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Regulation of Acute Lung Inflammatory Injury by Endogenous IL-13

Alex B. Lentsch,*† Boris J. Czermak,‡ Jacqueline A. Jordan,§ and Peter A. Ward¶

Using IgG immune complex deposition to trigger acute lung inflammation in rats, we have previously shown that exogenously administered IL-13 suppresses the acute inflammatory response. In the same model, expression of both mRNA and protein for IL-13 has now been detected. Treatment of rats with Ab to IL-13 accentuated the inflammatory response, with significant increases in lung vascular permeability and in the number of neutrophils in bronchoalveolar lavage fluids. In the presence of anti-IL-13, activation of the transcription factor, NF-κB, was significantly increased in lung. In addition, anti-IL-13 caused significant increases in bronchoalveolar lavage levels of TNF-α, macrophage inflammatory protein-2, and cytokine-inducible neutrophil chemoattractant but no changes in lung vascular ICAM-1. These data suggest that during lung inflammation endogenous IL-13 regulates NF-κB activation and related cytokine/chemokine generation, all of which determines the intensity of the lung inflammatory response. The Journal of Immunology, 1999, 162: 1071–1076.

Interleukin-13, which was originally identified as a product of activated Th2 cells, has been shown to possess a variety of immunomodulating properties for B cells and monocytes (1, 2). In vitro, IL-13 suppresses monocyte production of proinflammatory cytokines, including TNF-α, IL-1α and IL-1β, IL-6, IL-8, and macrophage inflammatory protein (MIP)-1α (3–5). In addition, IL-13 augments the production of IL-1 receptor antagonist (6). The anti-inflammatory effects of IL-13 are remarkably similar to those of IL-10: both down-regulate proinflammatory cytokine production by monocytes and macrophages (7–9). In vivo, both IL-10 and IL-13 (administered exogenously) have been shown to increase survival in a murine model of endotoxic shock by reducing systemic levels of TNF-α (10, 11). In a rat model of acute inflammatory lung injury induced by intrapulmonary deposition of IgG immune complexes, exogenous administration of either IL-10 or IL-13 reduced lung production of TNF-α and diminished ICAM-1 up-regulation, neutrophil recruitment, and lung injury (12, 13). These anti-inflammatory effects in lung were subsequently shown to be due to suppression of NF-κB activation (14).

Earlier studies have demonstrated that IL-10 function in vivo is an endogenous regulator of the lung inflammatory response (15). These studies demonstrated that IL-10 was up-regulated during lung inflammation and that blockade of IL-10 with Ab augmented pulmonary TNF-α production, neutrophil recruitment, and lung injury. In the current study we sought to determine whether IL-13 is an important endogenous mediator in the regulation of acute inflammatory lung injury induced by deposition of IgG immune complexes. Our findings indicate that IL-13 mRNA and protein are up-regulated during lung inflammation. Blockade of endogenous IL-13 with Ab augments NF-κB activation in lung and causes increased intrapulmonary production of TNF-α. These effects are associated with increased accumulation of neutrophils and increased vascular leak of albumin. The data suggest that IL-13 is an intrinsic regulator of the lung inflammatory response.

Materials and Methods

IgG immune complex-induced alveolitis
Pathogen-free male Long-Evans rats (275–300 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with ketamine HCl (150 mg/kg, i.p.). For measurement of pulmonary vascular permeability, rats received intratracheal administration of PBS, pH 7.4, 1.5 mg anti-BSA (ICN Biomedicals, Costa Mesa, CA), and 0.5 mg nonspecific goat IgG, or 1.5 mg anti-BSA and 0.25 or 0.5 mg goat polyclonal anti-mouse IL-13 (R&D Systems, Minneapolis, MN) in a volume of 0.3 ml of PBS. Immediately thereafter, 10 mg of BSA (<1 ng endotoxin/mg) containing trace amounts of 125I-labeled BSA in 0.5 ml of PBS was injected i.v. Four hours after IgG immune complex deposition, rats were exsanguinated, the pulmonary circulation was flushed with 10 ml of PBS by pulmonary artery injection, and the lungs were surgically dissected. The extent of lung injury was quantified by calculating the lung permeability index by dividing the amount of radioactivity (125I-labeled BSA) in the perfused lungs by the amount of radioactivity in 1.0 ml of blood obtained at the time the rats were sacrificed. For measurement of lung NF-κB activation, rats received intratracheal administration of PBS (negative control), 0.25 mg anti-BSA, and 0.5 mg nonspecific goat IgG, or 0.25 mg anti-BSA and 0.5 mg anti-IL-13 in a volume of 0.3 ml PBS. BSA (10 mg) was injected i.v., and 4 h later lungs were surgically removed and immediately frozen in liquid nitrogen. Unless otherwise indicated, n ≥ 5 for each experimental group described in Figs. 1–7.

