Two interferon-independent double-stranded RNA-induced host defense strategies suppress the common cold virus at warm temperature

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Most strains of rhinovirus (RV), the common cold virus, replicate better at cool temperatures found in the nasal cavity (33–35 °C) than at lung temperature (37 °C). Recent studies found that although 37 °C temperature suppressed RV growth largely by engaging the type 1 IFN response in infected epithelial cells, a significant temperature dependence to viral replication remained in cells devoid of IFN induction or signaling. To gain insight into IFN-independent mechanisms limiting RV replication at 37 °C, we studied RV infection in human bronchial epithelial cells and H1-HeLa cells. During the single replication cycle, RV exhibited temperature-dependent replication in both cell types in the absence of IFN induction. At 37 °C, earlier signs of apoptosis in RV-infected cells were accompanied by reduced virus production. Furthermore, apoptosis of epithelial cells was enhanced at 37 °C in response to diverse stimuli. Dynamic mathematical modeling and B cell lymphoma 2 (BCL2) overexpression revealed that temperature-dependent host cell death could partially account for the temperature-dependent growth observed during RV amplification, but also suggested additional mechanisms of virus control. In search of a redundant antiviral pathway, we identified a role for the RNA-degrading enzyme RNAseL. Simultaneous antagonism of apoptosis and RNAseL increased viral replication and dramatically reduced temperature dependence. These findings reveal two IFN-independent mechanisms active in innate defense against RV, and demonstrate that even in the absence of IFNs, temperature-dependent RV amplification is largely a result of host cell antiviral restriction mechanisms operating more effectively at 37 °C than at 33 °C.

apoptosis | RNaseL | innate immunity | rhinovirus | respiratory immunity

In the 1960s, rhinoviruses (RVs) were first cultured from patient samples and shown to cause acute upper respiratory infections (a.k.a. common colds). Early work showed that RV isolates replicated best in culture when incubated at temperatures slightly cooler than core body temperature (33–35 °C) (1, 2). This observation fit with the role of RV as a cause of disease in the nasal cavity, but not the lungs, as the nasal cavity is cooled by inhalation of environmental air, but the lungs are generally considered to be at core body temperature (37 °C). The primary target cells of RV infection are the airway epithelial cells that line the respiratory tract. Similar to all cells of the body, airway epithelial cells are equipped with a cell-intrinsic innate immune system that can sense the presence of a viral infection, using pattern recognition receptors such as toll-like receptors (TLRs) and RIG-I like receptors (RLRs), and can respond to the infection by inducing antiviral effector mechanisms (3). Recently, we used mouse primary airway cells and a mouse-adapted RV to show that replicating RV is recognized by the RLRs within airway epithelial cells, leading to two innate immune signaling events that occur more robustly at warm temperature than at cool temperature: activation of RLRs leading to induction of type 1 IFNs and induction of IFN-stimulated genes (ISGs) via signaling through the type 1 IFN receptor, IFN-αβR (4). However, some restriction of viral replication at 37 °C persisted even in the genetic absence of molecules required for the type 1 IFN response, including the RLR signaling adaptor, mitochondrial antiviral signaling protein (MAVS), and IFN-αβR. These observations prompted us to explore whether other additional host defense mechanisms also participate in the temperature-dependent restriction of RV replication.

In this study, we examined IFN-independent mechanisms involved in temperature-dependent control of RV replication. Our results reveal that, in the absence of IFN responses, rapid death of infected cells plays an important role in restricting RV replication, that the death rate of epithelial cells in response to diverse apoptotic stimuli is temperature-dependent, and that this temperature-dependent death rate contributes to the enhanced restriction of RV replication at 37 °C. Through mathematical modeling, we discovered that the increased rate of cell death could account largely, but not completely, for the temperature-dependent replication of RV. Furthermore, blockade of the apoptotic pathway alone was unable to close the temperature gap in RV replication. We found that RNAseL, together with apoptosis, controls temperature-dependent RV replication. These findings demonstrate the importance of two double-stranded RNA (dsRNA)-dependent host pathways for limiting RV replication and reveal mechanisms in addition to the IFN response whereby temperature affects host antiviral defense and replication of the common cold virus.

Significance

Most strains of rhinovirus (RV), the common cold virus, replicate better at cool temperatures found in the nasal cavity (33–35°C) than at lung temperature (37°C). Recently, we found that although 37°C temperature suppressed RV growth by promoting IFN secretion, some antiviral effects of temperature were IFN-independent. Here, we used H1-HeLa cells and human bronchial epithelial cells to explore additional mechanisms underlying temperature-dependent RV growth. Using mathematical modeling and genetic approaches, we show that together, enhanced host cell death and the double-stranded RNA (dsRNA)-dependent activity of RNAseL contribute to restriction of RV growth at 37°C. These findings show that even in the absence of interferons, temperature has profound effects on antiviral defense that affect the outcome of common cold infections.

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The authors declare no conflict of interest.

