Primming by Microbial Antigens from the Intestinal Flora Determines the Ability of CD4\(^+\) T Cells to Rapidly Secrete IL-4 in BALB/c Mice Infected with *Leishmania major*

Valérie Julia, Stephen S. McSorley, Laurent Malherbe, Jean-Philippe Breittmayer, Fernand Girard-Pipau, Alain Beck and Nicolas Glaichenhaus

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Valérie Julia,† Stephen S. McSorley,‡ Laurent Malherbe,* Jean-Philippe Breittmayer,† Fernand Girard-Pipau,‡ Alain Beck,§ and Nicolas Glaichenhaus*§

Infection of BALB/c mice with Leishmania major results in the rapid accumulation of IL-4 transcripts within CD4+ T cells that react to the parasite Leishmania homologue of mammalian RACK1 (LACK) Ag. Because memory/effector cells secrete IL-4 more rapidly than naive cells, we sought to analyze the phenotype of these lymphocytes before infection. Indeed, a fraction of LACK-specific CD4+ T cells expressed a typical CD62 ligandlowCD44highCD45RBlow phenotype in uninfected mice. LACK-specific T cells were primed in gut-associated lymphoid tissues by cross-reactive microbial Ags as demonstrated by their reactivity with bacterial extracts and by the ability of APCs from the mesenteric LN of BALB/c mice to induce their proliferation. Also, mice in which the digestive tract has been decontaminated exhibited a reduced proportion of LACK-specific T cells expressing a memory/effector phenotype and did not exhibit the early accumulation of IL-4 transcripts induced by L. major. Thus, LACK-specific T cells represent a subset of CD4+ T cells which have acquired the ability to rapidly secrete IL-4 as the result of their priming by cross-reactive microbial Ags. Tracking the fate of these cells may provide information about the regulation of cell-mediated immune responses to gut Ags in physiological and pathological situations.

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T helper cells play a critical role in regulating immune responses to intracellular pathogens. In many cases, the dominance of either one of the two Th subsets, Th1 and Th2, directly correlates with the outcome and the severity of the disease. Thus, in susceptible hosts infected with the intracellular parasite Leishmania major, Th2 lymphocytes which secrete IL-4, IL-5, IL-10, and IL-13, favor disease progression and fatal outcome. In contrast, Th1 cells secrete high levels of IFN-γ and IL-2 and mediate the control of pathogen replication and resistance to reinfection.

Although parasite-specific CD4+ T cells can be detected in infected mice, little is known about their specificity and their kinetics of activation and expansion. Early studies have demonstrated that L. major induces the expansion of CD4+ T cells which express the TCR Vα8 and Vβ4 variable regions (2). Accordingly, a significant fraction of parasite-specific T clone cassettes which were isolated from infected BALB/c mice used Vα8 and Vβ4, suggesting that a single Ag was the focus of the early immune response directed to the parasite. This latter hypothesis was confirmed by the cloning of the LACK5 Ag and the demonstration that LACK-specific CD4+ cells used Vα8 and Vβ4 (3).

Indirect evidence suggests that LACK-specific CD4+ T cells are rapidly activated to secrete IL-4 in infected animals. Thus, both L. major promastigotes and recombinant LACK Ag induced the early and transient accumulation of IL-4 transcripts within a subset of CD4+ T cells that expressed Vα8 and Vβ4 (4). This burst of IL-4 mRNA peaked in the draining LN of BALB/c mice 16 h after infection with L. major or s.c. injection of LACK. Moreover, a burst of IL-4 mRNA was also detected in the spleen of BALB/c mice 90 min after i.v. injection of LACK (4).

Previous studies have demonstrated that naive and memory CD4+ T cells exhibit distinct patterns and kinetics of cytokine secretion. Thus, naive CD4+ T cells secrete mainly IL-2 on initial stimulation, whereas memory CD4+ T cells synthesize a broader range of cytokines, including IFN-γ and IL-4 (5, 6). Although both naive and memory CD4+ T cells secrete IL-4 after in vitro stimulation, naive CD4+ T cells require at least 36 h to express IL-4 mRNA, whereas memory T cells synthesize IL-4 transcripts as early as 12 h after stimulation (5, 6). Because LACK-specific T cells accumulate IL-4 mRNA very rapidly in mice infected with L. major, we sought to characterize their phenotype in BALB/c mice before infection. Here, we demonstrate that the lymphoid organs of naive BALB/c mice contain microbial Ag-specific T cells that cross-react to LACK and that express a memory/effector phenotype. This phenomenon may account for the ability of the LACK-specific T cells to rapidly secrete IL-4 shortly after infection.

*a Centre National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France; † Institut National de la Santé et de la Recherche Médicale Unité 343, Nice, France; ‡ Service de Bactériologie, Hôpital de l’Arche, Nice, France; and § Centre d’Immunologie Pierre Fabre, Saint Julien en Genevois, France.

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2 Current address: DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304-1104.

