© Resistance to cutaneous leishmaniasis in nude mice injected with L3T4 + T cells but not with Ly-2 + T cells

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Summary. The importance of T cells in resistance to infection with the intracellular protozoan parasite *Leishmania major* is substantiated by the susceptibility to infection of athymic nude mice of both resistant and susceptible strains. However, the relative roles of different T cell subpopulations remain controversial. In order to address this issue, selected $L3T4^+ Ly-2^-$ or $L3T4^- Ly-2^+$ T cell subpopulations from normal mice were adoptively transferred into athymic nude recipients of the same strain, and their capacity to mediate host-protective immunity against infection with *L. major* promastigotes was determined. In experiments with mice of different inbred strains, reconstitution with $L3T4^+ Ly-2^-$ cells rendered the nude mice completely resistant to cutaneous leishmaniasis, whereas $L3T4^- Ly-2^+$ cells failed to do so. Partial protection in some recipients of large numbers of $Ly-2^+$ cells could be ascribed to contamination of the transferred inoculum with $L3T4^+$ cells. Thus, resistance to *L. major* infection in reconstituted nude mice can be promoted by $L3T4^+$ T cells in the absence of detectable $Ly-2^+$ T cells.

INTRODUCTION

Human isolates of the intramacrophage protozoan parasite, *Leishmania major*, can infect mice and produce a range of cutaneous disease patterns, similar to the situation in man, depending on the strain of inbred mouse. However, athymic nude mice of both resistant and susceptible genotypes are highly susceptible, showing the importance of T cell-dependent immunity in resistance to disease (1). All T cell-mediated activities in murine cutaneous leishmaniasis can be ascribed to $L3T4^+ Ly-2^-$ cells (2-4), though a recent study has documented responsiveness of $Ly-2^+$ cells in CBA mice to *L. major* antigens (5). Early studies showed that small numbers of T cells (10^5 to 10^6) were efficient in promoting resistance in athymic nude mice, and the phenotype of the resistance-promoting effector cell population was demonstrated by treatment with antibody and complement to be $Ly-2^-$ (6). The events leading to resistance in this and related models are presumably initiated by lymphokines with macrophage-activating properties produced by activated T cells responding to antigen on the infected macrophage surface and acting locally.

Cells treated with antiserum and complement such that they were predominantly $Ly-2^+$ (6), though ineffective at low numbers (10^5 to 10^6), were capable of promoting resistance when injected in high numbers ($>10^7$). It was not possible to determine whether high numbers of $L3T4^- Ly-2^+$ cells could indeed mediate protection or whether contamination with small numbers of $L3T4^+ Ly-2^-$ cells was responsible for this effect. With improved technologies it has become possible to determine whether mice injected with high numbers of purified $L3T4^- Ly-2^+$ cells contain sufficient numbers of contaminating $L3T4^+$ cells to account for their resistant phenotype. Furthermore, flow-cytometric sorting procedures allow the collection of highly purified lymphocyte subpopulations and their use for adoptive transfer experiments. In this report we present results using two different experimental protocols that support the notion that $L3T4^+ Ly-2^-$ T cells *alone* are responsible for host-protective immunity to *L. major* infection in BALB/c and CBA/H mice.

MATERIALS AND METHODS

Mice

Female mice of the inbred strains BALB/c and CBA/H were used at an age of 7 to 10 weeks; female athymic BALB/c.nu/nu and CBA/H.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 Hall Institute but, during experimentation, were maintained under conventional conditions in a small isolation facility.

Monoclonal antibodies, fluorescence and cytotoxic reagents

The monoclonal antibodies were prepared from supernatants of hybridomas grown at the Hall Institute, purified and conjugated where noted in this laboratory. Anti-Mac-1 antibodies, from the hybridoma M1/70 (7), and anti-Ly-2.2 antibodies, from the hybridomas HO 2/2 (8) or D9 (9), were used as culture supernatants. Anti-L3T4 antibodies, from the hybridoma GK1.5 (10), were used as a culture supernatant or, in the same way as anti-Ly-2 antibodies from the hybridoma 53-6.7 (11), as biotin-conjugated purified protein. A fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Silenus Laboratories, Melbourne, Australia) and FITC-conjugated avidin (Tago, Burlingame, CA, U.S.A) were used as second-stage reagents for fluorescence staining.

