

β_2 -Agonists Prevent Th1 Development by Selective Inhibition of Interleukin 12

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Abstract

Interleukin 12 (IL-12) plays a central role in the immune system by skewing the immune response towards T helper 1 (Th1) type responses which are characterized by high interferon- γ and low IL-4 production. In this report we present evidence that β_2 -agonists inhibit IL-12 production by both human monocytes in response to lipopolysaccharide (LPS) and dendritic cells stimulated via CD40. Inhibition of IL-12 production is selective, as other cytokines produced by monocytes are unaffected. IL-12 inhibition is dependent on β_2 -adrenoceptor stimulation and correlates with increased levels of intracellular cAMP. In conjunction with their ability to suppress IL-12 production, when β_2 -agonists are added at priming of neonatal T lymphocytes, they inhibit the development of Th1-type cells, while promoting T helper 2 (Th2) cell differentiation. Further, the in vivo administration of a therapeutic dose of salbutamol results in the selective inhibition of IL-12 production by whole blood lymphocytes stimulated in vitro with LPS. These findings provide new insight into the immunological consequences of the clinical use of β_2 -agonists and may suggest new approaches for the treatment of Th1-mediated diseases. (*J. Clin. Invest.* 1997. 100:1513–1519.) Key words: cytokines • T helper 2 • β_2 -adrenoreceptor • salbutamol • dendritic cells

Introduction

CD4⁺ cells can be subdivided into different subsets based on the kind of lymphokines they produce (1). T helper 1 (Th1)¹ cells secrete IFN- γ and TNF, whereas T helper 2 (Th2) cells secrete IL-4, IL-5, and IL-13. Th1 cells induce cellular-mediated inflammation and tissue injury, whereas Th2 cells upregulate IgE production and are prominent in the pathogenesis of allergic diseases (2, 3). Cytokines present during the initiation of a T cell response can determine the development of a particular Th subset. Th2 cells develop when naive T cells are

stimulated in the presence of IL-4 (4–6). Conversely, IL-12 is a critical factor driving the development of Th1 cells (7–10). Thus, a shift in the balance of these two cytokines appears to be an important element in the generation or correction of immune dysfunctions.

IL-12 is produced by myelomonocytic cells as a heterodimer composed of two disulfide-linked chains, p35 and p40, encoded by separate genes (11, 12). Simultaneous expression of the two genes is required for the production of the biologically active IL-12 heterodimer. IL-12 production by human macrophages has been shown to be inhibited by PGE₂ (13, 14), probably by increasing intracellular cAMP (13). Since β_2 -agonists also elevate intracellular cAMP levels (15), we speculated that β_2 -agonists could inhibit IL-12 production and thus the development of Th1 cells. In this paper, we report that β_2 -agonists, including salbutamol, a bronchodilator widely used in asthma, are potent and selective inhibitors of IL-12 production by human myelomonocytic cells when either added to in vitro cultures or when administered in vivo. β_2 -Agonists inhibit IL-12 production by both macrophages and dendritic cells (DC) stimulated with LPS or via CD40–CD40L interaction, respectively. These effects are dependent on β_2 -adrenoceptor stimulation and correlate with increased intracellular cAMP levels. In accord with their ability to suppress IL-12 production, when β_2 -agonists are added at priming of neonatal T cells, they selectively inhibit the development of Th1-type cells and enhance Th2-type cell development. These results indicate that β_2 -adrenergic compounds have important immunological effects which should be taken into consideration in the treatment of Th2-mediated human diseases.

Methods

Cytokines and reagents. The following reagents were purchased from commercial sources: PHA-M (Wellcome Diagnostics, Dartford, United Kingdom); LPS, PMA, ionomycin, actinomycin D, cycloheximide, salbutamol, isoproterenol, procaterol, propranolol, lebetalol, alprenolol, oxprenolol, pindolol, and DCI (Sigma Chemical Co., St. Louis, MO); brefeldin A (Sandoz, Basel, Switzerland); GM-CSF and IL-4 (PharMingen, San Diego, CA).

Cell lines and cultures. J558 cells transfected with mouse CD40 ligand (J558L-mCD40L) were from Dr. Peter Lane (Basel Institute for Immunology, Basel, Switzerland) (16). The cells were cultured in 75-cm² tissue culture flasks in IMDM medium supplemented with 5% fetal clone serum (Hyclone Labs., Logan, UT), L-glutamine (2 mM), streptomycin (10 μ g/ml), penicillin (10 U/ml), sodium pyruvate (1 mM) in the presence of L-histidinol dihydrochloride (5 mM) (Sigma Chemical Co.). The parental J558 cells were cultured in the same medium in the absence of L-histidinol. Human monocytes were purified from buffy coats as previously described (17). Supernatants were collected 12 or 24 h later for cytokines analysis.

DC preparation and stimulation. Immature DC were prepared as described (18). Briefly, PBMC were isolated from a buffy coat by Ficoll (Pharmacia Biotec AB, Uppsala, Sweden). T and B cells were depleted using an immunomagnetic method. PBMC were incubated for 30 min with anti-CD3 and anti-CD19 antibodies (PharMingen), then

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1. Abbreviations used in this paper: DC, dendritic cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Th, T helper.

