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L3T4$^+$ T cells promoting susceptibility to murine cutaneous leishmaniasis express the surface marker Ly-24 (Pgp-1)*

In a murine model of cutaneous leishmaniasis, the importance of T cell-dependent immunity has been documented by the susceptibility to parasite infection of athymic nude mice of both genetically resistant and genetically susceptible strains. T lymphocytes from uninfected mice have the capacity to promote resistance to *Leishmania major* infection in nude recipients, whereas T cells from mice chronically infected with *L. major* not only fail to mediate protection, but totally abrogate the host-protective effect of normal mouse lymphocytes. Both these effects are mediated by T cells which have the phenotype L3T4$^+$Ly-2$^-$. To discriminate between the two activities, we have tried to separate the L3T4$^+$ population on the basis of additional cell surface markers and we have found that peripheral L3T4$^+$ lymphocytes could be subdivided according to their expression of the Ly-24 (Pgp-1) surface marker. In adoptive transfer experiments, both the Ly-24$^+$ and the Ly-24$^-$ subset of L3T4$^+$ cells from uninfected mice had the capacity to mediate resistance to infection with *L. major*. However, disease-promoting activity was only found in the L3T4$^+$Ly-24$^+$ and not in the L3T4$^+$Ly-24$^-$ subset of cells from mice with chronic cutaneous disease. Moreover, Ly-24 expression was strongly increased in lymphocytes from chronically infected mice and *in vitro* limiting dilution analysis confirmed that the vast majority of *L. major*-reactive T cells was L3T4$^+$Ly-24$^+$. In genetically susceptible mice with chronic cutaneous leishmaniasis, Ly-24 therefore appears to be a marker for lymphocytes with the capacity to abrogate resistance to disease, these cells being activated and expanding in the course of progressive *L. major* infection. Ly-24 expression is a useful tool for phenotypic identification and selective enrichment of antigen-activated and possibly memory T cells. It may facilitate the isolation of *L. major*-specific T cell clones with defined activities.

1 Introduction

Human cutaneous leishmaniasis is caused by the protozoan parasite *Leishmania major* and the spectrum of clinical manifestations seen in humans can be reproduced experimentally in mice. The outcome of the disease in mice depends on the strain of animals used. In the course of infection *L. major* promastigotes invade host macrophages, inside which they transform to amastigotes and expand. Mice of genetically resistant inbred strains (*e.g.* C57BL/6) can control the infection with lesions healing completely, whereas genetically susceptible mice (*e.g.*, BALB/c) develop a progressive disease with lethal outcome. However, athymic nude mice of both healer and nonhealer genotypes are highly susceptible [1], demonstrating the significance of T cell-dependent immunity in resistance to cutaneous disease [2–4]. Early studies showed that T lymphocytes from uninfected mice have the capacity to promote resistance to *L. major* infection in nude recipients, whereas T cells from mice chronically infected with *L. major* generally not only fail to mediate protection, but can totally abrogate the host-protective effect of normal mouse lymphocytes [5]. T cells from uninfected mice with resistance-promoting activity and T cells from chronically infected mice with disease-promoting activity both express the L3T4$^+$Ly-2$^-$ phenotype.

Previous studies have indicated functional heterogeneity of L3T4$^+$ cells, showing that antigen-specific and major histocompatibility complex (MHC) class II-restricted cloned murine T helper (T_h) cells of this phenotype give rise to differential patterns of lymphokine production [6, 7]. The L3T4$^+$ T cell clones were classified into two categories [7], designated T_H1, producing interleukin (IL) 2 and interferon-γ (IFN-γ), and T_H2, producing IL 4 and IL 5. The different roles of these T cell types in the pathogenesis of *L. major* infections was suggested by the recent studies of Locksley et al. [8], which showed lymph node cells (LNC) from healer mice to be high producers of IFN-γ and those from nonhealer mice to release only minimal amounts of the same lymphokine. On the other hand, only lymphoid cells from nonhealer mice contained IL 4 mRNA.

Patterns of lymphokine production allow the identification of functionally distinct L3T4$^+$ cells. However, it would be particularly useful to be able to distinguish L3T4$^+$ subsets on the

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Abbreviations: **Con A SN:** Supernatant from concanavalin A-stimulated rat spleen cells **CTLL:** Cloned cytotoxic T lymphocyte line **FACS:** Fluorescence-activated cell sorter **FITC:** Fluorescein isothiocyanate **IFN-γ:** Interferon-γ **IL:** Interleukin **LD:** Limiting dilution **LNC:** Lymph node cell(s) **mAb:** Monoclonal antibody(ies) **MHC:** Major histocompatibility complex **PE-Av:** Phycoerythrin-conjugated avidin **SC:** Spleen cell(s) **SN:** Supernatant **T_h:** T helper (lymphocyte)

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basis of surface antigen expression, since this would allow their separation and functional characterization. Furthermore, it could facilitate the isolation of T cell clones with defined activities. During a screening for lymphocyte surface markers which might subdivide the L3T4$^+$ population, we observed that a monoclonal antibody (mAb) directed against Ly-24 (Pgp-1), previously shown to be expressed by a variety of cell types such as immature thymocytes and myeloid cells [9], could identify a subset of peripheral L3T4$^+$ lymphocytes. At the same time, this finding was reported by others [10, 11] who showed that Ly-24 expression was induced by mitogen or antigen stimulation suggesting that Ly-24 is a T cell activation antigen.

