Id1 Potentiates NF-κB Activation upon TCR Signaling

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E2A and HEB are basic helix-loop-helix transcription factors that play important roles in T cell development. Expression of Id1, one of their inhibitors, severely impairs T cell development in transgenic mice. Aberrant activation of NF-κB transcription factors has been shown to contribute to the developmental defects, but it is not clear if NF-κB activation is directly due to Id1 expression or is secondary to an abnormal thymic environment in Id1 transgenic mice. Here, by using a T cell line model, we demonstrate that Id1 expression stimulates basal levels of NF-κB activity and further enhances NF-κB activation upon TCR signaling achieved by anti-CD3 and anti-CD28 stimulation. Activation of NF-κB is partially mediated by the classical pathway involving the interaction between the regulatory subunit, NEMO, and the catalytic subunit, IκB kinase β (IKKβ). However, a NEMO-independent pathway also appears to be at play. Id1-potentiated activation of NF-κB leads to over production of cytokines such as tumor necrosis factor α (TNFα) and interferon-γ (IFNγ) in a T cell line as well as in thymocytes. Among members of the NF-κB family, c-rel appears to be preferentially activated by Id1, especially during TCR stimulation. Consistently, c-rel deficiency diminishes TNFα and IFNγ expression induced by Id1 and TCR signaling.

However, cross talk between different families of transcription factors also exists. In this report, we describe the influence of basic helix-loop-helix proteins on the activity of NF-κB transcription factors and the consequences of such an influence.

The NF-κB family consists of p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) (1). All members possess the Rel homology domain responsible for dimerization, DNA binding and interaction with IκB proteins. However, p65, RelB and c-Rel carry a transcription activation domain, which enable homodimers of these proteins or heterodimers between these proteins and p50 or p52 to activate transcription of target genes. In response to different stimuli, a large number of genes are regulated by NF-κB transcription factors, among which are various cytokines such as TNFα and interferon-γ (2-5). Although p65 and p50 are ubiquitously expressed, RelB, c-Rel and p52 are largely expressed by cells of hematopoietic lineages, possibly serving their specialized functions (6).

In the absence of stimulation, NF-κB transcription factors are retained in the cytoplasm through association with IκB proteins (1,7). Upon stimulation by a range of inducers, IκB proteins become phosphorylated and degraded through an ubiquitin-mediated process, thus allowing NF-κB proteins to translocate into the nucleus and function as transcription factors. Phosphorylation of IκB molecules is mediated by IκB kinases, IKKα and β, which are involved in two known pathways leading to NF-κB activation. In the classical pathway, NF-κB essential modulator (NEMO), which receives signals from various signaling pathways, forms complexes with IKKα and β and causes their activation (1,8). In the alternative pathway, IKKα homodimers are formed in response to inducers like LTβ and BAFF, and phosphorylate p100 causing its
cleavage to generate p52 (9). In T lymphoid cells, antigen binding to T cell receptors (TCR) leads to the activation of IKKβ/NEMO complexes through the interaction and function of a series of adapter and kinase molecules (10). Such complicated activation mechanisms provide ample opportunities for other cellular factors to intervene in the activation process. NF-κB activation has been shown to be important for thymocyte survival following pre-TCR signaling and for activation of peripheral T cells upon TCR stimulation (10,11), but aberrant activation of NF-κB could have additional consequences.

Basic helix-loop-helix transcription factors encoded by the E2A and HEB genes play crucial roles in T cell development (12,13). The function of these proteins, collectively called E proteins, can be diminished by a family of dominant negative inhibitors consisting of Id1 to Id4. While their activities are necessary for the earliest stage of T cell commitment and differentiation, E proteins also regulate the function of pre-TCR and TCR by controlling the threshold of signaling (14-17). T cell development constitutes a series of step-wise selection processes to check for the proper function of pre-TCR and TCR (18,19). Production of functional pre-TCR, monitored by the β selection checkpoint, allows the progression of double negative thymocytes to the CD4 and CD8 double positive (DP) stage. Thymocytes that express TCRs failing to recognize self-MHC molecules present in the thymus die in a process called “death by neglect”. In contrast, those thymocytes express TCRs that bind too strongly to self-MHC/peptide complexes in the thymus undergo apoptosis and are eliminated by negative selection. Thus only thymocytes that express TCRs capable of recognizing self-MHC/peptide complexes with limited affinity develop further as the result of positive selection (20-22). Disruption of the E2A gene enables RAG2 deficient thymocytes to pass the β selection checkpoint and differentiate to the DP stage without signaling from pre-TCR (15). E2A deficiency also leads to altered positive and negative selection of TCR receptors occurring at the DP stage (14). Interestingly, signaling from pre-TCR and TCR causes down-regulation of E2A and HEB function by transient stimulation of Id3 expression (15,23). This suggests that modulation of E protein function is an integral part of T cell differentiation driven by signaling from pre-TCR and TCR. However, the mechanism by which E proteins influence pre-TCR or TCR signaling remains poorly understood.

