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Tobacco Mosaic Virus Efficiently Targets DC uptake, Activation and Antigen-specific T Cell Responses *in vivo*

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Abstract

Over the past 20 years, dendritic cells (DCs) have been utilized to activate immune responses capable of eliminating cancer cells. Currently, *ex vivo* DC priming has been the mainstay of DC cancer immunotherapies. However, cell-based treatment modalities are inherently flawed due to a lack of standardization, specialized facilities and personnel, and cost. Therefore, direct modes of DC manipulation, circumventing the need for *ex vivo* culture, must be investigated. To facilitate the development of next-generation, *in vivo* targeted DC vaccines, we characterized the DC interaction and activation potential of the *Tobacco Mosaic virus* (TMV), a plant virus that enjoys a relative ease of production and the ability to deliver protein payloads via surface conjugation. In this study we show that TMV is readily taken up by mouse bone marrow-derived DCs, *in vitro*. Footpad injection of fluorophore-labeled TMV reveals preferential uptake by draining lymph node resident DCs *in vivo*. Uptake leads to activation, as measured by the upregulation of key DC surface markers. When peptide antigen-conjugated TMV is injected into the footpad of mice, DC-mediated uptake and activation leads to robust antigen-specific CD8⁺ T cell responses, as measured by antigen-specific tetramer analysis. Remarkably, TMV priming induced a greater magnitude T cell response than *Adenovirus* (Ad) priming. Finally, TMV is capable of boosting either Ad-induced or TMV-induced antigen-specific T cell responses, demonstrating that TMV,

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uniquely, does not induce neutralizing self-immunity. Overall, this study elucidates the *in vivo* DC delivery and activation properties of TMV, and indicates its potential as a vaccine vector in stand alone or prime-boost strategies.

Keywords

TMV; Viral vaccine; viral antigen carrier; Dendritic cell; Adenovirus prime-boost; T-cell activation

Introduction

While mammalian viral vectors currently present one of the most studied and efficient mechanisms of antigen and DC-enhancing system delivery *in vivo*, they also exhibit several disadvantages that hinder their utility as cancer vaccines. Chief among these is that the efficacy of several viral vectors for human application has been called into question due to high levels of pre-existing immunity from natural exposure. Certain types of poxvirus, adenovirus, measles virus, poliovirus, and hepatitis B virus have known pre-existing immunity in humans and their opsonization and clearance by neutralizing antibodies upon vaccine delivery renders them virtually unusable, at least in pre-exposed patients (2). Furthermore, immunogenicity leading to the induction of neutralizing antibodies limits the use of some viral vectors even in non-pre-exposed patients. While initial treatment may induce desired immune responses, subsequent treatments fail to boost immunity, as neutralized vectors are unable to deliver their payload to DCs. As a result, repeated injections of such a vector in an effort to prolong anti-tumor immune responses (a.k.a prime-boost strategies) are largely ineffective (9, 10, 19, 22). Third, the relative instability of some vectors necessitates storage at extreme temperatures and limits their shelf life, complicating their use for “off-the-shelf” therapies (14). Finally, complicated and time-consuming production procedures for some viruses inevitably result in expensive therapies which may not be accessible to all patients (2, 4). In an effort to overcome these challenges, this work describes the *in vivo* DC activation and T cell induction potential using antigen delivery by a plant virus particle unaffected by many of the issue that limit mammalian viral vector use.

Tobacco mosaic virus (TMV) is a self-assembling rod-shaped virus that only infects plants. Because of its inability to replicate in mammals, it possesses no known potential for adverse side effects. In fact, humans are repeatedly exposed to TMV in food and tobacco products (12) without adverse effects. Structurally, it consists of a single-stranded RNA molecule encapsidated by genetically modifiable coat protein (CP) discs. TMV’s assembly process normally occurs *in vivo* in plants and results in gram per kilogram virus accumulation that is easily purified from leaf tissue. Additionally, self-assembly also occurs *in vitro* provided RNA and CP are present in stoichiometric quantities (3, 15, 17). As a result, TMV virus particles are produced with relative ease and at low costs. Furthermore the virus is extremely stable, enabling storage at room temperature for decades (5). Most importantly however, DCs naturally and efficiently phagocytose or macropinocytose TMV (15). TMV’s ease of genetic modification enables the genetically engineered introduction of CP surface-exposed lysine residues that allow for covalent conjugation of immunodominant peptides and

