Id1 Potentiates NF-κB Activation upon T Cell Receptor Signaling*

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Yuanzheng Yang†, Hsiou-Chi Liou§, and Xiao-Hong Sun‡†

From the †Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104 and the ‡Division of Immunology, Department of Medicine, Weill Medical College of Cornell University, New York, New York 10021

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 whether 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 T cell receptor (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, NF-κB essential modulator (NEMO), and the catalytic subunit, IκB kinase β. However, a NEMO-independent pathway also appears to be at play. Id1-potentiated activation of NF-κB leads to overproduction of cytokines such as tumor necrosis factor α and interferon-γ 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 tumor necrosis factor α and interferon-γ expression induced by Id1 and TCR signaling.

Transcriptional regulation of gene expression plays crucial roles in cell proliferation, differentiation, and survival. This is orchestrated by coordinated efforts of various families of transcription factors. Each family of transcription factors is further regulated by specific stimuli or in a temporal and spatial-specific manner. 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 enables 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 tumor necrosis factor (TNFα)² and interferon-γ (IFNγ) (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 such as lymphoprotein β and B-cell-activating factor of the TNF family and phosphorylate p100, causing its cleavage to generate p52 (9). In T lymphoid cells, antigen binding to T cell receptors (TCRs) leads to the activation of IKKβ/NEMO complexes through the interaction and function of a series of adaptor 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. Although their activities are necessary for the earliest stage of T cell commitment and differentiation, E proteins also regulate the function of pre-TCR

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2 The abbreviations used are: TNFα, tumor necrosis factor-α; IFNγ, interferon-γ; TCR, T cell receptor; NEMO, NF-κB essential modulator; IKK, IκB kinases; DP, CD4 and CD8 double positive; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; MAP, mitogen-activated protein.
and TCR by controlling the threshold of signaling (14–17). T cell development constitutes a series of stepwise 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-major histocompatibility complex 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-major histocompatibility complex/peptide complexes in the thymus undergo apoptosis and are eliminated by negative selection. Thus only thymocytes that express TCRs capable of recognizing self-major histocompatibility complex/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, whereas inhibition of NF-κB with an 1kB 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 the 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, TNFα and 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.

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**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 RPMI 1640 medium supplemented with 10% fetal bovine serum. 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 Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum 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 medium, and viral stocks were obtained by harvesting culture supernatants after incubation for an additional 24 h. To transduce 16610D9 cells, cells were resuspended at a density of 5 × 10⁶ cells/ml in viral supernatants mixed with an 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 min 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 Nonidet P-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 min. Antibodies against p50, p65, c-Rel, relB, and E2A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Immunoblotting**—16610D9 cells with or without stimulation were lysed in radioimmuno-precipitation buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) plus a mixture of protease inhibitors, and supernatants were collected after centrifugation. Protein concentrations were determined by using the BCA reagents (Pierce). Thirty micrograms of each protein extract was analyzed using SDS-polyacrylamide gels. Antibodies against IKKβ, phospho-IKKα/β, IkBα, and phospho-IkBα were from Cell Signaling Technology (Beverly, MA).

**Transient Transfection**—1–2 × 10⁷ 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 1 ml of TS solution, DNA, 0.5 mg/ml DEAE-Dextran mixture solution, and kept at room temperature for 15 min before 10 ml of RPMI 1640 medium containing 10% fetal bovine serum and 100 μM chloroquine was added.
After incubation at 37°C for 30 min, cells were collected, washed with fresh medium, and then resuspended in fresh culture medium. After culturing for 20–40 h, cells were collected by centrifugation and lysed in 50 μl of 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 2 × 10^6/ml and chilled on ice. Antibodies against CD3 and CD28 (BD Biosciences) were added to a final concentration of 2 μg/ml and incubated with the cells on ice for 10 min before the addition of anti-hamster IgG (Sigma) 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). 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 fluorescence resonance energy transfer probes for IFNγ, TNFα, and glyceraldehyde-3-phosphate dehydrogenase 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 when compared with wild-type mice (25). To verify whether 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 when compared with wild-type T cells (Fig. 1A). As a control, Oct1 binding activity in both wild-type cells 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 whether 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 the 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 h 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-CD3 plus anti-CD28 antibodies resulted in slight increases in NF-κB activities in vector-transduced cells. In contrast, Id1 expression significantly potentiated NF-κB activation induced by TCR stimulation (Fig. 1C, upper panel). NF-κB binding complexes in anti-CD3 plus anti-CD28-stimulated and Id1-expressing cells primarily consists of homodimers of p50 or heterodimers between p50 and p65, c-Rel, or RelB as suggested by the ability of anti-p50 antibodies to supershift the majority of complexes. Antibodies against p65, c-Rel, or RelB each supershifted a fraction of the slower migrating complexes but not the faster migrating complexes mostly composed of p50 homodimers (Fig. 1C, lower panel).

