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# Influenza A Virus Negative Strand RNA Is Translated for CD8$^+$ T Cell Immunosurveillance

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**Probing the limits of CD8$^+$ T cell immunosurveillance, we inserted the SIINFEKL peptide into influenza A virus (IAV)–negative strand gene segments. Although IAV genomic RNA is considered noncoding, there is a conserved, relatively long open reading frame present in segment 8, encoding a potential protein termed NEG8. The biosynthesis of NEG8 from IAV has yet to be demonstrated. Although we failed to detect NEG8 protein expression in IAV-infected mouse cells, cell surface K$^b$–SIINFEKL complexes are generated when SIINFEKL is genetically appended to the predicted C terminus of NEG8, as shown by activation of OT-I T cells in vitro and in vivo. Moreover, recombinant IAV encoding of SIINFEKL embedded in the negative strand of the neuraminidase-stalk coding sequence also activates OT-I T cells in mice. Together, our findings demonstrate both the translation of sequences on the negative strand of a single-stranded RNA virus and its relevance in antiviral immunosurveillance.** ***The Journal of Immunology*****, 2018, 201: 000–000.**

Influenza A virus (IAV) causes significant worldwide morbidity and mortality due to its efficient evasion of humoral immune responses. IAV elicits a robust CD8$^+$ T cell response, and extensive evidence in humans and animals supports a role for CD8$^+$ T cells in limiting viral replication and reducing morbidity and mortality in hosts whose Ab responses fail to prevent infection (1–4).

CD8$^+$ T cell immunosurveillance encompasses oligopeptides encoded by each of the eight IAV gene segments. The exact peptides recognized by any infected individual is governed largely by their classical MHC class I genes (HLA-A, -B, and -C in humans and H-2 K, D, and L in mice) (5). In most species, each class I locus has thousands of alleles whose peptide specificity varies based on polymorphisms in and around the peptide-binding groove.

Following viral infection, viral peptide ligands are generated extremely rapidly with rates that parallel the translation of their source gene products (6–8). As viral proteins typically exhibit half-lives on the order of tens of h, the kinetic connection of peptide generation with protein synthesis, not degradation, strongly implies that viral peptides derive from a distinct pool of nascent gene products of defective ribosomal products (DRiPs) (9).

Although the biochemical nature of DRiPs generated from wild-type (wt) proteins remains largely undefined, viral peptides can clearly originate from noncanonical translation products (10). This includes downstream initiation on AUG codons (11), readthrough of stop codons (12–14), frame shifting (15, 16), initiation on CUG or other alternative start codons (17), and translation of viral RNA in the nucleus (18). Inasmuch as each of these mechanisms would likely generate a defective gene product that is rapidly degraded, they provide a clear source of immunologically relevant DRiPs.

Negative-strand viruses provide an intriguing opportunity for additional noncanonical translation in immunosurveillance, as the negative strand provides a potential source of peptides. Influenza viruses are of special interest because RNA transcription occurs in the nucleus, which may increase the possibility of noncanonical translation (18–21) or insertion of negative coding information into mRNA by RNA splicing, intended or otherwise. Adding to the interest, NEG8, a large open reading frame (ORF) encoding 167 or more residues (depending on the viral strain) is present in the genomic strand of segment 8 (Fig. 1A) that has been conserved in human IAV isolates for the last 100 years (22, 23). MHC peptide prediction algorithms have identified several NEG8-derived peptides potentially capable of eliciting T cell responses after IAV infection, and one peptide is reported to be immunogenic in IAV-infected mice (24). By contrast, bioinformatic evidence suggests that NEG8 is not under positive selection in nature among IAV isolates but rather is maintained because of constraints in maintaining NS1 and nuclear export protein ORFs on the opposite strand (25). Of note, however, this approach failed to detect positive selection in PB1-F2, despite abundant evidence of its function in IAV infection (26, 27).

In this study, we explore immunosurveillance of viral negative strand–encoded peptide by inserting the model peptide SIINFEKL into several locations in genomic noncoding RNA in IAV, including NEG8. SIINFEKL is a highly immunogenic peptide presented by H-2 K$^b$ to CD8$^+$ T cells (28), and its expression in vitro and in vivo

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Abbreviations used in this article: DRiP, defective ribosomal product; ER, endoplasmic reticulum; HA, hemagglutinin; IAV, influenza A virus; NA, neuraminidase; ORF, open reading frame; PEC, peritoneal exudate cell; PI, postinfection; rVACV, recombinant vaccinia virus; wt, wild-type.