In the present study, L3T4$^+$ cells expressing or lacking the Ly-24 alloantigen were separated by sorting in a fluorescence-activated cell sorter (FACS) and were examined for their ability to induce disease resistance, or abrogation of resistance, in nude mice infected with *L. major*. It was demonstrated that both L3T4$^+$Ly-24$^+$ and L3T4$^+$Ly-24$^-$ cells could mediate resistance to infection. On the other hand, only L3T4$^+$Ly-24$^+$ but not L3T4$^+$Ly-24$^-$ cells promoted permissiveness to disease. Furthermore, the proportion of T cells expressing Ly-24 was highly increased in mice chronically infected with *L. major*. The data are consistent with the notion that Ly-24 is a marker for disease-promoting T cells that have been activated in the course of *L. major* infection. Lymphocyte selection according to Ly-24 expression may provide an important tool for the isolation of *L. major*-specific T cell clones.

2 Materials and methods

2.1 Mice

Female mice of the inbred strains BALB/c and C57BL/6 were used at an age of 7 to 10 weeks; female congenitally athymic BALB/c.nu/nu (nude) mice were 6 to 8 weeks of age at the commencement of experiments. All mice were bred under specific pathogen-free conditions at The Walter and Eliza Hall Institute of Medical Research but, during experimentation, were maintained under conventional conditions in an isolation facility.

2.2 mAb and fluorescence reagents

The mAb were prepared from supernatants (SN) of hybridomas grown at the Hall Institute, purified and conjugated where noted in this laboratory. Antibodies directed against Ly-24 (Pgp-1), from the hybridoma IM7.8.1 [12], Mac-1, from the hybridoma M1/70 [13], and Ly-2.2, from the hybridoma HO2/2 [14] were used as culture SN. Anti-Thy-1.2 antibodies, from hybridoma F7D5 [15], were kindly provided by Dr. Phil Lake. Anti-L3T4 antibodies, from the hybridoma GK1.5 [16], and anti-Ly-2 antibodies, from the hybridoma 53-6.7 [17], were used as biotin-conjugated purified protein. A fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Silenus Laboratories, Melbourne, Australia), cross-reacting with rat immunoglobulin (Ig), and avidin conjugated to phycoerythrin (PE-Av; Becton Dickinson, Mountain View, CA) were used as second-stage reagents for fluorescence staining. Rat Ig, for blocking residual second-stage Ig reagents, was purchased from Miles Laboratories, Elkhart, IN.

2.3 Parasites and preparation of antigen

The cloned virulent *L. major* parasite line V121 was produced from the human isolate LRC-L137 [18] and maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood/agar cultures [1]. Stationary-phase promastigotes were washed and 2×10^6 organisms were injected in a volume of 50 µl intradermally (i.d.) on the dorsum of the mouse close to the base of the tail.

Antigen preparations for immunization and *in vitro* stimulation of lymphocytes were generated by UV-irradiation (254 nm) of *L. major* promastigotes for 20 min. This treatment resulted in killing of the parasites, as judged by staining with ethidium bromide/acridine orange [1 µg/ml in phosphate-buffered saline (PBS)] and their failure to infect mice, but did not cause disruption of the organisms. This preparation was found to be superior to parasites killed by freezing and thawing for use for *in vitro* stimulation of lymphocytes.

2.4 Immunization and cell preparations

Immunization of mice with *L. major* promastigotes was performed by i.v. injection with three weekly doses of 2×10^7 UV-irradiated promastigotes in 0.1 ml of PBS. Spleen cell (SC) suspensions were prepared 5–7 days after the last immunization and T cells were obtained by nylon wool purification [19] for use in *in vitro* cultures. For reconstitution of nude mice, SC and/or mesenteric, inguinal and axillary LNC were obtained from normal mice or from mice chronically infected with *L. major* parasites (*i.e.*, 60–100 days after infection). Lymphocyte subpopulations were purified by fluorescence staining and subsequent cell sorting and were injected i.p. in a volume of 0.2 ml.

2.5 Fluorescence staining and cell sorting

The basic procedures have been described in detail [20]. For phenotype analysis using two-color cytometry, 3×10^6 lymphocytes in 30 µl were stained sequentially with anti-Ly-24 (Pgp-1) antibodies, FITC-conjugated anti-Ig antibodies and rat Ig to block any free binding site on the latter reagent, followed by either biotin-labeled anti-Ly-2 or anti-L3T4 antibodies and PE-Av. Propidium iodide (0.5 µg/ml; Calbiochem, La Jolla, CA) was included in the final wash to stain dead cells and facilitate their exclusion from flow cytometric analysis. Cell sorting for LD cultures was performed after staining lymphocytes (10^8/ml) as described above. Cell sorting for adoptive transfer experiments was based on a one-color staining with anti-Ly-24 antibodies followed by FITC-conjugated anti-Ig. No propidium iodide was included when the sorted cells were prepared for nude mouse reconstitution. All reagents were used at near-saturating conditions and the reaction time was 20 min on ice for each step in siliconized glass tubes. The fluorescence-activated cell sorter was a modified FACS II (Becton Dickinson, Sunnyvale, CA). Details of the instrument and its use in two-color (red and green fluorescence) sorting have been given elsewhere [20]. Dead cells and debris were excluded from analysis and sorting on the basis of both low-angle light scatter and the strong red fluorescence from propidium iodide staining.

