Title:Expanding specificity of class I restricted CD8⁺T cells for viral epitopes following multiple inoculations of swine with a human adenovirus vectored foot-and-mouth disease virus (FMDV) vaccine.

Lasse E. Pedersen^{1,2}, Jared R. Patch^{1,3}, Mary Kenney¹, Raisa A. Glabman^{1,3}, Morten Nielsen^{2,4}, Gregers Jungersen², Soren Buus⁵, and William T. Golde¹.

¹Plum Island Animal Disease Center, Agricultural Research Service, USDA, Greenport, NY, USA; ²National Veterinary Institute and Centre for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark; ³ Department of Animal and Veterinary Sciences, University of Vermont, Burlington, VT, USA; ⁴ Instituto de Investigaciones Biotecnológicas, Universidad Nacional de San Martín, San Martín, Buenos Aires, Argentina; ⁵ University of Copenhagen, Copenhagen, Denmark.

Type of article:Original Article.

Abstract

The immune response to the highly acute foot-and-mouth disease virus (FMDV) is routinely reported as a measure of serum antibody. However, a critical effector function of immune responses combating viral infection of mammals is the cytotoxic T lymphocyte (CTL) response mediated by virus specific CD8 expressing T cells. This immune mechanism arrests viral spread by killing virus infected cells before new, mature virus can develop. We have previously shown that infection of swine by FMDV results in a measurable CTL response and have correlated CTL killing of virus-infected cells with specific class I major histocompatibility complex (MHC) tetramer staining. We also showed that a modified replication defective human adenovirus 5 vectorexpressing the FMDV structural proteins (Ad5-FMDV-Tvaccine) targets the induction of a CD8⁺ CTL response with a minimal humoral response. In this report, we show that the specificity of the CD8⁺ T cell response to Ad5-FMDV-T varies between cohorts of genetically identical animals. Further, we demonstrate epitope specificity of CD8⁺ T cells expands following multiple immunizations with this vaccine.

Introduction

Foot-and-mouth disease is a highly contagious viral infection of livestock causing significant loss of meat and milk productivity and restriction of trade of livestock in endemic countries. Many strains of the virus cause myocarditis and death, particularly in young animals. Control and eradication of this disease is a high priority to enable smallholder farmers in developing countries to have a reliable source of protein for consumption as well as supplemental income. In disease free countries, such progressive control reduces the risk of accidental or purposeful introduction of FMDV into the naïve herds and the economic impact of such an event (Grubman and Baxt, 2004).

Present killed virus vaccines have limited value in eradication efforts due to narrow protection across the many clades of viral strains and minimal duration of immunity. The recently licensed virus vectored vaccine for FMDV utilizes replication defective human adenovirus 5 to deliver an “empty capsid” (Moraes et al., 2002 and Grubman et al., 2010). This recombinant vaccine suffers the same performance shortfalls as the widely used killed virus vaccine. However, this vaccine platform presents an intriguing opportunity for addressing these performance deficits.

We have developed bioinformatic algorithms for the human leukocyte antigens (HLA) (*NetMHCpan*) to predict peptides within any pathogen proteome that hold the potential to be T cell epitopes within a given individual (Nielsen et al., 2007, Nielsen et al., 2008, Zhang et al., 2009, and Lundegaard, et al., 2011). This is based on the HLA molecules of that individual to bind pathogen derived peptides and “present” those to T cells. We have now applied this technology to the homologues of these proteins in both swine (swine leukocyte antigens; SLA) (Pedersen et al., 2011 and Pedersen et al., 2013) and cattle (bovine leukocyte antigens; BoLA) (Hansen et al., 2014, Pandya, et al., 2015, Svitek, et al., 2015). Analysis of the proteome corresponding to structural proteins (capsid) of FMDV, strain A24, identified 60 potential T cell epitopes for CD8⁺, cytotoxic T lymphocytes (CTL) predicted to be bound by two swine class I SLA proteins. Biochemical analysis determined that 20 of the 60 potential T cell

epitope peptides were efficiently bound by the swine SLA proteins (Pedersen et al., 2011 and Pedersen et al., 2013).

We altered the adenovirus vectored FMDV vaccine to target CTL responses by eliminating the 3C protease, the enzyme that processes the primary polypeptide product of the FMDV structural genes into the final protein components of the capsid (Patch et al., 2011). This new construct was termed Ad5-FMDV-T, as the unprocessed polypeptide was proposed to be targeted to the proteasome for digestion into peptides. Because this vector has the processing protease mutated, more than 95% of the P1 precursor protein remains unprocessed and this becomes a substrate for ubiquitination and degradation by the proteasome. The result of this is the generation of FMDV P1 peptides that can be bound by newly synthesized class I MHC (SLA-1, SLA-2 and SLA-3) proteins. Once the trimolecular complex of SLA alpha chain, beta-2 microglobulin (β_2m) and the peptide forms, the protein can be transported via the golgi apparatus and expressed on the cell surface. This cell now can induce a response from FMDV specific CD8⁺ T cells, and with other signals from primarily antigen presenting cells, expand that FMDV specific, CD8⁺ T cell population (Yewdell and Haeryfar, 2005, and Rock et al., 2010).

