Interleukin-27 Induces a STAT1/3- and NF-κB-dependent Proinflammatory Cytokine Profile in Human Monocytes

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IL-27 is a heterodimeric cytokine bridging innate and adaptive immunity by playing a role in the activation of naïve T cells and in development of Th1 cells. Additionally, recent evidence supports a role for IL-27 in the activation of monocytes. Both pro-inflammatory and anti-inflammatory activities have been attributed to IL-27; however, the role played by IL-27 in the activation of human monocytic cells in terms of cytokine production has not been well described. Our results show that IL-27 is a strong inducer of pro-inflammatory cytokine and chemokine expression, including enhancement of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression in human primary monocytes. Furthermore, we observed that IL-27-induced cytokine and chemokine production was mediated by STAT1, STAT3, and NF-κB activation. Understanding how IL-27 exerts its effects on monocytic cells will identify important molecular mechanisms in the regulation of immune responses, particularly in the modulation of monocyte activation.

Interleukin (IL)³–27 was identified in 2002 by Pflanz et al. (1–4) and is a heterodimeric cytokine composed of an IL-27p28 subunit and Epstein-Barr virus-induced gene 3 (EBI3) subunit. The heterodimeric IL-27 receptor (IL-27R) is composed of the IL-27pR (WSX-1/TCCR) subunit, unique for the binding of IL-27, and the gp130 subunit, which is shared with the receptor for IL-6 (5).

IL-27Rα has a short cytoplasmic domain compared with gp130 and has conserved tyrosines that impart the ability to bind STATs through SH2 domains and JAKs via a membrane proximal box 1 motif (6). IL-27Rα has been shown to directly associate with JAK1 as well as STAT1 (7, 8). In naive CD4 T cells, IL-27 induced the activation of both STAT1 and STAT3, but in fully activated T cells, IL-27-induced activation of STAT1 was lost, and IL-27-induced STAT3 activity was retained (9). This suggests that IL-27 may elicit differential signaling events dependent on cell type and activation state.

IL-27 is an immunoregulatory cytokine that has a role in initiation of the T cell response; however, several recent reports suggest that IL-27 also plays a key role in the regulation of mononcytic cell function. Primary human monocytes express the IL-27 receptor chains, and respond to IL-27 by the induction of STAT1 and STAT3 phosphorylation (5). In a mouse model of experimental tuberculosis, IL-27 inhibited the production of TNF and IL-12 in activated peritoneal macrophages (10). In addition, IL-27 can affect macrophage activation in a similar manner as IL-10 and IL-4 by inhibiting IL-12p40 expression in activated bone marrow-derived macrophages (11). The same study showed that alternatively activated macrophages exhibit up-regulated WSX-1 expression and as a result, enhanced IL-27-induced signals. These results strongly suggest a role for IL-27 in the regulation of inflammatory responses directed by macrophages.

Additionally, IL-27 induces a similar subset of IFN-α-inducible genes as IFN-α stimulation in human macrophages (12). Recently IL-27 was shown to enhance MHC class I and II expression in the human monocytic cell line, THP-1 (13). Another report indicates that IL-27 also induces IL-1α, IL-1β, IL-18, and TNF-α mRNA in mast cells, and IL-1β, TNF-α, IL-12p35, and IL-18 mRNA in monocytes (5). However, the molecular mechanisms utilized by IL-27 to induce cytokine production in human monocytic cells have not been well characterized.

In this study we describe a novel IL-27-induced cytokine profile in human monocytic cells. Our results show that IL-27 is a strong inducer of pro-inflammatory cytokine expression (IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α). We demonstrate for the first time that IL-27-induced cytokine expression is dependent on the activation of STAT1, STAT3, and NF-κB in human monocytic cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—THP-1 cells (pro-monocytic leu-kemic cells), were obtained from ATCC. Cells were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Recombinant IL-27 was used at a concentration of 120 ng/ml. Caffeic acid phenethyl ester (CAPE) was used as a potent and specific inhibitor of NF-κB binding activity.

Isolation of Primary Monocytes—Purified monocytes were isolated from whole blood obtained from healthy donors in agreement with the Queen’s University Research Ethics Board approval. Whole blood samples were diluted with an equal volume of PBS-EDTA (1 mM) + 2% fetal bovine serum and layered over Lympholyte (Cedarlane Laboratories) in a 4:3 ratio of diluted blood to lympholyte. Overlaid tubes were centrifuged for 20 min at 1200 × g, room temperature, with the brake off. The fraction of peripheral blood mononuclear cells (PBMCs)
was extracted, and primary monocytes were isolated by magnetic negative selection using the Human Monocyte Enrichment Kit (StemCell Technologies).

