IL-13 and IL-4 promote TARC release in human airway smooth muscle cells: role of IL-4 receptor genotype

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Submitted 17 April 2003; accepted in final form 14 July 2003

Faffe, Débora S., Timothy Whitehead, Paul E. Moore, Simonetta Baraldo, Lesley Flynt, Kerri Bourgeois, Reynold A. Panettieri, and Stephanie A. Shore. IL-13 and IL-4 promote TARC release in human airway smooth muscle cells: role of IL-4 receptor genotype. Am J Physiol Lung Cell Mol Physiol 285: L907–L914, 2003. First published July 18, 2003; 10.1152/ajplung.00120.2003.—The chemokine thymus- and activation-regulated chemokine (TARC) induces selective migration of Th2, but not Th1, lymphocytes and is upregulated in the airways of asthmatic patients. The purpose of this study was to determine whether human airway smooth muscle (HASM) cells produce TARC. Neither IL-4, IL-13, IL-1β, IFN-γ, nor TNF-α alone stimulated TARC release into the supernatant of cultured HASM cells. However, both IL-4 and IL-13 increased TARC protein and mRNA expression when administered in combination with TNF-α but not IL-1β or IFN-γ. Macrophage-derived chemokine was not expressed under any of these conditions. TARC release induced by TNF-α + IL-13 or TNF-α + IL-4 was inhibited by the β-agonist isoproterenol and by other agents that activate protein kinase A, but not by dexamethasone. To determine whether polymorphisms of the IL-4Rα have an impact on the ability of IL-13 or IL-4 to induce TARC release, HASM cells from multiple donors were genotyped for the Ile50Val, Ser478Pro, and Gln551Arg polymorphisms of the IL-4Rα. Our data indicate that cells expressing the Val50/Pro478/Arg551 haplotype had significantly greater IL-13- or IL-4-induced TARC release than cells with other IL-4Rα genotypes. These data indicate that Th2 cytokines enhance TARC release of TARC and MDC. IL-4 and/or IL-13, especially when administered in conjunction with TNF-α, increase TARC production in bronchial epithelial and nasal mucosal cells (4, 12, 16, 38, 41). In addition, transgenic overexpression of IL-13 in the lungs causes increased expression of TARC and MDC expression (44). Current evidence suggests that human airway smooth muscle (HASM) cells may orchestrate and perpetuate airway inflammation in asthma. Airway smooth muscle cells express numerous cytokines and chemokines and respond to a variety of cytokines, including IL-4 and IL-13, with changes in contractile function as well as changes in the release of chemokines (10, 18, 25). However, there are no reports on the ability of HASM cells to express TARC or MDC.

In HASM cells, IL-4 and IL-13 both act through the type II IL-4 receptor (R), a dimer consisting of the IL-4Rα and the IL-13Rα1 (10, 18). There are eight relatively common single-nucleotide polymorphisms (SNPs) in the IL-4Rα that result in amino acid substitutions in the coding region of the receptor (32). One SNP, an Ile-to-Val substitution at amino acid 50 in the extracellular domain of the receptor, is quite common (~50% of alleles) and occurs independently of the other polymorphisms. The other seven SNPs are in the in...
tracellular domain of the receptor, are in linkage disequilibrium, and occur with allele frequencies of 10–20%, except for the Ser727Ala and Ser761Pro SNPs, which are rare. Of these cytoplasmic SNPs, Ser478Pro and Gln551Arg are the most common. Note that two different conventions have been used for numbering amino acids in IL-4Rα, beginning either from the start of the signal peptide or from the start of the mature protein. We are using the latter method. The Ile60Val, Ser478Pro, and Gln551Arg variants of the IL-4Rα have been associated with atopy, asthma, and total serum IgE levels, although the results vary depending on the population studied and whether only single SNPs or haplotypes are considered (5, 8, 9, 11, 15, 23, 24, 30–32, 42). These SNPs have also been shown to affect responses to IL-4 in hematopoietic cells (9, 15, 24, 37, 42).

