International Journal of
*Molecular Sciences*

MDPI

*Supplementary Materials*

# CARD9 deficiency increases hippocampal injury following acute neurotropic picornavirus infection but does not affect pathogen elimination

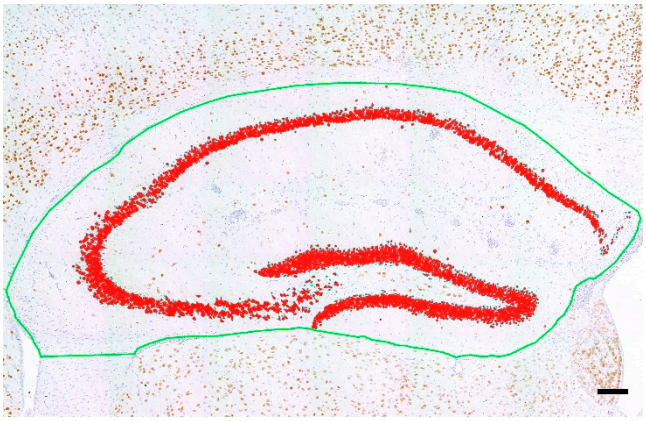

**Figure S1.** Densitometric analysis using the BZ-II Analyzer software (Keyence Corporation, Osaka, Japan). Representative image of a digitalized slide of a TMEV-infected wild type mouse at 7 days post infection (dpi), showing neuronal nuclear antigen (NeuN)-positive area (red area) in the cornu ammonis (CA) and dentate gyrus regions (brightness intensity extraction). The entire hippocampal area is determined by circumscribing the hippocampal area using the hybrid cell count function of the software (green line). NeuN-specific immunohistochemistry, scale bar: 100 μm.

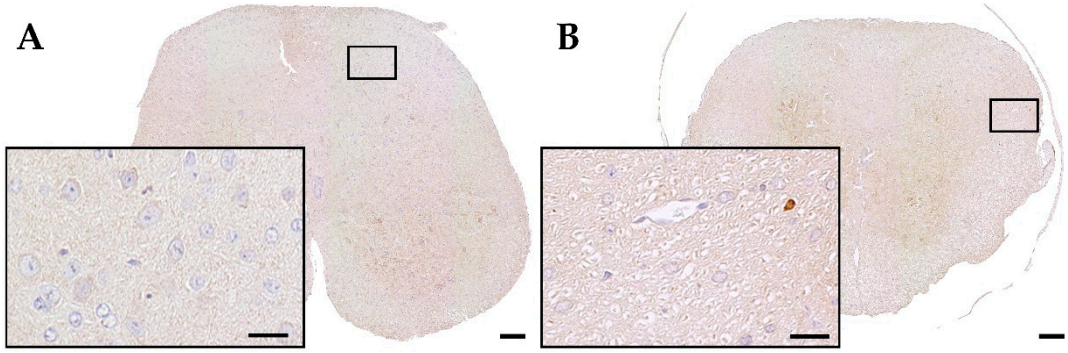


**Figure S2.** Detection of Theiler's murine encephalomyelitis virus (TMEV) antigen in the spinal cord of C57BL/6 wild type (WT) and CARD9$^{-/-}$ mice. (**A**) Lack of TMEV-infected cells in spinal tissue of a WT mice at 14 dpi. (**B**) Single TMEV-infected cell in the spinal cord of a CARD9$^{-/-}$ mouse at 14 dpi. The quantification of TMEV-infected cells in murine spinal cords revealed no significant difference between WT and CARD9$^{-/-}$ mice. TMEV-specific immunohistochemistry, scale bar: 100 μm (A-B) and 20 μm (A-B inserts).

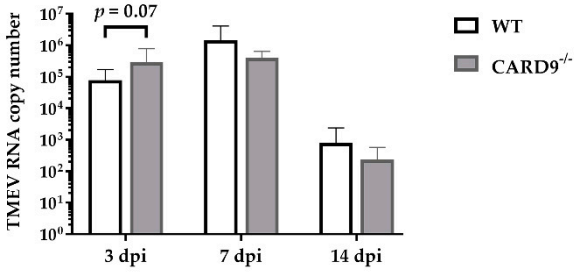


**Figure S3.** Quantification of Theiler's murine encephalomyelitis virus (TMEV) RNA copy numbers in the cerebrum of C57BL/6 wild type (WT) and CARD9$^{-/-}$ mice. Data were analyzed using Mann-Whitney *U*-test. 3 dpi: WT n=10, CARD9$^{-/-}$ n=10; 7 dpi: WT n=10, CARD9$^{-/-}$ n=9; 14 dpi: WT n=10, CARD9$^{-/-}$ n=10. Graphs show mean values with SD.

