

Pathogenesis of Steroid-Resistant Airway Hyperresponsiveness: Interaction between IFN- γ and TLR4/MyD88 Pathways¹

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Chronic inflammation and airway hyperresponsiveness (AHR) in asthmatics are usually managed effectively by treatment with glucocorticoids. However, a subset of patients remains refractory to therapy. The underlying mechanisms are largely unknown, although recruitment of neutrophils (rather than eosinophils) is strongly correlated, suggesting a role for nonallergic host defense responses. Increased levels of IFN- γ and endotoxins are also related to severe asthma and because these activate host defense pathways, we investigated a possible common etiologic link to steroid-resistant disease. To be able to unravel the complexity of asthmatic inflammation, we used two model systems which permitted dissection of the relevant molecular pathways. In the first of these, we transferred transgenic OVA_{323–339} peptide-specific IFN- γ -producing Th1 cells into mice. These animals were subsequently challenge via the airways with OVA_{323–339} peptide and/or LPS. Challenge with both components, but not with either one individually, induced AHR. Importantly, AHR was resistant to treatment with dexamethasone. Development of AHR was dependent on IFN- γ , inhibited by depletion of pulmonary macrophages (but not neutrophils) and abrogated in TLR4- or MyD88-deficient mice. In contrast, in the second model in which we transferred OVA_{323–339} peptide-activated Th2 cells, eosinophilic inflammation and AHR were induced, and both were suppressed by steroid treatment. We conclude that cooperative signaling between IFN- γ and TLR4/MyD88 constitutes a previously unrecognized pathway that regulates macrophage-dependent steroid-resistant AHR. *The Journal of Immunology*, 2009, 182: 5107–5115.

Asthma is clinically recognized as a chronic inflammatory condition of the airways, which predisposes to episodic breathlessness and wheezing, along with airway hyperresponsiveness (AHR)³ to a variety of spasmogenic stimuli (1). The mechanisms involved in development and persistence of AHR may be key factors in the perpetuation of disease. Furthermore, titration of therapy based on control of AHR may yield superior therapeutic outcomes (2). Although CD4⁺ T lymphocyte-driven inflammation plays a central role in the pathogenesis of AHR (3), the underlying regulatory mechanisms remain obscure.

Part of the reason for this is the complexity of inflammatory mechanisms that contribute to the lesions of asthma. Most asthma is allergic and involves activation of CD4⁺ Th2 lymphocytes (Th2 cells) in response to specific allergens. These cells release cytokines (e.g., IL-4, -5, -9, and -13) that promote eosinophil and mast cell influx, mucus hypersecretion, airway

wall remodeling, and AHR, which underpin the clinical manifestations of asthma (3). Th2 cells induce many of their clinical features via signaling through STAT6-regulated pathways (4), which appear to be especially important in regulation of AHR by IL-13 (5). Th2 cytokine-mediated eosinophilic inflammation and the clinical features of asthma can usually be effectively managed by combination therapy with broad-spectrum anti-inflammatory agents (typically inhaled glucocorticoids) and long-acting β agonists. There is, however, increasing evidence that asthma is a heterogeneous disorder to which other inflammatory mechanisms, associated with both innate and adaptive host defense mechanisms, contribute significantly (3, 6). Such mechanisms may be of particular relevance in severe asthma and acute exacerbations of asthma triggered by respiratory infection and allergens (7).

Acute exacerbations of asthma are an important clinical problem due to their severity, refractoriness to standard treatment, and cost to the health care sector. Episodes are characterized by an exaggerated inflammatory response with the recruitment of increased numbers of T lymphocytes, eosinophils, and neutrophils to the airways (8). Although there is considerable heterogeneity in the levels of eosinophil and neutrophil infiltrates, neutrophil recruitment is a prominent feature of acute exacerbations in these steroid-resistant patients, as it is in steroid-insensitive adult severe asthmatics and in children with severe asthma (8, 9). The presence of neutrophil infiltrates in these patients suggests that innate host defense pathways may be activated. This is consistent with evidence that infection and allergen exposure are synergistic in the pathogenesis of exacerbations of asthma (10). However, given the complex inflammatory environment, the relationship between neutrophil recruitment, steroid resistance, and the pathogenesis of airflow obstruction and AHR during asthmatic exacerbations remains poorly understood. Recently, in a mouse model, Th17 cells, which play a central role in regulating neutrophilic inflammation during infection, were linked to steroid-resistant AHR (11).

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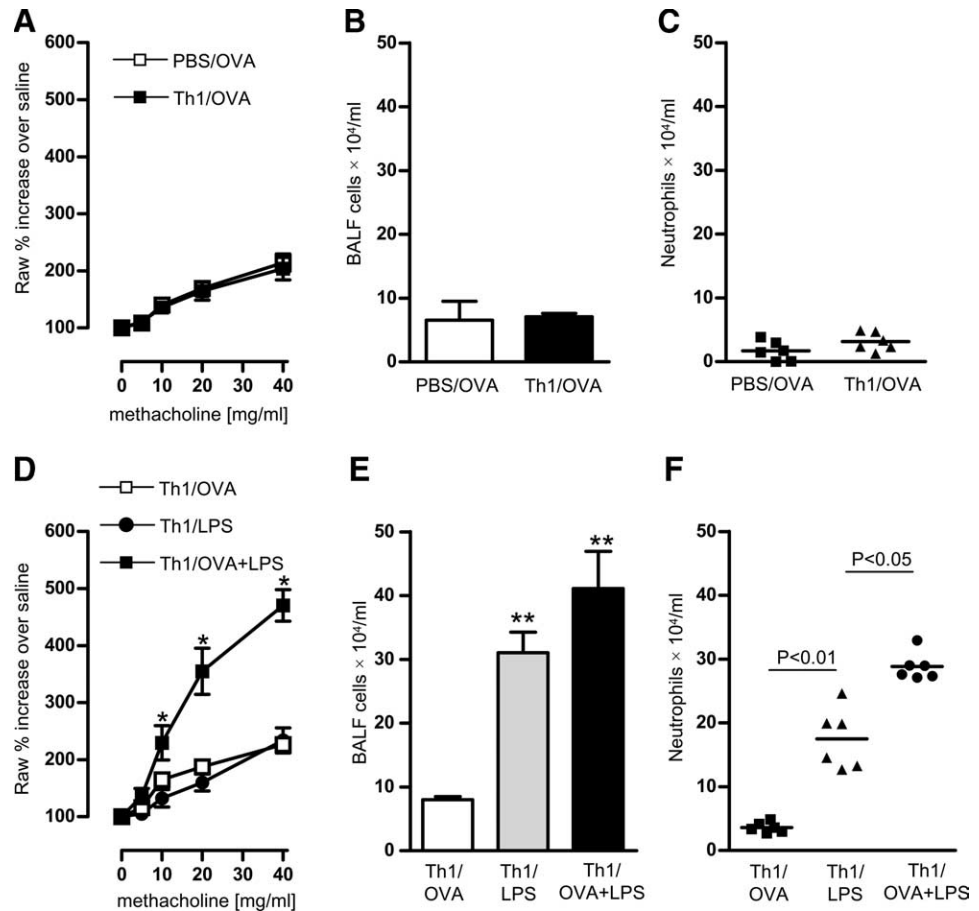
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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; i.n., intranasal; i.t., intratracheally; 2-CA, 2-chloroadenosine; DHR, dihydrodromamine 123; KO, knockout; WT, wild type; BALF, bronchoalveolar lavage fluid.

