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Molecular analysis of neutrophil spontaneous apoptosis reveals a strong role for the pro-apoptotic BH3-only protein Noxa

Susanne Kirschnek¹, Juliane Vier¹, Sanjivan Gautam^{1,5}, Tobias Frankenberg², Svetla Rangelova², Pedro Eitz-Ferrer^{1,2}, Francesca Grespi³, Eleonora Ottina³, Andreas Villunger³, Hans Häcker⁴ and Georg Häcker¹

¹Institute of Medical Microbiology and Hygiene, Universität Freiburg, Freiburg, Germany

²Institute of Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany

³Division of Developmental Immunology, Biocenter, Innsbruck Medical University, Innsbruck, Austria

⁴Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee, United States of America

⁵Spemann Graduate School of Biology and Medicine, Universität Freiburg, Freiburg, Germany

Running title: Noxa in neutrophil spontaneous apoptosis

Correspondence: Susanne Kirschnek, Institute of Medical Microbiology and Hygiene, Universität Freiburg, Hermann-Herder-Str. 11, 79104 Freiburg, Germany, Tel.: 0761-2035362, Fax: 0761-2036651, susanne.kirschnek@uniklinik-freiburg.de; and Georg Häcker, Institute of Medical Microbiology and Hygiene, Universität Freiburg, Hermann-Herder-Str. 11, 79104 Freiburg, Germany, Tel.: 0761-2036531, Fax: 0761-2036651, georg.haecker@uniklinik-freiburg.de

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Abstract

Neutrophils enter the peripheral blood from the bone marrow and die after a short time. Molecular analysis of spontaneous neutrophil apoptosis is difficult as these cells die rapidly and cannot be easily manipulated. We use conditional Hoxb8-expression to generate mouse neutrophils and test the regulation of apoptosis by extensive manipulation of Bcl-2 family proteins. Spontaneous apoptosis was preceded by down-regulation of anti-apoptotic Bcl-2 proteins. Loss of the pro-apoptotic BH3-only protein Bim gave some protection but only neutrophils deficient in both BH3-only proteins, Bim and Noxa were strongly protected against apoptosis. Function of Noxa was at least in part neutralization of Mcl-1 in neutrophils and progenitors. Loss of Bim and Noxa preserved neutrophil function in culture and apoptosis-resistant cells remained in circulation in mice. Apoptosis regulated by Bim and Noxa-driven loss of Mcl-1 is thus the final step in neutrophil differentiation, required for the termination of neutrophil function and neutrophil-dependent inflammation.

Introduction

Neutrophil granulocytes (neutrophils) are the main leukocyte population in human peripheral blood and represent, besides lymphocytes, a major leukocyte population in peripheral blood of mice. Neutrophils phagocytose and kill pyogenic bacteria¹ and are the first cellular line of defense to infections with many other pathogens, for instance *Chlamydia*² and *Leishmania*³. Neutrophils invade tissues from the bloodstream in response to infection or injury⁴. During the resolution of the infection they die by apoptosis and are taken up by macrophages⁵.

Neutrophils are constantly produced in the bone marrow and enter circulation where they die after relatively short time¹. Earlier studies have calculated a half-life of peripheral blood neutrophils in humans of significantly under a day⁶ although this has been challenged by a recent report that puts the half-life of human neutrophils at 5.4 d (mouse neutrophils around one day)⁷. In any case, neutrophils are ‘programmed to die’, i.e. apoptosis is their regular fate after this short time in circulation. The neutrophil life-span can be extended by inflammatory mediators either of host (e.g., GM-CSF, IL-3, TNF) or of pathogen origin (e.g., LPS). These signals probably operate to keep neutrophils alive in the inflammatory environment for long enough to be effective at combating bacteria^{8,9}. Experimental inhibition of neutrophil apoptosis can exacerbate tissue inflammation¹⁰ suggesting that apoptosis is the relevant mechanism to turn off neutrophil-mediated inflammation.

A number of studies have addressed the molecular regulation of spontaneous neutrophil apoptosis^{8,9}. Two major signaling pathways are thought to mediate apoptosis in mammalian cells, the death receptor pathway (triggered through surface receptors such as Fas and TRAIL)¹¹ and the mitochondrial pathway¹².

Mitochondrial apoptosis is regulated by the Bcl-2 family¹³. This family has eight pro-apoptotic BH3-only proteins (Bim, Puma, Noxa, Bad, Bmf, Bid, Bik, Hrk). BH3-only proteins are the upstream triggers, which can be activated by transcription, phosphorylation and probably other molecular mechanisms. BH3-only proteins activate the pro-apoptotic

effectors Bax and/or Bak, which permit the release of cytochrome *c* from mitochondria. In the cytosol, cytochrome *c* induces the formation of a large protein complex that activates caspases. The anti-apoptotic Bcl-2-like proteins (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1) inhibit this process, probably by direct binding of pro-apoptotic Bcl-2-family proteins. The exact mechanisms of Bax/Bak-activation and the action of Bcl-2-like proteins are under dispute¹³.

Although neutrophils express Fas on their surface and Fas-stimulation can induce neutrophil apoptosis, Fas-defective neutrophils die normally^{14,15}. On the other hand, mouse neutrophils over-expressing transgenic Bcl-2 or lacking the pro-apoptotic Bcl-2 protein Bim show reduced spontaneous apoptosis^{15,16}.

Neutrophils are relatively little understood, probably owing to the peculiarities that they die rapidly in culture, that relatively few cells can be isolated from a mouse and that human neutrophils cannot be genetically modified. However, an experimental system has recently been introduced that overcomes these limitations¹⁷. This system utilizes a conditional estrogen-dependent version of the homeobox protein Hoxb8 to expand mouse hematopoietic progenitors in the presence of stem cell factor (SCF). Deactivation of Hoxb8 initiates the differentiation of the progenitors into cells with all tested characteristics of neutrophils over about 4 days. As these cells can be generated from any genetically modified mouse and are amenable to gene over-expression and knock-down strategies, they provide a versatile experimental system for the study of neutrophil biology.

We used this system to analyze the regulation of spontaneous apoptosis in neutrophils. Using gene-deficient cells and cells over- or under-expressing Bcl-2-family genes we in particular describe an unexpectedly strong pro-apoptotic role of the BH3-only protein Noxa, which acts in concert with Bim to limit the life-span of neutrophils.

Results

Spontaneous apoptosis in Hoxb8-derived neutrophils is comparable to apoptosis in primary neutrophils

Progenitor cell lines were generated by transduction of bone marrow cells with a retrovirus driving expression of ER-Hoxb8 as described¹⁷. The progenitor lines were kept in long-term culture in the presence of estrogen and stem cell factor (SCF). These cells have a morphological promyelocyte-like appearance that did not change in culture over several months. Hoxb8-inactivation led to homogenous differentiation into cells that were morphologically indistinguishable from mouse primary neutrophils (Fig. S1A). All wt and mutant cell lines used for this study had the same morphological appearance in the progenitor stage and upon differentiation (Fig. S1B and data not shown).

Replicating their *in vivo* behavior, primary neutrophils rapidly undergo apoptosis in culture, and this cell death can be slowed down by pro-inflammatory mediators. Hoxb8-derived neutrophils (here simply referred to as neutrophils) also died rapidly (about 80 % were dead after 24 h; Fig. S1E). In the experiments in this study, neutrophils were used on day 4 of differentiation when they appeared homogeneously mature. At this point, SCF was removed to mimic the release from the bone marrow. The receptor of SCF, c-kit, was down-regulated during differentiation and was in differentiated cells expressed at levels comparable to primary neutrophils sorted from mouse bone marrow (Fig. S1C, D).

