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Rapid Expansion and IL-4 Expression by *Leishmania*-Specific Naive Helper T Cells In Vivo

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Summary

CD4 T cells are pivotal for effective immunity, yet their initial differentiation into effector subsets after infection remains poorly defined. We examined CD4 T cells specific for the immunodominant *Leishmania major* LACK antigen using MHC/peptide tetramers and IL-4 reporter mice. Comprising ∼15 cells/lymph node in naive mice, LACK-specific T cells expanded over 100-fold, and 70% acquired IL-4 expression by 96 hr. Despite their pathogenic role in susceptible mice, LACK-specific precursor frequency, expansion, and IL-4 expression were comparable between susceptible and resistant mice. When injected with unrelated antigen, *Leishmania* efficiently activated IL-4 expression from naive antigen-specific T cells. CD4 subset polarization in this highly characterized model occurs independently from IL-4 expression by naive T cells, which is activated indiscriminately after parasitism.

Introduction

The differentiation of naive helper T cells to cytokine-expressing effector cells is important for the orchestration of immunity. Th1 and Th2 cells, which express the canonical cytokines IFN-γ and IL-4, respectively, mediate defense against distinct types of challenges, with Th1 cells more typically involved in systemic and Th2 cells in mucosal immunity. Despite this dichotomy, it is not entirely clear where or when these distinct cytokine responses become established. Current models envision dendritic cells to be central in mediating these responses through their capacity to recognize conserved pathogen-specific recognition motifs by innate receptors, including Toll-like receptors (Reis e Sousa, 2001). Upon maturation and migration to lymph nodes, dendritic cells can subsequently influence T helper subset differentiation by the elaboration of specific gene programs, including cytokines and receptors (Huang et al., 2001). Distinct classes of dendritic cells may also influence Th development (Liu et al., 2001).

Examination of naive CD4 T cell responses in vivo has been hampered by the inability to identify the low numbers of cells within the endogeneous precursor population reactive to a given antigen. Methods for studying early antigen-specific CD4 T cell responses typically rely on the seeding of transgenic T cells of known specificity into animals prior to challenge (Garside et al., 1998; Reinhardt et al., 2001) or on analysis of effector functions revealed upon restimulation several days after challenge (Whitmire et al., 1998; Topham and Doherty, 1998). Modulation of activation markers has also been examined on normal T cells to define specificity, but few correlations with cytokine expression have been made (McHeyzer-Williams and Davis, 1995; McHeyzer-Williams et al., 1999; Panus et al., 2000). While such studies have elegantly documented the anatomy and dynamics of CD4 effector/memory T cells, the initial expansion and acquisition of effector fates after infection remain incompletely characterized.

Murine *Leishmania major* infection remains an exceptional model for T helper subset development because the outcome has been linked definitively to effector fate; resistant mice make a protective Th1 response, and susceptible mice make a nonprotective Th2 response (Reiner and Locksley, 1995). Further, the susceptible response in BALB/c mice is nucleated by an aberrant Th2 response to an immunodominant peptide from the parasite LACK antigen (Mougneau et al., 1995). Deleting or tolerizing T cells that respond to LACK protects subsequently infected BALB/c mice from infection by enabling the development of a protective Th1 response (Julia et al., 1996; Launois et al., 1997, Pingel et al., 1999). Despite the utility of this model, the events that specify the development of protective versus pathogenic T cells remain elusive because they occur early in infection, before significant expansion of rare naive precursors (Reiner and Locksley, 1995).

Here we use a flow cytometry strategy to visualize rare LACK-specific T cells and study their behavior after *L. major* infection in normal mice. Using bicistronic IL-4 reporter mice, we characterize the initial expression of IL-4 by these cells in the context of their early expansion. We find that despite the highly polarized responses that ultimately develop, early IL-4 expression is strikingly similar in MHC congenic susceptible and resistant mice. Indeed, *Leishmania* parasites inoculated with an unrelated antigen efficiently induced IL-4 expression from naive, antigen-specific T cells, suggesting that these parasites might activate the elusive "Th2-inducing" pathway in antigen-presenting cells.

Results

Generation of LACK-Specific Tetramers and Detection of LACK-Specific CD4 T Cells

The immunodominant peptide comprising amino acids 156–173 from the *L. major* LACK antigen (Pingel et al., 1999) was linked with the I-A^d β chain and used to generate peptide-specific tetramers after expression with I-A^d α chains in insect cells as described (Schatz, 1993; Scott

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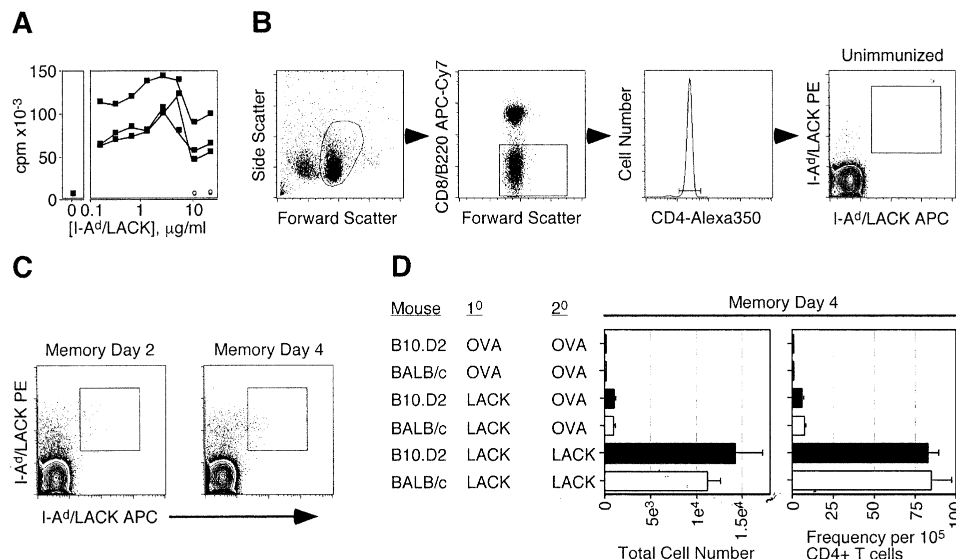

Figure 1. Specificity and Sensitivity of I-A^d/LACK Tetramers

(A) BALB/c ABLE TCR-C$\alpha^{-/-}$ (black squares) and DO11.10 TCR-C$\alpha^{-/-}$ (white circles) transgenic T cells were stimulated with plate-bound I-A^d/LACK at the indicated concentrations for 72 hr, and proliferation was measured by incorporation of ^{3}H-thymidine. Data for three independently produced batches of soluble I-A^d/LACK are shown.

