REVIEW ARTICLE

Interleukin-12: A Cytokine Produced by Antigen-Presenting Cells With Immunoregulatory Functions in the Generation of T-Helper Cells Type 1 and Cytotoxic Lymphocytes

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PHAGOCYTIC CELLS and natural killer (NK) cells are among the effector cell types of innate resistance that represent a first line of defense against infections or foreign pathogens. During the early inflammatory response to an infection, regulatory interactions between these cell types take place, mostly mediated by cytokines that regulate activation and migration of phagocytic cells and NK cells. One of these mechanisms, often referred to as T-cell–independent macrophage activation, is observed in T-cell–deficient SCID mice.[1] When these animals are infected with *Listeria monocytogenes* or other bacteria, a rapid production of interferon-γ (IFN-γ) is observed that acts as a potent activator for phagocytic cells, increasing their bacteriocidal activity as well as their ability to produce cytokines. The major factor produced by the infected phagocytic cells and responsible for induction of production of IFN-γ is interleukin-12 (IL-12), a heterodimeric cytokine that is a potent inducer of cytokine production, particularly IFN-γ, in T and NK cells, a growth factor for preactivated T and NK cells, and an enhancer of cytotoxic activity in both $CD8^+$ T cells and NK cells.[2-6] IL-12 is produced by phagocytic cells, B cells, and other antigen-presenting cell (APC) types.[7] In addition to its role in the phagocytic cell activation mechanism early in the inflammatory response to infections, APC-produced IL-12 has an obligatory role for the generation of T-helper type 1 (Th1) cells (producing IL-2 and IFN-γ)[8-10] and for optimal differentiation of cytotoxic T lymphocytes (CTL).[11] The early decision towards Th1 and Th2 cells in the immune response is dependent on the balance between IL-12, which favors Th1 responses, and IL-4, which favors Th2 responses.[10]

IL-12, A CYTOKINE WITH A UNIQUE HETERODIMERIC STRUCTURE

NK cell stimulatory factor (NKSF) or IL-12 was identified as a factor secreted by human Epstein-Barr virus (EBV)-transformed B cell lines and mediating several biologic activities on human T and NK cells, including induction of IFN-γ production, enhancement of cell-mediated cytotoxicity, and comitogenic effects on resting T cells.[2] NKSF/IL-12 was purified to homogeneity from the conditioned medium of the phorbol diester-stimulated RPMI-8866 EBV-transformed cell line and, unlike other cytokines, was shown to have a heterodimeric structure.[2] The genes encoding the two polypeptide chains of NKSF/IL-12 were cloned on the basis of partial amino acid sequences obtained from the purified proteins and biologically active recombinant NKSF/IL-12 was produced in eukaryotic cells transfected with the cDNA for both NKSF/IL-12 chains.[4] A cytotoxic lymphocyte maturation factor (CLMF) was also identified in the conditioned medium of an EBV-transformed B-cell line (NC37 line) on the basis of its ability to synergize with IL-2 in inducing the generation of lymphokine activated killer (LAK) cells.[3] Purification and cloning of the genes encoding CLMF showed that NKSF and CLMF are the same cytokine[4-6] and the unifying term of IL-12[5] is now widely accepted.

IL-12 is a heterodimer of 70 kD (p70) formed by two covalently linked glycosylated chains of approximately 40 kD (p40) and 35 kD (p35).[2] The p35 cDNA sequence encodes a 219 amino acid polypeptide[4,5] corresponding to a mature protein with a calculated molecular weight (M_r) of 27,500 containing 7 cysteine residues and 3 possible N-glycosylation sites. The p40 cDNA sequence encodes a 328 amino acid polypeptide with a 22 amino acid hydrophobic signal sequence, corresponding to a mature protein of calculated M_r of 34,700 with 10 cysteine residues, 4 possible N-linked glycosylation sites, and 1 consensus heparin binding site.[4,5] Transient transfection of COS cells or stable transfection of CHO cells with either p40 or p35 cDNA induces secretion of the respective IL-12 chains, but cotransfection with both cDNA in the same cells is required for secretion of the biologically active form of IL-12, the p70 heterodimer.[4,5]

The gene encoding the p40 chain has been mapped to human chromosome 5q31-q33, a region encoding several cytokine receptors and cytokines.[12] The p40 gene is closely linked to the monocyte colony-stimulating factor (M-CSF) receptor and, interestingly, is in the same chromosomal region encoding IL-4, the cytokine acting antagonistically to IL-12 in determining the Th1/Th2 dichotomy. The gene encoding the p35 chain is a completely unrelated gene that has been mapped to human chromosome 3p12-3q13.2.[12]

The two genes encoding the murine p40 and p35 chains were cloned by cross-hybridization with human cDNA clones and found to have 70% and 60% sequence homology, respectively, with the corresponding human genes.[6] Human IL-12 is not active on mouse cells, but murine IL-12 is active on both murine and human lymphocytes.[6] Interspecies heterodimers are active on both human and murine lymphocytes when the p35 chain is of murine origin, but are active only on human cells when the p35 chain is of human origin, regardless of the origin of the p40 chain, suggesting that the p35 chain has a determining effect on the species specificity of the heterodimer.[6]

The primary amino acid sequence of the IL-12 p35 chain indicates an α-helix–rich structure, similar to most cytokines. A comparison of the p35 amino acid sequence with

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those of IL-6 and granulocyte colony-stimulating factor (G-CSF) showed that many of the amino acid positions that are conserved between these two cytokines are also conserved in IL-12 p35.[13] The p40 sequence is not homologous to any other known cytokine, but appears related to the hematopoietic cytokine receptor family, which is characterized by 4 cysteine residues and 1 tryptophan residue in conserved positions in the extracellular portion of these receptors and by a WSXWS motif.[14] The p40 sequence has a particularly significant sequence homology with the extracellular portions of two members of this family, the IL-6 receptor (IL-6R) and the ciliary neurotrophic factor (CNTF) receptor.[6,14] IL-6R, CNTF-R, and IL-12 p40 have an N-terminal Ig-like domain followed by the sequence characteristics of the receptor family; the WSXWS motif in the p40 sequence is modified by the insertion of an alanine (WSEWAS) and is near the C-terminus in the p40 molecule. Most cytokine receptors can also be released by cells in soluble forms that usually terminate immediately after the WSXWS motif and are produced either by proteolytic digestion of the transmembrane form or by alternative splicing of the message that eliminates the exons encoding the transmembrane and C-terminal cytoplasmic portions.[15] The binding of IL-6 to the IL-6R is a low-affinity interaction, but upon association with a dimer of gp130, which is a nonligand binding signal-transducing transmembrane component of several cytokine receptors, a high-affinity complex is produced and signal transduction through the gp130 chain is triggered.[16] The soluble form of the IL-6R, unlike most other soluble receptors, does not compete for binding of IL-6 to the cellular receptors; rather, the soluble IL-6R binds in solution with IL-6 and this complex can bind to gp130 on the cell surface, mediating signal transduction and IL-6 biologic activities.[15,16] The CNTF-R is composed of three chains: gp130, shared with the IL-6R; the leukemia inhibitory factor (LIF) receptor β chain; and a CNTF-R α chain. Similar to the IL-6R, the CNTF-Rα chain is released as a soluble form that binds to CNTF and the complex mediates signal transduction on cell types expressing gp130 and LIF-Rβ chain.[17] It is therefore possible to hypothesize that heterodimeric IL-12 is evolutionarily derived from a primordial cytokine (p35 equivalent) that, similar to IL-6 or CNTF, had a multichain receptor. The transmembrane form of one chain of the receptor (p40 equivalent) was lost, but an efficient association of the primitive cytokine and the primitive soluble receptor was maintained through a covalent linkage between the two chains. This heterodimeric complex, similar to the soluble IL-6R/IL-6 or CNTF-Rα/CNTF complexes, would still be able to bind with high affinity to the one or more remaining transmembrane chains of the receptor, inducing signal transduction and biologic activity. If this hypothesis on the evolutionary origin of IL-12 is correct, one would assume that, like IL-6 and IL-6R or CNTF and CNTF-Rα, the p35 and p40 chains of IL-12 have maintained a ligand receptor-like affinity for each other, even in the absence of the covalent linkage between the two chains. Indeed, when monomeric recombinant IL-12 p40 and p35 are added together to responsive cells, all the biologic activities of IL-12 can be shown (Rengaraju et al, unpublished results), although at concentrations from 2 to 5 orders of magnitude higher than those effective for the covalently linked heterodimer.

Analysis of steady-state binding data of IL-12 by Scatchard analysis identified a single binding site on PHA-activated lymphoblasts with an equilibrium dissociation constant of 100 to 600 pmol/L and 1,000 to 9,000 sites/cell.[18] Cross-linking and immunoprecipitation experiments with anti–IL-12 antibodies identified a single protein of approximately 110 kD.[18] The cellular distribution of IL-12R was analyzed by identifying cell-bound IL-12 with fluorescent anti–IL-12 antibodies. The presence of the receptor was detected on activated T or NK cells, but neither on B cells nor on resting T or NK cells.[19] The available data on IL-12R only explain in part the cellular specificity and biologic activities of IL-12. Some of the biologic activities, eg, the proliferative effect or the induction of IFN-γ, can be shown at concentrations less than 1 pmol/L.[2,20,21] If the dissociation constant of the identified receptor is more than 100 pmol/L, it is necessary to assume either that signal transduction takes place at minimal occupancy of the receptors or that additional unidentified chains of the receptor are required for determining a low number of high-affinity binding sites. The other discrepancy with the functional data is that receptors cannot be identified on resting T and NK cells, whereas certain biologic activities of IL-12, eg, enhancement of cell-mediated cytotoxicity or induction of IFN-γ production, are mediated with a similar dose-response curve on both resting and activated NK and T cells.[21] It is not clear yet whether the p40 chain, the p35 chain, or both are directly involved in binding to the receptor. The observations that anti-p40 antibodies[22] and site-specific chemical modifications of a tryptophan residue on the p40 chain[23] block IL-12 binding to the receptor and that p35 is responsible for determining the species specificity of IL-12[6] suggest, however, that both chains may play a role.

