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## **Microbiota stimulation generates LCMV-specific memory CD8<sup>+</sup> T cells in SPF mice and determines their TCR repertoire during LCMV infection.**

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1 **Abstract**

2 Both mouse and human harbour memory phenotype CD8<sup>+</sup> T cells specific for antigens  
3 in hosts that have not been previously exposed to these antigens. The origin and the nature of  
4 the stimuli responsible for generation of CD44<sup>hi</sup> CD8<sup>+</sup> T cells in specific pathogen-free (SPF)  
5 mice remain controversial. It is known that microbiota plays a crucial role in the prevention  
6 and resolution of systemic infections by influencing myelopoiesis, regulating dendritic cells,  
7 inflammasome activation and promoting the production of type I and II interferons. By  
8 contrast, here we suggest that microbiota has a direct effect on generation of memory  
9 phenotype CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells. In SPF mice, it generates a novel GP33<sup>+</sup>CD44<sup>hi</sup>CD8<sup>+</sup>  
10 T cell sub-population associating the properties of innate and genuine memory cells. These  
11 cells are highly enriched in the bone marrow, proliferate rapidly and express immediate  
12 effector functions. They dominate the response to LCMV and express particular TCRβ  
13 chains. The sequence of these selected TCRβ chains overlaps with that of GP33<sup>+</sup>CD8<sup>+</sup> T cells  
14 directly selected by microbiota in the gut epithelium of SPF mice, demonstrating a common  
15 selection mechanism in gut and peripheral CD8<sup>+</sup> T cell pool. Therefore microbiota has a  
16 direct role in priming T cell immunity in SPF mice and in the selection of TCRβ repertoires  
17 during systemic infection. We identify a mechanism that primes T cell immunity in SPF mice  
18 and may have a major role in colonization resistance and protection from infection.

19

20 **Keywords:** TCR repertoires; microbiota; innate memory CD8<sup>+</sup> T cells; bone marrow; LCMV

21

22 **Abbreviations:** BM - bone marrow; GF - germ-free; HP - homeostatic proliferation; LCMV -  
23 Lymphocytic choriomeningitis virus; LN - lymph nodes; NKR - natural killer receptors; SP -  
24 spleen; SPF - specific pathogen free; TCR - T cell receptor; TLR4 - Toll-like receptor; T<sub>VM</sub> -  
25 virtual memory T cells; T<sub>RM</sub> - tissue resident memory cells

## 1 **1. Introduction**

2 Both mouse and human harbour memory phenotype CD8<sup>+</sup> T cells specific for antigens  
3 in hosts that have not been previously exposed to these antigens. In mice, memory phenotype  
4 (CD44<sup>hi</sup>) CD8<sup>+</sup> T cells are present in specific pathogen free (SPF) and even in germ-free (GF)  
5 mice (Haluszczak et al., 2009; Lee et al., 2013). Some researchers classified these cells as  
6 virtual memory CD8<sup>+</sup> T (T<sub>VM</sub>) and propose that they were generated in a TCR-independent  
7 manner (Haluszczak et al., 2009; White et al., 2016). The majority of CD44<sup>hi</sup> CD8<sup>+</sup> T cells are  
8 believed to be generated by homeostatic proliferation (HP) in the perinatal period. When the  
9 first mature thymocytes (putatively considered naïve cells) leave the thymus to colonize the  
10 “empty” peripheral T cell pool, a fraction is recruited to undergo HP reconstituting the  
11 CD44<sup>hi</sup>CD8<sup>+</sup> T cell compartment (reviewed in (Freitas and Rocha, 2000)). In adult mice,  
12 these CD44<sup>hi</sup> CD8<sup>+</sup> T cells are highly functional, exhibiting more rapid proliferation and  
13 cytokine production after TCR stimulation compared to true naïve CD44<sup>lo</sup>CD8<sup>+</sup> T cells (Duffy  
14 et al., 2012; Haluszczak et al., 2009; Lee et al., 2013; Raue et al., 2013), but the role of these  
15 cells during immune responses remain controversial. Recently a elegant study showed that  
16 early-life derived CD8<sup>+</sup> T cells preferentially become HP CD44<sup>hi</sup>CD8<sup>+</sup> T cells in adult SPF  
17 mice (Smith et al., 2018). These early-life derived CD44<sup>hi</sup>CD8<sup>+</sup> T cells exhibits rapid and  
18 innate-like functions in response to cytokine signals, expressed higher levels of CXCR3 and  
19 effector proteins IFN- $\gamma$  and granzyme B. In contrast, the adult derived CD8<sup>+</sup> T cells  
20 predominantly give rise to naïve CD44<sup>lo</sup>CD8<sup>+</sup> T cells with slower kinetics (Smith et al., 2018).  
21 The adoptive co-transfer of congenically labeled TCR transgenic CD8<sup>+</sup> T cells showed that  
22 early-life derived CD44<sup>hi</sup>CD8<sup>+</sup> T cells are the first CD8<sup>+</sup> T cells to respond to *Listeria*  
23 *monocytogenes* infection in adult mice (Smith et al., 2018). However, there was a substantial  
24 accumulation of memory CD8<sup>+</sup> T cells with age, consistent with cumulative antigen exposure  
25 over the mice (Quinn et al., 2018) and human (Su et al., 2013) life span, suggesting that HP

1 CD44<sup>hi</sup> CD8<sup>+</sup> T cell heterogeneity was underestimated in previous studies. The protective  
2 potential of CD44<sup>hi</sup>CD8<sup>+</sup> T cells was also mostly tested in model infections with TCR  
3 transgenic CD8<sup>+</sup> T cells (Smith et al., 2018). In addition, CD44<sup>hi</sup>CD8<sup>+</sup> T cells are particularly  
4 abundant in the bone marrow (BM) of unmanipulated SPF mice (Goncalves et al., 2017), the  
5 role of these polyclonal host CD44<sup>hi</sup>CD8<sup>+</sup> T cells during primary immune responses and their  
6 TCR repertoire remain unknown.

7         The origin and the nature of the stimuli responsible for HP of CD44<sup>hi</sup> CD8<sup>+</sup> T cells  
8 remain also controversial. Cytokine stimulation was claimed to generate HP CD44<sup>hi</sup> CD8<sup>+</sup>  
9 (Haluszczak et al., 2009; White et al., 2016), but other studies have shown that cytokines are  
10 necessary but not sufficient for HP (Freitas and Rocha, 2000). Besides cytokine stimulation,  
11 HP required TCR stimulation, the extent of HP being proportional to the extent of TCR cross-  
12 reactivity (Hao et al., 2006). HP was attributed to the recognition of cross-reactive self-  
13 antigens (Quinn et al., 2016; Surh and Sprent, 2000), since the OVA-specific CD44<sup>hi</sup>CD8<sup>+</sup>  
14 population in the spleen (SP) was not modified in germ-free (GF) conditions, and HP was  
15 maintained after microbiota elimination by antibiotic treatment (Haluszczak et al., 2009;  
16 Kawabe et al., 2017; Quinn et al., 2016). However, although antibiotics killed resident  
17 bacteria, they could not ensure that all bacteria components were eliminated. Other  
18 experiments supported a major role of microbiota in the generation of HP CD44<sup>hi</sup> CD8<sup>+</sup>  
19 (Beura et al., 2016; Feng et al., 2010; Kieper et al., 2005). Thus, when polyclonal CD4<sup>+</sup> T  
20 cells were adoptively transferred to severe combined immunodeficiency (SCID) germ-free  
21 (GF) mice they homeostatically proliferated slowly, while when transferred to SCID SPF mice  
22 they proliferated vigorously (Feng et al., 2010; Kieper et al., 2005). Also, the number of  
23 CD44<sup>hi</sup>CD8<sup>+</sup> T cells is much reduced in GF mice (Beura et al., 2016). Using different animal  
24 models studies showed that microbiota-derived antigens generate CD44<sup>hi</sup>CD8<sup>+</sup> T cells in part  
25 mediated by sustained TCR stimulation (Mani et al., 2019; Tanoue et al., 2019). Immediately

1 after birth, newborn mice experience rapid colonization by microbiota that participate in the  
2 development of their immune system and possible in generation of early-life derived HP  
3 CD44<sup>hi</sup>CD8<sup>+</sup> T cells (Smith et al., 2018). The existence of different types of CD8<sup>+</sup> T cells  
4 cross-reactive with self-antigens (Quinn et al., 2016; Surh and Sprent, 2000) or microbiota-  
5 antigens (Feng et al., 2010; Kieper et al., 2005; Mani et al., 2019; Tanoue et al., 2019) could  
6 explain the contradictory conclusions in the stimuli responsible for HP CD44<sup>hi</sup> CD8<sup>+</sup> T cells  
7 (White et al., 2017). Addressing the role of microbiota in generation of HP CD44<sup>hi</sup>CD8<sup>+</sup> T  
8 cells is an important issue since microbiota is known to have a major protective effect against  
9 systemic infections (Becattini et al., 2017).

10 We recently quantified precisely the number, organ distribution and the TCR $\beta$   
11 (TRBV) repertoire of CD8<sup>+</sup> T cells specific for the GP33 peptide of the LCMV (GP33<sup>+</sup> cells)  
12 in all lymphoid organs of SPF B6 mice. Surprisingly, we found that the majority of GP33<sup>+</sup>  
13 cells were not naïve, but rather expressed CD44, and were particularly abundant in the BM  
14 (Goncalves et al., 2017). Here, we study their phenotypic characteristics and directly track the  
15 effector GP33<sup>+</sup> cells numbers through the course of LCMV infection, together with  
16 clonotypic TCR repertoire analysis. We here showed that CD44<sup>hi</sup> GP33<sup>+</sup> T cells accumulate  
17 adaptive and innate functions. CD44<sup>hi</sup> GP33<sup>+</sup> T cells are preferentially selected during LCMV  
18 infection. This study is the first to our knowledge to demonstrate by TCR repertoire analyses  
19 that these cells are preferentially selected during LCMV infection. Our results suggest also  
20 that microbiota stimulation generates these cells in SPF mice. Their TCR $\beta$  repertoire is  
21 conditioned by microbiota stimulation. In conclusion, these data suggest that microbiota  
22 stimulates antigen-specific CD8<sup>+</sup> T cells, and selects their TCR repertoire during primary  
23 infection. These data reveal a new major mechanism conditioning the effective responses to  
24 infection in mice harbouring a normal microbiota.

25

## 1 **2. Materials and methods**

### 2 *2.1. Mice and immunization protocols*

3 C57BL/6 (B6) specific-pathogen-free (SPF-1 and SPF-2) or germ-free (GF) mice were  
4 obtained from the breeding colonies of the Pasteur Institute. CD45.1<sup>+</sup> Rag2<sup>-/-</sup> B6 mice  
5 expressing a transgenic TCR specific for LCMV epitope GP<sub>33-41</sub> (GP33<sup>+</sup>) P14 (P14) were  
6 obtained from our breeding colonies at the Centre de Distribution, Typage et Archivage  
7 (CDTA, Orleans, France). For LCMV infection, mice were injected i.p. with 2.5x10<sup>5</sup> PFU of  
8 LCMV Armstrong. Mice were kept in isolators until sacrifice.

9

### 10 *2.2. Quantification and characterization of antigen-specific CD8<sup>+</sup> T cells*

11 The method to the evaluation of the number of antigen-specific cells in spleen (SP) and the  
12 bone marrow (BM) was described in detail previously (Goncalves et al., 2017; Sung et al.,  
13 2013), multiple steps being used to optimize cell recovery and quantify cell loss during the  
14 preparation steps. The total number of BM cells was obtained by multiplying the number of  
15 cells recovered from two femurs by 7.9, as previously described (Slifka et al., 1995).  
16 Intraepithelial lymphocytes (IELs) of the murine small intestine were recovered as we  
17 described previously (Guy-Grand et al., 2013). Briefly, cell suspensions were depleted from  
18 non-CD8<sup>+</sup> T cells followed by staining with APC or PE-labeled H-2D<sup>b</sup> or H-2K<sup>d</sup> dextramers  
19 loaded with different peptides (Dextramers<sup>®</sup>, Immudex, Copenhagen, Denmark) (Goncalves  
20 et al., 2017; Sung et al., 2013). This was followed by the incubation at 4°C during 30 min  
21 with multiple MoAbs obtained from BD Pharmingen (San Diego, CA, USA): anti-CD45.1  
22 (A20), anti-CD3ε (145-2C11), anti-CD8β (H35-172), anti-CD44 (1M781), anti-CD62L  
23 (MEL-14), anti-CD5 (Ly-1), anti-CD122 (TM-B1), anti-CD25 (PC61), anti-PD-1 (29F.1A12),  
24 anti-KLRG1 (2F1), anti-CD69 (H1.2F3), anti-CD103 (M290), anti-CD49b (DX5), anti-  
25 CD160 (ebioCNIX46-3), anti-CD107a (1D4B), anti-TLR4/MD-2 (MTS510), anti-ASGM1,

1 anti-NKG2D (1D11), anti-CD183 (CXCR3-173), anti-V $\beta$ 13 (F23.1), anti-V $\beta$ 29 (TR310),  
2 anti-IFN- $\gamma$  (XM61.2), anti-GranzB (6B11). Dead cells were excluded by Sytox Green dead  
3 cell stain (Thermo Fisher Scientific, MA USA). Labelled populations were diluted in 0.5 ml  
4 of FACS flow buffer and acquired using the low-speed mode in a FACSCanto II or in a  
5 FACS LSRFortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Different  
6 single cell CD8<sup>+</sup> populations were sorted (FACS-Aria II system, Becton Dickinson, Franklin  
7 Lakes, NJ, USA) into 96-well PCR plates (purity  $\geq$  99%). The data analysis was performed  
8 using FlowJo software (TreeStar, Ashland, OR).

9

### 10 *2.3. Single-cell cDNA synthesis, nexted RT-PCR and sequencing*

11 Analysis of TRBV usage was carried out by a single-cell multiplex RT-PCR developed in our  
12 laboratory, followed by the direct sequencing of the PCR products was described in detail  
13 previously (Goncalves et al., 2017).

14

### 15 *2.4. Microbial DNA extraction, 16S rRNA sequencing and analysis*

16 Fresh mouse stool samples were also collected and were immediately stored at -80 °C until  
17 DNA extraction was performed. Total fecal DNA was extracted using the NucleoSpin® Soil  
18 kit (MACHEREY-NAGEL®) following the protocol using both physical and chemical lysis.  
19 DNA concentrations were determined using Nanodrop spectrophotometer (Thermo  
20 Scientific). The V3-V4 region of the bacterial 16S rDNA region were PCR amplified with  
21 V3-340F (CCTACGGRAGGCAGCAG) and V4-805R (GGACTACHVGGGTWTCTAAT)  
22 barcoded primers. PCR products were cleaned using Ampure magnetic purification beads  
23 (Agencourt AMPure XP Kit), quantified with the QuantiFluor® ONE dsDNA kit (Promega),  
24 and pooled in equal amounts of each PCR product. Library pools were loaded at 12pM with a  
25 15% PhiX spike for diversity and sequencing control, onto a v3 300-bp paired end reads



1 cartridge for sequencing on the Illumina MiSeq NGS platform. After removing reads  
2 containing incorrect primer or barcode sequences and sequences with more than one  
3 ambiguous base, we recovered from 16 samples a total of 2.467.335 reads (83% mapped,  
4 108.388 mapped reads on average). The bioinformatics analysis was performed as previously  
5 described (Quereda et al., 2016). Briefly, amplicons were clustered into operational  
6 taxonomic units (OTU) with VSEARCH (v1.4) and aligned against the SILVA database. The  
7 clustering was performed at 98% sequence identity threshold, producing 386 OTUs. The input  
8 amplicons were then mapped against the OTU set to get an OTU-abundance table containing  
9 the number of reads associated with each OTU. The normalization, statistical analyzes and  
10 multiple visualization were performed with SHAMAN (SHiny application for Metagenomic  
11 Analysis ([shaman.c3bi.pasteur.fr](http://shaman.c3bi.pasteur.fr)) based on R software.

12

### 13 *2.5. In vitro cytokine stimulation assay*

14 Bulk CD44<sup>+</sup> and CD44<sup>hi</sup> populations were sorted to purity  $\geq 99\%$ , and  $5 \times 10^5$  sorted cells  
15 plated on 12-wells plates in RPMI medium supplemented with 2% foetal calf serum. They  
16 were cultured overnight (18h) with medium supplemented with various cytokines: IL-12 (10  
17 ng/ml), IL-18 (10 ng/ml), IL-2 (10 ng/ml) and IL-15 (10 ng/ml) (R&D). For intracellular  
18 cytokines staining, cells were incubated in the presence of GolgiPlug<sup>TM</sup> (Brefeldin A solution,  
19 BD) for 4 h at 37°C, fixed, permeabilized, and stained by Cytotfix/Cytoperm Kit (BD  
20 Biosciences, San Jose, CA) according to the manufacturer's instructions, followed by the  
21 evaluation of the intra-cytoplasmatic expression of IFN- $\gamma$  or Granzyme B. Samples were  
22 acquired on LSRFortessa (BD) and barriers identifying positive cells were established in the  
23 same populations, labelled with isotype controls.

24

25

1 *2.6. Identification of in vivo dividing cells*

2 Bromodeoxy Uridine (BrdU) was purchased from Sigma, (St. Louis, MO). Mice at day 3 and  
3 5 post LCMV infections were injected i.p. with 1 mg BrdU. BrdU incorporation was studied  
4 in different organs one hour after injection using the Cytofix/Cytoperm kit (BD Biosciences,  
5 San Jose, CA) according to the manufacturer's instructions.

6

7 *2.7. Bioinformatic determination of cross-reactivity with self-antigens*

8 We used the iCrossR webserver, which performs assessment of cross-reactivity (CR) indices  
9 in different tissues (Jaravine et al., 2017).

10

11 *2.8. Statistical analysis*

12 Statistics were performed using Prism 5, GraphPad software (San Diego, USA). Statistical  
13 significance of the difference between two groups was evaluated by the Mann-Whitney U  
14 test. Single continuous variable data were analyzed by Kruskal-Wallis (KW) followed by  
15 Dunn's Multiple Comparison Test. For all these tests, a cut-off value of  $p \leq 0.05$  was chosen  
16 (\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ).

