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Interleukin 15–mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1

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Abstract

Interleukin 15 (IL-15) promotes the survival of natural killer (NK) cells by preventing apoptosis through mechanisms unknown at present. Here we identify Bim, Noxa and Mcl-1 as key regulators of IL-15-dependent survival of NK cells. IL-15 suppressed apoptosis by limiting Bim expression through the kinases Erk1 and Erk2 and mechanisms dependent on the transcription factor Foxo3a, while promoting expression of Mcl-1, which was necessary and sufficient for the survival of NK cells. Withdrawal of IL-15 led to upregulation of Bim and, accordingly, both Bim-deficient and *Foxo3a*^{-/-} NK cells were resistant to cytokine deprivation. Finally, IL-15-mediated inactivation of Foxo3a and cell survival were dependent on phosphatidylinositol-3-OH kinase. Thus, IL-15 regulates the survival of NK cells at multiple steps, with Bim and Noxa being key antagonists of Mcl-1, the critical survivor factor in this process.

Natural killer (NK) cells contribute to innate immune responses through the lysis of damaged cells and the production of cytokines and chemokines^{1,2}. NK cells recognize and rapidly respond to stressed or transformed cells by means of receptors that detect a lack of expression of major histocompatibility complex class I (ref. 3) and/or the expression of stress-inducible

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AUTHOR CONTRIBUTIONS

N.D.H. did experiments, analyzed data and wrote the manuscript; H.P., P.G., E.N., H.T., G.D. and S.N.W. did experiments; E.M.M. and N.M. provided unpublished genetically modified mice; M.A.D.-E., D.C.S.H. and M.J.S. designed research; and S.L.N., D.M.T. and A.S. designed research, analyzed data and wrote the manuscript.

COMPETING INTERESTS STATEMENT

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ligands. These attributes make NK cells important effectors in the clearance of virus-infected cells and certain tumors^{2,4}. Given the importance of NK cells to immunity in mammals, identifying mechanisms that mediate the survival of NK cells might assist in improving strategies for the immunotherapy of infectious diseases or cancer.

The lack of rearranged antigen receptors on NK cells means that unlike B cells and T cells, NK cells must rely on factors other than antigen for their development, activation and survival. One such factor is the pleiotropic cytokine interleukin 15 (IL-15). Mice deficient in IL-15 (*Il15*^{-/-}) lack peripheral NK cells⁵, as do mice deficient in any one of the three IL-15 receptor (IL-15R) subunits (α , β or γ)⁶⁻⁸ or those lacking the IL-15R signaling molecule Jak3 (refs. 9-10). IL-15 is critical not only for the development of NK cells but also for their survival *in vivo*, something best demonstrated by the observation that mature NK cells fail to survive when transferred into *Il15*^{-/-} mice^{11,12}.

Cell survival is controlled to a large extent by the balance between pro- and antiapoptotic members of the Bcl-2 protein family. Apoptosis signaling is initiated by the transcriptional or post-translational activation of proapoptotic Bcl-2 homology domain (BH3)-only proteins, such as Bim, Noxa and Puma¹³. These proteins bind to antiapoptotic Bcl-2 family members, such as Bcl-2 itself, Bcl-x_L and Mcl-1. This step is thought to ‘unleash’ the proapoptotic Bax and/or Bak proteins from their inactive state, leading to permeabilization of the outer mitochondrial membrane, release of apoptogenic molecules (such as cytochrome *c*) and activation of the caspase cascade¹⁴.

Bcl-2 has been proposed to be the critical factor by which IL-15 promotes the survival of NK cells, because IL-15 has been reported to maintain or increase Bcl-2 expression in human or mouse NK cells, respectively^{11,15,16}, and overexpression of Bcl-2 inhibits apoptosis of NK cells after their transfer into *Il15*^{-/-} mice. However, most resting NK cells have high constitutive expression of Bcl-2 (refs. 11-15) and experience only a slight decrease in Bcl-2 while undergoing apoptosis¹⁶, indicating that Bcl-2 may have only limited involvement in the survival of NK cells. Although so far none of the BH3-only proteins have been suggested as being critical initiators of NK cell apoptosis, Bim is a likely candidate because it is essential for the homeostasis of hematopoietic cells and cytokine deprivation-induced apoptosis of B lymphocytes and T lymphocytes¹⁷, osteoclasts¹⁸ and mast cells¹⁹. The phenotype of Bim-deficient lymphocytes has been reviewed²⁰.

Here we report the molecular basis by which IL-15 mediates the survival of NK cells. We found that IL-15 stimulation maintained Mcl-1 expression in NK cells and that IL-15 was still able to promote the survival of NK cells even when Bcl-2 and Bcl-x_L were blocked specifically by the BH3-mimetic compound ABT-737 but not when Mcl-1 was inhibited by the selective BH3 ligand Noxa. Moreover, Bim-deficient (*Bcl2l1*^{-/-}; called ‘*Bim*^{-/-}’ here) NK cells accumulated at a late maturation stage *in vivo* and were resistant to apoptosis caused by IL-15 deprivation. IL-15 inhibited Bim activation at many levels, including transcription initiation through phosphatidylinositol-3-OH kinase (PI(3)K)-dependent inactivation of transcription factor Foxo3a, and promoted proteasome-mediated degradation of Bim through activation of kinases Erk1 and Erk2 (called ‘Erk1/2’ here). Our results elucidate the pathway by which IL-15 regulates the survival of NK cells and identify Bim, Mcl-1 and, to a lesser extent, Noxa as the key factors in this process.