Following vaccination of genetically defined pigs (NIH mini) with the Ad5-FMDV-T vaccine, we have previously documented MHC restricted, antigen specific CTL killing of virus infected target cells specific for FMDV (Patch et al., 2011). These trials showed limited detection of T cell specificity using class I MHC tetramers, usually 2 or 3 of the twenty identified potential T cell epitope specificities. We further showed that animals infected with FMDV developed a clear and measurable CTL response, confirming the results generated using the altered Ad5-FMDV-T vaccine construct (Patch et al., 2013).

Here, we present further analysis of the fine specificity of reacting T cells from MHC homozygous pigs using MHC tetramers to characterize T cell specificities at a single cell level. The methodology was refined during these studies, leading to more sensitive and efficient detection of T cells. Results show that with each boost, the antigen specificity of T cells at this level of detection, expanded. Further, the specificities observed in the different trials were different, even though the genetics of the responding animals were identical in all trials and the

same lot of vaccine was used throughout these studies. These differences may indicate immunological history can influence specificities upon exposure to new antigens. This data supports the concept that targeting T cell responses, at least under a protocol of repeated vaccination, results in the antigen reactivity broadening within population of responding T cells over the course of vaccine application.

Materials and methods

Animals. National Institute of Health miniature (NIH mini) pigs, homozygous for MHC haplotype 4a.0/4a.0 (*0401) (previously d/d) were provided by Scott Arn and David Sachs (Transplantation Biology Research Center, Massachusetts General Hospital, Boston, MA). Animals ranged in age between 10 and 14 months, and weighed approximately 100 lbs. Experiments were performed in a secure biosafety level three laboratory on Plum Island Animal Disease Center (PIADC) with approval by the Institutional Animal Care and Use Committee, and according to guidelines of the United States Public Health Service, Department of Health and Human Services and the Animal Plant Health Inspection Service (APHIS) of the USDA.

Vaccinations. Animals described as vaccinated, boosted and then challenged with FMDV (shown in figure 1) were vaccinated as previously reported (Patch et al., 2013). Briefly, animals in this cohort (Cohort 1) were vaccinated with 2×10^9 PFU per animal and Ad5-FMDV-T at 5×10^9 per animal, intradermally with the DermaVac, needle free vaccination device. These animals were boosted 12 weeks after vaccination. Eight days following the boost, animals were challenged with 10^5 TCID₅₀ of FMDV, strain A24 Cruzeiro, by intradermal needle inoculation in the heel bulb (Patch et al., 2013).

In the subsequent experiments (Cohorts 2 and 3), all animals were vaccinated with Ad5-FMDV-B, “empty capsid” vaccine at 2×10^9 PFU per animal and Ad5-FMDV-T at 5×10^9 per animal, intradermally with the DermaVac, needle free vaccination device (Patch et al., 2013). Boost vaccinations were done identically as the first vaccination at the times indicated for each experiment. When to do boost vaccinations was determined by the antibody titer to Adenovirus. These titers needed to return to low levels so that circulating anti-adenovirus antibody did not

interfere with boost vaccinations. This timing varied between experiments.

Preparation of peripheral blood mononuclear cells (PBMC). Porcine blood was collected in Vacutainer® tubes containing heparin, then diluted with PBS and underlain with Lymphoprep(Axis-Shield, Oslo, Norway). After centrifugation at 1400 X g for 20 min at room temperature, the band of PBMC was removed and washed three times with PBS. Cells were finally suspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS and antibiotics/antimycotics.

CTL killing assay. Measurement of CTL cytolytic activity was carried out according to previously published procedures (Patch et al., 2011 and Patch et al., 2013). PBMC were cultured in the presence of the porcine kidney cell line, PK(15) cells infected with Ad5-FMDV-T for 3 days. PK(15) cells are class I SLA homozygous and match the SLA-1 haplotype of NIH d/d mini pigs. Following this stimulation, PBMC were gradient purified with Lymphoprep as described above, and then CD6⁺ cells were positively selected by incubating cells with anti-porcine CD6 antibody (clone MIL8, AbDSerotec, Raleigh, NC) followed by goat anti-mouse antibody coated microbeads, and then passaged through an LS column using the MidiMACS™ magnetic separator (MiltenyiBiotec, Gladbach, Germany). Purified CD6⁺ cells were incubated for 4 hr with target cells, PK(15) stably transfected with green fluorescence protein (PK(15)-EGFP cells) that had been infected overnight with Ad5-FMDV-T, Ad5-VSV-G, or mock infected. Effector and target cells were plated in E:T ratios of 50:1, 25:1, and 12:1, and incubation took place in the presence of 7-amino-actinomycin D (7-AAD) (BD Biosciences, San Jose, CA). Following incubation, percent killing was determined using flow cytometry by gating on EGFP⁺ cells and then measuring the percent 7-AAD⁺ cells. Flow cytometry was done using a FACS-Calibur flow cytometer with a high-throughput sampler (HTS) (BD Biosciences, San Jose, CA). Background lysis of target cells alone was subtracted from all data, and then for each time point and animal, the greatest mean lysis of control target cells (mock infected or Ad5-VSV-G infected) was subtracted from mean lysis of the antigen-specific target cells.

