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Notch1 augments NF-κB activity by facilitating its nuclear retention

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Notch1 specifically upregulates expression of the cytokine interferon-γ in peripheral T cells through activation of NF-κB. However, how Notch mediates NF-κB activation remains unclear. Here, we examined the temporal relationship between Notch signaling and NF-κB induction during T-cell activation. NF-κB activation occurs within minutes of T-cell receptor (TCR) engagement and this activation is sustained for at least 48 h following TCR signaling. We used γ-secretase inhibitor (GSI) to prevent the cleavage and subsequent activation of Notch family members. We demonstrate that GSI blocked the later, sustained NF-κB activation, but did not affect the initial activation of NF-κB. Using biochemical approaches, as well as confocal microscopy, we show that the intracellular domain of Notch1 ($N1^{IC}$) directly interacts with NF-κB and competes with IκBα, leading to retention of NF-κB in the nucleus. Additionally, we show that $N1^{IC}$ can directly regulate IFN-γ expression through complexes formed on the IFN-γ promoter. Taken together, these data suggest that there are two 'waves' of NF-κB activation: an initial, Notch-independent phase, and a later, sustained activation of NF-κB, which is Notch dependent.

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Introduction

Notch proteins are a family of large (300 kDa) single-pass type I transmembrane receptors, activated by regulated intramembrane proteolysis (Schroeter *et al*, 1998). They function as cell surface receptors and direct regulators of gene transcription, and are involved in a variety of cellular events. In vertebrates, there are four Notch genes, which encode receptors (Notch1–4) for at least five different Notch ligands (Jagged1/Serrate1, Jagged2/Serrate2, Delta1, Delta2 and Delta3) (Artavanis-Tsakonas *et al*, 1999). Notch is activated through binding of appropriate ligands on neighboring cells to the extracellular domain of the Notch receptors (Artavanis-Tsakonas *et al*, 1999). This culminates in proteolytic cleavage by γ-secretase, release and nuclear translocation of the Notch intracellular domain (N^{IC}) from the membrane, leading to the production of several downstream proteins (Artavanis-Tsakonas *et al*, 1999; Robey and Bluestone, 2004). The role of Notch signaling in early T- and B-cell development has been studied extensively, but its contribution to mature T-cell function is not fully understood.

In mammals, the NF-κB family of transcription factors contains five members: NF-κB_1 (p105/p50), NF-κB_2 (p100/p52), RelA (p65), RelB and c-Rel. NF-κB_1 and NF-κB_2 are synthesized as large polypeptides that are post-translationally cleaved to generate the DNA binding subunits p50 and p52, respectively (Caamano and Hunter, 2002; Ghosh and Karin, 2002). Members of the NF-κB family are characterized by the presence of a Rel homology domain, which contains a nuclear localization sequence, and are involved in sequence-specific DNA binding, dimerization and interaction with the inhibitory IκB proteins. Dimeric NF-κB complexes are associated with specific responses to different stimuli and differential effects on transcription. NF-κB1 (p50) and NF-κB2 (p52) lack transcriptional activation domains, and their homodimers are thought to act as repressors (Lee *et al*, 1995; Li and Verma, 2002) unless complexed with Bcl-3, a nuclear binding partner possessing a transactivational domain (Bours *et al*, 1993; Fujita *et al*, 1993). In contrast, RelA, RelB and c-Rel carry transcriptional activation domains, and are able to form homo- and heterodimers with other NF-κB family members with the exception of RelB, which cannot homodimerize. NF-κB is held inactive in the cytoplasm by interaction with an inhibitor of NF-κB (IκB). NF-κB can be activated by cellular exposure to inflammatory cytokines such as TNF or IL-1, by-products of bacterial and viral infection, radiation or T-cell costimulation (Beinke and Ley, 2004). This results in activation of the IκB kinase complex, leading to phosphorylation and degradation of IκB proteins and release of NF-κB. Free NF-κB translocates to the nucleus, activating downstream target genes, including IκBα. Several studies suggest that newly synthesized IκBα enters the nucleus and binds nuclear NF-κB, mediating export of the complex to the cytosol (Arenzana-Seisdedos *et al*, 1997; Rodriguez *et al*, 1999; Hoffmann *et al*, 2002; Nelson *et al*, 2004). Super-repressor IκBα (SR-IκBα) encodes a mutant IκBα protein, unable to be phosphorylated or degraded, which complexes with NF-κB in the cytoplasm keeping it inactive and resulting in decreased IFN-γ secretion (Ferreira *et al*, 1999).

The interaction between antigen-presenting cell (APC) and T cell also results in NF-κB activation in both cell types (Li and Verma, 2002). NF-κB activation is triggered in T cells by the engagement of the T-cell receptor (TCR) and the CD28 co-receptor with their respective ligands, the MHC class

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II-peptide complex and the costimulatory molecules CD80 and CD86, present on APCs (Schmitz *et al*, 2003). Signaling through the TCR and CD28 synergizes to induce the NF-κB-dependent genes required for T-cell activation and proliferation, such as IL-2, IL-2 receptor alpha chain and IFN-γ (Cross *et al*, 1989; Gerondakis *et al*, 1996; Sica *et al*, 1997; Zhou *et al*, 2002; Artis *et al*, 2003). Activated T cells, in turn, elicit NF-κB activation in APCs (Li and Verma, 2002).

We have previously shown that Notch signaling results in the activation of NF-κB, IFN-γ secretion and cell proliferation in murine T cells (Palaga *et al*, 2003). A pharmacological inhibitor specific for γ-secretase inhibitor (GSI) is a useful drug to block proteolytic cleavage of Notch proteins by γ-secretase and prevents the activation of all Notch isoforms. Using GSI treatment to block proteolytic cleavage of Notch receptors or using transgenic mice expressing an antisense Notch1 construct (Notch1AS Mice), we previously demonstrated downregulation of NF-κB activity, IFN-γ secretion and cell proliferation, suggesting that, in concert with TCR signaling, Notch1 induces NF-κB activation. However, precisely how Notch1 regulated NF-κB activity in peripheral T cells remained unclear. Recent studies have suggested possible interactions between Notch and NF-κB (Oswald *et al*, 1998; Espinosa *et al*, 2003; Oakley *et al*, 2003). In this study, we initiated a series of experiments designed to more clearly define the relationship between Notch and NF-κB activity.

Here, we show that GSI pretreatment abrogates the generation and nuclear translocation of active Notch1, leading to downregulation of NF-κB activity. Furthermore, pretreatment with GSI also attenuated the nuclear import and the sustained nuclear activity of NF-κB. Our data demonstrate that in splenocytes, IFN-γ is a direct target of Notch-regulated NF-κB activation. We present a model that suggests that Notch maintains NF-κB activity by direct interaction with p50/c-Rel in the nucleus. This interaction retains active NF-κB complexes in the nucleus, leading to sustained NF-κB activity over time and in the activation of NF-κB-regulated genes.

