NF-κB inhibits T-cell activation-induced, p73-dependent cell death by induction of MDM2

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NF-κB is a key transcription factor involved in the regulation of T-cell activation and proliferation upon engagement of the T-cell receptor (TCR). T cells that lack the IκB kinase (IKKβ) are unable to activate NF-κB, and rapidly undergo apoptosis upon activation. NF-κB activation following T-cell receptor engagement induces the expression of Mdm2 through interaction with NF-κB sites in its P1 promoter, and enforced expression of Mdm2 protected T cells deficient for NF-κB activation from activation-induced cell death. In T cells with intact NF-κB signaling, ablation or pharmacologic inhibition of Mdm2 resulted in activation-induced apoptosis. Mdm2 coprecipitates with p73 in activated T cells, and apoptosis induced by inhibition of Mdm2 was p73-dependent. Further, *Bim* was identified as a p73 target gene required for cell death induced by Mdm2 inhibition, and a p73-responsive element in intron 1 of *Bim* was characterized. Our results demonstrate a pathway for survival of activated T cells through NF-κB–induced Mdm2, which blocks Bim-dependent apoptosis through binding and inhibition of p73.

apoptosis | T lymphocyte | Bim

During the course of an immune response, activated T lymphocytes undergo cell death by a variety of mechanisms (1). Such activation-induced cell death (AICD) can be mediated by expression of death ligands of the TNF family, including CD95 ligand (1) and TNF-related apoptosis-inducing ligand (2), and the engagement of their respective receptors. An independent mechanism of AICD involves the expression and stabilization of the proapoptotic BCL-2 protein, BIM (3). Another, less well characterized mode of AICD in T cells involves engagement of the p53 family member p73 (4). Intriguingly, this mechanism of T-cell death appears to be inhibited by the function of the transcription factor NF-κB, as T cells expressing a nondegradable mutant of its inhibitor, IκB, undergo AICD in a p73-dependent manner (5). Upon ligation of the T-cell receptor (TCR) and coreceptors, T cells activate NF-κB via the IκB kinase (IKK) complex, composed of IKKα, IKKβ, and IKKγ/NEMO (6). T cells deficient in NF-κB components (7) or lacking IKKβ (8, 9) develop normally provided TNFR1 is disrupted (10), but display defective proliferation (11) and accumulation of memory T cells (12, 13).

The p53 family of proteins includes p53, p63, and p73, and forms of these proteins produced by alternative splicing and/or alternative translation-start sites (14). A critical regulator of p53 is the E3-ubiquitin ligase, murine double minute-2 (MDM2), which binds directly to the NH$_2$ terminus of p53 in the nucleus, inhibiting its transcriptional activity (15). The MDM2–p53 complex shuttles from the nucleus to the cytoplasm, where MDM2 targets p53 for proteasome-dependent degradation. MDM2 also binds to p73 and inhibits its transcriptional activity, but does not target p73 for degradation (16).

Expression of murine and human MDM2 is under the control of two promoters, P1 and P2 (17, 18). Whereas P2 is driven by p53 (17), transcription factors responsible for P1-mediated expression have not been identified to date. Interestingly, NF-κB has been shown to induce MDM2 expression in mouse embryonic fibroblasts (19), as an NF-κB–responsive element has been identified in intron 1 of MDM2.

Here, we explore the role of NF-κB in the expression of MDM2 in activated T cells and its role in preventing p73-dependent, activation-induced apoptosis. We further identify BIM as a p73 target in T lymphocytes, required for this form of AICD.

Results

To generate T cells defective in NF-κB signaling, we bred *Ikkβ$^{-/-}$Tnfr1$^{-/-}$* animals to rescue the embryonic lethality seen in *Ikkβ$^{-/-}$* mice. Splenic T cells from 7-d-old *Ikkβ$^{+/+}$Tnfr1$^{-/-}$* or *Ikkβ$^{-/-}$Tnfr1$^{-/-}$* mice (hereafter referred to as *Ikkβ$^{+/+}$* or *Ikkβ$^{-/-}$*) were stimulated with anti-CD3 plus anti-CD28, and as expected (5), apoptosis was dramatically elevated in activated *Ikkβ$^{-/-}$* T cells (Fig. S1 *A* and *B*). Similarly, we found that Jurkat T cells, stably expressing an IκBαM superrepressor (20), underwent apoptosis upon stimulation with anti-CD3, whereas cells stably transduced with a control vector did not (Fig. S1C).

