Respiratory Syncytial Virus Synergizes with Th2 Cytokines to Induce Optimal Levels of TARC/CCL17

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Respiratory syncytial virus (RSV) is a ubiquitous virus that preferentially infects airway epithelial cells, causing asthma exacerbations and severe disease in immunocompromised hosts. Acute RSV infection induces inflammation in the lung. Thymus- and activation-regulated chemokine (TARC) recruits Th2 cells to sites of inflammation. We found that acute RSV infection of BALB/c mice increased TARC production in the lung. Immunization of BALB/c mice with individual RSV proteins can lead to the development of Th1- or Th2-biased T cell responses in the lung after RSV infection. We primed animals with a recombinant vaccinia virus expressing either the RSV fusion (F) protein or the RSV attachment (G) protein, inducing Th1- and Th2-biased pulmonary memory T cell responses, respectively. After RSV infection, TARC production significantly increased in the vaccinia virus G-primed animals only. These data suggest a positive feedback loop for TARC production between RSV infection and Th2 cytokines. RSV-infected lung epithelial cells cultured with IL-4 or IL-13 demonstrated a marked increase in the production of TARC. The synergistic effect of RSV and IL-4/IL-13 on TARC production reflected differential induction of NFκB and STAT6 by the two stimuli (both are in the TARC promoter). These findings demonstrate that RSV induces a chemokine TARC that has the potential to recruit Th2 cells to the lung. The Journal of Immunology, 2007, 179: 1648–1658.

Respiratory syncytial virus (RSV) is a member of the Paramyxoviridae family of viruses (1). It preferentially infects airway epithelium and is responsible for significant pathology in infants, young children, asthmatics, and immunocompromised adults (1–4). Virtually all children become infected with RSV by the age of 2 years. In most cases, the virus remains localized to the nasopharyngeal epithelium and causes only mild disease. However, in a subset of individuals, RSV spreads to the lower respiratory tract, causing a severe acute bronchiolitis. In RSV-induced bronchiolitis, there is a strong inflammatory response mediated by both Th1 and Th2 cells with epithelial sloughing, eosinophilia, hypersecretion of mucus, edema, airflow obstruction, and wheezing (5, 6). Viral clearance and recovery from infection do not lead to prolonged resistance (1).

Asthma is an immune-mediated disease characterized by CD4+ T cells that secrete IL-4, IL-5, and IL-13 (Th2 cells), accumulation of eosinophils, circulating IgE Abs, and airway hyperresponsiveness (7). RSV infection has been linked to asthma and has been shown to cause asthma exacerbations (8–11). Less clear is the intriguing epidemiological link between infants who have severe RSV infections and develop asthma in subsequent years (10, 12–14).

The primary immune response to RSV is characterized by a generalized inflammatory response (15–23). Depending on the time and conditions of infection, both Th1 and Th2 chemokines (small secreted peptides that regulate leukocyte trafficking) can be induced by RSV (18, 24, 25). Th1- and Th2-associated chemokines are secreted at sites of inflammation and function to recruit and activate other immune cells. Recent data have suggested that production of these mediators not only is linked to classic immune cells (macrophages and T cells) but also comes from other cells such as epithelial and endothelial cells.

There is increasing evidence that thymus- and activation-regulated chemokine (TARC) is involved in the recruitment of Th2 cells during an allergic response (26–28). Th2 cells express the TARC receptor, chemokine (CC motif) receptor 4 (CCR4) and asthmatics have been shown to have increased levels of TARC in the airways (29). TARC can be produced by airway epithelial cells (30), but very little is known about how TARC production is regulated. For the human gene, two transcription factors have been shown to play a role in TARC production, NFκB and STAT6 (31, 32). In contrast to TARC, IFN-γ-inducible protein 10 (IP-10)/CXCL10 is a chemokine that preferentially attracts Th1 T cells via the receptor CXCR3. It is highly inducible by the Th1 cytokine, IFN-γ. IP-10 expression has also been shown to be up-regulated in...

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3 Abbreviations used in this paper: RSV, respiratory syncytial virus; HPRT, hypoxanthine guanine phosphoribosyl transferase; IP-10/CXCL10, human IFN-inducible protein 10; mox, multiplicity of infection; PI, propidium iodide; qRT-PCR, quantitative RT-PCR; TARC/CCL17, thymus- and activation-regulated chemokine; PVDF, polyvinylidene difluoride; CBP, CREB-binding protein.

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asthmatic airways, demonstrating the complex nature of the Th1/Th2 inflammation in that disease (33).