RESULTS

IL-15 blocks Bim accumulation in NK cells

To delineate the prosurvival versus mitotic effects of IL-15 on NK cells, we first established a concentration of IL-15 (5 ng/ml) that did not induce NK cell proliferation but was sufficient

to maintain viability above 95% for NK cell populations previously expanded with IL-15. NK cells cultured in the absence of IL-15 rapidly underwent apoptosis, with over 80% dead by 24 h (Fig. 1a). Given the critical function of the BH3-only protein Bim in the cytokine deprivation-induced apoptosis of lymphocytes, myeloid cells, osteoclasts and mast cells²⁰, we determined whether Bim was regulated by IL-15 in NK cells. Although we readily detected Bim_{EL} and Bim_L, the most highly expressed isoforms of Bim²¹, in IL-15-stimulated NK cells by immunoblot analysis, there was an increase in both isoforms after 2 h and 4 h of IL-15 deprivation, whereas Bcl-2 remained constant (Fig. 1b).

We next investigated the signaling pathways activated in NK cells by IL-15 that may have been connected with the regulation of Bim. IL-15 stimulation activated the mitogen-activated protein kinase and PI(3)K pathways in NK cells, with increases in phosphorylated c-Akt (protein kinase B) and phosphorylated Erk1/2 (p44/p42) within 30 min of exposure to IL-15 (Fig. 1c). During this interval, the amount of Bim in the IL-15 stimulated NK cells did not vary much. The Erk inhibitor U0126 completely blocked phosphorylation of both Erk1/2 and Bim (Bim_{EL} as well as Bim_L), indicating that Bim phosphorylation probably occurs 'downstream' of Erk1/2 in IL-15-stimulated NK cells (Fig. 1d) and that in NK cells, as in osteoclasts¹⁸, Bim may be a substrate for Erk. Moreover, Bim increased in IL-15-stimulated NK cells treated with the proteasome inhibitor PS341 (Fig. 1e), indicating that IL-15 primes Bim for proteasomal degradation, possibly through Erk-mediated phosphorylation and subsequent ubiquitination, as has been described for lymphocytes²². NK cells cultured in the presence of IL-15 and the Erk1/2 inhibitor U0126 were much less viable than cells cultured with IL-15 alone, confirming that mitogen-activated protein kinase activity contributes to the IL-15-mediated survival of NK cells (Supplementary Fig. 1a online).

Bim is not essential for NK cell effector function

Next we investigated the consequences of loss of Bim on the development and function of NK cells. The percentage of NK cells in the spleens of *Bim*^{-/-} mice was lower than that of control (wild-type) mice; however, given the greater cellularity of *Bim*^{-/-} spleens¹⁷, the total number of NK cells was similar, as it was in the liver and bone marrow (Figs. 2a,b). Bim-deficient NK cells proliferated normally in response to IL-15; they also responded normally to IL-18 and IL-12, as assessed by production of interferon- γ (IFN- γ) and modulation of the expression of NK1.1 or KLRG1, respectively (Figs. 2c-e). Moreover, cytotoxicity of major histocompatibility complex class I-deficient target cells and NKG2D ligand-positive target cells was unaffected by loss of Bim (Fig. 2f), indicating that Bim is not required for normal NK cell function.

Altered NK cell maturation and IL-15-regulated

As NK cell numbers were normal in *Bim*^{-/-} mice, we next investigated the maturation state of these NK cells. Mature (Mac-1⁺) NK cells can be categorized into subsets on the basis of their expression of CD27 and KLRG1, with KLRG1⁻CD27⁺ NK cells considered precursors to KLRG1⁺CD27⁻ NK cells²³⁻²⁵. Notably, *Bim*^{-/-} mice had a significantly higher percentage and total number of KLRG1⁺ NK cells in the spleen, liver and bone marrow than did wild-type mice ($P < 0.02$; Fig. 3a,b and data not shown). To determine whether the accumulation of KLRG1⁺ NK cells in *Bim*^{-/-} mice correlated with impaired apoptosis, we cultured wild-type and *Bim*^{-/-} NK cells in the presence or absence of IL-15. *Bim*^{-/-} NK cell populations expanded with IL-15 survived cytokine withdrawal significantly better than wild-type NK cells did ($P < 0.0008$; Fig. 3c). We obtained similar results with freshly isolated *Bim*^{-/-} and wild-type NK cells (Supplementary Fig. 1b).

To determine if such differential survival after IL-15 withdrawal occurred *in vivo*, we transferred equal numbers of *Bim*^{-/-} and wild-type NK cell populations expanded *in vitro* into *Il15*^{-/-} recipient mice and monitored their persistence over 72 h (Fig. 3d). During this time

essentially all wild-type NK cells disappeared and thus *Bim*^{-/-} NK cells comprised over 90% of the remaining population. The same ‘preferential’ survival of *Bim*^{-/-} cells occurred when we used primary NK cells isolated from spleen, although the kinetics of loss were different in that a greater fraction of wild-type NK cells survived at 24 h.

NK cells are important in controlling many viral infections. Infection of mice with mouse cytomegalovirus (MCMV) has provided useful insights into NK cell biology; in MCMV-resistant mouse strains, such as C57BL/6J, MCMV infection is controlled by activities mediated by NK cells²⁶. Notably, NK cell numbers expand in a MCMV-specific way²⁷ before contracting to homeostatic numbers by day 14 after infection²⁴. Thus, we next examined NK cell dynamics in the spleens of wild-type and *Bim*^{-/-} mice after infection with MCMV. At both day 10 and day 14 after infection, *Bim*^{-/-} mice retained significantly more NK cells than wild-type mice did ($P < 0.01$; Fig. 3e). Furthermore, the phenotype of the expanded NK cell population differed in wild-type versus *Bim*^{-/-} mice, with the latter showing a greater frequency of KLRG1⁺ NK cells (Fig. 3e). These results indicate that *Bim* is a key regulator of NK cell homeostasis and that enhanced survival of NK cells *in vivo* resulting from loss of *Bim* leads to the accumulation of late-stage KLRG1⁺ NK cells.

