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IL-12 Acts Directly on DC to Promote Nuclear Localization of NF-κB and Primes DC for IL-12 Production

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Summary

We analyzed the expression of an IL-12 receptor by fresh dendritic cells (DC) and a DC line. Using RT-PCR, RNAse protection, and electrophoretic mobility shift assay analysis, we found that DC possess an IL-12 receptor with β1 subunit (downstream box 1)-related differences from that on T cells. IL-12 signaling through this receptor involved members of the NF-κB but not STAT family. The unique properties of the IL-12 receptor on DC, characterized by a single class of binding sites with a K_d of about 325 pM, may underlie rather unique effects, such as IFNγ-independent augmentation of class II antigen expression and priming for LPS-induced production of IL-12.

Introduction

IL-12 serves a major role in the early inflammatory response to infections (Trinchieri, 1995). In addition, IL-12 produced by APC is critically involved in the generation of T helper type 1 (Th1) cells (Hsieh et al., 1993) and is required for optimal differentiation of CTL (Gately et al., 1992). These activities likely mediate or contribute to the antitumor effects observed in mice bearing a variety of malignancies and treated locally or systemically with IL-12 (Brunda et al., 1995; Zitvogel et al., 1995). Most of these effects are thought to involve direct actions on T (Seder et al., 1993) and NK cells (Kobayashi et al., 1989). Binding of IL-12 to human and murine B lymphocytes has recently been reported, suggesting that IL-12 can directly affect humoral immunity (Vogel et al., 1996), besides acting as an initiator of cell-mediated reactivity (Scott, 1993).

DC are the most potent cellular initiators of the immune response and, in particular, are responsible for the induction of primary antigen-specific immune reactions (Steinman and Cohn, 1973; Steinman, 1991). In keeping with this notion is their ability to secrete IL-12 and to direct the development of Th1 cells from naive $CD4^+$ T cells (Macatonia et al., 1995). Based on our previous finding that recombinant IL-12 will prime purified DC cultures pulsed with a tumor peptide in vitro for effective sensitization in vivo (Grohmann et al., 1995a, 1997, 1998; Bianchi et al., 1996), the present study has investigated the possible expression of an IL-12 receptor (IL-12R) by DC. The results indicate that DC express an IL-12R and that IL-12 signaling in these cells may involve early events that differ from those operating in T lymphocytes, including nuclear localization of members of the NF-κB family. These events may ultimately lead to functional responses that are independent of the presence of IFNγ, including increased expression of MHC class II antigens and priming of DC for LPS-induced production of IL-12.

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Results

Binding of IL-12 to DC

Transfer of DC exposed sequentially to IL-12 and a tumor peptide, P815AB, confers $CD8^+$ cell-mediated reactivity on prospective recipients of an intrafootpad challenge with the tumor peptide (Bianchi et al., 1996; Grohmann et al., 1997), which is accompanied by increased resistance to challenge with tumor cells expressing the otherwise poorly immunogenic rejection antigen P815AB (Grohmann et al., 1998). To investigate possible direct effects of IL-12 on DC, we first used flow cytometry for detection of IL-12 binding to highly purified DC cultures (as assessed by the presence of specific markers; Figure 1). Using double staining in a sandwich procedure with IL-12 and fluoresceinated IL-12-specific C17.8 MAb or control rat IgG, only the combination of externally added IL-12 and the IL-12-specific antibody resulted in a high proportion of cells (58%) staining positively for bound IL-12 and the specific DC marker CD11c (data not shown). We thus performed kinetic and equilibrium binding analysis of radiolabeled IL-12 binding to DC. The kinetics of association of $[^{125}I]$IL-12 was examined comparatively in DC and Con A–activated lymphoblasts. The binding of labeled IL-12 to both types of cells at room temperature appeared to be rapid, reaching equilibrium between 30 and 60 min for DC and between 60 and 120 min for Con A blasts. By plotting $-\ln[1-B/B_{max}]$ versus time (Coligan et al., 1994), where B represents bound cpm and B_{max} represents the maximum bound cpm achieved at equilibrium, the observed association rate constant for DC (slope of the resulting straight-line curve, indicating a single type of receptor) was 0.045 min^{-1} (Figure 2A). Consistent with the occurrence of two classes of IL-12 receptors on Con A–activated splenocytes (i.e., high- and low-affinity receptors) (Presky et al., 1996; Wu et al., 1997), two distinct curves and consequently, association rate constants were detected in these cells, 0.055 min^{-1} and 0.008 min^{-1} (data not shown). Binding assays were then performed with different concentrations of radioligand using DC and Con A blasts, and Scatchard plots of the specific binding data were analyzed by means of the LIGAND program, with a single (DC) or double (Con A blasts) site model. As expected, Con A blasts displayed both high (K_d = 14.4 ± 5.3 pM with 103 ± 25 sites/cell)- and low (K_d = 2.4 ± 0.4 nM with 1556 ± 740 sites/cell)-affinity IL-12 binding sites (data not shown), whereas DC (Figure 2B) expressed only high-affinity IL-12 binding sites (K_d =

