

# T Helper Lymphocyte Response to Respiratory Syncytial Virus and Its Components in Patients with Respiratory Allergy and Nonatopic Controls

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## Key Words

Respiratory syncytial virus · T cell line · Cytokine ·  
F protein · G protein · Allergy

## Abstract

**Background:** Respiratory syncytial virus (RSV) G protein is involved in Th2-shifted immune response, while F protein has a reverse effect on RSV infection in Th2-prone BALB/c mice. Studies on the human T cell response to F or G protein are few, and the relationship between the immune response to G protein and atopy is not known. **Methods:** We established CD4+ RSV-specific T cell lines (TCLs) from adult patients with respiratory allergic diseases (allergics) or non-atopic controls (controls), and examined proliferative responses and  $\gamma$ -interferon (IFN- $\gamma$ ) and interleukin 4 (IL-4) production in these TCLs upon stimulation with RSV, F or G proteins. **Results:** 32 and 29 RSV-specific oligoclonal TCLs were established from allergics and controls, respectively. IL-4/IFN- $\gamma$  in the culture supernatant of antigen-stimulated TCLs was significantly higher in allergics than in controls ( $p = 0.042$ ). IL-4/IFN- $\gamma$  ratios in the culture supernatants of G-protein-reactive TCLs were significantly higher in allergics than in controls ( $p = 0.016$ ), while no differences in IL-4/IFN- $\gamma$  in culture supernatants of F-protein-reactive TCLs were found between allergics and controls ( $p = 0.787$ ). IL-4/IFN- $\gamma$

in the culture supernatants of G-protein-reactive TCLs was significantly higher than those of F-protein-reactive TCLs in allergics ( $p = 0.023$ ) but not in controls ( $p = 0.768$ ). **Conclusion:** The results suggest that the T cell response to RSV is influenced by the atopic diathesis as well as by individual RSV antigens, and that G protein may be an important antigen involved in allergy in humans.

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## Introduction

Respiratory syncytial virus (RSV) infection is one of the major infectious diseases of the respiratory tract in infants and children [1]. Several prospective studies have suggested that severe RSV infection, such as bronchiolitis in infants, is a risk factor for recurrent wheezing and asthma later in childhood as well as in sensitization to allergens [2–4]. Sigurs et al. [3] examined the frequency with which children with severe RSV bronchiolitis developed allergic disease compared with a control group. At both 7 and 13 years of age, the incidence of allergic sensitization as defined by either a positive skin test or specific IgE in vitro blood test was significantly increased in children with a history of RSV hospitalization. Another cohort study by Schauer et al. [4] followed 42 infants who

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had been hospitalized with severe RSV bronchiolitis. At 1 year of age, 33% of infants who had been infected with RSV tested positive for serum IgE antibodies compared with only 2.3% of a matched control group [4]. Their data suggest that RSV itself is an independent factor for allergic sensitization and the development of asthma. This finding is supported by recent data in which anti-RSV immunoglobulin treatment was found to decrease the incidence of asthma [5].

Although 70–80% of previously healthy infants are infected with RSV during the first 2 years of life, only a small proportion develop bronchiolitis, with the vast majority experiencing upper respiratory tract infection alone [6]. Recent interest has, therefore, focused on the role of the host immune response in the pathogenesis of bronchiolitis [7]. It has been demonstrated that a preexisting reduced type 1 response to immunological stimuli is related to the development of RSV bronchiolitis [8]. Thus, newborns that later developed bronchiolitis showed low interleukin (IL) 12 levels in cord blood compared with newborns who did not develop the disease. One candidate host factor is a family history of atopy. In fact, it has been shown that a family history of atopy is associated with a risk for the development of severe RSV infection [9]. These data suggest the possibility that the immune response to RSV may depend on host factors such as presence or absence of atopy.

