

SUPPLEMENTAL MATERIAL

Materials and Methods

Animals, continued

The animals were caged at five to ten mice per cage and fed with a commercial diet (RM3 (p) PL IRR diet; Special Diet Service) with free access to food and water. All mice were 6-12 weeks old at the start of the experiments. All animal protocols were approved by the Ethical Committee for Animal Research at Karolinska Institutet. Methods were carried out in accordance with the approved guidelines.

Utilized DNA constructs, continued

A codon-optimized fusion construct of JFH-1 NS3/4A and stork HBcAg (co-Stork-HBcAg) was made synthetically (Retrogen, San Diego, CA) and inserted into the pVAX1 vector. Plasmids were amplified in TOP10 *E. coli* cells (Life Technologies, Carlsbad, CA) and purified using Qiagen EndoFree Plasmid purification Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Plasmid DNA concentration was determined spectrophotometrically and the purified DNA was dissolved in sterile PBS at a concentration of 1 mg/mL. Restriction enzyme digests were performed to ensure that the plasmid contained the gene of interest with the correct size. Nucleotide sequences of all DNA plasmids were validated (Eurofins MWG Operon, Ebersberg, Germany).

Peptides and proteins

A total of 75 20-mer peptides with 10 aa overlap covering the full-length NS3/4A-JFH-1 sequence were purchased from Sigma-Aldrich (St. Louis, MO). The 75 peptides were divided into either 5 or 15 peptide pools as outlined. Five pools (15 peptides in each pool): pool 1₁₋₁₅, pool 2₁₆₋₃₀, pool 3₃₁₋₄₅, pool 4₄₆₋₆₀, and pool 5₆₁₋₇₅. Fifteen pools (5 peptides in each pool): pool

1₁₋₅, pool 2₆₋₁₀, pool 3₁₁₋₁₅, pool 4₁₆₋₂₀, pool 5₂₁₋₂₅, pool 6₂₆₋₃₀, pool 7₃₁₋₃₅, pool 8₃₆₋₄₀, pool 9₄₁₋₄₅, pool 10₄₆₋₅₀, pool 11₅₁₋₅₅, pool 12₅₆₋₆₀, pool 13₆₁₋₆₅, pool 14₆₆₋₇₀, and pool 15₇₁₋₇₅. From the epitope screening using the outlined overlapping peptide pools, three 20-mer peptides were found to stimulate an NS3-specific immune response: #36:YGRAIPLSICKGGRHLIFCH, #57:LVAYQATVCARAKAPPPSWD, and #58:RAKAPPPSWDAMWKCLARLK (see supplementary figure 1). Fine mapping of the three 20-mer peptides was performed and led to the identification of two major histocompatibility complex (MHC) class I epitopes within the 20-mer peptide #58 (NS3 CTL epitope 1: APPPSWDAM, H-2D^b), and (NS3 CTL epitope 2: MWKCLARLK, H-2D^b).

The following negative controls were used: HBcAg (MGLKFRQL, H-2K^d), OVA CTL (SIINFEKL), media/DMSO (e.g. media containing the same DMSO concentration as in the respective peptide pools), and media only. Recombinant NS3-gt1 (rNS3-gt1) protein was obtained from Mikrogen (Neuried, Germany) and rNS3-gt2 was obtained from GenScript (Piscataway, NJ). Chicken egg albumin (OVA) and Concanavalin A (ConA) were purchased from Sigma Aldrich (St. Louis, MO).

Immunization protocols

Mice (5-20 per group) were immunized intramuscularly (i.m.) in the *tibialis cranialis* (TC) muscle one or two times with 0.5 to 50 µg plasmid DNA in a volume of 30-50 µL using the *in vivo* intracellular injection device (*IVIN*) or by regular needle injection. Immediately following administration of the plasmid DNA, tibialis cranialis muscles were subsequently electroporated (EP) using the Cliniporator² device (IGEA, Carpi, Italy) (25) with a 1 ms 600 V/cm pulse followed by a 400 ms 60 V/cm pulse pattern. Indicated groups did not receive EP. Mice were boosted at monthly intervals. A detailed description of the IVIN device, used for delivery of

DNA vaccine, has been described previously (26).

