In Vivo Treatment with Interleukin 12 Protects Mice from Immune Abnormalities Observed during Murine Acquired Immunodeficiency Syndrome (MAIDS)

By Ricardo T. Gazzinelli,* Nathalia A. Giese,‡ and Herbert C. Morse III‡

*From *the Section of Immunobiology and Cell Biology, Laboratory of Parasitic Diseases; and ‡the Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892*

Summary

Lymphoproliferation, chronic B cell activation resulting in hypergammaglobulinemia, and profound immunodeficiency are prominent features of a retrovirus-induced syndrome designated murine acquired immunodeficiency syndrome (MAIDS). In vivo treatment of infected mice with recombinant interleukin 12 (IL-12) beginning at the time of infection or up to 9 wk after virus inoculation markedly inhibited the development of splenomegaly and lymphadenopathy, as well as B cell activation and Ig secretion. Treatment with IL-12 also had major effects in preventing induction of several immune defects including impaired production of interferon γ (IFN-γ) and IL-2 and depressed proliferative responses to various stimuli. The therapeutic effects of IL-12 on the immune system of mice with MAIDS were also associated with reduced expression of the retrovirus that causes this disease (BM5def), with lesser effects on expression of ecotropic MuLV. IL-12 treatment was not effective in IFN-γ knockout mice or in infected mice treated simultaneously with IL-12 and anti-IFN-γ. These results demonstrate that induction and progression of MAIDS are antagonized by IL-12 through high-level expression of IFN-γ and may provide an experimental basis for developing treatments of retrovirus-induced immune disorders with similar immunopathogenic mechanisms.

Murine AIDS (MAIDS)[1] develops in some strains of mice infected with the LP-BM5 mixture of murine leukemia viruses (MuLV) originally isolated by Laterjet and Duplan (1). This mixture includes replication-competent, nonpathogenic helper viruses and etiologic replication-defective MuLV designated LP-BM5 def (2) or Du5H (3). MAIDS is characterized by an early, persistent polyclonal activation of CD4+ T cells accompanied by increasingly severe defects in cell-mediated immunity (4, 5). Abnormalities of B cells are another prominent manifestation of the syndrome and include polyclonal activation associated with B cell proliferation and differentiation to Ig secretion resulting in hypergammaglobulinemia (5, 6). As a consequence of B cell and CD4+ T lymphocyte proliferation, the susceptible strains of mice develop massive lymphadenopathy as well as splenomegaly and expression of these retroviruses increases progressively with time after infection (7, 8).

B lymphocytes are the major target for infection by LP-BM5 def (8) and their activation and expansion are essential components for the development of this disorder (9). In MAIDS, B cell activation, and lymphoproliferation are dependent on CD4+ T cells (10, 11) and are associated with increased production of IL-4, IL-6, and IL-10 (Type 2 cytokines)[2]; and reduced expression of IL-2 and IFN-γ proteins (Type 1 cytokines) (12). Type 2 cytokines efficiently stimulate B cell growth and differentiation to Ig secretion and may be of crucial importance to the chronic B cell activation and hypergammaglobulinemia, a central feature of this disease. High levels of such cytokines may also promote activation and replication of proviral DNA in B lymphocytes and therefore favor progression of MAIDS. Studies using IL-4–deficient mice suggest that there are strain-dependent differences in the importance of this particular Type 2 cytokine for devel-

[1] *Abbreviations used in this paper:* HPRT, hypoxanthine phosphoribosyltransferase; MAIDS, murine AIDS; MuLV, murine leukemia virus; RT, reverse transcriptase.

R. T. Gazzinelli and N. A. Giese contributed equally to this work.

[2] Studies identifying cells other than CD4+ T cells as important sources of IFN-γ (NK cells) or IL-10 (B cells, macrophages), for example, suggest the use of the terms Type 1 and Type 2 to designate certain cytokine profiles rather than Th1 and Th2 with their inherent implications of T cell origin. As many studies indicate that CD4+ T cells with Th1 and Th2 features exist in vivo, Th subset terminology will still be used to refer to these cell types.

opment of MAIDS. (B6 × 129)F_2 mice with an IL-4 knockout had a much-delayed course of disease (13), while progression of MAIDS was comparable in B6 knockout and wild-type mice (14).

Nevertheless, Type 1 cytokines, through their ability to activate macrophages, cytotoxic T cells, and NK cells, may be important components in resistance to this retrovirus mixture. If Type 1 responses are protective to virus-infected mice, it would be expected that manipulations which enhance induction of Th1 differentiation and Type 1 cytokine production would confer some measure of disease resistance to MAIDS-susceptible mice as manifested by reduced lymphoproliferation and improved cell-mediated immune function. We have explored this postulate by treating infected mice with IL-12.

IL-12 is a heterodimeric cytokine produced by macrophages in response to infection with various organisms. It acts in synergy with TNF-α and IL-2 to induce the generation of IFN-γ by NK cells (15–18) and acts on both $CD8^+$ T and NK cells to enhance their cytotoxic activity (19). Importantly, IL-12 also conditions naive helper cells to differentiate to the Th1 pathway (20–22) and stimulates Th2 cells to transiently produce IFN-γ (23).

In the results presented here, we show that chronic treatment with rIL-12 for 4 wk, initiated at either 0 or 4 wk post infection, resulted in significantly reduced spleen and cervical lymph node weights and marked reductions in the severity of histopathologic changes and immune abnormalities associated with the progression of MAIDS. IL-12 treatment also had an inhibitory effect on expression of the retrovirus that causes the disease (BM5def) but had less of an effect on the replication-competent, nonpathogenic, ecotropic helper virus transcripts. Finally, our results show that the beneficial effects of IL-12 were dependent on IFN-γ synthesis and were associated with inhibition of B cell proliferation and activation.

Materials and Methods

Mice and Viruses. 6-wk-old C57BL/6 (B6) female mice obtained from The Jackson Laboratory (Bar Harbor, ME) were injected intraperitoneally with 0.1 ml of stocks of LP-BM5 MuLV prepared as previously described (2). IFN-γ knockout mice (24) used in our studies were obtained from Genentech Inc. (South San Francisco, CA), were backcrossed into B6 genetic background for five to six generations, and typed individually for the wild-type and defective IFN-γ genes. Frequencies of spleen cells producing infectious ecotropic MuLV were determined in infectious center tests using mitomycin C–treated cells (2, 7).

