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Transient Neutropenia Following Granulocyte-Colony Stimulating Factor Administration is Associated with Neutrophil Accumulation in Pulmonary Vasculature

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Abstract

Objective—Transient neutropenia was observed shortly following granulocyte-colony stimulating factor (G-CSF) administration.

Methods—To evaluate this disappearance of neutrophils we investigated neutrophil trafficking. Ratios of neutrophil number to background cellularity for C57BL/6 LysM-EGFP knock-in mice and rhesus macaques were determined in the lung, liver, spleen, and kidney following G-CSF administration.

Results—For the C57BL/6 LysM-EGFP knock-in mice, the enhanced green fluorescent cells expressing (EGFP+) cells increased in the lung and spleen within 15 minutes of 50µg/kg SQ of G-CSF, and EGFP+ cells continued to increase in the lung and spleen from 15 minutes to 30 minutes. At 240 minutes, the pulmonary infiltrate declined to a level comparable to the level at 15 minutes, while in the spleen the EGFP+ cells continued the increasing trend to a higher level. For rhesus macaques, the CD18+ cells also significantly increased in the lung 30 minutes following the administration of 10µg/kg SQ of G-CSF compared to the control level.

Conclusion—These results suggest that the transient neutropenia following G-CSF administration in the mouse and non-human primate is associated with an accumulation of neutrophils within pulmonary and splenic vasculature.

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Conflict of Interest Disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared

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Introduction

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cell growth factor that is responsible for the progressive differentiation of myeloid progenitor cells into neutrophils (1–2). Neutrophils are considered to be the first line of defense against infection. Genetic deficiencies in neutrophil number or function, such as cyclic neutropenia or leukocyte adhesion deficiency, respectively, result in a high risk of contracting life threatening infections. When administered systemically, G-CSF increases neutrophil differentiation, proliferation, redistribution, and function (1,3). Cloned over 20 years ago, G-CSF is commonly used as a standard care for treatment to ameliorate genetic and chemotherapy related neutropenia (4). G-CSF is also used therapeutically to mobilize hematopoietic stem cells into the circulation for collection by leukapheresis (5).

Previously we observed that within 30 minutes of G-CSF administration there is a dramatic yet transient decline in circulating neutrophil count in non-human primates (NHP). Over the following two hours there is a subsequent recovery and expansion in neutrophils (5). During the first 30 minutes following G-CSF administration there is an 80–90% drop in circulating neutrophil count. This decline has been noted in other species and is believed to be related to neutrophil activation by G-CSF (6–8). G-CSF activates both mature and immature neutrophils to release secretory vesicles and alter surface adhesion molecules mediating adhesion to endothelial cells (9). Activated neutrophils can create a prothrombotic state (9) and induce release of lactoferrin causing leukoaggregation (10). One report shows that G-CSF activates plasma markers for coagulation (11).

Intrigued by this transient loss, we investigated neutrophil trafficking after G-CSF administration. Lung, spleen, liver, and kidney tissues were collected following G-CSF administration in both transgenic mice expressing EGFP off of the LysM promoter/enhancer and non-human primates. Immunohistochemistry (IHC) was performed on these tissues to determine temporal quantitative differences in neutrophil content. The ratio of neutrophil number to background cellularity was determined through threshold analysis of triplicate images of each organ at different time points, and compared to that of a control.

Methods and materials

Animals

All animals were housed and handled in accordance with the guidelines set forth by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Studies were performed according to approved protocols of the Animal Care and Use Committees of the National Heart, Lung, and Blood Institute (non-human primate studies) and the National Institutes of Allergy and Infectious Diseases (murine studies).