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Results

Temperature-Dependent Viral Replication in the Absence of IFN Induction During Single-Cycle RV Replication in Human Bronchial Epithelial Cells. First, we examined RV-1B replication in human bronchial epithelial cells during a single replication cycle by infecting all cells in the monolayer, using a high multiplicity of infection (MOI, 20). After inoculation, medium was added and plates containing multiple replicate wells of infected cells were incubated at 33 °C or 37 °C. Cells were collected at sequential 2-h intervals to assess the time course of viral replication by reverse transcription quantitative PCR (RT-qPCR). RV RNA level peaked earlier and at a lower viral load in host cells incubated at 37 °C compared with 33 °C (Fig. 1*A*, *Left*). During the single replication cycle, we did not observe induction of IFNs or of the ISG, *Oas1* (Fig. 1*A*, *Right*), or IFN secretion (*SI Appendix*, Fig. S1). These observations suggested the possibility of an IFN-independent viral restriction mechanism operating to limit viral replication at 37 °C under these conditions. Previously, we observed IFN secretion during RV replication in mouse primary airway epithelial cells, and previous studies have reported induction of type 1 and type 3 IFN expression during RV infection in human bronchial epithelial cells (4–6). Consistently, we observed induction of IFNβ mRNA in bronchial cells at 48 h after amplification of RV1B from a low MOI (0.1) (Fig. 1*B*). Similar to our observations in mouse airway cells, the induction of IFNβ mRNA was temperature-dependent (37 °C > 33 °C). Together with previous results, this indicates that cytoplasmic dsRNA generated during RV infection can trigger an RLR-dependent IFN response in human airway cells, but that amplification of RLR signaling capacity is required for robust IFN induction (7). Our results indicate that although RV can induce IFN expression in human bronchial epithelial cells during viral amplification (Fig. 1*B*), this is not the mechanism restricting viral replication at 37 °C at early times postinfection (Fig. 1*A*).

On the basis of these results, we hypothesized that this IFN-independent mechanism of virus restriction may be important in defense against RV strains that antagonize the IFN pathway. To this end, we used RV-14, which blocks interferon regulatory factor 3 (IRF3) activation and IFN induction in A549 cells (8). Unlike human bronchial epithelial cells, A549 cells respond to stimulation with small molecule ligands of the cytoplasmic RNA sensor retinoic acid inducible gene-I (RIG-I), which leads to IFN induction via IRF3 (9) (*SI Appendix*, Fig. S2*A*). However, on infection of A549 cells with RV-14, we observed no induction of IFNβ, IFNλ1, or Oas1 (*SI Appendix*, Fig. S1*B*), consistent with antagonism of IRF3 activation by RV-14 (8). Importantly, temperature-dependent RV14 replication in A549 cells (increase in viral load at 33 °C but not 37 °C) was preserved despite viral evasion of the IFN response. These data indicate that additional mechanisms operate to restrict viral replication at 37 °C, even in the face of antagonism of the IFN pathway.

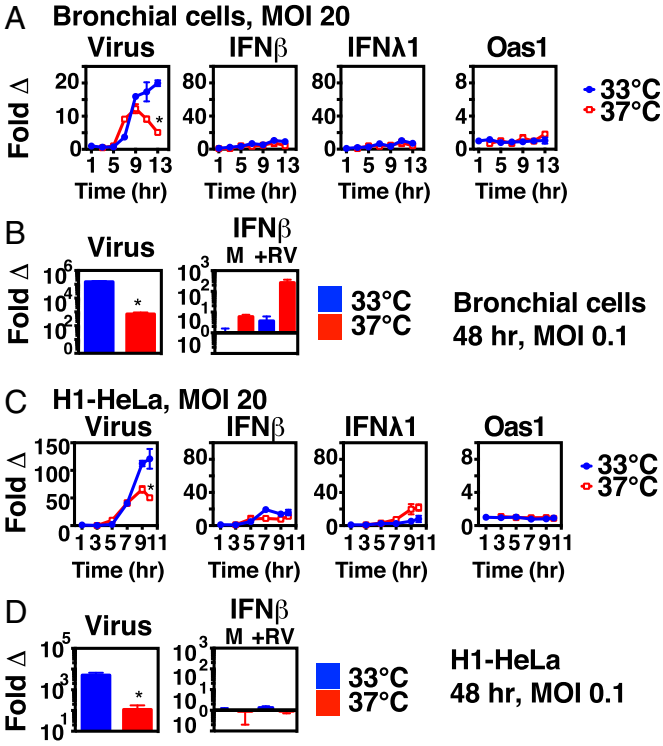


Fig. 1. IFN-independent RV restriction at 37 °C in primary human bronchial epithelial cells and in H1-HeLa cells. (*A*) Primary human bronchial epithelial cells (HBECs) were inoculated with RV-1B, MOI 20, for 1 h at 33 °C to achieve an equivalent inoculation of all cultures, then incubated at 33 °C (blue dots) or 37 °C (red squares). Starting with the postinoculation point (t = 1 h), cells were collected for RNA isolation and RT-qPCR at 2-h intervals. Graphs indicate fold change in viral RNA or mRNA level from postinoculation level (t = 1 h). (*B*) HBECs were inoculated with RV-1B, MOI 0.1, for 1 h at 33 °C to achieve an equivalent inoculation of all cultures, then incubated at 33 °C (blue bars) or 37 °C (red bars) for 48 h, at which time RNA was collected for RT-qPCR. Viral RNA is graphed as fold change from t = 1 h postinoculation time. Level of mRNA for IFNβ is graphed as fold change from mock-infected cells incubated at 33 °C. (*C*, *D*) H1-HeLa cells were infected with RV1B as in *A* and *B*, and fold change in viral RNA and host mRNAs is shown relative to postinoculation t = 1 h time point. Symbols and error bars represent mean and SD of two to three replicates per condition. Data are representative of at least three independent experiments. *P < 0.05.