As reported for primary neutrophils, cell death was reduced in the presence of SCF, GM-CSF or LPS (Fig. S1E). In primary neutrophils a correlation of factor-dependent survival with high expression of the Bcl-2-like protein Mcl-1 has been reported. We found that spontaneous cell death correlated with the loss of Mcl-1 while continued culture with pro-inflammatory factors increased Mcl-1 protein expression (Fig. S1F). Hoxb8-derived neutrophils also were capable of efficient phagocytosis of bacteria (Fig. S1G), displayed an

oxidative burst upon stimulation (Fig. S1H) and produced TNF and IL-6 upon stimulation with bacteria (Fig.S1I, K).

Loss of the BH3-only protein Bim or over-expression of Bcl-2 protects primary neutrophils against spontaneous apoptosis^{15, 16}. Neutrophils derived from cells that had either been established from bone marrow from Bim-deficient or from Bcl-2-transgenic mice were similarly protected (there was considerable protection in Bim-deficient cells and almost complete block of apoptosis by Bcl-2, Fig. 1A).

In lymphocytes, cell death in the absence of Bim depends at least in part on the BH3-only protein Puma¹⁸. We therefore included neutrophils from Bim/Puma-double-deficient mice in this study. As shown in Fig. 1A, the loss of Puma gave additional protection to Bim-deficient neutrophils although cell death was still considerably higher than in Bcl-2-transgenic cells.

Cell death was accompanied by the activation of caspase-3, which was again reduced in Bim-negative and Bcl-2-overexpressing cells (Fig. 1B), and the pan-caspase-inhibitor qVD-OPH blocked spontaneous cell death (Fig. 1C). These results reproduce for Hoxb8-derived neutrophils the results obtained in the past for primary neutrophils.

Regulation of Bcl-2-family proteins during spontaneous apoptosis in neutrophils

Bim is (based on analysis of gene-deficient mice) the most important BH3-only protein in the hematopoietic system. In lymphocytes, Bim-dependent apoptosis is associated with an induction of Bim-protein (T cells¹⁹, B cells²⁰, NK cells²¹). In neutrophils, spontaneous apoptosis was not accompanied with an increase in Bim protein levels, at least at early stages when the Bim-effect is most obvious (see below, Fig. 2). The apoptosis-associated induction of Bim (and of Puma) is at least in part driven by increased FOXO3a-dependent transcription^{18, 22}. We expressed FOXO3a-specific shRNA by lentiviral transduction of wt progenitor lines. This led to a substantial reduction of FOXO3a protein levels both in progenitors and in neutrophils differentiated from these cells (Fig. S2A). Down-regulation of FoxO3a did not

decrease Bim-levels (Fig. S2B), and neutrophils with reduced FoxO3a died at the same rate as control cells (Fig.S2C). Bim-dependent neutrophil apoptosis therefore appears to be independent of FOXO3a.

The near-complete inhibition of apoptosis by Bcl-2 demonstrates that spontaneous neutrophil-apoptosis is regulated through the mitochondrial apoptosis pathway.

We next determined the levels of Bcl-2-family proteins during spontaneous apoptosis in neutrophils. For this purpose, wt neutrophils were followed over 48 h and compared to Bcl-2-over-expressing neutrophils. Because of the massive apoptosis in wt neutrophils, events consecutive to cell death may confound expression levels of proteins. As Bcl-2-over-expressing cells show almost no cell death, changes observed in these cells are likely to be involved in the regulation of apoptosis rather than being a consequence.

Bcl-2-over-expressing neutrophils had higher levels of Bim and also of Bax and Bak (presumably because cells with higher levels of pro-apoptotic proteins can survive in the presence of high-level Bcl-2, Fig. 2). There was no clear increase in Bim-levels during spontaneous apoptosis (Fig. 2). Notably, there was a loss of Bax (and appearance of a faint band of smaller size) in wt but not in Bcl-2-over-expressing cells, which may reflect protein loss or consumption (and perhaps cleavage) during apoptosis. Bak-levels were slightly increased under these 'apoptosis-inducing' conditions in both cell lines (Fig. 2).

While these changes in levels of pro-apoptotic proteins appear fairly small, there were stronger changes in the levels of anti-apoptotic Bcl-2-like proteins. We found a moderate, relatively late but clearly detectable decline in Bcl-2 and Bcl-X_L-levels in both cells. The strongest and most rapid change occurred in Mcl-1 with a strong reduction already at 7 h, regardless of the over-expression of Bcl-2 and therefore independent of the occurrence of cell death (Fig. 2). Genetic loss of Mcl-1 causes the death of neutrophils already during differentiation²³. This makes Mcl-1 a prime candidate for the regulation of neutrophil spontaneous apoptosis. We were unable to detect endogenous A1 by Western blotting.

Regulation of Bcl-2-family proteins during neutrophil differentiation

In order better to understand the ‘starting point’ of spontaneous neutrophil apoptosis we followed the expression of Bcl-2 family proteins during differentiation of neutrophils from progenitor lines. As shown in Fig. 3, there was a considerable increase of the BH3-only protein Bim, a decrease of the BH3-only protein Bid, a small increase of Bax and (more strongly) of Bak. Expression of the BH3-only protein Puma, after initially increasing early in differentiation, was decreased in mature cells. Further, a decrease of the anti-apoptotic proteins Bcl-2, Bcl-w and Mcl-1 was seen, with only Bcl-X_L remaining at constant levels during differentiation. Although there was a small decline in Mcl-1-mRNA levels during differentiation (Fig. 3B) this seemed insufficient to explain the loss of Mcl-1 protein suggesting post-translational regulation. This protein expression pattern in mature neutrophils is suggestive of a state of high sensitivity to apoptosis.

Effect of the experimental over-expression of Bcl-2-like proteins

Since these results suggested a role of Mcl-1- (and Bcl-2-) down-regulation in spontaneous neutrophil apoptosis we tested the effect of over-expression of anti-apoptotic Bcl-2-like proteins in neutrophils. Progenitor lines were retrovirally transduced for stable expression of individual proteins. We had already observed the strong anti-apoptotic effect of Bcl-2-over-expression (see Fig. 1A). Surprisingly, there was no protection by either Mcl-1 or A1 expression in neutrophils (Fig. 4A). The same was seen when Mcl-1 or A1 was over-expressed in Bim/Puma-double deficient cells, suggesting a mechanism of independent regulation (Bcl-2 and Bcl-X_L efficiently protected also in Bim/Puma-double deficient neutrophils, Fig. 4A).

Analysis of protein levels showed that although strong over-expression of Mcl-1 protein was seen in progenitor cells transduced with Mcl-1-retrovirus, this over-expression was almost undetectable in neutrophils differentiated from these cells (Fig. 4B). Endogenous expression of A1 was undetectable by Western blotting but over-expressed A1 protein was

seen in both progenitors and differentiated neutrophils (Fig. 4B). Progenitor cells, which are constantly cultured in the presence of SCF, die rapidly when the factor is removed (Fig. S3A). In these cells, Mcl-1 provided strong protection while the protection by A1 was smaller but still considerable (Fig. S3A). Since both proteins were expressed as untagged proteins, we cannot directly compare the relative protein expression levels. We therefore cannot exclude the possibility that the differences in protection efficiency might be due to differing protein expression levels. All transgenic progenitor lines were sorted to achieve to similar GFP-levels (which is expressed from an IRES of the encoded mRNA). This makes it likely that the generation of protein is similar. In progenitor cells, apoptosis was further accompanied by a strong induction of Bim (Fig. S3B).

