Characterization of T Cells That Confer a High Degree of Protective Immunity against Tuberculosis in Mice after Vaccination with Tumor Cells Expressing Mycobacterial hsp65

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Mice vaccinated by injection with tumor cells expressing the *Mycobacterium leprae* gene for hsp65 acquire a remarkably high degree of protection against challenge with *Mycobacterium tuberculosis*. We used limiting-dilution analysis to assess the frequency of CD4⁺ CD8[−] and CD4[−] CD8⁺ splenocytes responding to mycobacterial hsp65 in such vaccinated mice. Cells of both phenotypes were present at very high and equal frequencies (approximately 1:100). Vaccination with live *Mycobacterium bovis* BCG also increased the frequencies of both phenotypes of hsp65-reactive cells equally (to approximately 1:2,500), whereas vaccination procedures that were not protective, with either dead BCG, hsp65 protein in incomplete Freund's adjuvant, or hsp65 mixed with tumor cells, resulted in preferential increase in CD4⁺ CD8[−] cells. Twelve CD4⁺ CD8[−] and twelve CD4[−] CD8⁺ hsp65-responsive T-cell clones were obtained and characterized. All showed conventional antigen recognition via major histocompatibility complex class II and class I pathways but differed in secretion of gamma interferon and interleukin 4 and cytotoxicity. In tests of antimycobacterial activity against *M. tuberculosis*, both in infected macrophages in vitro and by adoptive transfer of protection with T-cell clones injected into irradiated mice, the most effective clones were the most cytotoxic and secretion of gamma interferon made only a secondary contribution.

There is a major need for a new vaccine against tuberculosis. About 3 million people die from this disease every year, and the infection is increasingly resistant to all of the most useful antimycobacterial drugs (24). Although *Mycobacterium bovis* BCG vaccination is widely used, it is ineffective in some populations, and its deployment impairs the subsequent use of skin test hypersensitivity for diagnostic and epidemiological purposes. Until very recently, attempts to develop subunit vaccines that might overcome these deficiencies of BCG have been unsuccessful. Now, most remarkably, we have found that even a single antigenic protein can generate strong protection in mice against challenge with virulent *Mycobacterium tuberculosis*, provided that it is generated endogenously within the antigen-presenting cells (APC) (37).

We vaccinated mice by expressing the gene for the 65-kDa heat shock protein (hsp65) from *Mycobacterium leprae*, either in J774 macrophage-like tumor cells (39) or in bone marrow cells (40) which were then injected into the mice or by directly injecting plasmid DNA (21). Injections of protein were ineffective even with adjuvant. As a guide to future vaccine development, it is important to establish the basis of this superiority of endogenous over exogenous antigen. The finding is consistent with reports that T lymphocytes with the CD4[−] CD8⁺ phenotype make a substantial contribution to protective immunity to tuberculosis in mice (9, 10, 13, 26); endogenous antigen gains access to the major histocompatibility complex (MHC) class I antigen presentation pathway and thereby stimulates CD8⁺ lymphocytes. This view was sustained when we examined the properties of three T-cell clones that were ob-

tained from the spleens of mice that had been vaccinated with J774 cells expressing hsp65 (41). The clones represented the three main phenotypes; CD4⁺ CD8[−], CD4[−] CD8⁺, and T-cell receptor (TCR) γ/δ . In tests of adoptive transfer of protective immunity against challenge with *M. tuberculosis* to irradiated recipient mice, the CD4[−] CD8⁺ clone was the most effective. This clone was also able to selectively lyse macrophages that had phagocytosed *M. tuberculosis*. However, these clones might not be truly representative. The actual contribution of such T cells to protection in the actively immunized mice will depend on the absolute and relative frequencies of the cells. Here, we extend the investigation to assess this aspect. Furthermore, CD4⁺ CD8[−] lymphocytes can be cytotoxic and both CD4⁺ CD8[−] and CD4[−] CD8⁺ lymphocytes can release gamma interferon (IFN- γ), which enhances murine macrophage antimycobacterial activity (7, 16, 34), and interleukin 4 (IL-4), which down-regulates this aspect of cellular immunity in favor of expression of antibody response (36). Therefore, we have tested a range of CD4⁺ CD8[−] and CD4[−] CD8⁺ hsp65-specific clones to define the relationships among capacity to transfer protective immunity, antigen-specific cytotoxicity, and secretion of IFN- γ and IL-4.

MATERIALS AND METHODS

Immunization of mice. Young adult BALB/c and C57BL/6 mice were obtained from the vivarium of the School of Medicine of Ribeirão Preto, University of São Paulo, and were maintained under standard laboratory conditions. APC that endogenously produced *M. leprae* 65-kDa heat shock protein (J774-hsp65) were prepared by transfecting the macrophage-like BALB/c cell line J774.G8 with a retrovirus shuttle vector [pZIPNeoSV(X)] carrying the mycobacterial gene, and control cells were transfected with the vector only (J774-vector) as previously described (39). The mice received a regimen of weekly doses of intravenous (i.v.) 10⁵ J774-hsp65 cells for 4 weeks. This was the optimum to evoke cellular immunity to the protein and protection against challenge with *M. tuberculosis* (37). Challenge was with *M. tuberculosis* H37Rv obtained from the American Type Culture Collection (Rockville, Md.), grown in Dubos broth, washed, stored at

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TABLE 1. Frequencies of splenic T cells responding to J774-hsp65 or J774-vector^a

Immunization schedule ^b	Frequency for cells responsive to J774-hsp65		Frequency for cells responsive to J774-vector	
	CD4 ⁺ CD8 ⁻	CD4 ⁻ CD8 ⁺	CD4 ⁺ CD8 ⁻	CD4 ⁻ CD8 ⁺
J774-hsp65	1:128	1:64	1:43,214	1:76,800
J774-vector + rMLhsp65	1:8,950	1:48,970	1:76,540	<1:100,000
rMLhsp65 in IFA	1:2,670	1:25,950	<1:100,000	<1:100,000
Live BCG	1:2,370	1:2,960	1:69,800	1:91,400
Dead BCG	1:19,800	1:38,500	1:81,500	<1:100,000
J774-vector	<1:100,000	<1:100,000	<1:100,000	<1:100,000
<i>Listeria monocytogenes</i>	<1:100,000	<1:100,000	NT ^c	NT
Saline (control)	<1:100,000	<1:100,000	NT	NT

^a Thirty days after commencement of immunization, CD4⁺ CD8⁻ and CD4⁻ CD8⁺ splenocytes were prepared from immunized and control BALB/c mice by sequential negative and positive selection and were immediately titrated on J774-hsp65 or J774-vector feeder cells.