In mouse embryonic fibroblasts, NF-κB has been observed to drive the expression of MDM2, which regulates p53 function (19). Similarly, in both primary T cells (Fig. 1 *A* and *C*) and Jurkat cells (Fig. 1 *B* and *D*), activation led to increased mRNA and protein expression of MDM2. *Ikkβ$^{-/-}$* T cells and Jurkat cells expressing IκBαM did not display increased MDM2 following activation.

We next asked if the expression of MDM2 participates in NF-κB–dependent cell survival following T-cell activation. To ectopically express MDM2 in primary T cells, we optimized transduction to achieve expression of GFP in almost 50% of primary cells (Fig. S2C). We introduced a construct for transient expression of MDM2 in *Ikkβ$^{-/-}$* T cells before activation with anti-CD3 plus anti-CD28 (Fig. 1E and Fig. S2A). Although activation of *Ikkβ*-null T cells resulted in extensive cell death, enforced expression of MDM2 inhibited apoptosis.

Similarly, we stably enforced MDM2 expression in Jurkat cells with or without IκBαM superrepressor (Fig. S2B). Although activation of Jurkat cells with anti-CD3 resulted in apoptosis in cells expressing IκBαM superrepressor, this cell death was inhibited by MDM2, but not by stable transduction with the control vector (Fig. 1F).
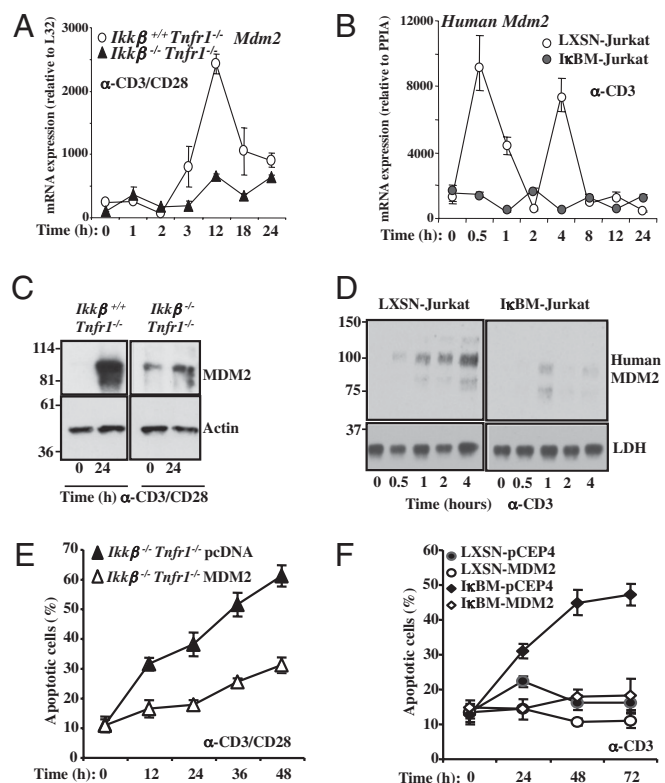
Fig. 1. Impaired Mdm2 up-regulation in primary $Ikk\beta^{-/-}Tnfr1^{-/-}$ and IκBM-Jurkat T cells upon TCR activation. (*A*) Total mRNA was isolated from anti-mCD3/anti-mCD28 activated $Ikk\beta^{+/+}Tnfr1^{-/-}$ and $Ikk\beta^{-/-}Tnfr1^{-/-}$ lymphocytes and assessed by real-time RT-PCR for *Mdm2* mRNA expression. Absolute mRNA values were determined, normalized to *L32*, and reported as arbitrary units. (*B*) Total mRNA was isolated from anti-hCD3 (OKT3) activated LXSN- and IκBM-Jurkat T cells and assessed by real-time RT-PCR for *human Mdm2* mRNA expression. Absolute mRNA values were determined, normalized to *Cyclophylin* (PPIA), and reported as arbitrary units. (*C*) $Ikk\beta^{+/+}Tnfr1^{-/-}$ and $Ikk\beta^{-/-}Tnfr1^{-/-}$ lymphocytes were activated for 24 h with anti-mCD3/anti-mCD28. MDM2 and Actin protein expression was detected by immuno-blotting. (*D*) LXSN- and IκBM-Jurkat T cells were activated with anti-hCD3 (OKT3) for the indicated times. Human MDM2 and lactate dehydrogenase protein expression were assessed by Western blotting. (*E*) Primary $Ikk\beta^{-/-}Tnfr1^{-/-}$ lymphocytes were transiently transfected with pcDNA or pcDNA-MDM2 for 13 h and then activated for the indicated times with anti-mCD3/anti-mCD28. Apoptotic T cells were determined visually by nuclear chromatin staining with Hoechst 33342 dye. (*F*) The indicated cell lines were activated with soluble anti-hCD3 (OKT3) for the indicated time and apoptotic cells were counted as in *E*.

