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Chemokines Acting via CXCR2 and CXCR4 Control the Release of Neutrophils from the Bone Marrow and Their Return following Senescence

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Summary

In this study we provide evidence that the SDF-1α/CXCR4 chemokine axis is involved in both the retention of neutrophils within the bone marrow and the homing of senescent neutrophils back to the bone marrow. We show that the functional responses of freshly isolated human and murine neutrophils to CXCR2 chemokines are significantly attenuated by SDF-1α, acting via CXCR4. As a consequence, the mobilization of neutrophils from the bone marrow in vivo by the CXCR2-chemokine, KC, was dramatically enhanced by blocking the effects of endogenous SDF-1α using a specific CXCR4 antagonist. As neutrophils age, they upregulate expression of CXCR4 and acquire the ability to migrate toward SDF-1α. We show here that these senescent CXCR4^{high} neutrophils preferentially home to the bone marrow in vivo in a CXCR4-dependent manner, suggesting a previously undefined mechanism for the clearance of senescent neutrophils from the circulation.

Introduction

SDF-1α/CXCL12 acts solely on one receptor CXCR4 that is expressed by CD34$^+$ progenitors, megakaryocytes, platelets, B cells, T cells, and mononuclear phagocytes (Bleul et al., 1996, 1997; Hori et al., 1998; Forster et al., 1998; Mohle et al., 1998; Kowalska et al., 1999). It has previously been reported that neutrophils express CXCR4 mRNA (Patel et al., 2001; Loetscher et al., 1998; Gonzalo et al., 2000). However, while some studies report surface expression of CXCR4 on neutrophils (Forster et al., 1998; Bruhl et al., 2001), others report its absence (Bleul et al., 1996; Nagase et al., 2002; Hori et al.,1998). In all of these studies, where chemotaxis of neutrophils toward SDF-1α was assessed, no response was observed. It has been reported that the expression of CXCR4 on neutrophils is dramatically upregulated as they age in culture (Nagase et al., 2002) and that senescent neutrophils acquire the capacity to migrate toward SDF-1α. However, the patho/physiological significance of this phenomenon is not known.

The bone marrow contains a large reserve of mature neutrophils and their rate of release from this tissue is a major determinant of the number of circulating neutrophils. Mature neutrophils reside within the hematopoietic compartment of the bone marrow and are intimately associated with stromal cells and/or components of the extracellular matrix. In response to inflammatory stimuli, circulating neutrophil numbers increase due to an acute release of neutrophils from the bone marrow reserve. There are many factors generated during an inflammatory reaction, e.g., C5a, LTB4, TNF-α, and CXC chemokines, that can stimulate the rapid mobilization of neutrophils from the bone marrow (Jagels and Hugli, 1992; Terashima et al., 1998). Indeed the intravenous injection of CXC chemokines promotes the rapid mobilization of neutrophils from the bone marrow reserve. We propose that the gradient of chemokine created across the sinus endothelium stimulates chemotaxis of neutrophils present in the hematopoietic compartment and induces their migration across the bone marrow sinusoidal endothelium, such that they can be liberated into the systemic circulation. In this study we tested the hypothesis that, in addition to factors such as chemokines, that are known to directly stimulate release (Jagels and Hugli, 1992; Terashima et al., 1998), there may also be mechanisms promoting the retention of neutrophils within the bone marrow, and it is the balance between these two opposing signals that determines the rate of neutrophil release from the bone marrow during an inflammatory episode. In particular we have examined the possibility that the chemokine SDF-1α, which is expressed constitutively in the bone marrow, acts as a retention factor for neutrophils in this tissue.

It is estimated that in humans 10^{11} neutrophils are released from the bone marrow per day. As these neutrophils have a short half-life ($t_{1/2} = 6$ hr) in the circulation, homeostatic mechanisms are essential to ensure their efficient clearance from the blood. It is thought that senescent neutrophils are cleared from the blood by the liver, spleen, and bone marrow, but the molecular mechanisms regulating this clearance have not been established (Thakur et al., 1977, Saverymuttu et al., 1985; Lovas et al., 1996; Suratt et al., 2001). At inflammatory sites as neutrophils age they become apoptotic, thereby changing their phenotype such that they are recognized and phagocytosed by tissue macrophages (Haslett, 1999). It is not known whether circulating neutrophils must also exhibit an apoptotic phenotype to promote their clearance, or whether there are other distinct mechanisms for the clearance of senescent neutrophils from the circulation. We hypothesized that as neutrophils age, upregulation of CXCR4 promotes the trafficking of se-

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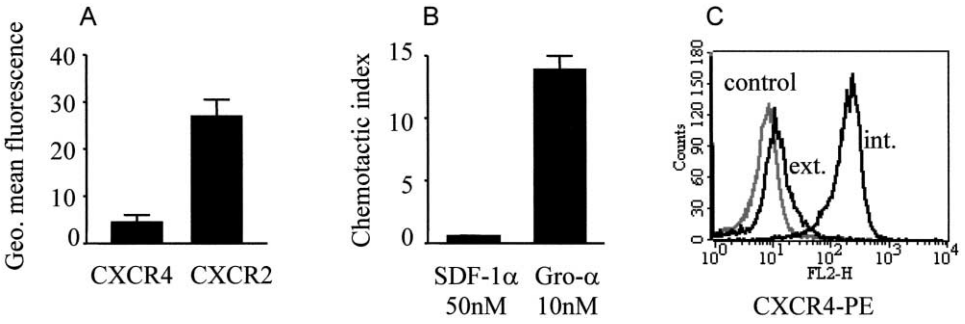

Figure 1. Neutrophil CXCR4 and CXCR2 Cell Surface and Intracellular Expression and Chemotactic Responses to SDF-1α and Gro-α

(A) Surface expression of CXCR4 and CXCR2 on freshly isolated human blood neutrophils measured by flow cytometry; mean ± SEM (n = 6 separate experiments performed in triplicate).

(B) Migration of freshly isolated human blood neutrophils to SDF-1α (50 nM) and Gro-α (10 nM) measured using a Transwell chemotaxis assay; mean ± SEM (n = 6 separate experiments performed in triplicate).

(C) Cell surface (ext.) and intracellular staining (int.) of CXCR4 on freshly isolated human blood neutrophils measured by flow cytometry; cont., isotype matched control mAb.

nescent/preapoptotic CXCR4^{high} neutrophils back to the bone marrow. We propose, therefore, that the homing of senescent neutrophils back to the bone marrow, in a CXCR4-dependent manner, may represent a novel pathway for the clearance of senescent neutrophils from the circulation.

In this study we have investigated the potential role of endogenous SDF-1α to act as a retention factor for mature neutrophils in the bone marrow. Furthermore, we have investigated the hypothesis that upregulation of CXCR4 on neutrophils as they age promotes the trafficking of senescent neutrophils back to the bone marrow. These data reveal a dynamic relationship between the chemokine receptors CXCR4 and CXCR2 and their ligands in determining both the release and clearance of neutrophils by the bone marrow.

Results

Freshly Isolated Human Blood Neutrophils Express Low Levels of CXCR4 and Do Not Migrate toward SDF-1α

Flow cytometry staining was performed to directly assess cell surface and intracellular levels of CXCR4 and CXCR2 on human blood neutrophils. Freshly isolated neutrophils were shown to express very low levels of CXCR4 on their cell surface while intracellular levels of CXCR4 were high (Figures 1A and 1C). In contrast, freshly isolated neutrophils expressed high cell surface levels of CXCR2 (Figure 1A). Examination of neutrophils stained with mAbs to CXCR4 by confocal microscopy, confirmed the flow cytometry data (data not shown). To determine whether neutrophils responded functionally via these receptors, chemotaxis assays were carried out. As shown in Figure 1B, freshly isolated neutrophils migrated in response to Gro-α but not to SDF-1α at 50 nM (Figure 1B) or over a concentration range 1–100 nM (data not shown).

