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Distinct Infiltration of Neutrophils in Lesion Shoulders in ApoE^{-/-} Mice

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Inflammation and activation of immune cells are key mechanisms in the development of atherosclerosis. Previous data indicate important roles for monocytes and T-lymphocytes in lesions. However, recent data suggest that neutrophils also may be of importance in atherogenesis. Here, we use apolipoprotein E (ApoE)-deficient mice with fluorescent neutrophils and monocytes (ApoE^{-/-}/Lys^{EGFP/EGFP} mice) to specifically study neutrophil presence and recruitment in atherosclerotic lesions. We show by flow cytometry and confocal microscopy that neutrophils make up for 1.8% of CD45⁺ leukocytes in the aortic wall of ApoE^{-/-}/Lys^{EGFP/EGFP} mice and that their contribution relative to monocyte/macrophages within lesions is approximately 1:3. However, neutrophils accumulate at sites of monocyte high density, preferentially in shoulder regions of lesions, and may even outnumber monocyte/macrophages in these areas. Furthermore, intravital microscopy established that a majority of leukocytes interacting with endothelium on lesion shoulders are neutrophils, suggesting a significant recruitment of these cells to plaque. These data demonstrate neutrophilic granulocytes as a major cellular component of atherosclerotic lesions in ApoE^{-/-} mice and call for further study on the roles of these cells in atherogenesis. (*Am J Pathol* 2010, 177:493–500; DOI: 10.2353/ajpath.2010.090480)

of macrophages and T-lymphocytes in promoting plaque development and destabilization.^{1,2} However, the most abundant white blood cell in the circulation, the neutrophilic granulocyte, has until recently rarely been associated with the development of atherosclerosis. Nonetheless, proteins typically secreted by neutrophils are abundant in lesions,^{3–7} and systemic neutrophil counts appear to correlate closely with severity of atherosclerosis in humans.⁸ Similar observations were also recently made in the murine system in which increased peripheral neutrophil count was associated with enhanced plaque size, whereas the opposite was true when neutrophils were depleted from the circulation.⁹ There are also data that indicate presence of neutrophils in lesions of low-density lipoprotein (LDL) receptor-deficient mice.⁵ Despite these findings, data on potential roles of neutrophils in atherogenesis are rare in the literature.

We recently crossed apolipoprotein E-deficient ApoE^{-/-} mice with mice carrying a knock-in mutation for enhanced green fluorescence protein (EGFP) in the lysozyme locus (Lys^{EGFP/EGFP} mice)¹⁰ generating ApoE^{-/-}/Lys^{EGFP/EGFP} mice, which allow for sensitive detection of neutrophils in atherosclerotic plaques.¹¹ Here, we study the presence and spatial distribution of neutrophils in atherosclerotic arteries of these mice. We demonstrate that neutrophils are present in substantial numbers in aortic plaque. Moreover, their contribution is higher in shoulder regions of plaque, which are areas of high inflammatory activity. Intravital microscopy further revealed that neutrophils are the main cell population that interacts with atherosclerotic endothelium, suggesting an ongoing recruitment of neutrophils to lesions. These data demonstrate that neutrophils represent a major

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Recruitment of immune cells to the arterial intima is central to atherogenesis. Current dogma emphasizes the role

population of cells in atherosclerosis and underscore the possibility that these cells may play previously underestimated roles in atherogenesis.

Materials and Methods

Animals

C57Bl/6 and ApoE^{-/-} mice were obtained from Bomholtgard, Denmark, Lys^{EGFP/EGFP} mice were kindly provided by Thomas Graf, Albert Einstein School of Medicine, Bronx, NY,¹⁰ and CX3CR1^{EGFP/EGFP} mice¹² were provided by Steffen Jung and Dan Littman, Skirball Institute, NYU. ApoE^{-/-}/Lys^{EGFP/EGFP} and ApoE^{-/-}/CX3CR1^{EGFP/EGFP} mice were generated by cross of strains in our own laboratory. Mice transgenic for human ApoB100 and crossed with LDL receptor deficient mice (hApoB100/LDLR^{-/-}) were provided by Jan Borén, Wallenberg Laboratory, Gothenburg. Atherosclerotic mice were fed either standard chow or Western diet containing 21% triglycerides and 0.15% cholesterol. CX3CR1^{EGFP/EGFP} mice and Lys^{EGFP/EGFP} mice were fed standard chow. All mice were provided water *ad libitum* and exposed to light-dark cycles, constant temperature, and humidity. All experiments were approved by the regional committee for animal experimentation.

