In Vivo Microbial Stimulation Induces Rapid CD40 Ligand–independent Production of Interleukin 12 by Dendritic Cells and their Redistribution to T Cell Areas

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Summary

The early induction of interleukin (IL)-12 is a critical event in determining the development of both innate resistance and adaptive immunity to many intracellular pathogens. Previous in vitro studies have suggested that the macrophage (MΦ) is a major source of the initial IL-12 produced upon microbial stimulation and that this response promotes the differentiation of protective T helper cell 1 (Th1) $CD4^+$ lymphocytes from precursors that are primed on antigen-bearing dendritic cells (DC). Here, we demonstrate by immunolocalization experiments and flow cytometric analysis that, contrary to expectation, DC and not MΦ are the initial cells to synthesize IL-12 in the spleens of mice exposed in vivo to an extract of *Toxoplasma gondii* or to lipopolysaccharide, two well characterized microbial stimulants of the cytokine. Importantly, this production of IL-12 occurs very rapidly and is independent of interferon γ priming or of signals from T cells, such as CD40 ligand. IL-12 production by splenic DC is accompanied by an increase in number of DCs, as well as a redistribution to the T cell areas and the acquisition of markers characteristic of interdigitating dendritic cells. The capacity of splenic DC but not MΦ to synthesize de novo high levels of IL-12 within hours of exposure to microbial products in vivo, as well as the ability of the same stimuli to induce migration of DC to the T cell areas, argues that DC function simultaneously as both antigen-presenting cells and IL-12 producing accessory cells in the initiation of cell-mediated immunity to intracellular pathogens. This model avoids the need to invoke a three-cell interaction for Th1 differentiation and points to the DC as both a sentinel for innate recognition and the dictator of class selection in the subsequent adaptive response.

Interleukin 12 (IL-12) is a key cytokine in the induction of cell-mediated immunity to intracellular pathogens. In the innate response to these microbial agents, IL-12 triggers the production of IFN-γ and TNF from unsensitized NK and T cells. At the same time, IL-12 selectively promotes the differentiation of Th1 $CD4^+$ cells, which produce the same effector lymphokines upon restimulation with antigen. Thus, the induction of IL-12 early in infection initiates innate resistance to the pathogen while ensuring the induction of the correct class of adaptive host response (1).

Macrophages (MΦ)[1] activated by microbial stimulation produce high levels of IL-12, and it has been assumed that these cells provide the major source of the cytokine in Th1 response initiation (2). Indeed, in vitro studies with TCR transgenic $CD4^+$ cells primed by antigen-pulsed cells showed IL-12–producing MΦ to be highly effective in inducing selective Th1 cell differentiation (3). However, the model of class selection suggested by these experiments requires that both antigen-bearing dendritic cells (DC) and IL-12–producing MΦ travel from the site of infection to lymphoid tissues where the responding T cells are found, and at present, no evidence exists for such MΦ migration. Furthermore, a model in which IL-12 produced by MΦ acts in a paracrine fashion to drive Th1 development of microbe-specific T cells would lead to Th1 differentiation of all recently activated precursors in the local microenvironment, thus limiting the ability of the immune system to independently control Th1 responses to different antigens and potentially leading to inflammatory responses to self-antigens. A similar objection applies to models of type 1 response

[1]*Abbreviations used in this paper:* DAB, diaminobenzidine; DC, dendritic cell(s); HRP, horseradish peroxidase; IDC, interdigitating DC; KO, knockout, L, ligand; LOD, low density spleen cells; MΦ, macrophage(s); OVA, ovalbumin; PALS, periarteriolar lymphoid sheath; PEC, peritoneal exudate cells; STAg, soluble *Toxoplasma gondii* tachyzoite extract; thio-, thioglycollate-elicited; Thp, Th precursor.

initiation involving other "third-party" IL-12–producing cells such as neutrophils (4).

In contrast to MΦ, DC constitute a highly efficient system for capturing antigens in the periphery and delivering them to the T cell areas of lymphoid tissues (5, 6). It is believed that this allows perusal of peripheral antigens by T cells that recirculate between the blood and lymphoid compartments. In addition, DC possess many specializations that allow them to function as efficient APCs, such as high levels of MHC products, adhesion and costimulatory molecules, extensive surface area, and high motility (5, 6). These properties suggest that DC act as the priming APC for most T cell responses and thus would be ideally placed to produce IL-12 at a site where it acts directly on those T cells responding to DC-presented immunogenic MHC–peptide complexes derived from infectious agents.

The early synthesis of IL-12 is of crucial importance in determining both innate and adaptive host resistance to the intracellular protozoan, *Toxoplasma gondii*, and live replicative forms (tachyzoites) as well as parasite extracts have been shown to be potent inducers of the cytokine from peritoneal inflammatory MΦ in vitro (7–10). Nevertheless, inflammatory MΦ populations that are elicited by local injection of an irritant, or, for that matter, any population obtained after in vivo or in vitro manipulation, may not be representative of the naive cells that initiate IL-12 responses. Therefore, we have used immunolocalization techniques to identify the cells first making IL-12 after in vivo stimulation with *T. gondii* products or LPS. Our results clearly demonstrate that DC, not MΦ, are the cells involved in the IL-12 response to these microbial stimuli, which also simultaneously trigger DC recruitment to the T cell areas of the spleen. Together, these findings support a two cell model of in vivo T cell activation in which the DC serves as both the APC and the IL-12–producing initiator of the Th1 response.

Materials and Methods

Experimental Animals. Female C57BL/6, CBA, and C3H/HeJ mice were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD). C57BL/6-SCID/SzJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at the National Institute of Allergy and Infectious Diseases (NIAID) animal facility (Bethesda, MD). IFN-γ gene-targeted (knockout; KO) mice (11) were originally provided by D. Dalton and T. Stewart (Genentech, Inc., South San Francisco, CA) and backcrossed for seven generations on the C57BL/6 background at Taconic Farms (Germantown, NY). IL-12 p40 KO mice (12) (backcrossed for five generations on the same B6 background) were originally donated by Jeanne Magram (Hoffman-La Roche, Nutley, NJ) and bred at the NIAID animal facility. CD40 ligand (CD40L) KO mice (13) maintained on a mixed B6 × 129/J background and control mice (B6 × 129)F_2 were purchased from The Jackson Laboratory. All animals were housed in specific pathogen-free conditions and were used at 6–9 wk of age.

Microbial Stimuli. Tachyzoites of the virulent RH *T. gondii* strain were maintained by passage on human foreskin fibroblasts and soluble tachyzoite antigen (STAg) was prepared as previously described (10). *Escherichia coli* LPS was purchased from Sigma Chemical Co. (St. Louis, MO).

