

**OMTM, Volume 15**

## **Supplemental Information**

### **Role of Regulatory T Cell and Effector**

#### **T Cell Exhaustion in Liver-Mediated**

#### **Transgene Tolerance in Muscle**

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## Supplementary Material

**Supplementary Table 1. Inflammatory markers expressed in liver of mice treated as described in Figure 2**

	CTRL	IM	IM/IV D0	IM/IV D15
<b>CD4</b>	49.13 ± 11.91	<b>135.73 ± 19.72</b>	36.61 ± 3.63	42.77 ± 14.01
<b>CD8</b>	3.45 ± 1.35	2.55 ± 0.59	15.17 ± 5.31	<b>26.36 ± 18.11</b>
<b>FOX-P3</b>	0.08 ± 0.09	0.06 ± 0.02	0.34 ± 0.13	<b>0.69 ± 0.38</b>
<b>LAG3</b>	1.73 ± 0.92	1.87 ± 0.70	2.63 ± 0.85	4.95 ± 3.14
<b>IFNg</b>	0.78 ± 0.16	0.43 ± 0.12	1.31 ± 0.46	<b>2.00 ± 0.64</b>
<b>PD-1</b>	0.23 ± 0.15	0.15 ± 0.04	2.84 ± 0.93	<b>6.84 ± 5.14</b>
<b>PD-L1</b>	3.40 ± 0.34	1.84 ± 0.32	<b>6.15 ± 1.20</b>	<b>10.45 ± 2.57</b>
<b>PD-L2</b>	0.04 ± 0.02	0.33 ± 0.62	0.08 ± 0.02	0.20 ± 0.06
<b>TIM3</b>	0.94 ± 0.48	0.51 ± 0.02	1.49 ± 0.56	3.05 ± 0.90
<b>CTLA-4</b>	1.43 ± 1.87	0.44 ± 0.39	0.16 ± 0.07	0.49 ± 0.22
<b>2B4</b>	0.34 ± 0.17	0.15 ± 0.06	0.47 ± 0.17	0.77 ± 0.12

Abundance values ( $2^{-\Delta\text{Ct}} \times 10^{-4}$ ) were expressed as mean ± standard deviation. Values in red are significantly different from control Group.

**Supplementary Table 2. Inflammatory markers expressed in muscle of mice treated as described in Figure 4**

	CTRL	Muscle	Liver	Muscle-Liver
<b>CD4</b>	0.65 ± 0.15	12.95 ± 5.02	0.90 ± 0.24	4.68 ± 0.58
<b>FOXP3</b>	1.15 ± 0.18	4.83 ± 2.39	1.21 ± 0.10	2.41 ± 0.50
<b>IL10</b>	0.02 ± 0.01	0.95 ± 0.49	0.05 ± 0.02	0.50 ± 0.33
<b>IL35</b>	0.29 ± 0.07	0.10 ± 0.01	0.53 ± 0.24	0.28 ± 0.04
<b>GITR</b>	0.20 ± 0.05	9.39 ± 5.43	0.30 ± 0.06	3.71 ± 1.48
<b>CTLA-4</b>	0.0013 ± 0.0005	0.1358 ± 0.1488	0.0021 ± 0.0012	0.0934 ± 0.0770
<b>AREG</b>	0.0005 ± 0.0001	0.0011 ± 0.0007	0.0004 ± 0.0000	0.0178 ± 0.0072

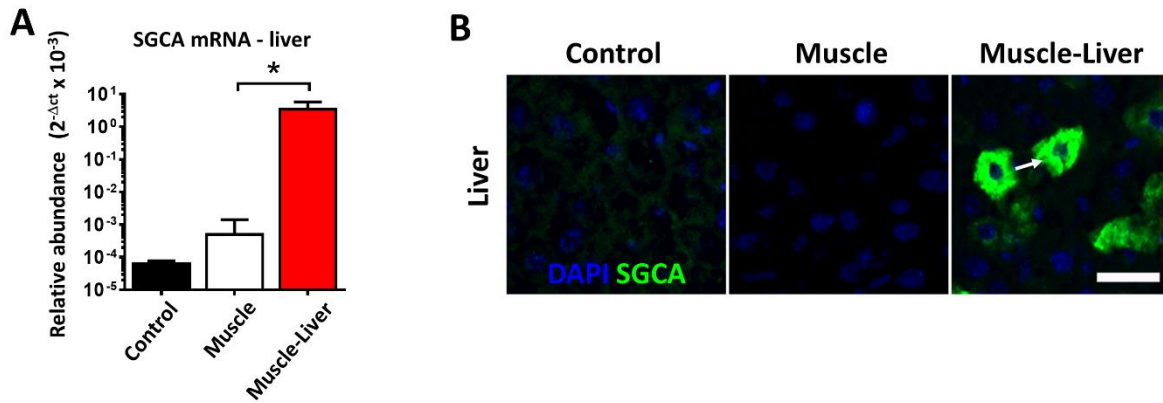
Abundance values ( $2^{-\Delta\text{Ct}} \times 10^{-3}$ ) were expressed as mean ± standard deviation. Values in red are significantly different from control Group.