PI(3)K facilitates IL-15-mediated NK survival

Given our finding that IL-15 activated the PI(3)K pathway in NK cells (Fig. 1c) and the importance of PI(3)K in lymphocyte signaling, homeostasis and survival²⁸, we investigated the requirement for PI(3)K in the IL-15-mediated survival of NK cells. LY294002, a PI(3)K inhibitor²⁹, reduced the survival of NK cells cultured in IL-15 in a dose-dependent way (Fig. 4a and Supplementary Fig. 1a) and required *Bim*, as *Bim*^{-/-} NK cells were significantly more resistant to LY294002-mediated apoptosis ($P < 0.003$; Fig. 4b). As anticipated, LY294002 decreased phosphorylated Akt in IL-15-stimulated NK cells without affecting the phosphorylation of Erk1/2 (Fig. 4c). Notably, LY294002 treatment of IL-15-stimulated NK cells increased *Bim* in cultured NK cells (Fig. 4c), suggesting a link between PI(3)K activity and *Bim* stability. Notably, inhibition of PI(3)K activity in IL-15-stimulated NK cells did not affect the amount of Mcl-1 (Fig. 4c). To further delineate the requirement for PI(3)K in the IL-15-induced survival of NK cells, we analyzed mice with an inactivating mutation in the p110 δ subunit of PI(3)K²⁸. NK cells with this inactivating mutation developed normally, proliferated in the presence of IL-15 and died after IL-15 withdrawal exactly as wild-type NK cells did (Supplementary Fig. 2a–c online). Finally, treatment with LY294002 in the presence of IL-15 killed NK cells with this inactivation mutation as effectively as it killed wild-type NK cells (Supplementary Fig. 2d). These experiments collectively indicate that PI(3)K signaling ‘downstream’ of the IL-15R complex does not require the p110 δ subunit.

IL-15 suppresses *Bim* in NK cells via PI(3)K-Foxo3a

Given that the inhibition of PI(3)K increased *Bim* protein concentrations and blocked IL-15-mediated survival of NK cells in a *Bim*-dependent way (Fig. 4), we next investigated whether PI(3)K-Akt signaling regulated *Bim* expression in IL-15-stimulated NK cells. Foxo3a (also called FKHR-L1), a member of the family of Forkhead box class O transcription factors, increases *Bim* transcription in certain hematopoietic cells when they are deprived of their requisite growth factors³⁰. In cytokine-stimulated cells, Foxo3a is inactivated through cytosolic sequestration after Akt-mediated phosphorylation^{30,31}. Indeed, treatment of NK cells with IL-15 increased Foxo3a phosphorylation, and this was blocked by LY294002 in a concentration-dependent way, indicating a relationship between PI(3)K and Foxo3a activity in NK cells (Fig. 5a). Mice deficient in Foxo3a (*Foxo3a*^{-/-} mice) had normal numbers of NK cells in the spleen that seemed grossly normal in phenotype except for a small but significant increase in the fraction that were KLRG1⁺ (less than that in *Bim*^{-/-} mice; $P < 0.01$; Fig. 5b). Notably, after IL-15 withdrawal in culture, *Foxo3a*^{-/-} NK cells failed to upregulate *Bim* (Fig.

5c) and survived significantly better than wild-type NK cells (albeit less well than *Bim*^{-/-} NK cells; $P < 0.05$; Fig. 5d). Moreover, LY294002 induced significantly less apoptosis of *Foxo3a*^{-/-} NK cells than of wild-type NK cells ($P < 0.01$; Fig. 5e), indicating that PI(3)K contributed to the survival of NK cells in part through the inhibition of Foxo3a-mediated *Bim* transcription.

IL-15-mediated maintenance of Mcl-1 and NK cell survival

Having identified *Bim* as the key proapoptotic protein after IL-15 withdrawal, we next sought to identify the prosurvival Bcl-2 family members that were responsible for the IL-15-mediated survival. It has been shown that Mcl-1 is critical for the survival of B cells and T cells *in vivo* and that IL-15 is a potent inducer of Mcl-1 in T cells³². Consistent with those findings, we noted that IL-15 was required for the maintenance of Mcl-1 in NK cells, as IL-15 withdrawal resulted in rapid loss of Mcl-1 (Fig. 6a). We assessed the importance of the various prosurvival Bcl-2 family members in IL-15-mediated survival of NK cells with two specific inhibitors: ABT-737, the BH3 mimetic that inhibits Bcl-2, Bcl-x_L and Bcl-w but not Mcl-1 (ref. 33), and enforced expression of Noxa, which acts selectively on Mcl-1 but not on Bcl-2, Bcl-x_L or Bcl-w³⁴. Treatment of NK cells with ABT-737 had no effect on their survival in the presence of IL-15, although it accelerated NK cell death after IL-15 withdrawal (Fig. 6b). Conversely, enforced expression of Noxa by retroviral gene transduction led to efficient killing of primary NK cells in the presence of IL-15. This finding was typified by the much lower frequency of cells positive for green fluorescent protein (GFP), a marker of retroviral infection, in Noxa-transduced NK cell cultures than in cultures of NK cells transduced with retrovirus encoding only GFP or GFP plus Noxa-3E, the inactive mutant form of Noxa ($P < 0.025$; Fig. 6c). Furthermore, GFP expression in the remaining Noxa-transduced NK cells was much lower than that of cells infected with the mutant Noxa-3E construct, consistent with selection against high Noxa expression in these cells (Fig. 6d). Notably, control fibroblasts refractory to the proapoptotic effects of Noxa³⁴ expressed similar amounts of retrovirally transduced Noxa or Noxa-3E (data not shown). These results indicate that Mcl-1 was critical for the survival of NK cells in the presence of IL-15 and that other prosurvival Bcl-2 family members had minor involvement, perhaps contributing to the survival of NK cells when IL-15 was scarce and Mcl-1 abundance diminished.

