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

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Abstract

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. The 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 following RSV infection. We primed animals with a recombinant vaccinia virus (vv) 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 vvG 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.

Keywords

Th1/Th2 Cells; Inflammation; Viral Infection

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Introduction

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 immuno-compromised adults (1-4). Virtually all children become infected with RSV by the age of two. In most cases, the virus remains localized to the nasopharyngeal epithelium and only causes 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 antibodies and airway hyper-responsiveness (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 has suggested that production of these mediators is not only 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 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, nuclear factor κ B (NFκB) and signal transducer and activator of transcription 6 (STAT6) (31, 32). In contrast to TARC, IP-10/CXCL10 is a chemokine that preferentially attracts Th1 T cells via the receptor, CXCR3. It is highly inducible by the Th1 cytokine, interferon γ. IP-10 expression has also been shown to be upregulated in asthmatic airways, demonstrating the complex nature of the Th1/Th2 inflammation in that disease (33).

[‖]The abbreviations used are: CCR4, chemokine (C-C motif) receptor 4; CXCR3, chemokine (C-X-C motif) receptor 3; ELISA, enzyme linked immunosorbant assay; HPRT, hypoxanthine guanine phosphoribosyl transferase; IFNα, interferon alpha; IFNβ, interferon beta ; IFNγ, interferon gamma; IκBα, Inhibitor of kappa B alpha; IL-1β, interleukin 1 beta; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-13, interleukin 13; IP-10/CXCL10, human interferon inducible protein 10; JAK 1, janus kinase 1; moi, multiplicity of infection; NFκB, nuclear factor kappa B; PI, propidium iodide; qRT-PCR, quantitative reverse transcribed-polymerase chain reaction; RSV, respiratory syncytial virus; STAT1, signal transducer and activator of transcription 1; STAT6, signal transducer and activator of transcription 6; TARC/CCL17, thymus and activation regulated chemokine; Th1, T helper lymphocyte type 1; Th2, T helper lymphocyte type 2; TNFα, tumor necrosis factor alpha.

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 towards 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. Following 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 the TARC is produced at low levels with primary RSV infection and that TARC production is markedly amplified in setting where both RSV and Th2 cytokines are present.

Materials and Methods

Materials

Chemicals were obtained from Sigma and Calbiochem, La Jolla, CA. Protease inhibitors were obtained from Roche Diagnostics, Indianapolis, IN. IkBα, p65 and STAT6 antibodies were from Santa Cruz, Santa Cruz, CA. Antibody to STAT6 phosphorylated on tyrosine 641 was from Cell Signaling, Beverly, MA. IL-4 and IL-13 Duoset ELISA's were from R&D Systems, Minneapolis, MN. qRT-PCR reagents are from Promega, Madison, WI. Primers were obtained from Integrated DNA Technologies, Iowa City, IA. Bay11-7082 and the JAK1 inhibitor (Catalog number 420099) were both from EMD Biosciences, La Jolla, CA.

Epithelial cell culture and viral infection

A549 lung epithelial cells were obtained from American Type Culture Collection (Mannassus, VA). A549 cells were used because they most closely mimic RSV observations in primary human airway cells (3, 4, 36). Cells were maintained in 75 cm^2 tissue culture flasks (Corning, Corning, NY) in minimal essential medium (Invitrogen, Grand Island, NY) with 10% fetal calf serum and gentamicin. For infection, cells at approximately 80% confluence were treated with human RSV, strain A-2 (multiplicity of infection (moi) of 2). Viral stocks were obtained from Advanced Biotechnologies Inc (Columbia, MD). 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 (1×10^9 $TCID_{50}$) was aliquoted and kept frozen at −135°. A fresh aliquot was thawed for each experiment. The virus was never refrozen.

RSV inactivation

RSV was inactivated using the Stratagene UV Stratalinker 1800 and radiating cell supernatant containing RSV with 450,000 uJoules of energy three times.

Mice and virus stocks

BALB/c mice were purchased from the National Cancer Institute (Frederick, MD). Mice were housed in a pathogen-free environment. All the animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. The A2 strain of RSV was a kind gift of Barney Graham (National Institutes of Health, Bethesda, MD). Recombinant vaccinia virus stocks (kind gifts from Gail Wertz (University of Virginia, Charlottesville, VA) and J.L. Beeler (Food and Drug Administration, NIH, Bethesda, MD)) were grown in BSC-40 cells (ATCC, Manassas, VA) and sucrose purified before use.

Infection of mice

Using a 25-gauge needle, mice were scarified on the rump by lightly scratching 10 μl of with (equivalent to 3×10^6 pfu/mouse) of a recombinant vaccinia virus expressing the attachment (G) protein of RSV, the fusion (F) protein of RSV, or β-galactosidase as a control. Three weeks later, mice were challenged intranasally with 100 μl of (2×10^6 pfu/mouse) RSV under light anesthesia with 30% halothane (Halocarbon Laboratories, River Edge, NJ) in mineral oil (Fisher Scientific, Fair Lawn, NJ). Mouse weights were recorded on a daily basis. Weight data (gms) was converted to a percentage of the starting (day 0) weight of each individual mouse. Day 0 is the day of RSV infection. Illness Scores for each mouse were recorded based on the following scale: 0, no apparent illness; 1, slightly ruffled fur; 2, an active mouse with ruffled fur; 3, inactive and ruffled fur; 4, hunched posture, gait, inactive and ruffled fur; 5, moribund or dead. For the acute RSV infection, mice were treated identically, except that there was no scarification with the recombinant vaccinia virus vectors.

Whole cell protein isolation

Whole cell protein was obtained by lysing the cells on ice for 20 minutes, in 300μl of lysis buffer (0.05M Tris pH 7.4, 0.15M NaCl, 1% NP-40, with added protease and phosphatase inhibitors: 1 protease minitab (Roche Biochemicals, Indianapolis, IN)/10 mls and 100ul 100X phosphatase inhibitor cocktail (#524625, Calbiochem)/10 mls. The lysates were sonicated for 20 seconds, kept at 4° for 30 minutes, spun at 15,000g for 10 minutes and the supernatant saved. Protein determinations were made using a protein measurement kit (Bradford Protein Assay, #500-0006) from BioRad (Hercules, CA). Cell lysates were stored at −70° until use.

Cytosolic and nuclear protein extracts

Experimental cells were resuspended in 0.4ml of lysis buffer (10mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA), placed on ice for 15 minutes, and then vigorous mixed after the addition of 25 ul of 10% Nonidet P-40. After a 30 second centrifugation (16,000g, 4° C), the supernatant was saved as the cytosolic fraction and the pelleted nuclei were resuspended in 50 ul of extraction buffer (50mM HEPES, pH 7.8, 50mM KCl, 300mM

NaCl, 0.1mM EDTA, 10% glycerol). The nuclei were incubated on ice for 20 minutes, vortexed and debris removed with a 16,000g, 4° C quick spin. Nuclear and cytosolic extracts were stored at 70° C.

