SUPPLEMENTARY APPENDIX

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

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 doi:10.3324/haematol.2014.121590
The online version of this article has a Supplementary Appendix.
Manuscript received on November 28, 2014. Manuscript accepted on May 8, 2015.
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SUPPLEMENTARY METHODS

Cell lines and cell culture

HT1080 and NIH3T3 cells were cultured and retrovirally transduced as described previously[22]. 32Dcl3 cells were grown in RPMI medium supplemented with 10% FCS and 2ng/ml murine IL-3 (PeproTech) and passaged every 2nd to 3rd day. For the CD63 knock down, 2x10^{6} cells were pre-incubated with 8 µg/ml Polybrene. Lentiviral particles were added and incubated for 2 h at 37°C in a 1 ml volume before addition of 4 ml fresh medium. Virus was washed off 24 h later, and transduced cells were selected with 2.5 µg/ml puromycin until non-transduced control cells were all dead.

Animals

Female CD1$^{nu/nu}$, Fox Chase SCID, DBA/2, C57Bl/6 and FVB/N mice were obtained from Charles River (Sulfeld). C57Bl/6-CD63$^{-/-}$ mice[35] were bred under pathogen-free conditions in the animal facility of Klinikum Rechts der Isar der TU München. Experiments were performed with mice at the age of 7 – 10 weeks.

NIH3T3 tumor model

For TIMP-1-secreting NIH3T3 tumors, 2*10^{6} cells in 100 µl PBS were subcutaneously injected into the neck of SCID mice. Tumor onset and growth was monitored every 2nd to 3rd day. After 28 days, when tumors had reached a diameter of approx.1 mm, mice were sacrificed, blood was collected and analyzed by ELISA and flow cytometry.

Adeno- and Lentiviral vectors

Construction and purification of adeno- and lentiviral vectors have been previously described.[22] Virus titers were determined as infectious units (ifu) by Hexon ELISA. For adenoviral transduction *in vivo*, resulting plasma levels of TIMP-1 or TIMP-1 variants were examined by ELISA for each virus batch, and adjusted if necessary.

ELISA

TIMP-1 and G-CSF plasma levels were determined by enzyme linked immunosorbant assay. Plasma was obtained as described and either frozen in liquid nitrogen until measurement, or immediately applied to ELISA plates in an appropriate dilution. TIMP-1 was quantified by Duo set Development kit (RnD Systems) calibrated against NSO-expressed recombinant human TIMP-1, and G-CSF by Murine G-CSF ELISA Development Kit (Peprotech) according to the providers instructions

Antibodies for Flow cytometry

PerCP-Cy5.5-conjugated antibody against murine CD45 (clone 30-F11), PE-Cy7-conjugated antibody against murine CD11b (clone M1/70) and APC-labeled antibody against murine CD63 (clone NVG-2) were obtained from eBioscience/Affimetrix, and PE-labeled anti-Ly6G antibody (clone 1A8) was received from BioLegend.

***Ex vivo* viability and migration assay**

Blood and BM were harvested and BM cells were forced through a 70µm cell strainer to separate cells. Erythrocytes were removed by hypotonic lysis in 1x multi-species RBC-lysis buffer (eBioscience). Neutrophils were purified from blood or BM by MACS using the mouse Neutrophil Isolation Kit (Miltenyi Biotec). Purity of neutrophils was confirmed by flow cytometry and was higher than 93% in all experiments.

For viability assays, 5,000 neutrophils were seeded in 100µl RPMI supplemented with 10% FCS, 1% Pen/Strep and 1000 ng/ml rTIMP-1 (where indicated), and incubated for up to 72 h. 10 µl AlamarBlue were added 30min before fluorescence measurement.

Migration assays were performed in Costar Transwell Permeable Supports with 3µm pore size. If indicated, neutrophils were pre-incubated with 1000 ng/ml rTIMP-1 for 1 h at 37°C. $5x10^5$ cells were seeded into the transwell insert, and medium containing the respective chemoattractant (1000 ng/ml rTIMP-1, 10% FCS or 100 ng/ml murine rCXCL6) was added to the lower well. Cells were allowed to migrate for 1h at 37°C before cells in the lower well were counted.

