μg/ml of puromycin for an additional 48 hours. These infectants were subjected to immunoblotting, EMSA and transient transfection with 2 μg of IgκCona-luc[30] and pEF1-LacZ[26] using DMRIE-C (Invitrogen) according to the manufacturer's instructions. Assays for luciferase and β-galactosidase were performed 48 hours after transfection by standard methods. Luciferase activity was normalized on the basis of β-galactosidase activity. The growth of lentivirus-infected cells was determined by the trypan blue staining method.

Immunoprecipitation

For the immunoprecipitation of endogenous NIK, approximately 2×10^7 cells were lysed in buffer A (20 mM Tris-HCl, pH7.5, 0.5% Nonidet P-40, 150 mM NaCl supplemented with 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.57 mM phenylmethanesulphonylfluoride (PMSF), 10 μM MG132, 10 μM MG115) followed by pre-clearing with purified rabbit IgG (Cedarlane Laboratories Ltd.) and protein G-Sepharose beads (Pierce, IL, USA). After centrifugation at 14,000 rpm for 3 minutes, supernatants were subjected to immunoprecipitation with purified non-immune rabbit IgG or anti-NIK antibody (#4994) (Cell Signalling Technology). Immunoprecipitates were washed 3 times with TNT buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl and 1% Triton X-100). Endogenous NIK proteins were detected by immunoblotting with anti-NIK antibody (#4994). For the immunoprecipitation of HA-tagged NIK, 750 μg of cell lysates prepared with buffer A were subjected to immunoprecipitation with anti-HA antibody (12CA5, a kind gift from Dr. A. Israël, Institut Pasteur Paris, France). Immunoprecipitates were washed 3

times with TNT buffer. HA-tagged NIK proteins were detected by immunoblotting with anti-NIK antibody. For immunoprecipitation of endogenous IKK1/2, 1,500 μg of cell lysates prepared with buffer A were subjected to immunoprecipitation with anti-IKK1 monoclonal antibody (B78-1) (Becton Dickinson Pharmingen, San Diego, CA) or purified mouse IgG2b (MI10-104) (Bethyl laboratories, Inc.). Immunoprecipitates were washed 3 times with TNT buffer. Expression of endogenous proteins was detected by immunoblotting with anti-phospho-IKK1/IKK2 (Ser180/Ser181) (#2681) (Cell Signalling Technology), anti-IKK1 (H-744) or anti-IKK2 (H-470) (Santa Cruz Biotechnology) antibodies.

Quantitative RT-PCR

Total RNA was extracted using Isogen reagents (Nippon Gene Co.) according to the manufacturer's instructions. Quantitative RT-PCR amplifications were performed with 100 ng of total RNA, 0.3 μM of each primer and 0.25 μM of TaqMan probe using an ABI-7700 sequence detector system (Applied Biosystems): reverse transcription was performed at 48°C for 30 minutes, Taq DNA polymerase was activated at 95°C for 10 minutes, followed by 45 amplification cycles of 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. The *nik*, *vegf*, *icam-1* and *mmp-9* mRNA levels were normalized based on the amount of 18S ribosomal RNA determined simultaneously by the real-time RT-PCR.

Mice and inoculation of cells

NOD/SCID/γc^{null} (NOG)[31] mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the Animal Center of Tokyo Medical and Dental University (Tokyo, Japan). The Ethical Review Committee of the institute approved the experimental protocol. ED40515(-) cells expressing Ctli or NIKi-1 and -2 were washed twice with serum-free RPMI 1640 and resuspended in the same medium. Mice were anesthetized with ether and inoculated subcutaneously in the postauricular region with 5×10^6 cells per mouse, as described previously.[31] We measured tumor size and weight 2 weeks after cell inoculation.

Statistics

Statistical significance was evaluated using a 2-tailed, unpaired Student's t test. P values of < 0.05 were considered to be significant.

