Supplementary Data.

Butyrate directly decreases human gut lamina propria T helper cell activation and cytokine production through histone deacetylase (HDAC) inhibition and GPR43 signaling.

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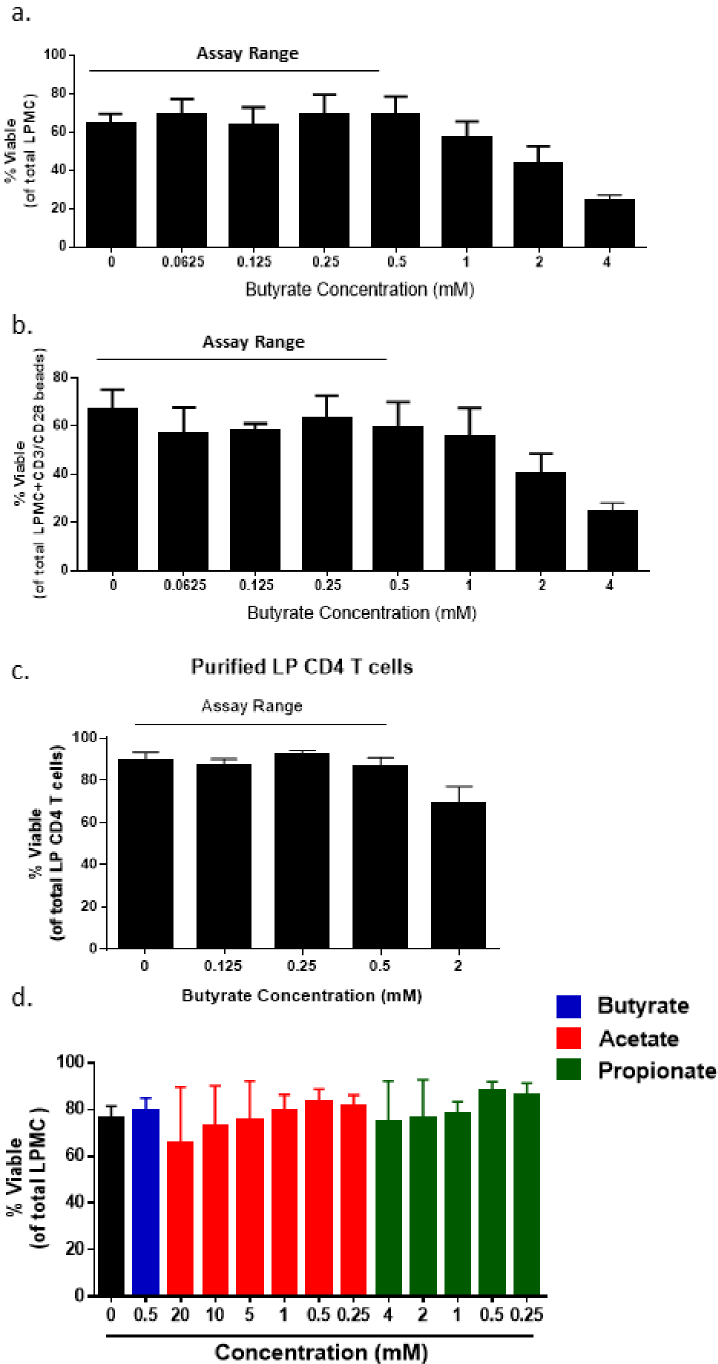
Supplementary Data.



Supplemental Figure 1. Viability after exposure to SCFA a.) LPMC were exposed to butyrate (0.0625mM - 4mM) or b.) butyrate (0.0625mM - 4mM) + CD3/CD28 beads for 4 days and total LPMC viability was determined by Trypan Blue and enumerated on a TC-120 cell counter (n=3). The assay range used in all subsequent assays is highlighted as 0-0.5mM. c.) Purified LP CD4 T cells were exposed to butyrate (0.0625mM - 2mM) for 4 days and total LPMC viability was determined by Trypan Blue and enumerated on a TC-120 cell counter (n=3). The assay range used in all subsequent assays is highlighted as 0-0.5mM. d.) LPMC were exposed to butyrate, acetate, or propionate for 4 days and total LPMC viability was determined by Trypan Blue and enumerated on a TC-120 cell counter (n=2-3).

