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Ubiquitous Transgenic Expression of the IL-23 Subunit p19 Induces Multiorgan Inflammation, Runtting, Infertility, and Premature Death

Maria T. Wiekowski,† Michael W. Leach,† Ellen W. Evans,‡ Lee Sullivan,* Shu-Cheng Chen,* Galya Vassileva,* J. Fernando Bazan,‡ Daniel M. Gorman,‡ Robert A. Kastelein,‡ Satwant Narula,* and Sergio A. Lira†*

p19, a molecule structurally related to IL-6, G-CSF, and the p35 subunit of IL-12, is a subunit of the recently discovered cytokine IL-23. Here we show that expression of p19 in multiple tissues of transgenic mice induced a striking phenotype characterized by runtting, systemic inflammation, infertility, and death before 3 mo of age. Founder animals had infiltrates of lymphocytes and macrophages in skin, lung, liver, pancreas, and the digestive tract and were anemic. The serum concentrations of the proinflammatory cytokines TNF-α and IL-1 were elevated, and the number of circulating neutrophils was increased. In addition, ubiquitous expression of p19 resulted in constitutive expression of acute phase proteins in the liver. Surprisingly, liver-specific expression of p19 failed to reproduce any of these abnormalities, suggesting specific requirements for production of biologically active p19. Bone marrow transfer experiments showed that expression of p19 by hemopoietic cells alone recapitulated the phenotype induced by its widespread expression, pointing to hemopoietic cells as the source of biologically active p19. These findings indicate that p19 shares biological properties with IL-6, IL-12, and G-CSF and that cell-specific expression is required for its biological activity. The Journal of Immunology, 2001, 166: 7563–7570.

Cytokines comprise a large family of secreted proteins that bind to and signal through defined cell surface receptors on a wide variety of target cells. Many cytokines share structural features and functions during development, immune response, or inflammation. Searching the databases with a computationally derived profile of IL-6, Oppman et al. (1) have recently identified a novel protein and named it p19. This molecule shares homology with members of the IL-6/IL-12 family of cytokines, which includes IL-6, oncostatin M, LIF, ciliary neurotrophic factor, cardiotrophin-1, novel neurotrophin-1, G-CSF, and p35. IL-6, IL-11, oncostatin M, LIF, ciliary neurotrophic factor, cardiotrophin-1, and novel neurotrophin-1 elicit multiple overlapping biological activities by signaling through specific receptors that share gp130 as signal transducer. These biological activities include stimulation of acute phase responses, hemopoiesis, thrombopoiesis, osteoclastogenesis, neuronal differentiation and survival, and cardiac hypertrophy (reviewed in Refs. 2–4). In contrast, G-CSF signals independently of gp130 and induces neutrophilic granulocytosis in transgenic mice (5). The last member of this family is p35, a molecule that is itself apparently devoid of biological activity. However, when p35 associates with p40, a soluble member of the cytokine receptor superfamily, it forms a powerful cytokine, IL-12, that induces Th1 differentiation and the release of IFN-γ from Th1 and NK cells (reviewed in Ref. 6).

To investigate the biological properties of p19, we generated transgenic animals expressing it ubiquitously or in a tissue-specific fashion. Phenotypic analysis of these transgenic animals indicates that p19 has biological properties related to IL-6, G-CSF, and IL-12 and that its expression from hemopoietic cells is a prerequisite for its biological activity.

Materials and Methods

Transgene construction and microinjection

A 0.5-kb cDNA encoding p19 was cloned as an EcoRI fragment into an expression vector containing the human CMV enhancer/chicken β-actin promoter and the rabbit β-globin polyadenylation signal (7). For liver specific expression, the cDNA for p19 was cloned into an expression vector containing the promoter for human α2-antitrypsin (HAT,2 bp 1–1976, GenBank accession number K02212) and 145 bp of a transcriptional enhancer from the human α2-microglobulin/bikunin gene (bp 2163–2308) (Fig. 5A). Transgenes were separated from vector sequences by zonal sucrose gradient centrifugation as described (8). Fractions containing the transgenes were pooled, microcentrifuged through Microcon-100 filters (Amicon, Bedford, MA), and washed five times with microinjection buffer (5 mM Tris-HCl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA).