Results

NIK is aberrantly expressed in both adult T-cell leukemia and Hodgkin Reed-Sternberg cells

The constitutive processing of p100 to p52 in ATL and H-RS cells[9,10] prompted us to examine whether NIK is aberrantly expressed in both established and primary ATL cells. Immunoblotting of whole-cell lysates prepared from ATL or H-RS cells did not show any detectable NIK signal (data not shown), however, when endogenous NIK was immunoprecipitated from approximately 20 million of these cells and subjected to

immunoblotting, NIK was specifically detectable in anti-NIK immunoprecipitates from ATL and H-RS cells, but not from control cells such as CEM and Jurkat (Figure 1A). Previous studies revealed that inhibition of the proteasome function allowed for detection of endogenous NIK in simple whole-cell lysates of B-cell lines.[19,20] Treatment of ED-40515(-) cells with the MG132 proteasome inhibitor for 3 hours prior to harvesting enabled us to observe robust endogenous NIK expression at the expected position (Figure 1B). Lysates of 293T cells with or without exogenous NIK expression were used as the positive and negative controls, respectively. We next examined the NIK expression levels as well as those of p100 phosphorylated at Serine residues 866 and 870 in a panel of ATL, H-RS and control cells (Figure 1C). No appreciable NIK expression could be observed in control CEM and Jurkat T-cell lines treated with MG132, in which NF-κB is not constitutively activated. Proteasome inhibition induced strong NIK expression in other Tax-negative ATL-derived cell lines, ATL-43Tb(-) and TL-Om1. Proteasome inhibition also strongly augmented NIK expression in H-RS cells, but only weakly so in the control B-cell lines, RG69. These results indicate that the steady state levels of NIK of the authentic size are elevated in ATL and H-RS cells, and suggest that NIK may be abundantly produced in ATL and H-RS cells, but is rapidly degraded by the proteasome. The levels of NIK expression well correlated with those of phosphorylated p100 (Figure 1C). Moreover, p52 and the phoshorylated form of IκBα were also abundant in ATL and H-RS cell lines, but not in the control T-cell lines (Figure 1C). These results indicate that the overexpression of NIK is closely linked to the downstream events leading to constitutive activation of the

canonical and non-canonical NF-κB pathways in ATL and H-RS cells. A previous study suggested that L428 cells express a C-terminally truncated form of IκBα and that phosphorylated form of this protein were accumulated after treatment of the cells with proteasome inhibitor or dexamethasone[32,33]. In agreement with this, we did not detect IκBα expression with the antibody used in this study, which recognizes the C-terminus of the protein, but detected the phoshorylated form of this IκBα only after treatment with MG132 (data not shown).

We next investigated *nik* expression at the mRNA level by quantitative PCR (Figure 1D) and found that that *nik* transcripts were at between 20- to 100-fold higher levels in ATL and H-RS cells, compared with CEM cells. Next, actinomycin D was used to block new mRNA synthesis, so that decay of existing transcripts could be detected. Quantitative PCR analyses revealed that the half-life of *nik* mRNA was ~3 hours both in the ATL and control T-cells (Figure 1E). Essentially similar results were obtained with the other cell lines shown in Figure 1D including H-RS cell lines (data not shown). A previous report has demonstrated that NF-κB is constitutively activated in primary ATL cells in the peripheral blood[34]. We therefore quantified the *nik* mRNA levels in peripheral blood mononuclear cells (PBMCs) from both healthy donors and ATL patients (Figure 2A), and found that *nik* mRNA is overexpressed in PBMCs of 15 out of 21 ATL patients. Actinomycin D treatment of PBMCs further revealed that *nik* mRNA was not apparently stabilized in primary ATL cells (Figure 2B). Moreover, fluorescence *in situ* hybridization (FISH) studies on primary ATL cells failed to detect amplification or translocation of the *nik* gene (Supplemental figure 1 and Supplemental

table 2). Finally, when PBMCs were cultured for 3 hours in the presence of MG132, NIK protein was detectable in cells from an ATL patient showing abundant *nik* mRNA expression, but not in those from a healthy donor (Figure 2C).

