

Preferential Role for NF- κ B/Rel Signaling in the Type 1 But Not Type 2 T Cell-Dependent Immune Response In Vivo¹

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T cell function is a critical determinant of immune responses as well as susceptibility to allergic diseases. Activated T cells can differentiate into effectors whose cytokine profile is limited to type 1 (IFN- γ -dominant) or type 2 (IL-4-, IL-5-dominant) patterns. To investigate mechanisms that connect extracellular stimuli with the regulation of effector T cell function, we have measured immune responses of transgenic mice whose NF- κ B/Rel signaling pathway is inhibited in T cells. Surprisingly, these mice developed type 2 T cell-dependent responses (IgE and eosinophil recruitment) in a model of allergic pulmonary inflammation. In contrast, type 1 T cell responses were severely impaired, as evidenced by markedly diminished delayed-type hypersensitivity responses, IFN- γ production, and Ag-specific IgG2a levels. Taken together, these data indicate that inhibition of NF- κ B can lead to preferential impairment of type 1 as compared with type 2 T cell-dependent responses. *The Journal of Immunology*, 1999, 163: 5116–5124.

T lymphocytes play a critical role in the development of acquired immune responses. Activation of T cells via the TCR and the costimulatory molecule CD28 induces nuclear forms of transcription factors that include members of the NF- κ B/Rel family (1–3). These data have raised the possibility that NF- κ B/Rel signaling is a key element in the modulation of T cell-dependent immunity. Specific features linking signal transduction with transcriptional regulation in this pathway have revealed it as an attractive target for therapeutic intervention. In quiescent cells, *trans*-activating members of the NF- κ B/Rel family of transcription factors are sequestered in the cytoplasm by inhibitory molecules such as the inhibitor of κ B α (I κ B α)³ (3, 4). Signal transduction pathways from many classes of receptor, including the TCR, converge on I κ B kinase complex(es) (4–6). I κ B α is subsequently phosphorylated by these kinases and degraded by the 26S proteasome, leading to translocation of NF- κ B to the nucleus, where it regulates gene transcription (1–4). NF- κ B induction is associated with proinflammatory stimuli that affect both lymphocytes and nonlymphoid cells, but it is not clear which immune

responses are affected after in vivo interruption of I κ B α degradation specifically in T cells.

Subsequent to antigenic stimulation, T cells acquire distinct patterns of function during immune responses (7–9). The function of these effector T cells is determined in part by their cytokine production profiles. Type 1 T cell effectors activate macrophages and serve as mediators of cell-mediated immune responses such as delayed-type hypersensitivity (DTH), in part through production of IFN- γ and TNF- β (8–11). In contrast, type 2 effector T cells induce IgE- and eosinophil-mediated reactions through production of IL-4, IL-5, and IL-13 (8, 9, 12, 13). These patterns of differential cytokine expression are controlled through transcriptional mechanisms in T cell clones and primary effector cells (14). Thus, a central question has been to identify transcription factors that are involved in differential regulation of type 1 and type 2 T cell responses in vivo. In this regard, Stat6, GATA-3, c-Maf, and NF-ATc all appear to make T cell-intrinsic contributions to the strength of type 2 T cell-dependent responses and to Th2 development (15–17), whereas only Stat4 and IFN regulatory factor-1 in T cells have been associated with type 1 T cell-dependent responses or Th1 effectors (18–20). NF- κ B induction can be correlated with the regulation of multiple gene products involved in inflammatory responses (3, 4, 21, 22). Moreover, Ag receptor cross-linking led to I κ B α degradation and nuclear induction of NF- κ B in Th1 but not Th2 clones, thus suggesting that type 1 (inflammatory) T cells might prove more dependent on NF- κ B than their type 2 counterparts (23, 24). However, it has been unclear whether the function of NF- κ B/Rel proteins in T lymphocytes could preferentially affect type 1 as compared with type 2 T cell-dependent responses in vivo.

To investigate the impact of impaired I κ B α degradation on T cell function in vivo, we generated transgenic mice in which NF- κ B induction is inhibited specifically in T cells by a mutated form of I κ B α (25). This mutant, termed I κ B α (Δ N), is resistant to signal-induced degradation and thus functions as a *trans*-dominant inhibitor of NF- κ B/Rel induction (23–25). Expression of the transgene was targeted specifically to the T lineage using a combination of the T cell-specific CD2 locus control region and the *lck*-proximal promoter (22, 26, 27). The CD4⁺ T cell population in these

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Received for publication May 14, 1999. Accepted for publication August 19, 1999.

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¹ This work was supported by grants from the National Institutes of Health (GM-15431, AI-36997, and HL-61752 to M.R.B., and Diabetes Research Training Center Grant P60 DK20593), a Glaxo-Wellcome Pulmonary Fellowship Award (M.A.A.), followed by an American Lung Association Research Training Fellowship Award (M.A.A.), and by a Leukemia Society of America Scholar's Award (M.R.B.).

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³ Abbreviations used in this paper: I κ B, inhibitor of κ B; BAL, bronchoalveolar lavage; BALF, BAL fluid; DTH, delayed-type hypersensitivity; HNT, anti-HA TCR.

⁴ Mora A., J. Youn, A. D. Keegan, and M. Boothby. NF- κ B/Rel participation in the lymphokine-dependent proliferation of T lymphoid cells. *Submitted for publication*.

transgenic mice was nearly normal, while the CD8⁺ T cell population was diminished to about one-quarter its normal size (23). In vitro characterizations of these cells demonstrated enhanced susceptibility of T cells to apoptosis after TCR ligation, defects in IL-2 production (23), and decreases in proliferation that were incompletely reversed by exogenous IL-2 (23). Moreover, mobility shift analyses demonstrate that a low but reproducible level of these transcription factors can be detected in nuclei from activated thymocytes and T cells of $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice, despite a substantial diminution of nuclear $\kappa\text{B}/\text{Rel}$ proteins (23). Of note, such a state of incomplete blockade to the nuclear induction of NF- κB has been reported after tolerance induction both in B and T lymphocytes (28, 29), suggesting that inhibition of NF- κB might create the equivalent of tolerance. To determine the effect of partial NF- κB inhibition on immune responses in vivo, we have used models characteristic of type 1 (DTH) and type 2 (allergic lung disease) T cell-dependent reactions to one Ag, OVA, and measured immune responses of $\text{IkB}\alpha(\Delta\text{N})$ mice.

