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Reprogramming of a subpopulation of human blood neutrophils by prolonged exposure to cytokines

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Essential cells of innate immunity, neutrophils are often considered to be a homogenous population of terminally differentiated cells. During inflammation, neutrophils are extravasated cells exposed to local factors that prolong their survival and activate their production of mediators implicated in disease progression. In this study, a phenotypically distinct subset of human neutrophils that appear after prolonged exposure to cytokines was characterized. Freshly isolated neutrophils from healthy donors were incubated with granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α and interleukin (IL)-4, three cytokines that are locally present in various inflammatory conditions. Eight to 17% of neutrophils survived beyond 72 h. This subset of non-apoptotic neutrophils, as evaluated by three different markers, was enriched by discontinuous Percoll gradient centrifugation before studying their phenotype. These viable neutrophils showed neoexpression of HLA-DR, CD80 and CD49d. Compared with freshly isolated neutrophils, they responded differentially to second signals similar to formyl-methionyl-leucyl-phenylalanine with three- to four-fold increases in production of superoxide anions and leukotrienes. These cells augmented their phagocytic index by 141%, increased their adhesion to human primary fibroblasts, but reduced their migration in response to chemotactic stimuli and decreased exocytosis of primary and secondary granules. In addition, they produced substantial amounts of IL-8, IL-1Ra and IL-1β. This neutrophil subset had a unique profile of phosphorylation of intracellular signaling molecules. In conclusion, the present identification of a novel neutrophil phenotype highlights the reprogammable character of the neutrophil. This aspect is crucial for our understanding of its contribution to disease pathogenesis and host defense.
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Neutrophils are the most abundant circulating leukocytes in human beings and are the first to migrate to inflammatory sites. They have a wide range of effector functions owing to preformed cytoplasmic and membrane proteins, enzymes and newly synthesized lipid and protein mediators that enable them to respond to diverse environmental triggers.[1] Neutrophils are, thus, an essential arm of the immune system, mounting the initial inflammatory response and participating in host defense. Peripheral blood neutrophils are certainly the most studied type of neutrophils. However, during inflammation, these leukocytes leave the circulation and enter new habitats, the tissues in which they are exposed for extended periods to multiple factors such as cytokines, endogenous growth factors, bacterial products as well as multiple other local products. Indeed, the activity of infiltrating neutrophils has been intimately linked to disease evolution in a variety of clinical conditions.[2–7] Thus, these cells contribute not only to acute inflammatory reactions, but also to the evolution of a variety of chronic inflammatory diseases.

Underlying the traditional definition of the neutrophil as a prototype effector of acute inflammation are two notions: that they are short lived, their lifespans being measured in hours, and that they have a predefined set of functions. As a corollary, they are most often considered all alike. Thus, functional and mechanistic studies on neutrophils generally treat them as a homogenous population of terminally differentiated cells. However, this concept is unsuited to several observations as evidence emerges that neutrophil lifespan is considerably prolonged in response to stimuli

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such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), interleukins (ILs), interferons and bacterial products.[8–12] Indeed, a percentage of neutrophils obtained from lungs in inflammatory lung injury and from synovial fluids of patients with rheumatoid arthritis have been seen to persist for days.[13–16] The dogma that neutrophils are all alike is countered by a growing number of studies showing the functional heterogeneity of neutrophils. Neutrophil subsets have been demarcated with identifiable characteristics in terms, for example, of their expression of formyl-methionyl-leucyl-phenylalanine (fMLP) receptors.[17] Recently, 5–8% of human neutrophils have been shown to express functional T-cell receptor.[18] In addition to normal neutrophils, two subsets of mouse neutrophils exhibiting different susceptibilities to methicillin-resistant *Staphylococcus aureus* have also been identified that have different cytokine and chemokine production, effect on macrophage activation, Toll-like receptors and surface antigen expression.[19]

In this work, we propose to extend the concept of neutrophil subsets to inflammatory situations in which neutrophils are major participants. In addition to the heterogeneity in circulating neutrophils evoked in earlier studies, specific inflammatory conditions can also favor the emergence of neutrophil subpopulations with distinct phenotypes. After a 72-h incubation of normal human blood neutrophils with a combination of GM-CSF, TNF-α and IL-4, mediators known to be present in inflammatory conditions such as early arthritis, allergic asthma and skin pustulosis, and to influence neutrophil survival and functions,[7,8,20–23] we isolated and characterized a subset of neutrophils with a functional profile that is markedly different from that of freshly isolated neutrophils. Constituting 8–17% of the global neutrophil populations exposed to the cytokines, these cells have substantially increased lifespan persisting beyond 72 h. They have a unique inflammatory profile seen in the pattern of surface marker expression, production of superoxide anions (O_2^-), phagocytosis, leukotriene biosynthesis, chemotactic responses and degranulation. In addition, this subset produces substantial amounts of cytokines, such as IL-1, IL-1 receptor antagonist (IL-1Ra) and IL-8, and interacts strongly with resident stromal cells. We conclude that certain inflammatory conditions can induce a phenotypic 'switch' in the circulating neutrophil toward a resident neutrophil with different and new functions.

MATERIALS AND METHODS

Reagents

Dextran T-500 and Percoll were obtained from GE Healthcare, Canada. Ficoll-Paque, RPMI 1640 and fetal bovine serum (FBS) were obtained from Wisent (St-Bruno, QC, Canada). Human recombinant GM-CSF, IL-4 and TNF-α were obtained from Peprotech (Rocky Hill, NJ, USA). FITC-conjugated annexin-V and propidium iodide (PI) were from Biovision (Mountainview, CA, USA). The fMLP, phorbol 12-myristate 13-acetate (PMA), A23187 calcium ionophore, pyruvate solution, human lactoferrin (LF), anti-human LF antibody and O-dianiside hydrochloride were obtained from Sigma Chemical Co. (St-Louis, MO, USA). FITC-conjugated zymosan particles, wheat germ agglutinin (WGA) lectin and 4′,6-diamidino-2-phenylindole (DAPI) were from Molecular Probes (Invitrogen Canada Inc. Burlington, ONT, Canada). Hema-Quick II was obtained from Biochemical Sciences (Swedesboro, NJ, USA). Chemotaxis plates (96 wells) were purchased from Neuroprobes (Gaithersburg, MD, USA). Cytochrome *c* was from MP Biomedicals (Solon, OH, USA). Anti-human HLA-DR, CD80, CD86, CD11b, CD18 antibodies (Ab) and IgG1, IgG2a, IgG2b were all purchased from BD Biosciences Pharmingen (San Diego, CA, USA) and peroxidase-conjugated anti-rabbit Ab was from Jackson ImmunoResearch (West Grove, PA, USA). Apostat intracellular caspase detection kit (FITC-VD-FMK), EIA kit for IL-1Ra (biotinylated secondary goat anti-human IL-1Ra Ab: DY280, #1144097) and Proteome Profiler: Human Phospho-MAP Kinase Array Kit were obtained from R&D Systems (Minneapolis, MN, USA). EIA kits for IL-8 (biotinylated secondary mouse IgG1 anti-human IL-8 Ab: CHC1303, #061901) and IL-1β (583311, #189338) were purchased from Biosource international (Camarillo, CA, USA) and Cayman Chemicals (Ann Arbor, MI, USA), respectively.

Preparation of Human Leukocytes

The institutional review board of the Université Laval (Québec, QC, Canada) approved this study and healthy adult volunteers signed a consent form. Peripheral blood neutrophils were prepared as described earlier in sterile conditions at room temperature.[24] Briefly, human venous blood collected on citrate phosphate dextrose adenine anticoagulant solution was centrifuged (250 *g*, 15 min) and the platelet-rich plasma was removed. After dextran sedimentation of the erythrocytes, neutrophils were purified by centrifugation over a Ficoll-Paque cushion (450 *g*, 20 min). Neutrophils were collected at the bottom and contaminating erythrocytes were eliminated by hypotonic lysis (15 s). After two washes, neutrophils were counted and resuspended in RPMI 1640 + 10% decomplemented FBS with or without 500 pM GM-CSF + 10 ng/ml IL-4 + 10 ng/ml TNF-α. Cell viability was routinely assessed by trypan blue dye exclusion test and was >99%. Neutrophils were incubated at 10^7 cells/ml, in 12-well plates (2 ml/well) at 37°C, 5% CO_2 for 3 days.

After 3 days of incubation, viable neutrophils were isolated from the incubated neutrophils by discontinuous Percoll density gradient centrifugation, as described earlier with slight modifications.[25] Briefly, neutrophil suspensions were added to discontinuous Percoll gradients created by gently layering equal volumes of 31, 42 and 51% of a standard isotonic Percoll solution (90% Percoll + 10% 10× phosphate-buffered saline (PBS)) in diluent (PBS) and centrifuged at 610 *g* for 28 min at 4°C. The 0/31 and 42/51% Percoll

interfaces contained the majority of necrotic, late and early apoptotic cells (>90% were annexin-V-positive cells or incorporated PI), whereas the viable cells were collected at the bottom. Percoll-enriched cells were washed thrice in excess of PBS and were used for subsequent tests after assessment of cell viability by annexin/PI, as described below.

Suspensions of peripheral blood mononuclear leukocytes (PBMLs) were prepared as described earlier.[24] PBMLs were enriched in their monocyte fraction by adherence on plastic petri dishes (5x10^6 cells/ml, 37°C, 90 min). The non-adherent fraction was removed by repeated washes, the adherent monocyte fraction was treated with accutase, counted and incubated with the combination of cytokines as above. Purity was 90–92% monocytes, as assessed by Wright–Giemsa and non-specific esterase staining.

Human blood eosinophils from healthy volunteers were purified as described earlier with slight modifications.[26] After dextran sedimentation and centrifugation on Ficoll-Paque cushions, eosinophils were isolated from the granulocyte pellet by negative selection using an anti-CD16 monoclonal Ab conjugated with magnetic beads and the magnetic Ab cell-sorting apparatus (Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity (Hema-Quick staining) and viability (trypan blue exclusion) of the eosinophil preparations were always >98.5 and 99%, respectively.