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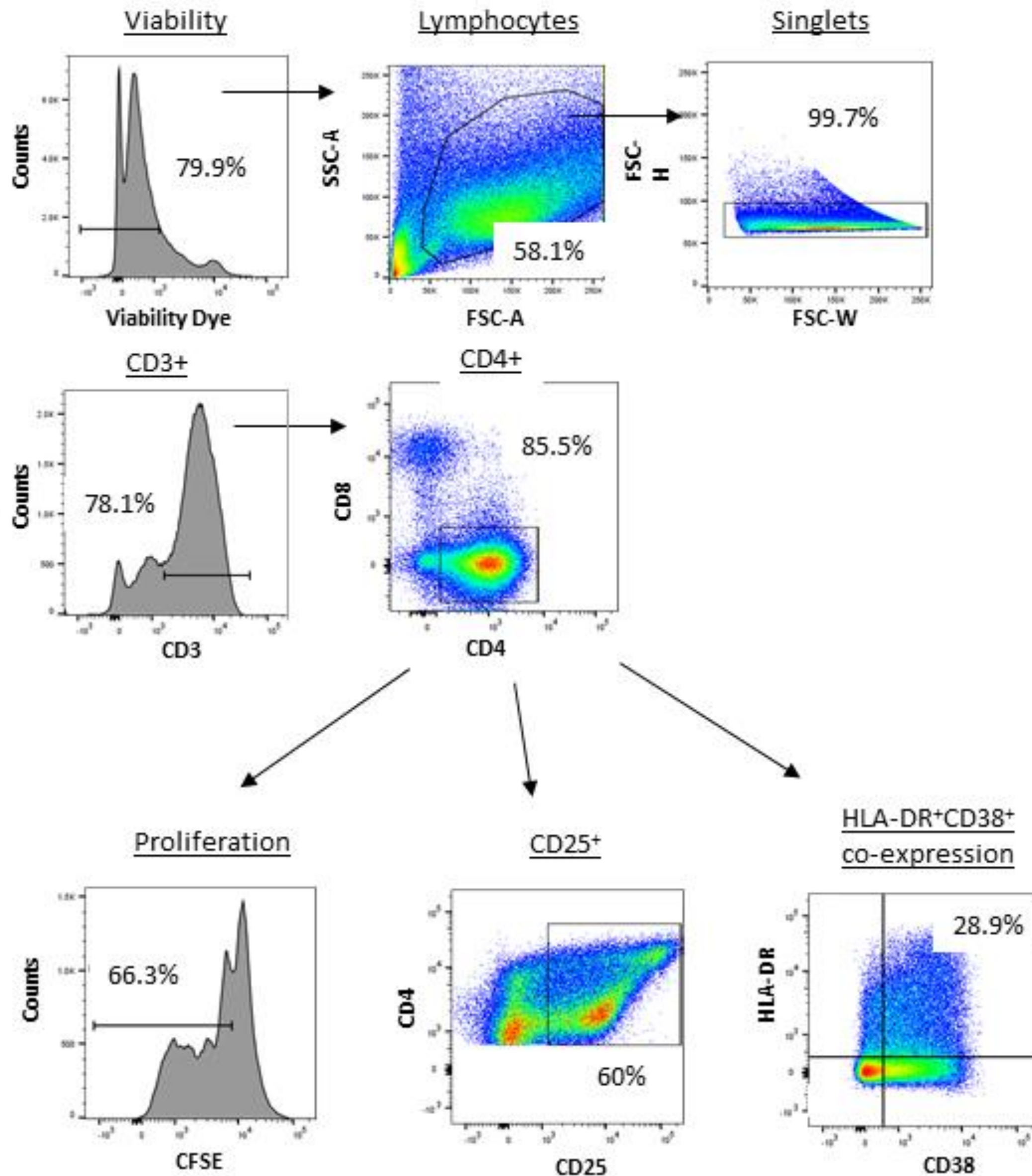

Supplemental Figure 2. Flow gating scheme for TCR stimulated LP CD4 T cell activation and proliferation studies.

