Supplementary Information

Supplementary Figure Legends

Supplementary Figure 1: Characterization of protein and TLR ligand encapsulated PLGA nanoparticles. Protein and TLR ligand encapsulated PLGA nanoparticle formulations were synthesized and characterized as explained in the materials and methods. This table summarizes the sizes, loading levels and efficiencies of proteins and TLR ligands (μg encapsulated per mg of formulation). Sizes or loading levels are represented mean ± s.d from various batches synthesized.

Supplementary Figure 2: Antigen and adjuvant in 2 separate particles induce significantly enhanced responses compared to antigen and adjuvant in the same particle. Graphs represent average titers (mean + s.e.m of 2 independent experiments, with 4 mice /treatment group in each experiment) at 4 weeks post primary immunization.

Supplementary Figure 3: Synergistic enhancement of antibody responses to immunization with PLGA(MPL+R837) plus PLGA(OVA). Dose of OVA is 50μg. Graphs in **a)** and **b)** represent average titers (mean + s.e.m of 2 independent experiments, with 4 mice /treatment group in each experiment) at 4 weeks post prime and boost immunizations. Boosting was done at 5 weeks post primary immunization. *** represents $p<0.001$ and ** represents $p<0.01$ and * represents $p<0.05$ (one way ANOVA with Bonferroni post hoc test).

Supplementary Figure 4: Synergistic enhancement of antibody responses to immunization with PLGA(MPL+R837) plus PLGA(OVA). Dose of OVA is 10μg. Graphs in **a)** and **b)** represent average titers (mean + s.e.m of 4 independent experiments, with 4 mice /treatment group in each experiment) at 4 weeks post prime and boost immunizations. Boosting was done at 5 weeks post primary immunization. *** represents $p<0.001$ and ** represents $p<0.01$ and * represents $p<0.05$ (one way ANOVA with Bonferroni post hoc test).

Supplementary Figure 5: Immunization with PLGA(MPL+R837) plus PLGA(PA) enhances the magnitude of high affinity antigen-specific antibody response. Graphs in **a)** and **b)** represent average titers (mean + s.e.m of 2 independent experiments, with 5 mice /treatment group in each experiment) at 4 weeks post prime and boost immunizations. Boosting was done at 5 weeks post primary immunization. *** represents $p<0.001$ and ** represents $p<0.01$ and * represents $p<0.05$ (one way ANOVA with Bonferroni post hoc test). **c)** BIACORE surface plasmon resonance (SPR) assay of antibody avidity. Individual dots in the graph represent pooled sera from 5 mice per group from one out of 2 independent experiments with identical results.

Supplementary Figure 6: DCs are required for induction of antigen-specific antibody responses to immunization with PLGA(MPL+R837) plus PLGA(OVA). (a) PLGA(MPL+R837) stimulates synergistic enhancement of cytokine production in splenic DCs in vitro. Graphs represent average cytokine response (pg/ml) (mean ± s.d of triplicate cultures) from 2-3 independent experiments. **(b, c)** Antigen-specific antibody responses to immunization with PLGA(MPL+R837) plus PLGA(OVA) are dependent on **b)** CD11c+DCs and **c)**

Langerhans cells. C57BL/6 mice, CD11c-DTRmice and Langerin-DTR mice were immunized with PLGA(MPL+R837) plus PLGA(OVA). Antibody titers are average titers (mean + s.e.m of 2 independent experiments, with 4 mice /treatment group in each experiment) measured at 4 weeks post primary immunization. *** represents $p < 0.001$, (One way ANOVA with Bonferroni post hoc test).

Supplementary Figure 7: Antigen-specific antibody responses stimulated by immunization with PLGA(MPL+R837) plus PLGA(OVA) is dependent on MyD88 and TRIF signaling.

(a, b) C57BL/6 mice, **a)** MyD88 or **b)** TRIF deficient mice were immunized with PLGA(MPL+R837) plus PLGA(OVA). Antibody titers are average titers (mean + s.e.m of 2 independent experiments, with 4 mice /treatment group in each experiment) measured at 4 weeks post primary immunization. *** represents $p < 0.001$ and ** represents $P < 0.01$, (One way ANOVA with Bonferroni post hoc test).

Supplementary Figure 8: Antigen-specific antibody responses stimulated by immunization with PLGA(MPL+R837) plus PLGA(OVA) is dependent CD4+ T helper cells. CD4+ T helper cells were depleted with anti CD4 (GK1.5) antibody as described in materials and methods. Mice were immunized with PLGA(MPL+R837) plus PLGA(OVA). Antibody titers are average titers (mean + s.e.m of 2 independent experiments, with 4 mice /treatment group in each experiment) measured at 4 weeks post primary immunization. *** represents $p < 0.001$, (One way ANOVA with Bonferroni post hoc test).

Supplementary Figure 9. Immunization with PLGA(MPL+R837) plus PLGA(OVA) stimulates persistent germinal centers. C57BL/6 mice were immunized as indicated in figure. Draining inguinal lymph nodes were excised at indicated time points, fixed, sectioned and stained as described in the materials and methods. Images are representative of sections from at least 4-6 lymph nodes per time point per treatment group. Scale bars represent 200μM for D7, 28, 42 and D14 treated group and 100 μM for D14 samples.

Supplementary Figure 10. Immunization with PLGA(MPL+R837) plus PLGA(OVA) stimulates enhanced antigen-specific memory B cell responses a) Gating strategy used to identify antigen specific B cells**.** Following live cell gating, T cells and CD11b+ myeloid cells were excluded, and the CD19+ cells were selected and further gated on IgD- IgG+ isotype switched cells. OVA-specific IgG cells were further identified as Alexa-488 labeled OVA+ cells, which were analyzed for expression of CD138+ and. GL-7 to differentiate plasma cell versus GC cells. **b)** Total numbers of TCRβ-CD11b-CD19+IgD-IgG+OVA+GL7+ antigen specific B cells per time point / draining lymph node, are averaged from pooled lymph node cells analyzed from at least 2-3 independent experiments per time point of analysis. Although at days 5 or 7, there were no differences in the numbers of such cells in mice immunized with PLGA(MPL) or PLGA(MPL+R837), at day 28 mice immunized with PLGA(MPL+R837) had significantly higher numbers of OVA-specific GC B cells. In addition, there were greatly enhanced numbers of OVA-specific memory B cells, after a secondary boost at day 35, in mice immunized with MPL+R837**.** **$p<0.01$ and * represents $p<0.05$ (one way ANOVA with Bonferroni post hoc test).

Supplementary Figure 11: Immunization with PLGA(MPL+R837) plus PLGA(OVA) induces a molecular signature characteristic of GC/memory B cells, in antigen activated B cells isolated early after immunization. At day 7 post-immunization, activated, isotype switched B cells (TCRβ-CD11b-CD19+IgD-IgG+ cells) were isolated from draining lymph nodes (pooled) from immunized mice (n=5/ treatment group), sorted by FACS, RNA extracted and analyzed by microarray analyses. **a)** Heat map shows the expression of differentially expressed genes (DEGs) in mice treated with MPL + R837 compared to those receiving single-treatment (either MPL or R837). Each duplicate column represents an independent experiment. Student's t-test ($p < 0.05$) was performed using z-score normalized expression values between MPL + R837 -treated samples versus MPL or R837 treated samples (see supplementary methods). **b)** Heat map of genes highly expressed in plasma, germinal center (GC) or memory B cells (see supplementary methods). Expression level of each gene (in rows) is represented by the number of standard deviations above (red) or below (green) the average value for that gene across all samples (in columns). DEGs related to B cell differentiation are depicted and colored accordingly to their expression pattern in B cell subsets (panel b). Genes represented on the figure have been reported before to be involved directly or are parts of signaling pathways critical in B cell survival, induction or maintenance of germinal centers or differentiation into memory B cells [39-56]. Genes colored in orange represent genes classified as "GC and Memory DEGs" (not shown in heat map), and were identified as up-regulated in germinal center compared to plasma and also up-regulated in memory compared to plasma. **c)** Bar graph shows the fold enrichment of genes up-regulated in MPL + R837 treatment (black bars) or MPL or R837 treatment (grey bars) among the genes highly expressed in different B cell subsets (panel b). Fold enrichment calculation is described in supplementary methods. The numbers of

overlapping genes are shown on each bar. Two independent experiments were performed and used as biological replicates.

Supplementary Figure 12. Immunization with PLGA(MPL+R837) plus PLGA(antigen) induces synergistic increases in frequencies of antigen specific memory CD4+ T helper cells, that last 1.5 years. **(a, b)** C57BL/6 mice were immunized with the indicated adjuvants plus PLGA(OVA), at day 0 and week 5. Mice were sacrificed at 8 weeks post boost immunization. Draining lymph node cells were isolated and restimulated in vitro with OVA, and IFN-γ producing CD4+ T cells identified as described in methods. **(a)** FACS plots are representative from one out of 2 independent experiments. **(b)** Graphs represent mean frequencies of IFN-γ producing memory CD4+ T cells ± s.d of triplicate cultures of pooled lymph node cells from one out of 2 independent experiments. **c)** Immunization with PLGA(MPL+R837) plus PLGA(HA) induces long lived antigen-specific memory CD4+ T cells for up to 1.5 years post immunization. Mice were immunized at day 0 and boosted at 5 weeks, and analyzed at 1.5 years. Lymph nodes were isolated restimulated in vitro with OVA, and IFN-γ producing CD4+ T cells identified as described in methods. Representative FACS plots of HA specific IFN-γ producing CD4+ T cells are shown. **(d)** Graphs represent mean frequencies of IFN-γ producing memory CD4+ T cells ± s.d of triplicate cultures of pooled lymph node cells from one out of 2 independent experiments. Balb/c mice were immunized with 10μg PA + TLR ligands twice 5 weeks apart. **(e)** FACS plots are representative from one out of 2 independent experiments. **(f**) Graphs represent mean frequencies of IFN-γ producing memory CD4+ T cells ±

s.d of triplicate cultures of pooled lymph node cells from one out of 2 independent experiments. *** represents p <0.001 (one way ANOVA with Bonferroni post hoc test).

