SUPPLEMENTAL MATERIAL

GL7 ligand expression defines a novel subset of $CD4^+$ T_{RM} cells in lungs recovered from pneumococcus.

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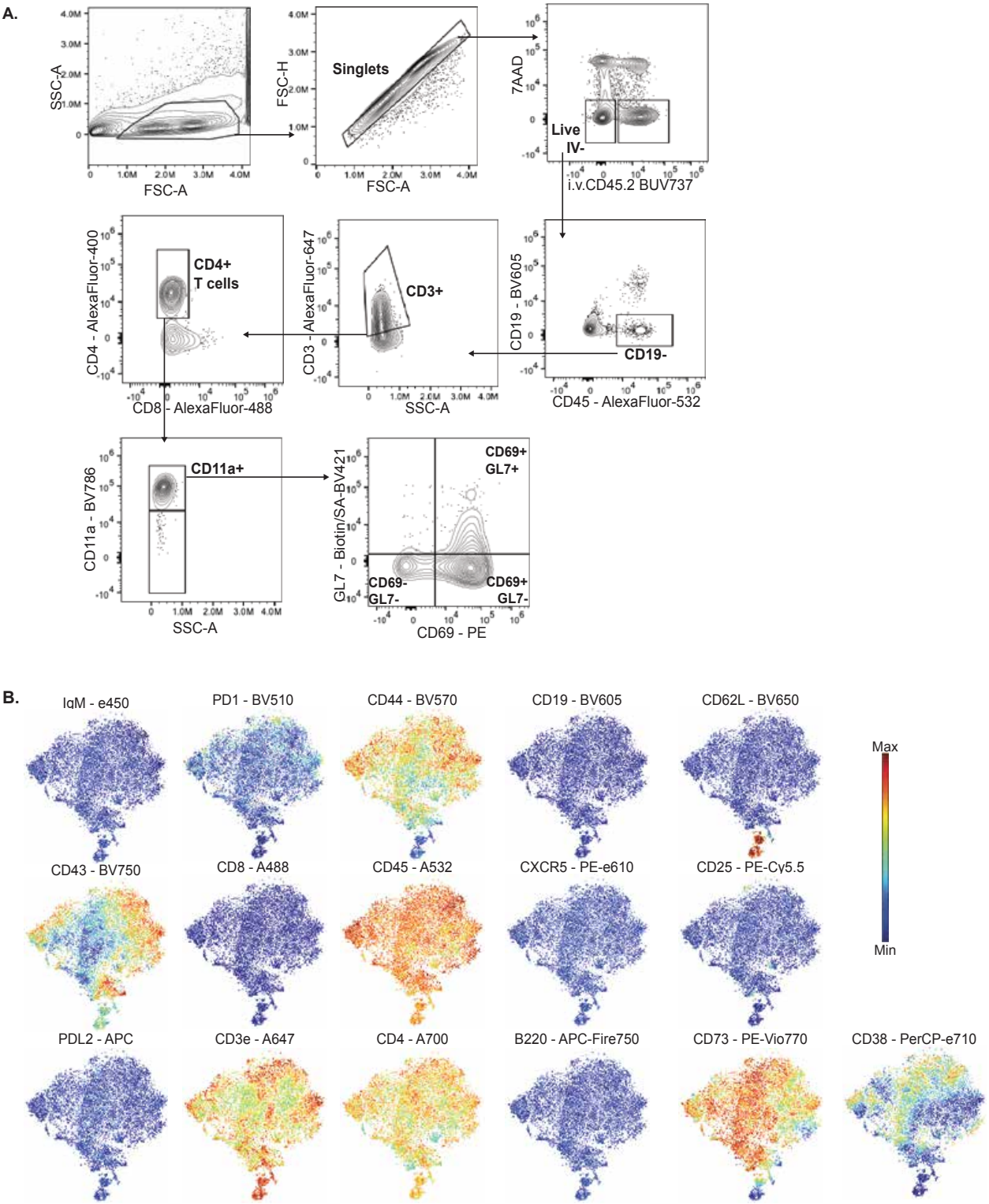
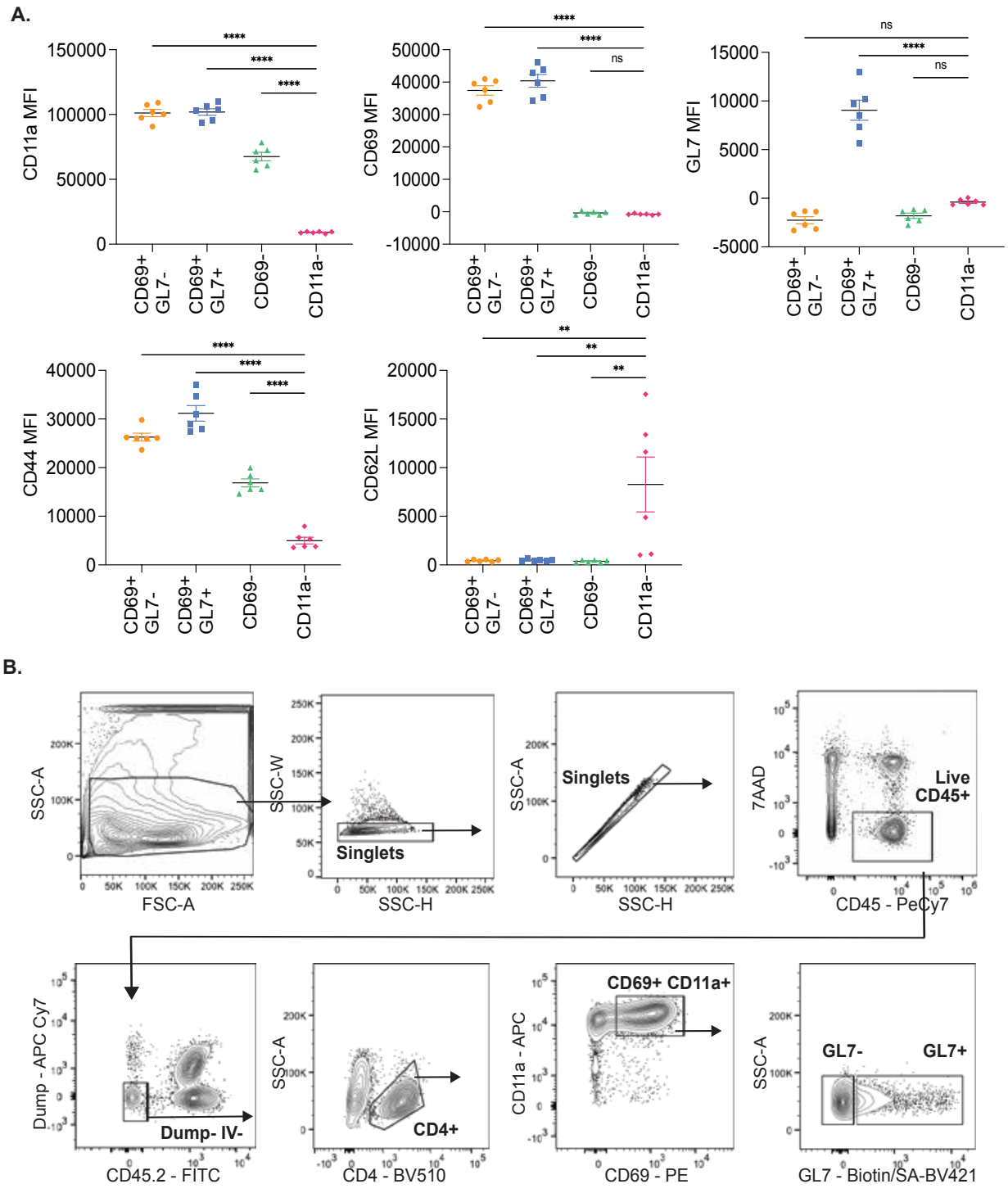
SUPPLEMENTAL FIGURES

