Leishmania major-specific, CD4⁺, Major Histocompatibility Complex Class II–restricted T Cells Derived In Vitro from Lymphoid Tissues of Naive Mice

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Summary

Several studies indicate that the outcome of experimental murine cutaneous leishmaniasis caused by *Leishmania major* (*Lm*) is determined by immunological events occurring shortly after infection. These events lead to outgrowth of either protective $CD4^+$ T cells in the C57BL/6 mouse, which cures, or exacerbative cells in the BALB/c mouse, which succumbs to disease. Potential factors influencing the outgrowth of protective or exacerbative T cells include antigen-presenting cells (APC), cytokines, and parasite antigens. An in vitro system, in which one could precisely control the factors shaping early events in the T cell response to *Lm*, would be very useful. To this end, we have examined the in vitro response of naive lymphocytes to *Lm* promastigotes. The data presented here show that *Lm*-specific $CD4^+$ T cell receptor α/β^+ T cells can be generated in vitro from spleen and lymph node cell populations of naive mice. Furthermore, they can be obtained from the $CD44^{low}$ (unprimed) population of T lymphocytes, indicating that in vitro priming occurs. The ability to generate these T cells is dependent on the presence of live parasites and is not due to a parasite-derived nonspecific T cell mitogen. Restimulation, as assayed by proliferation, requires APC bearing syngeneic I-A. Optimal restimulation of the in vitro derived T cells is achieved only when live promastigotes are used. The T cells do not proliferate in response to a frozen-and-thawed lysate of promastigotes, yet they exhibit mild reactivity to lysates prepared from heat-shocked promastigotes. Furthermore, they do not recognize two predominant antigens on the promastigote surface, lipophosphoglycan and gp63. T cells derived in vitro with *Lm* show crossreactivity with live *L. donovani*, less crossreactivity with live *L. mexicana*, and no crossreactivity with live Bacillus-Calmette-Guerin or live *Brugia malayi* microfilariae. Finally, these early T cells, whether derived from healing C57BL/6 or nonhealing BALB/c mice, produce interleukin 2 (IL-2), IL-4, and interferon γ.

In experimental murine cutaneous leishmaniasis caused by *Leishmania major (Lm)*,[1] it is well known that some strains of mice overcome the infection whereas others succumb to disease (1). Both cure and noncure responses are mediated by parasite-specific $CD4^+$ T cells that can protect against or exacerbate leishmaniasis (2, 3). There are several ways mice can be immunologically manipulated so as to change the course of disease. Noncuring BALB/c mice can be induced to control *Lm* infection after infusion of mAbs to IL-4 (4), IL-2 (5), or CD4 (6). In addition, treatment with anti-μ serum (7), sublethal irradiation (8), administration of cyclosporin A (9), or induction of partial tolerance to *Lm* Ags (10) results in control of the infection. Conversely, mouse strains that normally cure are unable to resolve infection after infusion of mAb to IFN-γ (11) or anti-μ treatment (12). Of particular interest to the present work is the observation that treatments with mAb to IL-4, IFN-γ, and CD4, as well as irradiation and administration of cyclosporin A, must be given during the early phase of infection to be effective (4, 6, 8, 9, 11). Similarly, treatment with anti-μ affects primarily the early phase of infection since μ-suppressed mice exhibit normal disease profiles after adoptive transfer of T cells from infected untreated animals (12). These observations suggest that immunological events occurring during the initial encounter between *Lm* and host immune system determine the subsequent outcome of infection.

[1] *Abbreviations used in this paper:* HSAg, heat-shock antigen; hsp, heat-shock protein; *Lm*, *Leishmania major*; LNC, lymph node cells; LPG, lipophosphoglycan; NMS, normal mouse serum; PIV, primary in vitro; SC, spleen cells.

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To study early events in the immune response to *Lm*, an in vitro model system that mimics the in vivo response would be very useful. Such a system would allow precise control over conditions, i.e., Ag, cytokines, and APC, that may lead to outgrowth of protective or exacerbative T cells.

To this end we have developed a procedure whereby *Lm*-specific T cells can be generated in vitro from naive mice. We have shown that these T cells generated by primary in vitro stimulation (PIV T cells) are analogous to those isolated from mice after infection in that they are $CD4^+$ TCR α/β^+, have strong *Lm*-specific responses, are I-A restricted, and produce IL-2, IL-4, and IFN-γ.

Materials and Methods

Animals. All mice were purchased as specific pathogen-free animals and were used as young adults. BALB/c mice were obtained from Taconic Farms (Germantown, NY). C57BL/6, C57BL/10, B10.BR/SgSn, B10.AQR, B10.A(5R)/SgSn, DBA/2, SWR/J, CBA/J, and CBA/CaH-T6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB.B and B10.D2/nSn mice were from Harlan Olac Ltd. (Oxon, UK).

Parasites, Antigens, Antibodies, and Cell Lines. *Lm* was a clone of LV39 and was maintained as previously described (13). *L. donovani* (Sudan strain; World Health Organization) and *L. mexicana amazonensis* (a clone of PH8[WR563] [14]) were maintained as for *Lm*. In all cases promastigotes from stationary phase cultures were used (15). Live *Mycobacterium bovis* BCG Montreal (lot 1012) was obtained from the Trudeau Institute (Saranac Lake, NY). *Brugia malayi* microfilariae were isolated from the peritoneal cavity of infected gerbils as described (16) and were cultured for 10 d at 37°C in DMEM supplemented with 10% FCS, penicillin, streptomycin, and glutamine. Live microfilariae were used and were separated from nonviable parasites by their ability to emerge after embedding in 0.5% low-melt agarose prepared in culture medium.