Supplementary Figure 13. Immunization with PLGA(MPL+R837) plus PLGA(OVA) induces a synergistic enhancement in the frequencies of antigen specific memory CD8+ T cell responses. C57BL/6 mice were immunized with PLGA(MPL+R837) plus PLGA(OVA) at day 0 and week 5. Peripheral blood mononuclear cells (PBMCs) were isolated from mice bled at day 7 post primary or secondary immunization. Cells from individual mice were cultured with ovalbumin specific immunodominant SIINFEKL peptide at (1 μg/ml) for 6 hours in the presence of brefeldin-A (5 μg/ml) and stained for intracellular IFN-γ. **(a)** FACS plots are representative from one out of 3 independent experiments. **(b)** Graphs represent frequencies of IFN-γ producing CD8+T cells (mean ± s.e.m; n=4 mice/treatment group) from one out of 3 independent experiments. *** represents p <0.001 (one way ANOVA with Bonferroni post hoc test).

Supplementary Figure 14. Immunization with PLGA(MPL+R837) induces enhanced polyfunctional CD8+ T cells. C57BL/6 mice were immunized with PLGA(MPL+R837) plus PLGA(OVA) at day 0 and week 5. Peripheral blood mononuclear cells (PBMCs) were isolated from mice bled at day 7 post primary or secondary immunization. PBMCs were pulsed ex-vivo with immunodominant SIINFEKL peptide for 6 hours in the presence of brefeldin A. Cells were intracellularly stained for IFN-γ, TNF-α, and IL-2 and acquired on a FACS Caliber. Data was analyzed on Flow Jo and multifunctional cytokine producers were plotted using the SPICE program. **(a)** PIE Charts represent the frequencies of various combinations of cytokine producing cells. **(b)** Bar graphs represent frequencies of various combinations of cytokine producing cells

normalized to represent cytokine producing cell numbers / 200,000 cells. *** represents p <0.001 (one way ANOVA with Bonferroni post hoc test).

Supplementary Figure 15. **Adjuvanting the 2009 pandemic H1N1 whole inactivated virus (WIV) with PLGA(MPL+R837) confers enhanced antigen-specific immunity in mice. (a-c)** Mice were immunized with different doses of whole WIV with or without PLGA(MPL+R837). WIV specific total IgG, IgG2a and IgG1 antibody concentrations were measured by ELISA as described in the materials and methods. Graphs represent average titers, total IgG, IgG2a and IgG1 (ng/ml) (mean ± s.e.m; n=5 mice per treatment group) from one out of 2 independent experiments. The adjuvant enhanced antibody responses relative to the unadjuvanted groups **(a-c)**. Furthermore, 0.05μg of WIV and PLGA(MPL+R837) induced antigen-specific total IgG titers similar to that obtained with a 100 fold higher antigen dose administered with alum **(a)**. **(d)** In addition, there was a greatly enhanced haemagglutinin (HAI) titers in mice immunized with PLGA(MPL+R837), relative to the unadjuvanted groups. Graphs represent HAI titers from 11 total mice per combined from 2 independent experiments (mean± s.e.m).

Supplementary Fig 16: Adjuvanting the 2009 pandemic H1N1 whole inactivated virus (WIV) with PLGA(MPL+R837), confers enhanced mortality and morbidity against infection in mice. (a, b) Survival of mice against challenge with mouse adapted 2009 H1N1 pandemic influenza virus. The table in **(a)** represents the cumulative survival percentages of mice immunized with 5, 0.5 and 0.05μg of WIV with or without the adjuvants as indicated. Mice were challenged with a lethal dose (20xLD50) of the homologous mouse adapted H1N1virus. All the groups that were immunized with a 5μg dose of WIV survived the challenge. When 0.5μg

antigen was used, only 72.7% mice immunized with the antigen alone survived the challenge, whereas 100% mice immunized with WIV plus PLGA(MPL+R837) survived. Curiously, only 4/11 (36.4%) of the mice immunized with 0.5 µg WIV +alum survived. Strikingly, at the lowest 0.05µg dose, 81.25% of mice immunized with WIV plus PLGA(MPL+R837) survived, whereas only 12.5% of mice survived in the absence of any adjuvant (**a, b**). Consistent with decreased protection observed in mice adjuvanted with alum, no mice survived when immunized with 0.05µg of WIV+alum (**a, b**). Percentage body weight loss post challenge in mice immunized with 0.05µg of WIV antigen alone, with alum or with PLGA(MPL+R837), is shown on the right panel of (**b**). Data is pooled from the total number of mice used in 2-3 independent experiments with 5-6 mice/treatment group. **(c)** Survival curves and body weight loss for mice immunized with 5µg of WIV antigen and adjuvants indicated. **(d)** Survival curves and body weight loss for mice immunized with 5µg of WIV antigen and adjuvants indicated.

Supplementary Figure 17. Survival and body weight loss of mice post challenge with a 1000x LD50 of H5N1 avian influenza post immunization **a)** Survival curve indicates protection against a challenge with 1000LD50 A/Vietnam/1203/2004 wild type H5N1 virus in mice immunized with H5HA adsorbed with alum, or encapsulated in PLGA nanoparticles and immunized with or without adjuvants as indicated. **b)** Graph represents percentage body weight loss post challenge in mice immunized with H5HA adsorbed with alum or encapsulated in PLGA nanoparticles with or without the adjuvants. Strikingly, mice immunized with the recombinant HA protein were protected when immunized with either single or combination of TLR ligands as well as alum adjuvant **(a)**. The enhanced protection mediated by the combination of TLR

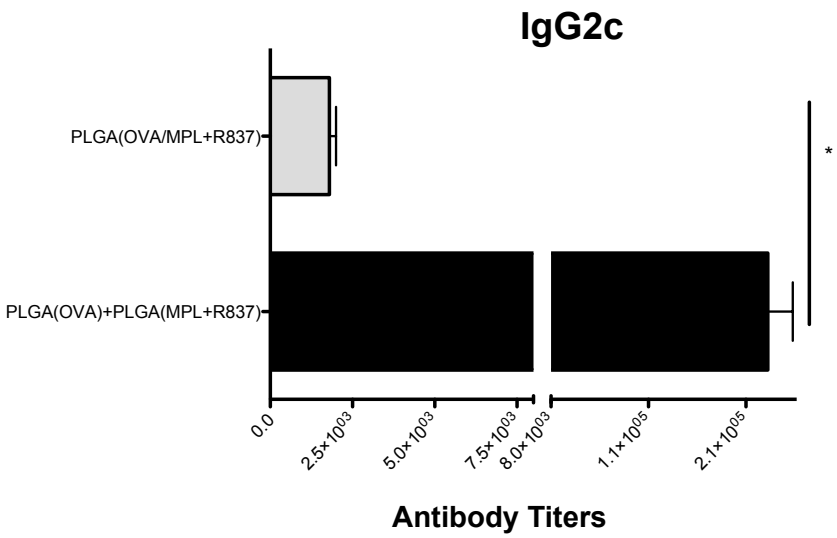
ligands was evident in increased resistance to morbidity during the first 5 days of wild type virus challenge (**b**).

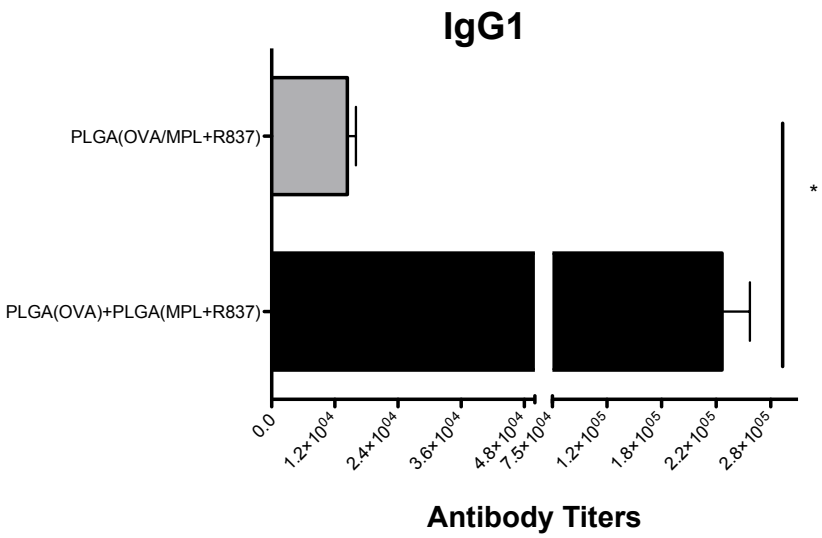
Supplementary Figure 18: Table summarizes the data demonstrating a preferential enhancement of persistent memory B cell responses stimulated by PLGA(MPL+R837), relative to immunization with PLGA(MPL) or PLGA(R837).