The BH3-only protein Noxa contributes to neutrophil spontaneous apoptosis

These results suggested that mechanisms exist that cause the down-regulation of Mcl-1 first during differentiation and later during spontaneous neutrophil death, and that this down-regulation is responsible for apoptosis and life-span restriction of neutrophils. One known mechanism regulating Mcl-1-levels is the induction of the BH3-only protein Noxa. Noxa can directly bind Mcl-1 (and A1), which can trigger the proteolytic degradation of Mcl-1 by the proteasome²⁴.

We were unable to detect Noxa protein consistently in neutrophils but some Noxa was detectable in progenitors by Western blotting. No increase of Noxa protein levels was seen during factor-deprivation-induced apoptosis of progenitors (Fig. S3B). This is however not conclusive evidence against a contribution of Noxa since Noxa is probably co-degraded with Mcl-1 and levels therefore could also go down.

To test the role of Noxa we generated Noxa-deficient cell lines. Neutrophils differentiated from lines that had been established from Noxa-deficient animals showed slightly improved survival (Fig. 5A). However, cells from Bim/Noxa double-deficient animals were almost completely protected against apoptosis over 72 h, substantially improved even

over Bim/Puma-deficient cells (Fig. 5A). The additional effect of Noxa-deficiency on the loss of Bim and Puma was further demonstrated by RNAi. Lentiviral transfer of Noxa-specific shRNA in Bim/Puma-double deficient progenitors led to a substantial reduction in Noxa-protein levels (Fig. S4B). Upon differentiation, Bim/Puma-double-deficient neutrophils carrying Noxa-specific shRNA were more strongly protected against spontaneous apoptosis than Bim/Puma-double-deficient controls (Fig. S4A). The cells had a moderately enhanced expression of Mcl-1 but the decrease of Mcl-1 during spontaneous apoptosis was clearly slower (Fig. S4C).

These results indicate that Noxa is an important mediator of spontaneous apoptosis in neutrophils, at least in part through its role of antagonizing Mcl-1. We confirmed these effects in primary neutrophils isolated from various gene-deficient mice. There was no significant survival advantage in Noxa-single-deficient cells, and we confirmed the reported moderate apoptosis-defect in Bim-deficient neutrophils (Fig. 5B). Cells from Bim/Puma-double-deficient animals showed slightly better survival compared to Bim-deficient cells but cells from Bim/Noxa-deficient mice demonstrated substantially reduced apoptosis compared to either Bim-deficient or Bim/Puma-double-deficient cells although Bim/Noxa double-deficiency was not quite as protective as transgenic Bcl-2 overexpression. Bim and Noxa thus cooperate to trigger spontaneous neutrophil apoptosis.

We further determined granulocyte numbers in peripheral blood of Bim/Noxa double-deficient mice compared to wt, Bim-deficient and Bcl-2 transgenic animals (Fig. 5C). Double-deficiency of bim and noxa led to an approximately 2.4-fold increase in granulocyte numbers compared to WT and to an 1.8-fold increase compared to bim^{-/-} (bim single-deficient mice had about 1.3 times as many neutrophils as wt mice). Neutrophil counts in bim/noxa double knock-out mice are thus comparable to those in vav-bcl-2 transgenic mice (about 2.3-fold over wt). Protection against spontaneous apoptosis and absolute numbers in peripheral blood therefore correlate.

During spontaneous apoptosis of differentiated neutrophils, the role of Noxa appeared to be the targeting and down-regulation of Mcl-1: comparison of Bim/Puma-deficient and Bim-Noxa-deficient cells showed a slower decrease of Mcl-1 in neutrophils lacking Noxa (Fig. 5D).

Intriguingly, despite its strong functional contribution the levels of Noxa mRNA declined during differentiation from progenitors to neutrophils (Fig. 5E). Relatively small amounts of Noxa mRNA therefore seem sufficient for Noxa function in neutrophils.

Proteasome inhibition showed that the loss of Mcl-1 protein was strongly determined by its proteolytic destruction (Fig. 5F). This was particularly apparent in Mcl-1-overexpressing cells. This strongly supports the view that in neutrophils Mcl-1-levels are regulated by mechanisms targeting its turnover, which are strong enough to counteract retroviral over-expression. One possible mechanism controlling Mcl-1 turnover is GSK3 mediated phosphorylation of Mcl-1 on specific serine sites, which destabilizes Mcl-1 by promoting proteasomal degradation of the protein²⁵. We tested this possibility by analyzing the effect of GSK3 inhibition on Mcl-1 turnover and cell death during spontaneous apoptosis of differentiated neutrophils. No protection from spontaneous apoptosis and no effect on Mcl-1 protein levels could be seen when the GSK3-specific inhibitor CT98104 was added to the culture (data not shown), which argues against a role of GSK3 for regulation of Mcl-1-levels in mature neutrophils.

The Noxa-Mcl-1-axis in progenitor cells

In progenitors, there was no significant protection by individual loss of Noxa or Bim or by combined loss of Bim and Puma. However, there was again a very strong protection by the combined loss of Bim and Noxa although this did not quite reach the protection level afforded by Bcl-2 over-expression (Fig. S4D). Again, death of these progenitors was accompanied by Mcl-1 loss, and this loss was slower in the absence of Noxa (Fig. S4E).

We further expressed A1 or Mcl-1 in Noxa-deficient progenitors. Loss of Mcl-1 during differentiation still occurred although it was slowed down in Noxa-deficient cells, indicating that mechanisms other than Noxa-binding contribute to the loss of Mcl-1 (Fig. S5A). Accordingly, there was no protection against apoptosis in neutrophils differentiated from Noxa-deficient, Mcl-1-overexpressing progenitors (Fig. S5B). In progenitors however, both Mcl-1 and A1 provided better protection in the absence of Noxa (Fig. S5C) than in its presence (wt progenitors Fig. S3A), suggesting that Noxa also in these cells exerts a pro-apoptotic effect through neutralizing A1 and Mcl-1.

Preliminary experiments indicate that down-regulation of Mcl-1 in progenitor cells by RNAi induced apoptosis in wt, Noxa- and Bim/Noxa-deficient progenitor cells to a similar extent (not shown). This supports the view that the loss of Mcl-1 (in part probably dependent on Noxa) is a contributing factor to spontaneous apoptosis.

Combined loss of Bim and Noxa prolongs neutrophil activity and their presence in peripheral blood

We have previously provided evidence suggesting that the inhibition of apoptosis by Bcl-2 also preserves functional activity of neutrophils¹⁰. To test the relevance of Bim/Noxa loss for neutrophil function, we cultured Bim/Noxa deficient neutrophils for 48 h (at this point almost all wt cells would be dead, Fig. 1A). When Bim/Noxa-double-deficient cells at 48 h were tested for functional parameters, we found that the cells were still fully capable of phagocytosis of bacteria (Fig. 6A), oxidative burst (Fig. 6B) and TNF- and IL-6-production (Fig. 6C, D). When Bim/Noxa-double-deficient neutrophils were injected into mice, they could be detected in peripheral blood much longer than wt cells suggesting better survival also *in vivo* (Fig. 6E). Apoptosis is thus the central process that regulates not only neutrophil apoptosis *in vitro* and *in vivo* but also their function. On a molecular level neutrophil apoptosis is regulated by the loss of Mcl-1 function and by the combined effects of Bim and Noxa.

Discussion

In this study we probe the role of the Bcl-2-family of proteins in Hoxb8-differentiated neutrophils. The comparison with primary, freshly isolated mouse neutrophils indicates the suitability of these cells for the study of primary neutrophil physiology. Our data confirm earlier results obtained with primary neutrophils but provide substantial new information about the molecular players regulating neutrophil spontaneous apoptosis.