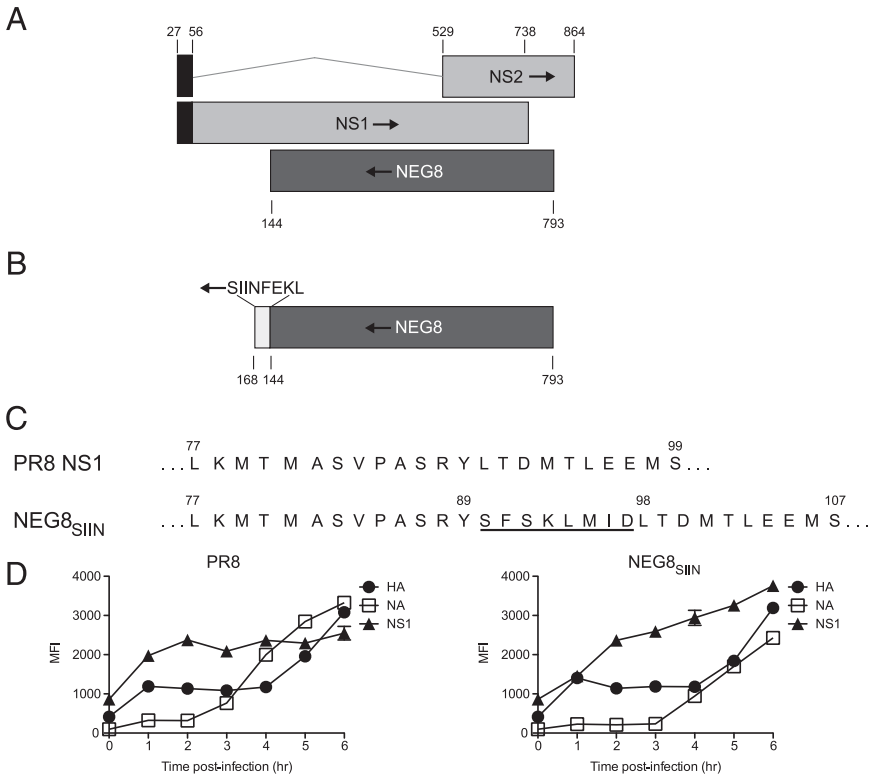


**FIGURE 1.** Production of recombinant virus expressing $NEG8_{SIIN}$. (**A**) NEG8 ORF on the negative, genomic strand of IAV segment 8 and its overlap with NS1/NS2. (**B**) Strategy for NEG8SIIN production. (**C**) Residues (SFSKLMID) inserted into NS1 by insertion of negative-orientation SIINFEKL. (**D**) Expression of viral proteins NA (open squares), HA (closed circles), and NS1 (closed triangles) after DC2.4 infection with PR8 (right) or NEG8SIIN (left). Error bars indicate mean ± SEM.

can be monitored at high sensitivity using OT-I–transgenic T cells (29). Our findings clearly show that immunosurveillance extends to genetic information encoded by the genomic strand of negative-strand RNA viruses.

## Materials and Methods

### *Confocal microscopy*

Hela cells were cultured on Nunc Lab-Tek chambered coverglass (Thermo Fisher Scientific, Waltham, MA) for 24 h in normal growth media. Cells were infected with rVV-NEG8-GFP for 12 h, fixed with cold acetone, and incubated with rabbit polyclonal Abs specific for TGN46 (NB110-40769; Novus Biologicals, Littleton, CO) and mouse protein disulfide isomerase (ab2792; Abcam, Cambridge, MA) followed by Alexa 594–conjugated anti-rabbit and Alexa 647–conjugated anti-mouse IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). Counterstaining was performed with Hoechst 33258 (Thermo Fisher Scientific). Stained cells were visualized by an SP8 confocal microscope system (Leica Microsystems, Mannheim, Germany) using 405-, 488-, 561-, and 633-nm excitation wavelengths for blue, green, red, and far-red channels, respectively.

### *In vitro OT-I activation*

Animal work was performed with the approval of the National Institute of Allergy and Infectious Diseases' animal care and use committee. Spleen and lymph nodes were removed from OT-I TCR-transgenic dsRed mice and homogenized to produce single-cell suspensions. RBCs were lysed, and samples were filtered through a 70-μm nylon filter, and cells were purified using an autoMacs Pro Separator and the $CD8^+$ T cell Negative Selection Kit (Miltenyi Biotec) according to the manufacturer's instructions. Purified cells were labeled in PBS with the CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Cells were plated at $5 \times 10^4$ cells per well in 96-well U-bottom plates. DC2.4 cells were infected at a multiplicity of infection of ∼10 for ∼1 h, then added at $1 \times 10^5$ per well. Some wells received uninfected DC2.4 cells pulsed with 0.1 μM SIINFEKL peptide as a positive control. Percentage of IAV infection was determined 8 h post-infection (PI) via flow cytometry for cell surface hemagglutinin (HA) expression using the H36-26 mAb (30). Twenty-four and forty-eight hours PI, cells were stained for CD8 (53-6.7), CD69 (H1.2F3), and CD25 (PC61) (all from eBioscience) and analyzed for CellTrace Violet fluorescence to determine cell division on a BD LSRII flow cytometer (BD Biosciences). Results were analyzed using FlowJo (Tree Star).