Tetramer construction and staining. Peptides and recombinant SLA-1*0401 and human beta-2-microglobulin (hβ₂m) were produced and complexed into tetramers as described previously

(Leisner et al., 2008 and Pedersen et al., 2011) with minor modifications. A list of peptides used to make MHC class I tetramers is given in Table I. Briefly, recombinant hβ₂m and peptides were added to 10-fold and 40-fold excesses, respectively, of SLA-1*0401 in a reaction buffer containing a cocktail of protease inhibitors [0.68mM EDTA, 4.8 μM pepstatin A, 94.4 μM 1-10 phenanthroline, 0.1 mM N-ethylmaleimide, 8.9 μM Tosyl-L-lysine chloromethyl ketone (TLCK), 9.3 μM Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 66.8 μM phenylmethylsulfonylfluoride (PMSF)] followed by the addition of streptavidin-PacificBlue (SA-PB), streptavidin-Allophycocyanin (SA-APC) (BD Biosciences, San Jose, CA) or streptavidin-Brilliant Violet (SA-BV-421) (Biolegend, San Diego, CA) for generating and labeling tetramers. The changes in the choice of fluorophore over the course of these experiments reflect the laser upgrades for the flow cytometer (B/D LSR II, San Jose, CA) and new fluorophores becoming available with stronger signal and better compatibility with other reagents being used.

Tetramer quality control.

All tetramer preparations were tested in ELISA before use in flow cytometry. 96 well plates were coated with mouse-anti-porcine SLA class I (AbDSerotec) diluted 1:500 in 1X carbonate buffer at 50 μl/well (final concentration; 0.5 μg/ml) and for control wells, mouse IgG1, κ isotype control diluted 1:250 (final concentration; 2 μg/ml). Plates were incubated overnight at 4 °C, then blocked with 5 % dried skim milk (100 μl/well) for 1 hour at 37 °C. Plates were washed in 1xPBS with Tween (0.05 %) (PBST). Tetramer samples were pre-diluted 1:20 in 1xPBS with PBST and added to respective wells (50 μl/well) and incubated at 37 °C for 1 hour. Plates were washed twice followed by mAb anti-beta2m (AbDSerotec) with an HRP label diluted 1:1000 in 1xPBST at 50 μl/well and incubated for 1 hour at 37 °C. Sureblue Substrate (KPL) was added to each well, 50 μl/well, and incubated at RT for 15 – 20 min. The reaction was stopped by adding 50 μl/well of 1M H₂SO₄ and plates read at 450 nm/ 570 nm.

The null tetramer. As a baseline control for the tetramer staining, we developed the null tetramer. This is a tetramer which binds and presents a synthetically designed peptide with strong SLA-1*0401 and SLA-2*0401 binding residues at position 2 and 9 and residues lacking side chains in most other positions of the nine amino acid peptide ligand. The sequence of the null peptide used

to **analyze cohort 1 and cohort 2**(832) is: ASYAGAGAY and has affinities of 40 and 117 nM for SLA-1 and -2, respectively. For experiments analyzing **cohort 3**, we used a peptide ligand with higher affinity, 833, ASYGAGAGY, that is bound with an affinity of 19nMfor SLA-1 *0401 and 60 nMfor SLA-2 *0401.

Gating Strategy for tetramer staining. CD6 enriched PBMC from the CTL killing assay were stained for the different tetramers under study. For tetramer staining, 2×10^5 to 5×10^5 cells from the CD6 column (see killing assay above) were washed with cold FACS buffer (PBS, 0.3% bovine serum albumin, 0.1% sodium azide) and resuspended in solution containing tetramer previously mixed 1:1 with 2x FACS buffer. Cells were incubated at room temperature with rocking for 20 min and then washed three times in cold FACS buffer. Cells were resuspended in FACS buffer containing PE-conjugated mAb against porcine CD8 α (clone 76-2-11, BD Pharmingen) and FITC-conjugated mAb against porcine CD4 (clone 74-12-4, BD Pharmingen) for additional cell surface staining, followed by incubation at room temperature for 20 min. In the final cohort of animals, we added live/dead staining, Live/Dead Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Molecular Probes). Each lot of the dead cell stain was titrated for use in these assay. After being washed three times in FACS buffer, cells were fixed in PBS containing 1% formaldehyde and stored at 4°C until being analyzed by flow cytometry.

In a 2D plot of forward and side scatter, the lymphocyte population was selected, followed by gating on single cells in a 2D plot of forward scatter area verses forward scatter height. Within the single cell population, we gated on CD4⁻/CD8 α ⁺. In the second and third trial, we gated on live cells before gating on the CD8 α ⁺ T cells. We then gated on the fluorophore that was used for making the tetramer for that study, APC, Pacific blue or in the final study, BV-421(an example of the gating is shown in Supplemental Figure 1). In the figures we show both the no tetramer control and the null tetramer control. The no tetramer control always yields an artificially low signal as there is no fluorophore present. Positive staining reported in Table II is statistically significant relative to the null tetramer.