Hoxb8-differentiated neutrophils are obviously not primary neutrophils. However, they have the morphological and functional hallmarks and express all the markers found in primary neutrophils¹⁷. Importantly for this study, they show the typical high rate of spontaneous apoptosis in culture. The possibility of generating cell lines from genetically modified mice and of over- and under-expressing genes in these neutrophils by transduction of progenitors allows a much more profound study of the regulation of neutrophil function and apoptosis.

There were subtle differences in apoptosis sensitivity between gene-modified Hoxb8-derived and primary neutrophils: the effect of the loss of Puma or Noxa on top of the Bim-deficiency was less pronounced in primary neutrophils. However, there is no unequivocal standard for the isolation or investigation of primary neutrophils. We used neutrophils sorted from the bone marrow of mice, a commonly used source, but these cells are heterogeneous in terms of maturation status, and they may have suffered during isolation. It is therefore difficult to know what in fact the 'best neutrophil' would be.

Human neutrophils undergoing apoptosis lose expression of Mcl-1, and in the presence of survival-prolonging factors this decrease is slower²⁶. It has recently also been shown that the genetic deletion of Mcl-1 prevented the generation of mature neutrophils, most likely due to apoptosis of immature myeloid progenitors²³. Our data show that Mcl-1 rapidly decreases in mature neutrophils also in situations where apoptosis is independently blocked

by over-expression of Bcl-2. This suggests that the loss of Mcl-1 and probably other anti-apoptotic Bcl-2 proteins is a primary event that triggers apoptosis.

The levels of Mcl-1 appear in many situations to be regulated by the turnover of the protein. Mcl-1-degradation has been shown to be regulated by the deubiquitinase USP9X²⁷ but also by ubiquitin-independent degradation²⁸. As upstream regulators of Mcl-1-degradation, glycogen synthase kinase-3 (GSK3)-dependent phosphorylation²⁵ and the binding by the BH3-only protein Noxa²⁴ have been identified. Noxa-deficient neutrophils (and progenitors) had slightly elevated Mcl-1 levels and, more clearly, a slower reduction in Mcl-1 levels during spontaneous apoptosis than wt cells. Noxa is therefore in part responsible for the loss of Mcl-1. As (slower) Mcl-1-degradation still occurred in the absence of Noxa, other factors such as PI3-kinase/GSK3-signalling also may be relevant.

Noxa is required for normal spontaneous neutrophil apoptosis. Indeed, the effect of the loss of Noxa in Bim-deficient cells may be the strongest effect of Noxa-loss reported to date. Bim and Noxa together were responsible for most of this form of apoptosis. In the absence of Noxa, there was only a small reduction in apoptosis; Bim therefore seems to be able to do most of the work. However, there was still considerable apoptosis in the single absence of Bim, and this apoptosis was mediated by Noxa.

Transgenic over-expression of Bcl-2 provided still somewhat stronger protection than the combined loss of Bim and Noxa. Perhaps additional BH3-only proteins such as Puma, Bmf or Bad have some small effect that is unmasked by the loss of Bim and Noxa. We think it also conceivable that a small amount of apoptosis occurs without contribution from BH3-only proteins, perhaps through cytosolic changes that directly activate Bax or Bak, and this small effect would also be blocked by direct binding of Bcl-2 to Bax/Bak.

The only molecular mechanism of Noxa action known is linked to the antagonism of Mcl-1 and A1. All our results are also consistent with this mode of action. Noxa very likely regulates the levels of Mcl-1, and in the absence of Bim this is necessary for full apoptosis.

Bim, on the other hand, can overcome the protection by Mcl-1. Authors are divided on the view whether Mcl-1 blocks apoptosis by binding to Bax and Bak or by binding to BH3-only proteins like Bim¹³. Either way, in neutrophils the activity of Bim is the main trigger of spontaneous apoptosis but apoptosis is modulated by Noxa, which in turn regulates the function of Mcl-1 and probably A1.

How Bim is activated in this situation is not entirely clear. There was no increase in Bim levels although Bim was clearly activated. There was, however, besides down-regulation of Mcl-1 also a moderate reduction in protein levels of anti-apoptotic Bcl-2 and Bcl-X_L. It seems likely that this down-regulation is involved in the activation of Bim. Moreover, Bim-induction does not necessarily correlate with its activation, as was previously demonstrated for LPS- or GM-CSF-induced survival^{29,30}.

Neutrophils in the Hoxb8-system differentiate from progenitors that morphologically resemble early promyelocytes. In these cells the effect of the loss of Bim or Noxa alone was marginal but a very strong effect was seen when both were missing.

On their way from these progenitors to neutrophils, the cells acquire a phenotype already inclined towards apoptosis: there was down-regulation of Bcl-2, Bcl-X_L and Mcl-1, together with up-regulation of Bax, Bak and Bim. One interpretation of these changes is that not only mature neutrophils are programmed to die but already a progenitor cell starts this program, perhaps when it stops dividing.

Neutrophil apoptosis is a program that is initiated at some point and that runs independently of programs that regulate functional capacity: when apoptosis was blocked by the loss of Bim and Noxa, cells were still fully functional. On a therapeutic level, this may be relevant since it indicates that strategies aiming at apoptosis inhibition will preserve neutrophil function and may be useful in situations of human neutropenia.

We believe that the Hoxb8-system is well-suited for the analysis not only of apoptosis but also for other investigations into neutrophil function. For apoptosis research, it has the

advantage of combining a near-physiological situation of apoptosis, a non-transformed cell and the ease of the generation of relatively large numbers of cells with the possibility of easy genetic manipulation.

Materials and Methods

Cell lines and cell culture

Hoxb8 neutrophil progenitors were derived from bone marrow of WT, Bim^{-/-}, Noxa^{-/-}, Bim^{-/-}/Puma^{-/-}, Bim^{-/-}/Noxa^{-/-} deficient and (human) *vav-bcl2*-transgenic mice on C57Bl/6 background. Polyclonal precursor cell lines were established by retroviral transduction of Hoxb8 and selection in the presence of stem cell factor as described¹⁷. Precursor cell lines were cultured in Optimem medium (Invitrogen) supplemented with 10% FCS (PAA, Coelbe, Germany), 30 mM β-mercaptoethanol (Sigma), antibiotics (100 IU/ml penicillin G and 100 IU/ml streptomycin sulfate, PAA), 1-4% supernatant from stem cell factor (SCF)-producing Chinese Hamster Ovarian cells, and 1 μM estrogen (Sigma). Neutrophil differentiation was induced by removal of estrogen, and subsequent culture for 4 days in medium containing 1-2% SCF-supernatant. To assess differentiation status, cells were subjected to cytopspin followed Giemsa staining and analyzed by light microscopy. In some experiments, cells were treated with LPS (Sigma), PMA (Sigma), the pan-caspase inhibitor qVD-OPH (R&D Systems) or MG-132 (Enzo Life Sciences).

Isolation of primary mouse neutrophils

Mouse neutrophils were positively selected from bone marrow of WT and mutant mice using the Miltenyi MACS Purification Kit (Miltenyi Biotech). Following harvest of bone marrow cells and red blood cell lysis, neutrophils were labeled with FITC-coupled anti-Gr-1 antibody (BD Biosciences), followed by anti-FITC antibody coupled to magnetic beads. Labeled cells were isolated by passage over a MACS column (Miltenyi Biotech). Gr-1-positive cells were cultured in Optimem medium (Invitrogen) supplemented with 10% FCS, 30 mM β-mercaptoethanol and antibiotics and analyzed as indicated.

Quantification of mouse granulocyte numbers in peripheral blood

100 μl of heparinized blood were collected from the orbital sinus of mice. Samples were analyzed in a scil Vet abc (Scil Animal Care Company GmbH; Viernheim, Germany).