In this study, we use both an in vivo murine model and an in vitro epithelial cell model to evaluate the expression of the chemokine TARC during RSV infection. We demonstrate that TARC production is a late event after RSV infection and that it occurs following expression of the Th1 chemokine, IP-10. We generated mice biased toward a Th1 or Th2 memory phenotype in the lung by priming with vaccinia vectors expressing either the RSV F (Th1) or G (Th2) protein followed by intranasal RSV infection. After challenge with RSV, there was considerably more TARC induction in the Th2-biased animals. In an in vitro model, we observed a super induction of TARC when RSV infection is combined with IL-4 or IL-13 exposure. No similar effect was observed when RSV infection was combined with Th1-like cytokines, nor did the Th2 cytokines affect IP-10 induction. This combined effect of RSV and Th2 cytokines was consistent with the effect of RSV and IL-4 or IL-13 on the relevant transcription factors (NFκB and STAT6). Binding sites for both NFκB and STAT6 are present in the TARC promoter region (30, 32, 34, 35). RSV activated only NFκB, and IL-4/IL-13 activated only STAT6. Only when both RSV and IL-4/IL-13 were present in the cultures was there activation of both NFκB and STAT6. Thus, the presence of both RSV and either IL-4 or IL-13 led to activation of both transcription factors needed for optimal TARC production. This study shows that TARC is produced at low levels with primary RSV infection and that TARC production is markedly amplified in settings where both RSV and Th2 cytokines are present.

Materials and Methods

Chemicals were obtained from Sigma-Aldrich and Calbiochem. Protease inhibitors were obtained from Roche Diagnostics. IbBr, p65, and STAT6 Abs were from Santa Cruz Biotechnology. Abs to STAT6 phosphorylated on tyrosine 641 was from Cell Signaling. IL-4 and IL-13 Duoset ELISAs were from R&D Systems. qRT-FCR reagents are from Promega. Primers were obtained from Integrated DNA Technologies. Bay11-7082 and the JAK1 inhibitor were both from EMD Biosciences.

Epithelial cell culture and viral infection

A549 lung epithelial cells were obtained from American Type Culture Collection. A549 cells were used because they most closely mimic RSV observations in primary human airway cells (3, 4, 36). Cells were maintained in 75-cm² tissue culture flasks (Corning) in minimal essential medium (Invitrogen Life Technologies) with 10% FCS and gentamicin. For infection, cells at ~80% confluence were treated with human RSV, strain A2 (multiplicity of infection [moi] of 2). Viral stocks were obtained from Advanced Biotechnologies Inc. Because of a report of possible adenovirus contamination in some RSV stocks (37), we tested our stock for adenovirus by PCR and found it to be completely free of adenoviral contamination. The initial stock (1 × 10⁸ g of protein were mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromphenol blue, and 1.25 M Tris, pH 6.8) and loaded onto a 10% SDS-PAGE gel and run at 110 V for 2 h. Cell proteins were transferred to Immuno-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) with a Bio-Rad semidytransfer system, according to the manufacturer’s instructions. Equal loading of the protein groups on the blots was evaluated using Porcineas S (Sigma-Aldrich), a staining solution designed for staining proteins on PVDF membranes. The PVDF was then blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 h, washed, and then incubated with the primary Ab at dilutions of 1/500 to 1/2000 overnight. The blots were washed four times with Tris-buffered saline with 0.1% Tween 20 and incubated for 1 h with HRP-conjugated anti-IgG Ab (1/5000–1/20000). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus or ECL (Amersham Biosciences). An autoradiograph was obtained, with exposure times of 10 s–2 min. Protein levels were quantified using a FluorS scanner and Quantity One software for data analysis (Bio-Rad). The data were analyzed and statistics performed using Graphpad software. Densitometry is expressed as fold increase (experimental value/control value).

Measurement of secreted proteins

A549 lung epithelial cells were plated at ~80% confluence. After designated culture, supernatants were collected and frozen at ~70°C. TARC and IP-10 concentrations in cell culture supernatants were determined using DuoSet ELISA kits from R&D systems.

Epithelial cell survival assays

Cell viability was analyzed by the Guava EasyCyte mini (Guava Technologies). The Guava ViaCount assay distinguishes between viable and non-viable cells based on the differential permeability of DNA-binding dyes in the ViaCount Reagent (Guava Technologies). Cell viability was also analyzed by monitoring ATP levels after RSV with and without IL-4 culture
performed in a 96-well plate; CellTiter-Glo Luminescent Cell Viability Assay (Promega). After incubation with virus and cytokine, cultures were brought to room temperature, and an equal volume of CellTiter-Glo reagent was added. After a 2-min mix, the plate was read on a Safire plate reader from Tecan, set for chemiluminescence.

