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 NFkB 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.


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3Abbreviations used in this paper: RSV, respiratory syncytial virus; HPRT, hypoxanthine guanine phosphoribosyl transferase; IP-10/CXCL10, human IFN-inducible protein 10; mos, 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 che-
mokine 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 ob-
served a super induction of TARC when RSV infection is com-
bined 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 acti-
vation 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, IkBα, p65, and STAT6
Abs were from Santa Cruz Biotechnology. Abs to STAT6 phosphorylated
on tyrosine 694 was from Cell Signaling. IL-4 and IL-13 Duoset ELISAs
were from R&D Systems. qRT-PCR 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 main-
tained in 75-cm² tissue culture flasks (Corning) in minimal essential me-
dium (Invitrogen Life Technologies) with 10% FCS and gentamicin. For
virus vectors. A549 cells were used because they most closely mimic RSV
observations in primary human airway cells (3, 4, 36). Cells were main-
tained in 75-cm² tissue culture flasks (Corning) in minimal essential me-
dium (Invitrogen Life Technologies) with 10% FCS and gentamicin. For

Infection of mice

With a 25-gauge needle, mice were scarified on the rump by lightly
scratching 10 μl of a recombinant vaccinia virus (equivalent to 3 × 10⁸
PFU/mouse) expressing the attachment (G) protein of RSV, the fusion (F)
protein of RSV, or β-galactosidase as a control. After 3 wk, mice were
challenged intranasally with 100 μl of 2 × 10⁹ PFU/mouse RSV under
light anesthesia with 30% halothane (Halocarbon Laboratories) in mineral
oil (Fisher Scientific). Mice were monitored daily for clinical symptoms.

Whole-cell protein isolation

Whole cell protein was obtained by lysis of the cells on ice for 20 min, in
300 μl of lysis buffer (0.05 M Tris (pH 7.4), 0.15 M NaCl, 1% Nonidet
P-40, with added protease and phosphatase inhibitors: 1 protease munitab
(Roche Biochemicals)/10 ml and 10 μl of 100× phosphatase inhibitor
mixture (Calbiochem)/10 ml. The lysates were sonicated for 2 s, kept at
4°C for 30 min, and spun at 15,000 × g for 10 min; the supernatant was
saved. Protein determinations were made using a protein measurement kit
(Bradford Protein Assay) from Bio-Rad. Cell lysates were stored at −70°C
until use.

Cytosolic and nuclear protein extracts

Experimental cell cultures were harvested in 0.4 ml of lysis buffer (10 mM
HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA), placed
on ice for 15 min, and then vigorously mixed after the addition of 25 μl of
10% Nonidet P-40. After a 30 s of centrifugation (16,000 × g at 4°C), the
supernatant was saved as the cytosolic fraction, and the pelleted nuclei
were resuspended in 50 μl of extraction buffer (50 mM HEPES (pH 7.8),
50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol). The nuclei
were incubated on ice for 20 min and vortexed, and debris was removed
with a 16,000 × g quick spin at 4°C. Nuclear and cytosolic extracts were
stored at 70°C.

Western analysis

Western analysis for the presence of particular proteins or for phosphory-
lated forms of proteins was performed as previously described (38).
Briefly, 40 μg 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 pellets were transferred to Immuno-Blot polyvinylidene difluo-
ride (PVDF) membrane (Bio-Rad) with a Bio-Rad semidy transfer system,
according to the manufacturer’s instructions. Equal loading of the protein
groups on the blots was evaluated using Ponceaus 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 (1/500–1/2000). 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 analysis (Bio-Rad). The data were analyzed
and statistics performed using Graphpad software. Densitometry is ex-
pRESSED as fold increase (experimental value/control value).

Measurement of secreted proteins

A549 lung epithelial cells were plated at ~80% confluence. After desig-
nated 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 Technol-
ologies). 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 ana-
yzed by monitoring ATP levels after RSV with and without IL-4 cultured
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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 (ΔCt) was calculated as the difference between Ct values.
FIGURE 2. RSV increases TARC protein and mRNA in lung epithelial cells. The increased TARC requires active viral replication. 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 fourth 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 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 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.
Mice primed to develop a memory Th2-biased response in the lung produce increased TARC in response to RSV infection compared with mice primed to develop a memory Th1-biased pulmonary response

Priming BALB/c mice with either the F or the G proteins from RSV has 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). We made use of this model to ask whether animals primed for a memory Th2 response would produce more TARC in the lung in response to a subsequent RSV infection. BALB/c mice were primed using recombinant vaccinia virus constructs expressing either the F or the G proteins according to a previously described protocol (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 IP-10, given that in both conditions there is increased IFN-γ production. The time point examined here (3 days) is 2–4 days before 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](http://www.jimmunol.org/)