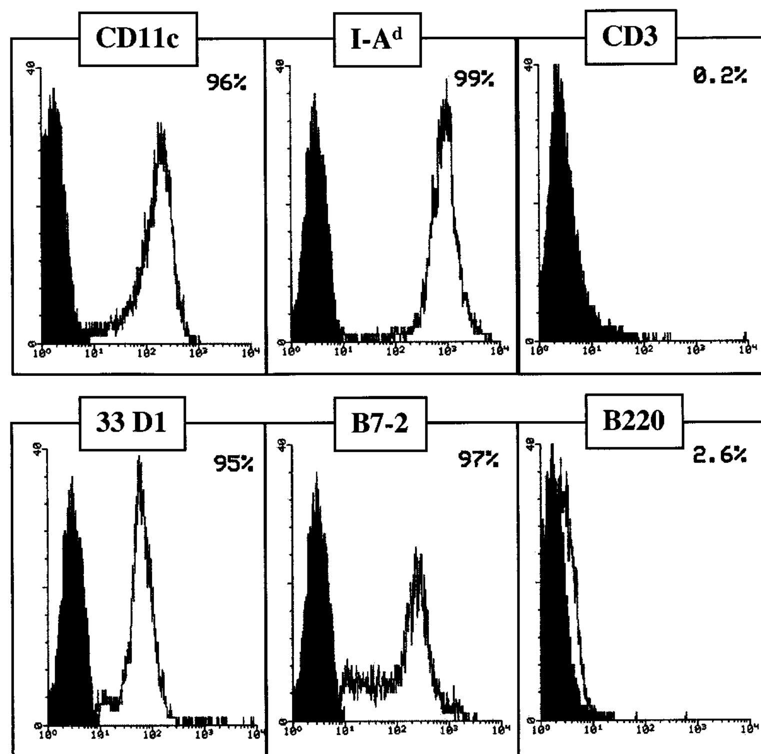

Figure 1. Cytofluorimetric Analysis of Purified DC Stained with a Set of MAbs Against Various Determinants Specific for DC or T and B Lymphocytes

Numbers within boxes indicate percentage of positive cells above background values (closed histograms).

325 ± 61 pM with 155 ± 9 sites/cell, mean ± SEM of three experiments). In competitive binding of labeled IL-12 to DC by unlabeled IL-12, we found a K_I value of 240 ± 42 pM with 130 ± 12 sites/cell (data not shown). In experiments not reported here, we also found that the presence of 100 nM concentrations of murine IL-1α, GM-CSF, IFNγ, and TNFα did not block binding of 500 pM labeled IL-12, thus demonstrating specificity of the IL-12 binding.

Analysis of IL-12R Subunit mRNA Expression by DC and a DC Line

IL-12 receptors, primarily expressed on activated T and NK cells, are gp130-like cytokine receptor superfamily members. Having the general makeup of β type cytokine receptor subunits, they are designated as IL-12Rβ1 and IL-12Rβ2. Coexpression of IL-12Rβ1 and IL-12Rβ2 leads to the formation of high-affinity IL-12-binding sites (Presky et al., 1996). Freshly isolated DC and the previously characterized DC line, CB1 (Paglia et al., 1993), were examined for the expression of the IL-12Rβ1 and β2 chains by RT-PCR and RNase protection analysis. In a first set of experiments, and using previously described β1 primers (Chua et al., 1995) (sense, nucleotides 1073–1092; antisense, 2220–2239), we comparatively analyzed the expression of IL-12Rβ1 and β2 messages in Con A lymphoblasts, fresh DC, the CB1 line, and the A20 B lymphoma line. The A20 cells were used as a negative control because preliminary experiments had shown failure of IL-12 to increase the APC ability of A20 cells in in vitro and in vivo assays. As expected (Chua et al., 1995; Presky et al., 1996), the Con A lymphoblasts expressed both β1 and β2 transcripts, but neither was expressed in A20 cells. In DC and CB1 cells, β2-specific transcripts were clearly expressed but no evidence was obtained for the presence of β1-specific messages (Figure 3A). These results were confirmed by the more stringent technique of RNase protection analysis, although the data for β1 transcript expression in DC were less clear (Figure 3B). This raised the possibility that our failure to detect β1 transcripts might be due to the use of inappropriate primers. We have thus begun to systematically examine a series of different β1 primers comprising regions encoding different domains of the β1 chain. We consistently found PCR products of the expected size using β1 primers spanning the extracellular, transmembrane, and cytoplasmic regions up to at least nucleotide 1865, belonging in the box 1 motif (Stahl and Yancopoulos, 1993). All of these amplicons were sequenced and proved to be identical to the corresponding published sequences (Chua et al., 1995). As an example, Figure 3A reports PCR data using a β1 sense primer consisting of nucleotides 239–259 and a β1 antisense primer comprising nucleotides 788–807.

IL-12 Does Not Activate Stat3 and Stat4 for DNA Binding in DC

The IL-12Rβ2 subunit is a signal-transducing component of the IL-12R (Presky et al., 1996), and IL-12 signaling in Th1 cells involves tyrosine phosphorylation of signal transducers and activators of transcription (Stat)3 and Stat4 (Jacobson et al., 1995; Szabo et al., 1997). To examine IL-12 signaling in DC, we wondered whether the cytokine might induce STAT factors capable of binding DNA (Figure 4). The FcγRI probe, from the IFNγ response region of the high-affinity FcγR promoter (Jacobson et al., 1995), has distinct binding specificity for various STAT family members and can be used as a probe in

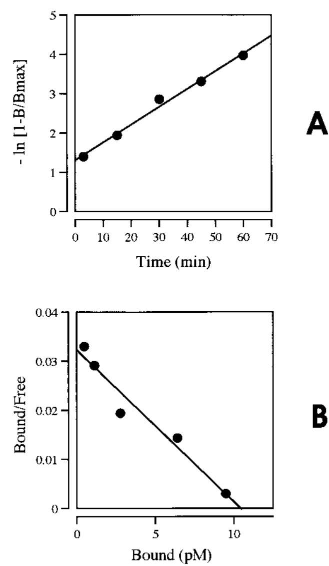

Figure 2. Kinetics and Equilibrium Binding Analysis of Radiolabeled IL-12 Binding to DC

(A) Kinetics of association of 500 pM [^{125}I]IL-12 at room temperature in the presence or absence of 50 nM unlabeled IL-12. The curve represents specific binding of labeled IL-12, where B is bound cpm and B_{max} is the maximum bound cpm at equilibrium.

(B) Scatchard analysis of [^{125}I]IL-12 binding. Cells were incubated for 2 hr at room temperature with various concentrations of radioligand. Results are expressed as specific binding data that were analyzed by the method of Scatchard. One experiment representative of three is shown for each assay.