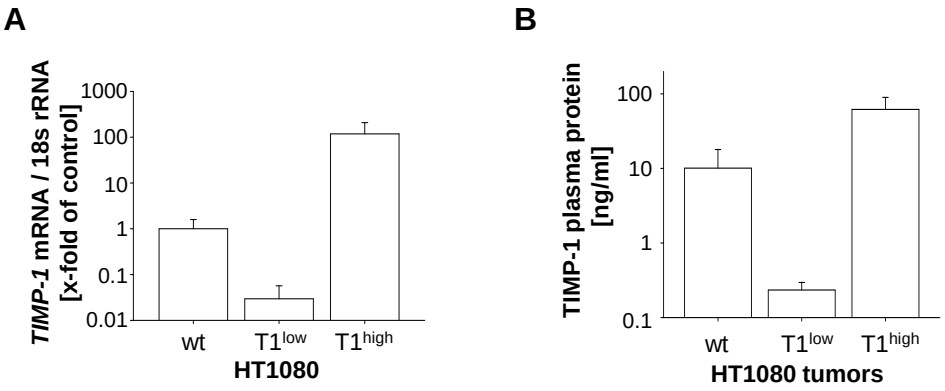
Gene expression analysis

BM cells were harvested, pelleted at 4°C and immediately re-suspended in Trizol® Reagent (LifeTechnologies). RNA was isolated by Trizol / Chloroform extraction as previously described[29]. Isolated RNA was run on agarose gels to control for degradation before transcription into cDNA using High-Capacity cDNA Reverse Transcription Kit (LifeTechnologies). Expression of the individual target genes was quantified by qRT-PCR using the Universal ProbeLibrary system (Roche).

Reagents

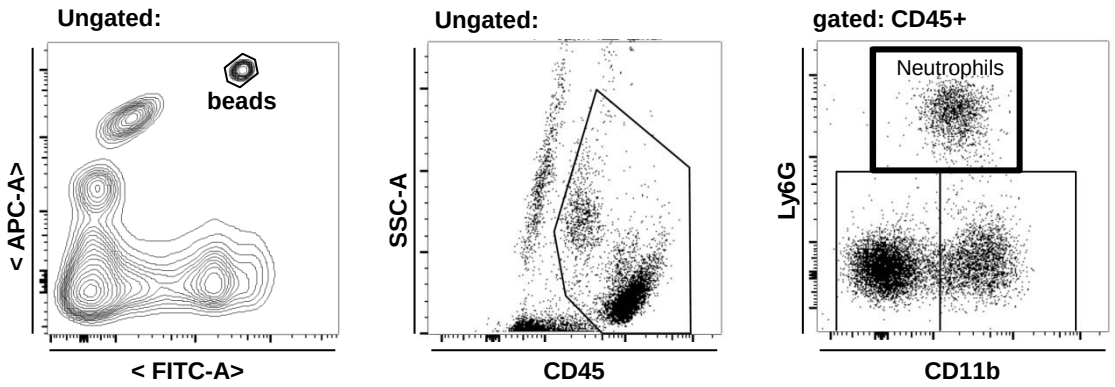
Recombinant, LPS-free TIMP-1 was a kind gift from Prof. Gillian Murphy (University of Cambridge, UK). All other recombinant proteins were obtained from Peprotech. Anti-Ly6G antibody (clone 1A8) for neutrophil depletion and the respective IgG control were obtained from Biolegend. For *in vivo* application, all reagents were administered in sterile, LPS-free PBS.

