

Local Blockade of Epithelial PDL-1 in the Airways Enhances T Cell Function and Viral Clearance during Influenza Virus Infection

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In order to maintain the gas exchange function of the lung following influenza virus infection, a delicate orchestration of positive and negative regulatory pathways must be maintained to attain viral eradication while minimizing local inflammation. The programmed death receptor 1 ligand/programmed death receptor 1 (PDL-1/PD-1) pathway plays an important immunoregulatory role, particularly in the context of T cell function. Here, we have shown that influenza virus infection of primary airway epithelial cells strongly enhances PDL-1 expression and does so in an alpha interferon receptor (IFNAR) signaling-dependent manner. PD-1 is expressed primarily on effector T cells in the lung, compared to effector memory and central memory cells, and shortly after influenza virus infection, an increased number of PD-1⁺ T cells are recruited to the airways. Using *in vitro* cocultures of airway epithelial cells and T cells and *in vivo* models of influenza virus infection, we have demonstrated that blockade of airway epithelial PDL-1 improves CD8 T cell function, defined by increased production of gamma interferon (IFN- γ) and granzyme B and expression of CD107ab. Furthermore, PDL-1 blockade in the airways served to accelerate influenza virus clearance and enhance infection recovery. Our findings suggest that local manipulation of the PDL-1/PD-1 axis in the airways may represent a therapeutic alternative during acute influenza virus infection.

The respiratory tract is constantly exposed to a broad range of foreign antigens due to respiration. The airway epithelium lining the respiratory tract not only represents the first barrier of defense against respiratory pathogens but is often the primary target of infection (1). Consequently, apart from a critical role in gas exchange function, airway epithelial cells additionally participate in host defense mechanisms by producing cytokines and chemokines (2–8). Since exacerbated responses by airway epithelial cells in response to innocuous antigens or pathogens can prove detrimental to lung function, airway epithelial cells have also evolved to regulate pulmonary homeostasis by multiple mechanisms, including, among others, expression of CD200 (9), MUC-1 (10), and surfactant proteins (11) and reduced expression of adaptor molecules (12–14).

Interaction of programmed cell death ligand 1 (PDL-1) with its receptor, programmed death 1 (PD-1), a member of the B7 family of signaling molecules, has been specifically linked to negative regulation of T cell immune responses (15, 16). Upon activation, PD-1 inhibits T cell receptor signaling by blocking phosphatidylinositol 3-kinase (PI3k)/Akt activation (17). During chronic viral infection and cancer, PD-1 expression leads to T cell dysfunction and exhaustion (15, 16, 18). Conversely, blockade of the PD-1/PDL-1 axis has been shown to either enhance T cell function or reduce the viral burden in a number of chronic viral infections (18–21). Our understanding of the role of the PD-1/PDL-1 pathway during acute viral infection, however, is just beginning to unfold. PD-1 expression is rapidly downregulated during acute lymphocytic choriomeningitis virus (LCMV) infection (22) but is expressed on functional CD8 T cells in other acute viral infections (23, 24). PD-1 has also been shown to regulate the development of T cell memory during vaccinia virus infection (25). Fairly recently, it has been shown that a number of acute respiratory viral infections, including human metapneumovirus, respiratory syncytial virus, and influenza virus, impair CD8 T cell function through PD-1 (26) (27). Although previous studies have shown that lack of

PD-1 function due to genetic mutation or to systemic blockade prevented CD8 T cell inhibition and enhanced viral control, the specific role that the airway epithelium plays during PDL-1/PD-1 regulation of pulmonary T cell function has not been fully elucidated.

Our study builds on the observations by Erickson et al. (27) to further show that PDL-1 is constitutively expressed in airway epithelial cells and its expression is upregulated by influenza virus and dependent on interferon alpha receptor (IFNAR) signaling. Coculture studies with airway epithelial cells and T cells in the presence of PDL-1 blocking antibody revealed that PDL-1 expression on airway epithelial cells repressed T cell function. Additionally, intranasal blockade of PDL-1 in influenza virus-infected mice resulted in increased levels of cytotoxic proteins and cytokines in the airways, enhanced CD8 T cell function, and reduced viral load. Our findings suggest that PDL-1 expression by airway epithelial cells during the early phase of the recall response to influenza virus infection represents a mechanism for regulating lung immune homeostasis and reveal a promising strategy to modulate local epithelial-T cell interaction in the airways.

MATERIALS AND METHODS

Mice. C57BL/6J, BALB/c, and IFNAR^{-/-} mice (BALB/c background) were obtained from The Jackson Laboratory (Bar Harbor, ME) or Harlan (Indianapolis, IN) or were bred at The Research Institute at Nationwide Children's Hospital. Mice were housed in BL2 containment under pathogen-free conditions. The Institutional Animal Care and Use Committee at

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The Research Institute at Nationwide Children's Hospital approved all of the animal studies described in this study.

Cell culture. Primary mouse tracheal epithelial cell (TEC) cultures were obtained from tracheas resected from 3- to 10-week-old wild-type BALB/c and IFNAR^{-/-} mice. Tracheal cell isolation and culture were performed as previously described (28). In short, tracheas were collected and incubated with 1.5 mg/ml pronase and 0.5 mg/ml crude pancreatic DNase I. Nonadherent cells were collected and seeded onto 24-mm coated membranes with 50 µg/ml type I rat tail collagen solution. Cells were incubated for 10 to 14 days postharvest and then allowed to differentiate by incubation under an air-liquid interface for an additional 14 days before use.

Virus infection. Influenza A viruses Udon, WSN, PR8, and X31 were grown in eggs, and virus titers were determined by an immunofluorescence focus assay. Male mice (6 to 12 weeks of age) were anesthetized with 2,2,2-tribromoethanol and intranasally inoculated with 3,000 50% egg infective doses (EID₅₀) influenza virus X31 and challenged with 1 × 10² PFU/mouse PR8 in a 30-µl volume. Organs were harvested on the indicated days posttreatment. Groups of three to six animals were used for each data point. For the analysis of PDL-1 transcript, TEC cultures derived from BALB/c and IFNAR^{-/-} mice were exposed to 2 × 10⁵ PFU WSN for 2 h or mock inoculated and harvested 24 h postinfection. Human airway epithelial (HAE) cultures were exposed to 2 × 10⁵ PFU influenza A virus Udon strain or mock treated for 2 h and harvested after 24 h of incubation as appropriate (28).

