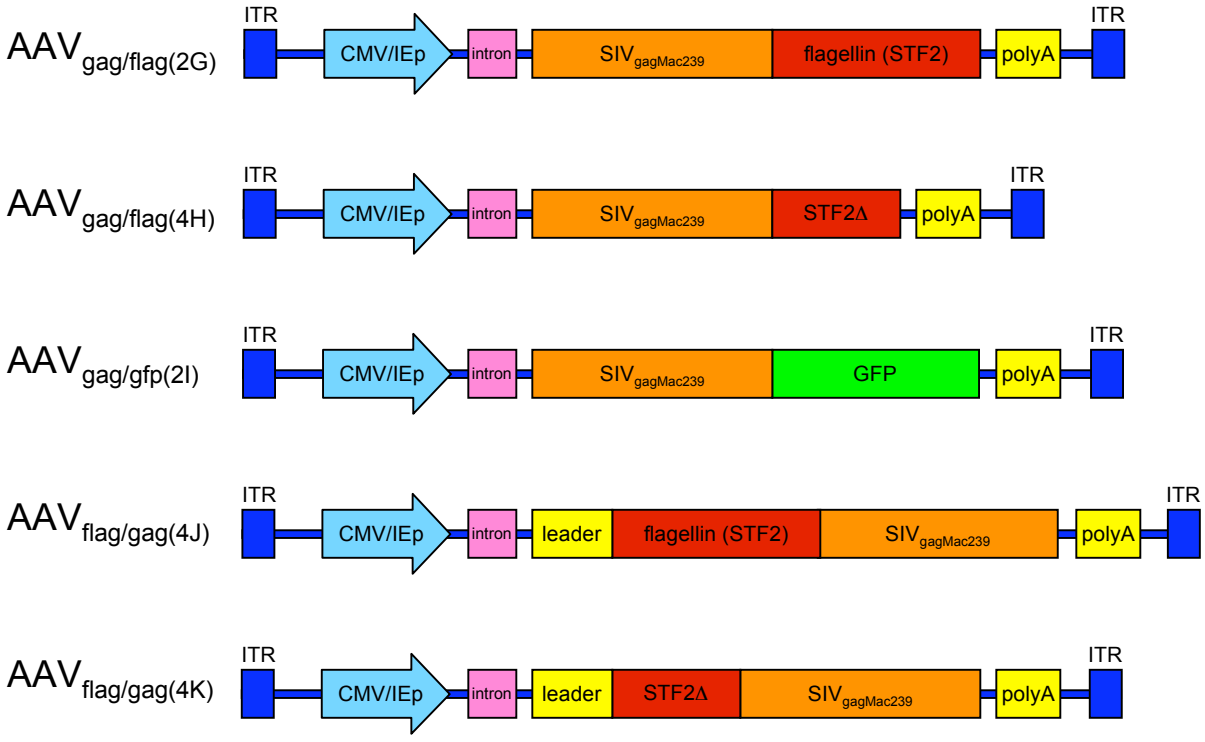
# ZAMB2008001: Immunogenicity of AAV vectors expressing flagellin/gag fusion proteins (peak response)

Mouse Immunology Laboratory
Collaboration for AIDS Vaccine Discovery

## Experimental Design:

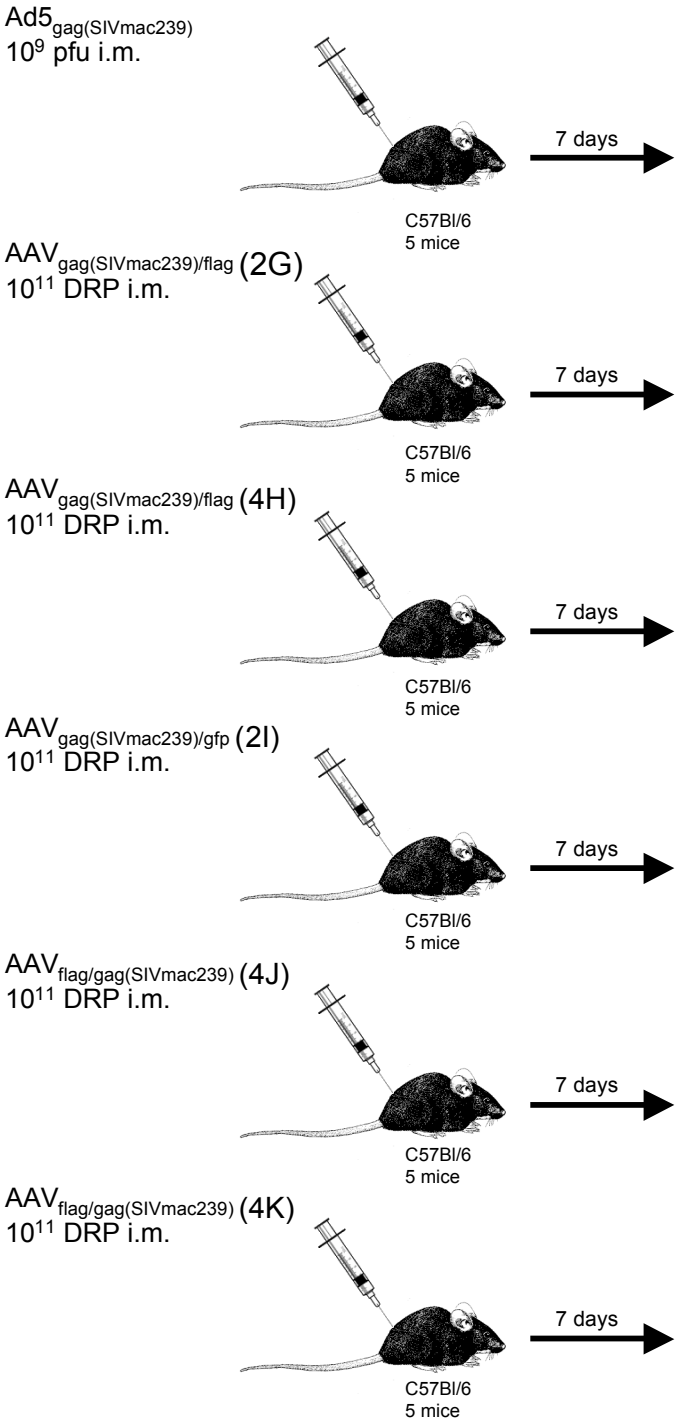
The purpose of this experiment is to compare the relative immunogenicity of Adeno-Associated Virus (AAV1) vectors expressing the gag protein from SIVmac239 fused to flagellin (STF2) or the minimal TLR5 binding domain of flagellin (STF2Δ) as a means to engage TLR5 and thereby increase innate and adaptive immune responses. An Adenovirus (Ad5) vector expressing the SIVmac239 gag protein with that has previously been shown to be immunogenic in mice was included as a reference for the relative immunogenicity of the AAVgag vectors. A control AAV expressing the SIVmac239 gag protein fused to green fluorescent protein, which should not engage TLR5, was also included for comparison. The different AAV constructs are shown below.

