Is IκBζ constitutively expressed in mammalian airway epithelium?

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Author Contributions: KS[1] and MDW conceived and designed experiments; KS[1] and MDW analyzed and interpreted data; KS[1] and MDW drafted the manuscript for important intellectual content; KS[1] and SM performed experiments; AF and FJ designed and performed immunohistochemistry; KSO and SL provided lung tissue from IκBζ knockout mice; PNB and HS provided lung tissue from gnotobiotic mice; AS provided lung tissue from normal wild type mice; KS[6] provided human lung tissue slides. All authors reviewed the final manuscript.

ABSTRACT

IκBζ is a transcriptional factor induced primarily in immune cells upon Toll-like receptor (TLR) activation that drives important cytokine responses. Recent studies have demonstrated constitutive IκBζ expression in the epithelial cells of mouse skin and eyes, possibly reflecting the activation of TLRs by pathogen-associated molecular patterns (PAMPs). In this context, another mucosal surface, the lung epithelium, may not be as actively exposed to the external environment as the skin and the conjunctiva, especially since the lower lung airways are typically conceived to be sterile. Whether IκBζ expression in the lungs is constitutive or induced remains largely unexplored. This is especially important since IκBζ has been shown to promote the expression of protective cytokine and antimicrobial peptide responses, supporting a role for IκBζ in lung host defense. We hence evaluated IκBζ expression in airway epithelia of both humans and mice using immunostaining with antiserum raised against recombinant IκBζ in our laboratory. We observed positive signal in the nuclei of ciliated epithelial cells lining the central airways. Airway cells of gnotobiotic mice also stained positive, suggesting that IκBζ expression does not require induction by bacterial PAMPs. Unexpectedly, we also observed staining in the lung epithelia of IκBζ knockout mice, indicating possible false positive signals from our immunohistochemistry experiments. In this context, 2D gel analysis followed by mass spectrometry revealed that our IκBζ antiserum also detected a nuclear protein lamin B1. Nevertheless, immunoblotting tissue homogenates from gnotobiotic mouse lungs and primary human airway epithelial cells showed the appropriate 86 kDa band for IκBζ. Together, these results demonstrate constitutive IκBζ expression in airway epithelium, suggesting that lung epithelial cells may depend upon IκBζ expression for airway protection.

Keywords: NFKBIZ, IκBζ, airway epithelium**,** constitutive expression.

INTRODUCTION

IκBζ, also known as MAIL (molecule with ankyrin repeats induced by LPS) or INAP (IL-1-inducible nuclear ankyrin-repeat protein), is a protein encoded by the gene *NFKBIZ* [1-3]. It predominantly functions as a transcription factor to regulate the expression of many downstream molecules that are involved in host defense, such as IL-6, IL-12 (p40), granulocyte-macrophage colony-stimulating factor, neutrophil gelatinase-associated lipocalin, human β defensin 2 and monocyte chemoattractant protein 1 [4-9]. IκBζ is a homolog of the IκB group of proteins, due to the presence of an ankyrin repeat region in its carboxy-terminus, with the help of which it binds to NFκB p50 homodimers, typically inside the nucleus of the cell [1, 3]. The amino-terminus of IκBζ encodes a transcriptional activation domain and a nuclear localization sequence. There are two known isoforms of the protein – long (IκBζ-L) and short (IκBζ-S), of which IκBζ-L is predominantly expressed at the protein level [1]. Apart from its role as a transcription factor, IκBζ is known to exhibit other functions too. For instance, IκBζ modulates chromatin remodeling, by selectively regulating H3K4 trimethylation and the assembly of the pre-initiation complex at the promoter of downstream genes [10].

A majority of studies have described IκBζ as a primary response factor induced rapidly following stimulation of immune cells including mononuclear phagocytes, NK cells, T and B lymphocytes [6, 8, 11]. Nevertheless, constitutive expression of IκBζ has been demonstrated in mucosal and epidermal sites that interact with the outside environment [12, 13], indicating the possibility of exogenous challenges inducing constant IκBζ expression in these cells. Additionally, IκBζ knock out mice display severe inflammation of the epithelium lining their eyes and skin [13-15], further supporting the importance of IκBζ in maintaining immune homeostasis of the host. Interestingly, IκBζ has been demonstrated to regulate the expression of

pro-inflammatory cytokines and anti-microbial peptides in airway epithelial cells that line the respiratory mucosal surface [4, 5, 16]. Studies have shown baseline *NFKBIZ* gene expression in lung tissues [3, 14], although the induction of IκBζ protein expression in the airway epithelium in response to environmental agonists remains understudied. The present study therefore explored the expression of IκBζ in both human and mouse airway epithelial cells. Using immunohistochemistry with our lab-made IκBζ antiserum, we detected positive staining in the nuclei of ciliated epithelial cells in the upper airway tract of human and wildtype gnotobiotic mice. Although for reasons unknown immunostaining was also obtained in *Nfkbiz* knockout mice, immunoblotting of tissue homogenates from gnotobiotic mice and primary human airway epithelial cells strongly indicated potential constitutive expression of IκBζ. Thus, our results support a role of IκBζ for airway protection.