17

18 **3. Results**

19

20 *3.1. Similarities and differences between CD44<sup>hi</sup>GP33<sup>+</sup> cells and virtual memory CD8<sup>+</sup> T*  
21 *cells.*

22 *3.1.1. CD44<sup>hi</sup>GP33<sup>+</sup> cells have characteristics of antigen-experienced memory CD8<sup>+</sup> T cells.*

23 The presence of CD44<sup>hi</sup> CD8<sup>+</sup> T cells with different antigen specificities was already  
24 described in SPF mice (Haluszczak et al., 2009; Lee et al., 2013). We studied different LCMV  
25 epitopes (NP376, GP33, GP276 and GP34) and we found CD44<sup>hi</sup> CD8<sup>+</sup> T cells specific for all

1 of these antigens, particularly abundant in the BM of SPF B6 mice (not shown) (Goncalves et  
2 al., 2017). Therefore, we studied the LCMV immune-dominant GP33<sup>+</sup> cells and an unrelated  
3 non-self non-microbial-associated antigen, ovalbumin (OVA) in the following experiences.  
4 First, we compare the phenotypic features of antigen-specific CD44<sup>hi</sup>GP33<sup>+</sup> and CD44<sup>-</sup>GP33<sup>+</sup>  
5 cells of naïve SPF-1 B6 mice (Figure 1). The method to identify antigen-specific CD8<sup>+</sup> cells  
6 was described in detail previously (Sung et al., 2013) (Supplementary Figure 1,  
7 Supplementary Note 1). Analysis of the CD44<sup>hi</sup>GP33<sup>+</sup> compound phenotype showed they  
8 correspond to a CD8<sup>+</sup> T cell subtype, different from previously described virtual memory T  
9 cells (T<sub>VM</sub>) (Haluszczak et al., 2009; White et al., 2016) or tissue resident memory cells  
10 (T<sub>RM</sub>). While T<sub>VM</sub> have a CD49d<sup>low</sup>CD62L<sup>+</sup>CD25<sup>low</sup>CD69<sup>low</sup>CD103<sup>low</sup> phenotype (Haluszczak  
11 et al., 2009), CD44<sup>hi</sup>GP33<sup>+</sup> were CD49d<sup>hi</sup> and comprised both CD62L<sup>+</sup> and CD62L<sup>-</sup> cells  
12 (Figure 1A). A fraction expressed the early activation markers CD25 and CD69 (Figure 1B)  
13 and the epithelial cell-binding integrin  $\alpha$ E $\beta$ 7 (CD103) (not shown), expressed by T<sub>RM</sub>, but lack  
14 killer cell lectin receptor G1 (KLRG1) (Figure 1C) (Tanoue et al., 2019). Interestingly, about  
15 half of total bone marrow (BM) CD8<sup>+</sup> CD44<sup>hi</sup> cells express also CD69 and the double  
16 labelling with CD69 and CD103 showed that these markers were co-expressed in 32% of the  
17 same cells (Figure 1D). Besides, CD44<sup>hi</sup>GP33<sup>+</sup> cells were CCR7<sup>+</sup>PD-1<sup>low</sup> (Figure 1E), while  
18 T<sub>RM</sub> are CD62L<sup>-</sup>CCR7<sup>-</sup>PD1<sup>hi</sup> (Debes et al., 2005). In addition, T<sub>RM</sub> cells have reduced  
19 expression levels of CD122, CD27, and Ly6C and CD44<sup>hi</sup>GP33<sup>+</sup> cells express these markers  
20 at higher levels (not shown).

21 Not all CD44<sup>hi</sup> CD8<sup>+</sup> T cells from SPF mice expressed the above markers. More than  
22 90% of the total CD44<sup>hi</sup> cells were CD49d<sup>low</sup> (Quinn et al., 2018) and express higher levels of  
23 CD5 and CD122 comparatively to CD44<sup>hi</sup>GP33<sup>+</sup> cells (results not shown), features of T<sub>VM</sub>  
24 cells (Drobek et al., 2018; Haluszczak et al., 2009). For example, CD44<sup>hi</sup>OVA<sup>+</sup> cells have the  
25 phenotype of T<sub>VM</sub> CD49d<sup>low</sup>CD62L<sup>+</sup> (Supplementary Fig. 2). These results suggest that

1 CD44<sup>hi</sup>CD8<sup>+</sup> T cell pool present in SPF mice is a heterogeneous population of T<sub>VM</sub> and other  
2 phenotypic CD44<sup>hi</sup> CD8<sup>+</sup> T cells.

3

4 *3.1.2. CD44<sup>hi</sup>GP33<sup>+</sup> cells are preprogrammed to mount a rapid innate-like effector response.*

5 Analysis of CD44<sup>hi</sup>GP33<sup>+</sup> showed they expressed multiple characteristics of innate  
6 lymphocytes (Seyda et al., 2016). Although lacking CD161 (NK1.1) (not shown) they  
7 expressed very high levels of several natural killer receptors (NKR). As compared to CD44<sup>-</sup>  
8 GP33<sup>+</sup> T cells, the % of cells expressing NKG2D and ASGM1 as well as its expression level  
9 was much increased (Figure 2A, B). As innate lymphoid cells (Seyda et al., 2016) a fraction  
10 up-regulated the expression of multiple receptors associated with effector functions: CD11a  
11 and CD11b, CD160 and CD107a (Figure 2A, B). They also expressed multiple receptors  
12 allowing a TCR-independent stimulation. Besides, CD44<sup>hi</sup>GP33<sup>+</sup> expressed ASGM1 and a  
13 fraction also expresses TLR4 (CD284) (Huggins et al., 2019; Loser et al., 2010) (Figure 2A,  
14 B), receptor for flagellin and LPS respectively. They express higher levels of CXCR3 (Figure  
15 3A, B), that defines a subset of innate cells that can be activated to mediate effector functions  
16 in the absence of TCR stimulation (Smith et al., 2018; Soudja et al., 2012). They up-regulated  
17 the expression of multiple cytokine receptors, CD121a (IL-1 $\beta$ R), CD218a (IL-18R) and  
18 CD122 (IL2 $\beta$ R, IL-15R) (Figure 3A, B), allowing them to receive inflammasome and IL-15  
19 signals. After overnight incubation with a cocktail of cytokines previously used (Haluszczak  
20 et al., 2009), CD44<sup>hi</sup>GP33<sup>+</sup> increase in numbers (Figure 3C) and mediated effector functions  
21 (Figure 3D, E), whereas naïve CD44<sup>-</sup>GP33<sup>+</sup> T cells failed to respond. We conclude that by  
22 their compound phenotype and TCR-independent effector functions the CD44<sup>hi</sup>GP33<sup>+</sup> cells  
23 from SPF mice differ from “conventional” memory CD8<sup>+</sup> T cells (White et al., 2017). These  
24 results suggest that CD44<sup>hi</sup>GP33<sup>+</sup> can provide protection against infections even in the  
25 absence of cognate antigen exposition.

1 *3.2. The role of CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells during primary immune responses.*

2 We observe that overnight incubation with GP33 peptide pulsed antigen presenting  
3 cells (APC) promote CD44<sup>hi</sup>GP33<sup>+</sup> increase in numbers (not shown) and induction of effector  
4 functions. More than 90% of CD44<sup>hi</sup>GP33<sup>+</sup> cells express CD69 (marker for early activation)  
5 and produce IFN- $\gamma$  (Supplementary Fig. 3). CD44<sup>hi</sup>GP33<sup>+</sup> cells express more CD69 and IFN- $\gamma$   
6 than naïve CD44<sup>+</sup>GP33<sup>+</sup> cells activated in the same conditions, suggesting a fast TCR-  
7 dependent induced activation and strong effector functions as demonstrated for other CD44<sup>hi</sup>  
8 antigen-specific cells (Duffy et al., 2012; Haluszczak et al., 2009; Lee et al., 2013; Raue et al.,  
9 2013).

10

11 *3.2.1. CD44<sup>hi</sup>GP33<sup>+</sup> cells are preprogrammed to mount a rapid early effector response.*

12 To evaluate the role of CD44<sup>hi</sup>GP33<sup>+</sup> T cells during systemic infection we first studied  
13 their accumulation kinetics and division rates after *in vivo* infection of SPF mice with LCMV  
14 (Figure 4A; Supplementary Fig. 4). The kinetics of the primary immune response of GP33<sup>+</sup>  
15 cells in the SP and LN of SPF mice (Supplementary Fig. 4) was as described previously  
16 (Murali-Krishna et al., 1998). By day 4 after priming the number GP33<sup>+</sup> cells was not  
17 significantly increased. This was expected since naïve T cells have a long-lag time before  
18 division (Veiga-Fernandes et al., 2000). Responses peaked at day 7, followed by a contraction  
19 phase where most cells disappeared, and generate the memory pool.

20 The responses in the BM were very different. CD44<sup>hi</sup>GP33<sup>+</sup> T cells were particularly  
21 abundant in the BM of SPF mice (Figure 4B) (Goncalves et al., 2017), a key site for priming  
22 of T cells specific for blood-borne antigens (Duffy et al., 2012; Feuerer et al., 2003).  
23 Interestingly, the number of GP33<sup>+</sup> cells was already 7 fold increased by day 4 after infection  
24 (Figure 4C). These results indicated a precocious activation and division of GP33<sup>+</sup> cells in the  
25 BM, a behaviour characteristic of memory cells (Veiga-Fernandes et al., 2000). Responses

1 also peaked at day 7, but the contraction phase was virtually absent (Supplementary Fig. 4B).  
2 We conclude that after LCMV infection, GP33<sup>+</sup> cells in the SP and LN behaved as expected  
3 from naïve cells in a primary immune response, while GP33<sup>+</sup> cells in the BM behaved as we  
4 previously described in conventional memory cells (Veiga-Fernandes et al., 2000).

5 To confirm these different behaviours, we determined the % of GP33<sup>+</sup> cells in the S  
6 phase of the cell cycle after a single pulse of BrdU (Figure 4D, E). In the SP and LN 31-42%  
7 of GP33<sup>+</sup> cells were BrdU<sup>+</sup> by day 3 after priming. The percentage of BrdU<sup>+</sup> cells in the SP  
8 declined to an average of 20% at day 5, when GP33<sup>+</sup> cells approached the response peak. By  
9 contrast in BM, an average of 66% of GP33<sup>+</sup> cells were in S phase at day 3, and yet 42% were  
10 cycling at day 5 after immunization. Less than 5% of CD44<sup>hi</sup>GP33<sup>-</sup> CD8<sup>+</sup> T cells were BrdU<sup>+</sup>  
11 in SP and LN, where almost 20% were cycling in BM at day 3 or 5 after priming, suggesting  
12 also a preferential proliferation of other antigen specificities (e.g. NP376, GP276) in BM.

13 These results confirm that GP33<sup>+</sup> cells located in the BM have a very different  
14 behaviour from those located in the SP and LN. They show an earlier activation, a much  
15 increased and persistent proliferation rates and increased survival. The different behaviour of  
16 GP33<sup>+</sup> cells located in the BM indicates an important advantage of CD44<sup>hi</sup>GP33<sup>+</sup> in the  
17 primary immune response to LCMV infection.

18

### 19 *3.2.2. CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells can trafficking from bone marrow to the periphery.*

20 A number of reports have indicated that BM is a major reservoir for recirculating  
21 CD44<sup>hi</sup>CD8<sup>+</sup> T cells (Klonowski et al., 2004; Stark et al., 2018; Thomas et al., 2011). Some  
22 other suggest that BM resident CD44<sup>hi</sup>CD8<sup>+</sup> T might be tissue resident memory cells (T<sub>RM</sub>)  
23 (Pascutti et al., 2019). CD44<sup>hi</sup>GP33<sup>+</sup> cells show an earlier activation and much increased  
24 proliferation rates in BM, therefore we considered the possibility of migration of these cells to  
25 the periphery. Because TCRβ (TRBV) repertoires of GP33<sup>+</sup> cells were essentially unique to

1 each individual mouse (“private”) and diverse (Goncalves et al., 2017), if this is the case, the  
2 TRBV repertoire in the periphery should significantly overlap with that in BM. We sorted  
3 CD44<sup>hi</sup>GP33<sup>+</sup> cells from BM, SP and LNs at different time points after LCMV infection and  
4 we analyzed their clonotypic TRBV repertoires (Goncalves et al., 2017). In total, we analyzed  
5 over 2,026 GP33-specific CD8<sup>+</sup> αβ<sup>+</sup> T cells at the single-cell level in all mice at different time  
6 points after LCMV infection. The sequences are shown in Supplementary Table 1. In the  
7 main text we show the modifications of the percentage of cells expressing different TRBV  
8 genes and the compound composition of all CDR3 regions. Interestingly, effector GP33<sup>+</sup>  
9 CD8<sup>+</sup> T cell clonotypes at day 7 post-infection are widely dispersed between different organs  
10 (Table 1, Supplementary Table 1), single-cell TRBV sequences that were prominent BM are  
11 also present in SP and LN, suggesting that T cell trafficking from and to BM might be  
12 precocious in early effector phase. These results suggest that after the early activation and  
13 proliferation in BM, effector GP33<sup>+</sup> cells were able to migrate to the infected organs and  
14 contribute to LCMV clearance.

15

16 *3.2.3. The TRBV repertoire of GP33<sup>+</sup> cells during LCMV infection converges to that of*  
17 *CD44<sup>hi</sup>GP33<sup>+</sup> cells already present in SPF mice.*

18 We next evaluated the relative contribution of CD44<sup>-</sup> and CD44<sup>hi</sup>GP33<sup>+</sup> cells to the  
19 immune response to LCMV infection. Since after *in vivo* infection naïve CD44<sup>-</sup>GP33<sup>+</sup>  
20 become activated expressing CD44<sup>hi</sup>, CD44 expression levels could no longer discriminate  
21 GP33<sup>+</sup> cells of naïve versus CD44<sup>hi</sup> origin. However, we described that CD44<sup>-</sup>GP33<sup>+</sup> T cells  
22 had a biased TCRB repertoire, different from that of CD44<sup>hi</sup>GP33<sup>+</sup> cells (Goncalves et al.,  
23 2017). The major differences between naïve and CD44<sup>hi</sup> GP33<sup>+</sup> cells were found in the  
24 expression frequencies of TRBV13 genes (coding for the Vβ8 proteins) and TRBV29 gene  
25 (coding for the Vβ7 protein). Up to 40-47% of CD44<sup>-</sup>GP33<sup>+</sup> cells expressed TRBV13/Vβ8, in

1 contrast to a 15-20% frequency in the CD44<sup>hi</sup>GP33<sup>+</sup> repertoires. By contrast, only 4% of  
2 CD44<sup>+</sup>GP33<sup>+</sup> expressed the TRBV29/V $\beta$ 7 chain while an averaged 12% of CD44<sup>hi</sup>GP33<sup>+</sup>  
3 cells expressed this TRBV (Figure 5A, B). Other TRBV genes showed but minor differences  
4 (Supplementary Table 1).

5 Next we evaluated how GP33<sup>+</sup> TRBV repertoires were modulated during LCMV  
6 infection (Figure 5C, Supplementary Table 1). As striking differences we found a major and  
7 progressive decline in the frequency of cells expressing TRBV13, concomitant with a major  
8 and progressive selection of TRBV29 expressing cells. Overall, the frequency of TRBV13<sup>+</sup>  
9 cells dropped from 47% of CD44<sup>+</sup>GP33<sup>+</sup> naïve cells to an average of 12% of secondary  
10 effectors (Figure 5C). TRBV29<sup>+</sup> cells followed the opposite trend. This TRBV, which was  
11 more abundant in the CD44<sup>hi</sup>GP33<sup>+</sup> in SPF mice, was further enriched in the effector phase,  
12 persisted in similar frequencies in the memory compartment, and was further increased in  
13 secondary immune responses (Figure 5C). While in SPF mice TRBV29 was expressed by  $\approx$   
14 12% of CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells,  $\approx$  38% of GP33<sup>+</sup> effectors cells expressed TRBV29 in a  
15 secondary immune response (Figure 5C). Of note, this modulation of the TRBV repertoire  
16 was similar in each animal, and common to all organs (Table 1, Supplementary Table 1).  
17 These results suggest that TCR repertoire of GP33<sup>+</sup> cells during LCMV response shifts from  
18 that of naïve CD44<sup>+</sup>GP33<sup>+</sup> (where TRBV13/V $\beta$ 8<sup>+</sup> cells predominate) to those expressing  
19 TRBV29/V $\beta$ 7<sup>+</sup>. The preferential selection of TRBV29/V $\beta$ 7 repertoire is compatible with a  
20 major advantage of CD44<sup>hi</sup>GP33<sup>+</sup> in the primary immune response to LCMV infection.

21  
22 *3.2.4. The role of TCR complementary-determining regions (CDRs) in selection of GP33<sup>+</sup>*  
23 *cells after LCMV infection.*

24 In general, it is assumed that the CDR1 and CDR2 loops of the TCR $\beta$  interact with the  
25  $\alpha$ 2 and  $\alpha$ 1 helix of MHC class I, while the highly diverse CDR3 loops are mainly responsible



1 for peptide recognition and repertoire selection (Das et al., 2015). Interestingly, we found a  
2 enrichment in asparagine (N) in CDR3 of TRBV29<sup>+</sup> cells. This amino acid was already  
3 overexpressed by TRBV29<sup>+</sup> primary effectors and was always dominant in position 7 in  
4 memory and secondary effectors (Supplementary Fig. 5A-E). These data suggest that the  
5 selection of TRBV29<sup>+</sup> cells during LCMV responses had features of a type 2 immune  
6 response (Turner et al., 2006). In type 2 responses antigen binds to the CDR1 and CDR2  
7 domains (encoded within the TCR germline variable sequence) of TRBV(29) gene, having  
8 conserved amino acids or “motifs” in the CDR3 (CASSxxxNxxEQYF) important to peptide  
9 recognition (Das et al., 2015) and involved in the selection of GP33<sup>+</sup> cells during LCMV  
10 response (Adams et al., 2016; Cole et al., 2009). Besides asparagine, glycines (coded by the  
11 germ line TRBD genes) were present at higher frequency, but this occurred in both TRBV29<sup>+</sup>  
12 and TRBV29<sup>-</sup> cells. The higher expression of glycines was maintained throughout the  
13 response (Supplementary Fig. 5A-E). A CDR3 region enriched in glycines increases the  
14 flexibility of the TCR reported to lead to greater promiscuity in antigen recognition (Chowell  
15 et al., 2015; Stadinski et al., 2016) and increases TCR cross-reactivity (Birnbaum et al.,  
16 2014).

17

### 18 *3.3. Nature of the stimuli responsible for generation of CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells.*

#### 19 *3.3.1. GP33 and OVA peptide molecular mimicry.*

20 To determine which type of cross-reactive antigens were responsible for the  
21 generation of different sub-populations of CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells we first performed an  
22 *in silico* search of molecular mimicry between GP33 and OVA peptides with self or  
23 microbiota-components. The BLAST search revealed that OVA peptide align 100% to  
24 multiple self components (Ge et al., 2004) and showed molecular mimicking with self-  
25 antigens present in different tissues (Supplementary Fig. 6A, B). OVA peptide showed also

1 molecular mimicking with microbiota components that besides are poorly represented within  
2 the microbiota (Supplementary Table 2). On the other hand, GP33 peptide showed low  
3 molecular mimicking with self-antigens (Supplementary Fig. 6A, B) (Quinn et al., 2016).  
4 However, the *in silico* search revealed that GP33 peptide showed molecular mimicking with  
5 multiple components present in bacterial strains (Supplementary Table 2). Bacterial strains  
6 predicted to have molecular mimicking with GP33 peptide represent  $\approx 4\%$  of the total mice  
7 fecal microbiota (*Clostridium*, *Staphylococcus* and *Peptococcus* genus) of SPF-1 mice  
8 determined by 16S ribosomal RNA (rRNA) sequencing (Supplementary Fig. 6H). These  
9 results suggest that CD44<sup>hi</sup>GP33<sup>+</sup> cells and CD44<sup>hi</sup>OVA<sup>+</sup> cells could be generated by cross-  
10 reactivity with different antigens. CD44<sup>hi</sup>GP33<sup>+</sup> cells may be mostly generated by cross-  
11 reactivity with microbiota components present in gut of SPF mice.