Retroviral transduction of neutrophil precursor lines

To generate Hoxb8 precursor lines stably overexpressing various anti-apoptotic proteins, mouse Bcl-2, Bcl-X_L, Mcl-1 and A1 were transferred from pENTR/SD/D-TOPO vector into MIGR1-GW retroviral vector by gateway-based LR recombination reaction according to the manufacturer's instructions (Invitrogen). Retroviral particles were produced by transient transfection of the corresponding retroviral expression plasmids together with packaging plasmid pCLEco into the ecotrophic Phoenix packaging cell line using Fugene HD transfection reagent (Roche). Retroviral supernatants were harvested on day 2 and 3, filtered through 0.45 µm membranes and used for infection of target cells at a cell density of 1-2 x 10⁵/ml in the presence of 5 µg/ml polybrene. GFP-positive cells were FACS-sorted and precursor lines with >90 % of GFP-positive cells were used for experiments.

Lentiviral transduction for generation of shRNA-mediated stable knockdown lines

For establishment of shRNA-mediated stable knockdown lines, a lentiviral system was used essentially as described³¹. Briefly, shRNA sequences were cloned into pENTR-THT and transferred via Gateway LR recombinase reaction (Invitrogen) into the lentiviral destination vector pHR-dest-SFFV-eGFP. Production of lentiviral particles was performed by transient transfection of shRNA expression vectors into HEK 293FT cells (Invitrogen) together with packaging plasmids psPAX2 and pMD2.G (Dr. Didier Trono, Lausanne) using Fugene HD transfection agent (Roche). Lentiviral supernatants were harvested on day 2 and 3, filtered through 0.45 µm membranes and used for infection of target cells at a density of 1-2 x 10⁵/ml in the presence of 5 µg/ml polybrene. GFP-positive cells were cell sorted and precursor lines with >90 % of GFP-positive cells were used for experiments.

Apoptosis assays

For analysis of apoptosis by AnnexinV-PI staining, Hoxb8 neutrophils were differentiated for 4 days, then washed in PBS/10 % FCS, and cultured in the absence of SCF for the indicated time periods. After washing with AnnexinV-binding buffer (10 mM Hepes, 140 mM NaCl,

2.5 mM CaCl₂), cells were incubated with AnnexinV-FITC or AnnexinV-APC (1:50, BD Pharmingen) and propidium iodide (1 µg/ml, Sigma) for 20 minutes, followed by flow cytometry analysis.

In some experiments, cell death was assessed by PI staining (1 µg/ml) for loss of cell membrane integrity followed by flow cytometry analysis.

For analysis of caspase-3 activation neutrophils differentiated for 4 days and washed as described above were cultured in the absence of SCF as indicated. Cells were washed with PBS, fixed in 3.7 % formaldehyde and permeabilized with 1 % Saponin (Sigma). Staining was performed with rabbit anti-active caspase-3 (BD Pharmingen) in PBS/0.5% BSA for 40 minutes, followed by incubation with secondary donkey anti-rabbit-FITC or anti-rabbit-Cy5 (Dianova) for 30 minutes, and samples were analyzed flow cytometry.

Analysis of neutrophil effector functions

Hoxb8 neutrophils were differentiated for 4 days and deprived of SCF for up to 48 hours as described for apoptosis assays. For analysis of phagocytic capacity, RFP- or GFP-expressing *E. coli* (DH5α) were grown overnight in liquid culture, harvested, washed with PBS and the optical density of the suspension at 600 nm was adjusted to 2.0. Neutrophils and *E. coli* were coincubated at a bacteria:cell ratio of 200:1 at 37°C for the indicated time periods. After extensive washing, cells were analyzed by flow cytometry. In case of adherence controls, cells were incubated at 4 °C. For analysis of oxidative burst, reactive oxygen species production was assessed by oxidation of dihydrorhodamine. Neutrophils (4×10^5) were stimulated with 5 µM PMA (Sigma) or with FITC-labeled pneumococci (bacteria:cell ratio 500:1), then 2 mM dihydrorhodamine (Sigma) was added and incubation was continued for another 30 minutes. Cells were analyzed by flow cytometry. For the measurement of TNF and IL-6 production, neutrophils were co-incubated with *E. coli* for 16 hours (bacteria: cell ratio 200:1). Supernatants were harvested and cytokines were quantified using mouse TNFα and IL-6 ELISA kits (eBioscience) according to the manufacturer's instructions.

RNA extraction and quantification

Total RNA was extracted and transcribed into cDNA with commercial kits (Roche) following the manufacturer's instructions. Samples were analyzed by quantitative RT-PCR using the Light Cycler TaqMan Master Kit and the Universal Probe Library system (Roche). Relative expression of the gene of interest was normalized to β -actin reference gene expression.

Immunoblot analysis

Cells were harvested and immediately lysed in Laemmli buffer ($3 \times 10^6/50 \mu\text{l}$). After separation of cell extracts by SDS-PAGE, proteins were transferred onto PVDF membranes. Loading of equal cell numbers was confirmed by probing for GAPDH or Tubulin using specific antibodies (Millipore, Sigma). Membranes were probed with antibodies against mouse Bim, Bcl-X_L, Bcl-w, Bax (all from Cell Signaling Technology), Noxa (Abcam), Bid, Puma (NT, ProSci), Bak (Millipore), Bcl-2 (BD Pharmingen), Mcl-1 (Rockland; Epitomics) or A1. Proteins were visualized using peroxidase-conjugated secondary antibodies against rabbit (Sigma), mouse, rat or hamster (all from Dianova) and an enhanced chemoluminescence detection system (GE Healthcare).

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Conflict of interest

The authors declare no conflict of interest.

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Titles and legends to figures

Fig.1 Spontaneous apoptosis of differentiated Hoxb8 neutrophils of various genotypes

(a,b) Analysis of spontaneous apoptosis of Hoxb8 neutrophils by staining with AnnexinV-FITC and propidium iodide (PI) (a) or staining for active caspase-3 (b). Mature neutrophils of the indicated genotypes (differentiated for 4 days by estrogen withdrawal) were incubated in the absence of SCF for the indicated time periods and analyzed by flow cytometry. Shown are all AnnexinV-positive cells including cells staining double-positive for AnnexinV and PI (a). Data are mean \pm SEM of three experiments.

(c) AnnexinV/PI staining of differentiated WT neutrophils SCF-deprived for 24h in the presence of the pan-caspase inhibitor qVD-OPH (50 μ M) or DMSO as vehicle control (veh). Data are mean/SEM (n=3 experiments).

Fig.2 Bcl-2 family protein expression levels during spontaneous apoptosis in Hoxb8 neutrophils

Immunoblot analysis of WT and Bcl-2-tg neutrophils. Mature neutrophils were SCF-deprived for the indicated time periods. Equivalents of 9×10^5 cells /sample were directly lysed in Laemmli buffer, analyzed by SDS-PAGE and immunoblotted for expression of Mcl-1, Bcl-2, Bcl-X_L, Bim, Bax or Bak. GAPDH served as loading control. Shown is one of two independent experiments with similar results.

Fig.3 Bcl-2 family protein levels during neutrophil differentiation

(a) Immunoblot analysis of Hoxb8 neutrophils during estrogen-withdrawal-induced differentiation. Equivalents of 6×10^5 cells /sample were directly lysed in Laemmli buffer, analyzed by SDS-PAGE and immunoblotted for protein expression of Bcl-2 family proteins. GAPDH served as loading control. Data are representative of a total of three experiments.

(b) Quantification of mRNA levels of Mcl-1 by real-time PCR in wt neutrophils during differentiation. mRNA levels are shown as “fold change” relative to mRNA expression in progenitors (day 0). Data are normalized to β -actin expression and represent mean/SEM of 4-6 experiments.