In H1-HeLa Cells, RV Exhibits Robust Temperature-Dependent Replication in the Absence of IFN Induction. Next we sought a model with which to study the IFN-independent mechanism or mechanisms that might contribute to restricting viral replication at 37 °C. To this end, we searched for a cell line that supports robust temperature-dependent RV replication and amplification similar to that observed in primary bronchial cells, in the absence of IFNs. H1-HeLa cells are a human cervical epithelial cell line developed for use in RV studies, and they have been reported to support high levels of RV replication with minimal IFN induction (10). RV replication in HeLa cells has been shown to recapitulate many aspects of RV infection in primary bronchial epithelial cells, including replication kinetics, viral protein processing, and viral protein localization, and effects of RV on the nucleo-cytoplasmic trafficking (11). Paralleling our observations in bronchial epithelial cells, IFN mRNAs were minimally induced, and *Oas1* mRNA was not induced (Fig. 1*C*). However, as in bronchial cells, viral replication plateaued earlier and at a lower viral load at 37 °C than at 33 °C (Fig. 1*C*). Furthermore, we observed temperature-dependent RV1B amplification, but no type 1 or type 3 IFN induction, by 48 h postinfection when H1-HeLa cells were challenged with a MOI of 0.1 (Fig. 1*D* and *SI Appendix*, Fig. S3 *A* and *B*). In addition, H1-HeLa cells did not express IFNs in response to transfection with RIG-I ligand (*SI Appendix*, Fig. S3*C*). Overexpression of RIG-I enabled IFN induction after RV1B infection in H1-HeLa cells and reduced viral replication (*SI Appendix*, Fig. S3 *D–F*). These data indicate that H1-HeLa cells are naturally deficient in RIG-I responsiveness, and therefore, we chose this cell type as a model in which to investigate IFN-independent mechanisms of temperature-dependent RV restriction.

Higher Temperature Leads to Earlier Onset of Death of Virus-Infected Cells. To gain insight into mechanisms limiting viral replication in the absence of IFN secretion, we examined RV replication and host cell responses. Notably, H1-HeLa cells infected with RV at 37 °C at a MOI of 20 underwent morphological changes consistent with cell death (cell retraction, rounding and detachment from the substratum) starting at 7 h postinfection, whereas no changes were evident in cells incubated at 33 °C until 9 h postinfection (Fig. 2*A*). Cell rounding and detachment were not accompanied by lactate

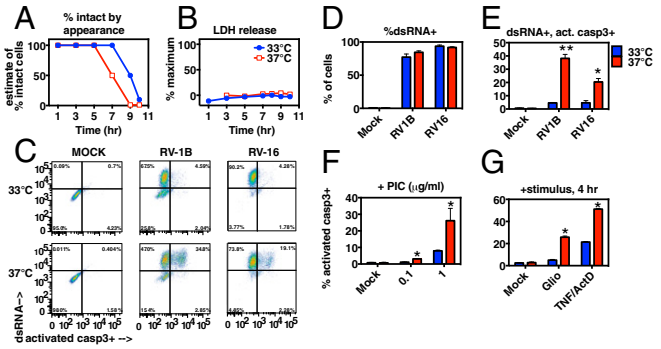


Fig. 2. Temperature-dependent cell death after infection with RV or exposure to apoptotic stimuli. (*A*) Time course estimating percentage of H1-HeLa cells affected by morphological changes (rounding and detachment of cells) following RV1B infection (MOI, 20). (*B*) LDH release into the medium during the same time course, expressed percentage of maximum LDH release from detergent-lysed cells [100*(LDH_{RV} − LDH_{MOCK})/(LDH_{MAX} − LDH_{MOCK})]. (*C*) H1-HeLa were infected with RV1B, MOI 20, for 1 h at 33 °C to ensure equivalent infection, then incubated at 33 °C or 37 °C for 8 additional h. Cells were collected, fixed, and costained intracellularly for the activated form of caspase-3 (*x* axis) and dsRNA (*y* axis). (*D* and *E*) Quantitation for experiment shown in *C*, indicating total cells positive for dsRNA (*D*) and percentage of cells positive for both dsRNA and activated caspase-3 (*E*). (*F*) H1-HeLa cells were transfected with PIC at the indicated concentrations for 1 h at 33 °C, then incubated for 4 h at 33 °C or 37 °C and stained intracellularly for activated caspase-3 or (*G*) exposed to medium only (mock), or medium containing gliotoxin (GLIO) or TNF+ actinomycin D for 4 h while incubated at 33 °C or 37 °C, then stained for activated caspase-3. Graphs show mean and SD of two to four replicates per condition. Results are representative of at least three independent experiments. Significant difference between 33 °C and 37 °C is indicated by asterisks, $*P < 0.005$; $**P < 0.001$.

dehydrogenase (LDH) leakage, indicating that cells were not necrotic or pyroptotic (Fig. 2*B*). Similarly, bronchial epithelial cells supporting RV1B replication exhibited retraction, rounding, and ultimately detachment from the substratum, with minimal LDH release, starting at 9 h postinfection in cells incubated at 37 °C, but not until 11 h at 33 °C (*SI Appendix*, Fig. S4 *A*–*C*). The timing of morphological changes for each cell type appeared to parallel the plateau in viral replication (Fig. 1 *A* and *C*), suggesting that effects of temperature on cell death rate might contribute to the differences in peak viral load observed in cells incubated at the different temperatures.

