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ARTICLE

Effect of the CXCR4 antagonist plerixafor on endogenous neutrophil dynamics in the bone marrow, lung and spleen

Janesh Pillay[1,2,3] | Nicola Tregay[1] | Goda Juzenaite[4] | Leo M. Carlin[5] | Chiara Pirillo[6] | David C. A. Gaboriau[7] | Neda Farahi[1] | Charlotte Summers[1] | Cristina Lo Celso[6] | Edwin R. Chilvers[1,4] | Sara Rankin[4] | Katia De Filippo[4]*

[1]Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge, UK

[2]Department of Intensive Care Medicine, University Medical Center, Groningen, The Netherlands

[3]Dept of Intensive Care Medicine, University Medical Center, The Netherlands, Groningen

[4]Department of Medicine, National Heart and Lung Institute (NHLI), Imperial College, London, UK

[5]Cancer Research UK Beatson Institute, Glasgow and Institute of Cancer Sciences, University of Glasgow, Glasgow, UK

[6]Department of Life Science, Imperial College, London, UK

[7]Facility for Imaging by Light Microscopy (FILM), NHLI, Imperial College, London, UK

Correspondence
Dr Katia De Filippo, National Heart and Lung Institute (NHLI), Imperial College London, UK, Sir Alexander Fleming Building, South Kensington Campus, London SW7 2AZ, UK.
Email: k.de-filippo@imperial.ac.uk

*Lead corresponding author.

Abstract

Treatment with the CXCR4 antagonist, plerixafor (AMD3100), has been proposed for clinical use in patients with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome and in pulmonary fibrosis. However, there is controversy with respect to the impact of plerixafor on neutrophil dynamics in the lung, which may affect its safety profile. In this study, we investigated the kinetics of endogenous neutrophils by direct imaging, using confocal intravital microscopy in mouse bone marrow, spleen, and lungs. Neutrophils are observed increasing their velocity and exiting the bone marrow following plerixafor administration, with a concomitant increase in neutrophil numbers in the blood and spleen, while the marginated pool of neutrophils in the lung microvasculature remained unchanged in terms of numbers and cell velocity. Use of autologous radiolabeled neutrophils and SPECT/CT imaging in healthy volunteers showed that plerixafor did not affect GM-CSF-primed neutrophil entrapment or release in the lungs. Taken together, these data suggest that plerixafor causes neutrophil mobilization from the bone marrow but does not impact on lung marginated neutrophil dynamics and thus is unlikely to compromise respiratory host defense both in humans and mice.

KEYWORDS

neutrophil activation, neutrophil dynamics, neutrophil mobilization

1 | INTRODUCTION

The CXCR4 antagonist plerixafor (AMD3100) is used clinically for the acute mobilization of HSPCs for bone marrow transplants.[1] Plerixafor also causes a dose- and time-dependent blood leukocytosis in humans.[2] Studies in both mice and humans have shown that plerixafor also affects neutrophil dynamics, most notably increasing circulating neutrophil numbers as early as an hour after administration.[3–5] Initial work from our group showed that when plerixafor was infused directly into the femoral artery of anaesthetized mice, there was an increase in the number of neutrophils collected via cannulation of the femoral vein, suggesting that plerixafor led to the mobilization of neutrophils from the bone marrow (BM).[3] This supported the hypothesis that the CXCR4-CXCL12 axis is important, not only for HSPC, but also neutrophil retention in the bone marrow.[3,6] Consistent with this hypothesis, a severe blood neutropenia is a characteristic of the Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis (WHIM) syndrome, a genetic disease associated with gain-of-function

Abbreviations: IVM, Intravital microscopy; L-IVM, Lung intravital microscopy; S-IVM, Spleen intravital microscopy; WHIM, Warts Hypogammaglobulinemia Immunodeficiency and Myelokathexis; PCLS, Precision Cut Lung Slices.

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mutations of CXCR4.[7,8] Indeed, patients and mouse models of this disease exhibit an increase in the numbers of mature neutrophils in the bone marrow reserve[7,8] and in patients with WHIM syndrome the blood neutropenia can be corrected by the administration of plerixafor.[9] Likewise, myeloid-specific deletion of CXCR4 in mice has been shown to result in reduced numbers of mature neutrophils in the BM and a blood neutrophilia.[10]

Contrary to these findings, an alternative view is that the blood neutrophilia induced by plerixafor may be due to neutrophil de-margination from the lung microvasculature and not due to mobilization from the BM.[11] Thus when Devi et al. imaged GFP$^+$ neutrophils that had been isolated from the BM of LysM-GFP$^+$ mice and adoptively transferred into WT recipients they observed neutrophil mobilization from the lung and not the BM.[11] Furthermore, they observed a transient (2–4 h) increase in neutrophil numbers in blood sampled from the carotid artery versus the vena cava following plerixafor treatment both in primates and mice, again consistent with mobilization of neutrophils from the lung.[11] These findings were subsequently challenged in a study by Liu et al., who quantified the numbers of endogenous neutrophils in tissues by flow cytometry.[12] They reported an increase in neutrophil numbers in the lung, spleen, and blood accompanied by a decrease in the BM following plerixafor treatment.[12] In addition, they reported that imaging of frozen sections of lung, post-plerixafor treatment, showed no evidence of neutrophil de-margination. Recent technological advances in intravital microscopy (IVM) now allow us to directly image endogenous neutrophil dynamics in the lung, spleen, and BM, thus providing the opportunity to characterize neutrophil migratory behavior within their anatomical location prior and subsequent to plerixafor administration.

Neutrophils in the pulmonary circulation play an important role in intravascular host defense.[13] Entrapment of systemically primed neutrophils within the lung microvasculature also facilitates de-priming, with these cells subsequently able to re-circulate.[14,15] In human studies, we have shown that radiolabeled neutrophils primed ex vivo with GM-CSF accumulate in the lung microvasculature, with 97% being retained in the lungs at first pass, versus <5% of un-primed neutrophils. It is not known, however, whether the retention of primed neutrophils or their subsequent time-dependent de-priming in the lungs is affected by plerixafor. Similarly, a proportion of murine neutrophils present at a site of experimental liver injury have been reported to re-enter the systemic circulation and subsequently lodge in the pulmonary vasculature, where they up-regulate CXCR4 before trafficking back to the BM for final clearance.[16] In this context plerixafor impaired trafficking of neutrophils from the lung to the BM, suggesting that this process is also mediated by CXCR4.[16]

Recently it was shown that chronic administration of plerixafor is a feasible strategy for long-term treatment of WHIM patients, reducing infection frequency and wart burden associated with the disease.[17] Chronic treatment with plerixafor is also being considered for additional clinical applications including pulmonary fibrosis, pancreatic cancer (Camplex-1 trial), and leukemia, where the mechanism of action appears independent of the effect of plerixafor on neutrophil dynamics.[18–20] However if plerixafor affects neutrophil dynamics in the lung, it has the potential to compromise lung host defense and this may have a detrimental impact on these patients especially if otherwise immunocompromised.