Selected non-toxic young rabbit serum had been prepared and absorbed as described (12) and was used as a source of complement.

Cytotoxic procedures

Single cell suspensions of unseparated spleen and lymph node cells or nylon wool-purified T cells (13) at a concentration of 10×10^6 per ml were incubated for 40 min on ice with either anti-L3T4 or anti-Ly-2.2 antibodies and for another 20 min at 37° with rabbit complement in a final dilution of 1:15 as previously described (12). 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. After incubation, the cells were pelleted and resuspended in metrizamide medium (density 1·091 g/cm³), and damaged cells were removed by centrifugation using the density-cut procedure (12).

Fluorescence staining and cell sorting

For phenotype analysis, 3×10^6 lymphocytes were incubated with biotin-conjugated anti-L3T4 or anti-Ly-2 antibodies in a total volume of 30 μl in small conical siliconized glass tubes. Reaction time was 20 min on ice. Thereafter, cells were washed and treated with FITC-conjugated avidin for another 20 min on ice. Propidium iodide (0·5 μg/ml; Calbiochem, La Jolla, CA, U.S.A.) was included in the final wash in order to stain dead cells and facilitate their exclusion from the flow cytometric analysis. For cell sorting, lymphocytes $(100 \times 10^6/ml)$ were stained with a mixture of anti-Mac-1, anti-L3T4 and FITC-conjugated anti-mouse immunoglobulin antibodies for 30 min on ice. All reagents were used at near-saturating conditions. Dead cells were excluded on the basis of low-angle light scatter and the fluorescence-activated cell sorter was a modified FACS II (Becton Dickinson, Sunnyvale, CA, U.S.A.).

Parasites and assessment of lesions

The cloned virulent *L. major* parasite line V121 was produced from the human isolate LRC-L137 (14) 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 on the dorsum of the mouse close to the base of the tail. 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 lesion less than 5 mm in average diameter; 3 = lesion 5-10 mm in diameter. Data are expressed as the arithmetic mean of the lesion scores for the groups of 4 to 6 mice, and the number of mice with lesions relative to the total number of mice per group is given for the last time point.

RESULTS

Reconstitution of BALB/c nude mice with graded numbers of L3T4-depleted or Ly-2-depleted lymphocytes from normal BALB/c mice.

For the selection of lymphocyte subpopulations, mesenteric, axillary and inguinal lymph node cells were incubated with either anti-L3T4 or anti-Ly-2.2 antibodies and with selected rabbit complement. We tested different sources of monoclonal antibodies and determined the purity of the resulting lymphocyte subpopulations by fluorescence staining of the cell surface markers and analysis in a fluorescence-activated cell sorter (FACS). Optimal purity was achieved using anti-L3T4 from the rat hybridoma GK1.5, or a mixture of anti-Ly-2.2 from the hybridomas HO 2/2 and D9, the contaminating cells of the depleted phenotype being below 5% or 1%, respectively, and having very low fluorescence intensity (data not shown).

BALB/c nude mice at an age of 6 to 8 weeks were injected intradermally with 2×10^6 promastigotes (in 50 μl) and intraperitoneally (in 200 μl) with either L3T4-depleted or Ly-2-depleted cells in numbers equivalent to their original proportion in 1×10^6, $4·5 \times 10^6$ or 20×10^6 cells treated with complement alone. As a control for the infectivity of the parasites,

one group of mice received promastigotes in the absence of lymphocytes. Fig. 1 shows the course of cutaneous lesion development. Depletion of Ly-2$^+$ cells (i.e., purified L3T4$^+$ cells) did not abrogate the capacity of normal mouse lymphocytes to promote resistance in nude mice, irrespective of the number of transferred cells. On the other hand, L3T4-depleted cells (i.e., purified Ly-2$^+$ cells) were not effective at protecting nude mice when injected at a low number (equivalent of 1×10^6) and all the mice developed severe lesions. However, increased cell numbers caused a delay of lesion development ($4 \cdot 5 \times 10^6$) or healing of lesions (20×10^6) and an increased proportion of mice per group to recover by the end of the experiment. The number of mice with lesions relative to the total number of mice per group is given for the last time point in Fig. 1.