It is known that intracellular dsRNA is a potent trigger of apoptosis (12). Although RV has a single-stranded RNA genome, dsRNA is generated as a viral replication intermediate in the cytoplasm of host cells (2). RV infection has been reported to trigger apoptosis in HeLa cells and airway epithelial cells (13). To investigate the possible relationship between viral RNA accumulation, temperature, and cell death, we costained RV1B-infected H1-HeLa cells for double-stranded RNA and an antibody specific for the activated form of caspase-3, an effector of apoptosis. Flow cytometric analysis of cells supporting RV1B replication revealed enhanced apoptosis of cells containing equivalent amounts of dsRNA when incubated at 37 °C compared with 33 °C (Fig. 2 *C*–*E*). To extend this observation to another strain of RV, we infected H1-HeLa cells with the major group RV16. Again, we observed a greater degree of activation of caspase-3 in cells containing similar levels of dsRNA when incubated at 37 °C compared with 33 °C (Fig. 2 *C*–*E*). These observations led us to hypothesize that a temperature of 37 °C restricts RV replication by enhancing apoptosis, thereby ending the replication cycle at a lower viral load in RV-infected epithelial cells incubated at 37 °C compared with cells incubated at 33 °C.

To remove the variable of viral replication and directly test whether an equivalent amount of dsRNA triggers different levels of caspase-3 activation at different ambient temperatures, we next transfected cells with Poly I:C (PIC), a synthetic mimic of viral dsRNA that has been reported to trigger apoptosis via multiple pathways (12). PIC transfection resulted in caspase-3 activation, with more robust activation of caspase-3 at 37 °C compared with 33 °C in both HeLa cells and primary human bronchial epithelial cells (Fig. 2*F* and *SI Appendix*, Fig. S5).

RV Infection Induces Apoptosis in Infected Cells. Next, we investigated the mechanism of cell death in RV1B-infected cells, using caspase inhibitors. RV1B infected cells with morphological changes did not exhibit significant LDH release (Fig. 2*B*) or staining with a cell-permeable dye (*SI Appendix*, Fig. S6*A*), indicating that cells were not undergoing necrosis or pyroptosis. Instead, we observed activation of the apoptotic caspase, caspase-3, in RV-infected cells (Fig. 2*C* and *SI Appendix*, Fig. S6*A*). In addition, cells that became membrane permeable after RV infection all had activated caspase-3 (*SI Appendix*, Fig. S6*A*), indicating that they underwent apoptosis followed by loss of membrane integrity. Further, the caspase-1 inhibitor VX-765 had no effect on the morphological changes after RV1B infection or the accumulation of dsRNA (*SI Appendix*, Fig. S6 *B* and *C*). In contrast, the pan-caspase inhibitor zVAD (which inhibits apoptotic caspases) profoundly inhibited RV1B-induced morphological changes (*SI Appendix*, Fig. S6*B*). In RV-infected H1 HeLa cells, we did not observe secretion of IL-1β (*SI Appendix*, Fig. S6*D*), indicating that this virus infection triggers minimal caspase-1 activation or pyroptosis.

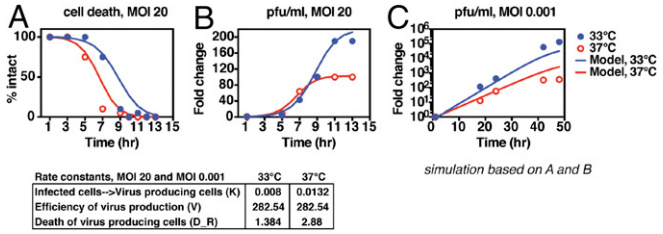
Apoptosis can occur via the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway. To better understand the contribution of these pathways to RV-induced death, we used specific inhibitors of the caspases. During RV1B infection, inhibition of either caspase-8 or caspase-9 diminished caspase-3 activation in dsRNA-positive cells, as did the pan-caspase inhibitor zVAD (*SI Appendix*, Fig. S7 *A* and *B*). Caspase inhibitors have previously been reported to partially cross-inhibit the RV 2A protease essential for the viral life cycle, because of similarities between caspases and RV cysteine proteases (14). Consistent with an inhibitory effect on viral replication, we observed that both zVAD and the caspase-8 inhibitor IETD reduced accumulation of dsRNA during the single replication cycle (*SI Appendix*, Fig. S7*C*). To assess the apoptotic pathway or pathways activated by dsRNA without the variable of viral replication, we next transfected cells with the PIC and assessed the effect of caspase-3 activation in the presence of caspase inhibitors. As in RV infection, inhibition of either caspase-8 or caspase-9 partially inhibited dsRNA-dependent caspase-3 activation (*SI Appendix*, Fig. S7*D*). Taken together, these results indicate that both extrinsic and intrinsic pathways contribute to RV- and dsRNA-triggered apoptosis.