Results

Stimulating splenocytes with anti-CD3ε and anti-CD28 induces and sustains activation of NF-κB and upregulates p50 and c-Rel

Our earlier studies demonstrated that active N^{IC} increases NF-κB DNA binding activity following stimulation with anti-CD3ε and anti-CD28 antibodies (Palaga *et al*, 2003). To identify the nature of the NF-κB subunits induced, nuclear extracts were prepared from splenocytes from C57BL/6 mice that were left unstimulated or were stimulated with anti-CD3ε and anti-CD28 antibodies for 48 h. EMSA was performed using the oligonucleotide probes corresponding to the NF-κB binding sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′). To characterize the composition of the DNA-bound NF-κB complexes found in activated splenocytes, we performed supershift analysis with antibodies specific for each NF-κB family member. Antibody stimulation enhanced NF-κB DNA binding activity compared to unstimulated controls, as previously shown (Palaga *et al*, 2003). These NF-κB complexes were identified as p50 and c-Rel, based on the ability of anti-p50 or anti-c-Rel to recognize the complexes, whereas binding of anti-p65 was not detected (Figure 1A). It has been suggested that the p65 component of NF-κB complexes is involved in early time points following T-cell activation, whereas c-Rel-containing NF-κB complexes dominate the later time points (Himes *et al*, 1996). To assess the temporal responses of NF-κB, we performed supershift analysis of p50, c-Rel and p65 subunits at several time points following splenocyte stimulation. A supershifted band consisting of

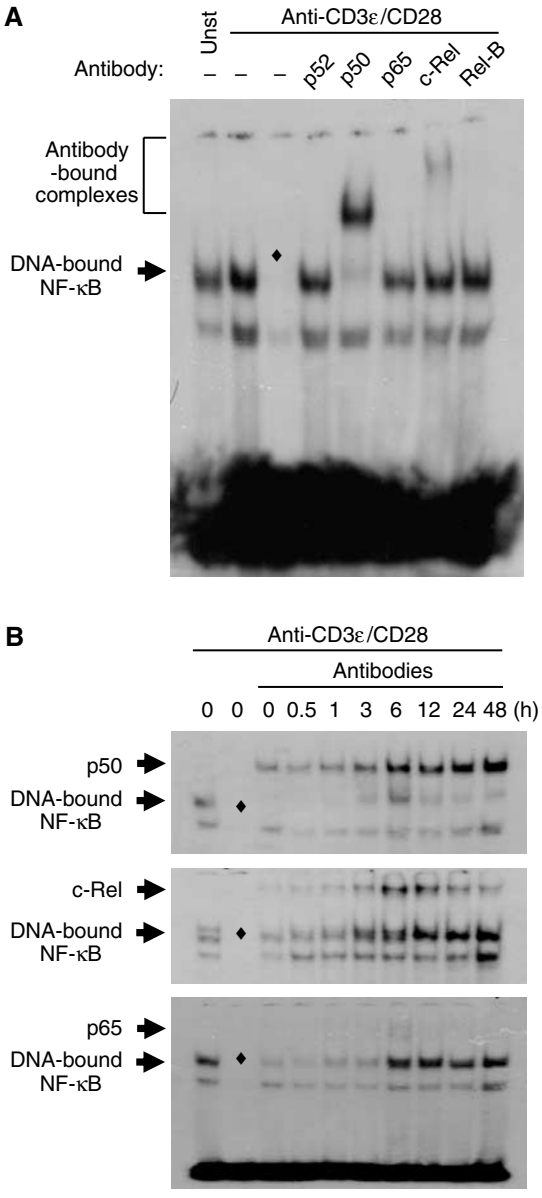

Figure 1 NF-κB expression during splenocyte activation. (**A**) Splenocytes isolated from C57BL/6 mice were stimulated with anti-CD3ε (1 μg/ml) and anti-CD28 (1 μg/ml) for 48 h. EMSAs were performed on nuclear extracts using radiolabeled oligonucleotides containing the NF-κB binding site and supershifted with specific NF-κB antibodies. The arrows indicate DNA-bound NF-κB complexes, and upper bands show specific antibody-bound DNA–NF-κB complexes. The lower band represents protein(s) present in the nuclear extracts that also bind the NF-κB consensus sequence of the oligonucleotides (Beverly and Capobianco, 2004), as addition of NF-κB-specific antibodies does not interfere or supershift this complex. (**B**) Splenocytes from C57BL/6 mice were stimulated as in panel A for the indicated time points, followed by EMSA, and supershifted with specific NF-κB antibodies (upper arrows). The lower arrows indicate DNA-bound NF-κB complexes and diamonds indicate competition with a 50-fold molar excess of unlabeled NF-κB oligonucleotides.

p50 steadily increased up to 48 h, whereas c-Rel activity peaked between 6 and 12 h and then diminished over time. In contrast, low levels of p65 activity could be detected only at 6 h (Figure 1B). These data indicate that NF-κB complexes present in stimulated splenocytes consist primarily of p50 and c-Rel.

During early splenocyte activation, temporal induction of phosphorylated IκBα leads to Notch-independent NF-κB activation

To further investigate the temporal expression of NF-κB and IκBα in activated splenocytes, we analyzed the expression of these proteins by immunoblot and EMSA. Phosphorylation of IκBα increased 30 min after treatment and gradually returned to basal levels by 24 h (Figure 2A, upper panel). The data in the upper panel of Figure 2A were scanned and the relative intensity was plotted as a function of time (Figure 2A, lower panel). Over this same time course, expression of total IκBα, p65 and c-Rel remained constant, but p50 expression increased gradually following antibody stimulation (Figure 2B). We observed peak NF-κB binding activity 12–24 h following splenocyte activation and this was maintained over 48 h, even in the presence of high levels of endogenous IκBα expression and low levels of phosphorylated IκBα (Figure 2A and B). Recent data from our lab suggested that

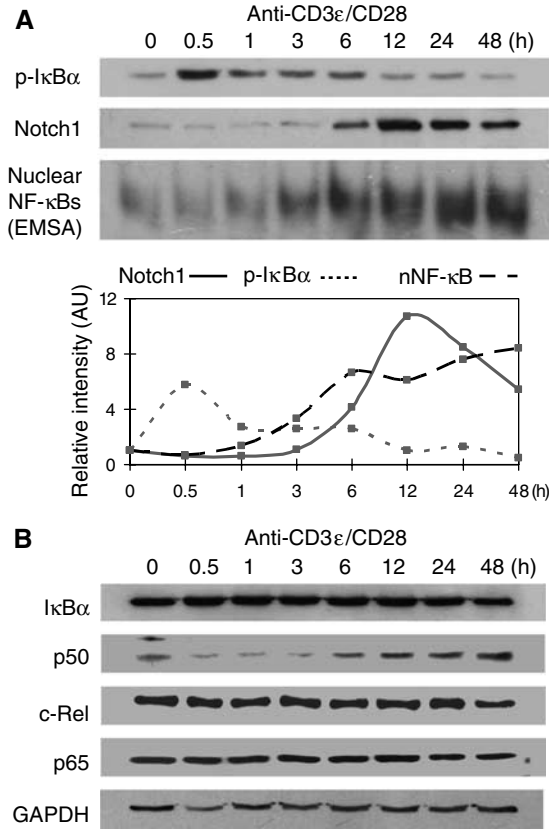

Figure 2 Temporal expression of NF-κB components and Notch1. (**A**) Splenocytes from C57BL/6 mice were stimulated as described for indicated time periods, followed by EMSA to determine NF-κB DNA binding activity, and immunoblotted for Notch1 and phospho-IκBα; calculated densitometric analyses were made using ImageJ software V. 1.31 supported by Wayne Rasband, NIH. The loading control of p-IκBα and Notch1 immunoblots is the same as in panel B. (**B**) Immunoblots were performed on whole-cell lysates from stimulated splenocytes, using indicated antibodies.