Microscopy

Cells were spun onto slides, air dried for 5 min, stained with Hema-Quick II solution and examined by light microscopy (magnification ×100). Neutrophils were resuspended in 50 μl PBS, placed on coverslips, air dried, fixed in 4% paraformaldehyde pH 7.4 for 15 min, washed two times, labeled with OG488-WGA-lectin (496/524 nm) and DNA-binding nuclear marker DAPI (358/461 nm) before epi-fluorescence microscopy. Images were visualized using Olympus BX-41TF microscope using the appropriate filter sets for the fluorochromes and analyzed by the Imagepro software. For transmission electron microscopy, cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde (100 mM sodium cacodylate buffer, pH 7.3) for 24 h at 4°C. After postfixation in 1% OsO_4 (in the same buffer) for 90 min at 20°C, the samples were dehydrated in an ethanol/propylene oxide series and embedded in Epon. Ultrathin sections of 80 nm were produced with a Reichert-Jung Ultramicrotome (Vienna, Austria). These sections were then transferred to a 200-mesh nickel/Formvar grid and stained with 3% uranyl acetate/lead citrate. Samples were examined in a JEM-1230 microscope (Jeol, Tokyo, Japan) at 80 kV.

Analysis of Viability

Neutrophil viability was evaluated by the lactate dehydrogenase (LDH) release assay.[27] After incubations, suspensions of neutrophils were centrifuged. Supernatants and cell pellets were collected separately. Cells were lysed with Triton X-100, and 1.25 ml of substrate solution (0.14 mg/ml NADH in 0.1 M sodium phosphate buffer, pH 7.35) together with 50 μl of pyruvate solution were added to 50 μl of lysed cells or supernatants; samples were analyzed by colorimetry at 340 nm. The results were expressed as percentages of the ratio of optical density values measured in supernatants by total optical density values (cells plus supernatant).

Neutrophils simultaneously labeled with FITC-conjugated annexin-V and PI were also evaluated for viability by flow-cytometry. Briefly, cells were collected by centrifugation and resuspended in 500 μl of binding buffer 1X supplied with the kit; 5 μl of FITC-labeled annexin-V and PI were then added and the cells were subsequently incubated for 5 min at room temperature in the dark. Ten thousand cells were counted and cell death was calculated as the number of cells stained by annexin-V or PI.

Apoptosis of neutrophils was also estimated by intracellular caspase activity, as determined by FITC-conjugated VD-FMK. Briefly, 10 μl Apostat reagent was added to 1 million cells in 1 ml. After incubation at 37°C for 30 min, cells were harvested, washed and resuspended in 500 μl of PBS for analysis by flowcytometry.

Analysis by Flowcytometry

For immunofluoresence staining, 100 μl of cell suspensions (10^7 cells/ml) were incubated for 30 min at 4°C with FITC-, PE-conjugated or unconjugated mAbs. For analysis using unconjugated mAbs, FITC-conjugated F(ab')$_2$ fragments of goat anti-mouse immunoglobulin antibodies were used as a second step reagent. The analysis was performed by flow-cytometry (EPICS-XL Beckman Coulter, Miami, FL, USA). Ten thousand events were counted and cell debris was excluded based on forward and side-light scatters. For cell cycle analysis, cell suspensions (2×10^6 cells/ml) were added dropwise to an equal volume of ice-cold absolute ethanol. After 24 h, cells were washed with 3 ml of cold buffer and stained with DNA-intercalating dye PI (40 μg/ml), 30 min at room temperature, in the presence of RNAase (200 μg/ml). DNA content was subsequently assessed by flowcytometry.

Evaluation of Neutrophil Functions

Production of O_2^-

O_2^- production was assessed by the superoxide dismutase-sensitive reduction of cytochrome *c*.[28] Neutrophils were suspended in Hanks' balanced salt solution (HBSS) containing calcium (1.6 mM). Cell suspensions (1×10^6 cells/ml) were then preincubated with 125 μg/ml of freshly prepared cytochrome *c* in HBSS and tubes were placed in a shaking water bath at 37°C for 5 min in the presence of fMLP, PMA or A23187 calcium ionophore (10^{-7}M final concentration). The reactions were terminated by rapidly transferring the tubes to an ice-cold water bath and adding superoxide dismutase (final concentration, 62.5 μg/ml), followed by centrifugation (1000 *g*, 30 min, 4°C). Supernatants were immediately transferred to cuvettes for measurement of reduced cytochrome *c* absorbance at 550 nm with a Beckman DU

spectrophotometer. The change in optical density was converted to nanomoles of O_2^- released into supernatants using the extinction coefficient $E_{550} = 21 \times 10^3$/M/cm.

Chemotaxis

Neutrophil migration assay was performed using disposable 96-well chemotaxis chambers with 3 μm pore membrane. Cells were exposed to 5 μg/ml of the fluorescent probe calcein-AM at 37°C for 30 min. Calcein-AM-loaded neutrophils were washed and resuspended in complete medium (3×10^6 cells/ml). The wells of the chemotaxis plates were filled in duplicate with a control solution (PBS), fMLP, LTB_4 or IL-8 (10^{-10}–10^{-6} mol/l). The polycarbonate membrane was placed over the wells and 60 000 neutrophils/chamber were added on top of the membrane. Cells were allowed to migrate for 60 min at 37°C. Cells that did not migrate through the filter were removed gently by scraping. The filter was analyzed using a fluorescence reader (Bio-Tek Instruments) at the excitation and emission wavelengths of 485 and 530 nm, respectively. Fluorescence intensities were proportional to the number of cells that crossed the filter.

Release of myeloperoxidase and LF

Myeloperoxidase (MPO), a marker for primary granules, was measured by the method of Bradley with slight modifications.[29] Five (5) million neutrophils/ml were stimulated with vehicle, fMLP, PMA or A23187 as above. The reactions were stopped by transferring the tubes to an ice-cold water bath. Supernatants and cells were collected in separate tubes after a quick centrifugation. Cells were lysed in a 50-mM phosphate buffer (pH6) containing 0.5% hexadecyltrimetylammonium bromide, and adjusted to a final volume of 1 ml. One hundred (100) μl of supernatants or cells were added to 2.4 ml of a mixture of potassium phosphate buffer, 0.2 mg/ml of O-dianiside hydrochloride and 500 μl of 0.003% H_2O_2. Absorbance was measured at 460 nm. MPO contents were calculated using an MPO calibration curve created with known dilutions of human MPO. The results are expressed as percentage of MPO released to total MPO.

Release of LF, a marker for specific granules, was measured by ELISA.[30] Briefly, 100 000 cells/ml were stimulated with vehicle, fMLP, PMA or A23187 (at a final concentration of 10^{-7} M each) for 5 min at 37°C in the presence or absence of 1 μg/ml cytochalasin B, an actin depolymerizing agent shown to amplify granule exocytosis. Supernatants were diluted 10- and 100-fold in 50 mM CO_2/HCO_3^- buffer (pH 9.6) and 100 µl of the diluted supernatants or of known concentrations of human LF were added to Nunc-Immuno 96 MicroWell Plates (Nalge Nunc International, Rochester, NY, USA) and incubated overnight at 4°C. Non-specific-binding sites were blocked with PBS supplemented with 0.5% BSA and 0.5% Tween-20. Plates were then treated with rabbit anti-human LF followed by peroxidase-conjugated anti-rabbit Ab. Each of the above steps was performed at room temperature, and between each step, the plates were repeatedly washed with TBS + 0.1% Tween-20. Color was developed with tetra-methyl-benzidene until optimum before the reaction was stopped with 50 μl of a 1-M sulphuric acid. Absorbance was read at 450 nm, and the LF concentration was calculated by comparison to the LF calibration curve.

Biosynthesis of leukotrienes

Cell suspensions (10^7 neutrophils/ml, 10^6 eosinophils or monocytes/ml) in HBSS with 1.6 mM calcium were prewarmed 10 min by placing in a shaking water bath at 37°C prior stimulation for 5 min with fMLP (10^{-7}M), A23187 (10^{-7}M) or arachidonic acid (2.5 μM) in the presence of 1 ng/ml of adenosine deaminase to neutralize the inhibitory effects of extracellular adenosine.[31] For the determination of 5-lipoxygenase (5-LO)-derived products, cell supernatants were denatured by the addition of 0.5 volume of ice-cold MeOH/CH_3CN (1:1, v/v) containing 12.5 ng each of PGB_2 and 19-OH-PGB_2 as internal standards, and the samples were processed and analyzed by reversed-phase (RP)-HPLC using an on-line extraction procedure, as described earlier.[32] The 5-LO products leukotriene (LT)B_4, 20-COOH-LTB_4, 20-OH-LTB_4, 6(E)-LTB_4, 6(E)-12-epi-LTB_4, peptido-leukotrienes (LTC_4, LTD_4 and LTE_4) and 5-hydroxy-eicosatetraenoic acid (5-HETE) were evaluated. Limits of detection for the different lipoxygenase products were 2–5 ng.

Nuclear translocation of the 5-LO was evaluated as described earlier.[33] Neutrophils (2×10^7/ml) stimulated with fMLP or A23187, as above, were pelleted and resuspended in 600 μl of ice-cold NP-40 lysis buffer (0.1% NP-40, 10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM PMSF). The cells were vortexed for 15 s, kept on ice for 5 min and centrifuged at 300 *g* (10 min, 4 °C). The supernatants, representing the non-nuclear fractions, and pellets, representing the nuclei-containing fractions, were then immediately solubilized in electrophoresis sample buffer and processed for SDS–PAGE and immunoblot determination of the 5-LO protein content using a rabbit polyclonal anti-5-LO antibody.

