Supplementary Data

Title: Intraperitoneal administration of a modified vaccinia virus Ankara confers single-chain interleukin-12 expression to the omentum and achieves immune-mediated efficacy against peritoneal carcinomatosis

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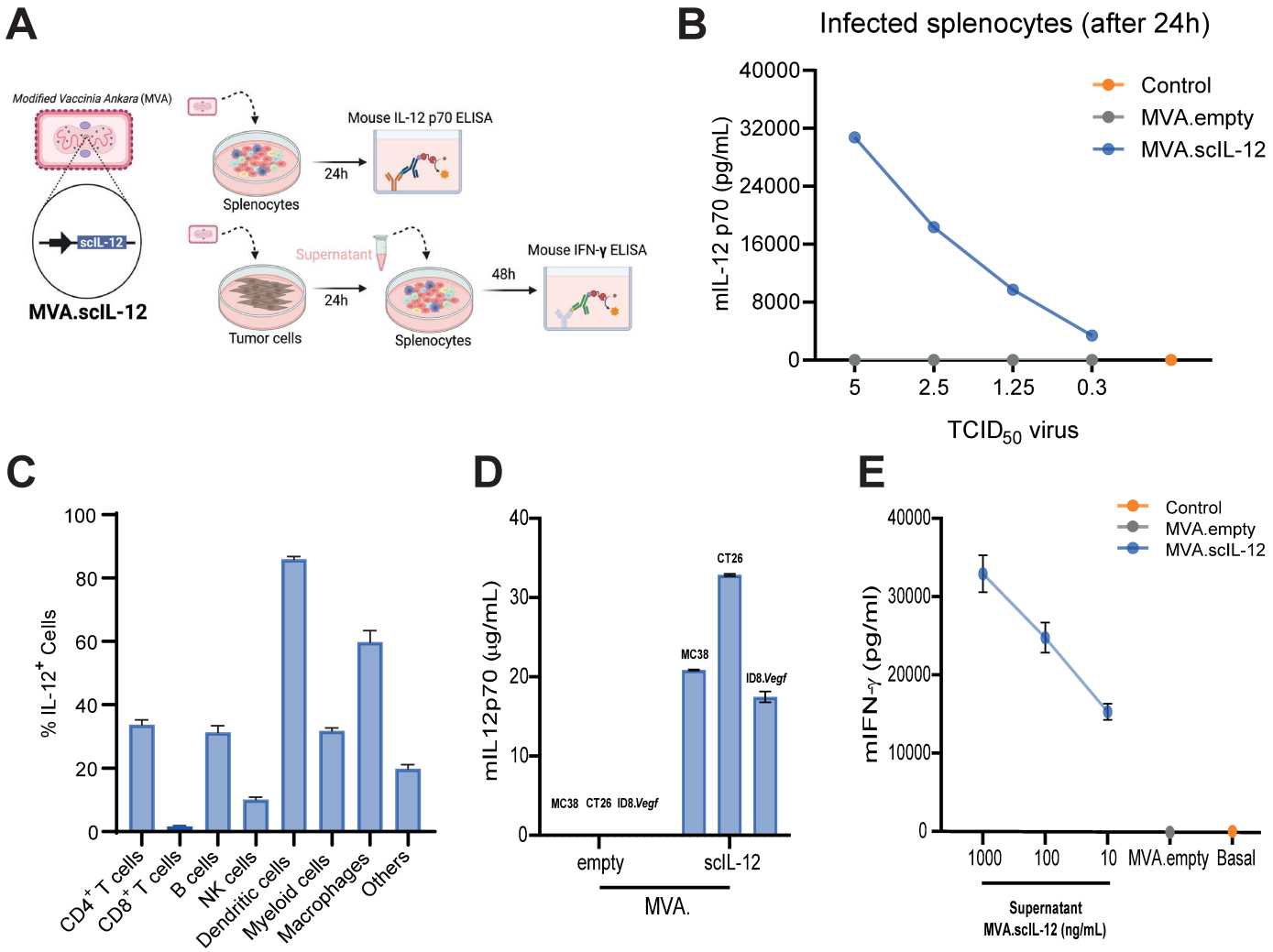
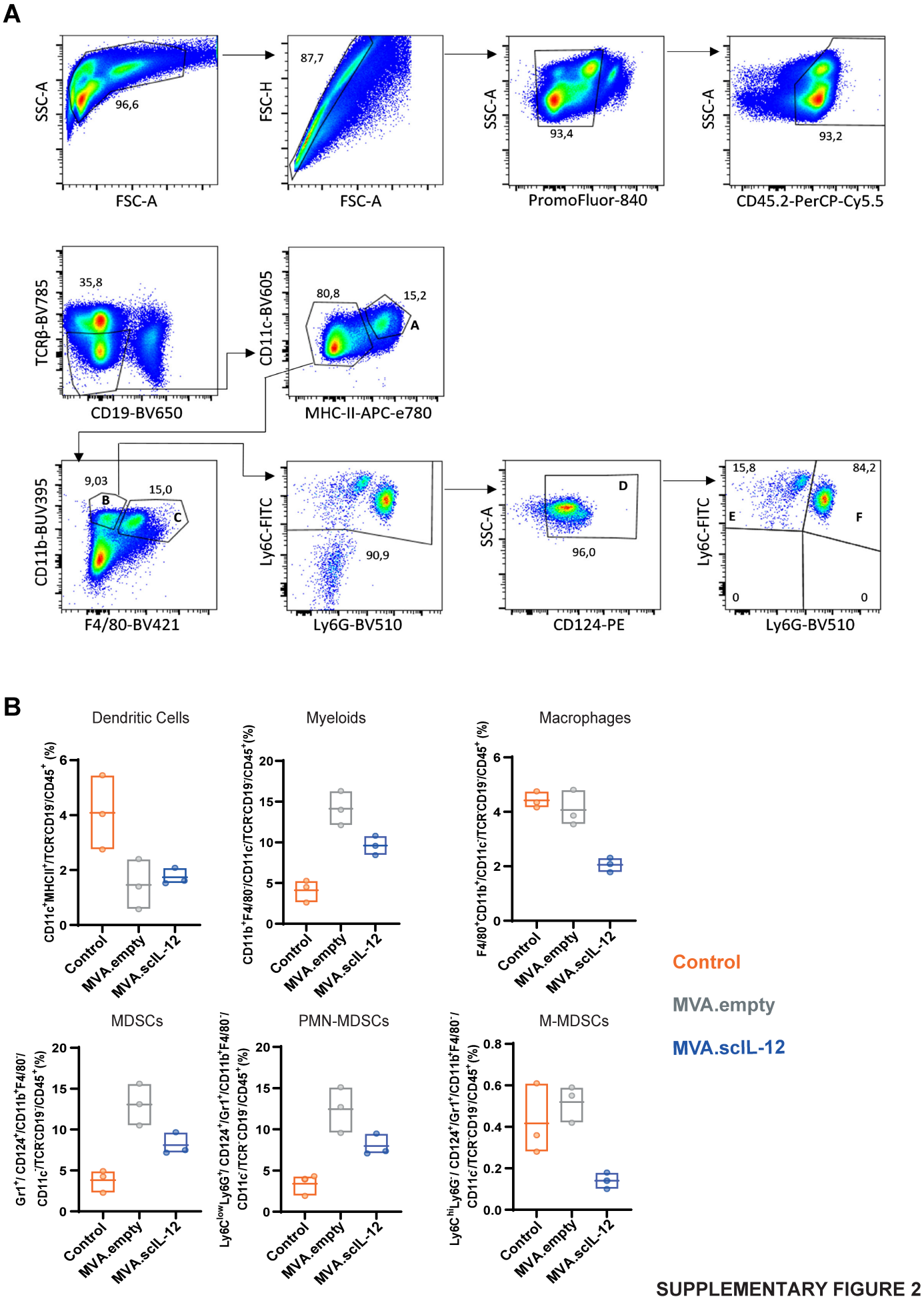