The DTH response is a classic example of T cell mediated immunity dependent on IL-12 induction of the type 1 immune response, leading in turn to T cell-derived IFN- γ and further macrophage activation (9–11, 30, 31). We show that the DTH response, IFN- γ production, and induction of Ag-specific IgG2a were all dramatically impaired in $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice compared with wild-type littermates. Type 2 immune responses are crucial in allergic responses to Ags and involve the production of IL-5 and IL-4 in association with eosinophil and mast cell activation and preferential switching to IgE production. In sharp contrast to the dramatic inhibition of type 1 responses, the $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice maintained a normal Ag-specific IgE response and mounted an inflammatory response in a model of allergic pulmonary inflammation. Taken together, our findings demonstrate a differential requirement for NF- $\kappa\text{B}/\text{Rel}$ signaling in type 1 T cell-mediated immune responses relative to the type 2 T cell-mediated reaction.

Materials and Methods

Mice

$\text{IkB}\alpha(\Delta\text{N})$ transgenic mice, in which expression of a FLAG-tagged *trans*-dominant inhibitor of NF- $\kappa\text{B}/\text{Rel}$ transcription factors is targeted specifically to the T lineage using the proximal *lck* promoter and coinjection of a CD2 locus control region, have been described previously (23). These mice were generated from F₁ zygotes derived by mating (DBA/2 $\times$ C57BL/6) breeders (low responders for Th2 development). As previously reported, tissue Northern blot analyses and immunoblots using T cell-depleted B cells failed to detect significant expression of $\text{IkB}\alpha(\Delta\text{N})$ outside of T lineage cells (23). In addition, immunoblotting of extracts from T cell-depleted splenocytes did not detect the FLAG-tagged $\text{IkB}\alpha(\Delta\text{N})$ protein (J. Chen, personal communication). The original lines, backcrossed three generations to C57BL/6, were backcrossed to BALB/c (susceptible to airway hyperreactivity induction), as indicated in the figure legends. In brief, for experiments quantifying the induction of allergy and airway hyperreactivity, data are presented on F₁ progeny generated by mating B6- $\text{IkB}\alpha(\Delta\text{N})$ -transgenic mice with BALB/c. Similar results have been obtained using progeny at the sixth sequential backcross to BALB/c. For experiments on cytokine production and DTH, $\text{IkB}\alpha(\Delta\text{N})$ -transgenic mice and wild-type littermates were the progeny resulting from three sequential backcrosses to BALB/c. HNT TCR transgenic mice extensively backcrossed to BALB/c (32) were obtained from D. Lo (Scripps Research Institute, La Jolla, CA). Adoptive transfer experiments were performed using donor cells from mice ($\text{IkB}\alpha(\Delta\text{N})$ transgenics and wild-type littermates) at the fifth backcross generation and BALB/c recipients. Breeding stock were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen-free conditions using microisolator cages and were used at 6–8 wk of age in accordance with the applicable regulations after institutional approval.

DTH protocol

An emulsion consisting of equal volumes of OVA 100 $\mu\text{g}/20\ \mu\text{l}$ in sterile PBS and CFA H37 Ra (Difco, Detroit, MI) was made by rapid mixing between two glass syringes connected by a three-way stopcock. Mice were anesthetized with methoxyflurane and then immunized by s.c. injection with 200 μl emulsion divided among three sites, one on each flank and a third at the base of the tail on day 0. One week later, mice were challenged with OVA (100 $\mu\text{g}/20\ \mu\text{l}$ in sterile PBS) given as a single injection into the left footpad. Footpad swelling was measured with a caliper micrometer (Mitutoyo, Cole-Parmer Instrument, Vernon Hills, IL) with baseline, $t = 24$ and $t = 48$ h measurements recorded. Data are expressed as the difference in mm between the left (challenged) footpad and the right (control) footpad. Control mice were sham immunized with an equivalent amount of sterile PBS and CFA and then challenged with either PBS alone or OVA. Control data from both $\text{IkB}\alpha(\Delta\text{N})$ and wild-type mice were combined, as there was no difference between these controls.

Cytokine analysis by ELISA

An emulsion consisting of equal volumes of CFA H37 Ra and Ag (OVA (100 $\mu\text{g}/50\ \mu\text{l}$ in sterile PBS) or HNT peptide (150 $\mu\text{g}/50\ \mu\text{l}$)) was prepared by rapid mixing between two glass syringes connected by a three-way stopcock. Mice were anesthetized with methoxyflurane and then immunized with 0.1-ml injection at the base of the tail. Alternatively, mice received an i.p. injection of 0.1 ml (100 μg) of OVA complexed with 20 mg of $\text{Al}(\text{OH})_3$ (alum) on day 0. One week later, mice were sacrificed and draining lymph nodes were harvested (inguinal and periaortic for OVA/CFA; periaortic and mesenteric for OVA/alum). Single cell suspensions were prepared and cultured in RPMI 1640 containing 10% FBS, as previously described (23). The cells were cultured in 96-well plates for 72 h at $2 \times 10^6/\text{ml}$ (200 $\mu\text{l}/\text{well}$) in medium alone, or with Ag (HNT peptide or OVA, as indicated). Supernatants were collected and analyzed for IFN- γ and IL-4 by sandwich ELISA using Ab pairs (PharMingen, San Diego, CA), according to the manufacturer's recommended procedures. The lower limits of sensitivity in the ELISA were 10 pg/ml (IL-4) and 60 pg/ml (IFN- γ), using mouse IL-4 and IFN- γ as standards (PharMingen).

Allergen sensitization protocol

Mice received an i.p. injection of 0.1 ml (10 μg) of OVA (chicken OVA, grade V; Sigma, St. Louis, MO) complexed with 20 mg of $\text{Al}(\text{OH})_3$ (alum) on day 0. On days 14 through 21, groups of mice were placed in an acrylic box so as to breathe an aerosol of OVA 1% w/v diluted in sterile PBS using an ULTRA-NEB 99 nebulizer (DeVILBISS, Somerset, PA) for 40 min each day. To minimize environmental variations between groups, transgene positive and negative littermates were maintained as cagemates and sensitized together. Changes in lung resistance caused by the bronchoconstrictor methacholine were measured, as described previously (33, 34). Control data from both $\text{IkB}\alpha(\Delta\text{N})$ and wild-type mice were combined, as there was no difference between these controls.