Since Ly-24 expression by peripheral T cells was continuous rather than bimodal, an appropriate control sample, stained

with the second-stage antibodies alone, was used to determine the cut-off point between Ly-24$^-$ and Ly-24$^+$ cells for each sorting procedure. The sorting windows were set to collect negative (see Fig. 1) and positive cells leaving a gap of 20 to 25 channels between the sorting windows. The purity of the sorted subpopulations was controlled by subsequent re-analysis in the FACS.

2.6 Cytotoxic procedures

Single-cell suspensions of SC or splenic T cells at a concentration of 10^7/ml were incubated for 40 min on ice with either anti-Thy-1.2 (for preparation of T cell-depleted stimulator cells) or anti-Ly-2.2 (for preparation of L3T4$^+$ responder cells) antibodies and for another 20 min at 37 °C with rabbit complement in a final dilution of 1 : 15 as described [20]. Allowance was made for the warm-up time in the second-incubation period, and DNase (50 μg/ml) was added to reduce clumping problems and improve recovery.

2.7 LD analysis

Splenic T cells, L3T4$^+$ T cells, or sorted L3T4$^+$ T cell subsets from *L. major*-immunized mice were cultured in limiting numbers in round-bottom microtiter wells (Linbro, Flow Laboratories, North Ryde, N.S.W., Australia) with 2×10^5 irradiated (3000 rad) syngeneic spleen accessory cells from normal mice in 0.2 ml of a modified RPMI culture medium (supplemented with 32 mM Hepes, 10^{-4} M 2-mercaptoethanol and 10% fetal calf serum) containing 15% of a SN from concanavalin A-stimulated rat SC cultures (Con ASN) as a source of IL 2. For each T cell concentration ranging from 10^4 to 150 cells/well, replicates of 24 wells were set up in the absence or in the presence of 2×10^5 UV-irradiated *L. major* promastigotes as a source of antigen. After 7 days of incubation (37 °C, 10% CO_2), microcultures were washed three times to remove Con ASN and were restimulated by adding 2×10^5 irradiated (3000 rad) T cell-depleted syngeneic SC in the absence or presence of antigen (2×10^5 UV-irradiated *L. major* promastigotes), respectively, in 0.2 ml culture medium. After 24 h of incubation, 150 μl of SN was removed for determination of IL 2 and IL 3 lymphokine activity. Cultures containing T cells and accessory cells but not antigen were used as controls for each T cell concentration. Antigen-stimulated microcultures were scored as positive when the values of lymphokine activity exceeded the arithmetic mean of the control wells by more than 3 SD. Minimal estimates of the precursor frequency for each activity were obtained by the maximum likelihood method from the Poisson distribution relationship between the number of responding cells and the logarithm of the fraction of negative cultures [21].

2.8 Assays for IL 2 and IL 3 activity

Microculture SN was tested for IL 2 or IL 3 activity by incubation with the cloned IL 2-dependent cytotoxic T lymphocyte line (CTLL) [22] or the IL 3-dependent hematopoietic cell line 32D [23, 24], respectively. The cell lines were kindly provided by Dr. Anne Kelso of this Institute. Of SN 50 μl were cultured with 4×10^3 CTLL cells or 2×10^3 32D cells in a total of 100 μl RPMI 1640 culture medium in flat-bottom microwells (Linbro, Flow Laboratories). After 20 h (CTLL assay) or 44 h (32D assay), 1 μCi = 37 kBq [^{3}H]thymidine was added for a further 4–5 h. Cells were harvested onto filter paper strips using an automated cell harvester and counted in a liquid scintillation β-counter.

2.9 Assessment of lesions

Lesion scores were determined at regular intervals according to the following system: 0 = no lesion or healed scar; 1 = small swelling (up to 5 mm in average diameter); 2 = large swelling (more than 5 mm in average diameter) or open lesion of less than 5 mm in average diameter; 3 = open lesion of 5–10 mm in diameter; 4 = open lesion greater than 10 mm in diameter and/or obvious metastases. Data are expressed as the arithmetic mean of the lesion scores for the groups of four to six mice, and the number of mice with lesions relative to the total number of mice per group as well as standard errors are given for the last time point of each experiment.

3 Results

3.1 Expression of the Ly-24 antigen on peripheral L3T4$^+$ T cells

Two-color flow cytometry demonstrated that peripheral L3T4$^+$ lymphocytes could be phenotypically subdivided according to expression of the Ly-24 surface marker. In normal BALB/c mice, 72% of L3T4$^+$ LNC were Ly-24$^+$ and 28% were Ly-24$^-$ (Fig. 1). This distribution of Ly-24 expression was very similar for SC of the same strain. SC and LNC from normal C57BL/6 mice can also be separated into a L3T4$^+$ Ly-24$^-$ and a L3T4$^+$ Ly-24$^+$ subset, the proportion of Ly-24$^+$ cells being lower than in BALB/c mice (data not shown). These findings are in agreement with recent reports by others showing that a minor population of L3T4$^+$ LNC and SC expresses Ly-24 in C57BL/6 mice [10, 25], whereas in BALB/c mice, the majority of peripheral L3T4$^+$ cells is Ly-24$^+$ [25].