Phenotype of lymphocytes from reconstituted BALB/c nude mice

In order to find out whether the host-protective effect of high numbers of L3T4-depleted lymphocytes could be attributed to a low-level contaminant population of L3T4$^+$ cells, expanded in the recipients, pooled lymph node and spleen cells from the individual groups of nude mice were subjected to phenotype analysis on day 50 after injection. The lymphocytes were stained by sequential treatment with biotin-conjugated anti-L3T4 or anti-Ly-2 antibodies and FITC-conjugated avidin, and the proportion of L3T4$^+$ or Ly-2$^+$ cells was determined in the FACS. Fig. 2 depicts the L3T4 fluorescence profiles of cells from mice which had been reconstituted with L3T4-depleted cells in
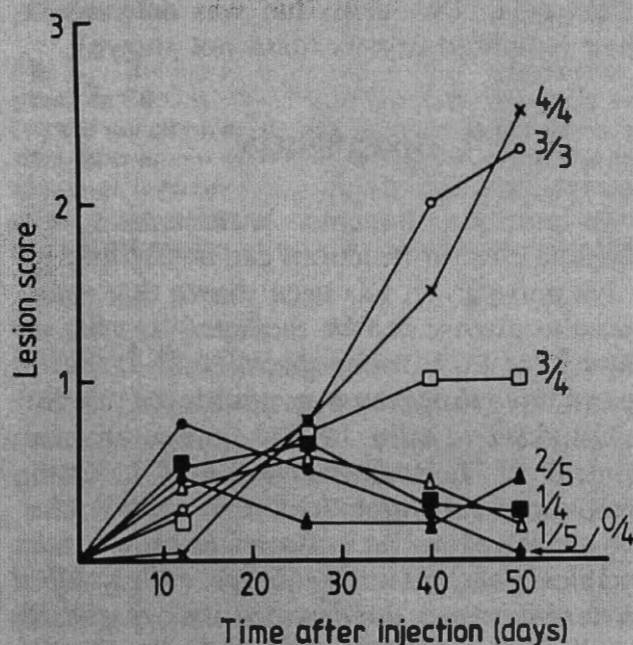