12

### 13 3.3.2. Microbiota stimulation generates CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells.

14 Given the immense antigenic load present in microbiota, we hypothesized that some  
15 microbiota mimotopes can be responsible for the generation of CD44<sup>hi</sup>GP33<sup>+</sup> cells in gut-  
16 associated lymphoid tissue (GALT) (Esterhazy et al., 2019; Harkioliaki et al., 2009; Li et al.,  
17 2019; Mani et al., 2019; Su et al., 2013; Tanoue et al., 2019; Yang et al., 2014). Activated  
18 CD8<sup>+</sup> T cells in GALT can migrate through the thoracic ducts to the blood vasculature,  
19 disseminating to peripheral lymphoid organs (Beura et al., 2018; Tanoue et al., 2019; Teng et  
20 al., 2016). In addition, microbiota mimotopes were shown to disseminate across the body and  
21 thus may stimulate recirculating CD8<sup>+</sup> T cells located elsewhere (Beura et al., 2018; Tanoue  
22 et al., 2019; Teng et al., 2016). To test this hypothesis we evaluated if the absence of  
23 microbiota under GF conditions decrease CD44<sup>hi</sup>GP33<sup>+</sup> numbers in peripheral lymphoid  
24 organs. The total number of CD8<sup>+</sup> T cells (in SP and BM) is not significant different between  
25 GF and SPF-1 B6 mice (not shown), suggesting that GF mice have a normal T cell poiesis

1 (Khosravi et al., 2014). However, the GF condition induced an overall reduction of  
2 CD44<sup>hi</sup>CD8<sup>+</sup> cells (Figure 6A) and the % and total number of CD44<sup>hi</sup>GP33<sup>+</sup> was much  
3 reduced in the BM (Figure 6B, C). The total number of CD44<sup>hi</sup>GP33<sup>+</sup> was reduced 80% in  
4 BM of GF mice. In accordance with the rare cross reactivity of OVA specific cells with  
5 microbiota components, we confirmed that the GF condition did not affect the number of  
6 CD44<sup>hi</sup>OVA<sup>+</sup> cells in the SP (not shown), as reported previously (Haluszczak et al., 2009). In  
7 GF mice the phenotype of CD44<sup>hi</sup>GP33<sup>+</sup> cells was also modified. In particular, almost all total  
8 CD44<sup>hi</sup>CD8<sup>+</sup> and CD44<sup>hi</sup>GP33<sup>+</sup> cells that remain in GF condition were CD49d<sup>low</sup> (T<sub>VM</sub>)  
9 (Figure 6D, E) (Drobek et al., 2018; Haluszczak et al., 2009). Interestingly, 92% of  
10 CD44<sup>hi</sup>CD8<sup>+</sup> in the BM failed to express CD69 (Figure 6F), and remaining CD44<sup>hi</sup>GP33<sup>+</sup>  
11 cells down-regulated CD69 expression levels (Figure 6G). The frequencies of BM  
12 CD44<sup>hi</sup>CD8<sup>+</sup> T cells CXCR3<sup>+</sup>, CD122<sup>+</sup>, NKG2D<sup>+</sup> and ASGM1<sup>+</sup> cells were also less abundant  
13 (Supplementary Fig. 7A). Importantly, the expression level of all these markers was reduced  
14 (Supplementary Fig. 7B), suggesting that microbiota stimulation is essential to induce their  
15 expression.

16 These results suggest that the presence of microbiota has an essential role in  
17 generation of CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells, and possibly also CD44<sup>hi</sup>CD8<sup>+</sup> T cells with other  
18 antigen specificities (Beura et al., 2016; Feng et al., 2010; Kieper et al., 2005). Moreover,  
19 microbiota stimulation also influences the properties of CD44<sup>hi</sup> CD8<sup>+</sup> T cells.

20

### 21 3.3.3. Microbiota with GP33 molecular mimicry generates CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells.

22 A different microbiota composition in different SPF breeding facilities (Thiemann et  
23 al., 2017) (or even cages (Constantinides et al., 2019)) can contribute to variable and  
24 sometimes contradictory experimental results obtained from genetically identical animals.  
25 Therefore, to confirm the role of microbiota in generation of CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells we

1 studied B6 mice from another breeding facility (SPF-2). The total number of CD44<sup>hi</sup>CD8<sup>+</sup>  
2 cells (in SP and BM) were not significant different between mice from the two breeding  
3 facilities (Figure 7A). The % and number of CD44<sup>hi</sup>OVA<sup>+</sup> cells were also not significant  
4 different (not shown). However, the % and total number of CD44<sup>hi</sup>GP33<sup>+</sup> were reduced in SP  
5 and BM of the SPF-2 (Figure 7B, C). The total number of CD44<sup>hi</sup>GP33<sup>+</sup> were reduced 40% in  
6 SPF-2 mice. Next, we performed 16S rRNA sequencing from fecal DNA to compare the  
7 microbiota composition between the two breeding facilities. Notably, beta diversity revealed a  
8 significant difference between the microbiota of SPF-2 and SPF-1, with samples clustering  
9 distinctively according to the breeding facility (Figure 7D). Interestingly, bacterial strains  
10 predicted to have molecular mimicking with GP33 peptide were significantly decreased in  
11 SPF-2 (Figure 7E, F, Supplementary Fig. 8). These results suggest that specific bacteria can  
12 promote the generation of some CD44<sup>hi</sup>GP33<sup>+</sup> cells in SPF-1 mice. Alpha diversity varied  
13 also between the two mouse lines, SPF-2 had lower Shannon diversity index (species richness  
14 combined with abundance and evenness) than SPF-1 (Figure 7G, H). Lastly, we found that  
15 microbiota stimulation had a continuous effect in CD44<sup>hi</sup>GP33<sup>+</sup> cells since their number  
16 increase progressively in the BM of SPF-1 mice throughout life (Supplementary Fig. 9).

17 These results suggest that the presence of microbiota with GP33 molecular mimicry  
18 has an essential role in generation of CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells. The microbiota stimulation  
19 is continuous as shown by the selective increase in the number of CD44<sup>hi</sup>GP33<sup>+</sup> cells  
20 throughout life.

21

22 *3.3.4. Microbiota stimulation is essential for the development of*  
23 *CD8 $\alpha\beta$ <sup>+</sup>CD44<sup>hi</sup>GP33<sup>+</sup>TRBV29<sup>+</sup> intraepithelial lymphocytes.*

24 Intraepithelial lymphocytes (IELs) represent about half of whole lymphocytes of all  
25 mouse organism (Rocha et al., 1991). CD8 $\alpha\beta$ <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> IELs develop through the continuous

1 interaction with intestinal antigens such as microbiota (Hegazy et al., 2017; Noble et al.,  
2 2019; Regnault et al., 1996; Umesaki et al., 1993). Therefore, we hypothesized that  
3 CD44<sup>hi</sup>GP33<sup>+</sup> cells would be enriched within the small intestine epithelium of SPF mice  
4 (Mani et al., 2019), and express particularly TRBV29/V $\beta$ 7. To test this hypothesis, we studied  
5 the CD8 $\alpha\beta$ <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> IELs of the murine gut of SPF and germ-free (GF) mice. OVA-specific  
6 dextramers only detected rare IELs, with very low labelling intensity (Figure 8A middle  
7 graphs). By contrast, CD44<sup>hi</sup>GP33<sup>+</sup>CD8 $\beta$ <sup>+</sup> IELs were clearly identified in SPF-1 mice (Fig.  
8 8A-left lower graphs). In germfree (GF) condition, only very rare events were dispersed in the  
9 CD44<sup>hi</sup>CD69<sup>+</sup>GP33<sup>+</sup>CD8 $\beta$ <sup>+</sup> quadrant, i.e., a clear-cut population could not be identified  
10 (Figure 8A-right lower graphs). Interestingly, CD44<sup>hi</sup>GP33<sup>+</sup>CD8 $\beta$ <sup>+</sup> IELs were clearly  
11 decreased in SPF-2 mice (Figure 8A-center lower graphs), suggesting that microbiota with  
12 GP33 molecular mimicry has an essential role in generation of these cells. The GF condition  
13 induced also an overall reduction of CD44<sup>hi</sup>CD8<sup>+</sup> cells and CD44<sup>hi</sup>GP33<sup>+</sup> cells in gLNs (not  
14 shown) and in the lamina propria (LP) (Figure 8B).

15 The immune response to the LCMV leads to the selection of GP33<sup>+</sup> cells expressing  
16 TRBV29/V $\beta$ 7 with a peculiar CASSxxxNxxEQYF CDR3 composition. Lastly, to study if  
17 microbiota help in the selection of these TRBV chains we studied the repertoire of the  
18 CD8 $\alpha\beta$ <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> IELs of SPF mice. Only a minority of CD8 $\alpha\beta$ <sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> IEL expressed  
19 V $\beta$ 7 but the vast majority of CD44<sup>hi</sup>GP33<sup>+</sup> were V $\beta$ 7<sup>+</sup> (Figure 8C). These data suggest that  
20 microbiota exposition can contribute to the selection of GP33<sup>+</sup>V $\beta$ 7<sup>+</sup> IELs in small intestine of  
21 SPF mice. Interestingly, the sequence of the CDR3 region of GP33<sup>+</sup>V $\beta$ 7<sup>+</sup> IELs of SPF mice  
22 showed a CDR3 composition (CASSxxxNxxEQYF), typical of the repertoire of  
23 GP33<sup>+</sup>selected during the LCMV response (Figure 8D). Notably, CD44<sup>hi</sup>GP33<sup>+</sup> IELs and  
24 peripheral CD44<sup>hi</sup>GP33<sup>+</sup> shared the dominant features, strongly suggesting that CD8<sup>+</sup> T cells  
25 activated in gut can migrate to the periphery (Beura et al., 2018; Tanoue et al., 2019; Teng et

1 al., 2016) and be a source of systemic CD44<sup>hi</sup>GP33<sup>+</sup> cells. These data suggest that microbiota  
2 contribute for the selection of the GP33<sup>+</sup> TRBV29<sup>+</sup> during the LCMV infection.

3

#### 4 **4. Discussion**

5 Microbiota protects against gastrointestinal pathogenic infections, which was named  
6 “colonization resistance” effect (Becattini et al., 2017). Microbiota employ an arsenal of  
7 mechanisms to influence development and specificity of host innate and adaptive immune  
8 system (Belkaid and Harrison, 2017). Therefore, microbiota plays also a crucial role in the  
9 prevention and resolution of systemic infections (Becattini et al., 2017; Tanoue et al., 2019).  
10 This effect was believed to be indirect. It was described to influence myelopoiesis (Khosravi  
11 et al., 2014), to regulate inflammasome activation (Ichinohe et al., 2011) and to promote the  
12 production of type I interferon and IFN- $\gamma$  (Abt et al., 2012; Thiemann et al., 2017). Some  
13 studies showed that microbiota generate conventional (Naik et al., 2012) and unconventional  
14 (Constantinides et al., 2019; Linehan et al., 2018) skin T cells, increasing their numbers, and  
15 these cells support mice skin repair. Recently, certain microbiota components presented by  
16 DCs were also shown to generate conventional CD44<sup>hi</sup>CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells in the gut and these  
17 cells are found also in several peripheral organs (e.g. LNs, SP and lung) (Huggins et al., 2019;  
18 Mani et al., 2019; Riquelme et al., 2019; Tanoue et al., 2019). These cells potentiate immune  
19 responses to tumours preventing the side effects of anti-PD1 administration (Tanoue et al.,  
20 2019). These later results highlight a potential therapeutic role of microbiota, and thus the  
21 importance of further studies on the impact of microbiota in immune responses.

22 We here describe a further major effect of microbiota: helping in the generation of a  
23 systemic subpopulation of CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells that have a major role in the response to  
24 LCMV infection. Our results suggest that the heterogeneity and nature of the stimuli  
25 responsible for HP and the development of CD44<sup>hi</sup> CD8<sup>+</sup> T cells was underestimated in the

1 previously described cell sets. Since T cells transferred into SCID SPF mice proliferate much  
2 more than those transferred to SCID GF hosts (Kieper et al., 2005) microbiota antigens was  
3 proposed to have a major role in HP. More recently, it was claimed that microbiota did not  
4 have any impact in this phenomenon, HP being strictly dependent on cross-reactivity with  
5 self-antigens (Haluszczak et al., 2009; Kawabe et al., 2017; Quinn et al., 2016). Our data  
6 suggest that the differences in the TCR cross-reactivity of the cells studied in each experiment  
7 explain these opposite conclusions. We found that the OVA peptide mimics self-antigens  
8 present in multiple tissues, OVA-specific CD8<sup>+</sup> T cells have the classical virtual memory T  
9 (T<sub>VM</sub>) phenotype (CD44<sup>hi</sup>CD49d<sup>low</sup>CD62L<sup>+</sup>) (Haluszczak et al., 2009; White et al., 2016) and  
10 are not reduced in the SP of GF mice. The OVA-specific HP CD44<sup>hi</sup>CD8<sup>+</sup> would be generated  
11 in conditions of T cell depletion (as found in neonatal and T cell deficient hosts) by the  
12 stimulation with cross-reactive self-antigens (Ge et al., 2004; Smith et al., 2018). By contrast,  
13 the GP33 peptide has no molecular mimicry with self-antigens but with multiple microbiota  
14 components. CD44<sup>hi</sup>GP33<sup>+</sup> cells have a TCR with a CDR3 region enriched in glycines which  
15 lead to greater promiscuity in antigen recognition (Chowell et al., 2015; Stadinski et al., 2016)  
16 indicating that these cells have a higher advantage to become activated by cross-reactive  
17 microbiota antigens and engaged in HP. Our results suggest that microbiota with GP33  
18 molecular mimicry generates these CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells. CD44<sup>hi</sup>GP33<sup>+</sup> were absent in  
19 the IELs of GF mice (where self and food antigens are present), and were hardly detected in  
20 other lymphoid organs, further suggest a role of microbiota antigens in their generation. In  
21 addition, the continuous microbiota stimulation induce a selective increase in peripheral  
22 CD44<sup>hi</sup>GP33<sup>+</sup> cells numbers throughout life (Quinn et al., 2018; Su et al., 2013). Besides  
23 GP33<sup>+</sup> cells, microbiota stimulation likely affects CD8<sup>+</sup> T cells with other antigen  
24 specificities, and also influences their properties. The total number of CD44<sup>hi</sup>CD8<sup>+</sup> T cells  
25 was reduced by 50% in GF mice and the phenotype of residual CD44<sup>hi</sup>CD8<sup>+</sup> T cells was

1 modified. The existence of these two sub-types of cross reactive CD44<sup>hi</sup>CD8<sup>+</sup> antigen-specific  
2 populations is supported by the fact that CD44<sup>hi</sup>CD8<sup>+</sup> peripheral T cell pool yet persist in GF  
3 conditions and immune responses to OVA are not affected by the absence of microbiota  
4 (Haluszczak et al., 2009), while responses to several pathogens (including *Listeria*  
5 *monocytogenes*, Influenza A and LCMV) are much reduced (Abt et al., 2012; Ichinohe et al.,  
6 2011; Khosravi et al., 2014; Thackray et al., 2018). In line with a further major effect of  
7 microbiota in generation of a systemic subpopulation of CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T cells, GF mice  
8 that lack the population of CD44<sup>hi</sup>GP33<sup>+</sup> T cells, exhibited a significant reduction in the  
9 number of effector GP33<sup>+</sup> T cells in multiple tissues 7 days post-LCMV infection and virus  
10 clearance is much delayed (Abt et al., 2012; MC, 2009).

11 CD44<sup>hi</sup>GP33<sup>+</sup> phenotype in SPF mice differs from T<sub>VM</sub> and T<sub>RM</sub> (Haluszczak et al.,  
12 2009; White et al., 2016). They share multiple characteristics of innate lymphocytes,  
13 including a very high expression of several NKR and CXCR3. The expression of CXCR3  
14 was shown to identify a cell subset able to mediate effector functions in the absence of TCR  
15 stimulation (Soudja et al., 2012). Indeed, CD44<sup>hi</sup>GP33<sup>+</sup> cells proliferate and express IFN- $\gamma$   
16 and granzyme B after overnight incubation with the cytokine cocktail, previously used to  
17 stimulate innate cells (Haluszczak et al., 2009). NKR receptors also mediate one prominent  
18 pathway for the crosstalk between CD8<sup>+</sup> T cells and surrounding tissues, activating effector  
19 functions independently of TCR stimulation (Seyda et al., 2016). Through their ubiquitous  
20 high expression of NKG2D activator receptor CD44<sup>hi</sup>GP33<sup>+</sup> cells can recognize and be  
21 activated by NKG2D ligands. These ligands are poorly expressed in healthy tissues, but are  
22 up-regulated on the surface of stressed, malignant or virus-infected cells (Raulet, 2003).  
23 Through this receptor, CD44<sup>hi</sup>GP33<sup>+</sup> cells may share the capacity of innate lymphoid cells  
24 (Seyda et al., 2016; Soudja et al., 2012) to interact with these cells in the absence of TCR  
25 recognition. Besides, their expression of activating NKRs confers them antigen-independent



1 bystander properties against target cells expressing stress markers, as well as a lower their  
2 TCR activation threshold (Seyda et al., 2016) allowing them to respond to lower affinity  
3 cross-reactive antigens. These CD44<sup>hi</sup>GP33<sup>+</sup> cells may act as early sensors to stress signals.  
4 Lastly, CD44<sup>hi</sup>GP33<sup>+</sup> cells expressed receptors allowing their direct stimulation microbiota.  
5 Their glycolipid receptor ASGM1 confers the capacity to respond to flagellin (McNamara et  
6 al., 2001), a major component of gram-negative bacteria. A fraction of CD44<sup>hi</sup>GP33<sup>+</sup> and of  
7 total CD44<sup>hi</sup>CD8<sup>+</sup> also expresses TLR4, suggesting that may be directly stimulated by  
8 bacterial LPS. It was shown that TLR4 can be expressed on the surface of activated mouse  
9 CD8<sup>+</sup> T cells (Huggins et al., 2019; Loser et al., 2010) and TLR4 activation enhances CD8<sup>+</sup> T  
10 cell proliferation, cytokine production and cytotoxicity (Wang et al., 2019). For example,  
11 microbiota exposition increase numbers of CD44<sup>hi</sup>CD8<sup>+</sup> T cells and CD44<sup>hi</sup>CD8<sup>+</sup> T  
12 expressing TLR4 (Huggins et al., 2019), but the CD8<sup>+</sup> sub-population expressing TLR4 was  
13 not characterized further.