Fig.4 Protection by antiapoptotic Bcl-2 family members in wt and $Bim^{-/-}/Puma^{-/-}$

neutrophils

(a) Analysis of spontaneous apoptosis of differentiated wt and $Bim^{-/-}/Puma^{-/-}$ neutrophils stably expressing either empty vector control (MIGR1) or antiapoptotic proteins Bcl-2, Bcl-X_L, Mcl-1 or A1. Mature neutrophils were incubated in the absence of SCF for the indicated time periods. Cell death was analyzed by PI staining. Data are mean /SEM (n=3 experiments for empty vector control, Bcl-X_L, Mcl-1 and A1; n=2 experiments for Bcl-2).

(b) Immunoblot analysis of wt and $Bim^{-/-}/Puma^{-/-}$ neutrophil progenitors and differentiated neutrophils stably expressing either empty vector control (MIGR1) or antiapoptotic proteins Mcl-1 or A1. Cells were harvested as progenitors or as mature neutrophils after estrogen-withdrawal-induced differentiation for 4 days. Laemmli lysates (9×10^5 cells /lane) were analyzed by SDS-PAGE and immunoblotted for protein expression of Mcl-1 and A1 as indicated. Tubulin served as loading control. Data are representative of three independent experiments.

Fig.5 Regulation of spontaneous apoptosis in neutrophils by Bim and Noxa.

(a) Spontaneous apoptosis of mature neutrophils of various genotypes (starting at d4 of estrogen withdrawal). Cell death was analyzed by PI staining followed by flow cytometry. Data are mean/SEM of three (Bcl-2-tg, Noxa^{-/-}, $Bim^{-/-}/Puma^{-/-}$) or four (WT, $Bim^{-/-}/Noxa^{-/-}$) independent experiments.

(b) Spontaneous apoptosis of primary neutrophils from wt, single ($Bim^{-/-}$, $Puma^{-/-}$, $Noxa^{-/-}$) or double ($Bim^{-/-}/Puma^{-/-}$, $Bim^{-/-}/Noxa^{-/-}$) gene-deficient mice or vav-Bcl-2-transgenic mice.

Neutrophils isolated from bone marrow were cultured in vitro and harvested at the indicated time points. Cell death was determined by PI staining and flow cytometry analysis. Data are mean/SEM of a total of three ($Bim^{-/-}$, $Puma^{-/-}$, $Noxa^{-/-}$, $Bim^{-/-}/Puma^{-/-}$, vav-Bcl-2) or four mice (wt, $Bim^{-/-}/Noxa^{-/-}$).

(c) Double deficiency of Bim and Noxa leads to accumulation of granulocytes in peripheral blood of mice. Comparison of peripheral blood granulocyte counts ($\times 10^6$ /ml) from wt (n=5), Bim-deficient (n=4), Bim/Noxa double-deficient (n=5) or vav-Bcl-2-tg mice (n=9).

Granulocyte numbers of peripheral blood were analyzed using a scil Vet abc blood cell counter. Data represent mean/SEM.

(d) Mcl-1-expression during spontaneous apoptosis of Bim/Noxa and Bim/Puma double-deficient neutrophils. Mature neutrophils (differentiated for four days) were SCF-deprived for the indicated time periods. Equivalents of 9×10^5 cells /sample were directly lysed in Laemmli buffer, analyzed by SDS-PAGE and immunoblotted for expression of Mcl-1. GAPDH served as loading control. Shown is one of three independent experiments with similar results.

(e) Quantification of mRNA levels of Noxa by real-time PCR in wt neutrophils during differentiation. mRNA levels are shown as “fold change” relative to mRNA expression in precursor cells (day 0). Data are normalized to β -actin expression and represent mean/SEM of 4 experiments.

(f) Immunoblot analysis of protein expression in neutrophils upon proteasome inhibition.

Differentiated WT neutrophils were either SCF-deprived or not for 14 hours, then treated with $10 \mu\text{M}$ MG-132 or solvent control for 6 hours. Nine times 10^5 cells/lane were lysed in Laemmli buffer and subjected to SDS-PAGE and immunoblotting for Mcl-1, A1 and Bim. Equal loading was confirmed by probing for GAPDH. Data are representative of two independent experiments.

Fig.6 Inhibition of apoptosis through combined loss of Bim and Noxa maintains neutrophil effector function

- (a) Phagocytic uptake of GFP-expressing *E. coli* by SCF-deprived Bim^{-/-}/Noxa^{-/-} neutrophils. Neutrophils were subjected to co-incubation at 37 °C or 4 °C (adherence control) with *E. coli* either immediately after differentiation (all genotypes) or after additional SCF-deprivation for 48 hours (Bim^{-/-}/Noxa^{-/-} and Bcl-2-tg). After one hour of co-incubation, cells were extensively washed and analyzed by flow cytometry. Uptake is indicated by GFP-fluorescence. Similar results were seen in 2 independent experiments.
- (b) Production of reactive oxygen species was measured by the capacity of neutrophils to oxidize dihydrorhodamine. Bim^{-/-}/Noxa^{-/-} or Bcl-2-tg neutrophils differentiated for 4 days were additionally SCF-deprived for 48 hours and analyzed in comparison to viable WT neutrophils (differentiated for 4 days). Neutrophils were incubated for 30 min with PMA (5 µg/ml, bold line). Unstimulated stained cells are shown as shaded histogram. Similar results were seen in two independent experiments.
- (c, d) Secretion of TNFα (c) or IL-6 (d) was measured by ELISA in supernatants of neutrophils stimulated with *E. coli* for 16 hours at a bacteria:cell ratio of 200:1. Data are means/SEM of three independent experiments.
- (e) *Ex vivo* analysis of neutrophil survival after intravenous injection into mice. Either wt or Bim/Noxa double-deficient neutrophils carrying GFP as a marker were differentiated *in vitro* for 4 days and then intravenously injected into wt C57Bl/6 mice (2 x 10⁷ cells/mouse). The percentage of GFP-positive cells in the blood, relating to total blood neutrophils, was analyzed by flow cytometry at the indicated time points. Shown are percentages of GFP-positive cells of all blood neutrophils after 24h (left panel), or after 24h, 4 and 7 days (right panel), respectively. Data represent mean/SEM of a total of 4 mice each group (n.d., not detectable).

