Supplementary Information for

Harnessing anti-cytomegalovirus immunity for local immunotherapy against solid tumors
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Supplementary Information Text

MCMV production and titration

MCMV Smith strain (ATCC, VR-1399) was amplified on M2-10B4 cells. The in vitro virus stock was used to infect (1 x 10^5 pfu, footpad) BALB/c mice and generate the first salivary gland homogenate followed by two serial passages (1 x 10^4 pfu, i.p.). Third passage salivary gland homogenates were resuspended in 50 mM Tris pH 7.8 supplemented with 12 mM KCl, 5 mM EDTA and 0.1% BSA, and stored in liquid nitrogen. All MCMV stocks were titrated by plaque assay on M2-10B4 cells.

Flow cytometry

Tetramer staining: APC-conjugated MHC-I tetramers were used to detect E7-, IE3-, m45-specific CD8^+ T cells and a PE-conjugated MHC-II tetramer to detect m25-specific CD4^+ T cells (NIH tetramer core facility). Cells were incubated in FACS buffer containing Fc receptor blocking anti CD16/32 antibody (24G2; Bio X Cell) with conjugated tetramers for 30min at 4°C, followed by an additional incubation of 30min at 4°C with anti CD3, CD4, CD8α, CD44, CD62L, CD69, CD103, CD127, and PD-1 antibodies (SI Appendix, Table 2).

Tumor immune infiltrate staining: After Fc receptor blocking, single cell suspensions were stained with anti B220, CD3, CD4, CD8α, CD11b, CD11c, CD19, CD45, F4/80, Ly6C, Ly6G, NK1.1 and MHC-II antibodies for 30min at 4°C (SI Appendix, Table S2).

All samples were fixed in paraformaldehyde solution (Cytofix, BD Bioscience) and acquired on a FACS Canto II flow cytometer using the BD High Throughput Sampler (BD Biosciences). Data were analyzed using FlowJo v10 (TreeStar).

In vitro stimulation and intracellular cytokine staining

In vitro antigen specific T cell stimulation was performed with the indicated MCMV-derived minimal epitope peptides (Genscript, SI Appendix, Table S1) alone or mixed at a concentration of 5µg/ml. Splenocytes were incubated for 6hrs at 37°C 5% CO2 in RPMI supplemented with 10% FBS, L-Glutamine, 2-mercaptoethanol, sodium pyruvate, antibiotics and brefeldin A + monensin (BD Bioscience). After incubation, cells were washed and labelled immediately with live/dead yellow dead cell stain kit (Invitrogen) followed by surface staining with CD3-BV421, CD4-APC.Cy7, CD8-PE and CD44-PE.Cy7 antibodies (SI Appendix, Table S2). Cells were then fixed and permeabilized using commercial buffers (BD Bioscience).

IFN-γ ELISPOT

CD8^+ T cell responses against two validated neoepitopes in the MC-38 colon cancer model (ADPGK and RESP1) were assessed by IFN-γ ELISpot (Diaclone) following manufacturer instruction. Briefly, microtiter plates (PVDF, Millipore) were coated overnight at 4°C with anti-IFN-γ
capture purified antibody then blocked with RPMI containing 10% FBS for 2hrs at 37°C. Splenic CD8\(^{+}\) T cells were enriched using mouse CD8\(\alpha^{+}\) T Cell Isolation Kit (Miltenyi Biotec), plated at 2x10\(^5\) cells per well and incubated with or without the MC-38 derived neoepitopes (Genscript, SI Appendix, Table S1) at 1\(\mu\)g/ml for 36 h. After incubation, plates were washed and incubated with biotin-conjugated IFN-\(\gamma\) antibodies. Spot forming units were developed following manufacturer's instructions using alkaline phosphatase-conjugate streptavidin and NBT/BCIP reagents. Spots were counted using an Immunospot CTL reader (CTL, USA) and expressed per 2x10\(^5\) CD8\(^{+}\) T cells.