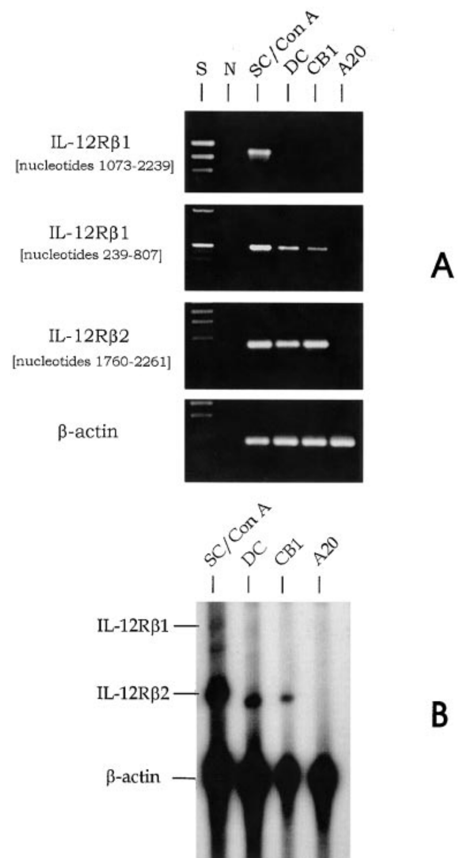

Figure 3. IL-12R Gene Transcription in DC

(A) RT-PCR analysis of IL-12Rβ1 and IL-12Rβ2 mRNA expression in Con A–activated splenic lymphoblasts (SC/Con A), DC, CB1 cells, and A20 cells. cDNA prepared from each cell population was PCR-amplified using β1 primer pairs that bridge different areas in the mRNA. PCR products were normalized to β-actin. Symbols: S, marker track; N, no DNA added to the amplification mix during PCR. Data are representative of eight separate experiments in which DC (96%–98% pure) were purified by either technique described in Experimental Procedures.

(B) RNase protection analysis of the IL-12Rβ1 and IL-12Rβ2 transcripts in the same cell populations. β-actin RNA was used as a loading control. One experiment representative of three.

electrophoretic mobility shift assay (EMSA) analysis. The assay was performed on fresh DC and CB1 cells using Con A blasts and IFNγ-treated DC as positive controls. Nuclear extracts of IL-12-treated or untreated cells were incubated with the labeled FcγRI probe prior to separation by native polyacrylamide gel. The results showed that nuclear complexes binding to FcγRI were clearly expressed by Con A blasts treated with IL-12 and IFNγ-treated DC but not by DC and CB1 cells exposed to IL-12. In accordance with previous evidence that IL-12 induces Stat4 homodimers and Stat3/Stat4 heterodimers in T cells (Jacobson et al., 1995), we found that the addition of anti-Stat antisera removed the majority (anti-Stat3) or the totality (anti-Stat4) of the nuclear complexes binding to FcγRI. Although the FcγRI probe may have different relative affinities for the various members of the STAT family, these results implied that the possible mechanisms of IL-12 signaling in DC would, at least in part, be different from those in T cells.

IL-12 Activates NF-κB for DNA Binding in DC

NF-κB is a ubiquitous transcription factor that is involved in the induction of several proinflammatory cytokines, including IL-12 (Baeuerle and Henkel, 1994). One of the currently known NF-κB family members, RelB, has a crucial role in the development and differentiation of DC (Burkly et al., 1995; Pettit et al., 1997). Recent evidence suggests that induction of the TNFα promoter in a murine dendritic cell line is controlled by the NF-κB proteins p50 (NF-κB1) and p65 (RelA) (Prieschl et al., 1996). It has been suggested that in DC, NF-κB factors play a crucial role in the activation of cytokine promoters (Prieschl et al., 1996). Using EMSA analysis, we investigated the possible nuclear uptake of NF-κB complexes in DC treated with IL-12. Nuclear extracts were obtained from normal spleen cells, Con A splenocytes, DC, and CB1 cells, treated or not with IL-12. A combination of PMA and ionomycin, known to activate NF-κB in DC (Prieschl et al., 1996), was also included in the assay as a positive control. Two different probes were used, one from authentic NF-κB sites from the mu heavy-chain enhancer (Baeuerle and Henkel, 1994) and a second probe (m12-27) that was derived from the IL-12 p40 promoter and contains an NF-κB half-site (Murphy et al., 1995). Figure 5 shows that using the NF-κB probe (from authentic NF-κB sites), both spleen cells and fresh DC displayed activation of NF-κB, presumably because the factor is easily activated upon physical manipulations of cell cultures (Baeuerle and Henkel, 1994). Nuclear uptake of NF-κB complexes after IL-12 treatment

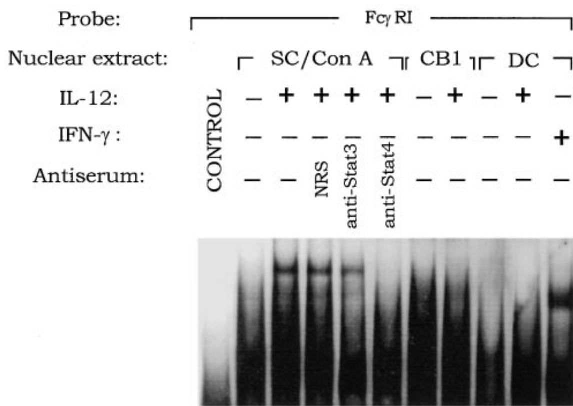

Figure 4. Failure of IL-12 to Induce Nuclear Complexes Containing Stat3 and Stat4 in DC

Nuclear extracts of Con A spleen cells, DC, and CB1 cells were prepared after IL-12 treatment. EMSA was performed with the FcγRI probe and antisera to Stat3 and Stat4 or normal rabbit serum (NRS). The control lane was loaded with probe but no nuclear extract. Free probe is not shown. One of three experiments with the same results.

