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# Serine protease inhibitors *serpina1* and *serpina3* are down-regulated in bone marrow during hematopoietic progenitor mobilization

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**Mobilization of hematopoietic progenitor cells into the blood involves a massive release of neutrophil serine proteases in the bone marrow. We hypothesize that the activity of these neutrophil serine proteases is regulated by the expression of naturally occurring inhibitors (*serpina1* and *serpina3*) produced locally within the bone marrow. We found that *serpina1* and *serpina3* were transcribed in the bone marrow by many different hematopoietic cell populations and that a strong reduction in expression occurred both at the protein and mRNA levels during mobilization induced by granulocyte colony-stimulating factor or chemotherapy. This decreased expression was restricted to the bone marrow as *serpina1* expression was maintained in the liver, leading to no change in plasma concentrations during mobilization. The down-regulation of *serpina1* and *serpina3* during mobilization may contribute to a shift in the balance between serine proteases and their inhibitors, and an accumulation of active neutrophil serine proteases in bone marrow extravascular fluids that cleave and inactivate molecules essential to the retention of hematopoietic progenitor cells within the bone marrow. These data suggest an unexpected role for *serpina1* and *serpina3* in regulating the bone marrow hematopoietic microenvironment as well as influencing the migratory behavior of hematopoietic precursors.**

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Abbreviations used:  $\beta$ 2m,  $\beta$ <sub>2</sub>-microglobulin; CG, cathepsin G; CY, cyclophosphamide; HPC, hematopoietic progenitor cell; NE, neutrophil elastase.

Hematopoietic progenitor cells (HPCs) are responsible for the renewal of all mature blood cells. In adult mammals, the majority of HPCs reside in the BM. Transient increases in the number of HPCs circulating in the peripheral blood (mobilization) occur in response to a wide variety of stimuli including strenuous physical exercise, myelosuppressive chemotherapy, polyanions, chemokines, and hematopoietic growth factors (1).

Mobilized HPCs are now the favored source of transplantable cells to reconstitute hematopoiesis after high-dose chemotherapy. Currently, the agent most commonly used to elicit HPCs mobilization is G-CSF used alone or in combination with myelosuppressive chemotherapy (2, 3). The administration of G-CSF induces a 10- to 100-fold increase in the level of circulating HPCs in both humans and mice. G-CSF-induced mobilization is time and dose dependent, involving a rapid neutrophilia (evident within hours) and a gradual increase in HPC numbers in the blood peaking between

4 and 7 d of G-CSF administration. Mobilization with chemotherapeutic agents such as cyclophosphamide (CY) occurs during the recovery phase after the chemotherapy-induced neutropenia, that is, days 6–8 in mice, and days 10–14 in humans. Although mobilized HPCs collected from the peripheral blood are extensively used to rescue hematopoiesis in patients undergoing high-dose myeloablative chemotherapy, the exact molecular mechanisms responsible for the mobilization of HPCs from the BM into the peripheral blood remain unclear.

Essential for the retention of HPCs in the BM are adhesive and chemotactic interactions. Particularly important are (a) the adhesive interaction between the vascular cell adhesion molecule VCAM-1 (CD106) expressed by the BM stroma with its counter receptor integrin  $\alpha_4\beta_1$  (VLA-4) expressed by HPCs, and (b) HPC chemotaxis due to binding of the chemokine CXCL12 (SDF-1) produced by the BM stroma, to its cognate receptor CXCR4 (CD184) expressed at the surface of HPCs.

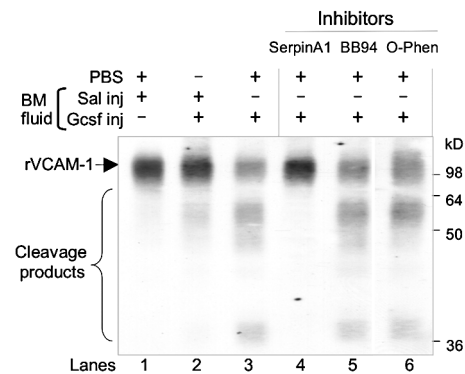
Blocking either of these interactions; the VCAM-1- $\alpha_4\beta_1$  adhesive interaction (4–6) or the CXCL12–CXCR4 chemotactic interaction (7, 8), by means of antibodies, antagonists, or tissue-specific gene-targeted deletion has been shown to result in mobilization of HPCs in vivo.

Our group has shown that HPC mobilization induced by G-CSF or CY coincides with the accumulation of high concentrations of active neutrophil serine proteases within the BM (9, 10). The predominant proteases were identified as neutrophil elastase (NE) and cathepsin G (CG), both released by neutrophils upon activation (9, 10). Once released into the BM extracellular fluid, these proteases can disrupt locally the two important mechanisms by which HPCs home to and remain within the BM: (a) adhesion to the BM stroma by cleaving the extracellular domain of VCAM-1 (9, 10), and (b) chemotaxis of HPCs by degrading and inactivating the chemokine CXCL12 (11, 12) and cleaving the 1st extracellular domain of CXCR4 expressed by human HPCs (12). As previous studies have shown, inactivation of either is sufficient to induce mobilization. However, a mechanism explaining how high concentrations of active neutrophil serine proteases accumulate in the BM during mobilization remains unclear.

Neutrophil serine protease activity is controlled in vivo by naturally occurring serine protease inhibitors or serpins (13, 14). Serpins function like mousetraps with a protruding reactive center loop. Cleavage of this reactive center loop by a specific protease triggers a spring-like mechanism, which entraps the protease within the serpin structure destroying the protease catalytic architecture (15–17). Once formed, the inactive serpin–protease complex is rapidly cleared from the body. Although serpin structure is very conserved, the reactive center loop sequence is highly variable between different serpins allowing the targeting of specific proteases. Two groups of serpins located on chromosome 14 in humans and chromosome 12 in mice (referred to as “clade A” serpins; 14, 18) can inactivate neutrophil serine proteases in blood and tissues. These are SERPINA1 (also known as  $\alpha_1$ -proteinase inhibitor or  $\alpha_1$ -antitrypsin: AAT) and SERPINA3 ( $\alpha_1$ -antichymotrypsin: ACT; 19, 20). Although humans express a single copy of each *SERPINA1* and *SERPINA3* gene, in the house mouse *Mus musculus* the *serpina1* gene has replicated five times (*serpina1a–e*; 21–23) whereas the *serpina3* gene has replicated 14 times (*serpina3a–n*; 23, 24).

To explain how high concentrations of active neutrophil serine proteases accumulate in the bone marrow during HPC mobilization, we hypothesize that (a) serine protease inhibitors, in particular serpins, are present in steady-state BM preventing the action of serine proteases released by BM neutrophils in steady-state conditions, and (b) that the balance between serine proteases and their inhibitors shifts in mobilized BM leading to the accumulation of active NE and CG.

Herein we report that, *serpina1* and *serpina3* are both transcribed in steady-state BM and are present at sufficient concentrations to inhibit local serine proteases. During mo-



**Figure 1. Extracellular fluids from steady-state BM contain serine protease inhibitors whereas those from mobilized BM do not.** Extracellular fluids from the BM of G-CSF-mobilized mice were mixed with an equal volume of PBS or extracellular fluids from saline-injected mice or with purified human SERPINA1, BB-94, or *O*-phenanthroline as indicated. The proteolytic activity of each sample was estimated by cleavage of recombinant human VCAM-1 visualized after immunoblotting with a goat anti-human VCAM-1 antibody.