**Cytokines and chemokines measurement**

Tumor tissues and EDTA-K plasma samples were obtained 24-36hrs after the last i.t. treatment and were kept frozen until further processing. Tumor tissue lysate were obtained as described\(^4\) by bead bashing (TissueLyser LT, Qiagen) in PBS, supplemented with 2mM Mg\(^{2+}\), Benzonase 25U/ml (Sigma) and a protease inhibitor cocktail (Complete mini, Roche). Protein content was assessed in each sample using bicinchoninic acid (BCA) assay kit (Pierce). Cytokine and chemokine content was measured using a cytokine release syndrome panel (Legendplex, Biolegend) following manufacturer's instructions. All data are expressed in pg per mg of protein content for tissue lysates and pg per ml for plasma samples.

**Whole tumor RNA gene expression analysis**

Tumor tissues were collected 48hrs after the last of three i.t. injections and flash-frozen in liquid nitrogen. Frozen tumors were pulverized in 1mL Trizol (Thermo Fisher) using ceramic beads in reinforced tubes and a Precellys24 Homogenizer (Bertin Instruments). A volume of 0.2mL of chloroform was added to the tumor lysate and mixed thoroughly before centrifugation at 12,000rpm for 15min at 4°C. Aqueous phase was further processed using the RNesy Mini QIAcube Kit (Qiagen). Purified RNA was analyzed and quantified using Nanodrop device (Nanodrop Products). A total of 200ng of RNA was used for gene expression analysis using the mouse nCounter PanCancer Immune Profiling Panel (Nanostring Technologies). Sample preparation and hybridization was done following manufacturer's instruction. The gene expression data were normalized using the nSolver Analysis Software 4.0 (Nanostring Technologies) before further analysis.

**Histology and immunofluorescence**

Fresh tumor tissues were embedded in OCT (Tissue-Tek) and stored frozen at -80°C. Ethanol-fixed 6-\(\mu\)m frozen sections were stained with Alexa-488 CD4 and Alexa-594 CD8 antibodies (Biolegend) and coverslips were mounted with an antifade reagent containing 4,6-diamidino-2-phenylindole for nuclei staining (Prolong Gold, Molecular Probes). Confocal images were acquired at the Confocal
Microscopy Core Facility, Center for Cancer Research, NCI, NIH, with Zeiss ZEN software on a Zeiss LSM 780 Confocal system using a 40X oil immersion objective and 364-nm, 488-nm, and 543-nm lasers. Images were analyzed using Adobe Photoshop, and color channel levels were adjusted uniformly across images.

