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Neutrophil-specific genes are abundant in PBMC microarrays from lupus patients because of the presence of low-density granulocytes (LDGs) in mononuclear cell fractions. The functionality and pathogenicity of these LDGs have not been characterized. We developed a technique to purify LDGs from lupus PBMCs and assessed their phenotype, function, and potential role in disease pathogenesis. LDGs, their autologous lupus neutrophils, and healthy control neutrophils were compared with regard to their microbicidal and phagocytic capacities, generation of reactive oxygen species, activation status, inflammatory cytokine profile, and type I IFN expression and signatures. The capacity of LDGs to kill endothelial cells and their antiangiogenic potential were also assessed. LDGs display an activated phenotype, secrete increased levels of type I IFNs, TNF-α, and IFN-γ, but show impaired phagocytic potential. LDGs induce significant endothelial cell cytotoxicity and synthesize sufficient levels of type I IFNs to disrupt the capacity of endothelial progenitor cells to differentiate into mature endothelial cells. LDG depletion restores the functional capacity of endothelial progenitor cells. We conclude that lupus LDGs are proinflammatory and display pathogenic features, including the capacity to synthesize type I IFNs. They may play an important dual role in premature cardiovascular disease development in systemic lupus erythematosus by simultaneously mediating enhanced vascular damage and inhibiting vascular repair.

elevated levels of these low-density granulocytes (LDGs) and developed a procedure to rapidly isolate highly enriched preparations of these cells by negative selection. This allowed us to directly assess the functional capacity of the LDGs relative to normal neutrophils isolated from healthy controls, as well as relative to lupus normal-density autologous neutrophils. We also assessed their pathogenic potential in SLE by measuring their production of proinflammatory cytokines and type I IFNs, as well as their ability to induce endothelial damage and disrupt endothelial repair.

**Materials and Methods**

**Abs**

For purification of LDGs, biotinylated Abs recognizing CD3, CD7, CD19, CD79b, CD56, MHC class II, CD86, and CD235a were obtained from Ancell (Bayport, MN). Characterization of surface molecule expression was performed using FITC-conjugated Abs recognizing CD15, CD16, MHC class II, CD11c, CD66bm and CD86; PE-conjugated Abs recognizing CD14 and CD11b; and PE/Cy5-conjugated Abs recognizing CD10 and CD33 (all from Ancell). L-selectin Ab (anti–CD62L-PE) was from Southern Biotechnology Associates (Birmingham, AL).

**Patient selection**

The University of Michigan institutional review board approved this study. Subjects gave informed consent in accordance with the Declaration of Helsinki. Patients fulfilled the revised American College of Rheumatology criteria for SLE (12) and were enrolled from the University of Michigan outpatient Rheumatology Clinic and from the Michigan Lupus Cohort. Age- and gender-matched healthy controls were recruited by advertisement. Lupus disease activity was assessed by SLE disease activity index (SLEDAI) (13).

**Purification of LDGs**

Peripheral venous blood from SLE patients was collected in heparinized vacuum containers and processed within 4 h of phlebotomy. PBMCs were isolated by Ficoll/Hypaque gradient, washed twice, and contaminating RBCs were lysed by incubation with ice-cold ammonium chloride solution followed by potassium bicarbonate solution or by the hypotonic/hypertonic sodium chloride method. Cell pellets were centrifuged, resuspended in PBS/2 mM EDTA/0.5% BSA, washed, and incubated with 10 µL LDG isolation mixture (mixture of equal volumes of biotinylated Abs recognizing human CD3, CD7, CD19, CD79b, CD56, MHC class II, CD86, and CD235a) for 30 min on ice to label T and B lymphocytes, NK cells, monocytes, and residual RBCs. The labeled cells were washed and cultured with 40 µL anti-biotin MACS beads for 10 min on ice, followed by the addition of a second volume of 40 µL anti-biotin MACS beads mix and 10 min incubation on ice. Cells were washed and applied to a MACS-LS column, and nonimmobilized cells were recovered by negative selection. The purity of the LDG fraction typically was >95% and was determined by staining with CD15-FITC, CD14-PE, and CD10-PE/Cy5 by flow cytometry. LDGs were identified as CD15+/CD14lo or CD10+/CD14lo.

**Isolation of neutrophils**

Normal-density neutrophils were isolated by dextran sedimentation of RBCs, monocytes, and residual RBCs. The labeled cells were washed and fixed with 2% paraformaldehyde, and the expression of L-selectin on the CD10+ cells was quantified by FACS.

**Phagocytosis of Staphylococcus aureus bioparticles**

LDGs and neutrophils (1 × 10^6 cells) were plated in 96-well plates in 30% autologous serum/HBSS and incubated at 37°C/5% CO_2 for 1 h for adhesion. pHrodo Staphylococcus aureus bioparticle conjugates (Invitrogen-A10010, Carlsbad, CA) were resuspended in HBSS (final concentration of 1 mg/ml) and sonicated for homogeneous dispersion. The pHrodo S. aureus particle suspension was added to the cell cultures, and plates were covered and incubated at 37°C for 2 h in the absence of CO_2. Fluorescence was read with a plate reader using 530/25 excitation and 590/55 emission at a sensitivity of 35–37, following the manufacturer’s instructions.

**Microbicidal assay**

Neutrophil and LDG microbicidal activity was measured using previously published protocols (14). In brief, 2.5 × 10^6 LDGs or neutrophils were incubated at 37°C for 20 min in HBSS/10% autologous serum with 2.5 × 10^7 S. aureus strain 502A bacteria (American Type Culture Collection, Manassas, VA). This was followed by the addition of 10 U lysozyme (Sigma-Aldrich) to the coculture to kill any nonengulfed bacteria. Cell aliquots were harvested and lysed at various time points following the addition of lysostaphin to release internalized viable bacteria. Dilutions of the cell lysates were plated onto Tryptic Soy Agar media (Acumedia, Lansing, MI) and incubated at 37°C for 16 h. The number of CFU was quantified using standard techniques.

**Flow cytometric analysis of cell surface L-selectin**

Flow cytometric analysis of cell surface L-selectin was performed, as previously described (15). In brief, a hydroxamic acid-based L-selectin shedding inhibitor, KD-IX-73-4 (TAPI-0) was purchased from Peptides International (Louisville, KY) and reconstituted in DMSO at 5 mg/ml. Lupus LDGs and lupus and control neutrophils were resuspended in PBS/10 mM glucose/0.5% FBS/20 Mm HEPES and incubated for 10 min at 37°C in the presence of vehicle (0.1% v/v DMSO) or freshly prepared TAPI-0 (50 µg/ml). The cells were then cultured in the presence or absence of 0.1 µg/ml PMA (Sigma-Aldrich) for 10 min at 37°C, followed by the addition of RPMI 1640/10% FBS and incubation on ice for 15 min. The samples were centrifuged at 1600 rpm for 5 min at 4°C, cell pellets were resuspended in PBS/1% horse serum/1% BSA and incubated on ice for 30 min with anti-L-selectin-PE and anti-CD10-PE/Cy5 (Biolegend, San Diego, CA) or isotype controls. Cells were washed, and MPO expression was determined by flow cytometric analysis, as described below.