Western analysis

Western analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed as previously described (38). Briefly, 40 μg of protein was mixed 1:1 with 2x sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue and 1.25M Tris pH 6.8) and loaded onto a 10% SDS-PAGE gel and run at 110V for 2 hours. Cell proteins were transferred to Immuno-Blot PVDF membrane (Bio-Rad) with a Bio-Rad semidry transfer system, according to the manufacturer's instructions. Equal loading of the protein groups on the blots was evaluated using Ponceaus S (Sigma Chemical Co.), a staining solution designed for staining proteins on PVDF membranes. The PVDF was then blocked with 5% milk in TTBS (tris buffered saline with 0.1% Tween 20) for 1 hour, washed, and then incubated with the primary antibody at dilutions of 1:500 to 1:2000 overnight. The blots were washed x4 with TTBS and incubated for 1 hour with horseradish-peroxidase conjugated anti-IgG antibody (1:5000 to 1:20,000). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus or ECL (Amersham Biosciences, Piscataway, NJ). An autoradiograph was obtained, with exposure times of 10 seconds to 2 minutes. 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 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 (Minneapolis, MN).

Epithelial cell survival assays

Cell viability was analyzed by the Guava EasyCyte mini (Guava Technologies, Hayward, CA). 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). Following incubation with virus and cytokine, cultures were brought to room temperature and equal volume of CellTiter-Glo reagent added. After a two minute mix the plate was read on a Safire plate reader from Tecam, set for chemiluminescence.

Realtime RT-PCR (qRT-PCR)

For cell cultures, total RNA was extracted using the Absolutely RNA Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA) RNA was quantified using the

RiboGreen Kit (Invitrogen, Eugene, OR). For mouse lungs, RNA was extracted with Invitorgen Trizol reagent, cleaned up with Stratagene Absolutely RNA and quantified with the Bio-Rad Experion. Total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit from Bio-Rad. The resulting cDNA (2uL) (from either mouse lungs or human cell line) was then mixed with 48 ul of PCR master mix consisting of Bio-Rad's iQ SYBR Green Supermix, 15 pmol of forward primer and 15 pmol of reverse primer in a 0.2-ml PCR tube (Bio-Rad, Hercules, CA). 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 Ct values, determined using the equation $2^{-\Delta Ct}$. The following primers were used for human TARC (agggacctgcacacagagac, aggtagtcccgggagacagt), IP-10 (aacctccagtctcagcaccatgaa, tgaagcagggtcagaacatccact) and HPRT (ttggaaagggtgtttattcctc, tcccctgttgactggtcatt). For the mouse lung samples, the following primers were used: TARC (ttgtgttcgcctgtagtgcata, caggaagttggtgagctggtata), IP-10 (aacctccagtctcagcaccatgaa, tgaagca gggtcagaacatccact), IL-13 (cagtcctggctcttgcttg, gcgaaacagttgctttgtgt), IFN-γ (gctttggagctcttcctc at, tgagctcattgaatgcttgg), HPRT (cctcatggactgattatggac, cagattcaacttgcgctcatc).

mRNA stability assay

A549 cells were stimulated with RSV (moi 2) with and without added IL-4 (10 ng/ml) for 24 hours and treated with 10 μg/ml actinomycin D to inhibit transcription. Additional harvests were then made at 3 and 6 hours following actinomycin D. Total RNA was isolated using Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA). RNA concentration was measured using QuantiT™ RiboGreen® RNA assay kit (Invitrogen, Carlsbad, CA). Total RNA (1 μg) was reverse transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad). Quantitative realtime RT-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 above. Relative gene expression was calculated and normalized to hypoxanthine phosphoribosyltransferase (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 Act D sample/Ratio of 24 hour RSV or RSV+IL-4 times 100) (GraphPad Software, San Diego, CA).

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. They represent the mean +/- SEM. These methods were performed using GraphPad Prizm 4 for Windows (GraphPad Software, San Diego, CA).

Results

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

RSV infection of airway epithelium is known to cause 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 post-infection, animals were sacrificed and lungs harvested for RNA isolation. Figure 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 upregulated for longer periods (staying slightly upregulated out through day 300). This data demonstrates 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 to 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 towards either a Th1 or a Th2 phenotype, respectively (1, 2, 43, 44). We made use of this model to ask if 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.

Figure 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 as in both conditions there is increased IFN-γ production. It is important to note that the time point examined here (3 days) is two to four days before the appearance of TARC mRNA in mice undergoing an acute RSV infection (Figure 1A).

In Figure 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 towards a Th2 bias leads to a significant increase in TARC production after RSV infection.

In the acute RSV infection shown in Figure 1A, we have no evidence that RSV alone induces Th2 cytokines. In fact, a recent study by N. Lukacs et al demonstrates that the A2 strain of RSV (used in this study) does not induce IL-4 or IL-13 in a Balb/c model (45). They show that, in contrast, the clinical isolate, Line 19, induces both IL-4 and IL-13. It will be of interest to determine if 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 Figure 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 hours (4, 36, 38, 46). We examined time points from 0 to 72 hours for protein production and 0 to 48 hours for mRNA production. In the mRNA studies, we stopped experiments at 48 hours of infection to avoid the variability that occurred with the onset of cell death between 48 and 72 hours. IP-10 mRNA began going up as early as 16 hours, while TARC accumulation began 24 hours post-infection. At 72 hours post-infection TARC protein was going up and at 48 hours post infection TARC mRNA was still rapidly increasing. In contrast, IP-10 mRNA amounts leveled at 48 hours and protein production was returning to baseline by 72 hours. As a composite, the data presented in Figure 2A, shows that in an in vitro model, RSV infection induces IP-10 and TARC in a sequential manner.

