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Crosstalk between CXCR4/Stromal Derived Factor-1 and VLA-4/VCAM-1 Pathways Regulates Neutrophil Retention in the Bone Marrow

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Neutrophil retention in and release from the bone marrow is a critical process that remains incompletely understood. Previous work has implicated the CXCR4/stromal derived factor-1 (SDF-1) chemokine axis in the marrow retention of neutrophils, yet the adhesion pathways responsible for this retention are unknown. Because α4β1 integrin (VLA-4) and its ligand VCAM-1 play a central role in the interactions of hematopoietic stem cells, lymphocytes, and developing neutrophils in the marrow, we investigated whether this integrin might be involved in marrow neutrophil retention and release. In this study, we show that VLA-4 is expressed on murine marrow neutrophils and decreases with maturation, whereas blockade of this integrin leads to the release of marrow neutrophils. Marrow neutrophils adhere via VLA-4 to VCAM-1, which is expressed on marrow endothelium and stroma, and inhibition of VCAM-1 causes release of marrow neutrophils. Furthermore, SDF-1 (CXCL12) signaling through neutrophil CXCR4 augments VLA-4 adhesion to VCAM-1 in vitro, an effect that is blocked by preincubation with pertussis toxin. In vivo blockade of both CXCR4 and α4 causes synergistic release of marrow neutrophils, showing that cross-talk between CXCR4 and VLA-4 modulates marrow retention of these cells. Taken together, these results indicate that the VLA-4/VCAM adhesion pathway is critical in the retention and maturation-controlled release of neutrophils from the marrow, while providing an important link between the CXCR4/SDF-1 signaling axis and the adhesion events that govern this process. The Journal of Immunology, 2009, 182: 604–612.

Homostastic control of circulating neutrophil levels is a critical process, because divergence of blood neutrophil content toward either extremely low or high levels may lead to overwhelming infection or inappropriate inflammatory states, respectively. Although the processes governing neutrophil recruitment to sites of inflammation have been extensively studied, comparatively little is known of the mechanisms responsible for neutrophil retention within the bone marrow and subsequent release into the circulation, particularly in the homostatic state (1). We (2) and others (3, 4) have demonstrated that the CXC cytokine stromal derived factor-1 (SDF-1; CXCL12) and its receptor, CXCR4, function to retain neutrophils in the marrow under normal conditions. Modulation of this chemokinereceptor axis by maturation-driven decrease in neutrophil surface CXCR4 expression and signaling in the marrow promotes orderly release of the retained cells to the periphery (2). This work provides insight into the cytokine pathways controlling the retention and release of marrow neutrophils, yet the corollary role of cell surface adhesion molecules in these processes remains unknown.

Several lines of evidence led us to hypothesize that the α4 integrin VLA-4 (α4β1, CD49d/CD29) might participate in the adhesion events governing homostatic retention and release of bone marrow neutrophils. This integrin has been implicated in the marrow homing and retention of hematopoietic stem cells through its binding of endothelial and stromal cell surface VCAM-1 (CD106) in the marrow (5–10), and both VLA-4 and VCAM appear to be critical for normal lymphopoiesis and myelopoiesis within the marrow (11–14). Furthermore, studies examining G-CSF-mediated hematopoietic stem cell mobilization from the marrow, a process that is accompanied by significant blood neutrophilia, have shown that its effects on the marrow, in part, are mediated by down-regulation of VCAM, presumably interrupting VLA-4 adhesion (15, 16).

Although VLA-4 expression on human blood neutrophils is controversial (17–20), marrow myeloid precursors have been shown to express high levels of VLA-4, which decrease during cell maturation (21–23), a finding that may suggest VLA-4 involvement in subsequent marrow neutrophil release. Yet, understanding of VLA-4 function in marrow neutrophils is limited. Recent studies have suggested a role for α4 integrins in regulating release of neutrophils from the marrow during inflammatory conditions (24). These authors demonstrated failure of MIP-2-mediated mobilization of neutrophils following blockade of CD49d (the α4 subunit of both VLA-4 (α4β1)) and lymphocyte Peyer’s patch adhesion molecule (LPAM) (α4β7) in an isolated perfused rat hind limb model of neutrophil release.