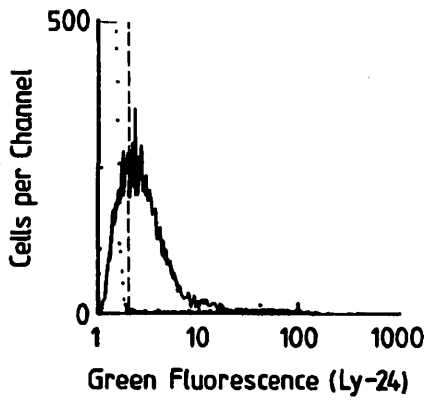

Figure 1. Subdivision of L3T4$^+$ lymphocytes according to Ly-24 expression. LNC from normal BALB/c mice were sequentially treated with anti-Ly-24 antibodies, FITC-conjugated anti-mouse antibodies, biotin-conjugated anti-L3T4 antibodies and PE-Av and were subjected to two-color fluorescence analysis in the FACS. As control, cells were treated only with anti-L3T4 antibodies and the second-stage reagents. The profiles show the green fluorescence (Ly-24 expression) of L3T4$^+$ (red positive) cells stained in the presence (solid line) or absence (dotted line) of anti-Ly-24 antibodies. The dashed line indicates the upper window used for sorting negative cells.

In the course of screening for lymphocyte surface markers which might subdivide peripheral L3T4$^+$ lymphocytes, we also identified two other antigens that define subsets of these lymphocytes in untreated mice (data not shown). These were the alloantigen Ly-6 [26] and a lymphocyte homing molecule defined by the antibody MEL-14 [27].

3.2 Reconstitution of BALB/c nude mice with Ly-24$^+$ cells or Ly-24$^-$ cells from normal BALB/c mice

We have previously shown that small numbers (10^5 to 10^7) of T lymphocytes from normal BALB/c mice have the capacity to induce resistance to infection with *L. major* parasites in syngeneic athymic nude mouse recipients and that these resistance-promoting T cells belong to the L3T4$^+$ subpopulation [5, 28]. To examine whether the host-protective activity resides in the Ly-24$^+$ or the Ly-24$^-$ subset of normal mouse lymphocytes, we separated these cells in the FACS and used them for adoptive transfer into nude mice.

For the selection of lymphocyte subsets, LNC from uninfected euthymic BALB/c mice were stained with anti-Ly-24 and anti-Mac-1 antibodies followed by FITC-conjugated anti-Ig. This staining procedure separated the brightly fluoresceinating B cells and macrophages from the less brightly stained Ly-24$^+$ T cells and the fluorescence-negative Ly-24$^-$ population. Unseparated lymphocytes (10^6), or the sorted Ly-24$^+$ and Ly-24$^-$ lymphocyte subsets, in numbers equivalent to their original proportion in 10^6 unseparated LNC, were injected into BALB/c nude mice that were subsequently challenged with 2×10^6 *L. major* promastigotes. As a control for the infectivity of the parasites, another group of mice received promastigotes in the absence of lymphocytes. The course of cutaneous lesion development (Fig. 2) shows that both Ly-24$^+$ and Ly-24$^-$ cells from normal BALB/c mice could transfer protective immunity as efficiently as unseparated cells. Since previous studies have documented that total resistance in reconstituted nude mice is promoted by L3T4$^+$ cells only [28], it can be concluded that host-protective cells occur amongst both L3T4$^+$ Ly-24$^+$ and L3T4$^+$ Ly-24$^-$ T cells.

3.3 Effect of Ly-24$^+$ and Ly-24$^-$ cells from chronically infected BALB/c mice on BALB/c nude mice reconstituted with normal BALB/c lymphocytes

In contrast to lymphocytes from uninfected euthymic mice, lymphoid cells from euthymic BALB/c mice chronically infected with *L. major* parasites generally fail to induce resistance in BALB/c nude mice. Moreover, an inoculum of 10^6 or more lymphoid cells from mice with progressive disease totally abrogates the protective effect of 10^5 to 10^7 normal mouse lymphocytes and this disease-promoting activity has been shown to be mediated by L3T4$^+$ T cells ([5] and Moll, unpublished observations). To determine whether disease-promoting cells are represented in both the Ly-24$^+$ and the Ly-24$^-$ population, we separated these cells by FACS sorting and injected them into nude mice that were reconstituted with unseparated lymphocytes from uninfected mice at the same time.

As a source of disease-promoting cells, LNC were obtained from euthymic BALB/c mice with chronic disease and large cutaneous lesions of score 3. These cells were stained by treatment with anti-Ly-24 and anti-Mac-1 antibodies followed by FITC-conjugated anti-Ig. As described above, Ly-24$^-$ cells and Ly-24$^+$ T cells could be clearly distinguished from B cells and macrophages and were collected separately. For injection into nude mice, 5×10^5 unseparated SC and LNC from unin-

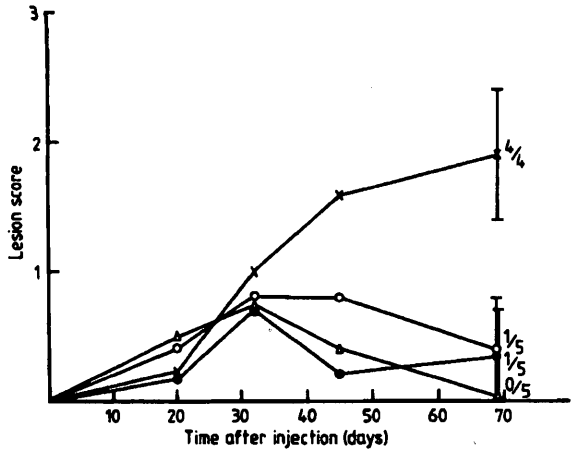

Figure 2. Resistance-promoting cells are present in both the Ly-24$^+$ and the Ly-24$^-$ subset of lymphocytes from uninfected mice. On day 0, four or five BALB/c nude mice were injected i.d. with 2×10^6 promastigotes of *L. major* and i.p. with LNC from uninfected euthymic BALB/c mice which were either unselected (10^6, △) or had been separated into the Ly-24$^+$ (●) and Ly-24$^-$ (○) subsets by sorting in the FACS and were given in numbers equivalent to their original proportion in 10^6 unselected cells. Control mice received promastigotes alone (X). Arithmetic means of the cutaneous lesion scores are given for certain time points and the number of mice with lesions relative to the total number of mice/group as well as SE are given for the last time point.