Supplementary figure 1:

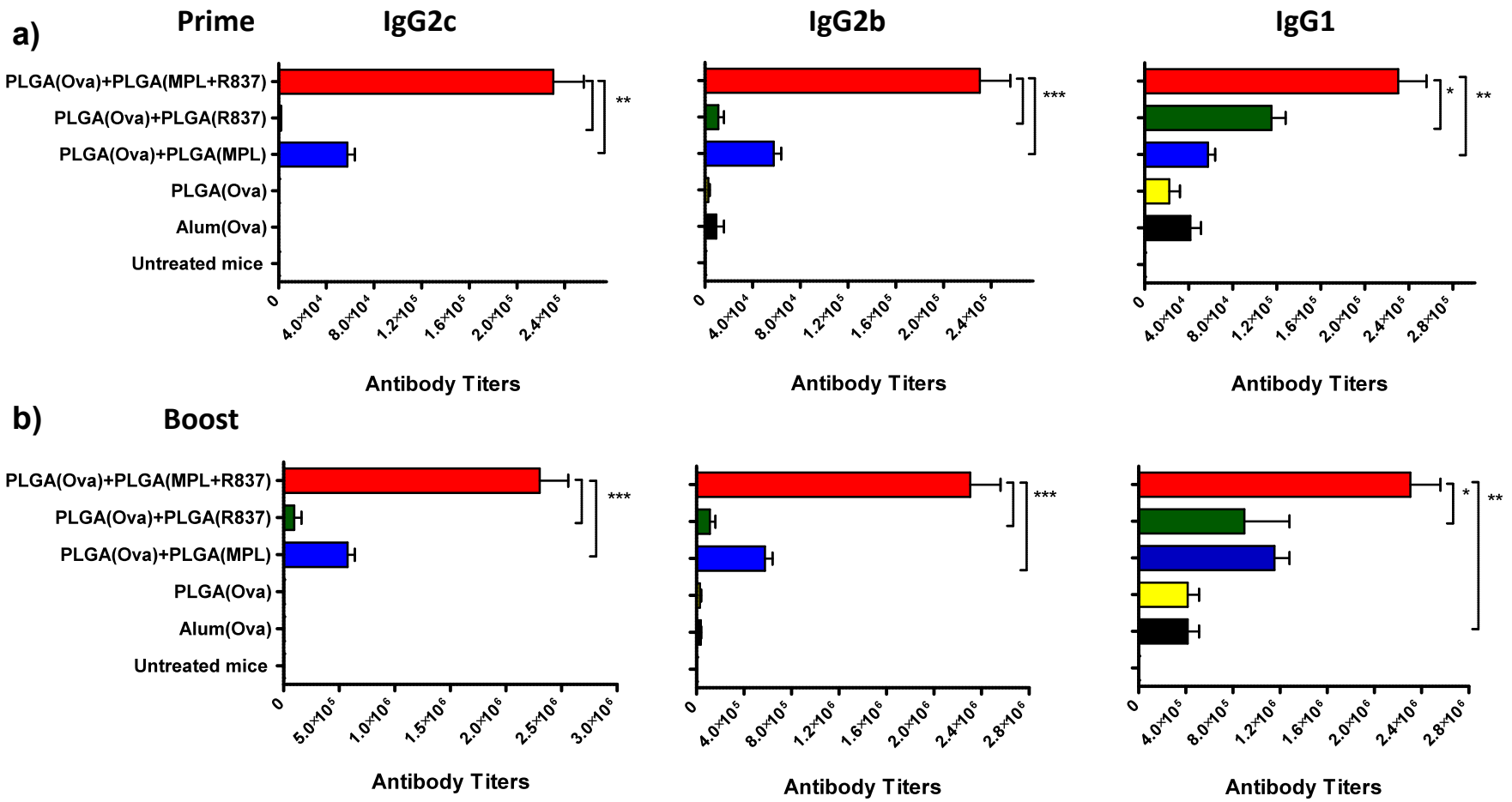
Formulation	Size (nm)	Protein loading (µg/mg)	TLR ligand loading (µg/mg)	Loading efficiency (%)
PLGA(OVA)	383.43 ± 134.45 (n=7)	44.11 ± 8.3	-	88.22
PLGA(PA)	322.1 (n=1)	11.88	-	79.2
PLGA(HA)	416.4 ± 36.20 (n=2)	9.695 ± 2.4	-	64.63
PLGA(MPL)	345.52 ± 136.05 (n=11)	-	12.5	100
PLGA(R837)	358.32 ± 96.80 (n=12)	-	19.86 ± 1.46	79.44
PLGA(MPL+R837)	374.74 ± 110.15 (n=14)	-	12.5 (MPL) 20.185 ± 1.33 (R837)	100 (MPL) 80.74 (R837)
PLGA(R848)	278.6 ± 117.097 (n=2)	-	19.6969 ± 0.791	49.2424
PLGA(MPL+R848)	375.1667± 147.153 (n=3)	-	12.5(MPL) 20.061 (R848)	100 (MPL) 50.0267 (R848)

Supplementary figure 2:

