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Resistance to murine cutaneous leishmaniasis is mediated by T_H1 cells, but disease-promoting $CD4^+$ cells are different from T_H2 cells*

A limiting dilution system has been used for quantitative analysis of antigen-reactive T cells producing interleukin (IL) 2, IL 4 and interferon (IFN)-γ in the course of murine infection with *Leishmania major*. The precursor frequencies of $CD4^+$ cells with the potential for production of IFN-γ, which has been associated with T_H1 cells, are much higher in resistant than in susceptible mice, whereas the reverse is found for $CD4^+$ cells secreting IL 4 which have been classified as T_H2 cells. Our results allow a better understanding of the relative contribution of these cell types at various stages of disease and can be summarized as follows: (a) secretion of IL 4 can be demonstrated in short-term clonal cultures of $CD4^+$ cells from *L. major*-infected mice, (b) $CD4^+$ cells releasing IL 2, suggested to be a characteristic of T_H1 cells that predominate in resistant mice, can also be detected in susceptible mice at any time of infection, (c) both IL 2 and IL 4 are released by the progeny of individual T cells from susceptible mice and (d) the kinetics of precursor frequencies in genetically susceptible mice protected against the disease by prophylactic treatment are different from those of congenitally resistant mice, thus indicating that the development of lymphokine-producing T cells and the establishment of protective immunity may be regulated differently in those mice. The data suggest that resistance to disease is correlated with the presence of IFN-γ-producing T_H1 cells, while susceptibility is associated with $CD4^+$ cells that do not segregate into the T_H1 or T_H2 subset but display an overlapping pattern of lymphokine activities.

1 Introduction

The protozoan parasite *Leishmania major* infects MΦ in mammalian hosts and produces a range of cutaneous disease manifestations. The outcome of experimental infection in mice is genetically determined. Mice of resistant inbred strains, such as C57BL/6, are able to control the disease with lesions resolving spontaneously, whereas genetically susceptible BALB/c mice develop disseminating skin ulcers with fatal impact. The ability to contain lesion development and complete healing can be induced by various treatments of BALB/c mice prior to infection, *e.g.* sublethal irradiation [1], injection of anti-IgM [2], or anti-CD4 [3] Ab, or immunization with purified parasite Ag [4–6]. The events leading to resistance are thought to be initiated by lymphokines with MΦ-activating properties, predominantly IFN-γ, that are generated by *L. major*-activated T cells [7–9]. Evidence points to the $CD4^+$ population as being the critical source of resistance-promoting T cells [10–12]. It has recently been shown that the levels of IFN-γ mRNA are greater in *L. major*-infected C57BL/6 mice than in infected BALB/c mice [13]. In contrast, IL 4 mRNA could only be detected in BALB/c mice [13]. These findings are consistent with the hypothesis that resistance-promoting T cells producing IFN-γ belong to the T_H subset of type 1 (T_H1), whereas disease-promoting T cells generating IL 4 would be T_H2 cells. This classification of $CD4^+$ subsets is based on studies with long-term cultured and cloned murine T cells [14]. In agreement with this model, *L. major* Ag-specific T cell lines mediating protection to cutaneous leishmaniasis displayed the T_H1 properties of secreting IL 2 and IFN-γ, while disease-exacerbating lines released IL 4 [15].

In the present study, we used an LD system for analysis of the frequencies and kinetics of *L. major*-reactive $CD4^+$ cells secreting lymphokines and we compared the activity patterns in resistant and in susceptible mice at various times of infection. In contrast to the study of long-term cultured T cell lines, LD analysis of freshly isolated lymphocytes provides information at the clonal level on the effector activities of the entire range of T cell populations that become activated during infection. In addition, it allows the quantitation of Ag-specific precursor T cells. As opposed to lymphocytes secreting IL 2 or IFN-γ, IL 4-producing cells are difficult to detect in LD cultures of freshly isolated lymphocytes [16]. However, IL 4 secretion can be demonstrated in LD cultures set up after a cycle of *in vitro* restimulation with Ag and an interval of rest in the absence of Ag in bulk cultures, as described previously [16, 17]. Using this protocol, we were able to estimate the frequencies in lymphocytes from *L. major*-infected mice of parasite-reactive precursor T cells with the potential to produce IL 2 and IFN-γ as well as those with the ability to secrete IL 4. Furthermore, it was possible to determine whether the distinct lymphokine activities were elicited by the same or by separate T cell populations.

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Abbreviations: **LNC:** LN cells **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **SC:** Spleen cells

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2 Materials and methods

2.1 Mice

Female mice of the inbred strains BALB/c and C57BL/6 were 5 to 7 weeks of age at the onset of experiments. All mice were purchased from Charles River Breeding Laboratories (Sulzfeld, FRG) and, during experimentation, were maintained under conventional conditions in an isolation facility.