Key points

- Using confocal intravital microscopy (IVM), neutrophils are observed to exit directly from the bone marrow following plerixafor treatment
- Plerixafor treatment does not result in demargination of neutrophils from the micro-vessels of the lung
- IVM shows a substantial increase in the number of splenic neutrophils after plerixafor treatment
- In human volunteers, plerixafor did not affect GM-CSF-primed neutrophil entrapment or release in the lungs

For this reason, in this study we used IVM of BM, lung, and spleen and precision cut lung slices (PCLS) to interrogate the effect of plerixafor on the dynamics of endogenous neutrophils in the mouse, and gamma scintigraphy (SPECT/CT) of radiolabeled neutrophils to investigate whether plerixafor impacts the trafficking of primed neutrophils in the lungs of humans. Our studies reveal that plerixafor has no effect on neutrophil dynamics in this organ, including neutrophil number and cell velocity in the lung, suggesting that it will not impact directly on lung host defense in patients.

2 | METHODS

2.1 | Mice

C57Bl/6J female mice between 6 and 8 weeks old were used in all the experiments. All mice were housed in specific pathogen free conditions at Imperial College London. All experiments were carried out in accordance with the recommendations in the Guide for the Use of Laboratory Animals of Imperial College London, with the ARRIVE (Animal Research Reporting of In Vivo Experiments) guidelines. All animal procedures and care conformed strictly to the UK Home Office Guidelines under the Animals (Scientific Procedures) Act 1986, and the protocols were approved by the Home Office of Great Britain. In vivo experiments were performed under the authority of our UK Home Office Project Licences that were reviewed by the Imperial College Animal Welfare Ethical Review Board (AWERB). BM, lung, and spleen IVMs of live mice were imaged under general, terminal anesthesia. BM IVM anesthesia was maintained with isoflurane in medical O_2 throughout the procedure. Lung IVM and spleen IVM anesthesia was maintained by alternating injections of either 50 mg/kg ketamine alone or in combination with 0.125 mg/kg medetomidine at predefined time points. O_2 was supplied throughout the procedure.

2.2 | Flow cytometry

Blood was collected in EDTA coated syringes by cardiac puncture under terminal anesthesia. Red blood cell lysis was carried out and samples were centrifuged at 450 × *g* for 5 min at 4°C. Single cell suspensions were stained with Live/Dead near-IR stain (Life Technologies) and Fc-Receptors block (using clone 93, BioLegend). Cell suspensions were incubated with directly conjugated fluorescent Abs for 30 min at 4°C. The following Abs were used: Ly6G (clone 1A8), CD45 (clone 30-F11), CD11b (clone M1/70), CD3e (clone 17 A2), CD19 (clone 6D5), Ter119 (clone TER-119), CD62L (clone MEL-14), and CXCR4 (clone 2B11). Acquisition was performed on BDFortessa using FacsDiva software (BD Bioscience) with further analysis by FlowJo software. Lung tissue was minced, digested with collagenase D, 20 U/ml DNase I for 25 min at 37°C, and filtered. To study lung marginated neutrophils by FACS, CD45 was pre-injected 3 min before sacrificing the mice, to label intravascular leukocytes.

In some experiments, low dose anti-Ly6G or IgG 3μg/mouse were i.v. injected before i.p. injection of plerixafor or PBS as control.

Sixty minutes following i.v. injection of Ly6G-PE blood, bone marrow, lungs, and spleen were harvested. Following tissue processing leukocytes were stained with mAbs for CD11b–PerCP Cy5.5 and Ly6G-BV605 and then using flow cytometry, we determined the percentage of $Ly6G^+$ and $CD11b^+$ cells that were $Ly6G\text{-}PE^+$. Our FACS graphs show that 99.2%, 99.7%, 99.1%, and 99.9% of BM, blood, splenic, and marginated lung neutrophils were Ly6G-PE positive respectively (Supplementary Fig. 1A–D); indicating that >99% of endogenous neutrophils are labeled by i.v. injection of Ly6G-PE, irrespective of the tissue examined.

2.3 | Calvarium BM intravital microscopy

BM intravital microscopy (IVM) was performed using a Zeiss LSM 780 upstanding confocal microscope supplied with Argon (488 and 561 and 633 nm lasers) as described by Duarte et al.[23] Neutrophils were labeled with 2–4 μg/mouse of Ly6G (clone 1A8)-647, the vasculature was labeled with CD31 (clone 390)-488, and Cy5-Dextran (Nanocs, MA). Videos were recorded for 40 min and treatment was administered by intraperitoneal (i.p.) injection during recording.

2.4 | Lung IVM

This method was first described in[21] with modifications.[22] Imaging was performed on an upright Leica SP5 confocal microscope using a 25 × 0.95na water immersion objective.

2.5 | Spleen IVM

This method is described in ref. 23 with modifications. Anesthetized mice were placed in the right lateral decubitus position and a small section of hair was removed from the left flank. A 5–8 mm abdominal incision on the left flank above the spleen was used to expose the surface of the spleen, which was mechanically stabilized with a gentle vacuum using the coverslip vacuum chamber used for lung IVM.

Imaging was performed on an upright Leica SP5 confocal microscope using a 25 × 0.95na water immersion objective. Images were acquired in 3 z-slices 5 μm apart. In both lung and spleen IVMs, neutrophils were labeled with 3 μg/mouse of Ly6G (clone 1A8)-PE, the vasculature was labeled with CD31 (clone 390)-488, fluorescent Abs were injected intravenous (i.v.) in a maximal volume of 50 μl ≈10–20 min before imaging commences.

While imaging both lung and spleen, i.p. injections of plerixafor at 5 mg/kg (AMD3100, Sigma–Aldrich) in vehicle PBS were performed and imaging continued non-stop until 90 min after treatment. At the end of the imaging session, mice were humanely killed by anesthetic overdose (Sodium-Pentobarbital) and blood was collected by cardiac puncture and lung and spleen were harvested.

2.6 | Cell tracking

BM IVM 3D time-series in .czi format were saved as 16bit TIFF and imported into NIS-Elements (Version 4.50, Nikon Instruments, UK). Files were processed with Advanced Denoising and saved as Maximum Intensity Projections. Neutrophils were tracked using the Spot Tracking plugin[24] in Icy, an open-source platform for bioimage analysis.[24] Tracks in the 200 frames sequence were checked manually to ensure they were correct.[25]

Lung IVM and spleen IVM 3D time-series in .lif format were analyzed using Imaris software (Bitplane, Oxford Instruments). The video was cropped in time to analyze 60 frames before and 30, 60, and 90 min after AMD3100/PBS application. Neutrophil tracking was performed automatically on Ly6G-positive cells transformed in spots. XYZ data were exported and track mean speed was plotted.

