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Evidence for IL-12-Activated Ca\(^{2+}\) and Tyrosine Signaling Pathways in Human Neutrophils

Kate Collison, Soad Saleh, Ranjit Parhar, Brian Meyer, Aaron Kwaasi, Khalid Al-Hussein, Sultan Al-Sedairy, and Futwan Al-Mohanna

The cytokine IL-12 is proposed to play a bridging role between innate and adaptive immunity. Here we demonstrate that IL-12 binds specifically to human neutrophils. This binding leads to a transient increase in 1) intracellular free calcium due to its release from membrane-enclosed stores and its influx from extracellular medium, 2) actin polymerization, and 3) tyrosine phosphorylation. IL-12 treatment also leads to a concentration-dependent increase in reactive oxygen metabolite production. The effect of IL-12 is blocked by neutralizing Abs to IL-12. Inhibition of either calcium transient or tyrosine phosphorylation causes inhibition of reactive oxygen metabolite production. However, inhibition of actin polymerization enhances IL-12-induced oxidative activation. Our data suggest 1) a direct role for IL-12 in the activation of human neutrophils, and 2) a calcium-dependent signaling pathway for IL-12.


Interleukin-12 is a heterodimeric cytokine known to be active early in innate and adaptive immunologic responses to bacterial and parasitic infections. It is produced primarily by phagocytic cells (1, 2) and has a broad range of activities, including enhancement of NK and CTL activities (3) and induction of cytokine production, particularly IFN-γ from NK and T cells (3). IL-12 and IL-12-induced IFN-γ prime CD\(^{4}\) T cells, resulting in differentiation and proliferation of Th1 cells (4). Three classes of receptor (IL-12R) with different affinities for IL-12 have been identified on T lymphoblast cell membranes (5–7). Their engagement has been associated with phosphorylation of two members of the Janus kinase (JAK)\(^{1}\) family, namely JAK2 and TYK2 tyrosine kinases (8), and three STATs, namely STAT1, -3, and -4 (9).

Neutrophils, the predominant circulating leukocytes, play a central role in the innate defense against invading micro-organisms. Neutrophil production of IL-12 is well established, and based on their sheer numbers, neutrophils are considered the major producer of IL-12 in vivo (10, 11). Although the immunoregulatory role of IL-12 is well characterized, its direct effect(s) on innate immune cells such as neutrophils has yet to be defined.

In this paper we show the direct binding of IL-12 to human neutrophils. This binding causes a transient calcium rise and is accompanied by actin polymerization, tyrosine phosphorylation, and increased production of reactive oxygen metabolites (ROM). Our data demonstrate the existence of IL-12R on human neutrophils and directly implicate IL-12 in calcium-dependent activation of neutrophils.

Materials and Methods

IL-12 was obtained from Genetics Institute (Cambridge, MA) and R&D Systems (Minneapolis, MN). Fura-2/AM, thapsigargin, and ionomycin were purchased from Molecular Probes (Eugene, OR). Ab to IL-12 (sheep anti-human) was a gift from Dr. S. Wolf (Genetics Institute). Abs to IL-12R and protein A/G PLUS agaro conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs to actin were purchased from Sigma (St. Louis, MO). Mouse anti-phosphotyrosine Abs (PY Plus, clones PY-7E1, PY-1B2, PY20) were purchased from Zymed (San Francisco, CA). Rabbit polyclonal anti-STAT1, -2, -3, and -4 Abs and monoclonal anti-STAT5 and -STAT6 Abs were purchased from Santa Cruz Biotechnology. Secondary horseradish peroxidase-conjugated Abs were purchased from Promega (Madison, WI) and used according to the manufacturer’s instructions. All other reagents were AnalR grade and were purchased from BDH (Poole, U.K.). Fura-2/AM, ionomycin, and thapsigargin were dissolved in DMSO and delivered to the cells at final concentrations of 1, 2, and 2 µM, respectively, in a final DMSO concentration of 0.1%. LPS was routinely tested for and was not found.