2.2 Parasites and infection of mice

The origin and propagation of the *L. major* isolate have been described in detail elsewhere [18]. The cloned virulent line used for this study was confirmed to be *L. major* by isoenzyme analysis (Dr. D. Evans, London School of Hygiene and Tropical Medicine, London, GB) and was maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood agar cultures [19]. Stationary-phase promastigotes were washed in PBS and 2×10^6 organisms were injected in a volume of 50 µl i.d. on the dorsum of the mouse close to the base of the tail.

2.3 Irradiation

For sublethal irradiation of mice, a dose of 450 rad of γ irradiation was used. For preparation of APC for *in vitro* cultures, spleen cells (SC) from untreated mice were irradiated with 2500 rad using a ^{137}Cs source (Gammacell, Frankfurt, FRG).

2.4 mAb and cytotoxic procedures

The mAb were prepared from hybridomas grown at this Institute. mAb directed against CD8 (Ly-2), from hybridoma YTS 169.4.2 [20], CD4 (L3T4), from hybridoma YTS 191.1.2 [20] and IL 2, from the hybridoma S4B6 [14] were used as ascites fluid. Anti-IL 4 mAb were used as culture SN from hybridoma 11B11 [21]. Rat mAb against mouse IFN-γ, from hybridomas R4-6A2 [22] and AN-18.17.24 [23], were purified from culture SN by affinity chromatography on protein G-Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden). The purified AN-18.17.24 mAb was conjugated with N-hydroxy-succinimid-biotin (Pierce, Weiskirchen, FRG), as previously described [24]. Anti-Thy-1.2 mAb, from hyridoma F7D5, were purchased from Camon (Wiesbaden, FRG).

For the preparation of lymphocyte subpopulations by cytotoxic procedures [25], single-cell suspensions of SC or LN cells (LNC) in RPMI 1640/1% FCS at a concentration of 10^7/ml were incubated for 40 min on ice with anti-CD8 (for preparation of $CD4^+$ responder cells), anti-CD4 (for preparation of $CD8^+$ responder cells) or anti-Thy-1.2 (for preparation of T cell-depleted APC) mAb. Thereafter, rabbit C (Cedarlane, Hornby, Ontario, Canada) at a final dilution of 1/15 was added and the suspensions were incubated for another 25 min at 37 °C. The cells were washed and the treatment with Ab and C was repeated once more. The contamination with cells of the depleted phenotype was < 5% as judged by staining with fluoresceinated Ab.

2.5 LD analysis

At various times after infection with *L. major*, three mice per experimental group were killed and the inguinal LN draining the cutaneous lesion were removed for the preparation of single-cell suspensions. Before conduction of LD analysis, the responder cells were subjected to a cycle of *in vitro* restimulation and rest [16, 17]. For this reason, cultures containing 3×10^6 cells were incubated with 5×10^6 syngeneic irradiated APC and 3×10^6 *L. major* promastigotes collected in the stationary phase of growth. The cultures were set up in flat-bottom tissue-culture wells (Nunc, Roskilde, Denmark) in a total volume of 1.5 ml of Click's RPMI 1640 culture medium (Gibco, Karlsruhe, FRG) supplemented with L-glutamine (2 mM), Hepes buffer (10 mM), $NaHCO_3$ (7.5%), 5×10^{-5} M 2-ME, penicillin (100 µg/ml), gentamycin (160 µg/ml) and 10% selected FCS. After 4 days (37 °C, 5% CO_2, humidified atmosphere), viable responder cells were isolated from the bulk cultures by centrifugation over Ficoll-Hypaque (Pharmacia) and placed into rest cultures consisting of 4×10^5 responder cells and 5×10^6 syngeneic APC, in the absence of Ag, for another 6 days. Thereafter, viable cells were again isolated over Ficoll-Hypaque and were separated into the $CD4^+$ or $CD8^+$ subpopulations by cytotoxic procedures prior to LD analysis.

Limiting numbers of lymphocytes or lymphocyte subpopulations were cultured in round-bottom microtiter wells (Nunc) with 2×10^5 syngeneic APC in 0.2 ml of culture medium containing 15% of an SN from PHA-stimulated human tonsil cell cultures as a source of IL 2. For each T cell concentration ranging from 2×10^4 to 40 cells/well, replicates of 24 wells were set up with 2×10^5 live *L. major* promastigotes as a source of Ag. After 7 to 10 days of incubation, microcultures were washed three times to remove exogenous factors derived from the tonsil cell culture SN and were restimulated by adding 2×10^5 T cell-depleted syngeneic APC in the presence of Ag (2×10^5 live *L. major* promastigotes), in 0.2 ml of culture medium. After 24 h, 0.16 ml of SN was collected from each well and split into four aliquots for the determination of lymphokine activities. Cultures containing APC and Ag but no T cells were used as controls. LD microcultures were scored positive when the values of lymphokine activity exceeded the arithmetic mean of the control wells by > 3 SD. For quantitation of lymphokine-producing T cells reacting to *L. major*, a second series of LD cultures was set up and restimulated without Ag for each responder cell population. The number of cultures that were positive in the absence of Ag was subtracted from the fraction of cultures responding in the presence of *L. major* Ag for each dose group. Minimal estimates of the precursor frequency for each lymphokine activity were obtained by the minimum χ^2 method from the Poisson distribution relationship between the number of responding cells and the logarithm of the fraction of negative cultures by using a computer program purchased from C. Taswell [26].