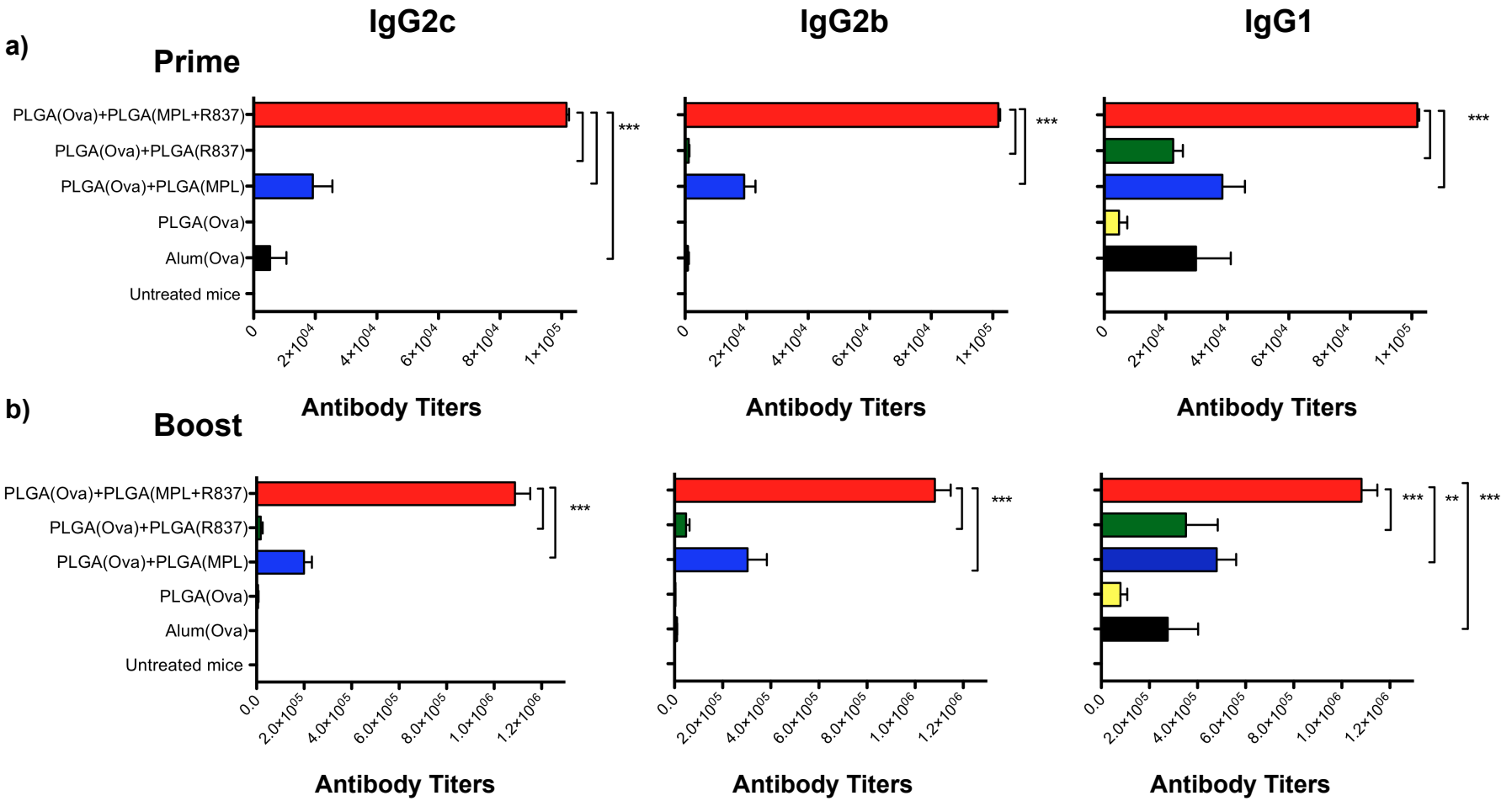




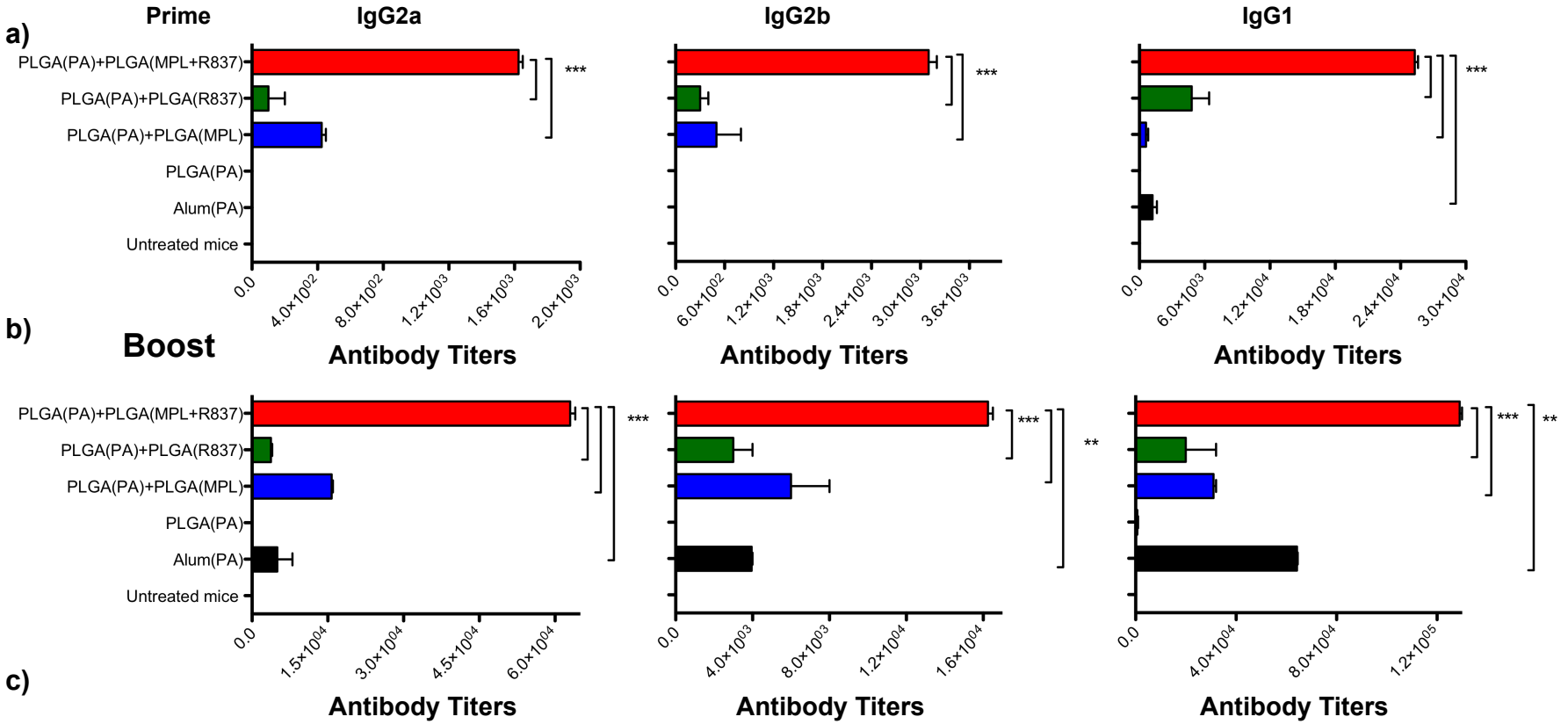
Supplementary figure 3

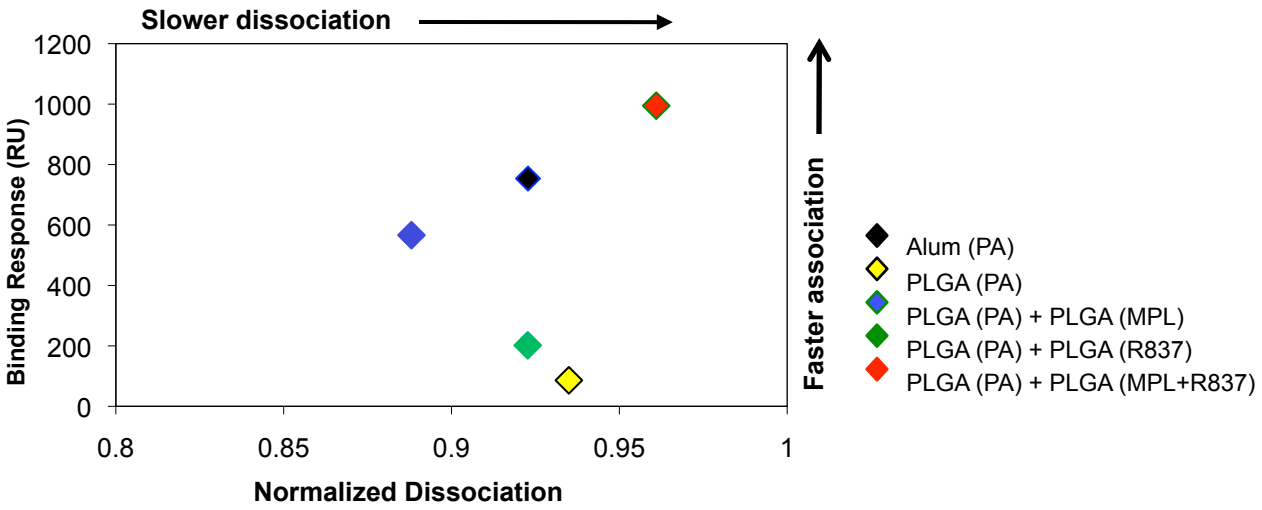


Supplementary figure 4

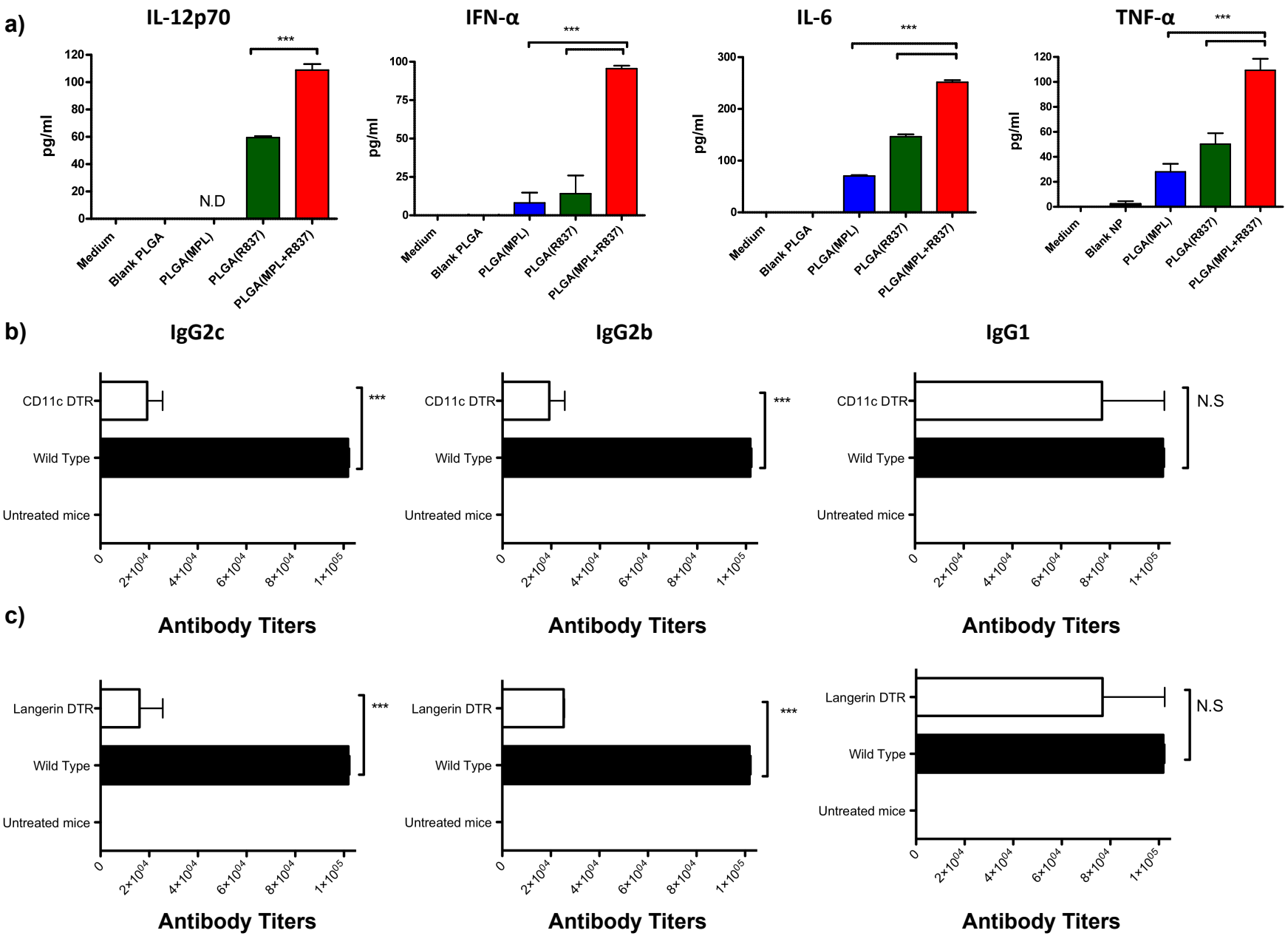


Supplementary figure 5