Statistical analysis of tetramer staining. Statistical significance of tetramer staining was determined for each group of animals using the Students t test where:

$$t = \frac{\text{mean of (\% tetramer/FMDV peptide staining - \% null tetramer staining)}}{\sqrt{n}}$$

A t value greater than 2 confirms statistically significant staining for that tetramer in that cohort of animals.

Results

Antigen specificity in animals vaccinated with Ad5-FMDV-T and challenged with live virus.

Our previous work in swine demonstrated antigen specific cytotoxic T cell killing of infected target cells by CD8 expressing T cells from peripheral blood of infected animals, 21 days after infection. In this report, we also describe the induction of FMDV specific CTL responses using the Ad5-FMDV-T vector to deliver the unprocessed precursor polypeptide of the FMDV, strain A24 structural proteins, termed FMDV P1 (Patch et al., 2011). In addition, PBMC from all swine vaccinated with Ad5-FMDV-T vaccine (**cohort 1**) showed virus specific CTL killing as well, 21 days after vaccination, and that response was increased following boost and again following live virus challenge four days following the boost with FMDV, strain A24. Finally, all tetramer staining data reported is restricted to samples from pigs that also showed CTL killing from the same sample.

Peripheral blood samples from these animals were also analyzed immediately *ex vivo* for antigen specificity using the class I MHC tetramer technology. PBMC from swine were stained for expression of CD4 and CD8 and gated on the CD4⁻/CD8⁺ cell population (Supplemental Figure 1). This was then analyzed to determine the percentage of tetramer-positive cells using tetramers made with peptides corresponding to the FMDV structural proteins and the swine class I protein, SLA-1*0401. Data in figure 1 show the reactivity of 3 class I FMDV tetramers and the null tetramer, 832. All animals in **cohort 1** were reactive with the peptide 121 at 21 days following vaccination. No animals reacted with peptide 120 after vaccination while four of the animals reacted with the FMDV 124 peptide and three others did not. Non-specific, background staining using the null tetramer was consistently around 1% of the CD8 expressing T cells in these

assays. These results were recorded in spite of the fact that these pigs are genetically identical. Following boost and challenge, all animals in cohort 1 remained negative for reactivity with peptide 120 and all animals were now reactive to both peptides 121 and 124, relative to staining with the null tetramer. This was the first indication that repeated exposure to FMDV P1 derived peptides could expand the peptide specific response of CD8⁺ T cells.

Statistical analysis of this tetramer reactivity for the entire cohort showed significant staining with SLA-1 121 after vaccination and with both SLA-1 121 and SLA-1 124 following challenge (Table II).

FMDV peptide specificity following repeated combined vaccination with Ad5-FMDV-B and Ad5-FMDV-T.

We tested the hypothesis that vaccination with Ad5-FMDV-T lacks optimal induction of FMDV specific, CD4 helper T cell responses as the design of the vaccine was to target the P1 protein to the proteasome for degradation and subsequent loading of peptides into SLA class I proteins. Vaccination with intact capsids leads to antigen uptake and processing by dendritic cells and other antigen presenting cells and subsequent stimulation of CD4 helper T cells via SLA class II proteins expressed by the antigen presenting cells. We proposed that combining the two vaccines would optimize the CTL response due to more optimal helper T cell responses provided by the empty capsid. In a preliminary analysis, 2 animals were vaccinated with the combination of the parent Ad5-FMDV-B vaccine, in which the structural proteins are processed into “empty capsids” by a functional protease, and the Ad5-FMDV-T vaccine, in which the P1 polyprotein is not processed, showed strong, CD8⁺ CTL responses. This CTL killing response was notably higher than in animals receiving the Ad5-FMDV parent vaccine alone (B cell vaccine) or the Ad5-FMDV-T vaccine alone (T cell vaccine) (Supplemental Figure 2).

In addition, before the next experiment analyzing the response of five MHC (SLA), homozygous mini pigs to combined vaccination, we synthesized the SLA-2*0401 protein and analyzed the peptide binding characteristics of this new molecule (Pedersen et al., 2013). This added four new peptide specificities for us to test when analyzing this new cohort.

Following vaccination with the combined Ad5-FMDV-B and Ad5-FMDV-T constructs, all animals in cohort 2 showed killing of target cells 14, 18 and/or 28 days following initial

vaccination (Supplemental figure 3A). Analysis of CD8⁺ T cells from cohort 2, 28 days after vaccination showed four of the five animals stained with the SLA-1 124 tetramer when compared to the SLA-1 832, null tetramer (Figure 2A). No animals had reactivity with any of the SLA-2 tetramers following initial vaccination.

These animals received a boost at 6 weeks after the first vaccination, and we again assessed the killing of infected target cells by CTL. The levels of target cell killing were detectable at day 7 in four of the five animals and all animals in cohort 2 showed CTL killing by 14 days after the boost vaccination. All but one animal showed decreased killing at 21 days after the boost (Supplemental Figure 3A), when the experiment was terminated.