Preparation of human neutrophils

Human peripheral blood neutrophils were prepared by dextran sedimentation of heparinized whole blood obtained from healthy donors and were centrifuged through Ficoll-Paque as described previously (12). Contaminating RBC were removed by hypotonic lysis with isotonic NH\(_{4}\)Cl. The remaining cells were suspended in Krebs-HEPES medium (pH 7.4) containing 120 mM NaCl, 1.3 mM CaCl\(_{2}\), 1.2 mM MgSO\(_{4}\), 4.8 mM KCl, 1.2 mM KH\(_{2}\)PO\(_{4}\), 25 mM HEPES, and 0.1% BSA and were further purified through neutrophil isolation medium (Cardinal Associates, Santa Fe, NM). Final purity and viability were both between 98 and 99% as indicated by flow cytometry and trypan blue dye exclusion tests.

Radioiodination and binding of IL-12

Preservative-free IL-12 was iodinated as described previously (13). The labeled protein was isolated on a Sephadex G-25 column, and its specific activity was determined (2.25 µCi/µg of protein). For binding experiments, 2.5 × 10\(^{5}\) neutrophils were seeded in 96-well, flat-bottom. Microtest III tissue culture plates (Becton Dickinson, Mountain View, CA) and allowed to adhere for 30 min at room temperature. Twenty microliters of radiolabeled IL-12 were added to each well in a total volume of 250 µl of cell suspension, and the cells were kept on ice for 1 h and washed thoroughly to remove any unbound radioactivity. The cells were harvested, and the radioactivity associated with them was measured in a gamma counter (CliniGamma 1272, LKB-Wallac, Turku, Finland) and expressed as a percentage of the total added radioactivity obtained from 20 µl of labeled protein. Specific binding was measured as the cell-associated radioactivity.

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\(3\) Abbreviations used in this paper: JAK, Janus kinase; ROM, reactive oxygen metabolite; \([\text{Ca}^{2+}]_{i}\), intracellular free Ca\(^{2+}\); LDCL, luminol-dependent chemiluminescence; F-actin, filamentous actin; \(K_{1/2}\), half-maximum response; BAPTA-AM, bis(2-aminophenoxy)ethane-N,N,N',N'-tetra-acetate acetyoxymethyl ester.
due to $^{125}$I}-rIL-12 alone minus that with $^{125}$I}-rIL-12 in the presence of a 50-fold molar excess of unlabeled rIL-12.

**Detection of IL-12R by indirect immunofluorescence**

Expression of IL-12R on neutrophils was investigated by FACS analysis (Becton Dickinson). Freshly isolated human neutrophils (2.5 x 10$^6$ cells in 250 $\mu$l) were incubated with anti-IL-12R Ab alone (10 ng/ml for 45 min on ice), with carrier-free human IL-12 (100 ng/ml, 30 min on ice) then with anti-IL-12R Ab as described above, or with nonimmune rabbit serum (non-specific binding). Cells were pelleted, washed, and incubated with FITC-labeled secondary Ab (Pierce, Rockford, IL) at a 1/50 dilution for 30 min on ice. Cells were pelleted, washed with PBS (three times), and resuspended in 500 $\mu$l of ice-cold PBS. Fluorescence analysis was performed using FACSscan (Becton Dickinson).

**Detection of IL-12R $\beta$1 chains by RT-PCR**

Quantitative extraction of total RNA from 5 x 10$^6$ neutrophils (>98% purity) or an equivalent number of mononuclear cells was performed using Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer's instructions. RT-PCR was used for semiquantitative analysis of transcript levels. cDNA was synthesized from the total RNA representative of neutrophils (2.5 x 10$^6$) or PBMCs (2.5, 0.125, 0.25, and 0.5 x 10$^6$), using AMV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's protocol. Sense and antisense primers for human IL-12R $\beta$1 chain were 5'-AGCTTCCAGAAGGCTGTCAAGG-3' (U03187; nucleotides 861–882) and 5'-GCTGCCATCTAATGCAATGC-3' (U03187; reverse complement of nucleotides 1159–1181), respectively. The total cDNA of each sample was amplified by PCR in a final volume of 50 $\mu$l containing 100 ng of each primer, 100 mM dNTPs, 50 mM KCl, 10 mM Tris, 1.5 mM MgCl$_2$, 50 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, and 1 U of Taq polymerase (Pharmacia, Uppsala, Sweden). Thirty-five cycles of denaturing for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C were used for amplification. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

**Measurement of calcium**

Neutrophils were loaded with fura-2/AM as described previously (14). The cells were observed on glass coverslips for 15 min at room temperature. Coverslips with adherent cells were rinsed with either Krebs-HEPES or modified Krebs-HEPES in which 1.3 mM CaCl$_2$ was substituted with 1 mM EGTA (calcium-free Krebs-HEPES). Coverslips were secured between two plates of a custom-designed coverslip holder and placed onto a heated microscope stage (33°C), and [Ca$^{2+}$]$\text{aq}$ measurements were performed on individual cells using the IonVision Dual excitation system (Immovision, Coventry, U.K.) as described previously (15).