Cytokine Array—The Human Cytokine Array Panel A kit was used to determine relative levels of cytokines released from primary human monocytes (R&D Systems), as per the manufacturer’s instructions. Briefly, primary monocytes were cultured in medium alone or in the presence of IL-27 for 24 h. After blocking the array membranes, culture supernatants were mixed with Cytokine Array Panel A Antibody Mixture and incubated for 1 h at room temperature. Supernatant-antibody mixture was then added to the membrane for an overnight incubation at 4 °C. After incubation membranes were washed and incubated with streptavidin-HRP for 30 min at room temperature. Membranes were then washed and incubated with chemiluminescent reagent. Chemiluminescent detection was performed using the AlphaInnotech HD2 Image (AlphaInnotech). Each cytokine was present in duplicate on the array and the average of both spots was taken for expression analysis of each cytokine. Fold increase in cytokine production was calculated over background using densitometry as measured by the analysis software on the HD2 imager.

ELISA—Primary monocyte and THP-1 cell culture supernatants were used in ELISA analysis to quantify expression of IL-27-induced cytokines as per the manufacturer’s instructions: IL-6 (CHC1263), IP-10 (CHC2363), MIP-1α (CHC2203), MIP-1β (CHC2293), and TNF-α (CHC1753). Briefly, 96-well microplates were coated overnight at 4 °C with capture antibodies (IL-6: 1 µg/ml, IP-10: 2 µg/ml, MIP-1α: 2 µg/ml, MIP-1β: 2 µg/ml, or TNF-α: 2 µg/ml). Plates were washed and blocked for 1 h at room temperature, followed by incubation with standards or sample (undiluted culture supernatants). Samples were added in duplicate. Immediately following standard and sample addition, detection antibody was added to microplates (IL-6: 0.16 µg/ml, IP-10: 0.2 µg/ml, MIP-1α: 0.16 µg/ml, MIP-1β: 0.16 µg/ml, or TNF-α: 0.32 µg/ml) and incubated for 2 h at room temperature with continual shaking. Wells were washed, incubated with streptavidin-HRP, washed again, and incubated with TMB Stabilized Chromogen (BioSource). Reactions were stopped with the addition of 1.8N H2SO4 and absorbencies were immediately measured on the BioTek ELx800 Absorbance Microplate Reader. ELISA results are shown as an average of the duplicate wells ± S.D.

RNA isolation and RT-PCR—Total RNA was extracted from cell pellets using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich). RNA (0.5 µg) was reverse-transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase Enzyme (Invitrogen). Equal aliquots of cDNA (2 µl) were used for IL-6, IP-10, MIP-1α, MIP-1β, TNF-α, and 18 S rRNA amplification using specific primers (Table 1) with 5 × Taq Polymerase Master Mix (New England Biolabs) containing dNTPs, MgCl2, KCl, and stabilizers. PCR cycles were performed on the P2x Thermal Cycler (Thermo Electron Corporation). The following annealing temperatures were used: IL-6, MIP-1β, RANTES, and 18 S rRNA at 55 °C; MIP-1α, and TNF-α at 65 °C; and IP-10 at 56 °C. The amplified products were resolved by electrophoresis on 1.2% agarose gels and visualized by UV detection of ethidium bromide intercalation on the AlphaInnotech HD2 Imager (AlphaInnotech).

Western Blotting—Levels of phosphorylated STAT1, STAT3, and NF-κB p50 were measured by Western blot analysis. Cell pellets were lysed in a buffer (1 M HEPES, 0.5 M NaF, 0.5 M EGTA, 2.5 M NaCl, 1 M MgCl2, glycerol, Triton X-100, and dH2O) containing a protease inhibitor mixture (Pierce). Protein concentrations were obtained using the Bradford Assay and absorbencies were measured using a Varioskan Rev. 2.0 (Thermo Electron Corporation) 96-well plate reader. Proteins were subjected to an 8% polyacrylamide SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore). The membranes were probed with monoclonal mouse anti-phospho-STAT1 (Santa Cruz Biotechnology), monoclonal mouse anti-phospho-STAT3, or polyclonal rabbit anti-phospho-NF-κB p50 (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology). The membranes were stripped (1 × Tris-HCl (pH 6.8), 2% SDS, and 0.7 M dithiothreitol) at 50 °C with gentle agitation and washed with TBST buffer (150 mM Tris-HCl, 1 M NaCl, and 1% Tween 20) seven times followed by re-probing with polyclonal rabbit anti-human-STAT1 or STAT3 (Cell Signaling) or polyclonal rabbit anti-human NF-κBp50 (Santa Cruz Biotechnology) to confirm equal loading. Monoclonal mouse anti-human β-actin (Sigma) was used as an additional loading control to pan-p50 for the NF-κB phospho-p50 immunoblots. All immunoblots were visualized by Enhanced Chemiluminescence (ECL) (Amersham Biosciences) on the AlphaInnotech HD2 Imager (AlphaInnotech).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA were performed to determine activation of STAT1, STAT3, and NF-κB DNA binding. Briefly, cells were stimulated with IL-27 and nuclear and cytoplasmic proteins were extracted from cell pellets using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), as per the manufacturer’s instructions. Nuclear protein concentration was measured by the Bradford method.