In Vitro Cell Culture and Cytokine Production Assays. Resident and inflammatory MΦ were obtained from animals that had been either untreated or inoculated intraperitoneally 4 d previously with 1.5 ml of 3% thioglycollate (Sigma Chemical Co.). Cells were plated in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) containing 2% FCS in 96-well plates for 2 h at 37°C. The medium and unbound cells were then removed and replaced with 200 μl of RPMI 1640 complete medium in the presence or absence of STAg or RH tachyzoites. In some experiments, cultures were supplemented with 100 U/ml of rMuIFN-γ (provided by Genentech, Inc.). Bulk splenocytes were cultured under the same conditions as the peritoneal cells. Supernatants were harvested at 6 h for TNF-α, at 18 h for IL-12 p40, and at 72 h for IFN-γ determinations. These measurements were performed by two-site ELISA, as previously described (14).

Flow Cytometry. Low density spleen cells (LOD) were prepared from collagenase-digested spleens as previously described (15), except that immediately after collagenase treatment, spleen digests were washed in PBS containing 5 mM EDTA (PBS/EDTA) and from this point onwards the cell suspension was always handled in buffers containing EDTA. This treatment allows for release of interdigitating DC (IDC) from the inner periarteriolar lymphoid sheaths (PALS) that are normally lost in the presence of Ca^{2+} (16, 17).

LOD and high-density spleen cells were fixed in 1% paraformaldehyde in PBS/EDTA for 10 min, washed, and kept overnight in washing solution (PBS/EDTA containing 1% FCS; WS). The next day, cells were stained with NLDC-145 (18) or anti–IL-12 p40 mAbs C17.15 or C17.8 (both rat IgG2a; reference 19); provided by Drs. G. Trinchieri and M. Wysocka, Wistar Institute, Philadelphia, PA), followed by FITC-conjugated mouse $F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). N418 supernatant (ATCC HB 224 [American Type Culture Collection, Rockville, MD]; reference 20) containing 25 μg/ml rat IgG was subsequently added, followed by biotin-conjugated goat $F(ab')_2$ anti–syrian hamster IgG cross-adsorbed against rat and mouse serum proteins (Jackson ImmunoResearch Laboratories); TriColor-streptavidin (Caltag, San Francisco, CA) was added, together with PE-conjugated 53-6.7 (anti-CD8α; PharMingen, San Diego, CA). Washes and reagent dilutions were in WS except before the N418 step when all reagents and WS also included 0.1% saponin to allow antibody access to intracellular compartments.

100,000 events were collected on a FACScan® cytometer and analyzed using CellQuest™ software (Becton Dickinson, Mountain View, CA).

Immunohistochemistry. Spleen fragments were frozen in embedding medium (Cryoform; International Equipment Co., Needham, MA). 6-μm frozen sections were cut, air-dried, fixed in acetone, and rehydrated in Tris-buffered saline (TBS) containing 0.05% Tween 20. Endogenous peroxidase was blocked with 0.3% H_2O_2. For IL-12 p40 staining, sections were incubated with C17.15 (10–20 μg/ml) or an isotype-matched control, followed by biotin-conjugated mouse $F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch Laboratories) and horseradish peroxidase (HRP)–streptavidin (DuPont, Boston, MA). HRP localization was revealed using a metal-enhanced diaminobenzidine (DAB) substrate (Pierce, Rockford, IL), resulting in brown/black staining. For double labeling with anti–T cell or anti–B cell markers, sections were first stained as above; after developing the HRP, sections were reblocked with 0.3% H_2O_2 and were further stained with either

FITC-H57-597 (anti–TCR-β; PharMingen) or FITC-RA3-6B2 (anti-B220; PharMingen), added in a solution containing 25 μg/ml of rat IgG; this was followed by HRP-conjugated rabbit antifluorescein (BIODESIGN International, Kennebunkport, ME). For double labeling with N418, sections were stained for IL-12 as above or with NLDC-145 and HRP-mouse $F(ab')_2$ anti–rat IgG (Jackson ImmunoResearch Laboratories); after developing HRP with metal-enhanced DAB, sections were reblocked with 0.3% H_2O_2 and were further blocked with avidin followed by biotin (both from Vector Laboratories, Burlingame, CA; used according to manufacturer's instructions). N418 supernatant containing 25 μg/ml rat IgG was subsequently added, followed by biotin-conjugated goat $F(ab')_2$ anti–syrian hamster IgG cross-adsorbed against rat and mouse serum proteins (Jackson ImmunoResearch Laboratories) and HRP-streptavidin.

HRP in the second label was developed using the VIP substrate (Vector Laboratories), resulting in purple staining. All staining reactions included appropriate negative and positive controls to account for any cross-reactivity. HRP activity in the first label, used to reveal IL-12 p40, is consumed during the first development reaction with metal-enhanced DAB and does not contribute to the purple VIP staining observed with the second label (Reis e Sousa, C., unpublished observations).

After staining, sections were washed in distilled water, dried, and mounted in Permount (Fisher Scientific Co., Fairlawn, NJ). Stained sections were photographed on an Axiophot microscope (Carl Zeiss Inc., Thornwood, NY) using Kodachrome 25 film (Eastman Kodak Co., Rochester, NY).

Results

Resting MΦ Populations Fail to Produce IL-12 in Response to T. gondii In Vitro. MΦ-derived IL-12 has traditionally been considered a major factor driving Th1 responses, based on the observation that MΦ produce high levels of IL-12 after exposure to microbial products or after microbial infection in vitro (1, 2). However, most experiments examining IL-12 synthesis by MΦ in response to microbial stimuli to date, including our own (10), have primarily made use of inflammatory cells such as those that can be isolated from the peritoneal cavity of mice after elicitation with thioglycollate. To study IL-12 production by resting, unactivated MΦ in response to microbial products, resident peritoneal exudate cells (PEC) from LPS hyporesponsive C3H/HeJ mice were compared to thioglycollate-elicited PEC (thio-PEC) from the same mouse strain for the ability to produce various monokines after in vitro infection with *T. gondii* or after exposure to a soluble *T. gondii* antigen extract (STAg). As shown in Fig. 1, infection of freshly isolated resident PEC with live *T. gondii* tachyzoites or incubation with STAg did not result in production of detectable IL-12 p40 despite the fact that the same cells produced TNF. In contrast, as previously reported (10), thio-PEC produced substantial levels of both IL-12 p40 and TNF in response to infection or exposure to STAg (Fig. 1). Addition of exogenous IFN-γ to the cultures, which dramatically augments production of IL-12 p40 by inflammatory MΦ stimulated with *T. gondii* (21), did not correct the selective defect in IL-12 p40 production by resident cells despite the fact that it increased their production of TNF by 10-fold (Fig. 1). Resident MΦ were able to produce IL-12 p40 upon exposure to STAg or after live infection only when incubated for 24 h before stimulation, especially if IFN-γ was included in the preculture (data not shown). Furthermore, preincubation in the presence of IFN-γ was required to obtain substantial levels of IL-12 p40 production in response to STAg with bone marrow–derived MΦ populations and monocytic cell lines (data not shown). These results suggest that resting, unprimed MΦ cannot serve as a significant source of IL-12 to drive Th1 responses to *T. gondii*. Thus, a model in which MΦ-derived IL-12 is a pivotal factor in driving Th1 responses to microbial infections requires that the infected MΦ be already "primed" for IL-12 production, in a manner analogous to thioglycollate elicitation or preculture in medium containing IFN-γ.

