ORIGINAL RESEARCH

House Dust Mite Allergens and the Induction of Monocyte Interleukin 1β Production That Triggers an IκBζ-Dependent Granulocyte Macrophage Colony-Stimulating Factor Release from Human Lung Epithelial Cells

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Abstract

Asthma is a chronic lung disease characterized by inflammation centered upon bronchial epithelium. House dust mite is one of the most common respiratory allergens that trigger exacerbations of asthma. IκBζ (gene *NFKBIZ*) is a recently recognized member of the NF-κB family that can be induced in mononuclear phagocytes and lung epithelial cells and has been shown to play a prominent role in epithelial cell function. We therefore analyzed the role of IκBζ in regulating lung epithelial cell cytokine responses to house dust mite mix (HDM). We found that human bronchial epithelial cells express IκBζ and release IL-6 and granulocyte macrophage colony-stimulating factor (GMCSF) when cocultured with human monocytes and HDM. This response is blocked in the presence of IL-1 receptor antagonist (IL-1Ra), indicating that it is IL-1 mediated. Neither HDM-stimulated macrophages nor dendritic cells release IL-1β and subsequently induce cytokine release from the bronchial epithelial cells. *Rhodobacter sphaeroides* LPS (RS-LPS), a TLR4 antagonist, blocks the ability of HDM to induce IκBζ and release GMCSF from epithelial cells cocultured with monocytes. Additionally, human bronchial epithelial cells show no induction of IκBζ or cytokine responses to direct HDM stimulation. Finally, *NFKBIZ* small interfering RNA–mediated knockdown in the bronchial epithelial cells suppresses the release of IL-1–induced IL-6 and GMCSF. Our findings indicate a possible role for monocyte recruitment and lung epithelial cell IκBζ in mediating asthma associated inflammation. Thus, IκBζ, IL-1Ra, and RS-LPS deserve future study as potential modulators of house dust mite–induced asthma.

Keywords: asthma; house dust mite; NFKBIZ; IL-1β; epithelium

Clinical Relevance

Inhalation of house dust mite antigens is an important asthma trigger. The present research identifies a novel pathway in this process. Infiltrating monocytes have the capacity to respond vigorously to house dust mites, releasing IL-1, which induces lung epithelial cells to produce granulocyte macrophage colony-stimulating factor via a recently recognized NF-κB family member, IκBζ.

Asthma is a chronic lung disorder characterized by airway inflammation, airway hyperresponsiveness, increased mucus production and airway remodeling (1). A combination of genetic predisposition and exposure to environmental allergens is considered the primary risk factor for developing the disease (2). House dust mites are one of the most common aeroallergens causing asthma (3). House dust mite–specific Th2 cells are recruited into the airways where they play a prime role in orchestrating the allergic response, which is characterized by increased production of IgE and Th2 cytokines, such as IL-4 and IL-13 (4). Although the main focus has been on the adaptive immune response to allergens (1, 5), an early innate immune response is being recognized as key to the pathogenesis of house dust mite–induced asthma (6–8).

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Bronchial epithelial cells, apart from acting as physical barriers, play a critical role in regulating asthma-associated inflammatory responses (9–12). House dust mites and their contaminants contain various allergenic epitopes and pathogen-associated molecular patterns that can bind to the pattern recognition receptors expressed in the lung epithelial cells (6) and the immune cells that inhabit the airway mucosa (13, 14), resulting in the production of proinflammatory cytokines, such as IL-6, granulocyte macrophage colony-stimulating factor (GMCSF), IL-25, IL-33, and TSLP (6). There is also evidence of cross-talk between epithelial cells and dendritic cells that enhances the allergic response in asthma (15). Exposure to allergic antigens is associated with increased influx of monocytes, which differentiate into macrophages and dendritic cells in the airway mucosa of both mice and humans (16–18). Thus, understanding the molecular mechanisms that regulate the innate immune response involved in house dust mite–induced asthma is fundamental to the development of new therapeutics.

NFKBIZ is a primary response gene, induced rapidly in monocytes and macrophages in response to LPS, which encodes the transcription factor IκBζ, also called MAIL or INAP (19–22). The molecule belongs to the IκB family due to the presence of multiple ankyrin repeat sequences at its carboxy terminus, with the help of which it binds to the subunits of nuclear factor κB (NF-κB) (19, 22). The amino-terminal portion of the protein encodes a transcriptional activation domain and a nuclear localization sequence. Unlike typical IκB homologs that are constitutively present in the cytosol where they complex to NF-κB Rel domains to inhibit nuclear localization, IκBζ is an inducible protein that binds to NF-κB inside the nucleus (20). IκBζ forms a complex with either p50 homodimers or p50-p65 heterodimers of NF-κB that bind to the promoter sequences of several secondary response genes, including *IL6*, *IL12*, *LCN2*, *IFNG*, and *DEFB4A* (23–26), and causes regulation of their transcription. Although there is a documented inhibitory role of IκBζ (22), its function as a transcriptional activator predominates. There are two known isoforms of the protein—long (IκBζ-L) and short (IκBζ-S)—of which IκBζ-L is more predominantly expressed (19). Apart from monocytes, IκBζ is also induced in epithelial cells in response to cytokines such as IL-1β, IL-18, and IL-17 (24, 25, 27, 28). IκBζ knockout mice display severe inflammation in the epithelial cells lining their skin and eyes (29, 30), suggesting that IκBζ is required for the homeostatic regulation of host defense at epithelial barriers.

Although IκBζ has been shown to regulate the production of proinflammatory cytokines in epithelial cells, its function in the context of house dust mite–induced asthma has not been previously evaluated. We therefore chose to test the hypothesis that IκBζ plays a prominent role in the regulation of lung epithelial cell innate immune function in response to house dust mites, using cocultures of bronchial epithelial cells with mononuclear phagocytes. We show that bronchial epithelial cells express IκBζ and release IL-6 and GMCSF when cocultured with monocytes that are stimulated with HDM. This induction was blocked in the presence of IL-1 receptor antagonist (IL-1Ra), suggesting that the effect is mediated through activation of the IL-1 receptor. We also show that neither HDM-stimulated macrophages nor dendritic cells have an effect on the cytokine release from bronchial epithelial cells. Similarly, supernatants from HDM-stimulated monocytes, but not dendritic cells or macrophages, stimulate IκBζ and cytokine expression in human bronchial epithelial cells (HBECs). We demonstrate that *Rhodobacter sphaeroides* LPS (RS-LPS), a TLR4 antagonist, blocks the effect of HDM on the monocytes and subsequently prevents the induction of IκBζ in bronchial epithelial cells. Interestingly, HBECs do not directly respond to HDM. Finally, *NFKBIZ* small interfering RNA (siRNA)-mediated knockdown in bronchial epithelial cells suppresses IL-6 and GMCSF release in response to IL-1β.

Materials and Methods

Preparation of HDM

Dermatophagoides pteronyssinus and *Dermatophagoides farinae* were mixed at a 1:1 ratio to yield a final HDM stock concentration of 25 μg/μl total protein.

Cell Culture

BEAS2B cells (ATCC, Manassas, VA) were cultured with or without FBS. Primary HBECs (Lonza, Basel, Switzerland), were allowed to differentiate in air–liquid interface in 24-well inserts as per the manufacturer's instructions. Monocytes purified from human blood were used in experiments immediately after isolation or were differentiated into macrophages and dendritic cells.

Epithelial Cell–Immune Cell Coculture

Supernatant add-back model. Overnight cultures of BEAS2B cells and primary differentiated HBECs were stimulated with supernatants from LPS- or HDM-treated monocytes, macrophages, or dendritic cells.

Contact-based model. Monocytes, macrophages, or dendritic cells were cocultured in contact with BEAS2B cells along with LPS, HDM, or HDM in the presence of IL-1Ra.

Contact independent model. Monocytes grown in transwell inserts were cocultured with BEAS2B cells grown in transfection wells. The monocytes were stimulated with LPS or HDM in the presence of IL-1Ra or RS-LPS.

siRNA-Mediated Knockdown of IκBζ

BEAS2B cells were transfected with 50 pmol of scrambled siRNA control or siRNA specific to IκBζ in the supernatant add-back model or with 100 pmol of each in the contact-independent model using lipofectamine. Undifferentiated HBECs were nucleofected with 100 pmol scrambled siRNA control or si-IκBζ. They were then stimulated with rIL-1β.

Preparation of Cell Lysates and Immunoblotting

Cells were lysed, and total protein in each cell extract sample was determined using Lowry assay. The proteins were separated on a gel and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with milk, incubated with the primary antibody, washed, and then stained with appropriate peroxidase-conjugated secondary antibody. The protein bands were visualized by autoradiography.