EMSA were performed using biotin-labeled probes specific for the STAT1, STAT3, and NF-κB binding sites, with the respective sequences as follows: STAT1: 5'-CAT GTT ATG
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CAT ATT CCT GTA AGT G-3, STAT3: 5'-GAT CCT TCT GGG AAT TCC TAG ATC-3', NF-κB: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Pierce) as per the manufacturer's instructions. Briefly, all binding reactions included 5 μg of nuclear extract, 10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol), 50% glycerol, 100 mM MgCl₂, poly(dIdC), 1% Nonidet P-40, and nuclear extract. Supershift reactions were performed for 1 h at room temperature using 2 μg of polyclonal rabbit anti-STAT1 and anti-STAT3 or 2 μg of polyclonal rabbit anti-NF-κB p50 and anti-NF-κB p65 (Santa Cruz Biotechnology). To determine the specificity of the proteins for the probe sequence, nuclear proteins were incubated for 30 min at room temperature with an excess (200-fold) of annealed, unlabeled, cold competitor probe. DNA-protein complexes were then resolved on a 5% non-denaturing polyacrylamide gel, transferred to nylon membranes (Pierce) and cross-linked (Spectroline UV Crosslinker, Fisher Scientific). Chemiluminescent detection was performed using the Lightshift Chemiluminescent EMSA kit (Pierce), as per the manufacturer's instructions, and visualized on the AlphaInnotech HD2 Imager (AlphaInnotech).

siRNA Transfections—THP-1 cells (0.1 x 10⁶) were transfected either with STAT1, STAT3, or control siRNA (Santa Cruz Biotechnology). Briefly, siRNA were incubated for 45 min at room temperature with transfection reagent (Santa Cruz Biotechnology) and serum-free IMDM media. The siRNA-transfection reagent mixtures were subsequently added to cell cultures and incubated at 37°C for 5 h. Serum-free media was then replaced with IMDM-10% fetal bovine serum media, and cells were cultured in the presence of IL-27 for 4 h to detect cytokine expression by RT-PCR.

Flow Cytometry Staining and Analysis—IL-27-induced intracellular cytokine production was measured using flow cytometry. Cells were cultured in 96-well plates in the presence or absence of IL-27 for 24 h. Brefeldin A (BFA) was added to cell cultures 12 h prior to harvesting for analysis. Cells were pelleted, washed twice with phosphate-buffered saline + 0.1% sodium azide, and fixed in 1% paraformaldehyde for 20 min. Pellets were then washed PBS/0.1% sodium azide and resuspended in 0.1% saponin. Cells were stained with PE-conjugated IL-6 (eBioscience), PE-conjugated IP-10 (R&D Systems), PE-conjugated MIP-1α (eBioscience), FITC-conjugated MIP-1β (R&D Systems), or PE-conjugated TNF-α (eBioscience). Data were acquired with the Epics XLMCL flow cytometer (Beckman Coulter, Miami, FL) and analyzed using the WinMDI version 2.9 software package (J. Trotter, Scripps Institute, San Diego).

RESULTS

IL-27 Induces mRNA and Protein Expression of Pro-inflammatoty Cytokines in Human Monocytes—To date, the IL-27-induced cytokine profile in human monocytes has not been well characterized, however, a role for IL-27 in modulating inflammatory responses via induction of cytokines has been demonstrated (5, 10). To more fully elucidate the IL-27-induced cytokine profile in primary human monocytes, we performed a cytokine array measuring expression levels of 36 different cytokines in response to IL-27. Monocytes from a healthy donor were either cultured in medium alone or in the presence of IL-27 for 24 h, and cell culture supernatants were analyzed for cytokine expression.