proteins thereby transforming TMV into an efficient, easily modified antigen-delivery vector (17, 20). Together with its ability to “cross-prime,” or facilitate MHC I presentation of its surface-conjugated payload, and induce DC activation, as measured by CD86 upregulation on *in vitro* splenocyte-derived DCs, TMV was shown to induce T cell responses that protect against subcutaneous antigen-specific tumor challenge and increase survival in a therapeutic model (15). To further characterize these *in vivo* dynamics of TMV, we set out to elucidate TMV uptake *in vivo* and the resulting DC activation profile. Finally, in an effort to augment and prolong T cell responses of the adenoviral-mediated immunotherapies, we utilized TMV-peptide fusion particles to induce secondary immune responses, thereby consolidating two vaccine approaches into one prime-boost strategy.

In the work that follows, we first confirmed previously reported observations regarding *in vitro* TMV DC-uptake dynamics (15), and expanded our observations to demonstrate that mouse footpad injection of TMV results in efficient relocation of the virus to draining lymph nodes (LNs) as well as secondary LNs and the spleen. Flow cytometric analysis of DC surface activation markers of LN resident DCs indicated a clear activation of DCs following mouse footpad injection of TMV. This activation translated into induction of antigen-specific CD8⁺ T cells following footpad injection of TMV conjugated to the H2-K^b-restricted immunodominant peptide of β -galactosidase (β -gal: ICPMYARV). Finally, TMV-ICPMYARV induced a strong cellular immune response, resulting in greater proliferation of antigen-specific CD8⁺ T cells than adenovirus-LacZ primed mice, with enhanced immunity in an adeno-LacZ priming/TMV-bGal boosting approach. Overall, this study details the *in vivo* uptake dynamics and DC activation profile of TMV and confirms its use as a highly promising vaccine carrier.

Materials and Methods

TMV, TMV-Alexa488, and TMV- β gal production

Virus particles were prepared and conjugated with Alexafluor488 (Invitrogen) or the immunodominant peptide for “ β -galactosidase, ICPMYARV as previously described (15).

TMV *in vitro* uptake and localization studies

Murine bone marrow-derived dendritic cells (mBMDCs) were prepared as previously described (8, 18). 5×10^5 adherent mBMDCs were co-incubated in triplicate with 2 μ g (8 μ g/ml) of TMVAlexa488 (TMV-488) in a 48 well cell culture plate for either 1, 2, 4, 8, 24, 48, and 96 hours. Uptake was first analyzed using fluorescence microscopy as described below. Then, cells were treated with 0.25% trypsin (GIBCO) to aid in harvest and remove surface adhered TMV-488, washed and resuspended in PBS prior to flow cytometric analysis using an LSRII flow cytometer (BD Biosciences). TMV co-localization within endocytic/acidic intracellular compartments was by Lysotracker Red staining (Invitrogen), along with with DAPI (DNA/nucleus), as per manufacturer’s instructions.

Fluorescence microscopy

The uptake of TMV-488 by mBMDCs *in vitro* as described above was directly visualized using the CKX41 fluorescence microscope (Olympus, Center Valley, PA). Pictures were obtained using a DP70 camera controlled by DPI controller.

Mice

Four- to six-week old female C57BL/6 mice were purchased from the Center for Comparative Medicine at Baylor College of Medicine and maintained under pathogen-free conditions in the Transgenic Mouse Facility at Baylor College of Medicine. Animal procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

TMV *in vivo* kinetics and uptake localization

Two mice were treated with bilateral footpad injections of 50 µg TMV-488 (25 µg into each footpad). Popliteal and inguinal lymph nodes (LNs, n=3–4) and spleens (n=2) were dissected 24, 48 and 96 hours after injection. LNs and spleens were mechanically disaggregated to yield single cell suspensions. Cells were then analyzed for the presence of TMV-488 using flow cytometry. Additionally, these cells were stained using PE-conjugated anti-CD11c (BD Pharmingen), CD19 (BD Pharmingen), or Gr-1 (BD Pharmingen) in three separate reactions and analyzed via flow cytometry.