ELISA

An ELISA kit for IL-6 was purchased from eBioscience (San Diego, CA) and one for GMCSF from BD Biosciences (San Jose, CA). The ELISAs for IL-1β and IL-18 were developed in our laboratory.

Quantitative PCR

BEAS2B cells were transfected with 100 pmol control siRNA or IκBζ siRNA, after which they were stimulated with rhIL-1β. Complementary DNA was made from the total RNA obtained from each sample and was used as the template for quantitative PCR by the SYBR Green method. Gene expression was normalized to the average of two housekeeping genes (GAPDH and CAP1).

Flow Cytometry

Monocytes, dendritic cells, and macrophages were stained with APC-conjugated CD14, APC-conjugated CD163, or FITC-conjugated CD1a. The surface antigen–specific staining of the gated population of cells was obtained and compared with appropriate IgG antibody staining and unstained controls.

Statistical Analysis

Results are expressed as mean ± SEM. For simple comparisons, Student's *t* test was used, and for multiple comparisons in experiments with immune cells, one-way ANOVA followed by Tukey's *post hoc* analysis was used. For experiments done using the supernatant add back model, a *t* test with the block design (SAS-JMP; SAS, Cary, NC) was used. Significance was defined as $P < 0.05$.

Information about the reagents and antibodies and details on each section are provided in the online supplement.

Results

IκBζ Knockdown Suppresses the Expression of IL-6 and GMCSF in BEAS2B Cells in Response to rhIL-1β

To look for a functional correlation between IκBζ and HDM-induced asthma-associated secondary response genes,

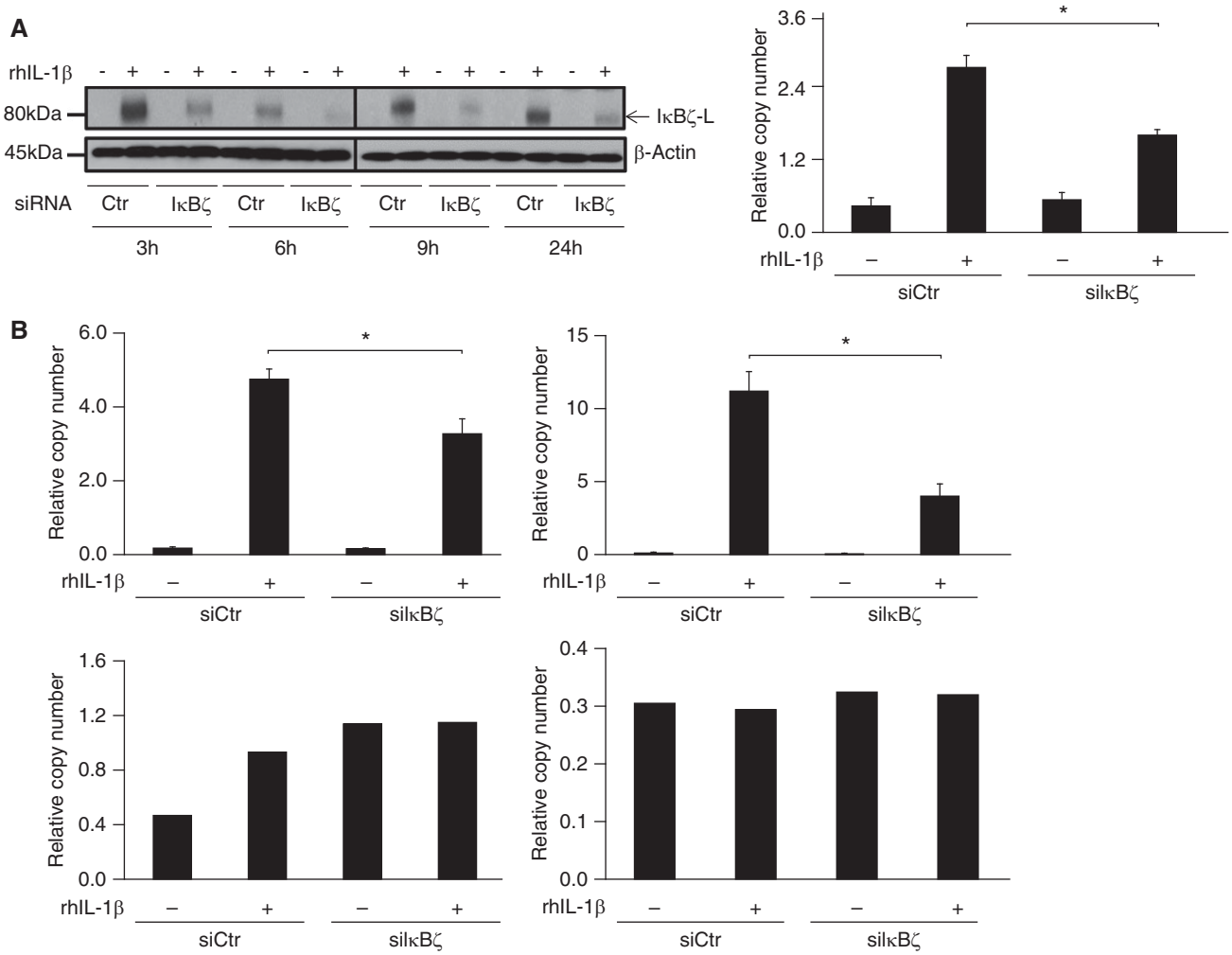

Figure 1. Genes regulated by IκBζ in lung epithelial cells in response to rhIL-1β. BEAS2B cells (10^5 cells/ml) were transfected with 50 pmol of scrambled small interfering (siRNA) control (siCtr) or siRNA specific to IκBζ (siIκBζ) and stimulated with rhIL-1β (10 ng/ml) for 3, 6, 9, and 24 hours. (*A*) Protein-matched total BEAS2B cell extracts were analyzed by immunoblotting for IκBζ and β-actin (*left*) and messenger RNA (mRNA) levels of IκBζ at 3 hours after stimulation evaluated by quantitative PCR (*right*). (*B*) mRNA levels of IL-6 (*top left*), granulocyte macrophage colony-stimulating factor (GMCSF) (*top right*), IL-18 (*bottom left*), and caspase 1 (*bottom right*) at 3 hours after stimulation evaluated by quantitative PCR. The immunoblot represents three independent experiments, and the *error bars* represent the mean ± SEM of three independent experiments for IκBζ, IL-6, GMCSF, and the mean of two experiments for IL-18 and caspase 1. $^*P < 0.05$.

we screened the HBEC line BEAS2B after siRNA knockdown of IκBζ and rhIL-1β stimulation. Small interfering IκBζ RNA inhibited IκBζ both at the messenger RNA level (Figure 1A, *right*) and at the protein level (Figure 1A, *left*). The expression of various downstream cytokines associated with asthma, such as IL-6 and GMCSF (Figure 1B) as well as CXCL5, MCP-1, and IL-8 (data not shown), was suppressed with IκBζ knockdown, thus implicating a strong correlation between IκBζ and asthma-associated cytokines. Importantly, this suppression was specific to IκBζ-mediated genes because inflammasome genes such as IL-18 and caspase1 remained unaffected. This result prompted us to study the role of IκBζ in regulating epithelial cell inflammatory responses to HDM treatment in a physiologically relevant coculture model of epithelial cells and mononuclear phagocytes.

HDM Induces Cytokine Production and IκBζ Expression in Monocytes, Macrophages, and Dendritic Cells

Infiltration of mononuclear phagocytes into asthmatic airways is well documented (16, 17), as is strong evidence for cross-talk between these cells and epithelial cells in asthma (15, 31). Therefore, we first evaluated the role of mononuclear phagocytes in responding to HDM in our system. Monocytes were freshly isolated from human blood and differentiated into macrophages and dendritic cells (*see* morphology [Figure 2A] and surface marker expression [Figure E1 in the online supplement]). Both LPS and HDM induced monocytes, macrophages, and dendritic cells to release IL-6, whereas IL-1β stimulation had only a minimal effect. In contrast, GMCSF responses were minimal from all cell types and stimuli (Figure 2C). On the other hand, strong IκBζ protein expression (IκBζ-L, ~81 kD) was induced by LPS, HDM, and rIL-1β in all immune cell types at 4 hours; this effect was gone by 24 hours (Figure 2B).