We next performed immunoblot assays to measure nuclear translocation of c-Rel, p65, and RelB using nuclear extracts isolated from vector- or 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 IκB—To obtain functional data about regulation of NF-κB activity by Id1, we utilized a luciferase reporter gene controlled by a promoter containing two NF-κB binding sites in transient transfection assays. This reporter, pBlx-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. When compared with 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 3–5-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 increased NF-κB activity by about 10-fold when compared with unstimulated vector-transduced cells (Fig. 2A). However, E47 reduced reporter expression to a similar level as unstimulated vector control. Co-expression of E47 and Id1 completely eliminated the stimulatory effect of Id1 (Fig. 2A). These results suggest that inhibition of endogenous E protein function by Id1 results in activation of NF-κB, whereas increasing E protein levels inhibits NF-κB activities.

To test whether Id1-induced activation of NF-κB can be inhibited by IkB, an IkBα super repressor (IkBα-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 IkBα-SR. Furthermore, activation of NF-κB caused by overexpression of Id1
can be completely blocked by co-expression of 1xBo-SR (Fig. 2B). These data demonstrated that activation of NF-κB by Id1 expression as indicated by the reporter activity could be inhibited by 1xB.

Id1 Potentiates IKK 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 min after stimulation of TCR, and the level began to decline after 1 h. 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.

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 (WT) 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 2 days before nuclear extracts were prepared. EMSA was performed using E-box and Oct1 probes. Anti-E2A antibodies (Ab) were added at the end of a binding reaction with extracts from non-transduced cells (-) as indicated. Supershifted complexes are labeled as SS. 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 h. 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 the indicated antibodies to the samples as indicated. D, nuclear extracts from 16610D9 cells were treated as described above and analyzed by immunoblotting with the indicated antibodies. A nonspecific 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 E47 and Id1 individually or together (A) or 1xBo-SR and Id1 individually or together (B). Transfected cells were incubated for 20 h and stimulated with or without 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28 antibodies for another 2 h 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.

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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, although Id1 could stimulate NF-κB reporter gene expression by severalfold (Figs. 2 and 3B), which suggests that 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 endoge-

uous IKKβ to interact with NEMO, thus acting as a dominant negative inhibitor (30). NBA has been developed to disrupt the interaction between NEMO and IKKβ, thus acting as a dominant negative inhibitor (30). NBA has been shown to inhibit NF-κB activation (31). However, the role of Id1 in this context remains to be elucidated.

Id1 Expression Induces Overproduction of TNFα and IFNγ

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 IFNγ and TNFα (31, 32). For example, IFNγ and TNFα are known to be regulated by IFNγ and TNFα. This result led us to suspect that Id1 expression might cause overproduction of IFNγ and TNFα 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. When compared with wild-type thymocytes, mRNA levels of both IFNγ and TNFα were increased in heterozygous Id1 transgenic mice but more dramatically in homozygous Id1 transgenic mice, suggesting a dose-dependent effect of Id1 expression (Fig. 4A). Alternatively, different levels of cytokine production may be explained by differences 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 IFNγ and TNFα mRNA were elevated in the latter (Fig. 4B). These results suggest that Id1 expression in thymocytes intrinsically causes up-regulation of IFNγ and TNFα. However, it is possible that other types of cells in the thymus may secondarily contribute to the overall production of IFNγ and TNFα because more dramatic differences were observed in total thymocytes.

To further test the direct stimulating effect of Id1 on TNFα and IFNγ 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. 5, A 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. IFNγ and TNFα secreted by Id1-expressing cells had minimal effects on their neighboring non-transduced cells.

**Id1 Enhances TNFα and IFNγ Expression through c-Rel Activation**—Since expression of TNFα and IFNγ is known to involve regulation by NF-κB (2, 5), we tested whether the effect of Id1 on TNFα and IFNγ 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 TNFα and IFNγ 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 IFNγ 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- and 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

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**FIGURE 4.** Id1 induces overproduction of TNFα and IFNγ 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 glyceraldehyde-3-phosphate dehydrogenase. Data are presented as relative levels to that detected in wild-type mice (WT) and averages of three experiments with standard deviations as shown. Id1tg and Id1tg/tg designate heterozygous and homozygous Id1 transgenic mice.

**FIGURE 5.** Id1 induces overproduction of IFNγ and TNFα in vitro. 16610D9 cells were retrovirally transduced with indicated viruses for 24 h and stimulated with anti-CD3 plus or minus anti-CD28 antibodies for an additional 2 h. 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 TNFα (A) and IFNγ (B) expression were as described in the legend for Fig. 4.
whether NF-κB activation was a direct consequence of Id1 expression or a secondary effect resulting 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). Id1 expression also enhances signaling through mitogen-activated protein kinase pathways mediated by Erk and p38. Therefore, it is not clear whether regulation of IKK and mitogen-activated protein 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
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of NF-κB was further demonstrated by the reduction of TNFα and IFNγ 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 to determine whether 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 hyperactivation 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 IFNγ 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.3 This may be due to multiple defects caused by Id1 expression of IFNγ, and TNFα utilization by these cells (34, 35). Whether Id1 plays a physiological role in IFNγ and TNFα expression in macrophages, especially in activated macrophages, is an interesting subject awaiting investigation.

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