

Interferon γ Inhibits Interleukin 10 Production by Monocytes

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Summary

Interleukin 10 (IL-10) was first described for its ability to inhibit interferon γ (IFN- γ) production. Herein, we studied the balance between IFN- γ and IL-10 production by human peripheral blood mononuclear cells (PBMC) in response to *Staphylococcus aureus* Cowan (SAC) or lipopolysaccharide (LPS). Monocyte depletion reduced IL-10 production by 90% and resulted in an increased IFN- γ production. Addition of anti-IL-10 antibody to PBMC cultures also strongly increased IFN- γ production. In contrast, among various cytokines, only IFN- γ strongly reduced IL-10 synthesis by SAC- or LPS-activated PBMC and monocytes. Thus, IFN- γ has proinflammatory effects through the combination of two mechanisms: (a) induction of early tumor necrosis factor α (TNF- α) and IL-1 β synthesis; and (b) inhibition of the delayed production of IL-10, an inhibitor of TNF- α and IL-1 β synthesis. Taken together, the present data indicate that IFN- γ and IL-10 antagonize each other's production and function.

Functions of immune cells are finely coordinated to ensure an adequate response to an antigenic stimulus, and part of this coordination is mediated by secreted cytokines. Among those, IL-10 (for review see reference 1) was recently discovered, as a mouse Th2 cell product, by its ability to suppress IFN- γ production by Th1 cells (2). Further reports indicated that IL-10 can also be produced by other cell types, such as normal and transformed B cells (3, 3a), activated T cell clones (4), and activated monocytes (5). In addition, IL-10 inhibits antigen presentation (6) and cytokine synthesis by monocytes (5), but induces B cell growth and differentiation (7).

In the present report, we studied the balance between IFN- γ and IL-10 in activated human normal PBMC and monocytes. We show that endogenous IL-10 downregulates the production of IFN- γ , and that IFN- γ , in a reciprocal fashion, inhibits the monocyte-dependent production of IL-10.

Materials and Methods

Isolation of Human Total PBMC and Subpopulations. Total PBMC were isolated from healthy donors by Ficoll-Hypaque centrifugation and separated into purified subpopulations of monocytes and PBL. Monocytes were isolated by elutriation centrifugation as previously described (8). The preparation was >90% pure, as controlled by flow cytometry (FACScan®; Becton Dickinson & Co., Mountain View, CA) with FITC-conjugated anti-CD14 antibody (Leu M3; Becton Dickinson & Co.) and by May Grünwald Giemsa staining. The monocyte population was >90% HLA-DR and <1% CD25⁺. To obtain purified PBL, total PBMC at 10⁶ cells/ml were

incubated with anti-CD14 antibody (IOM2; Immunotech, Luminy, France) at 1 μ g/ml for 45 min at 4°C. Cells were washed three times with RPMI 1640 and gently mixed for 45 min with ferrous beads (Dynabeads M450; Dynal, Oslo, Norway) (five beads per target cell) at 4°C. The bead-cell complexes were eliminated with a magnetic separator. The PBL population was >98% pure and usually contained 84% CD3⁺ (T cells) and 6–13% CD19⁺ (B cells). No change in lymphocyte phenotype was observed after immunodepletion.

Cell Culture. Cells were cultured in complete RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with heat-inactivated 10% FCS (Multiser; Cytosystems, Castle Hill, Australia), 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamycin, and 20 mM Hepes buffer. Cultures were performed in 96-well flat-bottomed plates (Falcon Labware, Oxnard, CA) at a final volume of 250 μ l with 2.5×10^5 cells/well for PBMC. For monocytes, these cultures were performed with an equivalent number of monocytes as in PBMC, calculated from the percent of monocytes. For the PBL population, the cell density was calculated as the total cell number minus the number of monocytes. Cells were stimulated with *Staphylococcus aureus* Cowan strain 1 (SAC) at a 1:200 dilution (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) or LPS (from *Escherichia coli*, serotype O111:B4, 1 μ g/ml; Sigma Chemical Co., St. Louis, MO), alone or in combination with various cytokines. Reagents to be tested were added at the onset of the culture and supernatants were harvested after 48 h unless otherwise indicated.

