Interactions Between Leukotriene C₄ and Interleukin 13 Signaling Pathways in a Mouse Model of Airway Disease

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**Context.**—During an asthmatic episode, leukotriene C₄ (LTC₄) and interleukin 13 (IL-13) are released into the airways and are thought to be central mediators of the asthmatic response. However, little is known about how these molecules interact or affect each other’s signaling pathway.

**Objective.**—To determine if the LTC₄ and IL-13 signaling pathways interact with each other’s pathways.

**Design.**—We examined airway responsiveness, cysteinyl LTs (Cys-LTs), and Cys-LT and IL-13 receptor transcript levels in wild-type mice and in mice that were deficient in γ-glutamyl leukotrienes (an enzyme that converts LTC₄ to LTD₄), STAT6 (signal transducer and activator of transcription 6 [a critical molecule in IL-13 signaling]), and IL-4Rxα (a subunit of the IL-13 receptor).

**Results.**—Wild-type (C57BL/129SvEv) and γ-glutamyl leukotrienes–deficient mice showed increased airway responsiveness after intranasal instillation of IL-13; similar results were observed after intranasal instillation of IL-13 or LTC₄ in a second wild-type strain (BALB/c). Interleukin 13 treatment reduced levels of Cys-LTs in bronchoalveolar lavage fluid. This change was unaccompanied by changes in other arachidonic acid metabolites or in RNA transcript levels of enzymes associated with Cys-LT synthesis. Interleukin 13 treatment also increased transcript levels of the Cys-LT 1 and Cys-LT 2 receptors, while LTC₄ increased transcript levels of the α chain of the IL-13 receptor. Furthermore, IL-4Rxα–deficient mice had increased airway responsiveness to LTC₄ but not to IL-13, whereas STAT6-deficient mice failed to respond to either agonist.

**Conclusions.**—These findings indicate that LTC₄ and IL-13 are dependent on or signal through STAT6 to increase airway responsiveness and that both agonists regulate expression of each other’s receptors.

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Asthma is a chronic inflammatory disorder of the airway in which T-helper 2 cytokines function as effectors of inflammation, remodeling, and airway responsiveness (AR). Interleukin 4 (IL-4) and IL-13 are T-helper 2 cytokines that regulate important aspects of the experimental asthma phenotype, including airway eosinophilia, mucus hypersecretion, and AR. Although IL-4 is important in the early phase of T-helper 2 cytokine inflammation, it has been shown to be unnecessary for the generation of the asthmatic phenotype. However, IL-13 is required and is sufficient to produce inflammation, mucus, and hyperresponsiveness in the airways. Interleukin 13 can be produced by a variety of cells, including CD4⁺ T cells, eosinophils, macrophages, and mast cells. The effects of IL-13 are mediated by its binding to a heteromeric receptor made up of IL-4Rxα and either IL-13Rxα1 or IL-13Rxα2. On binding, IL-13 transduces its signal predominantly by activating the signal transducer and activator of transcription 6 (STAT6) signaling pathway. STAT6 is a transcription factor that exists as latent cytoplasmic monomers until cytokine stimulation, which leads to phosphorylation by JAK1 or JAK3, dimerization, and translocation to the nucleus. Once in the nucleus, STAT6 binds certain DNA elements and initiates cytokine-specific gene transcription. The IL-13 and STAT6 signaling pathway appears to be a major and central mediator of allergic disease, but it is unclear how this pathway interacts with other allergic effector molecules such as leukotrienes (LTs).

Leukotriene C₄, LTD₄, and LTE₄ are products of the lipoxygenase pathway of arachidonic acid metabolism. Because these LTs contain a cysteine residue, they are designated cysteinyl LTs (Cys-LTs). Cysteinyl LTs have diverse biologic effects and are potent proinflammatory mediators that contribute to pathophysiologic features of asthma and other inflammatory diseases. They are produced largely by eosinophils, macrophages, and mast cells and, like IL-13, are implicated in the regulation of airway smooth muscle contraction, edema, and mucus hypersecretion during allergic inflammation. Cysteinyl LTs produce their effects by interacting with 2 known Cys-LT receptors, Cys-LT 1 and Cys-LT 2. These receptors are coupled to heterotrimeric G proteins with a Gₛ subunit that increases intracytoplasmic calcium fluxing through the phospholipase C and inositol triphosphate pathways. Each receptor has unique affinities for each Cys-LT and unique tissue distribution, which gives rise to the diverse biologic effects of LTs. Despite these observations, there has been little.