Bronchoalveolar lavage

Twenty-four hours following the final aerosol inhalation, the animals were anesthetized with pentobarbital. After placement of a tracheostomy tube, bronchoalveolar lavage (BAL) was performed by instilling 600 μl of 5% BSA diluted in normal saline and then withdrawing the fluid with gentle suction via the syringe. The typical BAL fluid return was 300–400 μl . White blood cells were counted on a hemocytometer, while cytologic examination was performed on cytopsin preparations fixed and stained using Diff Quick (American Scientific Products, McGaw Park, IL). Differential counts were based on counts of 100 cells using standard morphologic criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes). Counts were performed by a single observer who was blinded to the study group.

Lung histopathology

After BAL, the left lung was excised and placed directly in 10% phosphate-buffered Formalin, paraffin embedded, cut in 6-mm sections, mounted, and stained using hematoxylin and eosin for routine histology and Luna stains to specifically evaluate eosinophils. Quantification of eosinophils was performed using the Luna-stained slides by evaluating the three most inflamed arteries in each section. The total number of cells, and the percentage of eosinophils among these cells, were counted and a mean value of the percentage of eosinophils was calculated based on six vessels per group.

Ig assays by ELISA

Before sacrifice, sera were collected from sensitized and control mice, then analyzed by isotype-specific ELISA to determine levels of total and OVA-specific Abs. Briefly, ELISA plates (Corning Glass, Corning, NY) were incubated overnight at 4°C with 50 μ l of capture Ag solution (20 μ g/ml OVA for OVA-specific Ab measurements; sheep anti-mouse IgE (Serotec, Oxford, U.K.) or anti-mouse IgG2a (Southern Biotechnology, Birmingham, AL) for total isotype determinations). After discarding coating solutions, the plates were washed and blocked with 1% BSA in PBS (2 h at room temperature) and washed. Mouse serum or standard Abs diluted in PBS containing 1% BSA were added to each well (50 μ l). Antiserum against OVA, mouse IgE (affinity purified from mouse serum immunized with DNP; Sigma), or mouse IgG2a were used as standards for OVA-specific, total IgE, or total IgG2a ELISA, respectively. Plates were then incubated overnight at 4°C, washed, and incubated with detection Abs (rat monoclonal anti-mouse IgE-biotin (BioSource International, Camarillo, CA) or anti-mouse IgG2a-AP). IgE plates were washed and incubated with avidin-HRP (Zymed, San Francisco, CA) and washed, and HRP activity was determined with a tetra-methylbenzidine (Sigma) developing solution (1% TMB in DMSO, 0.001 M sodium acetate, and 0.45% H₂O₂ final concentration). Alkaline phosphatase activity was determined with phosphatase substrate tablets (Sigma). Both were assessed during the linear phase of the reaction using an ELISA reader (340 ATCC; SLT-Lab Instruments, Crailsheim, Germany) at 450 nm (IgE-HRP) or 420 nm (IgG2a-AP) and DeltaSoft 3 analytical software (BioMetallics, Princeton, NJ). Each sample was tested in duplicate, and the mean value was recorded.

Results

Impaired DTH responses in $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice

Our previous studies suggested that incomplete inhibition of NF- κ B in mature T cells may be sufficient to impair T cell function under in vitro conditions (23). In light of data implicating NF- κ B activation in inflammatory processes, we first tested whether the $\text{IkB}\alpha(\Delta\text{N})$ transgene leads to any impaired type 1 T cell-dependent response by using an assay of cell-mediated immunity. DTH is a T cell-dependent immune response that is manifested as an inflammatory recall response reaching peak intensity 24–48 h after antigenic challenge. The DTH response is dependent on the development of CD4⁺ T effector cells due to their interaction with APC resulting in T cell proliferation and the release of cytokines associated with type 1 but not type 2 T cell help (9–11, 30, 31, 35). Indeed, adoptively transferred type 1 T cells are sufficient to lead to DTH in recipient mice after Ag challenge, whereas normally a priming step followed by Ag challenge would be required (9–11). As shown in Fig. 1A, sham-sensitized mice developed no footpad swelling after Ag rechallenge. Unlike these nonsensitized controls, OVA-sensitized wild-type mice developed significant swelling in the footpad into which Ag was injected. In sharp contrast, the $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice had no significant increase in their re-challenged footpads and markedly reduced footpad swelling when compared with wild-type mice. These data indicate that NF- κ B induction in T cells is required for type 1 T cell-dependent inflammation.

Cytokine production after Ag priming

The DTH response after rechallenge with protein Ag is dependent on creation of a population of Th1 effector T cells, which produce IFN- γ (30, 31). To determine whether the observed impairment in an anti-OVA DTH response reflected a defect in type 1 cytokine production by T cells, mice were immunized with Ag in vivo under conditions similar to the DTH protocol. At the time in which DTH would be elicited by footpad injections of OVA, draining lymph nodes were isolated from these Ag-primed mice. Restimulation of cells from these draining lymph nodes in vitro demonstrated a dramatic defect in IFN- γ production (Fig. 1B). In contrast, IL-4 production by these draining lymph node cells was at or below the limit of detection (data not shown), perhaps reflecting the low frequency of OVA-specific type 2 cells recruited during the inflam-

