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Genetically defining the mechanism of Puma- and Bim-induced apoptosis

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Using genetically modified mouse models, we report here that p53 upregulated modulator of apoptosis (Puma) and Bcl-2 interacting mediator of cell death (Bim), two pro-apoptotic members of the B-cell lymphoma protein-2 (Bcl-2) family of proteins, cooperate in causing bone marrow and gastrointestinal tract toxicity in response to chemo and radiation therapy. Deletion of both *Puma* and *Bim* provides long-term survival without evidence of increased tumor susceptibility following a lethal challenge of carboplatin and ionizing radiation. Consistent with these *in vivo* findings, studies of primary mast cells demonstrated that the loss of *Puma* and *Bim* confers complete protection from cytokine starvation and DNA damage, similar to that observed for *Bax/Bak* double knockout cells. Biochemical analyses demonstrated an essential role for either Puma or Bim to activate Bax, thereby leading to mitochondrial outer membrane permeability, cytochrome *c* release and apoptosis. Treatment of cytokine-deprived cells with ABT-737, a BH3 mimetic, demonstrated that Puma is sufficient to activate Bax even in the absence of all other known direct activators, including Bim, Bid and p53. Collectively, our results identify Puma and Bim as key mediators of DNA damage-induced bone marrow failure and provide mechanistic insight into how BH3-only proteins trigger cell death.
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Programmed cell death, or apoptosis, is an evolutionary conserved process that regulates normal cellular homeostasis, and when deregulated results in developmental malformations, neurodegeneration, cancer and autoimmunity.[1] The initiation of apoptosis occurs through intrinsic and extrinsic pathways that can converge at the mitochondria.[2] Cellular stresses trigger mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c*, which complexes with APAF-1 and pro-caspase-9, leading to the activation of the caspase cascade and the eventual dismantling of the cell.[3]

Central to the regulation of MOMP is the B-cell lymphoma protein-2 (Bcl-2) family of proteins. The anti-apoptotic members Bcl-2, Bcl-X_L, Bcl-W, Mcl-1 and A1 each contain four BH domains (BH1–4), and maintain mitochondrial integrity by inhibiting the pro-apoptotic effectors Bax and Bak, which facilitate the release of cytochrome *c* into the cytoplasm. The BH3-only pro-apoptotic proteins Bim (Bcl-2 interacting mediator of cell death), Bid, Puma (p53 upregulated modulator of apoptosis), Noxa, Bad, Bik, Bmf and Hrk respond to cell death stimuli, including but not limited to DNA damage, growth factor withdrawal and ER stress.[4,5] These BH3-only proteins can be further subdivided into 'derepressors' (e.g. Bad, Bik and Noxa) that only bind the anti-apoptotic proteins and 'direct activators' (e.g. Bim and Bid) that can also directly trigger the activation of the pro-apoptotic effectors Bax and Bak.[6] The tumor suppressor p53, which initiates apoptosis through the transcriptional upregulation of *Puma*, *Noxa* and other target genes, has also been reported to antagonize Bcl-2, Bcl-X_L and Mcl-1, and to directly activate Bax/Bak.[7–9] Similarly, Puma may also act as a direct activator of Bax and Bak.[10]

We have taken a genetic and biochemical approach using knockout mice and primary cell lines to test the functional requirements of Puma, Bim, Bid and p53, the only known putative direct activators of Bax and Bak, in the induction of apoptosis. We report here that Puma and Bim cooperate in the activation of Bax/Bak in response to signals that trigger both p53-dependent and p53-independent cell death. Deletion of *Puma* and *Bim* protected mice long term without a significant increased risk of cancer from a lethal myelosuppressive regimen of carboplatin and ionizing radiation. Moreover, $Puma^{-/-}/Bim^{-/-}$ double knockout (DKO) primary bone marrow cells expanded in culture remained viable when deprived of growth factors and treated with irradiation (IR) or the BH3-mimetic drug ABT-737. The extent of survival was equivalent to that observed for $Bax^{-/-}/Bak^{-/-}$ cells. By contrast, cells lacking Bim, Bid and p53 either individually or collectively remained sensitive to growth factor deprivation and ABT-737 treatment. Our results demonstrate that the

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Abbreviations: Puma, p53 upregulated modulator of apoptosis; Bim, Bcl-2 interacting mediator of cell death; MOMP, mitochondrial outer membrane permeabilization; IR, irradiation; TPO, thrombopoietin; GI, gastrointestinal; SCF, stem cell factor; MCP, mast cell protease; CTMC, connective tissue mast cell
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BH3-only protein Puma is sufficient to induce Bax/Bak activation even in the absence of all other known direct activators. Most importantly, our findings provide a proof-of-principle that BH3 antagonists (e.g. small molecule inhibitors) could be developed to alleviate hematological toxicities commonly associated with cancer therapies without significantly increasing the risk of secondary malignancies.