Phagocytosis of zymosan

Phagocytic activity of neutrophils was measured using fluorescent zymosan A (membranes of *Saccharomyces cerevisiae*) bioparticles according to the manufacturer's instructions. Fluorescent particles were preopsonized in PBS containing 50% pooled human serum for 1 h at 37°C on a rotating mixer. Particles were washed, counted and added to 2.5×10^5 neutrophils (ratio cells/particles = 1/10) in 100 μl RPMI + 0.1% FBS for 10 min at 37°C. Cells were washed and resuspended in 300 μl RPMI + 0.1% FBS containing 10% trypan blue (added to quench the fluorescence of bioparticle conjugates bound to the surface, but not internalized). Ten thousand cells were evaluated by flowcytometry. The percentage of neutrophils that had internalized zymosan and the mean fluorescence intensity (MFI), a measurement of the number of particles taken up per cell, were assessed.

The results were then expressed as a phagocytic index of neutrophils = % of neutrophils that had internalized zymosan × MFI.[34] For microscopic evaluation of phagocytosis, cells were washed with PBS and labeled with 5 μg/ml of the calcein-AM probe at 37°C for 30 min, washed and subsequently incubated with opsonized AlexaFluor594-conjugated zymosan A Bioparticles, as above. The cells were disposed on 8-well labtek chamber slides for confocal laser microscopy using the appropriate filter sets for the fluorochromes. Red and green images were merged to visualize internalized zymosan. Images were analyzed by using Volocity LE software from Improvision Inc. (Waltham, MA, USA).

Biosynthesis of IL-1β, IL-1Ra and IL-8

Neutrophils were incubated (5×10^6/ml) in RPMI containing 10% decomplemented FBS in 12-well plates (2 ml/well) at 37°C, 5% CO_2, for 24 h. Cell-free supernatants were collected after centrifugation and immediately stored at −80°C until assayed for IL-1Ra, IL-1β and IL-8 by commercially available kits according to manufacturers' instructions. Cell pellets were washed in PBS, centrifuged, resuspended in 1 ml of PBS, sonicated (20 s, 4°C) and these intracellular materials were immediately stored at −80°C until assayed for cell-associated cytokines. Total cytokine production was obtained by addition of intracellular contents to supernatant contents. The IL-8 and IL-1Ra assays used HRP as tracer, whereas the enzyme immunometric assay for the IL-1β used acetylcholine esterase as tracer. Concentrations of cytokines were obtained from standard curves generated by known concentrations of respective cytokines. The detection limits were 31, 13 and 1.5 pg/ml for IL-1Ra, IL-8 and IL-1β, respectively.

Culture of Synovial Fibroblasts and Adhesion Assay

Primary synovial fibroblasts obtained from post-traumatic hip replacement were maintained in a humidified atmosphere containing 5% CO_2 in Dulbecco's minimum essential medium supplemented with 2 mM glutamine and 20% FBS. Fibroblasts were passaged by treatment with trypsin-EDTA and were used for tests within passage 5–15. For adhesion assays, fibroblasts were sub-cultured in 6-well plates (5×10^5 cells/well) for a 48-h period to obtain confluent monolayers. These fibroblasts were washed with PBS before adhesion assays. Neutrophils were labeled with 5 μg/ml of the fluorescent probe calcein-AM by incubation at 37°C for 30 min. Labeled neutrophils with and without prior fMLP stimulation were added at a neutrophil/fibroblast ratio of 5/1 followed by incubation at 37°C for 90 min. The non-adherent cells were removed by gentle aspiration. Fibroblasts and adherent neutrophils were washed repeatedly with PBS and observed under fluorescent microscope. The number of adherent cells were counted and presented as a mean of five separate fields/well.

Statistics

Values were expressed as means ± s.e.m. of n experiments performed with cells from different donors. Statistical analyses were performed using GraphPad Instat 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Groups were analyzed by using paired or unpaired Student's t-test. Significance was set at two-tailed P value < 0.05.

RESULTS

Individualization of a Neutrophil Subset Viable after Exposure to Cytokines

Survival of cytokine-activated neutrophils

Circulating neutrophils from healthy human subjects were incubated in the presence and absence of GM-CSF, TNF-α and IL-4 in RPMI 1640 supplemented with 10% decomplemented FBS. After 3 days of incubation, neutrophils were analyzed by flowcytometry after labeling with FITC-conjugated annexin-V and PI. Annexin-V binds to phosphatidylserine, a phospholipid externalized on the cell membrane of apoptotic cells, whereas necrotic cell nuclei are stained with PI, a DNA-chelating agent. Among the neutrophils exposed to cytokines for 3 days, a subset negative for annexin-V and PI that represented 13 ± 5% of the whole population was consistently observed. This subpopulation of neutrophils represented <2% of total cells in neutrophils incubated in RPMI 1640 + 10% FBS in the absence of the cytokines. Similar results were obtained with the release of cytosolic enzyme LDH, a cell death marker correlated with compromised membrane integrity.

To isolate this subset of neutrophils, cell debris, apoptotic and necrotic cells were separated from viable neutrophils by a discontinuous Percoll gradient centrifugation. Apoptotic and necrotic cells and cell debris were found at the cell suspension/31% Percoll interface and the 31/42% Percoll interface. A mixture of apoptotic and non-apoptotic cells was found at the 42/51% Percoll interface. The cell pellet was predominantly constituted of non-apoptotic and non-necrotic cells (99% of these cells routinely excluded trypan blue). Figure 1a shows the comparative profiles of annexin-V and PI labeling of cytokine-exposed cells before and after Percoll gradient centrifugation for a representative donor (9.3 and 84.1% viable cells, respectively). Similar results were obtained by testing the activity of neutrophil caspases, intracellular enzymes involved in apoptotic cell death. Before percoll separation, 88% of neutrophils showed positive caspase activity, whereas only 19% of Percoll-enriched cells had activated caspases (Figure 1b). DNA content was then analyzed in Percoll-enriched neutrophils using PI, a DNA-intercalating dye. A predominant G1 diploid peak was obtained with minimal apoptotic changes indicating that the identified neutrophil subset was terminally differentiated viable neutrophils with no cell division (results not shown). This viable subset of Percoll-enriched neutrophils isolated from cytokine-exposed neutrophils was routinely used in subsequent functional

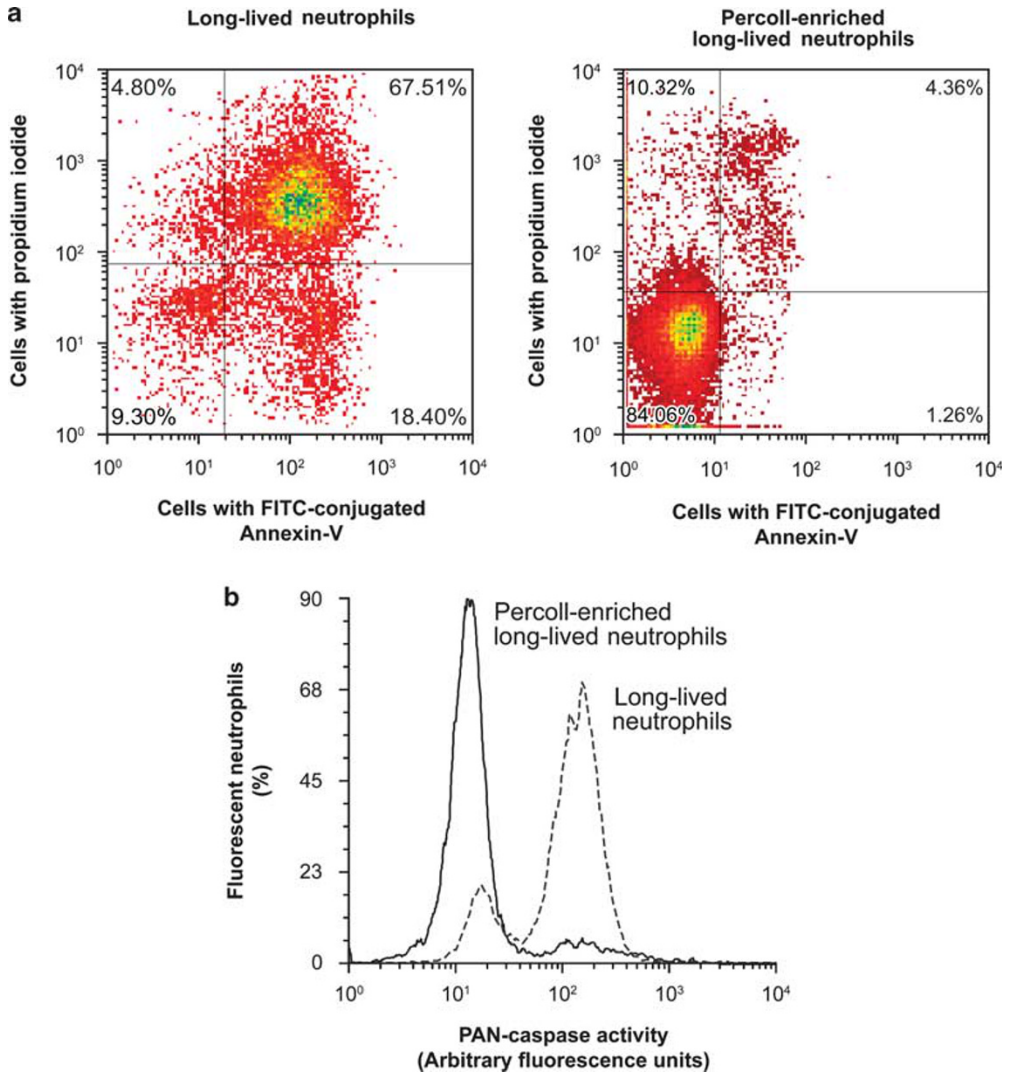


Figure 1 Enrichment of viable neutrophils by Percoll gradient centrifugation. Freshly isolated neutrophils (10^7 cells/ml) were incubated with GM-CSF, IL-4 and TNF-α for 3 days. The viable neutrophils were then enriched by eliminating dead cells and debris through a Percoll gradient centrifugation. (**a**) Viability of incubated neutrophils assessed before and after Percoll gradient centrifugation by annexin-V and PI labeling. (**b**) Viability of long-lived neutrophils before (dashed line) and after (plain line) enrichment by Percoll gradient centrifugation was also assessed by intracellular pancaspase activity. Figures (**a**) and (**b**) are representative of five different donors.

assays and referred to in the text below as long-lived neutrophils.

