TIMP-1 signaling via CD63 triggers granulopoiesis and neutrophilia in mice

Julia Kobuch,[1] Haissi Cui,[1] Barbara Grünwald,[1] Paul Saftig,[2] Percy A. Knolle,[1] and Achim Krüger[1]

[1]Institut für Molekulare Immunologie und Experimentelle Onkologie, Klinikum rechts der Isar, Technische Universität München; and [2]Institute of Biochemistry, Christian-Albrechts-Universität zu Kiel, Germany

ABSTRACT

The homeostasis of neutrophil granulocytes can affect the outcome of several inflammation-associated diseases including cancer. The regulation of this homeostasis is still not completely understood. We previously found that elevated systemic levels of tissue inhibitor of metalloproteinases-1 (TIMP-1) induce an increase of neutrophils in the liver, which in turn strongly promotes liver metastasis. Here, we report that increasing systemic TIMP-1 levels were sufficient to induce neutrophilia in mice. This was not attributed to prolonged survival or direct mobilization of neutrophils. However, TIMP-1 induced enrichment of myeloid progenitors and concomitant upregulation of granulopoiesis-associated genes in the bone marrow compartment. BrdU pulse-labeling confirmed that proliferating progenitors accounted for TIMP-1-induced neutrophilia. TIMP-1 variants that dissect its protease-inhibitory from its CD63 binding function relevant for cell signaling revealed that the TIMP-1 signaling domain was necessary and sufficient to augment granulopoiesis. Consequently, ablation of the TIMP-1 receptor CD63 abolished both neutrophilia and TIMP-1-enhanced granulopoiesis in the bone marrow. Our findings reveal that elevated levels of TIMP-1 impact on neutrophil homeostasis via signaling through CD63. This may provide a link to clinical observations, where TIMP-1 correlates with high severity and bad prognosis in inflammation-associated diseases.

Introduction

Activation of neutrophils can cause severe tissue damage due to the destructive capacity of these cells.[1] A fine-tuned regulation of the number of circulating neutrophils together with local control of neutrophil activation is important to balance pathogen defense, inflammation and tissue integrity. Imbalances are associated with infection and a number of inflammatory diseases including rheumatoid arthritis,[2] acute myocardial infarction,[3] sepsis,[4] or cancer.[5] In patients suffering from these diseases, TIMP-1 plasma levels are often elevated, and correlate with poor prognosis.[6-8]

TIMP-1 is a natural endogenous inhibitor of most secreted matrix metalloproteinases (MMPs) and the membrane-bound metalloproteinase ADAM-10.[9] MMPs are major players in remodeling and degradation of extracellular matrix and thereby regulate tissue composition and structure, the release of sequestered cytokines or growth factors, and are also involved in the shedding of cell surface molecules.[10] TIMP-1 as one important regulator of MMP activity can thus indirectly influence all these processes, thereby impacting on tissue homeostasis and cell behavior.[11] In addition, TIMP-1 exhibits a protease inhibition-independent cytokine-like function by directly inducing cell signaling via its receptor, the tetraspanin CD63.[12,11] The TIMP-1 protein consists of two separately folding domains.[9] While direct signaling interaction with CD63 requires the intact C-terminal domain, protease binding takes place with the N-terminal domain, which is sufficient to inhibit most MMPs.[9] Due to this two-pronged functional activity, TIMP-1 exerts a wide range of effects on tissue homeostasis, as well as cell behavior including inhibition of apoptosis,[13] promotion of cell proliferation and differentiation or the metastatic potential of tumor cells.[14,15] Separate investigation of the two functions of TIMP-1 can be achieved by employing variants either lacking the C-terminus (N-TIMP-1[9]) or with mutations in the N-terminal domain that diminish protease binding but retain signaling capacity (TIMP-1/T2G,[16] vvTIMP-1[17]). Although investigation of such variants helped to explain physiological effects of TIMP-1 *in vitro*, so far only limited knowledge is available on the relevance of signaling or protease inhibition for tissue homeostasis.

In patients, elevated TIMP-1 plasma levels are observed in inflammation-associated malignancies including fibrosis,[18] severe inflammatory response syndrome (SIRS) or sepsis,[7,19] artheriosclerosis[20] and cancer.[8] Especially in cancer, high systemic TIMP-1 levels are increasingly recognized as a bad prognostic factor, despite its inhibitory effect on tumor-promoting MMPs.[8,21] We have previously shown that high systemic TIMP-1 levels promote liver metastasis by altering the hepatic microenvironment, resulting in a strongly increased susceptibility of the liver towards metastasis of circulating tumor cells.[22] Neutrophils were shown to be involved in this enhanced metastasis.[22] High numbers of neutrophils are increasingly recognized to promote cancer progression, although they were originally believed to trigger cytotoxic responses against tumor cells.[23] Cancer-associated inflammation has recently been suggested to be a new hallmark of cancer[24] and clinical studies underline the critical role of neutrophils during cancer.[5] In fact, tissue-arrested neutrophils support tumor growth, angiogenesis, and metastasis by secreting cytokines, proteases[23] or neutrophil extracellular traps.[25] Furthermore, increase of metastasis was described to be associated with neutrophil interaction with circulating tumor cells in the blood stream.[26] It is still unclear at which level TIMP-1 impacts on neutrophils in the context of the TIMP-1-induced pre-metastatic niche in the liver.[22] In the cur-

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Correspondence: achim.krueger@lrz.tum.de

rent study, we investigated whether TIMP-1 affects neutrophil homeostasis. We show that TIMP-1 signaling via CD63 triggered granulopoiesis in the bone marrow (BM) resulting in increased systemic neutrophil blood counts. Our findings reveal a new function of TIMP-1 on immune cell homeostasis, and may provide a link to the frequently described correlation of high TIMP-1 levels with inflammatory diseases in the clinic.

