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IL-17 Stimulates the Production and Expression of Proinflammatory Cytokines, IL-β and TNF-α, by Human Macrophages

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IL-17 is a newly described, T cell-derived cytokine with ill-defined physiologic properties. As such, we examined the release of proinflammatory mediators by human macrophages in response to recombinant human (rh) IL-17. IL-1β and TNF-α expression and synthesis were up-regulated by rhIL-17 in a dose (ED_{50} was 50 ± 9 ng/ml)- and time-dependent fashion, with cytokine accumulation reaching a zenith after 9 h. Release of IL-6, PGE_2, IL-10, IL-12, IL-1R antagonist, and stromelysin was also stimulated by rhIL-17. IL-1β and TNF-α mRNA expression levels were controlled by rhIL-17 in a complex manner with an initial 30-min inhibitory phase, and then up-regulation beginning at 1 h and reaching a plateau at about 3 h. The latter expression pattern closely mirrored the nuclear accumulation of the transcription factor nuclear factor-κB. cAMP mimetics isobutyl-1-methylxanthine (IBMX), forskolin, PGE_2, and cholera toxin reversed rhIL-17-induced release of TNF-α, but had no consistent effect on induced IL-1β synthesis. Induced release of TNF-α was also inhibited by serine/threonine protein kinase inhibitors KT-5720 (protein kinase A) and Calphostin C (protein kinase C), mitogen-activated protein kinase kinase inhibitor PD098059, and a nonspecific tyrosine kinase inhibitor, genistein. Calphostin C alone abrogated the rhIL-17-induced release of IL-1β. The antiinflammatory cytokines IL-4 ($p < 0.01$) and IL-10 ($p < 0.02$) completely reversed rhIL-17-stimulated IL-1β release, while IL-13 and TGF-β_2 were partially effective (59 and 43% diminution, respectively). IL-10 exerted a significant suppressive effect on IL-17-induced TNF-α release (99%, $p < 0.02$), while the inhibitory effects of IL-4, IL-13, and TGF-β_2 on TNF-α secretion were partial (48, 10, and 23%, respectively). The data suggest a pivotal role for IL-17 in initiating and/or sustaining an inflammatory response.
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Macrophages are migratory phagocytic cells that play a central role in the host defense system. They are essential APCs that process Ags and present them to T cells, initiating an immune response. Macrophages are also an important source of cytokines, as well as of costimulatory molecules that play a crucial role in the efficient activation of T and B cells (1). Cytokines mediate so-called cross-talk between macrophages and Th cells, and are now widely accepted as the major factor involved in the communication between T cells, macrophages, and other immune cells in the course of an immune response (2). Monocyte/macrophage-produced cytokines, including TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, granulocyte-macrophage CSF, and IFN-γ, may affect differentiation toward Th1 and Th2 pathways in their own specific ways (2). Activated Th1 and Th2 $CD4^+$ lymphocytes also produce cytokines that, with cellular hormones of other cellular sources, alter macrophage function by causing selective changes in macrophage gene expression (3). The response of macrophages to any given stimulus is dependent upon the balance of exogenous ambient cytokines. Th1-associated cytokines, such as IL-2 and IFN-γ, are considered macrophage activators (4), whereas cytokines secreted by Th2 lymphocytes, such as IL-4, IL-10, IL-13, and TGF-β, exhibit suppressive activities on macrophage functions and antagonize the effect of Th1-secreted cytokines (5, 6). All of this may reflect the complexity of the feedback regulatory mechanisms that occur through the cytokine network in macrophages.

Human IL-17 is a 20- to 30-kDa homodimeric variably glycosylated polypeptide secreted by $CD4^+$ activated memory ($CD45^+RO^+$) T cells (7). There appears to be a ubiquitous nature to the tissue distribution of the IL-17R (8), although it is not yet known whether all cells expressing the IL-17R respond to IL-17. Murine rIL-17 exhibits biologic activity with a variety of cell types, including induction of IL-6 (8) and IL-8 (7) secretion from stromal cell elements such as fibroblasts, endothelial, and epithelial cells. IL-17 costimulates T cell proliferation induced by suboptimal amounts of PHA (9), increases surface expression of intracellular adhesion molecule-1 (ICAM-1)[2] by human fibroblasts (7), and stimulates the secretion of PGE_2. IL-17 up-regulates a number of gene products involved in cell activation, growth, and proliferation (10, 11), including the proinflammatory cytokines IL-1β, TNF-α, and IL-6. To date, there have been no reports alluding

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[2] Abbreviations used in this paper: ICAM, intracellular adhesion molecule; AP-1, activating protein-1; $[Ca^{2+}]_i$, intracellular calcium concentration; CalC, Calphostin C; $[cAMP]_i$, intracellular cyclic AMP; CREB, cyclic adenosine monophosphate-responsive element-binding protein; ; DIG, digoxigenin; IBMX, isobutyl-1-methylxanthine; IL-1Ra, interleukin-l receptor antagonist; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; rh, recombinant human.

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to a role for IL-17 in terms of macrophage activation. Fossiez et al. (9) found that hIL-17 did not alter the secretion of proinflammatory cytokines by blood monocytes, suggesting a limited proinflammatory role for IL-17 on T cell-driven inflammatory processes. Cytokines have no effect on nonactivated monocytes in vitro, and the latter observation may explain why circulating monocytes are not activated routinely by circulating cytokines (12). Freshly isolated monocytes and freshly seeded monocytes produce considerably less TNF-α and IL-6 than macrophages derived by monocyte adherence (13). It is known that adherence activates macrophages, inducing high steady state levels of mRNA for TNF-α, c-*fos*, and monocyte/macrophage CSF-1 (14), thereby enhancing their response to stimuli (15). The attachment occurs in vivo when the monocytes adhere to the vascular endothelium and migrate into the extravascular space (16), providing an activation signal that leads to biologic activation of the macrophages.

Knowing that macrophages and T cells are indissolubly linked in the activation of the immune system as a whole (1), and that all cells express the IL-17R (8), we have tested the effect of rhIL-17, the newly described T cell-derived cytokines, on macrophage function. In this work, we report for the first time the stimulation by rhIL-17 of IL-1β and TNF-α mRNA expression and synthesis, using activated human monocytes in primary culture. In addition, we show that the cytokine up-regulates IL-6, IL-10, IL-1Ra, PGE_2, and stromelysin (MMP-3) release, but does not induce nitric oxide (NO) synthesis. Using cell-permeable chemical inhibitors of protein kinases, we explored signaling pathways that may possibly mediate IL-17 action. We used the human peripheral blood monocytes isolated and activated by adherence, to examine the effect of the second activating signal on these cells provided by IL-17.