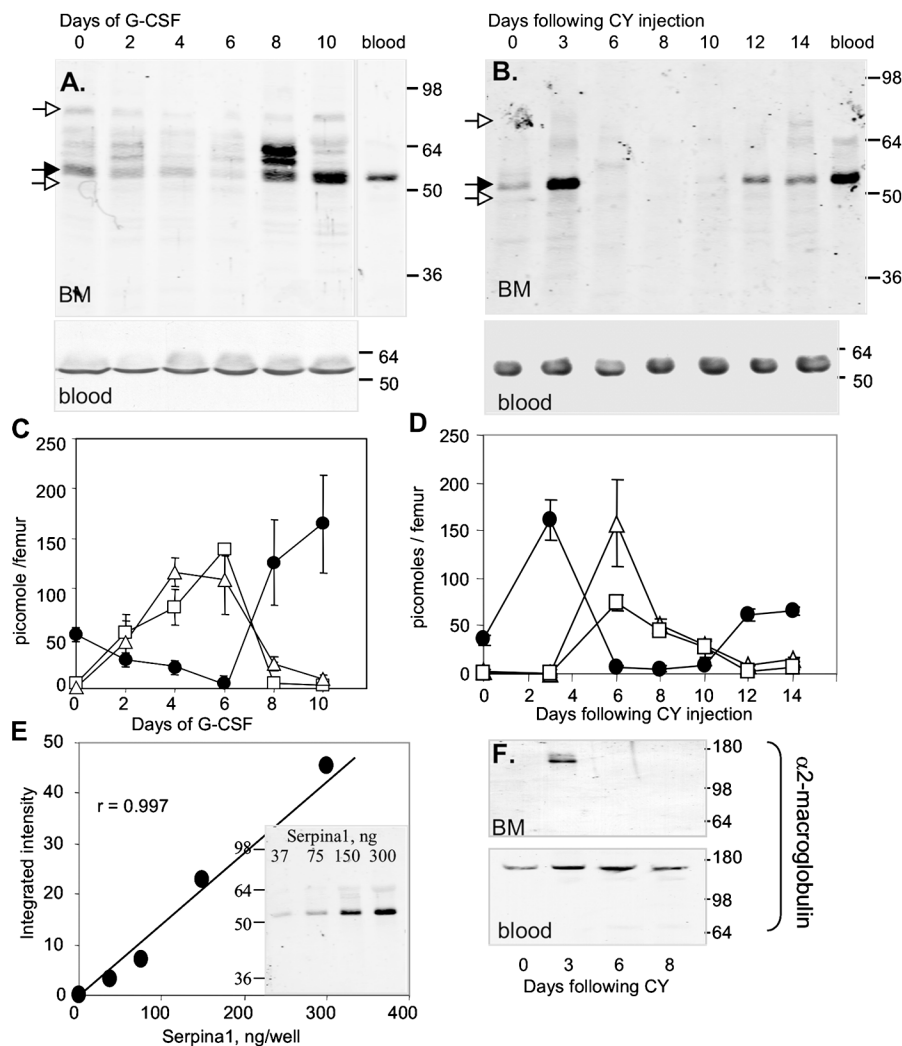
bilization induced by either G-CSF or CY, both protein and mRNA concentrations of *serpina1* and *serpina3* in the BM dramatically drop associated with an accumulation of active neutrophil serine proteases and concomitant cleavage and inactivation of molecules essential for the retention of HPCs within the BM.

## RESULTS

### Inhibitors of serine proteases are present in steady-state BM extracellular fluids

We have reported previously a dramatic increase in active serine protease levels in the BM at the peak of mobilization induced either by G-CSF or CY (9, 10, 12). This could be due to (a) a corresponding increase in protease production/release, and/or (b) a drop in the level of inhibitors that control the activity of these proteases. To determine whether naturally occurring protease inhibitors are involved in the regulation of protease activity in the BM, recombinant VCAM-1 was incubated with pooled BM fluids from three saline-injected mice (Fig. 1, lane 1), three G-CSF day 4 mice (lane 3), or a mixture of the two (lane 2). As expected from our previous work, no cleavage of VCAM-1 was observed when incubated with BM fluids from saline-injected nonmobilized mice, whereas cleavage of VCAM-1 did occur when incubated with BM fluids from G-CSF day 4 mice (9, 10). Interestingly, when both BM fluids were mixed together (lane 2) cleavage was significantly reduced. This result demonstrates that naturally occurring protease inhibitors are present in the BM fluids from steady-state (saline-injected) mice in sufficient quantities to significantly inhibit the proteolytic activity of BM fluids from mice mobilized with G-CSF.

When these same pooled BM fluids from G-CSF day 4 mice were incubated with purified human SERPINA1 ( $\alpha_1$ -antitrypsin), complete inhibition of VCAM-1 cleavage was



**Figure 2. Concentration of serpin1 protein in BM extracellular fluids drops during mobilization induced by G-CSF or CY.** Aliquots of BM extracellular fluids or blood plasma were taken at indicated time-points of mobilization induced by G-CSF (A) or by CY (B) and analyzed by immunoblotting with a rabbit anti-human SERPINA1 cross-reacting with mouse serpin1. The 53-kD intact, 50-kD cleaved, and 76-kD complexed forms of serpin1 are indicated by arrows as determined in Fig. 3 A. Panels C and D show the concentration of serpin1 (●), active NE (□), and CG (△) in BM extracellular fluids from G-CSF- (C) and CY-mobilized (D) mice. Results are expressed in picomoles per femur. Values are mean  $\pm$  SD of three mice per time-point. E shows a calibration curve for the quantification of serpin1 protein. Serial dilutions of mouse plasma were immunoblotted with serpin1 antibody and fluorescent intensity of the 53-kD serpin1 band measured using the Odyssey Infrared Imaging System. F shows the same CY-mobilized BM extracellular fluids and plasma as B, but immunoblotted with rabbit anti-human  $\alpha_2$ -macroglobulin.

observed, suggesting that murine steady-state BM may contain similar serine protease inhibitors (Fig. 1, compare lanes 1, 3, and 4). Inhibitors of matrix metalloproteinases such as BB-94 (Fig. 1, lane 5) or O-phenanthroline (lane 6) did not prevent VCAM-1 cleavage. Together these results indicate that the proteases in G-CSF-mobilized murine BM fluids able to cleave VCAM-1 are predominately serine proteases (inhibited by SERPINA1) and not matrix metalloproteinases. Consequently, the inhibition of protease activity by steady-state BM fluids is not due to tissue inhibitors of metalloproteinases but rather to endogenous serpins or nonserpin inhibitors of serine proteases.

(△) in BM extracellular fluids from G-CSF- (C) and CY-mobilized (D) mice. Results are expressed in picomoles per femur. Values are mean  $\pm$  SD of three mice per time-point. E shows a calibration curve for the quantification of serpin1 protein. Serial dilutions of mouse plasma were immunoblotted with serpin1 antibody and fluorescent intensity of the 53-kD serpin1 band measured using the Odyssey Infrared Imaging System. F shows the same CY-mobilized BM extracellular fluids and plasma as B, but immunoblotted with rabbit anti-human  $\alpha_2$ -macroglobulin.

### Levels of serpin1 protein drop in the BM during mobilization

Serpina1 protein, the main inhibitor of NE in tissues, was analyzed in BM extracellular fluids by immunoblotting. Whereas serpin1 (53 kD) was present in BM fluids before and after mobilization, levels were significantly decreased during HPC mobilization on days 2–6 after G-CSF administration (Fig. 2 A). Similarly, levels of serpin1 were significantly decreased in the BM fluids from CY-treated mice between days 6–10 again corresponding to the peak of mobilization in these mice (Fig. 2 B). This decrease in serpin1 was restricted to the BM as no corresponding

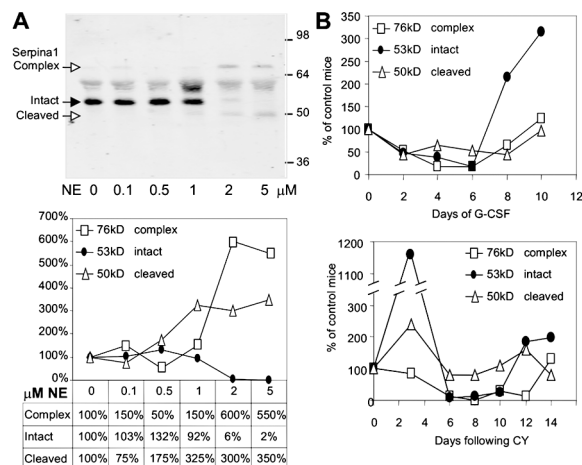
change in serpin1 was observed in the matching plasma from these same mice (Fig. 2, A and B, lower panels).

