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Tissue-Specific Targeting of Cytokine Unresponsiveness in Transgenic Mice

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Summary

The ubiquitous cellular distribution of certain cytokine receptors has hampered attempts to define the physiologically important cell-specific functions of cytokines in vivo. Herein, we report the generation of transgenic mice that express a dominant-negative IFNγ receptor α chain mutant under the control of either the human lysozyme promoter or the murine *lck* proximal promoter, which display tissue-specific unresponsiveness in the macrophage or T cell compartments, respectively, to the pleiotropic cytokine, IFNγ. We utilize these mice to identify previously undefined cellular targets of IFNγ action in the development of a murine antimicrobial response and the mixed lymphocyte reaction. Moreover, we identify the macrophage as a critical responsive cell in manifesting the effects of IFNγ in regulating CD4⁺ T helper subset development. These studies thus represent a novel approach to studying the cell-specific actions of an endogenously produced pleiotropic cytokine in vivo.

Introduction

The definition of the biologic roles of interferon γ (IFNγ) has been facilitated by the availability of purified recombinant IFNγ and neutralizing IFNγ-specific monoclonal antibodies (MAbs) (Farrar and Schreiber, 1993). Recently, the generation of mice lacking either the IFNγ or IFNγ receptor genes has allowed for a broader appreciation of the in vivo functions of IFNγ (Dalton et al., 1993; Huang et al., 1993). However, since IFNγ receptors are expressed on nearly all cells, the current technologies have been unable to define the cell-specific functions of this cytokine in vivo.

We have used dominant-negative mutant forms of the IFNγ receptor α chain to elucidate the cell-specific actions of IFNγ (Dighe et al., 1993, 1994). Overexpression of a cytoplasmically truncated form of the IFNγ receptor α chain in homologous cells eliminated cellular responses to IFNγ in vitro (Dighe et al., 1993). When this technique was used with cultured murine fibroblasts, the resulting cells became IFNγ unresponsive and failed to induce a variety of IFNγ-dependent cellular responses in vitro. These included induction of IRF-1 gene transcription, expression of inducible nitric oxide (NO) synthase, up-regulation of major histocompatibility complex (MHC) class I protein expression, and induction of an antiviral response. More recently, we expressed dominant-negative IFNγ receptors in a transplantable murine fibrosarcoma and used these cells to elucidate a novel role for IFNγ in the development of anti-tumor responses in mice (Dighe et al., 1994). Using Meth A tumor cells that overexpressed a cytoplasmically truncated IFNγ receptor α chain, we showed that IFNγ-insensitive Meth A cells displayed enhanced tumorigenicity compared with control Meth A cells and resisted rejection in endotoxin-treated and immune mice. In addition, IFNγ-insensitive tumor cells were poorly immunogenic. These results demonstrated that IFNγ has direct effects on tumor cell immunogenicity and thus plays an important role in promoting tumor cell recognition and elimination.

Although the two previous studies validated the use of dominant-negative IFNγ receptors as a means of selectively eliminating the response of specific cell populations to IFNγ, they required the use of transfected cultured cells. To explore whether tissue-specific IFNγ unresponsiveness could be induced in intact animals, we generated transgenic mice in which a dominant-negative IFNγ receptor mutant was targeted to specific cell types using tissue-specific promoters. Herein, we document the generation and characterization of transgenic mice with targeted IFNγ unresponsiveness in either the macrophage or T cell compartment. Moreover, we demonstrate the functional consequences of cell-specific IFNγ insensitivity and define the primary cellular targets of the actions of IFNγ in the development of antimicrobial and allogeneic responses and identify one of the cell-specific actions of IFNγ in facilitating development of CD4⁺ T helper (Th) cell subsets.

Results

Generation of Transgenic Mice Expressing a Dominant-Negative Form of the IFNγ Receptor α chain in Macrophages

Based on the well-established role of IFNγ in affecting macrophage function in vitro, we chose initially to target IFNγ unresponsiveness to the macrophage compartment. We used a cDNA encoding a previously characterized cytoplasmically truncated dominant-negative form of the IFNγ receptor α chain (Dighe et al., 1993). To enhance transgene detection, the cDNA was modified at its 5′ end to encode the myc proto-oncogene epitope recognized by the MAb 9E10. This cDNA was placed under the control of the human lysozyme promoter for selective expression in macrophages. FACS analysis of resident peritoneal macrophages derived from transgenic mice showed that the transgene was expressed at high levels on F4/80-positive cells (Figure 1). No significant 9E10 staining was seen on the F4/80-negative population, demonstrating selective expression in the resident macrophage lineage. Thioglycollate-elicited peritoneal exudate cells (PECs) did

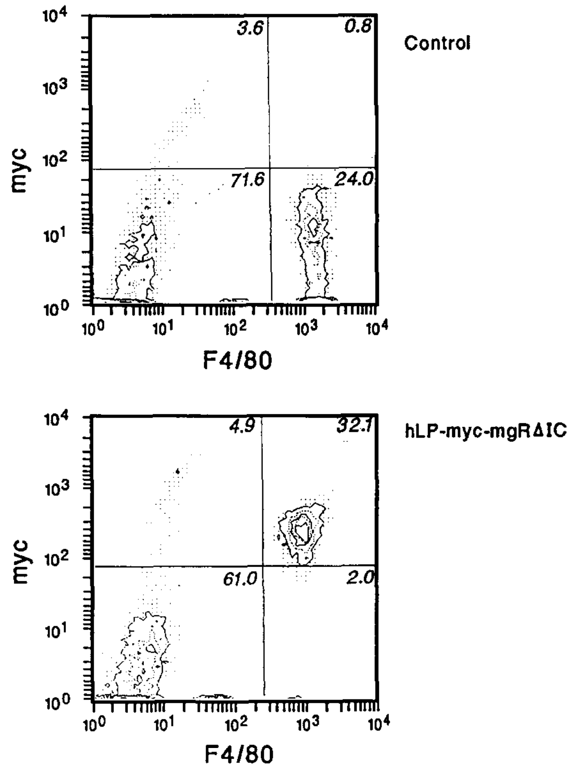

Figure 1. Expression of Epitope-Tagged Truncated Murine IFNγ Receptor α Chain in rPECs of Macrophage-Targeted Transgenic Mice

rPECs were obtained from transgene-positive hLP–myc–mgRΔIC mice and littermate controls. Expression of the epitope-tagged transgene was determined by two-color flow cytometry using biotinylated myc-tag-specific 9E10 MAb and streptavidin–PE (SAPE) and FITC-conjugated anti-F4/80. Flow cytometry was conducted as described in Experimental Procedures.

not show 9E10 staining, suggesting that transgene expression was limited to mature resident macrophages. Flow cytometric analysis of peripheral blood revealed low level 9E10 staining in a subset of the Mac-1$^+$ cells (3%–6% of total) but not on T cells (CD3$^+$), B cells (B220$^+$), neutrophils (Gr-1$^+$), or natural killer (NK) cells (5E6$^+$) (data not shown). In whole splenocyte populations, the epitope-tagged receptor was detectable only on Mac-1$^+$ cells, but at low levels. Identical patterns of expression were observed in three lines of mice derived from distinct founders. Normal numbers of CD3$^+$, B220$^+$, and NK cells were present in blood, thymus, and spleen in comparison to nontransgenic controls, demonstrating that no significant alteration of hematopoetic cell development had occurred secondary to transgene expression. Thus, the human lysozyme promoter-driven construct used in this study directed expression of the epitope-tagged truncated murine IFNγ receptor α chain specifically to a subset of cells of the macrophage lineage.