**Microbicidal assay**

Neutrophils and LDGs were fixed in 4% paraformaldehyde, washed, resuspended in PBS/10% DMSO (Sigma-Aldrich), and stored at −80°C prior to myeloperoxidase (MPO) staining, as described (14). Prior to staining, cells were thawed, permeabilized with 0.2% saponin/PBS (Sigma-Aldrich), washed, blocked with 1% horse serum/1% BSA/0.2% saponin/PBS, and stained at 4°C with anti-MPO-FTTC (BD Biosciences, San Jose, CA) or isotype control. Cells were washed, and MPO expression was determined by flow cytometric analysis.

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**Generation of H_2O_2**

The generation of H_2O_2 was quantified, as previously described (16). In brief, H_2O_2 secretion from LDGs or neutrophils (untreated or stimulated with 0.1 µg/ml FMA or 200 µg/ml BSA/anti-BSA immune complexes) was determined by colorimetric analysis using Amplex Red (Molecular Probes, Eugene, OR) reagent, according to the instructions of the manufacturer. A solution containing 50 µM Amplex Red reagent/10 U/ml HRP/PBS was prepared and added to neutrophil or LDG cultures at 37°C for 60 min. Absorbance at 560 nm was assessed, and the H_2O_2 concentration was determined using an H_2O_2 standard curve. The detection limit of this method was 0.625 nM H_2O_2.

**Generation of immune complexes**

BSA/anti-BSA immune complexes were generated, as previously described (17). In brief, BSA-1 (Sigma-Aldrich) was added to anti-BSA stock solution (MP Biomedicals, Solon, OH) at a ratio of 1:10, incubated for 30 min at 37°C, centrifuged at 2500 rpm for 5 min at room temperature, and washed twice with PBS. The complexes were resuspended in PBS for a final concentration of 2 mg/ml prior to their use.
Cytokine and eicosanoid quantification

Lupus LDGs, autologous neutrophils, and control neutrophils were cultured for 48 h in the presence or absence of 0.1 μg/mL PMA. Supernatants were harvested, and the concentration of the cytokines IL-1β, IL-6, IL-8, TNF-α, and IFN-γ, as well as the eicosanoids PGE2 and thromboxane B2, were quantified by a Bio-Plex mouse array kit (Bio-Rad Laboratories, Hercules, CA) as described by the manufacturer. Human IL-17 was quantified in cell supernatants by ELISA (eBioscience, San Diego, CA). Confirmatory experiments were performed for selected cytokines (IL-8 and TNF-α) by assessing their intracellular expression on CD10+ cells as follows. Lupus LDGs and control and lupus neutrophils were incubated with or without 1 μg/mL LPS and 1 × brefendin A (Biolegend) in IMDM/30% FBS (In-vitroGlo; Promega, Madison, WI). Cells were harvested twice with 0.5% horse serum/1% BSA containing 1 × brefendin A, and incubated with anti-CD10 (Biolegend) or respective isotype for 30 min on ice. Cells were then washed and resuspended in 2% paraformaldehyde overnight at 4°C. This was followed by three washes and incubation in 0.2% saponin/PBS/1% horse serum/1% BSA for 1 h on ice, then incubation with anti-IL-8-Alexa Fluor 488 (Biolegend), anti–TNF-α-PE (Miltenyi Biotec, Auburn, CA), or respective isotype control Abs on ice for 1 h. Cells were then washed and fixed in 2% paraformaldehyde, and the intracellular expression of IL-8 and TNF-α was assessed on the CD10+ cells by FACS.

Quantification of IFN-α mRNA

A total of 2 × 10^6 control or lupus neutrophils or autologous lupus LDGs were plated and left untreated or stimulated with PMA (100 ng/ml) for 1 h or G-CSF (50 ng/ml) for 16 h. Total RNA was prepared with Tripure (Roche, Indianapolis, IN). RNA template was digested with DNase I (Invitrogen), and then reverse-transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen). Oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) used in the reactions were as follows: universal IFNα: 5'-TCC ATG AGA TAT CGC AGC-3' (forward), 5'-ATT TCT CTT ACT CCA ACC TCC C-3' (reverse) and HPRT1: 5'-TCT GTC AOG CAG CAT TAT CCC-3' (forward), 5'-GGG CAT ATC CTA CAA ACA CAC-3' (reverse). PCR reactions were run on an ABI Prism 7900HT in duplicate using 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). These IFN-α primers essentially amplify all described forms of IFN-α (18). Because IFN-α genes are monoeixon, it is critical to remove all genomic DNA from RNA preparation, and this was verified prior to RNA use.

Type 1 IFN bioassay

LDGs, autologous neutrophils, or control neutrophils were isolated and cultured alone or in the presence of 0.1 μg/mL PMA for 1 h or 50 ng/mL rG-CSF for 16 h. In additional experiments, neutrophils and LDGs were transfected with polyinosinic:polycytidylic acid [poly(I:C); Calbiochem, San Diego, CA], using the Amaxa Biosystems nucleoporator (Lonza, Walkersville, MD) with program Y01, as previously reported (19). In brief, 2 × 10^6 neutrophils or LDGs were resuspended in 100 μl complete nucleofector solution (Lonza) containing 10 μg poly(I:C), transferred to a nucleofector cuvette, and immediately thereafter transferred into 24-well tissue culture plates. Supernatants were harvested 16 h after transfection. Induction of IFN-inducible genes by LDG or neutrophil supernatants was determined using a described bioassay (20, 21) that quantifies specific IFN-inducible genes by cell supernatants on cultured target epithelial cells, with some modifications. To this end, HeLa cells (American Type Culture Collection) were cultured in Difco/10% FBS/nonessential amino acids/10 mM HEPES at 37°C in 5% CO2, plated at 2 × 10^5 cells/well in a 24-well plate, and exposed to 100% LDG-supernatant or rIFN-α-2b (1 μg/well. Schering-Plough, Kenilworth, NJ, used as positive control) for 6 h. Triplicate was added, and cells were stored at −70°C until RNA extraction. cDNA was prepared, and real-time PCR reactions were run, as described above. The type 1 IFN-inducible genes quantified by this assay were IFN44, IFN44 and IFNIB-1. Primers for the genes were described previously (20). Samples were normalized to media alone after normalization to housekeeping gene HPRT1, and the results are reported as fold induction/media.