Flow Cytometry

Aorta

Mice were anesthetized and perfused through cardiac puncture (PBS with heparin; 20 U/ml). The aorta and the carotid arteries were harvested, carefully isolated from adjacent tissue, and digested according to a previously described protocol by Galkina et al.¹³ The obtained cell suspensions were filtered through a 40- μ m strainer and subsequently incubated with Fc-receptor blockage (anti-CD16/CD32, BD Pharmingen, Franklin Lakes, NJ) and after subsequent wash incubated with either primary conjugated antibodies or unconjugated Ly6G/1A8 (BD Pharmingen) for 20 minutes at room temperature and then washed twice. In experiments requiring secondary antibodies, incubation with primary and secondary antibodies was performed before staining with conjugated primary monoclonal antibodies (mAbs). Data were acquired immediately after staining using the CyAn ADP instrument (Dako, Denmark) and later analyzed and compensated with FlowJo Software (Three Star Inc., Ashland, OR) as described previously.¹⁴ The following antibodies were used: anti-CD45 APC-Cy7, anti-CD45 PE-Cy7, anti-Gr-1 PE-Cy7, anti-Gr-1 APC-Alexa Fluor 750, anti-Gr-1 PE-Cy5, anti-CD36 Alexa Fluor 647, anti-I-A/I-E PE, anti-I-A/I-E Pacific Blue, and anti-TER-119 PE (all BioLegend, San Diego, CA); anti-CD68 Alexa Fluor 647, anti-F4/80 Pacific Blue, and anti-F4/80 Alexa Fluor 647, anti-Rat F(ab')₂ IgG (FITC, DyLight 405, Pacific Blue, PE-Cy5) (all Serotec, Raleigh, NC). Anti-B220 APC-Alexa Fluor 750 and anti-CD3 APC-Alexa Fluor 750 were from eBioscience (San Diego, CA). Ethidium monoazide (EMA,

Molecular Probes, Eugene, OR) was used to identify dead cells in separate samples.

Blood Samples

Blood (75 μ l) obtained by tail vein incision was collected in 1 ml PBS supplemented with 5 mmol/L EDTA. After lysis of red blood cells, Fc-receptor blockage was performed with anti-CD16/CD32 (BD Pharmingen). Staining and analysis was conducted as described above.

Confocal Microscopy

Atherosclerotic lesions were isolated and fixed in 1% formaldehyde for 2 hours. Subsequently, lesions were either mounted *en face* for confocal microscopy on whole-mounted plaque as previously described or embedded with O.C.T. medium (Tissue-Tek), snap-frozen in liquid nitrogen, and stored at -80°C before sectioning. Cryosections (14 μ m) were cut in a cryostat (Microm; Heidelberg, Germany). Staining of sections or whole-mounted plaque was performed by similar protocols. Specimens were blocked with 5% donkey serum and incubated overnight at 4°C in a humid chamber with primary antiserum diluted in a solution containing bovine serum albumin and Triton X-100 in 0.01 M PBS. The following day, specimens were rinsed in PBS and incubated with a Cy3 or Cy5-conjugated polyclonal donkey secondary antibody (1:250 and 1:500 respectively; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 45 minutes in a humid chamber at room temperature. Specimens were then rinsed in PBS and mounted in a mixture of glycerol/PBS and examined in a laser scanning confocal microscope (Zeiss, LSM meta 510; Carl Zeiss, Germany). Staining was conducted using combinations of the following mAbs: F4/80, CD68 (Abd Serotec), Gr-1, CD36 (Biolegend), MOMA-2 (Abcam, Cambridge, MA) and Ly6G/1A8 (BD Pharmingen). For nuclear staining we used TOTO-3. For examination of lipid accumulation in lesions Nile Red (Invitrogen, Carlsbad, CA) dissolved in HBSS with 1% DMSO and pleuronic was used. Importantly, neutrophils were readily labeled by antibodies at a depth of at least 50 μ m in plaques (not shown). Analysis of whole mounted plaques was performed in LSM image browser (Carl Zeiss, Germany).

