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Research Article

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Interleukin-18 (IFNγ-inducing Factor) Induces IL-8 and IL-1β via TNFα Production from Non-CD14$^+$ Human Blood Mononuclear Cells

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Abstract

IL-18 is synthesized as a precursor molecule without a signal peptide but requires the IL-1β converting enzyme (ICE, caspase-1) for cleavage into a mature peptide. Human precursor IL-18 was expressed, purified, and cleaved by ICE into a 18-kD mature form. Mature IL-18 induced IL-8, macrophage inflammatory protein-1α, and monocyte chemotactic protein-1 in human peripheral blood mononuclear cells in the absence of any co-stimuli. Blocking IL-1 with IL-1 receptor antagonist resulted in a 50% reduction in IL-8. Neutralization of TNF with TNF binding protein resulted in a 66% reduction in IL-1β, an 80% reduction of IL-8, and an 88% reduction in mean TNFα mRNA. In purified CD14$^+$ cells but not CD3$^+$/CD4$^+$, IL-18 induced gene expression and synthesis of IL-8 and IL-1β. TNFα production was induced in the non-CD14$^+$ population and there was no induction of TNFβ by IL-18. In purified natural killer cells, IL-18 induced IL-8 that was also inhibited by TNF binding protein. IL-18 did not induce antiinflammatory cytokines, IL-1Ra, or IL-10, although IL-18 induction of TNFα was inhibited by IL-10. In the presence of IFNγ, IL-18–induced TNFα was enhanced and there was an increase in the mature form of IL-1β. We conclude that IL-18 possesses proinflammatory properties by direct stimulation of gene expression and synthesis of TNFα from CD3$^+$/CD4$^+$ and natural killer cells with subsequent production of IL-1β and IL-8 from the CD14$^+$ population. (*J. Clin. Invest.* 1998. 101:711–721.) Key words: inflammation • cytokines • T cells • chemokines • proteases

Introduction

IL-1β and TNFα require proteolytic enzymes for cleavage and release of their mature, active molecules (for review see reference 1). For IL-1β, a specific intracellular cysteine protease called the IL-1 converting enzyme (ICE)[1] cuts the precursor of IL-1β into an active mature form (2, 3). Specific inhibitors of ICE administered to mice reduce disease severity as effectively as does blocking IL-1 activity using specific antagonists (4), and ICE-deficient mice also exhibit decreased local and systemic inflammation after a generalized challenge (5–7). The increasing number of ICE-like intracellular proteases has been renamed caspase family to indicate cysteine proteases cleaving after an aspartic acid residue (8). Caspase-1 denotes the original ICE (these terms are often used interchangeably) and has the greatest specificity for cleaving proIL-1β. IL-18, formerly termed interferon-γ–inducing factor (9) is a new proinflammatory cytokine, structurally related to IL-1β (10).

The existence of a novel IFNγ-inducing cytokine was reported in 1995 in mice preconditioned with *Propionibacterium acnes* (9). In this model, the hepatic macrophage population (Kupffer cells) expands and mice become unusually susceptible to low doses of LPS, which in nonpreconditioned mice are not lethal. These results were consistent with the importance of IFNγ as a mediator of LPS lethality. For example, neutralizing anti-IFNγ antibodies protected mice against Shwartzman-like shock (11) and galactosamine-treated mice deficient in the IFNγ receptor were resistant to LPS-induced death (12). Anti-murine IL-18 antibodies also protect mice against severe hepatic cytotoxicity and purified IL-18 augments Fas ligand–mediated cytotoxicity of natural killer (NK) cells (13).

Murine IL-18 was purified and the NH_2-terminal amino acids used to clone the cytokine (14); the human cDNA sequence was reported subsequently (15). Recombinant human IL-18 was without direct IFNγ-inducing ability but acted as a co-stimulant for production of IFNγ and other Th1 cytokines independent of IL-12 (14). In addition, IL-18 together with IL-12 facilitates T lymphocyte activation and selection in *P. acnes*–primed mice (16). To date, IL-18 is thought to be primarily a costimulant for Th1 cytokine production and activity and to account for the role of IFNγ in LPS-mediated toxicity in preconditioned mice. Is there a broader role for IL-18 as a proinflammatory cytokine?

Similar to the IL-1β precursor, precursor IL-18 (proIL-18) lacks a signal peptide (14) and requires ICE (caspase-1) for cleavage and secretion and hence for biological activity (17, 18). The NH_2-terminal amino acid sequence of the secreted form of murine IL-18 (14) was consistent with cleavage after an aspartic acid residue, a typical cleavage site for ICE. It was clearly demonstrated that ICE (caspase-1) cleaved proIL-18

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1. *Abbreviations used in this paper:* ABS, AB serum; ECL, electrochemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICE, IL-1β converting enzyme; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; NK, natural killer; RT-PCR, reverse transcriptase–PCR; TNFbp, TNF-binding protein.

(after the aspartic acid 35) and resulted in a mature and active protein (17, 18) Relevant for inflammation is the fact that other caspases and particularly those cleaving intracellular proteins involved in apoptosis either did not cleave the proIL-18 or required a 100-fold greater concentration of enzyme compared with ICE (18).

In addition, a computer generated analysis of murine IL-18 predicted similarities to IL-1β in that the three dimensional structure of both cytokines are all β-pleated sheets forming a barrel configuration (10). This analysis prompted some investigators to name the IFNγ-inducing factor as IL-1γ but since IL-18 does not signal through the IL-1R type I, this name is not appropriate. Nevertheless, the similarities of IL-18 to IL-1 have some relevance since a functional component of the IL-18 receptor complex is the IL-1 receptor related protein (19), a member of the IL-1 receptor family (20). In this study, we have examined IL-18 as a direct-acting cytokine capable of initiating a cascade of proinflammatory cytokines.

Methods

Reagents and cytokines. Histopaque was purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640 culture medium (GIBCO BRL, Gaithersburg, MD) was supplemented with 10 mM L-glutamine, 24 mM $NaHCO_3$, 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). The endotoxin concentration as determined by the Limulus Amebocyte Lysate test (Associates of Cape Cod, Inc., Falmouth, MA) was below 10 pg/ml. Human AB serum (ABS) was obtained aseptically from volunteers and heat-inactivated for 1 h at 56°C. Recombinant human IL-10 was provided by Schering-Plough Research Institute (Kenilworth, NJ). Recombinant human TNF-binding protein (TNFbp) was a kind gift of Dr. Carl Edwards (Amgen, Boulder, CO). Recombinant human IL-1 receptor antagonist (IL-1Ra) was a kind gift of Dr. Daniel Tracey (Upjohn, Kalamazoo, MI). IFNγ was a kind gift of Dr. Michael Palladino (Genentech, Inc., South San Francisco, CA). PHA was purchased from Sigma Chemical Co. Recombinant human IL-18 was expressed in *Escherichia coli* as the 24-kD precursor molecule (proIL-18) (15) with histidine residues at the NH_2 terminus of the precursor. After purification using Ni affinity chromatography, proIL-18 was cleaved by recombinant ICE (18). The 18-kD mature IL-18 was then purified to homogeneity and the NH_2-terminal at tyrosine 37 confirmed by amino acids sequencing (15).