**Supplementary Table 3. Total or Dextramer+ CD8+, CD8+ PD1+, CD8+ PD1+ LAG3+ and CD8+ PD1+ TIM3+ cells measured in liver non-parenchymal cells of mice from the indicated groups.**

Group	CD8+		CD8+ PD1+		CD8+ PD1+ LAG3+		CD8+ PD1+ TIM3+	
	Total	Dextramer+	Total	Dextramer+	Total	Dextramer+	Total	Dextramer+
Control	2389 ± 560	44 ± 3	13 ± 10	5 ± 3	1 ± 1	0 ± 0	0 ± 1	0 ± 0
Muscle	3178 ± 1969	527 ± 235	31 ± 16	26 ± 13	2 ± 2	0 ± 0	1 ± 1	0 ± 0
Liver	3538 ± 346	46 ± 14	32 ± 11	6 ± 5	20 ± 6	2 ± 2	17 ± 3	1 ± 1
Muscle-Liver	2860 ± 342	40 ± 6	56 ± 28	9 ± 4	36 ± 22	3 ± 3	30 ± 17	2 ± 1

Absolute counts were reported as mean±standard deviation.

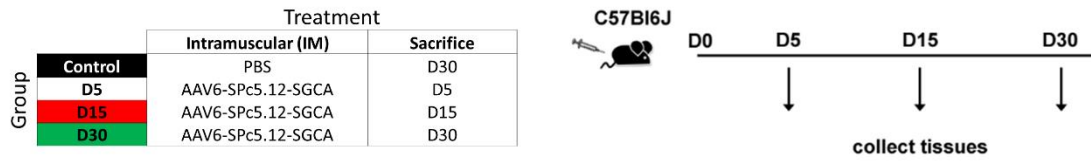
## Supplementary FIGURE 1



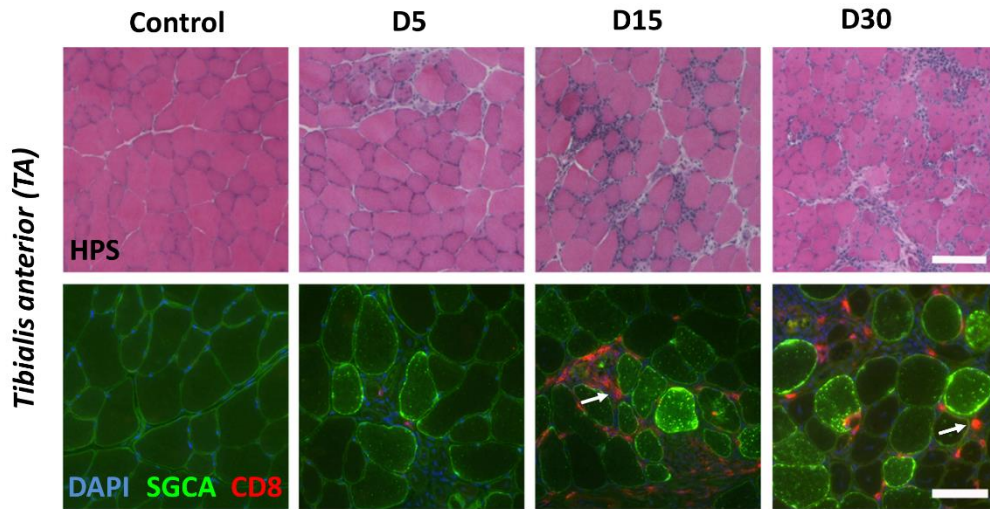
**Supplementary Figure 1.** (A) hSGCA mRNA measured in liver. (B) anti-hSGCA (green), and DAPI (blue) immunostaining performed in liver of mice treated as described in Figure 1 (Scale bar = 25 $\mu$ m). White arrow indicates SGCA-expressing hepatocyte. Data were expressed as mean  $\pm$  SD. Statistical analysis was performed by t-test (\* =  $p < 0.05$ , as indicated,  $n = 4$  per group).

