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T Helper 1 Background Protects Against Airway Hyperresponsiveness and Inflammation in Guinea Pigs With Persistent Respiratory Syncytial Virus Infection

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ABSTRACT: A family history of allergy has been implicated in children who develop post-bronchiolitis wheezing and asthma. In a guinea pig model of respiratory syncytial virus (RSV) lung infection, we evaluated the role of host Th1 background (either genetic or induced) on the development of a persistent infection, nonspecific airway hyperresponsiveness (AHR) and airway inflammation. Allergy resistant/T helper 1 (Th1)-skewed strain 2 guinea pigs (STR2) and cytosine phosphate guanine oligodeoxynucleotides (CpG-ODN) (Th1 stimuli) pretreated Cam Hartley guinea pigs (CH) were inoculated with RSV and compared with virus-inoculated allergy-susceptible/Th2-skewed CHs and to sham-inoculated STR2 and CH, 60 d post-inoculation. We measured titers of intrapulmonary RSV, lung interferon (IFN)-γ and interleukin (IL)-5 mRNA expression, AHR and airway T cells and eosinophils. All virus-inoculated groups of animals showed evidence of persistent RSV lung infection; however, Th2-skewed guinea pigs had virus-associated AHR and significantly greater levels of airway T cells and eosinophils. In conclusion, RSV can establish persistent infection of the guinea pig lung regardless of host Th1/Th2 background; however; a host Th1 background limits the extent of virus-associated AHR and airway inflammation. Heterogeneity in virus-host interactions may be relevant to understanding why some children hospitalized for RSV bronchiolitis go on to develop recurrent wheezing/asthma symptoms. **(*Pediatr Res* 61: 525–529, 2007)**

RSV is the most common cause of acute bronchiolitis, the leading cause of infant hospitalization in the developed world (1). A proportion of children hospitalized with acute bronchiolitis develop sequelae of recurrent wheezing and features of asthma for which allergic mechanisms (2–5) or a family history of allergy (6) have been implicated. In addition, studies in animal models have reported that RSV can establish a persistent, low-level infection in the lungs (7,8), and results of several cross-sectional (9–11) and longitudinal studies (12) suggest that RSV can persistently infect the human lung. However, the consequences of chronic intrapulmonary RSV persistence are unclear (13).

We have developed a guinea pig model of experimental RSV infection in which lung function and airway inflammation have been studied in association with RSV persistence (8,14). In this model, intranasal RSV inoculation of juvenile CH results in a productive lung infection that is associated with nonspecific AHR and airway inflammation by lymphocytes and granulocytes, including T cells and eosinophils (8,15,16). After resolution of the acute infection, CH develop a persistent lung infection characterized by ongoing, low-level RSV replication, AHR, and airway eosinophilia (8,14,15). These findings in guinea pigs suggest a possible role for RSV persistence in the pathogenesis of AHR and airway inflammation that have features similar to asthma (17).

Importantly, CH are a genetically outbred strain that is highly susceptible to allergic sensitization relative to inbred STR2 or STR13 (18). STR2 are comparatively allergy resistant, as these animals do not readily sensitize to foreign antigens (19) and release less histamine in response to ovalbumin (OA) challenge when passively sensitized with anti-OA IgG antibody (20). We have previously reported that RSV-inoculated STR2 develop an acute lung infection characterized by AHR, airway inflammation by T cells and eosinophils, and a Th2 shift in the lung gene expression ratios of IFN-γ (Th1 cytokine) to IL-5 (Th2 cytokine), but with lower titers of intrapulmonary replicating virus than observed in more allergy-susceptible animals (16,21). Long-term outcomes of RSV infection regarding the possibility of viral persistence, AHR, and airway inflammation in STR2 have not yet been reported. Because host Th1 responses are considered important for effective antiviral immunity and viral clearance (22,23), we were interested in determining whether STR2 can eradicate RSV from the lungs or whether they develop a persistent

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Abbreviations: ACh, acetylcholine; **AHR,** airway hyperresponsiveness; **AOI,** area of interest; **CH,** Cam Hartley guinea pigs; **CpG-ODN,** cytosine phosphate guanine oligodeoxynucleotides; **OA,** ovalbumin; **PC2,** provocative concentration of ACh associated with a doubling of baseline; **pfu,** plaque-forming units; $\mathbf{R_L}$**,** lung resistance; **RSV,** respiratory syncytial virus; **STR2,** strain 2 guinea pigs; **Th1 (2),** T helper 1 (2)

infection (and the virus-associated AHR and airway inflammation) that has been described in allergy-susceptible CH.