Two surface proteins, i.e. F and G proteins, among the 11 protein components of RSV are thought to be important in terms of anti-RSV immunity [10]. In animal models, viral challenge in mice previously sensitized with G protein results in severe pulmonary disease associated with the induction/recall of a strong Th2-type anti-RSV T cell response, whereas challenge in mice previously sensitized with F protein does not lead to Th2-type pulmonary inflammation [11]. These data suggest that the RSV G protein is involved in a Th2-shifted immune response while F protein has a reverse effect upon RSV infection. Interestingly, G-protein-associated skewing of the T cell immune response is observed in BALB/c mice but not in C57BL mice [12]. A difference in genetic susceptibility to RSV infection was recently confirmed by other investigators [13]. These data indicate that some host factors influence the immune response to RSV components such as F or G protein. Since it is known that the BALB/c mouse is a strain that favors a Th2-type immune response, an atopic diathesis may influence the response to F or G protein of RSV in humans. So far, there is only one report on how the T cell response to F or G protein is related to the Th1-type or Th2-type immune response in

humans, respectively [14]. However, in that report, the T cell immune response to RSV and its components was not studied with regard to atopy/nonatopy.

In this study, in order to elucidate the T cell response to RSV and its components in relation to an atopic diathesis, we established CD4<sup>+</sup> RSV-specific T cell lines (TCLs) from atopic and nonatopic adults, and examined the proliferative responses and balance of  $\gamma$ -interferon (IFN- $\gamma$ ) and IL-4 production of these TCLs upon stimulation with RSV F and G proteins.

## Materials and Methods

### *Human Donors*

Eight RSV-seropositive adult male and female volunteers were drawn from the staff of the Pediatric Department of Chiba University Hospital. Four of the 8 have allergic diseases (bronchial asthma and allergic rhinitis) and have specific IgE antibodies to house dust mite and Japanese cedar pollen. There was no history of bronchiolitis in their infancy. The other 4 have no history of allergy and no specific IgE antibodies to common aeroallergens. Their serum IgE levels were less than 100 IU/ml. Fifty-milliliter samples of peripheral blood were collected from each donor by venipuncture and collected into heparinized tubes.

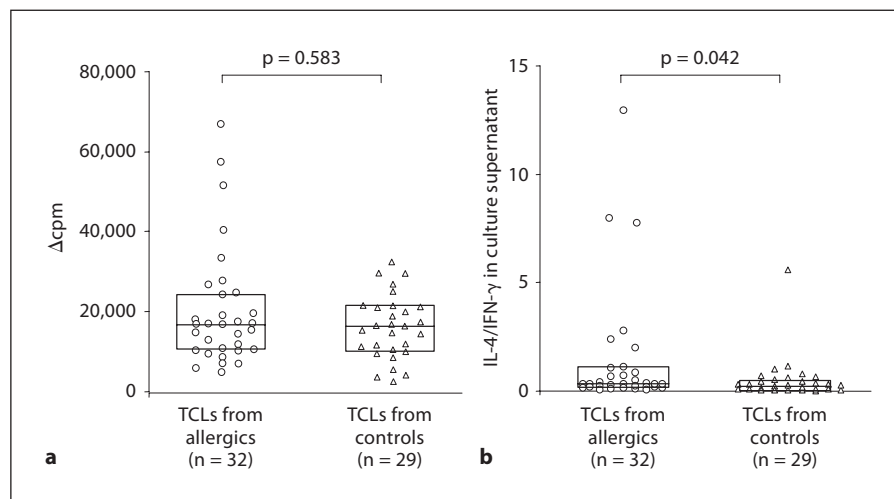
### *Preparation of Antigens*

The subtype A RSV (Long strain) antigen was obtained from Denka-Seiken Company, Tokyo, Japan. The virus was cultured on Vero cells and inactivated by exposure to UV light. Mock-infected Vero cells were treated in an identical way and used as a control antigen preparation. Purified RSV F and G proteins were kindly provided by Dr. J.A. Beeler (FDA, USA). The purity was determined to be 98 and 99%, respectively, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis.

