Immunity, Vol. 13, 715–725, November, 2000, Copyright ©2000 by Cell Press

Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12

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Summary

A novel sequence discovered in a computational screen appears distantly related to the p35 subunit of IL-12. This factor, which we term p19, shows no biological activity by itself; instead, it combines with the p40 subunit of IL-12 to form a novel, biologically active, composite cytokine, which we term IL-23. Activated dendritic cells secrete detectable levels of this complex. IL-23 binds to IL-12Rβ1 but fails to engage IL-12Rβ2; nonetheless, IL-23 activates Stat4 in PHA blast T cells. IL-23 induces strong proliferation of mouse memory ($CD4^+CD45Rb^{low}$) T cells, a unique activity of IL-23 as IL-12 has no effect on this cell population. Similar to IL-12, human IL-23 stimulates IFN-γ production and proliferation in PHA blast T cells, as well as in CD45RO (memory) T cells.

Introduction

The proliferation, differentiation, and effector functions of immune cells are regulated in part by a network of soluble protein factors. Discrete families of these molecules have been identified by sequence and structural analysis. Within the diverse set of sampled protein folds, the four–α helix bundle topology uniquely distinguishes members of the hematopoietic cytokine family (Rozwarski et al., 1994). Among the members of this large family, a further clustering is evident. Within these subfamilies, members often display faint but significant stretches of amino acid similarity that are indicative of a closer evolutionary and functional relationship.

A particularly important subfamily of helical cytokines encompasses a set of factors that are related to interleukin-6 (IL-6). These now include IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and novel-neurotrophin-1 (NNT-1). This group of cytokines exhibits a wide range of often overlapping biological functions that are transmitted via multichain cell surface receptors, which are typically formed by high-affinity, cytokine-specific receptor chains and lower-affinity, signal-transducing chains. The presence of shared signal-transducing receptors offers an explanation for the overlapping functions of IL-6-like cytokines.

Two other IL-6-like cytokines, G-CSF and the p35 subunit of IL-12, signal through private superfamily receptors. Within this outlier group, G-CSF is a biologically active protein that binds directly to a specific, signal-transducing G-CSFR. By contrast, IL-12p35 is not active on its own and instead forms part of a composite factor with a chain known as p40 (Kobayashi et al., 1989). Whereas p35 shows homology to the IL-6-type cytokines and G-CSF, p40 is a soluble member of the cytokine receptor superfamily. Only the composite p35p40 IL-12 molecule was found to display biological activity, although a p40 homodimer may function as an IL-12 antagonist by competing for the IL-12 receptor (Gately et al., 1996).

IL-12 plays a critical role in cell-mediated immunity (Gately et al., 1998; Trinchieri, 1995, 1998). Its activities are triggered through a high-affinity receptor complex that gathers two closely related subunits, IL-12Rβ1 and β2 (Chua et al., 1995; Presky et al., 1996b). Although no evidence exists for IL-12 receptor promiscuity, the p35 subunit has been suggested to bind to a second soluble cytokine receptor called EBI3 (Devergne et al., 1997). However, no biological activity has been reported for the p35-EBI3 pair. By contrast, phenotypic differences between IL-12 p40-deficient mice and IL-12 p35-deficient mice with respect to the clearance of bacterial infections (Decken et al., 1998; Brombacher et al., 1999) invite the possibility that p40 is involved in functional complexes with molecules other than p35.

In this report, we describe a novel helical cytokine that was identified by searching sequence databases with a computationally derived profile of IL-6 subfamily structures. This protein was designated p19, representing its approximate molecular weight. We further show that p19 is part of a novel composite factor that consists of a disulfide-bridged complex between p19 and the p40 subunit of IL-12. This novel p19p40 complex is naturally expressed by activated mouse and human dendritic cells and has biological activities that are similar to but distinct from IL-12. These activities result from interaction of the p19p40 complex with IL-12Rβ1 and an additional, novel receptor subunit.

Results

p19 Was Identified Computationally

We searched sequence databases with a computationally derived profile (Gribskov et al., 1987) of members of the interleukin-6 (IL-6) helical cytokine family. This search led to identification of a novel cytokine, which we named p19. The p19 cDNA sequences encode 189/196 amino acid polypeptides (human and mouse, respectively) corresponding to mature proteins with calculated molecular weights of 18.7 and 19.8 kDa. Both proteins contain five cysteine residues and no N-glycosylation sites. Human and mouse p19 are 70% identical and most

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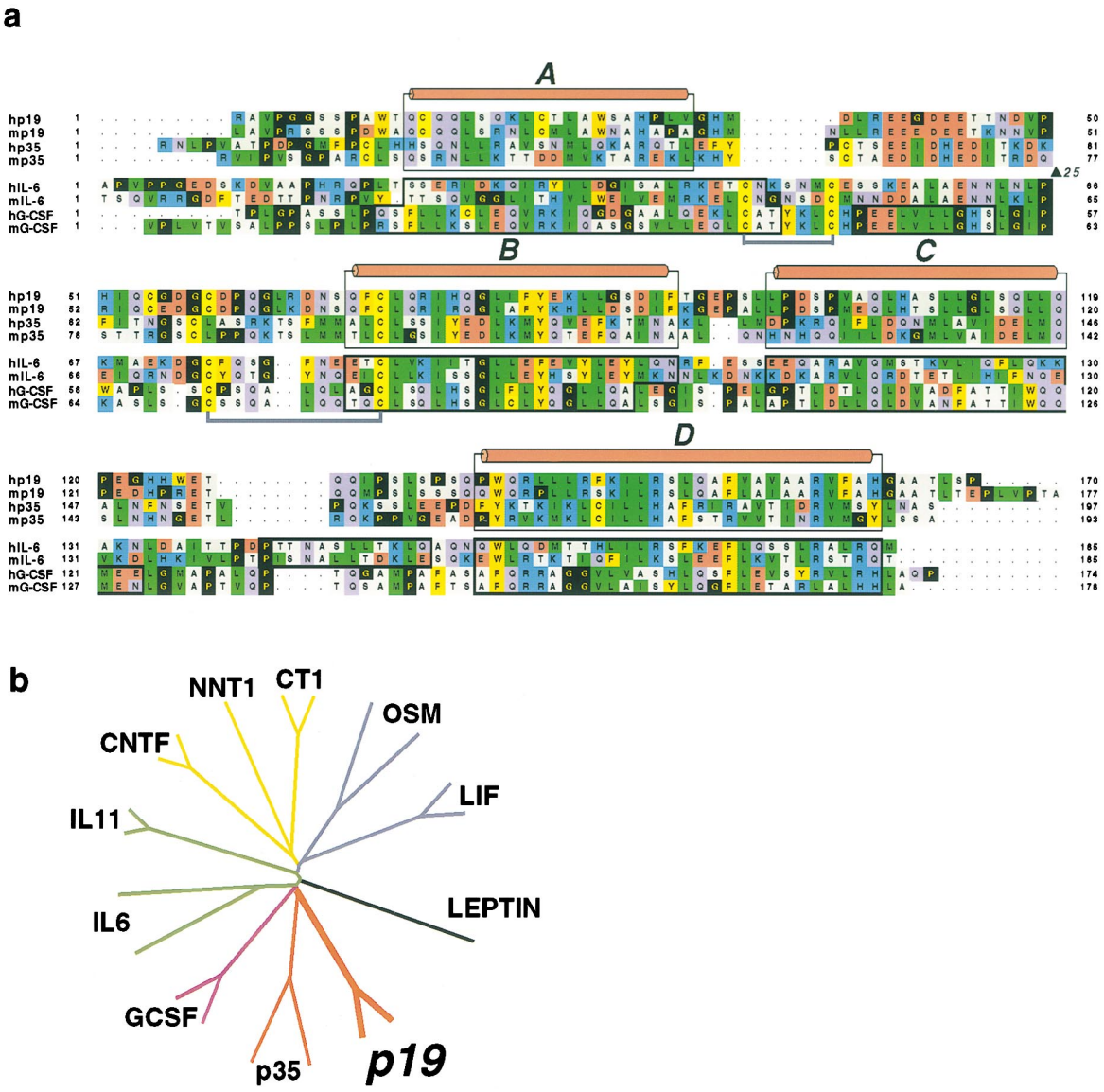

