ORIGINAL RESEARCH

Immune Modulation to Improve Survival of Viral Pneumonia in Mice

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Abstract

Viral pneumonias remain global health threats, as exemplified in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, requiring novel treatment strategies both early and late in the disease process. We have reported that mice treated before or soon after infection with a combination of inhaled Toll-like receptor (TLR) 2/6 and 9 agonists (Pam2-ODN) are broadly protected against microbial pathogens including respiratory viruses, but the mechanisms remain incompletely understood. The objective of this study was to validate strategies for immune modulation in a preclinical model of viral pneumonia and determine their mechanisms. Mice were challenged with the *Sendai paramyxovirus* in the presence or absence of Pam2-ODN treatment. Virus burden and host immune responses were assessed to elucidate Pam2-ODN mechanisms of action and to identify additional opportunities for therapeutic intervention. Enhanced survival of *Sendai virus* pneumonia with Pam2-ODN treatment was associated with reductions in lung virus burden and with virus inactivation before internalization. We noted that mortality in sham-treated mice corresponded with $CD8^+$ T-cell lung inflammation on days 11–12 after virus challenge, after the viral burden had declined. Pam2-ODN blocked this injurious inflammation by minimizing virus burden. As an alternative intervention, depleting $CD8^+$ T cells 8 days after viral challenge also decreased mortality. Stimulation of local innate immunity within the lungs by TLR agonists early in disease or suppression of adaptive immunity by systemic $CD8^+$ T-cell depletion late in disease improves outcomes of viral pneumonia in mice. These data reveal opportunities for targeted immunomodulation to protect susceptible human subjects.

Keywords: immunomodulation; immunopathology; $CD8^+$ T cells; viral pneumonia; inducible epithelial resistance

Viruses are the most frequent cause of community-acquired pneumonia in children and adults, resulting in significant morbidity in vulnerable subjects and exerting a tremendous healthcare burden (1–3). Seasonal influenza and emergent pandemic viruses, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), inflict particular mortality in susceptible individuals, with clinicians frequently lacking effective interventions to improve patient outcomes (4–7). Moreover, in addition to causing acute disease, respiratory virus infections are often complicated by chronic lung pathologies, such as exacerbation and progression of asthma and chronic obstructive pulmonary disease (8–10). Therefore, development of novel therapeutic antiviral strategies is required to effectively prevent and treat respiratory infections and their associated chronic complications (11, 12).

Although lung epithelial cells are the principal targets of most respiratory viruses (13), there is expanding evidence that lung epithelia themselves are capable of generating antimicrobial responses (10, 14,

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15). We hypothesized that lung epithelial cells can be harnessed to control virus replication, thereby enhancing acute survival and reducing chronic complications of virus infections (16–19). Our group has previously described the phenomenon of inducible epithelial resistance wherein the lungs' mucosal defenses can be broadly stimulated to protect against a wide range of respiratory pathogens, including viruses (16–21). This protection is induced by a single inhalation of a combination treatment consisting of Toll-like receptor (TLR) 2/6 and 9 agonists (Pam2-ODN) shortly before or after viral challenge. Although no individual leukocyte populations have been identified as critical for Pam2-ODN–induced resistance, lung epithelial cells are essential to the inducible antiviral response (16). More recently, we have shown prevention of chronic virus-induced asthma in mice treated with Pam2-ODN, but we have not clarified the antiviral mechanisms (22).

In this study, we investigated the mechanisms of Pam2-ODN–enhanced mouse survival of pneumonia caused by a paramyxovirus, *Sendai virus* (SeV). We found that Pam2-ODN treatment not only reduced lung SeV burden but also decreased epithelial cell injury and host immunopathologic leukocyte responses to SeV infections. Although $CD8^+$ T cells are known to contribute to antiviral immunity, it is shown here that $CD8^+$ T cells contribute substantially to mortality, and this effect can be prevented by Pam2-ODN treatment early in the course of infection or $CD8^+$ depletion late in the course. Furthermore, we demonstrate antiviral mechanisms of inducible epithelial resistance, where virus particles are inactivated before internalization by their epithelial targets.

Some of the results of these studies have been previously reported in the form of a preprint (23).

Methods

Mice

All *in vivo* experiments were performed using 6- to 10-week-old C57BL/6J mice. All groups in each individual experiment were composed of a single sex. Both sexes were tested in replicates of each experimental model. No sex-dependent differences were detected in any recorded outcome. Mice were handled according to the Institutional Animal Care and Use Committee of MD Anderson Cancer Center, protocol 00000907-RN01.

Cells

Mouse lung epithelial (MLE-15) cells were kindly provided by Jeffrey Whitsett, Cincinnati Children's Hospital Medical Center. Mouse tracheal epithelial cells were harvested and cultured as previously described (20, 24). *See* the online supplement for additional details.

TLR Treatments and Viral Challenges

Cells were treated with $Pam2CSK_4$ (2.2 μM) and ODN M362 (0.55 μM) as previously described (20, 21). For all *in vivo* experiments, mice were pretreated with 10 ml of $Pam2CSK_4$ (4 μM) and ODN M362 (1 μM) by nebulization 1 day before SeV challenge as previously described (20, 21). For *in vitro* challenges, SeV at multiplicity of infection = 1 was used. Unless otherwise stated, mice were challenged with 1×10^8 plaque-forming units inserted into the oropharynx as described (22). *See* the online supplement for additional details.