### *Generation of RSV-Specific TCLs*

RSV-specific TCLs were derived from adult peripheral blood mononuclear cells (PBMCs) by repeated antigen stimulation with RSV in vitro as previously described [15]. Briefly, PBMCs were incubated in 96-well round-bottom plates (Costar, Cambridge, Mass., USA) with  $5 \times 10^5$  PFU UV-irradiated RSV in 100  $\mu$ l of RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) containing 10% heat-inactivated human AB serum, 2 mM L-glutamine,  $2 \times 10^{-5}$  M 2-mercaptoethanol, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Fresh 30 IU/ml recombinant IL-2 (kindly provided by Takeda Chemical Industries, Osaka, Japan) was added on the third day of culture, and recombinant IL-2 was added every 3–4 days for a total period of 21 days. On day 21, the cells in the plate were washed to remove recombinant IL-2 and resuspended in 100  $\mu$ l of culture medium. The cells in each well were split into 2 adjacent wells. Autologous  $10^5$  PBMCs that had been irradiated were added to each of the 2 wells as antigen-presenting cells (APCs), and RSV antigen was added to only 1 cell. Outgrowing cells in the RSV-antigen-pulsed wells were transferred to a 24-well plate and stimulated with APCs and RSV antigen once a week.

**Fig. 1.** Response of TCLs to whole RSV antigen: allergics versus nonatopic controls. Proliferative responses ( $\Delta$ cpm, **a**) and cytokine secretion pattern, i.e. IL-4/IFN- $\gamma$  ratios, in the culture supernatant (**b**) of 32 RSV-specific TCLs established from 4 allergic subjects and 29 RSV-specific TCLs from 4 nonatopic adults. RSV specificity was evaluated by the stimulation index  $>2$ . The box blots show median and 1st and 3rd quartiles. The Mann-Whitney U test was used for statistical analysis.



#### T Cell Proliferation Assays

Cellular proliferation was measured by the tritiated methylthymidine ( $^3\text{H}$ ]TdR) assay, as has been described [15]. Briefly,  $2 \times 10^4$  T cells were mixed with  $5 \times 10^4$  APCs and antigen in flat-bottomed 96-well plates (Costar) in a total volume of 200  $\mu\text{l}$ . After 72 h of incubation at  $37^\circ\text{C}$ , 0.5  $\mu\text{Ci}$   $^3\text{H}$ ]TdR (New England Nuclear, Boston, Mass., USA) was added to each well. Sixteen hours later, the cells were harvested onto glass fiber strips, and the amount of incorporated  $^3\text{H}$ ]TdR was measured by liquid scintillation counting. The results were expressed as the delta counts per minute (cpm; mean cpm in culture stimulated with antigen – mean cpm in unstimulated culture)  $\pm$  standard deviation of triplicate cultures or stimulation index (counts in antigen-stimulated well/counts without antigen).

#### Determination of Cytokines in the Culture Supernatants

Twenty thousand T cells were stimulated with antigen in the presence of  $5 \times 10^4$  APCs in flat-bottomed 96-well plates in a final volume of 200  $\mu\text{l}$ . After 24-hour incubation, the supernatants were harvested and the cytokines secreted into the culture supernatant were measured using a solid-phase sandwich enzyme-linked immunosorbent assay, of which commercial kits (Cytoscreen Immunoassay Kits; BioSource International Inc., Camarillo, Calif., USA) were used to assay for IL-4 and IFN- $\gamma$ . The sensitivity of the assays was 10 pg/ml for both IL-4 and IFN- $\gamma$ . Th1/2 balance was compared by IL-4/IFN- $\gamma$  as reported by Ebner et al. [16].

#### Statistics

The Mann-Whitney U test was used for statistics.

#### Ethical Considerations

This study was approved by the ethical committee of the Graduate School of Medicine, Chiba University, and performed in accordance with the Declaration of Helsinki.

## Results

### Proliferation and Cytokine Production of RSV-Specific TCLs

Sixty-one RSV-specific TCLs (32 from allergics and 29 from controls) were established from the 3 donors by repeated restimulation with inactivated RSV antigen. The virus specificity of these lines was assessed initially at 2 weeks after the initial round of restimulation. Each line was found to proliferate specifically (stimulation index  $>2$ ) when challenged with inactivated virus antigen and APCs. A control antigen preparation plus APCs failed to induce proliferation in any of these lines. There was no difference between thymidine uptake between TCLs from allergic donors and those from nonatopic controls (fig. 1a). In contrast, the IL-4/IFN- $\gamma$  ratio in the culture supernatant was significantly higher in TCLs from allergic donors as compared to controls (fig. 1b,  $p = 0.042$ ).

