RSV-Induced Bronchial Epithelial Cell PD-L1 Expression Inhibits CD8$^+$ T Cell Nonspecific Antiviral Activity

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Respiratory syncytial virus (RSV) is a major cause of bronchiolitis in infants. It is also responsible for high morbidity and mortality in the elderly. Programmed death ligands (PD-Ls) on antigen-presenting cells interact with receptors on T cells to regulate immune responses. The programmed death receptor-ligand 1/programmed death receptor 1 (PD-L1-PD-1) pathway is inhibitory in chronic viral infections, but its role in acute viral infections is unclear. We hypothesized that bronchial epithelial cell (BEC) expression of PD-Ls would inhibit local effector CD8$^+$ T cell function. We report that RSV infection of primary human BECs strongly induces PD-L1 expression. In a co-culture system of BECs with purified CD8$^+$ T cells, we demonstrated that RSV-infected BECs increased CD8$^+$ T cell activation, proliferation, and antiviral function. Blocking PD-L1 on RSV-infected BECs co-cultured with CD8$^+$ T cells enhanced CD8$^+$ T cell IFN-γ, IL-2, and granzyme B production. It also decreased the virus load of the BECs. Based on our findings, we believe therapeutic strategies that target the PD-L1-PD-1 pathway might increase antiviral immune responses to RSV and other acute virus infections.

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Presented abstracts: ``Effector memory CD8$^+$T cell responses to RSV infected respiratory epithelial cells''

AG Telcian, LA Stanciu, and SL Johnston, Department of Respiratory Medicine, NHLI, Imperial College London, London, UK

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``Functional effect of RSV-induced PD-L1 on epithelial cell activation of CD8$^+$T cells''

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``CD8$^+$T cell responses to RSV infected respiratory epithelial cells''

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5th EAACI-GA2LEN-DAVOS Meeting, Basic Immunology on Allergy and Clinical Immunology, Davos, Switzerland, 1-4 February 2007; abstract A55

``Expression of PD-L1 on primary human bronchial epithelial cells and its regulation by respiratory syncytial virus, IFN-γ and IL-4''

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The programmed death ligand (PD-L)-PD-1 receptor pathway consists of the 2 ligands PD-L1 and PD-L2 and their common receptor PD-1. PD-Ls on antigen-presenting cells interact with PD-1 on T cells to regulate T cell responses [1]. In chronic viral infections, blocking PD-1 engagement to PD-L1 revived the ex vivo function of exhausted virus-specific $CD8^+$ T cells [2–4] and in vivo, restored $CD8^+$ T cell ability to proliferate, secrete cytokines, and kill infected cells [5].

The role of PD-L1-PD-1 interactions in acute viral infections is not yet well understood. When PD-1 KO mice were infected with adenovirus, there was hepatic T cell expansion and more rapid viral clearance [6]. Furthermore, during acute rabies virus infection, PD-L1 absence led to better virus control as well as greater local accumulation of $CD8^+$ T cells [7].

The role of the PD-1-PD-L1 pathway in acute respiratory viral infections is unknown.

Respiratory syncytial virus (RSV) is the major cause of respiratory morbidity in infants, children [8], and the elderly [9]. Immunity to RSV is incomplete, and re-infections may occur throughout life. RSV persistence has been reported in mice [10], guinea pigs [11], and rats [12]. Most importantly, it has been reported in humans with chronic obstructive pulmonary disease [13]. The mechanisms of deficient-memory immune response to RSV infection remain un-elucidated and demand further research.

Following recovery from a respiratory virus infection, an estimated ~50% of memory $CD8^+$ T cells established are effector-memory T cells that persist in non-lymphoid tissues [14, 15]. These local effector-memory $CD8^+$ T cells are involved in the initial response to re-infection, assisted by additional effector-memory $CD8^+$ T cells recruited from the circulation [16].

Bronchial epithelial cells (BECs) are the primary site of RSV infection and express molecules associated with antigen presentation [17], including the PD-Ls [18, 19]. The PD-L–PD-1 pathway is believed to be important in host defense as many viruses exploit the inhibitory PD-L1-PD-1 pathway to down-regulate T cell responses and promote pathogen persistence [1].

We reasoned that by expressing PD-L molecules BECs may directly influence antiviral activity of effector-memory $CD8^+$ T cells. The aim of our study, therefore, was to assess PD-L expression in primary human BECs and their modulation by RSV. Also, in a BEC/T cell co-culture system, we determined the influence of PD-1-PD-L pathways on $CD8^+$ T cell antiviral functions.

MATERIALS AND METHODS

Cell culture and virus infection

RSV A2 strain was grown in Hep-2 cells, and virus titer determined by plaque assay. Human primary BECs (Clonetics, Cambrex) and the BEAS-2B bronchial epithelial cell line (European Collection of Animal and Cell Cultures) were cultured with RSV, UV-inactivated RSV, and filtered RSV, as described elsewhere [19, 20].

BEC/$CD8^+$ T cell co-cultures

BEAS-2B cells were co-cultured with human $CD8^+$ T cells (ratio, BEC:CD8 = 1:1). $CD8^+$ T cells were isolated from peripheral blood of healthy donors by negative selection (Miltenyi Biotec). The study was approved by the local ethics committee, and we obtained informed consent.

BEAS-2B cells were seeded in 24-well plates and infected with RSV (multiplicity of infection [MOI], 1). Medium was removed 24 hrs after infection. Blocking antibodies to PD-L1, PD-L2, or isotype control (eBioscience, 10 μg/2.5 × 10^5 BECs) were added 3 hrs before our addition of $CD8^+$ T cells (purity, >95%). The moment when $CD8^+$ T cells were added to BECs was considered time 0.

