Neutrophils alter the inflammatory milieu by signal-dependent translation of constitutive messenger RNAs

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The mechanisms by which neutrophils, key effector cells of the innate immune system, express new gene products in inflammation are largely uncharacterized. We found that they rapidly translate constitutive mRNAs when activated, a previously unrecognized response. One of the proteins synthesized without a requirement for transcription is the soluble IL-6 receptor α, which translocates to endothelial cells and induces a temporal switch to mononuclear leukocyte recruitment. Its synthesis is regulated by a specialized translational control pathway that is inhibited by rapamycin, a bacterial macrolide with therapeutic efficacy in transplantation, inflammatory syndromes, and neoplasia. Signal-dependent translation in activated neutrophils may be a critical mechanism for alteration of the inflammatory milieu and a therapeutic target.

Neutrophils are essential cellular components of the innate immune system that have conserved roles in bacterial containment and wound surveillance and provide early links between innate and adaptive inflammatory responses (1–5). Paradoxically, however, neutrophils also mediate tissue injury in varied human diseases that include sepsis, inflammatory lung, bowel, and joint diseases, acute coronary syndromes and other sequelae of atherosclerosis, and additional conditions of dysregulated inflammation (4–9).

Neutrophils, also called polymorphonuclear leukocytes (PMNs), are the most numerous myeloid leukocytes and are first responders in microbial invasion and tissue injury (4, 5, 10). When activated by a variety of receptor-mediated agonists, PMNs are specialized for rapidly induced changes in phenotype and function including adhesion, migration, phagocytosis, synthesis of biologically active lipids, generation of reactive oxygen species, and degranulation of stored inflammatory peptides and proteins (4, 5, 11). Factors released by activated neutrophils or displayed on their surfaces then mediate local tissue responses to infection and injury and also provide molecular cues that orchestrate participation of the adaptive immune system (4, 5, 11).

Many PMN-activation responses use constitutive biochemical pathways and enzymes and do not involve altered gene expression or new protein synthesis (5). Until recently PMNs were in fact thought to have little or no capacity for regulated expression of new gene products after leaving the marrow, but it is now clear that they synthesize specific proteins when appropriately stimulated (12–15). Little is known regarding control of gene expression in PMNs. Mechanisms identified to date largely involve transcriptional regulation (12, 14, 15), although there is also evidence that posttranscriptional checkpoints are used (14, 16, 17).

Translational control of expression of specific proteins confers important biologic advantages, including the potential for rapid synthesis by using constitutive mRNAs without need for new transcript generation, processing, or nuclear export (18–22). In addition, translational regulation contributes to tight control of synthesis of cytokines, growth factors, angiogenic peptides, and other critical protein products when transcriptional and posttranscriptional checkpoints operate in concert (19–22). These features of gene regulation may be particularly important for the activities of specialized innate defensive cells such as PMNs because of the kinetics of their inflammatory responses and their production of potent mediators in response to outside-in signals. Recently, we found that human platelets and monocytes, which, like neutrophils, are acutely targeted to wounds and sites of inflammation (3, 5), rapidly synthesize highly regulated factors by using specialized translational control pathways (18, 19, 23–25). For specific proteins, translational control is accomplished by a pathway centered on mammalian target of rapamycin (mTOR) (18, 19). This intracellular kinase regulates initiation of translation of a subset of mRNAs, influences mitogenesis, growth, and metabolic control in response to signals from the environment in several cell types, and is a therapeutic target in transplantation and neoplasia (20, 26, 27). However, it was not previously known to influence acute changes in phenotype of terminally differentiated innate effector cells (18, 19). Here we demonstrate that PMNs have specialized signal-dependent translational control mechanisms and use regulation by mTOR for rapid synthesis and release of a key inflammatory modulator, IL-6 receptor α subunit (IL-6Rα). IL-6Rα is a central component in the IL-6/IL-6Rα trans-signaling system, which mediates complex inflammatory events, including a temporal switch in the pattern of cellular recruitment from PMN to mononuclear leukocyte accumulation that is critical for resolution of the acute inflammatory response and also for the transition from acute to chronic inflammation (7, 10, 28–31). Translation of IL-6Rα from constitutive mRNA is induced when neutrophils are activated by platelet-activating factor (PAF), a signaling phospholipid that is implicated in both homeostatic and injurious inflammation (32), as well as by other inflammatory agonists, and is specifically inhibited by the therapeutic macrolide rapamycin. Together, these observations identify a previously unrecognized mechanism by which PMNs express critical proteins by specialized signal-dependent translation and can thereby alter the acute inflammatory milieu.