Real-time RT-PCR (qRT-PCR)

For cell cultures, total RNA was extracted using the Absolutely RNA Kit according to the manufacturer’s instructions (Stratagene). RNA was quantified using the RiboGreen Kit (Invitrogen Life Technologies). For mouse lungs, RNA was extracted with Invitrogen Trizol reagent, cleaned up with Stratagene Absolutely RNA, and quantified with Bio-Rad Experion. Total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit from Bio-Rad. The resulting cDNA (2 μl; from either mouse lungs or a human cell line) was then mixed with 48 μl of PCR master mix consisting of iQ SYBR Green Supermix (Bio-Rad), 15 pmol of forward primer, and 15 pmol of reverse primer in a 0.2-ml PCR tube (Bio-Rad). PCR amplification was then performed in an iCycler iQ Fluorescence Thermocycler (Bio-Rad) (3, 4, 36). Chemokine gene expression was normalized to the housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), with approximately equal amplification efficiency. The Δ threshold cycle (Ct) was calculated as the difference between C, values,

FIGURE 1. RSV induces TARC protein and mRNA in BALB/c mice. In primed animals (Th1 or Th2), Th2 priming increases RSV induced TARC. A, BALB/c mice (two per group) were infected intranasally with RSV and followed up to 30 days postinfection. At designated time points, mice were euthanized as described in Materials and Methods, lungs were harvested, and RNA was isolated. TARC and IP-10 levels were measured using qRT-PCR. B, BALB/c mice (four per group) were scarified with 3 \times 10^6 PFU of a recombinant vaccinia virus (vv) expressing β-galactosidase (bgal), the attachment (G) protein of RSV or the fusion (F) protein of RSV. Three weeks later, mice were challenged intranasally with 2 \times 10^6 PFU of RSV under anesthetization. One lung was processed for RNA. TARC and IP-10 levels were measured by qRT-PCR. Significance was measured by using ANOVA followed by Bonferroni’s test for multiple comparisons and represents the mean ± SEM (GraphPad Prism). C, For the animals used in B, information was obtained on histology, weight and illness score changes, and mRNA (IL-4, IL-13, and IFN-γ). Weights and illness scores were kept from day 0 for all mice as described in Materials and Methods (data are summarized in two graphs, averages ± SE). From euthanized animals, one lung from each animal was fixed for histology (H&E stain), and one lung from each animal was processed for RNA. IL-4, IL-13, and IFN-γ mRNAs were measured by qRT-PCR. Significance was measured by using ANOVA followed by Bonferroni’s test for multiple comparisons and represents the mean ± SEM (GraphPad Prism).
In contrast to IP-10 induction, TARC is only minimally induced by TNF-α or TNF-α plus IFN-β. A. A549 cells were cultured at 80% confluence and then infected with RSV at moi 2. At selected time points, cells and supernatants were harvested for both RNA and protein analysis. Supernatants were analyzed using TARC- and IP-10-specific ELISAs. RNA was analyzed as described in Materials and Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. Significance was measured using a one-tailed Student t test (GraphPad Prism). B. A549 cells were cultured with and without RSV (moi 2) for 48 h. The supernatant was removed and split in half, and one half was UV treated. The four supernatants (Control, UV Control, RSV, and UV RSV) were then placed on fresh A549 cells, cultured to 80% confluence, and incubated for a further 48 h. The final supernatant was then harvested, and TARC and IP-10 were measured by ELISA. Significance (RSV supernatant vs UV RSV supernatant) was measured using a one-tailed Student t test (GraphPad Prism). C. A549 cells were cultured at 80% confluence and then treated with RSV (moi 2), TNF-α (10 ng/ml), IFN-β (1000 U/ml), or TNF-α and IFN-β together. After 48 h, supernatants were harvested, and TARC and IP-10 were measured by ELISA. Significance (all groups compared with RSV-infected sample) was measured using a one-tailed Student t test (GraphPad Prism).