TMV *in vivo* DC activation

Three mice were treated with footpad injection of PBS in one footpad and 25 µg of TMV488 into the other contralateral footpad. Popliteal lymph nodes were dissected 48 hours after injection and mechanically disaggregated to yield single cell suspensions. These cells were then double stained with CD11c-PE-Cy7 (BD Pharmingen) and either anti-CD40-PE (BD Pharmingen), anti-CD86-PE (BD-Pharmingen), anti-CD54-PE (BD Pharmingen), anti-H2-K^b-PE (BD Pharmingen) or anti-I-A^b-PE (BD Pharmingen). Cells were then analyzed using flow cytometry.

Adeno-TMV prime-boost immunization and bleeding schedule

Two groups of 30 mice each were injected into the footpad with 1×10^{10} VP of Ad-Empty (1.5×10^9 PFU) or Ad-LacZ (1.6×10^9 PFU). 10 mice were injected into the bilateral footpads with 50 µg of TMV-βgal as described above. 7 days later mice in the adenovector-injected groups were split into 3 groups of 10 mice each and injected with 1×10^{10} VP Ad-LacZ or 50µg of TMV (unconjugated) or TMV-βgal, respectively. TMV-βgal primed mice were also injected with a second dose of 50 µg TMV-βgal. All 7 groups were boosted with their respective vectors 2 additional times. Peripheral blood was obtained via retro-orbital bleed every 7 days (one day before boost), stained with anti-CD8 FITC-conjugated antibodies (BD Pharmingen) and PE-conjugated MHC I tetramers loaded with ICPMYARV (Baylor College of Medicine Tetramer Core) and analyzed via flow cytometry, as described below.

Tetramer analysis

Peripheral blood was obtained 7 days after vector injection via retro-orbital bleed of mice treated according to the immunization schedule above. Lymphocytes were extracted using Histopaque (Sigma-Aldrich) gradient. Cells were then stained using anti-CD8-FITC (BD Pharmingen, San Diego, CA) and ICPMYARV-H2K^b-PE tetramer for β -galactosidase immunizations (Baylor College of Medicine, Tetramer Core, Houston, TX). Stained cells were analyzed using an LSRII flow cytometer (BD Biosciences)

Statistics

All data are presented as the mean $\pm$ standard error of the mean (SEM). Data were analyzed using Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). An unpaired students *t*-test was used to calculate 2-tailed *P* values in order to determine statistical significance between 2 groups. One-way ANOVA with Bonferroni's post-test correction was used to determine statistical significance between 3 or more groups.

Results and Discussion

Fluorophore-conjugated TMV exhibits efficient uptake characteristics when co-incubated with mouse BMDCs *in vitro*

Before initiating *in vivo* studies, we set out to assess the uptake characteristics, and intracellular localization of TMV particles in DC's *in vitro* using an Alexafluor-488 conjugated TMV virus particle (TMV-488) described previously (15). In contrast to previous studies, we sought to understand and describe TMV/DC interactions, especially in the context of a pure population of mBMDCs, rather than splenocytes as previously reported [10]. Therefore, we added TMV-488 to adherent CD11c⁺ mBMDCs cultured in GM-CSF and IL-4 and measured their uptake kinetics by harvesting the cells at designated time points and analyzing the percentage of TMV-A488⁺ cells from using flow cytometry. TMV-488 was rapidly phagocytosed with 1.5% of cells staining positive within the first hour. This number increased to almost 40% by one day and reached almost 80% after 4 days of incubation (Figure 1a). Importantly, experiments with human DCs derived from peripheral blood yielded comparable results, supporting TMVs viability as a human vector (Figure 1b). Thus, TMV-488 exhibited similar DC uptake kinetics as previously described (1, 16, 17). Finally, in order to ascertain the mode of TMV uptake by DCs, we stained TMV exposed DCs with LysoTracker red to identify acidic vesicles. Localization of TMV within these vesicles confirmed phagocytosis of TMV by DCs (Figure 2). TMV-488 coat protein alone (not particulate) was not taken up by DC's (data not show), indicating the importance of particle size in driving passive uptake.