Methods

Animal experiments

Animal experiments were performed in compliance with the guidelines of the Tierschutzgesetz des Freistaates Bayern and approved by the Regierung von Oberbayern. For TIMP-1-secreting tumors, $2x10^6$ HT1080 cells were subcutaneously injected into the nuchal fold of $CD1^{nu/nu}$ mice and tumors were grown over ten days to a diameter of approximately 1 cm. Tumor size was monitored with a caliper. For adenoviral transduction, $3x10^9$ ifu were intravenously injected, as previously described.[22] Recombinant TIMP-1 protein was applied in LPS-free PBS by a single intraperitoneal (i.p.) injection of 2 mg/kg, as previously described.[22] Mice were killed with CO_2, blood was collected from the *vena cava inferior* using EDTA-coated syringes, and BM cells were obtained by flushing femurs and tibias with PBS.

Flow cytometry

Absolute quantification of blood neutrophils occurred in BD trucount™ tubes according to the manufacturer´s instructions. Briefly, antibody-cocktail was mixed with 50 µL fresh blood in trucount™ tubes and incubated for 15 min at RT. Subsequently, 450 µL FACS™ lysing solution (BD Bioscience) were added and incubated for 30 min at RT to lyse erythrocytes and fix the sample. For staining of BM, femurs and tibias were flushed with PBS and the obtained cell suspension was stained as blood. Samples were measured on a FACS Canto™II flow cytometer (BD) and analyzed using FlowJo software.

Immunohistochemical staining of neutrophils

For immunohistochemical analysis of BM, femurs were freed from tissue, fixed in 4% paraformaldehyde for 20 h, de-calcified in 0.5 M EDTA for five days, dehydrated and embedded into paraffin. Ly6G-positive cells were stained on 4 µm sections, as previously described.[22]

Colony formation assay

Bone marrow cells were harvested from AdCtrl or AdT1-transduced mice and erythrocytes were removed with RBC lysis buffer (eBioscience). $1x10^4$ BM cells were seeded in MethoCult™ medium (StemCell Technologies) containing 50 ng/mL murine rSCF, 10 ng/mL murine rIL-3, and 10 ng/mL murine rIL-6 into 6-well plates. Cells were incubated at 37°C for seven days and colonies per well were counted. For each mouse, analysis was performed in duplicates, and a total of 5 mice per group were analyzed.

32Dcl3 differentiation assay

32Dcl3 cells were seeded at a density of $8x10^5$ cells/mL in IL3-free medium supplemented with 100 ng/mL murine G-CSF (Peptrotech) +/- 1000 ng/mL rTIMP-1. Every two days, medium was replaced by fresh medium containing 50 ng/mL G-CSF +/- 1000 ng/mL rTIMP-1. At the indicated time points, cells were collected and spun on glass slides for QuickDiff staining. The percentage of differentiated cells was calculated by counting cells with segmented nuclei and total cells per image.

In vivo *BrdU assay*

DBA/2 mice were transduced with AdCtrl or AdT1. A single BrdU (Sigma Aldrich) dosage of 2 mg was i.p. injected three days post adenoviral transduction and 1 h before sacrifice to pulse-label proliferating neutrophil precursors. Newly generated mature neutrophils were identified by repeated BrdU injection starting 3 h after transduction with 2 mg and then 1 mg every 12 h. At day 3, mice were sacrificed, and blood and BM cells collected. Staining of BrdU-positive cells occurred after staining of cell surface markers, using FITC-BrdU Staining Kit (eBioscience). To determine background levels for gating of BrdU-negative cells, one mouse was treated with PBS instead of BrdU.

Statistical analysis

Data were tested for normal distribution and analyzed by Student *t*-test, which was preceded by one-way ANOVA, if more than two groups were compared.. $P<0.05$ was considered significant.

Results

TIMP-1 plasma levels positively correlate with peripheral blood neutrophils

Based on our reasoning that TIMP-1 is involved in neutrophil homeostasis, we analyzed peripheral blood neutrophils in mice bearing tumors that secrete different amounts of TIMP-1. We chose the human fibrosarcoma cell line HT1080, which endogenously expresses high TIMP-1 levels but does not metastasize.[22] HT1080 cells were lentivirally transduced either with a TIMP-1 overexpression ($T1^{high}$) or an anti-TIMP-1 shRNA ($T1^{low}$) vector, leading to strong upregulation or downregulation of TIMP-1 expression *in vitro* (*Online Supplementary Figure S1A*). Manipulation of TIMP-1 expression led to reduced or increased TIMP-1 plasma levels, respectively (*Online Supplementary Figure S1B*), but did not affect primary tumor growth (Figure 1A). Interestingly, flow cytometric quantification of blood neutrophils (*Online Supplementary Figure S2*) revealed a positive correlation between TIMP-1 plasma levels and the numbers of circulating neutrophils (Figure 1B). The same was observed for TIMP-1 secreted by NIH3T3 cells that grew to solid tumors upon subcutaneous injection (*Online Supplementary Figure S3C*), although endogenous TIMP-1 expression in this particular cell line also affected tumor growth (*Online Supplementary Figure S3B*).

To test whether this increase of neutrophils was a direct effect of TIMP-1, we employed a tumor cell-free model where we adenovirally transduced mice with control (AdCtrl) or TIMP-1 overexpression (AdT1) vectors. AdT1 infection of mice led to stably elevated TIMP-1 plasma levels (*Online Supplementary Figure S4*). Clearly, such systemic elevation of TIMP-1 was sufficient to significantly increase the numbers of blood neutrophils three days after AdT1 infection (Figure 1C). To demonstrate that neutrophilia was caused by TIMP-1 overexpression and not the immune response against viral infection or expression of a foreign protein, we injected adenovirus coding for N-TIMP-1/T2G, a mutant lacking both, the signaling and the protease-inhibitory function.[9] Neutrophil counts in AdCtrl- or AdN-T1/T2G-transduced mice were at the same level as in saline-treated mice (Figure 1D), indicating that functional TIMP-1 but not adenoviral infection itself induced neutrophilia. To substantiate our findings, we ele-

vated TIMP-1 levels in mouse strains of different genetic background. Although basal levels of blood neutrophils were slightly different in these strains, the TIMP-1-induced neutrophilia was consistently reproducible (Figure 1E). These findings indicate that high systemic levels of TIMP-1 are sufficient to increase the number of peripheral blood neutrophils.