Histological analyses (Histoserv, MD) of tumor tissue were performed on 4% PFA-fixed tumor tissue sections counterstained with hematoxylin and eosin. All tissue slides were analyzed with the Aperio software (Leica).
Fig. S1. The breadth of MCMV CD8+ T cell responses is shown by intracellular production of IFN-g staining by MCMV specific CD8+ T cells. Representative FACS plots of splenocytes stimulated in vitro for 6hrs with IE3b, m38, m57, m139, m141, m164 peptides from, C57BL/6 mice 2 weeks post MCMV infection.
Fig. S2. Absence of MCMV reactivation in tumor-bearing mice latently infected with MCMV. Mice latently infected with MCMV were transplanted with TC-1 tumors. When tumors reached 100 mm$^3$, they were injected i.t. 6 times every 2 days with saline, a mixture of poly I:C together with MCMV-derived MHC-I-restricted peptide alone or in combination with MCMV-derived MHC-II-restricted peptides. IE-1 gene expression was analyzed by (A) RT-PCR in tumor mRNA and (B) PCR in tumor DNA using nested MCMV IE-1 primer pairs. As controls RNA from salivary glands from uninfected, latently and acutely infected mice or from TC-1 tumor cells uninfected or infected in vitro with MCMV. PCR products were analyzed by agarose gel (0.8%) electrophoresis.
**Fig. S3.** In vitro multiplex chemokine and cytokine production analysis. TC-1 cells were exposed to low (LMW) and high (HMW) molecular weight poly I:C. PolyI:C complexed with lipofectamine is indicated (TFX). Lipofectamine alone is indicated (LIPO). After 48hrs incubation, culture supernatant was assessed with the inflammation Legendplex panel (Biolegend). Data are representative of 2 independent experiments (n=3) and presented as fold change versus conditioned medium with TC-1 alone.
Fig. S4. Intratumoral administration of MCMV epitopes induces broad local immune activation of the tumor microenvironment. Heatmap of log2 differential expression versus saline. MCMV infected C57BL6 mice were transplanted with TC-1 tumors. When tumors reached 100mm3 they were injected i.t. 3 times every 2 days with saline, poly I:C (50µg), MHC-I (1µg each, IE3, m38 and m45) and MHC-II (3µg, m139) peptides +/- pl:C. Tumor RNA was extracted 48hrs after the third injection and analyzed with the Nanostring PanCancer Immune Profiling panel (n=4). (A) Heatmap of differentially expressed genes compared to saline (log2 fold change). (B) Selected differentially regulated genes involved in T cell function, antigen presentation and tissue damage and inflammation (normalized count). Statistical significance was assessed by Dunn test (* * * *P<0.0001, ***P<0.001, **P<0.01, *P<0.05 n.s.: not significant), representative of a single experiment (n=4).
Fig. S5. Recall of inflationary and non-inflationary MCMV-specific CD8+ T cell responses in blood and E7 response in long-term survivors. (A) Experimental design. MCMV infected C57BL/6 mice were transplanted with TC-1 tumors. Tumors were injected on 6 consecutive times 2 days apart with MCMV-derived peptides + poly I:C. Group treatments as follow: saline (6x), MHC-I (IE3, m38, m45, 1µg each, 6x), sequentially MHC-II (m139, 3µg, 3x) then MHC-I (IE3, m38, m45, 1µg each, 3x), MHC-I (IE3, m38, m45, 1µg each, 3x) then MHC-II (m139, 3µg, 3x), or MHC-II (m139, 3µg, 6x), MHC-I (IE3, m38, m45, 1µg each) + MHC-II (m139, 3µg) (6x). (B) m45- and IE3-specific tetramer+CD8+ T cells in blood 48hrs after treatment. (C) E7-specific tetramer+CD8+ T cells MCMV peptide-treated partial responder versus complete responder/long-term survivors. Statistical significance compared to saline-treated was assessed by Dunn's test (***P<0.001, **P<0.01, *P<0.05, n.s.: not significant), representative of 2 experiments (n=6).
Fig. S6. Functional T cell avidity of MCMV-specific CD8+ T cells. C57BL/6 mice were infected with MCMV and T cell responses were analyzed in spleen cell suspensions by intracellular cytokine staining after in vitro titration with MCMV-derived non-inflationary (m45) and inflationary (IE3) and (m38). Production of (A) IFN-γ, (B) TNF-α by MCMV-specific CD8+ T cells with decreasing concentration of m45 (open square), IE3 (open circle) and m38 (open triangle) expressed as percent of maximum cytokine production.
**Fig. S7.** Tissue distribution and Ki67 expression of MCMV-specific T cells. MCMV-infected mice were treated i.t. 3 times with MCMV-derived peptides + pI:C (50µg). Group treatments as follow: saline, pI:C, MHC-II (m25, 1µg) + pI:C, MHC-I (IE3, m38, m45, 1µg) + pI:C or MHC-II (1µg) + MHC-I (1µg) + pI:C. (A and B) FACS analysis of MCMV CD8+ (IE3, m45) and CD4+ (m25) T cell responses in (A) tumor draining lymph nodes and (B) tumor tissue 48hrs after last intratumoral injection. Ki67 expression was analyzed by flow cytometry on MHC-I (IE3 and m45) and MHC-II (m25) MCMV tetramers (C) in tumor draining lymph nodes and (D) in tumors after i.t. injection. Data are expressed as mean fluorescence intensity of Ki67 by tetramer+ cells. Statistical significance was assessed by Dunn’s test for MCMV tetramer infiltration (**P<0.01, *P<0.05, n.s.: not significant), (n=4, 2 representative experiments).
Fig. S8. Intratumoral injection of poly I:C alone does not delay B16-F10 tumor growth. MCMV infected C57BL/6 mice were transplanted with B16-F10 tumors. Mice were treated 6 times with saline or pI:C (50 µg). Tumor growth was monitored until mice reached humane endpoint.
Fig. S9. Immune infiltrate analysis of B16 tumor tissues after intratumoral injection of MCMV-derived epitopes. Experimental design is as described in Fig 8. Mice were treated 2 times with saline, pI:C (50 µg) alone or admixed with MHC-I (m45, 1µg) and MHC-II (m139, 1µg) restricted peptides. Tumor tissues were collected between 24h and 36h after the second i.t. injection and cellular infiltrate was analyzed by multiparameter flow cytometry to assess the presence of the indicated cells: m45-tetramer+CD3+CD8+ (tetramer), CD3+CD8+ (CD8), CD3+CD4+ (CD4), CD3-CD4-CD8- (non-conventionnal T), CD3-NK1.1+ (NK), CD3+NK1.1+(NKT), CD19+ (B cells), CD11b+Ly6C-LyG+ (granulocytic) and CD11b+LyC+Ly6G- (monocytic) and MHC-II+CD11c+ (dendritic). Data are shown as individual values and mean +/- SEM (n=4-8, 2 independent experiments). Statistical significance was assessed by a one-way ANOVA followed by Tukey’s test for multiple comparison analysis (**P<0.01, *P<0.05, n.s.: not significant).
Fig. S10. Cytokine/chemokine production production in B16 tumor tissue and plasma after intratumoral injection of MCMV-derived epitopes. Experimental design is as described in Fig 8. Mice were treated 2 times with saline, pI:C (50 µg) alone or admixed with MHC-I (m45, 1 µg) and MHC-II (1 µg m139) restricted peptides. (A) Tumor tissues and (B) plasma were collected between 24h and 36h after the second i.t. injection. Cytokine and chemokine content analysis using Legendplex cytokine release syndrome panel. Data are shown as individual values and mean ± SEM (n=4-8, 2 independent experiments). Statistical significance was assessed by one-way ANOVA followed by Tukey’s test for multiple comparison analysis (**P<0.01, *P<0.05, n.s.: not significant).
**Fig. S11.** Intratumoral injection of MCMV MHC-I (0.1μg each, IE3, m38, m45) restricted and MHC-II (1μg each, m09, m25, m45, m83, m139, m142) restricted inhibits growth of MC38 tumors. (A) Experimental design, mice were treated on 6 times 2 days apart with saline, pI:C, MHC-II or MHC-II and MHC-I restricted MCMV epitope + pI:C small molecular weight. (B) Tumor volume was monitored 2-3 times a week. (C) ELISPOT IFN-γ production by CD8+ T cells against RPS1 and ADPGK MC38 neoepitopes. (B) Tumor growth is shown as mean tumor volume for each group and standard error. Statistical significance was assessed by Dunn’s test for tumor volume statistical analysis (**P<0.001, **P<0.01, *P<0.05, n.s.: not significant). Representative of 2 experiments (n=6).
Table S1. Peptides epitopes

