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IL-23 and IL-12 Have Overlapping, but Distinct, Effects on Murine Dendritic Cells¹

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Paolo Puccetti^{2*}

IL-23 is a recently discovered heterodimeric cytokine that shares biological properties with proinflammatory cytokines. The biologically active heterodimer consists of p19 and the p40 subunit of IL-12. IL-23 has been shown to possess biological activities on T cells that are similar as well distinct from those of IL-12. We have constructed single-chain IL-23 and IL-12 fusion proteins (IL-23-Ig and IL-12-Ig) and have compared the two recombinant proteins for effects on murine dendritic cells (DC). Here we show that the IL-23-Ig can bind a significant proportion of splenic DC of both the CD8 α^- and CD8 α^+ subtypes. Furthermore, IL-23- and IL-12-Ig exert biological activities on DC that are only in part overlapping. While both proteins induce IL-12 production from DC, only IL-23-Ig can act directly on CD8 α^+ DC to promote immunogenic presentation of an otherwise tolerogenic tumor peptide. In addition, the *in vitro* effects of IL-23-Ig did not appear to require IL-12R β 2 or to be mediated by the production of IL-12. These data may establish IL-23 as a novel cytokine with major effects on APC. *The Journal of Immunology*, 2002, 168: 5448–5454.

Cytokines are secreted proteins that have pleiotropic regulatory effects on hemopoietic and nonhemopoietic cells. They encompass families of critical regulators of development, immune response, and inflammation. Many cytokines share structural features and are characterized by overlapping effects on target cells, typically acting as cascades or networks. Redundancy is a recurrent motif among inflammatory cytokines. Despite the fact that these cytokines interact with structurally different receptors, cellular responses will be similar and in certain respects virtually superimposable (1).

p19, a molecule structurally related to IL-6, G-CSF, and the p35 subunit of IL-12, is a component of the recently discovered cytokine IL-23. p19 was identified by searching the databases with a computationally derived profile of IL-6 (2). p19 was found to lack biological activity *per se* and to combine with the p40 subunit of IL-12. The p19/p40 pair is naturally expressed by activated mouse and human dendritic cells (DC)³ and has biological effects on T cells that are similar to but distinct from those of IL-12 (2). Recently, the ubiquitous, transgenic expression of the IL-23 subunit p19 was found to result in multiorgan inflammation, runting, infertility, and premature death of mice (3). These activities probably result from interaction of IL-23 with the IL-12R β 1 and an additional, novel receptor subunit (2).

DC play a central role in activating and regulating T cell responses (4). Activation of DC by a variety of stimuli and cytokines

leads to the secretion of IL-12, which subsequently induces IFN- γ production by NK cells and directs Th1 development (5–7). We have recently described autocrine effects of IL-12 on murine myeloid APC, such as DC and macrophages (8, 9). In particular, IL-12 was found to interact with surface receptors expressed by the CD8 α^- subset of splenic DC, resulting in NF- κ B-mediated signaling and leading to the initiation of inflammatory and Th1-promoting actions (8, 10–12). In this study, using single-chain IL-23-Ig or IL-12-Ig fusion proteins, we have compared IL-23 and IL-12 for binding to murine DC and *in vitro* and *in vivo* effects mediated by the interaction with these cells. We found that the two cytokines elicit overlapping biological activities in DC by signaling through distinct receptor subunits expressed differentially by distinct DC subsets. These data add to the function of IL-23 as an initiator and regulator of T-dependent immunity in the mouse and highlight a potentially important role of the cytokine in the modulation of accessory cell function.

Materials and Methods

Construction and expression of single-chain mouse IL-23-Ig or IL-12-Ig fusion proteins

The cDNA encoding the p19 chain of mouse IL-23 was generated from resting cells of the macrophage cell line J774 that constitutively express p19 transcripts (2). The cDNA was amplified with primers specifying the mature protein-coding region and containing restriction site sequences (Table I). The cDNA encoding the p35 and p40 chains of murine IL-12 were amplified from plasmid DNA (13) with mutated primers that added appropriate restriction sites (Table I). The p40 sense mutated primer introduced a *Kpn*I 15-base sequence just before the start codon, and the antisense primer introduced a *Bsp*EI site just before the stop codon. For IL-12 p35 as well as IL-23 p19 cDNA, PCR amplification involved a sense-mutated primer introducing a *Bam*HI site just after the presumed 22-aa signal peptide sequence and an antisense-mutated primer introducing a *Bcl*I site just before the stop codon. Linker sequences were synthesized as sense and antisense oligonucleotides spanning the sequence reported by Huston et al. (14) encoding the (Gly₄Ser)₃ linker and adjacent sequences between convenient overhanging restriction enzyme sites in the 3' and 5' ends of the IL-12 or IL-23 subunit cDNA. Production of IL-23- or IL-12-Ig fusion proteins was further accomplished by PCR amplification of the region comprising the hinge, CH2, and CH3 domains of the murine IgG3 isotype heavy chain, using cDNA from the IgG3 anti-TNP hybridoma C3110 as a template with appropriate primers (Table I) (15). After amplification, PCR

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³ Abbreviations used in this paper: DC, dendritic cell; rIg, recombinant Ig protein.

Table I. Primers^a used for cloning of IL-23-Ig and IL-12-Ig into pEF-BOS^a

Fragment	Primer Orientation	Primer Sequence (5'-3')	Restriction Site ^b
p40	5'	CCAGGTACCTCGCAGCAAAGCAAG	<i>KpnI</i>
p40	3'	GCATCTCCGGATCGGACCCT	<i>BspI</i>
p19	5'	TCACTGGATCCCTGGCTGTGCCTAGG	<i>BamHI</i>
p19	3'	TGGGCTGATCAAAGCTGTTGGCACTA	<i>BclI</i>
p35	5'	TCAGTGGATCCAGGGTCATT	<i>BamHI</i>
p35	3'	CTTGATGATCAAGCGGAGCTCAG	<i>BclI</i>
Ig	5'	AAGACTGAGTTGATCAAGAGAATCGAGCCTAGA	<i>BclI</i>
Ig	3'	AATGCTCTAGATGCTGTTCTCATTACC	<i>XbaI</i>

^a GenBank accession numbers: p40, M86671; p19, AF301619; p35, M86672; Ig, D14625.