## Supplementary FIGURE 2

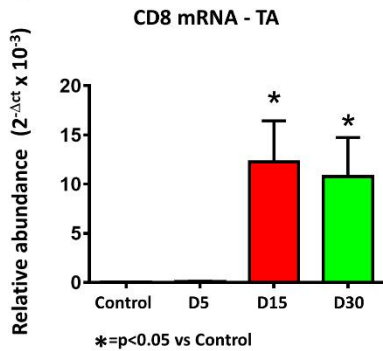
**A**



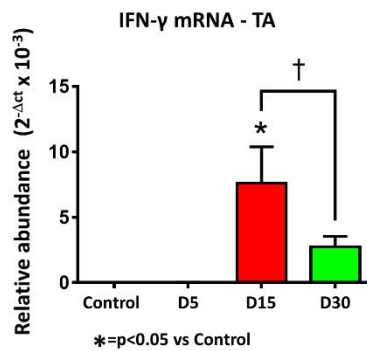
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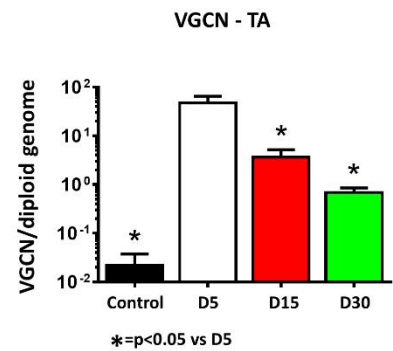
**C**



