Title: NIK controls classical and alternative NF-κB activation and is necessary for the survival of human T cell lymphoma cells

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STATEMENT OF TRANSLATIONAL RELEVANCE

Peripheral T cell lymphomas (PTCLs) are aggressive malignancies presenting poor clinical outcome. There are currently no effective treatments or targeted therapies available for these patients. Thus, studies aimed to elucidate the mechanisms contributing to these tumors and to identify new therapeutic targets, are required in order to improve their dismal prognosis. Here, we demonstrate for the first time that primary PTCLs expressing nuclear NF-κB are characterized by a significantly worse clinical outcome compared to NF-κB negative tumors, supporting a rationale for the exploration of NF-κB-interfering strategies. We further identify NIK, as a novel potential therapeutic target in T cell lymphomas, and show that targeting NIK might be more effective than previously suggested IKK inhibition. This study opens up opportunities for further translational studies and will hopefully contribute to the future development of new targeted drugs useful in PTCL.
ABSTRACT

Purpose: Peripheral T cell lymphomas (PTCLs) are a heterogeneous entity of neoplasms with poor prognosis, a lack of effective therapies, and a largely unknown molecular pathology. Deregulated nuclear factor κB (NF-κB) activity has been associated with several lymphoproliferative diseases, but its importance in T cell lymphomagenesis is poorly understood. We investigated the function of the NF-κB-inducing kinase, NIK, in this pathway and its role as a potential molecular target in T cell lymphomas.

Experimental Design: We used immunohistochemistry to analyze the expression of different NF-κB members in primary human PTCL samples and to study its clinical impact. With the aim of inhibiting the pathway, we used genetic silencing of NIK in several T cell lymphoma cell lines and observed its effect on downstream targets and cell viability.

Results: We showed that the NF-κB pathway was activated in a subset of PTCLs associated with poor overall survival. NIK was overexpressed in a number of PTCL cell lines and primary samples and a pivotal role for NIK in the survival of these tumor cells was unveiled. NIK depletion led to a dramatic induction of apoptosis in NIK-overexpressing cell lines and also showed a more pronounced effect on cell survival than IKK knockdown. NIK silencing induced a blockage of both classical and alternative NF-κB activation and a reduced expression of several prosurvival and antiapoptotic factors.

Conclusions: The results of the present study indicate that NIK could be promising therapeutic target in these aggressive malignancies.
INTRODUCTION

Peripheral T cell lymphomas (PTCLs) are a heterogeneous family of non-Hodgkin’s lymphomas often associated with an aggressive clinical course and poor outcome. First-line standard therapy is based on the combination chemotherapy regimens usually used in B cell lymphomas or solid tumors. As these regimens generally have poor response or high rates of recurrence, there is a need to develop targeted therapies based on the signaling pathways that are aberrantly expressed in these T cell malignancies (1, 2).

Deregulated nuclear factor κB (NF-κB) activity plays a key role in the development of multiple malignancies. Thus, constitutive activation of NF-κB signaling has been observed in various tumor types, including lymphomas, leukemias and solid tumors (3, 4). The NF-κB signaling pathway regulates the transcription of many genes involved in cancer initiation and progression events, such as apoptosis, proliferation, angiogenesis and metastasis and hence, several lymphoma types rely on NF-κB activity for their proliferation and survival (4, 5). Genetic changes leading to constitutive activation of the pathway have been detected in several hematological tumors, emphasizing its importance in the pathogenesis of these malignancies (6-9). NF-κB can be activated either through the classical (canonical) pathway or the alternative (non-canonical) pathway. Briefly, activation of the classical pathway results in nuclear translocation of mainly p50/p65 heterodimers through the phosphorylation of IκB and p105 by the IκB kinase (IKK) complex. Activation of the alternative pathway results in nuclear accumulation of p52/RelB heterodimers and depends on the activation of the NF-κB-inducing kinase (NIK) and IKKα phosphorylation (reviewed in (10)). NIK (MAP3K14) is a serine/threonine kinase described as critical for the activation of the alternative pathway by inducing phosphorylation of IKKα and p100, leading to p100 processing with subsequent p52 generation and nuclear translocation (11, 12). Nevertheless, the involvement of NIK in the activation of the classical pathway has also been noticed (13, 14). Signals from
CD40, B-cell activating factor receptor (BAFF-receptor) and lymphotoxin \( \beta \) receptor have been shown to induce NIK-dependent NF-\( \kappa \)B activation (15-18). In multiple myeloma, several genetic abnormalities, such as deletions and inactivating mutations of the TNF receptor-associated factor 3 (TRAF3), have been shown to lead to the stabilization of NIK (19). Similarly, in B cell lymphomas, activating mutations in the BAFF-receptor are known to activate NF-\( \kappa \)B in a NIK-dependent manner (20). Knockdown of NIK in several types of malignancies, such as multiple myeloma, diffuse large B cell lymphoma, adult T cell leukemia and melanoma, has been associated with antitumor effects (14, 16, 21, 22), suggesting that NIK could be a therapeutic target in some cancers. However, whether NIK is involved in NF-\( \kappa \)B activation and tumorigenesis or not, is dependent on the cell type and cellular context and has not been studied in PTCL. Cutaneous T cell lymphoma cell lines and primary samples undergo apoptosis upon treatment with the proteasome inhibitor bortezomib or an IKK\( \beta \) inhibitor (23-25), suggesting that NF-\( \kappa \)B inhibition could be employed as a therapeutic strategy. However, most studies performed address only the classical pathway, leaving the alternative pathway poorly described. There are no specific NF-\( \kappa \)B inhibitor drugs in clinical use for PTCL today, indicating a need for more detailed studies, the identification of new targets and the development of novel specific inhibitors in the pathway.