Groups of 5 C57Bl/6 mice received $10^{11}$ DNA-resistant particles (DRP) of each AAV vector intramuscularly (i.m.) or $10^{9}$ plaque forming units (pfu) of Ad5gag (i.m.). The peak of the T cell response was measured at day 7 post-vaccination in systemic and mucosal immune sites including spleen, inguinal lymph nodes, and mesenteric lymph nodes, by stimulation with known CD4 or CD8 T cell epitopes or by staining with MHC tetramers refolded with previously identified epitopes. To determine if inclusion of the flagellin domain resulted in an altered immunodominance hierarchy, compared to that seen with expression of the gag protein from the $Ad5_{gag}$ or $AAV_{gag/gfp}$ vectors, cells were stimulated with peptide pools containing 15-mer peptides (overlapping by 11 amino acids) that span the entire length of the gag gene. Responses to these peptide pools were then measured by intracellular cytokine staining.




**AAVgag vaccines**: AAV1-based vaccines expressing the gag protein from SIVmac239 fused to flagellin(STF2), the minimal TLR5 binding domain of TLR5 (STF2d), or gfp control protein under the control of a human cytomegalovirus immediate early promoter (CMV0IE).

# Experimental Design:




Tissues:
Spleen
Inguinal lymph nodes
Mesenteric lymph nodes

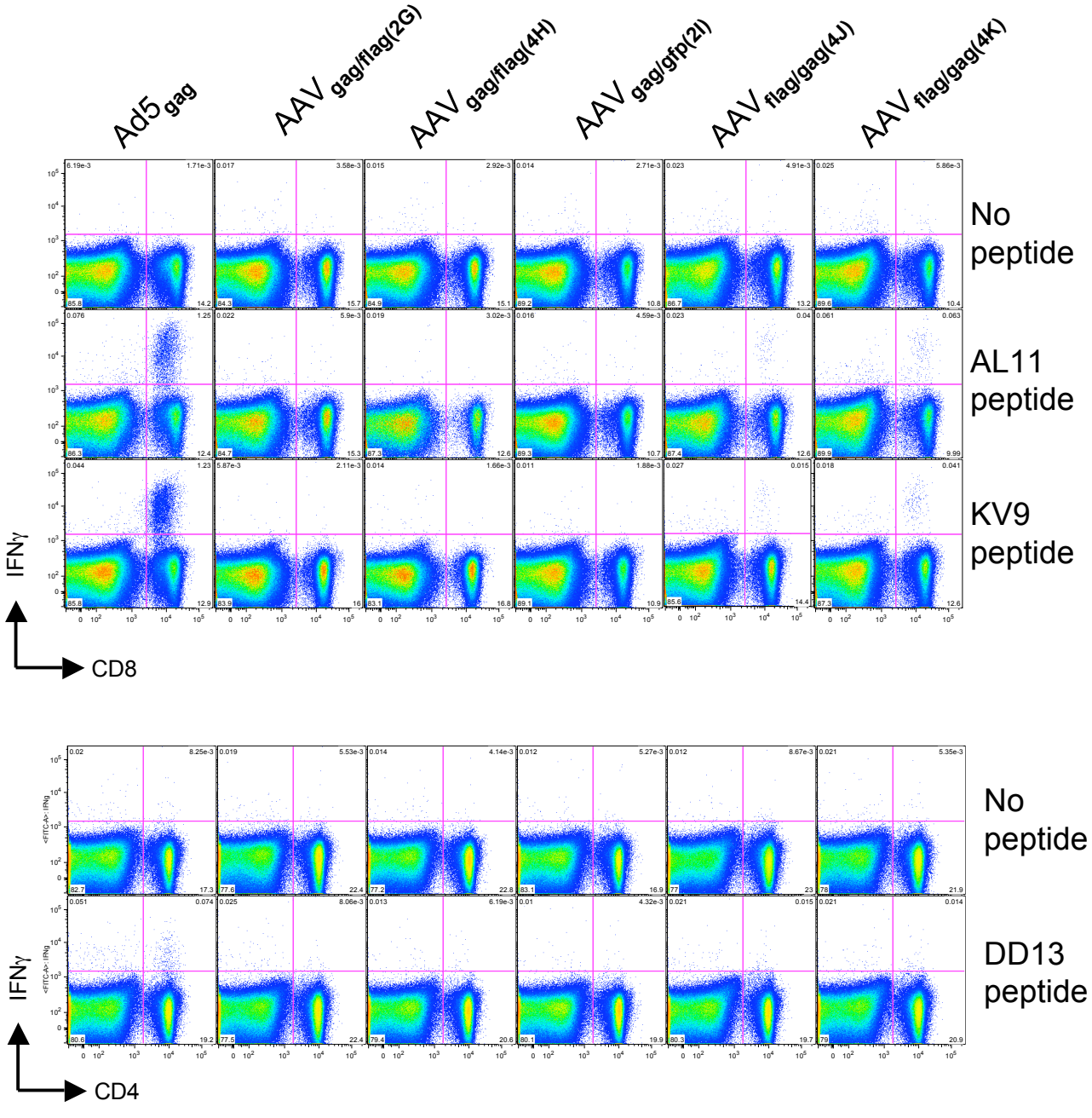
Assays:
1. Quantitation
(ICS & Tetramer)
2. Function
(ICS)
3. Breadth of Response
(Peptide pools)

**Immunization strategy**: Groups of 5 C57Bl/6 mice were immunized with the indicated constructs, and tissues harvested for measurement of immunogenicity after 7 days. pfu=plaque forming units, DRP=Dnase resistant particles, i.m.=intramuscular.

