Natural Killer Cells Are a Source of Interferon γ That Drives Differentiation of CD4+ T Cell Subsets and Induces Early Resistance to *Leishmania major* in Mice

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Summary

Infection of mice with the protozoan *Leishmania major* provides an excellent model to define the factors involved in T helper (Th) subset development, since Th1 cells confer protection in resistant strains of mice, whereas Th2 cells are associated with the fatal outcome of susceptible mice. We previously found that interferon γ (IFN-γ) was required for Th1 cell development after infection of mice with *L. major*. In this report, we evaluate the contribution of natural killer (NK) cells to IFN-γ levels early in *L. major* infection. NK cell activity was higher in resistant C3H/HeN mice than in susceptible BALB/c mice during the first week of infection, and removal of NK cells significantly decreased IFN-γ levels and promoted interleukin 4 (IL-4) production in both the draining lymph nodes and spleen. IFN-γ production by NK cells required the presence of CD4+ T cells or IL-2, but not CD8+ T cells. Enhanced disease, as measured by parasite numbers and lesion development, was observed in NK cell–depleted mice. Furthermore, a comparison of the NK cell response and the subsequent parasite burden in several inbred strains of mice demonstrated that NK cells mediate early resistance to *L. major*. Together, these data indicate that the stimulation of NK cells, through the production of IFN-γ, plays an important role in initiating Th1 cell differentiation in leishmaniasis and in controlling early resistance to *L. major*.

A major advance in immunology was the demonstration that CD4+ Th cells are composed of subsets based upon the cytokines produced after stimulation, and that these distinct T cell subsets often influence the outcome of infection (1–4). CD4+ Th1 cells produce IL-2 and IFN-γ, primarily mediate cell-mediated immunity, and are often associated with bacterial, protozoan, and viral infections (1, 2). In contrast, Th2 cells produce IL-4, IL-5, and IL-10, mediate humoral and allergic responses, and are often associated with helminth infections (1, 2). Observations from several in vitro and in vivo models indicate that the cytokines present during primary antigen stimulation are a major factor in determining the differentiation pathway of naive T cells (5–10). The role of cytokines has been most clearly shown in vivo in the murine model of cutaneous leishmaniasis, where a single administration of anti-IFN-γ mAb before *L. major* infection promotes susceptibility (11, 12) and inhibits the development of Th1 cells (10). Moreover, injection of IFN-γ with the parasites switches the early cytokine pattern in susceptible BALB/c mice from a Th2 to a Th1 profile (10). Importantly, the changes in the cytokine pattern after treatment with either anti-IFN-γ or IFN-γ are apparent within 3 d of infection, suggesting that the cytokine environment at the time of an-

tigen presentation is critical in Th cell subset development. The compelling evidence that IFN-γ levels modulate Th1 development in this model has led us to investigate the source of IFN-γ during the first few days of infection.

Two sources of IFN-γ have been described: T lymphocytes and NK cells. However, while T cells produce IFN-γ several weeks after *L. major* infection (13), the production of IFN-γ during the first week of infection occurs more rapidly than might be expected for a conventional primary T cell response. In contrast, NK cells have been implicated in early IFN-γ production after bacterial (14, 15), protozoal (16, 17), and viral (18, 19) infection of both conventional and T cell–deficient mice. Therefore, we investigated the role of NK cells in early cytokine production and in resistance to *L. major*. Our results show that NK cells are a primary source of IFN-γ early in *L. major* infection, indicating that NK cells participate in the early development of a Th1 response after infection. Furthermore, our results reveal that NK cells play a previously unappreciated role in early resistance to *L. major*.

Materials and Methods

Animals. Female BALB/cAnNCr, C57BL/6NCr, A/JCr, AKR/NCr, CBA/JCr, CBA/CaHNCr, B6C3F₁, CBA/NCr, C3H/

HeJNCr, and C3H/HeNCr mammary tumor virus–negative (MTV⁻) mice (6–8 wk old) were obtained from the National Cancer Institute (Bethesda, MD). Animals were maintained in a specific pathogen-free environment. The animal colony was screened regularly for the presence of murine pathogens and consistently tested negative.

Parasites and Antigen. A clone of *L. major* (WHO MHOM/ IL/80/Friedlin) was used for these studies. Parasites were grown in Grace's insect cell culture medium (Gibco Laboratories, Grand Island, NY) with 20% FCS (Advanced Biotechnologies Inc., Columbia, MD) and 2 mM glutamine. Stationary phase promastigotes were harvested and metacyclic stage parasites were lectin selected using *Arachis hypogaea* agglutinin (Sigma Chemical Co., St. Louis, MO) as described previously (20). Mice were injected in the hind footpads with 2 × 10⁶ metacyclic stage *L. major* parasites. Soluble leishmanial antigen (SLA)[1] was prepared as described previously (10).

Production and Analysis of Cytokines. Single-cell suspensions were prepared from popliteal LNs and spleens harvested at various times after footpad injection of *L. major* parasites. Popliteal LNs were pooled from animals killed within 1 wk of infection. LNs and spleens from mice killed 2 wk after infection were assayed individually. In all cases, cells were adjusted to a final concentration of 5 × 10⁶ cells/ml in DMEM containing 4.5 mg glucose/ml (Gibco Laboratories), 10% FCS, 2 mM glutamine, 100 U/ml penicillin 6-potassium, 100 µg/ml streptomycin sulfate, 25 mM Hepes, and 5 × 10⁻⁵ M 2-ME, and plated at 1 ml/well in 24-well tissue culture plates. Restimulated cell populations received 50 µg SLA/ml at the time of culture initiation. Supernatants were harvested 72 h later and were assayed for cytokine activity. IFN-γ production was measured using a specific two-site ELISA (21, 22). IL-4 levels were determined either by ELISA (9) or by the proliferative capacity of culture supernatants on the IL-4-responsive CT4S cell line (23). The levels of IFN-γ and IL-4 were calculated by comparison with a standard curve generated with rIFN-γ (1 U = 153 pg; generously provided by Genentech, So. San Francisco, CA) or rIL-4 (1 U = 45 pg; generously provided by DNAX, Palo Alto, CA).