Intravital Microscopy

Experiments were performed on either the abdominal aorta or the left carotid artery. The aorta was prepared as described previously.¹⁵ For intravital microscopy on the left carotid artery, the vessel was carefully exposed up to the carotid bifurcation. After surgical exposure of the aorta or carotid, the mouse was placed under the microscope. The exposed tissue was superfused with a thermostated (37°C) bicarbonate-buffered saline solution. Direct intravital microscopic observations were performed on shoulder regions of atherosclerotic plaques. Leukocyte rolling flux was determined as the average number of leukocytes rolling within a 10,000- μ m² area during 30

seconds within a total observation time of at least 180 seconds.

Bone Marrow Transplantation

Donor mice were sacrificed by an overdose of isofluran. The femur and tibia were subsequently isolated, immersed in ice cold PBS, and gently crushed. The slurry was then poured through a 70- μ m filter and stored on ice. Recipient mice were exposed to a single dose of lethal irradiation at 1400RAD. Within 6 hours after irradiation, recipient mice received a tail vein injection of 0.2 ml of donor bone marrow. The ratio between donors and recipients were 1:2. Mice were kept on trimetoprim/sulfamethoxazol in the drinking water for 3 weeks and experiments were performed >8 weeks after bone marrow transplantation (BMT).

Monocyte and Neutrophil Depletion

For depletion of neutrophils, ApoE^{-/-}/Lys^{EGFP/EGFP} mice were treated with an i.p injection of 20 μ g Gr-1 mAb (RB6-8C5, Nordic Biosite AB) 24 hours before intravital microscopy.¹⁶ Circulating monocytes/macrophages were depleted *in vivo* by i.v. injection of 200 μ l dichloromethylene-bisphosphonate (clodronate) liposomes as described previously.¹⁶ Blood samples were analyzed for WBC and flow cytometry before and after depletion.

Statistical Analysis

Data are displayed as mean $\pm$ SEM. Calculations were performed using *t* test (for normally distributed samples), the Mann-Whitney rank sum test, or one-way analysis of variance. Statistical significance was set at *P* < 0.05.

Results

Quantification of Neutrophils within the Atherosclerotic Vessel Wall

To assess the relative contribution of neutrophils to the total number of leukocytes within atherosclerotic vessels, we performed flow cytometry on cells derived from enzymatically digested aortas using a modified protocol according to that previously described by Galkina et al.¹³ We have previously shown that ApoE^{-/-}/Lys^{EGFP/EGFP} mice display similar development and composition of atherosclerotic lesions as ApoE^{-/-} mice.¹¹ The samples from the digested aortas contained cell types such as endothelial cells, smooth muscle cells, and leukocytes. In contrast, cell suspensions from aortas contained very few erythrocytes as assessed by anti-TER-119 staining, indicating minimal blood contamination (not shown). After elimination of double events (Figure 1A) all leukocytes were identified by staining with an antibody against the leukocyte common antigen (CD45) and subsequent analyses were performed on CD45⁺ cells (Figure 1B). Because myelomonocytic cells are positive for EGFP in

Figure 1. Neutrophils are present in significant numbers in aortas from ApoE^{-/-}/Lys^{EGFP/EGFP} mice. **A:** Representative gating algorithm with exclusion of double events (left) and FSC/SSC plotting of remaining events (right). **B:** Representative staining for CD45 (left), staining for MHC-II and EGFP on CD45⁺ cells (middle), and staining for CD68, F4/80, and CD36 on MHC-II⁺EGFP^{high}CD45⁺ cells (right). **C:** Representative staining for EGFP and Gr-1 (left) on CD45⁺ cells, and staining for CD68, F4/80, CD36, and MHC-II on Gr-1⁺EGFP^{low}CD45⁺ (middle) and Gr-1^{high}EGFP^{high}CD45⁺ (right) cells. **D-F:** Contribution of neutrophils of all CD45⁺ cells identified by the protocol outlined in **A** and **B** in aortas from different groups of mice as indicated. **G:** Contribution of neutrophils of total EGFP⁺ cells in the indicated mice. The number of mice in each group was: ApoE^{-/-}/Lys^{EGFP/EGFP} mice: 10 months chow, 3 mice, 12 months WD, 6 mice, 16 months WD, 4 mice. ApoE^{-/-} mice: 12 months chow, 3 mice. Lys^{EGFP/EGFP} mice: 12 months chow, 10 mice. C57BL/6 mice: 4 months chow, 4 mice. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