### *In vivo activation of OT-I cells*

Twelve to twenty-four hours prior to infection, we transferred $2 \times 10^5$ $CD45.1^+$ OT-I cells (purified as above) i.v. into C57BL/6 ($CD45.2^+$) mice. For analysis of IFN-γ production, mice were infected i.p. with ∼$1 \times 10^8$ 50% tissue culture–infective dose of each recombinant. Spleens or peritoneal exudate cells (PECs) were harvested at 7 d PI, homogenized, and cells were resuspended in RPMI-10 + 10 mM Hepes buffer and plated at $2 \times 10^6$ cells per well in U-bottom, 96-well plates along with SIINFEKL or an irrelevant control peptide (SSIEFARL) at a final concentration of 100 nM. Cells and peptide were incubated for 4 h at 37°C in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) to allow IFN-γ accumulation. For dead cell exclusion, cells were treated with ethidium monoazide (Invitrogen) before washing, incubated with Fc block (Ab 2.4G2 produced in-house), and stained with anti-CD8 (clone 53–6.7) and anti-CD45.1 (clone A20; eBioscience). After staining, cells were washed and fixed at room temperature for 15 min with 1% paraformaldehyde. Cells were incubated overnight at 4°C with Alexa Fluor 647 anti–IFN-γ (clone XMG1.2; eBioscience) diluted in PBS containing 0.5% saponin (EMD Biosciences). Cells were analyzed on a BD LSR II, and results were analyzed using FlowJo.

### *Peptide binding assay*

Peptide binding was determined as described (31). Briefly, highly purified synthetic peptides were dissolved at 1 mM in DMSO and diluted in FBS-free DMEM to limit proteolysis. RMA/S cells were cultured overnight at 27°C, washed, then incubated with indicated concentrations of peptides for 2 h at room temperature, incubated in the presence of 5 μg/ml brefeldin A at 37°C for 2 h, and then washed and stained with anti-Kb Ab AF6-88.5. Secondary staining was conducted with Alexa Fluor 647–coupled goat anti-mouse IgG(H+L) (Life Technologies).

### *Plasmid mutagenesis*

Mutant IAVs created were all based on the Mount Sinai PR8 strain molecular clone. Plasmid pDZ-PR8-NS was mutagenized by oligonucleotide-based, site-directed mutagenesis using the QuikChange system (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. Oligonucleotide sequences used for appending SIINFEKL to NEG8 were 5′-TTTCGAAGTTGATGATCGACCTAACTGACATGACTCTTGAG-3′ and 5′-CGATCATCAACTTCGAAAAGCTATAACGCGACGCAGGTACAGAG-G-3′; sequences used for appending a stop codon and SIINFEKL to NEG8 were 5′-TTTCGAAGTTGATGATCGATTACCTAACTGACATG-ACTCTTG-3′ and 5′-CGATCATCAACTTCGAAAAGCTATAACGCGA-CGCAGGTAC-3′; sequences used for appending a stop codon, ACG codon

(Thr), and SIINFEKL to NEG8 were 5′-TTTCGAAGTTGATGATCGACG-TTTACCTAACTGACATGACTCTTG-3′ and 5′-CGATCATCAACTT-CGAAAAGCTATAACGCGACGCAGGTACAGAG-3′; sequences used for mutagenizing the predicted AUG start codon of NEG8 to ACG were 5′-GAATAGTTTTGAGCAAATAACgTTTATGCAAGCCTTACATC-3′ and 5′-CTTATCAAAACTCGTTTATTGCAAATACGTTCGGAATGTAG-3′; sequences used for appending the myc tag to the predicted C terminus of NEG8 were 5′-ACAGGTCCTCCTCGGAGATGAGCTTCTGCTCCCT-AACTGACATGACTCTTG-3′ and 5′-CATCTCCGAGGAGGACCTGTAAC-GCGACGCAGGTACAGAGG-3′; and sequences used for appending three tandem myc tags to the predicted C terminus of NEG8 were 5′-CTTCTTCTGAAATCAACTTTTGTTCCAGATCTTCTTCAGAGATGAG-TTTCTGCTCtCCtCCCCTAACTGACATGACTCTTG-3′ and 5′-TGAT-TTCAGAAGAAGATCTGGAACAGAAGCTCATCTCTGAGGAAGATCTG-TTAATTAATTGACGCGACGCAGGTACAGAG-3′.