Noxa is upregulated after IL-15 withdrawal

Involvement of Noxa itself in NK cell apoptosis was suggested both by the importance of Mcl-1, a known target of Noxa^{34,35}, in the survival of NK cells and the proapoptotic effects of Noxa overexpression. Indeed, real-time PCR analysis showed that Noxa mRNA was significantly upregulated in NK cells after IL-15 withdrawal ($P < 0.04$; Fig. 7a). We therefore investigated the consequences of loss of Noxa on NK cell numbers *in vivo* and the survival of NK cells *in vitro*. Although Noxa-deficient mice (*Pmaip1*^{-/-}; called '*Noxa*^{-/-}' here) had normal numbers of NK cells in all organs analyzed (data not shown), both freshly isolated *Noxa*^{-/-} NK cells (Supplementary Fig. 3a online) and *Noxa*^{-/-} NK cell populations expanded *in vitro* survived significantly better than did wild-type NK cells when cultured without IL-15 ($P < 0.05$; Fig. 7b). Furthermore, a combined loss of *Bim* plus Noxa protected NK cells against IL-15 deprivation *in vitro* significantly better than did loss of *Bim* alone ($P < 0.015$; Fig. 7b). This effect of Noxa loss was specific, as *Bad*^{-/-} NK cells died normally in the absence of IL-15 and loss of *Bad* did not enhance the resistance of *Bim*-deficient NK cells to IL-15 deprivation (Supplementary Fig. 3b). These results demonstrate that Noxa acts together with *Bim* in inducing the apoptosis of NK cells after IL-15 withdrawal.

DISCUSSION

Unlike B cells and T cells, the development and survival of NK cells is not governed by signals generated from classic antigen receptors but instead relies on the cytokine IL-15 (ref. 36). The proapoptotic BH3-only protein Bim is known to be essential for developmentally 'programmed' and cytokine deprivation-induced death of B cells and T cells²⁰. Very little is known about the control of NK cell survival and the function that Bim might have in this process. Here we have identified Bim and the pathways that regulate its expression as the key molecular targets of the IL-15-mediated survival of NK cells.

The absolute dependence of NK cells on IL-15–IL-15R signaling and the ease with which they are grown *in vitro* have made these cells ideal for investigating pathways involved in cytokine-induced survival. The balance between prosurvival proteins, such as Bcl-2, Bcl-x_L, Bcl-w and Mcl-1, and proapoptotic proteins, such as Bim, Bid, Noxa, Puma and Bad, controls lymphocyte survival²⁰. Our main finding here was that stimulation with IL-15 limited the amount of Bim in NK cells by activating two distinct signaling pathways. First, Bim phosphorylated by an Erk1/2-dependent process was targeted for degradation by the proteasome. Second, IL-15 activated PI(3)K and its 'downstream' target Akt, which in turn phosphorylated Foxo3a, thereby preventing it from upregulating *Bim* transcription^{30,31}. IL-15 therefore joins IL-2 and IL-3 as a cytokine that regulates leukocyte survival by inactivating Foxo3a by means of PI(3)K–Akt–mediated phosphorylation^{37,38}. Notably, the p110δ subunit of PI(3)K was not required for the inhibition of Foxo3a activity, repression of *Bim* expression or survival of NK cells, although it is essential for normal survival of B cells and T cells²⁸. This result indicates that p110δ is critical in survival signaling from antigen receptors but not that from cytokine receptors.

Noxa and Puma are both BH3-only proteins that can be activated by p53-dependent apoptotic stimuli (such as DNA damage) as well as p53-independent apoptotic stimuli. Notably, our study has demonstrated that *Noxa* was also induced after IL-15 withdrawal and paralleled the induction of *Noxa* and resulting apoptosis seen in human T cells after glucose deprivation³⁹. Puma is critical for apoptosis of myeloid cells induced by IL-3 deprivation⁴⁰ and it is upregulated in T cells in a Foxo3a-dependent way when IL-2, which uses the same β- and γ-receptor subunits as IL-15, is withdrawn⁴¹. This suggests that Puma may also be involved in NK cell apoptosis and demonstrates that Noxa and Puma do not function exclusively in response to DNA damage but also participate in apoptosis elicited by the withdrawal of cytokines or other growth factors.

Given the critical involvement of Bim in apoptosis induced by IL-15 deprivation and the importance of IL-15 in NK cell development, the finding of normal numbers of NK cells in *Bim*^{−/−} mice was unexpected. In contrast, the development of B cells and T cells is greatly affected by the absence of Bim, with *Bim*^{−/−} mice having two- to threefold more B cells and T cells than control mice have¹⁷. Similarly, mice overexpressing Bcl-2 in all hematopoietic cells accumulate B cells and T cells but have normal numbers of NK cells, although these are also very resistant to IL-15 deprivation (N.H. and A.S., unpublished data). There are many potential explanations for the lack of NK cell accumulation in *Bim*^{−/−} and *Bcl2*-transgenic mice. First, it is possible that abnormal population expansion of B cells and T cells limits the availability of IL-15, thereby preventing abnormal accumulation of NK cells. Alternatively, it is possible that although NK cell numbers are normal in *Bim*^{−/−} and *Bcl2*-transgenic mice, their lifespan is abnormally long and, as a compensatory process, their production *in vivo* is lower. Such a scenario has been noted for *Bim*^{−/−} osteoclasts, which are resistant to cytokine deprivation but do not accumulate *in vivo*¹⁸. Notably, although *Bim*^{−/−} mice have normal overall numbers of NK cells, they accumulate at a late developmental stage, characterized as KLRG1⁺CD27[−], and are mostly post-mitotic^{23,24}. Notably, during the early stages of MCMV

infection, proliferation of NK cells results in the accumulation of KLRG1⁺ cells, which 'preferentially' downregulate Bcl-2 and undergo apoptosis during the contraction phase of the response²⁴. The accumulation of KLRG1⁺ NK cells in *Bim*^{-/-} mice could therefore reflect the normal upregulation of Bim in KLRG1⁺ NK cells in response to diminished IL-15R signaling. It will be useful to compare the lifespans of terminally differentiated *Bim*^{-/-} and wild-type NK cells and the production of such cells from their progenitors.