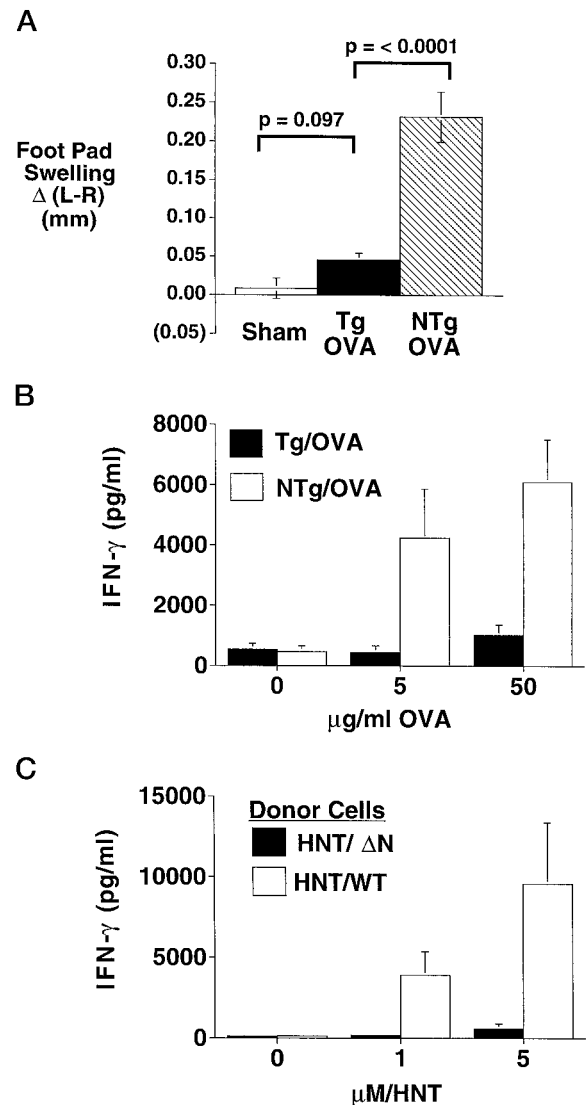


FIGURE 1. Impaired type 1 T cell-dependent responses in $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice. **A**, Diminution in DTH response. Mice (BALB/c F1N3) were immunized with OVA/CFA, and then challenged with OVA by s.c. injection in the left footpad, as described (Materials and Methods). Left footpad swelling was measured and compared with the unchallenged right footpad at 24 h; results were similar using measurement at 48 h. Data are expressed as the mean ($\pm$ SEM) of the difference between left and right footpad swelling in mm. Sham, OVA/CFA sensitized and PBS challenged and PBS sensitized and OVA challenged ($n = 10$); Tg/OVA, $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice, OVA/CFA sensitized and OVA challenged ($n = 24$); NTg/OVA, nontransgenic littermates, OVA/CFA sensitized and OVA challenged ($n = 25$). **B**, Deficient IFN- γ production in $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice after immunization with OVA/CFA. Cell suspensions were prepared from draining lymph nodes of individual $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice and wild-type littermates 7 days after immunization with OVA/CFA, and cultured for 72 h in the presence of 5 or 50 μ g/ml of OVA. Supernatants were assayed for IFN- γ and IL-4 by ELISA. Data are represented as the mean cytokine concentration ($\pm$ SEM). Tg/OVA, $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice; NTg/OVA, nontransgenic littermate controls. IL-4 production was at or below the limit of detection in both groups. **C**, T cell-intrinsic requirement for NF- κ B/Rel signaling in a type 1 response. Nylon wool-enriched T cells were prepared from BALB/c-HNT TCR transgenic mice expressing $\text{IkB}\alpha(\Delta\text{N})$ or littermates with normal NF- κ B signaling. Equal numbers of CD4⁺ T cells, as determined by flow-cytometric analysis, were transferred into naive syngeneic mice (2×10^6 cells per recipient). Mice were immunized with HNT peptide in CFA the next day, followed 1 wk later by in vitro restimulation of draining lymph node cells with the indicated concentrations of HNT peptide. Supernatants were assayed as in **B**.

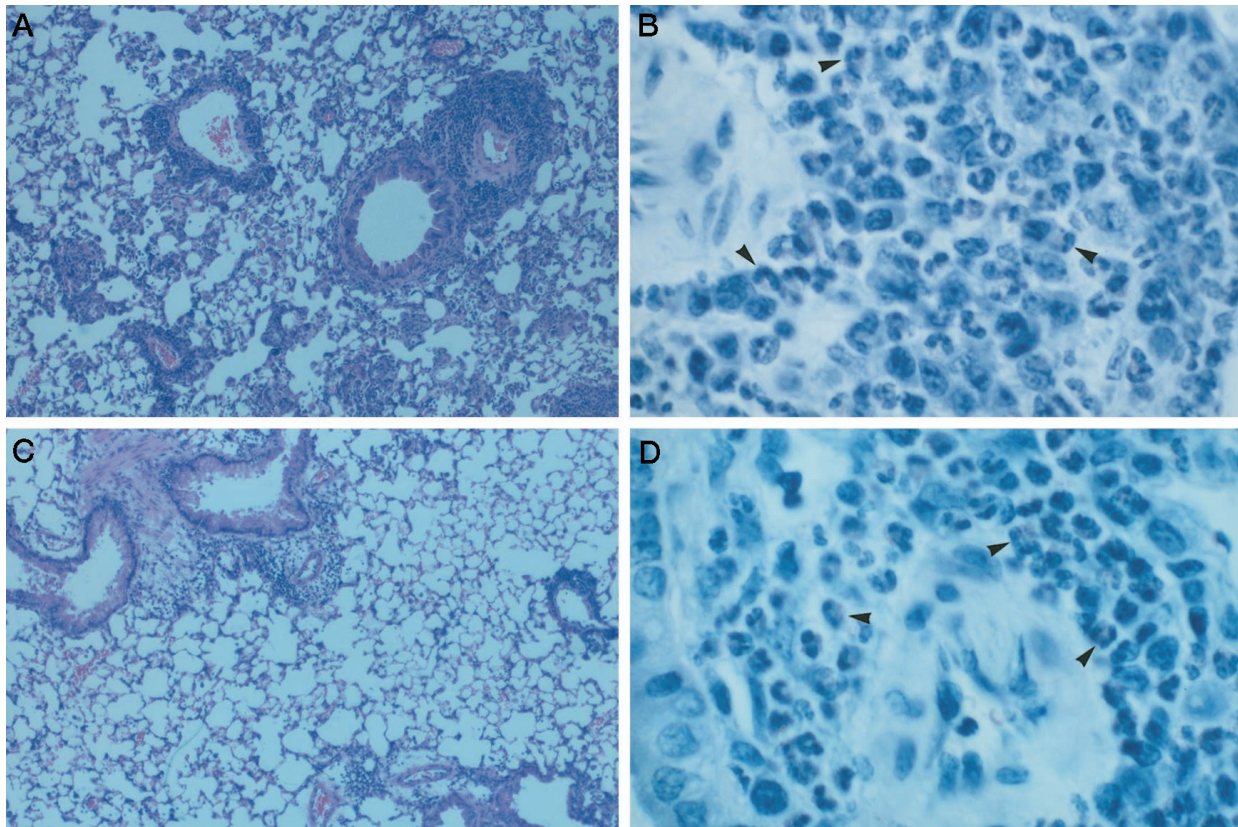


FIGURE 2. Recruitment of perivascular eosinophils in allergic pulmonary reaction of $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice. Wild-type (A and B) and $\text{IkB}\alpha(\Delta\text{N})$ (C and D) mice (F_1) were sensitized to OVA by a single i.p. injection ($10 \mu\text{g}$ in aluminum hydroxide), then subjected to a series of Ag inhalations, as described in *Materials and Methods*. After this induction of allergic lung disease, mice were subjected to BAL, and sacrificed for performance of lung histopathology. Representative lung sections from wild-type and transgenic OVA-sensitized mice after staining with H & E (A and C, respectively) or Luna stain (B and D, respectively). Data from these and similar animals are quantified in Table I.