Isolation of PBMC. These studies were approved by the Combined Colorado Investigational Review Board. All subjects gave consent. Platelet-depleted residual leukocytes obtained from healthy human volunteers were applied to Histopaque gradients (Sigma Chemical Co.) to obtain PBMC. The cells were aspirated from the interface, washed three times in pyrogen-free saline (Abbott Laboratories, North Chicago, IL), and resuspended at 2.5×10^6 per ml in RPMI 1640 containing 10 mM Hepes, 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% heat inactivated human ABS. The cells were cultured in flat-bottomed 24-well plates (Falcon Labware, Cockeysville, MD) with IL-18 (0.625-10 nM; Vertex Pharmaceuticals Inc., Cambridge, MA, or PeproTech, Inc., Princeton, NJ) in the presence or absence of the following: IL-1Ra (10 μg/ml), TNFbp, (soluble TNFRp55, 10 μg/ml), PHA (5 μg/ml, Sigma Chemical Co.), IFNγ (500 U/ml), IL-10 (5 ng/ml), LPS (100 ng/ml, Sigma Chemical Co.). Polymyxin B (1 μg/ml; Pfizer, New York, NY) was added to the IL-18 in certain experiments in order to ensure that the results were not due to endotoxin contamination either from the IL-18 preparation or from the RPMI 1640 culture medium. Cells were stimulated for 12–48 h. Similarly, for the detection of mRNA, incubation periods varied between 2 and 20 h. Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Isolation of $CD14^+$ and $CD3^+/CD4^+$. PBMC isolated from the Histopaque gradients were resuspended at 10^8 cells in 800 μl of PBS containing 2.5 mM EDTA and 0.5% BSA (Miltenyi buffer; Miltenyi Biotec, Auburn, CA) and incubated with 200 μl MACS CD14 Microbeads (Miltenyi Biotec) for 15 min at 8°C. The cells were washed in PBS, resuspended in Miltenyi buffer (2 ml) and applied to VS^+ positive selection columns (Miltenyi Biotec) in the presence of a magnetic field (VarioMACS separator; Miltenyi Biotec). The CD14 negative cells that passed through the column were collected as well as cells eluting during two washes of buffer (total volume of 12 ml). These cells were mostly the $CD3^+/CD4^+$ enriched fraction (see below). The column was removed from the magnetic separator and flushed with two 10 ml washes of Miltenyi buffer to obtain the $CD14^+$ cells. The purity of the two fractions was determined by FCM (Coulter Epics-XL; Coulter Immunology, Hialeah, FL) using the following antibodies: PE-anti-CD14 (Immunotech, Inc., Westbrook, ME), FITC-anti-CD3-RD-anti-CD4 (Coulter Immunology), and the Simultest (Becton Dickinson, Mountain View, CA) for NK cells (anti-CD3/CD16CD56). The cells were resuspended at 2.5×10^6 cells per milliliter in RPMI 1640 and cultured as described above.

Isolation of NK cells. PBMC (2×10^8) were resuspended in 800 μl of Miltenyi buffer and a cocktail of hapten-conjugated monoclonal antibodies (200 μl) against CD3, CD4, CD19, and CD33 (Miltenyi Biotec). The cells were incubated with these antibodies for 20 min at 8°C, washed in Miltenyi buffer, resuspended in 800 μl of buffer and incubated for 20 min at 8°C with 200 μl of colloidal supermagnetic MACS MicroBeads conjugated to an antihapten antibody. After incubation with the microbeads, the cells were washed and 2 ml cell suspension applied to a depletion column (CS; Miltenyi Biotec) in the presence of a magnetic field (VarioMacs; Miltenyi Biotec). The effluent and two rinses (12 ml) containing the enriched NK cell fraction was collected and the purity assessed as described for the $CD14^+$-$CD3^+/CD4^+$ cells. The cells were resuspended at a cell concentration of 2.5×10^6 cells per milliliter in RPMI 1640.

Cytokine assays. For the measurement of IL-8, an electrochemiluminescence (ECL) method was developed. Briefly, a purified mouse anti–human IL-8 antibody (R & D Systems, Inc., Minneapolis, MN) was labeled with biotin (Igen Inc., Gaithersburg, MD) and a goat anti–human IL-8 antibody (R & D Systems, Inc.) was labeled with ruthenium (Igen Inc.) according to the manufacturer's instructions. The biotinylated antibody was diluted to a final concentration 1 μg/ml in PBS, pH 7.4, containing 0.25% BSA, 0.5% Tween-20, and 0.01% azide, (ECL buffer). Per assay tube, 25 μl of the biotinylated anti–IL-8 was incubated at room temperature with 25 μl of a 1 mg/ml solution of streptavidin-coated paramagnetic beads (Dynal Corp., Lake Success, NY) for 30 min by vigorous shaking. Samples (25 μl) that had been diluted in RPMI 1640 containing 1% ABS or standards were added to tubes followed by 25 μl of ruthenylated antibody (final concentration 1 μg/ml, diluted in ECL buffer). The tubes were then shaken for an additional 2 h. The reaction was quenched by the addition of 200 μl per tube of PBS and the amount of chemiluminescence determined using an Origen Analyzer (Igen Inc.). Human TNFα and IL-1Ra specific ECL assays were developed using the same methods described above. Polyclonal and monoclonal antibodies used for biotinylation and ruthenium tagging of anti-TNFα and anti–IL-1Ra were obtained from R & D Systems Inc. For the detection of the IL-1β precursor in cell lysates, a specific ELISA for the IL-1β precursor was used (kindly provided by Cistron Biotechnology, Pine Brook, NJ). The IL-1β precursor ELISA detects less than 10% of mature IL-1β. A specific ELISA for mature IL-1β (Cistron Biotechnology) was used to measure the concentration of mature IL-1β in the supernatants. This ELISA detects less than 10% of the IL-1β precursor. Human MIP-1α, human IFNγ, human monocyte chemotactic protein-1 (MCP-1), human IL-10 and human TNFβ (lymphotoxin) were measured in cell supernatants or lysates using specific ELISA kits from R & D Systems, Inc.

Reverse transcriptase–PCR (RT-PCR). Total RNA was isolated from PBMC using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH). Briefly, cells were lysed in Tri-reagent and the RNA

was sequentially isolated following chloroform extraction and isopropanol precipitation. The RNA was dissolved in DEPC-treated water and quantitated (GeneQuant, Cambridge, United Kingdom). To prepare cDNA, 0.5 μg of total RNA was reverse transcribed using random hexamer primers as templates. The reaction took place in a total volume of 20 μl containing the following components (purchased from Perkin-Elmer Corp., Branchburg, NJ) at the final concentrations indicated: 5 mM $MgCl_2$, PCR buffer (1 ×, 50 mM KCl and 10 mM Tris-HCl, pH 8.3), 1 mM each of dNTPs, 2.5 μM random hexamer primers, 20 U RNAse inhibitor, and 50 U of MuLV reverse transcriptase. The reaction was incubated at 42°C for 30 min and terminated by 95°C for 5 min. For PCR, 2–4 μl of RT product was used in a total volume of 50 μl containing the following components (Perkin-Elmer Corp., final concentrations): 1.7 mM $MgCl_2$, PCR buffer (1 ×, 50 mM KCl and 10 mM Tris-HCl, pH 8.3) 0.2 mM each of dNTPs, 1 U of Ampli*Taq* polymerase and forward (F) and reverse (R) primers (20 μM each). The following sequence was performed on a thermocycler (Perkin-Elmer Corp.) for each PCR reaction: 90°C for 5 min and 60°C for 5 min (1 cycle), followed immediately by 72°C for 1 min, 90°C for 1 min and 60°C for 1 min (with variable number of cycles) and a final extension phase at 72°C for 10 min. The variable number of cycles was to ensure that amplification occurred in the linear phase and that differences between control and experimental conditions were maintained by adopting a limited number of cycles. To insure that differences between tubes were not the result of unequal concentrations of RNA, PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control were performed on each sample. The sequences and cycle number are indicated as follows (sequences obtained from Clontech, Palo Alto, CA): IL-8 (F): 5′ATGACTTCCAAGCTGGCCGTGGCT3′; IL-8 (R): 5′TCTCAGCCCTCTTCAAAACTTCTC3′ (24 cycles); IL-1β (F): 5′ATGGCAGAAGTACCTAAGCTCGC3′; IL-1β (R): 5′ACACAAATTGCATGGTGAAGTCAGTT3′; (24 cycles); TNFα (F): 5′GAGTGACAAGCCTGTAGCCCATGTTGTAGCA3′; TNFα (R): 5′GCAATGATCCCAAAGTAGACCTGCCCAGAC3′ (23 cycles); GAPDH (F): 5′ACCACAGTCCATGCCATCAC3′; GAPDH (R): 5′TCCACCACCCTGTTGCTGTA3′ (22 cycles).