**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. 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-4 alone did not induce TARC or IP-10. Supernatants were analyzed using TARC and IP-10 specific ELISA’s. 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.
FIGURE 4. IL-4 or IL-13 does not increase survival of RSV-infected cells. IL-4 or IL-13 has no effect on TARC or IP-10 mRNA stability in RSV-infected cultures. A, A549 cells were cultured at 80% confluence and then infected with RSV (moi 2) with and without added IL-4 (10 ng/ml). After 48 h of culture, cell viability was measured using either PI staining (Guava EasyCye mini) or ATP loss (ATP assay from Promega). There was no difference in viability with the addition of IL-4 or IL-13 to RSV-infected cultures. B, A549 cells were cultured at 80% confluence and then infected with RSV at a moi 2. After 24 h of culture, actinomycin D (ActD) was added (10 μg/ml), and cells were cultured for an additional 3 or 5 h. Cells were harvested for RNA. RNA was analyzed as described in Materials and Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. For calculations, mRNA levels at 24 h of RSV or RSV + ActD were set as 100%, and 3- or 6-h actinomycin percent was calculated as (ratio of ActD sample/ratio of 24-h RSV or RSV + IL-40) × 100. Significance was measured (each actinomycin sample was compared with the amount of mRNA (TARC or IP-10) present at 24 h (before the addition of actinomycin D) using a one-tailed Student t test (GraphPad Prism).

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 chemotactic 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 graps 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.
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-γ/H9253 minimally increased RSV-induced TARC protein. We demonstrated in Fig. 2C that TNF-α/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 that 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.

**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 the 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 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.
were cultured at 80% confluence and then infected with adenovirus (Ad) as previously described using a one-tailed Student’s t test (GraphPad Prism). RSV with and without IL-4 on NFκB was activated by RSV infection and STAT6 is known to be activated by RSV infection (Fig. 5A) but did not activate NFκB (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 (S3236A) (non-degradable IκBα mutant). After 16 h, cells were infected with RSV (moi 2) with and without added IL-4 (10 ng/ml). Cells were cultured with treatments for a further 48 h, supernatants were harvested, and TARC protein was measured by ELISA. A graph on the right shows an expanded version of the RSV alone data. Significance (RSV alone compared with RSV + JAK1 inhibitor and RSV + IL-4 alone compared with RSV + IL-4 + JAK1 inhibitor) was measured using a one-tailed Student’s t test (GraphPad Prism). Con, Control.

FIGURE 6. Inhibition of NFκB blocks TARC production after RSV infection and after RSV + IL-4 exposure. A, A549 cells were cultured at 80% confluence and then infected with RSV (moi 2) with and without added IL-4 (10 ng/ml). Some samples were also treated with an NFκB inhibitor, Bay11-7082 (50 μM). Cells were cultured with treatments for 48 h, supernatants were harvested, and TARC protein was measured by ELISA. A graph on the right shows an expanded version of the RSV alone data. Significance (RSV alone compared with RSV + Bay11-7082 and RSV + IL-4 alone compared with RSV + IL-4 + Bay11-7082) was measured using one-tailed Student’s t test (GraphPad Prism). B, A549 cells were cultured at 80% confluence and then infected with adenovirus (Ad) vectors expressing either BglII (negative control) or IκBα S3236A (non-degradable IκBα mutant). After 16 h, cells were infected with RSV (moi 2) with and without added IL-4 (10 ng/ml). Cells were cultured with treatments for a further 48 h, supernatants were harvested, and TARC protein was measured by ELISA. Significance (RSV alone compared with RSV + IκBα S3236A and RSV + IL-4 alone compared with RSV + IL-4 + IκBα S3236A) was measured using one-tailed Student’s t test (GraphPad Prism).

FIGURE 7. Inhibition of JAK1 blocks TARC production after RSV + IL-4 exposure but has no effect on TARC production after RSV alone. A549 cells were cultured at 80% confluence and then infected with RSV (moi 2) with and without added IL-4 (10 ng/ml). Some samples were also treated with a JAK1 inhibitor (10 μM). Cells were cultured with treatments for 48 h, supernatants were harvested, and TARC protein was measured by ELISA. The graph on the right shows an expanded version of the RSV alone data. Significance (RSV alone compared with RSV + JAK1 inhibitor and RSV + IL-4 alone compared with RSV + IL-4 + JAK1 inhibitor) was measured using a one-tailed Student’s t test (GraphPad Prism). Con, Control.

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 hematopoietic 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...
IL-4 or IL-13 exposure lead to activation of both NF-

resulting in high levels of TARC production.

cooperate to induce significant amounts of TARC (Fig. 8).

13) generates both a STAT6 and an NF-

B activation but not NF-

from mice) is optimally driven by a combination of STAT6 and NF-

outcome of IFN-

kines, the focus of this project is TARC. IP-10 is a well-described

induction of Th1 recruiting and Th2 recruiting chemokines, IP-10

In this study, we have examined the role of RSV infection in the

Discussion

TARC with IL-4 and RSV together. In contrast, STAT6 (the

transcription factor activated by both IL-4 and IL-13 signaling) is nec-

essary 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 asthmatic 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 NFxB to up-regulate TARC induction. Neither stimulus alone (RSV or IL-4/IL-

13) generates both a STAT6 and an NFxB signal; combined they cooperate to induce significant amounts of TARC (Fig. 8).

References


Disclosures

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FIGURE 8. TARC gene expression in lung epithelial cells (or in lungs from mice) is optimally driven by a combination of STAT6 and NFκB.

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/p300s 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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