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Identification of PP2A as a crucial regulator of the NF-κB feedback loop: its inhibition by UVB turns NF-κB into a pro-apoptotic factor

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Although nuclear factor-κB (NF-κB) usually exerts anti-apoptotic activity, upon activation by interleukin-1 (IL-1) it enhances ultraviolet-B radiation (UVB)-induced apoptosis. This paradoxical effect is associated with NF-κB-dependent pronounced secretion of tumour necrosis factor-α (TNF) which activates TNF-R1 in an autocrine fashion to enhance UVB-induced apoptosis. We demonstrate that sustained TNF transcription in UVB + IL-1-treated cells involves complete abrogation of the negative feedback loop of NF-κB preventing IκBα resynthesis, hence allowing uncontrolled NF-κB activity. We show that IκBα is not transcriptionally inhibited but resynthesized protein is immediately marked for degradation due to persistent inhibitor of κB kinaseβ (IKKβ) activity. Continuous IKKβ phosphorylation and activation is caused by UVB-mediated inhibition of the phosphatase PP2A. This study demonstrates that the cellular response to different NF-κB activators may be converted to the opposite reaction when both stimuli act in concert. Our data shed new light on the significance of negative feedback regulation of NF-κB and identifies PP2A as the key regulator of this process.
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The transcription factor nuclear factor-κB (NF-κB) is involved in many cellular responses. It comprises five proteins: p50/p105, p52/p100, p65, c-Rel and RelB that exist as homo- and heterodimers, p65/p50 being the most abundant form. In unstimulated cells, NF-κB is sequestered in the cytoplasm through interaction with the inhibitory protein IκBα that masks its nuclear localization signal.[1] NF-κB (p65/p50) is mostly activated by pro-inflammatory mediators, including tumour necrosis factor-α (TNF), interleukin-1 (IL-1) or LPS. Activated receptors mediate activation of a multi subunit inhibitor of κB kinase (IKK) complex consisting of IKKα, -β and -γ. Activated IKK acts through phosphorylation of IKKβ at Ser177/181, subsequently catalysing phosphorylation of inhibitor of κBα (IκBα) at Ser32/36, leading to its polyubiquitination and proteasomal degradation. Released NF-κB translocates into the nucleus to activate responsive genes, among these the one encoding IκBα.[2] Nuclear export of resynthesized IκBα is more potent than import, allowing cytosolic localization of the inactive complex, thus creating a negative feedback loop.[3]

As NF-κB serves many different functions, tight regulation by the negative feedback loop is crucial. Only highly controlled and transient expression of NF-κB-driven genes ensures proper function. Uncontrolled NF-κB activity is linked to transformation, proliferation, suppression of apoptosis and metastasis.[4,5] Thus, strategies interfering with signalling pathways activating NF-κB have become major targets for anticancer interventions.[6]

We have previously shown that stimulus-dependent activation of NF-κB can result in completely opposite effects. Stimulation with IL-1 protects keratinocytes and epithelial cells from cytotoxic effects of death ligands in an NF-κB-dependent manner by upregulation of anti-apoptotic cFLIP and cIAPs.[7] In contrast, stimulation with IL-1 and ultraviolet-B light (UVB) resulted in NF-κB-dependent downregulation of anti-apoptotic genes and simultaneous upregulation of pro-apoptotic TNF. Both events act in concert to enhance the predominantly intrinsic pathway triggered by UVB through additional activation of the extrinsic pathway by TNF-R1.[8] NF-κB-dependent repression of anti-apoptotic genes in response to DNA-damaging agents was shown to be facilitated by $p14^{ARF}$-induced association of p65 with the gene silencer histone deacetylase-1 (HDAC1) at different promoters,[9] turning NF-κB into a repressor of anti-apoptotic genes by ARF-induced ATR- and CHK1-dependent phosphorylation of p65.[10,11]

One proposed mechanism is that UVB drives the pro-apoptotic effect of NF-κB by general repression of anti-apoptotic genes. However, additional mechanisms exist, as NF-κB upon IL-1 + UVB co-treatment significantly enhances TNF expression compared to IL-1 alone. Although a mechanism underlying UV-mediated repression of NF-κB-regulated genes has been proposed, the mechanism by which UVB accelerates NF-κB-dependent TNF transcription remains unclear. Here we show that activation of *TNF* gene by UVB in IL-1-treated cells relies on complete inhibition of the

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Abbreviations: UVB, ultraviolet-B light; TNF, tumour necrosis factor-α; IL-1, interleukin-1; NF-κB, nuclear factor-κB; IκBα, inhibitor of κBα; IKKβ, inhibitor of κB kinaseβ
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negative feedback loop involving IκBα resynthesis, hence allowing uncontrolled NF-κB activity. Lack of IκBα production is not transcriptionally regulated but caused by persisting IKKβ activity, allowing continuous degradation of *de novo* synthesized IκBα. Enhanced activity of IKKβ is caused by UVB-mediated inhibition of phosphatase PP2A, a potential negative regulator of IKKβ.[12] This study identifies UVB to directly affect the negative feedback loop thereby changing the physiological properties of NF-κB. It also demonstrates that biologic effects induced by different NF-κB activators may tremendously differ when both act in concert.

Results

Co-stimulation with IL-1 inhibits TRAIL- but enhances UVB-induced apoptosis by NF-κB activation. Utilizing the epithelial cell line KB, co-stimulation with IL-1 was shown to desensitize cells from death receptor-induced apoptosis, whereas UVB-induced apoptosis was significantly enhanced due to NF-κB-dependent TNF production and downregulation of anti-apoptotic genes.[8] EMSA revealed that differential NF-κB responses (Figure 1a and c) were neither due to changes in intensity of activation nor to activation of alternative NF-κB activating pathways or to differences in heterodimer composition (Figure 1b). Accordingly, investigating the molecular mechanism underlying these differential cellular responses to NF-κB p50/p65 is of particular interest.

IL-1 enhances UVB-induced apoptosis by autocrine TNF-TNF-R1 signalling. Semiquantitative PCR analysis confirmed that enhanced TNF secretion upon IL-1 + UVB stimulation (Figure 1c) relies on sustained and elevated TNF transcription resulting in accumulation of TNF protein in supernatants over time, finally allowing autocrine TNF-R1 activation resulting in additive apoptotic signalling (Figure 2a).

TNF-R1-mediated apoptosis but not NF-κB-dependent, non-apoptotic responses depends on internalization of the receptor signalling complex.[13,14] Accordingly, FACS analysis revealed a non-apoptotic TNF dose (5 ng/ml) to not result in detectable loss of membrane TNF-R1 staining, whereas an apoptotic dose (100 ng/ml) caused a marked decrease of membrane TNF-R1 expression after 2 h, indicative of receptor internalization and induction of apoptotic pathways (Figure 2b). Thus, TNF-R1 internalization should precede IL-1 + UVB-induced induction of autocrine TNF-mediated apoptosis. UVB exposure alone produced a slow and only partial reduction in TNF-R1 membrane levels, whereas IL-1 + UVB caused a rapid loss of TNF-R1 at 2 h, which further decreased after 4 h (Figure 2b). Confirming the critical role of autocrine TNF signalling, IL-1-mediated enhancement of UVB-induced apoptosis was blocked with an antagonistic TNF-R1 antibody[15] (Figure 2c).