Lm antigen (*Lm*Ag) was prepared by rapid freezing and thawing as described (6). *Lm* heat-shock Ag (HSAg) was prepared by culturing parasites for 24 h at 34°C followed by freezing and thawing. *Lm* metacyclic lipophosphoglycan (LPG) was kindly provided by D. Sacks (National Institutes of Health, Bethesda, MD) and recombinant *Lm* gp63 was the gift of R. McMaster (University of British Columbia, Vancouver, Canada).

The mAbs used in this study are listed in Table 1 and were used as cell culture supernatants unless otherwise indicated.

The OVA-specific cell line was produced by immunizing BALB/c mice with OVA at the base of the tail as described (31). Briefly, 7 d after immunization, draining lymph nodes were removed and cultured for 5 d with OVA. Blast cells were isolated on discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradients and rested for 10 d. OVA-specific T cells were then isolated by passage over a discontinuous Ficoll gradient as previously described (32).

Priming of Lm-specific Cells In Vitro. Spleens from normal mice were aseptically removed and a single cell suspension was prepared. Cells were washed and resuspended at 5×10^6 cell/ml in culture medium consisting of DMEM (13) supplemented with 5×10^{-5} M 2-ME and 0.5% normal mouse serum (NMS). Stationary phase *Lm* promastigotes were then added at 5×10^5/ml. *Lm*Ag was used at a final concentration of 5×10^6 *Lm* equivalents/ml. 1 ml/well was then plated into 24-well culture plates (Costar, Cambridge, MA). After 3–7 d of incubation, blast cells were isolated on Percoll gradients. Lymph node cells (LNC) were prepared from popliteal, para-aortic, and inguinal lymph nodes and were processed as described for spleen cells (SC).

Priming of Thy-1.2^+CD44^{low} cells was accomplished as follows. Thy-1.2^+ cells from naive BALB/c spleens were prepared using a cocktail of mAbs to B cell surface antigens (RA3-3A1, J11d) and class II (MK-D6) followed by lysis with Low Tox Rabbit complement (Accurate Chem. & Sci. Corp., Westbury, NY) and removal of dead cells by passage over Ficoll. The remaining cells were >95% Thy-1.2^+ as assayed by flow cytometry. Cells were then stained with anti-CD44 mAb, 1M7.8.1, and secondary antibody, PE-labeled goat anti-rat IgG preabsorbed with mouse Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). $CD44^{low}$ cells were isolated on an Epics cell sorter (Coulter Electronics Inc., Hialeah, FL) by selecting the lower 50% of the $CD44^{low}$ population. These cells were combined with T cell–depleted splenocytes, <2% Thy-1.2^+, prepared by complement-mediated lysis using a mAb cocktail of NEI-001, GK1.5, and H35-17.5. The relative proportions of these two populations in the combined mixture was such that the original T cell/non-T cell ratio was preserved: in this case, 2:3. Cells were then cultured with *Lm* as described.

An additional round of restimulation of PIV T cells was achieved by replating 5×10^5 purified 7-d blasts, 5×10^6 irradiated (900 rad, ^{60}Co source) syngeneic SC, and 5×10^5 *Lm*/ml in 24-well culture plates. 5 d later, cells were harvested by passage over Ficoll.

Flow Cytometry. PIV T cells were incubated with hybridoma culture supernatants of the primary mAb supplemented with 0.02% azide and 1% NMS. Incubation with the appropriate secondary antibody, FITC-labeled goat anti-rat IgG preabsorbed with mouse Ig (Tago, Inc., Palo Alto, CA) or FITC-labeled goat anti–hamster IgG preabsorbed with rat and mouse IgG (Caltag Labs., San Francisco, CA), was performed in culture medium supplemented with 5% FCS and 0.02% azide. Cells were then fixed in PBS with 3.0% paraformaldehyde. Analysis was performed on either an Ortho 50-H or FACScan® (Becton Dickinson & Co., Mountain View, CA) flow cytometer.

Antigen Specificity and Cytokine Production of PIV T Cells. Before restimulation, 7-d PIV T cells were rested (5×10^5/ml) in DMEM supplemented with 0.5% NMS for 3–4 d. This allowed PIV T cells to become quiescent, thereby reducing background levels in proliferation assays. After rest, dead cells were removed by passage over Ficoll. Triplicate wells were prepared on 96-well microtiter plates (Costar) with 10^6 irradiated (1,500 rad) syngeneic SC and 5×10^4 PIV T cell blasts in 100 μl of culture medium with 0.5% NMS and appropriate Ag. All species of *Leishmania* were added at 10^5/well; *Lm*Ag and HSAg were added to a final concentration of 10^6 *Lm* equivalents/ml. *Lm* recombinant gp63 was used at 13 μg/ml and *Lm* LPG at 25 μg/ml. Bacillus-Calmette-Guerin (BCG) was used at 2×10^5 live BCG/well and live *B. malayi* microfilariae at 100 organisms/well. Proliferation was measured by addition of 1 μCi [^{3}H]thymidine/well 48 h after plating, which was determined to be optimal. Cultures were harvested 24 h later, and degree of proliferation was assessed by scintillation counting. Identical conditions were used for restimulating OVA-specific cells, with OVA used at 100 μg/ml.

To generate cytokines, 7-d PIV T cells (10^6/ml) were restimulated in 24-well plates with 5×10^6 irradiated (900 rad) syngeneic SC and 10^6 *Lm*/ml. Supernatants were harvested 24 h later.