Diverse Apoptotic Stimuli Trigger Temperature-Dependent Death of Epithelial Cells. Protection from cell death under conditions of mild hypothermia (32–35 °C) has been extensively studied in neurons and has been used therapeutically for neuronal cytoprotection after brain injury (15). Lowering temperature has also been reported to delay apoptosis in other cell types, including cardiomyocytes and fibroblasts, and diverse mechanisms have been implicated, but previous studies have not specifically addressed epithelial cells (16, 17). Therefore, we next examined whether temperature dependence of cell death tracked with a particular apoptotic pathway by treating cells with known triggers of extrinsic and intrinsic apoptosis. Tumor necrosis factor (TNF) + actinomycin D (AD), known to trigger extrinsic apoptosis, elicited temperature-dependent caspase-3 activation by 4 h (Fig. 2*G*). Consistent with apoptotic death, activation of caspase-3 was diminished by zVAD treatment, and no increase in cell permeability was observed (*SI Appendix*, Fig. S8*A*). The fungal toxin gliotoxin has been shown to trigger apoptosis via the intrinsic pathway (18). Similar to TNF + AD, gliotoxin triggered caspase-3 activation in a temperature-dependent manner (Fig. 2*G*).

Consistent with their roles in intrinsic and extrinsic apoptosis, respectively, gliotoxin-induced caspase-3 activation was inhibited by overexpression of the antiapoptotic mitochondrial membrane protein BCL2, whereas TNF + AD-induced caspase activation was not (*SI Appendix*, Fig. S8 *B* and *C*). Similar to TNF + AD, PIC triggered caspase-3 activation but did not increase cell permeability (*SI Appendix*, Fig. S8*D*), and cell death was inhibited by zVAD at both 33 °C and 37 °C (*SI Appendix*, Fig. S8*E*). Taken together, these results demonstrate a profound effect of incubation temperature on both the intrinsic and extrinsic apoptotic pathways in epithelial cells and support the hypothesis that 37 °C temperature restricts RV replication by potentiating rapid death of host cells during viral replication.

Temperature-Dependent Cell Death Rates Predict Viral Amplification Differences in a Mathematical Model. To test the hypothesis that death of the infected cells restricts RV replication at 37 °C temperature, we used mathematical modeling. We constructed a mathematical model based on single-step replication kinetics and asked whether observed rates of cell death could account for the differences in viral titer reached during viral amplification at 33 °C vs. 37 °C over multiple replication cycles (see model description in *SI Appendix*). The model was trained using data obtained from single-step replication experiments in H1-HeLa cells (Fig. 3 *A* and *B*). The mathematical model predicted two temperature-dependent rates that would fit the experimental data: the rate of cell death and the rate of initiation of virus production from infected cells. Both of these rates were ∼twofold higher at 37 °C than at 33 °C. To test the validity of this mathematical model, we simulated viral amplification over the course of 48 h from a low MOI, using these rates as the only variables affected by incubation temperature. The simulated viral amplification curves at 33 °C and 37 °C (lines, Fig. 3*C*) closely approximated the observed viral amplification curves [symbols, Fig. 3*C* (4)]. These results indicated that enhanced rate of death of infected cells at 37 °C compared with 33 °C could largely account for temperature-dependent RV growth in this cell type.

BCL2 Overexpression Partially Rescues RV Replication at 37 °C. Our mathematical model suggested that death of host cells is an important mechanism restricting RV growth, and may function in part to restrict viral growth preferentially at 37 °C compared with 33 °C. To test this experimentally, we stably expressed the antiapoptotic protein BCL2 in H1-HeLa cells and examined RV1B replication. H1-HeLa cells stably transduced with a lentivirus encoding BCL2 expressed BCL2 mRNA at levels six times higher than the parent



Rate constants, MOI 20 and MOI 0.001	33°C	37°C
Infected cells-->Virus producing cells (K)	0.008	0.0132
Efficiency of virus production (V)	282.54	282.54
Death of virus producing cells (D_R)	1.384	2.88

Fig. 3. Mathematical model and simulation of temperature-dependent RV amplification in H1-HeLa cells. (*A* and *B*) Mathematical model (solid lines) and experimental data (circles) indicating viral titer and cytotoxic appearance of H1-HeLa cells infected with RV-1B, MOI 20, and incubated at 33 °C or 37 °C. Best fit of model to data were used to derive temperature-dependent constants for K and D, as shown. (*C*) Simulation (solid lines) and data (circles) indicating viral titer for viral amplification at 33 °C or 37 °C in H1-HeLa cells infected with low MOI RV-1B. This simulation used temperature-dependent constants derived from high MOI data and included a term for increase in number of susceptible cells, as described in supplement. In *B* and *C*, *y* axis shows the ratio of the viral titer to the starting titer (titer immediately following inoculation).

