The importance of antiviral CD8+ T cell recognition of alternative reading frame (ARF)-derived peptides is uncertain. In this study, we describe an epitope (NS1-ARF21–8) present in a predicted 14-residue peptide encoded by the +1 register of NS1 mRNA in the influenza A virus (IAV). NS1-ARF21–8 elicits a robust, highly functional CD8+ T cell response in IAV-infected BALB/c mice. NS1-ARF21–8 is derived from a 14-residue peptide with no apparent biological function and negligible impacts on IAV infection, infectivity, and pathogenicity. NS1-ARF21–8 provides a clear demonstration of how immunosurveillance exploits natural errors in protein translation to provide antiviral immunity. We further show that IAV infection enhances a model cellular ARF translation, which potentially has important implications for virus-induced autoimmunity. The Journal of Immunology, 2019, 202: 3370–3380.

Damien J. Zanker,*1 Sara Oveissi,*1 David C. Tscharke,‡ Mubing Duan,* Siyu Yan,† Xiaomu Zhang,* Kun Xiao,* Nicole A. Mifsud,*,‡ James Gibbs,‡ Lenny Izzard,‡ Daniel Dlugolenski,‡ Pierre Faou,* Karen L. Laurie,|| Nathalie Vigneron,§ Ian G. Barr,‖ John Stambas,‡ Benoît J. Van den Eynde,‡ Jack R. Bennink,§ Jonathan W. Yewdell,‡,2 and Weisan Chen*,2

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Abbreviations used in this article: ACN, acetonitrile; ARF, alternative reading frame; BFA, brefeldin A; DRP, defective ribosomal product; FDR, false discovery rate; IAV, influenza A virus; ICS, intracellular cytokine staining; i.n., intranasal(ly); KO, knockout; MOI, multiplicity of infection; MS, mass spectrometry; MS/MS, tandem MS; ORF, open reading frame; PR8, A/Puerto Rico/8/34; RP, reverse phase; RV, recombinant vaccinia virus; TCD8+, CD8+ T cell; TFA, trifluoroacetic acid; wt, wild-type.

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Address correspondence and reprint requests to Dr. Jonathan W. Yewdell or Prof. Weisan Chen, Cellular Biology Section, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892 (J.W.Y.) or Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Victoria 3086, Australia (W.C.). E-mail address: jywdeell@niaid.nih.gov (J.W.Y.) or weisan.chen@latrobe.edu.au (W.C.).

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ORCID(s): 0000-0002-7353-5108 (S.O.); 0000-0001-6825-9172 (D.C.T.); 0000-0003-0855-5011 (M.D.); 0000-0001-8647-2102 (N.A.M.); 0000-0001-7436-9730 (L.I.); 0000-0002-2761-2083 (N.V.); 0000-0002-7351-418X (I.G.B.); 0000-0002-4995-3270 (B.J.V.d.E.); 0000-0002-3826-1906 (J.W.Y.); 0000-0002-5221-9771 (W.C.).
The mouse IAV infection model has played a key role in deciphering the rules of T<sub>CD<sub>8</sub></sub> immunodominance (8, 17–22). In studies on anti-IAV T<sub>CD<sub>8</sub></sub> responses in BALB/c mice, we observed an extremely robust response to a naturally processed peptide that we were unable to identify. After an arduous and extended hunt, we have finally caught our Moby Dick peptide, which provides the clearest example of a viral peptide generated from a natural ARF.

Materials and Methods

Mice

BALB/c mice were purchased from Walter Eliza Hall Institute of Medical Research (Kew, Melbourne, VIC, Australia). Mice were housed in specific pathogen-free isolators. Experiments were performed with animals aged at 6–12 wk, conducted under the auspices of the Austin Health and La Trobe University Animal Ethics Committee, and conformed to the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes.

Peptide and Abs

IAV peptides (please see Supplemental Table I) were synthesized by Mimotopes at >80% purity (Clayton, Melbourne, VIC, Australia). Peptides were used at 1 μM concentration of 0.5 μM purified following BsmBI restriction enzyme digestion of pCU57 vector (retroviral vector containing IRES-mCherry) were then subjected to BisIII and XhoI double digestion to linearize PMIM and to generate matching sticky ends in both the vector and the PCR products for directional cloning the PMIM-NS-Full and PMIM-NS-mut plasmids.

