

Differential cytokine mRNA expression in *Dermatophagoides farinae* allergen-sensitized and respiratory syncytial virus-infected mice

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ABSTRACT – The interaction between mite allergen sensitization and respiratory syncytial virus (RSV) infection at the level of cytokine mRNA expression was examined in a murine model in the present study. Primary RSV infection enhances expression of inflammatory cytokines such as IL-6, IFN- γ , and eotaxin in the lung and upregulates the expression of Th₂-like cytokines IL-10 and IL-13 in the spleen in BALB/c mice. Mite antigen-sensitized and RSV-infected (ASRSV) mice show enhanced ($P < 0.05$) total serum IgE compared to antigen-sensitized mice. However, the levels of viral mRNA in the lung tissues are comparable between RSV-infected and ASRSV mice. It is concluded that compartmentalization of cytokine expression following RSV infection plays a role in the augmentation of Th₂-like and IgE antibody response to RSV. © 2000 Éditions scientifiques et médicales Elsevier SAS

allergic inflammation / respiratory syncytial virus / *Dermatophagoides farinae*

1. Introduction

Bronchial asthma is characterized as a chronic inflammatory disease of the airways, and Th₂-like cytokines mediate this process [1–3]. Mite allergen exposure and viral respiratory tract infections in infancy and childhood are major risk factors for bronchial asthma [4, 5]. The respiratory syncytial virus (RSV) is the major respiratory pathogen of infants and young children [6, 7]. RSV causes lower respiratory tract infection in infants and induces Th₂-like immunity and an RSV specific IgE antibody response [8, 9]. Dust mites are an important source of the inhalant allergens implicated in asthma.

The mouse provides a good model to study the effect of viral infection. RSV infection prolongs methacholine-induced airway hyperresponsiveness and augments anaphylactogenic sensitization to aeroantigens in mice [10, 11]. RSV infection also enhances airway inflammation in guinea pigs [12]. Dust mite [*Dermatophagoides farinae* (*Df*)] protease enhances the infectivity and replication of influenza virus in the lungs of mice [13]. However, the

mechanism of interaction between allergen sensitization and RSV infection remains unclear. Acute RSV infection induces IFN- γ in mice [14], which is a suppressor of Th₂-like immune responses. Thus, RSV induction of Th₂-like immunity and its synergy with an allergic condition has remained paradoxical.

We hypothesized that the compartmentalization of cytokine expression during RSV infection may account for the RSV-induced Th₂-like immunity, which in turn, may synergize with allergen sensitization. We compared mRNA expression of a number of cytokines examined in both a local (e.g., lung) and a peripheral lymphoid organ (e.g., spleen) of mice exposed to RSV, allergens, or allergen plus RSV. The results indicate that acute RSV infection upregulates the expression of pro-inflammatory cytokines and eotaxin in the lung and Th₂-like cytokines in the spleen.

2. Materials and methods

2.1. Animals

Female BALB/c mice, 6–8 weeks of age, housed in pathogen-free conditions at the James A. Haley VA Hos-

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pital Animal Center, were used in the experiments. Procedures were reviewed and approved by the University of South Florida and James A. Haley VA Hospital Committee on Animal Research. All experiments were repeated at least twice.

2.2. RSV

The A2 strain of human RSV [American Type Culture Collection (ATCC), Rockville, MD] was propagated in HEp-2 cells (ATCC). For the preparation of a large stock of the virus, HEp-2 cells were infected with the ATCC stock virus and cell supernatant was collected 5 to 6 days postinfection, centrifuged at 2000 rpm for 10 min at 4 °C. The clear supernatant was collected and kept frozen at -70 °C until used. An aliquot of the viral suspension was irradiated with ultraviolet (UV) light for 15 min on ice to inactivate the virus.

2.3. Mode of infection

Using a well-characterized model of RSV infection [15], mice, under light anesthesia [pentobarbital (Nembutal®); Abbott Laboratories, North Chicago, IL; 0.06g/kg intraperitoneally (i.p.)], were inoculated intranasally (i.n.) with RSV in 50 µL of suspension containing 5×10^5 PFU/mL. RSV infection was confirmed by reverse transcription (RT)-PCR for RSV N protein mRNA expression in the lung. In some mice, the presence of RSV antigens in lung tissue was also confirmed by immunohistochemical methods.

