### Lineage-restricted Function of Nuclear Factor κB-inducing Kinase (NIK) in Transducing Signals Via CD40

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CD40 signaling in B cells and dendritic cells (DCs) is critical for the development of humoral and cell-mediated immunity, respectively. Nuclear factor κB (NF-κB)-inducing kinase (NIK) has been implicated as a central transducing kinase in CD40-dependent activation. Here, we show that although NIK is essential for B cell activation, it is dispensable for activation of DCs. Such data provide compelling evidence that different intermediary kinases are used by different cellular lineages to trigger NF-κB activation via CD40.

Key words: CD40 signaling • nuclear factor κB-inducing kinase • dendritic cell • nuclear factor κB activation • alymphoplasia mice

### Abstract

CD40 signaling in B cells and dendritic cells (DCs) is critical for the development of humoral and cell-mediated immunity, respectively. Nuclear factor κB (NF-κB)-inducing kinase (NIK) has been implicated as a central transducing kinase in CD40-dependent activation. Here, we show that although NIK is essential for B cell activation, it is dispensable for activation of DCs. Such data provide compelling evidence that different intermediary kinases are used by different cellular lineages to trigger NF-κB activation via CD40.

### Introduction

CD40 is a member of the TNFR family and plays a central role in the regulation of both humoral and cell-mediated immunity (1). Engagement of CD40 on B lymphocytes triggers the clonal expansion and differentiation of these cells and is an essential signal in the regulation of thymus-dependent humoral immunity (2–4). Furthermore, stimulation of APCs through CD40 promotes their differentiation and maturation into effective inducers of cell-mediated immunity, as manifested by enhanced production of cytokines and chemokines and expression of costimulatory molecules (5–7).

Although the functional significance of CD40–CD154 interactions in immunity has been studied extensively, the molecular components of the CD40 signal transduction cascade are still not thoroughly understood. One of the downstream events in CD40 signaling is activation of nuclear factor κB (NF-κB) (8), a transcription factor that promotes expression of genes involved in immune and inflammatory responses. The CD40-proximal event in NF-κB activation is recruitment of adaptor proteins called TNFR-associated factors (TRAFs) to the CD40 receptor complex; five of the six known TRAFs (TRAF1, 2, 3, 5, and 6 [9–14]) associate with CD40 upon stimulation by its ligand, CD154 (15). After recruitment to the receptor complex, one or more of the TRAFs activate NF-κB (10, 11, 16) via the IκB kinase (IKK) complex (17), a process that probably involves an intermediary kinase (18–20). The IKK complex then phosphorylates IκB, which triggers degradation of IκB via ubiquitin-mediated proteolysis (for a review, see reference 21).

Degradation of IκB releases NF-κB, and NF-κB then translocates to the nucleus and initiates transcription of genes involved in immune and inflammatory responses. Two serine/threonine kinases have been implicated as intermediary kinases between TRAF recruitment to TNFRs and activation of the IKK complex: NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase/extracellular signal regulatory kinase kinase (MEKK1) (18–20). However, most of the available data on the role of NIK and MEKK1 in NF-κB activation were derived from experiments using transfected cell lines.

Evidence that NIK is an important kinase in mediating TNFR family signal transduction in vivo has recently been deduced using alymphoplasia (aly) mice. Aly mice are characterized by the absence of Peyer’s patches and LNs, as well as by a loss of lymphoid organization in the spleen (22). Furthermore, aly mice have a severely reduced level of serum Ig, particularly IgA. This phenotype resembles the phenotype of the lymphotoxin (LT)βR (23) and LTα knockout mice (24). However, the aly mice have a more severe reduction in serum IgM levels than either the LTα or LTβR knockout mice. It has been demonstrated that the genetic lesion in the aly mouse is a point mutation that results in a single amino acid substitution in the COOH terminus of NIK, and that wild-type NIK expressed in transgenic (Tg) mice can restore a normal phenotype in these mice (25).

The similarity between the phenotypes of...
the aly, LTα knockout, and LTβR knockout mice suggests that the aly mutation interferes with LTβR signal transduction, but an involvement of other signal transduction cascades through other TNFR family members is likely. The studies described here were undertaken to determine whether or not NIK has a direct role in the CD40 signal transduction cascade by analyzing the biological responses of B cells and DCs from aly mice to stimulation through CD40.