Generation of PMIM-NS<sup><sup>-</sup></sub>′<sup>wt</sup>-WT and PMIM-NS<sup><sup>-</sup></sub>′<sup>mut</sup>-mut. A 1237-bp eGFP-specific primer set was constructed by inserting 623-bp wt or mutant NS intron sequences at 30 and 31 bp of the 744-bp eGFP fragment flank by 10-bp sequences at either side containing restriction enzyme sites (sequences not shown). The genes were synthesized (GenScript) and cloned into pUC57 vector. eGFP-NS<sup><sup>-</sup></sub>′<sup>mut</sup>-WT and PMIM-NS<sup><sup>-</sup></sub>′<sup>mut</sup>-mut fragments were PCR amplified using the eGFP-specific primer set (forward primer: 5′-agcGTATCTTGAGGCAAGG-3′ and reverse primer: 5′-actCTCGAGTATCTTGAGGCAAGG-3′). Both the NS PCR product (full and mut) and PMIM vector (retroviral vector containing IRES-mCherry) were then subjected to BisIII and XhoI double digestion to linearize PMIM and to generate matching sticky ends in both the vector and the PCR products for directional cloning of the PMIM-NS-Full and PMIM-NS-mut plasmids.

Generation of C-terminal truncated NS1 fragments. A 693-bp cDNA containing the wt PR8 NS1 gene was used as a template for PCR amplification of full-length as well as six 5′-terminal truncated fragments subcloned into pDNA3.1/V5-His TOPO vector (Life Technologies). NS1 truncation variants were PCR amplified using PR8 NS1-specific forward (5′-GGATCCATGCGATCCATCAGCGATCCATCAGCGATCCATCAGCG-3′) and reverse primer: 5′-GCATCCGCGGAGAATGCATGCGATCCATCAGCGATCCATCAGCG-3′) with 16-bp upstream and 17-bp downstream nucleotide sequences consisting of 16-bp upstream and downstream nucleotide sequences encompassing BsmBI restriction enzyme sites was designed (sequence not shown), synthesized, and cloned into pCU57 vector (GenScript, Nanjing, China). NS-Full and NS-mut fragments were PCR amplified from the pCU57-NS-full and pCU57-NS-mut template vectors, respectively, using the NS-specific primer set (forward primer: 5′-tattGATCTCTGGAGGCAAGG-3′ and reverse primer: 5′-acgCTCGAGTATCTTGAGGCAAGG-3′). Both the NS PCR product (full and mut) and PMIM vector (retroviral vector containing IRES-mCherry) were then subjected to BisIII and XhoI double digestion to linearize PMIM and to generate matching sticky ends in both the vector and the PCR products for directional cloning of the PMIM-NS-Full and PMIM-NS-mut plasmids.

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In vitro viral infection

For recombinant vaccinia virus (rVV) infection, cells were resuspended in 0.1% BSA/PBS and infected with rVV, as indicated in the figure legends, at a multiplicity of infection (MOI) of 10. For IAV infection, cells were resuspended in FCS-free, acidified RPMI medium and infected with IAV at 10 MOI. Cells were incubated in a 37°C water bath for 1 h with gentle agitation every 15 min. Infected cells were then washed twice with PBS.

Cell line culture

All cells were cultured at 37°C with 5% CO<sub>2</sub> in a humidified incubator. PR8, D25V, D25V transductants, and HEK293T transductants were cultured in RPMI 1640 containing 10% FCS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (RF-10). Platinum-E retroviral-producing cell line (Cell Biolabs) was cultured in DMEM with 10% FCS and the above supplements, plus 1 μg/ml purinecin and 10 μg/ml blasticidin. For reverse genetics, MDCK cells were maintained in MEM (Life Technologies, Australia) containing 10% FCS, 10 U/ml penicillin, and 100 μg/ml streptomycin (MEM-10), and HEK293T cells were cultured in Opti-MEM I Life Technologies, Carlsbad, CA) containing 5% FCS, 10 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged, and media were changed when cells became 80% confluent; cells were used during the exponential growth phase.

The mouse IAV infection model has played a key role in deciphering the rules of T<sub>CD<sub>8</sub></sub> immunodominance (8, 17–22). In studies on anti-IAV T<sub>CD<sub>8</sub></sub> responses in BALB/c mice, we observed an extremely robust response to a naturally processed peptide that we were unable to identify. After an arduous and extended hunt, we have finally caught our Moby Dick peptide, which provides the clearest example of a viral peptide generated from a natural ARF.
pHW2000 vector for construction of pHW2000-NS-ARF21–14 KO vector, which was then used in reverse genetics IAV production.

**Transfection and transductions**

One day before transfection, 2 × 10^6 retrovirus-producing cells were plated onto a 10-cm tissue culture dish in 10 ml of DMEM-10 without antibiotics. When cells reached 70–80% confluence, FuGENE HD (Promega) was used for transfecting Platinum-E cells with the bicistronic mammalian expression retrovector (PMIM) containing the gene of interest. Forty-eight hours later, retrovirus-containing supernatant was harvested, filtered (0.45 µm), and added to 3 × 10^5 D240 target cells in a 10-cm tissue culture dish. Polybrene was added into the retrovirus-containing supernatant at 8 µg/ml. Twenty-four hours later, the medium was replaced, and target cells were cultured for three more days. Transduced cells were then analyzed for mCherry reporter expression and were sorted using FACS-Saria III flow cytometer (Becton Dickinson).