Generation of transgenic mice

DNA containing the transgene was resuspended in microinjection buffer to a final concentration of 1–5 ng/ml and microinjected into mouse eggs (C57BL/6 × DBA/2 (B6D2) F2, The Jackson Laboratory, Bar Harbor, ME). The surviving eggs were transplanted into oviducts of ICR (Sprague Dawley) foster mothers, according to published procedures (9). By 10 days of life, a piece of tail from the resulting animals was clipped for DNA analysis. Founders carrying the p19 gene under control of the human CMV enhancer/chicken β-actin promoter (pC19) were identified by PCR amplification of a segment of the transgene using primers 5'-GCCCTCCTCTGACCAAGGGAT-3' and 5'-CCAGCCCCACATTGCTGATAAGGACGTGAC-3'. As an internal control for the amplification reaction, primers for the endogenous low density lipoprotein gene were used: 5'-ACCCAA

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2 Abbreviations used in this paper: HAT, human α2-antitrypsin; SAA, serum amyloid A; IGF-1, insulin-like growth factor-1; H&E, hematoxylin and eosin; EMH, extramedullary hemopoiesis; AGP-1, α2-acid glycoprotein.
IGF-1. Serum samples were acid-ethanol extracted according to instructions using a RIA for human IGF-1 that also recognizes murine IGF-1. Assays were performed using ELISA kits for murine IL-2 (sensitivity, 0.2 pg/ml), murine IL-12 p40 (sensitivity, 5.1 pg/ml), and murine IL-1 (sensitivity, 6 pg/ml) were purchased from Biosource International (Camarillo, CA). ELISA kits for murine IL-1 (sensitivity, <8 pg/ml) and murine serum amyloid A (SAA; sensitivity, <0.23 µg/ml) were purchased from Biosource International (Camarillo, CA). ELISA kits for murine IL-1α (sensitivity, <6 pg/ml) were purchased from Endogen (Cambridge, MA). Assays were performed according to the manufacturer’s instructions.

Levels of insulin-like growth factor-1 (IGF-1) in mouse serum were determined using a RIA for human IGF-1 that also recognizes murine IGF-1. Serum samples were acid-ethanol extracted according to instructions provided by the manufacturer (Nichols Institute, San Juan Capistrano, CA).

Histology

Mouse tissues were fixed by immersion in 10% phosphate-buffered formalin. Formalin-fixed tissues were routinely processed at 5 µm and were stained with hematoxylin and eosin (H&E). The number of megakaryocytes in transgenic and nontransgenic spleens was determined by counting the number of megakaryocytes in at least five different optical fields for each spleen.

Hematology

Blood samples were collected from the infraorbital sinus into sterile, evacuated tubes with added EDTA (Vacutainer Systems, Becton Dickinson, Rutherford, NJ). Hematological values were determined with an automated cell counter (Cell-Dyn 3500; Abbott Laboratories, Abbott Park, IL). Platelet counts were performed manually when the instrument was unable to provide accurate platelet counts due to excessive clumping or excessively large platelets. Blood smears were stained with Wright-Giemsa stain (Hema-Tek Stain Pack; Bayer, Elkhart, IN) using an automated stainer (Hema-Tek 2000; Bayer). Bone marrow smear were obtained from the sternum, fixed in methanol, and stained with Wright-Giemsa stains using a Midas II stainer (EM Diagnostics Systems, Gibbstown, NJ).

Bone marrow transfers

Bone marrow cells were flushed from femurs of transgenic and control mice under sterile conditions. Single-cell suspensions (10 million cells) were injected into the tail veins of lethally irradiated (1100 rad for 5 min) B6D2F1, mice (6–8 wk old).

Results

Animals expressing p19 in multiple tissues are runted, are infertile, and die prematurely.