Results

Cooperation of Puma and Bim in therapy-induced lethal myelosuppression. Previous studies demonstrated that the pathogenicity and mortality associated with the lethal myelosuppressive regimen of carboplatin plus γ-IR could be circumvented either by the deletion of the tumor suppressor *p53* or by the immediate treatment of the mice with recombinant cytokine thrombopoietin (TPO) post-therapy.[11] *p53*-deficient mice are presumably resistant to the myelosuppression regimen due to a failure to upregulate pro-apoptotic target genes, such as *Puma*, or to trigger cell death by directly activating Bax. TPO likely protects the hematopoietic compartment through the upregulation of the pro-survival proteins Bcl-2, Bcl-X_L and Mcl-1, which inhibit apoptosis by preventing Bax/Bak from inducing MOMP.[12,13] Furthermore, TPO may have a protective role by suppressing the expression of pro-apoptotics, such as Puma and Bim. To begin challenging these hypotheses, wild-type (WT), *Puma*$^{-/-}$, *Bim*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ mice were treated with carboplatin (80 mg/kg) immediately followed by 7.5 Gy total body IR and monitored for survival and recovery of hematopoiesis over time (Figure 1). Consistent with previous studies, all WT animals died by day 15 (median survival 12.5 days) due to bacteremia resulting from gastrointestinal (GI) damage, in combination with bone marrow failure (Supplementary Figure 1). Essentially, the damage to the GI tract leads to a breach in the colonic barrier, allowing resident bacteria to escape. Since the combined treatment immunocompromises the mice, they are unable to mount an effective immune response; hence, the animals develop disseminated bacteremia throughout a variety of tissues including the lungs, kidney and heart (Supplementary Figure 1 and data not shown). Similarly, the *Bim*$^{-/-}$ mice were also sensitive to colonic damage and myelosuppression and succumbed to bacteremia with a median survival of 13 days. The *Puma*$^{-/-}$ mice were remarkably resistant and 100% survived 4 weeks after treatment, most likely due to maintaining GI tract integrity (Supplementary Figure 1); however, $\sim$40% of the animals died between days 55 and 77 due to carditis, atherosclerosis, arteriosclerosis and unknown causes. By contrast, *Puma*$^{-/-}$/*Bim*$^{-/-}$ DKO mice were completely protected and survived long term without significant sequelae or increased tumor susceptibility (Figure 1a) when compared with untreated *Puma*$^{-/-}$/*Bim*$^{-/-}$ mice, which spontaneously develop lymphomas and sarcomas by $\sim$1 year of age.[14]

Bone marrow recovery was determined by assaying white blood cells (WBCs), platelets and hemoglobin in peripheral blood every 5 days after treatment (Figure 1b). All animals, regardless of genotype, displayed a sharp decrease in each of these parameters within the first 2 weeks. However, the *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ mice began recovering WBC, platelets and hemoglobin by day 15, whereas the WT and *Bim*$^{-/-}$ mice failed to restore hematopoiesis and died because of an insufficient immune response to bacteremia caused by GI syndrome. These results suggest that loss of *Puma* alone is adequate for protecting bone marrow progenitors and intestinal cells from DNA damage, and that combined deletion of *Puma* and *Bim* is required for long-term survival post-chemo and radiation therapy.

Functional redundancy of Puma and Bim in primary myeloid cells. To examine the molecular mechanisms by which Puma and Bim contribute to apoptosis, bone marrow cells derived from WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ mice were expanded in tissue culture in the presence of IL3, IL6 and stem cell factor (SCF) as previously described, resulting in Sca+, cKit+, Lin− cell populations.[15,16] Detailed phenotype analyses demonstrated that the primary cells express FcεR1 and MC-protease-5 (mMCP-5), but not MC-protease-1 (mMCP-1), which is indicative of connective tissue mast cells (CTMCs; Supplementary Figure 2).[17] Notably, deletion of *Bim* and/or *Puma* did not alter differentiation, resulting in the expansion of identical myeloid cell types regardless of genotype. Primary CTMCs require cytokines for proliferation and survival, and during growth factor starvation undergo G1 cell-cycle arrest and apoptosis in a p53-independent manner. Cell death during cytokine deprivation is accelerated by ionizing radiation and other DNA damaging agents, which occurs through a p53-dependent apoptotic mechanism.[18]

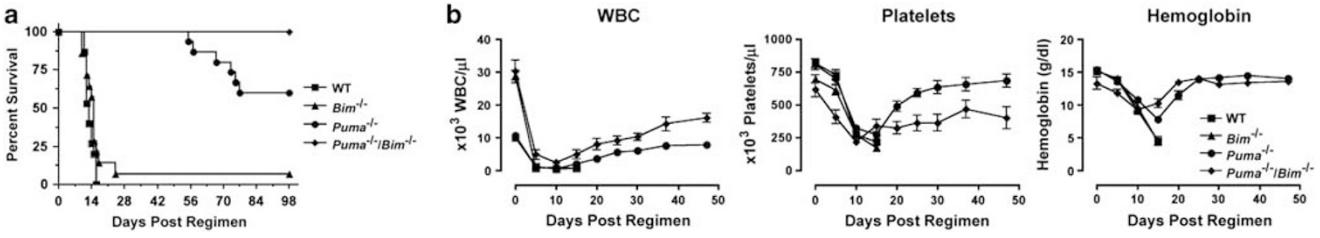

Figure 1 Co-deletion of *Bim* and *Puma* protects mice from lethal myelosuppression and is associated with recovery of the hematopoietic system. (**a**) Survival of wild-type (WT) (*squares*; $n=15$), *Bim*$^{-/-}$ (*triangles*; $n=14$), *Puma*$^{-/-}$ (*circles*; $n=15$) and *Puma*$^{-/-}$/*Bim*$^{-/-}$ (*diamonds*; $n=7$) mice treated with a myelosuppressive regimen of 80 mg/kg carboplatin and 7.5 Gy TBI. (**b**) The effects of 80 mg/kg carboplatin and 7.5 Gy TBI on white blood cell count (WBC; *left* panel), platelet count (*middle* panel) and hemoglobin concentration (*right* panel) in each mouse, from (**a**), determined at the indicated time points. Each data point in (**b**) represents the mean ± S.E.M.

