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# The pro-survival Bcl-2 family member A1 delays spontaneous and FAS ligand-induced apoptosis of activated neutrophils

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Neutrophils have a short lifespan that is extended after exposure to granulocyte macrophage colony stimulating factor (GM-CSF) or lipopolysaccharide (LPS)[1]. While the survival is regulated by BCL-2 family proteins[2], it is not known which pro-survival proteins are involved. GM-CSF stimulation in neutrophils upregulates A1, but *A1*-deficient mice showed no defects in this cell type[3]. MCL-1 is critical for the survival of quiescent neutrophils[4,5], but it is not known whether the same holds true after activation. We hypothesized that A1 and MCL-1 have overlapping roles in the survival of activated neutrophils.

We generated mutant mice deficient for A1 and lacking one allele of *Mcl-1* (*Mcl-1*$^{+/-}$*A1*$^{-/-}$). *Mcl-1*$^{+/-}$*A1*$^{-/-}$ mice are grossly normal in the haematopoietic compartment, with only a small reduction in lymphocyte numbers, similar to *Mcl-1*$^{+/-}$ mice[6] (Supplementary Fig. 1A). Loss of A1 did not cause a survival defect in GM-CSF-stimulated neutrophils. Here, we examined the survival of neutrophils activated with LPS plus GM-CSF from *A1*$^{-/-}$, *Mcl-1*$^{+/-}$, and *Mcl-1*$^{+/-}$*A1*$^{-/-}$ mice. Without stimulation, *Mcl-1*$^{+/-}$ neutrophils had a significant survival disadvantage compared to their wild-type and *A1*$^{-/-}$ counterparts and no further decrease in cell survival was observed in *Mcl-1*$^{+/-}$*A1*$^{-/-}$ neutrophils (Fig. 1a). Presumably, this increased apoptosis observed in *Mcl-1*$^{+/-}$ neutrophils is due to the in vitro conditions, as we saw normal neutrophil numbers in vivo in *Mcl-1*$^{+/-}$ or *Mcl-1*$^{+/-}$*A1*$^{-/-}$ mice (Supplementary Fig. 1B). After activation with LPS plus GM-CSF, the A1$^{-/-}$ and *Mcl-1*$^{+/-}$*A1*$^{-/-}$ neutrophils exhibited significantly poorer survival, whilst *Mcl-1*$^{+/-}$ neutrophils behaved similarly to wild-type cells (Fig. 1b). LPS treatment alone was ineffective at promoting a survival advantage and failed to induce neutrophil blasting or upregulate pro-survival MCL-1 expression (Supplementary Fig. 2A–C). GM-CSF treatment alone promoted survival, blasting, and MCL-1 upregulation in wild-type and *A1*$^{-/-}$ cells[3]. GM-CSF is known to induce expression of the TLR4 co-receptor CD14[7]. We observed marked upregulation of CD14 on neutrophils after GM-CSF stimulation, and more so after treatment with GM-CSF plus LPS (Supplementary Fig. 2C). Hence, the survival defect of LPS plus GM-CSF-stimulated *A1*$^{-/-}$ neutrophils could be due to a lack of increased A1 expression, contributing to the survival of activated neutrophils[8,9].

Neutrophils are highly sensitive to FAS-induced apoptosis[1], but this death is delayed when they are activated by LPS plus GM-CSF[1]. We analyzed FASL-induced apoptosis with and without LPS plus GM-CSF stimulation in neutrophils from *A1* and *Mcl-1* mutant mice. Additionally, FASL-induced apoptosis in neutrophils is dependent on caspase-8-mediated activation of the pro-apoptotic BCL-2 family member BID (called tBID)[10], which A1 binds to with high affinity[11]. We therefore also included *Bid*$^{-/-}$ mice[12] as a control in our experiments and, furthermore, generated *Bid*$^{-/-}$*A1*$^{-/-}$ mice in order to examine whether any effects seen in the *A1*$^{-/-}$ cells were dependent on A1–tBID interactions.

*Mcl-1*$^{+/-}$ (and *Mcl-1*$^{+/-}$*A1*$^{-/-}$) neutrophils died quicker than wild-type cells after FASL treatment (Fig. 1c). FASL-induced apoptosis was greater than basal apoptosis in culture (Supplementary Fig. 3). *Bid*$^{-/-}$ neutrophils were protected from FASL-induced apoptosis[10]. LPS plus GM-