Supplemental Figure 1



Supplemental Figure 1. Identification of lung CD4$^+$ T_{RM} subsets. A) Manual gating strategy to define live lung (i.v. CD45.2$^-$) CD4$^+$ T cell subsets in experienced mouse lungs after 3 min of i.v. injection of anti-CD45.2 antibody. **B)** Heat maps depiction of expression level of surface markers overlayed on opt-SNE plots of lung (i.v. CD45.2$^-$) CD4$^+$T cell from n=6 experienced mice.

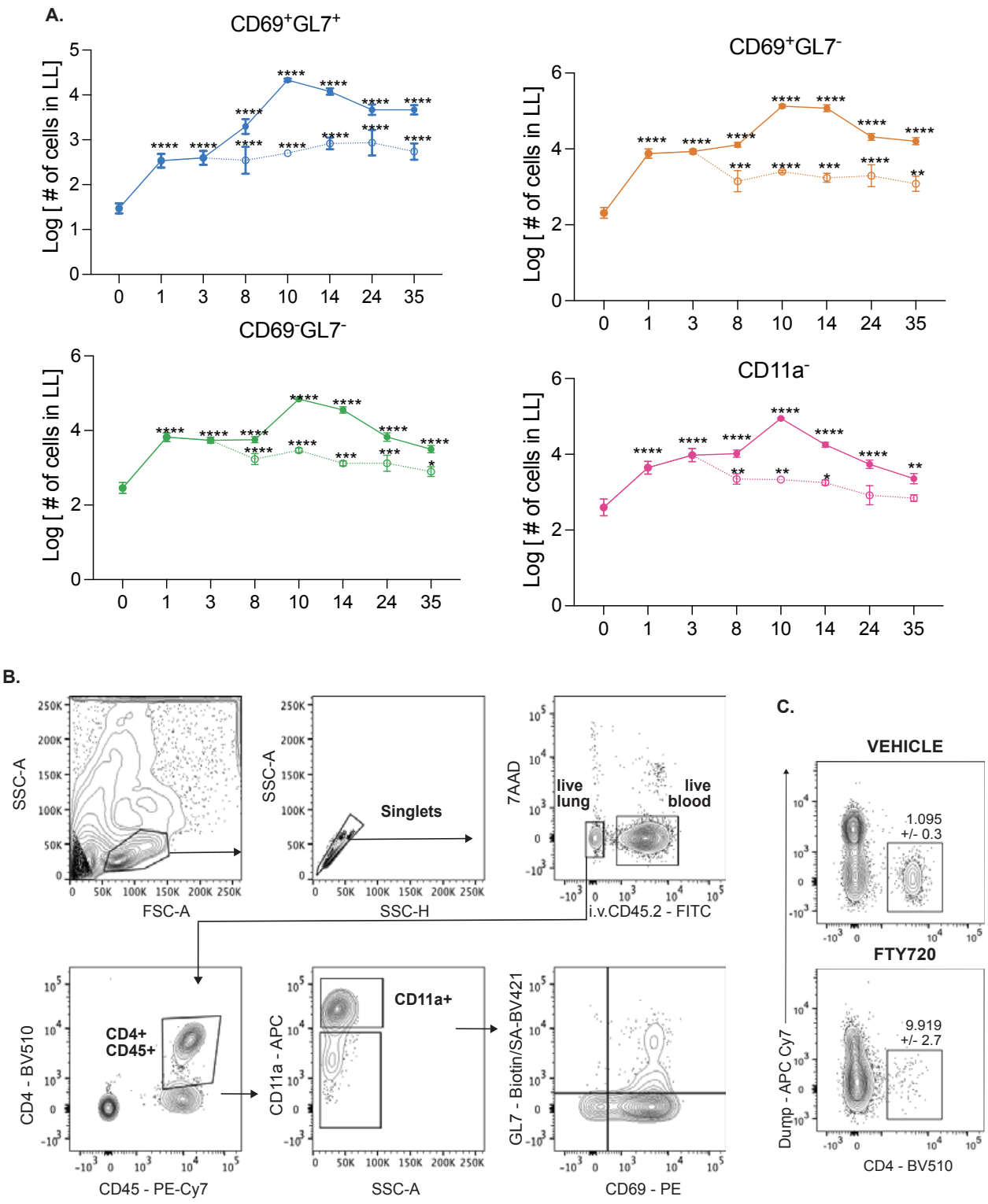
Supplemental Figure 2



Supplemental Figure 2. Characterization of lung CD4⁺ T_{RM} subsets. A) Mean fluorescence intensity (MFI) of expression in all subsets of lung (i.v. CD45.2⁻) CD4⁺ T cells. One-way ANOVA with Dunnett's multiple comparisons test against CD11alo subset, **p=0.002, ****p<0.0001. n=6, two independent experiments. **B)** Sorting gating

strategy to separate GL7+ and GL7- subsets of lung (i.v. CD45.2^{-}) CD4^{+} CD69^{+}CD11a^{+} cells. Sorted cells from 6 mice pooled during sort. Anti-CD8 and anti-CD11c were included in dump color cocktail.