**In vitro activation of Ag-specific TCD8+, assessed by Cr^51 release**

51Cr-release assays were performed as described (25). Data are expressed as the percentage of specific lysis = (TCD8+-induced lysis − spontaneous release)/(release by detergent − spontaneous release) × 100%

**In vitro activation of Ag-specific TCD8+ and enumeration by intracellular cytokine staining**

For ex vivo isolates, BAL wash and splenic cells were collected in RF-10. The Ag-specific TCD8+ were enumerated using intracellular cytokine staining (ICS) for the production of IFN-γ after being stimulated with 1 µg/mℓ antigen peptide in the presence of 10 µg/ml brefeldin A (BFA) for 5 h at 37°C, unless otherwise stated (22).

For BFA kinetics assay, P815 cells were infected with 10 MOI of PR8 for 1 h at 37°C, washed, and then added to monospecificity TCD8+ cultures, with BFA added at various time points. Following 4 h of exposure to BFA, TCD8+ were transferred onto ice, fixed with 1% paraformaldehyde, and stained in ICS for IFN-γ in the presence of 0.4% saponin (26).

**Peptide extraction and reverse phase–HPLC fractionation**

The method was described previously (25). Briefly, 5 × 10^5 cultured cells were infected at 10 MOI with PR8 for 5 h. Cells were washed, resuspended in trifluoroacetic acid (TFA)/H₂O₂, homogenized, and further sonicated. Peptide-containing supernatant was collected after ultracentrifugation and passed through a 3K cutoff filter. Samples were then dehydrated to <400 µl using a speed-vac, and the extracted peptides were then fractionated on an reverse phase (RP)-HPLC (Agilent 1100). Eluted fractions were collected between 10.0 and 38.8 min at 6-s intervals. Elution times of TCD8+ were transferred onto ice, fixed with 1% paraformaldehyde, and stained in ICS for IFN-γ in the presence of 0.4% saponin (26).

**RNA extraction and sequencing**

Cells were homogenized using the QIAshredder (Qiagen). Total RNA was extracted from 3 × 10^5 cells per sample using the Qiagen RNeasy Mini Kit, according to manufacturer instructions, with on-column DNase I digestion incorporated. Following RNA extraction, RNA integrity number was quality controlled using the Agilent 2100 bioanalyzer, and samples with a RIN score above 7 were used for sequencing. Following mRNA purification with Poly-T oligo-attached magnetic beads, strand-specific cDNA libraries were prepared using the NEBNext Ultra RNA Library Preparation Kit. cDNA libraries were sequenced on an Orbitrap Elite (Thermo Fisher Scientific) in data-dependent acquisition mode using massions 300–1500 as mass spectrometry (MS) scan range; collision-induced dissociation tandem MS/MS spectra were collected for the 20 most intense ions per MS scan. Dynamic exclusion parameters were set as follows: repeat count 1, duration 90 s, and the exclusion list size was set at 500 with a 10-s exclusion duration. Other instrument parameters for the Orbitrap were as follows: MS scan at 120,000 resolution, maximum injection time of 50 ms, automatic gain control target of 1 × 10^6, and collision-induced dissociation at 35% energy for a maximum injection time of 150 ms with an automatic gain control target of 5000. The Orbitrap Elite was operated in dual analyzer mode with the Orbitrap analyzer being used for MS and the linear trap being used for MS/MS.

**Liquid chromatography MS/MS data analysis and statistics**

Identification and isotopic quantification of proteins were performed on raw output files from liquid chromatography electrospray ionization MS/MS using MaxQuant (Version 1.5.1.6; Cox and Mann, 2008) together with its built-in search engine Andromeda. Uniprot using the Mouse Uniprot FASTA database (September 2015) together with common contaminants was used for this analysis. Carbamidomethylation of cysteines was set as a fixed modification, whereas acetylation of protein N termini and methionine oxidation were included as variable modifications, and dimethyllys0, dimethlys4, dimethNterm0, and dimethNterm4 were included as labels. Parent mass tolerance was set to 5 ppm (after refinement by MaxQuant), and fragment mass tolerance was set to 0.5 Da. Trypsin was set as the digestion enzyme with up to two missed cleavages allowed. Prior to searching, MS/MS spectra were filtered by retaining only the top eight peaks per 100 Da. The match between runs feature of MaxQuant was used to perform differential protein identification: peptide identifications from one run to another based on retention time and mass to charge ratio. Both peptide and protein identifications were reported as a P-value, and fragment mass tolerance was set to 0.1 Da. MS/MS spectra were filtered by retaining only the top eight peaks per 100 Da. The match between runs feature of MaxQuant was used to perform differential protein identification: peptide identifications from one run to another based on retention time and mass to charge ratio. Both peptide and protein identifications were reported as a FDR of 1%.

