

CD8⁺ T CELL CYTOKINE & CYTOTOXIC PROTEIN PRODUCTION AND PROLIFERATION IS DIFFERENTIALLY REGULATED BY NORMAL AND RSV-INFECTED HUMAN BRONCHIAL EPITHELIAL CELLS

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Abstract

It has been shown that epithelial cells have the capacity to present antigens to T lymphocytes. CD8⁺ T cell recognition of viral antigens presented by epithelial cells may be important in the clearance of respiratory viral infections, such as respiratory syncytial virus (RSV). Our aim was to investigate the effect of RSV-infected bronchial epithelial cells (BECs) on CD8⁺ T cell functions.

Methods. CD8⁺ T cells were cultured by themselves (in the presence of media or RSV) or co-cultured with un-infected or RSV-infected BECs and CD8⁺ T cells cytokine production and proliferation was determined.

Results. We found that CD8⁺ T cells co-cultured with normal BECs produced less cytokines and proliferated less compared with CD8⁺ T cells cultured with media alone, but in the CD8⁺ T cells co-cultured with RSV-infected BECs, cytokine production and proliferation were at high levels again.

Conclusion. Normal bronchial epithelial cells inhibit CD8⁺ T cell cytokine production and proliferation but after RSV infection this regulatory function is lost. These findings suggest an important role for respiratory epithelial cells in controlling T cell functions in healthy lungs. Studying the interactions between human bronchial epithelial cells and local lymphocytes will help understanding the pathogenesis of various airway diseases.

Keywords: CD8⁺ T, respiratory syncytial virus, bronchial epithelial cells.

PROLIFERAREA ȘI PRODUCȚIA DE CITOKINE ȘI PROTEINE CITOTOXICE DE CĂTRE CELULELE T CD8⁺ ESTE REGLATĂ DIFERENȚIAL DE CĂTRE CELULELE EPITELIALE BRONHIALE NORMALE ȘI INFECTATE CU VRS

Rezumat

Este demonstrat că celulele epiteliale au capacitatea de a prezenta antigeni limfocitelor T. Recunoașterea de către limfocitele T CD8⁺ a antigenilor virali prezentați de celulele epiteliale este importantă în cazul clearance-ului infecțiilor virale respiratorii, ca de exemplu virusul respirator sincițial (VRS). Obiectivul nostru a fost să investigăm efectul celulelor epiteliale bronhiale infectate cu VRS asupra funcțiilor limfocitelor T CD8⁺.

Metode. Limfocitele T CD8⁺ au fost cultivate singure (în mediu ca și control sau în prezența VRS) sau co-cultivate cu celule epiteliale bronhiale infectate sau nu cu VRS. Producția de citokine și proliferarea limfocitelor T CD8⁺ a fost determinată.

Rezultate. Limfocitele T CD8⁺ co-cultivate cu celule epiteliale bronhiale normale produc mai puține citokine și proliferază mai puțin comparativ cu limfocitele T CD8⁺ cultivate singure în mediu. Când limfocitele T CD8⁺ sunt co-cultivate cu celule epiteliale bronhiale infectate cu VRS, producția de citokine și proliferarea au fost din nou crescute la nivele similar cu cele din limfocitele T CD8⁺ cultivate singure în mediu.

Concluzii. Celulele epiteliale bronhiale normale inhibă producția de citokine și proliferarea limfocitelor T CD8⁺, însă prin infecția cu RSV această funcție regulatoare se pierde. Aceste rezultate sugerează un rol important pentru celulele epiteliale respiratorii în controlul funcțiilor limfocitelor T în plămânii normali. Descifrarea interacțiilor dintre celulele epiteliale bronhiale umane și limfocitele locale va contribui la înțelegerea patogenezei diferitelor boli respiratorii.

Cuvinte cheie: limfocite T CD8⁺, celule epiteliale bronhiale, virus respirator sincițial.