Recent detailed analysis of IL-12 binding sites on phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) has identified three binding sites with apparent affinities of 5 to 20 pmol/L, 50 to 200 pmol/L, and 2 to 6 nmol/L.[24] These data suggest the existence of different forms or different chains in the IL-12R, with the high-affinity form of the receptor possibly responsible for most of the biologic activities of IL-12. By screening of an expression cDNA library with an antibody able to precipitate the complex of IL-12R/^{125}I-IL-12 from cell lysates, a component of the human IL-12R was cloned.[24] This subunit is a 662 amino acid type I transmembrane protein with an extracellular domain of 516 and a cytoplasmic domain of 91 amino acids.[24] The subunit is a member of the hematopoietin receptor superfamily and most closely homologous, both in the extracellular and intracellular domain, to gp130, to the LIF-Rβ, and to the G-CSF-R.[24] Cells transfected with this chain bind IL-12 with an affinity of 2 to 5 nmol/L; covalently linked dimers or oligomers are responsible for the binding, unlike gp130, which is dimerized only after IL-6 binding.[24] A polyclonal antibody raised against this receptor inhibits IL-12–induced proliferation, suggesting that this chain is involved in mediating at least one of the functions of IL-12; however, the low-binding affinity of this receptor chain suggests that other chains may be required for formation of high-affinity receptors.[24]

The signal transduction events after interaction of IL-12 with its receptor are largely unknown. Within a few minutes of exposure to IL-12, phosphorylation of several proteins is observed in the treated cells.[25,26] Tyrosine kinase inhibitors suppress IL-12–mediated induction of CD69 expression and enhancement of cytotoxic activity in human NK cells.[27] A different phosphorylation pattern is apparently observed in NK cells and T cells. $p56^{lck}$ was reported to be primarily phosphorylated in resting and activated NK cells,[25] whereas, in activated T cells, phosphorylation of mitogen-activated protein (MAP) kinase is observed.[26] It has been suggested that these different signaling pathways may reflect the different activities of IL-12 on proliferation of T and NK cells. IL-12 increases IL-2–induced proliferation of activated T cells, but mostly inhibits IL-2–induced proliferation of NK cells.[20,28]

Production of IL-12

The requirement for expression of two different genes to produce the biologically active IL-12 heterodimer renders the genetic control of the production of this cytokine particularly complex. Originally, IL-12 was discovered and purified from the conditioned medium of EBV-transformed human cell lines and phorbol diesters were found to increase severalfold the production of IL-12.[2,3] A screening of many different cell lines showed that the large majority of EBV-transformed B-cell lines produced IL-12 either constitutively or after phorbol diester stimulation.[7] However, significant IL-12 production was not observed in cell lines of different origin, including Burkitt-lymphoma-derived lines, regardless of whether they expressed EBV.[7] All the cell lines producing the biologically active p70 heterodimer also produced a large excess, usually from 10:1 to 50:1, of the free p40 heavy chain.[3,7] The production of the p40 chain, either free or associated in the heterodimer, can be easily measured both in the human and in the mouse system by a two-determinant capture radioimmunoassay.[7] The production of the biologically active p70 heterodimer is more difficult to evaluate and relies on two different methods: (1) a radioimmunoassay using the 20C2 monoclonal antibody (MoAb),[22] which is relatively specific for the p70 dimer, but which also cross-reacts with the p40 chain, necessitating a correction factor that affects the reliability of the assay[7]; and (2) an antibody capture biologic assay, based on the capture of IL-12 on plates coated with a nonneutralizing anti-p40 MoAb, followed by the addition of indicator cells (NK cells or PHA blasts in humans, spleen cells in mice) and evaluation of either IL-12–induced proliferation[29] or IFN-γ production.[30] The physiologic significance of the production of the free p40 chain is not clear. It was reported that in the mouse recombinant free p40 inhibits the biologic activity of the p70 heterodimer, suggesting that p40 may act as a physiologic antagonist of IL-12.[31] However, we have observed that the inhibitory activity of recombinant murine p40 resides primarily in p40 homodimers formed by recombinant protein and it is not clear yet whether natural p40 also has antagonistic activity; furthermore, little if any antagonistic activity was demonstrable in human recombinant p40 (Wolf et al, unpublished observation and Gately, personal communication). The secretion of p40 homodimers, which may compete for IL-12 binding to its receptors, were, however, also described in cells transfected with human p40 cDNA.[32]

When expression of mRNA for the p40 and p35 chains was analyzed in cell lines, the expression of p40 transcripts correlated with the ability of the cell lines to produce IL-12, whereas the p35 transcripts were ubiquitously expressed in almost all cell lines, of various hematopoietic and nonhematopoietic origin.[7,33] Although sensitive radioimmunoassays able to detect the p35 chains are available, secretion of p35 free chain from either cell lines or normal cells has never been demonstrated, an indication possibly of the difficulty of secretion of p35 if not in association with p40, as suggested also by a poor production of p35 in cell lines transfected with the p35 cDNA.[7]

It is difficult at the present time to understand the apparently poor regulation of the expression of the two IL-12 genes, with overexpression of the p40 genes in the producer cells and expression of p35 transcripts in cells not expressing the p40 genes. It is possible that the individual p40 and/or the p35 chains have yet undemonstrated functions, different from those of the p70 heterodimer; alternatively, either chain may associate with unrelated polypeptides to form heterodimers with different functions. It is also possible that the apparently inefficient regulation of the expression of the two genes reflects the evolutionary origin of IL-12 genes from genes encoding a putative primordial receptor and a cytokine, which might have been in part expressed in different cell types and the expression of which did not need to be quantitatively correlated.

When the production of IL-12 from normal PBMC was analyzed, the major producer cells of IL-12 were found to be monocytes or monocyte-derived macrophages, although B cells and other major histocompatibility complex (MHC) class II-positive cell types were also found to be producers.[7] Surprisingly, phorbol diesters did not enhance production of IL-12 by peripheral blood cells, unlike what is observed in EBV-transformed B-cell lines, but the most efficient inducers of IL-12 production were found to be bacteria, bacterial products, and intracellular parasites.[7] Gram-positive and -negative bacteria, endotoxins, mycobacteria, and intracellular parasites such as *Toxoplasma gondii* were all efficient inducers of IL-12 production.[7,34] Because of the ability of endotoxin to induce IL-12 production, a high background production of IL-12 is observed when precaution is not taken to purify and culture PBMC in strictly endotoxin-free conditions.[7] In addition to PBMC, peripheral blood neutrophils were also shown to be able to produce IL-12 in response to lipopolysaccharide (LPS).[35]

As observed in cell lines, stimulated PBMC and neutrophils produced the free p40 chain in a 10- to 50-fold excess over the biologically active p70 heterodimer.[7,35] Both p40 and p35 mRNA were constitutively expressed at very low levels in unstimulated peripheral blood cells and accumulation of both was upregulated by stimulation with bacteria or bacterial products, although the induction of the p40 gene was much more marked, resulting in abundance of p40 transcriptions in stimulated cells up to 200-fold higher than that of p35 transcripts, explaining the excess production of the

p40 protein.[7,30,35] Production of IL-12 or accumulation of p40 mRNA have not been demonstrated in purified T or NK cells, which, however, similar to many other cell types, express low levels of p35 transcript.[7,34]

Besides LPS, the bacterial molecules responsible for induction of IL-12 or other monocyte-derived cytokines are poorly characterized. Because of the possibilities that production of IL-12 may be differentially regulated compared with that of other monokines such as IL-1, TNF-α, and IL-10[36,37] and because of the importance of cytokine-inducing molecules in the vaccine adjuvant effect of bacterial preparations, this field of research is receiving increasing attention. Various types of phagocytic cells respond to various inducer stimuli with different efficiency. For example, heat fixed *Staphylococcus aureus* is a much stronger inducer of IL-12 production in PBMC than LPS, whereas LPS is a stronger stimulus for neutrophils and myeloid cell lines.[7,35,36]

The initial analysis of human cell lines for constitutive or phorbol diester-induced IL-12 production identified only B-cell lines as producers.[7] However, by inducing the cell lines with LPS, several human myeloid leukemia-derived cell lines, including THP-1, ML-3, and HL-60 (but not U937 or other cell lines) were shown to be able to produce IL-12.[36] In certain experimental conditions, treatment with inducers of differentiation (eg, short treatment with dimethyl sulfoxide) enhanced the ability of the cell lines to produce IL-12.[36]

Similar to the human cells, both B and macrophagic murine cell lines have been shown to produce IL-12.[38,39] Both in vitro and in vivo bacteria and LPS induced production of IL-12 in mice[34,40]; because LPS induced similar IL-12 levels in normal and B-cell–deficient SCID mice, it is likely that macrophages rather than B cells are the major physiologic IL-12 producers.[40]

The ability of phagocytic cells to produce IL-12 is regulated by several cytokines with activating or deactivating effects on the producer cells. IFN-γ and GM-CSF are among the cytokines that enhance the production of IL-12 from phagocytic cells.[36] IFN-γ treatment of monocytes or neutrophils does not directly induce accumulation of p40 mRNA, but enhances the accumulation in response to other stimuli, eg, LPS; unlike p40, both constitutive and stimulated accumulation of p35 mRNA is enhanced by IFN-γ treatment.[39] Probably because of this direct effect of IFN-γ on p35 mRNA accumulation, the ratio of p70:free p40 is higher in cells stimulated in the presence of IFN-γ.[36] The cytokines with an inhibitory effect on IL-12 production include IL-10, IL-4, and TGF-β.[30,40a] These cytokines inhibit both IL-12 protein secretion as well as p40 and p35 mRNA accumulation in stimulated phagocytic cells. The ability of IL-10 to inhibit IL-12 production by accessory cells is the major mechanism by which IL-10 inhibits IFN-γ production by T and NK cells.[30,41]

In addition to phagocytic cells and B cells, other cell types are emerging as possible producers of IL-12. Mast cell growth factor (MGF)-induced murine mast cells, considered to represent connective tissue-like mast cells, express IL-12 p40 and p35 mRNA but not IL-4 mRNA; IL-3–derived mast cells, representing mucosal-like mast cells, express IL-4 but not IL-12 mRNA.[42] Because mucosal mast cells have been implicated in the development of a Th2 response because of their production of IL-4, these data suggest mast cells in different tissues may participate in the regulation of the Th1-Th2 balance. Keratinocytes, both normal and malignant, express IL-12 p40 and p35 transcript, but appear to produce only minute amounts of IL-12.[43] Similar expression of IL-12 mRNAs, but with secretion of physiologically relevant amounts of IL-12 has been observed in human skin Langerhans cells (Rook et al, unpublished results). Follicular dendritic cells are also potential producers of IL-12, as detected by immunocytochemistry and mRNA expression. Although they might produce only minute quantities of IL-12 compared with bacteria-activated macrophages, as discussed below, their ability to produce IL-12 locally while acting as APC is probably important in determining the differentiation of Th cells[44] (Schuler et al, unpublished results).