3 Current address: Microbiology Department, University of Minnesota, 312 Church Street, SE, Minneapolis, MN 55455.

4 Address correspondence and reprint requests to Dr. Nicolas Glaichenhaus, Center National de la Recherche Scientifique, Institut de Pharmacologie Moléculaire et Cellulaire, 660 Route des Lucioles, 06560 Valbonne, France. E-mail address: glaichenhaus@ipmc.cnrs.fr

5 Abbreviations used in this paper: LACK, Leishmania homologue of mammalian RACK1; LN, lymph node; GALTs, gut-associated lymphoid tissues; GF, germfree; SPF, specific pathogen free; HPRT, hypoxanthine-guanine phosphoribosyltransferase; CD62L, CD62 ligand.
Materials and Methods

Mice
Female BALB/c and BALB/c-ByJ nude mice were purchased from IFFA Credo (L’Arbresle, France). All animals were used between 6 and 8 wk of age. Mice were bred in our core animal facility and kept under specific pathogen-free (SPF) conditions. Germfree (GF) BALB/c mice were originally obtained from Dr. E. Balish and were maintained in isolators with sterile food and water. When indicated, mice were treated p.o. daily with 200 μl of a mixture of antibiotics containing kanamycin (4 mg/ml), gentamicin (0.35 mg/ml), colistin (850 μg/ml), metronidazole (2.15 mg/ml), and vancomycin (0.45 mg/ml). Treatment was started at 2 wk of age. Survival for bacterial contamination was performed by periodic bacteriologic examinations of feces (7). In experiments in which mice were infected with *L. major*, the treatment with antibiotics was stopped 1 day before infection. DO.11.10 (8), TRC-LACK Ca2+ (9), and JE-LACK transgenic mice have been described (10).

Parasites and infection
*L. major* (World Health Organization strain WHOM/JR/–/173) promastigotes were maintained in M199 medium containing 20% FCS as described (3). Unless otherwise stated, mice were infected in the footpads with the indicated numbers of stationary phase *L. major* promastigotes in 50 μl PBS. Footpad swelling was measured with a metric caliper. For IL-4 neutralization, mice were injected i.p. with 2 mg anti-IL-4, at day −1 and day 0 and subsequently infected with the indicated numbers of stationary phase *L. major* promastigotes at day 0. Parasite numbers were determined as described (11). Briefly, infected mice were killed 7 wk after infection. Single-cell suspensions were prepared from the spleen and the draining LN, and serial dilutions of these cells were incubated for 2–3 wk in flat-bottom 96-well plates containing complete medium 199. Parasite growth was determined by light microscopy.

Reagents and Abs
LACK recombinant protein was produced and purified as described (3). LACK (aa 158–173: FPSPLEHPIVSVGSWD) and OVA (aa 323–339: ISQAVHAAHAEINAG) peptides were produced from Chiron Mimotopes (Clayton, Australia). The following mAbs were purchased from PharMingen (San Diego, CA): 145-2C11, anti-CD3; GK1.5, anti-CD4 (12); 53-6.7, anti-CD8; 16A, CD45RB; IM7, anti-CD44; MEL-14, anti-CD54; BVD6-24G2, anti-IL-4; TRFK5 and TRFK4, anti-IL-5; R4-6A2 and XMG1.2, anti-IFN-γ. Recombinante mouse IL-2, IL-4, IL-5, and IFN-γ were purchased from Genzyme (Cambridge, MA).

Tissue culture medium
DMEM supplemented with 10% heat-inactivated FCS (Corning Costar France, Brumath, France), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM sodium pyruvate, L-glutamine (2 mM), and 2-ME (50 μg/ml), and 2-ME (50 μg/ml), was added, and absorbance was read on an ELISA plate reader (LabSystems, Issy-Les-Moulineaux, France) using a 410-nm pore size filter referenced to 510 nm.

Flow cytometry
Single-cell suspensions were prepared and stained with optimal concentrations of FITC-labeled and PE-conjugated mAbs for 30 min at 4°C in PBS containing 2% FCS. Analysis was performed on a FACSscan flow cytometer (Becton Dickinson). Data were collected on 4 × 105 viable cells as determined by forward light scatter and side scatter intensity and were analyzed using Lysis II software (Becton Dickinson).

Ab responses
Abs were measured by ELISA as described (15). Briefly, Immunosorb 96-well plates (Nalge Nunc International, Rochester, NY), were coated with LACK or OVA protein (10 μg/ml) overnight at 4°C. Plates were blocked with 10% FCS-PBS at room temperature for 30 min to prevent nonspecific binding. Serum was added at serial 3-fold dilutions (starting at 1:10) and incubated overnight at 4°C. HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich Chimie, St. Quentin Fallavier, France) was added for 45 min at room temperature. 2,2’-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate (Sigma-Aldrich Chimie, St. Quentin Fallavier, France) was added, and absorbance was read on an ELISA plate reader (LabSystems, Issy-Les-Moulineaux, France) using a 410-nm pore size filter referenced to 510 nm.