Notch1 specifically upregulates IFN-γ expression through NF-κB activation (Gottipati *et al*, in preparation); therefore, we also assessed the temporal induction of Notch1. Interestingly, Notch1 expression also peaked 12–24 h following stimulation; thus, it is unlikely that the early induction of NF-κB activity is mediated via Notch signaling. Furthermore, the fact that NF-κB binding activity was sustained at later time points, even when levels of IκBα were high, suggests a novel mechanism of maintaining NF-κB in activated T cells.

Blocking Notch signaling abrogates sustained but not initial NF-κB activation

As daily treatment with GSI blocked NF-κB activity at 48 h in antibody-stimulated splenocytes (Palaga *et al*, 2003), we examined the effects of GSI on the kinetics of NF-κB activation during splenocyte activation. Splenocytes from C57BL/6 mice were pretreated with GSI or DMSO as control, and then stimulated with anti-CD3ε and anti-CD28 antibodies. At the indicated time points, fractionated cytosolic and nuclear extracts were prepared for EMSA and immunoblotting. Within the first 12 h of stimulation, we found comparable NF-κB binding regardless of pretreatment (Figure 3A). In contrast, GSI pretreatment essentially abrogated NF-κB binding at later time points, compared to control-treated cells (Figure 3A). These data indicate that using GSI to block Notch upregulation abolishes the later, sustained wave of NF-κB activation, but not its initial induction (Figure 3A). We used a supershift assay to examine the effects of GSI pretreatment on individual components of the NF-κB signaling complex using extracts prepared after 24 h of stimulation. GSI pretreatment blocked formation of the nuclear NF-κB complex, which was shown to supershift with antibodies against p50 and c-Rel (Figure 3B). The immunoblot analysis with the cytosolic and nuclear fractions shows decreased nuclear p50 and c-Rel in cells pretreated with GSI (Figure 3C). Stimulation with anti-CD3ε and anti-CD28 antibodies for 24 h increased expression of both p50 and its precursor, p105, and markedly enhanced nuclear translocation of p50, whereas nuclear expression of c-Rel was only slightly increased. In a reciprocal fashion, inhibition of p50 nuclear localization following GSI pretreatment was more robust than that of c-Rel at 24 h. Regardless of treatment conditions, IκBα was found primarily in the cytosol. Taken together, these findings indicate that Notch exerts little, if any, influence on the early NF-κB response, and that Notch signaling is involved in sustaining NF-κB activity at later time points. Furthermore, our data suggest that Notch may modulate NF-κB activity by preferentially enhancing p50 nuclear distribution, maintaining NF-κB activity and prolonging splenocyte activation.

Direct interaction of NF-κB and $N1^{IC}$ promotes a synergistic signaling effect

Although earlier reports showed that Notch directly interacts with NF-κB, it remains controversial as to whether this interaction represses (Guan *et al*, 1996; Wang *et al*, 2001) or enhances NF-κB activity (Bellavia *et al*, 2000; Cheng *et al*, 2001; Palaga *et al*, 2003). Additionally, ectopic expression of $N1^{IC}$ in the T-cell hybridoma line, DO11.10, which lacks endogenous Notch1 expression, increases NF-κB response and IFN-γ production (Gotipatti *et al*, in preparation). To confirm the effects of Notch signaling on NF-κB activity, NF-κB luciferase reporter constructs were transfected into

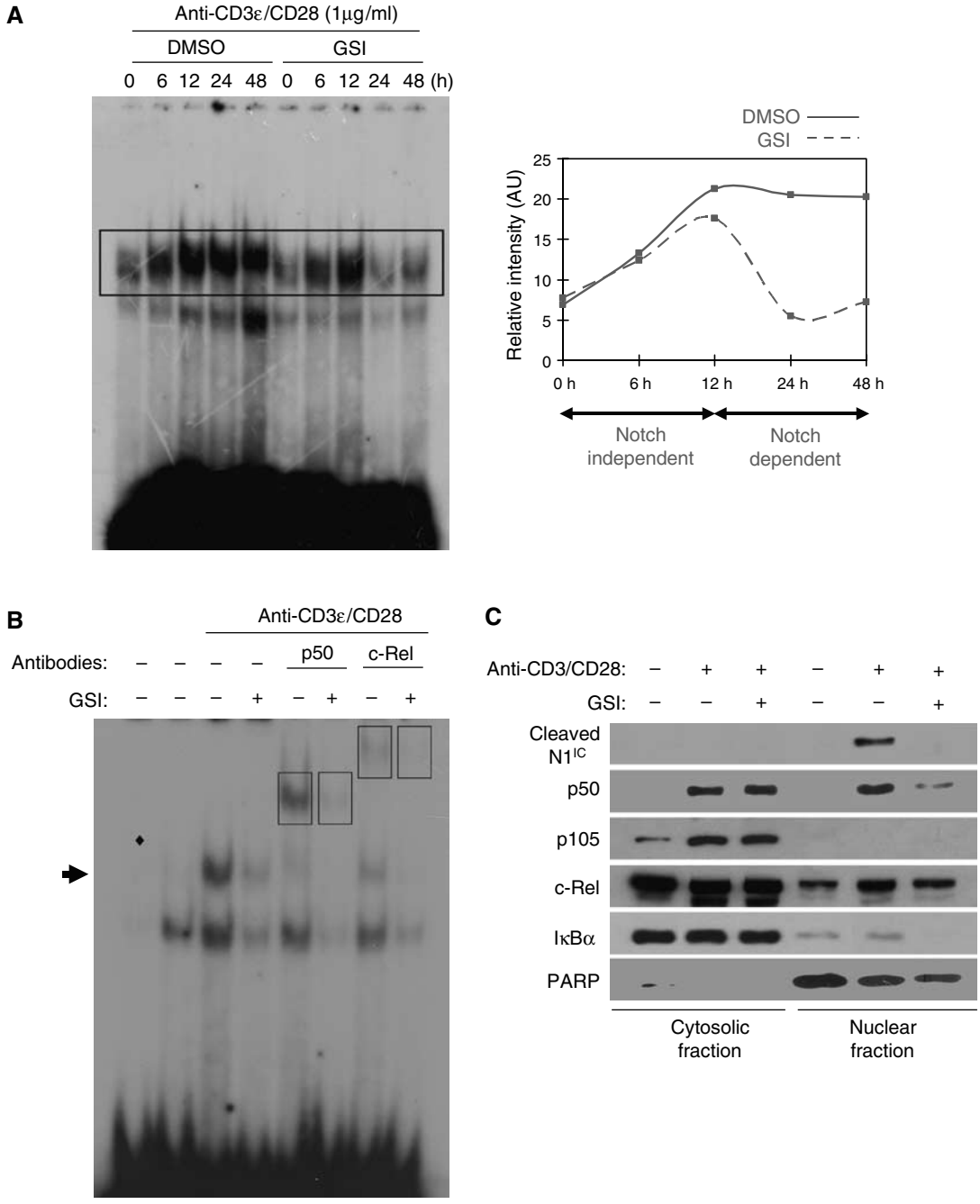

Figure 3 Blocking Notch signaling using GSI prevents sustained NF-κB activity. (**A**) Splenocytes from C57BL/6 mice were pretreated for 30 min with GSI (50 μM IL-CHO) or DMSO (vehicle control) before stimulation as previously described for the indicated time course. Nuclear extracts from treated splenocytes at each time point were harvested and analyzed by EMSA for DNA binding activity of NF-κB. The density of boxed bands (right upper panel) was calculated using ImageJ software. (**B**) Nuclear extracts from splenocytes were pretreated for 30 min with GSI (50 μM IL-CHO) or DMSO before stimulation as described for 24 h, followed by EMSA, and supershifted with p50 or c-Rel antibody. The arrow indicates DNA-bound NF-κB complexes and boxed bands show supershifted, antibody-bound DNA–NF-κB complexes. The diamond indicates competition with a 50-fold molar excess of unlabeled NF-κB oligonucleotides. (**C**) Splenocytes were pretreated for 30 min with GSI (50 μM IL-CHO) or DMSO before stimulation for 24 h, followed by immunoblotting of cytosolic and nuclear extracts with anti-cleaved Notch1, anti-p50, anti-c-Rel or anti-IκBα. Lack of expression of the nuclear protein PARP was used to assess purity of cytosolic fractions, whereas the lack of p105 (precursor of p50) in the nucleus indicated purity of the nuclear fraction.