Figure 1. Alignment of Amino Acid Sequences for Selected Members of the Long Chain α-Helical Cytokine Family

(A) Sequences for all cytokines are shown as mature peptides (human p35, GenBank accession number B38957; mouse p35, NP_032377; human IL-6, P05231; mouse IL-6, P08505; human G-CSF, NP_000750; and mouse G-CSF, NP_034101). The alignment is based on the overlapping structures of IL-6 (PDB entry code 1il6) and G-CSF (PDB entry code 1rhg). The α helices of hIL-6 and hG-CSF are boxed according to their secondary structures. The four helices predicted for p19 and p35 are labeled A, B, C, and D. The two disulfide bonds in IL-6 and G-CSF are numbered 1 and 2. Closed triangle indicates the position of 25 additional amino acids in human and mouse p35, not shown in the alignment, but that likely pack against the helix bundle. The amino acid coloring scheme depicts chemically similar residues: green (hydrophobic), red (acidic), blue (basic), yellow (C), orange (aromatic), black (structure breaking), and gray (small).

(B) Evolutionary dendrogram of selected cytokines, showing the branching pattern that captures the various IL-6-like subgroups. The tree is rooted by leptin.

closely related to IL-12p35, IL-6, and G-CSF (Figure 1A). An evolutionary dendrogram shows p19 and IL-12p35 as closest neighbors (Figure 1B).

p19 and IL-12p40 Form a Novel Composite Factor

The presence of a signal peptide suggested that p19 would be secreted when expressed in mammalian cells. However, upon transfection of 293T cells with tagged forms of human or mouse p19, only a small amount of mouse (Figure 2B, lane 2) but not the human protein (Figure 2A, lane 2) could be immunoprecipitated from the supernatant. Both proteins could be detected in the cellular lysates of transfected cells, indicating inefficient secretion (data not shown). mp19 transfection supernatant or semipurified mp19 protein was inactive when analyzed in various bioassays (data not shown). We next investigated the possibility that p19 is part of a composite factor like IL-12 (Gubler et al., 1991; Wolf et al., 1991) and requires coexpression with a partner protein to be efficiently secreted. p19 was coexpressed with various nonsignaling receptors of the IL-6 family, including

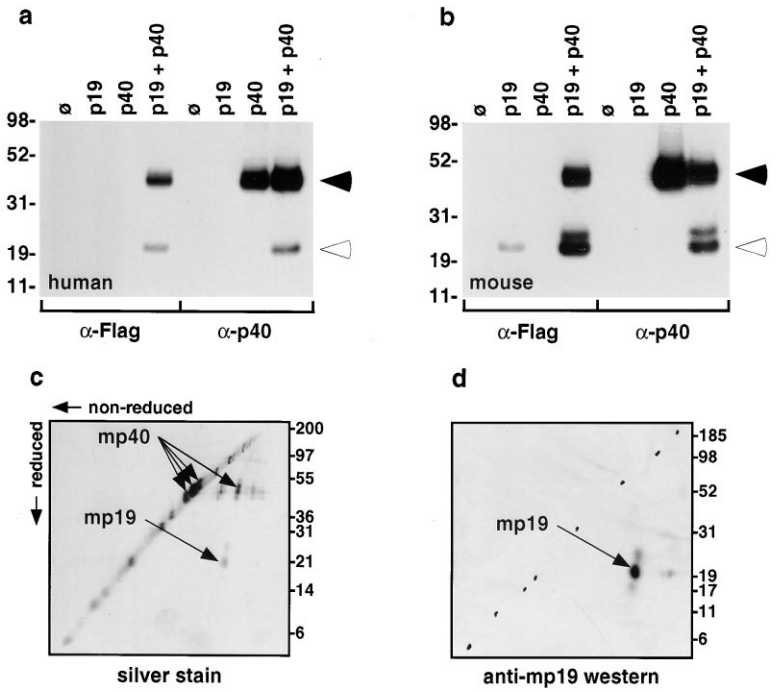

Figure 2. IL-12p40 and p19 Form a Soluble Disulfide-Linked Heterodimer

(A and B) 293T cells were transiently transfected with empty vector (lanes 1 and 5) or expression vectors for p19-Flag (lanes 2 and 6), IL-12 p40 (lanes 3 and 7), or both (lanes 4 and 8). Twenty-four hours after transfection, cells were metabolically labeled for 16 hr, and proteins were immunoprecipitated from supernatant with anti-Flag agarose or p40-specific antibodies coupled to protein G sepharose. Immunoprecipitation of the human (A) and the mouse (B) p19p40 complex.

(C and D) Nonreduced followed by reduced SDS-PAGE on mouse p19p40 supernatant purified from adenovirus coinfected 293T cells by heparin chromatography. (C) Silver stained gel. (D) Anti-mp19 Western blot. Molecular weight markers are indicated in kDa.

tagged forms of EBI3, soluble IL-6R, NR6 (CLF-1), and IL-12p40. Among these molecules, only the coexpression of p19 and IL-12p40 led to enhanced secretion of p19 (Figure 2, lanes 4 and 8). Moreover, both proteins were coimmunoprecipitated with antibodies against either tagged p19 or p40 (anti-Flag or anti-p40, respectively), indicating that p19 and IL-12p40 form a soluble cytokine/receptor complex similar to that of IL-12 p35p40. A small amount of mp19 migrates at a slightly higher molecular weight (Figure 2B). The higher band could result from O-linked glycosylation. Several potential O-linked glycosylation sites are predicted (Hansen et al., 1998). To investigate whether p19p40 is a disulfide-linked heterodimer like IL-12, the complex was purified from adenovirus p19 and p40 coinfected 293 cells and analyzed on two-dimensional SDS gels. Silver staining of the gel showed the presence of p19p40 disulfide-linked heterodimer in addition to free p40 monomers and dimers (Figure 2C). The identity of the mouse p19 spot on the silver stained gel was confirmed by anti-mp19 Western blot (Figure 2D).