In this study, we show that VLA-4 and its ligand VCAM are crucial in the homostatic retention and release of bone marrow neutrophils. We further demonstrate that signaling through
CXCR4 affects neutrophil release from the bone marrow by modulating VLA-4/VCAM adhesion.

Materials and Methods

Mice

Four to 8-wk-old female C57BL/6 mice were obtained from Harlan and housed in the animal facilities of the University of Vermont College of Medicine. All procedures that involved mice were approved by the University of Vermont Institutional Animal Care and Use Committee.

Reagents

Rat anti-mouse VCAM-1 (M/22; Serotec), CD49d (2B2.32; US Biological), LPAM-1 (DATK32; BD Pharmingen), CXL2L (MEL14; BioSource International), and beta 5 integrin (BD Biosciences) neutralizing Abs, and appropriate isotype control Abs were purchased. CXCR4 neutralizing polyclonal rabbit anti-mouse Abs (727/268b) (2, 25) were the gift of J.-A. Gonzalez (Millennium Pharmaceuticals, Cambridge, MA). All Abs used in the in vivo experiments were azide free. For flow cytometry and fluorescent immunohistology, rat anti-mouse CD16/CD32 (Fc block), FITC-conjugated rat anti-mouse CD49d (clone R1-2), and PE-conjugated rat anti-mouse Gr-1 (Ly-6C/G; RB6C6.8C5) mAbs and isotype control Abs were obtained from BD Pharmingen. Mouse anti-human/mouse SDF-1 mAb (clone 79018) was obtained from R&D Systems. Alexa Fluor 647-conjugated mouse anti-BrdU (PRB-1) and isotype control Abs, as well as Alexa Fluor 488-conjugated goat anti-rat IgG (H+L) were obtained from Invitrogen. Human rVCAM-1 (catalog ADP5) and mouse rCXCL12 (catalog 460-SDCWF) were purchased from R&D Systems.

Determination of neutrophil VLA-4 expression

Marrow and blood neutrophil expression of VLA-4 was determined by flow cytometry, whereas correlation between levels of VLA-4 expression and the maturational state of the marrow myeloid cells was examined using BrdU pulse labeling in vivo, as modified from Basu et al. (26). Briefly, mice were injected with BrdU (50 mg/kg in PBS) by tail vein infusion and 24 h later were euthanized. After whole blood and femoral marrow were harvested. After erythrocyte lysis, cells were fixed in cold 0.25% paraformaldehyde for 35 min, then treated with 2 N HCl plus 0.5% Tween 20 for 30 min at 37°C, neutralized with 0.1 M sodium borate, and washed with PBS/1% Tween 20.

The prepared samples were then Ab blocked before being stained with a mixture of FITC anti-CD49d (1:100), Alex Fluor 647 anti-BrdU (1:250), and PE-conjugated rat anti-Gr-1 (1:50), then washed and resuspended in 0.5% PBS for assay with a LSR II flow cytometer (BD Biosciences). Flow data were then analyzed by FlowJo software (Tree Star). Blood and marrow marrow neutrophils were identified by gating for Gr-1bright cells, whereas less mature forms of the myeloid lineage were examined by gating for all Gr-1+ cells (both Gr-1+ and Gr-1−) when examining marrow neutrophil maturation stages with BrdU. These cell populations were then analyzed for CD49d expression. Results were expressed as both percent positive (using isotype control) and a ratio of the mean fluorescence intensity of cells stained with CD49d mAb vs isotype control Ab.

Preparation of morphologically mature bone marrow neutrophils

To obtain marrow neutrophils from the postmitotic, morphologically mature pool, we used a discontinuous density gradient to separate whole marrow, as previously described (2, 27). These techniques have previously been shown not to cause substantial activation, damage, or apoptosis in the isolated cells (2, 27).

Evaluation of the effects of α4 and VCAM-1 neutralizing Abs on neutrophil marrow retention and release in vivo

Mobility of endogenous and labeled neutrophils from marrow.