(B) Flow cytometry strategy for detection of LACK-specific T cells. Intensity of I-A^d/LACK-PE and I-A^d/LACK-APC staining was monitored on [CD8/B220]−, CD4$^+$ lymphocytes. The right panel shows staining of 50,000 CD4 T cells from unimmunized BALB/c mice.

(C) BALB/c mice immunized 8 weeks earlier with LACK protein were rechallenged, and the emergence of LACK-specific T cells in the draining lymph nodes at days 2 and 4 of the recall response was enumerated. Each panel shows tetramer staining for 90,000 CD4 T cells.

(D) At day 4 of the recall response to the indicated proteins, the number and frequency of LACK-specific T cells in the draining lymph nodes of BALB/c and B10.D2 mice were enumerated. Mean and standard deviations of data from three to four mice per strain for each treatment are represented.

et al., 1998). Soluble I-A^d/LACK adsorbed to tissue culture plates stimulated the proliferation of LACK-specific but not ovalbumin-specific transgenic T cells, demonstrating that these molecules retain their specificity in vitro (Figure 1A).

To detect LACK-specific T cells by flow cytometry in normal mice, we simultaneously stained single-cell suspensions with nonsaturating concentrations of I-A^d/LACK tetramers coupled to both streptavidin-phycoerythrin (SA-PE) and SA-allophycocyanin (APC) (Figure 1B). Because PE and APC are excited by different lasers and their emission spectra do not overlap, a T cell must meet two independent criteria to be tetramer positive. This approach reduced the lower limit of detection to one in 3×10^5 CD4 T cells in normal mice. The lower limits of detection of PE- and APC-labeled tetramers used alone were one in 10^3 and one in 10^4, respectively. The frequency of DO11.10 transgenic T cells specific for ovalbumin peptide in I-A^d that stained with I-A^d/LACK tetramers was fewer than one in 4×10^6 (data not shown), confirming the specificity of the reagent revealed by the functional studies (Figure 1A).

We tested the sensitivity of this approach in vivo by analyzing the LACK-specific recall response 8 weeks after primary immunization with LACK or ovalbumin proteins in adjuvant. In both BALB/c and B10.D2 mice that were primed and rechallenged with LACK protein, we observed a 220-fold increase in both the total number of LACK-specific T cells and their frequency per 10^5 CD4 T cells (Figures 1C and 1D). Even at the peak of the memory response to saturating antigen doses, the frequency of LACK-specific T cells was fewer than 1 in 1000 CD4 T cells, a number that was undetectable with PE-labeled tetramers alone (Figure 1D). In contrast, LACK-specific T cell numbers remained at resting levels of 11–15 cells per lymph node in mice immunized and rechallenged with ovalbumin (Figure 1D). In mice immunized with LACK but rechallenged with ovalbumin, we detected resting, LACK-specific memory T cells that were 10- to 20-fold more numerous than in naive mice (Figure 1D). Thus, I-A^d/LACK tetramers detect an immune response after both primary and secondary immunization, and retain their sensitivity and specificity even during the peak response to immunization with an unrelated antigen.

I-A^d/LACK Tetramers Detect a Biologically Relevant Population of Precursor CD4 T Cells

The dual tetramer approach to identifying LACK-specific T cells in normal mice is sensitive to frequencies comparable to estimates of naive CD4 T cell precursors derived from direct sequencing approaches, suggesting numbers approximating 15 cells per lymph node or 200–250 per animal (McHeyzer-Williams and Davis, 1995; Casrouge et al., 2000). Recent estimates of naive CD8 T cell precursor frequencies suggest similar numbers (Blattman et al., 2002). The concern remains that this approach might fail to detect LACK-specific T cells in resting lymph nodes because the reagent lacks sufficient sensitivity to identify the true positives. Indeed, the electronic gates we use impose an arbitrary cutoff that excludes T cells with tetramer staining approaching back-

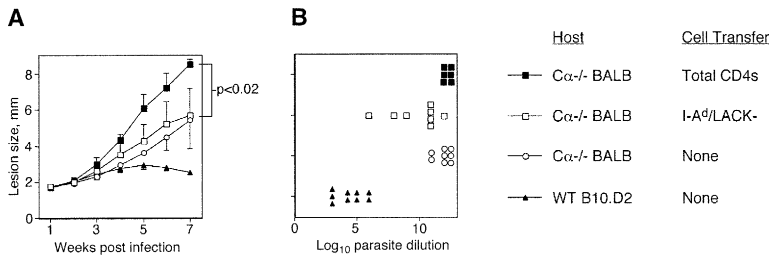

Figure 2. Depletion of I-A^d/LACK-Specific T Cells from the BALB/c CD4 Repertoire Ameliorates the Outcome of *L. major* Infection

Progression of footpad lesions (A) and parasite burdens in infected footpads (B) 7 weeks after infection in BALB/c TCR-C$\alpha^{-/-}$ mice receiving no cells (white circles), or 10^7 BALB/c CD4 T cells (black squares) or 10^7 BALB/c CD4 T cells depleted of I-A^d/LACK-tetramer$^+$ cells i.v. 24 hr prior to infection. Wild-type, resistant B10.D2 mice were infected concurrently (black triangles). The left panel depicts the mean and standard deviation for footpad lesions with parasite cultures represented as individual symbols from each infected mouse in the right panel. Data represent one of three comparable experiments. P values were calculated using the Student's t test.