was not modified in normal spleen cells and Con A blasts but was strongly induced (CB1) or enhanced (DC) in the other cell types to an extent similar to that induced by the combination of PMA and ionomycin. In both CB1 and DC, the presence of specific competitors removed the majority (m12-27) or the totality (NF-κB) of the nuclear complexes binding to NF-κB. In contrast, an oligonucleotide probe specific for a distinct transcription factor of the STAT family (FcγRI) failed to inhibit complex formation. Similar results were obtained using probe m12-27, although neither spleen cells in the absence of Con A activation nor fresh DC displayed complex formation in the absence of IL-12. Because the addition of cold NF-κB removed complex formation with m12-27, these results confirmed the previous finding that m12-27 interacts with selected members of the NF-κB family (Murphy et al., 1995).

p50 and RelB are Major Transcription Factors Activated by IL-12 in DC

We also used antisera to various NF-κB family members to analyze the constituents of the IL-12-induced EMSA complexes binding to authentic NF-κB sites (Figure 6). Nuclear extracts were obtained from IL-12-treated DC to be used in EMSA analysis with the NF-κB probe. Antisera were used to p50, p65 (RelA), RelB, or *c*-Rel in combination with nuclear extracts and the NF-κB probe. The p50 antiserum, but not normal rabbit serum, caused most of the band to shift significantly, whereas no such effect was observed with anti-p65 and anti-*c*-Rel antisera or with an irrelevant antibody to Stat4. Remarkably, the use of the anti-RelB reagent, but not control serum, greatly inhibited complex formation with the NF-κB probe (Figure 6A). Therefore, the p50 and RelB components of the NF-κB family appear to interact with authentic NF-κB sites. Analogous results (not reported in Figure 6A) were obtained when the m12-27 probe was used in the place of NF-κB. Nuclear translocation of p50 and RelB could also be demonstrated directly by immunofluorescence. Figures 6B and 6C report immunostaining of CB1 cells reacted with anti-p50 antibody, clearly demonstrating translocation of p50 from the cytoplasm to the nucleus after cell treatment with IL-12. Analogous results were obtained in studying nuclear translocation of RelB (data not shown).

Increased Class II Molecule Expression after Exposure of DC to IL-12

Because our previous experiments with peptide-pulsed DC had suggested improved presentation of class II-restricted epitopes of P815AB after sequential exposure of DC to IL-12 and peptide (Grohmann et al., 1997, 1998), we became interested in analyzing class II antigen expression and expression of costimulatory molecules in fresh DC treated with IL-12. We performed cytofluorimetric analysis of IL-12-exposed cells reacted with antibodies to I-A^d class II molecules (which are known to bind P815AB epitopes [Grohmann et al., 1997, 1998]),

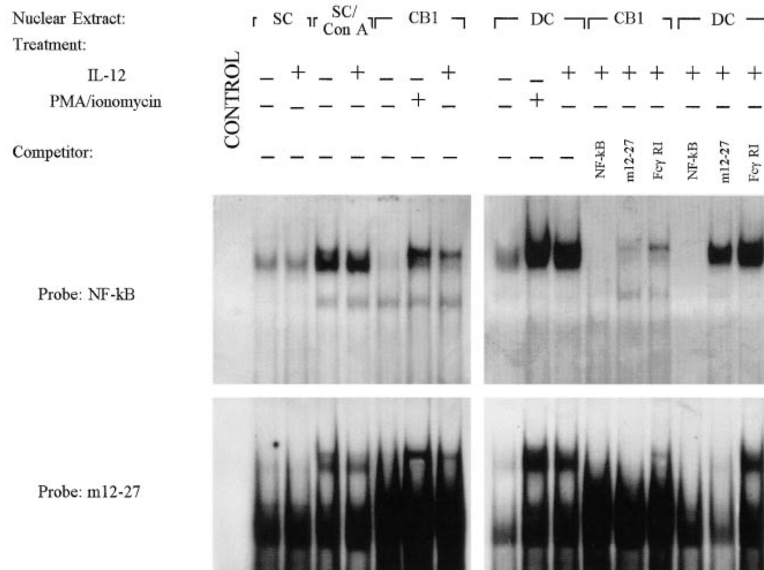

Figure 5. Ability of IL-12 to Induce Nuclear Uptake of NF-κB Complexes in DC

Nuclear extracts of spleen cells (SC), Con A blasts (SC/Con A), CB1 cells, and DC were obtained after treatment with either IL-12 or a combination of PMA and ionomycin. EMSA was performed with the NF-κB probe or the m12-27 probe. In assaying CB1 cells and DC, competitor probes were also used (indicated). The control lane was loaded with probe but no nuclear extract. Free probe is not shown. Additional controls (not reported in the figure) included DC stimulation with LPS in the absence of IL-12, and no effects were observed with LPS concentrations up to 1 μg/ml. One of four experiments with analogous results.

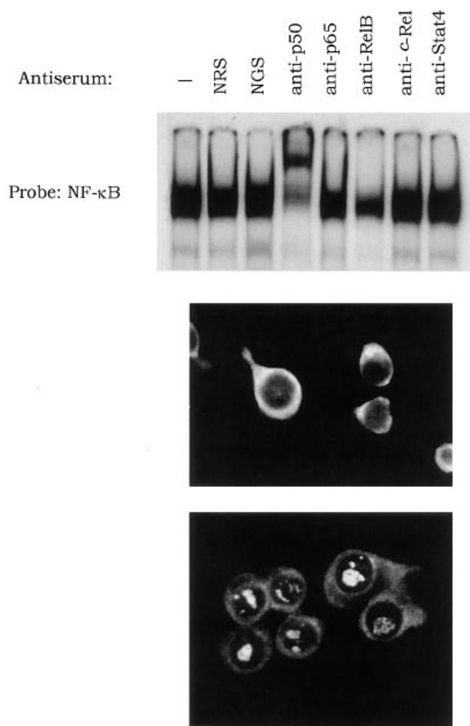

Figure 6. p50 and RelB are Major Transcription Factors Activated by IL-12 in DC and the DC Line

(A) Nuclear extracts from IL-12-treated DC were incubated with control antisera or antibody against NF-κB family members (p50, p65, RelB, or *c*-Rel). The control antibodies consisted of normal rabbit serum (NRS), normal goat serum (NGS), or irrelevant anti-Stat4 antibody. NF-κB was used as probe. Free probe is not shown. One of three experiments with the same results. (B) and (C) Effect of IL-12 on the translocation of NF-κB p50 from the cytoplasm to the nucleus. Untreated (B) and IL-12-exposed (C) CB1 cells were immunostained with anti-p50 antibody and visualized with rhodamine-conjugated goat anti-rabbit IgG antibody.