mRNA stability assay

A549 cells were stimulated with RSV (moi 2) with and without added IL-4 (10 ng/ml) for 24 h and treated with 10 μg/ml actinomycin D to inhibit transcription. Additional harvests were then made at 3 and 6 h after actinomycin D treatment. Total RNA was isolated using Absolutely RNA Miniprep kit (Stratagene). RNA concentration was measured using Quant-iT RiboGreen RNA assay kit (Invitrogen Life Technologies). Total RNA (1 μg) was reverse transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) in an iCycler IQ Fluorescence Thermocycler (Bio-Rad). The specific primer sets for TARC and housekeeping genes are shown in “Real-time RT-PCR (qRT-PCR).” Relative gene expression was calculated and normalized to HPRT or GAPDH mRNA as previously described (39). Cytokine mRNA stability was expressed as percent mRNA remaining at given time points after transcriptional inhibition relative to the mRNA abundance at t = 0 (ratio of actinomycin D sample/ratio of 24 h RSV or RSV + IL-4) × 100; GraphPad Software.

Statistical analysis

Statistical analysis was performed on ELISA results and real-time PCR data using either ANOVA followed by Bonferroni’s test for multiple comparisons or Student’s t test and are reported as the mean ± SEM. These methods were performed using GraphPad Prizm 4 for Windows (GraphPad Software).

Results

RSV induces both Th1-promoting and Th2-promoting chemokines in a murine infection model

RSV infection of airway epithelium causes the release of many cytokines and chemokines (25). One of the well-described RSV-induced chemokines is IP-10 (21, 40, 41). In contrast, the only report of TARC production after RSV is a microarray study, which lists TARC as one of many induced cytokine/chemokines (42). The focus of this paper is the Th2 cell-recruiting chemokine TARC; we show data on IP-10 to highlight the differences between TARC production and other better described inflammatory mediators. We were interested in how and when RSV induces these two potentially divergent mediators. BALB/c mice were infected intranasally with RSV, and at different time points postinfection, animals were sacrificed and lungs were harvested for RNA isolation. Fig. 1A shows that after RSV infection there is a peak of IP-10 induction at day 5. In contrast, TARC mRNA comes up at later time points (peaking at day 10) and stays up-regulated for longer periods (staying slightly up-regulated as long as 300 days postinfection). These data demonstrate in an animal model that RSV induction of the Th1-recruiting chemokine, IP-10, precedes induction of the Th2-recruiting chemokine, TARC. In addition, TARC mRNA remains elevated for a significantly longer time.
Primed BALB/c mice with either the F or the G proteins from RSV have been shown to bias the memory T cell response in the lung toward either a Th1 or a Th2 phenotype, respectively (1, 2, 43, 44). Three weeks after priming, some of the animals were challenged with RSV intranasally. Three days after RSV infection, the animals were sacrificed and blood and lungs harvested.

Fig. 1B demonstrates that priming with a construct that expresses the G protein from RSV (induces a Th2-biased memory T cell response in the lung) led to increased production of TARC after RSV infection. Priming with either the F or the G protein led to increased IFN-γ production. The time point examined here (3 days) is 2–4 days after the appearance of TARC mRNA in mice undergoing an acute RSV infection (Fig. 1A).

In Fig. 1C, we show that priming with either F or G protein increases systemic disease (as evidenced by decreased body weight and increased illness score). Both the F and G priming increase IFN-γ production by RSV. However, only in the animals primed with G protein was there an increase in the Th2 cytokines IL-4 and IL-13 after RSV. There was not a large increase in IL-4 and IL-13; however, the increase was enough to demonstrate a significant increase in the amount of TARC mRNA. These data demonstrate that in a murine model, skewing the RSV-specific memory T cell response toward a Th2 bias leads to a significant increase in TARC production after RSV infection.

In the acute RSV infection shown in Fig. 1A, we have no evidence that RSV alone induces Th2 cytokines. In fact, a recent study by Lukacs et al. (45) demonstrates that the A2 strain of RSV (used in this study) does not induce IL-4 or IL-13 in a BALB/c model. They show that, in contrast, the clinical isolate, line 19, induces both IL-4 and IL-13. It will be of interest to determine whether acute RSV infection with line 19 RSV increases TARC production in BALB/c mice.

**RSV induces both IP-10 and TARC in lung epithelial cells**

In the studies shown in Fig. 2A, we infected a human lung epithelial line, A549 cells with RSV (moi 2) and saved supernatants and RNA at various time points. We have found that the natural course of RSV infection in A549 cells starts with a period of rapid RSV replication, leading to cell death between 48 and 96 h (4, 36, 38, 46). We examined time points from 0 to 72 h for protein production and 0 to 48 h for mRNA production. In the mRNA studies, we stopped experiments at 48 h of infection to avoid the variability that occurred with the onset of cell death between 48 and 72 h. IP-10 mRNA began going up as early as 16 h, whereas TARC accumulation began 24 h postinfection. At 72 h postinfection, TARC protein was going up and at 48 h postinfection TARC mRNA was still rapidly increasing. In contrast, IP-10 mRNA amounts leveled at 48 h, and protein production was returning to baseline by 72 h. As a composite, the data presented in Fig. 2A show that in an in vitro model, RSV infection induces IP-10 and TARC in a sequential manner.