For analysis, the cytokines were grouped as follows: (i) chemokines, (ii) Th1 cytokines, (iii) anti-inflammatory cytokines, (iv) cytokines involved in inflammation and cell differentiation, (v) IL-12 and IL-17 family cytokines, and (vi) Th2 cytokines (Fig. 1A). IL-27-mediated induction of cytokine expression was observed in the chemokine (MIP-1α, MIP-1β, IP-10, GRO-α), proinflammatory (TNF-α, IL-1β), and IL-12 family (IL-6) groups of cytokines. Interestingly, decreases in cytokine levels in response to IL-27 treatment were also observed (Fig. 1A, panel i: serpinE, IL-8, panel ii: IL-16, and panel iv: MIF).

Because of the evidence that IL-27 is involved in mediating inflammatory processes (5, 10, 14, 15), we were interested in delineating the molecular mechanism used by IL-27 to induce expression of proinflammatory cytokines. Therefore, we focused on the chemokines and cytokines having a greater than 2-fold increase in response to IL-27 stimulation: IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α (Fig. 1A, asterisks). To quantitate levels of these cytokines induced by IL-27, we performed ELISAs for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α using the same culture supernatant as used for the cytokine array. Induction of all five cytokines was observed in response to IL-27 (Fig. 1B).

To further confirm these observations, we performed ELISAs to examine IL-27-induced cytokine kinetics (Fig. 2A) and intracellular cytokine expression by flow cytometry (Fig. 2B) on five separate primary monocyte donors (representative data from an individual donor is shown). ELISAs demonstrated that IL-27 induced rapid secretion of IL-6, MIP-1α, MIP-1β, and TNF-α after 4 h of IL-27 treatment and expression of these cytokines peaked between 8 and 24 h (Fig. 2A). We detected a similar increase in IP-10 expression after 4 h of IL-27 treatment, however expression of IP-10 further increased dramatically at 48 h. Additionally, we examined intracellular expression of the cytokines by flow cytometry in BFA-treated cells that were stimulated with IL-27 for 24 h. From these experiments, we detected IL-27-mediated enhancement of intracellular cytokines IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α (Fig. 2B). We then performed RT-PCR analysis on primary human monocytes treated with IL-27 for 2–8 h to determine whether IL-27 mediates cytokine production at transcriptional levels. Cytokine induction was observed after 2 h of IL-27 treatment for IL-6, IP-10, MIP-1β, and TNF-α, and MIP-1α induction was seen after 4 h (Fig. 2C).

To define the molecular mechanisms used by IL-27 to induce these cytokines, we used the human pro-monocytic cell line, THP-1 as our model system. An increase in protein expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α in response to IL-27 treatment was observed with ELISA (Fig. 3A) and flow cytometry (Fig. 3B). Similarly, in THP-1 cells treated with IL-27 for 2 to 16 h, mRNA levels of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α were up-regulated after 2 h of IL-27 treatment for all cytokines with the exception of IL-6, which was up-regulated after 4 h of treatment (Fig. 3C).
IL-27-induced STAT1 and STAT3 Are Required for Cytokine Induction—Primary human monocytes show tyrosine phosphorylation of STAT1 and -3 in response to IL-27 stimulation (5, 16). We hypothesized that STAT1 and/or STAT3 may be involved in IL-27-mediated cytokine induction. We confirmed the ability of IL-27 to induce STAT1 and STAT3 activation in THP-1 cells, detecting phosphorylation of STAT1 and -3 within 5–15 min of IL-27 treatment (Fig. 4A).

We also assessed whether IL-27 induced the activation of these transcription factors using gel shift assays. We observed an enhanced binding of STAT1 and STAT3 within 5 min of IL-27 stimulation (Fig. 4B). Cold competitor probes (CC) abrogated the STAT-DNA complexes. Supershift assays were also performed using anti-STAT1 or anti-STAT3 specific antibodies. Incubation with supershift antibodies resulted in disruption of the protein/DNA complexes. Interestingly, disruption of the STAT-DNA complexes was found with either STAT1 or STAT3 specific antibodies, indicating the formation of STAT1/STAT3 heterodimers.