matory response based on CFA as an adjuvant. To determine whether the defect in $\text{IFN-}\gamma$ production was intrinsic to the T lineage cells, we transferred naive TCR-transgenic cells into BALB/c recipients before immunization with peptide (36). The HNT TCR transgene generates a CD4^+ T cell population specific for an influenza hemagglutinin-derived peptide presented by I-A^d (32). After transfer of equal numbers of HNT T cells into recipients and immunization with this peptide, the $\text{IFN-}\gamma$ response of restimulated draining lymph node cells was abrogated in those samples whose $\text{NF-}\kappa\text{B}/\text{Rel}$ signaling was inhibited by the $\text{IkB}\alpha(\Delta\text{N})$ transgene (Fig. 1C). These data demonstrate that the inhibition of $\text{NF-}\kappa\text{B}$ signaling impaired the development of $\text{IFN-}\gamma$ -producing Ag-specific effector cells in vivo. This failure to develop a type 1 T cell response is not attributable to a lack of wild-type cells in vivo and instead reflects a T cell-intrinsic requirement for $\text{NF-}\kappa\text{B}$ during an inflammatory T cell-dependent response.

Recruitment of inflammatory cells to the lungs of $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice

Although the above data showed that inhibition of $\text{NF-}\kappa\text{B}$ abrogated the inflammatory type 1 T cell response, the outcome of type 2 T cell-dependent immune responses could not be determined under these experimental conditions. Mouse models of allergic pulmonary disease include a type 2 immune response (37–39). Such allergic pulmonary inflammation is dependent on CD4^+ T lymphocytes, in part due to their production and release of effector cytokines such as IL-4 and IL-5, which mediate IgE production and are essential for eosinophil cell recruitment (36–40). To investigate the role of the $\text{NF-}\kappa\text{B}/\text{Rel}$ signaling in Ag-induced type

2 responses, mice were sensitized using OVA in alum, then re-challenged by repeated inhalation of OVA. Histopathology was then performed on lungs from wild-type and $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice after OVA sensitization and airway challenge. Consistent with previous studies of this model, wild-type mice developed a marked inflammatory response in response to OVA, with evidence of large periarterial infiltrates composed predominantly of eosinophils and a lesser component of mononuclear cells (Fig. 2, A and B; Table I). Moderate perivenous infiltrates composed of lymphocytes and eosinophils were also observed. Finally, there was moderate intraalveolar inflammation composed of multinucleated giant cells and eosinophils with a sparse admixture of neutrophils and lymphocytes. Strikingly, the lungs of $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice

Table I. Quantitation of inflammation and eosinophils^a

Group	Periarterial Inflammation	Mean % Eosinophils	Interstitial Inflammation
Sham	0	<5	0
NTg/OVA	2+	62	1+
Tg/OVA	1+	77	0

^a The left lung was excised and placed in 10% phosphate-buffered formalin and processed as described in *Materials and Methods*. The entire section was evaluated, and then scored based on the most severe foci of inflammation, as follows: periarterial inflammation, 0–3 cells thick: 1+; 4–7 cells thick, 2+; >7 cells thick, 3+; interstitial inflammation, increased cellularity without widening, 1+; increased cellularity with widening, 2+; three or more cells wide, or intraalveolar edema or hemorrhage, 3+. Quantitation of eosinophils was performed using the Luna stain. In each section the three most inflamed arteries were evaluated. The total number of cells in the infiltrate was counted, and the percentage of cells that were eosinophils was calculated.

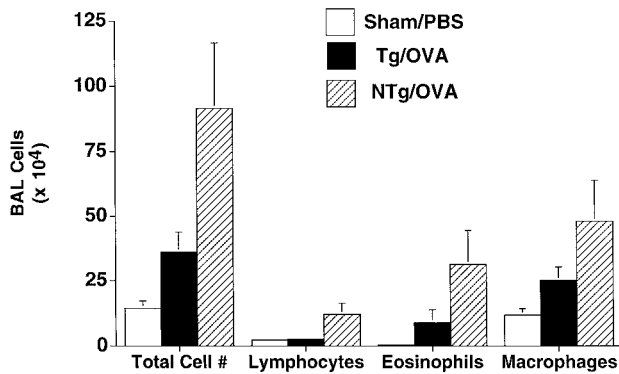


FIGURE 3. Eosinophils and inflammatory cells in BAL fluid from $\text{IkB}\alpha(\Delta\text{N})$ mice. Wild-type and $\text{IkB}\alpha(\Delta\text{N})$ mice were sensitized to OVA by i.p. injection and sequential inhalations as in Fig. 2. Negative control mice were sham sensitized using PBS; both transgenic and wild-type animals were used and their results were pooled, as there was no difference between these groups. Data are represented as mean values ($\pm$ SEM) for the numbers and distribution of cells recovered after BAL of the lungs of the mice.

sensitized and challenged with OVA revealed perivascular infiltrates that were similar to those of the wild-type mice (Fig. 2, C and D; Table I). The periaerial infiltrates were slightly smaller when compared with wild-type (1^+ vs 2^+ , respectively), but maintained a predominant eosinophilia (77% vs 62%), whereas intraalveolar inflammation was minimal. These data show that despite a dramatic decrease in DTH, the T cell-dependent steps leading to the perivascular recruitment of eosinophils were little affected by the inhibition of NF- κ B in $\text{IkB}\alpha(\Delta\text{N})$ mice.