Immunofluorescence. Influenza virus-infected HAE cultures maintained on Snapwell inserts were fixed in 10% paraformaldehyde and then washed and stored in 70% ethanol until paraffin embedded. Six-micrometer sections of HAE cultures or human trachea were deparaffinized for immunofluorescence. Following 30' antigen retrieval in sodium citrate (pH 6), tissues were incubated in 0.2% Triton X-100 for 15 min and blocked with 5% goat serum for 1 h. PDL-1 was visualized using rabbit polyclonal antibody to CD274 (Abcam, Cambridge, MA) and Texas-red goat anti-rabbit (Vector Laboratories, Burlingame, CA). Control sections were stained with rabbit immunoglobulin or with secondary antibody only. Tissues were mounted with Prolong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen/Molecular Probes). Immunofluorescent images were viewed on an Olympus BX61 microscope using a 40× objective and captured using a Hamamatsu ORCA-GR digital camera in concert with the Slidebook version 4.2 software program (Intelligent Imaging Innovations, Inc., Denver, CO).

RNA extraction, reverse transcription, and real-time PCR. RNA isolation from infected HAE cultures and TEC cultures was achieved by direct lysis of cells with TRIzol reagent (Invitrogen) and application of the resulting aqueous phase to Qiagen RNeasy minicolumns. Total RNA harvested from triplicate (TEC) or quadruplicate (HAE) cultures was processed and analyzed as described in reference 28. One microgram RNA extracted from infected lungs or cell cultures using a combination of TRIzol (Invitrogen) and/or the RNeasy minikit, (Qiagen) was also used for cDNA synthesis (high-capacity reverse transcription [RT] kit; Applied Biosystems), followed by quantitative PCR performed using Sybr green (Applied Biosystems) and primers for human or mouse PDL-1, the Flu NP gene, and human or mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data were analyzed using the Prism 7500 SDS software program. Mock-infected groups served as the calibrator sample, and all samples were normalized to the endogenous control, GAPDH.

TEC/T lymphocyte cocultures. TEC cultures derived from BALB/c and IFNAR^{-/-} mice were cocultured with splenic T lymphocytes at a 1:1 ratio (TECs/T cells). T lymphocytes were isolated from spleens of naive BALB/c mice by negative selection. TECs seeded in 24-well plates were exposed to 1 PFU/cell influenza virus WSN for 2 h. Fresh medium was added after the removal of inoculum, and panned T lymphocytes (purity, >95%) were added 24 h postinfection. Blocking antibodies to PDL-1 (clone MIH5; eBioscience) or control antibody (ChromPure rat IgG;

Jackson Immuno Research) were added 3 h before the addition of T lymphocytes such that 10 µg blocking antibody was added to 2.5 × 10⁵ TECs.

In vivo antibody blockade. Mice initially infected with influenza virus X31 were challenged 30 to 40 days later with influenza virus PR8 as described above and either intraperitoneally injected with 200 µg of functional-grade anti-mouse PDL-1 or 200 µg IgG or Hanks buffered salt solution (HBSS) or were intranasally treated with 150 µg anti-PDL-1 or control antibody administered successively over a 3-day time period in 50-µg (50-µl) doses. Sham-treated mice received the same volume of HBSS in lieu of antibody.

Plaque assay. Lung homogenates collected in 0.5 ml DMEM plus 0.1% bovine serum albumin (BSA) were used for viral titer determination. Plaque assays were performed by exposing 80 to 90% confluent MDCK cells grown in 12-well plates to 100-µl quantities of 10-fold serial dilutions of lung homogenates for 2 h at 37°C. The inoculated monolayers were covered with DMEM containing 0.8% methylcellulose, L-glutamine, 0.1% BSA, antibiotics, and 1 µg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma, St. Louis, MO) and incubated for 3 days at 37°C. After incubation, the monolayers were permeabilized with 0.2% Triton X-100. Virus-infected cells were immunostained using a monoclonal antibody that reacts with the influenza A virus nucleoprotein (Cal Bioreagents, San Mateo, CA), followed by a 1-h incubation with anti-mouse IgG peroxidase conjugate (Sigma, St. Louis, MO) and a 10- to 30-min incubation with True Blue substrate (KPL, Gaithersburg, MD). Quantification was performed by counting plaques using a dissecting microscope, and counts were expressed as log₁₀ PFU per milliliter.

Flow cytometry. (i) Epithelial cells. TECs were trypsinized to obtain a cell suspension and then stained and fixed with paraformaldehyde. Analysis of cell surface expression of PDL-1 on TECs was achieved using phycoerythrin (PE)-rat anti-mouse CD274 (BD Pharmingen, Franklin Lakes, NJ) and the BD LSRII flow cytometer with FlowJo software (TreeStar Inc., Ashland, OR). Results were expressed as median fluorescence intensities (MFI) after subtracting the MFI of control cells stained with the appropriate isotype control antibodies (BD Pharmingen).

(ii) T lymphocytes. Single-cell suspensions were obtained from the bronchoalveolar lavage (BAL) fluid, lung, mesenteric lymph nodes (MLN), and spleen. Red blood cells (RBCs) were lysed, and the number of cells per organ was determined. Cells were blocked with Fc-block (CD16/32) and washed and stained with a combination of NP₃₆₆₋₃₇₄/D^b tetramer and antibodies against CD3, CD4, CD8, CD44, CD62L, CD43 activation-associated glycoform, CD107ab, and PD-1 or combined with intracellular staining for cytokines/cytotoxic proteins: gamma interferon (IFN-γ), interleukin 2 (IL-2), perforin, and granzyme B (eBioscience). Flow cytometry data were acquired on a BD LSR II instrument (BD Biosciences) and analyzed using FlowJo software. Gates were set using negative controls and isotype controls.

ELISA for IFN-γ and granzyme B. Concentrations of IFN-γ and granzyme B were measured in BAL sample supernatants and cocultures with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Ready-SET-Go! kit; eBioscience). All assays were carried out according to the manufacturer's instructions, and 100 µl from each sample was assayed in duplicate. The lower limits of detection for these assays were 15 pg/ml for IFN-γ and 40 pg/ml (granzyme B). For statistical analysis, samples with optical density readings below the limit of the standard curve of the assay were assigned a value half that of the detection level.