The normalized protein group ratios values obtained from MaxQuant were then used to perform differential expression analysis with the limma package in R. A moderated statistic was calculated for each protein group to test for differences in expression between samples. Resulting p values were corrected for multiple testing and converted to an FDR. Missing values were excluded from the analysis, with df adjusted accordingly.

**Results**

Repeated in vitro T cell restimulation using IAV-infected P815 cells reveals a novel immunodominant T cell response

We previously reported NP147–155 as the most immunodominant of five immunodominant IAV peptides recognized by TCD8+ from IAV-infected BALB/c mice (22). Interestingly, when we used
IAV-infected P815 cells (PR8-P815) as APC to maintain a polyspecific T<sub>CD8</sub><sup>+</sup> line, the line gradually lost specificity for each of the five peptides yet remained highly specific for IAV (Fig. 1A). What did these T cells recognize?

To identify this potentially novel IAV epitope, we again raised a polyspecific T<sub>CD8</sub><sup>+</sup> line in the same manner. After the first round of stimulation, we determined recognition of 10 potential IAV gene products by infecting L929 cells (H-2<sup>K</sup>) expressing individual H-2<sup>d</sup> allomorphs with rVV<sub>s</sub> expressing individual IAV mRNA (as a strictly cytoplasmic virus, rVV-encoded mRNAs are not spliced). HA-, NP-, and PA-specific responses were stimulated by L929-K<sup>d</sup><sub>4</sub> cells, most likely representing the previously identified

**FIGURE 1.** PR8 restimulation reveals a novel immunodominant H-2L<sup>d</sup>–restricted response to NS1. (A) BALB/c mice were i.p. infected with 10<sup>7</sup> PFU PR8. Thirty days later, splenic cells containing memory T cells specific to IAV were harvested, and 3 × 10<sup>6</sup> splenic cells were infected with PR8 at 10 MOI for 5 h and used to restimulate 2.7 × 10<sup>7</sup> splenic cells. T cell cultures were restimulated every 14 d with PR8-P815 infected at 10 MOI. Ten days following restimulation, culture specificity was assessed by ICS for IFN-γ using synthetic peptides or PR8-P815. Black bars denote T cell Ag specificity following the first stimulation; white bars denote specificity following the fourth stimulation. (B) Assessment of T cell Ag specificity in a PR8-P815–restimulated T cell culture using single H-2<sup>d</sup> allele–transfected HEK293T cells infected with PR8 or rVV<sub>s</sub> encoding single IAV proteins. (C) NP<sub>147–155</sub> and a nine-round PR8-P815–restimulated polyspecific T<sub>CD8</sub><sup>+</sup> line were assessed for specificity using P815 and HEK293T-L<sup>d</sup> cells infected with PR8 or rVV<sub>s</sub> encoding single IAV proteins. All experiments are representative of at least two independent experiments. (D) Assessment for the presentation of the two predicted NS1 ARF peptides and IAV-infected P815 cells by IFN-γ ICS using a PR8-P815–restimulated T<sub>CD8</sub><sup>+</sup> Splenic cells were harvested from PR8-infected memory BALB/c mice and two separate T cell cultures raised by restimulating splenic cells with (E) ARF<sub>2</sub>–8 and (F) ARF<sub>2</sub>–11 synthetic peptides. Ag-specific T cell activation by ARF2 peptides or ARF2 peptides with an amino acid truncation or extension at either end was assessed by IFN-γ ICS using log-fold dilutions in serum-free condition. All experiments are representative of at least two independent experiments.
HA$_{518-526}$, NP$_{147-155}$, and PA-specific T cells, respectively (18). A modest response to rVV-PB2 infected L929-D$_{3}$ cells was observed (Fig. 1B), as originally reported (29, 30), and is likely PB$_{289-297}$-specific (21).

Critically, polyclonal T$_{CD8+}$ also recognized L929-D$_{3}$ cells infected with rVV-NS1, indicating the presence of novel NS1 epitope(s) (Fig. 1B). The L$_{d}$-restricted recognition of rVV-encoded NS1 was confirmed by a repeatedly PR8-P815 restimulated T$_{CD8+}$ line using H-2L$_{d}$ expressing HEK293T cells infected with PR8 or rVV-NS1 (Fig. 1C).

**Difficulties in identifying the NS1 L$_{d}$-restricted epitope**

To identify the novel NS1 T cell epitope(s), we initially cloned six truncated NS1 gene fragments into the pcDNA3.1/V5-His TOPO vector to express NS1/1-38, NS1/1-64, NS1/1-98, NS1/1-131, NS1/1-164, NS1/1-198, and full-length NS1/1-230. Following transfection of HEK293T-L$_{d}$ cells (31), none of the fragments displayed antigenicity (Supplemental Fig. 1A).