2.6 Assay for IL 2 and IL 4 activity

For concurrent determination of IL 2 and IL 4 activity, 40-µl aliquots of SN from individual LD cultures were tested for the ability to stimulate proliferation of 3×10^3 cells of the

mouse HT-2 T cell line [27] in a total volume of 0.1 ml in flat-bottom culture wells. After 48 h, responsiveness was measured by using a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [28]. One aliquot of SN was incubated in the absence of Ab, to test for IL 2 and IL 4 activities. A second aliquot was tested in the presence of anti-IL 2 mAb, for the determination of IL 4 activity, and a third aliquot in the presence of both anti-IL 2 and anti-IL 4 mAb. The detection threshold was 0.1 U/ml for IL 2 as well as for IL 4, as determined by standard titrations of recombinant lymphokines. Depending on the number of T cells and their lymphokine activity, up to 25 U/ml of IL 2 and 10 U/ml of IL 4 could be detected in LD cultures.

2.7 Assay for IFN-γ activity

For detection of IFN-γ activity in SN from LD cultures, an ELISA was used [29]. Round-bottom polyvinyl chloride microtiter plates (Dynatech, Denkendorf, FRG) were coated with anti-IFN-γ mAb R4-6A2 (10 μg/ml in PBS, 90 min at room temperature). Unsaturated binding sites were blocked with 1% BSA (Sigma) for 1 h at 37 °C. Plates were then washed with buffer (PBS/0.05% Tween 20) and 40-μl aliquots of SN from individual LD cultures were added to each microwell. After an overnight incubation at 4 °C in a humid box, the plates were washed and biotin-labeled anti-IFN-γ mAb AN-18.17.24 (2 μg/ml) in 50 μl PBS/1% BSA per well were added and incubated for 1 h at 37 °C. The plates wer washed again and wells were incubated with a conjugate of streptavidin-biotin-complex/alkaline phosphatase (Dakopatts, Hamburg, FRG) in PBS for 1 h. After another washing, the substrate p-nitrophenylphosphate (1 mg/ml; Sigma, St. Louis, MO) in diethanolamine buffer (pH 9.8) was added, and the absorbance in the wells was read 20 min later with a microplate reader (MR 700, Dynatech), using a test wavelength of 405 nm and a reference wavelength of 490 nm. The detection threshold was 2 U/ml, as determined by a standard titration of rIFN-γ. Depending on the number of T cells/well and their lymphokine activity, up to 60 U/ml could be detected in LD cultures.

3 Results

3.1 Frequencies of lymphokine-producing CD4+ cells in genetically resistant and genetically susceptible mice infected with *L. major*

At various intervals after i.d. infection of BALB/c and C57BL/6 mice with *L. major* promastigotes, lymphocytes from the inguinal LN draining the cutaneous lesions were collected and subjected to *in vitro* restimulation in bulk cultures followed by LD analysis, as described in Sect. 2.5. The production of IL 2 as well as IL 4 was assessed by the ability of LD culture SN to stimulate the proliferation of HT-2 cells which respond to both lymphokines. IL 2 activity was defined as the reduction of the HT-2 response in the

Table 1. Frequency of *L. major*-reactive precursor CD4+ cells with the potential for secretion of IL 2, IL 4 or IFN-γ[a)]

Strain	Weeks of infection	Reciprocal of precursor cell frequency[b)] HT-2 response in the presence of no mAb IL 2 + IL 4	HT-2 response in the presence of anti-IL 2 mAb IL 4	ELISA IFN-γ
BALB/c	1	48 (22–227)	129 (67–1562)	25 940 (13 513–33 330)
	3	191 (135–325)	2272 (1587–3846)	> 50 000
	5	107 (65–308)	585 (418–971)	> 50 000
	7	185 (142–267)	877 (671–1266)	20 325 (11 100–142 860)
	10	167 (128–240)	602 (450–917)	> 50 000
	13	2147 (1587–3330)	9091 (6667–14 286)	> 100 000
C57BL/6	1	68 (40–213)	143 (75–1733)	840 (463–4464)
	3	313 (183–1075)	2857 (2174–4167)	699 (535–1010)
	5	301 (226–450)	6667 (4545–12 500)	275 (188–513)
	7	1087 (787–1786)	> 50 000	1182 (855–1923)
	10	943 (662–1640)	> 50 000	1042 (787–1562)
	13	43 480 (21 276–200 000)	> 50 000	6250 (4000–14 286)

a) At various intervals after i.d. infection of mice with *L. major*, lymphocytes were collected from the inguinal LN. After a cycle of *in vitro* stimulation with *L. major* promastigotes followed by an interval of culture in the absence of Ag, limiting numbers of CD4+ cells selected by treatment with anti-CD8 mAb and C were cultured with syngeneic APC in the presence or absence of *L. major* promastigotes. LD cultures were restimulated 7 days later and, 24 h thereafter, SN from individual cultures were collected and split for determination of lymphokine activities. IFN-γ was measured by ELISA. Both IL 2 and IL 4 were assessed by the proliferation of HT-2 cells in the absence of mAb; IL 4 activity was defined by the HT-2 response in the presence of anti-IL 2 mAb. Minimal estimates of the precursor frequency were obtained by the minimum χ^2 method. The data represent mean values from two experiments. All p values > 0.1.