Extraction of RNA and DNA and quantitative real-time PCR

To allow for quantification of HCV RNA levels and to determine the total number of luciferase copies in tumor tissue or cells, purifications of RNA and DNA were performed using the AllPrep DNA/RNA kit (Qiagen, Hilden, Germany). In brief, tumor tissue or cells were homogenized and lysates were used for simultaneous DNA/RNA extraction using the AllPrep DNA/RNA kit according to the protocol of the manufacturer (Qiagen, Hilden, Germany). Isolated total RNA was used for quantification of HCV RNA copies and isolated DNA was used to quantify the number of luciferase copies. The latter was quantified by using an automated qPCR procedure (7500 fast real-time PCR system; Applied Biosystems) according to the manufacturer's instructions with the following primers and probe: 5'-TGCTGCAACACCCCAACATC-3'(forward primer), 5'-ACCTGGCTGGCCACATAGTCC-3'(reverse primer), and 5'FAM-GCAGTCGTC GTGCTGGAACAC-3'TAM (probe). Oligonucleotides were synthesized by Eurofins MWG Operon, Ebersberg, Germany. By using a standard dilution of the luciferase plasmid, the number of plasmid copies per cell could be calculated.

Quantification of HCV RNA amounts by qRT-PCR

Amplification was conducted in triplicate: five microliters of the RNA sample was used for qRT-PCR analysis using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Darmstadt, Germany). HCV-specific qRT-PCRs were conducted in triplicates using the One Step RT-PCR kit (Qiagen, Hilden, Germany). In brief, 15 μ l of reaction mixture contained 0.6 μ l enzyme mixture, 1.5 mM MgCl₂, 1.3 μ M of each JFH-1-specific primer (A246: 5'-TCT GCG GAA CCG GTG AGT A-3'; S146: 5'-GGG CAT AGA GTG GGT TTA TCC A-3',

Sigma Aldrich, Darmstadt, Germany) and the same concentration of each GAPDH-specific primer (GAPDH Taqman probe: 5'-VIC-CAA GCT TCC CGT TCT CAG CCT-TAMRA; GAPDH-S: 5'-GAA GGT GAA GGT CCGAGT C-3'; GAPDH-A: 5'-GAA GAT GGT GAT GGG ATT TC-3'), 0.67 mM of each dNTP, 0.27 μ M JFH-1-specific probe and GAPDH-specific probe (each) (JFH1 probe A195: 5'-6FAM-AAA GGA CCC AGT CTT CCC GGC AAT T- TAMRA -3', Sigma Alrich, Darmstadt, Germany; GAPDH Taqman probe: 5'-VIC-CAA GCT TCC CGT TCT CAG CCT-TAMRA, AppliedBiosystems, Darmstadt, Germany), 5 μ l template RNA and RNase-free water. To determine absolute RNA amounts, a serial dilution of an RNA standard (10^3 to 10^7 HCV RNA copies per reaction) was processed in parallel. Reactions were performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Darmstadt, Germany) using the following program: 50°C for 60 min, 95°C for 15 min and 40 cycles as follows: 95°C for 15 s, 60°C for 1 min.

Chromogenic in situ hybridization of formalin fixed paraffin embedded sections

Chromogenic in situ hybridization (CISH) was performed using the ViewRNA ISH Tissue Assay Kit and ViewRNA Chromogenic Signal amplification Kit provided by Affymetrix according to the manufacturer's protocol (Affymetrix UK Ltd., Cat. No. QVT0050 and QVT0200). In brief, 4 μ m sections of the respective specimen were mounted on glass slides. After fixation with 10% formaldehyde for 1h the specimen were deparaffinized in xylene baths. Heat treatment at 95°C in a water bath in the provided pretreatment solution was performed for 5min and consecutive protease digestion at 40°C was done for 10min. Signal detection with a probe set directed against the positive RNA strand of HCV gt2b (Affymetrix UK Ltd., Cat. No. VF1-10121-01) and subsequent signal amplification steps and signal development with FastRed were carried out according to the manufacturer's protocol using a ThermoBrite

hybridization chamber (Abbot Molecular, Germany). Samples were counterstained with hematoxyline and evaluated with an Olympus microscope.