2.4. Df sensitization and challenge

Four groups of mice were studied in this report (figure 1). Control animals were sham-infected with phosphate-buffered saline (PBS; Gibco BRL, Gaithersburg, MD) or UV-inactivated RSV. RSV-infected mice were infected with live RSV as described above. *Df* allergen-sensitized (AS) and *Df* allergen-sensitized plus RSV-infected (ASRSV) mice were immunized one time i.p. with 10 µg per mouse of *Df* allergens (Greer Laboratories, Lenoir, NC) in aluminum potassium sulfate (alum), 1 mg per mouse. Two weeks later these same mice were inoculated i.n. once daily with 50 µg of *Df* in 50 µL of PBS per mouse for three consecutive days. To facilitate pulmonary aspiration of *Df*, mice were lightly anesthetized, as described above, for RSV inoculation. ASRSV mice were infected with RSV 24 h after the last i.n. *Df* inoculation. Four days after the final *Df* inoculation, goblet cell metaplasia and peribronchovascular eosinophilic infiltration could be demonstrated in the lung tissue (data not shown).

2.5. Sample collection and preparation for evaluation

Half of each group of mice were killed with an overdose (0.6 g/kg) of pentobarbital four days after RSV infection, as described previously [15], or five days after the final *Df* sensitization. The lungs and spleens were removed for RNA isolation, quick-frozen, and stored at -70 °C.

2.6. Total RNA isolation and semiquantitative RT-PCR analysis

Lung or spleen tissue samples were thawed and homogenized using a tissue homogenizer. All reagents for RNA

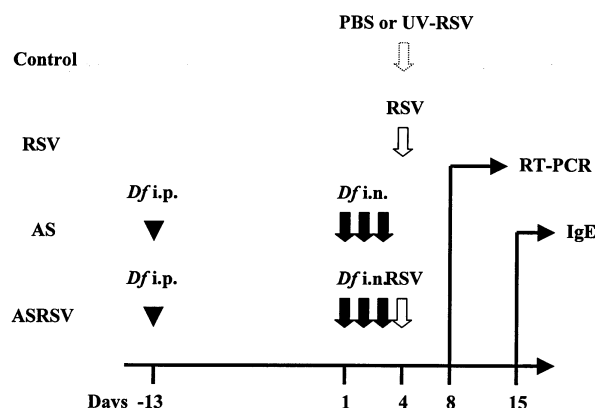


Figure 1. Study design. Control mice were sham-infected with PBS or UV-inactivated RSV on day 4. RSV-infected (RSV) mice were infected with live RSV on day 4. *Df* allergen-sensitized (AS) and *Df* allergen-sensitized plus RSV-infected (ASRSV) mice were immunized one time i.p. with *Df* plus alum, and two weeks later these mice were inoculated i.n. once daily with *Df* for three consecutive days (day 1 to 3). ASRSV mice were infected with RSV 24 hours after the final *Df* allergen inoculation. Mice were sacrificed on day 8 for removal of lungs and spleens for RT-PCR. For the IgE ELISA, sera were obtained from a separate set of mice on day 15.

extraction and RT-PCR were purchased from Gibco BRL. Total RNA was isolated with TRIzol® reagent. Five micrograms of total RNA was reverse transcribed in 20 µL of 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 75 mM KCl, 3 mM MgCl₂, 0.5 mM deoxyribonucleoside triphosphate (dNTP), 2 U/µL of Moloney murine leukemia virus reverse transcriptase, and 25 ng/µL of random hexamer primers. PCR was performed in 20 µL of reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton-X100, 1.25 mM dNTP], 1 µM each of sense-strand and antisense-strand primers, and 0.01 U/µL of *Taq* polymerase. For PCR, mouse gene specific primers were designed from the known sequence of the β -actin, IL-4, IL-5 and IFN- γ or from the previous reports (IL-1 α , IL-1 β , IL-6, TNF- α [16], IL-10, IL-13, eotaxin [17], RSV N protein [18]) (table I). Twenty-five to thirty-five amplification cycles (denaturation, 94 °C for 30 s; annealing, 54 °C for 30 s; extension 72 °C for 1 min) were used as previously reported [18]. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. PCR products were quantified by densitometry using the advanced quantifier software (BioImage, Ann Arbor, MI) as previously described [18], and results were shown as the band intensity relative to β -actin.