Activity of IL-12 on Hematopoietic Progenitor Cells

Although T and NK cells are the best-characterized target cells for IL-12 activity, IL-12 has also been shown to enhance the proliferation of early murine hematopoietic progenitor cells in culture in response to other growth factors, particularly Steel factor (SF), but also IL-3, M-CSF, and GM-CSF.[45-48] IL-12 acts directly on the progenitor cells, as indicated by its effect on single progenitor cell cultures, and induces an increase in the number of colonies formed as well as an increase of colony size.[45,48] Among the progenitor cells affected by IL-12 are early cells with multilineage differentiation potential, including cells able to differentiate to either myeloid or B-lymphoid cells, as well as single-lineage progenitor cells.[48] The most powerful effect of IL-12 on progenitor cell colony formation is observed when it is present with a combination of at least two other growth factors, eg, IL-3 + IL-11 or IL-3 + SF.[47] IL-12 synergizes with IL-3 alone or IL-3 + SF to increase the generation of late progenitors and the survival/proliferation of primitive long-term culture-initiating stem cells in liquid culture.[46]

IL-12 also enhances the colony formation by highly purified human progenitor cells, either multilineage or lineage-committed, stimulated by SF and IL-3.[49] All these stimulatory effects of IL-12 on hematopoiesis appear to be direct on the progenitor cells and are not mediated through other cytokines induced by IL-12. Thus, IL-12 appears to belong to the group of synergistic early acting hematopoietic cytokines, including IL-6, G-CSF, and IL-11, and distinct from cytokines such as SF, IL-3, or IL-4.[50] However, if IL-12 is added to human progenitor cells together with a small number of NK cells, it inhibits colony formation by inducing NK cells to produce the inhibitory cytokines IFN-γ and tumor necrosis factor-α (TNF-α).[49]

Because IL-12 has a direct stimulatory effect on hematopoietic stem cells and an indirect inhibitory effect by inducing production of inhibitory cytokines by NK cells and, possibly, by T cells,[49] it is difficult to predict the possible physiologic role of IL-12 in in vivo hematopoiesis. Treatment of mice with daily intraperitoneal (IP) doses of 1 μg IL-12 has been shown to have profound effects on hematopoietic homeostasis, with neutropenia, anemia, and appearance of extramedullary hematopoietic foci in the spleen and

liver.[51] The liver histology in IL-12–treated mice is characterized, in addition to the presence of hematopoietic foci, by focal hepatocyte necrosis and elevation of hepatic transaminases, with an increased number of macrophages, NK, and $CD8^+$ T cells, but not of $CD4^+$ T cells.[51] The extramedullary hematopoiesis is mostly responsible for the splenomegaly observed in the IL-12–treated mice.[51] It is therefore apparent that IL-12 treatment in vivo has a profound effect on hematopoietic cells, but it is difficult to identify the role of direct effects of IL-12 on progenitor cells, that of IL-12–induced cytokines (eg, IFN-γ and TNF-α), or that of other effects on the hematopoietic cells (eg, cell mobility and migration) or on other cell types (eg, endothelial cells). IL-12 induces a similar hepatotoxicity but a much more severe anemia than observed in IL-2–treated mice; unlike IL-2–treated mice, IL-12–treated mice did not develop mononuclear cell infiltrates and pulmonary edema.[51,52]

In addition to its effect on early hematopoietic cells and lineage-committed myeloid progenitor cells, IL-12 has been shown to influence intrathymic T-cell development.[53] In fetal organ cultures, IL-12 determined a decrease in most thymocyte subsets, but an increase, both proportional and absolute, of the number of $\alpha\beta TCR^+$ $CD4^-$ $CD8^+$ thymocytes.[53] When added to isolated thymocyte subsets, IL-12, in combination with IL-2 and IL-4, caused proliferation of $CD3^+$ $CD4^-$ $CD8^+$ cells and, in combination with SF, of early triple negative ($CD3^-$ $CD4^-$ $CD8^-$), $CD44^+$ $CD25^+$ pro-T cells.[53] Because stromal cells from both fetal and adult mouse thymuses express p35 and p40 mRNA and are a potential source of IL-12,[53] the physiologic role of IL-12 in regulating intrathymic T-cell development deserves to be investigated.

Induction of Lymphokine Production by IL-12

One of the most potent and probably physiologically relevant functions of IL-12 is its ability to induce both NK and T cells to produce lymphokines, particularly IFN-γ.[2] Like IL-2 and a few other cytokines, IL-12 induces NK and T cells to accumulate mRNAs and to secrete IFN-γ, TNF-α, GM-CSF, M-CSF, IL-3, IL-8, and IL-2.[2,4,20,21,34,54-59] Whereas the ability of IL-12 to induce most of these cytokines is lower or at most comparable with that of IL-2, IL-12 is selectively powerful in inducing IFN-γ production as a single stimulus and as a synergistic inducer together with other IFN-γ inducers. On T cells, IL-12 synergizes in inducing IFN-γ production with IL-2, phorbol diesters, mitogenic lectins, and T-cell receptor (TCR)/CD3 stimuli such as anti-CD3 antibodies, alloantigens, and specific antigens[20,21]; on NK cells with IL-2, phorbol diesters, Fc receptor ligands (immunocomplexes, IgG-coated cells, anti-CD16 antibodies), and NK susceptible target cells.[20,21] Both resting and activated NK and T cells are induced by IL-12 to produce IFN-γ, although maximal IFN-γ mRNA accumulation is reached in 2 to 4 hours in activated T or NK cells and in 18 to 24 hours in resting peripheral blood lymphocytes (PBL).[21] Within PBL, IL-12 induces mRNA accumulation, as detected by in situ hybridization, in a proportion of both NK and T cells,[21] although NK cells might be a major contributor to the early production of IFN-γ in response to IL-12 or IL-2.[60] As discussed above, the ability of IL-12 at concentrations as low as 1 to 5 pmol/L to induce IFN-γ on resting T and NK cells is difficult to reconcile with the fact that IL-12 receptor cannot be detected on resting PBL.[19] However, it is possible that a small number of high-affinity receptors, undetectable by the presently available methods, are expressed on a proportion of PBL and responsible for the IL-12 effect. It should also be noted that in peripheral blood $CD56^+$ NK cells, IL-12 has been shown to upregulate its own receptor,[61] which is perhaps a mechanism of regulation explaining the ability of IL-12 to induce resting PBL. Although NK and T cells are the IFN-γ producers in PBL preparations stimulated by IL-12, an accessory cell type (MHC class II-positive, non-monocyte, non-B cells) is required for optimal IFN-γ production by resting PBL.[21] Although the nature of these accessory cells has not yet been identified, they might provide costimulatory molecules for IFN-γ production. In murine spleen cells, it has been shown that IL-12 synergizes with TNF-α in inducing IFN-γ production.[34,62] This synergistic effect of TNF-α was not shown with human lymphocytes, but antibodies to TNF-α or IL-1β efficiently inhibited IL-12–induced IFN-γ production, suggesting that these two cytokines, endogenously produced in the PBL cultures, possibly by the class II-positive accessory cells, act as costimulatory molecules for IFN-γ production together with IL-12.[30] Another costimulatory signal possibly provided by the accessory cells is the B7 molecule, ligand for the CD28 receptors on T cells. Stimulation of T cells with B7-transfected cells or with anti-CD28 antibodies strongly synergized with IL-12 for induction of IFN-γ production[41,57] and blocking of B7-CD28 interaction with the hybrid recombinant molecule CTLA4-Ig significantly inhibited the ability of PBL to produce IFN-γ in response to IL-12.[57] These results suggest that TNF-α, IL-1β, and B7, possibly at least in part provided by the class II-positive accessory cells, are important costimulators for IFN-γ production in response to IL-12. The ability of IL-10 to inhibit IFN-γ production in T and NK cells is primarily due to its ability to suppress IL-12 production, but also, in part, because of its ability to suppress expression of these costimulatory molecules on accessory cells.[30,41,57]

The mechanisms by which IL-12 induces IFN-γ production and synergizes with IL-2 in this effect have been investigated.[54,56] Whereas IL-12 directly induced an increase in the transcriptional rate of the IFN-γ genes, the combination of IL-12 with IL-2 did not induce an additional increase in transcription, but increased the half-life of the IFN-γ mRNA severalfold.[54] Thus, both transcriptional and posttranscriptional mechanisms are involved in the regulation of IFN-γ gene expression by IL-12. IL-12 and IL-2 did not show the same strong synergistic effect in induction of other cytokines, either at the protein or the mRNA level,[20,56] although additive or, in some cases, more than additive effects were observed when IL-12 was used in combination with IL-2 or other stimuli.[56] For example, relatively high concentrations of TNF-α or GM-CSF were produced by T cells stimulated by IL-12 and anti-CD28 antibodies.[57]

Not only is IL-12 a potent inducer of IFN-γ production, but it is also most likely a required factor for efficient IFN-γ production depending in vivo and in vitro on accessory

cells. When human PBMC were treated in vitro with stimuli, eg, *S aureus,* that were able to induce production of IL-12, they rapidly produced large amounts of IFN-γ. This production of IFN-γ was almost completely inhibited by neutralizing antibodies against IL-12.[7] Even when IFN-γ inducers that are not known to stimulate IL-12 production were used (eg, IL-2 or anti-CD3 antibodies), the production of IFN-γ from PBMC was inhibited up to 80%, indicating that endogenously produced IL-12 is required for optimal IFN-γ production.[7] However, if purified T or NK cells, in the absence of IL-12–producing accessory cells, were stimulated to produce IFN-γ (eg, by IL-2 or anti-CD3 antibodies), no inhibitory effect of anti–IL-12 antibodies could be shown.[7]

Injection of mice with a daily IP injection of 1 μg of recombinant IL-12 induced high levels of serum IFN-γ, but only starting 48 hours after the first injection.[51] This delayed response was probably caused by the lack of appropriate costimulatory signals when only recombinant IL-12 was injected. The injected IL-12 had a serum half-life of 3.3 hours,[40] much longer than that of other cytokines. The ability of IL-12 to induce a rapid production of IFN-γ in vivo has been clearly shown in several experimental models of infectious diseases discussed below. A very informative experimental model for the understanding of the role of IL-12 in inducing IFN-γ in vivo is provided by the endotoxic shock in Bacille Calmette Guérin (BCG)-primed mice.[40] Several cytokines, particularly TNF-α and IFN-γ, have been shown to be responsible for pathologic reactions that may lead to shock and death observed in infection with gram-negative bacteria and in response to endotoxins. Priming of mice with the avirulent BCG vaccine strain of *Mycobacterium bovis* increases the sensitivity of mice to the lethal effect of LPS and results in an efficient priming for cytokine production in response to LPS. Mice injected with LPS produced IL-12 that controlled IFN-γ production, as shown by the ability of neutralizing anti–IL-12 antibodies to suppress IFN-γ production.[40] However, the concentration of biologically active IL-12 p70 heterodimer was similar in the serum of both BCG-primed or unprimed mice, reaching levels of 1 to 3 ng/mL at 3 to 6 hours after LPS injection, whereas IFN-γ production was observed only in BCG-primed mice.[40] TNF-α and other LPS-induced cofactors were required in cooperation with IL-12 to induce optimal IFN-γ production. The priming effect of BCG on IFN-γ production appears to be mostly caused by its ability to increase TNF-α production, which acts as cofactor with LPS-induced IL-12 in inducing IFN-γ production.[40] Neutralizing anti–IL-12 antibodies, in addition to inhibiting the in vivo LPS-induced IFN-γ production, also protected mice from septic shock-induced death.[40] Thus, IL-12 is required for IFN-γ production and lethality in an endotoxic shock model in mice.