^b Immunization was either by four i.v. injections of 10⁵ J774-hsp65 or J774-vector cells together with 25 µg of rMLhsp65 at weekly intervals, by two subcutaneous injections of 25 µg of rMLhsp65 in IFA a week apart and then of 15 µg in PBS on day 14, or by i.v. injection of 10⁴ live or dead BCG cells or 10⁶ live *L. monocytogenes* cells.

^c NT, not tested.

-70°C, and inoculated into mice as described elsewhere (37). Viability was checked by plating 10-fold serial dilutions on Middlebrook 7H11 agar (Difco) and counting the resultant colonies (in CFU) after 21 days of incubation at 37°C.

Preparation of T-cell subpopulations from spleens of vaccinated mice. Antigen-specific CD4⁺ CD8⁻ and CD4⁻ CD8⁺ T cells were obtained from splenocytes by negative and positive selection with specific antibodies and amplification by culture. In brief, groups of six immunized mice were killed 30 days after the first injection of J774-hsp65 cells, and their splenocytes were purified from homogenized spleens by centrifugation in complete RPMI 1640 medium (RPMI-C) on lymphocyte M, depletion of adherent cells on tissue culture plastic for 2 h at 37°C, and then by two passages through nylon wool columns (41). Selection for subpopulations was by sequential steps of complement-mediated lysis and panning, as previously described (41). The phenotype and purity of the T-cell subpopulations were checked with a FACScan by using fluorescein isothiocyanate immunofluorescence with rat monoclonal antibody (MAb) L3T4 or Lyt2 (Amersham) against surface markers CD4 and CD8 and hamster MAb H57-597 or GL3 (Pharmingen) against TCR α/β and γ/δ, respectively (41).

Frequencies of reactive CD4⁺ CD8⁻ and CD4⁻ CD8⁺ T cells in the spleen. Limiting-dilution analysis was used to determine the frequencies of hsp65-reactive splenocytes (15, 43). J774-hsp65 and J774-vector cells were irradiated (40 Gy) and used in parallel as accessory cells in RPMI-C with 5 × 10⁴ cells per V-bottom well in 96-well plates. Dilutions of subpopulations of splenocytes in RPMI-C were added to give final concentrations of 2 to 4,096 T cells per well in 0.2 ml with 24 replicates. The cells were cultured in the presence of recombinant IL-2 (rIL-2; 1 ng/ml) at 37°C with 5% CO₂ in air for 12 to 14 days. Cultures were then pulsed with 1 µCi (37 kBq) of [³H]thymidine (Amersham) per well for another 18 h, after which the cultures were harvested onto filter papers and radiolabel incorporation was assessed by liquid scintillation counting. Wells giving counts that exceeded the counts from controls (unstimulated cells) by 3 standard deviations were given positive scores. The frequencies of antigen-responsive T cells were estimated by plotting the percentage of wells that were negative against T-cell concentrations according to Poisson distribution and by the minimum χ² method (43). Cell concentrations at which all of the wells were positive or negative were not included in frequency calculations.

Isolation of T-cell clones. T-cell subpopulations obtained after negative and then positive selection as described above were seeded at 256 cells per well into V-bottom 96-well plates and were cultured in the presence of irradiated (40 Gy) J774-hsp65 cells (feeder cells; 5 × 10⁴ cells in 0.1 ml) and rIL-2 (1 ng/ml) for 14 days. The cells were then cloned at 0.3 cells per well in round-bottom 96-well plates in the presence of 10⁵ feeder cells and 2 ng of IL-2 per ml. Growing clones were restimulated every 2 to 3 weeks with phytohemagglutinin (0.5 µg/ml) in the presence of feeder cells. Twelve strongly growing CD4⁺ CD8⁻ and 12 CD4⁻ CD8⁺ clones were selected for characterization. All were TCR α/β positive by fluorescence-activated cell sorter analysis (41).

Characterization of CD4⁺ CD8⁻ clones. The antigen specificities and effects of inhibitors of antigen processing and presentation on clone proliferation were assessed with irradiated (40-Gy) J774-hsp65 and J774 vector cells as APC as previously described (38). The wells contained 1 × 10⁵ T cells and 5 × 10⁴ APC. After 72 h of incubation, the wells were pulsed with [³H]thymidine for 18 h. Antigens were present at a final concentration of 5 µg/ml (rMLhsp65) or 25 µg/ml (bovine serum albumin [BSA] or purified protein derivative [PPD]) and concanavalin A at 5 µg/ml. Chloroquine was tested by loading J774-hsp65 cells in a 12.5 µM solution for 4 h at 4°C before irradiation and by incubation continued at the same concentration. MAbs L3T4, Lyt2, and anti-I-A^d-I-A^k, and -H-2K^d were added to irradiated APC or to T cells as appropriate before mixing them together and were present for the duration of incubation. To assess cytokine secretion, clones were washed twice and suspended in RPMI-C supplemented with 10% fetal calf serum (FCS) and 10⁶ cells incubated for 24 h in 1 ml