RT-PCR
Total RNA from whole-lung tissue was extracted using a guanidinium-isothiocyanate method as described previously (16). RNA (1 μg) was reverse transcribed to cDNA. The cDNA products were amplified by PCR (35 cycles; 1 min each at 95°C, 55°C, and 72°C). The 5′ primer (5′-CCAGTGCGCCGTCATGCCCACTG-3′) and 3′ primer (5′-CTTTCCGGCTATGCCCCGCG-3′) were complementary to 5′ and 3′ regions of the open reading frame of the murine IL-13 sequence. PCR products were sequenced for verification. RT-PCR of the glyceralddehyde-3-phosphate dehydrogenase gene was performed under the same conditions described above to confirm equal loading of RNA. Ethidium bromide-stained PCR products were photographed, digitized, and analyzed using image analysis software (Adobe Systems, San Jose, CA).

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2 Address correspondence and reprint requests to Dr. Peter A. Ward, Department of Pathology, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109-0602. E-mail address: pward@umich.edu
3 Abbreviations used in this paper: MIP, macrophage inflammatory protein; EMSA, electrophoretic mobility shift assay; BAL, bronchoalveolar lavage; CINC, cytokine-inducible neutrophil chemoattractant.

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Western blot analysis
Lungs were homogenized in lysis buffer (10 mM HEPES, pH 7.9; 150 mM NaCl; 1 mM EDTA; 0.6% Nonidet P-40; 0.5 mM PMSF; 1 μg/ml leupeptin; 1 μg/ml aprotinin; 10 μg/ml soybean trypsin inhibitor; and 1 μg/ml pepstatin) on ice. Homogenates were sonicated and centrifuged at 5000 rpm to remove cellular debris. Protein concentrations were determined as described for nuclear extracts. Samples were separated in a 4% polyacrylamide gel and analyzed by autoradiography.

Assessment of NF-κB activation by electrophoretic mobility shift assay (EMSA)
Nuclear extracts of whole-lung tissues were prepared by the method of Deryckere and Gannon (17). Protein concentrations were determined by bicinchoninic acid assay with TCA precipitation using BSA as a reference standard (Pierce, Rockford, IL). Double-stranded NF-κB consensus oligonucleotide (5′-AGTGAGGGGACTTCCAGGC-3′; Promega, Madison, WI) was end-labeled with [γ-32P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham, Arlington Heights, IL). Binding reactions containing equal amounts of protein (10 μg for whole-lung extracts and 5 μg for alveolar macrophage extracts) and 35 fmol (≈50,000 cpm, Cherenkov counting) of oligonucleotide were performed for 30 min in binding buffer (4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, pH 8.0; 0.5 mM DTT; 50 mM NaCl; 10 mM Tris (pH 7.6); and 50 μg/ml poly(dI·dC); Pharmacia, Piscataway, NJ). Reaction volumes were held constant at 15 μl. Reaction products were separated in a 4% polyacrylamide gel and analyzed by autoradiography. NF-κB activation was quantitated from digitized autoradiography films using image analysis software (Adobe Systems).

Bronchoalveolar lavage (BAL) fluid cytokine content
BAL fluids were collected by instilling and withdrawing 5 ml of sterile PBS three times from the lungs via an intratracheal cannula. Cellular contents were recovered by centrifugation, and neutrophils were counted by microcytometry. BAL content of TNF-α was measured using a standard WEHI cell cytotoxicity assay as previously reported (18). Measurement of MIP-2 and cytokine-inducible neutrophil chemoattractant (CINC) in BAL fluids were performed using ELISA as described elsewhere (19).

Statistical analyses
All values were expressed as mean ± SEM. Data were analyzed with a one-way analysis of variance, and individual group means were then compared with a Student-Newman-Keuls test. Differences were considered significant when p < 0.05. For calculations of percentage change, negative control values were subtracted from positive control and treatment group values.