Expression of Truncated IFNγ Receptors in Resident Peritoneal Macrophages Results in Functional Nonresponsiveness to IFNγ

We next examined the in vitro responses of resident peritoneal exudate cells (rPECs) to IFNγ. Culture of rPECs derived from nontransgenic littermate control mice with the combination of IFNγ and lipopolysaccharide (LPS)-induced expression of NO synthase (iNOS), leading to the production of 5.4 nmol nitrite/10^5 cells (Figure 2A). In contrast, rPECs from the macrophage-targeted transgenic mice (hLP–myc–mgRΔIC) cultured with the combination of IFNγ and LPS produced only 0.3 nmol nitrite/10^5 cells, an amount that was comparable to the level of nitrite produced with LPS alone (0.2 nmol/10^5 cells). The lack of iNOS induction in rPECs from transgenic mice was specifically due to IFNγ unresponsiveness, since the cells responded to the combination of IFNα plus LPS in a manner that was comparable to rPECs isolated from littermate controls (4.1 and 5.4 nmol/10^5 cells, respectively). Thioglycollate-elicited PECs from transgene-positive and littermate control mice responded to IFNγ as monitored by induction of MHC class II proteins, a finding that was predicted by transgene expression data. IFNγ responsiveness was not altered in T or B cells from macrophage-targeted transgenic mice, as evidenced by monitoring IFNγ-dependent MHC class I protein enhancement (data

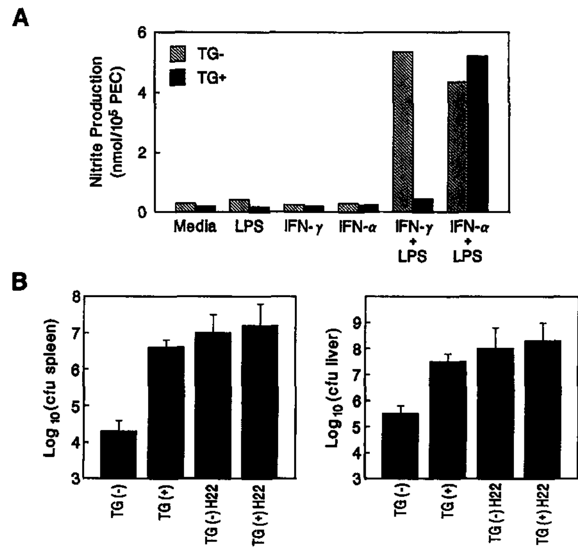

Figure 2. Functional Characterization of Mice with Targeted Expression of a Dominant-Negative IFNγ Receptor in Macrophages

(A) IFNγ unresponsiveness of rPECs in macrophage-targeted transgenic mice. PECs (10^5) from hLP–myc–mgRΔIC transgene-positive mice or littermate controls were seeded in a 96-well tissue culture plate and allowed to adhere for 2 hr. Adherent PECs were then washed and stimulated with either LPS (10 μg/ml), rMuIFNγ (1000 IRU/ml), or rHuIFNα$_{A-D}$ (1000 IRU/ml), or various combinations of the above reagents at the same concentration. After 48 hr of stimulation, supernatants were harvested and the level of nitrite determined by the Greiss reaction (Green et al., 1982).

(B) Increased susceptibility of macrophage-targeted transgenic mice to Listeria monocytogenes infection. Transgene-positive hLP–myc–mgRΔIC mice or littermate controls were infected with 5 × 10^5 cfu of Listeria monocytogenes intraperitoneally. On day 5, bacteria in the spleens and livers of infected mice were quantitated by colony counts of homogenates plated on BHI agar. At this time, 33% of the transgene-positive mice had died (3 of 9), whereas none of the transgene-negative group had died (0 of 10). In mice treated with the anti-IFNγ MAb H22, 44% (4 of 9) of the transgene-positive mice and 40% (4 of 10) of transgene-negative mice died by day 5.

not shown). Thus, expression of a dominant-negative IFNγ receptor α chain under control of the human lysozyme promoter produced a state of functional unresponsiveness to IFNγ, which was localized to resident macrophages.

Increased Susceptibility to Listeria monocytogenes in Mice Expressing Truncated IFNγ Receptors in Macrophages

To assess the in vivo consequences of functional IFNγ unresponsiveness of resident macrophages, we examined responses of transgene-positive and control mice to infection with Listeria monocytogenes. IFNγ is required for the initiation of antimicrobial responses and its site of action is presumed to be the macrophage (Buchmeier and Schreiber, 1985; Bancroft et al., 1991). To test this hypothesis formally, we infected transgene-positive or littermate control mice with 5 × 10^5 colony-forming units (cfu) of Listeria. This dose of Listeria was sublethal for control mice (0 of 10 dead by day 5). In contrast, 33% of transgene-positive animals (3 of 9) died by day 5. Bacterial titers in the spleen and liver of transgene-positive mice were approximately 100-fold greater than observed in control animals (Figure 2B). As expected, pretreatment of control mice with a neutralizing antibody to IFNγ increased their susceptibility to Listeria (4 of 10 dead by day 5). Surviving antibody-treated control mice had Listeria titers 100-fold higher than control mice. Anti-IFNγ treatment of transgene-positive animals did not significantly alter lethality (4 of 9 dead) or bacterial titers. Thus, whole animal neutralization of IFNγ produced similar effects to those seen by selectively inducing a state of IFNγ unresponsiveness in the resident macrophage. These results suggest that the resident macrophage is the principal cell required to initiate an IFNγ-dependent anti-Listeria response.