Analysis of NK Cell Activity. Specific cytotoxic activity of draining LN single-cell suspensions was measured against ⁵¹Cr-labeled YAC-1 targets in a standard 4-h chromium release assay at several E/T ratios (24). Briefly, 10⁷ target cells were labeled with sodium chromate at 500 µCi per pellet in 0.5 ml RPMI 1640 (Gibco Laboratories), 10% FCS, 2 mM glutamine, 100 U/ml penicillin 6-potassium, 100 µg/ml streptomycin sulfate, and 5 × 10⁻⁵ M 2-ME for 2 h. 5 × 10³ cells in 50 µl were then distributed into wells of round-bottomed microtiter plates containing effector draining LN cells at various concentrations. Each test sample was plated in triplicate. Microplates were incubated at 37°C in a CO₂ incubator for 4 h. The percent specific ⁵¹Cr release is equal to 100× [(cpm test sample − cpm medium control)/(cpm SDS control − cpm medium control)]. Spontaneous release never exceeded 30%. Significant NK cell responses were defined by a twofold increase of NK cell activity over background levels observed in the draining LN of uninfected mice. To confirm the responsiveness of BALB/c NK cells, mice were injected intraperitoneally with 100 µg poly(I:C) (Sigma Chemical Co.) or in the footpad subcutaneously with *Corynebacterium parvum* (100 µg; Wellcome Biotechnology, Beckenham, England) and 2 × 10⁶ metacyclic *L. major* promastigotes. Single-cell suspensions from spleens harvested 1 d after poly(I:C) injection or from draining LN harvested 2 d after *C.*

parvum/*L. major* injection were tested for NK cell cytotoxicity as described above.

In Vivo Cell Depletions. To deplete mice of CD4⁺ or CD8⁺ T cells, animals were injected intraperitoneally with 500 µg of anti-CD4⁺ (GK 1.5 [25]) or anti-CD8⁺ (H-35 [26]) antibodies, at both 2 and 1 d before infection. The efficiency of depletion was assessed by flow cytometric analysis (FACS®; Becton Dickinson & Co., Mountain View, CA) using two-color immunofluorescence with PE-labeled anti-CD8 (Boehringer Mannheim Biochemicals, Indianapolis, IN) and FITC-labeled anti-CD4 (Boehringer Mannheim Biochemicals). In all experiments mAb treatment depleted >98% of the target cell population. To deplete mice of NK cells, C3H/HeN mice were treated with rabbit anti-asialo (aAs) GM1 (Wako Bioproducts, Richmond, VA) at 7 and 4 d before injection of parasites (1.5 mg injected intravenously in 0.5 ml) and at the time of parasite challenge (100 µg in the footpad). Control mice were treated with either PBS or normal rabbit serum (Jackson Immunochemicals, West Grove, PA). Rabbit sera were screened to avoid using sera containing nonspecific inhibitory activity of NK cells, as has been previously described (27).

Preparation of CD4⁺ T Cells and CD4⁺ T Cell–depleted Populations. To isolate CD4⁺ T cells, B cells and adherent cells were first depleted by Ig and adherent cell panning at room temperature on goat anti–mouse IgG- and IgM- (Jackson Immunochemicals) coated petri dishes. Viable cells were isolated over lympholyte M (Cedarlane, Hornby, Ontario, Canada). Cells were next blocked for nonspecific staining with a cocktail of irrelevant antibodies, followed by specific staining with biotinylated anti-CD4 antibodies (RL172 [28] and GK1.5) at 0.5 µg/10⁶ cells for 30 min on ice. Cells were then stained with streptavidin-FITC (Jackson Immunochemicals) at 1 µg/10⁶ cells for 15 min, and finally stained with biotinylated iron-coated beads for 5 min on ice. CD4⁺ T cells were positively selected on a magnetic cell separator (MACS; Miltenyi, Biotec, Sunnyvale, CA). Efficiency of selection and depletion was assessed by FACS® analysis. The CD4⁺ T cell-enriched population contained >98% CD4⁺ T cells. In addition, <5% of the NK cell activity (>85% reduction at a 100:1 E/T ratio) was detected in the CD4⁺ T cell population. CD4⁺ T cell–depleted populations (CD4⁻) or NK cell–depleted cells were obtained by incubation of 2 × 10⁷ cells with anti-CD4 mAb (RL172) at 5 µg/ml or aAs GM1 at a 1:200 dilution and a 1:12 dilution of rabbit complement for 45 min at 37°C. Viable cells were isolated over lympholyte M and stained for FACS® analysis to assess the efficiency of depletion. RL172-treated populations contained <2% CD4⁺ T cells. Neither the CD4⁺ nor CD8⁺ T cell populations were affected by aAs GM1 treatment.

Reconstitution Experiments. 2 × 10⁶ CD4⁺ T cells from infected mice were cultured alone or with 3 × 10⁶ CD4⁺ T-depleted cells isolated from either infected or normal C3H/HeN mice. Cells were cultured with 50 µg/ml SLA at 5 × 10⁶ cells/ml in 24-well plates. Control CD4⁺ T cells and CD4⁺ T cell–depleted populations were cultured at both 2 × 10⁶ and 5 × 10⁶ cells/ml. No increase in IFN-γ production was observed at the higher cell concentration. IL-2 was added to the indicated cultures at 200 U/ml (Cetus Corp., Emeryville, CA). Anti-IL-2 mAb (S4B6 [1]) was added to the indicated cultures at a concentration of 25 µg/ml.

Measurement of Disease Progression. The number of parasites in the footpad was determined by a modified limiting dilution analysis of single-cell suspensions made from individual excised lesions taken from five mice. Briefly, single-cell suspensions of the lesions were plated in log-fold serial dilutions in Grace's insect tissue culture medium starting with a 1:1,000 dilution. Each sample was plated in quadruplicate and the mean of the negative log parasite

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[1] *Abbreviations used in this paper:* aAs, anti-asialo; SLA, soluble leishmanial antigen.

titer calculated 5 d after culture initiation. Footpad lesion development in *L. major*–infected mice was measured in groups of five mice by monitoring the increase in footpad thickness using a dial micrometer (L. S. Starett Co., Athol, MA) and calculating the difference against contralateral uninfected footpad measurements.