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FIGURE 1. Ag-specific Th1 cells induce AHR in the presence of LPS and neutrophils. OVA_{323–339}-activated Th1 cells did not alter airway reactivity (A), increase total numbers of inflammatory cells in BALF (B), or induce neutrophil recruitment (C). By contrast, OVA_{323–339}-activated Th1 cells in cooperation with LPS induced AHR (D) and increased the total number of inflammatory cells (E) and the number of neutrophils in BALF (F). Note that OVA refers to OVA_{323–339}. *, $p < 0.05$ compared with other groups and **, $p < 0.05$ compared with the Th1/OVA group.



Although the link to Th17 cells is an important observation, clinical data strongly suggest central roles for IFN- γ and endotoxin in the pathogenesis of exacerbations of asthma.

IFN- γ and endotoxin, which are key activating signals in the host defense response, have been linked to the severity of asthma (12, 13). IFN- γ is predominantly produced by activated T lymphocytes during infections, has been associated with development of AHR in chronic models of asthma (14), and is expressed by an increased percentage of cells in the airways of severe asthmatics (15). Indeed, in large-scale human studies, Th1 cytokines have also been shown to play a central role in asthma and AHR pathogenesis (16). Similarly, during infection, a primary mechanism for the recruitment of neutrophils into the airways is via activation of TLR4 and subsequently MyD88 by endotoxin (17). Whereas the IFN- γ R and TLR4/MyD88 pathways had until recently been regarded as independent modulators of host defense, *in vitro* studies now suggest that both signaling pathways may be integrated to regulate inflammation by increasing the expression of proinflammatory molecules (18). Importantly, TLR4 may also signal independently of MyD88 through the Toll/IL-1R domain-containing adaptor-inducing IFN- β pathway (19). To date, there are no data on the possible concurrent or synergistic roles of these molecules in the induction of airway inflammation and AHR or their potential contribution to steroid-resistant asthma.

To address the question of whether nonallergic mechanisms, including IFN- γ and TLR4-dependent signaling pathways, play a key role in the manifestations of steroid-resistant asthma, we performed experiments using two model systems which permitted dissection of the relevant molecular pathways. We demonstrate that activation of Th2 cells in the airways of mice results in eosinophilic inflammation and AHR that can be suppressed by treatment

with glucocorticoids. By contrast, Th1 cells induce steroid-resistant AHR through an IFN- γ /TLR4-MyD88-dependent mechanism after priming of innate host defense systems by LPS. This novel interaction between IFN- γ and LPS, which leads to activation of the immune system and induction of AHR, may have an important role in the pathogenesis of airflow obstruction and AHR in asthma that is insensitive or unresponsive to steroid therapy.

Materials and Methods

Animals

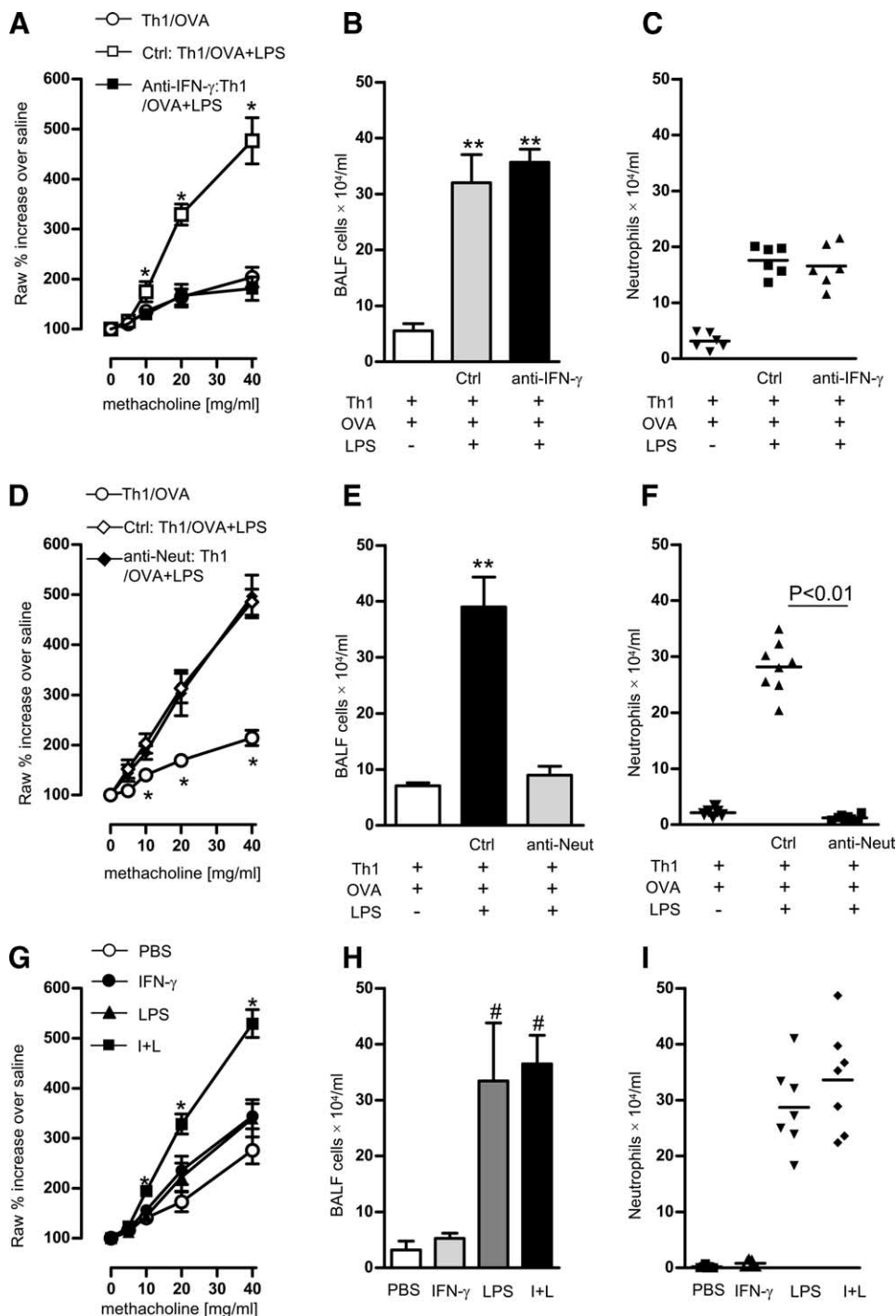
Wild-type (WT), DO10.11 (transgenic for the TCR recognizing OVA_{323–339}), TLR4 knockout (KO; TLR4^{−/−}), MyD88 KO (MyD88^{−/−}), and STAT6 KO (STAT6^{−/−}) BALB/c mice (6–8 wk) were obtained from the Special Pathogen Free Facility, University of Newcastle. All gene-targeted mice were backcrossed for 12 generations with the BALB/c strain.