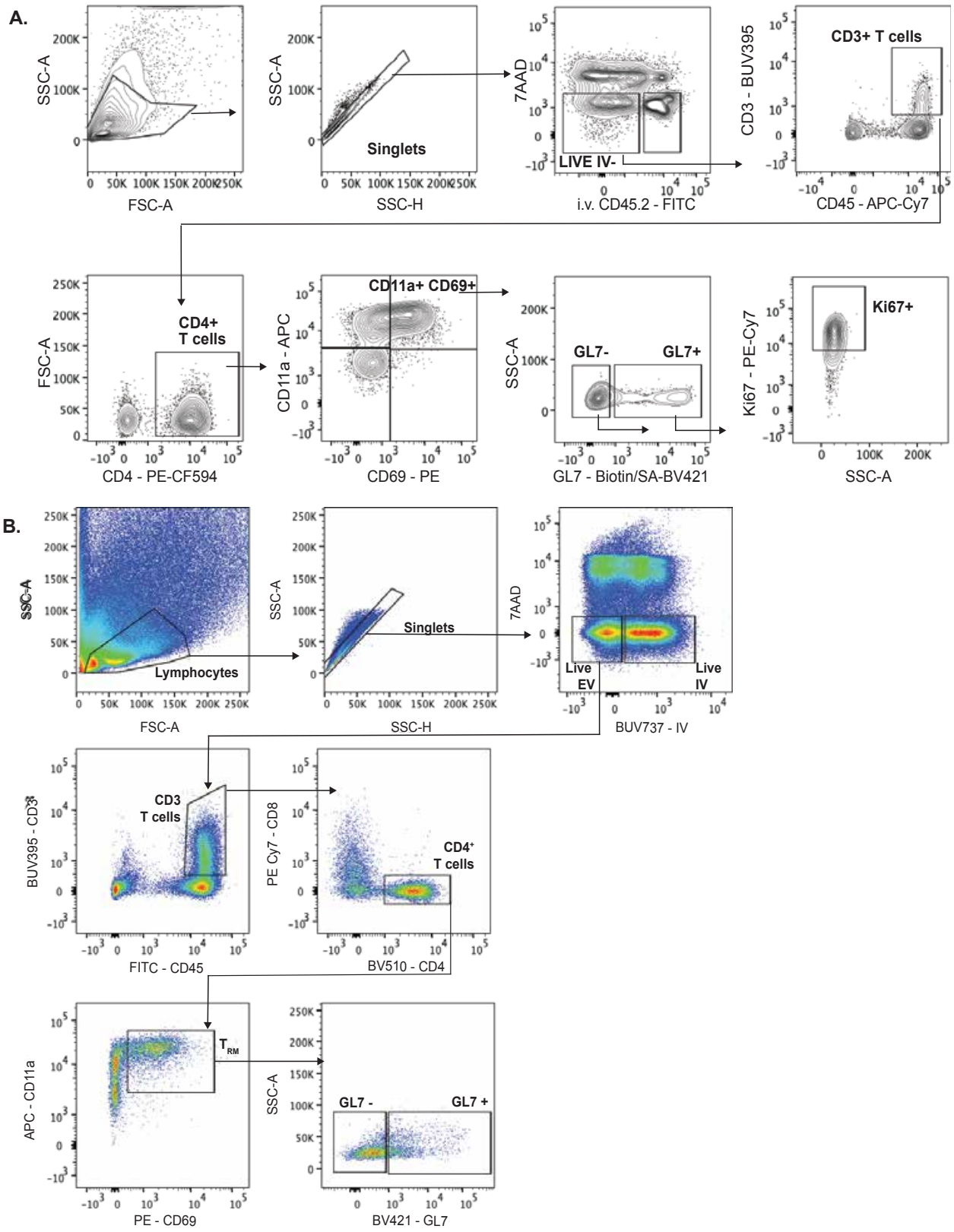
Supplemental Figure 3



Supplemental Figure 3. Dynamics of lung CD4⁺ T cell subsets. A) Cell counts of each subset of lung (i.v.CD45.2⁻) CD4⁺ T cells across heterotypic immune timeline after