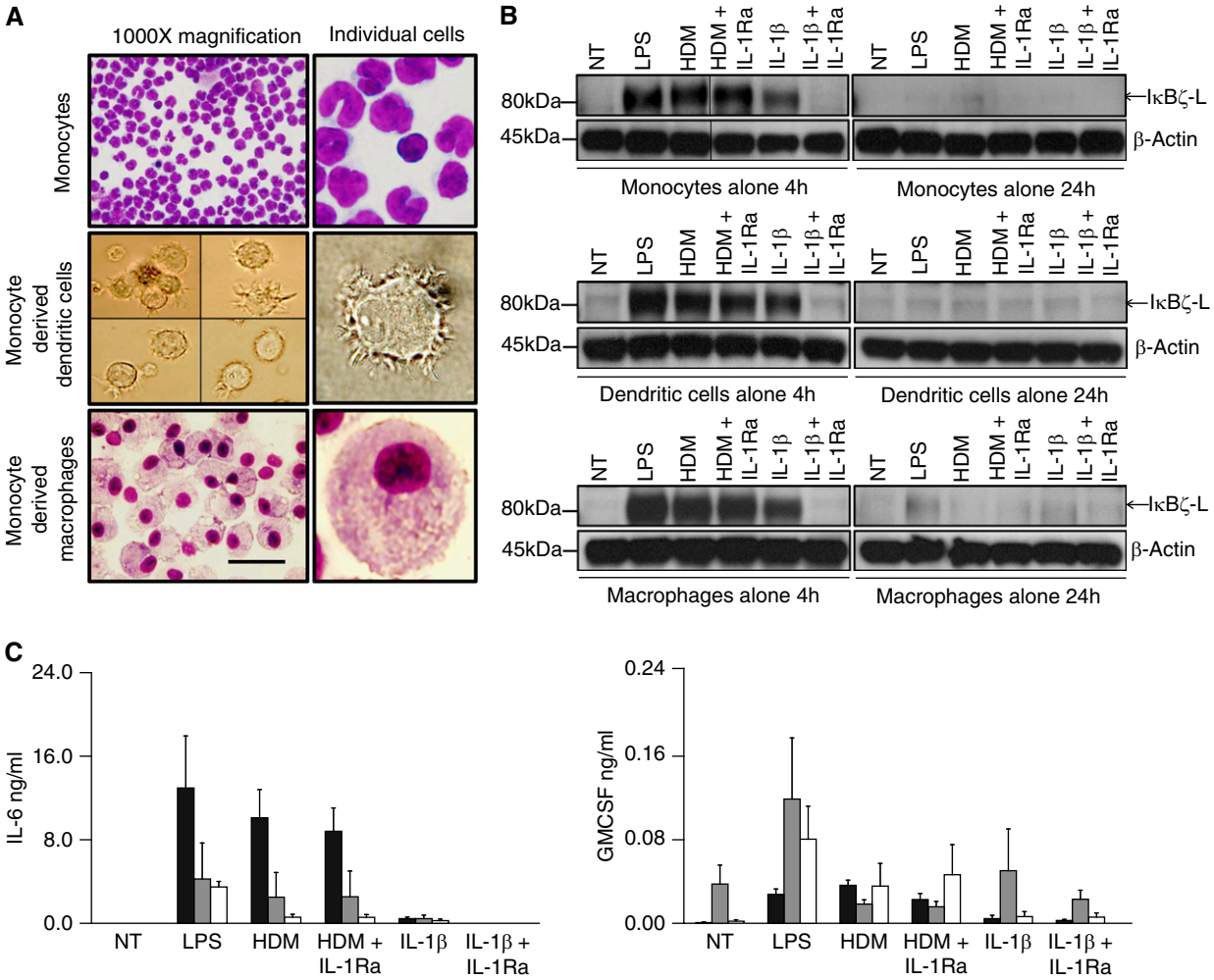

Figure 2. House dust mite mix (HDM) induces cytokine production and IκBζ expression in monocytes, macrophages, and dendritic cells. (*A*) Morphology of human monocytes, monocyte-derived macrophages, and monocyte-derived dendritic cells as observed through cytospin preparations of each. The *scale bar* denotes 50 μm for all three cells (*left panel*). (*B*) Human blood monocytes, dendritic cells, or macrophages (10^6 cells/ml each) were treated with LPS (1 μg/ml), HDM (100 μg of total protein/ml), or HDM in the presence of IL-1Ra (10 μg/ml), IL-1β (10 ng/ml), or IL-1β in the presence of IL-1Ra (10 μg/ml) for 4 and 24 hours. Immune cell lysates were analyzed by immunoblotting using antiserum against IκBζ and actin antibody at both time points. (*C*) IL-6 and GMCSF release from each of the immune cells was determined by ELISA at 24 hours after stimulation. The immunoblot represents three independent experiments, and the *error bars* represent the mean ± SEM of three experiments. $^*P < 0.05$. NT, no treatment.

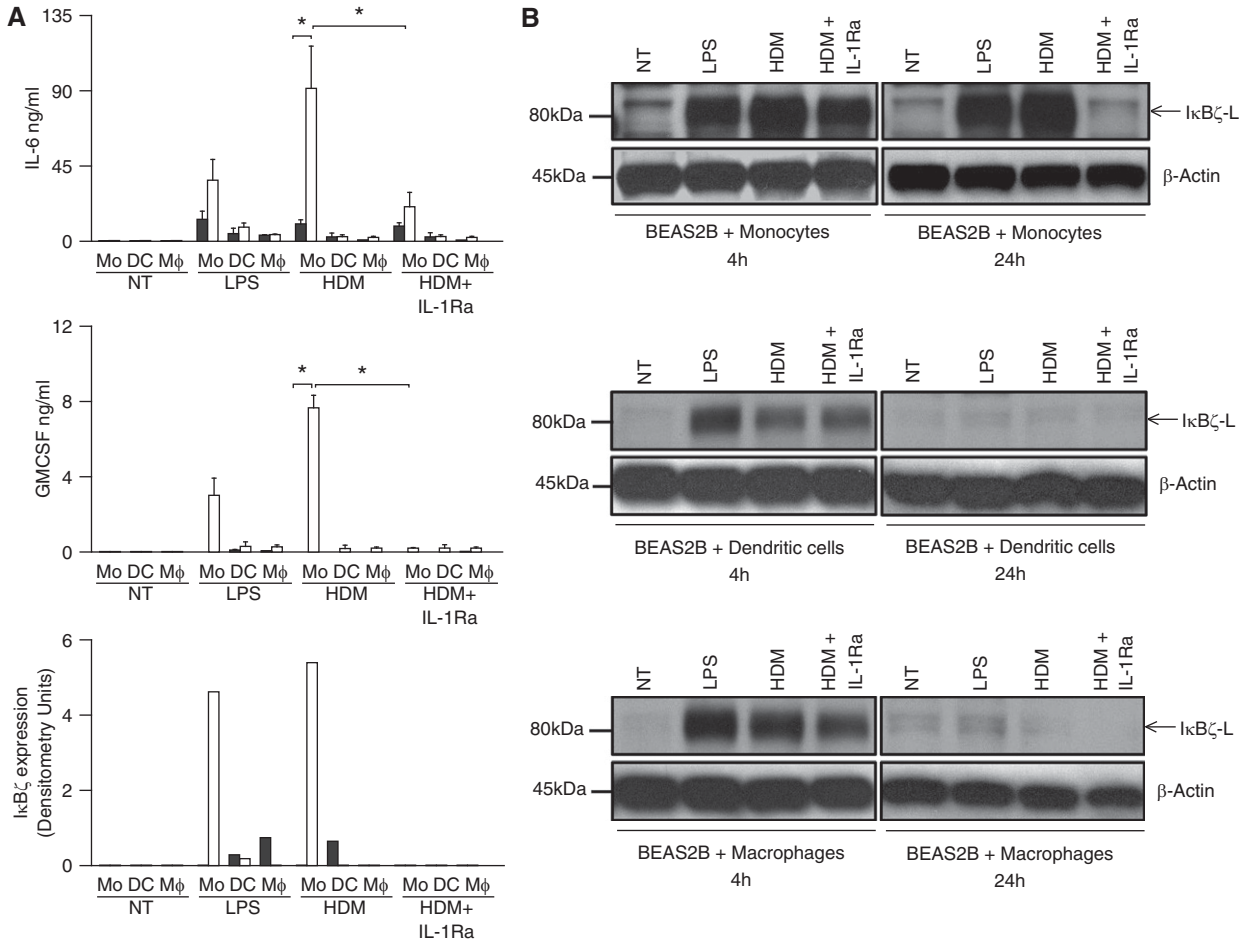

Figure 3. Epithelial cells express IκBζ, IL-6, and GMCSF in response to HDM when cocultured with monocytes (Mo) but not macrophages (Mϕ) or dendritic cells (DC). BEAS2B cells (1.5×10^5 cells/ml) were cocultured in contact with human Mo, DC, or Mϕ (10^6 cells/ml each), and the coculture was stimulated with LPS (1 μg/ml), HDM (100 μg of total protein/ml), or HDM in the presence of IL-1Ra (10 μg/ml) for 4 and 24 hours. (*A*) Supernatants from the cocultures were analyzed by ELISA for the release of IL-6 and GMCSF at 24 hours for cocultures (*open bars*) compared with that from the individual immune cells (*solid bars*). *Bottom panel*: IκBζ expression in the cocultures (*open bars*) or the individual immune cells (*solid bars*), measured at 24 hours as densitometry units. (*B*) IκBζ immunoblots of total cell extracts from the cocultures at 4 and 24 hours. The immunoblot representative of three independent experiments, and the *error bars* represent the mean ± SEM of four or five experiments. $^*P < 0.05$.

