

Original article

Selective blockade of NF- κ B by novel mutated I κ B α suppresses CD3/CD28-induced activation of memory CD4⁺ T cells in asthma

Background: Nuclear factor κ B (NF- κ B) overactivation plays a crucial role in T-helper 2 (Th2)-biased allergic airway inflammation by increased activation and decreased apoptosis of CD4⁺ T cells. We have shown that targeted NF- κ B suppression in dendritic cells by adenoviral gene transfer of a novel mutated inhibitor of NF- κ B (I κ B α) (AdI κ B α M) contributes to T-cell tolerance, but the immunosuppressive action of AdI κ B α M on memory (CD45RO⁺) CD4⁺ T cells remains enigmatic.

Methods: CD45RO⁺ T cells from *Dermatophagoides farinae*-sensitized asthmatic patients, untransfected or transfected with AdI κ B α M or AdLacZ (β -galactosidase) for 24 h, were stimulated with anti-CD3 (1.0 μ g/ml) plus anti-CD28 (0.5 μ g/ml) monoclonal antibody for an additional 24 h. I κ B α M transgene expression and NF- κ B activation were detected by polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), Western blot analysis, and electrophoretic mobility shift assay. Phenotype and apoptosis were measured by flow cytometry, annexin V binding, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling analyses. Cytokine production and cell proliferation were determined using enzyme-linked immunosorbent assay and [³H] thymidine incorporation.

Results: A unique 801-bp I κ B α M cDNA and a dose-dependent increase in I κ B α M transgene expression were observed in AdI κ B α M-transfected CD45RO⁺ T cells. Significantly, AdI κ B α M inhibited CD3/CD28-mediated NF- κ B activation in CD45RO⁺ T cells, leading to evident apoptosis, reduction of eotaxin, RANTES, Th1 [interferon (IFN)- γ and interleukin (IL)-2], and Th2 (IL-4, IL-5, and IL-13 despite a slight decrease in IL-10) cytokines and secondary proliferative response. AdI κ B α M also upregulated cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and downregulated CD69 besides no change in CD28.

Conclusion: I κ B α M might be beneficial to augment memory CD4⁺ T-cell tolerance through modulating B7-CD28/CTLA-4 co-stimulatory pathways and NF- κ B-dependent cytokine profiles in allergic inflammatory diseases including asthma.

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Key words: asthma; B7-CD28/ cytotoxic T lymphocyte-associated antigen 4; CD4⁺CD45RO⁺ T cells; immunotherapy; nuclear factor κ B/inhibitor of NF- κ B.

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Accepted for publication 5 October 2007

Abbreviations: ABIN-1, A20-binding inhibitor of NF- κ B activation; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; DCs, dendritic cells; Der f, *Dermatophagoides farinae*; EMSA, electrophoretic mobility shift assay; IFN, interferon; IgG, immunoglobulin G; IKK, I κ B kinase; IL, interleukin; I κ B, inhibitor of NF- κ B; mAb, monoclonal antibody; MHC, major histocompatibility complex; MOI, multiplicity of infection; NEMO, NF- κ B essential modulator; NF- κ B, nuclear factor κ B; PBMC, peripheral blood mononuclear cells; RANTES, regulated upon activation, normal T cell expressed and secreted; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCR, T-cell receptor; Th, T helper; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Asthma is a chronic respiratory disease that manifests airway inflammation, obstruction, and hyperresponsiveness to a variety of specific and nonspecific stimuli. Airway eosinophilia is central in the pathogenesis of asthma, which is driven primarily by CD4⁺ T cells (1). At the site of inflamed airways, T-cell accumulation correlates with a delayed apoptosis and an increased level of recruitment from the periphery (2). An aberrant allergen-specific T-helper type 2 (Th2) response occurs in atopic individuals with production of a distinct profile of cytokines during the effector phase, the most important of such cytokines being interleukin (IL)-4, IL-5, and IL-13, as well as chemokines like eotaxin and regulated

upon activation, normal T-cell expressed and secreted (RANTES)(3).

T-cell activation requires the engagement of T-cell receptor (TCR)-CD3 complex triggered by major histocompatibility complex (MHC) class II-associated antigenic peptides, and the co-stimulatory signals mediated by antigen-presenting cells such as dendritic cells (DCs) (4). The most potent co-stimulatory molecules for T-cell activation are CD28 and its structural homolog, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152) (5). CD28 is constitutively expressed both on resting and activated T cells, whereas CTLA-4 is rapidly expressed only on activated T cells. Counter-receptors B7-1 (CD80) and B7-2 (CD86) expressed on antigen-presenting cells manipulate T-cell activation by delivering active signals through CD28, and inhibitory signals through CTLA-4 that functions by directly influencing TCR ζ chain phosphorylation and immune synapse formation (6).