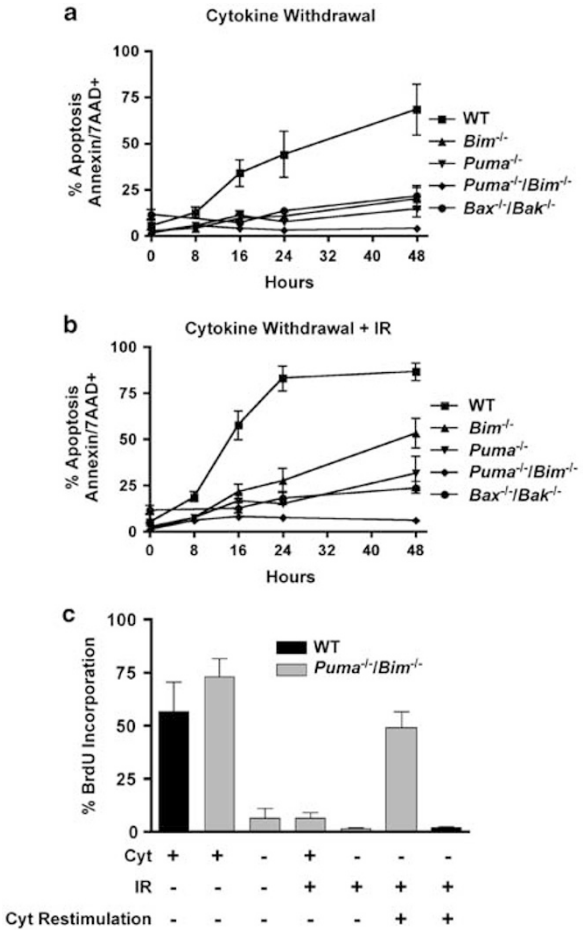

Figure 2 *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs are resistant to p53-dependent and p53-independent cell death. (**a** and **b**) WT (*squares*), *Bim*$^{-/-}$ (*triangles*), *Puma*$^{-/-}$ (*inverted triangles*), *Puma*$^{-/-}$/*Bim*$^{-/-}$ (*diamonds*) and conditionally deleted *Bax*/*Bak* double knockout (*circles*) cells were deprived of cytokines for 0–48 h (**a**) or in combination with 5 Gy IR (**b**). Apoptosis was assessed by flow cytometry of AnnexinV/7AAD-positive cells. (**c**) WT and *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells were cultured in complete media with and without cytokines (+ /− Cyt) for 48 h, treated with 5 Gy ionizing radiation (IR) at time zero, or restimulated with cytokines for 24 h following Cyt deprivation with. DNA synthesis was monitored by BrdU incorporation and FACS analysis, and quantified as the percent of BrdU-positive cells. Data are presented as the mean ± S.E.M. of at least three independent experiments, each performed with bone marrow-derived cells isolated from ($\geq$2) mice for each genotype

Consistent with these findings, cytokine deprivation blocked the proliferation and induced the death of primary WT cells (Figure 2a, $P \leq 0.012$ and data not shown), which was markedly enhanced by 5 Gy of ionizing radiation (Figure 2b, $P \leq 0.006$). By contrast, *Bim*$^{-/-}$ and *Puma*$^{-/-}$ cells maintained substantial viability for 48 h during cytokine starvation with and without IR (Figures 2a and b). Notably, primary *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells remained fully viable during cytokine starvation, even when treated with IR (Figure 2b), as demonstrated by the resumption of DNA synthesis following cytokine stimulation after 48 h starvation and IR treatment (Figure 2c). Indeed, the resistance of *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells to cytokine withdrawal plus IR was equivalent to conditional *Bax*$^{-/-}$/*Bak*$^{-/-}$ cells (Figures 2a and b), indicating that Puma and Bim are functionally redundant and account for essentially all of the apoptotic activity in DNA-damaged primary CTMCs deprived of growth factors.

Bid is dispensable for myeloid apoptosis during growth factor withdrawal and DNA damage. Bid, a pro-apoptotic BH3-only protein commonly associated with the extrinsic apoptotic pathway, activates Bax to induce apoptosis.[19] It has also been reported that the p53 tumor suppressor has a similar role in triggering cell death through Bax activation.[7–9] To address the role of these putative 'direct activators' in myeloid cell death during cytokine deprivation plus IR, *Bid*$^{-/-}$/*Bim*$^{-/-}$ and *p53*$^{-/-}$/*Bid*$^{-/-}$/*Bim*$^{-/-}$ (triple knockout, TKO) bone marrow cells were expanded in culture in the presence of IL3, IL6 and SCF. The *Bid*/*Bim*-deficient cells (Supplementary Figure 3) underwent apoptosis with the same kinetics and to the same degree as the *Bim*$^{-/-}$ cells (Figures 2a and b) in response to cytokine withdrawal alone or in combination with IR, excluding a role for Bid in the response to these death stimuli. In support of this observation, truncated Bid was not detected under these culture conditions (data not shown). The *p53*/*Bid*/*Bim* TKO primary myeloid cells were highly resistant to cytokine deprivation with or without IR, presumably due to the loss of Bim, p53 and indirectly Puma, which compromises the growth factor withdrawal- and DNA damage-induced apoptotic responses, respectively (Supplementary Figure 3). Together, these results demonstrate that the loss of p53 and Bim is sufficient to inhibit programmed cell death in these models of limiting cytokines and genotoxic stress.