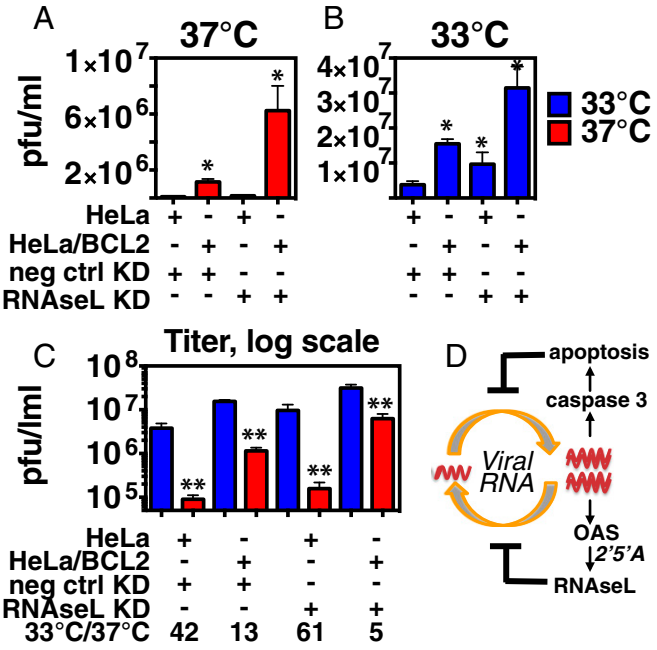


Fig. 4. Effect of BCL2 overexpression and RNAseL knockdown on RV amplification. RV1B titer at 48 h in H1-HeLa cells infected with RV1B, MOI 0.01. Apoptosis and RNAseL were antagonized in host cells as indicated with the + or − symbols. H1-HeLa: parent cell line or cell line stably overexpressing BCL2, pretreated with negative control siRNA or siRNA targeting RNAseL. After 1 h inoculation of all cells at 33 °C to achieve equivalent starting infection, cells were incubated at either 37 °C (*A* and *C*) or 33 °C (*B* and *C*) for 48 h, then cell lysates were collected and titer was determined by plaque assay. Bars show mean and SD of three replicates per condition. Asterisks indicate significant difference from H1-HeLa, negative ctrl (*$P < 0.05$; *A* and *B*) or significant difference between 33 °C and 37 °C titer for identical conditions (**$P < 0.05$; *C*). Ratio of average peak titer at 33 °C compared with 37 °C for each condition is indicated in bottom row. (*C*). Results are representative of at least three independent experiments. (*D*) Schematic representation of hypothetical relationship between cell death and RNAseL in limiting RV replication.

cells or vector-only transduced cells (*SI Appendix*, Fig. S8*B*). Consistent with our hypothesis, RV1B reached a higher peak titer in BCL2-overexpressing cells, and replication disproportionally increased in cells at 37 °C (Fig. 4 *A* and *B*). Examining the time course of viral replication revealed that BCL2-overexpressing cells had delayed appearance of cell rounding and detachment during the single replication cycle, and that single-step replication was somewhat delayed relative to control cells (*SI Appendix*, Fig. S9), although ultimately the virus reached higher titers in these cells (Fig. 4 *A* and *B*). RV1B replication in BCL2-overexpressing cells exhibited less temperature dependence than in the parent H1-HeLa cells, with a ∼10-fold higher final titer at 33 °C than at 37 °C in BCL2-overexpressing cells compared with a ∼40-fold difference in titer in RV1B replicating in the parent cell line (Fig. 4 *A* and *B*). However, the significantly enhanced replication at 33 °C compared with 37 °C in the BCL-2 overexpressing cell line suggested that additional mechanisms might also be contributing to the temperature dependence of viral replication.

RNaseL Replication and Cell Death Kinetics Restrict RV at Core Body Temperature. The data obtained in BCL2-overexpressing cells as well as the incomplete agreement of the mathematical model with the data, particularly underestimation of viral amplification at 33 °C (and overestimation at 37 °C), suggested that mechanisms in addition to temperature-dependent host cell death may differentially limit viral replication at distinct temperatures. We therefore examined IFN and cell death-independent mechanisms of viral

restriction that might contribute to the enhanced RV replication at 33 °C. In addition to initiating RLR signaling, leading to IFN secretion, dsRNA generated during viral infection can directly activate dsRNA-dependent antiviral effectors. These include protein kinase R (PKR), a dsRNA-dependent enzyme that halts protein synthesis upon dsRNA detection, and 2′5′ oligoadenylate synthetases (*OAS*). Upon binding of dsRNA, OAS enzymes OAS1–OAS3 generate oligomers of adenosine from ATP that then activate the RNA-degrading enzyme RNAseL, leading to degradation of viral RNA (19–21). Even though PKR, RNAseL, and OAS genes are ISGs (expression is enhanced by IFN signaling), they can also be constitutively expressed. To assess the effect of temperature on constitutive expression of dsRNA-dependent antiviral effectors, we performed qPCR to assess mRNA levels of these dsRNA-activated effectors in H1-HeLa cells, and we examined the effect of a 10 h temperature shift to 33 °C compared with continued incubation at 37 °C. All mRNAs were detectable in unstimulated cells (*SI Appendix*, Fig. S10), and mRNA level for RNAseL was ∼2.2-fold higher after 10 h incubation at 37 °C compared with 33 °C (*SI Appendix*, Fig. S11*A*). RNAseL mRNA was also enhanced at 37 °C relative to 33 °C in primary human bronchial epithelial cells after a 9 h incubation at 33 °C vs. 37 °C (*SI Appendix*, Fig. S11*A*). To test the contribution of these effectors to temperature-dependent viral restriction, we used siRNA knockdown of PKR or RNAseL (knockdown reduced expression to <10% of control levels; *SI Appendix*, Fig. S11*B*). Knockdown of PKR did not alter RV1B replication at either temperature (*SI Appendix*, Fig. S11*C*, *Left*). However, siRNA knockdown of RNAseL expression significantly increased viral replication (*SI Appendix*, Fig. S11*C*, *Right*).