The nuclear transcription factor nuclear factor κ B (NF- κ B), typical of p50 and p65 heterodimer, regulates over 150 genes involved in immune and inflammatory responses. Current evidence suggests an essential role of NF- κ B overactivation for increased expression of many inflammatory genes and for the perpetuation of eosinophilic airway inflammation in asthma (7, 8). One well established mechanism of NF- κ B suppression is the export of nuclear NF- κ B by inhibitor of NF- κ B (I κ B α), thereby rapidly repressing NF- κ B activation (9). We have recently constructed an adenoviral vector AdI κ B α M encoding novel truncated I κ B α (203–1003 bp) without Ser32/Ser36 (10). These mutations prevent I κ B α M phosphorylation, trapping NF- κ B in its inactivated cytoplasmic location complexed with I κ B α M. NF- κ B repression has been linked with T-cell tolerance and decreased capacity to produce TCR and effector cytokines, such as interferon (IFN)- γ , IL-2, and IL-4 (11). Targeted NF- κ B inhibition by AdI κ B α M induced apoptosis and downregulated B7 molecules in DCs, highlighting an important mechanism for T-cell tolerance (12).

Only activated/memory (CD45RO⁺) CD4⁺ T cells are the effector cells infiltrating into inflammatory tissues upon antigen exposure (13). Even then, it remains unclear whether AdI κ B α M could suppress the immune response in memory Th (CD4⁺CD45RO⁺) cells, and what this potentially implicates for immunotherapy of asthma. Here, we demonstrate a selective inhibition of memory CD4⁺ T cells by AdI κ B α M associated with block of NF- κ B activation.

Materials and methods

Cells

Peripheral blood mononuclear cells (PBMC) were obtained from 10 healthy volunteers and 11 house dust mite (*Dermatophagoides farinae*, Der f)-sensitized patients with mild intermittent asthma admitted to Jiangsu Province Hospital by Ficoll-Hypaque

(Pharmacia, Uppsala, Sweden) gradient centrifugation. CD4⁺ T cells were isolated and enriched using RosetteSep CD4 reagent from StemCell Technologies as described (14). CD45RO⁺ T cells (>95% purity) were negatively selected from the isolated CD4⁺ T cells with a MiniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). As all analyzed cells were CD4⁺ T cells, the CD45RO⁺ lymphocytes were considered naïve CD45RA⁺.

Vectors

Serotype 5-, E1-, and E3-defective adenoviral vectors AdI κ B α M and AdLacZ (β -galactosidase) were relevantly constructed with titers of 4.0×10^9 and 1.0×10^{10} plaque-forming units (pfu)/ml, as described previously (10). The molecular weight of I κ B α M is 30 kDa.

Gene transfer

CD45RO/RA⁺ T cells at 5×10^5 cells/dish were infected with AdLacZ or AdI κ B α M at a multiplicity of infection (MOI) of 25–100 pfu/cell for 4 h, serum-starved for 20 h, and then grown in RPMI 1640 containing 10% fetal bovine serum for an additional 24 h at 37°C in 5% CO₂. Immediately after 24 h of transfection, cells were stimulated with soluble anti-CD3 (1.0 μ g/ml) plus anti-CD28 (0.5 μ g/ml) monoclonal antibody (mAb; PharMingen, San Diego, CA, USA) for 24 h. The untransfected and unstimulated CD45RO/RA⁺ T cells from asthmatics were hereafter designated as the control (medium alone).

PCR and reverse transcription-PCR

DNA and RNA were extracted from cells using Wizard plasmid isolation and Trizol kits (Promega, Madison, WI, USA) following the manufacture's instructions, and the RNA was reverse transcribed into cDNA. Polymerase chain reaction (PCR) primers were sense 5'-CCTCTAGAATGAAAGACGAGGAGTACGAG-3', and antisense 5'-TGGTACCTCACAGCTCGTCCTCTGTGAACTC-CGTG-3', yielding an 801-bp product. The reaction started with an initial denaturation at 94°C for 5 min, followed by 35 cycles of 1 min each at 94, 54, and 72°C, and a final extension at 72°C for 10 min. Polymerase chain reaction products were quantified by 1% agarose gel electrophoresis.

Western blot analysis

Cytoplasmic and nuclear proteins were extracted as described previously (8). Denatured proteins (100 μ g) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto polyvinylidene difluoride (PVDF) membranes, and then probed with anti-human I κ B α Ab (1 : 1000) overnight at 4°C and with a recognizing horseradish peroxidase (HRP)-conjugated anti-goat IgG (1 : 5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Immunoblots were visualized with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA). The equal loading of proteins was ensured by Coomassie brilliant blue R staining.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using a commercial kit (Promega, Madison, WI, USA), as described elsewhere (15). The binding reaction was held in a final volume of 20 μ l containing nuclear proteins (5 μ g) and ³²P-labeled oligonucleotide

probe (30 fmol). Protein–DNA complexes were separated on 5% native PAGE and detected by autoradiography. To confirm the specificity, competition assay was conducted with a 100-fold excess of unlabeled wild-type (specific, cold) or mutant (nonspecific, NS) probes. For supershift assay, a 0.5 μ g of anti-p50 or anti-p65 Ab (Santa Cruz Biotechnology) was incubated with the nuclear extracts for 30 min prior to the radio-labeled probe.