mice on Lys^{EGFP/EGFP} background whereas lymphocytes are negative,¹⁰ CD45⁺ leukocytes were divided into EGFP⁻ and EGFP⁺ cells. EGFP⁺ cells were further subdivided into EGFP^{low} and EGFP^{high} cells. The majority of CD45⁺ EGFP^{high} cells lacked expression of monocyte/macrophage markers MHC-II, CD68, F4/80, and CD36 and were therefore regarded as neutrophils (Figure 1B). In contrast, EGFP^{low} cells consistently expressed these antigens, identifying them as monocyte/macrophages (Figure 1B). This is in agreement with previous studies, reporting that neutrophils express EGFP in higher levels as compared with monocytes and macrophages.¹⁰ Additional staining for Gr-1 was used to further validate a correct identification and assessment of neutrophils. Hence, Gr-1^{high}EGFP^{high}CD68⁻F4/80⁻CD36⁻MHC-II⁻ cells were considered as neutrophils and detected in

similar numbers as compared with when using the protocol based on EGFP without Gr-1 (Figure 1C). By applying the EGFP-based protocol, we found that neutrophils accounted for $0.12 \pm 0.034\%$ of all $CD45^+$ cells in the noninflamed aorta from 12-month-old $Lys^{EGFP/EGFP}$ mice. In contrast, $1.8 \pm 0.29\%$ of $CD45^+$ cells in atherosclerotic aortas obtained from 12-month-old $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice fed Western diet were neutrophils (Figure 1D). Hence, the data indicate that neutrophils accumulate in the aorta in atherosclerotic $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice. In a different approach, we used direct staining of neutrophils by using a neutrophil-specific antibody directed against Ly6G (clone 1A8). Using a protocol based on CD45, EGFP, CD68, F4/80, CD36 and 1A8, we studied 16-month-old $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice fed Western diet (see supplemental Figure S1 at <http://ajp.amjpathol.org>). Indeed, the contribution of neutrophils to $CD45^+$ cells in the aorta was $2.0 \pm 0.19\%$ and thus consistent to the data obtained by the staining protocols mentioned above. Moreover, to investigate whether neutrophil numbers are different in vessels carrying less advanced lesions we used the 1A8-based protocol also on 10-month-old $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice fed chow diet in which fatty streaks are the dominating stage of lesion development in most of the aorta. Interestingly, neutrophils were readily detected also in these mice $1.6 \pm 0.25\%$ of $CD45^+$ cells, $P = 0.22$ versus 16-month-old mice on WD, Figure 1E), demonstrating that neutrophil invasion is not radically different in mice with less advanced lesion burden. To certify that the presence of neutrophils in the vessel wall was not induced by the $Lys^{EGFP/EGFP}$ mutation, 1A8 was also used to stain cells derived from aortas of mice with wild-type lysozyme M. Here, the contribution of neutrophils in 4-month-old wild-type C57Bl/6 and 12-month-old $ApoE^{-/-}$ mice fed standard chow was $0.11 \pm 0.048\%$ and $1.5 \pm 0.75\%$ of $CD45^+$ cells, the latter similar to that found in $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice, which indicates that there is little relation between the $Lys^{EGFP/EGFP}$ mutation and the presence of neutrophils in the atherosclerotic aorta (Figure 1F). Of all $CD45^+$ cells obtained from 16-month-old $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice, $18 \pm 3.0\%$ of myelomonocytic cells ($EGFP^+$) were neutrophils. In contrast, in aortas from 12-month-old $Lys^{EGFP/EGFP}$ mice, no more than $2.9 \pm 0.97\%$ of myelomonocytic cells filled criteria as neutrophils (Figure 1G). These findings provide strong quantitative evidence for the relative abundance of neutrophils in the arterial wall in atherosclerotic mice.