Fig 1

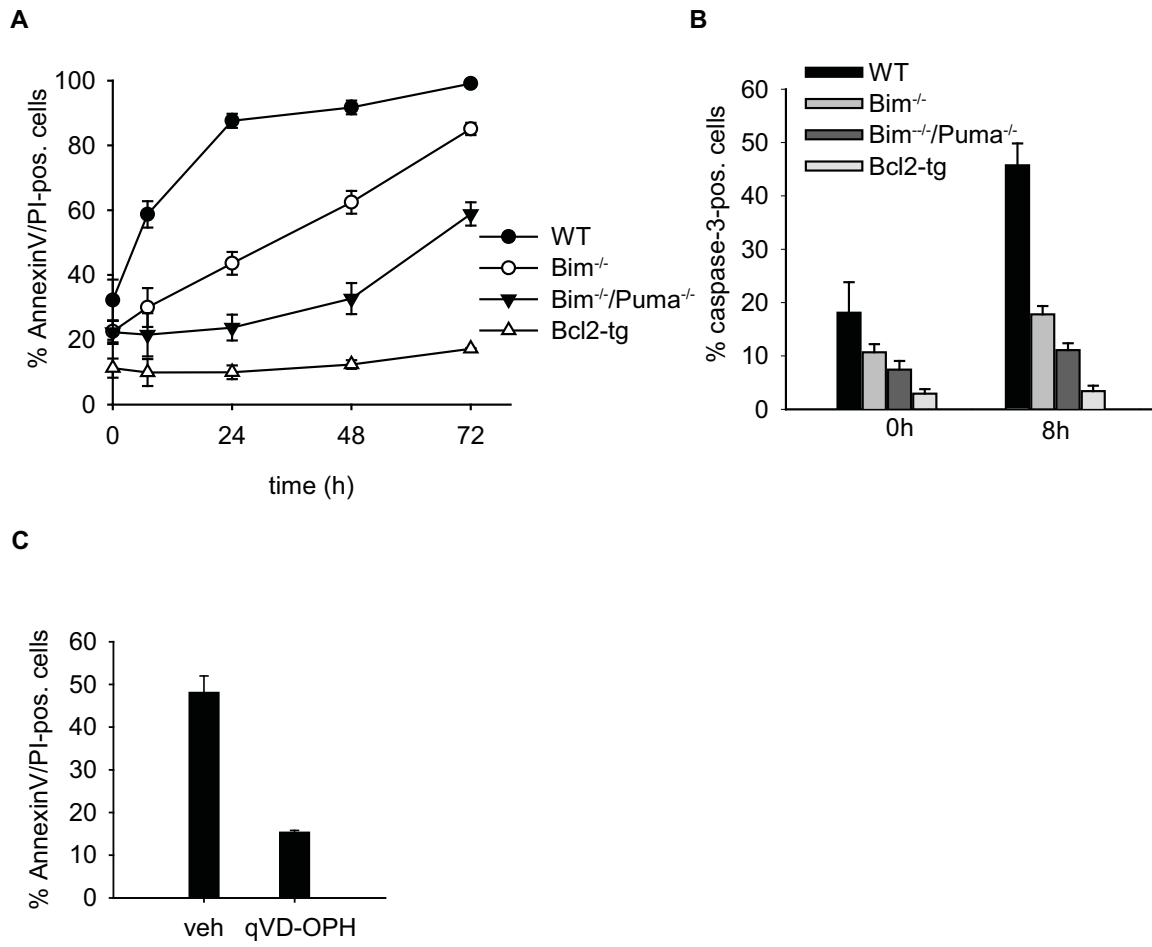
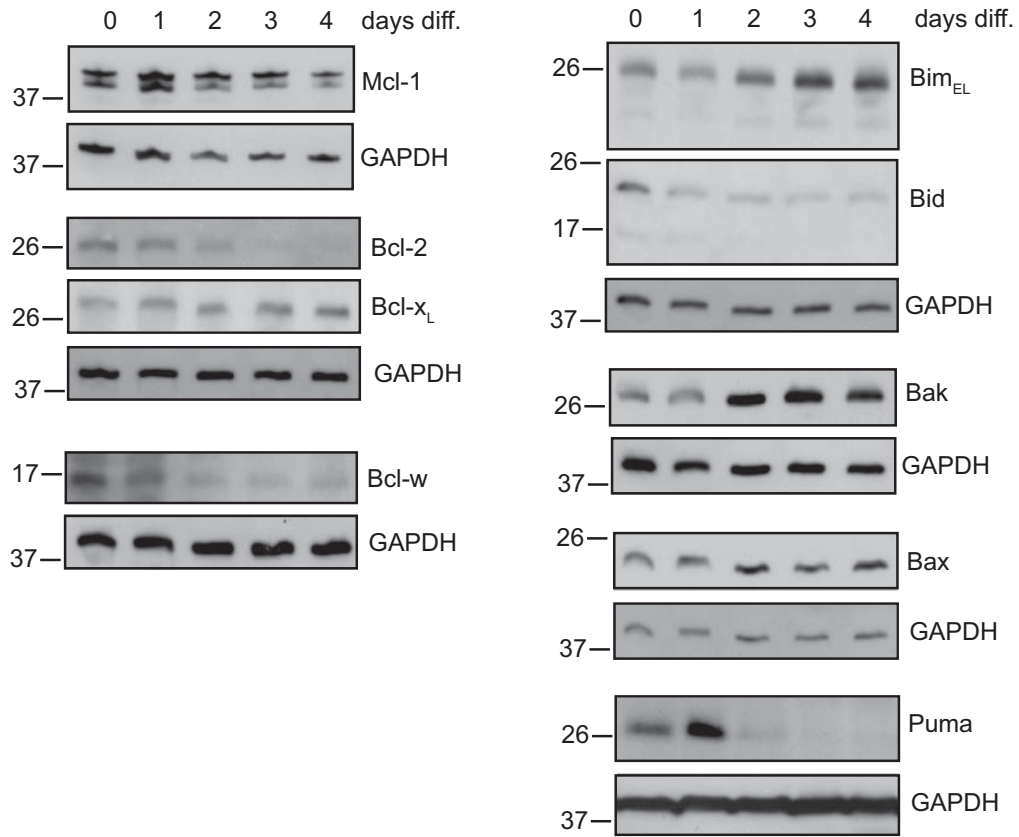