*T cell stimulation assays
For stimulation of *T* cell hybridomas, the indicated Abs were incubated in round-bottom 96-well plates with 2 × 105 LMR16.2 or LMR17.D12 T cell hybridomas and 3 × 105 mitomycin C-treated splenocytes from BALB/c or C57BL/6 mice. Supernatants were harvested 24 h later and assayed for IL-2 content by ELISA using anti-IL-2 JES6-5H4 and JES-1A12 as capturing and biotinylated detecting mAbs, respectively. The cytokine content of each sample was determined by comparison to a standard curve using recombinant mouse IL-2. The detection limit in all assays was 0.1 U/ml. For anti-CD3 stimulation experiments, ELISA grade 96-well plates were coated with 200 ng affinity-purified anti-CD3 mAbs in 50 μM Tris-HCl, 150 mM NaCl (pH 9.5) overnight at 4°C. After three washes in PBS, 2 × 105 splenocytes from SPF or GF mice were added to each well. Supernatants were harvested 48 h later and analyzed for IFN-γ and IL-4 content by ELISA. For T cell proliferation assays, 2 × 104 *T* cells from the TCR-LACK Ca2+ or DO.11.10 transgenic mice were incubated for 72 h with the indicated numbers of mitomycin C-treated APCs in round-bottom 96-well plates. Cells were pulsed with 1 μCi [3H]thymidine during the last 18 h of culture, and plates were harvested and counted on a Betaplate Reader (Amersham Pharmacia Biotech, Uppsala, Sweden). For other T cell assays, 106 CD4+ T cells from immunized or infected mice were incubated in flat-bottom 96-well plates with 105 mitomycin C-treated splenocytes and the anti-IFN-γ Abs were measured by ELISA. Supernatants were harvested 48 or 72 h later and analyzed for IFN-γ, IL-4, and IL-5 contents by ELISA.

Competitive RT-PCR
Total RNA was extracted using RNAeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription was performed using murine Moloney leukemia virus RT (Life Technologies, Cergy Pontoise, France) and oligo(dT) primers (Amersham Pharmacia Biotech). Semi-quantitative PCR was performed as described (16). In brief, cDNA was first assayed for levels of the constitutively expressed gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), by incubating different concentrations of the polycotector construct and determining the ratio of competitor to wild-type band intensity after amplification with HPRT-specific primers. The adjusted volumes of cDNA were used to measure IL-4 or IL-10 mRNA levels, using IL-4 or IL-10-specific primers. After separation of the PCR products by electrophoresis in agarose gel, the ratio of the relative concentration of IL-4 or IL-10 mRNA to the relative concentration of HPRT mRNA was calculated.

Results
*LACK*-specific CD4+ *T* cells express a memory/effector phenotype before infection with *L. major*
Naive and memory/effector *T* lymphocytes express different levels of surface molecules such as CD62L, CD45RB, and CD44 (17). Thus, in contrast to most naive *T* cells, which express high levels of CD62L and CD45RB and low levels of CD44, memory/effector *T* lymphocytes express low levels of CD62L and CD45RB and high levels of CD44. To analyze the phenotype of *LACK*-specific *T* cells in uninfected mice, CD4+ *T* cells from BALB/c mice were
sorted on the basis of CD62L expression and adoptively transferred into BALB/c nude recipients. Mice were immunized with LACK or OVA, and Ag-specific Ab responses were monitored by ELISA. As expected, OVA-specific Abs were detectable in the sera of the mice that had been injected with CD62L<sup>high</sup> or unseparated CD4<sup>+</sup> cells, but not in the sera of the recipients reconstituted with CD62L<sup>low</sup> cells (Fig. 1A). In contrast, LACK-specific Ig were detected in the sera of all reconstituted animals including those that had received the memory/effector CD62L<sup>low</sup> CD4<sup>+</sup> cells, but not in the sera of the recipients reconstituted with CD62L<sup>low</sup> cells (Fig. 1A). Thus, whereas all OVA-specific T cells expressed high levels of CD62L, LACK-specific T cells were found in both the CD62L<sup>low</sup> and CD62L<sup>high</sup> populations, suggesting that at least some of them had been stimulated by cross-reactive Ags before infection with the parasite.

To directly monitor the phenotype of LACK-specific T cells before infection, we used flow cytometry to analyze CD4<sup>+</sup> T cells in noninfected TCR-LACK Ca<sup>o</sup> transgenic mice in which all T cells express a single receptor recognizing a LACK-derived immunodominant peptide bound to I-A<sup>d</sup> molecules (9). In contrast to what was observed in other monoclonal TCR transgenic mice (18, 19), a majority of CD4<sup>+</sup> T cells in TCR-LACK Ca<sup>o</sup> transgenic mice were large CD62L<sup>low</sup> CD44<sup>high</sup> CD45RB<sup>low</sup> cells (Fig. 1B). Interestingly, the proportion of transgenic T cells exhibiting this large CD62L<sup>low</sup>CD44<sup>high</sup>CD45RB<sup>low</sup> phenotype was higher in the mesenteric LN than in other secondary lymphoid organs including the spleen (Fig. 2) or the popliteal and inguinal LN (data not shown).