Detection of Neutrophils in Sections of Atherosclerotic Plaques by Immunofluorescence

To visualize neutrophils in atherosclerotic lesions, cryosections of atherosclerotic plaques from $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice were investigated with confocal microscopy. $EGFP^+$ cells were readily identified. As shown above, combination of macrophage markers CD68, F4/80, and CD36 appeared to be sufficient to label all monocytes/macrophages (Figure 1C, middle panel). As expected, staining using all these markers together with the macrophage marker MOMA-2 revealed cells that stained for both EGFP as well as the

Figure 2. Representative immunofluorescence images on sections from atherosclerotic lesions from $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice after staining for the indicated antigens. Images show overlay EGFP in green and immunofluorescence in red. In the **top left**, EGFP fluorescence without staining is shown. In the **top right**, a section from an atherosclerotic lesion stained for F4/80, CD68, CD36, and MOMA-2 is displayed. **Arrows** indicate $EGFP^+$ cells negative for staining of these antigens. **Bottom** row shows staining for individual macrophage markers. Note the positive immunostaining for some, but not all, $EGFP^+$ cells. Scale bar, 10 μm .

combination of these antigens (Figure 2). However, some $EGFP^+$ cells were negative for monocyte/macrophage markers and thus appeared as the neutrophils that were detected by flow cytometry.

Neutrophils Accumulate in Lesion Shoulders and at Sites of High Monocyte Density

To quantify neutrophils in lesions and to investigate their spatial distribution in plaques, we studied whole mounted lesions from $ApoE^{-/-}$ and $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice by confocal microscopy. Borders of atherosclerotic lesions in both strains of mice were easily appreciated by their background fluorescence and this distinction of plaque was consistent with lipid staining with Nile red (see supplemental Figure S2, A–C at <http://ajp.amjpathol.org>). EGFP fluorescent cells were readily identified by their shape and size in serial confocal sections on plaque derived from $ApoE^{-/-}/Lys^{EGFP/EGFP}$ mice to a depth of at least 50 μm (see supplemental Figure S2D at <http://ajp.amjpathol.org>). Cells were not detected in $ApoE^{-/-}$ mice. All cells identified by EGFP were positive also for the nuclear marker TOTO-3, certifying their cellular identity (see supplemental Figure S2E at <http://ajp.amjpathol.org>). EGFP-positive cells were rare or absent in areas outside lesions and were thus not present in $Lys^{EGFP/EGFP}$ mice (see supplemental Figure S2F at <http://ajp.amjpathol.org>).

Staining of neutrophils in plaques was performed with anti-Ly-6G (1A8), which selectively labels neutrophils.^{5,9} The specificity of 1A8 was tested by flow cytometry and confocal microscopy on peripheral blood and atherosclerotic lesions from $CX3CR1^{EGFP/EGFP}$ and $ApoE^{-/-}/$

Figure 3. Neutrophil invasion varies between different regions of atherosclerotic lesions. **A:** Representative images from confocal microscopy of *en face* mounted atherosclerotic lesions from ApoE^{-/-}/Lys^{EGFP/EGFP} mice after staining with the neutrophil specific mAb 1A8. Plaques were analyzed in 100×100×40 μ m volume units. Volume units were characterized as low infiltration (≤ 5 EGFP⁺ cells), intermediate infiltration (6 to 15 EGFP⁺ cells), and high infiltration units (>15 EGFP⁺ cells). Scale bar, 100 μ m. **B:** Quantification of the number of neutrophils in percentage of total EGFP⁺ cells in areas of different infiltration of EGFP⁺ cells. *** $P < 0.001$.

CX3CR1^{EGFP/EGFP} mice (see supplemental Figure S3, A and B at <http://ajp.amjpathol.org>). Using staining with 1A8 on lesions derived from ApoE^{-/-}/Lys^{EGFP/EGFP} mice, we found that neutrophils make up for $23 \pm 1.6\%$ of EGFP⁺ cells in lesions from ApoE^{-/-}/Lys^{EGFP/EGFP} mice, thus supporting data obtained by flow cytometry (Figure 3A). Further, image stacks of plaque were by a grid divided into volume units of 100×100×40 μ m. Areas with low infiltration of EGFP⁺ cells (0 to 5 cells per volume unit) contained $17 \pm 1.9\%$ neutrophils of the total number of EGFP⁺ cells ($n = 202$ volume units). Notably, areas with intermediate (6 to 15 cells) and high infiltration (>15 cells) of EGFP⁺ cells displayed a relative neutrophil contribution of $29 \pm 2.6\%$ and $54 \pm 4.0\%$, respectively ($n = 101 + 23$ volume units, Figure 3B). These results indicate that neutrophils reside within lesions at a ratio to macrophages of about 1:3. However, the contribution of neutrophils is more prominent in areas that are rich in EGFP⁺ cells, presumably reflecting that neutrophils reside in regions of high inflammatory activity.