Expression of the Id1 gene in thymocytes of transgenic mice effectively inhibits all E proteins and severely impairs T cell development (24). Like E2A deficiency, Id1 expression enables RAG1 deficient thymocytes to differentiate to the DP stage in the absence of TCR (25). Furthermore, CD4 single positive thymocytes from Id1 transgenic but not wild-type mice proliferate vigorously upon signaling from TCR by anti-CD3 treatment without co-stimulation through CD28 (26). Id1 transgenic DP thymocytes are completely eliminated under positively selecting conditions created by expression of H-Y or AND TCR transgenes, a scenario reminiscent of negative selection. To address the underlying mechanisms, we have previously shown that NF-κB transcription factors, particularly c-rel, are aberrantly activated in Id1 transgenic mice (25). Activation of NF-κB by expression of a constitutively active form of IKKβ exacerbated T cell deficiency while inhibition of NF-κB with an IκB super-repressor partially alleviated the developmental defect in Id1 transgenic mice. However, it is not clear whether NF-κB activation is directly due to Id1 expression or secondary to the developmental abnormalities occurring in Id1 transgenic thymuses. Here, by using a DP T cell line model system, we demonstrate direct effects of Id1 expression or inhibition of E protein function on NF-κB activation before and after TCR stimulation and transcription of NF-κB downstream target genes, tumor necrosis factor α (TNFα) and interferon-γ (IFNγ). These effects are similar to those found in DP thymocytes isolated from Id1 transgenic mice. These findings suggest that aberrant activation of NF-κB potentiated by Id1 may be one of the mechanisms underlying the developmental defects seen in Id1 transgenic mice.

MATERIALS AND METHODS

Mice, cell line and plasmids-- Id1 transgenic mice were previously described as Id1-28 (24), in which expression of the Id1 cDNA is driven by a T cell-specific proximal promoter of the lck gene. Mice lacking c-rel were previously described by Tumang et al. (27). The CD4 and CD8 double positive 16610D9 T cell line was derived from a
p53 deficient T cell lymphoma (14) and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS). To generate retroviral vectors, cDNA fragments encoding Id1 or E47 were inserted into the EcoRI site of the MIGR vector (28), which carries EGFP coding sequence downstream of the internal ribosomal entry site. The NF-κB reporter construct, pBIIx-luc, contains two NF-κB binding sites upstream of the c-fos minimal promoter (provided by S. Ghosh, Yale University School of Medicine).

**Cell culture and retrovirus transduction** --

The Phoenix-E packaging cell line (29) was cultured in DMEM plus 10% FBS and transfected with retroviral vectors using the calcium-phosphate precipitation method in the presence of 25 µM chloroquine. Twenty-four hours later, cultures were given fresh media and viral stocks were obtained by harvesting culture supernatants after incubation for an additional 24 hours. To transduce 16610D9 cells, cells were resuspended at a density of 5x10^5 cells per ml in viral supernatants mixed with equal volume of fresh medium supplemented with polybrene at a final concentration of 4 µg/ml. The suspensions were then transferred to 6-well plates and spin-infection was performed by centrifugation at 2200 rpm for 90 minutes at room temperature followed by incubation at 37°C overnight and refreshment of medium. Typically, transduction efficiencies of 50-80% were obtained.

Electrophoretic Mobility Shift Assay (EMSA) -

Nuclear extracts were prepared from thymocytes or 16610D9 cells by using an NP-40 lysis method and used in EMSA as described (25). For supershift experiments, antibodies were added to the mixtures at the end of the binding reaction and incubated for an additional 5 minutes. Antibodies against p50, p65, c-rel, relB and E2A were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Immunoblotting --

16610D9 cells with or without stimulation were lysed in RIPA buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 1% NP40, 0.5% Na deoxycholate, and 0.1% SDS) plus a cocktail of protease inhibitors, supernatants were collected after centrifugation. Protein concentrations were determined by using the BCA reagents (Pierce, Rockford, IL). Thirty micrograms of each protein extract was analyzed using SDS-polyacrylamide gels. Antibodies against IKKβ, phospho-IKKα/β, IkBα and phospho-IκBα were from Cell Signaling Technology (Beverly, MA).