# I. Intracellular cytokine staining (ICS)

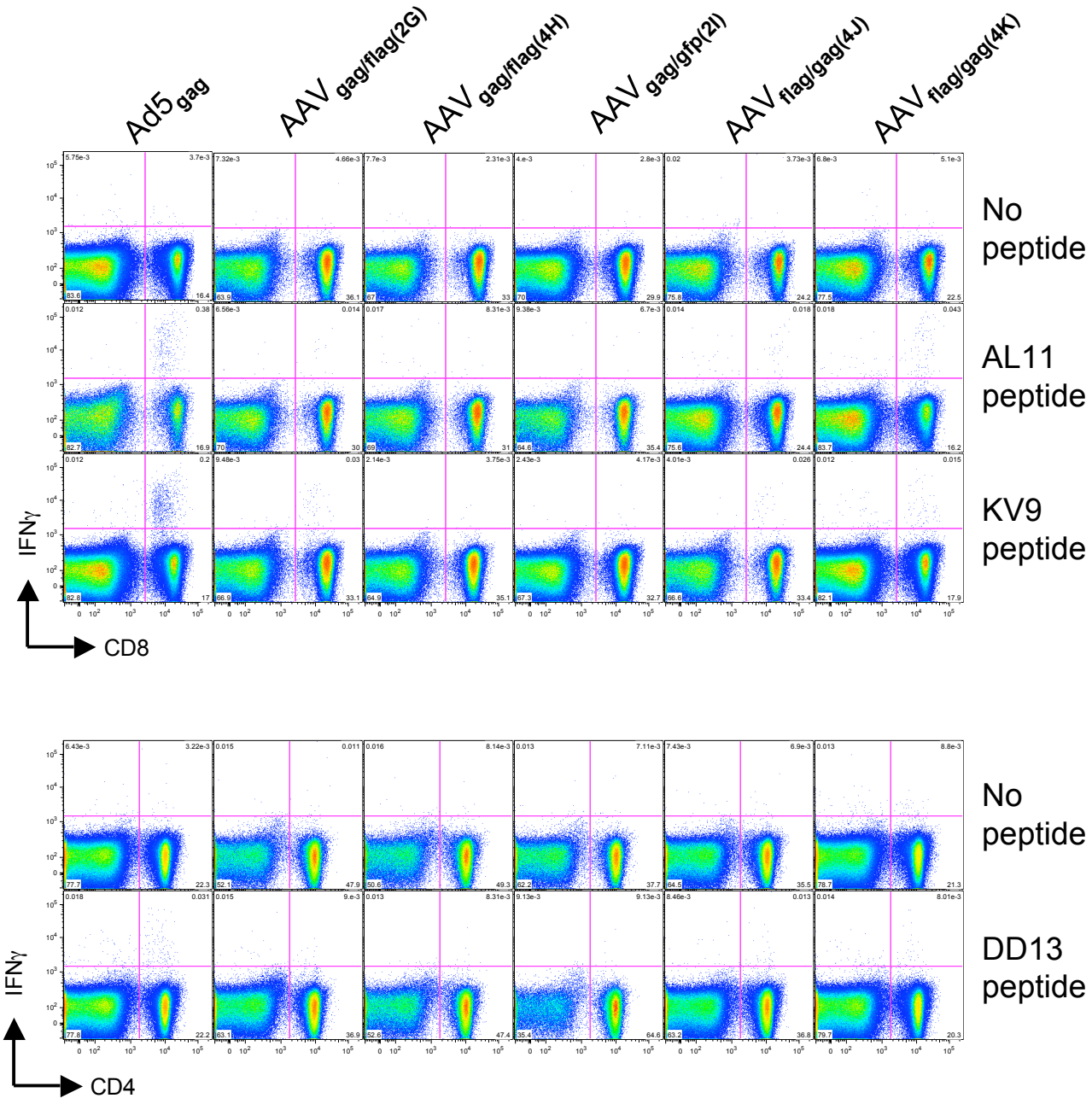
## A. Representative intracellular cytokine staining of splenocytes.

Single cell suspensions of splenocytes were left unstimulated or restimulated with either the AL11, KV9, or DD13 peptides for 5 hrs in the presence of Brefeldin A then stained in for CD8 and CD4, followed by permeabilization and staining for intracellular IFNγ.



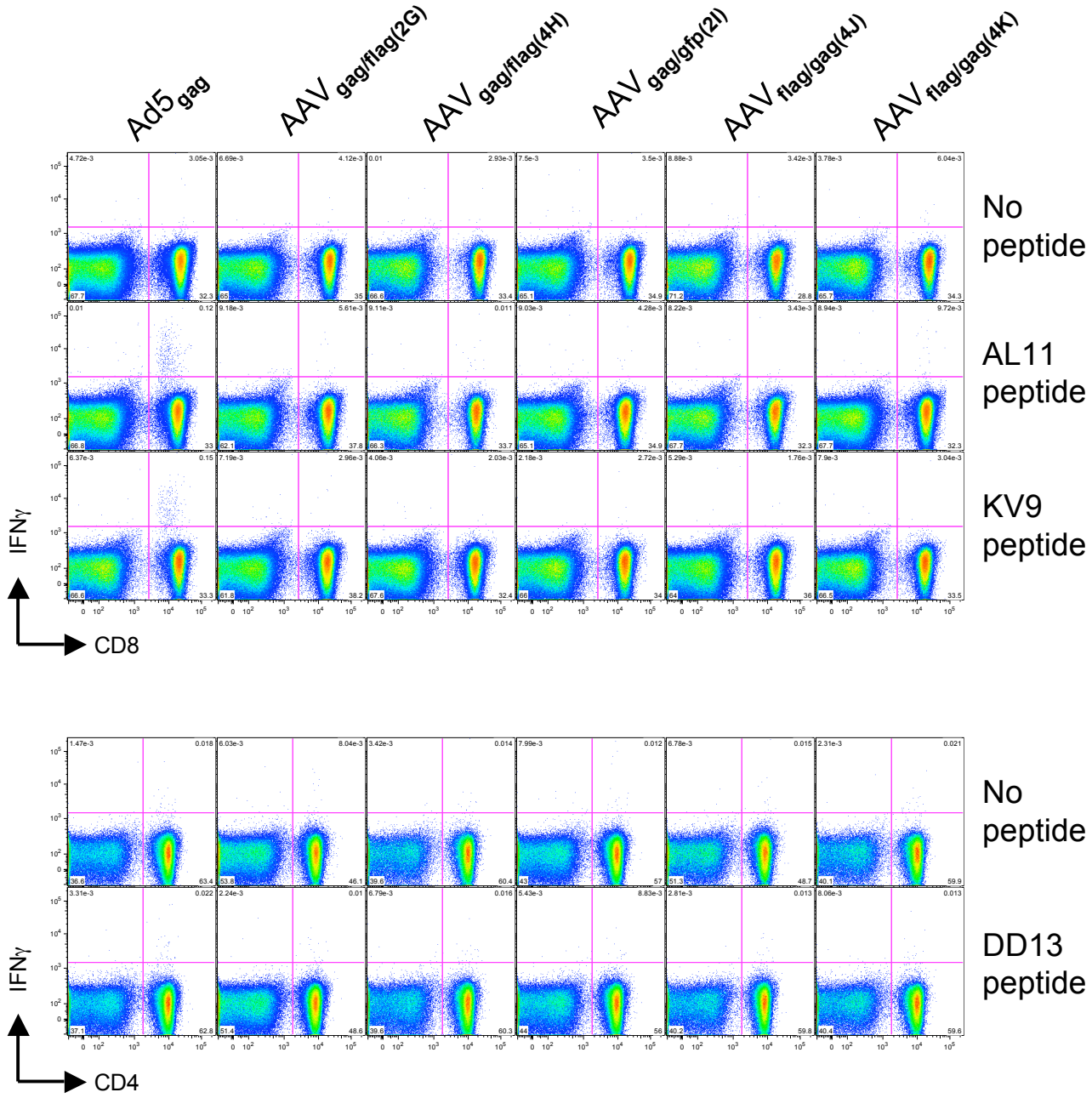
## B. Representative intracellular cytokine staining of inguinal lymph nodes

Single cell suspensions of inguinal lymph nodes were left unstimulated or restimulated with either the AL11, KV9, or DD13 peptides for 5 hrs in the presence of Brefeldin A then stained in for CD8 and CD4, followed by permeabilization and staining for intracellular IFNγ.