Reagents. Purified human cytokines from the following sources were used at the following concentrations: rIL-1 β (0.5 ng/ml), rIL-6 (40 ng/ml), and rTNF- α (10 ng/ml), from Genzyme (Boston, MA); rIFN- γ (0.01–100 ng/ml) and rIL-2 (100 U/ml), from Amgen Bio-

logicals (Thousand Oaks, CA); TGF- β (0.5 ng/ml), from Oncomembrane Inc. (Seattle, WA); IL-7 (10 ng/ml), from R&D Systems (Minneapolis, MN); rIL-4 (10 ng/ml), rIL-3 (10 ng/ml), and rGM-CSF (100 ng/ml), from Schering-Plough Research Institute (Bloomfield, NJ). Ascitic fluid containing a neutralizing antibody against IFN- γ B27 (9) and control ascitic fluid were used at the dilution 1:5,000. Neutralizing anti-IL-10 mAb 19F1 (1 μ g/ml) was kindly provided by Dr. J. S. Abrams (DNAX, Palo Alto, CA) (10).

Determinations of Cytokine Levels. The production of IL-10 was measured by IL-10-specific ELISA (sensitivity, 100 pg/ml) using two rat mAbs kindly provided by Dr. J. S. Abrams (DNAX) (10): 9D7 for coating and biotinylated 12G8 for detection. IFN- γ levels were measured by ELISA (sensitivity, 100 pg/ml) as previously described (9). IL-1- β , TNF- α , and IL-6 levels were detected with an enzyme amplified sensitivity immunoassay (sensitivity, 100 pg/ml) kindly provided by Medgenix Diagnostics (Brussels, Belgium).

Detection of Single Cell Cytokine Production (Enzyme-linked Immunospot [ELISPOT]). The cytokine ELISPOT assay, adapted from the method described by Czerkinsky et al. (11), was used to study cytokine synthesis by total PBMC, purified monocytes, and PBL at the single cell level. Wells were coated overnight at 4°C with 100 μ l of mAb 9D7 (30 μ g/ml) for IL-10 ELISPOT assay (10) and mAb BE8 (20 μ g/ml) for IL-6 ELISPOT assay (12). After washing and saturation with PBS/1% BSA, serial dilutions ranging from 2.5×10^4 to 3×10^2 cells/well were distributed in duplicate in a volume of 50 μ l. Cells were incubated with 50 μ l LPS (1 μ g/ml) in the absence or presence of IFN- γ (10 ng/ml) for 48 h at 37°C. To detect spot-forming cells, biotinylated antibodies (12G8 for IL-10 and BE4 for IL-6 ELISPOT, respectively) (10, 12) were then added for 2 h in 100 μ l of PBS/1% BSA. After incubation at room temperature and washing, plates were incubated for 2 h at room temperature with 100 μ l of alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). After washing, 50 μ l substrate solution (5-bromo-4-chloro-3-indolyl phosphate; 1 mg/ml; Sigma Chemical Co.), diluted in 2-amino-2-methyl-1-propanol buffer, in 3% agarose (Bethesda Research Laboratories, Gaithersburg, MD) was added to each well. The number of blue spots was enumerated after 12 h of incubation at room temperature. Frequencies were calculated as the ratio of the number of spots per 1,000 cells.

Results

Endogenous IL-10 Produced by Monocytes Inhibits IFN- γ Production. The production of IL-10 by total PBMC, purified monocytes, and PBL was measured using a specific ELISA after 48 h of culture with or without SAC, a polyclonal activator of both lymphocytes and monocytes (13), or LPS, an activator of monocytes. As shown in Fig. 1 A, low levels of IL-10 were produced by unstimulated cell populations (0.37 ± 0.12 ng/ml, mean \pm SEM of 10 different experiments). This spontaneous secretion was greatly enhanced by the addition of SAC (3.99 ± 0.42 ng/ml). Depletion of CD14⁺ cells (monocytes) by magnetic beads strongly decreased the SAC-induced IL-10 production to 0.27 ± 0.09 ng/ml. When seeded at a cell density corresponding to the number of monocytes present in PBMC, monocytes isolated by elutriation centrifugation also produced IL-10 after SAC activation, but levels were lower than those obtained with total PBMC (1.60 ± 0.27 ng/ml for monocytes). After LPS stimulation, the levels of IL-10 were 1.52 ± 0.28 ng/ml for PBMC, 0.68 ± 0.17 ng/ml