Data show that enhancement of TNF secretion is largely regulated by persistent transcription of *TNF* gene, suggesting sustained NF-κB activation.

Inhibition of IκBα reappearance is responsible for sustained NF-κB activity and elevated TNF production. IL-1 stimulation typically results in early, but transient NF-κB activation with maximum degradation of IκBα between 15 and 30 min. Rapid transcriptional activation of the IκBα gene and resynthesis of the protein result in replenishment of cytosolic IκBα after 1–2 h, finally terminating NF-κB activity.[3] This kinetics applies for KB cells upon co-treatment with IL-1 and TRAIL (Figure 3a). In contrast, co-stimulation with IL-1 + UVB completely abrogated reappearance of IκBα protein in the cytoplasm for at least 4 h (Figure 3a). At this time point cells are irreversibly committed to undergo apoptosis (data not shown). Correspondingly, EMSA revealed that high NF-κB levels persisted in the nucleus upon IL-1 + UVB co-treatment for several hours, whereas upon IL-1 treatment, NF-κB vanished from the nucleus with kinetics matching reappearance of IκBα in the cytoplasm (Figures 3a and b). This suggests that prolonged production of TNF is regulated at the level of IκBα turnover. Therefore, we mimicked inhibition of IκBα resynthesis by pre-incubation of cells with cycloheximide (CHX). At a concentration higher than 1 μg/ml, CHX completely inhibited IκBα *de novo* protein synthesis after 2 h (Figure 3c), when IκBα resynthesis is usually completed (Figure 3a). CHX-mediated inhibition of IκBα recurrence caused elevated TNF transcription in IL-1-treated cells even 4 h after stimulation. At this time point TNF transcription had almost ceased in the absence of CHX (Figure 3d) due to IκBα resynthesis and termination of NF-κB activity. These results support that inhibition of the negative feedback loop drives NF-κB-dependent TNF production.

Inhibition of IκBα reappearance is due to its immediate post-translational proteasomal degradation. As NF-κB can change from an activator to a repressor for selected genes,[8] loss of IκBα reappearance might be regulated at the transcriptional level. However, IL-1-mediated transcription pattern of IκBα remained unchanged upon co-stimulation with UVB (Figure 4a), suggesting that inhibition of IκBα reappearance is regulated post-transcriptionally, affecting either translation or protein stability.

Once designated by IKKβ-mediated phosphorylation IκBα is proteasomally degraded. Addition of proteasome inhibitor MG132 1 h before IL-1 or IL-1 + UVB stimulation prevented initial IκBα degradation. Adding MG132 15 min after stimulation and initial IκBα degradation, resynthesized IκBα reappeared within the cytoplasm (Figure 4b), indicating that translation was not impaired. Thus, failure to accumulate a critical level of IκBα sufficient to limit NF-κB activity appears to result from immediate and continuous proteasomal degradation of newly synthesized protein. To scrutinize whether immediate IκBα degradation follows conserved patterns or is due to any alternative mechanism the Ser32/36 phosphorylation status of newly synthesized IκBα captured in the cytoplasm by MG132 application was analysed. Corresponding to initial stimulation, resynthesized IκBα was phosphorylated at Ser32/36 marking it for degradation and causing sustained NF-κB activation (Figure 4c).

Assessing the role of IKKβ in continuous IκBα degradation, blocking its activity by the selective inhibitor BAY-11-7082 resulted in reappearance of IκBα in IL-1 + UVB-treated cells (Figure 4d), strongly suggesting constitutive IKKβ activation to be responsible for controlling IκBα reappearance. As IKKβ requires phosphorylation at Ser177/181 for activation,[2] we monitored IKKβ phosphorylation status (Figure 4e). Initial

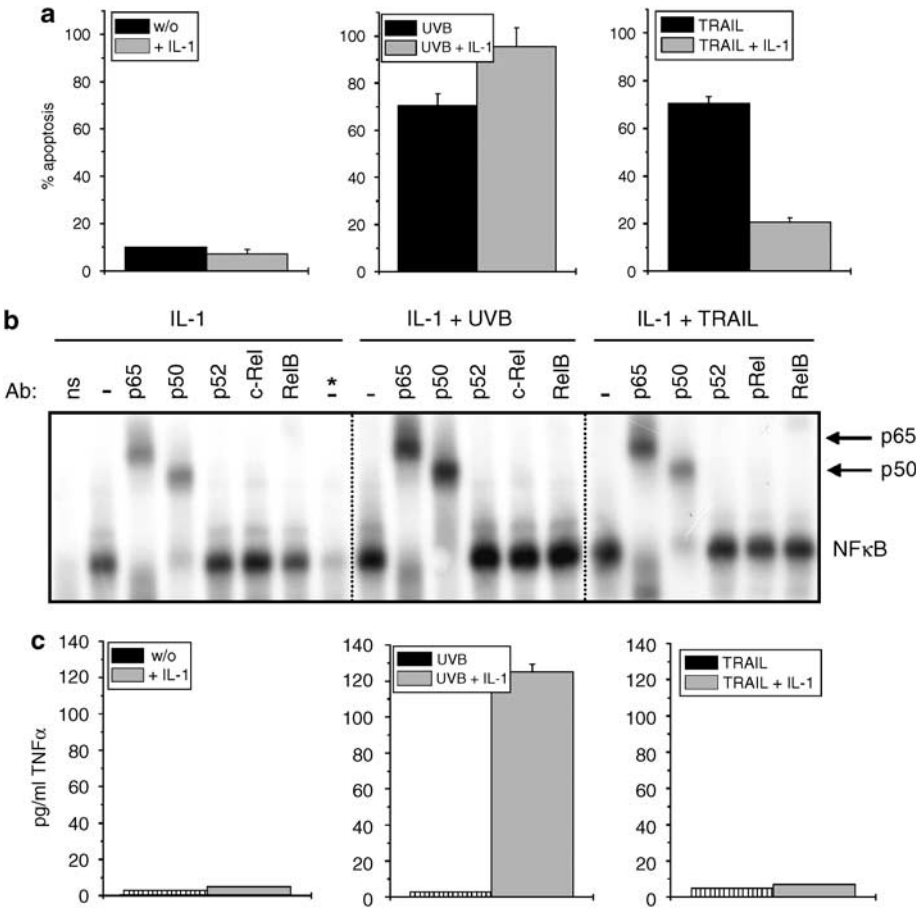

Figure 1 Interleukin-1 (IL-1) causes activation of the p65/p50 irrespective of the apoptotic co-stimulus. IL-1-induced enhancement of ultraviolet-B light (UVB)-induced apoptosis coincides with tumour necrosis factor-α (TNF) production. Cells were stimulated with IL-1, UVB, TRAIL, or co-stimulated with IL-1 and any of the apoptotic stimuli. (**a**) After 16 h apoptosis was determined in a cell death detection (CDD). (**b**) After identical stimulation for 30 min nuclear translocation of nuclear factor-κB (NF-κB) was analysed by EMSA and heterodimer composition in a supershift assay using antibodies against p65, p50, p52, cRel and RelB. ns, non-stimulated cells; *, UVB irradiation alone. (**c**) TNF in supernatants of cells analysed in (a) was determined in a TNF ELISA