Methods

Cells, Activation, and Inhibitors. PMNs were isolated from volunteer subjects, and primary human umbilical vein endothelial cell (HUVEC) monolayers were cultured in Transwell chambers

Abbreviations: PMNs, polymorphonuclear leukocytes; mTOR, mammalian target of rapamycin; IL-6Rα, IL-6 receptor α subunit; PAF, platelet-activating factor; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharide; fMLP, *N*-formylmethionylleucylphenylalanine; EC, endothelial cells.

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(Costar) by using minor modifications of previously described methods (13, 24, 28, 33). These protocols were approved by the University of Utah Institutional Review Board.

PMNs were activated with PAF (Biomol, Plymouth Meeting, PA) in concentrations of 10 or 100 nM for 30 min or 1 h except as indicated. In coincubations with HUVEC, they were separated from the endothelial monolayers by semipermeable membranes in Transwell incubation chambers (Costar). In specific experiments 100 fg/ml bacterial lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4, Sigma), 300 units/ml tumor necrosis factor α (TNFα) (Sigma), or 100 nM *N*-formylmethionylleucylphenylalanine (fMLP) (Sigma) was used as the PMN agonist. Ten or 100 nM rapamycin (Calbiochem) and 5 μg/ml actinomycin D (Sigma) were preincubated with PMNs for 30 min. Additional details are given below.

cDNA Array Analysis. PMNs (250×10^6 cells per ml) were activated with 100 nM PAF or left quiescent. RNA was isolated by using TRIzol (GIBCO/BRL) according to the manufacturer's recommendations. First-strand synthesis and labeling with [γ-^{32}P]dATP were performed with SuperScript II reverse transcriptase (GIBCO/BRL) by using a primer set supplied by the manufacturer of the cDNA array (Cancer Array 7742-1, Clontech). Probe purification, hybridization, washing, and developing (19, 24) were performed as specified by the manufacturer.

Two-Dimensional Gel Electrophoresis. Neutrophils were activated with 10 nM PAF in the presence of [^{35}S]methionine for 4 h at 37°C in a 5% CO_2 atmosphere alone or after pretreatment with rapamycin or actinomycin D. Control cells remained untreated. The PMNs were pelleted and immediately lysed in CHAPS lysis buffer (1% DTT/4% CHAPS/54% urea/5% Ampholine, preblended) in H_2O (Amersham Pharmacia). Isoelectric focusing was accomplished by using tube gels as described elsewhere (S.W.L. and A.S.W., unpublished work). The gels were removed, layered onto SDS/10% polyacrylamide slabs, dried in a vacuum dryer, and exposed to Kodak MS film.

RT-PCR. The primer pair for IL-6Rα was purchased from Stratagene, and RT-PCR was carried out as reported (18, 19, 24). First-strand synthesis was performed as described above by using SuperScript II reverse transcriptase and oligo(dT) primers (both from GIBCO/BRL). PCR for GAPDH was performed as described (18, 19, 24). Real-time quantitative RT-PCR was performed by using an ABI Prism 770 sequence detection system and a TaqMan MGB probe (Applied Biosystems).

Immunocytochemistry. PMNs (5×10^6 cells per ml) were incubated in control buffer or were activated with PAF for 30 min at 37°C. Equal volumes (50 μl) of PMN suspension and Hanks' balanced salt solution (HBSS) containing 0.2% human serum albumin were deposited onto glass coverslips by using a cytocentrifuge, fixed with 4% paraformaldehyde at room temperature, washed three times with PBS, permeabilized in PBS/0.05% Triton X-100 for 5 min, washed three additional times with PBS, and blocked with filtered PBS containing 5.0% goat serum. Antibodies against IL-6Rα (rabbit polyclonal anti-IL-6R, sc-661, C-2, Santa Cruz Biotechnology) or mTOR (rabbit polyclonal anti-mTOR, KAP-P1002, StressGen Biotechnologies, Victoria, BC, Canada) were incubated with the cells overnight in HBSS/5.0% goat serum at 4°C. In some experiments the peptide immunogen (SC-661P for anti-IL-6Rα and KPT-P100 2E for anti-mTOR), obtained from the manufacturer, was included in the overnight incubation to compete for antibody binding to cellular IL-6Rα or mTOR. After washing three times with PBS the PMNs were incubated with the secondary antibody (goat anti-rabbit, conjugated to Alexa 488, Molecular Probes), washed again three times with PBS, incubated in phalloidin conjugated to Alexa 617 for 30 min at room temperature, washed three times, and incubated with propidium iodide (15 μg/ml) for 5 min at room temperature for nuclear staining. After three washes in PBS the cells were stored until examined by confocal microscopy (18, 24, 25).

