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Activation or suppression of NFκB by HPK1 determines sensitivity to activation-induced cell death

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Restimulation of the T-cell receptor (TCR) in activated T cells induces CD95 (Fas/Apo-1)-mediated activation-induced cell death (AICD). The TCR-proximal mechanisms leading to AICD are elusive. Here we characterize hematopoietic progenitor kinase 1 (HPK1) as a differentially regulated TCR-proximal signaling protein involved in AICD of primary T cells. We show that HPK1 is a functional component of the endogenous IκB kinase (IKK) complex and is crucial for TCR-mediated NFκB activation. While full-length HPK1 enhances IKKβ phosphorylation, siRNA-mediated knockdown of HPK1 blunts TCR-mediated NFκB activation and increases cell death. We also demonstrate proteolytic processing of HPK1 into HPK1-C, specifically in AICD-sensitive primary T cells. The cleavage product HPK1-C sequesters the inactive IKK complex and suppresses NFκB upon TCR restimulation by binding to IKKα and IKKβ. T cells of HPK1-C transgenic mice are sensitized towards TCR-mediated AICD. Consequently, preventing HPK1-C generation in primary T cells by siRNA-mediated knockdown results in decreased AICD. Thus, these results show a novel mechanism of sensitization of T lymphocytes towards AICD by suppression of NFκB, and propose that HPK1 is a life/death switch in T lymphocytes.

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Introduction

Life and death of peripheral lymphocytes is strictly controlled to maintain physiological levels of T and B cells. The regulation of T-cell apoptosis during the immune response is controlled by activation-induced cell death (AICD) in response to T-cell receptor (TCR) triggering (reviewed in Green, 2003; Krueger *et al*, 2003). While initial stimulation of primary T cells from peripheral blood leads to proliferation, restimulation of expanded T cells results in AICD (Brunner *et al*, 1995; Dhein *et al*, 1995). Therefore, primary T cells shift from AICD resistance towards AICD sensitivity. In contrast to the well-documented role for death receptors (Zheng *et al*, 1995; Li-Weber and Krammer, 2003; Peter and Krammer, 2003) and mitochondrial pathways (Hildeman *et al*, 2002) in sensitization to AICD, little is known about the contribution of TCR-proximal signaling proteins, which modulate the TCR signal in a way that it induces activation and survival or death by AICD.

Regulation of lymphocyte fate and the execution of immune functions are connected to transcription factors of the NFκB family (Hildeman *et al*, 2002; Karin and Lin, 2002; Ruland and Mak, 2003). NFκB/Rel proteins determine life and death decisions in developing lymphocytes, immune responses and cell growth, and provide important signals in T cells to ensure cell survival (Ghosh and Karin, 2002; Kane *et al*, 2002; Schmitz *et al*, 2003). The inhibition of NFκB is considered to fulfill an important proapoptotic function (Pham *et al*, 2004; Kamata *et al*, 2005). NFκB family transcription factors are rendered inactive within the cytoplasm by interaction with IκB proteins. Upon TCR stimulation, a high-molecular-weight IκB kinase (IKK) complex is activated. The IKK complex is comprised of two enzymatic subunits IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). Activation of the IKK complex results in phosphorylation of the NFκB-inhibitory IκB proteins mediated by IKKβ and subsequent ubiquitination and degradation of IκB proteins. Derepressed NFκB dimers translocate into the nucleus and activate NFκB-regulated genes (Hayden and Ghosh, 2004).

Hematopoietic progenitor kinase 1 (HPK1) is comprised of a N-terminally located kinase domain and a C-terminally located regulatory domain, called citron homology domain. By ectopic expression in epithelial cells, full-length HPK1 is rendered active and selectively activates the SAPK/JNK and the NFκB pathways (Kiefer *et al*, 1996; Arnold *et al*, 2001). *In vitro*, the N-terminal kinase domain of HPK1 can be separated from the citron homology domain by a caspase-3 activity, resulting in suppression of NFκB (Arnold *et al*, 2001). HPK1 kinase activity is strongly enhanced by TCR crosslinking (Liou *et al*, 2000; Liu *et al*, 2000) and involves phosphorylation by protein kinase D1 (Arnold *et al*, 2005).

Here we show that HPK1 is a differentially regulated TCR-proximal signaling protein proteolytically processed into the C-terminal cleavage fragment, HPK1-C, in expanded AICD-sensitive primary T cells. HPK1-C suppresses NFκB and sensitizes T cells towards TCR-mediated cell death. For full length HPK1, we show a novel association to the endogenous IKK complex and a crucial role in TCR-mediated IKKβ activation. In contrast, HPK1-C leads to suppression of NFκB by sequestering the IKK complex. T cells of HPK1-C transgenic (tg) mice show a suppressed TCR-mediated IKK activity and are sensitized towards AICD. Furthermore, siRNA-mediated knockdown of HPK1 in Jurkat or in primary naive T cells

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results in enhanced AICD, whereas preventing the generation of proapoptotic HPK1-C in primary preactivated (day 6) T cells results in decreased AICD.

Results

AICD-sensitive primary T cells show conversion of HPK1 into the C-terminal cleavage fragment HPK1-C

We performed a microarray-based screen to identify differentially expressed molecules modulating TCR signaling. We compared AICD-resistant to AICD-sensitive primary human T cells and found upregulation of HPK1 specifically in AICD-sensitive cells. Increased expression of HPK1 was confirmed by real-time RT–PCR (Figure 1A) and by Western blotting (WB) (Figure 1B, upper panel). Upon activation and expansion, cultured T cells shift from an AICD-resistant to an AICD-sensitive phenotype (Peter *et al*, 1997). Interestingly, we detected conversion of full-length HPK1 into the C-terminal cleavage fragment, HPK1-C, at day 6 of culture, when T cells show the highest sensitivity towards AICD (Figure 1B, lower