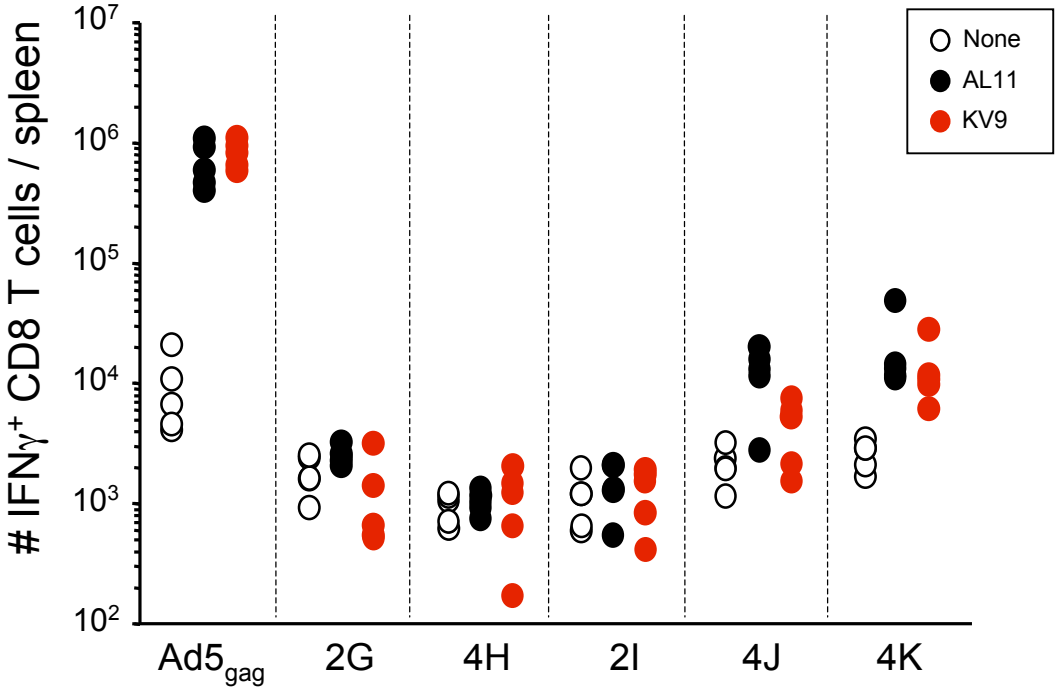
## C. Representative intracellular cytokine staining of mesenteric lymph nodes

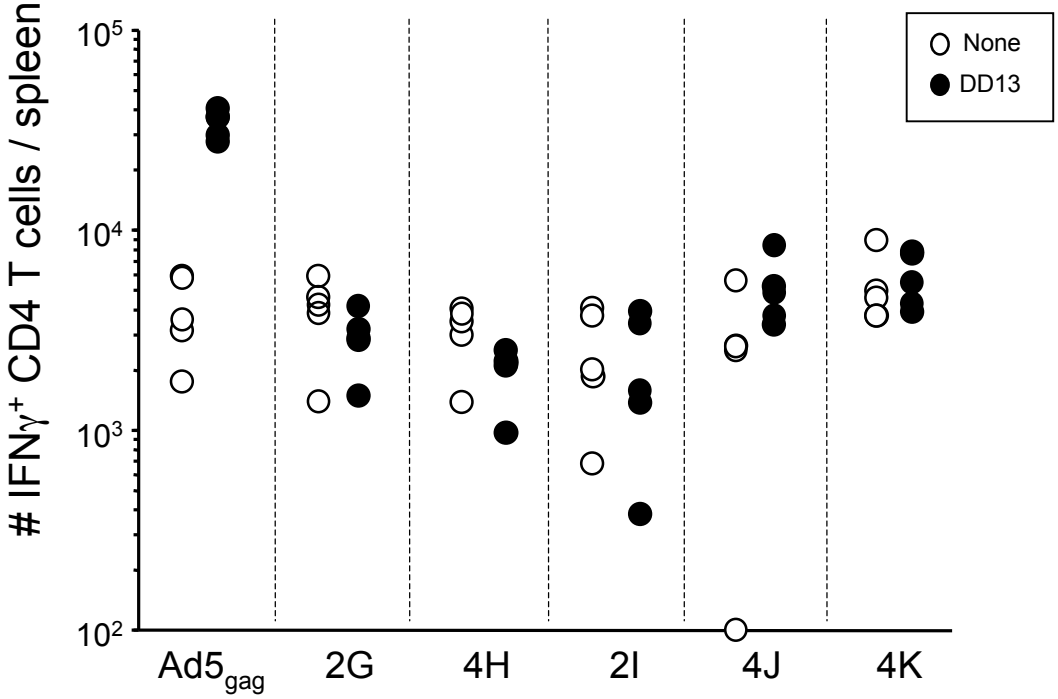
Single cell suspensions of mesenteric lymph nodes were left unstimulated or restimulated with either the AL11, KV9, or DD13 peptides for 5 hrs in the presence of Brefeldin A then stained in for CD8 and CD4, followed by permeabilization and staining for intracellular IFNγ.