We identified six potential NF-κB sites in the *human Mdm2* promoter (Fig. S3*A*) and found that two were active in reporter assays and electrophoretic mobility shift assays (EMSAs; Fig. 2 *A–C*). These sites in the P1 promoter, denoted κB1 and κB2 (Fig. S3*A*), drove reporter expression in activated Jurkat cells (Fig. 2*A*). EMSA analysis showed binding to κB1 following T cell activation (Fig. 2*B*). Consistent with earlier reports for other κB sites (21), NF-κB binding activity to the κB1 site appeared in two waves. Similarly, an observed background binding for κB2 was increased upon treatment with anti-CD3 (Fig. 2*C*). To explore the latter binding in more detail, we performed neutralization studies with antibodies to NF-κB components (Fig. 2*D*). We observed no role for c-Rel or RelB in the binding, but inhibition with anti-p50 antibody blocked both background and enhanced binding following activation. Addition of anti-RelA antibody caused the appearance of a different band, likely to be a p50 homodimer (22, 23). Thus, it is likely that, upon activation, p50/RelA binds to the κB2 site in the *Mdm2 P1* promoter.
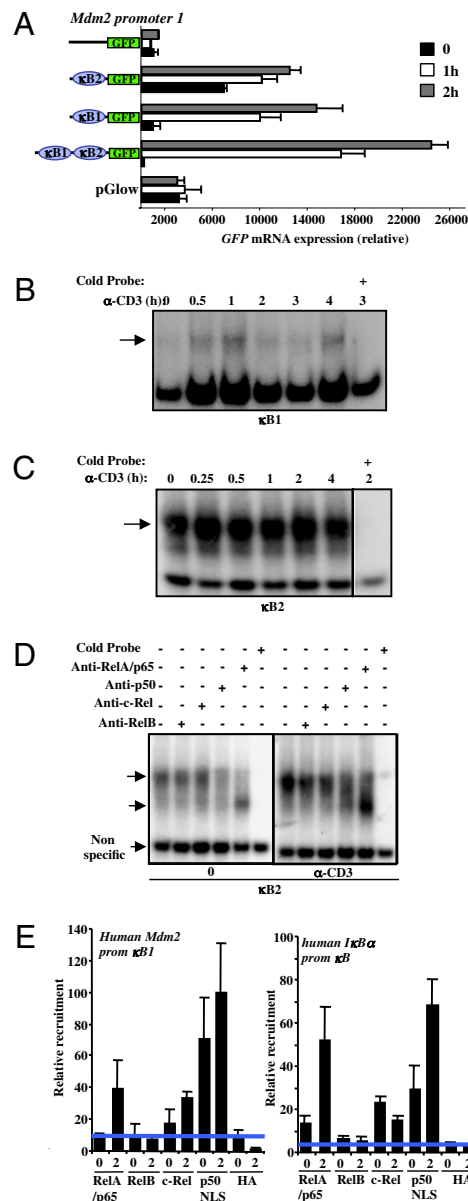