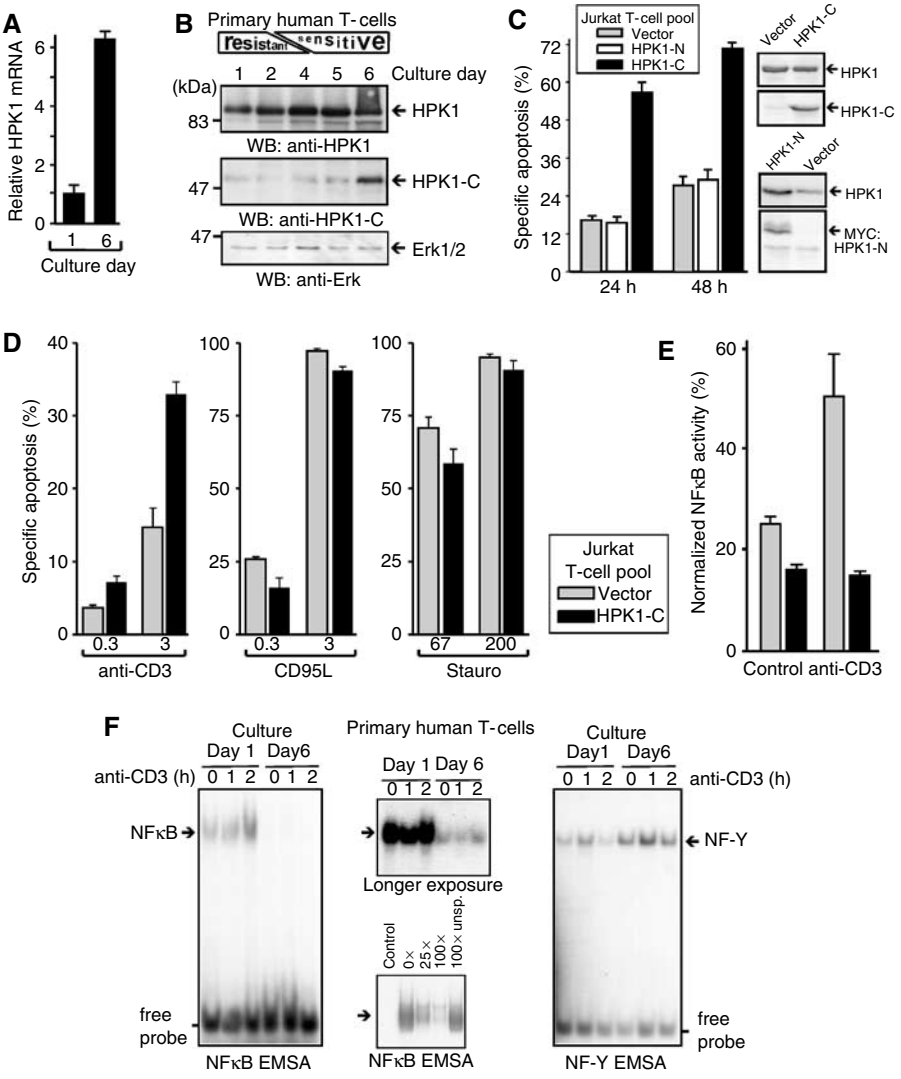


Figure 1 HPK1-C suppresses TCR-mediated NFκB activation and augments apoptosis. Expansion of T cells leads to increased HPK1 expression and conversion to HPK1-C. Primary human T cells isolated from peripheral blood were stimulated with PHA and expanded *in vitro*. Samples were taken at the indicated culture day and HPK1 was quantified by real-time RT–PCR (**A**) or by WB using the indicated Abs (**B**). Viability of the expanded T cells was higher than 90% at the time points when HPK1-C could be detected. (**C**) Jurkat T cell pools harboring stable integration of either empty vector or HPK1-N or HPK1-C expression vectors were analyzed for specific cell death after incubation with 10 μg/ml plate-bound anti-CD3 Abs for 24 or 48 h. Expression of HPK1 or HPK1-C or HPK1-N was analyzed in lysates from the indicated Jurkat T cell pools by WB using Abs against HPK1 or HPK1-C or MYC, respectively. (**D**) Jurkat T cell pools shown in C-expressing HPK1-C or control Jurkat T-cell pools (vector) were analyzed for specific cell death after incubation with 0.3 or 3 μg/ml plate-bound anti-CD3 Abs (left panel) or with dilutions of soluble CD95L at 0.3×10^{-4} or 3×10^{-4} (middle panel) or with 67 or 200 nM staurosporine (Stauro, right panel) for 18 h. (**E**) The Jurkat T cell pools shown in (D) were transiently transfected with an NFκB-specific reporter gene system and stimulated by plate-bound anti-CD3 or left nonstimulated (Control) for 8 h. Equal expression of CD3 and comparable stimulation by CD3 was controlled by flow cytometry or WB using anti-phospho-JNK1/2-specific Abs. (**F**) Primary human T cells at day 1 or day 6 of culture were stimulated by anti-CD3 Abs (CD3) for 1 or 2 h or left nonstimulated (0). Nuclear extracts were subjected to EMSA using ^{32}P-labeled oligonucleotides containing an NFκB (left panel) or an NF-Y (right panel)-binding site. Equal binding of the constitutively active transcription factor NF-Y to its binding site serves as a loading control. Longer exposure (top middle panel) and specificity control for the NFκB EMSA (bottom middle panel). EMSA reactions were coincubated with 25- or 100-fold excess of unlabeled NFκB-specific (spec.) or NFκB-unspecific NF-Y-binding site (unsp.). Values given depict the average and standard deviation of triplicate measurements.

panel). The cleavage of HPK1 therefore correlates with the sensitization of T cells towards AICD. Remarkably, HPK1-C does not result from background apoptosis of expanding cells since the viability during the culture period (day 1–6) is constantly increasing (data not shown).

HPK1-C suppresses TCR-mediated NFκB activation and augments apoptosis

To investigate whether HPK1 cleavage products have an impact on TCR-induced apoptosis, we generated stably transfected Jurkat T cell pools expressing HPK1-N or HPK1-C. HPK1-C- but not HPK1-N-expressing Jurkat T cells showed strongly enhanced TCR-mediated cell death (Figure 1C). This HPK1-C-mediated increase in cell death was only seen after TCR stimulation, while treatment with CD95L or staurosporine did not enhance cell death in HPK1-C-expressing Jurkat T cells (Figure 1D). As previously reported for non-T cells (Arnold *et al*, 2001), HPK1-C expressing Jurkat T cells showed suppressed TCR-mediated NFκB activation (Figure 1E). Therefore, sensitization towards cell death by HPK1-C correlates with suppression of NFκB. As reported previously, we did not find any influence of HPK1-C on the SAPK/JNK pathway upon stimulation of the TCR (Schulze-Luehrmann *et al* (2002) and Figure 6E). To further substantiate the involvement of HPK1 in TCR-mediated NFκB induction, we compared nuclear lysates of day 1–6 T cells by electrophoretic mobility shift assay (EMSA) (Figure 1F). While NFκB induction in day 1 T cells was clearly visible, NFκB induction, but not constitutive NF-Y activity, was significantly decreased in the HPK1-C containing day 6 T cells and could only detected upon longer exposure. In accordance with the prosurvival role for NFκB in T cells, the lack of NFκB activation in HPK1-C-expressing Jurkat T cells or primary day 6 T cells clearly correlates with enhanced TCR-mediated cell death. This result suggests a role for HPK1-C in sensitization towards AICD by suppression of the NFκB pathway.

Full-length HPK1 interacts specifically with IKKβ, while HPK1-C can associate with IKKα and IKKβ

While several studies demonstrated that HPK1 activates NFκB transcriptional activity (Arnold *et al*, 2001; Schulze-Luehrmann *et al*, 2002), the exact mechanism of the HPK1-mediated NFκB activation is not known. To delineate this mechanism, we tested for direct interaction of HPK1 with IKKs IKKα and IKKβ. COS-1 cells were transfected with IKKα or IKKβ alone or in combination with HPK1. Coimmunoprecipitation revealed a specific interaction of full-length HPK1 with IKKβ (Figure 2A). We did not detect interaction of full-length HPK1 with IKKα or IKKγ (Figure 2B). While HPK1-N neither interacts with IKKα nor with IKKβ (Figure 2C), HPK1-C does associate with IKKα and IKKβ (Figure 2D). Here, we show for the first time that HPK1 and HPK1-C interact with components of the NFκB-activating IKK complex. In addition, this result suggests that HPK1-mediated activation and HPK1-C-mediated suppression of the NFκB pathway share the same molecular targets. Furthermore, our data support the finding that HPK1-N does not influence NFκB activation (Arnold *et al*, 2001).

Preassociation of endogenous HPK1 with the IKK complex is released upon TCR stimulation

To test the interaction of IKKβ with endogenous HPK1, we used tg BJAB cells expressing different levels of FLAG-tagged

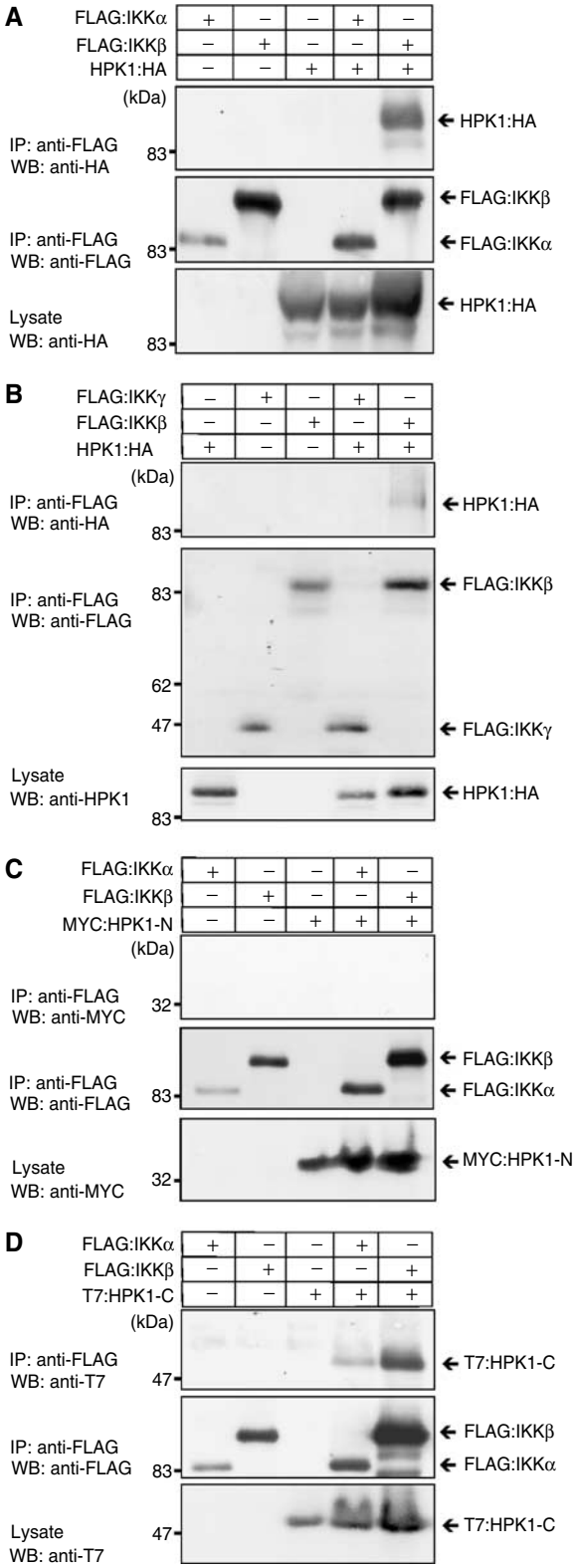


Figure 2 Full-length HPK1 interacts specifically with IKKβ, while HPK1-C can associate with IKKα and IKKβ. COS1 cells were transiently transfected with plasmids encoding FLAG-tagged versions of IKKα or IKKβ or IKKγ alone or in combination with HA-tagged full-length HPK1 (**A, B**) or MYC-tagged HPK1-N (**C**) or T7-tagged HPK1-C (**D**). Following anti-FLAG immunoprecipitation, the presence of coimmunoprecipitated HPK1 variants was analyzed by WB using tag-specific Abs.

