Characterization of an I-E-Restricted, gp63-Specific, CD4-T-Cell Clone from *Leishmania major*-Resistant C3H Mice That Secretes Type 2 Cytokines and Exacerbates Infection with *L. major*

Cynthia M. Theodos,¹ Robin V. Morris,² Jeanette V. Bishop,² Jeremy D. Jones,²
W. Robert McMaster,³ and Richard G. Titus^{2*}

Biogen, Inc., Cambridge, Massachusetts 02142¹; Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523²; and Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada³

Received 9 February 2004/Returned for modification 21 March 2004/Accepted 19 April 2004

A T-cell clone (designated KLmB-3) was derived from resistant C3H mice 2 weeks after infection with *Leishmania major*. KLmB-3 was a CD4-T-cell clone that utilized the V β 8.1 T-cell receptor. When adoptively transferred to naive C3H mice, KLmB-3 unexpectedly exacerbated infection with *L. major* (it increased the cutaneous lesion size and the parasite burden within the lesion). The ability of KLmB-3 to exacerbate disease correlated with its ability to produce the type 2-associated cytokines interleukin-4 (IL-4), IL-5, IL-10, and transforming growth factor beta. Interestingly, KLmB-3 was specific for an epitope in the amino-terminal end of the *L. major* surface gp63 zinc metalloproteinase (leishmanolysin) that has been shown to be capable of inducing a protective immune response. Moreover, KLmB-3 was activated when this epitope was presented in the context of H-2 I-E rather than H-2 I-A.

Leishmania major is a protozoan parasite that causes cutaneous leishmaniasis. Our knowledge of the immune response generated against this parasite has been significantly advanced through the use of suitable murine models of infection. Infection of highly susceptible BALB/c mice, which succumb to *L. major*, represent one end of the spectrum. In contrast, the other end of the spectrum is represented by the majority of mouse strains, including C57BL/6 and C3H, which are relatively resistant and heal their cutaneous lesions (reviewed in references 8, 19, and 34).

Of the T-cell lineages, CD4 T cells play an important role in determining the course of cutaneous leishmaniasis. Upon adoptive transfer into BALB/c mice, CD4 parasite-specific T cells can have either a beneficial (12, 28, 38, 39) or a detrimental (12, 39, 48, 49) effect on the outcome of infection. The observation that a single subset of parasite-specific T cells can have varied effects on the outcome of infection led to the current concept that *L. major*-specific CD4 Th1 T cells which secrete gamma interferon (IFN- γ) are protective in cutaneous leishmaniasis, while parasite-specific Th2 T cells which secrete interleukin-4 (IL-4) are detrimental in the disease (8, 19, 26, 34).

In contrast to adoptive transfer studies performed with BALB/c mice, relatively little work has been done with resistant mice, especially with highly resistant mice such as C3H (2). This is surprising since these mice more accurately mimic *L. major* infections that occur in humans. Thus, the present study was designed to characterize T-cell lines or clones derived from *L. major*-infected resistant C3H mice. The results presented here describe a CD4-T-cell clone (*L. major*-specific

CD4 T-cell clone B-3 from C3H [k haplotype] mice, designated KLmB-3) that unexpectedly has the Th2 phenotype and thus exacerbates infection with *L. major*. Interestingly, KLmB-3 is restricted to H-2 I-E, not H-2 I-A. In contrast to Th2-T-cell clones obtained from susceptible BALB/c mice, which are frequently specific for LACK (*Leishmania* homologue of receptors for activated C kinase [20, 27, 32]), KLmB-3 is specific for an epitope in the N terminus of the gp63 zinc metalloproteinase (leishmanolysin; promastigote surface protease) of *L. major*. This observation is significant since previous studies have shown that immunization with gp63 can lead to a protective Th1 immune response in resistant CBA mice (5), and thus gp63 is currently regarded as a vaccine candidate for leishmaniasis.

MATERIALS AND METHODS

Mice. In all cases young (6- to 10-week-old) adult female mice were used. C3H/HeN mice were purchased from the National Cancer Institute (Frederick, Md.). B10.BR and B10.A (4R) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). B10.AQR, B10.RFB3, and B10.Ea+ mice were obtained from the Mayo Clinic (Rochester, Minn.).

Parasites and antigens. Stationary-phase promastigotes of the *L. major* LV39 isolate (MRHO/Sv/59/P) were used and maintained as previously described (48). The metacyclic form of *L. major* promastigotes was produced as described elsewhere (36). In some experiments *L. major* strain A2 and a form of A2 in which all seven copies of the gp63 gene had been knocked out [*L. major* A2/gp63(1-7)KO (16)] were used. These parasites were cultured like strain LV39.

Recombinant gp63 (rgp63) and deletion polypeptides of the molecule were produced as described previously (41). Ovalbumin (OVA) (grade V; catalog no. A-5503) was purchased from Sigma, St. Louis, Mo.

Infection of mice and derivation and maintenance of T-cell lines and clones. C3H mice were infected subcutaneously with 5×10^6 *L. major* cells in one hind footpad, and 1 to 2 weeks later (a time when cutaneous lesions were progressing) the draining popliteal and inguinal lymph nodes were removed. A single-cell suspension was prepared, and the cells (5×10^6 cells/ml) were stimulated with *L. major* promastigotes (10^6 promastigotes/ml) in vitro to generate T-cell lines and subsequently T-cell clones (48). In certain cases, T-cell lines were derived by stimulating the cells with *L. major* rgp63 (10 μ g/ml) rather than with *L. major* promastigotes.