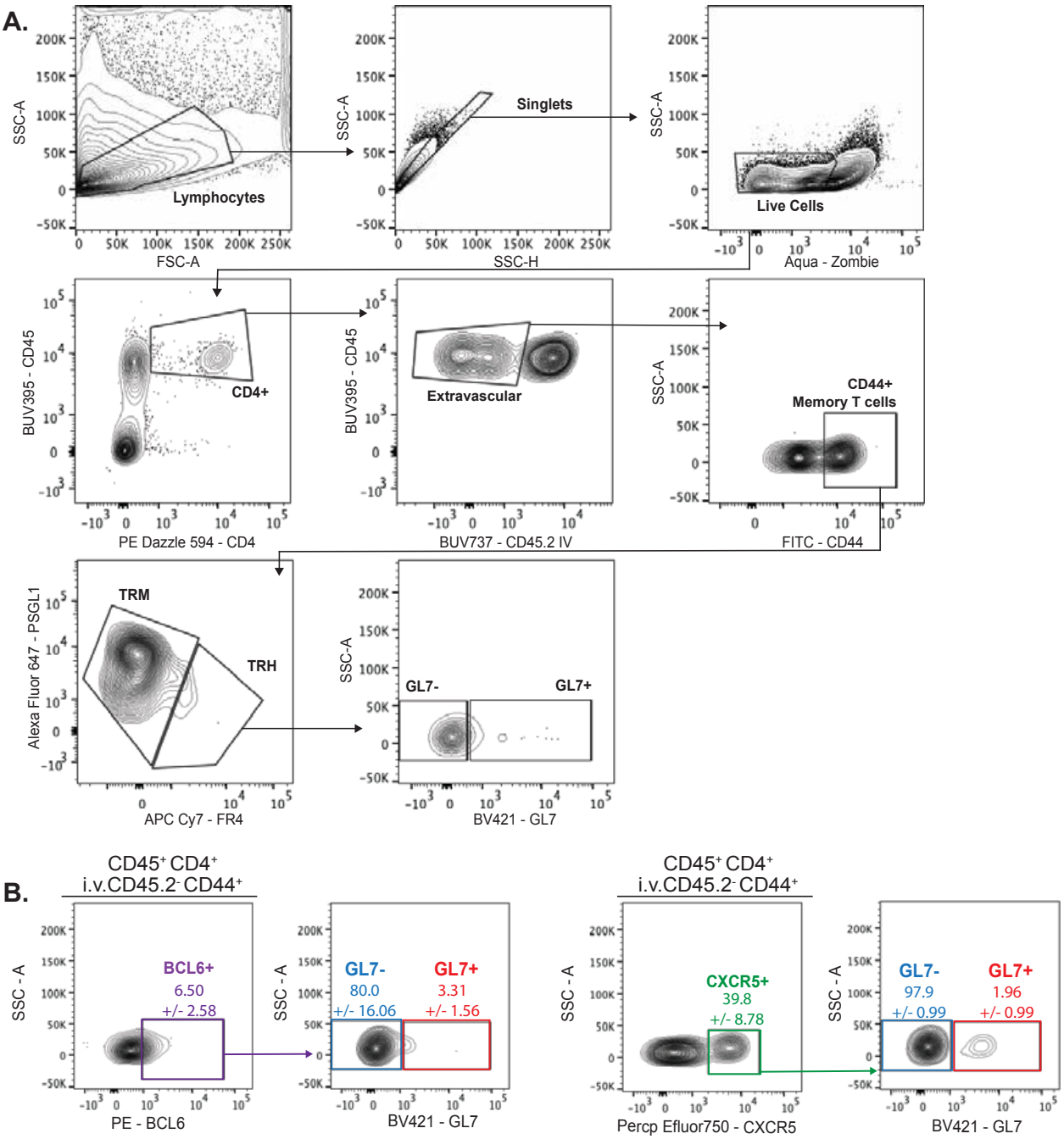
receiving 1x or 2x i.t. infections with Sp19F at days 0 and 7. Data shown as log transformed mean and SEM. n=6 per group, two independent experiments. 2-way ANOVA with Dunnett's multiple comparison tests against 0dpi, *p=0. 017 - 0. 049, **p=0.0017 - 0.0047, ***p=0.0001 – 0.0008, ****p<0.0001. FTY720 or vehicle i.p. to experienced mice at 1mg/kg every other day for 12 days. **B)** Gating strategy to identify lung (i.v.CD45.2^{-}) and blood (i.v.CD45.2^{+}) CD45^{+}CD4^{+} T cells in FTY720 or vehicle treated mice. **C)** Representative flow cytometry plots and mean ± standard deviation of frequency of live blood (i.v.CD45.2^{+}) CD45^{+}CD4^{+} from LL, n=10 per group, two independent experiments.