2.7 | Study participants

The human study was approved by the Cambridgeshire and Hertfordshire Research Ethics Committee (15/EE/0321) and the Administration of Radioactive Substances Advisory Committee of the United Kingdom (83/400/33731); all subjects gave written informed consent.

Healthy volunteers were excluded if there was a history of any acute lower respiratory tract illness within 4 weeks of screening, a current diagnosis of asthma, excluding childhood asthma, or an abnormal spirometry result defined as a forced expiratory volume in 1 s (FEV1) ≤80% of predicted or a FEV1 to forced vital capacity (FVC) ratio ≤70%.

2.8 | Human neutrophil isolation and radiolabeling

Neutrophils were isolated from 80 ml of autologous venous blood using discontinuous plasma-Percoll gradients and radiolabeled using 99mTc-hexamethylpropyleneamine oxime (GE Healthcare, Buckinghamshire, UK).[26] Administered activities were 114–200 MBq. Neutrophils were stimulated ex vivo for 15 min with either 1 ng/ml or 100 ng/ml GM-CSF (Bio-Techne, Abingdon, UK) at 37°C.

2.9 | Administration of plerixafor or placebo in human

Volunteers received either plerixafor or placebo, and neutrophils were reinfused 60 min following plerixafor administration at the circulating

pharmacological T_{max} for the drug. Plerixafor was administered in the clinically effective dose of 0.24 mg/kg (s.c.), which is used for mobilization of hematopoietic stem cells, which resulted in the expected marked leukocytosis at 3 h post-injection.[1] Re-infusion of neutrophils took place 60 min after plerixafor/placebo administration and was undertaken while the patients were on the SPECT-CT to allow for immediate imaging.

2.10 | Planar and SPECT/CT imaging

Volunteers were positioned in a double-headed SPECT/CT camera (GE Discovery 670, GE Healthcare), fitted with low-energy, parallel-hole collimators, and interfaced to a computer. After bolus intravenous injection of technetium-99 m-labeled neutrophils, the activities in the chest and upper abdomen (liver and spleen) were recorded by dynamic planar imaging with a frame time of 1 s for 2 min followed by 20 s for 38 min. At the later time-points, the frame times were 20 s for 10 min. SPECT images were acquired over 24 min at 45 min and 24 h post re-injection. A CT scan was performed at the end of the 45-min SPECT acquisition for anatomical co-registration with SPECT. To generate the organ time-activity curves, regions of interest (ROI) were drawn over the right lung (anterior projection), the liver (anterior and posterior), and spleen (anterior and posterior) using Xeleris software (Version 3.1, GE Healthcare). Mean counts per pixel or voxel in these ROIs were recorded and decay-corrected for physical decay of technetium-99 m.

Blood was collected at intervals up to 6 h post-injection and whole blood radioactivity measured in a gamma counter. The percentage of radiolabeled cells in the circulation 45 min post injection was calculated as follows: (Radioactivity in sample [kBq] / [injected activity (kBq)/total blood volume (ml)]) × 100; blood volume was estimated using height and weight.[27]

2.11 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc). A *P*-value of less than 0.05 was considered significant: $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$ ***, NS, not significant. Statistical tests used are as detailed in the figure legends.

3 | RESULTS

3.1 | IVM reveals direct mobilization of neutrophils from the BM and increased velocity of neutrophils after plerixafor treatment

In mice, circulating blood neutrophil numbers increased significantly 60 min after i.p. injection of plerixafor (Fig. 1A); this is accompanied by a concomitant reduction in neutrophil numbers in the BM (Fig. 1B). To directly test whether the blood neutrophilia was due to neutrophil release from the BM, we undertook IVM of the mouse calvarium, identifying BM vasculature by i.v. injection of Cy5-Dextran and endogenous neutrophils by i.v. injection of low dose anti-Ly6G-PE mAb. The use of low dose anti-Ly6G mAb (2–5 μg/mouse), as an imaging tool for neutrophil dynamics, has been widely reported and several studies have shown that it does not compromise neutrophil dynamics such as rolling, adhesion, and intravascular crawling in a number of tissues including the lung.[28,29] Although the same anti-Ly6G mAb is used to cause neutrophil-depletion, the dose to achieve this effect is much higher (100–500 μg/mouse).[13,30,31] Moreover, a previous study has compared the dynamics of LysM-GFP$^+$ and fluorochrome-conjugated Ly6G mAb neutrophils and results showed that Ly6G mAb did not cause change in their migratory behavior or in their recruitment during inflammation.[32] The mean speed of Ly6G neutrophils within the calvarium bone marrow (BM) parenchyma was calculated (Fig. 1C) and a significant increase in neutrophil speed was observed 20 min after a single i.p. injection of plerixafor (Fig. 1D). Tracking individual neutrophils in the BM showed that ≈60% of neutrophils under homeostasis have a speed ranging from 2 to 4 μm/min (Fig. 1E). Thirty minutes after injection of plerixafor, the percentage of neutrophils migrating at such speed significantly decreases, ≈40% with a concomitant significant increase in the percentage of neutrophils with a higher speed ranging from 4 to 6 μm/min, ≈30% (Fig. 1E). Moreover, the percentage of neutrophils with the lower speed ranging from 0 to 2 μm/min did not change after plerixafor, ≈30% (Fig. 1E). These results suggest that plerixafor causes an increase in the velocity of neutrophils that already showed a migratory behavior without altering the less motile cells. Analysis of IVM videos also revealed a decrease in the number of neutrophils in the calvarium BM 30 min after plerixafor injection (Fig. 1F and G) and an increase in neutrophils observed exiting from the BM stroma into the vasculature (Supplementary Video 1). Our findings provide direct visual evidence consistent with those of Liu et al. and Martin et al. supporting the ability of plerixafor to enhance the mobilization of neutrophils from the BM compartment by increasing cell velocity and egress from the BM compartment.