Production of IL-12 by Spleen Cells in Response to T. gondii Antigens. To identify a source of IL-12 not requiring priming, we tested unfractionated spleen cells because previous studies had identified them as a source of T-independent IFN-γ production (22). Surprisingly, whole splenocytes produced significant levels of IL-12 p40 after STAg exposure or *T. gondii* infection in vitro, but relatively little TNF (Fig. 1). This raised the possibility that the spleen contains a population of cells that can produce IL-12 directly in response to microbial stimuli, without priming (Fig. 1). When spleen cells were separated into adherent and nonadherent cells, STAg-induced IL-12 production was enriched in the adherent fraction, composed primarily of MΦ and DC, and was relatively depleted in the nonadherent lymphocyte fraction (data not shown). Adherent cells constitute only a small proportion of all splenocytes (<5%). Therefore, the high levels of IL-12 seen with unfractionated spleen cells, close to those elicited from the same

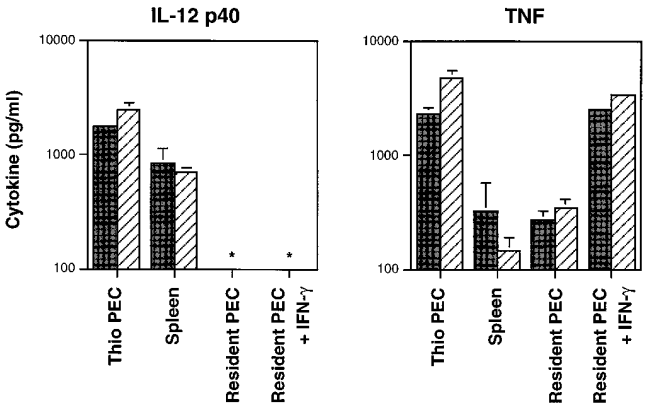

Figure 1. Comparison of IL-12 p40 production by resting PEC, thio-PEC, and spleen cells in response to *T. gondii*. Adherent resident or thio-PEC (2×10^5/well) or a similar number of whole spleen cells from C3H/HeJ mice were cultured overnight in the presence or absence of *T. gondii* tachyzoites (strain RH; 2×10^5/well) or STAg (5 μg/ml). Where indicated, resident PEC cultures were supplemented with 100 U/ml of IFN-γ. IL-12 p40 and TNF released into the supernatant were measured by ELISA. Spontaneous IL-12 production by cells cultured in medium alone was not detected. Results represent the mean of triplicate cultures. Error bars represent one SD from the mean. *Gray bars*, STAg; *hatched bars*, tachyzoites; *, not detectable.

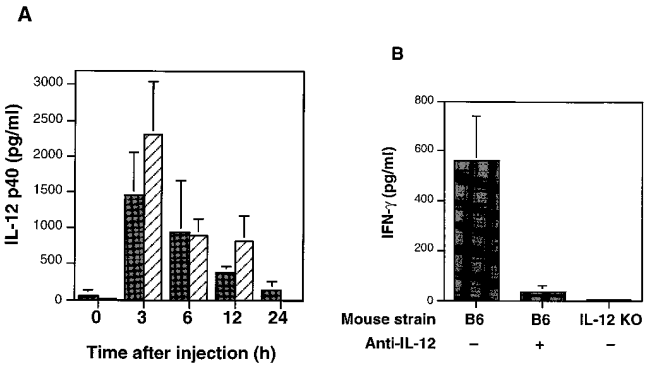

Figure 2. Intravenous administration of STAg causes production of IL-12 p40 that can prime an IFN-γ response. (*A*) Spontaneous IL-12 production by spleen cells isolated from C57BL/6 or IFN-γ KO mice injected with STAg for the indicated times. Cells were isolated by mechanical dissociation of spleens from control mice (0 h) or mice intravenously injected with 25 μg STAg and were cultured for 24 h in medium. IL-12 p40 released into supernatants was measured by ELISA. *Gray bars*, B6; *hatched bars*, IFN-γ KO. (*B*) Enhanced IFN-γ secretion by spleen cells is dependent upon STAg-elicited IL-12 in vivo. Mice from the indicated strains were intravenously injected with 25 μg STAg. Where indicated (+), mice were intraperitoneally treated with 1 μg anti–IL-12 (mAb C17.8; reference 19) immediately before injection of STAg by the intravenous route. Cells were isolated by mechanical dissociation of spleens 48 h after injection and were restimulated with 5 μg/ml STAg for 48 h. IFN-γ released into supernatants was measured by ELISA. Equivalent control cultures in medium alone did not produce IFN-γ (data not shown). Data in *A* and *B* are the average of three mice in each group except for the anti–IL-12-treated group in *B*, for which the data are the mean of two mice. Error bars represent one SD from the mean.

number of cells of homogeneous populations of thio-MΦ (Fig. 1), seem likely to reflect extremely high levels of production by a small subpopulation of cells.

To determine if IL-12 production by spleen cells in response to *T. gondii* also occurs in vivo, B6 mice were injected intravenously with STAg. Spleen cell suspensions from mice injected with STAg, but not from uninjected mice or from mice injected with PBS alone, spontaneously produced IL-12 p40 during overnight culture (Fig. 2 *A*). Maximal IL-12 p40 production could be seen as early as 3 h after systemic administration of STAg and declined progressively thereafter (Fig. 2 *A*). The IL-12 produced in vivo appeared to be bioactive because spleen cells from STAg-injected mice produced increased levels of IFN-γ upon in vitro restimulation of spleen cells with STAg or LPS. This enhancement of IFN-γ production was specifically dependent upon IL-12 induction in vivo because it was not seen in IL-12 p40–deficient mice or in wild type mice treated with anti–IL-12 antibodies at the time of STAg administration (Fig. 2 *B*). Together, these results demonstrate that spleen cells can synthesize IL-12 p40 in response to stimulation with *T. gondii* products in vitro or in vivo, and that IL-12 production elicited by these molecules in vivo can prime an IFN-γ response.