Unchanged survival of circulating neutrophils in the presence of TIMP-1

Circulating neutrophils are short-lived and quickly undergo apoptosis under non-inflammatory conditions. Delayed neutrophil death leads to neutrophilia and is typically associated with exaggerated inflammatory responses.[27] As TIMP-1 is known to inhibit apoptosis in several cell types,[11] we first examined the possibility that TIMP-1-induced neutrophilia resulted from prolonged survival of circulating neutrophils. To this end, we isolated neutrophils from blood of adenovirally transduced mice, confirmed purity of more than 95% (*Online Supplementary Figure S5A*), and analyzed their viability over time *ex vivo* (Figure 2A). Furthermore, we incubated isolated neutrophils with recombinant TIMP-1 protein (rTIMP-1) (Figure 2B). In both experimental setups, no prolongation of neutrophil viability by TIMP-1 was observed. As apoptotic blood neutrophils stain positive for Annexin V,[27] we analyzed their abundance *in vivo* by flow cytometry. The portion of Annexin V-positive neutrophils was unaltered in mice expressing high TIMP-1 levels, and absolute numbers were even slightly raised (Figure 2C and D) indicating increased rather than delayed neutrophil turnover.

TIMP-1 does not induce neutrophil mobilization from storage pools

We next investigated whether TIMP-1 triggered neutrophil mobilization from the BM pool. To examine whether TIMP-1 exhibits a direct chemotactic effect, we isolated neutrophils from BM and analyzed migration *ex vivo* towards rTIMP-1 (and rCXCL6 as positive control) (*Online Supplementary Figure S5C*). No increased migration was observed towards medium supplemented with rTIMP-1 (Figure 3A). Furthermore, pre-incubation of BM neutrophils with rTIMP-1 did not increase their motility, as measured by migration towards FCS (Figure 3B). Finally, we tested *in vivo* whether systemic application of rTIMP-1 would induce an immediate mobilization of neutrophils into the blood. We quantified blood neutrophils 2 h after rTIMP-1 injection, a time point at which a response to any mobilization signal could be expected.[28] rTIMP-1 did not induce alterations in neutrophil blood counts (Figure 3C), indicating that TIMP-1 itself does not directly mobilize neutrophils. However, these results still do not

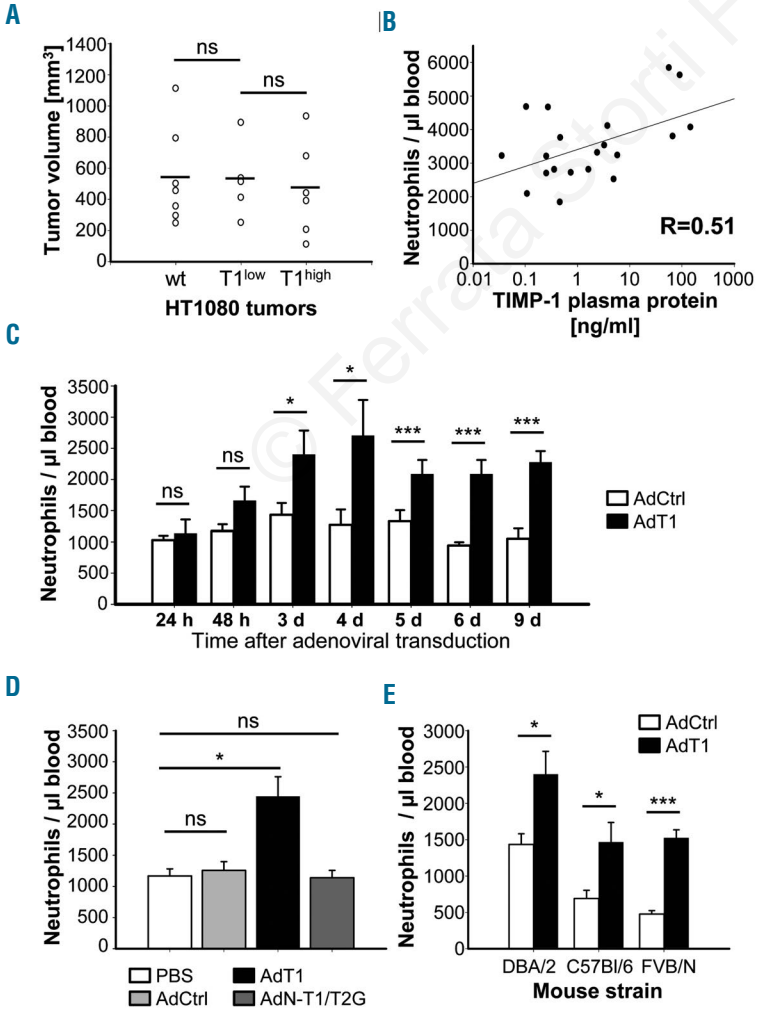

Figure 1. TIMP-1 plasma levels correlate with increased neutrophil counts in peripheral blood. (A) Volume of HT1080 tumors expressing different TIMP-1 levels. $2*10^6$ tumor cells were s.c. injected into the neck of CD1nu/nu mice and allowed to grow over ten days. (B) Correlation of TIMP-1 plasma levels with neutrophil blood counts in mice from (A). (C) Neutrophil blood counts in DBA/2 mice transduced to express high TIMP-1 levels. TIMP-1 coding (AdT1) or control (AdCtrl) adenovirus was i.v. injected into mice and peripheral blood neutrophils were quantified by flow cytometry at the indicated time points. (D) Neutrophil blood counts in DBA/2 mice three days after injection of adenovirus or PBS. (E) Neutrophil blood counts in different mouse strains three days after adenovirus injection. Columns: mean; Bars: SEM; Student's *t*-test. ns: not significant; $*P<0.05$; $***P<0.001$, n=5.

exclude the possibility that a TIMP-1-induced secondary effector triggered neutrophil release. Thus, we examined whether the number of neutrophils in typical storage pools would decrease over time. Immunohistochemical staining for the neutrophil marker Ly6G on BM sections revealed no reduction in AdT1 mice over time (Figure 3D and *Online Supplementary Figure S6A*). Flow cytometric analysis of BM leukocytes confirmed that the portion of mature neutrophils, characterized by high Ly6G surface expression, was unaltered (Figure 3E and *Online Supplementary Figure S6B*). In addition, neutrophil numbers in the spleen (*Online Supplementary Figure S7*) or the lung,[22] typical sites of marginated neutrophils, were unaltered. Taken together, these data indicate that neither TIMP-1 itself, nor any downstream effector induced immediate recruitment of mature neutrophils from storage sites.