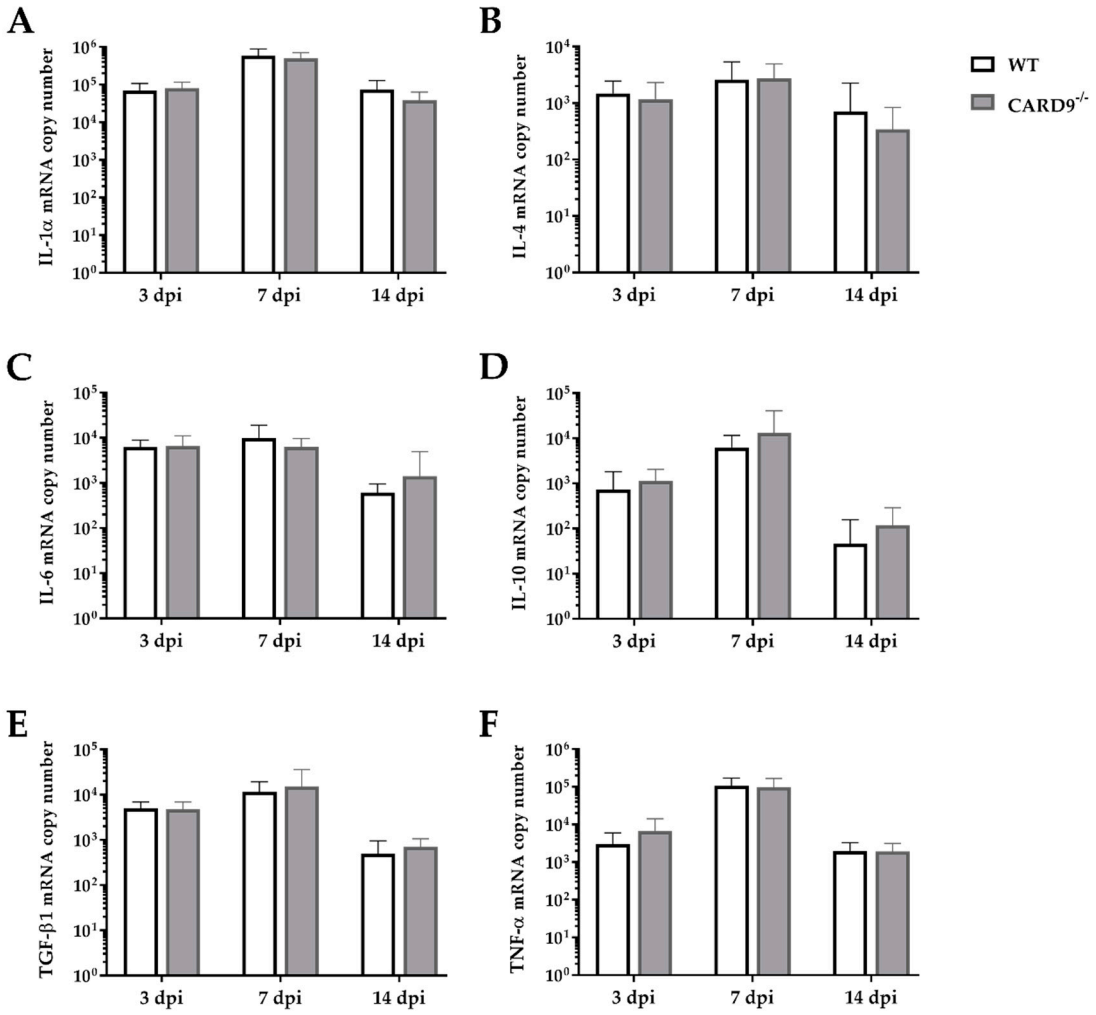


**Figure S4.** Quantification of cytokine mRNA expression in the cerebrum of TMEV-infected C57BL/6 wild type (WT) and CARD9$^{-/-}$ mice. Quantification of (**A**) interleukin (IL)-1$\alpha$, (**B**) IL-4, (**C**) IL-6, (**D**) IL-10, (**E**) transforming growth factor (TGF)-$\beta$1, and (**F**) tumor necrosis factor (TNF)-$\alpha$ mRNA levels in the cerebrum by reverse transcription quantitative polymerase chain reaction. Difference between WT and CARD9$^{-/-}$ mice were analyzed using the Mann-Whitney *U*-test. 3 dpi: WT n=10, CARD9$^{-/-}$ n=10; 7 dpi: WT n=10, CARD9$^{-/-}$ n=9; 14 dpi: WT n=10, CARD9$^{-/-}$ n=10. Graphs show mean values with SD.