In the present study, we show that both the alternative and classical NF-\( \kappa \)B pathways are activated in a subset of primary PTCL samples associated with poor clinical outcome. In order to block the NF-\( \kappa \)B signaling pathway in PTCL we demonstrated a key role for NIK as a regulator of both pathways and showed that NIK depleted PTCL cells present strongly reduced cell viability. These results highlight NIK as an attractive molecular target in T cell lymphomas.
MATERIALS AND METHODS

Patient samples and cell lines

Use of patient samples in this study was approved by the Clinical Research Ethics Committee of Hospital Universitario Marqués de Valdecilla, HUMV (Santander, Spain). Tumor biopsies before treatment were obtained from the CNIO Tumour Bank Unit (Madrid, Spain). T cells from peripheral blood of healthy donors or Sézary Syndrome patients were isolated through negative selection using the RosetteSep Kit (StemCell Technologies, Grenoble, France). The proportion of T cells (CD3+) in the samples was checked by flow cytometry and ensured to be >90%. For the gene expression data in primary samples, 37 frozen PTCL cases were used, including 19 PTCL-not otherwise specified (PTCL-NOS), 15 angioimmunoblastic T cell lymphomas (AITLs) and 3 anaplastic large cell lymphomas (ALCLs). The human T cell lymphoma cell lines DERL-7 (hepatosplenic gamma-delta T cell lymphoma) and SR-786 (ALCL) were obtained from the German Collection of Microorganism and Cell Cultures (DSMZ, Braunschweig, Germany). HuT 78 (Sézary Syndrome), HH (cutaneous T cell lymphoma) and MJ (PTCL, HTLV positive) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), and the cell line My-La (Mycosis fungoides) was obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). All cell lines were previously authenticated (year 2010-2011) by DSMZ.

Immunohistochemistry

Tissue microarrays of paraffin-embedded tumor biopsies or cell lines were used for immunohistochemistry and evaluated by the pathologists participating in the project (M.P. and S.M-M). Cases presenting nuclear staining in >20% of tumor cells were considered positive, grade 1 (pos +), while
cases with strong nuclear staining in >50% of cells were considered positive, grade 2 (pos ++). Antibodies and conditions are summarized in Supplementary Table S1.

**Survival and correlation analyses**

The Kaplan-Meier method applying the log-rank test was used to estimate the differences in overall survival between NF-κB-positive and negative cases in a series of 77 PTCL patients (Table 1). Samples presenting nuclear staining of NF-κB (p52, p50, RelB, p65 or c-Rel) in over 50% (pos++) of tumor cells were considered positive for NF-κB. Multivariate survival analysis including relevant clinical parameters (see Table 1) was performed using a Cox regression analysis. To determine the independence of variables, Pearson’s χ² test was used. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used to carry out these tests. Values of p<0.05 were considered statistically significant.

**Quantitative RT-PCR**

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 500 μg DNaseI-treated RNA with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers. Human GUSB and 18S expression were used as endogenous controls in samples and cell lines.