*Virus rescue and propagation*

Recombinant viruses were rescued using an eight-plasmid system. One microgram of each of the eight wt and mutant plasmids and 2 μg of pDZ-PR8-NP were mixed in 186 μl Opti-MEM medium (Life Technologies). Sixteen microliters of TransIt-LT1 (Mirus Bio, Madison, WI) was added and mixed, followed by incubation at room temperature for 1 h. Eight hundred microliters of Opti-MEM was added, and the mixture was pipetted onto an aspirated six-well well of cells plated the previous day at 0.6 × 106 293-T cells plus 0.2 × $10^6$ MDCK cells in DMEM with 7.5% FCS. Transfected cells were incubated overnight at 37°C. The following morning, medium was replaced with 3 ml DMEM without serum containing 50 μg/ml gentamicin (Quality Biological, Gaithersburg, MD), 3 μg/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone–treated trypsin (Worthington Biochemical, Lakewood, NJ), and 25 mM HEPES (Quality Biological). Over the following days, supernatant media was removed and assayed for HA activity. To grow virus further, 50 μl of rescued virus supernatant was injected into incubated 10-d-old embryonated pathogen-free hen eggs (Charles River, Norwich, CT). Forty eight hours PI, allantoic fluid containing the virus was removed and stored at −80°C.

## Results

*NEG8 encodes an endoplasmic reticulum–localizing protein undetected in IAV-infected cells in vitro*

We initially explored IAV negative-strand translation by attempting to demonstrate NEG8 translation, which has not been verified experimentally in the context of an IAV infection. We infected MDCK or HeLa cells with A/PR/8/34 (PR8) and used polyclonal rabbit Abs raised to synthetic peptides corresponding to the predicted $NH_2$ or C termini in immunoblots of whole cell lysates or in immunofluorescence of fixed and permeabilized cells. Although rabbit sera demonstrated high titers against the immunizing peptides in ELISA, they failed to specifically stain PR8-infected cells in immunofluorescence or to give a specific signal in immunoblots of total infected-cell lysates. We also failed to detect NEG8 in similar experiments using Abs to epitope tags (His, Myc, and Myc repeated three times to increase anti-Myc Ab binding) that we had genetically appended to the predicted C terminus of NEG8 in a PR8-recombinant virus.

By contrast, we could easily detect NEG8 with C-terminal GFP expressed from either a plasmid or a recombinant vaccinia virus (rVACV) consistent with a previous report that the protein is stably expressed by a recombinant baculovirus (32). As predicted (23), rVACV-expressed NEG8-GFP is present in the endoplasmic reticulum (ER) and post-ER compartments (Supplemental Fig. 1), as shown by clear localization to the nuclear membrane and colocalization of GFP signal with Abs specific for ER (protein disulfide isomerase) and *trans*-Golgi complex (TGN46). Reasoning that NEG8 might be metabolically unstable in IAV-infected cells, we treated PR8-infected cells with MG132 to inhibit proteasome-mediated degradation. This still did not enable detection of PR8-encoded native- or epitope-tagged NEG8 via immunoblotting or immunofluorescence.

Thus, although NEG8 is clearly expressed from a plasmid or rVACV as a reasonably stable protein, we repeatedly failed to find evidence that it is expressed by IAV-infected cultured cells.

*SIINFEKL tagging of NEG8 reveals its translation in IAV-infected cells in vitro*

Our failure to detect NEG8 protein could be due to a number of factors other than the complete absence of translation from genomic RNA. $CD8^+$ T cells can provide an exquisitely sensitive measure of translation, and indeed, Zhong et al. (24) reported that of four predicted $K^b/D^b$-binding peptides in the PR8 NEG8 ORF, one, corresponding to residues 33–40, binds to $K^b$ and activates IAV-induced $CD8^+$ T cells in vitro. We were unable to confirm that $NEG8_{33–40}$ binds $K^b$ by a standard flow-based $K^b$ stabilization assay (Supplemental Fig. 2).

To further examine potential immunosurveillance of IAV-genomic RNA, we generated $NEG8_{SIIN}$, a PR8-recombinant virus with SIINFEKL fused to the predicted NEG8 C terminus (Fig. 1B). SIINFEKL forms a highly stable complex with the mouse $K^b$ class I molecule that can be detected by either the 25D-1.16 mAb, or at highest sensitivity, by OT-I–transgenic T cells.

Inserting sequences into genomic RNA will, of course, alter proteins encoded by the positive-sense strand. Although PR8 $NEG8_{SIIN}$ has an eight-residue insertion between resides 89 and 90 of NS1, the virus replicates in embryonated chicken eggs at levels similar to wt virus. Sequencing of egg-grown stocks confirmed the presence of the SIINFEKL-encoding insert, demonstrating that NS1 can function with an essentially random insertion of eight amino acids after residue 89. Although the function of NS1 with an eight-residue insertion might seem surprising, it is likely explained by its location in the extended linker between the NS1 effector and RNA-binding domains (Supplemental Fig. 3). Following infection of DC2.4 cells [a B6 mouse–derived, dendritic cell–like cell line (33)] with $NEG8_{SIIN}$ or wt PR8, flow cytometry revealed similar expression kinetics of NS1 using fixed and permeabilized cells and HA or neuraminidase (NA) staining prior to fixation (Fig. 1D).