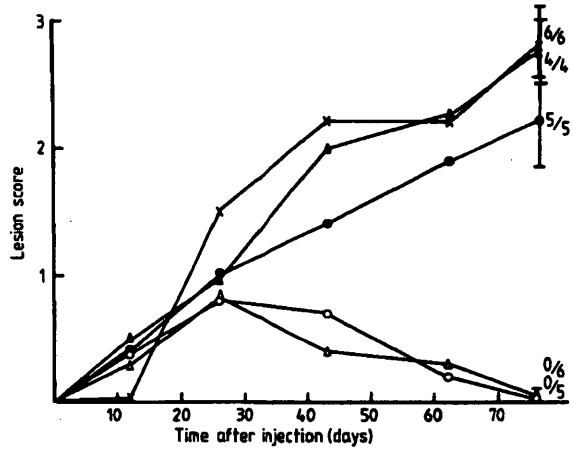

Figure 3. Disease-promoting cells can be found in the Ly-24$^+$ subset but not in the Ly-24$^-$ subset of lymphocytes from chronically infected mice. On day 0, four to six BALB/c nude mice were injected i.d. with 2×10^6 *L. major* promastigotes and i.p. with 5×10^5 normal BALB/c lymphocytes alone (△) or together with lymphocytes from BALB/c mice chronically infected with *L. major*. The latter were either unselected (10^6, ▲) or separated into the Ly-24$^+$ (●) and Ly-24$^-$ (○) subsets by FACS sorting and were given in numbers equivalent to their original proportion in 10^6 unselected cells. Control mice received promastigotes alone (X). Arithmetic means of the cutaneous lesion scores are given for certain time points and the number of mice with lesions relative to the total number of mice/group as well as the SE are given for the last time point.

fected euthymic BALB/c mice were mixed with sorted Ly-24+ or Ly-24− cells from infected mice in numbers equivalent to their original proportion in 10^6 unseparated cells. The nude mouse recipients were subsequently challenged with 2×10^6 *L. major* promastigotes and the course of cutaneous lesion development was determined. The data in Fig. 3 show that 5×10^5 normal mouse lymphocytes induced resistance to cutaneous disease and that a mixture of these cells and unseparated cells from diseased mice was not protective. Furthermore, it can be seen that the disease-promoting activity of lymphocytes from chronically infected mice resided in the Ly-24+ population, whereas Ly-24− cells failed to abrogate the host-protective effect of normal mouse lymphocytes.

3.4 Expression of Ly-24 on LNC from BALB/c mice chronically infected with *L. major*

In the adoptive transfer experiments using lymphocyte subsets from chronically infected mice, disease-promoting cells could only be detected in the Ly-24+ population. It was, therefore, of interest to examine whether Ly-24 expression was increased in BALB/c mice with late-stage cutaneous disease and with a very efficient resistance-inhibiting activity, as compared to uninfected mice which can give rise to resistance-promoting activity. For this experiment, we used euthymic BALB/c mice that had been infected with *L. major* for 3 months and showed severe cutaneous lesions (score 3 to 4). Lymphocytes were obtained from the inguinal LN that drain the lesion site and were analyzed by two-color immunofluorescence after staining with anti-Ly-24 and either anti-L3T4 or anti-Ly-2 antibodies.

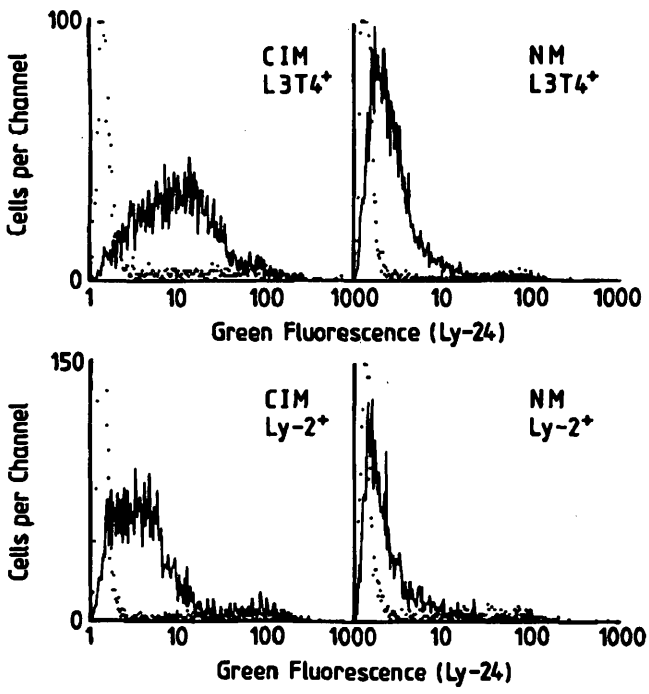

Figure 4. Ly-24 expression in L3T4+ and Ly-2+ lymphocytes from chronically infected mice. Inguinal LNC from uninfected BALB/c mice (NM) or from BALB/c mice chronically infected (CIM) with *L. major* were stained with anti-Ly-24 antibodies and either anti-L3T4 or anti-Ly-2 antibodies and were subjected to two-color fluorescence analysis in the FACS. The profiles show the Ly-24 expression by L3T4+ or Ly-2+ cells (solid lines). Controls were stained with anti-L3T4 or anti-Ly-2 alone (dotted lines).