Natural p19p40 Is Expressed by Activated Dendritic Cells

Expression analysis of mouse cDNA libraries showed the presence of p19 mRNA in various tissues and cell types (Figure 3A). Highest mRNA levels were found in polarized Th1 cells and activated macrophages. We could not detect p19 mRNA in bone marrow–derived dendritic cells. In contrast, both mouse and human dendritic cells derived from peripheral blood monocytes express high levels of p19 mRNA (Figure 3B). Monocyte-derived dendritic cells cultured in GM-CSF and IL-4 and activated via CD40 for 2 days also express high levels of p40 mRNA and to a lesser extent p35 mRNA (Figure 3B). We analyzed natural p19p40 in the supernatants of activated mouse and human dendritic cells. Mouse and human dendritic cells derived from peripheral blood adherent cells were stimulated with GM-CSF and IL-4 for 5 days. After culture with TNFα, LPS, and anti-CD40 antibody for 2 days, the culture supernatant was harvested. A native mouse p19p40 complex could be detected in a sandwich ELISA (Figure 4A) as well as by SDS-PAGE/anti-mp19 Western blot (Figure 4B). Similarly, a native human p19p40 complex was immunoprecipitated from activated human DC with anti-hp40 mAb. The precipitate was resolved on IEF/SDS-PAGE 2D gels (Figure 4D) and compared to the patterns obtained with similar precipitates from specific 293T cell cotransfection experiments (Figure 4C).

p19p40 Specifically Acts on Mouse Memory T Cells

To investigate the biological effects of the p19p40 complex, we initially engineered a soluble tagged fusion protein by flexibly linking the mouse p40 chain to mouse p19 (Hy-p40-p19). This chimeric molecule follows the design of the "hyper-IL-6" cytokine (IL-6Rα-IL-6, (Fischer et al., 1997) that shows greater solution stability, presumably because the cytokine and its cognate receptor domains are closely tethered and form a composite binding surface for the cellular receptors. A similar p40-p35 fusion protein shows specific activity identical to native IL-12 (Anderson et al., 1997). For our biological studies, we have used both mouse and human Hy-p40-p19 proteins. Experiments were also performed with conditioned medium of p19p40 cotransfected cells or adeno coinfected cells with identical results. First, we investigated the role of the Hy-p40-p19 on $CD4^+$ T cell subsets in comparison to IL-12. Naive T cells ($CD4^+CD45RB^{high}$) and memory/activated T cells ($CD4^+CD45RB^{low}$) were sorted from IL-10-deficient mice, which have elevated levels of memory T cells compared to wild-type mice (Davidson et al., 2000). The T cell subsets were stimulated with anti-CD3 mAb for 5 days

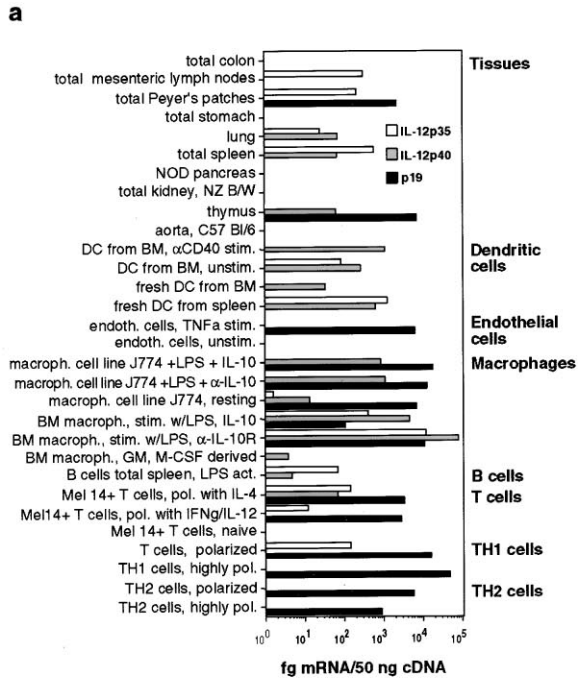

Figure 3. Distribution of Mouse p19, IL-12p35, and IL-12p40 mRNA in Various Tissues and Cell Types

(A) Quantitative PCR on various mouse libraries. (B) Quantitative PCR on human cultured dendritic cells. Dendritic cells were derived from human monocytes cultured in GM-CSF and IL-4 for 6 days and activated for 2 days in coculture with CD40L transfected L cells. Total RNA was isolated and analyzed for expression of p19, IL-12p40, and IL-12p35 mRNA by using the Taqman technique. mRNA levels are expressed in fg per 50 ng of cDNA.

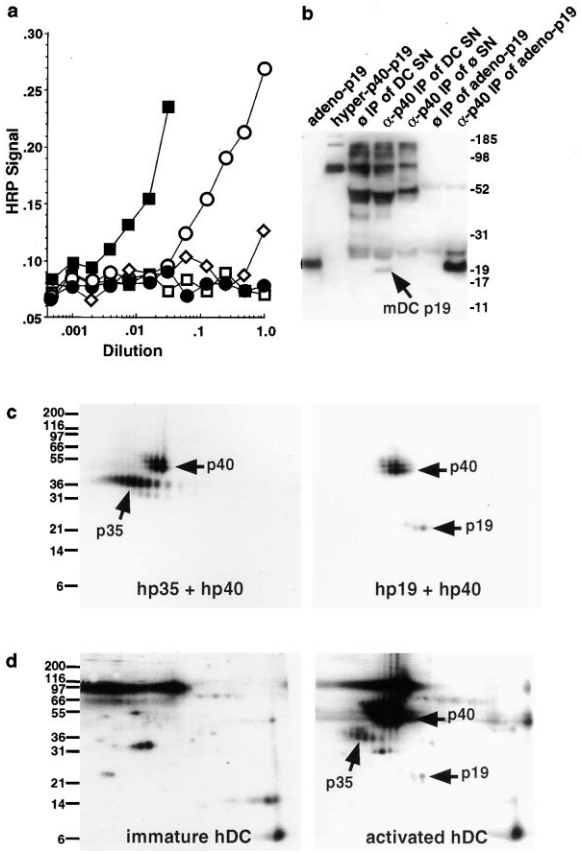

Figure 4. Natural p19p40 Heterodimer in Activated Mouse and Human Dendritic Cell Supernatants

DC supernatants and control medium were concentrated prior to detection by ELISA or immunoprecipitation (IP).

(A) Sandwich ELISA on activated (open circles) and immature (closed circles) mouse DC supernatant. Medium (open squares), 1000 ng/ml IL-12 (open diamonds), and 100 ng/ml Hy-p40-p19 (closed squares) were used as controls. Plates were coated with mp19-specific mAb 20C10. Bound cytokine was detected with biotinylated anti-mp40 mAb and HRP-streptavidin.

(B) Detection of mouse DC-produced p19 by Western blot. Control medium and DC supernatant were treated with rat anti-mp40 and isotype control beads, respectively. Blotted protein was detected with rat anti-mp19 mAb 10A11 and HRP-conjugated anti-rat antibody.

(C) IEF/SDS-PAGE 2D gels of human IL-12 p35p40 (left) and p19p40 (right) from 293T cotransfected cells.