Generation of Transgenic Mice Expressing a Dominant-Negative Form of the IFNγ Receptor Selectively in T Cells

Resolution of Listeria infection requires induction of cell-mediated immunity that is dependent on the induction of the $CD4^+$ Th1 subset. IFNγ has been suggested to regulate Th phenotype development at the level of the T cell (Gajewski and Fitch, 1988; Seder et al., 1993; Schmitt et al., 1994). To examine this issue in more detail, we generated transgenic mice with selective IFNγ unresponsiveness in T cells by driving the expression of the epitope-tagged dominant-negative IFNγ receptor α chain with the *lck* proximal promoter. Expression of the epitope-tagged, truncated IFNγ receptor in T cell–targeted transgenic mice (lck–myc–mgRΔIC) was restricted to the T cell lineage. Several lines of transgenic mice demonstrated expression of the epitope-tagged transgene in peripheral T cells. The *lck* proximal promoter has been previously demonstrated to direct the expression of heterologous cDNAs to the thymocyte compartment. The expression of the truncated IFNγ receptor in the peripheral T cells in several lines of lck–myc–mgRΔIC mice was likely due to the prolonged half-life of the mutant IFNγ receptor protein, which lacks those portions of the receptor required for efficient internalization and degradation. Peripheral blood $CD3^+$ cells

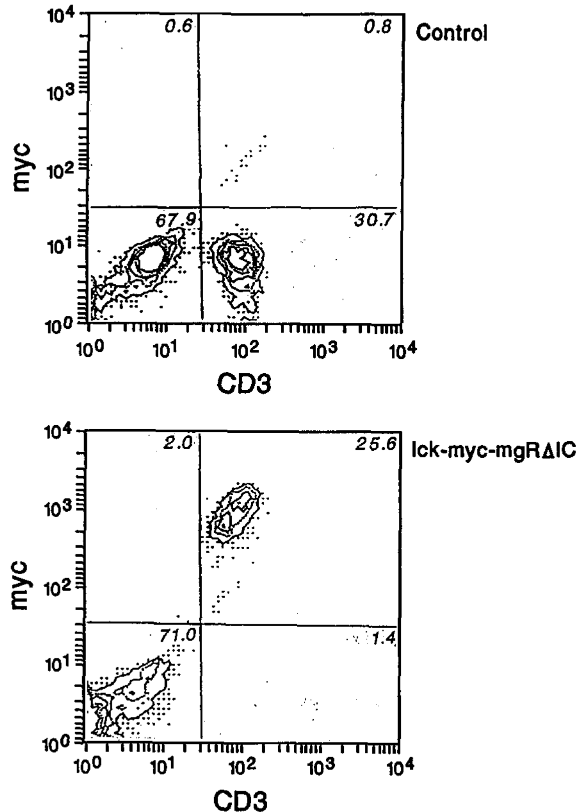

Figure 3. Expression of Epitope-Tagged Truncated Murine IFNγ Receptor α Chain in the Peripheral Blood of T Cell–Targeted Transgenic Mice

Peripheral blood was obtained from transgene-positive lck–myc–mgRΔIC mice and littermate controls. Expression of the epitope-tagged transgene was determined by two-color flow cytometry using biotinylated myc-tag-specific 9E10 MAb and SAPE and FITC-conjugated anti-CD3ε. Flow cytometry was conducted as described in Experimental Procedures.

from these lines expressed high levels of the epitope-tagged receptor (9E10 staining), as measured by flow cytometry (Figure 3). The epitope-tagged receptor was also expressed at high levels in the spleen and thymus on virtually all $CD3^+$ cells (data not shown). Normal distributions of $CD3^+$, $CD4^+$, $CD8^+$, $B220^+$, and NK cells were seen in thymus, spleen, and peripheral blood compared with nontransgenic controls. These results demonstrated that the epitope-tagged dominant-negative form of the IFNγ receptor was selectively targeted to the T cell lineage and did not effect either general hematopoietic or specific lymphocyte lineage development.

Expression of the Truncated IFNγ Receptor in T Cells Produces a State of T Cell Functional Nonresponsiveness to IFNγ

T cell responsiveness to IFNγ in T cell–targeted transgenic mice was initially assessed by monitoring IFNγ-dependent enhancement of MHC class I expression. Peripheral blood cells from nontransgenic littermates cultured in vitro in the presence of 1000 IRU/ml of murine IFNγ showed increased MHC class I expression on both the $CD3^+$ and

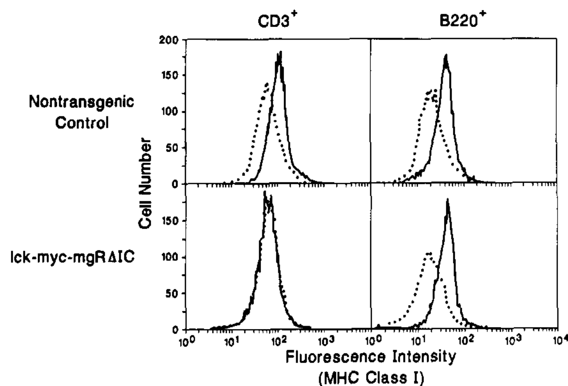

Figure 4. Specific Lack of IFNγ-Induced Enhancement of MHC Class I on T Cells from T Cell-Targeted Transgenic Mice

Peripheral blood of transgene-positive lck-myc-mgRΔIC and littermate control mice was cultured for 72 hr with 1000 IRU/ml of either murine IFNγ or human $IFN\alpha_{A-D}$. $CD3^+$ and $B220^+$ cells were then examined for MHC class I expression by flow cytometry using PE-conjugated anti-H-$2K^d$ MAb as described in Experimental Procedures. Dotted lines represent the constitutive level of MHC class I expressed on cells incubated with medium alone.

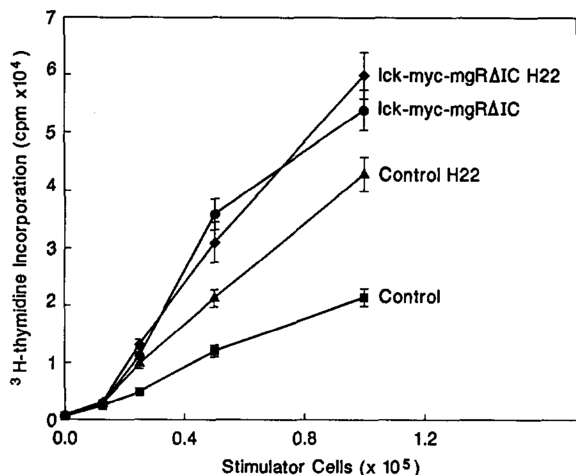

Figure 5. Enhanced Proliferative Responses in MLR Cultures Established with Splenocytes from T Cell-Targeted Transgenic Mice

MLRs were carried out by culturing 5×10^5 responder H-2^d splenocytes from transgene-positive lck-myc-mgRΔIC or littermate control mice with irradiated (2000 rads) C57BL/6 (H-2^b) splenocytes. Proliferation was assessed by thymidine incorporation as described in Experimental Procedures. Neutralization of IFNγ was carried out by the addition of 100 μg/ml of the anti-IFNγ MAb H22 at the initiation of the cultures.