TIMP-1 triggers granulopoiesis in the bone marrow

As neither reduced neutrophil turnover nor a decline within storage sites could explain TIMP-1-induced neutrophilia, we hypothesized that high systemic TIMP-1 levels enhance neutrophil generation. Indeed, neutrophil progenitors, characterized by intermediate Ly6G (Ly6G^{int}) surface levels (*Online Supplementary Figure S6B*), were enriched in the BM of AdT1-transduced mice (Figure 4A). As neutrophil maturation is characterized by sequential, differentiation stage-restricted expression of transcription factors[29,30] and granule proteins,[31,32] we examined expression of such genes in the BM. mRNAs for the surface marker Ly6G, the granule proteins myeloperoxidase (Mpo), lactoferrin (Ltf), and neutrophil elastase (Elane), as well as the granulopoiesis-regulating transcription factors C/EBPα, C/EBPβ, C/EBPε and PU.1, were up-regulated in AdT1 mice (Figure 4B). Furthermore, colony formation was higher with BM cells from these mice in *ex vivo* CFU assays (Figure 4C), indicating enhanced activity of myeloid progenitor cells. To check if TIMP-1 directly triggered proliferation of pre-mature neutrophils, we applied a single BrdU pulse to identify currently proliferating pre-mature neutrophils. Interestingly, no increase in BrdU-positive Ly6G^{int} cells was observed (Figure 4D), suggesting that TIMP-1 did not directly induce their proliferation, but rather shifted differentiation of early progenitors towards the granulocytic lineage.

We thus examined whether TIMP-1 was able to directly stimulate maturation of myeloid progenitors *in vitro*. The myeloid cell line 32Dcl3 is a well-established model system to study G-CSF-induced neutrophil maturation.[33] In this model, differentiated neutrophils can be identified by their characteristic segmented nuclei and the expression of proteins that are typical for neutrophils. Notably, rTIMP-1 alone was not sufficient to sustain viability of 32Dcl3 cells in the absence of IL-3 and G-CSF. However, stimulation with G-CSF in the presence of rTIMP-1 led to an increased portion of differentiated cells as quantified by CD11b surface expression (Figure 4E), segmentation of nuclei (Figure 4F), or expression of granulopoiesis-associated genes (*Online Supplementary Figure S8*).

Neutrophil generation from proliferating precursors accounts for TIMP-1-induced neutrophilia

To test whether enhanced generation of neutrophils accounted for the TIMP-1-induced neutrophilia in the periphery, we took advantage from the fact that mature circulating neutrophils do not proliferate.[34] Thus, BrdU would stain proliferating precursor cells only, allowing the differentiation of blood neutrophils that were mature or newly generated from progenitors at the time of BrdU application. We repeatedly injected BrdU into mice, commencing from the systemic elevation of TIMP-1, and analyzed blood neutrophils three days after transduction by flow cytometry (*Online Supplementary Figure S9A*). Indeed the portion of BrdU-positive blood neutrophils in AdT1 mice was significantly higher than in AdCtrl mice (Figure

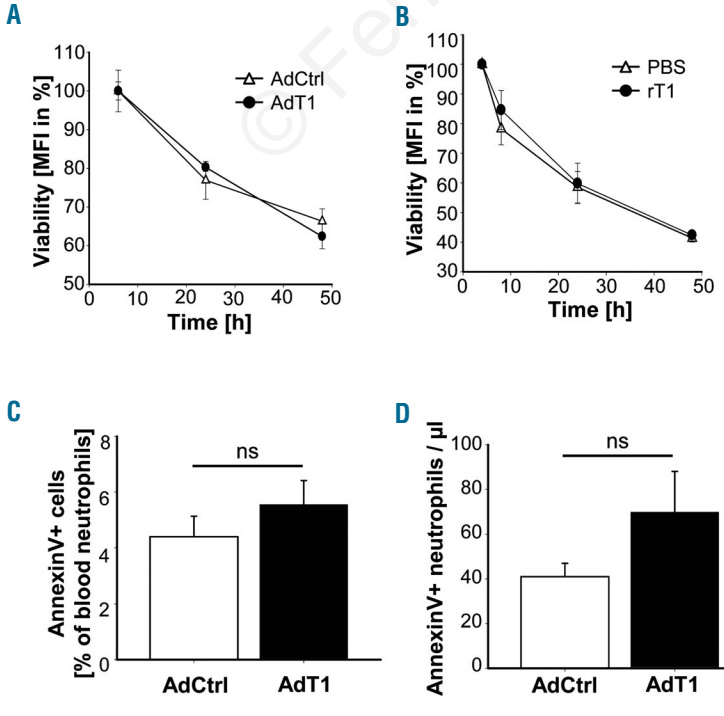

Figure 2. TIMP-1 does not prolong the survival of circulating neutrophils. (A) Viability of blood neutrophils isolated from AdT1-transduced mice. Neutrophils were isolated from DBA/2 mice (n=5 per group) three days after adenoviral transduction. Viability was analyzed *ex vivo* in an AlamarBlue assay. (B) Viability of isolated blood neutrophils exposed to recombinant TIMP-1 (rT1). Neutrophils were isolated from untreated DBA/2 mice and incubated with rT1 in AlamarBlue assays. (C and D) Annexin V staining of blood neutrophils in AdT1-transduced mice. Blood samples were taken three days after adenovirus injection, stained immediately with Annexin V and analyzed by flow cytometry. Relative (C) and absolute (C) quantification of apoptotic neutrophils. Columns: mean; Bars: SEM; Student's *t*-test. ns: not significant; n=5.

5A). Absolute quantification showed increased numbers of BrdU-positive neutrophils, whereas numbers of BrdU negative neutrophils remained unaltered (Figure 5B and C). Accordingly, the portion of BrdU-positive neutrophils in the BM was higher in AdT1-transduced mice (Figure 5D). Furthermore, recovery of blood neutrophils after systemic depletion seemed to be accelerated in AdT1 mice (*Online Supplementary Figure S9B*). These results indicate that increased generation of neutrophils from proliferating precursors in the BM accounted for the TIMP-1-induced neutrophilia.