The purpose of this study was to determine whether HASM cells have the capacity to express TARC and MDC in response to IL-13 and IL-4 and whether polymorphisms of the IL-4Rα have an impact on these responses. To that end, we measured IL-4- and IL-13-induced release of TARC into supernatants of HASM cells by ELISA. We studied cells from multiple donors and segregated the results by genotype. We also examined the effect of β-agonists and corticosteroids on TARC expression, since these agents are used in the treatment of asthma. Our results indicate that both IL-13 and IL-4 cause release of TARC, but not MDC, but do so only in the presence of TNF-α. Furthermore, β-agonists are effective in inhibiting TARC release. Our results also indicate that compared with wild-type cells, HASM cells from donors with the Val50/Pro478/Arg551 haplotype of the IL-4Rα have an increased ability to express TARC in response to either IL-4 or IL-13, whereas the presence of Val50 without Pro478/Arg551 or the presence of Pro478/Arg551 without Val50 has no effect. To our knowledge, this is the first report of the impact of IL-4Rα genotype on responses to IL-13 in any cell type. It is also the first report of the impact of IL-4Rα genotype on responses to IL-4 in any nonhematopoietic cell.

METHODS

Cell culture. Human tracheas were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was dissected under sterile conditions, and smooth muscle cells from the trachealis were isolated and cultured as previously described (18, 25, 33). Medium was replaced every 3–4 days. Cells were passaged with 0.25% trypsin and 1 mM EDTA every 14–21 days. Cells from 21 donors in passages 4–8 were used in the studies described below.

TARC and MDC release. Two weeks after passage, cells were serum deprived and supplemented with 5.7 μg/ml insulin and 5 μg/ml transferrin. After 48 h, cells were treated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), IFN-γ (10–100 ng/ml), IL-4 (0.3–30 ng/ml), IL-13 (1–50 ng/ml), or combinations of these cytokines. The supernatant was removed 24 h later and stored at −80°C. TARC; MDC; and regulated on activation, normal T cell expressed, and presumably secreted (RANTES) release were analyzed by ELISA (R&D Systems, Minneapolis, MN), following the manufacturer’s instructions. To examine the effect of isoproterenol (0.1–10 μM), forskolin (10 μM), or dibutyryl (db) cAMP (1 mM) on TARC release, we pretreated these agents at the same time as cytokines. To examine the effect of dexamethasone (1 μM), we treated HASM cells with this agent for 1 h before cytokine treatment.

RT-PCR. Total cellular RNA was extracted with the Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA). Total cellular RNA (1 μg) was reverse transcribed with the Advantage RT for PCR kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. cDNA was amplified by PCR using the following TARC-specific primers: sense 5′-CACGCAAGCTTGAGGACCAATGTT-3′, anti-sense 5′-TCAGACCTCTCAAGGCTTTG CAGG-3′. PCR reactions were carried out with puReTaq Ready-To-Go PCR Beads (Amersharm Biosciences, Piscataway, NJ) with 2 μg of cDNA in a total volume of 25 μl. The primers yielded a PCR product of 221 bp. RNA and cDNA integrity were confirmed by PCR using control GAPDH primers (BD Biosciences). PCR products were separated on a 1% agarose gel stained with ethidium bromide and photographed under UV illumination.