As an alternative, we turned to H-2L$_{d}$ peptide binding motif prediction of potentially antigenic peptides. Accordingly, we selected peptides with a position 2 serine or proline and position 8, 9, 10, and 11 isoleucine, phenylalanine, valine, leucine, arginine, and aspartic acid. Of the 17 peptides tested, none demonstrated antigenicity using a repeatedly PR8-P815–restimulated T$_{CD8+}$ line (Supplemental Fig. 1B).

Many well-characterized antigenic peptides do not match predictive algorithms (32). Taking a broader approach, we synthesized a set of 13 mer peptides with 10 overlapping amino acids to create a peptide panel spanning all possible 11mers and shorter linear epitopes and likely all potential 12mer epitopes, as most T$_{CD8+}$ recognize cognate peptides missing a single residue at linear epitopes and likely all potential 12mer epitopes, as most create a peptide panel spanning all possible 11mers and shorter (Supplemental Fig. 1B).

Although MHC class I ligands are typically ~9-10 aa and rarely longer than 12 aa, exceptions are known [e.g., 13mers from EBV BZLF1 Ag (33) or NY-ESO-1 (34) and a 14mer from M-CSF (35)]. To exclude the possibility of an unusually long epitope being recognized by our T$_{CD8+}$ line, we screened a set of 18 mer peptides with 12 aa overlapping. Once again, failure ensued (Supplemental Fig. 1D).

These findings were consistent with the idea that the L$_{d}$-restricted T$_{CD8+}$ recognize an unusual IAV peptide.

**Identification of the novel epitope by ARF peptides**

Failing to find the epitope in the standard NS1 reading frame, we searched for predicted L$_{d}$-binding peptides in the +1 or +2 reading frames downstream of potential Met initiating residues. This revealed two peptides that we designate as NS1-ARF1 (MSANELQTK) and NS1-ARF2 (MPHSLLIGFAEI). Testing synthetic peptides corresponding to the sequences, we found that synthetic NS1ARF2 activated a major population of the PR8-P815–stimulated polyclonal T$_{CD8+}$ (Fig. 1D).

Within ARF2$_{1-11}$, coincidentally, is an 8-mer peptide that matches the H-2L$_{d}$ binding motif xPxxxXXF. We compared the abilities of synthetic ARF2$_{1-8}$ versus ARF2$_{1-11}$ to generate T$_{CD8+}$ lines from splenocytes derived from PR8-prime BALB/c mice (Fig. 1E, 1F). Interestingly, each peptide could elicit T$_{CD8+}$ that greatly preferred their activating peptide compared with truncated versions, suggesting that each is naturally immunogenic in the context of PR8 infection in vivo.

To determine the extent to which cells naturally present ARF2$_{1-8}$ versus ARF2$_{1-11}$, we HPLC fractionated acid-soluble peptides from PR8-infected cells and assayed fractions for antigenicity against T$_{CD8+}$ lines (25). Using an IAV polyspecific T$_{CD8+}$ line, fractions eluting at 18.9, 21.2, 23.9, 24.3, and 25.0 min exhibited antigenic activity (Fig. 2Ai). Using monospecific T$_{CD8+}$ restimulated with synthetic peptides, it was clear that the two major peaks at 18.9 and 24.9 min are recognized by NP$_{147-155}$ and ARF2$_{1-8}$, respectively (Fig. 2Aii, iii). Corroborating this finding, this correlates perfectly with the elution of the cognate synthetic peptides. We fail to obtain evidence for natural presentation for ARF2$_{1-11}$ (Fig. 2Aiv), which is expected to elute at 26.6 min based on the behavior of the synthetic version. This is likely mostly due to inefficient ARF2$_{1-11}$ epitope presentation by PR8-P815 cells.

Titration of peak antigenic fractions (Fig. 2B) revealed that ARF2$_{1-8}$ is generated at least 3-fold more abundantly than NP$_{147-155}$ using the synthetic peptides as standards. We corroborated this finding with peptide generation kinetics, using BFA to block further transport of class I peptide complexes to the surface of PR8-infected P815 cells in conjunction with T$_{CD8+}$ lines raised to individual peptides (26). As the two T$_{CD8+}$ lines clearly had similar avidity shown by their cognate peptide titration (Fig. 2C), ARF2$_{1-8}$ is presented ~2- to 3-fold faster than NP$_{147-155}$ (Fig. 2D), which is consistent with the results of peptide elution and fraction titration results (Fig. 2B) and likely explains why multiple in vitro restimulated T$_{CD8+}$ lines become ARF2$_{1-8}$ specific.