b) 95% confidence limits.

presence of anti-IL 2 mAb. IL 4 activity was defined as HT-2 growth factor activity detectable in the presence of anti-IL 2 mAb but absent in the presence of both anti-IL 2 and anti-IL 4 mAb. The production of IFN-γ was measured by an ELISA. T cells from uninfected mice gave negligible responses in this system (data not shown).

Table 1 summarizes the frequencies (f) of *L. major*-reactive precursor $CD4^+$ cells with the potential for lymphokine secretion in infected BALB/c and C57BL/6 mice. During the entire course of infection, considerable frequencies of T cells mediating HT-2-stimulatory activity could be detected in both strains of mice (f = 1/48–1/2147), except for a decreased frequency in resistant C57BL/6 mice at 13 weeks after infection when their cutaneous lesions had been cured for several weeks (f = 1/43 480). When the LD culture SN were assayed in the presence of anti-IL 2 mAb, the frequency of *L. major*-reactive T cells mediating an HT-2 response was significantly reduced in susceptible and in resistant mice, suggesting that they both gave rise to substantial levels of IL 2 activity. With respect to the residual activity that had not been blocked by anti-IL 2 mAb, thus reflecting the production of IL 4, comparable levels were observed in both susceptible and resistant mice at the early stage of infection (1 and 3 weeks). At weeks 5 to 10 of infection, however, an increasing difference in the frequencies of IL 4-producing T cells became visible. Whereas frequencies of IL 4 activity declined in resistant C57BL/6 mice as their lesions healed, they remained unchanged in susceptible BALB/c mice (f = 1/585–1/877) and decreased only at a late stage of disease when these mice suffered from severe lesions (f = 1/9091). The remaining HT-2-stimulating activity was inhibited when the SN were tested in the additional presence of anti-IL 4 mAb (data not shown), indicating that it could in fact be attributed to IL 4.

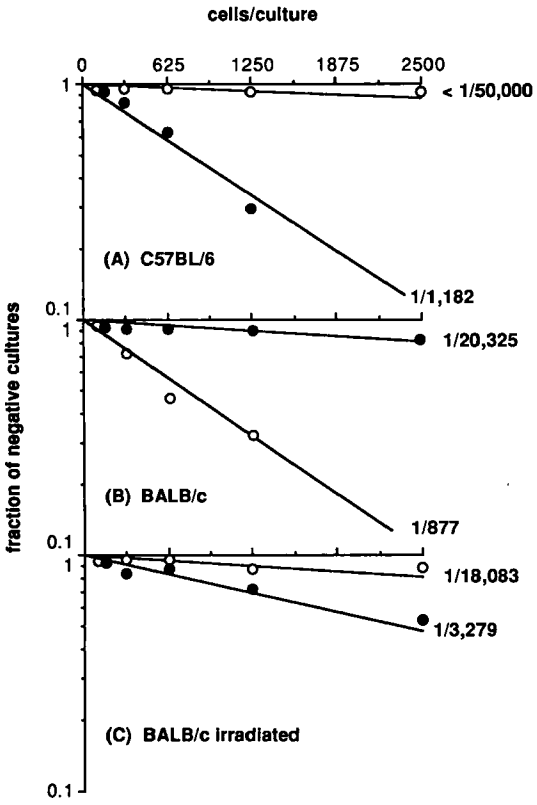

Figure 1. Precursor frequencies of *L. major*-reactive $CD4^+$ cells with the potential for production of IL 4 (○) or IFN-γ (●). Seven weeks after infection with *L. major*, LNC were collected from C57BL/6 mice (A), BALB/c mice (B), or from sublethally irradiated BALB/c mice (C) and were subjected to a cycle of *in vitro* restimulation and rest prior to LD analysis. The results shown represent the average values of two experiments. The numbers indicate the frequencies as estimated by the minimum χ^2 method. All p values > 0.35.

When aliquots of the LD culture SN were tested for the presence of IFN-γ, a different pattern of activity emerged (Table 1). As opposed to IL 4, the frequency of $CD4^+$ cells producing IFN-γ was very low in susceptible BALB/c mice (f = 1/20 325–< 1/100 000). In contrast, resistant C57BL/6 mice manifested significant frequencies of IFN-γ-secreting cells (f = 1/275–1/6250). The strain-dependent difference in the IL 4 and IFN-γ lymphokine activity patterns is illustrated in Fig. 1A, B for mice that had been infected with *L. major* for 7 weeks. It should be noted that the difference between susceptible and resistant mice in the potential for IFN-γ production was already discernible with lymphocytes collected at only 1 week of infection, whereas that for IL 4 activity developed as late as 5 weeks of infection (Table 1).