^b Restriction sites indicated by underlined sequence.

products and the linker sequence were digested with the appropriate restriction enzymes and cloned into the pEF-BOS plasmid (16). Clones with the correct inserts were stably transfected by the calcium phosphate method into P1.HTR cells, and supernatants were collected. Fusion proteins were affinity-purified by means of protein A-Sepharose. A sandwich ELISA was used to quantitate fusion proteins. The test was based on the use of p40-specific C15.6 and C17.8 mAb (BD PharMingen, San Diego, CA) and therefore detected p40 in both fusion proteins as well as the heterodimeric p35/p40 complex in the rIL-12 used as a control. Standard ELISA detection of IL-12 p70 involved the use of p70-specific 9A5 mAb (BD PharMingen) and p40-specific C17.8 mAb.

Western blot and bioassay of IL-23-Ig and IL-12-Ig fusion proteins

Purified samples containing fusion proteins were collected and stored at -70°C . Samples were concentrated 20- to 30-fold (Centricon 10; Amicon, Beverly, MA), and aliquots containing 10 ng fusion protein were run on 8% polyacrylamide gels. The primary Ab was p40-specific C17.8, and the secondary Ab was a sheep anti-rat peroxidase-conjugated Ab. Blots were developed using a chemiluminescent technique. Levels of IL-23/IL-12 bioactivity in fusion proteins were determined using mitogen activation of splenocytes by measuring IFN- γ production in response to IL-12 or IL-23 (2). In all in vitro experiments involving IL-23-Ig or IL-12-Ig, recombinant Ig protein (rIg) encompassing the Ig portion of the fusion proteins was used as a control (15). All recombinant proteins, including heterodimeric IL-12, were used at a concentration of 10 ng/ml unless otherwise indicated.

DC purification and binding analysis

DC were prepared and fractionated according to CD11c/CD8 α expression using positive selection columns in combination with CD11c and CD8 α microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described (17). The DC preparation consisted of 96–98% CD11c⁺, >99% Ia⁺, >98% B7-2⁺, and <0.1% CD3⁺ cells and appeared to consist of 90–95% CD8⁻ and 5–10% CD8⁺ cells. After DC fractionation, the recovered CD8⁻ cells typically contained <0.5% contaminating CD8⁺ DC, whereas the CD8⁺ fraction was made up of >90% CD8⁺ DC.

Flow cytometry was employed to assess the expression of cytokine receptors in freshly harvested DC or the DC cell line CB1 (8). Typically, 2×10^5 cells in 100 μl PBS/3% FCS were incubated with 1 μg FITC-conjugated fusion protein after blocking the Fc γ R on DC by means of 2.4G2 mAb (9). The stained cells were analyzed on a FACScan (BD Biosciences, San Jose, CA). To assess the specificity of the IL-12R staining, labeling was also performed in the presence or the absence of an excess of free rIL-12. In addition, in selected experiments FACS staining involved the use of unlabeled fusion proteins as the first step, followed by PE-labeled polyclonal anti-IgG3 as the second step.

Skin test assay

A skin test assay was employed for assaying the cytokine adjuvant potential for promotion of class I-restricted delayed-type hypersensitivity to synthetic peptides as previously described (18). Briefly, peptide-loaded DC (3×10^5), treated or not with cytokine/fusion proteins, were transferred into recipient hosts that were assayed for the development of peptide (P815AB)-specific delayed-type hypersensitivity at 2 wk. In selected experiments, a combination of CD8⁻ DC (3×10^5) and CD8⁺ DC (10^4) was transferred into recipient hosts (11, 12). To test for possible nonspecific effects of peptide challenge, specificity controls routinely involved the use of the antigenically unrelated P91A peptide, and no effects were found (9,

19). Results were expressed as the increase in footpad weight of peptide-injected footpads over that of vehicle-injected counterparts. Data are the mean \pm SD for at least six mice per group. The statistical analysis was performed using Student's paired *t* test by comparing the mean weight of experimental footpads with that of control counterparts (19).

Mice and additional reagents

Female DBA/2J mice (H-2^d) were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy) and were used at the age of 2–4 mo. Murine rIL-12 was a gift from Genetics Institute (Cambridge, MA). The characteristics and sp. act. of the purified rIL-12 preparation were previously described (20, 21). Murine rIFN- γ was obtained from Genzyme (Boston, MA). The Ab used for ELISA assessment of IFN- γ were R4-6A2 and biotinylated XMG1.2 mAb (both from BD PharMingen). Rabbit polyclonal IgG Ab specific for IL-12R β 2 was raised in our laboratory as previously described (9) and was used at a concentration of 10 $\mu\text{g}/\text{ml}$. Neutralizing, affinity-purified sheep anti-mouse IL-12 p70 polyclonal Ab was provided by Genetics Institute and was used at 10 $\mu\text{g}/\text{ml}$.

Results

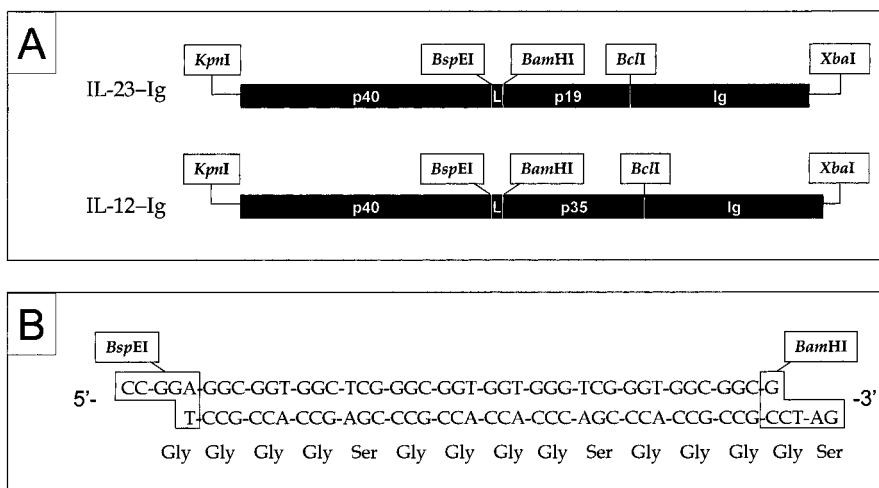
Expression of bioactive murine IL-23 and IL-12 fusion proteins