To elucidate the role of STAT1 and -3 in IL-27-induced cytokine expression, we transfected THP-1 cells with control (scrambled), STAT1, or STAT3 siRNA. To confirm specificity of the siRNA knockdowns, RT-PCR analysis of STAT1 and STAT3 expression showed that transfection of STAT1 siRNA inhibited STAT1 mRNA expression, but did not affect STAT3 expression. Similarly, transfection of STAT3 siRNA inhibited STAT3 mRNA expression but did not affect STAT1 expression. Transfection of control sequences did not affect either STAT1 or STAT3 (Fig. 4C).

To investigate the requirement of either STAT1 or STAT3 in IL-27-induced cytokine expression, we performed RT-PCR analysis on siRNA-transfected THP-1 cells stimulated with IL-27 for 4 h (Fig. 4D). As expected, in cells transfected with control siRNA, IL-27 treatment induced the up-regulation of all five cytokine mRNA levels. For IL-6 in the presence of STAT1 or STAT3 siRNA, IL-27 treatment resulted in a weaker induction of cytokine expression. In the case of IP-10, STAT1 siRNA transfection resulted in a stronger inhibition of mRNA expression compared with that observed for STAT3 siRNA knockdown. Transfection of STAT1 and STAT3 siRNA equally
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FIGURE 2. IL-27 induced expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α in primary human monocytes. Primary human monocytes were isolated by negative selection and were cultured in either media alone or IL-27 for the following conditions: (A) cells were treated with IL-27 for 0 to 48 h, and cell supernatants were analyzed for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression by ELISA. B, cells were treated with IL-27 for 24 h and IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression were detected by intracellular cytokine staining by flow cytometry. Representative histograms are shown with cells cultured in medium alone (shaded histogram) overlaid with the histogram for IL-27-treated cells (dark line). C, cells were treated with IL-27 for times ranging from 2–8 h. Levels of mRNA for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression were measured by RT-PCR. 18 S rRNA was used as a loading control. Results shown are from one donor and representative of five different donors.

Inhibition of NF-κB DNA Binding Activity Blocks IL-27-induced Cytokine Expression—In addition to the role played by STAT1 and STAT3, we investigated whether NF-κB activity we used the NF-κB-specific inhibitor CAPE. As previously described (22), 2-h preincubations with CAPE (dose ranges of 5–50 μg/ml) were performed to inhibit NF-κB activity prior to IL-27 stimulation. In agreement with others, the concentrations of CAPE and incubation times used did not down-regulated at 120 min. To confirm that IL-27-induced NF-κB DNA binding, we also performed EMSA analysis (Fig. 5B). The results demonstrate that IL-27 induced NF-κB binding after 5 min of IL-27 treatment, and peaked between 15 and 30 min of IL-27 treatment. This binding is specific to NF-κB as shown by inhibition by cold competitor probes (CC) as well as performing supershift analysis with antibodies to NF-κB p50 and p65 subunits (Fig. 5B).

To determine the role of NF-κB in IL-27-induced proinflammatory cytokine production, we initially attempted to knock down NF-κB expression using siRNA, however, because of the high levels of these proteins we were unable to achieve sufficient knock-down of NF-κBp50 or p65. Therefore, to block NF-κB activity we used the NF-κB-specific inhibitor CAPE. As previously described (22), 2-h preincubations with CAPE (dose ranges of 5–50 μg/ml) were performed to inhibit NF-κB activity prior to IL-27 stimulation. In agreement with others, the concentrations of CAPE and incubation times used did not
We pretreated THP-1 cells with CAPE, followed by stimulation with IL-27 for 15 min. Cell pellets were harvested and nuclear proteins were subjected EMSA to confirm the inhibitory action of CAPE on NF-κB DNA binding activity. CAPE inhibition was evident at concentrations from 15 μg/ml to 50 μg/ml (Fig. 5C). To confirm that CAPE did not affect STAT1 or STAT3 activation, EMSA analysis was performed for STAT1 and STAT3 DNA binding following CAPE inhibition, and no effect was observed (data not shown). Following CAPE pretreatment, THP-1 cells were stimulated with IL-27 for 4 h to investigate cytokine expression. RT-PCR analysis showed that CAPE blocked IL-27-induced mRNA expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α (Fig. 5D). IL-6 expression was abolished at 25 μg/ml whereas TNF-α expression was abolished at 50 μg/ml. MIP-1α expression was abolished at 15 μg/ml. IP-10 and MIP-1β expression showed partial inhibition at 15 μg/ml and were abolished at 50 μg/ml. To control for potential nonspecific effects of the CAPE inhibitor in THP-1 cells, we examined expression levels of RANTES, a cytokine not significantly induced by IL-27 treatment as observed by the cytokine array (Fig. 1A). Levels of RANTES mRNA did not change with IL-27 stimulation and were not significantly affected by CAPE treatment (Fig. 5D). This indicates that CAPE treatment specifically inhibits expression of IL-27-induced IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α and furthermore that
CAPE does not affect cytokine mRNA levels unaffected by IL-27 stimulation.