| Antigen | Organism | Ammino acid sequence     | MHC restriction |
|---------|----------|--------------------------|-----------------|
| m09     | MCMV     | GYLlYIPSAGNSFDL          | I-A<sup>b</sup> |
| M25     | MCMV     | NHlYETPISATAMVI          | I-A<sup>b</sup> |
| m45     | MCMV     | QTSPSTPIPIPAPRC          | I-A<sup>b</sup> |
| m83     | MCMV     | TLRyAKANGTPPDSL          | I-A<sup>b</sup> |
| m139    | MCMV     | TRPryPRVCDASLS           | I-A<sup>b</sup> |
| m142    | MCMV     | RSYrLTAAAVTAvlQ          | I-A<sup>b</sup> |
| m38     | MCMV     | LSPPMFRV                 | H2-K<sup>b</sup> |
| m45     | MCMV     | HGIRNASFI                | H2-D<sup>b</sup> |
| M57     | MCMV     | SCLEFWQRV                | H2-K<sup>b</sup> |
| M139    | MCMV     | TVYGFCll                 | H2-K<sup>b</sup> |
| M141    | MCMV     | VIDAFSRL                 | H2-K<sup>b</sup> |
| M164    | MCMV     | AGPPRYSRI                | H2-D<sup>b</sup> |
| IE3     | MCMV     | RALEyKNL                 | H2-K<sup>b</sup> |
| IE3b    | MCMV     | VRKAVDETRARmgMr          | H2-K<sup>b</sup> |
| e7      | HPV16    | RAHYNIvTF                | H2-D<sup>b</sup> |
| ADPGK   | MC38     | ASMTNMELM                | H2-K<sup>b</sup> |
| RESP1   | MC38     | AQLANDVVL                | H2-K<sup>b</sup> |