Administration of G-CSF to mice

Mice used in this study were C57BL/6 LysM-EGFP knock-in males between 6–12 weeks of age and were chosen due to their genetically engineered EGFP-expressing populations of neutrophils and monocytes (12). Four control mice were given a vehicle of phosphate-buffered-saline (PBS) + 0.1% bovine serum albumin (BSA) subcutaneously (SQ). Sixteen mice received 50µg/kg recombinant mouse granulocyte-colony stimulating factor (R&D Systems Inc, Minneapolis, MN, USA) SQ in 200ul PBS+ 0.1% BSA. The dose represents an equivalent surface area dosage for this species. Organs from six mice were harvested 15 and 30 minutes post G-CSF administration and organs from four mice were harvested 240 minutes post G-CSF administration. At each time point post G-CSF administration, the mice were euthanized by CO₂ asphyxiation. Controls were sacrificed 30 minutes after receiving

the vehicle. Lung, spleen, liver, and kidney were collected, sectioned, and fixed initially in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS overnight, then transferred to 30% sucrose (Sigma-Aldrich) overnight in PBS at 4°C for cryo-protection, and finally placed in Tissue-Tek OCT freezing medium (Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. The frozen tissues were kept frozen in a -80°C freezer until further processing was performed.

Administration of G-CSF to rhesus macaques

Four rhesus macaques (*Macaca mulatta*) were used in this study and were seronegative for simian T-cell leukemia, simian retrovirus type D, simian immunodeficiency virus, and herpes virus B. Blood samples were collected in 2ml ethylenediamine tetraacetic acid (EDTA) tubes prior to the subcutaneous administration of 10µg/kg of recombinant human G-CSF (Neupogen®, Amgen, Inc. Thousand Oaks, CA) SQ, and then 15 minutes and 30 minutes post G-CSF administration. The dose represents an equivalent surface area dosage for this species. The animals were euthanized 30 minutes post administration with tissues collected shortly thereafter. A control animal was given saline and the same procedure was followed. Tissues were placed into formalin or frozen in OCT at the time of the necropsy.

Staining of C57BL/6 LysM-EGFP knock-in mouse tissue

Sections of the lung, spleen, liver, and kidney were cut 10µm thick. For staining, tissues were air-dried for 7–10 minutes and washed three times in 1x PBS (pH 7.4 for 5 minutes at room temperature. A DAPI nuclear stain (VECTASHIELD Hard set Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA) was used to label nuclei of all cells. The slides were then mounted with a glass cover slip, placed in a cardboard slide tray, and stored in a 4°C refrigerator.

Immune Fluorescent Staining on frozen rhesus macaque tissue

Tissues used were of the left lower, left middle, left upper, right lower, right middle, and right upper lobes of the lung, as well as the spleen, liver, and kidney. Cryosections were cut 10µm thick.

Tissues harvested and frozen in OCT were stained using the NHLBI Pathology Core facility at NIH. For staining, tissues were dried for 7–10 minutes and washed three times in 1x PBS (pH 7.4) for 5 minutes at room temperature. The sections were then blocked with 10% normal horse serum for 1 hour at room temperature. Following this, the sections were incubated with a 1:100 dilution of the primary antibody, mouse anti-human CD18 (Clone 6.7; BD Pharmingen, San Diego, CA), overnight at 4°C. The dilution of the primary antibody for the spleen was 1:400. The sections were then washed in 1x PBS (pH 7.4) three times for 5 minutes at room temperature and incubated with a secondary antibody, an anti-mouse IgG FITC (Jackson ImmunoResearch, West Grove, PA), for 2 hours at room temperature. The dilution of the secondary antibody for the spleen was 1:200. Following incubation, the sections were washed in 1x PBS (pH 7.4) twice for 5 minutes at room temperature and a cover slip was mounted using VECTASHIELD Hard set Mounting Medium with DAPI (Vector Laboratories). The slides were placed in a cardboard slide tray, and stored at 4°C before examination under the microscope.

Immune Fluorescent Staining on paraffin embedded rhesus macaque tissue

Tissues were collected in formalin and sent to HISTOSERV, Inc. (Germantown, MD, USA) for paraffin-embedding, sectioning, and hematoxylin and eosin staining. Double-label immunofluorescence (IFA) was performed using a 1:100 dilution of an antibody against a myeloid/histiocyte antigen clone Mac387 (M0747, DAKO, Carpinteria, CA) which labels

neutrophils and monocytes and a 1:50 dilution of an anti-caveolin-1-Cy3 antibody (C3990, Sigma) to identify endothelium. The primary specific antibodies were applied sequentially followed by a secondary antibody. The secondary antibody was coupled with Alexa 488 (green) (Invitrogen, Molecular Probes). Following antibody treatment, sections were washed twice for 15 min in PBS with 0.2% fish skin gelatin. To-Pro3 (nuclear marker, Molecular Probes, Eugene, OR) was applied at 1 µg/ml, incubated for five minutes and tissues were then washed in PBS. Finally, the sections were rinsed in double distilled water and mounted with aqueous mounting medium according to a protocol described in Borda et al (13).