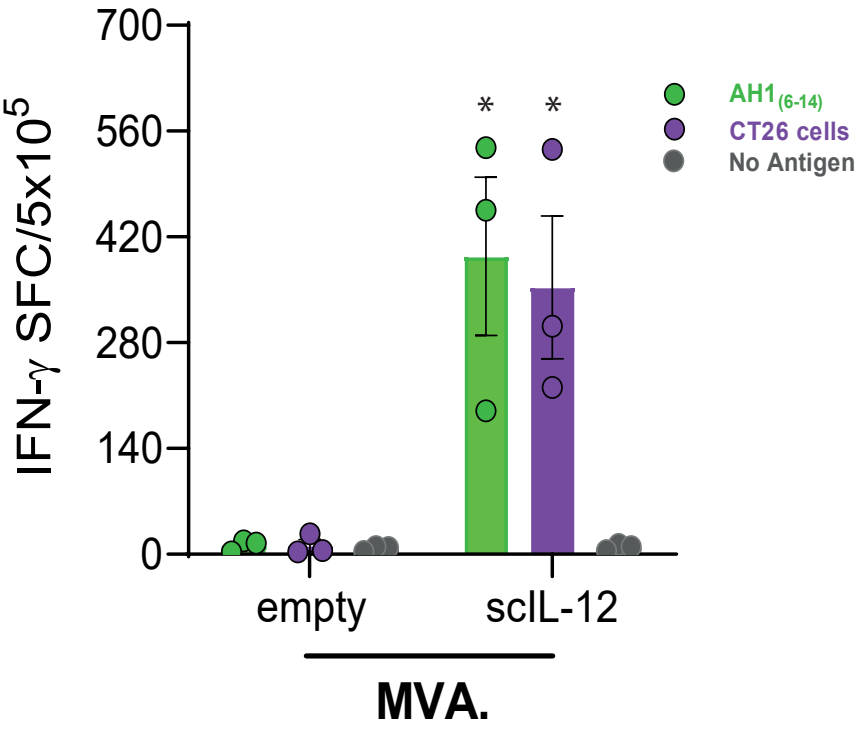
SUPPLEMENTARY FIGURE 1

Supplementary Figure 1. *In vitro* and *in vivo* characterization of MVA.scIL-12. (**A**) Schematic representations of experiments *in vitro*. **(B)** Mouse splenocytes were infected with different concentrations of MVA.scIL-12 (5; 2.5; 1.25 and 0.3 $TCID_{50}$), and 24h later, the concentration of IL-12 in the supernatant was analyzed by ELISA. **(C)** Splenocytes were infected with MVA.scIL-12 ($TCID_{50}$ 5). Two hours after infection, splenocytes were incubated with GolgiPlug™ for 5h to analyze the IL-12 intracellular production in different immune populations: $CD4^+$ T cells ($IL\text{-}12^+/CD4^+CD8^-/CD3^+CD19^-/CD45^+$) $CD8^+$ T cells ($IL\text{-}12^+/CD8^+CD4^-/CD3^+CD19^-/CD45^+$) B cells ($IL\text{-}12^+/CD19^+CD3^-/CD45^+$) NK cells ($IL\text{-}12^+/NK1.1^+/CD19^-CD3^-/CD45^+$), dendritic cells ($IL\text{-}12^+/CD11c^+/NK1.1^-/CD19^-CD3^-/CD45^+$), myeloid cells ($IL\text{-}12^+/CD11b^+F4/80^-/CD11c^-/NK1.1^-/CD19^-CD3^-/CD45^+$), macrophages ($IL\text{-}12^+/F4/80^+CD11b^+/CD11c^-/NK1.1^-/CD19^-CD3^-/CD45^+$) and other cells ($IL\text{-}12^+/F4/80^-CD11b^-/CD11c^-/NK1.1^-/CD19^-CD3^-/CD45^+$) using flow cytometry. **(D)** MC38, CT26 and ID8.*Vegf*/GFP tumor cells were infected with MVA.scIL-12, and 24h later, the concentration of IL-12 in the supernatant was analyzed by ELISA. **(E)** Splenocytes were stimulated with tumor-cell-derived-supernatant containing 1000, 100, or 10 ng/ml of IL-12 for 48h, and IFN-γ production was assessed by ELISA. **(F)** Six hours after PBS (Control), MVA.empty, or MVA.scIL-12, peritoneal washing fluid and serum were collected and analyzed by ELISA to determine the concentration of IL-12. **(G)** PBS, MVA.empty, or MVA.scIL-12 were injected *i.p.* in tumor-free C57BL/6 mice (n=3), and serum was collected at 6h, 24h, and 48h to analyze the concentration of IL-12 and IFN-γ by ELISA. Data are represented as mean ± SEM. Two-way ANOVA followed by Sidak's post-test. $*p<0.05$. MVA, modified vaccinia virus Ankara.