In the same LD system, $CD8^+$ cells obtained from *L. major*-infected mice at 25 and 70 days of disease, though capable of lymphokine production upon mitogenic stimulus with Con A, showed only marginal activities in response to *L. major* antigen. For any of the lymphokines tested, the frequency of *L. major*-reactive $CD8^+$ cells was 10 to 50 times lower than that of $CD4^+$ cells (data not shown).

3.2 Comparison of T cell lymphokine activities in individual microcultures of LD assays

For frequency determination of lymphokine-secreting T cells from *L. major*-infected mice, the responses for IL 4 and/or IL 2 as well as for IFN-γ were monitored simultaneously in individual microcultures at limiting cell numbers. Therefore, it was possible to analyze whether these activities were accomplished by the same or different populations of *L. major*-reactive T cells. At most time points of infection with *L. major*, C57BL/6 mice gave rise to similar frequencies of $CD4^+$ cells producing IFN-γ and those exhibiting HT-2 growth factor activity. As shown in Fig. 2A for 7 weeks after infection, virtually all microcultures that contained IFN-γ also promoted the growth of HT-2 cells in the absence of Ab. In the presence of anti-IL 2 mAb, on the other hand, the HT-2 response was completely abrogated (Fig. 2B), documenting that the HT-2-stimulating activity of $CD4^+$ cells from *L. major*-infected C57BL/6 mice reflected the production of IL 2 (see also Table 1). Thus, secretion of IFN-γ correlated closely with IL 2 production.

When the lymphokine secretion in individual microcultures of $CD4^+$ cells from BALB/c mice was analyzed, quite a different distribution emerged (Fig. 3A). The vast majority of microcultures was negative for IFN-γ (see also Table 1) and positive in the HT-2 assay in the absence of antibodies. The addition of anti-IL 2 mAb diminished the HT-2 growth factor activity in all microcultures, but a significant propor-

tion of wells was still positive, indicating residual IL4 activity (Fig. 3B). This was confirmed by the complete abrogation of the HT-2 response in the additional presence of anti-IL 4 mAb (data not shown). In all the LD assays performed with T cells from infected BALB/c mice, IL4 production was only detected in wells that also contained significant amounts of IL2. A representative example is depicted in Fig. 4 showing that not a single IL4-containing microwell could be detected in which the HT-2 response was not reduced by anti-IL2 mAb. This was true for all responder cell doses tested.

3.3 Frequencies of lymphokine-producing CD4+ cells in BALB/c mice protected against cutaneous leishmaniasis

The frequency analysis of CD4+ cells from *L. major*-infected mice of resistant or susceptible strains revealed significant differences in their potential for secretion of IL4 and IFN-γ. It was therefore of interest to examine whether the protective treatment of susceptible BALB/c mice would result in an alteration of their lymphokine secretion pattern. For this purpose, BALB/c mice were exposed to a sublethal dose of γ irradiation (450 rad) 20 h prior to infection with *L. major*. This regimen enables the mice to control the disease [1]. At various intervals of infection, their LNC were subjected to LD analysis of selected CD4+ cells, as described above. Fig. 1C shows the frequency of *L. major*-stimulated secretion of IL4 and IFN-γ for lymphocytes collected at 7 weeks of infection, when the cutaneous lesions of irradiated BALB/c mice were healing and significantly smaller than those of non-irradiated mice of the same strain. In comparison to non-irradiated mice, the frequency of IL4-producing cells was drastically reduced in CD4+ lymphocytes from irradiated BALB/c mice, whereas that of IFN-γ-releasing cells was highly