IKKβ. Immunoprecipitation of FLAG-tagged IKKβ showed copurification of endogenous HPK1, depending on the amount of IKKβ present in the BJAB cells (Figure 3A, top panels). Furthermore, immunoprecipitation of endogenous HPK1 revealed copurification of exogenous IKKβ depending on the amount of IKKβ expressed (Figure 3A, bottom panels). This demonstrates a constitutive and specific association of endogenous HPK1 with IKKβ in lymphoid cells.

To further confirm the involvement of HPK1 in NFκB activation in lymphoid cells, we pulled down all components of the IKK complex by precipitation of IKKγ (NEMO) (Tegethoff *et al*, 2003; Quirling *et al*, 2004). We detected endogenous HPK1 from nonstimulated DC27.1 T cells to be part of the endogenous IKK complex (Figure 3B, first lane). Surprisingly, the association of HPK1 with the IKK complex was lost immediately after TCR stimulation, while a re-association of HPK1 could be detected after 45 min of stimulation (Figure 3B). Neither HPK1 nor IKKβ show a significant activity in nonstimulated T cells, but both proteins are activated with a fast kinetic directly following TCR ligation (Liou *et al*, 2000; Liu *et al*, 2000). Whereas HPK1 activity is reported to peak at 2 min after stimulation, IKKβ activity is maximal after 15 min. According to our data, HPK1 dissociates from the IKK complex in a stimulation-dependent manner, while the reassociation correlates with the decline of IKKβ and HPK1 activity. Furthermore, we found that HPK1 could be specifically coimmunoprecipitated with the endogenous IKK complex from primary human day 1 T cells (Figure 3C). This result further supports the physiological relevance of the HPK1–IKKβ interaction.

HPK1 stimulates IKK activity by increasing phosphorylation of IKKβ

So far, the molecular mechanism for HPK1-mediated NFκB activation was unknown. To elucidate this, we expressed both proteins in COS1 cells and tested the immunoprecipitated IKKβ for its ability to phosphorylate the IKKβ-specific substrate, GST:IκBα. While IKKβ expressed alone showed a basal activity (Figure 4A, first lane), the coexpression of HPK1, but not the kinase-deficient mutant HPK1(K46E), strongly stimulated IKKβ activity (Figure 4A). This result indicates that HPK1 kinase can activate IKKβ.

We further analyzed whether HPK1 directly phosphorylates IKKβ and thereby enhances its activity. Therefore, we established a reconstituted *in vitro* kinase assay system by combining purified GST:HPK1 and FLAG-IKKβ. GST:HPK1 purified from COS1 cells is capable of autophosphorylation, but does not show kinase activity towards the IKKβ substrate GST:IκBα (Figure 4B, first lane). In contrast, IKKβ shows only marginal autophosphorylation and weak kinase activity towards its substrate GST:IκBα (Figure 4B, second lane). Already, traces of purified GST:HPK1 added to IKKβ lead to an increase in IKKβ phosphorylation and kinase

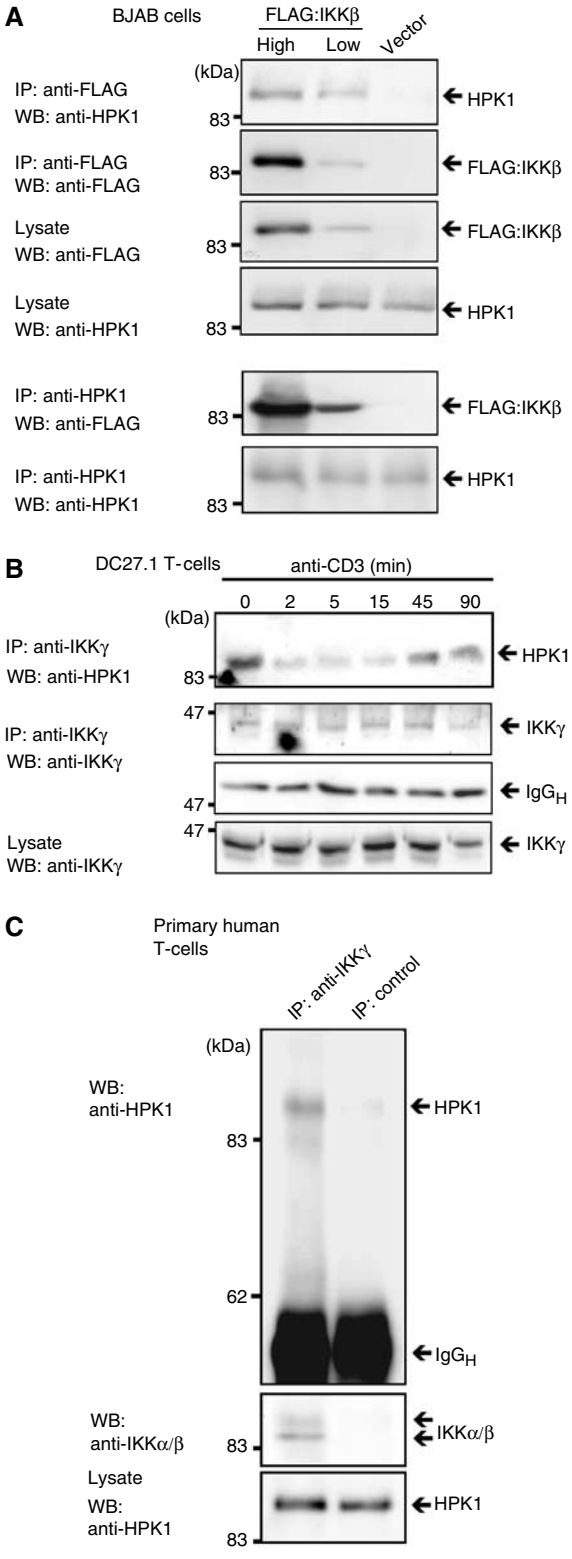


Figure 3 Preassociation of endogenous HPK1 with the IKK complex dissociates upon TCR stimulation. (**A**) BJAB cell pools selected for stable expression of high or low levels of FLAG-tagged IKKβ were subjected to anti-FLAG (upper panels) or anti-HPK1 (lower panels) immunoprecipitation. The presence of endogenous coimmunoprecipitated HPK1 was shown by WB using HPK1-specific Abs (upper panels). Precipitated IKKβ was analyzed by WB using a FLAG-tag specific Ab (lower panels). (**B**) DC27.1 T cells were stimulated with anti-CD3 Abs for the indicated time or left nonstimulated. The endogenous IKK complex was immunoprecipitated using anti-IKKγ Abs and tested for the presence of coimmunoprecipitated endogenous HPK1 by anti-HPK1 WB. (**C**) Primary human T cells at day 1 of culture were used to immunoprecipitate the endogenous IKK complex using anti-IKKγ Abs and tested for the presence of coimmunoprecipitated endogenous HPK1 by anti-HPK1 WB. A nonprecipitating Ab was used to control for specificity.