ground levels (Figures 1B and 1C). Activation of T cells can increase binding avidity to MHC-peptide tetramers (Fahmy et al., 2001), raising the possibility that resting naive T cells might not be reliably detected with the reagent. However, naive, LACK-specific transgenic T cells stained brightly with I-A^d/LACK tetramers, and the staining intensity did not change with proliferation in vivo (Figure 3C, see below). To address this issue further, we sorted CD4 T cells from naive BALB/c mice and excluded cells positive for both PE- and APC-labeled tetramers using our stringent gates. We reconstituted TCR C$\alpha^{-/-}$ BALB/c mice with the I-A^d/LACK tetramer-depleted cells and infected the mice with *L. major*. Depletion of the few I-A^d/LACK tetramer-positive cells prior to infection significantly ameliorated the outcome of infection in reconstituted TCR C$\alpha^{-/-}$ BALB/c mice, as evidenced by reduced footpad swelling ($p < 0.02$ at 7 weeks) and parasite burdens, when compared to mice reconstituted with the same number of total CD4 T cells (Figure 2). Given the stringent gating strategy, we were not surprised to recover I-A^d/LACK tetramer-positive cells after 7 weeks in the depleted mice, although these were reduced in number as compared to mice receiving total CD4 T cells (data not shown). Taken together, however, the data support our ability to detect a biologically relevant population of naive I-A^d/LACK-specific T cells that contributes to the susceptibility of BALB/c mice (Julia et al., 1996; Launois et al., 1997).

LACK-Specific CD4 T Cells Have a Naive Phenotype and Expand Rapidly after Infection

Prior to infection, both BALB/c and B10.D2 mice had an average of 11–15 LACK-specific T cells per lymph node. These cells were uniformly naive with respect to surface expression of the activation markers CD25, CD62L, and CD69 (Figure 3A). We focused on the LACK-specific response in the popliteal lymph nodes during the first week following *L. major* infection because treatments that reverse the susceptible phenotype of BALB/c mice are most effective when administered during this period (Reiner and Locksley, 1995). During the first 48 hr, a marginal increase in the number of LACK-specific T cells accompanied a 3-fold increase in popliteal lymph node total cellularity. Between 48 and 72 hr, however, LACK-specific T cell numbers expanded 15-fold in BALB/c mice and 50-fold over resting levels (Figure 3B). Expansion was not as pronounced in B10.D2 mice at 72 hr, potentially reflecting more efficient restriction of parasite dissemination to lymph nodes at early time periods in resistant mice (Laskay et al., 1995). By 96 hr, however, LACK-specific T cells peaked at similar numbers in BALB/c and B10.D2 mice and remained the same at 120 hr (Figure 3B). No increase in the numbers of LACK-specific T cells was detected in either mouse strain in spleen or cervical lymph nodes cells at this time (data not shown). By 192 hr, a decline in LACK-specific T cells occurred, consistent with migration from the draining lymph node as previously defined (Garside et al., 1998; Reinhardt et al., 2001).

To confirm our ability to identify naive LACK-specific T cells, we adoptively transferred CFSE-labeled, LACK-specific, transgenic T cells into BALB/c mice and monitored cell division at 24 hr intervals after infection by following the reduction in CFSE fluorescence intensity. Despite a >1000-fold increase in precursor frequency, cell division of the transferred population was minimal for the first 48 hr (Figure 3C). The transferred T cells divided extensively between 48 and 72 hr, followed by rapid disappearance from the draining lymph node, and was essentially restricted to the draining lymph node as compared to distal nodes (Figure 3C). Thus, the kinetics and expansion of transgenic T cells of known specificity mirrored the kinetics and expansion of rigorously gated, tetramer-positive T cells. Taken together, these data suggest that we have accurately identified the response of naive, LACK-specific, CD4 T cells in vivo.

Activation of IL-4 Expression from LACK-Reactive T Cells Is Comparable among Resistant and Susceptible Mice Infected with *L. major*

We next sought to track the emergence of IL-4-expressing cells during this early period of T cell expansion. To enumerate these cells in vivo, we used mice with a bicistronic IL-4/IRES/eGFP gene knocked into the IL-4 locus. These mice, designated 4get (IL-4 GFP enhanced transcript), maintain IL-4 production under endogenous regulation, and cells that activate IL-4 accumulate eGFP in their cytoplasm, allowing identification without the need for restimulation (Mohrs et al., 2001). 4get mice backcrossed onto BALB/c and B10.D2 backgrounds recapitulated the biology of infection when compared to their wild-type counterparts, as assessed by the kinetics of footpad swelling, parasite burden, and frequency of IL-4- and IFN-γ -producing cells 4 weeks after infection (Figure 4, and data not shown). We first characterized the emergence of IL-4 producing cells in susceptible, 4get BALB/c mice. Using I-A^d/LACK tetramers, we failed to detect a single LACK-specific, eGFP$^+$ T cell in the