Results

Characterization of IFN-γ Levels in BALB/c and C3H/HeN Mice Infected with Metacyclic Stage L. major Promastigotes. 3 d after subcutaneous inoculation of live or heat-killed stationary phase *L. major* promastigotes, or SLA, cells from the draining LNs of C3H/HeN mice produce significantly more IFN-γ than cells from similarly inoculated BALB/c mice (10). We now extend these findings by assessing the response to purified metacyclic stage promastigotes, the naturally transmitted form of the parasite (20). As shown in Fig. 1, IFN-γ levels were elevated in cultures of draining LN cells taken from both C3H/HeN and BALB/c mice when compared with uninfected controls. In cell cultures that were not restimulated in vitro, IFN-γ production by cells from C3H/HeN mice was dramatically higher than cells from BALB/c mice (Fig. 1 *A*). When SLA was added to the cultures, cells from both strains produced higher levels of IFN-γ (Fig. 1 *B*). In fact, the IFN-γ response of BALB/c LN cells taken 48 h after infection with metacyclic promastigotes was greater than we had previously observed after injection of either unfractionated promastigote populations or SLA (10), and the levels of IFN-γ approached those produced by C3H/HeN mice. Nevertheless, by 4 d after infection the IFN-γ response by cells from BALB/c mice was substantially reduced, while cells from C3H/HeN mice continued to produce high levels of IFN-γ. In all of our experiments IFN-γ levels were consistently higher and the production was sustained for a longer period of time in cell cultures from C3H/HeN mice, when compared with cells from BALB/c mice.

Activation of NK Cells in L. major–infected C3H/HeN and BALB/c Mice. The capacity of draining LN cells from infected C3H/HeN mice to produce IFN-γ appears earlier than might be expected from a conventional primary T cell response. However, similar kinetics for IFN-γ production have been documented in several infections where NK cells have been shown to be the source of IFN-γ (29, 30). To determine if NK cells were present in the draining LNs, we measured the NK cell cytotoxic activity of draining LN cells after infection of C3H/HeN and BALB/c mice with *L. major*. A striking elevation of NK cell activity in C3H/HeN, but not BALB/c, draining LN cells was apparent throughout the first 3 d of infection (Fig. 2, *A* and *B*). NK cell activity was measured immediately after isolation of LN cells (i.e., without in vitro restimulation with SLA). The levels of NK cell activity at 2 d correlated with the amounts of IFN-γ produced by unstimulated cells (Fig. 1 *A*). Only at 2 d after infection was there any significant NK cell cytotoxic response in the draining LNs of BALB/c mice. Notably, removal of adherent cells, before the NK cell chromium release assay, enhanced NK cell cytotoxicity, confirming that the observed cytotoxicity was NK cell, and not macrophage, mediated (data not

A

B
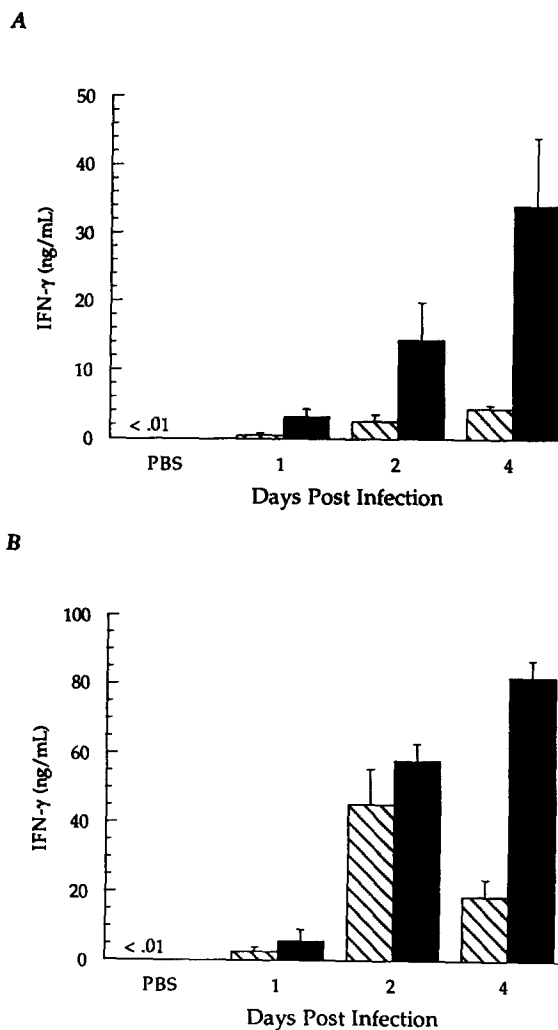
Figure 1. Kinetics of IFN-γ production in C3H/HeN and BALB/c mice after *L. major* infection. C3H/HeN (*filled bars*) and BALB/c (*hatched bars*) mice were injected in the footpad with 2 × 10⁶ metacyclic phase *L. major* promastigotes, and the popliteal LN cells were harvested 1, 2, or 4 d later, or from mice injected 1 d previously with PBS. IFN-γ production was measured by ELISA on 72-h supernatants from cell cultures of pooled popliteal LN cells. Cells were cultured without (*A*) or with (*B*) SLA at 50 μg/ml. Error bars reflect the SEM calculated from three measurements of IFN-γ in the supernatants. One representative experiment of three performed is shown.

shown). In contrast, treatment of C3H/HeN mice with aAsGM1 completely ablated the cytotoxic response (Fig. 2 *C*). This antiserum was used since no mAb with in vivo activity against C3H/HeN NK cells presently exists. While asialo GM1 is expressed at very low levels on cytotoxic T cells, as well as macrophages, in vivo aAsGM1 treatment has been shown to reduce the activity of NK cells while not affecting the cytotoxicity or IFN-γ-dependent activation of macrophages (31, 32) nor the specific cytotoxic or mitogenic response of T cells (31, 33).

One possible explanation for the lack of a significant NK cell response in BALB/c mice is that BALB/c mice are genet-