MATERIALS AND METHODS

Reagents and antibodies

The reagents were obtained from the following sources: IL-1 receptor antagonist, IL-1Ra (Amgen), penicillin-streptomycin (Invitrogen), fetal bovine serum FBS (Atlanta Biologicals), Dulbecco modified Eagle medium low glucose DMEM (Invitrogen), Bronchial Epithelial Growth Medium BEGM bullet kit (Lonza), Bronchial Air Liquid Interface B-ALI media (Lonza), Small airway epithelial cell media and detach kit (PromoCell), recombinant human IL-1β was purchased from R&D systems. Rabbit antiserum against IκBζ was generated in our laboratory using recombinant protein expressed in *Escherichia coli* [8].

Mouse lung tissue

Lungs were harvested from wildtype B10RIII mice weighing 17-20 grams and lungs and tracheae were isolated from 9-12 weeks old gnotobiotic male Swiss Webster mice, weighing 17-21 grams. Wildtype and *Nfkbiz* knockout mice [13] were allowed free access to food and water, while the gnotobiotics were bred and maintained in sterile isolators. All animal experiments were performed according to animal protocols approved by the Animal Care Use Committee of the Ohio State University College of Medicine. Lungs from wildtype mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma) to preserve the pulmonary architecture. The lungs were then embedded in paraffin. The sections of all organs collected were then stained with hematoxylin and eosin or with anti- IκBζ antiserum, along with appropriate controls.

Human lung tissue

Anonymized human lung tissue slides were provided by the Ohio State University Department of Pathology, Tissue Archive Service. Other investigators may have received specimens from the same subjects.

Immunohistochemistry

The lung tissue slides were labeled with IκBζ antiserum at the specified dilution with anti-rabbit secondary antibody at a 1:1000 dilution at the Comparative Pathology and Mouse Phenotyping Shared Resource, at the Veterinary school of OSU.

Cell culture

BEAS2B cells (purchased from ATCC) were maintained in DMEM low glucose, supplemented with 10% FBS and 1% penicillin-streptomycin, in a 37°C humidified incubator with 5% CO_2. Primary human bronchial epithelial cells (HBECs), purchased from Lonza were

allowed to differentiate in B-ALI media in 24-well inserts (6.5 mm) as per manufacturer's instructions. Human small airway epithelial cells (HSAECs) were maintained in their special growth media, both obtained from PromoCell. HSAECs ($2x10^5$ cells per ml) or HBECs ($5x10^4$ cells/$33mm^2$) were stimulated with rhIL-1β (10ng/ml) for 3 hours, with or without 30 minutes of pretreatment with IL-1Ra (10ug/ml). Cells are then lysed and immunoblotted.

Generation of recombinant IκBζ

IκBζ was cloned in pET32a bacterial vector with an N-terminal thioredoxin tag and a C-terminal histidine tag. This construct was over expressed in BL21DE3RIL strain of bacteria. The over expressed protein was purified using HisPur cobalt resin and concentrated by dialysis using slide-a-lyzer, both from Thermo Scientific. Expression of purified rIκBζ-S was confirmed using immunoblotting with both anti-IκBζ antiserum and His antibody.

2D gel analysis

Human monocytes isolated from peripheral blood were stimulated with LPS for 3 h. The cells were lysed, protein estimated and used for immunoprecipitation with anti-IκBζ antiserum. The immunoprecipitated samples were run on a 2D gel electrophoresis system at the OSU's protein analysis core facility, on an 18-cm 4-7 IPG strip, since the predicted isoelectric point for IκBζ was around 6. The separated proteins were then either transferred onto a PVDF membrane and immunoblotted with anti-IκBζ antiserum or were cut out from the gel and identified using mass spectrometry.

Tissue homogenization

Tissue chunks of lungs or trachea from mice were added to SDS lysis buffer (100 mM NaCl 500 mM Tris, pH 8.0 10% SDS) and vortexed for about 30 minutes till the chunks dissolved. The tissues were then syringed for about 20 times and boiled at 100°C for complete

extraction. The tissue extracts were then protein estimated and immunoblotted for IκBζ expression.

Preparation of cell lysates and immunoblotting

Cells were lysed in TN1 lysis buffer (50 mM Tris-HCl, pH 8.0, 125 mM NaCl, 10 mM ethylene diaminetetraacetic acid (EDTA), 10 mM NaF, 10mM sodium pyrophosphate,1% Triton X-100) with a protease inhibitor cocktail mix (Sigma-Aldrich), 10 mM methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 3 mM sodium orthovanadate. The extracts were incubated on ice for 15 minutes, syringed 10-15 times and centrifuged at 16,000g for 10 minutes. The lysates were transferred to a new Eppendorf tube and total protein in each sample was determined using Lowry assay (Bio-Rad). Equal protein (20ug per lane) was loaded onto a NuPAGE 7% Tris-acetate gel (Invitrogen). The separated proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 10% nonfat milk, incubated overnight at 4°C with the primary antibody, washed and then stained with appropriate peroxidase-conjugated secondary antibody for an hour. The protein bands were visualized by enhanced chemiluminescence substrate (GE Healthcare) by autoradiography.