Materials and Methods

Cell culture

The PBMCs, consisting of lymphocytes and monocytes, were isolated from heparinized blood samples of healthy donors ($n = 32$), ranging in age from 25 to 55 (38 ± 2). The samples were diluted 1/2 with PBS containing 3 U/ml preservative-free heparin (Sigma Chemical, St. Louis, MO), and centrifuged over Ficoll-Hypaque (Pharmacia Biotech, Baie D'Urfé, QC, Canada). PBMCs were washed three times in RPMI 1640 (Life Technologies, Grand Island, NY) containing penicillin and streptomycin, 100 U/ml and 100 μg/ml, respectively (Life Technologies), and supplemented with 2 mM L-glutamine (Life Technologies). The mononuclear cells were seeded in untreated plastic petri dishes, area per well approximately 28.2 cm (Flow Laboratories, McLean, VA), at a density of approximately 4×10^5 cells/cm^2, in a final volume of 5 ml. The cells were allowed to adhere to plastic dishes for 90 min at 37°C, 5% CO_2, in RPMI medium not supplemented with FCS (17). By vigorous washes with PBS (three times), the nonadherent cells (mainly lymphocytes) were removed. Macrophages obtained in this fashion are greater than 91% pure, as determined by FACS analysis (Becton Dickinson, Palo Alto, CA) with anti-CD14. Cell viability was determined by trypan blue exclusion.

Activation of macrophages with IL-17: effects of antiinflammatory cytokines, protein kinase inhibitors, and cAMP mimetics

For dose-response studies, macrophages were cultured in a total volume of 2 ml for 72 h in 12-well plates (Corning, Corning, NY) in RPMI supplemented with 10% of FCS at 37°C, 5% CO_2, in the presence of increasing concentrations (0, 2.5, 10, 20, 50, 100, and 200 ng/ml) of rhIL-17 (R&D, Minneapolis, MN). For the time-course experiments, macrophages were cultured for an increasing period of time (0, 0.5, 1, 3, 6, 9, 24, 48, and 72 h) in the presence of a fixed concentration of rhIL-17 (50 ng/ml). The supernatants were collected and cytokines were measured (see below).

To examine the blocking effect of antiinflammatory cytokines on rhIL-17-stimulated macrophages, the adherent cells were stimulated for 72 h with rhIL-17 (50 ng/ml) in the presence or absence of 10 ng/ml of the following cytokines: IL-4, IL-10, IL-13, or highly purified TGF-β_2 (all cytokines were from R&D). The proliferation of cells was measured by [^{3}H]thymidine incorporation, and cell viability was determined by trypan blue exclusion. The supernatants were collected, and the levels of cytokines were determined.

To explore, on a preliminary basis, possible signaling cascades activated by IL-17, we determined the effects of cell-permeable protein kinase inhibitors of diverse specificity and cAMP mimetics on the rhIL-17-induced release of TNF-α and IL-1β. Adherent cells were pretreated with the protein kinase inhibitors KT-5720 (PKA, 2 μmol/L), CalC (PKC, 250 nmol/L), PD098059 (mitogen-activated protein kinase, 50 μmol/L), or genistein (tyrosine kinase, 50 μmol/L), for 30 min before the addition of 50 ng/ml of rhIL-17 or vehicle for 24 h at 37°C. The protocol was repeated with the cAMP mimetics isobutyl-1-methylxanthine (IBMX; 200 μmol/L), forskolin (60 μmol/L), PGE_2 (1 μg/ml), or cholera toxin (200 ng/ml). Cell culture supernatants were recovered and cytokine levels were determined.

Cytokine detection

Measurement of IL-1β, TNF-α, IL-6, IL-10, IL-12, and IL-1Ra was performed using Quantikine 228 ELISA kits (R&D), and PGE_2 by using PGE_2 kit (Cayman Chemical Company, Ann Arbor, MI). This assay uses the quantitative sandwich immunoassay technique. By comparing the OD of the samples to the standard curve, the concentration of the cytokines in culture supernatant was determined. The minimum detectable dose of TNF-α was typically less than 4.4 pg/ml, 0.3 pg/ml IL-1β, 0.7 pg/ml IL-6, 1.5 pg/ml IL-10, 14 pg/ml IL-1Ra, and 80% B/Bo: 29 pg/ml PGE_2.

Northern blot analysis of cytokine mRNA

For time-course experiments, adherent cells were cultured for increasing periods of time on a short-term (0, 5, 10, 15, 30, 60 min) or long-term basis (0, 1, 2, 4, 8 h) in the presence of a fixed concentration of rhIL-17 (50 ng/ml). Cells were also treated with LPS for 8 h to serve as a positive control in all such experiments. Total cellular RNA was then isolated (1×10^6 cells = 10–20 μg RNA) using the Trizol reagent (Life Technologies). Generally, 2 to 5 μg of total RNA was resolved on 0.9% agarose-formaldehyde gels and electrophoretically transferred to Hybond-N nylon membranes (Amersham Canada, Oakville, ON) in 20× SSC buffer, pH 7. After prehybridization, hybridization was conducted in the same buffer containing digoxigenin (DIG)-labeled cRNA probes for human pro-IL-1β, or a DIG-labeled cDNA probe for TNF-α, for 24 h at 68°C or 50°C, respectively.

The human IL-1β cDNA (1.3-kb, *Pst*I fragment in a pSP64 vector, kindly provided by Dr. Katherine Turner (Genetics Institute, Cambridge, MA)) was subcloned into a Bluescript vector (Stratagene, La Jolla, CA), and an antisense cRNA IL-1β probe was generated, taking advantage of a T3 promoter site after linearization with *Sal*I restriction endonuclease. A 740-bp *Hin*dIII/*Bam*HI fragment from human TNF-α cDNA (1.38-kb insert; American Type Culture Collection, Rockville, MD) was excised, purified, and labeled by random priming with DIG-labeled dUTP (Boehringer Mannheim Corp., Laval, QC).

Stringent serial posthybridization washes were conducted at 68°C (IL-1β) or 50°C (TNF-α), with the final wash being in 0.1× SET (0.15 M NaCl, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), 0.1% SDS, and 0.1% sodium pyrophosphate. Following brief rinsing at room temperature in 3× SET, the membranes were subjected to autoradiography using Kodak XAR5 films (Eastman Kodak, Rochester, NY) and Cronex intensifying screens (DuPont Canada, Mississauga, ON) at −80°C. All blots were then subjected to the laser-scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA), and the results were expressed as the relative amount of IL-1β mRNA and TNF-α, normalized to the level of 28S rRNA.