TIMP-1 signaling but not protease inhibition triggers granulopoiesis

Next, we examined which of the two functions of TIMP-1, protease inhibition or direct signaling, were responsible for its effects on granulopoiesis and induction of neutrophilia. Therefore, we transduced mice to overexpress different TIMP-1 variants. N-TIMP-1 is sufficient to inhibit almost the full spectrum of TIMP-1 substrates,[9] but lacks the cytokine domain. TIMP-1/T2G[16] and vvTIMP-1[17] carry amino acid mutations in the N-terminus that impair or abolish the capability to bind proteases but retain full signaling function. Adenoviral transduction of mice with these variants revealed that N-TIMP-1 did not increase, but rather slightly decreased the numbers of circulating neutrophils, indicating that MMP inhibition was not sufficient to induce neutrophilia. In contrast, both, TIMP-1/T2G and vvTIMP-1 significantly increased blood neutrophil numbers to an even higher extent than wild-type TIMP-1 (Figure 6A and *Online Supplementary Figure 10*). Accordingly, immature neutrophil precursors were increased in BM of AdT1/T2G- and AdvvT1, but not in AdN-T1-transduced mice (Figure 6B), and TIMP-1/T2G was sufficient to up-regulate mRNA expression of neutrophil maturation markers and transcription factors (Figure 6C). These results indicate that the impact of TIMP-1 on neutrophil homeostasis relies on its signaling function, independent of protease inhibition.

TIMP-1-induced granulopoiesis is mediated through CD63

The tetraspanin CD63 is so far the only known membrane receptor to have been described as directly interacting with TIMP-1.[12] Since induction of neutrophilia and enhanced granulopoiesis relied on TIMP-1 signaling rather than protease inhibition, we next examined whether its receptor CD63 was involved. We thus induced increased TIMP-1 levels in CD63 knock-out mice[35] (-/-) and heterozygous (+/-) or wild-type (+/+) littermates by AdT1 infection. TIMP-1-induced neutrophilia was strongly reduced in CD63$^{+/-}$ mice and completely blocked in mice with total ablation of CD63 (Figure 7A). Furthermore, the observed increase in neutrophil precursors in the BM was not detectable upon reduction or ablation of CD63 (Figure 7B). In accordance with the cellular composition of the BM, expression of maturation markers and transcription factors was not up-regulated by TIMP-1 in CD63 knock-out mice (Figure 7C).

Interestingly, CD63 surface expression was higher in the Ly6G^{int} neutrophil precursor population than in mature BM neutrophils (Figure 7D). This suggested that TIMP-1

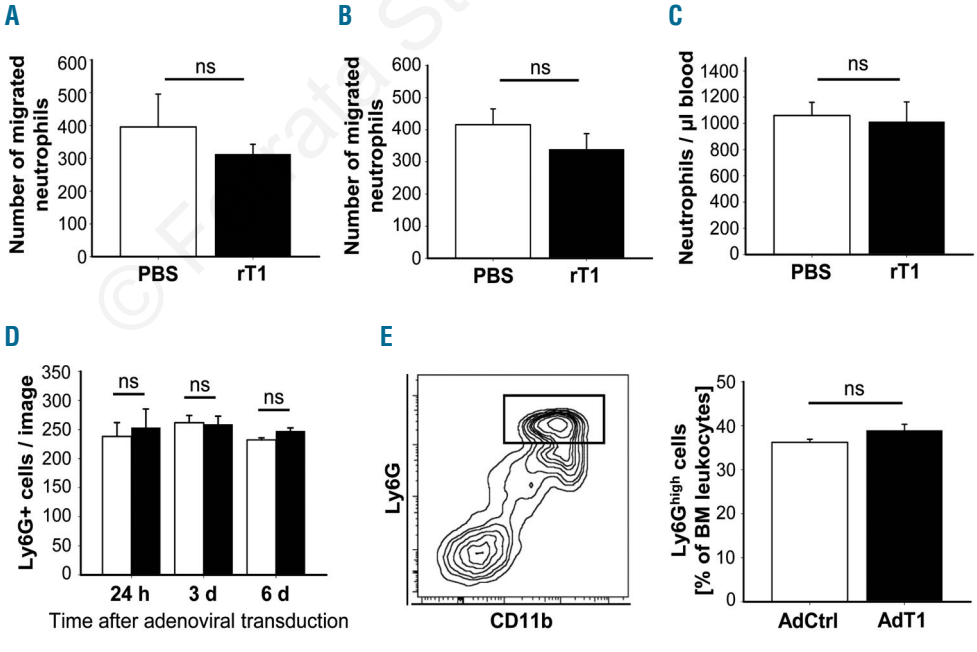

Figure 3. TIMP-1 does not directly mobilize neutrophils from the bone marrow. (A and B) Migration of isolated bone marrow (BM) neutrophils *ex vivo* towards rTIMP-1 (A) or towards FCS after pre-incubation with PBS or rTIMP-1 (B). (C) Mobilization of neutrophils by rTIMP-1 *in vivo*. rTIMP-1 protein was i.p. injected into DBA/2 mice and blood neutrophils were quantified 2 h later by flow cytometry using trucountTM tubes. Columns: mean; Bars: SEM; Student *t*-test. ns: not significant; n=5. (D) Quantification of neutrophils in BM. DBA/2 mice were transduced with AdCtrl (white) or AdTIMP-1 (black) and sacrificed 1, 3 or 6 days later. Ly6G-positive cells were immunohistochemically stained on femur sections and quantified per microscopic field. Columns, mean; Bars, SEM; Student *t*-test: ns: not significant; n=5. (E) BM cells were isolated from DBA/2 mice 3 days after adenoviral transduction. Gating (left) and relative quantification (right) of mature neutrophils (CD11b^{+} Ly6G^{high}) by flow cytometry. Columns: mean; Bars: SEM; Student's *t*-test. ns: not significant; n=5.