Detection of IFN γ -producing T cells by ELISpot

Splenocytes from each group of mice were pooled and tested for the presence of NS3/4A-specific T cells. Production of IFN- γ was determined by using a commercially available ELISpot assay (Mabtech, Nacka Strand, Sweden) exactly as described previously (28) using splenocytes from groups of immunized and/or tumor cell-challenged mice. Antigens used for stimulation of NS3/4A-specific immune responses are described under the heading “Peptides and proteins”. The number of spots was counted using the AID iSpot reader and software version 7.0 (AID, Strassberg, Germany). The number of spots (cytokine producing cells) was determined at each concentration of peptide or protein and the results given as the number of IFN- γ -producing cells per 10^6 cells. A mean number of cytokine-producing cells of 50 per 10^6 cells was considered as background.

Histopathological evaluation of the inflammatory response in tumor tissue

Tumor specimens were collected and fixed in 4% zinc paraformaldehyde solution (HistoLab, Gothenburg, Sweden) overnight and thereafter embedded in paraffin. For histopathological evaluation, deparaffinized sections (4 μ m, mounted on a glass slide) from at least two different tumor sections were stained, for each mouse, with Hematoxylin-Eosin, TROMA-I, and CD3. To retrieve antigens, tumor sections were boiled in a pressure cooker (TROMA-I: antigen retrieval solution in 0.01 M citrate buffer pH 6, CD3; antigen unmasking solution pH 9 [H-3301, Vector Laboratories, Burlingame, CA]). After blocking, the primary antibody solution

was added (TROMA-I; rat anti-TROMA-I/Cytokeratin 8 [Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA], CD3; polyclonal rabbit anti-human CD3 [A0452, Dakocytomation, Agilent Technologies, Santa Clara, CA]). Following incubation with the primary antibody, slides were incubated with DAB (ImmPACT DAB Peroxidase substrate kit, SK-4105, Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. The Hematoxylin-Eosin (H&E) staining was performed using standard techniques described in detail elsewhere.

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure 1. Clearance of HCV replicon cells is dependent on both HCV-specific CD4⁺ and CD8⁺ T cells. Kinetics (day 4, 8, 12, and 16) of tumor growth in groups (12 mice/group) of wild type, CD4^{-/-}, and CD8^{-/-} mice immunized twice with 50 µg of an NS3/4A-gt2a-DNA vaccine using *in vivo* EP or groups of non-immunized mice. All mice were challenged with 5x10⁶ tumor cells injected subcutaneously into the right flank. Tumor sizes were measured through the skin every second to third day using a sliding caliper. The tumor volume of individual mice is shown.

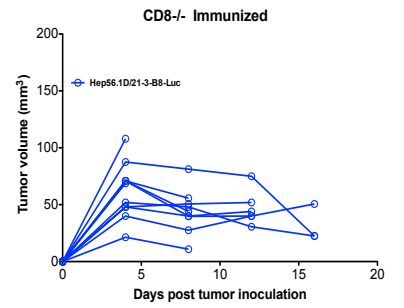
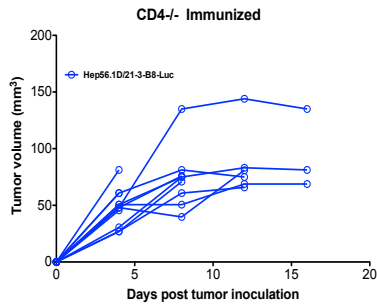
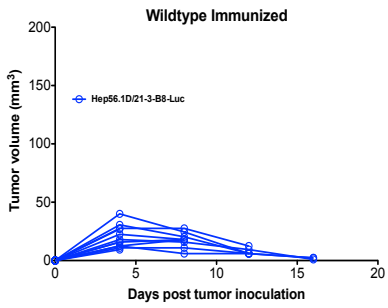
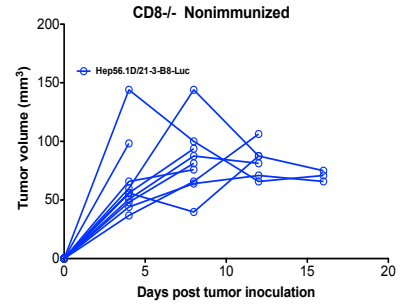
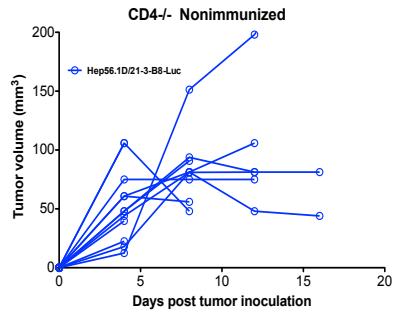
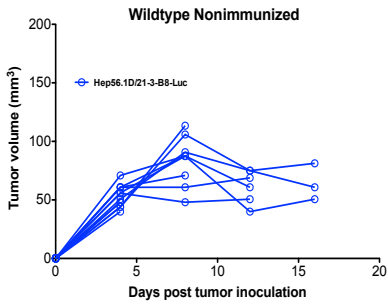
Supplementary Figure 2. Protection against HCV replicon cells *in vivo* requires optimal priming of HCV-specific T cells. Kinetics (day 4, 6, 8, 10, 12, 14, and 16) of tumor growth in groups (20 mice/group) of wild type mice immunized once with (a) an irrelevant HBV core control DNA (Ctrl.) administered intramuscularly (i.m.) in combination with *in vivo* electroporation (EP), (b) with 50 µg of an NS3/4A-gt2a-DNA vaccine administered i.m. in combination with *in vivo* EP, (c) with 50 µg of an NS3/4A-gt2a-DNA vaccine administered i.m. but not combined with EP. All mice were challenged with 5x10⁶ Hep56-derived tumor cells injected subcutaneously into the right flank. Tumors were measured through the skin every second to fourth day using a sliding caliper. The tumor volume of individual mice is shown. Statistically significant differences were found between the control group (Ctrl. DNA/EP) and both NS3/4A-immunized groups as well as between the two immunized groups (p<0.001, by AUC and ANOVA.). At indicated time-points, mice were sacrificed and splenocytes were harvested for determination of the number of IFN-γ spot-forming cells (SFCs) in wild type, CD4^{-/-}, and CD8^{-/-} mice. The production of IFN-γ was determined after *in vitro* stimulation of splenocytes with deescalating doses of recombinant NS3 gt1 or gt2 proteins (10, 2, 0.4 µg/ml),