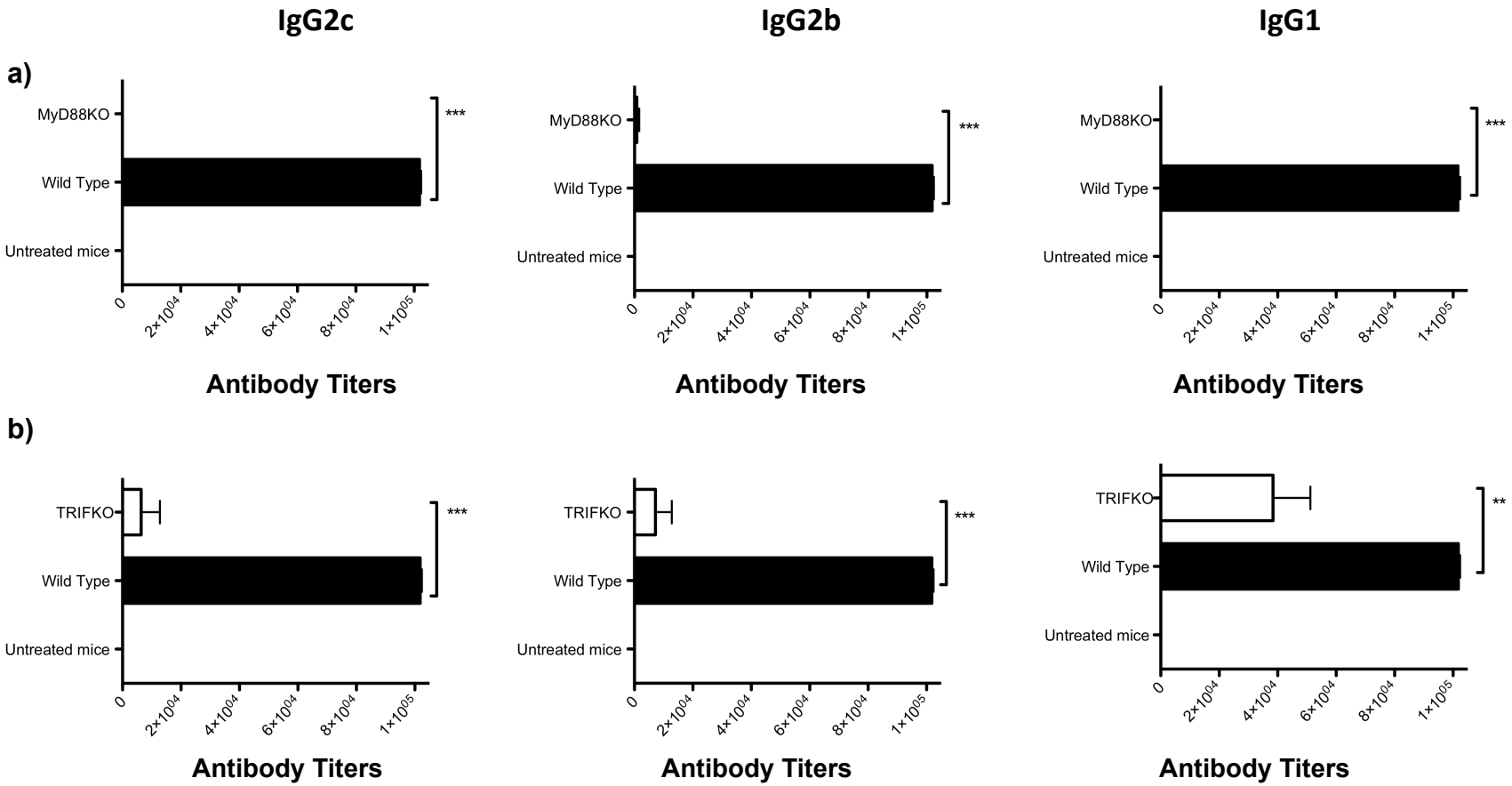




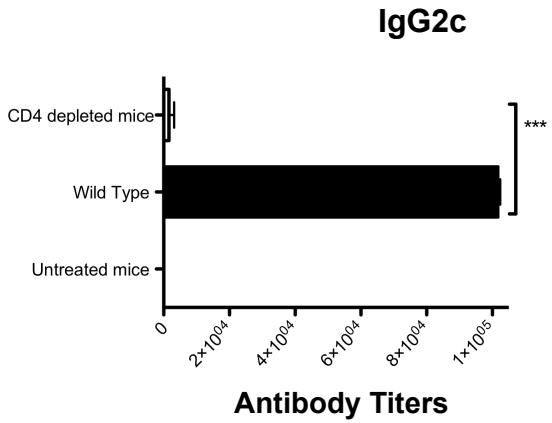
Supplementary figure 6

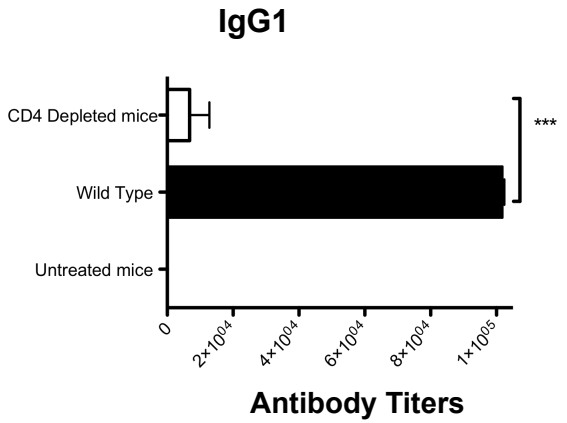


Supplementary figure 7

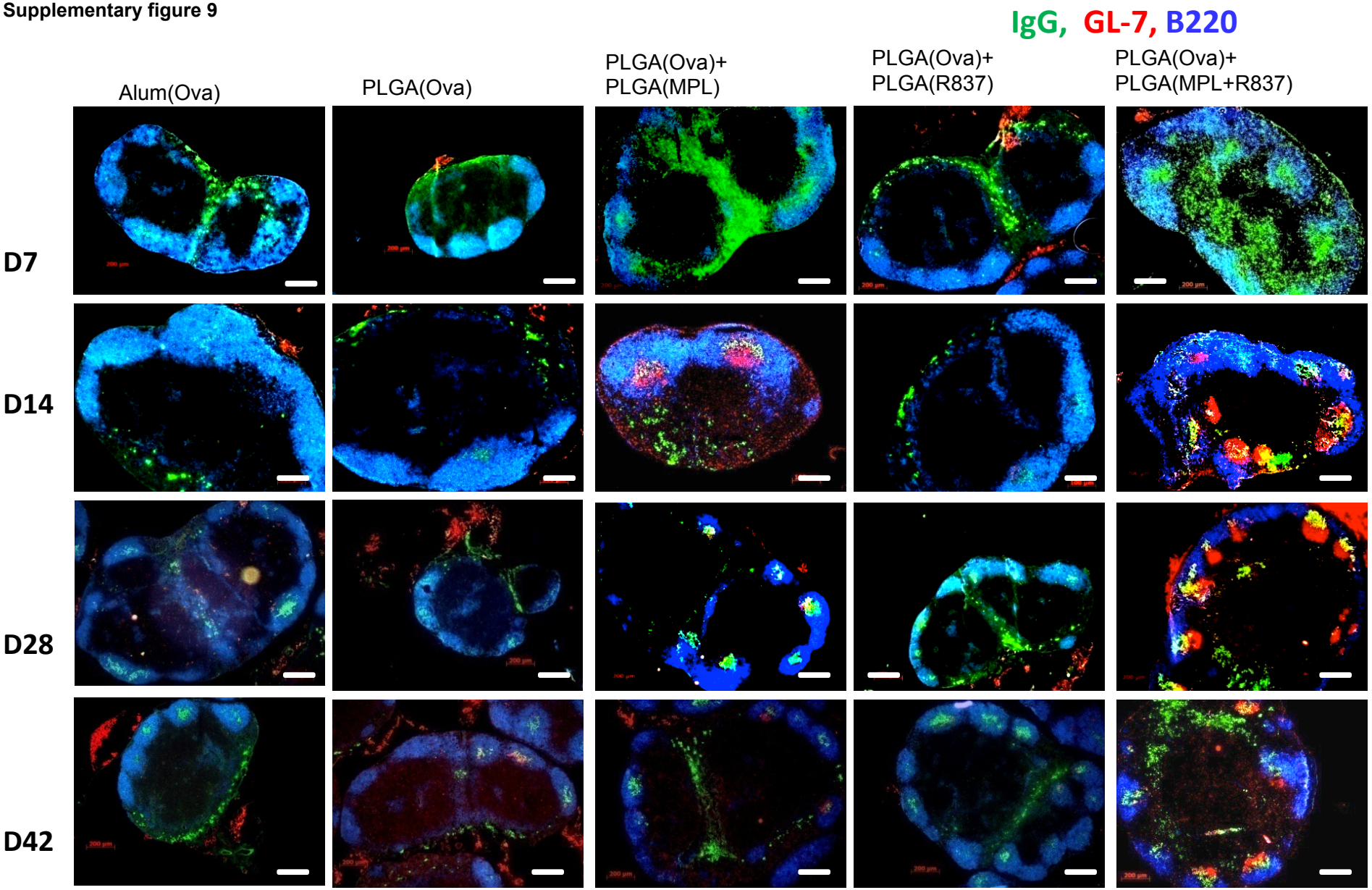


Supplementary figure 8