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. tumor necrosis factor α (TNF-α interleukin 1β (IL-1β) and interferon β (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 a moi of 2 for 48 hours. The supernatant was harvested, divided into two portions and half of each portion was UV treated to kill the RSV. The UV treated and not UV treated supernatants were then put on fresh A549 cells and the cells cultured for a further 48 hours. Final supernatants were harvested and IP-10 and TARC measured. Figure 2B demonstrates that the UV treated supernatant was incapable of inducing TARC in the secondary culture, while 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 or not. Note that both the TARC and IP-10 levels were greater than those shown in Figure 2A. This is because the ELISA is measuring chemokines produced in both the first supernatant generating incubation (200-400 pg/ml 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 Figure 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 interferons produced by RSV, does not induce any TARC, nor does it augment the low levels of TNFα induced TARC (55, 56). IP-10, on the other hand, 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, while 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 chemoattractant activity (57-59). We were interested in determining if 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 were added to epithelial cultures at the same time as RSV and the samples cultured for various times. Figure 3A demonstrates that exposing lung epithelial cells to both RSV and IL-4 has a dramatic 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 are dramatic and 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 (Figure 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-γ minimally increased RSV-induced TARC protein. We demonstrated in Figure 2C that TNFα induced small amounts of TARC protein and 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 Figure 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 Figure 3C demonstrates that 48 hours after infection, RSV has increased plasma membrane permeability (PI staining and FACS analysis). IL-4 and IL-13 have no effect on the viability changes due to the RSV infection (Figure 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 (Figure 4B). Because qRT-PCR does not detect any TARC transcript in unstimulated cells, statistically, we couldn't examine the ability of RSV to alter TARC mRNA stability compared to 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 hours, stopping transcription with actinomycin D and measuring mRNA levels at 3 and 6 hours 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. This data, in combination with the greatly increased TARC mRNA with the combination of RSV and Th2 cytokines, suggests 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. Figure 5A demonstrates that by 24 hours 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 Figure 5A, Figure 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 made use of NFκB inhibitors to study the effect of NFκB on TARC production. Figure 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). This IκBα cannot be ubiquitinated and degraded and so serves to keep NFκB in the cytosol. Figure 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 JAK 1 inhibitor was used to block STAT6 activity as 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) (63). The JAK1 inhibitor blocked production of TARC when cells were exposed to both RSV and IL-4 (Figure 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. It clearly shows that the JAK1 inhibitor had no effect on TARC production by RSV alone. This is 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, while not being necessary for RSV alone effects on TARC production.

Despite the fact that TARC has been shown to recruit Th2 cells via the CCR4 receptor and is upregulated 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 any significant TARC levels on their own but do provide a STAT6 signal that synergizes with RSV-induced NFκB to up regulate TARC induction. Neither stimuli alone (RSV or IL4/

IL13) generates both a STAT6 and NFκB signal, combined they cooperate to induce significant amounts of TARC (Figure 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. While 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 and it showed only that there was an increase in the transcript (42). We wanted to take a more comprehensive look at 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 upregulation (IP-10 first, TARC second) of the chemokines. Compared to 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 (three week incubation) and a three 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. While our present animal data does not support induction of a Th2 phenotype by RSV alone, it is 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 data 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.

It is interesting that 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 (ie 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 is consistent with this hypothesis.

One interesting hypothesis for the synergy between NFκB and STAT6 is the recruitment of CBP/p300 to promoters by NFκB. STAT6 in contrast to other STAT's has no binding site for and does not recruit CBP/p300 on its own (64). CBP/p300 are multi-functional co-activator 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 utilized 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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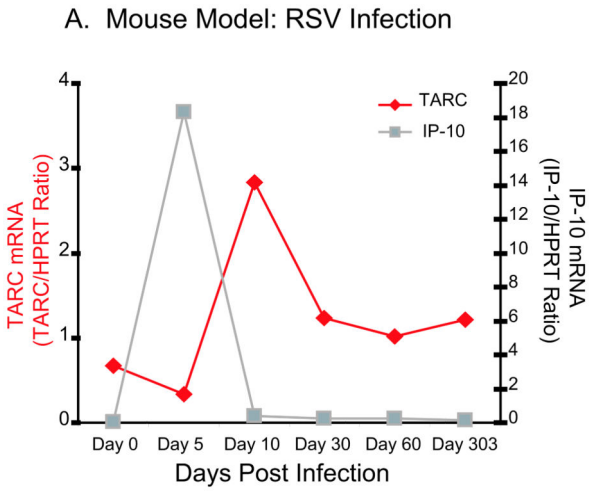