Constructs were built in the pEF-BOS vector for the expression of IL-23 and IL-12 fusion proteins (Fig. 1A). The cDNA were linked with a 45-bp linker encoding the 15-aa (Gly₄Ser)₃ linker (Fig. 1B). Constructs were compared for their ability to direct bioactive protein expression in side-by-side screening experiments with rIL-12 p70. Comparison of the results of the ELISA (Fig. 2A) specific for either the p70 IL-12 heterodimer or the p40 subunit in the IL-12 fusion protein suggested that the p40-specific ELISA quantitatively determines the subunit in IL-12 and IL-23 fusion proteins. In contrast, the p70-specific ELISA failed to detect IL-23. Comparable amounts of rIL-12 and fusion proteins were subjected to Western blot analysis under reducing conditions using a p40-specific Ab (Fig. 2B). The results showed that rIL-12 would run as a 45/50-kDa doublet, as expected (22). In contrast, the fusion proteins were expressed as single polypeptide chains of 115 and 120 kDa for IL-23 and IL-12 fusion proteins, respectively. We thus analyzed the ability of IL-23- and IL-12-Ig fusion proteins to induce IFN- γ production by Con A blast T cells. On comparing a range of concentrations of either rIL-12 or fusion proteins, we found that the IL-12 fusion protein induced IFN- γ levels similar to those of the native cytokine, and these levels were significantly higher than those induced by the IL-23-Ig (Fig. 2C). Similar results have previously reported for a human p19/p40 construct as compared with rIL-12 (2).

Binding of IL-23 to DC

Although most of the biological effects of IL-12 in vivo are thought to involve direct actions on T and NK cells (6, 7), we have recently described autocrine effects of murine IL-12 on myeloid APC, including DC (8) and macrophages (9). IL-12 was found to act on CD8 α ⁻ DC (11) leading to the activation of NF- κ B and the

FIGURE 1. Construction of vector inserts for expression of single-chain IL-23 or IL-12 fusion proteins. A, Schematic diagrams of the DNA constructs for single-chain IL-23 or IL-12 fusion proteins are reported, showing that the p40 and p19 subunits (for IL-23) and the p40 and p35 subunits (for IL-12) were genetically fused with a DNA linker (L) encoding the 15 aa (Gly₄Ser)₃ using appropriate restriction sites (shown in B). Both constructs were genetically fused with cDNA coding for the hinge, CH2, and CH3 domains of the murine IgG3 isotype heavy chain.



initiation of inflammatory and Th1-promoting actions (8, 11). We therefore became interested in analyzing the possible interaction of IL-23 on DC. We used flow cytometry for detection of IL-23-Ig binding to freshly harvested DC and a DC line. Using direct staining with fluoresceinated IL-23-Ig or IL-12-Ig, we detected a high proportion of cells staining positively for either fusion protein (Fig. 3). No significant difference was found between freshly harvested DC and the DC line. In contrast, there was no binding to either cell type by control fluoresceinated rIg.

Functional activity of IL-23 on DC

IL-12 actions on DC and macrophages include adjuvant effects that confer priming ability on these cells pulsed with a synthetic peptide (9, 11, 20, 21). In particular, DC and macrophages exposed sequentially to rIL-12 and a tumor peptide, P815AB, will confer T cell-mediated reactivity on prospective recipients of an intra-footpad challenge with the peptide (18, 20, 21). We therefore wanted to investigate whether similar adjuvant effects could be exerted by IL-23 on peptide presentation by myeloid APC. Fig. 4 shows the effects of sensitization with P815AB using freshly harvested DC exposed to IL-23-Ig or IL-12-Ig before peptide pulsing and transfer into hosts to be assayed for skin test reactivity at 2 wk. Similar to IL-12, IL-23 conferred priming ability on DC pulsed with the peptide. In experiments not reported in Fig. 4 we found that similar and comparable adjuvant activities were exerted by IL-23- and

IL-12-Ig on freshly harvested, highly purified peritoneal macrophages (>99% Mac-1⁺) (9). Therefore, both fusion proteins, under comparable experimental conditions, would exert adjuvant activity on myeloid APC.

In vitro induction of cytokines in DC treated with IL-23-Ig

We have previously demonstrated that autocrine IL-12 may act directly on DC to prime the cells for IL-12 production, with the cytokine priming DC for NF- κ B-induced transcription of the IL-12 p40 gene (8). We therefore wanted to investigate any possible autocrine effect of IL-23 on IL-12 production. We measured p70 production by ELISA in culture supernatants of freshly harvested DC treated for 2 h with 10 ng/ml IL-23-Ig or IL-12-Ig (Fig. 5A). Additional groups were treated with recombinant heterodimeric IL-12 or rIg. Culture supernatants were harvested at 1 and 24 h. According to previous results (8, 9), no p70 was found in any group at 1 h, thus indicating that the p70 measured at later time points was not derived from externally added IL-12. In contrast, considerable and comparable amounts of the cytokine were found at 24 h in DC exposed to recombinant (heterodimeric or single chain) IL-12 or to IL-23-Ig. When a range of concentrations (0.1–100 ng/ml) of the two fusion proteins were used, IL-23-Ig and IL-12-Ig were found to possess comparable effects at 10–100 ng/ml (Fig. 5B). However, Fig. 5B also shows that the combined

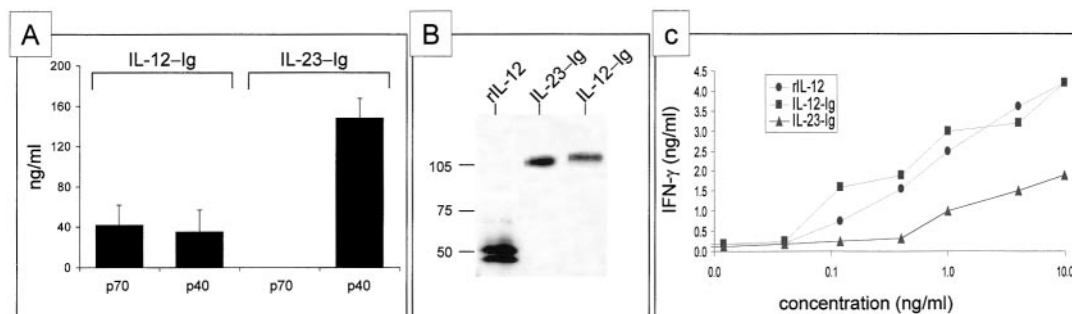


FIGURE 2. Expression of bioactive cytokine fusion proteins. A, Quantitative assessment of IL-12-Ig or IL-23-Ig by ELISA specific for IL-12 p70 or p40. Purified supernatants from vector-transfected P1.HTR cells were assayed for IL-12- or IL-23-Ig using rIL-12 as a standard. Data are the mean \pm SD of replicate determinations. B, Western blot analysis of recombinant heterodimeric and fusion protein form of murine IL-12 in comparison with IL-23-Ig. The primary mAb was p40-specific C17.8, and the secondary Ab was sheep anti-rat peroxidase-conjugated Ab. C, Levels of IL-23/IL-12 bioactivity in fusion proteins as determined by IFN- γ induction in Con A-activated splenocytes. Con A blasts were prepared by incubating spleen cells for 2 days with 2 μ g/ml Con A and 20 U/ml rIL-2, and cells were then exposed to a range of fusion protein concentrations for 48 h. Supernatants were assayed by ELISA for IFN- γ content. rIL-12 was used as a control.