Statistical analysis. *t* tests were used to compare both the percentage of cells positive for a given antigen of interest and the mean relative fluorescence intensity (MRFI) determined by flow cytometry analysis. Two-sample homoscedastic Student *t* tests were used for single comparisons in which a *P* value less than 0.05 was considered statistically significant. Results are representative of at least 2 to 3 separate experiments using 3 to 6 mice per group and are expressed as means ± standard deviations (SD). One-way analysis of variance (ANOVA) was used to determine statistical significance when comparing three or more independent groups (particularly in cases in which antibody treatment was compared to mock, Flu,

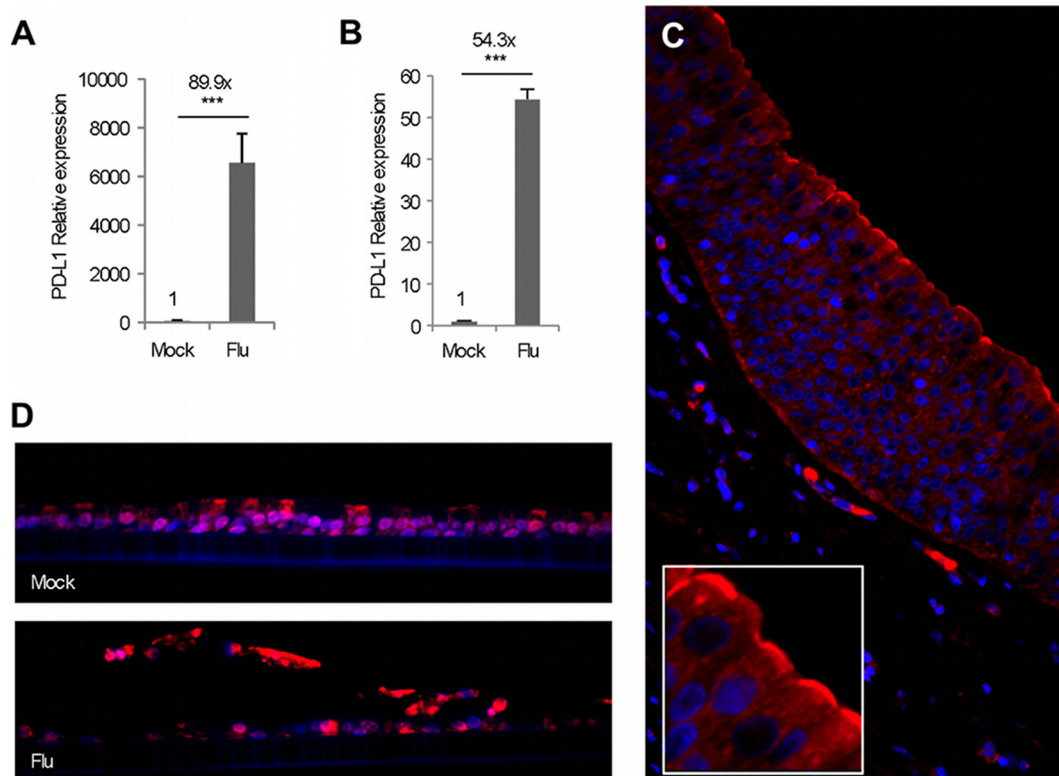


FIG 1 PDL-1 is constitutively expressed in the human airway epithelium and is upregulated by influenza infection in HAEs. (A) mRNA expression of PDL-1 in mock treated HAE cultures or in HAE cultures 24 h post-exposure to 2×10^5 PFU influenza virus (Udorn strain). (B) Relative mRNA levels of PDL-1 in influenza-virus-infected HAE cells, validated by qRT-PCR. (C) Immunofluorescence analysis of PDL-1 expression (red) in a paraffin-embedded human trachea section. The box inset represents a high-magnification detail of the apical side of the epithelium. (D) Immunofluorescence analysis of PDL-1 expression (red) in paraffin embedded HAE cultures either mock treated (upper panel) or infected with influenza virus (lower panel). In all immunofluorescent images, the nucleus is counterstained with DAPI (blue). Error bars indicate SD (***, $P \leq 0.0005$).

and IgG control treatments). When ANOVA demonstrated that the differences among means were statistically significant ($P < 0.05$), the Tukey posttest was used to correct for multiple comparisons and to generate multiplicity-adjusted P values (Prism GraphPad, version 6).

RESULTS

PDL-1 is constitutively expressed in airway epithelial cells, and influenza virus infection and alpha/beta interferon (IFN- α/β) signaling regulate its expression. The epithelial surfaces of the lung play an important role in regulating local immunity and homeostasis. In an initial attempt to analyze the role of regulatory factors critical to infection of the respiratory tract, we established polarized, well-differentiated human primary airway epithelial cell cultures (HAEs) and exposed them to influenza A virus. HAE cultures differentiate to form a pseudostratified mucociliary cell layer and effectively mimic the airway epithelial environment. A comparison of cluster of differentiation molecules following gene expression microarray-based screening revealed that one gene in particular, the PDL-1 gene, was drastically upregulated in HAE cultures 24 h post-exposure to influenza virus (data not shown). Our analysis showed that PDL-1 expression was enhanced 83.9-fold 24 h post-exposure to influenza virus compared to that of mock-treated HAEs (Fig. 1A). Quantitative RT-PCR (qRT-PCR) analysis corroborated the above findings, showing a 54.3-fold increase in PDL-1 expression in HAEs following influenza virus exposure (Fig. 1B). Additionally, immunofluorescent staining of

human tracheal sections showed that PDL-1 was not only constitutively expressed in airway epithelial cells but, perhaps more important, that the staining was prominent on the apical surface (Fig. 1C). To determine if influenza virus infection affected PDL-1 expression at the protein level, we performed immunofluorescent analysis of PDL-1 expression on HAE cultures infected or not with influenza virus. As we observed in human trachea epithelium, PDL-1 was constitutively expressed in control HAEs (Fig. 1D, upper panel). However, compared with mock-treated cultures, PDL-1 staining increased in intensity in HAEs 24 h post-exposure to influenza virus (Fig. 1D, lower panel). Altogether, these data indicate that PDL-1 is constitutively expressed by the human airway epithelium and that influenza virus infection increases the expression of PDL-1 in airway epithelial cells within 24 h of infection.