Confocal Microscopy

Confocal microscopy was used to observe the presence and location of neutrophils within the observed tissue. Slides of frozen tissues that underwent IHC were imaged using the Zeiss LSM-510-META (Carl Zeiss, Jena, Germany) confocal laser scanning microscope using a 20X objective. The resulting images were either of anti-CD18 immunostaining for the rhesus macaques or of EGFP expression for the mice, both imaged with a 488nm laser excitation and 505–550nm emission (displaying a green fluorescence) and DAPI nuclear stain imaged with a 405nm laser excitation and 420–480nm emission (displaying a blue fluorescence).

Slides produced from IHC staining of sections of paraffin embedded tissue underwent confocal microscopy using a Leica TCS SP2 confocal microscope (Leica Microsystems, Exton, PA) equipped with three lasers. Optical sections were collected at 512 × 512 pixels. Alexa-488 (488nm/517nm ex/em = fluorescence excitation and emission), Cy3 (514nm/540nm) and ToPro3 (642nm/661nm) were excited with the laser line 488nm, 514nm, and 633nm, respectively. NIH Image (version 1.62) and Adobe Photoshop (version 7.0) were used to process and assemble images. Pl Apo objectives 20x, 40x and 63x with a NA 0.7, 1.25 and 1.4, respectively, were used to image the tissues and the resulting magnification is in the images shown as a magnification bar.

Analysis

Complete blood counts (CBC) were performed using a Cell Dyn 3500 (Abbott Diagnostics, Santa Clara, CA, USA) on blood samples collected in EDTA tubes. Differentials were automated and determined based on cellular size and granularity according to parameters set by the manufacturer. Absolute neutrophil count (ANC) was calculated using the percentage of segmented neutrophils multiplied by the white blood cell count per microliter (µl) of blood.

Images were analyzed by obtaining the thresholded (to remove background) areas of the EGFP+ leukocytes or CD18+ cells and DAPI (nuclear stain) using Metamorph, software from Molecular Devices (Sunnyvale, CA, USA). By determining the ratio of the area expressing green fluorescence with that of blue fluorescence allowed us to standardize for the amount of tissue in a given field. The ratio of green to blue threshold per image was then calculated as a percentage. This volume was used to determine the normalized percentage of EGFP or CD18/FITC+ cells within the tissue. Evaluation was performed on single images from individual animals. Triplicate images per section per animal were evaluated. In the case of the non-human primates all six lobes of lung were evaluated in triplicate. Tissue sections from three non-human primates were compared with sections from one control.

Statistics

Bar charts of the threshold intensity data were constructed using mean and standard deviation (SD). The random effect analysis of variance (ANOVA) models were used to compare neutrophil percentages among the tissue samples taken at different time points for

both the C57BL/6 LysM-EGFP knock-in mice and rhesus macaques. The random effect models used individual image data from each animal, adjusting for the correlation among three images in each animal. A log-transformation was applied to the fluorescence data to achieve the normality if appropriate. The statistical significance was set at $p < 0.05$. Analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC).