Gel-retardation experiments

Macrophages in four-well cluster plates (2×10^6 cells/well) were treated with rhIL-17 (50 ng/ml) for 0 to 8 h, after which time the cells were gently scraped into 1.5 ml of ice-cold PBS and pelleted by brief centrifugation. The cellular pellet was carefully resuspended in 400 μl of ice-cold hypotonic lysis buffer containing 10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1 mM Pefabloc 228, and 1% Nonidet P-40. Cells were allowed to swell on ice for 10 min and vortexed vigorously for 10 s, and nuclei were recovered by brief centrifugation at $16,000 \times g$ for 20 s. The nuclear pellets were resuspended in 25 μl of high salt extraction buffer containing 20 mM HEPES-KOH, pH 7.9, 0.42 M NaCl, 1.2 mM $MgCl_2$, 0.5 mM DTT, 0.3 mM EDTA, 25% glycerol, 0.5 M Pefabloc 228, and 0.5 μg/ml each of aprotinin, leupeptin, and pepstatin, followed by incubation on ice for 45 min with intermittent vortexing. The nuclear extracts were recovered by centrifugation at $16,000 \times g$ for 30 min at 4°C and stored at −80°C until used.

Double-stranded oligonucleotides containing consensus sequences were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega Corp., Madison, WI). The sense sequences of the oligos used were as

Table I. *The effect of IL-17 on macrophage secretions*

Macrophages	IL-1β (ng/ml)	TNF-α (ng/ml)	IL-6 (ng/ml)	IL-10 (ng/ml)	IL-1Ra (ng/ml)	PGE_2 (ng/ml)	Stromelysin (U/ml)	Nitrite (nmol)	IL-12 (ng/ml)
Nonstimulated	0	0	0.04 ± 0.001	0	1.94 ± 0.60	0.13 ± 0.02	26.52 ± 2.64	0	0
Stimulated	0.62 ± 0.02	1.25 ± 0.23	4.19 ± 1.41 ($p < 0.007$)	0.21 ± 0.01	3.35 ± 1.23	5.10 ± 3.70 ($p < 0.05$)	87.35 ± 18.33 ($p < 0.005$)	0	0.21 ± 0.05

follows: AP-1, 5′-CGC TTG ATG AGT CAG CCG GAA-3′; NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; and CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′. Binding buffer consisted of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM $MgCl_2$, 4% glycerol, and 2.5 μg poly(dI-dC). Binding reactions were conducted with 15 μg of nuclear extract and 100,000 cpm of ^{32}P-labeled oligonucleotide probe at 22°C for 20 min in a final volume of 10 μl. Binding complexes were resolved by nondenaturing polyacrylamide gel electrophoresis at 4°C through 6% gels in a Tris-borate buffer system, after which the gels were fixed, dried, and prepared for autoradiography.

Estimation of intracellular calcium

Macrophages were loaded with 2 μM fura-2/AM in culture medium containing 1% FCS for 30 min at 37°C. They were then rinsed with HBSS, harvested with trypsin-EDTA, pelleted, and then washed twice with HBSS. Preliminary experiments revealed that loading macrophages while still in an adherent state was the most efficient procedure, and cells retained the fluorescent dye for several hours with no detectable leakage. Fluorometric measurements were made by the method of Grynliewicz et al. (18) using a Perkin-Elmer (Norwalk, CT) PE LS50B spectrofluorimeter coupled with a 386SX PC equipped with dual wavelength determination software. Cells were resuspended in a volume of 2 ml of HBSS (Ca^{2+} concentration, 1.3 mM) and equilibrated to 37°C, and 50 ng/ml of IL-17 was added, dissolved in minimal volumes of HBSS. Excitation wavelengths were set at 340 and 380 nm, and emission was detected at 510 nm. Readings were taken every 5 s for 10 min, and the intracellular calcium concentration $[Ca^{2+}]_i$ was computed from the fluorescence ratio data, as previously described (18).

cAMP RIA

Intracellular cAMP ($[cAMP]_i$) was measured in cellular extracts using a dual-range (Biotrak, Amersham, U.K.) RIA system. Following incubation for increasing periods of time (0–360 min) with IL-17 at 37°C, the cells were rinsed with ice-cold PBS, and the cultures were then snap frozen over a dry-ice/acetone mixture. Cells were scraped into 1 ml of ice-cold 75% alcohol solution containing 0.5 mM IBMX and, following low speed centrifugation to remove insoluble material, the supernatants were dried and redissolved in 1 ml of 50 mM sodium acetate buffer, pH 6.2. Data were expressed as fmol/10^6 cells.

Stromelysin activity and NO production

The level of stromelysin synthesis was measured because as a member of the metalloproteinase enzyme family (MMP), stromelysin (MMP-3) is implicated in extracellular matrix degradation, a process that is believed to contribute locally to the immune/inflammatory response. The stromelysin activity in the conditioned medium was measured by the method of Chavira et al. (19), using azocoll (Calbiochem-Novabiochem International, San Diego, CA) as substrate. The enzyme activity was expressed in arbitrary U/ml of supernatant, in which 1 U represents the micrograms of substrate digested in 1 h.

NO production was measured by determining the stable NO metabolite, nitrite, in conditioned medium, by modified Griess reaction (20).

DNA, protein, and data analysis

Values were expressed as mean ± SD of the mean, and *n* refers to the number of different cell lines (i.e., donors). Cellular DNA content was determined by the method of Burton (21) using salmon sperm DNA as a standard. Cytosolic protein was estimated by the Bio-Rad (Richmond, CA) protein assay reagent using a mixture of γ-globulin and BSA (80/20, respectively) as a standard. Statistical significance was assessed using the Student's *t* test. Significant differences were confirmed only when the probability was less than or equal to 5%. Where appropriate, ANOVA was performed.

Results

Products released by IL-17-stimulated macrophages

Many macrophage-secretory products (e.g., cytokines and prostaglandins) exert profound effects on the immune system and the inflammatory response. As such, we determined whether IL-17 had an impact on these response systems by modulating the release of macrophage products. Nonstimulated, adherent human monocytes released neither IL-1β, TNF-α, IL-12, nor NO; however, small amounts of IL-6 and PGE_2 were present in the cell culture supernatant (0.04 ± 0.001 ng/ml and 0.13 ± 0.02 ng/ml, respectively, mean ± SD, $n = 3$) (Table I). In contrast, considerably higher basal amounts of IL-1Ra (1.94 ± 0.60 ng/ml), and stromelysin (26.52 ± 2.64 U/ml) were detected.

IL-17 (50 ng/ml) induced an up-regulation in the release of IL-1β (0.62 ± 0.02 ng/ml), TNF-α (1.25 ± 0.23 ng/ml), IL-12 (0.21 ± 0.05 ng/ml), and IL-10 (0.21 ± 0.01 ng/ml), and a 1.7 (NS)-, 3.3 ($p < 0.005$)-, 39 ($p < 0.05$)-, and 104 ($p < 0.007$)-fold increase in IL-1Ra, stromelysin, PGE_2, and IL-6 concentrations, respectively. IL-17 had no effect on nitrite (NO*) production (Table I).

Time-course and dose-response studies

To better characterize the properties of the IL-17 signaling of human adherent monocytes, we performed both dose-response and time-course studies on IL-17-induced IL-1β and TNF-α release. As shown in Figure 1, *A* and *B*, both IL-1β and TNF-α release were dose dependent, and the effective concentration of IL-17 necessary to increase their release by 50% (ED_{50}) was 50 ng/ml ($n = 3$). At the highest concentration of IL-17 used (200 ng/ml), macrophages produced 1275 ± 202 pg/ml IL-1β and 2783 ± 876 pg/ml TNF-α.