in 24-well plates with phorbolmyristic acid (10 ng/ml) and anti-CD3 MAb (YCD3-1 [Gibco-BRL]; 50 ng/ml) to achieve maximal stimulation (11). Cell-free supernatants were harvested and kept at -20°C prior to assaying of IFN-γ and IL-4 by enzyme-linked immunosorbent assays (ELISA) and tests of the ability to activate macrophage antimycobacterial activity (see below). Antigen-specific cytotoxicity and cytotoxicity for cells infected with *M. tuberculosis* were determined as previously described (41). J774-hsp65 cells and J774-vector cells that had been pulsed with protein antigen (25 µg/ml) for 40 to 48 h were washed by centrifugation and were labelled with ⁵¹Cr (100 µCi/ml) for 1 h at 37°C. After three washes, 5 × 10³ target cells were incubated with serial dilutions of freshly cultured effector clones in a total volume of 200 µl of test medium in 96-well round-bottom plates. Target cells were also incubated with medium alone and with 0.5% Triton X-100 to determine the spontaneous and maximal ⁵¹Cr release, respectively. Putative inhibitors were either preincubated with the targets for 4 h at 4°C (chloroquine, 12.5 µM; brefeldin A, 100 µg/ml) or added immediately before effector cells (MAb) and were present throughout the assay. After 4 h at 37°C, the plates were centrifuged, cell-free supernatants were collected from the wells, and the ⁵¹Cr contents of the supernatants were determined with a γ counter. To test whether cytolytic clones also lysed cells infected with *M. tuberculosis*, J774 vector cells and macrophages from the thioglycolate-stimulated peritoneal cavities of normal BALB/c or C57BL/6 mice (given 3 ml of 3% Na-thioglycolate 3 days before harvesting) were used. Cells were loaded with ⁵¹Cr as described above and then allowed to phagocytose live bacteria in the presence of 5% fresh FCS overnight at a bacterium-to-cell ratio of 10:1 and were rinsed five times with medium to remove nonassociated bacteria. The removal of nonassociated bacteria was confirmed by Ziehl Neelsen staining, and 80 to 90% of the cells had cell-associated mycobacteria as determined by light microscopy. Lysis of infected cells by T-cell clones was assessed as ⁵¹Cr release as described above.

Characterization of CD4⁻ CD8⁺ clones. The specific cytotoxicity of CD4⁻ CD8⁺ clones was tested as described for CD4⁺ CD8⁻ clones, except that J774-vector target cells were preloaded with antigen by using liposomes to facilitate MHC class I presentation (41). Positively charged multilamellar liposomes were prepared with phosphatidylcholine-stearylamine-cholesterol (molar ratio, 63:18:19; liposome kit from Sigma, Poole, United Kingdom) by resuspending a thin lipid film in phosphate-buffered saline (PBS) with or without rMLhsp65 or BSA (30 mg/ml), ultrasonication, and then separating liposomes and entrapped antigen from the free antigen by gel filtration (Sephacrose 2B; Pharmacia). J774-vector cells were incubated with liposomes for 2 h at 37°C and then washed and loaded with ⁵¹Cr as described above. Cytokine production from maximally stimulated clones and cytotoxicity toward *M. tuberculosis*-infected macrophages and J774 cells were tested as described for CD4⁺ CD8⁻ clones.

Assays of antimycobacterial activity of T-cell clones in vitro. We tested the ability of supernatant cytokines and direct cell-cell contact to impair growth of *M. tuberculosis* in bone marrow-derived macrophages. Bone marrow cells were prepared as described elsewhere (40). Briefly, cells were flushed from the femurs of BALB/c mice and cultured in Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% FCS, 10% L-cell-conditioned medium, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. L-cell-conditioned medium was prepared by seeding 5 × 10⁵ L-929 cells in 50 ml of DMEM supplemented with 10% FCS and 150 µg of gentamycin per ml. After 1 week at 37°C, L-cell supernatants were harvested, cell debris was removed by centrifugation (3,000 × g, 10 min), and supernatants were filtered (0.45-µm-pore-size filters) and stored at -20°C until use. Bone marrow cells were plated at 5 × 10⁵ cells per well in 24-well plates and were cultured for 5 days at 37°C in 5% CO₂ in air. To test cytokines, the cells were washed, and fresh medium with or without 10% T-cell culture supernatant and with or without anti-IFN-γ MAb

FIG. 1. Antigen specificity (A) and effect of inhibitors (B) on proliferation of CD4⁺ CD8⁻ T-cell clones. Twelve clones raised from J774-hsp65-vaccinated mice were assessed for growth ([³H]thymidine uptake) by a standard lymphoproliferation assay with either J774-hsp65 or J774 vector as feeder cells. The results from 1 clone, CD4.1, are shown and were essentially identical with those from all 12. Chloroq., chloroquine.

was added on days 5 and 7. On day 8, 2×10^6 live *M. tuberculosis* cells were added, and after a further 5 days, the cells were washed and lysed with 0.1% saponin, 1 μ Ci of [³H]uracil was added, and radioactivity incorporation into the bacteria was determined 18 h later (6, 35). The numbers of live bacteria in lysates were determined as CFU on 7H11 agar in parallel (37). To test the effect of direct cell-cell contact, bone marrow-derived macrophages were infected as described above on day 5 of culture, and after a further 4 days, the cells were washed and 5×10^5 cells of hsp65-reactive T-cell clones with cytolytic activity were added, with or without anti-IFN- γ MAb. After 18 h of incubation, the cells were lysed with 0.1% saponin and the viability of the bacteria in the wells was determined by [³H]uracil uptake and CFU counts. CFU counts closely paralleled those of radiolabel incorporation, and only the latter are shown.

Adoptive transfer of immunity to *M. tuberculosis* with hsp65-reactive T-cell clones. The ability of clones to protect mice against challenge with *M. tuberculosis* was tested as previously described (41). Briefly, BALB/c mice were irradiated with gamma rays (50 Gy) and then injected i.v. with 5×10^6 cells of one of the hsp65-reactive CD4⁺ CD8⁻ or CD4⁻ CD8⁺ clones, which was immediately followed by 10^5 CFU of *M. tuberculosis* i.v. Some of the mice received anti-IFN- γ MAb (0.25 mg per dose) intraperitoneally 2 days before injection of the T cells, immediately before injection, and at 4-day intervals thereafter. Control mice either received whole CD4⁺ CD8⁻ or CD4⁻ CD8⁺ subpopulations purified from nonvaccinated mice or were untreated. The mice were killed after 3 weeks, and the numbers of CFU in livers were determined; earlier studies established that changes in the liver and spleen were similar (37).