IL-12 Treatment. rIL-12 (25) (5.6×10^6 U/mg) produced by the Mammalian and Microbial Cell Sciences and Process Biochemistry groups of Genetics Institute (Cambridge, MA; generously provided by Dr. Stan Wolf of that Institute) was diluted in PBS containing 0.1% BSA (PBS-BSA) and was inoculated intraperitoneally at 0.1–0.25 μg/mouse/d for 5 d, followed by 2 d with no inoculations. This cycle was repeated 4 or 8 times, with the first round starting at day 0 or day 1 after infection in protocols designed to test effects of IL-12 on early disease. Mice were tested at 8–10 wk post infection. For protocols designed to test effects of rIL-12 on established disease, mice were infected for 4 or 8 wk before starting treatment with rIL-12 for four cycles. Some infected mice were treated simultaneously with IL-12 and anti-IFN-γ mAb (XMG1.6) at a dose of 2 mg of partially purified (26) mAb/mouse/wk. Normal mice, uninfected mice treated with rIL-12, and infected mice treated with PBS-BSA served as controls.

Studies of Infected Mice. At autopsy, mice were bled, spleen and cervical lymph node (LN) weights were determined, and selected tissues were obtained for histopathologic studies. Single-cell suspensions prepared from spleen or pooled peripheral LN cells were treated by flow cytometry (FACS®; Becton Dickinson & Co., Mountain View, CA) using a panel of antibodies including Thy-1.2, κ, CD4, CD8, CD11b, CD32, CD43, and CD45R (B220). FACS® and histopathologic criteria used to stage the progression of MAIDS have been described in detail (7, 27). For proliferative responses, cultured spleen cells were stimulated with Con A (5 μg/ml), Con A plus PMA (10 ng/ml), PMA plus ionomycin, or LPS (20 μg/ml). Proliferative responses were measured after pulsing with [^{3}H]thymidine at 72 h. Supernatants were harvested at 24 h for measurements of IL-2 and at 48 h for IFN-γ. IL-2 was measured using the IL-2–dependent cell line CTLL or by ELISA, and IFN-γ by two-site ELISAs performed as previously described (28). Serum IgG and IgM levels were determined by ELISA as described (29).

Semiquantitative Reverse Transcriptase (RT)-PCR. The sequence of hypoxanthine phosphoribosyltransferase (HPRT) primers and probes and techniques used for PCR analysis of viral gene expression were published elsewhere (30). For detection of BM5def and ecotropic virus mRNAs, the following primers and probe were used respectively: 5′-CCTTTTCCTTTATCGACACT-3′, 5′-ACCAGGGGGGGAATACCTCG-3′, and 5′-CTCTGCCAAAGGGACCAGTT-3′; 5′-GGCCTAGAATATCGGGCTC-3′, 5′-TGTAGTCCTGGTCGTGGATG-3′, and 5′-CCCTGCTGTTCAGGAAGCA-3′. Briefly, total RNA was isolated from spleen or LN samples frozen in RNAzol, followed by extraction with chloroform and isopropanol. 1 μg of RNA was transcribed using MMLV-H^- reverse transcriptase (Promega Corp., Madison, WI). After this reaction, the cDNA-containing solution was used for specific sequence amplification using 1 U Taq DNA polymerase and 40 ng each of sense and antisense primers. The number of cycles was chosen experimentally for each gene product: ecotropic helper, 30 cycles and LP-BM5def, 23 cycles. As control for the RT-PCR, HPRT (23 cycles) was transcribed and amplified for all samples. Finally, PCR products were separated on 1% agarose gels and analyzed by Southern blot hybridization with fluorescein-labeled probes using an ECL-3′ oligolabeling and detection system and Hyperfilm-ECL (Amersham International, Little Chalfort, UK).

Results and Discussion

Two protocols were used to evaluate the effects of IL-12 during MAIDS. To determine if IL-12 would inhibit induction of disease, B6 mice were treated with rIL-12 for 4 or 8 wk beginning within a day after infection with LP-BM5 MuLV. At 4 and 8 wk after infection, mice were examined for progression of MAIDS. To evaluate if IL-12 would be useful in treatment of mice with established disease, cytokine injections were initiated 4 wk post virus inoculation and were continued for 4 wk before testing.

Chronic treatment with rIL-12 by either protocol had profound effects on the development of MAIDS. As shown in Table 1, rIL-12–treated mice had significantly reduced spleen and cervical LN weights and marked reductions in the severity of histopathologic changes in lymphoid and nonlymphoid

Table 1. *Effects of Treament with rIL-12 on Development of Lymphoproliferation and Other Manifestations of Disease Progression in B6 Mice infected with LP-BM5 MuLV**

LP-BM5 MuLV	Treatment with rIL-12 (wk post infection)	Tissue weight		Stage of disease‡	
		Spleen	Cervical LN	PATH	FACS®
		mg			
–	–	80 ± 10	<20	N	N
+	–	463 ± 12	213 ± 12	2	2
–	0–4	90 ± 10	<20	N	N
+	0–4	182 ± 33§	95 ± 42§	N-1	N-1
+	0–8	167 ± 45§	70 ± 36§	N-R	N-R
–	5–9	295 ± 63	33 ± 9	N	N
+	–	483 ± 163	293 ± 153	2-3	2-3
+	5–9	455 ± 54‖	66 ± 13	R-1	1

* Treatment with rIL-12 reduces development of splenomegaly and lymphadenopathy in mice infected with LP-BM5 MuLV. B6 mice were inoculated intraperitoneally at 6 wk of age with 0.1 ml of virus stocks prepared as previously described (7). Numbers indicate the mean ± 1 SE for data from four individual mice per group.

‡ For histopathologic (PATH) and flow cytometry (FACS) determinations of MAIDS progression, *N* indicates indistinguishable from normal; *R* indicates reactive to infection but with insufficient changes to be diagnostic of MAIDS; and 1, 2, and 3 are indicative of changes clearly diagnostic of MAIDS and of increasing severity. The criteria used for determining these stages of disease were detailed previously (6, 7).

§ Difference statisically significant (Schette test), $p < 0.05$ when compared with results obtained from mice infected with LP-BM5 MuLV and treated with rIL-12.

‖ Treatment with rIL-12 alone induces transient increases in spleen weight, mainly due to an expansion of the red pulp, which can be observed in animals killed shortly after cessation of immunotherapy. Staging of MAIDS by histopathology and FACS® analysis demonstrated that these increases in spleen weight could not be attributed to advanced MAIDS as seen in spleens of infected untreated mice in the same protocol.

tissues described previously for LP-BM5 virus–infected mice (7; Table 1). Spleens and LN of mice with MAIDS normally contain greatly increased numbers of immunoblasts, plasmablasts, and plasma cells (7). These populations are readily identified by their reduced expression of Ig κ and CD45(B220) and increased expression of CD43, an antigen absent on resting B cells but expressed on presecretory and secretory B lineage cells (Fig. 1). Both treatment protocols significantly reduced the proportions and total numbers of B cell blasts and plasma cells in spleen (Fig. 1 *A*) and LN (Fig. 1 *B*). IL-12 treatment also reduced the proportions and total numbers of the unusual subset of $CD4^+$ Thy-1^- TCR-α/β^+ T cells that accumulates progressively in spleen and LN of mice with MAIDS (27; Fig. 1).