As a control, LNC from uninfected euthymic BALB/c mice were treated in the same way.

The results show (Fig. 4) that Ly-24 expression was significantly increased in both the L3T4+ population and the Ly-2+ population of T cells from chronically infected mice. The increase in Ly-24 expression was more pronounced in L3T4+ cells than in Ly-2+ cells thus suggesting that it is predominantly the disease-promoting L3T4+ population that gets activated in the course of disease. No increase in Ly-24 expression could be found in SC from diseased mice (data not shown).

In a similar experiment, we also analyzed Ly-24 expression in genetically resistant C57BL/6 mice that were either untreated or infected with *L. major*. Mice of this strain usually develop only small cutaneous lesions that heal within 1 to 2 months after infection. At the peak of lesion development, there was a moderate increase in the proportion of Ly-24+ cells in both the L3T4+ and the Ly-2+ population of SC and LNC. However, no significant increase in Ly-24 expression could be found after healing of lesions (data not shown).

3.5 Frequency determination of *L. major*-specific precursor cells in T lymphocyte subpopulations from immunized mice

A LD system was established to estimate the frequency of *L. major*-specific precursor cells in different T lymphocyte subpopulations. We observed that T cells from mice infected with live parasites showed *in vitro* responses independent of the presence of antigen in culture. However, when responder cells were obtained from mice that had received several immunizations with UV-inactivated *L. major* parasites, antigen-specific T cell responses could be detected. Limiting numbers of T lymphocytes were stimulated *in vitro* with an optimal concentration of the same parasite antigen preparation and accessory cells in the presence of Con ASN. As a control, a second series of limiting dilution cultures was set up in the absence of antigen for each responder cell population. For detection of *L. major*-reactive T cells, the lymphokine production after restimulation of 7-day-old cultures was found to be much more antigen-specific and gave considerably lower background activity than lymphoproliferation. Hence, SN from individual microcultures were split and assayed for IL 2 and IL 3 activity as a read-out system for LD analysis.

T cell subpopulations were prepared either by cytotoxic treatment (anti-Ly-2.2 antibodies and complement) of nylon wool-purified T cells, to obtain L3T4+ lymphocytes, or by FACS sorting, to obtain Ly-24+ and Ly-24− subsets of L3T4+ lymphocytes after a two-color staining procedure. Table 1 summarizes the frequencies (f) of *L. major*-specific precursor cells in various T lymphocyte subpopulations from immunized or untreated C57BL/6 or BALB/c mice. The data demonstrate that *in vivo* immunization with *L. major* antigen resulted in a dramatic increase of the frequency of *L. major*-specific T cells (f = 1/4950, as compared to f = 1/80000 – 1/357000 in untreated mice) with antigen-reactive cells being enriched in the L3T4+ subpopulation (f = 1/1516 – 1/3472). For individual cell populations, frequencies based on IL 2 production were in the same range as those based on IL 3 production but the values were generally somewhat higher in C57BL/6 than in BALB/c mice. Correlation of the two lymphokine activities showed that virtually all microcultures with IL 2 activity also contained IL 3 and some additional cultures contained IL 3 only (data not shown).

Table 1. Frequency of *L. major*-specific precursor T cells[a)]

Treatment of mice	Cell population	Reciprocal of precursor frequency (95% confidence limits)			
		C57BL/6		BALB/c	
		IL 2	IL 3	IL 2	IL 3
Immunized	T	4950 (3865–7077)	ND[b)]	ND	ND
	L3T4$^+$	2271 (1770–3167)	1516 (1172–2145)	3453 (2577–5230)	3472 (2592–4995)
	L3T4$^+$Ly-24$^+$	1598 (1522–1682)	648 (622–677)	2962 (2260–4299)	2282 (1690–3533)
	L3T4$^+$Ly-24$^-$	35842 (35258–40322)	13263 (12438–14205)	ND	ND
None	T	82550 (47170–263158)	ND	ND	ND
	L3T4$^+$	80000 (42735–162500)	357000 (181800–995900)	80645 (48780–232560)	116280 (68490–384610)

a) LD analysis of precursor T cells with potential for IL 2 or IL 3 production in C57BL/6 or BALB/c mice which were either unprimed or had been immunized with 2×10^7 UV-irradiated *L. major* promastigotes three times at weekly intervals. Five days after the last immunization, T cell subpopulations were prepared either by treatment with anti-Ly-2.2 antibodies and complement, to obtain L3T4$^+$ lymphocytes, or by two-color FACS sorting, to obtain Ly-24$^+$ and Ly-24$^-$ subsets of L3T4$^+$ lymphocytes. Limiting numbers of T cells were cultured with syngeneic accessory cells in the presence or absence of *L. major* antigen and were restimulated 7 days later. After 24 h SN from individual cultures were tested for IL 2 and IL 3 activity and minimal estimates of the precursor frequency were obtained by the maximum likelihood method.

b) ND = Not determined.