Selective reduction of Bcl-X_L and Mcl-1 during cytokine starvation. To further define the biochemical mechanisms controlling apoptosis, the expression of pro-survival and pro-apoptotic Bcl-2 family members in WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs was examined during cytokine starvation with and without DNA damage. Western blot analysis demonstrated that cytokine deprivation resulted in the reduction of Bcl-X_L and Mcl-1 protein in each of the genetically altered cell lines (Figure 3). The decrease in Bcl-X_L and Mcl-1 expression is not a consequence of apoptosis *per se*, since the samples were harvested at early time points before cell death and occurred in fully viable *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells. The lower levels of Bcl-X_L and Mcl-1, in response to cytokine deprivation, presumably sensitize cells to apoptosis.

Puma or Bim is required for the activation of Bax, MOMP and cytochrome *c* release. Essential to programmed cell death is the activation of Bax and Bak to induce MOMP and cytochrome *c* release. Therefore, the level of Bax activation was determined as a surrogate marker of early apoptosis in WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs that were deprived of cytokines and/or subjected to IR at 16 h after treatment. Samples were subjected to immunoprecipitation using antibody 6A7, which is specific for the conformation-altered form of activated Bax, followed by western blot analysis (Figure 4a). WT, *Bim*$^{-/-}$ and *Puma*$^{-/-}$ cells that were deprived of cytokine (lane 2) and co-treated with IR (lane 4) exhibited a marked increase in activated Bax compared with untreated control cells (lane 1). DNA damage alone inhibited proliferation (Figure 2c) without

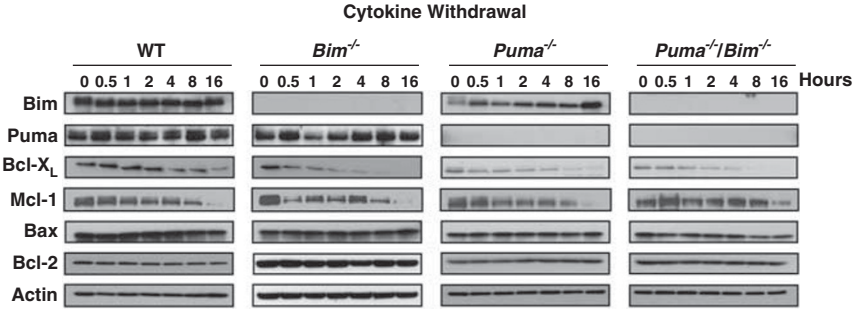

Figure 3 Cytokine withdrawal modulates the expression of both pro- and anti-apoptotic Bcl-2 family members in myeloid cells. WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells were deprived of cytokines for 0–16 h and Bcl-2-related proteins were evaluated by western blot analysis. Representative blot from at least three independent experiments is presented

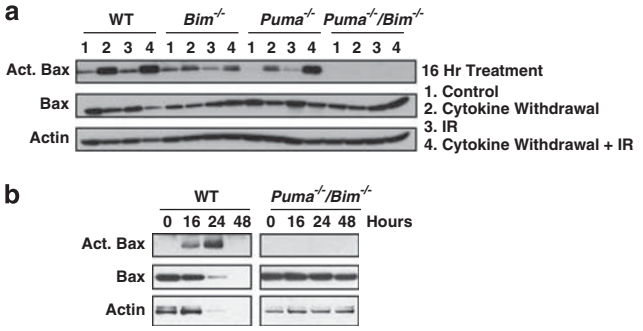

Figure 4 Bim and Puma are required for Bax activation in response to p53-dependent and p53-independent cell death. (**a**) WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs were deprived of cytokines for 16 h with or without 5 Gy IR. Bax activation was determined by immunoprecipitation with antibody 6A7, which detects Bax in its active confirmation, and subsequent western blot analysis. Actin levels were assessed in cell lysates before immunoprecipitation ($n=2$). (**b**) Western blot analysis of activated Bax in WT and *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells treated with cytokine withdrawal and 5 Gy IR for the indicated times ($n=2$)

significantly activating Bax (Figure 4a, lane 3 and data not shown). The degree of Bax activation corresponded to the level of cytochrome *c* release from the mitochondria (data not shown) and the extent of cell death (Figures 2a and b). Importantly, cytokine starvation with and without IR failed to activate Bax in the *Puma*/*Bim* DKO cells, which maintained mitochondrial integrity as determined by cytochrome *c* retention and cell viability (Figures 2a and b and data not shown).

The kinetics of Bax activation during cytokine withdrawal plus IR was also determined (Figure 4b). Treatment of WT cells resulted in significant Bax activation by 24 h, coinciding with rampant apoptosis (Figure 2b) and loss of intact Actin protein. By contrast, the *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells were completely devoid of activated Bax and remained fully viable following cytokine withdrawal and IR (Figure 2b). Therefore, either Puma or Bim is sufficient for the activation of Bax, MOMP and apoptosis in primary CTMCs deprived of cytokine and treated with IR.