Flow cytometry

Cells were incubated at a density of 1×10^6 cells/ml with 10 μ l of saturating concentrations of the fluorescent-labeled mAb, including anti-CD69-PE, anti-CD28-FITC, anti-CTLA-4-PE, and isotype control immunoglobulin G (IgG; eBioscience, San Diego, CA, USA) for 30 min at room temperature. Cells were washed once in phosphate-buffered saline, and the surface markers were assessed by FACScan cytofluorometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (Becton Dickinson). To identify apoptosis, cells were stained with 5 μ l of each annexin V-FITC and propidium iodide for 20 min at 4°C. Additionally, cells undergoing DNA fragmentation were detected with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method (Boehringer Mannheim, Indianapolis, IN, USA).

Enzyme-linked immunosorbent assay

Cellular supernatants were collected 24 h after CD3/CD28 stimulation to measure the levels of IFN- γ , IL-2, IL-4, IL-5, IL-10, IL-13, eotaxin, and RANTES using sandwich enzyme-linked immunosorbent assay (ELISA kits; R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocols, of which the minimum detectable levels were 1.5–5 pg/ml.

Proliferation assay

CD45RO/RA⁺ T cells were cultured in triplicate in 96-well flat-bottomed plates at 2×10^5 cells/well after CD3/CD28 stimulation for 24 h. For secondary proliferation, Der f-sensitized CD45RO⁺ T cells at 5×10^5 cells/well were cultured with 10 μ g/ml of Der f antigen for 7 days after 48 h of resting culture, and reactivated for 3 days with 10 μ g/ml of Der f in the presence of autologous PBMC treated with 50 μ g/ml of mitomycin C (A.G. Scientific Inc., San Diego, CA, USA) as antigen-presenting cells with or without adenoviral transfection and CD3/CD28 stimulation. Cells were pulsed with 0.5 μ Ci/well of [³H] thymidine (TdR) for the final 16 h prior to liquid scintillography.

Statistical analysis

Data were expressed as mean $\pm$ SD, and assessed by one-way ANOVA and paired Student's *t*-test with spss 11.0 software package (SPSS Inc., Chicago, IL, USA). A *P*-value < 0.05 was assumed statistically significant.

Results

AdI κ B α M inhibits CD3/CD28-mediated NF- κ B activation in CD45RO⁺ T cells

Electrophoresis analyses of PCR and RT-PCR products showed a unique 801-bp band in the AdI κ B α M-transfected

CD45RO⁺ T cells only, indicating the integration and transcription of I κ B α M gene in target cells (Fig. 1A). Western blot analysis showed no change in expression of wild-type I κ B α but an overt dose-dependent increase in I κ B α M transgene expression, peaking at MOI 100 pfu/cell and 48 h (Fig. 1B). As assayed by EMSA, pretreatment of PBMC or purified T cells from normals (data not shown) or asthmatics with AdI κ B α M (MOI, 100) markedly impeded the CD3/CD28-induced NF- κ B activation relative to AdLacZ (*P* < 0.01, Fig. 1C,D), demonstrating a powerful tool to block NF- κ B activation. Addition of 100-fold excess of unlabeled native but not mutant NF- κ B probe competed the NF- κ B-DNA complexes, ensuring the binding specificity. Addition of Ab against p50 and p65 resulted in retardation of the supershifted bands, confirming the classic subunits of NF- κ B (Fig. 1C).

NF- κ B repression in CD45RO⁺ is more prominent than in CD45RA⁺ T cells

As a disparity of NF- κ B activation between CD45RO⁺ and CD45RA⁺ T cells has been uncovered in normals (11, 16), we sought to determine whether this relationship would work in asthmatics. Interestingly, we observed that NF- κ B was more strongly activated in CD45RO⁺ T cells than in CD45RA⁺ T cells, possibly harmonizing the fact that there are higher numbers of inflammatory cytokine-producing cells within this subset from asthmatics (Fig. 2A,B). Also, lower doses of AdI κ B α M (50 pfu/cell) were needed to substantially attenuate the NF- κ B activation in CD45RO⁺ T cells compared with CD45RA⁺ T cells (*P* < 0.01). As anticipated, the complete T-cell activation exhibited an apparent proliferation of CD45RO⁺ T cells, which was not influenced by AdLacZ. Conversely, AdI κ B α M sharply blunted the proliferation in CD45RO⁺ T cells (*P* < 0.01, Fig. 2C).

Inhibitory effects of AdI κ B α M on phenotype of CD45RO⁺ T cells

Flow cytometry revealed similar amounts of CD28 in CD45RO⁺ T cells, irrespective of whether it was with CD3/CD28 stimulation or adenoviral transfection, implying that NF- κ B is not necessary for constitutive expression of CD28. As a result of the translocation from plasma to membrane, cells with CD3/CD28 stimulation boosted the appearance of surface CTLA-4 (17). Overexpression of I κ B α M, but not LacZ, clearly upregulated the level of CTLA-4 and downregulated the expression of activation marker CD69 in CD45RO⁺ T cells (Fig. 3).