RESULTS

To understand IκBζ's behavior in epithelial cells, we immunostained lung tissue slices from normal human subjects (**Figure 1A**) and from wild type mice (**Figure 1B**) with anti-IκBζ antiserum generated against recombinant IκBζ. We observed a significant increase in staining with anti-IκBζ antiserum compared to the preimmune control serum and the secondary antibody

negative control. This signal was predominantly present in the nuclei of ciliated epithelial cells lining the central airways.

Because IκBζ is considered to be a classic example of an inducible protein, we wanted to determine if the baseline IκBζ expression was due to exposure to bacterial PAMPs. Therefore, we examined lung tissue from gnotobiotic (germ-free) mice. Surprisingly, similar to what we observed previously, the ciliated epithelial cells lining the central airways immunostained for IκBζ expression, both in the trachea (**Figure 2A**) and major bronchi (**Figure 2B**) of the gnotobiotics, indicating possible constitutive expression of IκBζ in the airway epithelium.

To further validate these results, we immunostained lung tissue samples from IκBζ knockout mice (**Figure 3**). To our dismay, we obtained a positive signal from the IκBζ knockout tissue suggesting that our anti-IκBζ antiserum was also detecting other non-specific proteins.

We then tested the specificity of the anti-IκBζ antiserum. We used column-purified recombinant IκBζ-S expressed in bacterial cells (**Figure 4A**) to see if it can specifically block IκBζ detection by anti-IκBζ antiserum, without affecting the signal from the non-specific proteins. Purified rIκBζ-S, when incubated in excess with anti-IκBζ antiserum, blocked not only the detection of IκBζ but also that of all the non-specific proteins (**Figure 4A**). This was not surprising since purified rIκBζ-S was the antigen against which our anti-IκBζ antiserum was generated.

To identify the non-specific proteins detected by our anti-IκBζ antiserum, we performed 2D electrophoresis on samples obtained by immunoprecipitation using the anti-IκBζ antiserum from lysates of monocytes stimulated with LPS (**Figure 4B**). Mass spectrometry analysis of the different clusters of spots identified IκBζ-L at the expected size of 86 kDa and towards the acidic end of the pH strip. Interestingly, spots that ran at 68 kDa with isoelectric points close to neutral

pH were identified as the nuclear protein lamin B1. It is to be noted that the predicted size of IκBζ-S is also 68 kDa. This was further confirmed when lamin B1 was detected by anti-lamin antibody in the sample immunoprecipitated with the anti-IκBζ antiserum from lysates of BEAS2B cells stimulated with IL-1β (data not shown).

Since we could not resolve the issue of non-specific detection by our anti-IκBζ antiserum, we tested a panel of commercial antibodies to evaluate specific IκBζ detection in BEAS2B cells stimulated with IL-1β (**Figure 4C**). None of them specifically detected IκBζ alone. Anti-IκBζ antiserum was used as a positive control for detection.

We then resorted to immunoblotting with anti-IκBζ antiserum to perform size-based detection of specific IκBζ expression and hence explore its potential constitutive expression in lung epithelium. Tissue homogenates from gnotobiotic mice showed IκBζ-L expression both in the lungs and the trachea (**Figure 5A**), indicating that IκBζ is constitutively expressed in the lungs of mammals. As expected, we also observed bands at 68 kDa corresponding to IκBζ-S and/or lamin B1. We then compared the expression of the protein in differentiated primary human bronchial epithelial cells (HBEC) and in cultured primary human small airway epithelial cells (HSAEC). HBECs displayed baseline IκBζ expression in the untreated controls (**Figure 5B**) that was further induced by IL-1β and this induction was inhibited in the presence of IL-1 receptor antagonist (IL-1Ra). On the other hand, HSAECs did not express any IκBζ protein, even with IL-1β stimulation, further supporting the hypothesis of a specific geographic constitutive IκBζ expression in central airway epithelium.