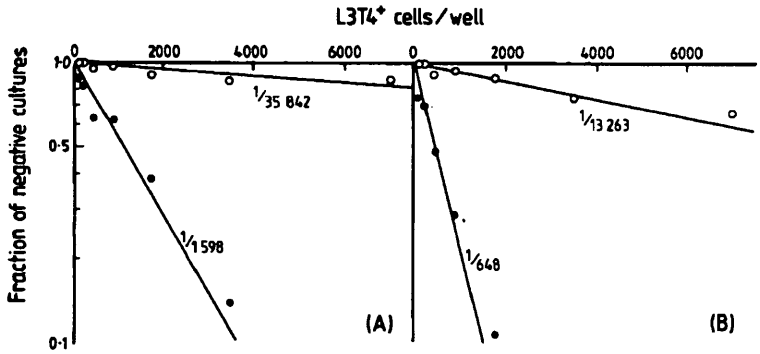

Figure 5. Comparison of frequencies of *L. major*-specific precursor T cells with the potential for production of IL 2 (A) and IL 3 (B) in L3T4$^+$ Ly-24$^+$ (●) and L3T4$^+$Ly-24$^-$ (○) subpopulations obtained from *L. major*-immune C57/BL6 mice. Each point is the mean of values from two experiments.

The collection of L3T4$^+$ subsets defined with Ly-24 was not useful for cells from immunized BALB/c mice, since over 90% of the L3T4$^+$ cells from these mice were Ly-24$^+$. In *L. major*-sensitized C57BL/6 mice, the proportion of Ly-24$^+$ cells was 60%–70%. This difference in Ly-24 expression between BALB/c and C57BL/6 mice was recently also reported for normal mice [25]. The frequency data obtained with T cells from immunized C57BL/6 mice (Table 1 and Fig. 5) demonstrated that *L. major*-specific lymphocytes were about 20-fold enriched in the Ly-24$^+$ subset as compared to the Ly-24$^-$ subset of L3T4$^+$ cells. These data from an *in vitro* culture system confirmed the results obtained from *in vivo* analysis and show that the vast majority of *L. major*-reactive L3T4$^+$ T cells in immunized mice express the Ly-24 marker.

4 Discussion

In murine cutaneous leishmaniasis, resistance to disease can be mediated by L3T4$^+$ cells from uninfected mice. On the other hand, L3T4$^+$ cells from genetically susceptible mice chronically infected with *L. major* are able to abrogate resistance and restore permissiveness to disease. Using a cell titration approach, previous studies have shown that both types of T lymphocytes (resistance-promoting *and* disease-promoting) can in fact be detected in uninfected mice of genetically susceptible genotypes [29] and in infected mice of genetically susceptible as well as genetically resistant mice [30], the proportion of the two functionally distinct cell populations being different in each case and determining the outcome of disease. The data are compatible with the notion that resistance-promoting T cells are of high frequency in uninfected mice relative to disease-promoting T cells but that this ratio is reversed in chronically infected mice. Since both T cell types are L3T4$^+$ Ly-2$^-$ and cannot to date be distinguished on the basis of surface marker expression, their isolation and functional characterization has not been possible.

The present study describes an attempt to subdivide L3T4$^+$ cells according to the expression of additional cell surface antigens, an approach that led to the identification of functionally

diverse subsets in human T4$^+$ cells [31–34]. Several markers were found to be expressed by a subset of L3T4$^+$ SC and LNC, including the alloantigens Ly-6 [26] and Ly-24 [12] and the lymphocyte homing antigen MEL-14 [27]. Transfer of the various subsets of L3T4$^+$ defined with these markers from either uninfected mice or mice chronically infected with *L. major* parasites, showed that only Ly-24 could split the resistance-promoting and the disease-promoting activities.

Using lymphocytes from uninfected BALB/c mice, both the Ly-24$^-$ and the Ly-24$^+$ subpopulations were able to establish resistance to *L. major* infection in otherwise susceptible nude mice. Since the studies of Budd et al. [10] suggested that Ly-24 acquisition *in vivo* is concomitant with primary antigenic stimulation, Ly-24$^+$ cells in untreated mice presumably represent a pool of T lymphocytes activated by immunization to environmental antigens. Resting T cells lacking the Ly-24 antigen, on the other hand, probably received their primary antigenic stimulus upon encounter of *L. major* antigens in the nude mouse host. The fact that this stimulation resulted in the emergence of resistance-promoting activity is in line with the notion that, in uninfected mice, the majority of *L. major*-reactive lymphocytes gives rise to this function. Although it is not known whether the Ly-24$^+$ and Ly-24$^-$ subsets of L3T4$^+$ cells have different activation requirements, the populations can be equally stimulated to mediate resistance to cutaneous disease since the development of lesions in the nude recipients showed a similar time course.

Using lymphocytes from mice chronically infected with *L. major* only Ly-24$^+$ cells but not Ly-24$^-$ cells could abrogate the resistance-promoting effect of normal mouse lymphocytes. It is likely that chronic disease with large concentrations of parasite antigen present induced the activation and expansion of *L. major*-specific disease-promoting cells in the genetically susceptible donor mice, these cells being represented in the Ly-24$^+$ subset. It is not clear whether the Ly-24$^+$ cells still have resistance-promoting activity, now masked by the expanded disease-promoting activity. The Ly-24$^-$ population in these mice presumably comprises resting lymphocytes with irrelevant specificities that do not affect the host-protective activity of normal mouse lymphocytes, all *L. major*-reactive cells having been activated. This was confirmed by the inability of Ly-24$^-$ cells from chronically infected donors to alter the outcome of cutaneous disease in nude recipients, *i.e.*, these lymphocytes exhibited neither disease-promoting nor resistance-promoting activity (Moll, unpublished data).