TMV-488 travels to draining lymph nodes and spleen upon footpad injection and is efficiently taken up by lymph node DCs

To further understand *in vivo* TMV interaction dynamics, we first wanted to assess the kinetics of TMV footpad delivery. Since DC-mediated antigen-specific T cell proliferation occurs in the T cell zones of lymph nodes, we removed and analyzed popliteal (proximal LN) and inguinal (distal LN) lymph nodes from mice 24, 48, and 96 hours after injection of TMV-488 into the footpad. We also removed and analyzed spleens to determine if any TMV

would accumulate in distal lymphoid tissue. While no cells containing virus particles were recovered at any of these sites at 24 hours (data not shown), after 48 hours approximately 1% of popliteal LN resident cells were positive for TMV-488. This uptake was dose dependent and increased to 12% when the amount of TMV injected was increased 5 fold (Figure 3a). Most of the TMV-488 virus particles were found in the proximal draining popliteal lymph node as expected, though some were also detected in the more distal inguinal lymph node and spleen (Figure 3b).

We next set out to determine the cellular uptake profile of TMV. Popliteal LNs were dissected 96 hours post footpad injection of TMV-488. Single cell suspensions were then stained for CD11c, CD19, and Gr1 using fluorescently labeled antibodies to detect the presence of DCs, B cells, and granulocytes, respectively. Subsequent flow cytometric analysis revealed that when gating on the TMV-488⁺ population as a whole, the vast majority of FITC⁺ cells were B cells with DCs and granulocytes representing a much smaller portion (Figure 3c). However, when the percentage of FITC⁺ cells within a specific population subset was assessed by gating on the specific cell population, an indirect measure of the uptake efficiency of a particular cell type, DCs showed a vastly superior ability to phagocytose TMV with nearly 60% uptake compared to less than 15% for B cells and less than 10% for granulocytes (Figure 3d). In conclusion, we show that TMV quickly travels to draining lymph nodes upon footpad injection and is phagocytosed by a variety of cell types, though most efficiently by DCs.

TMV-488 induces dose-dependent upregulation of MHC and co-stimulatory molecules in LN resident cells

To evaluate the overall immune activation potential of TMV, we measured the expression of immune-related surface markers by lymph node resident cells 48 hours after virus footpad injection. Compared to PBS controls, virus-exposed lymph node cells had an increased expression of CD40, CD86, and MHC I and II (Figure 4a and 4b). Furthermore, this response was dose dependent. When compared to the original dose, injection of five-fold the amount of virus particles induced a shift of each markers' flowcytometry histograms indicating increased upregulation of surface markers on a per cell basis (Figure 4a). This dose dependence provides further evidence that TMV possesses intrinsic *in vivo* immune activation properties.

In vivo DC uptake of TMV-488 upregulates ICAM-1, MHC, and costimulatory molecules

To gain further insight into the *in vivo* antigen-specific T cell induction properties of TMV, we analyzed the activation status of TMV-A488⁺ DCs in the popliteal LN, 48 hours after footpad injection. Compared to DCs from LNs of PBS-injected controls, TMV-488 containing DCs displayed several signs of induced activation. The T cell interaction molecule CD54 (a.k.a. ICAM-1), used by DCs for non-specific binding to passerby T cells for the purpose of TCR sampling, was significantly upregulated (Figure 5a). CD40, needed for crosstalk and stimulation of T cells via CD40L binding, was also significantly increased (Figure 5b). The co-stimulatory molecule CD86, required for potent CD4 and CD8 T cell activation, also displayed in higher amounts compared to PBS controls (Figure 5c). Finally, both MHC I and MHC II molecules were detected at increased levels on TMV-488

containing DCs (Figure 5d, e). These results indicated that not only was TMV efficiently taken up by DCs *in vivo* but also facilitated DC activation, indicating a credible potential for antigen-specific T cell proliferation.

TMV-βgal induces antigen specific CD8⁺ T cells in naïve and adenoviral pre-treated mice

To extend previously reported results that antigen-conjugated TMV induces the proliferation of antigen-specific T cells (13, 14), we treated naïve mice with footpad injections of TMV conjugated to the H2-K^b-restricted immunodominant peptide ICPMYARV of β-galactosidase (TMV-βgal) as well as empty- and β-galactosidase-expressing 1st generation adenovirus (Ad-Empty and Ad-LacZ, respectively). After 7 days, we extracted peripheral blood via retro-orbital bleeds and used flow cytometric analysis using fluorescently labeled anti-CD8 antibodies and ICPMYARV-loaded MHC I tetramers to detect the presence of antigen-specific T cells. Despite the differences in the type of antigen presentation (active viral replication of the whole antigen vs. passive peptide delivery), both Ad-LacZ and TMV-βgal induced significant CD8 T cell expansion compared to Ad-Empty. TMV-βgal induced robust T cell activation following DC uptake, with significantly more antigen-specific T cells (Figure 6a) than a dosage of Ad-LacZ that typically results in protection from tumor challenge (10).