To examine the mechanism of increased viral replication in RNaseL knockdown cells, we examined RV1B replication during the single replication cycle. Antagonizing RNASeL led to a faster rate of viral replication at both temperatures (*SI Appendix*, Fig. S11*D*). This was not the result of prevention of apoptosis. In fact, cell retraction, rounding, and detachment appeared earlier in RNaseL knockdown cells infected with RV (*SI Appendix*, Fig. S11*E*). During the single RV replication cycle, RNAseL knockdown led to faster accumulation of dsRNA within infected cells, and cells containing high levels of dsRNA rapidly became caspase-3 active (*SI Appendix*, Fig. S11*F*). These findings indicated that, on antagonism of RNAseL, rapid onset of apoptotic death in cells accumulating high levels of cytoplasmic dsRNA caused an earlier end to the replication cycle. DsRNA-induced apoptosis in this setting, therefore, appears to partially compensate for lack of RNAseL to limit viral replication (*SI Appendix*, Fig. S11 *D–F*). These findings suggested that two dsRNA-triggered host defense mechanisms, dsRNA-triggered apoptosis and RNASeL activity, play compensatory roles during RV infection, creating a resilient host defense strategy in case of viral evasion of either pathway.

Apoptosis and RNAseL Are Redundant Pathways that Control RV Replication at 37 °C. On the basis of the data indicating that apoptosis and RNAseL each contribute to viral control, particularly at lung temperature, we examined the effect of blocking both apoptosis and RNaseL simultaneously and compared this with blocking each mechanism separately. To do this, we used BCL2 overexpression and RNAseL knockdown in host cells. Antagonizing both apoptosis and RNAseL together dramatically increased replication of RV1B, particularly at 37 °C (Fig. 4*A*). We observed a 42-fold difference in peak viral titer at 33 °C compared with 37 °C during infection of control H1-HeLa cells, but only fivefold difference when both apoptosis and RNAseL were antagonized (Fig. 4*C*). These findings demonstrate that even in the absence of IFN production, temperature-dependent RV amplification is largely a result of host cell antiviral restriction mechanisms that operate more effectively at 37 °C than at 33 °C; namely, cell death and RNAseL. Our observations support a model in which each of these pathways, triggered by dsRNA, can compensate to limit RV replication when the other pathway is blocked (Fig. 4*D*); that is, as host cells accumulate viral dsRNA, this triggers the cell death pathway, limiting the duration of the replication cycle. However, if cells are able to survive longer, accumulating dsRNA also triggers the RNAseL pathway, leading to degradation of viral RNA. Conversely, antagonizing RNAseL leads to faster accumulation of dsRNA in the cytoplasm of host cells, which triggers cell death sooner (*SI Appendix*, Fig. S11 *D–F*).

Discussion

In this study, we investigated whether IFN-independent host defense mechanisms contribute to restricting replication of the common cold virus at lung temperature (37 °C) in human epithelial cells for the following reasons. First, our previous study found that some temperature dependence of RV replication remained even in host cells deficient in the IFN response (4). Second, recent evidence indicates that human bronchial epithelial cells have naturally attenuated IFN responses to some cytoplasmic nucleic acids, including RLR ligands (9). Third, RV strains have evolved to evade IFN responses (8). Finally, diminished IFN responses in the airway epithelium are associated with conditions such as asthma and cigarette smoke exposure, in which RV infection results in exacerbated disease (22–24). Although diverse lines of evidence suggest that virus-induced IFNs contribute to defense against RV infection, increasing evidence from studies in bronchial epithelial cells indicates that the mechanisms involved (i.e., the RLR signaling pathway) require time for up-regulation before mediating a robust IFN response (6, 7, 9). Consistently, we observed IFN induction in primary human bronchial epithelial cells supporting RV amplification by 48 h postinfection, but no induction of IFNs during the initial 12-h single replication cycle (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1). Together, previous studies and our data illustrate situations in which the IFN response to RV is attenuated in human epithelial cells, emphasizing the importance of investigating IFN-independent host defense mechanisms that restrict RV replication ((8, 25); Fig. 1 and *SI Appendix*, Figs. S1 and S2).

Our studies revealed two temperature-dependent effectors of host cell response to RV infection even in the absence of IFN induction: cell death and RNaseL. We found that in H1-HeLa cells, apoptosis was profoundly enhanced by 37 °C temperature in response to RV infection and cytoplasmic double-stranded RNA. We also observed greater apoptosis at 37 °C compared with 33 °C in response to diverse triggers including known stimuli of the extrinsic and intrinsic pathways of apoptosis. Through antagonism of apoptosis with BCL-2 overexpression and through mathematical modeling, we found that enhanced death rate of RV-infected cells at 37 °C compared with 33 °C largely accounts for the restriction of RV replication at this temperature. However, differences in cell death rates alone could not completely explain the differences in RV replication at the two temperatures. We found that the discrepancy is the result of an additional mechanism of RV restriction that depends on RNAseL. We did not test all possible dsRNA-dependent antiviral responses, including non-RNAseL-dependent antiviral responses of OAS proteins and the related OASL protein (26). Therefore, our study does not rule out additional mechanisms of dsRNA-dependent control of RV. Altogether our studies have identified three distinct pathways of RV control at core body temperature; namely, RLR signaling-dependent IFN responses (4), IFN-independent apoptosis of infected cells, and RNAseL-dependent degradation of RV RNA. We propose that the three distinct antiviral pathways provide the host with resilient defense against viruses that evade any one of these pathways.