**FIGURE 3.** The addition of IL-4 or IL-13 to a RSV-infected culture synergistically increases TARC production, while having no effect on IP-10 production. A. A549 cells were cultured at 80% confluence. Cells were infected with RSV (moi 2) at the same time as the addition of IL-4 (10 ng/ml). At selected time points, cells and supernatants were harvested for both RNA and protein analysis. For both protein and mRNA, IL-4 alone did not induce TARC or IP-10. Supernatants were analyzed using TARC and IP-10 specific ELISAs. RNA was analyzed as described in Materials and Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. Significance (RSV alone compared with RSV + IL-4) was measured using a one-tailed Student’s t test (GraphPad Prism). B. A549 cells were cultured at 80% confluence and then infected with RSV (moi 2) with and without added IL-13 (10 ng/ml). At selected time points, cells and supernatants were harvested for both RNA and protein analysis. For both protein and mRNA, IL-13 alone did not induce TARC or IP-10. Supernatants were analyzed using TARC and IP-10 specific ELISAs. RNA was analyzed as described in Materials and Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. Significance (RSV alone compared with RSV + IL-13) was measured using a one-tailed Student’s t test (GraphPad Prism). ns, Not significant.
RSV-induced TARC is dependent on ongoing viral replication while IP-10 is not

RSV infection induces a number of cytokines that are known to induce other cytokines and chemokines (i.e., TNF-α, IL-1β and IFN-β (1, 4, 21, 47–53). To examine whether either IP-10 or TARC production was secondary to a released cytokine, we performed the following experiment. A549 cells were infected with and without RSV at moi 2 for 48 h. The supernatant was harvested and divided into two portions, and one half of each portion was UV treated to kill the RSV. The UV-treated and not-UV-treated supernatants were then placed on fresh A549 cells, and the cells were cultured for a further 48 h. Final supernatants were harvested, and IP-10 and TARC were measured. Fig. 2B demonstrates that the UV-treated supernatant was incapable of inducing TARC in the secondary culture, whereas the supernatant with the live virus induced significant TARC levels. However, in contrast, IP-10 was induced by the supernatant whether or not the RSV in the supernatant was viable. Both the TARC and IP-10 levels were higher than those shown in Fig. 2A, because the ELISA is measuring chemokines produced in both the first supernatant-generating incubation (200–400 pg/ml quantities of both TARC and IP-10) and the second incubation.

We next examined two likely candidates for the IP-10-inducing effect of the supernatants. In Fig. 2C, we show that treating lung epithelial cells with TNF-α or IFN-β or a combination of the two has little effect on TARC production. TNF-α does induce low levels of TARC, which has been reported previously (54). IFN-β, one of two type I IFNs produced by RSV, does not induce any TARC, nor does it augment the low levels of TNF-α-induced TARC (55, 56). IP-10, in contrast, is induced by TNF-α to a greater extent than RSV, and there is significant synergy between TNF-α and IFN-β. As a composite, these data demonstrate that, whereas IP-10 can be induced via paracrine responses to RSV-induced mediators (possibly by both TNF-α and IFN-β), TARC induction requires active viral signals.

The Th2 cytokines IL-4 and IL-13 both significantly enhance production of TARC in RSV-infected cells

TARC is one of the few chemokines with demonstrable Th2 cell chemotactant activity (57–59). We were interested in determining whether the presence of Th2 cytokines (as can occur in an asthmatic’s lung or in individuals who have been previously infected with RSV) would have any effect on the production of IP-10 and TARC. We first examined the effect of IL-4 or IL-13 on RSV-induced TARC. IL-4 or IL-13 was added to epithelial cultures at the same time as RSV, and the samples were cultured for various times. Fig. 3A demonstrates that exposing lung epithelial cells to both RSV and IL-4 has a substantial effect on TARC production, while not increasing IP-10 production. The top two graphs show TARC protein release and mRNA production after IL-4. The differences completely overshadow TARC production by RSV alone. IL-4 alone produced no TARC protein and only very small increases in TARC mRNA. In the bottom two graphs, the effect of IL-4 exposure on RSV-induced IP-10 is shown. In contrast to TARC, IL-4 has no stimulatory effect on the production of IP-10 in RSV-infected cells. In fact, IL-4 appears to cause an initial inhibition of RSV-induced IP-10 protein. We next examined the effect of IL-13 on TARC and IP-10 production after RSV infection. Like IL-4, IL-13 caused a significant increase in TARC production (Fig. 3B). This was true both of TARC protein and TARC mRNA. Also like IL-4, IL-13 did not increase RSV-induced IP-10. Unlike IL-4, IL-13 did not inhibit RSV-induced IP-10. These data
show that either Th2 cytokine increases TARC production after RSV, while having no effect or inhibiting RSV-induced IP-10 production.