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washed. Goat anti-mouse IgG microbeads (Miltenyi Biotec GmbH, Bergish-Gladbach, Germany) were added and after 30 min the cell suspension was applied on the top of a MiniMACS column. Lymphocyte-depleted PBMC were plated in 25-cm² flasks at 6×10^6 cells/ml in complete medium supplemented with GM-CSF (800 U/ml) and IL-4 (1,000 U/ml). Cells were analyzed on day 6 for CD1a and CD40 expression using a FACScan[®] (Becton Dickinson, Mountain View, CA). 7×10^4 DC were incubated with 3.5×10^4 irradiated (10,000 rad) J558L-mCD40L and IFN- γ (1,000 U/ml) in the presence or absence of β_2 -agonists. After 24 h, supernatants were collected and IL-12 and p40 concentrations were measured by ELISA.

Cytokine assays. ELISAs for IL-12 p40 and p75 were performed as described (19). Human recombinant IL-12, IL-12 p40, and anti-IL-12 mAbs were kindly provided by Dr. Maurice Gately (Hoffman-La Roche Inc., Nutley, NJ). ELISAs for IFN- γ were performed using mAb clone 69 (20). ELISAs for human IL-6 were performed using mAb clone 16 and a sheep anti-IL-6-POD mAb (21). Human recombinant IL-6 and anti-human IL-6 mAb were received from Dr. Harald Gallati (INTEX, Muttenz, Switzerland). ELISAs for human IL-1 α and IL-1 β were performed using goat anti-human IL-1 α and IL-1 β mAbs, kindly provided by Dr. Richard Chizzonite (Hoffman-La Roche Inc.). Human IL-10 and TNF- α were detected using an ELISA kit (PharMingen) according to the manufacturer's instructions.

Measurement of cAMP accumulation in human monocytes. Human monocytes were isolated as described above and incubated at room temperature with salbutamol (10 nM) or Ro 19-8650 (1 nM) for different periods of time (ranging from 5 to 20 min). Forskolin (10 μ M) and dibutyl cAMP (5 mM) (dbcAMP) (both from Sigma Chemical Co.) were used as positive controls. To stop the reaction, the medium was discarded and the cells were lysed with 95% cold ethanol. After an incubation of 10 min on ice, the lysate was removed, dried in a SpeedVac, and resuspended in the radioimmunoassay buffer. cAMP was quantified in triplicate using a cAMP ¹²⁵I-labeled radioimmunoassay system (Amersham Life Science, Arlington Heights, IL).

Northern blot hybridization. Total RNA was extracted from induced and uninduced PBMC by TRIzol[™] (Gibco Laboratories, Grand Island, NY). Equal amounts of RNA (10 μ g/lane) were fractionated in a 1.5% agarose-formaldehyde gel. The specific RNA were detected by hybridization of nylon membranes (Hybond N⁺; Amersham Life Science) with ³²P-cDNA probes for IL-12 p40, p35, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The filters were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) between double intensifying screens (DuPont Co., San Diego, CA).

RNase protection assay. The vectors containing the entire coding regions of IL-12 p40 and p35 were kindly provided by Dr. Ueli Gubler (Hoffman-La Roche Inc.). 301-bp and 265-bp PCR products derived from p40 and p35 cDNA, respectively, were subcloned into pCR[™] II (Invitrogen, San Diego, CA). The vectors were linearized with appropriate restriction enzymes and transcribed with SP6 polymerase into complementary RNA (antisense) riboprobes using [³²P]CTP (Amersham Life Science) and a riboprobe kit (Promega, Madison, WI). Cells were collected by low-speed centrifugation and disrupted at 10^7 cells/ml lysis/denaturation solution final concentration, according to the instructions of the Direct Protect[™] lysate ribonuclease protection assay kit (Ambion Inc., Austin, TX). 45 μ l of cell lysate was hybridized in solution with an excess of riboprobes (10^5 cpm for p40, 2×10^5 cpm for p35, 10^4 cpm for 18S, 37°C overnight). 500 μ l of RNase solution was added for 30 min at 37°C and the instructions of the Direct Protect[™] kit were followed. The protected fragments were fractionated on 5% polyacrylamide/urea sequencing gel and detected by autoradiography.

In vitro differentiation of human naive T cells. Cord blood-derived mononuclear cells were incubated with anti-CD45RA and anti-CD4 monoclonal antibodies. After a 20-min incubation, cells were washed and incubated with goat anti-mouse Ig-coated magnetic beads. Positive cells were separated and seeded at 10^6 cells/ml in a 24-well plate, together with autologous adherent cells, PHA, and either anti-IL-12

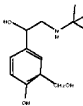
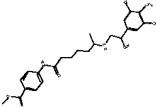
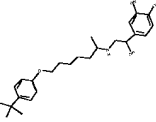
mAb or different concentrations of salbutamol or its analogs for 6 d. Cells were then washed and put back in culture in the presence of IL-2 (200 U/ml). After 10 d, cells were collected and restimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml) for 4 h. Brefeldin A (10 μ g/ml) was added in the last 2 h. Then the cells were fixed with 4% paraformaldehyde and permeabilized with saponin. Fixed cells were stained with FITC-anti-IFN- γ and PE-anti-IL-4 mAbs (PharMingen) and analyzed by FACS[®] (Becton Dickinson).