The in vivo effects of Ab-mediated CD49d (α4) blockade were initially assessed by examining the response of circulating neutrophil levels in treated mice. C57BL/6 mice were injected with either anti-CD49d mAb or isotype control Abs via tail vein (30 μg in 200 μl of PBS/BSA). After 4 h, 500 μl of peripheral blood was obtained by cardiac puncture under pentobarbital anesthesia and then analyzed using an Advia 120 hematology analyzer with veterinary software (Siemens/Bayer). Similar experiments were performed to evaluate the effects of Ab-mediated CD106 neutralization on marrow neutrophil release. To examine the non-specific effects of Ab binding of neutrophil or marrow cell surface Ags in this model, blocking Abs against CD62L, CD11a, and CD54 were infused in separate similar experiments and compared with isotype control Abs.

Calcium flux assays

To examine whether CD49d-blocking Ab might activate neutrophils, calcium flux assays were performed, as previously described (2). Cells were labeled with the fluorochrome Indo-1/AM (Invitrogen) and calcium mobilization in response to α4-blocking Ab (20 μg/ml) or fMLP (1 μM) was measured by flow cytometry. Results were expressed as the ratio of Indo-1 Violet to Indo-1 Blue against time.

Immunochemistry

Marrow plugs were flushed from mouse femurs with 4% formaldehyde and incubated for 30 min before being washed with PBS, embedded with OCT (Tissue Tek), and snap frozen before sectioning and mounting. For staining, slides were permeabilized with 0.1% Triton X-100 in PBS, blocked with 1% BSA followed by 10% goat serum, and then incubated with rat anti-VCAM-1 (1/50; MK2) for 60 min, followed by Alexa Fluor 488 goat anti-rat IgG.
anti-rat IgG (H + L; 1/400) for 30 min. Slides were then washed, counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), and coverslipped using Aqua PolyMount (Polysciences), before imaging with an Olympus BX50 upright light microscope (Olympus America) with attached Optronics MagnaFire digital camera (Optical Analysis) and associated MagnaFire software (version 2.0).

Marrow for dual VCAM-1/SDF-1 staining was prepared as above, except for the addition of 0.5% cetlypyridinium chloride to the fixation buffer, and the use of mouse on mouse immunodetection technique, as previously described (31) (M.O.M. Kit; Vector Laboratories). Slides were incubated with mouse anti-SDF-1 (1/200) and rat anti-VCAM-1 (1/50) overnight at 4°C, before being washed and then stained with biotinylated anti-mouse IgG reagent for 10 min, followed by streptavidin-Alexa Fluor 555 and Alexa Fluor 488 goat anti-rat IgG (H + L; 1/400) for 30 min. Slides were then washed, counterstained with DAPI, and imaged, as above.

**Neutrophil adhesion assays**

Neutrophil adhesion assays were performed using 111-indium-labeled morphologically mature marrow neutrophils, isolated, and labeled, as described above. Flat-bottom 8-well enzyme immunoassay/RIA strips (Corning Life Sciences) were coated with human rVCAM (at stated concentrations in 0.1 M sodium carbonate (pH 9.0)) overnight in a humidified chamber at 4°C. In experiments using comminibilized SDF-1, wells were first coated with murine rSDF-1 (0.2 μg/ml in carbonate buffer) for 30 min at room temperature. Wells were then washed with carbonate buffer and blocked with PBS/20% FBS for 1 h at 37°C before being washed with running buffer (1X HBSS with 2 mg/ml BSA, 10 mM HEPES, 1 mM CaCl2, and 1 mM MgCl2). A total of 1 × 10⁶ indium-labeled cells in 50 μl of running buffer was added to each well and allowed to adhere for 30 min at 37°C. Wells were then washed several times with running buffer and individually gamma counted. All samples are reported as percentages of total loaded cells by normalizing against separately assayed 50-μl aliquots of labeled cells. In experiments using Ab blockade, labeled cells were preincubated with CD49d neutralizing, LPAM neutralizing, or isotype control Abs (15 μg/ml) at 37°C for 30 min, before plating. In experiments using pertussis toxin, isolated neutrophils were incubated overnight at 37°C in DMEM/10% FBS with or without pertussis toxin (100 ng/ml) before being labeled with 111-indium and assayed (as above).

**Statistical analysis**

Analyses of differences in VLA-4 expression vs maturation state and neutrophil adhesion vs concentration of VCAM were both performed by one-way ANOVA using Prism 5 software (GraphPad). All other comparisons were performed with Student’s t test using Prism 5 software.