**Transient Transfection** --

1-2x10⁷ 16610D9 cells were transfected using a DEAE-DEXTRAN method. Briefly, cells were washed with TS solution (0.8% NaCl, 0.038% KCl, 0.01% Na₂HPO₄, 0.3% Tris HCl, pH to 7.4, 100 µg/ml each CaCl₂ and MgCl₂), resuspended in 1ml TS/DNA/0.5 mg/ml DEAE-DEXTRAN mixture solution and kept at room temperature for 15 minutes before 10 ml of RPMI1640 medium containing 10% fetal bovine serum and 100 µM chloroquine was added. After incubation at 37°C for 30 minutes, cells were collected, washed with fresh medium and then resuspended in fresh culture medium. After culturing for 20-40 hours, cells were collected by centrifugation and lysed in 50 µl lysis buffer (TROPIX, Bedford, MA). Luciferase assays were performed using a Luciferase Reporter Assay System (Promega, Madison, WI). Total amounts of transfected DNA were kept constant by supplementing appropriate amounts of empty vector DNA. For TCR stimulation, cells were resuspended at a density of 2x10⁶/ml and chilled on ice. Antibodies against CD3 and CD28 (BD bioscience, San Jose, CA) were added to a final concentration of 2 µg/ml and incubated with the cells on ice for 10 minutes before addition of anti-Hamster IgG (Sigma-Aldrich, St. Louis, MO) to a final concentration of 2 µg/ml. Cells were returned to 37°C and incubated for desired periods.

Real-time Quantitative PCR --

Total RNA was extracted from cells lysed with a Trizol reagent (Invitrogen, Carlsbad, CA). cDNA templates were produced by reverse transcription with Moloney murine leukemia virus reverse-transcriptase and DNase I treated RNA samples. PCR amplification was performed using Taqman Master Mix and the ABI Prism 7700 machine (Applied Biosystems, Foster City, CA). PCR primers and real time PCR FRET probes for Interferon-γ, TNF-α and GAPDH were from Biosource International (Camarillo, CA).

**Results**

Id1 expression activates NF-κB --

We have previously shown that the DNA binding activities of NF-κB transcription factors are dramatically increased in nuclear extracts of thymocytes from Id1 transgenic mice compared to wild-type mice.
To verify if the increased activities are intrinsic to T cells, nuclear extracts were prepared from sorted CD4 and CD8 DP T cells of wild type and Id1 transgenic mice and used in EMSA with NF-κB and Oct-1 probes. NF-κB binding activities were significantly increased in Id1 transgenic T cells compared to wild-type T cells (Fig. 1A). As a control, Oct1 binding activity in both wild-type and Id1 transgenic cells was similar. This result demonstrates that the increased NF-κB binding activity in Id1 transgenic thymocytes is contributed by DP thymocytes.

To determine if NF-κB is directly activated by Id1 expression or indirectly by an abnormal thymic environment in Id1 transgenic mice, we utilized a CD4+CD8+ T cell lymphoma cell line, 16610D9. To test the effect of Id1 expression, 16610D9 cells were transduced with Id1-expressing or control vector retroviruses. Id1 expression abolished the E-box binding activity by E2A homodimers or E2A/HEB heterodimers since antibodies against E2A supershifted the binding complexes (Fig. 1B). To examine effects of Id1 during TCR stimulation, vector or Id1 transduced cells were stimulated with 2 μg/ml anti-CD3 antibody plus or minus 2 μg/ml anti-CD28 antibody along with anti-Hamster IgG antibodies as cross-linkers of primary antibodies for 2 hours at 37°C. Nuclear extracts were prepared and analyzed using EMSA with NF-κB and Oct-1 probes. Stimulation of TCR by anti-CD3 or anti-CD28 antibodies increased NF-κB binding activities in vector-transduced cells. However, Id1-transduced cells experienced about 10-fold increases in NF-κB activity compared to unstimulated vector control. Co-expression of E47 reduced NF-κB activity by 60% in Id1-transduced 16610D9 cells treated with or without anti-CD3 alone or plus anti-CD28 (Fig. 1D). Although levels of nuclear p65 and RelB were similar in vector and Id1 transduced cells, the level of c-rel was markedly increased in Id1 transduced cells when stimulated with anti-CD3 or anti-CD3 plus anti-CD28 antibodies. Consistently, supershift assays using the same nuclear extracts also showed significant increases in c-rel containing NF-κB binding complexes in Id1 expressing cells upon stimulation with anti-CD3 plus or minus anti-CD28 (Fig. 1D). Together, these results suggest that Id1 expression specifically enhances the activation of c-rel transcription factors upon TCR signaling.