Figure 1, *C* and *D*, illustrates the time course of IL-17 stimulation of IL-1β and TNF-α, respectively, using a cytokine concentration equal to the ED_{50} (i.e., 50 ng/ml). Detectable levels of both cytokines were observed after 1 to 3 h, reached a zenith after 6 to 9 h ($n = 3$, $p < 0.001$), and remained stable for up to 24 h (IL-1β, 623 ± 157 pg/ml, and TNF-α, 1255 ± 234 pg/ml, $n = 3$). Forty-eight- and seventy-two-hour culture supernatants consistently showed lower levels of both cytokines than twenty-four-hour culture supernatants, although the data did not reach statistical significance. The same pattern of dose and time dependency was found for IL-6, IL-10, IL-12, IL-1Ra, PGE_2, and stromelysin (data not shown).

Despite the apparent absence of IL-1β and TNF-α in control culture supernatants, steady state levels of their respective mRNAs were relatively elevated. Figure 2 shows a representative time course of IL-17-stimulated IL-1β mRNA expression. Incubation of IL-17 with adherent monocytes resulted initially (i.e., 5–30 min) in a diminution of the steady state IL-1β and TNF-α mRNAs, with levels exceeding controls after 1 h and remaining relatively constant for up to 8 h.

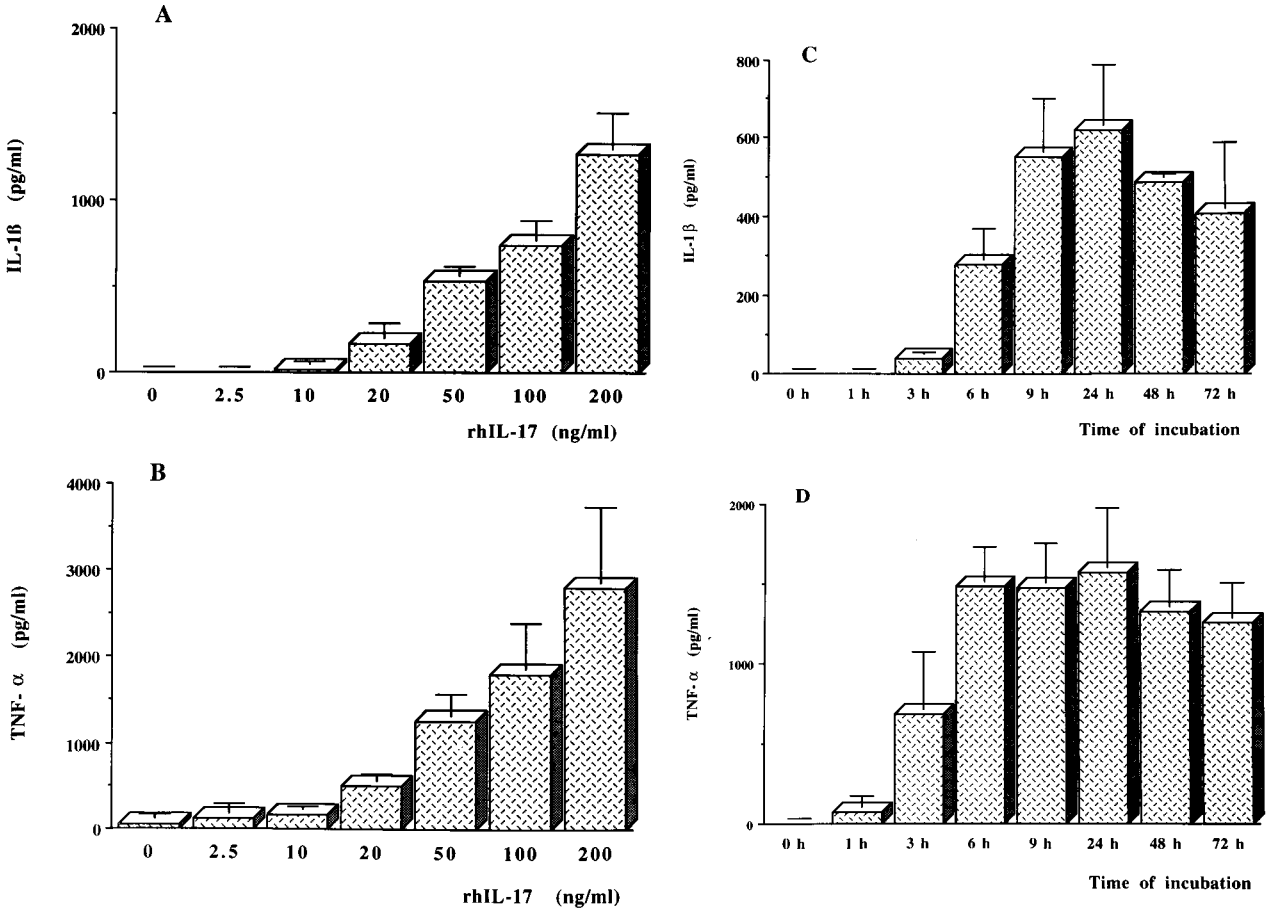

FIGURE 1. Dose-dependent stimulation by rhIL-17 of IL-1β (*A*) and TNF-α (*B*) release from adherent human monocyte in primary culture. Cells were incubated in the absence (control) or presence of increasing concentrations of rhIL-17 (0–200 ng/ml) for 24 h at 37°C. Conditioned medium was then collected and centrifuged briefly to remove floating cells, and IL-1β and TNF-α were quantitated by ELISA, as described in *Materials and Methods*. Time course of rhIL-17-activated IL-β (*C*) and TNF-α (*D*) release from adherent human monocyte in primary culture. Cells were incubated with 50 ng/ml (the ED_{50}) of rhIL-17 for 0 to 72 h, after which time the culture supernatant was collected and centrifuged, and IL-1β and TNF-α were quantitated by ELISA. Values are expressed as mean ± SD for three determinations.

Effect of antiinflammatory cytokines

In vivo, the effects of IL-17 on monocyte/macrophage activation are likely to be influenced by ambient cytokines, and indeed antiinflammatory cytokines such as IL-10 and IL-4 are known to exert a suppressive effect on macrophage function (5, 6). We addressed this issue in vitro by coincubating IL-17 in the absence or presence of IL-4, IL-10, IL-13, and TGF-β_2, and assessing macrophage activation by measuring IL-1β and TNF-α release. As shown in Table II, IL-4 essentially ($p < 0.01$) and IL-10 completely reversed the inductive effects of IL-17 in terms of IL-1β release, while IL-13 and TGF-β_2 were partly efficacious in this regard (59 and 43% diminution, respectively). In contrast, only IL-10 exerted a significant suppressive effect on IL-17-induced TNF-α release (99%, $p < 0.02$, $n = 3$), while the suppressive effects of IL-4, IL-13, and TGF-β_2 on TNF-α secretion were partial (48, 10, and 23%, respectively).