At 2 days of culture, supernatants were removed from cells stimulated with

* Corresponding author. Mailing address: Department of Microbiology, Immunology and Pathology, CVMB, Colorado State University, 1619 Campus Delivery, Fort Collins, CO 80523-1619. Phone: (970) 491-1607. Fax: (970) 491-0603. E-mail: richard.titus@colostate.edu.

rgp63 and from some of the cells stimulated with *L. major* promastigotes to determine their cytokine contents (for measurement methods see below). At 5 days of culture, blast cells in the remaining wells stimulated with *L. major* promastigotes were recovered by using Percoll gradients (48). These blast cells were rested for 7 to 10 days with irradiated syngeneic spleen cells as previously described (4). After resting, viable blast cells were recovered on Ficoll gradients (40). Clones were obtained from the rested T-cell blasts by the limiting dilution approach (28) in 96-well round-bottom microtiter plates (catalog no. 3799; Costar, Corning, N.Y.). The culture conditions for cloning were as follows: 0.3 rested T-cell blast, 10^6 irradiated syngeneic spleen cells, and 10^5 *L. major* promastigotes per well in complete Dulbecco's modified Eagle's medium (48) containing 10% fetal bovine serum (HyClone, Logan, Utah) and 5% P388D1 supernatant (30).

Wells that contained replicating T-cell blasts were identified by light microscopy and were expanded for further analysis. Complete characterization of one clone (KLmB-3) is described here. KLmB-3 was maintained through alternating cycles of stimulation in the presence of 10% (final concentration) concanavalin A supernatant (9) and resting. For all experiments, cells were collected from rested cultures and used as described below.

Fluorescence-activated cell sorter analyses. The surface phenotype (CD4 versus CD8, V β usage) of T cells was determined by using previously described techniques (40).

Adoptive transfer of KLmB-3 and its effect on infection with *L. major*. To determine the effect that KLmB-3 had on infection with *L. major* in mice, 2.5×10^5 KLmB-3 cells were coinjected with 2.5×10^5 *L. major* metacyclic promastigotes subcutaneously into one hind footpad of syngeneic C3H mice. Lesion development was monitored as previously described (46). Control mice were injected with *L. major* metacyclics alone.

Parasite burdens in footpad lesions of *L. major* were determined by using a limiting dilution assay as described previously (22).

KLmB-3 proliferation and cytokine production and measurement. As a source of antigen-presenting cells (APCs), 10^6 irradiated (1,500 rads) syngeneic C3H spleen cells (or congenic cells [see Fig. 5]) were seeded into the wells of 96-well flat-bottom microtiter plates (Costar 3997), and antigens (as described below) were added. The plates were incubated overnight at 37°C, and then KLmB-3 cells (40,000 cells/well; final volume, 100 μ l) were added to the wells. Twenty-four, 48, or 72 h later, the wells were pulsed for 18 h with 1 μ Ci of [3 H]thymidine (Amersham, Arlington Heights, Ill.) to determine the degree of cell proliferation (48).

To determine the cytokines elicited by KLmB-3, 1×10^6 KLmB-3 cells, 7×10^6 irradiated syngeneic C3H spleen cells, and 10^6 *L. major* promastigotes were plated into the wells (1 ml/well) of 24-well plates (Costar 3524). Supernatants were collected 12, 24, 48, and 72 h later, and their cytokine contents were assessed by using previously described techniques and commercial enzyme-linked immunosorbent assays (ELISAs) for cytokines (43).

Determining the isotypes of anti-*L. major* antibodies induced by KLmB-3. On the days of infection indicated below, sera were collected from mice infected with *L. major* alone or with *L. major* plus KLmB-3. The isotypes of the anti-*L. major* antibodies present in the sera were determined by an ELISA. Briefly, ELISA plates were coated with a frozen and thawed lysate of *L. major* promastigotes (10 μ g of *L. major* protein/ml [48]) by using standard techniques (40). The test sera were added to the plates, and the plates were then developed by using a monoclonal antibody isotyping kit (to quantify immunoglobulin G1 [IgG1], IgG2b, and IgG2a; catalog no. 37501; Pierce, Rockford, Ill.) and the manufacturer's directions. To detect IgE, the plates were incubated with biotin-labeled anti-IgE (catalog no. RMGE15; Caltag, Burlingame, Calif.), followed by avidin peroxidase (catalog no. A-3151; Sigma) and a TMB peroxidase development system (catalog no. 50-76-00; Kirkegaard & Perry, Gaithersburg, Md.).

Treatment of mice with anti-IFN- γ . Anti-IFN- γ antibodies were purified from ascites fluid as described elsewhere (7). Normal rat Ig was prepared from normal rat serum (Accurate, Westbury, N.Y.) by using the techniques employed to purify anti-IFN- γ antibodies. Mice were injected intraperitoneally with 2 mg of either normal rat Ig or anti-IFN- γ 3 h before infection with *L. major*.

Statistical analyses. Data for lesion progression were analyzed by using analysis of variance for repeated measure and for parasite burdens by using unpaired *t* tests. All experiments were repeated at least twice; most of the results presented below are representative of the results of three or four independent experiments.

RESULTS

***L. major*-infected resistant C3H mice produce less type 1 cytokines and more type 2 cytokines in response to gp63 in the first 2 weeks of infection.** Initially, we infected resistant C3H mice with *L. major* promastigotes in one hind footpad and

TABLE 1. gp63 induces less type 1 cytokine and more type 2 cytokine

Expt ^a	Stimulus	Response (pg/ml)		
		IL-4	IL-5	IFN- γ
1	<i>L. major</i> (10^6 promastigotes/ml) rgp63 (10 μ g/ml)	0 0	0 127	2,180 0
2	<i>L. major</i> (10^6 promastigotes/ml) rgp63 (10 μ g/ml)	9 213	6 87	2,560 264
3	<i>L. major</i> gp63(1-7)KO (10^6 promastigotes/ml) <i>L. major</i> A2 (10^6 promastigotes/ml)	1.8 5	7 50	18,340 1,908

^a In experiments 1 and 2, C3H mice were infected with 5×10^6 *L. major* promastigotes, and the draining lymph node cells were collected and stimulated with different amounts of *L. major* promastigotes or rgp63. Two days later the supernatants of the cultures were tested to determine their contents of IL-4, IL-5, and IFN- γ (see Materials and Methods for details of the techniques). The data were obtained with mice infected for 1 week; similar results were obtained with mice infected for 2 weeks (data not shown). Similar results were also obtained with lower and higher doses of rgp63 (data not shown). In experiment 3, cells from infected mice were restimulated with gp63-expressing (strain A2) or gp63-deficient (knockout) parasites. The cytokine responses were measured as described above for experiments 1 and 2.