There was also abnormal accumulation of KLRG1⁺ NK cells in *Foxo3a*^{-/-} mice, although to a lesser extent than that in *Bim*^{-/-} mice. This finding suggests that Foxo3a is responsible for a proportion of the Bim activation required for normal NK cell turnover *in vivo*. Notably, although Bim still underwent dephosphorylation after IL-15 withdrawal in *Foxo3a*^{-/-} NK cells, Bim did not increase appreciably. This result suggests that transcriptional induction has a dominant function in regulating Bim concentrations during IL-15 deprivation and posttranslational modifications have only a minor function. Our finding that *Foxo3a*^{-/-} NK cells were not as resistant to IL-15 deprivation as *Bim*^{-/-} NK cells were, however, demonstrates that the amount of Bim in *Foxo3a*^{-/-} NK cells was sufficient to trigger apoptosis when the concentration of Mcl-1 decreased.

Although *Mcl1*^{-/-} mice are not viable⁴², lineage-specific deletion of Mcl-1 has demonstrated a requirement for this molecule in the development and sustained survival of B lymphocytes and T lymphocytes³². There are no reports at present detailing a requirement for Mcl-1 in the survival or development of NK cells, presumably because mice expressing Cre recombinase under the control of an NK cell-specific promoter are not yet available. However, the requirement for IL-15 in maintaining Mcl-1 concentrations in NK cells and the upregulation of Mcl-1 in IL-15-stimulated thymocytes³² indicate that Mcl-1 may also be essential for the survival of NK cells. In support of that hypothesis, Noxa, which binds only Mcl-1 and Bcl-2-related protein A1 (refs. 34-35), was upregulated in IL-15-deprived NK cells, and enforced Noxa expression killed NK cells even in the presence of IL-15. Finally, Noxa-deficient NK cells were abnormally resistant to IL-15 withdrawal. These observations, along with the finding that combined inactivation of Bcl-2, Bcl-x_L and Bcl-w with the BH3 mimetic ABT-737 had no effect on the survival of IL-15-stimulated NK cells, indicate that Mcl-1 is probably the crucial inhibitor of apoptosis in NK cells.

On the basis of the findings reported here for NK cells and elsewhere for osteoclasts¹⁷ and lymphocytes²², we suggest that IL-15 maintains small amounts of Bim in NK cells by two complementary mechanisms. First, IL-15 activates the Erk1/2-mediated phosphorylation, ubiquitination and proteasomal degradation of Bim. Second, IL-15 represses transcription of *Bim* through the PI(3)K-Akt-mediated phosphorylation of Foxo3a. The small amounts of Bim coupled with the high expression of Mcl-1 after IL-15 exposure shift the balance toward survival. In contrast, withdrawal of IL-15 results in both inactivation of Erk1/2 and PI(3)K and accumulation of Bim in the cytoplasm. Noxa is also upregulated and, together with Bim induction and the decreasing abundance of Mcl-1, shifts the balance toward cell death.

Given that NK cells are critical for host defense against many infectious pathogens and transformed cells, understanding the mechanisms that govern the survival of NK cells could improve immunotherapeutic approaches to such diseases. Our finding that Bim and Mcl-1 are critical proapoptotic and antiapoptotic proteins, respectively, will allow research to focus on targeting these molecules in efforts to manipulate NK cell numbers and activity *in vivo*. For example, our finding that ABT-737 does not kill NK cells (even at concentrations used in preclinical cancer studies in mice) might have implications for the immunotherapy of cancer, as NK cell-mediated cytotoxicity would not be expected to be affected by treatment with ABT-737, as long as the concentration of IL-15 *in vivo* was not compromised.

METHODS

Mice

C57BL/6, *Bim*^{-/-} mice¹⁷, *Noxa*^{-/-} mice⁴³, *Bim*^{-/-}*Noxa*^{-/-} mice (E.M.M. and A.S., unpublished data), *Bad*^{-/-} mice⁴⁴, *Bim*^{-/-}*Bad*^{-/-} mice⁴⁵, *Foxo3a*^{-/-} mice (N.M., unpublished data), *Il15*^{-/-} mice⁵ and mice with an inactivating mutation in the p110δ subunit of PI(3)K²⁸ were bred and maintained at the Walter and Eliza Hall of Medical Research or the Ludwig Institute for Cancer Research (Melbourne, Australia). Mice of all mutant strains were generated on the C57BL/6 background or were backcrossed to this background for at least eight generations, except *Foxo3a*^{-/-} mice, which were backcrossed for five generations. The relevant Animal Ethics and Experimentation Committees approved animal experiments according to the guidelines of the National Health and Medical Research Council Australia.

Antibodies, flow cytometry and cell sorting

Antibodies specific for NK1.1 (PK136), Ly49C/I (SW5e6), CD11b (Mac-1; M1/70), TCR-β (H57-5921), IFN-γ (HB170 and XMG1.2), KLRG1 (2F1) and CD49b (DX5 and HMα2) were from BD PharMingen. Antibody to CD27 (anti-CD27; LG.7F9) was from eBioscience. Single-cell suspensions were prepared by forcing of organs through metal sieves. Lymphocytes from liver were isolated from a 40–80% Percoll gradient (Amersham Pharmacia Biotech) centrifuged for 20 min at 1,300g. For flow cytometry, single-cell suspensions were stained with the appropriate monoclonal antibody in PBS containing 2% (vol/vol) FCS. Biotinylated monoclonal antibodies were visualized with indodicarbocyanine- or phycorythrin-streptavidin (Southern Biotechnology Associates). LSR, FACSDiva (BD Biosciences) and MoFlo (Cytomation) apparatuses were used for cell sorting and analysis, with dead cells excluded by propidium iodide staining.