## D. Quantitation of gag-specific splenocytes by ICS

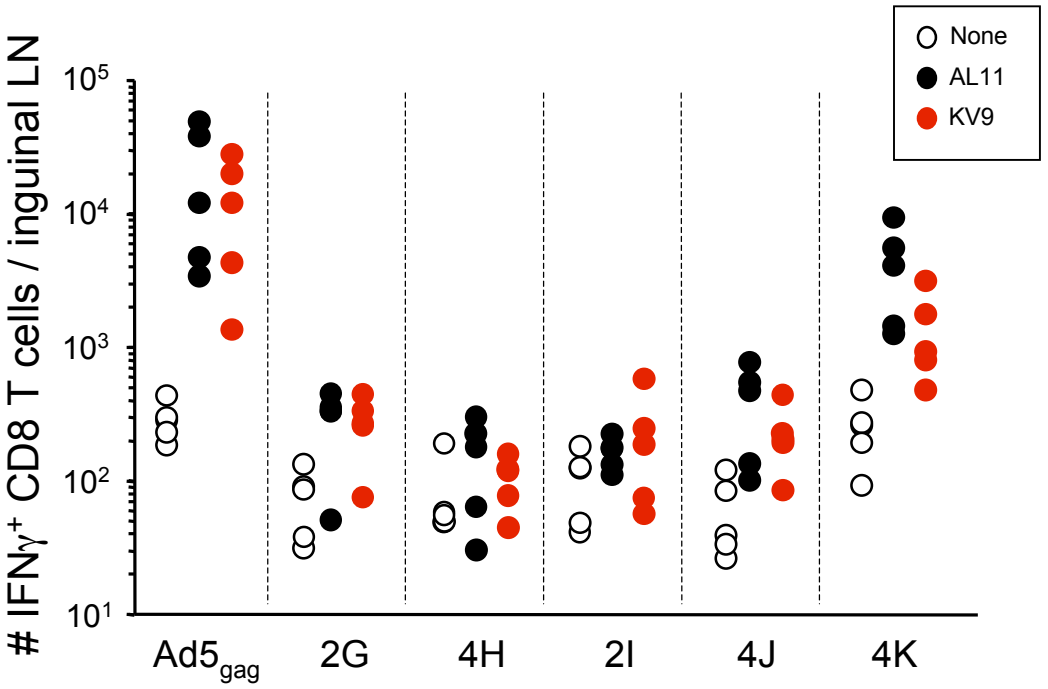
*Number of IFNγ⁺ cells is derived from cell recovery and % IFNγ⁺ of CD4 or CD8 T cells from ICS.*

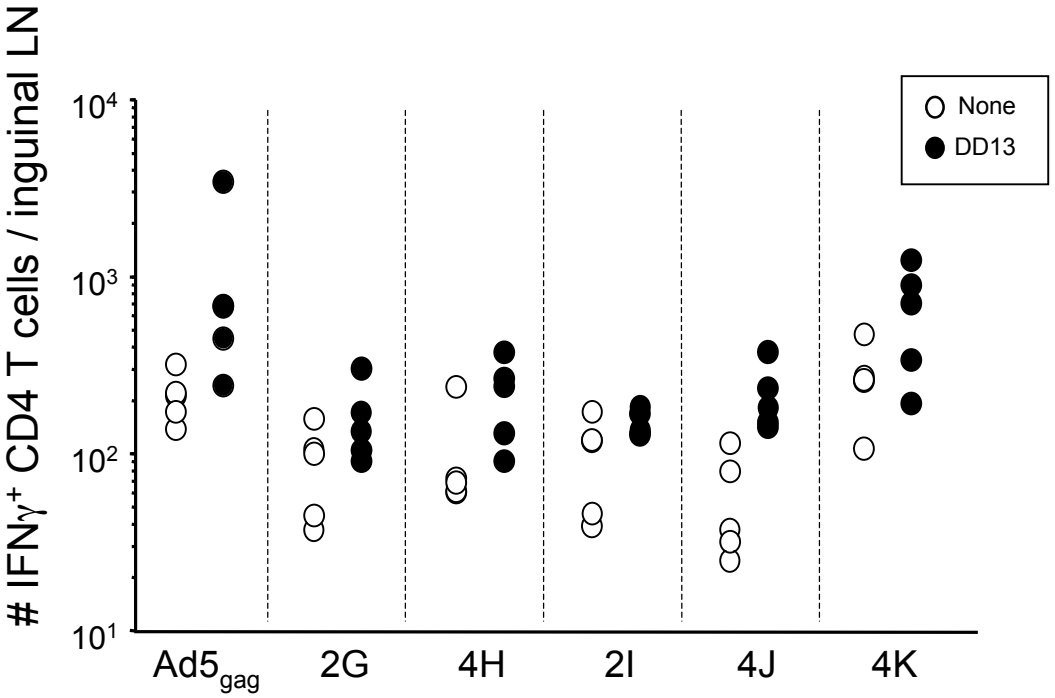


## D. Quantitation of gag-specific cells in inguinal lymph nodes by ICS

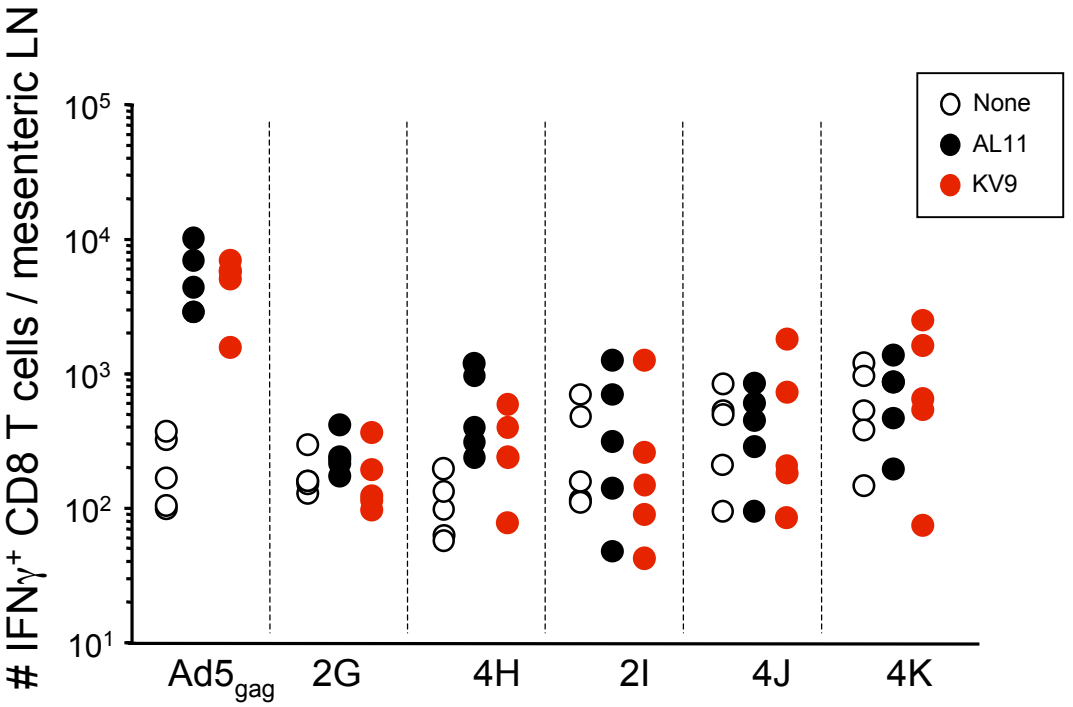
*Number of IFNγ⁺ cells is derived from cell recovery and % IFNγ⁺ of CD4 or CD8 T cells from ICS.*