SDF-1α Inhibits Responses of Human Neutrophils to CXCR2 Agonists, but Not CXCR1 Agonists, In Vitro

To determine the functional significance of CXCR4 expression on freshly isolated human neutrophils, a shape change assay was performed (GAFS assay, Sabroe et al., 1999). A robust concentration-dependent shape change of neutrophils was observed in response to both IL-8 and Gro-α, as has previously been reported (Figures 2A and 2B; Sabroe et al., 1999). In contrast to these chemokines, SDF-1α stimulated only a partial shape change. However, as shown in Figures 2A, 2C, 2D, and 2E the response of neutrophils to Gro-α, ENA-78, and NAP-2 was significantly reduced by their coincubation with SDF-1α. The inhibitory effect of SDF-1α on responses to Gro-α, ENA-78, and NAP-2 was completely abrogated by both the CXCR4 antagonist, AMD-3100 (Hatse et al. 2002), and a specific CXCR4 blocking mAb (Figures 2A, 2C, 2D, and 2E). In contrast, neutrophil responses to IL-8 were unaffected by SDF-1α coincubation (Figures 2B and 2F). Neutrophil shape change in response to Gro-α, ENA-78, and NAP-2 was markedly reduced by pretreatment with the CXCR2 antagonist SB225002, while the response to IL-8 was unaffected by this antagonist (Figures 2A–2F). Taken together, these results suggest that neutrophil responses to CXCR2 agonists, but not CXCR1 agonists, can be significantly attenuated by SDF-1α acting via CXCR4.

To determine whether SDF-1α downregulates surface expression of CXCR2, neutrophils were incubated with SDF-1α for 0–60 min at 37°C and levels of CXCR2 cell surface expression subsequently assessed by flow cytometry. As shown in Figure 2G, expression of CXCR2 on the surface of neutrophils was unaffected by their incubation with SDF-1α.

SDF-1α Suppresses Chemotaxis of Neutrophils in Response to Chemokines Acting via CXCR2

We next examined whether SDF-1α could also affect neutrophil chemotaxis toward chemokines acting via CXCR2. As a prelude to in vivo experiments, murine bone marrow neutrophils (which have CXCR2, but lack CXCR1) were used for these experiments. As shown in Figure 3A there was a very low level of CXCR4 expression on both murine blood and bone marrow neutrophils, comparable to the levels of expression observed on human blood neutrophils (Figure 1A). For the chemotaxis assays, a mixed population of murine bone marrow

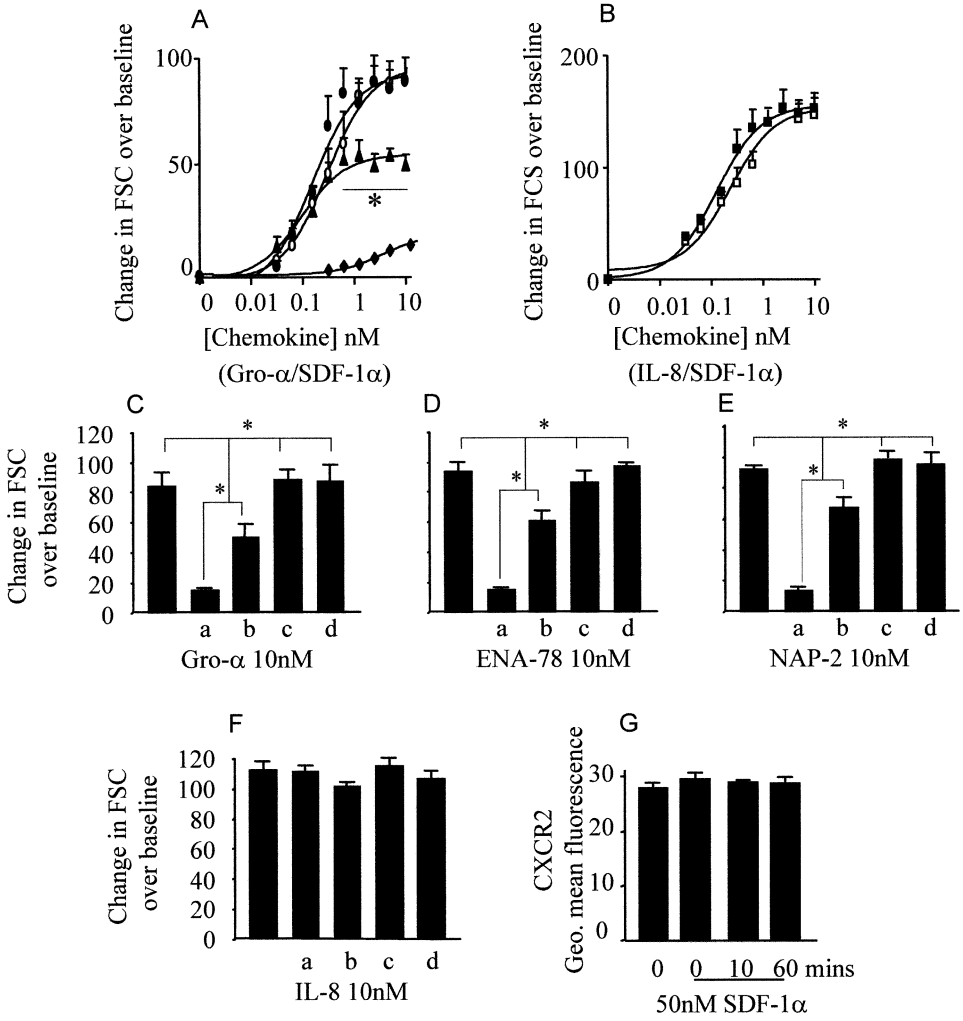

Figure 2. Neutrophil Shape Change Responses to the Chemokines Gro-α, ENA-78, NAP-2, IL-8, and SDF-1α

(A) Gro-α (closed circles), SDF-1α (closed diamonds), 25 nM SDF-1α + Gro-α (closed triangles), AMD-3100 (5 μg/ml) + 25 nM SDF-1a + Gro-α (open circles). Each point represents the mean ± SEM of n = 3 independent experiments. *, $P < 0.05$ (Kruskall Wallis H test) represents a significant difference between the groups (Gro-α alone or Gro-α + SDF-1α + AMD-3100) and the group (Gro-α + SDF-1α).

(B) IL-8 (open squares), 25 nM SDF-1α + IL-8 (closed squares).Each point represents the mean ± SEM of n = 3 independent experiments.

(C, D, E, and F) Neutrophil shape change in response to Gro-α, ENA-78, and NAP-2 and IL-8 (all at 10 nM) a, + 300 nM SB225002; b, + 25 nM SDF-1α; c, + 25 nM SDF-1α + 5 μg/ml AMD-3100; d, + 25 nM SDF-1a + 5 μg/ml anti-CXCR4 pAb. Each point represents the mean ± SEM of n = 3–5 independent experiments. *, $P < 0.05$ (Kruskall Wallis H test).

(G) Freshly isolated human blood neutrophils were incubated for 0–60 min with SDF-1α (50 nM). Surface expression of CXCR2 was then determined by flow cytometry. Results are expressed as mean ± SEM of n = 6 separate experiments performed in triplicate.

leukocytes was placed in the upper chamber of Transwell filters in the presence or absence of SDF-1α. The murine homolog of Gro-α, KC, was placed in the lower chamber. As shown in Figure 3B, the chemotaxis of neutrophils toward KC was significantly inhibited (59%) by the presence of SDF-1α in the upper well. This inhibition was reversed by pretreatment of the neutrophils with AMD-3100, demonstrating that the inhibitory effect of SDF-1α was mediated via CXCR4.