Endothelial cell cytotoxicity assay

The capacity of lupus LDGs and lupus and control neutrophils to induce HUVEC cytotoxicity was assessed by flow cytometry. In brief, HUVECs were cultured in MCDB131 (Life Technologies, Carlsbad, CA) basal media supplemented with microvascular endothelial growth medium-2 (without hydrocortisone) (Lonza) in 0.5% gelatin-coated 24-well tissue culture plates. Supernatants were harvested 16 h before exposure to neutrophils, as previously described (21). PMA-activated LDGs, autologous neutrophils, or control neutrophils were cocultured with HUVECs at a 1:2 E:T ratio for 16–20 h. LDGs and neutrophils were harvested, and HUVECs were exposed to 0.05% trypsin-EDTA (Life Technologies) and centrifuged at 1600 rpm for 5 min. Cells were resuspended in 2% horse serum/PBS, and 10^3–10^5 cells were incubated with PE-conjugated anti-human CD146 (BD Pharmingen, San Diego, CA; an endothelial cell marker), PECy5 anti-human CD10 (Biolegend; an LDG/neutrophil marker), and APC- or FITC-Annexin V (BD Pharmingen, an apoptosis marker). Cells were washed and fixed in 4% paraformaldehyde. The percentage of apoptotic endothelial cells was identified on cells that stained for CD146 and Annexin V, in the PECy5 anti-human CD10-negative gate. Experiments were performed in the presence or absence of a transwell (Corning, Lowell, MA) that separated HUVECs from LDGs/neutrophils. Findings were confirmed using a bioluminescence cytotoxicity assay (CellTox assay, Cell Technology, Mountain View, CA) that quantitatively measures GAPDH release, according to the manufacturer’s instructions.

Assessment of the capacity of endothelial progenitor cells and circulating angiogenic cells to become mature endothelial cells

Control or SLE PBMCs (4 × 10^6/ml) were cultured in endothelial cell-specific enrichment medium (Cambrex, East Rutherford, NJ) supplemented with 20% FBS, bovine brain extract, and epithelial growth factor, as described by our group (21). Media was changed 120 h after plating and then every 3 d. On day 15, cells were incubated with markers of mature endothelial cells, including 1 دي-3,3,3,3-4-tetramethylrhodamine carboxylic acid–acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, MA) and FITC–fibaes americanas agglutinin-1 (ICN, Irvine, CA). Cells were analyzed by fluorescence microscopy using a Leica DMIRB fluorescent inverted microscope (Leica Microsystems, Deerfield, IL). All images were acquired at room temperature using live cells in PBS without mounting media. Images were acquired with an objective lens of the fluorescent microscope were as follows: ×5, ×10, or ×20 (for ×50, ×100, or ×200 total magnification, respectively). The numeric aperture for the objective lenses of the fluorescent microscope were as follows: ×5 = 0.15, ×10 = 0.3, and ×20 = 0.4. Images were acquired with an Olympus DP300BW camera using the acquisition software Olympus-BSW (both from Olympus, Tokyo, Japan). Final processing was done with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA). In some of the experiments, PBMCs were depleted of plasmacytoid DCs (pDCs) using the anti-CD304 BDCA-4 Microbead Kit or depleted of LDGs using anti-CD10 beads (both from Miltenyi Biotec), prior to plating the rest of the PBMCs under proangiogenic conditions. The capacity of these culture supernatants to induce type 1 IFN-responsive genes on epithelial cell lines was measured, as stated above and as previously described (21).

Statistical analysis

The difference between means was analyzed using the Student t test or ANOVA with post hoc analysis with SPSS v.14 (SPSS, Chicago, IL). Univariate linear regression was performed to determine whether treatment with immunosuppressants was associated with phenotypic/functional abnormalities. Vascular repair markers were modeled separately as dependent variables, with medications modeled as dichotomous independent predictors. For the IFN-inducible gene response, two-group comparisons of continuous data that had a normal distribution were assessed using t tests. The Kruskal–Wallis nonparametric test was used to compare the study groups for the values of the IFN-inducible genes because the data were not normally distributed.

Results

Demographics and patient population

Demographic and clinical information for patients and controls enrolled in this study is included in Table 1. There were no significant differences between SLE patients and controls with regard to age or gender. Overall, 60% of SLE patients studied had serological and/or clinical evidence of active disease (measured as SLEDAI > 2). Thirty percent of the lupus patients included in the study had current lupus nephritis or a history thereof.

Characterization of lupus LDGs

The presence of LDGs was initially confirmed in adult SLE patients (Fig. 1). The LDG population segregated directly adjacent to the monocyte pool by flow cytometric analysis using a dual log scale of forward and side scatter intensity (Fig. 1A). The monocytes and LDGs could be clearly distinguished in lupus samples based on expression of the neutrophil marker CD15 (Lewisx) and the
monocyte marker CD14 (Fig. 1A). Monocytes were CD14\(^{+}/\)CD15\(^{{b}}\), whereas LDGs had a CD14\(^{b}/\)CD15\(^{+}\) profile. Indeed, >95% of the cells in the monocyte gate were CD14\(^{+}/\)CD15\(^{+}\). Monocytes could be further distinguished from LDGs by their expression of MHC class II and the costimulatory molecule CD86 (B7.2), as well as by their lack of expression of the membrane peptidase CD10 (Fig. 1A). Monocytes were CD14\(^{+}/\)CD15\(^{lo}\), whereas LDGs had a CD14\(^{lo}/\)CD15\(^{+}\) profile. Indeed, visible is the more activated phenotype of CD86\(^{+}/\)CD16\(^{+}\). In contrast, LDGs were CD16\(^{hi}/\)CD86\(^{+}\) (Fig. 1B).