**Immunoblotting**

Protein was extracted using RIPA lysis buffer supplemented with protease and phosphatase inhibitors and western blot was performed following standard protocols. For NIK detection, cell lines were treated for 3 hours with 20 nM proteasome inhibitor MG132 (Sigma, Missouri, USA). MG132 was exclusively
added to cells for NIK detection, and separate untreated cells were collected to detect other proteins. Western blots were quantified using the ImageJ program (National Institute of Health, Bethesda, MD, USA).

**Gene expression microarray analyses**

All gene expression experiments used 4x44K Whole Human Genome Oligo Microarrays (Agilent Technologies, Inc., Santa Clara, CA, USA). For gene expression analysis of primary PTCL samples, 37 frozen biopsies were used, and the Gene Set Enrichment Analysis (GSEA) tool (http://www.broad.mit.edu/gsea/) was applied to classify the expression profiles into functional pathways. The gene sets correlated with MAP3K14 (NIK) expression in primary samples were identified using Pearson correlation. Gene sets with an FDR<0.10 were considered significant. The microarray data are available at the Gene Expression Omnibus under accession number GSE36172. After NIK knockdown in cell lines, pellets were collected 48 hours after siRNA transfection and RNA was extracted. Three independent experiments were performed in My-La and 5R-786 cell lines, and each sample was hybridized onto a separate microarray. The gene sets up- or downregulated in NIK knockdown cells were identified with the GSEA tool by applying a limma t-test (FDR<0.10). Genes differentially expressed between control cells and NIK knockdown cells were identified using a paired t-test (http://pomelo2.bioinfo.cnio.es) and visualized using Gene Cluster and Treeview (http://rana.lbl.gov/EisenSoftware.htm).
RNA interference

Small interference RNAs (siRNAs) against MAP3K14 (NIK), IKBKB (IKKβ) and CHUK (IKKα), or a non-template control (100 nM stealth siRNA, Invitrogen) were used for genetic silencing. SiRNAs were introduced into the cells by microporation (Microporator MP-100, Digital Bio, Seoul, South Korea).

NF-κB binding activity

Nuclear cell fractions were isolated using the BioVision Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA, USA) and 5 μg of nuclear extract were used to quantify the NF-κB transcription activation using the ELISA-based TransAM NF-κB Family Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA), following the manufacturer’s instructions. Nuclear extracts were plated in triplicate.

Cell viability and cell cycle analysis

Cell viability and cell cycle were assessed in a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (FlowJo version 7.6.1, TreeStar Inc., Ashland, OR, USA) was used for data quantification. Cells staining negative for AnnexinV-APC and DAPI were considered viable. To estimate the number of cells in each stage of the cell cycle, cells were fixed with ethanol and stained with propidium iodide.

Reagent information, primer sequences and additional protocols are described in the online Supplementary Information.
RESULTS

**Nuclear NF-κB is present in primary PTCL samples and is associated with worse clinical outcome**

Even though NF-κB activation has been reported in PTCL, there are few studies of patient material, and the expression pattern of the various NF-κB members has not been described in detail. We performed immunohistochemistry on paraffin-embedded tissues from patients with the most common types of PTCL: angioimmunoblastic T cell lymphoma (AITL), anaplastic large T cell lymphoma (ALCL) and PTCL-not otherwise specified (PTCL-NOS), in order to examine the nuclear expression of NF-κB. Nuclear expression of components defining both the classical (p50 and c-Rel) and alternative (p52 and RelB) pathways (Figure 1A and 1B) was detected in the majority of PTCLs. To represent different levels of NF-κB expression, two positivity thresholds were used; 20% (pos +) and 50% (pos ++) positive nuclei. Surprisingly, the commonly used marker for NF-κB activation, p65, showed only cytoplasmic expression in most cases, suggesting that other factors of the family are more frequently involved in NF-κB signaling in PTCL. A subset of cases expressed highly elevated levels of NF-κB (pos ++), being most frequent in the CD30-positive ALCLs. Moreover, a significant positive correlation (p<0.05) was established between nuclear p50 and p52 expression (Figure 1C), indicating frequent activation of both pathways in the same sample.