When similar chemotaxis assays were performed with human neutrophils, we observed a significant inhibition (24%) of chemotaxis to Gro-α, with no effect on neutrophil chemotaxis to IL-8 (results not shown).

Taken together, these results show that neutrophil migration to CXCR2 ligands are attenuated by SDF-1α acting via CXCR4.

The CXCR2 Agonist, KC, and the CXCR4 Antagonist, AMD-3100, Act Cooperatively to Stimulate a Blood Neutrophilia

We hypothesized that endogenous SDF-1α, present in the bone marrow, might attenuate the release of neutrophils from the bone marrow in response to systemically acting CXCR2 chemokines. In order to investigate this possibility, mice were given a single i.v. injection of, PBS, SDF-1α, KC, AMD-3100, or KC plus AMD-3100. After 60 min, the mice were killed and the number of blood and bone marrow neutrophils were determined as shown in Figure 4. Our results showed that KC and AMD-3100 when injected alone stimulated a 5- and 3-fold increase in circulating numbers of neutrophils, respectively, while coinjection of KC together with AMD-3100 resulted in a 15-fold increase in circulating neutro-

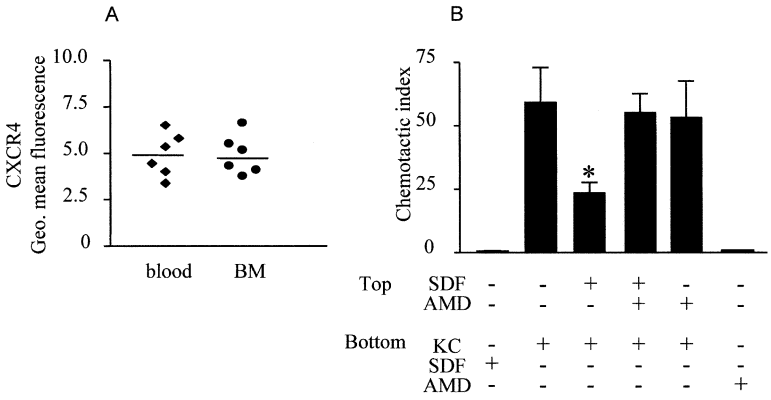

Figure 3. Murine Neutrophils Express Low Levels of CXCR4, but SDF-1α Inhibits KC Stimulated Neutrophil Chemotaxis

(A) Surface expression of CXCR4 on murine blood and bone marrow neutrophils measured by flow cytometry; n = 6 mice, bars express the median.

(B) Neutrophil chemotaxis assay. Murine bone marrow leukocytes were added to the upper chamber of the Transwell filters in the presence or absence of SDF-1α (10 nM), AMD-3100 (5 μg/ml), or both reagents. KC (50 nM), SDF-1α (10 nM), or AMD-3100 (5 μg/ml) was added to the lower chamber as indicated. Migration of neutrophils is expressed as the chemotactic index, mean ± SEM (n = 3–5 separate experiments performed in triplicate). *, $P < 0.05$ (Kruskall Wallis H test).

phil numbers (Figure 4A). The blood neutrophilia observed in response to KC, AMD-3100, and KC plus AMD-3100 was mirrored by a reduction in bone marrow neutrophil numbers (Figure 4B), suggesting that the increase in blood neutrophils was due to their release from the bone marrow. Administration of a single dose of SDF-1α intravenously did not stimulate the acute mobilization of neutrophils from the bone marrow.

Direct Evidence that the CXCR2 Agonist, KC, and the CXCR4 Antagonist, AMD-3100, Act Cooperatively to Mobilize Neutrophils from the Bone Marrow

To determine directly whether the increase in circulating neutrophils induced by the intravenous injections of KC, AMD-3100, or KC plus AMD-3100 is due to neutrophil release from the bone marrow, we set up an in situ perfusion system of the mouse femoral bone marrow. In this system, the femoral artery and vein are cannulated in situ such that the femoral bone marrow can be perfused in isolation and the leukocytes released from the bone marrow collected as they exit via the femoral vein. KC, AMD-3100, or KC and AMD-3100 were infused for 10 min directly into the mouse femoral artery and the leukocytes mobilized from the femoral bone marrow over a perfusion period of 60 min were collected. The number of neutrophils and B cells released into the perfusate were determined by Flow cytometry. B cells were quantified to determine whether the observed effects were cell-type specific. As shown in Figure 5A, there was a basal release of neutrophils (PBS, 1.35 ± 0.3 × 10^3/ ml perfusate) from the bone marrow over the 60 min perfusion period that was significantly enhanced by infusion of either KC or AMD-3100 alone (8.2 ± 1.4 × 10^3/ml perfusate, 8.6 ± 1.7 × 10^3/ml perfusate, respectively) while infusion of the two factors together significantly augmented neutrophil mobilization (23.4 ± 2.1 × 10^3/ ml perfusate).

In contrast, while AMD-3100 also mobilized B cells from the bone marrow KC alone did not mobilize B cells significantly. Furthermore, KC did not enhance B cell release stimulated by AMD-3100 (Figure 5B). Finally, infusion of SDF-1α (50 nM) did not stimulate the acute release of either B cells or neutrophils from the bone marrow (Figures 5A and 5B).

Collectively, these data support the hypothesis that endogenous SDF-1α plays a role in the retention of neutrophils in the bone marrow. Hence, neutrophil release from the bone marrow in response to KC is markedly increased by blocking CXCR4 with AMD-3100.

CXCR4 Is Upregulated on Murine Bone Marrow Neutrophils as They Age

As shown in Figure 6A, cell surface expression of CXCR4 was dramatically upregulated on murine bone marrow neutrophils after 6 and 20 hr in culture. This increase in expression was apparent on the total neutrophil population. These changes in levels of receptor expression were reflected by changes in functional responsiveness, as the ability of 6 and 20 hr cultured neutrophils to migrate toward SDF-1α was significantly increased as compared to freshly isolated bone marrow neutrophils (Figure 6B).

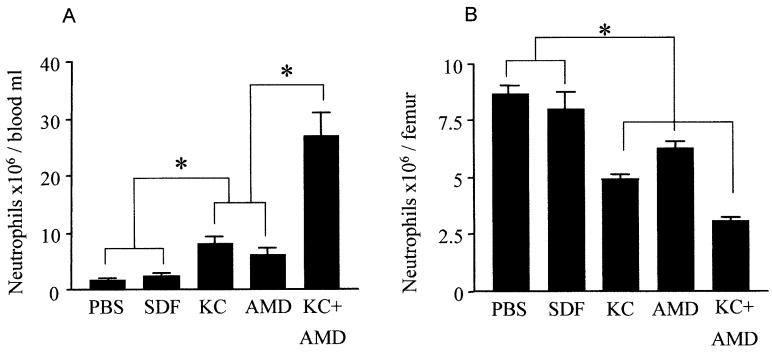

Figure 4. In Vivo Neutrophil Mobilization in Mice following the Intravenous Injection of KC and AMD-3100 Alone or In Combination

BALB/c mice were injected with either PBS, SDF-1α (40 mg/kg), KC (40 mg/kg), AMD-3100 (5 mg/kg), or AMD-3100 plus KC. The number of circulating neutrophils per ml of blood (A), and the number of bone marrow neutrophils per femur (B), 60 min following the i.v. injections. Results are expressed as mean ± SEM. (n = 6). *, $P < 0.05$ (Kruskall Wallis H test).