Regulation of NF-κB transcriptional activity by Id1, E47 and IkB--To obtain functional data about regulation of NF-κB activity by Id1, we utilized a luciferase reporter gene controlled by a promoter bearing two NF-κB binding sites in transient transfection assays. This reporter, pBIIx-luc, and a CMV-LacZ construct were co-transfected into 16610D9 cells with Id1 or E47 expressing constructs individually or together. Twenty-four hours after transfection, luciferase activities were measured and normalized against β-galactosidase activities, which served as internal controls for transfection efficiency. Compared to vector co-transfected cells, expression of Id1 caused over 4-fold increase in luciferase activity. In contrast, expression of E47 led to a 60% reduction in the basal level of reporter activity and an 85% reduction in Id1-stimulated expression of the reporter (Fig. 2A). When these transfected cells were stimulated with anti-CD3 and anti-CD28 antibodies, luciferase activity in cells transfected with the reporter alone typically increased 3 to 5 fold depending on the overall condition of the cells and their ability to respond to stimulation. Id1 expressing cells when stimulated with anti-CD3 and anti-CD28 antibodies, luciferase activity in cells transfected with the reporter alone typically increased 3 to 5 fold depending on the overall condition of the cells and their ability to respond to stimulation. Id1 expressing cells when stimulated with anti-CD3 and anti-CD28 antibodies, luciferase activity in cells transfected with the reporter alone typically increased 3 to 5 fold depending on the overall condition of the cells and their ability to respond to stimulation. Id1 expressing cells when stimulated with anti-CD3 and anti-CD28 antibodies, luciferase activity in cells transfected with the reporter alone typically increased 3 to 5 fold depending on the overall condition of the cells and their ability to respond to stimulation.
To test if Id1-induced activation of NF-κB can be inhibited by IκB, an IκBα super repressor (IκBα-SR), which is resistant to IKK-induced degradation, was co-transfected with NF-κB reporter and Id1 in the absence or presence of stimulation with anti-CD3 and anti-CD28 antibodies. Under both conditions, the basal level of NF-κB was inhibited by IκBα-SR. Furthermore, activation of NF-κB caused by overexpression of Id1 can be completely blocked by co-expression of IκBα-SR (Figure 2B). These data demonstrated that activation of NF-κB by Id1 expression as indicated by the reporter activity could be inhibited by IκB.

Id1 potentiates IKKβ activation--To delineate the signaling pathway involved in Id1-potentiated NF-κB activation, the kinetics of activation of IKK complexes were analyzed by immunoblotting with antibodies against phospho-IKK molecules. Retrovirally transduced 16610D9 cells were stimulated with 2 μg/ml plate-bound anti-CD3 and anti-CD28 antibodies and harvested at different time points. Whole cell lysates were analyzed by immunoblotting with antibodies against phospho-IKKα or β (Fig. 3A). The amount of total IKKβ was also measured as a loading control. Signaling through TCR caused a moderate level of IKKβ phosphorylation in vector-transduced cells starting at 2 minutes after stimulation of TCR and the level began to decline after 1 hour. In contrast, the magnitude of TCR-mediated phosphorylation of IKKβ was dramatically increased in Id1-expressing cells although the kinetics of IKKβ activation remained the same. Phosphorylation of IKKα was not affected by either anti-CD3 plus anti-CD28 stimulation or Id1 expression. Consistent with IKKβ activation, IκBα phosphorylation was also dramatically elevated in Id1 expressing cells, which led to a reduction in the level of total IκBα (Fig. 3A). Interestingly, the kinetics of IκBα phosphorylation followed IKKβ activation. These results suggest that Id1 expression enables the cells to vigorously respond to TCR signaling by activating excessive levels of IKKβ. It is worth noting that in the absence of TCR stimulation, Id1 had little effect on IKKβ or IκBα phosphorylation even though Id1 could stimulate NF-κB reporter gene expression by several-fold (Fig. 2 and 3B), which suggests additional mechanisms may be involved in NF-κB activation.