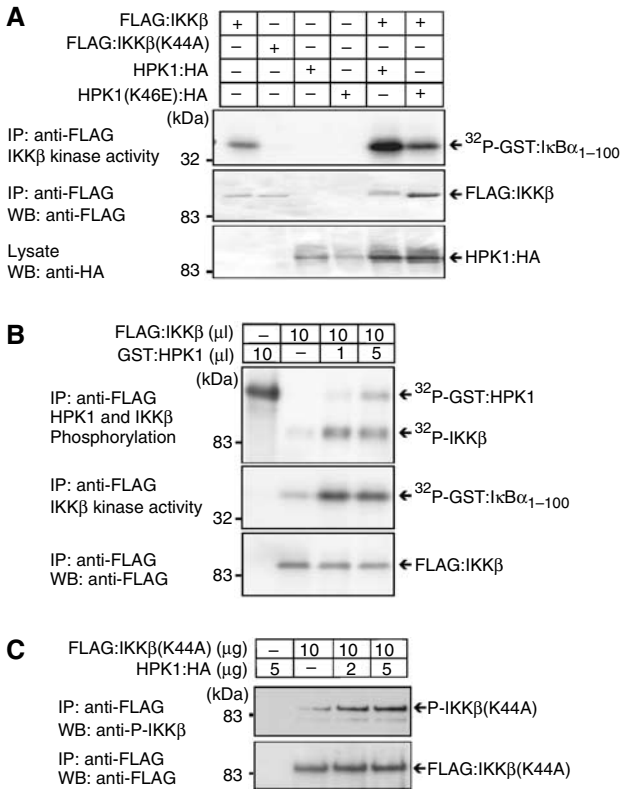


Figure 4 HPK1 stimulates IKK activity by enhancing phosphorylation of IKKβ. (**A**) COS1 cells were transiently transfected with plasmids encoding FLAG-tagged wt IKKβ or the ATP-binding site mutant IKKβ(K44A) alone or in combination with the HA-tagged wt HPK1 or the ATP-binding site mutant HPK1(K46E). After anti-FLAG immunoprecipitation, the IKKβ proteins were tested for their ability to transphosphorylate *in vitro* a recombinant GST:IκBα protein using ^{32}Pγ-ATP. Phosphorylated GST:IκBα was separated by SDS–PAGE and visualized by autoradiography (upper panel). The presence of immunoprecipitated IKKβ protein and the expression level of HPK1 were shown by WB (lower panels). (**B**) Purified GST:HPK1 expressed in COS 1 cells was used to set up a reconstituted *in vitro* kinase assay system. The kinase reaction was performed in the presence of recombinant GST:IκBα using various amounts of GST:HPK1 alone or in combination with purified FLAG-tagged IKKβ. Reaction products were separated by SDS–PAGE and visualized by autoradiography (upper panels). The presence of immunoprecipitated IKKβ protein is shown by WB (lower panel). (**C**) COS1 cells were transiently transfected with FLAG-tagged ATP-binding site mutant IKKβ(K44A) or the HA-tagged wt HPK1 alone or in combination. After anti-FLAG immunoprecipitation, the IKKβ proteins were tested for phosphorylation of Ser181 by WB using anti-phospho-IKKβ Abs. The presence of immunoprecipitated IKKβ protein is shown by anti-IKKβ WB. All experiments depicted show one out of three experiments with identical outcome.

activity towards GST:IκBα (Figure 4B). To further support a direct phosphorylation of IKKβ by HPK1, we expressed increasing amounts of HPK1 with the kinase-deficient IKKβ(K44A) harboring a point mutation in the ATP-binding site (Figure 4C). Indeed, we detected phosphorylation of IKKβ(K44A) in the presence of HPK1, which is likely to be mediated by HPK1 and cannot be caused by the kinase-deficient IKKβ(K44A). Therefore, we conclude that direct IKKβ phosphorylation by HPK1 contributes to the activation of the IKK complex. However, our experimental system does not firmly rule out the existence of copurified factors, which would help to positively regulate NFκB activity.

Full-length HPK1 is crucial for TCR-mediated NFκB activation and survival of T cells

Jurkat T cells express full-length HPK1 only. To investigate the role of full-length HPK1 in TCR-mediated IKK activation in Jurkat T cells, we used siRNA-mediated knockdown of endogenous human HPK1. Surprisingly, TCR-mediated IKK activation was completely blocked in HPK1-deficient Jurkat T cells (Figure 5A). Furthermore, upon prolonged TCR stimulation, NFκB activation was even dropping below baseline in HPK1-deficient Jurkat T cells, while the constitutive NF-Y activity was remaining constant (Figure 5B). These results imply that HPK1 is crucial for TCR-mediated NFκB activation and that the loss of HPK1 cannot be compensated by other NFκB-activating molecules in Jurkat T cells. Consistent with the antiapoptotic, prosurvival role of NFκB, TCR stimulation leads to enhanced cell death in HPK1-deficient Jurkat T cells (Figure 5C), further supporting the role of NFκB signaling pathways in AICD. Our siRNA approach did not prevent TCR signaling in general, as TCR-induced tyrosine phosphorylation detected by antiphosphotyrosine antibodies (Abs) was not impaired (data not shown). Furthermore, the cell death sensitization was specific for TCR-induced cell death as death via CD95 stimulation was not altered (Figure 5C). In summary, we propose a prosurvival role of full-length HPK1 due to activation of NFκB.

HPK1-C binding blocks TCR-mediated IKK activation

Full-length HPK1, capable of activating IKKβ, and the NFκB-inhibitory cleavage fragment HPK1-C are present at day 6 in primary human T cells (Figure 1B). To investigate whether HPK1-C has the capacity to competitively inhibit HPK1-mediated IKKβ activation, we transfected COS1 cells with HPK1 and IKKβ with or without HPK1-C and tested for activation of IKKβ (Figure 6A). While HPK1 led to enhanced phosphorylation of IKKβ and pronounced activation of IKKβ kinase activity, addition of HPK1-C resulted in a nearly complete suppression of IKKβ activity (Figure 6A, right lane). To elucidate the molecular mechanism of HPK1-C on modulation of the NFκB pathway, we analyzed HPK1-C-expressing Jurkat T cells (Figure 1) for association of HPK1-C with the endogenous IKK complex. As expected, the HPK1-C-expressing Jurkat T cells showed a pronounced suppression of IKK activity after TCR stimulation (Figure 6B, right panels) compared to the parental Jurkat T cells (Figure 6B, left panels). In contrast to full-length HPK1 that leaves the IKK complex upon activation (Figure 3B), HPK1-C remained bound to endogenous IKKβ after TCR stimulation (Figure 6C, top panel). As expected, the presence of HPK1-C did not interfere with the stimulation of the SAPK/JNK pathway by the TCR (Figure 6C, bottom panel). Besides a slight decrease in phosphorylated Akt, stimulation of various downstream signaling pathways by TNFα or PMA/ionomycin does not seem to be altered significantly in the presence of HPK1-C (Figure 6E). As already mentioned, TCR-mediated stimulation of the SAPK/JNK pathway in the presence of HPK1-C remained unaffected (Figure 6E). This result suggests that constant association of HPK1-C with IKKβ blocks specifically TCR-induced IKK activation and thereby directly modulates TCR-proximal signaling. We conclude that binding of HPK1-C to either IKKα or IKKβ is sufficient for suppression of the canonical, IKKβ-mediated pathway of NFκB activation.

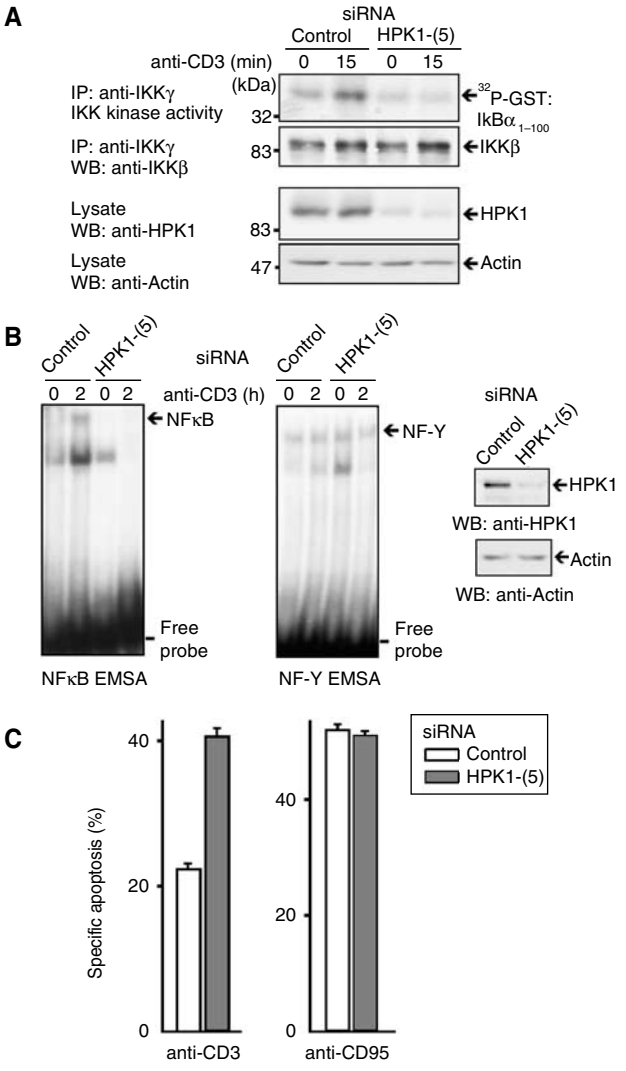


Figure 5 Full-length HPK1 is crucial for TCR-mediated NFκB activation and survival of T cells. (**A**) Jurkat T cells were transiently transfected with double-stranded siRNA oligonucleotides comprising a HPK1-specific sequence (HPK1-(5)) or a nonspecific sequence (control). At 48 h after transfection, cells were stimulated with anti-CD3 Abs for the indicated time or left nonstimulated; endogenous IKKγ proteins were immunoprecipitated and the co-precipitated IKK proteins were tested for their ability to phosphorylate *in vitro* a recombinant GST:IκBα protein using ^{32}Pγ-ATP. The siRNA-mediated downregulation of HPK1 was compared to actin by WB. (**B**) Jurkat T cells were transfected as in (A) and stimulated by anti-CD3 Abs for 2 h or left nonstimulated (0). Nuclear extracts were subjected to EMSA as described in Figure 1F. siRNA-mediated downregulation of HPK1 was compared to actin by WB. (**C**) Jurkat T cells were transfected as in (A) and stimulated with 30 μg/ml plate-bound anti-CD3 or 5 ng/ml soluble anti-CD95 Abs for 18 h and analyzed by flow cytometry. Values given depict the average and standard deviations of triplicate measurements. The experiment was repeated four times with similar outcomes.