Fig. 2. NF-κB regulates *Mdm2 promoter 1*. (*A*) LXSN-Jurkat T cells were cotransfected with the indicated GFP reporter plasmids with or without κB1 and κB2 sites and stimulated for 1 and 2 h with anti-hCD3 (OKT3). *GFP* mRNA was analyzed by real-time RT-PCR and normalized to *LacZ* mRNA expression as a transfection efficiency control. (*B*) NF-κB binding to a DNA probe containing the κB1-binding site in the *human Mdm2 P1* promoter. Nuclear extracts were prepared from LXSN-Jurkat T cells treated with anti-hCD3 (OKT3) for the indicated time and incubated with a γ^{32}P-labeled double-stranded oligonucleotide probe corresponding to the putative NF-κB site in the *P1 promoter* region. Specificity of binding to the κB sites was shown by competition with double-stranded oligonucleotides (cold probe). The arrow indicates specific binding. (*C*) As in *B*, use of a DNA probe containing the κB2-binding site in the *human Mdm2 P1* promoter region. The arrows indicate specific binding. (*D*) As in *C*, use of the DNA probe containing the κB2-binding site with the addition of antibodies against p65/RelA, p50, c-Rel, and RelB to identify the subunits present in the bound complexes (arrows indicate p50/p65, p50/p50, and nonspecific complexes). (*E*) ChIP of NF-κB proteins detects NF-κB site in the *human Mdm2 P1* promoter. Jurkat T cells were activated with anti-hCD3 (OKT3) for 2 h and ChIP was performed with the indicated antibodies. DNA was probed by quantitative PCR for the indicated sequences. Activation resulted in binding of p65 and p50 to both the established NF-κB site in the *human IκB* promoter and the κB1 site in the *human Mdm2* promoter.
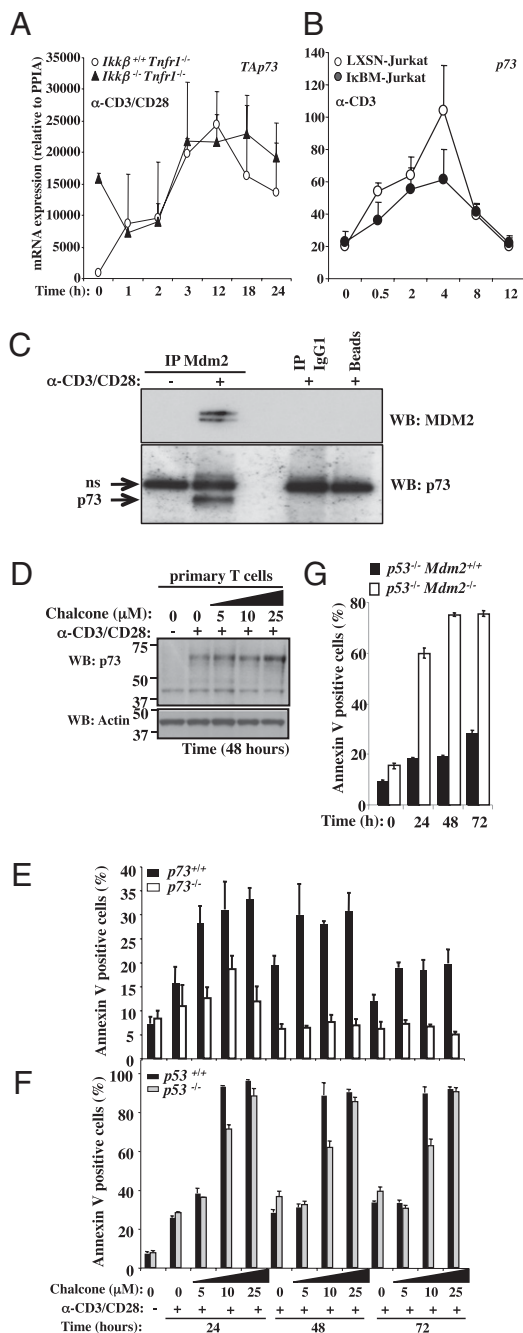
Fig. 3. *Ikkβ⁻/⁻Tnfr1⁻/⁻* and IκBM-Jurkat T cells express TAp73 upon TCR activation. (*A*) Primary *Ikkβ⁺/⁺Tnfr1⁻/⁻* and *Ikkβ⁻/⁻Tnfr1⁻/⁻* lymphocytes were stimulated with anti-mCD3/anti-mCD28 for the indicated times. Total mRNA was isolated and assessed by real-time RT-PCR for *TAp73* mRNA expression. Absolute mRNA values were determined, normalized to *Cyclophylin* (PPIA), and reported as arbitrary units. (*B*) LXSN- and IκBM-Jurkat T cells were stimulated with anti-hCD3 for the indicated times. Total mRNA was isolated and assessed by real-time RT-PCR for *p73* mRNA expression. Absolute mRNA values were determined, normalized to *Cyclophylin* (PPIA), and reported as arbitrary units. (*C*) Primary T cells from C57BL/6 mice were untreated or stimulated for 24 h with plate-bound anti-mCD3/anti-mCD28. Immunoprecipitations (IP) with an anti-MDM2 Ab or isotype control were followed by immunoblot detection of MDM2 and p73 with HRP-coupled antibodies. Controls include the Ab isotype alone (IP IgG1) and beads incubated in the absence of Ab (beads). (*Top*) IP, anti-Mdm2; WB, anti-Mdm2. (*Bottom*) IP, anti-Mdm2; WB, anti-p73. (*D*) WT primary T cells were activated for 48 h with plate-bound anti-mCD3/anti-mCD28 in the presence or absence of increasing concentrations of MDM2 inhibitor (chalcone). Total cellular proteins were isolated. p73 and Actin protein expression was assessed by immunoblotting.