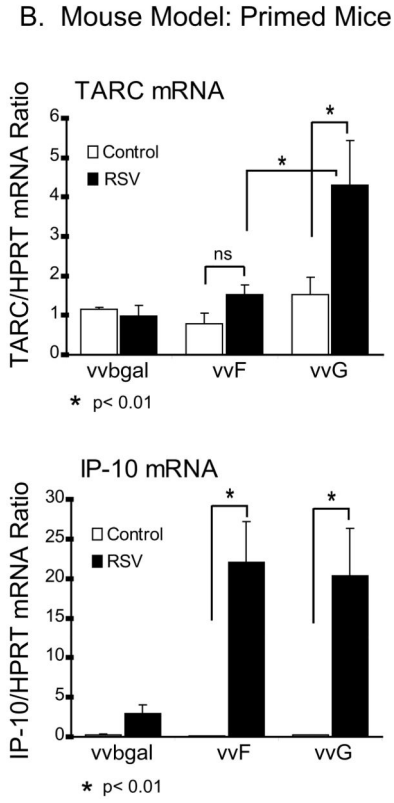

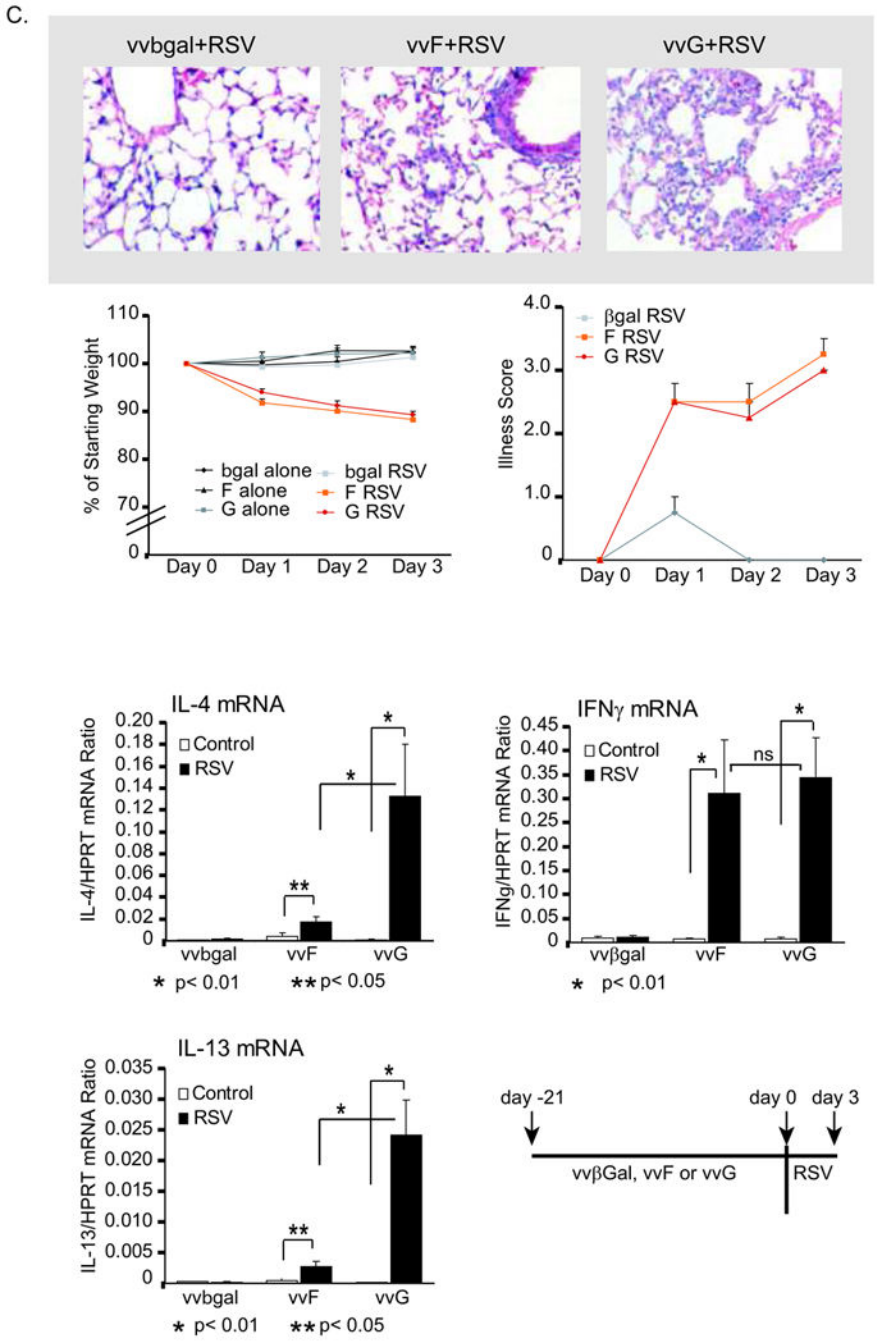

Figure 1.

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 300 days post infection. At designated time points, mice were euthanized as described in the Methods, lungs were harvested and RNA isolated. TARC and IP-10 levels were measured using qRT-PCR. **B.** BALB/c mice (4 per group) were scarified with 3×10^6 PFU of a recombinant vaccinia virus expressing β-galactosidase, the attachment (G) protein of RSV or the fusion (F) protein of RSV. Three

weeks later, mice were challenged intranasally with 2×10^6 PFU 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 Figure 1B, 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 the methods (data is summarized in two graphs, averages +/- standard error). From euthanized animals, one lung from each animal was fixed for histology (hematoxylin and eosin stain) and one lung from each animal was processed for RNA. IL-4, IL-13 and IFN-γ mRNA was 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).

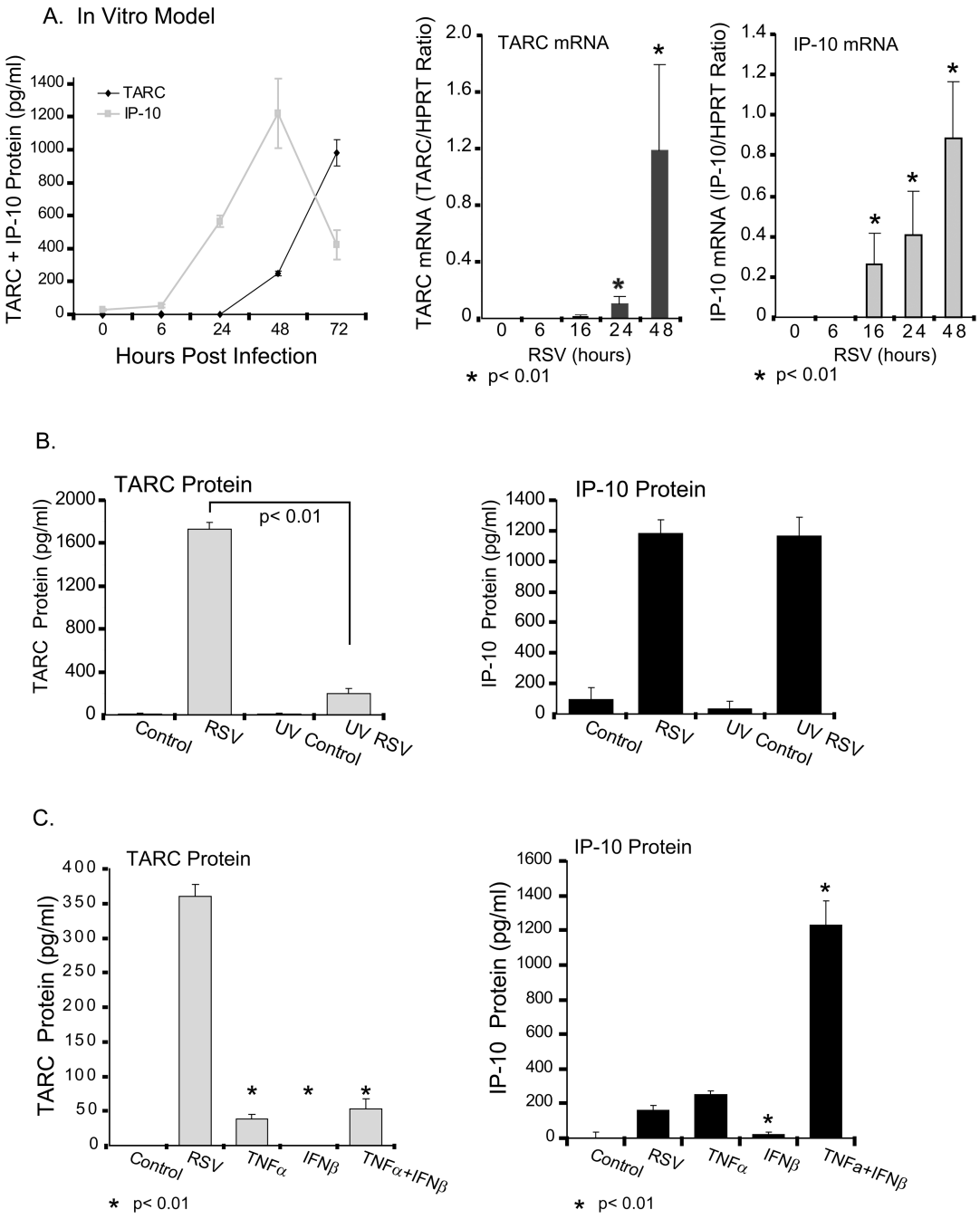

Figure 2.