A

B

C
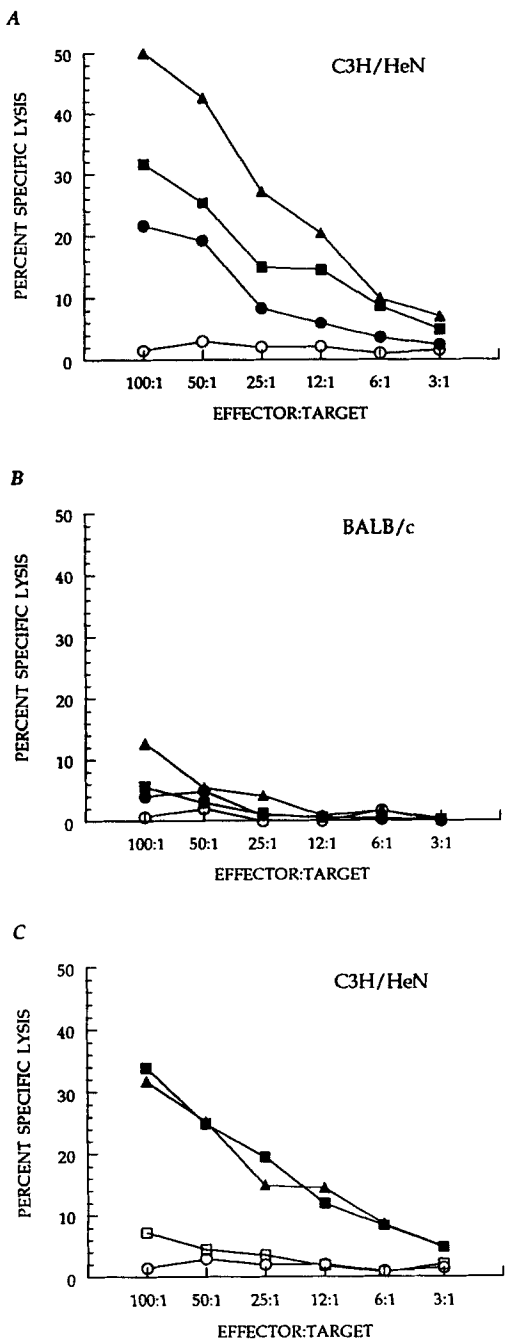
Figure 2. NK cell cytotoxicity in C3H/HeN and BALB/c mice after *L. major* infection. NK cell cytotoxic activity in the popliteal LNs of *L. major*-infected C3H/HeN (*A*) and BALB/c (*B*) mice killed 1 (■), 2 (▲), and 3 d (●) after infection, and uninfected controls (O) was assessed. Specific cytotoxic activity of popliteal LN single-cell suspensions was measured against ^{51}Cr-labeled YAC-1 targets in a standard 4-h chromium release assay at various effector cell ratios. (*C*) NK cell cytotoxicity in the draining LNs measured 1 d after infection. aAsGM1-treated (□), rabbit serum-treated (■), PBS-treated (▲), and uninfected control (O). The data shown are from one representative experiment of three performed.
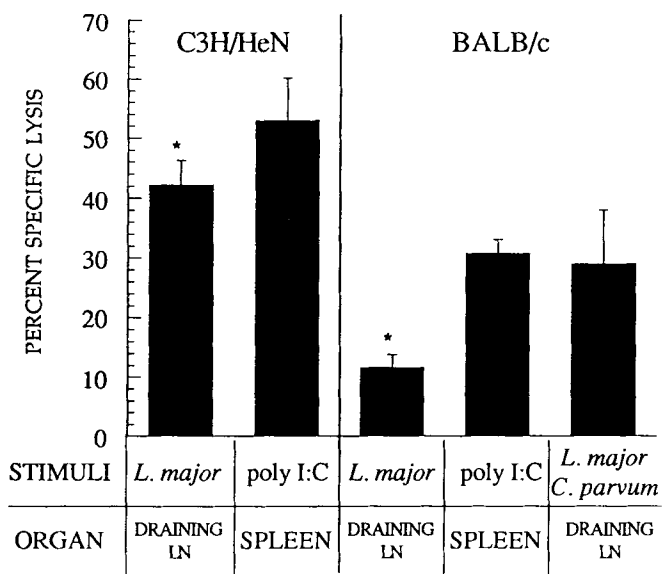
Figure 3. BALB/c and C3H/HeN NK cell responses to NK cell inducers. C3H/HeN and BALB/c NK cell cytotoxic responses were measured in the draining LNs after injection of *L. major* alone (C3H/HeN and BALB/c) or *L. major* and the bacterial adjuvant *C. parvum* (BALB/c only). Splenic NK cell responses were measured in animals treated intraperitoneally with 100 μg of poly(I:C) 24 h before death, as described in Materials and Methods. Data from the 50:1 E/T ratio are shown. The only significant difference observed between BALB/c and C3H/HeN NK cell response was the popliteal LN response to *L. major* injected in the footpad. LNs and spleen cells from unstimulated animals consistently exhibited <10% cytotoxicity at a 100:1 E/T ratio. Error bars reflect the SEM calculated from two or more experiments for each stimulus at a 50:1 E/T ratio. *Significant ($p \leqslant 0.05$, Student's *t* test) difference between the level of cytotoxicity in C3H/HeN and BALB/c LN from 2 d infected mice.

ically low NK cell responders, which has been suggested (34, 35). However, the ability of NK cells in BALB/c mice to respond to many agents has also been described (15, 36, 37). We confirmed that BALB/c mice have the capacity to exhibit an NK cell response in the spleen after systemic administration of poly(I:C) (Fig. 3). We also elicited a significant NK cell response in the draining LNs after footpad injection of *L. major* with the bacterial adjuvant *Corynebacterium parvum*, indicating that no deficit in the ability of BALB/c mice to exhibit an NK cell response exists in the LN given the appropriate stimulus (Fig. 3).