To further analyze the binding of NF-κB to the κB1 site, chromatin immunoprecipitation (ChIP) analysis was performed (Fig. 2*E*). The κB site in the *IκB* promoter (24) was used as a control. ChIP with anti-p50 precipitated both elements in resting and activated Jurkat T cells, whereas anti-RelA precipitated the elements only in activated T cells. Precipitation with anti–c-Rel was also observed. Together, these results implicate p50/RelA (and possibly p50/c-Rel) heterodimers in driving the *Mdm2 P1* promoter following T-cell activation. Consistent with a role for p50/RelA in *Mdm2* promoter activation, we found that both κB1 and κB2 promoter reporter expression was induced by coexpression of p50 and RelA in Jurkat cells (Fig. S3*B*) and primary T cells (Fig. S3*C*).

A role for p53 in this AICD, however, was unlikely, as Jurkat T cells lack p53 (25), and we were unable to detect significant p53 stabilization following activation of primary *Ikkβ*-deficient T cells. Previous studies (5, 26) had shown that cell death in activated T cells expressing an IκB superrepressor is dependent on the p53 family member p73. As described earlier (4), we detected induction of *p73* mRNA expression by T-cell activation in both primary T cells and Jurkat cells, and this was unaffected by NF-κB status (Fig. 3*A* and *B*). We therefore immunoprecipitated MDM2 from activated, primary WT T cells (Fig. 3*C* and Fig. S4 *A* and *B*). We found that p73 coprecipitated with MDM2 from activated but not resting T cells. The identification of p73 was validated using lysates from p73-deficient T cells (Fig. S4*A*). The effect of an MDM2 inhibitor on the binding of MDM2 to p73 was then examined. Chalcone is an MDM2 inhibitor that binds to MDM2 and competes for p53 binding (27). We found that chalcone effectively blocked the coprecipitation of p73 with MDM2 (Fig. S4*B*). The presence of chalcone had only a minor effect on levels of p73 following activation (Fig. 3*D*). This is consistent with the observations that MDM2 binds to and blocks the function of p73, but does not induce its degradation (16, 28). Further, we found that whereas the presence of chalcone promoted activation-induced apoptosis in WT T cells, T cells lacking p73 were strikingly resistant to this effect (Fig. 3*E*). In contrast, T cells lacking p53 were fully sensitive to activation-induced apoptosis in the presence of chalcone (Fig. 3*F*).

Genetic ablation of MDM2 is embryonically lethal, but can be rescued by deletion of p53 (29, 30). We therefore examined the role of MDM2 in activated T-cell survival by comparing T cells from *p53⁻/⁻Mdm2⁺/⁺* and *p53⁻/⁻Mdm2⁻/⁻* mice (Fig. 3*G*). Strikingly, the absence of MDM2 resulted in dramatic AICD in these T cells. Together, our results show that expression and function of MDM2 upon T-cell activation is required for survival of the cells.

The transcriptional targets of p53 and p73 are distinct, but overlapping (31). The proapoptotic BH3-only proteins PUMA (32) and BID (33) are p53 targets required for p53-mediated apoptosis in different tissues. PUMA is also a transcriptional target of p73 (34–36). We therefore examined the expression of *Puma* and *Bid* following activation of *Ikkβ⁺/⁺* and *Ikkβ⁻/⁻* T cells (Fig. 4*A*). In addition, we also examined the expression of another BH3-only protein, BIM, which is not a known p53 target,