removed the draining popliteal and inguinal lymph node cells to restimulate the cells in vitro. As reported by others (8, 19, 34), the cells produced little IL-4 or IL-5 when they were restimulated with *L. major* promastigotes in vitro (Table 1). However, curiously, when the cells were restimulated with *L. major* gp63, a quite different response was observed (Table 1). gp63, or leishmanolysin (25), is the major glycoprotein expressed on the surface of *L. major* promastigotes. Lymph node cells stimulated with gp63 produced 15- to 24-fold more IL-4 or IL-5 than cells stimulated with *L. major* promastigotes produced. The production of IL-4 by the cells was not as consistent as the production of IL-5, possibly due to consumption of the IL-4. gp63 also induced approximately 10-fold less IFN- γ (Table 1).

To corroborate and extend these results, we restimulated cells from *L. major*-infected C3H mice with either of a pair of *L. major* parasites, one that expressed gp63 and one that was genetically engineered so that all seven copies of the gp63 gene were deleted and thus did not produce gp63. As Table 1 shows, wild-type (gp63-expressing) *L. major* (*L. major* A2) induced more type 2 and less type 1 cytokines than the mutant parasite lacking gp63 [*L. major* gp63(1-7)KO] induced. Thus, the two experimental approaches (stimulating with rgp63 and stimulating with parasites genetically engineered to either express or not express gp63) yielded the same results: gp63 favored the development of a type 2 immune response.

Derivation of KLmB-3 and analysis of its surface phenotype. To characterize this phenomenon further, we infected C3H mice with 5×10^6 *L. major* promastigotes and 2 weeks later derived long-term T-cell lines from which we subsequently derived T-cell clones (for details of the techniques used see Materials and Methods). Clone KLmB-3 was selected for further analysis. Using flow cytometry, we determined that KLmB-3 was a CD4-T-cell clone that utilized the V β 8.1 T-cell receptor (data not shown).

Effect that adoptively transferring KLmB-3 has on a subsequent infection with *L. major*. To determine how KLmB-3 influenced an infection with *L. major* metacyclic promasti-

FIG. 1. Clone KLMb-3 exacerbates infection with *L. major* in C3H mice. Groups ($n = 5$) of C3H mice received a subcutaneous injection of either 2.5×10^5 *L. major* metacyclic promastigotes (control) or a mixture of parasites and 2.5×10^5 KLMb-3 cells in one hind footpad. The data show the development of lesions (increase in footpad thickness) over time. There was a significant ($P < 0.001$) difference in lesion size between groups. This effect of KLMb-3 on infection with *L. major* was not due to the nonspecific effect of simply adoptively transferring cells to recipient mice since adoptive transfer of a similarly restricted T-cell clone with a different specificity for molecules associated with the surface lipophosphoglycan of *L. major* had the opposite effect on infection (namely, it protected against challenge with the parasite) (47).

gotes, the parasite (2.5×10^5 cells) and KLMb-3 (2.5×10^5 cells) were coinjected into the hind footpads of syngeneic C3H mice. Controls were injected with the parasite alone. Lesion development (including the increase in footpad thickness and the parasite burden in the developing lesions) was then monitored over time. As Fig. 1 shows, lesions induced with promastigotes plus KLMb-3 were 2 to 10 times the size of lesions on control mice ($P < 0.001$). It is also important to mention that no delayed-type hypersensitivity response (i.e., footpad swelling) was observed at the site of injection of KLMb-3 and *L. major* promastigotes at either 24, 48, or 72 h postinjection (Fig. 1).

To verify that the increased lesion size observed in mice that received both KLMb-3 and parasites was due to an increase in the level of infection, parasite burdens were measured by using a limiting dilution assay (22). Our results indicated that coinjection of KLMb-3 and *L. major* resulted in a statistically significant increase in the number of parasites (2.6- to 940-fold more parasites) in the lesions of these mice compared to the number in control mice injected with the parasite alone (Table

TABLE 2. Parasite burden in lesions of C3H mice injected with KLMb-3

Day post-infection ^a	Parasites/lesion (10^6) ^b		Fold increase	<i>P</i>
	Control mice	Mice injected with KLMb-3		
8	8.63 ± 0.43	22.23 ± 2.13	2.6	<0.05
20	0.73 ± 0.10	46.31 ± 2.86	63.4	<0.01
45	0.01 ± 0.004	9.40 ± 0.73	940	<0.001

^a Parasite burdens were determined on days 8, 20, and 45 after infection for groups of three mice.

^b The data are the mean $\pm$ standard deviation number of parasites per footpad for each group.

TABLE 3. Cytokines induced in vitro after activation of KLMb-3

Time (h)	Concn (pg/ml) of ^a :					
	IL-4	IL-5	IL-10	TGF- β	IFN- γ	IL-12
12	ND ^b	$9,580 \pm 190$	836 ± 138	152 ± 62	ND	ND
24	97 ± 13	$16,830 \pm 210$	556 ± 22	5 ± 3	ND	ND
48	66 ± 7	$15,400 \pm 2,580$	560 ± 159	24 ± 6	ND	ND
72	81 ± 6	$13,200 \pm 30$	126 ± 18	ND	ND	ND

^a The data are means $\pm$ standard deviations.

^b ND, not detected.

2) (the *P* values ranged from <0.05 to <0.001). Thus, KLMb-3 significantly exacerbated *L. major* infection both with respect to lesion size and with respect to parasite burden.

Functional phenotype of KLMb-3. The fact that KLMb-3 exacerbated infection with *L. major* in C3H mice (Fig. 1 and Table 2) suggested that the cells might be Th2 cells. To test this hypothesis, we analyzed the cytokines that KLMb-3 induced after stimulation with *L. major*. As Table 3 shows, activation of KLMb-3 in vitro resulted in the secretion of substantial amounts of the type 2 cytokines IL-4, IL-5, IL-10, and transforming growth factor beta (TGF- β) into the culture supernatants. In contrast, we could not detect secretion of the type 1-associated cytokines IFN- γ and IL-12.