AdI κ B α M enhances apoptosis of CD45RO⁺ T cells

We next observed whether AdI κ B α M could aid apoptosis in CD45RO⁺ T cells. As measured by the annexin V binding method, CD45RO⁺ T cells underwent apoptosis in a dose- and time-dependent manner following exposure

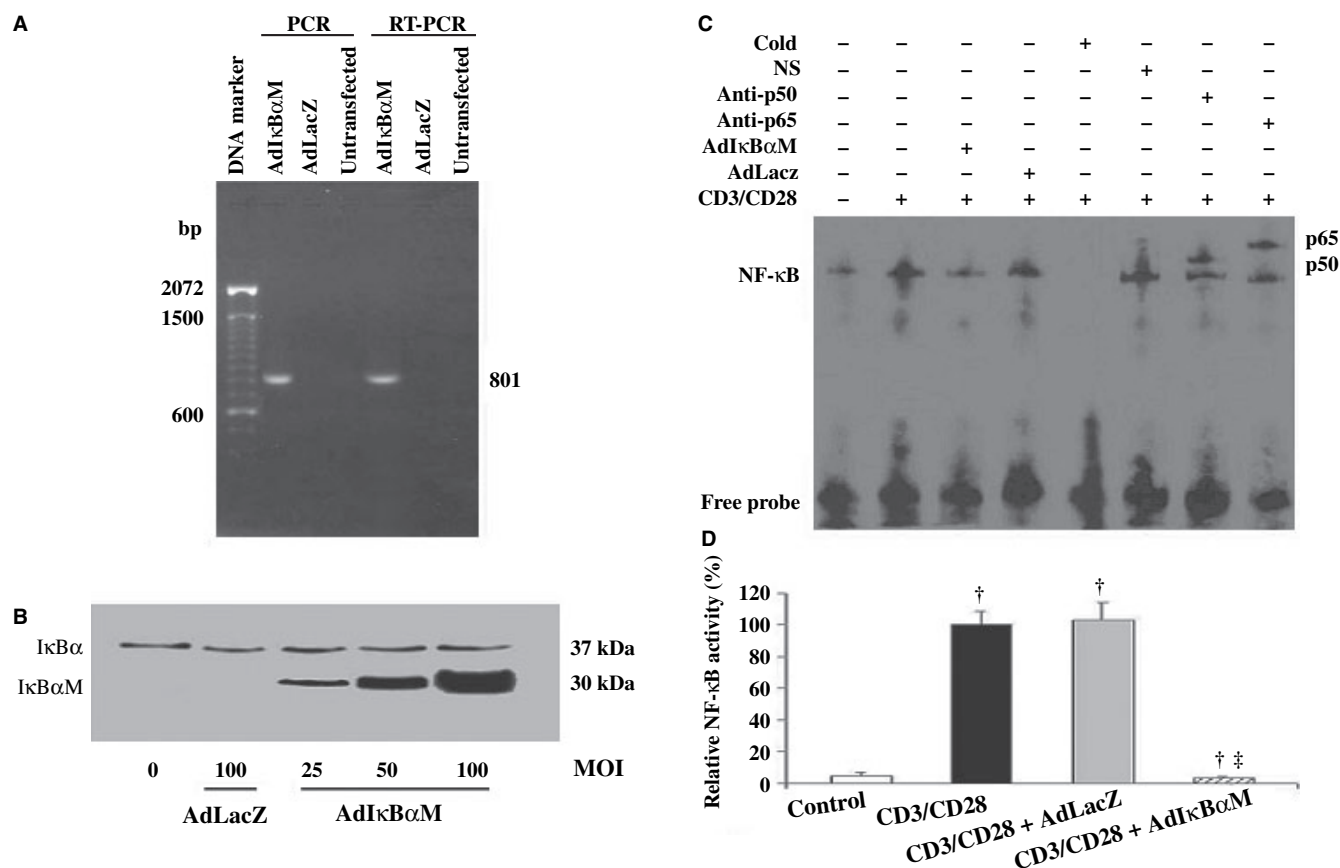


Figure 1. Expression and function of AdIkB α M in CD45RO⁺ T cells. (A) PCR and RT-PCR identified a unique 801-bp product in cells transfected with AdIkB α M. (B) Western blot analysis demonstrated an MOI-dependent increase in IkB α M transgene expression (MOI, 25–100). (C) EMSA analysis indicated a potent NF- κ B repression by AdIkB α M (MOI, 100). The competition and supershift assays verified the binding specificity and subunits of NF- κ B. (D) Densitometry of relative NF- κ B activity in EMSA. $n = 3$ (A–D). †, $P < 0.01$, vs control (untransfected and unstimulated CD45RO⁺ T cells from asthmatics); ‡, $P < 0.01$ vs CD3/CD28.

to AdIkB α M (Fig. 4A). In tandem with the corticosteroids (2), AdIkB α M significantly enhanced the apoptosis in CD3/CD28-stimulated CD45RO⁺ T cells, which was more sensitive in asthmatics than in normals (Fig. 4B). Remarkably, AdIkB α M caused a robust apoptosis in the apoptotic population of CD45RO⁺ T cells than did the AdLacZ in asthmatics. Although less so than in the apoptotic population, apoptosis in the apo-necrotic population was simultaneously heightened ($P < 0.01$, Fig. 4C). Likewise, similar results on the pro-apoptotic capability of IkB α M in CD45RO⁺ T cells were corroborated by the TUNEL assay (Fig. 4D).