Suppl. Figure 1



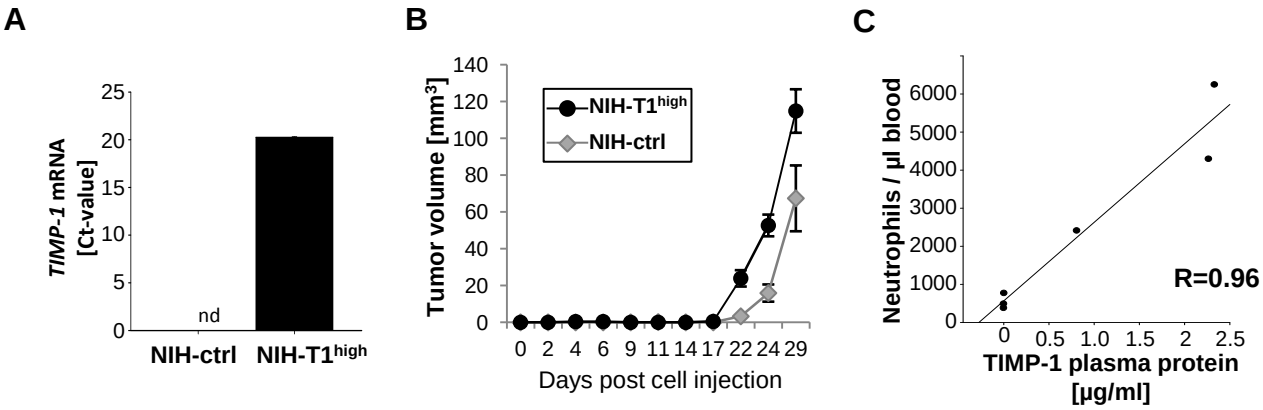
Supplementary Figure 1: TIMP-1 expression in HT1080 cells and in plasma of tumor bearing mice. (**A**) HT1080 cells were lentivirally transduced either to overexpress TIMP-1 ($T1^{high}$) or shRNA against TIMP-1 ($T1^{low}$). TIMP-1 mRNA relative to wild type (wt) cells, measured by qRT-PCR. (**B**) HT1080 tumors with cells from (A) were engrafted into the neck of $NMRI^{nu/nu}$ mice and resulting TIMP-1 plasma levels were measured by ELISA ten days after tumor injection. *bars*, mean, *errors*, SEM; $T1^{low}$, n=5; wt, n=7; $T1^{high}$, n=6.

Suppl. Figure 2



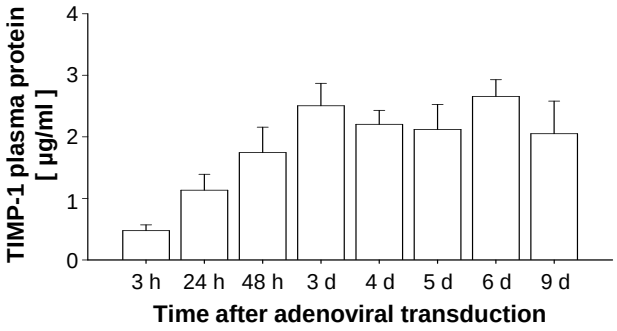
Supplementary Figure 2: Gating strategy for absolute quantification of blood neutrophils. Mouse blood was analyzed by flow cytometry using BD trucount™ tubes. Gating was done by FlowJo software, starting with trucount beads (*left*) and CD45 positive leukocytes (*middle*). CD45+ cells were sub-divided according to expression of CD11b and Ly6G (*right*), and double-positive cells were considered as neutrophils.