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 a moi of 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 ELISA's. RNA was analyzed as described in the Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. Significance was measured

using a one-tailed Student's *t* test (Graphpad Prism). **B.** A549 cells were cultured with and without RSV (moi of 2) for 48 hours. The supernatant was removed, split in half and one half UV treated. The four supernatants (Control, UV Control, RSV and UV RSV) were then put on fresh A549 cells cultured to 80% confluence and incubated a further 48 hours. The final supernatant was then harvested and TARC and IP-10 measured by ELISA. Significance (RSV supernatant versus UV RSV supernatant) was measured using a one-tailed Student's T test (Graphpad Prism). **C.** A549 cells were cultured at 80% confluence and then treated with either RSV (moi of 2), TNF-α (10 ng/ml), IFN-β (1000 units/ml), or TNF-α and IFN-β together. After 48 hours, supernatants were harvested and TARC and IP-10 measured by ELISA. Significance (all groups compared to RSV infected sample) was measured using a one-tailed Student's *t* test (Graphpad Prism).

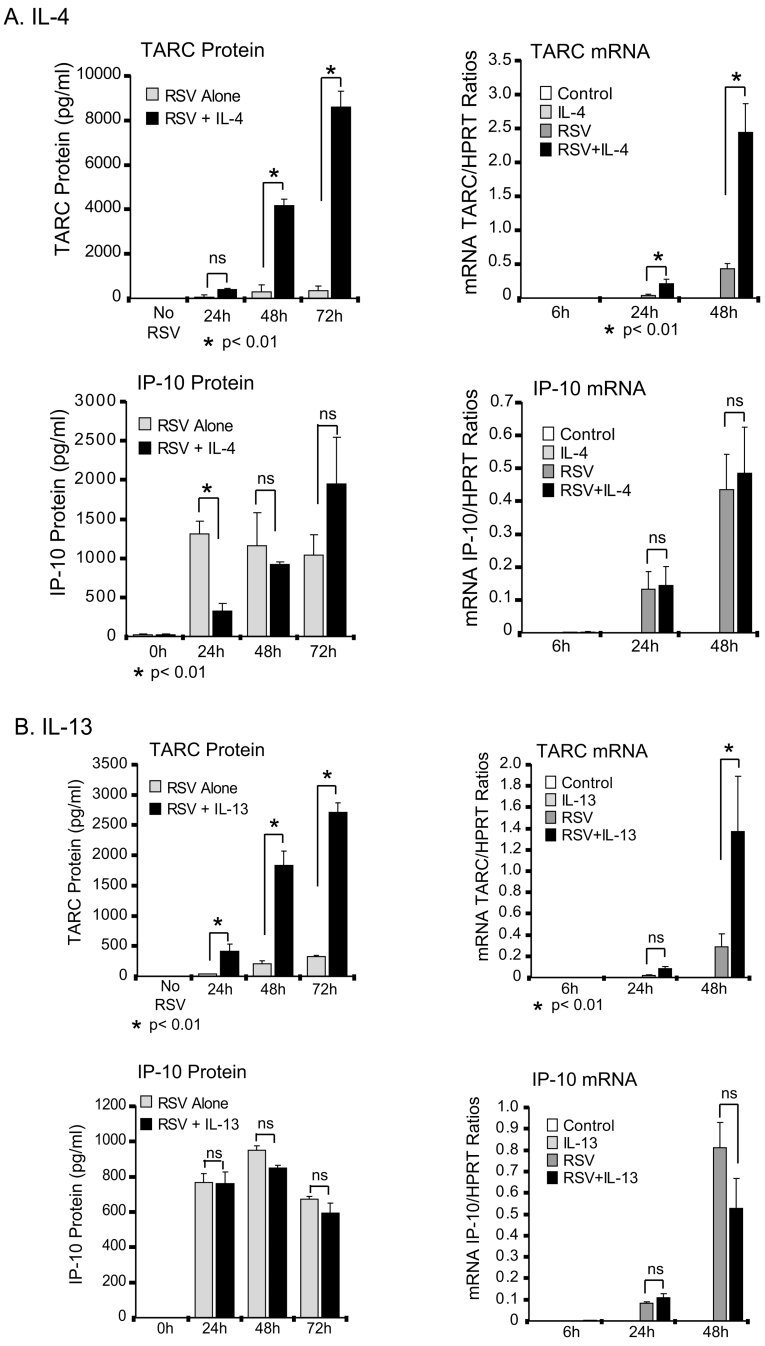

Figure 3.

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 of 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 ELISA's. RNA was analyzed as described in the Methods. Each graph (Tarc and IP-10) is a composite of three separate

experiments. Significance (RSV alone compared to 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 of 2) with and without added IL-13 (10 ng/ml). Cells were infected with RSV (moi of 2) at the same time as the addition of 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 ELISA's. RNA was analyzed as described in the Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. Significance (RSV alone compared to RSV+IL-13) was measured using a one-tailed Student's *t* test (Graphpad Prism).