To further confirm a role for NF-κB in IL-27-induced of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression, we examined the effect of CAPE treatment on IL-27-treated primary monocytes. As in the THP-1 cells, primary monocytes were pre-treated with CAPE followed by stimulation with IL-27 for 4 h. RT-PCR analysis showed that CAPE pretreatment blocked IL-27-induced mRNA expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α (Fig. 5E). With the exception of IP-10, expression of IL-27-induced cytokines was completely inhibited at 10 μg/ml of CAPE. Expression of IP-10 was partially inhibited at 10 μg/ml and completely abolished by 25 μg/ml. Interestingly, primary monocytes were more sensitive to the CAPE treatment as cytokine expression was blocked at lower doses compared with THP-1 cells. In contrast to THP-1 cells, mRNA expression levels of RANTES were slightly up-regulated by IL-27 treatment and partially inhibited by CAPE (15–50 μg/ml) in primary monocytes. However, at 10 μg/ml CAPE, RANTES levels remained consistent whereas levels of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α were significantly decreased. In stark contrast to IL-27-induced mRNA expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α, CAPE treatment at any dose did not fully abolish RANTES mRNA expression in primary monocytes. Taken together, these results indicate a role for NF-κB in IL-27-mediated proinflammatory cytokine expression.

**DISCUSSION**

In this study we identified a novel cytokine profile secreted in response to IL-27 in primary monocytes and THP-1 cells. Our results show that IL-27 is a strong inducer of pro-inflammatory cytokine expression (IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α). Furthermore, we observed that IL-27-mediated cytokine production required STAT1, STAT3, and NF-κB activation.

We initially measured IL-27-mediated cytokine production using an array for detection of 36 cytokines. Results from this experiment showed a strong induction of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α. Additional data from the cytokine array also indicated that IL-27 induced the expression of other cytokines and chemokines (GRO-α and IL-1β), while inhibiting basal expression of others (serpinE, IL-8, IL-16, and MIF). Pfanz et al. (5) demonstrated IL-27-induced increased mRNA expression of the inflammatory cytokines: IL-1β, TNF-α, IL-12p35, and IL-18 in primary human monocytes. Our study is in agreement with this work as we showed IL-27-induced TNF-α and IL-1β expression. The array did not include IL-18 detection antibodies. Although the array included detection spots for IL-12p70, we did not observe any induction for this cytokine. However the production of IL-12p70 does not correlate with IL-12p35 induction because of the requirement for IL-12p40 expression (23, 24).

The initial findings observed using the cytokine array were confirmed by ELISA, intracellular cytokine detection using flow cytometry, and RT-PCR analysis in primary human monocytes and THP-1 cells. Differences in kinetics observed in ELISA time courses between primary monocytes (Fig. 2A) and THP-1 cells (Fig. 3A) could be attributed to differences in developmental stages of the cells. Additionally THP-1 cells are transformed cells and as a result of this may exhibit different sensitivities to activation. However, the concentration of the cytokines induced by IL-27 is similar between the two cell types (Figs. 2A and 3A).

Several studies have characterized IL-27 as a negative regulator of macrophage function (10, 11). However, these studies examined the effects of IL-27 on activated macrophages, whereas experiments were performed on freshly isolated monocytes. Differences in the activation states between macrophages and monocytes may thus account for this apparent dichotomy in IL-27 function. Interestingly, a recent study by Kalliolias et al. (16) indicated that IL-27 can prime human monocytes in a STAT1-dependent manner resulting in enhanced TLR-mediated proinflammatory cytokine expression.

IL-27 is produced by monocytes and dendritic cells in response to bacterial antigens (4, 25–30). In this study we provide supporting evidence for the autocrine action of IL-27 as suggested by others (5, 10, 13). IL-27 may play a critical role in modulating the inflammatory response against bacterial infection via inducing proinflammatory cytokines and chemokines. IL-6 and TNF-α are key regulators of the inflammatory response and the production of MIP-1α and MIP-1β may serve to recruit cells to sites of infection. In addition, the expression of IP-10, previously shown to be induced by IL-27 in HUVECs (31), may serve to recruit activated Th1 cells and NK cells to sites of infection (32, 33).