Flow Cytometry

Single cells from disaggregated lungs or cell culture were stained as indicated in the antibody table (Table 1), fixed, and acquired on a BD LSRII (BD Biosciences). *See* the online supplement for additional details.

Epithelial Proliferation Assays

Epithelial proliferation was determined by staining lung sections for 5-ethynyl-2′deoxyuridine (EdU) 24 hours after intraperitoneal injection. *See* the online supplement for details.

$CD8^+$ T-Cell Depletion

Anti–CD8-β antibody (200 μg/mouse, clone 53-5.8; Bioxell) was delivered to mice intraperitoneally at indicated time points. $CD8^+$ T-cell depletion was confirmed by flow cytometry analysis 24 to 48 hours after depletion.

Viral Burden Quantification

Viral burden was determined by qRT-PCR of the Sendai Matrix (M) protein normalized to host housekeeping gene 18SRNA. For *in vivo* experiments, mouse lungs were collected 5 days after SeV challenge. For *in vitro* experiments, cell lysates were collected 24 hours after infection, unless otherwise indicated. *See* the online supplement for additional details.

Viral Attachment Assays

For most enveloped viruses, internalization into epithelial cells is inhibited at 4°C without affecting viral binding to epithelial cells (25–27). MLE-15 cells were infected with SeV at 4°C for 4 hours, washed to remove unattached virus, and then assessed for uninternalized SeV burden using immunofluorescence or flow cytometry. *See* the online supplement for additional details.

Results

Enhanced Mouse Survival of SeV Infection by Pam2-ODN Treatment

Aerosolized Pam2-ODN treatment 1 day before SeV challenge increased mouse survival of SeV challenge (Figure 1A), similar to the protection observed against

Table 1. Antibodies

Target Epitope	Vendors	Catalog Numbers
CD3	Tonbo	65-0031-U100
CD4	Tonbo	60-0042-U100
CD8	Tonbo	25-0081-U100
Live dead	Tonbo	13-0870-T500
CD25	Biolegend	102038
Foxp3 Treg kit	eBiosciences	72-5775
CD8-depleting Ab	Bioxell	BE0223-A025
CD19	Biolegend	115507
B220	BD Biosciences	562922
Anti-SeV Ab	MBL International	PD029
Ki67	Invitrogen	MA5-14520
cCasp3	Cell signaling	9662S

Definition of abbreviations: Ab = antibody; cCasp3 = cleaved caspase 3; SeV = Sendai virus.

lethal influenza pneumonia (16, 19, 20). The survival benefit was associated with reduced lung SeV burden, as measured by SeV M gene expression (Figure 1B). Investigating the natural progression of infection revealed that SeV lung burden was maximal on day 5 and gradually decreased until falling below the limit of quantification by day 11 (Figure 1C). Pam2-ODN pretreatment reduced SeV burden on all assessed days (Figure 1C). Although the lethality of SeV infection was exquisitely dependent on the inoculum size, we strikingly found that peak mortality paradoxically occurred around days 10 to 12 after infection irrespective of inoculum size, despite the fact that SeV is essentially undetectable that long after challenge (Figures 1A, 1C, and 1D).