We combined other cytokines with RSV and studied their effect on TARC production (data not shown). IL-6 and IL-10 had no effect (either positive or negative) on TARC protein production. IFN-\(\gamma\)/H9253 minimally increased RSV-induced TARC protein. We demonstrated in Fig. 2C that TNF-\(\alpha\)/H9251 induced small amounts of TARC protein and that in combination with RSV there was a 2- to 3-fold increase in TARC production. This was significantly lower than the 10- to 30-fold increase seen when IL-4 or IL-13 was combined with RSV infection. As a composite, the data in Fig. 3 demonstrate that both canonical Th2 cytokines, IL-4 and IL-13, significantly induce the production of TARC mRNA and protein during RSV infection.

**FIGURE 5.** RSV infection alone activates NF\(\kappa\)B and not STAT6. IL-4 exposure alone activates STAT6 and not NF\(\kappa\)B. A, A549 cells were infected with RSV (moi 2) with and without IL-4 (10 ng/ml) or IL-13 (10 ng/ml) for 24 h. Whole-cell lysates were obtained, and Western analysis was performed for IkBa (37 kDa) and phosphorylated (Phos) STAT6 (tyrosine 641). Equal loading was performed by analyzing the same blot for \(\beta\)-actin (42 kDa). Primary and secondary Ab dilutions for IkBa were 1/1,000 and 1/10,000, respectively. Primary and secondary Ab dilutions for phosphorylated STAT6 were 1/500 and 1/5000, respectively. Also shown is densitometry for both IkBa and phosphorylated STAT6 bands. B, A549 cells were infected with RSV (moi 2) with and without IL-4 (10 ng/ml) for 24 h. Cells were harvested, and nuclear and cytosolic fractions were isolated as described in Materials and Methods. Western analysis was performed for total p65 and total STAT6. Controls for protein loading were done by staining for \(\beta\)-actin. Control for the nuclear/cytosolic isolation was performed by staining for HDAC2, a nuclear protein. Also shown is densitometry for both p65 and STAT6 bands.

IL-4 and IL-13 have no effect on the viability of RSV-infected lung epithelial cells

One possible explanation for the increased TARC production with IL-4 or IL-13 in combination with RSV is that the cytokines increase survival of the RSV-infected cells. To rule out this possibility, we infected lung epithelial cells with RSV in combination with IL-4 or IL-13 and examined viability in two ways. The top graph in Fig. 3C demonstrates that 48 h after infection, RSV has increased plasma membrane permeability (propidium iodide (PI) staining and FACS). IL-4 and IL-13 have no effect on the viability changes due to RSV infection (Fig. 4A). The bottom graph examines total ATP levels, also as a marker of cell viability. As with the PI staining, the addition of IL-4 or IL-13 had no effect on the decrease in ATP due to RSV infection. Increased TARC mRNA and protein, if it did not result from changes in cell viability, could result from changes in mRNA stability or in TARC gene transcription. We next examined the effect of IL-4/IL-13 on mRNA stability in RSV-infected lung epithelial cells.

**IL-4 does not alter the stability of RSV-induced TARC mRNA**

We next examined mRNA stability of both TARC and IP-10 after exposure to RSV with and without IL-4 (Fig. 4B). Because qRT-PCR does not detect any TARC transcript in unstimulated cells, statistically, we could not examine the ability of RSV to alter TARC mRNA stability compared with baseline. We could look at whether IL-4 changes the stability of the RSV-induced TARC. We addressed this question by incubating cells with and without RSV and IL-4 for 48 h, stopping transcription with actinomycin D and measuring mRNA levels at 3 and 6 h after the stop of transcription. We found that IP-10, which has a long 3’-untranslated region, has a relatively short-lived mRNA species. This is supported by the IP-10 literature (60). TARC with its minimal 3’-untranslated region was a very stable transcript, and the TARC stability was not altered by RSV or IL-4. TARC mRNA has a very small 3’-untranslated region (201 nucleotides) with no demonstrable UAAAU sequences, consistent with both the long-term stability of the TARC transcript and the lack of changes in stability with RSV + IL-4 exposure. These data, in combination with the greatly increased TARC mRNA with the combination of RSV and Th2 cytokines, suggest that the increased TARC is the result of changes in transcription.
RSV alone induces NFκB activity; IL-4 alone induces STAT6 activity; together, RSV and IL-4 activate both NFκB and STAT6