The mesenteric LN of BALB/c mice contain APCs which can stimulate LACK-specific T cells in the absence of exogenously added Ags

The higher proportion of large CD62L<sup>low</sup>CD44<sup>high</sup>CD4<sup>+</sup> T cells found in the mesenteric LN of TCR-LACK Ca<sup>o</sup> mice could result from the preferential trafficking of these cells to the mesenteric LN. Alternatively, LACK-specific T cells could be primed in the mesenteric LN, possibly by exposure to cross-reactive Ags present in the digestive tract. To test this latter hypothesis, CD4<sup>+</sup> T cells from TCR-LACK Ca<sup>o</sup> transgenic mice were incubated with APCs purified from the secondary lymphoid organs of BALB/c mice. In contrast to APCs isolated from the spleen or the popliteal LN, APCs from the mesenteric LN induced the proliferation of LACK-specific CD4<sup>+</sup> transgenic T cells (Fig. 3). This proliferation was dependent on the number of APCs and was blocked by anti-I-A<sup>d</sup> mAbs (data not shown). In contrast, OVA-specific CD4<sup>+</sup> T cells from DO11.10 transgenic mice did not proliferate when incubated with mesenteric LN APCs. Thus, APCs from the mesenteric LN of naive BALB/c mice are loaded with peptides that can stimulate LACK-specific T cells.

Bacteria from the intestinal flora contain Ags that can stimulate LACK-specific T cells

Because Ags from the intestinal flora are the most abundant and diverse Ags to which T cells are exposed in SPF mice, we sought to determine whether bacteria isolated from the gut of BALB/c mice contained Ags that could stimulate LACK-specific T cells. To address this issue, we incubated five different LACK-specific T cell hybridomas with crude extracts from various aerobic and anaerobic bacteria. Extracts from Escherichia coli and Enterococcus faecalis, but not those from Proteus mirabilis and Clostridium perfringens induced IL-2 secretion by LMR16.2 and LMR17.1D12 T cell hybridomas in a dose-dependent manner (Fig. 4A and data not shown). In contrast, other LACK-specific T cell hybridomas, i.e., LMR8.1, LMR8.3, and LMR4.1, did not secrete IL-2 in response to these extracts (data not shown).

As expected, bacterial extracts were much less efficient than recombinant LACK Ag for inducing IL-2 secretion. Thus, similar amounts of IL-2 were secreted by LMR16.2 T cell hybridomas when incubated with either 10 μg/ml E. faecalis extracts or 25 ng/ml recombinant LACK protein (data not shown). Because the stimulatory effects of Escherichia coli or E. faecalis soluble extracts on LACK-specific T cell hybridomas were small, we sought to rule out the possibility that this phenomenon resulted from the activation of the APCs by bacterial products. To this end, LMR16.2 T cell hybridomas were incubated with an optimal concentration of E. faecalis extract in the presence of APCs from MHC class II-matched or unmatched mice, with or without anti-I-A<sup>d</sup> mAbs. Although IL-2 secretion was detected when T cell hybridomas were incubated in the presence of APCs from BALB/c mice, this was not the case when LACK-specific T cell LMR16.2 hybridomas were incubated with APCs from C57BL/6 mice. Moreover, IL-2 secretion which was induced by E. faecalis extracts in the presence of APCs from BALB/c mice was prevented by the addition of anti-I-A<sup>d</sup> mAbs (Fig. 4B). As an attempt to identify microbial proteins that could stimulate LACK-specific T cell hybridomas, soluble extracts from E. faecalis were fractionated on a gel filtration column, and individual fractions were tested for protein content and for the ability to stimulate LMR16.2 T cell hybridomas. Stimulatory activity was found in two groups of four consecutive fractions corresponding to proteins of 30–35 kDa and high molecular mass (>90 kDa), respectively (Fig. 4C).

**FIGURE 1.** LACK-specific Ab responses in BALB/c nude mice adoptively transferred with purified T cell subsets. Unseparated (△) or purified CD62L<sup>high</sup> (□) or CD62L<sup>low</sup> (●) CD4<sup>+</sup> T cells from the mesenteric LN of naive BALB/c mice were prepared, and 1.5 × 10<sup>6</sup> cells were injected i.v. into BALB/c nude recipients. Control animals were not injected (□). After 2 wk, mice were immunized i.p. with 50 μg of OVA (A) or LACK (B) in PBS. Mice were boosted 2 wk after the first immunization and analyzed 5 days later for the presence of serum IgG OVA (A)- or LACK (B)-specific Abs by ELISA.
Although soluble extracts from *E. faecalis* and *Escherichia coli* stimulated IL-2 secretion from two different LACK-specific T cell hybridomas, we sought to determine whether cross-reactivity between LACK and bacterial Ags could also be demonstrated using polyclonal populations of T cells. To this end, BALB/c mice were immunized with bacterial extracts from *E. faecalis*, and T cells from their LN were incubated with LACK or soluble bacterial proteins. We found that T cells from nonimmunized BALB/c mice did not secrete any detectable amount of IFN-γ or IL-4 when incubated with either LACK or *E. faecalis* extracts (Fig. 4D). In contrast, T cells from mice immunized with *E. faecalis* extracts secreted small but detectable amounts of IFN-γ in response to LACK peptide in vitro. As expected, T cells from immunized mice secreted high amounts of IFN-γ in response to soluble *E. faecalis* Ags (Fig. 4D).