How might the compensatory activities of cell death and RNAseL be coordinated to limit RV replication? Our data support a model in which antagonizing cell death leads to a higher level of dsRNA in infected cells, which in turn enhances synthesis of 2′-5′

oligoadenylate by OAS proteins interacting with dsRNA. This ligand activates RNAseL leading to enhanced degradation of viral RNA (Fig. 4*D*). Conversely, RNaseL inhibition permits more rapid accumulation of dsRNA during viral replication, triggering death of infected cells and thereby limiting RV replication (Fig. 4*D*). Only through the inhibition of both cell death and RNAseL did we observe a dramatic increase in RV titer at 37 °C. Redundancy of antiviral responses and the compensatory relationship among them creates resilient protection for the host, which is often critical for host defense, given the propensity of viruses to evolve mechanisms to evade host innate immune defenses (27). Although previous studies of RV biology showed no enhancement of viral entry or viral polymerase activity by cool temperature (28, 29), a recent study reported that cool temperature can promote IRES-driven translation required for the life cycle of picornaviruses (30). Therefore, future studies are needed to further understand how the host and viral mechanisms that promote RV replication at the cooler temperatures are coordinated within infected cells. The findings reported here highlight the profound impact of ambient temperature on host cell biology and innate immune defense, including, but undoubtedly not limited to, the capacity to effectively fight infection by the common cold virus.

Materials and Methods

Viruses and Cells. RV-1B, RV-16, and RV-14 were obtained from ATCC (ATCC VR-1645, ATCC VR-283, and ATCC VR-284, respectively) and amplified in H1-HeLa cells (a generous gift from W.M. Lee, University of Wisconsin). A549 cells (ATCC CCL-185) were cultured in F12K medium, 10% (vol/vol) FBS, penicillin-streptomycin, and infection medium was F12K medium, 5% (vol/vol) FBS, penicillin-streptomycin, and 30 mM $MgCl_2$. Primary human bronchial epithelial cells and bronchial epithelial growth medium (BEGM) were obtained from Lonza, and cells were cultured according to manufacturer's protocols.

Viral Infections. H1-HeLa cells were seeded in 6 -well (or 12-well) plates the day before experiment and infected at 80% confluence. All cells were plated and cultured identically at 37 °C, then inoculated simultaneously at 33 °C to ensure an equivalent infection of all cultures before the temperature shift. Cells were inoculated by incubating with 200 μL (or 100 μL) virus stock diluted in PBS/0.1% BSA for 1 h at 33 °C to achieve indicated MOI. At $t = 1$ h, inoculum was removed, cells were washed with warm PBS, medium was added, and plates were replaced in the 33 °C incubator or shifted to 37 °C until indicated time, at which times cells were collected to assay viral growth and/or host cell response to infection.

Intracellular Staining for Flow Cytometry. Cells were collected using trypsin/EDTA, washed, and fixed on ice with Fix/Perm buffer (BD Biosciences). Cells were stained with α-capsase-3-PE or α-capsase-3-FITC, using the manufacturer's protocol (1:10; BD Biosciences). To costain for double-stranded RNA, we used the α-dsRNA monoclonal antibody clone J2 (1:1,000; English & Scientific Consulting Kft) directly conjugated to Dy488 (Innova Biosciences). To detect cell permeability, we used the Far-Red Fixable Live/Dead Stain (Thermo-Fisher.)

Stimulation of Cells. Cells were transfected with PIC (Sigma P9582 or InvivoGen tlrl-picw) or small molecule ligands for RIG-I receptor, including 5ppp-RNA (InvivoGen) and the hairpin RNA 14hp [a generous gift from A. Pyle (31)]. Extracellular PIC was used to stimulate TLR3 (2 μg/mL added to the culture medium). For apoptotic stimuli, cells were incubated at 33 °C or 37 °C for 4 h before caspase-3 staining with gliotoxin (10 μM; Sigma), or TNF (50 ng/mL; eBioscience) + AD (0.5 μg/mL; MP Biomedicals). Mock-treated wells contained vehicle only (DMSO). Caspase inhibitors included: zVAD-FMK (InvivoGen), zIETD-FMK (BD Biosciences), zLEHD-FMK (BD Biosciences), and VX-765 (InvivoGen).

siRNA Knockdown. siRNAs were obtained from GE-Dharmacon as follows: RISC-free (D-001220-01), RNaseL (D-005032-02, CGACUAAGAUUAAUGAAUG), PKR (D-003527-01; CAAAUUAGCUGUUGAGAUA). They were transfected in H1-HeLa cells following the manufacturer's protocol. Subconfluent H1-HeLa cells were transfected with siRNA, then incubated at 37 °C in complete medium for 2 d before infection experiments. At the time of infection, HeLa cells were ∼80% confluent.

BCL2 Overexpression. BCL2-RFP-Bsd overexpressing (LVP553) and control (CMV-null-Bsd) lentiviral vectors were obtained from GenTarget, Inc. H1-HeLa cells were transduced and selected following manufacturer's protocols. Briefly, after drug selection (blasticidin), transduced RFP-high cells were selected using FACS, then cultured using conventional techniques.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc.).

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