To confirm these cytokine results and to obtain evidence that KLMb-3 was behaving like a Th2 cell in vivo, we next determined the isotypes of *L. major*-specific antibodies produced in mice coinjected with the parasite and KLMb-3. This information was useful since certain cytokines are known to be switch factors for particular antibody isotypes. For example, IL-4 is known to be a cytokine that preferentially induces an isotype switch to IgG1 and IgE, while TGF- β can induce a switch to IgG2b (14). As indicated above, both of these cytokines were secreted by KLMb-3 following in vitro restimulation with *L. major*. Therefore, we determined whether the levels of parasite-specific antibodies of these three isotypes were elevated in the sera of mice coinjected with *L. major* plus KLMb-3. Higher levels of parasite-specific IgG1 and IgE were seen in the sera of mice coinjected with KLMb-3 and the parasite (Fig. 2). Moreover, IgG2b was detected only in the sera of mice coinjected with KLMb-3 and *L. major* (Fig. 2). Finally, although parasite-specific IgG2a (an isotype stimulated by Th1-associated cytokines [14]) could be detected at days 13, 18, and 28 of infection in mice injected with *L. major*, this isotype could not be detected in the sera of mice coinjected with *L. major* and KLMb-3 until day 28 of infection (Fig. 2).

Antigenic specificity of KLMb-3. To determine the antigenic specificity of KLMb-3, we stimulated the cells in vitro with the unrelated and non-cross-reactive antigen OVA (49), with *L. major* promastigotes, or with a panel of gp63 recombinant proteins. The cultures were subsequently pulsed at different times with tritiated thymidine to determine the degree of proliferation in response to the stimuli. As expected, KLMb-3 did not respond to the non-cross-reactive antigen OVA (Fig. 3). In contrast, KLMb-3 proliferated vigorously in response to stimulation with *L. major* promastigotes. Importantly, KLMb-3 cells also responded vigorously to gp63 (Fig. 3), which is the same result which we found when we used gp63 to stimulate

FIG. 2. Clone *KLmB-3* modulates production of *L. major*-specific IgG1, IgG2b, IgE, and IgG2a in vivo. At 13, 18, and 28 days of infection, sera were collected from mice inoculated with *L. major* alone or *L. major* plus *KLmB-3*. The levels of parasite-specific IgG1, IgG2b, IgE, and IgG2a were then determined as described in Materials and Methods. IgG1, IgG2b, and IgG2a results were obtained with sera diluted 1:100; IgE results were obtained with undiluted sera. IgG2b was detected only in day 28 sera from mice with adoptively transferred *KLmB-3*; IgG2a was not detected until day 28 of infection in sera from mice with adoptively transferred *KLmB-3*. ND, not detected; OD, optical density.

lymph node cells obtained from C3H mice infected with *L. major* for 1 to 2 weeks (Table 1).

To define the exact specificity of *KLmB-3*, we used a series of recombinant deletion peptides of gp63 (Fig. 4) (41). TEX51, TEX82, and TEX84 are peptides of gp63 in which various amounts of the carboxy-terminal end have been deleted. In contrast, TEX50, TEX79, and TEX80 are peptides of gp63 in which various amounts of the amino-terminal end of the protein have been deleted. As a positive control we included TEX48, which is the full-length gp63 molecule. Similar to the data shown in Fig. 3, *KLmB-3* did not proliferate in response to stimulation with OVA, but it proliferated vigorously to response to stimulation with *L. major* promastigotes. Deleting various amounts of the C terminus of gp63 had no discernible effect on the proliferation of *KLmB-3* (Fig. 4A). In contrast, deleting the N terminus of gp63 profoundly inhibited the proliferation of *KLmB-3* (Fig. 4B). Based on the data shown in Fig. 4 as a whole, it appeared that the epitope that *KLmB-3* recognizes lies somewhere between amino acids 101 and 206 in the N-terminal region of the gp63 molecule.

MHC restriction of *KLmB-3*. Finally, we analyzed the major histocompatibility complex (MHC) restriction of *KLmB-3*. For these experiments we utilized a series of B10 congenic mice or F1 mice derived from crosses between various B10 congenic mice. The resultant mice varied in the ability to express functional H-2 MHC class I or MHC class II (I-A^k or I-E^k) molecules of the k haplotype. *KLmB-3* was derived from a C3H mouse and thus was H-2^k restricted. We first determined the proliferative response of *KLmB-3* using a k haplotype B10 series mouse (B10.BR) as a source of APCs. We found that

KLmB-3 proliferated vigorously when it was stimulated with *L. major* promastigotes and B10.BR spleen cells as a source of APCs (Fig. 5). *KLmB-3* also proliferated vigorously in response to *L. major* when spleen cells from B10.AQR mice were used as the APC source. These mice are of the k haplotype at the MHC class II loci but not at the MHC class I loci. Therefore, these results demonstrate that *KLmB-3* is not restricted to MHC class I and suggest that *KLmB-3* cells, as expected, are restricted by MHC class II (Fig. 5). To investigate this further, we used spleen cells from B10.A (4R) mice as a source of APCs to restimulate *KLmB-3*. These mice are of the k haplotype in the I-A region of MHC class II but not in the I-E region. Our results indicated that spleen cells from B10.A (4R) mice did not stimulate *KLmB-3* proliferation, suggesting that *KLmB-3* was restricted to I-E^k (Fig. 5). To test this hypothesis, we used APCs from two F1 mice (Fig. 5) that expressed a functional I-E^k (but not I-A^k) molecule through transcomplementation. To generate these mice, crosses were made between B10.A (4R) mice and either a B10.RFB3 mouse (1) or a B10 mouse transgenic for the k haplotype I-E^α molecule (B10.E^α+ mouse) (21). Both of these types of APCs supported vigorous proliferation by *KLmB-3* which was equivalent to that seen with APCs from the B10.BR H-2^k mouse. Together, these data demonstrate that *KLmB-3* cells are CD4 T cells that are surprisingly restricted to I-E^k rather than I-A^k.