RESULTS

Vaccination with J774-hsp65 cells promotes a high frequency of specific CD4⁺ CD8⁺ T cells. Our standard protocol of vaccination with J774-hsp65 cells resulted in a very high frequency of hsp65-reactive T cells in the spleen, and CD4⁺ CD8⁻ and CD4⁻ CD8⁺ phenotypes were about equally represented (Table 1). The second most effective protocol was vaccination with live BCG, for which the frequencies were about 10-fold lower, and again the frequencies of the phenotypes were equal. Injection of rMLhsp65 protein mixed with control J774-vector cells or emulsified in incomplete Freund's adjuvant (IFA) elicited predominantly CD4⁺ CD8⁻ cells; CD4⁻ CD8⁺ cells were about 10-fold less frequent. T cells reactive to the tumor cells (J774-vector) were elicited at low

frequencies by J774-hsp65, J774-vector plus rMLhsp65, or BCG, but not by rMLhsp65 in IFA or by J774-vector alone.

Antigen specificity of T-cell clones. Twelve CD4⁺ CD8⁻ clones were raised and compared for their abilities to proliferate in the presence and absence of hsp65 and unrelated antigen (BSA) and for the effects of inhibitors of antigen processing and presentation. The results were highly consistent between clones, and Fig. 1 shows the results from one of them. The cells did not proliferate with J774-vector unless rMLhsp65 was added; BSA and PPD were ineffective. Proliferation on J774-hsp65 was inhibited by chloroquine, which blocks antigen processing for presentation on MHC class II and by MAb directed against MHC class II of the correct haplotype (I-A^d) or against CD4 (MAb L3T4). MAb against irrelevant class II haplotype I-A^k or MHC class I (H-2K^d) or CD8 (Lyt2) had no effect.

Twelve clones with the CD4⁻ CD8⁺ phenotype were raised and tested for antigen-specific cytotoxicity and for effects of inhibitors of antigen processing, presentation, and recognition. The clones varied in their degrees of cytotoxic activity toward J774-hsp65, 2 of 12 being essentially noncytotoxic (Fig. 2); however, all showed the same pattern of effects of inhibitors, and results with 2 representative clones are shown (Fig. 3). Cytotoxicity was inhibited by brefeldin A, which blocks antigen processing for presentation on MHC class I but not by chloroquine; MAb against CD8 (Lyt2) but not against CD4 was inhibitory, and MAb against H-2K^d but not against H-2K^k or I-A was inhibitory. Seven of the 12 CD4⁺ clones were also cytotoxic toward J774-hsp65 (Fig. 2). These CD4⁺ clones also

FIG. 2. Comparison of cytokine secretion and cytotoxicity of the 12 CD4⁺ CD8⁻ and 12 CD4⁻ CD8⁺ clones. Cytokines were collected as 24-h supernatants from maximally stimulated clones and were assayed by ELISA. Similar results were obtained in two separate experiments. The sensitivities of the assays are indicated by the dashed line. Cytotoxicity was measured with ⁵¹Cr-loaded J774-hsp65 cells as targets at an effector-target ratio of 50:1, corrected for spontaneous radiolabel release, and expressed as percentages of maximal release with Triton X-100. Similar results were obtained in at least two experiments at a range of effector-to-target ratios.

FIG. 3. Effect of inhibitors on the cytotoxicity of CD4⁺ CD8⁺ clones. Clones were incubated with ⁵¹Cr-loaded J774-hsp65 target cells at an effector-to-target cell ratio of 50:1 with or without putative inhibitors, and percentages of cytotoxicity were calculated as described in the legend to Fig. 2. Similar results were obtained in at least two experiments. Chloroq., chloroquine.

lysed J774-vector cells when exogenous rMLhsp65 was provided (Fig. 4), whereas the CD8⁺ clones lysed J774-vector cells only when the antigen was introduced into the cells via liposomes. BSA was unable to substitute for rMLhsp65 (not shown).

Cytokine production by T-cell clones. Because the production of IFN-γ is a property of T cells that promote cellular immunity (i.e., of Th1 cells) and production of IL-4 is a property of cells that promote antibody-mediated immunity (i.e., of Th2 cells), we assessed the capacity of all 24 clones to produce these cytokines (Fig. 2). Three of the CD4⁺ clones did not produce IFN-γ and produced IL-4, and one CD4⁺ clone produced both IFN-γ and small amounts of IL-4. Ten of the CD8⁺ clones produced IFN-γ in amounts equivalent to those of the active CD4⁺ clones, and two produced a little. Four CD8⁺ clones produced IL-4 and two were low-level IFN-γ producers. Neither IFN-γ nor IL-4 production bore any clear relationship to cytotoxicity for J774-hsp65 cells (Fig. 2), although the IL-4 producers tended to be less cytotoxic.

Cytotoxicity and antimycobacterial activity against *M. tuberculosis*-infected cells. All of the CD8⁺ clones and the 7 CD4⁺ clones with cytotoxic activity were tested for ability to lyse infected cells (Fig. 5). J774-vector cells and BALB/c peritoneal macrophages were not lysed unless they were infected, in which case they were lysed as efficiently as J774-hsp65 cells. Macrophages from C57BL/6 mice were minimally lysed, even when infected, which is consistent with MHC restriction of cytolysis.

Antimycobacterial effects of T-cell clones in vitro. Four CD4⁺ clones and three CD8⁺ clones, representing the spectrum of cytokine and cytotoxic activity profiles that were observed, were tested for action against *M. tuberculosis* in monolayers of bone marrow-derived macrophages. Supernatants (lymphokines) from four of the clones were able to enhance macrophage antimycobacterial activity (Fig. 6); the ability of

the mycobacteria to incorporate [³H]uracil was impaired by 30 to 40% in treated compared with untreated macrophages, and the number of live bacteria (CFU) was restricted in parallel (not shown). Lysis of macrophages by exposure to clone supernatants was not detectable by nucleus counting with naphthol blue black (0.05%, pH 2.2; not shown). The effective clones were the ones that produced IFN-γ, and the antimycobacterial activity of the supernatants was neutralized with anti-IFN-γ Mab. Some IFN-γ-negative clones produced supernatants that had modest growth-enhancing effects. The basis of this was not explored.