**Decontamination of the digestive tract prevents both the development of memory/effector LACK-specific CD4+ T cells and the early burst of IL-4 mRNA induced by *L. major***

If exposure to bacterial Ags was responsible for the memory/effector phenotype expressed by LACK-specific T cells before infection, these cells should exhibit a naive phenotype in mice in which the digestive tract has been decontaminated with antibiotics. Indeed, treatment of TCR-LACK Cα° transgenic mice with antibiotics starting at 2 wk of age resulted in a dramatic reduction in the proportion of CD4+ T cells expressing low levels of CD62L (Fig. 5A). Moreover, the early accumulation of IL-4 mRNA which was detected 16 h after infection in the draining LN of SPF BALB/c mice was not observed in either antibiotic-treated SPF or GF mice following i.v. inoculation of *L. major* promastigotes (Fig. 5B, left). Similarly, no increase in IL-4 mRNA levels was observed in the spleens of antibiotic-treated SPF or GF mice following i.v. inoculation of *L. major* promastigotes (Fig. 5B, right). This latter phenomenon was not due to a defect in the ability of CD4+ T cells from GF mice to secrete IL-4. Indeed, CD4+ T cells from GF and SPF BALB/c mice secreted similar amounts of IL-4 and IFN-γ when stimulated in vitro with anti-CD3 mAbs (Fig. 5C).
Because CD4+ T cells that have been primed in GALTs often secrete IL-10 in addition to IL-4 (20), we sought to determine whether LACK-specific T cells could rapidly produce IL-10 in BALB/c mice infected with L. major. To address this issue, we compared the amount of IL-10 mRNA induced by L. major promastigotes. Unexpectedly, we found that both GF BALB/c mice and SPF BALB/c mice contained high numbers of parasites in their spleen and LN (Fig. 7A). In striking contrast, the early production of IL-4 is prevented in antibiotic-treated SPF and in GF BALB/c mice. Antibiotic-treated or untreated SPF, or GF BALB/c mice were either infected i.v. (left) or i.v. (right) with different L. major promastigotes ( ), or not infected ( ). RNA was extracted from the spleen (left) or from the spleen (right) 20 h and 90 min after infection, respectively. For each sample, the relative levels of IL-4 mRNA were determined by semiquantitative RT-PCR as described (16). Data are means of three individual mice per group and are representative of 3 different experiments.

GF BALB/c mice remain susceptible to L. major even when they are treated with anti-IL-4 Abs

Because GF BALB/c mice did not exhibit a parasite-induced IL-4 burst, we sought to determine whether these mice were resistant to L. major. To this aim, GF and SPF BALB/c mice were infected with L. major promastigotes. Unexpectedly, we found that both GF and SPF mice developed progressive lesions (Fig. 7A) and exhibited high numbers of parasites in their spleen and LN (Fig. 7B). Indeed, the LN and the spleens of GF mice contained 2.5- and 12-fold more parasites, respectively, than those of SPF animals, suggesting that GF mice were even more susceptible to L. major than SPF mice.

As the early burst of IL-4 mRNA that is induced by L. major in BALB/c mice has been associated with susceptibility, it was important to determine why GF mice were susceptible to L. major.
lesions developed by anti-IL-4-treated GF mice did not increase in size at later time points, they remained larger than those exhibited by anti-IL-4-treated SPF mice. In agreement with the latter result, the LN of anti-IL-4-treated GF mice contained 12-fold more parasites than those from anti-IL-4-treated SPF mice. Likewise, whereas parasites were not detectable in the spleens of anti-IL-4-treated SPF mice, the spleens of anti-IL-4-treated GF mice contained 3- to 4-fold more parasites than those from untreated SPF animals (Fig. 7B). Thus, whereas the early neutralization of IL-4 allowed SPF mice to control the replication of the parasite and to resolve the infection, anti-IL-4-treated GF mice remained highly susceptible to L. major.