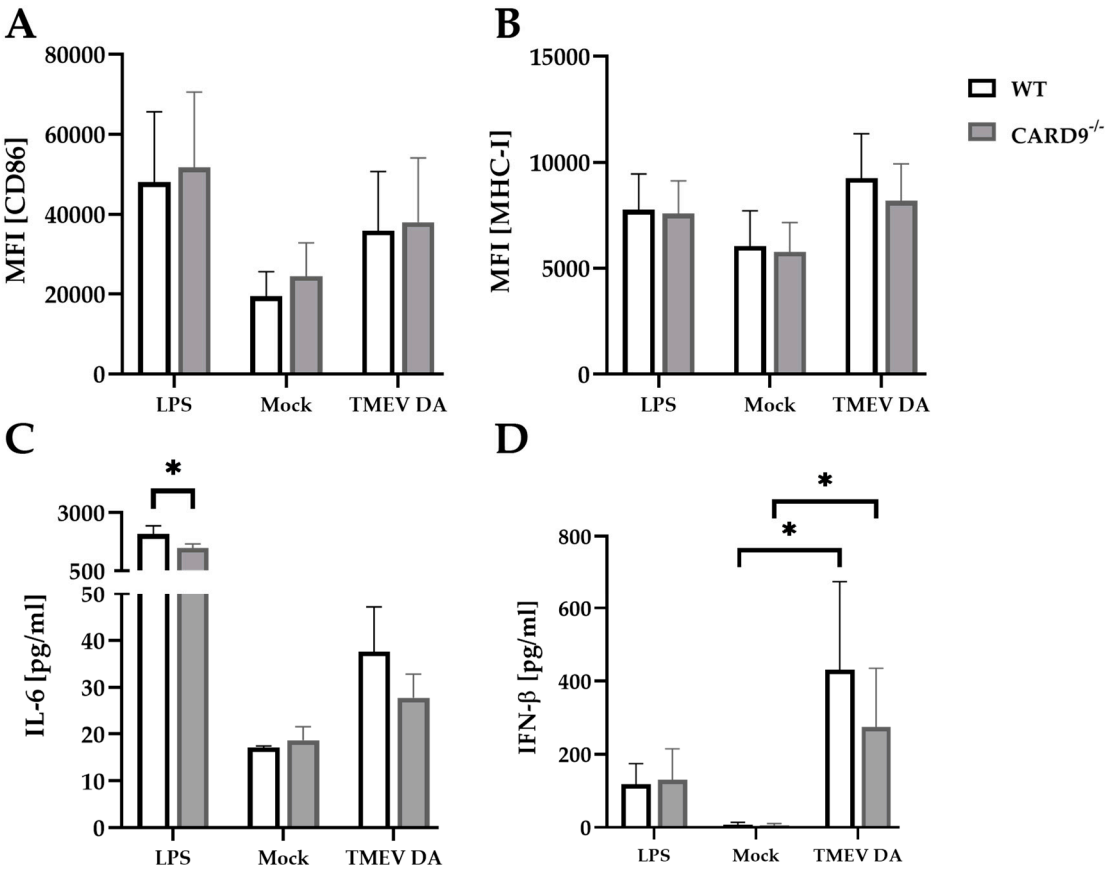


**Figure S5.** Activation of C57BL/6 wild type (WT) and CARD9$^{-/-}$ bone marrow-derived dendritic cells (BMDCs) following Daniels strain of Theiler's murine encephalomyelitis virus (TMEV DA) stimulation. The mean fluorescence intensity (MFI) of activation markers (**A**) CD86 and (**B**) MHC-I analyzed by flow cytometry. The cytokine productions: (**C**) interleukin (IL)-6 and (**D**) interferon (IFN)-β of stimulated BMDCs analyzed by ELISA. The BMDCs were stimulated by lipopolysaccharide (LPS, positive control), medium (Mock, negative control), or TMEV-DA. * Significant difference between WT and CARD9$^{-/-}$ mice ($p \leq 0.05$; Mann-Whitney *U*-test). 3 dpi: WT n=10, CARD9$^{-/-}$ n=10; 7 dpi: WT n=10, CARD9$^{-/-}$ n=9; 14 dpi: WT n=10, CARD9$^{-/-}$ n=10. Data are displayed as mean with SD.