Using surface expression of CD14 and CD15 as a guide, it was possible to construct scatter gates that discriminated LDGs from monocytes present in lupus PBMCs. The forward and side scatter profile was used to determine the relative levels of LDGs in PBMCs from healthy individuals and SLE patients, which was confirmed by CD14/CD15 coexpression. PBMCs from 22 healthy individuals showed an average of 5% LDGs. This likely represents contaminating mature degranulated neutrophils in the PBMC preparations, because most control samples were completely devoid of contaminating granulocytes. By comparison, all SLE preparations contained LDGs, which represented an average of 17% of total PBMCs, with a range of 1.2–54% (n = 65; p < 0.05). When total numbers of neutrophils present in the PBMC subset were compared, there was a highly significant difference between lupus patients and healthy controls (mean, 0.26 \(\pm\) 0.08 \(\times\) 10\(^{6}\) cells/ml of blood versus 0.0046 \(\times\) 10\(^{6}\) cells/ml of blood, respectively; p = 0.007), indicating a significant increase that was not related to the lymphopenia commonly observed in SLE. LDGs accounted for >25% of the total PBMCs in 12 of 65 SLE samples (19% of SLE patients). The clinical characteristics of this subset of SLE patients were examined in greater detail; 83% of patients with elevated levels of LDGs had skin involvement (including vasculitis) and/or synovitis. In contrast, these clinical complications were not observed in patients with PBMC profiles comparable to those of healthy controls. There was no significant correlation between age, disease duration, and/or use of immunosuppressive drugs or corticosteroid dose and the presence of LDGs, consistent with results from the two previous publications that reported this cell subset (2, 11). Indeed, these cells were present in SLE patients who were not using medications to treat their disease (either because of new onset or remission of disease), as well as in individuals with various doses of immunosuppressives, corticosteroids, or antimalarials (data not shown).  

The LDG population was examined in greater detail. To this end, a negative-selection approach to label and remove T and B lymphocytes, NK cells, monocytes, and erythrocytes from PBMCs by magnetic bead-assisted cell sorting was developed (Fig. 2). Negative selection was necessary because commercially available kits used to purify neutrophils using positive selection rely on
positive selection based on expression of CD15 or CD16, which could potentially interfere with subsequent cell-based assays of cell activation and cytotoxicity.

PBMCs from SLE patients were incubated with a mixture of biotin-conjugated mAbs recognizing CD3, CD7, CD19, CD79b, CD56, CD86, MHC class II, and glycophorin A; tagged with anti-biotin Ab-coupled superparamagnetic beads, and depleted in a magnetic field. The resulting cell suspension was highly enriched for LDGs (≈95%), as demonstrated by FACS analysis for granulocyte markers FcγRIII (CD16), CD15, and CD10. CD33, a marker expressed on developing or immature granulocytes was only very weakly expressed (Fig. 2A), and the expression of other early progenitor markers CD34 and Flt-3 (CD135) was not detected. Taken together, the profile of surface molecular expression was consistent with a mature neutrophil phenotype. In addition, LDGs expressed PECAM/CD31, CD11c (Fig. 2A, Table II), G-CSFR (CD114), and GM-CSFR (CD116) (Table II). IL-3Rα-chain (CD123) and M-CSFR (CD115) were not detectable.

There was a trend for CD10 and CD11c mean fluorescence intensity (MFI) to be lower in the lupus LDGs compared with autologous lupus neutrophils and control neutrophils, but the difference was not statistically significant (Table II). Overall, compared with autologous normal-density lupus neutrophils or control neutrophils, LDGs expressed comparable levels of the markers mentioned above (Table II). The phenotype of lupus LDGs was also confirmed by fluorescence microscopy (Fig. 2B).

To further assess activation status, SLE neutrophils, autologous LDGs, and neutrophils isolated from healthy volunteers were analyzed for surface expression of CD66b and CD11b. Elevated expression of these molecules on the cell surface is associated with cell activation due to vesicular membrane fusion during exocytosis of neutrophil granules (22). CD66b and CD11b were elevated on the cell surface of lupus neutrophils and LDGs compared with normal neutrophils (CD66b: 59.6 ± 0.5 [lupus LDGs], 49.1 ± 4.9 [lupus neutrophils], and 24.8 ± 0.5 [control neutrophils]; CD11b: 37.7 ± 3.6 [lupus LDGs], 24.7 ± 3.5 [lupus neutrophils], and 13.2 ± 1.1 [control neutrophils]; results represent MFI ± SEM of 5 controls and 13 lupus patients; \( p < 0.05 \) comparing control neutrophils to lupus LDGs and neutrophils; \( p = NS \) comparing LDGs and autologous neutrophils).

L-selectin is constitutively expressed at high levels on the surface of resting neutrophils; these molecules are shed upon neutrophil stimulation in a distinctly robust manner and play a role in regulating leukocyte rolling velocity in vivo (23). The shedding of l-selectin is distinct, because it is unusually rapid and resistant to common protease inhibitors (24). However, certain hydroxamic acid–base metalloproteinase inhibitors prevent l-selectin shedding from the surface of neutrophils (15). This was demonstrated by showing that exposure of neutrophils to various agents induces the rapid loss of l-selectin from the cell surface and that this is prevented by a metalloproteinase inhibitor. To additionally assess LDG activation status, we compared l-selectin shedding after PMA stimulation among the various cell subsets. Prior to stimulation, there were no significant differences in l-selectin expression among LDGs, lupus neutrophils, and control neutrophils when assessing the percentage of expression and MFI (data not shown). After stimulation, and in the absence of a metalloproteinase inhibitor, there was comparable downregulation of cell surface l-selectin expression among the three groups (−12.1% ± 2.5% for lupus LDGs, −12.4% ± 3% for lupus neutrophils, and −13.9% ± 4.2% for control neutrophils; results represent mean change (± SEM) in the percentage of cell surface l-selectin expression from baseline of four independent experiments; \( p = NS \)). Similar results were found when MFI was analyzed (−9.8 ± 3.5 for lupus LDGs, −10.4 ± 3.2 for lupus neutrophils, and −14 ± 4.2 for control neutrophils; results represent mean change (± SEM) in MFI cell surface l-selectin expression from baseline; \( p = NS \)). When a metalloproteinase inhibitor was added, shedding was abrogated in the three groups, and again there were no significant differences among the groups (17.3% ± 3% for lupus LDGs, 15.5% ± 2% for lupus neutrophils, and 13% ± 1.5% for control neutrophils; results represent mean change (± SEM) in the percentage of cell surface l-selectin expression from baseline of four independent experiments; \( p = NS \)). Similar results were found when MFI was analyzed (5.4 ± 3 for lupus LDGs, 5.3 ± 2.5 for lupus neutrophils, and 5.5 ± 1.8 for control neutrophils; results represent mean change (± SEM) in MFI cell surface l-selectin expression; \( p = NS \)). Thus, relative to healthy control neutrophils, LDGs and autologous lupus neutrophils have an activated phenotype based on the surface molecule expression of CD66b and CD11b ex vivo, but they do not differ with regard to l-selectin expression and shedding before and after stimulation.