Consistent with the reductions in cells with Ig-secreting phenotypes described above, IL-12–treated mice exhibited significantly reduced levels of serum IgG and IgM (Fig. 2). The concentrations of both Ig classes did not differ significantly from those of IL-12–treated uninfected mice (not shown) or normal controls (Fig. 2).

Treatment with rIL-12 also prevented virus-induced abnormalities in T cell proliferative responses and cytokine secretion. While spleen cells from mice with MAIDS were greatly impaired in their ability to proliferate or to produce IL-2 in response to various stimuli, the responses of cells from treated mice did not differ significantly from those of normal, uninfected mice or of uninfected mice treated with rIL-12 (Fig. 3).

The ability of rIL-12 treatment to prevent immune dysfunction in virus-infected mice also extended to the expression of IFN-γ protein following stimulation of spleen cells with mitogen (Table 2). In agreement with earlier studies (12; Giese, N., unpublished observations), production of IFN-γ by mitogen-stimulated spleen cells was progressively impaired following infection with LP-BM5 MuLV, whereas Type 2 cytokine (IL-4 and IL-10) synthesis was increased (Table 3). In contrast, spleen cells from either uninfected or infected mice treated with rIL-12 for the first 4 wk post inoculation produced cytokine levels comparable to those generated by cells from normal, uninfected animals (Tables 2 and 3).

We conclude that in vivo treatment of mice with rIL-12 significantly inhibited induction of MAIDS when used immediately after infection and was effective in reversing many manifestations of this syndrome when used to treat established disease. The blocking effects on progression of MAIDS were found to persist through 4 wk (Tables 1 and 2) and up to 10 wk (not shown) after cessation of treatment. Furthermore, marked reductions in lymphoproliferation and prolonged survival have been observed in mice treated as late as 8 wk post infection (data not shown).

In all infectious disease systems examined previously, protective effects of IL-12 have been dependent on IFN-γ (17, 31, 32). We examined the contribution of IFN-γ expression

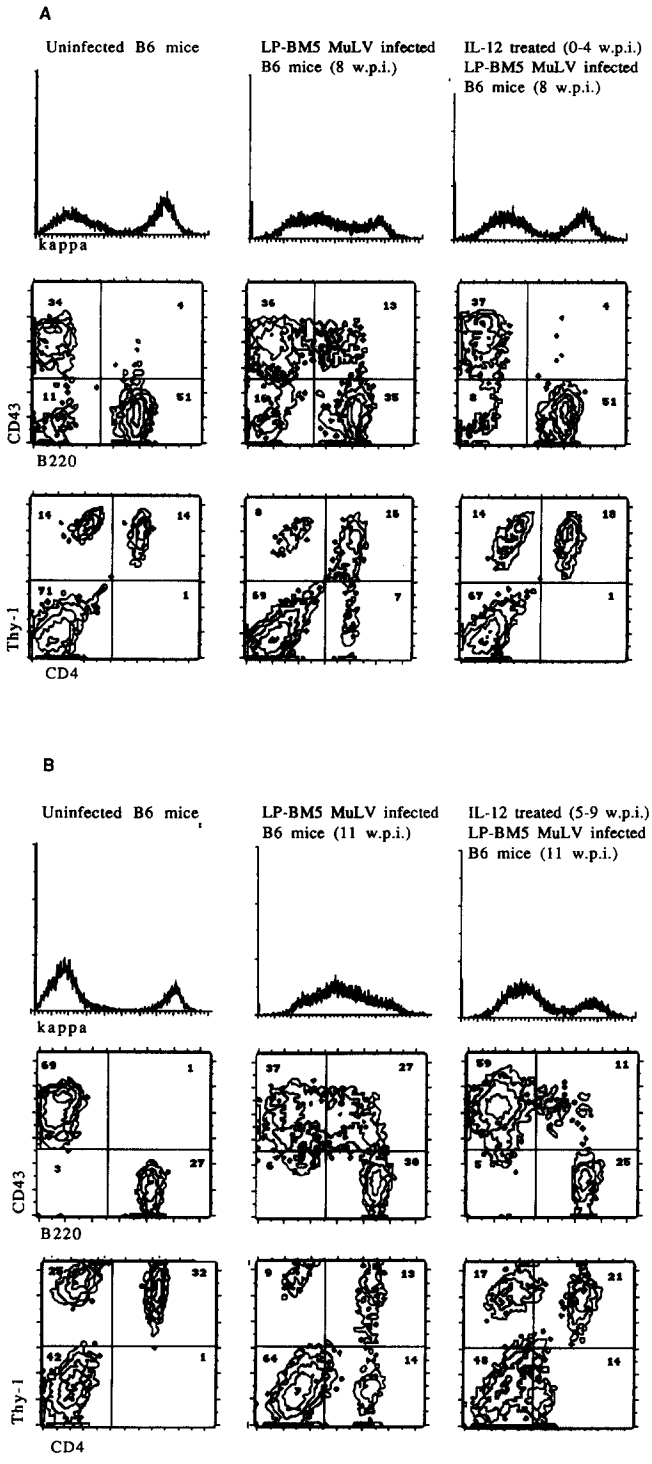

Figure 1. Flow cytometry analyses of spleen or LN cells from B6 mice. Mice were infected with LP-BM5 MuLV and treated with IL-12 for the next 4 wk (*A*) or treated from 5 to 9 wk (*B*) postinfection. Single cell suspensions were prepared from spleens (*A*) or LN (*B*) obtained at 8 or 11 wk post infection and stained with FITC-labeled antibodies to Ig κ light chain (*top*), FITC-labeled anti-CD45R(B220) mAb, and biotin/allophycocyanin (APC)-labeled anti-CD43 mAb (*middle*), or FITC-labeled anti-CD4 mAb and biotin/APC-labeled anti-Thy-1.2 mAb (*bottom*). All preparations were preincubated with unlabeled mAb to the IgG Fc receptor to prevent Fc binding. Cells were analyzed on a FACS® 440 (Becton Dickinson & Co.) on 3 × 10^5 viable cells as determined by narrow forward-angle light scatter and exclusion of propidium iodide. The numbers in the quadrants of the contour plots generated from the two-color analyses indicate the percent of cells bearing each of the phenotypes. Similar results were obtained in three separate experiments in studies of both spleen and LN cells.

