THE GENERATION OF ANTIGEN-SPECIFIC, MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED CYTOTOXIC T LYMPHOCYTES OF THE CD4+ PHENOTYPE

Enhancement by the Cutaneous Administration of Interleukin 2

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During the course of a cell-mediated immune response, effector mechanisms are induced that lead to the selective destruction of host cells. This was first brought to our attention during ultrastructural analysis of tuberculoid leprosy lesions. Hence, in the paucibacillary form of leprosy, parasitized and foamy macrophages and epithelioid cells were being destroyed in association with highly polarized lymphocytes of an unknown phenotype (1, 2). More recently, this has been observed in the lesions of lepromatous leprosy after the generation of an antigen driven purified protein derivative [PPD] cell mediated response. In this instance, infected macrophages liberate large numbers of Micobacterium leprae and their products into the extracellular environment (3). Parasitized macrophages are destroyed in a milieu containing many newly immigrated mononuclear cells, including CD4+ and CD8+ T cells, monocytes, and T6+ Langerhans' cells (4). This scenario has also been observed after the introduction of the recombinant lymphokines IFN-γ (manuscript in preparation) and IL-2 (5) into the lesions of lepromatous and borderline lepromatous leprosy patients. Under these conditions M. leprae is subsequently reingested and dismantled, leading to a striking local reduction in bacilli.

To examine the nature of the cytotoxic cell(s) involved and their recognition mechanism(s), we have now performed in vitro analyses. We have used human monocytes as targets and PBMC or enriched lymphocyte subsets as the source of killer cells. Both targets and effector cells have been exposed to antigens contained in PPD that crossreact with many species of Mycobacteria. In addition, we examine the role of IL-2 after its injection into lepromatous patients and added in culture on the generation of specific and nonspecific effector cells.

Materials and Methods

Tuberculin Skin-test Positive Donors. Donors were selected from healthy laboratory personnel.

Patient Population. Eight patients with lepromatous leprosy were seen at the All Africa Leprosy Rehabilitation and Training hospital and the Armauer Hansen Research Institute, Addis Ababa, Ethiopia. Each patient was injected intradermally with three doses of 10 μg human rIL-2 (Cetus Corp., Emeryville, CA) at 48-h intervals (5). Immediately before and
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8-14 d after the initiation of rIL-2 injections, 50 ml of peripheral blood was obtained from each patient. Antigen-dependent cytolytic activity (see below) was determined after 7 d stimulation in vitro with Bacillus Calmette-Guerin (BCG; Statens Serum Institut, Copenhagen, Denmark).

**Effector Cells.** Human PBMC from PPD-sensitized donors were isolated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). T lymphocytes were enriched by adherence to neuraminidase (Calbiochem-Behring Corp., La Jolla, CA)-treated SRBC and separated from nonadherent fractions over Ficoll-Hypaque. The T lymphocytes (2 × 10^6/ml) were cultured 0-8 d in 60-mm tissue culture dishes (Falcon Labware, Oxnard, CA) with 10 μg PPD/ml RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% human AB+ serum, 100 μg penicillin/ml, 100 μg streptomycin/ml, and 2 mM glutamine (R10). SRBC nonadherent fractions (0.8-1.2 × 10^6 cells) were added to facilitate antigen presentation. In some experiments, rIL-2 (40 U/ml) was added on days 0 and 3 of culture, (Collaborative Research, Lexington, MA) and in others, 1,000 U/ml was added at day 0 only (Cetus Corp., Emeryville, CA).

PBMC (1-2 × 10^6/ml) from lepromatous leprosy patients were stimulated in 24-well tissue culture dishes (Linbro; Flow Laboratories, Inc., McLean, VA) with BCG (2.5 μg/ml) or medium alone. After 7 d of culture, effector cells were harvested, washed in cold RPMI, and adjusted to a final concentration of 1-5 × 10^6/ml R10.