Correlation between NK Cell Activation and Early Parasite Burdens at the Site of Infection. Since *L. major* infection promoted NK cell activation primarily in resistant C3H/HeN mice, we investigated whether there was a correlation between NK cell activation early in infection and early resistance to *L. major* in several other inbred strains of mice. It has previously been reported that a spectrum of resistance to *L. major* exists among inbred strains. C3H and CBA strains are the most resistant, C57BL/6, C57BL/10, and A/J are somewhat less resistant although they eventually heal, and BALB/c is extremely susceptible (38). These inbred mouse strains, as well as the AKR strain and an F₁ cross between C57BL/6 and C3H/HeN (B6C3F₁), were infected with *L.*
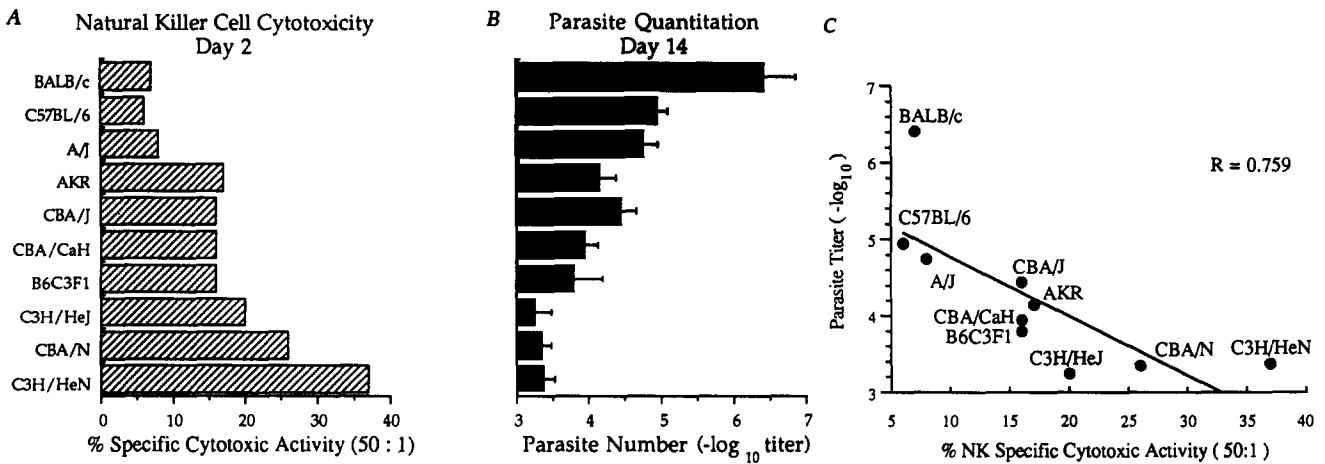
Figure 4. NK cell cytotoxicity and early parasite burdens in inbred mouse strains. BALB/c, C57BL/6, A/J, AKR, CBA/J, CBA/CaH, B6C3F₁, CBA/N, C3H/HeJ, and C3H/HeN mice were infected as described in Materials and Methods. The NK cell cytotoxicity (*A*) in pooled draining LNs harvested from two mice of each strain was measured 48 h after infection. The number of parasites at the site of parasite challenge (*B*) was determined by a modified limiting dilution analysis of individual excised lesions as described in Materials and Methods. Results shown are the mean ± SE of five mice. (*C*) Correlation between NK cell cytotoxicity levels and parasite titers 2 wk later.

major and their NK cell responses were measured 2 d later (Fig. 4 *A*). Three distinct groups of resistant mice were apparent based on their NK cell response. C3H/HeN, C3H/HeJ, and CBA/N mouse strains were all high NK cell responders with specific cytotoxic activity of ⩾20% at a 50:1 E/T ratio. In contrast, the *L. major*-susceptible BALB/c, as well as the resistant C57BL/6 and A/J mice, responded with <10% specific cytotoxic activities. The other strains exhibited an intermediate pattern. We next measured the number of parasites in the lesions of these mice at 2 wk of infection. As seen in Fig. 4 *B*, a relatively high parasite burden (>10⁴·⁵) was observed in the *L. major*-susceptible BALB/c mouse strain

and in the resistant C57BL/6 and A/J strains of mice. In contrast, a low parasite burden (<10³·⁵) was observed in the *L. major*-resistant C3H/HeN, C3H/HeJ, and CBA/N mouse strains. A statistical analysis of the data from Fig. 4, *A* and *B*, indicates that an inverse correlation exists between the day 2 NK cell response and the parasite burden 2 wk later (Fig. 4 *C*).

Effect of NK Cell Depletion on Early Cytokine Production in C3H/HeN Mice. To directly assess the role of NK cells in the production of IFN-γ, NK cells were removed from C3H/HeN mice by treatment with aAsGM1. Draining LN cells from aAsGM1-treated C3H/HeN mice produced significantly

Table 1. *The Effects of NK Cell Depletion on Cytokine Production in C3H/HeN Mice 2 d after L. major Infection*

		IFN-γ levels				IL-4 levels		
Exp.	In vitro restimulation*	PBS	Rabbit serum	Anti-asialo GM1	Percent reduction‡	PBS	Rabbit serum	Anti-asialo GM1
		ng/ml				*pg/ml*		
1	None	8.8	6.5	1.5	83.0	ND	ND	ND
	SLA	14.0	11.0	2.9	79.3	ND	ND	ND
2	None	8.4	10.0	ND	ND	<0.5	<0.5	<0.5
	SLA	24.0	59.0	4.4	81.7	<0.5	<0.5	40.5
3	None	10.0	6.8	0.7	93.0	<0.5	<0.5	<0.5
	SLA	9.9	14.0	0.8	91.9	<0.5	9.0	67.5

IFN-γ and IL-4 levels were measured in the culture supernatants of popliteal LN cells harvested from C3H/HeN mice 2 d after *L. major* infection. Mice were infected as described in Materials and Methods. C3H/HeN mice were treated with anti-asialo GM1 serum, normal rabbit serum, or PBS 4 d before infection.
* Cells were cultured with or without SLA at 50 µg/mL for 72 h.
‡ Percent reduction = 100 × [IFN-γ (PBS) − IFN-γ (anti-asialo GM1)]/IFN-γ (PBS).

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Table 2. *CD4⁺ Cells Are Required for the Induction of an NK Cell Cytotoxic Response and IFN-γ Production 2 d after L. major Infection of C3H/HeN Mice*

| In vivo treatment | | In vitro restimulation IFN-γ | | NK cell activity (50:1 E/T) |
		None	SLA	
		ng/ml		%
L. major	Control	10.1 ± 1.3	21.5 ± 3.0	32
L. major	Anti-CD4	1.9 ± 0.4	1.2 ± 0.6	4
L. major	Anti-CD8	9.0 ± 2.3	16.0 ± 2.9	40

C3H/HeN mice were treated intraperitoneally with anti-CD4 (GK1.5) or anti-CD8 (H-35) mAb at 2 and 1 d before *L. major* infection, as described in Materials and Methods. IFN-γ production and NK cell cytotoxicity of draining LN cells harvested from C3H/HeN mice 48 h after infection was assessed.

less IFN-γ at 2 d after infection when compared with controls. Furthermore, cells from the NK cell–depleted mice produced IL-4 (Table 1). Interestingly, draining LN cells from aAsGM1-treated BALB/c mice also exhibited a reduced capacity to produce IFN-γ and exhibited a fivefold increase in IL-4 levels over those detected in control mice, indicating that the IFN-γ produced in response to SLA in BALB/c mice on day 2 may also be NK cell derived (data not shown).