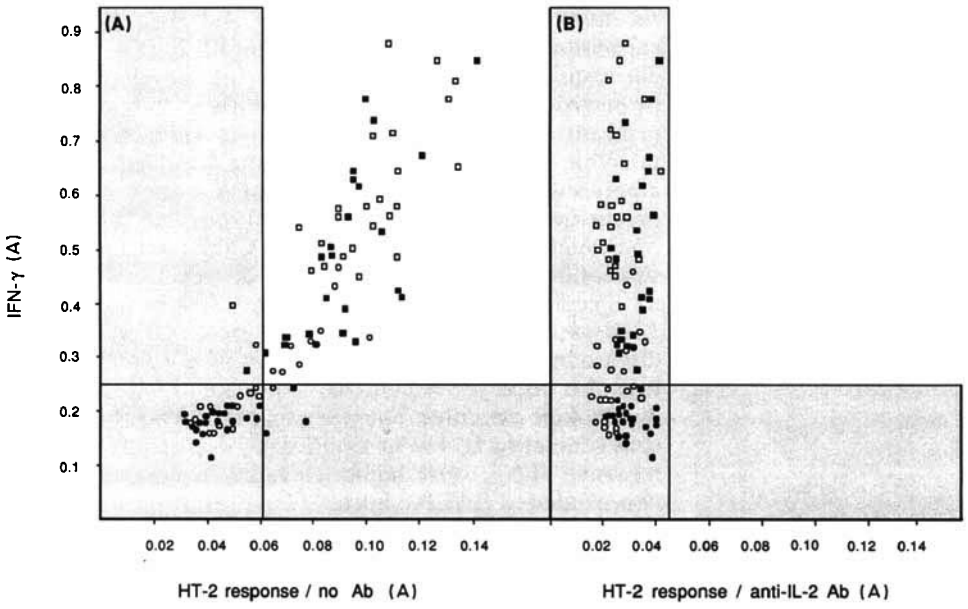

Figure 2. Comparison of lymphokine activities in LD cultures of CD4+ cells obtained from C57BL/6 mice at 7 weeks of infection with *L. major*. Correlation of IFN-γ production with IL2 as well as IL4 production, as indicated by the HT-2 response in the absence of mAb (A), and of IFN-γ production with IL4 production, as indicated by the HT-2 response in the presence of anti-IL2 mAb (B), in individual microcultures of T cells stimulated with *L. major* promastigotes. Twenty-four replicates were set up for each T cell dose and the values are given for 312 (●), 625 (○), 1250 (■) and 2500 (□) cells/microwell. The horizontal and vertical lines represent the mean absorbance values plus 3 SD of control cultures containing APC and Ag in the absence of T cells.

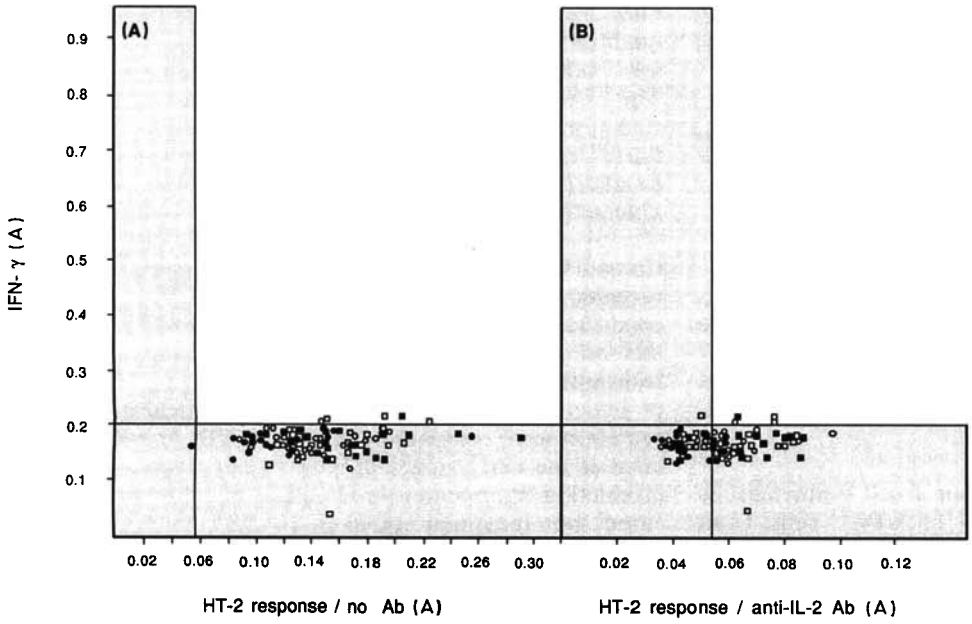

Figure 3. Comparison of lymphokine activities in LD cultures of CD4+ cells obtained from BALB/c mice at 7 weeks of infection with *L. major*. Correlation of IFN-γ production with IL2 as well as IL4 production, as indicated by the HT-2 response in the absence of mAb (A), and of IFN-γ production with IL4 production, as indicated by the HT-2 response in the presence of anti-IL2 mAb (B), in individual microcultures of T cells stimulated with *L. major* promastigotes. The symbols are as in Fig. 2.

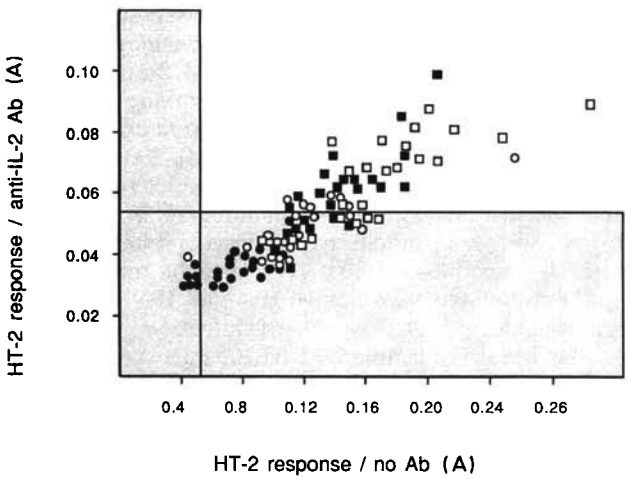

Figure 4. Correlation of the production of IL 2 as well as IL 4, as indicated by the HT-2 response in the absence of mAb, with the production of IL 4, as indicated by the HT-2 response in the presence of anti-IL 2 mAb, in individual microcultures of *L. major*-stimulated CD4+ cells from BALB/c mice that had been infected for 7 weeks. The values are given for 156 (●), 312 (○), 625 (■) and 1250 (□) cells per microwell. The horizontal and the vertical lines represent the mean absorbance values + 3 SD of control cultures containing APC and Ag in the absence of T cells.