Analysis of tetramer staining on day 7 and day 14 following the boost showed generally increased background with the null tetramers, SLA-1 832 and SLA-2 832. Samples from day 7 showed reactivity with SLA-1 124, as was detected after the first vaccination and that reactivity was the same or slightly less at day 14 (Figure 2B and 2C). Little reactivity was seen with any of the SLA-2 tetramers at day 7 after boost. All but one of the animals (#32) showed increased reactivity with SLA-1 312 at 14 days after boost. There was also increased reactivity with SLA-2 tetramers at day 14 including marginal staining with SLA-2 307 in all animals except animal 66 and 35 reacting with SLA-2 306. CD8⁺ T cells from animal 860 reacted with SLA-2 309 and animal 66 had marginal reactivity with SLA-2 310 at day 14 after the second vaccination. However, only SLA-1 124, SLA-1 312, and SLA-2 307 showed statistically significant staining at day 14 after the boost. Further, the SLA-2 832 null tetramer reactivity analyzing CD8⁺ T cells from animal 66 on day 7 was completely different than the other animals and the reactivity of that animal at day 14 indicating this to be an aberrant result. Even with these caveats, these data once again indicated that following a second vaccination with Ad5-FMDV-T in combination with Ad5-FMDV-B, the specificity of the CD8⁺ T cell response may be expanding. These results are summarized in Table II and show the expansion of reactivity of T cells from these animals following the boost.

Expansion of peptide specificities and analysis of T cell specificities after multiple vaccinations.

Before the final experiment, we updated the NetMHC-pan program with the results from these and other analyses. In addition, we initiated analysis of bovine leukocyte antigen (BoLA)

class I proteins for peptide binding, and that data was also used to update NetMHC-pan (Hansen et al., 2014 and Pandya et al., 2015). The updated algorithm identified multiple peptides from the precursor polyprotein, P1, of FMDV strain A24 that had not been detected in our earlier analysis. We tested SLA-1*0401 and SLA-2*0401 for binding these peptides and identified several new potential T cell epitopes. We now had 7 peptides bound by SLA-1*0401 and 8 peptides bound by SLA-2*0401 (Pedersen et al., 2013). In addition, we identified an improved null peptide with higher affinity for both SLA-1*0401 and SLA-2*0401. We anticipated that by using a new, stronger fluorophore, BV-421, this improved null peptide would allow more readily detected staining of FMDV specific CD8⁺ T cells in our system.

For the final cohort (cohort 3), we vaccinated four NIH mini pigs homozygous for SLA-1, SLA-2, and SLA-3*0401 with the combination of Ad5-FMDV and Ad5-FMDV-T as in the previous experiment. We then boosted all of the animals twice at 2 months and 6 months following the first vaccination. We analyzed the induction of CD8⁺ T cells to CTL killing of virus infected target cells following each vaccination. In supplemental figure 3B, we show that PBMC from all animals in this experiment had cytotoxic T cell killing activity following each vaccination.

We used the SLA class I tetramers, formed with the FMDV derived peptides, to analyze the T cell specificities of CD4⁻/CD8⁺ T cells from the PBMC of **animals in cohort 3** following each vaccination. Data in figure 3A show that peripheral blood T cells from all four animals reacted with the same three tetramers following the first vaccination, SLA-1 164, SLA-1 169 and SLA-2 173 as compared with the null tetramers SLA-1 833 and SLA-2 833. After the second vaccination at two months, all four animals showed the similar fine specificity of CD8⁺ T cells, reacting with the SLA-1 164, SLA-1 169 and SLA-2 173 tetramers. In addition, this cohort now had circulating CD8⁺ T cells reacting with six more tetramers, showing an expansion of T cell specificity (Table II).

Interestingly, when we vaccinated a third time, six months following the first vaccination, we saw expansion of the CD8⁺ T cell specificity in all four animals. T cells from all of these animals maintained reactivity with eight of the tetramers from the first boost (see Table II).

Staining CD8⁺ T cells from these samples no longer stained with SLA-2 119 and SLA-2 307 with statistical significance, but reactivity with SLA-1 312 was now significant. With the larger pool of FMDV derived peptides analyzed, the data in this last experiment confirms that multiple vaccinations with the Ad5-FMDV-T vaccine leads to expansion of the CD8⁺ CTL antigen specificity in these MHC homozygous pigs.

Discussion

The studies reported here are unique in that we tested a vaccine that was designed to induce a cytotoxic T cell response. Following the results we previously reported (Patch et al., 2011 and Patch et al., 2013), we optimized the vaccine strategy for the best adaptive immune response and we incorporated the new data into our bioinformatics analysis of potential T cell epitopes. This expanded our ability to follow T cell specificities from a few viral peptides (Patch et al. 2011) to a total of 60 predicted epitopes from over 1400 possible 9 and 10 amino acid peptides from the FMDV structural proteins. Of these, 15 were shown to be bound by the two class I MHC proteins we studied. Thus, these viral derived peptides were physically capable of being T cell epitopes in the animals expressing these MHC proteins, NIH *0401 minipigs.

Results of these analyses showed expansion of reactivity in all of the trials reported in this manuscript. In trials illustrated in figure 1 and figure 3, the tetramer staining was strong as compared to the null tetramer, i.e. tetramers assembled with peptides with no amino acid side chains for interaction with the T cell receptor. The trial shown in figure 2 had much lower reactivity in general, yet the trend of T cell staining in this trial also showed expansion with multiple vaccinations. Remarkably, within the group of animals in any given trial, the specificity was similar if not identical (n=7, n=5, and n=4). (Figures 1 through 3, Table II).