Ad α B α M alleviates NF- κ B-dependent cytokine profiles in CD45RO⁺ T cells

We evaluated whether AdIKB α M would affect the spectra of cytokine responses in CD45RO⁺ T cells. Compatible with the EMSA results, ELISA data validated how CD3/CD28-induced release of IFN- γ , IL-2, IL-4, IL-5, IL-13, eotaxin, and RANTES from CD45RO⁺ T cells was lost when cells were pretreated with AdIKB α M (Fig. 5). In

contrast, NF- κ B impairment by AdIKB α M did not statistically decrease IL-10 production ($P > 0.02$), in agreement with recent report that IL-10 is regulated by STAT3, and not by NF- κ B (18).

Adl κ B α M augments CD45RO⁺ T-cell tolerance in atopic asthma

Finally, we investigated whether NF- κ B repression by AdIkB α M in Der f-sensitized CD45RO⁺ T cells could expedite immune tolerance using the secondary proliferative assay and ELISA. In proof of previous report (19), culture of PBMC or CD45RO⁺ T cells with medium alone for 48 h resulted in spontaneous production of IL-10 but not of IL-4, IL-5, or IFN- γ (data not shown). Although IL-5 and IL-10 levels were enhanced by activation with Der f plus CD3/CD28 ($P < 0.01$), only IL-5 production was severely abated in CD45RO⁺ T cells by AdIkB α M (Fig. 6A,B), further confirming the NF- κ B-independent spontaneous secretion of IL-10. Activation of cells with Der f plus CD3/CD28 greatly potentiated the secondary proliferation which was abolished by AdIkB α M but not AdLacZ (Fig. 6C).

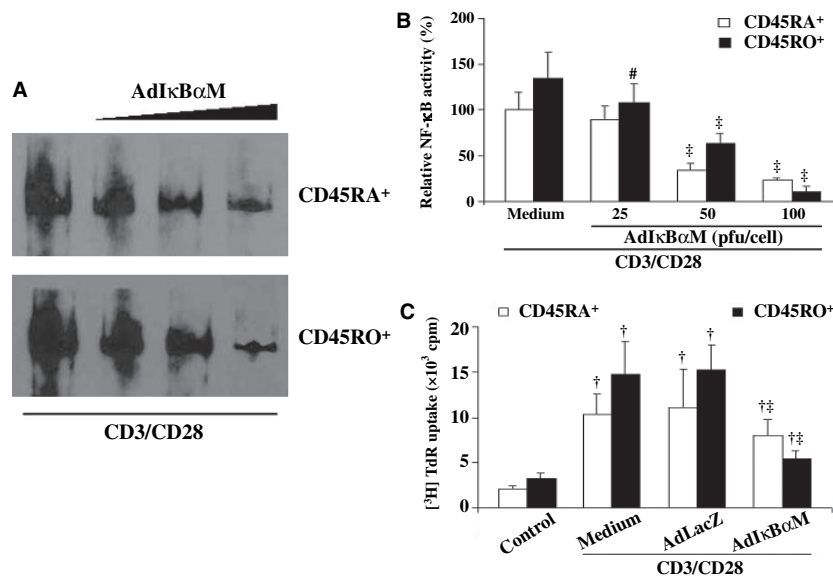


Figure 2. NF-κB impairment by AdIκBαM is more sensitive in CD45RO⁺ than in CD45RA⁺ T cells. (A) EMSA depicted that blockade of CD3/CD28-induced NF-κB activation occurred at lower MOI of AdIκBαM in CD45RO⁺ cells as compared with CD45RA⁺ cells. (B) Densitometry of relative NF-κB activity in EMSA. (C) Proliferation assay showed a stronger inhibition of cell proliferation in CD45RO⁺ than in CD45RA⁺ cells following NF-κB suppression by AdIκBαM (MOI, 100). *n* = 5–7 (A–C). †, *P* < 0.01 vs control (medium alone); #, *P* < 0.05 and ‡, *P* < 0.01 vs CD3/CD28.

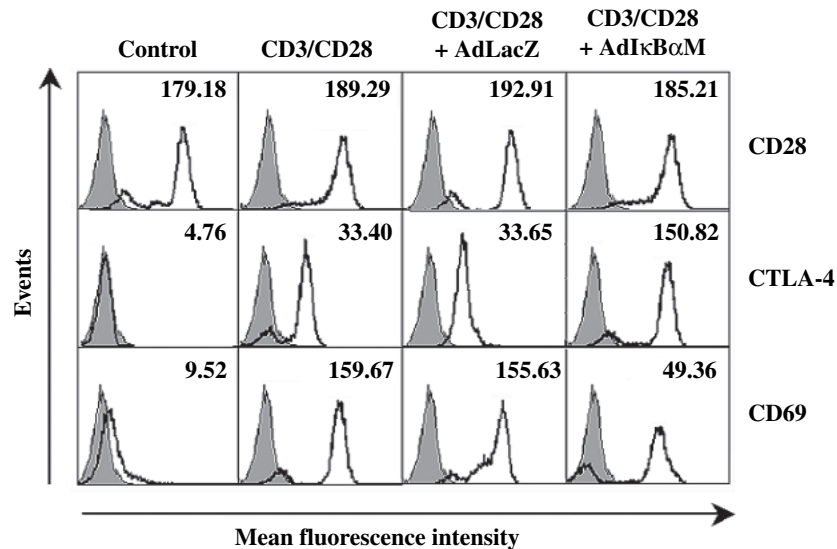


Figure 3. Effects of AdIκBαM on phenotype of CD45RO⁺ T cells. Phenotypic assays by flow cytometry demonstrated a significant difference between the control, CD3/CD28 or AdLacZ, and AdIκBαM (MOI, 100) groups in the expression of CTLA-4 and CD69 (*P* < 0.001) but not of CD28. *n* = 5. Gray histograms, isotype-matched cells; open histograms, AdLacZ- or AdIκBαM-transfected cells.