A separate step in the allergic lung infiltration after OVA inhalation is that eosinophils migrate into air spaces from which they can be recovered by lavage. Of note, recent experiments indicate that Th2-mediated eosinophil recruitment depends on Th1 cells (41). BAL fluid (BALF) from the mice in our experiments revealed that the total cell count recovered from the $\text{IkB}\alpha(\Delta\text{N})$ mice sensitized and challenged with Ag was approximately one-third that of their wild-type littermates treated in parallel, but substantially higher than in BALF from nonsensitized controls (Fig. 3). There was no increase in the number of lymphocytes recovered from the sensitized $\text{IkB}\alpha(\Delta\text{N})$ mice as compared with nonsensitized mice. In contrast, OVA sensitization and inhalation led to a substantial increase in the number of eosinophils recovered from $\text{IkB}\alpha(\Delta\text{N})$ mice, although the recovery of eosinophils in BALF was lower for $\text{IkB}\alpha(\Delta\text{N})$ mice than for wild-type littermates that had undergone allergy induction at the same time. Taken together, these findings reveal that eosinophils migrated to the air spaces and, despite a reduction in this migration, there was substantial recruitment of eosinophils to the perivascular spaces. Thus, the $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice were capable of developing an inflammatory response that requires type 2 T cell function.

Ab responses of $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice

The above data suggested that the inhibition of NF- κ B signaling in T cells had a greater effect on type 1 T cell responses as compared with a type 2 response. The development of allergic pulmonary inflammation in mice, as well as atopy in humans, is linked to the production of Ag-specific IgE (42, 43). The generation of Ag-specific IgE requires interaction between T cells and B cells and the production of IL-4, which in turn leads to Ab class switching from IgM to IgE (45, 46). In contrast, the production of IgG2a is augmented by the type 1 T cell-derived cytokine IFN- γ . Previous studies have shown that mice sensitized with OVA in alum and

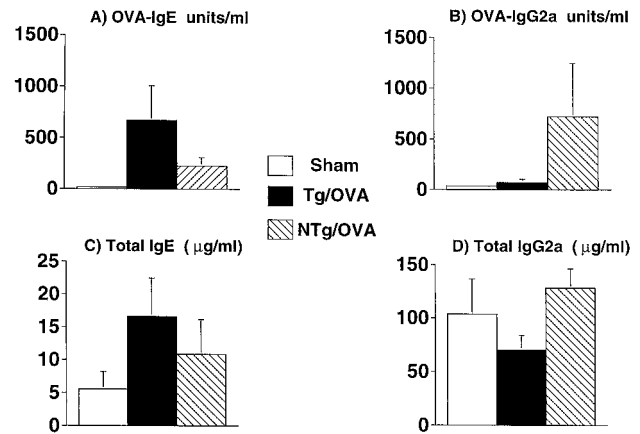


FIGURE 4. Preferential inhibition of IgG2a anti-OVA relative to an intact IgE response. Wild-type and $\text{IkB}\alpha(\Delta\text{N})$ mice were sensitized to OVA by i.p. injection and sequential inhalations as in Fig. 2. Serum was obtained from mice 24 h after their last inhalation challenge, and assayed for OVA-specific and total IgE (A and C, respectively) and OVA-specific and total IgG2a (B and D, respectively). Data are represented as the mean Ig concentration ($\pm$ SEM). Sham, NTg, and Tg littermates sensitized using PBS; Tg/OVA, $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice; NTg/OVA, nontransgenic littermates.

subsequently subjected to inhaled Ag challenge develop both OVA-specific IgE and IgG2a (47). Therefore, we measured the effect of the $\text{IkB}\alpha(\Delta\text{N})$ transgene on the humoral response to allergen exposure in vivo. Remarkably, the serum levels of OVA-specific IgE Abs were higher in sensitized $\text{IkB}\alpha(\Delta\text{N})$ mice than their cosensitized littermates, although the increase did not achieve statistical significance (Fig. 4A). In sharp contrast, the level of OVA-specific IgG2a after sensitization was markedly reduced in the transgenic mice compared with wild-type littermates (Fig. 4B). The observed difference was specific for Ag-specific Ab levels, because the steady state levels of Abs were essentially normal (Fig. 4, C and D). Taken together, these findings indicated that the defective Ab response is due to decreased T cell help, and the help required for Ag-driven Ig class switching to IgG2a was deficient, whereas the Th2-dependent process of Ag-specific IgE production was not (23, 48). To test whether this finding could be correlated with Ag-specific IL-4 production in $\text{IkB}\alpha(\Delta\text{N})$ transgenic mice, we performed immunizations similar to the OVA-priming step of the allergic lung disease model and measured cytokine production after restimulation in vitro. Whereas OVA-stimulated IFN- γ production by lymph node cells from $\text{IkB}\alpha(\Delta\text{N})$ mice was diminished compared with immunized littermate controls, IL-4 production was actually enhanced despite the transgenic inhibitor of NF- κ B (Fig. 5). We conclude that the type 2 T cell-dependent response can develop despite profound inhibition of the NF- κ B/Rel signaling pathway in T cells, whereas the type 1 T cell-dependent response during inflammation depends on NF- κ B.

Airway hyperreactivity in the allergic lung disease of $\text{IkB}\alpha(\Delta\text{N})$ mice

Ag-dependent allergic inflammation of mouse lungs depends on the type 2 T cell-dependent response, and many of its features can be mimicked when large numbers of activated allergen-specific Th2 effector cells are adoptively transferred before a prolonged course of allergen inhalation (49, 50). However, this process and an accompanying component of airway hyperreactivity to bronchoconstrictors may require additional T cell-dependent function(s), perhaps including type 1 T cells (41). Specifically, similar transfer experiments showed that Th1 cells potentiated eosinophil

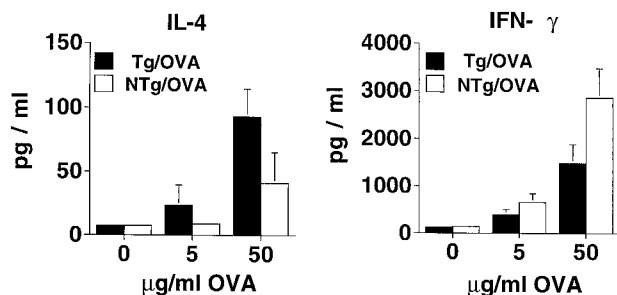


FIGURE 5. Cytokine production in $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice after immunization with OVA/alum. Cell suspensions were prepared from draining lymph nodes of $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice and nontransgenic littermates (BALB/c F1N3) 7 days after immunization with OVA/alum, and cultured for 72 h in the presence of 0, 5, or 50 $\mu\text{g/ml}$ of OVA. Supernatants were assayed for IFN- γ and IL-4 production by ELISA. Data are represented as the mean cytokine concentration ($\pm$ SEM). Tg/OVA, $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice; NTg/OVA, nontransgenic littermates.