14 SPF mice harbour CD44<sup>hi</sup>GP33<sup>+</sup> cells in SP, LN, LP, IELs and BM. However,  
15 CD44<sup>hi</sup>GP33<sup>+</sup> cells were particularly abundant in the BM of unmanipulated SPF mice. It must  
16 be noted that GP33<sup>+</sup>CD44<sup>hi</sup> populations located in the BM cannot be considered T<sub>RM</sub> since  
17 parabiosis experiments demonstrated that all CD8<sup>+</sup> T cells (including CD44<sup>hi</sup>CD69<sup>+</sup>CD8<sup>+</sup> T  
18 cells) present in the BM recirculate in-between different organs: they reach full chimerism in  
19 the two mice connected in each parabiotic pair (Klonowski et al., 2004; Stark et al., 2018;  
20 Thomas et al., 2011). We observed that CD44<sup>hi</sup>GP33<sup>+</sup> cells are selected during primary  
21 LCMV infection in SPF mice, as shown by their precocious accumulation, increased division  
22 and survival in the BM, where CD44<sup>hi</sup>GP33<sup>+</sup> cells predominate. These results show that the  
23 BM must also be considered as a secondary lymphoid organ with a major role even in the  
24 primary immune responses (Duffy et al., 2012; Feuerer et al., 2003). CD44<sup>hi</sup>GP33<sup>+</sup> cells are  
25 poised for greater bursts of proliferation when they encounter the antigen (Smith et al., 2018),

1 and they dominate the immune response to LCMV infection (Fulton et al., 2015). We here  
2 describe that the TCR repertoire of GP33<sup>+</sup> cells during LCMV response shifts from that of  
3 naïve CD44<sup>low</sup>GP33<sup>+</sup> (where Vβ8<sup>+</sup> cells predominate) to the repertoire of CD44<sup>hi</sup>GP33<sup>+</sup> cells  
4 particularly those expressing TRBV29/Vβ7<sup>+</sup>. We envisaged that microbiota stimulation could  
5 be responsible for the preferential selection of TRBV29/Vβ7<sup>+</sup> CASSxxNxxEQYF GP33<sup>+</sup>  
6 during the LCMV response. To evaluate this possibility we studied the repertoire of TCRαβ<sup>+</sup>  
7 GP33<sup>+</sup> IELs from SPF mice, which are in contact with microbiota, but are not stimulated by  
8 LCMV. We found selection mechanisms overlapping those found in the peripheral pools  
9 during the LCMV response. While the majority of IELs did not express Vβ7, the vast  
10 majority of GP33<sup>+</sup> IELs expressed Vβ7. Importantly, the CDR3 domains of GP33<sup>+</sup>Vβ7<sup>+</sup> IELs  
11 of SPF mice had the CASSxxNxxEQYF motif found in the GP33<sup>+</sup>Vβ7<sup>+</sup> selected in the  
12 peripheral T cell pools after LCMV infection. Taking into consideration the potential  
13 heterogeneity of TCRβ repertoires (Goncalves et al., 2017), the expression of equivalent  
14 TRBV chains in GP33<sup>+</sup> IELs of SPF mice and in GP33<sup>+</sup> peripheral T cells selected during  
15 LCMV infection can only be explained by a common selection mechanism. Interestingly,  
16 microbiota-reactive T cells were also prevalent in the human gut mucosa (Hegazy et al., 2017;  
17 Noble et al., 2019), and some circulating memory T cells recognize the same peptides derived  
18 from gut bacteria and can cross-react to other pathogens (Borbulevych et al., 2009; Hegazy et  
19 al., 2017; Noble et al., 2019; Su et al., 2013). These results suggest that microbiota can induce  
20 the expansion of antigen-specific memory phenotypic T cells in gut and periphery of both  
21 mice and human (Su et al., 2013).

22         Lastly, the influence of microbiota in the generation CD44<sup>hi</sup>GP33<sup>+</sup> cells must also be  
23 addressed. Microbiota antigens are continuously recognized by the adaptive immune system  
24 during homeostasis and some studies showed that mice antigen-specific T cells can have  
25 cross-reactivity to microbial mimotopes and be activated by these antigens (Beura et al.,

1 2016; Gil-Cruz et al., 2019; Harkiolaki et al., 2009; Tanoue et al., 2019; Yang et al., 2014).  
2 However, further studies will be required to investigate if microbiota mimotopes directly  
3 prime naïve CD44<sup>low</sup>GP33<sup>+</sup> cells induce their activation and promote the generation of  
4 CD44<sup>hi</sup>GP33<sup>+</sup> T cells in SPF mice. For example, microbiota can also regulate certain antigen-  
5 presenting cells and affect cross-presentation (Khosravi et al., 2014). In addition to  
6 mimotopes, microbiota-derived ligands (e.g. LPS) can also enter the systemic blood  
7 circulation induce innate stimulation (Feng et al., 2010) and promote bystander activation and  
8 proliferation/survival of CD44<sup>hi</sup> CD8<sup>+</sup> T cells (Tough et al., 1997). Besides structural  
9 components of bacteria, many of metabolites derived from microbiota metabolism (e.g. short-  
10 chain fatty acids, vitamins, bile acids, sterols, and xenobiotics) have been implicated in the  
11 development and function of diverse immune cells including antigen-presenting cells and  
12 CD8<sup>+</sup> T cells (Tanoue et al., 2019). Both microbiota-induced antigen-specific CD8<sup>+</sup> T cells  
13 activation and microbiota innate stimulation-driven CD44<sup>hi</sup> CD8<sup>+</sup> T cells proliferation and  
14 survival may be required for the generation/maintenance of innate CD44<sup>hi</sup>GP33<sup>+</sup> cells. In  
15 addition, cytokines stimulation were also involved in this process (Haluszczak et al., 2009;  
16 White et al., 2016).

17 To summarize, these results suggest a novel and direct function of microbiota in  
18 antigen-specific cells, which has a major impact in response to virus infection. This data  
19 suggest that microbiota components are responsible for the generation of a sub-population of  
20 CD44<sup>hi</sup>CD8<sup>+</sup> T cells in SPF mice, which can function as “innate guardians” to multiple types  
21 of aggressions. Besides, microbiota also plays a major role in the response of CD8<sup>+</sup> T cells  
22 and in the selection of their TCR $\beta$  repertoire during the response to LCMV infection.  
23 Microbiota can primes the peripheral T cell pools of naïve mice providing a pool of cross-  
24 reactive pre-activated memory cells that improve the efficiency of primary immune responses  
25 to virus infection.

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2

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15

16 P.G. conducted most of the experiments, analyzed the data and prepared the manuscript. S.D.,  
17 F.V., N.S., A.L. D.G. and O.A. performed experiments. H.D. prepared and titrated the LCMV  
18 virus. D.G, J.S., A.F and B.R. designed experiments and prepared the manuscript.

19

20 **Appendix A.** Supplementary data: Supplementary data associated with this article can be  
21 found, in the online version, at...

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1 **Table legend**

2 **Table 1 - The diversity of GP33-specific CD8<sup>+</sup> T cells in primary effector phase, memory**  
3 **and secondary effector phase after LCMV infection.**

4 CD8<sup>+</sup>GP33<sup>+</sup> cells from different LCMV infected mice were sorted from the SP, LN and BM  
5 to evaluate *Tcrb* expression in each individual cell from at different time points after LCMV  
6 infection. Results show a representative mouse at each time point. Remaining mice and full  
7 TCRB sequences of all individual cells in all mice are shown in Table S2. From left to right:  
8 the CDR3 region; CDR3 size; *Trbv* gene; *Trbj* genes; the number of cells expressing the same  
9 TCRB, localized in a single organ (SP or LN or BM) (**localized**) or present in different organs  
10 (**spread**).

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1 **Figure legends**

2 **Figure 1. CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> cells from SPF mice express a different phenotype from**  
3 **virtual memory cells (CD69<sup>-</sup>CD62L<sup>+</sup>CD49d<sup>low</sup>) or tissue resident memory cells (TRMs)**  
4 **(CD69<sup>+</sup>CD62L<sup>-</sup>CCR7<sup>-</sup>). (A-C, E) Results compare the expression of different cell surface**  
5 **markers in gated CD44<sup>-</sup>GP33<sup>+</sup> (black histograms) and CD44<sup>hi</sup>GP33<sup>+</sup> T cells (red**  
6 **histograms) from the spleen (SP) of the same mouse. We focus on phenotypic analysis in the**  
7 **SP since the numbers of CD44<sup>-</sup>GP33<sup>+</sup> recovered from the BM sometimes were insufficient to**  
8 **allow an accurate comparison between CD44<sup>-</sup> and CD44<sup>hi</sup>GP33<sup>+</sup> cells in the same organ. The**  
9 **numbers in each histogram show the average percentage of positive cells from 4-8 12 weeks**  
10 **old SPF B6 mice, studied in three independent experiments: upper numbers: CD44<sup>-</sup>GP33<sup>+</sup>;**  
11 **lower numbers: CD44<sup>hi</sup>GP33<sup>+</sup>. B and C,  $p \leq 0.05$  using the Mann Whitney U-test; D: CD69**  
12 **and CD103 co-expression by total bone marrow (BM) CD44<sup>hi</sup>CD8<sup>+</sup>T cells.**

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14 **Figure 2. CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells from SPF mice express markers of innate**  
15 **lymphocytes. A: MFI of different markers expressed by CD44<sup>-</sup>GP33<sup>+</sup> (black bars) and**  
16 **CD44<sup>hi</sup>GP33<sup>+</sup> T cells (grey bars). Results show the mean $\pm$ SEM from 4-8 mice studied in**  
17 **three independent experiments. The differences determined using the Mann Whitney U-test**  
18 **are: \* $p \leq 0.05$ , \*\*\*  $p \leq 0.001$ . B: Histograms compare the expression of different cell surface**  
19 **markers in gated CD44<sup>-</sup>GP33<sup>+</sup> (black lines) and CD44<sup>hi</sup>GP33<sup>+</sup> SP T cells (red lines) of the**  
20 **same mouse. The numbers in each histogram show the average percentage of positive cells**  
21 **obtained from 4-8 mice, studied in three independent experiments: CD44<sup>-</sup>GP33<sup>+</sup> (upper**  
22 **numbers) and CD44<sup>hi</sup>GP33<sup>+</sup> (lower numbers). All markers  $p \leq 0.05$  using the Mann Whitney**  
23 **U-test; C: Expression of TLR4 on total CD44<sup>hi</sup>CD8<sup>+</sup>T cells.**

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1 **Figure 3. CD44<sup>hi</sup> GP33<sup>+</sup> CD8<sup>+</sup> T cells present in SPF mice share properties of innate**  
2 **lymphocytes. A:** Results compare CXCR3, CD121a, CD218a, and CD122 expression by  
3 CD44<sup>-</sup> (black line) and CD44<sup>hi</sup>GP33<sup>+</sup> (red line) from the SP of same mouse. The numbers in  
4 each histogram show the average percentage of positive cells obtained from 4-8 mice, studied  
5 in three independent experiments: CD44<sup>-</sup>GP33<sup>+</sup> (**upper numbers**) and CD44<sup>hi</sup>GP33<sup>+</sup> (**lower**  
6 **numbers**). All markers  $p \leq 0.05$  using the Mann Whitney U-test. **B: Mean+/- SEM** of the  
7 MFI of these markers expression by CD44<sup>-</sup> (black bars) and CD44<sup>hi</sup> (grey bars) GP33<sup>+</sup> cells in  
8 5 mice studied in 3 experiments **C-E:** A pool of CD44<sup>-</sup> and CD44<sup>hi</sup> CD8<sup>+</sup> T cells from SP and  
9 BM of SPF mice were sorted and cultured overnight with cytokines. BFA was added for the  
10 last 3 h of culture. **C:** Number of GP33<sup>+</sup> cells in CD44<sup>-</sup> and CD44<sup>+</sup> populations cultured  
11 overnight without (upper graphs) or with cytokines (lower graphs); **D:** MFI of CD69,  
12 Granzyme B and IFN- $\gamma$  expression by CD44<sup>-</sup> (black bars) and CD44<sup>hi</sup> (grey bars) GP33<sup>+</sup> cells.  
13 Results are the mean+SEM of 4 independent experiments. Differences determined using the  
14 Mann Whitney U-test were: \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ . **E:** Intra-cytoplasmic expression of  
15 INF- $\gamma$  and Granzyme B by CD44<sup>-</sup> (left) and CD44<sup>hi</sup> CD8<sup>+</sup> T cells (right) in one representative  
16 experiment.

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18 **Figure 4. The kinetics of GP33<sup>+</sup> CD8<sup>+</sup> T cells response to LCMV infection. C57BL/6 (B6)**  
19 SPF mice were infected i.p. with  $2 \times 10^5$  PFU of LCMV Armstrong and studied at different  
20 time points after infection. **A:** Experimental protocol. **B:** Percentage of CD44<sup>hi</sup>GP33<sup>+</sup> cells in  
21 total GP33<sup>+</sup> cells in spleen (SP) or bone marrow (BM) of SPF mice. **C:** Numbers of GP33<sup>+</sup>  
22 CD8<sup>+</sup> T cells recovered at each time-point after infection in spleen (SP) (black) and bone  
23 marrow (BM) (red). Differences determined using the Mann-Whitney U test were: \* $p \leq 0.05$ ;  
24 \*\*\* $p \leq 0.005$ . Significant different from SP at each time-point. **D, E:** BrdU incorporation by  
25 GP33<sup>+</sup> cells during LCMV infection. Mice were injected i.p. with 1 mg/mouse of BrdU and

1 studied 1h after injection. Single-cell suspensions were obtained from inguinal (ING),  
2 brachial (BRA) and mesenteric lymph nodes (MES), SP and BM. Barriers defining BrdU<sup>+</sup>  
3 cells were determined in the same organs of control mice, which had not been injected with  
4 BrdU. **D:** Representative histograms of BrdU labelling by CD44<sup>hi</sup>GP33<sup>+</sup> from different  
5 lymphoid organs of the same mouse at day 3 post-infection. **E:** The kinetics of BrdU  
6 incorporation in different organs, during LCMV infection. Each dot represents the percentage  
7 of GP33<sup>+</sup>BrdU<sup>+</sup> cells found in the different organs from one individual mouse: **white dots:**  
8 CD44<sup>lo</sup>GP33<sup>+</sup> naïve cells in non-infected mice; **grey dots:** CD44<sup>hi</sup>GP33<sup>+</sup> cells at day 3 after  
9 infection; **black dots** CD44<sup>hi</sup>GP33<sup>+</sup> at day 5 after infection. Bars show the mean+/-SEM from  
10 3-4 mice studied in two independent experiments. The difference between two groups was  
11 evaluated by the Mann-Whitney U test. Significant different from SP at each time-point.  
12 Single continuous variable data were analyzed by Kruskal-Wallis (KW) followed by Dunn's  
13 Multiple Comparison Test. The differences in BrdU incorporation: BM versus any other  
14 organ, at day 3:  $p \leq 0.05$ ; at day 5: BM vs SP  $p \leq 0.001$ , BM vs MES  $p \leq 0.01$ ; BM vs other  
15 organs  $p \leq 0.05$ .

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17 **Figure 5. The TRBV repertoire of CD44<sup>hi</sup>GP33<sup>+</sup> cells is preferentially selected during**  
18 **LCMV infection.** Results show the % of GP33<sup>+</sup> cells expressing TRBV13 (V $\beta$ 8) and  
19 TRBV29 (V $\beta$ 7) determined by the sequence of the TCRB chain of individual cells (A, C) or  
20 by staining with TCRB specific antibodies (B). **(A, B):** SPF mice: left: CD44<sup>lo</sup>GP33<sup>+</sup> cells (5  
21 mice; 1.104 *tcrb* sequences), CD44<sup>hi</sup>GP33<sup>+</sup> cells (3 mice; 250 *tcrb* sequences). **(B):** % of  
22 GP33<sup>+</sup> cells expressing V $\beta$ 8 or V $\beta$ 7 (mean+/-SEM of 3-5 mice studied in three independent  
23 experiments). **(C):** Black bars: CD44<sup>lo</sup>GP33<sup>+</sup> cells from SPF mice; Dashed bars: CD44<sup>hi</sup>GP33<sup>+</sup>  
24 primary effector cells, at 7 days after infection (5 mice; 1097 *tcrb* sequences); white bars:  
25 memory cells 120 days after infection (4 mice; 795 *tcrb* sequences); and secondary effector

1 cells 7 days after infection (dashed bars) (white bars, 3 mice; 312 *tcrb* sequences). Differences  
2 by Kruskal-Wallis (KW) followed by Dunn's Multiple Comparison Test were: \* $p \leq 0.05$ , \*\*  
3  $p \leq 0.01$ \*\*\* $p \leq 0.001$ .

4

5 **Figure 6. Microbiota stimulation imprinted CD44<sup>hi</sup>GP33<sup>+</sup>CD8<sup>+</sup> T cells.** Results compared  
6 12 weeks old SPF and GF mice, studied simultaneously. **A:** Numbers of CD44<sup>hi</sup>CD8<sup>+</sup> T cells  
7 recovered from the spleen (SP) and bone marrow (BM) of SPF (black bars) and GF (white  
8 bars) mice. Percentage (**B**) and total number (**C**) of CD44<sup>lo</sup> and CD44<sup>hi</sup> GP33<sup>+</sup> cells recovered  
9 from the SP and the BM of SPF and GF mice. Each dot represents an individual mouse, and  
10 bars the SEM between different experiments. Representative histogram comparing the  
11 expression of CD49d in gated total CD44<sup>hi</sup> CD8<sup>+</sup> (**D**) and CD44<sup>hi</sup> GP33<sup>+</sup> cells (**E**) from SP of  
12 SPF and GF mice. The numbers in each histogram show the average percentage of positive  
13 cells from SPF (**upper numbers**) and GF (**lower numbers**) mice. **F:** Representative  
14 histogram comparing the expression of CD69 in gated BM CD44<sup>hi</sup> CD8<sup>+</sup> from SPF and GF  
15 mice. The numbers in each histogram show the average percentage of positive cells from SPF  
16 (**upper numbers**) and GF (**lower numbers**) mice. **G:** CD69 mean fluorescence intensity  
17 (MFI) on CD44<sup>+</sup> (black bars) and CD44<sup>hi</sup> GP33<sup>+</sup> cells (grey bars) from SPF and GF mice. The  
18 numbers in each panel are the mean $\pm$ SEM of 5-6 mice from each group, studied in three  
19 independent experiments. Differences evaluated using the Mann Whitney U-test were: \* $p \leq$   
20 0.05; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

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22 **Figure 7. Microbiota with GP33 molecular mimicry generates CD44<sup>hi</sup>GP33<sup>+</sup> CD8<sup>+</sup> T**  
23 **cells.** **A:** Numbers of CD44<sup>hi</sup>CD8<sup>+</sup> T cells recovered from the spleen (SP) and bone marrow  
24 (BM) of SPF-1 (dark blue) and SPF-2 (light blue) mice. Percentage (**B**) and number (**C**) of  
25 CD44<sup>lo</sup> and CD44<sup>hi</sup> GP33<sup>+</sup> cells recovered from the SP and the BM of SPF-1 (dark blue) and