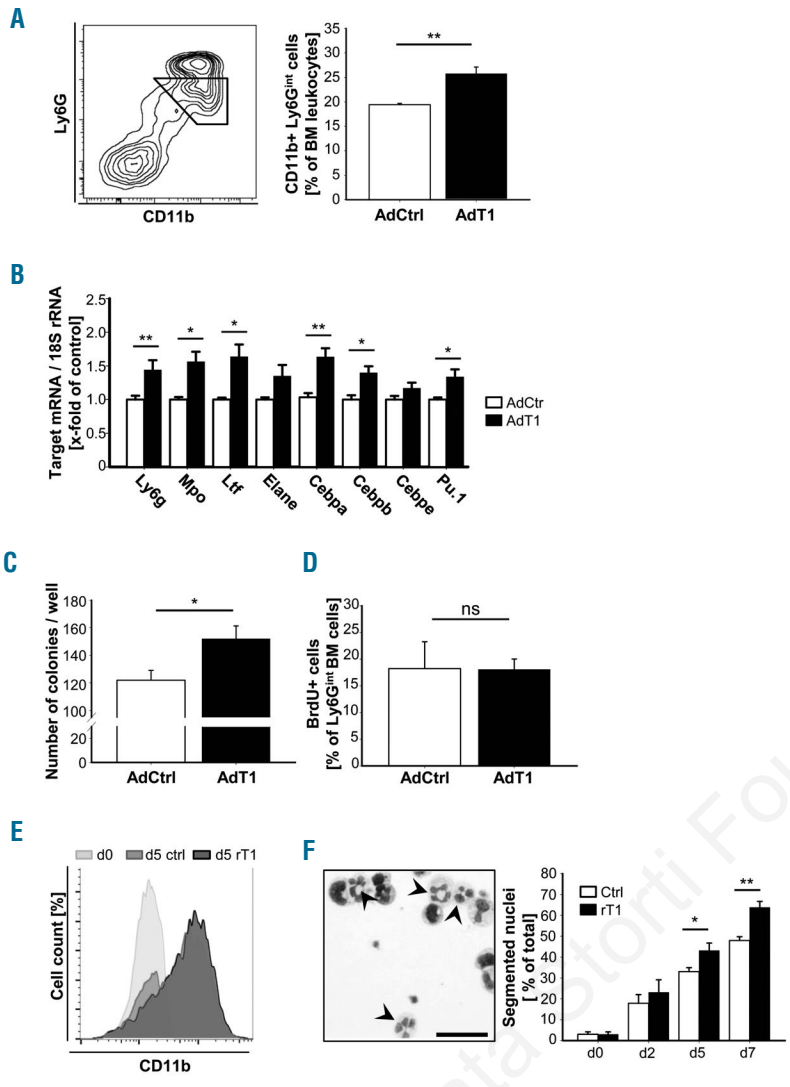

Figure 4. TIMP-1 triggers maturation of neutrophils in the bone marrow (BM). (A-D) DBA/2 mice (n=5) were adenovirally transduced and bone marrow (BM) was analyzed three days later. (A) Gating (left) and relative quantification (right) of pre-mature neutrophils (CD11b$^+$ Ly6G^{int}) by flow cytometry. (B) Expression of neutrophil maturation markers and granulopoiesis-inducing transcription factors in BM as analyzed by qRT-PCR. (C) CFU assay of BM isolates from AdCtrl or AdTIMP-1-transduced mice *ex vivo*. (D) A single BrdU pulse was injected 1 h before BM isolation. The proportion of BrdU-positive neutrophil progenitors (BM gated for CD11b$^+$ Ly6G^{int}) was analyzed by flow cytometry. Columns: mean; Bars: SEM; Student *t*-test. ns: not significant; **P*<0.05; ***P*<0.01. (E and F) G-CSF-induced differentiation of 32Dcl3 cells. Cells were stimulated with G-CSF for up to seven days in the absence or presence of rTIMP-1. (D) CD11b surface expression was analyzed by flow cytometry and (E) cells with segmented nuclei were counted on cytospins stained with DiffQuick. Representative image (left; arrows indicate cells with segmented nuclei; bar: 50 μm) and quantification of three independent experiments (right). Columns: mean; Bars: SEM; Student's *t*-test. **P*<0.05; ***P*<0.01.

might induce granulopoiesis by engaging CD63 directly on myeloid progenitors. We found that CD63 is expressed in the myeloid cell line 32Dcl3, where addition of rTIMP-1 led to an increased portion of cells with segmented nuclei (Figure 4E). To examine whether the capacity of TIMP-1 to induce differentiation of these precursor cells was CD63-dependent, we reduced CD63 levels by lentiviral transduction with two separate anti-CD63 shRNAs (*Online Supplementary Figure S11A*). Clearly, the increased differentiation in response to TIMP-1 was abolished in 32Dcl3 cells with CD63 knockdown upon G-CSF stimulation (Figure 7E and *Online Supplementary Figure S11B*).

Discussion

Neutrophil homeostasis is tightly regulated at several levels, and neutrophilia is increasingly seen as a negative prognostic factor in several inflammatory diseases.[3-5] Here, we identify elevated TIMP-1 as a new factor to directly induce neutrophilia in mice. Interestingly, TIMP-1 is associated with a number of inflammatory diseases including fibrosis,[18] sepsis,[7,19] artheriosclerosis,[20] and cancer.[8] We show that high systemic TIMP-1 levels trigger neutrophil production in the BM, resulting in increased neutrophil blood counts, which may provide a new link for the observed association of TIMP-1 with inflammation in patients.

Neutrophil homeostasis is maintained by an equilibrium between proliferation and differentiation from myeloid precursors in the BM, retention or release into the blood stream, and finally, apoptosis and phagocytic clearence.[36] We observed that TIMP-1-induced neutrophilia did not occur immediately upon elevation of TIMP-1, but with a 3-day delay, arguing against a quick release of stored mature neutrophils. This was confirmed *in vitro* and *in vivo*, where analysis of neutrophil storage pools or short-term application of rTIMP-1 did not indicate direct mobilization. Neutrophil apoptosis was also not affected by TIMP-1. Instead, we observed raised numbers of pre-mature neutrophils in the BM and increased mRNA expression of genes that are typically transcribed during specific stages of granulopoiesis, but not in mature neutrophils,[29,37] indicating enhanced granulopoiesis. This increased neutrophil production was not reflected in a higher capacity of the BM pool of mature neutrophils, but led to the observed neutrophilia in blood, as confirmed by BrdU pulse labeling experiments.