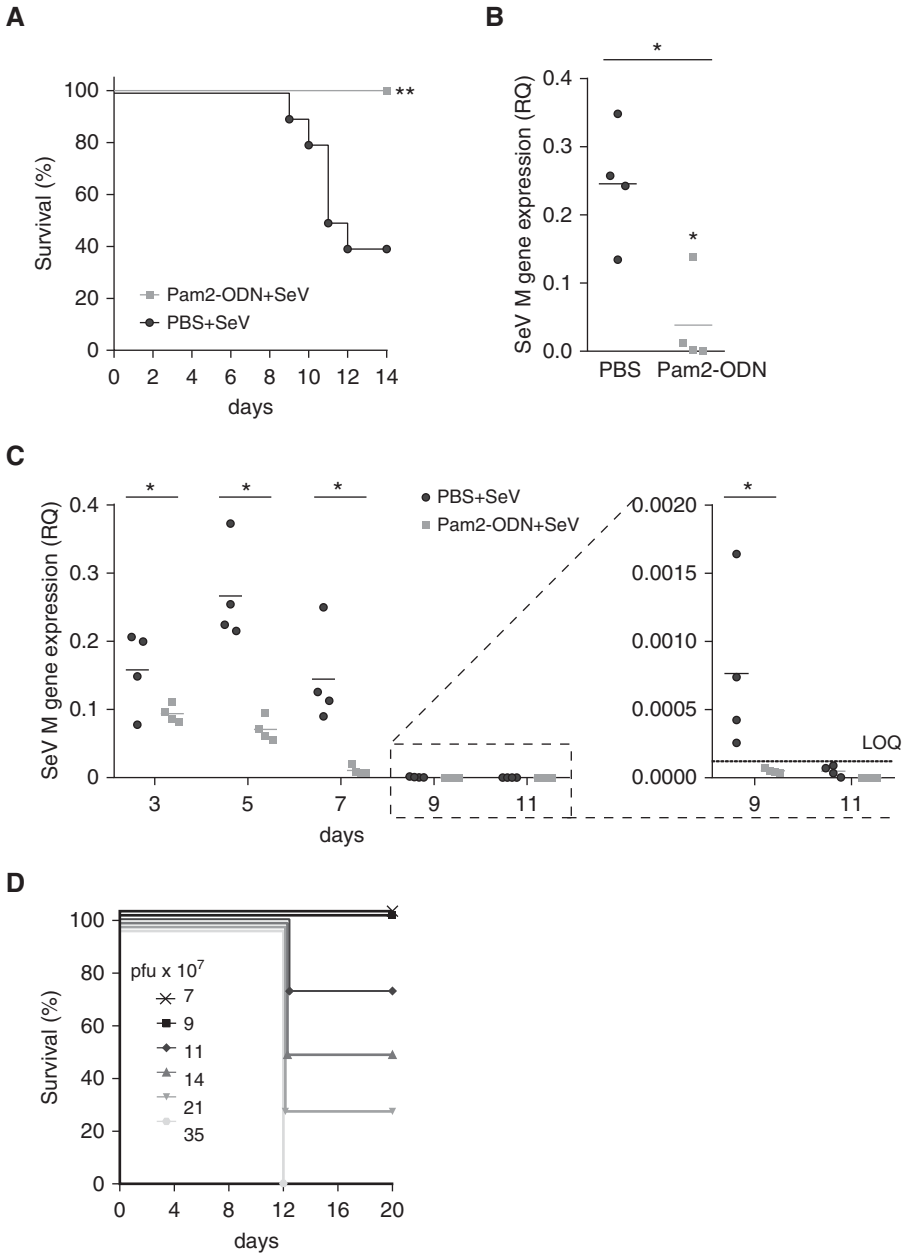

Figure 1. Pam2-ODN enhances mouse survival of *Sendai virus* (SeV) infection and reduces lung virus burden. (*A*) Survival of mice treated with PBS or combination therapy with Pam2CSK4 and ODN M362 (Pam2-ODN) day before SeV virus challenge. (*B*) Mouse lung SeV burden 5 days after infection assessed by qPCR for M gene (RQ to 18S) relative to 18S. (*C*) Time course of lung SeV burden in mice treated with PBS or Pam2-ODN. (*D*) SeV inoculum–dependent mouse survival. Data shown are from one representative experiment of three independent experiments. $n = 10$ mice per group in survival plots (*A* and *D*). $n = 4$ mice/group in virus burden experiments. $^{*}P < 0.05$, Student's *t* test and $^{**}P < 0.005$, Log-rank (Mantel-Cox) test. LOQ = limit of quantification; M = Sendai Matrix; pfu = plaque-forming unit; RQ = relative quantification.

Pam2-ODN Treatment Attenuates SeV-induced Epithelial Injury

This temporal dissociation between peak virus burden and peak mortality led to the hypothesis that SeV-induced mortality may not be exclusively driven by excessive virus burden but may also result from untoward SeV-induced host immune responses. Therefore, the acute changes in mouse lungs following SeV infection were characterized. We found increases in lung epithelial cleaved caspase 3, a marker for programmed cell death, on days 7 to 11 after SeV infection (Figure 2A, upper panel). Virus infection–related epithelial cell injury and death is typically associated with proliferative repair mechanisms (28, 29). Staining the infected mouse lung tissue for Ki67 and EdU revealed maximum signals for both markers in the second week after infection (Figure 2B–2E, upper panel). These events of lung epithelial cell death and proliferation coincided with the peak of mortality (day 12, Figure 1E). Furthermore, hematoxylin and eosin staining of lung tissues infected with SeV showed profound increases in inflammatory cells from days 7 to 10 with evidence of damaged airway and parenchymal tissue (Figure 2F). However, Pam2-ODN pretreatment of mice reduced epithelial cell injury and proliferation (Figures 2A–2E, lower panel). This temporal association of epithelial injury and death after viral clearance supported our hypothesis that mouse mortality caused by SeV infection is due in part to the host immune response to SeV infections.

Pam2-ODN Attenuates SeV-induced Lymphocytic Lung Inflammation

To explore this hypothesis, the host leukocyte response to SeV infection was characterized. Differential Giemsa staining of BAL cells revealed increased neutrophils on days 2 to 5 and increased macrophages on days 5 to 8 (Figure 3A, left and middle panel, solid gray line) after SeV challenge. Congruent with our prior studies, inhaled treatment with Pam2-ODN in the absence