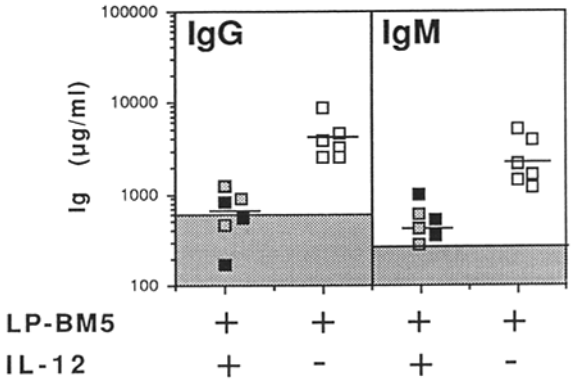

Figure 2. In vivo treatment with rIL-12 prevents and reverses hypergammaglobulinemia induced by infection with LP-BM5 MuLV. B6 mice were infected with virus and treated either with PBS-BSA (□) or with rIL-12 diluted in PBS-BSA (0.1–0.25 μg/mouse/d for 5 d followed by 2 d without inoculation) for four cycles starting at the time of infection (▩) or at 4 wk post infection (■). Sera were collected at 9–10 wk post infection, and serum IgG and IgM levels were determined by ELISA as previously described (29). The *horizontal lines* crossing the boxes represent the mean Ig levels in sera from uninfected controls, which did not differ from the Ig levels in sera from uninfected mice treated with rIL-12 under the same protocols used for infected mice (data not shown). The *short horizontal bars* represent the mean for the experimental group. The differences in the Ig levels for both classes were significantly different ($p < 0.05$) for treated and untreated, infected animals.

to IL-12–induced resistance to MAIDS in two systems. First we studied the effects of IL-12 treatment on disease in IFN-γ knockout mice. IL-12–treated infected knockout mice showed no significant reduction in splenomegaly (Fig. 4) or improvement in FACS® profiles (data not shown) as a result of treatment. In addition, serum IgG levels of the knockout mice infected with LP-BM5 and treated with IL-12 were the same as those mice infected but untreated (Fig. 4). In the second system, IL-12–inoculated mice were treated simultaneously with high levels of neutralizing mAb to IFN-γ. Treatment with the mAb completely blocked the ability of IL-12 to inhibit lymphoproliferation (Table 4). In addition, there were no differences in the histopathologic changes or abnormalities of cell surface antigen expression exhibited by infected, untreated mice and infected mice treated simultaneously with rIL-12 and anti-IFN-γ (data not shown). The combined results from these systems demonstrated that the therapeutic effects of rIL-12 were highly dependent on expression of IFN-γ.

To determine if treatment with rIL-12 altered expression of the MuLV involved in MAIDS, splenic mRNAs were examined by RT-PCR techniques for transcripts from both ecotropic helper and BM5def viruses (Fig. 5). Studies of spleens from infected mice treated with rIL-12 for 4 wk post infection and studied at 4 wk after virus inoculation revealed re-

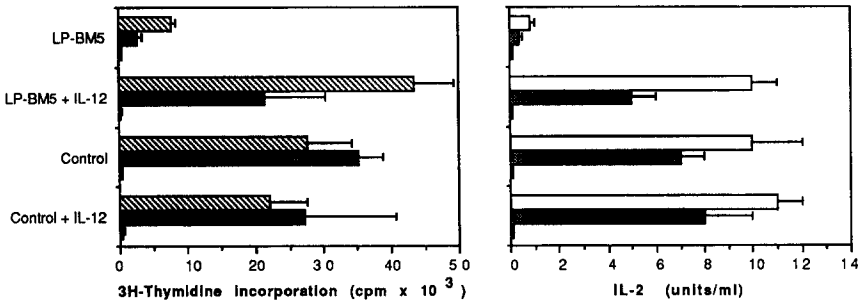

Figure 3. In vivo treatment with rIL-12 restores both proliferation and IL-2 production to spleen cells of mice infected with LP-BM5 MuLV. Uninfected mice and virus-infected mice were treated with rIL-12 (0.1 μg/mouse/d) beginning at the time of infection and continuing for 4 wk. Controls included mice injected with virus but not with rIL-12 and uninfected animals. Spleen cells obtained at 9 wk post infection were cultured with medium alone (*solid bars*), Con A (*hatched bars*), PMA plus ionomycin (*slashed bars*), and Con A plus PMA (*open bars*).

duced expression of BM5def as well as ecotropic virus in the spleens of treated mice (Fig. 5 *A*, experiment *I*). Similar effects of treatment were seen with mRNA from spleens of animals treated for 4 or 8 wk post infection and examined at 9 wk after virus inoculation (Fig. 5 *A*, experiment *II*). To quantitate the effects of IL-12 treatment on expression of the helper and defective virus, we determined the ratio of BM5def or ecotropic helper virus mRNAs to HPRT transcripts in each of the samples from 14 untreated and 22 treated mice (Fig. 5 B). Transcripts of the defective virus were present in all preparations from treated animals but were clearly reduced (10-fold difference in means) below the levels of untreated animals. Reduced expression of BM5def could not be attributed solely to inhibition of helper virus replication by IL-12, as the levels of ecotropic virus mRNA for IL-12–treated mice were reduced about threefold compared with levels for untreated, infected mice (Fig. 5 *B*). Further studies of the effects of IL-12 treatment on expression of ecotropic MuLV by infectious center tests showed that the frequencies of virus-producing spleen cells were generally comparable for IL-12–treated or untreated, infected mice (data not shown).

Table 2. *Effects of Treatment with rIL-12 on Induced Production of IFN-γ by Spleen Cells from Mice Infected with LP-BM5 MuLV**

Wk post infection		IFN-γ	
LP-BM5 MuLV	Treatment with rIL-12	Media	Con A
		ng/ml	
–	–	0.3 ± 0.2	25.8 ± 1.9
4	–	0.3 ± 0.1	12.2 ± 6.0‡
4	0–4	4.7 ± 1.5	25.8 ± 4.8
–	0–4	2.8 ± 0.9	30.3 ± 3.1
8	–	<0.1	6.2 ± 1.6‡
8	0–4	0.7 ± 0.3	26.5 ± 3.8

* Treatment with rIL-12 restores inducibility of IFN-γ. The groups of mice presented received the cytokine treatment from week 0 to 4 on the same schedule and dose decribed in Table 1. Groups of uninfected B6 mice treated with rIL-12 were used as controls. Numbers indicate the mean for four mice per group. Spleen cells were cultured in media alone or Con A (5 μg/ml) as detailed elsewhere (12). Supernatants were harvested at 48 h for assay of IFN-γ. Cytokine measurements were performed by two-site ELISA tests as previously described (12, 28).
‡ Difference statistically significant (Schette test), $p < 0.05$ when compared with results obtained from infected or control mice treated with rIL-12.