Morphology of long-lived neutrophils

Microscopy after different staining methods of long-lived neutrophils revealed a morphology similar to that of freshly isolated neutrophils with hypersegmented nuclei and cytoplasmic granules. Promyelocytes, myelocytes and band cells that lack this polylobar nuclear morphology[35] were absent from our neutrophil preparations. Non-specific esterase-positive cells were absent, whereas eosinophils represented 2–5% in freshly isolated neutrophils. After 3 days of incubation, monocytes and lymphocytes were not detectable by microscopy, whereas eosinophils represented 2–4% of long-lived neutrophils. Of note and in concordance with viability results (see above), long-lived neutrophils lacked morphological alterations of apoptosis such as chromatin condensation and nuclear pyknosis.[36] Transmission electron micrographs of freshly isolated neutrophils (Figure 2a) and long-lived neutrophils (Figure 2b) showed similar morphology of plasma membranes, intra-cytoplasmic organites and nuclear segmentation.

Modification of surface markers of long-lived neutrophils

Cytokine exposure modifies surface molecule expression in neutrophils.[37] The majority of long-lived neutrophils (98–99%) expressed CD11b, CD18 and CD32 as analyzed by flowcytometry (Table 1). The percent expression was similar to that of freshly isolated neutrophils, whereas the MFI of these molecules was decreased. These cells also showed a

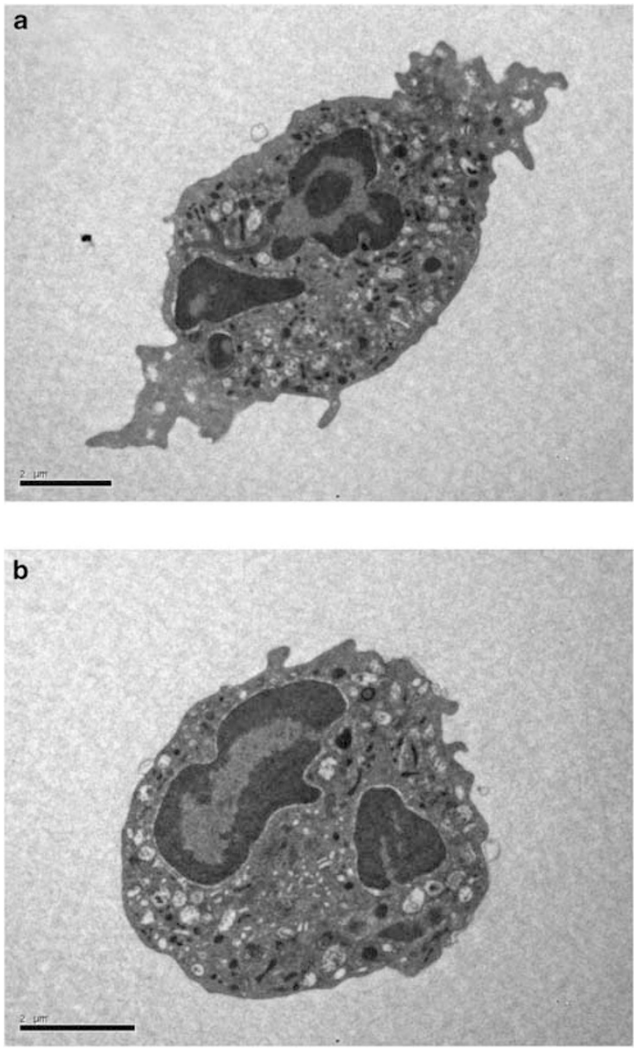


Figure 2 Electron microscopy of human neutrophils. Freshly isolated (**a**) and long-lived neutrophils (**b**) were spun onto slides and fixed in 4% paraformaldehyde and 2.5% glutaraldehyde. After postfixation in 1% OsO_4, the samples were dehydrated and embedded in Epon. Ultrathin sections were transferred to a 200-mesh nickel/Formvar grid and stained with 3% uranyl acetate/lead citrate to be examined in a JEM-1230 microscope.

unique profile of expression of integrins with neoexpression of CD49d, the α4 subunit of the integrin VLA-4 (Figure 3). In comparison to freshly isolated neutrophils, long-lived neutrophils had an augmented MFI of CD54 (ICAM-1) and a strong reduction of CD29 (β1 integrin) surface expression (Table 1). Interestingly, long-lived neutrophils had a neoexpression of HLA-DR and CD80 (Figure 3; Table 1), molecules that characterize antigen-presenting cells.

Functional Characterization of Long-Lived Neutrophils

Production of O_2^-

The fMLP increased the production of O_2^- in long-lived neutrophils three to four times more efficiently than in freshly isolated neutrophils (40.7 ± 10.7 nM/10^6 cells/ml *vs* 11.0 ± 2.9 nM/10^6 cells/ml, respectively; $P = 0.019$). Superoxide generation by freshly isolated neutrophils was in the range of results from other groups.[38] Interestingly, long-lived neutrophils produced similar amounts of O_2^- under fMLP and under PMA (Figure 4a). PMA stimulated the O_2^- production to a similar extent in the two neutrophil populations. In contrast, the O_2^- generation in response to A23187 was greatly diminished in long-lived neutrophils. As 2–4% (20–40 000) eosinophils were present in long-lived neutrophils, similar experiments were also performed with this cell type to confirm that long-lived neutrophils were different from eosinophils. After 3 days of exposure to cytokines, pure eosinophils were, however, incubated at concentrations similar to those of long-lived neutrophils to be able to detect O_2^- production. Cyokine-exposed eosinophils in presence of fMLP produced three times lower quantities of O_2^- (16.3 ± 3.3 nM/10^6 cells/ml) than did long-lived neutrophils (Figure 4b). Moreover, their response to PMA was also lower than that of long-lived neutrophils. In addition, monocytes under fMLP or PMA produced negligible O_2^- (1.5 ± 2.9 nM/10^6/ml and 4.1 ± 0.4 nM/10^6/ml, respectively). These results indicate that long-lived neutrophils have a distinct capacity to produce O_2^- depending on the stimulatory agents.

Table 1 Cell surface phenotype of freshly isolated and long-lived neutrophils

Classification	Surface marker	A (percentage expression)[a]	B (mean fluorescence intensity)	C (percentage expression)	D (mean fluorescence intensity)
Neutrophil surface markers	CD18	99.5 ± 0.1	49.6 ± 0.1	98.8 ± 1.0	34.0 ± 10.5
	CD11b	99.8 ± 0.2	155.9 ± 33.2	98.3 ± 0.1	20.8 ± 13.2**
	CD32	99.9 ± 0.1	46.8 ± 8.9	99.0 ± 0.1	16.0 ± 2.9
APC-related surface markers	CD86	1.4 ± 0.4	1.6 ± 0.5	2.9 ± 0.2	8.2 ± 1.6
Adhesion molecules	CD54	88.9 ± 1.6	5.6 ± 0.7	86.5 ± 4.9	13.7 ± 4.4*
	CD29	93.6 ± 0.4	3.4 ± 0.9	7.8 ± 2.4***	3.8 ± 1.6

[a]Freshly isolated neutrophils (A, B), long-lived neutrophils (C, D). Antibodies are directly labeled, except IgG2b isotype that required staining with FITC-labeled secondary antibody. Data represent mean ± s.e.m. ($n = 3$ independent experiments). Statistical analysis: Student's paired *t*-test, long-lived neutrophils *vs* freshly isolated neutrophils (*$P < 0.02$, **$P < 0.005$, ***$P < 0.0001$).

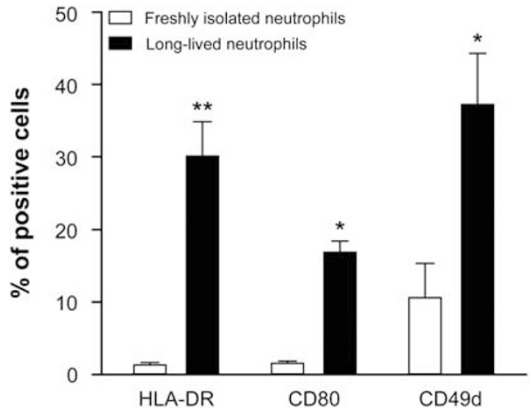


Figure 3 Surface expression of HLA-DR, CD80 and CD49d by neutrophils. Percoll-enriched long-lived neutrophils (10^6 cells) were marked with unconjugated primary antibody against HLA-DR and CD80 followed by staining with FITC-labeled secondary antibody, or directly stained with FITC-labeled anti-CD49d antibody before flowcytometry. Data are expressed in percentage of cells and represent mean ± s.e.m. ($n = 3$). Statistics: Student's paired two-tailed *t*-test (freshly isolated neutrophils *vs* long-lived neutrophils); $^*P < 0.05$, $^{**}P < 0.02$.