Fig. 1. Titration of L3T4- or Ly-2-depleted lymphocytes in nude mice. On day 0, four or five BALB/c nude mice per group were injected intradermally with 2×10^6 promastigotes of *L. major* (V121) and intraperitoneally with either L3T4-depleted (open symbols) or Ly-2-depleted (closed symbols) normal BALB/c lymph node cells in numbers equivalent to their original proportion in 1×10^6 (○, ●), $4 \cdot 5 \times 10^6$ (□, ■) or 20×10^6 (△, ▲) unselected cells. Control mice received promastigotes alone (×). Depletion of lymphocyte populations was performed by treatment with anti-L3T4 or anti-Ly-2.2 antibodies and complement. Arithmetic means of the cutaneous lesion scores are indicated at certain time points and the number of mice with lesions relative to the total number of mice per group is given for the last time point. Standard errors were below $0 \cdot 4$.
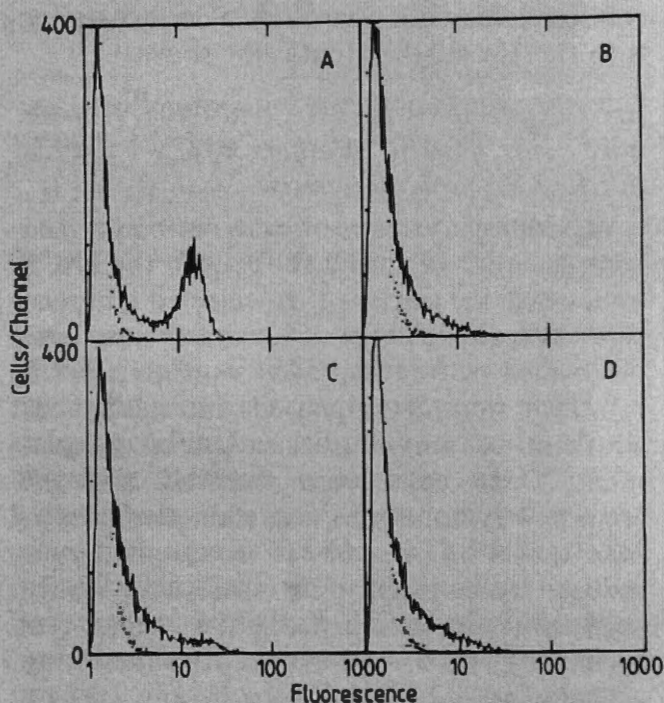

Fig. 2. L3T4 fluorescence profiles of lymphoid cells from reconstituted nude mice. 50 days after injection of BALB/c nude mice with *L. major* promastigotes alone (B) or together with L3T4-depleted normal BALB/c lymph node cells in numbers equivalent to 20×10^6 (C) or 1×10^6 (D) unselected cells (courses of lesion development are depicted in Figure 1), the proportion of L3T4$^+$ cells in their lymph nodes and spleens was determined by staining with biotin-conjugated anti-L3T4 antibodies plus FITC-conjugated avidin and analysis in the FACS (solid lines). Lymph node and spleen cells from normal BALB/c mice were used as a control (A). Dotted lines represent the background of cells stained with FITC-conjugated avidin alone.

numbers equivalent to 20×10^6 (C) or 1×10^6 (D). It can be seen that transfer of the low cell number did not cause a significant increase of L3T4$^+$ cells in the recipients (D) when compared to cells from *L. major*-infected nude mice that were not reconstituted with any lymphocytes (B). Cells that were stained by the anti-L3T4 antibodies had a low fluorescence intensity and were probably haemopoietic cells similar to the low L3T4$^+$ cells found in normal bone marrow (10). However, lymphocytes from mice that had received a high number of supposedly L3T4-depleted cells (C) showed an evident increase (5-6%) of L3T4$^+$ cells with a fluorescence intensity like that of normal mouse lymphocytes (A). Lymph nodes and spleens from nude mice which had been reconstituted with Ly-2-depleted cells at any number did not contain detectable quantities of cells bearing that surface marker (data not shown).

Reconstitution of BALB/c and CBA/H nude mice with graded numbers of Ly-2$^+$ cells purified by flow cytometry

In addition to treatment with antibodies and complement, we sorted the cells in the FACS to increase the purity of the selected lymphocyte subpopulations. Prepurification was performed by treating nylon wool-purified T cells from normal lymph node and spleen cells with anti-L3T4 antibodies and rabbit complement. Dead cells were removed and the remaining lymphocytes were stained with anti-Mac-1, anti-L3T4 and FITC-conjugated anti-mouse immunoglobulin antibodies. Subsequently, fluorescence-negative cells were sorted by flow cytometry. The resulting subpopulation did not contain any L3T4$^+$ cells detectable by FACS analysis (<0.5%) and therefore was highly enriched for cells bearing the Ly-2 marker. These cells in numbers equivalent to their proportion in 1×10^6 or 15×10^6 unseparated cells, or 1×10^6 or 15×10^6 unselected lymph node and spleen cells, were injected into nude mice that were subsequently challenged with 2×10^6 *L. major* promastigotes.