The basis for the dissociation between expression of BM5def and ecotropic helper virus transcripts in infected mice treated with IL-12 is unclear. Studies of B cell lymphomas that contain and express BM5def showed that treatment with IL-12 in vitro did not affect cell viability or proliferative rate and did not reduce the levels of defective virus mRNA (data not shown), suggesting that the cytokine did not act directly on B cells by eliminating targets for BM5def expression or altering the rate of virus transcription. It is known, however, that BM5def is expressed at highest levels in B lymphocytes, whereas the helper virus might be less specific in terms of its target cells. Therefore, an inhibitory effect of IL-12 treatment on B lymphocyte activation and expansion could explain this discrepancy. We examined these possibilities in studies of mice tested immediately after completion of treatment with IL-12. It should be noted, first, that splenomegaly, due primarily to expansion of the red pulp and congestion, develops in mice treated with IL-12, with the increases being more prominent in infected animals (Table 5 and data not shown). These IL-12–induced changes resolve rapidly after cessation of treatment of both infected and uninfected mice. By 8 wk post infection, the beneficial effects of IL-12 in preventing development of splenomegaly due to virus were again evident (Table 5).

Further studies showed that the frequencies of B cells in spleens of mice treated with IL-12 were reduced, particularly in tissues of infected mice (Table 5). The increases in spleen size associated with IL-12 treatment did not compensate for these reductions, as lymphocyte recovery from the congested spleens was reduced (data not shown). In contrast, LN from IL-12–treated mice had twice the normal frequency of B cells for both infected and uninfected mice, indicating that IL-12 altered the trafficking of B cells (Giese, N., unpublished observations). The demonstration of disproportionately reduced proliferative responses to LPS by spleen cells from infected, IL-12–treated mice (Table 5) indicated that treatment altered not only the total numbers but the biologic responses of B cells.

Treatment with rIL-12 dramatically changed the cytokine profile of infected mice, which in untreated animals was characterized by a dominance of Type 2 cytokines (Tables 2 and 3). During IL-12 treatment, we observed greatly enhanced

Table 3. *In Vivo Treatment with IL-12 Inhibits IL-4 and IL-10 Synthesis Produced by Lymphocytes Obtained from Mice Infected with LP-BM5**

Weeks post infection		IL-4		IL-10	
LP-BM5 MuLV	Treatment with rIL-12	Media	Con A/P	Media	Con A/P
		U/ml		*U/ml*	
–	–	<0.1	4.5 ± 0.5	1 ± 1	7.4 ± 0.5
4	–	0.5 ± 0.1‡	7.5 ± 0.5‡	<1.0	35.5 ± 4.5‡
4	0–4	<0.1	2.4 ± 0.4	<1.0	5.0 ± 0.8
–	0–4	<0.1	2.6 ± 1.2	<1.0	12.3 ± 2.5
8	–	0.6 ± 0.3‡	8.5 ± 0.9‡	<1.0	21.6 ± 3.4‡
8	0–4	<0.1	2.5 ± 1.2	<1.0	4.5 ± 0.5

* The results presented in this table were obtained from animals of the same experimental group described in Table 1. Numbers indicate the mean for four mice per group. Spleen cells were cultured in media alone or with Con A (5 μg/ml) plus PMA (10 ng/ml) as detailed elsewhere (12). Supernatants were harvested at 24 h and 72 h for assay, respectively, for IL-4 and IL-10. Cytokine measurements were performed as previously described (12, 28).

‡ Difference statistically significant (Schette test, $p < 0.05$) when compared with results obtained from infected or control mice treated with rIL-12.

expression of IFN-γ accompanied by decreased levels of IL-4 and IL-10. It is noteworthy that spleen cells from infected mice treated with IL-12 not only gained the ability to produce high levels of IFN-γ upon Con A stimulation but produced remarkable amounts of IFN-γ spontaneously—a phenomenon that has been seen after infection of resistant strains of mice including A/J, BALB/c, and 129 (Giese, N., and R. Morawetz, unpublished observations). After cessation of cytokine therapy, spontaneous production of IFN-γ gradually decreased with time followed by a period of "remission" in development of MAIDS lasting up to 10 wk. Subsequently, as noted above, the mice developed the full spectrum of features characteristic of MAIDS including progressive lymphoproliferation, immunodeficiency, and increased expression of Type 2 cytokines.

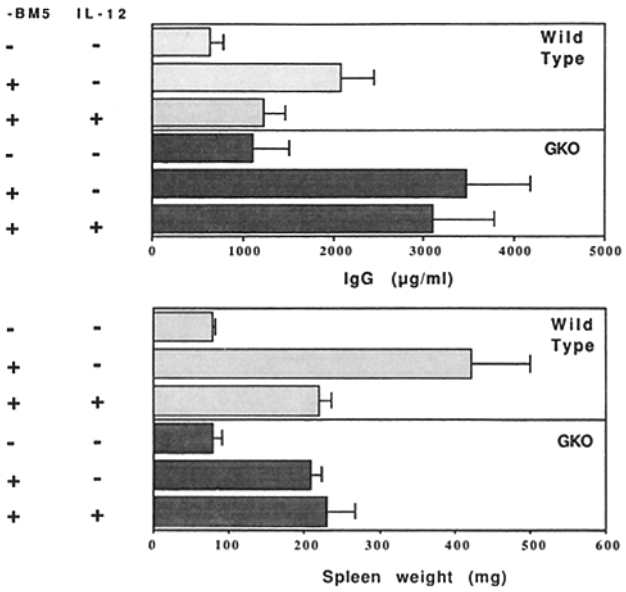

Figure 4. In vivo treatment with rIL-12 does not prevent hypergammaglobulinemia and splenomegaly induced by infection with LP-BM5 MuLV in IFN-γ knockout mice. IFN-γ knockout mice or the wild-type controls (which include animals containing one or two alleles from the normal IFN-γ gene) were infected with virus and either untreated or treated with rIL-12 diluted in PBS-BSA (0.25 μg/mouse/d for 5 d followed by 2 d without inoculation) for four cycles starting at the time of infection. Spleens and sera were collected at 9–10 wk post infection. Serum IgG levels were determined by ELISA as previously described (29). The differences in the Ig levels and spleen weights were significantly different ($p < 0.05$) for treated and untreated wild-type infected animals.

Based on the findings presented here as well as on studies performed with the IL-4 knockout B6 mice (14), we believe that induction of a vigorous Type 1 response featuring high-level expression of IFN-γ and NK cell activation, rather than inhibition of Type 2 cytokine synthesis, is the basis of IL-12 therapeutic effects during MAIDS. Thus, treatment with IL-12 would result in strong cell-mediated immunity, control of virus replication, and delay of pathologic manifestations characteristic of MAIDS rather than causing a permanent switch from Th2 to Th1 cell activation and resistance to disease. It is likely that in vivo treatment with IL-12 would affect B cell activation and proliferation by inducing high levels of IFN-γ (33) and/or restricting proliferation of Th2 lymphocytes (34) and, secondarily, the production of Type 2 cytokines that are essential for B lymphocyte expansion (35, 36). Since B cells are the primary targets for infection and expression by BM5def, control of their proliferation would restrict BM5def virus expression, thus limiting the drive for CD4 T cell polyclonal activation and progression of MAIDS. In addition, by acting directly on NK cells (15) and $CD8^+$ lymphocytes (19), as well as stimulating Th1 expansion and limiting Th2 development (20–23), IL-12 could exert other beneficial effects favoring development of effective cell-mediated functions against BM5def. A mechanism involving $CD8^+$