The PCR products were separated on a 2% agarose gel containing 0.5 × TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.3) with 0.5 μg/ml ethidium bromide, visualized by UV illumination and photographed. The predicted sizes of the PCR products were 289 bp, 802 bp, 444 bp, and 452 bp for IL-8, IL-1β, TNFα, and GAPDH, respectively. Densitometry was performed on the negative image (ImageQuant software; Molecular Dynamics, Sunnyvale, CA) and the relative absorbances of the cytokine PCR products were corrected against the absorbances obtained for GAPDH.

Statistical analyses. Data are expressed as the mean±SEM. Group means were compared by analysis of variance (ANOVA) using Fisher's least significant difference.

Results

Induction of IL-8. IL-18 (concentration range of 0.625–10 nM) induced production of IL-8 in unfractionated human PBMC in the absence of a co-stimulus (Fig. 1). At a concentration of 2.5 nM, the fold increase was 5.2, $P < 0.05$ compared with unstimulated PBMC. The effect of IL-18 on IL-8 synthesis was not the result of endotoxin contamination as assessed by pre- or co-incubation of IL-18 with polymyxin B (2.5 μg/ml) (data not shown). In the same donors, IL-1β at 10 ng/ml induced 198±95 ng/ml of IL-8. The stimulatory effects of IL-18 on PBMC did not extend to the oxidative burst using oxidation of Cytochrome-C compared with incubation with *Candida albicans* in these same donors (data not shown).

In order to define the specific cell type(s) participating in the synthesis of IL-8, cell separation procedures were performed using magnetic microbead selection using anti-CD14 antibodies. Using three different donors, the average purity of the $CD14^+$ fraction (monocytes) as determined by cell sorting was 85±5%. In these preparations of $CD14^+$ monocytes, $CD3^+/CD4^+$ cells accounted for 7–15%. However, the $CD3^+/CD4^+$ enriched fraction did not contain $CD14^+$ cells (< 0.5%). As shown in Fig. 1, using these $CD14^+$ and non-$CD14^+$ cells preparations, IL-18–induced IL-8 production was primarily from $CD14^+$ cells. However, on a per cell basis, the amount of IL-8 produced in the $CD14^+$ cell population was 66% less than that from unfractionated PBMC. This suggested that a non-

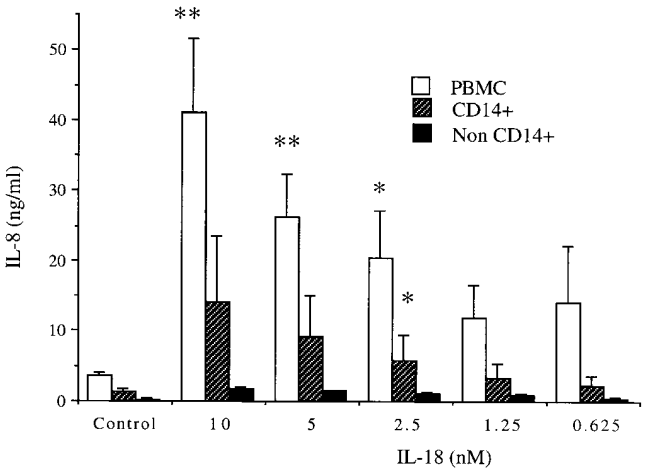

Figure 1. IL-18 induction of IL-8. Unfractionated PBMC and $CD14^+$ and non-$CD14^+$ cells obtained from the same donors. Cells were stimulated with IL-18 at various concentrations indicated under the horizontal axis. Cell supernatants were collected after 20 h and assayed for IL-8. The cell concentration of each fraction was adjusted to 2.5×10^6 cells per milliliter. The data represent the mean±SEM, $n = 3$. *$P < 0.05$; **$P < 0.01$ compared with control for each cell type.

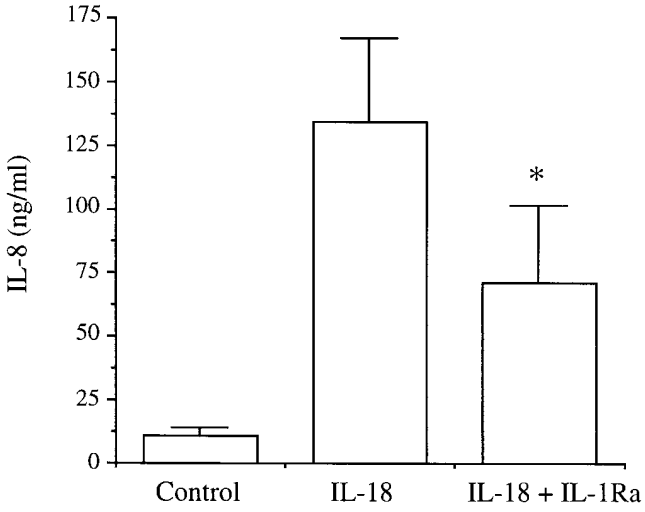

Figure 2. IL-1Ra inhibition of IL-18–induced IL-8. Unfractionated PBMC were stimulated with IL-18 (10 nM) in the presence or absence of IL-1Ra (10 μg/ml) for 20 h. The cell supernatants were collected and assayed for IL-8. The data represent the mean±SEM, $n = 5$. *$P < 0.004$, difference with or without IL-1Ra.

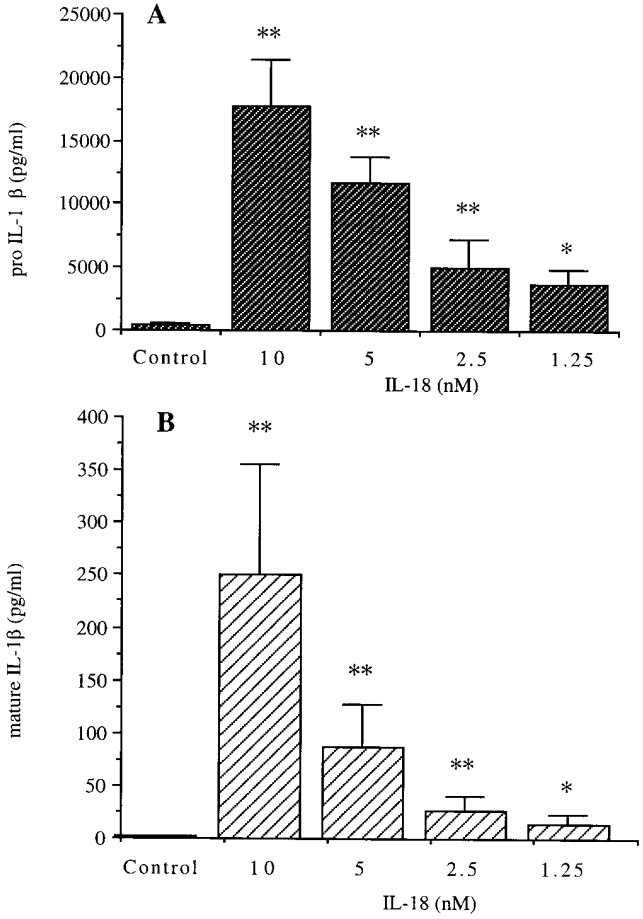

Figure 3. IL-18 induces proIL-1β and mature IL-1β. (*A*) Unfractionated PBMC were stimulated with IL-18 as indicated for 20 h. After the incubation, the cell supernatants were removed and replaced with the same volume of fresh medium. The cells were subjected to three freeze–thaw cycles and then assayed for proIL-1β by specific ELISA (see Methods). (*B*) The mature form of IL-1β was detected in cell supernatants by a specific ELISA. The data represent the mean±SEM, $n = 4$. *$P < 0.04$; **$P < 0.01$ compared with control.