$B220^+$ populations, as detected by flow cytometry (Figure 4). In contrast, $CD3^+$ cells from T cell-targeted transgenic mice did not increase MHC class I expression in response to murine IFNγ. This loss of IFNγ responsiveness was specific to the $CD3^+$ population, since $B220^+$ cells from lck-myc-mgRΔIC transgenic mice remained fully responsive to IFNγ. The lack of IFNγ responses in the transgenic T cells was not due to a generalized defect in the MHC class I synthesis pathway, since both $CD3^+$ and $B220^+$ cells from transgene-positive and control mice showed enhanced MHC class I expression in response to IFNα (data not shown). Like peripheral blood leukocytes, splenocytes from transgenic mice also showed specific IFNγ unresponsiveness that was restricted to the $CD3^+$ population (data not shown). Peritoneal macrophages derived from the T cell-targeted transgenic mice showed normal induction of iNOS in response to the combination of IFNγ and LPS (data not shown). Thus, transgenic mice that express the dominant-negative form of the IFNγ receptor under the control of the lck proximal promoter show a selective state of functional unresponsiveness to IFNγ, which localizes to the T cell compartment.

To assess the functional consequences of eliminating IFNγ responses in T cells, we examined allogeneic T cell proliferative responses in splenocytes derived from T cell-targeted transgenic and littermate control mice. Previous reports established that global elimination of cellular responses to IFNγ in vitro (either using neutralizing MAbs or by targeted disruption of the IFNγ gene) augmented the proliferative responses of T cells to allogeneic stimuli (Bucy et al., 1988; Dalton et al., 1993). We therefore examined responses of splenocytes from our H-2^d haplotype transgenic mice or controls to irradiated H-2^b haplotype allogeneic stimulator cells (Figure 5). Nontransgenic littermate control splenocytes proliferated when cultured with allogeneic stimulator cells. As expected, neutralization of IFNγ with the anti-IFNγ MAb H22 increased the proliferative response of responding cells by 3- to 4-fold. In contrast, T cells from H-2^d lck-myc-mgRΔIC transgenic mice displayed an increased proliferative response, comparable to that of the H22-treated control cell cultures. No additional increase in proliferation of IFNγ-unresponsive T cells occurred when IFNγ was neutralized. This result thus demonstrates that the anti-proliferative action of IFNγ in mixed lymphocyte reaction (MLR) cultures is the result of a direct effect of IFNγ on T cells.

To assess the in vivo consequences of T cell-specific IFNγ unresponsiveness on the development of antimicrobial responses, we infected T cell-targeted transgene-positive or littermate control mice with 5×10^5 cfu of Listeria. None of the mice in either group died of the infection, indicating that both groups were capable of developing sterilizing anti-Listeria immunity (0 of 8 and 0 of 10 dead by day 5 for transgenic and control mice, respectively). Bacterial titers on day 5 in the spleen and liver of transgene-positive mice were similar to that of littermate control animals (data not shown). The ability of both groups of mice to produce sterilizing immunity to Listeria was demonstrated by the absence of Listeria cfu in the spleen and liver of the mice on day 10. As expected, pretreatment of transgene-positive or control mice with a neutralizing antibody to IFNγ increased susceptibility to Listeria (4 of 8 transgene-positive and 4 of 10 control mice dead by day 5). Surviving H22-treated mice had Listeria titers 100-fold higher than untreated transgene-positive or control mice (data not shown). Thus, in contrast with the results obtained using mice with targeted macrophage IFNγ unresponsiveness, mice with targeted T cell IFNγ unresponsiveness had no defect in the ability to mount a full curative

anti-Listeria response. Since sterilizing immunity to Listeria is known to require development of a cell-mediated immune response, and since cell-mediated immunity is promoted by the Th1 subset, our results suggest Th1 subset induction occurs normally in transgenic mice with T cell-targeted IFNγ responsiveness.

Normal Th Phenotype Development of DO11.10 TCR Transgenic T Cells Lacking IFNγ Responsiveness

A role for IFNγ in Th phenotype development has been predicted from in vitro experiments that utilize T cells derived from TCR transgenic mice that express the DO11.10 KJ1-26⁺ TCR (Murphy et al., 1990). This system allows for experimental control over the antigen-presenting cell (APC), cytokine environment, and exposure to pathogens during primary T cell activation (Hsieh et al., 1992, 1993b). Using this system, IFNγ has been found to be necessary but not sufficient for induction by heat-killed Listeria monocytogenes (HKLM) of a Th1 phenotype (Hsieh et al., 1992, 1993a, 1993b). Additionally, IFNγ has been reported to exert a selective antiproliferative effect on Th2 and not Th1, thereby leading to the suggestion that IFNγ may regulate Th phenotype development by inhibiting Th2 formation (Gajewski and Fitch, 1988). However, based on in vivo anti-Listeria responses manifest in mice with T cell-specific IFNγ unresponsiveness, we questioned whether IFNγ played a physiologically important role in Th phenotype development.

To explore this issue, we crossed the lck-myc-mgRΔIC transgenic mice to αβ TCR transgenic mice expressing the DO11.10 KJ1-26⁺ TCR. T cells derived from DO11.10 αβ TCR transgenic mice are naive clonally restricted CD4⁺ T cells specific for a peptide of ovalbumin (323–339) (Murphy et al., 1990). The majority of the T cells derived from these doubly transgenic mice were CD4⁺, clonotype positive, phenotypically naive (i.e., MEL-14⁺), and expressed high levels of the epitope-tagged dominant-negative IFNγ receptor α chain. On the basis of IFNγ-dependent MHC class I enhancement, these cells were IFNγ unresponsive. Clonotype-positive CD4⁺ T cells from the doubly transgenic mice were present in the periphery in amounts comparable to mice transgenic only for the DO11.10 TCR.

We examined Th development in these cells using an in vitro assay system. In this system, Th1 and Th2 subsets are defined by the polarized production of IFNγ but not interleukin 4 (IL-4) and of IL-4 but not IFNγ, respectively. Purified T cells from T cell-targeted IFNγ-unresponsive doubly transgenic mice or control mice expressing only the DO11.10 transgenes were activated in vitro with OVA peptide and irradiated BALB/c splenocytes and allowed to develop for 7 days. Following restimulation with antigen, doubly transgenic T cells showed no substantial differences in IL-4 or IFNγ production compared with control DO11.10 transgenic T cells (Figure 6, top bars). IL-12 induced Th1 development of doubly transgenic T cells lacking IFNγ responsiveness in a manner similar to that observed with control TCR transgenic T cells. Furthermore, IFNγ-unresponsive T cells expressed a Th1 cytokine profile when differentiated in the presence of HKLM in a manner that was indistinguishable from control TCR transgenic T cells. Neutralization of either IFNγ or IL-12 during primary stimulation prevented HKLM-induced Th1 development, as observed previously (Hsieh et al., 1993a, 1993b). Addition of IL-4 to primary cultures induced Th2 development in both doubly transgenic T cells and control IFNγ-responsive T cells. Neutralization of IL-4 during primary activation produced a Th1 phenotype in both doubly transgeneic and control T cells. Thus, regulation of Th phenotype development in IFNγ-unresponsive T cells is unaltered in comparison to that of IFNγ-responsive T cells. These results demonstrate that IFNγ is not required by these T cells for IL-12-induced Th1 development.