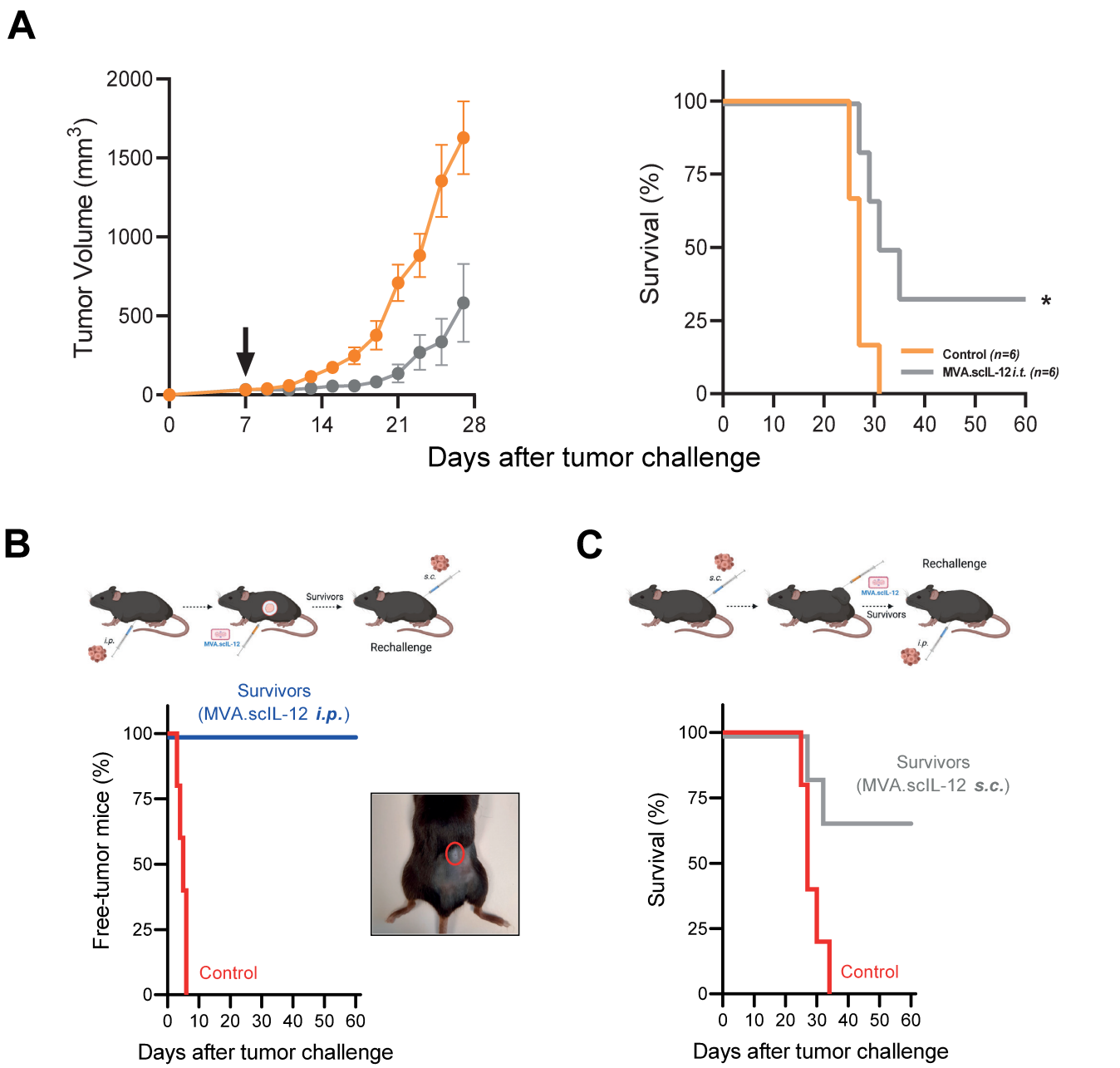
SUPPLEMENTARY FIGURE 2

Supplementary Figure 2. Flow cytometry to examine the myeloid cell compartment in mice bearing MC38 peritoneal carcinomatosis, both in untreated mice and 24 hours after treatment with either MVA.empty or MVA.scIL-12. Peritoneal washing single-cell suspensions were initially stained with PromoFluor 840 (#840301; PromoCell, Heidelberg, Germany) as a live/dead marker before staining surface markers using the following fluorochrome-labeled antibodies (BioLegend, San Diego, CA, unless otherwise specified): anti-CD19-BV650 (6D5), anti-CD45.2-PerCPCy5.5 (104), anti-CD11b-BUV395 (M1/70; BD Biosciences, San Diego, CA), anti-CD11c-BV605 (N418), anti-F4/80-BV421 (BM8), anti-TCR-β-BV785 (H57-597), anti-Ly6G-BV510 (1A8), anti-Ly6C-FITC (HK1.4), anti-CD124-PE (CTLL-19.4; BD Biosciences, San Diego, CA) and anti-MHC-II (I-A/I-E)- APC-eFluor780 (AF6-120.1; eBioscience, Thermo Fisher Scientific, Waltham, MA). **(A)** Schematic representation of the gating strategy to define the myeloid compartment in the peritoneal cavity. Cells were first selected for live cells (PromoFluor-840$^-$), CD45$^+$, CD19$^-$ and TCR-β$^-$. A) Dendritic cells (CD11c$^+$MHCII$^+$/TCR$^-$CD19$^-$/CD45$^+$); B) Myeloid cells (CD11b$^+$F4/80$^-$/CD11c$^-$/TCR$^-$CD19$^-$/CD45$^+$); C) Macrophages (F4/80$^+$CD11b$^+$/CD11c$^-$/TCR$^-$CD19$^-$/CD45$^+$); D) Myeloid-derived suppressor cells, MDSCs (Gr1$^+$/ CD124$^+$/CD11b$^+$F4/80$^-$/CD11c$^-$/TCR$^-$CD19$^-$/CD45$^+$); E) Monocytic-MDSCs (Ly6C^{hi}Ly6G$^-$/CD124$^+$/Gr1$^+$/ CD11b$^+$F4/80$^-$/CD11c$^-$/TCR$^-$CD19$^-$/CD45$^+$); F) Polymorphonuclear-MDSCs (Ly6C^{low}Ly6G$^+$/ CD124$^+$/Gr1$^+$/CD11b$^+$F4/80$^-$/CD11c$^-$/TCR$^-$CD19$^-$/CD45$^+$). **(B)** Percentage of dendritic cells, myeloid cells, macrophages, MDSCs, PMN-MDSCs, and M-MDSCs.