Suppl. Figure 3



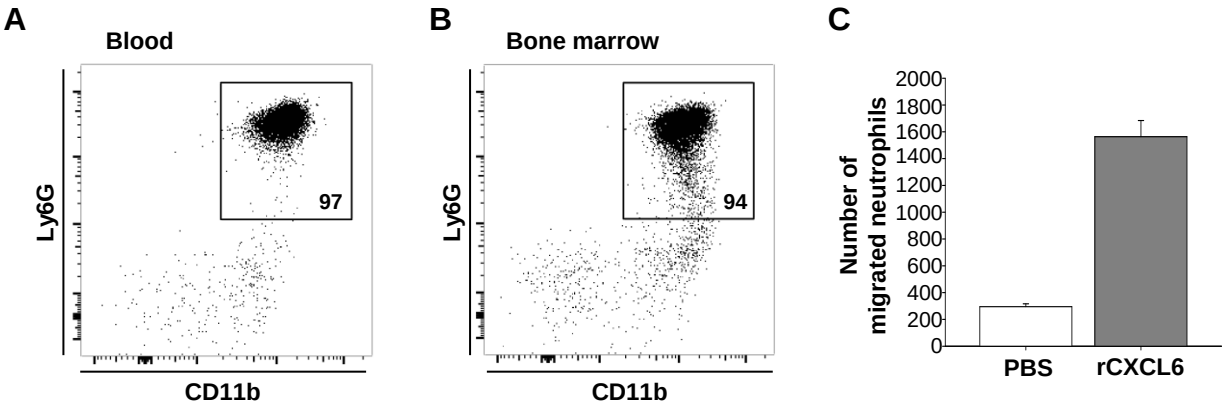
Supplementary Figure 3: Correlation of TIMP-1 plasma levels and blood neutrophil counts in NIH3T3 tumor bearing mice. NIH3T3 cells were lentivirally transduced with a control vector (NIH-ctrl) or a TIMP-1 overexpression vector (NIH-T1^{high}), and were subcutaneously injected into Scid mice, where they formed solid tumors. After 4 weeks, when tumors reached a diameter of approx. 10-15 mm, mice were sacrificed, blood was collected, TIMP-1 was measured by ELISA, and neutrophils were quantified by flow cytometry using BD trucount^{TM} tubes. (**A**) TIMP-1 mRNA as quantified by qRT-PCR. (**B**) Tumor growth upon subcutaneous injection. (**C**) Correlation of TIMP-1 plasma levels and blood neutrophil counts 4 weeks after tumor injection.

Suppl. Figure 4



Supplementary Figure 4: TIMP-1 expression in adenovirally transduced mice. TIMP-1 coding adenovirus (AdT1) was i.v. injected into mice and plasma TIMP-1 protein levels were measured by ELISA at the indicated time points. *bars*, mean, *errors*, SEM; n=5.

Suppl. Figure 5



Supplementary Figure 5: Purity and of isolated blood and BM neutrophils after MACS and migration towards rCXCL6. Neutrophils were isolated from whole blood or BM by MACS. Purity was confirmed by flow cytometric quantification of $Ly6G^+$ $CD11b^+$ cells. Representative plots for blood (**A**) and BM (**B**) are shown. Numbers within gates represent percentages. **(C)** Migration of BM neutrophils towards medium supplemented with 100 ng/ml rCXCL6.

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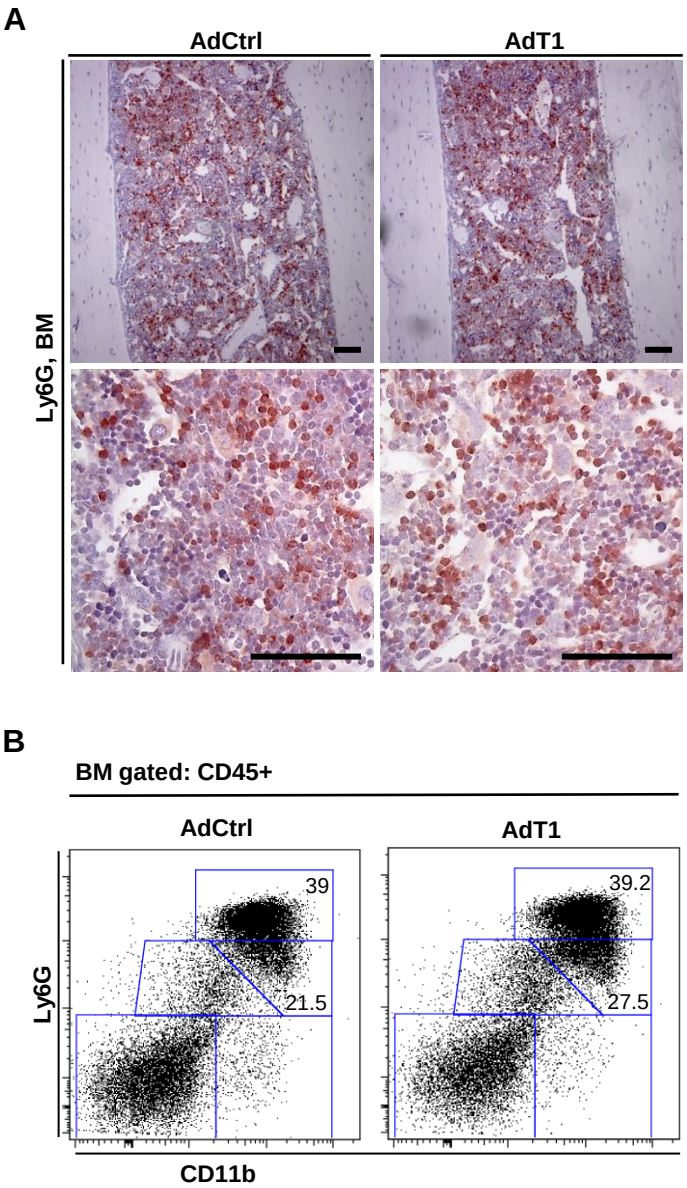