or OVA protein (10 µg/ml) as indicated. As control antigens OVA-CTL (1 µg/ml), HBcAg-CTL (HBV-CTL, 10, 1, 0.1, 0.01 µg/ml), and ConA (2, 1, 0.5 µg/ml) were used. Results are given as the mean SFCs/10⁶ (+SD) splenocytes with a cut-off set at 50 SFCs/10⁶ splenocytes.

Supplementary Figure 3. Identification of two MHC class I restricted CTL epitopes within the HCV genotype 2a NS3/4A protein complex. From an epitope screening, using a total of 75 20-mer peptides with 10 aa overlap covering the full-length NS3/4A-JFH-1 sequence, we were able to identify three 20-mer peptides which stimulated an NS3-specific immune response: #36:YGRAIPLSCIKGGRHLIFCH, #57:LVAYQATVCARAKAPPSWD, and #58:RAKAPPSWDAMWKCLARLK. Fine mapping revealed two MHC class I epitopes within the 20-mer peptide #58: NS3 CTL epitope 1, APPPSWDAM, H-2D^b and NS3 CTL epitope 2, MWKCLARLK, H-2D^b. Wild type mice were immunized twice with 50 µg of an NS3/4A-gt2a-DNA vaccine intramuscularly (i.m.) in combination with *in vivo* electroporation. Two weeks post last immunization, mice were sacrificed and splenocytes harvested for determination of the number of IFN-γ spot-forming cells (SFCs) by ELISpot assay. The production of IFN-γ was determined after *in vitro* stimulation of splenocytes with deescalating doses of the following antigens: (a) five NS3/4A overlapping peptide pools (15 peptides per pool, total concentration 75 µg/ml) and 15 NS3/4A overlapping peptide pools (5 peptides per pool, total concentration 75 µg/ml); (b) individual 20-mer (#36, #57, and #58) peptides (20 or 2 µg/ml), and two CTL peptides (NS3-CTL epitope 1 and epitope 2; 20 µg/ml). As control antigen ConA (2 or 1 µg/ml) was used. Results are given as the mean SFCs/10⁶ (+SD) splenocytes with a cut-off set at 50 SFCs/10⁶ splenocytes.