CFSE-labelled LPMC were stimulated with TCR-stimulatory beads or left untreated for 4 days. LPMC were collected and LP CD4 T cell proliferation and levels of activation determined using multi-color flow cytometry. Live cells were gated using a viability exclusion dye (viability dye^{-}) with lymphocytes identified based on size (Forward Scatter Area; FSC-A) and granularity (Side Scatter Area (SSC-A) and doublets excluded (Forward Scatter Width [FSC-W] versus Forward Scatter Height [FSC-H]). CD4 T cells were identified as CD4^{+}CD8^{-} within total CD3^{+} T cells. Proliferation (CFSE^{dim}) was gated based on corresponding conditions without exogenous stimulation. Activation (CD25^{+}, HLA-DR^{+}CD38^{+}) were gated using FMO (CD25, HLA-DR) and isotype (CD38) controls.

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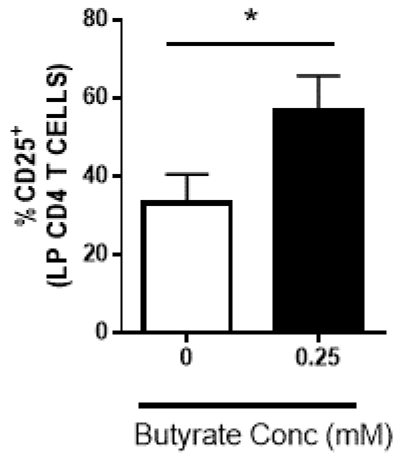

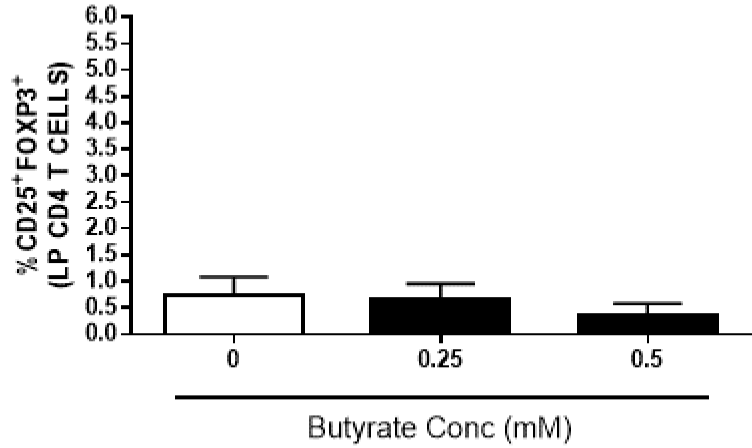

Supplemental Figure 3. Examination of FoxP3+ CD25+ LP CD4 T cells after the addition of butyrate. LPMC (N=3) were cultured with butyrate for 4 days and frequency of CD4 T cells a.) expressing CD25 and b.) co-expressing FoxP3 and CD25 were determined by multicolor flow cytometry. CD4+ CD8- cells expressing CD25 or CD25 and Foxp3 were identified in viable, single T cells. Isotypes (background) values were removed from final reported data. Bars represent mean+SEM values. Statistical significance was determined using a.) paired t-tests *P<0.05.