IL-12 Production by DC In Situ in Response to Systemic Administration of Microbial Products. The ability of STAg to induce IL-12 p40 production in the spleen in vivo offered an opportunity to phenotype the IL-12–producing cells in situ. Spleen sections from STAg-injected C57BL/6 mice showed numerous intensely stained IL-12 $p40^+$ cells (Fig. 3 *B*) that were not seen in sections from uninjected control mice (Fig. 3 *A*). Induction of IL-12 p40 was specifically dependent on exposure to the parasite extract because it was not found in sections from animals injected with PBS, hen egg lysozyme, or ovalbumin (OVA; Fig. 3 *C* and data not shown). Intensely stained IL-12 $p40^+$ cells could be detected as early as 3 h after STAg injection and the staining peaked between 6 and 12 h, and then declined progressively. 24 h after injection, staining was barely visible and the sections resembled those of control animals (data not shown). IL-12 $p40^+$ cells had dendritic profiles and formed abundant "nests" surrounding central arterioles (Fig. 4 *D*), suggesting that they might represent IDC. Indeed, staining of serial sections demonstrated that IL-12 $p40^+$ cells localized exclusively in the T cell areas of the white pulp and were excluded from the red pulp or the B cell areas (Fig. 4, *D–F*). This was true except at 3 h after injection, when IL-12 $p40^+$ cells showed a more diffuse location, with many cells found at the edge of the T cell area and others interspersed with B cells in the marginal zone (Fig. 4, *A–C*, and data not shown). This picture is consistent with the IL-12–producing cells being in the process of migrating into the T cell area (see below). LPS coinjected intravenously with OVA also induced the appearance of $IL\text{-}12^+$ nests of dendritic profiles surrounding central arterioles in spleen sections (Fig. 3 *D*). These were not seen in control sections injected with OVA alone (Fig. 3 *C*). Nevertheless, IL-12 p40 staining of putative IDC in response to LPS injection was consistently weaker and involved fewer cells than in response to STAg (Fig. 3 *D*). Importantly, IL-12 staining could not be detected in MΦ in the red pulp or those in the marginal zone (including MOMA-1^+ [23] metallophils and marginal zone MΦ), either at early times or up to 96 h after injection of STAg (Figs. 3 and 4, and data not shown). IL-12 p40 staining by MΦ was also not detected in LPS-injected animals (Fig. 3 *D* and data not shown).

To demonstrate that the IL-12 $p40^+$ cells were indeed DC, sections were double stained with anti–IL-12 p40 and N418, a marker for mouse DC (20). As shown in Fig. 4 *G*, $IL\text{-}12^+$ cells were also positive for N418 (*white arrow*) although some $N418^+$ cells did not appear to stain for IL-12, particularly those $N418^+$ cells at the edge or outside the T cell area (Fig. 4 *G*, *black arrow*). Furthermore, staining of serial sections with different antibodies demonstrated that $IL\text{-}12^+$ cells colocalized with cells positive for the NLDC-145 marker (18), also known as DEC-205 (24), which is highly expressed by IDC in situ (data not shown). To confirm these results and to allow more accurate immunophenotyping of IL-12–producing cells, LOD suspensions were prepared in Ca^{2+}-free media from mice injected with STAg or from controls injected with PBS, and then analyzed by flow cytometry. A distinct subpopulation of LOD from STAg-injected but not from PBS-injected mice could be stained intracellularly with anti–IL-12 p40 antibodies (Fig. 5, *A* and *B*). All $IL\text{-}12^+$ cells were also $N418^{bright}$, confirming the observations from immunohistochemistry that $N418^{-/dull}$ splenic MΦ, which, like DC, are enriched in

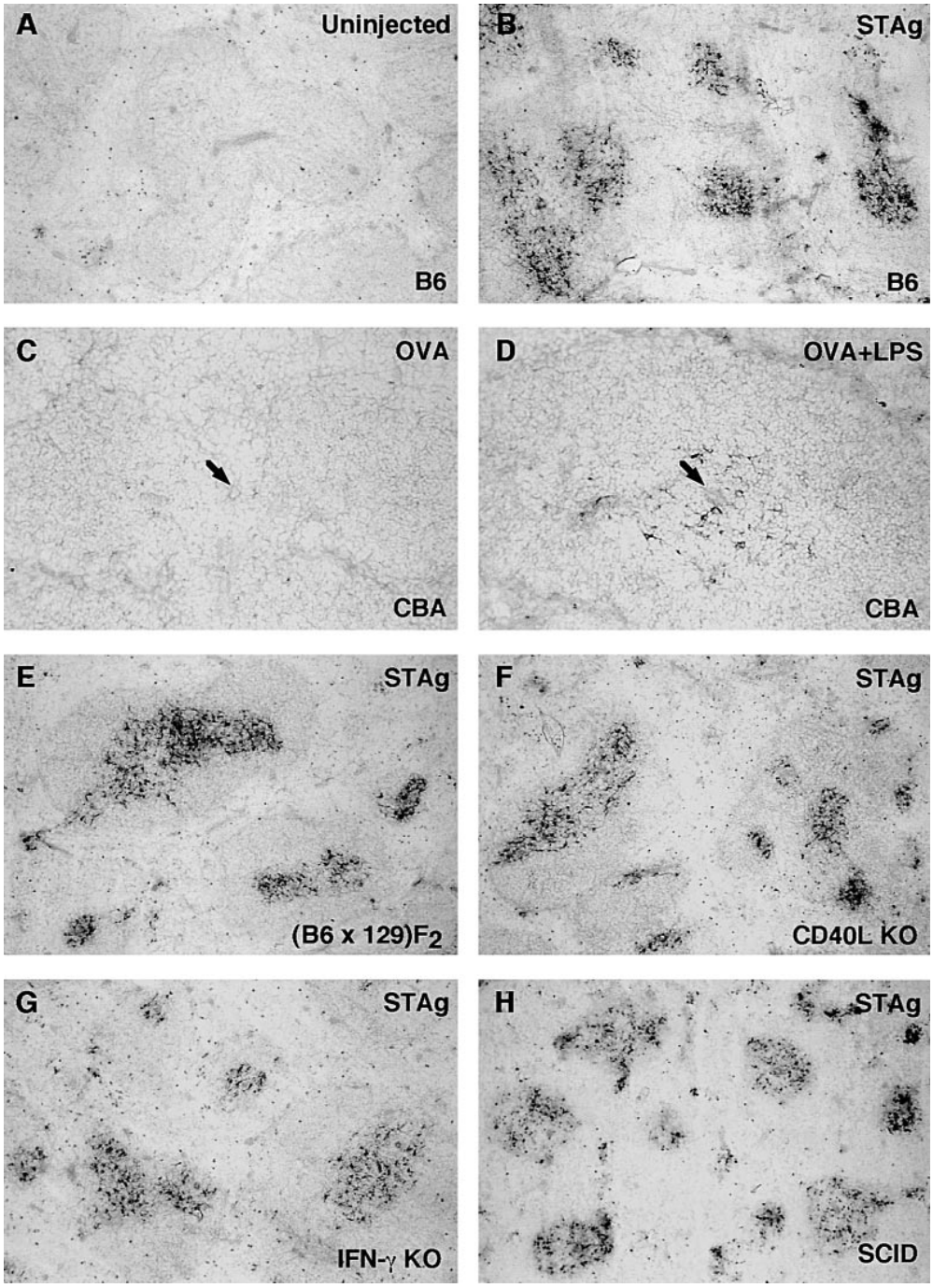

Figure 3. IL-12–producing cells are found in the spleens of mice injected systemically with STAg or LPS. Mice of the indicated strains were left untreated (*A*), or were intravenously injected with 0.5 μmol OVA (*C*), OVA + 40 μg LPS (*D*), or with 25 μg STAg (*B, E–H*). Animals were killed 6 h (*B, E–H*) or 4 h (*C* and *D*) after injection. Spleens were frozen, sectioned, and stained with anti–IL-12 p40 as detailed in Materials and Methods. Note "nests" of IL-12–producing cells after STAg or LPS injection. Arrows in *C* and *D* indicate the central arterioles. Original magnification: *A*, *B*, and *E–H*, ×100; *C* and *D*, ×200.