Interestingly, a large increase in serpin1 was observed specifically on day 3 after CY injection. As the BM is very damaged at this time-point, we hypothesized that this peak in serpin1 in the BM could be due to blood infiltration across the endothelium into the BM. This was confirmed by immunoblotting the same BM fluids as above for  $\alpha_2$ -macroglobulin, a major plasma protein. Because of its large size (four 160-kD subunits linked by disulfide bridges),  $\alpha_2$ -macroglobulin cannot normally diffuse through vascular walls from the blood into tissues. Although  $\alpha_2$ -macroglobulin was readily detected in all blood samples, it was only detected in the BM extravascular fluids of mice at day 3 after CY administration, but absent from all other BM fluids (Fig. 2 F).

In another series of experiments, serpin1 concentration in BM fluids from individual mice were directly measured from immunoblots using a secondary antibody conjugated to the infrared fluorescent dye Alexa Fluor 680 and the Odyssey Infrared Imaging System. The advantage of this system is that the fluorescent intensity of a band is directly proportional to serpin1 concentration (Fig. 2 E, see standard curve). Large amounts of serpin1 ( $54.3 \pm 1.3$  picomole [ $2.9 \mu\text{g}$ ]/femur) were measured in steady-state BM fluids (Fig. 2 C), dropping to  $11.0 \pm 1.2$  picomole [ $0.5 \mu\text{g}$ ]/femur on day 6 of G-CSF mobilization and rebounding (to  $185 \pm 49$  picomole [ $9.8 \mu\text{g}$ ]/femur) during the recovery from mobilization. A similar drop in serpin1 concentration (down to 5.1–7.6 picomole/femur) was observed in the BM of CY-mobilized mice at days 6–10 during mobilization, with serpin1 levels again rebounding to 61–66 picomole/femur on days 12–14 (Fig. 2 D).

As no specific antibodies for murine NE and CG are available, it was not possible to directly measure changes in protease concentration during mobilization. Instead we measured proteolytic activity using specific chromogenic substrates and then calculated the molar concentration of each within a femur using purified human NE and CG as standards (Fig. 2, C and D). As serpin1 inhibits both NE and CG with a 1:1 stoichiometry (19, 20), the concentration of serpin1 (53 kD) in steady-state BM fluids is sufficient to inhibit all NE and CG present in a steady-state femur. However, at the peak of mobilization, serpin1 decreases and active NE and CG in the BM are in large molar excess (up to 45 times over serpin1 on day 6 of G-CSF and 36 times on day 6 after CY injection).

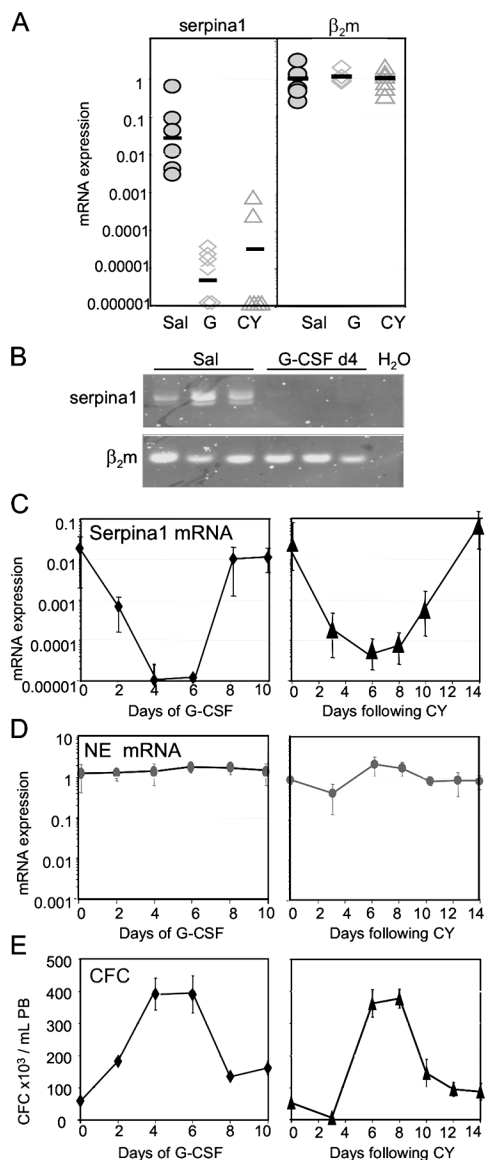
Serpin1 within the BM may originate from two possible sources. It may derive from blood slowly diffusing through the BM endothelium into the BM extracellular fluid, or alternatively, serpin1 may be endogenously produced by BM cells. If continuous diffusion from blood was the predominant source of BM serpin1, it is likely that this process would continue during mobilization with the “fresh” serpin1 being continuously cleaved and complexed by the increased active neutrophil serine proteases. This would result in the accumulation of cleaved and complexed serpin1 in the BM fluid. Alternatively, a decrease in endogenous



**Figure 3. Relative variations in intact serpin1, cleaved serpin1, and serpin1-protease complexes during mobilization.** (A) Mouse plasma was incubated with increasing concentrations of purified human NE and then immunoblotted with rabbit anti-SERPINA1 to reveal the disappearance of intact 53-kD serpin1 and appearance of cleaved 50-kD and complexed 76-kD serpin1 band in the presence of excess serine protease. The relative intensities of 50-, 53-, and 76-kD bands were measured using the Odyssey Infrared Imaging System. (B) BM extracellular fluids were taken from mice mobilized with G-CSF or CY at indicated time-points and then immunoblotted and the relative intensities of the 50, 53, and 76-kD serpin1 bands measured as described above. Values from non-mobilized control mice were used to calculate the 100% baseline.

production of serpin1 by BM cells during mobilization would also result in the observed decrease in intact serpin1 levels within the BM fluid but without an associated increase in cleaved or complexed serpin1 forms.

We first confirmed the identity of the 50- and 76-kD bands detected on previous blots (Fig. 2, A and B), by incubating mouse plasma with increasing concentrations of NE for 30 min at 37°C (Fig. 3 A). Indeed the 53-kD “intact” serpin1 band disappeared when incubated with increasing concentrations of NE, with a concurrent 3.5-fold increase in the 50-kD band (cleaved serpin1) and sixfold increase in the 76-kD band (complexed serpin1). The concentration of 50-kD cleaved and 76-kD complexed serpin1 bands were then quantified in BM extracellular fluids from G-CSF- and CY-mobilized mice (Fig. 3 B). This quantification revealed that the concentration of cleaved and complexed serpin1 did not increase in BM extracellular fluids during mobilization suggesting that serpin1 diffusion from the blood through the BM endothelium is limited and below the rate required to replenish the pool of intact serpin1 cleaved by NE and CG released by BM neutrophils. This conclusion is supported by the fact that when the endothelium barrier is disrupted at day 3 after CY injection (Fig. 2, B and D), serpin1 diffusion into the BM extracellular fluid dramatically rises resulting in an 11.5-fold increased serpin1 concentration compared with nonmobilized mice. Taken together, these data indicate that serpin1 diffusion through the BM endothelial barrier is limited and endogenous production within the BM is likely to



**Figure 4. Reduced *serpina1* mRNA levels in the BM during mobilization induced by G-CSF or CY.** (A) Total RNA was isolated from BM cells of mice injected for 4 d with saline (Sal), G-CSF (G), or 8 d after a single cyclophosphamide injection (Cy). Murine *serpina1a-e* (left) and  $\beta_2m$  mRNA levels were measured by quantitative real-time RT-PCR. Results are expressed as mRNA amount relative to mRNA for the cellular cytoskeleton protein vimentin (on a log scale). Each symbol represents the mRNA level from a different mouse. Black bars represent average of each group. (B) RNA was extracted from the BM cells of six mice injected with saline or G-CSF for 4 d. Products after RT-PCR for *serpina1a-e* (30 cycles) or  $\beta_2m$  (25 cycles) were loaded on 8% PAGE and visualized by ethidium bromide staining. (C) Kinetics of *serpina1* mRNA levels during the course of G-CSF- (left) and CY-induced (right) mobilization. Concentrations of *serpina1* mRNA were measured by real-time RT-PCR from whole BM cells and are relative to vimentin mRNA. Data are mean  $\pm$  SD of six to nine mice per time-point. (D) Kinetics of NE mRNA levels during the course of G-CSF-induced (left) and CY-induced (right) mobilization. Concentrations of mRNA were measured as described in C. (E) Kinetics of CFC mobilization into the peripheral blood induced by G-CSF (left) and CY (right). Data are mean  $\pm$  SD of six to nine mice per time-point.

be the predominant contributor to the large amounts of *serpina1* observed within BM.