INTRODUCTION

The mucosal surface of the respiratory tract represents a major portal of entry for many pathogenic agents. Epithelial cells line the mucosal surface of airways and form a mechanical barrier that is important in repulsion and removal of airborne pathogens by means of the mucociliary escalator. It is also notable that substantial numbers of T cells are found in association with epithelium in the respiratory and gastrointestinal systems [1]. Several studies have shown that epithelial cells have the capacity to present antigens to T lymphocytes and to stimulate them *in vitro* [2]. This exposed epithelium poses tremendous problems for an immune system charged with maintaining a sterile environment in the lungs. Pathogens and aeroallergens that are not eliminated may elicit T and B cell immune responses.

Respiratory syncytial virus (RSV) is an enveloped, negative-strand RNA virus belonging to the *Paramyxoviridae* family. RSV infects nearly all infants in their first or second winter [3], and represents a major cause of respiratory morbidity in infants, children under 5 years of age [4] and of morbidity and mortality in the elderly worldwide [5,6]. Currently, there is no effective vaccine, and antiviral drugs to control infection are limited.

CD8⁺ T cell recognition of viral antigens presented by epithelial cells may be important in the clearance of respiratory viral infections such as RSV. CD8⁺ T cells employ a variety of cytolytic (granzyme B-dependent mechanisms) and noncytolytic effector mechanisms (production of antiviral cytokines, such as interferon- γ) that contribute to viral clearance, but may also contribute to lung injury in the process [7,8].

Our aim was to investigate the interactions between human bronchial epithelial cells (BECs) and CD8⁺ T cells and the effect of RSV-infected BECs on CD8⁺ T cell functions. We developed an *in vitro* co-culture system

in which RSV-infected BECs were interacting directly with human CD8⁺ T cells. We reasoned that un-infected epithelium would not stimulate resting CD8⁺ T cells to induce cytokine or cytotoxic protein secretion or to proliferate, as this would lead to uncontrolled activation of any CD8⁺ T cells in contact with respiratory epithelium, but in contrast that under infective conditions CD8⁺ T cells would be stimulated to induce cytokine or cytotoxic protein secretion and to proliferate.

MATERIAL AND METHODS

Cell culture and virus infection

BEAS-2B cells, a bronchial epithelial cell line (European Collection of Animal and Cell Cultures) were cultured and infected with RSV as previously described [9]. RSV A2 strain (gift from Prof. PJ Openshaw, Imperial College London, UK) was grown in Hep-2 cells and virus titre determined by plaque assay.

Human CD8⁺ T cells were co-cultured with BEAS-2B cells (ratio BECs:CD8 = 1:1) or alone in the presence of media or RSV. CD8⁺ T cells were isolated from peripheral blood of healthy donors by negative selection (Miltenyi Biotec). This study was approved by St Mary's NHS Trust Ethics Committee and all subjects gave informed consent. The purity of CD8⁺ T cell population was routinely found to be over 95%.

For co-culture, BECs were seeded in 24-well plates and infected with RSV (at a multiplicity of infection, MOI of 1). After 24 hours medium was removed and CD8⁺ T cells were added; this was considered time-point 0.

Flow-cytometry

CD8⁺ T cells (Carboxyfluorescein succinimidyl ester, CFSE, labelled, FITC) [10] were stained for intracellular IFN- γ , IL-2, IL-4 and granzyme B expression, 48 hours after co-culture [11]. Analysis was performed on CyAN (DakoCytomation) with Summit V4.3 software; at least 20,000 events were acquired. Antibodies were from BDPharmingen except granzyme B (eBioscience), all phycoerythrin conjugated. Appropriate isotype controls were included to assess background fluorescence.

To assess cell proliferation, PHA 1µg/mL (Sigma) was added to medium when total peripheral blood mononuclear cells (PBMCs) or purified CD8⁺ T cells were added to the co-culture system. After 6 days of co-culture with BECs, non-adherent lymphocytes were removed along with supernatants and analysed by flow-cytometry.

Statistical analysis

The results were analysed using GraphPad Prism version 4.00 (GraphPad Software, California). Results of at least three separate experiments were expressed as means±standard errors of the means. When analysing multiple groups, a one way ANOVA of all pairs and columns was used, followed if significant by paired *t* test for paired comparisons. *P* values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We used a co-culture system of CD8⁺ T cells freshly isolated from human peripheral blood and BECs (BEAS-2B cells) treated with media as control or infected with RSV, in parallel with CD8⁺ T cells co-cultured with media alone or with RSV in order to determine the role of BECs on CD8⁺ T cells capacity of cytokine and cytotoxic protein production or on CD8⁺ T cells proliferating properties.