Cytokine Analysis. IL-2 and IL-4 were measured in supernatants by bioassays that used indicator cells whose proliferation is affected by the presence of a particular cytokine: IL-2 was detected using CTLL-2 (33; CTLL was maintained in recombinant human [rh]IL-2; Cetus Corp., Emeryville, CA); IL-4 was detected using 11.6 (34; 11.6 was maintained in recombinant murine [rm]IL-4; A.

Glasebrook, Eli Lilly, Indianapolis, IN). The effect of a sample supernatant on the proliferation of CTLL or 11.6 was assessed by [^{3}H]thymidine incorporation, and units per milliliter of a particular cytokine in the test supernatant were calculated from a standard curve obtained with either rmIL-2 or rmIL-4 (Genzyme, Cambridge, MA). Specificity was assessed using blocking anti-IL-2 mAb S4B6 or anti-IL-4 mAb 11B11.

IFN-γ activity was detected by capture ELISA performed on 96-well Falcon® 3912 Micro Test III™ plates (Becton Dickinson Labware, Oxnard, CA) using the rat anti-IFN-γ mAb XMG1.2 as the primary antibody and hamster anti-IFN-γ mAb 5.102.12 as the secondary antibody. XMG1.2 and 5.102.12 were prepared by ammonium sulfate precipitation from cell culture supernatant and ascites, respectively. Binding of the secondary mAb was detected using horseradish peroxidase–conjugated goat anti–hamster Ig preabsorbed to rat Ig (Caltag Labs.), and the ELISA was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Units per milliliter were calculated from a standard curve obtained with rmIFN-γ (a gift of Genentech, South San Francisco, CA).

Results

Naive SC Undergo T Cell Blastogenesis in the Presence of Live Lm Promastigotes. SC from naive BALB/c mice were coincubated with live *Lm* promastigotes. The kinetics of the ensuing blastogenic response are shown in Fig. 1. As early as day 3, a blastogenic response could be observed; small numbers of blast cells, 0.3–1.0% of initial splenocytes plated, could be isolated and restimulated to proliferate in the presence of *Lm* and fresh irradiated syngeneic SC as APC. By day 7, a potent blastogenic response was clearly discernable. Analysis by flow cytometry showed that these 7-d blast cells were 60–75% Thy-1.2$^+$CD4$^+$. This generally represented a yield of 2–7% of the cells present in the initial culture, or ~5–17% of T cells initially present. After an additional 5 d of stimulation using fresh syngeneic irradiated SC as APC and *Lm*, the blast cells were >95% Thy-1.2$^+$CD4$^+$ and >95% TCR α/β$^+$. This potent blastogenic response was not observed if naive splenocytes were cocultured in the absence of any exogenous Ag or in the presence of 100 μg/ml OVA.

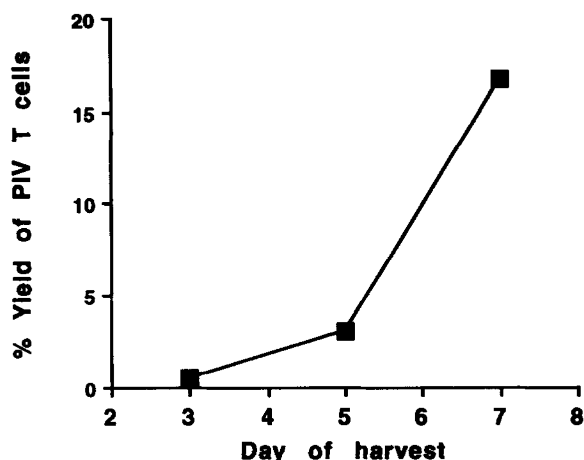

Figure 1. Kinetics of the PIV response to BALB/c mice. The development of PIV T cells from BALB/c SC was monitored by harvesting cultures at the indicated time points and isolating blast cells over Percoll gradients as detailed in Materials and Methods. The number of blast cells was determined by counting in a hemacytometer. Calculations were based on the assumption that spleen cell populations are composed of 40% T cells. Therefore, the percent yield = 100× [blasts/(SC plated)(0.4)].

It was possible that the PIV response to *Lm* was a characteristic of only certain inbred mouse strains. To test this we examined several strains of mice (Table 2) differing in genetic

Table 1. *mAbs to Murine Antigens Used in This Study*

Antibody	Specificity	Reference
AF6-120.1.2	I-A^{bku}	17
GK1.5	CD4	18
H35-17.2	CD8	19
H57-597	TCRαβ	20
J11d	Heat-stable antigen	21
M1/70	Mac-1	22
M5/114	I-A^{bd}I-E^{dk}	23
M5/49.4	Thy-1	23
MK-D6	I-A^d	24
NEI-001	Thy-1.2	New England Nuclear (Boston, MA)
RA3-3A1	B220	25
S4B6	IL-2	26
XMG 1.2	IFN-γ	27
1M7.8.1	CD44	28
5.102.12	IFN-γ	29
11B11	IL-4	30

Table 2. *Mouse Strains from which PIV T Cells Could Be Obtained*

Strain	Response* to *Lm*	H-2‡	I-E‡	Mls type§
BALB/c	S	d	+	b
BALB.B	S	b	–	b
SWR/J	S	q	+	b
DBA/2	S/R	d	+	a
C57BL/6	R	b	–	b
B10.A(5R)/SgSn	R	b	+	b
B10.D2/nSn	R	d	+	b
CBA/CaH-T6J	R	k	+	b
CBA/J	R	k	+	a

* Compiled from references 1 and 35; S, susceptible (noncuring); R, resistant (curing).
‡ H-2 haplotype as indicated in reference 36.
§ Conventional Mls type as compiled in reference 37.

background, resistance or susceptibility to *Lm*, H-2 haplotype, expression of I-E, and Mls type. As indicated, all mouse strains were capable of generating a potent T cell blastogenic response.