IκBα degradation coincided with strong IKKβ Ser177/181 phosphorylation, irrespective of co-stimulation with UVB, indicating IL-1 to be the predominant trigger. IKKβ phosphorylation stayed stable for up to 1 h. After 2 h of IL-1 stimulation, IKKβ phosphorylation had completely vanished allowing stabilization of resynthesized IκBα. In contrast, IKKβ remained phosphorylated in cells co-stimulated with IL-1 + UVB after 2 h, preventing IκBα resynthesis. *In vitro* kinase assay with IKKβ immunoprecipitated from cells 2 h after co-stimulation with IL-1 + UVB, using a purified IκBα(5–55) fragment confirmed high IKKβ activity at this time point (Figure 4f). Together, these data demonstrate that phosphorylation of resynthesized IκBα upon stimulation with IL-1 + UVB for 2 h is a direct downstream effect of continuous IKKβ phosphorylation and activity.

Cooperation of IL-1 and UVB signalling is a prerequisite for enhanced apoptosis. Inhibition of IκBα reappearance seems to be the molecular trigger efficiently enhancing UVB-induced apoptosis. As UV may induce NF-κB activation itself,[3,16] prolonged activation might result from additive IκBα degradation following the transient IL-1 signal. Investigating the kinetics of IκBα degradation and NF-κB activity at 300 J/m^2 UVB used in this study, UVB alone caused a rather weak and delayed NF-κB response after 10–16 h, coinciding with only partial IκBα degradation (Figure 5a). However, at 2 h neither IκBα degradation nor NF-κB translocation into the nucleus was detected, suggesting cooperative signalling.

Hence, we investigated the time window of cooperative signalling required to convert IL-1 signalling to pro-apoptosis by UVB. Cells pre-exposed for various length of time (15 min to 8 h) to IL-1 were irradiated with UVB. Alternatively, IL-1 was added after irradiation for identical time intervals (15 min to 8 h) and apoptosis measured 16 h following UVB. IL-1-mediated enhancement of apoptosis was recorded in a narrow window around UVB treatment, ranging from 30 min before to 1 h after irradiation (Figure 5b, upper panel) and coincided with pronounced TNF release (Figure 5b, lower panel). This confirms the close interrelationship between TNF production and IL-1-mediated acceleration of UVB-induced apoptosis, revealing critical temporal constraints of cooperative signalling.

To translate tight co-stimulation to interference with IκBα reappearance, cells were stimulated with IL-1 and harvested after 2 h, when cytosolic IκBα levels have regained pre-

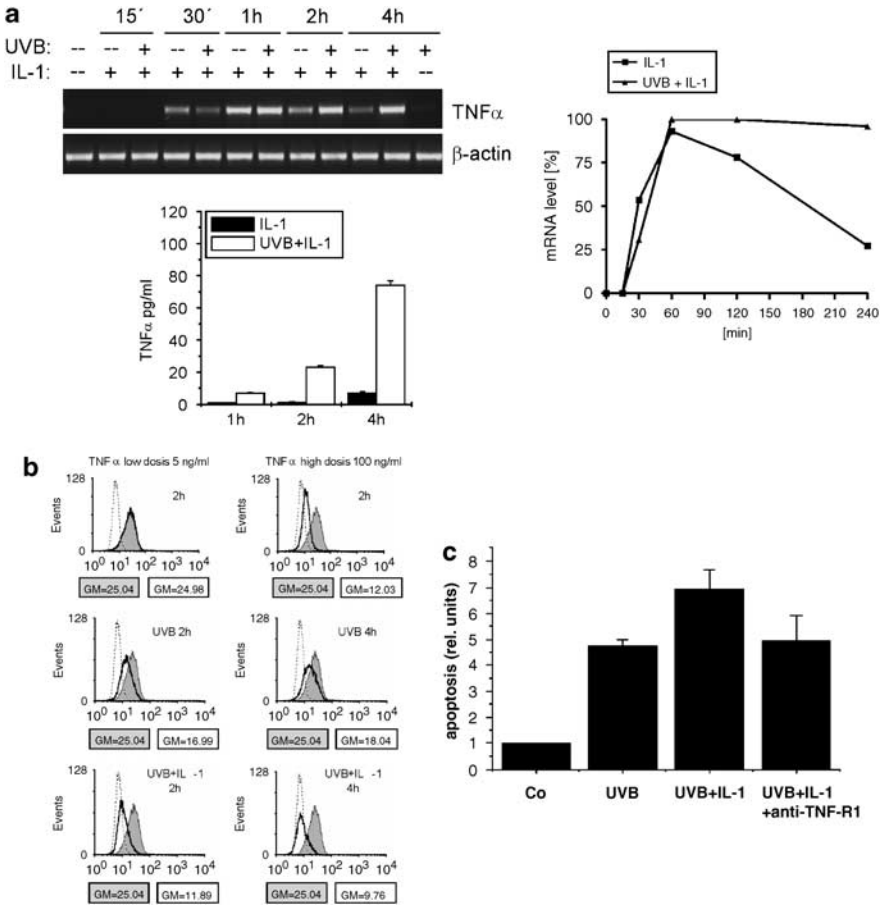

Figure 2 Sustained transcription upon ultraviolet-B light (UVB) + interleukin-1 (IL-1) causes release of tumour necrosis factor-α (TNF) and autocrine activation of TNF-R. (**a**) Cells were stimulated with IL-1 or in combination with UVB as denoted. TNF transcription was determined by semiquantitative RT-PCR and analysed densitometrically. Release of TNF to supernatants of the cells was determined in a TNF ELISA. (**b**) Cells were exposed to UVB, co-treated with IL-1 + UVB or stimulated with 5 ng/ml or 100 ng/ml TNF for 2 h. Cell-surface expression of TNF-R1 was determined by FACS analysis. Dashed line presents isotype control. Grey-shaded curve shows basic TNF-R1 expression (projected into every histogram) and the bold line TNF-R1 expression of treated cells. (**c**) Cells were stimulated with UVB or UVB + IL-1 with or without pre-incubation for 1 h with an antagonistic TNF-R1 antibody (H-398, 20 μg/ml). After 16 h, apoptosis was measured by cell death detection (CDD)-ELISA

stimulation levels. In addition, cells were UVB irradiated in intervals from 5 min up to 120 min prior to harvest (Figure 5c). WB analysis revealed that IκBα reappearance was only inhibited when the time span between IL-1 and UVB stimulation did not exceed 30 min, closely matching the temporal constraints for TNF production and apoptosis (Figure 5b).