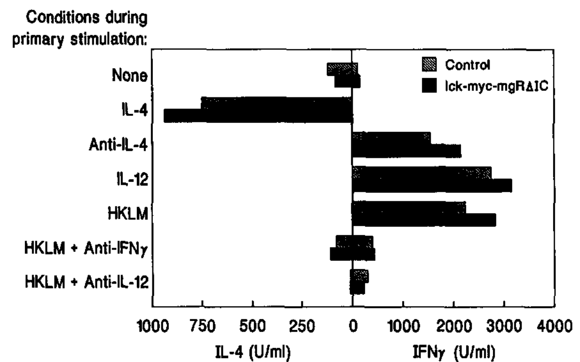

Figure 6. Normal Th Cell Phenotype Development of T Cells Lacking IFNγ Responsiveness

Purified T cells (2.5 × 10⁵/well) from DO11.10 lck-myc-mgRΔIC mice or DO11.10 control mice were stimulated with 0.3 μM OVA peptide presented by 6 × 10⁶ BALB/c splenocytes. Conditions added in the primary culture were media, IL-4 (20 U/ml), anti-IL-4 (10 μg/ml), IL-12 (5 U/ml), HKLM (1 × 10⁷/ml), HKLM plus anti-IFNγ (10 μg/ml), and HKLM plus anti-IL-12 (1:500 dilution polyclonal rabbit anti-IL-12). On day 7, the T cells were harvested, washed, counted, and restimulated at 2.5 × 10⁵ cells/well using 6 × 10⁶ BALB/c splenocytes presenting 0.3 μM OVA peptide in the absence of experimental conditions. Supernatants were collected at 48 hr and cytokine profiles assayed by ELISA.

IFNγ Responsiveness by APCs Is Required for HKLM-Induced Th1 Development

The lack of role for T cell IFNγ responsiveness suggested that the APC may be the physiologic target of the actions of IFNγ during Th phenotype development. Because of limited expression of the dominant-negative transgene in splenic macrophages, we were unable to use APC derived from the macrophage-targeted mice. To circumvent this problem, we took advantage of the observation that DO11.10 transgenic T cells respond to OVA peptide presented either by H-2ᵈ or H-2ᵇ splenocytes. In initial control experiments, we determined that under a variety of culture conditions there were no significant differences in proliferation or cytokine production using either BALB/c (H-2ᵈ) or 129 Sv (H-2ᵇ) splenocytes as the APCs (data not shown). We then examined whether IFNγ-unresponsive APC derived from spleens of 129Sv mice homozygous for the targeted disruption of the IFNγ receptor α chain (Huang et al., 1993) could effect normal Th phenotype development.

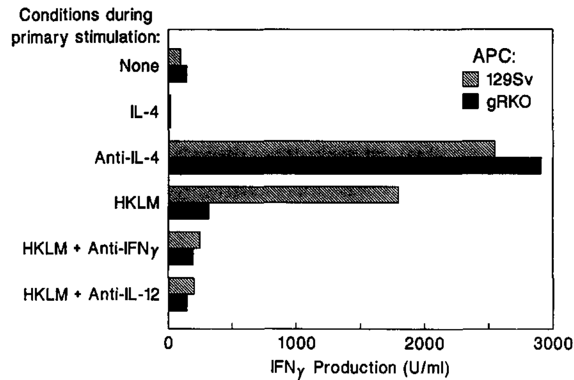

Figure 7. Th1 Phenotype Development in Response to Heat-Killed Listeria Requires IFNγ Responsive APCs

T cells (2 × 10^5/well) were stimulated in the presence of added cytokines or anti-cytokine antibodies in 24-well plates with 0.3 μM OVA peptide presented by either 6 × 10^6 129 Sv splenocytes or splenocytes from mice lacking the IFNγ receptor α chain. Cultures were maintained during the primary stimulation in the presence of the indicated conditions. On day 7, the T cells were harvested, washed, counted, and restimulated at 2.5 × 10^5 cells/well using 6 × 10^6 129 Sv splenocytes presenting 0.3 μM OVA peptide in the absence of experimental conditions. Supernatants were collected at 48 hr and cytokine profiles assayed by ELISA.

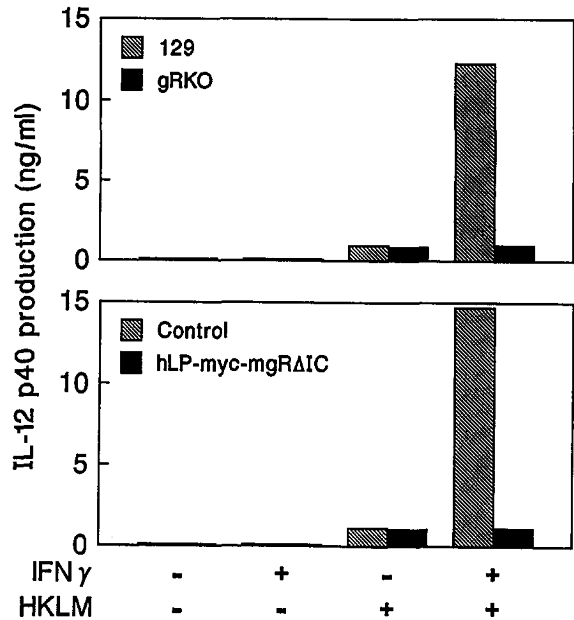

Figure 8. IFNγ Enhances Macrophage IL-12 Production Induced by Heat-Killed Listeria monocytogenes

PECs (5 × 10^5) derived from 129 Sv IFNγ receptor-deficient mice (γRKO), or hLP-myc-mgRΔIC transgenic mice were stimulated with media or rMuIFNγ (1000 IRU/ml) for 1 hr. PECs were then washed three times with media and HKLM (1 × 10^7/ml) was added. Supernatants were harvested 48 hr following HKLM addition and IL-12 production quantitated by IL-12 p40 ELISA (Tripp et al., 1994).

DO11.10 transgenic T cells were activated in vitro with OVA peptide and irradiated splenocytes derived from wild-type 129 Sv mice or 129 Sv mice lacking the IFNγ receptor (γRKO). After incubation for 7 days, the T cells were restimulated with OVA peptide and irradiated wild-type 129 Sv splenocytes. No substantial differences in IL-4 or IFNγ production were observed between T cells primed using either control 129 Sv splenocytes or gRKO splenocytes (Figure 7). Thus, the lack of APC IFNγ responsiveness produced no significant differences in the default development of TCR transgenic T cells.