B7-1, or B7-2. In several independent experiments, we consistently observed a reproducible increase in mean fluorescence units for I-A^d expression. In contrast, no reproducible effects were afforded by IL-12 on B7-1 or B7-2 expression (data not shown). Although the high baseline expression of I-A^d molecules on DC (Figure 1) would impede full appreciation of IL-12 effects in individual experiments, the mean change in linear fluorescence units in eight different experiments was 209 $\pm$ 102 (mean $\pm$ SD, $p = 0.001$) after exposure of $>$96% pure DC to IL-12. When IFNγ concentration was measured in DC culture supernatants at the end of the 18 hr incubation with IL-12, we found that IFNγ levels were always below the detection limit of the assay (i.e., 20 pg/ml). In addition, no IFNγ transcripts were revealed by RT-PCR following exposure of DC to IL-12 (data not shown). These results suggest that a biologically relevant effect of IL-12 on DC might be increased class II antigen expression independent of the presence of IFNγ.

Induction of Endogenous IL-12 Production in DC by IL-12 and LPS

In monocytic cells, IFNγ enhances IL-12 production mostly by priming cells for LPS-induced transcription

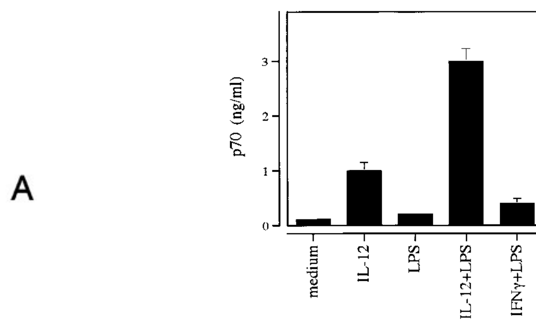

Figure 7. IL-12 Production by IL-12-Treated CB1 Cells

CB1 cells were exposed overnight to IL-12 or IFNγ followed by extensive washing and LPS stimulation. At 1 and 24 hr of LPS stimulation, supernatants were assayed for IL-12 p70 contents by ELISA. The 1 hr IL-12 titers were below the detection limit of the assay. One of three experiments.

of the IL-12 p40 gene (Ma et al., 1996). Because in our experiments exposure of DC to IL-12 enhanced nuclear uptake of NF-κB complexes binding to a p40 promoter NF-κB half-site, we investigated the possible production of IL-12 by DC exposed to a combination of IL-12 plus LPS or IFNγ plus LPS. We measured p70 production by ELISA in culture supernatants of CB1 cells treated overnight with IFNγ or IL-12 (Figure 7). Cells were extensively washed and then incubated in fresh medium with or without LPS. Culture supernatants were harvested at 1 and 24 hr. No IL-12 was found in any group at 1 hr, whereas considerable amounts of the cytokine were found at 24 hr in CB1 cells exposed to the combination of IL-12 and LPS. Lack of IL-12 detection at 1 hr clearly indicated that the p70 measured at 24 hr was not derived from externally added IL-12, bound and/or internalized in CB1 cells. Of interest, significant production of p70 was also detected by treatment with IL-12 alone or, to a lesser extent, with a combination of IFNγ and LPS.

Discussion

Our data demonstrate that highly purified murine DC and a DC line can directly bind IL-12, thus providing the first evidence that IL-12 may act directly on professional APC that are regarded as the most potent initiators of the immune response and are responsible for the induction of primary antigen-specific immune reactions (Steinman and Cohn, 1973; Steinman, 1991). Using flow cytometric analysis, we found that a great proportion of purified splenic DC expressing very high levels of surface markers typical of late DC such as class II (99%) and B7-2 (97%) (Banchereu and Steinman, 1998) (Figure 1) constitutively express a receptor for IL-12. The addition of anti-IL-12 alone to DC ablated the detection of IL-12 binding (data not shown), thus excluding that the positive staining was due to the occurrence of DC-derived IL-12. Experiments of receptor affinity and concentration dependence, competition, and ligand specificity with labeled IL-12 in kinetic and equilibrium binding analysis revealed that the binding of [^{125}I]IL-12 to DC was saturable and specific, because the binding of radiolabeled ligand was only inhibited by IL-12 and not

by other cytokines. The kinetics of [^{125}I]IL-12 binding to DC was rapid at room temperature, reaching equilibrium within 60 min (Figure 2A). Analysis of steady-state binding data by the method of Scatchard (Figure 2B) identified a single binding site with an apparent equilibrium dissociation constant of 325 pM and 155 sites/cell. The equilibrium dissociation constant for competing unlabeled IL-12 was 240 pM with 130 sites/cell. Parallel experiments of [^{125}I]IL-12 binding to Con A lymphoblasts gave results (i.e., occurrence of high- and low-affinity binding sites) consistent with previous data in the literature (Presky et al., 1996; Wu et al., 1997).