Two independent adoptive transfer experiments with different strains of mice were performed using either lymphocytes from normal BALB/c mice and BALB/c nude recipients or lymphocytes from normal CBA/H mice and CBA/H nude recipients. The course of cutaneous lesion development in the reconstituted nude mice (Fig. 3) shows that, irrespective of their genotype, recipients of both low and high numbers of Ly-2$^+$ cells were susceptible to disease. At the end of the experiment (day 50 after injection), all the mice of these groups had developed severe lesions, whereas only a small proportion of the recipients of unseparated lymphocytes showed resolving symptoms in the form of small swellings. The proportion of mice with lesions per group is given for the last time points in Fig. 3. The slight reduction of the mean lesion scores in BALB/c mice that received Ly-2$^+$ equivalents of 15×10^6 cells (Fig. 3, left panel) could again be explained by a very small population of L3T4$^+$ cells that was detected in their lymphoid organs (data not shown).

DISCUSSION

In murine cutaneous leishmaniasis, all immune effector functions can be ascribed to T lymphocytes. It has been shown that resistance to disease can be mediated by cells of the L3T4$^+$Ly-2$^-$ subpopulation (2-4), but a recent report suggested an additional role for L3T4$^-$Ly-2$^+$ cells in the immunological control of *L. major* infection (5). Using lymphocyte subpopulations from normal mice for adoptive transfer into susceptible athymic recipients of the same genotype, early studies in our laboratory documented the capacity of small numbers (10^5 to 10^6) of Ly-2$^-$ T cells to promote resistance in athymic nude mice (6). On the other hand, Ly-2$^+$ cells were not able to mediate a host-protective effect when used at the same numbers, but caused the resolution of cutaneous lesions when injected at a high dose (>10^7). The T cell subpopulations used in these experiments were selected by treatment with antisera and complement and it was not possible to determine whether high numbers of L3T4$^-$Ly-2$^+$ cells could indeed mediate resistance to disease or whether contamination with L3T4$^+$Ly-2$^-$ cells was responsible for
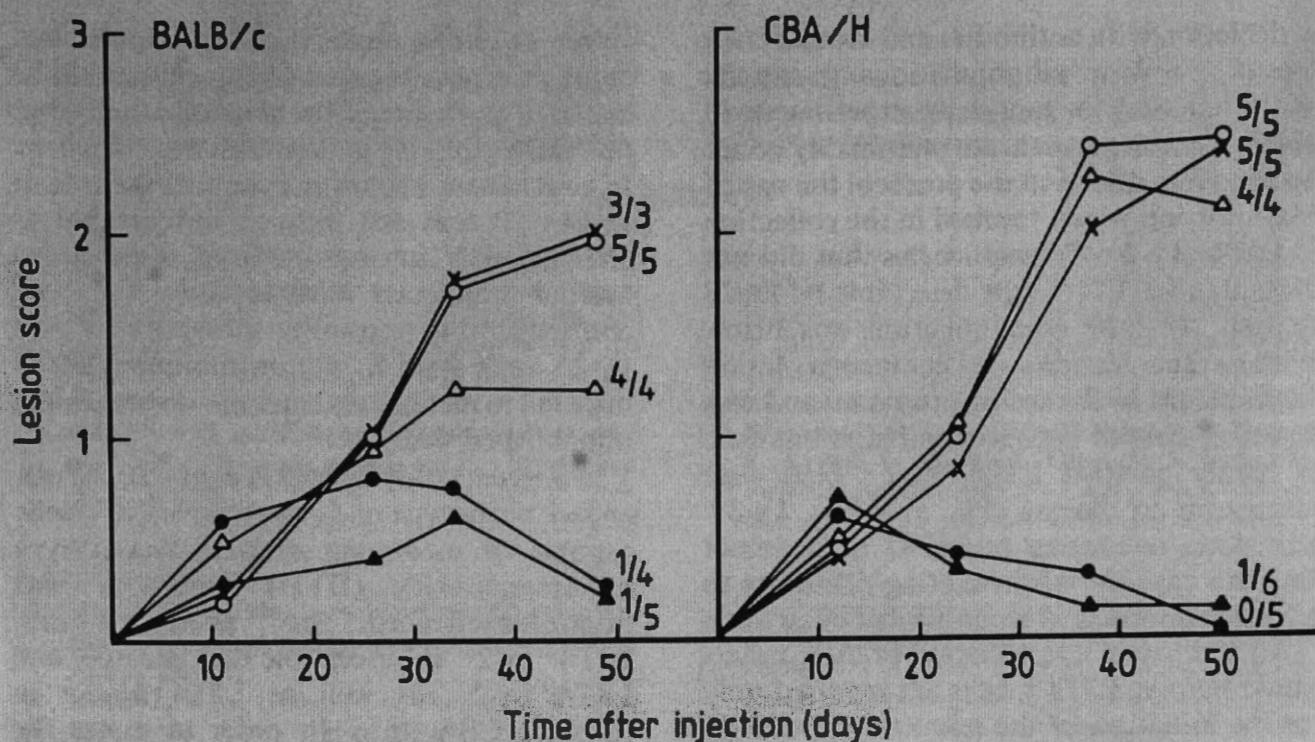
Fig. 3. Titration of unselected or Ly-2⁺ lymphocytes in nude mice. On day 0, five or six BALB/c nude mice (left panel) or CBA/H nude mice (right panel) per group were injected intradermally with 2×10^6 *L. major* promastigotes (×) and intraperitoneally with lymph node and spleen cells from normal BALB/c or CBA/H mice, respectively, which were either unselected (1×10^6, ●; 15×10^6, ▲) or had been highly purified for Ly-2⁺ cells by sorting in the FACS and were given in numbers equivalent to their original proportion in 1×10^6 (O) or 15×10^6 (△) unselected cells. Arithmetic means of the cutaneous lesion scores are indicated at certain time points and the number of mice with lesions relative to the total number of mice per group is given for the last time point. Standard errors were below 0.3.