### Components Recognized by RSV-Specific TCLs in Allergics and Controls

These 61 RSV-specific TCLs were analyzed for their RSV surface protein recognition by challenging the cells in vitro with RSV F protein and RSV G protein in the presence of APCs. With a stimulation index above 2 indicating a positive result, 14 TCLs (5 from allergics and 9 from controls) proliferated in response to F protein, 5 TCLs in response to G protein (3 from allergics and 2 from controls), 26 TCLs in response to both F and G proteins (15 from allergics and 11 from controls) and 16 TCLs in response to whole RSV antigen but neither F or G proteins (9 from allergics and 7 from controls) as shown in

**Fig. 2.** Cytokine secretion pattern of F- or G-reactive TCLs in allergics versus controls. **a** IL-4/IFN- $\gamma$  ratios in the culture supernatant of RSV F-reactive TCLs were compared between allergics (n = 20) and nonallergics (n = 20). RSV F protein reactivity was evaluated by the stimulation index >2. **b** IL-4/IFN- $\gamma$  ratios in the culture supernatant of RSV G-reactive TCLs were also compared between allergics (n = 18) and controls (n = 13). RSV G protein reactivity was evaluated by the stimulation index >2. The box blots show median and 1st and 3rd quartiles. The Mann-Whitney U test was used for statistical analysis.

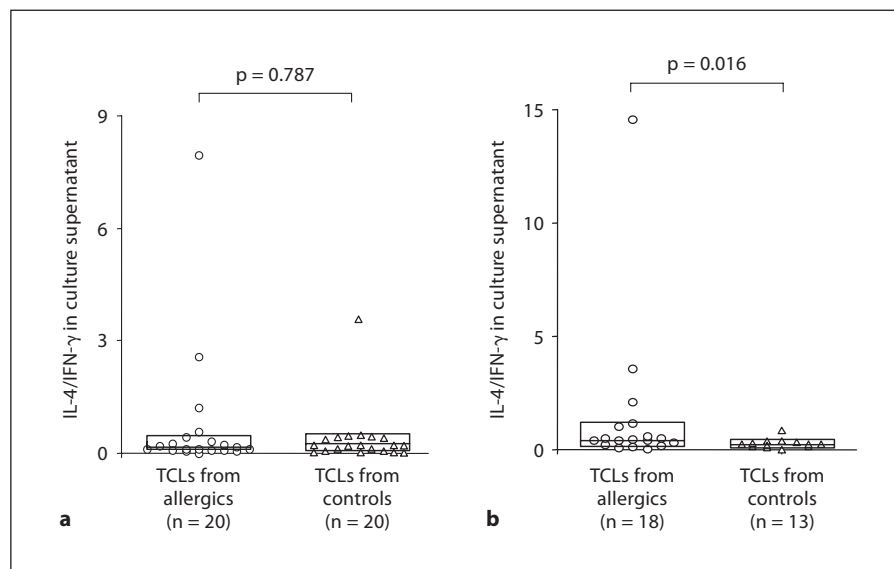


table 1. There were no statistical differences in numbers of F- or G-protein-reactive TCLs between allergics and controls.

#### *Cytokine Secretion Profile of F- and G-Protein-Stimulated TCLs in Allergics versus Controls*

We measured IL-4 and IFN- $\gamma$  in the culture supernatants of F- or G-protein-reactive TCLs (stimulation index >2) 24 h after stimulation with F or G protein, respectively. There was no difference in the IL-4/IFN- $\gamma$  ratios of F-protein-reactive TCLs from allergic donors (n = 20) and those from controls (n = 20; p = 0.787; fig. 2a). In contrast, the IL-4/IFN- $\gamma$  ratios in the culture supernatant of G-protein-stimulated TCLs from allergic donors (n = 18) were significantly higher than those of TCLs from controls (n = 13; p = 0.016; fig. 2b).