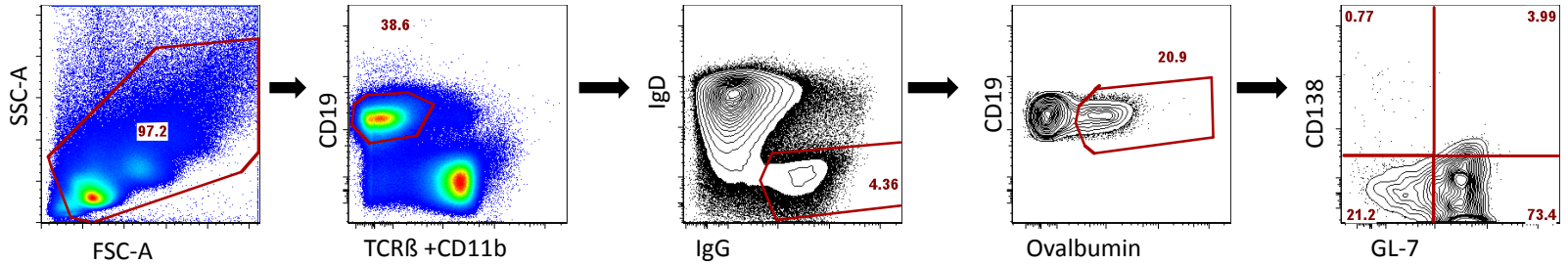


Supplementary figure 9

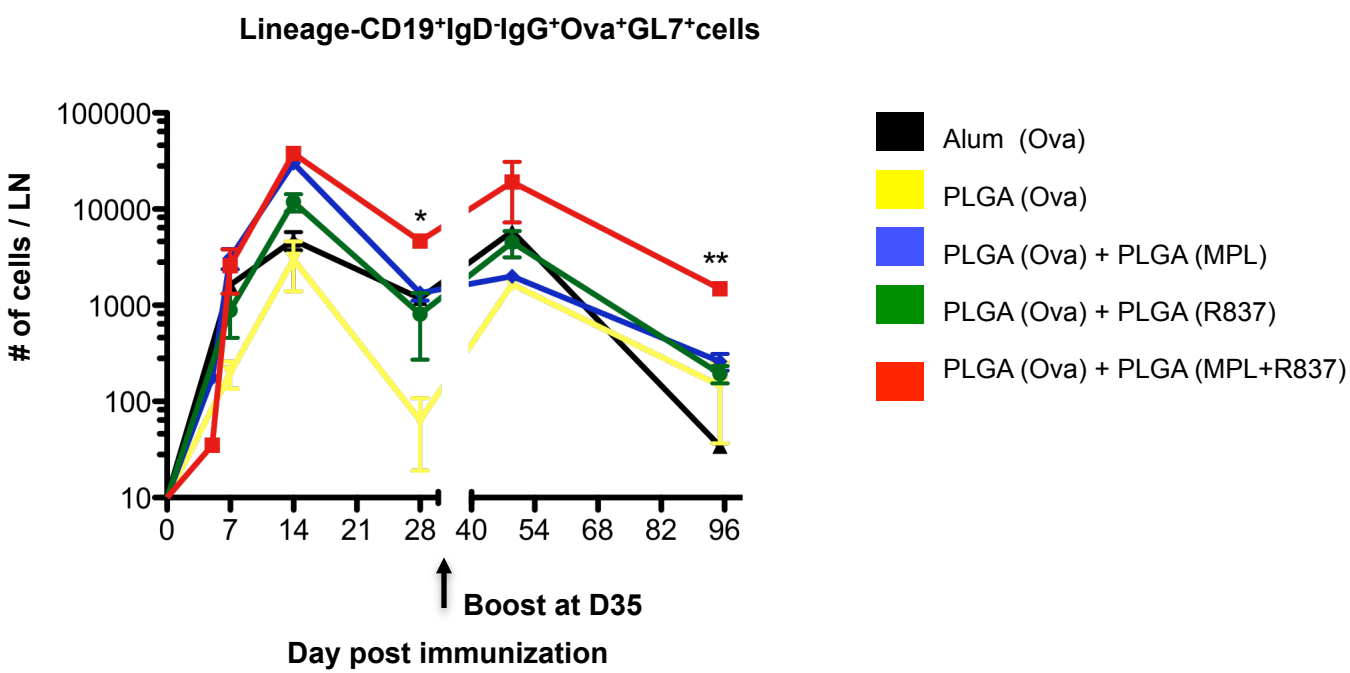


Supplementary figure 10

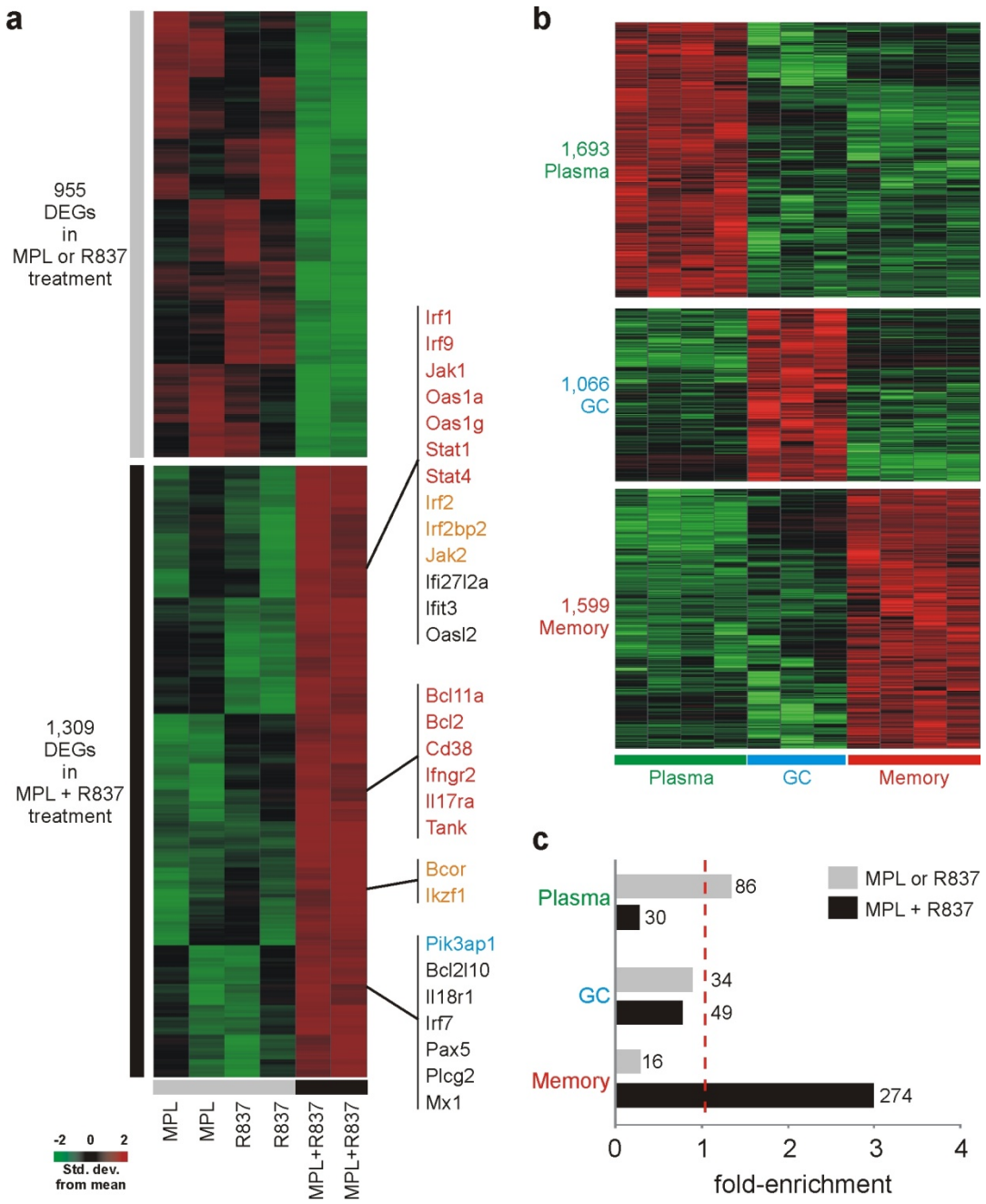
a)



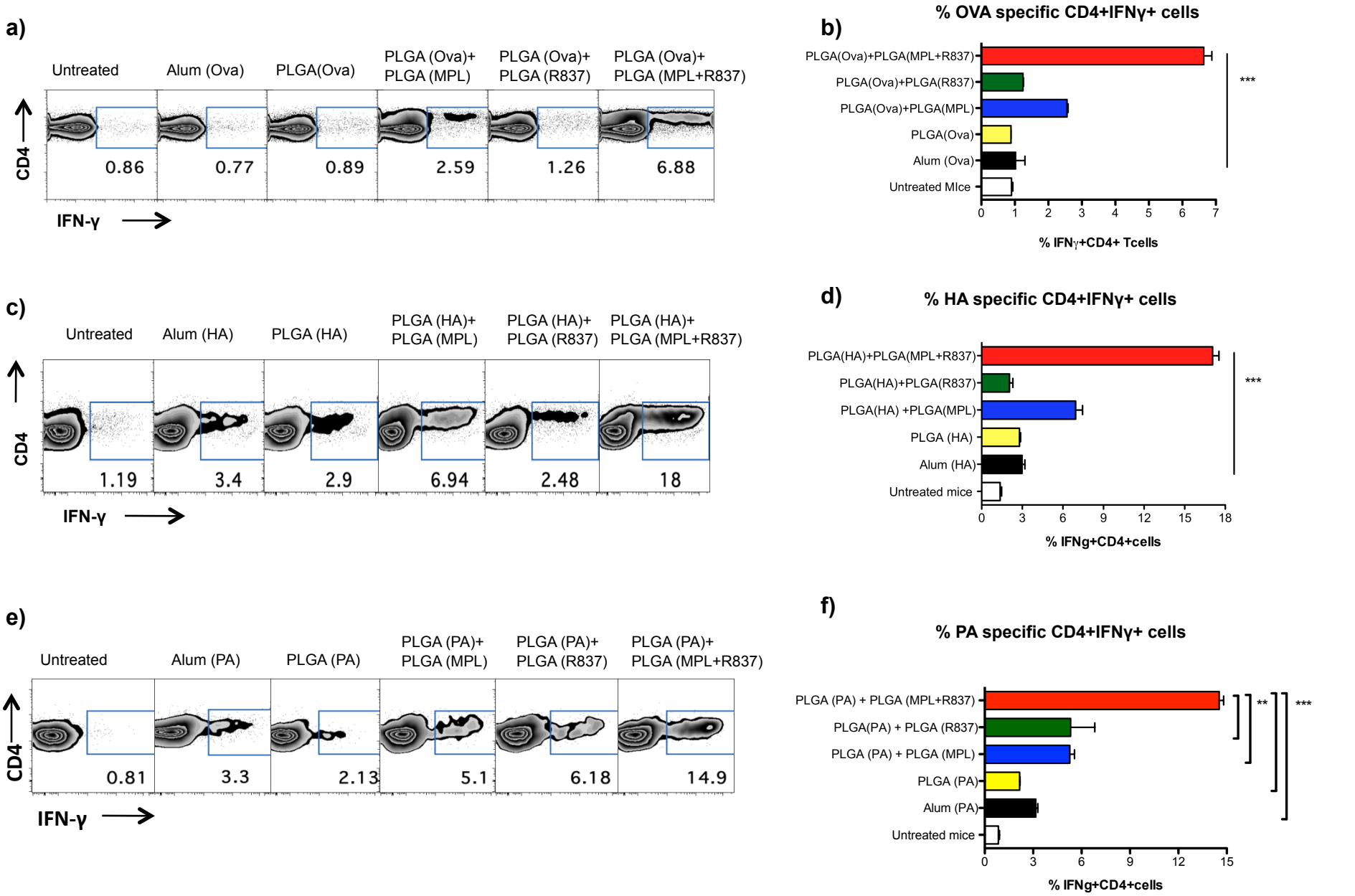
b)