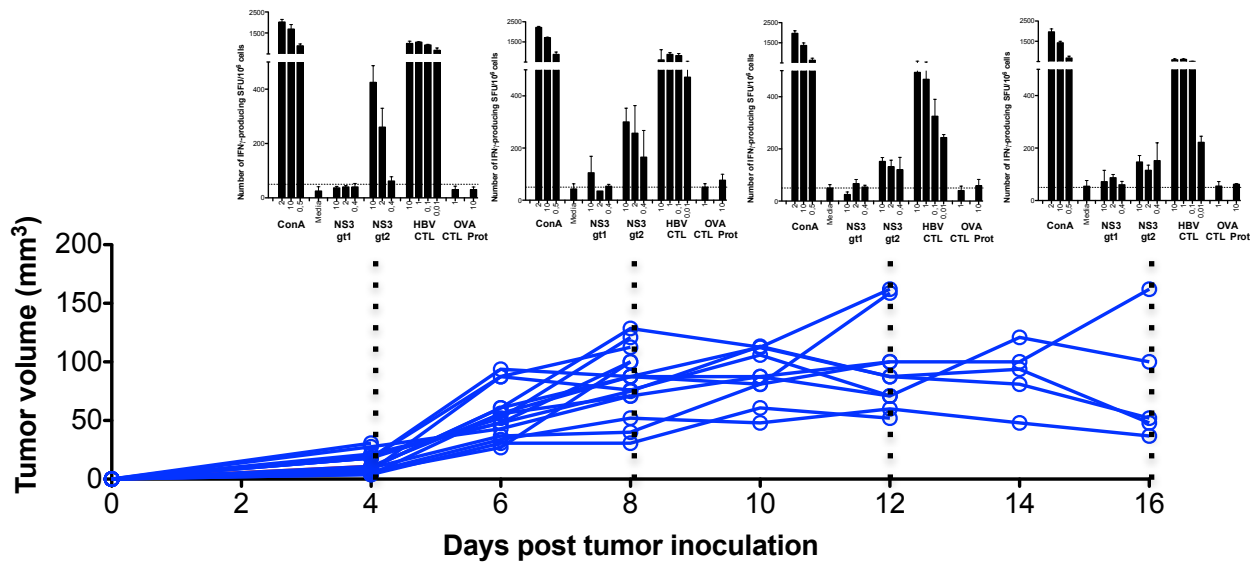
Supplementary Figure 4. Quantification of NS3-specific CD8⁺ T cell responses in wild type mice immunized with an improved NS3/4A-gt2a-stork HBcAg vaccine. The expansion

of NS3-specific CD8⁺ T cells in wild type mice was determined using direct *ex vivo* staining of splenocytes with the DimerX technology, where an antibody has been grafted with parts of the H-2^b molecule to allow for binding of CTL peptides. Mice (5 per group) were immunized once with 50 µg of an improved NS3/4A-gt2a-stork HBcAg-DNA vaccine or left non-immunized. Mice were immunized intramuscularly (i.m.) using the *in vivo* intracellular injection (*IVIN*) device directly followed by *in vivo* electroporation (EP). Quantification of APPPSWDAM epitope-specific CD8⁺ T cells was performed two weeks post immunization. APPPSWDAM epitope-specific CD8⁺ T cells are shown as the mean percentage of NS3-specific CD8⁺ T cells (+SD). The statistical difference between the groups is indicated as $p < 0.05$ as determined with the Mann-Whitney U test.