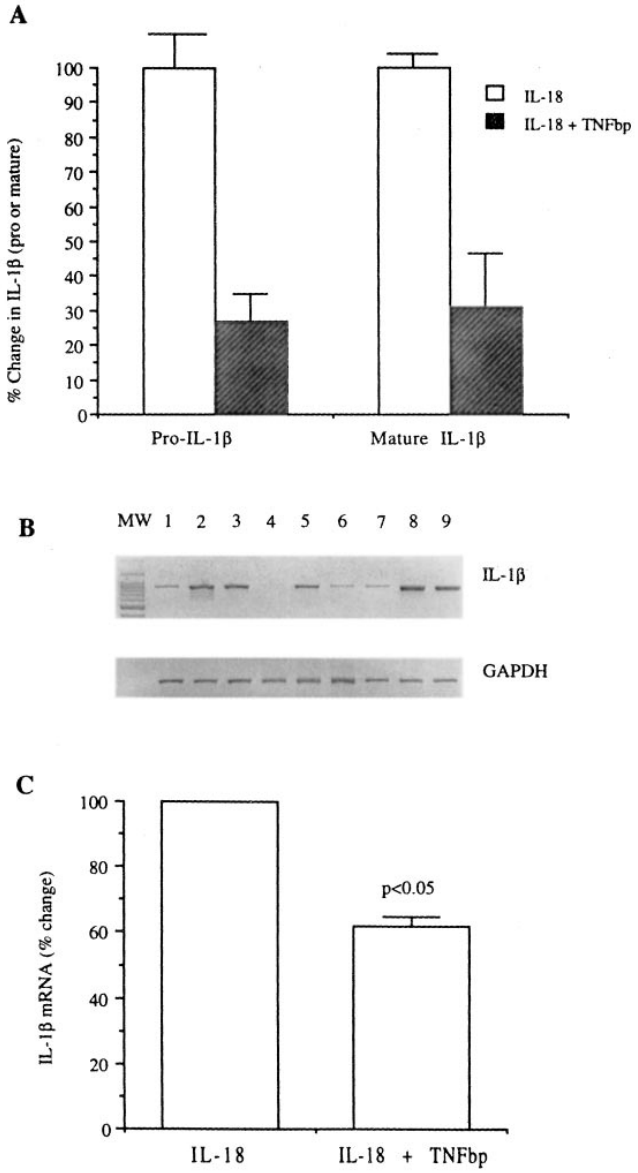

Figure 4. TNFbp inhibition of IL-18 induced IL-1β. (*A*) Unfractionated PBMC were incubated with IL-18 (10 nM) in the presence or absence of TNFbp (10 μg/ml). ProIL-1β was assayed in the cell lysates and mature IL-1β in the cell supernatants as described in Fig. 3. The reduction in IL-1β by TNFbp was significant in each case ($P < 0.05$, $n = 3$). (*B*) Effect of TNFbp (10 μg/ml) on steady state IL-1β mRNA after a 20-h incubation with IL-18 as determined by RT-PCR. Results of three donors are presented. *MW*, 100-bp molecular marker ladder; lanes *1*, *4*, and *7* are unstimulated cells from each of three donors, respectively; lanes *2*, *5*, and *8* are IL-18 (10 nM) stimulated cells from each donor; lanes *3*, *6*, and *9* are IL-18–stimulated cells in the presence of TNFbp (10 μg/ml). GAPDH served as the internal control. (*C*) Quantification of the PCR products as determined by densitometry for the three donors in *B*. IL-18–stimulated cells were set at 100%. Results represent the mean±SEM percent change in the presence of TNFbp shown in *B*.

$CD14^+$ cell in PBMC was contributing to the total production of IL-18–induced IL-8.

IL-18 induction of IL-8 requires IL-1. During a 24-h culture, the elaboration of cytokines in response to any initial stimulus can be influenced by the production of other cytokines in the culture. For example, using endotoxin stimulated PBMC, over 50% of the 24-h production of IL-8 is IL-1 mediated (21). We explored the possibility that the upstream cytokines, namely, IL-1β or TNFα, may influence IL-18 induction of IL-8. PBMC stimulated with IL-18 in the presence of IL-1Ra showed a significant decrease (∼ 50%) in IL-8 release 24 h after stimulation (Fig. 2). At lower concentrations of IL-18 (and hence lower levels of stimulation), this effect of IL-1Ra did not reach statistical significance. Next, we investigated the ability of IL-18 to induce IL-1β (both the mature and precursor forms) based on the above observed inhibition by IL-1Ra. IL-18 (concentration range: 0.625–5 nM) stimulated the release of mature IL-1β into the cell supernatants (Fig. 3 *B*). There was a corresponding concentration-dependent increase in the synthesis of the IL-1β precursor detected in cell lysates after 24 h of stimulation with IL-18 (Fig. 3 *A*).

Induction of IL-1β is TNF dependent. Since TNFα can induce IL-1 (22), experiments were performed in the presence or absence of the p55 TNF soluble receptor (TNFbp). In unfrac-

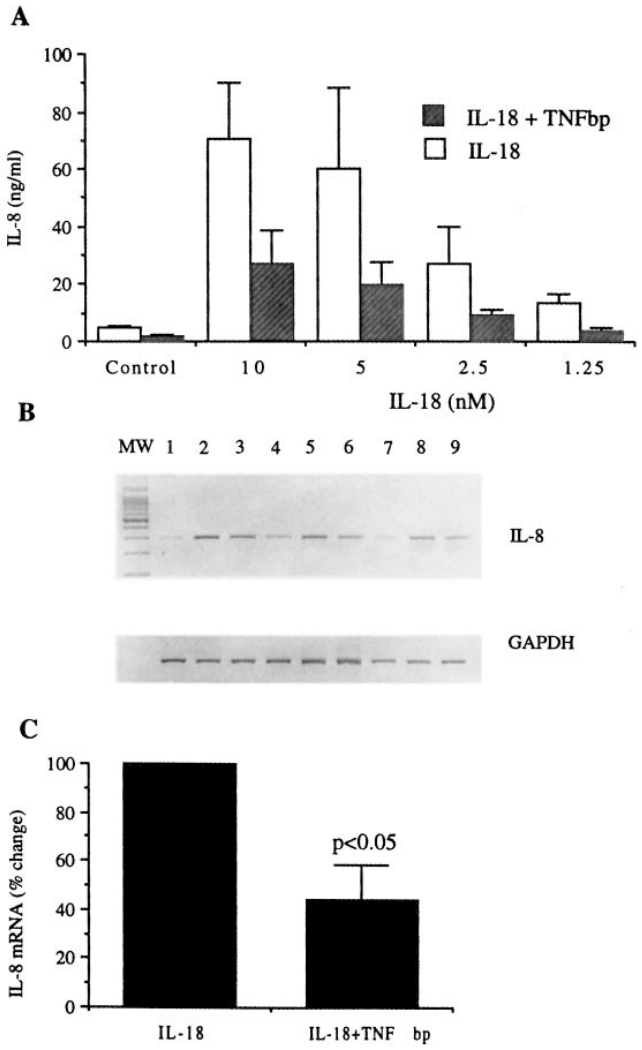

Figure 5. TNFbp inhibits IL-18 induction of IL-8. (*A*) Unfractionated PBMC were incubated with IL-18 at the indicated concentrations in the presence or absence of TNFbp (10 μg/ml). IL-8 was measured in the cell supernatants. The data represent the mean±SEM, $n = 4$. The reduction in IL-18 by TNFbp was significant at each concentration tested compared with IL-18 only ($P < 0.05$). (*B*) Effect of TNFbp (10 μg/ml) on steady state IL-8 mRNA after a 20-h incubation as determined by RT-PCR. Results of three donors are presented. *MW*, 100-bp molecular marker ladder; lanes *1*, *4*, and *7* are unstimulated cells from each of three donors, respectively; lanes *2*, *5*, and *8* are IL-18 (10 nM)–stimulated cells from each donor; lanes *3*, *6*, and *9* are IL-18–stimulated cells in the presence of TNFbp (10 μg/ml). GAPDH served as the internal control. (*C*) Quantification of the PCR products as determined by densitometry for the three donors shown in *B*. IL-18–stimulated cells were set at 100%. Results represent the mean±SEM percent change in the presence of TNFbp shown in *B*.

tionated PBMC cultures, the presence of TNFbp significantly inhibited (73%) the release of mature IL-1β by IL-18 (Fig. 4 *A*). The neutralization of TNFα activity was also observed on the synthesis of the IL-1β precursor in response to IL-18 stimulation (Fig. 4 *A*). As shown in Fig. 4 *B*, IL-18 increased the steady state levels of IL-1β mRNA (sevenfold increase above basal levels) and this was reduced when the cells were cultured in the presence of IL-18 plus TNFbp. In Fig. 4 *C*, the mean reductions in IL-18–induced IL-1β mRNA levels are shown.