Because Con A–activated splenocytes are known to express both the β1 and β2 subunits of the IL-12R, we comparatively analyzed these cells with fresh DC and the CB1 line in RT-PCR and RNase protection assays for receptor subunit expression. Using RT-PCR, we found that Con A lymphoblasts expressed β1 and β2 transcripts in contrast to the murine B lymphoma A20 cell line. Both DC and CB1 cells clearly expressed β2-specific transcripts by RT-PCR and RNase protection analysis (Figure 3). However, DC and CB1 cells appeared to express a different β1 isoform relative to Con A blasts. Systematic RT-PCR analysis with β1 primers bridging different regions of the mRNA combined with sequencing of PCR products has so far revealed identity up to at least nucleotide 1865, belonging in the intracellular box 1 motif. In contrast, the use of a β1 antisense primer complementary to nucleotides 2220–2239 resulted in no mRNA amplification (Figure 3A). Recently, several classes of IL-12Rβ1 cDNA clones have been found to result from alternate splicings by comparison to the genomic sequence (Showe and Trinchieri, 1995). We are currently attempting the production of a specific anti-murine IL-12R β1 chain antibody that may enable us to perform Western blot and immunoprecipitation experiments.

In T cells, IL-12 selectively induces nuclear DNA-binding complexes that contain Stat3 and Stat4, recently cloned members of the STAT family (Jacobson et al., 1995; Szabo et al., 1997). As a first approach to the identification of the early events in IL-12 signaling in DC, we investigated the possible induction of STAT factors capable of binding DNA in nuclear extracts of DC and CB1 cells exposed to IL-12 for 15 min. Using EMSA analysis and the FcγRI probe from the IFNγ response region of the high-affinity FcγR promoter, we failed to detect nuclear complexes binding to the probe, in contrast to that observed with Con A blasts, in which IL-12 clearly induced Stat4 homodimers and Stat3/Stat4 heterodimers (Figure 4). In experiments not reported here using Western blot and RT-PCR analysis, we found that DC and CB1 cells express Stat3 but not Stat4. Although these results might indicate inability of the IL-12Rβ2 subunit to transduce activating signals in DC, we explored the possibility that the early events of IL-12 signaling in DC may be different from those in T cells.

Several considerations led us to examine the possibility that NF-κB might be involved in IL-12 signaling in DC. (1) NF-κB is involved in the induction of several proinflammatory cytokines (Baeuerle and Henkel, 1994), and regulation of IL-12 p40 expression occurs through an NF-κB half-site (Murphy et al., 1995); (2) RelB, one of the known members of the NF-κB family, is involved in the development and differentiation of DC (Burkly et al., 1995), and nuclear localization of RelB is associated with effective APC function (Pettit et al., 1997); (3) two NF-κB proteins, NF-κB1 and RelA, control the induction of the TNFα promoter in a murine DC line (Prieschl et al., 1996) and, in general, NF-κB factors appear to play an important role in the activation of cytokine promoters in DC (Baeuerle and Henkel, 1994). Using probes from authentic NF-κB sites or the IL-12 p40 promoter in EMSA analysis, we found that IL-12 treatment of DC resulted in the appearance of NF-κB nuclear complexes in a fashion similar to that observed with a combination of PMA and ionomycin (Figure 5). The presence of specific competitors removed most or all of the nuclear complexes binding to NF-κB in both fresh DC and CB1 cells treated with IL-12. These findings demonstrated that IL-12 induces or strongly enhances activation of NF-κB in fresh DC and the DC line, but no such effects were observed with spleen cells, whether or not activated by Con A. Subsequent experiments, using antisera to various NF-κB family members, showed that the p50 and the RelB components of the NF-κB family bind authentic NF-κB sites and the p40 promoter NF-κB half-site in DC (Figure 6A; data not shown). Nuclear uptake of NF-κB p50 and RelB in IL-12-treated CB1 cells could also be demonstrated directly by immunofluorescence staining (Figures 6B and 6C; data not shown).

Apart from the involvement of an NF-κB half-site in the regulation of IL-12 p40 expression (Murphy et al., 1995), members of the NF-κB family are strongly implicated in the control of basal and induced MHC class I gene expression (Ting and Baldwin, 1993), and NF-κB binding sites may be involved in *I-A* (Benoist and Mathis, 1990) and *I-E* (Baeuerle and Henkel, 1994) gene regulation. It has been recently shown that inflammatory stimuli such as TNFα, IL-1, and LPS (all of which can activate NF-κB [Baeuerle and Henkel, 1994]) induce accumulation of MHC class II complexes on DC (Cella et al., 1997). Because indirect evidence in our previous experimentation had suggested that IL-12 may increase the number and/or stability of MHC class II/P815AB complexes on DC (Grohmann et al., 1997, 1998), we became interested in examining class II antigen expression in fresh DC treated with IL-12. In several independent experiments, we obtained consistent evidence for increased I-A^d expression following exposure of DC to IL-12 for 18 hr.

IFNγ is one of the most potent class II MHC inducers (Moses et al., 1992). TNFα, TGFβ, and IFNα and -β, which are activators of protein kinase C and cAMP, are other modulators of class II MHC genes, although their role varies with experimental conditions (Ting and Baldwin, 1993). Modulation of the *I-A* gene by TNFα is mediated through an NF-κB site (Freund et al., 1990). Our present finding of no detectable IFNγ in culture supernatants of DC exposed to IL-12 tends to exclude a major involvement of the former cytokine in the increased class II molecule expression. However, whether other cytokines participate in the inducible class II gene expression resulting from DC exposure to IL-12 remains to be clarified. As regards the possible role of TNFα as a mediator of IL-12 effects, we have found that IL-12 does not induce TNFα message expression by RT-PCR in DC,

as opposed to the strong induction observed with IFNγ, and in fact the crucial promoter element for TNFα induction in DC is controlled by the NF-κB protein p65 (RelA) (Prieschl et al., 1996), which does not appear to be activated by IL-12 (Figure 6). Therefore, it would appear that neither IFNγ nor TNFα plays a major role in the induction or accumulation of MHC class II complexes on DC by IL-12.