the protective effect. In the present study, however, using highly purified lymphocyte subpopulations, we provide evidence for an exclusive role of L3T4⁺ cells in promoting protection to *L. major* infection.

After a protocol had been worked out for a very efficient selection of L3T4⁺ Ly-2⁻ or L3T4⁻ Ly-2⁺ lymphocyte subpopulations by treatment with monoclonal antibodies and complement, BALB/c nude mice were reconstituted with graded numbers of L3T4-depleted or Ly-2-depleted lymphocytes from normal BALB/c mice and were infected with *L. major* parasites. The course of cutaneous lesion development in the different groups of mice shows (Fig. 1) that L3T4⁺ Ly-2⁻ cells promoted resistance in nude recipients at any of the cell numbers used. Thus, L3T4⁺ cells have the capacity to transfer protection to *L. major* infection in the absence of detectable Ly-2⁺ cells. On the other hand, L3T4⁻ Ly-2⁺ cells were not effective at protecting nude mice when injected at a low

number, whereas increased cell numbers caused a delay of lesion development or even healing of lesions. These results were therefore comparable to our early findings as described above (6). However, phenotype analysis of T lymphocytes recovered from the individual groups of mice at the end of the experiment (i.e., 50 days after injection) revealed that the host-protective effect of L3T4-depleted lymphocytes could be attributed to a low-level contamination with L3T4⁺ cells. Lymphoid cells from recipients of high numbers of supposedly L3T4-depleted cells contained a slightly increased (5-6%) proportion of L3T4⁺ cells as compared to the controls (Fig. 2). Such a contamination with L3T4⁺ cells could well account for the resistance to cutaneous lesions, since this can be induced in nude mice by reconstitution with as few as 10^5 cells (6; 15) which presumably expand in the course of infection.