(D) Human immature (left) and activated (right) dendritic cell supernatants.

in the presence of anti-IL-2 mAb and various amounts of IL-12 or Hy-p40-p19. Upon stimulation in the presence of IL-12, naive ($CD4^+CD45RB^{high}$) but not memory T cells ($CD4^+CD45RB^{low}$) proliferated (Figure 5A). In contrast, Hy-p40-p19 protein had no effect on naive T cells but strongly induced proliferation of memory T cells. The stimulatory effect on the memory T cells was 100-fold lower in the presence of a neutralizing anti-IL-12p40 antibody. The same proliferative signal could be induced by stimulation with conditioned medium of p19p40 cotransfected cells (Figure 5B). Similar results were obtained with memory T cells from wild-type mice; however, their response to Hy-p40-p19 was always less than

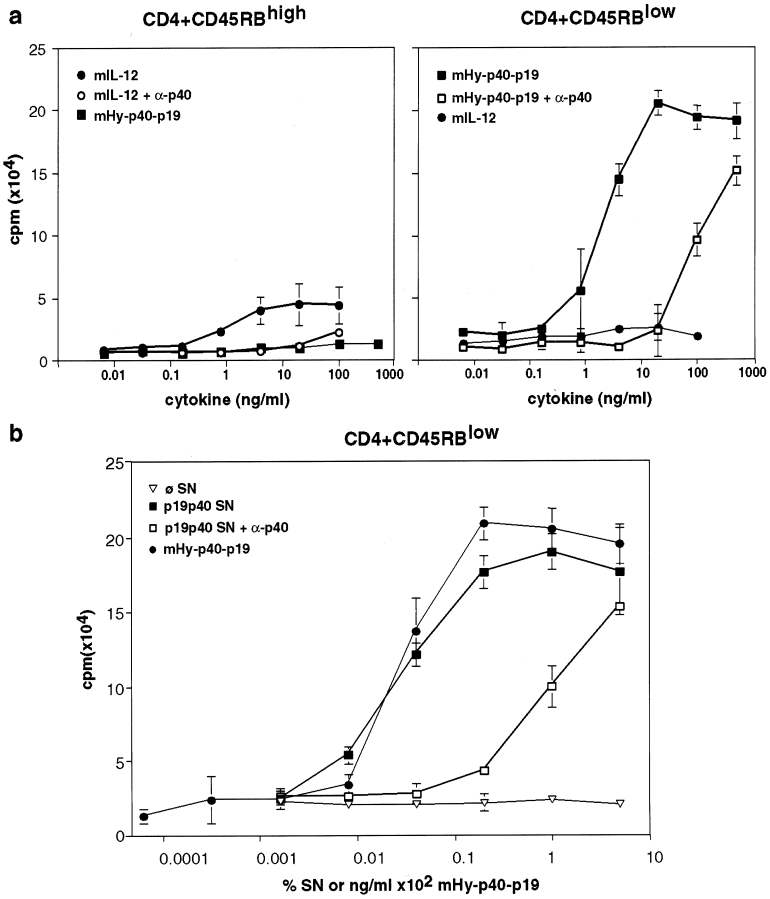

Figure 5. p19p40 Stimulates the Proliferation of Mouse Activated/Memory T Cells but Not Naive T Cells

(A) Sorted $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cells were cultured with plate-bound anti-CD3 mAb. Anti-IL-2 mAb (100 μg/ml) was added to all cell cultures. Mouse IL-12 or purified mouse Hy-p40-p19 was titrated into the cultures, ± anti-IL-12(p40) mAb (250 μg/ml). [3H]TdR incorporation (counts per minute) was assessed after 5 days of culture. Data are mean ± SD of triplicate wells and are representative of three experiments.

(B) Sorted $CD4^+CD45RB^{low}$ similarly treated. Purified mouse Hy-p40-p19 (started at 500 ng/ml) or supernatant from 293T cells cotransfected with p19 and p40 (started at 5% by volume) was titrated into the culture, ± anti-IL-12(p40) mAb (250 μg/ml). [3H]TdR incorporation (counts per minute) was assessed after 5 days of culture. Data are mean ± SD of triplicate wells.

the response of T cells from IL-10-deficient mice (data not shown).

p19p40 Acts on Human Memory and Naive T Cells

We next analyzed the ability of human Hy-p40-p19 to induce proliferation and IFN-γ production in human PHA blast T cells. Hy-p40-p19 significantly enhanced the production of IFN-γ by 7-day-old PHA blasts activated by plate-bound anti-CD3 and soluble anti-CD28 mAbs (Figure 6A). The maximal levels of IFN-γ production induced by saturating amounts of Hy-p40-p19 were lower compared to those induced by saturating amounts of IL-12. The IFN-γ enhancing effects of both Hy-p40-p19 and IL-12 could be blocked by anti-IL-12Rβ1 or anti-p40/p70 antibodies. However, the anti-p35 antibody only blocked the IFN-γ production induced by IL-12 and not that induced by Hy-p40-p19. The low level IL-4 production by PHA blasts was not affected by Hy-p40-p19 or IL-12 (data not shown). Hy-p40-p19 and IL-12 enhanced the proliferation of PHA blasts (Figure 6B). Again, the effects of Hy-p40-p19 on proliferation could be blocked by anti-IL-12Rβ1 or anti-p40/p70 but not by anti-p35 antibodies.

We also compared the activity of Hy-p40-p19 and IL-12 on CD45RA (naive) and CD45RO (memory) T cell populations isolated from PBMC. FACS-sorted CD45RA or CD45RO T cells were activated by plate-bound anti-CD3 antibodies and soluble anti-CD28 mAbs in the absence or presence of Hy-p40-p19, IL-12, or IL-2. Activated CD45RA T cells did not produce IFN-γ in the absence of Hy-p40-p19 or IL-12, whereas CD45RO T cells produced moderate amounts at day 3 and day 6 (Figure 6C). Addition of Hy-p40-p19 to these cultures did not enhance IFN-γ production by CD45RA T cells at 3 days after activation but caused a modest increase in IFN-γ production at day 6. In contrast, Hy-p40-p19 enhanced IFN-γ production by CD45RO T cells at both day 3 and day 6 after activation. Addition of IL-12 slightly enhanced production of IFN-γ by CD45RA T cells at day 3 but induced a significant increase at day 6, which is in agreement with the previously described requirement for upregulation of IL-12Rβ2 expression on naive T cells (Rogge et al., 1997). In addition, IL-12 significantly enhanced the production of IFN-γ by CD45RO T cells at both day 3 and day 6. Expression of IFN-γ was in all cases strongly dependent on the endogenous production of IL-2 since neutralization with anti-IL-2 and anti-IL-2Rα mAbs abolished it completely (data not shown). Neither Hy-p40-p19 nor IL-12 induced IFN-γ production by CD45RA or CD45RO T cells in the absence of activation by anti-CD3 and anti-CD28 mAbs (data not shown).