Discussion

One of the hallmarks of the immune system is that responses to an Ag are stronger and occur more quickly in individuals who have already been exposed to the same Ag. Although the phenomenon of immunological memory has been initially defined as an operational property of the whole immune system, several studies have shown that memory cells are intrinsically and functionally different from naive T cells. Thus, in contrast to naive CD4+ T cells which often express a typical CD62LhighCD45RBhighCD44low phenotype, memory/effector CD4+ T cells are characterized by the down-regulation of CD62L and CD45RB and the up-regulation of CD44 (21). Most importantly, memory/effector CD4+ T cells secrete a wider variety of cytokines than do naive cells, and responses of memory/effector T cells peak earlier than those of naive T cells (5, 6). Because infection of susceptible BALB/c mice with L. major results in the very rapid production of IL-4 by CD4+ T cells that react to the parasite LACK Ag (4), we sought to determine whether these cells express a memory/effector phenotype before infection.

Because molecular probes to visualize the LACK-specific T cells were not available, we used an adoptive transfer system to analyze the surface phenotype of these cells in BALB/c mice. In contrast to OVA-specific T cells which were only found within the CD4+CD62Llow population, LACK-specific T cells were found in both the CD4+CD62Lhigh and CD4+CD62Llow subsets. Although this result suggested that at least some LACK-specific T cells had been exposed to an Ag, we did not know which proportion of these cells expressed a naive or a memory/effector phenotype, respectively. For this reason, we have analyzed the surface phenotype of CD4+ T cells in TCR-LACK Caα transgenic mice in which all CD4+ T cells expressed a single TCR recognizing a LACK-derived peptide bound to I-Aα molecules (9). Although the proportion of CD4+ cells expressing a memory/effector phenotype were variable from one mouse to another, the spleen and the LN of these mice always contained a large fraction of CD4+ T cells expressing low levels of CD62L and CD45RB and high levels of CD44. Thus, in TCR-LACK Caα transgenic mice, 40–50% and 60–70% of splenic CD4+ T cells expressed low levels of CD62L and high levels of CD44, respectively. Moreover, because late memory/effector cells can sometimes revert to a naive phenotype, it is possible that some of the LACK-specific T cells which expressed high levels of CD62L and/or low levels of CD44 were not true naive cells, but had reverted to this phenotype after a first antigenic stimulation.

Several piece of evidence suggests that the memory/effector phenotype expressed by LACK-specific T cells results from their priming by cross-reactive microbial Ags derived from the gut flora. First, the fractions of CD4+ T cells expressing low levels of CD62L or high levels of CD44 were higher in the mesenteric LN of TCR-LACK Caα transgenic mice than in any other secondary lymphoid organs. In mesenteric LN, most LACK-specific T cells
expressed a large phenotype typical of actively dividing cells. The latter phenomenon was not observed in either BALB/c mice or DO.11.10 TCR transgenic mice in which a large number of T cells reacted to an OVA peptide bound to I-A<sup>d</sup> molecules. Secondly, APCs from the mesenteric LN of BALB/c mice, but not those from the spleen or the popliteal LN, were able to stimulate the proliferation of transgenic LACK-specific T cells in the absence of exogenously added Ags. In contrast, no proliferation was observed when OVA-specific CD4<sup>+</sup> T cells from DO.11.10 TCR transgenic mice were incubated with mesenteric LN APCs. In this respect, it is noteworthy that the TCR transgenic mice that we have used in this study have been maintained as heterozygotes by successive crosses to the same genetic stock of BALB/c mice. Thus, the fact that T cells from TCR-LACK C<sub>a</sub>-<sup>scid</sup> mice proliferated in response to BALB/c APCs was not due to subtle mismatches in minor histocompatibility Ags selectively expressed by mesenteric and not popliteal APCs. Thirdly, cells from two of five different LACK-specific T cell hybridomas secreted IL-2 when incubated with crude or partially purified soluble extracts from *Escherichia coli* or *E. faecalis*. Although the amounts of IL-2 secreted in response to bacterial Ags were small compared with those secreted in response to recombinant LACK protein, IL-2 secretion was observed only in the presence of syngeneic APCs and was blocked by anti-I-A<sup>d</sup> Abs. Moreover, only two of five LACK-specific T cell hybridomas secreted IL-2 in response to bacterial extracts, ruling out the possibility that this phenomenon was the result of the activation of APCs by bacterial products such as LPS or lipoteichoic acid. Although we were not able to detect a T cell response against either LACK or *E. faecalis* soluble proteins in naive BALB/c mice, this could easily be explained if the frequency of the LACK-specific T cells was not high enough. In contrast, T cells from mice that had been immunized with *E. faecalis* extracts responded to LACK in vitro. Although it may be surprising that IFN-γ but not IL-4 was detected in this assay, it could be that s.c. immunization with bacterial extracts favored the development of LACK-specific Th1 cells, as opposed to natural priming in GALTs. Thus, experiments performed with T cell hybridomas, T cells from TCR transgenic mice, or polyclonal populations of T cells pointed to cross-reactivity between LACK and bacterial soluble proteins. Fourthly, the fraction of LACK-specific T cells expressing a memory/effector phenotype was reduced in TCR-LACK C<sub>a</sub>-<sup>scid</sup> mice in which the digestive tract had been decontaminated with antibiotics. Lastly, we have recently shown that a subset of CD4<sup>+</sup> T cells from TCR-LACK C<sub>a</sub>-<sup>scid</sup> mice, but not from DO.11.10 *scid* mice, adoptively transferred colitis when injected into C.B.-17. *scid* mice (unpublished results).