Table 4. *Ability of rIL-12 to Inhibit Development of Lymphoproliferation and Other Manifestations of Disease Progression in B6 Mice Infected with LP-BM5 MuLV Is Blocked by Simultaneous Treatment with Anti-IFN-γ mAb**

LP-BM5 MuLV	Treatment with rIL-12	Treatment with anti-IFN-γ	Tissue weight		Stage of disease‡	
			Spleen	Cervical LN	PATH	FACS®
			mg			
–	–	–	70 ± 8	<20	N	N
+	–	–	407 ± 20	145 ± 9	1	1
+	–	+	346 ± 20	130 ± 20	1	1
+	+	–	158 ± 19	64 ± 13	R	R
+	+	+	454 ± 53§	218 ± 40§	1	1

* Simultaneous treatment with anti-IFN-γ antibodies blocks effects of IL-12 treatment on progression of MAIDS. B6 mice were inoculated intraperitoneally at 4–6 wk of age with 0.1 ml of virus stocks prepared as previously described (7). Mice were treated with rIL-12 (0.25 μg/mouse/d) and/or anti-IFN-γ (2 mg/mouse/wk) for the first 4 wk of infection, as indicated in the table.
‡ For histopathologic (PATH) and flow cytometry (FACS®) determination of MAIDS progression, see Table 1.
§ Difference statistically significant (Schette test, $p < 0.05$) when compared with results obtained from mice infected with LP-BM5 MuLV and treated with rIL-12.

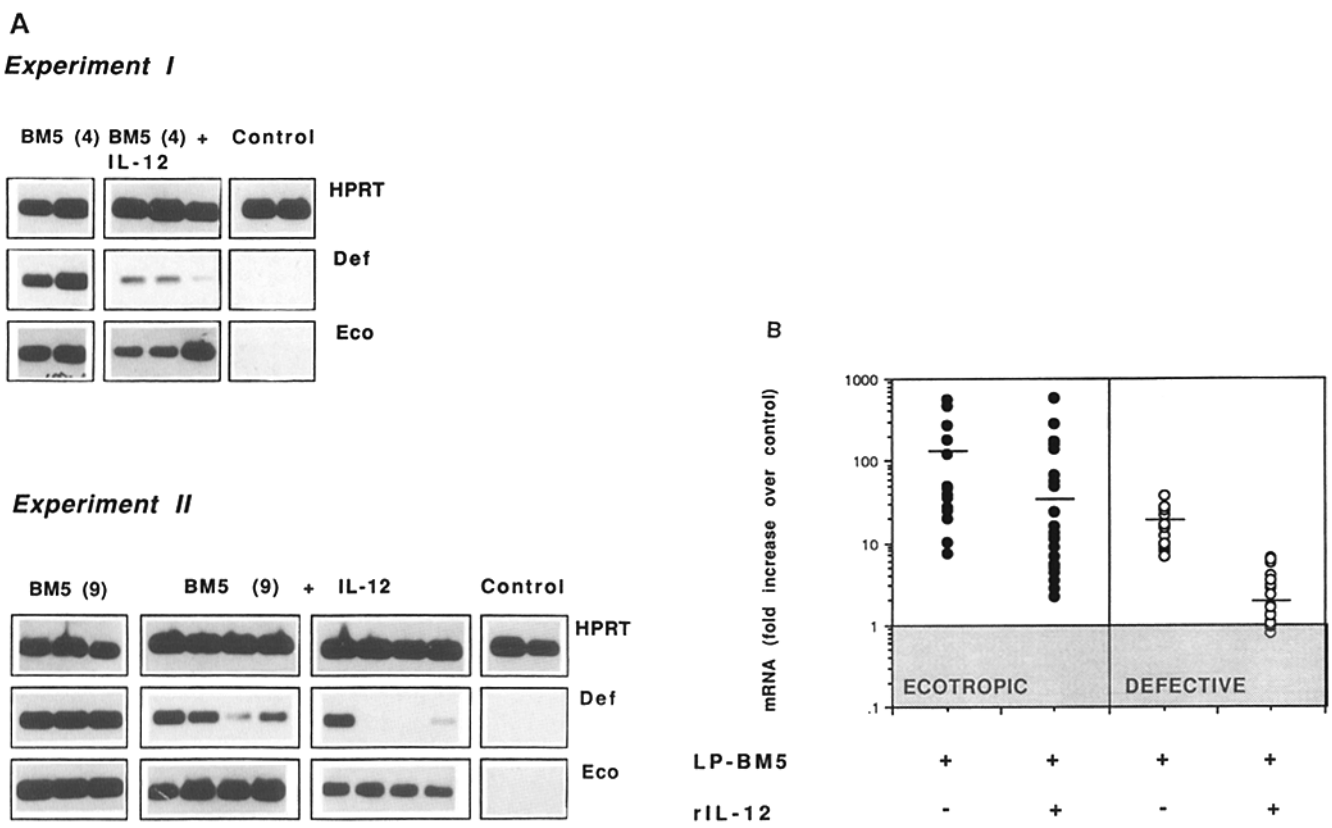

Figure 5. In vivo treatment with rIL-12 alters expression of LP-BM5 def transcripts in cells from mice infected with LP-BM5 MuLV. RT-PCR assays were used for measurement of the housekeeping gene HPRT, ecotropic helper virus, and the LP-BM5-defective virus. (*A*) Effects of rIL-12 on spleen cells from virus-infected mice (BM5) or uninfected mice (control). In experiment *I* mice were treated with 0.25 μg/mouse/d of rIL-12 during the first 4 wk of infection, and virus mRNA transcripts measured at 5 wk post infection with LP-BM5 MuLV mixture. In a second experiment, mice were treated with rIL-12 at 0.1 μg/mouse/d for 4 or 8 wk, and mRNA transcripts from spleen were tested for expression of BM5def at 9 wk post infection. Spleen weights (mg) at the time of testing were: LP-BM5–infected: 430, 450, 470; infected + rIL-12 for 4 wk: 200, 220, 170, 160; infected + rIL-12 for 8 wk: 230, 120, 160, 160. (*B*) Quantitation of ecotropic helper and BM5def transcripts in the spleen of 14 control infected mice and in 22 infected mice treated with IL-12. Quantitation was performed by determining the ratios of BM5def or ecotropic virus to HPRT transcripts expressed in spleen cells. Background values obtained from control mice were arbitrarily defined as 1 and did not differ from control mice treated with IL-12. Data were obtained from mice in three separate experiments.