When the clones with cytotoxic activity were incubated in contact with infected macrophages, bacterial radiolabel incorporation was reduced by 22 to 80%, compared with macrophages that were not exposed to lymphocytes (Fig. 6); the least effective clone was the CD8⁺ clone with only slight cytotoxic activity.

In theory, the impaired bacterial viability in monolayer wells treated with clone supernatants could have been an artifact if bacteria were more readily lost from the wells during the rinsing procedure prior to assaying, either because more of the bacteria were extracellular or more of the macrophages detached. Furthermore, conventional concentrations of antibiotics were included in cell culture media to minimize extraneous microbial contamination. However, macrophage activation

FIG. 4. Antigen dependence of cytotoxicity. J774 vector cells were used as targets either directly (open bars) or after incubation with antigen (shaded bars). Antigen (rMLhsp65) either was initially present in the medium for 48 h at 25 μg/ml or was introduced in positively charged liposomes for 2 h before the targets were loaded with ⁵¹Cr and tested at an effector-to-target ratio of 50:1. Similar results were obtained in two separate experiments.

FIG. 5. Cytotoxicity towards infected cells. J774-vector cells and peritoneal macrophages from BALB/c and C57BL/6 mice were loaded with ^{51}Cr and then infected by incubation with *M. tuberculosis* overnight at a bacterium-to-cell ratio of 10:1 before use as targets in the standard cytotoxicity assay at an effector-to-target ratio of 50:1. Uninfected J774 vector cells and macrophages (open bars) and J774-hsp65 cells were tested in parallel. Subtracted spontaneous radiolabel release values were 4 to 5% for noninfected cells and 10 to 12% for infected cells. Similar results were obtained in two separate experiments.

might promote antibiotic action against intracellular bacteria, perhaps through enhanced antibiotic uptake (31), and macrophage damage or lysis by cytotoxic T cells might expose intracellular bacteria to the extracellular antibiotics. Tests showed that neither of these potential artifacts contributed significantly to the antimycobacterial activities of the clones (Table 2). Our tests have not distinguished whether the antimycobacterial effects were due to mycobacterial growth inhibition only or included killing.

Adoptive transfer of protective immunity by injecting T-cell clones into naive mice. When each of the seven T-cell clones representing the spectrum of phenotypes of hsp65-reactive $\text{CD4}^+ \text{CD8}^-$ or $\text{CD4}^- \text{CD8}^+$ clones was separately injected into BALB/c mice before challenge infection with *M. tuberculosis*, they differed in their protective efficacy (Fig. 7). The most protective clone tested (CD8.4) reduced the numbers of live bacteria in the liver at 3 weeks postchallenge by 2 orders of magnitude (99%) compared with untreated mice or mice that had been irradiated and then injected with nonspecific CD8^+ or CD4^+ T cells. This clone both was cytotoxic and produced $\text{IFN-}\gamma$, and its effect was moderately reduced by coinjection of anti- $\text{IFN-}\gamma$ MAb. The second most protective clone (CD8.5; 94% reduction) did not produce $\text{IFN-}\gamma$, and coinjection of anti- $\text{IFN-}\gamma$ did not reduce protection. The CD8^+ clone that produced $\text{IFN-}\gamma$ but was only slightly cytotoxic (CD8.8) had only a slight protective effect (30% reduction) that was neutralized by anti- $\text{IFN-}\gamma$. The most protective CD4^+ clone (CD4.3; 84% reduction) was cytotoxic and produced $\text{IFN-}\gamma$; anti- $\text{IFN-}\gamma$ partially abrogated protection. The CD4^+ clone with cytotoxicity and no $\text{IFN-}\gamma$ production had a small protective effect (51%) that was not affected by anti- $\text{IFN-}\gamma$. The clone with $\text{IFN-}\gamma$ production and no cytotoxicity (CD4.1) also had a small protective effect (34%), and this was neutralized by anti- $\text{IFN-}\gamma$. The clone that had neither cytotoxicity nor $\text{IFN-}\gamma$ production (CD4.5) had virtually no protective effect either.

DISCUSSION

These results indicate that the very high level of protective efficacy of J774-hsp65 vaccination can be ascribed to two features of the immune response: induction of hsp65-reactive T cells to a very high frequency in the lymphocyte population and induction of cells with an appropriate phenotype. These findings have implications for both understanding the mechanisms of protection and the development of practical vaccines to replace BCG.

Hsp65 is the first individual mycobacterial antigen to be shown capable of eliciting a high degree of protective immunity against tuberculosis. However, it is only one of many potentially protective antigens (28). Although hsp65-reactive T cells were about 20 times more frequent after J774-hsp65 vaccination than after vaccination with live BCG, only about 1/20 of T cells recognizing BCG-infected cells after BCG vaccination were specific for hsp65 (data not shown). A similar proportion of mycobacterium-specific T cells recognized hsp65 after vaccination with killed *M. tuberculosis* in IFA (6). T cells with a mixture of mycobacterial antigen specificities can adoptively transfer protection (19, 27). Whether a similar vaccination protocol can achieve equivalent protection with any of the other individual antigens remains to be seen; however, this seems likely. There may be three basic prerequisites for the expression of protection: (i) there must be a high frequency of reactive T cells, (ii) the T cells must have an appropriate

FIG. 6. Antimycobacterial effect of clone supernatants and direct cell-cell contact with bone marrow macrophages infected in vitro. Bone marrow macrophage monolayers were either activated by exposure to T-cell supernatants (10% [vol/vol]) for 3 days before infection with *M. tuberculosis*, bacterial viability being measured 5 days later (A), or were infected for 4 days and then incubated in contact with T cells for 18 h (B). Antimycobacterial activities were measured as inhibition of bacterial capacity to take up ^3H uracil and were expressed as percentages of the radiolabel uptake by bacteria from control cells that had not been exposed to either supernatants or clones (None). The effects of adding anti- $\text{IFN-}\gamma$ to either supernatant or cell mixture were tested in parallel (shaded bars). The results are means ($n = 3$ [$\pm$ standard deviations]). The abbreviated clone properties are capacity to secrete $\text{IFN-}\gamma$ or IL-4 or to exhibit antigen-specific cytotoxicity (CytX). The dashed line represents 0% inhibition for convenient reference.