Table 3. *CD4⁺ T Cells Are Required for, but Are Not the Source of, IFN-γ Produced by Draining LN Cells 2 d after L. major Infection of C3H/HeN Mice*

| Exp. | Source of fractionated cell populations* | | In vitro addition | IFN-γ levels | Percent control† IFN-γ level | NK cell cytotoxicity (50:1 E/T) |
	CD4⁺	CD4⁻				
				ng/ml	%	%
1	Infected	Infected		52.9		–
	Infected	–		0.0	0	5
	Infected	Normal		0.0	0	0§
	–	Infected		0.6	1	43
2	Infected	Infected		14.2		–
	Infected	–		0.0	0	5
	Infected	Infected	Anti-asialo GM1	5.5	39	7‖
	Infected	Infected	S4B6 (25 μg/ml)	2.2	15	–
	–	Infected		0.0	0	26
	–	Infected	IL-2 (200 U/ml)	5.5	39	–
3	Infected	Infected		78.0		–
	Infected	–		0.0	0	4
	Infected	Normal		5.7	7	5§
	Infected	Infected	Anti-asialo GM1	31.5	40	9‖
	–	Infected		8.9	11	20
	–	Infected	IL-2 (200 U/ml)	39.6	51	–

* LN cells from normal C3H/HeN mice (normal) or from mice 2 d after *L. major* infection (infected) were fractionated into CD4⁺ T cell–enriched populations (CD4⁺) or a CD4 T cell–depleted population (CD4⁻) and cultured with SLA at 50 μg/ml for 72 h. Culture supernatants were then assayed for IFN-γ. Cell fractionations were done as in Materials and Methods.
† Percent control = 100 × {[IFN-γ (cell fraction)]/[IFN-γ (CD4⁺ infected + CD4⁻ infected) reconstitution]}.
§ NK cell activity in the CD4⁺ T cell–depleted population from normal mice.
‖ NK cell activity in the anti-AsGM1-treated cell population from infected mice.

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IFN-γ Production and NK Cell Cytotoxic Function in the Draining LN of L. major–infected C3H/HeN Mice Requires CD4+ T Cells. We previously reported that in vivo depletion of CD4+ T cells abrogates IFN-γ production by C3H/HeN LN cells taken 3 d after *L. major* infection (10). To investigate the relationship between CD4+ T cells and NK cells in the draining LNs of C3H/HeN mice, we evaluated both IFN-γ production and NK cell cytotoxic activity in the draining LNs of 2 d infected C3H/HeN mice treated with either anti-CD4+ or anti CD8+ mAb before infection (Table 2). CD4+ T cell depletion diminished IFN-γ production by >80% in both background and restimulated cultures of LN cells, whereas CD8+ T cell depletion had no effect on IFN-γ production. CD4+ T cell depletion also diminished NK cell cytotoxic activity in the draining LN to background levels. In contrast, CD8+ T cell depletion slightly enhanced NK cell activity in the draining LN.

The Role of CD4+ T Cells in the Production of IFN-γ by Draining LN NK Cells. To evaluate the source of IFN-γ in the LN, LN cells were fractionated into CD4+ enriched (>98% CD4+) and CD4+ T cell–depleted, NK cell–enriched (<2% CD4+ T cells; ⩾100% NK cell activity of unfractionated cells) populations (Table 3). When both CD4+ T cells and CD4+ T cell–depleted cells from infected mice were combined, IFN-γ levels were comparable to those observed in unfractionated LN cell cultures (data not shown). However, while CD4+ T cells did produce IL-2 (data not shown), they did not produce IFN-γ when cultured alone, and produced minimal levels (<7% of control) of IFN-γ when cultured with normal cells (Table 3, exps. 1 and 3). To determine whether NK cells were the source of IFN-γ in the CD4+ T cell–depleted population, the effect of NK

cell depletion on IFN-γ production by the CD4+ T cell–depleted population was evaluated (Table 3, exps. 2 and 3). IFN-γ levels were reduced 60% when NK cells were depleted using aAsGM1. These results suggest that most of the IFN-γ produced by draining LN cells taken 2 d after *L. major* infection is NK cell derived, but that CD4+ T cells provide a factor required for IFN-γ production. Because CD4+ T cells from infected mice produced IL-2 both when cultured alone or with normal CD4+ T-depleted cells, and IL-2 promotes IFN-γ production by NK cells, we tested the effect of IL-2 depletion on IFN-γ production. Addition of anti-IL-2 mAb (S4B6) (Table 3, exp. 2) reduced IFN-γ production by >80%. In contrast, addition of IL-2 to CD4+ T cell–depleted populations restored 40% (Table 3, exp. 2) and 50% (Table 3, experiment 3) of the IFN-γ levels observed in control cultures. Thus, IL-2 derived from CD4+ T cells is required for IFN-γ production by NK cells in this infection.