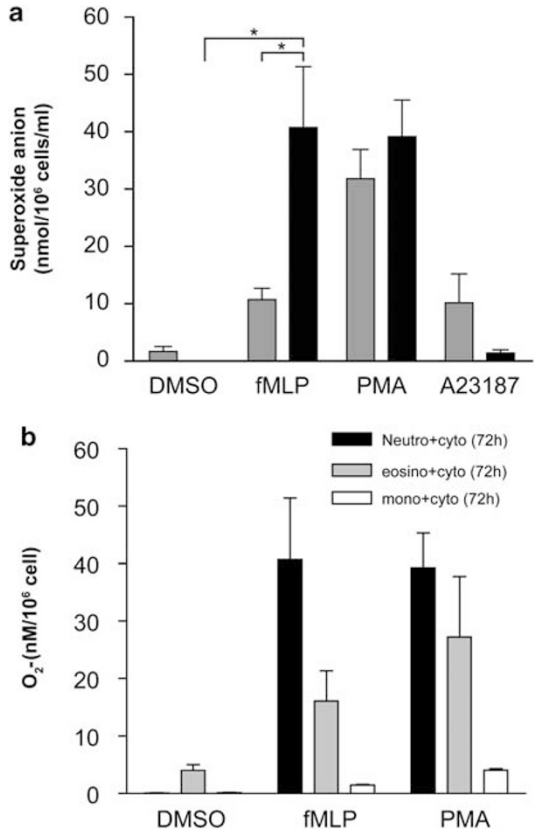


Figure 4 O_2^- production by human leukocytes. (**a**) After preincubation with cytochrome *c*, freshly isolated neutrophils (gray columns) and long-lived neutrophils (black columns) were stimulated with vehicle (DMSO), 10^{-7}M fMLP, PMA or ionophore A23187 for 5 min at 37°C. The release of extracellular reactive oxygen (O_2^-) was calculated in nmol of oxygen for 10^6 cells (see details in Materials and methods). The results are means ± s.e.m. ($n = 5$). Statistics: Student's paired two-tailed *t*-test ($^*P < 0.05$: unstimulated *vs* fMLP-stimulated long-lived neutrophils or fMLP-stimulated freshly isolated neutrophils *vs* fMLP-stimulated long-lived neutrophils). (**b**) Incubations with cytokines similar to those of long-lived neutrophils were conducted with human eosinophils and monocytes. Cells were then stimulated with DMSO, 10^{-7}M fMLP or PMA for 5 min at 37°C. The release of extracellular reactive oxygen (O_2^-) was calculated in nmol of oxygen for 10^6 cells. The results are means ± s.e.m. ($n = 5$: PMN, $n = 3$: eosinophils and monocytes).

Chemotaxis

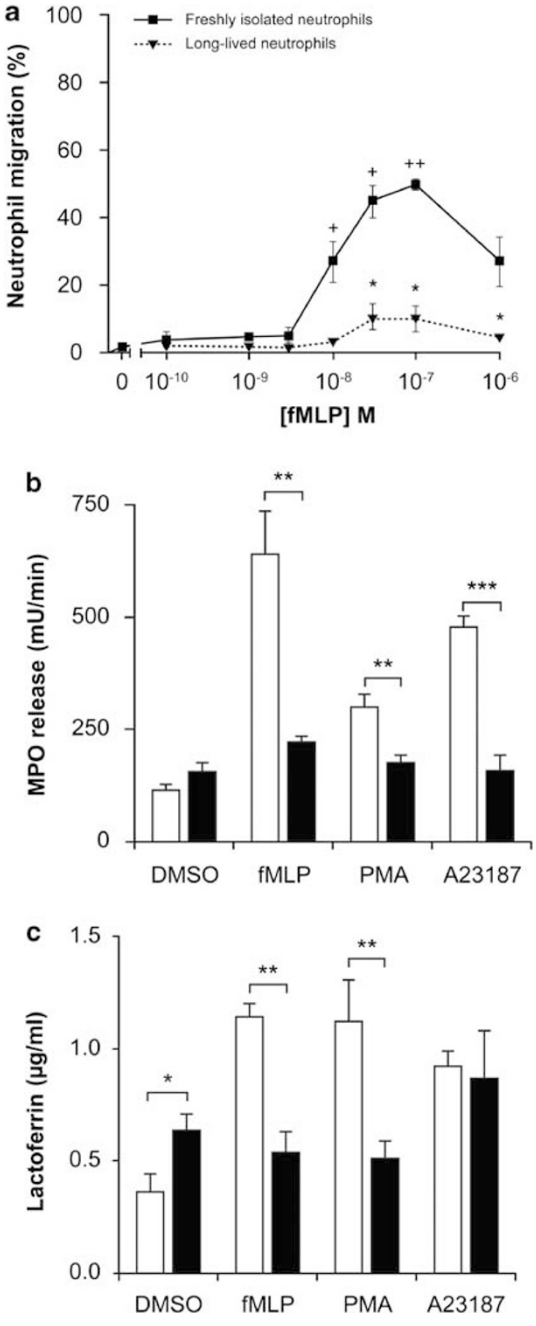
Chemotactic responses of long-lived neutrophils to fMLP, LTB_4 and IL-8 were studied. These factors are known to efficiently activate chemotaxis of human blood neutrophils.[39] Freshly isolated neutrophils responded to these chemoattractants with characteristic biphasic curves that showed maximal neutrophil responses at 10^{-7}M for fMLP (Figure 5a) and LTB_4 (data not shown) and 10^{-8}M for IL-8 (data not shown). In contrast, long-lived neutrophils migrated significantly less than freshly isolated neutrophils toward chemotactic factors. Percentages of neutrophil migration were greatly reduced for the three agonists tested. For example, 10.2 ± 3.9% long-lived neutrophils migrated in response to 10^{-7}M fMLP instead of 49.8 ± 1.6% of freshly isolated neutrophils (Figure 5a). Similarly, long-lived neutrophils *vs* freshly isolated neutrophils had a reduced migration response toward LTB_4 (5.3 ± 1.1% *vs* 51.2 ± 1.2%, respectively) and IL-8 (4.1 ± 1.6% *vs* 49.8 ± 0.3%, respectively). Chemotactic data obtained using polycarbonate filters with 8 μm pore size were similar to those obtained with 3 μm pore size (data not shown), indicating that the observed reduction of migration was not related to changes in cell size of long-lived neutrophils.

Exocytosis of primary and secondary granules

The release of MPO contained in primary granules and of LF prestocked in secondary granules by long-lived and freshly isolated neutrophils was evaluated in response to fMLP, PMA or A23187. Freshly isolated neutrophils released MPO in supernatants when stimulated by fMLP (639 ± 96 mU/min), PMA (300 ± 29 mU/min) and A23187 (476 ± 24 mU/min) with respect to vehicle (155 ± 22 mU/min) alone. In contrast, none of the abovementioned agonists significantly affected MPO release by long-lived neutrophils (Figure 5b). On the other hand, long-lived neutrophils had an increased spontaneous release of LF, but none of the agonists triggered an augmented release of LF by long-lived neutrophils, even in the presence of cytochalasin B (Figure 5c). This is in contrast to freshly isolated neutrophils in which LF release is significantly augmented with stimulations (213, 209 and 154% of vehicle for fMLP, PMA and A23187, respectively, in the presence of cytochalasin B used to amplify degranulation response). These results indicate that long-lived neutrophils have a low spontaneous release of MPO similar to that of circulating neutrophils, have an increased spontaneous release of LF, but remain insensitive to exogenous stimuli to increase this release.

Phagocytosis of opsonized zymosan particles

Neutrophils are avid phagocytes, phagocytosis of particles being a function essential to host defense during immune and inflammatory reactions.[1] The capacity of long-lived and freshly isolated neutrophils to phagocytize opsonized fluorescent zymosan bioparticles was compared by flow cytometry (Figure 6a and b) and confocal laser microscopy (Figure 6c). The fluorescence of non-internalized, surface-bound bioparticle conjugates was quenched by trypan blue. The percentage of neutrophils that had internalized zymosan, as well as MFI directly related to the number of particles taken up per cell, were assessed. Phagocytosis of opsonized zymosan by long-lived neutrophils was more efficient than phagocytosis of these particles by freshly isolated neutrophil (Figure 6a). A 141% increase of the phagocytic index of long-lived neutrophils was noted relative to freshly isolated neutrophils (Figure 6b). Both the percentage of phagocytozing cells and the MFI per cell were augmented with respect to freshly isolated cells. Of note, Figure 6c shows multiple zymosan particles (seen in red) easily visualized in a single phagocytozing long-lived neutrophil (seen in green). Thus, long-lived neutrophils have an augmented capacity to phagocytize particles.



Production of leukotrienes

Neutrophils are major purveyors of LTB_4, a bioactive lipid derived from arachidonic acid that is strongly involved in patho-physiology.[40,41] The profile of 5-lipoxygenase products was characterized by RP-HPLC in long-lived neutrophils.[32] Freshly isolated and long-lived neutrophils incubated in control conditions with the diluent DMSO did not produce any detectable amounts of LTA_4-derived metabolites (data not shown). Freshly isolated neutrophils responded minimally to fMLP in terms of leukotriene synthesis. In contrast, long-lived neutrophils stimulated with fMLP produced large amounts of LTA_4-derived metabolites (Figure 7a). Surprisingly, the most important part of these LTA_4-derived metabolites was composed of the $\Delta 6$-*trans* isomers of LTB_4 (35.8 ± 14.9 pmol), compared with 5.8 ± 2.9 pmol LTB_4 and 2.7 ± 0.9 pmol 20-OH-LTB_4 ($n = 5$). Long-lived neutrophils did not respond to exogenous arachidonic acid (Figure 7b), and their response to A23187 was significantly diminished (Figure 7c). Finally, long-lived neutrophils also generated 5-HETE with, however, a very different profile of synthesis compared with freshly isolated neutrophils (Figure 7d). The

Figure 5 Chemotactic and exocytosis functions of long-lived neutrophils. (**a**) Neutrophils charged with calcein-AM were stimulated to migrate across filters using concentration gradient of fMLP. The results are expressed in percentages (mean ± s.e.m. of triplicates) of fluorescent cells that migrated through the filter with respect to the total number of cells loaded. Statistics: Student's unpaired *t*-test of two separate experiments ($^{+}P<0.05$, $^{++}P<0.01$ with respect to vehicle; $^{*}P<0.05$ with respect to fMLP stimulation of freshly isolated neutrophils at equal molar concentrations of fMLP). (**b**) Secretion of MPO by neutrophils. Freshly isolated neutrophils (□) and long-lived neutrophils (■) at 5×10^6 cells/ml were stimulated with vehicle, fMLP, PMA or ionophore A23187 at a final concentration of 10^{-7}M for 5 min in the presence of 1 μg/ml cytochalasin B. The results are expressed in mU/min of MPO released in the supernatants and are displayed as means ± s.e.m. ($n = 4$). Statistics were performed using Student's unpaired *t*-test ($^{**}P< 0.02$, $^{***}P<0.001$). (**c**) Secretion of LF by neutrophils. Freshly isolated neutrophils (□) and long-lived neutrophils (■) at 10^5 cells/ml were stimulated similar to (**b**). LF released in supernatants was determined by ELISA. The results are expressed in ng/ml as means ± s.e.m. of four donors (freshly isolated neutrophils) and five donors (long-lived neutrophils). Statistics were performed using Student's unpaired *t*-test ($^{*}P<0.05$, $^{**}P<0.02$).