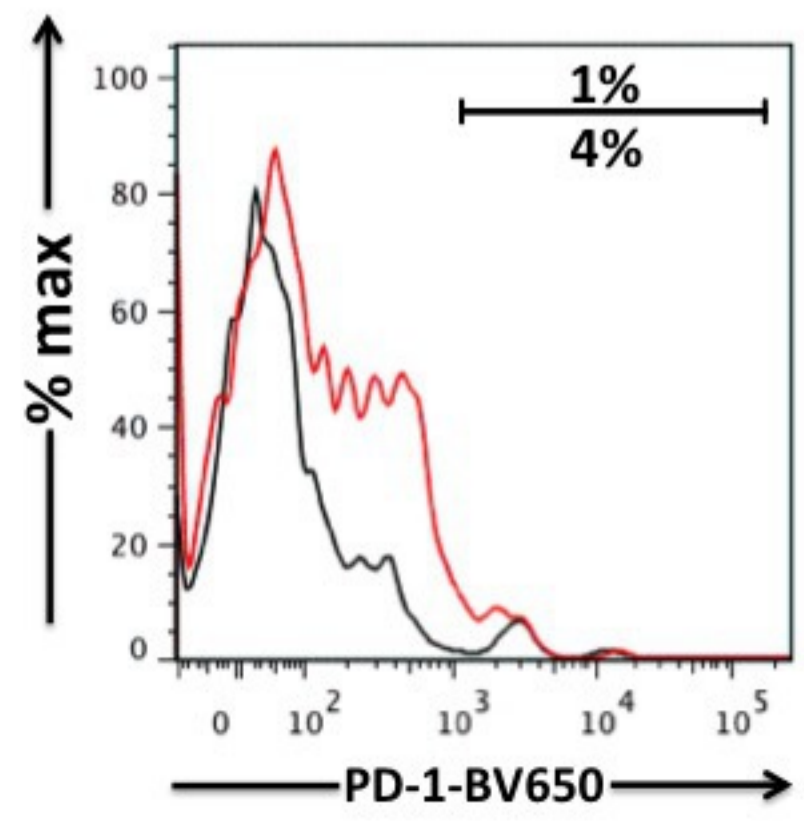
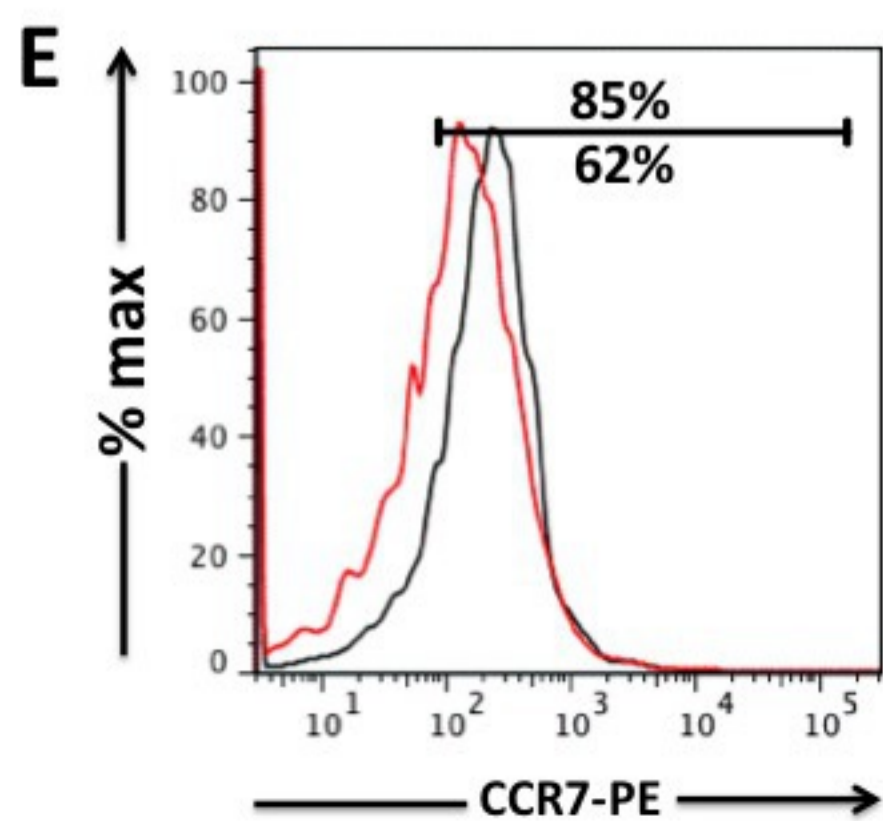
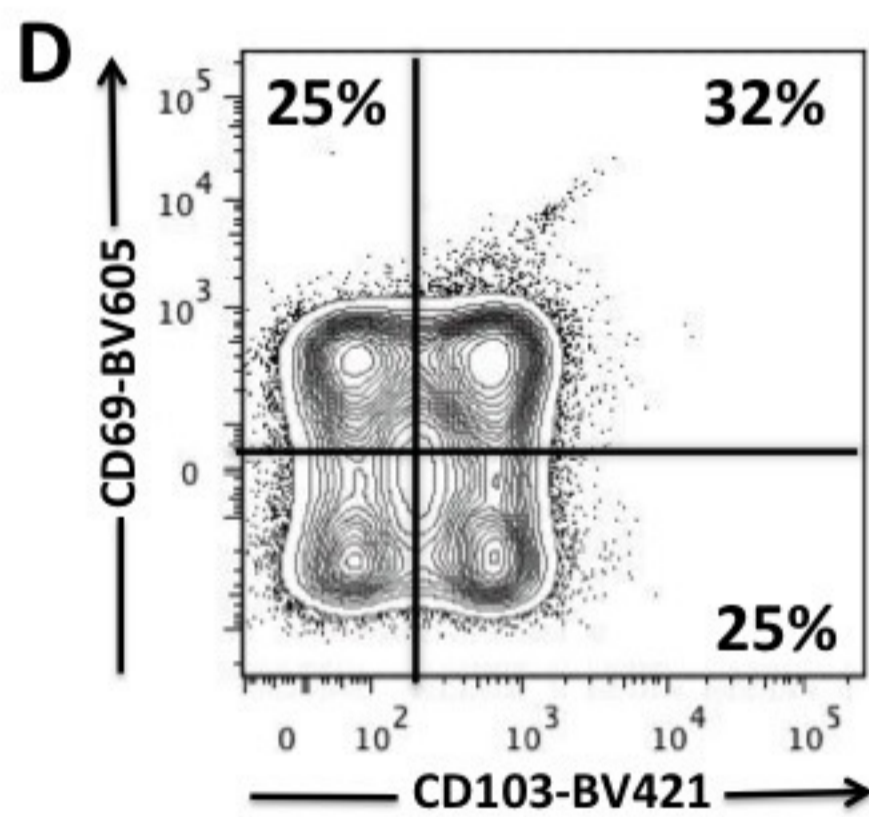
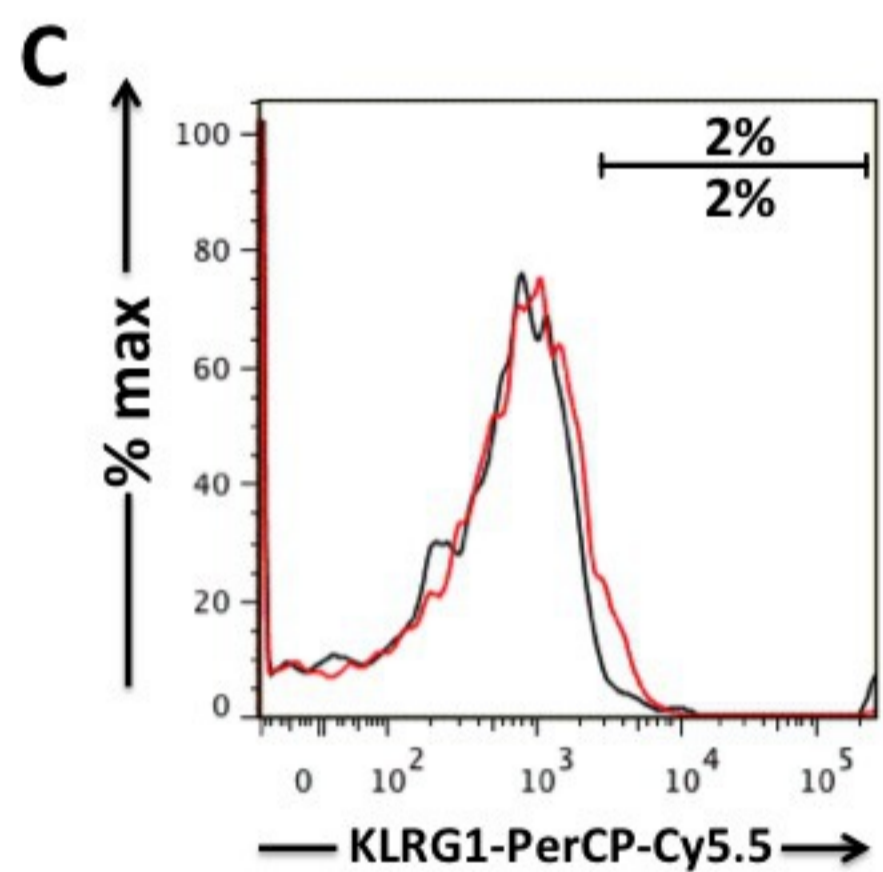
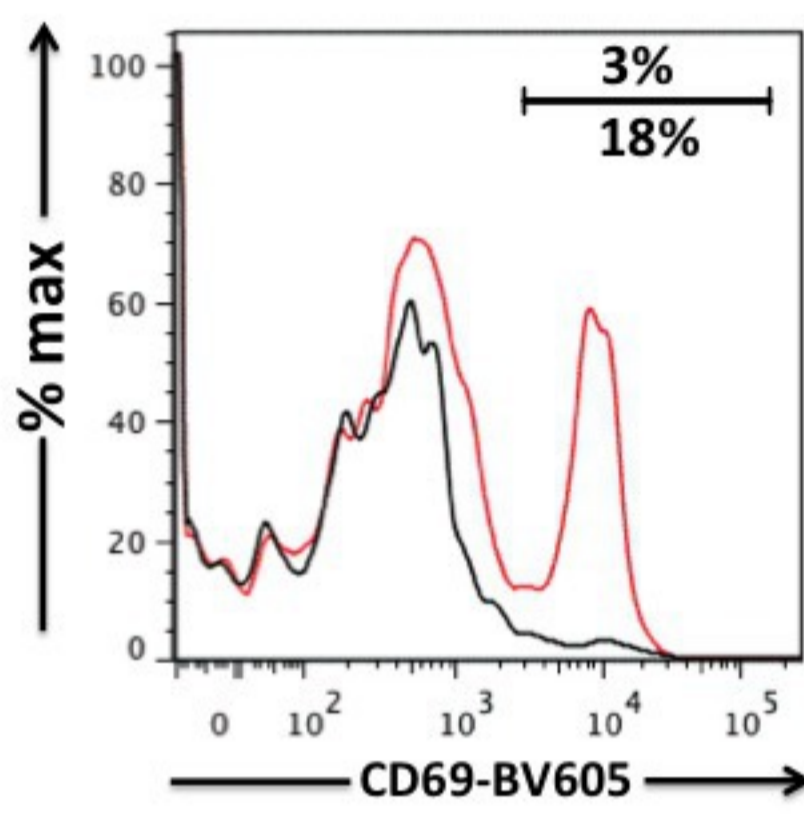
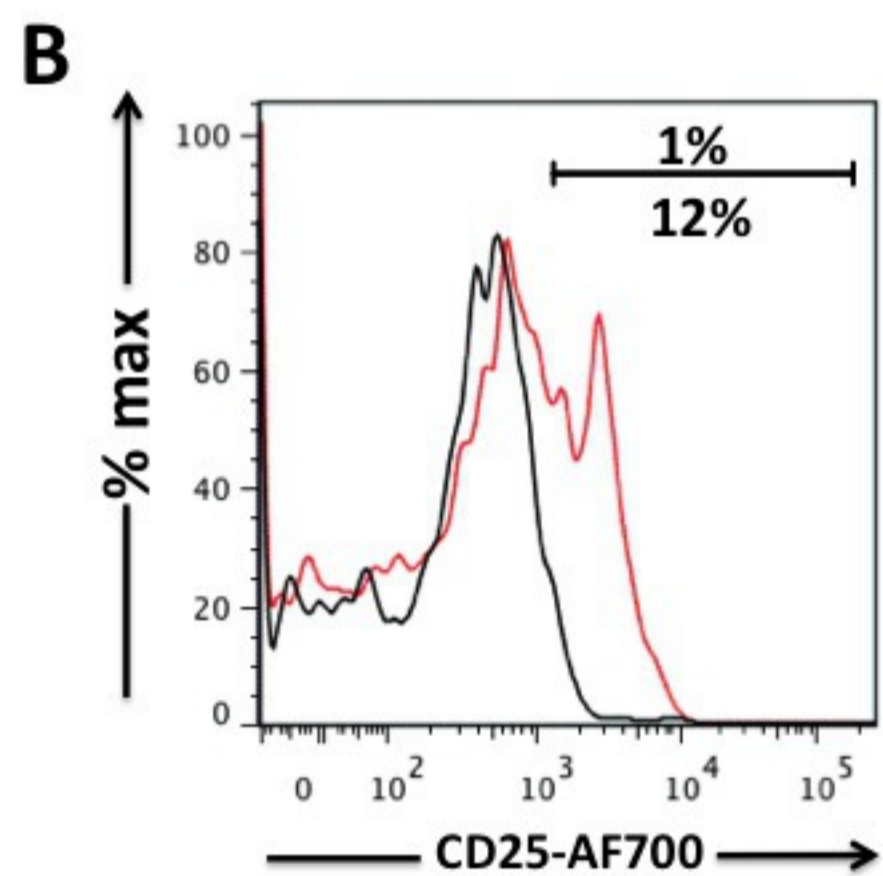
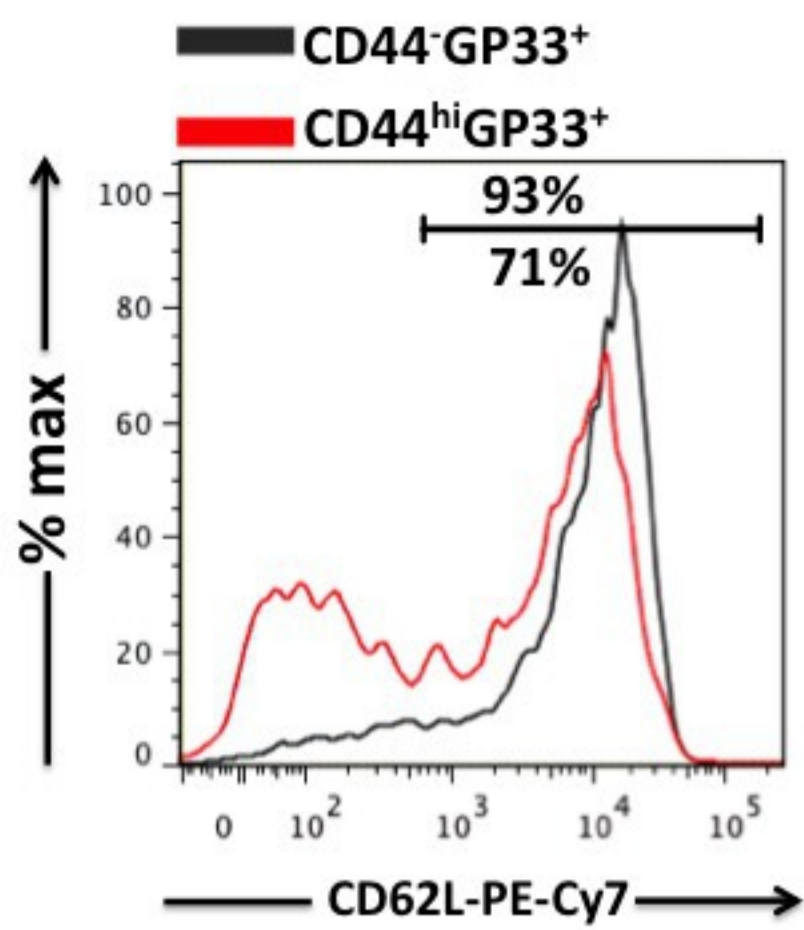
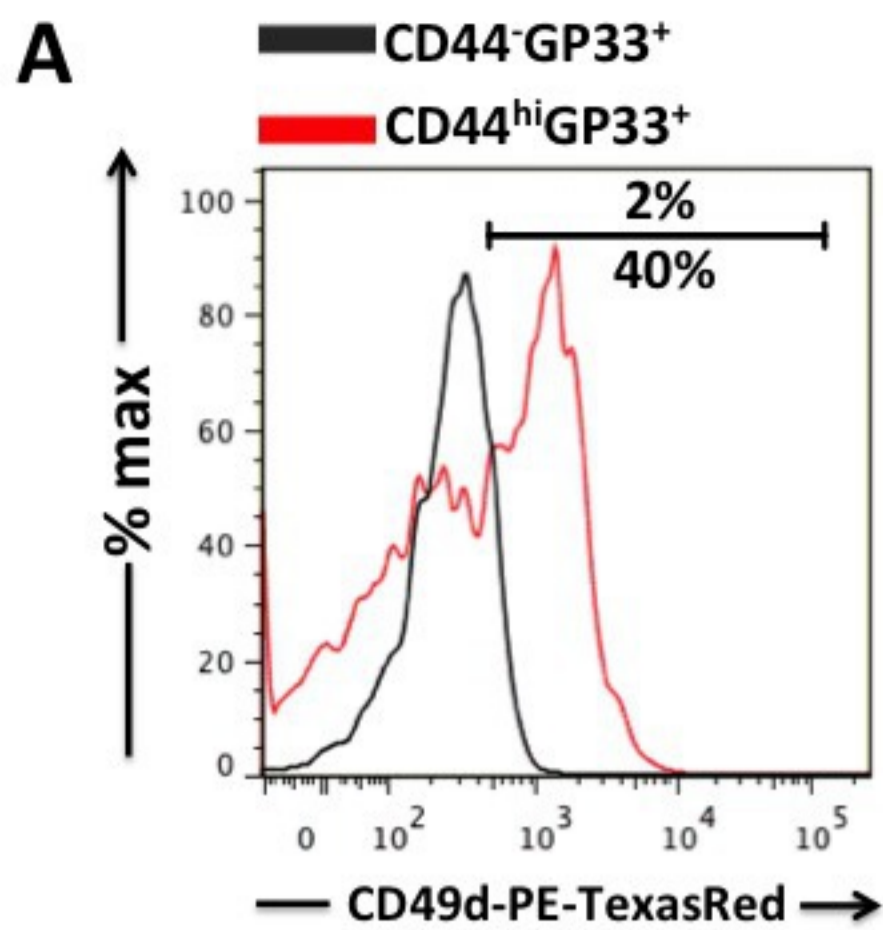
1 SPF-2 (light blue) mice. Each dot represents an individual mouse, and bars the SEM between  
2 different experiments. **D:** Principal coordinate analysis (PCoA) of 16S RNA sequencing  
3 Operational Taxonomic Units (OTU) of the SPF-1 (dark blue) and SPF-2 (light blue) mice,  
4 along the first two principal coordinate (PC) axes based on Bray-Curtis distances and the  
5 respective PERMANOVA test. **E:** Relative abundance of some genus predicted to have cross-  
6 reactivity with GP33 peptide in the feces of SPF-1 (dark blue) and SPF-2 (light blue) mice.  
7 **H:** Volcano plot displaying differential microbiota genus between SPF-2 and SPF-1 mice.  
8 The vertical axis (y-axis) corresponds to the mean expression value of log<sub>10</sub> (q-value), and  
9 the horizontal axis (x-axis) displays the log<sub>2</sub> fold change value. The red dots represent the  
10 up-regulated genus in SPF-2 mice; the blue dots represent the genus downregulated in SPF-2  
11 mice. Positive x-values represent up-regulation and negative x-values represent down-  
12 regulation. Fecal microbiota alpha diversity (**G**), calculated using the Shannon index (**H**), in  
13 SPF-1 (dark blue) and SPF-2 (light blue) mice. The numbers in each panel are the mean+/-  
14 SEM of 5 mice from each group, studied in two independent experiments. Differences  
15 evaluated using the Mann Whitney U-test were: \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