In addition to the endotoxic shock model, the important role of IL-12–induced IFN-γ production was demonstrated in the generalized Shwartzman reaction in mice.[63] In this model, mice are sensitized to an intravenous (IV) injection of LPS with a local injection, 24 hours earlier, of a low LPS dose in the footpad. The importance of the LPS-induced IL-12 and of IL-12–induced IFN-γ in the sensitization phase was demonstrated by the observations that (1) the sensitization was blocked by neutralizing antibodies to either IL-12 or IFN-γ and that (2) LPS sensitization could be replaced by injection of either IL-12 or IFN-γ.[63]

Enhancement of NK and T-Cell–Mediated Cytotoxicity by IL-12

The ability of IL-12 to enhance lymphocyte-mediated cytotoxicity was one of the first IL-12 activities to be described and responsible for the original designation of IL-12 as NK cell stimulatory factor[2] and cytotoxic lymphocyte maturation factor.[3] Incubation of PBL or purified NK cells with IL-12 for incubation times of 8 hours or longer enhanced severalfold NK cell cytotoxic activity against both NK cell-sensitive and -resistant target cells.[2,4,28,55,64-66] The ability of NK cells to lyse virus-infected target cells, including human immunodeficiency virus (HIV)-infected cells[66] and, to a lesser extent, antibody-coated target cells (antibody-dependent cell-mediated cytotoxicity [ADCC])[28,66] was also enhanced by IL-12. The enhancement of NK cell-mediated cytotoxicity was paralleled by increased binding to the target cells and by an increase in granularity of NK cells,[65,66] probably reflecting an effect of IL-12 on the expression of adhesion molecules and granule-associated proteins, as discussed below. IL-12-treatment also had positive modulatory effects on NK cell granule exocytosis induced by CD16/FcγRIII triggering, activation of protein kinase C, or stimulation of G proteins.[67] The effect of IL-12 on cytotoxicity appears to be direct on NK cells, because it was observed with highly purified preparations of NK cells[28,55,66] and, unlike the IFN-γ production by PBL,[21] does not require the participation of accessory cells.[28,66] The activation of NK cells by IL-12 was not dependent on the production by NK cells of cytokines with NK cell enhancing activity such as IL-2, IFN-α, or IFN-γ.[2,66,68] Unlike IL-2–mediated enhancement of NK cell-mediated cytotoxicity, the effect of IL-12 was not inhibited by IL-4.[27,28] Antibodies against TNF-α have been shown to inhibit the enhancing effect of IL-12 on NK cell cytotoxicity in one study,[68] but not in another one,[66] suggesting that endogenously produced TNF-α may cooperate with IL-12 on some NK cell subsets or experimental conditions, but not in others.

Even when optimal concentrations of IL-12 are used, the enhancement of NK cytotoxicity induced by IL-12 is usually lower than the optimal enhancement obtained with IL-2 or with IFN-α.[2] However, enhancement of NK cell-mediated cytotoxicity was observed with concentrations of IL-12 of less than 1 pmol/L, whereas concentrations of IL-2 or IFN-α up to 3 orders of magnitude higher were required for similar effects.[2] Unlike the induction of IFN-γ production from PBL or NK cells, stimulation of NK cells with combination of IL-12 and IL-2 resulted in an additive, but not a synergistic, effect on cytotoxic activity.[66]

In vivo treatment of mice with daily IP injection of IL-12 determined a striking increase of NK activity in spleen and liver with a maximum after the second injection and declining thereafter.[51] This decrease in NK cells activity during continuous cytokine treatment was previously observed with IFN-α treatment[69] and it is not caused by a decreased

number of NK cells, because they increase rather than decrease with continuous IL-12 treatment.[51] It is possible that inhibitory mechanisms, possibly dependent on IFN-γ–mediated macrophage activation, were responsible for the observed decline in NK activity, as previously observed during IFN-α treatment.[69]

In addition to short-term activation of NK cells, IL-12 also induces generation of LAK cells in culture of PBL[11] or purified NK cells.[55] The IL-12 effect of IL-12 on the generation of LAK cells was not blocked by antibodies to IL-2 or IFN-γ, but was significantly inhibited by antibodies to TNF-α.[11,55] Because IL-12 induces only minor levels of TNF-α from purified NK cells and TNF-α alone is not an inducer of LAK cell generation, it is likely that endogenously produced TNF-α acts as a cofactor together with IL-12 in the generation of LAK cells.[55] The requirement for production of TNF-α and, possibly, of other costimulatory molecules probably explains the observation that, in the presence of hydrocortisone, which inhibits endogenous cytokine production, IL-12 alone was unable to induce generation of LAK cells, but synergized with IL-2 in this effect.[3,5] It is noteworthy that anti–IL-12 antibodies decreased the generation of LAK cells induced by IL-2, suggesting a role for endogenously produced IL-12 in this phenomenon.[11]

In addition to its effect on NK cell cytotoxicity, IL-12 also enhances T-cell–mediated cytotoxicity and has an enhancing effect on the generation of CTL. IL-12, similar to, although not as powerfully as IL-2, induced in peripheral blood T cells the ability to lyse anti-CD3 antibody-coated Fc-receptor–positive target cells (reverse ADCC).[66] The spontaneous cytotoxicity mediated by acute T-cell leukemia (T-ALL)-derived cell lines was enhanced by short-term treatment with IL-12.[65] IL-12 enhanced 10- to 20-fold the generation of cytotoxic cells lytic for anti-CD3 hybridoma target cells from human $CD8^+$ cells stimulated by immobilized anti-CD3 antibodies.[70] This effect of IL-12 was not dependent on secretion of IFN-γ or IL-2.[70] The in vitro generation of both human and murine allospecific CTL was enhanced by IL-12.[11,71] The mechanism of CTL enhancement was shown to be IL-2 dependent in human T cells,[11] but not in murine T cells.[71] The increase in mouse allospecific CTL activity was equivalent either when IL-12 was added at the beginning or during the last day of the 5-day mixed leukocyte cultures used for CTL generation, suggesting that IL-12 enhances both the generation and the cytotoxic activity of CTL.[71]

The rapid enhancement of NK cell cytotoxicity by IL-12, observed within a few hours of treatment, and the modest effect of IL-12 on cell proliferation, particularly of resting NK cells, suggest that proliferation plays a minor role in the IL-12–mediated enhancement of cytotoxicity. The increased ability of IL-12–treated NK cells to form conjugates with target cells[65,72] is probably caused by an upregulation of NK cell adhesion molecules. IL-12 induces an upregulation on NK cells of the following activation markers and cell-adhesion molecules: CD2, CD54, CD56, CD69, and CD71.[28,55,72] Within the β_2 integrins, IL-12, similar to IL-2, upregulates CD11a but not CD11b expression[28,72]; unlike IL-2, IL-12 did not upregulate any of the β_1 integrins on NK cells.[72] Although IL-2–activated NK cells have increased integrin-dependent adhesion to fibronectin- or laminin-coated surfaces, IL-12–activated NK cells were unchanged in their adhesion to laminin and were less adherent than IL-2–activated cells to fibronectin[72]; although overall the effect of IL-12 on adhesion functions of NK cells was more moderate than that of IL-2, IL-12 was active at much smaller doses than IL-2,[72] suggesting a possible physiologic relevance of these effects. The changes in adhesion properties of IL-12–treated NK cells together with an enhanced migratory activity[72] and with a chemotactic effect of IL-12 on NK cells[73] are likely to play a role in vivo in the NK response to endogenous or administered IL-12. In addition to the upregulation of adhesion-related molecules, IL-12 induced or enhanced expression of cytokine receptors on NK cells, in particular the α (p55) and β (p75) chains of the IL-2 receptor[28,55] and the p75 TNF receptor.[55,68] Anti–IFN-γ antibodies inhibited the enhancement of the p75 TNF-R in IL-12–treated NK cells, but induced expression of the p55 TNF-R, suggesting that the effect of IL-12 on the p75 TNF-R is mediated by IFN-γ and that IFN-γ has opposite effects on the expression of the two TNF-R.[68] The ability of IL-12 to modulate the expression of cytokine receptors on lymphocytes has probably a role in affecting the responsiveness of the lymphocytes to the cytokine cascade during inflammation or immune response. IL-12 treatment also resulted in an increase in the number of cytoplasmic granules in NK cells,[66] concomitant with an increased expression of proteins and mRNA for the granule-associated serine esterases, granzyme A and granzyme B, and for the pore-forming protein perforin, dependent, at least in part, on transcriptional activation.[56,65,70,74] The ability of IL-12 to increase the expression of these cytotoxicity-related proteins, which was observed in NK cells,[56,65,74] $CD8^+$ cells,[70] and T-ALL cells,[65] as well as to modulate the stimulus-dependent granule exocytosis[67] is likely to play a key role in the mechanisms by which IL-12 treatment enhances the cytotoxic potential of NK cells and CTL.

Effect of IL-12 on T and NK Cell Proliferation

IL-12 has very little, if any, effect on the proliferation of resting peripheral blood T and NK cells. However, it enhances the proliferation of PBL induced by mitogens such as lectins and phorbol diesters.[2,4] This enhancing effect on PBL proliferation reaches maximal levels at IL-12 concentrations of 1 pmol/L. Similarly, IL-12 enhances T-cell proliferation in response to anti-CD3 antibodies or to allogeneic cells.[20] EBV-transformed human B-cell lines have often been used as feeder cells for optimal growth in culture of human NK and T cells[75-77]; the endogenous IL-12 produced in the culture medium by many of these cell lines was shown to significantly enhance proliferation and cytotoxic activity of both T and NK cells.[78]

Unlike resting lymphocytes, T cells and NK cells that have been preactivated in vitro in different culture conditions (eg, after PHA stimulation of T cells or coculture of NK cells with EBV-transformed B-cell lines) proliferated in response to IL-12.[3-5,20,22,79] The ability of PHA blasts to proliferate in response to IL-12 paralleled the expression of IL-12 receptors as detected by ^{125}I–IL-12 binding. Maximal

proliferation was observed at 2 to 4 days after PHA stimulation and then declined rapidly after day 6, similar to the expression of detectable IL-12R.[19] IL-12 also synergizes with low doses of IL-2 in inducing proliferation of PBMC from day 7 to 11 of culture, whereas at earlier times of culture no cooperation between IL-12 and IL-2 was observed[79]; these kinetics also correlate with the ability of IL-2 to induce IL-12R expression on PBL from day 4 to 10 of culture.[19] Thus, it appears that for IL-12 to induce T-cell proliferation, IL-12R needs to be induced above the level present on resting peripheral blood T cells. The ability of PHA blasts to proliferate in response to IL-12 and the enhancing effect of IL-12 on the proliferation of PBL induced by PHA on day 4 to 6 of culture and induced by IL-2 on day 7 to 11 is compatible with this hypothesis. Whether the ability of IL-12 to enhance the proliferation of PBL induced by phorbol diesters already at day 3 of culture[2] is caused by the ability of these compounds to rapidly induce IL-12R expression remains to be investigated. However, the available data on expression of IL-12R and responsiveness to IL-12 are difficult to interpret. The half-maximal proliferative response to IL-12 was observed at concentrations of the cytokine between 1 and 10 pmol/L,[20,79] much lower than the observed Kd of IL-12 binding to PHA blasts[18,19]; furthermore, resting T and NK cells, which do not proliferate in response to IL-12, are rapidly induced to produce IFN-γ by picomolar concentrations of IL-12.[2]

Human CTL lines, which proliferated in response to IL-12 alone, proliferated in response to IL-12 only when activated by anti-CD3 antibodies[80]; the proliferative response to IL-12 was independent of endogenously produced IL-2.[80] Human (Kubin et al, unpublished results) and murine[81] Th1 clones also proliferated in response to IL-12 only when costimulated by antigen, anti-CD3 antibodies, or mitogens; unlike the human CTL lines, the IL-12–induced proliferation of murine Th1 clones was dependent on IL-2 production in the cultures.[41] Murine Th1 clones anergized in vitro by treatment with soluble anti-CD3 antibodies as well as anergized $CD4^+$ T cells isolated from mice tolerized to the Mls-1^a antigen in vivo showed defective induction of proliferation to IL-12 upon restimulation with antigens, indicating that T-cell clonal anergy results not only in failure to produce the autocrine growth factor IL-2, but also in lack of responsiveness to the APC-derived cytokine IL-12.[82]