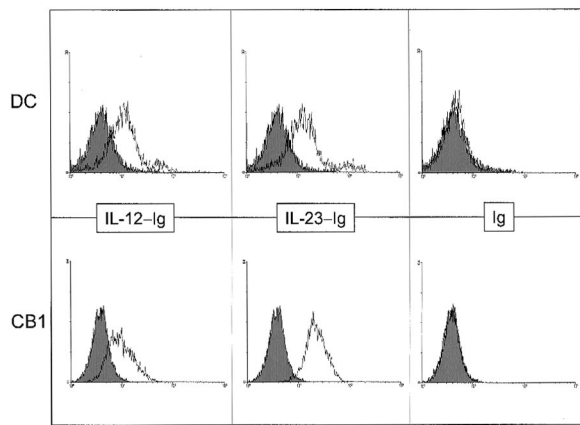


FIGURE 3. Cytofluorometric analysis of freshly harvested DC or CB1 cells stained with fluoresceinated IL-12-Ig, IL-23-Ig, or rIg. Splenic DC and CB1 cells were reacted with the labeled protein after blocking the Fc γ R on DC by means of 2.4G2 mAb. Filled histograms indicate control background values in the absence of fluoresceinated reagent. Cells treated with rIg were used as a negative control. One experiment representative of three is shown.

effects of IL-23-Ig and IL-12-Ig would result in sustained IL-12 p70 production at 1–100 ng/ml of each fusion protein.

We next became interested in ascertaining other possible effects of IL-23 in vitro. We tested IL-12-Ig and IL-23-Ig comparatively for a variety of different effects on splenic DC, including production of IFN- γ and TNF- α and expression of phenotypic markers such as CD40, CD80, and class II molecules. Under the experimental conditions employed (i. e., 24- to 48-h exposure to a fusion protein), neither IL-23-Ig nor IL-12-Ig significantly or reproducibly affected the expression of CD40, CD80, or class II molecules (data not shown). Similarly unaffected by either fusion protein appeared to be the production of TNF- α (data not shown). In contrast, both fusion proteins induced considerable production of IFN- γ at 0.1–10 ng/ml (Fig. 6). Again, the combined exposure of DC to IL-12-Ig and IL-23-Ig appeared to result in additive effects on cytokine production.

Modulation of different DC subsets by IL-23

The similarities between the activities of IL-23-Ig and IL-12-Ig prompted us to investigate whether the two cytokines may be acting on the same target cells. In fact, IL-12 is known to act selectively on CD8 α ⁻ DC to enhance presentation of P815AB in vivo (11). To this purpose splenic DC were fractionated to yield a pop-

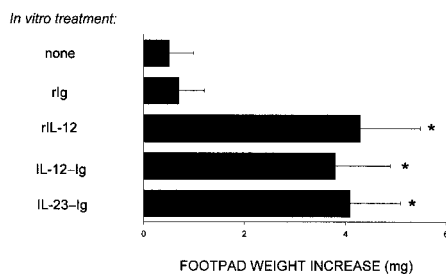


FIGURE 4. Ability of IL-23-Ig fusion protein to prime DC for induction of skin test reactivity to a synthetic peptide. DC were exposed sequentially in vitro to a recombinant protein (as indicated, 10 ng/ml for 18 h) and P815AB (5 μ M for 2 h) before transfer into recipient hosts. Two weeks after cell transfer, mice were assayed for skin test reactivity in vivo. *, significant difference ($p < 0.01$) in increase in footpad weight between experimental and control footpads. One experiment of four is shown.

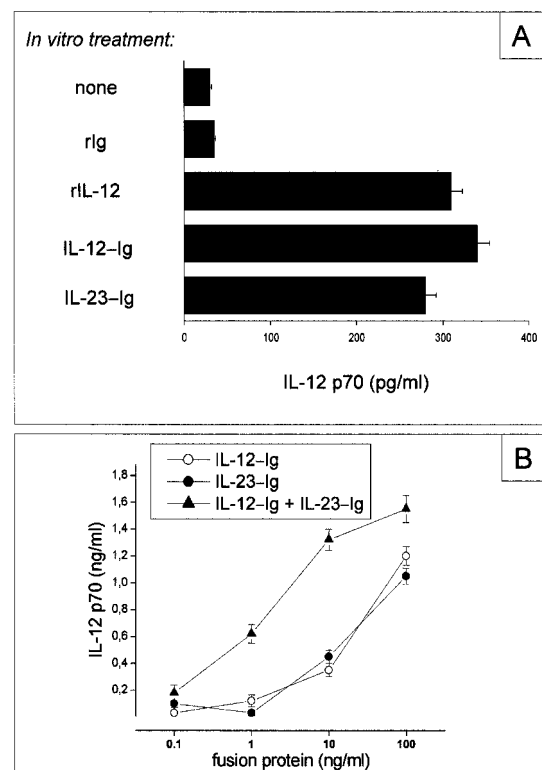


FIGURE 5. IL-12 production by DC-treated with fusion proteins. Freshly harvested DC were exposed for 2 h to different recombinant proteins (as indicated), followed by extensive washing. At 1 and 24 h supernatants were assayed for IL-12 p70 content by ELISA. The 1-h IL-12 titers were below the detection limit of the assay. A, IL-12-Ig, IL-23-Ig, rIL-12, and rIg were used at a concentration of 10 ng/ml. One experiment of four is shown. B, IL-12-Ig and IL-23-Ig, either singly or in combination, were used at different concentrations (as indicated). One experiment of two is shown.