TABLE 2. Contribution of monolayer rinsing prior to assaying and inclusion of antibiotics in the medium to apparent macrophage antimycobacterial activity induced by T-cell clones

T-cell clone	Monolayer treatment		Testing clone supernatant ^a		Testing clone contact ^b	
	Rinsing	Antibiotics	[³ H]uracil uptake	% Inhibition	[³ H]uracil uptake	% Inhibition
None	—	—	12,832 ± 1,040	0.0	15,370 ± 1,612	0.0
	—	+	12,131 ± 854	5.5	14,090 ± 1,213	8.4
	+	—	12,540 ± 917	2.1	14,832 ± 1,257	3.7
	+	+	11,721 ± 864	8.7	13,738 ± 1,475	10.7
CD4.3	—	—	10,322 ± 1,351	20.0	9,372 ± 1,072	40.4
	—	+	9,724 ± 816	25.3	8,512 ± 861	45.7
	+	—	10,114 ± 1,203	21.2	9,131 ± 1,130	40.6
	+	+	8,917 ± 921	31.6	8,049 ± 917	47.7
CD8.4	—	—	9,070 ± 1,120	30.4	4,720 ± 561	70.3
	—	+	8,564 ± 952	34.3	3,384 ± 617	78.0
	+	—	8,871 ± 947	31.9	3,748 ± 386	75.7
	+	+	8,085 ± 789	37.0	2,830 ± 416	81.6

^a Monolayers were exposed to 10% clone supernatant for 3 days then incubated with bacteria for 5 days and then either rinsed to remove extracellular bacteria or not rinsed. Maintenance medium was either antibiotic free or contained 100 U of penicillin and 100 µg of streptomycin per ml throughout.

^b Monolayers were infected on day 5 of culture and then either rinsed or not rinsed on day 9 and exposed to T cells for 18 h. Medium either contained or did not contain antibiotics throughout.

phenotype (function), and (iii) the antigen(s) recognized must be presented in adequate amounts at the surfaces of infected cells. The precise identity of the antigen recognized, whether one or many, may be essentially irrelevant, provided that these requirements are met.

Others have argued on theoretical grounds that the immune recognition of hsp65 is irrelevant to protective immunity (28). The argument seems to be (i) that the antigen will not be released by live bacteria in infected cells but will only appear late in the infection when the bacteria are being killed following immune recognition of some other antigen(s), and then (ii) that the phenotype of the T cells generated at that stage is not appropriate for protection. Our experiments show that both this conclusion and its premises are mistaken.

Regarding antigen availability, we see that the prominence of CD4⁺ CD8⁺ hsp65-reactive T cells in BCG-vaccinated mice, the lysis of infected macrophages by such T cells in vitro, and the protective effect in vivo on adoptive transfer all imply that the hsp65 protein is produced in substantial amounts by intracellular mycobacteria. It is possible that cross-reactive endogenous murine hsp60 induced by the stress of infection could also play a part (17). However, we previously showed by competitive inhibition that the cytotoxic activity of a CD4⁺ CD8⁺ clone was specific for mycobacterial hsp65 (41). It is further implied by our findings that the protein produced by the intracellular mycobacteria, or fragments of the protein, readily gains access to the intracellular antigen-processing pathway leading to presentation on MHC class I. Since this pathway favors antigen originating within the cytosol (12), the question arises of whether there are intracellular transport routes whereby antigen released by microorganisms living in endosomes can access that pathway or whether the mycobacteria themselves enter the cytosol. There is evidence in both directions (2, 22, 32), and further study is needed.

Regarding the necessary phenotype of the protective T cell, debate initially revolved around the relative contributions of subgroups distinguished as using either CD4 or CD8 in response to antigen (9, 10, 13, 26, 28, 29). Indeed, our initial hypothesis was that endogenous generation of antigen within APC would bias the immune response toward the CD8/MHC class I pathway and that this might be advantageous for pro-

FIG. 7. Adoptive transfer of protective immunity against tuberculosis by transfer of T-cell clones to naive mice. Mice were irradiated with gamma rays and then injected i.v. with 5×10^6 clone cells and 1×10^5 *M. tuberculosis* cells. Some mice were in addition treated intraperitoneally with anti-IFN- γ MAb (shaded bars). The numbers of live bacteria in the liver were determined 3 weeks after infection. Control mice were either untreated or were irradiated and reconstituted with nonspecific splenic T cells enriched from normal mice (CD4-ns and CD8-ns). Percentages of inhibition of bacterial numbers were calculated relative to untreated controls. Results are means ($\pm$ standard deviations) from groups of five animals.

tection. Our results are consistent with this hypothesis; both J774-hsp65 and live BCG favored the development of CD8 responses. However, it is clear from our experiments that, at least in this model, it is not primarily the presence or absence of CD4 or CD8 molecules (and resultant antigen recognition on MHC class II or class I, respectively), but rather the degree of associated cytotoxic activity that most closely predicts the protective efficacy of the T cell (Fig. 7). A higher proportion of CD8⁺ clones were cytotoxic (Fig. 2), and they were on average more cytotoxic toward infected macrophages than were the CD4⁺ clones (Fig. 5). Presumably, infected macrophages are lysed by these cells *in vivo*, but how this results in protection is not clear. The antimycobacterial effect observed *in vitro* (Fig. 6) may reflect the poor ability of the bacteria to multiply extracellularly in tissue culture medium rather than a bactericidal event. *In vivo*, additional factors such as rephagocytosis and concomitant killing by freshly activated macrophages may play a part (6). Indeed, CD8 cells may be better able to provide or activate the additional factors since, both *in vitro* with infected macrophages and in the adoptive transfer experiments, cytotoxic CD8 clones were substantially more antibacterial than cytotoxic CD4 clones (Fig. 6 and 7) despite having only slightly greater cytotoxic activity against infected cells (Fig. 5). It may be relevant that T cells with different phenotypes can use different mechanisms of cytotoxicity, including ATP production (3, 33); BCG is killed by human macrophages when they undergo ATP-triggered apoptotic death but not necrosis (23).