**Panning.** Effector cells were enriched for CD4+ and CD8+ cells as described (6, 7). Briefly, effector cells were incubated 1 h on ice with OKT4 (CD4) (CRL 8002; American Type Culture Collection, Rockville, MD) or OKT8 (CD8) (CRL 8014; American Type Culture Collection) mAb containing supernatants, washed, and panned on 100-mm petri dishes (1005; Falcon Labware) coated with 60 μg of the IgG fraction of goat anti-mouse Fc (0211-0121; Cappel Laboratories, Cochranville, PA). Cells bearing the Fc receptor were depleted from effector populations by incubation for 30 min in 100-mm Petri dishes coated with human Ig (10 mg/ml) (0001-0910; Cappel Laboratories) (7).

**Preparation of Monocyte-enriched Targets.** Autologous monocyte-enriched targets (SRBC nonadherent PBMC) were isolated from donors either 1 d before the cytotoxicity assay, or at the time the PBMC were obtained for in vitro stimulation with antigen, cultured (10^6/ml R10) in Teflon beakers (Savillex Corporation, Minnetonka, MN) and incubated, before assay, on ice for 1 h to increase cell yields. In some experiments, monocyte targets were recovered from the Teflon beakers and added (10^4/well) to 96-well round-bottomed tissue culture dishes for overnight adherence in the presence of antigen. In other experiments, the targets were pulsed overnight in the Teflon beakers with antigen, labeled with 51Cr, and then added to 96-well round-bottomed tissue culture dishes (10^6/well) with antigen immediately before initiation of the assay.

**Antigens.** The antigens used were PPD of tuberculin (Statens Serum Institut, Copenhagen, Denmark) (40 μg/ml overnight and 1 μg/well during the assay, or 1 μg/well overnight); M. leprae soluble filtrate (40 μg/ml overnight and 4 μg/ml during the assay, or 4 μg/well overnight); and tetanus toxoid (TT; 1:300 final dilution overnight or 1:500 final dilution during the assay of a 770 LF/ml solution).

**Determination of Percent Specific 51Cr Release.** 51Cr (New England Nuclear, Boston, MA) release was determined after 4 or 15 h of incubation.

**4-h Assay (7).** Briefly, 50 μl (10^6 cells) of 51Cr-labeled monocytes were incubated (37°C, 5% CO2) with increasing concentrations of effector cells (in 100 μl volumes) in triplicate round-bottomed microwells (Costar, Cambridge, MA). At the end of incubation, culture supernatant was measured for 51Cr release and compared with spontaneous release (monocytes cultured with medium alone, 15-25%) and total release (monocytes cultured in medium containing 1% SDS, vol/vol in PBS). Percent specific 51Cr release was determined by: 100 × [(mean cpm experimental) - (mean cpm spontaneous release)]/[(mean cpm total release) - (mean cpm spontaneous release)].

**15-h Assay.** Specific 51Cr release after 15 h incubation was determined in a modified assay. Briefly, PBMC isolated at the time the PBMC were obtained for in vitro stimulation with antigen were cultured (5 × 10^6/well) in 96-well round-bottomed dishes, and the adherent cells served as monocyte targets. Alternatively, SRBC nonadherent monocyte targets, also
isolated at the initiation of antigen stimulation in vitro, were cultured in Teflon beakers, harvested 1 d before the cytotoxic assay, adjusted to 2 × 10^6/ml R10, added to 96-well round-bottomed tissue culture dishes (50 µl, 10^4/well), and incubated 2 h (37°C, 5% CO₂) to allow monocyte target adherence. After incubation, the nonadherent cells were removed and 50 µl warm R10 was added. The monocyte targets were charged with antigen overnight. The monocytes were then washed with warm RPMI (5% A⁺ human serum) and labeled with ^51^Cr (1.25 µCi/well) for 6–7 h, washed, and incubated 15 h with increasing numbers of effector cells. After supernatant harvest, 100 µl of 1% Triton X (vol/vol, in PBS) was added to each well, incubated 3 h (37°C), and collected. Percent specific ^51^Cr release was determined by: 100 × (test cpm)/(test cpm + cpm from the same Triton X-treated well) – percent spontaneous release. Percent spontaneous release was usually 20–30%.