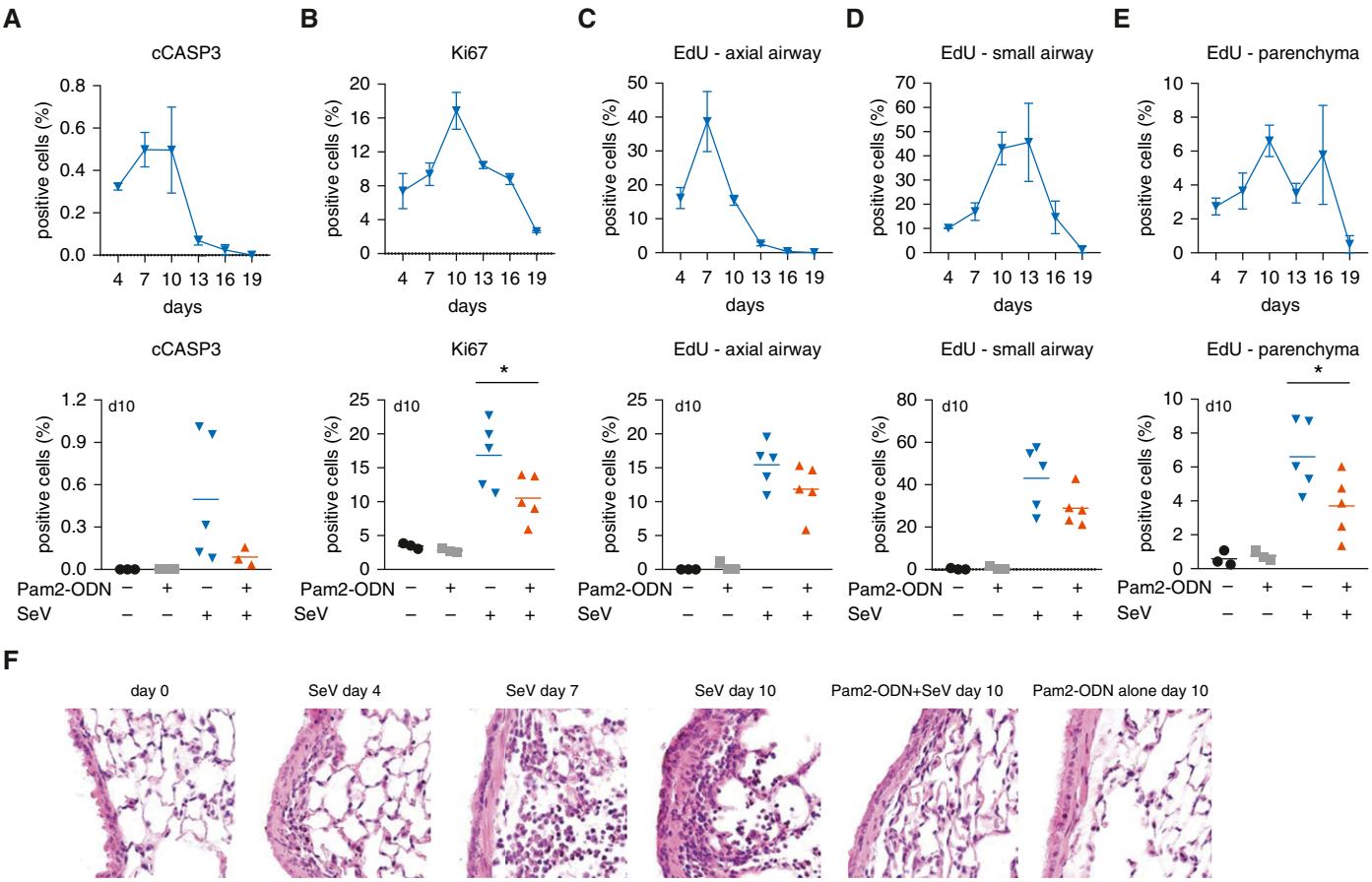

Figure 2. Pam2-ODN pretreatment reduces epithelial cell death and proliferation during acute SeV infection. (*A*) cCasp3- or (*B*) Ki67-positive cells in mouse lung epithelium after SeV infection (upper panel) with or without Pam2-ODN treatment (lower panel, day 10). EdU-positive cells in (*C*) axial airways, (*D*) small airways, and (*E*) parenchyma after SeV infection (upper panel) with or without Pam2-ODN (lower panel, day 10). (*F*) Mouse lung histology following SeV challenge with or without Pam2-ODN. $n = 5$ mice per condition. Data shown are from one representative experiment of two independent experiments. Scale bars, 100 μm. *$P < 0.05$, Student's *t* test. cCasp3 = cleaved caspase 3; EdU = 5-ethynyl-2′deoxyuridine.

of infection led to a rapid rise in neutrophils that was resolved within 5 days (Figure 3A, dashed line) (30). The neutrophil response to SeV challenge was modestly increased among mice pretreated with Pam2-ODN (Figure 3A, left panel, solid dark line). Pam2-ODN–treated, SeV-challenged mice showed almost no difference in macrophage number compared with PBS-treated, SeV-challenged mice (Figure 3A, middle panel, solid dark line). A rise in lymphocytes was observed on days 8 to 11 in PBS-treated, SeV-challenged mice (Figure 3A, right panel, solid gray line), temporally corresponding with peak mortality. However, Pam2-ODN–treated, SeV-challenged mice displayed significantly reduced lymphocyte numbers at every time point assessed (Figure 3A, right panel, solid dark line). The gating strategy for lymphocyte subsets by flow cytometry is shown in Figure E1 in the online supplement. A modest reduction in $CD4^+$ T cells was observed in Pam2-ODN–treated, SeV-challenged mice compared with PBS-treated, SeV-challenged mice (Figure E2). We also found the percentage of $CD19^+$ $B220^+$ B cells was reduced after SeV infection in comparison with Pam2-ODN–treated and uninfected mice (Figure E2), as has been seen with other viral models (31, 32). However, the biggest difference between groups was in $CD8^+$ T cells, with Pam2-ODN–treated, SeV-challenged mice displaying a significantly lower number and percentage of $CD8^+$ T cells than PBS-treated, SeV-challenged mice (Figures 3B and 3C). Because the greatest difference after Pam2-ODN treatment was in $CD8^+$ T-cell levels and there was a tight correlation between peak mortality and the increase in lung $CD8^+$ T cells on days 8 to 11, we investigated the role of $CD8^+$ T cells in SeV-induced mortality.