#### Reduction of *serpina1a-e* mRNA levels in the BM during mobilization

To further investigate endogenous BM production of *serpina1*, total RNA was extracted from BM cells of six individual mice injected with either saline for 4 d, G-CSF for 4 d, or 8 d after a single injection of CY, and quantitative real-time RT-PCR performed. Results were standardized using primers specific for murine vimentin, a cytoskeleton protein ubiquitously expressed.

Using primers that amplify transcripts from the five murine *serpina1* genes (*serpina1a-e*), we observed a dramatic decrease in *serpina1a-e* mRNA levels in the BM of mice mobilized with G-CSF (8,000-fold decrease) or CY (790-fold decrease; Fig. 4 A). When primers to  $\beta_2$ -microglobulin ( $\beta_2m$ ), another ubiquitously expressed gene, were substituted for the pan-*serpina1* primers, there was no difference between saline-, G-CSF-, or CY-injected mice. These results confirm that *serpina1* genes are transcribed in the BM, and that the concentration of transcripts decreases during mobilization. Furthermore, the decrease in concentration of mouse *serpina1a-e* transcripts is specific (no changes are found with other RNAs such as  $\beta_2m$ ) when compared with the vimentin.

To confirm real-time RT-PCR results, a repeat PCR was stopped at 30 cycles for *serpina1* (mid-log phase of reaction) or 25 cycles for  $\beta_2m$ , run on 8% PAGE and bands visualized with ethidium bromide. After 30 cycles, *serpina1* product (140 bp) was detectable in RNA from the BM of three individual saline-injected mice but not in the three G-CSF-mobilized mice (Fig. 4 B).

Real-time RT-PCR was performed from BM at different time-points of G-CSF-induced mobilization. The decrease in *serpina1* mRNA on days 2, 4, and 6 (Fig. 4 C) corresponds to the timing of decreased *serpina1* protein in BM fluids (Fig. 2) as well as the rise in colony-forming cells mobilized into the peripheral blood (Fig. 4 E).

Similar decreases in *serpina1a-e* mRNA levels were observed in the BM at the peak of CY-induced mobilization (days 6–8). Together these data indicate that a significant two to three log decrease in *serpina1a-e* mRNA concentration occurs in the BM at the peak of mobilization even when two completely different mobilization protocols (either G-CSF, a cytokine, or CY, a cytotoxic agent) are used.

#### Transcription of the neutrophil proteases NE, CG, as well as VCAM-1 and CXCR4 in the BM remains unchanged during mobilization

In sharp contrast to *serpina1*, no significant change in NE or CG transcripts was observed during mobilization induced by G-CSF or CY (Fig. 4 D and Fig. 5). However this is not surprising as these two proteases are transcribed in myeloid progenitors (see Fig. 6 E and reference 25) and stored in

azurophil granules where they are released upon granulocyte activation (26) or in response to mobilizing agents (27).

No significant changes in the transcription of other molecules reported to be important for the retention of HPCs in the BM such as VCAM-1 or CXCR4 were observed (Fig. 5). Taken together, these results support the notion that a significant drop in serpin production combined with neutrophil activation and degranulation (27), both contribute to the increased active serine protease levels observed in mouse BM at the peak of G-CSF- and CY-induced mobilization.

### Serpina3 mRNA decreases in the BM at the peak of mobilization

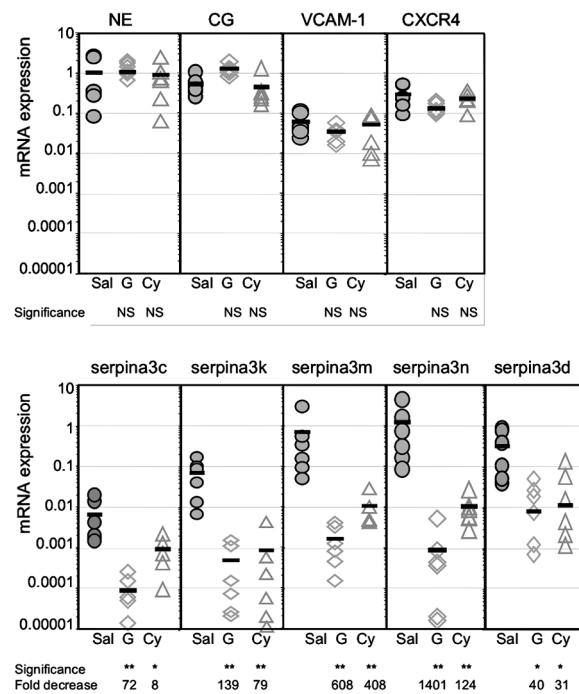
Using the same approach, we followed the mRNA levels of six murine *serpina3* genes that encode serpins with an NH<sub>2</sub>-terminal secretion sequence and thus are potentially secreted by cells (*serpina3b*, *serpina3c*, *serpina3d*, *serpina3k*, *serpina3m*, and *serpina3n*; 23). A similar decrease in transcripts (between 8- and 1,400-fold) was found in the BM at the peak of mobilization induced by either G-CSF or CY (Fig. 5, bottom) with the exception of *serpina3b*, which was not transcribed in the BM of BALB/c mice. We also analyzed by real-time RT-PCR, transcripts of another five murine *serpina3* genes that have no secretion sequences and are thus presumed to remain cytoplasmic (*serpina3e*, *serpina3f*, *serpina3g*, *serpina3h*, and *serpina3i*), the majority of which were detected in the BM of steady-state BALB/c mice (Table I). Similar to the secreted *serpina3*, the mRNA levels of these other *serpina3* genes were significantly decreased at the peak of mobilization (-fold reduction in BM transcription at day 4 of G-CSF was 85 with  $P < 0.001$  for *serpina3e* and *serpina3i*, 621 with  $P < 0.01$  for *serpina3g*, and 1,592 with  $P < 0.01$  for *serpina3h*). *Serpina3f* was not expressed in steady-state or mobilized BALB/c BM.

### Decrease in *serpina1* and *serpina3* transcripts within sorted BM cell populations during mobilization

The exact identity of cell types expressing *serpina1* and *serpina3* in mouse BM has not been thoroughly investigated. We sorted BM cells from saline- and G-CSF-treated mice at day 4 based on lineage markers to determine which cell types were expressing *serpina1* and *serpina3* and whether serpin transcription in these cells was down-regulated during mobilization.

We first examined myeloid cells as they are the predominant cell type accumulating in the BM at the peak of mobilization (Fig. 6 A). Bone marrow myeloid cells were sorted on the basis of positive CD11b (Mac-1) expression and relative maturity on the basis of Gr-1 expression (Fig. 6, B and C) with primitive-mono/myeloid cells being Gr-1<sup>dim</sup> (sort gate R3), immature myeloid intermediate for Gr-1 staining (sort gate R4), and mature BM neutrophils Gr-1<sup>bright</sup> (sort gate R2). With G-CSF-induced mobilization a twofold increase in both the proportion and total cell number of primitive Mac-1<sup>+</sup>Gr-1<sup>dim/intermediate</sup> myeloid cells (R3 plus R4 regions) was found in the BM.