RNA extraction, reverse transcription, and TaqMan real-time PCR

RNA was extracted (RNeasy Mini Kit, Qiagen), and 1 μg was used for cDNA synthesis (Omniscript RT Kit, Qiagen). Quantitative PCR was performed using primers and probes for PD-L1 (forward, 5′-GAATTGGTCATCCCAGAACTACCT-3′; reverse, 5′-GCATAATAAGATGGCTCCCAGAAT-3′; probe, FAM 5′-TGGCACATCCTCCAAATGAAAGGACTCA-3′ TAMRA), PD-L2 (forward, 5′-GCTGTGGCAAGTCCTCATATCA-3′; reverse, 5′-GCTGCAATTCCAGGCTCAAC-3′; probe, FAM 5′-ATACAGAACATGATCTTCCTCCTGCTAA-3′ TAMRA), RSV-L gene and 18S rRNA [20, 21].

Data were analyzed using Prism 7500 SDS software (version 1.4, ABI), normalized to 18S rRNA and presented as 45-δCT for PD-L1 and PD-L2 or converted to copy number using a standard curve for a plasmid of known concentration containing the amplified region of the RSV genome.

Flow cytometry

Epithelial cells were trypsinized to obtain a cell suspension and processed as described elsewhere [19]. Surface expression of PD-L1 and PD-L2 was detected on BECs by direct staining using mouse anti-human antibodies from BD Pharmingen: PD-L1 (PE) and PD-L2 (FITC or APC). Results were expressed as mean fluorescence intensity (MFI) after subtracting the MFI of control cells stained with the appropriate isotype control antibodies (BD Pharmingen). We acquired at least 10,000 events using a BD LSR flow cytometer with CellQuest software (BD Bioscience) or a CyAN with Summit software (version 4.3, DakoCytomation).

FITC-carboxyfluorescein succinimidyl ester-(CFSE)-labeled $CD8^+$ T cells [22] were stained for surface markers only (CD45RA [APC] and CCR7 [PECy7], as well as CD69, CD25, or PD-1 [all PE]) or combined surface and intracellular staining for cytokines/cytotoxic proteins: IFN-γ, IL-2, perforin (BD Pharmingen), and granzyme B (eBioscience) (all PE)] [23]. Analysis was performed on a CyAN 7 or 9 with Summit software (version

4.3, DakoCytomation) after we acquired at least 20,000 events. Appropriate isotype controls were included to assess the background fluorescence. Results were expressed as the percentage of all $CD8^+$ T cells expressing a certain marker and as the distribution of these readouts in $CD8^+$ T cell–specific subpopulations.

To assess cell proliferation, we added PHA (1 μg/mL, Sigma) to medium when we added $CD8^+$ T cells to the co-culture. In different experiments we incubated $CD8^+$ T cells overnight in plates coated with anti-CD3 monoclonal antibody (10 μg/mL, BD Pharmigen) in the presence of IL-2 (50 U/mL, Biosource). After 4–6 days of co-culture with BECs, we removed the lymphocytes along with supernatants and analyzed them by flow cytometry.

ELISA for IFN-γ, IL-2, and granzyme B

Supernatants from the co-cultures were used to measure IFN-γ and IL-2 levels using paired Abs and standards (CytoSet-s from Biosource). Assay sensitivities were 8 pg/mL (IFN-γ) and 15 pg/mL (IL-2). Granzyme B was tested by direct ELISA using an anti-human granzyme B antibody, followed by a biotinylated antibody (R&D Systems) with a sensitivity of 2 ng/mL.

Statistical analysis

Results were analyzed using Prism (version 4.00, GraphPad). Results of at least 3 separate experiments were expressed as means ± standard errors of the means (SEM). When analyzing multiple groups, we used a 1-way ANOVA of all pairs and columns, followed, if significant, by a paired *t* test for paired comparisons. We considered $P < .05$ to be statistically significant.

RESULTS

PD-L1 is constitutively expressed by human primary BECs and is up-regulated by RSV infection

We previously reported the effect of RSV infection on PD-L1 and PD-L2 expression on cultured respiratory epithelial cell lines [19]. Here, we confirm that human primary BECs constitutively express PD-L1 (Figure 1A) but not PD-L2 (Figure 1B).

Exposure of human primary BECs to increasing doses of RSV up-regulated PD-L1 mRNA expression and surface protein levels in a dose-responsive manner (Figure 1C and 1E). RSV up-regulation of PD-L1 was replication-dependent (UV-inactivated RSV had no effect on PD-L1 expression) (Figure 1C and 1E). PD-L2 mRNA expression was up-regulated by RSV, but surface protein was not found at 24 hrs after infection (Figure 1D and 1F).

RSV infection of BECs activates and increases proliferation of $CD8^+$ T cells

Having demonstrated that RSV up-regulates PD-L1 expression on human primary BECs, we next investigated whether this molecule is functional in interacting with human $CD8^+$ T cells in an in vitro co-culture system in which RSV-infected BEAS-2B cells directly interact with $CD8^+$ T cells.

We initially confirmed our previous findings [19] that BEAS-2B cells express PD-L1/PD-L2 and that RSV up-regulated them (Figure S1A-D). We also confirmed the specificities of blocking antibodies to PD-L1 and PD-L2 (Figure S2E-F). As RSV-induced PD-L1/PD-L2 expression in these cells was present from 24 hrs until 96 hrs, BECs were infected with RSV alone for 24 hrs and then co-cultured for 72 hrs, so that induced PD-L expression would occur during BEC/$CD8^+$ T cell co-cultures.

We first determined whether RSV infection of BECs results in $CD8^+$ T cell activation in co-culture by determining CD69, CD25, and PD-1 levels. $CD8^+$ T cells expressed activation markers CD69 and CD25 (Figure 2A-B) significantly more when co-cultured with RSV-infected BECs compared to co-culture with uninfected BECs. We detected no significant difference in PD-1-positive $CD8^+$ T cells (Figure 2C). $CD8^+$ T cell activation was not a result of direct stimulation by RSV, as CD69 positivity on $CD8^+$ T cells exposed to RSV alone was 2.4 ± .8%, similar to $CD8^+$ T cells co-cultured with uninfected BECs (2.1 ± .3%) or $CD8^+$ T cells in media alone (1.9 ± .8%, P = ns between groups).