To further analyze regulation of PDL-1 expression on airway epithelial cells, we generated primary murine tracheal epithelial cell cultures (TECs). As we observed with the HAEs, exposure of TECs to influenza virus resulted in a significant increase (27.6-fold) in PDL-1 mRNA expression by 24 h (Fig. 2A). Since alpha interferon receptor (IFNAR) signaling is critical for the antiviral response of the airway epithelium (28), we also analyzed the role of IFNAR on the regulation of PDL-1 expression in airway epithelial cells during the response to influenza virus. The data show that there was a 21.1-fold difference in PDL-1 mRNA expression when

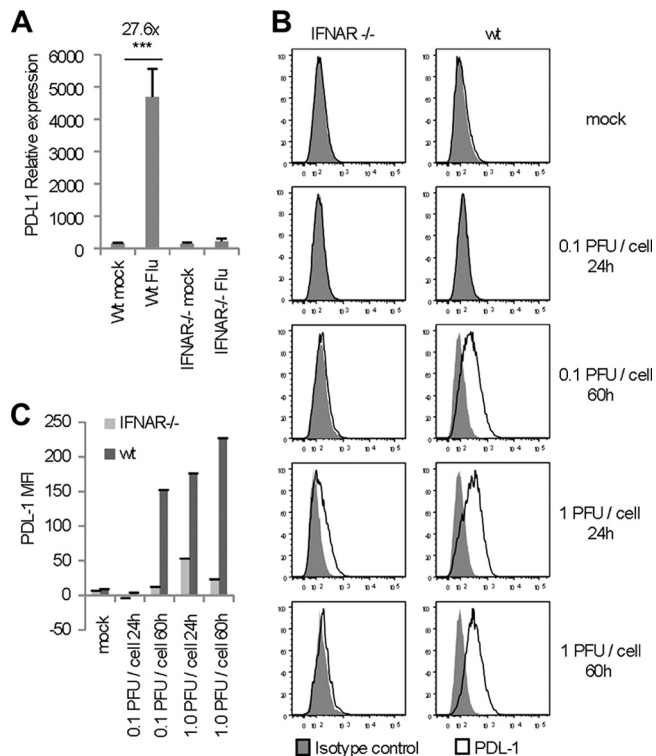


FIG 2 IFNAR signaling regulates PDL-1 expression in TEC cultures. (A) PDL-1 mRNA expression analyzed in TEC cultures derived from BALB/c (wt) and IFNAR^{-/-} mice 24 h after exposure (or not) to 2×10^5 PFU influenza virus (WSN). (B and C) Cell surface expression of PDL-1 on wt or IFNAR^{-/-} TEC cultures infected with 0.1 PFU/cell or 1.0 PFU/cell influenza virus 24 and 60 h postinfection. (B) Representative histograms. (C) Median fluorescent intensity (MFI), in which values used for the bar graph represent the MFI of PDL-1 minus the MFI of the isotype control. All panels are representative of 2 or 3 separate experiments in which TEC cultures were performed in triplicate. Error bars indicate SD (***, $P \leq 0.0005$).

influenza virus-infected IFNAR^{-/-} TECs were compared to wild-type TECs, indicating that PDL-1 mRNA expression is dependent on signaling. We next corroborated these findings at the protein level by fluorescence-activated cell sorting (FACS) analysis of PDL-1 cell surface expression. The data show that influenza virus infection enhanced the cell surface expression of PDL-1 on TECs but that PDL-1 expression on IFNAR-deficient TEC cultures was not different from isotype control staining (Fig. 2B and C). Altogether, these data indicate that the influenza virus-induced increase in PDL-1 expression on epithelial cells is IFNAR signaling dependent.

PD-1⁺ T cells are present in the lung early during influenza virus infection. In order for PDL-1 to be functional on airway epithelial cells, contact with cells expressing its receptor is necessary. Therefore, we investigated whether or not a significant population of PD-1-expressing cells was available in the respiratory tract. In order to do this, cohorts of mice were intranasally infected with influenza A virus, and cell populations in the bronchoalveolar lavage (BAL) fluid and lung parenchyma were analyzed at different time points after infection. The data show that PD-1 was expressed in 10 to 40% of the CD4 and CD8 T cells present in the airways and lung parenchyma of naive mice and that there were not significant changes in the frequency of PD-1-expressing T cells

during the first week after influenza virus infection (Fig. 3A and B). The analysis of T cell numbers shows that a small population of PD-1-expressing CD4 and CD8 T cells was present in the lung parenchyma ($\sim 1,000$ cells), while the number of PD-1⁺ T cells in the airways was very low (10 CD8 T cells and 100 CD4 T cells) (Fig. 3C and D). However, we observed a significant increase in the absolute number of PD-1⁺ CD4 and CD8 T cells present in the airways by day 3 after influenza virus infection (Fig. 3C). Additionally, the number of PD-1⁺ T cells in the lung parenchyma also increased over time, though the differences were not statistically significant (Fig. 3D). Although the number of PD-1-expressing T cells returned to baseline levels in the lung parenchyma by day 12 postinfection, greater numbers of PD-1⁺ CD4 and CD8 T cells remained in the airways of influenza virus-infected mice up to 1 month after infection. To more precisely determine the expression pattern of PD-1 on different populations of influenza virus-specific effector and memory T cells, influenza virus memory mice (40 or more days after inoculation with influenza virus) were intranasally stimulated with poly(I-C) to induce nonspecific activation of T cells in the respiratory tract. We found that PD-1 was expressed on effector CD8 T cells but was absent from the cell surface of effector memory and central-memory CD8 T cells as defined by the expression of L-selectin and CD43 activation-associated glycoform (Fig. 4). This pattern of expression was found on influenza virus NP₃₆₆₋₃₇₄-specific CD8 T cells as well as NP tetramer-negative CD8 T cells. Altogether, these data indicate that the majority of influenza virus-specific effector CD8 T cells present in the lung express PD-1.