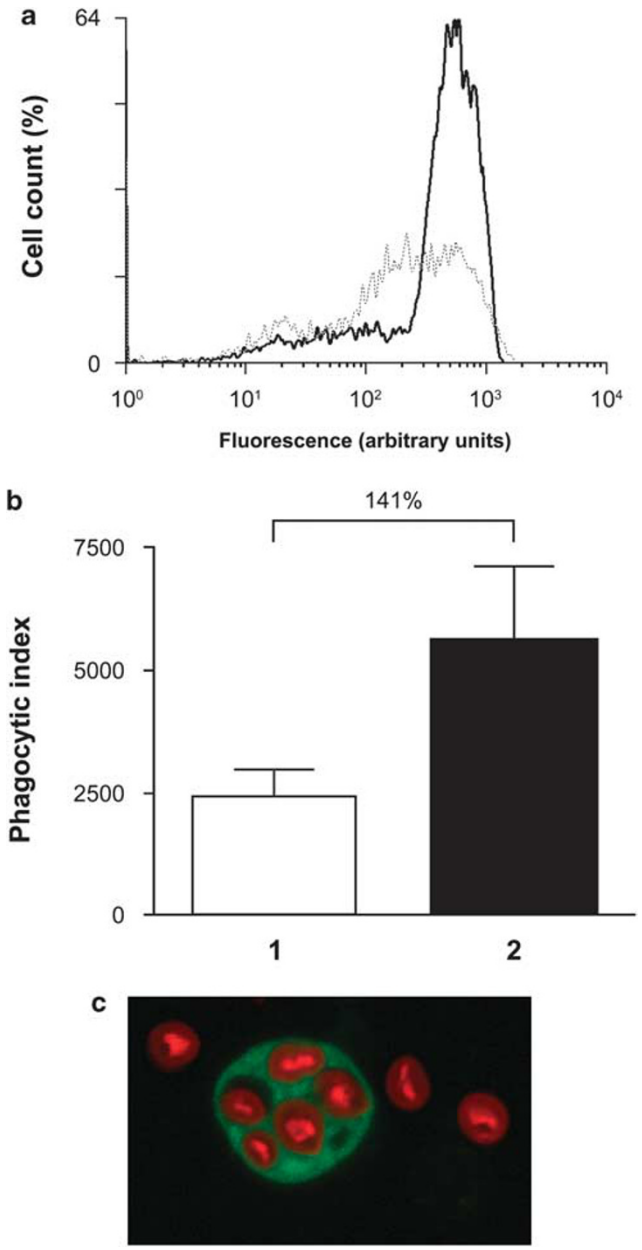


Figure 6 Phagocytosis of opsonized zymosan particles by neutrophils. (**a**) Flowcytometry of freshly isolated neutrophils (dashed tracing) and long-lived neutrophils (solid tracing). Cells were incubated with opsonized FITC-conjugated zymosan bioparticles for 10 min at 37°C using a neutrophil/zymosan ratio of 1/10. Data shown are representative of three separate experiments. (**b**) Histograms show the phagocytic index (calculated as % of neutrophils that had internalized zymosan × MFI) of freshly isolated neutrophils (bar 1) and long-lived neutrophils (bar 2). The results are means ± s.e.m. Statistics: phagocytic index of long-lived *vs* freshly isolated neutrophils is significantly different with $P < 0.02$ (Student's unpaired *t*-test, $n = 3$). (**c**) Visualization of zymosan phagocytosis by long-lived neutrophils. Cells labeled with calcein-AM (490/515 nm) were incubated with opsonized Alexa Fluor 594-conjugated zymosan bioparticles (590/617 nm) for 10 min similar to (**a**). Cells were observed by confocal laser microscopy using appropriate filters (magnification × 100).

fMLP stimulation was associated with a significant increase of 5-HETE that was not present in freshly isolated neutrophils. A23187 minimally induced 5-HETE production, as compared with the freshly isolated counterpart. Moreover, no accumulation of 5-HETE was observed in long-lived neutrophils in the presence of arachidonic acid, a pattern completely different from that of freshly isolated neutrophils. Similar experiments were also conducted with cytokine-exposed eosinophils (results not shown). In comparison to long-lived neutrophils, cytokine-exposed eosinophils synthesized six times less LTB_4 in response to A23187, and their LTB_4 production in response to fMLP and arachidonic acid was undetectable by RP-HPLC. However, they generated peptido-leukotrienes (3.6 ± 0.9, 13.5 ± 1.0 and 2.6 ± 1.5 ng in response to fMLP, A23187 and arachidonic acid, respectively, $n = 3$). Long-lived neutrophils did not produce peptido-leukotrienes in any of the above conditions, as analyzed by RP-HPLC.

Activation of 5-LO induced by A23187 or fMLP is associated with the nuclear translocation of the enzyme.[42] The next step was to verify whether the augmented leukotriene synthesis observed in fMLP-stimulated long-lived neutrophils was related to the activation of 5-LO through its translocation to the nuclear fraction. Immunoblot determination of the 5-LO protein content revealed an increased translocation of the 5-LO to the nuclear fraction of long-lived neutrophils compared with that of freshly isolated neutrophils (Figure 7e). In contrast, the nuclear fractions of A23187- and arachidonic acid-stimulated long-lived neutrophils were almost devoid of 5-LO. It is also interesting to note that, in long-lived neutrophils, a portion of 5-LO was already associated to the nuclear membrane in the absence of stimulation.

Production of IL-1β, IL-8 and IL-1Ra

Neutrophils can be a source of various cytokines during the inflammatory process,[43] and given the sheer number of neutrophils at an inflammatory site, such neutrophil-derived cytokines can greatly impact on the local process. The presence of cytokines produced was evaluated in cell-free supernatants and cell-associated materials of long-lived neutrophils and freshly isolated neutrophils incubated with or without fMLP.

Unstimulated long-lived neutrophils (column 2 of histograms) had a remarkable capacity for biosynthesis of the three cytokines (Figure 8). Production of IL-1Ra by long-lived neutrophils was 20 times higher than that of freshly isolated cells, and production of IL-8 was 32 times higher in long-lived neutrophils (Figure 8a and b). In addition, whereas there was no detectable IL-1β synthesis in freshly isolated neutrophils, long-lived cells produced 24 ± 11 pg/ml of this cytokine (Figure 8c). The fMLP stimulation, however, did not affect release of IL-1β and IL-1Ra in both types of neutrophils (results not shown). Though fMLP robustly increased IL-8 release in both freshly isolated and long-lived

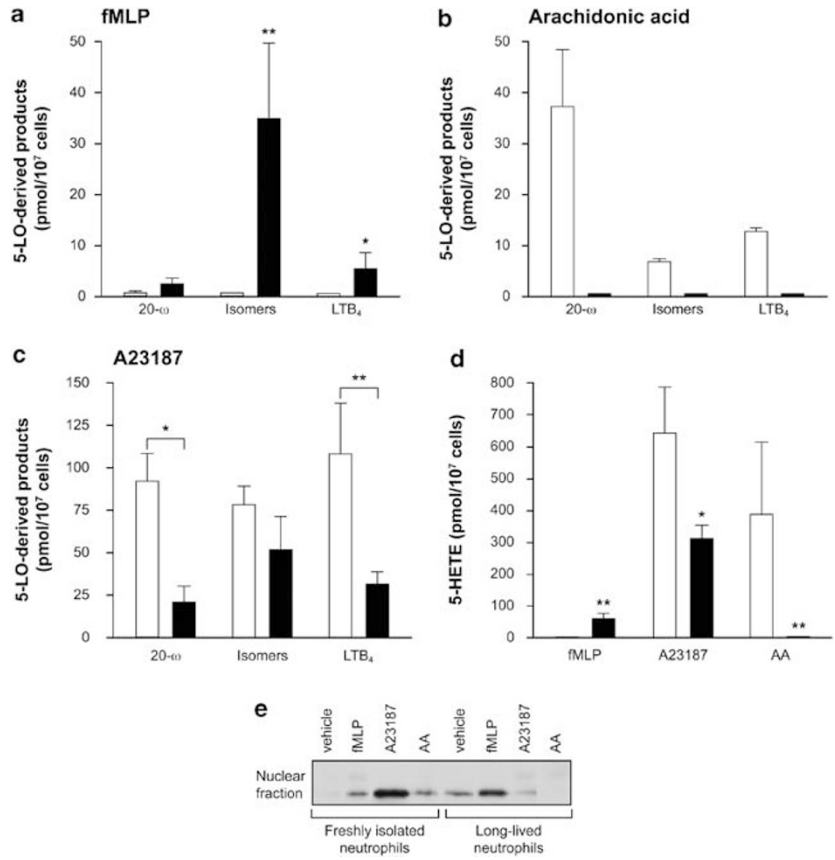


Figure 7 Leukotriene biosynthesis by neutrophils. Biosynthesis of LTA_4-derived metabolites was evaluated in freshly isolated neutrophils (□) and long-lived neutrophils (■) stimulated by 10^{-7}M fMLP (**a**), 2.5 μM arachidonic acid (**b**) and 10^{-7}M ionophore A23187 (**c**) using RP-HPLC. (**d**) 5-HETE accumulated by freshly isolated and long-lived neutrophils in conditions (**a**, **b**, **c**) was also assessed by RP-HPLC. The results are means ± s.e.m. of five independent experiments for fMLP and A23187, and three independent experiments for arachidonic acid (AA). Statistics: Student's unpaired two-tailed *t*-test: long-lived neutrophils *vs* freshly isolated neutrophils (*$P<0.05$, **$P<0.02$). (**e**) Nuclear fractions of cells (2×10^7) were prepared by lysis in NP-40 buffer and centrifugations. Subsequently, SDS–PAGE and immunoblots were performed with rabbit polyclonal anti-5-LO antibodies. The figure is representative of three different experiments.