**Highly functional ARF2$_{1-8}$-specific T$_{CD8+}$ are a major component of BALB/c mice anti-IAV cellular immunity**

Having defined NS1-ARF2$_{1-8}$ as the major peptide recognized by L$_{d}$-restricted IAV-specific T$_{CD8+}$, we next used the synthetic peptide to measure the magnitude of the in vivo response 7 d post-i.p. infection. In mouse groups infected with IAV doses lower than 5 × 10$^{5}$ PFU, NS1-ARF2$_{1-12}$ T$_{CD8+}$ responses were slightly smaller than that of T$_{CD8+}$ specific to the immunodominant NP$_{147-155}$. Remarkably, NS1-ARF2$_{1-8}$ response ascended to the α-position in the immunodominance hierarchy at higher virus doses (Fig. 3A).

In mice 10 d post-i.n. infection with 100 PFU of IAV, in both spleen and lungs, NS1-ARF2$_{1-8}$-specific cells rank second in the immunodominance hierarchy to NP$_{147-155}$ (Fig. 3B, 3C).

To be certain that recognition of NS1-ARF2$_{1-8}$ is a bona fide measure of its in vivo immunogenicity, we identified a 2009 pandemic H1N1 virus, A/Tasmania/09 (Pan09) (H1N1), possessing a phenylalanine → serine substitution in the C-terminal anchor residue of NS1-ARF2$_{1-8}$ that obviates high-affinity binding to L$_{d}$. Pan09 induced a vigorous response to all of the major epitopes except NS1-ARF2$_{1-8}$, confirming its status as a naturally processed epitope in vivo (Fig. 3B, 3C).

We next compared the effector function of T$_{CD8+}$ specific for NS1-ARF2$_{1-8}$ versus NP$_{147-155}$ after their ex vivo activation with cognate peptide. This revealed that T$_{CD8+}$ populations expressed similar amounts of effector cytokines IFN-γ and TNF-α and were equally cytolytic, as judged by expression of the degranulation marker, CD107a (Fig. 3D).
not, however, exclude a role for splicing in generating the peptide in IAV-infected cells, particularly given the evidence for nuclear translation as a source of peptides from IAV (36) and from intronic regions of plasmid-encoded genes (37).

To examine the role of mRNA splicing in NS1-ARF2-1–8 generation, we cloned the PR8 NS gene into the retroviral vector PMIM as wt sequence (NS-full) or modified to abrogate splicing (NS-mut) (Fig. 4Aii). D2SV cells transduced with either NS-full or NS-mut equally activated an ARF2-1–8–specific TCD8+, demonstrating that mRNA splicing does not enhance ARF epitope generation (Fig. 4B). We noted that in our NS1 constructs, ARF2-1–8 epitope presentation is clearly weaker compared with IAV-infected cells. The likely explanation is that NS1 expression levels are lower from our constructs compared with that from IAV infection (Supplemental Fig. 2).

We further examined the contribution of NS1 splicing to peptide generation using NS1-GFP IAV (23), a recombinant IAV whose NS segment is modified to express NS1-GFP, followed by an FMDV 2A ribosome stop-and-go site (38) that releases NS2 (also called NEP) as it is synthesized. Two synonymous mutations are also present to prevent NS mRNA splicing. P815 cells infected with NS1-GFP IAV efficiently presented ARF2-1–8 epitope (Fig. 4B).

**FIGURE 2.** ARF2-1–8 is presented more abundantly than NP-147–155. (A) P815 cells were infected with PR8 at 10 MOI for 5 h and lysed, and peptides were extracted by acid and fractionated by RP-HPLC. Fractions were collected at 0.1-min intervals and pulsed onto low temperature–induced P815 cells and used to assess for IFN-γ production in an ICS by (i) PR8-P815–polyspecific, (ii) NP-147–155–specific, (iii) ARF2-1–8–specific, and (iv) ARF2-1–11–specific TCD8+ to confirm epitope presentation following a natural IAV infection. (B) Peak fractions corresponding to synthetic peptide elution times for NP-147–155 and ARF2-1–8 were titrated with NP-147–155–specific and ARF2-1–8–specific TCD8+, respectively, by IFN-γ ICS. (C) Synthetic peptide titration curves for NP-147–155– and ARF2-1–8–specific TCD8+. (D) P815 cells were infected with PR8 at 10 MOI for 1 h. Cells were washed and added to NP-147–155–specific (triangles) or ARF2-1–8–specific (square) TCD8+ lines, with BFA added at specific time points postinfection and T cell lines assessed by IFN-γ ICS for peptide presentation kinetics. Max IFN-γ response determined by synthetic peptide is shown in (C). All experiments are representative of at least two independent experiments.
Together, these results demonstrate that the ARF21–8 epitope is generated through the translation of the ARF2 of the NS1 mRNA and that mRNA splicing plays, at most, a minor role in generating this peptide.