In vitro analysis of NK cell function

NK cell proliferation was assayed by culture for 72 h of 3×10^4 cells in 100 μl of Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) FCS plus gentamycin (50 ng/ml; Sigma) and a 'titration' of recombinant human IL-15 (0.5–50 ng/ml), mouse IL-21 (100 ng/ml), IL-12 (2 ng/ml) and IL-18 (10 ng/ml; R&D Systems), in 96-well, flat-bottomed plates. Plates were then pulsed with 1 μCi [³H]thymidine (NEN) and samples were collected after 8 h onto glass-fiber filters (Packard), followed by scintillation counting. Survival was assessed by culture for 72 h of freshly sorted or IL-15-expanded NK cell samples at a density of 3×10^4 cells per well or 1×10^6 cells per well in 100 μl of Iscove's modified Dulbecco's medium plus FCS, in 96-well, flat-bottomed plates. Live and dead cells were discriminated by staining with propidium iodide and annexin V–fluorescein isothiocyanate (BD PharMingen) followed by analysis on a FACScan (Becton-Dickinson). Induction of KLRG1 expression was analyzed by expansion of sorted NK cell populations in IL-15 (50 ng/ml) with cells at a density of 1×10^6 cells per ml in Nunclon six-well flat-bottomed tissue culture plates (Nunc). After 5 d, IL-12 (2 ng/ml) and IL-18 (10 ng/ml) were added alone or in combination and KLRG1 expression was monitored by flow cytometry after 24 and 48 h. Analysis of IFN-γ production and cytotoxicity assays were done as described⁴⁶. The kinase and proteasome inhibitors U0126, LY294002 and PS341 were dissolved in dimethyl sulfoxide and were added for 6–24 h. The BH3 mimetic ABT-737 was from Abbot Laboratories.

Immunoblot analysis

Sorted NK cells were grown for 5–8 d in six-well tissue culture plates with IL-15 (50 ng/ml). For IL-15 withdrawal, 5×10^6 NK cells were washed twice and were cultured for various times in Iscove's modified Dulbecco's medium plus FCS. Cells were collected and were washed in PBS and total cell lysates were prepared in lysis buffer (20 mM Tris-HCl, pH 7.4, 135 mM

NaCl, 1.5 mM MgCl₂, 1% (vol/vol) Triton X-100 and 10% glycerol) supplemented with Pefabloc (0.5 mg/ml) and 1 mg/ml each of leupeptin, aprotinin, soya bean trypsin inhibitor and pepstatin (Sigma). Proteins were separated by electrophoresis through either 12% or 4–20% gradient Novex gels (Invitrogen) and were transferred to Hybond-C extra nitrocellulose membranes (Amersham) with an Excell II blot module (Novex, Invitrogen). Proteins were detected as described⁴⁷. Nuclear proteins were obtained with the NucBuster Extraction kit (Novagen). Antibodies used included rat monoclonal anti-Bim (3C5; Alexis Biochemicals), rabbit anti-Mcl-1 (600-401-394; Rockland Immunochemicals; hamster monoclonal anti-Bcl-2 3F11; BD Pharmingen); mouse monoclonal anti-Hsp70 (N6); rabbit polyclonal antibodies to phosphorylated Akt (9271), total Akt (9272), phosphorylated Erk1/2 (9102) and total Erk1/2 (9101; all from Cell Signaling); antibodies to phosphorylated Foxo3a (06–952) and total Foxo3a (06–951; both from Upstate); and anti- β -actin (sc-7210; Santa Cruz Biotechnology). Band intensities were measured by densitometry (Molecular Dynamics) and were analyzed with ImageQuant v5 software. Densities are expressed as the ratio of staining relative to staining with anti- β -actin and the ‘fold change’ in this ratio relative to that of unstimulated samples.

Cell transfer

NK cell populations from control (wild-type) or *Bim*^{-/-} bone marrow were expanded for 7 d with IL-15 and then were labeled with CFSE (carboxyfluorescein succinimidyl diester) at a concentration of 10 μ M or 1 μ M, respectively. Labeled wild-type and *Bim*^{-/-} NK cells were mixed at near-equal numbers and were placed in culture with or without IL-15 (10 ng/ml) or were injected into *Il15*^{-/-} recipient mice. Survival of NK cells was determined 24 and 72 h later by analysis of cultures and spleens of recipient mice to determine the ratio of *Bim*^{-/-} to wild-type NK cells, identified as CFSE⁺CD49b⁺. The proportion of persisting wild-type NK cells was determined as follows: (percent wild-type NK) / (percent wild-type NK + percent *Bim*^{-/-} NK). *Ex vivo* wild-type and *Bim*^{-/-} NK cells were isolated from donor spleens with DX5 MACS beads (Miltenyi Biotec), were differentially labeled with CFSE and then were injected into *Il15*^{-/-} recipient mice. Recipient mice were analyzed at 24 and 72 h after injection and NK cell persistence was determined as described above.

Retroviral transduction of primary NK cells

Retroviral expression vectors pMIG-Noxa and pMIG-Noxa-3E have been described^{34,35}. Retroviral constructs were transiently transfected into Phoenix Ecotropic packaging cells⁴⁸ with Lipofectamine (Invitrogen) and viral supernatants were used to infect cells with a combination of RetroNectin (Takara) and spin inoculation. Twelve-well non-tissue culture plates were coated for 12 h at 4 °C with RetroNectin (4 μ g/cm² in PBS) before being blocked with PBS containing 2% (wt/vol) BSA. Viral supernatant (2 ml) was added and plates were then centrifuged for 15 min at 4 °C and 1,200g. Bone marrow cells were cultured for 48 h in IL-15 (50 ng/ml) and 2 $\times$ 10⁶ cells per ml were added to each well after the viral supernatant was removed. Cells were then cultured for 6 h at 37 °C and 2 ml fresh viral supernatant was added together with IL-15 (50 ng/ml) and two rounds of ‘spin infection’ 18 h apart were done as described⁴⁹. Plates were returned to 37 °C and infection efficiency was determined by flow cytometry of GFP expression.

MCMV infection

Mice were infected by intraperitoneal administration of 5 $\times$ 10³ plaque-forming units of MCMV (K181 Perth strain) diluted in PBS containing 0.05% (vol/vol) FCS. Control mice received PBS containing 0.05% (vol/vol) FCS only. Organs were collected at various times and were processed for further analysis.