Discussion

A better understanding of the nature of T-cell tolerance may help exploit novel approach to abrogate the NF-κB-dependent Th2-biased inflammation in asthma. Antigen-experienced CD45RO⁺ T cells are functionally suppressed in the tumor milieu by oxidative stress associated with blocking NF-κB activation (16). However, much of the

evidence supporting T-cell tolerance upon NF-κB repression stems from experiments in a Jurkat T-cell line (20), as little is known about memory CD4⁺ T cells in the pathogenesis of asthma. The present study demonstrates that selective inhibition of NF-κB by AdIκBαM augments CD45RO⁺ T-cell tolerance in atopic asthma. To the best of our knowledge, it is the first time to address the specific IκB interfering with NF-κB signaling in CD45RO⁺ T cells.

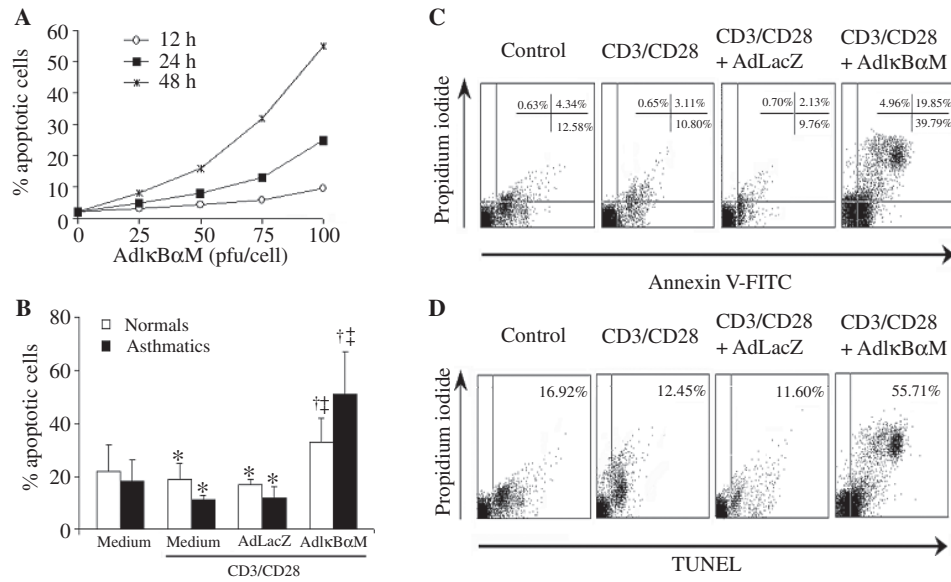


Figure 4. AdIkBαM increases the susceptibility to CD45RO⁺ T cell apoptosis. (A) Flow cytometric analysis using annexin V binding assay displayed an IkBαM-induced apoptosis in CD45RO⁺ T cells in a time- and dose-dependent fashion. (B) Overexpression of IkBαM (MOI, 100) clearly enhanced the apoptosis of CD45RO⁺ T cells from asthmatics than from normals ($P < 0.01$). (C) Annexin V binding illustrated that the difference between AdIkBαM and AdLacZ groups in both apoptotic and apo-necrotic populations is highly significant ($P < 0.001$). (D) TUNEL analysis confirmed the similar results as annexin V binding. $n = 4-6$ (A-D). * $P < 0.05$ and †, $P < 0.01$ vs control; ††, $P < 0.01$ vs CD3/CD28.