Depleting $CD8^+$ T Cells after Viral Clearance Enhances Survival of SeV Infection

To understand the apparent contributions of host immunopathology to mouse outcomes, we depleted $CD8^+$ T cells on day 8—after virus burden was substantially reduced but before peak mouse mortality (Figures 1 and 4A). Mice depleted of $CD8^+$ T cells displayed significantly enhanced survival of SeV challenge compared with mice with intact $CD8^+$ T cells (Figure 4B). Depletion of $CD8^+$ T cells was confirmed by flow cytometry in disaggregated lung cells 10 days after SeV challenge (Figures 4C and E3A). We also assessed lung injury by hematoxylin and eosin staining of lung tissue 10 days after SeV challenge and found increased inflammation and

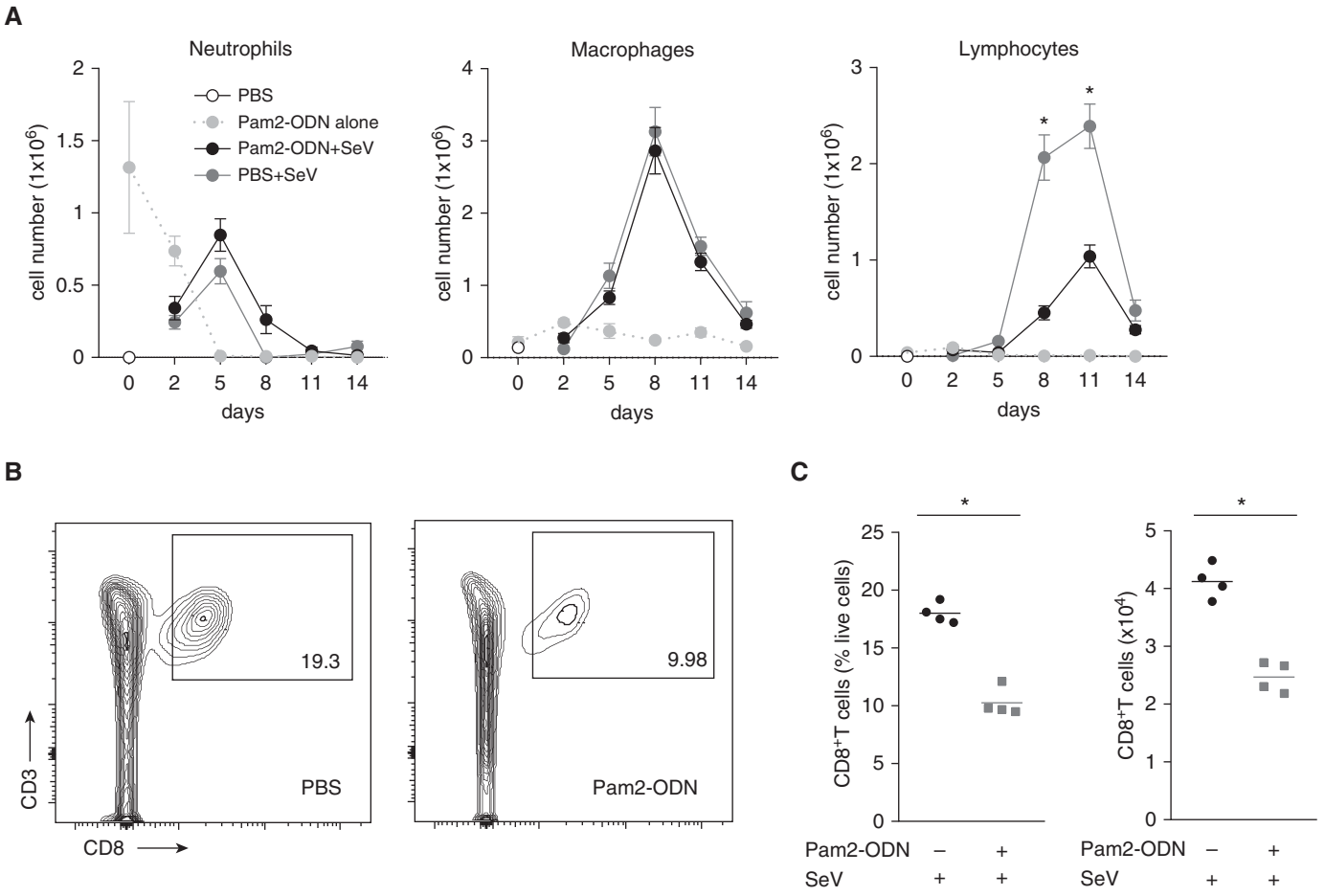

Figure 3. Pam2-ODN pretreatment reduces SeV-induced lung CD8$^+$ T cells. (*A*) Differential Giemsa staining of BAL cells from mice challenged with SeV with or without Pam2-ODN pretreatment. (*B*) Flow cytometry for CD8$^+$ T cells from disaggregated mouse lungs 11 days after SeV infection with or without Pam2-ODN. (*C*) Lung CD8$^+$ T cells 11 days after SeV challenge in mice pretreated with PBS or Pam2-ODN. Data shown are from one representative experiment of three independent experiments for *A* and of five independent experiments for *B* and *C*. *$P < 0.05$ compared with PBS+SeV, Student's *t* test.

epithelial cell damage in undepleted mice compared with CD8$^+$ T-cell–depleted mice (Figure 4D). This supported our hypothesis that CD8$^+$ T cells contribute to fatal SeV-induced immunopathology.