Results

Presence of neutrophils in C57BL/6 LysM-EGFP knock-in mice tissue

Tissues from six mice were used for the 15 and 30 minute post G-CSF administration time points and tissues from four mice were used for the 240 minute post G-CSF administration and the control. EGFP+ cells displayed a green fluorescence and DAPI nuclear stain displayed a blue fluorescence (Figure 1). For each tissue, three regions were evaluated. The mean percentage of area occupied EGFP+ cells relative to the area occupied by nuclei was significantly increased 30 minutes following G-CSF in the lung tissue compared to the control lung tissue (mean $\pm$ SD: $15.9 \pm 6.8\%$ vs. $6.3 \pm 2.6\%$, respectively, $p < .001$) (Figure 2). There was a significant decrease from 30 to 240 minutes post G-CSF administration in the percentage of EGFP+ in the lung (from $15.9 \pm 6.8\%$ to $9.2 \pm 5.1\%$, $p = 0.002$, Figure 2). The sequential percentage of EGFP+ area in the spleen also significantly increased following G-CSF administration, from a control value of $3.2 \pm 1.1\%$, to $11.3 \pm 7.3\%$ at 15 minutes, $14.7 \pm 6.3\%$ at 30 minutes, and $19.4 \pm 7.7\%$ at 240 minutes (ANOVA $p < .001$, Figure 2). Other tissues, such as the kidney and liver also demonstrated significant differences; however, the percentage of area occupied within these tissues was consistently very low (less than 1%, Figure 2). For example, the percentage area of EGFP+ in the kidney 240 minutes following G-CSF administration was somewhat higher than the control at the same time point. ($0.17 \pm 0.07\%$ vs. $0.08 \pm 0.08\%$, respectively, $p = 0.03$). For the liver, the mean percentage of EGFP+ area was also increased from the control $0.2 \pm 0.1\%$, compared to percentages at 15 minutes ($0.4 \pm 0.2\%$), 30 minutes ($0.6 \pm 0.3\%$), and 240 minutes ($0.4 \pm 0.2\%$), ANOVA $p < .001$.

Decline in circulating neutrophils after administration of G-CSF to Rhesus Macaques

Three animals (RQ6681, RG6697, and RQ6707) were evaluated after receiving a single 10 μ g/kg SQ dose of G-CSF. One animal (RQ6720) served as a control, receiving saline alone. All animals receiving G-CSF demonstrated an average decline of 90% in circulating neutrophil count 15 minutes following G-CSF administration, from an mean(SD) neutrophil count of $4853(2248)/\mu$ l to $469(334)/\mu$ l (Figure 3). The mean neutrophil count 15 and 30 minutes following G-CSF administration for the experimental animals were $469(334)$ and $857(476)/\mu$ l, respectively. The neutrophil count for the control at 15 and 30 minutes remained steady at $2661/\mu$ l and $2627/\mu$ l, respectively (Figure 3). No significant changes in lymphocyte or monocytes numbers were observed.

Presence of neutrophils in rhesus macaque tissue

Confocal microscopy was used to directly examine neutrophil trafficking shortly following G-CSF administration in tissues. IHC performed on tissues frozen in OCT were analyzed for threshold intensity within a given area of $636.4\mu\text{m} \times 636.4\mu\text{m}$ which is $10\mu\text{m}$ thick. The threshold area computes the area in which the pixels of a certain light intensity occupy.

The mean percentage of CD18+ area in the lungs of the rhesus macaques receiving G-CSF at 30 minutes was significantly greater compare to the control animal ($3.8 \pm 3.1\%$ ($n=54$ images from six lobes) vs. 1.5 ± 1.1 ($n=18$ images from six lobes), respectively, $p < .001$) (Figure 4). No statistically significant differences were observed between lobes in area of CD18 fluorescence when compared with nuclear fluorescence. There was an upward trend

between the percent of CD18+ area in the spleen of the experimental group at 30 minutes ($16.5 \pm 8\%$) and that of control animal ($9.1 \pm 3.1\%$), $p=0.09$. The mean percent of CD18+ area in the liver of primates receiving G-CSF was significantly greater than that of the control liver ($1.5 \pm 1.5\%$ vs. $0.002 \pm 0.003\%$, respectively, $p<0.05$). Finally, the mean percentages of CD18+ area were not significantly different in the kidney between the control and primates receiving G-CSF ($0.03 \pm 0.02\%$ vs. $0.14 \pm 0.14\%$, respectively).

Morphological evaluation of paraffin embedded lung tissue stained with hematoxylin and eosin demonstrate neutrophil accumulation within the pulmonary vasculature 30 minutes following G-CSF administration is shown in Figure 5. Lung tissues were further evaluated in order to define better cellular relationships within the vasculature utilizing multiple antibodies capable of identifying neutrophils and endothelial cells as well as a nuclear stain. Confocal images of the lungs of the non-human primate demonstrated a close association of calgranulin-Mac387+ neutrophils to the endothelial cells lining the pulmonary vasculature (Figure 6). This was not the case in the control animal in which hematopoietic cells remained within the lumen of the vasculature with no evidence of margination.