Supplemental Figure 4



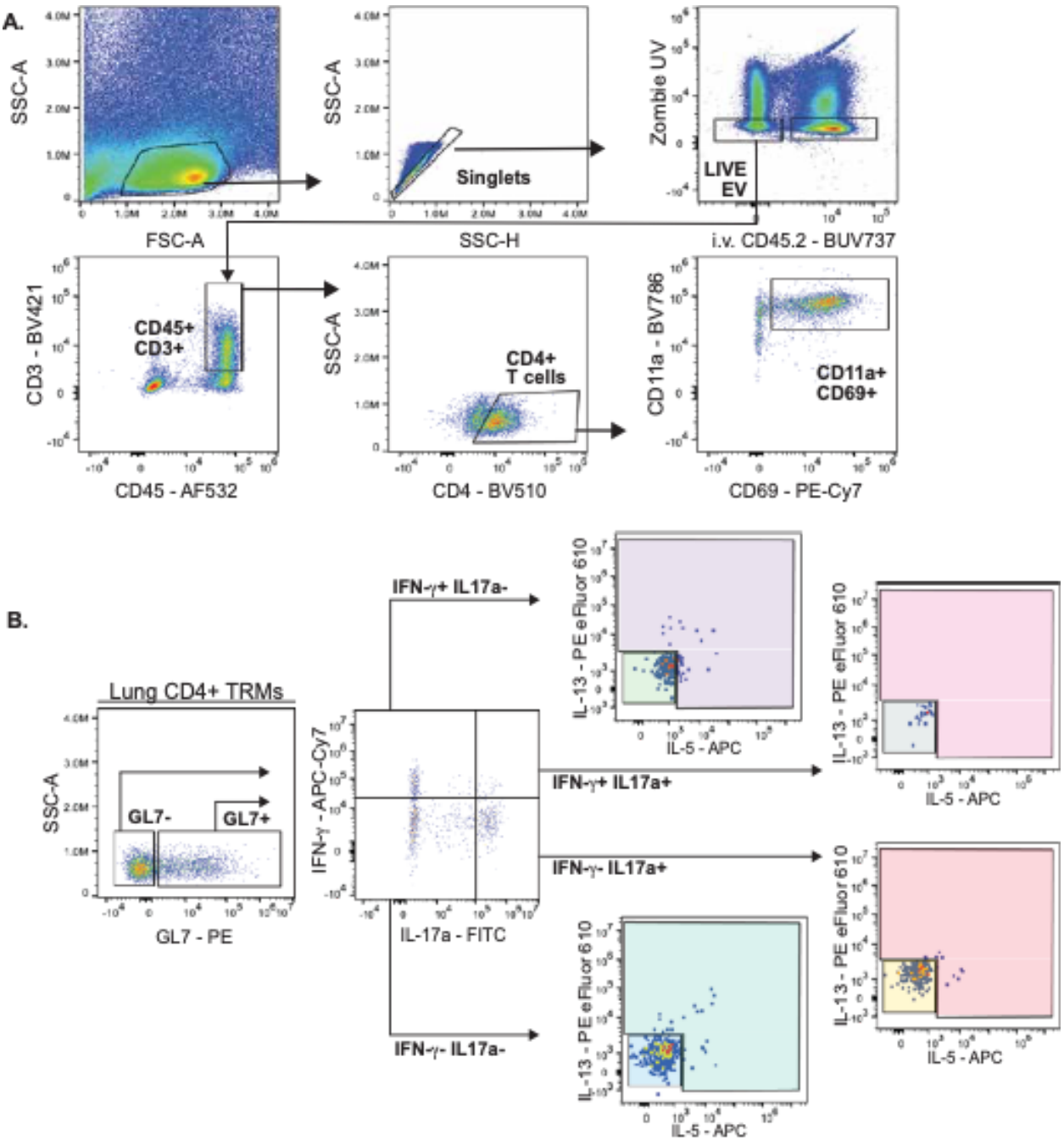
Supplemental Figure 4. Ki67 Characterization of GL7$^+$ T_{RM} subsets. A) Gating strategy to evaluate Ki67 expression in GL7$^+$ and GL7$^-$ subsets of lung (i.v.CD45.2$^-$) CD4$^+$ CD69$^+$CD11a$^+$ T cells. **B)** Gating strategy to evaluate percent of GL7$^+$ and GL7$^-$ cells of lung (i.v.CD45.2$^-$) CD4$^+$ CD69$^+$CD11a$^+$ T cells in cells infected with Sp19F or killed KPN or IAV.

Supplemental Figure 5



Supplemental Figure 5. Gating strategy to evaluate TRM vs TRH phenotypes in memory T cells. A) Gating strategy used to evaluate expression of GL7 in PSGL1 and FR4 subsets of lung (i.v.CD45.2$^-$) CD4$^+$CD44$^+$ memory T cells in mouse lungs processed with manual digestion method. **B)** Representative flow cytometry plots and mean +/- standard error of mean of GL7 subsets in BCL6+ and CXCR5+ populations in lung (i.v.CD45.2$^-$) CD4$^+$CD44$^+$ memory T cells in mouse lungs processed with manual digestion method, n=6, two independent experiments.