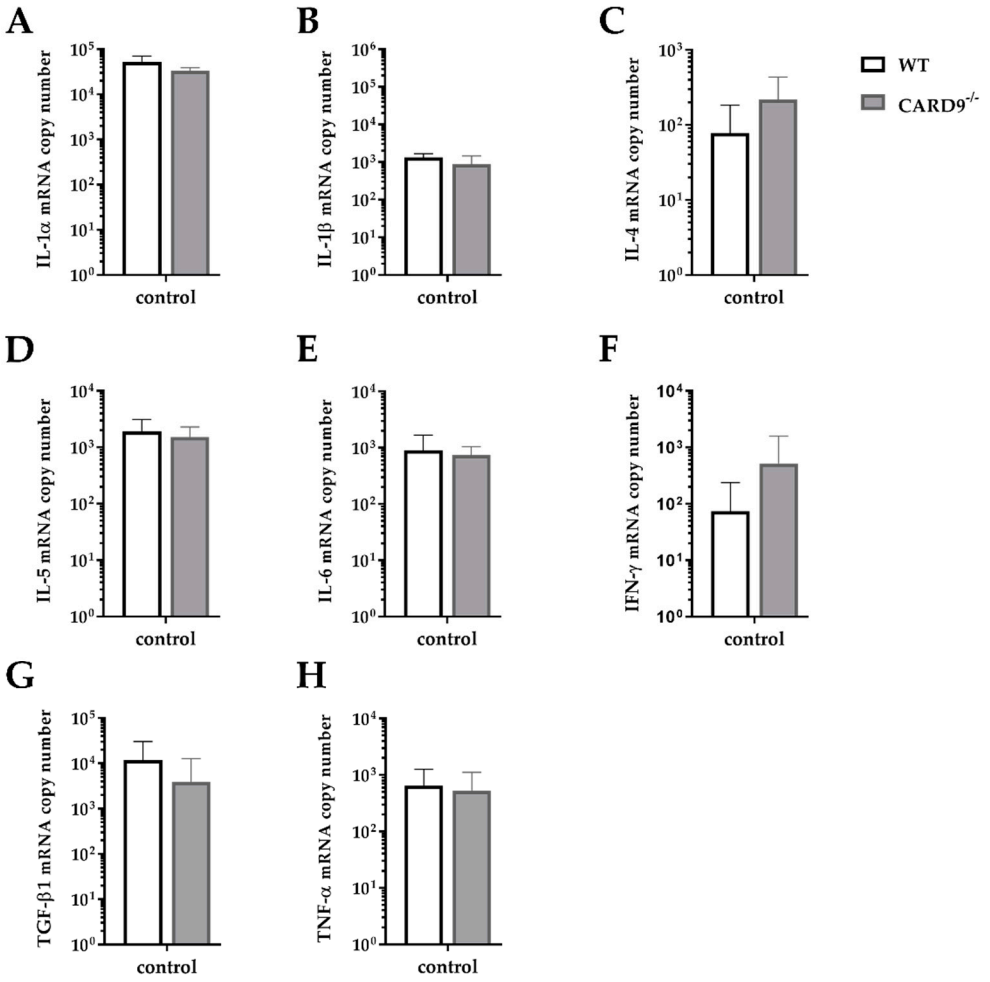


**Figure S6.** Quantification of cytokine mRNA expression in the cerebrum of non-infected C57BL/6 wild type (WT) and CARD9$^{-/-}$ mice. Quantification of (**A**) interleukin (IL)-1$\alpha$, (**B**) IL-1$\beta$, (**C**) IL-4, (**D**) IL-5, (**E**) IL-6, (**F**) interferon (IFN)-$\gamma$, (**G**) transforming growth factor (TGF)-$\beta$1, and (**H**) tumor necrosis factor (TNF)-$\alpha$ mRNA levels by reverse transcription quantitative polymerase chain reaction. Difference between WT and CARD9$^{-/-}$ mice were analyzed using the Mann-Whitney *U*-test. IL-10 mRNA was not detectable in WT and CARD9$^{-/-}$ mice. Graphs show mean values with SD. n: 5 WT and 5 CARD9$^{-/-}$ mice.