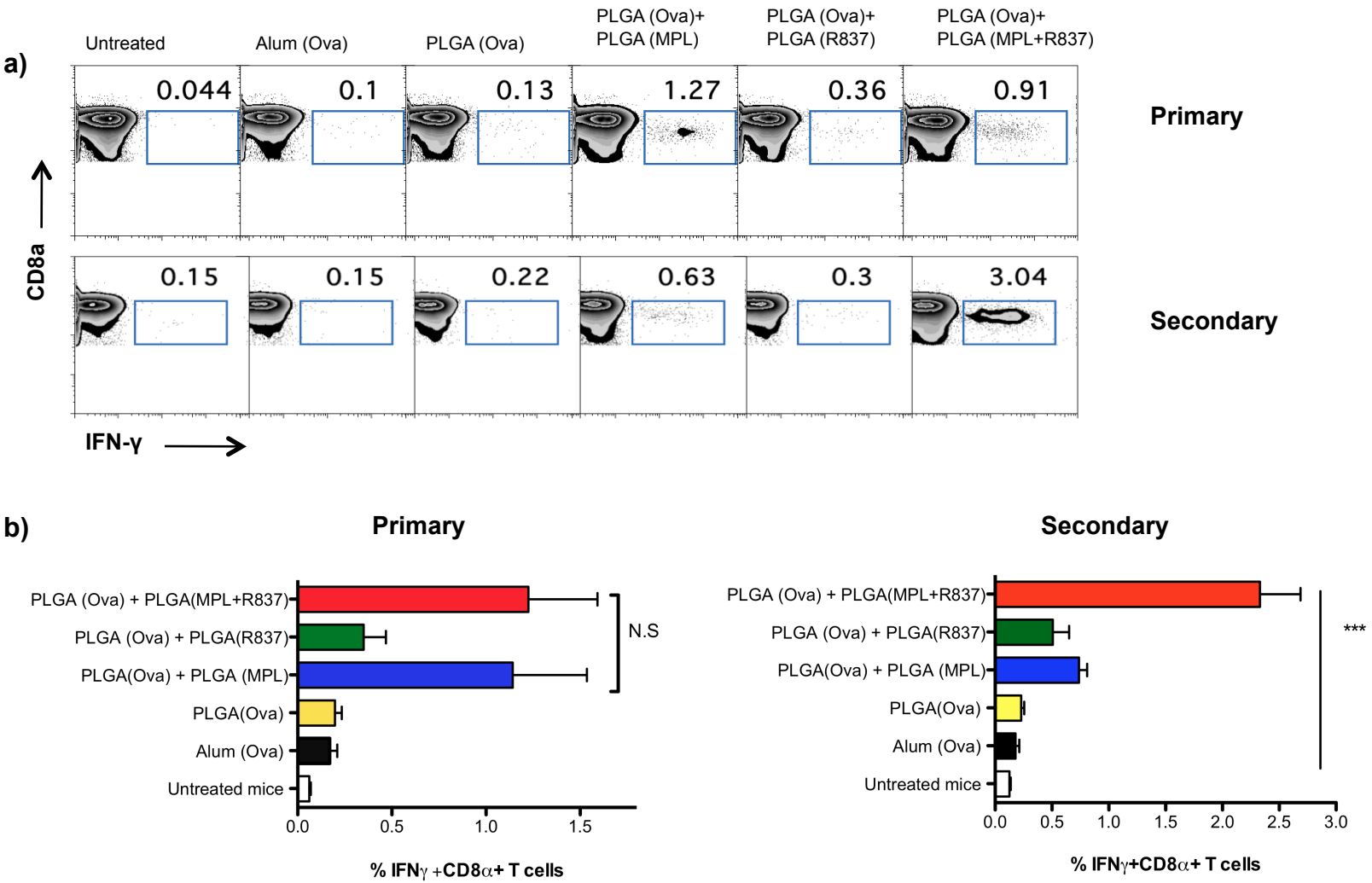
Supplementary figure 11



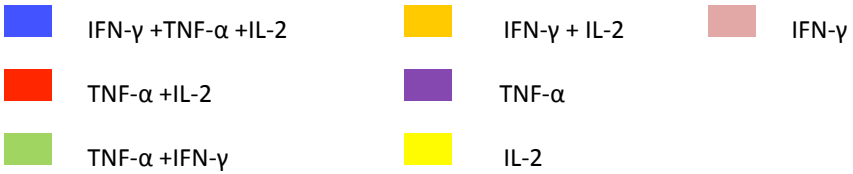
Supplementary figure 12



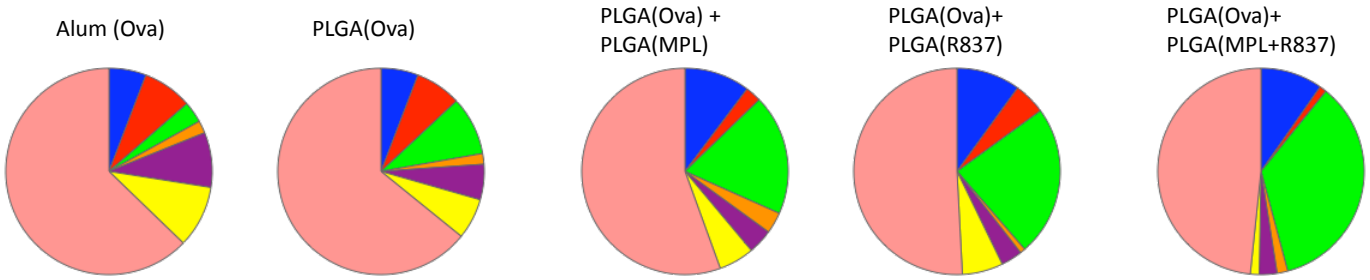
Supplementary figure 13



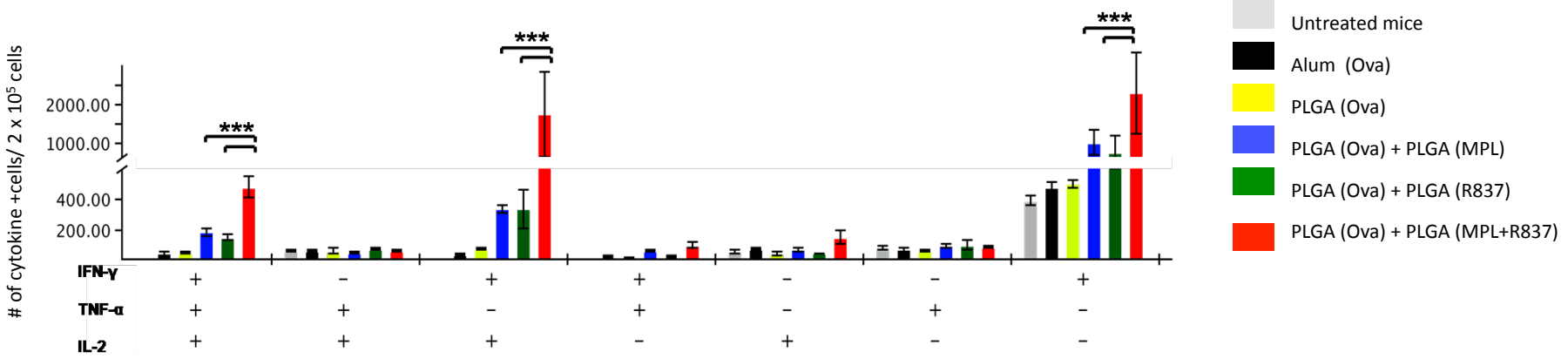
Supplementary figure 14



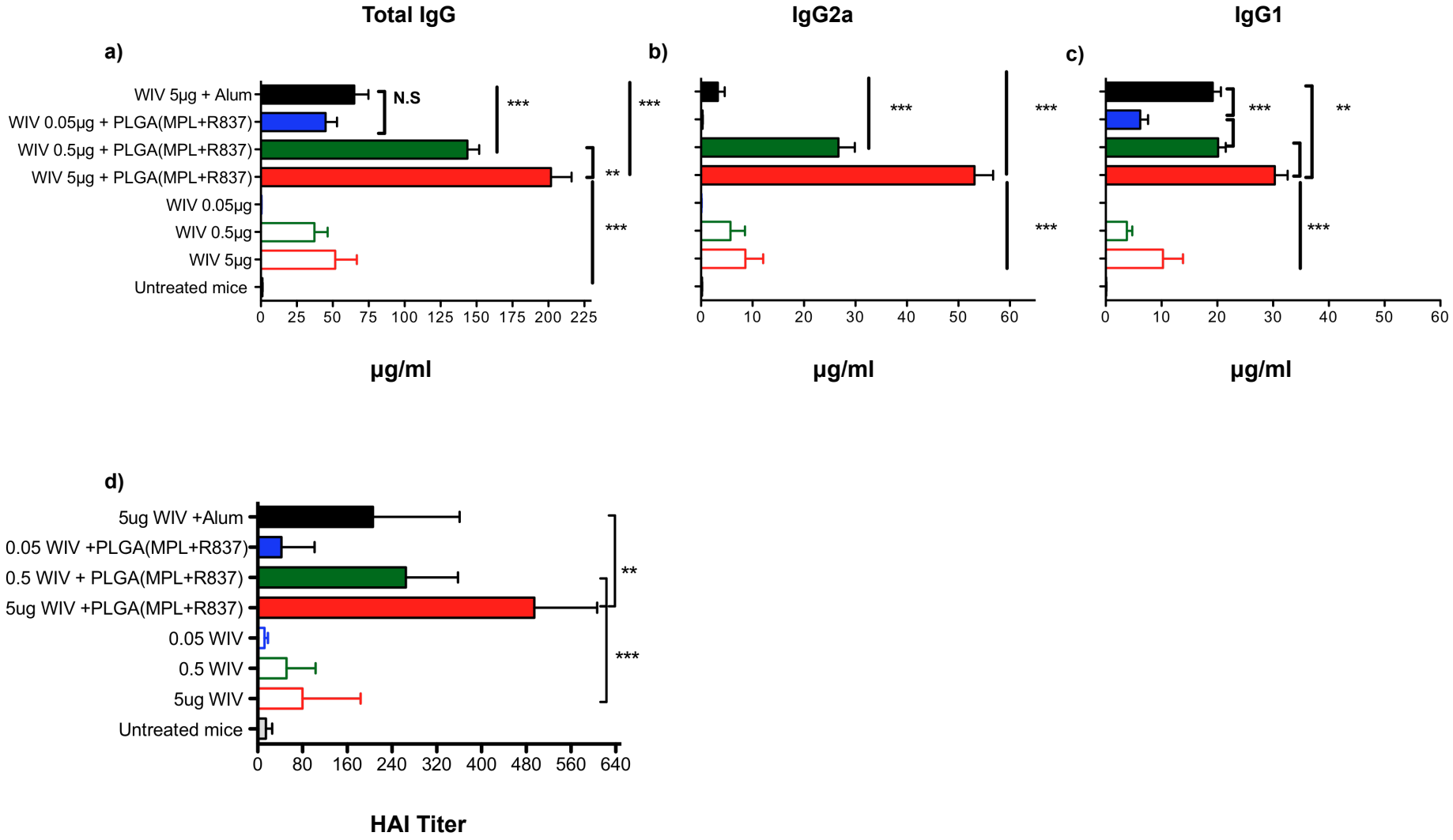
a)



b)