3.2 | Spleen is not a source of blood neutrophilia following plerixafor treatment

The spleen is an alternative site of significant neutrophil margination.[33,34] Mobilization of neutrophils from the spleen might therefore also contribute to the increase in circulating neutrophil numbers seen after plerixafor treatment.[33,34] However, when quantifying cell numbers by flow cytometry, we noted a significant increase in the absolute number of splenic neutrophils 60 min after plerixafor administration (Fig. 2A); this is in agreement with previous reports.[12] Moreover, analysis of neutrophil dynamics using spleen IVM showed that as early as 30 min after plerixafor injection there was an increase in the number of splenic neutrophils and that this increase was maintained up to 90 min after treatment (Fig. 2B and C; Supplementary Video 2). Tracking individual neutrophils in the spleen showed that <40% of neutrophils under homeostasis have a speed ranging from 0 to 1 μm/min while >40% of neutrophils have a speed ranging from 1 to 2 μm/min and 20% from 2 to 3 μm/min (Fig. 2D). Sixty minutes after injection of plerixafor, the percentage of less motile neutrophils decrease significantly with a concomitant increase in the percentage of cells with a greater migratory speed (Fig. 2D).

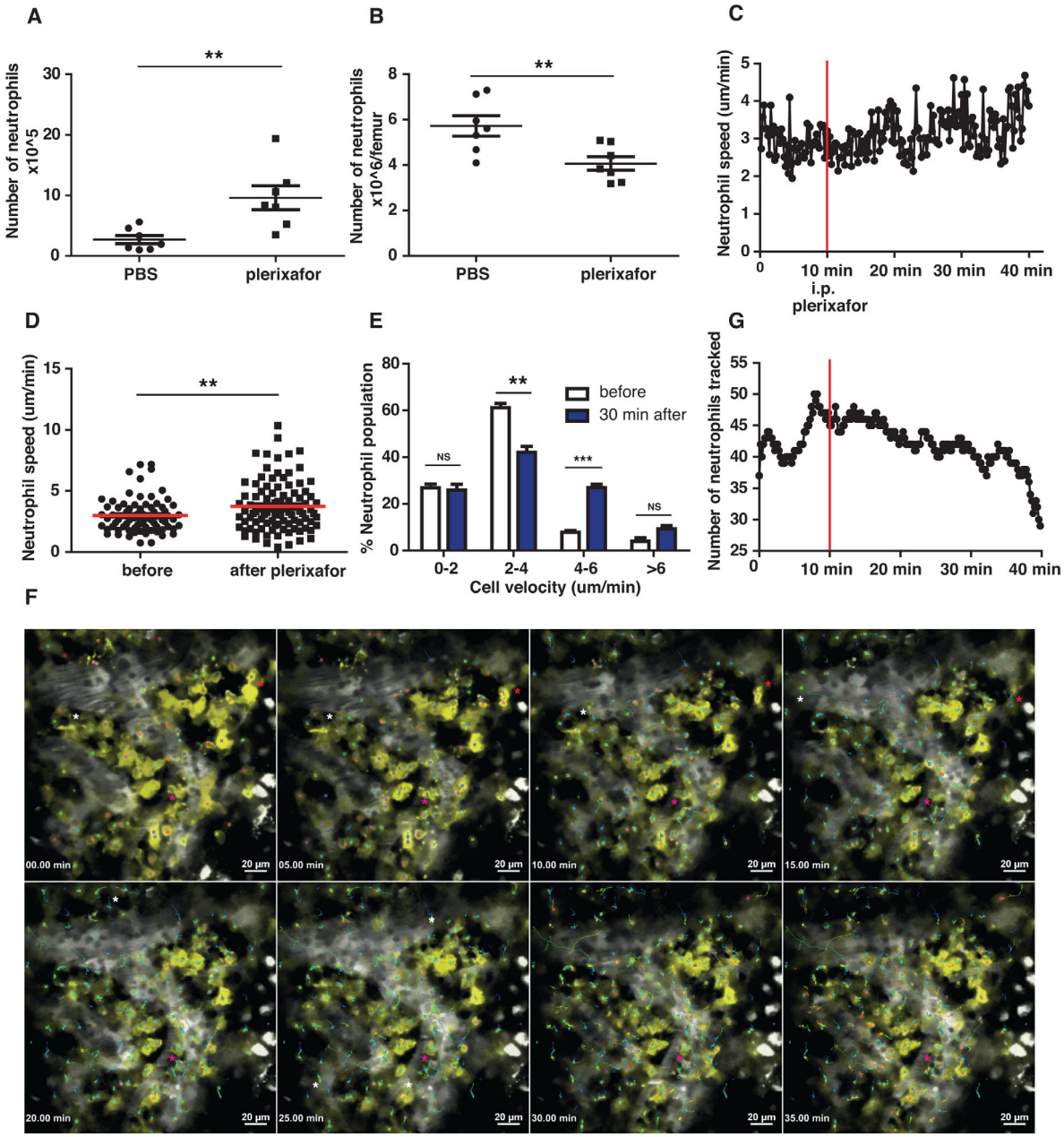

FIGURE 1 Plerixafor treatment causes neutrophil mobilization and blood neutrophilia. (**A**) FACS analysis of total number of circulating neutrophils 60 min after i.p. injection of PBS or plerixafor ($N = 7$). (**B**) FACS analysis of neutrophils/femur 60 min after i.p. injection of PBS or plerixafor ($N = 7$). (**C**) Mean of instantaneous speed of all neutrophils per frame representative of 3 experiments. BM-IVM was imaged for a total of 40 min, 10 min after start, plerixafor was i.p. injected, and BM-IVM was further recorded for 30 min. (**D**) Mean speed of each neutrophil tracked before and after i.p. plerixafor were compared. (**E**) Tracking quantification of neutrophil cell velocity before and after plerixafor treatment, representative of 3 experiments. (**F**) Panel showing the tracking region at different time points during the experiment. Vasculature (white) and neutrophils (yellow) are shown with their respective tracking tails. Panel shows the disappearance of neutrophils marked with white asterisks (*) from the imaged region. (**G**) Quantification of number of neutrophils tracked over a period of 40 min. Data were analyzed by unpaired *t*-test. $^{**}P < 0.01$; NS, not significant

We also analyzed the phenotype of neutrophils mobilized from the BM to the blood, those remaining in the BM and in the spleen after plerixafor treatment. The MFI levels of CD11b, CD62L, CXCR4, CXCR2, and ICAM-1 on neutrophils did not change significantly after plerixafor treatment (Supplementary Fig. 1E–G). Specifically, plerixafor treatment did not increase the expression level of CD11b and decrease expression on CD62L suggesting that circulating neutrophils did not get activated by this drug (Supplementary Fig. 1E). These results suggest that plerixafor treatment causes an increase in the velocity of splenic neutrophils but not neutrophil activation. Of note, CXCR4 levels were significantly higher on neutrophils remaining in the BM 60 min after plerixafor administration (Supplementary Fig. 1F) suggesting that plerixafor does not mobilize these senescent neutrophils. These data suggest that plerixafor did not cause activation of mobilized neutrophils nor change in their phenotype. Together, these data suggest that the spleen functions