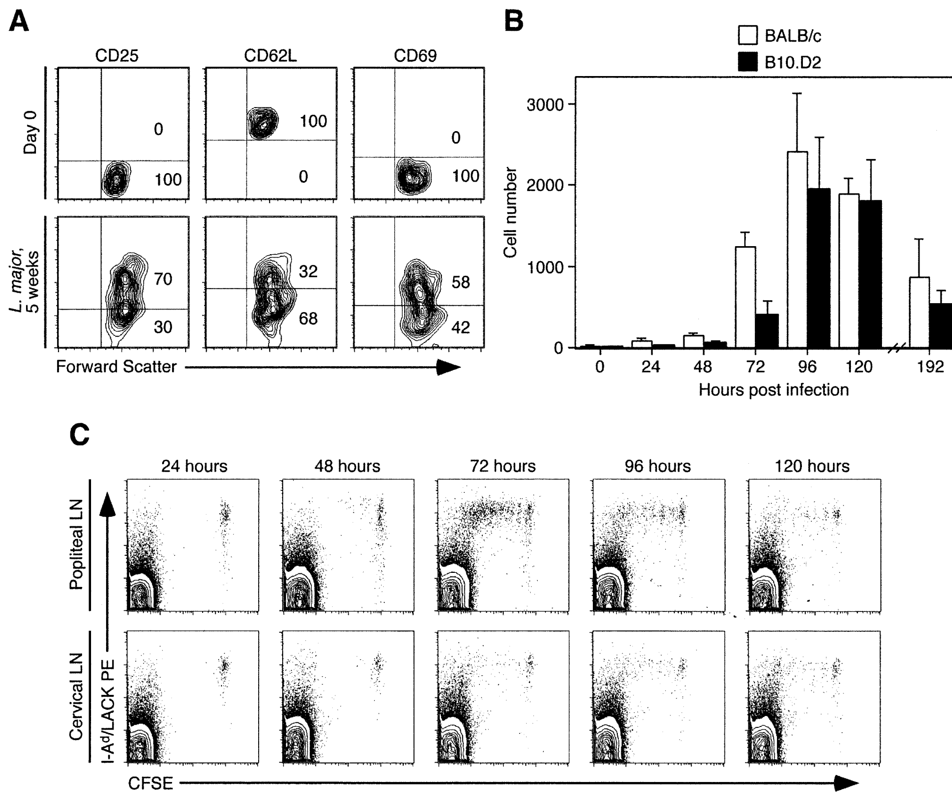

Figure 3. Kinetics of LACK-Specific T Cell Expansion after *L. major* Infection

(A) Surface expression of CD25, CD62L, and CD69 was compared on I-A^d/LACK$^+$ T cells from uninfected (top panels) and 5-week-infected (bottom panels) BALB/c mice. Cells were pooled from peripheral and mesenteric lymph nodes and spleen.

(B) Quantitation of LACK-specific T cell numbers in the draining, popliteal lymph nodes of BALB/c (white bars) and B10.D2 (black bars) mice at 24 hr intervals after infection with *L. major*. Data represent the mean and standard deviation of 4–11 mice of each strain per time point.

(C) 10^6 CFSE-labeled, BALB/c ABLE TCR-C$\alpha^{-/-}$ T cells were transferred intravenously into normal BALB/c recipients 24 hr prior to infection, and dilution of CFSE fluorescence intensity in the I-A^d/LACK tetramer$^+$ donor T cells was analyzed at 24 hr intervals. The plots represent 10^5 [CD8/B220]− T cells and are representative of four mice per time point.

spleen and pooled lymph nodes (including mesenteric) of uninfected 4get BALB/c mice (Figure 5A). Postinfection, none of the LACK-specific T cells were eGFP$^+$ after 48 hr (Figure 5A). By 72 hr, coincident with their abrupt proliferation (Figure 3B), 50% of I-A^d/LACK$^+$ T cells were eGFP$^+$ (Figure 5A). At 96 hr, this frequency peaked at 70% and remained similar at 120 hr (Figures 5A and 5B).

We next compared the frequency of eGFP-expressing T cells in susceptible 4get BALB/c and resistant 4get B10.D2 mice. Unexpectedly, the frequency of LACK-specific, eGFP-expressing cells in 4get B10.D2 mice was not different from 4get BALB/c mice at the peak of cellular expansion in the draining lymph node (Figure 5B, left panel). Moreover, the percentage of tetramer-negative T cells that activated IL-4 expression was even higher in infected 4get B10.D2 mice (Figure 5B, right panel). Neither strain of mice developed significant numbers of tetramer-positive, eGFP$^+$ CD4 T cells in the spleen during this period (data not shown). To assess whether the IL-4-expressing cells from susceptible or healer mice expressed differing amounts of IFN-γ, the eGFP$^+$ CD4$^+$ T cells were sorted from the lymph nodes of 120 hr infected 4get BALB/c and 4get B10.D2 mice. Total RNA was isolated and used for reverse-tran-

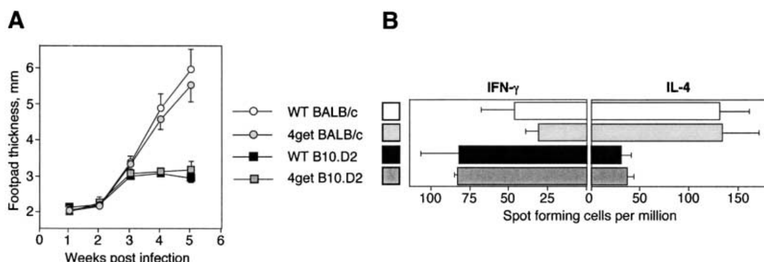

Figure 4. Characterization of 4get BALB/c and 4get B10.D2 Mice

(A) Progression of footpad lesions after *L. major* infection of BALB/c and B10.D2 mice with wild-type or 4get homozygous *Il-4* alleles. Mean and standard deviation of four mice per group are shown and are representative of three experiments.

(B) The frequency of IL-4 and IFN-γ-secreting cells from dissociated popliteal lymph nodes was determined by ELISPOT 4 weeks after infection. Mean and standard deviations of four mice per group are shown.