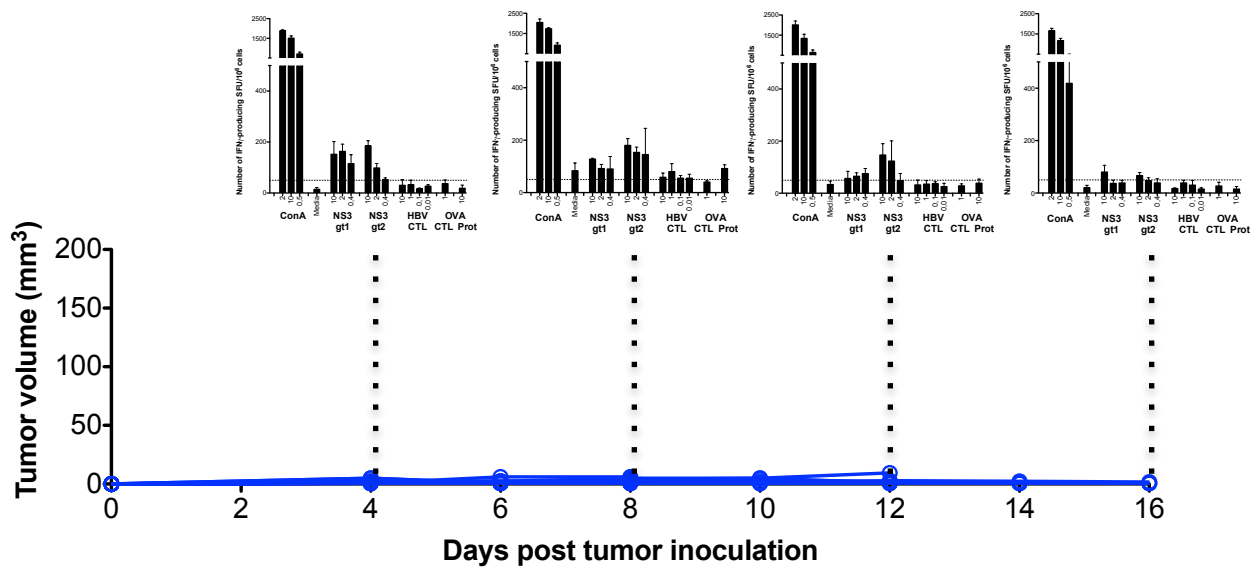


Supplementary Figure 1

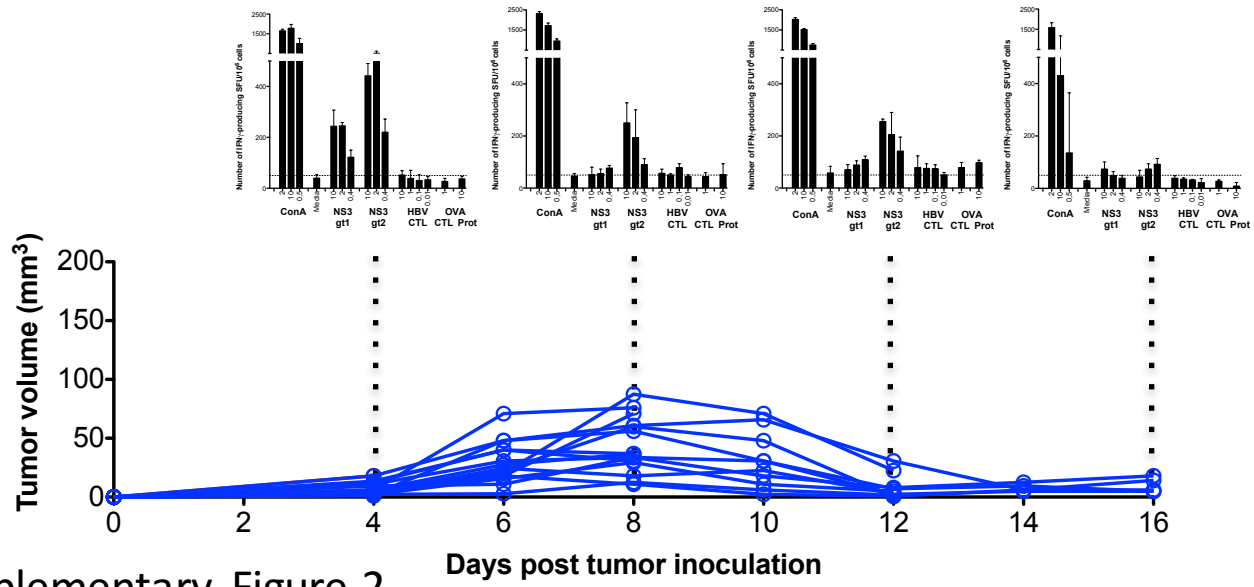
a 50 μ g Ctrl. DNA/EP



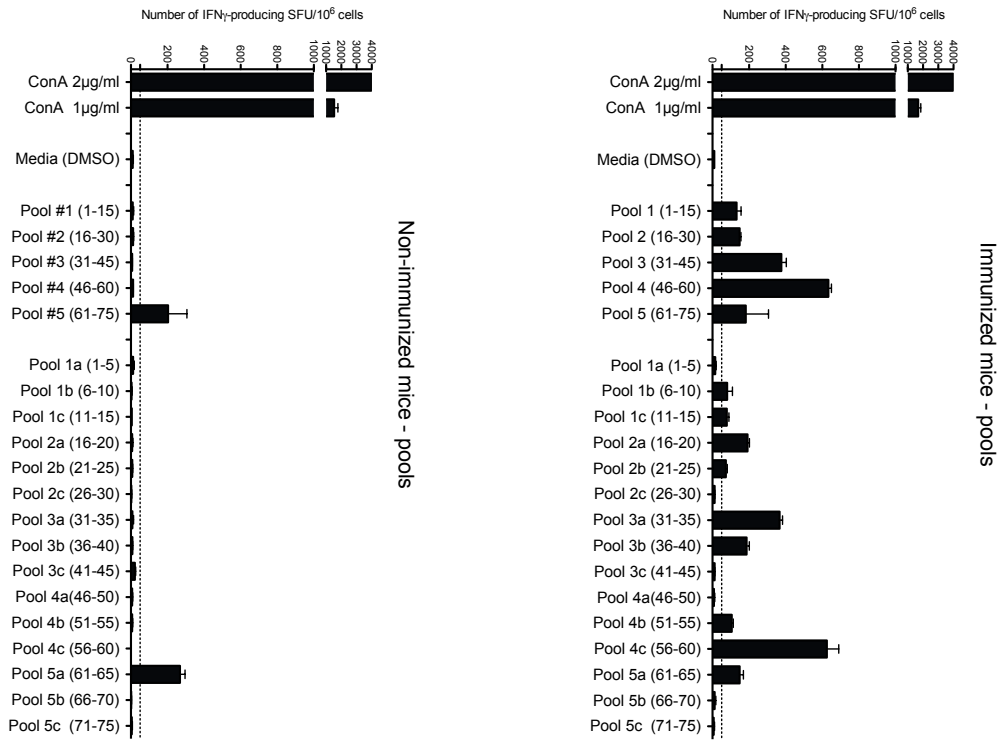