In vivo administration of salbutamol. 12 nonsmoking apparently healthy volunteers, aged 27–43 participated in the study. Six subjects received salbutamol tablets (4 mg) (Ventolin; Glaxo, Verona, Italy), and six subjects received no treatment and served as controls. Blood samples were obtained before and 2 h after treatment, i.e., at the time of the expected maximum salbutamol plasma concentration of ~ 12 –14 μ g/liter (22). Whole blood was seeded in 24-well plates and stimulated with IFN- γ (1,000 U/ml) and LPS (1 μ g/ml) for 24 h. Supernatants were harvested and cytokines measured as described above.

Results

β_2 -Agonists selectively inhibit IL-12 production. We examined the effect of three β_2 -agonists on the production of IL-12 by freshly isolated human monocytes primed with IFN- γ for 16 h and stimulated with LPS for 24 h (Table I). Salbutamol inhibited the production of IL-12 in a dose-dependent manner with a 50% inhibitory concentration (IC₅₀) of 30 nM. The inhibition of IL-12 was specific, since salbutamol did not influence IL-1 α , IL-1 β , IL-6, or IL-10 production by LPS-stimulated monocytes (Fig. 1 A). Salbutamol inhibited TNF- α production albeit with higher IC₅₀. Ro 19-8650 and Ro 19-0865 were more potent inhibitors of IL-12, with IC₅₀ of 0.1 and 0.05 nM, respectively (Fig. 1 B). Similar results were observed using whole peripheral blood instead of purified monocytes (data not shown). Further, β_2 -agonists inhibited neither IFN- γ nor IL-4 production by a series of human Th1 and Th2 clones activated by ConA (data not shown).

Table I. β_2 -Agonists Bind with High Affinity to β_2 -Adrenoreceptor and Are Potent Inhibitors of IL-12 Production by Human Monocytes

Compound	Structure	Binding to β_2 -adrenoreceptor*	Inhibition of IL-12 (IC ₅₀)
salbutamol		n.d.	30 nM
19-8650		91.56%	0.1 nM
19-0865		74.58%	0.05 nM

β_2 -Binding assay was performed by Novascreen (Hanover, MA) according to established receptor binding techniques. *Values are expressed as the percent inhibition of specific binding of [¹²⁵I]pindolol and represent the average of replicate tubes at 10^{-7} M concentration. Alprenolol was used as the reference compound.

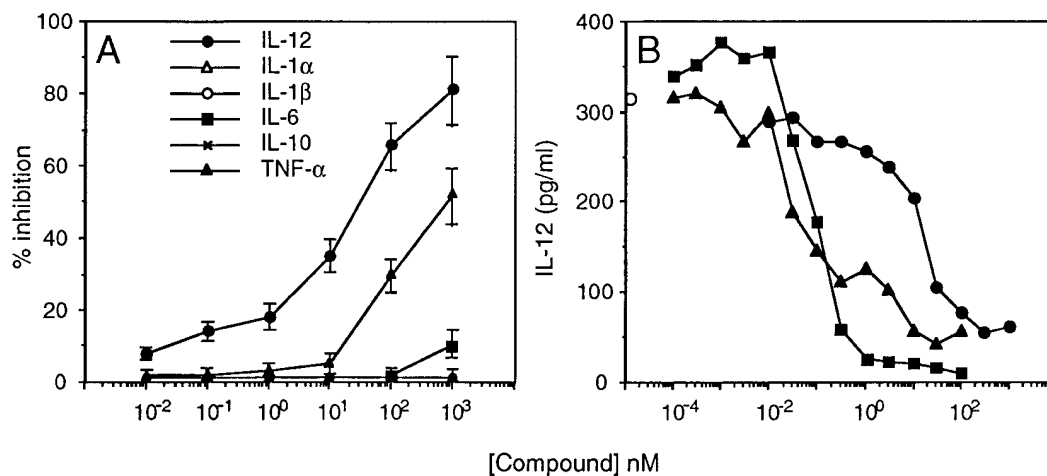


Figure 1. β_2 -Agonists specifically inhibit IL-12 production by human monocytes. (A) Human monocytes were primed with IFN- γ (1,000 U/ml) for 16 h and stimulated with LPS (1 μ g/ml) in the presence of different concentrations of salbutamol. Supernatants were harvested 12 h after stimulation with LPS for TNF- α analysis, and 24 h after stimulation with LPS for IL-12, IL-1 α , IL-1 β , IL-6, and IL-10 analysis. Cytokines levels in the absence of salbutamol were

as follows: IL-1 α , 35 ng/ml; IL-1 β , 2 ng/ml; IL-6, 11 ng/ml; IL-10, 1.4 ng/ml; TNF- α , 7 ng/ml. Cytokine concentrations were evaluated by ELISA. Results represent four independent experiments (mean \pm SD). (B) Human monocytes were primed with IFN- γ (1,000 U/ml) for 16 h and stimulated with LPS (1 μ g/ml) in the presence of medium alone (open circle) or different concentrations of salbutamol (filled circles), Ro 19-8650 (filled squares), Ro 19-0865 (filled triangles). Supernatants were harvested 24 h after stimulation with LPS and IL-12 concentrations were evaluated by ELISA. Results from a single representative experiment are given as the mean of triplicate cultures (SD < 5% of mean).