CpG-ODN (DNA sequences rich in cytosine-phosphate-guanine motifs) are potent adjuvants and strong Th1 stimuli and have been used in immunotherapy of allergic conditions (24–26). In naïve CH, intranasal administration of CpG-ODN is well tolerated and produces a Th1 shift in the lung IFN-γ/IL-5 gene expression ratio (21). We have recently shown that CpG-ODN immunoprophylaxis of CH inhibits the viral component of RSV-enhanced allergic sensitization to aerosolized OA (21), suggesting that CpG-ODN could potentially be useful for limiting the extent of RSV infection in these animals.

In the current study, we examined the role of host Th1 background, either genetic (STR2) or induced (CpG-ODN immunoprophylaxis of CH) on the development of RSV persistence and virus-associated AHR and airway inflammation 60 d post-inoculation (7). CH were used as the reference allergy-susceptible strain and STR2 were used as the reference allergy-resistant strain. We performed viral culture on lung homogenates to quantify titers of intrapulmonary, replicating RSV; semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) to measure IFN-γ and IL-5 mRNA levels in lung homogenates, with calculation of IFN-γ/IL-5 mRNA ratios to assess host Th1/Th2 balance; inhalational acetylcholine (ACh) challenge to test for RSV-associated nonspecific AHR; and quantitative histology for T cells and eosinophils (14,16) to measure the extent of airway inflammation by these cells.

METHODS

Animals. Female, juvenile CH and STR2 (1 mo old, body weight 250–300 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada) and the Cancer Research Centre (Frederick, MD), respectively. Animals were allowed to acclimatize for 5 d before use. Animals receiving RSV were housed separately from sham-inoculated animals in large polycarbonate cages. Otherwise, all animals were kept under similar conditions of alternating 12-h light-dark cycles and free access to guinea-pig chow (Ralston Purina Corp., St. Louis, MO) and water. This study was approved by the Committee on Animal Care of the University of British Columbia, and all animals were kept in accordance with standards of the Canadian Council on Animal Care (27).

Study design. Animals were divided as follows: (n = 10–12/group): (1) sham-inoculated CH (CH); (2) RSV-inoculated CH (CH+RSV); (3) CpG-ODN pretreated, RSV-inoculated CH (CH+CpG+RSV); (4) sham-inoculated STR2 (STR2); (5) RSV-inoculated STR2 (STR2+RSV).

Figure 1 shows a schematic diagram of the experimental design of this study.

Virus preparation and inoculation. Human Long strain, type A RSV (American Type Culture Collection, Manassas, VA) was propagated on HEp-2 cell monolayers (28). Working stocks of RSV were prepared by collection of the supernatant from lysed RSV-infected HEp-2 cells and stored at −70°C. Preparations were free of *Mycoplasma* as determined by culture of aliquots (Microbiology Laboratory, St. Paul's Hospital, Vancouver, BC, Canada), and endotoxin levels were below the limits of detection of *Limulus* E-Toxate assay (Sigma Chemical Co.). Guinea pigs were anesthetized *via* inhalation of 3%–5% halothane and received 300 μL of either RSV-containing inoculum [5.3×10^4 plaque-forming units (pfu)] or sham vehicle (uninfected HEp-2 cell culture supernatant prepared similarly to viral stocks) delivered to the lungs by intranasal instillation, as described elsewhere (15). As in previous studies with this animal model (8), guinea pigs that received the same volume of a tracer substance (India ink) confirmed that the inocula were delivered to all lung lobes (not shown).

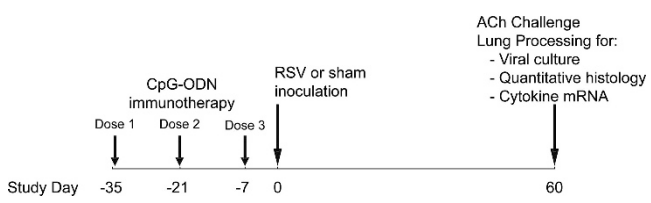

Figure 1. Schematic diagram of experimental design.