Generation and transfer of Th1 or Th2 cells

Th1 or Th2 cells were generated from naive CD4⁺ T cells isolated from splenocytes of DO10.11 mice. Cultures contained CD4⁺ T cells and mitomycin C-treated CD4⁺-depleted splenocytes (used as APCs) at 0.5×10^6 cells/ml. OVA_{323–339} peptide-specific Th1 and Th2 cells were generated as previously reported (20). Cultured Th1- or Th2-cells were harvested after 4 days, confirmed >95% CD4 and TCR-transgenic positive by FACS, and 5×10^6 cells were injected *i.v.* into WT mice.

One day later, 5 μ g of OVA_{323–339} in 50 μ l of saline was *i.n.* instilled into the airways daily for 4 consecutive days to recruit and activate OVA_{323–339} peptide-specific Th1 or Th2 cells. Some groups of mice that received Th1 cells and OVA_{323–339} were also treated with 50 ng of LPS during the last *i.n.* exposure with OVA_{323–339} (Th1/OVA plus LPS). Twenty-four hours after the last treatment, airway responsiveness and the inflammatory response were characterized (end points).

FIGURE 2. IFN- γ but not neutrophils are critical for Th1- and LPS-induced AHR. Anti-IFN- γ mAb (A) inhibited OVA_{323–339}-activated Th1 cells and LPS-induced AHR but did not affect the total number of inflammatory cells (B) or the number of neutrophils in the BALF (C). Depletion of neutrophils with NIMP-R10 mAb (D) did not block OVA_{323–339}-activated Th1 cells and LPS-induced AHR, but decreased the total number of inflammatory cells (E) and neutrophils in the BALF to baseline levels (F). Cooperation between IFN- γ and LPS was required for the induction of AHR (G). LPS but not IFN- γ increased the total number of inflammatory cells in BALF (H), which reflected a selective neutrophil influx in BALF (I). As before, OVA refers to OVA_{323–339}. *, $p < 0.05$ compared with other groups; **, $p < 0.05$ compared with the Th1/OVA group; and #, $p < 0.05$ compared with the PBS or IFN- γ groups.



Administration of IFN- γ and/or LPS

Optimal doses of murine rIFN- γ (1.5 μ g/mouse; PeproTech) and/or LPS (50 ng/mouse; Sigma-Aldrich) were instilled intratracheally (i.t.). For temporal analysis in WT mice, airway reactivity and inflammation were characterized at 0, 6, 12, 24, or 48 h, 4 or 8 days after treatment. For studies in TLR4^{-/-}, MyD88^{-/-}, and STAT6^{-/-} mice, end points were assessed 12 h after treatment with IFN- γ and LPS.

Neutralization of IFN- γ and depletion of neutrophils and macrophages in the lung

For neutralization of IFN- γ , mice that received Th1/OVA plus LPS treatments were i.p. injected with 100 μ g of anti-IFN- γ mAb (R46A2) or isotype control 1 day after the first OVA_{323–339} i.n. exposure, daily for 3 consecutive days, after which end points were assessed.

For depletion of neutrophils, mice were i.p. injected with 2 μ g of NIMP-R10 2 days before, on the day of, and 2 days after the first OVA_{323–339} i.n. exposure. For depletion of macrophages, 2-chloroadenosine (2-CA; 50 μ l/mouse of 1 mM dissolved in saline; Sigma-Aldrich) or vehicle (50 μ l of PBS) was i.t. instilled daily for 4 consecutive days, then vehicle or IFN- γ and LPS were i.t. administered 1 day later and end points were assessed after a further 12 h. Cellular depletion was confirmed by examination of bronchoalveolar lavage fluid (BALF).

Administration of dexamethasone

Dexamethasone (3 mg/kg; Sigma-Aldrich) was administered by daily i.p. injections for 3 consecutive days, starting 1 day after the first OVA_{323–339} i.n. instillation in mice exposed to Th1/OVA plus LPS or Th2/OVA.