Effect of NK Cell Depletion on the Course of Infection and Cytokine Profile in C3H/HeN Mice. The effect of NK cell depletion on cytokine responses after the first week of infection and on the overall course of infection was evaluated in aAsGM1-treated C3H/HeN mice. IFN-γ production by cells taken 2 wk after *L. major* infection was reduced by 75% in both the spleen and draining LNs of aAsGM1-treated, infected C3H/HeN mice when compared with control mice, while IL-4 levels were threefold higher (Fig. 5, *A* and *B*). Consistent with the change in cytokine production after aAsGM1 treatment, limiting dilution analysis of the parasites in the footpad revealed 1.5 (exp. 1) and 3.2 (exp. 2) log higher titers of parasites in the aAsGM1-treated mice when compared with C3H/HeN controls (Fig. 5 *C*). Lesions in treated mice were also significantly larger than controls
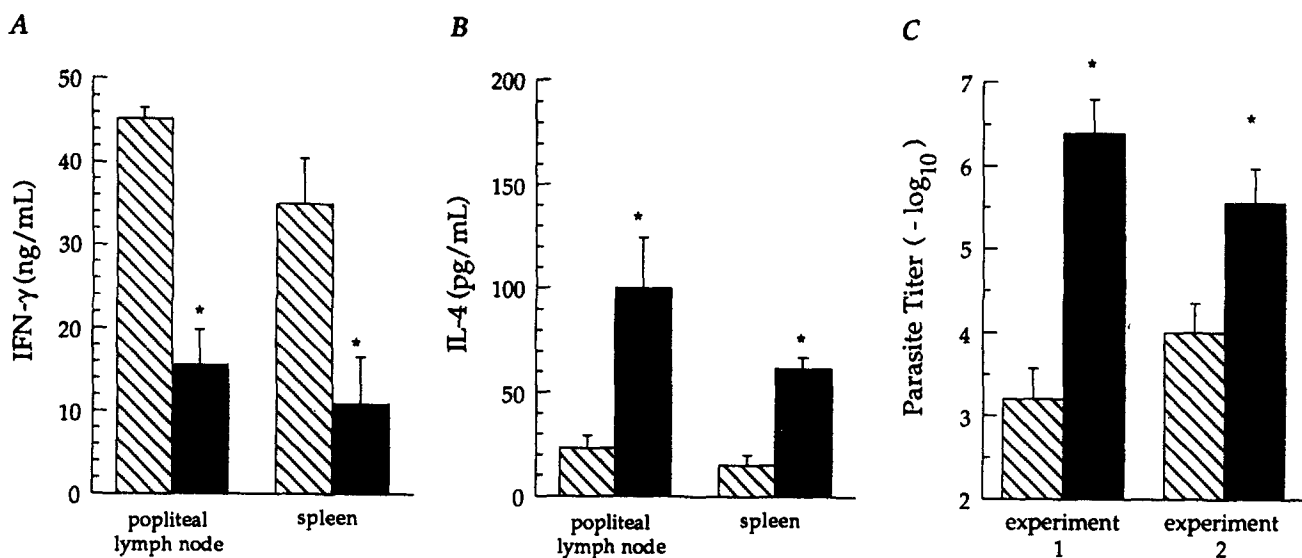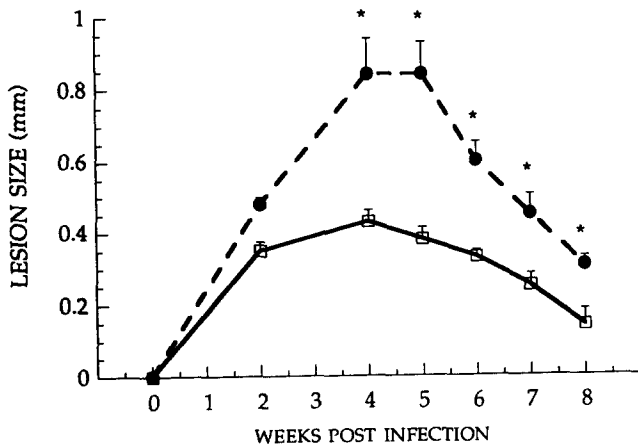
Figure 5. The effect of NK cell depletion on cytokine responses and parasite numbers in C3H/HeN mice 2 wk after *L. major* infection. C3H/HeN mice were treated with aAsGM1 as described in Materials and Methods. Animals were killed 2 wk after infection, and the IFN-γ (*A*) and IL-4 (*B*) levels were measured in spleen and draining LN cell cultures from control- (*hatched bars*) and aAsGM1- (*filled bars*) treated C3H/HeN mice as described for Fig. 1. Cells were cultured with or without SLA (50 μg/ml). Only results with SLA are shown. Parasites were quantitated as described in Materials and Methods. Results shown are the mean ± SE of four mice. *Statistical significance (*p* <0.05, Student's *t* test) between aAsGM1-treated and control responses.

throughout the 8 wk of investigation. However, aAsGM1-treated mice began healing between 5 and 8 wk postinfection (Fig. 6). Associated with healing was a reversion to a Th1 cytokine profile in the spleen and draining LNs (data not shown).

Discussion

Cytokines are a major influence on Th cell subset differentiation (5–10). IFN-γ is required for the differentiation of Th1 cells in leishmaniasis, since a single injection of anti-IFN-γ mAb at the time of parasite inoculation abrogates the development of this subset (10). In this report, we identify NK cells as the primary source of IFN-γ during the initial phase of the immune response to L. major in C3H/HeN mice. While NK cells have been suggested to influence the selection of human Th1 cell clones in vitro (40), the current report represents the first direct evidence that NK cells responding during the innate phase of immunity influence Th subset differentiation in vivo.

NK cells are a lymphocyte population that was initially defined by the ability of these cells to mediate spontaneous cytotoxicity of a variety of target cells, including the murine T cell lymphoma, YAC-1 (41, 42). Over the past decade, the study of NK cell function in tumorigenesis and infectious disease has focused primarily on their lytic capacity. However, the principle function of NK cells in infectious disease may be primarily mediated by their production of cytokines, such as IFN-γ, TNF-α, GM-CSF, and IL-3 (43). Based upon our results, we propose that NK cells participate in Th cell differentiation by providing an early source of IFN-γ. Similarly, the ability of naive T cells to differentiate into Th1 cells after transfer into L. major–infected scid mice has also been

attributed to the production of IFN-γ by NK cells (44). One might expect, then, that NK cell and IFN-γ depletion would have similar effects. This was the case during the first several weeks of infection (10–12). However, unlike anti-IFN-γ treated mice, which succumb to L. major infection, aAsGM1-treated mice began healing between 5 and 6 wk after infection (Fig. 6). The fact that aAsGM1-treated C3H/HeN mice resolved their L. major infections may be attributed to several factors. First, since NK cells rapidly proliferate in the bone marrow and in the spleen during viral infections (29), aAsGM1 may not deplete NK cells for a sufficient time period to permanently alter the response. In this regard, mice were only treated with aAsGM1 before and at the time of infection, and the circulatory half life of this antiserum has been reported to be <1 h (45). Multiple injections of this antisera were not possible, since a wasting type syndrome develops in mice given repeated treatments (44). Alternatively, other IFN-γ-producing cells, such as CD8$^+$ T cells or γ/δ T cells, may compensate for the loss of NK cells. Finally, we found that some resistant mouse strains only exhibit a predominant Th1 response after several weeks of infection. Implicit in this observation is the possibility that a delayed IFN-γ-dependent mechanism operates to drive the eventual predominance of Th1 cells, and hence healing, in these animals. For example, in contrast to C3H/HeN mice, C57BL/6 mice produce elevated levels of IL-4 during the first 2 wk of infection and both Th1 and Th2 cells can be cloned from the draining LNs (46, 47) (our unpublished observations). However, the eventual predominance of Th1 cells in C57BL/6 mice, as well as healing, is still dependent upon IFN-γ, since anti-IFN-γ treatment renders them susceptible to L. major (11). This delayed mechanism would appear to be NK cell independent, since we found that continuous treatment of C57BL/6 mice with a mAb directed against NK cells (anti-NK1.1) failed to alter the course of infection (data not shown). Based on the similarities in the course of infection and cytokine profiles between C57BL/6 mice and aAsGM1-treated C3H/HeN mice, we currently favor the possibility that the capacity of NK cell–depleted C3H/HeN mice to heal is related to such a mechanism. This interpretation implies that although NK cells control early parasite numbers and facilitate early Th1 cell development, NK cells are not an absolute requirement for resolution of leishmanial lesions or the development of Th1 cells.