HPK1-C mediates AICD in primary T cells by inhibition of IKK activation

As demonstrated for primary human T cells (Figure 1B), AICD-sensitive mouse T cells at culture day 4 (Baumann *et al*, 2005) also show conversion of full-length HPK1 into HPK1-C (Figure 7A). To clarify how HPK1-C would influence IKK activation and AICD of primary T cells, we generated HPK1-C tg mice and compared primary mouse T cells from these mice to their wild-type (wt) littermates. As expected, analysis of IKK activation after TCR stimulation showed a strong reduction of IKK kinase activity and NFκB activation in primary HPK1-C tg T cells (Figure 7B and C). The HPK1-C-mediated suppression of IKK activation, which results in decreased NFκB activation (Figure 7C), is reflected in decreased expression of the NFκB target gene *Bcl-2A1* in tg mouse T cells (Figure 7D) upon TCR stimulation. Besides a slight decrease in phosphorylated Akt, TCR-mediated stimulation of various downstream signaling pathways does not seem to be altered in the presence of HPK1-C (Figure 7E), demonstrating that HPK1-C is specifically suppressing the NFκB signaling pathway. Suppression of NFκB activation by HPK1-C again correlated with enhanced cell death in the AICD-sensitive HPK1-C tg T cells (culture day 5) compared to their wt littermates (Figure 7E). According to the previous finding showing that the sensitization to cell death by HPK1-C does not depend on higher sensitivity towards CD95L (Figure 1), also primary HPK1-C tg T cells do not show elevated apoptosis in response to CD95L (data not shown). In conclusion, this result implies that HPK1-C enhances AICD in primary T cells by inhibition of NFκB activation.

Knockdown of full-length HPK1 enhances cell death sensitivity, but prevention of HPK1-C generation leads to AICD resistance

To investigate the roles of HPK1 and HPK1-C in TCR-induced cell death in Jurkat T cells, we used siRNA-mediated knockdown of endogenous human HPK1 in the Jurkat T-cell pool stably expressing mouse HPK1-C (Figure 1C). Expression of endogenous human HPK1 was greatly decreased, while the level of the mouse HPK1-C transgene was not altered (Figure 8A, left panels). In addition, siRNA-mediated knockdown of HPK1 did not influence the expression of downstream signaling proteins IκBα or JNK. Also, in primary human T cells siRNA-mediated knockdown resulted in clear reduction of endogenous HPK1 levels (Figure 8A, right panels). HPK1-deficient, but not the mouse HPK1-C-containing, Jurkat T cells showed strongly enhanced TCR-induced cell death. Death via CD95 stimulation, however, was not affected (Figure 8B). In conclusion, loss of full-length HPK1 sensitizes Jurkat T cells to cell death by blunting NFκB activation, while the HPK1-C-expressing Jurkat T cells cannot be further sensitized by the loss of HPK1. This finding suggests that HPK1-C-mediated suppression of NFκB alone is already sufficient to fully sensitize Jurkat T cells to apoptosis. To further support the physiological role of HPK1 and HPK1-C, we analyzed primary human day 1 T cells, harboring only full-length HPK1. Comparable to our results in parental Jurkat T cells, siRNA-mediated knockdown of full-length HPK1 sensitizes towards TCR-mediated cell death in day 1 T cells (Figure 8C, left diagram). In contrast, the prevention of HPK1-C generation by siRNA-mediated knockdown in preactivated day 6 T cells results in resistance towards AICD (Figure 8C, middle diagram) independent of enhanced sensitivity towards CD95. These results clearly show that endogenously processed HPK1-C is causing sensitization towards AICD (by inhibition of NFκB), while full-length HPK1-mediated NFκB activation is needed for T-cell activation and survival.

Discussion

In the present study, we describe the differential involvement of HPK1 and HPK1-C in the regulation of the NFκB pathway.

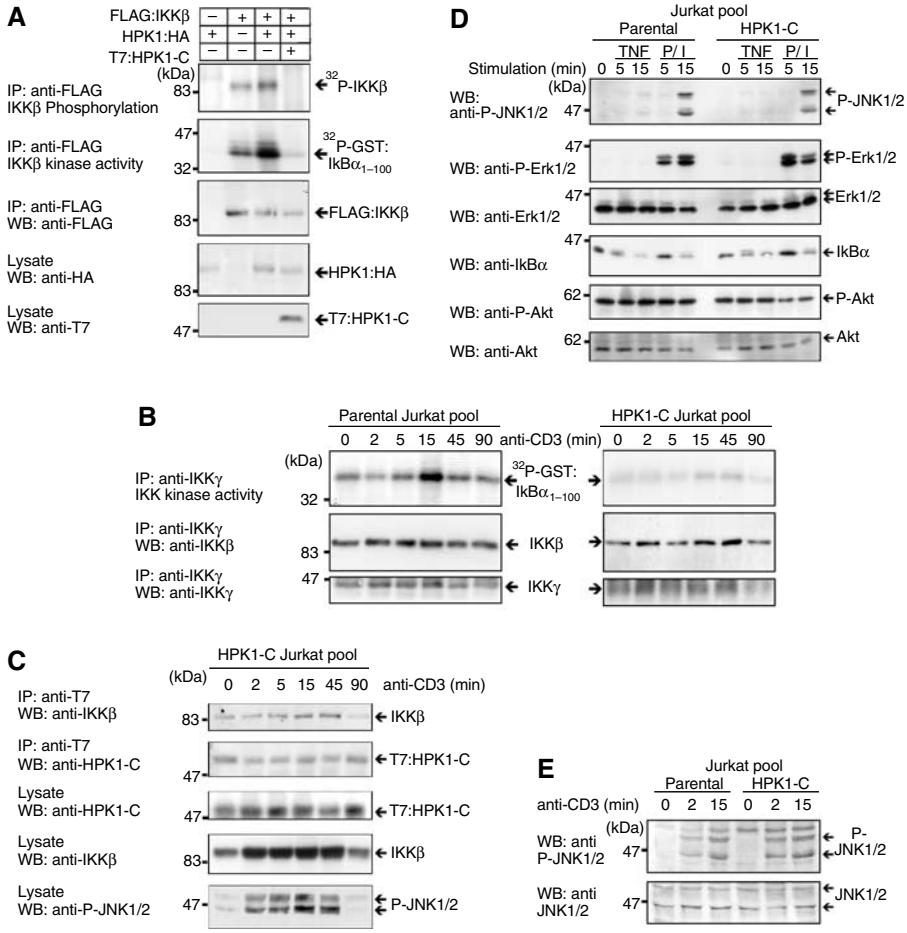


Figure 6 HPK1-C binding blocks TCR-mediated IKK activation. (**A**) COS1 cells were transiently transfected with plasmids encoding FLAG-tagged IKKβ with or without HA-tagged HPK1 or the T7-tagged HPK1-C. After anti-FLAG immunoprecipitation, the IKK proteins were tested for their ability to autophosphorylate and to transphosphorylate *in vitro* a recombinant GST:IκBα protein using ^{32}Pγ-ATP. (**B**) The HPK1-C stably expressing the Jurkat T-cell pool depicted in Figure 1C or parental Jurkat T cells were stimulated with anti-CD3 Abs for the indicated time or left nonstimulated. Endogenous IKKγ proteins were immunoprecipitated and the co-precipitated IKK proteins were tested for their ability to phosphorylate *in vitro* a recombinant GST:IκBα protein using ^{32}Pγ-ATP. (**C**) HPK1-C stably expressing Jurkat T cells were stimulated as in B, T7-tagged HPK1-C proteins were immunoprecipitated and the co-precipitated endogenous IKKβ proteins were shown by WB. Protein expression levels in the lysates were controlled by WB. Efficiency of TCR stimulation was controlled by WB using anti-phospho-JNK1/2 (P-JNK1/2) Abs. (**D**) HPK1-C stably expressing or parental Jurkat T cells were stimulated with TNFα or PMA/ionomycin (P/I) for the indicated time or left nonstimulated. Lysates were subjected to WB using the indicated Abs. (**E**) HPK1-C stably expressing or parental Jurkat T cells were stimulated as in (B) and lysates were controlled by WB using anti-phospho-JNK1/2 (P-JNK1/2) and anti-JNK1/2 Abs.