Contradictory results have been reported for the clinical correlation of NF-κB in different tumors (5, 26-28). To estimate the impact of nuclear NF-κB expression on the clinical outcome of patients with PTCL, we did a Kaplan-Meier analysis to compare the overall survival (OS) between NF-κB positive and negative tumors in a series of 77 PTCL cases (Figure 1D, see patient characteristics in Table 1). Tumors with >50% positive nuclei for any NF-κB factor were considered positive. Similar thresholds have previously been used for immunohistochemistry of NF-κB in other studies in the lymphoma field (6, 28, 29). Only PTCL-NOS and AITL subtypes were included in the survival analysis, since these patients did not
present any significant difference in basal OS due to PTCL subtype (Supplementary Figure S1). When NF-κB was taken into account, however, patients with NF-κB-positive tumors had a significantly inferior OS than the negative group (log rank test, p=0.003) with a 2-year OS of 41.7% compared with 67.9%. NF-κB activation in PTCL was still significantly associated with inferior OS when the impact of other clinical covariates was taken into account, although the International Prognostic Index (IPI) was still a more accurate estimate of prognosis in our series (Supplementary Table S2). The observation that NF-κB signaling is activated in a subset of T cell lymphomas with very poor clinical outcome, suggests the merit of evaluating NF-κB inhibition strategies in these tumors.

NIK is overexpressed in PTCL cell lines and primary samples and its expression is correlated with NF-κB activation

Since NIK is involved in the activation of NF-κB in some cells and settings, we examined whether NIK was involved in NF-κB activation in T cell lymphomas. We observed a remarkable overexpression of NIK mRNA in several PTCL cell lines and primary Sézary Syndrome samples compared with T lymphocytes from healthy donors (Figure 2A). The cell lines expressing high levels of NIK mRNA, also expressed elevated protein levels of NIK (Figure 2B). While DERL-7 lacked nuclear expression of alternative NF-κB factors (p52 and RelB), the other cell line with low NIK levels, HuT 78, expressed both nuclear p52 and RelB (Supplementary Table S3). However, HuT 78 expresses a truncated form of p100 (Figure 2B) which has previously been described to activate the alternative pathway in a NIK-independent manner (30). In order to detect NIK protein levels in primary PTCL samples, we tested a handful of different NIK antibodies in paraffin-embedded tissues, but none of these provided consistent results. For that reason we used gene expression microarray data to compare the expression of NIK (MAP3K14) and NF-κB target genes (6) in a series of 37 PTCL samples. Using Gene Set Enrichment
Analysis, we found a significant positive correlation between the expression of NIK and NF-κB target genes (Figure 2C), suggesting that NIK possibly is involved in NF-κB signaling in these tumors. Other gene sets significantly correlated with the expression of NIK are listed in Supplementary Table S4.

NIK is involved in both classical and alternative NF-κB signaling in T cell lymphomas

Whether NIK participates in classical NF-κB pathway activation as well as in alternative pathway regulation is currently unclear. In order to study the role of NIK in NF-κB signaling in T cell lymphoma cells, we knocked down NIK using two different siRNAs in two PTCL cell lines (My-La and SR-786). The two siRNA sequences induced different levels of knockdown efficiencies (Figure 3A), allowing us to study the effect of a dose-dependent decrease in NIK. As expected, the levels of p52 were reduced and the levels of p100 were initially increased after NIK knockdown indicating an attenuation of p100 processing. We also observed a similar but somewhat delayed decrease of p50 levels and an increase of the levels of p105, linking NIK to the regulation of classical NF-κB activation as well (Figure 3A, C and Supplementary Figure S2). Figure 3B confirms that the knockdown of NIK gave rise to decreased nuclear levels of both p52 and p50. The NF-κB DNA-binding activity of all five proteins, measured by TransAM ELISA assay, was decreased, supporting a role for NIK in both classical and alternative NF-κB regulation in PTCL (Figure 3D).