Table 2. Frequency of *L. major*-reactive precursor CD4+ cells from irradiated BALB/c mice with the potential for secretion of IL 2, IL 4 or IFN-γ[a)]

Weeks of infection	Reciprocal of precursor cell frequency[b)] HT-2 response in the presence of no mAb IL 2 + IL 4	anti-IL 2 mAb IL 4	ELISA IFN-γ
3	73 (54–112)	43 480 (30 300–83 300)	> 50 000
5	106 (78–166)	15 150 (9 091–55 500)	629 (457–1020)
7	360 (256–602)	18 083 (10 100–83 330)	3 279 (2439–5000)
13	2083 (1562–3125)	> 50 000	2 778 (1887–5263)

a) BALB/c mice were exposed to 450 rad γ-irradiation 20 h before infection with *L. major* promastigotes. At various intervals after infection, lymphocytes were collected from the inguinal LN. See Table 1 for *in vitro* culture conditions and LD analysis of lymphokine activities. All p values > 0.1.
b) 95% confidence limits.

increased. Thus, the ability of genetically susceptible BALB/c mice to contain a *L. major* infection after prophylactic treatment is reflected by an *L. major*-stimulated lymphokine secretion pattern resembling that of genetically resistant C57BL/6 mice.

The entire time course of precursor T cell frequencies is given in Table 2. The frequency of CD4+ cells from irradiated mice inducing HT-2 growth was very similar to that of cells from non-irradiated mice. It was predominantly based on IL 2 because there was a pronounced reduction in the presence of anti-IL 2 mAb; *i.e.* the frequency of IL 4-producing cells remained very low during the entire course of disease. This time course was in contrast to genetically resistant C57BL/6 mice which gave rise to considerable frequencies of IL 4-producing cells at the onset of infection. On the other hand, IFN-γ production could not be detected in irradiated BALB/c mice at 3 weeks of infection, but was elicited by a high number (f = 1/699, Table 1) of T cells from C57BL/6 mice at that time. In conclusion, even though the lymphokine secretion patterns of protected BALB/c mice and genetically resistant C57BL/6 mice were similar at later stages of disease and after healing, the frequencies of CD4+ cells producing IL 4 and IFN-γ were divergent at the early phase of infection.

4 Discussion

In the present study, an LD system has been used for evaluation of the T cell reaction to *L. major* both in terms of the numbers of responding T cells and their functional capacities. Since CD4+ cells secreting IL 2, IL 4 or IFN-γ are considered to be crucial for the development of protective immunity, we compared their frequencies in resistant and in susceptible mice at various times of infection with *L. major*. The results revealed different kinetics with regard to the release of IL 4 and IFN-γ. The precursor frequencies of CD4+ cells producing IFN-γ were much higher in genetically resistant C57BL/6 mice than in susceptible BALB/c mice, whereas the reverse was found for T cells with the capacity to secrete IL 4 in response to *L. major*. BALB/c mice that were protected against the disease by pretreatment with a sublethal dose of γ irradiation also displayed healer characteristics in terms of their lymphokine activities. The precursor frequencies of CD4+ cells generating IL 4 were significantly decreased and those releasing IFN-γ were highly elevated as compared with non-irradiated BALB/c mice.

The present study documents for the first time that IL 4 is expressed not only on the mRNA level [13] but can be detected as biologically functional lymphokine secreted by freshly isolated lymphocytes from susceptible BALB/c mice infected with *L. major*. Furthermore, we provide quantitative data by determining the frequencies of precursor T cells with the potential for lymphokine production. The LD system used for this purpose allows the examination of purified T cell subpopulations at the clonal level and the selective quantitation of T cells responding to *L. major*. In addition, it enables the comparison of different lymphokine activities raised by individual T cell populations.

Comparison of the time course of precursor frequencies revealed some remarkable differences for IL 4 and IFN-γ production in susceptible and resistant mice. The strongly increased potential for IFN-γ production of T cells from resistant C57BL/6 mice was already detectable at the onset of infection, whereas the precursor frequencies for IL 4 secretion were comparable in both C57BL/6 and BALB/c mice at the early stages of disease. At later stages, IL 4 production disappeared in C57BL/6 mice as their lesions cured, but remained stable in BALB/c mice with well-established skin ulcers. These findings indicate that the production of IFN-γ may be crucial for the generation of an effective anti-leishmanial immune response immediately

after inoculation of the parasites. The presence of precursor cells for IL 4 production does not seem to interfere with this effect in resistant mice but may sustain the infection in IFN-γ-deficient mice of susceptible strains. However, the kinetics of precursor frequencies at the early stage of infection were quite different in congenitally resistant C57BL/6 as compared with protectively pretreated BALB/c mice, even though their lymphokine secretion patterns were similar at later stages of disease and after healing. These results strongly suggest that the development of lymphokine-producing T cells is regulated differently in these mice and that as yet unknown and independent mechanisms may exist for the establishment of protective immunity.