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Fig. 4. p73 regulates *Bim* gene expression after TCR costimulation. (*A*) Primary lymphocytes from *Ikkβ⁺/⁺Tnfr1⁻/⁻* and *Ikkβ⁻/⁻Tnfr1⁻/⁻* mice were activated with plate-bound anti-mCD3/anti-mCD28 for the indicated times. Total mRNA was isolated and assessed by real-time RT-PCR for *Puma*, *Bid*, and *BimEL* mRNA expression. Absolute mRNA values were determined, normalized to *L32*, and reported as arbitrary units. (*B*) Primary *Bim⁻/⁻* and *Bim⁺/⁺* T cells were activated with plate-bound anti-mCD3/anti-mCD28 for 18 h in the presence or absence of MDM2 inhibitor (chalcone). Total mRNA was isolated and assessed by real-time RT-PCR for *BimEL* mRNA expression. (*C*) Primary T cells isolated from *Bim⁻/⁻* and *Bim⁺/⁺* mice were activated with plate-bound anti-mCD3/anti-mCD28 for the indicated times in the presence of the indicated concentration of MDM2 inhibitor (chalcone) and were analyzed by flow cytometry for APC-conjugated annexin V staining. Percentages of annexin V-positive cells are indicated. (*D*) Primary *p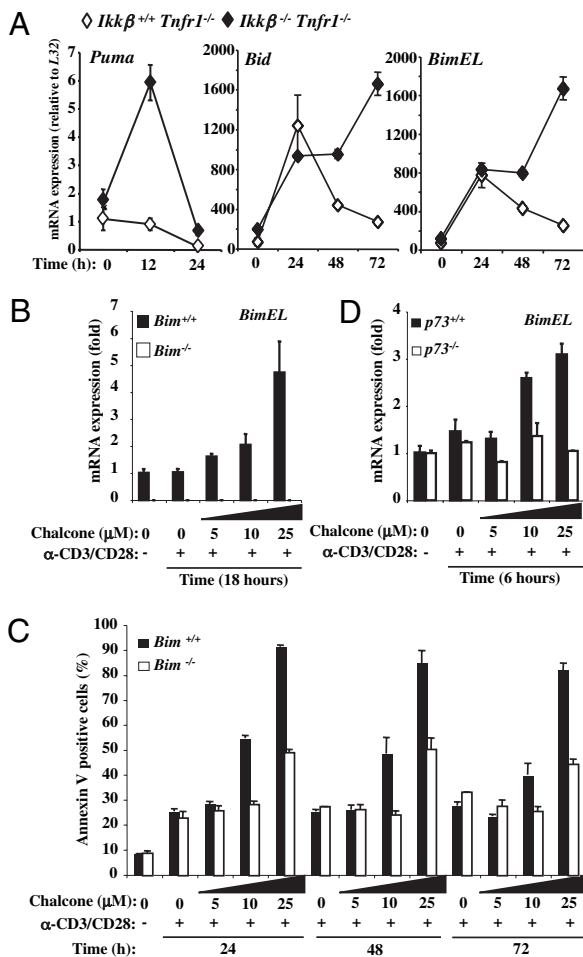73⁻/⁻* and *p73⁺/⁺* T cells were activated with plate-bound anti-mCD3/anti-mCD28 for 6 h in the presence of the indicated concentration of chalcone. Total mRNA was isolated and assessed by real-time RT-PCR for *BimEL* mRNA expression.

but has been implicated in activation-induced apoptosis in thymocytes (37) and mature T cells (38). *Puma* mRNA was selectively induced in activated T cells lacking IKKβ. Although both *Bid* and *Bim* were transiently induced following activation of *Ikkβ⁺/⁺* T cells, levels of expression of both were dramatically enhanced in T cells lacking IKKβ (Fig. 4*A*).

To determine if any or all of these BH3-only proteins play roles in p73-dependent, chalcone-promoted death of activated T cells, we used primary T cells from mice deficient in PUMA, BID, or BIM. Treatment with chalcone promoted activation-induced T-cell apoptosis, regardless of the status of PUMA (Fig. S5*A*) or BID (Fig. S5*B*). In contrast, T cells lacking BIM were resistant to the effects of chalcone (Fig. 4*C*). Consistent with these findings, *Bim* mRNA was elevated in primary T cells, activated in the
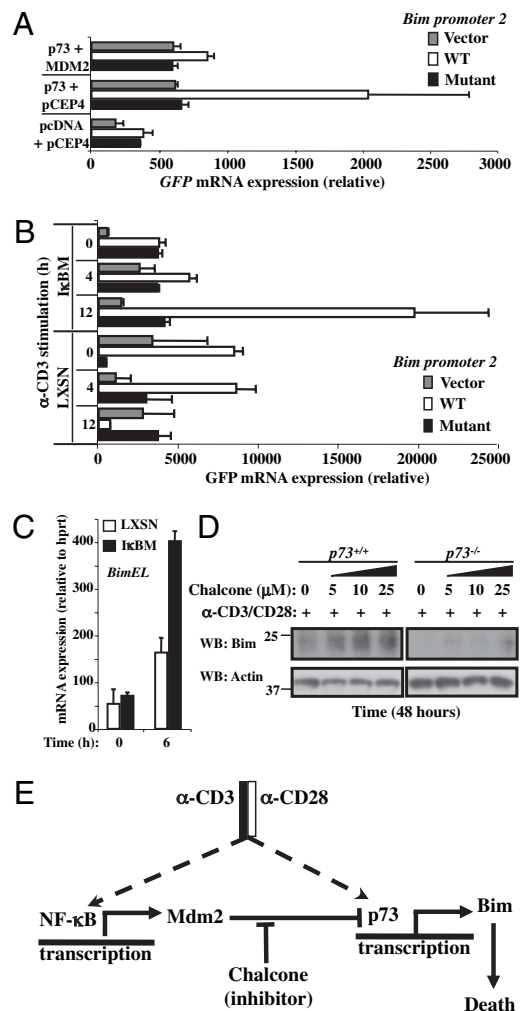