UVB-induced inhibition of PP2A is responsible for persistent IKKβ activity. We next examined how IKKβ phosphorylation is maintained in IL-1 + UVB co-treated cells. Screening for IKKβ upstream targets that might become either activated or inhibited by UVB, thereby causing chronic IKKβ phosphorylation, revealed that chemical inhibition or siRNA knockdown of potential IKKβ activators (in particular TAB1/TAK, JNK, RIP1, MAPK, AKT, PKD, PKCδ, PI3K, CK II, DNA-PK (including ATM and ATR) IKKγ and p38) as well as ROS scavenging (PDTC, GSH, NAC, Trolox), IKKγ Ser85Ala mutation and overexpression of inhibitors CYLD and A20 all failed to allow IκBα reappearance in IL-1 + UVB-treated cells (see Supplementary Figure S1).

Therefore it seemed that IL-1 + UVB cooperate to maintain IKKβ activity by inhibiting its dephosphorylation. So far inactivation[12] as well as activation[17] of IKKβ have been described to be mediated by the Ser/Thr-phosphatase PP2A *in vitro*. PP2A is a multi-subunit protein with PP2Ac being the catalytic subunit. Indeed, co-immunoprecipitation revealed that endogenous PP2Ac was constitutively associated with IKKβ, proposing a functional relationship (Figure 6a). To elucidate the role of PP2A as a potential negative regulator of IKKβ and to mimic the assumed UVB-mediated inactivation, the effect on IκBα degradation in IL-1-treated cells was studied upon specific inhibition of PP2Ac by calyculin A and/or siRNA-mediated knockdown. The results fully confirm the critical role of PP2A as a negative regulator of IKKβ. Each, knockdown of PP2Ac and specific inhibition resulted in persistent IKKβ phosphorylation and IκBα degradation upon IL-1 stimulation, with combined treatment producing the strongest effect (Figure 6b). *In vitro* kinase assay with PP2Ac knockdown cells confirmed that IKKβ in IL-1-treated cells induced a persistent IκBα phosphorylation (Figure 6c). Furthermore, knockdown of PP2Ac allowed a sustained TNF production at the mRNA and the protein level

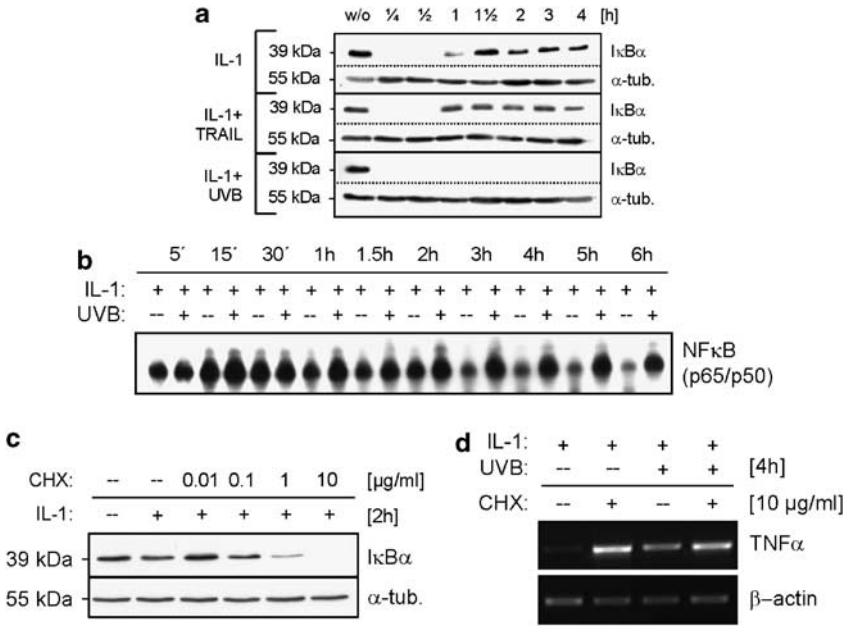

Figure 3 Sustained tumour necrosis factor-α (TNF) transcription is due to lack of IκBα resynthesis. (**a**) Cells were stimulated with interleukin-1 (IL-1), or in combination with either ultraviolet-B light (UVB) or TRAIL. Cytosolic protein extracts were analysed for IκBα degradation and resynthesis by WB. (**b**) Cells were treated with IL-1 or co-stimulated with IL-1 + UVB as indicated. Nuclear localization of nuclear factor-κB (NF-κB) was monitored by EMSA. (**c**) Cells were pre-incubated with cycloheximide (CHX) for 1 h and stimulated with IL-1. After 2 h, the IκBα status was documented by WB. (**d**) Cells were stimulated with IL-1 with or without UVB for 4 h. Resynthesis of IκBα protein was blocked by pre-treatment with CHX (10 μg/ml) for 1 h. Transcription efficacy of TNF was determined by semiquantitative RT-PCR

(Figures 6d and e), supporting the functional relationship between cooperative inhibition of PP2Ac and TNF production finally leading to enhancement of apoptosis.

Finally, we investigated the effect of UVB on PP2Ac activity. *Ex vivo* irradiation of whole-cell lysates with UVB followed by an *in vitro* phosphatase assay with a threonine phosphopeptide revealed overall phosphatase activity to be reduced to approximately 80% of untreated controls (Figure 6f). Phosphatase activity of PP2Ac immunoprecipitated from UVB-exposed cells was found to be strongly reduced to about 35% of the activity in control cells, supporting the concept that continuous IKKβ phosphorylation and subsequent IκBα degradation in IL-1 + UVB-treated cells is a result of PP2Ac inhibition. Conclusively, our data reveal two major findings: First they identify PP2Ac to be a novel cellular target of UVB and second they unravel chronic NF-κB activation to be involved in cell killing instead of cell survival. Consequently, combination of PP2Ac inhibition and DNA-damaging chemotherapeutic drugs may have broader implications in cancer therapy.

Discussion

UVB radiation may serve as a carcinogen by activation of skin oncogenes or inactivation of tumour suppressors and by repression of cell-based immune responses that are generally able to eliminate highly antigenic skin tumours.[18] In contrast, UVB-induced DNA damage is a prerequisite for execution of apoptosis, leading to elimination of cancer-prone cells, thereby protecting the skin from keratinocyte transformation.[19,20] The molecular switch, however, has not yet been identified. It has long been known that human keratinocytes are a potent source of IL-1[21] that becomes activated and released in response to UVB.[22,23] In this respect keratinocytes serve an important role in the induction of skin immune responses under physiological and pathological conditions like sunburn and other inflammatory skin diseases.[22,24] According to our findings, UVB-induced apoptosis of keratinocytes and epithelial cells is significantly enhanced upon co-treatment with IL-1 *in vitro*.[7,8,25] Correspondingly, UVB-induced apoptosis of keratinocytes *in vivo* may be facilitated by concomitant IL-1 signals from neighbouring cells. We therefore propose that the signal cross talk at the level of keratinocytes is of patho/physiological relevance in differential skin responses to UVB.