recruitment and allergic manifestations. Moreover, recent experiments using physiologic numbers of cells demonstrated that airway hyperreactivity could develop despite a lack of both IL-4 and IL-5 function and in the absence of significant eosinophil recruitment (51). These findings and others raise the possibility that inhibition of T cell-dependent functions other than eosinophil recruitment and type 2 help might attenuate airway hyperreactivity. Accordingly, we measured the bronchoconstrictor response after OVA sensitization of $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice and their littermates. As shown in Fig. 6, $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice developed airway reactivity greater than nonsensitized controls, consistent with other evidence of type 2 responses (Figs. 2–5). Of note, however, perivascular eosinophilia had been slightly diminished (Table I), and alveolar eosinophil recruitment (interstitial inflammation and

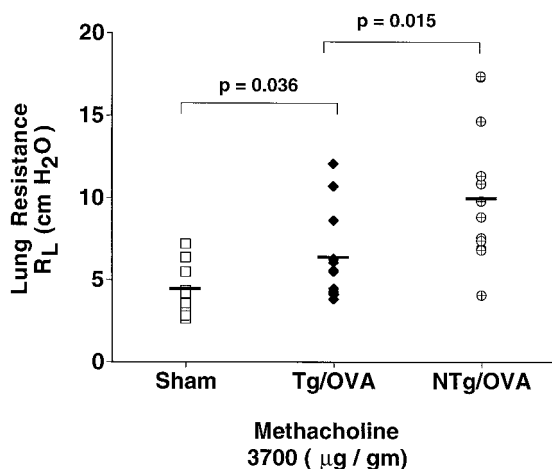


FIGURE 6. Attenuation of airway hyperresponsiveness in allergen-sensitized $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ transgenic mice. Wild-type and $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice were sensitized to OVA by a single i.p. injection (10 μg in aluminum hydroxide), then subjected to a series of Ag inhalations, as described in *Materials and Methods* and Fig. 2. Negative control mice (SHAM) were sham sensitized using PBS. Mice then underwent measurements of lung resistance to airflow after each of a series of methacholine injections. Results are from five separate experiments, each with two to three mice per group (SHAM, Tg/OVA, and NTg/OVA), using a (BALB/c $\times$ B6) F_1 background, as outlined in *Materials and Methods*. Similar results were obtained using mice at the sixth backcross to BALB/c. Each symbol represents an individual mouse, and solid lines (—) indicate the mean value for each group. SHAM, sham-sensitized littermates ($n = 12$); Tg/OVA, $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$, OVA sensitized ($n = 14$); NTg/OVA, nontransgenic, OVA-sensitized littermates ($n = 12$).

bronchoalveolar eosinophil count) was also diminished in $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice compared with sensitized controls (Table I and Fig. 3), despite intact IL-4 and IgE production (Figs. 4 and 5). Consistent with this evidence, the inhibition of NF- κB in T cells resulted in a less pronounced airway hyperreactivity of $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice when compared with littermates. As with other lines of evidence (41, 51–56), these data suggest that the magnitude of airway reactivity may be regulated through T cell populations in addition to type 2 T cells.

Discussion

Two polarized states of differentiation have been characterized among populations of the T lymphocyte effectors that arise during acquired immune responses (7–9, 57). Type 1 T (Th1 and Tc1) cells govern inflammatory responses and immunity against intracellular pathogens, whereas type 2 T (Th2) cells promote Ab production, antihelminthic immunity, and allergic diseases, and may attenuate Th1-mediated tissue injury. The strength of type 1 and type 2 responses, and thus the balance between Th1 and Th2 cells in vivo, appears dependent on the degree to which particular cell surface receptors are stimulated, and consequently on differential activation of signaling pathways (57–59). Ultimately, these differences lead to the selective induction of specific transcription factors that then amplify the differentiative process (14, 18, 58, 59). Thus, the identification of transcription factors whose regulation in T cells can influence the balance between type 1 and type 2 T cell-dependent responses is crucial for understanding immune regulation. However, few factors have been found to play a preferential role in Th1 as compared with Th2 development.

Activation of the NF- κB /Rel transcription factor family in non-lymphoid cells (e.g., monocytes and endothelial cells) has been associated with inflammation (3, 4). In this study, we have investigated the role of this signal-dependent transcription factor during immune responses in vivo. Using transgenic mice whose NF- κB /Rel signaling pathway is inhibited in T cells, we have measured both allergic pulmonary inflammation (type 2) and DTH (type 1) induced by the Ag OVA. We found that inhibition of NF- κB /Rel signaling in T cells had little effect on the recruitment of eosinophils to the perivascular and peribronchial areas of allergen-challenged lungs, and the Ag-specific IgE response in transgenic animals was unimpaired. In contrast, $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice had severely impaired DTH responses and diminished induction of Ag-specific IgG2a. These results provide strong evidence for a critical role for T cell NF- κB /Rel signaling in the development of type 1 immune responses. Consistent with these observations, transgenic mice expressing a mutant $\text{I}\kappa\text{B}\beta$ exhibited decreased contact hypersensitivity, although the effect on IFN- γ or other cytokine production was not determined (60). $\text{I}\kappa\text{B}\alpha(\Delta\text{N})$ mice are protected against autoimmune diabetes (S. Stanley, J. W. Thomas, and M. Boothby, unpublished observations) and collagen-induced arthritis (61), an effect associated with a profound decrease in Ag-induced IFN- γ production. This decrease in type 1 T cell effector function may arise due to the role of NF- κB in IL-18 signal transduction (62), enhanced apoptosis of effector Th1 cells (23, 63), direct regulation of the IFN- γ promoter by NF- κB (64), or some combination of these mechanisms. In addition, we have observed a decrease in the IL-2 responsiveness and Stat5 induction in T cells with impaired NF- κB signaling, despite normal expression of IL-2R subunits (23; A. L. Mora, et al., manuscript submitted). Such a decrease in IL-2 responsiveness may also contribute to the dependence of Th1 development on NF- κB , inasmuch as there is evidence suggesting that the Th1 response in vivo is enhanced by IL-2R α (65). As

suggested by differences between Th1 and Th2 clones in their induction of NF- κ B by TCR ligation in vitro (21, 22), however, our results further indicate that type 2 responses arising from naive T cells in vivo are far less dependent on NF- κ B/Rel signaling than their type 1 T cell-dependent counterparts. Our in vitro studies also indicate that the proliferative response of T cells to IL-4 in vitro and induction of the IL-4R α -chain are regulated by NF- κ B (A. L. Mora, et al., manuscript submitted). Of note, however, activation of the two IL-4 signal transduction pathways implicated to date in Th2 development, Stat6 and insulin receptor substrate-2, was decreased only to about 50% of control levels (A. L. Mora, et al., manuscript submitted). Taken together with the present data on type 2 responses in vivo, these observations suggest that Th2 development can be normal, despite decreased expression of IL-4R α , particularly when the concurrent Th1 response is attenuated so that less IFN- γ is present (Figs. 1 and 5).