The proliferation induced by IL-12 is largely IL-2 independent and anti–IL-2 or anti–IL-2R antibodies minimally inhibit proliferation in most experimental systems.[20,79] However, the maximal proliferation induced by IL-12 in preactivated T or NK cells was usually only between 10% and 50% of the maximal proliferations induced by IL-2.[3,20,79] When IL-12 and IL-2 were added together to PHA blasts, an additive effect was observed on proliferation, unlike the strong synergistic effect of these two cytokines on IFN-γ production.[20,79] However, on NK cells and on T cells with $\gamma\delta$TCR, IL-12 inhibited IL-2–induced proliferation to the level of proliferation induced by IL-12 alone, especially when high doses of IL-2 ($\geq$100 U/mL) were used.[20,28] The antagonistic effect of IL-12 on IL-2–induced proliferation of NK cells depended on the activation state of the cells: highly activated NK cells, expressing high levels of CD25 (IL-2Rα) and other activation markers, were not inhibited by IL-12, whereas NK cells collected from later times of culture, past the peak of proliferation or expression of activation antigens, were sensitive to the inhibitory effect of IL-12.[20] IL-12 was also inhibitory for the IL-2–induced proliferation of purified fresh NK cells,[28] although IL-12 present during coculture of PBL with EBV-transformed B-cell lines had a strong enhancing effect on the endogenous IL-2–dependent expansion of NK cells in these cultures.[78] The effect of IL-12 on the proliferation of $\gamma\delta TCR^+$ T cells was confirmed by its ability to suppress the IL-2–dependent proliferation of a $\gamma\delta TCR^+$ T-ALL–derived cell line.[20] Although IL-12 was shown to enhance IL-2–dependent proliferation of both $CD4^+$ and $CD8^+$ T cells contained in PHA blast preparations,[20] IL-12 inhibited the proliferation of anti-CD3 activated $CD8^+$ T cells induced by high ($\geq$4.5 ng/mL) concentrations of IL-2, whereas it enhanced the proliferation induced by low concentrations.[70] The inhibitory effect of IL-12 on IL-2–induced proliferation of NK cells and $\gamma\delta TCR^+$ T cells was completely prevented by neutralizing anti–TNF-α antibodies, although TNF-α, in the absence of IL-12, did not affect proliferation.[20]

IL-4 inhibited proliferation, cytokine production, enhancement of cytotoxic activity, and induction of CD69 antigens in NK and/or T cells induced by IL-2, but not the same effects mediated by IL-12.[27,28,61] In addition, IL-4 and IL-12 synergized in inducing proliferation of $CD56^+$ PBL (NK cells), but not of $CD56^-$ PBL.[61] Although the mechanism of this synergistic effect is not known, it is noteworthy that IL-4 potentiated the IL-12–induced upregulation of the IL-12R and that IL-12 treatment upregulated the expression of IL-4R, suggesting that a reciprocal regulation of each other cytokine receptor may play a role in this synergy.[61]

Although IL-12 was able to induce proliferation of activated T and NK cells at very low concentrations, the reduced levels of proliferation compared with those induced by IL-12 raise concern over the physiologic relevance of IL-12 as a proliferative stimulus in vivo for T and NK cells. It was therefore particularly interesting the finding that the costimulation of either PHA blasts or even PBL with antibodies against the T-cell surface receptor CD28 or with one of the CD28-ligand, B7.1, transfected on L cells, strongly synergized with IL-12 in inducing lymphocyte proliferation.[57] In the presence of B7/CD28 costimulation, IL-12 induced proliferation of T cells higher than that obtained with maximal IL-2 stimulation, and it was effective at concentrations 100- to 1,000-fold lower than effectual concentrations of IL-2; the proliferative effect of anti-CD28 and IL-12 was resistant to moderate doses of cyclosporin A and was largely independent of endogenous IL-2.[57] Equivalent results with murine Th1 $CD4^+$ clones indicate that the proliferation of the clones in response to antigen and spleen APC was dependent on B7 (expressed on APC or on B7-transfected third party L cells) and IL-12 (either produced by the APC or exogenously added).[41] Interestingly, the induction of IL-12 responsiveness in murine Th1 clones by antigen and APC was inhibited by cyclosporin A, but the IL-12–induced proliferation of clones preactivated by antigen and APC was cyclosporin A insensi-

tive.[82] These results suggest that TCR stimulation by antigen induces IL-12 responsiveness, possibly dependent on induced IL-12R by a mechanism that may require IL-2– and/or other cyclosporin A-sensitive signals, whereas the IL-12–induced proliferation of the responsive cells is cyclosporin A-resistant and IL-2–independent. These in vitro results suggest that the synergy between B7 and IL-12, a surface antigen and a soluble product, respectively, of APC may have a central role in regulating T-cell activation and immune response in the microenvironment of inflamed tissues.

Role of IL-12 in the Development of Th1 Cells

The powerful effect of IL-12 in rapidly inducing IFN-γ production both in vitro and in vivo raised the question whether this cytokine was also involved in the differentiation or selection of the major T-cell type responsible for IFN-γ production during an immune response, the Th1 cells.[83] Several recent studies both in humans[8,59,84,85] and in the mouse[9,86,87] have indeed identified IL-12 as a factor facilitating and probably required for Th1 cell development, acting in an antagonistic equilibrium with IL-4, which favors differentiation of the Th2 cells.[10] These in vitro observations have been fully confirmed by in vivo experimental models of infection or immunization, reviewed below.

Stimulation in vitro of PBL from atopic patients with allergens such as *Dermatophagoides pteronyssinus* group 1 (Der p.1) resulted in the generation of T-cell lines and clones with high IL-4 and low IFN-γ production typical of Th2 cells, whereas PBL stimulation with bacterial products (eg, purified protein derivative [PPD]) generated Th1-type T-cell lines and clones that produced IFN-γ but not IL-4. When PBL were stimulated with Der p.I in the presence of IL-12, T-cell lines were generated that exhibited a reduced ability to produce IL-4 and an increased ability to produce IFN-γ.[8] These cell lines developed into Der p.I-specific $CD4^+$ T-cell clones exhibiting a Th0- (producing both IFN-γ and IL-4) or Th1-phenotype (producing only IFN-γ). This Th1-inducing effect of IL-12 was not inhibited by anti–IFN-γ, but was reduced by removal of NK cells from the PBL preparations. Thus, the effect of IL-12 is at least in part independent of IFN-γ production, but might require the participation of NK cells. PPD-specific T-cell lines generated in the presence of anti–IL-12 antibodies during the initial antigenic stimulation produced significant levels of IL-4, unlike the cell lines generated in the absence of antibodies, and gave rise to PPD-specific $CD4^+$ cell clones showing a Th0/Th2 phenotype rather than a Th1 phenotype.[8] These results indicate not only that IL-12 is able to facilitate proliferation and activation of Th1 cells in a memory response in vitro, but also that, as shown by the effect of anti–IL-12 antibodies, endogenously produced IL-12 is an obligatory factor for Th1 generation in vitro in response to bacterial antigens.

The results obtained with the analysis of human T-cell response to recall antigens clearly showed that memory T cells with a predominant Th0/Th1 phenotype in the case of bacterial antigens and a Th0/Th2 phenotype in the case of allergens can be modulated in vitro to generate either Th1 or Th2 clones depending on the cytokine pattern present during the in vitro restimulation. Whether this ability to shift cytokine production phenotype during in vitro stimulation reflected a plasticity of still incompletely differentiated Th cells or rather the selective expansion of few clones of phenotypes different from the predominant ones remained undetermined.

To determine whether IL-12 has an effect on the maturation of naive human $CD4^+$ T cells, neonatal (cord blood) $CD4^+$ T cells were studied.[59,85] Culture for 1 week in the presence of IL-12 rendered cord blood $CD4^+$ cells able to produce IFN-γ, whereas freshly purified $CD4^+$ cord blood cells were unable to produce IFN-γ even in response to phorbol diesters and Ca^{2+} ionophore.[59] This IL-12–mediated priming of naive cord blood $CD4^+$ cells for IFN-γ production was enhanced severalfold when irradiated cord blood mononuclear cells, IL-2, TNF-α, or IL-1 were added to the cultures.[59] Interestingly, if cord blood $CD4^+$ cells were expanded for 3 weeks in IL-4, they produced Th1 cytokines (IFN-γ and IL-2) but not Th2 cytokines (IL-4 and IL-5).[85] If IL-12 and IL-4 were presented during the priming, cell cultures that produced very high levels of both IFN-γ and IL-4 were obtained, indicating that IL-12 in this experimental system may allow the IL-4–mediated priming for IL-4 production to develop in the culture, while concomitantly inducing priming for IFN-γ production.[85]

The experimental systems used to date have not permitted determination of whether the different cytokines affecting Th cell development, IL-12 in particular, induce differentiation of bipotential Th precursors or rather a selective priming and/or expansion of already committed Th1 and Th2 precursor cells.[59,88-90] This question is particularly relevant in the case of human studies that have analyzed clonal expansion of memory Th cells.[8,91] However, once a Th1 or Th2 response has been established, it appears to be relatively stable, and no factors capable of inducing qualitative changes in the cytokine profile of established murine or human T-cell clones have been reported.

In the experiments of analysis of cytokine production from human T cells stimulated with recall antigens (PPD) or allergens (Der P1), the expansion of a small proportion of memory T cells was first obtained in polyclonal T-cell cultures, from which single antigen-specific clones were obtained only after several weeks of culture of the polyclonal cell line.[8,91] During this culture period, emergence of Th cell subsets, with characteristic cytokine production profiles, could be caused by differentiation of precursor Th cells, as well as by positive selection (growth advantage) of certain Th subsets or negative selection (cytotoxicity, antiproliferative effect) of other subsets. When the Th response in vitro to PPD and Der p1 was analyzed, the presence of endogenous or added recombinant IL-12, respectively, during antigenic stimulation was observed to induce a striking decrease in IL-4 production and a more modest enhancement of the ability of the T cells to produce IFN-γ.[8] The results were equivalent when the polyclonal T-cell culture or the antigen-specific $CD4^+$ T-cell clones derived from them were analyzed.[8]

To analyze whether the effect of IL-12 on the cytokine profile of T cells was due to differentiation of single cells or selective mechanisms, clonal growth of virtually every T

cell from PBL was obtained by a limiting dilution method using PHA stimulation in the presence of accessory cells.[84] Thus, progeny of both naive and memory T cells was analyzed and the possibility that selection of precommitted Th precursors play a role in determining the characteristics of the clones generated was excluded. IL-12 present during the cloning procedures endowed both $CD4^+$ and $CD8^+$ clones with the ability to produce IFN-γ at levels severalfold higher than those observed in clones generated in the absence of IL-12. This priming was stable, because the high levels of IFN-γ production were maintained when the clones were cultured in the absence of IL-12 for 1 or 2 weeks. The $CD4^+$ and some of the $CD8^+$ clones also produced variable amounts of IL-4. Unlike IFN-γ, IL-4 production was not significantly different in clones generated in the presence or absence of IL-12. These data suggest that IL-12 primes the clone progenitors, inducing their differentiation to high IFN-γ–producing clones. The suppression of IL-4–producing cells observed in polyclonally generated T cells in vivo and in vitro in the presence of IL-12 was not observed in this clonal model, suggesting that the apparent suppression most likely results from positive selection of non—IL-4–producing cells.[84] In the same experimental system, when IL-4 and IL-12 are present during the T-cell cloning, only a minimal inhibitory effect on the IL-12–mediated priming for high IFN-γ production was found (Gerosa and Trinchieri, unpublished results).