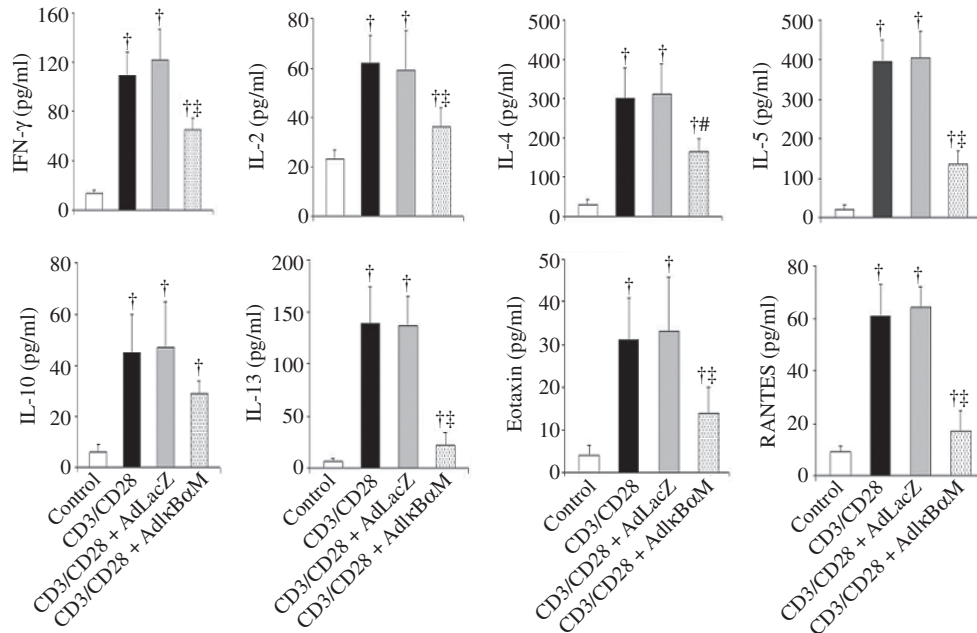


Figure 5. AdIkBαM inhibits NF-κB-dependent cytokine profiles, but not IL-10, in CD45RO⁺ T cells. ELISA analyses of eotaxin, RANTES, Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5, IL-10 and IL-13) cytokine levels under the control, CD3/CD28 stimulation, and CD3/CD28 stimulation plus transfection with AdLacZ or AdIkBαM (MOI, 100). $n = 4$. †, $P < 0.01$ vs control; #, $P < 0.05$ and ††, $P < 0.01$ vs CD3/CD28.

In the context of B7-CD28/CTLA-4 pathways, blockade of B7 co-stimulation contributes to periphery tolerance via downregulating CD28 or upregulating

CTLA-4 (21). CD45RO⁺ T cells can be activated by the TCR stimulation only, whereas CD45RA⁺ T cells require co-stimulatory signals for activation (11). IL-10

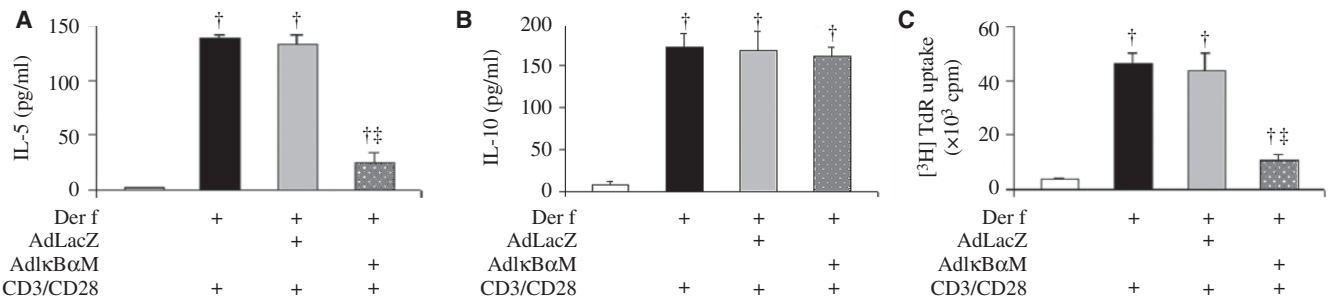


Figure 6. AdIkB α M conduces a memory Th2 cell tolerance in atopic asthma. (A and B) ELISA verified that AdIkB α M (MOI, 100) diminished the production of IL-5 not IL-10 in Der f-sensitized CD45RO⁺ T cells stimulated with Der f and CD3/CD28 after 48 h of resting culture. (C) Proliferation assay validated that AdIkB α M reversed the Der f and CD3/CD28-induced secondary proliferative response in CD45RO⁺ T cells from Der f-sensitized asthmatics. $n = 3-5$ (A–C). †, $P < 0.01$ vs control (medium alone); ‡, $P < 0.01$ vs Der f plus CD3/CD28.

acts *per se* as an immunosuppressive Th2 cytokine irrespective of its pro-inflammatory and anti-inflammatory roles in asthma (22). Existing data indicate that IL-10 inhibits both antigen-specific and anti-CD3 antibody-mediated proliferation and cytokine production by Th2 cells, playing a critical role in T-cell unresponsiveness through restraining the expression of B7 co-stimulatory molecules and MHC class II antigens that are important for T-cell activation (23). We and others have reported that targeted NF- κ B inhibition either upstream or downstream of the I κ B kinase (IKK) complex in DCs contributes to T-cell tolerance (12, 24). A differential regulation of Th1 (IL-2, IFN- γ , and TNF- α) vs Th2 (IL-10) cytokine production by oxidative stress associated with block of NF- κ B activation has been demonstrated in CD4⁺CD45RO⁺ T cells from healthy donors (16). T-cell tolerance is generally relevant to decreased Th1 (IL-2 and IFN- γ) cytokine production and increased production of IL-10 or CTLA-4 (25), in accordance with our data showing that targeted NF- κ B repression by AdIkB α M might conduce CD45RO⁺ T-cell tolerance in atopic asthma.