Materials and Methods

B Cell Activation Studies. aly/aly and aly/+ mice were obtained (Clea Japan) and bred in the Dartmouth College Animal Resource Center. B cells were isolated from spleens of aly/aly and aly/+ mice, cultured in vitro, and assayed for their ability to proliferate, produce Ig, and upregulate cell surface markers in response to CD40 stimulation as described (26). Induction of proliferation by anti-CD40 (10 μg/ml FGK115 [27]), LPS (50 μg/ml), and anti-IgM (goat anti–mouse IgM) was measured by the incorporation of [3H]thymidine from 66 to 72 h after initiation of culture. All cultures contained 10 ng/ml of IL-4. Induction of Ig secretion was performed using soluble, recombinant CD154 (28) or LPS in combination with IL-4 (10 ng/ml) and IL-5 (10 ng/ml). Ig secretion was measured using an isotype-specific ELISA, as described (4). Expression of cell surface molecules on splenic B cells was measured by flow cytometry as described previously (15).

Phospho-IκBα Immunoblotting. 10 × 10⁶ cells/ml were left unstimulated or stimulated with the optimal dose of CD8–CD154 (COS cell supernatant) or TNF-α (PeproTech) for the indicated times. After stimulation, cells were lysed in lysis buffer containing 1% IGEPAL, 50 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM NaF, 0.4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PM SF. Lysates were spun to remove nuclei and cell debris. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Phosphorylation of IκBα was assayed by immunoblotting with a phospho-specific anti-IκBα antibody (New England Biolabs) according to the manufacturer’s instructions.

Results and Discussion

To investigate the function of NIK in CD40-mediated B cell activation, B cells from aly/+ and aly/aly mice were assessed for their ability to proliferate, produce Ig, and upregulate costimulatory molecules in response to soluble sCD154 and other polyclonal activators. B cells from aly/aly mice displayed a significant reduction in proliferative capacity in response to CD40, LPS, and anti-Ig activation relative to B cells from aly/+ mice (Fig. 1 A). Similarly,

![Figure 1. Role of NIK in activation of B cells aly/aly and aly/+ splenic B cells were cultured in vitro and assayed for their ability to proliferate (A), produce Ig (B), and upregulate cell surface markers (C) in response to CD40 stimulation. (A) Induction of proliferation by anti-CD40 (10 μg/ml), FGK115, LPS (50 μg/ml), and anti-IgM (goat anti–mouse IgM) was measured by the incorporation of [3H]thymidine from 66 to 72 h after initiation of culture. All cultures contained 10 ng/ml of IL-4. (B) Induction of Ig secretion was performed using sCD154 or LPS in combination with IL-4 (10 ng/ml) and IL-5 (10 ng/ml). Ig secretion was measured using an isotype-specific ELISA. (C) Expression of cell surface molecules on splenic B cells from aly/+ (top) or aly/aly (bottom) mice after culture with (purple outline histogram) or without (green outline histogram) sCD154 for 48 h was measured by flow cytometry.

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the capacity of B cells from aly/aly mice to produce IgM and IgG in response to sCD154 and LPS was also reduced (Fig. 1 B). In contrast to the diminished proliferation and Ig production observed in the aly/aly B cells, upregulation of several surface molecules (MHC class II, intercellular adhesion molecule 1 [ICAM-1], CD23, and B7.2) that are hallmarks of B cell activation in response to CD40 stimulation appeared intact. However, upregulation of one surface marker, B7.1, was impaired by the aly mutation (Fig. 1 C).

To further elucidate the molecular basis of the defects in biological responses of aly/aly B cells to CD40 stimulation, phosphorylation of IκBα in response to CD40 stimulation was analyzed as an indicator of NF-κB activation. B cells from spleens of aly/+ and aly/aly mice were stimulated in vitro with sCD154, LPS, or PMA plus ionomycin and assayed for phosphorylation of IκBα by Western blot analysis (Fig. 2). A significant reduction in CD40-stimulated phosphorylation of IκBα was observed in B cells from aly/aly mice relative to B cells from aly/+ animals. Interestingly, LPS-induced NF-κB activation was similar in B cells from aly/aly mice, even though the aly mutation did impact the biological responses to LPS. Finally, no difference in phosphorylation of IκBα was observed when B cells from aly/aly mice were pharmacologically activated with PMA plus ionomycin.

The direct effects of the aly NIK mutation on CD40-dependent B cell proliferation and Ig production indicate that NIK is an important intermediary of B cell activation. Since the aly NIK mutation did not have a noticeable effect on upregulation of several surface proteins, other signaling cascades such as c-Jun N-terminal kinase (JNK)- or tyrosine kinase-mediated pathways (31, 32) may be necessary for complete B cell activation. Alternatively, the aly mutation may have left some aspects of NIK function intact.