Confocal calcium measurements were performed essentially as described previously (16). Images were acquired at 140-ms intervals, on the average, using a Leica DM IRBE inverted microscope attached to TCS confocal system (Leica-Kaki, Riyadh, Saudi Arabia). Images were converted into PICT format and analyzed by IonVision software (Immovision). Each image was divided (pixel by pixel) by an averaged image acquired before challenge with IL-12. The changes in calcium in divided images were calculated as described previously (16).

**Gel electrophoresis and Western blotting**

Unless otherwise stated, 10% SDS-PAGE was used throughout. SDS-PAGE and Western blotting were performed as described previously (17, 18).

**Actin polymerization measurements**

The extent of actin polymerization was measured by the increase in binding sites for rhodamine phallacidin as described previously (19, 20). Briefly, neutrophils (10$^6$/ml) were incubated at 37°C for 10 min in a stirred temperature-controlled chamber. Samples (100 $\mu$l) were drawn, and cells were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature. IL-12 (1 ng/ml) was added to the cells, and 100-$\mu$l samples were drawn at different time intervals and fixed as described above. Fixed samples were washed and permeabilized in 0.5% Triton X-100 in PBS for 5 min, washed three times with PBS, and then stained with rhodamine phallacidin (0.33 $\mu$m) for 1 h at room temperature. The fluorescence intensity of washed cells was measured using flow cytometry (FACScan, Becton Dickinson). Actin polymerization was also measured by SDS-PAGE and immunoblotting of the actin associated with the Triton X-100-insoluble cytoskeleton. SDS-PAGE of actin was performed essentially as described previously (21). Separated protein bands were electrotransferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA), and blots were washed twice with double-distilled water, preblocked with 5% nonfat milk in PBS for 1 h at room temperature, and rinsed with double-distilled water. Incubation with primary anti-actin Ab (4°C, overnight) was followed by two rinses in double-distilled water and three washes in PBS containing 0.02% Tween-20. Incubation with secondary horseradish peroxidase-conjugated Ab (1 h, 4°C) was followed by washing as described above and detection using enhanced chemiluminescence (ECL, Amersham, Aylesbury, U.K.).

**Measurement of tyrosine phosphorylation**

For phosphotyrosine and STAT Western blotting, the protocol used was essentially that supplied by the Ab manufacturer. Briefly, cells were lysed in a medium containing 20 mM Tris-HCl (pH 7.4); 1% Nonidet P-40; 1 mM sodium orthovanadate; 150 mM NaCl; 10 mM EGTA; 1 $\mu$m each of aprotonin, leupeptin, pepstatin A, and chymostatin; and 1 mM PMSF. The lysates were separated by SDS-PAGE and transferred onto nitrocellulose. Blots were washed and blocked with 4% BSA in PBS for 1 h at room temperature. Primary Abs were incubated at 4°C for 18 h at a concentration of 1 $\mu$g/ml. Secondary horseradish peroxidase-conjugated Abs were incubated with the blots for 2 h at the concentrations recommended by the manufacturer.

**Immunoprecipitation**

Cell lysates were prepared from 10$^7$ cells/ml in ice-cold cell lysis buffer. Debris was pelleted (15,000 x g), and lysates were precleared using protein A/G plus agarose (50 $\mu$l of a 40% slurry). Supernatant was incubated with 5 $\mu$g/ml of the designated primary Ab or serum at 4°C for 4 h, then with protein A/G plus agarose (30 $\mu$l of 40% slurry) at 4°C overnight. Immunoprecipitates were collected, washed, pelleted, and resuspended in electrophoresis sample buffer containing 20% glycerol, 8% SDS, 125 mM Tris-HCl, 10% β-mercaptoethanol, and 0.05% bromophenol blue. Finally, the samples were subjected to SDS-PAGE as described above.