Although most LACK-specific T cells express V<sub>Aa</sub>8 and V<sub>β</sub>4, LACK-specific TCR can use very distinct CDR3 regions (our unpublished results). Thus, the T cell response directed to the LACK immunodominant peptide is highly polyclonal. In this respect, it would be interesting to determine which proportion of the LACK-specific T cells react to gut flora Ags. Although we do not have the answer to this question, it is noteworthy that only two of five LACK-specific T cell hybridomas secreted IL-2 in response to *E. faecalis* extracts. Thus, although our results suggest that the parasite-induced IL-4 burst depends on the priming of LACK-specific T cells by microbial Ags, it is likely that only a fraction of the LACK-specific T cells are cross-reactive to these Ags and eventually contribute to the rapid production of IL-4 induced by *L. major*. Experiments are in progress to identify structural features of LACK-specific TCR which would allow them to respond to bacterial extracts.

In an attempt to identify the gut flora Ags which were responsible for the priming of the LACK-specific T cells, we have searched *Escherichia coli* open reading frames for sequences exhibiting similarities to the LACK immunodominant peptide (SLE HIPVVSGLSW) using the BLAST program (22). We identified 61 sequences and synthesized the corresponding peptides. Although one of these peptides (VLQHPLVTATFAVS) was derived from the *Escherichia coli* *lil* gene product, stimulated LMR16.2 T cell hybridomas in vitro, we do not know whether this peptide was actually presented to T cells in vivo and, even if this was the case, how many other microbial peptides were able to stimulate LACK-specific T cells. Indeed, several fractions from *E. faecalis* extracts were capable of stimulating the LACK-specific hybridomas LMR16.2 and LMR17.1D12, suggesting that these bacteria contained more than one LACK mimicry Ag. Also, additional experiments showed that only high concentrations of the *lil*-derived peptide were able to stimulate LMR16.2 T cell hybridomas and that this peptide bound poorly to I-A<sup>d</sup> molecules. Thus, the bacterial Ags which were responsible for the priming of LACK-specific T cells in vivo remain to be identified.

LACK-specific CD4<sup>+</sup> T cells are rapidly activated to secrete IL-4 in BALB/c mice infected with *L. major*. Indeed, as early as 16 h after infection, a burst of IL-4 mRNA could be detected in the draining LN of BALB/c mice, but not in animals that had been made tolerant to LACK by immunological manipulations or transgenic expression of this Ag in the thymus (23). Here, we found that decontamination of the digestive tract of SPF mice with antibiotics caused a dramatic reduction in the early burst of IL-4 induced by the parasite. Likewise, no early IL-4 burst was observed in GF mice. As expected for T cells that have been primed in GALTs (20), we found that *L. major* induced the rapid accumulation of IL-10 mRNA in SPF BALB/c mice. However, this IL-10 burst was dramatically reduced in both BALB/c GF mice and IE-LACK transgenic SPF mice that were tolerant to LACK as the result of the expression of this Ag in the thymus. Thus, our results suggest that the priming of LACK-specific T cells by microbial Ags from the gut flora is critical in determining their ability to rapidly produce IL-4 and IL-10 mRNA in response to *L. major*.