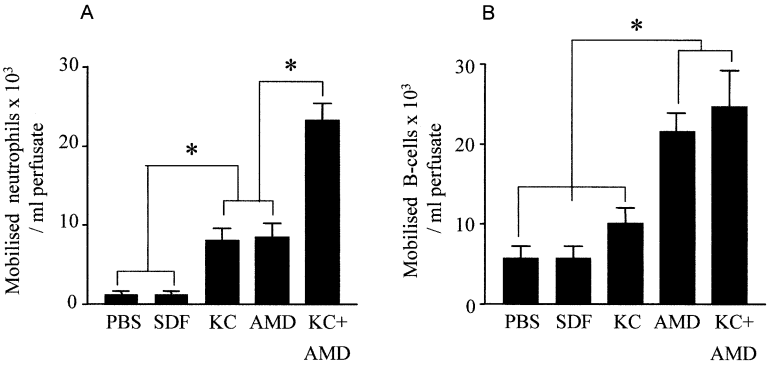

Figure 5. Mobilization of Neutrophils and B Cells from the Perfused Femoral Bone Marrow in Mice following the Infusion of KC and AMD-3100 alone or In Combination

(A) The number of neutrophils (Gr-1+) and (B) the number of B cells (B220+) mobilized from the bone marrow following 10 min infusion of PBS, SDF-1α (50 nM), KC (50 nM), AMD-3100 (5 μg/ml), or AMD-3100 plus KC. Results expressed as mean ± SEM (n = 5 separate perfusions). *, P < 0.05 (Kruskall Wallis H test).

As neutrophils age in culture they become apoptotic and can be readily identified by their binding of annexin V. Analysis of the neutrophils cultured for 20 hr revealed that 76 ± 5% of these neutrophils were apoptotic (as assessed by FITC-annexin V binding). However, expression levels of CXCR4 were not significantly different between the apoptotic and nonapoptotic populations. Interestingly, we observed that in the chemotaxis assays it was only the nonapoptotic/annexinV negative neutrophils that migrated toward SDF-1α (data not shown).

Senescent CXCR4^{high} Neutrophils Home to the Bone Marrow and Traffic into the Hematopoietic Compartment

As SDF-1α is expressed constitutively in several tissues including the bone marrow, it is feasible that senescent, preapoptotic neutrophils that express CXCR4 and have the capacity to migrate toward SDF-1α preferentially home to these tissues in vivo. Experiments were therefore carried out to determine whether senescent neutrophils home to the bone marrow. Murine bone marrow

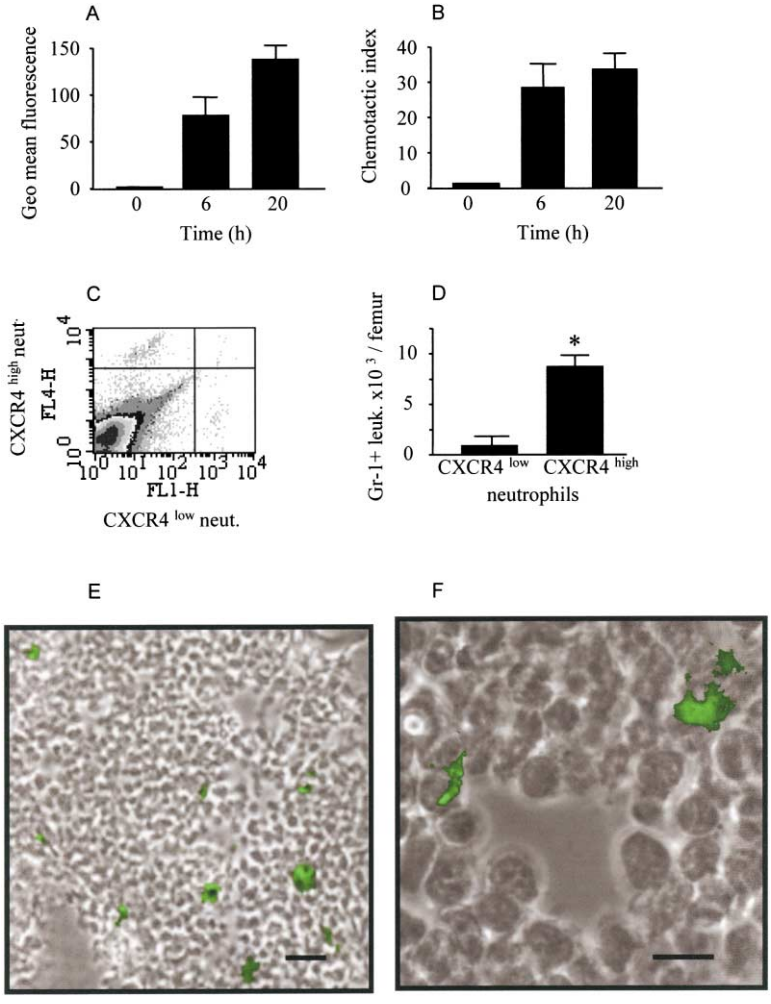

Figure 6. Trafficking of Neutrophils to the Bone Marrow In Vivo

(A) Surface expression of CXCR4 on freshly isolated (0 hr), 6 hr, and 20 hr cultured murine bone marrow neutrophils measured by flow cytometry. Results represent the mean ± SEM (n = 5).

(B) Chemotaxis assay of freshly isolated (0 hr), 6 hr, and 20 hr cultured neutrophils to SDF-1α (50 nM). Results represent the mean ± SEM (n = 3 separate experiments performed in triplicate).

(C and D) Bone marrow leukocytes cultured for 0 hr (CXCR4^{low}) or 6 hr (CXCR4^{high}) at 37°C were labeled with FITC- and APC-conjugated anti-Gr-1 mAb, respectively.

(C) A representative flow cytometry plot of bone marrow harvested from a BALB/c mouse 60 min following an i.v. injection with a 1:1 mix of CXCR4^{low} FITC neutrophils and CXCR4^{high} APC neutrophils.

(D) Results show the number of CXCR4^{low} and CXCR4^{high} neutrophils per femur as determined by flow cytometry. Mean ± SEM of n = 5 experiments.

(E and F) Confocal microscopy showing the localization of CXCR4^{high} neutrophils in the recipient bone marrow 20 hr after iv injection of CXCR4^{high} neutrophils alone. Bars, 25 μm (E) and 10 μm (F).

leukocytes were harvested from donor mice and incubated in RPMI 1640 for 6 hr at 37°C or used immediately. The level of apoptosis, as judged by annexin V staining was <24% of the population of neutrophils that had been cultured for 6 hr. CXCR4 expression (geo mean fluorescence) on the freshly isolated and 6 hr cultured neutrophils was 5 ± 0.4 and 76.3 ± 12.7, respectively (Figure 6A); hence these two neutrophil populations were termed CXCR4^{low} and CXCR4^{high}. Bone marrow CXCR4^{low} and CXCR4^{high} neutrophils were then labeled with FITC- and APC-conjugated Gr-1 mAb, respectively. The two leukocyte populations were subsequently mixed 1:1 and then injected intravenously into a recipient mouse. After 1 hr the mice were killed, femurs removed, and the bone marrow harvested. The number of FITC-labeled neutrophils and APC-labeled neutrophils in the harvested bone marrow was then determined by Flow cytometry. From Figures 6C and 6D it is clear that there are significantly more APC-labeled CXCR4^{high} neutrophils present in the bone marrow than FITC-labeled CXCR4^{low} neutrophils.