**Results**

**Binding of IL-12 to human neutrophils**

Incubation of freshly prepared human neutrophils with $^{125}$I}-rIL-12 at 4°C resulted in a time-dependent specific binding (difference between binding observed with $^{125}$I}-rIL-12 and that in the presence of a 50-fold excess of unlabeled rIL-12) of the cytokine that reached a maximum within 1 h (Fig. 1A). This binding was concentration dependent, with half-maximum receptor occupancy occurring at 280 ± 80 PM (Fig. 1B). The kinetics of this binding fit a one-binding site model with a maximum of 1.7 ± 0.25 x 10$^5$ binding sites/neutrophil.

The presence of IL-12R on neutrophils was further demonstrated by indirect immunofluorescence and FACS analysis using anti-IL-12R Ab and by detection of IL-12R $\beta$1 mRNA by RT-PCR. FACS analysis revealed increased fluorescence intensity (relative to control) of neutrophils labeled with anti-IL-12R Ab, which was reversed by prior treatment of the cells with IL-12 (Fig. 1C). RT-PCR revealed the presence of IL-12R-$\beta$1 mRNA in human neutrophils (Fig. 1D). Since the neutrophil preparations are typically 98 to 99% pure (as detected by FACS analysis) with 1 to 2% contamination of other cell types, namely PBMC, the possibility existed that the IL-12R $\beta$1 monomers revealed by RT-PCR were a product of the contaminating PBMC rather than of neutrophils. This was tested by running RT-PCR of purified neutrophils (2.5 x 10$^5$ cells; Fig. 1D, lane 1) and various numbers of PBMC prepared from the same blood donor. Figure 1D, lanes 2, 3, 4, and 5, shows the IL-12R $\beta$1 monomers obtained from 2.5 x 10$^4$, 1.25 x 10$^4$, 2.5 x 10$^3$, and 5 x 10$^3$ PBMC, respectively. Even with 5 x 10$^4$ PBMC equivalent (Fig. 1D, lane 5), the amount of IL-12R mRNA
amplicons was less than that obtained from routinely prepared neutrophils (Fig. 1D, lane 1), suggesting that the signal in lane 1 is predominantly derived from mRNA of neutrophil origin.

**IL-12 evokes a transient rise in $[Ca^{2+}]_i$.**

Quiescent human neutrophils exhibit a resting calcium level of $81 \pm 7 \text{ nM}$. Upon stimulation with IL-12, a transient rise in $[Ca^{2+}]_i$ was evoked (Fig. 2), reaching a maximum of $663 \pm 119 \text{ nM}$. The calcium rise was both asynchronous and heterogeneous, with a dose-dependent increase in the number of cells displaying a calcium transient that was at least twofold higher than the resting levels. At IL-12 concentrations of 0.02, 0.1, and 1 ng/ml, the percentages of cells exhibiting a calcium transient were 32, 56, and 85%, respectively. Removal of the extracellular calcium reduced the extent of the IL-12-induced calcium rise. However, the rate of decay of the calcium transient was higher in the absence of extracellular calcium than in normal extracellular calcium concentrations (Fig. 3, a and b), suggesting an extracellular calcium influx component to the intracellular transient. Calcium influx was tested directly by using Mn$^{2+}$ as the surrogate ion following IL-12 challenge. In a series of experiments, the fluorescence of fura-2-labeled neutrophils was monitored using two excitation wavelengths of 340 nm (calcium sensitive) and 360 nm (calcium insensitive). Under such conditions IL-12 caused a sharp rise in fura-2 emission.