Recent debate has concerned the role of T-cell cytokine phenotype in protective immunity both in humans (4, 25) and mice (14, 30). IFN- γ is one of the main factors that activate macrophage antimycobacterial activity in mice, primarily enhancing intracellular mycobacteriostasis but also being capable of causing killing of some mycobacteria during phagocytosis by murine macrophages (1, 16). The majority of the CD4⁺ and CD8⁺ clones were able to produce this cytokine to a similar extent (Fig. 2), and the IFN- γ in supernatants of CD4⁺ and CD8⁺ clones was equally effective in activating macrophage antimycobacterial activity *in vitro* (Fig. 6). However, the small contribution made by any clone's IFN- γ production to transferred protection (Fig. 7) suggests that either substantial IFN- γ is already available from background sources in irradiated naive mice or IFN- γ makes little contribution to expression of immunity in this model. The lack of effect of anti-IFN- γ MAb on the infection in control mice or in mice protected by clone CD8.5 (a cytotoxic but IFN- γ -negative clone) is consistent with a lack of contribution by background sources of IFN- γ . If this interpretation is correct, then the essential role of IFN- γ in protection against tuberculosis in mice, which is demonstrated by the susceptibility of IFN- γ gene knock-out mice (8), would be largely restricted to the amplification to high frequency of antigen-specific cytotoxic T cells (42), in other words, to the acquisition rather than the expression phase of acquired immunity. The small beneficial effect that IFN- γ from transferred clones did have (as judged by the effect of anti-IFN- γ [Fig. 7]) was at its greatest when the IFN- γ was produced by the clones with cytotoxic activity. This is consistent with its having a secondary role of activating macrophages to kill the mycobacteria liberated from the lysed macrophages during the expression phase.

Although a third of the clones that we obtained secreted IL-4, irrespective of CD4 or CD8 expression, and several of these produced both IFN- γ and IL-4, this had little bearing on the cytotoxicity or antimycobacterial function of the clones (Fig. 2 and 7). Therefore, the stable differentiation for coordinate expression of cytokines favoring cellular immunity (Th1

or Th2; typified by IFN- γ , IL-2, and IL-12) or antibody response (Th2 or Th1; typified by IL-4, IL-6, and IL-10) occurs independently of differentiation for the expression of the final effector stage of immunity, for which we have at present no marker except cytotoxicity.

It remains to be seen if the provision of protection against tuberculosis by cytotoxic T cells represents a general principle that will apply in other animal models and in humans. There is evidence that it might (5, 18). The discovery that direct injection of plasmid DNA can be used to test this and other mycobacterial antigens (20, 21) means that we might know relatively quickly.

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REFERENCES

- Andrew, P. W., and D. B. Lowrie. 1988. Role of interferon in immunity to mycobacteria, p. 263–286. In G. I. Byrne and J. Turco (ed.), *Interferon and nonviral pathogens*. Marcel Dekker, New York.
- Armstrong, J. A., and P. D. Hart. 1975. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Reversal of the usual non-fusion pattern and observations on bacterial survival. *J. Exp. Med.* **142**:1–16.
- Blanchard, D. K., S. Wei, C. N. Duan, F. Pericle, J. I. Diaz, and J. Y. Djeu. 1995. Role of extracellular adenosine triphosphate in the cytotoxic T-lymphocyte-mediated lysis of antigen presenting cells. *Blood* **85**:3173–3182.
- Boom, W. H., R. S. Wallis, and K. A. Chervenak. 1991. Human *Mycobacterium tuberculosis*-reactive CD4⁺ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. *Infect. Immun.* **59**:2737–2743.
- Bothamley, G. H., F. Festenstein, and A. Newland. 1992. Protective role for CD8 cells in tuberculosis. *Lancet* **339**:315–316.
- DeLibero, G., I. Fleisch, and S. H. E. Kaufmann. 1988. Mycobacteria-reactive Lyt-2⁺ T cell lines. *Eur. J. Immunol.* **18**:59–66.
- Fleisch, I. E. A., and S. H. E. Kaufmann. 1990. Activation of tuberculostatic macrophage functions by gamma interferon, interleukin-4, and tumor necrosis factor. *Infect. Immun.* **58**:2675–2677.
- Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* **178**:2249–2254.
- Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom. 1992. Major histocompatibility complex class-I-restricted T-cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* **89**:12013–12017.
- Guleria, L., R. Mukherjee, and S. H. E. Kaufmann. 1993. *In vivo* depletion of CD4 and CD8 T-lymphocytes impairs Mycobacterium-w vaccine-induced protection against *M. tuberculosis* in mice. *Med. Microbiol. Immunol.* **182**:129–135.
- Haanen, J. B. A. G., R. W. Malefijt, P. C. M. Res, E. M. Kraakman, T. H. M. Ottenhof, R. R. P. Vries, and H. Spits. 1991. Selection of a human T helper type 1-like T cell subset by mycobacteria. *J. Exp. Med.* **174**:583–592.
- Howard, J. C. 1995. Supply and transport of peptides presented by class IMHC molecules. *Curr. Opin. Immunol.* **7**:69–76.
- Hussein, S., J. Curtis, H. Akuffo, and J. L. Turk. 1987. Dissociation between delayed type hypersensitivity and resistance to pathogenic mycobacteria demonstrated by T cell clones. *Infect. Immun.* **55**:564–567.
- Huygen, K., D. Abramowicz, P. Vandenbussche, F. Jacobs, J. DeBruyn, A. Kentos, A. Drowart, J.-P. VanVooren, and M. Goldman. 1992. Spleen cell cytokine secretion in *Mycobacterium bovis* BCG-infected mice. *Infect. Immun.* **60**:2880–2886.
- Kaufmann, S. H. E., U. Vath, J. E. R. Thole, J. D. A. VanEmbden, and F. Emmrich. 1987. Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur. J. Immunol.* **17**:351–357.
- Khor, M., D. B. Lowrie, and D. A. Mitchison. 1986. Effects of recombinant interferon-gamma and chemotherapy with isoniazid and rifampicin on infections of mouse peritoneal macrophages with *Listeria monocytogenes* and *Mycobacterium microti* *in vitro*. *Brit. J. Exp. Pathol.* **67**:707–717.
- Koga, T., A. Wand-Wurtenberger, J. DeBruyn, M. E. Munk, B. Schoel, and S. H. E. Kaufmann. 1989. T cells against a bacterial heat shock protein recognize stressed macrophages. *Science* **245**:1112–1115.
- Kumararatne, D. S., A. S. Pithie, P. Drysdale, et al. 1990. Specific lysis of