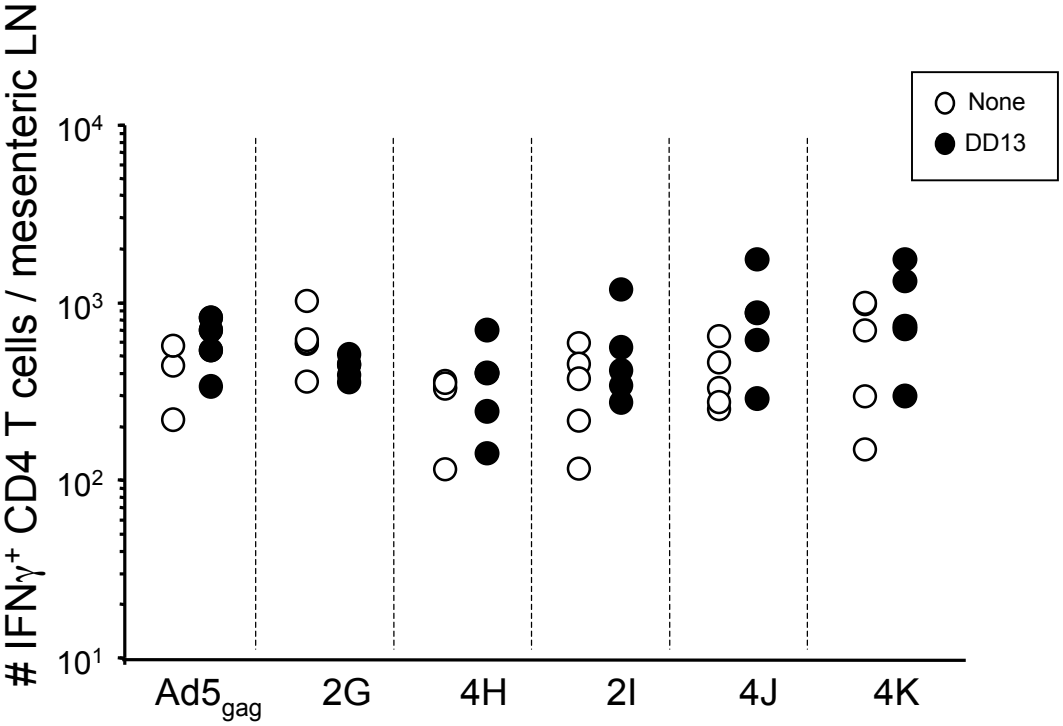


## D. Quantitation of gag-specific mesenteric lymph nodes cells by ICS

*Number of IFNγ⁺ cells is derived from cell recovery and % IFNγ⁺ of CD4 or CD8 T cells from ICS.*



**Summary intracellular cytokine staining:**

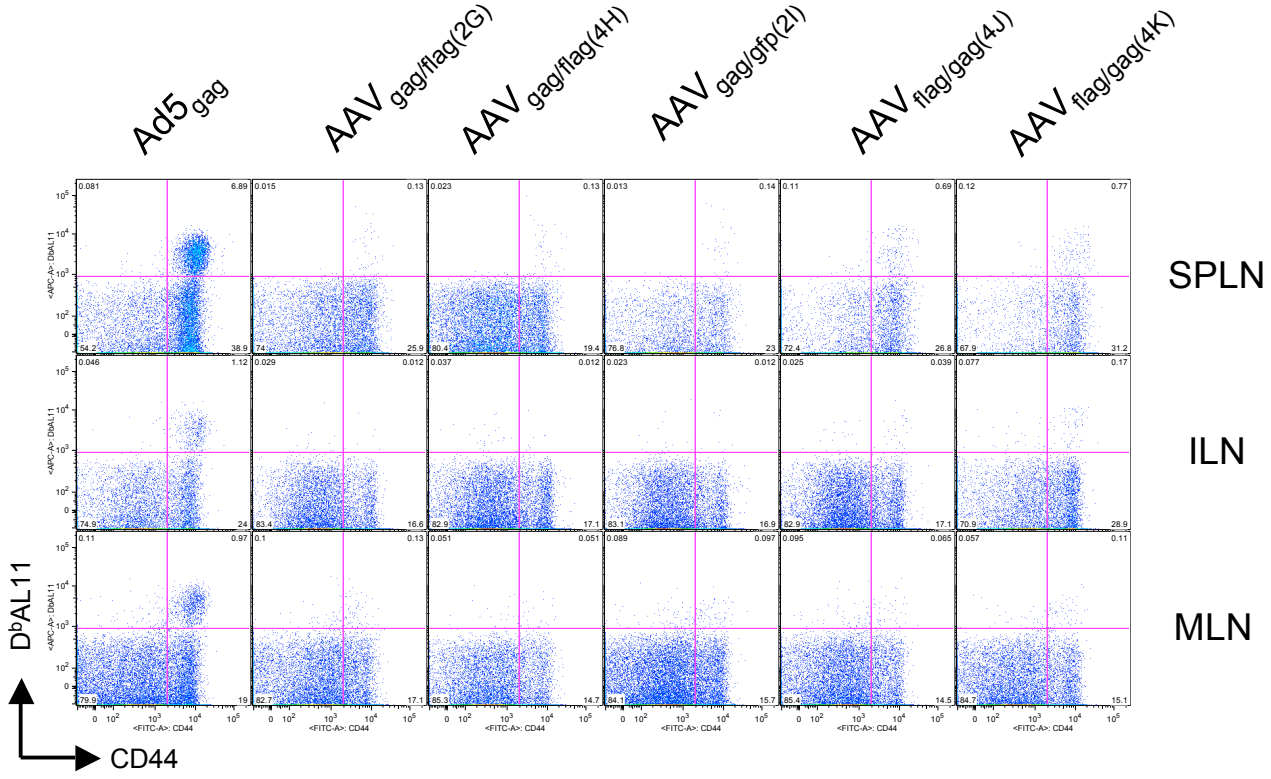
In all cases the Ad5gag positive control gave the highest magnitude CD8 T cell responses in the tissues examined. Among the AAV vaccines examined, immunization with the AAV expressing the STF2 or STF2Δ fused to the N-terminus of the gag protein, resulted in higher magnitude CD8 T cell responses specific for the AL11 and KV9 epitopes than did immunization with the control AAV expressing the gag/gfp fusion protein or AAV expressing the STF2 or STF2Δ fused to the C-terminus of the gag protein. This was apparent in the spleen and draining (inguinal) lymph node, but responses in the mesenteric lymph nodes were at or below background staining levels in all AAV immunized groups. Particularly in the draining lymph nodes, fusion of the STF2Δ minimal TLR5 binding domain to the gag protein resulted in higher magnitude T cell responses than did fusion of the entire STF2. As these experiments did not include the 12L gfp control AAV virus that includes a leader sequence similar to the seen in the 4J and 4K versions, and which might also alter expression levels resulting in higher responses, future experiments should also include this control.

CD4 T cell responses to the DD13 epitope were detectable in the spleens, and to a lesser extent inguinal lymph nodes, from Ad5gag immunized mice. CD4 T cell responses to the DD13 epitope were also visible, although at very low levels, in draining inguinal lymph nodes from mice immunized with the AAV expressing the STF2 or STF2Δ fused to the N-terminus of the gag protein. Responses were at or below background staining levels in other tissues in these mice as well as all tissues examined in other groups immunized with the other AAV constructs.