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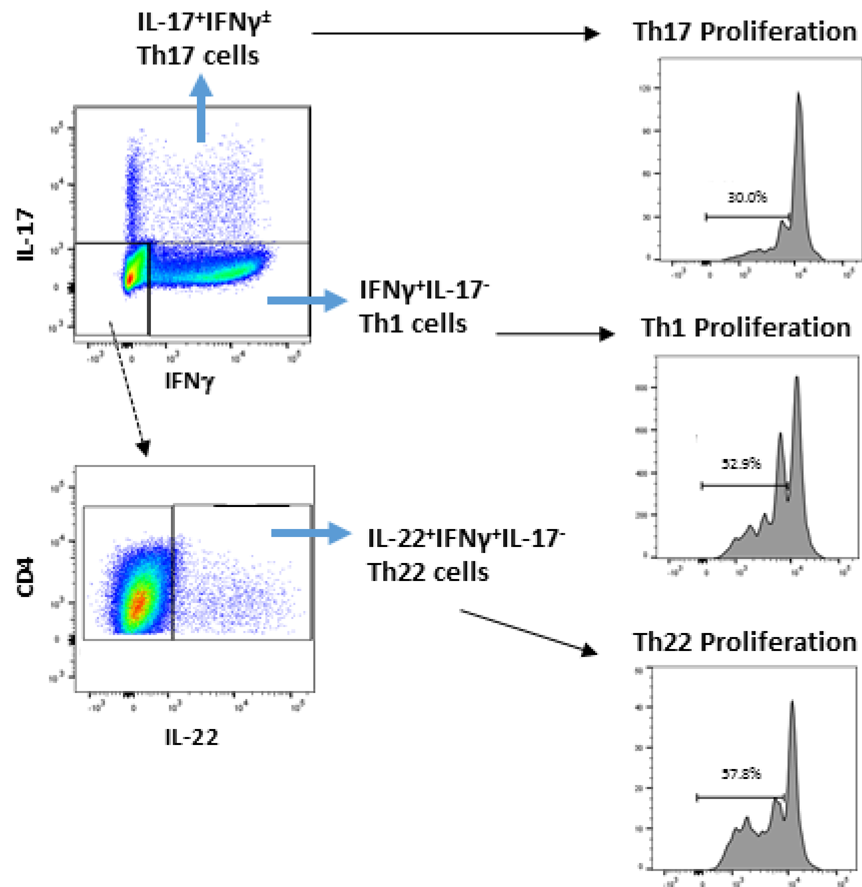

Supplemental Figure 4. Gating scheme to determine percentages of proliferating Th cell subsets. CFSE-labelled LPMC were stimulated with TCR-stimulatory beads or left untreated for 4 days. In the final 4hrs, all cultures were stimulated with PMA and ionomycin. LPMC were collected and measurement of proliferating Th cells determined using multi-color flow cytometry. Flow gating scheme for the identification of proliferating T helper (Th) subsets in LPMC stimulated with TCR beads. LP CD4 T cells were identified within viable, $CD3^{+}$ single lymphocytes and levels of proliferation ($CFSE^{dim}$) of Th17 ($IL\text{-}17^{+}IFN\gamma^{\pm}$), Th1 ($IFN\gamma^{+}IL\text{-}17^{-}$),Th22 ($IL\text{-}22^{+}IL\text{-}17^{-}IFN\gamma^{-}$) determined.