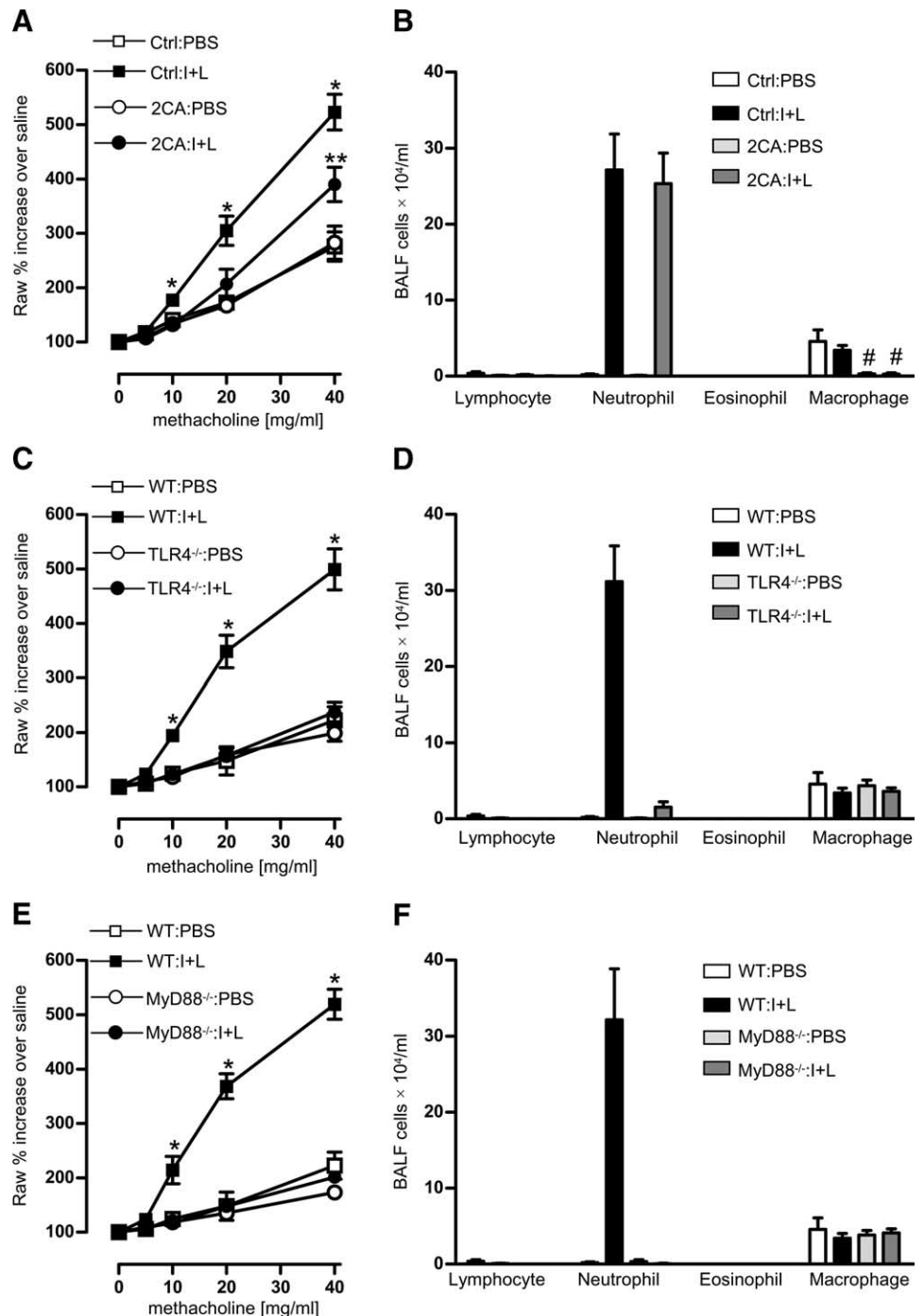


FIGURE 3. Macrophages and TLR4/MyD88 signaling are critical for IFN- γ - and LPS-induced AHR. Depletion of airway macrophages by treatment with 2-CA (A) resulted in marked attenuation of IFN- γ and LPS-induced AHR but (B) did not affect neutrophil numbers in the BALF. In TLR4^{-/-} (C and D) and MyD88^{-/-} (E and F) mice, instillation of IFN- γ and LPS into the airways did not induce AHR or a pronounced neutrophilia. I + L refers to the combination treatment with IFN- γ and LPS. *, $p < 0.05$ as compared with other groups; **, $p < 0.05$ compared with the PBS treatment groups; and #, $p < 0.05$ compared with the control groups.

Characterization of lung morphology and bronchoalveolar lavage

Numbers of mucus-staining cells in the airways were determined by periodic acid-Schiff staining. The numbers of neutrophils and eosinophils in the BALF were identified by morphologic criteria as previously described (21).

Measurement of airway resistance

Airway reactivity (resistance: Raw) was measured using a modification of the low-frequency forced oscillation technique as previously described (21). Briefly, once mice were stably anesthetized, they were challenged with a saline aerosol followed by increasing concentrations of β -methacholine (5, 10, 20, and 40 mg/ml; Sigma-Aldrich). Changes in Raw were calculated as percentage increase over saline control. Although Raw purports to measure changes in lung function related to conducting airways, evidence is emerging that it may also be influenced by peripheral airway

inflammation/cellular activation and the resultant closure of small airways (22).

Flow cytometry

Lung, blood, spleen, and bone marrow were isolated from mice after treatment with vehicle or LPS and IFN- γ . Samples were dissociated into single-cell suspensions and depleted of erythrocytes using 0.86% (w/v) ammonium chloride. Cells were then washed and immediately stained for surface marker expression using the following fluorescent mAbs: anti-F4/80, anti-CD11b, and anti-CD44 (BD Pharmingen). Anti-F4/80 and anti-11b were used to detect monocytes/macrophages. Anti-CD44 was used to examine macrophage activation. Dihydrorhodamine 123 (DHR; Sigma-Aldrich) oxidation was validated as the index of macrophage activation/phagocytosis (23). DHR is metabolized to rhodamine, which is fluorescent and measured at wavelength of 530 nm. Briefly, 0.75 μ g of DHR was added into 50 μ l of cell suspensions, incubated at room temperature for 20 min, and then centrifuged at 500 $\times$ g for 5 min. The supernatant was discarded and cells

were resuspended in PBS and again centrifuged. After the second centrifugation, the supernatant was discarded and replaced with 100 μ l of 1% paraformaldehyde in PBS to stabilize the cells. All samples were analyzed using a BD FACSCanto flow cytometer (BD Biosciences).

Data analysis

An initial one-way ANOVA (or a Kruskal-Wallis test for nonparametric data) was followed by appropriate comparisons to test for differences between means of groups. Values are reported as the mean $\pm$ SEM. The number of mice in each group ranged from 8 to 10. Differences in means were considered significant if $p < 0.05$.

Results

Activation of host defense pathways by IFN- γ -producing Th1 cells in combination with LPS induces neutrophil accumulation and AHR

To examine the relationship between activation of the innate immune system and Th1 cells for the generation of neutrophilic inflammation and AHR, we adoptively transferred Th1 cells to naive mice and i.n. administered OVA peptide 323–339 (OVA_{323–339}) and/or LPS (Fig. 1). Th1 cells were generated from naive OVA_{323–339} peptide-specific TCR-transgenic mice and predominantly produce IFN- γ on activation with OVA_{323–339} (data not shown). In mice that received OVA_{323–339} peptide-specific Th1 cells and OVA_{323–339}, there was no significant increase in the total numbers of cells or numbers of neutrophils in the BALF, and airway reactivity to methacholine was unchanged (Fig. 1, A–C). Similar results were obtained after treatment with OVA_{323–339} alone. Delivery of LPS to the lungs of mice that received OVA_{323–339} peptide-specific Th1 cells induced a selective neutrophil accumulation in the airways (no eosinophil infiltrates were observed) but did not induce AHR (Fig. 1, D–F). However, in mice given OVA_{323–339} peptide-specific Th1 cells, concurrent exposure of the airways to OVA_{323–339} and LPS significantly amplified neutrophil recruitment and induced pronounced AHR to methacholine (Fig. 1, D–F). Thus, concurrent activation of both the innate host defense pathways and Ag-specific Th1 cells was required for the induction of AHR and enhanced neutrophil recruitment.