At first sight it appears counterintuitive that an MMP inhibitor promotes leukocyte differentiation and release from the BM, as HSPC activity and mobilization is known

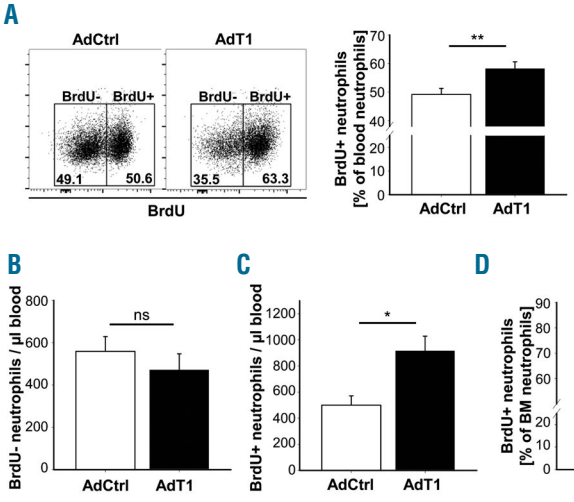

Figure 5. Increased neutrophil production accounts for TIMP-1-induced neutophilia. (A-C) DBA/2 mice (n=5) were transduced with AdTIMP-1 and BrdU was repeatedly injected, starting with elevation of TIMP-1 levels. Three days later, BrdU positive neutrophils in blood were analyzed by flow cytometry. (A) Representative plots (left) and relative quantification (right) of BrdU- and BrdU$^+$ blood neutrophils. (B and C) Absolute quantification of BrdU- (B) and BrdU$^+$ (C) neutrophils in peripheral blood. (D) Relative quantification of BrdU$^+$ neutrophils in BM. Columns: mean; Bars: SEM; Student's *t*-test. ns: not significant; **P*<0.05; ***P*<0.01.

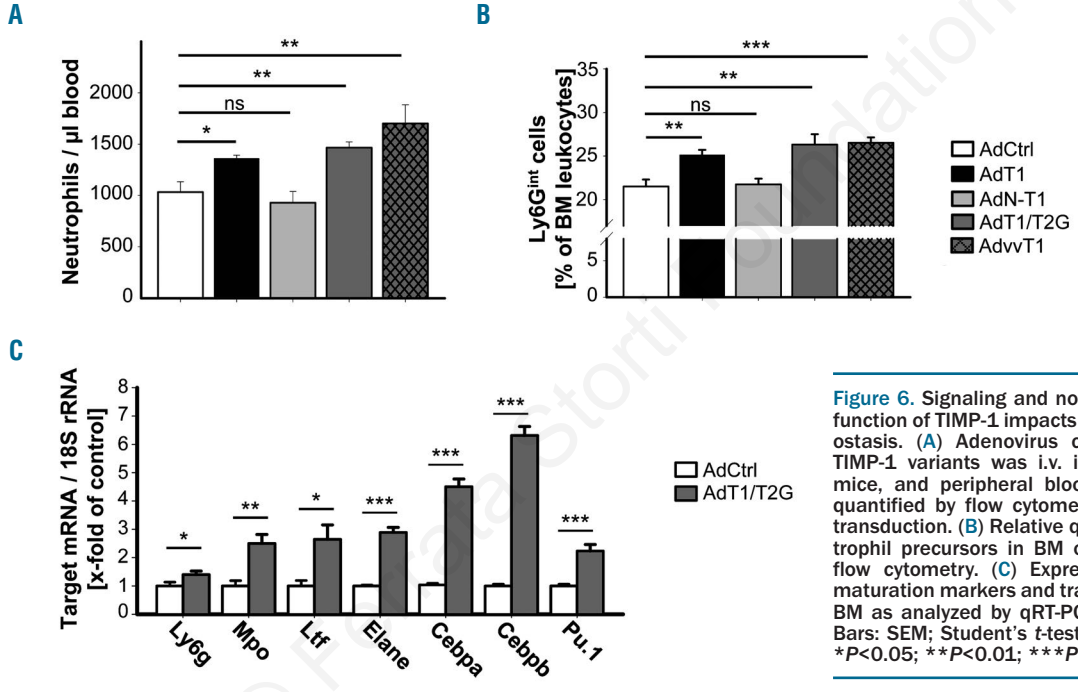

Figure 6. Signaling and not protease inhibitory function of TIMP-1 impacts on neutrophil homeostasis. (A) Adenovirus coding for different TIMP-1 variants was i.v. injected into DBA/2 mice, and peripheral blood neutrophils were quantified by flow cytometry three days after transduction. (B) Relative quantification of neutrophil precursors in BM of mice from (A) by flow cytometry. (C) Expression of neutrophil maturation markers and transcription factors in BM as analyzed by qRT-PCR. Columns: mean; Bars: SEM; Student's *t*-test. ns: not significant; **P*<0.05; ***P*<0.01; ****P*<0.001, n=5.

to require MMP activity.[38-42] In fact, the protease-inhibitory N-terminal domain of TIMP-1 alone was unable to induce neutrophilia, but rather led to a slight decrease in circulating neutrophils. In contrast, expression of TIMP-1 mutants that are incapable of binding proteases but contain an intact C-terminal domain, were sufficient to trigger granulopoiesis and neutrophilia with even stronger effects than wild-type TIMP-1. The lack of protease interaction of these mutants might result in an increased amount of proteins available for receptor binding and signal transmission, a perspective that has been recently proposed.[43]