Apart from the use of *trans*-dominant inhibitors, the immunologic function of the NF- κ B/Rel family has been studied by inactivation of its specific members through homologous recombination/gene targeting. In considering the overall role of NF- κ B in T cell function, it is important to compare the results presented in this study with the available data on in vivo models of immune responses and inflammatory disease in κ B/Rel knockout mice. There are two important differences between such knockout mice and animals expressing a *trans*-dominant inhibitor that are intrinsic to these comparisons. First, in vivo studies of inflammation in knockout animals typically involved a perturbation present in all cells. Effects on T cell function during disease evolution may reflect a requirement for NF- κ B in the APC population in vivo (35, 57). In contrast, expression of the I κ B α (Δ N) transgene has only been detectable in T lineage cells, perhaps reflecting the combined lineage specificity of the *lck*-proximal promoter and the CD2 locus control region. Moreover, *trans*-dominant inhibition requires a sufficient level of mutant I κ B α to block most NF- κ B binding to endogenous I κ B α . Accordingly, it is likely that substantial inhibition of NF- κ B is achieved only in T cells. Our cell transfer results demonstrate that the inhibition of NF- κ B in T cells creates a cell-intrinsic defect in Th1 development, because all other cells in the recipient mouse have normal NF- κ B/Rel signaling. Second, the biochemical effects of *trans*-dominant I κ B transgenes differ from those achieved by targeted inactivation. Thus, knockout mice selectively lack expression of a single member of the NF- κ B/Rel family. Because I κ Bs interact with multiple forms of NF- κ B/Rel dimers, there is little evidence of such a selective effect under conditions of physiologic regulation. In contrast, the I κ B α (Δ N) transgene leads to diminished nuclear levels of both inducible *trans*-activators in the NF- κ B/Rel family, RelA and c-Rel (23). However, this inhibition is incomplete: trace levels of complexes containing these proteins can be detected in nuclear extracts after T cell activation. The impact of these differences includes the finding that mice expressing this *trans*-dominant inhibitor of NF- κ B/Rel signaling experience increased apoptosis after T cell activation, whereas NF- κ B1-deficient, c-Rel-deficient, and RelA-deficient T cells apparently do not (23, 66–69). For several of the knockout lines, little information on T cell effector function is available. The embryonic lethality of RelA (p65) deficiency has limited investigation of the immunologic consequences of this knockout (67, 68). RelB-deficient mice have impaired development and function of dendritic cells, a crucial APC population, but apparently no cell-intrinsic defect of T lymphocytes (70). Inactivation of c-Rel leads to a broad impairment of Ag-specific Ab responses, but little is known about the susceptibility of such mice to inflammatory or allergic diseases (66, 69).

In this regard, susceptibility to allergic lung disease has been reported for the NF- κ B1 (p50) $-/-$ model. p50 knockout (KO) mice probably have impaired Th cell function (65), although the relative contributions of defective APC function and T cell-intrinsic defects are not clear. Unlike our results (Fig. 4), B lymphoid cells from NF- κ B1^{KO} mice exhibit a selective defect in their ability to switch Ab isotype to IgE (54). Such mice were protected against eosinophilic airway inflammation after OVA sensitization (68), due either to the lack of allergen-specific IgE (42, 54) or to a decrease in IL-5, macrophage-inflammatory protein-1 α , and macrophage-inflammatory protein-1 β production (71), or to other defects in nonhemopoietic cell types. The profound decrease in perivascular eosinophils observed in NF- κ B1^{KO} mice after induction of allergic lung disease (71) presents a striking contrast with the preservation of this recruitment step in I κ B α (Δ N) mice (Fig. 2). We speculate that recruitment of eosinophils into air spaces sampled by BAL must involve at least two steps. The first, trans-endothelial migration, may require a low level of p50, which can translocate to the nuclei of I κ B α (Δ N) but not NF- κ B1^{KO} mice. The second step would be migration from perivascular sites into air spaces and is inhibited by I κ B α (Δ N) expression, perhaps due to the decrease in cytokine or chemokine production by type 1 T cells. It is equally intriguing to note that airway hyperreactivity developed in I κ B α (Δ N) mice and indicators of type 2 T cell function were undiminished (Figs. 4, 5, and 6), yet airway hyperreactivity was decreased. T cells make a contribution to airway hyperresponsiveness that can be achieved despite neutralization of both IL-4 and IL-5 (51). The adoptive transfer of supraphysiologic numbers of activated allergen-specific Th2 cells can prime airway hyperreactivity in immunologically intact recipient mice (49, 50, 72). However, these mice were subjected to prolonged antigenic stimulation after transfer and may have generated pulmonary Th1 cells as a consequence: under physiologic conditions, allergic sensitization activates a substantial population of allergen-reactive Th1 cells (53–55; reviewed in Ref. 52). Interestingly, other studies employing adoptive transfers of Th1 or Th2 cells generated in vitro suggest that collaboration between these subsets is also important for eosinophil immigration (41, 73). These findings raise a question as to whether the decreased airway hyperreactivity and migration of eosinophils into air spaces in I κ B α (Δ N) mice may be due to their attenuated type 1 T cell function. Although these hypotheses remain to be tested in each model, the observed differences between NF- κ B1^{KO} and I κ B α (Δ N) mice in their allergic pathophysiology are probably due to differences in the cellular and biochemical patterns of perturbation in the NF- κ B1^{KO} and I κ B α (Δ N) models.

Acknowledgments

We thank J. Chen for helpful discussions, technical advice, and open sharing of data before publication; D. Lo for the gift of HNT TCR transgenic mice; J. Chen and G. Oltz for critical comments on the manuscript; and W. Armistead and S. Stanley for technical assistance.

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