Two transcription factors have demonstrated importance in TARC production, NFκB and STAT6 (30–32, 34, 35). NFκB is known to be activated by RSV infection and STAT6 is known to be activated by IL-4 and IL-13 (38, 61, 62). We next evaluated the effect of RSV with and without IL-4 on NFκB activation and STAT6 activation. Fig. 5A demonstrates that by 24 h post infection, RSV induced degradation of IκBα (consistent with NFκB activation) but had no effect on STAT6 activation (phosphorylation of tyrosine 641). In contrast, IL-4-induced phosphorylation of STAT6 on tyrosine 641 but did not activate NFκB. When RSV and IL-4 were combined, both transcription factors were activated. We also looked at nuclear localization of p65 NFκB subunit and STAT6 total protein. Consistent with the data in Fig. 5A, Fig. 5B demonstrates that RSV induces p65 nuclear translocation and IL-4 induces STAT6 nuclear translocation. Both exposures combined resulted in nuclear localization of both p65 and STAT6. These data suggest that the synergy between RSV and IL-4 results, in part, from the fact that neither exposure (RSV or IL-4) induces both of the transcription factors needed for optimal TARC production. However, in combination, there is activation of both NFκB and STAT6 and significantly increased TARC production. RSV alone was capable of inducing low levels of TARC without a STAT6 signal. We have no evidence that RSV alone induces IL-4 or IL-13 in BALB/c mice, and it does not induce these cytokines in lung epithelial cells. We are examining the hypothesis that RSV, via NFκB and some as yet unidentified factor(s), induces transcription of the TARC gene. Further, we hypothesize that RSV alters the environment at the TARC promoter, allowing for the STAT6 effect seen when both RSV and IL-4 are present.

Inhibition of NFκB inhibits TARC production

We used NFκB inhibitors to study the effect of NFκB on TARC production. Fig. 6A demonstrates that inhibiting NFκB with the translocation inhibitor, Bay11-7082, blocks both the RSV-induced TARC and the synergistic increase in TARC with IL-4 exposure. The graph on the right shows only the RSV-induced TARC. With a smaller y-axis, it is clear that the NFκB inhibitor blocks TARC production by RSV alone. We repeated these experiments using an adenovirus vector containing a mutant IκBα (S32/36A), kindly provided by J. Engelhardt (Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA). This IκBα cannot be ubiquitinated and degraded and thus serves to keep NFκB in the cytosol. Fig. 6B shows, consistent with the chemical inhibitor data, that NFκB activity is essential for TARC production by RSV and by the combination of RSV and IL-4.

Inhibition of JAK1 inhibits RSV and IL-4 TARC production, but not TARC production by RSV alone

The JAK1 inhibitor was used to block STAT6 activity because it would block IL-4 signaling downstream of either the classic IL-4 receptor (found primarily in hemopoietic cells (signaling via JAK1 and JAK3) and the combination IL-13 and IL-4 receptor found in a wider range of cell types (signaling via JAK1 and JAK2 or Tyk2; Ref. 63). The JAK1 inhibitor blocked production of TARC when cells were exposed to both RSV and IL-4 (Fig. 7). However, inhibiting STAT6 had no effect on the production of TARC by RSV alone. The graph on the right is an expanded version of the RSV alone data showing that the JAK1 inhibitor had no effect on TARC production by RSV alone, in contrast to the significant fall in TARC produced with a combination of RSV and IL-4.

As a composite, these data demonstrate that NFκB is essential for TARC production after RSV and for the synergistic increase in
TARC with IL-4 and RSV together. In contrast, STAT6 (the transcription factor activated by both IL-4 and IL-13 signaling) is necessary for the IL-4/IL-13 synergistic effect on RSV-induced TARC, although not being necessary for the effects of RSV alone on TARC production.