Most experiments were carried out with SC since they were easily obtained in large numbers. However, it was conceivable that the observed effect was unique to the spleen and not obtainable with LNC. Since LNC and not the spleen would be the site of initial exposure to *Lm* during natural infection, this was an important consideration. Therefore, we cultured naive LNC from C57BL/6 and BALB/c mice with live *Lm*. A similar blastogenic response was obtained, confirming that this effect was not unique to SC.

We next wanted to ascertain if the responding cells were indeed naive lymphocytes that were primed in vitro or memory cells responding to a crossreactive antigen present in *Lm*. This was determined by incubating purified $CD44^{low}$ unprimed (38) T cells, prepared from spleen, with Thy-1.2-depleted splenocytes and live *Lm*. *Lm*-specific T cell blasts were again obtained, confirming that T cell priming was taking place.

Finally, we wanted to determine if the PIV response required the presence of live *Lm* or if certain *Lm* Ag drove this response. We, therefore, incubated naive SC with freeze-thawed *Lm* promastigote Ag. No blastogenic response was observed even when the amount of *Lm*Ag added was 10 times that present when live parasites were used.

Since it is normally difficult to obtain Ag-specific primary T cell responses in vitro, we wanted to determine if the $CD4^+$ PIV T cells were conventional T cells with respect to their MHC restriction, Ag specificity, and cytokine production.

Conditions Required for Restimulation of PIV T Cells. 7-d PIV T cells were restimulated under various conditions to assess their proliferative requirements. As shown in Fig. 2 *A*, PIV T cells did not proliferate constitutively, nor did they respond to either *Lm* or syngeneic SC alone. Only when both APC and *Lm* were present did the cells proliferate. This was also observed for PIV T cells derived from LNC and $CD44^{low}$ T cells (data not shown).

The data presented above did not eliminate the possibility that PIV T cells were being stimulated by a T cell mitogen produced by living parasites in conjunction with SC. To test this, we incubated an OVA-specific $CD4^+$ T cell line with live *Lm* and irradiated SC. As can be seen in Fig. 2 *B*, OVA-specific cells did not proliferate in response to *Lm* yet responded strongly to OVA. This indicates that the PIV response is not driven by a nonspecific mitogen.

Analysis of PIV T Cell MHC Restriction. We then determined if the MHC restriction pattern of PIV T cells was identical to that observed with conventional $CD4^+$ T cells, i.e., (*a*) inhibition of proliferation by anti-MHC class II mAb; (*b*) inhibition by anti-CD4 mAb; and (*c*) H-2-restricted recognition of Ag. Results shown in Fig. 3 indicate that proliferation of PIV T cells was inhibited by anti-I-A/I-E and anti-CD4 antibodies. In addition, proliferation was unaffected by controls consisting of either an isotype-matched antibody, anti-CD8, or another antibody that binds to the cell surface of macrophages, anti-Mac-1.

Results concerning the MHC restriction of PIV T cells are presented in Table 3. In experiment 1, PIV T cells were obtained using naive spleens from B10.BR mice. These cells were restimulated with *Lm* and APC consisting of irradiated SC from various H-2 congenic mice of the B10 series. APC from B10.AQR mice supported proliferation of B10.BR PIV T cells, indicating that class I loci K and D were not involved in Ag recognition. This would be anticipated given that PIV T cells are $CD4^+8^-$, although class I–restricted $CD4^+$ cells have been observed (39). Furthermore, APC from B10.D2 mice did not effectively present Ag to B10.BR PIV T cells, suggesting that class II is involved, as would be expected from Fig. 3. Finally, APC from CBA/CaH-T6J mice did support proliferation, confirming that these PIV T cells were MHC restricted and were not being stimulated by genes outside the MHC region. This requirement for H-2-compatible APC