Epithelial Cells Express IκBζ, IL-6, and GMCSF in Response to HDM When Cocultured with Monocytes but Not Macrophages or Dendritic Cells

To determine if lung epithelial cells might be triggered by the pathogen-sensing capabilities of neighboring phagocytes, BEAS2B cells cocultured in contact with human monocytes, dendritic cells, or macrophages were stimulated with LPS or HDM. Interestingly, HDM- or LPS-stimulated monocyte-lung epithelial cell cocultures synergistically increased the production of IL-6 and GMCSF when compared with the immune cells cultured alone (Figure 3A). However, this effect was not observed in the dendritic cell or macrophage cocultures. IκBζ expression at 24 hours paralleled the IL-6 and GMCSF responses (i.e., it was sustained in the monocyte–lung epithelial cell sample but not in the macrophage or dendritic cell cocultures) (Figure 3B). Importantly, IL-1Ra blocked HDM-induced IκBζ expression completely at 24 hours and suppressed the HDM-induced production of IL-6 and GMCSF from the monocyte coculture. Together these results support the possibility that IL-1 released by immune cells induces the prolonged induction of *NFKBIZ* in the lung epithelial cells and the subsequent release of IL-6 and GMCSF.

Supernatants from HDM-Treated Monocytes Induce IκBζ Expression and Cytokine Production in Lung Epithelial Cells

To determine the source of the IκBζ expression in the coculture and to examine if contact was required between the epithelial and the immune cells for HDM-induced IκBζ expression, human monocytes, dendritic cells, or macrophages were stimulated with LPS or HDM. After 24 hours, the supernatants were added to primary differentiated HBECs for 4 hours or to BEAS2B cells for another 24 hours. Consistent with monocytes as the HDM responder cells, only stimulated monocyte supernatants induced IκBζ expression in lung epithelial cells (Figure 4A). The proportion of IL-6 and GMCSF released by the epithelial cells was determined by comparing the release before and after addition to BEAS2B cells (Figure 4B). Again only monocyte supernatants from HDM or LPS treatment induced a robust release of cytokines from epithelial cells,

which suggests that IκBζ expression and cytokine production can be induced in bronchial epithelial cells through HDM-induced monocyte factors.

IL-1β Released from HDM-Induced Monocytes Is Sufficient To Activate Epithelium

To confirm that IL-1β released from the HDM responder cells was sufficient to induce IκBζ in the epithelium, human monocytes, dendritic cells, and macrophages were stimulated with LPS or HDM for 24 hours. Monocytes released 0.5 to 0.7 ng/ml of IL-1β in response to 100 μg/ml of HDM total protein, whereas dendritic cells and macrophages released undetectable amounts (Figures 5A and E2A). HDM-stimulated HBECs did not release IL-1β (Figure 5A), eliminating the possibility of IL-1 feeding back into the epithelial cells. We also observed IL-1α release only from HDM-treated monocytes but not from macrophages, dendritic cells, or HBECs (Figure 5A). IL-1β (0.05 ng/ml) was sufficient to induce IκBζ expression in the epithelial cells (Figure E2B). Of note, dendritic cells and macrophages produced intracellular proIL-1β in response to LPS and HDM (Figure 5B) but needed an activator of the inflammasome, such as *Burkholderia cepacia*, to release mature IL-1β (Figure E2C).

LPS Present in HDM Is Responsible for IκBζ Expression and Cytokine Production in Lung Epithelial Cells via Monocyte-Derived IL-1β

To investigate the connection between the ability of LPS in the HDM to induce monocyte IL-1β release and HDM's ability to induce IκBζ and cytokine expression in lung epithelial cells, BEAS2B cells were cultured in the supernatants of monocytes that had been induced by LPS or HDM in the presence of the TLR4 antagonist RS-LPS. IL-1Ra was added to the BEAS2B cells before addition of HDM-treated monocyte supernatants. Monocyte supernatants induced by LPS or HDM with RS-LPS or IL-1Ra no longer induced IκBζ protein (Figure 5C) or IL-6 and GMCSF release (Figure 5D) in BEAS2B cells. This effect was confirmed using a transwell model that allowed cell-to-cell feedback (Figures E2E and E2F). Although the TLR4 antagonist RS-LPS (Figure E2D) and the LPS inhibitor polymyxin B (data not shown) abolished HDM-induced IL-1β release from monocytes, there was no significant reduction in the release of IL-1β with heat inactivation of HDM compared with crude HDM (data not shown). Taken together, these results confirm that LPS present in HDM causes IκBζ expression and cytokine production in epithelium through monocyte-derived IL-1β.

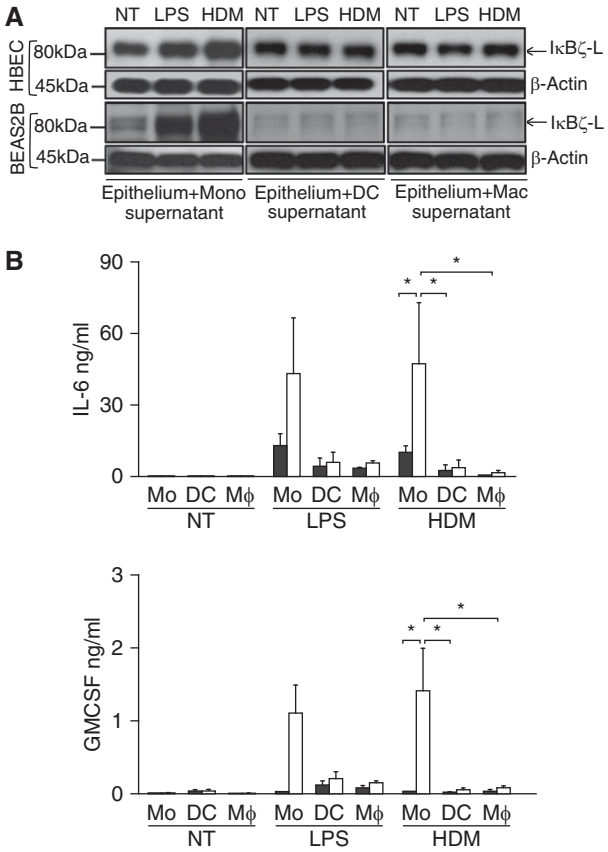

Figure 4. Supernatants from HDM-treated monocytes induce IκBζ expression and cytokine production in lung epithelial cells. Human Mo, DC, or Mϕ (10^6 cells/ml each) were stimulated with LPS (1 μg/ml) or HDM (100 μg of total protein/ml). Twenty-four hours after treatment, the supernatants collected from the immune cells were added to BEAS2B cells (1.5 × 105 cells/ml) for another 24 hours or to primary differentiated human bronchial epithelial cell (HBECs) (5×10^4 cells/33 mm^2) for 4 hours. (*A*) Protein-matched total BEAS2B cell extracts or HBEC extracts were analyzed by Western blotting using antiserum against IκBζ and actin antibody. (*B*) The supernatants from the LPS- or HDM-treated immune cells were analyzed by ELISA for IL-6 and GMCSF release before (*solid bars*) and after (*open bars*) adding to BEAS2B cells. The immunoblot represents three independent experiments, and the *error bars* represent the mean ± SEM of three experiments. $^*P < 0.05$.