A

Compound	Effect on β_2 -adrenoceptor	IL-12 inhibition IC ₅₀ (nM)
Isoproterenol	agonist	30
Procaterol	agonist	30
Salbutamol	agonist	30
Propranolol	antagonist	>1000
Labetalol	antagonist	>1000
Alprenolol	antagonist	>1000
Oxprenolol	antagonist	>1000
Pindolol	antagonist	>1000
DCI	antagonist	>1000

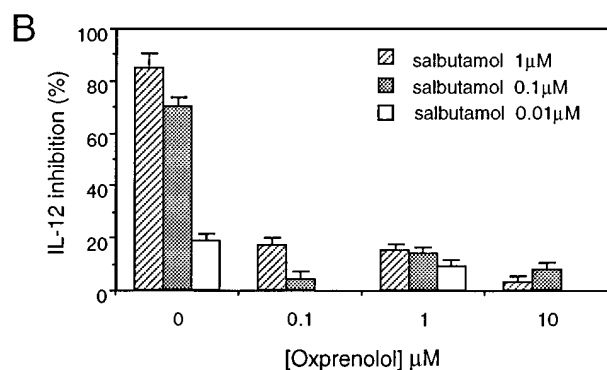


Figure 2. β_2 -Agonists inhibit IL-12 production through binding to the β_2 -adrenoreceptor. (A) Human monocytes were primed with IFN- γ (1,000 U/ml) for 16 h and stimulated with LPS (1 μ g/ml) in the presence or absence of different concentrations of β_2 -agonists and antagonists. (B) Human monocytes were primed with IFN- γ and stimulated with LPS in the presence or absence of different concentrations of salbutamol and the β_2 -receptor antagonist oxprenolol. Supernatants were harvested 24 h after stimulation with LPS and IL-12 was evaluated by ELISA. The results are given as the mean \pm SD of three independent experiments.

Inhibition of IL-12 production by β_2 -agonists depends on β_2 -receptor stimulation and correlates with increased intracellular cAMP levels. The inhibition of IL-12 production by β_2 -agonists is dependent on β_2 -receptor stimulation, since all the β_2 -agonists tested, but not β_2 -antagonists, inhibited IL-12 production (Fig. 2 A). Furthermore, the inhibition of IL-12 production was completely reversed by oxprenolol, a β_2 -receptor antagonist (Fig. 2 B).

β_2 -Agonists elevate intracellular cAMP levels through the activation of the membrane-bound enzyme adenylate cyclase. In accord with earlier studies showing a correlation between cAMP levels and the inhibition of IL-12 production (13), we found a perfect match between increased intracellular cAMP levels induced by β_2 -agonists and the inhibition of IL-12 secretion (Fig. 3). In addition to the β_2 -agonists, both forskolin, which elevates cAMP by direct activation of adenylate cyclase, and dbcAMP, a cell permeable form of cAMP, inhibited IL-12 production.

β_2 -Agonists inhibit IL-12 p40 and p35 mRNA. To determine whether the inhibition of IL-12 secretion was the result of decreased mRNA production, we analyzed the effect of salbutamol on the expression of IL-12 p40 and p35 mRNA in LPS-stimulated monocytes. The percentage of inhibition of steady state mRNA accumulation is shown in Fig. 4 A. Salbutamol tested at a concentration of 100 nM induced \sim 40% inhibition of p40 and p35 mRNA, as compared with the untreated control. The effect of β_2 -agonists on IL-12 p40 mRNA stability was also examined by blocking transcription with actinomycin D 4 h after stimulation with LPS. As shown in Fig. 4 B, salbutamol did not accelerate IL-12 p40 mRNA degradation. Finally, inhibition of IL-12 p40 mRNA in LPS-stimulated monocytes was evaluated in the presence of cycloheximide, a protein synthesis inhibitor (Fig. 4 C). When cycloheximide was added to the cells, salbutamol still inhibited IL-12 p40 mRNA, suggesting that IL-12 inhibition by β_2 -agonists does not require de novo protein synthesis.

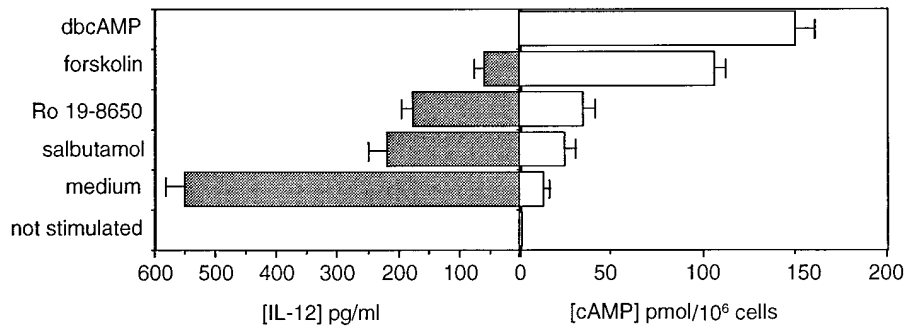


Figure 3. The inhibition of IL-12 production by β_2 -agonists correlates with increased levels of intracellular cAMP. Monocytes were primed with IFN- γ and stimulated with LPS in the presence of medium alone, salbutamol (10 nM), Ro 19-8650 (1 nM), forskolin (10 μ M), or dbcAMP (5 μ M). For IL-12 production, cells were incubated for 24 h at 37°C, and IL-12 was measured in the supernatants by ELISA. For intracellular cAMP determination, cells were incubated for 10 min at room temperature. Cell lysate was pre-

pared by lysis in cold ethanol and dried. cAMP determination was performed by a radioimmunoassay. Results are given as the mean \pm SD of three independent experiments.