CPG-ODN immunoprophylaxis. CPG-ODN, Type B (single-stranded, code 2007, sequence 5′-TCGTCGTTGTCGTTTTGTCGTT-3′) was purchased from Coley Pharmaceuticals, Inc. (Wellesley, MA). Based on our previous work with CpG-ODN administration to guinea pigs (21), animals underwent anesthesia with 3%–5% inhalational halothane and were intranasally inoculated with 100 μg of CpG-ODN dissolved in 300 μL of sterile saline, given biweekly over 4 wk, for a total of three doses. CpG-ODN-treated animals were inoculated with RSV 7 d after receiving the final dose of CpG-ODN (21).

ACh challenge. Sixty days post-RSV inoculation, all animals from each group underwent ACh challenge in a whole-body plethysmograph and lung resistance (R_L) was calculated as described elsewhere (29). Dose-response curves were generated using R_L values at baseline (no aerosol exposure) and after challenge with 0, 0.5, 1.5, 5, 15, 50, and 150 mg/mL of ACh in saline. After completion of the ACh challenge, animals underwent euthanasia with an overdose of sodium pentobarbital (120 mg/kg, intraperitoneally). Lungs were extracted under aseptic conditions, weighed, and processed for viral plaque assays, nucleic acid extraction, and quantitative histology.

Viral plaque assays. To determine titers of replicating RSV within guinea pig lung specimens obtained at 60 d post-RSV inoculation, one lung lobe was frozen at −70°C in 5 mL of minimal essential media (GIBCO, Burlington, Ontario, Canada). Subsequently, serial 10-fold dilutions of lung homogenates were subject to viral plaque assay on HEp-2 cell monolayers with an agarose overlay, as previously described (8,15). For each animal, results were reported as number of pfu/g of lung tissue.

Semiquantitative RT-PCR. Lung tissue was stored in RNAlater (Qiagen, Mississauga, Ontario, Canada) and total RNA was extracted from 150 mg of tissue using an RNeasy Midi Kit (Qiagen) with on-column DNase digestion (Qiagen). RT was performed on 1.0 μg aliquots of total RNA using random primers (Invitrogen Canada, Inc., Burlington, Ontario, Canada) according to the manufacturer's instructions with Superscript II (Invitrogen). PCR for guinea pig-specific IFN-γ, IL-5, and β-actin (constitutively expressed housekeeping gene) was performed in duplicate for each animal using cycling conditions published elsewhere (21). PCR products underwent electrophoresis on 2% agarose gels stained with ethidium bromide. The optical density (OD) of each band was measured under ultraviolet light using an Eagle Eye digital image capture and analysis system (Stratagene, La Jolla, CA). For each animal, OD values for IFN-γ and IL-5 were normalized to OD signals of the corresponding β-actin signal, with results expressed as the average for duplicate runs.

Quantitative histology. Formalin-fixed, paraffin-embedded lung slices were cut into 4-μm serial sections and mounted onto glass slides. T cells were stained with anti-CD3+ polyclonal antibody (1:250 dilution) (Cell Marque, Hot Springs, AR) by an automated immunostainer (Dako, Mississauga, Ontario, Canada), and eosinophils were stained by a modified Hansel's method (14). Slides were coded to mitigate against observer bias. Digital images of five to 10 membranous bronchioles per animal were captured using a digital camera mounted on a light microscope (Nikon Corporation, Kanagawa, Japan) (21). Image Pro PLUS image analysis software (Media Cybernetics, Inc., Silver Spring, MD) was used to place a grid (60 × 60 pixels) over each image. The circumference of the airway was traced to designate an area of interest (AOI) and a computer macro calculated the number of grid points within the AOI. The grid points that were on positively stained cells and on the luminal space were then counted, and the percentage of airway wall occupied by CD3+ T cells or eosinophils was calculated as follows: [positive points/(total number of grid points in AOI − luminal points)] × 100. For each animal, the results for individual airways were averaged and expressed as the percentage of airway wall occupied by T cells or eosinophils (21).