In this study we shed light on the molecular mechanism underlying the paradoxical finding that IL-1-mediated NF-κB activation causes acceleration of UVB-induced apoptosis. Our findings account for sustained transcriptional upregulation of TNF to be a prerequisite for TNF secretion and autocrine activation of TNF-R1 to additively enhance UVB-induced apoptosis, corroborating previous findings.[7,8] Opposing effects of TNF coincide with compartmentalization of activated TNF-R1 complexes.[13,14] TNF-R1 mediates a pro-apoptotic response only after internalization of the complex, whereas membrane-anchored TNF-R1 generates NF-κB activation.[14] The observed TNF-R1 internalization upon IL-1 + UVB treatment is in full accordance with a predominating apoptotic response initiated by autocrine TNF. UVB alone also induced TNF-R1 internalization, however with a different kinetics and to a lesser extent, which might result from the ability of UV to induce ligand-independent activation of death receptors.[26,27]

We show that initial IL-1-governed degradation of IκBα is required for NF-κB activation. Upon co-stimulation with UVB

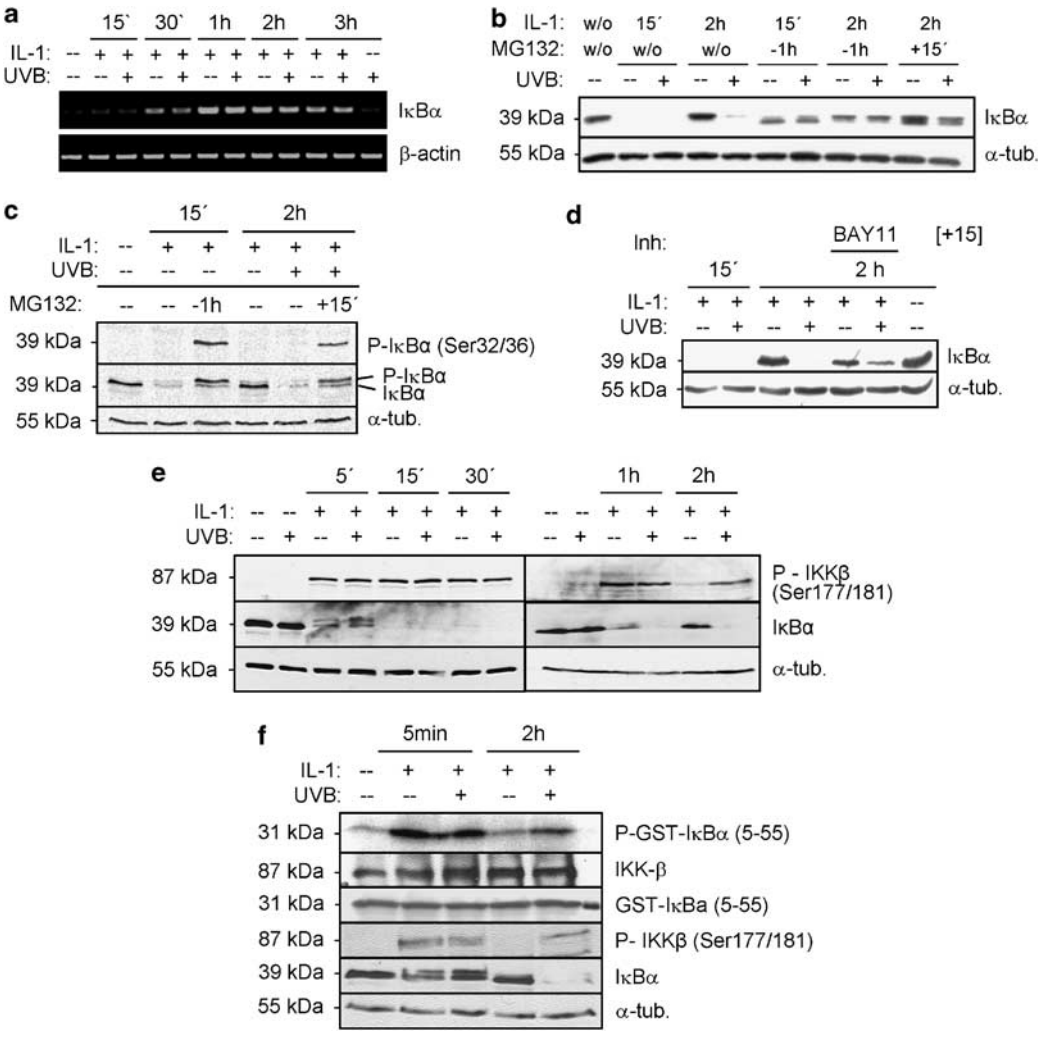

Figure 4 Failure of IκBα resynthesis is due to continuous inhibitor of κB kinaseβ (IKKβ) phosphorylation. (**a**) Cells were stimulated with interleukin-1 (IL-1) or IL-1 + ultraviolet-B light (UVB). At the indicated time points IκBα transcription was analysed by semiquantitative RT-PCR. (**b**) Cells were stimulated with IL-1 or co-stimulated with IL-1 + UVB. MG132 was added 1 h prior to (−) or 15 min after (+) IL-1/UVB stimulation. Cytosolic IκBα status was determined by WB. (**c**) Cells were stimulated with IL-1 or IL-1 + UVB. MG132 was added 1 h prior to (−) or 15 min after (+) IL-1/UVB stimulation. Specific Ser-Phosphorylation of IκBα was monitored and confirmed by reprobing the stripped membrane with an IκBα antibody. (**d**) Cells were stimulated with IL-1 or IL-1 + UVB as indicated. BAY-11-7082 (30 μM) was added 15 min following initial IL-1/UVB stimulation. Resynthesis of IκBα was detected by WB. (**e**) Cells were stimulated with IL-1 with or without UVB as annotated and the phosphorylation status of IKKβ as well as IκBα degradation determined by WB. (**f**) Cells stably expressing IKKβ-GFP were stimulated with IL-1 or IL-1 + UVB for 5 min or 2 h. IKKβ-GFP was immunoprecipitated and subjected to an *in vitro* kinase assay with a purified GST-IκBα(5–55) peptide. IκBα, and phospho-IKKβ statuses were determined by WB

slowly increasing amounts of resynthesized IκBα protein continuously become degraded due to constitutive IKKβ phosphorylation caused by UVB-induced inhibition of its negative regulator PP2Ac. Through this mechanism the negative feedback loop for NF-κB is abolished resulting in sustained NF-κB activation. Under these specific conditions NF-κB persists to upregulate TNF and to repress anti-apoptotic genes finally producing pro-apoptotic effects (Figures 7a and b).

As TNF is not a strong inducer of apoptosis but of NF-κB-mediated survival signals in epithelial cell lines, downregulation of anti-apoptotic proteins (FLIP, cIAP) and signal transducers (TRAF 1, -2, -6) complement to trigger apoptotic pathways under the given conditions.[8] Our findings account for selective transcriptional upregulation of TNF as a prerequisite for TNF secretion and autocrine TNF-R1 activation.