Results
Expression of IL-13 during IgG immune complex-induced lung inflammatory injury
To determine whether IL-13 is up-regulated during the lung inflammatory response, lung extracts were assessed for content of IL-13 mRNA and protein. Pulmonary expression of IL-13 mRNA in rats undergoing IgG immune complex-induced lung injury was determined by RT-PCR. The presence of a clearly detectable PCR product in lung RNA obtained at time 0 indicated that IL-13 mRNA was constitutively expressed in the noninflamed lung (Fig. 1). IL-13 mRNA expression was increased 30 min after initiation of the lung inflammatory response. mRNA levels decreased somewhat at 1 h, increased to near-maximal levels again at 2 h, and returned to baseline 4 h after initiation of injury. In contrast to IL-13 mRNA, protein levels of IL-13 were undetectable by Western blot in noninflamed lung extracts (time 0) (Fig. 2). However, IL-13 protein was detectable within 30 min after initiation of injury, with increased expression at 1, 2, and 4 h.

Augmentation of IgG immune complex-induced neutrophil recruitment and lung injury by anti-IL-13
Because exogenous administration of IL-13 has been shown to suppress lung injury induced by IgG immune complexes (13), we designed experiments to assess the role of endogenous IL-13 in the lung inflammatory response. The extent of lung injury was determined by extravascular leak125I-labeled albumin. Intrapulmonary deposition of IgG immune complexes in the presence of 500 μg of irrelevant goat IgG mixed with anti-BSA resulted in a more than twofold increase in the lung permeability index (Fig. 3). Intrapulmonary administration of Ab to IL-13 (along with the IgG anti-BSA) caused a dose-dependent increase in the lung permeability index. In the presence of 250 μg or 500 μg anti-IL-13, the permeability index was increased 36% (p = 0.029) and 62% (p = 0.002), respectively. To determine whether the increased lung injury induced by anti-IL-13 was related to enhanced pulmonary recruitment of neutrophils, the effects of anti-IL-13 on the number of BAL neutrophils recovered 4 h after IgG immune complex deposition was determined. Intrapulmonary deposition of IgG immune complexes caused an almost sixfold increase in BAL neutrophil numbers (Fig. 4). In the presence of 500 μg of anti-IL-13, the number of neutrophils present in BAL fluids was increased by 65% (p = 0.005).
Effects of anti-IL-13 on IgG immune complex-induced lung injury. Pulmonary vascular permeability was assessed 4 h after intratracheal administration of 1.5 mg of anti-BSA (with 500 μg of normal goat IgG) followed by i.v. infusion of 10 mg of BSA together with trace amounts of 125I-labeled albumin. Anti-IL-13 (250 or 500 μg) was administered intratracheally with the IgG anti-BSA. Values represent mean ± SEM; n = 5–10 for each group.

**FIGURE 3.** Effects of anti-IL-13 on IgG immune complex-induced lung injury. Pulmonary vascular permeability was assessed 4 h after intratracheal administration of 1.5 mg of anti-BSA (with 500 μg of normal goat IgG) followed by i.v. infusion of 10 mg of BSA together with trace amounts of 125I-labeled albumin. Anti-IL-13 (250 or 500 μg) was administered intratracheally with the IgG anti-BSA. Values represent mean ± SEM; n = 5–10 for each group.

Effects of anti-IL-13 on BAL levels of TNF-α and CXC chemokines and pulmonary vascular expression of ICAM-1

Intrapulmonary production of TNF-α is known to drive the lung inflammatory response induced by deposition of IgG immune complexes (20). Because exogenous administration of IL-13 has been shown to suppress lung inflammation in this model with an associated reduction in TNF-α levels in BAL fluids (13), we assessed whether endogenous IL-13 serves to regulate lung production of TNF-α. BAL fluids were obtained 4 h after intrapulmonary deposition of IgG immune complexes were analyzed by WEHI assay for TNF-α bioactivity. For each group, n = 5 or 6. B, Lung vascular ICAM-1 expression was determined 4 h after IgG immune complex deposition using vascular fixation of 125I-labeled anti-rat ICAM-1. For each group, n = 4. All values represent mean ± SEM.

**FIGURE 5.** Effects of anti-IL-13 on IgG immune complex-induced BAL TNF-α and lung vascular ICAM-1 expression. A, BAL fluids harvested 4 h after intratracheal administration of PBS or intrapulmonary deposition of IgG immune complexes were analyzed by WEHI bioassay for TNF-α bioactivity. For each group, n = 5 or 6. B, Lung vascular ICAM-1 expression was determined 4 h after IgG immune complex deposition using vascular fixation of 125I-labeled anti-rat ICAM-1. For each group, n = 4. All values represent mean ± SEM.