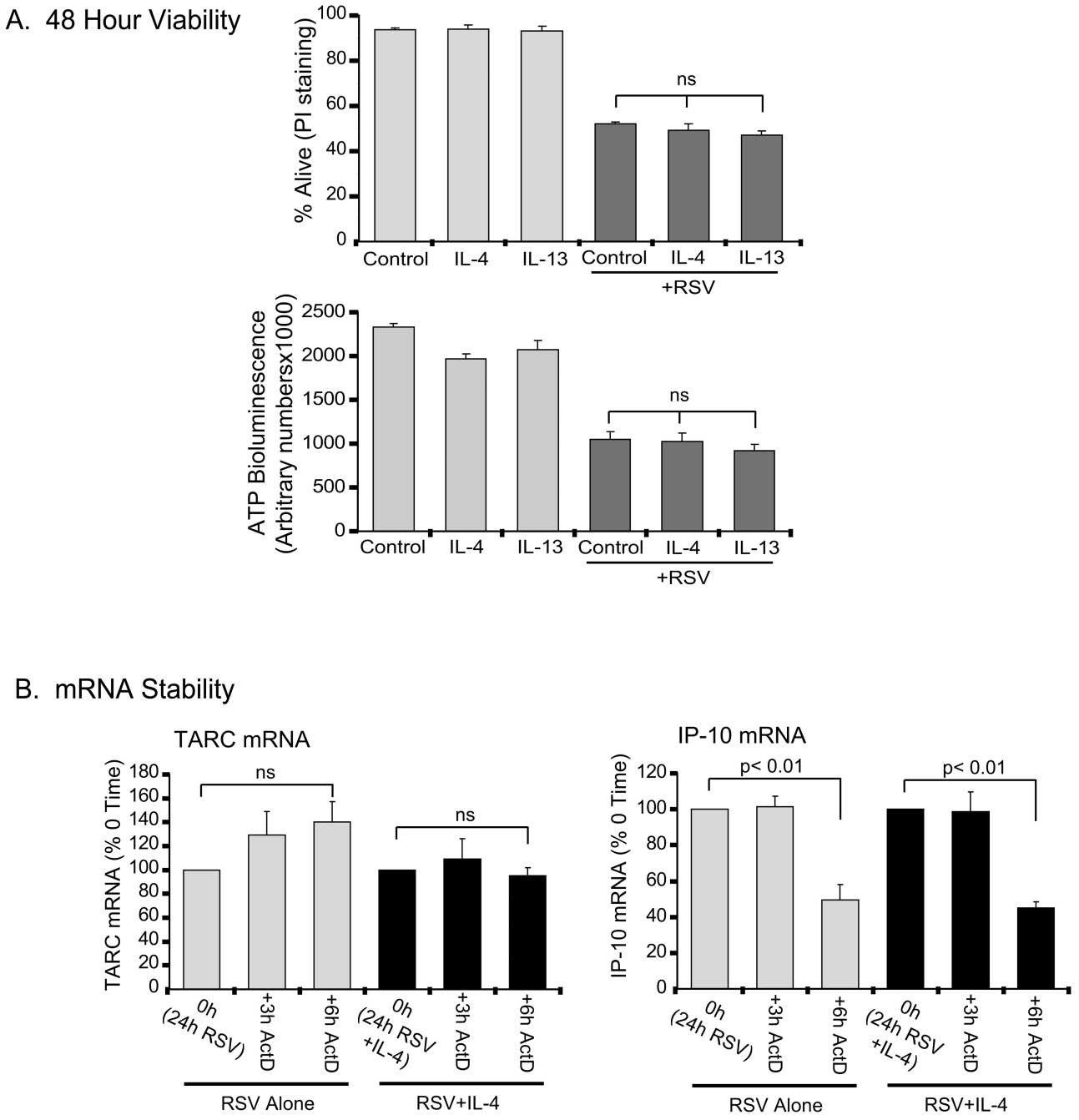

Figure 4.

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 of 2) with and without added IL-4 (10 ng/ml). After 48 hours of culture, cell viability was measured using either PI staining (Guava EasyCyte 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 of 2. After 24 hours of

culture, Actinomycin D was added (10 ug/ml) and cells cultured for an additional three or six hours. Cells were harvested for RNA. RNA was analyzed as described in the Methods. Each graph (Tarc and IP-10) is a composite of three separate experiments. For calculations, mRNA levels at 24 hours of RSV or RSV+IL-4 were set as 100% and 3 or 6 hour actinomycin % calculated (Ratio of Act D sample/Ratio of 24 hour RSV or RSV+IL-4 times 100). Significance was measured (each actinomycin sample was compared to the amount of mRNA (TARC or IP-10) present at 24 hours (before the addition of Actinomycin D) using a one-tailed Student's *t* test (Graphpad Prism).

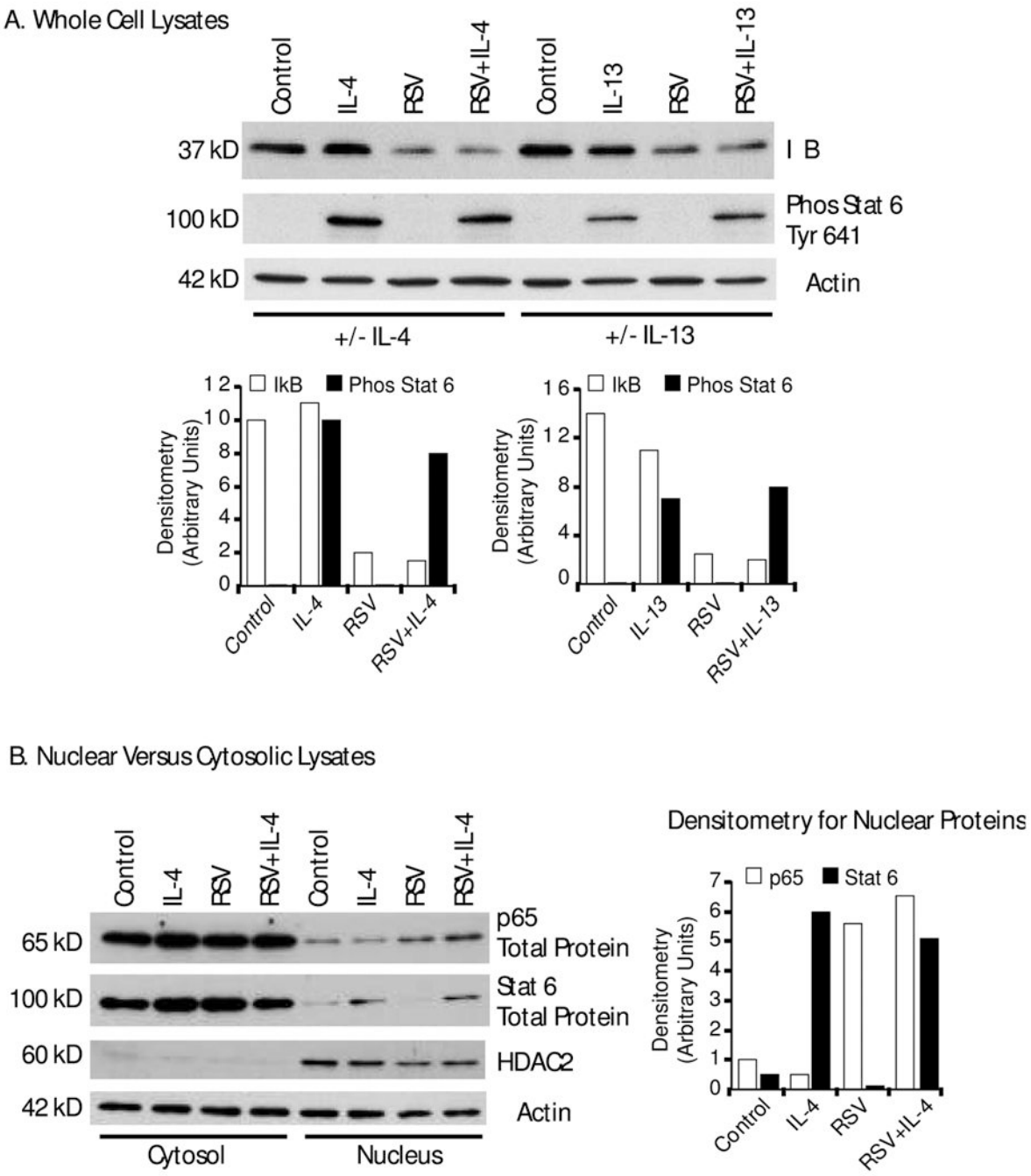

Figure 5.