2.7. Quantitation of total and *Df*-specific serum IgE

Sera were obtained from the rest of the mice 11 days after RSV infection or 12 days after the final *Df* inoculation in a separate set of experiments. Diluted sera were first incubated in rat anti-mouse IgE antibody (clone R35-72, PharMingen, San Diego, CA) or *Df* extract (5 µg/mL) ovalbumin (used as a control antigen, 5 µg/mL) coated

Table I. Primer sequences for cytokines, eotaxin, and RSV N protein.

Primers		Sequence
β-actin	(sense)	5-GAC ATG GAG AAG ATC TGG CAC-3
	(antisense)	5-TCC AGA CGC AGG ATG GCG TGA-3
IL-1α	(sense)	5-GTT CTG CCA TTG ACC ATC CTC-3
	(antisense)	5-CCA GAA GAA AAT GAG GTC GGT C-3
IL-1β	(sense)	5-TGA CGG ACC CCA AAA GAT GAA G-3
	(antisense)	5-CTG CTT GTG AGG TGC TGA TGT A-3
IL-4	(sense)	5-GAC AAA AAT CAC TTG AGA GAG A-3
	(antisense)	5-ACG AGT AAT CCA TTT GCAT GAT-3
IL-5	(sense)	5-AAG GAT GCT TCT GCA CTT GA-3
	(antisense)	5-ACA CCA AGG AAC TCT TGC-3
IL-6	(sense)	5-TTG CCT TCT TGG GAC TGA TGC T-3
	(antisense)	5-GTA TCT CTC TGA AGG ACT CTG G-3
IL-10	(sense)	5-GGA CTT TAA GGG TTA CTT GGG TTG CC-3
	(antisense)	5-CAT TTT GAT CAT CAT GTA TGC TTC T-3
IL-13	(sense)	5-TCT TGC TTG CCT TGG TGG TCT CGC-3
	(antisense)	5-GAT GGC ATT GCA ATT GGA GAT GTT G-3
IFN-γ	(sense)	5-TCT GGA TCC ATG AAC GCT ACA CAC TG-3
	(antisense)	5-CAC CTC GAG TCA GCA GCG AC-3
TNF-α	(sense)	5-CCA GAC CCT CAC ACT CCA GAT-3
	(antisense)	5-AAC ACC CAT TCG CTT CAC AG-3
Eotaxin	(sense)	5-GGG CAG TAA CTT CCA TCT GTC TCC-3
	(antisense)	5-CAC TTC TTC TTG GGG TCA GC-3
RSV N	(sense)	5-GCG ATG TCT AGG TTA GGA AGA A-3
	(antisense)	5-GCT ATG TCC TTG GGT AGT AAG CCT-3

ELISA plates (Costar, Cambridge, MA), and then incubated with biotin-conjugated rat anti-mouse IgE monoclonal antibody (clone R35–92, PharMingen). The color reaction was developed using streptavidin-horseradish peroxidase conjugate and ABTS as substrate. The optical density (OD) of the reaction was determined using an automatic ELISA plate reader at 405 nm. The results of total IgE were expressed as µg/mL utilizing mouse IgE standard (PharMingen), and those of *Df*-specific IgE were expressed as OD₄₀₅ values.