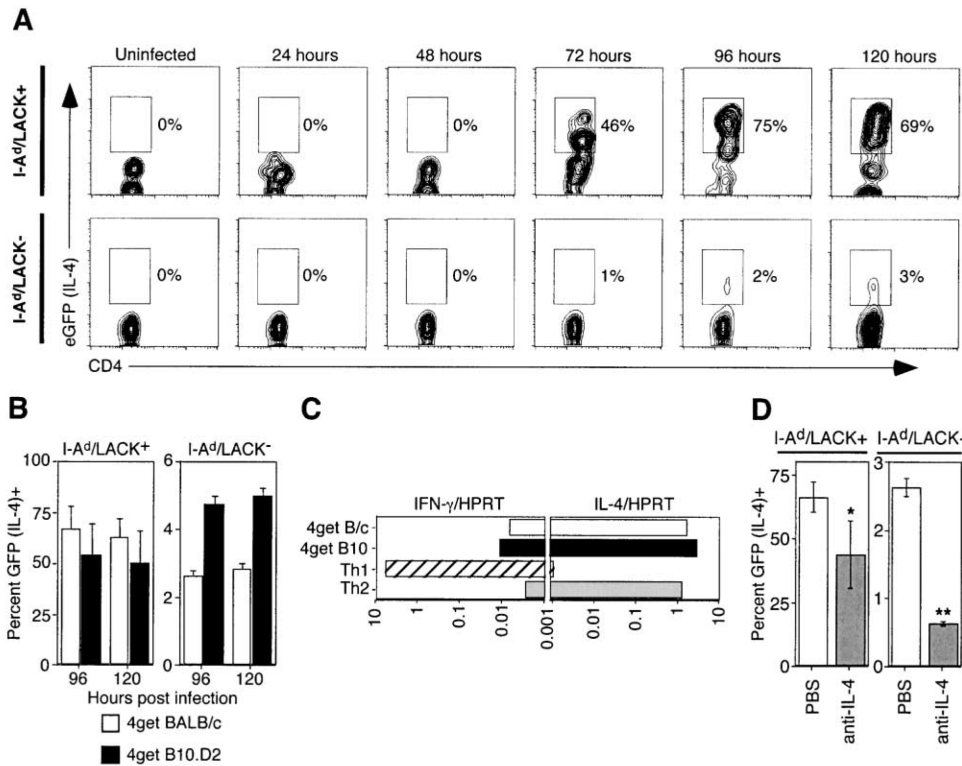

Figure 5. Emergence of IL-4-Expressing Cells In Vivo

(A) Representative FACS plots of eGFP expression in LACK-tetramer positive (top panels) and LACK-tetramer negative (bottom panels) CD4 T cells from 4get BALB/c mice before and at 24 hr intervals after infection. Data are representative of 4–12 mice at each time point.

(B) The percentage of $eGFP^{+}$, LACK-tetramer positive (left panel), and LACK-tetramer negative (right panel) CD4 T cells in infected fifth backcross 4get BALB/c (white bars) and 4get B10.D2 (black bars) at 96 and 120 hr after infection. Data represent means and standard deviations for four mice per strain at each time point.

(C) 5′ nuclease fluorogenic RT-PCR for IL-4 and IFN-γ was performed on purified $CD4^{+}$ $eGFP^{+}$ T cells from 4get BALB/c and 4get B10.D2 mice infected 120 hr earlier with *L. major*. Data are represented as the ratio of cytokine message to HPRT message and depict data from one of two comparable experiments. Established Th1 and Th2 cell lines generated as described (Grogan et al., 2001) were compared for reference.

(D) Cohorts of 4get BALB/c mice were treated with neutralizing IL-4 antibody or vehicle control the day of infection. After 4 days, draining lymph node cells were harvested and analyzed for eGFP expression among LACK tetramer-positive and tetramer-negative populations. Means and standard deviations are shown for four mice per group and are representative of three experiments. P values were calculated using the Student's t test. *, $p = 0.11$; **, $p = 0.0005$.

scriptase-polymerase chain reaction (RT-PCR) using fluorogenic probes. Quantitative RT-PCR revealed that IL-4 and IFN-γ transcripts were comparable, respectively, in the $eGFP^{+}$ T cells from 4get BALB/c and 4get B10.D2 mice, and similar to the transcript abundance from Th2 cells generated in vitro (Figure 5C). Thus, $eGFP^{+}$ $CD4^{+}$ T cells from healer mice isolated early after infection contain cytokine transcripts consistent with their identification as Th2 cells.

We next examined the emergence of eGFP expression among LACK tetramer-positive and -negative cells in susceptible BALB/c 4get mice treated at the time of infection with neutralizing IL-4 antibody. A cohort of mice was followed for 8 weeks and confirmed that the anti-IL-4 intervention conferred a healer phenotype (data not shown). When examined at 96 hr after infection, IL-4 expression from tetramer-positive cells was slightly, but insignificantly, attenuated in mice that received anti-IL-4 compared to control mice (Figure 5D). In contrast, IL-4 expression from tetramer-negative cells was reduced 4-fold, consistent with a role for LACK-specific cells in nucleating a broader Th2 response.

Leishmania Activate Antigen-Dependent IL-4 Expression from Naive T Cells

Prior studies have suggested that the early IL-4 made by CD4 T cells after *Leishmania* infection derives from crossreactive memory cells that might more efficiently activate cytokine gene expression (Julia et al., 2000). Three observations in the 4get mice suggested that *Leishmania* efficiently activated IL-4 expression from naive T cells. First, the few (∼0.5%) spontaneously $eGFP^{+}$ T cells in resting 4get mice were uniformly $CD62L^{lo}$, consistent with their identification as effector/memory cells (Figure 6A). Second, eGFP expression early after infection occurred exclusively among cells that were $CD62L^{hi}$, consistent with their naive phenotype, as demonstrated definitively for the LACK tetramer-positive subpopulation (Figures 6A and 3A). Finally, none of the $eGFP^{+}$ cells that appeared in the lymph