We subsequently analyzed the density of neutrophils and monocytes in shoulder regions of plaques (Figures 4, A–C). In areas within 150 μ m of the plaque border (areas regarded as shoulder regions), there were on average 9.1 ± 0.72 EGFP⁺ cells per volume unit ($n = 112$) whereas only 5.2 ± 0.40 EGFP⁺ cells per volume unit were found in central regions of plaque ($n = 198$). In these central regions, $18 \pm 1.8\%$ of EGFP⁺ cells were neutrophils whereas $38 \pm 2.9\%$ of EGFP⁺ cells in shoulder regions were positive for 1A8, indicating that neutrophils are located primarily in these areas.

A Majority of Leukocytes Rolling on Atherosclerotic Lesions Are Neutrophils

We also investigated neutrophil recruitment by studying leukocyte–endothelial interactions on atherosclerotic lesions. Rolling of leukocytes occurred predominantly in shoulder regions of lesions,¹⁵ which corresponds well to

the high density of EGFP⁺ leukocytes in these areas. In ApoE^{-/-} mice treated with rhodamine 6G, which labels all circulating leukocytes, the number of rolling cells was 23 ± 2.6 cells/min (Figure 5A). Rolling flux was similar on

Figure 4. Neutrophils and monocytes invade lesion shoulders. Graphs show the quantitative contribution of EGFP⁺ cells (**A**) and relative neutrophil contribution (**B**) in shoulders and central regions of plaque. *** $P < 0.0001$. **C:** Representative image of an atherosclerotic lesion stained by 1A8 as Figure 3. The healthy aortic wall and the lesion border are clearly visible in the lower left. The area between the dashed line and the lesion border is 150 μ m wide and represents the plaque shoulder.

Figure 5. Leukocytes rolling on atherosclerotic endothelium are primarily of myelomonocytic origin. **A:** Leukocyte rolling in rhodamine 6G (R6G)-treated ApoE^{-/-} mice ($n = 17$) and untreated ApoE^{-/-}/Lys^{EGFP/EGFP} mice ($n = 25$). Rolling was analyzed in a $100 \times 100 \mu\text{m}$ endothelial surface area on shoulder regions of plaque ($P = 0.62$). **B:** Leukocyte rolling flux in ApoE^{-/-}/Lys^{EGFP/EGFP} mice before and after treatment with R6G ($n = 5$, $P = 0.59$).

lesions in ApoE^{-/-}/Lys^{EGFP/EGFP} mice (25 ± 2.9 cells/min, Figure 5A) and rolling did not increase after subsequent treatment with rhodamine (Figure 5B), indicating that leukocytes of non-myelomonocytic origin (i.e., lymphocytes) do not contribute in large numbers to leukocyte rolling on atherosclerotic plaque *in vivo*.

To further discriminate between different subclasses of rolling myelomonocytic cells, ApoE^{-/-} mice were subjected to BMT with Lys^{EGFP/EGFP} or CX3CR1^{EGFP/EGFP} mice as donors. We have previously shown that EGFP⁺ cells in both these strains including also EGFP^{low} cells in CX3CR1^{EGFP/EGFP} mice can be detected by intravital microscopy,¹⁶ and EGFP^{low} cells were also visualized rolling along endothelium in the inferior vena cava in experiments in which they were first sorted from CX3CR1^{EGFP/EGFP} mice and subsequently injected into the femoral vein of C57Bl/6 mice (data not shown). Experiments on mice exposed to BMT were performed >8 weeks after transplantation at which time >95% of leukocytes were of donor origin (data not shown). As shown in Figure 6A, a significantly higher number of fluorescent cells were rolling in ApoE^{-/-} mice that received BM from Lys^{EGFP/EGFP} mice (27 ± 4.6 cells/min) than in mice receiving BM from CX3CR1^{EGFP/EGFP} mice (2.4 ± 4.6 cells/min). There were