the DO11.10 cells expressing $N1^{IC}$ or empty control. $N1^{IC}$ increased luciferase activity approximately four-fold above empty control (Figure 4A). To confirm physical interaction between Notch1 and components of the NF-κB complex, we performed co-immunoprecipitation experiments. Vectors expressing $N1^{IC}$ and either p50 or c-Rel were co-transfected into 293T cells and subjected to co-immunoprecipitation with antibodies against specific NF-κB subunits. Consistent with earlier reports, we detected direct interaction between $N1^{IC}$ and p50, as well as with c-Rel (Figure 4B).

To further investigate the effects of Notch signaling on NF-κB activity, $N1^{IC}$ and p50 and c-Rel expression vectors were

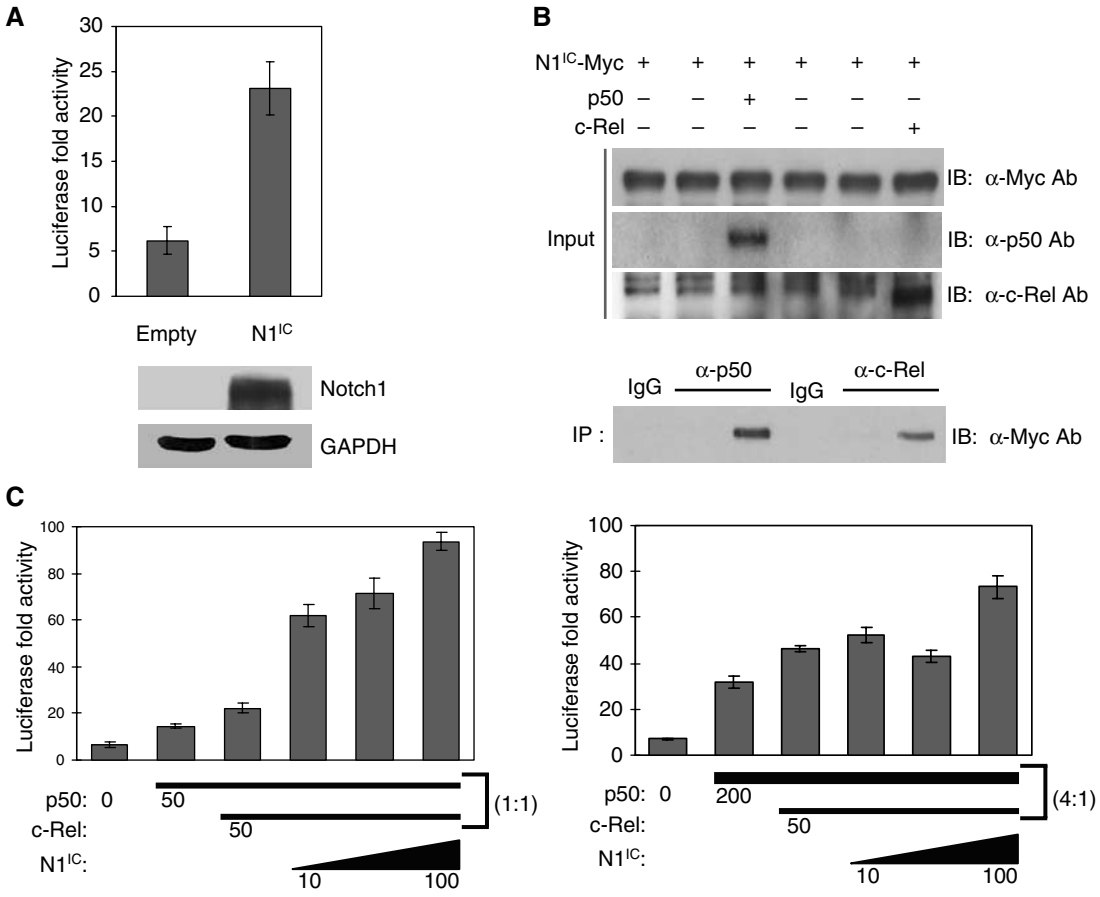

Figure 4 Synergistic effect of $N1^{IC}$ on NF-κB is mediated by their direct interaction. (**A**) NF-κB luciferase reporter plasmid (2 μg) and pRL-CMV (50 ng) plasmid of an internal control were transiently transfected in DO11.10 cells expressing $N1^{IC}$ or empty control and harvested 48 h later for dual luciferase assays. The relative luciferase values were calculated by dual luciferase assays as described in Materials and methods. Values shown are averages of at least two separate experiments. Harvested lysates were immunoblotted with anti-Notch1 and anti-GAPDH antibodies. (**B**) $N1^{IC}$ expression plasmids were transiently co-transfected with p50 or c-Rel expression plasmids and 293T cells were harvested 24 h later and subjected to co-immunoprecipitation as described in Materials and methods. 1/100 of input shown was immunoblotted with anti-Myc, anti-p50 and anti-c-Rel. (**C**) NF-κB luciferase reporter (400 ng) plasmid and pRL-CMV (100 ng) plasmid of an internal control were transiently transfected with the indicated plasmids into 293T cells. In the upper graph, the ratio of p50 to c-Rel expression plasmids was 1:1 (50 ng:50 ng) and in the lower graph, p50:c-Rel was 4:1 (200 ng:50 ng) with increasing amounts of $N1^{IC}$ expression plasmids (10, 50 and 100 ng). Transfected cells were incubated for 48 h and harvested for dual luciferase assays. The relative luciferase values were calculated by dual luciferase assays. Values shown are averages of at least three separate experiments.

co-transfected into 293T cells along with an NF-κB luciferase reporter construct. Expression of p50 alone resulted in slightly increased reporter activity, suggesting possible interaction with endogenous c-Rel or p65. Coexpression of c-Rel and p50, increased luciferase activity approximately two-fold above non-transfected controls (Figure 4C). However, co-expression of $N1^{IC}$ increased NF-κB-dependent transactivation of the luciferase reporter gene in a dose-dependent manner, up to nearly 10-fold over controls, as shown in Figure 4C (upper panel). Based on the expression levels of nuclear p50 and c-Rel present in EMSAs (Figure 1A), we adjusted the ratio of p50 and c-Rel expression vectors (p50: c-Rel, 4:1). At these ratios, we observed similar synergistic effects of $N1^{IC}$ on NF-κB-dependent gene expression (Figure 4C, right panel), although the maximal fold induction was somewhat less than 1:1 ratios. $N1^{IC}$ induced NF-κB-dependent reporter activity in a dose-dependent manner up to a concentration of 100 ng. Together, these data suggest that $N1^{IC}$ can synergize with p50 and/or c-Rel to positively modulate NF-κB-dependent gene expression and this may be mediated by direct molecular interaction between these proteins.