TCR-mediated activation of IKKβ is known to be mediated through the classical pathway involving the interaction between the regulatory subunit of IKK complexes, NEMO, and the catalytic subunit, IKKβ, (8). Inhibitors of IKK complexes have been developed to disrupt the interaction between NEMO and IKKβ. NBA is a cell permeable polypeptide containing the NEMO-binding domain of IKKβ and competes with endogenous IKKβ to interact with NEMO, thus acting as a dominant negative inhibitor (30). NBD is a control peptide containing mutations in the binding site. To test whether Id1-mediated activation of NF-κB utilizes the classical pathway, we measured Id1-stimulated NF-κB reporter expression in the presence of NBD or NBA, as well as antibodies against CD3 and CD28 (Fig. 3B). Upon stimulation with anti-CD3 or anti-CD3 plus anti-CD28 for 2 hours, NF-κB activities in Id1 expressing cells were significantly increased. The same treatments caused slight increases in NF-κB activities in control cells under this assay condition where 0.5 μg/ml anti-CD3 antibody was used to best observe the stimulatory effect of Id1. Addition of NBD but not NBA inhibited NF-κB activation by about 50% in stimulated Id1-expressing cells, suggesting that the classical pathway for NF-κB activation following TCR signaling was partially involved. The residual NF-κB activity in Id1 expressing cells could be due to an incomplete inhibition of the classical pathway by NBD or contributed by a different activating mechanism independent of the classical pathway. Consistent with the second possibility, the NBD inhibitor had minimal effect on Id1-mediated activation of NF-κB in the absence of TCR signaling. The exact nature of such a mechanism remains to be elucidated.

Id1 expression induces overproduction of TNF-α and interferon-γ--In an attempt to understand the molecular mechanism underlying the inhibitory effect of Id1 on T cell development, we performed microarray analysis to compare gene expression profiles between DP thymocytes from wild-type and Id1 transgenic mice and obtained two major groups of genes regulated by interferon-γ and TNF-α (Yang and Sun, unpublished data). For example, IRF-1, IRF-7, ISGF3, ICSBP, STAT1 MX2, MyD88 and SOCS-3 are known to be
regulated by interferon-γ and/or TNF-α (31,32). This result led us to suspect that Id1 expression might cause overproduction of interferon-γ and TNF-α in the thymus. To test this hypothesis, we performed real-time PCR assays using Ficoll-purified thymocytes from wild type, heterozygous and homozygous Id1 transgenic mice. Compared to wild type thymocytes, mRNA levels of both interferon-γ and TNF-α were increased in heterozygous Id1 transgenic mice but more dramatically in homozygous transgenic mice, suggesting a dose-dependent effect of Id1 expression (Figure 4A). Alternatively, the different levels of cytokine production may be explained by a difference in the proportion of various cell populations present in heterozygous or homozygous transgenic thymuses, which may be different in their ability to produce these cytokines.

We next sorted CD4+CD8+ thymocytes from wild type and heterozygous Id1 transgenic mice and found that levels of both interferon-γ and TNF-α mRNA were elevated in the latter (Fig. 4B). These results suggest that Id1 expression in thymocytes intrinsically causes up-regulation of interferon-γ and TNF-α. However, it is possible that other types of cells in the thymus may secondarily contribute to the overall production of interferon-γ and TNF-α because more dramatic differences were observed in total thymocytes.

To further test the direct stimulating effect of Id1 on TNF-α and interferon-γ production, we analyzed the transcript levels of the two cytokines in Id1-expressing 16610D9 cells. Cells were transduced with vector or Id1-expressing retroviruses and stimulated with anti-CD3 plus or minus anti-CD28 antibodies. Transduced cells were then separated from non-transduced cells based on EGFP expression produced from the retrovectors. Real-time PCR analyses were performed using total RNA isolated from EGFP positive and negative cells (Fig. 5A and B). Stimulation with anti-CD3 and especially with both anti-CD3 and anti-CD28 antibodies increased TNFα and IFNγ expression in vector transduced cells or non-transduced cells. However, the same treatments resulted in much more dramatic increases in the production of both cytokines in Id1-expressing cells. Interestingly, non-transduced cells co-cultured with Id1-transduced cells did not exhibit such dramatic increases in TNFα and IFNγ production, suggesting that the synergistic stimulatory effects by TCR signaling and Id1 expression are intrinsic to Id1-expressing cells. Interferon-γ and TNF-α secreted by Id1-expressing cells had minimal effects on their neighboring non-transduced cells.