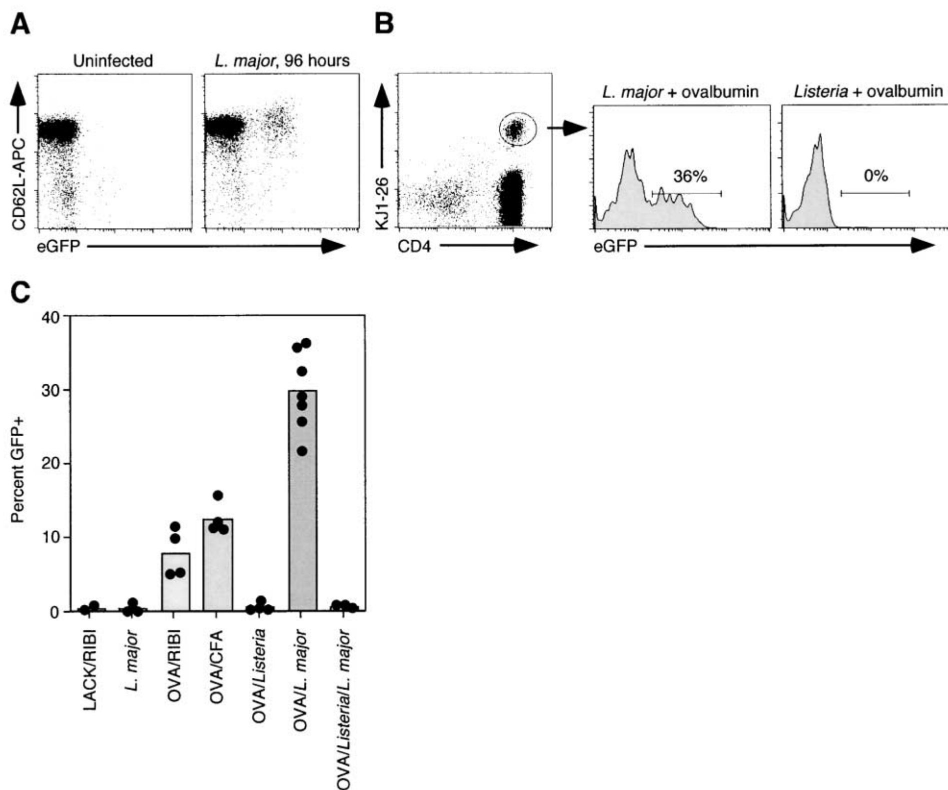

Figure 6. *Leishmania* Activate IL-4 Expression from Naive T Cells

(A) CD62L and eGFP expression were examined on CD4 T cells from uninfected (left panel) and 96 hr infected (right panel) 4get BALB/c mice. Ten thousand cells per plot are shown and are representative of eight mice per group. Analysis of 4get B10.D2 mice gave identical results.

(B) $CD4^+$, KJ1-.26^+ donor T cells were enumerated from the draining lymph nodes of wild-type BALB/c mice that received 10^6 highly purified, naive DO11.10 4get T cells 24 hr before inoculation. The FACS plot on the left is of [CD8/B220]− lymphocytes and indicates the gate used for analysis of eGFP expression (right panels).

(C) eGFP (IL-4) expression by KJ1-26^+ DO11.10 T cells was analyzed 96 hr after inoculation with the indicated antigens. The bars are means, and the dots represent individual mice.

nodes over the initial 120 hr stained with α-galactosylceramide/CD1 tetramers which identify canonical NK T cells (data not shown) (Matsuda et al., 2000). NK T cells can rapidly secrete IL-4 after activation, although these cells are unlikely to contribute to the early IL-4 production after *L. major* infection (Brown et al., 1996).

Whereas the specificity of the tetramer-negative, eGFP-expressing cells is not known, their phenotype is consistent with a de novo response to parasite antigens distinct from LACK. To assess directly whether *L. major* infection could elicit IL-4 expression from naive CD4 T cells of known but unrelated specificity, naive, ovalbumin-specific D011.10 × 4get T cells were transferred into BALB/c recipients before challenge with ovalbumin in adjuvant or with the pathogens *L. major* or *Listeria monocytogenes*. *Listeria* is a potent inducer of IL-12 from antigen-presenting cells (Hsieh et al., 1993). Draining lymph node cells were recovered after 96 hr and donor T cells were assessed for specificity using the clonotypic mAb KJ1-26 and for IL-4 gene activation by eGFP fluorescence (Figures 6B and 6C). *L. major* potently induced IL-4 expression from OVA-specific T cells when coinjected with ovalbumin but not when injected in the absence of ovalbumin. Whereas CFA or RIBI adjuvants elicited intermediate levels of IL-4 expression when injected with ovalbumin, *Listeria* coinjection resulted in no IL-4 expression from OVA-specific 4get T cells. Interestingly, coinoculation of *Listeria* with *L. major* and ovalbumin completely abrogated the IL-4 response elicited by *L. major* alone (Figure 6C). Thus, activation of the IL-4 gene is not the result of bystander stimulation of 4get T cells in lymph nodes but is powerfully activated by *Leishmania* parasites by an antigen-dependent process.

Discussion

L. major infection results in a highly stereotyped host response characterized by a protective Th1 response in resistant inbred strains and an aberrant Th2 response in susceptible strains. The use of MHC congenic mice and analysis of a dominant peptide-specific response allowed careful assessment of host-specific determinants that might ultimately underpin the dichotomous T helper subset differentiation that determines the outcome of disease. A number of novel findings were uncovered. First, we used a dual fluorescent labeling approach to identify a biologically relevant naive CD4 T cell precursor population for the LACK epitope that comprises approximately 15 cells in the popliteal lymph node

of resting mice. Second, we identified the kinetics of early expansion, peaking at approximately 125-fold by 4 days, and demonstrated that cell expansion is coordinately regulated with IL-4 gene expression in the majority of peptide-specific responding cells. Third, despite the clear pathogenic nature of these cells in susceptible mice as shown here (Figure 2) and elsewhere (Julia et al., 1996; Launois et al., 1997; Pingel et al., 1999), we could discern no differences in the precursor frequency, expansion, or IL-4 gene expression in comparing susceptible and resistant mice. Last, *Leishmania* parasites efficiently activate IL-4 gene expression from naive T cells even when administered with exogenous antigen. Taken together, these findings suggest that the ultimate differentiation into Th1 and Th2 cells that determines the outcome of infection must occur independently from the initial activation of naive T cells in draining lymph nodes.

Our flow cytometry approach allowed us to characterize the initial expansion of LACK-specific T cells in the draining lymph nodes of normal mice. During the first 48 hr after infection, LACK-specific T cells expanded minimally, and none activated IL-4 expression (Figures 3B and 5A). Seeding recipient mice with a $>$1000-fold excess of antigen-specific cells did not enable significant cell division during the first 2 days (Figure 3C). These data suggest that transit of antigen-presenting cells to the draining lymph node and maturation of MHC-peptide complexes on the surface of APC, rather than T cell precursor frequency, are rate limiting during the initiation of the response to *L. major* in both susceptible and resistant mice. Additionally, *Leishmania* does not appear to interfere with MHC class II/LACK peptide presentation in vivo (Prina et al., 1996).