DISCUSSION

Various groups have explored the role of IκBζ in regulating respiratory immunity. IκBζ has been demonstrated to modulate the expression of pro-inflammatory cytokines like IL-6 and GMCSF and anti-microbial peptides like NGAL in airway epithelial cells, that line the respiratory mucosal surface [4, 5, 7, 16]. Recently, we demonstrated IκBζ's role in regulating inflammatory responses to pneumococcus infection [17], further emphasizing the importance of IκBζ in lung immune defense. Original studies reported that mononuclear phagocytes which exist in the pristine vascular environment require additional TLR or IL-1R agonists to induce IκBζ expression [8]. However, surprisingly, constitutive expression of IκBζ messenger RNA levels in epithelial cells lining various mucosal surfaces, including the eyes and the skin have been reported [13, 14]. These findings suggest the likelihood that local recognition of PAMPs in exposed tissues can induce a steady state expression of IκBζ. However, here for the first time, we provide strong evidence suggesting potential constitutive expression of IκBζ protein in airway epithelium, in the absence of exogenous agonist activity. This unique expression of IκBζ has been consistently observed in all of our previous studies in which we used immunoblotting to study IκBζ expression in primary respiratory epithelial cells cultured in the absence of known PAMPs and IL-1 [7, 17].

Although our immunoblotting data strongly indicated constitutive IκBζ protein expression in airway epithelium, we could not be conclusive due to the presence of additional non-IκBζ epitopes detected by our antisera. The non-specific protein detected by the anti-IκBζ antiserum, lamin B1, predominantly localizes in the nucleus, where functional IκBζ also accumulates. This made the interpretation of our immunostaining data much more difficult. Our attempts to specifically block only IκBζ detection by preblocking our anti-IκBζ antiserum with

recombinant IκBζ failed (**Figure 4A**). We hence tried to remove all the non-specific IgG molecules in our anti-IκBζ antiserum, leaving the IgGs specific to IκBζ available for binding. To do this, we incubated anti-IκBζ antiserum with an excess of nuclear extracts (NE) from unstimulated THP-1 cells that contained the non-specific antigens detected by the anti-IκBζ antiserum but no IκBζ protein. We then immunostained BEAS2B cells containing IκBζ with this "blocked" antiserum. After removal of the non-specific signal with the THP-1 NE, we observed basal IκBζ expression in the unstimulated cells and further induction of its expression after IL-1β stimulation (data not shown). Future studies will focus on repeating this procedure on lung tissue slices from humans and mice to confirm constitutive expression of IκBζ. Immunoblotting tissue homogenates from IκBζ knockout mice with anti-IκBζ antiserum will also provide vital information on the expression of IκBζ in airway epithelium.

If proven conclusively, IκBζ's constitutive expression in airway epithelium suggests a function and regulation that may differ from its classical TLR/IL-1 induction seen in immune cells. Interestingly, the baseline IκBζ expression had no apparent function in terms of activating cytokine release from primary human bronchial epithelial cells. However, IL-1 was able to induce further IκBζ protein beyond the baseline expression which was associated with subsequent cytokine release in these cells [7]. Future studies evaluating IκBζ's signal specific interactions with its known partners like NFκB or akirin2 [18] or its post-translational modification as an activation step, may be able to identify the exact function of the uniquely expressed IκBζ in lung epithelium. Nevertheless, our current data support a vital role for IκBζ in maintaining homeostasis of lung immunity.

The reason behind the difference in expression of IκBζ between central and lower airway epithelial cells is unknown but supports a likely difference between central ciliated cells and

lower airway roles in host defense. For example, bronchial and alveolar epithelial cells have been demonstrated to express different sets of genes and to respond differently to danger signals [19, 20]. It is likely that central airway cells are prepared constitutively to defend the lower respiratory tract from pathogen challenges. In conclusion, our data strongly suggests potential constitutive IκBζ protein expression in the airway epithelium, although further investigation is required to prove this conclusively.

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FIGURE LEGENDS

Figure 1. IκBζ expression in normal lung tissue. (A) Lung tissue slices from healthy human individuals immunostained with secondary antibody alone (1:1000), rabbit control IgG (1:1000), or anti-IκBζ antiserum (1:1000). **(B)** Lung tissue slices from wild type normal mice immunostained with secondary antibody alone (1:1000), IgG isotype control (1:2000), or anti-IκBζ antiserum (1:2000). The results are representative of 2-3 different mice/humans.

Cellular localization of IκBζ. BEAS2B cells ($5x10^5$ cells per ml) were stimulated with rhIL-1β (10ng/ml) for 4 and 24 h. Cells were then fractionated into cytosol and nucleus. Protein matched cytosolic and nuclear extracts were analyzed for IκBζ, lamin B (nuclear marker) and pro IL-1β (cytosolic marker) expression using immunoblotting. The results are representative of 2 independent experiments. * represents non-specific band.