SUPPLEMENTARY FIGURE 3

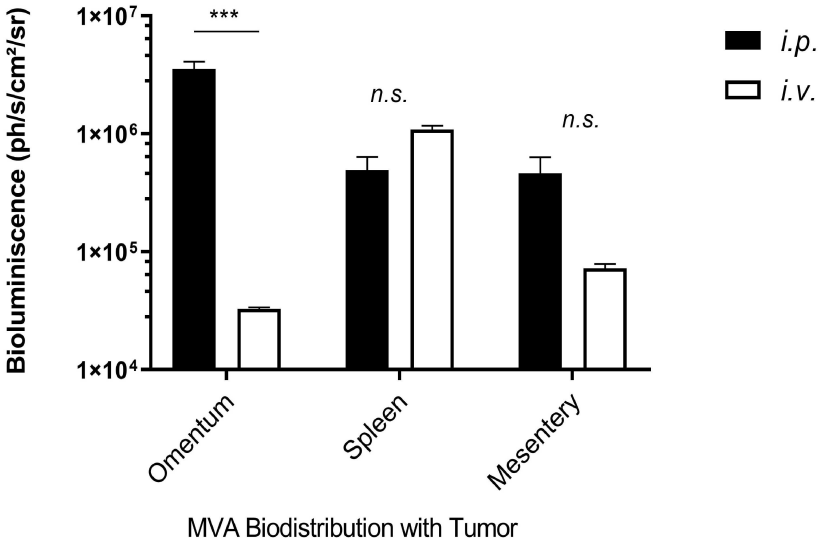
Supplementary Figure 3. Quantification of IFN-γ-producing T lymphocytes in the spleen from mice bearing intraperitoneal CT26 tumors that were intracavitary treated with MVA.empty or MVA.scIL-12. BALB/c mice were challenged *i.p.* with 2 x 10^5 CT26 colon cancer cells. Seven days later, mice were treated *i.p.* with 5 x 10^7 $TCID_{50}$ of MVA.empty or MVA.scIL-12. After one week, mice were sacrificed, and IFN-γ–producing cells were measured by ELISpot in splenocytes stimulated with the $AH1_{6-14}$ antigen, or with CT26 colon cancer cells.



SUPPLEMENTARY FIGURE 4

Supplementary Figure 4. Differences in systemic immune memory after eradication of peritoneal carcinomatosis by *i.p.* MVA.scIL-12 or rejection of subcutaneous tumors induced by *i.t.* MVA.scIL-12. **(A)** C57BL/6 mice were challenged *s.c.* with 5 x 10^5 MC38 colon cancer cells. Seven days later, mice were treated *i.t.* with 5 x 10^7 $TCID_{50}$ of either MVA.empty or MVA.scIL-12. The mean tumor volume over time and the Kaplan-Meier survival curve are presented. **(B)** Mice that survive after an *i.p.* MC38 inoculation treated with *i.p.* MVA.scIL-12 were rechallenged with 5 x 10^5 MC38 tumor cells injected subcutaneously. Kaplan-Meier survival curves are shown. **(C)** Mice that survived after a subcutaneous MC38 inoculation treated with *i.t.* MVA.scIL-12 were rechallenged with 5 x 10^5 MC38 tumor cells injected *i.p*. Kaplan-Meier survival curves are shown.