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investigation of possible cross talk between Cys-LT signaling pathways and other signaling pathways.

Asthma is a complex inflammatory disease that involves the interactions of several different cell types and numerous chemical mediators. Cross talk between LT and IL-13 signaling pathways may be involved in the pathogenesis of asthma. To examine possible Cys-LT and IL-13 interactions, we used mice that were deficient in γ-glutamyl leukotrienease (GGL) (which results in systemic elevation of Cys-LTs), IL-4Ra (which is required in IL-13 receptor signaling), or STAT6 (which is essential in IL-13 signal transduction). Our data demonstrate complex cross-regulatory interactions between these 2 pathways and a crucial role for the STAT6 receptor in LTC4 function.

MATERIALS AND METHODS

Transgenic Mice

Mice that were deficient in GGL were generated in our laboratory as previously described.22,23 Wild-type (WT or GGL+) and GGL-deficient mice were of a C57BL/129SvEv mixed background, and genotypes were determined by Southern blot or polymerase chain reaction (PCR) analysis of genomic DNA obtained from tails at weaning. Wild-type mice of a BALB/c background were obtained from the Jackson Laboratory (Bar Harbor, Me). IL-4 and IL-13 receptor-deficient and STAT6-deficient mice also of BALB/c background were obtained from one of the authors (D.B.C.). All mice were housed in cages equipped with microisolator lids and were maintained under strict containment protocols.

Intranasal Treatment of Mice

Eight- to 12-week-old naïve WT and GGL-deficient mice were treated by intranasal instillation (2 µL/g) of 0.9% sterile saline (vehicle) or recombinant IL-13 (0.35 µg) in the same vehicle. Eight- to 12-week-old naïve WT, IL-4Ra-deficient, and STAT6-deficient mice were treated by intranasal instillation (2 mL/g) of 0.9% sterile saline and 10% alcohol (ETOH), LTC4 (1 µg), or IL-13 (0.116 µg) and 10% ETOH. Sensitization was performed at room temperature after sedation with isoflurane; respiration rate was monitored using the volumetric method in a closed chamber plethysmograph as previously described.24 Lung resistance was monitored using the SmartCycler 2 quantitative reverse transcription–PCR machine (Cepheid, Sunnyvale, Calif) according to manufacturer’s directions. Data were analyzed using SmartCycler software. The presence of amplified products was confirmed when the fluorescent signal exceeded an automatic noise-based defined threshold. To generate a standard curve, PCR amplification was performed with template dilutions for each transcript, ranging from 100 pg per reaction to 0.001 pg per reaction. A standard curve was created by the SmartCycler program according to the transcript’s concentration versus the cycle number at which it crosses the threshold. The final data were normalized to β-actin and are presented as picograms of transcript per picograms of β-actin × 100. Results are expressed as mean ± SEM.

Statistical Analysis

The significance of differences between experimental groups was analyzed using Student t tests. Differences in mean values were considered significant at P < .05.

RESULTS

Effect of Recombinant IL-13 on AR and Cys-LT Production in WT or GGL-Deficient Mice

Wild-type (GGL+) and GGL-deficient mice were treated with 0.35 µg of IL-13 or with 0.9% sterile saline (vehicle) twice a day for 3 days, and AR was measured 18 to 20 hours after the last treatment (Figure 1, A). After administration of vehicle, GGL-deficient mice, which have constitutively elevated levels of LTC4, show greater AR than GGL+ mice as measured by a decrease in acetylcholine; this confirms an earlier finding in our laboratory.25 We also found that treatment of GGL+ and GGL-deficient mice with IL-13 resulted in greater decreases in acetylcholine compared with vehicle treatment. We examined Cys-LT levels in lung supernatant and in cell-free BALF (Figure 1, B and C). As expected, we observed a significant increase in Cys-LTs in GGL-deficient mice compared with GGL+ mice after vehicle treatment. This increase results from LTC4 accumulation due to the absence of GGL. However, we observed a significant decrease in Cys-LTs in both experimental groups after IL-13 challenge. Therefore, IL-
13 negatively regulates Cys-LT levels in BALF and lung tissue, despite its effect on AR.