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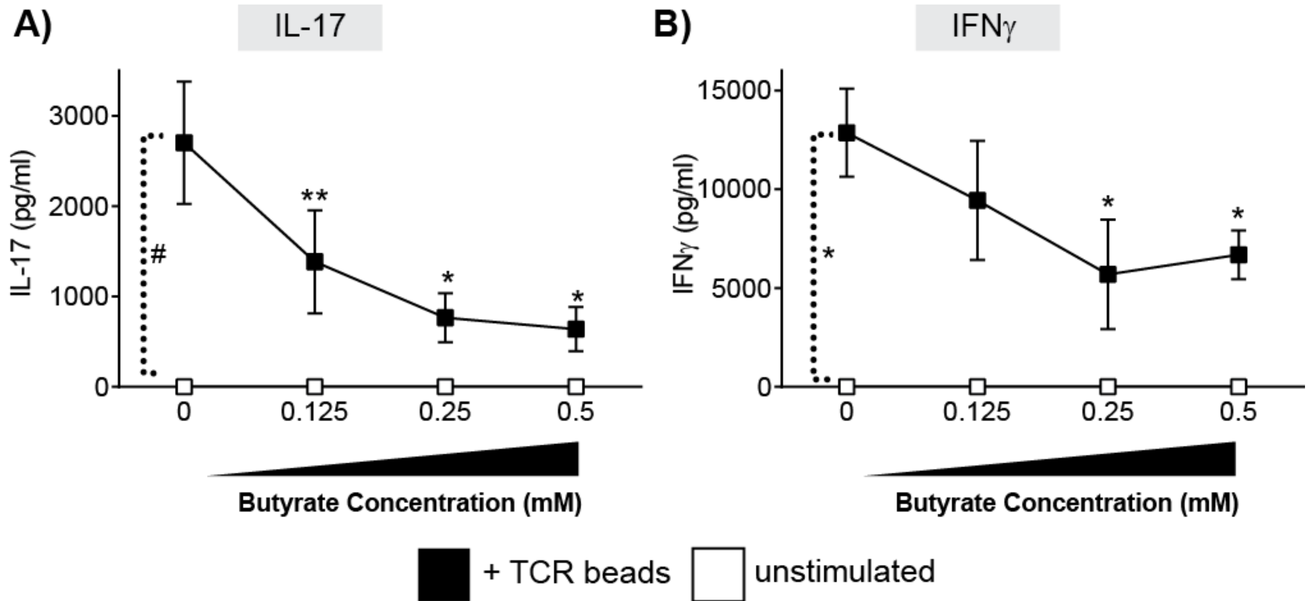

Supplemental Figure 5. Butyrate directly decreases LP CD4 T cell IL-17 and IFNγ production in a dose dependent manner. Purified LP CD4 T cells (N=3) were cultured with or without TCR-activating beads (TCR beads) and exogenous butyrate (0.125-0.5mM) for four days and levels secreted (**A**) IL-17 and (**B**) IFNγ in culture supernatants determined using cytokine ELISAs. Values are shown as mean±SEM. Statistical analysis: Paired t tests were conducted to determine differences in proliferation or activation between no stimulated and TCR-stimulated conditions and between butyrate concentrations versus no butyrate conditions. *P<0.05. $^{\#}$P<0.06.