Western Blot Analysis. Neutrophils in suspension were activated with PAF, fMLP, TNFα, or LPS for the designated time periods alone or after pretreatment with rapamycin or actinomycin D, treated with Protease-Arrest (Geno Technology, St. Louis) to prevent degradation of neutrophil proteins (12, 13), centrifuged, and lysed. The pellet was resuspended in Laemmli gel-loading buffer and boiled for 5 min. The IL-6Rα and S6K1 proteins were separated by using SDS/8% PAGE and 4E-BP1 by using SDS/12% PAGE and blotted onto poly(vinylidene difluoride) (PVDF) membranes (Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 (Merck). The following antibodies were used: sc-661 for IL-6Rα, sc-230 for S6K1 kinase, and sc-6025 for 4E-BP1 (all from Santa Cruz Biotechnologies). A goat horseradish peroxidase-conjugate (AMI0404/ALI3404, BioSource International, Camarillo, CA) was used as the secondary antibody. Detection was performed as described (24, 25).

Results and Discussion

PMNs Synthesize New Proteins in Response to Cellular Activation. We first examined mRNA profiles in freshly isolated human PMNs under basal conditions and after activation by PAF. Many RNAs were present in unstimulated PMNs when both a small commercial arrayed cDNA library and a larger in-house library displaying over 10,000 cDNAs were interrogated, similar to reports by others (14, 15, 34). When PMNs were activated with nanomolar concentrations of PAF for 2 h the transcripts for only 12 known genes were increased $\geq$4-fold when the in-house library was used ($n = 3$) and an equally small number was up-regulated by using the commercial array ($n = 2$). Whereas this result likely underestimates the total number of transcripts induced or amplified by engagement of the PAF receptor at this and later time points, it suggested an abbreviated acute transcriptional response. In contrast, proteomic analysis using radiolabeling with [^{35}S]methionine and separation of products by two-dimensional gel electrophoresis revealed that many new proteins are synthesized within 2–4 h of stimulation with PAF (Fig. 1 *A* and *B* and data not shown). The disparity between the patterns of transcript and protein expression suggests translational control of some of the protein products (18, 19, 24, 35, 36) and that the PAF receptor delivers outside-in signals to translational checkpoints in addition to its known capacity to activate transcriptional pathways (32). Consistent with this, PAF triggers translocation of mRNAs from monosomes to polyribosomes, a feature of actively translated mRNAs (36), in a surrogate neutrophil system (unpublished data). Synthesis of some newly expressed radiolabeled proteins by PAF-stimulated neutrophils was preserved after treatment of the PMNs with the transcriptional inhibitor actinomycin D (Fig. 1*D* and data not shown; also see below), suggesting that new generation of the mRNA is not required.

IL-6Rα Is a Candidate for Translational Regulation in PMNs. To examine the physiologic significance of these findings, we identified rapidly synthesized proteins in neutrophils stimulated with PAF and mechanisms that regulate their production. The mRNA for IL-6Rα was basally present in PMNs in the expression-array analysis outlined above, suggesting that it can be used as a template for rapid synthesis of IL-6Rα protein. We then examined two-dimensional gels from PAF-activated neutrophils and found that mobility characteristics of one radiolabeled protein were consistent with IL-6Rα (Fig. 1*B*). The roles of