Cytosolic sequestering of NF-κB by IκBα is reversed by nuclear $N1^{IC}$

Given that $N1^{IC}$ can physically interact with p50 and c-Rel proteins and that increased $N1^{IC}$ expression correlated with sustained NF-κB activity in stimulated splenocytes even in the presence of high levels of non-phosphorylated IκBα, we asked whether $N1^{IC}$ might somehow prevent IκBα-mediated inhibition of NF-κB. SR-IκBα was co-transfected along with p50 and c-Rel expression constructs and an NF-κB reporter plasmid in the absence or presence of a vector expressing $N1^{IC}$. As expected, SR-IκBα efficiently abrogated transactivation of the NF-κB reporter (Figure 5A). Remarkably, expression of $N1^{IC}$ restored NF-κB transcriptional activity in a dose-dependent manner (Figure 5A). To assess the influence of $N1^{IC}$ on the subcellular localization of NF-κB, we generated dsRed and GFP chimeras of p50 and c-Rel. The dsRed-p50 and GFP-c-Rel constructs were co-transfected into 293T cells in the absence or presence of vectors expressing SR-IκB-α or $N1^{IC}$. As indicated in Figure 5B, transfected p50 and c-Rel were localized predominantly to the nucleus, but were sequestered in the cytosol in the presence of SR-IκBα. Addition of

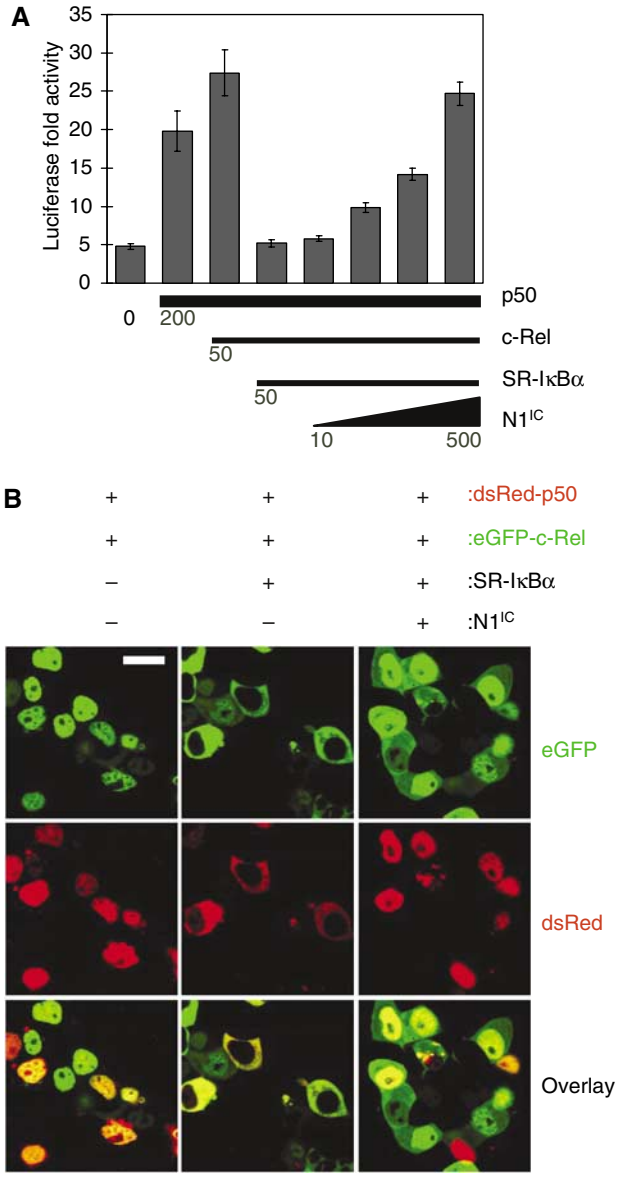

Figure 5 Notch1 rescues NF-κB activity from suppression by SR-IκBα. (**A**) NF-κB luciferase reporter (400 ng) plasmid and pRL-CMV (100 ng) of an internal control were transiently co-transfected with the indicated plasmids into 293T cells. Ratio of p50 expression plasmid to c-Rel expression plasmid was 4:1 (200 ng:50 ng) with SR-IκBα expression plasmid (50 ng) and increasing amounts of $N1^{IC}$ expression plasmids (10, 50, 100 and 500 ng). Transfected cells were incubated for 48 h and harvested for dual luciferase assays. The relative luciferase values were calculated as described. Values shown are averages of at least three separate experiments. (**B**) Indicated expression plasmids were transiently transfected into 293T cells. Transfected cells were incubated for 24 h and examined using confocal microscopy in green channel (488 nm), red channel (560 nm) and overlay. Scale bar represents 20 μm.

$N1^{IC}$, however, completely abolished cytosolic sequestering of p50 by SR-IκBα and promoted its nuclear relocalization (Figure 5B). Similarly, cytosolic sequestration of c-Rel by SR-IκBα was also reversed when $N1^{IC}$ was coexpressed, although to a lesser extent (Figure 5B; also see Figure 2). Collectively, these data demonstrate that $N1^{IC}$ is capable of sustaining the nuclear activity of NF-κB through its direct interactions with p50 and c-Rel subunits, by increasing nuclear retention of NF-κB subunits.

Impaired nuclear localization of $N1^{IC}$ correlates with decreased transcriptional activity of NF-κB

To further investigate the influence that subcellular localization of $N1^{IC}$ has on NF-κB signaling, GFP-$N1^{IC}$ constructs were modified so as to increase or decrease their nuclear retention. Specifically, GFP-$N1^{IC}$ was modified by inclusion of either an additional nuclear export signal to reduce nuclear levels (GFP-$N1^{IC\text{-}NES}$) or an additional nuclear localization signal to increase its nuclear retention (GFP-$N1^{IC\text{-}NLS}$) (Jeffries and Capobianco, 2000). These expression vectors were then used in fluorescence imaging to study their effects on localization of $N1^{IC}$ and on NF-κB reporter activity. When the GFP-$N1^{IC\text{-}NES}$ or GFP-$N1^{IC\text{-}NLS}$ expression vectors were transfected into 293T cells, addition of the NES sequence to GFP-$N1^{IC}$ promoted its cytosolic retention, whereas addition of the NLS sequence enhanced nuclear localization of GFP-$N1^{IC}$ (Figure 6A, upper panel). When an NF-κB reporter was co-transfected into 293T cells with these vectors, we found that enhanced nuclear localization of GFP-$N1^{IC\text{-}NLS}$ resulted in increased activity of the NF-κB luciferase reporter, whereas impaired nuclear localization, as seen with expression of GFP-$N1^{IC\text{-}NES}$, reduced NF-κB reporter activity to basal levels (Figure 6A, lower panel). We next examined whether subcellular localization of $N1^{IC}$ could also influence cytosolic versus nuclear localization of p50. The dsRed-p50 vector was co-transfected with GFP-$N1^{IC\text{-}NLS}$ or GFP-$N1^{IC\text{-}NES}$ into 293T cells and analyzed by confocal microscopy. As expected, GFP-$N1^{IC\text{-}NLS}$ was predominantly located in the nucleus where it colocalized with dsRed-p50 (Figure 6B). In contrast, a large proportion of GFP-$N1^{IC\text{-}NES}$ was retained in the cytosol and also colocalized with dsRed-p50. These data strongly suggest that cellular distribution of Notch plays a critical role in its ability to sustain NF-κB transcriptional activity, by further influencing subcellular localization of NF-κB components.