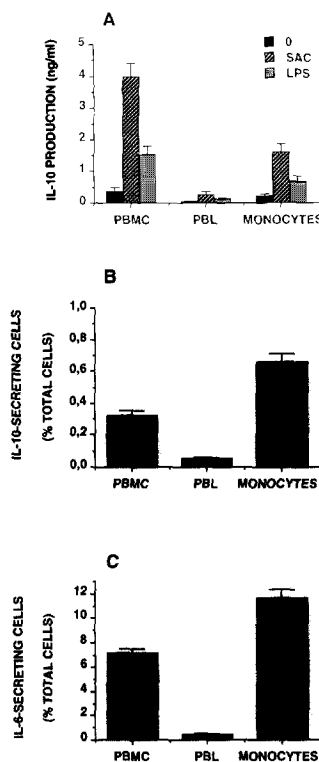


Figure 1. Role of monocytes in the production of IL-10. (A) Production of IL-10 by total PBMC (2.5×10^5 cells/well), PBL, and monocytes in response to SAC or LPS, was measured by ELISA in 48-h supernatants. PBL were seeded at a cell density equivalent to the total cell number minus the number of monocytes. Monocytes were seeded at a cell density equivalent to the number of monocytes present in PBMC. Results are expressed in nanograms per milliliter as the mean \pm SEM of 10 independent experiments, each in triplicate. The production of IL-10 (B) and IL-6 (C) was measured at the single cell level by using the cytokine ELISPOT assay. Cells were activated for 48 h with LPS (1 μ g/ml). The results are expressed as the mean \pm SEM of the percent of cytokine-producing cells in three independent experiments, each in duplicate.

for purified monocytes, and 0.13 ± 0.04 for PBL, results that are significantly lower than obtained with SAC stimulation ($n = 10$, $p < 0.01$, by the Wilcoxon T test).

The production of cytokine at a single cell level was estimated by the calculation of the frequency of spot-forming cells using the ELISPOT assay. Since SAC particles cannot be used in the ELISPOT assay because they bind the conjugate, cells were activated with LPS (1 μ g/ml). The frequency of IL-10-secreting cells was $0.32 \pm 0.03\%$ for PBMC, $0.66 \pm 0.05\%$ for monocytes, and $0.05 \pm 0.01\%$ for PBL (mean \pm SEM of three independent experiments; Fig. 1 B). The frequency of IL-10-secreting cells was ~ 20 -fold lower than that of IL-6-producing cells (IL-6 spots: $7.1 \pm 0.4\%$ for PBMC, $11.6 \pm 0.7\%$ for monocytes, and $0.4 \pm 0.1\%$ for PBL) (Fig. 1 C).

Since IL-10 has been identified by its ability to inhibit the IFN- γ synthesis, we have examined whether endogenous IL-10 might downregulate IFN- γ production by SAC-activated PBMC. As shown in Fig. 2 A, removal of monocytes from PBMC reduced by 16-fold the amount of secreted IL-10 and increased by 10-fold that of IFN- γ , and this effect persisted over a 10-d culture period. Furthermore, PBMC cultures carried out in the presence of purified neutralizing anti-IL-10 19F1 produced eightfold more IFN- γ levels (mean of three experiments), whereas a purified isotype control antibody failed to do so (Fig. 2 B).

IFN- γ Inhibits IL-10 Production. In as much as IL-10 inhibited the production of IFN- γ , we considered whether IFN- γ was able, in a reciprocal fashion, to inhibit IL-10 secretion. Thus, we tested the effect of various cytokines on IL-10

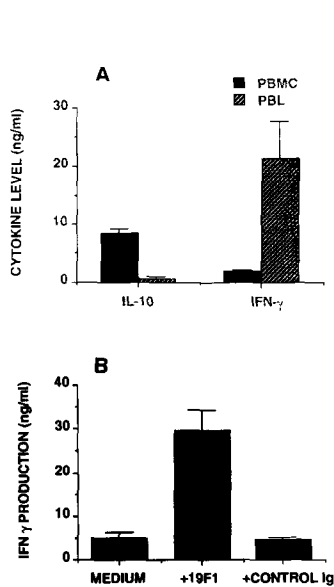


Figure 2. Endogenous IL-10 produced by monocytes inhibits IFN- γ synthesis. (A) PBMC and PBL activated by SAC particles were tested for their capacity to produce IL-10 and IFN- γ . Levels of IL-10 and IFN- γ were measured at day 10 by ELISA. The results are expressed in nanograms per milliliter as the mean \pm SEM of three independent experiments, each in triplicate. (B) PBMC were activated by SAC particles (1:200) in the absence or presence of purified anti-IL-10 mAb (19F1) and a control antibody, both used at a concentration of 1 μ g/ml. Levels of IFN- γ were measured at day 10 by ELISA. The results are expressed in nanograms per milliliter as the mean \pm SEM of three independent experiments, each in triplicate.