**ARF21–8 epitope is also presented when the intronic sequence from NS is cloned into eGFP gene**

Given the location of ARF2 1–8 epitope in the NS gene intron, we investigated whether its synthesis is influenced by regulatory sequences outside the intronic sequence by transplanting the NS-intronic sequence into the eGFP gene (Fig. 4Aiii). The construct was then used to transduce D2SV cells, and transduced cells were enriched based on mCherry expression. Interestingly, the ARF2 1–8 epitope was generated more efficiently by eGFP-NS intron–transduced cells than those transduced with NS-full (Fig. 4B), showing that ARF21–8 is generated in its local translational context. Again, when the splicing sites were eliminated in the eGFP-NS intron, the generation of ARF21–8 epitope was not affected.

**ARF21–8 epitope presentation from autoantigen eGFP-NSintron is enhanced by IAV infection**

Although the ARF21–8 epitope was generated from D2SV cells transfected with full-length NS gene or transduced with eGFP-NS intron, in which either the NS or NS-intronic sequence is essentially converted to a self-gene by chromosomal integration, its presentation was clearly less efficient than in the context of IAV infection. We therefore questioned whether such ARF DRiP generation is enhanced with IAV infection.

To explore this, we generated the above-mentioned transfectant and transductant in the P815 cell line expressing either full-length NS or eGFP-NS intron and then superinfected these cells with Pan09 IAV. As this IAV strain does not generate ARF2 1–8 from its own genome, all detected ARF2 1–8 is derived from the autoantigens expressed in these cell lines. As shown in Fig. 4C, Pan09 IAV infection clearly enhanced ARF21–8 presentation, especially from cells that expressed eGFP-NS intron. Again, such enhanced presentation of endogenous DRiPs did not require mRNA splicing as the epitope presentation was equally efficient in the splicing mutants. The infection by Pan09 IAV was as efficient as those by PR8, as shown by equal NP147–155 epitope presentation (Fig. 4D). **ARF21–8 epitope is a bona fide DRiP as no biological function was detectable for ARF21–14**

As very short peptides can have significant biological functions (39), to formally exclude the possibility that ARF21–14 functions in IAV infection, we generated ARF21–14 KO IAV viruses using the reverse genetics approach (24). We then compared the mutant IAV to a control wt recombinant generated in parallel for in vitro infectivity and ability to trigger immunopathology and cellular immunity in two mouse strains. First, we infected the

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**FIGURE 3.** Polyfunctional ARF21–8–specific T cell responses occur in IAV-infected mice. (A) BALB/c mice were i.p. infected with $5 \times 10^6$ to $2 \times 10^8$ PFU of PR8 IAV. Seven days later, i.p. wash cells were isolated and enumerated for T cell Ag specificity using synthetic peptides by IFN-γ ICS. BALB/c mice were i.n. infected with 100 PFU of PR8 or $10^7$ PFU of Pan09 (both H1N1) IAV. Ten days later, (B) bronchoalveolar lavage and (C) splenic cells were isolated and enumerated for T cell Ag specificity using synthetic peptides by IFN-γ ICS. (D) IFN-γ+ (i) NP147–155+ and (ii) ARF21–8+–specific TCD8+ were assessed for TNF-α production and (iii) cell degranulation as measured by surface CD107a expression (n = 4 mice per group). Error bars indicate SEM. All experiments are representative of at least two independent experiments.

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3376 INFLUENZA A VIRUS INFECTION INCREASES DRiPs

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mouse lung epithelial adenoma cell line LA-4 (40) and measured the production of NP, M1, M2, and NS1 via flow cytometry with the appropriated mAbs. As shown in Fig. 5A (i–iv), LA-4 cells infected with wt or the ARF2 mutant virus generated equal amounts of each of the proteins measured, indicating that the small ARF is not required for viral mRNA synthesis or translation. Further, when we infected P815 cells with wt versus mutant viruses and characterized the transcriptome and proteome, we detected only very minor differences. Only 12 out of 13,167 genes and two out of 4419 proteins were differentially expressed. Moreover, the differentially detected genes and proteins did not overlap between replications, and no biological signaling pathways were overrepresented (Supplemental Fig. 3A, 3B).

Second, when we infected C57BL/6 and BALB/c mice i.n. with 100 PFU with wt or mutant viruses, mice showed identical weight loss profiles, indicating that short ARF is not required for viral pathogenesis. Finally, we assessed the cellular immune responses following i.n. infection of mice. In C57BL/6 mice, we found a nearly identical TCD8+ response to seven different peptides in spleen, with relatively minor variation in the BAL response to some peptides (Fig. 5B, 5D, 5F). Similarly, in BALB/c mice, with the obvious exception of the ARF21-8 response, there were only minor differences in splenic or BAL responses to six other peptides (Fig. 5C, 5E, 5G). Higher infecting doses of 500 PFU of ARF21-14 KO virus did not demonstrate any alteration in lethality or the resulting immune response (data not shown).