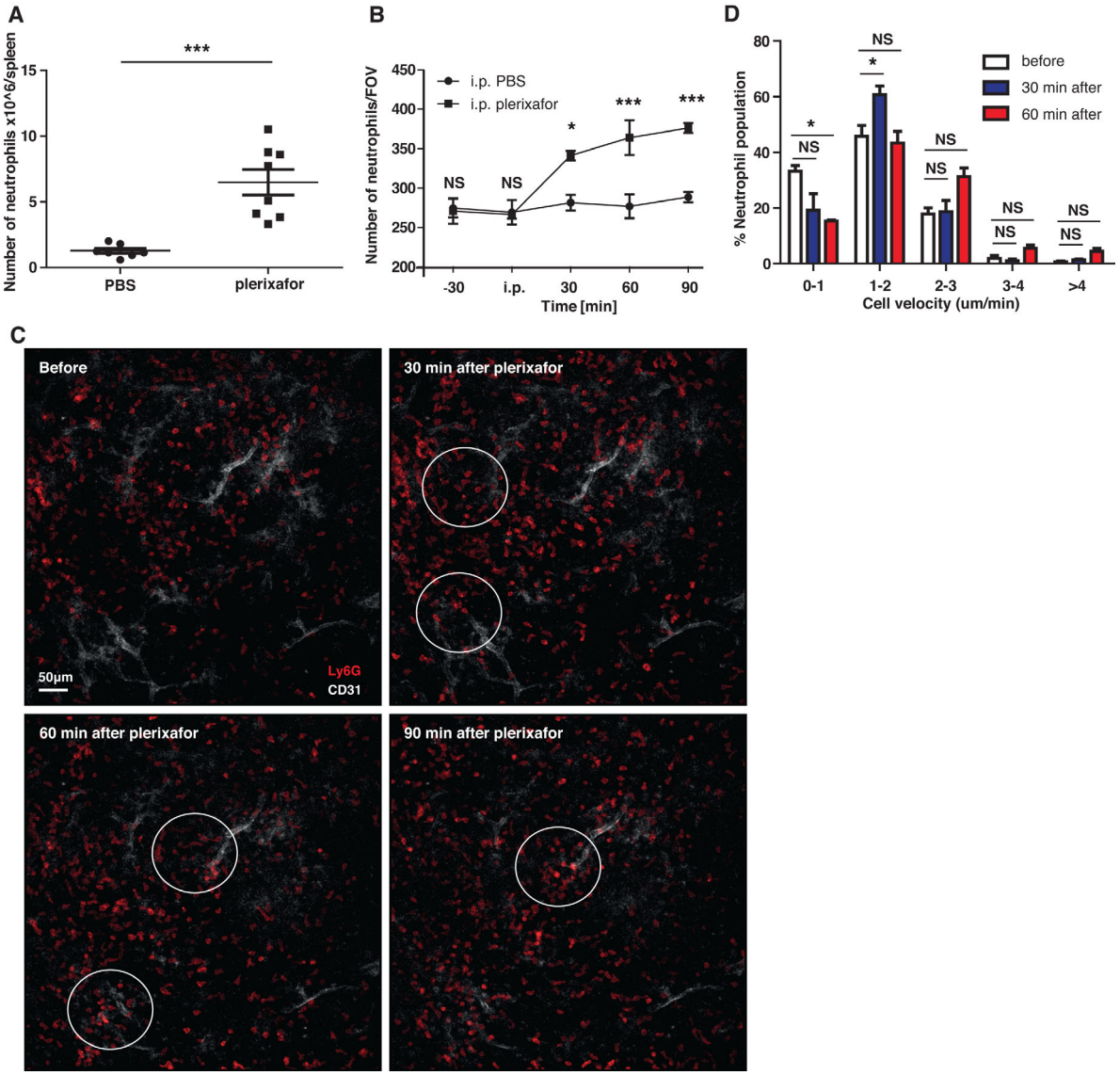

FIGURE 2 **Number of resident neutrophils increases within the spleen after plerixafor treatment.** (**A**) FACS analysis of total number of splenic neutrophils 60 min after i.p. injection of PBS or plerixafor ($N = 7$). Data were analyzed using unpaired *t*-test. (**B**) Neutrophil number over the time-course of spleen IVM were quantified and compared between i.p. plerixafor versus i.p. PBS. Data are presented as mean ± SEM (N = 3). Data were analysed using 2way ANOVA, Sidak's multiple comparison test. (**C**) Representative images of (N = 3) C57Bl/6J mouse spleen IVM time course of before, 30 min, 60 min and 90 min after plerixafor treatment. The vasculature and neutrophils were labelled by i.v. injection of CD31 mAb (white) and Ly6G mAb (red). Plerixafor was injected i.p. during imaging. Some of the neutrophils reaching the spleen are highlighted within the white circles. (**D**) Tracking quantification of neutrophil cell velocity before and 30 min and 60 min after plerixafor treatment, representative of 3 experiments. Data were analyzed using 2-way ANOVA, Tukey's multiple comparison test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant

as a "sink" when neutrophil numbers in the blood are elevated, and that the size of splenic marginated pool in this setting simply reflects circulating neutrophil numbers. These data are consistent with the blood neutrophilia seen in response to plerixafor administration in splenectomised mice.[12]

3.3 | Plerixafor does not cause neutrophil de-margination from the pulmonary vasculature

Devi et al. proposed that s.c. injection of plerixafor in mice and primates caused neutrophil de-margination from the pulmonary vasculature.[11] If this is the case, this may impair lung host defense in patients treated with plerixafor. To address this issue directly, endogenous marginated neutrophils were labeled by i.v. injection of low dose anti-Ly6G-PE mAb and the lung imaged by IVM for 90 min following administration of plerixafor or PBS, or using precision cut lung slices (PCLS). When the numbers of marginated neutrophils were quantified under homeostatic conditions, these two alternative imaging strategies gave comparable numbers (Fig. 3A).