HDM Does Not Directly Induce IκBζ Expression and Cytokine Production in Human Lung Epithelial Cells

Because lung epithelial cells directly mediate immune responses to house dust mite in murine asthma models (9, 12), we examined the expression of IκBζ in human lung epithelial cells in response to direct HDM treatment. BEAS2B cells were treated with HDM, LPS, or rhIL-1β for 4 and 24 hours. Although these epithelial cells expressed IκBζ in response to rIL-1β, they did not respond to HDM or LPS (Figure 6A). Importantly, IL-6 and GMCSF release correlated with the IκBζ protein expression (Figure 6C). The IκBζ expression and the downstream cytokine production were blocked in the presence of IL-1Ra at 4 and 24 hours of rIL-1β stimulation. This observation confirmed that IL-1β directly induces a human lung epithelial cell inflammatory response. To determine if this differential response to IL-1β versus LPS was true for primary lung epithelial cells, primary HBECs were grown to confluence in air–liquid interface and treated with rIL-1β, HDM, or LPS. Interestingly, we observed background IκBζ expression in the untreated control sample

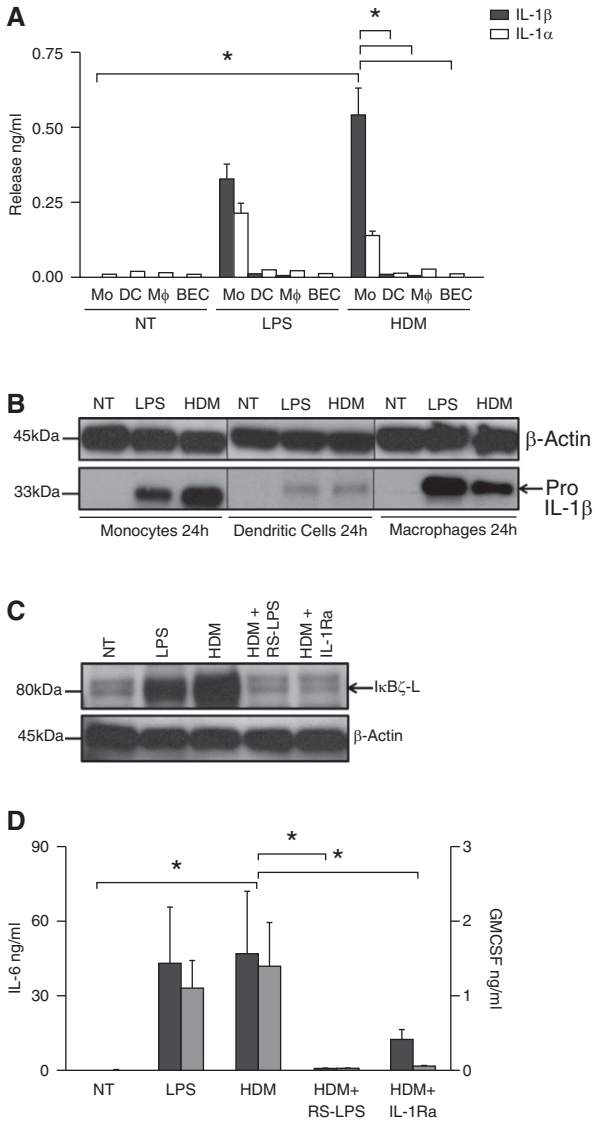

Figure 5. LPS present in HDM induces IκBζ expression and cytokine production in lung epithelial cells through IL-1β released from monocytes. Human Mo, DC, Mϕ (10^6 cells/ml each), or HBECs (5×10^4 cells/33 mm^2) were stimulated with LPS (1 μg/ml) or HDM (100 μg of total protein/ml) for 24 hours. (*A*) The supernatants from the LPS- or HDM-treated Mo, DC, Mϕ, and HBECs were analyzed for IL-1β (*solid bars*) and IL-1α (*open bars*) release using ELISA. (*B*) IL-1β immunoblots of immune cell extracts. (*C*) BEAS2B cells (1.5×10^5 cells/ml) were cultured for 24 hours in supernatants from monocytes that had been treated with LPS (1 μg/ml), HDM alone (100 μg of total protein/ml), or HDM in the presence of *Rhodobacter sphaeroides* LPS (RS-LPS) (10 μg/ml), or these epithelial cells were treated with IL-1Ra (10 μg/ml) before being cultured in HDM-treated monocyte supernatants. BEAS2B cell extracts were analyzed by immunoblots using antiserum against IκBζ and actin antibody. (*D*) The monocyte supernatants added to BEAS2B cells were analyzed by ELISA for IL-6 (*black bars*) and GMCSF (*gray bars*) 24 hours after addition. The immunoblots represent three independent experiments, and the *error bars* represent the mean ± SEM of six or seven experiments for Mo and three or four experiments for DC and Mϕ (*A*) and three experiments (*D*). $^*P < 0.05$.

that was further induced by rIL-1β (Figure 6B). There was a parallel increase in downstream cytokine production (Figure 6D), and these responses were blocked by IL-1Ra. LPS and HDM did induce modest IκBζ expression and downstream cytokine production, but the induction was subtle and not blocked by RS-LPS. Thus, HDM effects on bronchial epithelial cells are small but the epithelial responses are significantly enhanced by inflammatory cell cytokines such as IL-1.

IκBζ Accumulates in the Nucleus and Regulates the Release of IL-6 and GMCSF from Bronchial Epithelial Cells in Response to IL-1β

To confirm the role of IκBζ in regulating the release of IL-6 and GMCSF from lung epithelial cells in response to the IL-1β from HDM-stimulated monocytes, we used several approaches that directed knockdown of *NFKBIZ* specifically to the epithelial cells. Using monocyte supernatants on BEAS2B cells (Figures E3A and E3B) or the contact-independent transwell coculture with BEAS2B cells (Figures 7A and 7C) or rIL-1β–stimulated primary HBECs (Figures 7B and 7D), IκBζ knockdown, specifically to epithelial cells, demonstrated that the epithelial cell *NFKBIZ* is primarily responsible for the IL-6 and GMCSF release. Overexpression of green fluorescent protein–tagged IκBζ-L showed nuclear localization of the fluorescence compared with pEGFP C2 vector control (Clontech, Mountain View, CA) (Figure 7E), confirming nuclear accumulation of IκBζ. We also observed the characteristic speckled pattern of staining for IκBζ as described in previous reports (19, 32).

Discussion

IκBζ, also called MAIL or INAP (19, 20), is a transcription regulator that belongs to the IκB family of proteins. Although there have been studies suggesting that IκBζ inhibits NF-κB activity (22, 23), the predominant function of IκBζ is believed to be transcriptional activation of secondary response genes through NF-κB binding (21, 27, 33). In this report, we propose a model for house dust mite–induced asthma (Figure 7F) in which IκBζ plays a critical role in regulating inflammatory responses in the lung epithelium. Notably, at the protein level, IκBζ-L is the predominantly expressed isoform in lung epithelial cells. This preferential expression of IκBζ-L over IκBζ-S is consistent with previous publications from our group and others (21, 26, 34). To our knowledge, we are the first to demonstrate a role for IκBζ in mediating inflammation associated with house dust mite–induced asthma. IL-6 and GMCSF are important proinflammatory cytokines released into the bronchoalveolar lavage fluid of patients with asthma (35). GMCSF is required for the activation, proliferation, and function of dendritic cells