According to CD45RA and CCR7 expression, $CD8^+$ T cells are classified into the following subsets: effector memory (EM), terminally differentiated (TD), central memory (CM), and naïve [24]. Because $CD8^+$ T cells present in the respiratory epithelium are likely to be either EM or TD cells, we investigated the ability of RSV-infected BECs to activate these 2 specific subpopulations. Significantly more EM and TD $CD8^+$ T cells expressed CD69 when co-cultured with RSV-infected BECs compared to EM or TD $CD8^+$ T cells in co-culture with uninfected BECs (Figure 2D). We observed no significant difference in $CD25^+$ cells (Figure 2E), but we saw frequencies of PD-1-positive cells that were higher in both $CD8^+$ T cell subpopulations during exposure to RSV-infected BECs (Figure 2F).

We next compared proliferation of CFSE-labeled $CD8^+$ T cells in co-culture with uninfected or RSV-infected BECs. The percentage of non-proliferating PHA-stimulated cells was higher in $CD8^+$ T cells co-cultured with uninfected BECs (94.8 ± 1.2%) compared to co-cultures with RSV-infected BECs (21.09 ± 4.9%, n = 4, $P < .001$), where cells underwent proliferation of 2–8 generations. We obtained similar results when we pre-stimulated $CD8^+$ T cells with anti-CD3/IL-2 before adding them to the co-culture (Figure 2G).

RSV infection of BECs increases antiviral cytokine production and cytotoxic capacity in $CD8^+$ T cells

IFN-γ and IL-2 production are key elements of $CD8^+$ T cell antiviral function. We determined the frequencies of IFN-γ^+CD8^+ and IL-2^+CD8^+ cells in co-culture by intracellular staining (plots of IFN-γ and IL-2 staining in $CD8^+$ T cells are

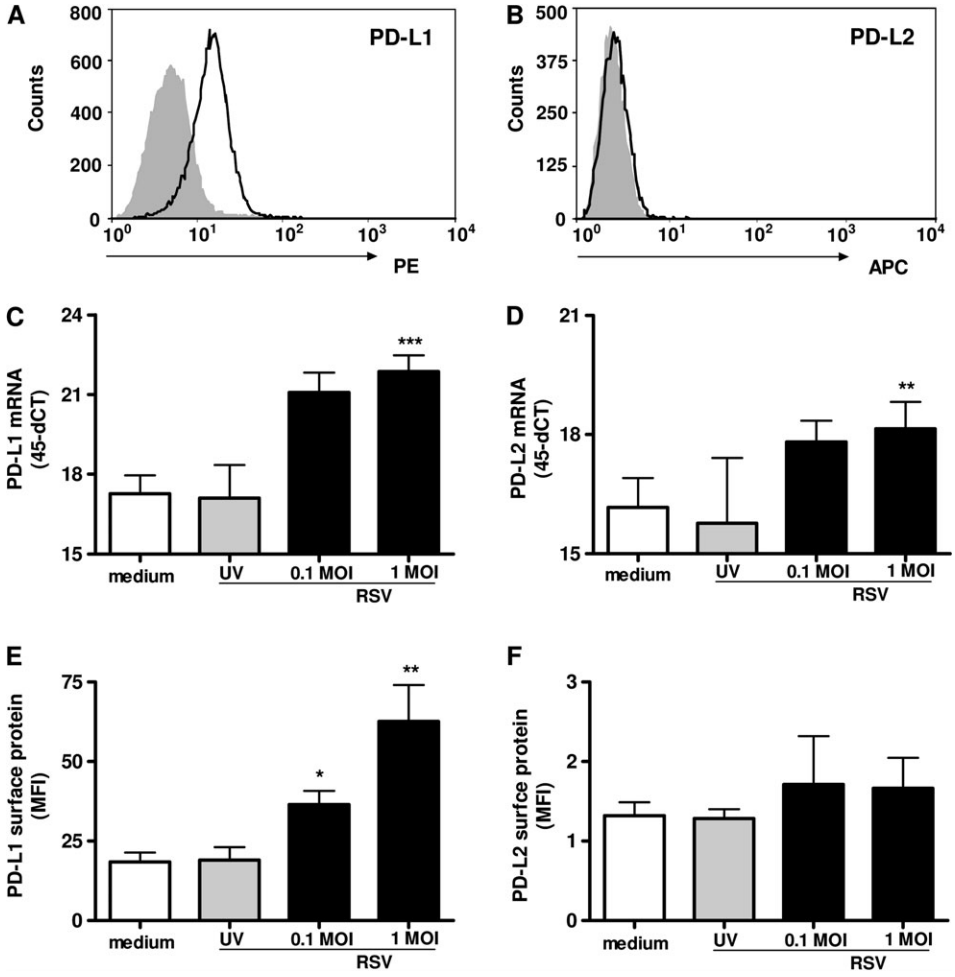

Figure 1. Constitutive expression of PD-L1 and PD-L2 in human primary BECs and response to RSV infection. *A-B*: Cells cultured in medium alone were stained with isotype control (gray filled) or specific antibody (black line) to PD-L1 and PD-L2. C-F: Cells cultured in medium (white bars) or treated with UV-inactivated virus (gray) or with RSV at MOI of .1 or 1 (black) were harvested at 8 hrs for mRNA expression [*C-D*] and 24 hrs for surface protein expression [*E-F*]. Data are means ± SEMs of 5–7 experiments; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

shown in Figure 3A). The percentage of $CD8^+$ T cells positive for IFN-γ or IL-2 was significantly higher when cells were co-cultured with RSV-infected BECs compared with uninfected BECs (Figure 3B-C).

Among the effector subpopulations, both EM and TD $CD8^+$ T cells showed significantly increased frequencies of IFN-γ^+ cells, but not of IL-2^+ cells, when co-cultured with RSV-infected BECs compared with uninfected BECs (Figure 3D-E).