LOD, do not produce significant levels of IL-12 after exposure to STAg in vivo (Fig. 5 *B*). Remarkably, most IL-12–producing N418$^+$ cells were also positive for CD8α (Fig. 5, *C* and *D*), a marker that, like DEC-205, is expressed by IDC (16, 17). 67% of all the CD8α$^+$ N418$^+$ DC in STAg-injected animals stained for IL-12; in contrast, only a few (12%) of the CD8α$^-$ N418$^+$ DC were positive for IL-12 and the intensity of IL-12 staining of these cells was lower than that of the CD8$^+$ DC (mean fluorescence 223 versus mean fluorescence 644; see Fig. 5 *D*). We conclude that high levels of IL-12 production in mouse spleen can be detected shortly after systemic administration of two different microbial products, STAg and LPS. This IL-12 production almost exclusively involves a large proportion of CD8α$^+$ DEC-205$^+$ IDC, under conditions in which production of the same cytokine by splenic MΦ cannot be detected.

DC Production of IL-12 Is Not Dependent on Priming by IFN-γ or Activation by CD40L$^+$ T Cells. IL-12 production by DC in vivo in response to exposure to STAg was independent of the mouse strain used. It could be seen in both C57BL/6 and BALB/c mice, two strains known to vary in

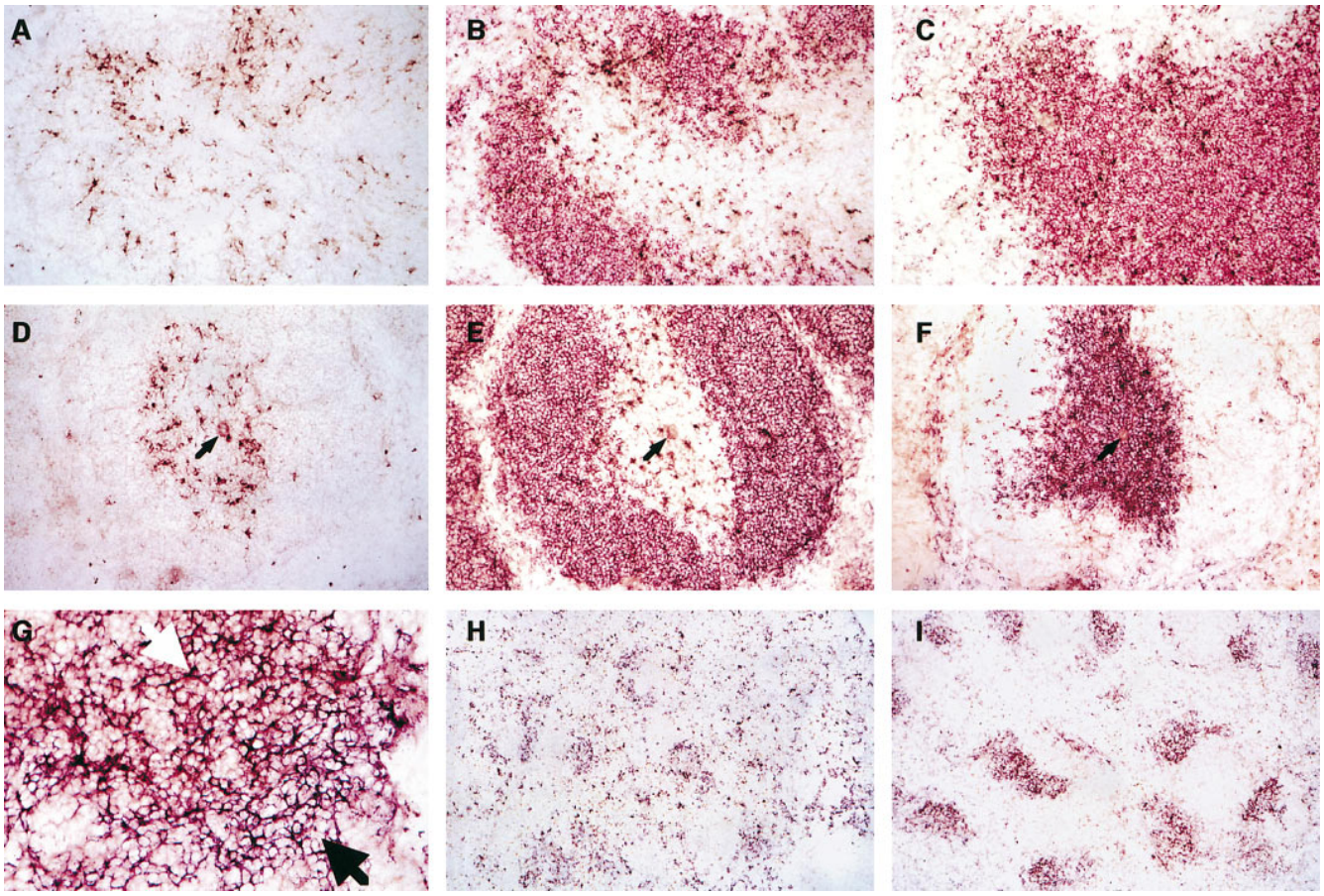

Figure 4. IL-12–producing cells migrate into the inner PALS to become IDC in response to STAg. Spleen sections from C57BL/6 mice intravenously injected 3 (*A–C*) or 6 h (*D–G*, *I*) previously with 25 μg STAg, or sections from uninjected mice (*H*), were stained for IL-12 p40 (*A–G*; *dark brown*) or DEC-205 (*H* and *I*; *dark brown*) and double stained (*purple*) for B220 (*B* and *E*), TCR-β (*C* and *F*), or N418 (*G–I*). *A–C* and *D–F* are serial sections through the same white pulp nodule. Note: IL-12–producing cells are found in the marginal zone and outer PALS 3 h after injection (*A–C*), but are seen in the inner PALS (T cells area) 6 h after injection (*D–F*; *arrows* indicate the central arteriole); IL-12 $p40^+$ cells are also positive for N418 (*G*, *white arrow* indicating brown and purple stain) although not all $N418^+$ cells are IL-12 $p40^+$ (*G*, *black arrow* indicating purple only stain; see also Fig. 5); after STAg administration (*H*), there is an apparent increase in the number of DC in the spleen and redistribution of these cells to the inner PALS (compare *H* with *I*; see also Table 1). Original magnification: *A–F*, ×200; *G*, ×400; *H–I*, ×100.

their predisposition to Th2 responses (25), and was also seen in (B6 × 129)F_2 (data not shown and Fig. 3 *E*). To determine whether STAg-induced IL-12 production by DC in vivo is dependent on priming by IFN-γ, responses to STAg were compared between B6 and IFN-γ KO mice. Spleen cells from both strains isolated after STAg injection spontaneously produced comparable amounts of IL-12 p40 in culture (Fig. 2 *A*). Similarly, IL-12 staining in the spleens of IFN-γ KO mice intravenously injected with STAg was indistinguishable from that in wild-type B6 controls, being restricted to dendritic profiles surrounding central arterioles (Fig. 3 *G*). Furthermore, the kinetics of IL-12 induction in vivo were identical between the two mouse strains, with staining peaking at 6–12 h and disappearing by 24 h after injection (data not shown).