Requirement for Puma or Bim in Bax activation and apoptosis during ABT-737 treatment. The neutralization model proposes that the anti-apoptotic Bcl-2 proteins bind and sequester Bax and Bak, preventing its activation and hence apoptosis.[20,21] Therefore, inhibition of Bcl-2 and Bcl-X_L with the BH3-mimetic ABT-737 should permit efficient Bax activation and cell death in the absence of Bim and Puma, analogous to WT cells. To test this hypothesis, WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs were treated with either ABT-737 (1 μM) alone or in combination with cytokine withdrawal, and assayed for apoptosis by flow cytometry (Figure 5). WT and *Bim*$^{-/-}$ cells treated with ABT-737 in the presence of cytokines displayed a modest induction of apoptosis, whereas *Puma*/*Bim* DKO cells were completely resistant (Figure 6a, left panel: $P \leq 0.026$). Despite the induction of extensive apoptosis of WT, *Bim*$^{-/-}$ and *Puma*$^{-/-}$ cells during cytokine withdrawal plus ABT-737 treatment, the *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary cells remained fully viable similar to *Bax*$^{-/-}$/*Bak*$^{-/-}$ cells (Figure 5a, middle panel: $P \leq 0.036$). By contrast, cells only deprived of cytokines for 16 h maintained substantial viability, demonstrating the efficacy of ABT-737 (Figure 5a, right panel *versus* middle panel). Consistent with these results, Bax activation was rapid and robust in WT and *Bim*$^{-/-}$ cells, slightly delayed in *Puma*$^{-/-}$ cells and absent in *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells deprived of cytokines and treated with ABT-737 (Figure 5c). Therefore, the diminished expression of Bcl-2 survival proteins during cytokine withdrawal (Figure 3) in combination with ABT-737 to further antagonize Bcl-2 and Bcl-X_L function is not sufficient to activate Bax/Bak nor to induce apoptosis in *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells. Finally, the extent of apoptosis at 48 h in primary CTMCs lacking *Bid, Bim* and *p53* during cytokine withdrawal plus ABT-737 is indistinguishable from WT cells (Figures 5a and b, middle panels). These findings establish Puma as an essential activator of Bax/Bak and cell death even in the absence of all other reported direct activators.

ABT-737 induced MOMP during growth factor withdrawal requires *Puma* and *Bim*. The biochemical roles of Puma and Bim and the effect of ABT-737 on MOMP were assessed in WT, *Puma*$^{-/-}$/*Bim*$^{-/-}$ and *Bax*$^{-/-}$/*Bak*$^{-/-}$ primary CTMCs deprived of cytokines for 6 h. Cells were then permeabilized with digitonin and incubated with Bim, Puma or a non-specific peptide, caspase-8 cleaved Bid

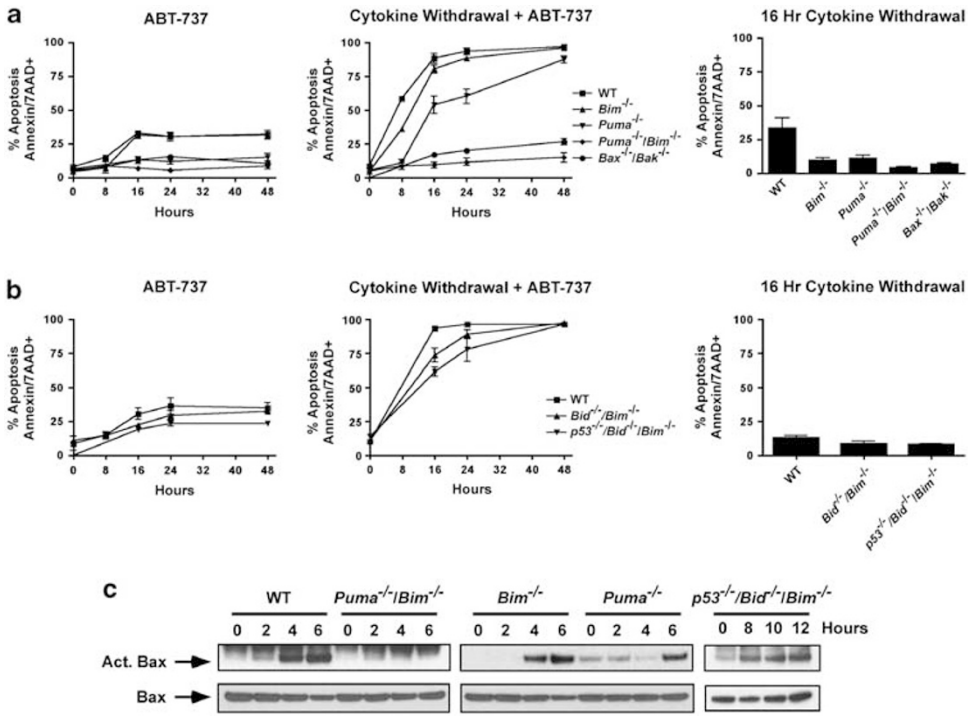

Figure 5 Bax activation and apoptosis in response to Bcl-2 and Bcl-X_L inhibition is dependent upon Bim and Puma. (**a**) WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ or *Puma*$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs were treated with 1 μM ABT-737 for the indicated times in fully supplemented media (left panel) and under conditions of cytokine withdrawal (middle panel), or treated with cytokine withdrawal alone for 16 h (right panel). Apoptosis was determined by flow cytometric evaluation of AnnexinV/7AAD-positive population. (**b**) WT, *Bim*$^{-/-}$/*Bid*$^{-/-}$ or *p53*$^{-/-}$/*Bim*$^{-/-}$/*Bid*$^{-/-}$ cells were treated with 1 μM ABT-737 for the indicated times in fully supplemented media (left panel) and under conditions of cytokine withdrawal (middle panel) or treated with cytokine withdrawal alone for 16 h (right panel). (**c**) IP/western blot analysis of Bax activation in cells deprived of cytokines and treated with ABT-737 as described in Materials and Methods ($n = 2$). Data in (**a** and **b**) are presented as the mean ± S.E.M. of three independent experiments