These trials were conducted over the course of a number of years and this allowed the refined analysis to include more and more peptide specificities as the bioinformatics matured during the course of this work. As a result, the early trials already published (Patch et al., 2011) analysis included only a few FMDV derived peptides using a single SLA protein, SLA-1 *0401. At the time we conducted the final trial, a total of 15 peptides were determined by biochemical analysis to be bound by the SLA class I proteins, SLA-1*0401 and SLA-2*0401, from the pool

of FMDV derived peptides that were predicted to be bound. SLA-1*0401 and SLA-2*0401 are the MHC class I proteins. We were then able to analyze all 15 tetramers based on the bound FMDV peptides in the final trial.

Results presented here suggest a hypothesis that the specificity of T cell responses following vaccination is strongly influenced by previous exposure to multiple protein antigens from the environment, from infections passing through the herd and from the standard vaccines the animals received as a matter of normal, approved animal care. The husbandry involved with these animals was such that each group of animals was housed together from the time of weaning. Litters from NIH minipigs sows are usually small, 1 to 3 piglets, so litters were combined to create groups of 6-10 animals, gender sorted, of similar age and of the same genotype. Once groups were arranged, animals were kept separate from other groups until entry into a study. Further, due to logistics, these animals were not entered into any of the studies reported here until age 10 to 14 months. This means that these animals had at least a year of environmental exposure in high security barns with daily traffic of animal caretakers and maintenance personnel. Finally, these trials were carried out over 4 years and a new cohort of animals was not born when the previous cohort was transferred to PIADC for vaccination, further separating their specific exposures. Therefore, the immunological history of these separate groups of pigs was uniquely different.

This hypothesis is consistent with these results reported here, that these different immunological histories predisposed the fine specificity of the T cell response in these animals that we report here. The response in the context of this “normal” immunological experience is therefore not uniquely a primary T cell response, but may involve cross reactivity with antigens from various sources previous to entry into these vaccine studies. This is rarely seen in mouse studies, as they are not only more genetically limited, but raised in extremely clean environments and routinely used within weeks of birth for study, severely limiting any immunological history. This is also rarely analyzed in humans due to genetic diversity of the responder population, but technologies are evolving that should make such analyses possible.

Results from these trials will give vaccine scientists new information to consider in vaccine design. First, design of vaccine vectors to provide proteins that can be targeted for degradation in the proteasome and result in peptides being loaded into newly synthesized, class I MHC proteins can stimulate a strong CD8⁺ T cell response. While we did not investigate the cellular physiology to formally demonstrate the non-processed FMDV P1 polypeptide was polyubiquitinated, leading to degradation via the proteasome, the induction of class I MHC restricted T cell responses in all animals receiving the vaccine are consistent with that prediction. While formal demonstration of proteasome degradation will provide important supporting data for this conclusion, the definitive data is the induction of a class I MHC restricted T cell response that we report here, in combination with prior work (Patch et al., 2011 and Patch et al., 2013).

Further, we show that repeated vaccination with these constructs, providing processed empty capsid in combination with the unprocessed polypeptide sourcing peptides for incorporation into MHC class I proteins, shows expanding T cell reactivity (Table I). B cells that are stimulated by antigen undergo affinity maturation within the germinal centers of lymph nodes, during which cells expressing high-affinity antibodies are selected for survival (Talmadge, 1957, Tonegawa, 1988, Cruse and Lewis, 1994, and reviewed by Allen et al., 2007, Victora and Mesin, 2014, and De Silva and Klein, 2015). The resulting antibody repertoire is relatively narrow with high affinity. These data suggest a method of targeting vaccines to broaden the reactivity to antigens following multiple vaccinations by activating more CTL precursors with broader specificity. We are actively pursuing studies to test the relative breadth of B cell specificity in a field setting in cohorts of animals vaccinated multiple times, but a formal study awaits funding.

Regardless, the data presented here are consistent with the T cell response expanding fine specificity following multiple vaccinations with the Ad5-FMDV-T vaccine construct. **Protection against FMD following multiple vaccinations with the standard killed virus vaccine seldom shows any cross-protection between strains of the virus (reviewed by Grubman and Baxt, 2004). Vaccination with Ad5-FMDV-B alone is likely to yield similar results. Demonstration that protection following multiple vaccinations with this vaccine formulation, Ad5-FMDV-B in combination with Ad5-FMDV-T, shows broader protection would require challenge following**

multiple vaccinations of cohorts of animals with homologous and other cohorts with heterologous strains. These will be very informative studies.

These data provide analysis of a unique approach to FMDV vaccines and viral vaccines in general. By combining the Ad5-FMDV-B empty capsid vaccine with the Ad5-FMDV-T vaccine, the safety of the replication defective viral vector is combined with the advantage of induction of a cellular immune response mediated by CD8⁺, MHC class I restricted T cells. The same advantage inherent in live, attenuated virus vaccines. This approach yields a more complete, and we propose, effective immune response. Testing for protection from infection as well as disease following vaccination with the combined vaccine will show whether there is improved effective immunity as well as duration of protection. Such vaccine trials are critical to confirm the value of this approach to vaccination.