IL-12 production by both murine and human DC in vitro has been previously reported (26–32). The major mechanisms involved in IL-12 induction appear to be signaling through DC surface CD40 molecules after cross-linking by T cell–expressed CD40L, or direct signaling through MHC class II molecules on DC, cross-linked by the TCR (30, 31, 33). Spleen cells from CD40L KO mice secreted substantial levels of IL-12 p40 in response to live parasite infection or STAg stimulation in vitro, although these levels were somewhat lower than those produced by cells from (B6 × 129)F_2 control mice (Fig. 6). To examine the CD40L dependence of IL-12 production by DC in response to STAg in vivo, CD40L KO mice were injected with STAg as before and spleens removed 6 h later. Staining of sections from CD40L KO mice was comparable to that of sections from B6 mice or (B6 × 129)F_2 controls, suggesting that STAg-induced IL-12 production by IDC does not require cross-linking of CD40 on DC by CD40L on T cells (Fig. 3, *E* and *F*). To exclude the possibility that other cognate T cell–DC interactions or T cell–derived cytokines might be responsible for the STAg effect, SCID mice were similarly injected with STAg and analyzed in parallel to the CD40L KO mice. Again, SCID spleen sec-

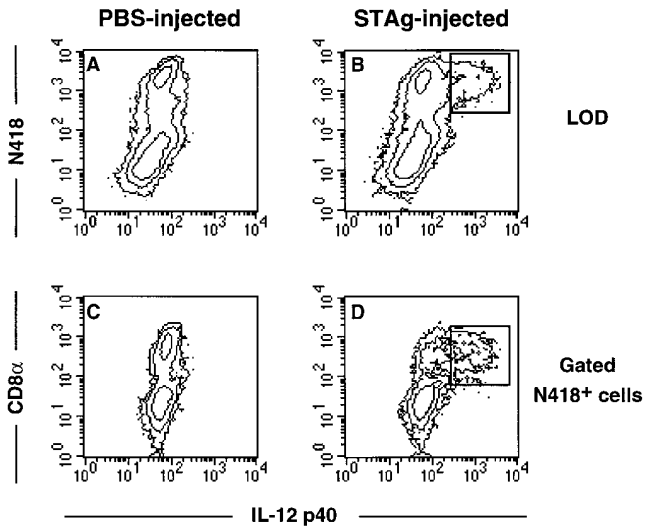

Figure 5. IL-12 production by LOD in response to systemic administration of STAg is restricted to CD8α$^+$N418$^+$ DC. LOD prepared from groups of STAg- or PBS-injected C57BL/6 mice were triple stained for IL-12 p40, N418, and CD8α or for DEC-205, N418, and CD8α. (*A* and *B*). IL-12$^+$ cells are only seen in STAg-injected animals and are all bright for N418 (*box*). (*C* and *D*) Gating on N418$^+$ cells demonstrates that IL-12$^+$ cells seen in response to STAg (*box*) are part of the CD8α$^+$ DC subset. All CD8α$^+$ N418$^+$ cells were also positive for NLDC-145, as reported (16, 17). Data are representative of three independent experiments. Other data from the same experiment are shown in Table 1.

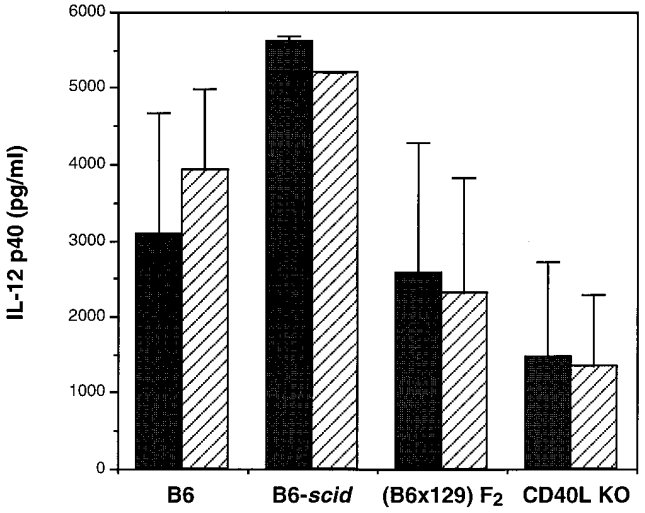

Figure 6. IL-12 production by spleen cells in response to stimulation with *Toxoplasma* antigens in vitro is independent of CD40L or of signals from lymphocytes. Spleen cells from the indicated mouse strains were cultured in the presence of STAg or live *T. gondii* tachyzoites as in Fig. 1, and IL-12 p40 secreted into supernatants was measured by ELISA after 24 h. C57BL/6 served as controls for the B6-SCID and (B6 × 129)F_2 for the CD40L KO mice. The apparently higher levels of IL-12 production from SCID spleen probably reflect their enrichment for nonlymphoid cells relative to wild-type spleens. Spontaneous IL-12 production by spleen cells cultured in medium alone was not detected. Results represent the mean of triplicate cultures from two to three animals per group. Error bars represent one SD from the mean. *Gray bars*, STAg; *hatched bars*, tachyzoites.

tions showed IL-12 p40 staining comparable to wild-type controls (Fig. 3 *H*). SCID spleen cells also made high levels of IL-12 p40 in response to STAg or live infection in vitro (Fig. 6). Thus, microbe-induced IL-12 production by spleen cells in vitro or by DC in vivo does not require interactions with lymphocytes and is likely to reflect a direct effect on the cells of one or more components of the microbial extract, or an indirect effect through induction of DC-activating inflammatory cytokines produced by nonlymphoid cells.