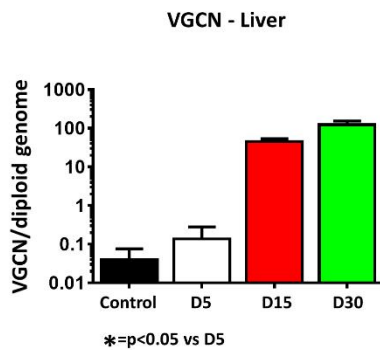
**D**



**E**



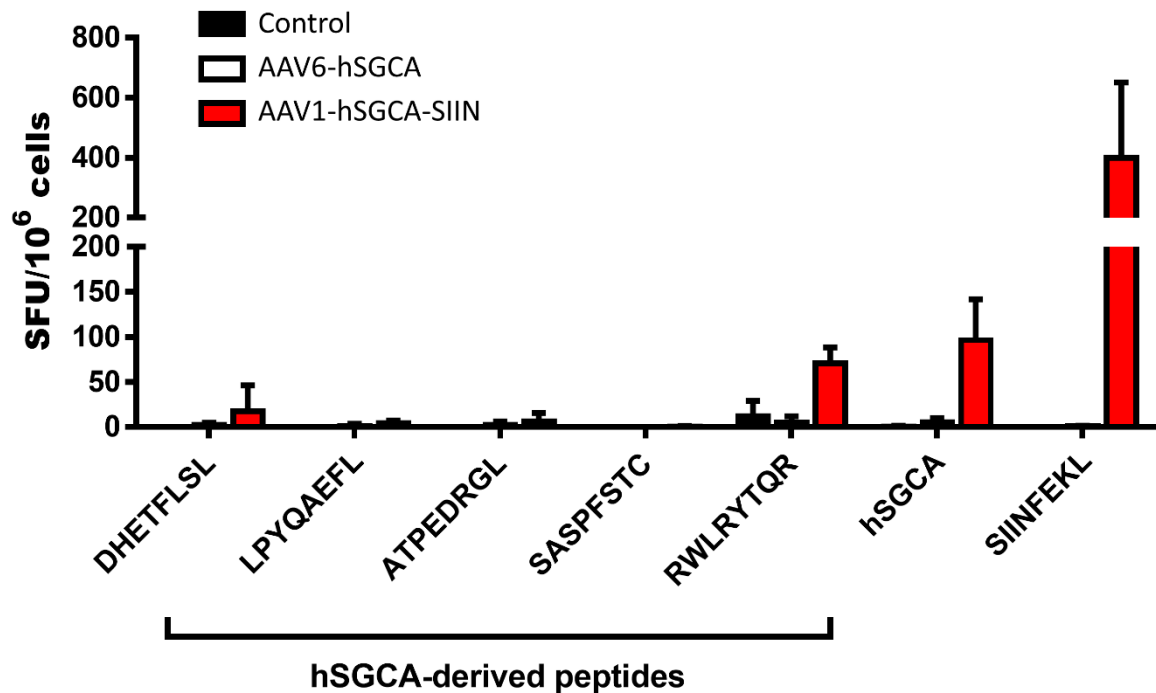
**F**



**Supplementary Figure 2.** (A) Eight-week old C57BL/6J mice received at day 0 an intramuscular injection (IM, *Tibialis Anterior*, TA) of  $2.5 \times 10^9$  vg/mouse of AAV6-SPc5.12-

hSGCA vector. Mice were sacrificed and tissues collected at day 0 (D0), day 5 (D5) and day 30 (D30) after vector injection. PBS-injected mice sacrificed 30 days after vector injection were used as controls (Control). **(B)** Hematoxylin phloxine saffron staining (HPS, upper panel, scale bar = 100 $\mu$ m) and anti-hSGCA (green), CD8 (red) and DAPI (blue) immunostaining (lower panel, scale bar = 20 $\mu$ m) performed in TA. White arrows indicate CD8 cells. **(C, D)** CD8 and IFN $\gamma$  mRNA measured in TA. **(E)** Vector genome copy number (VGCN) per diploid genome measured in TA. **(F)** Vector genome copy number (VGCN) per diploid genome measured in liver. Data were expressed as mean  $\pm$  SD. Statistical analyses were performed by ANOVA (\* =  $p < 0.05$ , † =  $p < 0.05$  as indicated, n=4 per group).