PDL-1 expression on airway epithelial cells controls CD8 T cell function. To determine if PDL-1 expression on the airway epithelium modulated the function of T cells, we used a memory system that allowed us to analyze the function of virus-specific T cells. We incubated T cells isolated from the spleens of day 30 to 40 influenza virus-infected mice with primary, polarized TECs in the presence or absence of PDL-1-blocking antibodies. TECs were first exposed to influenza virus for 24 h to induce the upregulation of PDL-1 on the epithelial cell surface and then cocultured with T cells for an additional 36 h. T cell function was assessed by measuring the levels of granzyme B secreted into the culture medium. As shown in Fig. 5, granzyme B was undetectable in uninfected cocultures regardless of the presence or not of anti-PDL-1 antibodies. However, in the presence of influenza virus, granzyme B was released into the culture medium, and PDL-1 blockade resulted in significantly higher levels of granzyme B in culture supernatants. These data indicate that PDL-1 expressed on airway epithelial cells can inhibit the cytotoxic activity of T cells through its interaction with PD-1 and that this process can be reversed *in vitro* by blockade of PDL-1/PD-1 interaction. To address the functional role of PDL-1/PD-1 interactions *in vivo* during influenza virus infection, we performed PDL-1 blockade studies and monitored parameters of T cell function in the airways. For these studies, we used influenza virus memory mice to ensure that an elevated number of virus-specific T cells were present in the respiratory tract very early after challenge. The memory mice were intranasally challenged with a heterosubtypic influenza virus (PR8) infection and injected with PDL-1 blocking antibody or with isotype control antibody, and the levels of granzyme B and of IFN- γ were assayed in BAL fluid 3 days after viral challenge. As shown in Fig. 6, intraperitoneal administration of anti-PDL-1 blocking antibody resulted in a significant increase in the concen-

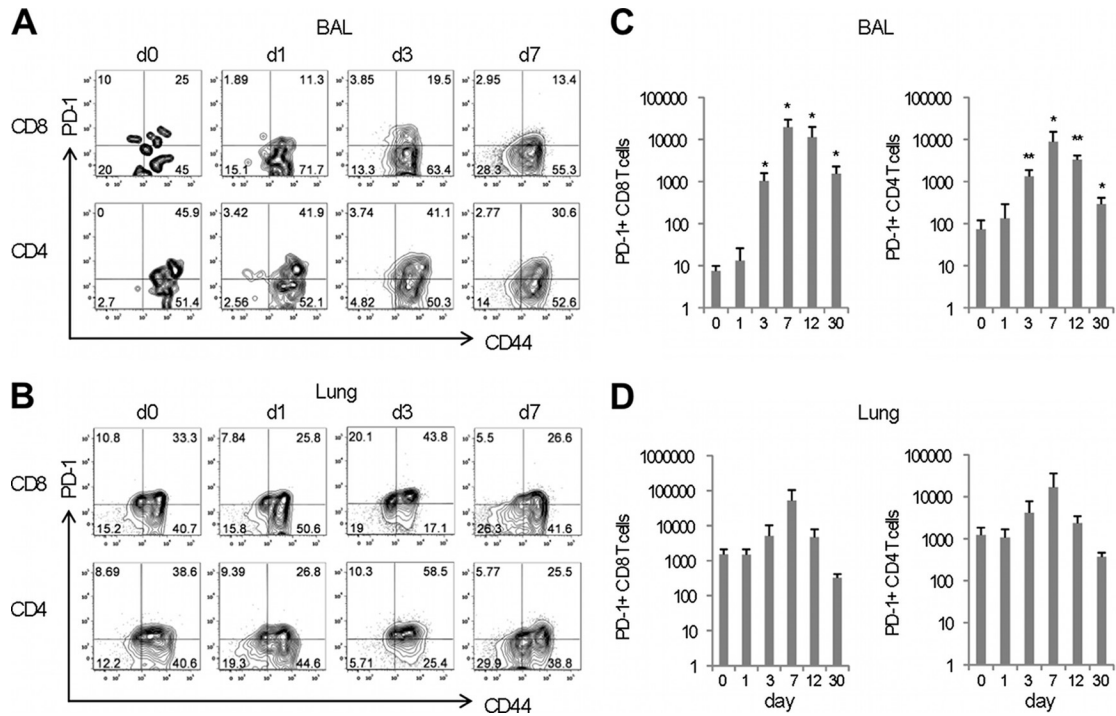


FIG 3 Influenza virus infection induces early, nonspecific recruitment of PD-1⁺ effector T cells into the lung airways. Cohorts of naive C57BL/6 mice were infected with influenza virus X31, and the T cell phenotype was assessed at the indicated time points. Representative dot plots show the frequency of PD-1⁺ CD44⁺ T cells present in the CD4 and CD8 subpopulations of the BAL fluid (A) or lung parenchyma (B) at the indicated time points after infection. Bar diagrams show the numbers of antigen-experienced PD1⁺ CD4 and PD-1⁺ CD8 T cells present in the BAL fluid (C) or lung parenchyma (D). Data are representative of 3 independent experiments with 3 mice per group; error bars indicate SD (*, $P \leq 0.05$; **, $P \leq 0.005$).

trations of granzyme B and IFN- γ in the airways compared to results for mice treated with control IgG.

Although these *in vivo* experiments demonstrated that PDL-1/PD-1 interaction regulates the effector functions during influenza

virus infection, they did not specifically address the role of the airway epithelium in this process. This is important because the PDL-1/PD-1 pathway has been shown to impact the priming of T cell responses (29–34). Thus, in order to elucidate the role of the