production by SAC-stimulated PBMC. As shown in Fig. 3 A, IFN γ was the only cytokine among the 10 tested able to significantly inhibit IL-10 synthesis. A 50% inhibition of IL-10 production was induced with 0.45 ± 0.28 ng/ml IFN- γ (mean of three independent experiments), and a maximal inhibition (80–90%) with ~ 5 –10 ng/ml (Fig. 3 B). The inhibitory effect of IFN- γ was completely reversed in the presence of anti-IFN- γ antibody (data not shown). At the single cell level, 10 ng/ml IFN- γ reduced by twofold the number of IL-10 spots generated by LPS-activated PBMC and monocytes (Fig. 3 C). Kinetic studies indicated that the secretion of IL-10 could be detected 9 h after SAC induction, reaching its maximum after 36 h. The inhibitory effect of IFN- γ was observed over the entire culture period (Fig. 3 D). Finally, whereas IFN- γ inhibited IL-10 production by PBMC and purified monocytes, it increased the production of TNF- α and IL-1 β , but only weakly affected the IL-6 production (Table 1).

Taken together, these data indicate that endogenous IL-10 has an inhibitory effect on endogenous IFN- γ synthesis and

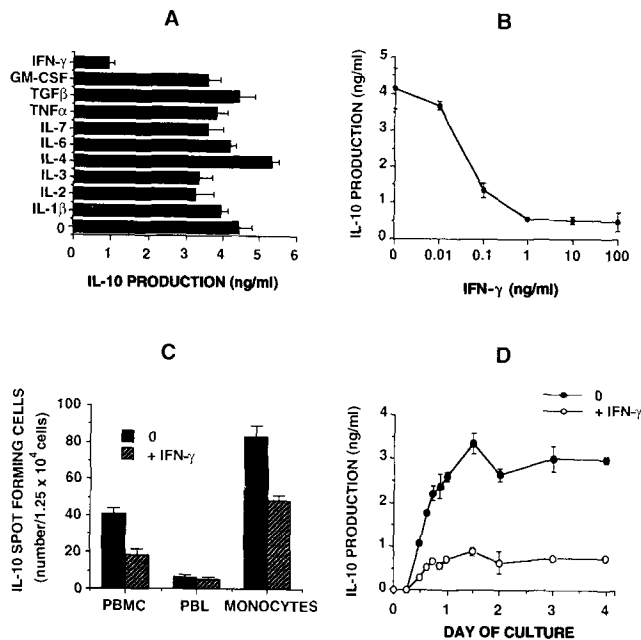


Figure 3. IFN- γ inhibits IL-10 production. (A) PBMC (2.5×10^5 cells/well) were activated by SAC particles in the absence or presence of purified recombinant cytokines, used at the following concentrations: rIL-1 β (0.5 ng/ml), rIL-2 (100 U/ml), rIL-3 (10 ng/ml), rIL-4 (10 ng/ml), rIL-6 (40 ng/ml), rIL-7 (10 ng/ml), rTNF- α (10 ng/ml), TGF- β (0.5 ng/ml), rGM-CSF (100 ng/ml), and IFN- γ (5 ng/ml). After 48 h, production of IL-10 was determined in the culture supernatants by IL-10 ELISA. Results are expressed in nanograms per milliliter as the mean \pm SEM of culture triplicates of three independent experiments, performed under identical conditions. (B) PBMC were stimulated in the absence or presence of increasing concentrations of IFN- γ . Results are expressed in nanograms per milliliter as the mean \pm SD of culture triplicates of one experiment representative of three. (C) The inhibitory effect of IFN- γ on IL-10 production was investigated at the single cell level using IL-10 ELISPOT assay. PBMC, monocytes, and PBL were activated by LPS (1 μ g/ml) for 48 h in the absence or the presence of IFN- γ (10 ng/ml). Results (spot number for 1.25×10^4 cells) are expressed as the mean \pm SEM of duplicate determinations of two independent experiments. (D) Monocytes (10^5 cells/well) purified by elutriation centrifugation from normal PBMC were stimulated with SAC particles in the absence or the presence of 5 ng/ml IFN- γ . Supernatants were collected at the indicated times and assayed for IL-10 levels. Results are expressed in nanograms per milliliter as the mean \pm SD of culture triplicates.

Table 1. Selective Effects of IFN- γ on Cytokine Production

	IFN- γ	IL-10 (n = 6)*	IL-6 (n = 5)	IL-1 β (n = 5)	TNF- α (n = 5)
PBMC	-	4.02 \pm 0.48	636 \pm 230	9.12 \pm 1.89	17.92 \pm 3.82
	+	1.01 \pm 0.26	576 \pm 182	21.86 \pm 3.93	36.37 \pm 3.51
Monocytes †	-	1.87 \pm 0.35	560 \pm 172	4.47 \pm 0.71	15.30 \pm 3.36
	+	0.72 \pm 0.13	935 \pm 250	12.30 \pm 3.23	40.16 \pm 2.36

Total PBMC (2.5×10^5 cells/well) and purified monocytes were incubated with SAC particles for 48 h in the absence or presence of IFN- γ (5 ng/ml). Levels of IL-10, IL-6, IL-1 β , and TNF- α were determined by specific ELISA. Results are expressed in nanograms per milliliter as the mean \pm SEM of culture triplicates.