To express p19 in transgenic animals, we initially used the Cp19 transgene (Fig. 1A). The enhancer/promoter cassette used directs expression of the transgene to virtually all organs (10). Twenty-four founder mice (referred to as Cp19) were generated; of these, 10 died during the first 3 wk of life before data could be collected. Of the remaining 14 founders, 3 grew normally but 11 were small and failed to thrive (Fig. 1B). Reduced body weight was evident in the first 10 days of life (average weight for Cp19, 5.1 ± 1.9 g, n = 8; controls, 8.6 ± 1.4 g, n = 23; p = 0.0007). Four of the 11 small founders (founders 25, 36, 51, 88) were severely runted, appeared moribund, and were sacrificed before weaning. To determine whether there was a correlation between the appearance of this phenotype and transgene expression, we performed Northern blot analysis of skeletal muscle RNA (Fig. 1C). Transgene expression was detected in all 11 Cp19 founders showing stunted growth, whereas no expression was detected in controls or in the 3 remaining, normally growing founders. All seven expressing Cp19 founders that survived beyond weaning age were clearly affected by the expression of the transgene; all had impaired growth, a swollen abdomen, and ruffled fur. These animals were infertile, and none survived beyond 90 days of age. Thus, ubiquitous expression of p19 resulted in stunted growth, infertility, and death.

FIGURE 1. Impaired growth of Cp19-transgenic animals. A, Schematic representation of the Cp19 transgene with the human CMV enhancer (hCMV), chicken β-actin promoter (c β-actin), the murine p19 cDNA, and the rabbit β-globin polycadenylation signal (r globin(A)). B, Small body weight and stunted growth of Cp19-transgenic mice. Data were collected from 11 expressing Cp19 founders and from 69 nontransgenic littermates (−). Error bars, SD. C, Northern blot analysis of skeletal muscle RNA from transgenic founders and nontransgenic littermates (−) hybridized with p19 cDNA. The arrow indicates the major transcription product of the transgene.
Cp19-transgenic mice develop inflammation in multiple tissues and increased extramedullary hemopoiesis (EMH)

Tissues were collected from 10 of the 11 Cp19 founders described in Fig. 1, routinely fixed, processed to slides, and examined microscopically. Tissues collected from age- and gender-matched nontransgenic littermates were used as reference. Minimal to moderate inflammation (Fig. 2), sometimes associated with epithelial hyperplasia, was detected in all 10 Cp19 animals (age 15–85 days) in one or multiple sites, including the esophagus, stomach, small intestine, large intestine, skin, lungs, liver, and pancreas. In general, the inflammatory infiltrates consisted of lymphocytes and macrophages, sometimes accompanied by varying numbers of neutrophils. In the esophagus (Fig. 2, A and B), stomach, and intestines (Fig. 2C), the infiltrates were minimal to moderate, multifocal, and primarily localized in the epithelium, lamina propria, and submucosa and were often associated with epithelial hyperplasia. The hyperplasia resulted in lengthening of intestinal glands and shortening or loss of villi in the small intestine (Fig. 2C). Inflammation in the skin was multifocal, involved the epidermis and dermis, and was sometimes associated with acanthosis and/or ulceration (Fig. 2D). In the lungs (Fig. 2, E and F), findings consisted of peribronchial/perivascular mononuclear cell infiltrates; neutrophils were not a prominent component of the pulmonary inflammation. In addition to the inflammation, the epithelium lining bronchi and bronchioles often had minimal to mild hyperplasia, sometimes with eosinophilic intracytoplasmic material. Inflammation in the liver consisted of minimal to mild periportal inflammatory infiltrates (data not shown). Pancreatic inflammatory infiltrates were minimal and consisted primarily of lymphocytes (data not shown).

In addition to inflammation, a few additional changes were noted. Minimal to mild EMH was observed in the liver and in medullary cords of lymph nodes, and mild to marked EMH was observed in the spleen of all 10 Cp19 founders examined (Fig. 2H). The splenic capsule was sometimes thickened (Fig. 2H). The cortex of lymph nodes was sometimes sparsely cellular and lacked secondary follicles. Microscopic changes were not observed in the skeletal muscle, heart, kidney, and brain, despite high levels of transgene expression in these organs (Fig. 1 and data not shown).