**NK and Lymphokine-activated Killer (LAK) Cell Assay.** NK (K562) (8) and LAK (Daudi) (9) targets were obtained from C-C Liu (The Rockefeller University, New York, NY) and cultured in RPMI 1640 supplemented with 10% heat-inactivated (56°C, 30 min) FCS (HyClone Laboratories, Logan, UT), 100 µg penicillin/ml, 100 µg streptomycin/ml, and 2 mM glutamine. The targets were harvested, labeled 1–2 h with ^51^Cr (100 µCi/10^7 targets), washed, added to 96-well round-bottomed tissue culture dishes (10^4/well), and incubated 4 h (37°C, 5% CO₂) with increasing numbers of effector cells. Percent specific ^51^Cr release was determined as above.

**Immuno fluorescence.** The cellular composition of the PPD-stimulated effectors obtained from the tuberculin-sensitive donors and their enriched subpopulations was determined by staining in suspension (2 × 10^5 cells/round-bottomed microwell; Costar) with Leu-3a/Leu-2a "Simultest" (Becton Dickinson & Co., Mountain View, CA) and immunofluorescence was analyzed with FACScan flow cytometer (Becton Dickinson & Co.).

**Inhibition of Cytolytic Activity with mAb Directed against MHC Determinants.** The enriched effectors and monocyte targets (E/T ratio 20:1) were incubated during the cytotoxic assay with increasing concentrations (percent, vol/vol) of hybridoma supernatants containing mAbs 9.3C9 (anti-class II MHC products), L243 (anti-HLA-DR), or W6/32 (anti-class I, A, B, C) (HB 180, HB 55, and HB 95, respectively; American Type Culture Collection).

**Results**

**PPD-stimulated PBMC Yield Cytotoxic Cells for Antigen-pulsed Monocytes.** PBMC from previously sensitized normal donors were stimulated in vitro with PPD. After 8 d of culture with PPD, the PBMC killed monocytes that had been pulsed with the sensitizing antigen, but not the monocytes pulsed with nonrelated antigen (tetanus toxoid) or medium alone (Fig. 1). Only at very high E/T ratios (50:1) was some antigen-independent killing of targets observed. PBMC that had not been stimulated with PPD were not cytotoxic. PPD-stimulated PBMC also demonstrated cyto-

![Figure 1](https://example.com/figure1.png)

**Figure 1.** PPD-stimulated PBMC contain antigen-dependent cytotoxic cells for autologous monocyte targets. Monocyte targets were pulsed overnight with PPD ( ), TT ( ), or medium alone ( ) and then exposed to PBMC stimulated 8 d with either PPD (circle) or medium alone (triangle) at the indicated ratios. After 4 h incubation, percent specific ^51^Cr release was determined.
lytic activity against monocytes pulsed with the crossreactive antigen \textit{M. leprae} (Table I). Stimulation of the PBMC with both PPD and rIL-2 in vitro did not enhance the \textit{M. leprae}-specific cytotoxic activity but did induce antigen-independent nonspecific cytolytic activity against monocytes. This data suggested that specific and nonspecific cytolytic subpopulation(s) were generated during the in vitro stimulation with PPD.

\textbf{Antigen-specific CTL Are Largely of the CD4\textsuperscript{+} Subset.} To characterize the cell population that mediated antigen-specific monocyte killing, PPD-stimulated PBMC were enriched for CD4\textsuperscript{+} or CD8\textsuperscript{+} cells. The bulk PBMC killed antigen-pulsed mono-

\begin{table}[h]
\centering
\caption{PPD-stimulated PBMC Contain Cells Cytotoxic for Monocyte Targets Pulsed with \textit{M. leprae}}
\begin{tabular}{|l|c|c|c|}
\hline
Effector (PBMC) & \textbf{Percent specific release\textsuperscript{*} from monocytes pulsed with:} & PPD & \textit{M. leprae} & T. toxoid \\
stimulation & & & & \\
\hline
PPD & 17 & 18 & 0 \\
PPD + IL-2 & 29 & 28 & 8 \\
IL-2 & 2 & 11 & 0 \\
None & 0 & 10 & 0 \\
\hline
\end{tabular}
\textsuperscript{*} E/T ratio is 15:1.
\end{table}