However, examination of LDGs by differential staining and microscopy suggested an immature phenotype consistent with a previous report on pediatric lupus patients (2). Using microscopy, the stages of neutrophil development are distinguished by changes in nuclear morphology. Early or immature neutrophils possess round or ovoid nuclei, whereas more developed cells have indented, band, or segmented nuclei. Despite the apparent mature phenotype demonstrated by surface molecule expression (Figs. 1, 2), differential staining of the enriched LDGs revealed a mixed population with cells with band, lobular, or myelocyte-like nuclei (Fig. 2C).

The distribution of cells displaying the different nuclear morphologies varied among samples, but the LDGs were always less segmented and more lobular than the corresponding lupus autologous normal-density neutrophils or the neutrophils isolated from healthy individuals (data not shown). Approximately 60% of the LDGs were polymorphonuclear, with the rest of the cells having a band, lobular, or myelocyte-like nuclei. This is in contrast with the autologous normal-density neutrophils and the control neutrophils in which >90% were polymorphonuclear. Overall, although LDGs and their autologous lupus neutrophils displayed surface markers of activation, their nuclear morphology differed significantly.
**LDGs display neutrophil function but have decreased phagocytic potential**

Next, the functional capacity of LDGs was evaluated. Data included the capacity to engulf and kill bacteria, the intracellular levels of MPO granules, and the ability to mount a respiratory burst and to generate ROS. The phagocytic function of neutrophils is crucial for host defense and is mediated by FcγR and complement receptors (25). As shown in Fig. 3A, LDGs displayed decreased phagocytosis of *S. aureus* compared with healthy control or lupus autologous neutrophils. However, the bactericidal activity of the engulfed bacteria was comparable among LDGs, autologous neutrophils, or healthy control neutrophils (Fig. 3B). Furthermore, LDGs displayed comparable levels of intracellular MPO expression (Fig. 3C), as well as comparable degrees of respiratory burst, as assessed by H₂O₂ synthesis after PMA or immune complex stimulation (Fig. 3D, 3E and data not shown; normal-density neutrophil isolation by dextran or by negative selection). Overall, these results indicate that LDGs have phenotypic and functional features of normal neutrophils; however, despite an overall activated phenotype based on the expression of surface receptors, their capacity to phagocytose bacteria seems to be significantly impaired.

**LDGs synthesize increased levels of proinflammatory cytokines**

During migration, neutrophils are activated, resulting in the generation of arachidonic acid metabolites, ROS, proteases (26), and various cytokines that may play an additional role in the progression of inflammation to resolution or to a chronic inflammatory response (27). The secretion of nine cytokines and two eicosanoids was assessed in the supernatants of resting or PMA-activated LDGs, autologous neutrophils, or healthy control neutrophils (Fig. 3B). Furthermore, LDGs displayed comparable levels of intracellular MPO expression (Fig. 3C), as well as comparable degrees of respiratory burst, as assessed by H₂O₂ synthesis after PMA or immune complex stimulation (Fig. 3D, 3E and data not shown; normal-density neutrophil isolation by dextran or by negative selection). Overall, these results indicate that LDGs have phenotypic and functional features of normal neutrophils; however, despite an overall activated phenotype based on the expression of surface receptors, their capacity to phagocytose bacteria seems to be significantly impaired.

**LDGs synthesize increased levels of type I IFNs**

Several studies have implicated a key role for IFN-α, and potentially other type I IFNs, in SLE pathogenesis (2, 4, 6, 28). Although the exact sources of the increased levels of IFN-α in SLE are not known, depletion experiments demonstrated that pDCs contribute only part to the increased expression of this molecule in this disease (6). Because mature neutrophils can produce this cytokine in response to specific stimuli (18, 29), we quantified IFN-α mRNA levels in SLE LDGs, their autologous neutrophils, and healthy control neutrophils in response to PMA and G-CSF. As shown in Fig. 5A, upon activation with PMA, LDGs expressed significantly higher levels of IFN-α mRNA than control or autologous lupus neutrophils. Furthermore, LDGs and autologous lupus neutrophils expressed higher levels of IFN-α mRNA upon stimulation with G-CSF. Because these cells could synthesize other type I IFNs in addition to IFN-α, and to confirm that increased levels of type I IFNs were being synthesized by LDGs, we assessed the capacity of LDGs and neutrophil supernatants to induce type I IFN signatures on an epithelial cell line, using a bioassay reported by Hua et al. (20) and previously used by our group with some modifications (21). In this bioassay, an epithelial cell line is exposed to the supernatants of lupus or control cells to assess the induction of type I IFN-inducible genes on the cell line. Supernatants from unstimulated, PMA-activated, and poly(I:C)-transfected LDGs caused a significant induction of type I IFN signatures on epithelial cell lines compared with control and autologous lupus neutrophils (Fig. 5B, 5C, 5E). This was significantly enhanced upon stimulation with G-CSF (Fig. 5D). Overall, these data indicated that lupus neutrophils synthesize higher amounts of type I IFNs than control neutrophils, and this is significantly enhanced in the LDG group. These results were confirmed when normal-density neutrophils were isolated by negative selection after gradient centrifugation (Supplemental Fig. 1), indicating that the differences observed among groups were not due to the isolation techniques.

**LDGs alter the balance of endothelial cell damage and repair**

SLE patients develop a striking increase in the risk for premature cardiovascular (CV) complications as the result of accelerated atherosclerosis (30). As a potential mechanism leading to enhanced CV damage, our group showed that SLE patients develop an imbalance between vascular damage and repair that is characterized by accelerated endothelial cell apoptosis and abnormal phenotype and function of the cells involved in vascular repair (21, 31). These abnormalities correlate with the development of endothelial dysfunction (31), a predictor of atherosclerosis development in the

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**Table II. Expression of cell surface markers (%+SEM [MFI+SEM]) in LDGs and neutrophils**