NIK transforms rat fibroblasts in an NF-κB-dependent manner

To further explore the roles for NIK during cell transformation, we infected the 3T3-like rat fibroblast cell line Rat-1 with a retroviral vector expressing human NIK and examined its oncogenic activity. As expected, cells transduced with this NIK vector exhibited strong NF-κB DNA binding activity within 36 hours (data not shown). Rat-1 cells transduced with a control retrovirus became resistant to the selection marker puromycin approximately 24 hours after infection and continued to proliferate rapidly. In contrast, Rat-1 cells transduced with the NIK expression vector expressed a readily detectable level of NIK, had a transformed morphology, but ceased proliferating and died within 3 to 4 days after becoming resistant to puromycin. Cells that survived two weeks of puromycin selection following NIK transduction eventually appeared indistinguishable from those transduced with the control vector, and showed no detectable NIK expression nor NF-κB DNA binding activity (data not shown).

Based upon these observations, we speculate that the retroviral over-expression of NIK is toxic to the cells so that only cells that had lost its expression could emerge from the puromycin-resistant pools. To address this problem, we employed B5 and h12 cells carrying an integrated Igκ2bsrH plasmid that confers resistance to the antibiotic Blasticidin S when cells are constitutively expressing active NF-κB.[26] B5

cells are derived from Rat-1 cells, and h12 cells are from 5R cells that lack NEMO expression. When the B5 and h12 cells were transduced with the wild type NIK retroviral expression vector and subjected to selection with both puromycin and blasticidin S, the majority of the resultant cell clones maintained both the initial transformed cell morphology (Figure 5B) and detectable NIK expression (Figure 3A). On the other hand, when B5 and h12 cells were transduced with a retrovirus vector expressing a catalytically inactive mutant form of NIK and selected with puromycin alone, the cells successfully expressed this protein (Figure 3A) without significant morphological change (Figure 5B) or constitutive NF-κB activation (Figure 3C). As expected, these cells failed to survive selection with blasticidin S (data not shown).

The expression of wild type NIK in B5 and h12 cells potently induces p52 expression and NF-κB DNA binding activity, whereas the catalytically inactive NIK mutant does not (Figure 3B-C). We also found a specifically phosphorylated form of IκBα in cells expressing wild type NIK (Figure 5A). Super-shift experiments revealed that the NF-κB-DNA binding complexes in B5 and h12 cells expressing NIK involve p50, RelB and RelA (Figure 3D). The presence of p52 in the DNA binding complexes could not be examined, however, because an antibody recognizing rat p52 in super-shift assay is not currently available. Instead, we analyzed DNA-binding complexes induced by NIK expression in wild type mouse embryonic fibroblasts (MEFs) (Supplemental figure 2). Retroviral overexpression of NIK indeed induced DNA-binding NF-κB complexes containing p52, and enhanced expression of p52 and phosphorylated form of IκBα. We have previously demonstrated that the treatment of

ATL cells with MG132 greatly enhances IKK activity, whereas protein synthesis inhibition quickly abolished this activity.[11] Figure 4 shows that the IKK activity in B5 cells stably expressing NIK (NIK#1) is modulated by MG132 and cycloheximide (CHX) in a manner that is very similar to that seen in ATL cells. In addition, treatment of NIK#1 cells with MG132 remarkably elevates the level of exogenous NIK expression. The constitutive NF-κB activation caused by the presence of exogenous NIK was found to be abolished by the retroviral expression of a super-repressor form of IκBα (SR-IκBα), without affecting exogenous NIK expression (Figure 5A). Interestingly, the forced expression of SR-IκBα also diminishes the p52 and p100 expression levels.