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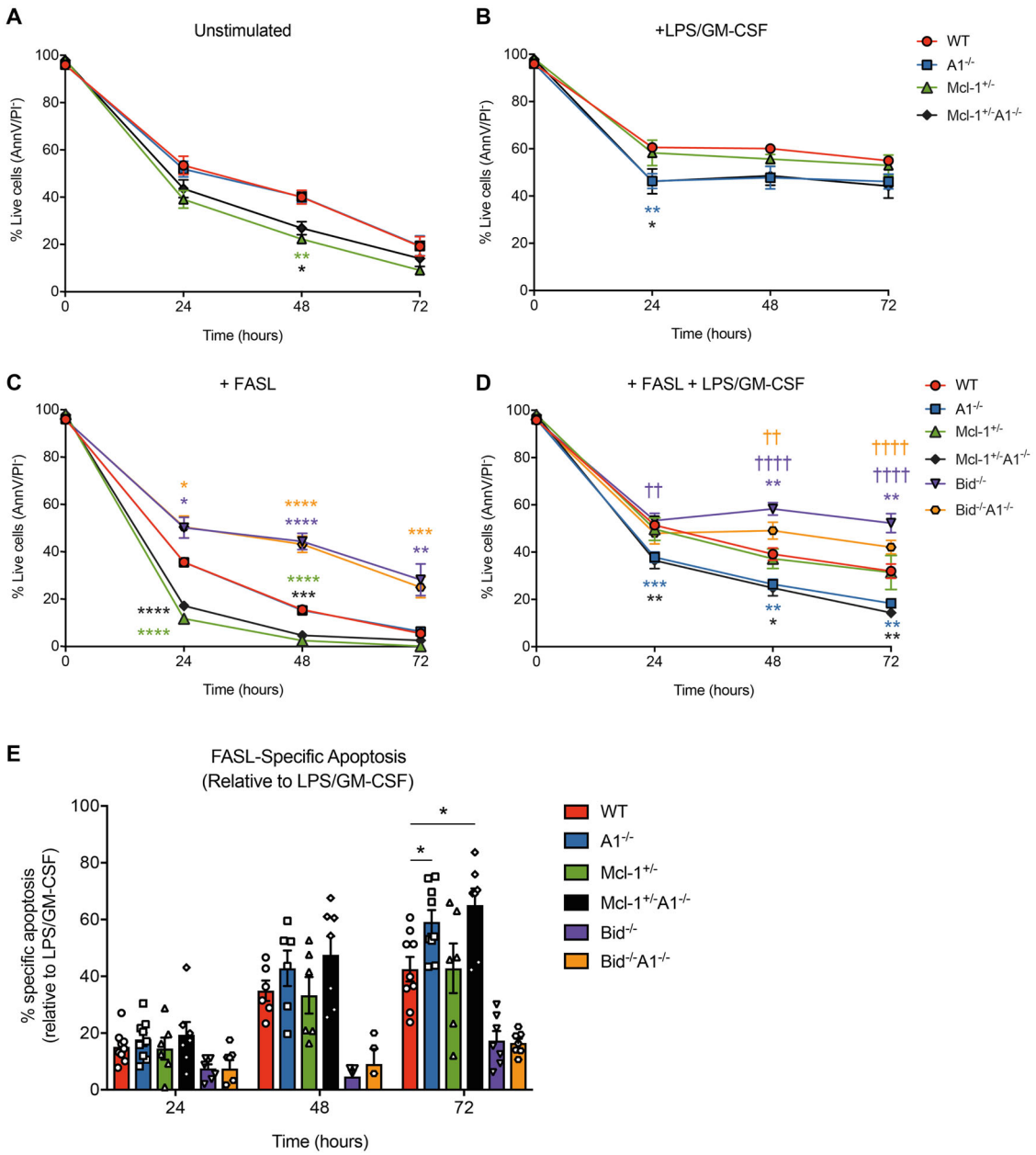


**Fig. 1** Survival analysis of neutrophils from mice with the indicated genotypes cultured in **a** simple medium (no added cytokines), **b** after stimulation with 10 ng/mL GM-CSF plus 10 ng/mL LPS, **c** after treatment with Fc-FASL (0.6 ng/mL), and **d** after stimulation with LPS plus GM-CSF (10 ng/mL each) and Fc-FASL (0.6 ng/mL). **e** FASL-specific apoptosis when compared to survival of cells stimulated with LPS plus GM-CSF. Data are from five combined experiments (WT $n = 9$, $A1^{-/-}$ $n = 9$, $Mcl\text{-}1^{+/-}$ $n = 6$, $Mcl\text{-}1^{+/-}A1^{-/-}$ $n = 7$, $Bid^{-/-}$ $n = 7$, and $Bid^{-/-}A1^{-/-}$ $n = 7$ mice). Statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) was determined using Student's *t*-test at each timepoint compared to WT (*) or $A1^{-/-}$ (†).

CSF protected both wild-type and $Mcl\text{-}1^{+/-}$ neutrophils against FASL-induced killing (Fig. 1d). In contrast, $A1^{-/-}$ and $Mcl\text{-}1^{+/-}A1^{-/-}$ neutrophils exhibited significantly more apoptosis across all time points after treatment with FASL in LPS plus GM-CSF-activated neutrophils. Taking into account the increase in apoptosis after LPS plus GM-CSF stimulation in $A1^{-/-}$ neutrophils. We observed a trend towards more FASL-specific apoptosis in the A1-deficient cells, although this only reached statistical significance at 72 h (Fig. 1e). The amount of FASL-specific apoptosis did not differ between $Bid^{-/-}$ and $Bid^{-/-}$ $A1^{-/-}$ cells, indicating that the increased sensitivity of activated $A1^{-/-}$ neutrophils to FASL killing is mediated by tBID. $Bid^{-/-}A1^{-/-}$ neutrophils displayed lower viability than their $Bid^{-/-}$ counterparts, both after LPS plus GM-CSF stimulation (Supplementary Fig. 4) and with the

combination of LPS, GM-CSF, and FASL (Fig. 1d), fitting with the role we showed for A1 in promoting cell survival after LPS plus GM-CSF stimulation alone.

Collectively, we demonstrate that upregulation of A1 after stimulation imparts a survival advantage in neutrophils, including FASL-induced apoptosis. However, A1's role is relatively small, and other factors must also regulate the survival of activated neutrophils. These results suggest a previously unrecognized role for A1 in promoting neutrophil survival in an inflammatory context.

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**Author contributions**
R.L.S. performed and designed most experiments and wrote the manuscript; L. G. helped to perform experiments and write the manuscript; K.E.L. helped with discussions and advice on neutrophil experiments and write the manuscript; L. A.O. provided reagents and helped with advice on FASL experiments and write the manuscript; A.S. and M.J.H. planned the project, were involved in experimental design and helped to write the manuscript.

**Conflict of interest**
The authors declare that they have no conflict of interest.

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