Human antigen-specific established Th2 clones that were unable to produce IFN-γ with any other stimulator did produce IFN-γ at low but significant levels when stimulated with IL-12 in combination with specific antigen or insoluble anti-CD3 antibodies.[84] This induction of IFN-γ gene expression was transient, because culture of the established clones with IL-12 for up to 1 week did not convert them into IFN-γ producers when stimulated in the absence of IL-12. These results suggest that Th clones can respond to IL-12 treatment with either a stable priming for IFN-γ production or with only a transient low level expression of the IFN-γ gene, depending on the stage of differentiation.

To determine whether IL-12 directly initiates Th1 cell development in naive murine T cells, Hsieh et al[9] showed that $CD4^+$ T cells derived from mice transgenic for an anti-ovalbumin TCR were induced by ovalbumin to develop into Th1 cells in the presence of IL-12, whereas they developed into Th2 cells in the presence of IL-4. The effect of IL-4 was dominant over that of IL-12 when both cytokines were present. *Listeria*-infected macrophages, by producing IL-12, also induced Th1 cell development in $CD4^+$ cells cultured from these mice.[9] Unlike in the human system, the IL-12–induced development of Th1 cells in these TCR transgenic mice was abolished by anti–IFN-γ antibodies.[86] The ability of IL-12 to induce development of Th1 cells and priming for high IFN-γ production was confirmed using another strain of TCR (anti-cytochrome C) transgenic mice.[87] In this experimental system,[87] IL-12 did not prevent the ability of IL-4 to prime T cells for IL-4 production, but, unlike the results of Hsieh et al[9] and in agreement with the human data, IL-4 only partially diminished, but did not prevent the IL-12–induced priming for IFN-γ production. Furthermore, anti–IFN-γ antibodies did not prevent the Th1 development in response to antigen and APC in the presence of IL-12 in these mice.[87] In the absence of accessory cells, IL-12 is able to induce Th1 development in naive murine $CD4^+$ T cells stimulated with anti-CD3 antibodies. In these experimental conditions, Th1 development was suppressed by anti–IFN-γ antibodies.[87,92] The discordant results on the requirement for IFN-γ in the IL-12–mediated induction of Th1 development are difficult to interpret. IFN-γ in the absence of IL-12 is unable to induce Th1 development or priming for IFN-γ production.[86,87,92] Thus, in certain conditions, both IL-12 and IFN-γ are needed for this effect; because IL-12 is a potent inducer of IFN-γ production, its presence may be sufficient to provide both factors needed for Th1 development, but its effect can be blocked by neutralizing anti–IFN-γ antibodies. The fact that a requirement for IFN-γ has not been shown in all in vitro experimental conditions[84,87] is possibly in part explained by the recent results by Macatonia et al[44] showing that, in the antiovalbumin TCR transgenic mice, IFN-γ is required for the IL-12–induced Th1 development of LECAM-1^{bright} naive $CD4^+$ T cells, whereas IFN-γ is not required for the Th1 development of a subset of LECAM-1^{dull} $CD4^+$ T cells with phenotype of ''memory/activated'' T cells. These results are compatible with the human data showing that the effect of IL-12 on the Th1 development of memory $CD4^+$ cells does not require the participation of IFN-γ.[9]

Macrophages, especially when infected by intracellular bacteria, are potent producers of IL-12, directing the Th cell development toward Th1 responses.[9] In the absence of a source of high concentrations of IL-12, production of IL-4 by subsets of $CD4^+$ cells[93,94] or other cell types[95] may prevent Th1 cell development. Indeed, when dendritic cells were used for antigen presentation to antiovalbumin TCR-transgenic $CD4^+$ T cells, development of Th1 cells was observed only if endogenous IL-4 was neutralized with specific antibodies.[9] This dendritic cell-driven Th1 development was inhibited by anti–IL-12 antibodies, showing that the low concentration of IL-12 produced by dendritic cells was efficient to induce Th1 development only when the antagonistic effect of endogenous IL-4 was abrogated.[44] It remains to determine which conditions induce production of IL-12 by dendritic cells, although preliminary evidence suggests that cognate interaction of $CD4^+$ cells and APC in the presence of antigen might result in stimulation of IL-12 production.[44,96,97]

Differentiated Th1 cells may still require IL-12 for optimal IFN-γ production and, at least in part, proliferation. Germann and Rüde partially purified a T-cell stimulatory factor (TSF), a soluble mediator involved in the proliferation and IFN-γ production of murine Th1 cells,[96,97] which later they proved to be IL-12.[81,98] TSF/IL-12 has proliferative activities, in part through induction of IL-2Rα, and induces IFN-γ production in Th1, but not in Th2 clones.[81,96-99] Furthermore, it was shown that proliferation and IFN-γ production by Th1 clones in response to antigen and splenic APC was dependent on expression of B7 on APC, synergizing with IL-12.[41] Prevention of B7-CD28 interaction with the chimeric recombinant molecules CTLA4-Ig, abrogation of IL-12 activities by specific antibodies, or inhibition of IL-12 secretion

and B7 expression on APC by IL-10 inhibited proliferation and IFN-γ production in Th1 clones.[41]

IL-1 has similar costimulatory effects on Th2 clone activity as IL-12 has on Th1 clones; Th2 but not Th1 clones express IL-1R.[81,100] However, costimulation by IL-1 has been shown to be required for IL-12–induced IFN-γ production by human PBL,[30] for the IL-12 priming for IFN-γ production in cord blood $CD4^+$ cells,[101] and for the proliferation and IFN-γ production in Th1 clones in the presence of macrophage accessory cells, both in antigen-independent[96] and -dependent[41] experimental systems. However, it is unclear whether in these conditions the effect of IL-1 is directly on T cells or, more likely, mediated by the accessory cells.

Effects of IL-12 on Humoral Immunity

In vitro, IL-12 suppressed the synthesis of IgE by IL-4–stimulated B cells in the presence of T cells.[102] Although receptors for IL-12 have not been shown on B cells,[19] IL-12 has been shown to act as a growth factor for *S aureus* or anti-μ antibody–stimulated human B cells,[103] suggesting the possibility that IL-12 may affect B-cell activity both through its effect on T-helper cells and directly on B cells. However, the ability of IL-12 to suppress IgE production in vitro appears to be mediated through T cells, because no effect was observed with purified B cells stimulated by IL-4 and anti-CD40 antibodies.[102] Although IFN-γ may in part reproduce the effect of IL-12, the IL-12–induced inhibition of IgE production in vitro is not mediated through IFN-γ secretion because (1) anti–IFN-γ antibodies did not inhibit the IL-12 effect and (2) IL-12, but not IFN-γ, was effective in inhibiting IgE production in PBMC stimulated by IL-4 and anti-CD40 antibodies.[102] Thus, IL-12 may suppress IgE synthesis by inducing secretion of factors other than IFN-γ or by modulating the expression on T-helper cells of surface molecules, eg, the CD40-ligand, required for B-cell activation.

In vivo IP injection of IL-12 in mice resulted in enhanced IFN-γ and IL-10 gene expression, reduced basal levels of IL-3 and IL-4 gene expression, and increased serum IgG2a concentration.[104] The induction by IL-12 of IL-10 gene expression was unexpected and suggests that IL-12 in vivo activated the production of a cytokine that profoundly inhibits IL-12 production by a negative feedback mechanism.[30] It remains to be shown whether IL-12, directly or indirectly, induces production of IL-10 from T cells or from other cell types, eg, monocyte/macrophages. In mice that have been injected with goat antimouse IgD antibody, the simultaneous injection of IL-12 suppressed IgG and IgE response, whereas IL-12 had little effect on the IgE response in mice injected with anti-IgE antibodies.[104] When mice were immunized with a hapten-protein conjugate and treated with IL-12, a marked inhibition of IL-4 secreting cells and of antihapten serum IgG1 and IgG2b and an enhancement of IgG2a antibodies were observed, whereas little effect on serum IgG3 was noted.[105] The in vivo effects of IL-12 on Ig isotypes were either not affected[105] or incompletely inhibited[104] by anti–IFN-γ antibodies, indicating that the effect of IL-12 is not uniquely mediated by in vivo induction of IFN-γ. Consistent with these observations, the IFN-γ–inducing effect of IL-12 in vivo, but not its effects on Ig isotypes, were partially blocked by treatment of the mice with antiasialo GM1 (anti-NK cell) antibodies.[105] It is noteworthy that anti–IL-12 antibodies in vivo significantly blocked Th1 response to antigen, as evaluated by either IFN-γ production or serum IgG2a antibody response.[105] These results strongly suggest that IL-12 has an obligatory role for antigen-induced Th1 differentiation in vivo and its effects on Ig isotypes.

Role of IL-12 in Infectious Disease Models in Experimental Animals

The ability of IL-12 to induce acute production of IFN-γ and other phagocytic cell activating cytokines (eg, GM-CSF and TNF-α) is particularly important during acute bacterial infection as part of the innate resistance mechanisms. In these defensive mechanisms, NK cells are often responsible for the early production of IFN-γ; T cells, although probably also involved, are not essential.[64] The acute production of TNF-α, IL-12, and IFN-γ in the endotoxic shock model in mice[40] most likely represents an exaggeration of these innate resistance mechanisms, with uncontrolled and often lethal production of these inflammatory cytokines. In chronic infections, IL-12 is responsible for the development of Th1 responses that are generally protective for intracellular parasites, but ineffective against other parasites, eg, nematodes. Several experimental models of infectious diseases, reviewed below, are shedding light on these functions of IL-12 in innate and adaptive immune responses to infections.

Listeria monocytogenes. IFN-γ production by NK cells is necessary in combatting infection in SCID mice, which lack T and B cells, and also in immunocompetent mice.[1] Heat-killed *L. monocytogenes* in vitro induced splenocytes and macrophages from SCID mice to produce IL-12 and anti–IL-12 antibodies suppressed IFN-γ production by *L monocytogenes*-treated splenocytes, indicating that IFN-γ production in response to *Listeria* infection is mediated by IL-12.[62] TNF-α, a macrophage factor previously shown to be required for IFN-γ production in this experimental system,[106] is now identified as a costimulatory factor for IL-12–mediated induction of IFN-γ production in NK cells.[62] Endogenous production of IL-12 is critical for the survival of both immunocompromised SCID mice and normal C.B-17 control mice during a primary infection with a normally sublethal dose of *L monocytogenes*, because anti–IL-12 antibody-treated mice showed a decreased macrophage expression of class II MHC antigens and an increased *Listeria* burden in the spleen and eventually died.[107] The effect of IL-12 was likely mediated by IFN-γ production, because IFN-γ treatment of anti–IL-12—treated mice limited the spread of the infection and resulted in survival of the SCID mice.[107]

Toxoplasma gondii. *T gondii* is a protozoan parasite normally controlled by a strong and persistent cell-mediated immune response resulting in an asymptomatic chronic infection maintained by dormant tissue cysts. Similar to *L monocytogenes, T gondii* tachyzoites trigger IFN-γ production by NK cells through a pathway that involves the production from macrophage accessory cells of TNF-α and IL-12, cytokines that synergistically trigger the NK cell IFN-γ response.[34] Immunocompetent mice acutely infected with

sublethal doses of *T gondii* and treated with either anti–IL-12 or anti–IFN-γ were unable to develop an efficient Th1 response to the parasite and died within 2 weeks of the infection.[108] In contrast, neutralization of endogenously produced IL-12 had no effect when the antibodies are administered during chronic infection.[108] In agreement with the survival data, treatment with anti–IL-12 resulted in decreased IFN-γ and enhanced Th2 cytokine synthesis by splenocytes when administered during acute but not chronic toxoplasmosis.[108] However, the production of IFN-γ by Th1 $CD4^+$ cells was still required for resistance to chronic infection with *T gondii* because all mice with chronic infection treated with anti–IFN-γ antibodies, unlike those treated with anti–IL-12 antibodies, died within 2 weeks.[108] Overall, these data suggest that the early stimulation of IL-12 plays a major role in both the induction of resistance and Th1 cell subset selection in *T gondii* infection but it is not required for maintenance of established Th1 immunity.