Although many studies indicate a role for IL-27 in inducing cytokine production, few indicate the signaling pathways used by IL-27 to modulate cytokine expression. Previous studies have shown IL-27-mediated effects on monocytic cells resulting from the activation of STAT1 and STAT3 (5, 16). IL-27Rα physically associates with JAK1 and upon phosphorylation of tyrosine residues can activate STAT1 (3, 7, 34). In monocytes, we and others have demonstrated that IL-27 activates JAK/STAT signaling cascades via STAT1 and STAT3 (5, 16). The role of JAK activation in monocytic cells in IL-27-mediated production of cytokines has yet to be determined.

Interestingly, we demonstrate IL-27-induced formation of STAT1/STAT3 heterodimers by EMSA as shown via disruption of complex formation with both STAT1 and STAT3 antibodies. This is in agreement with other studies showing STAT1/STAT3 heterodimer formation with EMSA analysis (35–37). This phenomenon explains the dependence of both STAT1 and STAT3 for IL-27-induced cytokine expression.

Additionally, we show for the first time the ability of IL-27 to activate NF-κB in human monocytes. Taken together, our results demonstrate that STAT1, STAT3, and NF-κB are required for IL-27-induced expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α. We observed inhibition of IL-27-induced cytokine and chemokine levels in the presence of siRNA for STAT1 or STAT3, but induction of mRNA levels was never completely abolished. Additionally, our results clearly demonstrate a differential requirement for STAT1 or STAT3 in IL-27 induced cytokine and chemokine expression. These observations led us to investigate the role of NF-κB, as we hypothesized that STAT1 and STAT3 were not the sole requirements for IL-27-mediated cytokine expression. Upon CAPE-mediated inhibition of NF-κB activity, we observed abrogation of IL-27-
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induced proinflammatory cytokine mRNA in both THP-1 cells and primary human monocytes. Interestingly, CAPE treatment of primary monocytes resulted in inhibition of cytokine and chemokine production at lower doses compared with that observed in THP-1 cells. The difference in sensitivity between these cell types may be due to inherent differences between primary monocytes due to the tumorigenic nature of the THP-1 cell line. These findings indicate a role for NF-κB in mediating IL-27-induced cytokine production in addition to that played by STAT1 and STAT3. Additionally, the role of other transcription factors required for cytokine expression cannot be ruled out at this point.

It is important to note that in our study, EMSA analysis was performed using consensus sequences for STAT1, STAT3, and NF-κB binding. It is likely that the exact sequences to which NF-κB, STAT1, STAT3, and NF-κB bind in the promoter regions of the individual cytokine and chemokine genes may differ and may require additional protein interactions. This may explain the differential requirement observed for each of these transcription factors in IL-27-induced cytokine expression.

Understanding how IL-27 exerts its effects on monocytic cells will identify key signaling mechanisms in the regulation of immune responses, particularly in the development of the Th1 and inflammatory responses. Our study supports the idea that IL-27 can activate the inflammatory response in resting monocytic cells. We demonstrate for the first time that IL-27 treatment of primary human monocytes elicits the induction of proinflammatory cytokines and chemokines that is mediated by STAT1, STAT3, and NF-κB signaling cascades.