#### *Comparison of Cytokine Secretion Pattern between F- and G-Protein-Reactive TCLs*

We compared the cytokine secretion patterns of F-protein-reactive TCLs and G-protein-reactive TCLs in allergics and controls. The IL-4/IFN- $\gamma$  ratio was significantly higher in G-protein-reactive TCLs than in F-protein-reactive TCLs from allergics (p = 0.023; fig. 3a) but not from controls (p = 0.768; fig. 3b). When TCLs that dominantly recognized either the F or G protein were studied, the G-protein-reactive T cells again showed significant Th2-skewing compared to F protein-reactive T cells only in allergics (data not shown).

**Table 1.** Reactivity of RSV-specific TCLs to F or G protein of RSV (defined as reactive when stimulation indices >2)

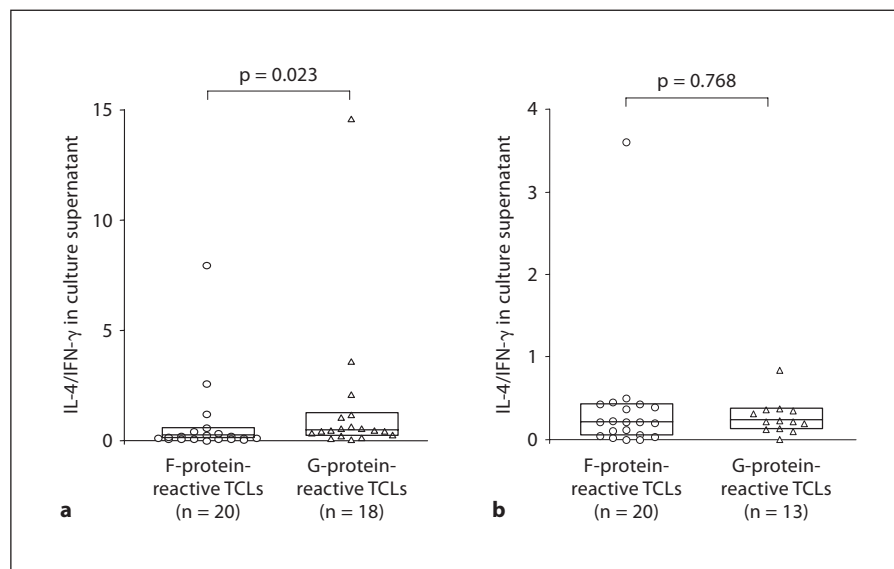
| Donors    | F protein | G protein | F and G protein | Neither |
|-----------|-----------|-----------|-----------------|---------|
| Allergics | 5         | 3         | 15              | 9       |
| Controls  | 9         | 2         | 11              | 7       |

Values indicate the numbers of TCLs responding to antigens.

## **Discussion**

There have been many studies on the deviation of antigen-nonspecific human T cell function in acute RSV infection based on cytokine production [7]. In contrast, the T cell response to RSV and its components has been studied mainly in animals such as mice [17], and a few studies on memory T cell response to RSV and its components in humans have been reported [14, 18–20]. Knowledge about RSV-component-specific memory T cell immunity in humans is quite limited, especially with regard to host factors. In this study, we demonstrate for the first time that the T cell response to RSV whole virus is skewed to a more Th2-type response in patients with respiratory allergy compared to nonatopic controls and that the G protein is a responding antigen for this shift. There was no difference in the cytokine production patterns in F-protein-reactive T cells between allergics and nonatopic controls.

**Fig. 3.** Cytokine secretion pattern of F- versus G-reactive TCLs in allergics and controls. **a** IL-4/IFN- $\gamma$  ratios in the culture supernatants of RSV F-reactive TCLs (n = 20) and RSV G-reactive TCLs (n = 18) were compared in allergics. **b** IL-4/IFN- $\gamma$  ratios in the culture supernatants of RSV F-reactive TCLs (n = 20) and RSV G-reactive TCLs (n = 13) were compared in controls. The box blots show median and 1st and 3rd quartiles. The Mann-Whitney U test was used for statistical analysis.