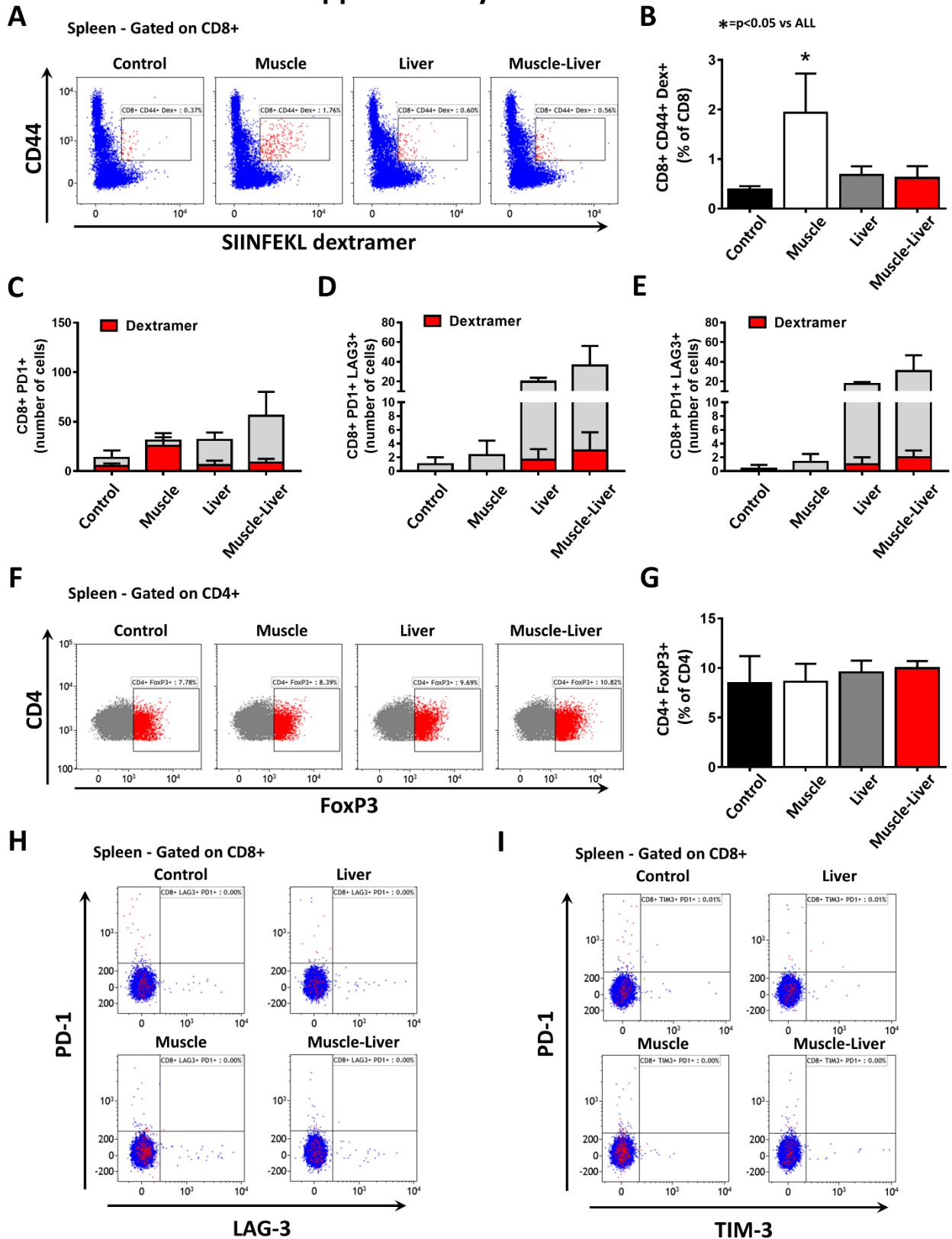
### Supplementary FIGURE 3



**Supplementary Figure 3.** Identification of peptide epitopes binding to class I murine MHC in hSGCA protein. Epitopes of hSGCA with the highest probability of presentation by H-2Kb MHC class I molecules were identified by Immune Epitope Database ([www.iedb.org](http://www.iedb.org)). Five peptides with the highest score were synthesized by GeneCust and used to stimulate splenocytes obtained from C57BL/6J mice intramuscularly injected with PBS (Control), AAV6 expressing hSGCA under the control of SPc5.12 promoter (AAV6-hSGCA) or AAV1 expressing hSGCA-SIINFEKL transgene under the control of SPc5.12 promoter (AAV1-hSGCA-SIIN). Splenocytes were stimulated in parallel with hSGCA recombinant protein or with the SIINFEKL peptide. Data were expressed as mean  $\pm$  SD.