Induction of IL-8 is TNF dependent. Since TNFbp reduces IL-18–induced IL-1β, we studied the effect of TNF neutralization on IL-18 induction of IL-8 to determine whether a similar control mechanism existed. PBMC were stimulated with increasing concentrations of IL-18 in the presence of TNFbp and IL-8 production was measured after 24 h of incubation. As shown in Fig. 5 *A*, TNFbp significantly inhibited the concentration-dependent induction of IL-8 by IL-18. In PBMC stimulated with 1.25 nM IL-18, the presence of TNFbp reduced IL-8 production to the level in unstimulated control cultures. The inhibitory effect of TNFbp on IL-18 action extends to the β chemokine MCP-1. IL-18 alone (10 nM) induced 10.6±0.27 ng/ml compared with < 200 pg/ml in unstimulated PBMC ($n = 3$). In the presence of TNFbp (10 μg/ml), MCP-1 was reduced to 2.3±0.6 ng/ml.

As shown in Fig. 5 *B*, IL-18 increased steady state levels of IL-8 mRNA (fourfold above basal levels) as determined by RT-PCR. In each of the three donors' cells, the presence of TNFbp reduced mRNA levels. The mean reduction in IL-8 mRNA levels was nearly 60% (Fig. 5 *C*).

IL-18 induces TNFα in the CD3⁺/CD4⁺–enriched subset. To date, the biological properties of IL-18 have been attributed to being primarily a helper molecule in the induction of IFNγ and other cytokines from T lymphocytes. In this study, we observed the induction of IL-8 by IL-18 in the absence of other stimuli. Although others have reported the production of IL-8 from $CD8^+$ T cells (23), IL-18 did not induce IL-8 from the $CD14^+$–depleted population. The primary cellular source of the IL-8 was the $CD14^+$ cells, not the $CD3^+/CD4^+$ cells (Fig. 1). In contrast, as shown in Table I, the cellular source of IL-18–induced TNFα was the non-$CD14^+$ population. Therefore, IL-18–induced IL-8 in PBMC is from $CD14^+$ cells but through the intermediate production of TNFα (and/or a member of the TNF family) from non-$CD14^+$ cells. We tested IL-18–stimulated PBMC for the induction of TNFβ (lymphotoxin). At 5 nM IL-18, in the absence of another stimulant, there was no TNFβ present in either the supernatants or the lysates. For positive controls in PBMC cultures from these donors, PHA induced 1796±311.6 pg of TNFβ per milliliter, $n = 3$.

We also sought to identify the cell source of IL-1β in the PBMC population. As shown in Fig. 6, *A* and *B*, the $CD14^+$ cell population was the primary source of IL-1β in IL-18 stimulated cultures. However, we cannot rule out that the 7–15% contamination of the $CD14^+$ population with $CD3^+$ cells was providing a source of IL-18–induced TNF in these cultures.

Effects of IL-18 on purified NK cells. NK cells were purified as described in Methods and stimulated with IL-18. As

Table I. TNFα Production in PBMC and Purified Cell Subsets Stimulated with IL-18

Experiment	PBMC	$CD14^+$	Non-$CD14^{+*}$
Donor 1‡	32334	244	3029
Donor 2‡	3838	403	2434
Donor 2§	4215	1704	2607

*> 90% enriched $CD3^+/CD4^+$ as assessed by FACSCAN; ‡IL-18 at 5 nM; §IL-18 at 10 nM; ‖TNFα concentration in pg/2.5 × 10^6 cells per milliliter.

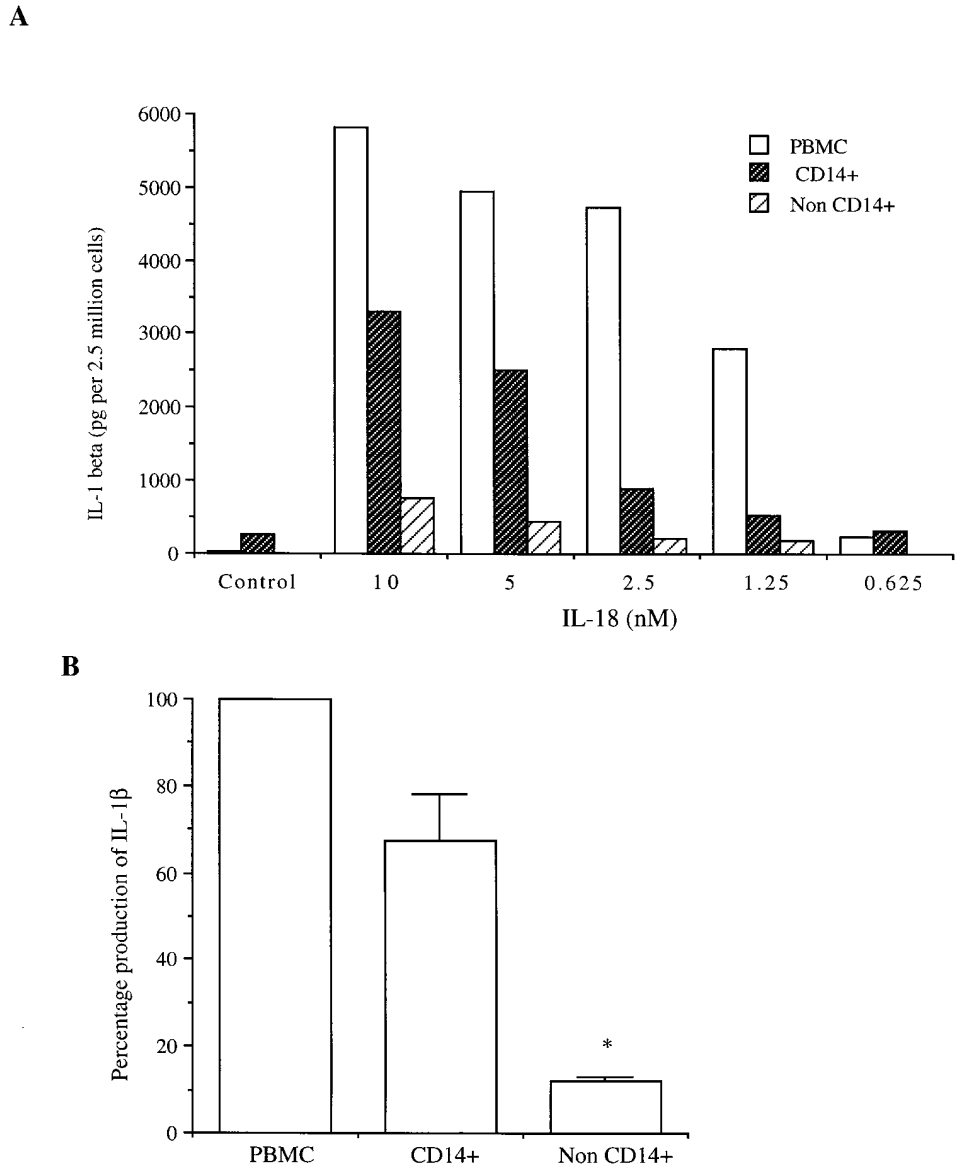

Figure 6. IL-18 induction of IL-1β in $CD14^+$ cells. (*A*) Unfractionated PBMC, $CD14^+$, and non-$CD14^+$ cells obtained by microbead antibody selection procedures (see Methods) were incubated with IL-18 at the indicated concentrations for 20 h. Mature IL-1β was assayed in cell supernatants by specific ELISA. The results of a single donor are shown. (*B*) Unfractionated PBMC, $CD14^+$, and non-$CD14^+$ cells were stimulated with IL-18 (10 nM) and after 20 hours, mature IL-1β was assayed in cell supernatants by specific ELISA. Results represent the mean±SEM in three donors. The levels of mature IL-1β were set at 100% in PBMC stimulated with IL-18. $*P < 0.05$.