In monocytic cells, IFNγ enhances IL-12 production mostly by priming cells for LPS-induced transcription of the p40 gene (Ma et al., 1996), and the induction of IFNγ by IL-12 acts in a positive feedback loop that represents an important amplifying mechanism in the inflammatory response to infection (Romani et al., 1997). The finding of an IL-12R in DC capable of activating transcription factors for the p40 promoter NF-κB half-site prompted us to investigate the possibility that IL-12 might be involved in the regulation of its own production. We comparatively analyzed IL-12 and IFNγ for ability to prime DC for LPS-induced production of IL-12 p70. In fact, murine splenic DC are known to express low levels of LPS receptor CD14 under resting conditions (Kato et al., 1997). We obtained evidence that LPS alone or a combination of LPS and IFNγ induced only a limited production of p70 (Figure 7). A greater effect was observed with IL-12 alone. However, the combination of IL-12 and LPS resulted in the production of very high levels of IL-12 p70. The failure of IFNγ plus LPS to induce high levels of IL-12 in DC is in line with recent observations by others (Kato et al., 1997). This may be consistent with the notion that the various APC serve very different immune functions and that DC may be unique in their ontogenetic and functional characteristics (Peters et al., 1996). It would be tempting to speculate that IL-12 contributes to effective APC function in DC by activating NF-κB, increasing class II antigen expression, and regulating the production of IL-12.

In conclusion, we have shown that DC express a single class of high-affinity IL-12R on their surface. We have provided evidence that IL-12 binding on DC transduces activating signals via nuclear localization of p50 and RelB, two members of the NF-κB family. This unique feature of IL-12 effects on DC may confer unique actions on the target cells, including increased class II antigen expression and enhanced production of endogenous IL-12. Therefore, our present data demonstrate a novel possible cellular target for IL-12, suggest the existence of IL-12 receptors structurally and functionally distinct from those in T and NK cells, and demonstrate the involvement of members of the NF-κB family in IL-12 signal transduction. Because until now DC have only been thought to make rather than respond to IL-12, our present finding of a functional high-affinity IL-12R on DC may have a number of important implications in the general pathways of immunoregulation.

Experimental Procedures

Mice

DBA/2J (H-2^d) were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy). Male mice were used at the age of 2 to 4 months.

Cell Lines

The immortalized dendritic cell line CB1 (Paglia et al., 1993) was a generous gift of Dr. P. Ricciardi-Castagnoli, CNR Center of Cytopharmacology, University of Milan, Milan. The murine B cell lymphoma A20 was from the American Type Culture Collection (ATCC), Rockville, MD.

Cytokines and Antibodies

Murine rIL-12 was a generous gift from Dr. B. Hubbard (Genetics Institute, Inc., Cambridge, MA). IL-12 was 98.8% pure, as assessed by SDS-PAGE, and endotoxin contamination was <0.9 EU/mg on Limulus amoebocyte assay. The specific activity of the purified rIL-12 preparation, measured as ability to stimulate proliferation in human phytohemoagglutinin-activated blasts, was 3.1×10^6 U/mg. Endotoxin was removed from all solutions containing IL-12 with Detoxi-gel (Pierce Chemical Co., Rockford, IL), resulting in endotoxin contamination below the detection limit (0.05 EU/ml) of the assay (Coatest Endotoxin, Chromogenix AB, Mölndal, Sweden). Murine rIFNγ was from Genzyme Corp. (Boston, MA).

Affinity purified anti-CD11c (N418, rat IgG2a), anti-I-A^d (MK-D6, mouse IgG2a), and anti-mouse DC (33D1, rat IgG2b) MAbs were obtained from ATCC. Rat anti-mouse IL-12 p40 MAb C17.8 and hamster anti-mouse IL-12 p35 (clone Red-T) were from Genzyme Corp. N418, MK-D6, 33D1, and C17.8 MAbs were conjugated to FITC or biotin using conventional methods. FITC-labeled anti-mouse B7-1 and B7-2 and anti-mouse CD3 (145-2C11) were from Pharmingen. PE-labeled anti-mouse B220 was a gift from Dr. L. D'Adamio (NIH, Bethesda, MD).

Dendritic Cell Preparation

DC were prepared from collagenase-treated spleens (collagenase type IV, Sigma Chemical Co., St. Louis, MO), as described (Steinman et al., 1986). In brief, total spleen cells were suspended in dense BSA (ρ = 1.080), overlaid with 1 ml of RPMI medium, and centrifuged in a swingout bucket rotor at 7,500 rpm for 20 min at 4°C. The low-density fraction at the interface was collected and washed several times. The recovered cells were resuspended in RPMI medium supplemented with 10% FCS and allowed to adhere for 2 hr, and this was followed by an additional 18 hr incubation to allow DC to detach. Contaminating B cells were further removed by one round of panning on polyvalent goat anti-mouse Ig (Sigma Chemical Co.). The recovered cells were routinely >96% N418$^+$. Cytofluorimetric analysis of these cells with several MAbs revealed a characteristic DC phenotype (Figure 1). In addition, no detectable IFNγ was present in culture supernatants from IL-12-treated DC, thus excluding a significant contamination by NK cells. In selected experiments, DC were purified from collagenase-treated spleens using a positive selection column and CD11c MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The two purification procedures led to comparable results when cells were assayed in parallel in in vivo and in vitro experiments.

Binding Assays

Purified rIL-12 was labeled with ^{125}I by a modification of the IodoGen method (Pierce Chemical Co., Rockford, IL) as previously described (Chizzonite et al., 1992; Wu et al., 1997). Activation of splenocytes with Con A (Presky et al., 1996) and [^{125}I]IL-12 binding assays (Wu et al., 1997) was performed as previously reported. All binding assays were performed in duplicate (DC) or triplicate (Con A blasts) using 5×10^6 DC and 3×10^6 Con A blasts in a volume of 100 μl. Receptor binding data were analyzed using the LIGAND nonlinear regression program (Munson, 1983) and plotted by the method of Scatchard (1949).