Taken together, our findings do not support an important biological role for ARF21-14 in the IAV infection of cultured cells or infected mice.

Discussion

In this study, we show that BALB/c mice mount a robust TCD8+ response to NS1-ARF21-8, which under some conditions, ascends to the top of the immunodominance hierarchy. We previously systematically searched for potential ARF epitopes presented by H-2Kb, Db, or Dd. Although 9 of the 35 synthetic peptides screened efficiently stimulated TCD8+ elicited with the autologous peptide, only a single peptide could stimulate or induce IAV-specific TCD8+ response for (B) determined by synthetic peptide: ARF21-8: 60%; NP147-155: 75%. All experiments are representative of at least two independent experiments.
ARF/ORF is >30 residues, which is of sufficient length to form a membrane spanning oligomer, or at the very least, associate with a viral or cellular protein to modulate its function.

NS1-ARF2 is but 14 residues. Although not impossible, it is unlikely that such a short peptide could be functional. A *Drosophila* 11-mer seems to have a function (13), and plant 18- and 20-mer microproteins were recently described to possess biological activity (48, 49). The shortest viral gene products with known biological activity are >40 residues. Out of >5000 NS1 sequences available online, only one (A/chicken/Jiangsu/IT34/2011[H9N2]) lacks the start codon of the ARF21–8 epitope. All others have sequences highly similar (or identical) to PR8 or Pan09. Interpreting this sequence conservation is muddied by the high conservation of the corresponding overlapping NS1 sequence, which encodes residues ~30–43 of the RNA-binding domain critical for sequestering IAV dsRNA during its replication (50, 51). To formally exclude the possibility that NS1-ARF21–14 might be an intended viral product with biological function, we generated NS1-ARF21–14 deletion mutant and its control wt IAV virus by reverse genetics. In our parallel in vitro, in vivo, and systems biology experiments, we were not able to detect any function or influence of NS1-ARF21–14 during IAV infection. We therefore conclude that it is likely that NS1-ARF21–14 is a bona fide viral DRiP.

Although 14 residues may be too short to generate a functional gene product, we have not completely ruled out that NS1-ARF2 is synthesized as something more than a 14-mer. Our evidence conclusively demonstrates NS1-ARF21–14 deletion mutant and its control wt IAV virus by reverse genetics. In our parallel in vitro, in vivo, and systems biology experiments, we were not able to detect any function or influence of NS1-ARF21–14 during IAV infection. We therefore conclude that it is likely that NS1-ARF21–14 is a bona fide viral DRiP.

**FIGURE 5.** ARF21–8 is a nonfunctional DRiP. (A) LA-4 cells were infected with 10 MOI wtPR8 or ARF21–8 KO PR8 IAV and assessed for (i) NP, (ii) M1, (iii) M2, and (iv) NS1 protein production by flow cytometry. (B) C57B6 and (C) BALB/c mice were infected i.n. with 100 PFU of wtPR8 or ARF21–8 KO PR8 IAV, and daily body weight was recorded. Following 10 d, (D and E) BAL wash and (F and G) splenic cells were isolated and enumerated for T cell Ag specificity using synthetic peptides by IFN-γ ICS (*n* = 4 mice per group). Error bars indicate SEM. All experiments are representative of at least two independent experiments.
little room upstream of NS1-ARF2 for frame-shifting, with only a Val residue before encountering a stop codon and no known frame shift abetting sequence. Although it seems unlikely, it is possible that the NS1-ARF2 is extended by readthrough of the stop codon or by shifting back into the NS1 frame between residue 8 and the stop codon after residue 14.

Our findings clearly demonstrate the potential importance of unusual translation events in generating biologically important viral peptides, extending the recent exciting evidence for the potential involvement of nuclear translation in generating viral (36, 52) and cell peptides, in the latter case from intronic regions (37). More importantly, we further demonstrated that IAV infection enhances cellular ARF DRiP generation, a finding very similar to that recently reported by Prasad et al. (53), in which the generation of a model T cell epitope translated via alternative start codon was enhanced by viral infection. These findings may help us to further understand the relationship between virus infection and autoimmunity. Perhaps virus infection could trigger autoimmunity by simply increasing DRiP Ag presentation?

Pragmatically, it is important to reconsider strategies for identifying immunogenic and antigenic peptides. In our own specific circumstances, we would have found NS1-ARF2$_{1-8}$ had we synthesized all potential ARF peptides at their predicted full length, using serum proteases to liberate antigenically active fragments. Such an approach would have allowed us to define complete immunodominance hierarchies of CD8$^+$ T-cell responses to influenza virus, extending the voluminous evidence from studies of peptide generation in cultured cells.

Disclosures
The authors have no financial conflicts of interest.

References


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