<table>
<thead>
<tr>
<th>Marker</th>
<th>LDGs</th>
<th>Lupus Neutrophils</th>
<th>Control Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD10</td>
<td>93.7 ± 11 (121 ± 20)</td>
<td>95.8 ± 1.5 (265 ± 50)</td>
<td>97 ± 0.5 (255.7 ± 80)</td>
</tr>
<tr>
<td>CD11c</td>
<td>97.3 ± 0.1 (37.8 ± 1)</td>
<td>98.6 ± 0.1 (91.3 ± 30)</td>
<td>98.2 ± 0.3 (73 ± 20)</td>
</tr>
<tr>
<td>CD14</td>
<td>68.08 ± 10 (63 ± 20)</td>
<td>83.6 ± 3 (71.4 ± 20)</td>
<td>76.7 ± 8 (56.7 ± 11)</td>
</tr>
<tr>
<td>CD15</td>
<td>97.3 ± 0.7 (359.9 ± 200)</td>
<td>98 ± 0.2 (200 ± 90)</td>
<td>97.8 ± 0.2 (198 ± 70)</td>
</tr>
<tr>
<td>CD16</td>
<td>98 ± 0.01 (373 ± 80)</td>
<td>99 ± 0.03 (482 ± 30)</td>
<td>97 ± 0.6 (384 ± 100)</td>
</tr>
<tr>
<td>CD31</td>
<td>98 ± 0.4 (138 ± 10)</td>
<td>99 ± 0.08 (162 ± 30)</td>
<td>99 ± 0.08 (240 ± 40)</td>
</tr>
<tr>
<td>CD114</td>
<td>72 ± 16 (30 ± 10)</td>
<td>65 ± 13 (26 ± 6)</td>
<td>63 ± 18 (25 ± 4)</td>
</tr>
<tr>
<td>CD116</td>
<td>5.6 ± 0.5 (2.5 ± 1.2)</td>
<td>7 ± 2.2 (2.9 ± 1.1)</td>
<td>4.2 ± 2.5 (3.6 ± 1.5)</td>
</tr>
</tbody>
</table>

MFI, mean fluorescence intensity.
general population. The mechanisms that induce accelerated endothelial cell apoptosis in SLE remain to be determined. Given the potential role of neutrophils in the induction of damage to the endothelium in other pathologic conditions (32), we examined whether LDGs can harm endothelial cells. Lupus neutrophils induced significantly higher levels of cytotoxicity of endothelial cells than control neutrophils (Fig. 6). However, the induction of endothelial cytotoxicity was much more marked by LDGs compared with lupus or control neutrophils. Endothelial cytotoxicity was completely abolished with the use of a transwell, indicating that direct contact between the neutrophil effectors and the endothelial cell target is required to induce cell death. Similar results were obtained when normal-density neutrophils were isolated using negative selection after gradient centrifugation: lupus LDGs, 28.2% ± 5.8%; lupus neutrophils, 9.1% ± 4.1%; and control neutrophils, 2.5% ± 2.5%. Results represent the mean percentage
of apoptotic HUVECs after overnight exposure to LDGs, autologous lupus neutrophils, or control neutrophils for six SLE and four control samples; $p < 0.05$ comparing lupus neutrophils with control neutrophils and lupus LDGs with lupus neutrophils; $p < 0.01$ comparing LDGs with control neutrophils. These findings were confirmed using a bioluminescence cytotoxicity assay (data not shown).

Previously, our group and other investigators showed that SLE patients display abnormal phenotype and function of cells involved in blood vessel repair (endothelial progenitor cells [EPCs] and myeloid circulating angiogenic cells [CACs]) due to the antiangiogenic effects of IFN-α and, potentially, other type I IFNs (21, 33). Among various abnormalities, we previously reported that lupus PBMCs containing EPCs/CACs that are plated under proangiogenic stimulation strikingly fail to form a mature endothelial cell monolayer after 7–14 d in culture (21). This is in contrast with healthy control PBMCs that typically differentiate into mature endothelial cells during proangiogenic stimulation. Given that LDGs contaminate lupus PBMCs and synthesize increased levels of IFN-α, we assessed whether LDG depletion from EPC/CAC-containing PBMCs prior to plating of proangiogenic cultures would promote the normal phenotype and capacity to form an endothelial cell monolayer. In contrast to pDC depletion from lupus PBMC preparations, which did not restore the functional capacity of PBMCs to differentiate into mature endothelial cells and did not decrease type I IFN synthesis, LDG depletion led to a striking restoration of the normal capacity of the PBMCs to differentiate into mature endothelial cells and a pronounced decrease in type I IFN synthesis (Fig. 7). These results indicate that LDGs contribute to type I IFN production in the proangiogenic cultures and seem to be an important factor that leads to the aberrant phenotype and function of EPCs/CACs in this disease.

**Discussion**

Despite considerable advances identifying the importance of adaptive immunity in the pathogenesis of lupus, the study of innate immune responses in SLE was neglected for many years. However, in recent years, experimental evidence has indicated that lupus patients also have significant disruptions in innate immunity (2, 6). The role of neutrophils in the pathogenesis of SLE, particularly

### Table III. Intracellular cytokine expression in LDGs and neutrophils

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LDG Unstimulated</th>
<th>LDG Stimulated</th>
<th>Lupus Neutrophils Unstimulated</th>
<th>Lupus Neutrophils Stimulated</th>
<th>Control Neutrophils Unstimulated</th>
<th>Control Neutrophils Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 (%)</td>
<td>9.3 ± 1</td>
<td>11.6 ± 3</td>
<td>6.5 ± 2</td>
<td>8.3 ± 1.5</td>
<td>5 ± 1.5</td>
<td>6.9 ± 2.4</td>
</tr>
<tr>
<td>IL-8 (MFI; mean ± SEM)</td>
<td>33 ± 13.2</td>
<td>42 ± 10.9</td>
<td>29 ± 9.2</td>
<td>40 ± 3.46</td>
<td>27 ± 11.7</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>TNF-α (%)</td>
<td>49.6 ± 10</td>
<td>57 ± 8</td>
<td>48 ± 2.5</td>
<td>52 ± 1.5</td>
<td>45 ± 10</td>
<td>54.3 ± 8</td>
</tr>
<tr>
<td>TNF-α (MFI; mean ± SEM)</td>
<td>70 ± 25</td>
<td>2688 ± 960*</td>
<td>39.3 ± 80</td>
<td>62.6 ± 18</td>
<td>38 ± 0.1</td>
<td>50 ± 0.2</td>
</tr>
</tbody>
</table>

$p < 0.05$, LDGs compared with control or lupus neutrophils.

MFI, mean fluorescence intensity.
their potential role in nephritis, was proposed decades ago, (7); however, the exact role that these cells play in the pathogenesis of autoimmune responses and organ damage in this disease has not been completely characterized.