previously demonstrated, NK cells produce various cytokines when incubated with other cytokines. For example, NK cells stimulated with IL-2 produce IL-8 (24, 25) As shown in Table II, the NK cell fractions obtained in three experiments were relatively pure with minimal contamination by either $CD3^+$/$CD4^+$ or $CD14^+$ cells. These NK cell preparations, when stimulated with IL-18, produced large amounts of IL-8 ranging from 22- to 153-fold over IL-8 production from unstimulated NK cells. In addition, the presence of TNFbp (10 μg/ml) added to these cultures attenuated the production of IL-8 induced by IL-18, ranging 6- to 43-fold less than cultures stimulated with IL-18 only.

*Table II. IL-8 Production from Purified NK Cells Stimulated with IL-18**

Experiment	NK‡	CD3/CD4‡	CD14‡	IL-8§	+ TNFbp‖¶
Donor 1	97.8	0.3	0.6	10	6.4
Donor 2	92.7	1.2	0.2	171	35
Donor 3	97.6	0.3	1.6	88	43

*IL-18 at 10 nM; ‡percent NK, CD3/CD4, or CD14 positive cells by FACS; §IL-8 fold increase over unstimulated cells; ‖TNFbp added to cultures at 10 μg/ml; ¶fold decrease compared to IL-18 alone.

Effects of IFNγ on IL-18 activity. We confirmed that IL-18 induction of IFNγ requires the presence of a co-stimulus such as the T cell mitogen PHA. As shown in Fig. 7, the IL-18 preparation used in these studies did not itself induce IFNγ but markedly enhanced IFNγ from PHA primed PBMC. In the presence of LPS (100 ng/ml), the addition of IL-18 enhanced IFNγ production four- and fivefold over that induced by LPS in PBMC from two donors, respectively. Next, we investigated the influence of exogenously added IFNγ to cells stimulated with IL-18. For example, presence of exogenously added IFNγ enhances endotoxin-induced cytokine production (26) but abrogates IL-1–induced IL-1 in these same cultures (27). As shown in Fig. 8 *A*, IL-18 induction of TNFα was enhanced by

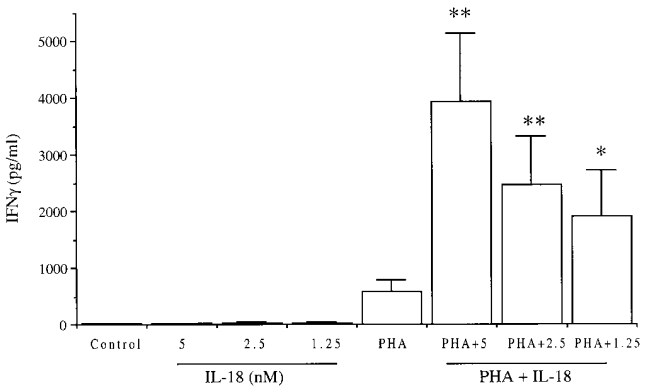

Figure 7. IL-18 co-induction of IFNγ production. Unfractionated PBMC were incubated with IL-18 at the indicated concentrations in the presence or absence of PHA (10 μg/ml) for 48 h. IFNγ was detected in cell supernatants by ELISA. The data represent the mean±SEM, $n = 4$. $^{**}P < 0.02$, $^{*}P < 0.05$ compared with PHA for each concentration of IL-18.

exogenous IFNγ. Although there was a small decrease in the production of the IL-1β precursor in the presence of IFNγ, this was accompanied by an increase of the mature form of IL-1β in cell supernatants of IL-18-treated cells (Fig. 8, *B* and *C*). This observation may be a direct effect of IFNγ but it is also consistent with the ability of IFNγ to increase ICE gene expression and activity as well as increase IL-1β processing (28, 29). Since MIP-1α is an important chemokine for T cell–mediated autoimmune disease (30), we examined the effect of IFNγ on IL-18 induction of MIP-1α. IFNγ did not affect IL-18 induction of the chemokine MIP-1α (Fig. 8 *D*).

Effect of TNF blockade on TNFα mRNA. There was a twofold increase above basal levels of steady state TNFα mRNA induced by IL-18. As shown in Fig. 9, *A* and *B*, TNFbp significantly attenuated (83% reduction) IL-18 induction of TNFα mRNA in PBMC. These findings are consistent with the ability of exogenous TNF to induce TNFα (31). However, in this study, the initiating stimulus is IL-18 induction of endogenous TNF; hence, by blocking the TNF activity produced in the PBMC cultures with TNFbp, any further induction of TNF is curtailed.

IL-1Ra and IL-10 induction. The argument for measuring IL-1Ra and IL-10 in response to IL-18 was based on the observation that when the endogenous activity of these antiinflammatory cytokines is specifically neutralized in vivo, disease worsens (32, 33) or the spontaneous in vitro production of IL-1 and TNFα is increased (34). TNFα is a potent stimulus of IL-1Ra production (35). Therefore, PBMC were stimulated in the absence of human serum (36) and after 24 h, a small, concentration-dependent increase in IL-1Ra production was observed (maximal increase of 55% over control, $n = 4$). Although the increase was statistically significant ($P < 0.05$), the amount (less than twice background) of IL-1Ra induced by IL-18 is of questionable biological significance in comparison to the 11-fold increase in IL-18–induced IL-8 in the same cultures.

The amount of IL-10 induced by IL-18 was also examined. Using a broad dose–response, levels of IL-10 were below the detection limit of the IL-10 assay (< 10 pg/ml) in unfractionated PBMC of four donors. In addition, we observed that IL-18 did not augment LPS-induced IL-10 production as it did in the case of IFNγ production, i.e., IL-18 was synergistic with LPS in the production of IFNγ. In contrast, IL-10 effectively inhibited TNFα production by 88% in response to IL-18 (Fig. 10). The suppression by IL-10 of IL-18–induced cytokine synthesis was also observed for IL-1β and IL-8 in these same cultures (data not shown).

Discussion

We have demonstrated that IL-18, in the absence of a co-stimulus, triggers a cascade of proinflammatory cytokines in freshly obtained human PBMC. Previous descriptions of the biological activities of IL-18 have relied on the presence of a co-stimulus to induce cytokines, and in particular, the induction of IFNγ and other events in promoting Th1 responses. In contrast, we investigated the properties of IL-18 in the induction of the proinflammatory, neutrophil chemokine, IL-8. In models of local and systemic, neutrophil-mediated inflammation, blocking IL-8 dramatically reduces the severity of inflammation and tissue damage (for review see reference 37). IL-8 has also been shown to be an angiogenic cytokine that may be involved in the growth of certain tumors. In the process of inducing IL-8 in PBMC, IL-18 appears to first recruit the cytokines, TNFα and IL-1β. It became clear early in these studies that IL-18 induction of IL-8 involved the biological activity of IL-1 but more impressively, a TNF-like molecule appeared to be essential since TNFbp reduced nearly all the IL-18 induced IL-8 production. This property of IL-18 (induction of TNF) is consistent with the ability of IL-18 to activate the translocation of NFκB in T cells (19, 38) and accounts for the production of TNF in the absence of other stimuli.