Supplementary Figure 6: Immunohistochemical and flow cytometric analysis of bone marrow neutrophils. DBA/2 mice (n=5) were adenovirally transduced and sacrificed 3 days later. (**A**) Paraffin sections of decalcified femurs were stained with anti-Ly6G antibody (clone 1A8). Representative images in two magnifications are shown for AdCtrl- and AdTIMP-1-transduced mice, respectively. *Bars,* 100µm. (**B**) Flow cytometric analysis of bone marrow. Total BM cells were gated for CD45-positive cells and further analysed according to their surface expression of Ly6G and CD11b. Cells with high Ly6G expression were regarded as mature neutrophils and CD11b+ Ly6G^{int} cells as neutrophil precursors. A representative dot plot including percentage of the populations of interest is shown for AdCtrl and AdTIMP-1 transduced mice, respectively.

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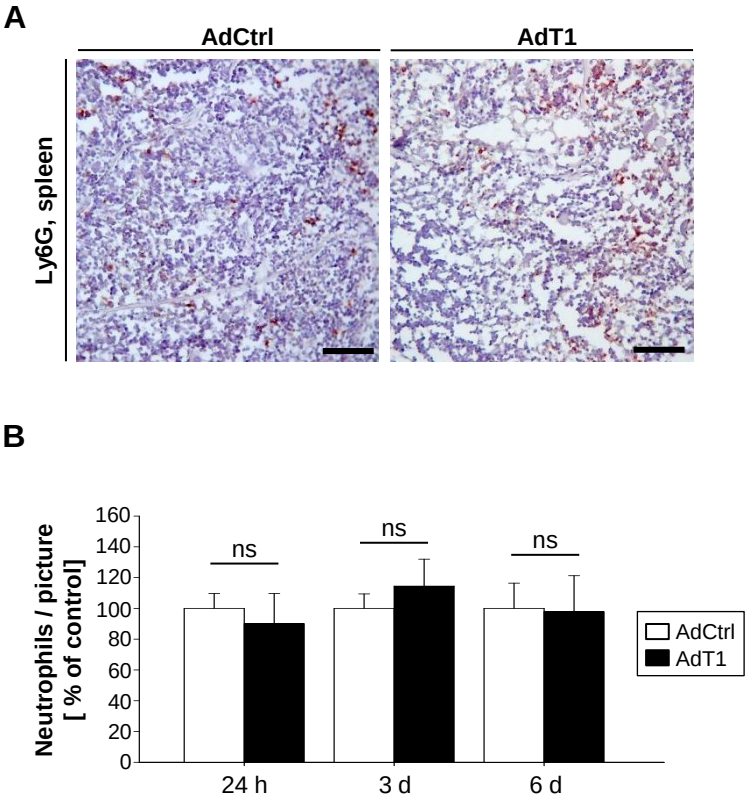
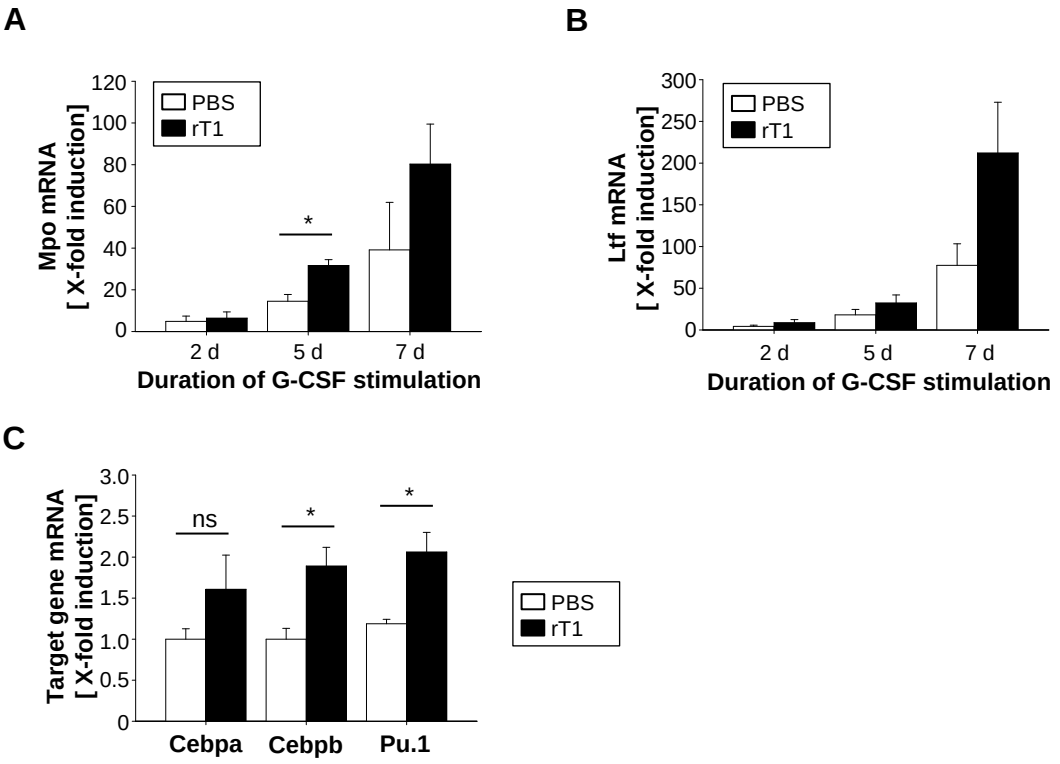


Supplementary Figure 7: TIMP-1 has no effect on neutrophil numbers in the spleen. DBA/2 mice (n=5) were adenovirally transduced and sacrificed one, three or six days later, respectively. Cryosections of TissueTek-embedded spleens were stained with anti-Ly6G antibody (clone 1A8). (**A**) Representative images of AdCtrl- or AdT1-transduced mice at day 3. *Bars,* 100µm. (**B**) Quantification of Ly6G positive cells per microscopic field at the indicated time points. *Columns*, mean; *Bars*, SEM; Student *t* test. ns, not significant