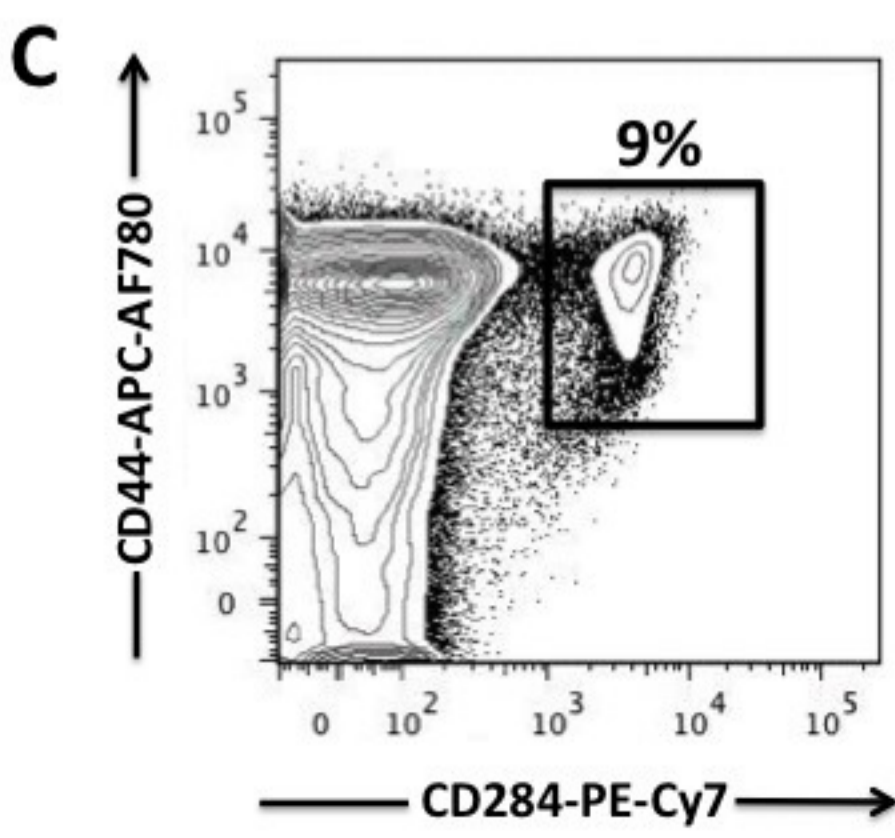
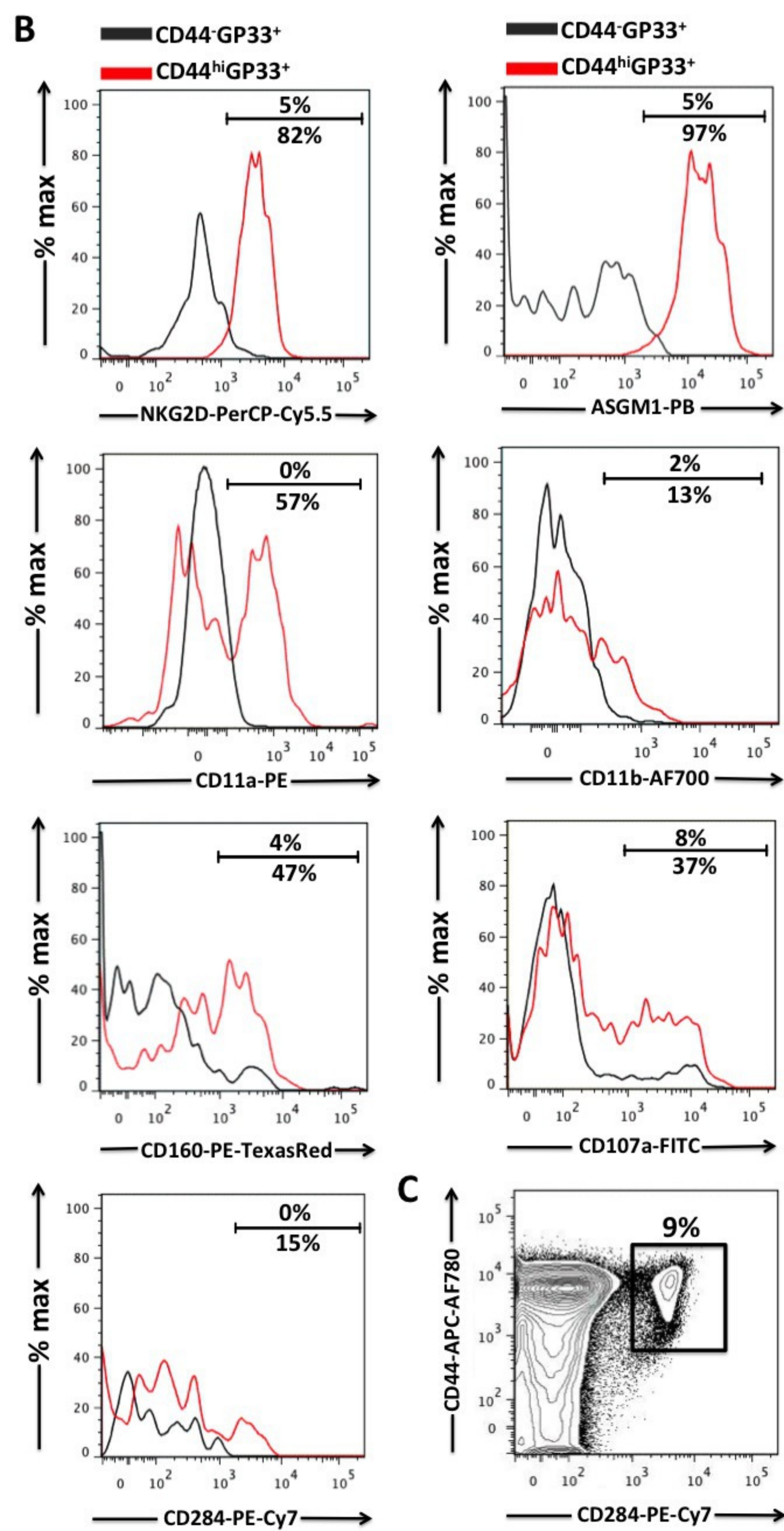
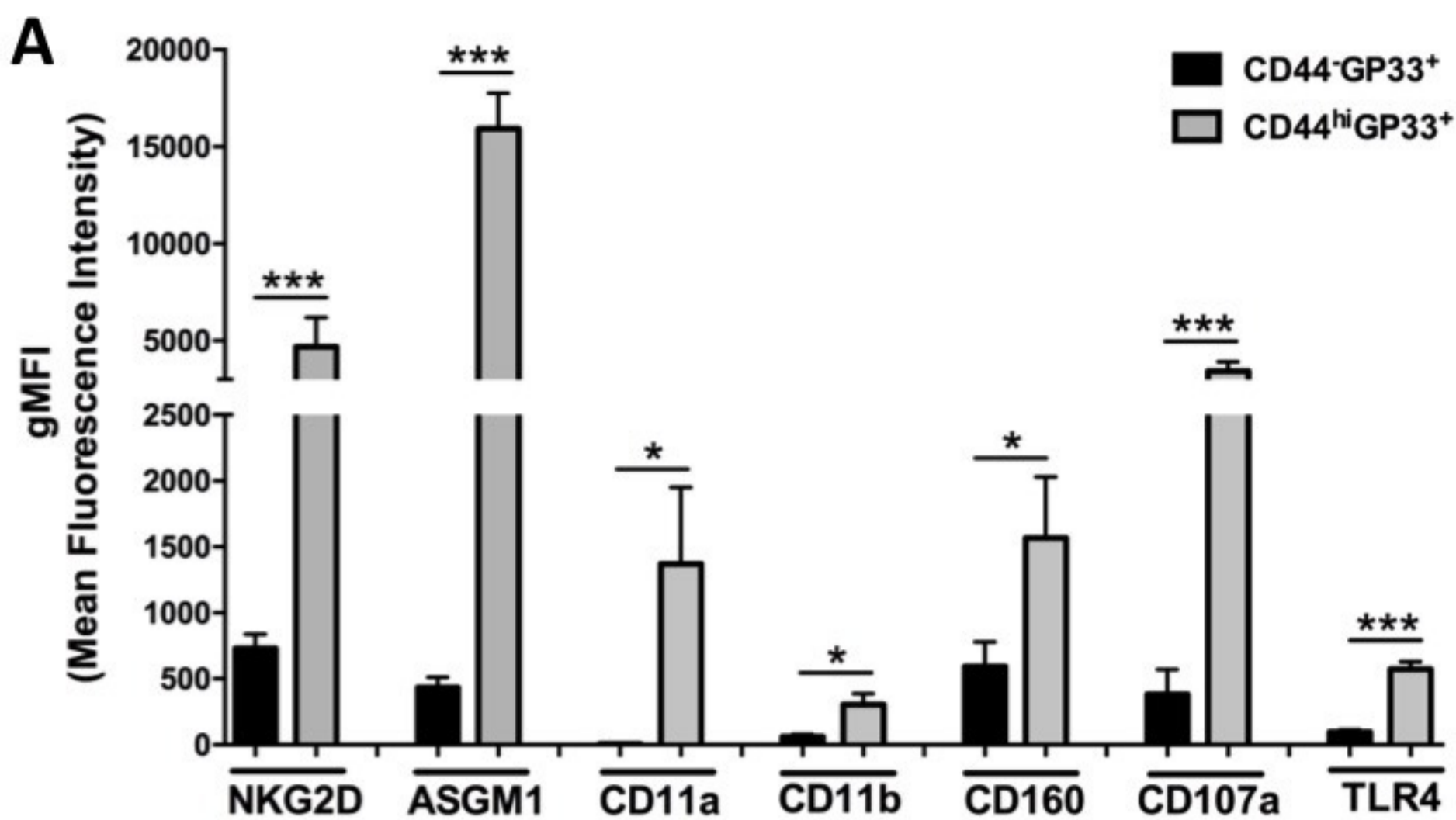
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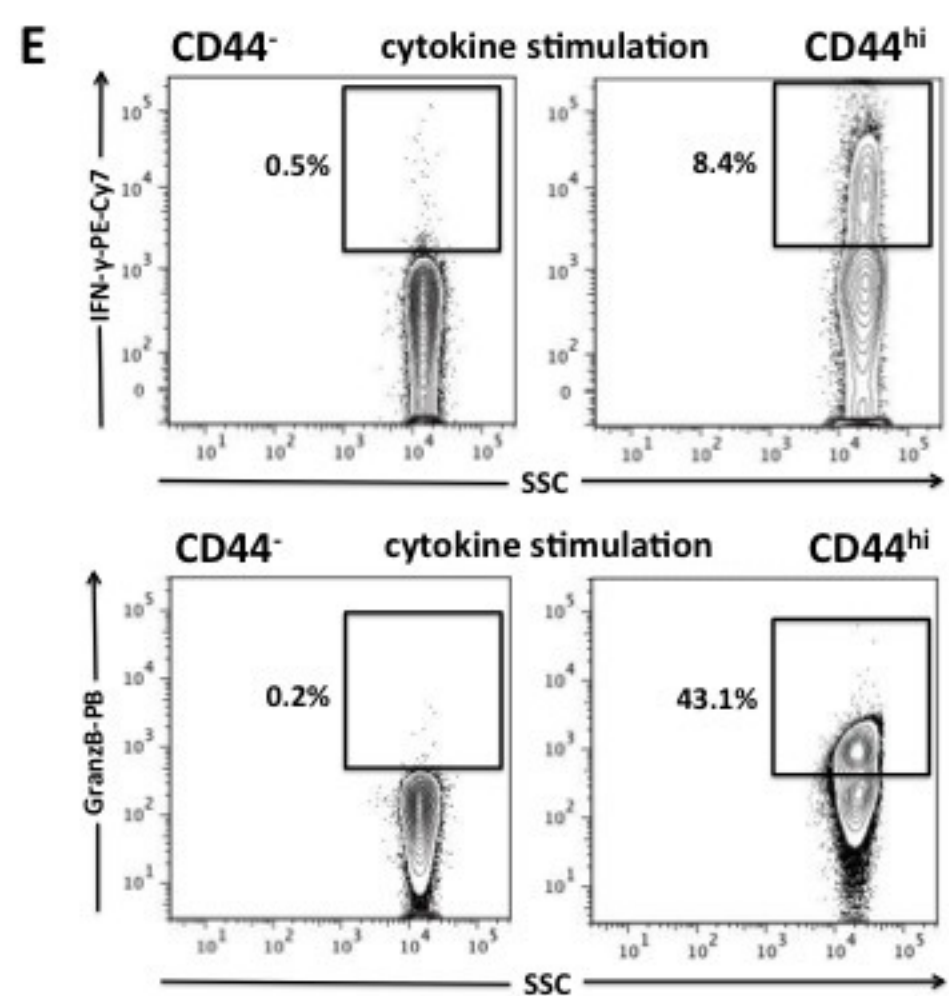
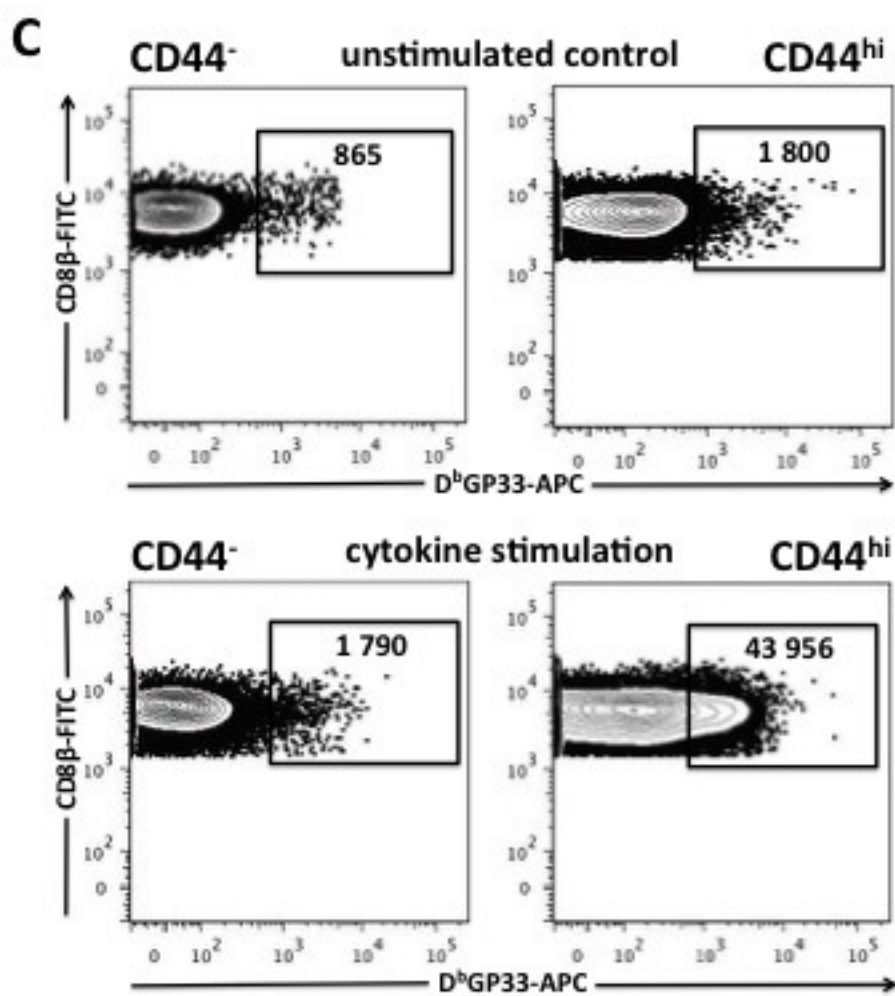
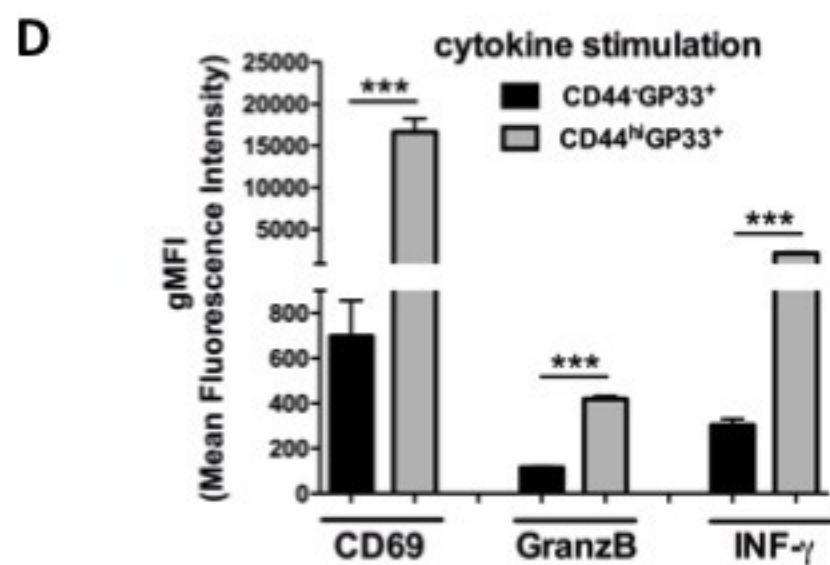
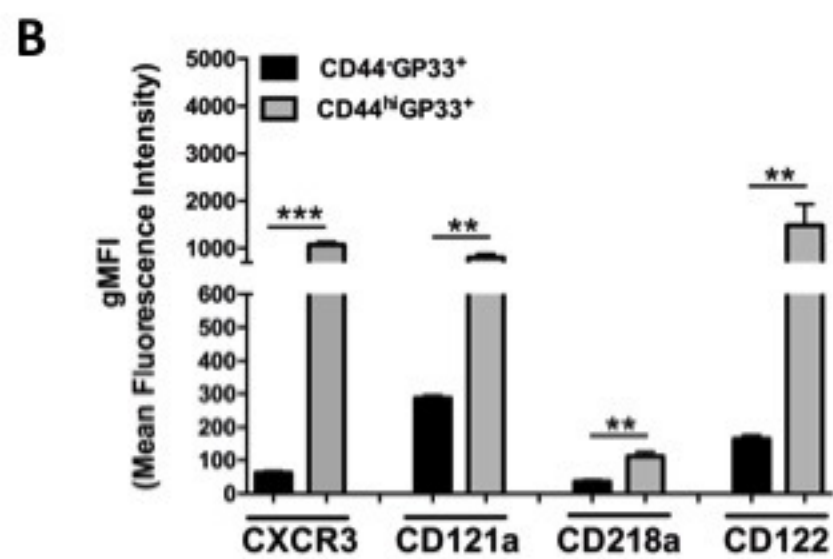
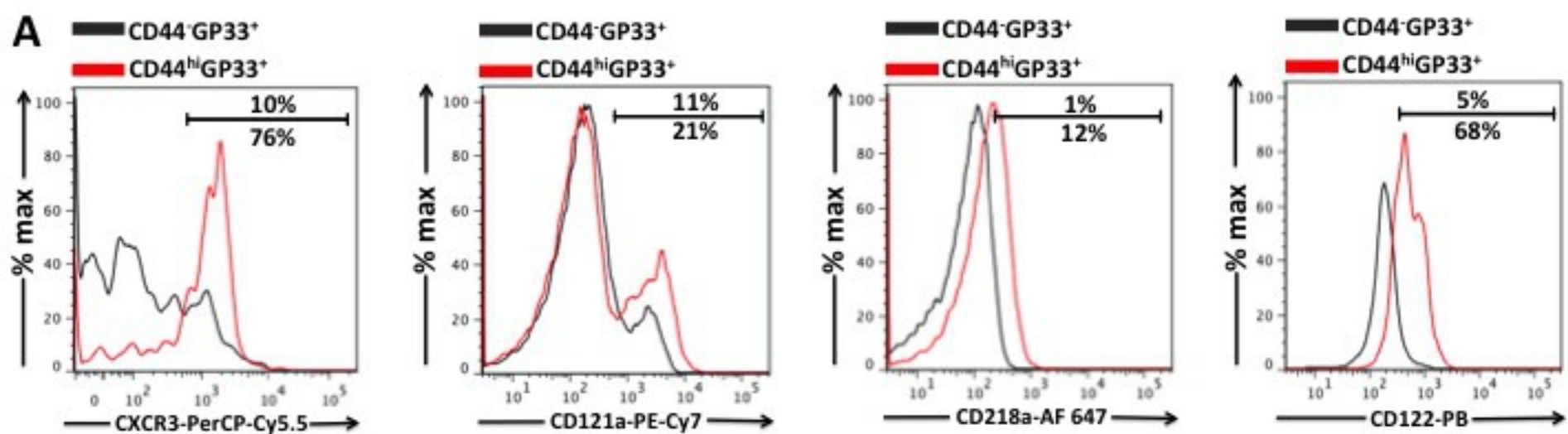
17 **Figure 8. Microbiota induce CD44<sup>hi</sup>GP33<sup>+</sup>TRBV29<sup>+</sup> IELs expansion in SPF mice. (A, B):**  
18 Results compared 12 weeks old SPF-1, SPF-2 and GF mice, studied simultaneously. **A:** IELs:  
19 representative dot plots. The upper panels show the gates used to select CD69<sup>+</sup>CD8β<sup>+</sup>IEL  
20 followed bellow by the identification of K<sup>b</sup>-OVA-specific (Ova<sup>+</sup>) and D<sup>b</sup>-GP33-specific  
21 (GP33<sup>+</sup>) IELs. The numbers in each panel are the mean+/-SEM (**upper numbers**) and %  
22 (**lower numbers**) of antigen-specific cells after acquisition of total IELs of 5-6 mice from  
23 each group, studied in three independent experiments. **B:** Numbers of CD44<sup>hi</sup>CD8<sup>+</sup> T cells  
24 recovered from the IEL, lamina propria (LP) of SPF-1 (dark blue) SPF-2 (light blue) and GF  
25 mice (white). **(C):** Vβ7 expression by GP33<sup>-</sup> and GP33<sup>+</sup> IELs from 12 SPF-1 mice, studied in