The removal of most but not all of the nonadherent population decreased the production of IL-8. To determine the cellular sources of IL-8, IL-1, TNF, and the possible interplay of the different subsets of cells responding to IL-18, cell purification steps were employed. By comparison to unfractionated PBMC, purified $CD14^+$ monocytes did not achieve the same degree of IL-8 production in response to IL-18. In keeping with the general view that monocytes are the main producers of IL-8, IL-8 was not detectable in $CD3^+/CD4^+$ cells stimulated with IL-18 but rather in the $CD14^+$ population. From these data we surmised that either $CD3^+/CD4^+$ contact with a monocyte or soluble factors from the T cells or both were essential in optimal IL-18 induction of IL-8. To determine a role for IL-1, we observed that IL-1Ra inhibited IL-18–induced IL-8 secretion by 50%. This is similar to the ability of IL-1Ra to inhibit ~ 43% of LPS-induced IL-1β and 20% of TNFα produced in PBMC (39, 40). More dramatic results were achieved with the use of TNFbp; maximal inhibition of 80% was measured for IL-18 induction of IL-8 and IL-1β precursor and mature forms. The interpretation of these data would be that TNFα is the driving mediator of IL-18 induction of IL-1β and chemokines. Although TNFβ could also account for this observation, we were unable to demonstrate that IL-18 induced TNFβ in these studies.

Furthermore, part of the TNF induction of IL-8 could be IL-1 dependent. However, our findings do not exclude a role for a direct effect of IL-18 in IL-8 production. It is likely that, similar to LPS-induced cytokines, IL-18 induction of TNF, IL-1, and IL-8 occurs in several overlapping waves of cytokines. The

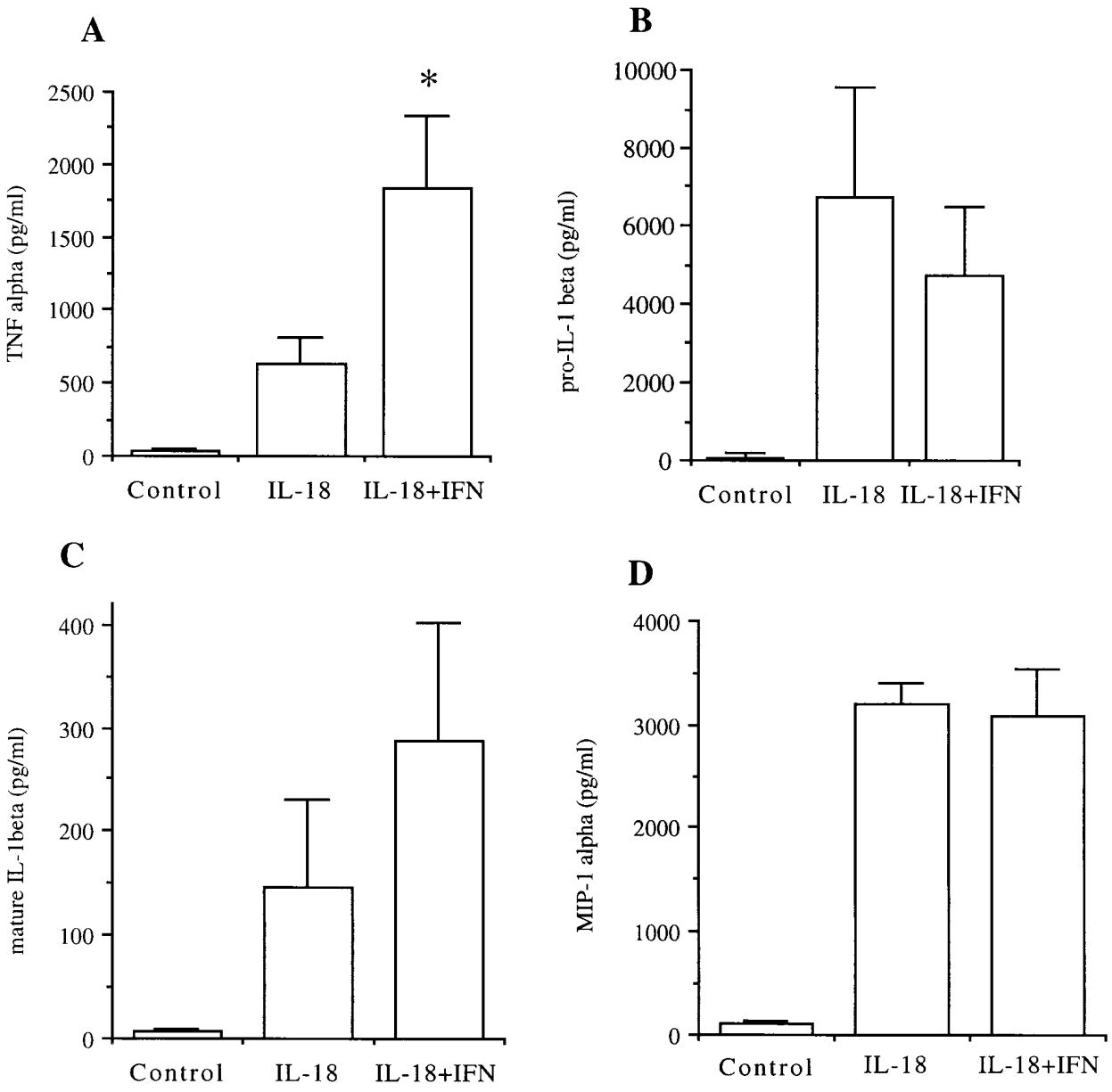

Figure 8. Effect of exogenously added IFNγ on IL-18 induction of cytokines. Unfractionated PBMC were incubated with IL-18 (10 nM) in the presence or absence of IFNγ (500 U/ml) for 12 h in the case of TNFα production and for 20 h for IL-1β and MIP-1α. Cytokines were detected in cell supernatants in the case of (*A*) TNFα, (*C*) mature IL-1β, and (*D*) MIP-1α. ProIL-1β (*B*) was assayed in cell lysates. The data represent the mean±SEM. For *A*, $n = 4$, $*P < 0.02$ compared with IL-18 only; for *B*, $n = 4$; for *C*, $n = 3$; and for *D*, $n = 4$ for each cytokine, respectively.

first wave of IL-18 activity is a direct response but a second and subsequent wave may be mediated by endogenous stimulation by TNF and IL-1. Our results are in keeping with the recent findings that anti–IL-18 prevents the second peak of TNFα production in endotoxin-induced liver damage (13).

When we measured TNFα in unfractionated PBMC and the various cell subsets, we discovered that the source of measurable TNFα was not the $CD14^+$ cells as expected but the $CD3^+/CD4^+$ enriched fraction. It would appear that the production of IL-8 begins with IL-18 induction of a TNF family molecule from the $CD3^+/CD4^+$ cells followed by IL-1 production and IL-1 and TNF that act in synergy. Recent studies have shown that IL-18 directly activates nuclear translocation of NFκB in T cells (38). The addition of a co-stimulus under these circumstances does not affect the translocation by IL-18. Since the role of NFκB in TNFα gene expression is relatively well established (41), our data suggest that the IL-18 signal alone is sufficient for TNFα synthesis in contrast to IFNγ production. Although the cell source of IL-18–induced IL-1β was the $CD14^+$ cell, the $CD14^+$ population contained from 7–15% $CD3^+/CD4^+$ cells that could be the cell source for TNF. It became important to establish what influence IFNγ exerts on IL-18–induced synthesis of cytokines. The regulation of cytokine synthesis by IFNγ is determined by the nature of the stimulus or the disease model. IFNγ augments LPS-induced TNF and IL-1 (26, 27) and TNF-induced IL-1 (22) but suppresses