Discussion

G-CSF is known to stimulate neutrophil production through the differentiation of myeloid progenitors and used clinically to accelerate neutrophil recovery. The effects of G-CSF appear restricted to neutrophils and not other hematopoietic lineages. Intrigued by an acute but transient loss of neutrophils from the circulation shortly following G-CSF administration, we were interested in determining what happened to these neutrophils. To do this, two animal model systems were utilized, a transgenic C57BL/6 LysM-EGFP knock-in mouse model and a large animal model using rhesus macaques. Previously we demonstrated in rhesus macaques that there was a 90% decrease in circulating neutrophil count within the first 15 minutes following G-CSF administration (5), consistent with the observations made in this study. Similar transient losses in circulating neutrophil numbers have also been reported in rats and humans (6–8). The mechanism behind this loss remains unclear. G-CSF has been observed to alter neutrophil cell surface protein expression, as well as function and behavior (1,2,7). In our previous study, concurrent with the drop in circulating neutrophil count there was also an increase in cell surface expression of CD11a, CD18, CD31, and CD49d (5). CD11a, CD18, and CD49d are involved in cellular adhesion and CD31 is involved in transendothelial migration. An increase of CD11 and CD18 expression has also been reported by others (6,7,9). This implies that G-CSF may alter and/or activate cellular membrane associated adhesion proteins enhancing vascular margination.

Confocal microscopy was used in this study to examine neutrophil trafficking into the lung, spleen, liver, and kidney tissues in murine and non-human primate animal models. In both model systems the greatest accumulation of neutrophils shortly following G-CSF administration occurred within the pulmonary vasculature. In the murine study, thirty minutes following G-CSF administration the presence of EGFP+ expressing cells utilizing the LysM promoter/enhancer increased from a control value of $6.3(2.6)\%$ ($n=12$) to $15.9(6.8)\%$ ($n=18$) ($p \leq 0.001$) in the lung. Similarly thirty minutes following G-CSF administration in the non-human primate lung there was an increase in neutrophil percentage from $1.5(1.1)\%$ ($n=18$) for the control to $3.8(3.1)\%$ ($n=54$) for the three experimental animals ($p \leq 0.001$). The accumulation of neutrophils in the lung shortly following G-CSF administration is interesting as there has been reported a diminishment of pulmonary function in rats (14), rabbits (15), and humans (16). Although rare, G-CSF is known to have adverse effects on the respiratory system on healthy donors, such as acute lung injury, pulmonary edema, cough, and dyspnea (16–17). These side effects are believed to be attributable to sequestration of neutrophils to the lung, release of active superoxides by

activated neutrophils, or the release of pro-inflammatory cytokines caused from cell stimulation by G-CSF leading to damage of the endothelial and epithelial layers of the lung (14). As our results show that neutrophils shortly following G-CSF administration sequester to the lung this suggests a potential concern for those having pulmonary infections. It also suggests potential therapeutic applications for targeting neutrophils to the lung. Further investigation into neutrophil activation and presence of superoxides as an effect of G-CSF administration is merited.

A marked increase in neutrophil numbers following G-CSF administration was observed in the spleen for mouse and monkey based on nuclear morphology and morphological evaluation, respectively. Although not reaching significance in the non-human primate at 30 minutes there was a trend for an increase, and the difference was significant in the murine study. With additional time in the non-human primates this increase may have proven significant. In the murine studies it is interesting to note that at later time points there was a decline in number of EGFP+ cells within the lung, however, there was an increase in the spleen. Nuclear morphology suggests that these cells are polymorphonuclear leukocytes, or neutrophils. One can speculate that unless there is no secondary stimulus for adherence and transmigration within the tissue, the neutrophils will subsequently return to the circulation. Values from other tissues, such as the kidney and liver, although significantly changed, were proportionally low in EGFP+ cell numbers. An increase in neutrophils within the spleen following G-CSF administration is consistent with previous observations in the human and rat (18–21). This can be attributable to increase of circulating neutrophils passing through the spleen, and the potential for extramedullary hematopoiesis in the spleen (20–21). Although rare, some reports show that enlargement of the spleen due to G-CSF administration may lead to splenic rupture (10,18–20).