Analysis of marginated neutrophil behavior by IVM showed the ability of these cells to tether, crawl, and adhere to the microvasculature of the lung (Supplementary Video 3) as reported previously.[13,21]

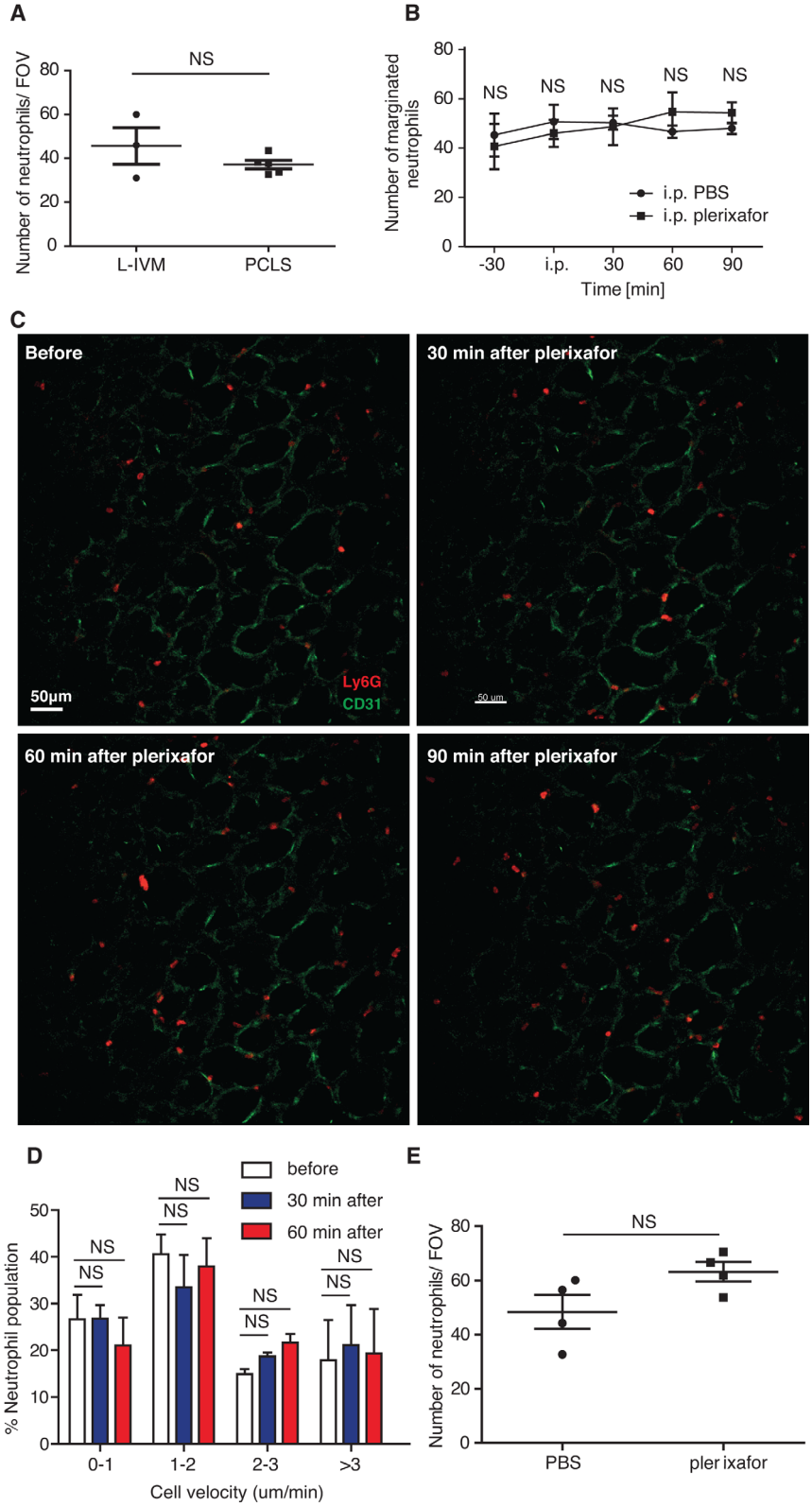

FIGURE 3 Lung marginated neutrophils are insensitive to plerixafor treatment. (**A**) Number of neutrophils were counted in lung-IVM or PCLS of homeostatic mice and expressed as mean per field of view, $N = 3$ for the IVM and $N = 5$ for the PCLS. Data were analyzed using unpaired *t*-test. (**B**) Neutrophil numbers over the time-course of lung IVM were quantified and compared between i.p. plerixafor versus i.p. PBS as control. Data are presented as mean ± SEM. Lung IVM experiment is $N = 3$, and data were analyzed using 2-way ANOVA, Sidak's multiple comparison test. (**C**) Representative images of C57Bl/6J mouse lung IVM time course of before and 30, 60, and 90 min after plerixafor (AMD3100). The vasculature and neutrophils were labelled by i.v. injection of CD31 mAb (green) and Ly6G mAb (red). Plerixafor was injected i.p. during imaging. (**D**) Tracking quantification of neutrophil cell velocity before and 30 and 60 min after plerixafor treatment, representative of 3 experiments. Data were analyzed using 2-way ANOVA, Tukey's multiple comparison test. (**E**) Number of neutrophils were counted in PCLS of PBS or plerixafor treated mice ($N = 4$). Data were analyzed using unpaired *t*-test. $^{*}P < 0.05$; NS, not significant

Critically, data analysis of lung-IVM and PCLS revealed no significant change in the number of intravascular marginated neutrophils up to 90 min following i.p. administration of plerixafor (Fig. 3B and C). Furthermore, plerixafor did not influence the velocity of lung intravascular marginated neutrophils, suggesting that CXCR4 signaling is not involved in homeostatic neutrophil migration within the murine lung (Fig. 3D). By IVM, an increase in circulating neutrophils flowing within and not getting in contact with the lung microvasculature was apparent 20 min following plerixafor treatment consistent with the blood neutrophilia (Supplementary Video 3). PCLS allowed us to image

neutrophils located deeper in the lung, but this again showed that marginated neutrophil numbers were not significantly affected by plerixafor treatment (Fig. 3E). Thus, direct imaging of endogenous marginated neutrophils in the pulmonary vasculature of the mouse indicates that contrary to Devi et al., plerixafor does not cause the de-margination of neutrophils from the lung microvessels nor a change in migratory behavior. However, it is beyond the technical capacity of our current IVM system to phenotypically correlate migratory behaviors. This is an area that clearly warrants further investigation given the current interest in neutrophil subsets.[35,36]

3.4 | Low dose anti-Ly6G mAb does not interfere with neutrophil redistribution after plerixafor treatment

In a model of arthritis, Cunin et al. have shown that Ly6G ligation has no effect on the integrin-independent migration of neutrophils but attenuates integrin-dependent migration.[37] To test whether Ly6G ligation has any impact on neutrophil redistribution following plerixafor treatment in our system, we i.v. injected low dose (3 μg/mouse) anti-Ly6G mAb or IgG2A mAb as a control prior to plerixafor administration. Our data show that the low dose of anti-Ly6G mAb has no effect on neutrophil mobilization from the BM and the increase in circulating and splenic neutrophils after plerixafor treatment (Supplementary Fig. 1H–J). These data suggest that low dose anti-Ly6G mAb, used in this study, does not interfere with neutrophil redistribution after plerixafor treatment. More studies are needed to directly prove whether integrins are involved in this response.