NF-κB activity could escape termination upon IL-1 + UVB stimulation due to lack of IκBα resynthesis. The IκBα gene itself is regulated by NF-κB and post-degradational *de novo* synthesis of IκBα serves as a negative feedback regulation terminating NF-κB.[28] Consequently, failure of IκBα resynthesis coincided with prolonged nuclear localization of NF-κB. Sustained NF-κB activity seems to account for elevated TNF transcription, as inhibition of IκBα protein synthesis with CHX after initial degradation resulted in increased TNF mRNA expression upon IL-1 stimulation.

Unlike the TNF transcription pattern, the IL-1-dependent IκBα transcription profile remained unchanged in UVB-irradiated cells. Peculiarly, immediate post-translational degradation of resynthesized IκBα was found to cause lack of its cytosolic reappearance. UVC and UVB alone can induce NF-κB activation, but no common mechanism became

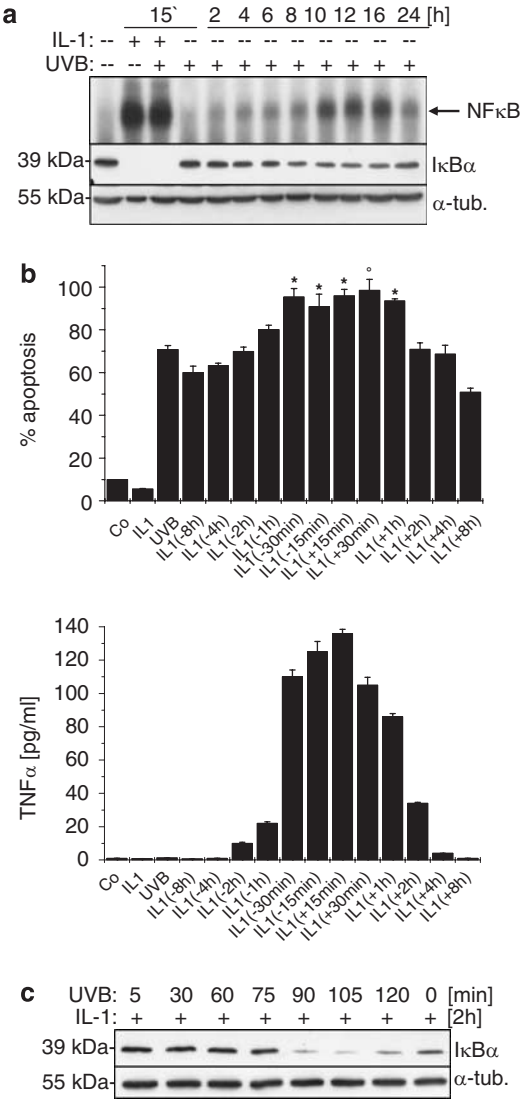

Figure 5 Cooperation of interleukin-1 (IL-1) and ultraviolet-B light (UVB) affects apoptosis, tumour necrosis factor-α (TNF) production and inhibition of IκBα resynthesis. (**a**) Cells were stimulated with IL-1, UVB or with IL-1 + UVB for 15 min. Nuclear translocation of nuclear factor-κB (NF-κB) was determined by EMSA. In parallel, corresponding cytosolic IκBα status was determined by WB. (**b**) Cells were stimulated with UVB and co-treated with IL-1 for 8, 4, 2, 1 h, 30 min, 15 min before (−) as well as after (+) UVB exposure. At 16 h after UVB exposure apoptosis was measured by cell death detection (CDD)-ELISA and TNF secretion analysed in a TNF ELISA. $^{*}P<0.05$; $^{\circ}P<0.01$. (**c**) Cells were treated with IL-1 for 2 h and exposed to UVB at the indicated time points prior to cell harvest. Cytosolic IκBα status was determined by WB

apparent. IKK and IκBα degradation-dependent and -independent mechanisms have been described and the time courses vary remarkably.[16,28,29] In our hands, UVB-alone-triggered NF-κB activation only strongly delayed and was accompanied by only partial degradation of IκBα, excluding the effect of IL-1 + UVB-mediated instability of resynthesized IκBα to be just additive.

Our kinetic studies identified a critical time window (30 min before until 1 h after UVB) of co-stimulation to be essential for the enhancing effect of IL-1 on UVB-induced TNF secretion and apoptosis. Identical kinetics applied for IκBα degradation, revealing that the temporal constraints of this cooperative signalling affect IκBα protein levels.

HDAC inhibitor trichostatin A was reported to potentiate TNF-mediated activation of several NF-κB-driven genes, being associated with delayed reappearance of IκBα and prolonged IKK activity.[30] Enhanced proteasomal degradation of IκBα in our setting also appeared to be linked to signals upstream of its phosphorylation, because it could be counteracted by inhibition of IKKβ. Correspondingly, resynthesized IκBα captured by proteasome inhibition after IL-1 + UVB treatment displayed phosphorylation of Ser32/36 residues. This implied that IκBα degradation was not due to alternative, possibly UVB-induced, cleavage of IκBα, and was consolidated by the observation that stability of IκBα Ser32/36Ala mutants remained unaffected by IL-1 + UVB (Supplementary Figure S2). Kinetic analysis finally revealed that IKKβ dephosphorylation at Ser177/181 in IL-1-treated cells fully matched reappearance of IκBα in the cytoplasm after 2 h. In contrast, IKKβ remained phosphorylated in cells treated with IL-1 + UVB, thereby preventing IκBα resynthesis.

Data allowed two interpretations: either UVB activates an upstream kinase that constitutively phosphorylates IKKβ or it inactivates a phosphatase, thereby preventing its dephosphorylation. PP2A is known to modulate NF-κB activity,[31] its precise role in NF-κB-dependent cellular responses, however, remains controversial. Although IKK–PP2A complex formation was suggested to be required for TNF-induced IKKβ phosphorylation and IκBα degradation,[17] more evidence exists connecting inhibition of PP2A to NF-κB activation: PP2A inhibition with calyculin A or ocadaic acid both resulted in IκBα phosphorylation and proteasomal degradation without additional cytokine treatment.[32,33] Correspondingly, ocadaic acid treatment of monocytes caused IL-1 production linking PP2A inhibition to NF-κB-dependent responses.[34] Moreover, PP2A was shown to interact with IKKγ to downregulate activation of IKKβ following TNF treatment.[35,36] We show that the catalytic subunit PP2Ac associates with IKKβ. Furthermore, siRNA-mediated knockdown of PP2Ac and specific inhibition by calyculin A resulted in degradation of IκBα 2 h after IL-1 stimulation *in vivo* and *in vitro*, in both cases coinciding with chronic IKKβ phosphorylation, mimicking the cellular response to IL-1 + UVB. Stimulus-dependent recruitment of PP2A to IKK can be ruled out, as endogenous PP2Ac co-precipitated with IKKβ even in unstimulated cells. But, irradiation of cell lysates with UVB reduced total cellular phosphatase activity by ∼20%, indicating that UVB generally affects phosphatase activity. However, the majority of phosphatases remained unaffected, provided by the fact that the substrate used was not PP2A specific, but can be processed by other phosphatases present in cell lysates. Yet, immunoprecipitated PP2Ac proved to be particularly sensitive to UVB, resulting in ∼65% inhibition of its catalytic activity.