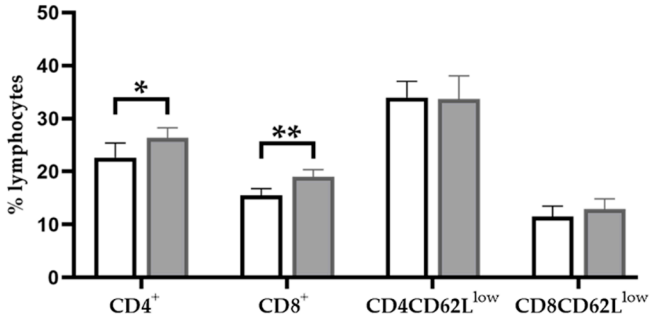


**Figure S7.** Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cell responses of non-infected C57BL/6 wild type (WT) and $CARD9^{-/-}$ mice. Quantification of $CD4^+$, $CD8^+$, $CD4^+CD62L^{low}$, and $CD8^+CD62L^{low}$ T cells in the spleen of non-infected C57BL/6 wild type (WT) and $CARD9^{-/-}$ mice. (* = $p \leq 0.05$; ** = $p \leq 0.01$, Mann-Whitney *U*-test). Data are shown as mean with SD. n: 5 WT and 5 $CARD9^{-/-}$ mice.

**Table S1.** Details of antibodies used for immunohistochemistry

| **Primary antibody** | **Clonality/clone** | **Supplier; catalog number** | **Dilution** | **Pre-treatment** | **Blocking serum** | **Secondary antibody** | **Specificity** |
|---|---|---|---|---|---|---|---|
| arginase 1 | polyclonal | Thermo Fisher Scientific PA5-29645 | 1:200 | HIER | Goat | Goat anti-rabbit | M2-macrophages/ microglia |
| CD107b | monoclonal M3/84 | AbD Serotec MCA2293 | 1:200 | HIER | Rabbit | Rabbit anti-rat | macrophages/ microglia |
| CD3 | polyclonal | DakoCytomation A0452 | 1:500 | HIER | Goat | Goat anti-rabbit | T cells |
| CD45R | monoclonal RA3-6B2 | BD Biosciences 553085 | 1:1000 | HIER | - | - | B cells |
| DCX | monoclonal E-6 | Santa Cruz Biotechnology sc-271390 | 1:100 | HIER | Goat | Goat anti-mouse | neuronal progenitor cells |
| Foxp3 | monoclonal FJK-16s | eBioscience 14-5773-82 | 1:50 | HIER | Rabbit | Rabbit anti-rat | regulatory T cells |
| GFAP | polyclonal | DakoCytomation Z0334 | 1:1000 | - | Goat | Goat anti-rabbit | astrocytes |
| GrB | polyclonal | Abcam ab4059 | 1:400 | HIER | Goat | Goat anti-rabbit | effector T cells and NK cells |
| NeuN | monoclonal A60 | Merck/Millipore MAB377 | 1:1600 | HIER | Goat | Goat anti-mouse | neurons |
| TMEV | polyclonal | Kummerfeld et al. 2009 | 1:2000 | - | Goat | Goat anti-rabbit | TMEV |
| *β*-APP | monoclonal 22C11 | Merck/Millipore MAB348 | 1:2000 | HIER | Goat | Goat anti-mouse | axonal damage |

DCX = doublecortin; Foxp3 = forkhead box protein P3; GFAP = glial fibrillary acidic protein; GrB = granzyme B; NeuN = neuronal nuclear protein; TMEV = Theiler's murine encephalomyelitis virus; β-APP = beta-amyloid precursor protein; HIER = heat induced epitope retrieval (microwave heating, citrate buffer)