Similar to the experiments in the mouse, Hy-p40-p19 induced a more pronounced effect on proliferation of CD45RO T cells as compared to CD45RA T cells at day 6 after activation in the absence of endogenous IL-2 (Figure 6D). However, both CD45RA and CD45RO T cells could respond to IL-12 under these conditions. These results indicate that Hy-p40-p19 acts on human CD45RO

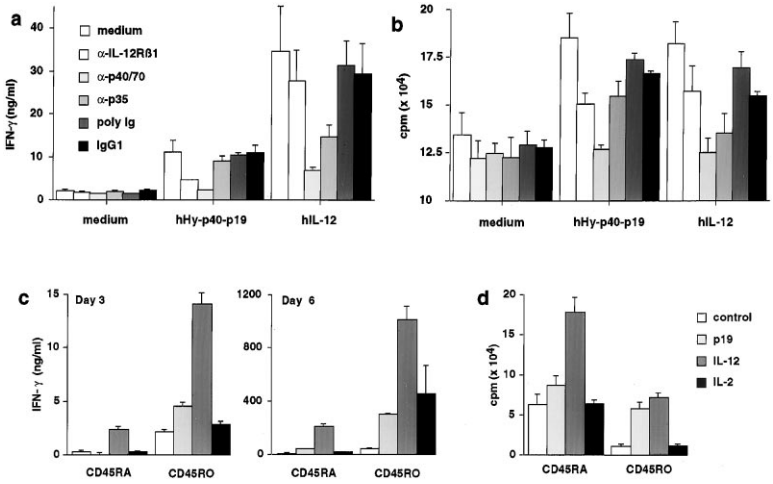

Figure 6. p19p40 Stimulates the IFN-γ Production and the Proliferation of Human PHA Blasts and Activated/Memory T Cells

(A) PHA blasts were derived from cultured PBMC in PHA (0.1 μg/ml)- and IL-2 (10 U/mL)-containing medium. After 7 days, cells were stimulated for 60 hr with 40 ng/ml hHy-p40-p19 or 1 ng/ml IL-12 and 1 μg/ml soluble anti-CD28 in 96-well plates coated with 10 μg/ml anti-CD3 mAb. IFN-γ was measured by ELISA.

(B) Remaining cells were then pulsed with 1 μCi/well ^{3}H-thymidine (NEN) for 6 hr, and incorporation of ^{3}H-thymidine was determined by scintillation counting. All blocking antibodies and isotype controls were used at 10 μg/ml.

(C and D) p19p40 has a more pronounced effect on human CD45RO (memory) than on CD45RA (naive) T cells. FACS-purified CD45RA and CD45RO T cells were cultured on anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) coated 96-well plates and stimulated with 40 ng/ml hHy-p19-p40, 1 ng/ml IL-12 (R&D Systems), or 100 U/ml IL-2 (R&D Systems) for 60 or 136 hr. IFN-γ production (C) and proliferation (D) were determined as in (A) and (B).

memory T cells and on CD45RA naive T cells but on the latter population only after a prolonged activation period.

Hy-p40-p19 Binds to IL-12Rβ1 but Not to IL-12Rβ2

To investigate the molecular basis of the effects described above, we analyzed the signaling receptors used by p19p40. Ba/F3 cells were infected with retroviral constructs encoding human or mouse forms of IL-12Rβ1 or -β2 (Gubler and Presky, 1996; Wu et al., 1996) (Figure 7). Ba/F3 cells expressing hIL-12Rβ1 bound human Hy-p40-p19 and hIL-12 (Figure 7A). Binding of mouse Hy-p40-p19 could be detected only on cells expressing the IL-12Rβ1 but not on cells expressing the IL-12Rβ2 (Figures 7B and 7C). Ba/F3 cells tranfected with both receptor subunits proliferated in response to IL-12 but not upon addition of Hy-p40-p19 stimulation (Figure 7D). Taken together, our data show that p19p40 and IL-12 share IL-12Rβ1 but not IL-12Rβ2 in their respective signaling complexes.

Hy-p40-19 Activates Stat4

IL-12 stimulation results in the activation of Stat4 (Bacon et al., 1995; Jacobson et al., 1995). IL-12Rβ2 has been identified as the subunit that links IL-12 signaling to Stat4 activation (Naeger et al., 1999). Since p19p40 does not share this receptor with IL-12 but presumably uses its own unique receptor subunit in addition to IL-12Rβ1, we asked whether p19p40 can activate Stat4. Hy-p40-p19 significantly induced the activation of Stat4 in human PHA blast T cells (Figure 7E). The level of activation was always less than that observed with IL-12 stimulation, which is in agreement with the lower levels of IFN-γ induced by p19p40 in these cells. This result suggests that p19p40 is linked to the Stat4 signaling pathway. The identification of a specific p19p40 receptor is currently under investigation.

Discussion

We describe a novel four–α helix cytokine called p19 that is most closely related in structure to IL-12p35. This cytokine is also similar to IL-12p35 in other ways: (1) p19 cosecretes with the p40 subunit of IL-12; (2) formation of biologically active p19p40 heterodimer requires synthesis of both subunits within the same cell; (3) the p19p40 interaction is stabilized by an interchain disulfide bond; and (4) p19p40 binds to IL-12Rβ1, the p40-specific component of IL-12R (Presky et al., 1996a). p19p40 differs from p35p40 in requiring an as yet unidentified transmembrane receptor subunit to complete the p19p40 signaling receptor complex. The identification of a receptor common to both signaling complexes provides a molecular rationale for our finding that p19p40 shows both overlapping and unique functions in comparison with IL-12. The interleukin designation for p19p40 is interleukin-23.

IL-12 p35p40 is mainly produced by monocytes, macrophages, and other antigen-presenting cells. Similarly, p19p40 is produced by activated human and mouse dendritic cells. In fact, in the supernatant of activated human dendritic cells we find evidence for the simultaneous production of both IL-12 and p19p40 heterodimers (Figures 4C and 4D). Although p19 mRNA is expressed in endothelial cells and polarized T cells, p40 is not found in these cells. The availability of functional IL-12 is also limited by the expression of p40 and not by p35, which is expressed in most cell types at low levels (D'Andrea et al., 1992). This suggests that functional expression of both composite factors is regulated in a similar fashion. p19 has no N-glycosylation sites. In contrast, p35 is extensively posttranslationally modified by N-linked glycosylation. Recent studies show that these modifications of p35 are a required step in the secretion of the p35p40 complex (Carra et al., 2000). Secretion of p40 does not require such modifications. Since secretion of neither p40 nor p19 depends on the addition of N-linked oligosaccharides, it is possible that upon activation of antigen-presenting cells capable of producing both factors p19p40 is produced at earlier times.

Our initial attempts to detect a biological activity for p19 by itself failed. Partially purified mouse p19 had no biological activity on its own when tested in the same

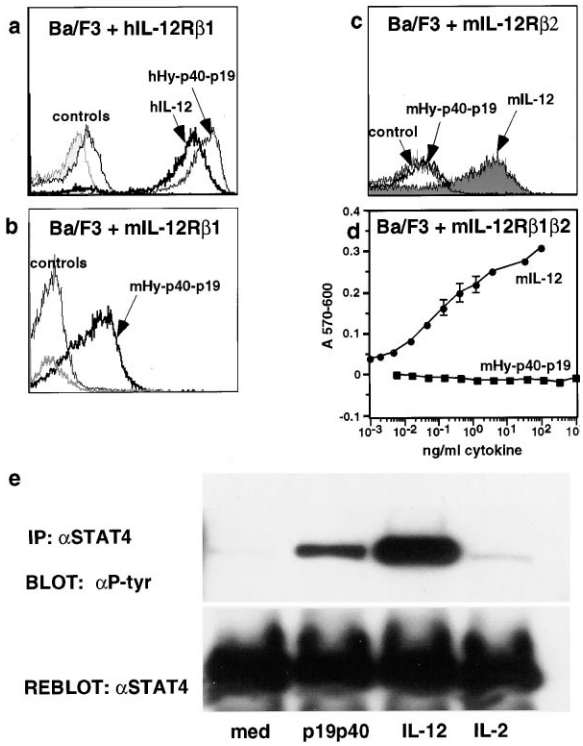

Figure 7. The p19p40 Complex Binds to IL-12Rβ1 but Not to IL-12Rβ2 and Activates Stat4

Cells were infected with retroviral supernatants for 24–48 hr and then incubated with cytokines. Bound Hy-p40-p19 was detected using an anti-Flag mAb, bound IL-12 with anti-p40 mAb.