CD8⁺ T cells cultured with media alone had increased frequency of cytokines or cytotoxic protein compared to CD8⁺ T cells co-cultured with un-infected BECs

Cytokine production and cytotoxic activity are key elements of CD8⁺ T cell anti-viral function. The frequencies of IFN-γ, IL-2, IL-4 and granzyme B-positive CD8⁺ T cells were therefore determined by intracellular staining. CD8⁺ T cells were co-cultured for 48 hours with BECs or alone and for the last 4 hours before staining were re-stimulated with general stimuli, PMA and ionomycin, in the presence of brefeldin (Golgi stop, so the newly formed proteins will be kept intracellularly). As shown in Fig 1,

in each case, the percent of positive cells is significantly lower when CD8⁺ T cells were co-cultured with un-infected BECs compared to CD8⁺ T cells co-cultured by themselves in the presence of media (first and third column of each graph in Fig. 1) suggesting that healthy BECs have a role in inhibiting CD8⁺ T cell activation.

Respiratory viruses are major triggers of airways inflammation and asthma exacerbations. Bronchial epithelial cells (BECs) represent the primary site of RSV infection and this leads to enhanced T cell responses an inflammation. We therefore examined whether RSV infection of BECs interferes with their ability to inhibit T cell cytokine/cytotoxic protein production. We found that RSV infection of BECs reduces their inhibitory capacity and CD8⁺ T cells have an increased frequency of cytokines (IFN-γ, IL-2, and IL-4) or cytotoxic protein (granzyme B) when co-cultured with RSV-infected BECs compared to when co-cultured with un-infected BECs (first and second column of each graph in Fig. 1). We confirmed this increased frequency was not a result of lymphocyte stimulation due to RSV released by the BECs in the co-culture interacting directly with the lymphocytes, as in each case, the percent of cytokine/cytotoxic protein-positive cells on CD8⁺ T cells co-cultured with the same titer of RSV alone, in the absence of BECs, was non-significant compared with CD8⁺ T cells cultured by themselves with media alone (last two columns of each graph in Fig. 1). As CD8⁺ T cells were re-stimulated with un-specific stimuli (PMA and ionomycin) it not surprising they are producing a wide range of cytokines, type 1 and type 2. Optimal CD8⁺ T cell responses to RSV are predominantly type 1 (antiviral cytokine IFN-γ production and cytolysis of virus-infected cells) and over exuberant type 2 responses are detrimental [12,13]. Accordingly, in our experiments, the percent of type 1 cytokines (15.3±4.2% for IFN-γ and 12.8±4.7% for IL-2), is predominantly over type 2 cytokines (IL-4, 0.8±0.2%) when CD8⁺ T cells were cultured with media alone. In