Id1 enhances TNF-α and interferon-γ expression through c-rel activation--Since expression of TNF-α and interferon-γ is known to involve regulation by NF-κB (2,5), we tested if the effect of Id1 on TNF-α and interferon-γ expression is due to the ability of Id1 to potentiate TCR-stimulated NF-κB activation. Because we found that Id1 expression led to a marked activation of c-rel upon TCR signaling, we examined TNF-α and interferon-γ expression in c-rel deficient thymocytes. Total thymocytes were isolated from wild-type or c-rel deficient mice and transduced with Id1-expressing or control retroviruses. Twenty-four hours later, the cells were stimulated with antibodies against CD3 and CD28. Transduced cells were then sorted for EGFP expression and they displayed a CD4+CD8+ phenotype. TNFα and Interferon-γ mRNA levels were measured using real-time PCR. TCR signaling stimulated TNFα expression in wild-type cells transduced with vector viruses but Id1 expression greatly enhanced this stimulation (Fig. 6A). However, the effects of both TCR signaling and Id1 expression were nearly abolished in c-rel deficient cells, suggesting that c-rel plays an important role in Id1-potentiated and TCR-stimulated expression of TNFα. In addition, c-rel also contributed to basal levels of TNFα expression because lack of c-rel led to reduction of TNFα expression in both vector or Id1-transduced cells in the absence of TCR signaling (Fig. 6A).

Interferon-γ expression was also stimulated by TCR signaling and further potentiated by Id1 expression. Loss of c-rel also diminished these effects (Fig. 6B). However, c-rel appeared to partially contribute to the effect of Id1 in the presence of TCR stimulation because a modest reduction in interferon-γ expression was observed in c-rel deficient mice. Whether the residual level of expression is due to the function of other NF-κB family members or other unrelated transcription factors remains to be determined.


Discussion

Our previous analyses of Id1 transgenic mice, in which Id1 is expressed under the control of lck promoter, revealed aberrant NF-κB activation in total thymocytes, which contributed to the T cell defects seen in these mice (25). However, it was not clear whether NF-κB activation was a direct consequence of Id1 expression or a secondary effect resulted from abnormal thymic environment. For example, inappropriate levels of cytokines in Id1 transgenic thymuses could lead to aberrant activation of NF-κB. Here, we demonstrate the intrinsic effect of Id1 expression on NF-κB activation using a cell culture system. Although TNFα can stimulate NF-κB activation, TNFα expression occurs downstream of Id1 expression and TCR signaling, which lead to NF-κB super-activation. Furthermore our results indicate that cells co-cultured with Id1 expressing cells fail to produce TNFα at the level found in Id1 expressing cells, thus ruling out an extrinsic effect of TNFα on NF-κB activation (Fig. 5A).

Id1 expression acts synergistically with TCR stimulation to activate NF-κB in 16610D9 cells, which is reminiscent of the situation in the thymus where developing thymocytes receive signals from antigen presenting cells through their TCR. The effect of Id1 expression, at least in part, involves the classical pathway of NF-κB activation through the interaction between NEMO and IKKβ, which is known to be responsible for TCR-mediated NF-κB activation (1,10). This is consistent with the observation that IKKβ phosphorylation is rapidly and dramatically increased in Id1-expressing cells upon anti-CD3 and anti-CD28 stimulation. However, part of the activating effect of Id1 appeared to be NEMO-independent, especially in the absence of TCR signaling. The inhibitor, which interferes with NEMO and IKKβ interaction, had minimal effects on the basal level of Id1-induced NF-κB activation as indicated by the luciferase activity produced by a NF-κB reporter gene. In contrast, IkBα super repressor almost completely diminishes NF-κB reporter activity. Taken together, these data suggest that Id1 not only potentiates NF-κB activation through the classic pathway initiated by TCR signaling but also causes NF-κB activation through an unknown alternative pathway. This phenomenon was not only observed in the 16610D9 cell line but also in CD4+ thymocytes of Id1 transgenic mice (26). The molecular nature of this pathway and its interaction with the classical pathway remains to be elucidated.