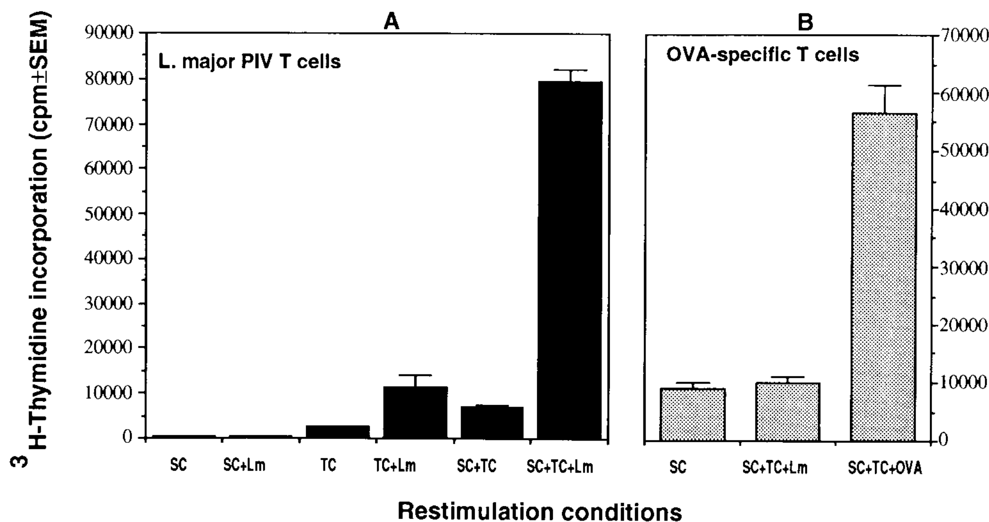

Figure 2. Restimulation of PIV T cells requires APC and *Lm* and is not the result of a nonspecific mitogen. (*A*) 7-d PIV T cells from BALB/c mice were obtained and rested as described in Materials and Methods. The cells were restimulated as indicated: *SC*, 10^6 irradiated (1,500 rad) BALB/c SC; *TC*, 5×10^4 PIV T cells; *Lm*, 10^5 live stationary phase *Lm* promastigotes. (*B*) OVA-specific T cells were generated from BALB/c mice as described in Materials and Methods. The cells were restimulated using conditions given for *A*: *TC*, 5×10^4 OVA-specific T cells; *OVA*, 100 μg/ml OVA. Proliferation was assessed as described in Materials and Methods.

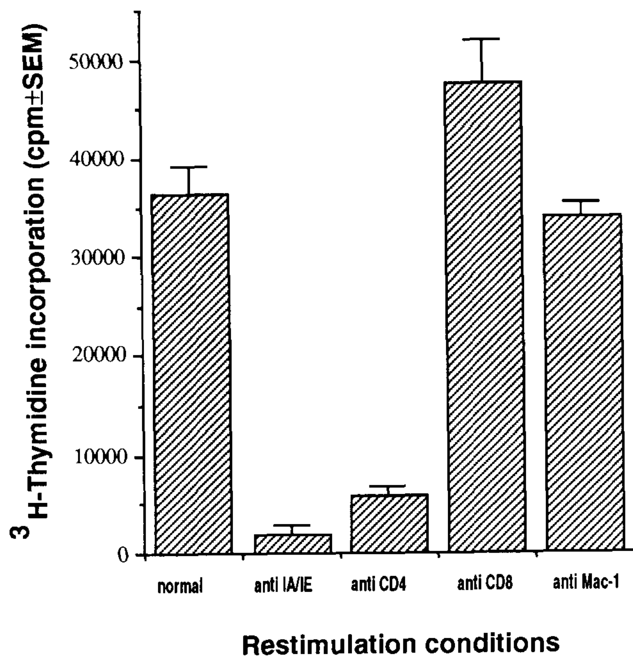

Figure 3. Restimulation of PIV T cells is inhibited by anti-CD4 and anti-I-A/I-E antibodies. 7-d PIV T cell blasts from BALB/c mice were obtained, rested, and restimulated as described in Materials and Methods. All antibodies were added as 1:10 dilutions of hybridoma culture supernatant. *Anti-I-A/I-E*, M5/114; *anti-CD4*, GK1.5; *anti-CD8*, H35-17.2; *anti-Mac-1*, M1/70.

was a characteristic of PIV T cells derived from all mouse strains tested.

Experiment 2 (Table 3) determined if PIV T cells were I-A or I-E restricted. PIV T cells from B10.A(5R) mice were restimulated with syngeneic APC and *Lm* in the presence or absence of anti-I-A^b mAb. The proliferative response in the presence of anti-I-A^b was only 10% of the control. In addition, B10.A(5R) PIV T cells were restimulated using APC from C57BL/10 (I-A^bI-E^{null}) mice, and the proliferative response was virtually identical to that observed with syngeneic APC. The role of I-A was also observed with B10.D2 (I-A^d) and BALB/c (I-A^d) mice in that anti-I-A^d antibody inhibited restimulation when syngeneic APC were used (data not shown). Last, PIV T cells were readily obtained from C57BL/10 and BALB.B mice, both of which do not express I-E. Taken together, these results indicate that PIV T cells are I-A restricted, as are $CD4^+$ *Lm*-specific T cells isolated from infected mice (40).

Antigenic Specificity of PIV T Cells. We next examined the antigenic specificity of PIV T cells (Fig. 4). Although PIV T cells did proliferate vigorously upon restimulation with live *Lm*, they did not proliferate to a preparation of total *Lm* promastigote Ag (*Lm*Ag). No significant responses were obtained to either the major surface glycoprotein of *Lm* (gp63) or to the major surface glycoconjugate of *Lm* (LPG), although both of these Ag preparations strongly stimulated LNC isolated from infected mice (data not shown). Since *Lm* promastigotes undergo heat shock in the PIV system, we also examined the response of PIV T cells to Ag prepared from heat-shocked promastigotes. Interestingly, a response to this preparation was observed. Finally, PIV T cells elicited with *Lm* were found to proliferate to live *L. donovani* and to a lesser extent to live *L. m. amazonensis*. No proliferation was obtained with either an unrelated intracellular organism, live BCG, or with an unrelated extracellular organism, live *B. malayi* microfilariae.

Cytokine Production by PIV T Cells. For these studies, cytokine-containing cell culture supernatants were generated by restimulating 7-d PIV T cells with live *Lm* and low dose–irradiated (900 rad) SC as APC. Low dose irradiation was used so that the radiosensitive APC functions of B cells would

Table 3. *PIV T Cells Exhibit I-A-restricted Antigen Recognition*

Exp.	PIV T cell	APC	H-2 region K	A	E	D	mAb	Proliferation response
								% ± SEM
1	B10.BR/SgSn	B10.BR/SgSn	k	k	k	k	–	100 ± 2
		B10.AQR	q	k	k	d	–	115 ± 3
		B10.D2/nSn	d	d	d	d	–	1 ± 3
		CBA/CaH-T6J	k	k	k	k	–	99 ± 3
2	B10.A(5R)/SgSn	B10.A(5R)/SgSn	b	b	b	d	–	100 ± 3
		B10.A(5R)/SgSn	b	b	b	d	Anti-I-A^b	10 ± 3
		C57BL/10	b	b	–	b	–	92 ± 3

7-d PIV T cells from the indicated mouse strains were derived and restimulated with *Lm* promastigotes as detailed in Materials and Methods. Anti-I-A^b antibody AF6-120.1.2 was added at a 1:10 dilution of hybridoma culture supernatant. Proliferation was assessed as described in Materials and Methods. Data from two independent experiments have been normalized for comparison. Proliferative values ranged from 5 × 10^4 to 10^5 cpm.