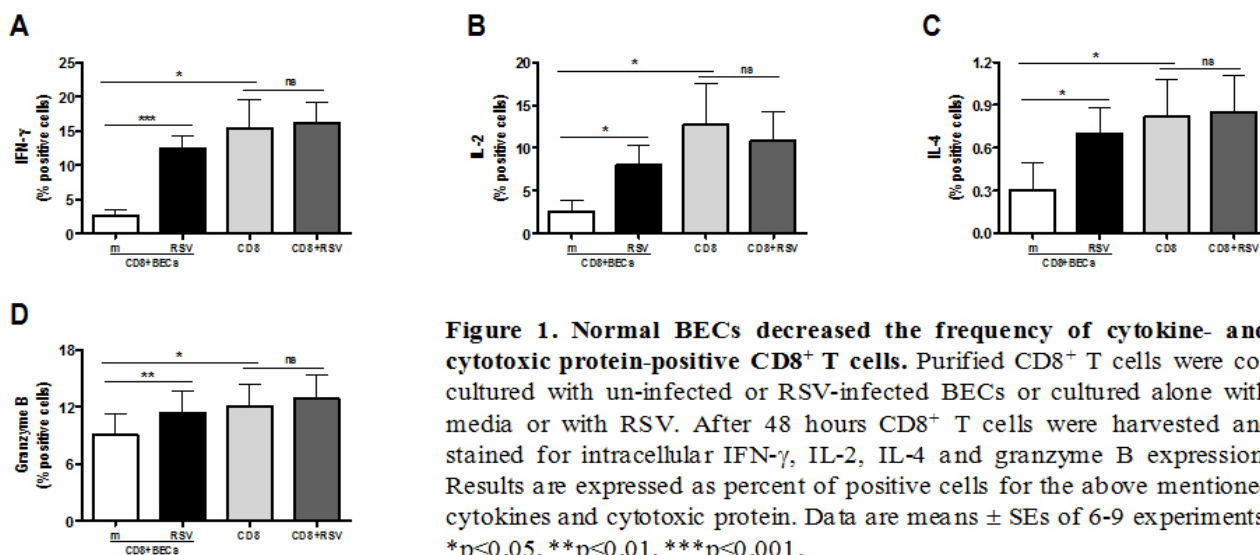


Figure 1. Normal BECs decreased the frequency of cytokine- and cytotoxic protein-positive CD8⁺ T cells. Purified CD8⁺ T cells were co-cultured with un-infected or RSV-infected BECs or cultured alone with media or with RSV. After 48 hours CD8⁺ T cells were harvested and stained for intracellular IFN-γ, IL-2, IL-4 and granzyme B expression. Results are expressed as percent of positive cells for the above mentioned cytokines and cytotoxic protein. Data are means ± SEs of 6-9 experiments; **p*<0.05, ***p*<0.01, ****p*<0.001.

the case of granzyme B, the difference in the percent of positive CD8⁺ T cells when cultured with media alone was marginally, but significantly greater compared with CD8⁺ T cells co-cultured with un-infected BECs ($12 \pm 2.3\%$ vs $9 \pm 2.1\%$, $p < 0.05$, Fig. 1D) due to the high baseline of granzyme B in these cells. This cytolytic mechanism could be activated in CD8⁺ T cells due to allogeneic stimulation by un-infected BECs.

CD8⁺ T cells cultured with media alone proliferated more compared to CD8⁺ T cells co-cultured with un-infected BECs

We next compared the proliferation of CD8⁺ T cells when cultured by themselves or co-cultured with un-infected BECs. When co-cultured with un-infected BECs, the great majority of CD8⁺ T cells did not proliferate ($\sim 80.68\%$, Fig. 2A, region 1, very light grey histogram), while CD8⁺ T cells cultured alone underwent proliferation, with very few cells remaining non-proliferative ($\sim 10.73\%$, Fig. 2A, region 1, black histogram), suggesting that healthy BECs inhibit CD8⁺ T cell proliferation. When CD8⁺ T cells were co-cultured with RSV-infected BECs, inhibition of CD8⁺ T cell proliferation by BECs was lost and most of them underwent proliferation, with only $\sim 33.7\%$ remaining non-proliferative (Fig. 2A, region 1, light grey histogram). Similarly with cytokine production, proliferation of CD8⁺ T cells cultured by themselves, was not influenced directly by RSV, as proliferation of CD8⁺ T cells cultured in the presence of RSV was not different compared to proliferation of CD8⁺ T cells cultured in the presence of media (Fig. 2A, dark grey and black histograms).

We obtained similar results when we used total PBMCs cultured alone or co-cultured with BECs; histograms presented in Fig. 2B show the proliferation of CD8⁺ T cells gated from total PBMCs. The patterns of proliferation are even more clear compared with Fig. 2A. Region 1 in Fig. 2B was set to include un-proliferated cells plus cells that underwent a low degree of proliferation, and

regions 2 and 3 were set to include the cells that underwent higher degrees of proliferation. When lymphocytes were co-cultured with un-infected BECs the percent of cells situated in region 1 was $\sim 74.57\%$ compared with only 1.46% when they were cultured alone with media (very light grey versus black histogram). When BECs were infected with RSV they lost the capacity of inhibiting CD8⁺ T cell proliferation in co-culture ($\sim 16.65\%$ cells in region 1, light grey histogram). Again, RSV didn't influence directly CD8⁺ T cell proliferation as there is no difference between histograms when PBMCs are cultured by themselves, with or without RSV (Fig. 2B, dark grey and black histograms).