RNA Preparation and PCR

These procedures were previously described in detail (Grohmann et al., 1995b; Bianchi et al., 1996). β-actin primers were purchased from Clontech Laboratories (Palo Alto, CA), and IL-12R primers were synthesized according to published sequences (Chua et al., 1995; Presky et al., 1996). The sequence of 5′ sense primer and 3′ antisense primer of IL-12Rβ1 and IL-12Rβ2 were as follows:

5′ IL-12Rβ1 [239–259], TAT-GAG-TGC-TCC-TGG-CAG-TAT;

3′ IL-12Rβ1 [787–807], GGC-ATG-CTC-CAA-TCA-CTC-CAG;
5′ IL-12Rβ1 [1073–1092], AAT-GTG-CTC-GCC-AAA-ACT-CG;
3′ IL-12Rβ1 [2220–2239], CGC-AGT-CTT-ATG-GGT-CCT-CC;
5′ IL-12Rβ2 [1760–1779], ACA-TTA-CTG-CCA-TCA-CAG-AG; and
3′ IL-12Rβ2 [2242–2261], AGG-AGA-TTA-TCC-GTA-GGT-AG.

The positive control for both IL-12Rβ1 and IL-12Rβ2 was cDNA from mouse Con A lymphoblasts.

RNase Protection Analysis

Antisense RNA probes for IL-12Rβ1 and IL-12Rβ2 were prepared using the T7 promoter in PCRII (Invitrogen, San Diego, CA). The probes were labeled with [^{32}P]UTP, purified on a G50 Sephadex column, and used the same day. Total RNA (40 μg) was hybridized with 10^5–10^6 cpm antisense probe in 80% (w/v) formamide buffer at 60°C. IL-12Rβ1 and IL-12Rβ2 probes, generated from mouse cDNA clones, were a generous gift from Dr. Louise Showe (The Wistar Institute, Philadelphia, PA). The protected fragments for IL-12Rβ1 and IL-12Rβ2 probes were 330 bp (occasionally, an additional fragment may be present a few bp apart; Dr. L. Showe, personal communication) and 263 bp, respectively. A mouse β-actin anti-sense probe (Ambion, Austin, TX) was used as an internal control for standardization of expression levels between samples. Samples were processed as described (Gilman, 1996) and fractionated on a 6% polyacrylamide sequencing gel. Autoradiography was performed at −70°C for 3 days using Kodak X-OMAT AR films (Eastman Kodak, Rochester, NY) with an intensifying screen.

Nuclear Extracts and Electrophoretic Mobility Shift Assay

Cells were stimulated for 15 min with rIL-12 (100 ng/ml), rIFNγ (200 U/ml), or 12.5 ng/ml PMA and 100 ng/ml ionomycin, and nuclear extracts were prepared as previously described (Musso et al., 1994). All DNA binding reactions were conducted for 20 min at room temperature in a final volume of 20 μl. The reactions were started by adding 10 μg of nuclear protein extract to a reaction mix containing 1 μg of poly (dI.dC).(dI.dC) (Pharmacia), 4 μl of 5× binding buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 5 mM EDTA, 25% (v/v) glycerol, and 5 mM dithiothreitol), and approximately 20,000 cpm (approximately 0.1 ng) of the respective [γ-^{32}P]ATP-labeled dsDNA oligonucleotide. Cold competitor oligonucleotides were added to the reaction mix before the radiolabeled probe. For supershift experiments, protein extracts were incubated with polyclonal anti-Stat3, anti-Stat4, anti-p50, anti-p65, anti-RelB, or anti-*c*-Rel (all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min at room temperature after the addition of the radiolabeled probe. The whole sample was then loaded on a 5% native polyacrylamide gel in Tris-borate-EDTA buffer. After electrophoresis, gels were dried and separated protein-DNA complexes were visualized by autoradiography using Kodak XAR5 films. The following dsDNA oligonucleotides were used in EMSA analysis as labeled or competitor probes: FcγRI (Larner et al., 1993), 5′-TCGACGCATGTTTCAAGGATTTGAGATGTA TTTCCCAGAAAAGGCTCGA-3′; NF-κB (Promega), 5′-AGAGGG GACTTTCCGAGAGGC-3′; and m12-27 (Murphy et al., 1995), 5′-CTT AAAATTCCCCCAGAATGTTTTG-3′.

Intracellular Immunofluorescence Staining

CB1 cells were grown on coverslips for 24 hr prior to 15 min exposure to 100 ng/ml IL-12 at 37°C. Cells were then fixed with methanol at −20°C for 7 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The fixed cells were blocked in 3% BSA and 1% glycine in PBS overnight at 4°C. Cells were incubated with anti-p50 antibody (1:400) in blocking solution for 1 hr. The cells were washed with 0.1% Tween-20 in PBS and then incubated for 1 hr with rhodamine-conjugated goat anti-rabbit IgG (Sigma Chemical Co.). Coverslips were mounted onto slides using glycerol containing 20% PBS, 1 mg/ml *p*-phenylendiamine, and 0.02% sodium azide, and the immunofluorescence staining was examined with an Olympus BX20 fluorescence microscope.

Detection of IL-12 in CB1 Cell Culture Supernatants

CB1 cells (1.5 × 10^6) were incubated with medium alone, IL-12 (100 ng/ml), or IFNγ (1000 U/ml) at 37°C. After 18 hr, cells were extensively washed and resuspended in 1 ml of RPMI medium containing 10% FCS and LPS (1 μg/ml). Supernatants were collected at 1 and 24 hr prior to assaying IL-12 p70 contents by ELISA using the hamster anti-mouse p35 MAb and biotinylated anti-mouse p40 (C17.8) MAb. The sensitivity limit of this assay was approximately 100 pg/ml for IL-12 p70.

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