Quantitative PCR

Total RNA from 1×10^6 cells was purified and treated with DNase I with RNeasy mini columns (Qiagen). SuperScript II reverse transcriptase (Invitrogen) was used for first-strand cDNA synthesis according to the manufacturer's instructions. Samples without reverse transcriptase were used as controls for contaminating DNA. One tenth of the reverse-transcription reaction product was amplified by quantitative PCR with Quantitect SYBR Green PCR Master Mix (Qiagen) and the ABI PRISM system (Applied Biosystems). Primers used included β -actin (5'-AGTGTGACGTTGACATCCGTA-3' and 5'-CCAGAGCAGTAATCTCCTTC-3') and *Noxa* (5'-GCAGAGCTACCACCTGAGTTC-3' and 5'-ATCTGCGCCAGAACCACAG-3'). A 100-base pair DNA template (Geneworks) spanning each primer set was purified by high-performance liquid chromatography and was used as a standard. The amount of *Noxa* in each sample was normalized to the amount of *Actb* present.

Statistical analysis

A standard Student's *t*-test with two-tailed distributions for two-samples with equal variance was used for statistical analysis. *P* values of 0.05 or less were considered significant; exact *P* values are provided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IL-15 deprivation causes accumulation of Bim in NK cells

(a) Viability of NK cell populations (NK1.1⁺CD49b⁺) expanded with IL-15, then cultured with (+) or without (-) IL-15 (5 ng/ml) and assessed by flow cytometry. *, $P = 0.014$; **, $P = 0.001$; ***, $P < 0.001$. (b) Immunoblot analysis of Bim in NK cell populations expanded with IL-15, then washed and cultured without IL-15 (time, above lanes). Bcl-2, loading control. The *Bim*^{-/-} NK cell lysate demonstrates the specificity of the antibody to Bim. (c) Immunoblot analysis of phosphorylated Akt (p-c-Akt) and Erk1/2 (p-Erk1/2) and total Erk1/2 and Bim in lysates of NK cell populations expanded with IL-15 and cultured in cytokine-free medium for 6 h before the addition of IL-15 (50 ng/ml; time, above lanes). β-actin, loading control. (d) Immunoblot analysis of phosphorylated Erk, total Erk and Bim in NK cell populations expanded with IL-15, then lysed immediately (0 h) or cultured for 2 h or 4 h with IL-15 alone or with IL-15 plus the Erk inhibitor U0126 (10 μM). (e) Immunoblot analysis of Bim in NK cell populations expanded with IL-15 and then cultured (time, above lanes) with IL-15 (50 ng/ml) with or without the proteasome inhibitor PS341 (1 μM). β-actin, loading control. Below lanes, 'fold change' in Bim relative to that at 0 h after normalization for β-actin. Data are representative of six (a), three (b) or two (c-e) experiments.

Figure 2. Bim is not essential for development or function of NK cells

(a) Flow cytometry of NK cells (NK1.1⁺CD49b^{hi}) from the spleens, livers and bone marrow (BM) of *Bim*^{-/-} or control, wild-type (WT) mice. Numbers beside boxed areas indicate percent CD49b⁺NK1.1⁺ cells. Data are representative of four mice per genotype. (b) NK cell numbers in the spleens of *Bim*^{-/-} and wild-type mice. Data (mean ± s.e.m.) are representative of three experiments. (c) Proliferation of *Bim*^{-/-} and wild-type NK cells sorted and cultured with IL-15 alone or in combination with IL-21 or with IL-18 plus IL-12; [³H]thymidine was added for the final 8 h of culture and labeled DNA was assessed by scintillation counting. Data (mean + s.e.m.) are representative of three experiments with least three mice per genotype. (d) ELISA of IFN-γ production by IL-15-expanded *Bim*^{-/-} and wild-type NK cell populations cultured for 24 h with IL-15 alone, with IL-15 plus IL-21 or with IL-18 plus IL-12. Data (mean ± s.e.m.) are representative of three independent experiments. (e) Flow cytometry of the expression of NK1.1 and KLRG1 by *Bim*^{-/-} and wild-type NK cells cultured as described in d. (f) ⁵¹Cr-release assays with IL-15-expanded *Bim*^{-/-} and wild-type NK cell populations as effector cells and YAC-1 cells (deficient in major histocompatibility complex class I; solid lines) or RMA-S cells (positive for NKG2D ligand; dashed lines) as target cells. E:T, effector/target. Data are representative of two independent experiments.

Figure 3. Apoptosis-resistant NK cells accumulate in *Bim*^{-/-} mice

(a,b) Flow cytometry of the surface expression of various antigens (horizontal axes) by *Bim*^{-/-} and wild-type NK cells (NK1.1⁺CD49b^{hi}) from the spleen (a) or the liver and bone marrow (b). (a) *, *P* = 0.0026; **, *P* = 0.0008. (b) *, *P* = 0.0113; **, *P* = 0.0031. Data (mean ± s.e.m.) are representative of four mice per genotype. (c) Viability of IL-15-expanded *Bim*^{-/-} and wild-type NK cell populations from the spleen, recultured in the absence of IL-15 and assessed by flow cytometry. *, *P* < 0.001; **, *P* = 0.0001; ***, *P* < 0.0001. Data (mean ± s.e.m.) are representative of three independent experiments. (d) Survival of *Bim*^{-/-} and wild-type NK cells after culture or after injection into *Il15*^{-/-} recipient mice. NK cell populations from bone marrow were expanded, differentially labeled with CFSE, mixed in equal numbers, then cultured with IL-15 (+ IL-15 *in vitro*) or without IL-15 (- IL-15 *in vitro*) or injected into recipient mice (*in vitro* → *Il15*^{-/-} mice); NK cells purified from the spleen were differentially labeled with CFSE, mixed in equal numbers, then injected into recipient mice (*ex vivo* → *Il15*^{-/-} mice). Each data point indicates the proportional survival of wild-type NK cells relative to that of *Bim*^{-/-} NK cells for a single mouse, resolved by CFSE fluorescence intensity; data are representative of two experiments. (e) Numbers and developmental stages of NK cells in the spleens of *Bim*^{-/-} and wild-type mice after MCMV infection. Cell numbers for NK cell subsets (NK1.1⁺TCR⁻KLRG1⁺ or NK1.1⁺TCR⁻KLRG1⁻) were determined by flow cytometry and spleen cellularity. *, *P* < 0.01. Data (mean ± s.e.m.) are representative of two experiments with three or four mice at each time point in each experiment.