Acknowledgements: The authors would like to thank Drs. David Sachs, Scott Arn and Mr. Robert Billiter of the Transplantation Biology Research Center, Massachusetts General Hospital, for supplying the NIH mini-pigs. We would also like to thank the staff at the Animal Resources Unit at Plum Island Animal Disease Center for their professional assistance in these studies. This work was funded by CRIS #1940-32000-053/57-00D from the Agricultural Research Service, USDA (WTG); by the Danish Council for Independent Research, Technology and Production Sciences (274-09-0281) (GJ); and by the United States National Institutes of Health (NIH) (HHSN266200400025C) (SB).

References:

- Allen, C.D., Okada, T., 2007. Cyster JG. Germinal-center organization and cellular dynamics. *Immunity*. 2:190-202.
- Cruse, J M and R E Lewis, Jr. 1994. David W. Talmage and the advent of the cell selection theory of antibody synthesis. *J Immunol* 1994; 153:919-929.
- De Silva, N.S., Klein, U., 2015. Dynamics of B cells in germinal centres. *Nature Reviews of Immunology*. 15:137-148.
- Grubman, M.J., Baxt, B., 2004. Foot-and-mouth disease. *ClinMicrobiol Rev*. 1:465-493.
- Grumban, M.J., Moraes, M. P., Schutta, C., Barrera, J., Neilan, J., ETTYREDDY, D., BUTMAN, B. T., BROUGH, D. E., BRAKE, D. A., 2010. Adenovirus serotype 5-vectored foot-and-mouth disease subunit vaccines: the first decade. *Future Virology* 2010 5:1, 51-64.
- Hansen, A.M., Rasmussen, M., Svitek, N., Harndahl, M., Golde, W.T., Barlow, J., Nene, V., Buus, S., Nielsen, M., 2014. Characterization of binding specificities of bovine leucocyte class I molecules: impacts for rational epitope discovery. *Immunogenetics*. 66:705-718.
- Leisner, C., Loeth, N., Lamberth, K., Justesen, S., Sylvester-Hvid, C., Schmidt, E.G., Claesson, M., Buus, S., Stryhn, A., 2008. One-pot, mix-and-read peptide-MHC tetramers. *PLoS One*. 3(2):e1678.
- Lundegaard, C., Lund, O., Nielsen, M., 2011. Prediction of epitopes using neural network based methods. *J. Immunol. Methods*. 374(1-2):26-34.
- Moraes, M.P., Mayr, G.A., Mason, P.W., Grubman, M.J., 2002. Early protection against homologous challenge after a single dose of replication-defective human adenovirus type 5

expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. *Vaccine* 20:1631–1639

Nielsen, M., Lundegaard, C., Blicher, T., Lamberth, K., Harndahl, M., Justesen, S., Røder, G., Peters, B., Sette, A., Lund, O., Buus, S., 2007. NetMHCpan, a method for quantitative predictions of peptide binding to any HLA-A and -B locus protein of known sequence. *PLoS One*. 2(8):e796.

Nielsen, M., Lundegaard, C., Blicher, T., Peters, B., Sette, A., Justesen, S., Buus, S., Lund, O., 2008. Quantitative predictions of peptide binding to any HLA-DR molecule of known sequence: NetMHCIIpan. *PLoS Computational Biology*. 4(7):e1000107.

Pandya, M., Rasmussen, M., Hansen, A., Nielsen, M., Buus, S., Golde, W., Barlow, J., 2015. A modern approach for epitope prediction: identification of foot-and-mouth disease virus peptides binding bovine leukocyte antigen (BoLA) class I molecules. *Immunogenetics*. 67:691-703.

Patch, J.R., Kenney, M., Pacheco, J.M., Grubman, M.J., Golde, W.T., 2013. Characterization of cytotoxic T lymphocyte function after foot-and-mouth disease virus infection and vaccination. *Viral Immunol*. 26(4):239-249.

Patch, J.R., Pedersen, L.E., Toka, F.N., Moraes, M., Grubman, M.J., Nielsen, M., Jungersen, G., Buus, S., Golde, W.T., 2011. Induction of foot-and-mouth disease virus-specific cytotoxic T cell killing by vaccination. *Clin. Vaccine Immunol*. 18(2):280-288.

Pedersen, L.E., Harndahl, M., Nielsen, M., Patch, J.R., Jungersen, G., Buus, S., Golde, W.T., 2013. Identification of peptides from foot-and-mouth disease virus structural proteins bound by class I swine leukocyte antigen (SLA) alleles, SLA-1*0401 and SLA-2*0401. *Anim. Genet*. 44(3):251-258.

Pedersen, L.E., Harndahl, M., Rasmussen, M., Lamberth, K., Golde, W.T., Lund, O., Nielsen, M., Buus, S., 2011. Porcine major histocompatibility complex (MHC) class I molecules and analysis of their peptide-binding specificities. *Immunogenetics*. 2011. 63(12):821-834.

Rock, K.L., Farfán-Arribas, D.J., Shen, L., 2010. Proteases in MHC class I presentation and cross-presentation. *J Immunol*. Jan 1;184(1):9-15.