Examination of Lung Inflammation and Mucus in GGL* and GGL-Deficient Mice After Intranasal Recombinant IL-13 Treatment

We also examined inflammatory cell recruitment to the airways of GGL* and GGL-deficient mice in response to IL-13. Total bronchoalveolar lavage inflammatory cells were increased to a similar degree after IL-13 challenge of GGL* and GGL-deficient mice (Figure 2, A). Furthermore, the total number of eosinophils, macrophages, neutrophils, and lymphocytes recruited to the airways after IL-13 treatment did not vary with mouse genotype (Figure 2, B). We evaluated lung sections stained with periodic acid–Schiff to detect mucus-containing cells (goblet cells). This analysis showed similar increases in periodic acid–Schiff–positive airway cells in IL-13-treated GGL* and GGL-deficient mice compared with the control group. Therefore, GGL deficiency did not alter the ability of IL-13 to induce airway inflammation and goblet cell metaplasia.

Role of Cyclooxygenase Pathway and Histamine in AR After IL-13 Treatment

To evaluate the mechanism of IL-13–induced decreases in airway Cys-LT levels, we examined the possibility that IL-13 might shunt intermediates in the Cys-LT synthesis pathway away from Cys-LT synthesis and toward PG and thromboxane synthesis pathways. When levels of PGF_2α, PGE_2, PGD_2, and thromboxane B_2 were measured after IL-13 treatment, we found no changes (Figure 3, A through D). These findings suggest that shunting of arachidonic acid metabolic intermediates to alternate synthetic pathways is unlikely to explain the IL-13–dependent decrease in airway Cys-LT levels. Although histamine is not strongly correlated with allergic responses in murine models, we evaluated histamine levels after IL-13 treatment and found no changes (data not shown).

Effect of IL-13 on LTA₄ Hydrolase and LTC₄ Synthase, Arachidonate 5-Lipoxygenase–Activating Protein RNA in Mouse Lung

We further considered the possibility that IL-13 diminished expression of enzymes related to the synthesis of Cys-LTs. To determine this, we examined RNA expression of enzymes that convert LTA₄ to LTB₄ (LTA₄ hydrolase) and LTA₄ to LTC₄ (LTC₄ synthase), as well as a protein that functions with 5-lipoxygenase to synthesize LTs (arachidonate 5-lipoxygenase–activating protein using quantitative reverse transcription–PCR analysis). We found that with our standard protocol (3 days of IL-13 treatment), RNA levels of these enzymes were unchanged (Figure 4, A through C), suggesting that decreases in Cys-LT levels after IL-13 treatment are not the result of altered transcription of other enzymes in the Cys-LT production pathway.
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Figure 3. Profile of prostaglandins (PG) and thromboxane B2 (TXB2) in bronchoalveolar lavage fluid (BALF) samples from mice that were interleukin 13 (IL-13) sensitized. Levels of PGF₂₅ (A), PGE₂ (B), PGD₂ (C), or TXB₂ (D) were analyzed by enzyme-linked immunosorbent assay in BALF from wild-type (GGL⁺/⁻) and γ-glutamyl leukotrienease (GGL⁻/⁻) mice. n = 5–10. Values are mean ± SEM.

Increases in AR to LTC₄ and IL-13 Are Additive and Dependent on STAT6 Signaling

BALB/c mice treated with IL-13 (0.116 mg) twice a day for 3 days exhibited a significant increase in AR compared with the control group (Figure 5, A). This finding is similar to results with C57BL/129SvEv mice (Figure 1, A). A similar increase in AR was observed when BALB/c mice were treated with 0.5 μg of LTC₄ twice a day for 3 days. When mice were treated with LTC₄ and IL-13 together, a greater increase in AR was observed than that produced by either agent alone, demonstrating an additive effect of LTC₄ and IL-13. To examine a possible role for IL-13 signaling in LTC₄–induced AR, IL-4R⁻/⁻ deficient mice (of a BALB/c background) were challenged with 1 μg of LTC₄ or 0.0116 mg of IL-13 (Figure 5, B). As expected, IL-4R⁻/⁻ deficient mice failed to respond to IL-13, confirming the previous observation that IL-13 uses this receptor to promote AR. However, LTC₄ treatment of IL-4R⁻/⁻ deficient mice resulted in a significant increase in AR to acetylcholine compared with the control group. To determine if LTC₄–induced AR was STAT6 dependent, STAT6-deficient mice were also treated with LTC₄ or IL-13 (Figure 5, C). In contrast to IL-4R⁻/⁻ deficient mice, STAT6-deficient mice failed to respond to LTC₄ or IL-13. This finding demonstrates that LTC₄ and IL-13–induced AR requires STAT6. Cys-LTs were also measured in the BALF of these mice (Figure 6); our data show that the decrease in Cys-LT after IL-13 treatment is also dependent on STAT6.