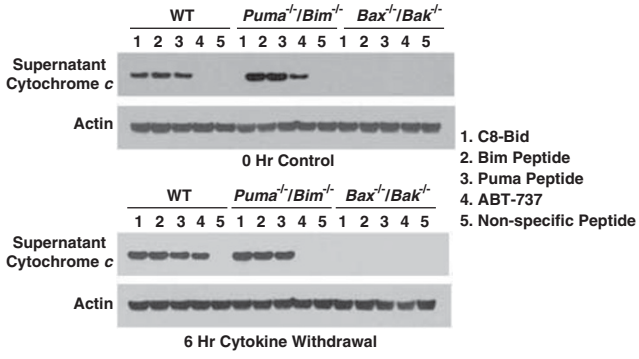

Figure 6 ABT-737 induced cytochrome *c* release is Bim and Puma dependent. Primary WT, *Puma*$^{-/-}$/*Bim*$^{-/-}$ and *Bax*$^{-/-}$/*Bak*$^{-/-}$ CTMCs were cultured in the presence or absence of cytokines for 6 h, permeablized in 0.015% digitonin and treated with C8-Bid (25 nM), Puma or Bim peptide (10 μM) or ABT-737 (0.75 μM) for 75 min. Cytochrome *c* release was determined by western blot analysis and actin was used as a loading control. Representative blot from three independent experiments is presented

or ABT-737 and supernatants were collected after centrifugation. The presence of cytochrome *c* in the supernatant was then assayed by western blot as an indicator of MOMP (Figure 6). Primary cells maintained in the presence of cytokines served as controls. Regardless of the culture conditions, WT and *Puma*$^{-/-}$/*Bim*$^{-/-}$ CTMCs incubated with either Puma or Bim peptides released cytochrome *c* from the mitochondria, while the *Bax*$^{-/-}$/*Bak*$^{-/-}$ cells were fully resistant. Importantly, the BH3-mimetic ABT-737 induced MOMP in WT, but not *Puma*$^{-/-}$/*Bim*$^{-/-}$ or Bax/Bak DKO cells deprived of cytokines. These results suggest that cytokine starvation sensitizes cells to MOMP primarily through Bim and/or Puma in a Bax/Bak-dependent manner.

Discussion

We addressed the physiological functions of Puma and Bim and the mechanisms by which they induce cell death using genetically engineered mouse models. Previous studies examining the loss of *Puma*, *Bim* or in combination have demonstrated that these proteins are mostly redundant in the context of growth factor withdrawal or glucocorticoid treatment in mast, T and B cells.[14,22] However, our results clearly demonstrate that *Bim*$^{-/-}$ and *Puma*$^{-/-}$ mice respond differently to carboplatin and IR-induced lethal myelosuppression. *Puma* knockouts were highly resistant to both GI syndrome and hematopoietic failure with 100% of the animals surviving past 30 days, whereas WT and *Bim*-deficient mice displayed marked hyperplasia in the colon with systemic bacteremia, in combination with a failure to recover hematopoiesis and died within 2 weeks of treatment (Figure 1). In support of this finding, two independent studies demonstrated that the loss of

Puma conferred a similar survival advantage during high-dose IR due to hematopoietic stem cell protection.[23,24] However, as we show here, deletion of both *Puma* and *Bim* was required for long-term survival, suggesting that each may have a defined role in hematopoietic stem and/or progenitor cells.

Cytokine signaling pathways maintain elevated levels of Bcl-2, Bcl-X_L and Mcl-1, while limiting the expression of Puma, Bim and other pro-apoptotics. Therefore, cytokine withdrawal sensitizes primary cells to apoptosis by altering the balance between pro-survival and pro-apoptotic proteins. *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells deprived of cytokines express lower levels of Bcl-X_L and Mcl-1, but maintain viability, demonstrating that the decrease in Bcl-X_L and Mcl-1 occurs independently of binding to Bim or Puma, and cell death (Figures 2 and 3). Primary CTMCs deprived of cytokines and treated with IR undergo p53-dependent apoptosis. Although deletion of *Bim* or *Puma* conferred only partial protection, *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells were completely resistant to cytokine starvation and IR due to a failure to activate Bax and MOMP (Figure 4 and data not shown). The DKO cells remain completely viable, but undergo cell-cycle arrest and efficiently resume DNA synthesis when restimulated with cytokines (Figure 2c). Consistent with these results, recombinant TPO, or the deletion of either *p53* or *Puma,* fully protects mice from a lethal myelosuppressive regimen of carboplatin and IR (Figure 1).[11] These findings establish a clinically relevant link between cytokines, DNA damage and p53 signaling pathways.