Svitek, N., Awino, E., Nene, V., Steinaa, L., 2015. BoLA-6*01301 and BoLA-6*01302, two allelic variants of the A18 haplotype, present the same epitope from the Tp1 antigen of *Theileriaparva*. *Vet.Immunol. Immunopathol*. 167:80-85.

Tonegawa, S. 1988. Somatic generation of immune diversity. *Cell*. 8:3-26.

Talmadge, D.W., 1957. Allergy and Immunology. *Annual Review of Medicine*. 8:239-256.

Victoria G.D., Mesin, L., 2014. Clonal and cellular dynamics in germinal centers. *Current Opinion of Immunology*. 28:90-6.

Yewdell, J.W., Haeryfar, S.M., 2005. Understanding presentation of viral antigens to CD8+ T cells in vivo: the key to rational vaccine design. *Annual Review of Immunology*. 23:651-682.

Zhang, H., Lundegaard, C., Nielsen, M., 2009. Pan-specific MHC class I predictors: a benchmark of HLA class I pan-specific prediction methods. *Bioinformatics*. 25(1):83-89.

Figure Legends:

Figure 1. Analysis of peripheral blood CD8⁺ T cells from cohort 1 following vaccination with Ad5-FMDV-T, the day before challenge compared to 7 days following challenge (Patch et al., 2013) of 7 NIH minipigs homozygous for MHC. Staining for FMDV reactive T cells was carried out with four SLA-1 *0401 tetramers; 832, the null tetramer, and 120, 121, and 124, peptides from FMDV strain A24 P1 protein (see Table I). Four SLA-2 *0401 tetramers were also tested but all showed no staining (data not shown).

Figure 2. Analysis of peripheral blood CD8⁺ T cells from cohort 2 at day 28 following vaccination with Ad5-FMDV-B plus Ad5-FMDV-T (2A) and days 7 (2B) and 14 (2C) following boost with the same vaccine. Staining for FMDV reactive T cells was carried out with three SLA-1 *0401 tetramers; 832, the null tetramer, and 124 and 312, peptides from FMDV strain A24 P1 protein (see Table I) and five SLA-2 *0401 tetramers; 832, the null tetramer, and 306, 307, 309 and 310, peptides from FMDV strain A24 P1 protein (see Table I).

Figure 3. Analysis of peripheral blood CD8⁺ T cells from cohort 3 at day 28 following vaccination with Ad5-FMDV-B plus Ad5-FMDV-T (3A) and days 7 following boost with the same vaccine (3B) and 14 days following a second boost with the same vaccine (3C). Eight SLA-1 *0401 tetramers; 833, the null tetramer, and 120, 124, 162, 164, 169, 172 and 312, peptides from FMDV strain A24 P1 protein (see Table I) and eight SLA-2 *0401 tetramers; 833, the null tetramer, and 119, 120, 125, 173, 306, 307, 309 and 310, peptides from FMDV strain A24 P1 protein (see Table I).

Figure1

Tetramer Staining of CD8+ CTL's

Figure 2A

Animal 32

Animal 35

Animal 66

Animal 127

Animal 860

2B**Animal 32** **Animal 35** **Animal 66** **Animal 127** **Animal 860**

2C

Animal 32

Animal 35

Animal 66

Animal 127

Animal 860

Figure

3A

Animal 26

Animal 59

Animal 586

Animal 886

3B

3C

Animal 26

Animal 59

Animal 586

Animal 886

Table I. Peptides used to make tetramers with SLA-1*0401 and SLA-2 *0401.

Tetramer		
ID	Peptide sequence	Peptide origin
120	MTAHITVPY	FMDV A24 P1
121	SSVGVTHGY	FMDV A24 P1
124	NMTAHITVPY	FMDV A24 P1
162	RVVQAERFY	FMDV A24 P1
164	GSTDSKARY	FMDV A24 P1
173	YSGTINLHF	FMDV A24 P1
306	INNYYMQQY	FMDV A24 P1
307	TNTQNNDWF	FMDV A24 P1
309	SKFTFSIPY	FMDV A24 P1
310	DSYAYMRNGW	FMDV A24 P1
312	KVYANIPTY	FMDV A24 P1
832	ASYAGAGAY	No origin - Synthetic design
833	ASYGAGAGY	No origin - Synthetic design

Table II. Summary of tetramer reactivity with CD6 enriched CD8 cytotoxic T cells.

Experiment	Prime	Challenge	
Cohort from Figure 1	SLA-1 121*	SLA-1 121, SLA-1 124	
	Prime	Day 7 after Boost	Day 14 after Boost
Cohort from Figure 2	SLA 1-124	SLA-1 124, SLA-1 312	SLA-1 124, SLA-1 312, SLA-2 307
	Prime	First Boost	Second Boost
Cohort from Figure 3	SLA-1 164, SLA-1 169, SLA-2 173	SLA-1 120, SLA-1 124, SLA-1 162, SLA-1 164, SLA-1 169, SLA-2 119, SLA-2 120, SLA-2 173, SLA-2 307	SLA-1 120, SLA-1 124, SLA-1 162, SLA-1 164, SLA-1 169, SLA-1 312, SLA-2 120, SLA-2 173

*tetramers listed had statistically significant staining compared to the null tetramer.