While full-length HPK1 leads to activation of the IKK complex upon TCR stimulation, the proteolytic fragment HPK1-C blocks IKK activation after TCR ligation (Figure 8D). We show for the first time association of full-length HPK1 with the IKK complex and phosphorylation-dependent enhancement of IKKβ activity by HPK1. By siRNA-mediated knockdown, we show that full-length HPK1 is crucial for TCR-mediated IKK kinase activity and NFκB activation. Shutting down TCR-mediated NFκB activation consequently leads to enhanced sensitivity towards cell death (Figure 8D, left panel). We find HPK1-C to be specifically present in AICD-sensitive preactivated primary (day 6) T cells. Expression of HPK1-C sensitizes T cells towards TCR-mediated AICD independent of altered CD95 sensitivity, but involving suppression of NFκB (Figure 8D, right panel). We show tight binding of HPK1-C to the IKK complex as a novel mechanism of regulation of IKK activity. In tg mice, we demonstrate that HPK1-C leads to enhanced AICD by inhibition of IKK activation in primary T cells. Preventing HPK1-C generation in primary preactivated (day 6) T cells by siRNA-mediated knockdown of HPK1 results in resistance towards AICD (Figure 8D, right panel). Therefore, we conclude a novel role for HPK1 as a life/death switch in T lymphocytes.

Previously, we and others have demonstrated activation of NFκB by full-length kinase-active HPK1 (Arnold *et al*, 2001; Tsuji *et al*, 2001). However, the responsible mechanism for activation of NFκB by HPK1 was still elusive. Our results show enhanced phosphorylation of IKKβ by kinase-active HPK1 and therefore explain why HPK1 kinase activity is needed for NFκB activation. Furthermore, we show that HPK1 is a functional, TCR-inducible component of the IKK complex and that the C-terminal region of HPK1 is mediating the association with the IKK complex.

HPK1 was shown to be recruited to the contact site of an antigen-presenting cell–T-cell conjugate (Le Bras *et al*, 2004; Arnold *et al*, 2005). Also, IKKβ membrane recruitment was defined to be a prerequisite for activation of the IKK complex (Khoshnan *et al*, 2000). Very rapidly after TCR stimulation, HPK1 is fully activated (Liou *et al*, 2000), while full activation of IKKβ takes several minutes (Lin *et al*, 2000). Therefore, it is

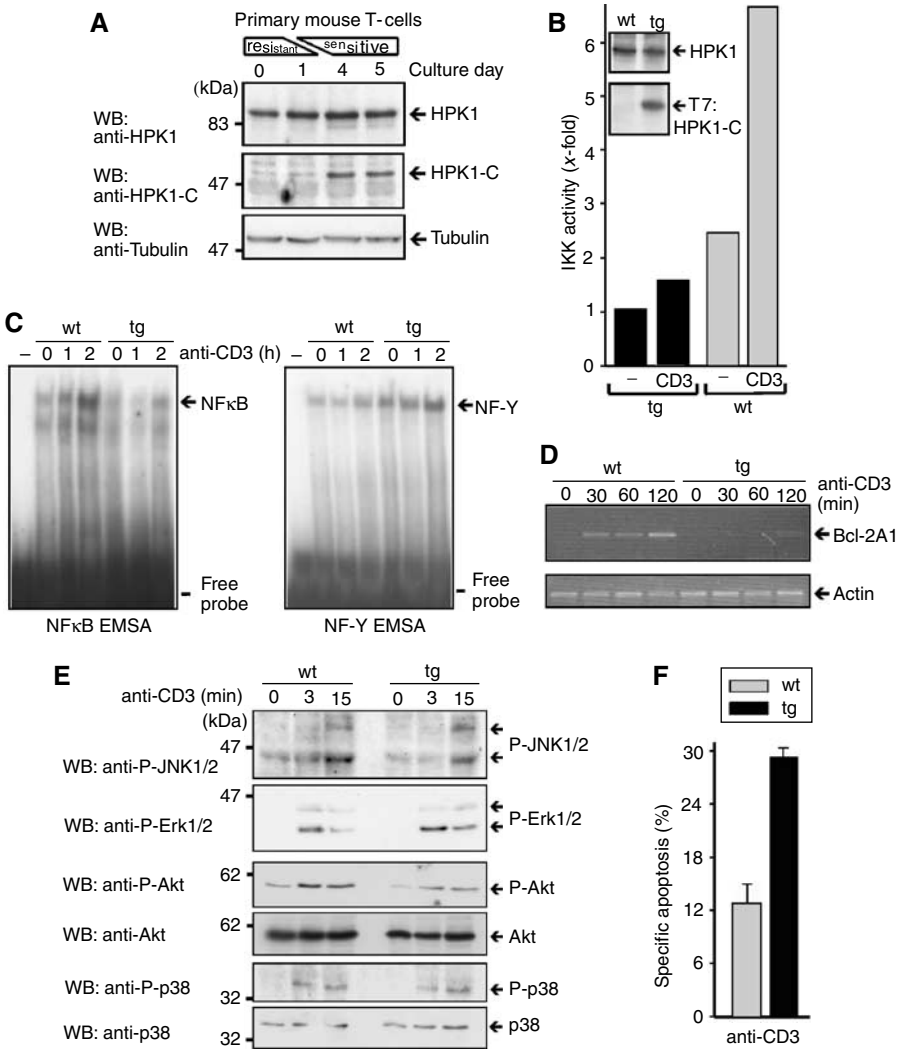
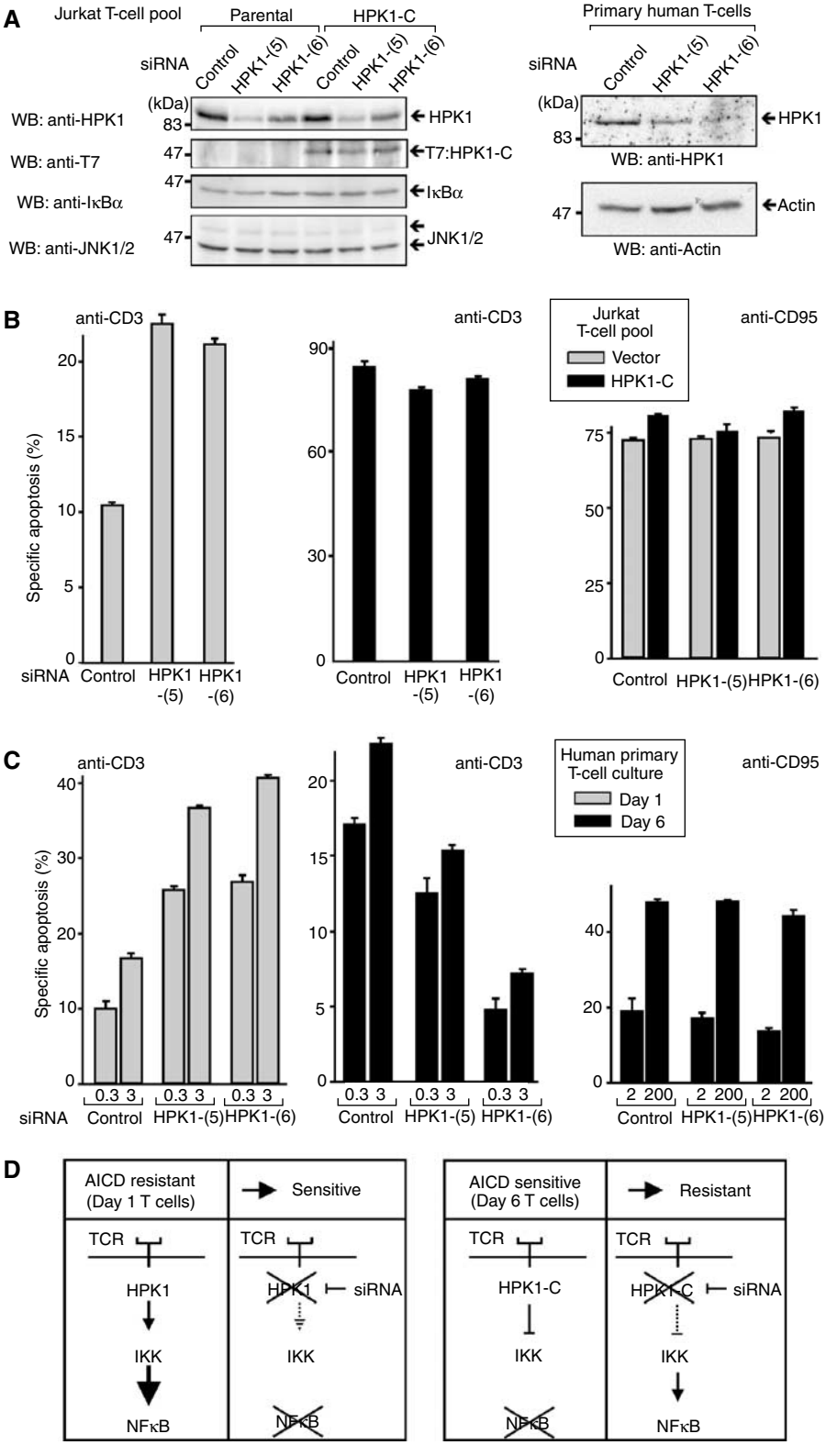