The mechanisms by which neutrophils are sequestered into the lung and spleen require further investigation. Our study demonstrates conclusively that shortly following a subcutaneous administration of G-CSF there is a rapid accumulation of neutrophils in both the lung and spleen. Although the neutrophil accumulation within the lung may be transient, as suggested by the murine study, this observation may have clinical ramifications should a patient or donor have a subclinical pulmonary infection. There is no evidence that the neutrophils transmigrate into the pulmonary space based on our studies. The neutrophils appear to strictly marginate along the pulmonary vasculature. Should, however, the neutrophils become activated, transmigration may occur and pulmonary damage and pathology ensue. Side effects associated with G-CSF administration typically are minor, however, our results highlight the need for concern of the pulmonary health of a donor or patient as an accumulation of neutrophils and their subsequent activation could lead to unforeseen pulmonary complications. Caution should be taken in evaluating donors and patients receiving G-CSF as an underlying pulmonary illness may result in the activation of the accumulating neutrophils resulting in adverse side effects.

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Figure 1. Nuclear stain of lung and spleen C57BL/6 LysM-EGFP knock-in mouse tissues
Images captured using a x20 objective. Lung tissues from C57BL/6 LysM-EGFP knock-in mice administered G-CSF and a vehicle control were stained with DAPI. The green fluorescence is a result of the EGFP+ cells and the blue fluorescence results from the DAPI of the DNA counterstain.

Figure 2. Standardized image threshold quantification of EGFP-expressing cells in images of the C57BL/6 LysM-EGFP knock-in mouse lung, spleen, liver, and kidney

Control, 15, 30, and 240 minutes post administration 50 μ g/kg recombinant mouse G-CSF the lung, spleen, liver, and kidney tissue was harvested. Tissues were examined with LSM-510-META confocal microscope and analyzed with Metamorph. A ratio of green fluorescence (EGFP+) to blue fluorescence (DAPI) within a fixed area was obtained. N is the number of animals evaluated, n the total number of tissue samples. Error bars represent SD. Compared to the control: *: P<.05, **: P<.01, ***: P<.001

Figure 3. Changes in circulating neutrophil count for rhesus macaques receiving a single subcutaneous dose of 10 µg/kg of recombinant human G-CSF (RQ6707, RQ6681, and RQ6697) or saline (RQ6720).

Figure 4. Standardized image threshold area quantification for CD18+ in images of the rhesus macaque lung, spleen, liver, and kidney

Thirty minutes post administration of G-CSF the lung, spleen, liver, and kidney tissue were harvested and frozen in OCT. IHC was performed on all tissue. Detection for neutrophils was performed with affinity purified mouse anti-human CD18 and a secondary anti-mouse FITC. A DAPI nuclear stain was performed as well. Tissues were examined with LSM-510-META confocal microscope and analyzed with Metamorph, an image acquisition and analysis software. The threshold area was obtained for the DAPI nuclear stain and CD18-FITC. The ratio CD18 area and DAPI area was determined for each image. Error bars represent SD. N is the number of animals evaluated, n the total number of tissue samples. Compared to the control: *: $P < .05$, **: $P < .01$, ***: $P < .001$

Figure 5. Neutrophil accumulation in non-human primate lung tissue (G-CSF treated RQ6707)
Images of hematoxylin and eosin stained lung tissue of a non-human primate (RQ6707) thirty minutes following administration of recombinant human G-CSF using a 60X objective. Neutrophils are identified with arrows.

Figure 6. Neutrophil, endothelial, and nuclear immunostaining of non-human primate lung tissue (control RQ6720; G-CSF treated RQ6707)

Immune Fluorescence of lung tissue of non-human primates receiving G-CSF and a control. ToPro-3 (blue), Calgranulin Mac387 (green), and caveolin (red) cells of stained rhesus macaque lung tissue.

(A) Confocal image of the lung of the control non-human primate (RQ6720) showing neutrophils predominantly in vessels using a 20X objective. (B) Confocal images of the lungs of non-human primates receiving G-CSF 30 minutes before euthanasia (RQ6707) demonstrated an adherence of Mac387+ neutrophils to the endothelial cells using a 20X objective. (C and D) The neutrophils appear elongated and adhered to the endothelium in those animals receiving G-CSF (RQ6707). 50X and 100X objectives were used, respectively.