Given that the aly mutation exerted a severe biological phenotype in B cells, the impact of the aly mutation in another CD40-responsive cell type was also evaluated. It is known that the APC capacity of DCs is influenced by CD40 ligation, as is the capacity of DCs to produce cytokines (IL-12 [33]) and chemokines (regulated on activation, normal T cell expressed and secreted [RANTES]) (34). To evaluate whether DC responses were impaired by the aly mutation, DCs from the spleens of aly/aly and aly/+ mice were assayed in vitro for their ability to produce IL-12 and present antigen in response to CD40 engagement. DCs from both aly/aly and aly/+ mice produced IL-12 at comparable levels in response to CD40 triggering (Fig. 3 A). To assess DC maturation, DCs from aly/aly mice and aly/+ mice were used as a source of APCs for Tg T cells that express a TCR that specifically recognizes a peptide (amino acids 323–339) from OVA. DCs were pulsed with OVA peptide and cultured with OVA-specific Tg T cells for 2 d, and release of IL-2 into the culture medium was measured by ELISA. DCs from aly/aly and aly/+ mice were equally effective at stimulating IL-2 release from Tg T cells (Fig. 3 B). Furthermore, the CD40-induced upregulation of B7.1, B7.2, ICAM-1, and MHC class II by aly/+ and aly/aly DCs was indistinguishable (data not shown). To evaluate if the CD40-stimulated NF-κB activation pathway in DCs is impaired as a result of the aly mutation, DCs from aly/aly and aly/+ mice were assayed for phosphorylation of IκBα by Western blot analysis (Fig. 4). Phosphorylation of IκBα in response to sCD154 was indistinguishable in DCs from both aly/aly and aly/+ mice. Since TNF-α is a known inducer of NF-κB, and NIK has been implicated in TNFR signaling, we evaluated whether the activation of NF-κB by TNF-α in aly/aly DCs was impaired. As observed with CD40 activation, the activation of NF-κB by TNF-α was indistinguishable between the aly/aly and aly/+ DCs. These results indicate that NIK is not essential for CD40-induced IL-12 production or maturation or for NF-κB activation in DCs. It further shows that NIK is dispensable for activation of NF-κB in DCs by TNF-α.

Although experiments with transfected cell lines have suggested that NIK is involved in CD40 signaling, the data presented in this report demonstrate that NIK plays an essential role in vivo in CD40-dependent biological responses.
of B cells, but not in DCs. B cells from aly mice were largely unresponsive to CD40-induced proliferation, Ig production, and NF-κB activation. However, induced expression of several early activation molecules (e.g., CD23, B7.2) was not impaired by the aly mutation. In contrast, DCs from aly/aly mice were indistinguishable from aly/+ mice in their capacity to produce IL-12 in response to CD40. Furthermore, aly/aly-derived DCs were able to present antigen efficiently as DCs from aly/+ mice. Direct assessment of NF-κB activation in aly-derived DCs demonstrated that NF-κB was intact. Hence, the function of NIK as a mediator of NF-κB activation via CD40 is lineage restricted, and it is likely that another mitogen-activated protein kinase (MAPK) is critical for NF-κB activation via CD40 in DCs. Since MEKK1 has also been shown to be an IKK, it is possible that DCs may utilize MEKK1 to trigger CD40-dependent responses.

Responses to LPS and anti-Ig were also significantly reduced, suggesting that NIK plays a role in these biological responses as well. The role of NIK as a kinase involved in signal transduction through non-TNFR family members has been suggested by studies showing that NIK may regulate CD28-induced IL-2 production (35). Hence, NIK may have multiple roles in the regulation of lymphocyte activities that are not directly related to its ability to associate with TRAFs.

Interestingly, the aly mutation that has been reported in mice bears a striking resemblance to a mutation observed in humans. Although most hyper-IgM (HIM) patients have a mutation in the CD154 gene, there is a cohort of HIM patients that have an autosomal recessive mutation that manifests as a defect in CD40 signaling (36, 37). One such patient (38) was shown to have defective responses in B cell activation, but normal responses within the DC compartment in response to CD40 signaling. In addition, these patients do not exhibit an overt enhanced susceptibility to opportunistic infections, unlike HIM patients with mutations in the CD154 gene. It is possible that mutations in NIK may account for the selective loss of CD40-dependent immunity in the humoral, but not cellular, compartments of the immune system.

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