Cp19 mice are anemic and have increased numbers of neutrophils in the peripheral blood

The effect of p19 on peripheral blood was analyzed in three independent founders and compared with data from three nontransgenic littermates. All Cp19 mice examined had mild to moderate microcytic hypochromic anemia. The mean hematocrit values in the Cp19 animals were 37–70% lower than that of controls (Cp19, 23.9 ± 8.3%; controls, 47 ± 2.4%, p = 0.029). Microscopic analysis of blood smears revealed erythrocytes of abnormal shape (poikilocytosis) and/or fragments of erythrocytes (schistocytosis). Slight to moderate polychromasia and variation in the size of erythrocytes (anisocytosis) suggested the presence of regeneration. Microcytosis (small average RBC size) and poikilocytosis and/or fragments of erythrocytes (schistocytosis). Slight to moderate polychromasia and variation in the size of erythrocytes (anisocytosis) suggested the presence of regeneration. The presence of microcytosis (small average RBC size) and hypochromia (diminished erythrocyte hemoglobin concentration) was assessed by the granulocytic/erythroid ratio) was increased in the bone marrow relative to the erythroid component. Interestingly, the number of circulating platelets in Cp19 animals was slightly increased over the number found in control littermates (Cp19, 599; control, 821). Although these differences were not statistically significant, the presence of excessive clumping and platelets of unusual morphology in Cp19 blood smears suggested increased platelet production. To determine whether the increased number of platelets in Cp19 founders was caused by accelerated platelet production by megakaryocytes or by an increase in the number of megakaryocytes, we examined the spleen and bone marrow of Cp19 mice microscopically. In these tissues, megakaryocytes were enlarged, but their numbers were not increased (Cp19 (n = 11), 7566 TRANSGENIC EXPRESSION OF THE IL-23 SUBUNIT p19

Table I. Analysis of leukocyte populations in blood of Cp19 and control animals

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Controls</th>
<th>Cp19 Founders</th>
</tr>
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<tbody>
<tr>
<td>Neutrophils</td>
<td>0.60 ± 0.14</td>
<td>4.71 ± 3.24*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>4.91 ± 1.40</td>
<td>2.79 ± 1.47*</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.29 ± 0.24</td>
<td>0.69 ± 0.6</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.11 ± 0.02</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.002 ± 0.005</td>
<td>0.09 ± 0.11</td>
</tr>
</tbody>
</table>

*Values are expressed as 10^6 cells/µl. Results for Cp19 founders (n = 3) and nontransgenic controls (n = 3) are expressed as mean ± SD, *p < 0.05.

FIGURE 3. Cytokine expression in Cp19 founders. Concentrations of TNF-α (A), IFN-γ (B) and p40 (C) were determined in serum of Cp19 founders and wild-type (wt) animals by ELISAs specific for the selected cytokine. Bars in A represent the average TNF-α concentrations found in wild-type and Cp19 animals (p = 0.008).

Acute phase protein genes are chronically expressed in the livers of Cp19 animals

During inflammation, genes encoding acute phase proteins are up-regulated in the liver. Because Cp19 mice exhibit a phenotype characterized by systemic inflammation, we investigated whether acute inflammation by LPS (M. T. Wiekowski, unpublished observation).

In addition, concentrations of IFN-γ were increased in 3 of 13 founders tested (Fig. 3). Another cytokine involved in an inflammatory response is IL-12, a heterodimeric cytokine formed by association of the subunits p40 and p35 (6, 11, 12). Therefore, we determined the concentration of p40 in serum of eight Cp19 founders. With the exception of a single Cp19 founder, the levels of p40 did not differ from controls (Fig. 3C). This indicated that overexpression of p19 in Cp19 animals did not elevate the concentrations of circulating p40.

Surprisingly, IL-6 protein could not be detected in the peripheral blood of Cp19 animals, despite the high circulating concentrations of TNF-α and IL-1, cytokines that directly induce IL-6 production (13). This was especially surprising considering the fact that IL-6 is expressed during systemic inflammation (14, 15).