By real-time RT-PCR, *serpina1a-e* mRNA transcripts were greatest in BM neutrophils (Mac-1<sup>+</sup> Gr-1<sup>bright</sup>) with



**Figure 5. mRNA levels of secreted murine serpin3, NE, CG, VCAM-1, and CXCR4 at the peak of HPC mobilization induced by G-CSF or CY.** mRNA levels were measured by real-time RT-PCR from BM cells isolated from mice injected for 4 d with saline (Sal) or G-CSF (G) or at day 6 after a single CY injection (Cy). Results are expressed as mRNA level relative to mRNA for the cytoskeleton protein vimentin (on a log scale). Each symbol represents the mRNA level from a different mouse. Black bars represent average of each group. Fold decreases between saline-treated groups and G-CSF- or CY-treated groups, together with corresponding significance levels (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  and NS for nonsignificant) are indicated underneath.

one or two log less mRNA in more primitive Gr-1<sup>intermediate</sup> and Gr-1<sup>dim</sup> cells, respectively (Fig. 6 D). Importantly, at the peak of G-CSF mobilization, a dramatic and significant transcriptional down-regulation of *serpina1a-e* genes was observed when compared with saline-injected BM in all myeloid subsets. This transcriptional down-regulation was not just specific to *serpina1a-e* genes, but was also found with the secreted  $\alpha_1$ -antichymotrypsin *serpina3c* (mRNA concentration was 5-fold lower in BM neutrophils and 10-fold lower in more primitive myeloid cells) during mobilization. However, no change was found for other mRNAs transcribed by these cells during mobilization. For example, although transcription of the protease CG is higher in more primitive myeloid cells than in mature BM neutrophils (consistent with packaging in primary azurophil granules), administration of G-CSF was not found to alter CG transcript levels in any of the three myeloid cell populations sorted from the BM (Fig. 6 E).

A similar 100–1,000-fold decrease in *serpina1a-e* mRNA levels was also observed during mobilization for other sorted BM cell populations such as B220<sup>+</sup> B lymphocytes, CD4<sup>+</sup>

**Table I.** Murine serpin genes and primer sequences

	Old nomenclature	Sequence of reactive loop, P <sub>1</sub> P <sub>1</sub> '	NH <sub>2</sub> -terminal secretion sequence	Oligonucleotide sequences for real-time RT-PCR	RT-PCR BALB/c BM
<i>Serpina1</i>					
<i>Serpina1a</i>	Spi1-1	MS <sup>a</sup>	Yes	f 5'-gaagctgcagcagctacagtc-3' 5'-tgtgggatctaccactttcc-3'	+
<i>Serpina1b</i>	Spi1-2	MS <sup>a</sup>	Yes	"	"
<i>Serpina1c</i>	Spi1-3	YS	Yes	"	"
<i>Serpina1d</i>	Spi1-4	YS	Yes	"	"
<i>Serpina1e</i>	Spi1-5	LS <sup>b</sup>	Yes	"	"
<i>Serpina3</i>					
<i>Serpina3a</i>	Unknown1	QS	Yes		ND
<i>Serpina3b</i>	6A1	MS <sup>a</sup>	Yes	f 5'-TCATGTCTGCAAACTTAAAC-3' 5'-TGTGGGGTTGATAACCTTCC-3'	- <sup>c</sup>
<i>Serpina3c</i>	1A1	LS <sup>b</sup>	Yes	f 5'-CTTAGTAGAAGAACCAGTCTG-3' 5'-CTTAGGGTGAGTGATTTTGGC-3'	+
<i>Serpina3d</i>	2B1	LS <sup>b</sup>	Yes	f 5'-AGATTCAAATTTGCCCCACTG-3' 5'-CTTGGGATCAAGACTTTCAC-3'	+
<i>Serpina3e</i>	2B2(b)	RC	?	f 5'-AGGAGTTAAAGTTAATCTACG-3' 5'-CTTGGGATTTGTAACCTTGGC-3'	+ <sup>d</sup>
<i>Serpina3f</i>	2A1	CC	No	f 5'-ATCTCCAATGTTGTCAAGGTG-3' 5'-TG(T/G)AACTTTTCCATAAAGAG-3'	- <sup>c</sup>
<i>Serpina3g</i>	2A2	CC	No	f 5'-CAGGAATGGCAGGTGTCGG-3' 5'-TG(T/G)AACTTTTCCATAAAGAG-3'	+ <sup>c</sup>
<i>Serpina3h</i>	6C28	CC	No	f 5'-CCACAGGGTCAAATTAATTC-3' 5'-CTTGGGATTTGTAACCTTGGC-3'	+
<i>Serpina3i</i>	2B2	RC	No	f 5'-AGGAGTTAAAGTTAATCTACG-3' 5'-CTTGGGATTTGTAACCTTGGC-3'	+ <sup>d</sup>
<i>Serpina3j</i>	Unknown2	LS <sup>b</sup>	Yes		ND
<i>Serpina3k</i>	MMCM	RK	Yes	f 5'-GTTATTGGTGGCATTTCGTAAG-3' 5'-CTTGGGGTTA(T/G)TGACTTTGGC-3'	+
<i>Serpina3l</i>	3E2	QS	Yes		ND
<i>Serpina3m</i>	3e46	RS	Yes	f 5'-GCTTCGTTCTAGAAGATTAC-3' 5'-CTTGGGGTTA(T/G)TGACTTTGGC-3'	+ <sup>c</sup>
<i>Serpina3n</i>	EB22.4	MS <sup>a</sup>	Yes	f 5'-CAATGTCTGCGAAACTGTACC-3' 5'-TTGGGGTTGGCTATCTTGGC-3'	+

<sup>a</sup>"MS" is the canonical human  $\alpha_1$ -antitrypsin-reactive center loop P<sub>1</sub>P<sub>1</sub>'.

<sup>b</sup>"LS" is the canonical human  $\alpha_1$ -antichymotrypsin-reactive center loop P<sub>1</sub>P<sub>1</sub>'.

<sup>c</sup>Denotes that PCR was performed with a lower annealing temperature of 55°C.

<sup>d</sup>Oligonucleotides for *serpina3e* and *serpina3i* do not distinguish between these two transcripts.

and CD8<sup>+</sup> T lymphocytes, and total CD45<sup>+</sup> hematopoietic cells (unpublished results).

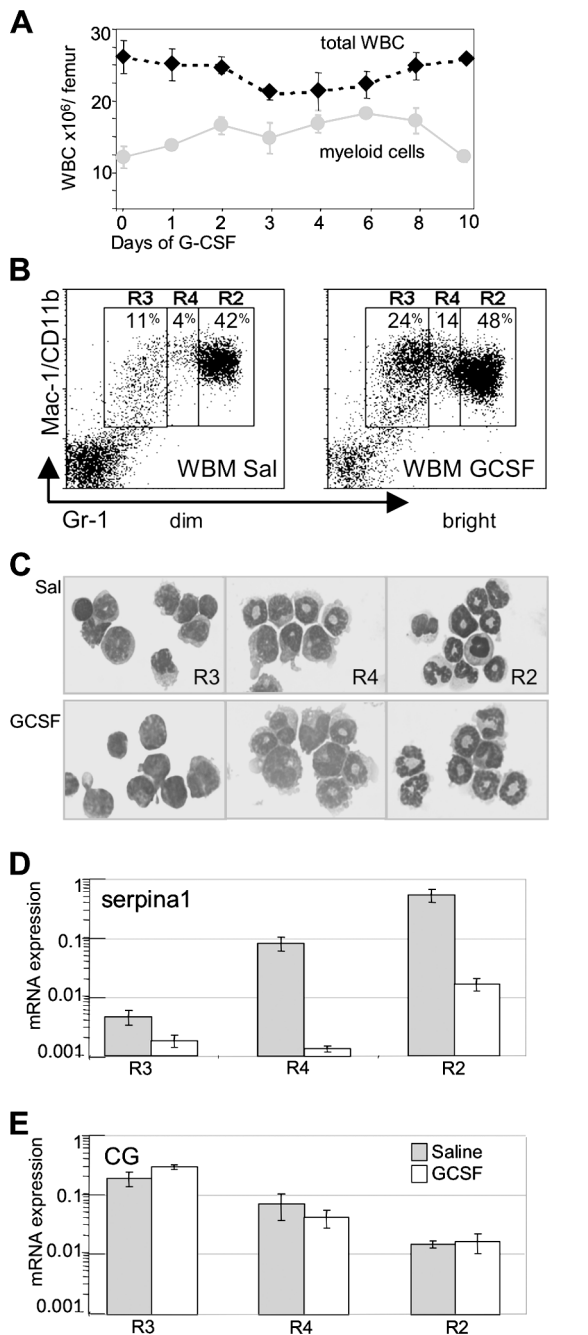
### Decreases in *serpina1* transcripts is restricted to the BM of mobilized mice

During mobilization, a large number of the mobilized HPCs home to the spleen. We therefore queried whether the drop in *serpina1* and *serpina3* transcripts during mobilization was a general phenomenon or restricted to the BM. For this purpose, we analyzed *serpina1* mRNA levels in liver, BM, and spleen by real-time RT-PCR and confirmed these results at the protein level by immunohistochemistry of tissue sections.