**FIGURE 1.** Binding of IL-12 to human neutrophils. Binding experiments were performed as described in Materials and Methods. A. A representative experiment showing the time-dependent binding of $[^{125}\text{I}]$IL-12 to human neutrophils. B. Specific binding of $[^{125}\text{I}]$IL-12 to neutrophil membrane. Specific binding was calculated as the difference between radioactivity in the absence and that in the presence of a 50-fold excess of unlabeled IL-12. Nonspecific binding accounted for $20.8 \pm 1.4\%$ at the highest concentration of labeled IL-12. Each point represents the mean $\pm$ SEM ($n = 3$). Analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). C. Expression of IL-12R in human neutrophils as revealed by FACS analysis. Cytofluorograms of neutrophils incubated with nonimmune rabbit serum (control), anti-IL-12R Ab (αIL-12R), or anti-IL-12R Ab after treatment with IL-12 (αIL-12R after IL-12). The data show a representative experiment in which binding of αIL-12R Ab to human neutrophils was reversed by prior treatment of the cells with IL-12. The experiment was performed as described in Materials and Methods. The graph on the right shows the same data expressed as mean fluorescence of cells per treatment. D. RT-PCR of IL-12R β1-chain transcripts from human neutrophils and PBMC isolated from the same donor. Amplicons were from: lane 1, purified neutrophils (98–99% purity by FACS analysis); lane 2, equivalent number of PBMC ($2.5 \times 10^5$); lane 3, $1.25 \times 10^5$ PBMC; lane 4, $2.5 \times 10^4$ PBMC; and lane 5, $5 \times 10^4$ PBMC.
due to the 340-nm excitation wavelength and a concomitant decrease in emission due to the 360-nm excitation (Fig. 3c). The former result suggests the release of calcium from an internal store(s), and the latter result suggests a direct influx of calcium from the extracellular medium. The ability of IL-12 to cause calcium influx was confirmed by the partial inhibition of IL-12-induced calcium transients in the presence of Cd\(^{2+}\) and Ni\(^{2+}\) (data not shown). Depletion of the [Ca\(^{2+}\)]\_i store(s) by pretreatment of neutrophils with thapsigargin caused a rapid increase in [Ca\(^{2+}\)]\_i, reaching a sustained level of 407 \pm 50 nM. Further addition of IL-12 failed to induce any significant calcium rise (Fig. 3d). The IL-12-induced calcium rise was also inhibited by pretreatment of neutrophils with pertussis toxin (1 \mu g/ml), suggesting the involvement of heterotrimeric G proteins (Fig. 3e). The IL-12-sensitive calcium store(s) was visualized directly using fast (temporal resolution of 140 ms/frame) confocal laser scanning microscopy of fluo-3/AM-labeled neutrophils. Treatment of these cells with IL-12 revealed a small, rapid, and diffused increase in cytosolic calcium followed by larger increases within punctate areas with the eventual global rise throughout the cytosol (Fig. 3f).

**Induction of tyrosine phosphorylation by IL-12**

Since IL-12-mediated responses in a variety of cell types have been associated with tyrosine phosphorylation (8, 26, 27), the possibility existed that similar effects may be seen with human neutrophils. IL-12 elicited an increase in tyrosine phosphorylation of a number of proteins compared with that in unstimulated control cells (Fig. 5A). Five major bands of relative molecular masses of 78, 75, 52, 36, and 26 kDa and several minor bands were detected with antiphosphotyrosine Abs following IL-12 challenge. The occurrence of these bands followed similar kinetics, with maximal phosphorylation occurring within 1 to 5 min of stimulation before decaying to prestimulatory levels within 20 min. Interestingly, the phosphotyrosine inhibitor genistein (20 ng/ml) attenuated the phosphorylation state of some, but not all, bands induced by IL-12 (Fig. 5B). Since the IL-12-induced calcium transient preceded tyrosine phosphorylation, the possibility existed that the calcium transient was a prerequisite for the phosphorylation step. IL-12-induced tyrosine phosphorylation was inhibited in neutrophils treated with the calcium chelator BAPTA-AM before IL-12 encounter (Fig. 5B).

**Direct activation of human neutrophils by IL-12**

The effect of IL-12 on neutrophil function was tested by monitoring the production of ROM. In a series of experiments human neutrophils were treated with IL-12 (1 ng/ml), and the actin associated with the Triton-insoluble cytoskeleton was run on SDS-PAGE followed by Western blotting and probing with anti-actin mAb. Under such conditions a transient rise in the amount of actin associated with the cytoskeleton was observed, reaching a maximum at about 60 s (Fig. 4B).