Acute phase protein genes are chronically expressed in the livers of Cp19 animals

During inflammation, genes encoding acute phase proteins are up-regulated in the liver. Because Cp19 mice exhibit a phenotype characterized by systemic inflammation, we investigated whether...
the expression of acute phase genes was altered in their livers. As shown in Fig. 4A, the acute phase genes AGP-1, haptoglobin, and hemopexin were highly expressed in the liver of all four transgenic founders tested, whereas no expression of these genes was detected in nontransgenic livers. To test whether the concentration of acute phase proteins was also increased in the circulation, blood from C19 founders was tested for the presence of SAA. The average level of circulating SAA (248 ± 159 μg/ml, n = 10) was significantly increased over the levels found in controls (8 ± 5.1 μg/ml, n = 8, p = 0.05) (Fig. 4B). These results indicate that acute phase liver genes are chronically expressed in C19 animals.

**Impaired growth and infertility of C19 animals are associated with decreased circulating concentrations of IGF-1**

Growth impairment caused by chronic inflammatory conditions (16, 17) or by overexpression of cytokines in transgenic animals (18) is associated with a decrease in the circulating levels of IGF-1, a hormone that regulates postnatal growth (19) and influences fertility (20). To test whether the impaired growth of C19 animals was associated with reduced levels of IGF-1, serum samples of transgenic animals were assayed for IGF-1. In all founders tested, the amount of IGF-1 in the serum was reduced to 12–14% of the concentrations found in nontransgenic, age-matched littermates (Table II). This suggests that overexpression of p19 may directly or indirectly reduce IGF-1 concentrations, resulting in impaired growth and infertility of transgenic animals (20).

**Liver-specific expression of p19 in transgenic mice does not result in a detectable phenotype**

The infertility and premature death seen in C19 transgenic animals precluded further analysis of the biological function of p19. Therefore, we attempted to generate another transgenic model expressing p19 using a tissue-specific promoter. To this end, we made transgenic animals carrying the p19 gene under the control of the liver-specific human α1-antitrypsin promoter (Hp19 animals). We generated eight founders, from which seven transgenic lines were derived. Transgene expression was detected by Northern blot analysis in all mice analyzed, but not in controls (Fig. 5B).

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Expression of acute phase liver genes in C19 animals. **A,** Total RNA (20 μg) extracted from the liver of C19-transgenic animals (founders 89, 78, and 95; 35–85 days old) and nontransgenic littermates (−) was probed with radiolabeled PCR fragments for the murine genes AGP-1, hemopexin, and haptoglobin. Equal loading of RNA for each sample was verified by reprobing with a radiolabeled PCR fragment for the murine β-actin gene after the blot had been stripped. **B,** Levels of the acute phase protein SAA in serum of 10 C19 founders (age 16 days–3 mo) and nontransgenic animals (wild-type (wt), n = 8; p = 0.05). Bar represents the average SAA level in C19 animals.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Transgenic expression of p19 in liver does not lead to inflammation. **A,** Schematic representation of the Hp19 transgene with HAT, the SV40 polyadenylation signal (SV40 (A)) and a transcriptional enhancer from the human α1-microglobulin/bikunin gene (AMBP). **B,** Northern blot analysis of liver RNA from Hp19-transgenic lines and nontransgenic littermates (−) hybridized with cDNA for p19. For comparison, skeletal muscle RNA from a C19 founder (+) was included. **C** and **D,** H&E-stained liver from control (C) and transgenic (D) Hp19 animal (magnification, ×75). Normal appearance of liver with central vein (arrows) and several portal areas.

<table>
<thead>
<tr>
<th>Founder</th>
<th>Age (days)</th>
<th>IGF-1 (ng/ml)</th>
<th>% of Control</th>
</tr>
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<tbody>
<tr>
<td>36</td>
<td>15</td>
<td>25</td>
<td>12.1</td>
</tr>
<tr>
<td>89</td>
<td>31</td>
<td>28</td>
<td>12.2</td>
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<tr>
<td>60</td>
<td>54</td>
<td>59</td>
<td>14.5</td>
</tr>
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*Absolute levels of IGF-1 in serum of transgenic mice were determined and compared with those of age-matched control littermates (n = 6).*
The two highest expressing lines were expanded, and a detailed analysis of growth patterns, blood parameters, expression of acute phase genes, and histology of several organs was performed. Surprisingly, these transgenic animals grew normally and had no signs of hepatic (Fig. 5, C and D) or systemic inflammation (data not shown). In addition, we were unable to detect any changes in the peripheral blood cell composition or in the expression of acute phase genes (data not shown). These results suggest that there are requirements for the biological activity of p19 that are not satisfied by its production in liver cells.