2.8. Quantitative competitive RT-PCR

A quantitative competitive (QC) RT-PCR was used to quantitate the amount of RSV mRNA according to the previous report [19]. Briefly, the competitive template was generated from the lung cDNA library from RSV-infected BALB/c mice using RSV *ns1-ns2* gene specific primers, sense; 5'-GCA GCA ATT CAT TGA GTA TG-3' and anti-sense; 5'-TAA TTT TCA GGC TCC ATC TG-3'. The PCR product (983 bp) was cloned into pCR2.1 (Invitrogen, San Diego, CA) and then digested with *AccI* and *HincII* to delete the 408-bp fragment. After ligation, the plasmid contained NS1-NS2 sequences, which were recognized by the same primers to produce a 575-bp PCR product. QC-RT-PCR was performed in 20 µL of reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton-X100, 1.25 mM dNTP], 250 µM each of primers, 1 µL of RT product, dilutions of the competitive template (0.5 to 2 500 pg/mL) and 0.01 U/µL of *Taq* polymerase. QC-RT-PCR products were separated by ethidium bromide-stained gels and scanned by densitometry as described above for semiquantitative RT-PCR. The amount of RSV mRNA was determined relative to the known amount of competitive

plasmid according to the previous report [20]. In brief, the log₁₀ of the ratio of the band intensity of the competitive plasmid over the wild-type fragment is plotted as a function of the log₁₀ of the concentration of the competitive plasmid. This yields a linear plot and interpolation on the plot for Y value; 0 gives the amount of RSV mRNA present in the test sample. The assay was reproducible and quantitation was linear (not shown). The coefficient of variation between experiments was less than 10%.

2.9. Statistical analysis

All data were expressed as mean ± SEM. For comparison of the two different groups, Student's *t* test was used. Differences between groups were considered significant at *P* < 0.05. All analyses were performed on a Macintosh computer (Apple Computer Inc., Cupertino, CA) with Statview II software (Abacus Concepts, Berkeley, CA).

3. Results

3.1. RSV infection enhances the expression of specific cytokines

The RSV infection of BALB/c mice has been established previously [15]; i.n. administration of 50 µL of RSV (5 × 10⁵ PFU/mL) induces an infection of lung cells that reaches a peak at 4–6 days after infection. N protein mRNA was detected in the lung tissues of all live RSV-infected BALB/c mice and not detected in sham- or UV-inactivated RSV-infected mice by RT-PCR on day 2 after infection. RSV antigen was detected immunohistochemically in lung tissues at day 4 in RSV-infected mice but not in sham- or UV-inactivated RSV-infected mice (data not shown).

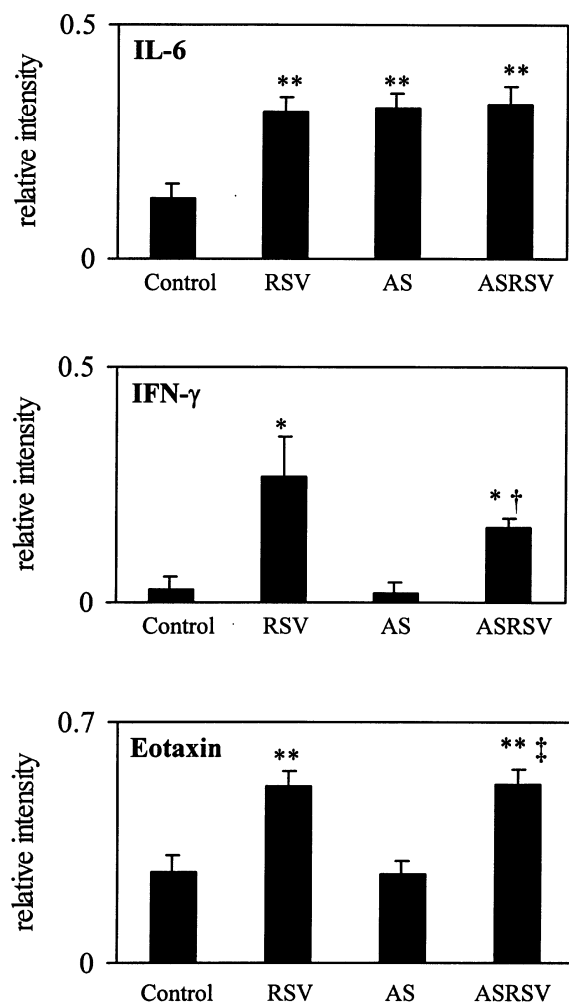


Figure 2. The expression of IL-6, IFN- γ and eotaxin mRNA in lung tissue. Total RNA was extracted from lung tissues of four groups of mice [control, RSV-infected (RI), *Df*allergen-sensitized (AS) and *Df*allergen-sensitized plus RSV-infected (ASRSV)] four days after RSV infection or 5 days after the final *Df* inoculation. PCR amplifications were performed using mouse gene specific primers. The products were separated on agarose gels and quantified by densitometry. All data were normalized to β -actin. Bars represent mean ($n = 13$) $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, NS: not significant.