$N1^{IC}$ can directly regulate IFN-γ expression through complexes formed with p50 and c-Rel on the IFN-γ promoter

NF-κB and Notch1 regulate the expression of IFN-γ, and inhibiting Notch activation abrogates INF-γ production (Palaga *et al*, 2003; Minter *et al*, 2005; Gottipati *et al*, in preparation). We next sought to confirm the influence of Notch1 on IFN-γ expression at either the protein or mRNA transcript level. Splenocytes from C57BL/6 mice were pretreated with GSI or DMSO, and then stimulated with anti-CD3ε and anti-CD28 antibodies. At the indicated time points, we assessed mRNA transcription of IFN-γ by RT–PCR and assayed the culture supernatants for IFN-γ secretion using standard enzyme-linked immunosorbant assay (ELISA) methods. Following splenocyte stimulation, transcription of IFN-γ increased as did secretion of IFN-γ in culture supernatants. GSI pretreatment decreased IFN-γ transcripts and abrogated IFN-γ secretion (Figure 7A and B). To further investigate whether NF-κB and $N1^{IC}$ directly interact to form a nuclear complex on the IFN-γ promoter, we examined NF-κB binding sites within the IFN-γ promoter using chromatin immunoprecipitation (ChIP) assays. Splenocytes from C57BL/6 mice were pretreated with GSI or DMSO and then stimulated with anti-CD3ε and anti-CD28 antibodies for 24 h, at which point ChIP assays were performed using anti-p50, anti-c-Rel or anti-Notch1 antibodies to precipitate proteins crosslinked to DNA. After releasing proteins, PCR was used to

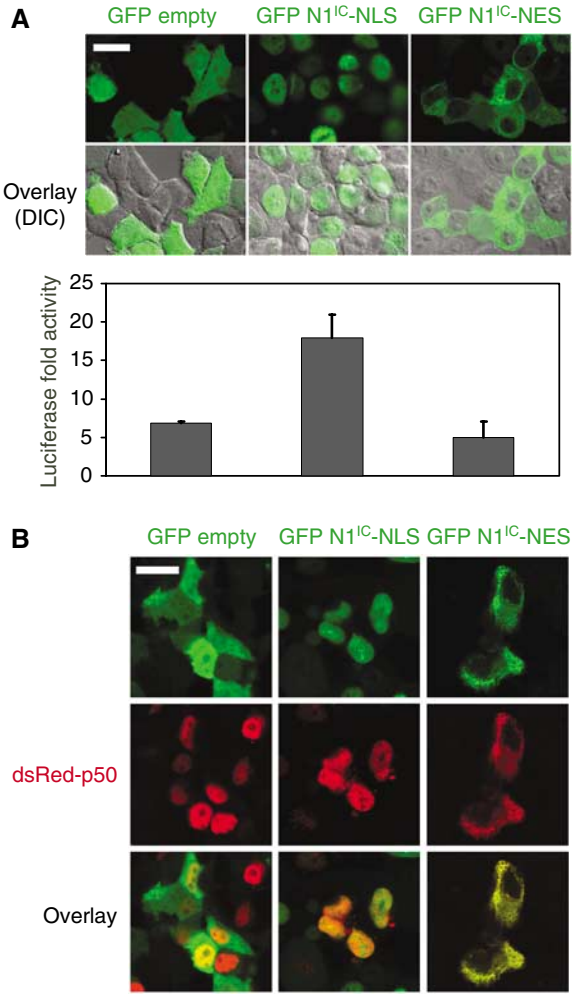

Figure 6 Cellular distribution of NF-κB p50 is regulated by Notch1. (**A**) 293T cells were transiently transfected with eGFP empty vector as a control, eGFP $N1^{IC\text{-}NLS}$ or eGFP $N1^{IC\text{-}NES}$ and examined 24 h after transfection using laser-scanning confocal microscopy. Composite panel, top row: images collected in green channel; center row: DIC images; bottom row: overlay. NF-κB luciferase reporter (400 ng) plasmid and pRL-CMV (100 ng) of an internal control were transiently transfected with the eGFP empty vector as a control, eGFP $N1^{IC\text{-}NLS}$ or eGFP $N1^{IC\text{-}NES}$ into 293T cells. Transfected cells were incubated for 24 h and harvested for dual luciferase assays. The relative luciferase values were calculated as described. Values shown are averages of at least three separate experiments. Scale bar represents 20 μm. (**B**) dsRed-p50 expression plasmids were transiently transfected with eGFP empty as a control, eGFP $N1^{IC\text{-}NLS}$ or eGFP $N1^{IC\text{-}NES}$ into 293T cells. Transfected cells were incubated for 24 h and examined using confocal microscopy. Images were captured in the green channel (488 nm), red channel (560 nm) and then merged in overlay. Scale bar represents 20 μm.

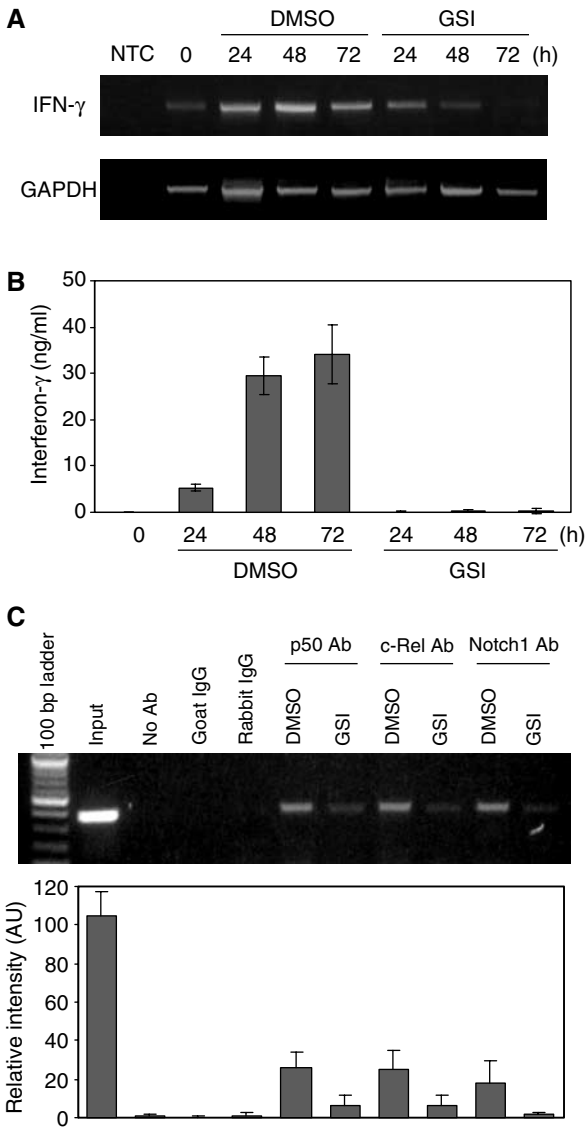

Figure 7 Notch1 forms a nuclear complex with NF-κB subunits on the IFN-γ promoter. (**A**) Expression of IFN-γ was assessed by RT–PCR of total RNA isolated at the indicated time points from GSI- or DMSO-pretreated splenocytes from C57BL/6 mice, stimulated as described. GAPDH (bottom): loading control. Results are representative of two independent replicates for each time point. (**B**) Culture supernatants from splenocytes stimulated as in panel A for the indicated time points were harvested and IFN-γ levels were determined by ELISA. Results shown are averages of three separate experiments. (**C**) Splenocytes from C57BL/6 mice were stimulated as described for 24 h and harvested for ChIP assay using goat anti-p50, rabbit anti-c-Rel, rabbit anti-Notch1 and goat isotype and rabbit isotype control immunoglobulins as negative controls. Recruitment of Notch1 to a 388 bp region of the IFN-γ promoter containing NF-κB binding sites was amplified by PCR using the primers described in Materials and methods; calculated densitometric analysis was made using ImageJ software V. 1.31 supported by Wayne Rasband, NIH. Equal volumes of input controls from stimulated splenocytes or immunoprecipitates were used. NTC: non-template control.

amplify a 388 bp region of the IFN-γ promoter containing NF-κB binding sites. The PCR bands in three separate ChIP experiments were scanned and the relative intensities plotted (Figure 7C, lower panel). Although, as expected, p50 and c-Rel proteins formed a complex, along with Notch1, in this region of the IFN-γ promoter, using GSI pretreatment to inhibit Notch1 activation abrogated this co-recruitment of p50, c-Rel and Notch1 to these NF-κB binding sites (Figure 7C). Together, these data demonstrate that p50 and c-Rel are recruited to the IFN-γ promoter in a nuclear complex with Notch1 to directly regulate IFN-γ production.