The mechanism by which Id1 expression, likely through inhibiting E protein functions, influences these NF-κB activating pathways is an interesting issue to be addressed. Because E proteins are transcription factors, it is possible that these proteins activate transcription of genes encoding proteins normally involved in suppressing NF-κB activation. However, such genes have not been identified but their products likely act upstream of IKKβ complexes (Fig. 3). Our unpublished data suggested that Id1 expression also enhances signaling through MAP kinase pathways mediated by Erk and p38. Therefore, it is not clear whether regulation of IKK and MAP kinases involves similar mechanisms or separate events. Interestingly, Id1 expression also causes NF-κB activation in a prostate cancer cell line (33). Whether a similar molecular mechanism is involved in different cell types also remains to be determined.

c-rel is the member of the NF-κB family primarily affected by Id1 during TCR signaling in 16610D9 cells. This is consistent with our previous finding in Id1 transgenic thymocytes. The role of c-rel in Id1-potentiated and TCR-mediated activation of NF-κB was further demonstrated by the reduction of TNFα and interferon-γ expression in c-rel deficient thymocytes. Interestingly, c-rel is known to be preferentially expressed in lymphoid cells and its expression is auto-regulated by NF-κB (6). It will be interesting if initial activation of NF-κB lead to up-regulation of c-rel gene expression or Id1 expression directly causes selective activation of c-rel.

NF-κB hyper-activation has been attributed to the developmental defect found in Id1 transgenic mice (25,26). Since NF-κB transcription factors control the expression of a large number of genes, it is difficult to pinpoint a single specific factor responsible for the developmental defect. Indeed, although expression of IFN-γ and TNFα has been shown to be stimulated by Id1 via NF-κB activities, disruption of receptors for these two cytokines could not significantly rescue T cell development (Yang and Sun, unpublished results). This may be due to multiple defects caused by Id1 expression or even by NF-κB hyper-activation. However, the
finding that Id1 expression influences IFN-γ and TNFα expression has biological implications beyond the T cell lineage. For example, Id1 is found to be expressed at relatively high levels in macrophages (Cochrane and Sun, unpublished results), IFN-γ and TNFα are cytokines produced or utilized by these cells (34,35). Whether Id1 plays a physiological role in IFN-γ and TNFα expression in macrophages, especially in activated macrophages, is an interesting subject awaiting investigation.

Acknowledgement

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Reference


Figure Legends

Figure 1. NF-κB is activated by Id1 expression both in vivo and in vitro. (A) Nuclear extracts were prepared from CD4+CD8+ thymocytes of wild type and heterozygous Id1 transgenic mice. NF-κB activities were detected by using EMSA with NF-κB and Oct-1 probes as labeled. (B) 16610D9 cells transduced with Id1-expressing (Id1) or vector (V) control retroviruses were sorted for EGFP expression and cultured for two days before nuclear extracts were prepared. EMSA was performed using E-box and Oct1 probes. Anti-E2A antibodies were added at the end of a binding reaction with extracts from non-transduced cells (-) as indicated. (C) 16610D9 cells transduced with Id1-expressing or control retroviruses were then stimulated with 2 µg/ml anti-CD3 plus or minus 2 µg/ml anti-CD28 antibodies for another 2 hours. Nuclear extracts prepared from these cells were used in EMSA with NF-κB and Oct-1 probes. Supershift experiments were performed using extracts from Id1 transduced and anti-CD3 plus anti-CD28 stimulated cells by adding indicated antibodies to the samples as indicated. Supershifted complexes are labeled as “SS”. (D) Nuclear extracts from 16610D9 cells were treated as described above and analyzed by immunoblotting with indicated antibodies. A non-specific band was used as a loading control. Supershift assays with anti-c-rel antibodies were performed using the same nuclear extracts.

Figure 2. NF-κB reporter gene expression. 16610D9 cells were transiently transfected with a NF-κB luciferase reporter, pBIIx-Luc, and CMV-lacZ along with: (A) E47 and Id1 individually or together or (B) IκBα-SR and Id1 individually or together. Transfected cells were incubated for 20 hours and stimulated with or without 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28 antibodies for another 2 hours as labeled. Luciferase activities were normalized against that of β-galactosidase and expressed as fold of activation relative to activities in cells transfected with reporter alone without stimulation. Data presented are averages of at least three experiments with standard deviations.

Figure 3. Id1-potentiated NF-κB activating pathways. (A) Retrovirally transduced 16610D9 cells were stimulated with 2 µg/ml plate-bound anti-CD3 and anti-CD28 antibodies and harvested at
different time points as indicated. Whole cell lysates were immunoblotted with antibodies against indicated proteins. (B) 16610D9 cells were transiently transfected with pBI1x-Luc and CMV-LacZ with or without Id1 for 20 hours. Subsequently, 300 µM NBD or NBA polypeptides or DMSO vehicle control was added and incubated for 2 hours before the cells were stimulated with 0.5 µg/ml plate-bound anti-CD3 plus or minus 0.5 µg/ml anti-CD28 antibodies for another 2 hours. Reporter expression was analyzed as described for Fig. 2.