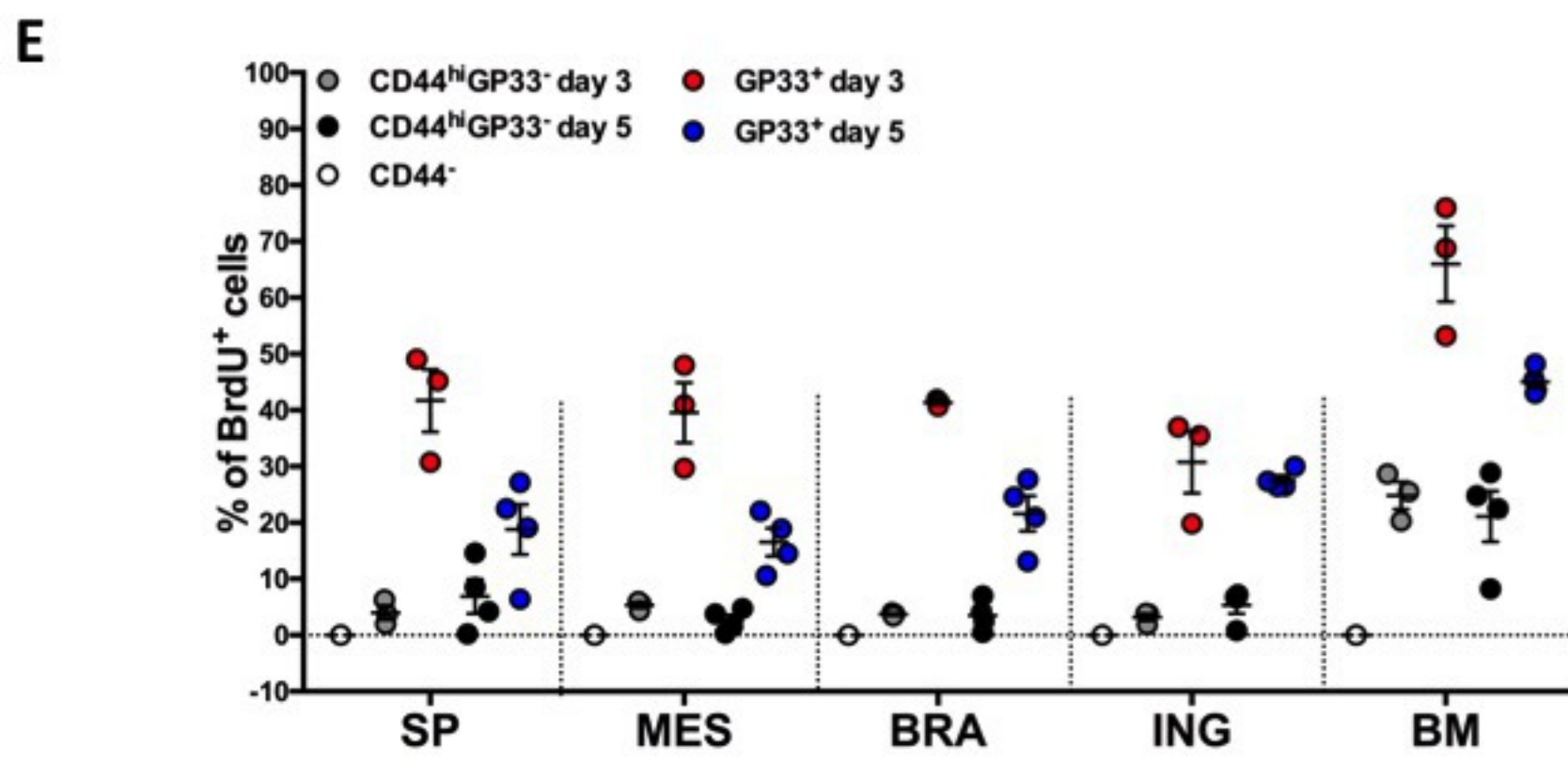
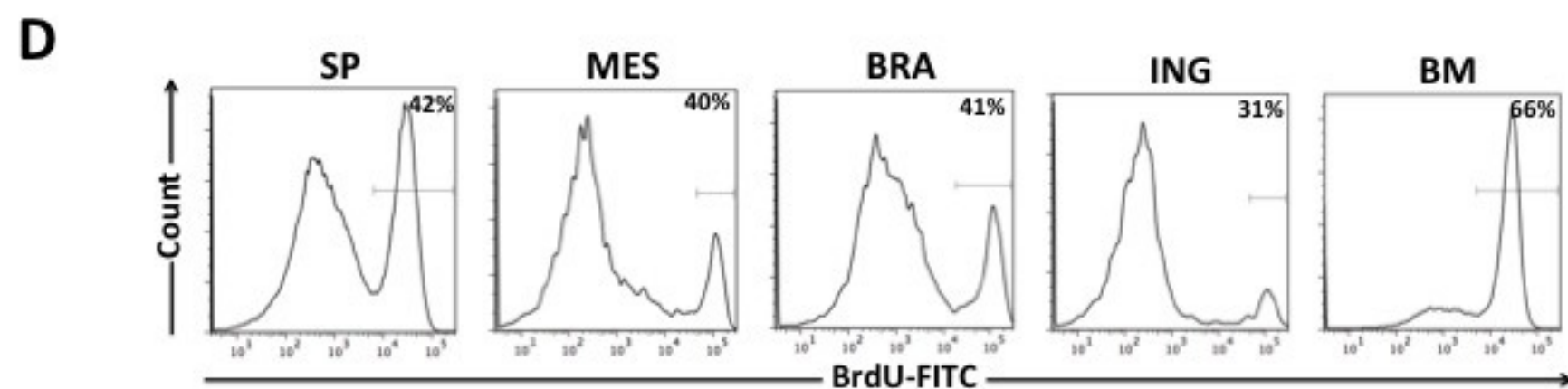
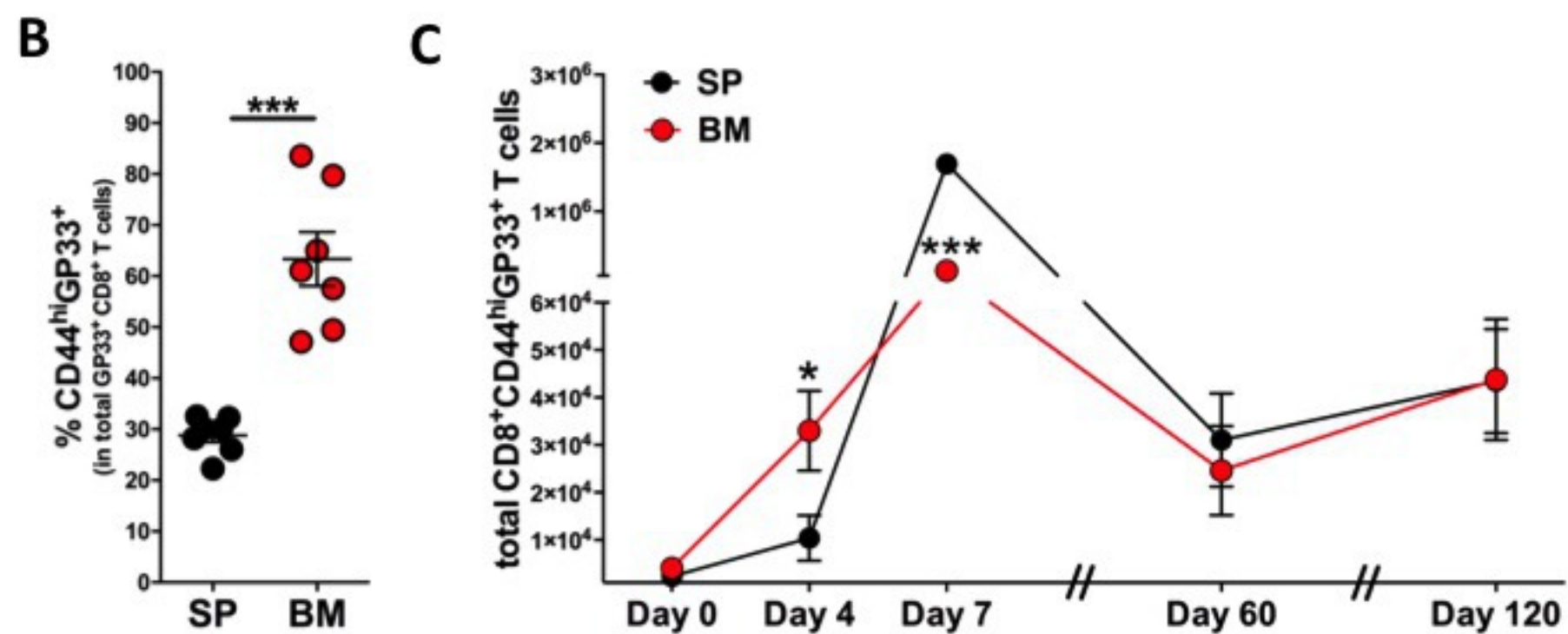
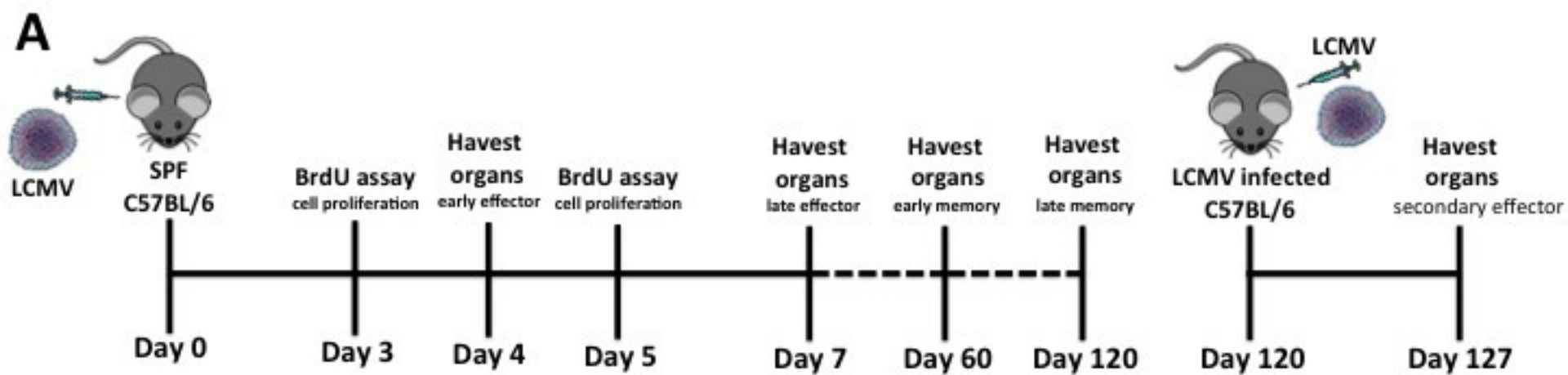


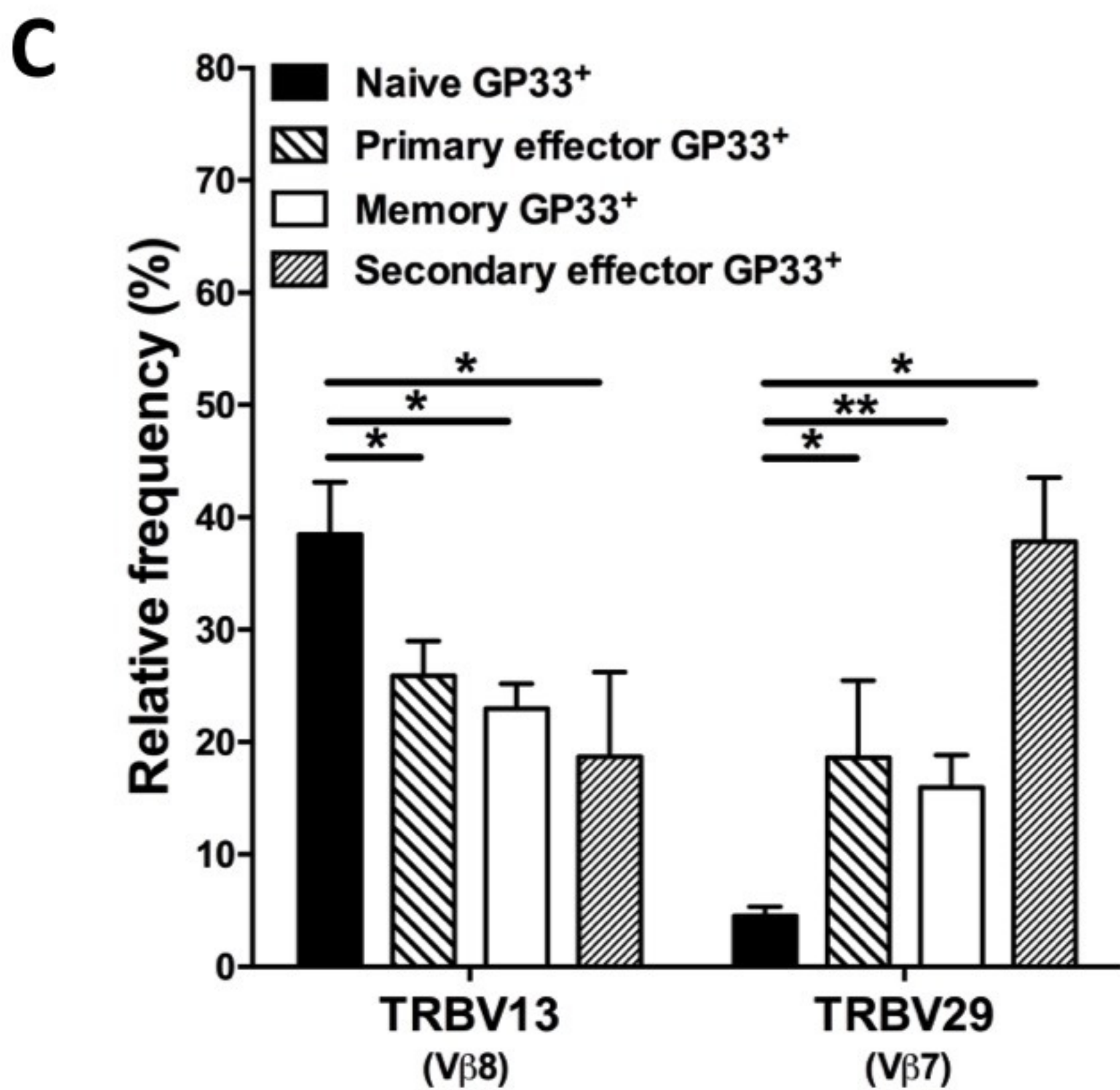
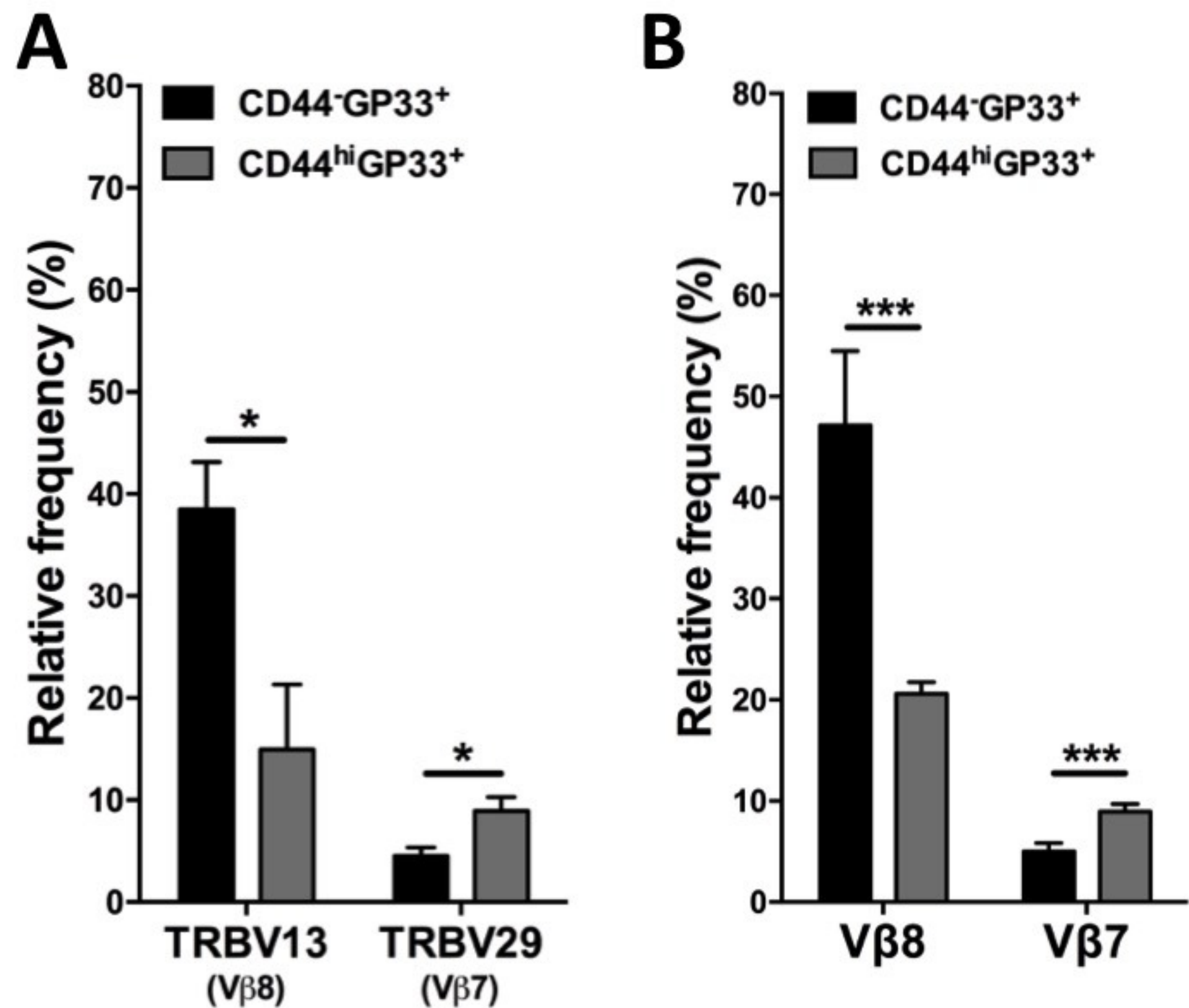
1 three independent experiments. **(D)** The CDR3 a.a. composition of IELs from SPF-1 mice.  
2 Results correspond to CDR3 with 12 a.a. sequenced from 114 single-cells, from three mice  
3 studied in two independent experiments. The difference evaluated using the Mann Whitney  
4 U-test were: \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