One of the main goals of this study was to determine the utility of TMV to act as an antigen carrier in a prime-boost strategy, to enhance and prolong adenoviral-mediated transgene-specific immune responses. Therefore, we assessed the ability of TMV-βgal to boost antigen-specific immune responses in adenovirus-exposed mice. Mice were pre-treated with Ad-Empty and 7 days later re-injected with Ad-Empty, Ad-LacZ or TMV-βgal. As expected Ad-LacZ failed to induce a primary immune response to the β-galactosidase transgene, demonstrating the major drawback in the failure of Adenovirus boosting after pre-exposure. However, tetramer analysis identified a significant proliferation of antigen-specific CD8⁺ T cells in TMV-βgal treated mice (Figure 6b) thereby illustrating the feasibility of an adeno-TMV prime-boost strategy.

Adeno-TMV prime-boost regimens enhance and prolong adenoviral-mediated transgene-specific immune responses

To evaluate the boosting capacity of TMV-βgal and compare it to that of adenovirus, mice primed via footpad injections of Ad-Empty, Ad-LacZ, or TMV-βgal from the experiment shown in Figure 6a were subsequently treated with a second footpad injection of Ad-LacZ, or TMV-βgal 8 days after the first injection. As expected, TMV-βgal was able to boost the antigen-specific T cell response of Ad-LacZ pre-treated mice. However, a second or third injection with Ad-LacZ induced no response in Ad-Empty pretreated mice as shown before and had no boosting and even a potential negative effect on T cell proliferation in Ad-LacZ pretreated mice (Figure 6c and d).

Wild-type TMV is known to exhibit excellent humoral immuno-induction potential leading to antibodies that may neutralize the virus, including by human exposure in food and tobacco products (12, 21). However, TMV-lysine covalently linked to an immunodominant peptide (15) or protein (13) has not been observed to induce vector-mediated immune

suppression, and we have not observed an effect of anti-TMV antibodies on DC uptake (data not shown). To indirectly illustrate that such antibody responses are either not induced or generally irrelevant to peptide-bound TMV, we compared the ICPMYARV-tetramer⁺ T cell populations of mice primed and boosted with only TMV-βgal to those of treated only with Ad-LacZ. While Ad-LacZ treated mice exhibited a decrease in their ICPMYARV-tetramer⁺ population after only 2 boosts, TMV-βgal treated mice demonstrated a marked increase in antigen-specific T cells after a second boost, though a primary boost was unable to illicit this response (Figures 6c and 6d). Overall, these results indicate that Adeno-TMV prime-boost strategies are a viable option for not only prolonging but also enhancing adenoviral-mediated transgene-specific T cell responses, and that this phenomenon is made possible by conjugated-TMV's ability to escape neutralizing antibodies.

Conclusion

This paper is the first report of the *in vivo* cellular uptake profile of TMV, and the first to detail DC activation *in vivo*. Although other studies have shown plant virus/immune system interactions (7, 11), we are the first to show in detail the *in vivo* DC uptake and immune activating potential of TMV specifically. Notably, this study is also the first to demonstrate uptake into human DC, and the first to compare the T-cell activation potential of TMV compared to Adenovirus antigen delivery. Lastly, TMV potentiates adeno-primed responses using a TMV boost strategy, with potential utility in combined vaccination strategies against weakly immunogenic cancer antigens. TMV used in this study and others have been described as VLP's, however because TMV does contain an RNA genome (albeit inactive), it is more similar to an inactive virus than a VLP (6, 16, 17). Indeed, the presence of this RNA may be one of the characteristics that make TMV effective for *in vivo* vaccine immunotherapy. Ongoing investigations will focus on the molecular basis of immune activation, including the likely role of TLR activation in DC and other APC populations. Because of its ease of production, safety and potency, these studies re-confirm TMV antigen delivery as a stand-alone vaccine that can be given without adjuvant.