In contrast to $CD4^+$ cells secreting IL 4 or IFN-γ, the frequencies of precursor cells for IL 2 production were very similar in resistant and in susceptible mice at any time of infection with *L. major*. This observation explains why Heinzel et al. [13] could not find a relationship between IL 2 mRNA expression and the outcome of Leishmania infection. These authors suggested that the generation of IL 2 in susceptible mice may be predominantly associated with $CD8^+$ cells. However, our present study clearly demonstrates that a high number of $CD4^+$ cells from these mice are capable of producing IL 2 and that the contribution of $CD8^+$ cells seems to be of little importance. Thus, $CD4^+$ cells from resistant mice generate mainly IL 2 and IFN-γ, whereas $CD4^+$ cells from susceptible mice give rise to IL 2 as well as IL 4.

It has been proposed that resistance in experimental leishmaniasis is conferred by T_H1 cells secreting IL 2 and IFN-γ, while susceptibility is associated with T_H2 cells secreting IL 4 and IL 5 [13, 15]. The segregation analysis performed in the current study allowed the comparison of different lymphokine activities exhibited by the progeny of individual T cells responding to *L. major*. It was found that $CD4^+$ cells from resistant C57BL/6 mice displayed the properties of T_H1 because secretion of IFN-γ closely correlated with IL 2 production. In contrast, susceptible BALB/c mice gave rise to precursor $CD4^+$ cells with potential for IL 4 production as well as to those able to generate IL 2. Surprisingly, IL 4 secretion was only detected in microwells that also contained IL 2 activity. It may be argued that two distinct *L. major*-reactive precursor T cells expanded in these cultures and that the proliferation of IL 4-secreting T cells was dependent on IL 2 produced by the other T cell population [16]. However, in the present study this seems unlikely because exogenous IL 2 had been provided to LD cultures during the expansion phase, before the final restimulation, and because the co-existence of IL 2 and IL 4 production was also observed with very low numbers of responder cells ensuring the clonal origin of *L. major*-specific T cells. Alternatively, the functionally defined T_h subsets may not be members of separate $CD4^+$ cell lineages but may reflect consecutive differentiation stages of $CD4^+$ cells with T_H1 cells developing into T_H2 cells upon restimulation. This possibility has previously been discussed by several authors [16, 30, 31]. It would be in analogy to the human system, where the functionally distinct T cell subsets $CD45R^+$ and $CDw29^+$ have been shown to represent distinct activation stages but not separate cell lineages [32]. In murine cutaneous leishmaniasis, we have recently shown that a disease-promoting effect can only be mediated by activated $CD4^+$ cells from chronically infected BALB/c mice, expressing the surface marker Ly-24 (CD44), but not by resting $CD4^+Ly\text{-}24^-$ cells [33]. In the present LD studies, it is notable that IL 2 production by $CD4^+$ cells from susceptible mice was never paired with significant secretion of IFN-γ. Thus, these $CD4^+$ cells do not represent typical T_H1 cells which have originally been classified on the basis of studies with long-term cultured T cell clones. The development of $CD4^+$ cells into T_H1 or T_H2 cells, or intermediates thereof, may depend on the strain of mice infected with *L. major*, the composition of the antigen [15, 34], or the type of APC. The LD system used here for studying the T cell response to whole *L. major* promastigotes is well suited to test these possibilities.

The role of IFN-γ as a MΦ-activating factor that enhances the anti-leishmanial defence has been described repeatedly [7–9]. The functions of IL 2 and IL 4 in leishmaniasis, on the other hand, are still not understood. Some studies suggested that a MΦ-mediated suppression of the IL 2 response may contribute to disease progression in infected BALB/c mice [35, 36], whereas another report showed exacerbation of lesions after treatment with IL 2 [37]. IL 4 has been shown to induce both protective immunity to Leishmania infection [38] and impairment of the anti-leishmanial effects of IFN-γ [39]. It is conceivable that these lymphokines are able to mediate quite different effects depending on the availability of other cytokines. Such a multipotential activity has recently been shown by us for TNF which was found to be expressed in *L. major*-infected mice of both resistant and susceptible strains [40]. *In vitro* studies demonstrated that in combination with IFN-γ, TNF induced the elimination of parasites in infected MΦ, whereas it promoted parasite burden in the presence of IL 4 [41]. These findings emphasize the need to study the complex interactions between T cell subsets and cytokines at the clonal level as well as the nature of the antigens that induce them.

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