We next tested the ability of NIK to induce anchorage-independent growth of rat fibroblasts. B5 and h12 cells transduced with the control vector did not form colonies of significant size in soft agar, whereas those transduced with wild type NIK expression vector formed a number of large colonies, as shown in Figure 5B and Table 1. Cells expressing catalytically inactive NIK failed to form colonies in soft agar. The expression of SR-IκBα completely abolished NIK-induced colony formation and also the morphological alterations of B5 and h12 cells. Given that SR-IκBα specifically suppresses NF-κB activation, we conclude from these results that NIK transforms rat fibroblasts in an NF-κB-dependent manner.

NIK mediates constitutive NF-κB activation in ATL cells

The similar modulation of IKK activity by CHX or MG132 in both ATL and B5 cells expressing NIK (Figure 4) suggests that NIK plays an important role in constitutive

NF-κB activation in ATL cells. We therefore examined whether the RNA interference-mediated silencing of endogenous *nik* gene expression would lower NF-κB-dependent transcription in these cells. ED-40515(-) and ATL-43Tb(-) cells were infected with lentiviral constructs that express short hairpin RNA (shRNA) molecules that target mRNA for either *renilla luciferase* (Ctli) or *nik* (NIKi), and then subjected to puromycin selection for 2 days. To suppress NIK expression maximally, we used independently or in combination two shRNAs (NIKi-1 and -2) that target different *nik* sequences and reduce NIK expression. The infected cells were then assayed for transcriptional activity by transient transfection with an NF-κB-dependent reporter gene (Figure 6A). Lentiviral expression of NIKi constructs resulted in suppression of NF-κB-dependent reporter gene expression in ATL cells when independently used, and the combined use of the two NIKi constructs (NIKi-1 and -2) was found to be more effective. We then examined ATL cells transduced with NIKi-1 and -2 constructs for the expression of endogenous NIK and specifically phosphorylated forms of p100, IκBα and IKKs by immunoblotting (Figure 6B) and for NF-κB DNA binding activity by EMSA (Figure 6C). NIK expression in ATL cells was found to be down-regulated by the shRNA-mediated silencing (Figure 6B). As expected, p52 and phosphorylated p100 were also reduced by NIK depletion, and interestingly, phoshorylation of IκBα was also suppressed. This is consistent with the results observed in NIK-transduced rat fibroblasts that express phoshorylated form of IκBα (Figure 5A), indicating that NIK, when aberrantly and stably expressed, induces phosphorylation of IκBα. In addition, NIK depletion suppressed phosphorylation of

the Serine residues in the activation loop of IKKs, suggesting a key role for NIK in constitutive activation of IKKs in ATL cells (Figure 6B). Moreover, depletion of NIK resulted in suppression of NF-κB DNA binding activity (Figure 6C). Super-shift assays revealed that DNA-binding of NF-κB components, p50, p52, RelA and RelB, was reduced by NIK depletion (Figure 6D). As shown previously, c-Rel was not detected in ATL cells.[34] We further investigated alterations in the expression of NF-κB target genes by NIK depletion. Vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9) and intracellular adhesion molecule-1 (ICAM-1), the expression of which has been reported to be under the control of NF-κB,[35-37] are highly expressed in ATL cells and suggested to contribute to their invasive properties.[38-41] Quantitative RT-PCR studies reveal that depletion of NIK results in down-regulation of the expression of these NF-κB target genes (Figure 6E).

NIK regulates tumorigenicity of ATL cells *in vivo*

We finally investigated biological effects of NIK depletion in ATL cells. NIK depletion did not significantly influence the growth of cells in culture (Figure 7A). We then examined if depletion of NIK affects the tumorigenicity of ATL cells in a mouse model. NOD/SCID/γc^{null} mice were subcutaneously inoculated with ED-40515(-) cells that express Ctli or NIKi and are characterized in Figure 6B-C, and tumor formation was evaluated 2 weeks later. As expected, ED-40515(-) cells expressing Ctli efficiently formed large tumors, while tumors formed in mice inoculated with ED-40515(-) cells expressing NIKi were significantly smaller (Figure 7B-D), suggesting that NIK supports

efficient tumor cell growth *in vivo*.