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REFERENCES

1. Batten, M., and Ghilardi, N. (2007) J. Mol. Med. 85, 661–672
2. Kastelein, R. A., Hunter, C. A., and Cua, D. J. (2007) Annu. Rev. Immunol. 25, 221–242
3. Villarino, A. V., Huang, E., and Hunter, C. A. (2004) J. Immunol. 173, 715–720
4. Pflanz, S., Timans, J. C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenstein, W. M., Mattson, J. D., Wagner, J. L., To, W., Zurawski, S., McClanahan, T. K., Gorman, D. M., Banaz, I. F., de Waal, M. R., Rennick, D., and Kastelein, R. A. (2002) Immunity 16, 779–790
5. Pflanz, S., Hibbert, L., Mattson, J., Rosales, R., Vaisberg, E., Banaz, J. F., Phillips, J. H., McClanahan, T. K., de Waal, M. R., and Kastelein, R. A. (2004) J. Immunol. 172, 2225–2231
6. Sprecher, C. A., Grant, F. J., Baumgartner, J. W., Presnell, S. R., Schrader, S. K., Yamagiwa, T., Whitmore, T. E., O’Hara, P. J., and Foster, D. F. (1998) Biochem. Biophys. Res. Commun. 246, 82–90
7. Takeda, A., Hamano, S., Yamanaka, A., Hanada, T., Ishibashi, T., Mak, T. W., Yoshimura, A., and Yoshida, H. (2003) J. Immunol. 170, 4886–4890
8. Takeda, A., Hamano, S., Shiraishi, H., Yoshimura, T., Ogata, H., Ishii, K., Ishibashi, T., Yoshimura, A., and Yoshida, H. (2005) Int. Immunol. 17, 889–897
9. Yoshimura, T., Takeda, A., Hamano, S., Miyazaki, Y., Kinjyo, I., Ishibashi, T., Yoshimura, A., and Yoshida, H. (2006) J. Immunol. 177, 5377–5385
10. Hölscher, C., Hölscher, A., Ruckerl, D., Yoshimoto, T., Yoshida, H., Mak, T., Saris, C., and Ehlers, S. (2005) J. Immunol. 174, 3534–3544
11. Ruckerl, D., Hessmann, M., Yoshimoto, T., Ehlers, S., and Hölscher, C. (2006) Immunobiology 211, 427–436
12. Imamichi, T., Yang, J., Huang, D. W., Brann, T. W., Fullmer, B. A., Adelsberger, J. W., Lempicki, R. A., Baseler, M. W., and Lane, H. C. (2008) AIDS 22, 39–45
13. Feng, X. M., Liu, N., Yang, S. G., Hu, L. Y., Chen, X. L., Fang, Z. H., Ren, Q., Lu, S. H., Liu, B., and Han, Z. C. (2008) Biochem. Biophys. Res. Commun. 367, 553–559
14. Awasthi, A., Carrier, Y., Peron, J. P., Bettelli, E., Kamanaka, M., Flavell, R. A., Kuchroo V. K., Oukka, M., and Weiner, H. L. (2007) Nat. Immunol. 8, 1380–1389
15. Ru¨ckerl, D., Hessmann, M., Yoshimoto, T., Ehlers, S., and Ho¨lscher, C. (2008) Immunobiology 213, 779–790
16. Imamichi, T., Yang, J., Huang, D. W., Brann, T. W., Fullmer, B. A., Adelsberger, J. W., Lempicki, R. A., Baseler, M. W., and Lane, H. C. (2008) AIDS 22, 39–45
17. Kalliolias, G. D., and Ivashkiv, L. B. (2008) J. Immunol. 180, 922–930
18. Donnelly, R. P., and Kotenko, S. V. (2004) J. Biol. Chem. 279, 2041–2046
19. Martinasso, G., Saracino, S., Maggiora, M., Oraldi, M., Canuto, R. A., and Muzio, G. (2009) Cancer Lett. 287, 62–66
20. Bernardino, A. L., Kaushal, D., and Phillipp, M. T. (2009) J. Infect. Dis. 199, 1379–1388
21. Pearson, A. L., Colville-Nash, P., Kwan, J. T., and Dockrell, M. E. (2008) J. Nephrol. 21, 887–893
22. Takeda, Y., Takeda, Y., Luster, A. D., Mizuguchi, J., and Yoshimoto, T. (2006) J. Immunol. 177, 7317–7324
23. Shimizu, M., Shimamura, M., Owaki, T., Asakawa, M., Fujita, K., Kudo, M., Ikawara, Y., Takeda, Y., Luster, A. D., Mizuguchi, J., and Yoshimoto, T. (2006) J. Immunol. 177, 7317–7324
24. Bonecchi, R., Bianchi, G., Bordignon, P. P., D’Ambrosio, D., Lang, R., Borst, A., Sozzani, S., Allavena, P., Gray, P. A., Mantovani, A., and Sagnaglia, F. (1998) J. Exp. Med. 187, 129–134
25. Charo, I. F., and Ransohoff, R. M. (2006) N. Engl. J. Med. 355, 610–621
26. Hunter, C. A. (2005) Nat. Rev. Immunol. 5, 521–531
27. Sato, T., Selleri, C., Young, N. S., and Maciejewski, J. P. (1997) Blood 90, 4749–4758
28. Sheikh, F., Baurin, V. V., Lewis-Antes, A., Shah, N. K., Smirnov, S. V., Anantha, S., Dickensheets, H., Dumoutier, L., Renauld, J. C., Zdanov, A., Donnelly, R. P., and Kotenko, S. V. (2004) J. Immunol. 172, 2006–2010
29. Stancato, L. F., David, M., Carter-Su, C., Larner, A. C., and Pratt, W. B. (1996) J. Biol. Chem. 271, 4134–4137