Mobilization of DC to the Inner PALS after Systemic Administration of STAg. Mouse spleen contains a subset of relatively immature DC outside or at the margin of the T cell area that may act as precursors for IDC and that probably represent the bulk of DC in conventionally prepared spleen cell suspensions (20, 34). In vivo, LPS induces functional maturation of these marginal zone DC and causes them to move into the PALS area (35 and Reis e Sousa, C., unpublished data). The striking restriction of IL-12 p40 staining to CD8α$^+$ DEC-205$^+$ DC in the T cell areas, seen as early as 6 h after STAg or LPS injection, could be due to stimulation of resident IDC by the microbial products and/or to a rapid redistribution of IL-12–producing IDC precursors to the inner PALS. Consistent with the latter, IL-12 p40$^+$ DC could be seen at early times after STAg injection outside the T cell area (see above). Examination of spleen sections from STAg-injected animals demonstrated a striking accumulation of double-stained N418$^+$ NLDC-145$^+$, as well as single-stained N418$^+$ cells, in nests surrounding the central arterioles (Fig. 4 *I*), corresponding to the locations where IL-12–producing cells were found. This contrasted markedly with the more diffuse distribution of DC seen in sections from control animals (Fig. 4 *H*). At earlier times after injection, the distribution of splenic DC was intermediate between that seen in control animals and the exclusive localization in the T cell areas seen after 6 h, much like the distribution of IL-12$^+$ cells at 3 h (Fig. 4, *A–C*). The overall number of DC in spleen sections from STAg-injected mice also appeared to be greater than in control animals (compare Figs. 4, *H* and *I*). This was confirmed by counting different cell subpopulations in the low density fraction of spleen. 6 h after STAg administration, STAg-injected mice had ~50% more N418$^+$ cells in their spleens than control PBS-injected animals, whereas no change was seen in the relative number of B220$^+$ cells between the two groups (Table 1). The increase in splenic DC in STAg-injected mice reflected an increase in the number of both NLDC-145$^+$ and NLDC-145$^-$ cells (Table 1), consistent with the results obtained by immunohistochemistry, which suggested that many of these recruited cells were moving into the T cell areas of spleen and becoming DEC-205$^+$ IDC. The increase in DC numbers did not explain the 40% total increase in spleen cellularity seen after STAg injection, suggesting that other cell types were also being recruited to the spleen (Table 1). Some of these cells appeared to be MΦ (Reis e Sousa, C., unpublished data) although, unlike DC, they did not produce IL-12 (see Fig. 5). Thus, like LPS (35), STAg increases immigration of DC into the spleen

Table 1. *Systemic Administration of STAg Increases the Number of N418$^+$ DC in Mouse Spleen*

	Number of cells ($\times 10^6$)		
	PBS-injected	STAg-injected	Ratio
Whole spleen	570	770	1.4
LOD	36	46	1.3
LOD B220$^+$ cells	16.3	16.0	1.0
LOD N418$^+$ cells	8.4	12.9	1.5
LOD N418$^+$ DEC-205$^-$ cells	6.5	10.1	1.6
LOD N418$^+$ DEC-205$^+$ cells	2.0	2.8	1.4

Groups of four C57BL/6 mice were intravenously injected with either PBS or 25 μg STAg. Spleens were removed 6 h later, digested with collagenase, treated with EDTA, and fractionated over dense BSA. The numbers of nucleated cells in the unfractionated spleen cell suspension and LOD fraction were counted with a hemocytometer. The number of cells in LOD subpopulations was determined by multiplying the frequency of each subpopulation, as determined by flow cytometry, by the absolute number of LOD cells in each group. Ratios represent the number of cells in the STAg-injected group divided by the corresponding number in the PBS-injected control group. No N418$^+$ cells were found in the high density fraction of spleen, even after STAg injection (data not shown). All DEC-205$^+$N418$^+$ cells were also CD8α$^+$. Intracellular staining for IL-12 p40 from the same experiment is shown in Fig. 5.

and induces a redistribution of these immigrants and of resident splenic DC to the inner PALS.

Discussion

Understanding the regulation of IL-12 production during the course of immune responses is the focus of much research because of the importance of this cytokine in driving the development of Th1 cell responses, as well as in regulating innate immunity (1). Two pathways for IL-12 production by MΦ have been clearly identified, one involving stimulation by T cell–derived, membrane-bound, or soluble CD40L during MΦ–T cell interactions, and another through direct stimulation of MΦ cells by microbial products (1). In contrast, little is known about the ability of DC to produce IL-12 in response to microbial stimuli. Several reports have shown that interaction with T cells appears to be the main pathway for induction of IL-12 production by DC, and that MΦ activators such as LPS or bacteria have much less effect on these cells (26, 28, 30, 31). T cell–dependent DC-derived IL-12 is induced during cognate interactions, through ligation of CD40 on DC by CD40L expressed on the activated T cells, as well as through direct signaling by MHC class II molecules cross-linked by the TCR (30, 31). This has suggested a model in which the Th1 development often associated with microbial infections is attributed to two separate sources of IL-12. Initial IL-12 production is presumed to occur in response to microbial stimulation of MΦ and predispose for Th1 development (2, 3, 36). Later IL-12 production by antigen-presenting DC interacting with antigen-specific T cells is thought to synergize with MΦ-derived IL-12 to promote Th1 differentiation (37). In this model, MΦ act as a bridge between the innate and the adaptive immune systems, sensing infection and helping to induce protective Th1 responses to microbes.

However, several lines of evidence suggest that maximal IL-12 production by MΦ in response to bacteria or microbial products requires prior activation of the cells (38–40). This is particularly evident in the inability of freshly isolated, resting peritoneal cells, bone marrow–derived MΦ, or MΦ cell lines to produce IL-12 in response to infection with live *Toxoplasma* or to stimulation with *Toxoplasma* antigens, as reported in this study, despite being able to produce other cytokines such as TNF (Fig. 1 and data not shown). The selective inability of resting MΦ to produce IL-12 even after infection with live parasites raises questions about a model in which MΦ-derived IL-12 is crucial for inducing IFN-γ–mediated protective responses to infection.

We have found an alternative source of IL-12 in response to microbes in the adherent fraction of mouse spleen cell suspensions stimulated in vitro. This source was independent of pretreatment of mice with inflammatory stimuli and appeared to represent DC rather than splenic MΦ in preliminary fractionation studies (data not shown). This is consistent with our observations that noninflammatory MΦ such as those found in the spleen do not produce appreciable levels of IL-12 in direct response to microbial stimuli (Fig. 1). Furthermore, we have found no evidence for high levels of IL-12 production by splenic MΦ after STAg injection in vivo, even using a sensitive flow cytometric technique (Fig. 5). In contrast, we clearly demonstrate that DC in the spleen can produce high levels of IL-12 p40 in response to two microbial products delivered systemically, in circumstances in which neither T cell help nor IFN-γ is available. Production of IL-12 p40 is transient and extremely rapid and involves a significant fraction of splenic DC. This hitherto undiscovered abundant source of IL-12, independent of IFN-γ, could be responsible for the increase in serum levels of IL-12 found after systemic administration of LPS, which is seen in wild type as well as IFN-γ–deficient mice (19, 41).