Discussion

Here, we propose that, following TCR engagement, Notch is required for sustained NF-κB activity leading to upregulation of IFN-γ production. Our data demonstrate a direct interaction between Notch signaling and NF-κB activation as

determined by both confocal microscopy and biochemical methods. We show that there are two waves of NF-κB induction in stimulated splenocytes, and pretreating cells with GSI to block Notch activation suggests that the later, sustained wave of NF-κB activity is Notch dependent. Our data further confirm a direct interaction between $N1^{IC}$ and NF-κB subunits to enhance NF-κB signaling. Finally, we show that NF-κB and $N1^{IC}$ form a complex on the IFN-γ promoter to positively regulate production of this cytokine. Taken together, these data support a model whereby Notch retains active NF-κB complexes in the nucleus, facilitating sustained NF-κB activity over time.

TCR engagement allows antigen-specific proliferation and maturation of lymphocytes to effector cells through the activation of multiple signaling pathways, including NF-κB activation, and triggers production of several types of cytokines. IFN-γ and IL-2 are hallmark cytokines generated by effector T cells in response to immunological stimuli. Single deletions of NF-κB components ($p50^{-/-}$, $p65^{-/-}$ or c-$Rel^{-/-}$) partially attenuate proliferative responses in T cells (Zheng *et al*, 2001, 2003). However, T cells lacking both p50 and c-Rel show impaired cytokine profiles (Zheng *et al*, 2001, 2003). Previous reports have revealed that production of IFN-γ and IL-2 is regulated by multiple mechanisms involving a wide variety of transcription factors (Penix *et al*, 1996; Schwartz, 1997; Sica *et al*, 1997). Among these, NF-κB plays a pivotal role in transcriptional regulation of these cytokines (Kane *et al*, 2002; Caamano and Hunter, 2002). Previous studies have shown that Notch proteins also regulate cytokine production mediated by NF-κB activation (Bellavia *et al*, 2000; Adler *et al*, 2003; Palaga *et al*, 2003; Gottipati *et al*, in preparation).

We previously showed that using GSI to block Notch activation *in vitro* or *in vivo* abolishes IFN-γ production in splenic CD4 and CD8T cells (Palaga *et al*, 2003; Minter *et al*, 2005). Furthermore, exogenous expression of $N1^{IC}$ could restore the defect in IFN-γ production in GSI-treated CD4T cells. When using GSI, we cannot exclude the possibility of its action on undefined substrates of γ-secretase during the immune response. However, exogenous expression of $N1^{IC}$ by retroviral gene transfer and overexpression of $N1^{IC}$ in DO11.10 cells revealed that Notch1 is sufficient to elicit IFN-γ secretion and to increase NF-κB activity (Cheng *et al*, 2001; Gottipati *et al*, in preparation). As GSI inhibits the activation of all four Notch proteins, and multiple Notch proteins may redundantly regulate T-cell activation, it is possible that several Notch proteins lead to IFN-γ production via NF-κB activation. It remains to be determined whether different combinations of Notch receptors and ligands have discrete functions to promote NF-κB responses, leading to the production of cytokines. Additional studies in our lab revealed that Notch1 can directly regulate T-bet expression at the transcriptional level, directing T_H1 differentiation (Minter *et al*, 2005). Moreover, ectopic expression of $N1^{IC}$ in CD4T cells restored T-bet expression, leading to IFN-γ production in the presence of GSI. This rescue experiment suggests that although compensatory functions may exist among Notch proteins, $N1^{IC}$ expression is sufficient to induce IFN-γ production.

There are conflicting reports in the literature as to the effects of Notch interaction with NF-κB. It has been suggested that Notch inhibits NF-κB activity in the nucleus via direct interaction mediated by the N-terminal portion of $N1^{IC}$ (Guan *et al*, 1996; Wang *et al*, 2001). However, this observed negative regulation of NF-κB by $N1^{IC}$ might be due, in part, to the size of the $N1^{IC}$ construct used, which is distinctly different from the physiological, *in vivo*-generated $N1^{IC}$. Notch1 protein is proteolytically cleaved by γ-secretase between amino acids G1743 and V1744 (Schroeter *et al*, 1998). Furthermore, V1744 resides within or very close to the cytoplasmic side of the transmembrane domain. All identified Notch proteins possess this conserved cleavage site that produces the N^{IC} that translocates to the nucleus (Schroeter *et al*, 1998; Mizutani *et al*, 2001). Data from our lab indicate that $N1^{IC}$ effects on NF-κB activity and production of IFN-γ can be vastly different depending on the $N1^{IC}$ construct used (Cheng *et al*, 2001; Palaga *et al*, 2003; Gottipati *et al*, in preparation). Therefore, we expect that size discrepancies between active Notch constructs may lead to distinctly different effects in T cells and may account for the contradictory results reported previously.

There are also conflicting reports about crosstalk between Notch1 and NF-κB (Oswald *et al*, 1998; Bash *et al*, 1999; Espinosa *et al*, 2003; Oakley *et al*, 2003; Aguilera *et al*, 2004). It has been suggested that NF-κB expression and/or activation by Notch1 may be cell type dependent, and nuclear accumulation of Notch1 may lead to NF-κB inhibition, suggesting a mechanism for signal termination (Cheng *et al*, 2001). Our model predicts that NF-κB activation, controlled by Notch protein, is likely to be time and dose dependent. It has been reported that active Notch1 may strongly activate $NF\text{-}\kappa B_2$ (p100/p52) expression at the level of transcription by converting the repressor complex to an activator complex to overcome CBF-1-mediated repression (Oswald *et al*, 1998). On the other hand, IκBα and p65 may regulate cytosolic retention of nuclear corepressors such as N-CoR (nuclear receptor corepressor) or SMRT (silence mediator for retinoic acid and thyroid receptors), which are thought to recruit HDACs, permitting upregulation of Notch-dependent genes (Espinosa *et al*, 2003). Our studies have attempted to elucidate more precisely the regulation of NF-κB activity by Notch. Temporal induction of NF-κB activity following TCR engagement and CD28 co-stimulation showed continued NF-κB activation beyond 48 h, in contrast to TNF-1-induced NF-κB asynchronous oscillation that decreases its nuclear function over time (Hoffmann *et al*, 2002; Nelson *et al*, 2004). The data presented in our study also suggest that $N1^{IC}$ can override the cytoplasmic sequestration of NF-κB by IκB proteins, by promoting nuclear retention of NF-κB. Enhanced relocation of p50 to the cytoplasm when coexpressed with $N1^{IC\text{-}NES}$, and to the nucleus when co-expressed with $N1^{IC\text{-}NLS}$, further supports a role for Notch as a means of physically retaining NF-κB in the nucleus. Moreover, our ChIP data confirm the interaction of p50/c-rel/$N1^{IC}$ on the promoter region of IFN-γ, lending support to the notion that $N1^{IC}$ may contribute to the DNA binding/transcriptional capacity of NF-κB.