Genotyping. Genomic DNA was extracted from HASM cells using DNA spin columns (DNeasy; Qiagen, Valencia, CA) according to the manufacturer’s specifications. Genomic DNA was amplified by PCR using two different oligonucleotide primer pairs: Ile60Val, 5′-TCATGTTGTAAGGAGCTGTGG (forward) and 5′-TCAGCCCGAGCAGTGGT (reverse); intracellular domain (Glu375Ala, Cys346Arg, Ser411Leu, Ser478Pro, and Gln551Arg), 5′-GAGGACAGCAGGAT-GACTTT (forward) and 5′-TCCAGGATGACAAACTC (reverse). For PCR, each 50 μl of reaction mixture contained 200 μM dNTP, 20 μM of each primer, 5 μl of 10× PCR buffer, 1.5 mM MgCl2, 250 ng DNA, 2 units Taq polymerase (Perkin-Elmer, Foster City, CA), and 31.6 μl H2O. Taq was added to the mixture after a 3-min hot start at 94°C. PCR conditions were as follows: 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min by 35 cycles, followed by a 7-min extension at 72°C. PCR products were purified by use of QIAquick PCR Purification Kit (Qiagen). Analysis of the polymorphism at position 50 (Ile60Val) was performed either by direct sequencing or by restriction fragment length polymorphism as follows: 15 μl of the PCR product were incubated with 3 μl of restriction enzyme BsmFI (New England BioLabs, Beverly, MA), 3 μl of the recommended buffer, 3 μl of BSA, and 6 μl of water for 3 h at 65°C. The fragments were resolved on a 1% agarose gel and stained with ethidium bromide. The wild type was characterized by two fragments (292 and 135 bp), the homozygous mutant as Pro478/Arg551 alleles are in very strong linkage disequilibrium (32), and the heterozygous by four fragments (292, 209, 135, and 83 bp). Determination of the polymorphisms in the intracellular domain was performed by sequencing.

The frequency of alleles in our bank of HASM cell donors was as follows: Val50 0.49, Ala375 0.10, Arg406 0.14, Leu411 0.07, Pro478 0.23, and Arg551 0.22, and was consistent with the literature (32). From this cell bank, cells from 21 donors with relevant genotypes at amino acids 50, 478, and 551 were selected and analyzed for their ability to produce TARC in response to TNF-α plus IL-13 or IL-4. Table 1 lists the precise genotypes of the cells we examined. Because the Pro478 and Arg551 alleles are in very strong linkage disequilibrium (32), all cell donors chosen for assay that had at least one Pro478 allele also had at least one Arg551 allele, and these cells were designated as Pro478/Arg551.

Statistics. Data were analyzed by t-test, paired t-test, or ANOVA as appropriate. TARC measurements in isoproterenol-
Table 1. IL-4Rα genotypes at amino acids 50 and 478/551 of HASM cell donors used

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IL-4Rα, interleukin 4 receptor-α; Wt, wild type; He, heterozygous; Mu, mutant; aa, amino acid. *One of these donors was heterozygous at 478, but mutant at 551.

... of TARC than cells with any other genotype. In contrast, there was no significant difference in TARC release between wild-type cells and cells with at least one Val50 allele that were wild type in the cytoplasmic domain of the IL-4Rα. Similarly, there was no significant difference in TARC release between wild-type cells and cells with at least one Pro478/Arg551 allele that were wild type at amino acid 50. Note that because we did not use any cells from donors who were both heterozygous at amino acid 50 and heterozygous at amino acids 478/551 (Table 1), all donors who had at least one Val50 allele and one Pro478/Arg551 allele had at least one Val50/Pro478/Arg551 allele. Therefore, the results suggest that the Val50/Pro478/Arg551 haplotype is associated with increased IL-4- or IL-13-stimulated TARC release in HASM cells.

We also sought to determine whether β-agonists or glucocorticoids, therapies used for the treatment of asthma, had any impact on TARC release from HASM cells. When administered at the same time as cytokines, the β-agonist isoproterenol caused a concentration-related decrease in TARC release induced by either TNF + IL-13 or TNF + IL-4 (Fig. 5). Forskolin, which directly activates adenylyl cyclase to produce cAMP, and db cAMP, a cell-permeable agonist of cAMP, virtually abolished TARC release. Similar effects of β-agonists were obtained for TARC mRNA (data not shown). In contrast, the corticosteroid dexamethasone was without effect on TARC release. In cells treated with dexamethasone (10 μM) 1 h before TNF-α (10 ng/ml) and IL-13 (30 ng/ml), TARC release averaged 73 ± 18% of the TARC released measured in cells treated with vehicle (not significant). Similarly, in cells treated with dexamethasone (10 μM) 1 h before TNF-α (10 ng/ml) and IL-4 (3 ng/ml), TARC release averaged 99 ± 12% of the TARC released measured in cells treated with vehicle. Results were obtained in cells from five donors, each studied in duplicate on one to two occasions. Dexamethasone still failed to inhibit TARC release in cells treated with TNF-α (10 ng/ml) in...
conjunction with lower concentrations of IL-13 (10 ng/ml) or IL-4 (1 ng/ml).