Figure 7 HPK1-C mediates AICD in primary T cells by inhibition of IKK activation. (**A**) HPK1 is converted to its C-terminal fragment, HPK1-C, during expansion of primary mouse T cells after concanavalin A stimulation *in vitro*. Samples were taken at the indicated culture day and analyzed for expression of HPK1 or HPK1-C by WB using the indicated Abs. Viability of the expanded cells was higher than 90% at the time points where HPK1-C could be detected. The experiment presented is representative of four repeats. (**B**) Purified T cells of wt mice or HPK1-C tg mice were stimulated by anti-CD3 Abs for 15 min (CD3) or left nonstimulated (−). Endogenous IKKγ proteins were immunoprecipitated and the co-precipitated IKK proteins were subjected to an *in vitro* kinase assay using recombinant GST:IκBα as substrate. The kinase reaction was separated by SDS–PAGE and quantified using a Phosphor-Imager. Values given are expressed relative to the basal activity of nonstimulated cells. (Inset) Expression of HPK1 and HPK1-C was analyzed in lysates from primary mouse T cells by anti-HPK1 and anti-T7 tag WB. (**C**) T cells of wt or HPK1-C (tg) mice as seen in (B) were stimulated by anti-CD3 Abs for 1 or 2 h or left nonstimulated (0). Nuclear extracts were subjected to EMSA as described in Figure 1F. (**D**) T cells of wt or HPK1-C (tg) mice were stimulated by anti-CD3 Abs for the indicated times or left nonstimulated (0). RNA was extracted and subjected to RT–PCR analysis using gene-specific primers. Expression of actin is shown as a control. (**E**) T cells of wt or HPK1-C (tg) mice were stimulated by anti-CD3 Abs for the indicated times or left nonstimulated (0). Lysates were subjected to WB using the indicated Abs. (**F**) T cells of wt or HPK1-C (tg) mice were analyzed at culture day 5 for AICD after incubation with plate-bound anti-CD3 Abs for 18 h. Values given depict the average and standard deviations of triplicate measurements. The experiments were repeated five times with identical outcome.

Figure 8 Knockdown of full-length HPK1 enhances cell death sensitivity, but prevents HPK1-C generation and results in AICD resistance. (**A**) HPK1-C stably expressing Jurkat T cells depicted in Figure 1C or parental Jurkat T cells or primary human T cells were transiently transfected with double-stranded siRNA oligonucleotides comprising HPK1-specific sequences (HPK1-(5) and HPK1-(6)) or a nonspecific sequence (control). At 24 h after transfection, human T cells were activated as described. At 48 h after transfection, cells were divided into two fractions and subjected to WB using the indicated Abs or further analyzed as depicted in (B) or (C). (**B, C**) siRNA-transfected HPK1-C stably expressing or parental Jurkat T cells were incubated with 3 μg/ml of plate-bound anti-CD3 Abs or 50 ng/ml of soluble anti-CD95 Abs for 18 h and analyzed by flow cytometry. Primary human day 1 or day 6 T cells, transfected 48 h before with the siRNA oligonucleotides, were incubated with 0.3 or 3 μg/ml of plate-bound anti-CD3 Abs or 2 or 200 ng/ml of soluble anti-CD95 Abs for 18 h and analyzed by flow cytometry. Values given depict the average and standard deviation of triplicate measurements. The experiments were repeated four times with identical outcome. (**D**) Model for the involvement of HPK1 in TCR-mediated resistance and sensitivity towards AICD. While HPK1-mediated IKK activation in AICD-resistant T cells leads to NFκB activation, the presence of HPK1-C blocks IKK activation downstream of the TCR and leads to sensitivity towards AICD. siRNA-mediated knockdown of full-length HPK1 in Jurkat T cells or in primary naive T cells blunts TCR-proximal NFκB signaling and leads to enhanced cell death. Preventing HPK1-C generation in primary preactivated (day 6) T cells by siRNA-mediated knockdown results in decreased AICD.

likely that, upon recruitment to the immunological synapse in lymphocytes, HPK1 activity precedes IKKβ activation and full-length HPK1 initiates activation of the IKK complex. Independent of its enzymatic function, HPK1 might also contribute to the activity of the IKK complex by coordinating its localization. While activated HPK1 would recruit the IKK complex to a TCR-proximal membrane region, binding of an inactive HPK1 might also be a mechanism for the shutdown of IKK activity. Therefore, HPK1 might be a crucial regulator of IKK activity, which is further supported by our data that siRNA-mediated knockdown of full-length HPK1 results in a complete block of IKK activity. It is tempting to speculate that HPK1 also contributes to the clustering of critical regulators of NFκB activation, like PDK1-associated PKCθ (Wang *et al*, 2004; Lee *et al*, 2005), Bcl10 (Ruland *et al*, 2001; Zhou *et al*, 2004), MALT1 (Ruefli-Brasse *et al*, 2003; Che *et al*, 2004, Sun



et al, 2004) or lipid raft-associated CARMA1/CARD11 (Gaide *et al*, 2002). As HPK1 is activated very fast upon TCR stimulation (Liou *et al*, 2000), it might well be that HPK1 initiates the IKK activation and then leaves the signaling complex, which would explain the stimulation-dependent dissociation of HPK1 and the IKK complex seen in DC27.1 T cells.

We provide evidence for the function of HPK1-C as a suppressor of IKK activity and suggest sequestration of the inactive IKK complex by bound HPK1-C. A similar mechanism for inhibition of NFκB activation was defined for the negative-regulatory domain of NIK (Xiao and Sun, 2000). Negative regulation of IKK activity was also reported by several mechanisms, including autophosphorylation of the C-terminal tail of IKKβ (Delhase *et al*, 1999) or degradation of Bcl10 (Scharschmidt *et al*, 2004). In addition, oligomerization of IKKγ (NEMO) (Tegethoff *et al*, 2003) and homotypic interaction and transautophosphorylation of IKKβ were reported to be required for activation (Tang *et al*, 2003). Thus, constitutive binding of HPK1-C might interfere with the composition of the IKK complex and disturb activation by sterical hindrance. A similar model for inhibition of IKK activity was suggested for p65 bound to IKKβ (May *et al*, 2004). Thus, inhibition of IKK activity by direct binding of HPK1-C to the IKK complex presents a likely and effective means of NFκB regulation.

Suppression of NFκB leads to downregulation of several molecules implicated in cell survival, like X-IAP, Bcl-x_L, c-FLIP and A20. This downregulation results in susceptibility towards cell death (Campbell *et al*, 2004; Shishodia and Aggarwal, 2004; Golks *et al*, 2005). The NFκB-dependent inhibition of p73 expression was shown to be required for survival of antigen-stimulated T cells (Wan and DeGregori, 2003), while expression of p73 has been shown to be essential for AICD (Lissy *et al*, 2000). Therefore, alteration of the NFκB response through HPK1-C interferes with known prosurvival mechanisms. This is reflected by our results showing suppressed induction of the antiapoptotic Bcl-2 family protein Bcl-2A1 in HPK1-C tg mice. The definition of the molecular mechanisms by which HPK1 regulates IKK activity increases our understanding of the NFκB pathway as a differential signal integrator and helps to explain the switch of a T cell from AICD resistance towards AICD sensitivity.

We show that HPK1 mediates AICD of primary T cells. This finding corroborates a previous report showing that HPK1 supports apoptosis of T lymphocytes (Schulze-Luehrmann *et al*, 2002). In this study, the retroviral transduction of wt HPK1 or an HPK1 antisense construct was shown to influence H_2O_2-mediated cell death of EL-4 cells, but the mechanism remained elusive (Schulze-Luehrmann *et al*, 2002). Our study explains the modulation of TCR-induced AICD mechanistically by suppression of IKK activity through HPK1-C. In addition, we have shown for the first time that HPK1 is cleaved towards HPK1-C in AICD-sensitive primary human and murine T cells. We defined that a caspase 3-like activity generates HPK1-C (Arnold *et al*, 2001). Also, in expanded day 5/6 T cells, we detected a caspase 3 activity generating HPK1-C *in vitro* (unpublished results), whereas we have no indication that caspase-8 is activated. As demonstrated in T cells of HPK1-C tg mice, the conversion of HPK1 towards HPK1-C contributes to the sensitization to AICD. In contrast to the study of Schulze-Luehrmann *et al*, we did not observe alterations in TCR-mediated cell death in HPK1-N-expressing T cells. This could result from differences in the experimental systems and one might speculate that depending on the cell type HPK1-N also contributes to AICD.