SERPINA1 and SERPINA3 proteins are present in the blood at high concentrations at 1.5 and 0.5 mg/ml, respec-

tively, in humans (28). They are mainly produced by the liver and released into the blood. The regulation of expression has not been exhaustively studied in the mouse. As expected, we found high levels of *serpina1a-e* mRNAs (Fig. 7 A, left) and *serpina1* protein expression (Fig. 7 B, left) in liver cells, with no change in mRNA or protein expression during G-CSF-induced mobilization. Conversely, *serpina1a-e* transcripts dropped dramatically (1,000-fold) in mouse BM during G-CSF-induced mobilization (Fig. 7 A) with a corresponding sharp drop in protein levels (Fig. 7 B, middle).

Interestingly, although *serpina1a-e* transcription in the spleen of mice was minimal, it increased during mobilization (Fig. 7 A). This finding was confirmed at the protein level by immunohistochemistry showing accumulation of cell clusters producing *serpina1* proteins within the myeloid areas of the



**Figure 6. Serpina1 transcripts are decreased in all BM mono/myeloid populations during HPC mobilization.** (A) Number of nucleated cells and Mac-1<sup>+</sup> (CD11b<sup>+</sup>) mono/myeloid cells in the femoral BM during the course of G-CSF administration. Data represent mean  $\pm$  SD from six to nine mice per group. (B) Phenotypic expression and sorting of primitive mono/myeloid Mac-1<sup>+</sup> Gr-1<sup>dim</sup> cells (gate R3), immature myeloid Mac-1<sup>+</sup> Gr-1<sup>intermediate</sup> cells (gate R4), and mature Mac-1<sup>+</sup> Gr-1<sup>bright</sup> neutrophils (gate R2) from mouse BM after 4 d of saline (left) or G-CSF (right) injection. Percentages in sorting gates indicate the proportion of these cells among total BM-nucleated cells. (C) Giemsa staining of the R3, R4, and R2 populations sorted as above. Top row corresponds to cells sorted from BM of saline-injected mice whereas the bottom row corresponds to cells sorted from the BM of G-CSF-injected mice. (D) Serpina1 mRNA expression

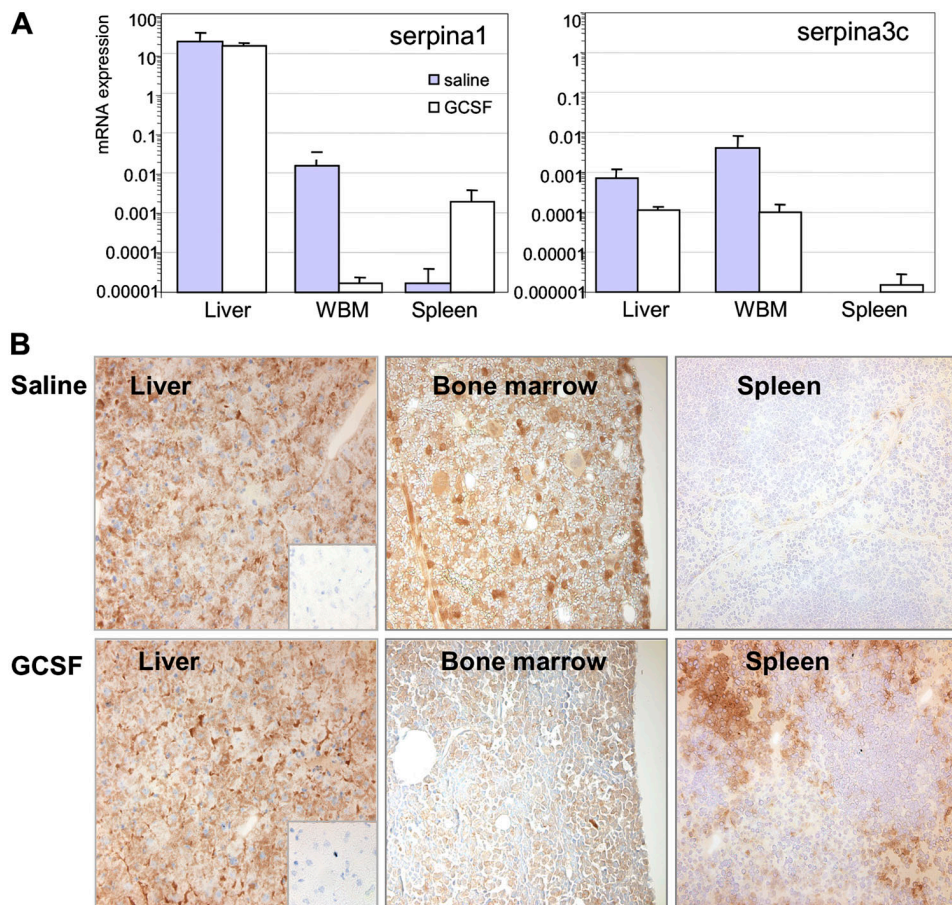
spleen from mobilized mice but barely detectable in the spleens from nonmobilized mice. However, no increase in *serpina1a–e* mRNA was found in Gr-1<sup>+</sup> cells sorted from spleens of mobilized versus nonmobilized mice (unpublished results) suggesting that the increase in *serpina1* protein and mRNA levels in the spleen after the administration of G-CSF is most likely due to the migration of serpin-producing cells (such as myeloid cells) from the BM into the spleen. This is supported by the findings that the spleen of saline-injected mice contained  $6 \pm 2\%$  Gr-1<sup>+</sup> neutrophils increasing to  $25 \pm 5\%$  Gr-1<sup>+</sup> neutrophils after 4 d of G-CSF. When absolute numbers are considered, this corresponds to  $5 \pm 2 \times 10^6$  neutrophils/spleen in nonmobilized animals compared with  $38 \pm 7 \times 10^6$  neutrophils/spleen with mobilization.

RT-PCRs using primers for *serpina3c* revealed a similar phenomenon, that is a drop in mRNA levels in the BM and increased mRNA concentrations in spleen during mobilization (Fig. 7 A, right). In the liver, mobilization also resulted in a 10-fold decrease in *serpina3c* transcripts.