Does gp63 contribute to the progression of disease in C3H mice treated with anti-IFN- γ ? Treating mice that resist infection with anti-IFN- γ reverses the phenotype of the mice, causing the mice to develop a progressive disease similar to that seen in susceptible BALB/c mice (3, 37). To determine whether gp63 drives a Th2 response in C3H mice treated with anti-IFN- γ , we challenged treated mice with *L. major* and 1 week later harvested the draining lymph nodes. The lymph node cells were stimulated in the fashion shown in Table 1, and the supernatants of the cultures were tested to determine their IL-4, IL-5 and IFN- γ contents. The results of these experiments (data not shown) were very similar to the results shown

FIG. 3. Specificity of clone *KLmB-3*. *KLmB-3* was cocultured with irradiated syngeneic spleen cells as a source of APCs for different times. The cultures were stimulated with nothing (control, medium alone), OVA (200 μ g/ml), *L. major* promastigotes (2×10^5 promastigotes/ml) (*Lm*), or gp63 (10 μ g/ml) (*gp63*). At the end of the times indicated the cultures were pulsed with tritiated thymidine to determine the degree of proliferation of *KLmB-3*.

FIG. 4. Fine specificity of clone KLmB-3. Cultures were constructed as described in the legend to Fig. 3. All TEX polypeptides were added to the cultures at a final concentration of 10 μ g/ml. *Lm*, *L. major* promastigotes.

in Table 1. Next, we infected anti-IFN- γ -treated C3H mice with *L. major* and removed the draining lymph nodes 6 weeks later, a time when the infection was completely uncontrolled in the mice. The lymph node cells were again stimulated with either *L. major* or gp63, and the levels of IL-4, IL-5, and IFN- γ present in the culture supernatants were determined 48 h later. The results are presented in Table 4. At this point in the infection control mice (normal rat Ig-treated mice) produced considerably more IFN- γ (~1,000-fold more) than they produced after 1 week of infection (compare Tables 1 and 4). Anti-IFN- γ treatment led to a greatly decreased ability to

produce IFN- γ and to a modest increase in production of the Th2 cytokines IL-4 and IL-5 when cultures were stimulated with *L. major* (Table 4). Similar observations have been reported by other workers (37). Approximately the same amounts of IL-4 and IL-5 were produced when the cultures were stimulated with gp63. Thus, the capacities of *L. major* and gp63 to induce Th2 cytokines in anti-IFN- γ -treated C3H mice were similar. Perhaps the most striking aspect of the data shown in Table 4 is that gp63 is a very poor inducer of the Th1 cytokine IFN- γ (gp63 induced 431-fold less IFN- γ than *L. major* induced in anti-IFN- γ -treated mice and 4,128-fold less

FIG. 5. Genetic restriction of clone KLmB-3. Cultures were constructed as described in the legend to Fig. 3. Cultures were stimulated for 48 h and then pulsed with tritiated thymidine for 18 h.

TABLE 4. Response to gp63 in mice treated with anti-IFN- γ

Treatment ^a	Stimulus	Response		
		IL-4 (pg/ml)	IL-5 (ng/ml)	IFN- γ (ng/ml)
Normal rat Ig	<i>L. major</i> (10 ⁶ promastigotes/ml) rgp63 (10 μ g/ml)	1.0	16.6	2,477
		0.8	1.86	8
Anti-IFN- γ ^b	<i>L. major</i> (10 ⁶ promastigotes/ml) rgp63 (10 μ g/ml)	5.4	14.3	259
		5.3	4	0.6

^a C3H mice were injected with 2 mg of either normal rat Ig or anti-IFN- γ (XMG 1.2), and 3 h later the mice were infected with 5×10^6 *L. major* promastigotes. Six weeks later the draining lymph node cells were collected and stimulated with the amount of *L. major* promastigotes or rgp63 indicated. Two days later the supernatants of the cultures were tested to determine their IL-4, IL-5, and IFN- γ contents (see Materials and Methods for details of the techniques). The data are averages of three experiments.

^b Anti-IFN- γ treatment fully reversed the outcome of infection in treated mice as the animals developed lesions that resembled those that occur in susceptible BALB/c mice (7).

IFN- γ than *L. major*-stimulated cells from normal rat Ig-treated mice induced [Table 4]). Finally, it should be mentioned that since the strain of *L. major* used in these studies, LV39, can induce IL-13 (29), we also measured IL-13 levels in culture supernatants but found no consistent differences between groups (data not shown).

DISCUSSION

We describe here the derivation and characterization of a *L. major*-specific Th2 CD4-T-cell clone (KLmB-3) from resistant C3H mice. C3H mice were selected for this study since they are highly resistant (2) to infection with *L. major* and therefore behave as most humans do when they are infected with the parasite. Thus, C3H mice normally develop a protective Th1 response when they are infected with *L. major*. The fact that KLmB-3 was an exacerbative Th2-T-cell clone was unexpected. Although this phenotype of KLmB-3 was not expected, this observation is at least consistent with previously described results. Susceptible BALB/c mice and resistant C57BL/6 mice do not become polarized to a Th2 or Th1 phenotype until after 1 and 2 weeks of infection with *L. major* (31). Since KLmB-3 was isolated from infected resistant C3H mice after 2 weeks of infection, this isolation may correlate with a time when *L. major*-specific Th2 cells were still active in these mice. The functional capacity of KLmB-3 is clearly that of a classical Th2-T-cell clone; that is, this cell clone does not mediate a delayed-type hypersensitivity reaction (6, 13), secretes exacerbative type 2 cytokines (IL-4, IL-5, IL-10, TGF- β) but not protective type 1 cytokines (IFN- γ , IL-12), and stimulates the production of type 2-associated Ig isotypes.