We next incubated NEG8-SIIN– or PR8-infected DC2.4 cells with OT-I $CD8^+$ T cells and measured T cell activation after 1 d by induction of the CD69 or CD25 activation markers or by cell division (measured by dilution of CFSE label) (Fig. 2). Two positive controls, SIINFEKL peptide-supplemented cultures and cells infected with PR8 with SIINFEKL inserted into the NA stalk (PR8 $NA_{SIIN}$) (34, 35), confirmed that DC2.4 expressing $K^b$–SIINFEKL can robustly activate OT-I cells under these conditions. By contrast, uninfected cells or cells infected with PR8 lacking SIINFEKL failed to express activation markers or to divide, demonstrating the expected requirement for DC2.4 cell presentation of $K^b$–SIINFEKL complexes. Remarkably, $NEG8_{SIIN}$-infected cells activated OT-I cells at similar or even higher levels relative to the positive controls, as assessed by activation markers or cell division. To our knowledge, this provides the first direct evidence of translation of genetic information from the IAV-negative strand.

Generating additional recombinant PR8 viruses (Fig. 3) revealed that a start codon at the predicted N terminus of $NEG8_{SIIN}$ is not required for generation of SIINFEKL from infected DC2.4 cells, as determined by in vitro induction of CD69 on OT-I T cells using a virus in which the initiating Met is replaced by Thr ($Met(-)NEG8_{SIIN}$). Further, a stop codon immediately upstream of SIINFEKL ($stop\text{-}NEG8_{SIIN}$) abrogates $K^b$–SIINFEKL generation, although not completely, consistent with readthrough translation. Note that flow cytometric detection of HA on live cell surfaces confirmed that cells were well infected by the various PR8 viruses used (all >90% positive).

These findings indicate that initiation can occur downstream of the first AUG in the NEG8 ORF. ATGpr bioinformatic software (http://atgpr.dbcls.jp/) predicts that initiation is nearly equally likely to occur on the second of the six NEG8 AUG codons (reliability score of 0.14 versus 0.12 for first versus second AUG),

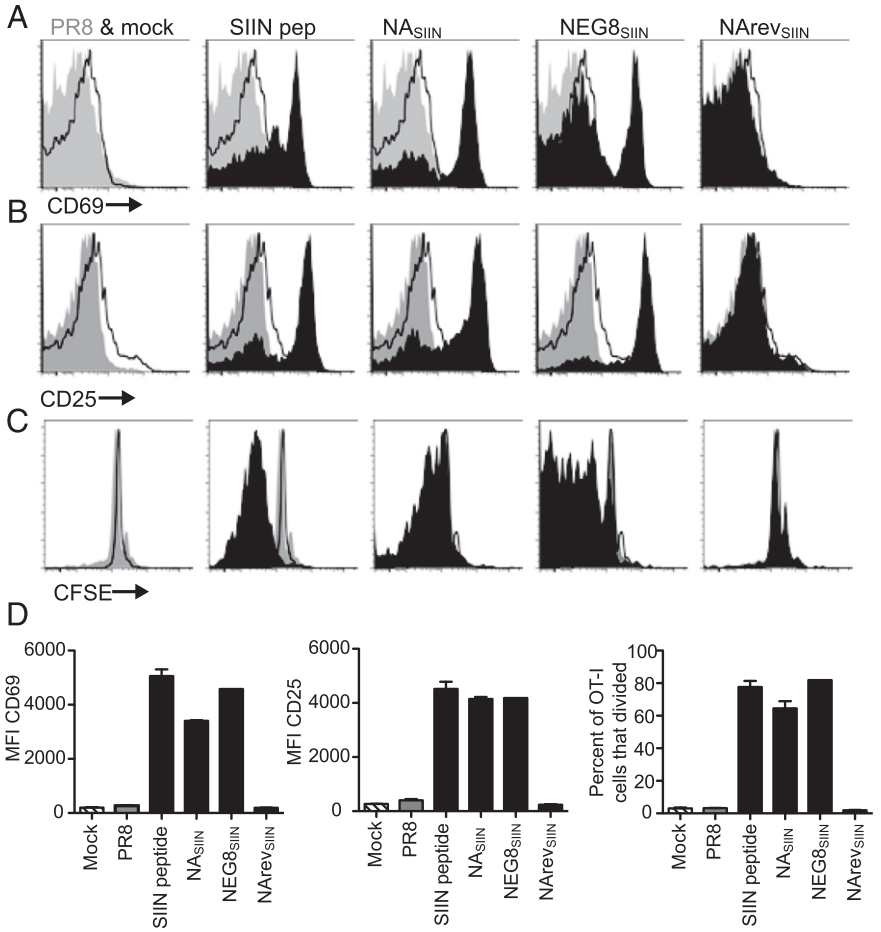


**FIGURE 2.** SIINFEKL is produced in vitro after NEG8$_{SIIN}$ infection and activates OT-I CD8$^+$ T cells. (**A** and **B**) CD69 (A) or CD25 (B) expression 24 h after coculture with infected cells. Right, Mean fluorescence intensities. (**C**) CFSE dilution (indicating cellular division) 48 h after coculture. Error bars indicate mean ± SEMs. Statistics were measured by two-tailed Student *t* test. Experiments were repeated twice with two to three replicates per group.

which would encode a 93-residue polypeptide. Initiation could also occur on any of other four AUG codons, despite their low score (all at background levels), because the accuracy of the algorithm falls off at the bottom end with a specificity of only 20%. In addition, we note the presence of four CUG codons that could support initiation, particularly because CUG initiation is likely favored during IAV infection (17, 36).