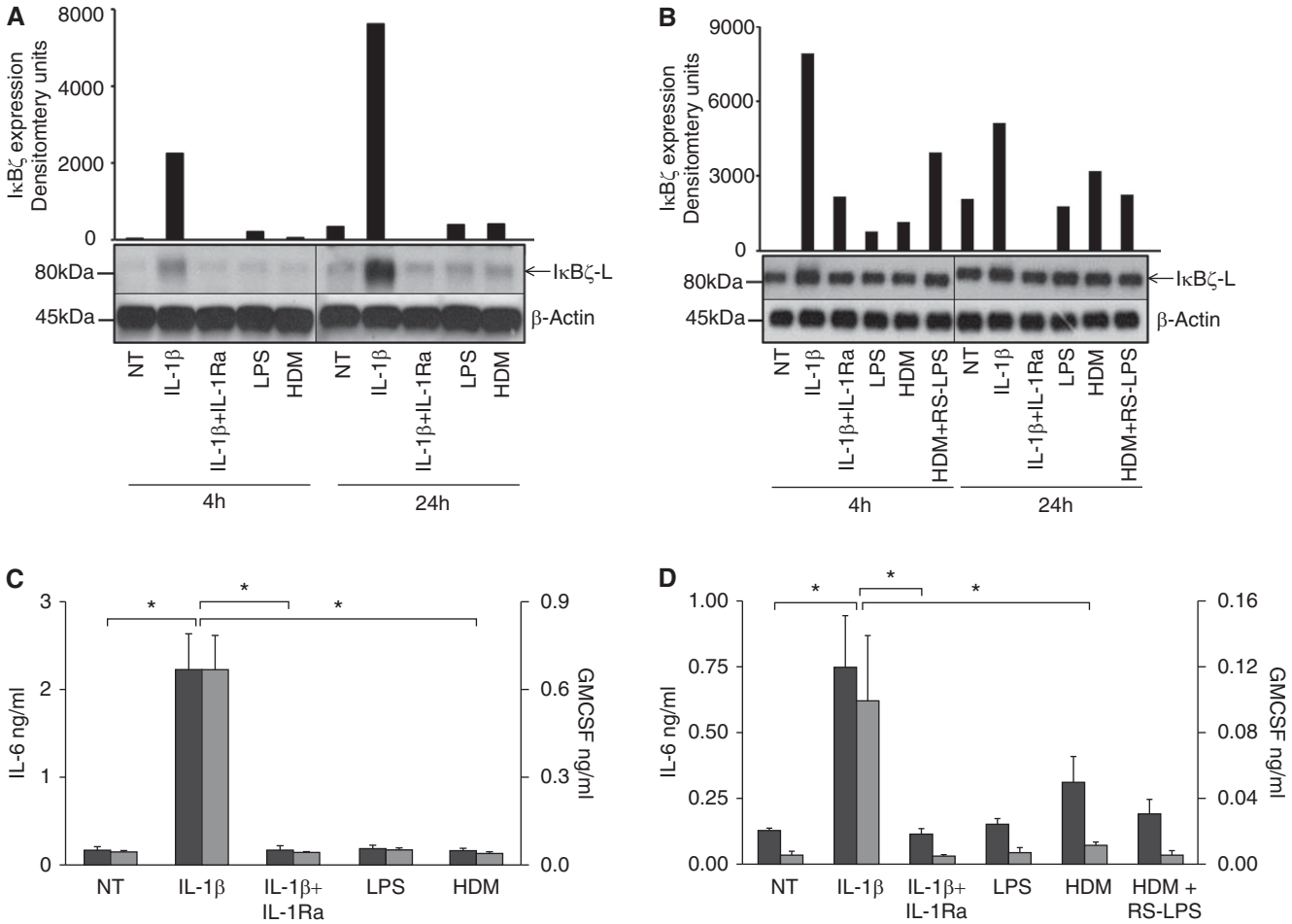

Figure 6. HDM does not directly induce IκBζ expression and cytokine production in lung epithelial cells. BEAS2B cells (1.5×10^5 cells/ml) and primary differentiated HBECs (5×10^4 cells/33 mm^2) were treated with LPS (1 μg/ml), HDM (100 μg of total protein/ml), or HDM in the presence of *R. sphaeroides* LPS RS-LPS (10 μg/ml), IL-1β (10 ng/ml) or IL-1β in the presence of IL-1Ra (10 μg/ml) for 4 and 24 hours. (*A*) BEAS2B cell extracts and (*B*) HBEC extracts were analyzed by immunoblots using antiserum against IκBζ and actin antibody. (*C* and *D*) IL-6 (*black bars*) and GMCSF (*gray bars*) release from BEAS2B cells and HBECs, respectively, was determined by ELISA at 24 hours after stimulation. The immunoblot and the corresponding densitometry graph represent three independent experiments, and the cytokine release *error bars* represent the mean ± SEM of three experiments. $^*P < 0.05$. For the densitometry graphs, the value corresponding to the sample with the lowest expression at each time point was subtracted out from the rest of the samples.

and eosinophils in the lungs (36), whereas IL-6 induces proliferation and activation of T cells (37). Thus, these cytokines are essential for linking the early innate immune response to the late adaptive immune response of asthma. We report a vital role for IκBζ in mediating the release of these cytokines from the lung epithelial cells in the context of HDM-induced asthma, making it a potential target for therapy.

We show that HBECs express IκBζ, IL-6, and GMCSF in response to HDM when cocultured with monocytes but not with dendritic cells or macrophages (Figure 3). We deduced that the expression of IκBζ in BEAS2B cells cocultured in contact with monocytes was predominantly from the BEAS2B cells because the pattern of IκBζ was characteristic of two BEAS2B responses: (*1*) the expression was sustained for 24 hours and (*2*) the expression was completely blocked in the presence of IL-1Ra, patterns not seen with monocytes. We also show that contact is not required between monocytes and bronchial epithelial cells to induce IκBζ expression in the epithelial cells (Figure 4) because supernatants of HDM-treated monocytes induced IκBζ expression in the epithelial cells. IL-1β was released by monocytes in response to HDM (Figure 5), which induced the expression of IκBζ in BEAS2B cells, and this induction was blocked in the presence of IL-1Ra. RS-LPS blocked IκBζ expression in BEAS2B cells in response to HDM-treated monocytes (Figure 5), demonstrating that LPS is the prime component of HDM that elicits a response, as previously reported (6, 9). We then show that HBECs do not express IκBζ, IL-6, and GMCSF in response to HDM in the absence of monocytes (Figure 6). Finally, siRNA-mediated knockdown of IκBζ in bronchial epithelial cells resulted in decreased release of IL-6 and GMCSF (Figure 7) from the cells in response to IL-1β, demonstrating that IκBζ regulates the production of these cytokines in the lung epithelium.

It is important to fit our model into the context of what is known about asthma pathophysiology *in vivo*. Our model