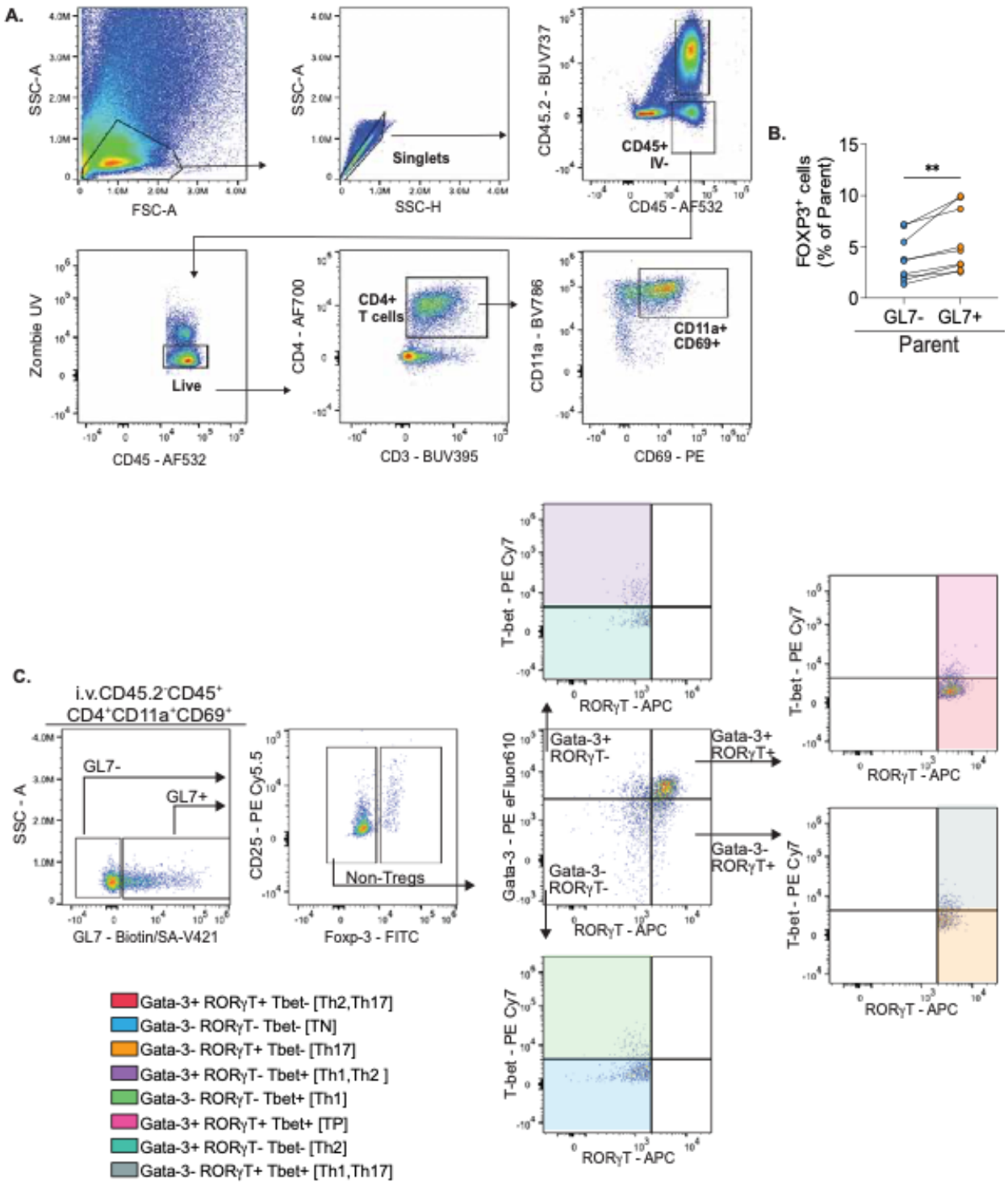
Supplemental Figure 6



Supplemental Figure 6. Gating strategy to evaluate intracellular cytokine staining profiles of GL7$^+$ T$_{RM}$ subsets.

A) Intracellular cytokine staining (ICS) gating strategy used to evaluate cytokine secretion in GL7$^+$ and GL7$^-$ subsets of lung (i.v.CD45.2$^-$) CD4$^+$ T$_{RM}$ cells. **B)** Nested gating strategy to identify expression of one, two, three, or four cytokines in GL7$^+$ and GL7$^-$ subsets of lung (i.v.CD45.2$^-$) CD4$^+$ T$_{RM}$ cells.

Supplemental Figure 7



Supplemental Figure 7. Gating strategy for functional evaluation of GL7$^+$ T_{RM} subsets.

A) Manual gating strategy to identify expression of transcription factors in subsets of lung $CD4^+$ T_{RM} cells. **B)** Quantification of percent of FOXP3+ cells in parent population ($GL7^+$ or $GL7^-$ T_{RM}), n=9, two independent experiments, paired T-test, **p=0.0032. **C)** Nested gating strategy to identify expression of one, two or more transcription factors in both $GL7^+$ and $GL7^-$ subsets of lung (i.v.$CD45.2^-$) $CD4^+$ T_{RM} cells. Lung $CD4^+$ T_{RM} cells gated as shown in **A**. Subsets labeled based on the lineage-defining transcription factors (LDTF) they express.