**FIGURE 4.** Effects of 500 μg of anti-IL-13 on BAL neutrophils 4 h after intrapulmonary deposition of IgG immune complexes. Anti-IL-13 (500 μg) was administered intratracheally with the IgG anti-BSA. Values represent mean ± SEM; n = 6 for each group.

**FIGURE 3.** Effects of anti-IL-13 on IgG immune complex-induced lung injury. Pulmonary vascular permeability was assessed 4 h after intratracheal administration of 1.5 mg of anti-BSA (with 500 μg of normal goat IgG) followed by i.v. infusion of 10 mg of BSA together with trace amounts of 125I-labeled albumin. Anti-IL-13 (250 or 500 μg) was administered intratracheally with the IgG anti-BSA. Values represent mean ± SEM; n = 5–10 for each group.

Since TNF-α is known to regulate the expression of lung vascular ICAM-1 (21), we assessed the effects of 500 μg of anti-IL-13 or normal goat IgG on ICAM-1 expression in the pulmonary vasculature. Expression of ICAM-1 in lung was determined by the binding index of 125I-labeled anti-ICAM-1. As expected, intrapulmonary deposition of IgG immune complexes caused a significant increase in the ICAM-1 binding index (Fig. 5B). In spite of the effects of anti-IL-13 on BAL levels of TNF-α, there was, unexpectedly, no effect on the binding index for ICAM-1. Because
TNF-α may facilitate expression of the CXC chemokines MIP-2 and CINC, which, like ICAM-1, are required for pulmonary neutrophil recruitment (19), the effects of anti-IL-13 on lung production of MIP-2 and CINC were determined. BAL levels of MIP-2 and CINC were measured by ELISA. In the presence of irrelevant IgG, IgG immune complexes caused dramatic increases in the content of MIP-2 and CINC in BAL fluids (Fig. 6). The presence of anti-IL-13 modestly but significantly augmented BAL levels of MIP-2 and CINC by 18% (p = 0.048) and 20% (p = 0.003), respectively.

**Effects of anti-IL-13 on lung NF-κB activation**

We have previously shown that exogenous administration of IL-13 suppresses lung NF-κB activation induced by intrapulmonary deposition of IgG immune complexes (14). Since anti-IL-13 augmented IgG immune complex-induced BAL TNF-α, MIP-2, and CINC, and in view of the fact that these mediators are regulated by NF-κB (22–24), we sought to determine whether endogenous IL-13 might regulate lung NF-κB activation during the inflammatory response. For these experiments, the intratracheal dose of anti-BSA employed was very low (250 μg), designed to produce minimal activation of NF-κB so that effects of anti-IL-13 might be more sensitively evaluated. Lung nuclear extracts obtained 4 h after initiation of lung injury were assessed by EMSA. Intrapulmonary deposition of IgG immune complexes (using 250 μg of anti-BSA and 500 μg of normal goat IgG) resulted in little, if any, activation of lung NF-κB (Fig. 7A). However, in the presence of 500 μg of anti-IL-13, there was a measurable increase (~40%) in the amount of lung NF-κB activation. Image analysis of digitized EMSA blots indicated that anti-IL-13 significantly increased nuclear localization of lung NF-κB (p = 0.003) (Fig. 7B).

**Discussion**

The acute lung inflammatory response induced by intrapulmonary deposition of IgG immune complexes is driven by induction of the “early response” cytokines, TNF-α and IL-1, which are generated by lung macrophages (20, 25). These cytokines promote lung inflammation by stimulating pulmonary vascular endothelial cells to express adhesion molecules, which mediate the adhesion and transmigration of neutrophils from the vascular lumen into the lung interstitial spaces. Both E-selectin and ICAM-1 seem to play
In vitro, IL-13 prevents production of proinflammatory cytokines by activated macrophages and monocytes (3–5). In vivo, IL-13 has been shown to protect against LPS-induced lethality and to suppress lung inflammatory injury following deposition of IgG immune complexes (11, 13). In both models, the protective effects of IL-13 were associated with reduced production of TNF-α. In the current studies, we show that neutralization of endogenous IL-13 caused enhanced production of TNF-α, suggesting that intrinsic IL-13 regulates the production of early response cytokines during acute lung inflammation. In addition, BAL levels of MIP-2 and CINC are increased under these same conditions, although much more modestly when compared with larger increases in BAL levels of TNF-α. The mechanism(s) by which IL-13 regulates proinflammatory cytokine production seems to involve suppression of the transcription factor, NF-κB. We have recently shown that IL-13 inhibits NF-κB activation in alveolar macrophages in vitro and in vivo (14). In addition, those studies demonstrated that exogenously administered IL-13 inhibited nuclear translocation of NF-κB in whole-lung nuclear extracts. The NF-κB-suppressing effects of IL-13 were found to be mediated by stabilization of the cytoplasmic NF-κB inhibitory protein, IκBα (14). In the current studies, we found that neutralization of endogenous IL-13 caused significantly more NF-κB activation in lung. These data suggest that endogenous IL-13 regulates pulmonary NF-κB activation and subsequent proinflammatory mediator expression during the development of lung injury.