Leishmania major. Infection of mice with the protozoan parasite *L major* is an established in vivo model for the definition of factors that contribute to $CD4^+$ Th cell subset development. Mice that naturally resolve their lesions, such as C3H or CD57BL/6, exhibit a dominant Th1 response, whereas mice that succumb to the infection, such as BALB/c, exhibit a dominant Th2 response to *L major.*[109] Treatment of susceptible BALB/c mice with daily doses of IL-12 for at least 1 week starting from the beginning of the *L major* infection resulted in the survival of most of the mice with a dramatic reduction of lesion size (footpad thickness) and parasite burden and provided durable resistance against reinfection.[110,111] Associated with these protective effects, IL-12 treatment induced a decreased production of IL-4 by the draining lymph node cells and increased production of IFN-γ, suggestive of a shift from a Th2 type of response to a predominantly Th1-type.[110,111] Delay of IL-12 treatment of 1 week or more resulted in ineffective protection against *L major* infection, suggesting that, similar to the observation with the Th1 response in *T gondii* infection, it is difficult to change the cytokine production phenotype of an established Th2 response.[111]

The development of a vaccination protocol for *Leishmaniasis* infection has encountered many difficulties both in experimental animals and in the clinical practice. A partial protection of BALB/c mice against *L major* infection was obtained by vaccination with a soluble leishmanial antigen in conjunction with IFN-γ and the bacterial adjuvant *Corynebacterium parvum.*[112] Vaccination of BALB/c mice with leishmanial antigen and IL-12 promoted the development of leishmanial-specific memory $CD4^+$ Th1 cells;[113] these vaccinated mice were completely resistant to subsequent infection with *L major.* Thus, IL-12 is an effective adjuvant for the initiation of protective cell-mediated immunity against leishmaniasis and may be an important component in other vaccines that need to induce cell-mediated immunity. This potential clinical use of IL-12 is particularly promising because only single or a few local injections of limited doses of IL-12 are required for vaccination, likely overcoming possible complications caused by toxicity of the cytokine. It should also be noted that the bacterial adjuvant *C parvum* is a powerful inducer of IL-12 production, suggesting the possibility that its adjuvant activity is at least in part mediated by IL-12 production.

The ability of IL-12 to render BALB/c mice resistant to *L major* infection raises the question whether IL-12 plays a role in the induction of a Th1 response in resistant strains of mice. In vitro, *L major* amastigotes, but not promastigotes, efficiently induced IL-12 production from macrophages;[114,115] because amastigotes are not present in vivo for several days after natural or experimental infection with metacyclic promastigotes, it was suggested that *L major* evade IL-12 induction by macrophages in the first few days of infection.[114] This hypothesis was supported by the observation that, in BALB/c and C57BL/6 mice injected with metacyclic *L major,* IL-12 p40 mRNA was not measurable in the draining lymph nodes until 7 days after infection.[114] However, IL-12 p40 protein was released by lymph node cells of C3H and BALB/c mice 1 day after infection with *L major,* but not in B57BL/6 mice.[115] IL-12 production declined at later times (7 to 14 days) in BALB/c mice, but not in C3H mice.[115] Neutralizing anti–IL-12 antibodies injected in resistant strains of mice at the time of infection prevented the early (3 days after infection) production of IFN-γ and appearance of NK cell cytotoxic activity in the lymph node; the development of a protective Th1 response was also prevented and the animals became unable to resist the *L major* infection.[111,115] These data suggest that C3H and BALB/c mice, but possibly not C57BL/6, produce IL-12 early during infection with *L major* and that IL-12 production is required for development of a protective Th1 response in C3H mice. Because no difference was observed in production of IL-12 or in the ability of NK and T cells of the resistant C3H or susceptible BALB/c to respond to IL-12, the genetic difference in the susceptibility of the two strains to *L major* is probably not due to IL-12. Preliminary evidence suggests the possibility that factors inhibitory of the IL-12 activity such as IL-4, IL-10, and, particularly, TGF-β could be responsible for the failure of BALB/c mice to develop a Th1 response.[115] TGF-β was indeed shown to inhibit in vitro the generation of a Th1 response induced by IL-12.[92] Once a Th2 response is established in BALB/c mice, Th2 cytokines such as IL-4 and IL-10 are probably responsible for the downregulation of IL-12 production.

Schistosoma mansoni. Schistosomiasis is a chronic helmintic disease, with morbidity primarily caused by fibrosis mostly resulting from the granulomatous response to parasite eggs in tissues.[116] Unexpectedly, the $CD4^+$ T-cell–dependent delayed-type hypersensitivity reaction responsible for granuloma formation is of Th2 type, although a Th0 response precedes the Th2 response and both IL-2 and IL-4 are required for granuloma formation.[117] Inhibition of IFN-γ or IL-12 using neutralizing antibodies in mice infected with *S mansoni* eggs resulted in a marked enhancement of granuloma formation, concomitant with an increased Th2- and decreased Th1-cytokine mRNA expression.[118] In contrast, treatment with IL-12 profoundly inhibited granuloma formation and increased Th1 cytokine expression, while decreasing Th2 cytokines.[118]

IL-12 could be used also in this experimental system on

an adjuvant in vaccination. Immunization with *S mansoni* eggs and IL-12 induced a commitment of their T cells to Th1 responses, which allowed only minimal granuloma formation upon subsequent egg challenge.[118] These results suggest that the use of IL-12 as adjuvant may allow the development of ''antipathology'' vaccines[118] that could prevent schistosoma egg pathology as well as other diseases caused by the production of Th2 cytokines. It is of interest that IL-12 inhibited secondary granuloma formation in mice presensitized with *S mansoni* eggs.[118] These latter results indicating that IL-12 can induce a Th1 response even in the presence of memory T cells generated during a Th2 biased immune response, confirm previous results in vitro in the human system[8,84] and indicate that the use of IL-12 as a therapeutical agent or an adjuvant in vaccination for inducing a Th1 response could be effective even in the presence of Th2-biased memory T cells.

Nippostrongylus brasiliensis. The immune response to helminths is often characterized by a bias to development of Th2 cells. The question of whether these responses are helpful or harmful has been a controversial issue; the emerging evidence is pointing to the conclusion that Th2 cells and IL-4 play an important role in the control of egg laying and expulsion of the worms.[119] *N brasiliensis* is an intestinal nematode parasite; when infective larvae of the nematode are inoculated orally, they stimulate IL-3, IL-4, IL-5, and IL-9 cytokine production that induces IgE, eosinophil, and mast cell responses. Systemic treatment with daily doses of IL-12 from the beginning of the infection inhibited IL-4, IL-5, and IL-9 gene expression and induced enhanced expression of IFN-γ and IL-10.[120] This effect of IL-12 on cytokine expression during infection was paralleled by a profound inhibition of IgE production and of the intestinal mast cell and eosinophil responses.[120] The inhibition of eosinophil response was sensitive to lower doses of IL-12 than the other responses. Anti–IFN-γ antibodies reversed IL-12 inhibition of mast cell and IgE responses, but have little effect on the eosinophil response.[120] If IL-12 treatment was delayed to 6 days after treatment, it was mostly ineffective, but the eosinophil response was still partially suppressed when the treatment was initiated as late as day 8.[120] IL-12 treatment, initiated within the first 6 days of infection, increased egg production and suppressed adult worm expulsion in *N brasiliensis*-infected mice.[120] Thus, IL-12 early treatment can suppress the Th2 response to the nematode and prevent the resolution of the infection; when a Th2 response is established, IL-12, as already shown in other systems, is ineffective in modulating the Th response. Interestingly, the IL-12 treatment during a primary infection partially prevented the development of Th2 responses during a secondary infection, whereas the IL-12 treatment during secondary infection was only partially effective in preventing the development of a Th2 response, possibly because it was antagonized by high endogenous levels of IL-4.[120]

Candida albicans. The outcome of systemic challenge of mice with the fungus *C albicans* is determined by immunologic events occurring shortly after infection; as in the case of *L major* infection, development of a Th1 response is protective, whereas an exacerbative Th2 response is observed in mice challenged with virulent *C albicans* strain or in the susceptible DBA/2 mice challenged with vaccine strain infection.[121] The expression of IL-12 p40 mRNA was readily detected in macrophages from healing mice, but was detected only early in infection in mice with progressive disease.[122] Although the mutually exclusive production of IL-4/IL-10 and IFN-γ by early $CD4^+$ T cells is likely to be the major discriminative factor of healing or nonhealing responses in murine candidiasis, IL-12 rather than IFN-γ production appears to be an indicator of early Th1 differentiation.[122] In vivo neutralization of IL-12 with specific antibodies ablated the development of anticandidal resistance and showed the requirement for IL-12 production in resistance to *C albicans* infection in mice.[123] However, in mice with progressive systemic disease as well as in a mucosal infection model, administration of IL-12 did not result in therapeutic activity, demonstrating the difficulty in affecting in vivo established or heavily biased Th2 responses.[123]

Virus infections. IL-12 as a potentiator of delayed type of hypersensitivity and of cytotoxic lymphocyte responses should be expected to play a role in the resistance against virus infections. However, surprisingly little information is available on either the importance of endogenous IL-12 or the effect of IL-12 treatment in these infections. Only the effect of repeated daily injections with IL-12 on lymphocyte choriomeningitis virus (LCMV) and murine cytomegalovirus (MCMV) infections in mice has been analyzed.[124] Surprisingly, IL-12 inhibited rather than enhanced CTL generation during LCMV infection.[124] This inhibitory effect, evident particularly at high doses of IL-12 ($\geq$0.1 μg daily), was paralleled by an inhibition of the LCMV-induced expansion of $CD8^+$ cells.[124] This decrease of $CD8^+$ cells was not observed in control or in MCMV-infected mice, although in these latter animals a significant decrease of splenic $CD4^+$ cells was observed at high IL-12 doses (1 μg daily).[124] Although an enhancing effect of IL-12 on the generation of antiviral CTL activity was not observed, a significant reduction in LCMV titer was observed in mice treated with low doses of IL-12 ($\leq$10 ng daily), whereas a 2 log increase was observed in mice treated with high doses (1 μg daily).[124] The mechanism of these effects have not been investigated, even if the induction of serum IFN-γ and TNF-α especially in the LCMV-infected animals treated with high IL-12 doses may explain some of the lymphotoxic effect of IL-12.[124] It is clearly important to extend the analysis of the role of IL-12 to other viruses and to investigate whether virus infection affects IL-12 production and whether IL-12 plays a role in the cell-mediated resistance against virus infections.