To further investigate the subpopulations of $CD8^+$ T cells producing each cytokine, we back-gated $CD8^+$ T cells producing each cytokine and plotted these positive cells with markers for $CD8^+$ T cell subpopulations (Figure 3F-G, Table 1). The majority of IFN^+CD8^+ T cells were EM and TD, whereas very few were naïve or central memory. The majority of IL-2-producing cells were naïve, although substantial numbers of cells were EM and smaller numbers were TD or central memory.

We also assessed IFN-γ and IL-2 release into supernatants from the co-cultures and found a significant increase in IFN-γ and IL-2 levels when $CD8^+$ T cells were co-cultured with RSV-infected BECs compared with uninfected BECs (Figure 5A-B, left 2 columns). Peak levels were at 48 hrs after co-culture (Figure S1G-H). Therefore we used this time point to determine most outcomes.

Because cytotoxic activity is also an important antiviral function of $CD8^+$ T cells, we next investigated how RSV-infected BECs influence $CD8^+$ T cell cytotoxic activity. Representative plots of granzyme B and perforin-staining gated on $CD8^+$ T cells are shown in Figure 4A. Frequencies of granzyme B- and perforin-positive $CD8^+$ T cells were marginally—and this is significantly—greater in co-culture with RSV-infected BECs compared with uninfected BECs (Figure 4B-C).

When we examined effector subpopulations, we observed similar small, but significant, increases in granzyme B- and perforin-positive cells among TD $CD8^+$ T cells when co-cultured with RSV-infected BECs. However, we observed no significant differences in the EM populations (Figure 4D-E).

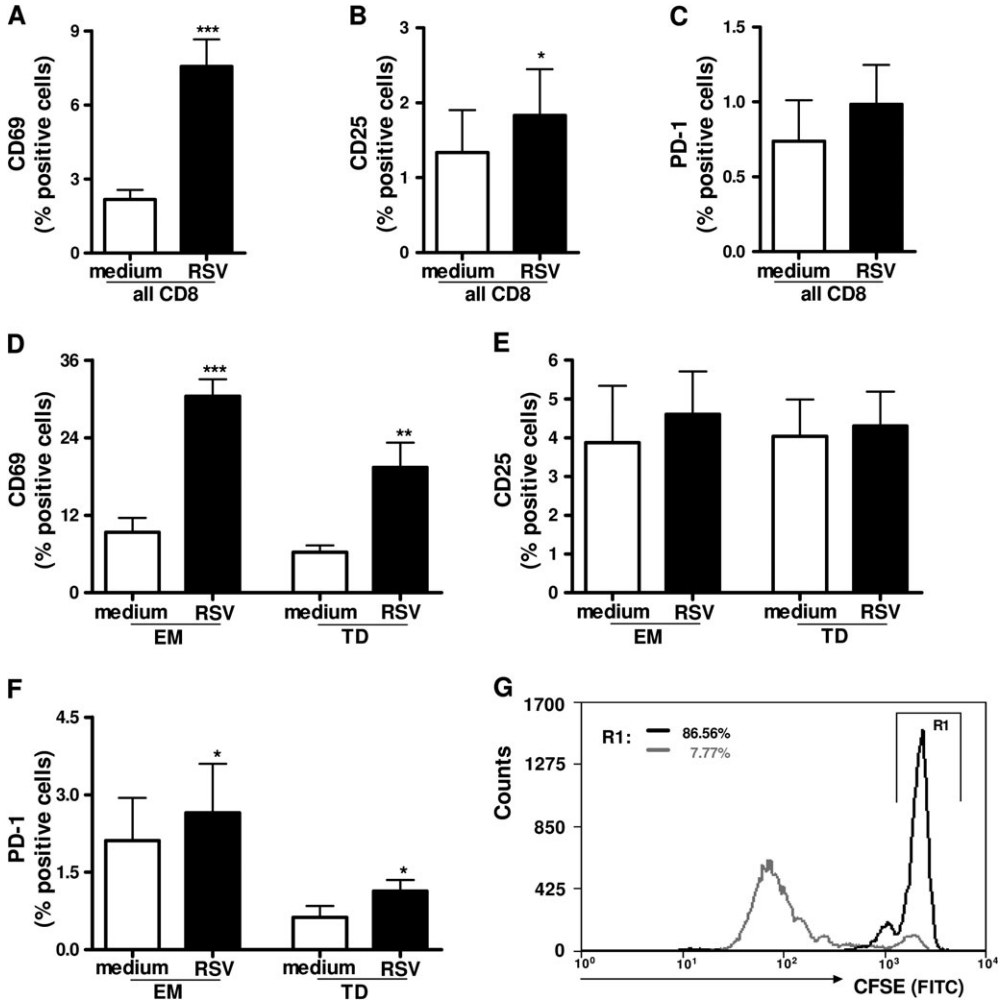

Figure 2. RSV infection of BECs induces activation of co-cultured $CD8^+$ T cells. *A-F*: $CD8^+$ T cells (CFSE labeled) were harvested at 48 hrs from the co-culture system and stained for CD69, CD25, and PD-1. CD69, CD25, and PD-1 expression on $CD8^+$ T cells co-cultured with uninfected (white bars) or RSV-infected BECs (black) gated on total $CD8^+$ T cells [*A-C*] or effector memory and terminally differentiated subsets [*D-F*]. Results are expressed as percentage of positive cells. Data are means ± SEMs of 4–9 experiments; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. *H*: $CD8^+$ T cells (CFSE labeled) stimulated with anti-CD3/IL-2 overnight were co-cultured with uninfected (black histogram) or RSV-infected BECs (gray histogram) for 4 days.

After back-gating the $CD8^+$ T cells that produced each cytotoxic protein from uninfected and RSV-infected BEC co-cultures, we confirmed that the majority of granzyme B- and perforin-producing cells were EM and TD $CD8^+$ T cells (Figure 4F-G, Table 1).