## II. Tetramer staining

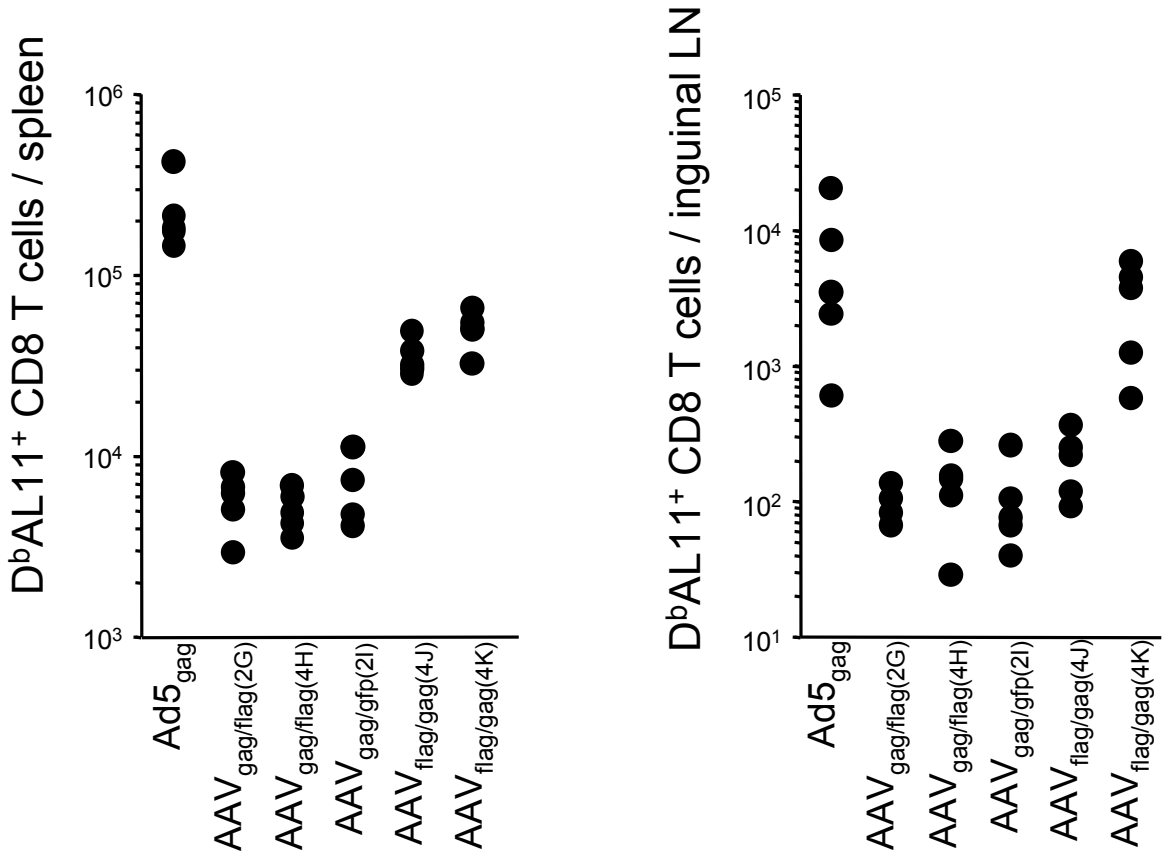
### A. Representative $D^bAL11$ tetramer staining of splenocytes

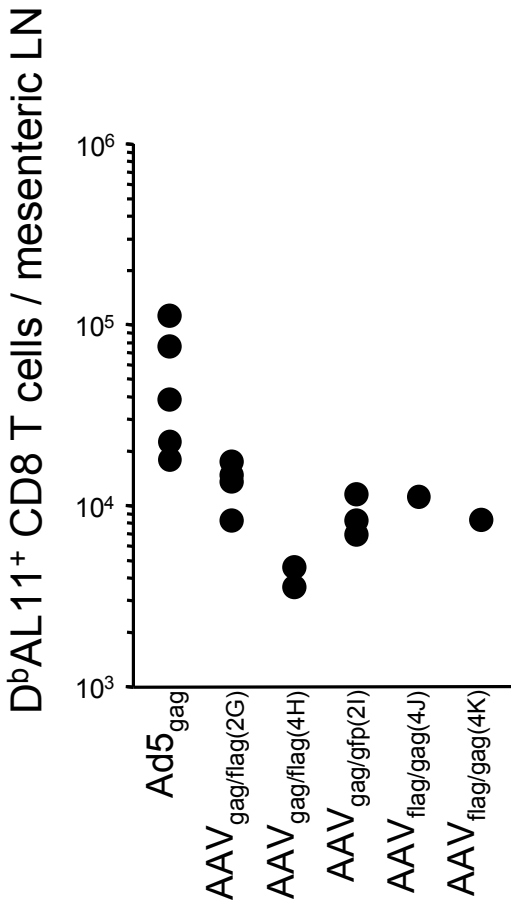
Single cell suspensions of splenocytes were stained with the $D^bAL11$ tetramer and antibodies to CD8α and the activation marker CD44. FACS plots are gated on $CD8^+$ T cells. SPLN=spleen, ILN=inguinal lymph nodes, MLN=mesenteric lymph nodes.



## B. Quantitation of $D^bAL11$ tetramer positive cells

Cell numbers are derived from cell recoveries and $\%CD8^+D^bAL11^+$ T cells. Due to technical difficulties encountered, several samples were not analyzable from the mesenteric lymph nodes.






**Summary Tetramer staining:**

Highest magnitude responses to the DbAL11 epitope were always detected in Ad5gag immunized mice, but responses were still detectable in AAV immunized mice. Similar to results from intracellular cytokine staining, higher responses were seen when immunizing with AAV expressing the STF2 or STF2delta fused to the N-terminus of the gag protein compared to the control gag-gfp fusion protein or the STF2/stf2delta fused to the C-terminus of the gag protein. Particularly in the draining inguinal lymph node, the STF2delta/gag fusion protein gave higher responses than the STF2/gag fusion protein. Again, since the control 12L AAV expressing the gag-gfp fusion protein with a leader sequence similar to that in the 4J & 4K vaccines was not used in this experiment, and this difference could result in higher responses due to differences in epitope presentation, this control should be included in future experiments. Due to technical issues, many of the tetramer staining samples from the mesenteric lymph nodes were not analyzable, and so only analyzable samples are shown.