One of the main messages of the present report is that atopy may be related to a Th2-type immune response to RSV. Trefny et al. [9] reported that a family history of atopy is associated with a risk for the development of severe RSV infection. In mice, the primary RSV infection in the absence of allergen sensitization induces an aggressive cytokine response, typical of a Th0-like response, and further infections induce decreased Th2 or increased Th1 dominance. In contrast, in allergen-sensitized mice, the primary infection induces IFN- $\gamma$  production, which downregulates Th2-like cytokines. Further infections in these mice reversed the cytokine dominance from a Th1-like to a Th2-like response [21]. The effect of prior exposure to allergen on RSV-induced bronchial hyperresponsiveness was recently confirmed by Makela et al. [22]. These data suggest that the immune status of the host is an important factor in determining the immune response to RSV.

In these experiments, we stimulated PBMCs from adult allergics and healthy controls with whole inactivated RSV and examined their specificity for the RSV F or G protein. The results are consistent with the general consensus that the F and G proteins are major targets for RSV immunity [1]. Our data suggest that the T cell cytokine production pattern to G protein is skewed more to Th2 compared to that to F protein only in allergic subjects and the cytokine production pattern in response to the F protein does not differ from that to G protein in nonatopic controls. Jackson and Scott [14] showed several years ago that T cells responding to the F and G pro-

teins show Th1-type and Th2-type responses, respectively, by establishing TCLs specific for RSV components from PBMCs from adult volunteers. In their study, F- or G-protein-specific T cell lines were generated by repeated stimulation with each antigen with IL-2 while we established RSV-specific T cell lines by the stimulation of PBMC with whole RSV. The difference in methodology to establish T cell lines and/or host factors such as atopy may be a reason for clear dichotomy in T cell lines in Jackson and Scott experiments although in their report it was not demonstrated whether the donors were atopic or not. De Waal et al. [18] recently reported on 2 CD4+ T cell clones established from infants who suffered from RSV bronchiolitis and 3 clones from adult volunteers. They showed that one clone from an adult produced IFN- $\gamma$ , IL-4, IL-5 and IL-13 in response to RSV G protein peptide, suggesting that G-specific T cells may produce Th1 cytokines in addition to Th2 cytokines. In their study, the number of clones was limited, and there was no information provided about the atopic status of the donors. The present data not only support the importance of the RSV G protein in Th2-type immune shift, which has been shown in an animal model of RSV infection [17], but also suggest that the G protein in combination with host factors such as atopy is related to the Th2-type T cell response to RSV.

The G protein of RSV has been shown to be deeply involved in the Th2-type immune response in mice [23–25]. Although it is still not clear why the RSV G protein induces a Th2-type response in some mice, a secreted

form of the G protein was demonstrated to be critical in promoting the Th2-type immune response [26]. Since the Th2-shift in response to the G protein is dependent on a Th2 cytokine, IL-13 [27], Th2-type memory T cells may play some role in the development and exacerbation of allergic airway inflammation upon recurrent RSV infection. In this sense, it is important to reduce the G-protein-induced Th2 response, especially at the time of the primary infection. Although RSV with the G protein deleted induces severer pulmonary lesions [28], pulmonary eosinophilia in mice was significantly reduced by immunization with a recombinant RSV depleted of the region that induces an unbalanced T cell response to the G protein [26]. The clinical availability of this recombinant RSV remains to be determined.

It has been shown that evaluating effector/memory function in systemic compartments such as peripheral blood may not provide an adequate assessment of the local response to RSV [29, 30]. Although the studies reported here were carried out in adult human peripheral blood

and not in the human lung and numbers of donors are limited, we suggest that the production of Th1- or Th2-type cytokines in response to RSV is controlled differentially by the nature of the RSV antigenic stimulus and host immune status. In this study, we could not establish RSV-specific TCLs from children because of difficulty in obtaining large amounts of blood. It is interesting and necessary to study T cell response to G protein in allergic and nonatopic children since Pala et al. [19, 20] reported that the T cell response to RSV might be different between children and adults.

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