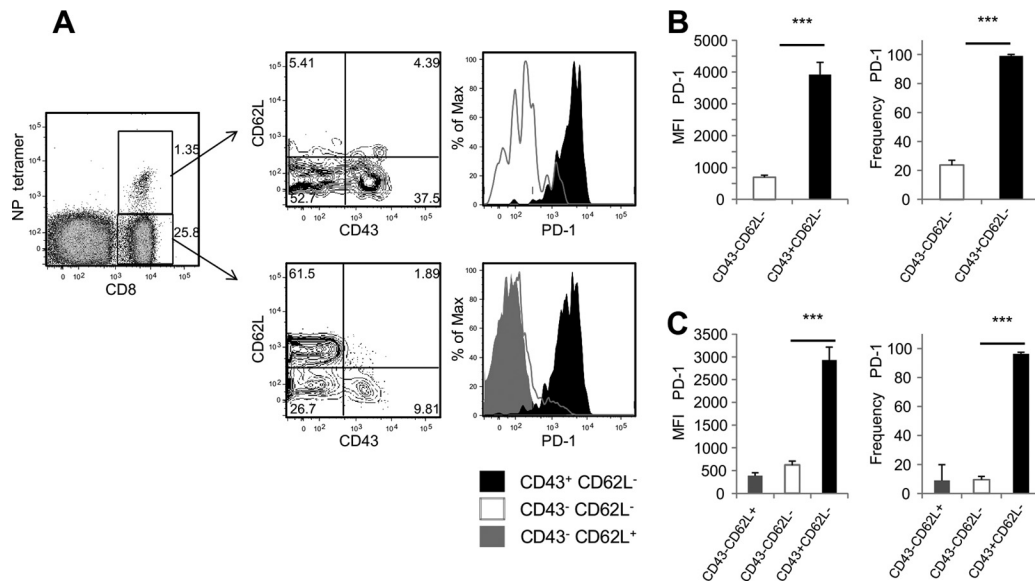


FIG 4 PD-1 is mostly expressed on effector CD8 T cells. Cohorts of mice previously infected with influenza virus X31 received intranasal administration of 50 μ g poly(I:C). (A) Representative dot plots and histograms show PD-1 expression on effector and memory T cell subsets within NP tetramer-positive and -negative CD8 T cell populations in the lung parenchyma 3 days poststimulation. Bar diagrams show the MFI and frequency of PD-1⁺ effector (CD43⁺ CD62L⁻), effector-memory (CD43⁻ CD62L⁻), and central-memory (CD43⁺ CD62L⁺) CD8 T cells residing in the NP_{366–374}/D^b tetramer-positive subset (B) or in the tetramer-negative subset (C). Data are representative of 2 independent experiments with 3 mice per group. Error bars indicate SD (***, $P \leq 0.0005$).

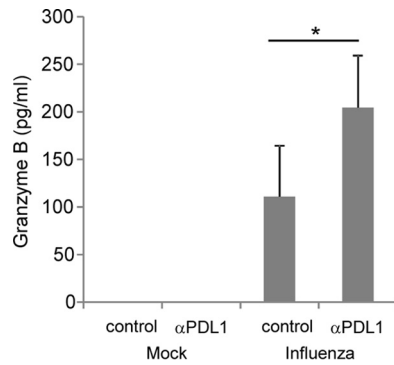


FIG 5 PDL-1 blockade on airway epithelial cells enhances CD8 T cell function. T cells isolated from the spleens of influenza virus-infected mice were cocultured with TEC cells infected or not with influenza virus WSN in the presence of anti-PDL-1 blocking antibody or control IgG antibody. Thirty-six hours after coculture, supernatants were harvested for the determination of granzyme B secretion by ELISA. Data are combined from 2 separate experiments employing triplicate wells per group. Error bars indicate SD (*, $P \leq 0.05$).

PDL-1/PD-1 axis in regulating intraepithelial T cell function, we administered anti-PDL-1 blocking antibodies intranasally to induce blockade in the airways. Preliminary studies demonstrated that intranasal administration of antibodies had a specific local effect in the airways but not in the lung parenchyma, draining

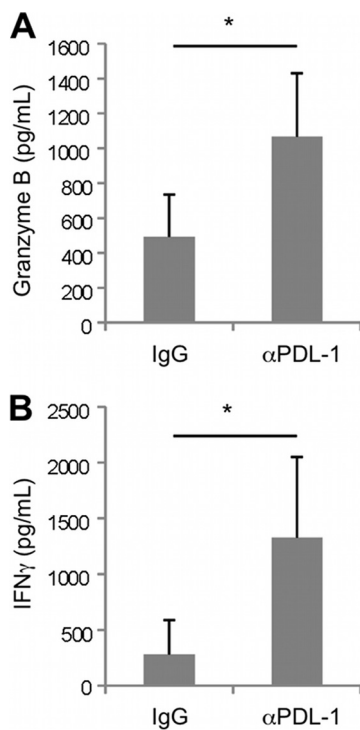


FIG 6 PDL-1 blockade increases the concentration of granzyme B and IFN-γ in the airways during recall. Influenza virus memory mice were secondarily challenged with heterosubtypic influenza virus PR8 and intraperitoneally treated with anti-mouse PDL-1 blocking antibody or rat IgG control. Three days postinfection, BAL fluid samples were assayed for the presence of granzyme B (A) or gamma interferon (B) by ELISA. Data are representative of 3 independent experiments (3 mice per group). Error bars indicate SD (*, $P \leq 0.05$).