To assess the role of CD8$^+$ T cells throughout the course of infection, mouse CD8$^+$ T cells were depleted before and during SeV challenge (Figures 4A, E3A, and E3B). This depletion resulted in significantly reduced survival of SeV infection (Figure E3C), compatible with the known antiviral functions of CD8$^+$ T cells (33–35). However, it is notable that Pam2-ODN treatment still significantly enhanced survival of SeV challenge even in the absence of CD8$^+$ T cells (Figure E3C). This finding was congruent with our previous studies showing Pam2-ODN–inducible resistance against bacterial pneumonia despite the lack of mature lymphocytes (*Rag1*$^{-/-}$) (16).

Pam2-ODN Treatment Leads to Extracellular Inactivation of Virus Particles

As the antiviral protection consistently correlated with reduced viral burden *in vivo*, and as the reduced virus burden likely contributes to the reduced CD8$^+$ T-cell levels, we sought to determine how Pam2-ODN–induced responses cause antiviral effects. Assessing the effect of Pam2-ODN on SeV burden in immortalized mouse epithelial cells (MLE-15) and primary mouse tracheal epithelial cells, we found that Pam2-ODN treatment reduced SeV burden at every time point measured, reflecting the inducible antiviral capacity of isolated epithelial cells (Figure E4).

Furthermore, we investigated whether the principal Pam2-ODN effect occurred before (extracellular) or after (intracellular) virus internalization into their epithelial targets. SeV inoculation was performed at 4°C, preventing SeV internalization while allowing SeV attachment to epithelial cells (25–27). Using multiple methods to determine the effect of Pam2-ODN on SeV attachment, we found no differences in attachment (Figures 5A–5D). However, even though similar numbers of virus particles were attached to epithelial cells, when these attached virus particles were liberated from the epithelial cell targets, virus particles from Pam2-ODN–treated epithelial cells were less able to subsequently infect other naive epithelial cells (Figures 5E and 5F). As the number of attached virus particles was the same, this