Four days after RSV inoculation, cytokine mRNA expression in both the lung and spleen tissues of both control and RSV-infected mice were examined by RT-PCR using primers for IL-1 α , IL-1 β , IL-4, IL-5, IL-6, IL-10, IL-13, eotaxin, TNF- α , and IFN- γ . There was a significant difference between control and RSV-infected mice in lung samples for the cytokines, IL-6 ($P < 0.01$), eotaxin ($P < 0.01$) and IFN- γ ($P < 0.05$) (figure 2), and in spleen tissues, for IL-10 ($P < 0.01$) and IL-13 ($P < 0.01$) (figure 3). The other cytokines in both the lung and spleen tissues did not show any significant differences (data not shown).

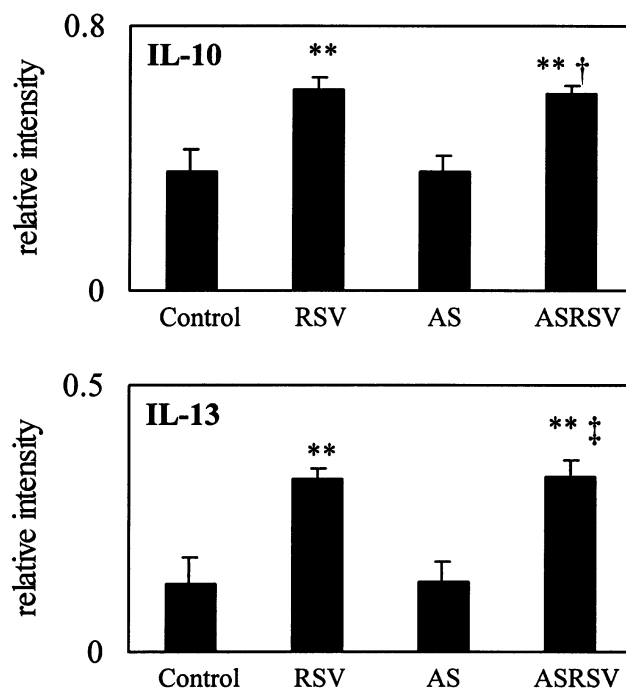


Figure 3. The expression of IL-10 and IL-13 mRNA in spleen tissue. PCR amplifications were performed with total RNA isolated from spleen tissues. The bands were quantified by densitometry and normalized to β -actin. Bars represent mean ($n = 13$) $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, NS: not significant.

3.2. RSV infection enhances the mRNA expressions of IFN- γ and eotaxin in the lungs and IL-10 and IL-13 in the spleens of *Df*-sensitized mice

To determine the molecular mechanism of synergy between allergic predisposition and RSV infection, cytokine mRNA expression was compared between *Df*allergen-sensitized AS and ASRSV mice. IL-6 in lung tissue was the only cytokine examined here, which was significantly enhanced by only *Df* inoculation compared to control mice (figure 2) ($P < 0.01$) four days after the final *Df* challenge. However, subsequent RSV infection did not show additional effects on *Df*-induced IL-6 mRNA expression. The mRNA expression of IFN- γ ($P < 0.05$) and eotaxin ($P < 0.01$) in the lungs and IL-10 ($P < 0.01$) and IL-13 ($P < 0.01$) in the spleens of AS mice was significantly enhanced by subsequent RSV infection (figures 2 and 3).