Many studies have suggested that there is a causal relationship between the early accumulation of IL-4 transcripts in the draining LN of BALB/c mice and the development of a counterprotective Th2-like immune response against *L. major*. Thus, the early burst of IL-4 mRNA induced by the parasite in susceptible BALB/c mice was not observed in resistant strains including C57BL/6, C3H/He, and CBA/Ca. No accumulation of IL-4 mRNA was found in BALB/c mice that were made resistant by the early administration of IFN-γ or IL-12 (24). In contrast, treatment of resistant C57BL/6 mice with neutralizing anti-IFN-γ mAbs induced both the early accumulation of IL-4 mRNA and susceptibility (24). Despite these data showing that IL-4 is critical for susceptibility, some other studies have suggested that other yet unidentified factors may be capable of causing susceptibility to *L. major* in the absence of IL-4 or IL-4 signaling (25, 26). At least two hypotheses could account for these apparently conflicting data. Thus, it is possible that the very rapid accumulation of IL-4 transcripts which is induced by the parasite in BALB/c mice would not be required for susceptibility as was previously thought. In this case, priming of LACK-specific T cells by *L. major* cross-reactive intestinal microbial Ags would not be causally related to susceptibility but might be an innocent bystander effect resulting from a possible high frequency of LACK-specific CD4<sup>+</sup> T cells. We think this hypothesis is unlikely for two reasons. First, it was recently found that administration of IL-4 to BALB/c mice that were depleted from LACK-specific T cells was sufficient to instruct Th2 cell development and to restore susceptibility to *L. major* (27). Secondly, we have tried to determine the proportion of CD4<sup>+</sup> T cells reacting to LACK in BALB/c mice before and 1 day after infection with *L. major*. Decontamination of the digestive tract of SPF mice with antibiotics (22) made tolerant to LACK by immunological manipulations or transgenic expression of this Ag in the thymus (23). Here, we found that decontamination of the digestive tract of SPF mice with antibiotics caused a dramatic reduction in the early burst of IL-4 induced by the parasite. Likewise, no early IL-4 burst was observed in GF mice. As expected for T cells that have been primed in GALTs (20), we found that *L. major* induced the rapid accumulation of IL-10 mRNA in SPF BALB/c mice. However, this IL-10 burst was dramatically reduced in both BALB/c GF mice and IE-LACK transgenic SPF mice that were tolerant to LACK as the result of the expression of this Ag in the thymus. Thus, our results suggest that the priming of LACK-specific T cells by microbial Ags from the gut flora is critical in determining their ability to rapidly produce IL-4 and IL-10 mRNA in response to *L. major*.
major. Although LACK-specific T cells could not be detected in naive mice, the frequency of these cells among CD4+ T cells was below 1 of 5 × 10⁴ in infected animals as determined by an IL-4 ELISPOT assay. Thus, although we cannot rule out that the number of LACK-specific T cells would be underestimated using this assay, this frequency does not seem to be different from those reported for other Ag-specific CD4+ T cells (28). Alternatively, the lack of an intestinal flora in antibiotic-treated SPF and GF mice may result in complex physiological alterations that could impair their ability to control the infection. In agreement with this latter hypothesis and in contrast to what was observed in SPF BALB/c mice, we found that anti-IL-4-treated GF BALB/c mice did not control the replication of the parasite and remained susceptible to *L. major* infection. Likewise, a recent study has shown that GF Swiss mice developed progressive lesions when infected with *L. major*, whereas SPF Swiss mice only make small lesions and eventually heal (29).

Lastly, macrophages derived from the spleen of GF mice expressed reduced amounts of MHC class II molecules (30) and exhibited impaired ability to secrete IL-1, IL-6, and IL-12 (31–33). Thus, although T cells from GF mice differ from those from SPF mice because they have not been exposed to gut flora Ags, GF mice exhibit many other defects that could explain why they remained susceptible to *L. major* despite the lack of the parasite-induced IL-4 burst.

It would be interesting to know whether bacteria-cross-reactive LACK-specific T cells also develop in mice from resistant strains. Although we have not directly addressed this issue, we recently found that mice from the resistant B10.D2 strain exhibited an early burst of IL-4 mRNA when infected with *L. major* (23). Moreover, similarly to what we have observed in BALB/c mice, the early production of IL-4 was completely abolished when B10.D2 mice were made tolerant to LACK (23). Although we do not know whether antibiotic-treated or GF B10.D2 mice would exhibit a burst of IL-4 mRNA, our data suggest that priming of the LACK-specific T cells by cross-reactive bacterial Ags may not be restricted to susceptible BALB/c mice, but rather to mice of the d haplotype.

Because of their location, it is not surprising that bacteria from the intestinal flora play a critical role in both the development and the function of the mucosal immune system. Thus, as compared with SPF mice or GF mice colonized with single or multiple species of bacteria, GF mice exhibit smaller Peyer’s patches, reduced numbers of IgA-secreting plasmacytes (34) and intraepithelial lymphocytes (35, 36), and are partially or totally resistant to the induction of oral tolerance to some Ags (37–40). In contrast to this large body of literature, very few data are available on the role of the intestinal flora on systemic Ag-specific immune responses (41).

In this respect, the results reported here are the first to demonstrate that priming of helper T cells by cross-reactive bacterial proteins in GALTs may be critical in determining their ability to rapidly respond to their cognate Ag in peripheral lymphoid organs. Thus, a key parameter of the immune response to a cutaneous parasite may be a consequence of T cell cross-priming by microbial Ags from the indigenous intestinal flora.

Although several studies have demonstrated that the mucosal immune system of healthy individuals is unresponsive to bacterial Ags from the gut flora, the molecular and cellular mechanisms that are responsible for the establishment and/or the maintenance of tolerance are still poorly understood (42–44). Thus, it is not known when and where bacteria-specific T cells encounter these Ags for the first time and how these T cells behave when they have been primed. Although more studies are needed to generalize these findings, our results suggest that bacteria-specific CD4+ T cells that have been primed in GALTs are not deleted but recirculate through the spleen and the LN, where they can rapidly produce IL-4 and IL-10 when stimulated by cross-reactive Ags. Thus, tracking the fate of the LACK-specific T cells in BALB/c mice may provide important information about the regulation of cell-mediated immune responses to gut Ags in both physiological and pathological conditions.

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**References**