IL-17 reduces macrophage $[cAMP]_i$: effects of cAMP mimetics on IL-1β and TNF-α release

Incubation of adherent human monocytes with IL-17 (50 ng/ml) resulted in time-dependent decrease in the levels of $[cAMP]_i$ (Fig. 3*A*). Control cells exhibited levels of cAMP exceeding 5000 fmol, which were diminished steadily by IL-17, with significant reductions observed after 30 min and the nadir occurring between 4 and 6 h ($n = 3$, $p < 0.005$). The latter time frame corresponded to the period in which IL-17 activated IL-1β and TNF-α release (see above). Furthermore, cAMP mimetics such as IBMX, forskolin, PGE_2, and cholera toxin reversed the IL-17-induced increase in TNF-α release (Fig. 3*B*); basal levels of TNF-α release were abrogated completely by the latter cAMP-elevating substances. Contrary to this effect, IL-17-stimulated IL-1β release did not respond to treatments with aforementioned mimetics, as shown in Figure 3*C*.

IL-17 stimulates Ca^{2+} flux in human macrophages

When the cells were exposed to IL-17 (50 ng/ml, $n = 3$), there was a transient increase in the free cytoplasmic $[Ca^{2+}]_i$ (Fig. 4). The increase in $[Ca^{2+}]_i$ was nearly twofold, rising from a basal value of 150 to 285 nM. The rate of rise of $[Ca^{2+}]_i$ was rapid, reaching the peak within 5 s, and lasting for nearly 10 s before returning to pre-IL-17 values.

Effects of protein kinase inhibitors on IL-17-stimulated IL-1β and TNF-α release

To delineate postreceptor signaling pathways activated by IL-17, we chose as a first approach to use cell-permeable chemical inhibitors of intracellular protein kinases. The cAMP protein kinase (PKA) inhibitor KT-5720 (2 μM) suppressed the stimulatory effects of IL-17 in terms of TNF-α release by 96.5% ($p < 0.003$,

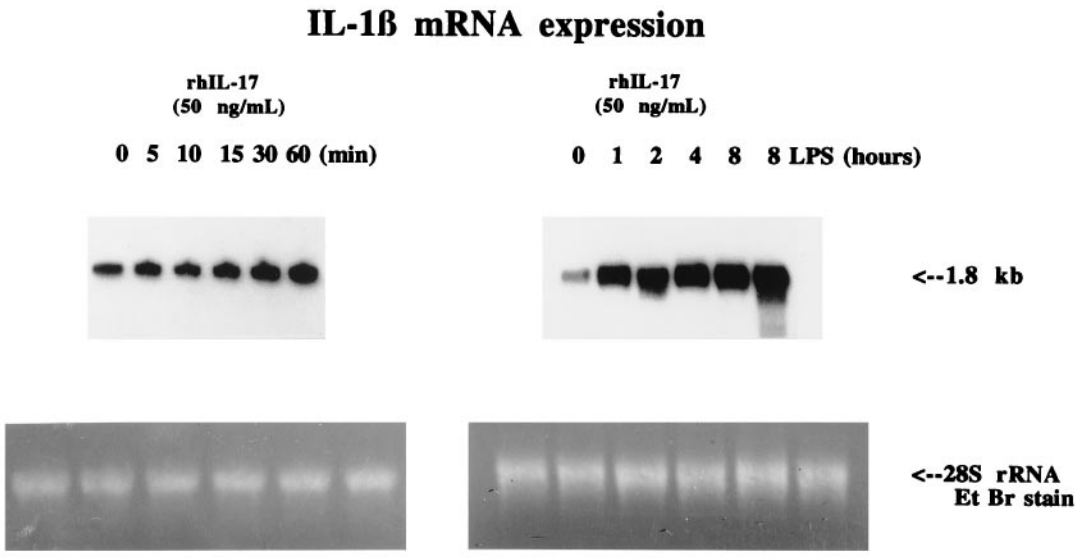

FIGURE 2. Time course of rhIL-17 stimulation of IL-1β mRNA expression. Adherent monocyte/macrophage cultures were incubated in the absence (control) or presence of 50 ng/ml of rhIL-17 for 5, 10, 15, 30, and 60 min or, over a longer time course of 1, 2, 4, and 8 h. LPS (8-h)-stimulated macrophages were used as a positive control. Monolayers were rinsed in ice-cold PBS, total RNA was extracted (2 μg/lane), and Northern analysis was performed using an IL-1β antisense cRNA probe, as described in *Materials and Methods*. Also shown is the 285 rRNA ethidium bromide (EtBr) stain for each gel.

$n = 4$), while the nonspecific tyrosine kinase inhibitor genistein (50 μM) inhibited IL-17 action by 89% ($p < 0.001$, $n = 4$) (Fig. 5*A*). When macrophages were pretreated for 30 min before the addition of IL-17 with 50 μM of PD098059, a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo (22), 60% of the up-regulation of TNF-α by IL-17 was abrogated ($p < 0.03$). The specific inhibitor of Ca^{2+} phospholipid-dependent protein kinase (PKC), CalC (250 nM) (23), inhibited IL-17-induced TNF-α release by 65% ($p < 0.04$).

With the exception of CalC, none of the inhibitors had a statistically significant effect on IL-17-stimulated IL-1β release (Fig. 5*B*). Indeed, the inhibitor blocked IL-17-activated IL-1β release by 92.3% ($p < 0.001$, $n = 3$).

IL-17 induces NF-κB, AP-1, and CREB nuclear DNA-binding proteins in human macrophages

The promoter regions of human IL-1β and TNF-α genes harbor *cis*-acting enhancer elements that are recognized by *trans*-acting transcription factors such as NF-κB, AP-1, CREB, and related family members. The latter factors are believed to mediate gene transcription induced by a variety of external signals including cytokines (24–26). Indeed, IL-17 affected the level of nuclear DNA-binding proteins, recognizing the ^{32}P-labeled NF-κB (identical to Ig enhancer NF-κB site) oligonucleotide in a complex fashion. The first 60 min were characterized by an initial decrease in NF-κB binding compared with controls, followed by a dramatic time-dependent rise, which reached a zenith at 4 h and declined thereafter (Fig. 6). The gel-shift pattern with ^{32}P-labeled AP-1 oligonucleotide was identical ($n = 3$, data not shown). In contrast, ^{32}P-labeled cAMP response element (CRE) binding was present in control nuclear extracts, but was down-regulated dramatically in a time-dependent fashion up to 8 h (Fig. 6).

Discussion

Macrophages are ubiquitously distributed mononuclear phagocytic cells responsible for numerous homeostatic, immunologic, and inflammatory processes (27). These cells are the essential APCs, and an important source of costimulatory molecules and cytokines (3).