<sup>a</sup>Genscript Biotech
### Table S2. Antibodies and tetramer

| Antigen   | Clone     | Fluorochrome | Application | Company          |
|-----------|-----------|--------------|-------------|------------------|
| CD3       | 17A2      | BV421        | FC          | Biolegend        |
| CD3       | 145-2C11  | PE/Cy7       | FC          | Biolegend        |
| CD3       | 17A2      | PE.Cy7       | FC          | Biolegend        |
| CD4       | RM4-5     | APC/Cy7      | FC          | Biolegend        |
| CD8       | 53-6.7    | PE           | FC          | Biolegend        |
| CD8       | 53-6.7    | BV570        | FC          | Biolegend        |
| CD11b     | M1/70     | FITC         | FC          | Biolegend        |
| CD11c     | HL3       | PE           | FC          | Biolegend        |
| CD16/CD32 | 24G2      | Purified     | FC<sup>a</sup> | BioXcell |
| CD19      | 1D3       | Percp.Cy5.5  | FC          | Biolegend        |
| CD25      | PC61      | Percp.Cy5.5  | FC          | Biolegend        |
| CD44      | IM7       | PE/Cy7       | FC          | Biolegend        |
| CD44      | IM7       | Percp/Cy5.5  | FC          | Biolegend        |
| CD45      | 30-F11    | BV421        | FC          | Biolegend        |
| CD62L     | MEL-14    | FITC         | FC          | Biolegend        |
| CD69      | H1.2F3    | PE/Cy7       | FC          | Biolegend        |
| CD103     | 1E7       | Percp/Cy5.5  | FC          | Biolegend        |
| CD127     | SB/199    | PE           | FC          | Biolegend        |
| CD223     | C9B7W     | APC          | FC          | Biolegend        |
| CD279     | 29F.1A12  | FITC         | FC          | Biolegend        |
| F4/80     | BM8       | APC          | FC          | Biolegend        |
| H-2D<sup>b</sup>/E7<sup>49-57</sup> | N/A<sup>b</sup> | PE | FC | NIH Tetracer Core |
| H-2D<sup>b</sup>/E7<sup>49-57</sup> | N/A<sup>b</sup> | APC | FC | NIH Tetracer Core |
| H-2D<sup>b</sup>/m45<sup>985-993</sup> | N/A<sup>b</sup> | APC | FC | NIH Tetracer Core |
| H-2K<sup>b</sup>/m122<sup>390-404</sup> | N/A<sup>b</sup> | APC | FC | NIH Tetracer Core |
| IA<sup>b</sup>/m25<sup>409-423</sup> | N/A<sup>b</sup> | PE | FC | NIH Tetracer Core |
| I-A/I-E   | M5/114.15.2 | Percp.Cy5.5 | FC | Biolegend |
| IFN-γ     | XMG1.2    | FITC         | ICFC<sup>c</sup> | Biolegend |
| IL-2      | JES6-1A12 | Percp/Cy5.5  | ICFC        | Biolegend        |
| Ki-67     | 16A8      | FITC         | ICFC        | Biolegend        |
| Ly6C      | HK1.4     | PE.Cy7       | FC          | Biolegend        |
| Ly6G      | 1A8       | APC.Cy7      | FC          | Biolegend        |
| NK1.1     | PK136     | PE           | FC          | Biolegend        |
| TNF-α     | MP6-XT22  | APC          | ICFC        | Biolegend        |

<sup>a</sup>Flow Cytometry  
<sup>b</sup>Not Applicable  
<sup>c</sup>Intra-Cellular Flow Cytometry
SI References

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DATASETS (included as separate files)

Dataset S1. Cell_type profiling z-score shown in Fig. 3C (Nanostring Pancancer Immune Profiling)

Dataset S2. Nanostring Pancancer Immune Profiling Differential Expression Analysis shown in Fig. 4B (Nanostring Pancancer Immune Profiling)