Data analysis. For all analyses, SPSS v.11 (Statistical Products and Services Solutions, Chicago, IL) software was used, and, when warranted, data underwent logarithmic transformation to better approximate a normal distribution (16). For the ACh challenge, dose-response curves for the experimental groups were compared using general linear model with repeated-measures and analysis of variance (ANOVA), followed by *t* tests for pairwise group comparisons at individual ACh doses (14,29). The provocative concentration of ACh associated with a doubling of the value of baseline R_L (PC2) was determined from each animal's ACh dose-response curve (29). Group comparisons of mean PC2, titers of intrapulmonary replicating RSV, lung IFN-γ/β-actin, IL-5/β-actin,and IFN-γ/IL-5 mRNA ratios and the percentage of the airway wall occupied by T cells and eosinophils were per-

formed using ANOVA. Whenever significant differences ($p < 0.05$) were found by ANOVA, groups underwent further pairwise comparisons using t tests, with a Bonferroni procedure used to account for multiple comparisons (30). In all analyses, a two-tailed p value <0.05 was considered to be statistically significant.

RESULTS

During the experiments, animals tolerated all CpG-ODN and sham inoculations. With the exception of one Cam Hartley guinea pig that completed a full course CpG-ODN immunoprophylaxis but died during the subsequent RSV inoculation procedure, all animals survived for the duration of the study.

Viral plaque assays. Persistent RSV infection was documented by positive viral plaque assays of lung homogenates in all groups of animals that received virus. Replicating RSV was isolated from the lungs of animals within the CH+RSV (12/12), STR2+RSV (9/10), and CH+CpG+RSV (11/11) groups. No RSV was isolated from the lungs of any of the animals within the sham-inoculated CH ($n = 12$) or STR2 ($n = 10$) groups. The CH+RSV group had significantly higher levels of replicating RSV (45.7 ± 9.8 pfu/g lung, mean ± SE) compared with the CH+CpG+RSV (17.5 ± 3.8 pfu/g lung, $p < 0.02$) and STR2+RSV groups (12.6 ± 2.4 pfu/g lung, $p < 0.01$). There were no significant differences in the RSV titers between the STR2+RSV and CH+CpG+RSV groups ($p = 0.30$).

Semiquantitative RT-PCR. Figure 2 summarizes the results of semiquantitative RT-PCR for lung IFN-γ/β-actin, IL-5/β-actin, and IFN-γ/IL-5 mRNA ratios. For IFN-γ, the CH+CpG+RSV, STR2 and STR2+RSV groups had similar levels to each other and were significantly higher than the mean levels of the CH and CH+RSV groups ($p < 0.01$). For IL-5, the CH+RSV group had significantly higher expression in comparison with all other groups ($p < 0.001$). Further, the CH and CH+CpG+RSV groups had similar levels of IL-5 expression that were significantly higher compared with the STR2 and STR2+RSV groups ($p < 0.001$). There were no significant differences in mean IL-5 mRNA levels between the STR2 and STR2+RSV groups ($p = 0.11$).

Comparison of mean ratios of lung IFN-γ/IL-5 gene expression between the five groups of animals showed that the CH+RSV group had a significantly lower ratio compared with all other groups ($p < 0.001$), indicative of a Th2 shift. The STR2 and STR2+RSV groups had similar mean IFN-γ/IL-5 ratios to each other, and these were significantly higher than the ratios in the three other groups ($p < 0.001$), indicative of a comparatively Th1 phenotype that was not affected by RSV persistence. The CH+CpG+RSV group had an "intermediate" mean IFN-γ/IL-5 ratio, significantly higher than those of the CH+RSV and CH groups ($p < 0.001$) and significantly lower than the STR2 and STR2+RSV groups ($p < 0.01$).