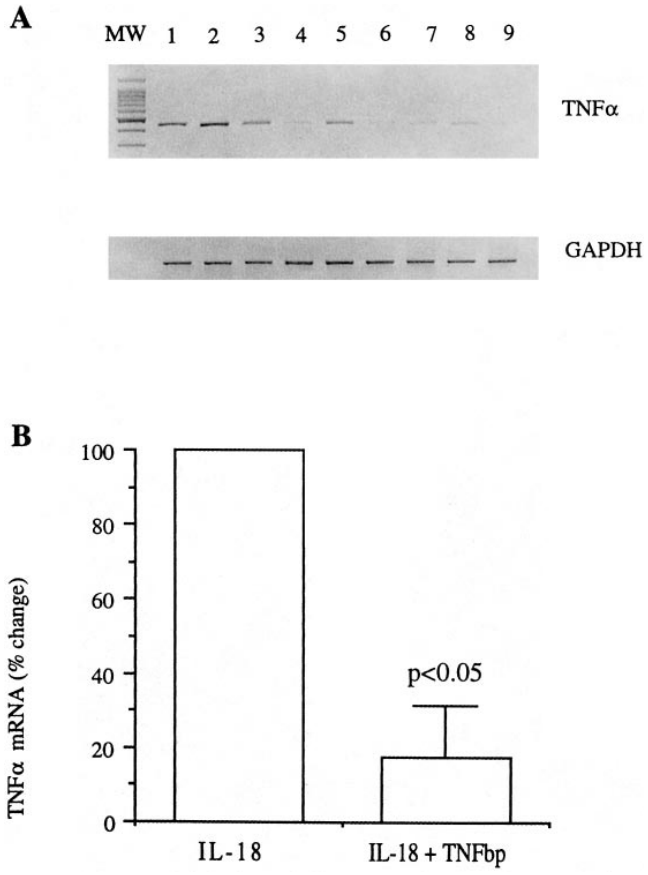

Figure 9. Inhibition of IL-18 induction of TNFα mRNA by TNFbp. (*A*) Unfractionated PBMC were incubated with IL-18 (10 nM) and the effect of TNFbp (10 μg/ml) on steady state TNFα mRNA was determined by RT-PCR. Results of three donors are presented. *MW*, 100-bp molecular marker ladder; lanes *1*, *4*, and *7* are unstimulated cells from each of three donors, respectively; lanes *2*, *5*, and *8* are IL-18 (10 nM)–stimulated cells from each donor; lanes *3*, *6*, and *9* are IL-18–stimulated cells in the presence of TNFbp (10 μg/ml). GAPDH served as the internal control. (*B*) Quantification of the PCR products as determined by densitometry for the three donors in *A*. IL-18–stimulated cells were set at 100%. Results represent the mean±SEM percent change in the presence of TNFbp shown in *A*.

IL-1–induced IL-1 (27, 42). The enhancement by IFNγ of TNFα and IL-1β production induced by IL-18 is in line with that of the effects of IFNγ on LPS and TNFα induction of cytokines. The augmentation of IL-1β release may be related to the ability of IFNγ to enhance ICE expression (29). When the production of chemokines is considered and the effects of IFNγ are taken into account the picture is complex. IFNγ inhibits IL-8 release in response to LPS, IL-1 (43) and IL-2 (44). However, IFNγ independently is able to induce the expression of IL-8 in U937 cells (45). With respect to the β chemokine RANTES, IFNγ was shown to enhance TNFα and IL-1β responses in synovial fibroblasts (46) We confirmed (43) that IFNγ suppressed IL-1β induction of IL-8 and MIP-1α (our data not shown). However, we extended these observations to show that IFNγ partially abolished IL-18 induction of IL-8 (data not shown) but had no effect on MIP-1α production. The lack of an effect on MIP-1α production is analogous to IL-2 induction of IL-6 (47) and IL-8 (44) which is independent of IL-1 and not affected by IFNγ. That IL-18 may act independently of IFNγ was demonstrated by, for example, IL-18–induced osteoclast multinucleated cell formation via GM-CSF, which is unaffected by IFNγ (48).

The triad of TNFα, IFNγ, and IL-10 have an interesting relationship of regulation. In PBMC it has been proposed that TNFα is the major cytokine that induces IL-10 production (49–51). IL-10 downregulates TNFα production (52) whereas IFNγ, on the other hand, inhibits IL-10 production in monocytes (53). The importance of this interaction is demonstrated by the addition of IFNγ to LPS-stimulated cells where the predicted enhancement of TNFα production by IFNγ was observed with the concomitant decrease in IL-10 synthesis (52, 54). Based on these latter data, we predicted that IL-18 could induce IL-10 production but we did not observe this. One explanation is that the concentrations of TNFα produced in response to IL-18 do not always match the optimum concentrations (5–10 ng/ml) of TNF required to induce IL-10 (51). The lack of IL-1Ra and IL-10 production supports a view that the monocyte is not directly or minimally stimulated by IL-18. Furthermore, these data suggest that IL-18 is principally a proinflammatory cytokine in nature.

We investigated the level of IL-18 regulation of cytokine production by examining the expression of mRNA for IL-8, IL-1β, and TNFα. IL-18 enhanced steady state levels of the transcripts for these cytokines but we did not explore whether the effect was on direct gene expression, mRNA stabilization or both. Given that IL-18 stimulates the nuclear translocation of NFκB (38), we postulate that at least part of the enhancement in mRNA expression is a result of gene transcription. Moreover, the effect of IL-18 on gene expression was dependent on TNFα since a significant reduction of mRNA levels was observed in the presence of TNFbp. These data further reinforce the notion of a central role for TNFα in mediating the effects of IL-18.

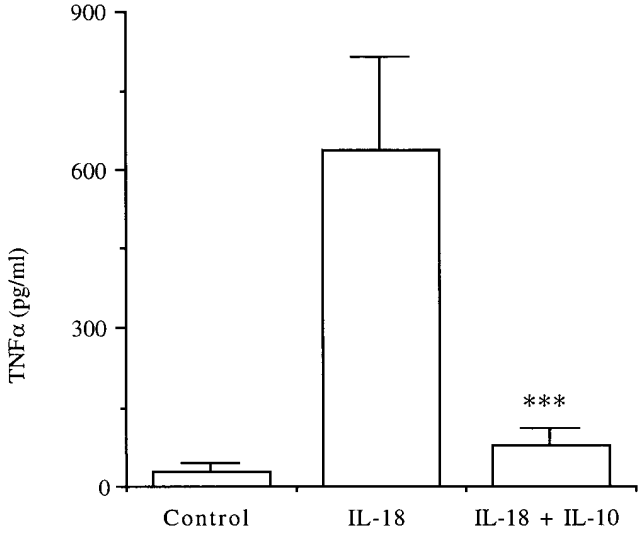

Figure 10. Inhibition of IL-18 induced TNFα by IL-10. Unfractionated PBMC were incubated with IL-18 (10 nM) in the presence or absence of IL-10 (10 ng/ml) for 12 h. TNFα was detected in cell supernatants. The data represent the mean±SEM, $n = 3$. ***$P < 0.03$ compared with IL-18 only.

We suggest that IL-18 is able to act directly on $CD3^+/4^+$ T lymphocytes and NK cells to initiate a chemotactic response and that IL-18 may have a key role in the inflammatory cascade. IL-18 induces the production of the immediate early cytokines, namely, TNFα and IL-1β, pivotal in the cytokine hierarchy. This is followed by production of the downstream chemokines IL-8, MIP-1α, and MCP-1. The nature of IL-18 pleiotropism is proinflammatory since there is minimal or no induction of IL-1Ra and IL-10 respectively. This is consistent with an increase in IL-18–induced TNFα by IFNγ. On the other hand, an inhibitory effect of IFNγ on IL-18–induced IL-8 release was observed (data not shown), similar to other reports (43). This latter observation may imply an attempt to limit IL-18–induced inflammation.

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