3.5 | Plerixafor does not perturb pulmonary sequestration of primed neutrophils in humans

Undertaking similar experiments in a human setting is essential, however obviously more challenging. Neutrophil priming, occurring either systemically or ex vivo under experimental conditions results in neutrophil retention in the lungs.[38] This process is transient and proposed to be driven by priming/activation-related changes in neutrophil shape and deformability.[14,15] Hence, to investigate whether plerixafor administration interfered with either the initial entrapment of primed neutrophils within the pulmonary circulation, or the subsequent de-priming and release events, we examined the effect of plerixafor on the pulmonary sequestration of autologous radiolabeled neutrophils that had been primed ex vivo with GM-CSF (1 or 100 ng/ml).[14] Neutrophils from healthy volunteers were isolated, dual radio-labeled, and re-infused (Fig. 4). Immediate dynamic planar gamma scintigraphy was undertaken to monitor early neutrophil bio-distribution, with lung, spleen, and liver time-activity curves generated. Volunteers received either plerixafor (0.24 mg/kg s.c.) or placebo (double-blinded) and autologous radiolabeled neutrophils were injected as a single bolus at the T_{max} (60 min) following plerixafor administration. Plerixafor resulted in the expected marked leucocytosis in all subjects at 3 h post-injection. Specifically, neutrophils increased from 4.3×10^9/L ± 0.7 (mean ± SEM) before injection of plerixafor to 9.9×10^9/L ± 1.1 (mean ± SEM), 180 min after injection. Robust and immediate sequestration of radiolabeled neutrophils was seen in the lungs of both groups, with 63.5 ± 4.3% versus 65.6 ± 3.5% (plerixafor vs. placebo; mean ± SEM) of the peak pulmonary signal still present at 40 min (Fig. 4A–C). No difference in the neutrophil signal could be distinguished when quantifying transaxial 45 min SPECT/CT of spleen and liver (Fig. 4B and C) in either saline or plerixafor-dosed subjects. The proportion of cells remaining within the left and right lung compared with peak levels of ^{99m}Tc-neutrophils, again showed no difference between plerixafor and saline treated subjects (Fig. 4D). In addition, the percentage recovery of the injected radiolabeled neutrophils from the peripheral blood at 40 min was identical 6.9 ± 3.7% versus 6.1 ± 1.9% (plerixafor vs. placebo; mean ± SEM; Fig. 4E). Taken together these data show that pulmonary sequestration of primed neutrophils in humans is not affected by plerixafor administration.

4 | DISCUSSION

The CXCR4 antagonist, plerixafor, is used clinically as a single dose to mobilize stem cells for bone marrow transplants. Recently, plerixafor has been trialed in WHIM patients,[17] where it has been shown that chronic administration reverses the blood neutropenia and reduces the high rate of infections in these patients. Preclinical studies in mice also indicate that chronic administration of plerixafor reduces lung fibrosis.[39]

It is important to note that unlike other CXCR4 antagonists, plerixafor binds to the transmembrane region of CXCR4[40] and when the mechanism of action of plerixafor has been investigated it has been shown, unexpectedly, to reverse the gradient of CXCL12 across the bone marrow endothelium.[5,41] Moreover, neutralizing CXCL12 was shown to inhibit plerixafor mobilization of both murine leukocytes and HSPCs.[5,41] Thus plerixafor does not appear to be working as a classical competitive antagonist.

The lung has a marginated pool of neutrophils present under homeostatic conditions that have recently been proposed to function as an important site of intravascular immunity in mice, for example, playing an essential role in bacterial surveillance in the lung.[13,42] Additionally, it has also been proposed that the lung constitutes a unique niche where neutrophil de-priming takes place under inflammatory conditions.[14] The evidence presented by Devi et al. indicate that plerixafor stimulates the demargination of neutrophils from the lung microvasculature, raising concerns that host lung immunity may be compromised in patients administered plerixafor chronically,[11] for example for the treatments of WHIM syndrome or lung fibrosis. In the study, by Devi et al. de-margination of neutrophils from the lung was shown by IVM in mouse lungs, imaging GFP$^+$ neutrophils that had been isolated from the BM of LysM-GFP$^+$ mice and then adoptively transferred into WT recipients and by measuring the difference in neutrophil numbers in the blood collected from the carotid artery and the vena cava of mice and nonhuman primates following plerixafor treatment.

In contrast in this study, IVM of the bone marrow, lung, and spleen of mice was carried out imaging endogenous neutrophils labeled by i.v.