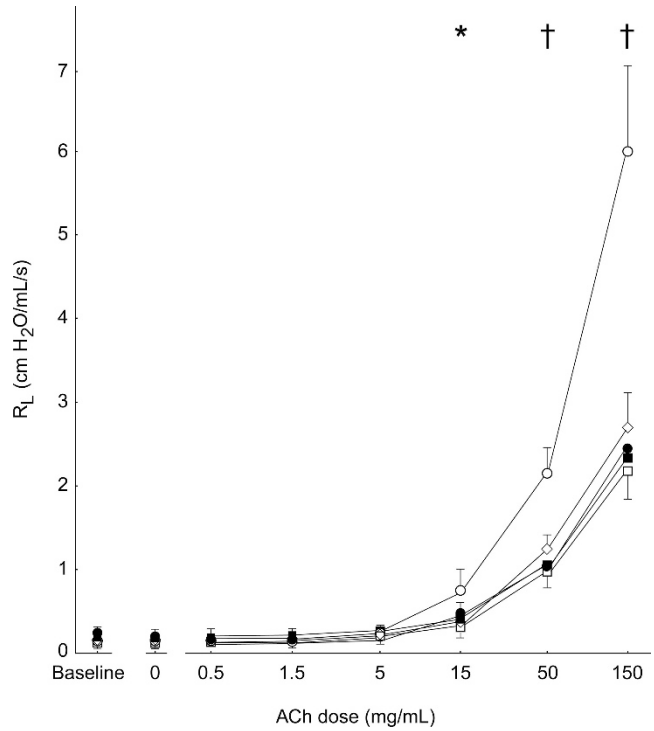

Figure 3. R_L *vs* ACh dose-response curves (mean ± SE) for the five groups of experimental animals. Groups are denoted as follows: CH (□) ($n = 12$), CH+RSV (○) ($n = 12$), CH+CpG+RSV([graphic]) ($n = 11$), STR2 (■) ($n = 12$), STR2+RSV (●) ($n = 12$). *$p < 0.05$, CH+RSV group *vs* all other groups; †$p < 0.01$, CH+RSV group *vs* all other groups.

ACh challenge. Figure 3 shows the ACh dose-response curves for the five groups of animals studied. The ACh

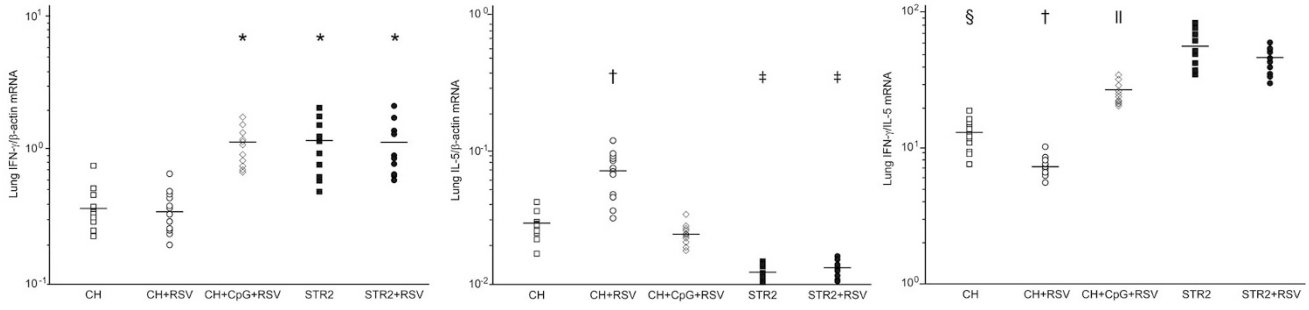

Figure 2. Lung IFN-γ/β-actin (*left*), IL-5/β-actin (*middle*), and IFN-γ/IL-5 (*right*) ratios of gene expression in the five groups of experimental animals [CH ($n = 12$), CH+RSV ($n = 12$), CH+CpG+RSV ($n = 11$), STR2 ($n = 10$), STR2+RSV ($n = 10$)]. Note higher IFN-γ expression in the CH+CpG+RSV, STR2, and STR2+RSV groups; highest IL-5 expression in the CH+RSV group; lowest IL-5 expression in STR2 and STR2+RSV groups; Th2 shift in IFN-γ/IL-5 ratio for CH+RSV group and Th1 skewing in CH+CpG+RSV, STR2, and STR2+RSV groups. *$p < 0.01$ in comparison with CH and CH+RSV groups; †$p < 0.001$, CH+RSV *vs* all other groups; ‡$p < 0.001$ *vs* CH and CH+CpG+RSV groups; §$p < 0.001$, CH *vs* all other groups; ‖$p < 0.01$, CH+CpG+RSV *vs* all other groups.