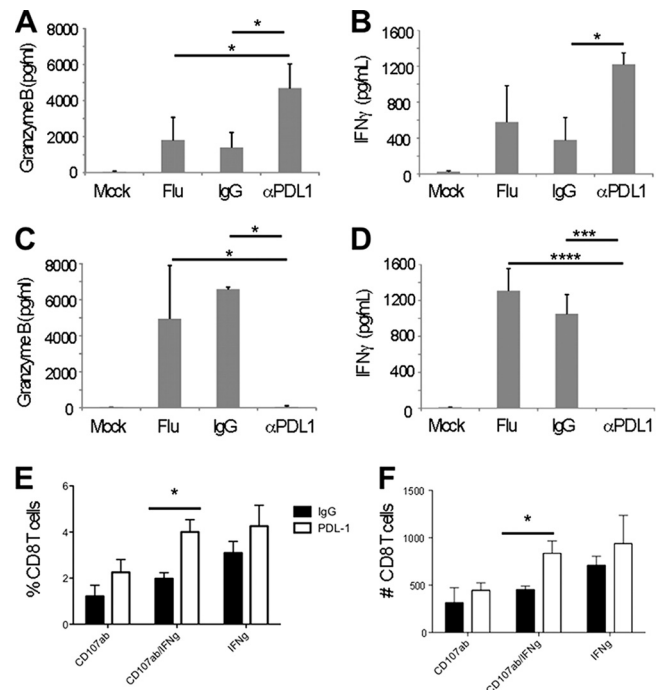


FIG 7 Intranasal PDL-1 blockade enhances the secretion of granzyme B and IFN-γ in the airways during influenza virus recall. Influenza virus memory mice were challenged with influenza virus PR8 (or mock challenged with HBSS) and treated intranasally with anti-mouse PDL-1 blocking antibody or IgG control or HBSS (Flu). ELISA was used to determine the levels of granzyme B or IFN-γ present in the BAL fluid on days 3 and 5 post-PDL-1 blockade (A to D). One-way ANOVA was used to compare the differences between means, and when these differences were significant ($P \leq 0.05$), the Tukey test was used to correct for multiple comparisons and to generate the multiplicity-adjusted P values shown. (A) Granzyme B, day 3 (ANOVA; **, $P < 0.01$). (B) IFN-γ, day 3 (ANOVA; **, $P < 0.01$). (C) Granzyme B, day 5 (ANOVA; **, $P < 0.01$). (D) IFN-γ, day 5 (ANOVA; ****, $P < 0.0001$). (E) Frequency of IFN-γ and CD107ab-expressing NP₃₆₆₋₃₇₄-specific CD8 T cells in lung. (F) Number of IFN-γ- and CD107ab-expressing NP₃₆₆₋₃₇₄-specific CD8 T cells in lung. Data are representative of 2 to 4 independent experiments (3 mice per group). Error bars indicate SD (*, $P \leq 0.05$; **, $P \leq 0.0005$; ****, $P \leq 0.0001$).

lymph nodes, or spleen (data not shown). The results show that intranasal administration of anti-PDL-1 blocking antibodies resulted in significantly higher concentrations of granzyme B and IFN-γ in the airways 3 days after influenza virus infection (Fig. 7A and B). By day 5 after influenza virus infection, the levels of granzyme B and IFN-γ in mice treated with anti-PDL1 antibodies were comparable to those of nonchallenged mice (Fig. 7 C and D). These results indicate that intranasal PDL-1 blockade can enhance and accelerate the cytotoxic function of immune cells in the airways.

We next analyzed the effect of intranasal PDL-1 blockade on CD8 T cell function. Influenza virus-challenged mice treated with anti-PDL-1 antibodies had a statistically significant higher frequency and absolute number of IFN-γ CD107ab-doubly positive cells than mice treated with control antibody or mock treated (Fig. 7E and F). The percentage of IFN-γ and of CD107ab-singly positive CD8 T cells was also higher in mice treated with anti-PDL-1 antibodies, but the differences were not statistically significant. We did not observe any differences in CD8 T cell function in the draining lymph nodes or the spleen between mice treated or not

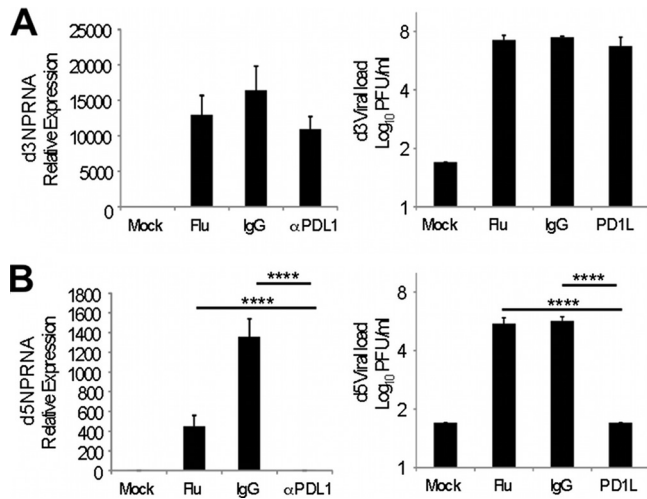


FIG 8 Reduced viral load in anti-PDL-1-treated mice. Influenza virus memory mice (30 to 40 days post-intranasal inoculation with the influenza virus X-31 strain) were intranasally challenged with 1×10^2 PFU/ml influenza virus PR8 (or mock challenged with HBSS) and treated with intranasal instillations of anti-mouse PDL-1 blocking antibody, rat IgG, or HBSS (Flu). On days 3 and 5 after initiation of PDL-1 blockade, the influenza virus load in lung homogenates was determined by qRT-PCR for the NP transcript (left panels) and by plaque assay (right panels). (A) Day 3. (B) Day 5. Values for plaque assay represent the means (\pm SD) of RSV \log_{10} PFU/ml, where 1.7 \log_{10} PFU/ml represents the limit of detection. Comparisons were made by one-way ANOVA (****, $P < 0.0001$ for all panels), and the Tukey posttest was used to correct for multiple comparisons and to generate the multiplicity-adjusted P values shown. Data are representative of at least 2 independent experiments (3 mice per group). Error bars indicate SD (****, $P \leq 0.0001$).

with anti-PDL-1 antibodies (data not shown). Altogether, our data indicate that preventing the PDL-1/PD-1 interaction between the airway epithelia and T cells results in enhanced IFN- γ production and cytolytic function by influenza virus-specific CD8 T cells.

PDL-1 blockade in the airways reduces influenza virus load and ameliorates disease. We next analyzed the consequences of intranasal PDL-1 blockade on influenza virus control on days 3 and 5 following a secondary viral challenge. The viral loads were measured in lung homogenates using both qRT-PCR and infectious plaque assays. The data show that PDL-1 blockade resulted in a slight reduction of influenza virus nucleoprotein (NP) transcripts and of infectious viral titers by day 3 postinfection (Fig. 8A). However, by day 5 postinfection, NP transcript levels and infectious virus titers were below the limit of detection in the anti-PDL-1 treated group (Fig. 8B). Conversely, untreated or control IgG-treated mice had significantly greater levels of expression of NP RNA in the lungs as measured by qRT-PCR and several orders of magnitude (10^2 to 10^5) higher viral titers measured by plaque assay. We also subjected each mouse from each group to whole-body plethysmography on day 5 post-infection with influenza virus. Enhanced pause (Penh) values in influenza virus memory mice exposed to a heterosubtypic influenza virus challenge but treated with anti-PDL-1 registered values equivalent to those for influenza virus memory mice that did not receive the secondary influenza challenge. Conversely, Penh values were significantly elevated in influenza virus memory mice that were exposed to a heterosubtypic influenza virus challenge but were left untreated or treated with control anti-IgG (data not shown). Last, to determine