The factors responsible for the induction of NK cells in C3H/HeN mice and the absence of this response in BALB/c mice are unknown. However, NK cell activation by other intracellular organisms, such as Listeria or Toxoplasma, requires the production of cytokines from infected macrophages (48, 49). One required cytokine is TNF-α, and another is likely to be natural killer cell–stimulating factor (NKSF) or IL-12. IL-12 is produced by macrophages and B cells and stimulates the production of IFN-γ by NK and T cells (50, 51). Preliminary data from this laboratory show that both TNF-α and IL-12 are, in fact, produced in the draining LNs of L. major–infected mice soon after infection, and studies are currently in progress to assess their role in NK cell development in

Figure 6. Course of infection in L. major–infected control and aAsGM1-treated C3H/HeN mice. Mice were infected and treated as described in Materials and Methods. The course of infection was measured in groups of five mice by monitoring the increase in footpad thickness using a dial micrometer and calculating the difference against the contralateral uninfected footpad. Levels of significance for each time point were determined by the Student's t test and considered significant at p <0.05. Results shown are the means of five mice $\pm$ SE of footpad swelling.

leishmaniasis. Interestingly, treatment of C3H/HeN mice with an anti-TNF-α mAb enhanced susceptibility to *L. major*, although whether this was due to a deficit in the NK cell response was not assessed (52).

We previously reported that in vitro or in vivo depletion of CD4⁺ T cells eliminated early IFN-γ production in *L. major*–infected C3H/HeN mice (10). We have now found that CD4⁺ T cell depletion also reduces NK cell responses in our system, and that IL-2 can substitute for the presence of CD4⁺ T cells. Purified CD4⁺ T cells from the draining LNs of 2 d infected C3H/HeN mice fail to make IFN-γ when cultured with normal LN cells, but CD4⁺ T cell–depleted populations that are enriched for NK cells produce IFN-γ when cultured with IL-2 (Tables 2 and 3). These observations are consistent both with the known capacity of IL-2 to induce NK cell function (37, 53), and with the synergistic effect IL-2 has on the capacity of IL-12 to stimulate NK cells (50). It would appear, then, that *Leishmania* infection in C3H/HeN mice differs from the T cell–independent, NK cell–dependent production of IFN-γ observed in *scid* mice infected with *Listeria* or *Toxoplasma* (48, 49). One explanation for these differences is that lower numbers of NK cells or higher levels of inhibitory cytokines may necessitate the presence of IL-2 to enhance NK cell function in conventional mice. Alternatively, *Leishmania* may induce less IL-12 than other pathogens, such as *Listeria* and *Toxoplasma*, and require IL-2 for amplification of its activity. In this regard, the inability of *L. donovani* to activate NK cells in *scid* spleen cell cultures suggests a role for T cells for the induction of an NK response to *Leishmania* (54).

It remains to be determined if the absence of a positive signal or the presence of an inhibitory signal leads to the low NK cell response in BALB/c mice after *L. major* infection. The fact that BALB/c mice exhibit some NK cell activation (10–15%) and a burst of IFN-γ on day 2 when stimulated in vitro with SLA may be interpreted to suggest that the positive signals for activation of NK cells are present in *L. major*–infected BALB/c mice, but that the response is inhibited. Two candidate inhibitory cytokines are IL-4 and TGF-β. IL-4, which is produced very early in both susceptible BALB/c mice and resistant, low NK cell responder C57BL/6 mice, has been shown to directly inhibit NK cell activity (55, 56). Alternatively, TGF-β, which has recently been shown to augment susceptibility in *Leishmania* (57), and also downregulates NK cell function in viral infections (58), might also prematurely inhibit NK cell function in these animals.

The higher number of parasites observed in the footpad at 2 wk and the larger lesion sizes of mice treated with aAsGM1 indicate that NK cells not only modulate Th subset development, but also contribute to the early resistance to *L. major*. This hypothesis is supported by the observation that C3H and CBA mice, which exhibit a strong NK cell response early in infection, exhibit significantly lower parasite burdens at 2 wk compared with C57BL/6, A/J, and BALB/c mice, which lacked a significant NK cell response. In fact, an analysis of the responses of several inbred strains to *L. major* indicates that an inverse correlation exists between the NK cell response and parasite burden. These results are in agreement with previous studies demonstrating that C3H and CBA mice are highly resistant to *L. major*, while C57BL/6 and A/J mice develop lesions that are larger and take longer to heal (38). We suggest that these differences are directly attributable to the induction of an NK cell response that controls early parasite burdens. In support of this hypothesis was the finding that the course of infection in NK cell–depleted *L. major*–infected C3H/HeN mice was strikingly similar to that observed in C57BL/6 mice. This is the first study to demonstrate a role for NK cells in resistance to cutaneous leishmaniasis. Previous studies of the function of NK cells in leishmaniasis revealed a role for NK cells in visceral, but not cutaneous, leishmaniasis (59–61). However, the prior studies either failed to assess NK cell activity in the LN or used mice with the beige (*bg/bg*) defect, which although influencing NK cell cytolytic function, does not affect NK cell production of IFN-γ (53). Our results caution against the use of mice with the beige defect for the study of NK cell function in diseases where IFN-γ production, and not cytotoxicity, may represent the physiological function of NK cells.

Efforts to define the factors responsible for the induction of distinct Th subsets have accelerated over the last year. One of the primary advances in the field was the recognition that components of the innate immune response signal the adaptive immune response how to appropriately react to infection (40, 62). Our results demonstrate that NK cells, through the production of IFN-γ, are one such mediator. Defining the mechanisms involved in the selective development of Th1 or Th2 cells will have a direct and fundamental influence on future vaccine development and the design of effective therapies for infectious diseases. Our results provide a rationale for targeting NK cells during vaccination, in order to augment IFN-γ at the time of antigen challenge, and thus subsequently promote cell-mediated immunity. In fact, the adjuvant properties of some bacteria, such as *C. parvum*, may in part be mediated by their ability to activate an early NK cell response.

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