IFN- γ is critical for Th1 cell and LPS-induced AHR

Activation of Th1 cells with OVA_{323–339} in culture predominantly results in the secretion of IFN- γ (the Th2 cytokines IL-5 and IL-13 were not detected; data not shown). Therefore, this cytokine may be the critical T cell-derived cofactor that cooperates with LPS for the induction of AHR and enhancement of neutrophil recruitment. To test this, mice supplemented with Th1 cells were treated with inhibitory IFN- γ mAbs and then concurrently challenged with OVA_{323–339} and LPS (Th1/OVA plus LPS; Fig. 2, A–C). Depletion of IFN- γ did not affect neutrophil numbers in the BALF, but AHR was abolished. This result establishes a critical role for IFN- γ secreted by Th1 cells in the regulation of AHR. Furthermore, Th1 cells can amplify neutrophil recruitment in the presence of LPS independently of IFN- γ (Fig. 1F vs 2C). To determine whether IFN- γ induces AHR by activating neutrophils, we depleted these cells in Th1/OVA plus LPS-treated mice (Fig. 2, D–F). It was noteworthy that reduction of neutrophils to baseline levels did not affect the development of AHR (Fig. 2, D–F).

To confirm the relationship between IFN- γ and LPS for the generation of AHR, we delivered these molecules either alone or in combination to the airways of naive mice (Figs. 2, G–I and 3). We performed a temporal analysis of the effects of these molecules alone and in combination on the induction of neutrophil recruitment and AHR (Fig. 4 and data for the 12-h time point is shown in Fig. 2, G–I). As observed in studies with Th1 cells and/or LPS,

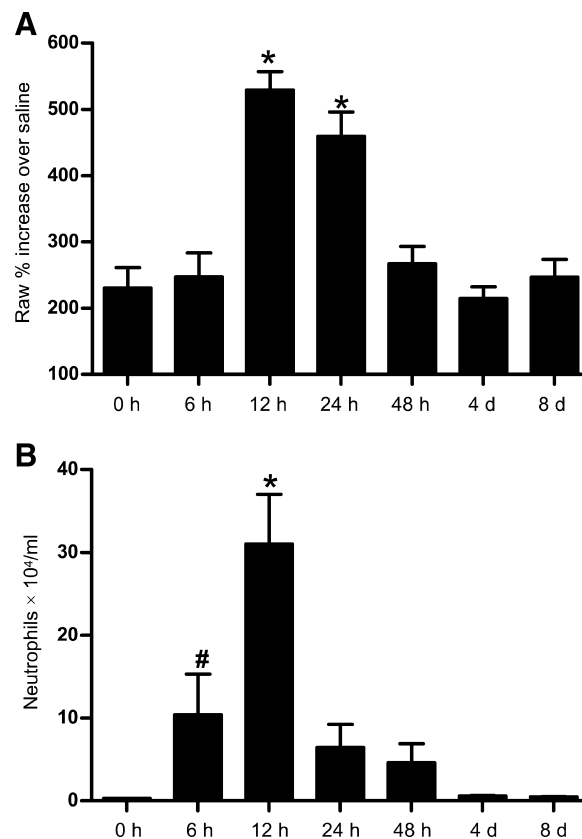


FIGURE 4. Temporal analysis of IFN- γ - and LPS-induced AHR and neutrophil recruitment. Time course of IFN- γ - and LPS-induced AHR (A), expressed as maximal responses at 40 mg/ml β -methacholine challenge, and neutrophil numbers in BALF (B). *, $p < 0.05$ compared with the other groups and #, $p < 0.05$ compared with the 0-h, 4-day, and 8-day groups.

only in combination did IFN- γ and LPS induce AHR (Figs. 2, G–I, and Fig. 4). Similarly, LPS but not IFN- γ was responsible for driving the specific neutrophilic response. The recruitment of neutrophils to the lungs peaked 12 h after administration and was sustained for more than 48 h (Fig. 4). AHR also peaked 12 h after administration and returned to baseline levels within 48 h (Fig. 4). Collectively, these data established that IFN- γ was crucial for Th1 cells in triggering AHR. Furthermore, these studies demonstrated that AHR could be temporally dissociated from pulmonary neutrophil recruitment, implying that resident lung cells were likely to play a role.

FACS analysis of lung macrophages and blood, spleen, and bone marrow monocytes

Administration of LPS to the lung may also become systemic and not only affect the activation status of pulmonary macrophages but also monocytes in the blood and bone marrow compartments. Thus, we isolated by FACS CD11b- and F4/80-positive cells from these compartments in mice treated with IFN- γ and LPS or vehicle and subsequently determined the percentages that were positive for the activation makers CD44 and DHR. In sham-treated mice, 43, 89, 8.4, and 10.5% of CD11b⁺F4/80⁺ cells were CD44⁺ and DHR⁺ in the lung, blood, spleen, and bone marrow, respectively. By contrast 57, 41, 12.4, and 20.2% of CD11b⁺ and F4/80⁺ cells were CD44⁺ and DHR⁺ in the lung, blood, spleen, and bone marrow, respectively, of mice treated with IFN- γ and LPS. These data are representative of experiments with six mice in each group. A greater frequency of activated macrophages was observed in the lung in response to IFN- γ and LPS treatment. Furthermore, greater