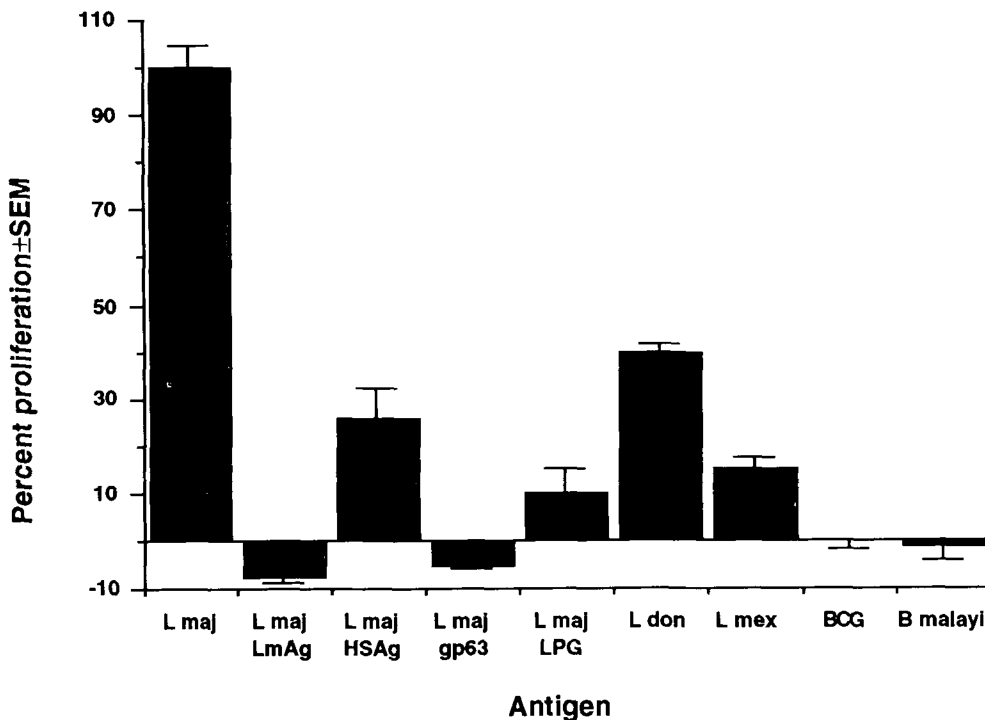

Figure 4. Antigenic specificity of PIV T cells. 7-d PIV T cells from BALB/c mice were restimulated with the indicated antigens: *L maj*, *L don*, *L mex*, 10^5 live stationary phase promastigotes/well; *L maj LmAg*, 10^6 *Lm* equivalents/well of freeze-thawed promastigotes; *L maj HSAg*, 10^6 *Lm* equivalents/well of freeze-thawed heat-shocked promastigotes; *L maj gp63*, recombinant *Lm* gp63 at 13 μg/ml; *L maj LPG*, purified *Lm* metacyclic promastigote LPG at 25 μg/ml; BCG, 2 × 10^5 live Bacillus-Calmette-Guerin/well; *B malayi*, 100 live *B. malayi* microfilaria/well. Results are a composite of three independent experiments that have been normalized for comparison. Proliferative values ranged from 5 × 10^4 to 10^5 cpm.

remain intact (41), thereby mimicking as much as possible the in vivo environment. Representative results are shown in Fig. 5. 7-d PIV T cell blasts from both nonhealing BALB/c and healing C57BL/6 mice produced IL-2, IL-4, and IFN-γ. Interestingly, when high dose (1,500 rad)–irradiated SC were used as APC, no IL-4 could be detected in culture supernatants, although IL-2 and IFN-γ were still produced.

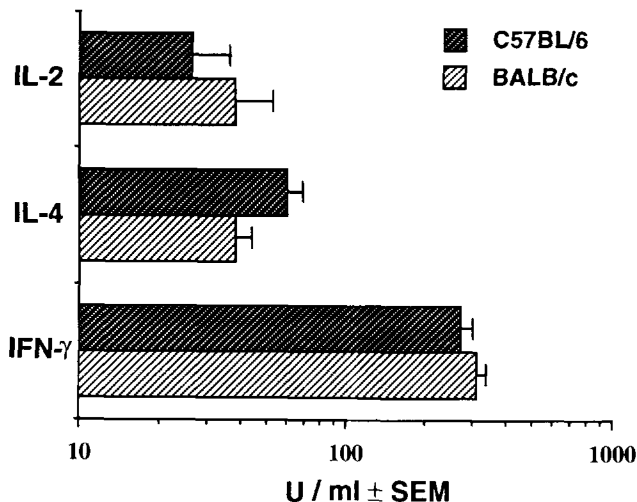

Figure 5. Cytokine production of PIV T cells. 7-d PIV T cell blasts from C57BL/6 and BALB/c mice were isolated and restimulated with fresh syngeneic irradiated (900 rad) spleen cells and live *Lm* as described in Materials and Methods. Supernatants were collected 24 h after restimulation and examined for cytokine activity by bioassay or ELISA as outlined in Materials and Methods.