To determine whether the CXCR4^{high} neutrophils traffic into the hematopoietic compartment of the bone marrow, 6 hr cultured neutrophils were incubated with unlabeled Gr-1 mAb or an isotype control and then injected intravenously into mice. After 12 hr the mice were killed and the bone marrow harvested and processed for microscopy according to Experimental Procedures. The injected neutrophils were detected with a FITC-labeled secondary mAb. No staining was observed in bone marrow harvested from mice injected with leukocytes treated with the control mAb and then processed and stained with secondary mAb (data not shown). Figures 6E and 6F show representative sections of bone marrow harvested from mice injected with Gr-1 labeled CXCR4^{high} neutrophils at low and high power. It can be seen from these sections that the CXCR4^{high} neutrophils that home to the bone marrow are observed in the hematopoietic compartment of the bone marrow; i.e., these cells are able to attach to and migrate through the sinus endothelium into the stroma.

Sequestration of Neutrophils within the Bone Marrow Is Mediated via CXCR4

When neutrophils are injected intravenously into mice they distribute predominantly between the lung, bone marrow, liver, and spleen (Suratt et al., 2001). Thus, it is possible that we observed relatively more CXCR4^{high} neutrophils homing to the bone marrow because the CXCR4^{low} neutrophils were selectively taken up into other tissues. The next set of experiments were therefore designed to compare the sequestration of neutrophils by the bone marrow alone. Freshly isolated (CXCR4^{low}) or 6 hr cultured (CXCR4^{high}) neutrophils labeled with APC-conjugated Gr-1 mAb were infused directly into the femoral bone marrow of separate mice. Leukocytes were infused via the femoral artery over a period of 30 min using the in situ perfusion system of the mouse femoral bone marrow. After a further 30 min perfusion, the femur was removed and the femoral bone marrow harvested. This system allowed us to determine directly the relative number of CXCR4^{low} and CXCR4^{high} neutrophils that were sequestered in the bone marrow, without the possibility of differential sequestration in other tissues. Under these conditions, 5.6 times more CXCR4^{high} neutrophils were sequestered in the bone marrow than CXCR4^{low}neutrophils (Figures 7A–7C). To determine whether sequestration is mediated via CXCR4, CXCR4^{high} Gr-1^{+} leukocytes were either pretreated with anti-CXCR$_4$ blocking pAb (10 μg/ml) or desensitized by stimulation with 50 nM SDF-1α for 10 min. Some of these leukocytes were taken for in vitro chemotaxis assays. These studies showed that chemotaxis of CXCR4^{high} neutrophils toward 50 nM SDF-1α in vitro was reduced by 55% by pAb pretreatment and by 85% by SDF-1α desensitization. The remaining leukocytes (untreated, desensitized with SDF-1α, treated with control or anti-CXCR4 blocking pAbs) were infused directly into the femoral bone marrow of individual mice using the in situ perfusion system (Figures 7D–7I). Sequestration of CXCR4^{high} neutrophils was significantly reduced by pAb pretreatment (57%) and by SDF-1α desensitization (79%). Thus, sequestration of CXCR4^{high} neutrophils within the bone marrow was significantly reduced by blocking the SDF-1α/CXCR4 axis. These data indicate that the homing of senescent neutrophils back to the bone marrow is mediated via CXCR4.

Discussion

In this study we have examined the functional significance of CXCR4 expression on neutrophils. Consistent with previous reports, we have shown that freshly isolated human blood neutrophils and murine blood and bone marrow neutrophils express low levels of CXCR4 and do not migrate in response to SDF-1α. In a more sensitive shape change assay a small, but consistent, response of human neutrophils to SDF-1α was observed. While CXCR4 was expressed at extremely low levels on freshly isolated neutrophils we observed high levels of intracellular staining for this receptor. This receptor distribution may be explained by a high level of spontaneous endocytosis of CXCR4, as has previously been reported in other cell types (Tarasova et al., 1998).

We show here, for the first time, that the response of human and murine neutrophils to CXCR2-acting chemokines is significantly inhibited when the neutrophils were costimulated with SDF-1α. The results presented show inhibition using optimal concentrations of SDF-1 α, as determined in preliminary experiments. Thus, maximal inhibition was never more than 60%. We have shown that this inhibition is mediated via CXCR4 as it is abrogated both by the specific CXCR4 antagonist, AMD-3100, and the CXCR4 blocking mAb (Hatse et al., 2002). The ability of SDF to mediate this effect despite the low level of CXCR4 expression on freshly isolated neutrophils is consistent with a previous report of crossregulation of CXCR4 by CCR5 in B cell progenitors that express barely detectable levels of CCR5 (Honczarenko et al., 2002). Our data are in contrast to a recent report that shows that IL-8 acting via CXCR1, but not CXCR2, reduces the responsiveness of monocytes to SDF-1α (Richardson et al., 2003); this is most likely a reflection of the difference in the cell type used for each study.

The crossregulation of CXCR2 by CXCR4 observed in this study does not appear to be the result of heterolo-