NIK is necessary for the survival of PTCL cell lines with high NIK levels

According to our data, My-La and SR-786 are characterized by NIK overexpression and an active NF-κB pathway (Figure 2A,2B and Supplementary Table S3). We measured cell viability after NIK
silencing in these cells by flow cytometry using AnnexinV and DAPI staining. NIK knockdown led to a
dramatic increase in cell death compared with non-template control (NTC) transfected cells, suggesting
that NIK is necessary for the survival of these cells. The cell death observed after NIK knockdown
increased as knockdown became more efficient (Figure 4A). To study the effect of NIK knockdown over a
longer period, we repeated the siRNA transfection 4 days after the first microporation. Strikingly, after 1
week of NIK depletion, nearly all cells had undergone apoptosis, demonstrating the essential role of NIK
in the viability of these tumor cells (Figure 4B). The appearance of cleaved caspase 3 after NIK
knockdown in both My-La and SR-786, indicates an induction of a caspase-dependent apoptotic
pathway (Figure 4C). Interestingly, NIK silencing in DERL-7 or HuT 78 cell lines, presenting low NIK levels,
had no effect on cell survival (Figure 4D), suggesting that NIK targeting has a selective effect only on the
cells presenting elevated NIK levels. No obvious alterations of the cell cycle distribution were observed
after NIK knockdown, apart from there being more cells in sub-G1 (Supplementary Figure S3).

To rule out whether the toxicity of NIK knockdown was due to a blockade of the classical or
alternative NF-κB pathway, we knocked down either IKKα, IKKβ, or both, in these cell lines (Figure 4E
and Supplementary Figure S4). Surprisingly, IKKα and IKKβ knockdown (separately or in combination)
only led to a slight increase in apoptosis compared with NIK knockdown. Moreover, only the knockdown
of NIK, but not the knockdown of IKK, was able to strongly reduce the levels of p52 and p50 (Figure 4E
and F). These results suggest that NIK might have, at least in part, IKK-independent roles that confer
survival on these cells, and that targeting NIK could be a more effective therapeutic approach than IKK
inhibition in these tumors.
NIK knockdown leads to decreased expression of NF-κB target genes and downregulation of prosurvival genes

To gain a better insight into the mechanisms involved in NIK-dependent survival, we analyzed the gene expression profile induced 48 hours after NIK knockdown in My-La and SR-786 cells. Knockdown efficiencies are shown in Supplementary Figure S5. Gene Set Enrichment Analysis applying a limma t-test comparing siNIK1 and siNIK2 with control cells was performed. The gene sets significantly lost (FDR<0.10) in the siNIK cells included the NF-kB target genes, the JAK-STAT pathway and targets of XBP1, and are described in Supplementary Table S5. NF-κB target genes were significantly underrepresented after NIK knockdown, indicating again a pivotal role for NIK in NF-κB activation (Figure 5A). A paired t-test revealed 395 genes in My-La, and 94 genes in SR-786, that were significantly and differentially expressed (FDR<0.05 and log₂ foldchange >0.6 in either direction) between NIK knockdown and control cells (Figure 5B, see Supplementary Table S6 for the complete list of genes). RT-qPCR was performed on selected genes to validate the gene expression data (Figure 5C and D). Several NF-κB target genes involved in cancer cell survival were downregulated upon NIK silencing, such as the antiapoptotic BCL2L1 (Bcl-x(L)) and CFLAR (c-FLIP) as well as several interleukins. Interleukin 6 (IL-6) and interleukin 21 (IL-21) are cytokines with known functions in cell proliferation and survival of cancer cells and were strongly downregulated after NIK depletion in both cell lines. Curiously, in spite of an activated NF-κB pathway in DERL-7 and HuT 78 as well, only the NIK-expressing My-La and SR-786 express high levels of these interleukins (Supplementary Figure S6), indicating a different NF-κB transcriptional program in NIK-expressing versus NIK-non-expressing cells. Apart from known NF-κB target genes, NIK depletion also modulated the expression of other genes involved in tumorigenesis, such as Yes-associated protein 1 (YAP1), paraoxonase 2 (PON2) and Kruppel-like factor 2 (KLF2). In summary, NIK
knockdown leads to a decrease in the expression of NF-κB target genes and modulates the expression of many genes involved in tumor growth and survival.
DISCUSSION

The molecular pathology of peripheral T cell lymphomas has been poorly characterized, while the dismal prognosis of these neoplasms and the lack of efficient therapies demand further studies to clarify their molecular background and to identify new therapeutic targets. Previous data suggest that NF-κB activation is a key step in T cell lymphoma pathogenesis (21, 23). NF-κB activation has previously been reported in a subset of human PTCLs using gene expression data and immunohistochemistry for classical NF-κB subunits (24, 27, 29, 31). However, only a few studies have described the alternative pathway status or the clinical impact of this activation. Here, we found markers of activation of the classical and alternative pathways in a subset of PTCL samples, characterized by the nuclear expression of p50, p52, RelB and c-Rel, while nuclear expression of p65 was usually absent. Nuclear expression of classical or alternative components was significantly associated with worse overall survival in PTCL patients, suggesting that aberrant NF-κB activation may confer enhanced survival or treatment-resistance on these tumors, and supports the exploration of therapies that interfere with NF-κB activation. These findings mimic those already described in diffuse large B cell lymphoma, where the more aggressive activated B-cell-like (ABC) subtype is distinguished by increased NF-κB activation (5, 26).