Effects of TIMP-1 on proliferation and differentiation have been described for several cell types, including tumor cells,[44] neuronal cells,[45] and mesenchymal stem cells.[17] Interestingly, TIMP-1 was concurrently discovered as erythroid potentiating activity (EPA),[46] a factor that stimulated proliferation of human erythroid progenitors,[47] and this effect was found to be independent of protease inhibition.[48] Furthermore, the hematopoietic stem cell (HSC) compartment was shown to be functionally impaired in TIMP-1 knockout mice[49] and engraftment of HSPCs after BM transplantation was promoted by TIMP-1.[50] These studies are in line with our finding that TIMP-1 impacts on the hematopoietic system, particularly *via* its signaling domain. We show that TIMP-1-induced granulopoiesis required the presence of its receptor, the tetraspanin CD63. Although it needs to be clarified in more detail, on which cell type and differentiation state this interaction takes place *in vivo*, we propose a direct effect on HSPCs. We found CD63 surface levels to be high on neutrophil progenitors compared to mature neutrophils, and CD63 has been identified as a TIMP-1 receptor on CD34$^+$ hematopoietic progenitors.[50] *In vitro*, where BM stromal cells, which might secrete granulopoiesis stimulating cytokines, were absent, rTIMP-1 had a direct effect on 32Dcl3 cells. Furthermore, cytokine profile

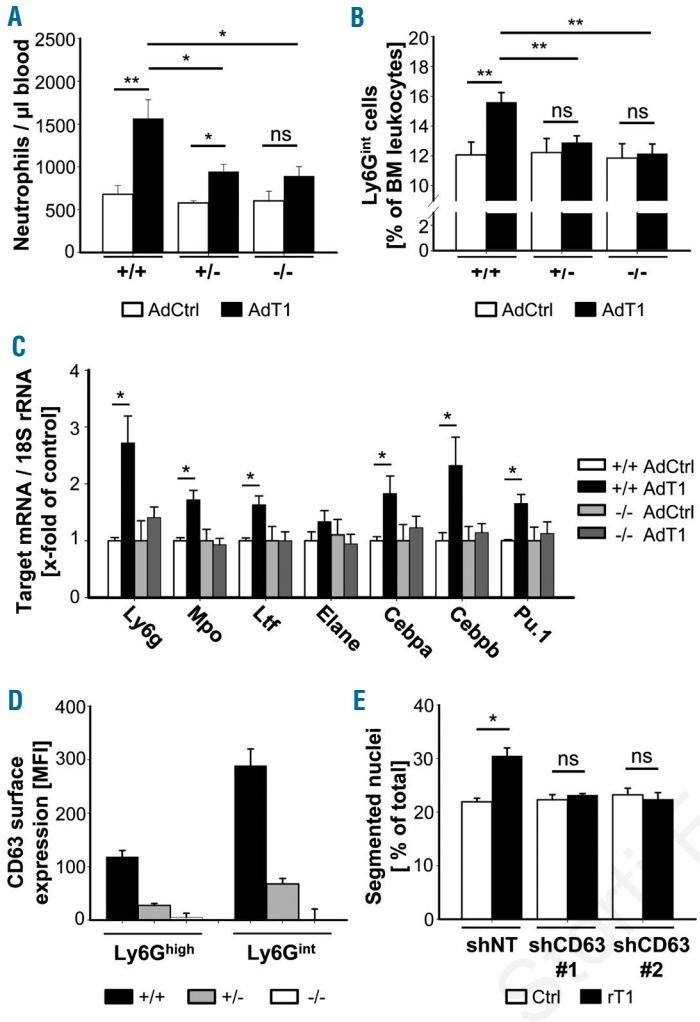

Figure 7. TIMP-1 impact on neutrophil homeostasis is CD63-dependent. (A-D) C57Bl/6 wild-type (+/+, n=6), heterozygous (+/-, n=7) or homozygous (-/-, n=5) CD63 knock out mice were transduced with AdCtrl or AdTIMP-1, and blood or bone marrow (BM) was analyzed three days later by flow cytometry. (A) Absolute quantification of blood neutrophils. (B) Relative quantification of neutrophil precursors in BM. (C) Expression of neutrophil maturation markers and transcription factors in BM as analyzed by qRT-PCR. Columns: mean; Bars: SEM; Student *t*-test. ns: not significant; **P*<0.05; ***P*<0.01. (D) CD63 surface levels of mature BM neutrophils (Ly6G^{high}) and neutrophil precursors as analyzed by flow cytometry. (E) G-CSF-induced differentiation of 32Dcl3 cells expressing non-targeting shRNA (shNT) or two different shRNA against CD63. Cells were stimulated with G-CSF for five days in the absence or presence of rTIMP-1, and cells with segmented nuclei were counted upon DiffQuick staining of cytospins. Columns, mean of four experiments; Bars, SEM; Student's *t*-test. ns: not significant; **P*<0.05; n=4.

analysis in mice with high TIMP-1 levels revealed no remarkable changes in granulopoiesis-regulating cytokines (*Online Supplementary Figure S12*). Our BrdU pulse-labeling experiment indicated that TIMP-1 did not directly trigger proliferation of pre-mature neutrophils, and overall cellularity in the BM was not increased in mice with high TIMP-1 levels. Nevertheless, the increase in mature neutrophils could be attributed to increased neutrophil generation, and Ly6G-expressing lineage-committed precursors were found to be enriched. It is, therefore, likely that TIMP-1 directly acts on hematopoietic progenitors, triggering their differentiation towards the granulocytic lineage. As CD63 itself is described to regulate activation and adhesion of mature neutrophils to endothelium,[51] granule targeting and release,[52] and is up-regulated on apoptotic neutrophils,[53] TIMP-1 /CD63 interactions may have additional effects on mature neutrophils.

Our observations were made on mice, where neutrophil blood counts are generally lower than in humans.[34] Therefore, it remains to be shown whether high TIMP-1 levels have a similar effect on the human hematopoietic system. Although no clinical study has so far directly correlated blood neutrophil counts to serum TIMP-1 in patients, elevated TIMP-1 levels are observed in a variety of inflammatory diseases that are accompanied by neutrophilia, and increased neutrophil activity was observed in patient groups with high TIMP-1 expression.[54]

Taken together, our study uncovers a so far unknown regulatory effect of TIMP-1 on neutrophil homeostasis, which corroborates the growing appreciation of the TIMP family of proteins not only as important regulators in the proteolytic network, but as protease-independent signaling molecules. The direct link between TIMP-1 and increased neutrophil generation leading to neutrophilia may provide new insights into the frequently described association of this protein with inflammatory diseases in the clinic.

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