A



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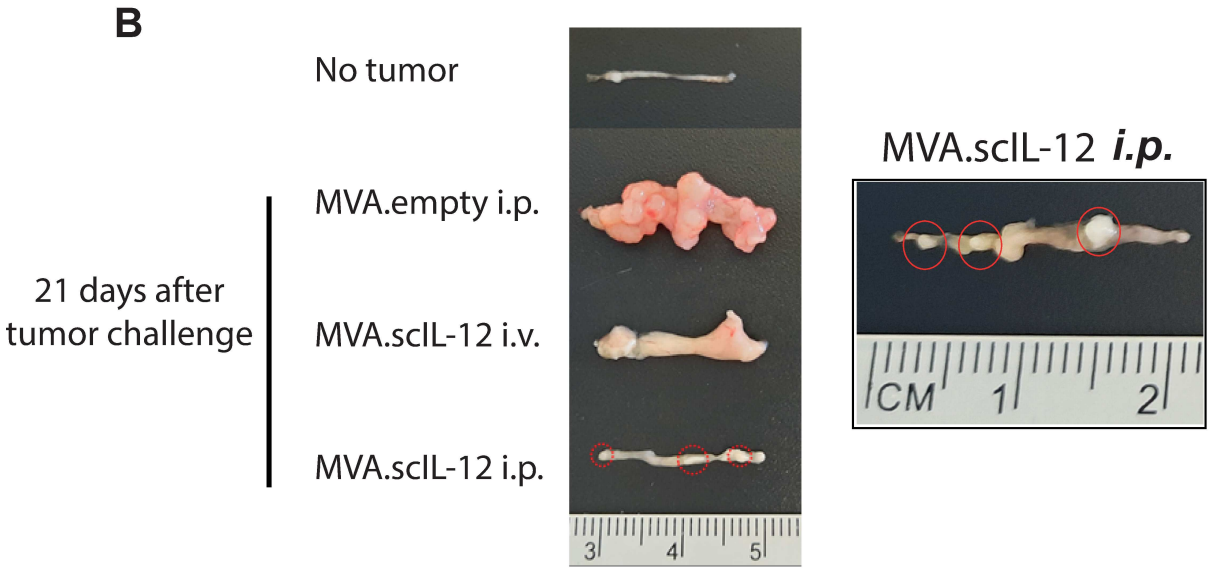
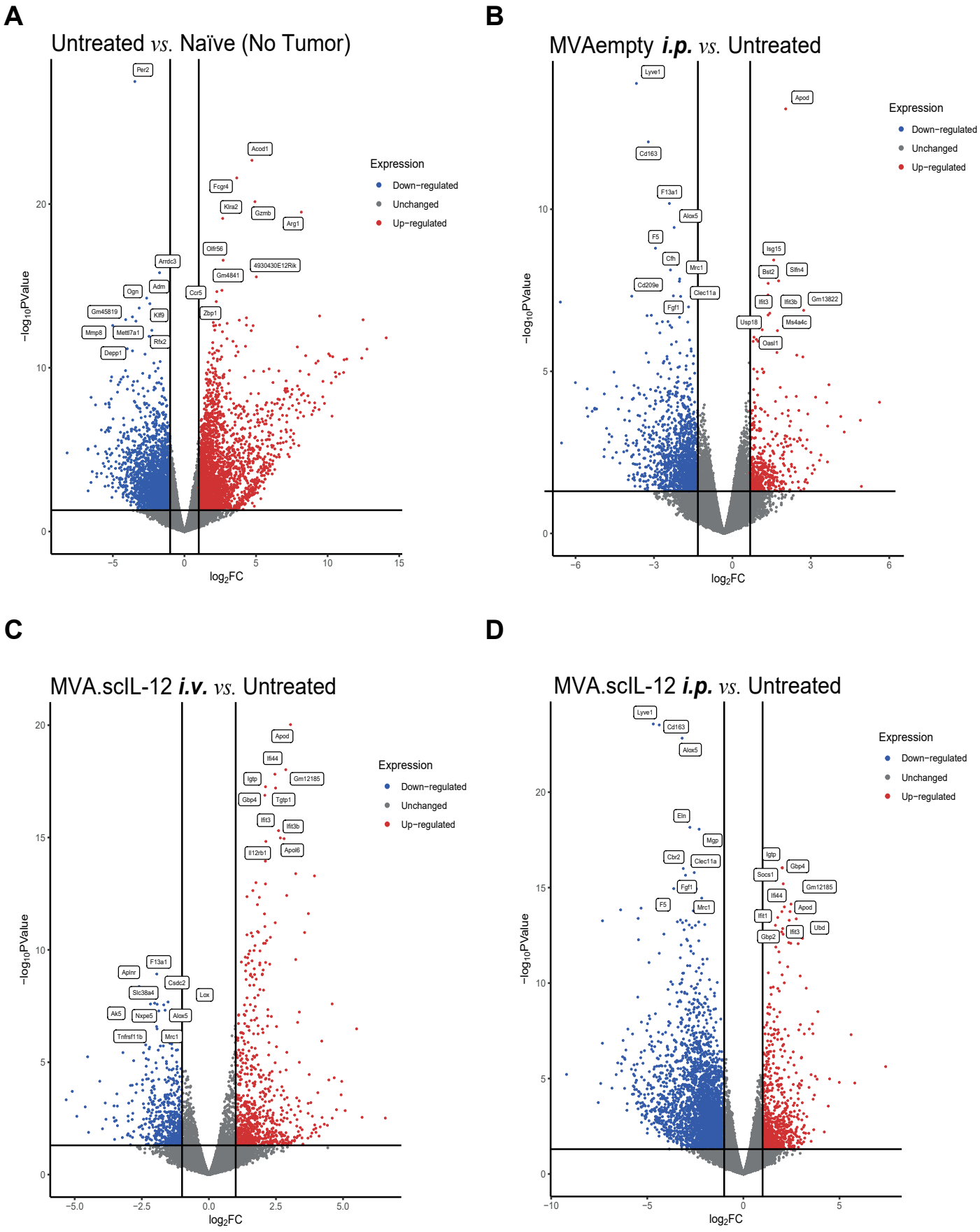