Discussion

This report demonstrates that *Lm*-specific T cells can be generated in vitro from lymphoid tissues of normal mice. Importantly, we have further shown that the T cells generated in the PIV system arise from the naive $CD44^{low}$ T cell population, indicating that genuine priming occurs. Furthermore, PIV T cells resemble conventional *Lm*-specific T cells in that they: (*a*) are $CD4^+$ TCR α/β^+; (*b*) are restricted to the I-A determinant of MHC class II; and (*c*) produce IL-2, IL-4, and IFN-γ.

The in vitro priming phenomenon described here does not appear to be unique to *Lm*, as we have also observed in vitro blastogenic responses to *L. m. amazonesis*, *L. b. brasiliensis*, and *L. donovani* using culture conditions identical to those described here for *Lm*. Live *L. donovani* amastigotes can also induce the proliferation of parasite-specific T cells if coincubated with naive murine SC (Paul Kaye, personal communication). Interestingly, in vitro stimulation of naive lymphocytes is not restricted to either T cells or to murine systems. For example, polyclonal B cell activation using normal murine splenocytes and *Lm* promastigote lysates has been observed (42). In addition, cells from nonexposed human beings have been reported to respond in vitro to *L. amazonesis*, *L. donovani*, or *Lm* (43, 44), to *Plasmodium falciparum* (45), and to *Trypanosoma cruzi* (46). Thus, it is possible that the ability to generate primary T and B cell blastogenic responses in vitro from naive hosts is not only a capability of *Leishmania* but may well be a common property of protozoan parasites. This could account for the potent immunogenic response to many of these infections. For example, in mice it has been observed that as early as 3 d after infection with *Lm*, parasite-

specific production of cytokines can be detected in the draining LNC (47).

The facility with which *Lm*-specific T cell blasts can be obtained by primary in vitro immunization is remarkable. The blastogenic response to live parasites can occur even in the absence of specialized conditions such as oxygenated media, rocking of cultures, daily addition of nutrients (48), or even 2-ME, an important supplement for other in vitro priming systems (49). Therefore, it would be useful to know how such a response is generated and which factors, such as cytokines, accessory cells, or antigens, may be involved. Second, the in vivo effects of those factors governing the PIV response should be examined.

Initially, we speculated that the *Lm*-specific T cells generated in the PIV system were responding to a parasite superantigen. Superantigens are highly immunogenic proteins produced by some pathogenic bacteria, and are capable of stimulating naive T cells in vitro by activating cells bearing certain V_β regions regardless of the antigenic specificity of the TCR itself (50). However, several observations argue against a leishmanial superantigen being responsible for the PIV response. First, PIV T cells exhibit clear I-A-restricted recognition of antigen (Table 3). Although superantigens may prefer certain I-A types more than others, strict syngeneic requirements are not observed (51, 52). Second, whereas the PIV T cells generated here are exclusively $CD4^+$, foreign superantigens generally stimulate both $CD8^+$ and $CD4^+$ T cells (52). In addition, if a superantigen were responsible for the PIV response, one would expect to see a change in the proportional representation of predominant V_β regions in stimulated T cell populations as compared with naive ones. In the naive BALB/c mouse, ~25% of splenic T cells express $V_\beta8.1$, 8.2, or 8.3, and 10% express $V_\beta6$, accounting for ~35% of all splenic T cells. Therefore, by examining the percentage of cells bearing $V_\beta8$ or $V_\beta6$ and the change in the $V_\beta8/V_\beta6$ ratio, most superantigen effects can be detected. For example, when BALB/c splenocytes are exposed in vitro to the superantigen staphylococcal enterotoxin B, which binds $V_\beta3$, 7, 8.1, 8.2, 8.3, and 11 (53), the $V_\beta8/V_\beta6$ ratio of 4-d T cell blasts increased sevenfold (53). Similarly, when *Y. enterocolitica* superantigen containing lysates are used, which bind $V_\beta3$, 7, 8.1, 9, and 11 (54), the $V_\beta8/V_\beta6$ ratio decreased 40% (54). In our PIV system, however, we find that V_β usage of 7-d PIV T cells is 25% $V_\beta8.1$, 8.2, or 8.3, and 10% $V_\beta6$, giving a $V_\beta8/V_\beta6$ ratio of 2.5:1, identical to that of naive BALB/c splenic T cells. Collectively, these data do not formally rule out a role for a superantigen-like molecule in our system but they do suggest that if a superantigen is acting, a novel mechanism is involved.

Given that a superantigen activity is unlikely, the mechanism(s) underlying the PIV phenomenon and the parasite antigen(s) that the PIV T cells recognize remain unknown. It has been shown, however, that in vitro infection of macrophages from both curing and noncuring mouse strains results in production of large amounts of IL-1 (55; H. R. Chakkalath and R. G. Titus, unpublished data), which could facilitate T cell priming. With regard to antigens, it has been demonstrated that live *Lm* can act as an adjuvant when coinjected with peptides (56). This effect can be mimicked by substituting for live organisms the 70-kD heat-shock protein (hsp) of *M. bovis*, *M. tuberculosis*, or the GroEL hsp of *Escherichia coli* (56). Since the 70-kD hsp's are among the most conserved proteins throughout evolution (57) and are potent antigens of many pathogens, including *Lm* (58, 59), it is possible that hsp's are partially responsible for stimulating the PIV response. The recognition of heat-shocked promastigote antigen by PIV T cells (Fig. 4) is consistent with this idea. Along with hsp's other proteins may be expressed during transformation from promastigote to amastigote. Given these considerations, we are currently examining the roles of IL-1, hsp, and amastigote antigens in the PIV system.