**Table S2.** Details of primer sequences used for reverse transcription quantitative polymerase chain reaction

| Gene | Acc. No. | Primer direction | Primer position in mRNA | Primer sequence from 5′→ 3′ | Amplicon length |
|---|---|---|---|---|---|
| GAPDH | AY618568 | Forward | 1-20 | GAG GCC GGT GCT GAG TAT GT | 288 bp |
| | | Reverse | 288-269 | GGT GGC AGT GAT GGC ATG GA | |
| HPRT | NM_013556.2 | Forward | 646-665 | GGA CCT CTC GAA GTG TTG GA | 188 bp |
| | | Reverse | 814-833 | TCG TAT TTG CAG ATT CAA CT | |
| β-Actin | AY618569 | Forward | 1-20 | GGC TAC AGC TTC ACC ACC AC | 233 bp |
| | | Reverse | 214-233 | ATG CCA CAG GAT TCC ATA CC | |
| TMEV-DA | M20301.1 | Forward | 1596-1615 | TGG TCG ACT CTG TGG TTA CG | 238 bp |
| | | Reverse | 1814-1833 | GCC GGT CTT GCA AAG ATA GT | |
| IFN-γ | NM_008337.4 | Forward | 176-195 | CAC GGC ACA GTC ATT GAA AG | 144 bp |
| | | Reverse | 300-319 | AAT CTG GCT CTG CAG GAT TT | |
| IL-1α | NM_010554.4 | Forward | 294-313 | AAG CAA CGG GAA GAT TCT GA | 179 bp |
| | | Reverse | 453-472 | TGA CAA ACT TCT GCC TGA CG | |
| IL-1β | NM_008361.4 | Forward | 311-330 | AGC TAC CTG TGT CTT TCC CG | 150 bp |
| | | Reverse | 439-460 | AGT GCA GTT GTC TAA TGG GAA C | |
| IL-4 | NM_021283.2 | Forward | 224-245 | CCT CAC AGC AAC GAA GAA CAC C | 156 bp |
| | | Reverse | 358-379 | CAT CGA AAA GCC CGA AAG AGT C | |
| IL-5 | NM_010558.1 | Forward | 104-123 | ATG GAG ATT CCC ATG AGC AC | 180 bp |
| | | Reverse | 264-283 | CCC ACG GAC AGT TTG ATT CT | |
| IL-6 | NM_031168.2 | Forward | 250-269 | GTT CTC TGG GAA ATC GTG GA | 176 bp |
| | | Reverse | 404-425 | CCA GAG GAA ATT TTC AAT AGG C | |
| IL-10 | NM_010548.2 | Forward | 307-326 | CCA AGC CTT ATC GGA AAT GA | 162 bp |
| | | Reverse | 449-468 | TTT TCA CAG GGG AGA AAT CG | |
| TGF-β1 | NM_011577.2 | Forward | 1719-1738 | TTG CTT CAG CTC CAC AGA GA | 183 bp |
| | | Reverse | 1882-1901 | TGG TTG TAG AGG GCA AGG AC | |
| TNF-α | NM_013693.3 | Forward | 268-287 | GCC TCT TCT CAT TCC TGC TT | 203 bp |
| | | Reverse | 451-470 | CAC TTG GTG GTT TGC TAC GA | |

Acc. No. = GenBank®-accession-number; bP = base pair; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HPRT = Hypo-xanthine-guanine phosphoribosyl transferase; TMEV-DA = Daniels strain of Theiler's murine encephalomyelitis virus; IFN-γ = interferon-gamma; IL = interleukin; TGF-β1 = transforming growth factor-β1; TNF-α = tumor necrosis factor-α.

**Table S3.** Details of antibodies used for flow cytometry

| Antibody | Conjugate | Supplier | Clone | Dilution | Specificity |
|---|---|---|---|---|---|
| CD4 | FITC | Thermo Fisher Scientific, Invitrogen, eBioscience™, Waltham, USA | RM4-5 | 1:100 | $CD4^+$ helper T cells |
| CD4 | PerCP Cy5.5 | Thermo Fisher Scientific, Invitrogen, eBioscience™, Waltham, USA | RM4-5 | 1:200 | $CD4^+$ helper T cells |
| CD8a | APC | BD Biosciences, BD Pharmingen™, Heidelberg, Germany | 53-6.7 | 1:200 | $CD8^+$ cytotoxic T cells |
| CD8a | PE | BD Biosciences, BD Pharmingen™, Heidelberg, Germany | 53-6.7 | 1:200 | $CD8^+$ cytotoxic T cells |
| CD62L | PE-Cy7 | Thermo Fisher Scientific, Invitrogen, eBioscience™, Waltham, USA | MEL-14 | 1:200 | Naïve T cells, downregulation upon T cell activation, L-selectin |
| CD69 | APC | Thermo Fisher Scientific, Invitrogen, eBioscience™, Waltham, USA | H1.2F3 | 1:200 | Early T cell activation, upregulation upon T cell activation |
| CD69 | PerCP Cy5.5 | Thermo Fisher Scientific, Invitrogen, eBioscience™, Waltham, USA | H1.2F3 | 1:200 | Early T cell activation, upregulation upon T cell activation |