Using sensitive IL-4 reporter mice, we found that $>$70% of LACK-specific T cells in draining lymph nodes activated IL-4 expression by 96 hr in both susceptible and resistant mice. These findings are in agreement with earlier studies demonstrating that a broad range of cytokines, including IL-4, are induced at early time points after *L. major* infection, regardless of genetic background (Morris et al., 1992; Reiner et al., 1994; Sommer et al., 1998), although we add quantitative insights to these earlier analyses. The finding that fully 5% of lymph node T cells activate IL-4 in resistant mice suggests alternative roles for this cytokine in the subsequent response. IL-4 promotes the maturation and survival of myeloid dendritic cells (Rissoan et al., 1999) and has been implicated in paradoxically enhancing production of the prototypic type 1-enhancing cytokine, IL-12, from dendritic cells (Hochrein et al., 2000). Indeed, pretreatment of BALB/c mice with a high dose of recombinant IL-4 protein directed development of stable protective immunity, presumably through effects on dendritic cells (Biedermann et al., 2001). IL-4-deficient mice have impaired type 1 immune and cytotoxic CD8 T cell responses in some experimental systems (Schuler et al., 1999, 2001), including *L. major* infection (Noben-Trauth et al., 1996). Such data suggest that early IL-4 production may serve an important role in enhancing elements of antigen presentation required not only for type 2 but also for type 1 responses. Importantly, exogenous IL-4 at the time of infection does not reverse the inherent resistance of healer mice (Sadick et al., 1991).

We focused our studies on LACK-reactive cells because of prior evidence that IL-4 production by these cells is required for the BALB/c susceptible phenotype (Julia et al., 1996; Launois et al., 1997; Pingel et al., 1999). Despite their pathogenic role, these cells comprise less than 5% of the total CD4 T cells that express IL-4 early after infection. How then to explain their central role? As shown here, treatment with anti-IL-4 had a greater impact on IL-4 expression from the LACK tetramer-negative population than the LACK tetramer-positive population, which was only modestly affected (Figure 5D). These findings are consistent with the interpretation that naive LACK-reactive cells may rely less on exogenous IL-4 for their initial activation of Th2 cytokine expression. In turn, these cells might serve to prime the spread of IL-4 production to CD4 clones of other specificities, as demonstrated elsewhere (Launois et al., 1997). It is also possible that differences may occur at the level of IL-4 mRNA translation into protein. Since the 4get mice were generated to mark transcriptional activation of the IL-4 gene, it is conceivable that translational differences occur among LACK-specific and nonspecific cells or when comparing resistant and susceptible strains. Further work will be required to address these possibilities.

These studies leave open the question of when and from which precursors do protective IFN-γ-producing Th1 cells emerge in resistant mice? Quantitative RT-PCR analysis of transcripts from eGFP$^+$ cells from resistant and susceptible mice revealed no significant IFN-γ mRNA, consistent with the activation of bona fide Th2 cells in both inbred strains (Figure 5C). Moreover, in preliminary experiments, the transfer of eGFP$^+$ cells from 120 hour-infected B10.D2 mice into TCR $C\alpha^{-/-}$ B10.D2 recipients did not confer protection upon subsequent infection (data not shown). These data suggest that IFN-γ-expressing effector cells either diverge from a common precursor before detectable IL-4 expression in resistant mice or emerge independently from the IL-4-expressing cells. Our findings differ from prior studies using single-chain LACK-specific TCR transgenic mice demonstrating that IL-4 expression correlated inversely with TCR affinity (Malherbe et al., 2000). Although we do not quantitate TCR affinity/avidity here, we find no differences in activation of the IL-4 gene when comparing either LACK-tetramer-specific cells or total CD4 T cells between resistant and susceptible mice. Despite these unexpected findings, we emphasize that the 4get backcrossed mice respond faithfully to *L. major* infection (Figure 4) and we identify rigorously a biologically important subset of T cells as defined by their capacity to mediate disease progression in BALB/c mice (Figure 2).

Together with data from the infections, our experiments with DO11.10 x 4get T cells demonstrate that *Leishmania* parasites powerfully activate IL-4 expression from naive T cells. It is thus tempting to speculate that elements of *L. major* direct a Th2-inducing pathway in antigen-presenting cells. Coinoculation of *L. major* with *Listeria* completely abrogated IL-4 expression from naive T cells in vivo, presumably through a Toll-like receptor (TLR)-dependent response to bacterial products. We are investigating whether the IL-4 response induced by *L. major* reflects a default response in the absence of TLR-dependent activation or is an active process that is suppressed by Toll-like receptor recognition (Schnare

et al., 2001). Despite the capacity of *L. major* to activate indiscriminately an IL-4 response from naive T cells, resistant mice are able to constrain parasite growth by developing a polarized Th1 response while extinguishing the early Th2 response we document. Whether this switch is kinetically displaced or played out in peripheral tissues will be important issues that could be addressed with similarly designed IFN-γ reporter mice.

Experimental Procedures

Mice

4get mice containing a mutated knockin gene consisting of IL-4 linked through a viral IRES element to enhanced green fluorescent protein (eGFP) were generated as described (Mohrs et al., 2001). Mice were backcrossed five generations to either BALB/c or B10.D2 mice and mated to achieve homozygosity. BALB/c ovalbumin-peptide-specific DO11.10 T cell receptor transgenic mice (Murphy et al., 1990) and LACK-peptide-specific ABLE T cell receptor transgenic mice (Reiner et al., 1998) were crossed to tenth backcross BALB/c x TCR-Cα-deficient mice prior to use. DO11.10 mice were crossed to N8 4get BALB/c mice. BALB/c and B10.D2 mice (Jackson Laboratories, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the animal care facility at the University of California, San Francisco according to institutional guidelines.

Parasites and Infection

L. major (strain WHOM/IR/$-$/173) was prepared and injected as 10^6 metacyclic promastigotes in Hank's balanced salt solution into the hind footpads of mice as described (Fowell et al., 1999). Designated mice were treated the day of infection with 3 mg neutralizing IL-4 antibody, 11B11, or isotype control. The course of disease was followed by measuring footpad thickness using a metric caliper. At the end of the experiments, dispersed popliteal lymph nodes, footpads, and spleens were serially diluted into culture media and assayed for parasite growth after 2 weeks as described (Fowell et al., 1999).