It is important to mention that although PR8 NEG8$_{SIIN}$–infected cells consistently activated OT-I CD8$^+$ T cells, we only detected K$^b$–SIINFEKL complexes on the cell surface using the K$^b$–SIINFEKL complex-specific 25-D1.16 mAb (37) in a minor fraction of experiments, and always perilously close to the limits of detection. As OT-I cells are ∼30-fold more sensitive than 25-D1.16–based detection (37), this is consistent with the generation of low amounts of K$^b$–SIINFEKL from PR8 NEG8$_{SIIN}$–infected cells.

### *NEG8$_{SIIN}$ is sufficiently translated in vivo to enable immunosurveillance*

To establish the in vivo relevance of these findings, we transferred $2 \times 10^5$ CD45.1$^+$ OT-I CD8$^+$ T cells into C57BL/6 (CD45.2$^+$) mice and infected them 1 d later via i.p. injection with the various recombinant PR8 viruses (Fig. 4). Seven days PI, we enumerated OT-I (CD45.1$^+$) cells in spleens and PECs by flow cytometry. In control PR8-infected mice, there were few (<0.2%) OT-I T cells present in the spleen or PECs. In contrast, PR8 NA$_{SIIN}$ infection increased OT-I T cell percentages in both the spleen and PECs (20.8 ± 1.6 and 31.9 ± 2.5% or total cells, respectively). In NEG8$_{SIIN}$-infected mice, 2.8 ± 0.2% of splenocytes and 5.5 ± 1.2% of PECs were OT-I cells, ∼10–20 times greater than background. Notably, the total number of CD45.1$^+$ OT-I T cells recovered was far greater than the number adoptively transferred, indicating OT-I proliferation in response to SIINFEKL rather than solely altered recruitment (Fig. 4A, 4B, far right panels).

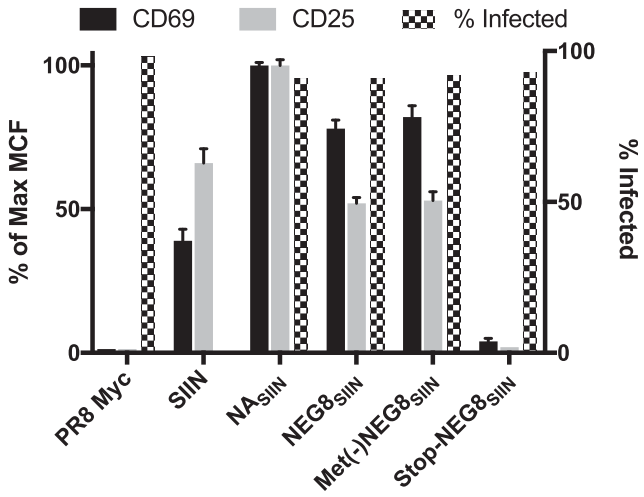


**FIGURE 3.** Characterization of NEG8$_{SIIN}$ translation. DC2.4 cells were infected with the rPR8 viruses indicated and tested 8 h PI for NA expression (percentage infected) by flow cytometry using directly conjugated NA2-1C1 mAb. PI, a separate aliquot of cells were coincubated for 24 h with OT-I T cells, which were then stained for expression of CD69 and CD25 using directly conjugated mAbs. Data represent the mean channel fluorescence determined by flow cytometry and were normalized to maximal expression, and error bars represent the SEM. Two additional experiments gave highly similar results.