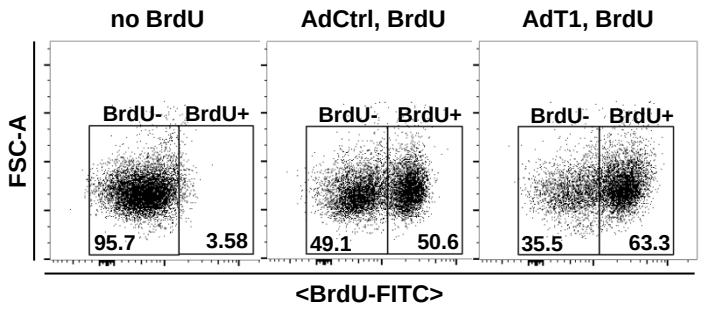
Suppl. Figure 8



Supplementary Figure 8: TIMP-1 increases expression of differentiation-associated genes in 32Dcl3 cells. (A) Cells were stimulated with G-CSF for up to 7 days in the absence or presence of rTIMP-1. mRNA was isolated at the indicated time points and gene expression was quantified by qRT-PCR. Expression relative to unstimulated 32Dcl3 cells is shown. (**B**) 32Dcl3 cells were incubated with rTIMP-1 or PBS for 12 h before mRNA was isolated and analyzed by qRT-PCR. Expression relative to saline-treated cells is shown. *Columns*, mean; *Bars*, SEM; n=3; Student *t* test. ns, not significant; *, $p<0.05$.

Suppl. Figure 9

A



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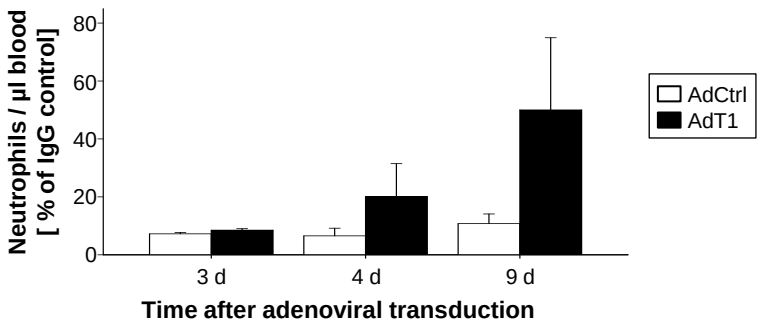
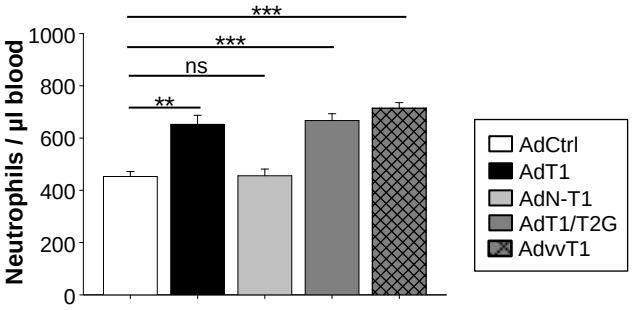


Supplementary Figure 9: TIMP-1-induced neutrophilia is due to enhanced neutrophil production in BM. (**A**) Gating of BrdU-positive and -negative neutrophils. Representative plots for BrdU-stained blood neutrophils (gated: Ly6G+ CD11b+) in untreated mice (*left*) and AdT1-transduced mice repeatedly injected with BrdU (*right*). (**B**) Recovery of blood neutrophils after depletion. Anti-Ly6G depletion antibody was injected 24 h before adenoviral elevation of TIMP-1 in DBA/2 mice. Blood neutrophils were quantified 3, 4 and 9 days after adenoviral transduction by flow cytometry using trucount™ tubes. Neutrophil counts relative to IgG control antibody-treated mice are shown. *Columns*, mean; *Bars*, SEM; n=4

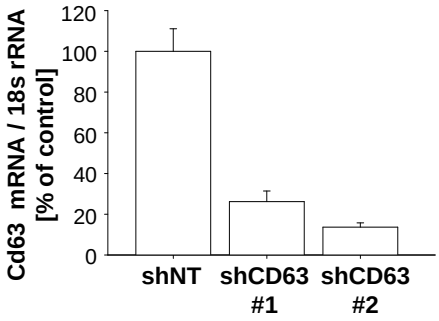
Suppl. Figure 10



Supplementary Figure 10: The signaling function of TIMP-1 induces neutrohpilia in C57Bl/6 mice. C57Bl/6 mice were adenovirally transduced to express TIMP-1 or the respective TIMP-1 variant. 3 days after transduction, mice were sacrificed, blood was collected and neutrophils were quantified by flow cytometry. *Columns*, mean; *Bars*, SEM; Student *t* test. ns, not significant; **, $P<0.01$; ***, $P<0.001$, n=6.