Discussion

Persistent activation of NF-κB has previously been reported to play an essential role in the growth and survival of specific cancer cell types, including ATL, H-RS, melanoma and prostate cancer cells.[9,42-45] Inappropriate NF-κB activation can also contribute to the resistance to the apoptotic responses induced by certain anti-cancer drugs.[46] On the other hand, cancer cell apoptosis can be induced when persistent NF-κB activity is blocked by inhibitors such as SR-IκBα, by drugs targeting IKK or the proteasome, via peptides targeting p50 or NEMO, and by double-stranded oligonucleotides containing NF-κB binding sites.[47,48] One problem with such inhibitors, however, is their lack of specificity to cancer cells, because they also necessarily block normal NF-κB activation. Hence, it would be desirable to specifically inhibit NF-κB activation in cancer cells by identifying molecular targets in each cancer type. Virally transformed cancer cells express a virus-derived regulatory protein(s) that targets critical molecules in a variety of key signaling pathways. Cytokine autocrine loops or genetic alterations to genes regulating the NF-κB signaling mechanisms that lead to persistent NF-κB activation have also been identified in some cancer cells.[16,17,32,47,49] However, the mechanisms underlying persistent NF-κB activation in many types of cancer remain unknown.

Most primary ATL cells, although infected with HTLV-I, are characterized by the loss of viral protein expression including Tax, probably due to the host immune surveillance during the long period of latency.[50] Nevertheless, NF-κB is strongly and

persistently activated in ATL cells through IKK,[9] although the mechanism of IKK activation has remained unknown. The findings in our present study demonstrate the aberrant expression of NIK at the pre-translational level in ATL cells derived from 15 out of 21 patients. This overexpression does not seem to correlate with the patients' age, sex, disease type or percentage of abnormal lymphocytes (supplemental table 1). Further studies will be required to clarify potentially NIK-independent NF-κB activation in the other six cases. The stable expression of functional NIK in fibroblasts, but not that of its catalytically inactive mutant, causes cellular transformation and persistent NF-κB activation with molecular features quite similar to those reported previously in ATL cells. These include the rapid loss of IKK activity after protein synthesis inhibition, and the super-induction of IKK activity in the presence of MG132.[11] Moreover, RNA interference studies have also indicated that the deregulated NIK expression is the principal cause of constitutive NF-κB activation in ATL cells. In line with a previous report by Ramakrishnan et al., which showed that the induction of IκBα degradation by CD70, CD40 ligand, and BLyS/BAFF is dependent on the function of NIK,[18] we find in our present experiments that the stable expression of NIK induces IκBα phosphorylation and the formation of DNA binding complexes containing not only p50 and RelB, but also RelA both in wild type and in NEMO-deficient rat fibroblasts. This indicates that NIK can stimulate the canonical pathway characterized by IκBα phosphorylation and RelA activation and that NIK does not require NEMO for it. Interestingly, the forced expression of SR-IκBα in these fibroblasts abolishes the transformed phenotype and suppresses constitutive NF-κB activity, with the p100 and

p52 expression levels being diminished simultaneously, probably because p100 expression is largely dependent on NF-κB activity.[51] RelB expression is also known to be controlled by NF-κB,[52] suggesting that the non-canonical pathway of NF-κB activation does not work independently, but rather coincides with NF-κB activation through the canonical pathway under stable conditions.