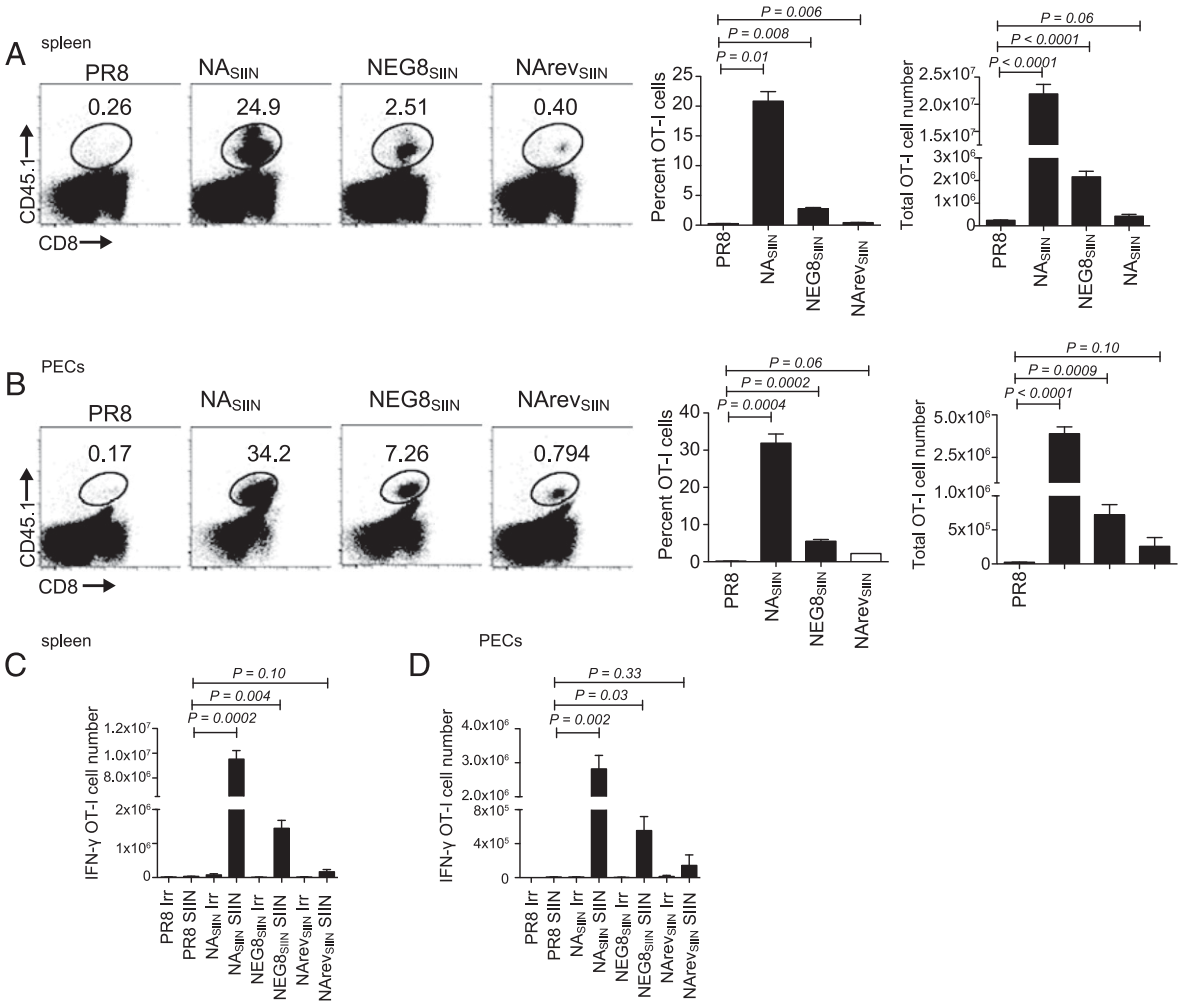


**FIGURE 4.** NEG8$_{SIIN}$ is produced in vivo and recognized by the antiviral immune response. (**A** and **B**) Flow cytometric dot plots showing gates for CD8$^+$ CD45.1$^+$-transferred OT-I cells in the spleen (A) or PECs (B) 7 d PI. Means and SEMs for percentage of cells (middle) and total cell number (right panels) are indicated. (**C** and **D**) Numbers of OT-I T cells producing IFN-γ in the spleen (C) and PECs (D). Means and SEMs are shown. Statistics were measured by two-tailed *t* test. Experiment was performed twice with three mice per group.

To confirm OT-I activation by NEG8$_{SIIN}$, we assayed spleens and PECs for IFN-γ synthesis by stimulating ex vivo with SIINFEKL or an irrelevant peptide (Fig. 4C, 4D). A majority of OT-I cells in either NA$_{SIIN}$- or NEG8$_{SIIN}$-infected mice produced IFN-γ in response to restimulation with SIINFEKL but not an irrelevant peptide, which is indicative of Ag-experienced, effector CD8$^+$ T cells.

Taken together, these data clearly demonstrate that NEG8$_{SIIN}$ activates CD8$^+$ T cells in vivo, establishing that translation from the IAV genomic strand can occur in vivo.

### *Immunosurveillance extends to peptides inserted elsewhere in genomic RNA*

To extend these findings, we inserted SIINFEKL-encoding nucleotides elsewhere in genomic RNA. To minimize the perturbation to coding sequences, we used the basic local alignment search tool on reverse-strand ORF amino acid sequences against SIINFEKL to find the most homologous naturally encoded sequence and then changed the negative-strand sequence to encode SIINFEKL. Unfortunately, inserting SIINFEKL at a position equivalent to residue 154 in HA (H3 numbering) or residue 809 of PA, which in each case changed only four residues, was incompatible with recovering infectious viruses.