- mycobacterial antigen-bearing macrophages by class-II MHC-restricted polyclonal T-cell lines in healthy donors or patients with tuberculosis. *Clin. Exp. Immunol.* **80**:314–323.
19. **Lefford, M. J.** 1975. Transfer of adoptive immunity to tuberculosis in mice. *Infect. Immun.* **11**:1174–1181.
 20. **Lowrie, D. B., M. J. Colston, and R. Tascon.** 1995. Protection against tuberculosis by immunization of mice by intramuscular injection of naked DNA expressing *M. leprae* hsp65. *J. Cell Biochem.* **19B**:95.
 21. **Lowrie, D. B., R. E. Tascon, M. J. Colston, and C. L. Silva.** 1994. Towards a DNA vaccine against tuberculosis. *Vaccine* **12**:1537–1540.
 22. **McDonough, K. A., Y. Kress, and B. R. Bloom.** 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect. Immun.* **61**:2763–2773.
 23. **Molloy, A., P. Laochumroonvorapong, and G. Kaplan.** 1995. Apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular bacillus Calmette-Guerin. *J. Exp. Med.* **180**:1499–1509.
 24. **Murray, C. J. L., K. Styblo, and A. Rouillon.** 1990. Tuberculosis in developing countries: burden, intervention and cost. *Bull. Int. Union Tuberc. Lung Dis.* **65**:6.
 25. **Mutis, T., Y. E. Cornelisse, and T. H. M. Ottenhoff.** 1993. Mycobacteria induce CD4⁺ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. *Eur. J. Immunol.* **23**:2189–2195.
 26. **Orme, I., and F. M. Collins.** 1984. Adoptive protection of the *Mycobacterium tuberculosis* infected lung: dissociation between cells that passively transfer protective immunity and those that transfer delayed type hypersensitivity to tuberculin. *Cell. Immunol.* **84**:113–120.
 27. **Orme, I. M.** 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *Mycobacterium tuberculosis*. *J. Immunol.* **138**:293–298.
 28. **Orme, I. M., P. Andersen, and W. H. Boom.** 1993. T cell response to *Mycobacterium tuberculosis*. *J. Infect. Dis.* **167**:1481–1497.
 29. **Orme, I. M., and F. M. Collins.** 1983. Protection against *Mycobacterium tuberculosis* infection by adoptive immunotherapy. *J. Exp. Med.* **158**:74–83.
 30. **Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams.** 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. *J. Immunol.* **151**:518–525.
 31. **Patterson, R. J., and G. P. Youmans.** 1970. Multiplication of *Mycobacterium tuberculosis* within normal and “immune” mouse macrophages cultivated with and without streptomycin. *Infect. Immun.* **1**:30–40.
 32. **Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Findlay, S. J. Normark, and C. V. Harding.** 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature (London)* **361**:359–362.
 33. **Podack, E. R.** 1995. Execution and suicide: cytotoxic lymphocytes enforce Draconian laws through separate molecular pathways. *Curr. Opin. Immunol.* **7**:11–16.
 34. **Rook, G. A. W., B. R. Champion, J. Steele, A. M. Valey, and J. L. Stanford.** 1985. I-A restricted activation by T cell lines of anti-tuberculous activity in murine macrophages. *Clin. Exp. Immunol.* **159**:414–420.
 35. **Rook, G. A. W., and S. Rainbow.** 1981. An isotope incorporation assay for the antimicrobial effects of human monocytes. *Ann. Immunol. Paris* **132D**:281–289.
 36. **Sad, S., R. Marcotte, and T. R. Mossman.** 1995. Cytokine-induced differentiation of precursor mouse CD8⁺ T cells into cytotoxic CD8⁺ T cells secreting Th1 or Th2 cytokines. *Immunity* **2**:271–279.
 37. **Silva, C. L., and D. B. Lowrie.** 1994. A single mycobacterial protein (hsp65) expressed by a transgenic antigen-presenting cell vaccinates mice against tuberculosis. *Immunology* **82**:244–248.
 38. **Silva, C. L., K. Lukacs, and D. B. Lowrie.** 1993. Major histocompatibility complex non-restricted presentation to CD4⁺ T lymphocytes of *Mycobacterium leprae* heat shock protein 65 antigen by macrophages transfected with the mycobacterial gene. *Immunology* **78**:35–42.
 39. **Silva, C. L., A. Palacios, M. J. Colston, and D. B. Lowrie.** 1992. *Mycobacterium leprae* 65hsp antigen expressed from a retroviral vector in a macrophage cell line is presented to T cells in association with MHC class II in addition to MHC class I. *Microb. Pathog.* **12**:27–38.
 40. **Silva, C. L., R. L. R. Pietro, A. Januario, V. L. D. Bonato, V. M. F. Lima, M. F. DaSilva, and D. B. Lowrie.** 1995. Protection against tuberculosis by bone marrow cells expressing mycobacterial hsp65. *Immunology* **86**:519–524.
 41. **Silva, C. L., M. F. Silva, R. C. L. R. Pietro, and D. B. Lowrie.** 1994. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. *Immunology* **83**:341–346.
 42. **Simon, M. M., U. Hochgeschwender, U. Bruger, and S. Landolfo.** 1986. Monoclonal antibodies to interferon-gamma inhibit interleukin 2-dependent induction of growth and maturation in lectin/antigen-reactive cytolytic T lymphocyte precursors. *J. Immunol.* **136**:2755–2762.
 43. **Taswell, C.** 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126**:1614–1619.

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