IL-12 is composed of two subunits, p40 and p35, which can form a bioactive heterodimer or a p40 homodimer that acts as an IL-12 receptor antagonist (1). We have not been able to stain spleen sections or cell suspensions for IL-12 p75, even under conditions in which IL-12 p40 was clearly induced (data not shown). This is likely due to the fact that the heterodimer is made at levels 10–50-fold lower than those of the IL-12 p40 subunit (1). Staining for IL-12 p35 did not help resolve whether IDC produced the heterodimer since the p35 subunit was found to be constitutively expressed in spleen white pulp of unstimulated mice, particularly in B cell areas, and expression did not change upon injection of STAg (data not shown). This result is

consistent with a previous report that used in situ hybridization with probes for the p35 subunit of IL-12 and also detected hybridization in the B cell area (42). Interestingly, in the same report it was shown that probes for the IL-12 p40 subunit hybridized strongly to the T cell areas of spleen after intraperitoneal injection of LPS (42), a result entirely consistent with the observations made here that both LPS and STAg induce production of high levels of IL-12 p40 on IDC. However, in spite of our inability to stain for IL-12 p75, IL-12 production in animals injected with STAg was bioactive in that neutralization of the cytokine in vivo abrogated the enhancement of IFN-γ production during subsequent in vitro restimulation of spleen cells with STAg or LPS (Fig. 2 *A*). Thus, we believe that the production of IL-12 p40 by DC is likely to translate into bioactive p75 cytokine that can act on Th precursors (Thp) to drive Th1 development, and on NK cells to elicit IFN-γ secretion.

Although the main DC population staining for IL-12 p40 in situ was found deep in the T cell areas (Fig. 4, *D–F*) and expressed the IDC markers CD8α and DEC-205 (Fig. 5), occasional dendritic profiles scattered among marginal zone B cells also stained for IL-12 in situ at early times (3 h) after intravenous injection of STAg (Fig. 4, *A–C*). As these profiles were not seen at later times after injection (≥6 h), it is probable that they represent marginal zone DC and/or newly arrived immigrant DC that are in the process of migrating to the inner PALS where the bulk of the IL-12$^+$ DC were found. This interpretation is consistent with the redistribution of DC to the T cell areas observed after administration of STAg (Fig. 4, *H* and *I*) or LPS (35 and Reis e Sousa, C., unpublished data). It is also consistent with the increase in DC number in the spleen observed after STAg injection (Table 1), which probably represents an influx of blood DC released from nonlymphoid organs, as seen in response to systemic LPS (43, 44). Together, these results suggest that the effect of microbial products is, in part, on IDC precursors, and that IL-12 production is another consequence of the general DC activation induced by STAg or LPS that leads to maturation of nonlymphoid DC with recruitment to lymphoid organs and migration to the T cell areas (6).

The redistribution of activated IDC precursors to the T cell areas may explain why maximal in vitro IL-12 production by spleen cells isolated from STAg-treated animals is seen earlier (3 h after injection; Fig. 2 *B*) than the maximal IL-12 staining of IDC observed in situ (6–12 h after injection; Figs. 3 and 4): as DC move into spleen T cell areas to become IDC, they become increasingly resistant to isolation by simple mechanical dissociation of spleens into a cell suspension (17). However, the 40% increase in numbers of splenic CD8α$^+$ DEC-205$^+$ N418$^+$ DC seen after STAg injection (Table 1), presumed to be due to immigration/maturation of IDC precursors, is not sufficient to explain the fact that 67% of these cells produce IL-12 p40 by flow cytometric analysis (Fig. 5). Thus, in addition to activating IDC precursors, it is likely that microbial products also act on small numbers of preexisting IDC in the T cell areas.

Production of IL-12 by DC in response to microbial products such as STAg and LPS is likely to be important in the development of cell-mediated immunity to infection. IL-12 production by MΦ at the site of infection (see below) is unlikely to influence the development of Th1 cells taking place at a distance in draining lymph nodes. In contrast to MΦ, DC are specialized APC for transporting antigens from the periphery to lymphoid tissues (5, 6). IL-12 production by lymphoid DC derived from recent immigrants that brought microbial antigens from the site of infection would be more likely to influence Th1 development in the lymphoid microenvironment than would the IL-12 produced by MΦ that remain at the peripheral site of invasion. This is especially true because these DC would be presenting microbial peptides in association with MHC molecules to the responding antigen-specific T cells, ensuring juxtaposition of the IL-12 source and the responding Thp. In fact, even in cases in which there is abundant draining of microbial products to lymphoid tissues, such as those mimicked here by intravenous injection of STAg or LPS, DC rather than MΦ appear to be the main source of IL-12 (Figs. 3–5). Thus, we can propose a revised model for the role of MΦ- versus DC-derived IL-12 in immunity to microbial infections. At the site of infection, local signals trigger the migration of nonlymphoid DC bearing microbial antigens to lymphoid tissues. These cells produce IL-12 due to direct stimulation by microbial products and present antigen to microbe-specific T cells, triggering clonal expansion and predisposing the Thp to differentiate towards Th1 effectors. During the DC–T cell interaction, engagement of CD40 on the DC by CD40L upregulated on the T cell (30, 31), as well as direct signaling through MHC molecules (31), serve to sustain IL-12 production by DC, further driving Th1 development of the responding cells. DC-derived IL-12 might also activate NK cells in lymphoid tissues, which then produce IFN-γ locally, further predisposing Thp to differentiate towards Th1 effectors.

On the other hand, IL-12 produced by tissue MΦ in response to the infectious organism could be important at the site of infection. This might require IFN-γ as a priming signal that, during the innate phase of the immune response, could be provided by recruited NK cells that were activated in lymphoid tissues by DC. Alternatively, inflammation itself could prime tissue MΦ to make IL-12 in response to microbial products, consistent with our observations that inflammatory thio-MΦ in vitro do produce IL-12 in response to *Toxoplasma*, even when derived from IFN-γ KO mice (Fig. 1 and reference 14). In either case, MΦ-derived IL-12 could then further stimulate NK cells at the site of infection. This would lead to increased secretion of IFN-γ by these cells, which, in turn, would potentiate the microbicidal activity of these MΦ, as well as increase their ability to produce additional IL-12 (38, 39). This establishes a positive feedback loop that would ensure maximal activation of MΦ effector cells at the site of infection. That loop can eventually be broken by the known ability of MΦ to also produce the antiinflammatory cytokine, IL-10, ensuring that full-blown activation does not lead to debilitating immunopathology (1, 9). In addition, during the adaptive

phase of the immune response, IL-12 production by MΦ may help maintain the differentiated phenotype of Th1 effectors at the site of infection.

This model postulates that the priming APC produces IL-12 directly and that *trans*-acting MΦ-derived IL-12 is not essential for Th1 development. Dispensing with the need for *trans*-acting IL-12 ensures that IL-12 produced in response to infection acts specifically on the T cells responding to microbial antigens rather than on bystander T cells, and explains how Th1 and Th2 responses to different antigens could occur simultaneously in the same microenvironment. Thus, in this model, DC and not MΦ provide the bridge between adaptive and innate immunity.

We are grateful to Drs. G. Trinchieri and M. Wysocka for providing antibodies against IL-12, and to R. Dreyfuss and S. Everett for photography.

C. Reis e Sousa is supported by a Visiting Fellowship from the Fogarty International Center, National Institutes of Health.

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Received for publication 5 September 1997 and in revised form 6 October 1997.

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