\textbf{Figure 2.} In vitro stimulation of PBMC with PPD generates antigen-dependent CD4\textsuperscript{+} CTL. Fig. 2 represents data from two experiments, experiment 1 (\textbf{A}, \textbf{B}) and experiment 2 (\textbf{C}, \textbf{D}). The percent CD4\textsuperscript{+} and/or CD8\textsuperscript{+} T lymphocytes contained within the bulk PBMC or enriched populations was determined on the day of assay. Monocyte targets were charged overnight and during the assay with PPD (filled symbols) or medium alone (open symbols). The effectors were PBMC not stimulated with PPD (\textbf{A}), bulk PBMC stimulated with PPD (\textbf{B}), or enriched PPD-stimulated T lymphocytes (\textbf{C}, \textbf{E}). Percent specific \textsuperscript{51}Cr release was determined after 15 h incubation. In \textbf{C}, CD8\textsuperscript{+} cells were enriched by depletion of CD4\textsuperscript{+} cells by panning with OKT8 mAb; in \textbf{D}, CD4\textsuperscript{+} cells that were pre-incubated with OKT4 mAb were positively selected by adherence to pans coated with goat anti-mouse mAb; and in \textbf{E}, CD4\textsuperscript{+} cells were enriched by depletion of CD8\textsuperscript{+} cells by panning with OKT8 mAb.
cytes only if the PBMC had been cultured for 7 d with antigen (compare Fig. 2 A and B). If CD4⁺ cells were removed by panning with OKT4 mAb, the CD4⁻ PBMC retained only low levels of killing capacity (Fig. 2 C). In contrast, depletion of the CD8⁺ subset with OKT8 mAb did not reduce cytolytic activity (Fig. 2 E). The data in Fig. 2 C and E suggested that CD8⁺ suppressor/cytotoxic T cells were not the mediators of monocyte killing in this system. Positively selected CD4⁺ T cells showed active killing (Fig. 2 D). Taken together, these observations suggested that the CD4⁺ population was the antigen-specific cytolytic population. In addition, these studies suggested that the PPD-stimulated PBMC also contained CD4⁻,
CD8- cells that mediated nonspecific killing. NK or LAK cells could be the mediators of this effect.

**PPD-stimulated CTL Are MHC Class II Restricted.** CD4+ CTL are known to be MHC class II restricted (10). To verify this in our system, killing assays were carried out in the presence of mAb specific for class I (W6/32) or class II antigens (9.3C9 and L243; Fig. 3). Only the latter mAb blocked. CD4+ T cell cytotoxicity was inhibited by the presence of anti-class II (Fig. 3, A and B), but not by the anti-class I mAb (Fig. 3 D). The low level of antigen-independent cytotoxicity in our system was not affected by either anti-class I or anti-class II mAb (Fig. 3, A, C, and E). These observations indicated that antigen-specific CD4+ T cells were class II restricted, while antigen-independent CD4- , CD8- cells were not MHC restricted.

**Antigen-independent Cytotoxic Activity Generated by PPD Stimulation of PBMC.** Simultaneously with the generation of the CD4+ CTL (Fig. 4 C), PPD stimulation of PBMC generated a minor population of cytotoxic cells. This activity was more easily demonstrated by enrichment of the CD4- effector population (Fig. 4 D). The CD4- cells, 8% of the PPD-stimulated PBMC (Fig. 4 A), were enriched five- to sixfold in order to demonstrate the nonspecific killing of both PPD-pulsed and medium-pulsed monocyte targets.

Because NK and LAK cells exhibit antigen-independent killing (10), we tested if such killers might be present using standard target cells. Significant NK and LAK activity was generated after PPD stimulation (Fig. 4 B). This finding suggested, during the PPD response, that IL-2 was released and expanded NK and LAK cells that were then capable of killing monocyte targets. Since these populations were minor components of 7-d antigen-stimulated PBMC, we next compared the kinetics of their activity with that of antigen-dependent cytotoxicity after PPD, IL-2, PPD + IL-2, or medium stimulation (Table II). Peak LAK activity was observed after 4 d of culture. In contrast, antigen-dependent cytotoxicity was not detected until day 7, when nonspecific LAK cell activity was reduced.

![Figure 4](https://example.com/figure4.png)
In Table II, kineticsof antigen-dependent, NK, and LAK cell activity in PBMC cultures stimulated with PPD, IL-2, PPD + IL-2, or medium alone are presented.