RSV infection alone activates NFκB and not STAT6. IL-4 exposure alone activates STAT6 and not NFκB. **A.** A549 cells were infected with RSV (moi of 2) with and without IL-4 (10 ng/ml) or IL-13 (10 ng/ml) for 24 hours. Whole cell lysates were obtained and Western analysis performed for IκBα (37 kD) and phosphorylated STAT6 (tyrosine 641). Equal loading was performed by analyzing the same blot for β actin (42 kD). Primary and secondary antibody dilutions for IκBα were of 1:1000 and 1:10000 respectively. Primary and secondary antibody dilutions for phosphorylated STAT6 were 1:500 and 1:5000

respectively. Also shown is densitometry for both IκBα and phosphorylated STAT6 bands. **B.** A549 cells were infected with RSV (moi of 2) with and without IL-4 (10 ng/ml) for 24 hours. Cells were harvested and nuclear and cytosolic fractions isolated as described in the methods. Western analysis was performed for total p65 and total STAT6. Controls for protein loading were done by staining for β 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.

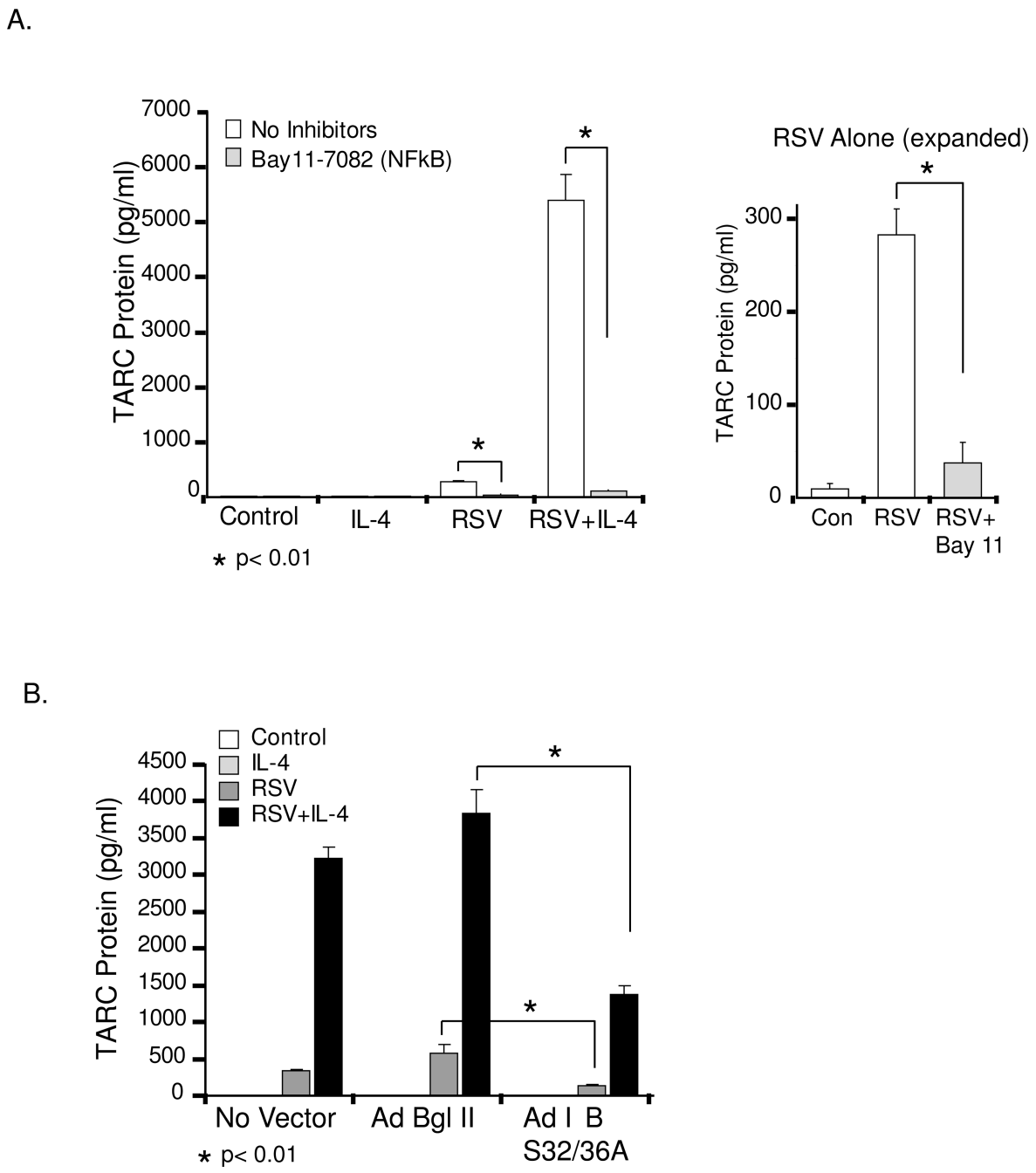

Figure 6.

Inhibition of NFκB blocks TARC production after RSV infection and after RSV plus IL-4 exposure. **A.** A549 cells were cultured at 80% confluence and then infected with RSV (moi of 2) with and without added IL-4 (10 ng/ml). Some samples were also treated with an NFκB inhibitor, Bay11-7082 (50 uM). Cells were cultured with treatments for 48 hours, supernatants harvested and TARC protein measured by ELISA. On the right is graph showing an expanded version of the RSV alone data. Significance (RSV alone compared to RSV+Bay11-7082 and RSV+IL-4 alone compared to RSV+IL-4+Bay11-7082) was

measured using a one-tailed Student's *t* test (Graphpad Prism). B. A549 cells were cultured at 80% confluence and then infected with adenovirus vectors expressing either Bgl II (negative control) or IκBα S3236A (non-degradable IκBα mutant). 16 hours later cells were infected with RSV (moi of 2) with and without added IL-4 (10 ng/ml). Cells were cultured with treatments for a further 48 hours, supernatants harvested and TARC protein measured by ELISA. Significance (RSV alone compared to RSV+ IκBα S3236A and RSV+IL-4 alone compared to RSV+IL-4+ IκBα S3236A) was measured using a one-tailed Student's *t* test (Graphpad Prism).