In contrast to our results, Martínez-Delgado et al (27) associated the expression of NF-κB-related genes, using gene expression microarrays, with a favorable clinical outcome in PTCL. These differences might be explained by the genes included in the NF-κB signature (not restricted to NF-κB target genes) and the signals from the tumor stroma in Martínez-Delgado et al., versus the nuclear expression of NF-κB subunits as assessed by IHC in our study.

Even though there is evidence that NF-κB inhibition has an antitumor effect in some circumstances (5, 32), there is still a need to identify therapeutic targets in the pathway and to develop specific NF-κB inhibitors. NF-κB inhibitors targeting mutated or aberrantly expressed molecular targets
in a particular tumor might be preferable to broad NF-κB inhibition that also abolishes normal NF-κB activation. We showed that NIK was highly overexpressed in a subset of PTCL cell lines and tumor samples and that its expression was significantly associated with NF-κB activation. NIK has previously been found to be overexpressed at the RNA and/or protein level in other cancers, such as melanoma, multiple myeloma, diffuse large B cell lymphoma, MALT lymphoma and adult T cell leukemia (14, 16, 21, 22, 33). In some cases, genetic alterations such as gene amplifications, translocations or mutations in NIK, or alterations in genes regulating the stability of NIK protein, are described, emphasizing the role of NIK in tumorigenesis (14, 19, 34). From previous reports, the role of NIK in the alternative NF-κB pathway is clear, but the involvement of NIK in the regulation of the classical pathway appears to be signal- and cell type-dependent (13, 35). For example, in melanoma and pancreatic cancer, NIK only affects the alternative pathway (22, 36), while NIK regulates both pathways in DLBCL and multiple myeloma (14, 16). In PTCL, we demonstrated that NIK is involved in both classical and alternative pathway activation, since NIK knockdown led to decreased expression and DNA-binding activity of both classical (p65, p50 and c-Rel) and alternative (p52 and Rel-B) NF-κB transcription factors. Moreover, NIK knockdown also altered the expression of many genes commonly associated with classical NF-κB activation, such as IL6, IL10 and NFKBIA (37, 38). However, NIK does not appear to be strictly essential for NF-κB activation in all PTCL cells, since we found that nuclear NF-κB was not exclusively expressed in NIK-overexpressing cell lines. Thus, the pathway may be activated by different mechanisms in these cells and a proper identification of upstream mechanisms is important for efficient NF-κB inhibition. The fact that NIK knockdown led to a more efficient reduction of p50 and p52 compared with IKKα or IKKβ knockdown, suggests that NIK can, at least in part, regulate the NF-κB pathway by IKK-independent mechanisms. Consistent with these results, direct phosphorylation of p100 in an IKKα-independent manner has been reported (11). It is not clear whether the effect on p105 processing is a direct effect of NIK or an indirect effect of reduced alternative activity.
In order to investigate the possible downstream effectors of NIK-regulated tumorigenesis, we performed gene expression analysis of NIK-silenced T cell lymphoma cells. As expected, many of the genes differentially expressed between NIK-silenced and control cells were NF-κB target genes known to be important in regulating tumor growth and survival. The expression of anti-apoptotic proteins is one of the mechanisms by which tumor cells manage to survive in the environment and to resist chemotherapy (39). After NIK knockdown, we observed a downregulation of anti-apoptotic genes such as CFLAR (Bcl-xl), BIRC3 (cIAP2) and BCL2L1 (c-FLIP), which might explain the strong induction of apoptosis observed in these cells after NIK inhibition. The interleukins IL-6 and IL-21, which were highly expressed in cell lines that were sensitive to NIK knockdown but not in resistant cell lines, were strongly downregulated after NIK depletion. These cytokines are important for B and T cell development and a proper regulation of the immune response, but have also been widely studied in several tumors because of their protumorigenic activity and their roles as targets for cancer therapy (40, 41). Both these cytokines are NF-κB target genes but are also involved in JAK-STAT signaling, which also fits our observation that NIK knockdown leads to downregulation of the JAK/STAT pathway. The expression of other genes involved in tumorigenesis, not described as NF-κB targets, was also modulated after NIK knockdown. For example, the expression of the oncogene YAP1 (42) was reduced and an upregulation of KLF-2, a tumor suppressor with antiproliferative effect known to be silenced in tumor cells (43), was observed. This can indicate either NF-κB-independent functions of NIK or interaction between the NF-κB pathway and other pathways (44).