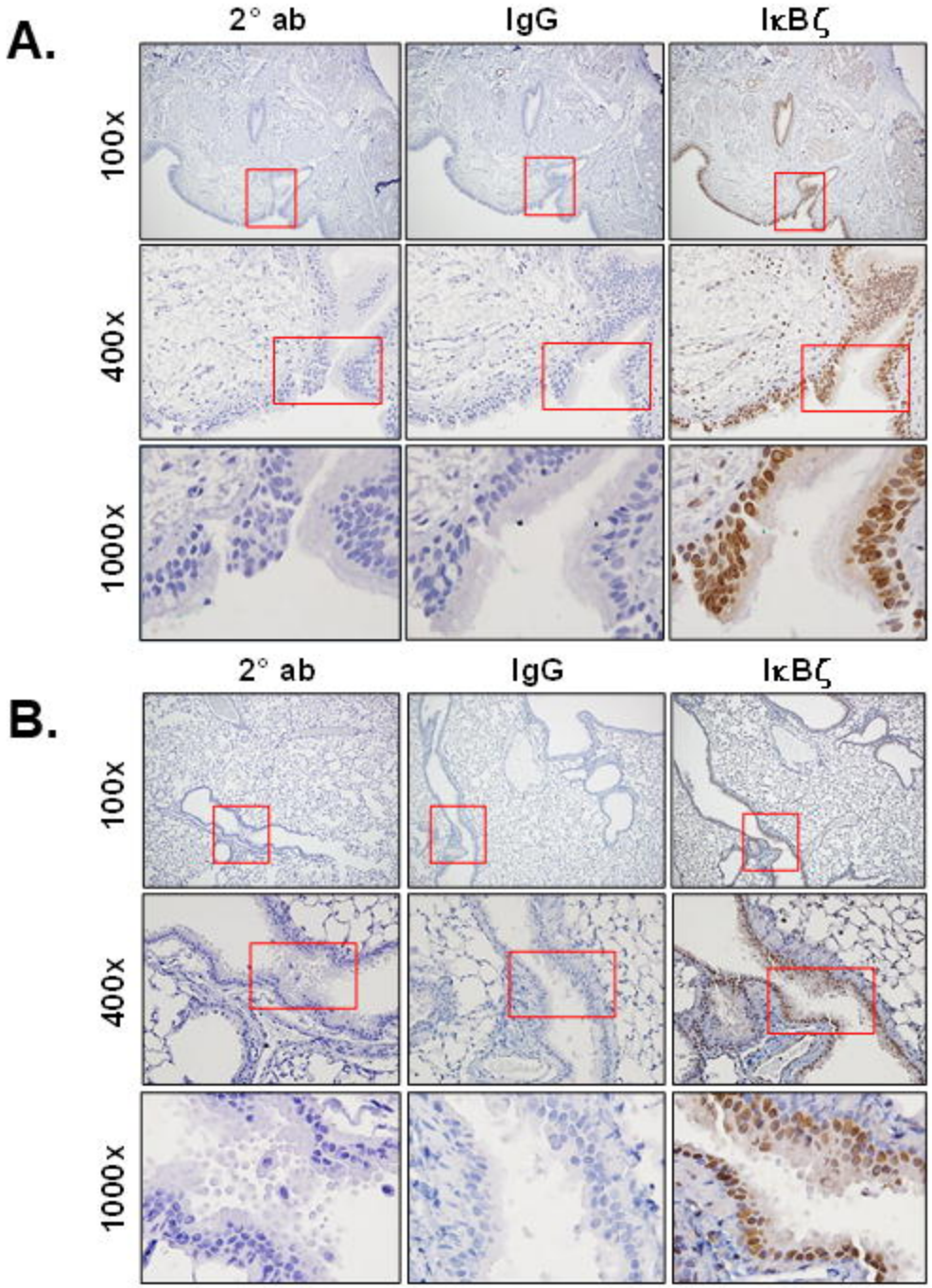
Figure 2. IκBζ expression in lung tissue from gnotobiotic mice. (A) Tracheal slices immunostained with secondary antibody alone (1:1000), IgG isotype control (1:1500), or anti-IκBζ antiserum (1:1500). **(B)** Lung tissue slices immunostained with secondary antibody alone (1:1000), IgG isotype control (1:1500), or anti-IκBζ antiserum (1:1500). The results are representative of 2 different mice.

Figure 3. IκBζ expression in IκBζ knockout mice. Lung tissue slices from IκBζ knockout mice immunostained with IgG isotype control (1:2500), or anti-IκBζ antiserum (1:2500). The results are representative of 2 different mice.

Figure 4. Lack of antibody to detect specific IκBζ expression. (A) Left panel: Immunoblot of column purified rIκBζ-S running at ~82kDa due to the presence of His and thioredoxin tags, expressed in bacterial cells. Right panel: IL-1β-stimulated BEAS2B cell extracts immunoblotted

with anti-IκBζ antiserum alone or with anti-IκBζ antiserum incubated with excess rIκBζ-S protein. **(B)** 2D gel electrophoresis of sample immunoprecipitated using anti-IκBζ antiserum from lysates of monocytes stimulated with LPS. The labels on the blot represent identification of the proteins using mass spectrometry. **(C)** Extracts from BEAS2B cells untreated (NT) or stimulated with IL-1β were immunoblotted with rabbit polyclonal antibody from LS Bio Sci or Thermo Scientific, mouse monoclonal from Abcam or our lab-made anti-IκBζ antiserum. * represents non-specific band.