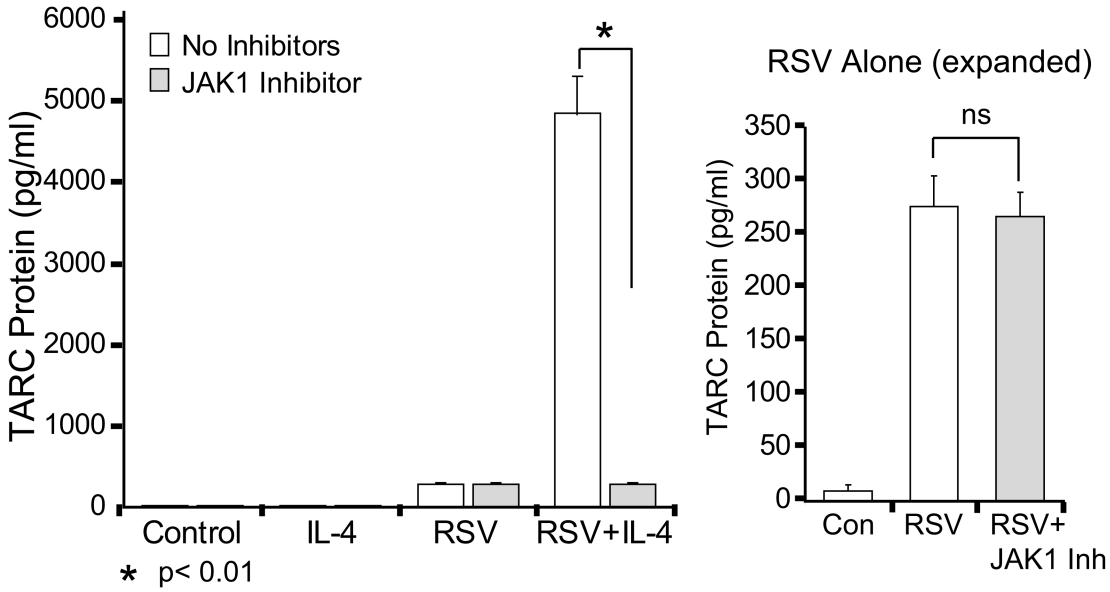

Figure 7.

Inhibition of JAK1 blocks TARC production after RSV plus 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 of 2) with and without added IL-4 (10 ng/ml). Some samples were also treated with a JAK1 inhibitor (10 uM). Cells were cultured with treatments for 48 hours, supernatants harvested and TARC protein measured by ELISA. On the right is a graph showing an expanded version of the RSV alone data. Significance (RSV alone compared to RSV+JAK1 inhibitor and RSV+IL-4 alone compared to RSV+IL-4+JAK1 inhibitor) was measured using a one-tailed Student's *t* test (Graphpad Prism).

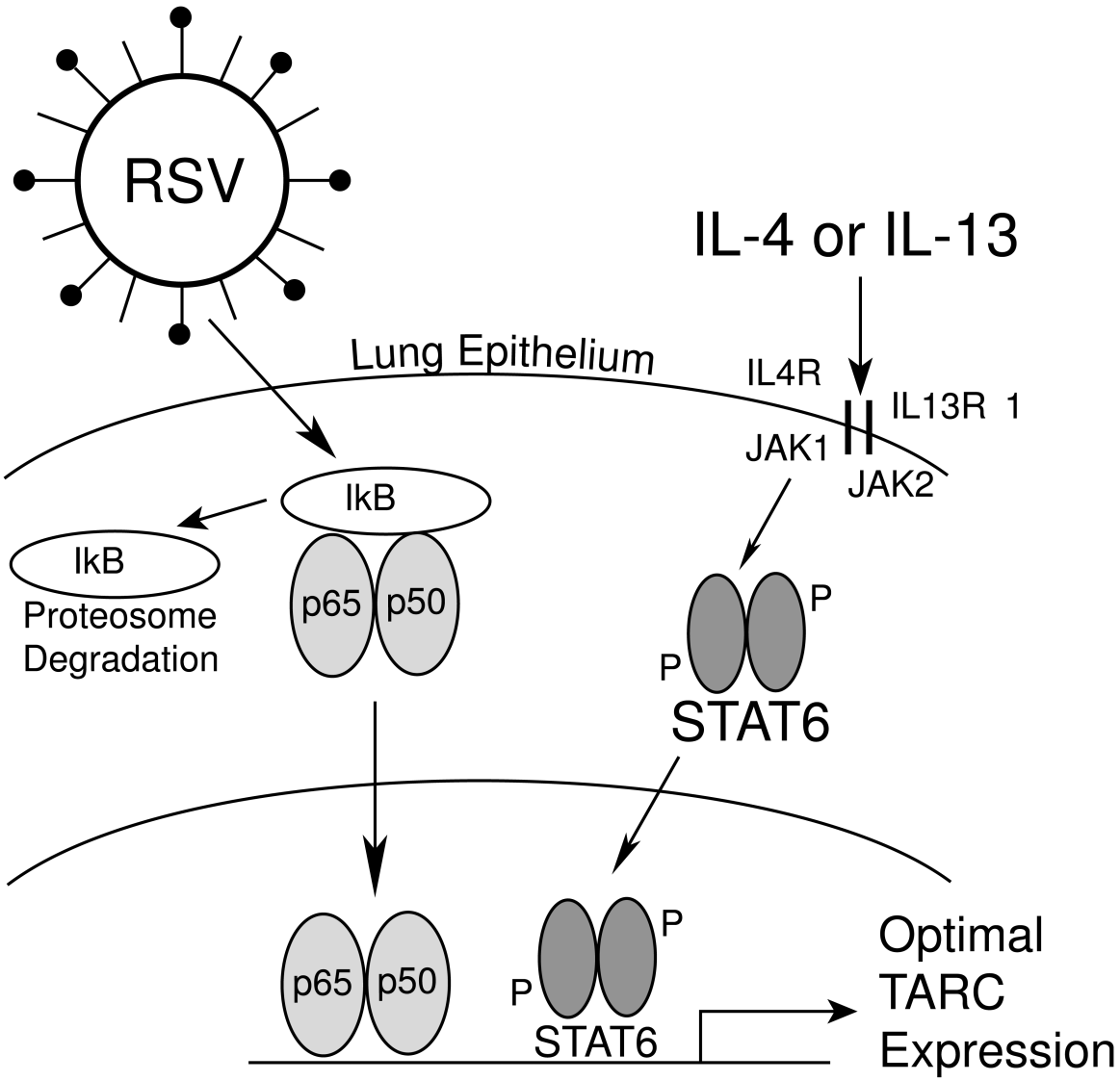

Figure 8.

Summary of Findings: *TARC* gene expression in lung epithelial cells (or in lungs from mice) is optimally driven by a combination of STAT6 and NFκB. RSV induces NFκB and low levels of TARC. IL-4 or IL-13 induces STAT6 activation but not NFκB activation. Together RSV infection and IL-4 or IL-13 exposure lead to activation of both NFκB and STAT6, resulting in high levels of TARC production.