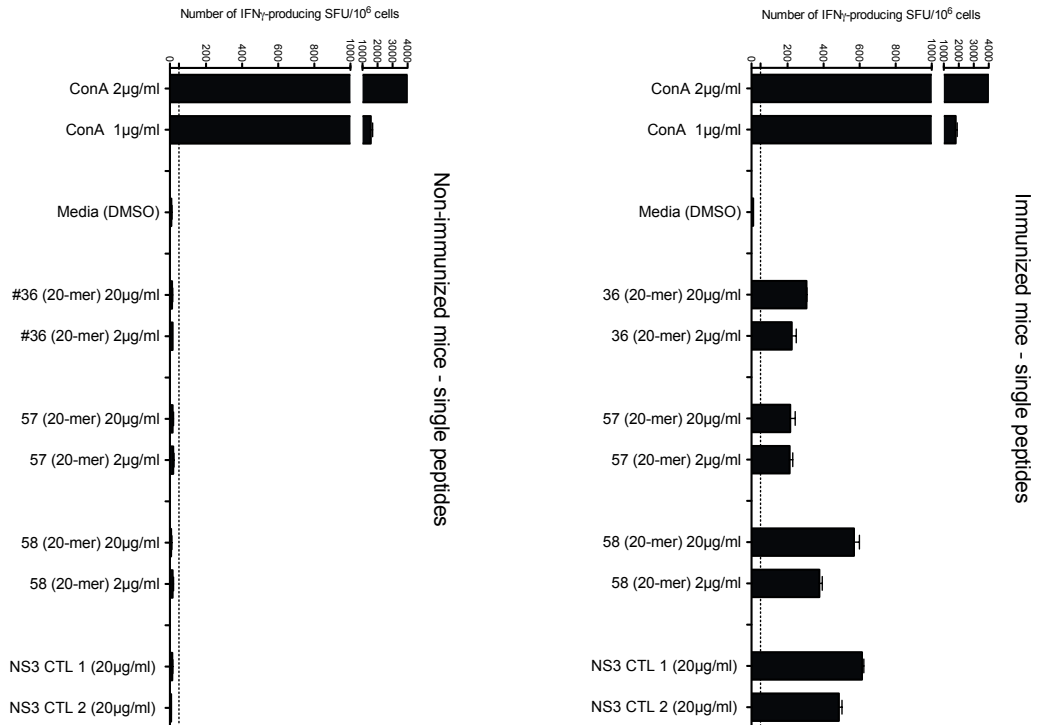
b 50 μ g NS3/4A DNA/EP



c 50 μ g NS3/4A DNA



Supplementary Figure 2

a**b**

Ex vivo frequency of NS3-specific CD8⁺ T cells