A complex interaction exists between macrophages and T cells. Macrophages may control the expression of many of the T cell cytokines, and T cell factors control macrophage activation. Macrophages can release cytokines associated with Th1 (IL-12, TNF-α) or Th2 (IL-1, IL-10) responses, and sometimes both simultaneously (6). Th1-derived cytokines (IFN-γ, IL-2, TNF-α) favor macrophage activation, whereas the Th2 cytokines (IL-4, IL-10, IL-13) exhibit suppressive activities on macrophage functions (2). A limited number of studies have shown that human IL-17 is capable of inducing the production of other cytokines from stromal cell elements, such as fibroblasts, endothelial cells, and epithelial cells (7, 9). In this study, we have tested the effect of rhIL-17, the newly described T cell-derived cytokines, on macrophage function.

We found that IL-17 increased the synthesis of IL-1Ra and stromelysin, and stimulated the secretion of IL-1β, TNF-α, IL-6, IL-10, IL-12, and PGE_2 by human macrophages. This secretion was dose dependent and time related. Contrary to our findings, Fossiez et al. (9) have reported that IL-17 had no effect on the secretion of proinflammatory cytokines by blood monocytes, suggesting a limited proinflammatory role for IL-17 on T cell-driven

Table II. *Antiinflammatory cytokine effect on IL-17 activity*

Cytokine	Control	IL-17	IL-17 + IL-4	IL-17 + IL-10	IL-17 + IL-13	IL-17 + TGF-β2
IL-1β (pg/ml)	0	346.2 ± 35.0	25.3 ± 13.5 ($p < 0.01$)	0	141.4 ± 48.5	197.3 ± 32.5
TNF-α (pg/ml)	12.0 ± 1.2	552.3 ± 85.9	285.2 ± 64.7	5.3 ± 1.3 ($p < 0.02$)	494.9 ± 95.1	424.1 ± 96.1

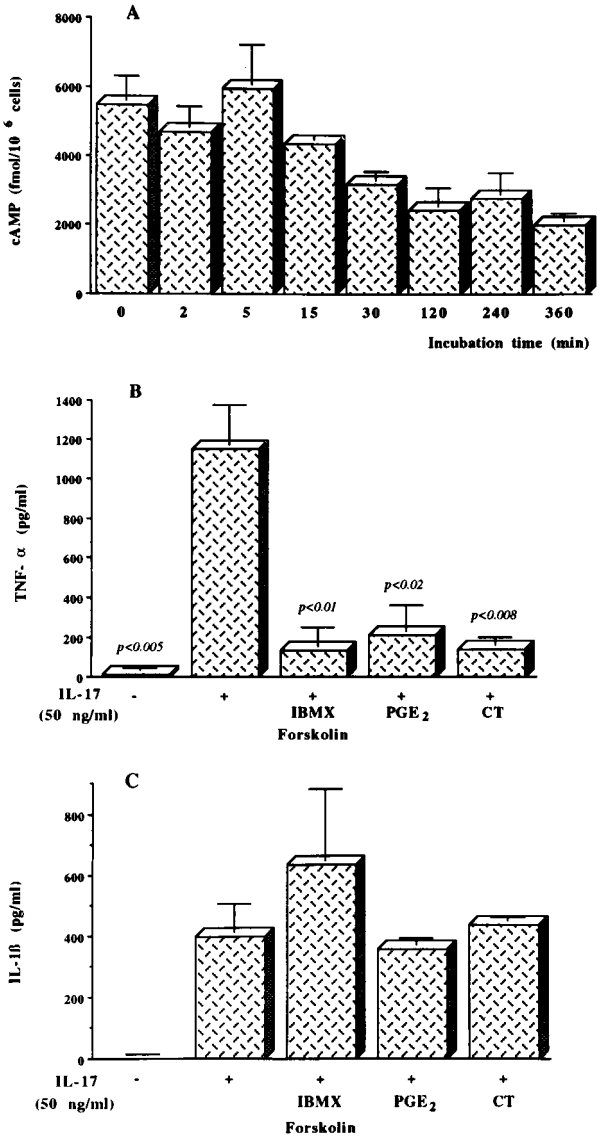

FIGURE 3. *A*, Time course of rhIL-17 suppression of $[cAMP]_i$. Cells were incubated in the absence (control) or presence of 50 ng/ml for 2 to 360 min at 37°C. $[cAMP]_i$ levels were extracted and quantitated as described in *Materials and Methods.* Results are expressed as fmol/10^6 cells. cAMP mimetic effects on rhIL-17 stimulation of TNF-α (*B*) and IL-1β (*C*) release. Where indicated, cells were preincubated for 30 min with IBMX (200 μmol/L) + forskolin (60 μmol/L), 1 μg/ml of PGE_2, or cholera toxin (CT, 200 ng/ml), and then stimulated for 24 h at 37°C with 50 ng/ml of rhIL-17. Medium was collected and analyzed for IL-1β and TNF-α by ELISA, as described in *Materials and Methods.*

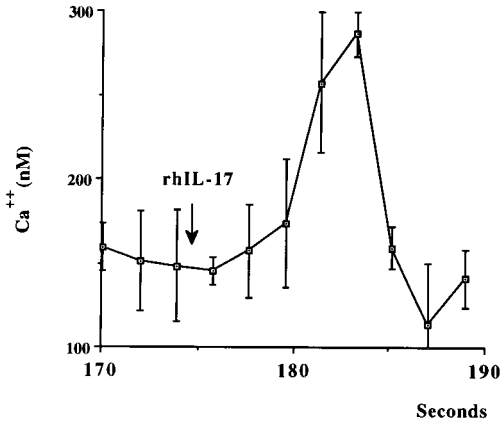

FIGURE 4. Stimulation of Ca^{2+} flux by rhIL-17. Macrophages were loaded with 2 μM fura-2/AM in culture medium containing 1% FCS for 30 min at 37°C. They were then rinsed with HBSS, harvested with trypsin-EDTA, pelleted, and then washed twice with HBSS. Cells were resuspended in a volume of 2 ml of HBSS (Ca^{2+} concentration, 1.3 mM) and equilibrated to 37°C, and 50 ng/ml of IL-17 was added dissolved in minimal volumes of HBSS. Fluorometric measurements were made with a Perkin-Elmer PE LS50B spectrofluorimeter coupled with a 386SX PC equipped with dual wavelength determination software. Excitation wavelengths were set at 340 and 380 nm, and emission was detected at 510 nm. Readings were taken every 5 s for 10 min, and the $[Ca^{2+}]_i$ was computed from the fluorescence ratio data, as previously described (Grynliewicz et al., Ref. 18).