Several types of cancer are thought to be associated with alterations in NF-κB or Notch signaling pathways. In a number of instances, constitutive nuclear NF-κB activity results from constitutive activation of upstream signaling kinases or mutations that inactivate IκB subunits and may contribute to oncogenesis (Rayet and Gelinas, 1999). Moreover, activation of the Notch pathway may influence tumorigenic transformation progression, and activating mutations of Notch1 are associated with T-cell acute lymphoblastic leukemia (Capobianco *et al*, 1997; Jeffries and

Capobianco, 2000; Weng *et al*, 2004; Curry *et al*, 2005). It has been suggested that activated Notch in T cells may result in constitutive NF-κB activation, leading to increased proliferation and T-cell leukemia (Bellavia *et al*, 2000; Palaga *et al*, 2003). Although the precise mechanism defining Notch regulation of NF-κB is still controversial, our study helps to elucidate how Notch may mediate NF-κB activation during T-cell activation. It remains to be determined whether different combinations of Notch receptors and ligands have discrete functions in promoting NF-κB signaling and in mediating transcription of NF-κB target genes.

Materials and methods

Plasmid DNA

The Notch1^{IC} constructs were generated by subcloning into pcDNA3.1 (Jeffries and Capobianco, 2000) and pEGFP vector (BD Co.) into *Bam*HI and *Sal*I sites (described in Supplementary Figure 1). cDNAs of p50 and c-Rel were kindly provided by GE Sonenshein (Boston University) and subcloned into *Bam*HI and *Xho*I sites of pcDNA3.1 and *Bgl*II and *Sal*I sites of pdsRed-N1 or pEGFP-C1 vector, respectively (BD Biosciences Co.) using PCR. SR-IκBα, kindly provided by BJ Nickoloff (Loyola University Medical Center) and previously described (Qin *et al*, 1999), and mouse cDNA of wt IκBα, generated by RT–PCR, were subcloned into pcDNA3.1 (Invitrogen Co.). The reporter construct of NF-κB × 3 luc was purchased from Clontech Co. All cDNA constructs generated by PCR were confirmed by sequencing and by immunoblot following overexpression in 293T cell line.

Antibodies

Anti-p50 (sc-1190X), anti-c-Rel (sc-71X and sc-6955X), anti-p65 (sc-372X), anti-RelB (sc-226X), anti-p52 (sc-298X), anti-IκBα (sc-371), anti-Notch1 (sc-6014-R) and anti-GFP (sc-8334) antibodies, normal goat IgG (sc-2043) and normal rabbit IgG (sc-2027) were purchased from Santa Cruz Biotechnology; cleaved N1 (#2421) and phospho-IκBα antibody were from Cell Signaling Technologies; PARP antibody (SA250) was from Biomol; and GAPDH antibody (MAB374) was from Chemicon International.

Activation of splenocytes

C57BL/6 mice were housed in the animal care facility at the University of Massachusetts/Amherst in accordance with IACUC guidelines. Splenocytes were stimulated to proliferate with soluble anti-CD3ε plus anti-CD28 (both BD Pharmingen), at 1 μg/ml each, for the indicated time periods. For experiments using the GSI, IL-CHO (previously described by Palaga *et al*, 2003), cells were pretreated for 30 min at 37°C with 50 μM of the inhibitor, or with DMSO as vehicle control. The inhibitor was present during the time course of the experiment.

RT–PCR and cytokine analysis using ELISA

Total RNA was isolated from splenocytes stimulated as described above using RNA-BEE (Tel-Test), following the manufacturer's directions. Reverse transcription was performed with oligo-dT primers and cDNAs were analyzed by PCR using the following primers: IFN-γ forward, 5′-TGAACGCTACACACTGCATCTTGG-3′; IFN-γ reverse, 5′-CGACTCCTTTTCCGCTTCCTGAG-3′; GAPDH forward, 5′-ACTTTCGATCAAGGATCAGCA-3′; GAPDH reverse, 5′-ACG GAAGGCCATGCCAGTGAGCTT-3′. Conditions used were 94°C for 2 min, 94°C for 30 s, 55°C for 30 s and 68°C for 30 s (35 cycles). Supernatants from splenocytes stimulated as described above were analyzed by standard ELISA for the secretion of IFN-γ with paired capture and detection antibodies (BD Pharmingen).

Co-immunoprecipitation assays

293T cells were transfected with indicated plasmids using Fugene 6 (Roche Co.). After 2 days, cells were lysed for 30 min at 4°C in 500 μl of 1% NP-40 lysis buffer (10 mM Tris–HCl pH 7.8, 0.5 mM EDTA, 250 mM NaCl and protease inhibitor cocktail). Supernatants were incubated with 10 μl of normal serum and 50 μl of protein G-Sepharose beads (Pharmacia Co.) at 4°C on rotator for the preclearing step. After centrifugation, precleared supernatants were incubated at 4°C on rotator overnight with p50 or c-Rel antibodies (Santa-Cruz Co.). For immunoprecipitation samples, goat normal IgG or rabbit normal IgG (Santa-Cruz Co.) served as negative controls. Then, 50 μl of protein G-Sepharose beads was added and the incubation allowed to proceed for an additional 60 min at 4°C on rotator. The beads were washed five times with 1% NP-40 lysis buffer containing 10 mM Tris–HCl pH 7.8, 0.5 mM EDTA, 250 mM NaCl and protease inhibitor cocktail and were then boiled in Laemmli buffer and assayed by Western blot.

Chromatin immunoprecipitation

ChIP analysis was performed on 2×10^6 splenocytes stimulated as describe above for 24 h using the ChIP Assay Kit (Upstate Cell Signaling Solutions) following the manufacturer's instructions. For PCR, 5 μl from a 50 μl DNA extraction and 30 cycles of amplification were used. In the mouse IFN-γ promoter, a consensus binding site of NF-κB was amplified by PCR. The following primer set (388 bp) was used: forward, 5′-ATCACCTCCATTGAAGGGCTTCCT-3′; reverse, 5′-AGTTTCCTTTCGACTCCTTGGGCT-3′. Conditions used were 94°C for 2 min, 94°C for 30 s, 55°C for 30 s, 68°C for 30 s (30 cycles) and 68°C for 5 min.

Subcellular fractionation

293T cells transfected with indicated plasmids and splenocytes stimulated as described above were harvested and washed once in cold PBS. Cells were incubated in buffer A (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and protease inhibitor cocktail) for 30 min on ice. After centrifugation at 3500 r.p.m. at 4°C for 20 min, cytosolic fractions were collected and the pellets washed once in buffer A, resuspended in 1% NP-40 lysis buffer (10 mM Tris–HCl pH 7.8, 0.5 mM EDTA, 250 mM NaCl and protease inhibitor cocktail) and incubated for an additional 30 min on ice. After centrifugation at full speed at 4°C, nuclear factions were collected and protein concentrations of all fractionated samples were estimated using BCA protein assay according to the manufacturer's protocol. All fractionated samples were separated by SDS–PAGE and immunoblots were performed as indicated above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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