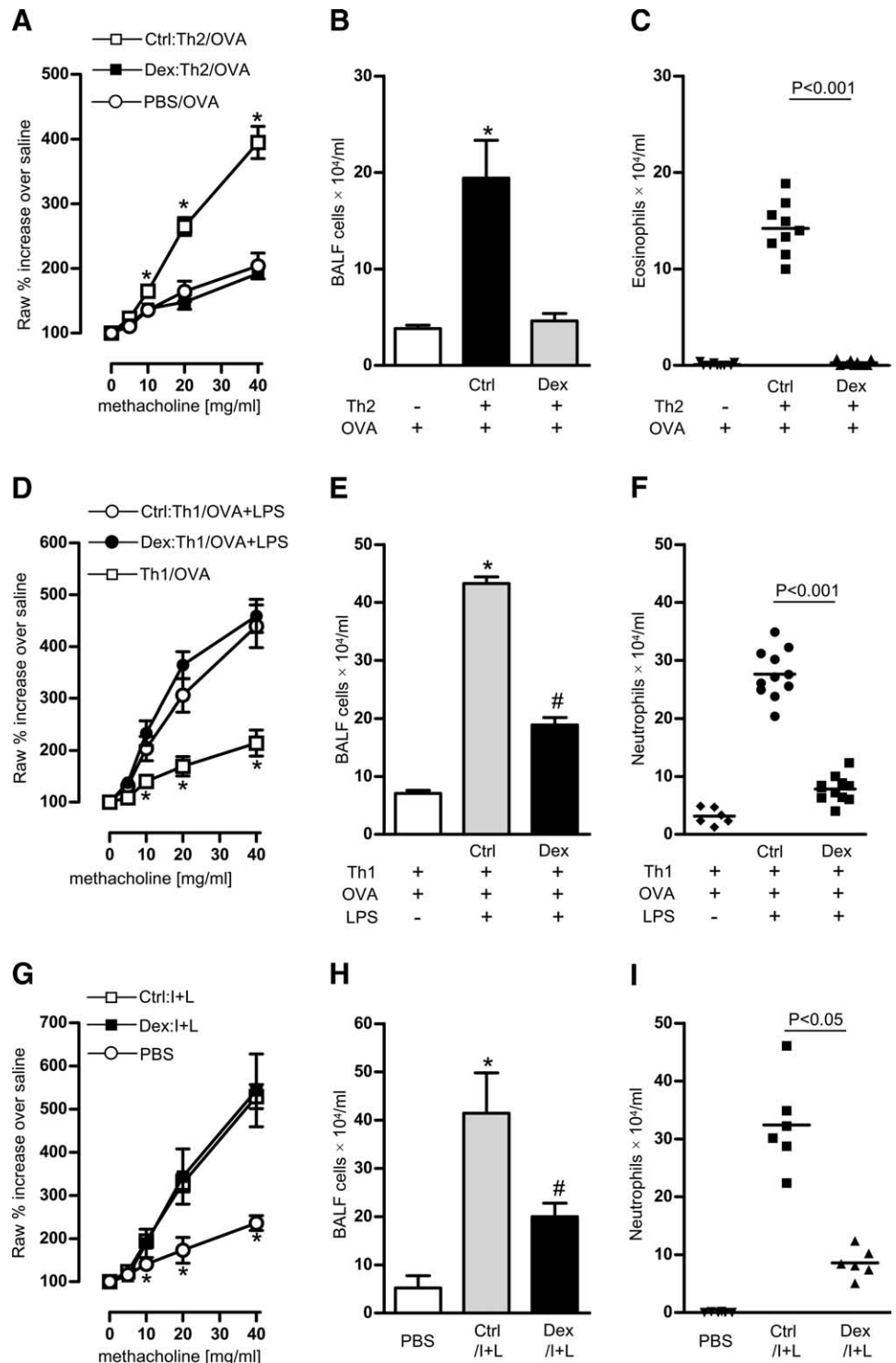


FIGURE 5. Dexamethasone treatment suppresses Th2 but not Th1- and LPS-induced inflammation and AHR. Dexamethasone inhibited activated Th2 cell-induced AHR (A), the total number of inflammatory cells in BALF (B), and eosinophil recruitment into the airways (C). By contrast, dexamethasone treatment did not inhibit activated Th1 cells and LPS-induced AHR (D), but partially suppressed the total numbers of inflammatory cells (E) and the numbers of neutrophils in BALF (F). Treatment with dexamethasone did not suppress IFN- γ and LPS-induced AHR (G) but partially suppressed the numbers of inflammatory cells in BALF (H) and partially suppressed neutrophil recruitment (I). Again, I + L refers to the combination treatment with IFN- γ and LPS. *, $p < 0.05$ compared with the other groups and #, $p < 0.05$ compared with the Th1/OVA (E) or PBS (H) groups respectively.

cell numbers were observed in the lungs of IFN- γ - and LPS-treated mice in contrast to sham-exposed mice (data not shown). However, systemic activation of monocytes was also observed in the bone marrow and spleen. Although the total numbers of cells were low in the blood, a decreased frequency of activated monocytes was observed in IFN- γ - and LPS-treated mice, suggesting that they were recruited from the circulation into the lung.

Macrophages and the TLR4/MyD88 signaling pathways are critical for IFN- γ - and LPS-induced AHR

Resident lung macrophages have been implicated in the pathogenesis of asthma and are key targets of both IFN- γ and LPS. To

determine the potential role of macrophages in regulating IFN- γ and LPS-induced AHR, we depleted these cells in the pulmonary compartment (24). Treatment of mice with 2-CA resulted in $>90\%$ reduction (control: $3.41 \pm 0.64 \times 10^4$ cells/ml; 2-CA: $0.26 \pm 0.11 \times 10^4$ cells/ml; data expressed as mean $\pm$ SEM, $n = 8$) in numbers of macrophages and a significant attenuation in airway reactivity to methacholine, with only the maximal dose significantly different from baseline levels (Fig. 3, A and B). Treatment with 2-CA did not affect the recruitment of neutrophils or other cells to the airways or baseline reactivity to methacholine in IFN- γ - and LPS-treated mice, indicating that attenuation of AHR was due to the specific depletion of macrophages (Fig. 3, A and B).