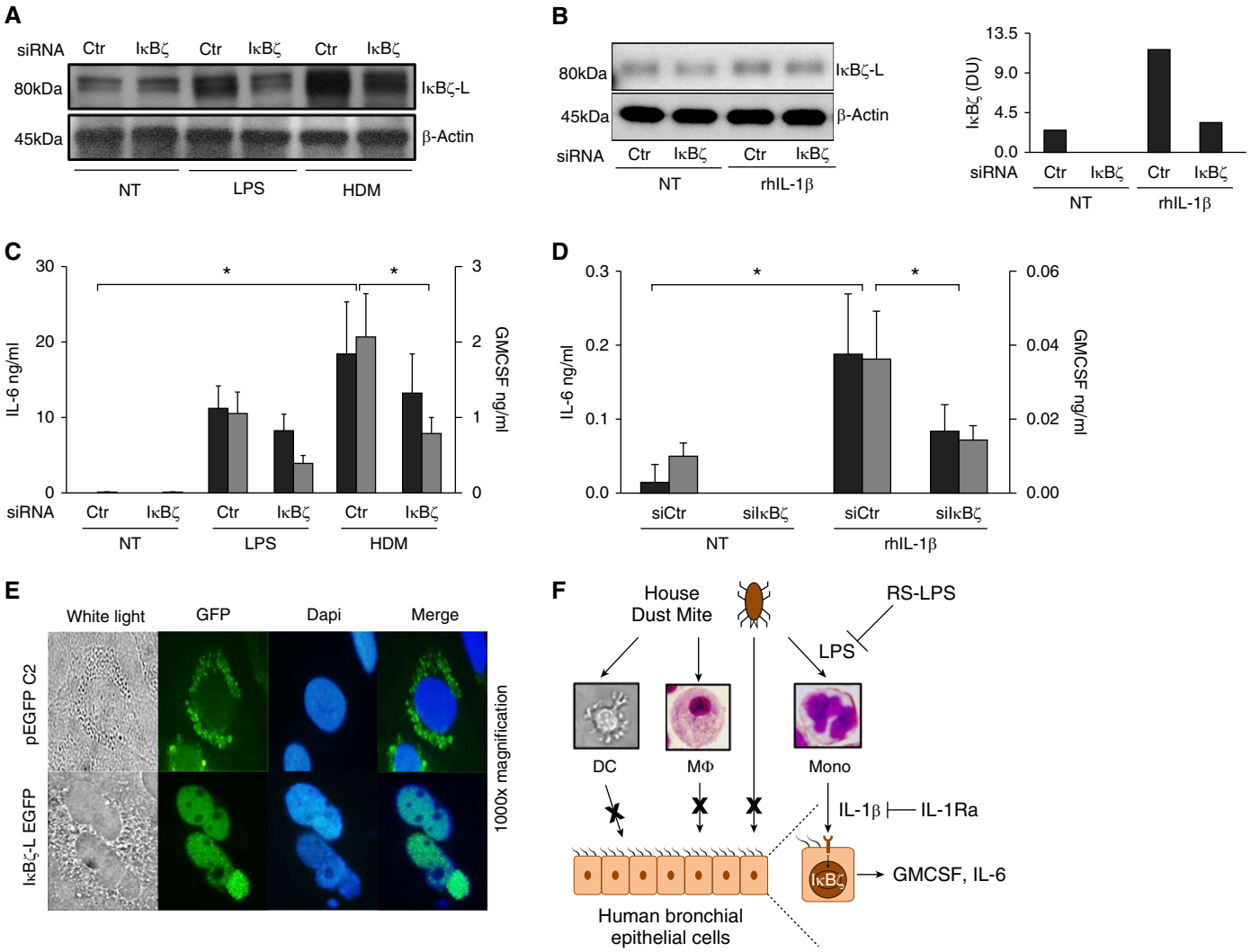

Figure 7. IκBζ accumulates in the nucleus and regulates the release of IL-6 and GMCSF from lung epithelial cells in response to IL-1β from HDM-treated monocytes. BEAS2B cells (1.5×10^5 cells/ml) were transfected with 100 pmol of scrambled siRNA control or siRNA specific to IκBζ in culture wells and were cocultured with monocytes (10^6 cells/ml) grown in transwell inserts stimulated with either LPS (1 μg/ml) or HDM (100 μg of total protein/ml) for 24 hours. HBECs (5×10^5 cells) were nucleofected with 100 pmol siCtr and siIκBζ. The cells were plated in transwell inserts (24 mm) and stimulated with rIL-1β (10 ng/ml) for 4 and 24 hours. (*A* and *B*) BEAS2B cell extracts (*A*) and HBEC extracts (*B*) were analyzed by immunoblotting for IκBζ and actin. (*C*) The supernatants from BEAS2B cells in the culture wells and (*D*) HBECs in transwell inserts were analyzed by ELISA for IL-6 (*black bars*) and GMCSF (*gray bars*) release. The immunoblot represents three independent experiments, and the cytokine release bar graphs represent the mean ± SEM of four or five experiments for BEAS2B cells and three experiments for HBECs. $^*P < 0.05$. For the densitometry and the cytokine graphs of HBECs, the value corresponding to the sample with the lowest expression (siIκBζ) was subtracted from the rest of the samples. (*E*) BEAS2B cells (2×10^5 cells) were transfected either with pEGFP C2 vector control or green fluorescent protein (GFP)-tagged IκBζ-L and were stimulated with rIL-1β for 4 hours. Nuclear accumulation of IκBζ was visualized using fluorescent microscopy. (*F*) HDM asthma model. House dust mite antigens contain LPS, which affects monocytes preferentially, inducing IL-1β, which drives primary lung epithelial cell expression of IκBζ-dependent GMCSF and IL-6. These events can be blocked through LPS receptor inhibition by *Rhodobacter sphaeroides* LPS (RS-LPS) or IL-1β receptor inhibition by IL-1Ra. DAPI, 4',6-diamidino-2-phenylindole.

demonstrates induction of IκBζ in bronchial epithelial cells in response to HDM-induced monocytes but not dendritic cells or macrophages. However, dendritic cells have been reported to be the predominant innate immune cells lining the lung epithelium that respond to antigens (14, 18, 38). Notably, pulmonary dendritic cells described in these studies are naive, not mature, and are more like the monocytes used in our model. Additionally, our dendritic cells express surface CD1a (Figure E1) and CD83 (data not shown), similar to mature, differentiated dendritic cells as described in previous studies (39), and hence are probably not responsive. The other reason for the dendritic cells used in our model to be unresponsive could be that they are differentiated *in vitro* from human monocytes in the presence of GMCSF and IL-4. Resident dendritic cells in the lungs may be exposed to many other inflammatory

factors and hence may respond better to antigens.

In support of our monocyte-dependent model, monocyte influx into the lungs of patients with asthma and allergen-exposed mice is well documented (16, 17). Furthermore, monocytes are demonstrated to be the prime producers of IL-1β in the lungs of patients with asthma (40). In this context, we have shown previously that monocytes from human whole blood are the main cell type to synthesize and release IL-1β, compared with neutrophils and lymphocytes (41). Moreover, unlike monocytes, macrophages have been shown to require a second signal that activates the inflammasome to release IL-1β (42–44). Thus, macrophages abundantly release IL-1β when treated with *B. cepacia* (Figure E2C), which provides two signals but not when given the single signal, LPS, or HDM. It is therefore likely that the IL-1β that induces IκBζ expression in the lung epithelial cells is released only by the monocytes present in the lungs when exposed to HDM.

IL-1α is also known to be released by monocytes in response to LPS (45) and has been shown to regulate allergic responses to HDM in murine lung epithelial cells (12). Similar to IL-1β, we observed IL-1α release only from HDM-treated monocytes but not from macrophages, dendritic cells, or HBECs (Figure 5A). It is thus probable that IL-1α also can cause IκBζ and cytokine expression in bronchial epithelial cells.

IκBζ is induced in BEAS2B cells cocultured with (*1*) monocytes in contact, (*2*) monocytes in transwell inserts, or (*3*) monocyte supernatants in response to both LPS (1 μg/ml) and HDM (final concentration of 100 μg/ml total protein). In all the three models, HDM induced a stronger expression of IκBζ when compared with LPS alone, although the amount of endotoxin present in 100 μg/ml of total protein in the HDM was only 70 ng/ml, which is approximately 15 times less than the 1 μg/ml LPS (Figures 3–5 and 7). We speculate that the presence of other allergens in HDM enhances the effect of the endotoxin. The HDM allergen Der p2 has been reported to mimic MD-2, the LPS-binding accessory TLR4 coreceptor (46), resulting in the augmentation of TLR4-associated immune responses. It is possible that a similar phenomenon exists in our model whereby Der p2 amplifies the TLR4 signaling in monocytes, resulting in the stronger induction of IκBζ in BEAS2B cells compared with LPS alone. It is also possible that the other components of the mix can be sources of the second signal required for IL-1β processing and release (47) because monocytes release more IL-1β in response to HDM when compared with LPS alone (Figure E2A) but not increased GMCSF and IL-6 (Figure 2).

Murine lung epithelial cells have been shown to release proinflammatory cytokines in response to HDM (9, 12). However, we observed no IL-6 and GMCSF release from BEAS2B cells cultured in DMEM with FBS in response to crude HDM (Figure 6C). Although there have been previous reports that demonstrate the expression of proinflammatory cytokines, including IL-6 and GMCSF in BEAS2B cells in response to direct HDM treatment, they all used recombinant or purified HDM-associated allergens Der p1, Der p2, and Der p9 (10) as inducers and not the crude mix (48). Der p2, for instance, is shown to reconstitute TLR signaling in the absence of MD-2 (46). It is intriguing that this putative MD-2 mimicry is not observed in our BEAS2B cells treated with HDM. The amount of Der p2 present in our HDM preparation may not be sufficient to reconstitute the TLR4 signaling by itself. Because our model focuses on LPS as the prime component of the crude HDM that elicits a response, we believe that our model still fits with these studies. Moreover, individual allergens of HDM can have responses different from the crude mix that has all the allergens together in different proportions. Additionally, BEAS2B cells grown in the presence of serum have been shown to undergo differentiation into squamous cells (49). To eliminate the possibility of squamous differentiation of BEAS2B cells as a reason for the lack of IκBζ and cytokine expression in response to HDM, we treated BEAS2B cells cultured in serum-free BEGM media, as suggested by ATCC, with rIL-1β, LPS, or HDM as done in Figure 6. We did not observe any induction of IκBζ and cytokine expression in the cells in response to HDM (Figure E4), similar to what we observed with the serum-grown BEAS2B cells, confirming that BEAS2B cells grown in either culture conditions behave the same. This lack of response in the HBECs to HDM treatment is likely due to the fact that human lung epithelial cells do not express surface TLR4. For example, there are several reports demonstrating intracellular compartmentalization of TLR4 and the absence of MD-2 in human lung epithelial cells (50–52). A recent study demonstrates the action of LPS on mononuclear phagocytes to release IL-1β and TNF-α that induce proinflammatory cytokine release in lung epithelial cells due to the lack of a direct effect of LPS on the epithelial cells, further validating our model (53). In another report, in the context of viral infections, lung monocytes sense antigens and release soluble factors that induce cytokine release from bronchial epithelial cells (54).

In the primary differentiated HBECs, although there was an induction of IκBζ and cytokine expression in response to direct HDM stimulation, it was insignificant. Moreover, this HDM-induced release of cytokines from the primary HBECs was not blocked by the TLR4 antagonist RS-LPS, suggesting that the response is not due to LPS but rather is due to other HDM allergens like Der p2. Interestingly, both primary differentiated HBECs (Figure 6B) and BEAS2B cells grown in serum-free conditions (Figure E4A) expressed IκBζ even in the unstimulated control sample. This IκBζ expression in the lung epithelial cells could possibly be constitutive and required for homeostatic regulation of host defense, as reported in previous studies that observed constitutive expression of IκBζ in the skin and the eyes, parts of the host that are constantly exposed to antigens (29, 55).