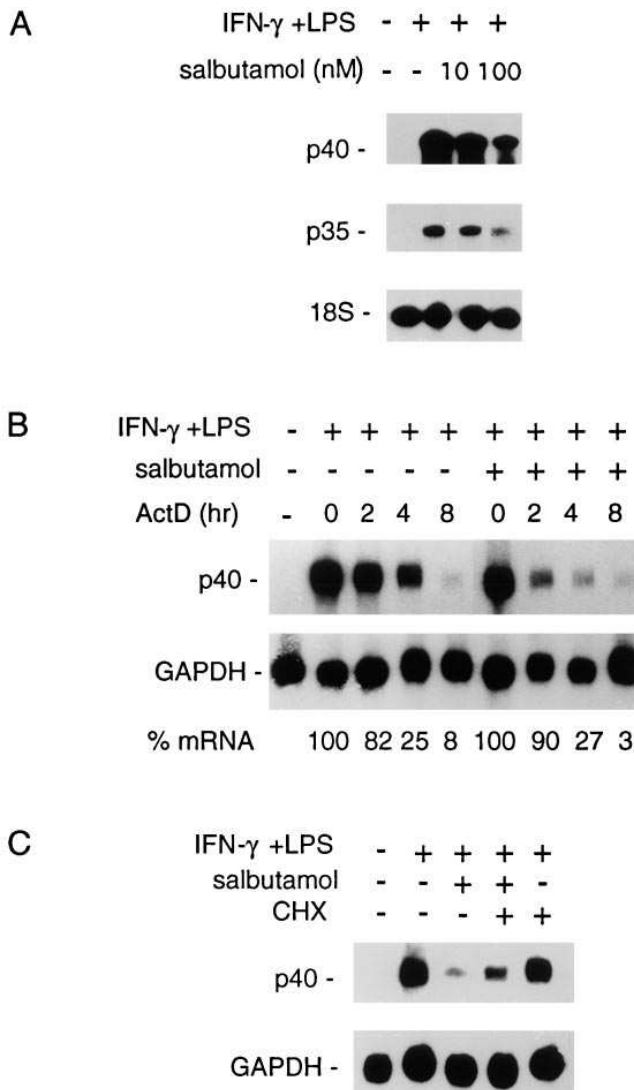


Figure 4. (A) β_2 -Agonists inhibit accumulation of IL-12 p40 and p35 mRNA in human monocytes. The inhibition of IL-12 p40 and p35 mRNA accumulation by β_2 -agonists was analyzed in human monocytes primed with IFN- γ and then stimulated with LPS in the presence or absence of different concentrations of salbutamol. RNase protection assay using the indicated riboprobes was performed as indicated in Methods. The protected fragments were precipitated and fractionated on 5% polyacrylamide/urea sequencing gel. Gels were

We observed that p40 mRNA accumulation and p40 protein secretion are similarly inhibited in the presence of salbutamol (Fig. 5). However, salbutamol at 100 nM induced 40% inhibition of p35 mRNA and complete inhibition of IL-12 protein secretion, thus suggesting that β_2 -agonists, in addition to having a transcriptional effect, may also influence posttranslational processing steps in IL-12 biosynthesis and secretion.

β_2 -Agonists inhibit IL-12 production by DC. DC represent an important source of IL-12 in vivo (23, 24). DC can be triggered to produce very high levels of IL-12 upon ligation of CD40 by CD40L (25, 26). To address whether β_2 -agonists could also inhibit IL-12 production by DC, we stimulated human monocyte-derived DC with IFN- γ and J558 cells expressing the CD40 ligand in the presence or absence of salbutamol (Fig. 6 A). Production of IL-12 was inhibited in a dose-dependent manner. Total RNA was isolated from four DC preparations stimulated as indicated above, and accumulation of IL-12 p40, p35, and 18S mRNA was evaluated by RNase protection assay. The complete inhibition of both p40 and p35 mRNA was observed when salbutamol was added at a concentration of 10 μ M, although inhibition was already evident at a concentration of 1 μ M of salbutamol (Fig. 6 B).

β_2 -Agonists inhibit in vitro differentiation of Th1 cells. IL-12 drives the differentiation of naive T cells toward IFN- γ -producing Th1 (7, 8, 27). Because of the inhibitory effect of β_2 -agonists on IL-12 production, we investigated the possibility

dried and exposed to PhosphorImager screens and the p40 and p35 messages were normalized against the 18S message. (B) β_2 -Agonists do not accelerate degradation of p40 mRNA. Human monocytes were primed with IFN- γ and then stimulated with LPS in the presence or absence of salbutamol (10 nM). Actinomycin D (10 μ g/ml) was added 4 h after LPS stimulation. mRNA was isolated by Trizol at 0, 2, 4, and 8 h after addition of actinomycin D, and subjected to Northern blot analysis. Gels were dried and exposed to PhosphorImager screens and the p40 and p35 messages were normalized against the GAPDH message. The amount of mRNA is expressed as a percentage of the mRNA levels at the time of addition of actinomycin D. (C) The inhibitory effect of β_2 -agonists on p40 mRNA accumulation does not require de novo protein synthesis. Human monocytes were primed with IFN- γ for 16 h and then stimulated with LPS and salbutamol (100 nM) for 4 h in the presence or absence of cycloheximide (15 μ g/ml). mRNA was isolated by Trizol and subjected to Northern blot analysis. The results shown are from one representative experiment out of three performed with similar results.