**IL-12 induces transient actin polymerization in human neutrophils**

One of the earliest events following neutrophil activation is a rapid and transient increase in actin polymerization (20). Using flow cytometry to measure the binding of rhodamine phallacidin to F-actin, IL-12 (1 ng/ml) caused a rapid and transient increase in the number of binding sites for rhodamine-phallacidin (Fig. 4A). This is consistent with a transient rise in actin polymerization, which reached a maximum within 60 s and decayed slowly to prestimulatory levels. This rise was only partially affected by inhibition of the calcium transient with bis(2-aminophenoxy)ethane-N,N',N',N'\_-tetra-acetate acetoxymethyl ester (BAPTA-AM) or by inhibition of tyrosine phosphorylation by the phosphotyrosine inhibitor genistein (Fig. 4A). To further confirm the effect of IL-12 on actin polymerization we measured the amount of actin associated with the Triton X-100 insoluble cytoskeleton. In a series of experiments neutrophils were treated with IL-12 (1 ng/ml), and the actin associated with the Triton-insoluble cytoskeleton was run on SDS-PAGE followed by Western blotting and probing with anti-actin mAb. Under such conditions a transient rise in the amount of actin associated with the cytoskeleton was observed, reaching a maximum at about 60 s (Fig. 4B).
FIGURE 3. Averaged calcium responses to IL-12 in the presence (a) and the absence (b) of extracellular calcium. Each point represents the mean ± SEM. Arrowheads indicate the time of addition of IL-12. Calcium release and influx are illustrated in c, where fura-2 emission was monitored following excitation at 340 nm (squares, calcium sensitive) and 360 nm (diamond, calcium insensitive) in neutrophils bathed in Krebs-HEPES medium containing 100 μM Mn²⁺. The first arrowhead indicates the time of introduction of Mn²⁺ to the medium, and the second arrowhead indicates the addition of IL-12. Each point represents the mean ± SEM. d, Depletion of the intracellular calcium store by thapsigargin (10 μg/ml) inhibits the IL-12-induced calcium rise. The first arrow indicates the time of thapsigargin addition, and the second arrow indicates the time of IL-12 addition. Each point represents the mean ± SEM. e, Inhibition of IL-12-induced calcium transient in pertussis toxin-treated neutrophils. The arrowhead indicates the time of addition of IL-12. f, Calcium release from the intracellular store(s) in response to IL-12. Confocal micrographs of intracellular free calcium before (0 s) and at the indicated time intervals after IL-12 treatment. High levels of calcium are indicated by “hot” colors (color bar). The scale bar is 7 μm. Data are representative of four experiments with cells obtained from four individuals.
the presence of neutralizing Ab to IL-12 (Fig. 6b). Since neutrophil ROM production is intimately linked to both the \([\text{Ca}^{2+}]_{i}\), transient and actin polymerization (14, 28, 29), we investigated the effects of inhibition of IL-12-induced calcium transients and IL-12-induced actin polymerization on ROM production. Whereas inhibition of the \([\text{Ca}^{2+}]_{i}\) transient by preincubation with BAPTA-AM totally abolished ROM production (Fig. 6c), inhibition of actin polymerization by cytochalasin B enhanced ROM production (Fig. 6d). This enhancement was also abolished by inhibition of the IL-12-induced calcium rise with BAPTA-AM. Similarly, genistein caused inhibition of IL-12-induced ROM production (Fig. 6e), suggesting an intimate correlation between tyrosine phosphorylation and IL-12-induced oxidase activation. The potency of IL-12 to induce LDCL was compared with that of other known neutrophil activators, namely the chemokine IL-8 and the chemotactic peptide FMLP. IL-12 was several orders of magnitude more potent than either IL-8 or FMLP, with half-maximum responses \((K_{1/2})\) occurring at 6.0 ± 1.4 pM, 2.0 ± 0.9 nM, and 0.24 ± 0.06 \(\mu\)M for IL-12, IL-8, and FMLP, respectively.

**Discussion**

IL-12 is a heterodimeric proinflammatory and immunoregulatory cytokine produced by many cells in response to soluble and particulate stimuli (13, 30, 31). Its production by phagocytic and APCs has been demonstrated in early stages of infection (10, 32–36). Biologically active IL-12 induces the production of IFN-\(\gamma\) and TNF-\(\alpha\) from NK and Th1 cells, both of which are potent regulators of phagocytic cell function, and further enhances cell-mediated immunity by activation of macrophages and production of opsonins such as IgG2a isotype (38–41). IL-12 also induces the expansion of Th1 cells, which produce more IL-2 and IFN-\(\gamma\), and inhibits IL-4-producing cells (1, 42, 43). It has been proposed that immune responses are regulated by the balance between IL-12, which favors Th1 responses, and IL-4, which favors Th2 responses (43). IL-12 involvement in the activation of professional phagocytes such as macrophages and neutrophils has been suggested (44, 45), and directional migration of human neutrophils in response to IL-12 has been reported (45). In addition, IL-12 administration to mice is known to cause a decrease in the number of circulating leukocytes/neutrophils (46, 47), whereas administration of IL-12 together with *Candida albicans* causes an impaired neutrophil response (47).