inflammatory processes. The activation of macrophages is defined as a two-stage process (28), the first being a primed state in which macrophages exhibit enhanced MHC class II expression, Ag presentation, alteration in cell morphology, rearrangement of the cytoskeleton, and oxygen consumption, but reduced proliferative capacity (29). In our experiments to isolate the macrophages, we have used the adherence, a process known to activate these cells and enhance their response to stimuli (15). In vivo extravasation into areas of acute and chronic inflammation resulted in subsequent adherence of monocytes to the surface of capillary and postcapillary vascular endothelial cells (16). Adherence is thus likely to play a pivotal role in the earliest events in macrophage maturation and activation (14). The cytokines had no effect on nonadherent cells, and that may be one reason that circulating monocytes are not routinely activated by circulating cytokines (12). For example, TNF-α and IL-6 production by in vitro cultured differentiated macrophages is considerably higher than by freshly seeded monocytes (13). It is clear that adherence markedly influences in vitro studies of macrophage function (30). Although the same technique to enrich the monocyte population from PBMCs was used by Fossiez et al. (9), their results are in contrast to ours. This could be related to differences between the two experimental protocols that are somewhat difficult to analyze since little information was provided about their experimental conditions. As such, it is in point of fact quite difficult to make direct comparison of the results and to rationalize the obvious discrepancies between the two studies.

In this study, we have shown that human activated monocytes, when stimulated with IL-17, did not produce a measurable amount of NO. In contrast to rodent mononuclear phagocytes, the presence of inducible high output NO in human mononuclear phagocytes has been questioned by a number of investigators (31, 32). In mice and rats, the synthesis of NO is induced by several cytokines, including IL-1 and TNF-α, by activation of the inducible form of NO synthase. In humans, however, specific living microorganisms, rather than cytokines, may induce the formation of NO in macrophages (33). Fieren (34) found no evidence that the NO system is an important part of the antimicrobial arsenal of peritoneal macrophages.

As an excessively high response of macrophages to secretion of proinflammatory cytokines may result in serious injury, a strict control of their secretion and action is required (34). Deactivation may occur indirectly via the induction of antiinflammatory cytokines such as IL-10 and IL-4 (35). We found that IL-17-stimulated macrophages secreted IL-10 and IL-1Ra, two molecules with antiinflammatory properties. Our results have shown that IL-10 completely blocked the IL-17-stimulated secretion of IL-1β and TNF-α by human macrophages, whereas the blocking effects of IL-4, IL-13, and TGF-β_2 were partial. IL-4, IL-10, IL-13, and TGF-β comprise the quartet of defined macrophage-deactivating

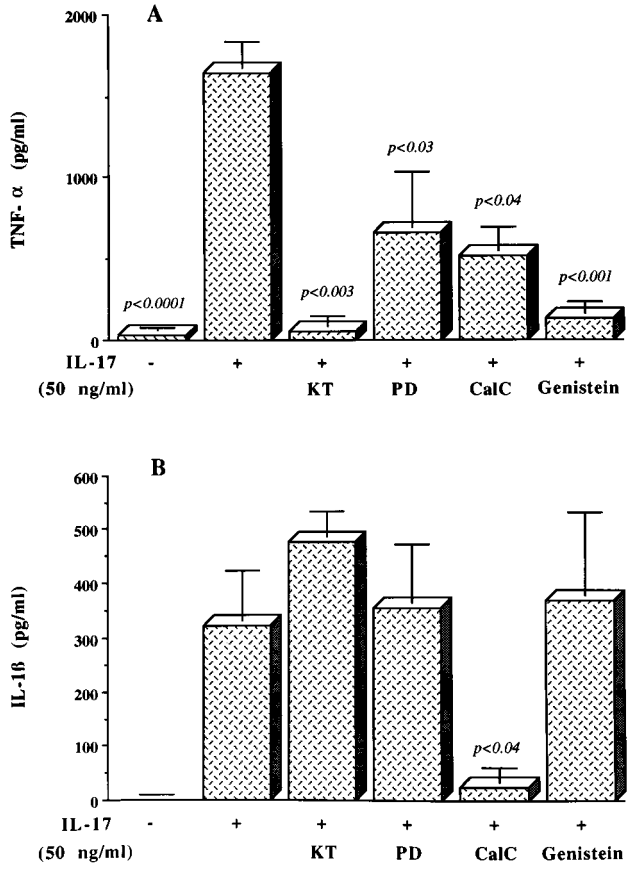

FIGURE 5. Effect of protein kinase inhibitors on rhIL-17-stimulated TNF-α (*A*) and IL-1β (*B*) release. Where indicated, cells were preincubated for 30 min with KT-5720 (2 μmol/L), PD098059 (PD, 50 μmol/L), CalC (250 nmol/L), and Genistein (50 μmol/L), and then stimulated with 50 ng/ml of rhIL-17 for 24 h at 37°C. Medium was collected and analyzed for IL-1β and TNF-α by ELISA, as described in *Materials and Methods.*

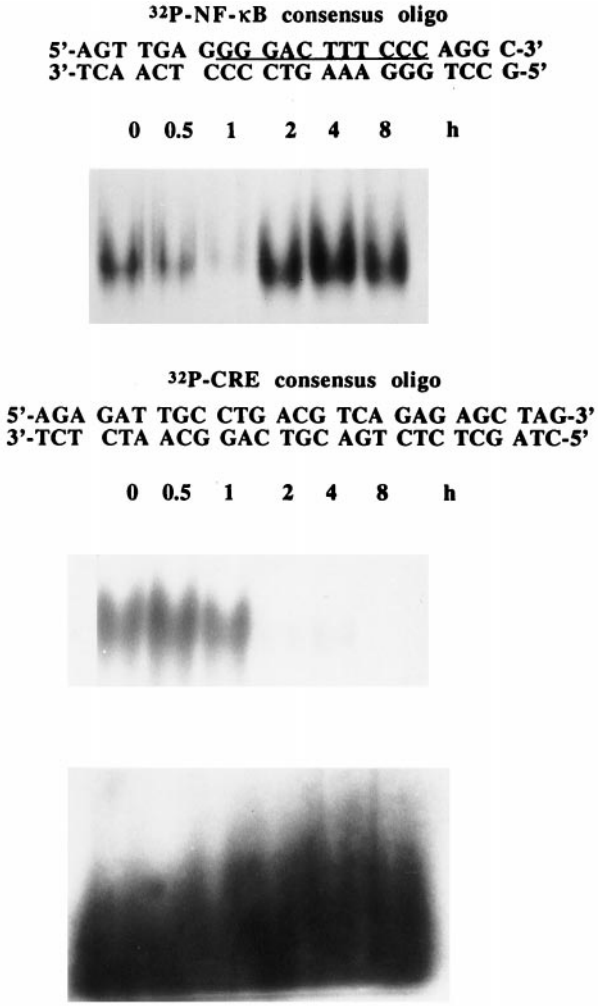

FIGURE 6. Induction of NF-κB and cAMP response element (CRE) consensus oligonucleotide nuclear binding activity by rhIL-17 in human macrophages. Cells were incubated in the absence (control) or presence of 50 ng/ml of rhIL-17 for 0.5, 1, 2, 4, and 8 h at 37°C. Nuclear protein extraction procedures and gel-shift analysis were performed as described in *Materials and Methods.*

cytokines, and each inhibits some, but not all macrophage function (6). IL-10, produced by both macrophages and T cells (36), has been shown to be a potent inhibitor of T cell responses, acting indirectly by suppression of multiple macrophage functions (5, 6). It is important to note that the same stimulus that induces proinflammatory cytokines (i.e., IL-1, IL-6, and TNF-α) in macrophages is also capable of inducing IL-10 and IL-1Ra, which in turn act as negative regulators of cytokine synthesis (2, 37). IL-4 and recently described IL-13 do not act as general macrophage deactivators. These cytokines inhibit the production of proinflammatory cytokines, while the production of IL-1Ra is increased (2). All of this may reflect the complexity of the feedback regulatory mechanisms that occur through the cytokine network in macrophages (2).