In conclusion, our findings indicate a role for IκBζ in mediating asthma-associated inflammation in the lung epithelium, thus making it a potential novel target for therapy. Furthermore, our results suggest that IL-1Ra and RS-LPS deserve further study as possible therapeutic asthma modulators. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

1. Fireman P. Understanding asthma pathophysiology. *Allergy Asthma Proc* 2003;24:79–83.
2. Hogg JC. The pathology of asthma. *APMIS* 1997;105:735–745.
3. Sporik R, Holgate ST, Platts-Mills TA, Cogswell JJ. Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood; a prospective study. *N Engl J Med* 1990;323:502–507.
4. Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255–281.
5. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012;18:673–683.
6. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 2011;32:402–411.
7. Minnicozzi M, Sawyer RT, Fenton MJ. Innate immunity in allergic disease. *Immunol Rev* 2011;242:106–127.
8. Hams E, Fallon PG. Innate type 2 cells and asthma. *Curr Opin Pharmacol* 2012;12:503–509.
9. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410–416.
10. King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998;161:3645–3651.
11. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 2011;242:205–219.
12. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med* 2012;209:1505–1517.
13. Dean S. Dust mites' dirty dealings with dendritic cells. *Nat Med* 2008; 14:487–488.
14. Lambrecht BN, Hammad H. Biology of lung dendritic cells at the origin of asthma. *Immunity* 2009;31:412–424.
15. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;8: 193–204.
16. Schaumann F, Müller M, Braun A, Luettig B, Peden DB, Hohlfeld JM, Krug N. Endotoxin augments myeloid dendritic cell influx into the airways in patients with allergic asthma. *Am J Respir Crit Care Med* 2008;177:1307–1313.
17. Tomita K, Tanigawa T, Yajima H, Fukutani K, Matsumoto Y, Tanaka Y, Sasaki T. Identification and characterization of monocyte subpopulations from patients with bronchial asthma. *J Allergy Clin Immunol* 1995;96:230–238.
18. Rate A, Upham JW, Bosco A, McKenna KL, Holt PG. Airway epithelial cells regulate the functional phenotype of locally differentiating dendritic cells: implications for the pathogenesis of infectious and allergic airway disease. *J Immunol* 2009;182:72–83.
19. Kitamura H, Kanehira K, Okita K, Morimatsu M, Saito M. MAIL, a novel nuclear I kappa B protein that potentiates LPS-induced IL-6 production. *FEBS Lett* 2000;485:53–56.
20. Haruta H. Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. *J Biol Chem* 2001;276:12485–12488.
21. Seshadri S, Kannan Y, Mitra S, Parker-Barnes J, Wewers MD. MAIL regulates human monocyte IL-6 production. *J Immunol* 2009;183: 5358–5368.
22. Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J Biol Chem* 2001;276: 27657–27662.
23. Motoyama M, Yamazaki S, Eto-Kimura A, Takeshige K, Muta T. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. *J Biol Chem* 2005;280:7444–7451.
24. Cowland JB, Muta T, Borregaard N. IL-1beta-specific up-regulation of neutrophil gelatinase-associated lipocalin is controlled by IkappaB-zeta. *J Immunol* 2006;176:5559–5566.
25. Kao CY, Kim C, Huang F, Wu R. Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium. *J Biol Chem* 2008;283:15309–15318.
26. Kannan Y, Yu J, Raices RM, Seshadri S, Wei M, Caligiuri MA, Wewers MD. IkappaBzeta augments IL-12- and IL-18-mediated IFN-gamma production in human NK cells. *Blood* 2011;117:2855–2863.
27. Lorenz J, Zahlten J, Pollok I, Lippmann J, Scharf S, N'Guessan PD, Opitz B, Flieger A, Suttorp N, Hippenstiel S, *et al*. Legionella pneumophila-induced IkappaBzeta-dependent expression of interleukin-6 in lung epithelium. *Eur Respir J* 2011;37:648–657.
28. Karlsen JR, Borregaard N, Cowland JB. Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor-alpha is controlled by IkappaB-zeta but neither by C/EBP-beta nor C/EBP-delta. *J Biol Chem* 2010;285:14088–14100.
29. Shiina T, Konno A, Oonuma T, Kitamura H, Imaoka K, Takeda N, Todokoro K, Morimatsu M. Targeted disruption of MAIL, a nuclear IkappaB protein, leads to severe atopic dermatitis-like disease. *J Biol Chem* 2004;279:55493–55498.
30. Ueta M, Hamuro J, Ueda E, Katoh N, Yamamoto M, Takeda K, Akira S, Kinoshita S. Stat6-independent tissue inflammation occurs selectively on the ocular surface and perioral skin of IkappaBzeta−/− mice. *Invest Ophthalmol Vis Sci* 2008;49:3387–3394.
31. Hammad H, Lambrecht BN. Dendritic cells and airway epithelial cells at the interface between innate and adaptive immune responses. *Allergy* 2011;66:579–587.
32. Totzke G, Essmann F, Pohlmann S, Lindenblatt C, Janicke RU, Schulze-Osthoff K. A novel member of the IkappaB family, human IkappaB-zeta, inhibits transactivation of p65 and its DNA binding. *J Biol Chem* 2006;281:12645–12654.
33. Hildebrand DG, Alexander E, Horber S, Lehle S, Obermayer K, Munck NA, Rothfuss O, Frick JS, Morimatsu M, Schmitz I, *et al*. IkappaBzeta is a transcriptional key regulator of CCL2/MCP-1. *J Immunol* 2013; 190:4812–4820.
34. Kitamura H, Matsushita Y, Iwanaga T, Mori K, Kanehira K, Fujikura D, Morimatsu M, Saito M. Bacterial lipopolysaccharide-induced expression of the IkappaB protein MAIL in B-lymphocytes and macrophages. *Arch Histol Cytol* 2003;66:53–62.
35. Marini M, Vittori E, Hollemborg J, Mattoli S. Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 1992; 89:1001–1009.
36. Park CS, Choi YS, Ki SY, Moon SH, Jeong SW, Uh ST, Kim YH. Granulocyte macrophage colony-stimulating factor is the main cytokine enhancing survival of eosinophils in asthmatic airways. *Eur Respir J* 1998;12:872–878.
37. Doganci A, Sauer K, Karwot R, Finotto S. Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. *Clin Rev Allergy Immunol* 2005;28:257–269.
38. Masten BJ, Olson GK, Tarleton CA, Rund C, Schuyler M, Mehran R, Archibeque T, Lipscomb MF. Characterization of myeloid and plasmacytoid dendritic cells in human lung. *J Immunol* 2006;177: 7784–7793.
39. Zhou LJ, Tedder TF. CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells. *Proc Natl Acad Sci USA* 1996;93:2588–2592.
40. Mattoli S, Mattoso VL, Soloperto M, Allegra L, Fasoli A. Cellular and biochemical characteristics of bronchoalveolar lavage fluid in symptomatic nonallergic asthma. *J Allergy Clin Immunol* 1991;87: 794–802.
41. Allen JN, Herzyk DJ, Allen ED, Wewers MD. Human whole blood interleukin-1-beta production: kinetics, cell source, and comparison with TNF-alpha. *J Lab Clin Med* 1992;119:538–546.
42. Wewers MD, Rennard SI, Hance AJ, Bitterman PB, Crystal RG. Normal human alveolar macrophages obtained by bronchoalveolar lavage have a limited capacity to release interleukin-1. *J Clin Invest* 1984;74: 2208–2218.
43. Wewers MD, Herzyk DJ. Alveolar macrophages differ from blood monocytes in human IL-1 beta release: quantitation by enzyme-linked immunoassay. *J Immunol* 1989;143:1635–1641.
44. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, van de Veerdonk FL, Ferwerda G, Heinhuis B, Devesa I, *et al*. Differential requirement for the activation of the inflammasome for

processing and release of IL-1beta in monocytes and macrophages. *Blood* 2009;113:2324–2335.
45. Conti P, Reale M, Nicolai M, Barbacane RC, Placido FC, Iantorno R, Tenaglia R. Bacillus Calmette-Guerin potentiates monocyte responses to lipopolysaccharide-induced tumor necrosis factor and interleukin-1, but not interleukin-6 in bladder cancer patients. *Cancer Immunol Immunother* 1994;38:365–371.
46. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, *et al*. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;457:585–588.
47. Jacquet A. Innate immune responses in house dust mite allergy. *ISRN Allergy* 2013;2013:1–18.
48. Riechelmann H, Deutschle T, Grabow A, Heinzow B, Butte W, Reiter R. Differential response of Mono Mac 6, BEAS-2B, and Jurkat cells to indoor dust. *Environ Health Perspect* 2007;115: 1325–1332.
49. Ke Y, Reddel RR, Gerwin BI, Miyashita M, McMenamin M, Lechner JF, Harris CC. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. *Differentiation* 1988;38:60–66.
50. Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, Hunninghake GW. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* 2003;278:53035–53044.
51. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, McCray PB Jr. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L428–L437.
52. Lambrecht BN, Hammad H. The airway epithelium in asthma. *Nat Med* 2012;18:684–692.
53. Tsutsumi-Ishii Y, Nagaoka I. Modulation of human beta-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. *J Immunol* 2003;170:4226–4236.
54. Korpi-Steiner NL, Valkenaar SM, Bates ME, Evans MD, Gern JE, Bertics PJ. Human monocytic cells direct the robust release of CXCL10 by bronchial epithelial cells during rhinovirus infection. *Clin Exp Allergy* 2010;40:1203–1213.
55. Oonuma T, Morimatsu M, Ochiai K, Iwanaga T, Hashizume K. Role of NF-kappaB in constitutive expression of MAIL in epidermal keratinocytes. *J Vet Med Sci* 2007;69:279–284.

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