The p53 tumor suppressor induces apoptosis through the transcriptional induction of downstream target genes, such as *Puma*, *Noxa* and *Bax*.[25] It has also been proposed that p53 directly activates Bax and this transcription-independent pro-apoptotic activity is inhibited by binding to Bcl-2, Bcl-X_L and Mcl-1.[7–9] In this model, the induction of Puma during cellular stress competitively binds Bcl-X_L, which free p53 to activate Bax and trigger apoptosis.[9] As shown in Figure 5b, TKO primary CTMCs deficient in *Bid*, *Bim* and *p53* underwent apoptosis to the same extent as WT cells when deprived of cytokines and treated with ABT-737, demonstrating that these three putative direct activators of Bax are dispensable for cell death under these conditions. In light of these results and the findings that *Puma/Bim* DKO cells are completely resistant to cytokine starvation plus ABT-737 (Figure 5a), we propose that endogenous Puma is sufficient to activate Bax *in vivo* (Figure 5c). In support of this conclusion, recent studies have reported a direct interaction between Puma with Bax in cell-free systems and yeast.[26,27]

Puma$^{-/-}$/*Bim*$^{-/-}$ primary CTMCs provided a unique opportunity to challenge the 'direct activator/derepressor' *versus* 'neutralization' models of Bax activation utilizing the BH3-mimetic drug ABT-737.[19] We hypothesized that if the neutralization model was operative, then treatment of *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells with ABT-737 in the absence of cytokines should induce apoptosis by releasing Bax and Bak from sequestration by the anti-apoptotic Bcl-2 family members. Although Mcl-1 protects cells from apoptosis mediated by ABT-737,[28–30] our data demonstrate that the expression of this anti-apoptotic protein is markedly diminished upon cytokine withdrawal. Furthermore, WT cells deprived of cytokines and treated with ABT-737 rapidly underwent apoptosis, suggesting that Mcl-1 and other survival factors, such as A1, are not sufficient to block MOMP and cell death. By contrast, *Puma*$^{-/-}$/*Bim*$^{-/-}$ cells were completely protected from Bax activation and apoptosis, demonstrating an essential role for Bim and Puma in triggering cell death. Our findings provide genetic and biochemical evidence in support of the direct activator/derepressor model of apoptosis.[19–21] Similar results and conclusions have been generated using *Bid*$^{-/-}$/*Bim*$^{-/-}$/*Puma*$^{-/-}$ TKO mice and primary cells.[31]

Puma expression is naturally selected against during lymphomagenesis in c-*Myc* transgenic mice and primary human Burkitt lymphomas.[32,33] Therefore, restoring Puma apoptotic function would be a reasonable goal for treating specific malignancies, and BH3 mimetics (e.g. ABT-737, SAHBs) could provide such an opportunity.[26,34] Interestingly, Strasser and Villunger recently reported the surprising findings that *Puma* deficiency suppresses lymphomagenesis in response to low-dose, fractionated ionizing radiation, raising the cautionary flag that BH3 mimetics could promote secondary malignancies in the context of cancer therapies.[35,36] Our studies complement these findings and show that *Puma* deficiency also protects against a lethal dose of carboplatin and ionizing radiation without increasing tumor susceptibility.

In summary, we demonstrated that Puma and Bim cooperate in activating Bax, MOMP and apoptosis both *in vitro* and *in vivo* in response to cell stress. We also provided mechanistic insight into how these pro-apoptotic BH3-only proteins trigger cell death, supporting the 'direct activator' of apoptosis model. Most importantly, we identified Puma and Bim as key targets that limit chemo and radiation therapy due to GI syndrome and bone marrow toxicity. These findings provide a proof-of-principle that BH3 antagonists, such as small molecule inhibitors, could be developed to protect hematopoiesis and the GI tract from DNA damaging agents commonly used in cancer treatments without significantly increasing susceptibility to secondary malignancies. Given the scope of Puma in other pathological diseases, such as heart ischemia, stroke and neurodegeneration, the application of BH3 antagonists could have broader clinical implications.

Materials and Methods

Reagents. Caspase-8 cleaved mouse BID (C8-BID; R&D Systems, Minneapolis, MN, USA), anti-cytochrome *c* (clone 7H8.2C12, BD Pharmingen, San Jose, CA, USA), anti-BAK (clone NT, Millipore, Billerica, MA, USA), anti-Mcl-1 (Rockland, Gilbertsville, PA, USA), anti-Bcl-X_L (clone 4) and anti-Bcl-2 (clone 7; both from BD Transduction Laboratories, San Diego, CA, USA), anti-Bax (N-20) and anti-Bim (H-191) were from Santa Cruz Biotechnology (La Jolla, CA, USA), anti-Bax (clone 6A7) anti-Puma (N-terminus) and anti-β-actin (clone AC-15, Sigma-Aldrich, St. Louis, MO, USA). ABT-737 was a gift from Dr. Stephen Fesik, Abbott Laboratories (Abbott Park, IL, USA). BH3 domain peptides, Bim, and PUMA, sequences as described.[37] Peptides were resuspended in anhydrous DMSO, stored at $-80\,^{\circ}C$, and thawed only once.