Granzyme B protein release into supernatants of co-cultured $CD8^+$ T cells and RSV-infected BECs also showed an increase compared with co-cultures $CD8^+$ T cells and uninfected BECs (Figure 5C, left 2 columns).

PD-L1 blockade increases antiviral cytokine and cytotoxic protein levels in $CD8^+$ T cells co-cultured with RSV-infected BECs

To investigate the functional roles of PD-L1/PD-L2-PD-1 pathways in BEC/$CD8^+$ T cell interactions, we co-cultured $CD8^+$ T cells with RSV-infected BECs in the presence of blocking antibodies to PD-L1. These co-cultures released increased IFN-γ, IL-2, and granzyme B levels into supernatants, compared with co-cultures in the absence of anti-PD-L1 antibodies (Figure 5A-C, middle 2 panels). In contrast, anti-PD-L2 antibodies had no influence on IFN-γ, IL-2, or granzyme B released in $CD8^+$ T cell/RSV-infected BEC co-cultures (Figure 5A-C, second and fourth columns). The isotype controls showed no effect on released cytokines compared with $CD8^+$ T cells co-cultured with RSV-infected BECs with no blocking antibodies (IFN-γ, 139.5 ± 28.3 vs 129.6 ± 22.5 pg/mL; IL-2, 43.7 ± 5.5 vs 35.1 ± 4.5 pg/mL; granzyme B, 15.7 ± 3.8 vs 16.8 ± 2.9 ng/mL; P = ns in all cases).

PD-L1 blockade decreases RSV gene expression in BECs

Finally, we assessed RSV replication by quantifying RSV polymerase L gene expression in BECs in the co-culture system (Figure 5D) and observed a significant reduction (Figure 5D, left 2 columns) in RSV L gene expression when RSV-infected

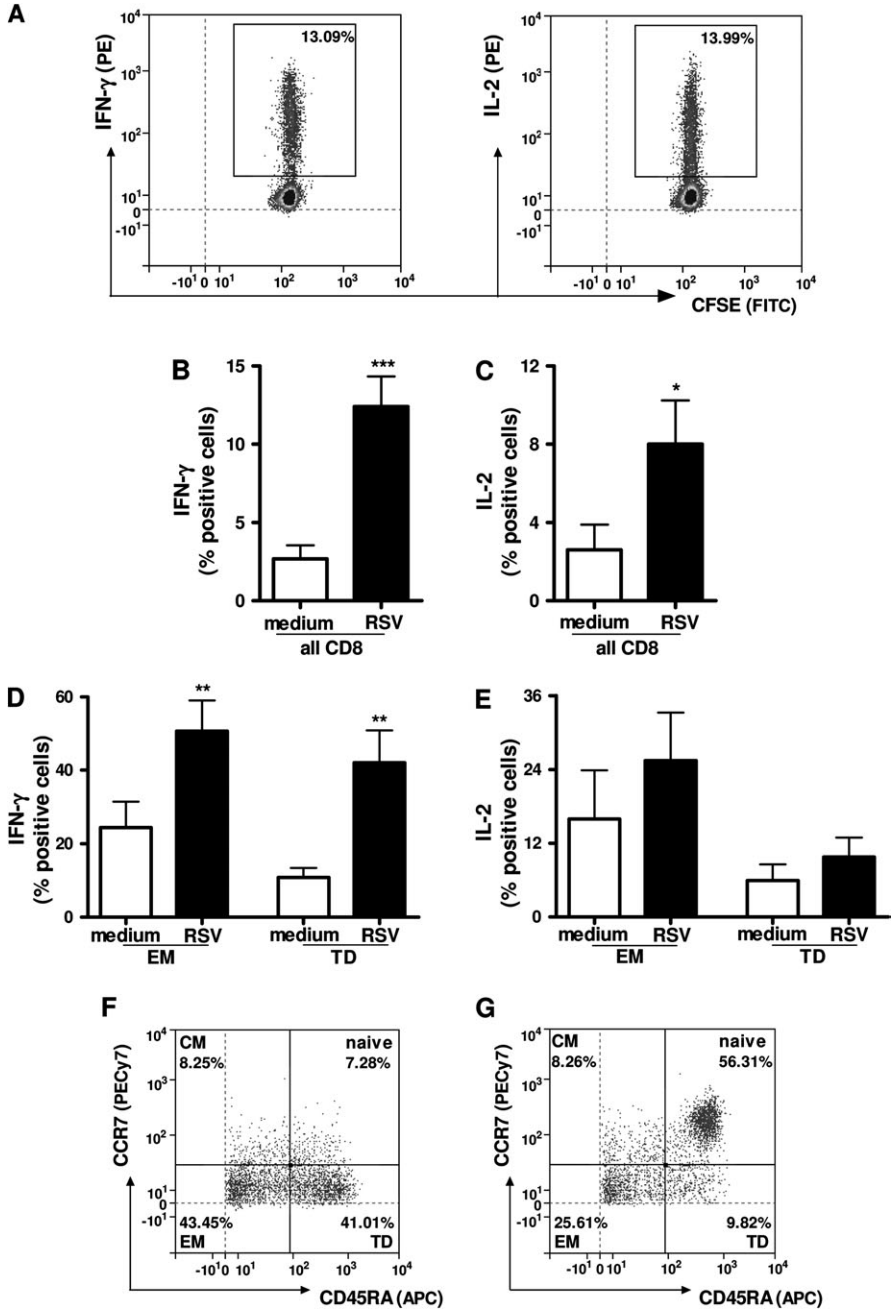

Figure 3. RSV infection of BECs induces antiviral cytokine production in $CD8^+$ T cells. $CD8^+$ T cells (CFSE labeled) were harvested at 48 hrs from the co-culture system and stained for intracellular IFN-γ or IL-2 (PMA and ionomycin in the presence of brefeldin were used to re-stimulate the cells for the last 4 hrs before staining). *A*: Representative histograms of IFN-γ and IL-2 expression by total $CD8^+$ T cells (samples co-cultured with RSV-infected BECs). *B-E*: IFN-γ and IL-2 positivity of $CD8^+$ T cells co-cultured with uninfected (white bars) or RSV-infected BECs (black) gated on total $CD8^+$ T cells [*B-C*] or gated on effector memory and terminally differentiated subsets [*D-E*]. Results are expressed as percentage of positive cells gated as shown in Figure 3A. Data are means ± SEMs of 6–7 experiments; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$. *F-G*: Cells positive for IFN-γ or IL-2 were back-gated into a plot with markers for $CD8^+$ T cells subsets to identify the subtype of the producing cells.