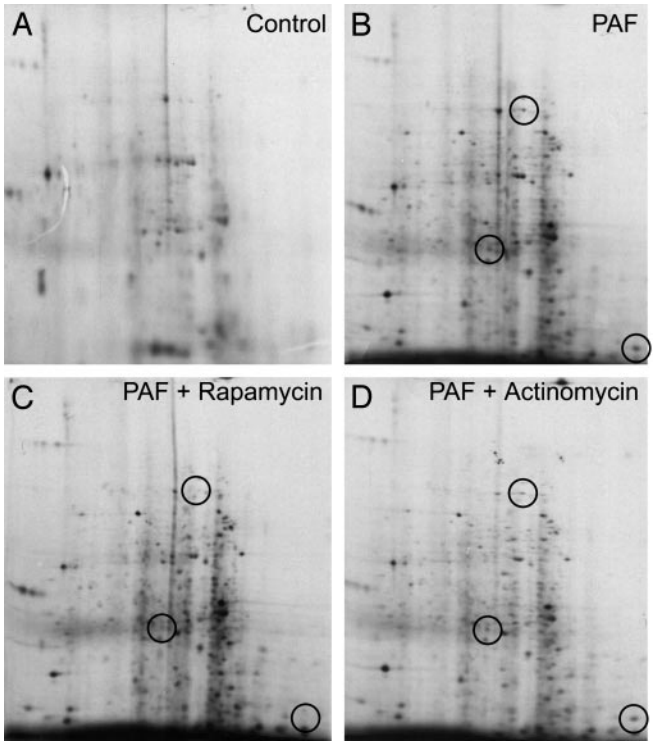

Fig. 1. Signal-dependent translation controls expression of proteins in activated human neutrophils. PMNs were incubated in suspension for 4 h with control buffer (*A*) or PAF (10 nM) (*B*) and [^{35}S]methionine alone or after pretreatment with rapamycin (*C*) or actinomycin D (*D*) and newly synthesized radiolabeled proteins were detected by two-dimensional gel electrophoresis. Expression of three prominent protein products (*B*) was blocked by rapamycin (*C*) but not by actinomycin D (*D*) (circles). One of these (the uppermost circled product) had molecular weight and pI consistent with IL-6Rα. Activation of PMNs with PAF also induced new synthesis of multiple labeled proteins in two additional experiments (data not shown).

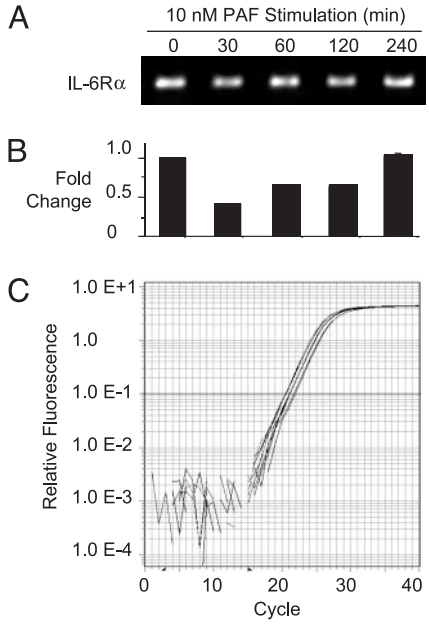

Fig. 2. IL-6Rα mRNA is constitutively present in human PMNs and is not increased in response to activation with PAF. Freshly isolated human PMNs were lysed immediately (time 0) or after activation with 10 nM PAF in suspension for the times shown, and transcript levels were analyzed by real-time PCR using probes specific for IL-6Rα or GAPDH (data not shown). (*A*) PCR amplification products for IL-6Rα mRNA at baseline and after activation with PAF. The levels of IL-6Rα mRNA did not change when PMNs were incubated for the same time intervals in the absence of PAF (data not shown). (*B*) The relative change in IL-6Rα transcript abundance as analyzed by real-time PCR is shown compared with that present at time 0 by using template from the same PCR analysis in *A*. The level at time 0 was arbitrarily set as 1. (*C*) Fluorescence curves used in the real-time analysis of IL-6Rα transcript levels in *B*. This pattern of IL-6Rα mRNA expression in unstimulated and PAF-activated PMNs was seen in five additional experiments.

IL-6Rα in inflammation also suggest that it is subject to specialized regulation. PMNs express IL-6Rα as a transmembrane protein that can be rapidly cleaved and released in response to activating signals (7, 10, 28–31), one of two soluble IL-6Rα isoforms (31). IL-6Rα released by activated PMNs associates with a transmembrane homodimer of two gp130 chains (sometimes termed IL-6Rβ) that is constitutively present on the plasma membranes of human endothelial cells (EC), forming a functional heterotrimeric complex that is competent to transmit outside-in signals (10, 28–31). This trans-signaling (10, 31) confers new recognition of IL-6, a critical modulator of inflammation and systemic responses to injury that is released endogenously by EC and other cell types (10, 28–31, 37). T lymphocytes, mesothelial cells, and vascular smooth muscle cells also respond to trans-signaling (7, 38, 39). One consequence of trans-signaling of EC is expression of chemokines and adhesion molecules that mediate additional PMN targeting and transition to the mononuclear cell phase of inflammation (7, 10, 28–31). Microarray analysis of human EC incubated with IL-6Rα or supernatants from PAF-stimulated PMNs (see below) indicates that mRNAs that code for proteins with other functions are also induced or amplified (S.W.L., A.S.W., and G.A.Z., unpublished data). Whereas generation of IL-6Rα is rate limiting in IL-6 signaling (31) and its release by neutrophils may be a key event critical in the regulation of acute and chronic inflammation (10, 31), the mechanisms that control synthesis of IL-6Rα by human PMNs have not been characterized.

When we examined freshly isolated unstimulated human PMNs by microarray analysis or by standard or real time RT-PCR, IL-6Rα transcripts were constitutively present (Fig. 2 and data not shown), consistent with an earlier report (40). The dominant mRNA detected in resting neutrophils codes for the transmembrane isoform of IL-6Rα that is released by proteolytic cleavage, with little of the soluble alternatively spliced isoform (7, 31) present (data not shown). In contrast to the presence of IL-6Rα mRNA, little or no IL-6Rα protein was detected in quiescent, unactivated neutrophils (Figs. 3 and 4). Differential expression of the transcript and protein further suggested the possibility of specialized translational regulation (18, 19, 24, 35, 36).