Transplantation of transgenic hemopoietic cells into wild-type mice results in the development of multiorgan inflammation

The absence of a phenotype in mice overexpressing p19 in liver and the observation by Oppmann et al. (1) that p19 is expressed by a subset of hemopoietic cells led us to examine whether overexpression of p19 by hemopoietic cells would be sufficient to induce a phenotype similar to the one described for Cp19 animals. We observed previously that the CMV/β-actin promoter targets expression of transgenes to a variety of cells within the immune system, including T and B lymphocytes and dendritic cells (21). Thus, we generated a new set of Cp19 founders and transferred their bone marrow (Fig. 6A) into lethally irradiated wild-type recipient mice. The health of these Cp19 bone marrow recipients deteriorated within 35–66 days posttransfer, as judged by the appearance of ruffled fur and inflamed skin around the snout and ventral neck. In contrast, recipients of wild-type bone marrow did not develop an obvious phenotype. Analysis of the bone marrow recipients showed p19 expression in the bone marrow (data not shown) and spleens of Cp19, but not control, bone marrow recipients (Fig. 6A). In Cp19 bone marrow recipients, the acute phase liver genes hemopexin and AGP-1 were highly expressed (not shown), and the serum levels of SAA were elevated (Fig. 6B), but again no IL-6 could be detected in circulation (data not shown). As in Cp19 donor animals, skin, lung, liver, and the gastrointestinal tract were inflamed in recipients of Cp19 bone marrow, but not in wild-type bone marrow recipients. Perivascular/peribronchial infiltrates of lymphocytes and macrophages and slight epithelial hyperplasia were observed in the airways of Cp19 bone marrow recipients (Fig. 6, C and D). These results indicate that p19 produced by bone marrow cells is biologically active and can induce a phenotype of systemic inflammation similar to that obtained by ubiquitous expression of p19.

Discussion

To characterize the biological activity of p19, we expressed it in transgenic mice. Widespread expression of p19 led to a phenotype of systemic inflammation, impaired growth, and premature death. Tissue-specific expression of p19 yielded two outcomes. Animals expressing p19 in liver were fertile, had a normal life span, and did not present signs of systemic or localized inflammation. In contrast, animals expressing p19 in bone marrow-derived cells presented a phenotype of systemic inflammation that was similar to the one observed when p19 was expressed ubiquitously. These results indicate that hemopoietic expression of p19 is necessary and sufficient to induce systemic inflammation, impaired growth, and premature death, phenotypes that are strikingly reminiscent of those observed in mice overexpressing cytokines structurally related to p19 (Table III). For example, runting or failure to thrive has been reported for transgenic animals expressing IL-6 in skin (22), astrocytes (23), neurons (18), and in the airway epithelium (24); and for transgenic animals expressing oncostatin M in the skin (25). Similar to observations in mice overexpressing IL-6, Cp19 mice had reduced levels of IGF-1, which may have contributed to their failure to thrive and their reduced fertility (18). It is unclear whether the reduced levels of IGF-1 were directly caused by p19 or caused by other factors secreted during systemic inflammation.

Other striking findings in Cp19 animals were the inflammation affecting multiple organs and the high levels of circulating TNF-α.
We suggest that the absence of a phenotype in the animals expressing p19 is the function of the simultaneous production of p40, and p40 up-regulates IL-6 expression. This in vivo result differs from the in vitro results obtained by Oppmann et al. (1), who reported induction of IFN-γ by T-cells after IL-23 (p19/p40) treatment. Unfortunately, this discrepancy could not be satisfactorily resolved because of the short life span of the transgenic mice, which precluded analysis of specific immune responses. Thus, the function of p19 on expression of IFN-γ in vivo and its functional relationship to IL-12 remains to be determined in mice deficient for p19 and in transgenic mice expressing p19 conditionally.

Our results show that overexpression of p19 in vivo induces a phenotype resembling that observed on overexpression of the structurally related cytokines IL-12, IL-6, and G-CSF. Further studies will be necessary to understand how expression of p19 leads to these phenotypes and the molecular nature of the receptor(s) mediating these responses.

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