3.3. RSV infection enhances IgE production in *Df* allergen-sensitized mice

The total and *Df*-specific serum IgE levels were determined by ELISA in each of the four groups of mice. Neither control nor RSV-infected mice showed any detectable total and *Df*-specific IgE. Ova specific IgE was not detected in the four groups of mice, suggesting the specificity of *Df*-specific IgE ELISA. Total serum IgE increased significantly ($P < 0.05$) in ASRSV mice compared to AS mice, whereas *Df*-specific IgE was comparable between AS and ASRSV mice (figure 4).

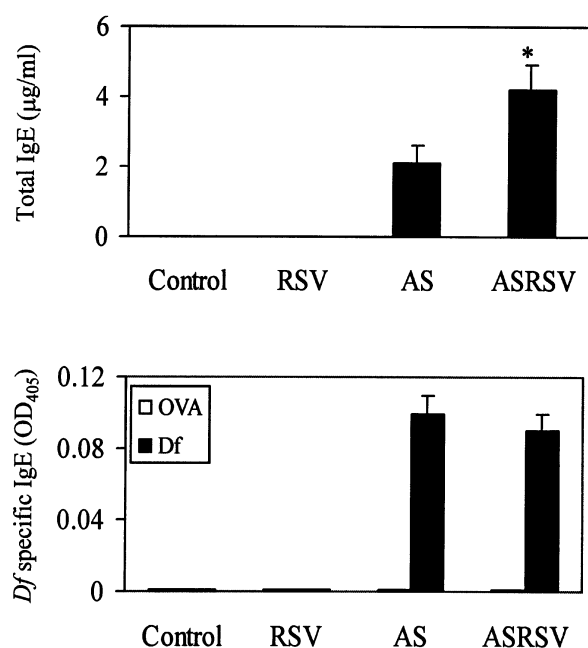


Figure 4. Total and *Df*-specific serum IgE value. Total and *Df*-specific IgE levels were determined in AS and ASRSV mice 11 days after RSV infection or 12 days after the final *Df* inoculation, by ELISA. Bars represent mean ($n = 4$) $\pm$ SEM. * $P < 0.05$, NS: not significant.

3.4. *Df* allergen sensitization does not increase RSV replication in lung tissue

To compare the RSV replication between RSV and ASRSV mice, QC-RT-PCR was performed in the presence of a plasmid containing the NS1-NS2 sequence. The amount of RSV mRNA was determined by taking the $\log_{10}$ of the ratio of the band intensity of the competitive plasmid over the wild-type fragment, and plotting it as a function of the $\log_{10}$ of the concentration of the competitive plasmid. This yields a linear plot and interpolation on the plot for Y value: 0 gives the amount of RSV mRNA present in the test sample. The assay was reproducible and quantitation was linear (not shown). The coefficient of variation between experiments was less than 10%. The amount of RSV mRNA was comparable between RSV and ASRSV mice [RSV: 49.8 ± 5.6 pg/mL ($n = 8$); ASRSV: 44.9 ± 3.6 pg/mL ($n = 8$); $P > 0.1$].

4. Discussion

A major finding of this study is that the lung and the peripheral lymphoid organ tissues respond differently to acute RSV infection in terms of their cytokine gene expression. The RSV-infected mice showed significant increases in the expression of IL-6, eotaxin, and IFN- γ in their lung tissue. The RSV-induced IL-6 in the lung is consistent with the increased production of IL-6 by RSV infection in vitro of the human bronchial epithelial cell [21] and human alveolar macrophages [22]. In addition, increased expres-

sion of eotaxin, which is chemotactic for eosinophil [25], is found only in the lungs of RSV-infected mice.

In contrast to the lung, the transcripts for two important cytokines, IL-10 and IL-13, are detected in the spleen tissues of RSV-infected mice. The detection of these transcripts in spleen cells suggests the possibility of upregulation of these cytokine mRNAs. IL-10 functions as an immunosuppressive regulator of Th₁-like cells; however, the role of IL-10 in RSV infection is unknown [26, 27]. IL-13 stimulates IgE synthesis by B cells and upregulates the expression of surface antigens, CD23, CD71, and HLA-DR [28], raising the possibility that an important physiological function of this Th₂-like cytokine is to stimulate IgE dominant humoral immunity in response to RSV infection.