Over the last couple of decades, two studies reported the presence of an abnormal subset of neutrophils in the peripheral circulation of SLE patients. The results of these studies suggest that these cells are aberrant in SLE. In the first study, Hacbarth and Kajdacsy-Balla (11) reported that Ficoll-Hypaque density gradient preparations of PBMCs from adult patients with SLE were highly contaminated with low buoyant density neutrophils. These neutrophils were proposed to be activated, and activation was considered secondary to a plasma effect of an uncharacterized molecule. In the second study by Bennett et al. (2), gene-array analysis of PBMCs from pediatric lupus patients revealed increased expression of genes related to the development and function of granulocytes, which was not associated with medication use. These neutrophils were proposed to be activated, and activation was considered secondary to a plasma effect of an uncharacterized molecule. In the second study by Bennett et al. (2), gene-array analysis of PBMCs from pediatric lupus patients revealed increased expression of genes related to the development and function of granulocytes, which was not associated with medication use. This granulocyte signature present in lupus PBMCs was confirmed by the presence of highly granular cells in the mononuclear cell subset that covered all stages of granulocyte development, including pro, myelo-, and meta-myelocytes; bands; and segmented neutrophils, similar to what we are now reporting in adult SLE. Interestingly, the granulocyte signature in the pediatric lupus PBMCs was coincident with an IFN signature. Although not usually considered IFN-α–producing cells, mature neutrophils are capable of producing IFN-α in response to certain stimuli (29). Thus, the coassociation of the granulocyte and IFN signatures could have indicated neutrophil-derived IFN-α expression and/or IFN-α–mediated inhibition of neutrophil maturation (34–36). From our study in adult SLE, it seems that LDGs and, to a lesser extent, normal-density lupus neutrophils, are capable of synthesizing and secreting higher amounts of IFN-α (and potentially other type I IFNs) and that LDGs account for significant type I IFN activity in lupus PBMCs.

Several studies have indicated a key role for IFN-α in SLE pathogenesis, both in disease initiation and the development of flares and organ severity in human and murine systems (6, 37–41). Although the exact source of increased IFN-α in SLE has not been completely characterized, depletion experiments demonstrated that pDCs contribute only part of the IFN-α (6, 42, 43). The expression of FcγRs on the surface of LDGs suggests that anti-DNA immune...
complexes, present in the blood of patients with SLE, could be delivered to intracellular TLR9 via Fcγ-mediated activation and internalization, as described for DCs (44–46). Although a previous study suggested that neutrophils are not responsive to TLR9 agonists alone (47), this may reflect the inability of the ligands to be properly internalized (48–50). Thus, TLR9 ligands delivered to the receptor as part of immune complexes present in lupus serum (51) could be an additional source that stimulates LDGs to synthesize IFN-α. A recent report indicated that neutrophils are able to activate type I IFN responses via helicase recognition, because poly(I:C)-transfected human neutrophils express elevated mRNA levels of type I IFN responses via helicase recognition, because poly(I:C)-transfected human neutrophils express elevated mRNA levels of IFN-α, IL-12, and IFN-γ secretion that may promote and enhance tissue damage. Although the beneficial versus deleterious role of TNF-α in SLE remains a matter of discussion, animal models show that this molecule can be harmful in murine lupus (62, 63), and TNF-α blockade in MRL/lpr mice and other lupus murine models was proved to decrease disease severity (64, 65). TNF-α is overexpressed in human lupus nephritis (66–68) and refractory cutaneous disease (69), and this increase is associated with worsening kidney histological activity (70). It remains to be determined whether LDGs represent an important source of this cytokine in blood or specific tissues in which TNF-α may have deleterious effects. With regard to IFN-γ, levels of this cytokine in serum and tissue were found to be elevated in SLE and correlate with the development of nephritis (71, 72) and the overproduction of autoantibodies (73). Taken together, our results indicate that LDGs secrete enhanced levels of proinflammatory cytokines that were reported to potentially play an important role in tissue damage in SLE.

Aberrant apoptotic cell death, phagocytic uptake, and their interplay may induce autoantibody production and autoimmunity (74). Neutrophil phagocytosis and chemotaxis were reported to be impaired in SLE patients (75), with different mechanisms proposed, including autoantibodies and serum cytokines (76–78). We confirmed a trend for decreased phagocytosis of bacteria in normal-density lupus neutrophils, although there was no significant difference compared with control neutrophils. In contrast, LDGs display a profound decrease in their capacity to phagocytose bacteria. These results may indicate a potential deficient ability for the clearance of infectious agents and increased predisposition for infections in SLE. Indeed, patients with this disease have a higher infection rate than the general population, and episodes of bacteremia are associated with an unfavorable long-term outcome in this patient population (79). It remains to be determined whether this increased predisposition and poor outcome to infection is mainly secondary to immunosuppressive drugs or to abnormalities in bacterial phagocytosis/clearance secondary to the disease.

Patients with SLE have a strikingly higher risk for developing CV complications compared with age- and gender-matched controls (30, 80). Our group and other investigators proposed that this is due to
ABNORMAL LUPUS NEUTROPHILS INDUCE VASCULAR DAMAGE

A strong imbalance between vascular damage (endothelial cell apoptosis) and repair (by EPCs, CACs, and other cells crucial for vascularogenesis) (21, 31, 81). We and other investigators proposed that type I IFNs play a crucial role in the induction of aberrant vascular repair because neutralization of IFN-α or type I IFNR leads to abrogation of the abnormal capacity of lupus EPCs/CACs to become mature endothelial cells (21, 33). Furthermore, IFN-α is clearly cytotoxic to EPCs (21). Other groups showed that pDCs are not the primary source of enhanced IFN-α synthesis in SLE, but the precise subset(s) involved in humans with this disease remain unclear (6, 82). We showed that LDGs, not pDCs, seem to induce the enhanced IFN-α production that leads to abnormal EPC/CAC function in vitro and, potentially, in vivo in SLE. This and the observation that LDGs are cytotoxic to the endothelium suggest that this neutrophil subset may play an important role in the induction of premature vascular damage in SLE. Indeed, in ischemic heart disease, apoptosis of endothelial cells and aberrant vascular repair were shown to contribute to disease progression and events (83–85). Therefore, future strategies aimed at characterizing the origin of these cells and therapeutic mechanisms to deplete them are warranted.

The mechanisms by which LDGs, and lupus neutrophils in general, induce enhanced damage of endothelial cells remains to be fully characterized and are likely to be multifactorial, given the proinflammatory profile of these cells. Neutrophils can directly cause damage to vascular endothelium through a variety of mechanisms (9, 86–89). This typically does not occur when neutrophils are suspended in the bloodstream but rather when they are adherent to endothelium or are in contact with extracellular matrix proteins in the interstitium. Thus, adhesion of neutrophils is crucial in inflammatory injury, and it is possible that enhanced proinflammatory cytokine synthesis, as well as other yet unidentified molecules, could play a role in enhancing LDG adherence to endothelium and promoting enhanced cytotoxicity (89). Indeed, endothelial cell death induced by LDGs was blunted when their contact with endothelial cells was eliminated with a transwell. Furthermore, high LDG levels correlated with vascular inflammation in SLE patients, which further indicates that these cells may contribute to aspects of lupus related to vascular damage or inflammation.