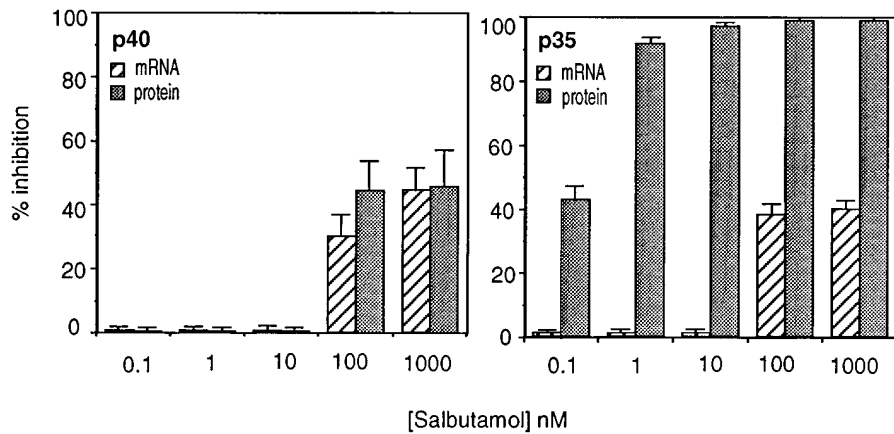


Figure 5. Percentage of inhibition of steady state mRNA accumulation and protein secretion for IL-12 p40 and p35 by β_2 -agonists. The percentages of inhibition of steady state mRNA accumulation and protein production are shown for IL-12 p40 and p35 in monocytes primed with IFN- γ and stimulated with LPS in the presence of different concentrations of salbutamol for 4 h for RNA analysis and for 24 h for protein production. Values shown represent the mean \pm SD of the three experiments. Because free p35 is not detectable in the supernatant fluid, values for the p75 heterodimers were used to evaluate production of the p35 protein.

that β_2 -agonists affect Th1 development. We analyzed the effect of salbutamol and Ro 19-8650 on mitogen-induced differentiation of neonatal CD4⁺ T cells at the single-cell level by measuring their intracellular production of IL-4 and IFN- γ by flow cytometry. Neonatal CD4⁺ T cells stimulated with PHA (1 μ g/ml) preferentially develop into IFN- γ -secreting cells (49%) (Fig. 7 a). In addition, a population of Th2 cells that produced only IL-4 and no IFN- γ (13%) and a small population of double-positive cells (3%) were detected. The addition of β_2 -agonists to the cell cultures resulted in a marked inhibition of the development of IFN- γ -producing cells (from 49 to 3–5%) (Fig. 7, c and d), which was comparable to the reduction

induced by a neutralizing anti-IL-12 Ab (Fig. 7 b). In contrast, \sim 30% of neonatal CD4⁺ T cells stimulated in the presence of β_2 -agonists differentiated into Th2 cells producing IL-4, but not IFN- γ . Thus, priming of naive T cells in the presence of β_2 -agonists leads to the inhibition of Th1 and the enhancement of Th2 cell development.

In vivo administration of salbutamol inhibits IL-12 production upon in vitro stimulation with LPS. β_2 -Adrenergic agonists are commonly used as inotropic agents in the treatment of allergic asthma. To study the effect of β_2 -agonists in vivo, salbutamol tablets (4 mg) were administered to six healthy volunteers. Six healthy subjects received no treatment and served as controls. Peripheral blood was taken before and 2 h after administration of salbutamol (or no treatment) and stimulated in

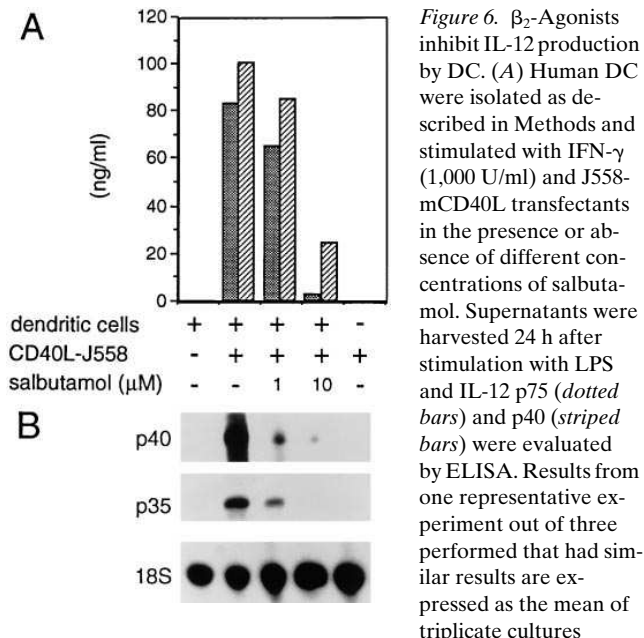


Figure 6. β_2 -Agonists inhibit IL-12 production by DC. (A) Human DC were isolated as described in Methods and stimulated with IFN- γ (1,000 U/ml) and J558-mCD40L transfectants in the presence or absence of different concentrations of salbutamol. Supernatants were harvested 24 h after stimulation with LPS and IL-12 p75 (dotted bars) and p40 (striped bars) were evaluated by ELISA. Results from one representative experiment out of three performed that had similar results are expressed as the mean of triplicate cultures