IL-12 and HIV

Infection with HIV induces an early deficiency in $CD4^+$ Th cell response and, primarily at late stages of the disease, a depression in NK cell cytotoxic activity and in vitro production of IFN-γ was reported.[125-127]

The depressed NK cytotoxic activity observed in HIV-seropositive patients at different stages of the disease is upregulated within a few hours of in vitro treatment with IL-12.[64] The cytotoxic activity of IL-12–treated PBL from HIV-infected patients was also efficiently enhanced, not only

against tumor-derived target cells but also against CMV-infected target cells, to levels similar or slightly higher than those mediated by untreated PBL from healthy donors.[64] IL-12 also induces IFN-γ production from PBL of AIDS patients, although at lower levels than from cells from healthy controls.[64,128] Furthermore, addition to IL-12 to PBL from HIV-infected patients in vitro restores in part their defective ability to respond to HIV peptides and to recall antigens or alloantigens.[128]

The ability of PBMC from HIV-infected patients to produce IL-12 in vitro was evaluated in response to *S aureus* stimulation.[37] On average, PBMC from the patients produced approximately 10-fold less IL-12 p40 and fivefold less IL-12 p70 than a panel of healthy donors. Unlike *S aureus*-induced p40 production, the low constitutive production of p40 detected in unstimulated cultures was not statistically different from controls. In contrast and under the same culture conditions, PBMC from HIV-infected patients produced threefold to fourfold more IL-6 both constitutively and after stimulation than control donors. The production of IL-10, a cytokine able to inhibit IL-12 production, as well as TNF-α and IL-1β, was not significantly different in PBMC from HIV-seropositive patients and from control donors. IL-4, a Th-2 cytokine reported to be overproduced in HIV-infected patients, was not detectable in *S aureus*-stimulated PBMC cultures. These data suggest that the defect in IL-12 production by PBMC of HIV-infected patients is relatively specific and not secondary to a generalized inability of their monocytes to produce cytokines or to overproduction of antagonistic Th-2 cytokines (eg, IL-4 and IL-10).[37]

Although some studies described a dysregulation of production of cytokines typical of Th-1 or Th-2 responses in PBMC or T-cell clones from HIV-infected patients assayed in vitro,[129-131] contradictory results have been reported by others analyzing cytokine transcript expression.[132,133] Several factors can influence the pattern of cytokines expressed or secreted and therefore might explain these disparate results: anatomic locations and differences in tissue or organ systems and source of APC. In addition, cell type, culture conditions, cloning procedures, cytokine detection assays, and the nature of the stimulus may also play a role in the production of cytokines and proliferation of T-helper cells.

Because IL-12 has an important role for macrophage activation, generation and stimulation of cytotoxic T cells[11] and NK cells,[2] immune responses to tumors,[134] and generation of IFN-γ–producing Th-1 cells,[10] the deficiency of this cytokine in HIV-infected individuals may play a role in progression of immunodeficiency. Several of the deficiencies in immune response parameters in HIV-infected individuals are compatible with a defect in IL-12 production early in the infection. The low NK cytotoxicity activity and impaired ability to produce IFN-γ often observed in HIV$^+$ patients could reflect the absence of sufficient levels of IL-12 during in vivo differentiation of NK and T cells. Although several studies[129-131,135,136] reported a defect in IFN-γ production from PBL and T cells during HIV infection, others described an elevated IFN-γ gene expression in PBMC and CD4$^+$ cells from HIV-infected donors as compared with control donors[133] and the presence of IFN-γ in the serum of HIV$^+$ donors.[137] However, these contrasting findings are not incompatible, because, due to activation by HIV infection or other HIV-associated factors or infections, T cells from patients might constitutively express IFN-γ transcripts and secrete IFN-γ. At the same time, because of a relative anergy[138] associated with the infection and possibly due, at least in part, to lack of priming for high IFN-γ production by IL-12, they might be partially unresponsive to stimulation by IFN-γ inducers. The decreased ability of T cells from HIV$^+$ patients to proliferate in response to recall antigens or alloantigens could also be compatible with a deficiency in IL-12 production, because IL-12, in addition to being an initiation factor in the immune response, favoring Th-1 cell development, is also an important and in some experimental conditions an essential growth factor for differentiated Th-1 cells.[41,81,82] Defective IL-12 production is likely to result in inefficient cell-mediated immune responses, although not necessarily in a dominance of Th-2 versus Th-1 cell development. The regulation of Th-1 and Th-2 responses in vivo is complex and requires the participation of several other factors in addition to IL-12, none of which probably plays an irreplaceable role. Several factors may render the analysis of the Th-1/Th-2 response in patients difficult. These factors include the possibility that anergy induction in cells with Th-0 or Th-1 characteristics, suggested as one of the possible mechanisms of the defective T-cell response in HIV$^+$ patients, might induce in these cells a cytokine profile and other characteristics typical of Th-2 cells.[139] A deficient production of IL-12 or a defective Th1 response may be responsible for the reduced resistance of AIDS patients to opportunistic infections. The requirement for IL-12 production and Th1 response in the resistance to *Toxoplasma* and *Candida*, two opportunistic pathogens in AIDS, has been reviewed above. Furthermore, it has been shown that IL-12 was produced in the pleural fluid of patients with *Mycobacterium tuberculosis*-induced tuberculous pleuritis and that anti–IL-12 antibodies partially inhibited the proliferative response of the pleural fluid lymphocytes of the patients in response to *M tuberculosis*.[140] These results indicate the possible importance of IL-12 production in the immune response to another opportunistic pathogen important in AIDS patients.

IL-12 and Antitumor Immunity

The ability of IL-12 to facilitate cell-mediated immune responses, including enhancement of NK cytotoxicity, generation of CTL, and macrophage activation, suggests that it could have a role in both the innate and adaptive resistance mechanisms against tumors. In vitro, IL-12 was shown to enhance the cytotoxicity mediated by NK cells from healthy donors against colon carcinoma and neuroblastoma cell lines,[141,142] to enhance cytotoxicity of NK cells from most hairy cell leukemia patients,[143] and to stimulate proliferation and cytotoxicity against autologous tumor cells mediated by lymphocytes infiltrating different types of tumors.[144,145]

Studies using transplantable tumors in experimental animals have shown a dramatic effect of IL-12 in decreasing tumor growth and metastasis formation and in significantly delaying death.[134] Systemic daily treatment (5 days per week) had a significant inhibitory effect on the growth of metastasis

induced by intravenous injection of B16 melanoma cells and efficiently inhibited the growth of subcutaneously injected tumors, even when treatment was initiated 2 weeks after tumor inoculation.[134] An inhibitory effect of IL-12 on tumor growth, with a greater than twofold increase in survival of inoculated animals, was also observed with the reticulum cell sarcoma M5076 and with the renal cell adenocarcinoma Renca.[134] In this latter tumor, complete remission, especially with peritumoral injection of IL-12, was observed in some animals; reinjection of the Renca cells in the ''cured'' animals resulted in delayed growth of the tumor, suggesting that IL-12 may induce a memory immune response against the tumor.[134] The antitumor effect of IL-12 is mostly independent of NK cells, but requires $CD8^+$ cells for an optimal response.[134]

The effect of paracrine secretion of IL-12 was examined using the poorly immunogenic BL-6 murine melanoma cell line admixed with allogeneic fibroblasts transfected with cDNAs encoding both chains of IL-12 and secreting biologically active IL-12 heterodimer.[146] The emergence of detectable tumors was significantly delayed in mice receiving injections of BL-6 admixed with transfected fibroblasts, but not in those receiving BL-6 admixed with control fibroblasts not producing IL-12; the delay was proportional to the amount of produced IL-12.[134] Immunization with irradiated tumor cells admixed with fibroblasts producing low levels of IL-12 followed by a subsequent tumor challenge resulted in delay of tumor appearance, indicating the possibility of using IL-12 in the preparation of cancer vaccines.[146] The tumors growing after injection of tumor cells admixed with IL-12–producing fibroblasts were characterized by an extremely reduced lymphocyte infiltrate and by a characteristic fibroblastic capsule around the tumor.[146] The C26 murine colon carcinoma induces tumors that are minimally sensitive to in vivo systemic treatment with IL-12; transduction into C26 cells of both IL-12 genes using a polycistronic retroviral vector resulted in cells that produced low levels of IL-12 and that were significantly delayed in inducing tumor formation in vivo.[147] The delayed growth of the IL-12–producing C26 cells was due to NK cells and, in part, to $CD8^+$ cells.[147] The in vivo growing tumors was characterized by an extremely poor lymphocytic infiltrate; however, in vivo depletion of $CD4^+$ cells resulted in a significant increase in tumor infiltration with $CD8^+$ and NK cells and in complete remission of the tumor in approximately half of the animals.[147] These results suggest that, as observed in other experimental tumor systems,[148,149] the IL-12–transduced C26 cells may activate $CD4^+$ cells that have an inhibitory activity on the tumor infiltration and antitumor activity of $CD8^+$ cells; the complexity of the cellular mechanisms involved in tumor immunity cautions against premature generalizations of the results obtained in few experimental models for the planning of possible therapeutic manipulation in cancer patients.

CONCLUSION

In the short time since its first description in 1989,[2] IL-12 has been emerging as a central cytokine in immune response, with the potential of therapeutic application in a variety of disease states. Being produced by phagocytic cells and other antigen-presenting cell types and involving in many aspects of its immunomodulating functions the participation of NK cells, IL-12 represents a bridge between innate resistance and adaptive immune response. IL-12 is produced early during the response to infectious agents or to other antigens and induces production of IFN-γ first primarily by NK cells and then by T cells. This early response is important for the activation of the phagocytic cell system as a first line of defense against infections, but the IL-12 produced in this early phase, often acting in combination with the induced IFN-γ, is also required for optimal generation of Th1 $CD4^+$ cells and CTL. The present evidence suggests that the bias of the immune system to either Th1 or Th2 response is regulated by the balance of IL-12 and IL-4 early during the immune response. IL-12 is therefore a true initiation cytokine for cell-mediated responses[150] and its production is a requirement for early activation of the phagocytic cell system and generation of Th1 responses. IL-12 is also a costimulatory factor for differentiated Th1 cells and required for optimal proliferation and IFN-γ production by Th1 cells in response to antigens. However, an established Th1 response is stable and IL-12 does not appear to be an absolute requirement for its maintenance. The central role of IL-12 in the biology of immune response suggests the possibilities of its therapeutic use in infectious diseases, allergic diseases, tumors, immunodeficiencies, and as an adjuvant in vaccination; conversely, the antagonists of IL-12 action might have indications in certain autoimmune diseases or parasitic infections. Because of the biologic and possibly clinical relevance of this cytokine, it is important that its biologic functions continue to be investigated and, in particular, that the molecular mechanisms leading to its production are elucidated.

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