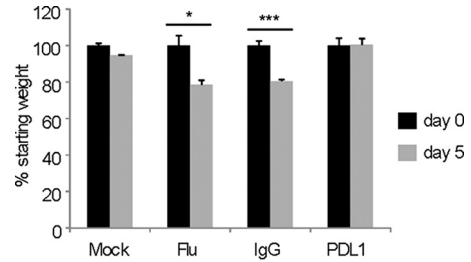


FIG 9 Intranasal PDL-1 blockade prevents influenza-associated weight loss. Percent weight loss in mice inoculated and treated as for Fig. 8 was determined by dividing the average starting weight of each group (day 0, preinoculation) by the average weight of each treatment group on day 5 postinfection. Data are representative of 3 independent experiments with 3 mice per group each. Error bars indicate SD (*, $P \leq 0.05$; ***, $P \leq 0.0005$).

the effect of intranasal anti-PDL-1 treatment on acute respiratory disease, we monitored weight loss. We observed that mice treated with anti-PDL-1 antibodies during influenza virus challenge did not present any significant weight loss between day 0 and day 5 of treatment, while untreated or control IgG-treated mice had a significant decrease in weight loss (Fig. 9), suggesting that intranasal anti-PDL-1 treatment can influence acute respiratory disease severity. Altogether, our data indicate that blockade of PDL-1/PD-1 interaction in the airways during influenza virus infection can result in enhanced viral control and disease recovery.

DISCUSSION

In this report, we showed that influenza virus infection of primary airway epithelial cells strongly enhances PDL-1 expression and does so in an IFNAR signaling-dependent manner. Most important, we observed that intranasal administration of anti-PDL-1 blocking antibodies to induce local blockade of PDL-1 in the airways improved T cell function in the acute phase of infection as defined by increased production of IFN- γ , granzyme B, and cell surface mobilization of CD107ab. PDL-1 blockade in the airways resulted in accelerated influenza virus clearance and enhanced infection recovery as measured by weight loss. These data indicate that at the onset of influenza virus infection, the PD-1/PDL-1 axis is available to limit effector T cell function in the airways.

Our findings showing that PDL-1 was constitutively expressed on airway epithelial cells, prompted us to hypothesize that the PDL-1/PD-1 axis could be regulating local lung immune responses as a means to maintain the integrity of the epithelial lining of the respiratory tract during infection. The epithelial surfaces of the lung have been shown to express molecules such as CD200 among others to regulate the function of immune cells and maintain lung homeostasis (9). The observation that expression of PDL-1 in airway epithelial cells is controlled by viral infection and IFNAR signaling suggests that the PDL-1/PD-1 pathway plays an important role during inflammatory responses in the lung. Innate interferons are critical for the response of airway epithelial cells to respiratory viral infection (28). These observations indicate that interferon signaling also contributes to the regulation of inflammatory homeostasis in the lung by enhancing the expression of PDL-1 in airway epithelial cells, adding another layer of complexity to the regulatory pathways operated by interferon-stimulated genes. Our findings with airway epithelial cells are in accordance with observations in other systems. Mice lacking Toll-like receptor 3 (TLR3) had reduced PDL-1 expression (35), and a major

downstream product of TLR3 signaling, IFN- β , has been shown to enhance PDL-1 expression on monocytes, dendritic cells, and hippocampal cultures (35, 36). Furthermore, synthetic double-stranded RNA (dsRNA) has been shown to trigger PDL-1 expression on epithelial cells (37, 38). Taken together, the above findings suggest that PDL-1 expression in the periphery during the effector phase of the response to virus infection likely represents a generalized host regulatory response to viral infection rather than a virus-specific immunoevasion strategy.

The PD-1/PDL-1 signaling cascade is a prominent mechanism by which T cell nonresponsiveness occurs in the face of chronic viral infections and cancer. The role of PD-1-expressing T cells during acute infection has remained controversial (39), although recent observations report increased primary T cell responses in the absence of PD-1 signaling (25, 27, 40). Additionally, a recent study showed that the majority of PD-1-expressing T cells in healthy human adults do not exhibit an exhausted phenotype (41). Our data show that PD-1 is primarily expressed on effector T cells in the lung, compared to findings for effector memory and central memory cells, and that shortly after influenza virus infection, an increased number of PD-1⁺ T cells are recruited to the airways. Zelinsky et al. (23) also demonstrated that during acute Friend retrovirus infection, the PD-1-expressing CD8 T cells were terminally differentiated cytotoxic effectors. These studies indicate that PD-1 is not a marker of T cell exhaustion *per se* and suggest a likely scenario during acute infection in which the PD-1/PDL-1 axis modulates inflammation and ultimately serves to prevent tissue damage from activated effector T cells.

Using cocultures of primary airway epithelial cells and T cells and a mouse model of influenza virus infection, we determined that blockade of airway epithelial PDL-1 improves CD8 T cell function, defined by increased production of IFN- γ , granzyme B, and expression of CD107ab. Importantly, therapeutic PDL-1 blockade in the airways accelerated influenza virus clearance and enhanced infection recovery as measured by weight loss. Our results are in accordance with recent findings by Erickson (27) showing that viral acute respiratory infections impair CD8 T cells through PD-1 and that systemic inhibition of PD-1 prevented CD8 T cell impairment. Our study adds that PD-1/PDL-1 regulation of CD8 T cell effector function is mediated locally by the epithelial cells of the lung, is regulated by IFNAR signaling, and can be therapeutically manipulated in the airways by intranasal administration of anti-PDL-1 blocking antibodies. Since PD-1 signaling has been shown to impact dendritic cell maturation, antigen presentation, and T cell priming and expansion (34, 42–44), it is important to differentiate the systemic role of PD-1/PDL-1 during the generation of adaptive immune responses from the specific role at the mucosal site of infection during epithelial-T cell interaction cross talk.

Our findings are in line with those of several studies demonstrating that loss of PDL-1 during acute viral infection can promote viral clearance (45, 46) and that PDL-1 blockade at the onset of infection may serve as a potential therapeutic treatment (27, 35). Clearly, further work is needed to optimize the timing of therapy and to address role of other cell populations expressing PD-1/PDL-1 in the lung. Nevertheless, our findings indicate that local manipulation of the PDL-1/PD-1 pathway in the airways may represent a therapeutic alternative during acute influenza virus infection.

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