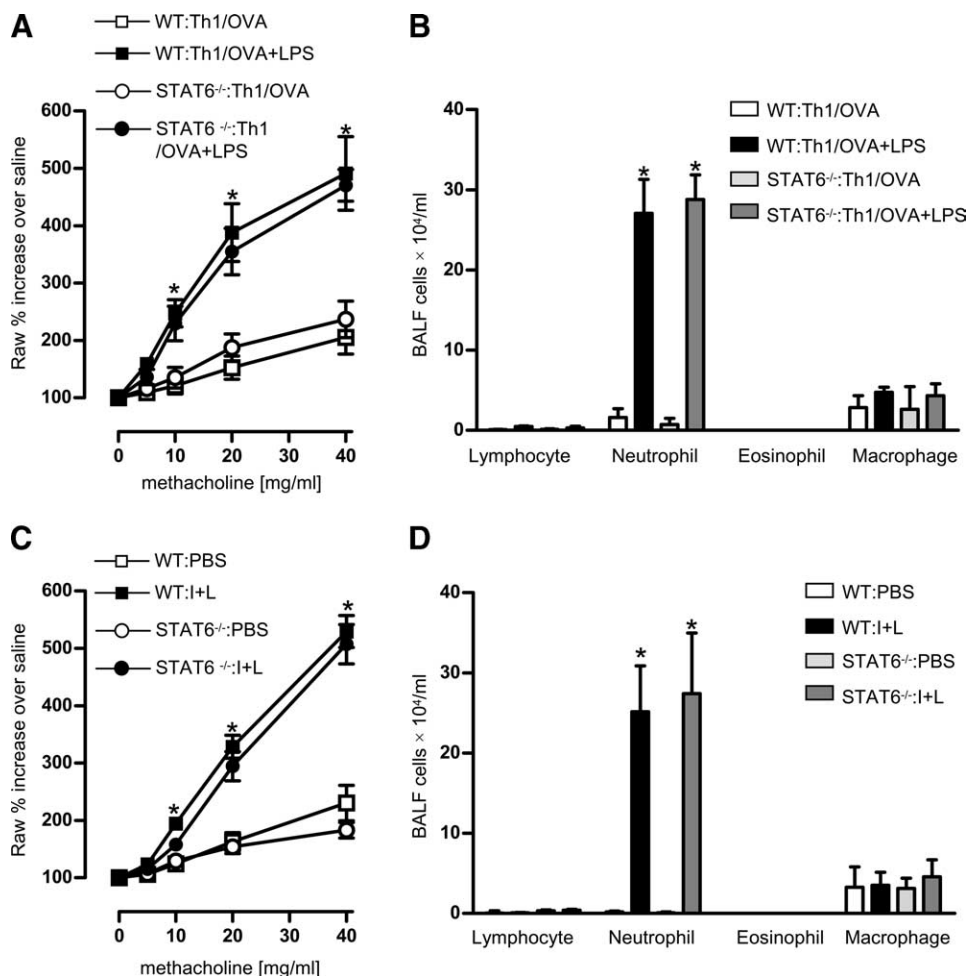


FIGURE 6. STAT6 is not involved in AHR and neutrophil accumulation induced by activated Th1 cells and LPS or IFN- γ and LPS. Activated Th1 cells and LPS-induced AHR (A) and neutrophil accumulation did not depend on activation of STAT6 (B). IFN- γ - and LPS-induced AHR (C) and neutrophil accumulation did not need the activation of STAT6 (D). I + L refers to the combination treatment with IFN- γ and LPS. *, $p < 0.05$ compared with the respective control group.

LPS is primarily thought to signal via the TLR4- and MyD88-dependent pathways (25). However, an alternative signaling mechanism through IFN regulatory factor 3 has been described (26). Thus, to confirm the specificity of the LPS response and the role of TLR4/MyD88 signaling in the induction of AHR, we treated mice deficient ($-/-$) in these molecules with both IFN- γ and LPS (Fig. 3, C–F). IFN- γ and LPS could not induce AHR in TLR4 $^{-/-}$ or MyD88 $^{-/-}$ mice, and lung macrophage numbers were normal in these mice. Baseline reactivity to methacholine in TLR4 $^{-/-}$ or MyD88 $^{-/-}$ mice was also equivalent to responses in WT mice (Fig. 3, C–E). Activation of the TLR4/MyD88 pathway in macrophages was therefore central to the induction of AHR by IFN- γ and LPS.

AHR induced by Th1 cells and LPS, but not Th2 cells, is steroid resistant and STAT6 independent

To be functionally relevant to acute exacerbations of asthma, the mechanism by which Th1/OVA plus LPS treatment induces AHR should be, at least in part, insensitive to steroid treatment. By contrast, Th2-induced eosinophilic inflammation and AHR would be expected to be steroid sensitive. Therefore, to compare the effect of steroid treatment on Th2-regulated allergic inflammation and AHR, we generated OVA_{323–339} peptide-specific Th2 cells as previously reported (20). It has previously been shown that activation of these Th2 cells with OVA_{323–339} results in the secretion of IL-4, IL-5, and IL-13 (no IFN- γ) and in the induction of hallmark features of allergic disease after adoptive transfer to naive WT mice (20). Exposure of the airways to OVA_{323–339} in mice that received Th2 cells (Th2/OVA) induced a pronounced allergic inflammatory

response characterized by eosinophil accumulation, mucus hypersecretion (data not shown), and AHR (Fig. 5, A–C). In mice that only received Th2 cells (data not shown) or OVA_{323–339}, allergic inflammation and AHR were not induced (Fig. 5, A–C). Notably, treatment of Th2/OVA_{323–339}-exposed mice with dexamethasone resulted in complete suppression of eosinophil recruitment and AHR (Fig. 5, A–C). By contrast, treatment of Th1/OVA plus LPS-exposed mice with dexamethasone partially suppressed neutrophil recruitment but did not affect the induction of AHR (Fig. 5, D–F). A similar effect of dexamethasone was observed in mice exposed to IFN- γ plus LPS (Fig. 5, G–I).

In models of allergic asthma induced either by allergen sensitization or by Th2 cell adoptive transfer, AHR is critically dependent on IL-13 signaling through STAT6 (4). In some settings, Ag-specific Th1 cells may induce AHR and features of allergic inflammation by IFN- γ and IL-13 production (27). To determine the role of the IL-13/STAT6 pathway as a common downstream effector mechanism for AHR induced by Th2 and Th1 cells, we compared airway reactivity and inflammation in WT and STAT6 $^{-/-}$ mice treated with Th1/OVA plus LPS or IFN- γ plus LPS (Fig. 6). AHR and neutrophil recruitment were not attenuated and the numbers of pulmonary macrophages or other cell types were not affected in STAT6-deficient animals.

Discussion

In this study, we have identified novel molecular mechanisms that may help to explain the development of steroid-resistant neutrophilic inflammation in acute severe asthma. Because of the complexity of the inflammatory process in such a setting, in which a

triggering infection may be superimposed on a background of allergic inflammation, we deliberately used a reductionist approach to be able to dissect the contributions of individual mediators and signaling pathways. Therefore, using OVA_{323–339} peptide-specific T cells from transgenic BALB/c mice, we transferred either Th1 or Th2 CD4⁺ T cells into animals that were then challenged with OVA peptide. Whereas this was sufficient to induce characteristic features of asthma (eosinophil accumulation, mucus hypersecretion, and AHR) in Th2 recipients, it had little effect in mice that received Th1 cells. However, if the latter animals were concurrently challenged with LPS, they exhibited marked neutrophil recruitment (greater than induced by LPS alone) and AHR. Strikingly, whereas the inflammation and AHR associated with antigenic challenge in Th2 recipient mice could be suppressed by treatment with glucocorticoids, cellular recruitment in the Ag plus LPS-challenged Th1 recipients was relatively steroid resistant.