Table 5. *Effects of rIL-12 Treatment on B Cell Frequencies and Proliferative Responses to LPS**

Wk post infection	Treatment	Spleen wt.	B220	Kappa	LPS
		mg	*Percent positive cells*		*cpm* × 10^{-3}
0	–	70	56 ± 1	49 ± 4	173
4	BM5	160 ± 10	63 ± 2	49 ± 0	181 ± 5
0	IL-12 (4 wk)	193 ± 18	44 ± 1	35 ± 1	108 ± 13
4	BM5 + IL-12 (4 wk)	397 ± 123	29 ± 9	24 ± 4	43 ± 24
0	–	75 ± 5	57 ± 3	51 ± 3	297 ± 1
8	BM5	463 ± 7	46 ± 1	45 ± 2	153 ± 17
0	IL-12 (4 wk)	90 ± 10	56 ± 1	47 ± 4	185 ± 36
8	BM5 + IL-12 (4 wk)	197 ± 15	56 ± 1	48 ± 2	192 ± 13

* Mice, normal or infected with LP-BM5 MuLV, were treated with IL-12 for 4 wk. Tests were performed immediately after cessation of treatment or 4 wk later (8 wk post infection). Numbers indicate the mean ± 1 SE for groups of two to four mice. The percentages of cells expressing B220 or κ were determined by FACS®. Similar results were obtained in three different experiments.

lymphocytes clearly contributes to virus clearance in MAIDS-resistant A/J mice (37).

Recent studies show that treatment with rIL-12 can also enhance immunity to other viral infections such as lymphocytic choriomeningitis virus (38). It is well documented that the course of HIV infection has certain features, other than immunodeficiency, that parallel observations made in MAIDS. These include chronic B cell activation, hypergammaglobulinemia, and persistent generalized lymphadenopathy due to B cell proliferation (39, 40). In addition, different studies have demonstrated that a functional defect in the synthesis of Type 1 cytokines by T lymphocytes may occur during the later stages of HIV infection preceding the decrease in $CD4^+$ lymphocyte counts (41, 42). In conjunction with recent studies showing impaired IL-12 production in HIV-infected patients (43), IL-12 stimulation of IL-2 and IFN-γ synthesis by peripheral blood cells from HIV-infected individuals (44, 45), enhanced NK cytotoxicity (44), and enhanced HIV-specific responses of PBC from HIV^+ individuals (46), the results presented here support the concept that IL-12 may be useful in the treatment of retrovirus-induced immunodeficiencies.

We gratefully acknowledge Sara Hieny, Patricia Caspar, Thomas McCarty, and Catherine de Rivera for technical assistance; Ruth Swofford for FACS® assistance; and Nan Lofgren and Brenda Rae Marshall for preparing this manuscript. We are indebted to the Mammalian and Microbial Cell Sciences and Process Biochemistry groups of Genetics Institute for production and purification of rIL-12. We also acknowledge Dr. Torgny Fredrickson for help and advice with histopathology; Dr. Sisir Chattopadhyay for supplying the primer sequences used to detect both ecotropic helper and BM5def transcripts, and Drs. Wendy Davidson, Alan Sher, Gene Shearer, Robert Coffman, Janet Hartley, and Stan Wolf for critical reading of this manuscript.

This work was supported in part by Contract NO1-AI-45203 to Microbiological Associates, Inc. (Bethesda, MD).

Address correspondence to Dr. Ricardo T. Gazzinelli, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, Building 4, Room 126, Bethesda, MD 20892.

Received for publication 16 May 1994 and in revised form 28 July 1994.

References

1. Laterjet, R., and J.F. Duplan. 1962. Experiment and discussion on leukaemogenesis by cell-free extracts of radiation-induced leukaemia in mice. *Int. J. Rad. Biol.* 5:339.
2. Chattopadhyay, S.K., H.C. Morse III, M. Makino, S.K. Ruscetti, and J.W. Hartley. 1989. Defective virus is associated with induction of murine retrovirus-induced immunodeficiency syndrome. *Proc. Natl. Acad. Sci. USA.* 86:3862.
3. Aziz, D.C., Z. Hanna, and P. Jolicoeur. 1989. Severe immunodeficiency disease induced by a defective murine leukemia virus. *Nature (Lond.).* 338:505.
4. Morse, H.C., III, S.K. Chattopadhyay, M. Makino, T.N. Fredrickson, A.W. Hügin, and J.W. Hartley. 1992. Retrovirus-