no significant differences in rolling between the two groups after treatment with rhodamine 6G (Figure 6A). In parallel, rolling in hApoB100/LDLR^{-/-} mice displayed similar characteristics between mice receiving Lys^{EGFP/EGFP} (23.6 ± 4.0 cells/min) or CX3CR1^{EGFP/EGFP} BM (1.5 ± 1.1 cells/min, Figure 6B). Hence, neutrophils appear to contribute largely to leukocyte rolling on lesions whereas rhodamine does not increase rolling in mice receiving Lys^{EGFP/EGFP} BM, further indicating that lymphocytes do not roll along atherosclerotic endothelium in high numbers.

In a third approach, ApoE^{-/-}/Lys^{EGFP/EGFP} mice were selectively depleted of monocytes or neutrophils by clodronate liposomes or Gr-1 antibody, respectively.^{16–18} As seen in Figure 6C, depletion of monocytes did not affect the number of cells interacting with atherosclerotic endothelium (33 ± 3.5 cells/min) compared with untreated ApoE^{-/-}/Lys^{EGFP/EGFP} mice despite that monocytes were reduced by $80 \pm 5.1\%$. In contrast, anti-Gr-1 treatment, which decreased neutrophil counts by $90 \pm 3.4\%$, reduced the number of rolling cells by >80% (4.7 ± 0.72 cells/min, Figure 6C). Importantly, and in agreement with previous reports,^{9,16} there was no effect on the number of monocytes in peripheral blood after treatment with anti-Gr-1 (see supplemental Figure S4, A–C at <http://ajp.amjpathol.org>). Treatment with control mAb (IgG2b) or PBS-filled liposomes did not alter the number of rolling cells from that of ApoE^{-/-}/Lys^{EGFP/EGFP} mice (data not shown).

Discussion

Recent data suggest an important role for neutrophils in atherogenesis and plaque rupture.^{19,20} A major problem with studies addressing the importance of neutrophils in atherosclerosis has been the fact that neutrophils are seldomly detected in lesions. Here, we use flow cytometry on the whole aorta and confocal microscopy on whole-mounted plaque in ApoE^{-/-} and ApoE^{-/-}/Lys^{EGFP/EGFP} mice for sensitive detection of neutrophils in lesions. Our data demonstrate that neutrophils are abundant in shoulder regions of plaques, especially at sites of high monocyte content and thus likely in places of high inflammatory activity. Neutrophils also make up the main population of leukocytes that interact with lesion endothelium, suggesting that

Figure 6. Neutrophils account for most leukocyte-endothelial interactions in shoulder regions on lesions. Leukocyte rolling flux in ApoE^{-/-} mice (**A**) or hApoB100/LDLR^{-/-} mice (**B**) after BMT with Lys^{EGFP/EGFP} (fluorescent neutrophils and monocytes, $n = 6$ and 4) or CX3CR1^{EGFP/EGFP} (fluorescent monocytes, $n = 7$ and 4) bone marrow before and after treatment with R6G. **C:** Leukocyte rolling on atherosclerotic endothelium after depletion of circulating monocytes ($n = 5$) or neutrophils ($n = 5$). ** $P < 0.01$, *** $P < 0.0001$.

invasion and turnover of neutrophils could be even more significant compared with the contribution of these cells to overall leukocyte numbers in plaque. These findings have several implications on our understanding of atherogenesis.