Our data support previous observations that Ag-specific Th1 cells alone do not induce AHR (20, 28). However, unlike previous studies with transferred Ag-specific Th1 cells, we did not see pronounced neutrophil recruitment, presumably because we challenged the airways with OVA_{323–339} rather than OVA protein; this limited the potential for exogenous contamination with LPS and permitted clear separation of the effects of LPS and allergen in our model. Thus, we were able to demonstrate that an important second signal, associated with the activation of innate host defense pathways by LPS, contributes to a mechanism whereby Th1 cells induce AHR and enhance the recruitment of pulmonary neutrophils. LPS has been previously shown to cooperate with Th cells of a mixed phenotype, which secrete both IFN- γ and type 2 cytokines (e.g., IL-13 and IL-9) and induced eosinophilic (similar to Th2-induced responses) and neutrophilic inflammation in response to Ag challenge (27). In this model, IL-18 played a critical role in promoting the mixed Th phenotype and AHR but the role of neutrophils and macrophages and the steroid sensitivity of the response was not determined. By contrast we demonstrate that polarized Th1 cells in association with LPS induce neutrophilia and macrophage-dependent steroid-insensitive AHR.

Taking advantage of the model system we had used, we then showed that IFN- γ secreted by Th1 cells was the critical T cell-derived mediator contributing to the induction of AHR, because neutralization of this cytokine by administration of a mAb completely abrogated the increase in airway reactivity to methacholine and delivery of IFN- γ plus LPS to the airways could induce AHR. However, AHR clearly was not dependent on the associated neutrophilic influx. Although neutrophils were the only cells recruited to the airways of Th1/OVA plus LPS cell-treated mice, depletion of these cells to baseline levels did not prevent the development of AHR. This result strongly suggested that IFN- γ plus LPS induced AHR by activating resident lung cells.

In additional experiments, we established that macrophages were likely to be the key population of resident cells that contributed to AHR. Like neutrophils, lung macrophages are crucial in host defense against infections and have long been recognized as undergoing activation in asthma (29). Recently, Schaumann et al. (30) have shown that LPS increases influx of macrophage-like cells into alveolar space and enhances allergic inflammation in the lung of asthmatics. Indeed, macrophage activation is tightly regulated by TLRs and IFN- γ , and these molecules have been shown to increase the production of proinflammatory cytokines (e.g., macrophage inflammatory protein 1 α , GM-CSF, and IL-12) and to activate transcriptional programs (e.g., NF- κ B and ERK1/2 MAPK) (31) in these cells. A significant increase in the frequency and total number (data not shown) of CD11b⁺F4/80⁺CD44⁺ and DHR⁺ were observed in the lung after IFN- γ and LPS, indicating

that these molecules directly activated pulmonary macrophages. Although the total numbers of cells were low, systemic activation of monocytes was also observed after treatment. Notably, the frequency of activated circulating monocytes decreased after exposure to IFN- γ and LPS, suggesting that activated monocytes may be recruited from the blood and contribute to the pulmonary macrophage pool.

LPS is primarily thought to signal by binding to TLR4 and subsequently activating MyD88-dependent pathways (25), although an alternative signaling mechanism through IFN regulatory factor 3 has also been described (26). We showed that deletion of TLR4, or of MyD88, resulted in attenuation of AHR induced by IFN- γ plus LPS. Thus, our data collectively indicate that this pattern of AHR is dependent on activation of TLR4/MyD88 signaling in macrophages. Importantly, LPS-induced neutrophil recruitment was critically dependent on the MyD88 signaling pathway, as has previously been observed (32).

Although allergic asthma is usually well controlled by treatment with glucocorticoids and bronchodilators (3), in acute exacerbations of asthma and in a subset of patients with chronic severe asthma who are difficult to manage with conventional therapy, a steroid-resistant component of disease exists (7, 33). The observation that neutrophil recruitment is a prominent feature in these situations suggests that activation of host defense pathways, similar to those activated by infection, may contribute to the clinical expression and AHR in these patients (34, 35). Consistent with this, we found that although Th2-mediated eosinophil recruitment and AHR were suppressed by treatment with dexamethasone, Th1/OVA plus LPS-induced AHR (which by contrast was independent of STAT6 signaling) was resistant to dexamethasone therapy. Similar effects were observed in mice exposed to IFN- γ plus LPS, directly confirming that the mechanism for the induction of AHR by these molecules was resistant to steroid treatment. Furthermore, whereas treatment with dexamethasone completely suppressed Th2-driven inflammation, Th1-driven neutrophil recruitment was only partially inhibited, supporting clinical observations that neutrophilic inflammation in asthma is relatively resistant to steroid therapy.

In conclusion, by using models that allow dissection of complex molecular pathways, we have described a novel mechanism for the induction of AHR which involves collaborative interaction between effector mechanisms of the innate and adaptive host defense response. This mechanism requires concurrent activation of the IFN- γ R and TLR4/MyD88 pathways in macrophages, is independent of neutrophils, is steroid resistant, and utilizes a pathway that is distinct from the steroid-sensitive IL-13/STAT6 pathway associated with induction of AHR and eosinophilic inflammation by Th2 cells. Expressed another way, two separate T cell-dependent mechanisms can induce AHR and inflammation under specific conditions.

How might these apparently redundant and overlapping pathways contribute to the pathogenesis of treatment-unresponsive airflow obstruction which characterizes chronic severe asthma and acute exacerbations of asthma? Although speculative, one possibility is that activation of the Th1 cell/TLR4/MyD88 pathway may primarily be oriented toward limiting infection and its effects, in the process activating proinflammatory pathways in macrophages, recruiting neutrophils and increasing airway responsiveness to cholinergic stimuli. In asthmatics who develop superimposed infections, activation of this pathway on a background of aberrant Th2 cytokine-driven chronic inflammation may elicit the features of an acute exacerbation. This notion is consistent with evidence

that infection synergizes with allergen exposure in the pathogenesis of acute exacerbations (10) and that Th cells of an intermediate phenotype (secreting IFN- γ and Th2 cytokines) can induce pronounced AHR (36). It is also consistent with the relative steroid resistance of both chronic severe asthma and acute exacerbations. Our findings identify a hitherto unrecognized role for macrophages, Th1 cells, and the IFN- γ R and TLR4/MyD88 pathways in the pathogenesis of steroid-resistant AHR. Potentially, understanding these cellular and molecular mechanisms may provide new targets for therapy of difficult asthma.

Although our model does not reflect the complexities associated with the inflammatory response in human asthma, it does provide an important step in identifying the role of, and relationship between, two critical immune factors that have been clinically linked to the pathogenesis of severe and difficult-to-manage asthma. Our data suggest that further analysis of the cooperative elements of the IFN- γ and TLR4 pathways in relevant patient populations may lead to new insights into pathogenesis and potentially new targets for the clinical management of steroid-refractive asthma.

Disclosures

The authors have no financial conflict of interest.

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