In contrast, HKLM-induced Th1 development required IFNγ-responsive APCs. DO11.10 transgenic T cells activated with HKLM and control 129 Sv splenocytes developed a strong Th1 phenotype, as evidenced by the high levels of IFNγ produced upon restimulation (Figure 7). In contrast, HKLM-induced Th1 development was nearly abrogated when γRKO splenocytes were used as APCs during the primary stimulation. Neutralization of IFNγ or IL-12 inhibited HKLM-induced Th1 development as described (Hsieh et al., 1993a, 1993b). Furthermore, IL-4-induced Th2 development and anti-IL-4-induced Th1 development proceeded in a similar fashion regardless of whether the APCs were derived from 129 Sv control mice or IFNγ receptor–deficient mice. These results thus show that IFNγ-responsive APCs are required for HKLM-induced Th1 development.

IFNγ Increases IL-12 Production Induced by HKLM-Treated Macrophages

The inability of IFNγ-unresponsive APCs to support HKLM-induced Th1 development suggested that IFNγ may influence IL-12 production. This question was examined by quantitating IL-12 production by HKLM-stimulated 129 Sv PECs that were cultured in the absence or presence of IFNγ. HKLM induced production of 0.95 ng/ml of IL-12 p40 in untreated 129 Sv PEC (Figure 8, top). In contrast, HKLM induced 13 times more IL-12 p40 in IFNγ-treated PECs (12.3 ng/ml of IL-12 p40). The enhancement of HKLM-induced IL-12 production specifically required the presence of IFNγ, since IFNα had no potentiating effects (data not shown). IFNγ failed to enhance HKLM-induced IL-12 production in PEC derived from mice lacking the IFNγ receptor (γRKO, Figure 8, top). In addition, IFNγ failed to enhance HKLM-induced IL-12 production in PEC derived from the hLP-myc-mgRΔIC transgenic mice with macrophage-specific IFNγ unresponsiveness (Figure 8, bottom). Thus, we propose that IFNγ acts to augment macrophage production of IL-12 and that this effect explains the observed requirement of IFNγ for Th1 phenotype development.

Discussion

In this study, we demonstrate the generation of transgenic mice that express a dominant-negative IFNγ receptor α chain mutant under the control of tissue-specific promoters and document that these mice exhibit tissue-specific unresponsiveness to IFNγ. Using these mice, we identify the primary cellular targets of IFNγ action in the well-defined models of murine Listeriosis and the MLR. Moreover, we establish a role for IFNγ in influencing CD4+ Th subset development. These studies thus represent a novel method to define the cell- or tissue-specific actions of an endogenously produced pleiotropic cytokine in vivo.

Many cytokines such as IFNγ, IL-6, and tumor necrosis factor (TNF) induce their pleiotropic effects on a wide variety of target cells due to the nearly ubiquitous expression of their respective cellular receptors. The widespread expression of certain cytokine receptors in vivo is a major obstacle preventing the definition of the cell-specific physiologic roles of these cytokines. However, recent studies have begun to define the structure–function relationships of many cytokine receptors, including those for IFNγ, IL-6, and TNF, and this information can be used to develop novel methodologies to study the functions of specific cytokines in vivo. In the case of the IFNγ receptor, it is now known that at least two receptor subunits are required for IFNγ signaling: the IFNγ receptor α chain, which is required for ligand binding, ligand trafficking, and signal transduction, and the IFNγ receptor β chain, which is required primarily for signal transduction (Farrar and Schreiber, 1993). Moreover, the functionally important sequences within the intracellular domains of the IFNγ receptor α and β chains and the molecular interactions that effect initiation of the signal transduction cascade have been identified (Farrar et al., 1991, 1992; Cook et al., 1992; Greenlund et al., 1994, 1995).

In the course of our structure–function studies of the IFNγ receptor α chain, we observed that functionally inactive IFNγ receptor α chain intracellular domain mutants, when overexpressed in homologous cells, functioned to inhibit cellular responses to ligand in a dominant-negative manner both in vitro (Dighe et al., 1993) and in vivo (Dighe et al., 1994). The observations suggested that it might be possible to target IFNγ unresponsiveness to specific tissues by generating transgenic mice that utilize tissue-specific promoters to direct expression of the dominant-negative receptor. For this purpose, we used a cytoplasmically truncated mutant IFNγ receptor α chain, since we had previously established that it was unable to traffic efficiently through the cell and accumulated at high levels on the plasma membrane (Dighe et al., 1993). To facilitate analysis of transgene expression, we used an epitope-tagged form of the mutant polypeptide. This epitope tag did not effect the ability of the mutant receptor to bind ligand or interact with the receptor β chain.

The experiments presented herein demonstrate the validity of this approach using two different cellular targets. The human lysozyme promoter was used to direct expression of the dominant-negative IFNγ receptor to the macrophage compartment and the *lck* proximal promoter was used for T cell–specific expression. This technique represents a novel approach for disrupting cellular responsiveness to a cytokine ligand in a tissue-specific manner. One advantage of this system is that it can accommodate promoters that drive transgene expression to a low degree in cell types other than those that have been targeted. Induction of unresponsiveness to ligand occurs only in cells expressing high levels of the dominant-negative receptor mutant. In the case of the IFNγ receptor α chain dominant-negative mutant, an approximately 100:1 ratio of mutant to endogenous receptor is required for induction of IFNγ unresponsiveness (Dighe et al., 1993). Therefore, low level expression of mutant receptors in nontargeted cell types will be functionally silent. This protocol may be generalizable to a variety of different receptor systems, especially systems in which functionally active receptors are composed of at least two subunits.

The mice generated in our study have been useful in defining the physiologic cellular targets of the actions of IFNγ in three different models. First, although macrophages have been assumed to be a critical target of IFNγ action in anti-Listeria responses, this concept has never been formally tested. The results reported here unequivocally demonstrate this concept in vivo. Moreover, owing to the specific targeting of IFNγ unresponsiveness to resident macrophages, this study reveals the novel role played by these cells in initiating anti-Listeria responses.

The pattern of expression observed in the human lysozyme promoter transgenics in this study differs in some respects from that observed in another study (S. C., D. R. G., L.-P. Chung and S. G., unpublished data), in which transgenic mice were generated using the human lysozyme promoter fragment placed upstream of a bacterial chloramphenicol acetyltransferase (CAT) reporter gene. In these animals, CAT enzyme activity was detected in both thioglycollate-elicited macrophages and in cultured, but not freshly isolated, resident peritoneal macrophages, resembling the expression pattern of the endogenous murine lysozyme gene (Keshav et al., 1991). The human lysozyme promoter also directed CAT expression in peritoneal thioglycollate-elicited PMN. In the transgenic mice analyzed in the current study, the human lysozyme promoter did not direct expression of the truncated IFNγ receptor α chain to circulating PMN in normal uninfected mice. It is possible that the differences in expression patterns seen in these two studies reflect the different reporter genes and 3′ untranslated regions present in the two constructs.