SUPPLEMENTAL TABLES

Supplemental Table 1: List of antibodies used in this study				
Marker	**Conjugate**	**Clone**	**Vendor**	**Catalogue #**
B220	APC-Fire750	RA3-6B2	Biolegend	103259
BCL6	AF488	K112-91	BD Biosciences	561524
BCL6	PE	K112-91	BD Biosciences	561522
CD103	BV711	2E7-	Biolegend	121435
CD11a	BV786	M17	BD Biosciences	740866
CD11a	APC	M17/4	invitrogen	17-011-82
CD19	BV605	6D5	Biolegend	115539
CD25	PE-Cy5.5	PC61.5	Thermo Fisher	35-0251-80
CD3	AF647	145-2C11	Biolegend	100322
CD3	BV421	145-2c11	Biolegend	100335
CD3	BUV395	145-2c11	BD Biosciences	563565
CD38	PerCP-e710	90	Thermo Fisher	46-0381-80
CD4	AF700	RM4-4	Biolegend	116021
CD4	BV510	GK1.5	Biolegend	100449
CD4	PE-CF594	RM4-5	BD Biosciences	562314
CD4	PE-Dazzle594	GK1.5	Biolegend	100456
CD43	BV750	S7	BD Biosciences	747277
CD44	BV570	IM7	Biolegend	103037
CD44	FITC	IM7	Biolegend	103006
CD45	AF532	30-F11	Thermo Fisher	58-0451-82
CD45	APC Cy7	30-F11	Biolegend	103115
CD45	BUV395	HI30	BD Biosciences	563791
CD45.2	BUV737	104	BD Biosciences	612778
CD62L	BV650	MEL-14.	BD Biosciences	564108
CD69	PE	H1.2F3	Biolegend	104508
CD69	PECY7	H1.2F3	Biolegend	104512
CD73	PE-Vio770	REA778	Miltenyi	130-111-519
CD8	AF488	53-6.7	Biolegend	100723
CXCR5	PE-e610	SPRCL5	Thermo Fisher	61-7185-82
CXCR5	PerCP-eFluor750	SPRCL5	Thermo Fisher	46-7185-82
FOXP3	FITC	FJK-16s	Invitrogen	2126755
FR4	APC Fire-750	12A5	Biolegend	125013
FR4	PerCP Cy5.5	12A5	Biolegend	125018
Gata-3	PE-e610	TWAJ	Invitrogen	2187622
GL7	Biotin	GL7	Biolegend	144616
GL7	PE	GL7	Biolegend	144608
ICOS	BV650	C398.4	BD Biosciences	568041
IFN-γ	APC Cy7	XMG1.2	Biolegend	505850
IgD	AF488	11-26c.2a	Biolegend	405717
IgM	e450	eB121-15F9	Thermo Fisher	48-5890-82
IL-13	PE E610	eBIO13A	Thermo Fisher	61-7133-82

IL-17a	FITC	TC11-18H10.1	Biolegend	506908
IL-5	APC	TRFK5	Biolegend	504306
Ki67	PeCy7	16A8	Biolegend	652426
PD1	BV510	29F.1A12	Biolegend	135241
PD1	PE-Cy7	29F.1A12	Biolegend	135216
PDL2	APC	TY25	Biolegend	107210
PSGL1	Alexa Fluor 647	4RA10	Thermo Fisher	16-6763-84
RORγT	APC	B2D	Invitrogen	17-6981-80
Streptavidin	BV421	n/a	Biolegend	405225
Streptavidin	APC-Fire750	n/a	Biolegend	405260
T-bet	PE-Cy7	4B10	Biolegend	644823
ZOMBIE	AQUA	n/a	Biolegend	423101
ZOMBIE	UV	n/a	Biolegend	423107
	7AAD	n/a	BD Biosciences	559925

Supplemental Table 2: Parameters for duplex fluorescent IHC assay performed in this study

Sequence Order	Antigen Target	Species Origin	Clone	Manufacturer	Catalog	Primary probe or antibody Dilution	Antigen Retrieval (Ventana)	Incubation Temperature	Incubation Period	Fluorophore
1	CD4	Rabbit	D7D2Z	CST	25229	1:50	CC1	RT	24 Hrs.	Opal 570 1:100
2	GL7	Rat	GL7	BioLegend	144602	1:100	CC1	RT	2 Hrs.	Opal 520 1:100

CC1-cell conditioning 1-Tris based antigen retrieval buffer; CST-cell signaling technology