ulation of >99% CD8⁻ cells and >90% CD8⁺ cells (17). The two subsets were analyzed by flow cytometry for the ability to bind fluoresceinated IL-23-Ig in vitro (Fig. 7A) and to produce IL-12 p70 in response to IL-23-Ig (Fig. 7B). We found that IL-23 would bind the two DC subsets equally well and that CD8⁻ and CD8⁺ DC subsets would produce comparable amounts of IL-12 p70 in response to 10 ng/ml IL-23-Ig. In addition, we wanted to examine CD8⁻ and CD8⁺ DC for the ability to prime the host in vivo following sequential exposure to IL-12-Ig or IL-23-Ig and P815AB (Fig. 8). In line with previous data (11), we found that

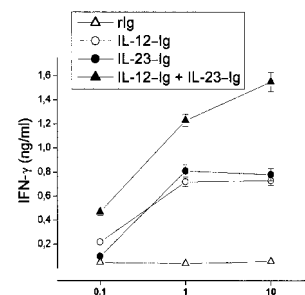


FIGURE 6. Production of IFN- γ by DC treated with IL-12-Ig, IL-23-Ig, or a combination of both. Freshly harvested DC were exposed for 72 h to different concentrations of the recombinant proteins. Supernatants were then assayed for IFN- γ levels by ELISA. Data are the mean \pm SD of replicate samples. One experiment of two is shown.

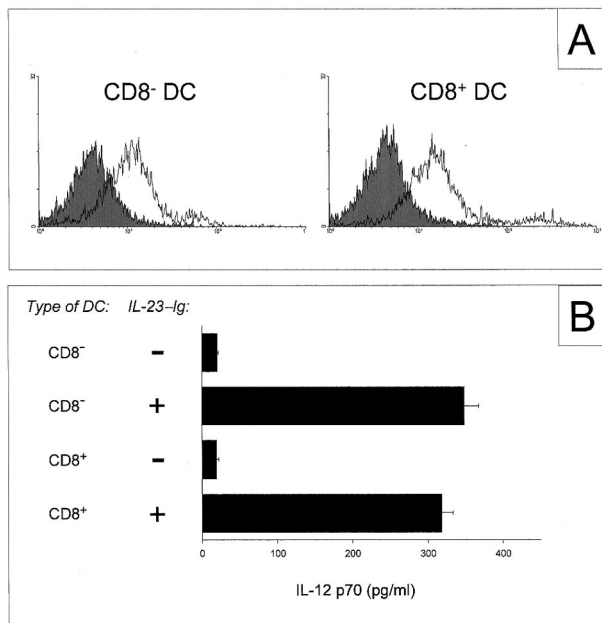


FIGURE 7. Interaction of IL-23 with distinct DC subsets. *A*, Binding of fluoresceinated IL-23-Ig to CD8 α ⁻ and CD8 α ⁺ DC. Fractionated CD8⁻ and CD8⁺ DC were reacted with the labeled protein after blocking the Fc γ R. Filled histograms indicate control background values treated with labeled rIg. One experiment representative of three is shown. *B*, Production of IL-12 p70 by CD8⁻ and CD8⁺ DC in response to IL-23-Ig. Cultures were established using fractionated DC subsets and were assayed for IL-12 p70 content as indicated in Fig. 5. One experiment of two is shown.

IL-12-Ig showed selective ability to activate CD8⁻ DC for presentation *in vivo* of the peptide (Fig. 8*A*). In contrast, IL-23-Ig appeared to be able to interact with both DC subsets to prime the host *in vivo* for skin test reactivity to the tumor peptide (Fig. 8*B*).

Ability of anti-IL-12R β 2 subunit Ab to block the activity of IL-12, but not IL-23

The experiments reported above indicated that IL-23 may exert biological effects on DC that are similar to but distinct from those of IL-12, although a portion of IL-23 effects on DC could be mediated by the induction of IL-12. In addition, it has been suggested that the biological activity of naturally expressed IL-23 relies on interaction with the IL-12R β 1 subunit and an additional, novel receptor subunit (2). This would exclude a major role for the IL-12R β 2 subunit, which, in contrast, plays a major role in IL-12 signaling in T cells (23). We have recently generated an IL-12R β 2 chain-specific Ab that detects this subunit in the IL-12R expressed by different cell types (9). We took advantage of this reagent to comparatively investigate the role of the β 2 subunit in the signaling of IL-23 and IL-12 in DC. According to the experimental design illustrated above, the two fusion proteins were assayed for adjuvant ability resulting from the activation *in vitro* of DC in the presence or the absence of anti- β 2 Ab. The β 2-specific Ab or control rabbit Ig were present during the *in vitro* exposure of DC to IL-23-Ig or IL-12-Ig before peptide pulsing and transfer into recipient hosts to be assayed for skin test reactivity at 2 wk. Fig. 9 shows that the anti- β 2 Ab completely blocked the effect of IL-12 on DC. In contrast, there was no effect of the Ab added to cultures of DC exposed to IL-23-Ig. In line with the lack of effect of anti- β 2 Ab on the activity of IL-23-Ig was the observation that an Ab to IL-12 p70 present in the coculture in place of the anti- β 2 reagent was equally devoid of any effect on the expression of IL-23-Ig activity (Fig. 9). These findings confirm the lack of a role for the

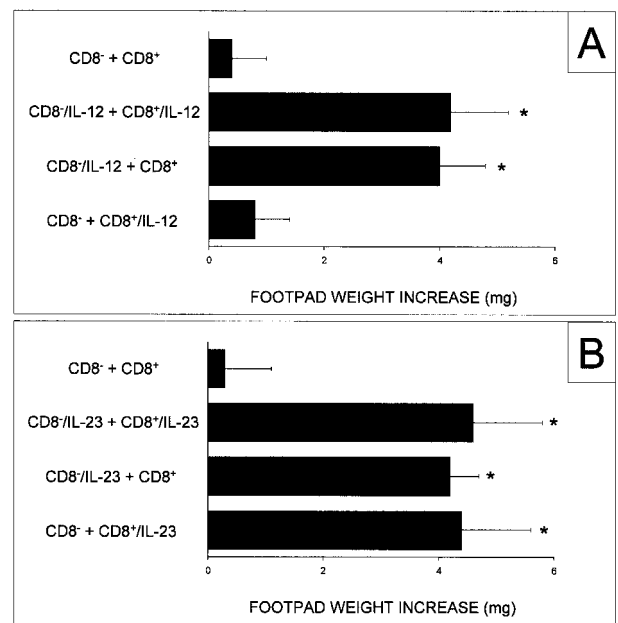


FIGURE 8. IL-23 acts on different DC subsets to exert adjuvant activity. *A*, Induction of skin test reactivity to P815AB by host transfer with DC subtypes treated with IL-12-Ig. DC fractionated according to CD8 expression and treated (CD8⁻/IL-12, CD8⁺/IL-12) or not (CD8⁻, CD8⁺) with IL-12-Ig were mixed after peptide pulsing and transfer into recipient hosts. P815AB-specific skin test reactivity was assessed at 2 wk. *B*, Induction of skin test reactivity to P815AB by host transfer with DC subtypes treated with IL-23-Ig. Parallel experimental groups were established using IL-23-Ig in place of IL-12-Ig. *, $p < 0.001$ (experimental vs control footpads). One experiment representative of three is shown.