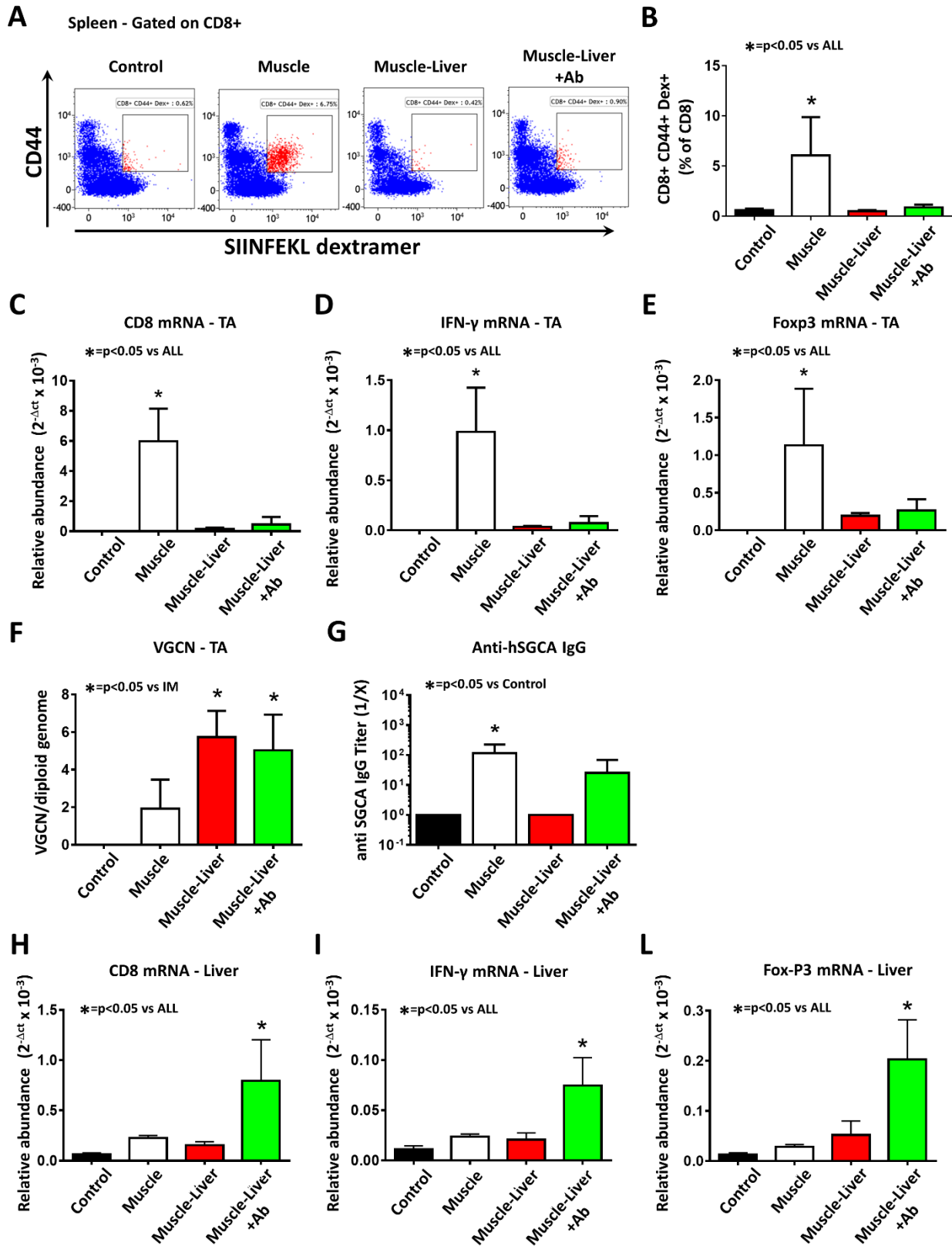
## Supplementary FIGURE 4



Supplementary Figure 4. (A,B) Flow cytometry dot plots representing CD8+CD44+Dextramer+ cells gated on CD8+ cells in splenocytes. The histogram shows the

quantification of the dot plots. **(C)** Absolute counts of CD8<sup>+</sup> PD1<sup>+</sup> cells (in gray) and CD8<sup>+</sup> PD1<sup>+</sup> DEX<sup>+</sup> (in red) measured in liver. **(D)** Absolute counts of CD8<sup>+</sup> PD1<sup>+</sup> LAG3<sup>+</sup> cells (in gray) and CD8<sup>+</sup> PD1<sup>+</sup> LAG3<sup>+</sup> DEX<sup>+</sup> (in red) measured in liver. **(E)** Absolute counts of CD8<sup>+</sup> PD1<sup>+</sup> TIM3<sup>+</sup> cells (in gray) and CD8<sup>+</sup> PD1<sup>+</sup> TIM3<sup>+</sup> DEX<sup>+</sup> (in red) measured in liver. **(F,G)** Flow cytometry dot plots representing CD4<sup>+</sup> Foxp3<sup>+</sup> cells gated on CD4<sup>+</sup> cells in splenocytes. The histogram shows the quantification of the dot plots. **(H)** Flow cytometry dot plots representing CD8<sup>+</sup> PD1<sup>+</sup> LAG3<sup>+</sup> cells gated on CD8<sup>+</sup> cells in splenocytes. **(I)** Flow cytometry dot plots representing the CD8<sup>+</sup> PD1<sup>+</sup> TIM3<sup>+</sup> cells gated on CD8<sup>+</sup> cells in splenocytes. Data were expressed as mean  $\pm$  SD. Statistical analyses were performed by ANOVA (\* =  $p < 0.05$ ,  $n = 3$  per group).