Suppl. Figure 11

A



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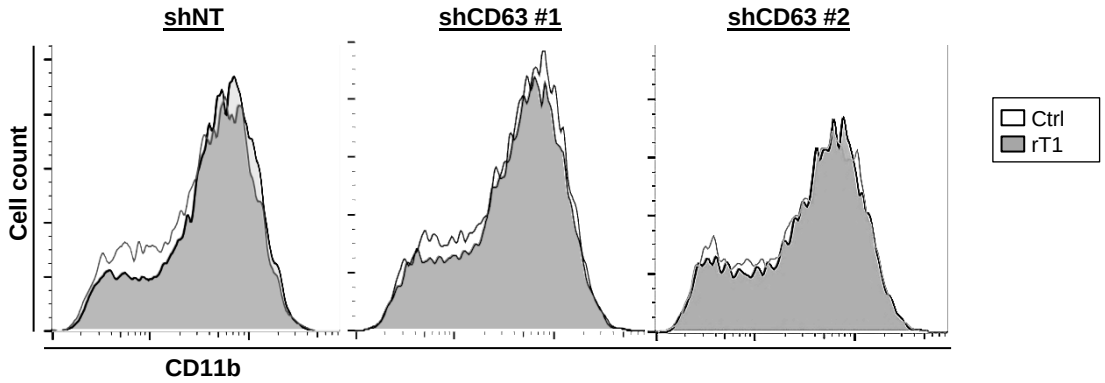
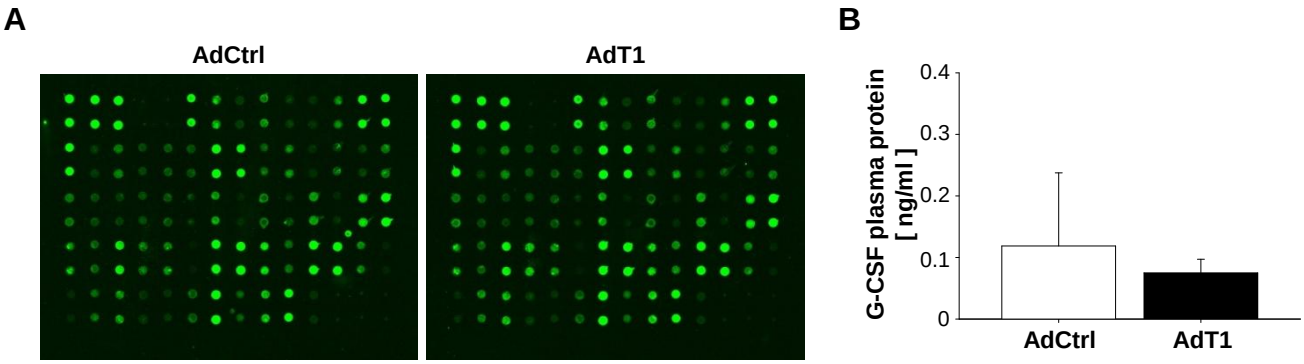


Supplementary Figure 11: Knock down of CD63 in 32Dcl3 cells. (**A**) mRNA expression in un-stimulated 32Dcl3 cells stably transduced with lentiviral vectors coding for non-targeting (shNT) or CD63-shRNA as quantified by qRT-PCR. (**B**) CD11b surface expression in cells from (A) after G-CSF stimulation for 5 days in the presence or absence of rTIMP-1 as measured by flow cytometry.

Suppl. Figure 12



Supplementary Figure 12: TIMP-1 does not induce granulopoiesis-regulating cytokines. Cytokine array (A) and G-CSF protein ELISA (B) on plasma isolated from mice three days post adenoviral transduction.