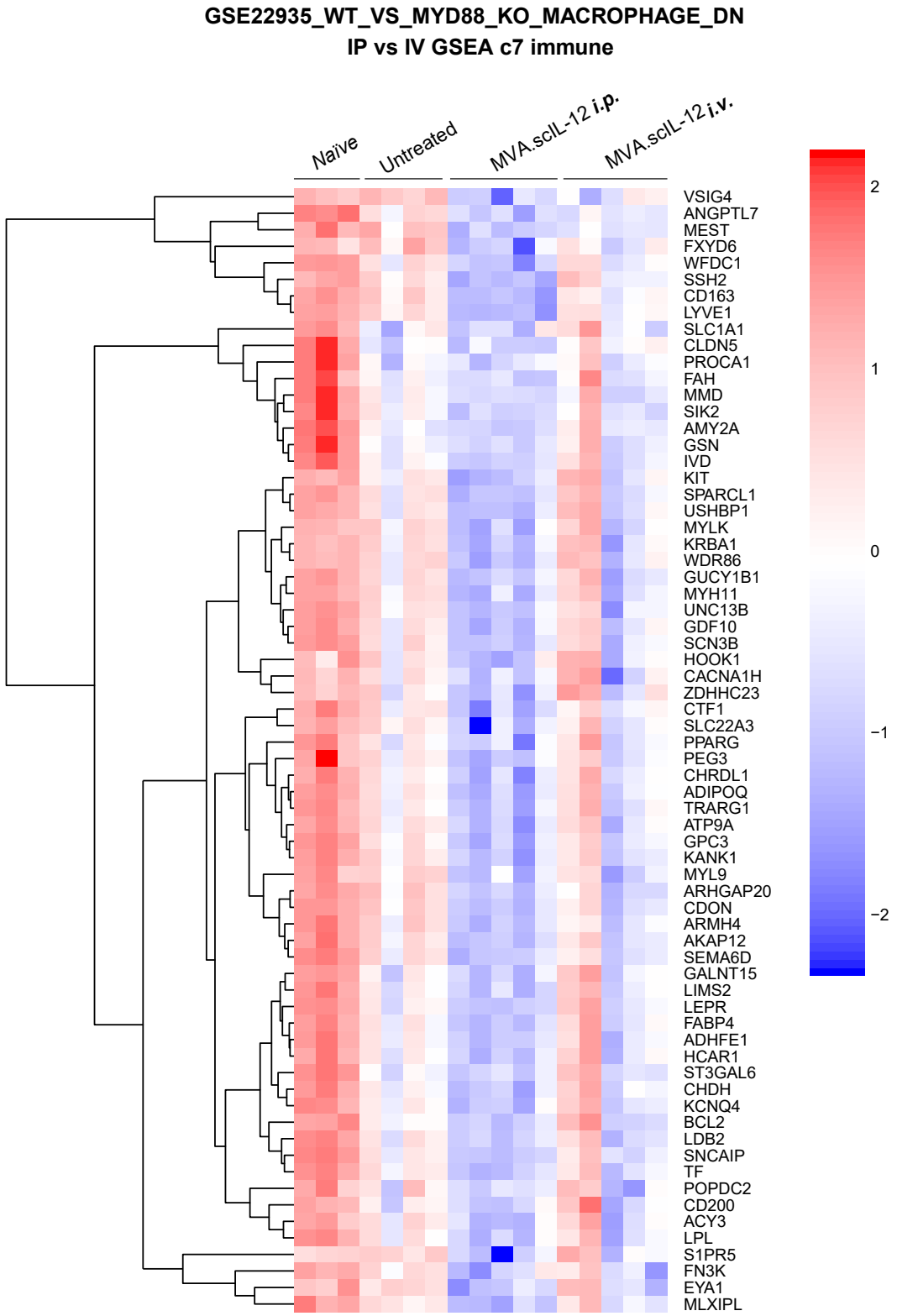
SUPPLEMENTARY FIGURE 5

Supplementary Figure 5. MVA biodistribution in tumor-bearing mice and representative photographs of omenta from different experimental groups of treatment. **(A)** C57BL/6 mice were challenged with PCa. Seven days after the tumor challenge, mice were injected *i.p.* or *i.v.* with 5 x 10^7 $TCID_{50}$ of MVA.Luc. Six hours later, the omentum, spleen, and mesentery were isolated and bioluminescence was quantified. **(B)** Omenta from mice inoculated with 5 x 10^5 MC38 cells *i.p.* and treated seven days later with 5 x 10^7 $TCID_{50}$ MVA.empty or MVA.scIL-12 *i.p.* or 5 x 10^7 $TCID_{50}$ MVA.scIL-12 *i.v.*