Figure 5. IκBζ expression in airway epithelial tissue and cells using immunoblotting: (A) Immunoblot of tissue homogenates from gnotobiotic mice with anti-IκBζ antiserum. **(B)** HSAECs or HBECs ($2x10^5$ cells per ml) were stimulated with rhIL-1β (10ng/ml) for 3 hours, with or without the pretreatment with IL-1Ra (10 µg/ml). IκBζ protein expression in the cell extracts was analyzed using immunoblotting with anti-IκBζ antiserum. The results are representative of 2-3 independent experiments. * represents non-specific band.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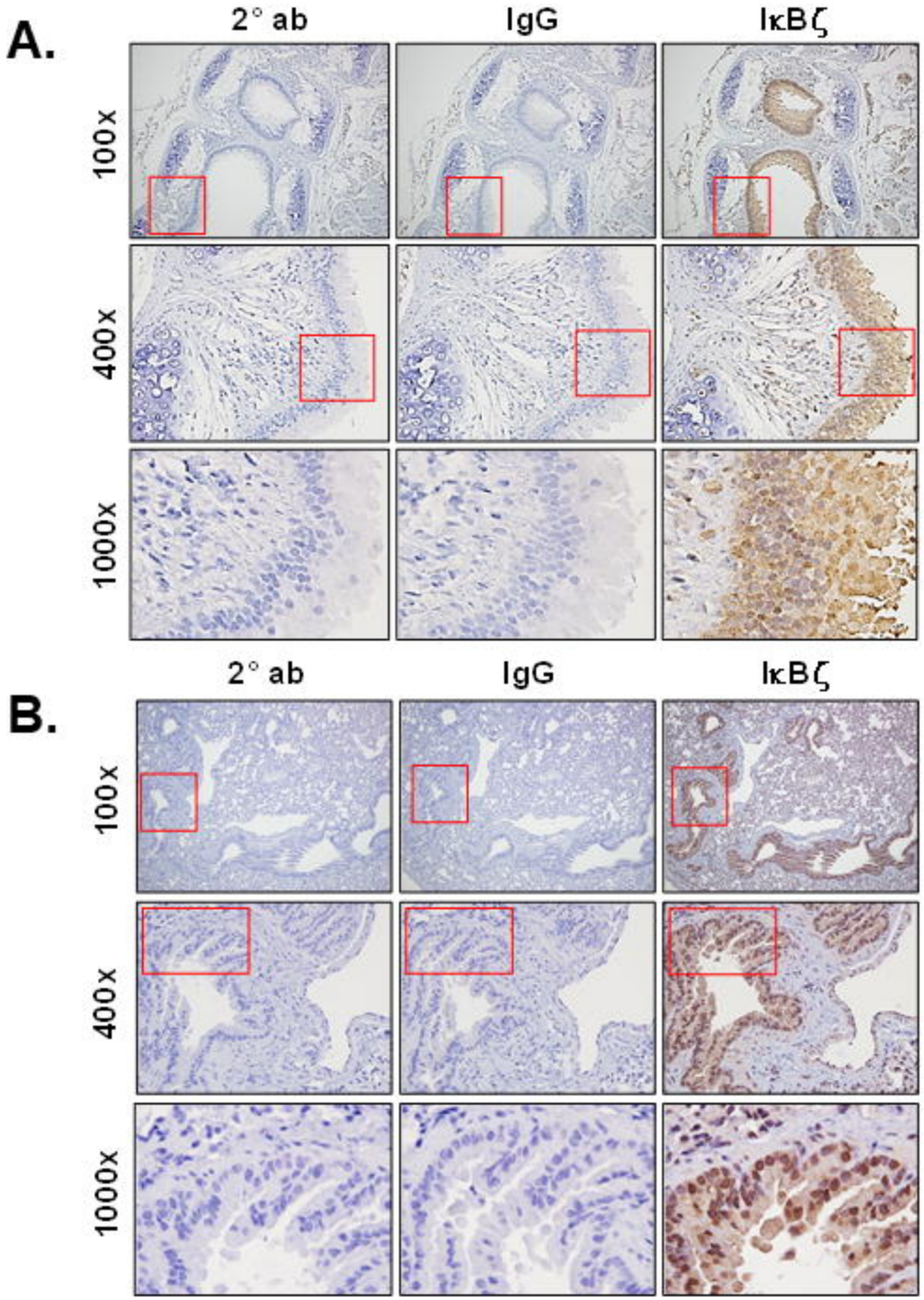
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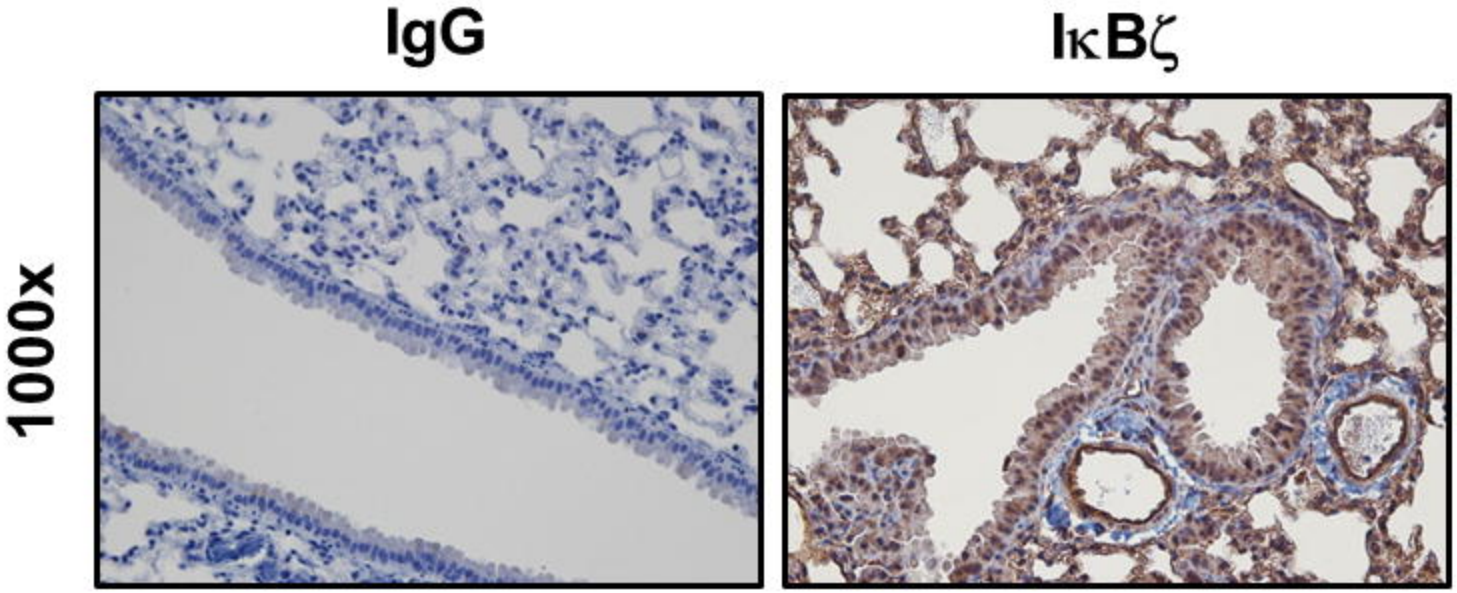
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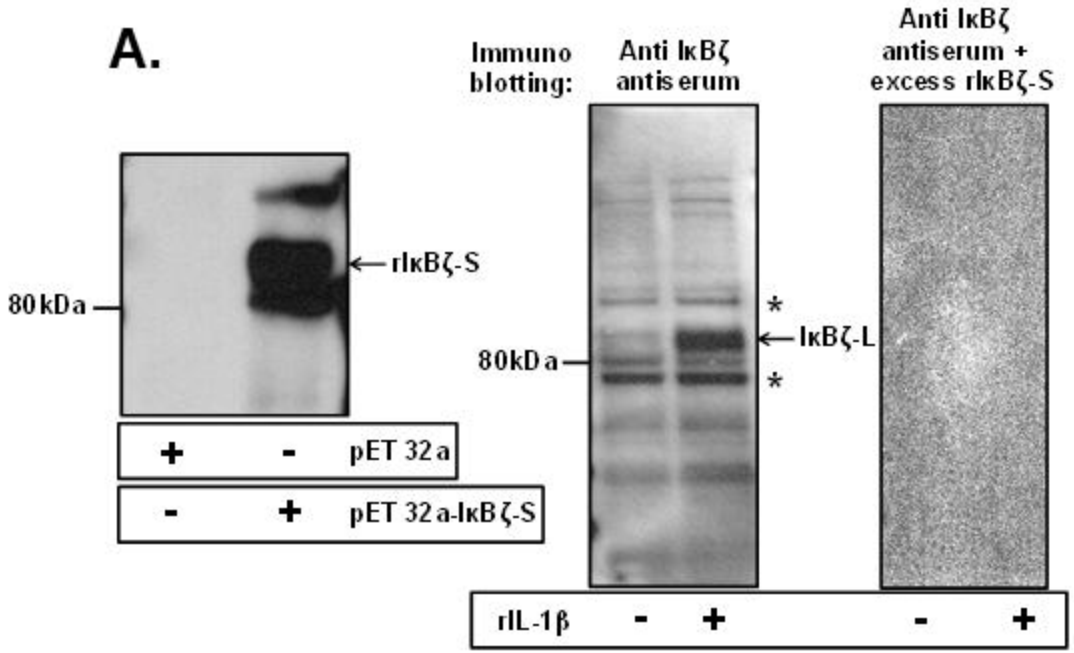
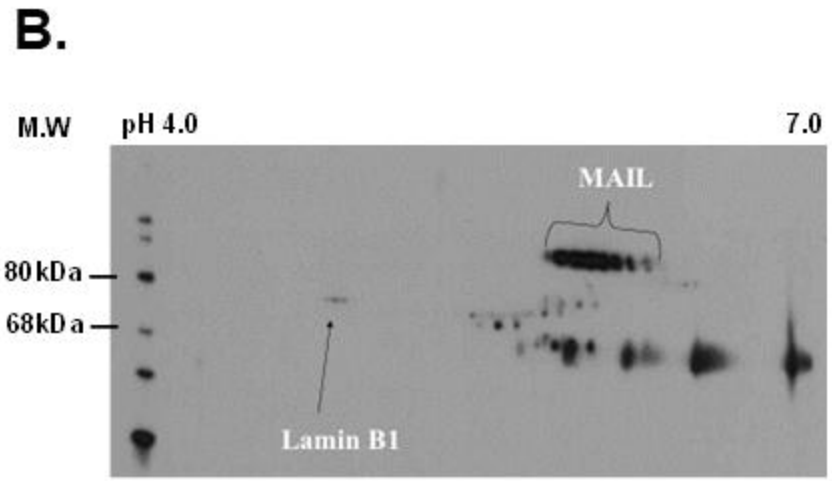
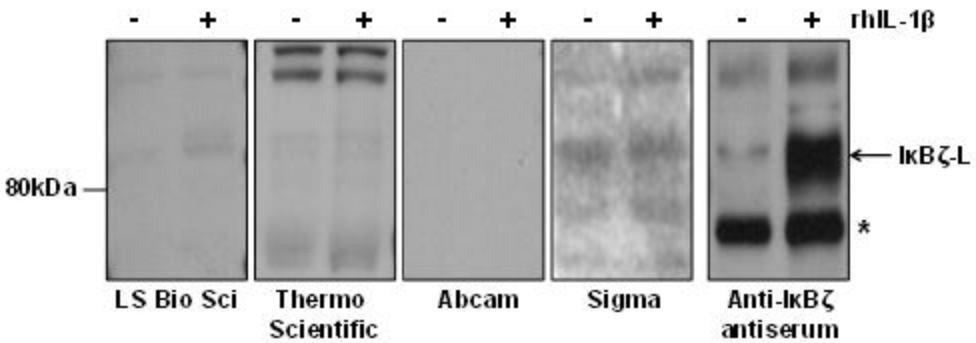
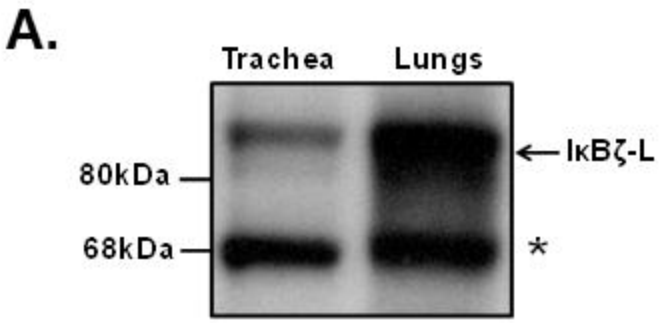