STAT6 Signaling Is Involved in LTC₄- and IL-13–Induced Lung Inflammation

Bronchoalveolar lavage fluid was collected from BALB/c and STAT6-deficient mice treated with LTC₄ or IL-13 to determine the degree of airway inflammation induced by these agonists. Similar to experiments in mixed background mice, IL-13 increased the total cells entering the airways of WT mice (Figure 7, A), whereas STAT6-deficient mice showed no airway inflammation. Similarly, BALB/c mice, but not STAT6-deficient mice, treated with exogenous LTC₄ showed a significant increase in total airway cells relative to control mice. Interleukin 13 and LTC₄ enhanced the total numbers of macrophages, lymphocytes, eosinophils, and neutrophils obtained from BALF of WT mice (Figure 7, B). Airway lymphocytes, eosinophils, and neutrophils were also increased in STAT6-deficient mice challenged with LTC₄ and IL-13 but to a far less degree than in BALB/c mice. These data strengthen the argument that LTC₄ actions in AR and pulmonary inflammation are dependent on the STAT6 signaling pathway.

STAT6 Modulates Cys-LT and IL-13 Receptor Expression

We also examined the RNA levels of LTC₄-treated or IL-13–treated BALB/c and STAT6-deficient mice. In WT mice
Figure 5. Airway reactivity to acetylcholine in response to interleukin 13 (IL-13) or leukotriene C₄ (LTC₄) in BALB/c mice (A), IL-4 receptor α-deficient (IL-4Rα⁻/⁻) mice (B), or signal transducer and activator of transcription 6 (STAT6)–deficient mice (C). A, Effect of IL-13 and LTC₄ treatment on airway reactivity to ACh in BALB/c wild-type mice. IL-13 + alcohol (ETOH) versus vehicle, *P < .01. LTC₄ versus vehicle, †P < .01. IL-13 + LTC₄ versus 0.9% sterile saline + ETOH, **P < .01. n = 8–10. Values are mean ± SEM. Evaluation of the airway reactivity as PC₂₀₀ to ACh in (B) IL-4Rα⁻/⁻–deficient mice or (C) STAT6-deficient mice treated with IL-13 or LTC₄. Vehicle versus IL-13 + ETOH treated wild-type mice, *P < .05, and wild-type versus STAT6-deficient mice treated with IL-13, **P < .05. n = 10. Values are mean ± SEM.

(BALB/c), the number of Cys-LT receptor 1 transcripts is increased by LTC₄ or IL-13 treatment (Figure 8, A). Mice that are STAT6 deficient show a constitutively higher level of Cys-LT receptor 1 transcripts than WT mice, but administration of LTC₄ or IL-13 does not further increase this value. Transcript levels of Cys-LT receptor 2 in WT mice were unresponsive to LTC₄ but showed an increase after IL-13 treatment (Figure 8, B). However, STAT6-deficient mice again showed higher basal levels of the receptor

Figure 6. Analyses of endogenous cysteinyl leukotriene (cys-LT) levels (LTC₄, LTD₄, and LTE₄) were evaluated in bronchoalveolar lavage fluid samples in BALB/c or signal transducer and activator of transcription 6 (STAT6)–deficient mice treated with 0.9% sterile saline + alcohol (ETOH), LTC₄ or interleukin 13 (IL-13) + ETOH. Vehicle versus IL-13 + ETOH treated wild-type mice, *P < .05, and wild-type versus STAT6-deficient mice treated with IL-13, **P < .05. n = 10. Values are mean ± SEM.

Figure 7. Pulmonary inflammation in response to leukotriene C₄ (LTC₄) and interleukin 13 (IL-13). Changes in total cells (A) and in profiles of cells (B) recovered in bronchoalveolar lavage fluid from BALB/c or signal transducer and activator of transcription 6 (STAT6)–deficient mice treated with 0.9% sterile saline, LTC₄ or IL-13 for 3 days, twice a day. Wild-type (WT) vehicle versus WT LTC₄, or IL-13, *P < .05. Wild-type versus STAT6-deficient, †P < .05. n = 6–7. Values are mean ± SEM.
C4 increased transcript levels of IL-13Rα1 and IL-4Rα. Results are expressed as the ratio of each transcript/β-actin. Wild-type (WT) vehicle versus WT LTC4 or IL-13, * P < .05; WT versus signal transducer and activator of transcription 6 (STAT6)–deficient, † P < .05. Values are mean ± SEM.