IL-6Rα Protein Is Synthesized in a Signal-Dependent Fashion from Constitutive mRNA in Human PMNs. We found that IL-6Rα protein is rapidly synthesized in response to activation of PMNs with PAF and was detected within 30–60 min and, in some cases, even more rapidly (Figs. 3 and 4 and data not shown). Recombinant PAF acetylhydrolase, a highly specific antiinflammatory enzyme that degrades PAF and PAF-like lipids (32), inhibited its synthesis (Fig. 4*B*). Blocking the neutrophil PAF receptor with a specific competitive antagonist also inhibited IL-6Rα synthesis (data not shown). IL-6Rα released by PAF-stimulated neutrophils associated with human EC, consistent with previous studies (28–31) (Fig. 4*C*; also see below). These experiments establish a mechanism linking PAF signaling and IL-6/IL-6Rα trans-signaling, two pathways that mediate complex inflammatory and immune events in a variety of human diseases and syndromes

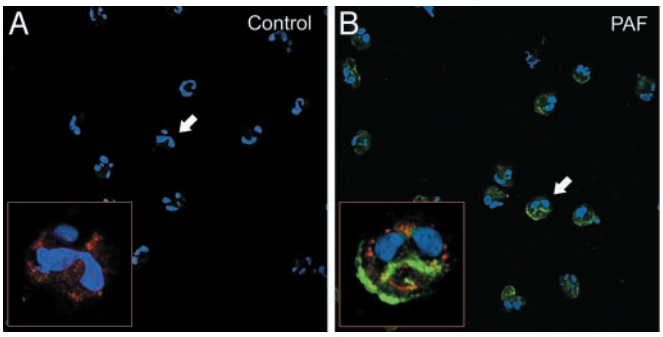

Fig. 3. IL-6Rα protein is rapidly synthesized by activated human PMNs. Freshly isolated PMNs were left quiescent or were activated with 100 nM PAF for 30 min and stained for IL-6Rα (green immunofluorescence). Nuclei were identified by propidium iodide (blue). (*A*) Quiescent, unactivated PMNs. (*Inset*) Higher magnification of a PMN from the group shown in *A* (indicated by arrow) stained for polymerized actin (red) in addition to IL-6Rα. (*B*) PMNs stimulated with PAF. (*Inset*) Higher magnification of a PMN from the group shown in *B* (arrow) stained for IL-6Rα and actin (red). In a second experiment with the same antibody against IL-6Rα, PMNs were fixed in suspension without permeabilization after stimulation with PAF and stained for IL-6Rα and β_2 integrins, which demonstrated surface display of both proteins (data not shown). Competition with the peptide against which the IL-6Rα antibody was raised completely quenched the immunofluorescence, indicating specificity of the staining.

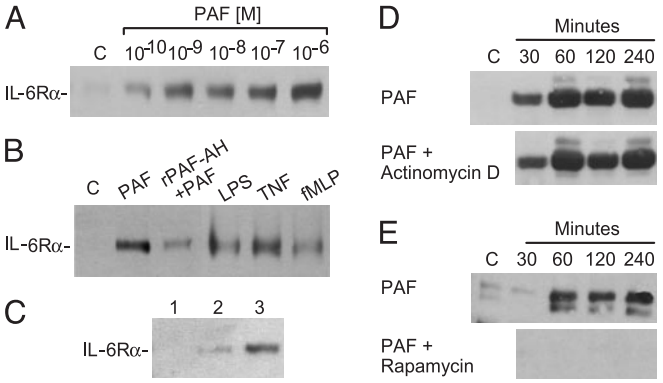

Fig. 4. IL-6Rα is synthesized from constitutive mRNA by human PMNs in response to inflammatory signaling to mTOR. PMNs were incubated with control buffer, PAF, or other inflammatory agonists in suspension, and IL-6Rα protein was detected by Western analysis with the same antibody used for immunocytochemical studies (Fig. 3). (*A*) PMNs were incubated with control buffer (lane C) or increasing concentrations of PAF for 1 h. (*B*) PMNs were incubated with control buffer, 100 nM PAF, PAF that had been preincubated with recombinant human PAF acetylhydrolase (rPAF-AH), or the indicated agonists. (*C*) HUVEC monolayers were incubated with PMNs activated with 100 nM PAF alone or after pretreatment with rapamycin by using separated chambers and were then blotted for IL-6Rα. Control HUVEC were incubated in the absence of PAF-stimulated PMNs. Lane 1, control HUVEC; lane 2, HUVEC incubated with PMNs pretreated with rapamycin before stimulation with PAF; lane 3, HUVEC incubated with PAF-stimulated PMNs. (*D*) PMNs were pretreated with control buffer or actinomycin D followed by activation with 100 nM PAF for the times shown. Blots from PMNs activated with PAF alone or after pretreatment with actinomycin D are shown above and below one another at each time point. Control PMNs were lysed in the absence of actinomycin D or PAF and blotted (leftmost lane in *Upper*). In some experiments IL-6Rα stains as a doublet or triplet on immunoblotting, as shown. (*E*) PMNs were pretreated with control buffer or rapamycin and then activated with 100 nM PAF for the times indicated. In a control experiment, neither actinomycin D nor rapamycin alone in the absence of PAF induced an IL-6Rα band (data not shown).