**Results**

**VLA-4 expression on murine neutrophils is down-regulated during maturation**

To examine the expression of VLA-4 on murine peripheral blood and morphologically mature, Gr-1-high bone marrow neutrophils, we examined α₄ integrin using flow cytometry. As shown in Fig. 1A, α₄ staining profiles for peripheral blood and bone marrow-derived Gr-1 high neutrophils demonstrate that α₄ is highly expressed in both populations (98–99% of cells). Although α₄ expressed in the context of the α₄β₇ integrin complex (LPAM) has also been found at low levels on certain neutrophil populations (32), using Ab specific for the α₄β₇ heterodimer, we found it to be expressed at very low levels on murine neutrophils (Fig. 1B). Thus, the high levels of α₄ seen on both blood and marrow neutrophils are presumably expressed in the context of the α₄β₁ heterodimer (VLA-4). Blood neutrophils show lower levels of α₄ expression compared with marrow neutrophils (Fig. 1C), suggesting that VLA-4 expression decreases during neutrophil maturation.

To further explore the regulation of VLA-4 expression during marrow neutrophil maturation, we examined marrow from mice pulse labeled with the radiolabel analog BrdU (26). This technique allows for relative determination of cell maturation state in the marrow using the intensity of BrdU nuclear staining, such that at the time point examined (24 h after BrdU pulse) three populations of Gr-1-positive cells are identifiable, as follows: (1) cells that were in the postmitotic compartment of the marrow (metamyelocytes/bands) at the time of pulse and therefore incorporate no BrdU label (BrdU negative, the most mature cell population); (2) cells that were in the last phase of the mitotic compartment (myelocytes) and incorporate BrdU just before ceasing division, and hence, have high levels of BrdU incorporation (BrdU bright, the second most mature cell population); and (3) cells still in the mitotic compartment (myeloblasts/myelocytes) that have undergone multiple divisions and thus diluted their nuclear BrdU content (BrdU dim, the least mature cell population). We find that α₄ expression is highest in the least mature (dim) cells and lowest in the most mature (negative) (Fig. 1D), suggesting that marrow neutrophil VLA-4 expression decreases with maturation.

**Neutralization of VLA-4 results in neutrophil release from marrow**

To determine whether neutrophil retention in the marrow during homeostatic conditions requires VLA-4, neutralizing Ab to CD49d was i.v. infused in mice. Animals injected with anti-CD49d (α₄) Ab demonstrate a marked blood neutrophilia 4 h after injection compared with isotype control (Fig. 2A), suggesting release of marrow neutrophils into the circulation.

We next sought to confirm that the peripheral neutrophilia observed following systemic α₄ blockade reflected accelerated release of postmitotic marrow neutrophils and not demargination of
neutrophils already in the blood granulocyte pool. To examine this possibility, we conducted neutrophil adoptive transfer experiments in which labeled morphologically mature marrow neutrophils were infused into untreated animals and allowed to home to marrow. We have used this technique previously to examine neutrophil kinetics during inflammation and interruption of CXCR4/SDF-1 binding, and it has been found to be both sensitive and specific for marrow release of these cells (2, 27). In the current experiments, after marrow retention was achieved, blocking Ab was infused and the labeled cells released from marrow were quantified 2 h later. Neutralizing Ab infusion (but not isotype control) results in decreased numbers of labeled neutrophils found in bone marrow and increased numbers in the liver, spleen, and lung (Fig. 2B). To determine whether the effect of α4 blockade in this model was specific and not an artifact of Ab-mediated neutrophil activation or opsonization, the effect of α4 Ab binding on isolated neutrophils was examined by calcium flux, whereas the effects of blocking Abs to other neutrophil surface receptors not believed to participate in neutrophil marrow retention (L-selectin and integrin αL; CD62L and CD11a, respectively) were examined in marrow mobilization experiments, as described above. No neutrophil calcium flux was seen in response to α4 Ab binding (Fig. 2F), whereas L-selectin and αL-binding Abs failed to mobilize labeled neutrophils from the marrow (Fig. 2E). Taken together, these results suggest that VLA-4 blockade induces a specific, substantial release of neutrophils from the marrow, with subsequent localization of these cells to other tissues following circulation, as we have seen previously following CXCR4 blockade (2).