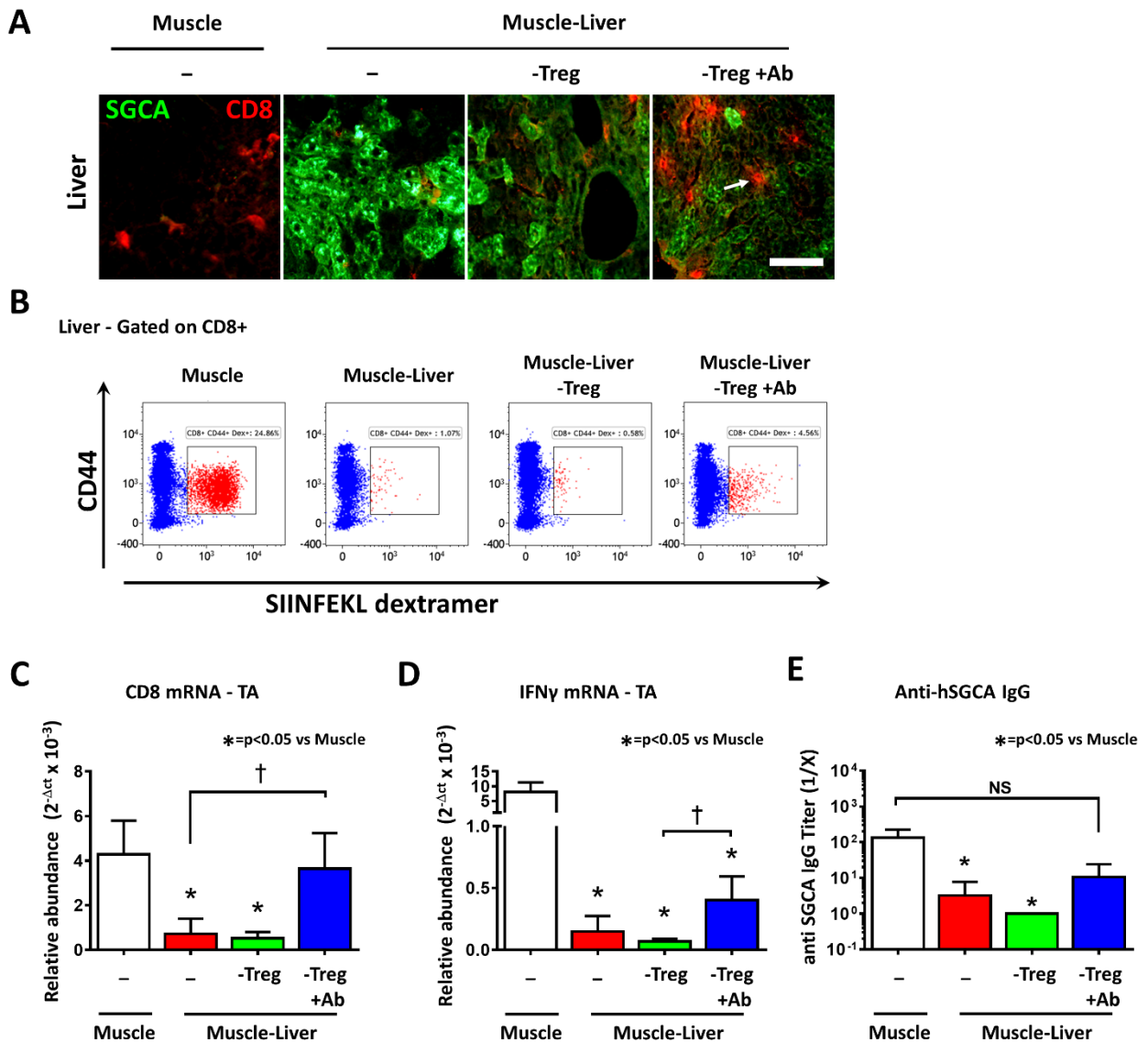
## Supplementary FIGURE 5



Supplementary Figure 5. (A,B) Flow cytometry dot plots representing CD8+CD44+Dextramer+ cells gated on CD8+ cells in splenocytes. The histogram shows the