The upstream signaling events leading to NIK overexpression and/or NF-κB activation in T cell lymphomas remain to be clarified. Several factors are known to trigger NF-κB activation in other tumors, including signals provided by the tumor microenvironment or mutations that give rise to a constitutive activation of the pathway. In PTCL, apart from the previously reported p100 truncations (7, 45), genetic lesions in NIK or other NF-κB pathway genes have still not been reported. Other possible contributions
to NF-κB activation in T cell lymphomas could be oncogenic viruses (46), signals from CD30 or the T cell receptor (47, 48), or epigenetic mechanisms, such as the previously reported Polycomb-mediated repression of mir31(49). Even though we observed a lack of mir31 in our cell lines, this absence was independent of the levels of NIK (data not shown). It could also be hypothesized that the activation of NIK and NF-κB can be an effect of deregulated signaling of other pathways linked to T cell lymphomagenesis, such as the PI3K/AKT, Notch or JAK/STAT pathway, which can all result in NF-κB activation (44).

The present study reveals a pivotal role for NIK in the survival of T cell lymphoma cells. NIK knockdown strongly reduces the cell viability of PTCL cell lines and is shown to be more effective than IKK inhibition in these cells. Importantly, the fact that cells with a low level of expression of NIK were not affected by NIK knockdown suggests a selective toxicity of NIK inhibition in NIK-overexpressing lymphoma cells. Moreover, although NIK-deficient mice exhibit defects in lymphoid organogenesis, they do not present any gross phenotypic changes (50), suggesting that pharmacological inhibition of NIK might be safer than broad NF-κB inhibition. To develop NF-κB-based cancer therapies efficiently and safely, it is necessary to identify molecular targets as well as biomarkers with which to stratify patients who are likely to benefit from the therapy. Peripheral T cell lymphomas are highly aggressive malignancies that currently lack efficient therapies. Our findings indicate that NIK is a promising molecular target in NIK-overexpressing PTCL, a conclusion that should be taken into account in further validation and development of specific NIK inhibitors.
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REFERENCES


### Table 1. Characteristics of patients included in the survival analysis

<table>
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<th>Characteristics</th>
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*Note: IPI = International Prognostic Index, ECOG = Eastern Cooperative Oncology Group, LDH = Lactate dehydrogenase, AITL = Angioimmunoblastic T cell lymphoma, PTCL-NOS = Peripheral T cell lymphoma, not otherwise specified.*
FIGURE LEGENDS

Figure 1. Expression of NF-κB in human PTCL samples. (A) Immunohistochemical staining of p100/p52, p105/p50, p65, RelB and c-Rel in primary paraffin-embedded PTCL samples shows nuclear staining of NF-κB in most cases (B). The category pos + indicates nuclear staining of >20% of the tumor, while pos ++ indicates nuclear staining in >50% of the tumor. The percentage of the total number of cases (57 PTCL-NOS, 42 AITLs and 28 ALCLs) in each PTCL subtype is shown. Comparison of the numbers of tumors with high levels of nuclear expression (pos ++) of p50 or p52 reveals a significant positive correlation between their expression, in which the majority of cases positive for p52 also were positive for p50 (C). (D) Kaplan-Meier curve representing the overall survival times of NF-κB-positive and NF-κB-negative PTCL patients. The NF-κB-positive group (represented in red) has a significantly worse overall survival (p<0.05, log rank test) than the NF-κB-negative group (blue line).

Figure 2. NIK expression in peripheral T cell lymphoma cell lines and samples. (A) RT-qPCR analysis of NIK expression in PTCL cell lines and isolated T cells from Sézary Syndrome patients (SS1-SS5) showed strongly elevated levels of NIK compared with T cells from healthy donors (Control 1-3). (B) NIK protein levels were detected after MG-132 treatment and a comparable expression pattern was seen between NIK mRNA and protein levels among the cell lines. (C) Gene Set Enrichment Analysis of gene expression microarray data from 37 PTCL cases shows a significant positive correlation (FDR<0.10) between the expression of NIK (MAP3K14) and NF-κB target genes.