(SD < 5% of mean). (B) The induction of IL-12 p40 and p35 mRNA accumulation was analyzed in DC stimulated with IFN- γ (1,000 U/ml) and J558-mCD40L cells in the presence or absence of salbutamol. Cell pellet was lysed with lysis solution 4 h after stimulation with LPS, and subjected to RNase protection analysis using the riboprobes as indicated in Methods. Protected fragments were precipitated and fractionated by PAGE. The 18S message was used as a control. A representative experiment is shown out of three performed that had similar results.

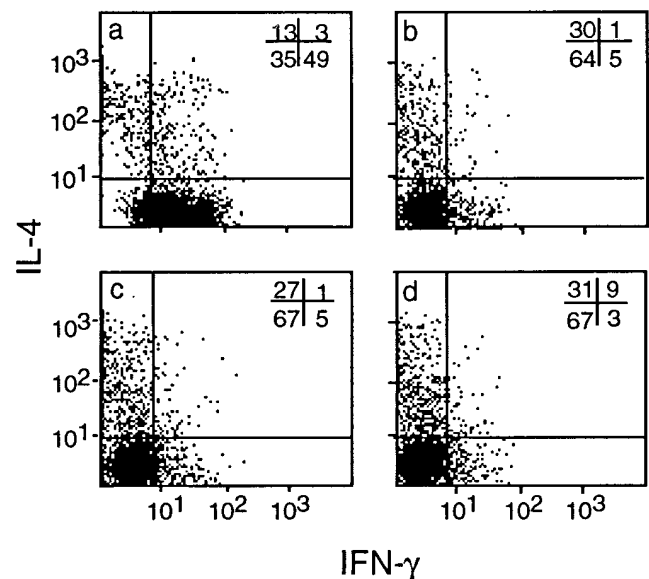


Figure 7. β_2 -Agonists inhibit in vitro differentiation of Th1 cells. Cord blood-purified CD4⁺ CD45RA⁺ T cells were stimulated with PHA (1 μ g/ml) in the presence of medium alone (a), anti-IL-12 Ab (b), 1 μ M salbutamol (c), or 100 nM Ro 19-8650 (d), and autologous adherent cells. Cells were washed after 6 d and cultured in medium containing IL-12 (200 U/ml). After 10 d, cells were washed, stimulated with PMA and ionomycin and stained for intracellular IFN- γ and IL-4. Cells were then analyzed by FACS[®] analysis. One representative experiment out of five is shown.

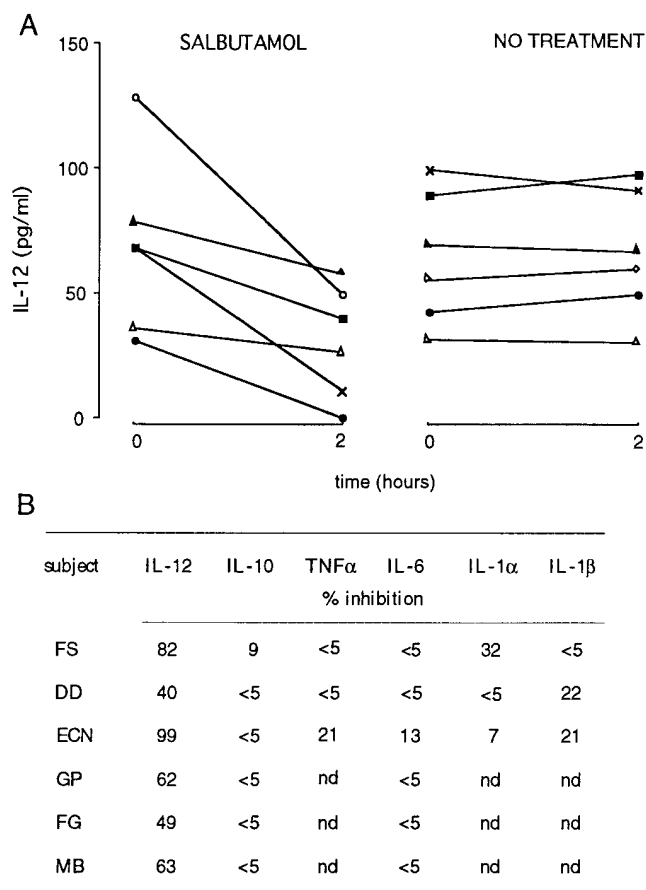


Figure 8. Oral administration of salbutamol inhibits IL-12 production in vitro stimulation with LPS. Salbutamol tablets (4 mg) were administered to six healthy volunteers. Six healthy controls received no treatment. Peripheral blood was taken before (0) and 2 h after (2) salbutamol (or no treatment) and stimulated in vitro with IFN- γ (1,000 U/ml) and LPS (1 μ g/ml). Cell-free supernatants were harvested 12 h after stimulation with LPS for TNF- α analysis, and 24 h after stimulation with LPS for IL-12, IL-1 α , IL-1 β , IL-6, and IL-10 analysis.

in vitro with IFN- γ (1,000 U/ml) and LPS (1 μ g/ml). The concentration of IL-12 was markedly decreased 2 h after salbutamol administration in each of the six treated volunteers, whereas it did not change significantly in any of the control volunteers (Fig. 8 A). The inhibitory effect on IL-12 was selective, as the production of other cytokines was either unaffected or only marginally inhibited (Fig. 8 B).