Despite the fact that TARC has been shown to recruit Th2 cells via the CCR4 receptor and is up-regulated in asthmaic airways, very little is known about TARC regulation. In this paper, we demonstrate that RSV induces TARC production in the lung and in lung epithelial cells. Beyond this observation, we show that RSV and Th2 cytokines (IL-4 or IL-13) synergize to significantly increase the magnitude of TARC production. IL-4 or IL-13 do not produce significant TARC levels on their own but do provide a STAT6 signal that synergizes with RSV-induced NFκB to up-regulate TARC induction. Neither stimulus alone (RSV or IL-4/IL-13) generates both a STAT6 and an NFκB signal; combined they cooperate to induce significant amounts of TARC (Fig. 8).

Discussion

In this study, we have examined the role of RSV infection in the induction of Th1 recruiting and Th2 recruiting chemokines, IP-10 and TARC. Although we examined production of both chemokines, the focus of this project is TARC. IP-10 is a well-described outcome of IFN-β exposure, and RSV induces IFN-β. We examined IP-10 in parallel as an aid in determining what was unique about TARC mRNA and protein generation.

The only description of viral-induced TARC is a microarray study examining induction of a number of chemokines; it showed only that there was an increase in the transcript (42). We wanted to more comprehensively examine TARC production and whether RSV, the only virus known to induce a Th2-like response, produced TARC. We used a variety of models to examine the effect of RSV on TARC production. We first infected BALB/c mice with RSV and examined mRNA of both IP-10 and TARC. We found a sequential up-regulation (IP-10 first, TARC second) of the chemokines. Compared with IP-10, there was a prolonged expression of TARC. To study the effect of a Th2 bias, we made use of a murine model, in which priming by either F or G RSV proteins sets up a Th1- or Th2-biased pulmonary memory T cell response following RSV infection. After priming (3-wk incubation) and a 3-day RSV infection, we found significantly greater expression of TARC in the lungs of RSV-infected animals that were primed to express a memory Th2 cell phenotype.

We then found a synergistic effect of RSV and IL-4 exposure on TARC production in an in vitro model of lung epithelial cells. These data suggest a possible positive amplification loop between RSV and IL-4 or IL-13. Although our present animal data do not support induction of a Th2 phenotype by RSV alone, they are suggestive of in vivo interactions between IL-4 or IL-13 and RSV, increasing TARC production.

When we examined the transcription factors involved in TARC production (NFκB and STAT6), we found that each stimulus activated only one of these factors; RSV activates NFκB, and IL-4 activates STAT6. Only when both RSV and IL-4 were present together was there activation of both NFκB and STAT6. This observation leads to the conclusion that IL-4 and RSV synergize in inducing TARC by each providing one of the transcription factors needed for optimal activation of the TARC promoter.

In both the animal- and cell-based models, RSV alone, with no evidence of IL-4 to activate STAT6, still induces TARC. It is our hypothesis that low levels of TARC can be produced by RSV-induced NFκB and an as yet unidentified RSV-induced factor. The reason we believe that there is an unidentified RSV-induced factor is that some other inducers of NFκB do not produce TARC (i.e., TLR ligands; data not shown). The inhibitor data showing that a JAK1 inhibitor blocks the IL-4 effect on TARC in the presence of RSV but not RSV-induced TARC are consistent with this hypothesis.

One interesting hypothesis for the synergy between NFκB and STAT6 is the recruitment of CREB-binding protein (CBP)/p300 to promoters by NFκB. STAT6 in contrast to other STATs has no binding site for and does not recruit CBP/p300 on its own (64). CBP/p300 are multifunctional coactivator proteins that act as bridging factors to the basal transcription machinery, including RNA polymerase II. They also remodel chromatin by acetylating nucleosomal histones. CBP/p300 is essential for STAT-driven transcription (65–67), and STAT6 does not recruit it on its own. One hypothesis that fits our data (no TARC with IL-4 or IL-13 alone and high TARC with RSV and IL-4/IL-13) is that RSV brings CBP/p300 to the transcription start site where it is also used by STAT6, allowing for an IL-4 response where there was none (or only an extremely minimal response) before. We are at present pursuing this hypothesis.

As a composite, these data demonstrate that infection with RSV induces the Th2 chemokine TARC via a mechanism distinct from RSV-induced IP-10. Furthermore, the synergistic increase in TARC production with IL-4/IL-13 and RSV suggests that RSV infection of individuals who already have an increased capacity to generate a Th2 immune environment may have more severe disease after RSV than individuals without a Th2 bias in the lung.

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Disclosures

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