induced immunodeficiency in the mouse: MAIDS as a model of AIDS [editorial]. *AIDS.* 6:607.
5. Mosier, D.E., R.A. Yetter, and H.C. Morse III. 1985. Retroviral induction of acute lymphoproliferative disease and profound immunosuppression in adult C57BL/6 mice. *J. Exp. Med.* 161:766.
6. Klinman, D.M., and H.C. Morse III. 1989. Characteristics of B cell proliferation and activation in murine AIDS. *J. Immunol.* 142:1144.
7. Hartley, J.W., T.N. Fredrickson, R.A. Yetter, M. Makino, and H.C. Morse III. 1989. Retrovirus-induced murine acquired immunodeficiency syndrome: natural history of infection and differing susceptibility of inbred mouse strains. *J. Virol.* 63:1223.
8. Huang, M., C. Simard, D.G. Kay, and P. Jolicoeur. 1991. The majority of cells infected with the defective murine AIDS virus belong to the B-cell lineage. *J. Virol.* 65:6562.
9. Cerny, A., A.W. Hügin, R.R. Hardly, K. Hayakawa, R.M. Zinkernagel, M. Makino, and H.C. Morse III. 1990. B cells are required for induction of T cell abnormalities in a murine retrovirus-induced immunodeficiency syndrome. *J. Exp. Med.* 171:315.
10. Yetter, R.A., R.M.L. Buller, J.S. Lee, K.L. Elkins, D.E. Mosier, T.N. Fredrickson, and H.C. Morse III. 1988. $CD4^+$ T cells are required for development of a murine retrovirus-induced immunodeficiency syndrome (MAIDS). *J. Exp. Med.* 168:623.
11. Mosier, D.E., R.A. Yetter, and H.C. Morse III. 1987. Functional T lymphocytes are required for a murine retrovirus-induced immunodeficiency disease (MAIDS). *J. Exp. Med.* 165:1737.
12. Gazzinelli, R.T., M. Makino, S.K. Chattopadhyay, C.M. Snapper, A. Sher, A.W. Hügin, and H.C. Morse III. 1992. $CD4^+$ subset regulation in viral infection. Preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. *J. Immunol.* 148:182.
13. Kanagawa, O., B.A. Vaupel, S. Gayama, G. Koehler, and M. Kopf. 1993. Resistance of mice deficient in IL-4 to retrovirus-induced immunodeficiency syndrome (MAIDS). *Science (Wash. DC).* 262:240.
14. Morawetz, R.A., T.M. Doherty, N.A. Giese, J.W. Hartley, W. Muller, R. Kuhn, K. Rajewsky, R. Coffman, H.C. Morse III. 1994. Resistance to murine acquired immunodeficiency syndrome (MAIDS). *Science (Wash. DC).* 265:264.
15. Kobyashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827.
16. D'Andrea, A., M. Rengarajau, M.N. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387.
17. Gazzinelli, R.T., S. Hieny, T. Wynn, S. Wolf, and A. Sher. 1993. IL-12 is required for T cell-independent induction of IFN-γ by an intracellular parasite and induces resistance in T cell deficient hosts. *Proc. Natl. Acad. Sci. USA.* 90:6115.
18. Tripp, C.S., S.F. Wolf, and E.R. Unanue. 1993. Interleukin-12 and tumor necrosis factor α are costimulators of interferon-γ production by natural killer cells in severe combined immunodeficiency syndrome mice with listeriosis and interleukin-10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA.* 90:3725.
19. Stern, A.S., F.J. Podlaski, J.D. Hulmes, Y.C. Pan, P.M. Quinn, A.G. Wolitzky, P.C. Familletti, D.L. Stremlo, T. Truitt, R. Chizzonite, et al. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 87:6808.
20. Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science (Wash. DC).* 260:547.
21. Seder, R.A., R.T. Gazzinelli, A. Sher, and W.E. Paul. 1993. IL-12 acts directly on $CD4^+$ T cells to enhance priming for IFN-γ production and diminishes IL-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA.* 90:10188.
22. Manetti, R., P. Parronchi, M.G. Giudizi, M.-P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* 177:1199.
23. Manetti, R., F. Geroza, M.G. Giudizi, R. Biagiotti, P. Parronchi, M.-P. Piccini, S. Sampagnoaro, E. Maggi, S. Romagnani, and G. Trinchieri. 1994. Interleukin 12 induces stable priming for interferon γ (IFN-γ) production during differentiation of human T helper (Th) cells and transient IFN-γ production in established Th2 cell clones. *J. Exp. Med.* 179:1273.
24. Dalton, D., S. Pitts-Meek, S. Keshav, I.S. Figari, A. Bradley, and T.A. Stewart. 1993. Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science (Wash. DC).* 259:1739.
25. Schoenhaut, D.S., A.O. Chua, A.G. Wolitzky, P.M. Quinn, C.M. Dwyer, W. McComas, P.C. Familletti, M.K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. *J. Immunol.* 148:3433.
26. Gazzinelli, R.T., Y. Xu, S. Hieny, A. Cheever, and A. Sher. 1992. Simultaneous depletion of $CD4^+$ and $CD8^+$ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J. Immunol.* 149:175.
27. Holmes, K.L., H.C. Morse, M. Makino, R.R. Hardy, and K. Hayakawa. 1990. A unique subset of normal murine $CD4^+$ T cells lacking Thy-1 is expanded in a murine retrovirus-induced immunodeficiency syndrome, MAIDS. *Eur. J. Immunol.* 20: 2783.
28. Mosmann, T., and T.A.T. Fong. 1989. Specific assays for cytokine production by T cells. *J. Immunol. Methods.* 116:151.
29. Rizzo, L.V., R.H. DeKruyff, and D.T. Umetsu. 1992. Generation of B cell memory and affinity maturation: induction with Th1 and Th2 cell clones. *J. Immunol.* 148:3733.
30. Gazzinelli, R.T., I. Eltoum, T.A. Wynn, and A. Sher. 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J. Immunol.* 151:3672.
31. Heinzel, F.P., D.S. Shoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. *J. Exp. Med.* 177:1505.
32. Tripp, C.S., M.K. Gately, J. Hakimi, P. Ling, and E. Unanue. 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and CB-17 mice, reversal by IFN-γ. *J. Immunol.* 152:1883.
33. Reynolds, D.S., W.H. Boom, and A.K. Abbas. 1987. Inhibition of B lymphocyte activation by interferon-γ. *J. Immunol.* 139:767.
34. Gajewski, T.F., and F.W. Fitch. 1988. Antiproliferative effect of IFN-γ in immune regulation. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T cell clones. *J. Im-*

munol. 140:4245.
35. Mosmann, T.R., and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145.
36. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, M.P. Beckmann, L.S. Park, K.A. Schooley, R.L. Coffman, T.R. Mosmann, and W.E. Paul. 1989. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303.
37. Makino, M., S.K. Chattopadhyay, J.W. Hartley, and H.C. Morse III. 1992. Analysis of role of $CD8^+$ T cells in resistance to murine AIDS in A/J mice. *J. Immunol.* 149:1702.
38. Orange, J.S., S.F. Wolf, and C.A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. *J. Immunol.* 152:1253.
39. Fauci, A.S. 1993. Multifactorial nature of human immunodeficiency virus disease: implications for therapy. *Science (Wash. DC).* 262:1011.
40. Metroka, C.E., S. Cunningham-Rundles, M. Krim, and M.S. Pollack. 1984. Generalized lymphadenopathy in homosexual men: an update of the New York experience. *Ann. N.Y. Acad. Sci.* 437:400.
41. Murray, H.W., B.Y. Rubin, H. Masur, and R.B. Roberts. 1984. Impaired production of lymphokines and immune (γ) interferon in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* 310:883.
42. Lane, H.C., J.M. Depper, W.E. Greene, G. Whalen, T.A. Waldmann, and A.S. Fauci. 1985. Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome. Evidence for a selective defect in soluble antigen recognition. *N. Engl. J. Med.* 313:79.
43. Chehimi, J., S.E. Starr, I. Frank, A. D'Andrea, X. Ma, R.R. MacGregor, J. Sennelier, and G. Trinchieri. 1994. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *J. Exp. Med.* 179:1361.
44. Chehimi, J., S.E. Starr, I. Frank, M. Rengaraju, S.J. Jackson, C. Llanes, M. Kobayashi, B. Perussia, D. Young, E. Nickbarg, et al. 1992. Natural killer (NK) cell stimulatory factor increases the cytotoxic activity of NK cells from both healthy donors and human immunodeficiency virus–infected patients. *J. Exp. Med.* 175:789.
45. Clerici, M., D.R. Lucey, J.A. Berzofsky, L.A. Pinto, T.A. Wynn, S.P. Blatt, M.J. Dolan, C.W. Hendrix, S.F. Wolf, and G.M. Shearer. 1993. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. *Science (Wash. DC).* 262:1721.
46. Chehimi, J., N.M. Valiante, A. D'Andrea, M. Rengaraju, Z. Rosado, M. Kobayashi, B. Perussia, S.F. Wolf, S.E. Starr, and G. Trinchieri. 1993. Enhancing effect of natural killer cell stimulatory factor (NKSF/interleukin-12) on cell-mediated cytotoxicity against tumor-derived and virus-infected cells. *Eur. J. Immunol.* 23:1826.