The data presented here show that both curing and noncuring mouse strains produce PIV T cell blasts (Table 2), which secrete IL-2, IL-4, and IFN-γ (Fig. 5), upon exposure to *Lm* in vitro. Several studies indicate that IL-2 (60, 61), IL-4 (62), and other cytokines (63–65) can promote *Leishmania* survival both in vitro and in vivo. It is, therefore, conceivable that the early immune response to *Leishmania* could directly promote parasite survival. If an initial parasite-specific immune response benefits the parasite, inhibition of this early response might benefit the host. It is noteworthy that all treatments that enable noncuring BALB/c mice to control a primary infection (4–10) would inhibit T cell priming and expansion. Likewise, partial tolerance to *Lm* antigens in curing mouse strains C3H/HeN and (B6 × B10.D2)F_1 also results in less severe disease (A. Shankar, unpublished data). Moreover, both irradiated and $CD4^+$ T cell–depleted mice harbor fewer parasites than untreated controls during the early phase of infection (66, 67). Hence, attenuating the initial immune response to *Lm* does appear to facilitate cure. As mentioned, the early response should, therefore, be exacerbative. In fact, we have adoptively transferred 7-d PIV T cell blasts from both BALB/c and C57BL/6 mice into naive syngeneic recipients and observed that lesion development was potentiated (data not shown). Thus, *Lm* can foster development of exacerbative cells shortly after infection such that its survival and replication is facilitated. This early response, though beneficial for the parasite in all mouse strains, may differentially influence the subsequent cure or noncure response observed in mice of different genotypes. Therefore, a better understanding of factors governing the PIV response and its potential effects in vivo may have implications for beneficial immunomodulation of leishmaniasis.

As mentioned, 7-d PIV T cell populations from both resistant and susceptible mice produced IL-2, IL-4, and IFN-γ (Fig. 5). Hence, early in a PIV response, the responding cells are either Th0 cells or some mixture of Th1 and Th2. This is consistent with recent data examining the early response to *Lm* in which both curing and noncuring mouse strains produced IL-4 and IFN-γ (47, 68). These studies were performed with lymph node cells taken shortly after infection and stimulated in vitro. Since varying potentials for cytokine production can exist between peripheral lymph nodes and SC (69, 70), we are currently examining LNC-derived PIV T cells for the cytokine these cells produce and the effect the cells have on infection with *Lm* in vivo. In addition, cytokine

secretion patterns in long-term cultures of PIV T cells are being monitored to detect differentiation into Th1 or Th2 phenotypes.

In experimental murine cutaneous leishmaniasis caused by *Lm*, a correlation exists between Th1 parasite–specific T cell responses and disease resolution, and Th2 responses and disease progression (reviewed in reference 5). However, experiments involving T cell clones and lines from several different strains of mice indicate that a Th1 or Th2 phenotype is not an indicator of protective or exacerbative function (13, 31, 71–73). Attempts to more precisely identify Th1 or Th2 cytokines regulating disease outcome have also yielded conflicting results. Infusing mAb to IFN-γ is detrimental while mAbs to IL-4 or IL-2 can be protective (4, 5, 11, 74). However, injection of either IFN-γ or IL-4 has little effect on disease (4, 47, 74, 75) unless coadministered with adjuvant, in which case both are protective (47, 76). Thus, a complex picture is emerging with respect to cytokines and preferential outgrowth of protective or exacerbative T cells after *Lm* infection of mice.

Clearly, the ability to precisely manipulate in vitro the conditions under which *Lm*-specific T cells first encounter antigen would facilitate the study of the complex interactions leading to outgrowth of protective or exacerbative T cells. The in vitro system involving *Lm* that has been developed and analyzed in this study shares many characteristics with the natural in vivo response to *Lm* during the early phase of infection. Therefore, this system may offer a tool to investigate factors responsible for development of various Th cell subsets and protective or exacerbative T cells. For example, since cytokines have been shown to affect outgrowth of Th cell subsets (77–81), exogenously added cytokines, both alone and in combination, can be included at the inception of a PIV response to determine whether this alters the nature of the responding *Lm*-specific T cell. In this respect, it is intriguing that the PIV T cells produce appreciable amounts of IL-4 since recent antigen-specific in vitro priming studies indicate that IL-4 is produced only when it is also present during priming (82), suggesting that *Lm* may induce IL-4 production by some novel mechanism. Last, APC function can be examined. A role for B cells in the PIV system is clearly suggested by the fact that IL-4 was not produced by PIV T cells restimulated with 1,500-rad irradiated SC that are deficient in B cell antigen presentation, but was produced when 900-rad irradiated SC were used in which B cell presentation does occur. Several laboratories have, in fact, demonstrated effects of APC type on cytokine production by T cells (83–85). We can, therefore, test various APC such as Langerhans cells, monocytes, or B cells in the PIV system to determine whether certain APC selectively activate protective or exacerbative *Lm*-specific T cells.

The advantage of the PIV approach is that control can be maintained over early events that shape the *Lm*-specific T cell response, i.e., T cell priming and outgrowth. The influence of factors such as cytokines, APC, and parasite antigens can, therefore, be precisely determined. Such an experimental approach may help unravel the mechanisms determining susceptibility and resistance in cutaneous leishmaniasis.

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