The possible contribution of neutrophils in atherosclerosis is not a novel idea, as their presence in atherosclerotic plaques in primates was demonstrated as early as 1982.²¹ However these findings were for long isolated in the literature, much of which is due to their low occurrence in human plaques. Apart from the notion that recruitment of neutrophils to lesions is indeed restricted, several reasons may have contributed to limited detection of neutrophils in atherosclerosis. For example, neutrophils, which are cells with a short life span both in the circulation and in inflamed tissues, will likely remain in the lesion for a shorter time than T-lymphocytes or monocytes. Another possibility for their rare appearance in lesions could relate to technical limitations in the detection of these cells or to difficulties in discriminating them from macrophages or dendritic cells after they have undergone phenotypic changes on extravasation and activation.^{22,23} Previous data suggesting roles for neutrophils in atherogenesis include those that show correlation between neutrophil counts in peripheral blood and lesion development. Friedman et al showed an epidemiological connection between peripheral neutrophil count and risk for coronary atherosclerosis and acute myocardial infarction (AMI).²⁴ Huang et al demonstrated a correlation between neutrophil counts and the presence of carotid atherosclerosis in humans.²⁵ Moreover, association between atherosclerosis and peripheral neutrophils was also shown recently in ApoE^{-/-} mice.⁹ In conjunction with the current data that demonstrate that a majority of leukocytes rolling along atherosclerotic endothelium are neutrophils, and that this results in significant recruitment of neutrophils to plaque, it is reasonable to anticipate that increased numbers of neutrophils in blood leads to increased accumulation in lesions and subsequent aggravation of atherosclerosis. Effects of inhibition or deficiency of molecules involved in the recruitment process in mouse models may thus have effects on neutrophils rather than on other hematopoietic cells. For instance, the strong protective effects by genetic deficiency of P- and/or E-selectin in mice are likely to be at least in part dependent on inhibition of the recruitment of neutrophils.²⁶ This is highlighted by the fact that leukocyte rolling along atherosclerotic endothelium is critically dependent on P-selectin.¹⁵ Moreover, absence of the common β_2 integrin chain CD18 as well as its counter receptor ICAM-1 is also likely to attenuate atherogenesis by effects on neutrophils.²⁶ As for chemokines, chemokine receptors (CRs), and chemoattractants, the reduction of atherosclerosis seen in CXCR2-deficient mice are perhaps the effects that are most likely to be mediated by inhibition of neutrophil trafficking.²⁶ However, blockage of the receptor for leukotriene B4 (BLTR) may also influence the disease by acting on neutrophils.²⁶ Still, the molecules that guide recruitment of individual subclasses of leukocytes in atherogenesis are as yet unclear and other chemotactic molecules may be involved.

There are several mechanisms by which neutrophils may influence atherogenesis and increase plaque vulnerability. Neutrophil granule proteins, which are released on neutrophil activation, may efficiently perpetuate lesion growth and contribute to destabilisation and rupture.^{27,28} Granule contents activate endothelial cells leading to subsequent induction of adhesive²⁹ and chemotactic proteins³⁰ directed at recruiting other leukocytes. Neutrophil proteins may also influence antigen-presenting cells and T-lymphocytes to enhance antigen-presentation.^{31,32} Neutrophil-triggered cytokine release by mononuclear cells may further result in sustained inflammation.³³ Neutrophils also produce and secrete large amounts of oxygen radicals and defensins that have been proposed to be involved in the oxidation and sub-endothelial entrapment of LDL particles.^{34–36} Nonetheless, previous findings of products typically secreted by neutrophils in plaque have regularly been attributed to the possibility that these compounds may be derived from macrophages. For instance, matrix metalloproteinases 8 and 9, myeloperoxidase, and neutrophil elastase which are all strongly expressed in neutrophils are also found in lesions.^{37–40} According to our data it is clear that neutrophils may be a major source of these compounds, and the colocalization of neutrophils and monocytes demonstrated here emphasizes that neutrophil-derived products may be mistakenly interpreted as being of macrophage origin. The fact that neutrophils are distinct proinflammatory cells with few anti-inflammatory properties also suggests that they are important in lesion expansion rather than acting as modulators of inflammation in atherogenesis. This is supported by the finding that neutrophils accumulate in shoulder regions of lesions where they may influence both disease progress as well as triggering the events that are related to plaque rupture. Those data are strengthened by previous findings showing that neutrophils accumulate in ruptured plaque and at sites of erosions, indicating that neutrophil infiltration in atherosclerotic lesions are associated with acute events related to atherosclerosis.^{20,41} Clearly, the addition of the neutrophil as a previously not fully recognized player in atherosclerosis increases the complexity of disease pathogenesis. In fact, it is possible that the full perspective of neutrophil biology is only beginning to emerge.

In summary, our data provide strong evidence for the presence and invasion of significant numbers of neutrophils in atherosclerotic lesions. These findings call for further investigation of the functional importance of neutrophils and their interplay with complex immunological processes in atherogenesis.

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