(A) Ba/F3 cells expressing hIL-12Rβ1 bind human Hy-p40-p19. Controls, no antibody and secondary antibody only; hIL-12, 1 μg/ml; hHy-p40-p19, 1 μg/ml.

(B and C) Ba/F3 cells expressing mIL-12Rβ1 but not mIL-12Rβ2 bind mouse Hy-p40-p19. (B) Controls, Ba/F3-mIL-12Rβ1, no antibody and secondary antibody only; Ba/F3-mIL-12Rβ1, 1 μg/ml mouse Hy-p40-p19. (C) Control, Ba/F3-mIL-12Rβ2, secondary antibody only; mIL-12, 1 μg/ml; mouse Hy-p40-p19, 1 μg/ml.

(D) Ba/F3 cells expressing mIL-12Rβ1 and β2 respond to IL-12 but not Hy-p40-p19. Cells were stimulated with varying cytokine concentrations, and proliferation was measured as metabolic activity visualized by the reduction of a colorimetric REDOX indicator. (Closed circles) and (closed squares), stimulation of Ba/F3-IL-12Rβ1β2 with mIL-12 or mHy-p40-p19, respectively.

(E) Hy-p40-p19 induces phosphorylation of Stat4. Day 3 PHA blasts were incubated with medium, Hy-p40-p19 (1 μg), IL-12 (50 ng), or IL-2 (1000 U) for 20 min and lysed. Stat4 immunoprecipitates were run on denaturing gels, transferred and blotted with anti-phosphotyrosine mAb 4G10, and stripped and reblotted with anti-Stat4 mAb.

in vitro assays that later identified p19p40 as the active complex (data not shown). Additionally, administration of p19 adenovirus to mice did not reveal a biological response. Similarly, transgenic mice expressing mouse p19 under the control of a liver-specific promoter showed no obvious abnormalities (M. Wiekowski, unpublished data). In contrast, transgenic mice expressing mouse p19 mRNA in multiple tissues display a striking phenotype characterized by runting, systemic inflammation, infertility, and premature death (M. Wiekowski et al., unpublished data). Moreover, this phenotype can be transferred to irradiated mice transplanted with p19 transgenic bone marrow cells indicating that hematopoietic cells from the bone marrow produce biologically active p19. Together, these experiments indicate that p19, either administered as protein or expressed via mRNA on its own, is not sufficient to elicit a biological response.

In characterizing the biological activity of p19p40, we initially compared it to IL-12. IL-12 is a cofactor that synergizes with IL-2 to enhance the proliferation, cytotoxicity, and production of cytokines, in particular IFN-γ, by T cells and NK cells (Trinchieri, 1995; Gately et al., 1998). Furthermore, IL-12 induces the differentiation of naive T cells into Th1 cells (Hsieh et al., 1993; Manetti et al., 1993). Like IL-12, p19p40 enhanced proliferation and production of IFN-γ by activated human PHA blast T cells. The maximum levels of IFN-γ production induced by p19p40 were always lower than those induced by IL-12, even at saturating levels of the added cytokines. This characteristic may make p19p40 a more suitable entity to stimulate the cell-mediated immune response in cancer patients, as IL-12 administration leads to severe cytotoxicity associated with extremely high IFN-γ levels in the serum of such patients (Leonard et al., 1997). The immunoregulatory effects of p19p40 were also examined on $CD4^+$ naive and memory T cell subsets in both mice and human. In mice, p19p40 did not enhance proliferation of IL-12-responsive naive CD45RB high cells. Similar observations were made on human CD45RA naive T cells. However, CD45RA T cells could respond to p19p40 by increasing production of IFN-γ after prolonged stimulation. This might be explained if, like IL-12Rβ2, the p19p40- specific receptor subunit is not present on freshly isolated naive T cells but is induced following TCR triggering. In contrast, both mouse and human memory T cells respond strongly to p19p40 by enhanced proliferation and, in the case of human cells, by enhanced IFN-γ production. Interestingly, human memory T cells but not mouse memory T cells were also very responsive to IL-12. This may represent a true species difference or more likely indicates that the subpopulations of T cells as defined by these CD45 isoforms are not equivalent.

IL-12 plays an important role in promoting cell-mediated immunity against microbial pathogens. Endogenous IL-12 is essential for the host defense against *Mycobacterium tuberculosis*, *Leishmania major*, *Listeria monocytogenes*, and *Klebsiella pneumoniae*, as determined by neutralization with blocking anti-IL-12 p40 antibodies (Tripp et al., 1994; Greenberger et al., 1996; Cooper et al., 1997; Mattner et al., 1997). However, these same antibodies, as shown here, also block the activity of p19p40. Thus, studies using anti-p40 antibodies address the role of both IL-12 and p19p40. Analyses of IL-12p35- and IL-12p40-deficient mice have shown that p35-deficient mice are less susceptible to *L. monocytogenes* infection than p40-deficient mice (Brombacher et al., 1999); in addition, IL-12 p40-deficient mice died earlier and developed higher organ burdens following infection by *Cryptococcus neoformans* than p35-deficient mice (Decken et al., 1998). Since p35-deficient mice are unable to produce IL-12 but should still be capable of making p19p40, this observation suggests that p19p40 can contribute to clearance of *Listeria* and *Cryptococcus* and argues for a protective role of p19p40 in bacterial infection. Similarly, the phenotype of IL-

12Rβ1-deficient mice is more severe than that of IL-12Rβ2-deficient mice (Wu et al., 1997, 2000). In particular, IL-12Rβ1 animals are more severely impaired in their ability to produce IFN-γ following endotoxin administration in vivo and following ConA or anti-CD3 activation of splenocytes in vitro. Together, these results indicate that elimination of both IL-12 and p19p40 pathways results in a more severe disturbance of the immune system than loss of only IL-12 signaling.

Although IL-12 plays a key role in the generation of protective immunity, it has also been implicated in the immunopathology of organ-specific autoimmune diseases in humans such as diabetes, arthritis, and Crohn's disease. Direct evidence that IL-12 plays a major role in generating Th1-mediated diseases has been provided by rodent studies in which treatment with a neutralizing anti-IL-12 mAb prevents the onset of diseases such as collagen-induced arthritis (Adorini et al., 1997), experimental autoimmune encephalomyelitis (EAE) (Leonard et al., 1995), and chronic IBD (Blumberg et al., 1999). Because antibodies used in these studies block the function of both IL-12 and p19p40, these studies do not address the role of each individual factor in these diseases. Support for a unique role of p19p40 in chronic inflammation comes from our observation that p19p40 has a much more pronounced effect on the proliferation of memory T cells isolated from IL-10-deficient mice with inflammatory bowel disease as compared to memory T cells from wild-type mice or normal human donors. Thus, the greatly increased number of Th1 memory/activated cells present in chronically inflamed intestines and draining lymph nodes of IL-10-deficient mice may reflect their selective expansion in response to p19p40 (Davidson et al., 2000). The predominance of Th1 cells with a memory/activated phenotype has also been observed in chronic lesions of rodents with various forms of autoimmune disease (TNB-induced colitis, EAE, and collagen-induced arthritis). Whether p19p40 plays a critical role in the maintenance and or function of these T cells remains to be determined. Thus, studies are in progress to establish the importance of p19p40 as a mediator of immunological disease and conversely, as a costimulator of protective immune responses.