IL-12R β 2 subunit in IL-23 signaling. Furthermore, these data strongly suggest that most of the activity of IL-23 on DC is not mediated by the induction of autocrine IL-12. However, it should be noted that binding of the blocking Ab may not be durable *in vivo* and/or that up-regulation of IL-12R β 2 may occur after the adoptive transfer. Finally, it is also possible that, although not

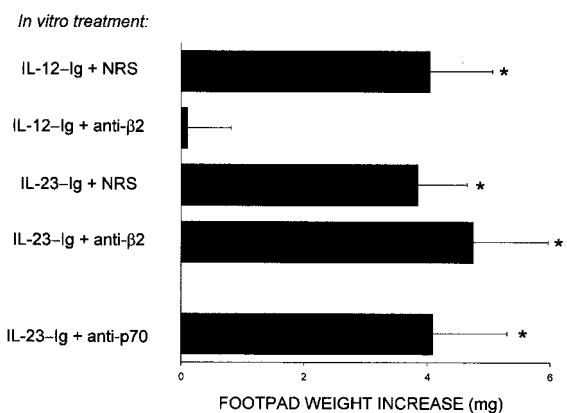


FIGURE 9. Ability of anti- β 2-chain Ab to block the adjuvant activity of IL-12, but not IL-23, on DC. Freshly harvested DC were exposed sequentially to IL-23- or IL-12-Ig and peptide before transfer into hosts to be assayed for Ag-specific skin test reactivity at 2 wk. Exposure to either fusion protein was conducted in the presence or the absence of Ab directed to the β 2-chain of the IL-12R. Controls included the use of DC exposed to either fusion protein in the presence of normal rabbit serum (NRS). As an additional control DC were also exposed to anti-IL-12 p70 in place of the anti- β 2-chain Ab. *, $p < 0.001$ (experimental vs control footpads). One experiment representative of three is shown.

strictly in an autocrine fashion, IL-23-stimulated DC may produce IL-12 *in vivo* that may act on other native DC.

Discussion

A major subfamily of helical cytokines encompasses a set of factors related to IL-6. These factors include mediators that share 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 may account for the overlapping functions of IL-6-like cytokines (2). IL-6-like cytokines include the p35 subunit of IL-12, which is not active on its own, but forms part of a composite factor with a chain known as p40. p40 is a soluble member of the cytokine receptor superfamily, and heterodimeric IL-12 (p35/p40) plays a critical role in the initiation of cell-mediated immunity (6), encompassing actions on both T cells (7, 24) and myeloid accessory cells, such as DC (8, 25) and macrophages (9, 26, 27). IL-12 activities are mediated via a high affinity receptor complex that gathers two closely related subunits, IL-12R β 1 and IL-12R β 2 (23). p40, however, may be involved in functional complexes with molecules other than p35 (28, 29), and in fact a novel p19 protein has recently been described that engages p40 to form the newly described cytokine, IL-23 (2, 3). The p19/p40 complex is naturally expressed by activated human and murine DC and exerts activities on T cells that, although similar to those of IL-12, can nevertheless be differentiated from the latter.

In addition to direct actions on T cells, IL-12 may critically affect the functions of accessory cells of the immune response, including DC and macrophages (27). We have recently shown that rIL-12 acts selectively on CD8 α ⁻ DC (11) and that autocrine production of IL-12 may be involved in DC modulation via different immunotherapeutic maneuvers (10, 30). In contrast, IL-6 will act on CD8 α ⁺ DC to down-modulate the tolerogenic properties of this subset of DC (17) and may thus complement the adjuvant effect of IL-12 acting on CD8⁻ DC. Therefore, a complex interplay takes place among inflammatory cytokines that regulate the functional activity of DC (31). Because it has been shown that the biological activities of IL-23 on T cells result from interaction of the p19/p40 complex with IL-12R β 1 (2), we became interested in ascertaining any possible direct effect of IL-23 on murine splenic DC, the possible interplay and cross-regulation of IL-12 and IL-23, and the possible sharing of receptor subunits with particular regard to the β 2 subunit. In fact, the β 2 subunit is a major component of the IL-12 signaling pathway in T cells (23, 32).

By using single-chain IL-23-Ig or IL-12-Ig fusion proteins, we have comparatively analyzed IL-23 and IL-12 for *in vitro* and *in vivo* effects on DC. Analogous to their activities on T cells, we found that the two cytokines may have similar as well as distinct effects on myeloid APC. These would include increased production of endogenous IL-12 and IFN- γ *in vitro* as well as induction *in vivo* of a combined CD4⁺/CD8⁺ T cell response to an otherwise poorly immunogenic tumor/self peptide (18). However, in contrast to IL-12, which lacks direct actions on CD8⁺ DC, the adjuvant effect *in vivo* of IL-23 probably resulted from combined actions on CD8⁻ and CD8⁺ DC, and in fact each subset alone, when treated with IL-23-Ig, was capable of mediating the immunogenic presentation of the tumor peptide.

The adjuvant effect of IL-23 on the otherwise tolerogenic CD8⁺ subset of murine DC is remarkably similar to that of IL-6 (17), which we have previously shown to mediate the effect of CD40 activation on these cells (30) and to oppose the tolerogenic properties of IFN- γ (12). CD8⁺ DC in the mouse may correspond in humans to the progeny of DC precursors with a characteristic sur-

face phenotype and a plasmacytoid appearance (33) and may represent a unique type of regulatory APC involved in tolerance and immunosuppression (34). The finding that IL-23 may act directly on those cells to promote the immunogenic presentation of an otherwise tolerogenic tumor/self peptide (21) might implicate a role of the cytokine not only in the generation of protective immunity, but also in the promotion of autoimmunity, according to a pattern similar to that of IL-12 (35). Because most experimental studies aimed at assessing the role of IL-12 in autoimmunity have relied on the use neutralizing Ab that would block the function of both p35/p40 and p19/p40, our present data may raise further questions about the role of each individual cytokine in these models of autoimmunity (2).