IL-1β and TNF-α are key mediators of the body's response to inflammation, and important messengers in the communication between a wide variety of cells and tissues involved in host defense (34). In our study, we have examined these two cytokines to delineate the mechanism by which IL-17, as the second signal, affects macrophage function. Primed macrophages respond to second stimuli to become fully activated, a state characterized by maximal secretion of inflammation mediators such as TNF-α, IL-1, PGE_2, reactive oxygen products, and NO (38). In this study, we have found that the adherence is sufficient to induce high steady state levels of mRNA for IL-1β and TNF-α, the results of which are consistent with those reported by Haskill et al. (14). There is little, if any, constitutive expression of any of the monokines in cells freshly isolated by nonadherent techniques (14). The induction of transcription of the IL-1β and TNF-α genes occurred rapidly upon adherence, but there was no significant secretion of these cytokines. In human monocytes, limited translational activity is initiated in the absence of a second signal (30). Only a small and transient intracellular level of IL-1β was observed by Newton (39), whereas Matsushima et al. (40) failed to detect any. In both instances, however, the secretion of these cytokines was not observed in the absence of a second signal. We have detected high levels of mRNA for IL-1β and TNF-α 60 min after stimulation with IL-17, with significant amounts of secreted cytokines found at 3 h, and the maximum amounts detected at 6 h for TNF-α and at 24 h for IL-1β. These differences most likely reflect posttranscriptional regulation, as suggested by Haskill et al. (14).

Signaling in stimulated cells may be initiated at the cell surface by the direct activation of receptor protein tyrosine kinases (41, 42). We have found that the nonspecific tyrosine-blocking agent genistein almost completely inhibited TNF-α secretion, while mitogen-activated protein kinase blockade by PD resulted in partial inhibition. These two inhibitors had no detectable effect on IL-1β

secretion, results that concur with the findings of Shapira et al. (43), again suggesting a difference in the signaling pathways of IL-1β and TNF-α. In addition, we have found that blocking PKC and PKA differentially regulated IL-1β and TNF-α. As the cytoplasmic region of IL-17R lacks an identifiable tyrosine kinase homology region, it was suggested that signaling through IL-17 membrane receptor may proceed indirectly via activation of membrane-associated, cytoplasmic, nonreceptor protein tyrosine kinases (44). Protein tyrosine kinase activation is the initial, rapid event linked to one of several downstream signaling elements. Activation of phospholipase C-γ by tyrosine kinase activation leads to generation of the PKC activator diacylglycerol, and the endogenous Ca^{2+}-mobilizing agent inositol (1, 4, 5)-triphosphate (45). This leads to the activation of Ca^{2+} and phosholipid-dependent PKC, as well as other Ca^{2+}-regulated protein kinases. Our results are consistent with the findings of Oliver et al. (46), who reported that IL-17 induced the rapid release of Ca^{2+} from intracellular stores, which was associated with oxidative burst and other functional responses, including cytokine production. In our preliminary experiments (data not shown) using the substances that inhibited extra (EGTA)- or intracellular (thapsigargin) Ca^{2+} mobilization, we found that the secretion of TNF-α was inhibited significantly, whereas the secretion of IL-1β was not affected. Moreover, exposure to CalC (an agent that inhibits Ca^{2+}-regulated PKC) abrogated changes in macrophage functions induced by IL-17, decreasing both IL-1β and TNF-α secretion, which is in concordance with the results of Kemmerich et al. (47) and Ohmori et al. (48).

IL-17 stimulation of macrophages was followed by a gradual decrease in cAMP. We have used different cAMP mimetics to examine the effect of cAMP levels on IL-1β and TNF-α secretion by IL-17-stimulated macrophages. We have found that PGE_2, which raises $[cAMP]_i$ levels (49), decreased TNF-α release from IL-17-stimulated peripheral blood macrophages, but exhibited no evidence of IL-1β release control. These results are consistent with the hypothesis that PGE_2 exerts a selective regulation of cytokine gene expression and production (50, 51). As the highest concentration of cAMP analogues induced a modest decrease in the secreted IL-1β, and an increase in cell-associated IL-1β concentration, it has been suggested that $[cAMP]_i$ elevation selectively suppresses the release of IL-1β (52). Our results with inhibition of cAMP-dependent PKA using KT-5720 also showed different effects on IL-1β and TNF-α secretion, which is in line with the findings of distinct regulation of these two cytokines.

The control of protein secretion involves regulation at a variety of levels. Transcriptional regulation is the area of gene expression control most clearly understood. Transcriptional regulatory factors, such as AP-1, CREB, and NF-κB, bind to enhancer/promoter sequences in the promoter region of a number of proinflammatory cytokines, such as IL-1β (53), TNF-α (54), and IL-6 (55). This leads to enhanced expression of these genes (56). Eugui et al. (57) have shown that, in isolated human monocytes, the genes for IL-1β, TNF-α, and IL-6 are coordinately expressed when stimulated with endotoxin. Rather than a single cytokine, multiple cytokines appear to be released simultaneously following monocyte stimulation (58). The genes for different cytokines are thought to function as a cassette (59). There are synergic interactions between different transcriptional factors (60, 61), with a marked increase in the expression of genes containing binding motifs for these factors, as compared with gene expression seen when one factor alone is active (58).

Although IL-1β and TNF-α share many activities, their expression and secretion are quite distinctly regulated. This phenomenon has in vivo significance, as it would be difficult for microorganisms to eliminate both cytokines simultaneously (34), and may explain why, in the case of therapeutic interest, it is difficult to manipulate the host defense system (62). T cells secreting IL-17 are among the first to be activated during immune responses (7), suggesting IL-17 may play an important role in the early stages of inflammation. It would be interesting to explore whether the inhibition of early secretion IL-17 by memory T cells could decrease the proinflammatory cytokine secretion by activated macrophages, as this could be a mechanism through which one inflammatory process may be controlled.

Acknowledgments

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