The discovery of p19p40 as a novel composite factor closely related to IL-12 in structure and with biological activities similar as well as distinct from IL-12 raises important issues. The anti-p40 antibodies used in critical studies to address the role of IL-12 in immunity and immunopathology do not discriminate between IL-12 and p19p40. From the work presented here, as well as from studies based on mice deficient in either shared or unique IL-12 and p19p40 pathway components, evidence is emerging that the biological functions of p19p40 and IL-12 are intimately related. We are currently focusing on the development of reagents to discriminate between the biological functions of p19p40 and IL-12 and their specific contributions to immunity and immunopathology.

Experimental Procedures

Identification of Human p19

A structural alignment of available IL-6 family cytokine folds (CNTF, LIF, IL-6, OSM, and GCSF) from FSSP (Holm and Sander, 1998) was profile aligned to other sequences (including distant species variants of the aforementioned cytokines, plus CT-1, GPA, and viral IL-6's) with Clustal X (Thompson et al., 1997)—with some manual adjustment. A weighted profile (Thompson et al., 1994) of the most conserved region of the fold, the C-terminal D helix segment, an ~40 amino acid block, was created. Fast scans of sequence databases on a Bioccelerator machine (Compugen, Tel Aviv, Israel) with the Profilesearch program (Gribskov et al., 1987) identified two IMAGE ESTs (AA18955 and AA418747) that were used for the computational reconstruction of the human p19 sequence, and the cloning of its full-length cDNA.

Identification of Mouse p19

Based on a strong cross-species hybridization signal, pools of a RAW library (activated monocyte cell line) were generated and screened with a human p19 probe. The full-length 1353 bp cDNA encodes a 189 aa protein. Both human and mouse p19 cDNA sequences have been deposited at GenBank (accession numbers AF301619 and AF301620). Identical sequences were deposited by Y. Hirata and Y. Kosuge (accession numbers NM_016585 and AB030001).

Transient Transfection, Metabolic Labeling, and Immunoprecipitation

Cells (1×10^6) were transiently transfected with 5 μg empty vector or expression vectors encoding human or mouse forms of p19-N-Flag, IL-12p40, or both. Cells were cultured for 24 hr and then metabolically labeled for 16 hr with 50 μCi/ml Pro-mix L-[^{35}S] in vitro cell labeling mix (Amersham Pharmacia) in cysteine/methionine free MEM. Proteins were precipitated from 300 μl supernatant with either the anti-Flag M2 agarose (Sigma), anti-hp40/p70 antibody C8.6 (Pharmingen), or anti-mp40/p70 C17.8 (Genzyme) bound to protein G sepharose (Amersham Pharmacia).

2D-PAGE

For nonreducing/reducing PAGE, purified p19p40 heterodimer was run in two lanes of a nonreducing 10% NuPage gel in MES running buffer (Novex). The lanes were excised, reduced in sample buffer containing DTT, laid horizontally on two-well 10% gels, and run reduced in a second dimension. One gel was silver stained (Daiichi) while the other was blotted to a PVDF membrane and developed using the mouse p19-specific rat mAb 19A11 (1 μg/ml) and the HRP-coupled sheep anti-rat Ig NA932 (Amersham). For isoelectric focusing, immunoprecipitated ^{35}S-labeled heterodimer was run on pH 3–10 immobiline drystrips (Amersham Pharmacia) followed by SDS-PAGE and autoradiography.

Quantitation of mRNA Expression

cDNAs from various libraries or cultured macrophages and dendritic cells were prepared as described (Bolin et al., 1997) and used as templates for quantitative PCR. Fifty nanograms cDNA was analyzed for expression of p19, IL-12p35, or IL-12p40 by the fluorogenic 5′-nuclease PCR assay (Holland et al., 1991) using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Primers and probes for human and mouse IL-12p35 and IL-12p40 were obtained as Taqman PDAR's (Perkin-Elmer, Foster City, CA). Analysis of cDNA samples from cultured cells was corrected for expression of 18S rRNA using a VIC-labeled probe (Perkin-Elmer, Foster City, CA) in multiplex reactions.

Expression of mp19 IgG and E Tag Fusion Proteins

A HindIII-XhoI fragment encoding the mp19 protein was inserted into a modified form of pCDM8 encoding a C-terminal hIgG fusion protein. A vector encoding E Tag fusion protein was similarly constructed using pCDM8 (Pharmacia). Proteins were produced via transient transfection of COP5 or 293 cells followed by affinity chromatography purification via Protein A or Anti-E Tag. The Etag fusion protein was blotted onto an PVDF membrane (0.2 μm, Biorad, Hercules, CA) and subjected to Edman degradation. The derived sequence was L-A-V-P-R-S-S-S-P-D, indicating that the mature N-terminal mp19 residue is L20.

Development of Rat Anti-Mouse p19 Monoclonal Antibodies

Rat anti-mouse p19 monoclonal antibodies (mAbs) were produced from splenocytes of a female 8-week-old Lewis rat (Harlan Sprague Dawley, Indianapolis, IN) immunized with mouse p19:Ig fusion protein. The rat was primed with 50 μg of fusion protein in Complete Freund's Adjuvant and boosted three times. Splenocytes were fused with the mouse myeloma P3X63-AG8.653 using PEG 1500 (Boehringer-Mannheim). Hybridoma supernatants were screened in indirect ELISA on PVC plates coated with 50 μl of 0.5 μg/ml p19:Etag fusion protein. Selected positive hybridoma lines were subcloned, grown in serum-free medium supplemented with SITE (Sigma), and purified.

Purification of Natural Mouse and Human p19p40 Heterodimers

Dendritic cell supernatants and control medium were concentrated to 45× using Centriprep 10 concentrators (Millipore). Immunoprecipitations of recombinant and natural human p19p40 heterodimers were performed using goat-anti-mouse Ig beads preloaded with the mouse anti-hp40/p70 antibody C8.6 (Pharmingen), while isotype-control beads contained mouse IgG1 349,040 (Beckton-Dickinson). Mouse p19p40 heterodimers and fusion protein were detected in solution using a sandwich ELISA in which plates were coated with the p19-specific monoclonal antibody 20C10, and detection was performed with the biotinylated p40-specific monoclonal antibody 18482D (Pharmingen) and HRP-streptavidin 016-030-084 (Jackson). Mouse p19 Western blots were performed with the rat-anti-p19 monoclonal antibody 19A11.

Recombinant Adenovirus and Protein Production

m19 cDNA and mIL-12p40 cDNA were inserted separately into the transfer vector pQB1-AdCMV5-GFP (Quantum Biotechnologies, Montreal, Canada) by PCR. Recombinant adenovirus was produced as described in Quantum applications manual 24AL98. Recombinant virus (moi 100 mp19 virus plus moi 1000 mp40 virus) were used to infect 5×10^8 cells in 1 L CMF-1 with culture in a Nunc Cell Factory (Nalge Nunc, Naperville, IL) for 3 days. The culture medium was clarified by centrifugation and filtered prior to application to a 1 ml NHS-activated HiTrap (Pharmacia, Uppsala, Sweden) column coupled to 1 mg of mAb 20C10. The column was washed with PBS and eluted with 100 mM glycine (pH 3.0), and the eluate was immediately loaded onto a 4.6 × 100 mm Poros R2/H column (PerSeptive Biosystems, Cambridge, MA) with elution via a 80 ml linear gradient from 20% to 50% acetonitrile/0.1% trifluoroacetic acid.