We were, however, able to generate a virus (NArev$_{SIIN}$) encoding negative-strand SIINFEKL inserted in a position corresponding with residue 42 in the NA stalk (Supplemental Fig. 4). NA stalk length is naturally variable in nature, and NA is able to accept SIINFEKL in the positive strand with little consequence (34, 35). Indeed, NArev$_{SIIN}$ grew to wt titers. SIINFEKL synthesis could potentially initiate at an AUG three codons upstream and would be terminated by a stop codon after translating five additional residues. The next potential start site is located dozens of codons upstream and is unlikely to be relevant because there are six intervening stop codons.

Although we failed to detect OT-I T cell activation with NArev$_{SIIN}$-infected DC2.4 cells in vitro (Fig. 2), we observed weak but consistent OT-I T cell activation in vivo following i.p. infection, as assessed either by splenic and PEC OT-I T cell proliferation or IFN-γ production ex vivo (Fig. 4).

Thus, even in the absence of any selective pressure for gene expression as might occur with NEG8 SIINFEKL, there is sufficient translation from essentially a random sequence in genomic IAV RNA to enable CD8$^+$ T cell immunosurveillance in vivo.

## Discussion

Despite lurking for decades in the published IAV segment 8 sequence (38, 39) in full view of clever students of IAV, evidence supporting the translation of the NEG8 ORF in the context of a bona fide influenza infection is lacking. Using multiple Ab-based approaches to detect NEG8 (raising rabbit Abs to predicted $NH_2$- and C-terminal

peptides, generating NEG8 fused genetically to Ab epitope tags [Myc, HA, and SIINFEKL itself using a SIINFEKL specific mAb (40)]), we failed to detect NEG8 in PR8-infected HeLa or MDCK cells (including cells treated with MG132 to block potential proteasome-mediated degradation) by immunofluorescence or immunoblotting using extracts prepared by immersing cells in boiling SDS extraction buffer to maximize protein solubilization. It is likely that if the protein is synthesized in these cells, it is only in minute quantities.

Given the unusual nature of its encoding RNA, it would not be surprising if NEG8 is only expressed physiologically under special circumstances in vivo. Perhaps expression is limited to a subset of the wide variety of cell types that can be infected by IAV, which include epithelial, endothelial, and hematopoietic cells. It is clearly of interest in future experiments to examine possible NEG8 translation in whole organ extracts and frozen sections of lungs and immune tissues of infected animals. Note that the putative positive selection of NEG8 in human and animal viruses requires that NEG8 translation enhances IAV transmission in the corresponding hosts.

Our findings show that ribosomes in cultured cells and in vivo can initiate downstream of the first AUG on the NEG8 ORF and continue translating to the predicted C terminus of the appended model $CD8^+$ T cell peptide SIINFEKL. Such translation, however, may be exclusively related to immunosurveillance, in which case, the translation products may be truncated and degraded rapidly, with encoded peptides being rescued from typically rapid destruction by binding to MHC class I molecules and potentially class II molecules as well (41).

How might negative-strand IAV RNA be translated? Viral RNA is synthesized from an intermediate form of RNA (cRNA) dedicated to viral RNA synthesis (42). In theory, negative-sense RNA should not be capped, polyadenylated, or exported from the nucleus, except for incorporation into budding virions as NP-coated genomic segments. These events may, however, occur at a level with functional consequences but are still below the radar of current methodologies. Further, none of these events are absolutely required for translation, particularly for viruses. There are many examples of cap- and polyA-independent mRNA translation, including the translation of peptides embedded in introns, which appears to occur in the nucleus (19). Viruses are virtuosos at manipulating the rules of translation, typically exploiting noncanonical translation while shutting down canonical translation to monopolize ribosomes and also manipulate innate host antiviral responses (43).

The nuclear localization of IAV transcription may be key to negative-strand translation. We previously reported that upon drug blockade of NA mRNA export from the nucleus of cells infected with NA-SIINFEKL PR8, NA protein expression is reduced to a far greater extent than $K^b$–SIINFEKL generation (18), consistent with peptide generation from nuclear translation of the NA-positive strand. It would be interesting to determine if $NEG8_{SIIN}$ is translated/immunogenic in the context of a typical cytoplasmic negative-stranded virus such as vesicular stomatitis virus. If so, this would undermine the potential contribution of nuclear translation or aberrant splicing of genomic RNA and mRNA to the SIINFEKL synthesis in $NEG8_{SIIN}$-infected cells.

In summary, we show that, through an unknown mechanism, immunosurveillance extends to the negative strand of at least a subset of RNA viruses. If this is a general phenomenon, it implies negative-strand information can potentially double the peptides presented for T immunosurveillance of viruses and cancer cells. If such T cells exert sufficient antiviral activity to limit transmission, they could select for escape mutants that will contribute to viral evolution and whose significance will not be apparent from standard analysis of viral ORFs.

## Disclosures

The authors have no financial conflicts of interest.

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