To further investigate the importance of VLA-4 in the retention of neutrophils within the marrow, we examined whether α4 blockade might block homing of infused neutrophils to the marrow. Ab directed against α4 was infused before injection of the labeled cells, and the number of labeled neutrophils sequestered in marrow was quantified at 4 h. In this model, α4 blockade attenuates homing

**FIGURE 2.** VLA-4 integrin blockade results in significant neutrophil release from bone marrow. A, Mice were infused with either CD49d (α4) neutralizing or isotype control Abs (30 μg), and blood neutrophil levels were measured 4 h later. B, This effect was further examined using neutrophil adoptive transfer in which 111indium-labeled marrow neutrophils were injected into naive mice and allowed to localize to marrow for 4 h before infusion of anti-CD49d or control Ab. Thirty minutes after Ab infusion, blood and tissue 111indium-neutrophil content was determined by gamma counting. C, The role of α4 in neutrophil homing to marrow was investigated in mice injected with blocking Ab or isotype control 30 min prior to labeled cell infusion. Blood and tissue 111indium-neutrophil content was determined 4 h after cell infusion. D, To determine whether the effects of α4 blockade reflect disruption of α4β1 (VLA-4) or α4β7 (LPAM), neutralizing Ab specific for LPAM was used in similar homing experiments. E, To examine the nonspecific effects of Ab binding of neutrophil cell surface Ags, blocking Abs against CD62L (L-selectin) or CD11a (αL) were infused in separate experiments similar to those reported in B. Marrow levels of labeled neutrophils are shown. Data points are the means of three to five mice/condition (±SEM). Significantly different when compared with control-treated animals: *, p < 0.005; **, p < 0.02. F, To determine whether neutrophil might be activated by binding of the CD49d-blocking Ab, calcium flux was assayed in response to α4-blocking Ab (20 μg/ml), isotype control, or fMLP (1 μM; as a positive control).
of neutrophils to marrow (Fig. 2C), and induces similar retention in liver, spleen, and lung, as seen in the release studies.

As noted previously, α4 may be expressed in the context of two different integrin heterodimers, α4β1 (VLA-4) and α4β7 (LPAM). Although LPAM appears to be expressed at very low levels on murine neutrophils compared with VLA-4, we wished to exclude its participation in the marrow retention of these cells. We therefore tested the effect of a LPAM-specific neutralizing Ab on neutrophil marrow homing and release. As compared with isotype control Ab, there was no effect of LPAM Ab (Fig. 2D). These data suggest that LPAM is not involved in neutrophil interaction with marrow ligands under homeostatic conditions, and that the above demonstrated effects of α4 blockade reflect a critical role for α4β1 (VLA-4) in such interactions.

Marrow neutrophil sensitivity to VLA-4 neutralization increases with cell maturation

To examine the relationship between neutrophil maturation state and sensitivity to VLA-4 neutralization, in vivo BrdU pulse-labeling experiments were conducted (as described above). Animals were pulsed with BrdU and, 48 h later, were then injected with either anti-CD49d Ab or isotype control. Blood and marrow were then examined 4 h after Ab injection. Significant decreases in marrow neutrophil content were seen in anti-CD49d-treated animals relative to control, with the greatest decrease being seen in the most mature neutrophil population (BrdUnegative), followed by less mature population (BrdU dim), with relatively little change seen in immature (BrdU dim) neutrophil content (Fig. 3A). Similarly, significant increases in neutrophil levels were seen in blood following CD49d neutralization, with the largest increases seen in the more mature neutrophil populations (BrdU negative and BrdU bright), indicating release of these cells from the marrow to the blood. This pattern of release suggests that neutrophil susceptibility to VLA-4 blockade increases with maturation, a finding that is consistent with our finding that marrow neutrophil surface levels of VLA-4 decrease with maturation (Fig. 1C), but may also suggest other binding interactions predominate in the retention of myeloid progenitors.