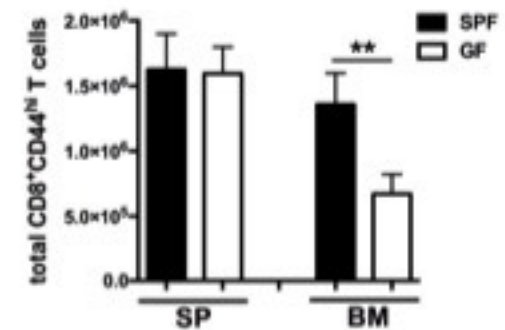
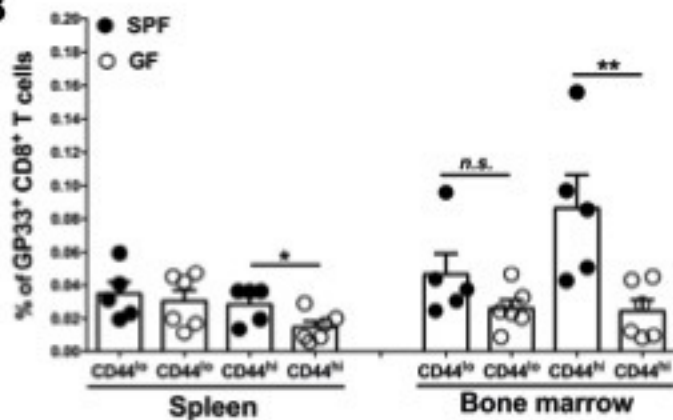
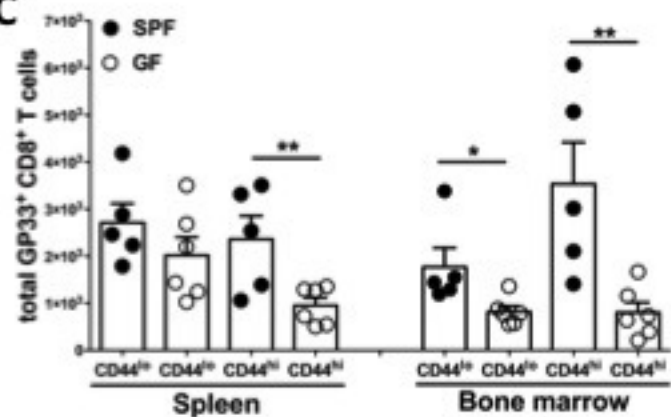
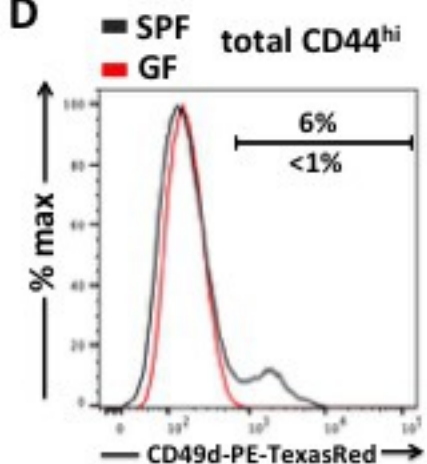
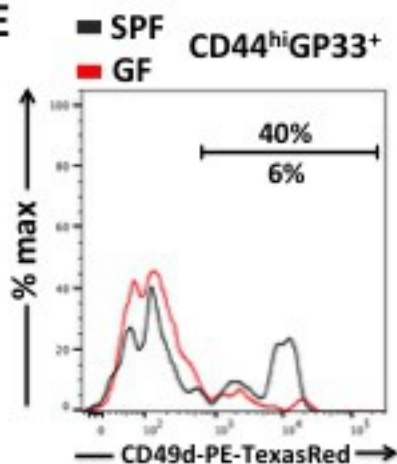
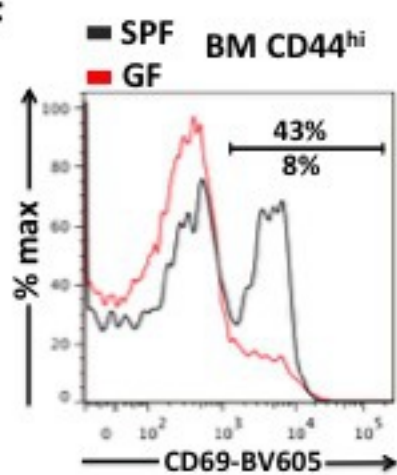
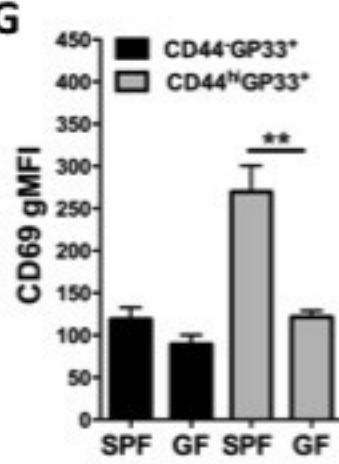






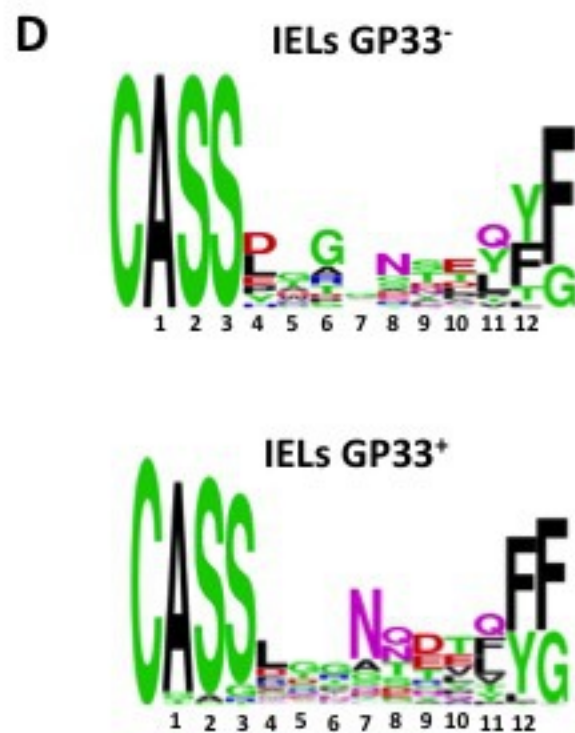
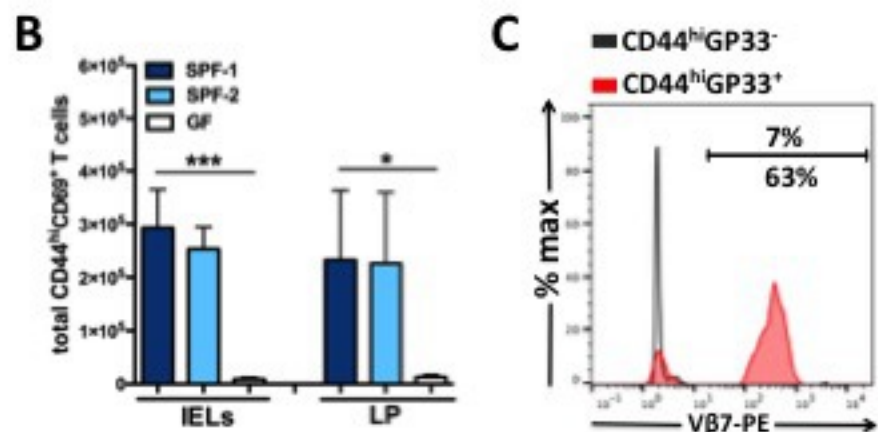
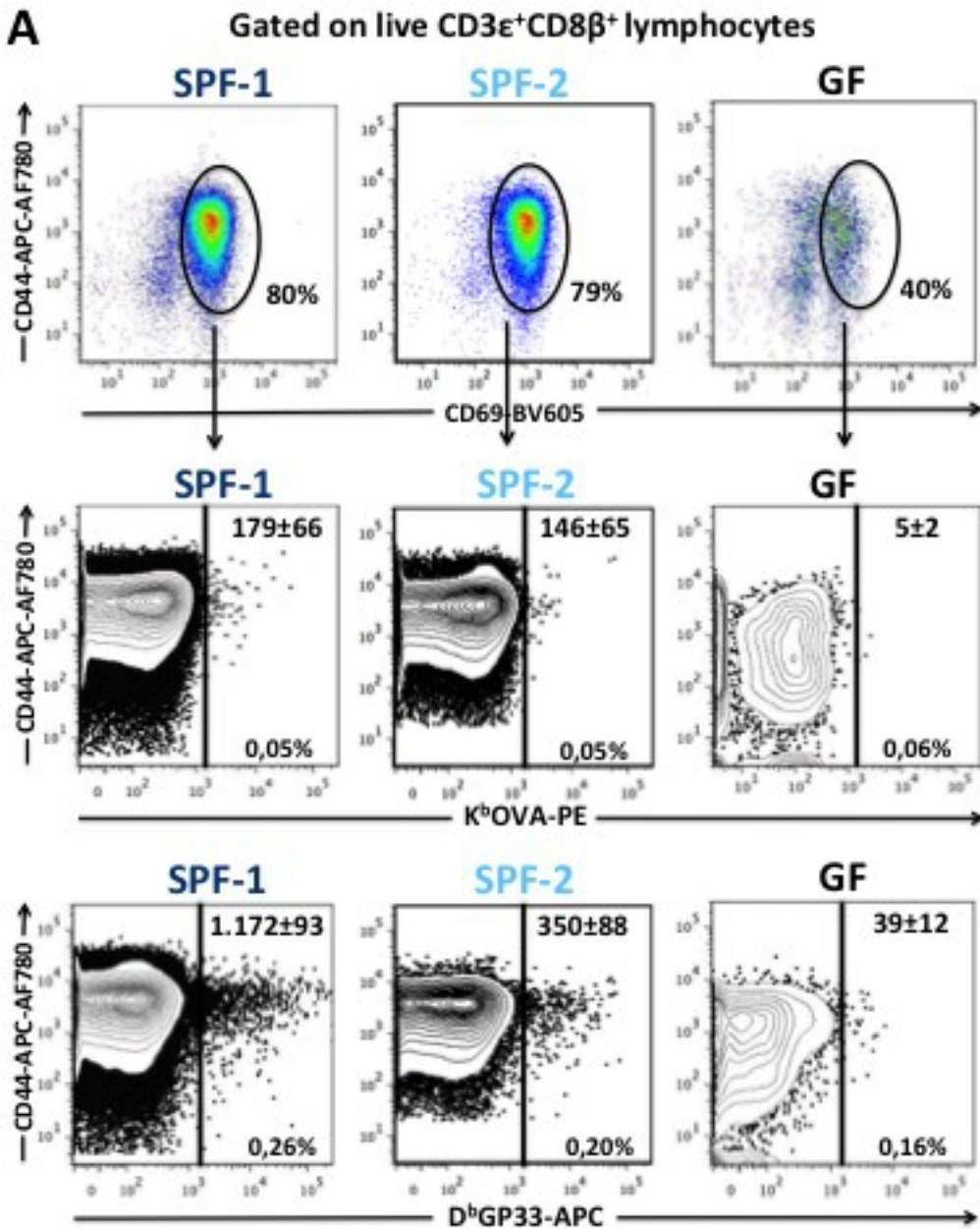




**A****B****C****D****E****F****G**







**Table 1.** The diversity of GP33-specific CD8<sup>+</sup> T cells in primary effector phase, memory and secondary effector phase after LCMV infection.

CDR3 $\beta$ region	a.a.	TRBV	TRBJ	Total cells	Clones localized	Clones spread
<b>Effector</b>						
CASSFRDRGVAEQFF	13	4	2.1	3		x
CASSYSYEQYF	9	4	2.7	6	x	
CASSLDRGLEQYF	11	4	2.7	5		x
CASSSGTYNQAPLF	12	12.2	1.5	7	x	
CASSLGAGQLYF	10	12.2	2.2	8		x
CASSLWGTSAETLYF	13	12.2	2.3	2	x	
CASSDHTNTEVFF	11	13.1	1.1	3		x
CASSDNGNTEVFF	11	13.1	1.1	11		x
CASSDWTNTEVFF	11	13.3	1.1	8		x
CASSDWGNTEVFF	11	13.3	1.1	2	x	
CASSPGSYNSPLYF	12	16	1.6	2	x	
CASSLDGTSYAEQFF	13	16	2.1	13		x
CASSPWGAGEQYF	11	16	2.7	2		x
CASSLDDWGSYEQYF	13	16	2.7	3	x	
CASSPGTGGGYAEQFF	15	17	2.1	2	x	
CATGTTNTEVFF	10	19	1.1	2	x	
CASSIQGNYAEQFF	12	19	2.1	2	x	
CASSIQGNYAEQFF	14	19	2.7	5		x
CASSLGGGDTEVFF	12	29	1.1	5	x	
CASSLWANSDYTF	11	29	1.1	13		x
CASSLVSNERLFF	11	29	1.4	22		x
CASSLWRGGAEQFF	12	29	2.1	5		x
CASSLFRDAEQFF	11	29	2.1	14		x
CASSLTPAEQFF	10	29	2.1	3	x	
CASSYWGSNTGQLYF	13	29	2.2	3		x
CASTPTSQNTLYF	11	29	2.4	5	x	
CASSLVTNQDTQYF	12	29	2.5	5	x	
CASSLTLNQDTQYF	12	29	2.5	13		x
CASSFTQGRDTQYF	12	29	2.5	3		x
CASSLGGAQDTQYF	12	29	2.5	12	x	
CASSLGGNQDTQYF	12	29	2.5	4	x	
CAWSLPGGNQDTQYF	13	31	2.5	22		x
CASSFRDRGVAEQFF	13	4	2.1	3		x
CASSYSYEQYF	9	4	2.7	6	x	
CASSLDRGLEQYF	11	4	2.7	5		x
<b>Total clonotypes</b>				<b>112</b>		
<b>Total cells studied</b>				<b>259</b>		
<b>Sample size (x10<sup>-3</sup>)</b>				<b>11.7</b>		
<b>Memory</b>						
CTCSAAGEGSQNTLYF	14	1	2.4	6		x
CASSQEDSISNERLFF	14	2	1.4	2		x
CASSMTGGAREQYF	12	3	2.7	2	x	
CASSPTDRGDTGQLYF	14	5	2.2	2	x	
CASSLGESQNTLYF	12	12.2	2.4	4		x
CAS TTGGEEQYF	10	12.2	2.7	4		x
CASSPGAPFEQYF	10	12.2	2.7	2		x
CASSPWGYEQYF	10	12.2	2.7	2	x	
CASSDWTDTTEVFF	11	13.1	1.1	3		x
CASSDAANTEVFF	11	13.1	1.1	3	x	
CASGDPIQYF	8	13.2	2.7	2		x
CASSDNSNTEVFF	11	13.3	1.1	4		x
CASSPGNSDYTF	10	13.3	1.2	4		x
CASSDAGGAREQYF	12	13.3	2.7	6		x
CARKGGGGEQYF	10	13.3	2.7	2	x	

CASSSGLGGLYAEQFF	14	15	2.1	3		x
CASSLLGVYEQYF	11	15	2.7	3	x	
CASSLDGGSYEQYF	12	16	2.7	10		x
CASSRARDNTEVFF	12	17	1.1	6		x
CASSYRQQYF	9	17	2.7	2		x
CASSLGGDTEVFF	12	29	1.1	2	x	
CASSLYPQDTQYF	12	29	2.5	4		x
CASSLGGNQDTQYF	12	29	2.5	9		x
CASSLSGDEQYF	10	29	2.7	3	x	
CASSLFRGYEQYF	11	29	2.7	2		x
CASSLLPGKYEQYF	12	29	2.7	2		x
<b>Total clonotypes</b>				<b>123</b>		
<b>Total cells studied</b>				<b>165</b>		
<b>Sample size</b>				<b>0.26</b>		
<b>Secondary effector</b>						
CASGDWGAYAEQFF	12	13.2	2.1	2	x	
CASSDAGGRDEQYF	12	13.3	2.1	2	x	
CASSFRDRGHAEQFF	13	14	2.1	2		x
CASSLRDRGHAEQFF	13	14	2.1	9		x
CASSPWDSNYAEQFF	13	14	2.1	16		x
CASSFWGTS AETLYF	13	15	2.3	2	x	
CASSRTEQDTQYF	11	15	2.5	4		x
CASSLDDWGSSYEQYF	14	16	2.7	2	x	
CGARGPQGNTEVFF	12	20	1.1	2		x
CGAREGNQAPLF	10	20	1.5	5	x	
CASSLFTNTEVFF	11	29	1.1	4	x	
CASSLSGGDTEVFF	11	29	1.1	2		x
CASSLSTGANSDYTF	11	29	1.2	9		x
CASSLSANTGQLYF	12	29	2.2	2		x
CASSLSLNTGQLYF	12	29	2.2	3	x	
CASSLFRDSAETLYF	13	29	2.3	3	x	
CASSWTGGSQNTLYF	13	29	2.4	8		x
CASSFSSQNTLYF	11	29	2.4	2	x	
CASSFGGNQDTQYF	12	29	2.5	6		x
CASSFVINQDTQYF	12	29	2.5	2		x
CASSLSRRVEQYF	11	29	2.7	5		x
<b>Total clonotypes</b>				<b>87</b>		
<b>Total cells studied</b>				<b>144</b>		
<b>Sample size (x10<sup>-3</sup>)</b>				<b>16.6</b>		

CD8<sup>+</sup>GP33<sup>+</sup> cells from different LCMV infected mice were sorted from the SP, LN and BM to evaluate *Tcrb* expression in each individual cell from at different time points after LCMV infection. Results show a representative mouse at each time point. Remaining mice and full TCRB sequences of all individual cells in all mice are shown in Supplementary Table 2. From left to right: the CDR3 region; CDR3 size; *Trbv* gene; *Trbj* genes; the number of cells expressing the same TCRB, localized in a single organ (SP or LN or BM) (**localized**) or present in different organs (**spread**).