Figure 3. Knockdown of NIK in T cell lymphoma cell lines. (A) NIK was knocked down using two siRNAs (siNIK1 and siNIK2) or a non-template control (siNTC) in My-La and SR-786. After NIK knockdown, the expression of NIK, p100/p52 and p105/p50 was analyzed by western blot. (B) Nuclear expression of p50 and p52 after NIK knockdown in My-La. The quantification of the signal is represented under each blot.
as a ratio against the siNTC transfected cells. (C) Quantification of the immunoblots confirms a decrease in the levels of both p50 and p52 after NIK knockdown. The data are represented as the mean ± SD of three independent experiments. (D) DNA binding activity of p52, RelB, p50, p65 and c-Rel 48 hours after NIK knockdown was measured using the ELISA-based TransAM assay and reveals a reduction of classical and alternative NF-κB activation.

**Figure 4. The effect of NIK knockdown on PTCL cell survival.** (A) NIK knockdown (siNIK) induces cell death in My-La and SR-786 cells compared with untransfected (MOCK) or non-template transfected (siNTC) cells, as measured by flow cytometry using DAPI and AnnexinV staining. The data are represented as the mean ± SD of three independent knockdown experiments. (B) Only 1.26% of cells remain viable 7 days after NIK knockdown in My-La (DAPI/AnnexinV negative). (C) The cleavage of caspase-3 was detected after NIK depletion in both MyLa and SR-786 cells. (D) Increase in cell death in cell lines with high (My-La and SR-786) or low (HuT 78 and DERL-7) NIK expression, represented as the ratio between the values of cell death in NIK knockdown (siNIK2) and control (siNTC) cells. (E) Knockdown of IKKα, IKKβ or NIK in My-La and evaluation of the expression of p100/p52 and p105/p50. (F) Percentage of non-viable SR-786 cells after IKKα, IKKβ or NIK silencing demonstrate a more potent induction of apoptosis after NIK knockdown compared with IKK knockdown.

**Figure 5. Gene expression profile after NIK knockdown.** Whole genome microarray analysis was performed 48 hours after NIK knockdown in My-La and SR-786. (A) GSEA enrichment plot of the NF-κB target genes. The NF-κB target genes were significantly underrepresented in the NIK knockdown cells. (B) Heatmaps of differentially expressed genes (FDR<0.05 and log₂ foldchange >±0.6) between control and NIK knockdown cells. Negative log₂ foldchanges (ratio siNIK/control) are represented in green (downregulation in siNIK cells) and positive foldchanges are represented in red (upregulation in siNIK
cells). (C and D) Relative gene expression in siNIK cells compared with the non-template control (NTC) measured by RT-qPCR.
Figure 2

A

NIK expression in PTCL cell lines

Control

NIK expression in Sézary Syndrome T cells

Control

B

NIK

α-tubulin

p100

p52

p105

p50

α-tubulin

C

Enrichment plot NF-κB target genes

Rank in Ordered Dataset

Enrichment score (ESS)

zero score at 10,000

Enrichment profile

Ranking metric scores

MAP3K14 (positively correlated)

MAP3K14_neg (negatively correlated)
Figure 3

A

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- NIK
- α-tubulin
- p100
- p52
- α-tubulin
- p105
- p50
- α-tubulin

B

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- NIK
- α-tubulin
- p100
- p52
- α-tubulin
- p105
- p50
- α-tubulin

C

p52 and p50 levels in My-La after NIK knockdown

D

NIK knockdown in My-La

DNA binding activity (OD 450nm)
NIK knockdown in My-La

NIK knockdown in SR-786

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Cleaved Caspase 3
α-tubulin

My-La

Cleaved Caspase 3
α-tubulin

SR-786

Increase in cell death (Fold change siNIK/siNTC)

My-La

SR-786

NIK knockdown in SR-786

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IKK-β
IKK-α

p100
p52
p105
p50
α-tubulin

Research.
Figure 5

A
Enrichment plot NF-κB target genes

B

My-La

SR-786

C

My-La

SR-786

D

KLF-2 expression
NIK controls classical and alternative NF-kB activation and is necessary for the survival of human T cell lymphoma cells

Lina Odqvist, Margarita Sanchez-Beato, Santiago Montes-Moreno, et al.

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