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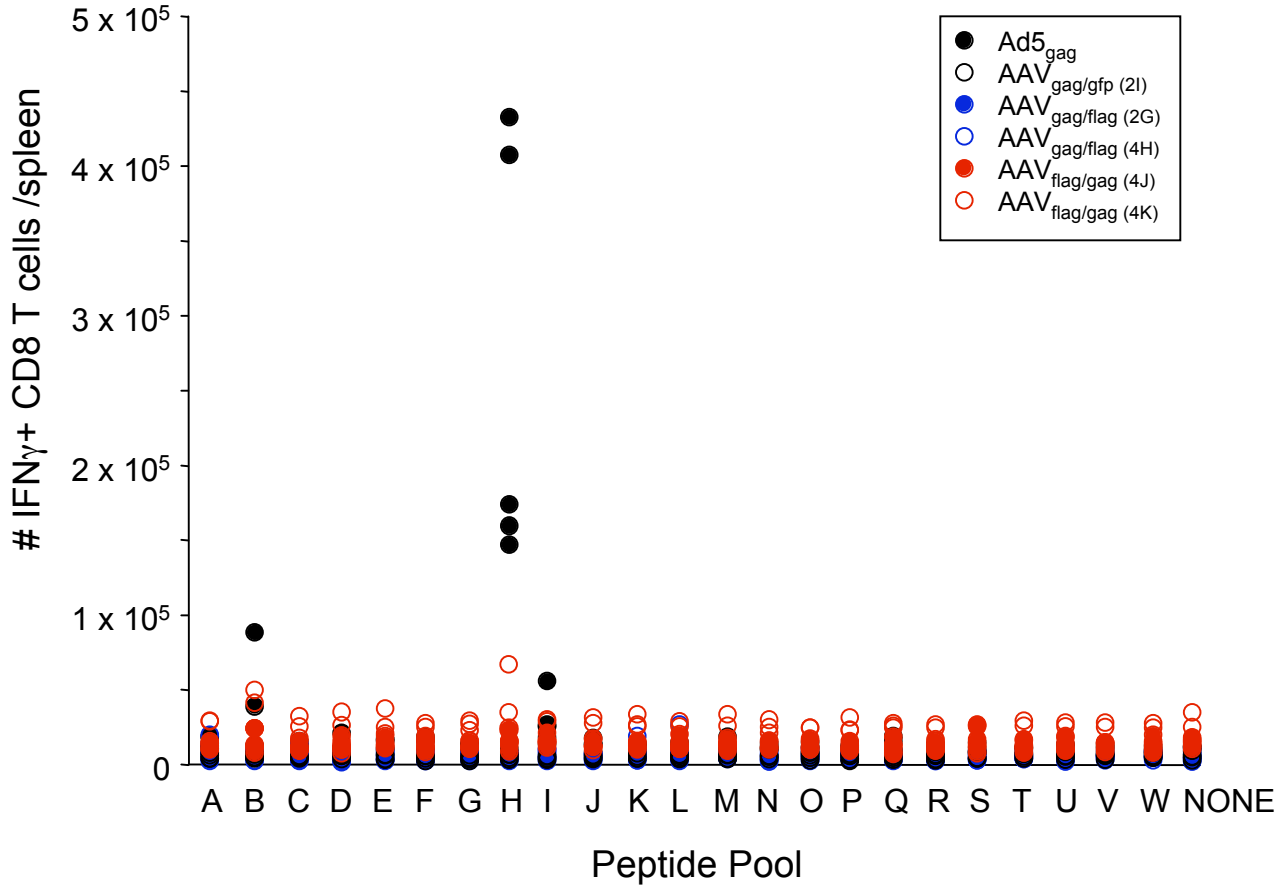
## E. ICS of peptide pool stimulated splenocytes.

Splenocytes were stimulated with pools of 15-mer peptides (overlapping by 11 amino acids) spanning the entire length of the SIVmac239 gag protein. Peptides were combined in pools such that any single peptide was only in two pools (figure next page). Following stimulation, cells were stained for CD4 or CD8α and intracellular IFNγ. Quantitation of IFNγ$^+$ cells is based on cell recoveries and flow cytometry analysis. No IFNγ+ CD4 T cells were observed in this assay, and so that data is not shown.



## Peptide pools

| Peptide pools | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| K | 5211 | 5212 | 5213 | 5214 | 5215 | 5216 | 5217 | 5218 | 5219 | 5220 |
| L | 5221 | 5222 | 5223 | 5224 | 5225 | 5226 | 5227 | 5228 | 5229 | 5230 |
| M | 5231 | 5232 | 5233 | 5234 | 5235 | 5236 | 5237 | 5238 | 5239 | 5240 |
| N | 5241 | 5242 | 5243 | 5244 | 5245 | 5246 | 5247 | 5248 | 5249 | 5250 |
| O | 5251 | 5252 | 5253 | 5254 | 5255 | 5256 | 5257 | 5258 | 5259 | 5260 |
| P | 5261 | 5262 | 5263 | 5264 | 5265 | 5266 | 5267 | 5268 | 5269 | 5270 |
| Q | 5271 | 5272 | 5273 | 5274 | 5275 | 5276 | 5277 | 5278 | 5279 | 5280 |
| R | 5281 | 5282 | 5283 | 5284 | 5285 | 5286 | 5287 | 5288 | 5289 | 5290 |
| S | 5291 | 5292 | 5293 | 5294 | 5295 | 5296 | 5297 | 5298 | 5299 | 5300 |
| T | 5301 | 5302 | 5303 | 5304 | 5305 | 5306 | 5307 | 5308 | 5309 | 5310 |
| U | 5311 | 5312 | 5313 | 5314 | 5315 | 5316 | 5317 | 5318 | 5319 | 5320 |
| V | 5321 | 5322 | 5323 | 5324 | 5325 | 5326 | 5327 | 5328 | 5329 | 5330 |
| W | 5331 | 5332 | 5333 | 5334 | 5335 | - | - | - | - | - |

**Summary peptide scanning:**

Since fusion of the gag protein to the STF2 flagellin might result in an altered immunodominance hierarchy and thus the epitopes that were examined would not be representative of the overall response, cells from immunized mice were stimulated with peptides spanning the entire length of the gag protein to provide a picture of the total response. Similar to previous data, although Ad5 immunized mice had the highest responses, mice immunized with either the AAVflag/gag 4J or 4K constructs also had detectable responses. Only responses to pool H and pool B were observed. Since there is no peptide common to these pools, these represent two responses distinct response. Pool H contains peptide 5288 encompassing the entire AL11 epitope, while pool B contains the 5222 peptide encompassing most of the KV9 epitope. It is not clear why no responses were detected in pools R or M, respectively, which also contains these peptides. This may indicate that there are problems with the peptide pool reagents, and these will be replaced with new peptides in subsequent experiments. Also, the individual peptides containing each of these epitopes will be included in separate stimulations to confirm that these are indeed responsible for the IFNγ production observed. However, taken together, these results indicate that there is not a gross change in the immunodominance hierarchy, and that conclusions drawn from results obtained with the AL11 and KV9 epitopes are valid for the entire response. As no IFNγ+ CD4 T cells were detected in this assay, presumably due to the low frequency of these cells, it is unclear if there is a change in the CD4 immunodominance hierarchy.