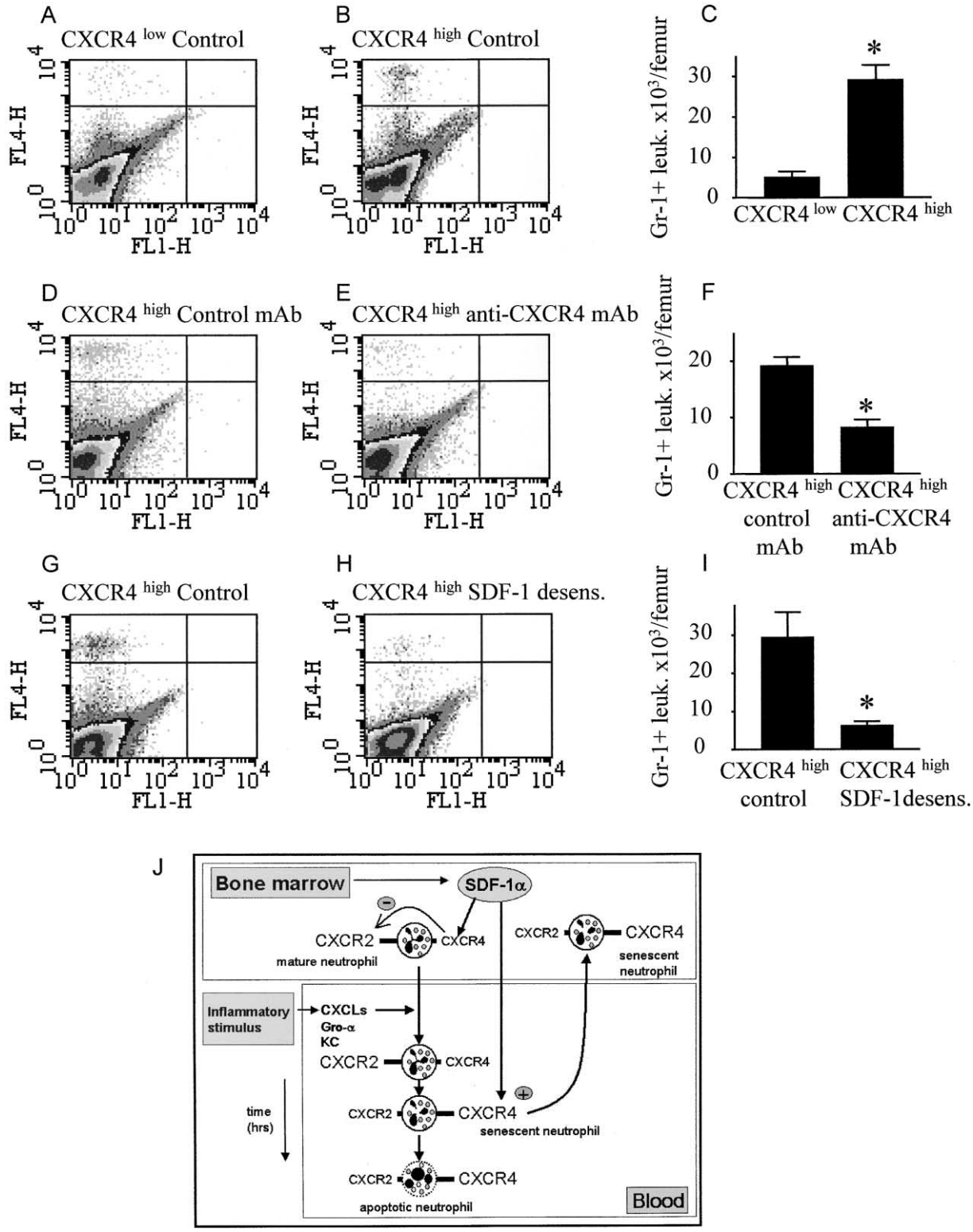

Figure 7. Sequestration of CXCR4^{high} Neutrophils within the Bone Marrow Is Mediated via CXCR4

Murine bone marrow leukocytes were cultured at 37°C for 0 hr (CXCR4^{low}) (A) or 6 hr (CXCR4^{high}) (B, D, E, G, and H). Neutrophils were subsequently left untreated (A and B) or preincubated with a CXCR4 blocking Ab (E) or an isotype matched control Ab for 30 min (D), or incubated for 10 min in the presence (H) or absence (G) of SDF-1α (50 nM). All neutrophil populations were then labeled with APC-conjugated GR-1mAb. Using the in situ perfusion system of the mouse femoral bone marrow the different populations of bone marrow leukocytes were infused directly into the femoral bone marrow over a period of 30 min. The femoral bone marrow was harvested and the number of Gr-1$^+$ neutrophils determined by flow cytometry. Results show the number of Gr-1 labeled neutrophils per femur (C, F, and I) Mean ± SEM of n = 5 experiments. A representative flow cytometry plot from a BALB/c mouse injected with Gr-1$^+$ APC conjugated leukocytes is shown (A, B, D, E, G, and H). (J) A model for the role of the CXCR4/SDF-1α pathway in neutrophil release and return to the bone marrow is shown schematically.

gous receptor downregulation, as cell surface expression of CXCR2 was unaffected by incubation of neutrophils with SDF-1α. It seems likely, therefore, that the crosstalk between CXCR4 and CXCR2 is mediated either via receptor desensitization or via inhibition of specific downstream signaling elements. Interestingly, in human neutrophils, we found that SDF-1α reduced responses to chemokines acting via CXCR2 but not CXCR1. This difference may reflect a difference in the signal transduction pathways utilized by these two receptors or a difference in the susceptibility of these two receptors to desensitization (Haribabu et al., 2000). The biological significance of this finding remains to be established, although it may relate to the distinct functions suggested for these two receptors (White et al., 1998).

During acute inflammatory reactions, an elevation of systemic levels of inflammatory mediators such as the CXC chemokines stimulates the acute mobilization of neutrophils from the bone marrow. Based on our in vitro findings showing a negative crosstalk between CXCR4 and CXCR2, we hypothesized that endogenous SDF-1α, present in the bone marrow, acts as a retention factor for bone marrow neutrophils, limiting neutrophil release in response to elevated systemic levels of CXCR2-acting chemokines. To test this hypothesis, we examined whether blocking CXCR4 signaling, using AMD-3100, would potentiate neutrophil mobilization stimulated by the CXCR2-acting chemokine KC. Our data showed that neutrophils may be mobilized either directly by stimulating neutrophil chemotaxis or indirectly by blocking the effect of SDF-1α. Furthermore, we showed that AMD-3100 and KC acted synergistically with respect to neutrophil mobilization when coinjected. These results indicate that maximal mobilization of neutrophils, stimulated by CXCR2-acting chemokines, is dependent on blockade of the CXCR4-dependent pathway that promotes the retention of neutrophils in the bone marrow. We observed that the cooperative effect of AMD-3100 and KC was selective for neutrophils, as B cell release was only enhanced by AMD-3100 and not KC and was not potentiated when AMD and KC were infused together.

While it is likely that neutrophil mobilization stimulated by KC is mediated via a direct action of KC on neutrophil motility, it is also possible that indirect mechanisms operate to effect this release as has been proposed for the basal release of neutrophils stimulated by G-CSF (Semerad et al., 2002). For example, it has recently been shown that $CD34^+$ cells are mobilized through inactivation of SDF-1α and CXCR4 by serine proteases, such as neutrophil elastase or cathepsin G released from neutrophils (Levesque et al. 2003). These proteases may, therefore, similarly be involved in neutrophil release in response to KC.

When SDF-1α was administered acutely as a single i.v. injection in vivo or as a 10 min infusion in the bone marrow perfusion system in situ, it did not stimulate neutrophil mobilization, measured over 60 min. This indicates that intravascular SDF-1α does not promote the rapid mobilization of bone marrow neutrophils, when a chemotactic gradient is established across the sinus endothelium. This result is consistent with our in vitro findings that SDF-1α does not stimulate neutrophil chemotaxis. Previous studies have shown that chronic elevation of plasma levels of SDF-1α effects a leukocytosis (Hattori et al., 2001). However, the mechanism underlying this response appears to be indirect.

Our results support the hypothesis that endogenous bone marrow SDF-1α plays an important role in the retention of mature neutrophils within the bone marrow. There are currently four other indirect lines of evidence that support this hypothesis. First, it has been shown that irradiated WT mice reconstituted with bone marrow derived from CXCR4-deficient mice have fewer mature neutrophils in the bone marrow and higher numbers of circulating myeloid cells (Ma et al. 1999), suggesting that in the absence of CXCR4 mature neutrophils are not retained within the bone marrow reserve. Second, treatment of mice with G-CSF reduces bone marrow SDF-1α levels and this is associated with the mobilization of bone marrow neutrophils (Semerad et al., 2002). Third, an i.v. injection of the CXCR4-antagonist, AMD-3100, has been shown to induce a leukocytosis in humans (Hendrix et al., 2000). Finally, our results would explain the data recently reported by Hernandez et al. that patients with WHIM syndrome express an activating mutation of CXCR4 (Hernandez et al. 2003). Thus cells expressing this mutated CXCR4 receptor were shown to exhibit enhanced signaling in response to SDF-1α. These patients exhibit a blood neutropenia despite having normal numbers of mature neutrophils in the bone marrow. Thus, it has been suggested that they suffer with a defect in neutrophil release from the bone marrow. The results of Hernandez et al. suggest that neutrophils derived from patients with WHIM syndrome would exhibit a hyperresponsiveness to SDF-1α as compared to those derived from normals. Hence we would predict that the ability of endogenous SDF-1α to retain mature neutrophils in the bone marrow would be enhanced in these patients, thus resulting in a blood neutropenia.

In this study, consistent with the report of Nagase et al. (2002), we showed that expression of CXCR4 was dramatically upregulated on murine bone marrow neutrophils cultured for 6 and 20 hr. Moreover, these neutrophils gained the ability to migrate toward SDF-1α. On human neutrophils we have shown that upregulation of CXCR4 occurs when these cells are cultured in autologous serum, suggesting that this process may occur in vivo in the circulation (C.M., unpublished data). Importantly, upregulation of CXCR4 on murine neutrophils preceded neutrophil apoptosis (as defined by annexin V staining) and we showed that it was the nonapoptotic, $CXCR4^{high}$ neutrophils that had the capacity to migrate toward SDF-1α. Apoptotic neutrophils did not migrate toward SDF-1α.