Inhaled corticosteroids remain the mainstay of asthma management, and most of the anti-inflammatory activities of corticosteroids derive from by inhibiting mainly NF- κ B and AP-1-dependent transcription (26). Corticosteroids, particularly at high doses, have significant and severe adverse effects that could limit their therapeutic usefulness because of the concomitant transactivation (27). In addition, a group of steroid-dependent and -resistant asthmatics poorly responded to corticosteroid treatment (28). We speculate that direct inhibition of NF- κ B could therefore be an alternative way to selectively reproduce the key anti-inflammatory actions of corticosteroids.

Both pharmacologic and gene transfer strategies have been applied to target blockade of NF- κ B. Antagonizing NF- κ B activity by antisense oligonucleotides to p65 or double-stranded decoy oligonucleotides in airway immune cells alleviates the effector phase of experimental asthma (27). Nuclear factor κ B repression in DCs by

targeting IKK complex with the NF- κ B essential modulator (NEMO)-binding domain peptide blocks maturation and prevents T-cell proliferation, accompanied by less Th1 and Th2 polarization (24). Nef-associated factor 1/A20-binding inhibitor of NF- κ B activation (ABIN-1) binds to NEMO/IKK γ , and coordinately elicits NF- κ B impairment with A20 (29). I κ B α superrepressor (I κ B α SR) and I κ B α M are resistant to phosphorylation-induced degradation by site-directed mutagenesis and deletion of Ser32/36, respectively (12). Intrapulmonary delivery of I κ B α M ameliorated airway eosinophilia, but not hyper-reactivity, through facilitating eosinophil apoptosis in allergic mice (unpublished data), consistent with previous findings that adenoviral gene delivery of either ABIN-1 or I κ B α SR attenuated the eosinophilic airway inflammation in a murine asthma model (29).

It is clear that IL-5 augments eosinophil differentiation, proliferation, activation and survival, and thus plays an essential role in the initiation and chronicity of airway eosinophilia (30). IL-13 boosts eosinophil egression, mucus production, and airway hyperresponsiveness (31). Chemokines eotaxin and RANTES have been identified as the most potent chemoattractants for eosinophils and Th2 cells, and potentially for the development of pulmonary fibrosis as a result of sensitization and challenge with allergen (8, 22). Indeed, apoptosis plays a fundamental role in controlling homeostasis at inflamed sites, and tends to confine inflammatory tissue injury, and to urge resolution rather than progression of inflammation (15). The persistence of CD4⁺ T-cell activation in bronchial mucosa is strongly related to delayed apoptosis, enabling prolonged release of inflammatory soluble mediators that amplify both asthma-associated inflammation and airway damage (2). Notably, AdIkB α M was more responsive in promoting the apoptosis of CD3/CD28-stimulated CD45RO⁺ T cells in asthmatics than in normals. This suggests that in CD45RO⁺ T cells, constitutively active NF- κ B is required to maintain a survival response, and NF- κ B inhibition by AdIkB α M unmasks a more potent pro-apoptotic signal in asthmatics as opposed to normal

subjects (32). Given that CD45RO⁺ T cells represent the activated CD4⁺ T cells at sites of inflammation, the combined deficiency of these NF- κ B-dependent Th2 cytokines and chemokines in AdI κ B α M-treated CD45RO⁺ T cells and their increased apoptosis would explain the subsequent magnitude of reduced inflammatory response evoked by inhaled antigen. As allergen-specific Th2 cells generate exacerbated responses and induce skin inflammation, it would be beneficial to interfere with the process of skin homing of memory Th2 cells by selective blockade of NF- κ B with AdI κ B α M for the treatment of inflammatory skin diseases including atopic dermatitis (33).

In conclusion, our data support the proposal that the novel NF- κ B inhibitory protein I κ B α M promotes CD45RO⁺ T-cell tolerance, which correlates with increased apoptosis, decreased proliferation and function via modulation of the B7-CD28/CTLA-4 co-stimulatory pathways and NF- κ B-dependent cytokine profiles. Further investigations are warranted to dissect the complex molecular and cellular interactions implicated in the I κ B α M-induced CD45RO⁺ T-cell tolerance when activated by antigen-pulsed DCs, especially in a trans-endo-

thelial trafficking model (34). I κ B α M offers a potential strategy for future immunotherapy of asthma, as well as the infectious, inflammatory, cancerous, and autoimmune disorders associated with NF- κ B overactivation.

Acknowledgments

We thank all subjects enrolled in this study. We also feel indebted to Drs Hakon Hakonarson, Hengjiang Zhao, Christopher Kirby, Michael Mittelman, Guang Yang, Qing Lin, and Phyllis A. Dennerly (University of Pennsylvania School of Medicine, Philadelphia, PA, USA), Jijun Xu (University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA) and Guangyong Peng (Baylor College of Medicine, Houston, TX, USA) for constructive comments, and David J. Erle (University of California, San Francisco, CA, USA) and Bruce S. Bochner (Johns Hopkins University School of Medicine, Baltimore, MD, USA) for critically reading the manuscript. L.F.Z. is a visiting scientist of Global Health Programs at University of Pennsylvania School of Medicine. This work was supported by grants from the National Natural Science Foundation of China 30400191 and 30570797 (to L.F.Z. and K.S.Y.), and in part from the Summit Project of Jiangsu Personnel 06B035 and the Jiangsu Key Principal Investigator of Medicine RC2007043 (to L.F.Z.).

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