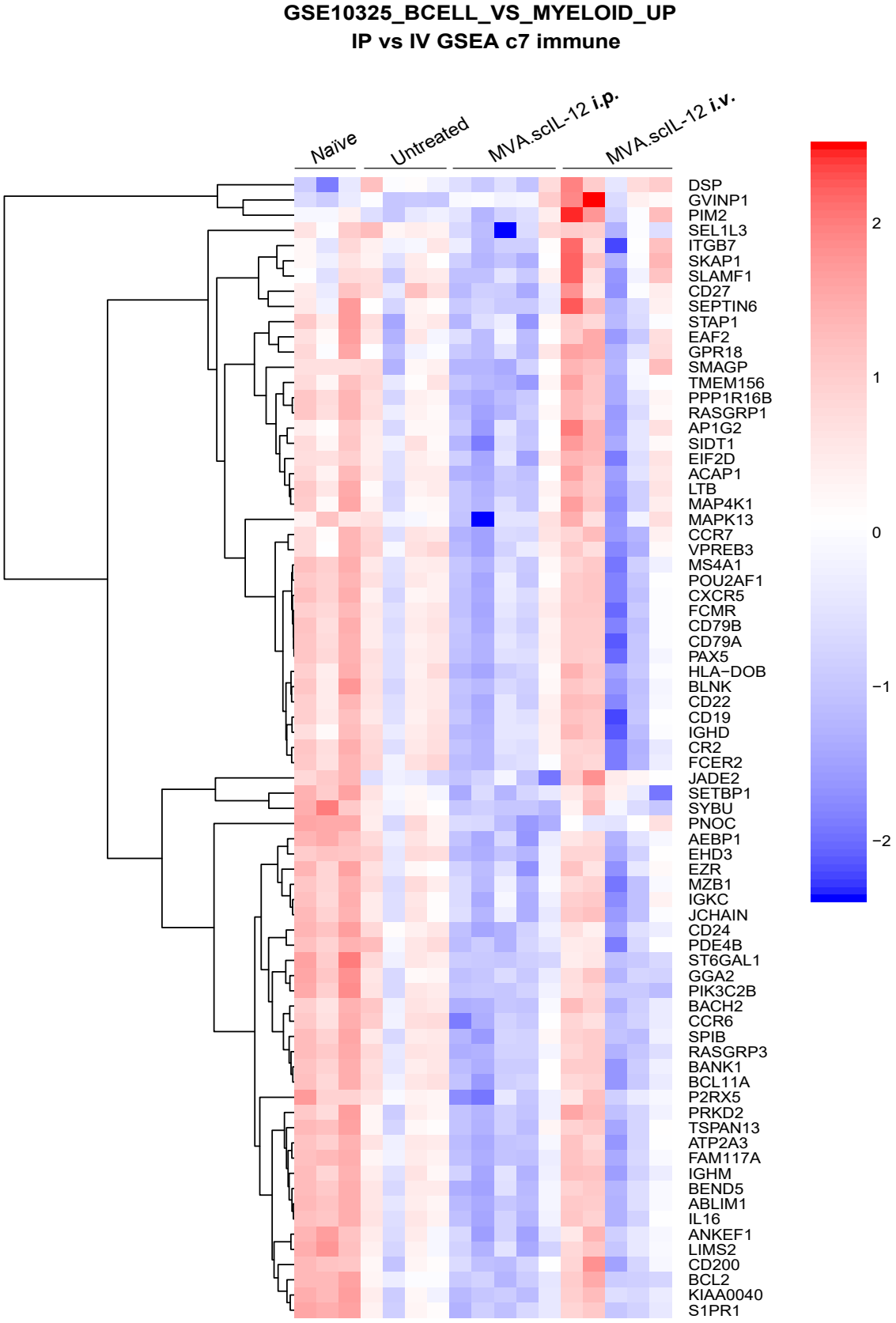
SUPPLEMENTARY FIGURE 6

Supplementary Figure 6. Volcano plots representing the differentially expressed genes among different experimental groups. **(A)** PBS-treated tumor-bearing mice vs. *naïve* (non-tumor bearing mice). **(B)** MVA.empty *i.p. vs.* PBS-treated tumor-bearing mice. **(C)** MVA.scIL-12 *i.v. vs.* PBS-treated tumor-bearing mice. **(D)** MVA.scIL-12 *i.p. vs.* PBS-treated tumor-bearing mice. Red and blue dots indicate up-regulated or down-regulated genes, respectively, with a false discovery rate (FDR) <0.05.



SUPPLEMENTARY FIGURE 7

Supplementary Figure 7. Differentially expressed genes related to macrophages comparing MVA.scIL-12 *i.p.* and MVA.scIL-12 *i.v.* GSEA was performed against C7 IMMUNESIGDB reference giving GSE22935_WT_VS_MYD88_KO_MACROPHAGE_DN gene set as enriched (*padj* < 1 x 10^{-4}; NES= -1.4827974). The heatmap shows the z-scored log2CPM normalized expression of differentially expressed genes (*p.val* < 0.05% and FDR < 0.05%). The heatmap shows the z-scored log2CPM normalized expression of differentially expressed genes (*p.val* < 0.05% and FDR < 0.05%).



SUPPLEMENTARY FIGURE 8

Supplementary Figure 8. Differentially expressed genes related to B cells comparing MVA.scIL-12 *i.p.* and MVA.scIL-12 *i.v.* GSEA was performed against C7 IMMUNESIGDB reference giving GSE10325_BCELL_VS_MYELOID_UP gene set as enriched (*p.adj* < 3 x 10^{-4}; NES=-1.4577). The heatmap shows the z-scored log2CPM normalized expression of differentially expressed genes (*p.val* < 0.05% and FDR < 0.05%).

Supplementary Video 1. Intravital microscopy of ID8.*Vegf*/GFP omental tumor lesions in a transgenic mouse with fluorescent $CD2^+$ T cells and macrophages two weeks after tumor inoculation in the peritoneum. Tumor ID8.*Vegf*/GFP lesions (Green), T cells, hCD2RFP (Red) and macrophages were stained with an antibody against F4/80-Alexa Fluor 647 (blue).

Supplementary Video 2. Intravital microscopy of T-cell infiltrating in omental lesions from MVA.empty and MVA.scIL-12 treated mice. Tumor ID8.*Vegf*/GFP lesions (Green), T cells, hCD2RFP (Red) and macrophages, antibody against F4/80-Alexa Fluor 647 (blue). MVA.empty (MVA-Control) and MVA.scIL-12 were administered *i.p.* 48h before intravital imaging in two-weeks- ID8.*Vegf*/GFP-bearing mice.