When equal numbers of $CXCR4^{low}$ and $CXCR4^{high}$ neutrophils were injected i.v. into mice or infused directly into the bone marrow using the in situ perfusion system, significantly more $CXCR4^{high}$ neutrophils were present in the femoral bone marrow as compared to $CXCR4^{low}$ neutrophils after one hour. This data suggests that $CXCR4^{high}$ neutrophils preferentially home to the bone marrow. Furthermore using either anti-CXCR4 blocking pAb or SDF-1α desensitization protocol we have shown that the sequestration of senescent $CXCR4^{high}$ neutrophils in the femoral bone marrow is CXCR4 dependent.

Confocal microscopy of the bone marrow revealed that $CXCR4^{high}$ neutrophils traffic into the hematopoietic

compartment of the bone marrow. This is evidence that these senescent neutrophils are not merely being lodged in the bone marrow sinuses but are migrating across the sinusoidal endothelium into the hematopoietic compartment. These data suggest that the SDF-1α-stimulated migration of CXCR4^{high} neutrophils may play a role in the homing of senescent neutrophils back to the bone marrow. It is important to point out that our study does not exclude the possibility that senescent neutrophils may also home to other tissues expressing SDF-1α, such as the spleen and liver. Hence this mechanism may also contribute to neutrophil clearance via these tissues.

The fact that neutrophils expressing high levels of CXCR4 have not been observed in the circulation suggests that, if these cells do exist in vivo, then they are rapidly cleared from the circulation. To date, the only report of neutrophils expressing high levels of CXCR4 in vivo is on neutrophils present in the synovial fluid of patients with inflammatory joint diseases (Bruhl et al., 2001). It is likely that these neutrophils do not reenter the circulation, but are cleared via tissue macrophages.

Our results provide a mechanism to explain the results of Suratt et al. (2001), who showed that up to 65% of purified ^{111}In-labeled bone marrow neutrophils were sequestered in the bone marrow 4 hr following their i.v. injection in mice. They are also consistent with the observations of Saverymuttu et al. (1985), who studied the fate of ^{111}In-labeled autologous granulocytes following their i.v. injection in humans. 24 hr after their injection the distribution of the granulocytes was visualized using a γ camera and a signal was observed from the bone marrow, indicating homing of mature granulocytes back to the bone marrow in humans.

The results of this study show that the SDF-1α/CXCR4 chemokine axis serves as an important retention pathway for bone marrow neutrophils, regulating the extent of neutrophil mobilization that occurs in response to an elevation in systemic levels of CXC chemokines (Figure 7J). At a cellular level, we have shown that this effect is due to the ability of SDF-1α to reduce the responsiveness of neutrophils to CXCR2-acting chemokines. The expression of CXCR4 on senescent neutrophils and their ability, unlike their nonsenescent counterparts, to migrate toward SDF-1α, suggested that these cells may preferentially traffic to tissues expressing SDF-1α. Indeed, we have shown here that CXCR4^{high} senescent neutrophils preferentially home to the bone marrow in a CXCR4-dependent manner (Figure 7J). We propose that this pathway may represent a previously undefined pathway for the basal clearance of senescent neutrophils from the circulation and thus is of fundamental importance for neutrophil homeostasis.

Experimental Procedures

Clinical Samples and Animals

Whole blood was obtained by venipuncture from healthy human volunteers. Female BALB/c mice were purchased from Harlan (Oxford, UK) and used at 6 weeks old.

Reagents

General laboratory and cell culture reagents were purchased from either Life Technologies (Paisly UK) or Sigma Chemical Co. (Poole, UK). Recombinant chemokines, KC, Gro-α, ENA-78, NAP-2, IL-8, and SDF-1α were from PeproTech EC Ltd. (London, UK). The PE-conjugated mouse anti-human CXCR4 mAb (clone 12G5) and mouse IgG2a isotype control (clone 20102.1), the FITC-conjugated mouse anti-human CXCR2 mAb (clone 48311.211) and mouse IgG2a isotype control (clone 20102.1) were obtained from R&D Systems. Purified mouse anti-human CXCR4 mAb (clone 12G5), PE-conjugated rat anti-mouse CXCR4 mAb (clone 2B11/CXCR4) and rat IgG2b isotype control (clone A95-1), purified, FITC-conjugated or APC-conjugated rat anti-mouse Ly-6G (Gr-1) mAb (clone RB6-8C5), PE-conjugated rat anti-mouse CD45R/B220 mAb (clone RA3-6B2), rat anti-mouse CD16/CD32 (Fcγ III/II Receptor) mAb (clone 2.4G2) were obtained from BD Biosciences. FITC-conjugated mouse anti-human CD16 mAb (clone DJ130c) was obtained from DAKO (Ely, UK). The CXCR2 antagonist SB225002 was obtained from Calbiochem. ACK lysis is composed of 0.15 M NH4Cl, 1 mM KHC03, and 0.1 mM Na2EDTA. Perfusion buffer is a modified Krebs-Ringer bicarbonate buffer supplemented with 10 mM D-Glucose, 2.50 mM CaCl2, 0.49 mM MgCl2.6H20, 4.56 mM KCl, 120 mM NaCl, 0.7 mM Na2HPO4, and 24 mM NaHC03, and Ficoll T-70 4% and BSA 0.1%. The CXCR4-antagonist, AMD-3100, was a generous gift from Dr. Gary Bridger, AnorMED Inc., British Columbia, Canada. The rabbit polyclonal anti-mouse CXCR4-blocking Ab (Gonzalo et al., 2000) was a generous gift from Dr. Jose-Carlos Gutierrez-Ramos (Millennium Pharmaceuticals Inc., Cambridge, Massachusetts).

Isolation of Human Peripheral Blood PMNs

Human PMNs were isolated from the peripheral venous blood as previously described (Haslett et al., 1985). In brief, blood was collected into citrate (3.8%) and centrifuged to remove the plasma. The cell pellet was resuspended in 6% dextran solution and left for 30 min to sediment the erythrocytes. The leukocyte layer was carefully removed and layered onto Histopaque-1077 and centrifuged. The pellet containing erythrocytes and PMNs was lysed by hypotonic shock.

Preparation of Murine Bone Marrow Leukocytes

The right femur was removed, and the femoral bone marrow was harvested by flushing with 5 ml HBSS buffer (Hanks Balanced Salts with Ca^{2+}/$Mg2^{+}$, 30 mM Hepes, 0.1% low endotoxin BSA [pH 7.4]). The erythrocytes were removed using hypotonic shock and the leukocyte population was resuspended in HBSS buffer.

Chemokine Receptor Expression on Neutrophils

Neutrophil surface CXCR4 expression was examined on (1) freshly isolated blood human PMNs, (2) human PMNs cultured for 20 hr in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, (37°C), (3) murine whole blood and bone marrow leukocyte suspension, and (4) murine bone marrow cultured for 6 or 20 hr. Human neutrophils were identified by their characteristic forward/side scatter (FCS/SSC) and/or CD16-positive staining. Neutrophils were preincubated with human serum for 15 min and then stained with anti-CXCR4-PE (50 μg/ml) or a PE-labeled isotype matched control mAb and anti-CD16-FITC (3.33 μg/ml) for 20 min. CXCR2 expression was also analyzed using anti-CXCR2-FITC (5 μg/ml) or a FITC-labeled isotype matched control mAb. Murine neutrophils were identified by their characteristic FCS/SSC and Gr-1 positive staining. Neutrophils were preincubated with murine Fc block and then stained with anti-CXCR4-PE (20 μg/ml) or a PE-labeled isotype matched control mAb and anti-Ly-6G (Gr-1)-APC (20 μg/ml). The samples were run on a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK), and analyzed using CellQuest software.