Discussion

In this study, we have demonstrated that β_2 -agonists are potent and selective inhibitors of IL-12 production by myelomonocytic cells when either administered in vivo or when added to in vitro cultures. Occupation of a β_2 -receptor by an agonist results in a conformational change that leads to G protein activation. This in turn activates adenylate cyclase, which results in the conversion of ATP to cAMP, the second messenger of β_2 -receptor function. Our data demonstrate that increased intracellular cAMP levels, induced by β_2 -agonists, correlate with the inhibition of IL-12 secretion. This is in accord

with previous observations that the elevation of intracellular cAMP levels by PGE2 leads to the effective inhibition of IL-12 by LPS-stimulated monocytes (13). The inhibition of IL-12 production by β_2 -agonists is selective. Other proinflammatory cytokines, such as IL-1 α , IL-1 β , and IL-6 are not inhibited, while TNF- α , in agreement with previous observations (28, 29), is partially inhibited. Most importantly, production of the antiinflammatory cytokine IL-10 is not affected. This is particularly relevant in view of the fact that IL-10 negatively regulates IL-12 production by inhibiting IL-12 p40 and p35 gene transcription (29a).

In addition to inhibiting IL-12 production by LPS-stimulated monocytes, β_2 -agonists also inhibit IL-12 produced by DC upon CD40-CD40 ligand interaction. DC play a critical role in initiating primary immune responses (30). Recent reports indicate that DC costimulate T cell functions by producing high levels of IL-12 upon antigen-specific interaction (24). This interaction involves the binding of CD40 to its ligand on T cells (25, 26). DC can thus direct the development of antigen-specific CD4⁺ T cells towards the Th1 phenotype through the secretion of IL-12 (23). Salbutamol inhibits p40 and p35 mRNA accumulation and IL-12 protein secretion by immature DC stimulated via CD40. The LPS-induced IL-12 production by monocytes is significantly more sensitive to inhibition by β_2 -agonists than the CD40-mediated IL-12 production by DC. The difference in the degree of inhibition may reflect differences in kinetics or depend upon the type of activation. Indeed, we observed a 300-fold higher production of IL-12 by DC stimulated with J558 cells expressing the CD40 ligand, as compared with LPS-stimulated monocytes.

The in vitro differentiation of neonatal CD4⁺ T cells into IFN- γ -producing cells is almost completely inhibited by β_2 -agonists. Analysis of cytokine production at the single-cell level indicates that neonatal CD4⁺ T cells activated in the presence of β_2 -agonists differentiated mainly into Th2 cells producing high levels of IL-4 but no IFN- γ , as compared with the mixed phenotype displayed under neutral conditions. This effect clearly can be attributed to IL-12 inhibition since β_2 -agonists do not directly inhibit IFN- γ production by a series of human T cell clones, and since comparable results are achieved when a neutralizing anti-IL-12 mAb is used.

It is now generally accepted that allergic respiratory diseases, including allergic asthma, are associated with active T cell immunity to common allergens skewed toward the Th2 cytokine phenotype (3). It has been suggested that the key cytokine-mediated events which control the selection of relevant Th memory cells occur during the early phase of allergen exposure, i.e., in humans, in conjunction with the perinatal period and particularly during late gestation (31). Interestingly, the maturation of the capacity to produce IFN- γ is delayed in children genetically at risk of developing allergic diseases (32). Oral β_2 -agonists are frequently used in mothers as a tocolytic to prevent abortion and to treat premature labor (33), and inhaled as well as oral β_2 -agonists are the most widely used anti-asthma medication used in children to reverse bronchoconstriction (34). The inhibitory effect of β_2 -agonists on IL-12 production reported in this study, together with the observation that, in vitro, these agents block Th1 while enhancing Th2 differentiation, suggest that the use of β_2 -agonists during pregnancy and early life may increase the risk of developing allergic diseases by skewing the immunologic response to allergen towards the Th2 cytokine profile. In addition, the inhibitory ef-

fect of β_2 -agonists on IL-12 production might be at least partially responsible for the enhanced late asthmatic response to inhaled allergens reported in asthmatic subjects regularly treated with inhaled β_2 -agonists (35). In this respect, it is significant that the *in vivo* administration of a therapeutic dose of salbutamol resulted in the selective inhibition of IL-12 production by whole blood lymphocytes stimulated *in vitro* with LPS.

Our findings may also provide a rationale for the observation that treatment with β_2 -antagonists can exacerbate psoriasis, a Th1-type skin disorder (36). It may be that β_2 -antagonists counteract the regulatory role that circulating catecholamines have on IL-12 produced by antigen-presenting cells in psoriatic skin.

β_2 -Agonists which selectively inhibit the production of IL-12 may, on the other hand, be potentially effective in the treatment of Th1-mediated human diseases (37). An interesting case could be, for example, multiple sclerosis. Supporting this hypothesis are results showing that β_2 -agonist treatment suppresses chronic/relapsing allergic encephalomyelitis in Lewis rats (38).

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