Despite the fact KLmB-3 is a classical Th2-T-cell clone, the cells were still only capable of exacerbating infection for the first 4 weeks following adoptive transfer; beyond this time lesions began to heal. It has been shown in *L. major*-infected mice that adoptively transferred parasite-specific T cells migrate to developing cutaneous lesions of *L. major*, where they are functionally active for the first few weeks of infection. After this the cells can no longer be detected (49). It appears that the same phenomenon occurred in the experiments described here. In mice that received KLmB-3, lesions began to heal and

the Th1-associated antibody isotype IgG2a was produced by 4 weeks of infection. This suggests that KLmB-3 was not active beyond 4 weeks of infection.

Considerable work has been done to characterize the specificity of Th2 T cells that respond to infection to *L. major* in susceptible BALB/c mice. These cells utilize a V β 4 T-cell receptor and are specific for the leishmanial LACK antigen (19). In contrast, KLmB-3 utilizes a V β 8.1 T-cell receptor and is specific for an epitope in the amino terminus of the surface gp63 molecule of *L. major* promastigotes. Thus, the parasite epitopes that can trigger Th2 cells and the T-cell receptor utilized by the T cells can differ in BALB/c and C3H mice.

Although it is clear that gp63 can elicit a type 2 response in C3H mice infected with *L. major* and that gp63-specific Th2 T cells can be isolated from infected C3H mice, our results suggest that these cells are not able to completely skew the immune response in these mice to a Th2 phenotype and thus cause the ultimate death of the animals. The reason(s) for this is not known, but it may be due to the inability of gp63-specific cells to elicit an IL-4 response that has the same kinetics and is of the same magnitude as the response seen with LACK-specific cells in susceptible BALB/c mice infected with *L. major*. In response to LACK, BALB/c mice produce IL-4 within hours of infection with *L. major*, but this does not occur in resistant mice infected with the parasite. Thus, BALB/c mice ultimately die of infection, whereas resistant mice (e.g., C3H mice) cure their infection (2, 19). Since resistant mice can be made susceptible to *L. major* by treatment with anti-IL-12 (11, 45) or anti-IFN- γ (3, 37), we treated C3H mice with anti-IFN- γ to determine whether gp63 could become a strong inducer of a Th2 response in this Th2 environment. We found that gp63 induced Th2 cytokines with the same potency as *L. major*. Since we and other workers (37) have found that the principal effect of anti-IFN- γ treatment is to reduce IFN- γ production rather than increase Th2 cytokine production, there may be no epitope of *L. major* that induces a strong Th2 response in C3H mice. In this vein, however, Launois et al. (18) recently demonstrated that the LACK antigen becomes a strong inducer of IL-4 in C57BL/6 mice treated with anti-IFN- γ antibody.

KLmB-3 is activated when the amino-terminal epitope of gp63 is presented in the context of the MHC class II protein I-E and not in the context of I-A. Louis et al. (23, 24) were the first workers to report the derivation of *L. major*-specific T-cell lines and clones. An analysis of the MHC restriction of these cells revealed that they were restricted to I-A and not I-E. Subsequently, T-cell clones were derived from susceptible BALB/c mice and semiresistant C57BL/6 mice by a number of laboratories (28, 32, 38, 39, 49). The MHC restriction of these cells was not examined, but many of the clones were found to be LACK specific and to utilize the V β 4 T-cell receptor (32). Thus, to the best of our knowledge, the MHC restriction (I-E), antigenic specificity (gp63), and T-cell receptor usage of KLmB-3 (V β 8.1) of KLmB-3 are unique.

Previous immunization studies with gp63 have had diverse outcomes. gp63 has been shown either to induce protection (33, 44) or to not induce protection (10) in mice, and adjuvant has been shown to augment protection (15, 44) or to reverse protection (33, 35). In addition, when gp63 is transfected into bacterial delivery systems, this can lead to protection (5, 51). Thus, as pointed out by Soares et al. (42), it is crucial to

carefully select a proven protective epitope of gp63 or any potential immunogen. The present studies show that following infection with *L. major* promastigotes, gp63 can induce an exacerbating Th2 response. Taken together, the results imply that the context of the initial exposure to gp63, the epitope of gp63 presented, and the manner in which it is presented may determine whether a (protective) Th1 or a (exacerbative) Th2 response evolves following activation by this molecule.

Finally, the fact that C3H mice (the mouse model used in this study) are resistant to infection with *L. major* (2) and thus accurately mimic *L. major* infection in most humans adds particular significance to the results presented in this paper. gp63 is currently considered a vaccine candidate for *L. major* infection of humans. The results presented in this report indicate that it is possible that gp63 may not induce the desired protective type 1 response in some humans. Indeed, peripheral blood mononuclear cells from patients with visceral leishmaniasis produce IL-4 when they are restimulated with gp63 in vitro (17, 50). Therefore, gp63 may be an inappropriate choice for a globally effective vaccine formulation for humans.

ACKNOWLEDGMENTS

We thank C. David (Mayo Clinic, Rochester, Minn.) for providing the B10.AQR, B10.RFB3, and B10.Ea+ mice. We thank Joseph Sypek (Genetics Institute) for helpful suggestions and for critically reading the manuscript.

This work was supported by NIH grants AI 27511 and AI29955 (to R.G.T.) and in part by grant MT-7399 from CIHR (to W.R.M.).