**Figure 4.** Id1 induces overproduction of TNF-α and interferon-γ in vivo. Total RNA was extracted from Ficoll-purified total thymocytes (A) or sorted CD4+CD8+ thymocytes (B) of mice with indicated genotypes. Real-time PCR analyses were performed using cDNAs prepared from total RNA. Levels of TNFα or IFNγ mRNA were normalized against that of GAPDH. Data are presented as relative levels to that detected in wild-type cells and averages of three experiments with standard deviations as shown.

**Figure 5.** Id1 induces overproduction of IFN-γ and TNF-α in vitro. 16610D9 cells were retrovirally transduced with indicated viruses for 24 hours and stimulated with anti-CD3 plus or minus anti-CD28 antibodies for an additional 2 hours. Transduced and non-transduced cells were separated based on EGFP expression using a cell sorter and used to prepare total RNA. Real-time PCR analyses for TNF-α (A) and IFN-γ (B) expression were as described for Fig. 4.

**Figure 6.** Loss of c-rel diminishes overproduction of TNFα and IFNγ stimulated by TCR signaling and Id1 expression. Fresh isolated thymocytes from wild-type or c-rel−/− mice were retrovirally transduced with Id1 or control retroviruses for 24 hours. After stimulation with anti-CD3 plus anti-CD28 antibodies for an additional 2 hours, cells were sorted for EGFP expression and used for isolation of total RNA. Real-time PCR analyses for TNF-α (A) and IFN-γ (B) expression were as described for Fig. 4.
Figure 1

A.  

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<td>NF-κB</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Oct1</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

B.  

| Ab: α-E2A | -  | -  | -  | -  | -  | - |
| cells:    | -  | -  | V  | Id1 |
| SS        | ![Image] |
| E2A-      | ![Image] |
| Oct1      | ![Image] |

C.  

<table>
<thead>
<tr>
<th></th>
<th>vector</th>
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</tr>
</thead>
<tbody>
<tr>
<td>α-CD3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>α-CD28</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NF-κB</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Oct1</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Supershift ab: p50 p65 c-rel RelB

D.  

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<tbody>
<tr>
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<td>+</td>
</tr>
<tr>
<td>α-CD28</td>
<td>-</td>
<td>+</td>
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<tr>
<td>IB:</td>
<td>c-rel</td>
<td>![Image]</td>
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<tr>
<td>p65</td>
<td>![Image]</td>
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<tr>
<td>RelB</td>
<td>![Image]</td>
<td></td>
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<tr>
<td>Loading control</td>
<td>![Image]</td>
<td></td>
</tr>
</tbody>
</table>

EMSA/supershift: w/ α-c-rel

\{ SS \}
Figure 2

A. No stimulation

α-CD3 + α-CD28

Fold of activation

B. No stimulation

α-CD3 + α-CD28

Fold of activation
Figure 3

A.

<table>
<thead>
<tr>
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<td><img src="image6" alt="image" /></td>
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</tr>
<tr>
<td>60</td>
<td><img src="image9" alt="image" /></td>
<td><img src="image10" alt="image" /></td>
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<tr>
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<td>240</td>
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<td><img src="image14" alt="image" /></td>
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B.

Luciferase activities

<table>
<thead>
<tr>
<th>α-CD3</th>
<th>α-CD28</th>
<th>DMSO</th>
<th>NBD</th>
<th>NBA</th>
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<tbody>
<tr>
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<td><img src="image16" alt="image" /></td>
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<tr>
<td>+</td>
<td>-</td>
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<tr>
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<td><img src="image22" alt="image" /></td>
<td><img src="image23" alt="image" /></td>
</tr>
</tbody>
</table>

- CD3            - +        +        - +        +       - +       +
- CD28          - - +        - - +       - +       +

DMSO NBANBD
Figure 4

A.

B.

Relative expression level

WT  Id1tg  Id1tg/tg

0  2  4  6  8  10  12

TNFα

IFNγ

Relative expression level

WT  Id1tg

0  1  2  3

TNFα

IFNγ

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Figure 5

A. Fold of increase in TNFα mRNA

B. Fold of increase in IFNγ mRNA