H-RS cells were also found to overexpress NIK, including its transcripts, in this study. Earlier reports have described two potential mechanisms of constitutive NF-κB activation in H-RS cells: persistent signaling from receptors that cause NF-κB activation, such as CD30, CD40 and RANK as well as a CD40-like molecule LMP1 of the EB virus; and disruption of IκBα-dependent suppression due to the mutation of this gene.[32,48] The H-RS cell lines used in this study are EB virus-negative, and neither HDLM-2 nor L540 cells harbor mutations in their *iκb* genes. Indeed, CD30, CD40 and RANK were all found to be expressed in the H-RS cell lines used in this study, but we envisage that the aberrant expression of NIK is a distinct mechanism underlying the persistent NF-κB activation in these cells. It is partly because these TNF family receptor molecules, when stimulated or overexpressed transiently in cultured cells, elevates the NIK protein expression levels with a concomitant reduction in TRAF3, but does not increase *nik* mRNA.[19,20]

Whereas the transient stimulation of a B-cell line with BAFF or anti-CD40 antibody stabilizes the NIK protein at the post-translational level and does not up-regulate its mRNA expression,[20] NIK was observed to be constitutively overexpressed in ATL and H-RS cells at the pre-translational level. These differing mechanisms of NIK

regulation may not be all that surprising, however, in light of the transient versus persistent nature of the activation of NF-κB. The barely detectable levels of steady state NIK protein expression and its robust accumulation following proteasome inhibition in ATL and H-RS cells further suggest that the proteasome-dependent degradation of NIK occurs rapidly in tumor cells as in normal cells, although we cannot rule out the possibility that TNF family receptors known to be overexpressed in H-RS cells influence the stability of NIK to some extent. This point is currently very difficult to address because the protein amount of NIK in the absence of the proteasome inhibitor is quite limited. At least three mechanisms of pre-translational induction of NIK are plausible: the stabilization of *nik* transcripts, transcriptional activation and/or amplification of the *nik* gene. It should be noted that the stability of *nik* mRNA in ATL cells was similar to that in control cells, suggesting that NIK expression is deregulated in ATL cells at the level of mRNA production. In this regard, we are currently analyzing the regulatory region of the *nik* gene in normal and cancer cells.

We detected NIK in whole-cell lysates only when the cells themselves were treated with the proteasome inhibitor, MG132. It is possible that the expression of the NIK protein is tightly regulated under detectable levels in resting normal cells. However, in ATL and H-RS cells, enhanced NIK production, although still not detectable by simple immunoblotting, may be sufficient to cause its deregulated activity toward IKK. During the manuscript preparation, two reports demonstrated deregulated expression of NIK due to mutations in *traf3, cyld or nik* itself in multiple myeloma cells.[16,17] In case of ATL cells, formation of a fusion protein following genomic rearrangement seems to be

unlikely based on the apparently normal size of the protein. At present, the mechanism of overproduction of *nik* mRNA in ATL cells remains to be determined, but the FISH results suggest that aberrant NIK expression in ATL cells is not due to genomic abnormalities such as amplification or translocation.

Successful anti-cancer drug or gene therapies can be conducted in a number of ways, including the general administration of particular reagents that mechanistically work exclusively on cancer cells, or delivering conventional anti-cancer reagents specifically to cancer cells. The former strategy is likely to be more promising in the case of hematopoietic cancers. In this regard, NIK could be an attractive molecular target for ATL and Hodgkin lymphoma therapy, although the physiological functions of NIK in human adults remain unknown. Suppressing high NF-κB activity levels by targeting NIK may also sensitize these cancer cells to commonly used anti-cancer agents.

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Authorship

Contribution: Y.S., T.S. and S.Y. designed the study; Y.S., Norio Yamamoto, H.S., V.J.M.B., Y.I., K.M., X.Q., I.I., J.I., and S.Y. carried out the research; MD.Z.D. carried out the animal experiments; A.U. and T.W. collected and analyzed sample blood from ATL paptients; T.M. contributed to lentiviral vector constructions; Y.S. and S.Y. analyzed the data; T.S., Naoki Yamamoto and S.Y. controlled the data; Y.S. and S.Y. wrote the paper; and all authors checked the final version of the manuscript.

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