Marrow neutrophils adhere to VCAM in vitro through VLA-4

Given the established role of VCAM as a ligand for VLA-4 (5, 7) (9, 10), we hypothesized that neutrophil adhesion in the marrow might be mediated by VLA-4/VCAM interactions. To initially investigate this hypothesis, we examined marrow neutrophil adhesion to plates coated with varying concentrations of rVCAM. We found high levels of neutrophil adhesion at VCAM concentrations as low as 500 ng/ml, with a saturation point at ~5 μg/ml, and with little nonspecific binding (Fig. 4A). This binding is almost completely reversed by α4 blocking Ab (Fig. 4B), whereas LPAM blockade has no effect on binding, indicating that neutrophil adhesion to VCAM is mediated by VLA-4.

VCAM-1 is expressed widely in murine marrow, and VCAM neutralization induces release of neutrophils from the marrow

To examine the distribution of marrow VCAM expression in adult mice, we performed immunofluorescent staining in fixed femoral marrow plugs. VCAM expression in the marrow is widespread
with strong staining found on both venous sinusoidal endothelium (white arrow), and large interdigitating stromal cells (yellow arrow). Blue staining (DAPI) indicates cell nuclei. B, Secondary control staining. Magnification ×400.

(Fig. 5A), with strong staining found on both venous sinusoidal endothelium (white arrow), and large interdigitating cells (yellow arrow), morphologically consistent with marrow stromal or nurse cells (33). To determine the role of VCAM in the retention of bone marrow neutrophils, we next conducted VCAM-blocking experiments similar to those performed for VLA-4 (above). Infusion of VCAM-neutralizing Ab into mice results in marked blood neutrophilia (Fig. 6A) 4 h after injection compared with isotype control. That this finding represents release of neutrophils from the marrow was confirmed by measuring the effects of VCAM blockade in a neutrophil adoptive transfer model, as above. Ab infusion results in substantial release of cells from marrow, and subsequent localization in liver, spleen, and lung (Fig. 6B). Control experiments using blocking Ab against another marrow-expressed integrin (ICAM-1; CD54) showed no evidence of labeled neutrophil mobilization, suggesting that the effects of VCAM blockade are not due to steric or other nonspecific effects of marrow cell binding (Fig. 6D). Pretreatment of animals with VCAM neutralizing Ab was found to block marrow homing of subsequently infused labeled neutrophils (Fig. 6C) as well, confirming a role for the VLA-4/VCAM adhesion pathway in the marrow retention of neutrophils.

**Neutrophil VLA-4/VCAM adhesion is modulated by SDF-1**

Given our previous findings that neutrophil surface CXCR4 signaling is critical to marrow retention (2), and reports that CXCR4 signaling in hematopoietic stem cells (34) significantly increases surface VLA-4-binding affinity, we hypothesized that a similar process might modulate neutrophil VLA-4 adhesion in the marrow. We first examined the effects of SDF-1 on neutrophil adhesion in vitro using VCAM-coated plates in the presence or absence of coplated SDF-1. SDF-1 significantly augments neutrophil binding to VCAM (Fig. 7A), and this appears to be specific because
SDF-1 does not increase binding to serum-coated (blank) wells. The effects of SDF-1 were attenuated when performing the assay at 4°C (data not shown), and were completely abrogated by pre-incubation of the cells with pertussis toxin (PTX), a compound that inhibits G_i protein-coupled signaling and has been shown to block CXCR4 (35). These findings indicate that the observed SDF-1-driven augmentation of VLA-4/VCAM adhesion is dependent on intracellular signaling.

**FIGURE 7.** Neutrophil VLA-4/VCAM-1 adhesion is modulated by SDF-1/CXCR4 signaling in vitro. A, Neutrophil adhesion to VCAM-coated wells was performed, as described, in the presence or absence of coimmobilized SDF-1. B, Labeled neutrophils were incubated overnight with the G_i protein-coupled signaling inhibitor pertussis toxin (PTX) before VCAM adhesion. Data points are the means of four to five separate experiments (±SEM). *, Significantly different, p < 0.0001.