It is worth noting that the results of present studies, although revealing, suffer from two drawbacks. First, cytokine expression was examined only at the mRNA level and these cytokines may be regulated at the posttranslational level. Second, the cytokine expression of the lung differs from that of spleen, presumably due to different cell populations, but these cell populations remain to be identified. For instance, epithelial cells and the macrophages are the major targets of RSV infection and are likely to contribute to this cytokine increase in the lung, whereas the lymphocytes contribute to the production of IL-13 and IL-10 in the spleen.

Another important finding is the interaction between allergen sensitization and RSV infection. Because the default immune response in utero and at birth are Th₂-like [29] and infants may be exposed to allergens in utero and immediately following birth before they are exposed to RSV, we chose to compare cytokine and antibody responses in mice exposed to RSV or allergens or allergens plus RSV. Consistent with the upregulation of Th₂-like cytokine mRNAs, total serum IgE antibody level is significantly enhanced in ASRSV mice compared to AS mice. Several investigators reported an antigen-specific IgE enhancement in animals with prior RSV [30, 31], influenza [32] and parainfluenza-3 virus [33] infection. In contrast, in this study, a general increase in total IgE was not accompanied by an increase in *Df*-specific IgE, suggesting that this increase is probably due to IgE antibodies specific to RSV. However, the detection of RSV-specific IgE is difficult and has been controversial, although recently RSV and RSV-G protein-induced IgE antibodies were reported [34, 35].

Acute RSV infection also induces the expression of IFN- γ mRNA, which plays an important regulatory role in the differentiation of CD4⁺ T cells to Th₁-like cells and decreased allergic response [36]. RSV infection increases IFN- γ protein in murine lung tissue [37, 38]. The total serum IgE level measured at four days after RSV infection showed a decline in IgE in the ASRSV mice compared to AS mice (unpublished data). This is in contrast to the findings in children [8, 9] that RSV infection induces a Th₂-like immunity. The role of IFN- γ in immunity to RSV remains unclear. There are two possibilities. First, the short-term IFN- γ production caused by RSV infection does not differentiate in specific CD4⁺ T cells into Th₁ or Th₂ cells in mice [39]. It is thus likely that the increase in IFN- γ production in response to RSV infection is transient and

RSV subsequently induces a Th₂-like response. Secondly, in BALB/c mice, IFN- γ expression is elevated locally in the lung but not in spleen tissues; this lung expression of IFN- γ does not affect the ability of spleen to induce a Th₂-like cytokine response to RSV. In this study, the cytokine mRNA expression in whole lung and spleen tissues was examined by a semiquantitative RT-PCR analysis, with an intent to determine the *in vivo* cytokine profiles. Although RSV infection induces secretion of a wide range of pro-inflammatory cytokines and chemokines in an *in vitro* cell culture [21–24], these results may not really reflect the real *in vivo* situation, wherein not only epithelial cells but also macrophages and fibroblasts are infected with RSV. However, the cell-specific expression of the cytokines was not examined.

The replication of influenza virus in the lungs of mice is enhanced by Df protease [13]. Thus, prior Df sensitization might enhance RSV replication and thereby increase lung inflammation. However, the amount of RSV mRNA in lung tissues of ASRSV mice was not enhanced compared to only RSV-infected mice by QC-RT-PCR. This result suggests that the immunological response to RSV is not determined by the amount of live virus.

In conclusion, RSV infection may contribute to ongoing allergic inflammation in three ways. First, RSV infection induces Th₂-like cytokine expression in the spleen. This may synergize with mite allergen-induced inflammation by the bystander effect. Second, RSV infection-induced IFN- γ may prolong the survival of eosinophils in the lung. IFN- γ produced by activated T cells following rhinovirus infection prolongs the survival of human peripheral eosinophils [40]. Third, IFN- γ also upregulates the expression of cell adhesion molecules including ICAM-1, which, in turn, may be important in the recruitment of eosinophils. The combination of Df sensitization and RSV infection enhances the ICAM-1 expression on epithelial cells (unpublished data). Together, these molecules may aid in the recruitment into and survival of eosinophils in the lung, which further pulmonary inflammation and may cause asthma.

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