Fig. 5. p73 binds and activates the *bim* promoter. (*A*) LXSN-Jurkat T cells were cotransfected with different constructs expressing *human p73-α* in the presence or not of *Mdm2* with the indicated *GFP reporter* plasmids with WT p73 site (white bars), mutated p73 site (black bars), or empty vector (gray bars). *GFP* mRNA was analyzed by real-time RT-PCR and normalized to *LacZ* mRNA expression as a transfection efficiency control. (*B*) LXSN- and IκBM-Jurkat T cells were cotransfected with the indicated *GFP reporter* plasmids without (gray bars) or with WT p73 site (white bars) or mutated p73 site (black bars) from *Bim P2* promoter region. *GFP* mRNA was analyzed by real-time RT-PCR and normalized to *LacZ* mRNA expression as a transfection efficiency control. Anti-hCD3 (OKT3) stimulation. (*C*) LXSN- and IκBM-Jurkat T cells were activated with anti-hCD3 (OKT3) for 6 h. Total mRNA was isolated and assessed by real-time RT-PCR for *BimEL* mRNA expression. (*D*) Primary T cells from *p73⁺/⁺* and *p73⁻/⁻* were activated with plate-bound anti-mCD3/anti-mCD28 for 48 h in presence or not of the indicated concentration of chalcone. Total cellular proteins were isolated and Western blots were probed with antibodies against Bim and Actin. (*E*) Model of the NF-κB/Mdm2/p73/Bim pathway for survival and death of activated T cells.

presence of chalcone (Fig. 4*B*). T cells lacking p73 did not show this effect (Fig. 4*D*).

This suggested that *Bim* is expressed in response to p73 in activated T cells, provided the function of NF-κB or MDM2 is disrupted. We therefore examined the *Bim* promoter and identified a potential p73 target element (Fig. S6*A*). A reporter of 70 bp of the *human Bim* promoter containing the potential p73 binding site was cotransfected with p73 into Jurkat cells (Fig. 5*A*). Although p73 drove expression of this reporter, a mutation in the p73 site rendered the promoter unresponsive to p73. Coexpression of

MDM2 prevented this activity of p73 on the *Bim* promoter. In keeping with these observations, activation of primary T cells in the presence of chalcone effectively induced expression from the *Bim* promoter reporter (Fig. S6B).

We then examined the effect of NF-κB status on activation-induced expression of the *Bim* promoter reporter. Although only marginal reporter expression was observed upon activation of Jurkat cells with anti-CD3, this was dramatically enhanced in Jurkat expressing the IκBαM superrepressor (Fig. 5B). Mutation of the p73 site in the *Bim* promoter negated this effect. Consistent with these observations, we found that Jurkat cells expressing the IκBαM superrepressor induced expression of *Bim* mRNA (Fig. 5C). In primary WT T cells, a low level accumulation of BIM was observed following activation, and this was dramatically enhanced by chalcone (Fig. 5D). In contrast, activation of p73$^{-/-}$ T cells did not result in BIM expression with or without chalcone (Fig. 5D).

Discussion

We have defined a pathway in activated T cells in which NF-κB induces the expression of MDM2, which in turn binds to and inhibits p73-induced BIM expression, thereby preventing BIM-mediated apoptosis (Fig. 5E). NF-κB, induced upon T-cell activation, directly engages the *Mdm2 P1* promoter, and in the absence of NF-κB function, T-cell activation leads to cell death that can be inhibited by enforced expression of MDM2.

NF-κB can drive MDM2 expression, without a direct interaction with the *P2* promoter of *Mdm2*. Recently, an NF-κB component, BCL3, was found to bind in the first intron of *Mdm2*. We have found (Fig. S7A) that *Bcl3* is expressed in activated WT but not *Ikkβ*-deficient T cells, but our results directly implicate p50/RelA (and perhaps p50/c-Rel) in binding to and promoting expression from the *P1* promoter of *Mdm2*. This does not rule out a role for BCL3 in MDM2 expression as well. T cells from mice lacking p50 and c-Rel develop normally but die upon activation (39). This was attributed to a requirement for NF-κB for expression of the anti-apoptotic protein BCL-2 and IL-2, either of which prevented AICD in the deficient T cells, as did ectopic expression of active AKT. As BCL-2 blocks BIM-dependent apoptosis (40) (implicated in the pathway we have elucidated), the effect of ectopic BCL-2 is not unexpected. Similarly, the ability of IL2 or AKT signaling to prevent apoptosis in this setting may involve the role of cytokines and AKT in inhibiting BIM expression via phosphorylation of FOXO3a (41). Recently, however, the absence of FOXO3 in T cells was observed to have no effect on the expansion or contraction of T cells in response to viral infection (42). At present, we do not know if FOXO3a (or another FOXO protein) participates in optimal BIM expression induced by p73.