Second, we have defined the role of IFNγ in regulating the MLR. Previous studies showed that proliferation of T cells during a MLR was suppressed as a result of IFNγ production in the culture (Bucy et al., 1988; Huang et al., 1993; Dalton et al., 1993). However, since MLR cultures contain multiple functionally important cell types, these earlier studies did not identify the cellular target(s) of IFNγ action. In the current report, we show that the effects of IFNγ are directed at the T cell. These results suggest that IFNγ may play an important regulatory role during CD8$^+$ T cell generation. We speculate that this action of IFNγ may serve to prevent CD8$^+$ T cell responses from causing immunopathologic reactions. Mice that specifically lack T cell IFNγ responsiveness may be useful to examine the potential autoregulatory action of IFNγ during CD8$^+$-dependent antiviral or antitumor responses.

Finally, the mice generated in the current study have been used to elucidate a role for IFNγ in CD4$^+$ Th cell phenotype development. IFNγ is necessary for efficient Th1 development (Hsieh et al., 1992, 1993a, 1993b), and was suggested to regulate the development of Th2 responses by exerting an antiproliferative action specifically on Th2 cells (Gajewski and Fitch, 1988). This concept has been supported by studies of experimental murine Leishmaniasis. Neutralization of IFNγ using either neutralizing

anti-IFNγ MAbs (Scott, 1991) or targeted disruption of the IFNγ gene (Wang et al., 1994) leads to an exacerbating disease in normally resistant mice. In contrast, administration of IFNγ in vivo fails to cure susceptible BALB/c mice (Scott, 1991). Therefore, IFNγ appears to be necessary but not sufficient for the development of Th1 cells both in vitro and in vivo. However, because of the ubiquitous cellular expression of IFNγ receptors, these studies were unable to define the cellular site of IFNγ action under physiologic circumstances.

In this report, we demonstrate that T cells made unresponsive to IFNγ by the targeted specific in vivo expression of dominant-negative IFNγ receptors maintain the capacity to differentiate towards both the Th1 and Th2 phenotypes. The in vitro development of Th1 cells induced by IL-12 or HKLM remains intact regardless of whether these T cells can respond to IFNγ. These results are supported by the demonstration that mice with T cell–targeted IFNγ unresponsiveness had no defect in their ability to develop sterilizing immunity to infection by Listeria monocytogenes, a process known to require intact Th1 responses.

Previous work demonstrated that IFNγ is necessary, but not sufficient, for HKLM-induced Th1 development (Hsieh et al., 1993b). The actions of pathogens such as Listeria for promoting Th1 development are macrophage dependent, and rely on the production of IL-12 (Hsieh et al., 1993a, 1993b). Since we had demonstrated that T cell IFNγ responsiveness was not required for HKLM-induced Th1 development, we asked whether IFNγ acted on APCs to enhance Th1 development. Herein, we show that the APC responsiveness to IFNγ is indeed required for HKLM-induced Th1 development. Using splenocytes from mice homozygous for the targeted disruption of the IFNγ receptor and control 129 Sv mice, we demonstrate that T cells activated in the presence of IFNγ-unresponsive APCs fail to develop towards the Th1 phenotype in response to HKLM. Further, we show that optimal IL-12 production by macrophages requires IFNγ. Pretreatment of wild-type macrophages with IFNγ before addition of HKLM strikingly enhanced IL-12 production. In contrast, IFNγ-unresponsive macrophages failed to augment IL-12 production when exposed to IFNγ plus HKLM. The IL-12 potentiating function of IFNγ observed in this study is similar to that recently observed by Flesch et al. (1995) using IFNγ receptor–deficient mice.

In sum, our results identify the macrophage as a critical site of the action of IFNγ in pathogen-induced Th1 development. IFNγ acts by enhancing the capacity of macrophages to produce IL-12. We find no role for IFNγ in these responses at the level of the T cell. Thus, we predict that an obligate role for IFNγ in directing Th1 phenotype would be observed only under conditions where IL-12 production is limiting.

Experimental Procedures

Reagents

Recombinant murine IFNγ was provided by Genentech, Incorporated (South San Francisco, California) and displayed a specific antiviral activity of 5.2 × 10^6 IRU/mg. Recombinant Human IFNα_{A-D} (specific activity 1.6 × 10^8 IU/mg), a nonspecies-specific form of human IFNα, was provided by Dr. G. Garotta of Hoffman-LaRoche AG (Basel, Switzerland). Hamster MAb specific for murine IFNγ (H22) was produced and purified as described (Schreiber et al., 1985). The anti-myc 9E10 MAb (Evan et al., 1985) was conjugated to biotin using the ENZO biotinylating reagent (ENZO Biochemicals) as described (Sheehan et al., 1988). The IL-4-specific MAb 11B11 (Ohara and Paul, 1985) has been previously described. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated anti-murine H-$2K^d$, anti-murine H-$2K^b$, anti-CD3, anti-CD4, anti-B220, anti-Gr-1, and anti-NK cell (derived from clone 5E6) were purchased from Pharmingen (San Diego, California). F4/80 is an antigen specifc for cells of the macrophage lineage (Austyn and Gordon, 1981). FITC-conjugated anti-murine F4/80 MAb was purchased from Caltag. Hamster MAbs specific for p35 (Red-T) and p40 (TOSH) subunits of IL-12 were provided by Drs. E. Unanue and C. Tripp (St. Louis, Missouri) (Tripp et al., 1994).

Mice

Female BALB/c ByJ and C57BL/6 mice 5–7 weeks of age were obtained from Jackson Labs (Bar Harbor, Maine). Female C3H/C57BL/6 F1 hybrid mice 3–4 weeks of age used in generation of transgenic mice were obtained from Harlan Sprague Dawley (Indianapolis, Indiana). 129 Sv and mice with a homozygous deletion of the IFNγ receptor gene (γRKO) (Huang et al., 1993) were provided by Dr. M. Aguet (Genentech, South San Francisco, California). Mice transgenic for the DO11.10 αβ TCR (Murphy et al., 1990) were selected by staining peripheral blood leukocytes with the anti-clonotype MAb KJ-126 (Marrack et al., 1983).

Plasmid Construction

Plasmids were constructed using standard procedures (Sambrook et al., 1989). We previously generated and characterized a cDNA encoding a cytoplasmically truncated form of the murine IFNγ receptor α chain, which contains only three intracellular domain amino acids (Dighe et al., 1993). The truncated murine IFNγ receptor mutant cDNA was subcloned into the pBSSKII(−) vector (Stratagene) and modified at its 5' end by insertion of nucleotides that encode 13 residues of *c-myc* (SMEQKLISEEDLN), against which the 9E10 MAb is directed (Evan et al., 1985). The myc sequences were inserted into the receptor such that they followed the signal sequence of the reeptor and first seven N-terminal amino acids to create a plasmid encoding the myc-tagged truncated murine IFNγ receptor α chain (pBS.myc–mgRΔIC). To ensure the myc tag did not interfere with the expression or the dominant-negative function of the truncated murine IFNγ receptor, the myc–mgRΔIC cDNA was subcloned into the pSFFV eukaryotic expression vector (Farrar et al., 1991) and stably transfected into murine L929 fibroblast cells to create the L929.myc–mgRΔIC cell line. This cell line expressed high levels of the myc-tagged truncated receptor as assessed by flow cytometry, using either the 9E10 MAb or the anti-murine IFNγ receptor MAb GR-20. As expected, the transfected cells were completely unresponsive to murine IFNγ but maintained responsiveness to IFNα.