## DISCUSSION

In this paper we report that steady-state BM contains a molar excess of *serpina1* compared with the serine proteases NE and CG. As serpins irreversibly block serine proteases with 1:1 stoichiometry (19, 20), this concentration of *serpina1* is sufficient to inhibit neutrophil serine proteases released in steady-state BM. Furthermore, we report that mobilization is associated with a sharp drop in the concentration of both *serpina1* protein and mRNA levels in the BM, which may contribute to the accumulation of active NE and CG within this tissue. The decrease in intact 53-kD *serpina1* protein in mobilized BM was not accompanied by a rise in *serpina1*–protease complexes nor in cleaved *serpina1* levels, which normally occurs when *serpina1* is cleaved by serine proteases. Therefore our data suggests that the decrease in transcription of endogenous *serpina1* in mobilized BM was not compensated by liver-derived *serpina1* diffusion from the blood. Taken together, these data support the hypothesis that the rise in NE and CG proteolytic activity in the BM during mobilization is likely to be due to the dual effects of both an increase in neutrophil activation as well as a decrease in the replenishment of serine protease inhibitors such as *serpina1* and *serpina3*. Furthermore after stopping G-CSF treatment, a rebound in *serpina1* and *serpina3* mRNA and protein levels in the BM is observed possibly contributing to the cessation of mobilization. Of note, we have also found similar results in other inbred mouse strains such as C57BL6/J, C3H/HEJ, and 129SvJ confirming that this phenomenon is not restricted to a particular mouse strain (unpublished results).

levels in R3, R4, and R2 populations sorted from the BM of mice injected for 4 d with saline (gray columns) or G-CSF (white columns). Results are expressed as mRNA concentration relative to vimentin mRNA on a log scale. (E) Cathepsin G (CG) mRNA expression levels in R3, R4, and R2 populations sorted from the BM of mice injected for 4 d with saline (gray columns) or G-CSF (white columns). Results are expressed as mRNA concentration relative to vimentin mRNA on a log scale.



**Figure 7. Down-regulation of serpina1 is restricted to the BM during HPC mobilization.** (A) Serpina1a–e (left) and serpina3c (right) mRNA levels were measured in liver, whole BM, and spleen cells from mice injected for 4 d with saline (gray columns) or G-CSF (white columns). Results are expressed as mRNA concentration relative to vimentin mRNA on a log scale. (B) Immunohistochemical staining of liver, BM, and spleen sections with a

rabbit anti-human SERPINA1 antibody cross-reacting with mouse serpina1. In the top row are sections from mice injected for 4 d with saline whereas in the bottom row are sections from mice injected for 4 d with G-CSF. The small inserts shown in the liver sections are negative controls performed with nonimmune control rabbit IgG used at the same concentration as the rabbit anti-human SERPINA1. All sections are magnified  $\times 200$ .

Interestingly, hepatic transcription of *serpina1* was not altered during HPC mobilization, resulting in constant blood plasma levels. This would explain in part why there is no active NE or CG in the blood and no cleavage of VCAM-1 on the lumen of BM sinuses that are in direct contact with blood (9). VCAM-1 expression in the spleen does not change during mobilization (9). In this study using immunohistochemistry as well as real-time RT-PCR, we show that cells producing serpina1 proteins accumulate in the spleen during mobilization, possibly protecting VCAM-1 from proteolytic cleavage by neutrophil serine proteases in this tissue.

The human genome has only one copy of the *SERPINA1* and *SERPINA3* genes and their target protease specificity is well established (29). Both human SERPINA1 and SERPINA3 proteins can inhibit NE and CG in vitro, however human SERPINA1 preferentially targets NE (with the reactive center loop P<sub>1</sub>P<sub>1</sub>' amino acids "MS"; 19) whereas SERPINA3 preferentially targets CG (with reactive center loop P<sub>1</sub>P<sub>1</sub>' amino acids "LS"; 20). The reactive center loop

of many of the murine serpina1 and serpina3 proteins have diverged (30), although several still exhibit the canonical  $\alpha_1$ -antitrypsin reactive center loop (murine serpina1a, serpina1b, serpina3b, and serpina3n) whereas others have the canonical  $\alpha_1$ -antichymotrypsin-reactive center loop (murine serpina1e, serpina3c, serpina3d, and serpina3j; Table I). Therefore members of both the murine serpina1 and serpina3 families are likely to be involved in the inhibition of serine protease activity in steady-state BM as shown in Fig. 1.

An interesting finding of this study is that although the level of serpina1a–e transcripts dropped dramatically in the BM of mice during mobilization, hepatic mRNA levels did not alter resulting in no changes in plasma serpina1 concentrations. Earlier studies on human *SERPINA1* transcription/expression reveal five alternative transcription starts can be used depending on the tissue in which expression occurs (with three transcription start sites in leukocytes, one in cornea and one in hepatocytes; 31). In this study we found the reduction in serpina1 transcripts was specific to BM leukocytes during

mobilization whereas hepatic transcription remained unchanged. Although we cannot exclude mechanisms involving mRNA destabilization, this suggests that different tissue-specific *serpina1* transcriptional start sites are used by murine leukocyte and/or hepatic cells. In contrast to *SERPINA1*, the human *SERPINA3* gene appears to use a single promoter regardless of tissue-type in which it is expressed (31).

Both human *SERPINA1* and *SERPINA3* genes are reported to be “acute phase proteins” as expression increases two- to fivefold in the liver during inflammation leading to increased levels of SERPINA1 and SERPINA3 proteins in plasma (32, 33). Whether a similar increase in the transcription of hepatic murine *serpina1* occurs during inflammation remains controversial (34, 35). A few studies have reported that several cytokines (such as IL-6, IL-1 $\beta$ , or TNF- $\alpha$ ) increase *SERPINA1* transcription in human monocytes (36, 37). However, to our knowledge, no down-regulation of human or mouse SERPINA1 or SERPINA3 in response to cytokines/growth factors has been reported previously in leukocytes or even hepatocytes.

An intriguing finding of this study is that the same murine BM Gr-1<sup>+</sup> myeloid cells contain large amounts of serine proteases as well as large amounts of serine protease inhibitors, which would suggest that the serpins and proteases, although present in the same cells, are stored in different compartments. This is likely to be the case as *serpina1* transcription is greatest in BM neutrophils, whereas serine protease (NE and CG) transcription was highest in primitive mono/myeloid cells (Fig. 6). It is known that in granulocyte precursors, the timing of transcription determines in which granules the protein products are stored (25, 38). For instance, the serine proteases synthesized by primitive myeloid cells are packaged predominantly in primary azurophil granules, whereas granule proteins synthesized by more mature BM neutrophils are more likely packaged in secondary or tertiary granules (39). The release of these distinct granules by a neutrophil occurs at different stages during the inflammatory process (26), so although contained within the one cell, both the serine proteases and their specific inhibitors may be released separately. Alternatively, serpins may be constitutively secreted with the proteases remaining sequestered in granules.

In conclusion, our study is the first to suggest a novel role of *serpina1* and *serpina3* proteins in regulating the homeostasis of the hematopoietic microenvironment, which ultimately determines the fate of hematopoietic stem cells. This conclusion is strengthened by other studies using genetic linkage analysis showing that the *Aat* locus containing the *serpina1* genes, is significantly linked to BM cellularity in inbred mouse strains (40). As reported previously, many proteoglycans (41), extracellular matrix proteins (42–44), cytokines, and chemokines (45, 46) expressed in the BM hematopoietic microenvironment and playing a major role in determining the fate of hematopoietic stem cells, are substrates of the neutrophil serine proteases that these serpins inhibit. Similarly, an earlier report has shown that the addition of either recombinant human SERPINA1 or antileukoproteinase

to serum-free culture media boosts in vitro proliferation and survival of HPCs, probably by protecting cytokines from the proteases released by differentiated leukocytes (47). Therefore we propose that (a) *serpina1* and *serpina3* proteins play a major role in maintaining the molecular and functional integrity of the BM hematopoietic microenvironment, (b) the stoichiometric balance between these serpins and neutrophil serine proteases is inverted locally in the BM during systemic administration of G-CSF or chemotherapy, and (c) the reversal of the serpin–protease balance allows proteases to evade their naturally occurring inhibitors, accumulate in active form and to contribute to the mobilization of HPCs.

## MATERIALS AND METHODS

**Mobilization of mice, tissue collection, and clonogenic assays.** All procedures on animals have been approved by the Animal Experimentation Ethics Committee of the Peter MacCallum Cancer Centre. Male 10–12-wk-old BALB/c mice were injected with 125  $\mu$ g/kg recombinant human G-CSF (Neupogen Filgrastim; Amgen) twice daily (days 0–5) or an equal volume of saline (control mice). Mice were harvested at days 2, 4, and 6 after G-CSF injection or allowed to rest and harvested at days 8 and 10. Cyclophosphamide-treated mice received a single intraperitoneal injection of 200 mg/kg CY (Amersham Biosciences) on day 0.