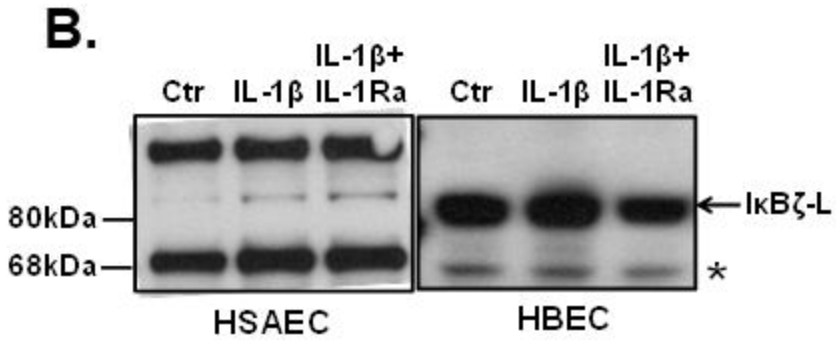

A.
Immuno blotting:
Anti IκBζ antiserum
Anti IκBζ antiserum + excess rIκBζ-S
rIκBζ-S
80kDa
+ - pET 32a
- + pET 32a-IκBζ-S
*
IκBζ-L
80kDa
*
rIL-1β - + - +


B.
M.W
pH 4.0
7.0
MAIL
80kDa
68kDa
Lamin B1


C.
- + - + - + - + - + rhIL-1β
80kDa
IκBζ-L
*
LS Bio Sci
Thermo Scientific
Abcam
Sigma
Anti-IκBζ antiserum


A.
Trachea
Lungs
80kDa
68kDa
IκBζ-L
*


B.
Ctr
IL-1β
IL-1β+ IL-1Ra
Ctr
IL-1β
IL-1β+ IL-1Ra
80kDa
68kDa
IκBζ-L
*
HSAEC
HBEC