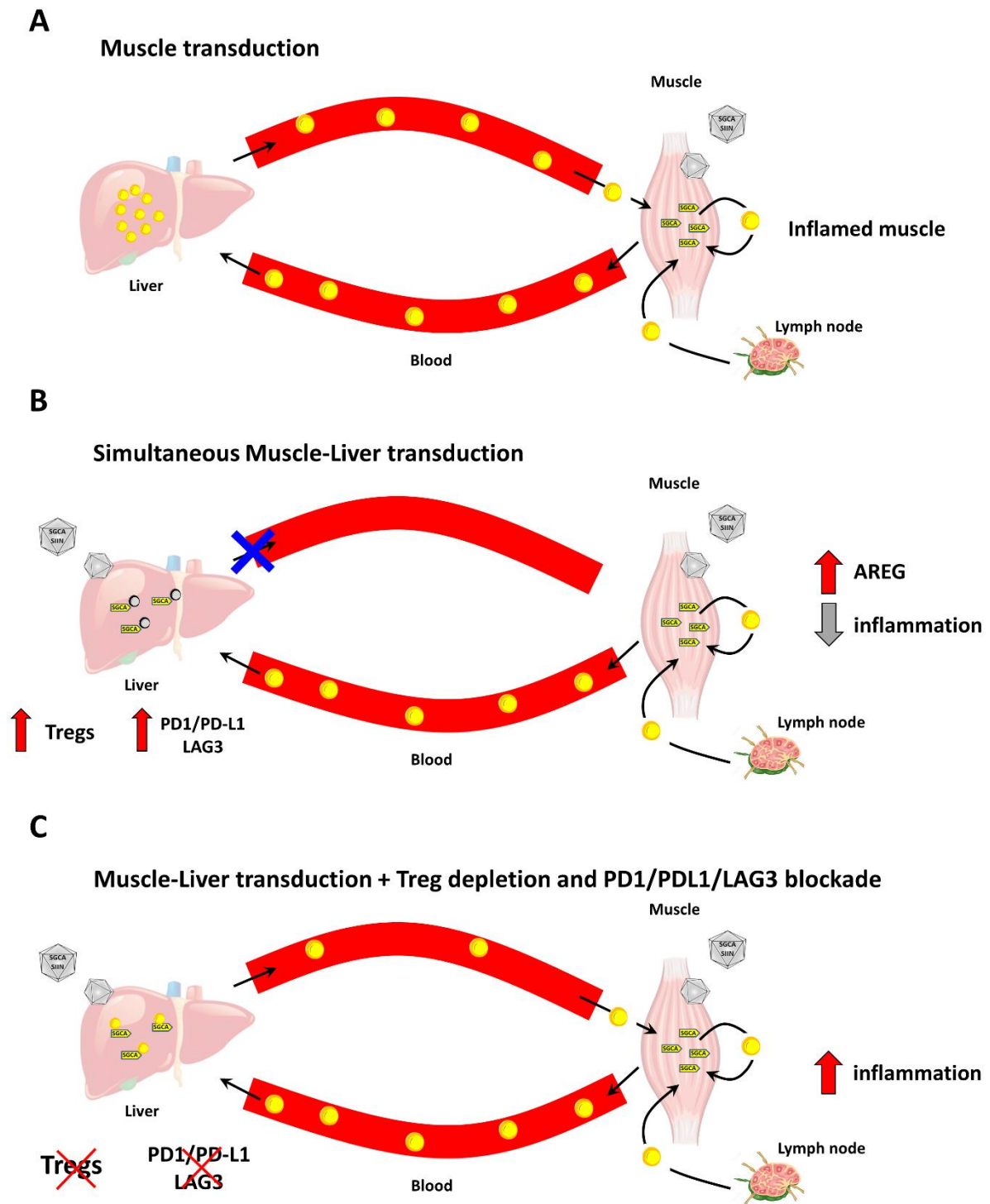
quantification of the dot plots. **(C-E)** CD8, IFN $\gamma$  and FoxP3 mRNA measured in *Tibialis anterior* (TA) muscle. **(F)** Vector genome copy number (VGCN) per diploid genome measured in TA muscle. **(G)** Anti-hSGCA IgG titers measured by ELISA using recombinant hSGCA protein. **(H-L)** CD8, IFN $\gamma$  and Foxp3 mRNA measured in liver. Data were expressed as mean  $\pm$  SD. Statistical analyses were performed by ANOVA in all panels except for panel G where a Kruskal-Wallis test was used (\* =  $p < 0.05$ ,  $n = 4$  per group).

## Supplementary FIGURE 6



**Supplementary Figure 6.** (A) Immunostaining anti-hSGCA (green), CD8 (red) performed in liver (scale bar = 50  $\mu$ m). White arrow indicates CD8 cell. (B) Flow cytometry dot plots representing liver non-parenchymal CD8+CD44+Dextramer+ cells gated on CD8+ cells. (C, D) CD8 and IFN $\gamma$  mRNA measured in *tibialis anterior*. (E) Anti-hSGCA IgG titers measured by ELISA using recombinant hSGCA protein. Data were expressed as mean  $\pm$  SD. Statistical analyses were performed by ANOVA in all panels except for panel E where a Kruskal-Wallis test was used (\* =  $p < 0.05$ ,  $n = 4$  per group).

## Supplementary FIGURE 7



**Supplementary Figure 7.** (A) After intramuscular AAV-mediated delivery of a transgene, antigen presentation occurs directly in muscle or in lymph nodes. Circulating activated T cells

home to the liver with little effect on their activation state. **(B)** In case of simultaneous liver and muscle transduction, activated T cells home to the liver and do not participate in the ongoing immune response in muscle. **(C)** When Tregs depletion is combined with anti PD1/PDL1 and LAG3 inhibition, a partial rescue of the immune response in muscle is observed.