Figure 8. Examination of normalized cysteinyl leukotriene (Cys-LT) receptor transcripts in the lung after LTC4 or interleukin 13 (IL-13) administration. Total messenger RNA was isolated from the lung tissue and reverse transcribed to complementary DNA (cDNA). The cDNA was amplified by specific primers for (A) Cys-LT receptor 1 and (B) Cys-LT receptor 2. Results are expressed as the ratio of each transcript/β-actin × 100 (% β-actin). Wild-type (WT) vehicle versus WT LTC4 or IL-13.

Figure 9. Analysis of normalized interleukin 13 receptor α1 (IL-13Ra1) transcripts in the lung after leukotriene C4 (LTC4) or IL-13 treatment. Total messenger RNA was isolated from the lung tissue and reverse transcribed to complementary DNA (cDNA). The cDNA was amplified by specific primers for IL-13Ra. Results are expressed as the ratio of each transcript/β-actin × 100 (% β-actin). Vehicle versus LTC4 or IL-13 for individual mouse strains, * P < .05. Values are mean ± SEM.

Recently, other investigators observed a connection between LTs and IL-13. Vargaftig and Singer33 showed that intratracheal instillation of IL-13 caused an increase in Cys-LTs in the BALF of BP2 mice as soon as 15 minutes after a single installation and persisted for 72 hours. This result is different from ours and may reflect differences in the mouse strains used or in the experimental design. We used BALB/c and C57BL/129SvEv mice, treated them twice a day for 3 days by intranasal instillation, and made our measurements 18 to 20 hours later. In our studies, we found that treatment with IL-13 caused a decrease in Cys-LTs in BALF. Vargaftig and Singer used a dose of 4.0 μg, while our dose was less than 10% of this amount (0.035 μg). Interactions between the LT and IL-13 pathways are complex and will require further study.

We also demonstrated that IL-13 caused an increase in the Cys-LT receptors 1 and 2 RNA in WT mice (Figure 8). The relationship of this finding to our data about IL-13–induced decreases on LTs is unclear, but other investigators have found increases in the Cys-LT receptor 1 in fibroblast and macrophage cell lines.34,35 These studies suggest that IL-13 can affect LT signaling by modulating the levels of Cys-LT or Cys-LT receptors.

Our study demonstrates that LTC4 induces AR and lung inflammation through an IL-4– and IL-13–independent but a STAT6-dependent pathway. This finding can be explained in 2 ways. First, there may be an as yet unrecognized Cys-LT receptor that signals through STAT6. Second, because Cys-LT receptors 1 and 2 are known to be Gαq–coupled receptors,19 another possibility is that somehow Gαq activation activates the STAT6 pathway. There is increasing evidence for both of these possibilities. Since the discovery of Cys-LT receptor agonists and antagonists, there has been controversy about the inability to produce or inhibit specific responses. These experiments led to the idea that there must be other receptors that bind Cys-LTs.19,21,26 Although there is no direct evidence of a Gαq–coupled receptor activating STAT6, there are several studies showing that G protein–coupled receptors activate other STAT molecules.37–41 This underscores the need for fur-
ther studies examining the complex interaction between these signaling pathways.

In summary, we demonstrated that intranasal instillation of IL-13 causes decreases in Cys-LT production but an increase in Cys-LT receptors 1 and 2 RNA. Furthermore, the changes in Cys-LT production are not accompanied by an increase in other arachidonic acid by-products or by a change in RNA levels for Cys-LT-associated enzymes. In addition, endogenously and exogenously elevated levels of LTC4 caused an increase in AR. This AR by LTC4 is dependent on STAT6 activation. Conversely, the ability of LTC4 to increase the RNA level of IL-13R α3 was not dependent on STAT6. These findings suggest that there is significant regulation of LTC4 and IL-13 signaling pathways by LTC4 or by IL-13. A better understanding of these effects and of the cross-regulation of these pathways could provide the basis for new treatments for asthma.

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References