Fig. 3

A



B

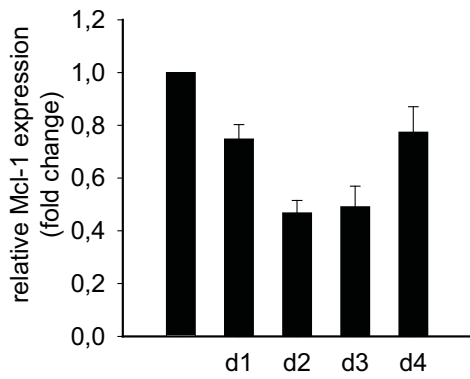


Fig.5

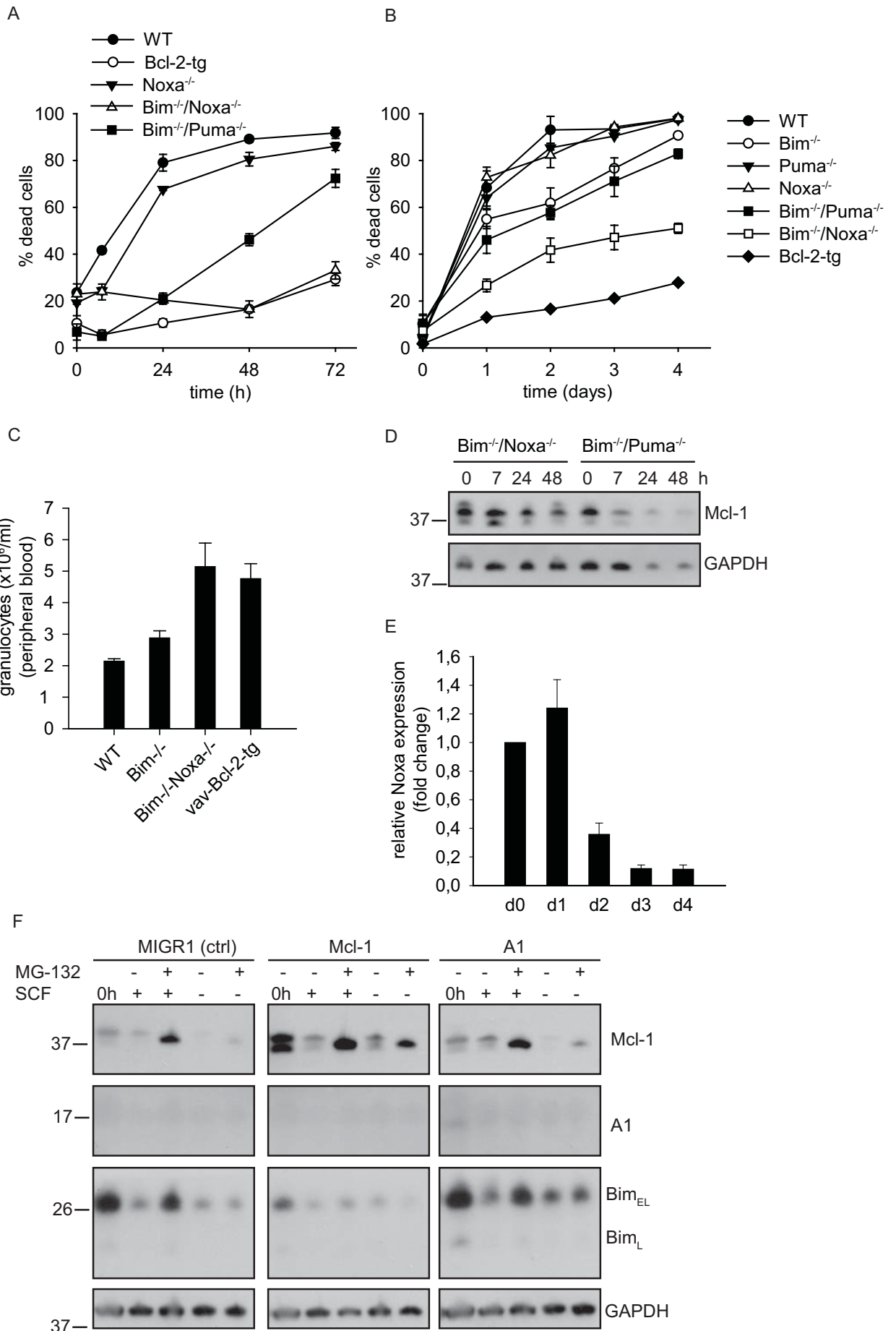
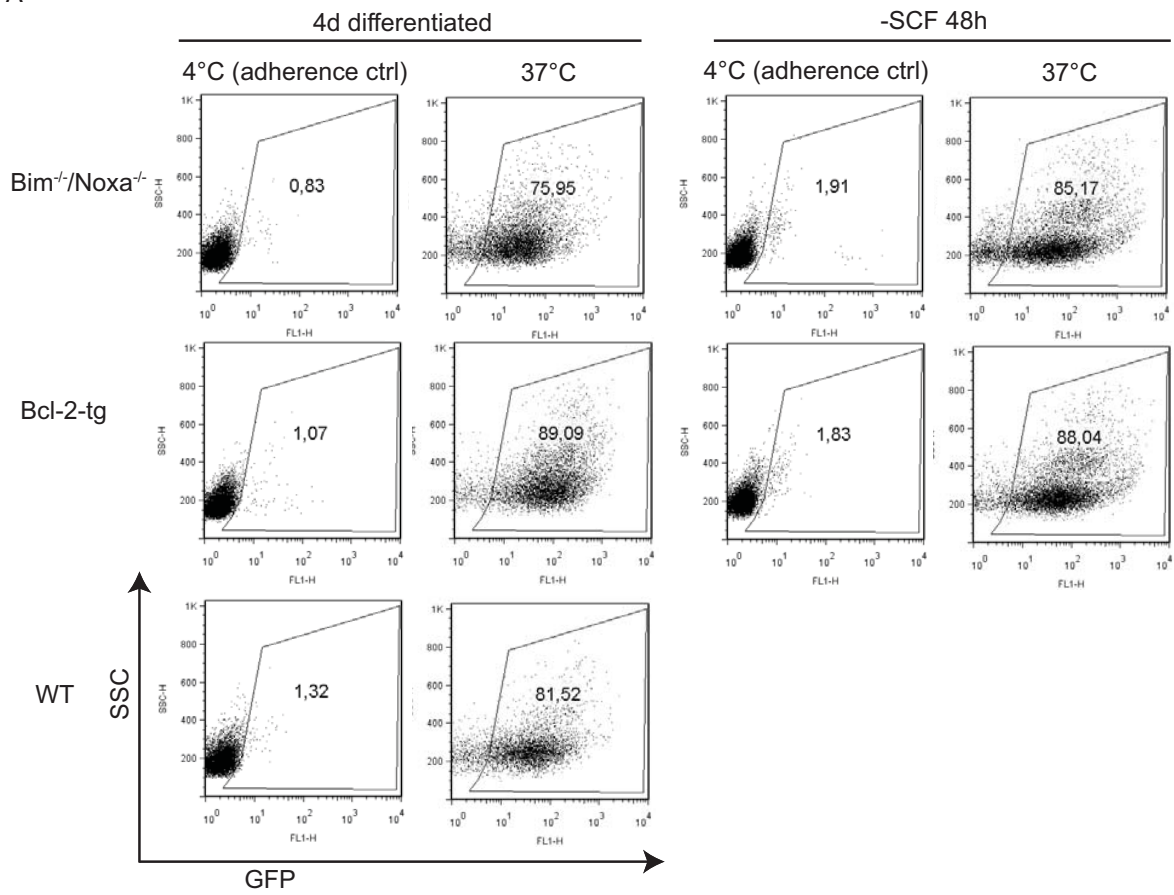


Fig. 6

A



B

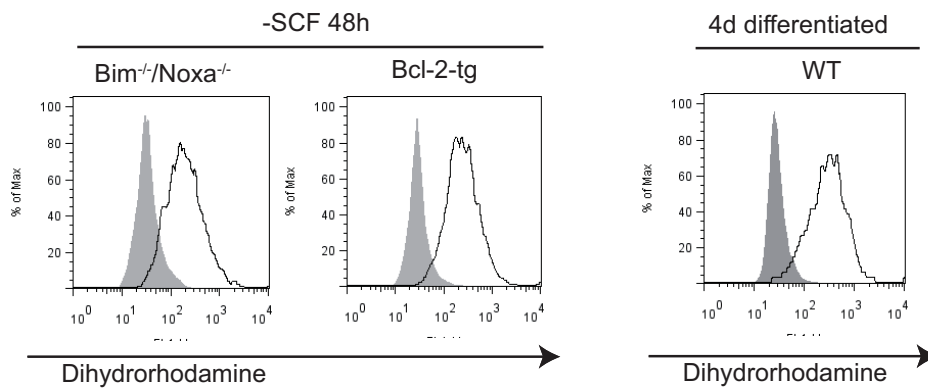
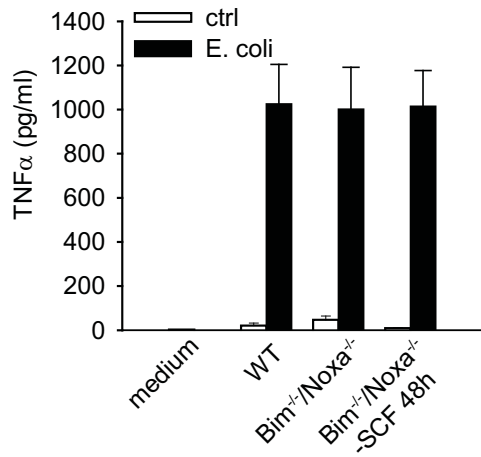
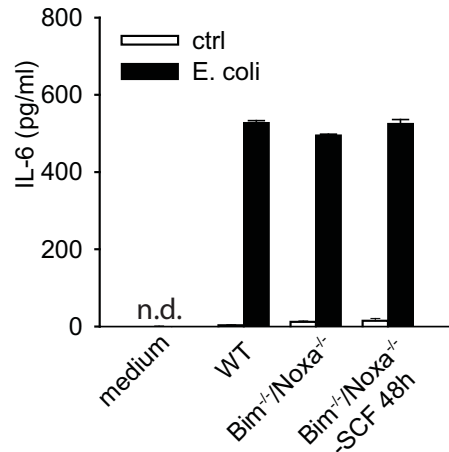


Fig. 6

C



D



E