In contrast to TNF-α/H9251, IL-1β/H9252 did not result in significant release of TARC either alone or in IL-13- or IL-4-treated cells, even though in the same supernatants IL-1β did cause RANTES release, as reported by others (10), confirming the efficacy of the IL-1β in these cells. We used cells from the same donors for experiments examining TNF-α or IL-1β effects on TARC release, so the lack of effect of IL-1β is not related to differences in IL-4Rα genotype of the cells used in these experiments. IFN-γ either alone or in combination with TNF-α or TNF-α + IL-4 also had no effect on TARC release, even though IFN-γ did cause a concentration-related increase in the ability of TNF-α or TNF-α + IL-4 to induce RANTES release, as reported by others (13).

Given the effects on TARC expression of agents that activate cAMP (Fig. 5), we considered the possibility that the inability of IL-1β to induce TARC expression, even when administered with IL-13 or IL-4, might be the result of the ability of IL-1β to induce cyclooxygenase (COX)-2 expression and to augment PGE2 in these cells. PGE2 causes a very robust increase in cAMP in HASM cells (40), which could inhibit TARC release in IL-1β-treated cells. In contrast, TNF-α does not induce COX-2 expression in HASM cells (26, 34). To examine this possibility, we treated HASM cells with indomethacin (10^{-6} M) before cytokine treatment. We have previously reported that at this concentration, indomethacin virtually abolishes PGE2 release from IL-1β-treated cells (17). IL-1β given in conjunction with IL-13 still failed to increase TARC release (Fig. 6) even after treatment with indomethacin, indicating that differences in their ability to activate COX-2 do not contribute to the differential effects of TNF-α vs. IL-1β on TARC release. Interestingly, indomethacin caused a significant (P < 0.05) increase in TARC release in TNF-α + IL-13-treated cells, which do not express COX-2. We have previously reported that indomethacin decreases basal cAMP formation even in the absence of COX-2, presumably as a result of effects on COX-1-mediated PGE2 release (19). Thus the effect of indomethacin on TARC release in TNF-α + IL-13-stimulated cells may be due to inhibition of basal cAMP levels.

We also examined the impact of IL-13 and IL-4 either alone or in conjunction with TNF-α to induce release of another chemokine that induces migration of Th2 cells, MDC. In contrast to TARC, MDC could not be detected under any of these conditions or in cells bearing any IL-4Rα genotype.

DISCUSSION
The novel findings of this study are that HASM cells express TARC at both the mRNA and protein levels, that polymorphisms in the IL-4Rα have a profound effect on IL-13- and IL-4-induced TARC release, and that agents that activate protein kinase A (PKA) inhibit TARC release. Because IL-4 or IL-13 is required for TARC release and since TARC is a chemotactic factor for the Th2 cells that express IL-4 and IL-13, these results suggest that airway smooth muscle par-
ticipates in a positive feedback loop that promotes the recruitment of Th2 cells to the airways.

Other cellular sources of TARC include monocytes, dendritic cells, fibroblasts, bronchial, and nasal epithelial cells (4, 12, 38). The concentration ranges over which IL-4 and IL-13 induce their effects on TARC release in HASM cells are similar to the concentrations that are effective in bronchial epithelial cells (38) and similar to concentrations that are required for eotaxin production by HASM cells (10, 25). For similar types of stimulation, the concentration of TARC in the supernatant of confluent bronchial epithelial cells (38) is greater than for HASM cells. Nevertheless, it is likely that production of TARC by smooth muscle contributes to the recruitment of lymphocytes towards these cells.