SDF-1 and VCAM-1 are colocalized in murine marrow, and combined CXCR4/VCAM neutralization is synergistic in causing release of neutrophils from the marrow

Based on previous cell culture studies (36), we hypothesized that SDF-1 and VCAM-1 might be closely localized in the marrow stroma and examined this question using dual immunofluorescent staining of fixed femoral marrow. SDF-1 and VCAM-1 are found to be widely expressed in the marrow. Although rare cells show single expression of either SDF-1 (Fig. 8A, white arrows) or VCAM-1 (Fig. 8B, light blue arrows), expression of both is predominantly found to colocalize in the marrow (Fig. 8C, yellow arrows), particularly on the surface of what morphologically appear to be marrow stromal cells. Given this finding and the apparent modulation of VLA-4/VCAM adhesion by CXCR4 simulation (Fig. 7), we examined whether the mobilizing effects of VCAM-1 neutralization and CXCR4 signaling inhibition might interact in vivo. We performed neutrophil adoptive transfer experiments, as detailed above, in which we then administered very low doses of both CXCR4- and VCAM-1-blocking Abs (that by themselves had no mobilization effect) simultaneously. Marrow neutrophil release was determined 2 h after infusion. Under these circumstances, labeled neutrophil release was greatly augmented compared with either low-dose CXCR4 or VCAM-1 Ab infusion alone (Fig. 9).

**FIGURE 8.** SDF-1 is closely colocalized with VCAM-1 on the surface of bone marrow stromal cells. Dual fluorescent immunohistology for SDF-1 and VCAM-1 was performed on fixed whole bone marrow plugs from mouse femurs. Samples were imaged by fluorescent microscopy for each fluorophore individually and together. A, SDF-1. Red fluorescence denotes SDF-1 staining (white arrowheads indicate cells staining solely for SDF-1). B, VCAM-1. Green fluorescence indicates VCAM-1 staining (whereas light blue arrowheads indicate cells staining solely for VCAM-1). C, Merged image. Yellow fluorescence indicates large areas of colocalization between SDF-1 and VCAM; yellow arrowheads indicate examples of colocalization. D, Dual secondary control staining. Blue staining (DAPI) indicates cell nuclei. Magnification ×400.

**FIGURE 9.** Neutralization of CXCR4 and VCAM-1 is synergistic in vivo. Cross-talk between CXCR4 and VLA-4 was examined in vivo. Mice were treated with low doses of either VCAM-blocking or CXCR4-blocking Abs, both Abs simultaneously, or isotype control 4 h after labeled marrow neutrophils were sequestered to the marrow, as described. Subsequent marrow content of labeled neutrophils was assayed 2 h after Ab infusion. Results are expressed as percentage of decrease compared with control-treated animals. Data points are the means of five separate experiments (±SEM). *, Significantly different when compared with control-treated animals, p < 0.01; †, significantly different when compared with low-dose VCAM-blocking Ab or low-dose CXCR4-blocking Ab alone, p < 0.05.

Thus, these results suggest a synergistic effect of such blockade, and further support the role of cross-talk between neutrophil CXCR4 and VLA-4 in the regulation of marrow retention.
Discussion

In the present study, we demonstrate a role for integrin $\alpha_4\beta_1$ (VLA-4) and its ligand VCAM-1 in the homeostatic control of neutrophil release from the bone marrow. We further implicate cross-talk between neutrophil surface CXCR4 and VLA-4 in the modulation of VCAM binding, suggesting a direct link between the SDF-1/CXCR4 chemokine axis and the adhesion events retaining neutrophils in the marrow.

Although previous work has shown VLA-4 to be critical in the marrow retention of hematopoietic stem cells and normal hematoepoietic development (5, 6, 9–14), its role in subsequent marrow neutrophil retention and release has not been investigated. Although neutrophil expression of VLA-4 has been debated (17–20), neutrophil retention and release has not been investigated. Although neutrophil expression of VLA-4 has been debated (17–20), particularly regarding mature neutrophils in the circulation, our data suggest that this integrin is expressed at high levels during murine neutrophil development and declines with maturation, a pattern similar to that described with human neutrophil development (21–23). The finding that VLA-4 expression is progressively lost during neutrophil maturation suggests a role for this integrin in the choreography of neutrophil release from the marrow.