Table 1. *Mean PC2 values for the five groups of guinea pigs*

Group	PC2 (mg/mL ACh), mean ± SE
CH ($n = 12$)	53.91 ± 12.85
CH+RSV ($n = 12$)	19.70 ± 1.83*
CH+CpG+RSV ($n = 11$)	52.22 ± 5.46
STR2 ($n = 10$)	55.83 ± 9.77
STR2+RSV ($n = 10$)	52.19 ± 9.96

* $p < 0.03$, CH+RSV*vs* all other groups.
SE, standard error.

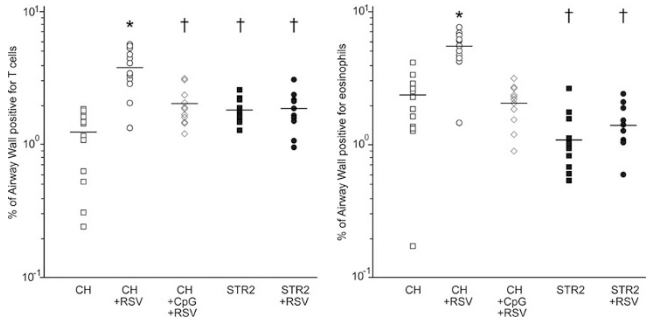

Figure 4. Percentage of the airway wall (mean ± SE) occupied by T cells (*left*) and eosinophils (*right*) in the five groups of experimental animals [CH ($n = 12$), CH+RSV ($n = 12$), CH+CpG+RSV ($n = 11$), STR2 ($n = 10$), STR2+RSV ($n = 10$)]. Note the highest mean percentages of airway wall T cells in the CH+RSV group and lowest percentages of eosinophils in the STR2 and STR2+RSV groups. *$p < 0.001$, CH+RSV group *vs* all other groups; †$p < 0.05$ *vs* CH group.

dose-response curve for the CH+RSV group showed significantly higher mean R_L at the three highest ACh challenge doses than in each of the other experimental groups. There were no significant differences in the mean R_L values at any ACh challenge dose between the CH, STR2, STR2+RSV, and CH+CpG+RSV groups.

Table 1 shows comparisons of mean PC2 for the experimental groups. The CH+RSV group had a significantly lower mean PC2 in comparison with all other groups ($p < 0.03$), consistent with this group having increased sensitivity to ACh. There were no statistically significant differences in mean PC2 between the CH, CH+CpG+RSV, STR2, and STR2+RSV groups.

Quantitative histology. Figure 4 summarizes the results of quantitative histology for the percentage of the airway wall occupied by T cells and eosinophils. The CH+RSV group had significantly higher levels of airway T cells and eosinophils compared with all other groups of animals ($p < 0.001$). In examining T cell levels, the STR2, STR2+RSV, and CH+CpG+RSV groups had an intermediate percentage of the airway wall occupied by T cells that was significantly lower than that of the CH+RSV group ($p < 0.001$) and significantly higher than that of the CH group ($p < 0.01$). There were no significant differences in the percentage of the airway wall occupied by T cells between the STR2, STR2+RSV, and CH+CpG+RSV groups.

Concerning airway eosinophils, the CH+RSV group had significantly higher mean percentage of airway wall occupied by eosinophils compared with all other groups ($p < 0.001$). The CH+CpG+RSV group had similar levels of airway eosinophils to the CH group ($p = 0.67$). The STR2 and STR2+RSV groups had similar levels of airway eosinophils ($p = 0.27$) that were significantly lower than all CH groups ($p < 0.05$), indicative of no significant effect of RSV persistence on airway eosinophils in STR2. In comparing the two groups of uninfected animals, the STR2 group had significantly lower levels of airway eosinophils than the CH group ($p < 0.01$), consistent with allergy-resistant STR2 having a lower baseline level of airway eosinophils than allergy-susceptible CH.