The origin of LDGs remains unclear. Although the cells display some phenotypic properties of activated neutrophils, they do not differ in their activation status from autologous lupus neutrophils, and they express comparable levels of MPO and generate equivalent levels of ROS compared with control and lupus neutrophils. These observations indicate that these cells do not represent a population of in vivo-activated and degranulated lupus neutrophils. Furthermore, the nuclear morphology indicates potential disruptions in their development and the presence of more immature forms. GM-CSF–secreting PBMCs have been identified in SLE patients, and GM-CSF levels were reported to be elevated in SLE (90); therefore, it is possible that this cytokine could play a role in the accelerated mobilization of neutrophil precursors from the bone marrow (91). This seeming contradiction in the assessment of the development state of LDGs, depending upon surface molecule expression and nuclear morphology, may also be indicative of disruptions in neutrophil

FIGURE 7. LDG depletion improves the capacity of EPCs/CACs to become mature endothelial cells by abrogating type I IFN activity. Lupus PBMCs were cultured under proangiogenic conditions and incubated at different time points during culture with 9,10-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–acylated LDL and FITC–Ulex europaeus agglutinin-1. Mature endothelial cells were identified by the coexpression of these markers. A, Bar graphs represent the number of mature endothelial cells per power field at day 7 comparing the initial plating of unfractionated lupus PBMCs versus PBMCs depleted of pDCs [pDC(−)] (top panel) or LDGs [CD10 depletion, CD10(−)] (bottom panel). Results are mean ± SEM of four independent experiments for pDC depletion and six independent experiments for LDG depletion; *p < 0.05 for LDG depletion. B, Representative images of mature endothelial cells obtained after 7 d in culture comparing initial plating of lupus unfractionated PBMCs versus PBMCs depleted of pDCs (top panels) or LDGs (bottom panels) exposed to proangiogenic stimulation. Lupus CD10 depletion, but not pDC depletion, resulted in enhanced numbers of mature endothelial cells. DiI-LDL+ cells are red and UEA-1+ cells are green. Images were acquired with an objective magnification of 310 (×100 total magnification). C, Bar graphs represent the capacity of supernatants obtained at day 7 from lupus unfractionated PBMCs or PBMCs depleted of LDGs (all cultured under proangiogenic stimulation) to induce type I IFN-inducible genes in epithelial cell lines. Results are mean ± SEM of four independent experiments for pDC depletion and six independent experiments for LDG depletion. *p < 0.05, type I IFN induction compared between total PBMCs and CD10-depleted PBMC cultures.
development in SLE, possibly due to the effects of type I IFNs and/or other yet unidentified mechanisms. Overall, it seems that LDGs differ from their autologous normal-density neutrophils in that they have a different nuclear morphology and show strikingly diminished phagocytic potential, enhanced capacity to kill endothelial cells, and significant increases in TNF-α and IFN-α synthesis upon stimulation. However, more studies are needed to assess whether LDGs represent a distinct population of lupus neutrophils with specific disruptions in neutrophil development or merely a more aberrant/activated subset within the spectrum of polymorphonuclear cells present in patients with this disease. Similarly, the significance of the trend observed for decreased CD10 expression in LDGs, as assessed by MFI by FACS, compared with control or autologous lupus neutrophils is unclear. CD10 is a cell surface enzyme with neutral metalloendopeptidase activity that cleaves and inactivates multiple proinflammatory and vasoactive molecules (92) and whose expression is directly related to neutrophil mean age (93). There is evidence that neutrophil CD10 expression decreases significantly in response to in vivo inflammatory challenges (94). Importantly, CD10 is only expressed by segmented neutrophils and not by earlier myeloid progenitors. Although a CD10^+/CD16^low phenotype was proposed to identify greater numbers of phenotypically immature neutrophils than does cellular morphology alone (93), the vast majority of LDGs expressed CD10, even if possibly at lower levels than the other neutrophils subsets. Nevertheless, this observation further suggests that potential abnormalities in neutrophil development may be involved in the generation of LDGs.

Our results also indicate that the differences observed between LDGs and normal-density neutrophils are not due to the isolation technique, because similar results were observed when different isolation methods were used to obtain the normal-density cells. This is in agreement with previous studies that showed that magnetic bead-isolation techniques do not lead to changes in neutrophil activation compared with other isolation methods (95).

Future studies should also investigate whether these aberrant cells are also present in individuals with other systemic autoimmune diseases (without representing mere activated degranulated cells). Over the last few years, evidence has accumulated indicating that increased type I IFN signatures are present in patients with other systemic autoimmune diseases, including primary Sjögren’s syndrome (96, 97), progressive systemic sclerosis (98, 99), psoriasis (100, 101), and inflammatory myopathies (102). Interestingly, all of these conditions are associated with an increased risk for CV complications (103–106). Therefore, it will be important to assess the role of neutrophils in enhanced type I IFN activity and the role of these cells in vascular damage in these diseases.

Overall, we characterized, in detail, the phenotype of a low-density neutrophil subset that seems to be present in higher numbers in SLE patients with distinct clinical manifestations. LDGs have preserved neutrophil function overall, but they display impairments in phagocytic potential, have a proinflammatory phenotype, and induce vascular damage, suggesting that they may contribute to the accelerated atherosclerosis observed in SLE patients. The potential role of these cells in lupus pathogenesis (in part mediated by enhanced type I IFN synthesis) and on organ damage warrants further investigation.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In Fig. 1B, the y-axis was incorrectly labeled. The correct y-axis label should denote CD86, not CD14. The correct figure is shown below. The figure legend is correct as published but is shown below for reference.

**FIGURE 1.** Identification of LDGs in lupus PBMC fractions. Healthy control or SLE PBMCs were stained for markers of the monocyte or granulocyte lineages and analyzed by FACS. A, Gates that contained predominantly lymphocytes, monocytes, and granulocytes were established in dual-log scattergrams. Granulocytes (blue) and monocytes (pink) are distinguished based on CD14, CD15, CD86, and MHC class II expression. Monocytes express high levels of CD14 and are positive for CD86 and MHC class II, whereas CD15 is weak or absent. Granulocytes present in the PBMC fraction are CD15hi, CD14lo, and negative for CD86 and MHC class II. Similar results were seen in two additional controls and five additional SLE patients. B, Analysis of CD86 and CD16 revealed several subpopulations. Most healthy control monocytes display the resting phenotype of CD86−CD16− (light blue), whereas SLE monocytes have the more activated phenotype of CD86+CD16+ (blue). The CD16hi cells can be divided based on CD86 expression. The CD16hi/CD86− pool (yellow) likely represents LDGs, whereas the CD86hi/CD16hi population (pink) possibly reflects conjugates of CD16hi granulocytes and CD86+ monocytes.

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