HASM cells produced TARC only when stimulated with IL-4 or IL-13 in combination with TNF-α (Fig. 1). Similar results were obtained for TARC mRNA (Fig. 2). The essential role of the Th2-derived cytokines IL-4 and IL-13 for TARC production has also been observed in monocytes, dendritic cells, corneal, and dermal fibroblasts (6, 12). TNF-α, IL-1β, and IFN-γ on their own did not evoke TARC release, and IL-13 and IL-4 were effective only in combination with TNF-α and not when combined with IFN-γ or IL-1β. In contrast to HASM cells, bronchial epithelial cells produce TARC protein and mRNA even when stimulated with TNF-α or IL-1β alone (4), and IFN-γ enhances production of TARC induced by TNF-α or IL-1β in these cells (4, 38). Together, the results suggest that the regulation of TARC expression is highly cell type dependent. The cell specificity of regulation of TARC release is further emphasized by the observation that corneal and dermal fibroblasts express TARC in response to TNF-α and IL-13 or IL-4, whereas lung fibroblasts do not (6).

We do not know why TNF-α but not IL-1β enhances TARC release in HASM cells. The lack of effect of IL-1β is not due to lack of efficacy, since in the same cells, IL-1β enhanced RANTES secretion. The lack of effect of IL-1β is also not related to the genotype of the cell donors used, since cells from the same donors were...
used in experiments with TNF-α and IL-1β. We considered the possibility that IL-1β-induced PGE_2 release was inhibiting the production of TARC, but that was not the case (Fig. 6). In bronchial epithelial cells, NF-κB activation is required for TNF-α-induced TARC release (4). Both TNF-α and IL-1β are effective in activating NF-κB in HASM cells (2, 27), although it is possible that there are differences in the time course of activation of NF-κB that might explain differences in the efficacy of these cytokines with respect to TARC expression. Aside from the importance of NF-κB, virtually nothing is known about the regulation of TARC in any cell type, although it is likely that STAT6 plays an important role, given the dependence of TARC release on IL-4 and IL-13 (Fig. 1).

Our results also indicate that the β-agonist isoproterenol causes a concentration-dependent decrease in IL-4- and IL-13-induced TARC release in HASM cells (Fig. 6), as well as a decrease in TARC mRNA expression. Db cAMP, a cell-permeant analog of cAMP, and forskolin, which directly activates adenyl cyclase to produce cAMP, had similar effects. The latter were administered at concentrations that are maximally effective in terms of their ability to relax HASM cells in culture (40). β-Agonists and other agents that activate cAMP have also been shown to inhibit the release of a variety of other cytokines and chemokines from HASM cells (2, 20, 35). The mechanism(s) regulating effects of β-agonists and other agents that activate PKA on TARC expression remains unknown. However, taken in conjunction with the ability of β-agonists to inhibit granulocyte-macrophage colony-stimulating factor, RANTES, and eotaxin release from HASM cells (2, 20, 35), the results add to an increasing body of evidence that indicates the effects of β-agonists on airway smooth muscle are not limited to relaxation, but also include anti-inflammatory actions.

In contrast to the effects of β-agonists, dexamethasone was without effect on the release of TARC from HASM cells, whereas it has been shown to inhibit TARC release in bronchial epithelial cells (38). One factor that could account for the different effects of dexamethasone in these two cell types is its differential effects on NF-κB activation, an event that has been shown to be important for TNF-α induction of TARC (4). Upon binding to glucocorticoids, cytoplasmic glucocorticoid receptors (GRs) can interact with other transcription factors. For example, GRs have been shown to interact with NF-κB in some cell types, including epithelial cells, inhibiting NF-κB activation (1). In contrast, dexamethasone has no effect on binding of nuclear proteins from IL-1β-treated HASM cells to consensus NF-κB-binding sequences (27). Similarly, glucocorticoids do not influence luciferase activity in HASM cells transfected with a luciferase reporter driven by multiple NF-κB elements and stimulated with TNF-α (3).