In this model of acute lung injury, it is likely that TNF-α stimulates ICAM-1 expression on vascular endothelial cells via activation of NF-κB (21, 28, 29). Despite increases in BAL TNF-α and lung NF-κB activation induced by anti-IL-13, there were no detectable increases in expression of pulmonary vascular ICAM-1. However, lung accumulation of neutrophils was increased by 65%. Under the experimental conditions employed, it is possible that ICAM-1 expression was maximally stimulated. Alternatively, augmented lung neutrophil accumulation might be explained by enhanced E-selectin up-regulation and enhanced chemokine generation. At present there is no reliable methodology for the quantitative assessment of E-selectin expression on the rat pulmonary vasculature. Like TNF-α and ICAM-1, gene expression of the CXC chemokines MIP-2 and CINC is regulated by NF-κB (23, 24). In parallel with increased lung NF-κB activation, BAL levels of both MIP-2 and CINC were augmented (~20%) in the presence of anti-IL-13. These data suggest that the increased number of neutrophils in lung induced by anti-IL-13 could be attributed to augmented production of MIP-2 and CINC. Furthermore, the data suggest that intrinsic IL-13 may regulate CXC chemokine production via effects on NF-κB.

IL-10 and IL-13 appear to be the most powerful of the “regulatory cytokines.” Exogenous administration (intratracheally) of these ILs in very low amounts (1–5 μg) profoundly suppresses lung inflammatory responses by blocking NF-κB activation (this report and Refs. 12–14, 26, and 28). In turn, production of TNF-α is reduced, vascular ICAM-1 expression diminishes, and fewer neutrophils are recruited into lung. It is now clear that both IL-10 and IL-13 interfere with NF-κB activation by causing retention of IκBα, which prevents nuclear translocation of NF-κB (14). How the proteolysis of IκBα is prevented is not known. Exogenous administration of regulatory ILs into lungs of rats undergoing deposition of IgG immune complexes reveals the following rank order of inhibitory activity (in descending order): IL-10 > IL-13 > IL-4 > IL-6 > IL-12 (13). Our recent studies together with the current report indicate that, except for IL-4, which does not appear to be expressed in the lung, each of these ILs is up-regulated during the inflammatory response and each is playing a regulatory role, as defined by a more intense inflammatory response when any of these ILs is blocked by the presence of an Ab. The lack of available reagents has not allowed for precise measurements of each of these ILs in lung during initiation of the inflammatory response as well as over the course of the next several hours. Consequently, we cannot yet quantify the precise contributions for each of these ILs. On the other hand, it might be predicted that blockade of all relevant regulatory ILs might cause the inflammatory response to be uncontrolled, resulting in a greatly increased intensity and, perhaps, in irreversible lung damage, proceeding to interstitial fibrosis.

The source of these regulatory ILs in lung is not completely known. In the case of IL-13, our own attempts to resolve this issue have employed immunostaining techniques. BAL macrophages obtained from normal lung have failed to demonstrate any staining, while BAL macrophages obtained 4 h after initiation of the inflammatory response have shown positive staining (A. B. Lentsch, R. L. Warner, and P. A. Ward, unpublished observation). It is possible that there are additional sources of IL-13, such as T cells or epithelial cells, but this has not yet been determined.

The current studies suggest that IL-13 is an important endogenous regulatory cytokine during IgG immune complex-induced lung injury. The anti-inflammatory properties of endogenous IL-13 seem to operate by suppressing activation of lung NF-κB and the subsequent production of the early response cytokine TNF-α and the CXC chemokines MIP-2 and CINC. These combined effects appear to contribute to the resolution of lung inflammation and may represent a potential point of therapeutic intervention in humans.

References


ENDOGENOUS IL-13 REGULATES LUNG INJURY