr later and IL-2 content were measured by ELISA.

Flow Cytometry Analysis

For I-A^d/LACK multimer staining, 10^6 purified CD4$^+$ T cells were stained with 60 μg/ml of I-A^d/LACK multimers for 1 hr on ice in PBS supplemented with 0.5% bovine serum albumin (BSA). Cells were washed twice and incubated with cychrome-conjugated anti-CD4 and with the indicated phycoerythrin-conjugated anti-B220, anti-CD44, or anti-CD69 mAbs. Lymphocytes, gated by forward- and side-scatter analysis, were analyzed on a FACScan flow cytometer (Becton Dickinson SA, Le Pont de Claix, France).

For the detection of BrdU$^+$ cells, *Leishmania*-infected mice were fed continously cells with drinking water containing BrdU (0.8 mg/ml). On day 3 postinfection, draining LN were harvested and purified CD4$^+$ T cells were stained with I-A^d/LACK multimers followed by cychrome-conjugated anti-CD4 mAbs. Staining for BrdU with PE-conjugated anti-BrdU mAbs was performed as described (Tough and Sprent, 1994) using PE-conjugated isotypic mAbs as a control for nonspecific binding.

Staining decay assays and scatchard analysis were performed as described (Savage et al., 1999) with the difference that cells were stained for 1 hr on ice. For staining decay assays, cells were stained with I-A^d/LACK multimers, washed three times, and resuspended in 100 μl BSA-containing PBS and 100 μg/ml of M5/114 anti-I-A^d mAbs.

T Cell Repertoire Analysis

RNA was extracted from T cell hybridomas or from sorted cell populations, and cDNA was synthesized as described (Pannetier et al., 1993). To analyze Vα chain usage by LACK-specific hybridomas, PCR was performed on cDNA using a set of 19 AV segment-specific primers and analyzed on a 2% agarose gel stained with ethidium bromide. AV-ACa PCR product (5 μl) was incubated for 40 min at 37°C with 0.5 U of Shrimp Alkaline Phosphatase and 10 U of Exonuclease I (Amersham Pharmacia Biotech Europe GbmH) in 10 μl. Sequencing reactions were performed using ACb internal primer and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Biosystems, Foster City). Sequences were run on a 373A DNA sequencer (Perkin Elmer Biosystems). Immunoscope analysis was performed as described (Pannetier et al., 1993) using AV- and AC-specific (Casanova et al., 1991).

Acknowledgments

This work was supported by grants from the Ministère de l'Education Nationale, de la Recherche et de l'Enseignement Supérieur and the EU (contract QLGI-CT-1999-00050). L. M. and V. J. were supported by fellowships from the Ligue Nationale Contre le Cancer. M. M. was supported by the Association Italiana for Cancer Research (AIRC). K. W. was supported by a grant from the National Institutes of Health (PO1 AI45757). We thank Nigel Killeen for helpful discussions and critical comments of the manuscript, Luc Teyton for reagents, and Jean-Philippe Breittmayer for help with flow cytometric analysis. We would also like to thank Franck Aguila for designing the cover image and Dr. Jean-Claude Antoine for providing us with the photograph of *Leishmania* metacyclic promastigotes.

Received April 21, 2000; revised November 2, 2000.

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