Supplementary figure 15

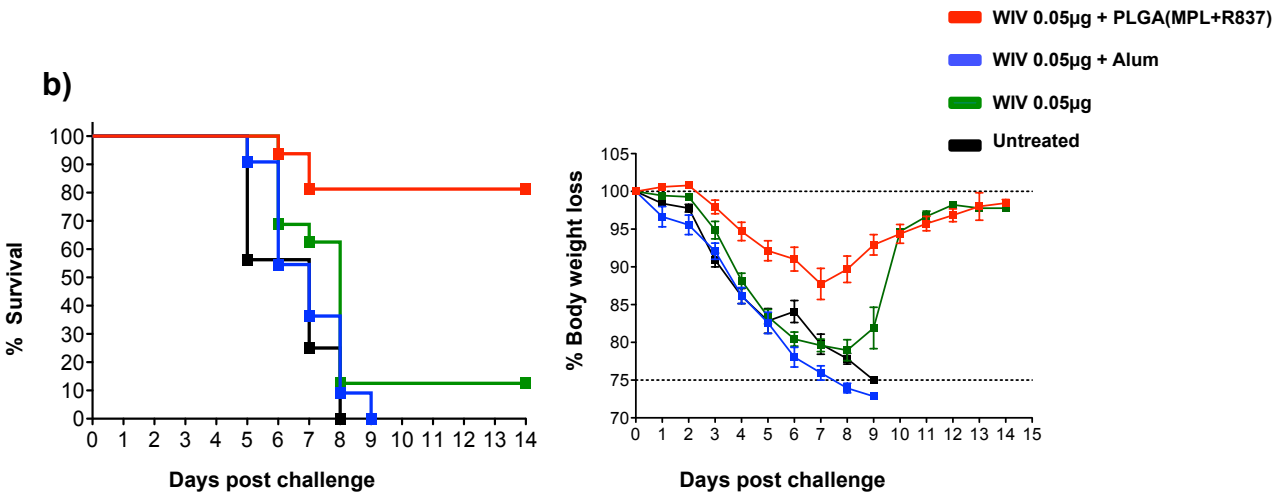


Supplementary figure 16

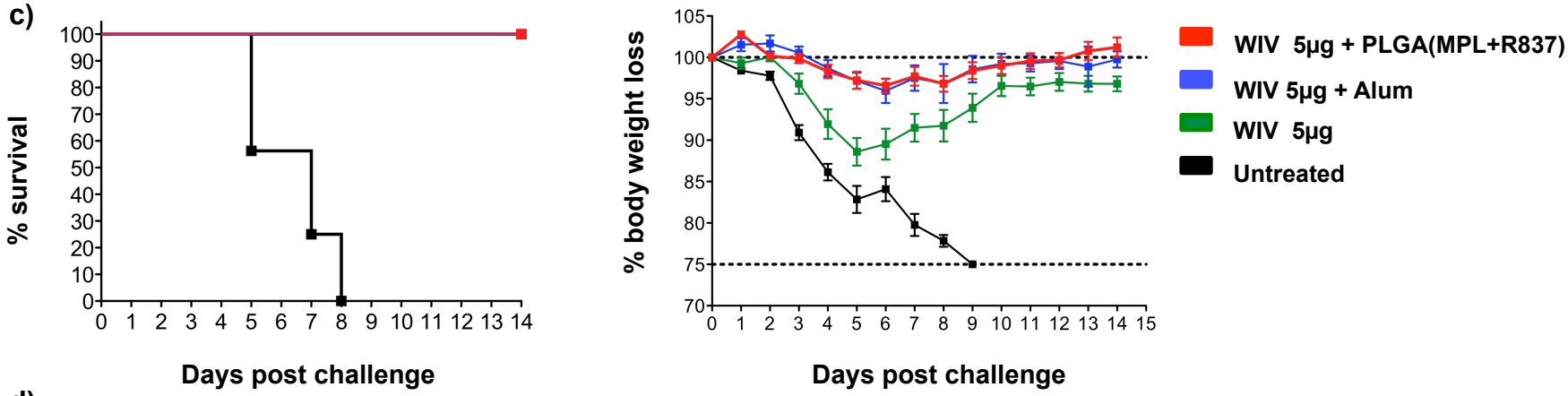
a)

Experimental group	5µg WIV	0.5µg WIV	0.05µg WIV
Untreated mice 0/16 **(0%)**	----	-----	----
No adjuvant	11/11 **(100%)**	8/11 **(72.7%)**	2/16 **(12.5%)**
PLGA(MPL+R837)	11/11 **(100%)**	11/11 **(100%)**	13/16 **(81.25%)**
Alum	11/11 **(100%)**	4/11 **(36.4%)**	0/11 **(0%)**

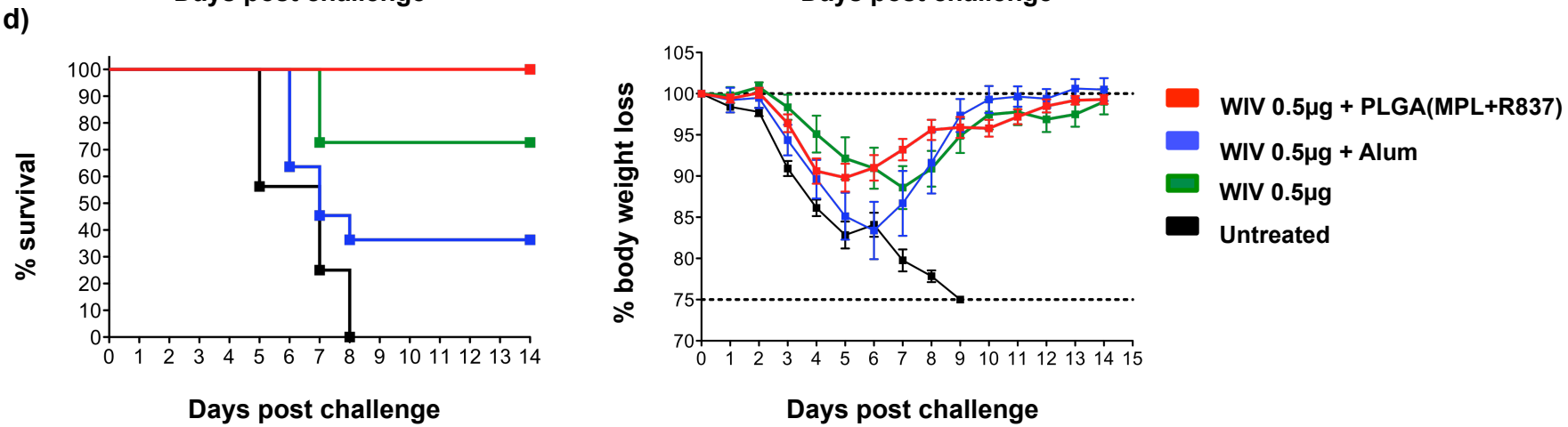
b)



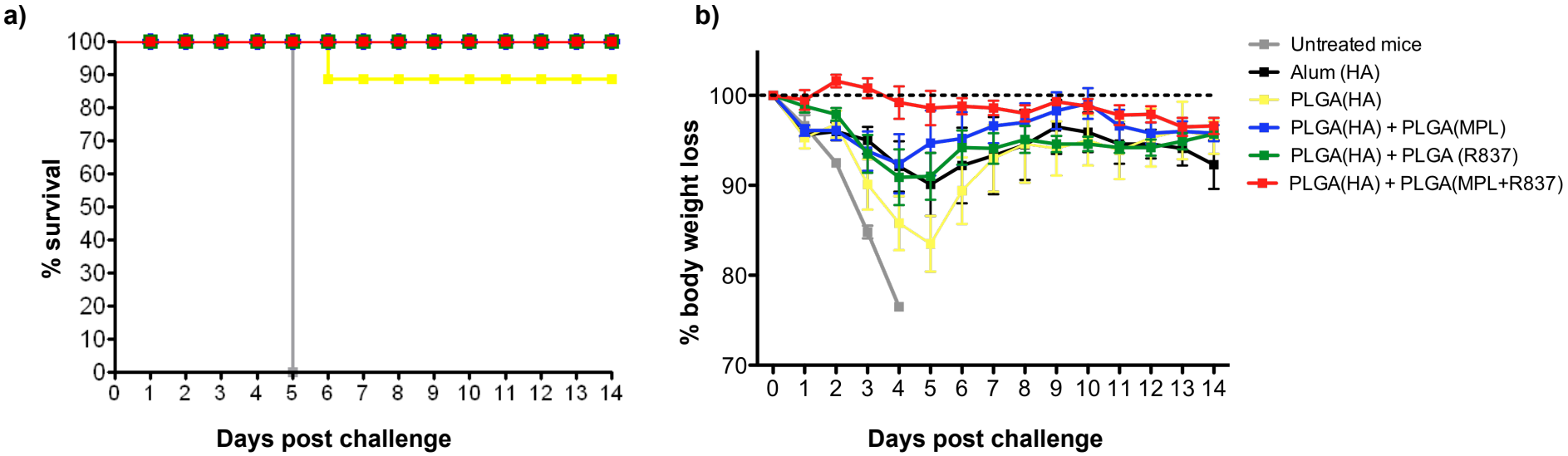
c)



d)



Supplementary figure 17



Supplementary figure 18

Experiment	Treatment with individual MPL or R837	Treatment with combination of MPL and R837	Reference Figure
Persistence of germinal centers	+/-	+++	Fig 3. a,b
Persistence of antibody secreting cells	+/-	+++	Fig 3.c
Persistence of antigen specific B cells	+/-	+++	Suppl Fig. 10
Enrichment of genes in activated B cells towards memory	+/-	+++	Suppl Fig. 11
Memory CD4+ T cell responses	+/-	+++	Suppl Fig. 12
Memory CD8+ T cell responses	+/-	+++	Suppl Fig. 13