Measurement of Neutrophil Shape Change Using Flow Cytometry

5×10^5 purified PMNs in cell buffer (PBS, 10 mM HEPES, 10 mM glucose, and 0.1% BSA [pH 7.2–7.4]) were incubated with chemokines or buffer in a final volume of 100 μl, at 37°C for 4 min. The cells were then fixed on ice by addition of 250 μl of ice-cold fixative (a 1/4 dilution of Cellfix from BD Biosciences, Oxford, UK). In some experiments, PMN were preincubated with SDF-1α (25 nM) or CXCR2 antagonist (300 nM) for 5 min, prior to the addition of chemo-

kines. Some cells were preincubated for 10 min with the CXCR4 antagonist AMD-3100 (5 μg/ml) or anti-human CXCR4 blocking mAb (5 μg/ml) before being incubated with SDF-1α (25 nM) for a further 5 min and then with the chemokines. The samples were run on a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK), and analyzed using CellQuest software as previously described (Sabroe et al., 1997). Acquisition was terminated after 5000 neutrophil events. Results are expressed as the change from baseline in forward scatter of neutrophil population in response to chemokines, and is the mean of three separate experiments.

Neutrophil Chemotaxis Assay

Murine bone marrow leukocytes (3×10^6 cells in 0.2 ml assay buffer) or isolated human PMNs (0.5×10^6 in 0.2 ml assay buffer) were placed in the upper chamber of Transwell filters, Millipore (3 μm pore diameter) that were in turn placed in individual wells of a 24-well cell culture plate containing 0.3 ml of assay buffer with or without chemokine (KC 50 nM, SDF-1α 50 nM or 10 nM, IL-8 1 nM, and Gro-α 10 nM). In some experiments, the cells were preincubated at 37°C with SDF-1α (50 nM) for 5 min and/or AMD-3100 (5 μg/ml) for 15 min before being placed in the upper Transwell chamber. Chambers were incubated for 60 min at 37°C. The number of neutrophils that migrated into the bottom chamber were determined by a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK), with relative cell counts obtained by acquiring events for a set time period of 30s.

In Vivo Neutrophil Mobilization in Mice

BALB/c mice were injected with either KC (40 μg/kg), AMD-3100 (5 mg/kg), SDF-1α (40 μg/kg), or PBS only via the tail vein. A fifth group of mice received an i.v.injection of both AMD-3100 (5 mg/kg) and KC (40 μg/kg). In this case mice were injected with AMD-3100 20 min prior to the administration of KC. After 60 min, the mice were anaesthetized with intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg). Blood (0.5–1 ml) was collected by cardiac puncture into EDTA (100 mM), the erythrocyte population was removed using ACK lysis, and the sample was centrifuged and resuspended in RPMI 1640. Femoral bone marrow cells were harvested and the total number of nucleated cells was determined by light microscopy using a Neubauer haematocytometer. For differential cell counts, blood or bone marrow leukocytes were preincubated with 10 μg/ml rat anti-mouse CD16/CD32, washed, and then stained with 10 μl anti-Ly-6G (Gr-1)-APC (20 μg/ml) or 10 μl anti-B220-PE (20 μg/ml). The samples were analyzed by flow cytometry.

In Situ Perfusion of the Murine Femoral Bone Marrow

The guinea pig hind limb perfusion technique previously described (Palframan et al., 1998) was adapted to the mouse. Female BALB/c mice were anaesthetized with intraperitoneal injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) and the external femoral artery and veins were exposed. The hind limb was isolated by occlusion of the external iliac artery, superficial epigastric and muscular branch with 5/0 braided silk suture. The animals were killed with Expiral (250 mg/kg by intracardiac injection), and polyethylene cannulae (0.61 mm OD, Portex, London, UK) were immediately inserted into the femoral artery and vein and tied in place with 5/0 braided silk suture. Perfusion buffer at 37°C was infused (0.1 ml/min) via the arterial cannula and removed from the venous cannula using a Minipuls peristaltic pump (Anachem, Luton, UK). The hind limb was perfused for an initial 10 min to remove blood from the vasculature and then perfused for a further 60 min. The perfusate was collected onto ice and centrifuged (200g for 10 min, 4°C). Erythrocytes were lysed using hypotonic shock and the cell pellet was resuspended and stained as previously described with anti-Ly-6G (Gr-1)-APC and anti-B220-PE. The samples were analyzed by flow cytometry. The total number of events recorded by the flow cytometer was used to calculate the number of cells mobilized from the hind limb during the 60 min perfusion. In some experiments, KC (50 nM), AMD-3100 (5 μg/ml), SDF-1α (50 nM), or buffer alone were infused over a 10 min period using an infusion/withdrawal pump (Harvard Instruments, UK). In other experiments, bone marrow leukocytes (prepared as described below) were infused over a 30 min period using the same pump.

Comparison of Homing Capacity of Bone Marrow of Neutrophils Expressing High or Low Levels of CXCR4

20×10^6 murine bone marrow leukocytes prepared as above (at least 40% neutrophils) were cultured for 0 or 6 hr in RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (37°C). 0 and 6 hr cultured bone marrow leukocytes were then incubated with 50 μl anti-Ly-6G (Gr-1)-FITC (20 μg/ml) or 50 μl anti-Ly-6G (Gr-1)-APC (20 μg/ml), respectively, for 30 min on ice. Leukocytes were washed twice with RPMI 1640 and a sample of each preparation was processed by flow cytometry analysis to check the percentage of Gr-1+ neutrophils. The two populations were mixed 1:1 in a final volume of 0.2 ml RPMI 1640 and were injected in BALB/c mice via the tail vein. After 1 hr the femoral bone marrow was removed and analyzed by flow cytometry for either Gr-1-APC positive neutrophils or Gr-1-FITC positive neutrophils.

For infusion directly into the bone marrow 0 or 6 hr cultured leukocytes were labeled with 50 μl anti-Ly-6G (Gr-1)-APC (20 μg/ml) for 30 min on ice and resuspended in 0.5 ml perfusion buffer. Some 6 hr cultured leukocytes were preincubated for 30 min with anti-CXCR4 mAb or rat isotype matched control mAb, or preincubated with SDF-1α (50 nM for 10 min at 37°C) and then washed. The hind limb was perfused as described above and leukocytes were infused over 30 min. After 60 min the femoral bone marrow was removed and the number of Gr-1 positive neutrophils retained in the bone marrow was determined by flow cytometry.

Localization of CXCR4 High Expressing Neutrophil within the Haematopoitic Compartment via Confocal Laser Microscopy

6 hr cultured murine bone marrow neutrophils were incubated with unlabeled Gr-1 mAb or an isotype control mAb. These leukocytes were then injected intravenously into mice. After 12 hr the mice were killed and the bone marrow harvested, embedded in gelatin, and snap frozen. 3 μm sections were incubated for 1 hr at room temperature with PBS supplemented with 5% BSA, washed with PBS, and then incubated with a FITC-labeled secondary rabbit anti-rat antibody (diluted 1/50) for 1 hr at room temperature. Slides were washed 3 times in PBS and then mounted in Immuno Fluor medium (ICN Biomedicals) and observed using a Leica DM-IRBE confocal microscope. Images were processed using Leica TCS-NT software associated with the microscope and Adobe Photoshop 4.0 software.

Statistics

The nonparametric Kruskall-Wallis H test and the Mann-Whitney U test were used to assess nonnormally distributed parameters.

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