(5, 10, 28–32, 38). EC rapidly produce PAF in response to inflammatory and thrombotic agonists and use it as a juxtacrine signal for neutrophils (5, 32), providing a mechanism for local synthesis and release of IL-6Rα and retrograde signaling of endothelium (28–31). In addition to PAF, LPS, TNFα, and the bacterial peptide mimetic fMLP induced rapid synthesis of IL-6Rα by PMNs (Fig. 4*B*), indicating that diverse receptor-mediated agonists also trigger this response.

Abundance of the IL-6Rα mRNA was not increased in activated PMNs when analyzed by real-time quantitative PCR and instead transiently decreased over 4 h of stimulation with PAF or LPS (Fig. 2 and data not shown). This finding suggested that synthesis of IL-6Rα protein can be rapidly accomplished without transcription in response to outside-in signals. Consistent with this, production of IL-6Rα was not diminished by actinomycin D although it was inhibited by the translational blocker cycloheximide (Fig. 4*D* and data not shown). Thus, rapid expression of IL-6Rα protein by PMNs is mediated by signal-dependent translation of the constitutive mRNA. It remains possible that coordinate transcriptional and translational control, and additional posttranscriptional mechanisms such as mRNA stabilization, influence synthesis of IL-6Rα at later time points. Expression of other proteins with potent biologic activities is regulated at multiple checkpoints in this fashion (35).

Synthesis of IL-6Rα Is Regulated by mTOR in Stimulated Human PMNs. Because synthesis of IL-6Rα is translationally regulated in a signal-dependent fashion in activated PMNs we considered the possibility that its expression is controlled by mTOR (18–20). We examined the reported sequence of the 5′ UTR of the IL-6Rα mRNA (41) for characteristics that indicate translational regulation, and found extensive predicted secondary structure (calculated energy of unwinding of −320 kcal/mol; 1 kcal = 4.18 kJ) and four tracts of five to six consecutive pyrimidine bases. These features strongly suggest the possibility of translational control by mTOR (18–20, 42). Preliminary analysis of the 5′ UTR of the dominant IL-6Rα transcript present in freshly isolated unstimulated PMNs by using 5′ RACE also demonstrates extensive secondary structure. In addition, introduction of this sequence into a reporter construct causes its translational repression when transfected into a heterologous cell line (M.M.D., T.M.M., A.S.W., and G.A.Z., unpublished data).

In parallel, we documented that mTOR is present in freshly isolated human PMNs (Fig. 5 *A* and *B* and data not shown), excluding the possibility that it is eliminated from these cells as they become terminally differentiated during myelopoesis. Two variants of TOR kinase have been reported (20). PCR analysis and sequencing of the PCR product identified mTOR 1 in human PMNs (data not shown). Rapamycin, a highly specific probe and inhibitor of mTOR (20, 21, 26, 42), abolished IL-6Rα synthesis (Fig. 4*E*). This result contrasts with preserved synthesis when neutrophils were pretreated with actinomycin D (Fig. 4*D*). In addition, synthesis of IL-8 by PAF-stimulated neutrophils was inhibited by actinomycin D, whereas there was no significant effect of rapamycin or FK506 (see below) (data not shown). The synthesis of many other radiolabeled proteins was also preserved in activated neutrophils in the face of rapamycin treatment (Fig. 1*C* and data not shown), further demonstrating its selective activity and consistent with the observation that mTOR controls translation of a subset of mRNAs in human cells (18–21). Therefore, rapamycin, although best known for its therapeutic inhibition of T cell proliferation and cell-cycle progression (20, 21, 27), also inhibits rapid expression of new proteins by activated PMNs.