BECs were co-cultured with $CD8^+$ T cells, compared with RSV-infected BECs alone ($[17.6 \pm 2.7]\times10^6$ vs $[31.9 \pm 8.3]\times10^6$ copy numbers, 40% reduction, $P < .05$). This assessment confirms that $CD8^+$ T cells have an antiviral effect in this co-culture system. RSV L gene expression was further decreased in the presence of PD-L1 blocking antibodies in co-cultures compared with RSV-infected BECs/$CD8^+$ T cells without blocking antibody ($[11.1 \pm 2.7]\times10^6$ vs $[17.6 \pm 2.7]\times10^6$ copy numbers, $P < .05$) (Figure 5D, middle 2 columns), whereas the isotype control antibody had no effect ($[14 \pm 3.4]\times10^6$ vs $[17.6 \pm 2.7]\times10^6$ copy numbers, $P =$ ns). As we expected based on their failure to influence type 1 cytokine and cytotoxic protein production, PD-L2

Table 1. Distribution of cytokine- and cytotoxic protein–positive $CD8^+$ T cells within $CD8^+$ T cell subpopulations. $CD8^+$ T cells were co-cultured with uninfected or RSV-infected BECs, harvested at 48 hrs from the co-culture system, and stained for intracellular IFN-γ, IL-2, granzyme B, and perforin. Positive cells were back-gated into a plot with markers for $CD8^+$ T cells subsets to identify the type of the producing cells. Results are expressed as percentage of positive cells for each of the $CD8^+$ T cell subpopulations (effector memory [EM], terminally differentiated [TD], central memory [CM], and naïve). Data are means ± SEs of 5–7 experiments.

	EM (%)	TD (%)	CM (%)	Naïve (%)	
IFN-γ	41.2±3.5	32.9±4.4	9.6±2.05	16.3±3.7	+ uninfected BECs
	48.9±2.1	30.3±2.8	13.02±2.6	7.7±1.1	+ RSV-infected BECs
IL-2	26.3±4.9	17.3±4.4	16.6±2.1	39.7±7.6	+ uninfected BECs
	37.6±5.3	12±2.2	19.5±4.1	30.9±5.5	+ RSV-infected BECs
Granzyme B	37.7±2.5	58.1±3.2	1.5±.4	2.7±.6	+ uninfected BECs
	43.3±3.1	49.1±3.5	3.9±.9	3.7±1.04	+ RSV-infected BECs
Perforin	24.5±5.6	68.1±5.7	0.25±.2	7.1±3.9	+ uninfected BECs
	31.4±5.1	60.6±6.2	3.3±2.3	4.6±2.2	+ RSV-infected BECs

blocking antibodies did not influence the RSV gene expression in co-culture ($[15.6 \pm 4.1]\times10^6$ vs $[17.6 \pm 2.7]\times10^6$ copy numbers, P = ns) (Figure 5D).

DISCUSSION

We report here that RSV infection of primary human BECs strongly up-regulated PD-L1 expression and that in $CD8^+$ T cell/BEC co-cultures, RSV-infected BECs increased $CD8^+$ T cell activation, proliferation, and antiviral cytokine and cytotoxic protein production. We observed the latter chiefly among effector-memory and terminally differentiated $CD8^+$ T cell subsets. PD-L1 blockade enhanced $CD8^+$ T cell secretion of IFN-γ, IL-2, and granzyme B and suppressed RSV replication. These data indicate that virus induction of PD-L1 expression on BECs can inhibit local $CD8^+$ T cell antiviral activities in the context of an acute respiratory viral infection.

PD-L1 is constitutively expressed in primary nasal cells and BECs [18, 19, 25]. Poly I:C and rhinovirus-16 induce both PD-L1 and PD-L2 on primary BECs and nasal epithelial cells [25]. We found that RSV infection of primary BECs increased PD-L1-expression robustly, ~30-fold for mRNA and ~4-fold for protein. In contrast, rhinovirus-16 up-regulated PD-L1 protein only ~2-fold on human primary BECs [25]. These data, together with the known inhibitory effects of PD-L1 in the context of chronic viral infections [2–4], prompted us to investigate in vitro the functional importance of PD-L1 induction in the antiviral immune responses to RSV infection.

We first hypothesized that uninfected epithelial cells would not activate $CD8^+$ T cells, but in contrast RSV-infected BECs would stimulate $CD8^+$ T cells to express activation markers, proliferate, and induce cytokine and cytotoxic protein secretion.

CD69 is one of the earliest activation markers on T cells. The peak proportion of $CD69^+$ T cells in response to alloantigen stimulation is reported to be <3% [26]. Consistent with this, in our studies, CD69 was present on 2–3% of $CD8^+$ T cells co-cultured with uninfected BECs. But this percentage was significantly increased (~3 times) on $CD8^+$ T cells co-cultured with RSV-infected BECs. We noticed a weaker induction for CD25 and PD-1 (which is persistently up-regulated on exhausted lymphocytes in chronic viral infections) [2–4]. PD-1 was found in low percentages (< 0.5%) on our $CD8^+$T cells as they were isolated from normal donors, and was not significantly increased on total $CD8^+$T cells co-cultured with RSV-infected BECs.