Suppression of PP2A was shown to cooperate with oncogenic changes to promote cell transformation.[37] Suppression of PP2A in IL-1-treated cells presumably could also result in tumour progression due to constitutive NF-κB activation. In combination with UVB, however, NF-κB promotes pro-apoptotic pathways and may exert completely different effects on tumour growth.

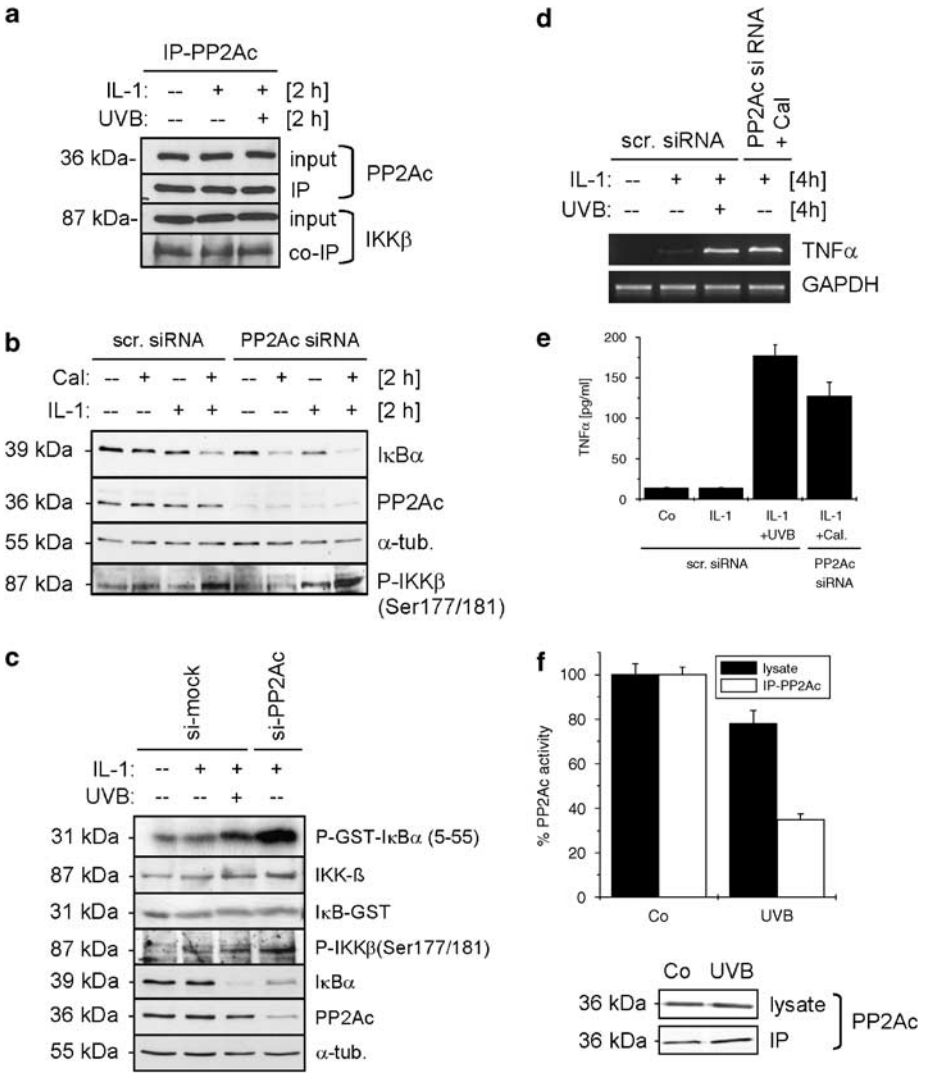

Figure 6 Loss of PP2Ac results in constitutive activation of inhibitor of κB kinaseβ (IKKβ) and production of tumour necrosis factor-α (TNF). (**a**) KB cells were stimulated with interleukin-1 (IL-1) or IL-1 + ultraviolet-B light (UVB) for 2 h. PP2Ac was immunoprecipitated and co-precipitation of IKKβ is shown by WB. (**b**) Cells were transfected with scrambled- or PP2Ac-siRNA and stimulated with IL-1 and/or calyculin A (5 nM). After 2 h cytosolic IκBα and phospho-IKKβ statuses as well as the PP2Ac knockdown were documented by WB. (**c**) Cells stably expressing IKKβ-GFP were transfected with scrambled or PP2Ac-siRNA and stimulated with IL-1 or UVB + IL-1 for 2 h. IKKβ-GFP was immunoprecipitated and subjected to an *in vitro* kinase assay with a purified GST-IκBα(5–55) peptide. IκBα and phospho-IKKβ status were determined by WB. (**d**) Cells were transfected with scrambled- or PP2Ac-siRNA and stimulated with IL-1 and/or calyculin A (5 nM) and stimulated as indicated. After 4 h semiquantitative RT-PCR was performed to determine the efficacy of TNF transcription. (**e**) Cells were treated as in (**d**). After 16 h TNF secretion was measured in a TNF ELISA. (**f**) Whole-cell lysates were left untreated or irradiated *ex vivo* with UVB. In parallel cells were mock treated or irradiated with UVB and PP2Ac was immunoprecipitated. Lysates and precipitates were subjected to an *in vitro* phosphatase assay using a threonine phosphopeptide as a substrate

In conclusion, the present study attributes a crucial role to PP2A in maintaining the negative feedback loop terminating NF-κB. In addition, we unravel a novel mechanism by which persistent NF-κB activation plays an unexpected role as a pro-apoptotic mediator and is a vivid example for the importance of signalling contexts for overall cellular responses.

Because of NF-κB's crucial role in physiology and pathophysiology, NF-κB-activating pathways are in the focus of therapeutic interventions.[38] Combinations of targeted therapies with radio- and chemotherapeutics are in progress to advance the treatment of cancer and inflammatory diseases.[38,39] Therefore, an integrated understanding of cellular responses to NF-κB appears mandatory. This requires knowledge about the context-dependent behaviour of NF-κB-triggered signalling pathways and molecules to pinpoint suitable targets, like PP2Ac, to improve therapeutic strategies.

Materials and Methods

Unless otherwise stated, results of CDD- and TNFα-ELISA are presented as mean ± S.D. of three independently performed experiments. For statistical analysis Student's *t*-test was performed. WB analysis, EMSAs, *in vitro* kinase assays and RT-PCR analysis shown represent one out of three independently performed experiments. For details see Supplemental Data.