The involvement of the NFκB pathway in regulation of AICD was recently emphasized by the finding that CD28-mediated upregulation of Bcl-x_L leads to survival of primary T cells (Noel *et al*, 1996; Kirchhoff *et al*, 2000; Kerstan and Hunig, 2004). T-cell costimulation with CD28 is known to cause IKK activation (Coudronniere *et al*, 2000; Hehner *et al*, 2000) and the survival factor Bcl-x_L is a *bona fide* NFκB target gene. Thus, suppression of the NFκB pathway seems to be required for efficient AICD to occur. We find that conversion of full-length HPK1 to HPK1-C is dependent on T-cell activation, but independent of apoptosis induction. Similar to our finding, caspase activity was recently reported to be required for T-cell activation and NFκB signaling (Misra *et al*, 2005; Su *et al*, 2005). By interfering with TCR-mediated NFκB activation, caspase inhibition blocks the expansion of primary T cells towards day 5/6 (where HPK1 cleavage is seen). Therefore, we have used siRNA-mediated knockdown of HPK1-C in day 6 primary T cells to show a crucial role for HPK1-C in execution of AICD. These findings clearly indicate a physiological role for caspase activity distinct from the execution of apoptosis. Based on our results, we suggest HPK1 as a molecular target for caspase activity regulating NFκB signaling in T cells.

Materials and methods

Abs and reagents

The HRPO-conjugated Abs anti-mouse IgG1 or IgG2b or anti-rabbit IgG were purchased from Southern Biotechnology Associates. Polyclonal rabbit Abs anti-HPK1 (#2, #7 and #9/10) have been described previously (Arnold *et al*, 2001). Mouse monoclonal Abs anti-CD95 (anti-Apo-1) and recombinant CD95L were described previously. The Abs used were anti-T7-tag (Novagen), anti-FLAG-tag (M2, Sigma), anti-Erk, anti-P-Erk1/2, anti-P-Jnk1/2, anti-P-IKKα/β, anti-p38, anti-P-p38, anti-Akt and anti-P(Ser473)-Akt (all from Cell Signaling Technology), anti-α-tubulin (Sigma), anti-β-actin (Sigma), anti-IKKγ (B3 and FL-419, Santa Cruz), anti-IKKβ (159A, Imgenex), anti-IKKα/β, anti-IκB and anti-JNK1/2 (all from Santa Cruz), anti-human CD3 (OKT3, BD Bioscience) and anti-mouse CD3 (145-2C11, PharMingen). HA- or MYC-tagged proteins were detected by hybridoma supernatants anti-HA (12CA5) or anti-MYC (9E10). FITC-coupled Abs anti-mouse IgG1 or PE-coupled anti-mouse IgG2b were from Caltag Laboratories. All chemicals used were of analytical grade and purchased from Merck or Sigma.

Expression plasmids

The following expression plasmids were described previously: HPK1:HA(wt), HPK1:HA(K46E), T7:HPK1-C and HPK1:HA(Y379F) (Kiefer *et al*, 1996; Arnold *et al*, 2001; Tsuji *et al*, 2001), FLAG:IKKα and FLAG:IKKβ (Tsuji *et al*, 2001), NIK and FLAG:IKKβ(K44A) (Ling *et al*, 1998), GST:IKKβ and GST:IKKβ(S177A,S181A) (Nemoto *et al*, 1998). The MYC:HPK1-N constructs, comprising amino acids 1–382 of murine HPK1, were cloned into the pEF4 expression vector (Invitrogen) using standard PCR technique and the primers 5′-ATAA GAATGCGGCCGCATGGAACAAAAACTCATCTCAGAAGAGGATCTGG GCGCAGGCGCCCTTGTGGACCCCGACATTT-3′ and 5′-GCTCTAGATT AATCATAGTCGTCATCAGAATGGGGG-3′.

Primary T cells and cell lines

Human and murine peripheral T cells were prepared and cultivated as described previously (Peter *et al*, 1997; Baumann *et al*, 2005). For activation and expansion, primary human T cells were stimulated with 1 μg/ml PHA and murine T cells were stimulated with 5 μg/ml concanavalin A (Pharmacia) for 18 h. The purity of the obtained

T cells by either method was >90%, as determined by flow cytometry. The HPK1-C tg mice were provided by FK. Details regarding the human Jurkat T-cell clone J16–145, the human B lymphoblastoid cell line BJAB, the murine DC27.1 T-cell line and COS1 cells were published elsewhere.

Apoptosis assays

For TCR-mediated apoptosis induction, cells were seeded in triplicates on culture dishes precoated with the indicated concentrations of anti-CD3 Abs. Cell death of unstained cells was quantified by FSC/SSC analysis or by staining with 2.5 μg/ml propidium iodide and analyzed by flow cytometry. Specific apoptosis was calculated as (% of induced apoptosis−% of spontaneous apoptosis)/(100−% of spontaneous apoptosis) × 100.

Transfections, stable cell lines and siRNA-mediated knockdown

COS1 cells were transfected by Ca^{2+}-phosphate co-precipitation using 3–10 μg of plasmid DNA. After 48 h, COS1 cells were lysed in lysis buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, protease inhibitor cocktail (Roche), 1% Triton X-100 (Serva) and 10% glycerol). 1×10^7 Jurkat T cells or BJAB cells were transfected by electroporation (250 V, 950 μF) in 400 μl of culture media using 10–30 μg plasmid DNA. To obtain stable transfections, cells were incubated 48 h after transfection with culture media containing 1 mg/ml neomycin and selected for 2 weeks. Jurkat T cells and primary human T cells were transfected by nucleofection (AMAXA) or lipofection (HiPerfect, Qiagen) with negative control or validated siRNA oligonucleotides specific for human HPK1 (Qiagen; HPK1#5: MAP4K1_5_HP, HPK1#6: MAP4K1_6_HP). In all, 2 μg (750 nM) siRNA was used for nucleofection of 5×10^6 cells and 0.2 μg (75 nM) was used for lipofection of 2×10^5 cells. Transfected cells were rested for 48 h before subjecting to further analysis.

NFκB reporter assays

10^7 Jurkat T cells were cotransfected with pGL8xNFκB-fos and pfos-LacZ as described previously (Arnold *et al*, 2001). Luciferase activity is presented as the percentage of induction obtained with PMA and ionomycin set to 100%.

RT–PCR analysis

Quantitative RT–PCR was performed with the SYBR green system using an ABI Prism 7700 Sequence Biodetector (Applied Biosystems). Bars depict the average value and the standard deviations of three independent experiments. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions and 2 μg of RNA was reverse transcribed using Superscript II (Invitrogen) and oligo $(dT)_{15}$ primers (MWG). Reactions were normalized to glyceraldehyde-3-phosphate dehydrogenase or actin expression. Primers used: human HPK1: 5′-ACCAAGGACCAGCACC TGC-3′; 5′-CGGTTCAGGATGAAGATGCC-3′, mouse Bcl-2A1: 5′-CAG TATGTGCTACAGGTACCC-3′; 5′-TTGAGGAGAAAGAGCATTTCCC-3′, mouse β-actin 5′-TGACGGGGTCACCCACAATGTGCCCATCTA-3′; 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′.

Immunoprecipitation and WB

A measure of 2 μg of Ab coupled to protein A sepharose was used to immunoprecipitate proteins from cell lysates for 2–18 h at 4°C. Proteins were resolved by SDS–PAGE and transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech), incubated with 5% nonfat dry milk in PBS/T (PBS with 0.05% Tween-20) for 1 h, washed with PBS/T, and incubated with the primary Abs diluted in PBS/T for 18 h at 4°C. Blots were developed using chemoluminescence following the manufacturer's protocol (Perkin-Elmer Life Sciences).

In vitro kinase assays

All *in vitro* kinase assays were performed as described previously (Kiefer *et al*, 1996). An reconstituted *in vitro* kinase assay was set up using various amounts of purified, COS1-expressed GST:HPK1 and 10 μl immunoprecipitated FLAG:IKKβ bound to sepharose beads. FLAG:IKKβ was immunoprecipitated by anti-FLAG Abs and GST:HPK1 was pulled down by glutathione Sepharose beads (Pharmacia) and eluted with reduced glutathione.

Electrophoretic mobility shift assay

Soluble nuclear proteins were prepared and used for EMSA as described previously (Arnold *et al*, 2001). For each reaction 10–20 fmol of ^{32}P-labeled oligonucleotides comprising an NFκB- or NF-Y- (5′-CACCTTTTAACCAATCAGAAAAAT-3′) binding site were employed.

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