In the present work we report that human neutrophils express functional IL-12Rs, as illustrated by 1) the presence of IL-12R \(\beta 1\) mRNA (Fig. 1D), 2) the expression of IL-12R on the neutrophil membrane (detected by anti-IL-12R Ab) and the ability of IL-12 to attenuate the binding of anti-IL-12R Ab to neutrophils (Fig. 1C),
3) the specific binding of IL-12 to neutrophils (Fig. 1B), and 4) the ability of IL-12 to activate human neutrophils. IL-12 specifically binds and activates human neutrophils in a concentration-dependent manner. The $K_d$ for binding was 280 ± 80 pM. Three binding sites for IL-12 exist on T lymphoblast cell membranes; high, medium, and low affinity binding sites (5, 6). Because of the low $K_d$ for binding, the neutrophil membrane is likely to have the medium to low affinity receptors. It is noteworthy, however, that the difference between the reported kilodaltons may be due to differences in the specific activity associated with the recombinant proteins.

Whereas IL-12-induced responses in a variety of cell types are known to be mediated via the JAK/STAT signaling pathway (8, 9, 27), the existence of other signaling pathways for IL-12 has not been reported. Here we demonstrate that treatment of neutrophils with IL-12 causes a concentration-dependent and transient rise in intraneutrophil-free calcium. This rise is due to the release of $[\text{Ca}^{2+}]_i$ from an intracellular membrane-enclosed store(s) and its influx from the extracellular medium. Evidence for this derives from the fact that 1) removal of extracellular calcium reduces the amplitude of the IL-12-induced calcium transient; and 2) experiments in which calcium was substituted for the surrogate ion Mn$^{2+}$ revealed a decrease in fura-2 fluorescence due to the calcium-independent excitation wavelength of 360 nm, suggesting the influx of Mn$^{2+}$. The calcium rise was inhibited by treatment of neutrophils with pertussis toxin (1 mg/ml) before IL-12 challenge, suggesting the involvement of heterotrimeric G proteins. The ability of IL-12 to release calcium from an intracellular store(s) was further confirmed using confocal laser scanning microscopy at temporal and spatial resolutions of 140 ms and 3 μm, respectively. The fast temporal acquisition was needed to directly demonstrate the IL-12-induced release of the $[\text{Ca}^{2+}]_i$ store. Challenging fluo-3/AM-loaded neutrophils with IL-12 revealed a small global rise in cytosolic calcium followed by a localized and punctate rise within the cytosol, suggesting areas of $[\text{Ca}^{2+}]_i$ storage. The source of the initial small global rise before emptying of the calcium store(s) by IL-12 has yet to be determined.

Since calcium transients are intimately linked to the tyrosine phosphorylation signaling pathway, we tested the effect of IL-12 on phosphotyrosine levels. We found that IL-12 treatment induced phosphorylation of a number of proteins.

Neutrophil activation is normally coupled to transient actin polymerization in response to many soluble stimuli (28, 29). Here we demonstrate that IL-12 causes actin polymerization, as detected by increased binding sites for rhodamine phallacidin and by an increase in the amount of actin associated with the Triton X-100-insoluble cytoskeleton. Following IL-12 treatment, a biphasic actin response occurred, with polymerization reaching a maximum within 60 s, followed by a slow depolymerization. F-actin levels were still significantly higher than the resting levels after 5 min of IL-12 challenge. Inhibition of the IL-12-induced calcium rise by BAPTA-AM had no apparent effect on the extent or the kinetics of actin polymerization. Furthermore, inhibition of tyrosine phosphorylation had no significant effect on actin polymerization. It is therefore reasonable to speculate that at least two independent intracellular signals occur following IL-12R engagement on the neutrophil membrane: one for calcium transients and another for actin polymerization. It is noteworthy that both calcium changes and actin polymerization exhibited slow kinetics, which may suggest an indirect effect of IL-12 on the neutrophils, perhaps through the generation of secondary signals induced by IL-12.