Under viral infection, BECs can undergo apoptosis and necrosis. We therefore assessed cell death by trypan blue exclusion assay over few days period, when BECs were cultured in control medium or infected with RSV (MOI=1). In co-culture with CD8⁺ T cells, BECs formed initial a confluent monolayer, but in time, in the case of RSV-infected BECs due to cell death the confluence decreased dramatically, in contrast to uninfected BECs were the cells were overgrown. Indeed, without RSV infection BECs are little variable as number (3×10^5 cells/well at time 0; 3.5×10^5 on day 1; 2.8×10^5 day 2 and 2.5×10^5 cells/well on day 3) but when BECs are infected with RSV the number is decreasing rapidly (1.5×10^5 day 1; 1×10^5 day 2 and 0.6×10^5 cells/well on day 3). The substantial rate of cell death in BECs after RSV infection probably contributed to the reduction in CD8⁺ T cell inhibition when co-cultured with RSV-infected BECs compared with CD8⁺ T cells co-cultured with un-infected BECs.

Similar results were reported based on *in vitro* studies with murine cells [14]; they found that dendritic cell induced antigen-specific T cell proliferation and cytokine production is inhibited by lung epithelial cells. Here, we tried to create *in vitro* an environment similar to that found in the human respiratory tract *in vivo* by using human bronchial epithelial cells and human CD8⁺ T cells. We

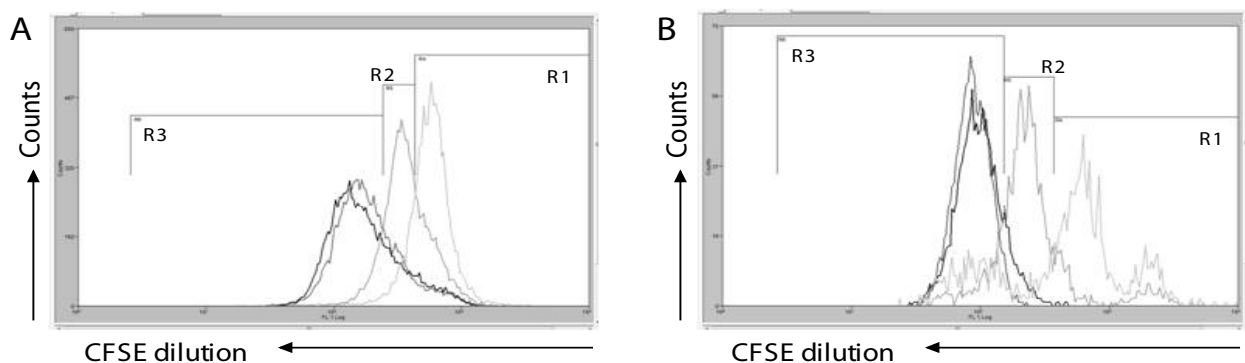


Figure 2. Normal BECs inhibit CD8⁺ T cell proliferation. A. CD8⁺ T cells (CFSE labelled and stimulated with PHA) were co-cultured with un-infected BECs (very light grey histogram) or RSV-infected BECs (light grey histogram) or cultured alone with media (black histogram) or with RSV (dark grey histogram) for 6 days. **B.** Same conditions as above, only total PBMCs were used and the results were gated on CD8⁺ T cells.

found that co-culture of CD8⁺ T cells with confluent layers of BECs reduced CD8⁺ T cell proliferation and significantly decreased the percent of cytokine- and cytotoxic protein-positive CD8⁺ T cells, indicating that BECs provided inhibitory signals that prevented CD8⁺ T cell cytokine production and proliferation. This could simulate the healthy lower respiratory tract mucosa which contains CD8⁺ T cell lymphocytes and provides a tolerogenic environment, minimising inappropriate immune response to harmless inhaled antigens such as allergens. During natural RSV infection extensive destruction of lower respiratory tract epithelium is induced. In affected areas normal inhibition of CD8⁺ T cells may be lost, resulting in excessive local CD8⁺ T cell responses and subsequent inflammation.

Further analysis of the interactions between human bronchial epithelial cells and local lymphocytes will provide an understanding of the human airway mucosal system and the pathogenesis of various airway diseases.

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