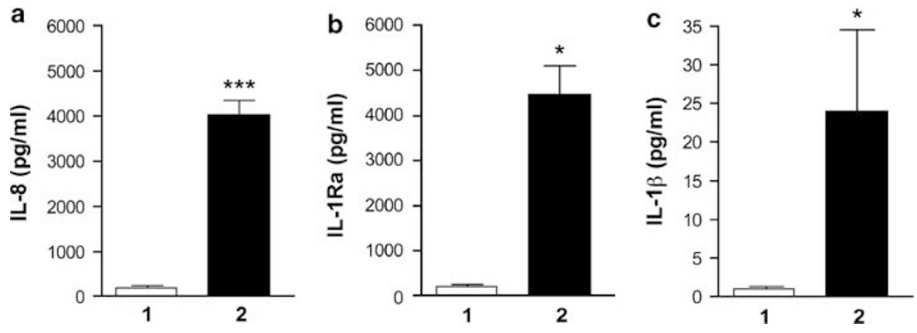


Figure 8 Release of IL-1β, IL-8 and IL-1Ra by long-lived neutrophils. Freshly isolated neutrophils (bar 1) and long-lived neutrophils (bar 2) were incubated at 5×10^6/ml for 24 h. IL-1β (**c**), IL-1Ra (**b**) and IL-8 (**a**) were measured in supernatants using enzyme immunometric assays. The results are means ± s.e.m. of three donors (freshly isolated neutrophils) and five donors (long-lived neutrophils). Statistics: Student's unpaired *t*-test for freshly isolated neutrophils *vs* long-lived neutrophils (*$P<0.05$, ***$P<0.001$).

neutrophils, this was accompanied by significant decline in intracellular IL-8 stores (results not shown). Thus, fMLP acted only as a secretagogue and did not influence *de novo* synthesis of IL-8 in these cells.

In addition to cytokine release shown in Figure 8, the total production of these cytokines by long-lived neutrophils was also greatly increased relative to freshly isolated neutrophils. Whereas freshly isolated neutrophils synthesized 868 ± 203 pg/ml of IL-8,

production of IL-8 was about 9.25 times more elevated in long-lived cells (8025 ± 482 pg/ml). IL-1Ra production was about 5.7 times increased in long-lived cells (9513 ± 2603 pg/ml) compared with freshly isolated cells (1659 ± 367 pg/ml). IL-1β was not detectable in the cell-associated materials of both neutrophil populations. This points to a *de novo* biosynthesis of IL-8, IL-1Ra and IL-1β in these cells.

Adhesion to primary human fibroblasts

The interactions between immune cells and fibroblasts have a critical function in host defense or tissue injury.[44–46] The next set of experiments was aimed at verifying whether long-lived neutrophils have the potential to interact with primary human fibroblasts. Neutrophils prelabeled with calcein-AM were incubated with confluent synovial fibroblasts and adherent neutrophils were observed by fluorescent microscopy. Freshly isolated neutrophils had a very low capacity to adhere on fibroblasts with or without stimulation by fMLP (Figure 9a and b). In contrast, long-lived neutrophils spontaneously adhered on cultured fibroblasts (58 ± 3 cells), and the number of adherent neutrophils was drastically increased in the presence of fMLP (364 ± 15 cells). The adhesion induced by fMLP was 50 times more elevated for long-lived neutrophils than for freshly isolated neutrophils. Interestingly, similar results were obtained by studying the adherence of long-lived neutrophils on MG-63 cells, another stromal cell type with osteoblastic characteristics (data not shown).

Proteome profile of phosphorylated serine-threonine kinases

The prolonged survival of long-lived neutrophils suggests cytokine-induced activation of intracellular signaling cascades delivering anti-apoptotic signals. As several protein kinases of the serine/threonine family are implicated in neutrophil survival,[10,47] phosphorylation of these kinases was compared in long-lived neutrophils *vs* freshly isolated neutrophils.

Lysates of long-lived neutrophils and freshly isolated neutrophils were incubated on nitrocellulose membranes impregnated with capture and control antibodies. Membranes were then probed with a cocktail of phospho-site-specific biotinylated antibodies and the relative amounts of phosphorylation of serine-threonine kinases were quantitated by densitometric measurements of the dot blots using ImageJ software. A robust phosphorylation of p38δ MAP kinase, GSK (glycogen synthase kinase)-3α/β, RSK (ribosomal protein S6 kinase)-1 and Akt1 and 2 was identified in long-lived neutrophils compared with freshly isolated neutrophils (Panel 2 *vs* 1, Figure 10a). The relative change in phosphorylation status of these kinases (long-lived neutrophils *vs* freshly isolated neutrophils) shows a nine-fold increase in p38δ MAP kinase, a 2.3-fold increase in RSK-1, a 2.6-fold increase in GSK-3α/β, a 12-fold increase in Akt1 and a 82-fold increase in Akt2 phosphorylation (Figure 10b). MSK2 and Akt pan phosphorylation are increased in freshly isolated neutrophils only. ERK1, 2, JNK1, 2, 3 and p38α, β and γ MAP kinases are minimally stimulated in both types of neutrophils.

DISCUSSION

The ability of leukocytes to switch phenotype is an essential property of the adaptive immune system. In particular, the microenvironment can activate such switch that drives cells to change their effector responses orienting host defenses and disease evolution. In this work, we show reprogramming in a cell traditionally associated with the innate immune system: the neutrophil. In response to combined cytokines enhancing survival, a subset of normal human blood neutrophils changes its effector functions. Derived from highly pure neutrophil preparations, this CD66b-positive population resembling the circulating neutrophil in morphology and staining characteristics, lives longer, responds differentially to second stimuli and has a phenotype vastly different from starting populations. Centrifugation on discontinuous Percoll gradients allowed isolation of this subpopulation of neutrophils from contaminating dead cells, debris, necrotic and apoptotic cells that could interfere with functional studies.

Inflammatory reprogramming increases neutrophil viability. Indeed, they exclude markers of different stages and different mechanisms of cell death, annexin-V and caspases being early cell death markers and PI incorporation being indicative of late apoptosis and necrosis. As shown herein, 8–17% neutrophils of the global neutrophil population have the potential to persist for >72 h under inflammatory conditions. This is in stark contrast to the circulating neutrophils whose survival is measured in hours. What would be the mechanisms of this persistence? We opted to explore this issue through global measure of intracellular protein phosphorylation, given that the protein kinases have been largely implicated in neutrophil survival.[21,48] The results obtained using the Proteome Profiler (human phospho-MAP kinase array kit) indicated a basal phosphorylation of the serine-threonine kinases GSK(glycogen synthase kinase)-3α/β, RSK(ribosomal protein S6 kinase)-1 and Akt in long-lived neutrophils. In contrast, the above kinases were not activated in non-stimulated circulating neutrophils. Significantly, these downstream components of the PI3-kinase pathway are involved in transmission of anti-apoptotic survival messages induced by chemotactic agents in neutrophils.[49] Thus, the present results suggest a probable role for these kinases in the extended lifespan of long-lived neutrophils.

The phenotype of long-lived neutrophils differs substantially from that of the circulating neutrophils. The surface phenotype of long-lived neutrophils, for instance, is indicative of the acquisition of novel 'non-classic' neutrophil functions. Although still expressing the usual neutrophil cell surface markers CD32, CD18 and CD11b, they acquired new surface proteins, such as HLA-DR and the co-stimulation molecule CD80, which are associated with antigen presentation. We note a similar instance of inflammatory