The mTOR Pathway Is Activated in a Signal-Dependent Fashion in Human PMNs. mTOR regulates phosphorylation of S6 kinase 1 (S6K1) and eIF4E binding protein 1 (4E-BP1), which are key downstream signaling intermediates (Fig. 5*C*) and indicators of

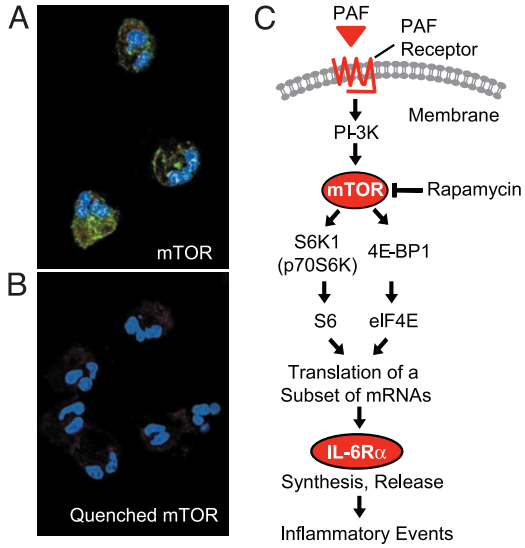

Fig. 5. The mTOR pathway regulates signal-dependent translation in human neutrophils activated via the PAF receptor. (*A*) Freshly isolated PMNs were activated with 100 nM PAF for 30 min followed by staining for mTOR. (*B*) The peptide immunogen against which the anti-mTOR antibody was raised completely quenched the immunofluorescent staining. (*C*) Components of the mTOR pathway in human neutrophils activated by PAF (also see Fig. 6). S6K1 and related members of this family are also called p70S6 kinases. In other cell types, phosphatidylinositol 3-kinase (PI-3K) lies upstream of mTOR and may also mediate parallel signaling to S6K1 and 4E-BP1 (20), but this has not been formally demonstrated for signaling to the mTOR pathway via the PAF receptor.

mTOR activity (20, 21, 42, 43). Inhibition by rapamycin selectively blocks phosphorylation of these regulatory proteins and consequent initiation of translation of mRNAs with appropriate cis-acting features in their 5′ UTRs (18–22, 42, 43) (see above). Activation of neutrophils with PAF triggered phosphorylation of S6K1 within 1–5 min. (Fig. 6 *A* and *B* and data not shown), as did their activation with fMLP (data not shown). In addition, adhesion of PMNs to P-selectin, which acts in concert with PAF (5, 32), triggered S6K1 activation (data not shown), consistent with our previous observation that engagement of the myeloid ligand for P-selectin, PSGL-1, transmits outside-in signals that activate mTOR (19). Pretreatment of neutrophils with rapamycin blocked phosphorylation of S6K1 in response to PAF at all time points (Fig. 6 *A* and *B*). In contrast, FK506, which binds to the same intracellular protein that recognizes rapamycin, FKBP12, but does not inhibit signaling via mTOR (44), had no effect (Fig. 6*B*). Phosphorylation of S6K1 in PMNs stimulated with fMLP was also inhibited by rapamycin (data not shown).

Activation of neutrophils by PAF induced phosphorylation of 4E-BP1, detected by a phosphospecific antibody (Fig. 6*C*). Phosphorylation of 4E-BP1 and related 4E-BPs in response to activation of mTOR causes these proteins, which are translational repressors, to dissociate from eukaryotic initiation factor-4E (eIF4E) (20, 25). This phosphorylation allows eIF4E, which is central to translation control in many circumstances (20, 21) and is present in freshly isolated PMNs (data not shown), to recognize the 7-methyl cap of mRNAs and to then organize an initiation complex that includes a helicase that unwinds inhibitory secondary structure in the 5′ UTR (20, 25, 42). Phosphorylation of 4E-BP1 in response to PAF was rapid (Fig. 6*C*), with a time course similar to phosphorylation of S6K1 (Fig. 6 *A* and *B*). Basal and stimulated phosphorylation of 4E-BP1 was inhibited by rapamycin (Fig. 6*C*), consistent with the pattern in other cell types (43, 44) and with inhibition of phosphorylation of S6K1 (Fig. 6 *A* and *B*) and synthesis of IL-6Rα (Fig. 4*E*). Thus, the mTOR-signaling cascade is linked to the PAF receptor and is triggered in activated human PMNs, and blocking mTOR inhibits synthesis of IL-6Rα. These observations identify a previously unrecognized innate signaling pathway in neutrophils (Fig. 5*C*).

Signal-Dependent Translation in Human Myeloid Leukocytes: An Inflammatory Mechanism and Therapeutic Target. About 20% of the newly synthesized IL-6Rα partitioned into solution when PMNs were activated by PAF for 1 h ($n = 3$; data not shown). When released, IL-6Rα associated with EC monolayers as predicted (28–30), an event that was inhibited by blocking its synthesis with rapamycin (Fig. 4*C* and data not shown). This result suggests that trans-signaling of endothelium and other cell types by activated neutrophils in complex inflammatory syndromes (7, 10, 28–31) may be inhibited in a similar fashion.