The conclusions drawn from the adoptive transfer experiment are supported by the finding that Ly-24 expression is highly increased in L3T4$^+$ cells from chronically infected BALB/c mice and that this increase is confined to cells from the LN draining the site of lesion where large amounts of *L. major* antigen are present. Furthermore, *in vitro* LD analysis using T lymphocyte subpopulations from mice immunized with *L. major* confirmed that the vast majority of *L. major*-reactive L3T4$^+$ T cells express the Ly-24 marker. For these experiments, mice were immunized with a preparation of killed parasites since T cells from mice infected with live parasites showed *in vitro* responses independent of the presence of antigen in culture. It must be emphasized that the injection regime used results in protective immunization of the lymphocyte donors. The cells responding in culture, therefore, are probably not disease-promoting cells. However, we have no idea of the relative ability of the disease-promoting and resistance-promoting cells to respond *in vitro*, so this remains an assumption. In the same LD system, Ly-2$^+$ cells from *L. major*-immune mice, though capable of lymphokine production upon mitogenic stimulus, fail to show any activity in response to *L. major* antigen (Moll, unpublished data).

The results described in the present study are in agreement with recent reports by others [10, 25] showing that Ly-24 expressed by peripheral T lymphocytes is a marker for antigen-stimulated (memory?) T cells. Furthermore, the present data extend this notion to L3T4$^+$ T lymphocytes with functional activity in an *in vivo* infectious disease model. The only other report of highly increased expression of Ly-24 in an immunopathological situation deals with autoimmune lpr/lpr mice [35], where most lymphocytes in the peripheral lymphoid tissues have the unusual phenotype Ly-2$^-$ L3T4$^-$.

With respect to the functionally distinct L3T4$^+$ subpopulations that determine the outcome of *L. major* infections, it should be noted that expression of the Ly-24 surface marker *per se* does not distinguish resistance-promoting and disease-promoting T cells since, in lymphoid organs from uninfected mice, both the Ly-24$^-$ and the Ly-24$^+$ subset can mediate resistance. Therefore activation alone (assuming all Ly-24$^+$ cells have been previously activated) is not sufficient to generate disease-promoting activity. In mice chronically infected with *L. major*, on the other hand, Ly-24 can be regarded as a marker for lymphocytes with the capacity of abrogating resistance to disease, these cells being activated and expanding in the course of progressive infection. Ly-24 is thus not a marker identifying distinct lineages of L3T4$^+$ lymphocytes but an antigen expressed as a result of antigenic stimulation. Expression of Ly-24 is independent of DNA synthesis and the majority of peripheral Ly-24$^+$ T cells are in the G_0/G_1 phase of the cell cycle [11, 36] showing that Ly-24 is not merely a marker of actively dividing cells. Although the function of the Ly-24 glycoprotein is not known, Ly-24 expression provides an extremely useful tool for phenotypic identification and selective enrichment of antigen-activated and possibly memory T cells. This may facilitate the study of immunopathological processes in the course of *L. major* infection and, most importantly, the isolation of *L. major*-specific T cell clones with defined activities.

It has been suggested that the functionally different types of T cells involved in the immune response to *L. major* belong to the same subpopulation and that promotion of either protection or disease may merely be dependent on the number of activated *L. major*-specific L3T4$^+$ cells present in the host [5, 37, 38]. According to this hypothesis, small numbers of L3T4$^+$ cells would be host protective, whereas excessive numbers of the same cell type would have a detrimental effect. However, there is increasing evidence supporting the alternative possibility that resistance to disease or its abrogation are induced by distinct subsets of L3T4$^+$ cells [8, 39, 40]. In particular, a recent study by Locksley et al. [8] has provided direct evidence for differences in lymphokine production by lymphocytes from healer and those from nonhealer mice infected with *L. major*. The findings are consistent with the interpretation that inability to control cutaneous disease is reflected by expansion of type 2 T helper cells (T_H2), whereas effective cellular immunity is accompanied by expansion of type 1 T helper cells (T_H1). However, this classification of L3T4$^+$ subsets [7] is based on studies with long-term cultured and cloned murine T cells and its validity for freshly isolated lymphocytes has yet to be con-

firmed. Moreover, not all T cell clones fit into the T_H1 and T_H2 groups ([41] and Anne Kelso, personal communication) and, in the human system, equivalent types of T_h cells cannot be detected. To date, there is no cell surface marker for phenotypic distinction of the two types of T cells in the mouse but recent evidence suggests that rat T cells can be phenotypically subdivided into functionally similar subsets [42].

Finally, the possibility exists that $L3T4^+$ cells mediating resistance to cutaneous leishmaniasis and $L3T4^+$ cells promoting disease differ in antigen specificity rather than in surface marker expression [43]. This concept is supported by the finding that a lipophosphoglycan (LPG) isolated from *L. major* induces resistance to leishmanial infection [44], whereas a carbohydrate (CHO) produced by enzymatic cleavage of the same LPG can exacerbate subsequent disease [45]. It is conceivable that LPG-specific $L3T4^+$ cells activate *L. major*-infected macrophages for killing of intracellular organisms, resulting in the resolution of disease, whereas activation of CHO-specific $L3T4^+$ cells may result in further inflammation and recruitment of immature macrophages that serve as targets of infection [46]. The implication of this model is that different antigens or classes of antigens activate T cells with quite different functions, a point that has a precedent in the case of T cells being MHC class I or class II restricted.

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