REFERENCES

- Anderson, G. D., and C. David. 1989. In vivo expression and function of hybrid Ia dimers (EaAb) in recombinant and transgenic mice. *J. Exp. Med.* **170**:1003–1008.
- Behin, R., J. Mauel, and B. Sordat. 1979. *Leishmania tropica*: pathogenicity and in vitro macrophage function in strains of inbred mice. *Exp. Parasitol.* **48**:81–91.
- Belosevic, M., D. S. Finbloom, P. H. Van Der Meide, M. V. Slayter, and C. A. Nacy. 1989. Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. *J. Immunol.* **143**:266–274.
- Boom, W. H., L. Lieberman, A. K. Abbas, and R. G. Titus. 1990. Patterns of lymphokine secretion in murine leishmaniasis: correlation with disease progression or resolution. *Infect. Immun.* **58**:3863–3870.
- Connell, N. D., H. Medina-Acosta, W. R. McMaster, B. R. Bloom, and D. G. Russell. 1993. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guérin expressing the *Leishmania* surface proteinase gp63. *Proc. Natl. Acad. Sci. USA* **90**:11473–11477.
- Convit, J., M. E. Pinardi, and A. J. Rondon. 1972. Diffuse cutaneous leishmaniasis: a disease due to an immunological defect of the host. *Trans. R. Soc. Trop. Med. Hyg.* **66**:603–610.
- DeKrey, G. K., H. C. Lima, and R. G. Titus. 1998. Analysis of the immune responses of mice to infection with *Leishmania braziliensis*. *Infect. Immun.* **66**:827–829.
- Etges, R., and I. Muller. 1998. Progressive disease or protective immunity to *Leishmania major* infection: the result of a network of stimulatory and inhibitory interactions. *J. Mol. Med.* **76**:372–390.
- Fitch, F. W., and T. F. Gajewski. 1994. Production of T cell clones, p. 3.13.6–3.13.8. In J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), *Current protocols in immunology*. John Wiley and Sons, Inc., New York, N.Y.
- Handman, E., L. L. Button, and W. R. McMaster. 1990. *Leishmania major*: production of recombinant gp63, its antigenicity and immunogenicity in mice. *Exp. Parasitol.* **70**:427–435.
- Heinzel, F. P., R. M. Rerko, F. Ahmed, and E. Pearlman. 1995. Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. *J. Immunol.* **155**:730–739.
- Holaday, B. J., M. D. Sadick, Z.-E. Wang, S. I. Reiner, F. P. Heinzel, T. G. Parslow, and R. M. Locksley. 1991. Reconstitution of *Leishmania* immunity in severe combined immunodeficiency mice using Th1- and Th2-like cell lines. *J. Immunol.* **147**:1653–1658.
- Howard, J. G., C. Hale, and F. Y. Liew. 1980. Immunological regulation of experimental cutaneous leishmaniasis. III. Nature and significance of specific suppression of cell-mediated immunity in mice highly susceptible to *Leishmania tropica*. *J. Exp. Med.* **152**:594–607.
- Janeway, C. A., and P. Travers. 1997. The humoral immune response, p. 8.8–8.9. In *Immunobiology, the immune system in health and disease*. Current Biology Ltd./Garland Publishing, New York, N.Y.
- Jardim, A., J. Alexander, H. S. The, D. Ou, and R. W. Olafson. 1990. Immunoprotective *Leishmania major* synthetic T cell epitopes. *J. Exp. Med.* **172**:645–648.
- Joshi, P. B., B. L. Kelly, S. Kamhawi, D. L. Sacks, and W. R. McMaster. 2002. Targeted gene deletion in *Leishmania major* identifies leishmanolysin (GP63) as a virulence factor. *Mol. Biochem. Parasitol.* **120**:33–40.
- Kurtzals, J. A., A. S. Hey, A. Jardim, M. Kemp, K. U. Schaefer, E. O. Odera, C. B. Christensen, J. I. Githure, R. W. Olafson, T. G. Theander, and A. Kharazmi. 1994. Dichotomy of the human T cell response to *Leishmania* antigens. II. Absent or Th2-like response to gp63 and Th1-like response to lipophosphoglycan-associated protein in cells from cured visceral leishmaniasis patients. *Clin. Exp. Immunol.* **96**:416–421.
- Launois, P., A. Gumy, H. Himmelrich, R. M. Locksley, M. Rocken, and J. A. Louis. 2002. Rapid IL-4 production by *Leishmania* homolog of mammalian RACK1-reactive CD4(+) T cells in resistant mice treated once with anti-IL-12 or -IFN-gamma antibodies at the onset of infection with *Leishmania major* instructs Th2 cell development, resulting in nonhealing lesions. *J. Immunol.* **168**:4628–4635.
- Launois, P., F. Tacchini-Cottier, C. Parra-Lopez, and J. A. Louis. 1998. Cytokines in parasitic diseases: the example of cutaneous leishmaniasis. *Int. Rev. Immunol.* **17**:157–180.
- Launois, P., I. Maillard, S. Pingel, K. G. Swihart, I. Xenarios, H. Acha-Orbea, H. Diggelmann, R. M. Locksley, H. R. MacDonald, and J. A. Louis. 1997. IL-4 rapidly produced by Vβ4 Vα8 CD4+ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. *Immunity* **6**:541–549.
- LeMeur, M., P. Gerlinger, C. Benoit, and D. Mathis. 1985. Correcting an immune-response deficiency by creating Ea gene transgenic mice. *Nature* **316**:38–42.
- Lima, H. C., J. Bleyenbergh, and R. G. Titus. 1997. A simple method for quantifying *Leishmania* in tissues of infected animals. *Parasitol. Today* **13**:80–82.
- Louis, J. A., E. Moedder, H. R. MacDonald, and H. D. Engers. 1981. Recognition of protozoan parasites by murine T lymphocytes. II. Role of the H-2 gene complex in interactions between antigen-presenting macrophages and *Leishmania*-immune T lymphocytes. *J. Immunol.* **126**:1661–1666.
- Louis, J. A., R. H. Zubler, S. G. Coutinho, G. Lima, R. Behin, J. Mauel, and H. D. Engers. 1982. The in vitro generation and functional analysis of murine T cell populations and clones specific for a protozoan parasite, *Leishmania tropica*. *Immunol. Rev.* **61**:215–243.
- McMaster, W. R., C. J. Morrison, M. H. MacDonald, and P. B. Joshi. 1994. Mutational and functional analysis of the *Leishmania* surface metalloproteinase GP63: similarities to matrix metalloproteinases. *Parasitology* **108** (Suppl.):S29–S36.
- Mosmann, T. R., and R. L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**:145–173.
- Mougeon, E., F. Altare, A. E. Wakil, S. Zheng, T. Coppola, Z. Wang, R. Waldmann, R. M. Locksley, and N. Glaichenhaus. 1995. Expression cloning of a protective *Leishmania* antigen. *Science* **268**:563–566.
- Muller, I., and J. A. Louis. 1989. Immunity to experimental infection with *Leishmania major*: generation of protective L3T4+ T cell clones recognizing antigen(s) associated with live parasites. *Eur. J. Immunol.* **19**:865–871.
- Noben-Trauth, N., W. E. Paul, and D. L. Sacks. 1999. IL-4- and IL-4 receptor-deficient BALB/c mice reveal differences in susceptibility to *Leishmania major* parasite substrains. *J. Immunol.* **162**:6132–6140.
- Nordan, R. P., C. D. Richards, and J. Gauldie. 1994. Measurement of interleukin 6, p. 6.6.1–6.6.5. In J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), *Current protocols in immunology*. John Wiley & Sons, Inc., New York, N.Y.
- Reiner, S. L., S. Zheng, Z. Wang, L. Stowring, and R. M. Locksley. 1994. *Leishmania* promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. *J. Exp. Med.* **179**:447–456.
- Reiner, S. L., Z.-E. Wang, F. Hatam, P. Scott, and R. M. Locksley. 1993. TH1 and TH2 cell antigen receptors in experimental leishmaniasis. *Science* **259**:1457–1460.
- Rivier, D., P. Bovay, R. Shah, S. Didisheim, and J. Mauel. 1999. Vaccination against *Leishmania major* in a CBA mouse model of infection: role of adjuvants and mechanism of protection. *Parasite Immunol.* **21**:461–473.
- Rogers, K. A., G. K. DeKrey, M. L. Mbow, R. D. Gillespie, C. I. Brodskyn, and R. G. Titus. 2002. Type 1 and type 2 responses to *Leishmania major*. *FEMS Microbiol. Lett.* **209**:1–7.
- Russell, D. G., and J. Alexander. 1988. Effective immunization against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. *J. Immunol.* **140**:1274–1279.
- Sacks, D. L., S. Hieny, and A. Sher. 1985. Identification of cell surface

- carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania major* promastigotes. *J. Immunol.* **135**: 564–569.
37. Scott, P. 1991. IFN- γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J. Immunol.* **147**: 3149–3155.
 38. Scott, P., P. Caspar, and A. Sher. 1990. Protection against *Leishmania major* in BALB/c mice by adoptive transfer of a T cell clone recognizing a low molecular weight antigen released by promastigotes. *J. Immunol.* **144**:1075–1079.
 39. Scott, P., P. Natovitz, R. L. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper cell subsets and respond to distinct parasite antigens. *J. Exp. Med.* **168**:1675–1684.
 40. Shankar, A. H., and R. G. Titus. 1995. T cell and non-T cell compartments can independently determine resistance to *Leishmania major*. *J. Exp. Med.* **181**:845–855.
 41. Shreffler, W. G., J. M. Burns, R. Badaro, H. W. Ghalib, L. L. Button, W. R. McMaster, and S. G. Reed. 1993. Antibody responses of visceral leishmaniasis patients to gp63, a major surface glycoprotein of *Leishmania*. *J. Infect. Dis.* **167**:426–430.
 42. Soares, L. R., E. E. Sercarz, and A. Miller. 1994. Vaccination of the *Leishmania major* susceptible BALB/c mouse. I. The precise selection of peptide determinant influences CD4⁺ T cell subset expression. *Int. Immunol.* **6**:785–794.
 43. Soares, M. B. P., J. R. David, and R. G. Titus. 1997. An in vitro model for infection with *Leishmania major* that mimics the immune response in mice. *Infect. Immun.* **65**:2837–2845.
 44. Spitzer, N., A. Jardim, D. Lippert, and R. W. Olafson. 1999. Long-term protection of mice against *Leishmania major* with a synthetic peptide vaccine. *Vaccine* **17**:1298–1300.
 45. Sypek, J. P., C. L. Chung, S. E. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. F. Wolf, and R. G. Schaub. 1993. Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. *J. Exp. Med.* **177**:1797–1802.
 46. Theodos, C. M., J. M. C. Ribeiro, and R. G. Titus. 1991. Analysis of the enhancing effect of sand fly saliva on *Leishmania* infection in mice. *Infect. Immun.* **59**:1592–1598.
 47. Theodos, C. M., D. L. Sacks, W. R. McMaster, and R. G. Titus. 1992. Analysis of the antigenic specificity of T cell clones derived from *L. major*-infected, genetically-resistant C3H mice. *FASEB J.* **6**:A1797.
 48. Titus, R. G., G. Lima, H. D. Engers, and J. A. Louis. 1984. Exacerbation of murine cutaneous leishmaniasis by adoptive transfer of parasite-specific helper T cell populations capable of mediating *Leishmania major*-specific delayed-type hypersensitivity. *J. Immunol.* **133**:1594–1600.
 49. Titus, R. G., I. Muller, P. Kimsey, A. Cerny, R. Behin, R. M. Zinkernagel, and J. A. Louis. 1991. Exacerbation of experimental murine cutaneous leishmaniasis with CD4⁺ *Leishmania major*-specific T cell lines or clones which secrete interferon- γ and mediate parasite-specific delayed-type hypersensitivity. *Eur. J. Immunol.* **21**:559–567.
 50. Vinhas, V., M. Freire, O. Bacellar, S. Cunha, H. Rocha, and E. M. Carvalho. 1994. Characterization of T cell responses to purified leishmania antigens in subjects infected with *Leishmania chagasi*. *Braz. J. Med. Biol. Res.* **27**:1199–1205.
 51. Yang, D. M., N. Fairweather, L. L. Button, W. R. McMaster, L. P. Kahl, and F. Y. Liew. 1990. Oral *Salmonella typhimurium* (AroA⁻) vaccine expressing a major leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against leishmaniasis. *J. Immunol.* **145**:2281–2285.

Editor: W. A. Petri, Jr.