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Highlights

- Fluorescently labeled TMV is actively taken up by primary DC's *in vitro*, and co-localizes in acidic endosomes.
- *After food pad injection*, TMV is actively taken uptake and migrates to draining lymph node cells, primarily B cells and Dendritic cells. TMV uptake leads to upregulation of surface markers important for robust antigen presentation.
- TMV delivers peptide antigens to DC's and induces potent T cell responses measured by tetramer analysis.
- Compared to active viral antigen delivery by Adenovirus, TMV induces a higher percentage of antigen-specific T cells after a single dose.
- TMV antigen boosting is synergistic with Adenovirus priming.

Figure 1.

In vitro uptake into mouse and human DCs. Mouse (A) or Human (B) DC's were prepared and incubated with 2ug/ml TMVA488. Samples were analyzed by Flow cytometry at indicated time points. Error bars represent SEM.

Figure 2. TMV is co-localized in acidic endosomes after DC uptake

Cells were incubated with 8ug/ml TMV-A488 for 4h. Cells were washed with PBS and incubated for 24h, then stained with Lysotracker red (for vesicles) and DAPI (for nuclei). The cells were visualized by fluorescence microscopy. Cells (A) in light phase, (B) with DAPI (C) with Lysotracker red, (D) TMV-A488, or (E) a merged image of B, C and D.

Figure 3. TMV migrates to draining lymph nodes and spleen after footpad injection and is efficiently taken up by DCs

2 mice were injected into each footpad with TMV-488 and one with PBS. Lymph nodes were then dissected and treated as separate data point. Spleens were also dissected. **(A)** Presence of TMV-488 in the popliteal lymph node observed at 48 hours after footpad injection. **(B)** Distribution of TMV-488 in proximal and distal draining lymph nodes and spleen 48 hours after injection of 10ug TMV-488 per footpad. **(C)** Surface marker staining of FITC⁺ popliteal lymph node cells 96 hours after initial injection. DCs (CD11c), B cells (CD19) and granulocytes (Gr-1) were analyzed. This data represents the proportion of FITC⁺ cells that stain positive for each indicated surface marker and is thus a depiction of overall TMV distribution within the lymph node. **(D)** Same as C. However this data represents the proportion of FITC⁺ cells within a given cell population and is thus a depiction of TMV uptake efficiency by a given cell type. Error bars represent SEM.

Figure 4. In vivo surface marker upregulation of LN resident cells on day 2

Mice were injected with either PBS (red), 10ug TMV per footpad (blue and white), or 50 ug TMV per footpad (green). Single cell suspensions of dissected popliteal lymph nodes were then stained with anti-CD40, CD86, MHCI and MHCII and analyzed via flowcytometry. Data are represented here as A. histograms and B. overall relative mean fluorescence intensity (MFI) of each population.

Figure 5. TMV induces DC activation in vivo as indicated by surface marker upregulation
 4 mice were injected into each footpad with TMV-488 and 2 with PBS. Popliteal lymph nodes were dissected at 48 hours, and each resulting single cell suspension was treated as separate sample. Single cell suspensions were then stained with PE-Cy7-CD11c⁺ and PE-
 (A) CD54, (B) CD40, (C) CD86, (D) H2-K^b or (E) I-A^b and cells analyzed via flow cytometry. *p<0.05, **p=0.0556, ***p=0.11. Error bars represent SEM.

Figure 6. TMV conjugated with b-galactosidase induces antigen-specific CD8⁺ T cells even in the context of adenoviral pre-existing immunity

30 mice (Ad-Empty), 30 mice (Ad-LacZ) and 10 mice (TMV-βgal) were injected via both footpads with a total of 1×10^{10} virus particles of the respective adenoviruses (Ad-Empty = 1.5×10^9 PFU; Ad-LacZ = 1.6×10^9 PFU) and with a total of 50 μg of TMV or TMV-βgal (25 μg per footpad). **(A)** 7 days later peripheral blood from all mice were analyzed for CD8⁺ ICPMYARV:MHC I tetramer⁺ cells via flow cytometry. One day later, Ad-Empty and Ad-LacZ injected mice were split into three groups of 10 mice each and injected with TMV (unconjugated), Ad-LacZ, or TMV-βgal. The TMV-βgal prime group was also injected with TMV-βgal on that day. **(B&C)** 8 days after the injection tetramer analysis was performed on peripheral blood from all mice as before. **(D)** The next day mice were again injected (boosted) with the same vector as before and tetramer analysis performed on peripheral blood 7 days later as before. * $p < 0.05$. Data shown represent mean +SEM of all mice in the group ($n=30$ or $n=10$) at each time point of analysis.