Figure 4. PI(3)K is essential for IL-15-mediated survival of NK cells

(a) Viability of wild-type NK cell populations expanded with IL-15, then cultured in the presence of IL-15 (10 ng/ml) plus carrier (dimethyl sulfoxide (DMSO)) or the PI(3)K inhibitor LY294002 (10 or 100 μ M), assessed by flow cytometry. *, $P < 0.05$; **, $P < 0.001$; ***, $P = 0.0017$; and ****, $P < 0.0001$, compared with dimethyl sulfoxide. (b) Viability of *Bim*^{-/-} and wild-type NK cell populations expanded with IL-15, then cultured in the presence of IL-15 (10 ng/ml) plus either dimethyl sulfoxide or 10 μ M LY294002 and assessed by flow cytometry. *, $P < 0.01$; and **, $P < 0.0001$; LY294002 treatment of *Bim*^{-/-} cells versus wild-type NK cells. (c) Immunoblot analysis of phosphorylated Akt and Erk1/2 as well as total Erk1/2, Mcl-1 and Bim in lysates of wild-type NK cell populations expanded with IL-15, then cultured for 2 h in IL-15 (50 ng/ml) in the presence (+) or absence (-) of LY294002 (10 μ M). Hsp70, loading control. Below lanes, change in Bim relative to untreated cells and normalized for loading. Data are representative of two experiments.

Figure 5. IL-15 suppresses Foxo3a-mediated Bim upregulation

(a) Immunoblot analysis of phosphorylated Foxo3a (p-Foxo3a) and total Foxo3a in nuclear extracts of wild-type NK cell populations expanded with IL-15, cultured in medium alone (-IL-15) or in medium with IL-15 (50 ng/ml; + IL-15) plus either carrier (0) or LY294002 (LY; 10 or 100 μ M). Data are representative of two experiments. (b) Flow cytometry of KLRG1 expression by NK cells (NK1.1⁺CD49b^{hi}) from the spleens of $Foxo3a^{-/-}$ and wild-type mice. Numbers above boxed areas indicate percent CD49b⁺NK1.1⁺ cells; numbers in histograms indicate average percent KLRG1⁺ cells in boxed areas ($\pm$ s.d.). *, $P < 0.01$. Data are representative of three mice per genotype. (c) Immunoblot analysis of Bim in lysates of $Foxo3a^{-/-}$ and wild-type NK cell populations expanded with IL-15, then washed and cultured

for 0, 3 or 6 h in medium lacking IL-15. β -actin, loading control. Data are representative of two experiments. **(d,e)** Viability of *Foxo3a*^{-/-} and wild-type NK cell populations (NK1.1⁺CD49b⁺) expanded with IL-15, then cultured in the absence of IL-15 (**d**) or cultured for 12 h in the presence of IL-15 (10 ng/ml) plus dimethyl sulfoxide or 10 μ M LY294002 (**e**) and assessed by flow cytometry. (**d**) *, $P < 0.05$; **, $P = 0.025$. (**e**) *, $P = 0.0047$. Data (mean $\pm$ s.e.m., **d**; mean + s.e.m., **e**) are representative of three independent experiments.

Figure 6. IL-15-induced upregulation of Mcl-1 promotes the survival of NK cells

(a) Immunoblot analysis of Mcl-1 in lysates of wild-type NK cell populations (NK1.1⁺CD49b^{hi}) expanded with IL-15 (50 ng/ml), then deprived of IL-15 for 3 or 6 h. β-actin, loading control. Data are representative of two experiments. (b) Viability of wild-type NK cell populations expanded with IL-15, then cultured for 24 h in the presence or absence of IL-15 (20 ng/ml) and the BH3 mimetic ABT-737 (concentration, horizontal axis), assessed by flow cytometry. Data are normalized to the viability of untreated cells (0 nM ABT-737), set at 100%. Data (mean ± s.e.m.) are representative of three independent experiments. (c) Flow cytometry of IL-15-stimulated bone marrow cells transduced with retrovirus encoding Noxa plus GFP (from an internal ribosomal entry site), inactive mutant Noxa-3E plus GFP or GFP alone; GFP⁺ NK cells (CD49b^{hi}) were analyzed at 1 d and 4 d after transduction. Data (mean + s.e.m.) are from two independent experiments. (d) Transduction of NK cells with Noxa plus GFP or Noxa-3E plus GFP at day 1. Numbers in histograms of GFP⁺ cells (bottom row) indicate mean fluorescence intensity of GFP. Data are representative of two individual experiments with each retroviral construct.

Figure 7. Noxa acts together with Bim in mediating NK cell apoptosis

(a) Real-time PCR analysis of Noxa in wild-type NK cell populations expanded with IL-15, then washed and cultured (time, horizontal axis) in medium lacking IL-15. Noxa expression is presented as 'fold induction' relative to that at time 0 after normalization to β -actin expression. *, $P = 0.04$; **, $P = 0.016$. Data (mean $\pm$ s.e.m.) are from two independent experiments. (b) Viability of *Noxa*^{-/-}, *Bim*^{-/-}, *Bim*^{-/-}*Noxa*^{-/-} and wild-type NK cells expanded in IL-15 (50 ng/ml), then washed and cultured without IL-15 and assessed by flow cytometry. *, $P = 0.019$, and **, $P = 0.023$, wild-type versus *Noxa*^{-/-}; ***, $P = 0.024$, and ****, $P = 0.014$, *Bim*^{-/-}*Noxa*^{-/-} versus *Noxa*^{-/-}. Values for both *Bim*^{-/-} and *Bim*^{-/-}*Noxa*^{-/-} NK cells are significantly different from those of wild-type NK cells at all time points. Data (mean $\pm$ s.e.m.) are representative of two experiments with three mice of each genotype.