One major finding in this study was the ability of the IL-23-Ig fusion protein to trigger the release of IL-12 from DC *in vitro* to an extent comparable to that of rIL-12. This raised the issue of possible bidirectional influences between the productions of IL-23 and IL-12. In particular, we were concerned with the possibility that at least a portion of the adjuvant effect of IL-23-Ig on DC could be mediated by the endogenous production of IL-12. To investigate this issue, we took advantage of the recent availability in our laboratory of neutralizing Ab raised to the β 2-chain of the IL-12R (9). We found that the presence of this Ab as well as that of an anti-p70 reagent during activation *in vitro* of DC with IL-23- or IL-12-Ig would selectively block the adjuvant effect of the latter protein. Besides emphasizing the critical role of β 2 as a signal-transducing element in IL-12 signaling, these data demonstrate that, similar to T cells (2), β 2 is not involved in the biological effects of IL-23 on DC, and a significant portion of these effects is independent of endogenous IL-12. On the other hand, in experiments not reported here, we found that rIL-12 would increase the transcriptional expression of murine p19 in DC, thus suggesting the occurrence of mutual regulation between IL-12 and IL-23. As a corollary to the current findings, our data suggest that the use of β 2-specific Ab *in vivo* may represent a suitable means of discriminating between the relative roles of endogenous IL-12 and IL-23 in experimental models of autoimmunity.

It has been suggested that IL-12 may represent a unique cytokine that bridges innate resistance and Ag-specific adaptive immunity (6). However, the critical β 2 subunit required for IL-12 signaling is not present on freshly isolated native T cells and is induced following TCR triggering (32). This condition is different from that of DC, which constitutively express the β 2-chain (8). Although the putative receptor subunit involved in IL-23 signaling in T cells has not been identified, there is evidence that the latter chain, like IL-12R β 2, may not be expressed by naive T cells and may be induced following TCR engagement. Our data suggest that DC do express this subunit constitutively. This again underlines the similarities between IL-12 and IL-23 and further suggests that the latter cytokine may be involved early in the initiation of an Ag-specific immune response. Interestingly in this regard we have found that IL-23-Ig will induce IFN- γ production by DC to an extent similar to IL-12 and that the combination of the two fusion proteins will result in very high levels of IFN- γ production by DC. Because early production of IFN- γ by APC is pivotal in determining the effectiveness of an immune response to pathogens (27), these data may underscore the potential role of IL-23 at the initiation of immunity against microbial pathogens.

In conclusion, the recent discovery of p19/p40 as a novel composite factor closely related to IL-12 capable of critically affecting T cell functions raises important issues. We here show that, analogous to their effects on T cells, IL-12 and IL-23 may also exert similar as well distinct activities on DC, which play a central role

in activating and regulating T cell responses. However, the activities of IL-23 may be even more complex than those of IL-12, encompassing effects on both CD8⁻ and CD8⁺ DC. Given the crucial roles of these subsets in regulating the balance between immunogenic and tolerogenic stimuli in the early response to Ag, our data propose a critical role for IL-23 at the interface of innate and adaptive immunities.