Peripheral blood was collected in EDTA. Plasma was separated and red cells lysed with 10 mM NaHCO<sub>3</sub>, pH 7.4, and 150 mM NH<sub>4</sub>Cl. Nucleated cells were plated in double-layer nutrient agar clonogenic cultures (1,500 cells/dish) in the presence human IL-1 $\alpha$ , mouse IL-3, mouse CSF-1, and rat KIT ligand as described previously (9). Colonies were scored after a 2-wk culture at 37°C in the presence of 5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>.

The BM content of one femur from each mouse was flushed into 1 ml PBS on ice. After centrifugation for 5 min at 400 g, the supernatant fraction (BM extracellular fluid) was removed and used directly in Western blots or for the digestion of recombinant human VCAM-1. The BM cell pellet, as well as the liver and spleen were used for RNA extraction, immunostaining and cell sorting, or to generate frozen sections.

**Flow cytometry and cell sorting.** Bone marrow and spleen cells were stained using 1  $\mu$ g/ml of directly conjugated mAb (BD Biosciences) for myeloid cells (Gr-1-FITC and CD11b-PE), for B lymphocytes (B220-PE), for T lymphocytes (mix of CD3-FITC, CD4-FITC, CD5-FITC, and CD8a-FITC), and for total hematopoietic cells (CD45-PE). Cells positive for each category were sorted and RNA extracted as described below.

**RNA extraction and quantitative real-time RT-PCR.** Total RNA was extracted using Trizol (Invitrogen). After DNase treatment and reverse transcription using random hexamers, quantitative real-time PCR with SYBR green (ABI Systems) was performed after manufacturer’s instructions using the oligonucleotide combinations shown in Table I. RNA levels were standardized by parallel RT-PCRs using primers to two different house-keeping genes, vimentin (a cytoskeletal protein, forward primer 5′-CACCTGCA-GTCATTCAGACA-3′; and reverse 5′-GATTCCACTTTCCGTTCAA-GGT-3′) and  $\beta_2$ -microglobulin (forward primer 5′-TTCACCCCACT-GAGACTGAT and reverse 5′-GTCTTGGGCTCGGCCATA-3′). Additional RT-PCRs for the serine proteases NE (forward primer 5′-ACCCTCAT-TGCCAGGAAGCTTC-3′ and reverse 5′-CCTGCACTGACCGGAAATTT-3′), CG (forward primer 5′-AGGCAGGGAAGATCATTGGA-3′ and reverse 5′-TGGATCAGAAGAAATGCCATGT-3′) as well as VCAM-1 (forward primer 5′-CTGGGAAGCTGGAACGAAGTA-3′ and reverse 5′-GCCACTGAATTGAATCTCTGGAT-3′) and CXCR4 (forward primer 5′-GGCTGACTGGTACTTTGGGAAA-3′ and reverse 5′-CCGGTCCAGGCTGATGAA-3′) were also performed. A PCR from each sample before reverse transcription was also performed to confirm the absence of contaminating genomic DNA.

**VCAM-1 cleavage and immunoblotting.** BM extracellular fluids collected from three mice at day 4 of G-CSF-induced mobilization were pooled and mixed with an equal volume (10  $\mu$ l) of either pooled BM fluids from three nonmobilized mice or with PBS. Where indicated, protease inhibitors were added at the following concentrations: 1 mg/ml human  $\alpha$ 1-antitrypsin (Sigma-Aldrich), 1  $\mu$ M BB-94/Batimastat (British Biotech Pharmaceuticals), 1 mM *O*-phenantroline. After a 20-min incubation at room temperature, 10 ng recombinant human VCAM-1 (98-kD glycosylated extracellular domain; R&D Systems) was added and the mixture reincubated at 37°C for 20 min. An equal volume of loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS) was then added and samples boiled for 3 min, before separation by electrophoresis on 10% SDS-PAGE and transfer onto nitrocellulose membrane. Membranes were immunoblotted using a goat anti-human VCAM-1 antibody as described previously (9, 10).

**Immunoblotting for serpin1 and  $\alpha$ <sub>2</sub>-macroglobulin, and measurements of serpin1 concentration.** 10  $\mu$ l aliquots of pooled BM extracellular fluids at indicated time-points of G-CSF or saline administration were mixed with an equal volume of loading buffer with 10 mM dithiothreitol, boiled for 3 min, and separated by electrophoresis on 10% SDS-PAGE. After transfer onto nitrocellulose membranes and blocking in Odyssey blocking buffer, membranes were incubated for 1 h with an anti-human  $\alpha$ <sub>1</sub>-antitrypsin (SERPINA1) Ab cross-reacting with mouse serpin1 diluted 1/3,000 in Odyssey blocking buffer with 0.5% Tween 20. After extensive washes with PBST, membranes were incubated with a 1/3,000 dilution of Alexa Fluor 680-conjugated goat anti-rabbit IgG (Molecular Probes). Visualization and quantification were performed on the Odyssey Infrared Imaging System (LI-COR Biosciences) equipped with two solid phase lasers at 680 and 800 nm with a resolution of 169  $\mu$ m. The integrated intensity of the serpin1 band in the samples was compared with that of mouse plasma serpin1 and the moles per femur calculated, assuming the concentration of murine serpin1 in blood to be the same as in humans, 1.5 mg/ml (28), with a molecular weight of 53,000. Some experiments were performed with BM extracellular fluids from three individual mice from each time-point group and average concentration  $\pm$  SD were calculated.

Immunoblotting for  $\alpha$ <sub>2</sub>-macroglobulin were performed by incubating the membranes with a 1/3,000 dilution of a rabbit anti-human  $\alpha$ <sub>2</sub>-macroglobulin (Sigma-Aldrich) followed by an incubation with a 1/10,000 dilution of a horseradish peroxidase-conjugated donkey F(ab)'<sub>2</sub> anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Blots were revealed by enhanced chemoluminescence.

**Measurement of protease catalytic activity.** The concentrations of active NE and CG in BM extracellular fluids were measured using the chromogenic substrates methyl-*O*-succinyl (MetOSuc)-Ala-Ala-Pro-Val-paranitroanilide (pNA) and Suc-Ala-Ala-Pro-Phe-pNA (Calbiochem-Novabiochem) as described previously (9, 10). Measurements were calibrated using serial dilutions of purified human NE and CG (Elastin Products) and molar concentrations were calculated using molecular weight of 30,000 and 29,000 for murine NE and CG, respectively.

**Immunohistochemistry.** Frozen sections of mouse liver and spleen were air dried and then fixed for 30 min in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> to destroy endogenous peroxidases. Slides were then blocked in 4 $\times$  SSC containing 5% BSA, 5% skim milk powder, and 0.05% Triton X-100 for 2 h before incubation with either 25  $\mu$ g/ml purified rabbit anti-human  $\alpha$ <sub>1</sub>-antitrypsin or nonimmune-purified rabbit IgG in PBST with 5% skim milk and 5% BSA overnight at 4°C. After extensive washes, slides were incubated with a 1/500 dilution of horseradish peroxidase-conjugated donkey F(ab)'<sub>2</sub> anti-rabbit IgG with minimal cross-reactivity to mouse proteins for 2 h at room temperature, and then washed. Staining was revealed using 0.5 mg/ml 3,3'-diaminobenzidine in 50 mM Tris-HCl, pH 7.4, 0.3% H<sub>2</sub>O<sub>2</sub> before counterstaining with hematoxylin and mounting with Aquamount (BDH).

For BM wax-embedded sections, femoral BM was fixed by perfusing 0.05% glutaraldehyde, 2% paraformaldehyde into the descending femoral aorta and femurs processed exactly as described by Nilsson et al. (48). Longitudinal 3.5- $\mu$ m sections were cut, dewaxed in xylene, and then rehydrated using progressive baths from ethanol to water. After antigen retrieval (12 min boiling in 10 mM sodium citrate, pH 6.0), endogenous peroxidase elimination and antigen labeling were performed as described above.

**Statistics.** Levels of significance were calculated using the nonparametric Mann-Whitney test.

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