The p1017 vector used for transgenic expression in the T cell compartment consists of the 3.2 kb *lck* proximal promoter, which has been demonstrated to direct the expression of cDNAs to the thymocyte compartment (Cooke et al., 1991). The transgenic construct was generated by subcloning the myc-tagged truncated murine IFNγ receptor cDNA into the BamHI site of the p1017 vector to create p1017.myc–mgRΔIC. To generate the fragment used for microinjection, p1017.myc–mgRΔIC was digested with NotI and the 6.3 kb transgenic fragment isolated and purified by standard protocols (Cooke et al., 1991).

The 3.5 kb human lysozyme promoter fragment has been demonstrated to direct expression of genes to the macrophage compartment (S. C., D. R. G., L.-P. Chung, and S. G., personal communication). The 2.1 kb BamHI–NotI fragment (containing the human growth hormone gene) of p1017 (Cooke et al., 1991) was subcloned into the BamHI and NotI sites of pBSSKII(−) to create pBS.hGH. The 3.5 kb KpnI–HincII human lysozyme promoter fragment from pUC19.hLP was then subcloned into the KpnI and HincII sites of pBS.hGH to create pBS.hLP–hGH. The myc-tagged truncated murine IFNγ receptor cDNA was then subcloned into the BamHI site of this plasmid to create

pBS.hLP–myc–mgR–hGH. To generate the microinjected fragment, pBS.hLP–myc–mgRΔIC–hGH was digested with KpnI and Not I and the 6.8 kb transgenic fragment was isolated and purified.

Generation of Transgenic Mice
Transgenic mice were generated using standard techniques (Hogan et al., 1986) by microinjection of embyros derived from a BALB/c × C57BL/6/C3H F1 breeding. Transgene-positive animals were identified by Southern blot analysis using a 0.6 kb PstI–EcoRI fragment of the human growth hormone gene as a probe. Following the generation of founder animals, subsequent generations were maintained by breeding to BALB/c mice. Founder lines with the highest relative expression of the myc-tagged truncated IFNγ receptor transgene were used for the experiments presented here. Shown are representative data using the transgenic lines lck–myc–mgRΔIC–hGH–(1) and hLP–myc–mgRΔIC–hGH–(9).

Analysis of Transgene Expression by Flow Cytometry
Two-color flow cytometry of peripheral blood, splenocytes, or PECs utilized biotinylated 9E10 and FITC-conjugated CD3, CD4. CD8, B220, Gr-1, 5E6, or F4/80-specific MAb.

Analysis of Macrophage Responsiveness to Murine IFNγ
For analysis of NO production, 1 × 10^5 PECs were seeded in a 96-well tissue culture plate (Costar, Cambridge, Massachusetts) and allowed to adhere for 2 hr. Adherent PECs were then washed three times with warmed media. PECs were stimulated with either LPS (10 μg/ml), rMuIFNγ (1000 IRU/ml), or rHuIFNα_{A-D} (1000 IRU/ml), or various combinations of the above reagents at the same concentration. After 48 hr of stimulation, supernatants were harvested and the level of nitrite determined by the Greiss reaction (Green et al., 1982). IL-12 production by PEC was performed essentially as previously described (Tripp et al., 1993). PEC (5 × 10^6) were seeded in a Costar 24-well tissue culture plate and allowed to adhere for 2 hr. Adherent PECs were washed three times with warmed media and were then stimulated with rMuIFNγ (1000 IRU/ml) or rHuIFNα_{A-D} (1000 IRU/ml) for 1 hr. PECs were then washed three times with warmed media and HKLM (1 × 10^7/ml) was added. Supernatants were harvested 48 hr following HKLM addition and IL-12 production was quantitated using a SCID splenocyte bioassay (Ishida et al., 1992) and by capture ELISA for the IL-12 p40 subunit (Tripp et al., 1994).

Listeria
Viable and heat-killed Listeria monocytogenes was provided by Dr. E. Unanue (St. Louis, Missouri). Listeria was diluted in saline and injected intraperitoneally at an infecting dose of 5 × 10^5 cfu in 0.4 ml. Bacteria in the spleens and livers of infected mice were quantitated by colony counts of homogenates in PBS with 0.05% Triton X-100 plated on BHI agar.

MLR
MLR were carried out by culturing 5 × 10^5 responder H-2^d splenocytes with irradiated (2000 rads) C57BL/6 (H-2^b) splenocytes (stimulator cells) in a 96-well plate. Proliferation was assessed by thymidine incorporation (Rogers et al., 1992). IFNγ was neutralized by addition of 100 μg/ml of anti-IFNγ MAb H22 to the culture.

Transgenic T Cell Purification
$CD4^+$ T cells were purified by FACS sorting with FITC-conjugated anti-CD4 and PE-conjugated Mel-14 (Pharmingen) as previously described (Hsieh et al., 1995). Sorted T cells were routinely >98% $CD4^+$/Mel-14^+.

T Cell Culture
T cells were cultured in Iscove's complete media as previously described (Hsieh et al., 1992). T cells (2.5 × 10^5/well) were stimulated in the presence of added cytokines or anti-cytokine antibodies in 24-well plates with 0.3 μM OVA peptide presented by 6 × 10^6 splenocyes derived from BALB/c, 129 Sv, or γRKO mice (Dalton et al., 1993). Recombinant murine IL-4 (Genzyme, Cambridge, Massachusetts) was used at 20 U/ml. IL-12 (Hoffman-LaRoche) was used at 5 U/ml. The anti-IL-4 MAb 11B11 and anti-IFNγ MAb H22 were used at 10 μg/ml. Rabbit anti-murine IL-12 polyclonal antisera (Hoffman-LaRoche) was used at a 1:500 dilution. Heat-killed Listeria monocytogenes was used at 1 × 10^7/ml. On day 7, the T cells were harvested, washed, counted, and restimulated at 2.5 × 10^5 cells/well by 6 × 10^6 splenocytes presenting 0.3 μM OVA peptide in the absence of experimental conditions to determine the resulting Th cell phenotype. Supernatants were collected at 48 hr and cytokine profiles assayed by ELISA.

Cytokine Assays
Quantitation of IL-4 and IFNγ were quantitated by ELISA as previously described (Hsieh et al., 1992). Standard curves were generated using recombinant cytokines from Genzyme.

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