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References

- Mantovani, A., C. A. Dinarello, and P. Ghezzi. 2000. Cytokines: a world apart? In *Pharmacology of Cytokines*. Oxford University Press, New York, pp. 1–18.
- Oppmann, B., R. Lesley, B. Blom, J. C. Timans, Y. Xu, B. Hunte, F. Vega, N. Yu, J. Wang, K. Singh, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13:715.
- Wiekowski, M., M. W. Leach, E. W. Evans, L. Sullivan, S.-C. Chen, G. Vassileva, J. F. Bazan, D. M. Gorman, R. A. Kastelein, S. Narula, et al. 2001. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J. Immunol.* 166:7563.
- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
- Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. IL-12 acts directly on CD4⁺ T cells to enhance priming for IFN γ production and diminishes IL-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 90:10188.
- Trinchieri, G. 1995. Interleukin 12: a proinflammatory cytokine with immunoregulatory functions that bridges innate resistance and antigen specific adaptive immunity. *Annu. Rev. Immunol.* 13:251.
- Macatonia, S. E., N. A. Hosken, M. Litton, P. Vieira, C.-S. Hsieh, J. A. Culpepper, M. Wysocka, G. Trinchieri, K. M. Murphy, and A. O'Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4⁺ T cells. *J. Immunol.* 154:5071.
- Grohmann, U., M. L. Belladonna, R. Bianchi, C. Orabona, E. Ayroldi, M. C. Fioretti, and P. Puccetti. 1998. IL-12 acts directly on DC to promote nuclear localization of NF- κ B and primes DC for IL-12 production. *Immunity* 9:315.
- Grohmann, U., M. L. Belladonna, C. Vacca, R. Bianchi, F. Fallarino, C. Orabona, M. C. Fioretti, and P. Puccetti. 2001. Positive regulatory role of IL-12 in macrophages and modulation by IFN- γ . *J. Immunol.* 167:221.
- Bianchi, R., U. Grohmann, C. Vacca, M. L. Belladonna, M. C. Fioretti, and P. Puccetti. 1999. Autocrine IL-12 is involved in dendritic cell modulation via CD40 ligation. *J. Immunol.* 163:2517.
- Grohmann, U., R. Bianchi, M. L. Belladonna, C. Vacca, S. Silla, E. Ayroldi, M. C. Fioretti, and P. Puccetti. 1999. IL-12 acts selectively on CD8 α ⁻ dendritic cells to enhance presentation of a tumor peptide in vivo. *J. Immunol.* 163:3100.
- Grohmann, U., R. Bianchi, M. L. Belladonna, S. Silla, F. Fallarino, M. C. Fioretti, and P. Puccetti. 2000. IFN- γ inhibits presentation of a tumor/self peptide by CD8 α ⁻ dendritic cells via potentiation of the CD8 α ⁺ subset. *J. Immunol.* 165:1357.
- Gajewski, T. F., J.-C. Renauld, A. van Pel, and T. Boon. 1995. Costimulation with B7-1, IL-6, and IL-12 is sufficient for primary generation of murine anti-tumor cytolytic T lymphocytes in vitro. *J. Immunol.* 154:5637.
- Huston, J. S., D. Levinson, M. Mudgett-Hunter, M.-S. Tai, J. Novotny, M. N. Margulies, R. J. Ridge, R. E. Brucoleri, E. Haber, R. Crea, et al. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 85:5879.
- Gajewski, T. F., F. Fallarino, C. Uyttenhove, and T. Boon. 1996. Tumor rejection requires a CTLA4 ligand provided by the host or expressed on the tumor: superiority of B7-1 over B7-2 for active tumor immunization. *J. Immunol.* 156:2909.
- Grohmann, U., P. Puccetti, M. L. Belladonna, F. Fallarino, R. Bianchi, L. Binaglia, K. Sagakuchi, M. G. Mage, E. Appella, and M. C. Fioretti. 1995. Multiple point mutations in an endogenous retroviral gene confer high immunogenicity on a drug-treated murine tumor. *J. Immunol.* 154:4630.
- Grohmann, U., F. Fallarino, R. Bianchi, M. L. Belladonna, C. Vacca, C. Orabona, C. Uyttenhove, M. C. Fioretti, and P. Puccetti. 2001. IL-6 inhibits the tolerogenic function of CD8 α ⁺ dendritic cells expressing indoleamine 2,3-dioxygenase. *J. Immunol.* 167:708.
- Grohmann, U., M. C. Fioretti, R. Bianchi, M. L. Belladonna, E. Ayroldi, D. Surace, S. Silla, and P. Puccetti. 1998. Dendritic cells, interleukin 12, and CD4⁺ lymphocytes in the initiation of class I-restricted reactivity to a tumor/self peptide. *Crit. Rev. Immunol.* 18:87.
- Puccetti, P., R. Bianchi, M. C. Fioretti, E. Ayroldi, C. Uyttenhove, A. van Pel, T. Boon, and U. Grohmann. 1994. Use of a skin test assay to determine tumor-specific CD8⁺ T cell reactivity. *Eur. J. Immunol.* 24:1446.
- Bianchi, R., U. Grohmann, M. L. Belladonna, S. Silla, F. Fallarino, E. Ayroldi, M. C. Fioretti, and P. Puccetti. 1996. IL-12 is both required and sufficient for initiating T cell reactivity to a class I-restricted tumor peptide (P815AB) following transfer of P815AB-pulsed dendritic cells. *J. Immunol.* 157:1589.
- Grohmann, U., R. Bianchi, E. Ayroldi, M. L. Belladonna, D. Surace, M. C. Fioretti, and P. Puccetti. 1997. A tumor-associated and self antigen peptide presented by dendritic cells may induce T cell anergy in vivo, but IL-12 can prevent or revert the anergic state. *J. Immunol.* 158:3593.
- Wilkinson, V. L., R. R. Warrior, T. P. Truitt, P. Nunes, M. K. Gately, and D. H. Presky. 1996. Characterization of anti-mouse IL-12 monoclonal antibodies and measurement of mouse IL-12 by ELISA. *J. Immunol. Methods* 189:15.
- Presky, D. H., H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C.-Y. Wu, M. K. Gately, and U. Gubler. 1996. A functional interleukin 12 receptor complex is composed of two β -type cytokine receptor subunits. *Proc. Natl. Acad. Sci. USA* 93:14002.
- Hsieh, C., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. *Listeria*-induced Th1 development in $\alpha\beta$ -TCR transgenic CD4⁺ T cells occurs through macrophage production of IL-12. *Science* 260:547.
- Nagayama, H., K. Sato, H. Kawasaki, M. Enomoto, C. Morimoto, K. Tadokoro, T. Juji, S. Asano, and T. A. Takahashi. 2000. IL-12 responsiveness and expression of IL-12 receptor in human peripheral blood monocyte-derived dendritic cells. *J. Immunol.* 165:59.
- Fukao, T., D. M. Frucht, G. Yap, M. Gadina, J. J. O'Shea, and S. Koyasu. 2001. Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune response. *J. Immunol.* 166:4446.
- Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea, and S. Koyasu. 2001. IFN- γ production by antigen-presenting cells: mechanisms emerge. *Trends Immunol.* 22:556.
- Decken, K., G. Kohler, K. Palmer-Lehmann, A. Wunderlin, F. Mattner, J. Magram, M. K. Gately, and G. Alber. 1998. Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. *Infect. Immun.* 66:4994.
- Brombacher, F., A. Dorfmueller, J. Magram, W. J. Dai, G. Kohler, A. Wunderlin, K. Palmer-Lehmann, M. K. Gately, and G. Alber. 1999. IL-12 is dispensable for innate and adaptive immunity against low doses of *Listeria monocytogenes*. *Int. Immunol.* 11:325.
- Grohmann, U., F. Fallarino, S. Silla, R. Bianchi, M. L. Belladonna, C. Vacca, A. Micheletti, M. C. Fioretti, and P. Puccetti. 2001. CD40 ligation ablates the tolerogenic potential of lymphoid dendritic cells. *J. Immunol.* 166:277.
- Drakesmith, H., B. Chain, and P. Beverley. 2000. How can dendritic cells cause autoimmune disease? *Immunol. Today* 21:214.
- Jacobson, N. G., S. J. Szabo, R. M. Weber-Nordt, Z. Zhong, R. D. Schreiber, J. E. Darnell, and K. M. Murphy. 1995. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J. Exp. Med.* 181:1755.
- Banchereau, J., J. Pulendran, R. Steinman, and K. Palucka. 2000. Will the making of plasmacytoid dendritic cells in vitro help unravel their mysteries? *J. Exp. Med.* 192:F39.
- Fallarino, F., C. Vacca, C. Orabona, M. L. Belladonna, R. Bianchi, B. Marshall, D. B. Keskin, A. L. Mellor, M. C. Fioretti, U. Grohmann, et al. 2002. Functional expression of indoleamine 2,3-dioxygenase by murine CD8 α ⁺ dendritic cells. *Int. Immunol.* 14:65.
- Adorini, L., F. Aloisi, F. Galbiati, M. K. Gately, S. Gregori, G. Penna, F. Ria, S. Smirolto, and S. Trembleau. 1997. Targeting IL-12, the key cytokine driving Th1-mediated autoimmune diseases. *Chem. Immunol.* 68:175.