Recently, p73-induced expression of BIM was implicated in cell death induced by prolonged mitotic arrest in fibroblasts (43). Three p73-binding elements distinct from that we found in the promoter region were identified in the first intron of *Bim* by ChIP analysis. Thus, although *Bim* does not appear to be a target of p53, it is a target of p73. Direct induction of *Bim* by p73 accounts for the role of BIM in p73-dependent apoptosis we observe in activated T cells with defective NF-κB activation, or in which MDM2 is inhibited.

We have found that T cells with normal NF-κB function but deficient in MDM2 (either by genetic ablation or pharmacologic inhibition) undergo cell death upon activation. Mice carrying one hypomorphic allele of MDM2 have been noted to display a marked lymphopenia that was attributed to p53 hyperactivation (44). This p53-dependent destruction of lymphoid cells was also observed in *Mdm2*$^{-/-}$ mice carrying a tamoxifen-responsive *p53ER*^{tam} knock-in allele upon treatment with tamoxifen (45). In our studies, however, treatment with the MDM2 inhibitor chalcone resulted in activation-induced apoptosis in T cells lacking p53, but not in T cells lacking p73. Thus, T-cell activation appears to engage p73 but not p53, and the former is inhibited by NF-κB–induced MDM2. Intriguingly, we have found that T cell activation also engages expression of another p53 inhibitor, MDMX [also called MDM4 (46); Fig S7B]. At present, we do not know if MDMX can inhibit p73, or if chalcone interferes with MDMX function in this context. However, such expression of MDMX to inhibit p53 might explain why inhibition of MDM2 by chalcone does not appear to induce p53-dependent apoptosis in p73-deficient activated T cells. Indeed, irradiation of activated T cells results in p53-independent apoptosis. Interestingly, irradiation of activated T cells recruits p73 to the *Bax* promoter, and this effect is antagonized by p50/RelA (47).

When might a failure to engage NF-κB during T-cell activation occur during normal immune responses? Although engagement of the CD3-TCR complex activates IKK, coreceptor (e.g., CD28) signaling enhances NF-κB activation. Thus, at the initiation of immune responses, a failure to costimulate T cells may result in inefficient NF-κB activation, thereby engaging the death pathway we have elucidated. If so, the lack of p73 may allow such cells to persist. Intriguingly, p73-deficient mice often show early postnatal lethality, in part because of systemic inflammatory responses. The possible contribution of T cells to this effect has not been rigorously explored.

Materials and Methods

Cell Lines and Animals. All mice (described in detail in *SI Materials and Methods*) were bred and housed under specific pathogen-free conditions in our animal facilities at La Jolla Institute for Allergy and Immunology (La Jolla, CA) and at St. Jude Children' Research Hospital (Memphis, TN).

T Cells and T-Cell Lines. Briefly, T cells were enriched from spleens by negative selection using a MACS system with microbeads conjugated to monoclonal anti-mouse CD45R (B220; isotype: rat IgG2a; clone:RA3-6B2; Miltenyi Biotec) following the manufacturer's instructions. LXSN-Jurkat and IκBM-Jurkat cells have been previously described (20). Primary T cells were stimulated in 96-well flat-bottom plates with plate-bound anti-mCD3 (clone 145–2C11) and anti-mCD28 (clone 37.51; antibodies coated in PBS solution at 10 μg/mL overnight at 4 °C; BD Pharmingen) and 0.5–1 × 10^6 T cells were added per well or Jurkat T cells were stimulated with soluble anti-hCD3 (OKT3; eBioscience), in the presence or absence of an MDM2 inhibitor [Chalcone (*trans*-4-Iodo, 4′-boranyl-chalcone); Calbiochem] was added as indicated. Following culture, cells were harvested at indicated times and apoptosis assessed as described (48).

Plasmid Constructs and Transfections. Murine *mdm-2* cDNA was amplified by RT-PCR from total RNA (*SI Materials and Methods* provides details). Human *Mdm2* promoter constructs (κB1–κB2, κB1, κB2, and no κB sites) and *human Bim* promoter constructs were generated by PCR (*SI Materials and Methods*). Transient expression experiments were performed as described previously (2) (*SI Materials and Methods*).

Real-Time RT-PCR. Total RNA isolation, reverse transcription, and real-time PCR were assessed as described previously (2). Primer sequences are available upon request.

EMSA. Extracts and probes were prepared as previously described (49). *SI Materials and Methods* provides more details on EMSA.

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