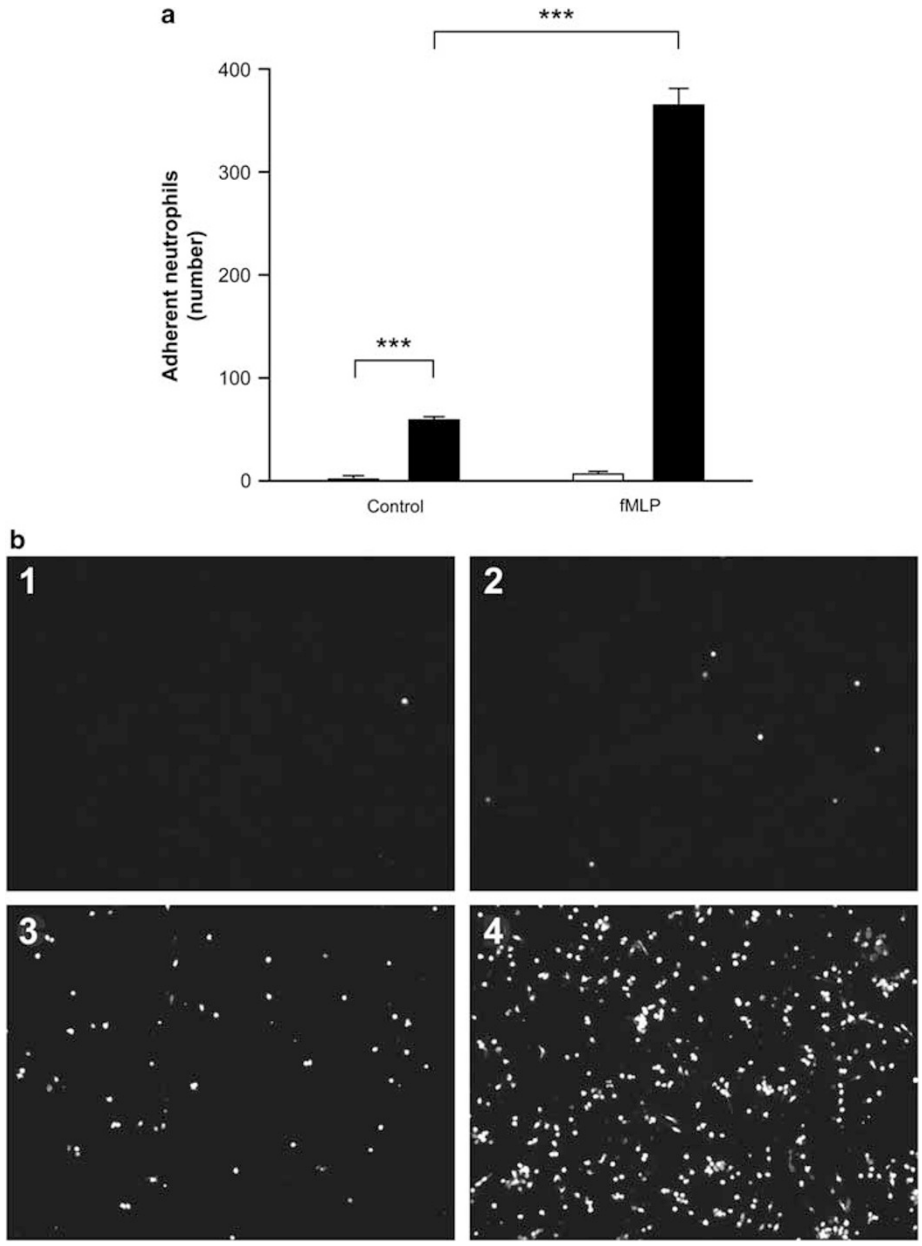


Figure 9 Adhesion of neutrophils to fibroblasts. Freshly isolated neutrophils (□) and long-lived neutrophils (■) with calcein-AM as tracer were added to confluent monolayers of primary normal synovial fibroblasts in culture in the presence or absence of pre-stimulation with 10^{-7}M fMLP for 5 min at 37°C. Adhesion was evaluated by fluorescence microscopy (magnification × 100). (**a**) The results are means ± s.e.m. of adherent cells counted in five separate fields/well. Statistics: Student's paired *t*-test for unstimulated freshly isolated neutrophils *vs* unstimulated long-lived neutrophils, unstimulated *vs* fMLP-stimulated long-lived neutrophils (***$P < 0.001$). (**b**) Panels 1 and 2 show adhesion of freshly isolated neutrophils before and after fMLP stimulation; Panels 3 and 4 show adhesion of long-lived neutrophils before and after fMLP stimulation. The results are representative of three different donors.

reprogramming in synovial fluid neutrophils, a percentage of which expressed functional HLA-DR.[50] Neutrophils from patients with vasculitis and systemic bacterial infections also acquire novel surface receptors and functions, such as expression of CD64, CD14 and neoexpression of HLA-DR and CD83.[51,52] In addition, we showed that, relative to freshly isolated neutrophils, long-lived neutrophils have increased cell surface ICAM-1, neoexpression of CD49d with concomitant diminution in expression of the traditional neutrophil adhesion integrins β_1 and β_2. This unique pattern of expression of adhesion molecules might underlie the robust adhesion of this subset of long-lived neutrophils to stromal cells. Neutrophils have, in other respects, a strong capacity to functionally interact with immune cells, fibroblasts, endothelial cells and osteoblasts.[53–57]

What would be the functional correlates of this cytokine-induced reprogramming? We show that long-lived neutrophils have diminished migration to certain chemotactic

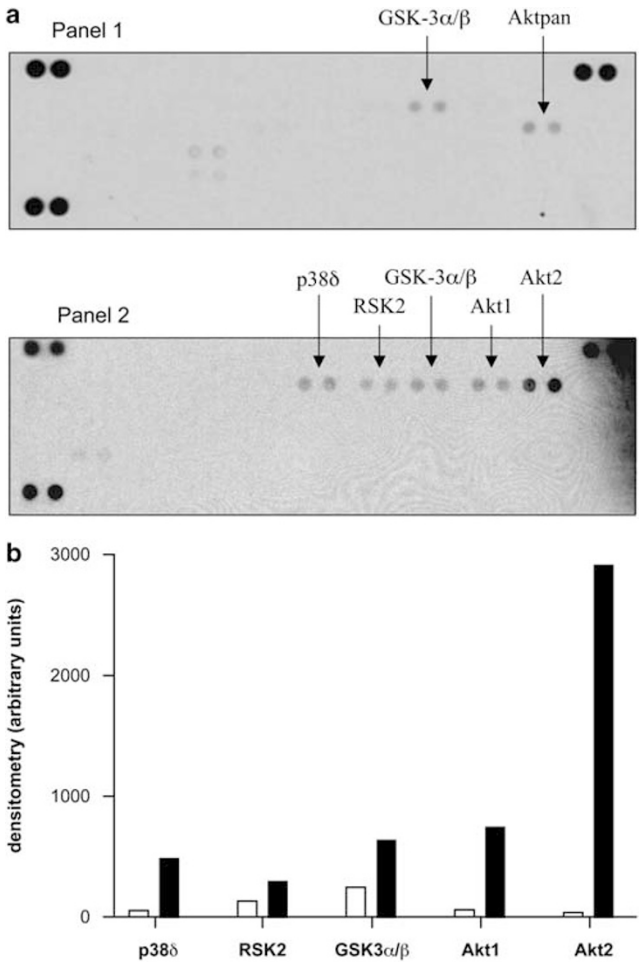


Figure 10 Human phospho-MAPK proteome profile of neutrophils. (**a**) Equal amounts of cell lysates of freshly isolated neutrophils (Panel 1) and long-lived neutrophils (Panel 2) were deposited on nitrocellulose membranes spotted in duplicate with capture and control antibodies. A cocktail of phospho-site-specific biotinylated antibodies was used to detect phosphorylated kinases. The arrays were exposed to Streptavidin-HRP and the ECL detection system before development on X-ray films. Spots were identified by alignment with grid provided in the kit. Arrays shown are representative of three different neutrophil donors. (**b**) Densitometric analysis of phosphorylation states of serine/threonine kinases in freshly isolated neutrophils (□) and long-lived neutrophils (■). The results are expressed in arbitrary units of densitometry. Data shown are representative of three different donors. GSK, glycogen synthase kinase; RSK, ribosomal protein S6 kinase.

stimuli, phagocytize more particles and release less of destructive enzymes. Moreover, they show a high capacity for cytokine synthesis such as the chemoattractant IL-8, the antagonist of IL-1R and IL-1β. The IL-1Ra/IL-1 ratio is 238 ± 99 ($n = 3$ paired donors), and neutrophil IL-1Ra is thus in a range to effectively neutralize IL-1 bioactivity.[58] As tissue-infiltrated neutrophils are in a cytokine-rich milieu that may potentially change their responsiveness to second signals, we studied the effects of a subsequent stimulation on neutrophils pre-exposed to prolonged incubation with cytokines. They showed a very characteristic profile, particularly evident in O_2^- production. Responses to fMLP were about five times greater in long-lived neutrophils than in freshly isolated neutrophils. The response to the ionophore A23187 that increases intracellular calcium concentration was, however, diminished whereas response to the PKC activator, PMA, remained unchanged. The above agonists elicit their responses by triggering specific signaling pathways and our results suggest qualitative and quantitative differences in the intracellular mechanisms between these long-lived neutrophils and circulating neutrophils. The phospholipid metabolic pathway leading to leukotriene B_4 synthesis also illustrates such differences. Interestingly, a significant amount of the 5-LO localized to the nuclear membrane in long-lived neutrophils in basal conditions, a phenomenon absent in circulating neutrophils. Furthermore, stimulation with fMLP had a much stronger effect on 5-LO translocation to membrane in the long-lived *vs* circulating neutrophils, a marked difference that might underlie the much greater capacity of the long-lived neutrophils to produce LTs on fMLP stimulation. Notably, instances of altered LT generation were also seen in pathological situations similar to SF neutrophils from patients with RA.[59,60] However, long-lived neutrophils under fMLP challenge showed a dramatic increase in the LTA_4-derived non-enzymatic hydrolysis products. This could suggest that LTA_4 from this neutrophil subpopulation is exported and hydrolyzed to the isomers *in vitro*. This increase of LTA_4 production by long-lived neutrophils could be destined for transcellular synthesis of LTA_4. This finding would be consistent with the drastic effect of fMLP on the adherence of long-lived neutrophils to fibroblasts, and with the phenotype switch in neutrophil-derived lipid mediators identified during the course of inflammation and its programmed resolution. Long-lived neutrophils could increase their LTA_4 biosynthesis without a concomitant increase in the production of LTA_4 hydrolase required for LTB_4 generation. LTA_4 could, thus, be converted into bioactive products other than LTB_4, such as lipoxins known to have actions different from those of leukotrienes. Moreover, the local microenvironment of these long-lived neutrophils would dictate the conversion fate of the high amounts of LTA_4 generated.

The results presented here might be a specific example of a more generalized phenotype-switching process. Many other groups have shown various cytokine combinations to influence neutrophil surface marker expression differentially, though these studies lacked functional characterization. TNF-α and IFN-γ induce the expression of CCR6 and CD83,[61] whereas GM-CSF and IFN-γ favor the expression of HLA-DR without concomitant expression of costimulatory molecules.[37] Functional modulation also suggests underlying transcriptome regulation similar to that seen in infiltrating neutrophils in endotoxin-mediated lung injury and in GM-CSF-stimulated neutrophils.[12,13]

In conclusion, cytokine-induced emergence of neutrophil subsets indicates that neutrophils, far from being terminally

differentiated cells, should be considered as a versatile long-lived entity. A better characterization of different long-lived neutrophils may enable new approaches to target these subpopulations of neutrophils with minimal effects on the immunologically indispensable circulating cells. As evidence accumulates on the role of the neutrophil in disease pathogenesis, further comprehension of the different aspects of neutrophil biology will aid in developing target specific and disease-specific tools.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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