These data demonstrate that even a carefully optimised protocol for lymphocyte selection

by depletion with antibodies and complement does not result in subpopulations having the purity required for long-term experiments in which clonal expansion can presumably occur. We therefore added to the protocol the use of FACS sorting which resulted in the collection of L3T4⁻Ly-2⁺ T lymphocytes that did not contain any L3T4⁺ cells detectable by FACS analysis (i.e., the contamination was below 0·5%). The course of cutaneous lesion development in the reconstituted mice shows that recipients of both low and high numbers of highly purified L3T4⁻Ly-2⁺ cells were susceptible to disease (Fig. 3). Thus, Ly-2⁺ cells alone, in contrast to L3T4⁺ cells, do not have the capacity of promoting resistance to *L. major* infection in reconstituted nude mice.

An alternative interpretation of these results would be that L3T4⁺ cells are required only for the initiation of the immune response to *L. major* and allow Ly-2⁺ cells to differentiate and to contribute to the mediation of resistance. However, the experiment shown in Fig. 1 strongly argues against this possibility. In this experiment, nude mice that had been reconstituted with Ly-2-depleted (i.e., purified L3T4⁺) lymphocytes in numbers equivalent to their original proportion in as few as 1×10^6 unseparated cells were fully resistant to disease. Since the lymphocyte population used for this transfer contained less than 0·5% of contaminating Ly-2⁺ cells as shown by FACS analysis (data not shown), it is most unlikely that Ly-2⁺ cells of donor origin have contributed to the host-protective effect. This conclusion is further substantiated by phenotype analysis of lymphocytes recovered from the reconstituted nude mice at the end of the experiment. 50 days after the injection of L3T4⁺ Ly-2⁻ cells, the recipients' lymph nodes and spleens did not contain detectable quantities of cells bearing the Ly-2⁺ surface marker (data not shown). This evidence also argues against the possibility that the transferred L3T4⁺ cells may have recruited Ly-2⁺ cells of host origin by inducing the maturation of nude pre-T cells, a mechanism that is furthermore excluded by other experiments in which the injection of normal mouse T cells into Thy-1 congenic nude mice did not increase the number of host T cells in

spleen or lymph nodes (Scollay, unpublished data). It is possible that Ly-2⁺ cells could be localised at the site of the skin lesion only, but this seems unlikely in their absence elsewhere. In conclusion, our results support the role of L3T4⁺ T cells not only as inducer but as effector cells in the immune response to murine cutaneous leishmaniasis. We have confirmed this notion by showing that even Ly-2⁺ cells from *L. major*-immune CBA/H mice fail to mediate resistance to disease (Moll, unpublished data).

In a recent study by Milon *et al.* (5), the cell surface phenotype of *L. major*-specific T cells capable of mediating specific delayed-type hypersensitivity (DTH) reactivity was documented to be predominantly L3T4⁺Ly-2⁻ in susceptible BALB/c mice and L3T4⁺Ly-2⁻ as well as L3T4⁻Ly-2⁺ in resistant CBA mice. In order to assess the capacity of L3T4⁻Ly-2⁺ T cells from CBA mice in promoting resistance in nude mice, we included mice of this inbred strain in our adoptive transfer experiments using highly purified lymphocytes selected by FACS sorting. L3T4⁻Ly-2⁺ cells of CBA origin, like those from BALB/c mice, were unable to mediate resistance in the nude mice transfer system. It therefore remains to be determined whether *L. major*-specific DTH-mediating T cells expressing the L3T4⁻Ly-2⁺ phenotype are able to exert a host-protective role in cutaneous leishmaniasis.

In the present study, no evidence has emerged for a role for Ly-2⁺ T cells in mediating resistance to cutaneous leishmaniasis in either BALB/c or CBA/H mice. If any function exists for this subpopulation in control of disease (in any mouse strain), then it is not likely to be revealed by the approaches used in this study. A demonstration of resistance-promoting activity by *in vitro*-derived Ly-2⁺ T cell clones or lines could alter the interpretation drawn from this and other previous studies, although the problem would then be relating such cells to lymphocytes found *in vivo*.

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