It is reported that healthy epithelium can suppress local lymphocyte activation, whereas infected epithelium becomes an activator [27]. The mechanisms through which $CD8^+$ T cells get activated by virus-infected BECs are not fully understood. It was shown that activation is partly contact-dependent and partly mediated by transforming growth factor β [27]. We have observed that RSV up-regulates MHC class I molecule levels on the surface of infected BECs (data not shown). It is also likely that RSV up-regulates CD80 and CD86 molecules. These molecules have been shown to be induced by rhinovirus infection on respiratory epithelial cells [17]. So far, there are no data to support that $CD8^+$ T cell activation by virus-infected BECs occurs via these molecules.

The $CD8^+$ T cells most likely found in contact with BECs in vivo are the EM and TD subpopulations [14, 15]. We observed that the levels of expression of CD69, CD25, and PD-1 were all higher in these subpopulations than in total $CD8^+$ T cells co-cultured with uninfected BECs. In addition their induction by RSV-infected BECs was greater in the EM and TD subpopulations than in total $CD8^+$ T cells. These data suggest the $CD8^+$ T cells, which respond to RSV-infected BECs by activation, are the EM and TD $CD8^+$ T cell subsets.

Nonspecific-stimulated $CD8^+$ T cells did not proliferate in uninfected BEC/$CD8^+$ T cell co-cultures. However, the majority of nonspecific-stimulated $CD8^+$ T cells co-cultured with RSV-infected BECs underwent >1 round of proliferation. Nonspecific-stimulated $CD8^+$ T cells cultured alone proliferated in a manner similar to $CD8^+$ T cells co-cultured with

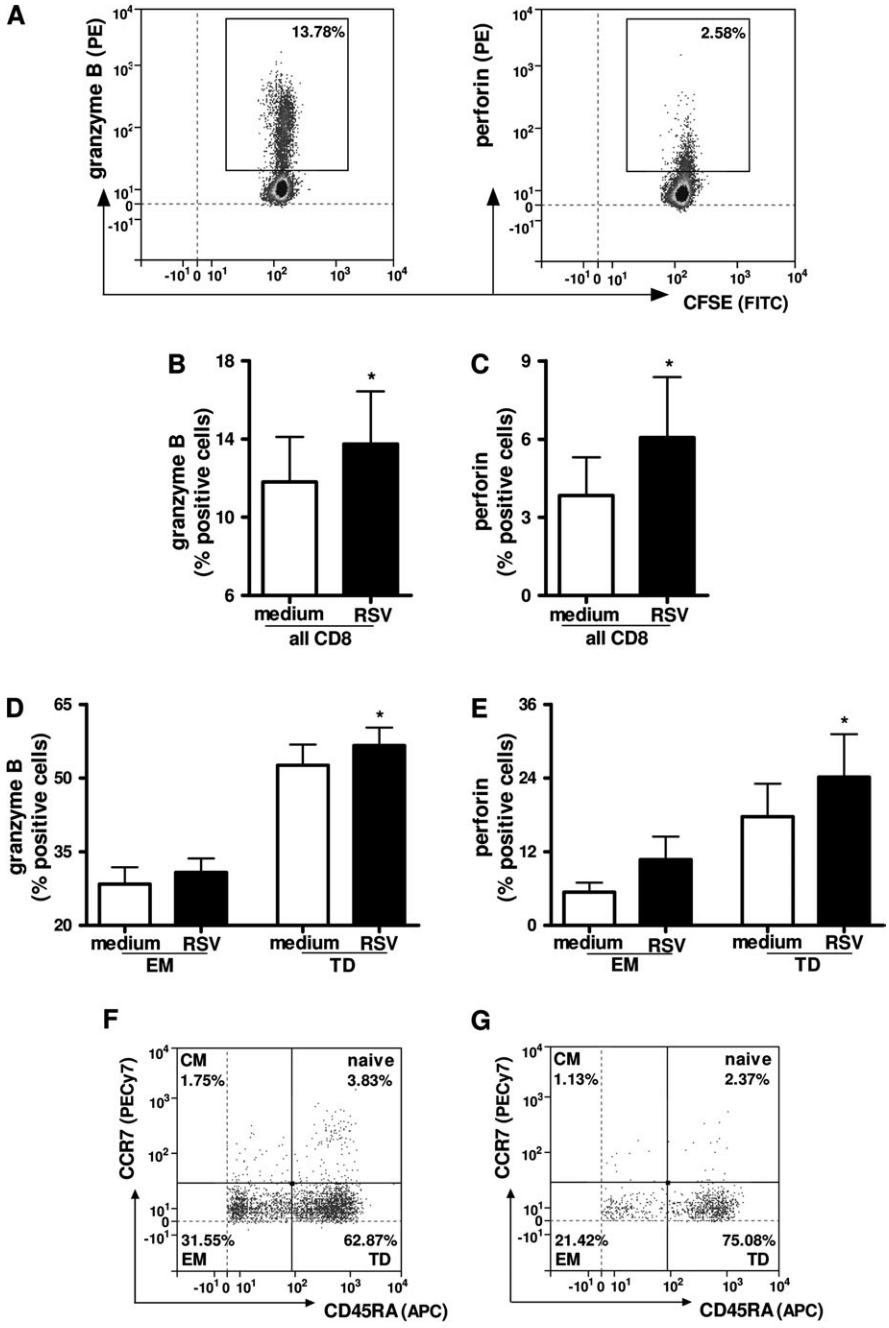

Figure 4. RSV infection of BECs induces antiviral cytotoxic granule protein production in $CD8^+$ T cells. $CD8^+$ T cells (CFSE labeled) were harvested at 48 hrs from the co-culture system and stained for intracellular granzyme B or perforin (brefeldin was used for the last 4 hrs before staining for cytotoxic proteins). *A*: Representative histograms of granzyme B and perforin expression by total $CD8^+$ T cells co-cultured with un-infected BECs. *B-E*: Granzyme B and perforin positivity in $CD8^+$ T cells co-cultured with un-infected (white bars) or RSV-infected BECs (black) gated on total $CD8^+$ T cells [*B-C*] or gated on effector memory and terminally differentiated subsets [*D-E*]. Results are expressed as percentage of positive cells gated as shown in Figure 4A. Data are means ± SEMs of 6–7 experiments; $*P < .05$. *F-G*: Cells positive for granzyme B or perforin were back-gated into a plot with markers for $CD8^+$ T cells subsets to identify the subtype of the producing cells.