Animals. *Puma*$^{-/-}$ (C57BL6/SV129 mixed) mice were previously reported.[15] *Bim*$^{-/-}$ (C57BL6/SV129 mixed) mice were a kind gift from Andreas Strasser (WEHI) and have been reported elsewhere.[38] The *Bid*$^{-/-}$ (C57BL6/SV129 mixed) and conditional *Bax/Bak* knockout mice were generously provided by Stanley Korsmeyer and have been reported elsewhere.[39,40] *Puma*$^{+/-}$ and *Bim*$^{+/-}$ mice (C57BL6/SV129 mixed) were intercrossed to generate *Puma*$^{-/-}$/*Bim*$^{-/-}$ (DKO) littermates. *p53*$^{-/-}$ (C57/BL6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Bid*$^{-/-}$/*Bim*$^{-/-}$ (C57BL6/SV129) mice have been reported

elsewhere.[41] *Bid*/*Bim*/*p53*-deficient mice were generated by crossing *Bid*$^{-/-}$/*Bim*$^{-/-}$ and *p53*$^{-/-}$ animals. Resulting progeny was intercrossed to obtain (C57BL6/SV129) TKO mice. All animal procedures were approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

Myelosuppressive regimen. Mice were given 80 mg/kg carboplatin (SICOR Pharmaceuticals, Inc., Irvine, CA, USA) intravenously immediately followed by 7.5 Gy total body ionizing radiation for the indicated period while on the antibiotic Baytril.[11] All mice were observed daily for signs of morbidity and tumor development. Tissues from morbid or moribund mice were fixed in 4% paraformaldehyde for subsequent histopathological evaluation.

Blood counts. Blood was collected into EDTA-coated 20 l microcapillary tubes (Fisher Scientific, Pittsburgh, PA, USA). WBC counts, hemoglobin concentration and platelet counts were determined using a FORCYTE Hematology System (Oxford Science, Inc., Oxford, CT, USA).

Bone marrow-derived primary myeloid cell isolation, culture and treatment. Bone marrow was isolated from the hind femur and tibias of WT, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Puma*$^{-/-}$/*Bim*$^{-/-}$ mice. Primary myeloid cells were consequently generated as described elsewhere and cultured in RMPI-1640 supplemented with 15% FBS, 1% P/S, 10 mM glutamine, 10 ng/ml mouse recombinant SCF (R&D), 10 ng/ml mouse recombinant IL-6 (R&D) and 40 U/ml mouse recombinant IL-3.[15,16] Following 3 weeks of culture with regular changes of media, the immunophenotype was determined by flow cytometry. Established homogenous cultures of bone marrow-derived cells were Sca-1$^+$cKit$^+$, negative for F4/80, GR-1/Ly6G, CD4, CD8, B220 and Ter-119 and positive for FcR1. Primary CTMCs were washed twice with HBSS and resuspended in cytokine-free media before IR (5 Gy) exposure or ABT-737 (1 μM). Cells were incubated for indicated time periods and cell responses compared with cells cultured in the presence of cytokines.

Cell viability and apoptosis determination. Cell concentration and viability was evaluated by trypan blue exclusion utilizing a Vi-CELL automated analyzer (Beckman Coulter, Brea, CA, USA). Apoptosis was determined by flow cytometric evaluation of the AnnexinV-positive population. Samples were washed twice with ice-cold PBS and stained with AnnexinV-APC and 7-AAD according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

Protein isolation and western blot analysis. Cells were harvested, washed in PBS and resuspended in CHAPS buffer (1% CHAPS, 10 mM HEPES, pH 7.4, 150 mM NaCl) containing Complete Mini protease inhibitor pill (Roche, Applied Science, Indianapolis, IN, USA), phosphatase inhibitors and 1 μM PMSF (Sigma-Aldrich). The lysates were incubated on ice for 30 min and supernatants harvested following centrifugation at 13 000 $\times$ *g* for 15 min. Protein concentrations were determined by the BCA assay (Thermo Scientific, Rockford, IL, USA) as described by the manufacturer's instructions. Protein expression (50 μg/lane for both cellular and isolated mitochondria experiments) was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequent western blot analysis (Invitrogen, Carlsbad, CA, USA). Western blot filters were probed with the indicated primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (KPL, Inc., Gaithersburg, MD, USA). Proteins were visualized using the enhanced chemiluminescence (ECL) system (Thermo Scientific) followed by exposure to CL-Xposure films (Kodak, Rochester, NY, USA).

Immunoprecipitation of activated Bax. To detect the active form of Bax, primary CTMCs were collected and lysed in CHAPS lysis buffer on ice. Briefly, activated Bax was immunoprecipitated from 500 μg of whole cell lysates with the monoclonal antibody clone 6A7 (Sigma-Aldrich), which reacts only with Bax in its conformationally active state, and Protein G Sepharose 4 Fast Flow Beads (Amersham, Little Chalfont, UK). Isolated active Bax samples were then subjected to western blot analysis and identified with a rabbit polyclonal anti-Bax (N-20; Santa Cruz Biotechnology) antibody and ECL as described above.

Cellular permeablization and MOMP analysis. Primary CTMCs (5×10^6/sample) were collected, washed in PBS and incubated on ice for 5 min in permeability buffer (20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose and 0.015% digitonin from 10% DMSO stock) containing Complete Mini protease inhibitor pill. Proteins, peptides and ABT-737 (final concentrations indicated in the figure legends) were then added and the cells were incubated at 30 °C for 1.25 h. Samples were centrifuged for 10 min in 4 °C at 15 000 $\times$ *g*. Supernatants were collected and analyzed by SDS-PAGE and western blot with anti-cytochrome *c* (clone 7H8.2C12).

BrdU incorporation. Primary myeloid cells were labeled with 10 μM 5-bromo-2′-deoxy-uridine (BrdU) for 24 h at 37 °C under aseptic conditions. Cells were harvested and BrdU incorporation was determined using the 'APC BrdU Flow Kit' according to the manufacturer's instructions using a FACScalibur flow cytometer (BD Biosciences).

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)