* Number of experiments, each in triplicate.

† Monocytes were seeded at a cell density equivalent to the number of monocytes present in PBMC.

that exogenous IFN- γ inhibits specifically the production of IL-10 by monocytes.

Discussion

IL-10 was initially described as cytokine synthesis inhibitory factor (CSIF), a product of mouse CD4⁺ Th2 cells with regulatory effects on Th1 cells (2). The results presented in this report demonstrate the role of monocytes, within SAC- or LPS-activated human PBMC, in the production of IL-10. Levels of secreted IL-10 were in the nanogram range, a quantity that is \sim 100-fold lower than that of IL-6 but similar to that of IL-1 β . The ELISPOT assay indicated that only a small proportion of PBMC and monocytes produced IL-10. The frequency of IL-10-secreting cells was \sim 20-fold lower than that of IL-6-producing cells. Removal of monocytes from SAC-activated PBMC resulted in an \sim 16-fold decrease of IL-10 production and, conversely, in a 10-fold increase in IFN- γ production. Furthermore, addition of anti-IL-10 antibody also resulted in an increase of IFN- γ production. These observations are in line with the initial description of Th1 cell inhibition by Th2 cell-derived CSIF, but they suggest a balance occurring between T cells and monocytes rather than between T cell subsets (14). Note, however, that purified monocytes produced less IL-10 than PBMC containing an equivalent number of monocytes. This suggests a possible positive cooperation between monocytes and lymphocytes for the production of IL-10.

One of the most striking findings of the present study is the demonstration that exogenous IFN- γ inhibits the production of IL-10 by SAC-activated PBMC and monocytes. The inhibition of IL-10 secretion by monocytes appears to be specific of IFN- γ , as GM-CSF (15), TNF- α (16), and IL-1 (17), known stimulators of monokine production, or IL-4, a known inhibitor of monokine secretion (18), had no significant effect on IL-10 production. The IFN- γ -dependent inhibition of IL-10

production was obtained both at the culture supernatant level and at the single cell level. Furthermore, the downregulation of IL-10 synthesis by IFN- γ did not result from a nonspecific inhibition of protein synthesis, as it increased SAC-induced IL-1 β and TNF- α production by PBMC and purified monocytes while downregulating IL-10 production. Thus, IFN- γ acts as a monocyte-activating factor (for review see reference 19), not only by enhancing: (a) the release of oxygen radicals, (b) their tumoricidal activity, (c) their secretion of proinflammatory cytokines such as TNF- α , IL-1, and IL-6 (20), and (d) their MHC class II antigen expression and their antigen-presenting activity, but also by blocking the production of IL-10, which decreases all the functions of monocytes described above (5, 6, 21). The IFN- γ -dependent downregulation of IL-10 production by monocytes demonstrated here would support the speculation that the stimulatory effect of IFN- γ on these cells may be a consequence of a decreased production of endogenous IL-10. However, the stimulatory effect of IFN- γ on the early TNF- α and IL-1 β synthesis does not appear to be linked to the inhibition of IL-10 production, which occurs in the later stages of the culture. Thus, IFN- γ activates monocytes/macrophages in a direct as well as an indirect fashion by inhibiting the production of IL-10, which acts as a monocyte-deactivating factor (21).

An altered balance in the production of either IL-10 or IFN- γ will result in altered monocyte activation, including the secretion of monokines and the presentation of antigen and thus lymphocyte activation. For example, at the onset of autoimmune diseases, the role of IFN- γ has been suggested for the increased MHC class II expression and proinflammatory cytokine production (22). In this situation, the inhibitory effect of IFN- γ on IL-10 production would reduce the antiinflammatory properties of IL-10, leading to disease perpetuation. In conclusion, the present study demonstrated that IFN- γ and IL-10 antagonize each other's production and function.

We thank Dr. J. Chiller and Pr. J. P. Revillard for reading the manuscript, Drs. J. Abrams and J. Wijdenes for providing mAbs, and N. Courbière and M. Vatan for manuscript preparation.

P. Chomarot was supported by the Fondation Mérieux.

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Received for publication 17 August 1992 and in revised form 28 October 1992.

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