RSV-infected BECs (data not shown). This proliferation suggests that un-infected epithelium actually has the capacity to inhibit $CD8^+$ T cell proliferation, similar to results obtained in vitro with murine cells [27].

Cytokine (IFN-γ and IL-2) and cytotoxic protein (granzyme and perforin) production showed similar patterns for the activation markers, with higher induction occurring in $CD8^+$ T cells co-cultured with RSV-infected BECs compared with

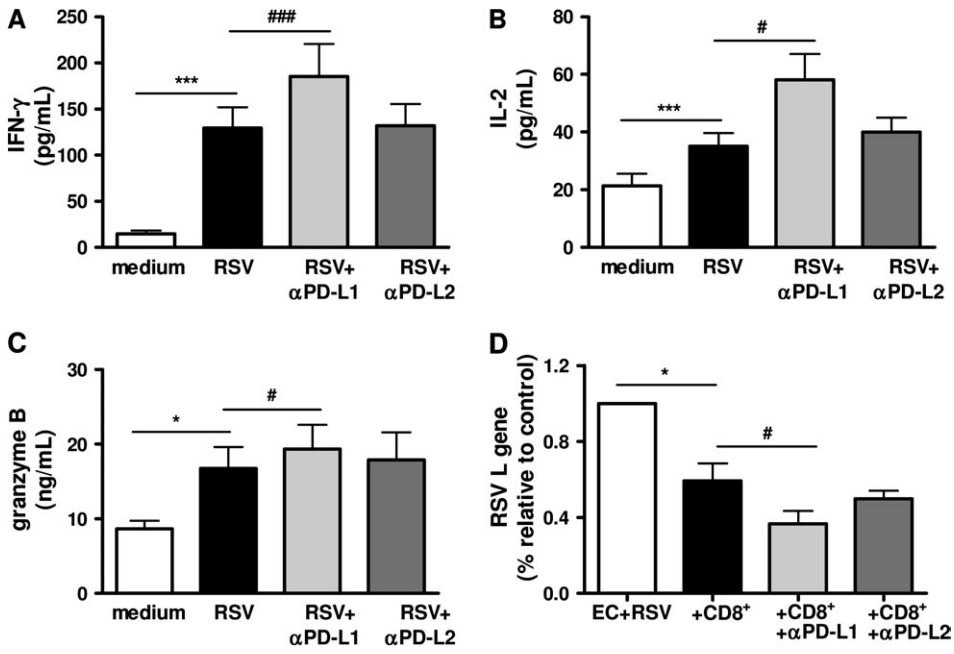

Figure 5. Blocking PD-L1 in co-culture enhances $CD8^+$ T cell antiviral cytokine production and reduces viral load in BECs. *A-C*: Effect of PD-L blockage on the levels of cytokines and cytotoxic proteins released by $CD8^+$ T cells in CD8/BEC co-culture. $CD8^+$ T cells were co-cultured with un-infected (white bars), RSV-infected, (black) or RSV-infected BECs in the presence of anti-PD-L1 (light gray) or anti-PD-L2 (dark gray) blocking antibodies, and supernatants were harvested at 48 hrs after co-culture. IFN-γ, IL-2, and granzyme B were determined by ELISA. Data are means ± SEMs of 7–10 experiments. *D*: Effect of PD-L blockade on RSV replication in BECs in co-culture. BECs were harvested at 72 hrs from the co-culture system, and RSV polymerase was determined by TaqMan. Shown are RSV-infected BECs alone (white bar) and RSV-infected BECs with $CD8^+$ T cells alone (black) or in the presence of blocking antibodies to PD-L1 (light gray) or PD-L2 (dark gray). Data are means ± SEs of 3 experiments; $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

un-infected BECs. Induction of IFN-γ in $CD8^+$ T cells occurred chiefly among the effector cell populations, whereas induction of IL-2 occurred mostly among naïve cells. Granzyme B and perforin production was found almost exclusively in EM and TD subpopulations. But $CD8^+$ T cells expressed high levels of cytotoxic proteins, especially granzyme B, even when co-cultured with un-infected BECs. Thus, the increase in their numbers was relatively modest.

These data are in accordance with anticipated function: Resting BECs did not activate $CD8^+$ T cells, but RSV-infected BECs equipped local $CD8^+$ T cells for appropriate expansion and antiviral effector function.

We next hypothesized that increased PD-L1 expression in this context might prevent full activation of antiviral effector functions. Indeed, we found a further increase of IFN-γ, IL-2, and granzyme B levels in the presence of PD-L1 (but not PD-L2) blocking antibody, confirming that increased expression of PD-L1 acts as an inhibitor in this system.

Relatively few models have investigated functional memory T cell responses to live virus infections. We therefore developed this in vitro co-culture system to study these events in a system modeling epithelial/$CD8^+$ T cell interactions as they might occur in vivo. T cells are found in association with epithelium in the respiratory tract, and it has been demonstrated that human bronchial epithelial cells can present antigens and directly activate cytotoxic $CD8^+$ T cells [28]. Substantial evidence underscores the fact that effector-memory $CD8^+$ T cells do not proliferate in the lung or airways, suggesting that new cells continually come from the circulation [16]. By using $CD8^+$ T cells isolated from peripheral blood, we tried to create an in vitro environment similar to that found in the human respiratory tract. Our data confirm that PD-L1 can indeed inhibit local antiviral interactions between BECs and $CD8^+$ T cells in acute respiratory infections.

These findings may have important implications in increased susceptibility to RSV infection in infants, as well as in incomplete memory immune responses to RSV in older children, adults, and the elderly. Blocking PD-L1 may be a useful therapeutic approach to augment antiviral immunity in acute respiratory viral infections such as RSV.

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