<table>
<thead>
<tr>
<th>Effector stimulation</th>
<th>$^{31}$Cr release targets</th>
<th>Percent specific $^{31}$Cr release* with effectors at:</th>
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<tr>
<td>PPD</td>
<td>MO + PPD</td>
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<td>PPD</td>
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<td>Medium</td>
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* Percent specific $^{31}$Cr release was determined at an E/T ratio of 40:1.
† Monocyte targets were pulsed overnight with PPD, T. toxoid, or medium alone.

Intraleisional Injection of IL-2 Enhances Cytotoxic T Cell Precursor Activity. The effect of rIL-2 injections on the ability to generate antigen-induced cytotoxic activity of PBMC in vitro was tested. In 2:8 lepromatous leprosy patients, PBMC stimulated with BCG demonstrated antigen-specific cytolytic activity against monocytes charged with PPD, but not against monocytes pulsed with medium alone (Fig. 5, B and D). As with the PBMC isolated from PPD sensitive donors, unstimulated PBMC showed no cytolytic activity. If the patients were given three doses of 10 μg rIL-2 (3 × 10⁶ U/mg) intradermally at 48-h intervals, and their PBMC were then isolated and stimulated with BCG, antigen-specific cytotoxicity against PPD-charged monocyte targets was enhanced, compared with pre-IL-2 bloods, in 4:8 of the individuals tested (Fig. 5, A'-D' vs. A-D). In 3:8 lepromatous leprosy patients, no cytolytic activity was detected from PBMC isolated either pre- or post-IL-2 injection after stimulation with BCG (data not shown). High levels of nonspecific killing of medium-pulsed monocytes were also observed in 1:8 individuals examined (Fig. 5, C and C'). In vivo rIL-2 administration did not affect the number of T cells (CD3⁺ cells) nor did it affect the CD4⁺/CD8⁺ ratios in the blood of the individuals tested (mean, 67 ± 8% and 68 ± 4% CD3⁺ cells of PBMC before and after rIL-2 injection, respectively, and mean, 1.4 ± 0.1 and 1.5 ± 0.2 CD4⁺/CD8⁺ ratios of PBMC before and after rIL-2 injection, respectively). These observations suggested that in vivo rIL-2 administration modulated precursor cytotoxic cells such that antigen and rIL-2 treatment of PBMC in vitro induced an increase in cytolytic activity.
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Figure 5. Intradermal injection of rIL-2 into lepromatous leprosy lesions enhances in vitro antigen-dependent cytolytic activity. PBMC were isolated from four lepromatous leprosy patients before (A, B, C, and D) and 8 (A) 9 (B), 10 (C), and 14 (D') d after intradermal injection of rIL-2. Both pre and post-IL-2 PBMC were cultured in vitro for 7 d with BCG (●, ○) or medium alone (▲, △). Filled symbols denote monocyte targets pulsed overnight with PPD. Open symbols denote monocytes pulsed with medium alone. In 2/4 cases (A and C), the pre-IL-2 PBMC stimulated with BCG did not show killing of PPD-charged monocytes. In 4/4 cases the post-IL-2 PBMC demonstrated enhanced killing of monocytes as long as the PBMC were boosted with BCG (circles) and the monocytes were pulsed with PPD (closed symbols).

Discussion

Under steady state conditions and influenced by the specific unresponsiveness of T cells to M. leprae antigens, the dermis of lepromatous leprosy patients is relatively immunologically quiescent. Other than a small number of CD8+ T cells, the major cellular population consists of mononuclear phagocytes containing intact and fragmented M. leprae (1, 2).