DISCUSSION

The mechanisms of post-bronchiolitis wheezing and asthma are poorly understood, but in some cases may involve allergic mechanisms in genetically predisposed children (3,4,6). Previous work in allergy-susceptible CH has shown that RSV can cause a persistent lung infection associated with AHR and airway inflammation (7,8,14), consistent with a possible role of RSV persistence in the pathogenesis of post-bronchiolitis sequelae. In contrast, other studies have implicated RSV persistence in the lungs but without a clear relationship to a particular clinical phenotype (9,10,12,13). Because Th1 responses are considered important for antiviral immunity, we hypothesized that a host genetic background for allergy susceptibility, manifested by Th2 skewing, would confer more severe consequences of RSV persistence.

Consistent with our previous studies in CH (7,8,14), we found that 60 d post-inoculation, RSV-inoculated animals developed persistent lung infection in association with AHR and airway eosinophilia. Here, for the first time, we show that persistently infected CH also had associated increases in airway T cells and pulmonary IL-5 mRNA levels with no changes in the levels of IFN-γ mRNA, resulting in a Th2 shift of IFN-γ/IL-5 mRNA ratios. This finding is consistent with some of these T cells having a Th2 phenotype. Unfortunately, quantitative histology for other inflammatory cell types potentially relevant to mechanisms of post-bronchiolitis wheezing (*e.g.* B cells, natural killer cells, macrophages, dendritic cells) was precluded by the lack of available guinea pig–specific reagents for immunohistochemical staining in paraffin-embedded sections. However our findings of elevated airway T cells and eosinophils in the CH+RSV group are similar to changes described in the small airways of human asthmatic patients (17). Furthermore, airway mucus was not assessed because airway goblet cell metaplasia is not a feature of the guinea pig model of experimental RSV infection (15).

By contrast, neither RSV-exposed STR2 nor virus-exposed CpG-ODN pretreated CH developed AHR or airway eosinophilia, despite having a persistent RSV lung infection. Overall, our findings suggest that it is not RSV persistence *per se*, but rather the characteristics of the infected host that determine whether animals will develop long-term AHR and increased airway inflammation. Furthermore, as animals with a Th1 background (either genetic or induced) had lower titers of intrapulmonary RSV than the CH+RSV group, it appears that the host background may also control the extent of viral replication during persistent infection.

A recent study has compared effects of RSV persistence between Balb/c (Th2-skewed) and C57BL/6 (Th1-skewed) mice (31). Both mouse strains showed transient AHR that lasted longer in Balb/c mice (up to 42 d post-viral inoculation) than in C57BL/6 mice (up to 28 d post-inoculation); both mouse strains had elevated histopathological scoring at all time points studied (up to d 77 post-inoculation) and had similar levels of persistent RSV RNA in the lungs from d 12 onward. These apparent discrepancies between the findings in mice and our current findings in guinea pigs may be attributed to methodological differences including use of unrestrained *versus* anesthetized restrained whole-body plethysmography (32), use of histological scoring *versus* quantitative histology by image analysis, and potential species differences in permissiveness and responses to human RSV.

To our knowledge, this is the first study to show an association between RSV persistence, host T helper background, and the development of sequelae of AHR and airway inflammation. Given the similarities of ACh dose-response curves between the CH, CH+CpG+RSV, STR2, and STR2+RSV groups, despite differences in results of viral plaque assays, cytokine gene expression, and airway T cells and eosinophils, our results suggest that there could be threshold levels of viral replication, Th2 shift, or airway inflammation required to produce AHR. Further studies are required to investigate this possibility. Concerning the potential relationship of our findings to humans, RSV-associated airway eosinophilia in guinea pigs is consistent with so-called eosinophilic bronchiolitis that develops in 20%–25% of children with acute bronchiolitis (33). Furthermore, consistent with epidemiological studies, our findings indicate that an allergic background may predispose the host to developing post-bronchiolitis sequelae (3,4,6).

In conclusion, RSV is capable of establishing a persistent lung infection in guinea pigs regardless of host Th1/Th2 background. Guinea pigs with a genetic Th2 background develop chronic virus-associated AHR, significantly greater airway inflammation by T cells and eosinophils, and Th2 shift in the balance of lung Th1/Th2 gene expression. Our findings suggest that host background to allergy is important in modulating long-term outcomes of RSV persistence and might be relevant to RSV-infected children who develop recurrent wheezing and asthma symptoms as sequelae of acute bronchiolitis.

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