FIGURE 6. ROM production in human neutrophils in response to IL-12 treatment. a, Concentration-dependent increase in LDCL obtained from 10$^6$ neutrophils. The arrowheads show the time of addition of the indicated concentrations of IL-12. The inset shows a representative experiment examining the IL-12-induced increase in oxygen consumption rate as measured by a Clark-type oxygen electrode. The arrowhead indicates the time of addition of medium alone (upper trace) or with IL-12 (lower trace). The data were obtained from 1.3 × 10$^6$ cells in a total volume of 1.5 ml. b, ROM production by IL-12 and its inhibition by mAb to IL-12. c, Inhibition of IL-12-induced ROM production by pretreatment with the intracellular calcium chelator BAPTA-AM. d, Enhancement of IL-12-induced ROM production by cytochalasin B (CB) and its inhibition by BAPTA-AM. e, Inhibition of IL-12-induced ROM production by genistein (20 ng/ml).
The involvement of calcium transients in activation of the neutrophil oxidase system to produce ROM is well established. We tested the hypothesis that the IL-12-induced calcium rise was necessary for ROM production by neutrophils. In a series of experiments we demonstrated that IL-12 caused ROM production in a dose-dependent manner. The $K_{1/2}$ for ROM production was 6.0 ± 1.4 pM, suggesting that only a relatively small number of the total IL-12Rs need to be occupied to evoke ROM response. Using pulse binding techniques, Painter et al. (48) found that for $[Ca^{2+}]$, cAMP, and membrane potential responses, <5% of the total FMLP receptors need to be occupied. For superoxide production, however, the receptor occupancy needed was around 30%. Sklar et al. (49) reported that for the initial phase of actin polymerization in response to FMLP, only 100 receptors/neutrophil (of 50,000) are required. Based on the $K_{1/2}$ for ROM and the $K_d$ for binding, it is estimated that <2% of human IL-12R need to be engaged to evoke 50% of the maximum IL-12-induced ROM production.

That ROM induction was due to activation of the neutrophil oxidase system was confirmed by the increased rates of oxygen consumption following neutrophil challenge with IL-12. ROM production was inhibited in cells pretreated with calcium chelator BAPTA/AM. Similarly, the tyrosine kinase inhibitor genistein inhibited the IL-12-induced ROM production. The question therefore arose as to whether genistein also inhibited the IL-12-induced calcium transient. Genistein alone had no apparent effect on resting neutrophil calcium (data not shown). Furthermore, pretreatment of neutrophils with genistein at concentrations known to inhibit tyrosine phosphorylation had no apparent effect on the IL-12-induced calcium transient.

Since actin polymerization occurs within the time of $[Ca^{2+}]$, changes, the possibility of a direct role for actin polymerization in the activation of the neutrophil oxidase system has been suggested (29). It is thought that the degree of ROM production is inversely proportional to the amount of F-actin within an activated neutrophil (29). Our present findings show that inhibition of actin polymerization enhances IL-12-induced ROM production and also confirm the role of F-actin in oxide activation.

In this paper we demonstrate that human neutrophils express functional IL-12Rs that upon engagement by IL-12 activate the cells via a calcium-dependent mechanism(s). This activation leads to increased ROM production. Human IL-12R can exist as human IL-12Rβ1 and human IL-12β2, each independently exhibiting low affinity binding to IL-12, and/or a human IL-12β1β2 complex, exhibiting high affinity binding (50–54). Neither human IL-12Rβ1 nor human IL-12Rβ2 contains the seven-span membrane motif characteristic of G protein-dependent and pertussis toxin-sensitive receptor signaling, yet their engagement by IL-12 exhibits both G protein-dependent and pertussis toxin-sensitive signaling. This apparent anomaly is by no means restricted to human IL-12R. Hepatocyte growth factor induces phospholipid signaling and calcium changes in a pertussis toxin-sensitive fashion (55). Furthermore, epidermal growth factor has been shown to stimulate inositol 1,4,5-trisphosphate production and $[Ca^{2+}]$, which is inhibited by pretreatment with pertussis toxin (56). Neither the hepatocyte growth factor receptors nor the epidermal growth factor receptors exhibit the seven-transmembrane motif. The membrane events leading to stimulus-response coupling for this class of receptors have yet to be defined. Although neutrophil migration in response to IL-12 has previously been demonstrated (45), to our knowledge our work is the first to report a direct effect of IL-12 on calcium homeostasis, actin polymerization, and ROM production in phagocytic cells. The existence of a calcium signaling pathway in IL-12-mediated responses has many implications, especially with respect to the JAK/STAT signaling pathway. It is noteworthy that the calcium ionophore ionomycin “interrupts” JAK/STAT signaling in mononuclear cells following IL-6 stimulation (57). A similar mechanism may exist in human neutrophils following the IL-12-induced rise in calcium.

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