Three general situations are known to disrupt the quiescent immunity leading to a massive influx of mononuclear cells into the dermis. The first are those factors leading to reactive states, in which for as yet unknown reasons, there is a widespread immigration of both CD4+ and CD8+ T cells and monocytes into the dermis (11). This massive response, often associated with systemic symptoms and evidence of advancing peripheral nerve damage, is associated with local cell destruction (12). A second method is to introduce crossreactive antigens, e.g., PPD, into the skin to initiate a typical, delayed-type, cell-mediated immune response. Here, extensive destruction of parasitized macrophages occurs, a reaction that we believe is critical for the subsequent disposal of bacilli (3, 4, 13). In our experience, Schwann cells and axons of peripheral nerves are spared in the PPD response, but are heavily involved in reactive states. In a third situation, lymphokines, such as IFN-γ (manu-
script in preparation) and particularly IL-2 (5), distal products of antigen-driven CD4+ cells, by themselves can initiate a local mononuclear cell infiltrate and reduction in acid-fast bacilli.
In this study we have identified one mechanism that could explain the destruction of parasite-laden mononuclear phagocytes. Since we could not isolate sufficient cells from the lepromatous lesions to use for this analysis, peripheral blood cells were used as a source of monocyte targets and cytotoxic cells. We selected PPD as the test antigen since large numbers of leprosy patients demonstrate sensitivity to PPD and because our results suggest that the dermal response to PPD in situ is associated with the destruction of macrophages (3).

Our results indicate that antigen-specific and nonspecific effector cells can be identified in peripheral blood and that these vary in numbers, potency, and inducibility. Antigen specificity is indicated because both effectors and targets must be exposed to antigen to detect killing. The major cell type involved in antigen-specific cytotoxicity is a T cell of the CD4* phenotype that recognizes class II-compatible targets. This response requires 4–7 d of exposure to PPD in vitro to be detectable. Prior studies in analogous murine (14) and human systems (15) have identified CD4* antigen-dependent killer cells. In addition, CD8* effector cells have been isolated (14). We do not find such a cytotoxic effector population in this system. A second group of cytotoxic cells is also generated during the PPD response, but killing is observed on targets that are not exposed to exogenous antigen and are required in high multiplicity to demonstrate their effectiveness. It appears that this "antigen-independent" population develops earlier than antigen-dependent populations under in vitro conditions.

It is likely that only very small numbers of PPD-reactive T cells (1% or less) are present in the circulation or tissues of sensitized individuals. Their response to injected PPD would require clonal expansion, the generation of lymphokines, and differentiation of CTL. This process requires time and would generate cells only after 7 d in the dermal site, which would have the properties of the CD4* antigen-specific, MHC class II-restricted T cell. This is about the time of extensive macrophage destruction in the PPD reaction (3). Earlier macrophage destruction has been noted after IL-2 administration (5). We suspect this represents a direct IL-2 effect on pre-existing and/or rapidly emigrating cells of the NK or LAK lineage (16), cells capable of amplifying the response with IFN-γ production and with the ability to carry out antigen-independent cytotoxicity. Examination of this temporal sequence of CTL generation requires direct analysis of the dermal populations isolated from biopsy specimens and these studies are currently in progress.

The following scenario would combine the in vitro and in vivo results from antigen- and lymphokine-driven systems. The proposed function of the cytolytic populations would be to kill the M. leprae parasitized monocytes and release the bacteria into the extracellular space. Once exposed to the extracellular environment, the bacilli could then be phagocytosed and killed by newly recruited, more activated macrophages and thus provide, in addition to IFN-γ activation, a mechanism to eradicate the parasites that have adapted to the intracellular environment of the very cells responsible for their destruction.

Summary

We have examined an in vitro system in which PBMC from purified protein derivative (PPD)-sensitized patients generate CTL after in vitro activation with antigen. These cells selectively destroy mycobacterial antigen PPD-pulsed monocyte targets.
These CTL are of the CD4+ phenotype and exhibit MHC class II restriction. After exposure to antigen these cells require 5–7 d for maximal development, whereas, a separate antigen-independent population is generated within 3–4 d. CD8+ cells are poorly, if not at all, cytotoxic under similar conditions. Cells with properties of the NK and LAK lineage are also present in these cultures and kill other specific targets.

Human rIL-2 was injected into the skin of lepromatous patients at 10-µg doses, given at 48-h intervals, for three doses. Peripheral blood cells obtained 8–14 d after the initiation of IL-2 injection demonstrated enhanced antigen-dependent destruction of monocyte targets.

The efficacy of antigen-dependent and -independent populations and their amplification by IL-2-dependent mechanisms is discussed in terms of the local destruction of parasitized macrophages and the subsequent disposal of M. leprae.

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