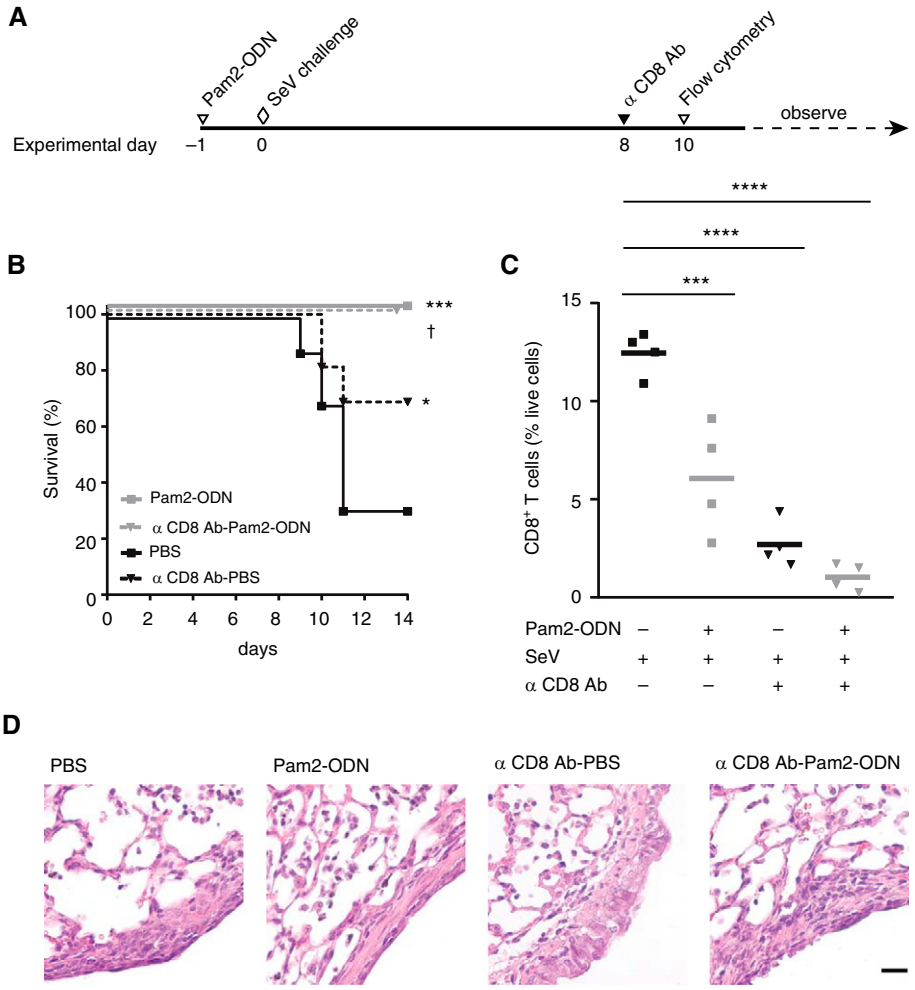

Figure 4. Pam2-ODN treatment reduces $CD8^+$ T-cell–associated SeV-induced immunopathology. (*A*) Experimental outline, (*B*) survival, and (*C*) percentage of $CD8^+$ T cells from disaggregated mouse lungs 10 days after SeV challenge following pretreatment with PBS or Pam2-ODN and with or without $CD8^+$ T cells depleted on day 8 of SeV challenge. (*D*) Lung histology 10 days after SeV challenged with or without Pam2-ODN treatment and/or $CD8^+$ T cells. Data shown are one representative experiment of two independent experiments. Scale bars, 100 μm. $n = 16$ mice/group for survival in experiment *A* and $n = 4$ mice/group in experiment *B*. $^{****}P < 0.0001$ compared with PBS in *C*, $^{***}P < 0.0005$ compared with PBS in *B* and *C*, $^{\dagger}P < 0.05$ compared with CD8 Ab-PBS and $^{*}P < 0.05$ compared with PBS. Mouse survival analysis in *B* were analyzed using log-rank, Mantel-Cox test. One-way ANOVA with multiple comparisons was used in *C*. Ab = antibody.

difference in SeV burden in cells that received liberated virus particles from PBS- versus Pam2-ODN–treated cells indicated that SeV is inactivated before epithelial internalization.

Discussion

In this study, we find that therapeutic stimulation of lung epithelial cells enhances mouse survival of acute SeV infections by both reducing the virus burden and attenuating host immunopathology. Although our group has previously demonstrated inducible resistance against multiple respiratory pathogens including viruses (16–21, 30), this study demonstrates for the first time when in the virus lifecycle the antiviral effects begin (i.e., before internalization).

We observed that PBS-treated mice died of SeV infection after they had almost completely cleared the virus. This observation prompted the hypothesis that host mortality is not the exclusive result of direct viral injury to the lungs but is primarily due to the host response to the virus infection. Supporting this, we found enhanced survival in mice depleted of $CD8^+$ T cells 8 days after infection (Figures 4A and 4B), revealing the importance of balancing the dual functions of $CD8^+$ T cells in antiviral immunity and in causing fatal immunopathology. Our findings further suggest that the surge in $CD8^+$ T cells within the lungs after most virus has been cleared causes physiologic impairment via lung injury and cell death (Figure 4D). These findings are potentially informative in the context of treating viral pneumonia in human subjects, including those suffering lung injury associated with SARS-CoV-2. Of note, the median period from SARS-CoV-2 exposure to respiratory distress is 9 to 13 days, identical to the period from viral inoculation to death from pneumonitis in this study of SeV pneumonia in mice (36). Furthermore, it was recently reported that dexamethasone treatment confers a survival advantage to patients with coronavirus disease (COVID-19) requiring respiratory support and in those recruited after the first week of their illness (36). This suggests a stage of disease dominated by immunopathology (37), rather than active viral replication, similar to our observation in the SeV model 9 days after viral challenge. It will be interesting to test the effects of dexamethasone on $CD8^+$ T-cell activity and host mortality in the SeV model.

Previous reports support the concept of counter-balanced immune protection and immunopathology by $CD8^+$ T cells during virus infections (35, 38–42). These include studies showing that antigen-experienced memory $CD8^+$ T cells enhance respiratory syncytial virus clearance but also mediate severe immunopathology (40, 43). Our findings are also congruent with reports on the role of $CD8^+$ T cells in nonrespiratory viral infection models, such as in West Nile virus infection, where $CD8^+$ T-cell–deficient mice display decreased mortality (41). Although these studies and ours reveal that the harmful effects of $CD8^+$ T-cell–mediated immunopathology can supersede the benefits of T-cell–mediated viral clearance, the question arises of what might be the adaptive value of the vigorous late $CD8^+$ T-cell response. A recent study showing that sites of viral RNA remnants following influenza infection are linked to chronic lung disease in mice may offer an answer (44). Thus, a trade-off may exist between the adaptive