In this study, we show that $\alpha_4$ integrin-mediated adhesion events are critical to the retention of neutrophils within the bone marrow. Although $\alpha_4$ may exist in two different heterodimers on the surface of neutrophils ($\alpha_4\beta_1$/VLA-4 and $\alpha_4\beta_2$/LPMAM) (32), we find very low levels of LPAM expression on marrow neutrophils, and no effect of specific blockade of this integrin on neutrophil release from the marrow. We therefore believe the effects of $\alpha_4$ blockade are specifically attributable to interruption of VLA-4 adhesion. This finding, in conjunction with our evidence that marrow neutrophil maturation is associated with increasing sensitivity to $\alpha_4$ blockade, is consistent with the hypothesis that maturing neutrophils are retained within the marrow under the influence of VLA-4, and that such retention wanes with cell maturation-associated down-regulation of VLA-4, leading to controlled release into the circulation.

The best-described ligands for VLA-4 are VCAM and fibronectin. Previous studies using radioautography of whole marrow (8) have suggested that VCAM is expressed widely in the marrow, and more recent work in newborn mice has shown that the advent of stromal and endothelial VCAM expression coincides with the initiation of granulopoiesis in the marrow (14). Based on these studies and our own work suggesting that postmitotic neutrophils in the marrow are located in close proximity to the venous sinusoids and the dendritic processes of stromal cells (27), we hypothesized that VCAM might be a critical target for neutrophil adhesion. We show in this study that VCAM is strongly expressed on both stromal cells and the venous endothelium in murine marrow, and that neutrophil interaction with both marrow stroma and endothelium is most likely mediated by the VLA-4/VCAM pathway. It is important, however, to recognize that our studies do not rule out a concomitant role for fibronectin in this process, as has previously been suggested in the marrow retention of hematopoietic progenitors (14).

VLA-4 exists in low- and high-avidity states (37), and this is regulated in part by inside-out signaling, in which cytokine receptor activation leads to changes in the binding affinity state of the integrin, modulating cell adhesion (38). One such cytokine receptor is CXCR4, which when activated by the binding of SDF-1 has been shown to significantly increase VCAM binding by VLA-4 in hematopoietic stem cells, lymphocytes, and tumor cell lines (34, 39, 40). This process appears to be dependent on close coexpression of SDF-1 and VCAM on cell surfaces (34), a relationship that has been shown to exist on cultured bone marrow stromal cells (36), and is further suggested in vivo in our present study. As a continuation of our previous work implicating CXCR4 signaling in the retention of marrow neutrophils (2), we show in this study that the neutrophil VLA-4/VCAM adhesion pathway is significantly augmented by SDF-1. This alteration in adhesion appears to require $G_{\beta\gamma}$ protein-coupled intracellular signaling, consistent with a dependence on CXCR4 signaling in this process (35). The importance of this interaction in vivo is suggested by the synergistic effects of very low doses of neutralizing Abs against both CXCR4 and VCAM in the mobilization of labeled marrow neutrophils. Thus, the retention and release of marrow neutrophils appear to be governed by both SDF-1/CXCR4 signaling and VLA-4/VCAM adhesion events in the marrow, and these processes may be, to a degree, interdependent.

Interestingly, recent studies have suggested that the neutrophilic growth factor G-CSF may act to drive the release of the marrow neutrophils, in part, through down-regulation of marrow SDF-1 (41, 42), whereas G-CSF modulation of the combined axis (SDF-1/CXCR4 and VLA-4/VCAM) has been implicated in the release of hematopoietic stem cells (15, 43, 44). In total, this suggests the possibility that maturation-related loss of both VLA-4 and CXCR4 may govern the orderly release of neutrophils during the homeostatic setting, whereas more profound alterations to this complex axis may lead to massive release, as in the case of systemic inflammation.

In summary, we find an important role for VLA-4 and VCAM, in the homeostatic retention and release of neutrophils from the bone marrow. This adhesion interaction appears to be modulated by cross-talk from neutrophil surface CXCR4, and may account, at least in part, for our previous findings that marrow neutrophil retention is dependent on the SDF-1/CXCR4 axis. The orderly release of mature cells from the marrow then may reflect a programmed down-regulation of both neutrophil CXCR4 and VLA-4, whereas the massive release of often less mature neutrophils that accompanies systemic inflammatory states may reflect direct interruption of these interactions under the influence of mediators such as G-CSF. Further work will be required to delineate these possibilities.

Disclosures

The authors have no financial conflict of interest.

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