Whereas the transition from accumulation of neutrophils to mononuclear leukocytes induced by IL-6/IL-6Rα signaling (7, 10, 31) together with other molecular cues (4, 5, 10) is central to resolution of acute inflammation, it is also a key feature of persistent inflammation in many pathologic syndromes (4, 5, 10, 11). A topical example is restenosis after interventional angioplasty and intravascular stent placement in atherosclerotic vascular disease, a common syndrome of dysregulated inflamma-

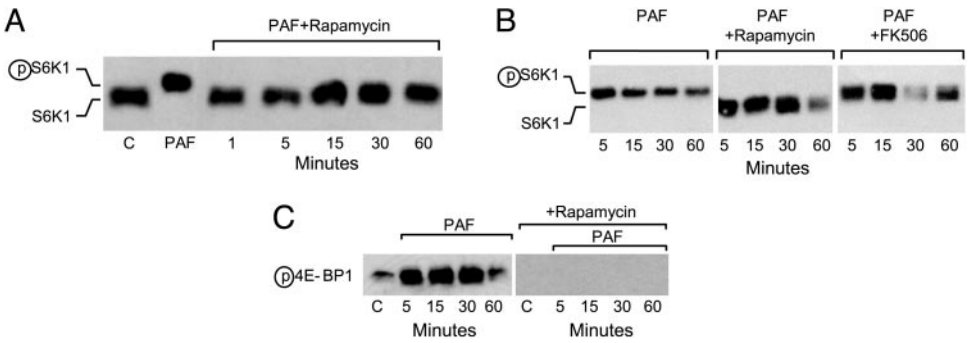

Fig. 6. Activation of human PMNs by PAF triggers rapid mTOR-dependent phosphorylation of S6K1 and 4E-BP1. (*A*) Freshly isolated human PMNs were lysed immediately (control; lane 1) or were activated with 10 nM PAF (lane 2) and the mobility of S6K1 was assayed by immunoblotting. In parallel, PMNs were pretreated with rapamycin and activated with PAF for the times shown (lanes 3–7). (*B*) Freshly isolated PMNs were pretreated with control buffer (lanes 1–4), rapamycin (lanes 5–8), or FK506 (lanes 9–12) and activated with PAF for the times shown followed by immunoblotting. More slowly migrating bands represent phosphorylated S6K1, as in *A*. (*C*) Freshly isolated human PMNs were lysed immediately (control; lane 1) or after activation with 10 nM PAF for the times shown after pretreatment with control buffer (lanes 2–5) or rapamycin (lanes 6–10). The lysates were then probed for 4E-BP1 by using a phosphospecific antibody that recognizes phosphorylation on serine-65, a critical residue (20).

tion (45–47). Restenosis is a complication of a temporally defined iatrogenic injury that induces accumulation of platelets and neutrophils followed by a transition to mononuclear leukocyte accumulation, altered matrix turnover, and smooth muscle cell hyperplasia (8, 9, 48, 49). IL-6 signaling may be a central component in this process (39, 48). Local delivery of rapamycin (also called sirolimus) dramatically reduces both experimental and clinical restenosis by incompletely defined mechanisms (48, 49). It is possible that inhibition of synthesis of IL-6Rα and other translationally regulated products (see below) by activated PMNs may be one means by which rapamycin favorably modifies the transition to chronic inflammation, smooth muscle proliferation, and recurrent occlusion of the injured vessel (39, 48) although this remains to be determined *in vivo*. Rapamycin also inhibits mTOR-dependent translation in human monocytes (19), a second key effector cell in restenosis (9, 39, 48). Inhibition of synthesis of key mediators by PMNs, monocytes, or macrophages may additionally contribute to beneficial activities of rapamycin in other conditions that have inflammatory components (4, 5), including neoplasia (21, 27, 50).

Rapamycin selectively inhibits expression of several radiolabeled proteins synthesized by activated neutrophils in addition to IL-6Rα (Fig. 1*C* and data not shown), and we subsequently documented that translation of other key transcripts is controlled by mTOR (unpublished results). Thus, this previously unrecognized mechanism of gene regulation may influence synthesis of multiple neutrophil proteins in defensive and dysregulated inflammation. It is also likely that activated PMNs use diverse mechanisms of signal-dependent translational control for expression of specific gene products (21, 22), as do other innate defensive cells (18, 19, 23–25), adding to the repertoire of specialized responses of this critical innate immune effector and potentially identifying new therapeutic targets for modifying acute and chronic inflammatory diseases.

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