Cells and reagents. The human epithelial carcinoma cell line KB (ATCC) was cultured in RPMI 1640, 10% FCS. Subconfluent cells were stimulated in colourless

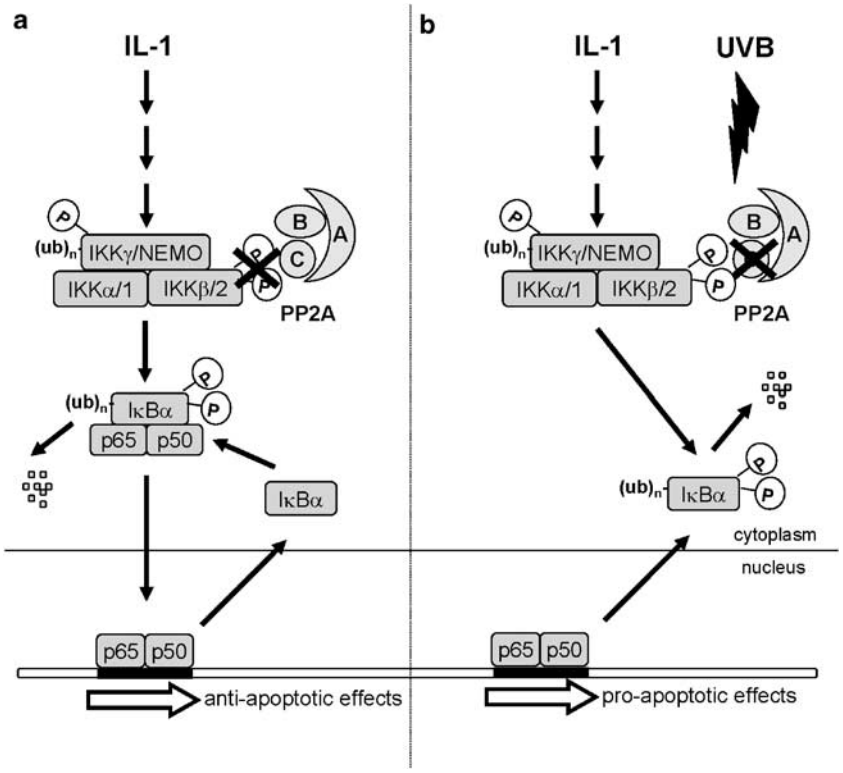

Figure 7 Scheme of interleukin-1 (IL-1) + ultraviolet-B light (UVB)-induced interruption of the negative feedback loop of nuclear factor-κB (NF-κB). (**a**) Upon IL-1 stimulation inhibitor of κB kinaseβ (IKKβ) becomes phosphorylated at Ser 177/181 followed by subsequent phosphorylation of IκBα at Ser 32/36 causing its proteasomal degradation. Released NF-κB translocates into the nucleus to mediate resynthesis of IκBα. In parallel, PP2A dephosphorylates IKKβ that allows for stabilization of resynthesized IκBα within the cytoplasm. (**b**) In case of co-stimulation with UVB, initial IκBα degradation remains unaffected but UVB-mediated inactivation of PP2Ac results in continuous phosphorylation and activation of IKKβ. Consequently, persistent phoshorylation and degradation of IκBα disrupts the negative feedback loop of NF-κB. Prolonged NF-κB activation in this specific physiological context triggers enhancement of apoptosis by repression of anti-apoptotic genes and continuous and elevated expression of tumour necrosis factor-α (TNF)

medium with 2% FCS. UVB irradiation (300 J/m^2) was performed with TL12 fluorescent bulbs (290–320 nm; Philips). Apoptosis was triggered with 80 ng/ml lz-TRAIL.[40] Recombinant human IL-1β (R&D Systems) was applied at 10 ng/ml. Transfection of cells with siRNA (MWG) was carried out in Lipofectamin2000 (Invitrogen). For semiquantitative RT-PCR, cDNA was generated as described before[25] using the RedTaq system (Sigma).

Proteasome inhibition was achieved with MG132 (25 μM; Merck). IKKβ-inhibitor BAY-11-7082 was applied at 30 μM (Merck). PP2Ac inhibitor calyculin A (Cell Signaling) was added at 5 nM. TNF was measured with a TNF ELISA (BioSource), apoptosis with a Cell Death Detection (CDD)-ELISA (Roche). The enrichment of nucleosomes released into the cytoplasm by factor 2 corresponds to 10% apoptotic cells as determined by AnnexinV FACS analysis.

EMSA and supershift analysis. Following stimulation cells were harvested and nuclear extracts were generated as described before.[25] EMSA was performed using an NF-κB consensus oligonucleotide (sc-2505; Santa Cruz). For supershift assays the following antibodies recognizing p65, p50, p52, c-Rel and RelB were used: sc-109X, sc-7178X, sc-7386, sc-70X, sc-226X (Santa Cruz).

Immunoprecipitation, WB analysis and *in vitro* kinase assay. Cells were lysed in lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1.5 mM $MgCl_2$; 1 mM EGTA; 100 mM NaF; 10 mM pyrophosphate, 0.01% NaN_3 and Complete® protease inhibitor cocktail (Roche) for 15 min on ice. Endogenous PP2Ac was immunoprecipitated using a specific antibody (no. 05-421, clone 1D6; Upstate) and A/G-plus agarose (Santa Cruz) overnight. Precipitates were analysed by WB using antibodies against PP2Ac and IKKβ (10A9B6; Imgenex). For WB analysis cytosolic and nuclear protein extracts were detected with antibodies against IκB-α, P-IκBα-Ser32/36, P-IKKβ-Ser177/181, PP2Ac (L35A5, 5A5, 16A6, no. 2038; Cell Signaling), IKKβ (10A9B6; Imgenex) and α-tubulin (DM1A; Neomarkers). For kinase assay immunoprecipitation of IKKβ-YFP was carried out as above. GST-IκBα (5-55) was purified with GSH-sepharose-4B (Amersham) and incubated with IKKβ and [^{32}P]-γ-ATP in kinase buffer and analysed by SDS-PAGE and autoradiography.

FACS analysis. To monitor membrane-bound TNF-R1, FACS analysis was performed as described before[25] using 1 μg of anti-TNFα T1 mouse IgG1 (H398) per 2×10^5 cells and an goat anti-mouse IgG conjugated to Phycoerythrin (Santa Cruz). Cells were analysed in FACSCalibur Flow Cytometer (Becton Dickinson) and using WinMDI 2.8 software.

Phosphatase assay. Cell lysates (10 μg) or immunoprecipitated PP2Ac were diluted in 74 μl phosphatase assay buffer (50 mM Tris-HCl, pH 7.0; 100 μM $CaCl_2$) and incubated with 6 μl threonine phosphopeptide (final concentration 75 μM) for 5 min at 30 °C. Malachite green solution (20 μl) (BioAssay Systems) was added and absorption measured at different time points at 650 nm. Phosphatase activity of unirradiated cells was determined to be 100%. As an assay standard a serial dilution of 40 μM phosphate (BioAssay Systems) was used.

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