In HASM cells, both IL-13 and IL-4 act at the type II IL-4R, a dimer composed of the IL-4Ra and the IL-13Rα1 (10, 18). IL-13 and IL-4 signaling occur through ligand-induced dimerization of these receptors, JAK activation, and consequent phosphorylation of both receptors (28). Our results (Fig. 4) demonstrate that genetic variation in the IL-4Ra affects IL-13- and IL-4-induced TARC release from HASM cells. To our knowledge, this is the first report in any cell type indicating functional differences in the response to IL-13 associated with these polymorphisms, although others have reported genotype-related differences in responses to IL-4 (9, 15, 24, 37, 42). It is also the first report of the impact of IL-4Ra polymorphisms on cell function in any nonhematopoietic cell. This is particularly important because hematopoietic cells also express the type I IL-4 receptor, a dimer composed of the IL-4Ra and the common gamma chain of the IL-2 receptor (γc), and it is possible that the impact of IL-4Ra polymorphisms depends on whether the receptor forms dimers with IL-13Rα1 or γc.

Our results suggest that the presence of the Val50/Pro478/Arg551 haplotype is required for maximal TARC induction by IL-4 and IL-13 in HASM cells. Amino acid 478 of the IL-4Ra is within the IL-4R motif that has been shown to be necessary for IL-4-induced STAT6 binding to the receptor (43), whereas amino acid 551 is immediately adjacent to one of the tyrosines that form the STAT6 binding site (9). The 14R motif of the IL-4R is also required for insulin receptor substrate binding and subsequent ERK phosphorylation (29). Although the mechanistic basis for regulation of TARC by IL-13 and IL-4 remains unknown, it is possible that changes in the conformation of the Val50/Pro478/Arg551 variant of the IL-4Ra result in altered STAT6 or ERK phosphorylation. Both STAT6 and ERK have been shown to be important for IL-13- and IL-4-induced release of other chemokines, such as eotaxin (10, 21, 22, 25). However, it is also possible that the Val50/ Pro478/Arg551 allele is in linkage disequilibrium with some other SNP that is affecting IL-4Ra function. For example, a relatively common SNP in the promoter of the IL-4Ra that could impact IL-4Ra expression has been shown to be in strong linkage disequilibrium with the Ile50Val SNP (7).

The Ile50Val, Ser478Pro, and Gln551Arg variants of the IL-4Ra have each been reported to associate with atopy, atopic asthma, and/or total serum IgE levels, although the results of such studies have not been consistent (5, 8, 9, 11, 15, 23, 24, 30–32, 37, 42). One potential explanation for this inconsistency is that in most of these studies, each of the IL-4Ra SNPs was considered independently. Our data suggest that the Val50/Pro478/Arg551 IL-4Ra haplotype is associated with increased IL-4- or IL-13-induced TARC release, whereas neither the Val50 allele nor the Pro478/Arg551 allele on its own is sufficient to confer differences in TARC expression. Similarly, Risma et al. (37) reported that the Val50/Arg551 haplotype is required for maximal induction of CD23 by IL-4 in a murine B cell line transfected with a Val50/Arg551 variant of the IL-4Ra created by site-directed mutagenesis. In that study, as in ours, neither the Val50 nor the Arg551 mutations alone had any effect. Hence it is likely that functional alterations in the IL-4Ra will be observed.
only in the presence of specific IL-4Rx haplotypes. If so, it seems imperative that association studies examining the impact of IL-4Rx SNPs on disease prevalence consider haplotypes and not just single SNPs.

In summary, our results indicate that Th2 cytokines enhance TARC protein and mRNA expression in HASM cells and that the ability of these cells to express TARC is strongly dependent on the IL-4Rx haplotype. Our data suggest that HASM cells participate in a positive feedback loop that promotes the recruitment of Th2 cells into asthmatic airways.

The authors thank Igor Schwartzman, Karry Muzzey, and Matthew McKenna for technical assistance.

DISCLOSURES

This study was supported by National Institutes of Health Grants HL-67664, HL-67663, and ES-00002. D. Faffe was supported by Consejo Nacional de Desenvolvimento Cientifico e Tecnologico, Brazil.

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AJP-Lung Cell Mol Physiol • Vol. 285 • October 2003 • www.ajplung.org