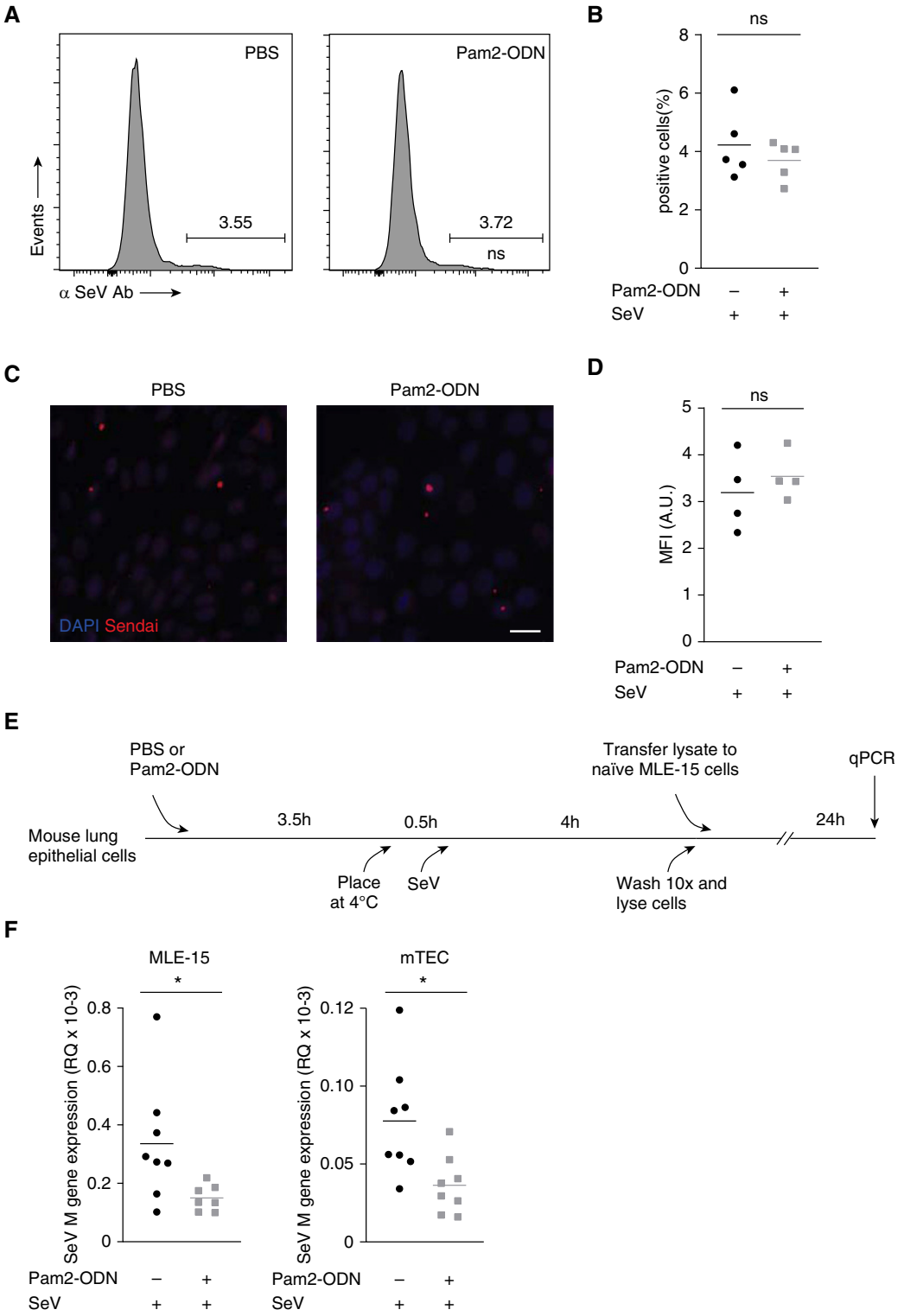

Figure 5. Pam2-ODN inhibits SeV without altering attachment. (*A*) Flow cytometry to measure virus attachment to epithelial cells 4 hours after SeV challenge. (*B*) Percentage of SeV-positive epithelial cells from *A*. (*C*) Representative examples of immunofluorescence for virus attachment. (*D*) Mean fluorescence intensity of SeV-exposed epithelial cells 4 hours after SeV challenge. (*E*) Experimental outline showing viral attachment and prevention of virus internalization by epithelial cells. (*F*) SeV M gene expression in untreated MLE-15 cells (left) or primary tracheal epithelial cells (right) challenged with liberated virus (uninternalized virus particles) from cultures that had been pretreated with PBS or Pam2-ODN before SeV infection 24 hours after transfer of liberated virus to new cells. Data shown are one representative experiment of five independent experiments. *$P < 0.05$, Student's *t* test. A.U. = arbitrary units; MFI = mean fluorescence intensity; MLE = mouse lung epithelial; mTEC = mouse tracheal epithelial cell ns = nonsignificant.

value of a vigorous $CD8^+$ T-cell response to prevent chronic lung disease and the acute mortality it can cause. Manipulating this balance therapeutically will need to account for both the benefits and costs of the response.

Together, these observations demonstrate an advantage of early immune stimulation to enhance viral clearance and late immune suppression to prevent immunopathology and enhance overall outcome of respiratory infections. Based on this reasoning and evidence of protection against other coronaviruses (45, 46), clinical trials of the use of Pam2-ODN to prevent or treat early COVID-19 have been launched (NCT04313023, NCT04312997), and we suggest that therapeutic targeting of $CD8^+$ T cells later in COVID-19 be considered.

Although the $CD8^+$ T-cell depletion studies enhanced our understanding of immunopathology in virus infections, much of the survival benefit against SeV infection was mediated by rapid antiviral effects induced by Pam2-ODN. This led us to investigate the mechanisms of these inducible antiviral effects. Given the multiple steps in the virus life cycle, it was not known at what stage Pam2-ODN exerted its antiviral effect. Exploring this, we found no differences in the attachment of SeV to epithelial cells induced by Pam2-ODN treatment (Figures 5A–5D). However, attached virus particles that were liberated from Pam2-ODN–treated cells retained less infective capacity when added to naive epithelial cells, revealing preinternalization virus inactivation by Pam2-ODN treatment (Figures 5E and 5F).

Conclusions

Taken together, these findings provide mechanistic insights into the antiviral responses generated by the lung epithelium and the prevention of host immunopathology that may inform future therapeutics to target immunomodulation as a means to improve the survival of respiratory infections in vulnerable populations. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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