Expression of Human and Mouse Hy-p40-p19

HindIII-NotI fragments were generated encoding the mature coding sequence of either human or mouse IL-12p40, followed by the synthetic linker GSGSSRGGSGSGGSGGGGSKL and by the mature coding sequence of either human or mouse p19. Fragments were inserted into pFLAG-CMV-1 (Sigma). Proteins were produced via transient transfection of 293 cells followed by affinity chromatography purification via anti-FLAG M2-agarose (Sigma).

Mouse T Cell Proliferation Assay

$CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cell subsets were purified from the spleen and mesenteric lymph nodes of >6- month-old *IL-10*$^{-/-}$ C57/B6 N12 mice as described (Davidson et al., 1998). Cells were fractionated into $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cell populations by two-color sorting on a FACSTAR plus (Beckton Dickinson). All populations were >99% pure upon reanalysis. $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ were put into a proliferation assay with plate-bound anti-CD3 (145.2C11) stimulation as described (Davidson et al., 1998). Additions to the growth media included anti-IL-2 mAb (JES6-1A12) 100 μg/ml, anti-IL-12(p40) mAb (C.17.8), 250 μg/ml, IL-12 (R&D), purified recombinant Hy-p40-p19, 293T cell supernatant containing mouse p19p40 heterodimer or mock supernatant. Cells were incubated for 5 days in a humidified chamber (37°C, 5% CO_2) with [^{3}H]TdR (Amersham) added at a final concentration of 1 μCi/well for the last 24 hr of incubation.

Cell Culture

PBMC were isolated from buffy coats of healthy donors (Stanford Blood Bank). Human monocytes were obtained from PBMC by negative selection using Dynabeads M-450 (by Dynal A.S., Oslo. Norway). Purified monocyte populations (85%–90% $CD14^+$) were cultured in GM-CSF (800 U/ml) and IL-4 (300 U/ml) (Schering-Plough, Kenilworth, NJ) at 5×10^5 cells/ml in RPMI + 10% FBS for 6 days, with a change of medium at day 3. At day 6, dendritic cells were induced to mature by coculture with irradiated (7000 rad) CD40 ligand transfected L cells, LPS (1 μg/ml), IFN-γ (100 U/ml), TNFα (100 U/ml) (R&D Systems), or combinations of these.

PHA Blast Generation and Activation

PHA blasts were derived by culture of PBMC in Yssel's medium with 0.1 μg/ml PHA (Wellcome) at 10^6 cells/ml. Cells were plated at a density of 2×10^4 cells per well with or without 40 ng/ml hHy-p19-p40 or 1 ng/ml huIL-12 on a 96-well plate coated with 10 μg/ml anti-CD3 and 1 μg/mL soluble anti-CD28. After 60 hr, IFN-γ production was determined by ELISA. Cells were then pulsed with 1 μCi/well with ^{3}H-thymidine (NEN) for 6 hr, harvested, and incorporation of ^{3}H-thymidine determined. In blocking experiments, the following antibodies were used: polyclonal goat anti-human IL12Rβ1 (R&D cat#AF839), mouse anti-human p40/p70 C8.6 (Pharmingen cat#20510D), mouse anti-human p35 (DIACLONE cat#855.120.010), mouse anti-human p40 (Pharmingen cat#20711D), polyclonal goat Ig (Jackson Immuno Research code 0055-000-003), and monoclonal mouse IgG1 (Pharmingen cat#20800D) as isotype controls at 10 μg/ml. Western blots were performed following immunoprecipitation of PHA blast lysates with anti-stat4 mAb (UBI cat # 06-788) and blotting with anti-phosphotyrosine mAb 4G10 (UBI cat # 05-321) or reblotting with anti-stat4.

CD45RA/CD45RO T Cell Proliferation and IFN-γ Production

FACS-purified CD45RA and CD45RO T cells (purity > 99%) were cultured at a density of 4×10^4 cells/well in a 96-well plate previously coated with anti-CD3 antibody at 10 μg/ml and soluble anti-CD28 at 1 μg/ml with or without 40 ng/mL hHy-p40-p19, 1 ng/mL hIL-12 (R&D Systems), or 100 U/ml hIL-2 (R&D Systems). Anti-hIL-2 mAb 17H12 and anti-hIL-2R Mab B-B10 (Diaclone) were added at 10 μg/ml where indicated. Cells were incubated for 60 or 136 hr, and 100 μl aliquots were collected to determine IFN-γ production by ELISA. Proliferation was measured as described for PHA blasts.

FACS Analysis and Proliferation Assay

Human IL-12Rβ1 cDNA (kindly provided by Dr. Xiaojing Ma) was cloned in the retroviral vector pMX (Kitamura, 1998). Ba/F3 cells were infected with retroviral supernatants for 24–48 hr on petri dishes coated with 30 μg/ml recombinant fibronectin fragments (Retronectin, TaKaRa). Expression of hIL-12Rβ1 was detected using goat anti-hIL-12Rβ1 (R&D Systems) followed by phycoerythrin (PE)-conjugated donkey anti-goat IgG (Jackson Immunoresearch). Mouse IL-12Rβ1 and IL-12Rβ2 cDNAs in pMX-IRES-EGFP were provided by A. O'Garra and L. Showe (Showe et al., 1996; Heath et al., 2000). Cells expressing both receptors were prepared by coinfection with both retroviruses. Hy-p40p19 and IL-12 binding was assessed by FACS. Cells were incubated with 0–10 μg/ml (0–168 nM) Hy-p40p19 or 0–1 μg/ml IL-12 for 30 min on ice, followed by a single wash. Bound Hy-p40p19 was detected using 10 μg/ml anti-FLAG M2 mAb (Sigma) followed by PE-conjugated anti-mouse IgG (Jackson Immunoresearch), or biotinylated M2 (Sigma) followed by streptavidin-PE (Pharmingen). Results were similar using the two procedures. Human IL-12 binding was detected using anti-IL-12p40 mAb C11.5 (Pharmingen) followed by PE-conjugated goat anti-mouse IgG. Mouse IL-12 binding was detected using rat anti-mIL-12 (p40) mAb C17.15 (R&D Systems) followed by goat anti-rat IgG-PE (Jackson Immunoresearch). Proliferation responses were measured as described (Ho et al., 1993).

Acknowledgments

We thank Deborah Ligett and Sylvia Lo for synthesizing oligonucleotides, Terri McClanahan for providing cDNA libraries, Steve Ho, Svetlana Antonenko, and Thomas J. Cradick for technical support, and

Gary Burget and Maribel Andonian for graphics help. DNAX is supported by Schering Plough.

Received June 13, 2000; revised October 3, 2000.

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GenBank Accession Numbers

The GenBank accession numbers for the human and mouse p19 sequences reported in this paper are AF301619 and AF301620, respectively.