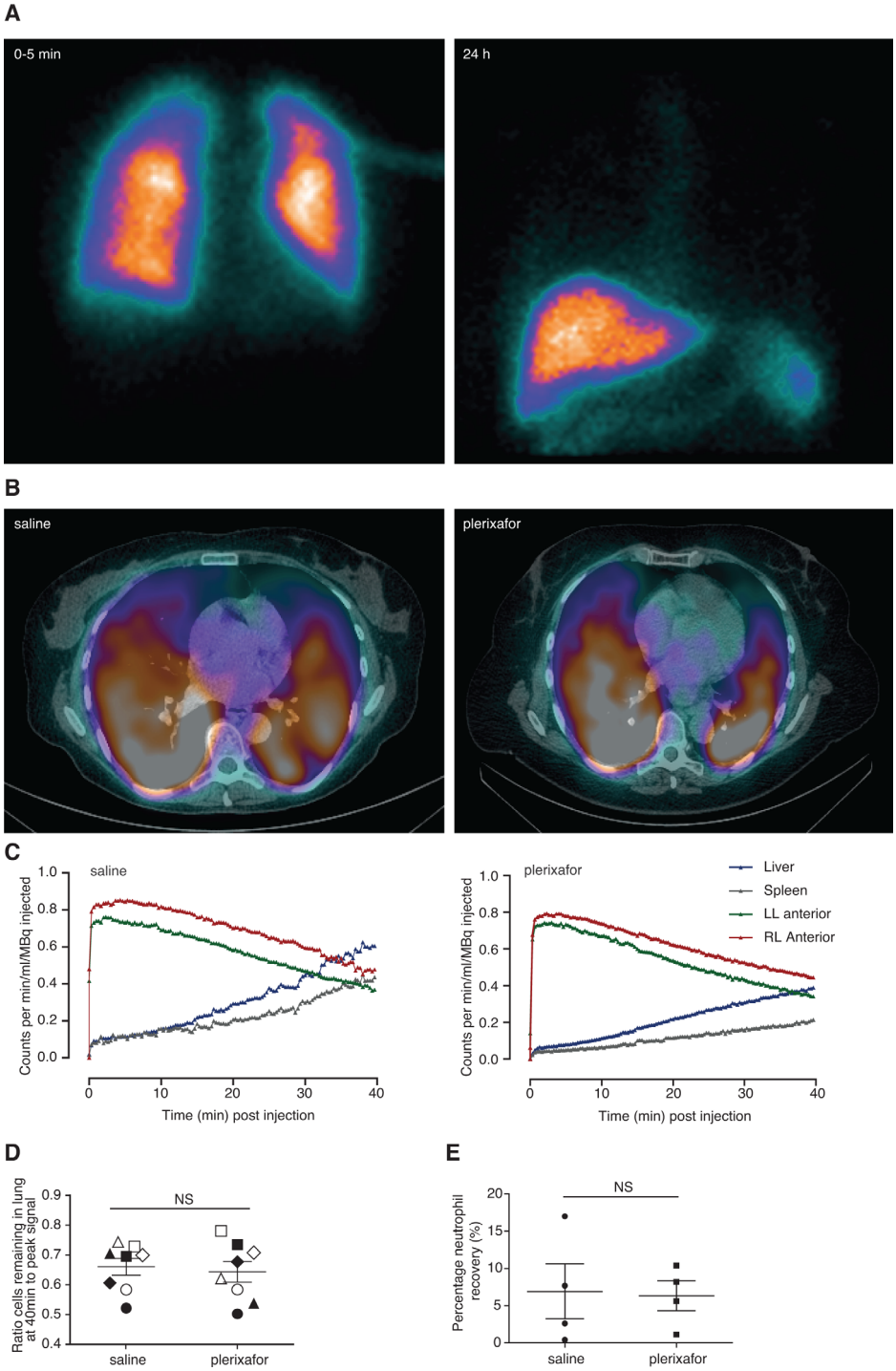

FIGURE 4 **The CXCR4 inhibitor plerixafor does not affect the retention or release of GM-CSF primed neutrophils in the human lung.** (**A**) Anterior 0–5 min reframed image of the dynamic planar gamma scintigraphy (representative of $N = 8$), brighter color signifies a more intense signal. This illustrates the almost complete immediate retention of GM-radiolabeled neutrophils within the lung regions of interest seen following the injection of ex vivo GM-CSF primed neutrophils. For comparison, the anterior 24 h planar image shows labeled neutrophils present in the liver and spleen. (**B**) No difference can be distinguished when viewing transaxial 45 min SPECT/CT in either saline or plerixafor treated subjects. (**C**) Representative organ time-activity graphs post-injection of primed radiolabeled neutrophils in subjects treated with either saline or plerixafor. All data are corrected for radioisotope decay and cross-talk. No difference in the distribution of ^{99m}Tc-neutrophils is detected between spleen, liver, and lung (left or right) in either group. (**D**) Ratio of cells remaining within the left (closed symbols) and right (open symbols) lung compared with peak levels ^{99m}Tc-neutrophils, each individual subject is represented by a different symbol with mean and standard error displayed. No difference was seen between the plerixafor ($N = 4$) and saline ($N = 4$) treated subjects, or between neutrophils pretreated ex vivo with a high (100 ng/ml, $N = 2$) or low (1 ng/ml, $N = 2$) concentration of GM-CSF. (**E**) Percentage of injected radio-labeled cells remaining within the circulation at 45 min post-injection in both saline ($N = 4$) and plerixafor ($N = 4$) treated subjects. NS, not significant

injection of a specific fluorescently labeled mAb. Our BM IVM study shows that plerixafor enhances neutrophil motility within the bone marrow environment and increased numbers of neutrophils that can be seen exiting the BM compartment. In contrast, our data show clearly that plerixafor does not alter lung marginated neutrophil migratory behavior and does not cause de-margination of neutrophils from the lung microvasculature. Thus the data presented here are consistent with our previous work and that of Liu et al., indicating that plerixafor stimulates neutrophil mobilization from the BM into the blood, with no evidence for de-margination of lung neutrophils,[3,12] These results are at odds with those reported by Devi et al. possibly due to the difference in experimental approaches.[11]

Liu reported that plerixafor increased neutrophil numbers in the spleen and they also showed that plerixafor could increase circulating neutrophil numbers in splenectomized mice, indicating that the spleen was not a source of mobilized neutrophils. Our data are consistent with these finding in which IVM of the spleen revealed that plerixafor caused a significant increase in splenic neutrophil velocity, while flow cytometry showed an increase neutrophil numbers in the spleen, suggesting that the spleen may function as a pool for excessive numbers of neutrophils in the blood.

Critically, although it is not possible to recapitulate this exact approach in humans, we have been able to address whether plerixafor affects neutrophil de-priming in human lungs, using nuclear imaging techniques to follow the trafficking of GM-CSF primed neutrophils through the lungs. Our data show that plerixafor does not impact the kinetics of retention or release of primed neutrophils in humans.

This is the first in vivo imaging study to comprehensively assess the effect of plerixafor on neutrophil kinetics in humans and mice. Our IVM data in mice show that plerixafor increases neutrophil motility and mobilization them from the bone marrow, and causes neutrophil accumulation in the spleen, while have no effect on numbers or migratory behavior of marginated intravascular neutrophils in the lung. Further in the human lung plerixafor did not affect the accumulation and release of GM-CSF-primed neutrophils. This study therefore adds to our knowledge of how plerixafor redistributes neutrophils from the BM into the blood with the resulting pooling of excess numbers of neutrophils in the spleen. Importantly while we observed an increase in the number of circulating neutrophils there was no evidence that these leukocytes were activated or primed and no evidence that plerixafor stimulates neutrophil de-margination in the lung.

In conclusion, our results suggest that it is unlikely that plerixafor will compromise respiratory host defense, however further experiments assessing the impact of chronic plerixafor treatment in models of respiratory disease are required to further determine the safety of this drug in the context of its clinical use in WHIM patients[17] and patients with cancer or lung fibrosis.[19]

AUTHORSHIP

K.D.F. and S.R. conceived the mouse project; J.P., C.S., and E.R.C. conceived the human project. L.M.C. established lung imaging, K.D.F. established spleen imaging, and C.L.C. established BM imaging platforms. K.D.F. performed lung and spleen imaging experiments and analyzed the data. C.P. performed BM experiments. G.J. performed flow cytometry analysis. J.P., N.F., N.T., and C.S. performed and analyzed the human gamma scintigraphy experiments. D.C.A. performed BM IVM imaging analysis and generated BM video. J.P., E.R.C., S.R., and K.D.F. wrote the manuscript, which was edited by all authors.

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DISCLOSURE

The authors declare no conflict of interest.

ORCID

Katia De Filippo https://orcid.org/0000-0002-2863-8888

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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