I-A^d/LACK Tetramers

Expression constructs for Aα^d and Aβ^d were as described (Scott et al., 1998). The Aβ^d cDNA was modified to contain coding sequence for the LACK peptide—ICFSPSLEHPIVVSGSWD—followed by an SGSGS linker preceding the N terminus, and for biotinylation sequence number 85 at the C terminus (Schatz, 1993). For protein expression, stable lines of transfected *Drosophila* SC2 cells were expanded to 13 liters in 850 cm^2 roller bottles in Insect Xpress serum-free media (Biowhittaker, Walkersville, MD) by splitting at a 1:1 ratio every 2 days. After induction of protein expression by 0.7 mM copper sulfate for 3 days, cells were cleared by centrifugation, and the supernatants were concentrated to 400 ml by tangential flow. Soluble I-A^d-peptide molecules were purified by affinity chromatography after overnight incubation at 4°C with Ni-NTA agarose beads and further purified by anion exchange chromatography on a Resource Q column (Pharmacia, Peapack, NJ). Elution fractions were checked by SDS-PAGE for purity and stoichiometric expression of each chain. Following buffer exchange to 10 mM Tris/50 mM arginine, soluble I-A^d-peptide molecules were biotinylated at 1.8 mg/ml for 24 hr at 27°C according to the manufacturer's instructions (Avidity, Denver, CO). The extent of biotinylation was determined following extensive buffer exchange to PBS using the HABA colorimetric reagent (Pierce, Rockford, IL) and depletion with streptavidin beads followed by SDS-PAGE. Biotinylation was 70%–100% efficient for each batch of protein. Tetrameric complexes were generated by incubation of biotinylated monomers with streptavidin-PE (Biosource, Camarillo, CA) or streptavidin-APC (Molecular Probes, Eugene, OR) at a molar ratio of 6:1.

To assess T cell activation, biotinylated I-A^d-peptide molecules and purified anti-CD28 (37N51.1, 5 μg/ml) in PBS were incubated overnight at 4°C in 96-well U-bottom plates. After washing, 2×10^5 DO11.10 x TCR-C$\alpha^{-/-}$ or ABLE x TCR-C$\alpha^{-/-}$ T cells were added to each well in complete RPMI media (10% FCS, penicillin/streptomycin, L-glutamine, and 2-mercaptoethanol) with 50 U/ml recombinant human IL-2. ^{3}H-thymidine was added after 48 hr of incubation at 37°C, and cells were harvested 18 hr later for analysis on a scintillation counter.

Cells and Procedures

For immunizations, 140 μg recombinant LACK protein, prepared as described (Mougneau et al., 1995), or ovalbumin (Sigma Chemical Co., St. Louis, MO), was injected subcutaneously at the base of the tail in MPL+TDM RIBI adjuvant (Corixa, Seattle, WA). Mice were rested for 8 weeks before reimmunization with the same dose of the designated protein in adjuvant. Following immunization or infection, mice were killed at the indicated time points and single-cell suspensions were prepared from draining lymph nodes by dispersal through a 70 μm mesh. Live cell counts were estimated by trypan blue exclusion, and cells were washed and resuspended at 4×10^8/ml in 5% FCS/PBS. Tetramers were prepared as described above, and optimal, nonsaturating concentrations were determined for each batch by staining of ABLE x TCR-C$\alpha^{-/-}$ transgenic T cells specific for I-A^d/LACK. PE- and APC-labeled tetramers were mixed at [2×] and added to cells at a 1:1 ratio for a final cell concentration of 2×10^8/ml. Cells were stained at 25°C for 45 min before transfer to ice and addition of the following antibodies: Alexa$_{350}$-GK1.5 (anti-CD4; Molecular Probes), FITC-anti-CD25 (Pharmingen, San Diego, CA), APC-Cy7-anti-CD8 and APC-Cy7-anti-B220 (Caltag, Burlingame, CA). Cells were incubated with antibodies for 45 min on ice, washed, and resuspended at 4×10^7/ml in 5% FCS/PBS. Data acquisition and sorting were performed using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO) using an ultraviolet laser for excitation of Alexa$_{350}$, a 488 nm laser for excitation of FITC, PE, and Cy5-PE, and a 647 nm krypton-argon laser for APC and APC-Cy7 excitation. Elliptical beam shaping optics were used to minimize physical overlap in laser interrogation of the stream, and crossbeam compensation was performed electronically. For each sample, a 500 thousand event file was collected to set gates. A second file was then collected in which 100% of dual tetramer-positive events were saved with 1.0% of events that failed the gating criteria to allow accurate enumeration of tetramer-positive cells in a file of manageable size. Routinely, three to ten million live events were analyzed per sample. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

For analysis of ovalbumin specific responses, DO11.10 × 4get CD4$^+$, GFP-negative, T cells were sorted, and an aliquot was stained with KJ1-26 to determine the frequency of clonotypic T cells in the sorted population. Cell volumes were adjusted to contain 10^6 KJ1-26$^+$ cells in 250 μl PBS and were injected intravenously into BALB/c recipients. After 1 day, mice were immunized in the hind footpad with 100 μg ovalbumin protein mixed with RIBI or CFA adjuvants, 10^4 *Listeria monocytogenes*, or 10^6 *L. major* in a final volume of 50 μl. Popliteal lymph nodes were harvested after 96 hr and prepared for FACS analysis as described above.

Staining with α-galactosylceramide/CD1 tetramers was performed as described (Matsuda et al., 2000).

Quantitative Fluorogenic RT-PCR for Cytokines

Cells of the desired phenotype were sorted to >99% purity, and RNA was prepared using RNAzol according to the manufacturer's instructions (Biotecx, Houston, TX). RT reactions were performed using Sensiscript RT (Qiagen, Valencia, CA). Primers and probes for quantitative PCR were as described (Grogan et al., 2001). Quantitative PCR was performed on an ABI-Prism 7700 DNA sequence analyzer, and signals for each cytokine were normalized to HPRT levels.

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