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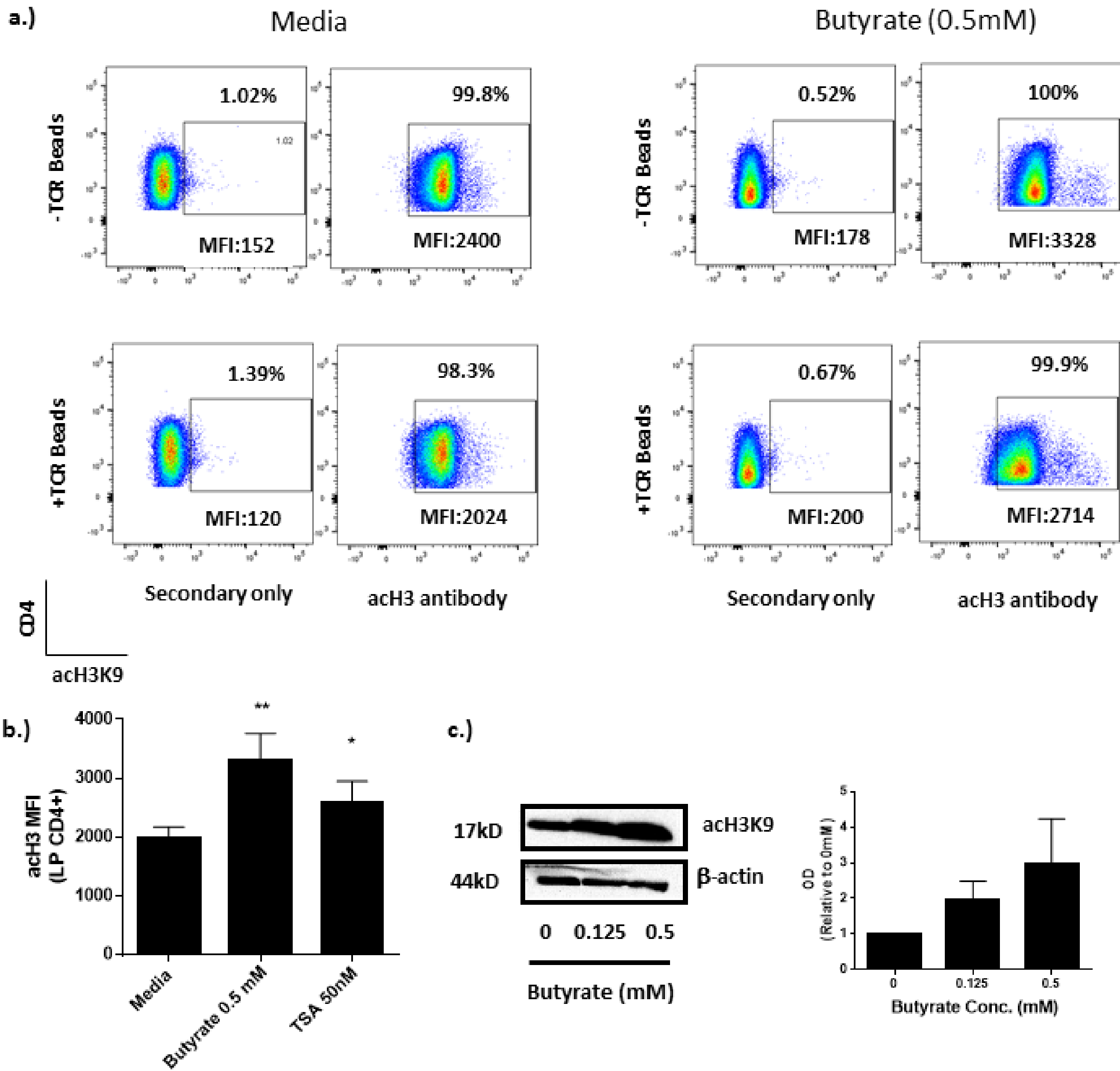

Supplemental Figure 6. Butyrate increases LP CD4 T cell histone acetylation levels (acH3K9) in the setting and absence of T cell activation.

A) Example flow plots for LPMC treated with butyrate 0.5mM or without butyrate (media; 0mM) in the presence or absence of TCR bead stimulation (24hrs). LP CD4 T cells were identified within viable CD3+ single lymphocytes and acH3 expression was determined based on control staining using secondary only antibody. B.) The ability of butyrate (0.5 mM) to increase hyperacetylation of histone H3 in LP CD4 T cells was compared to TSA at 50nM (N=6). Values are shown as Mean Fluorescence Intensity (MFI) of acH3 expression with secondary only values removed. (C) Purified LP CD4 T cells (N=3) were exposed to exogenous butyrate (0.125 and 0.5mM) for 24 hours and H3K9 levels were evaluated by western blot. OD values were determined using Image J software comparing acH3 and b-actin expression in each sample and relative OD values are displayed in the bar graph. Bars represent mean+SEM values. Statistical significance was determined using paired t-tests comparing the addition of butyrate or TSA to 0mM butyrate. *P<0.05, **P<0.01.

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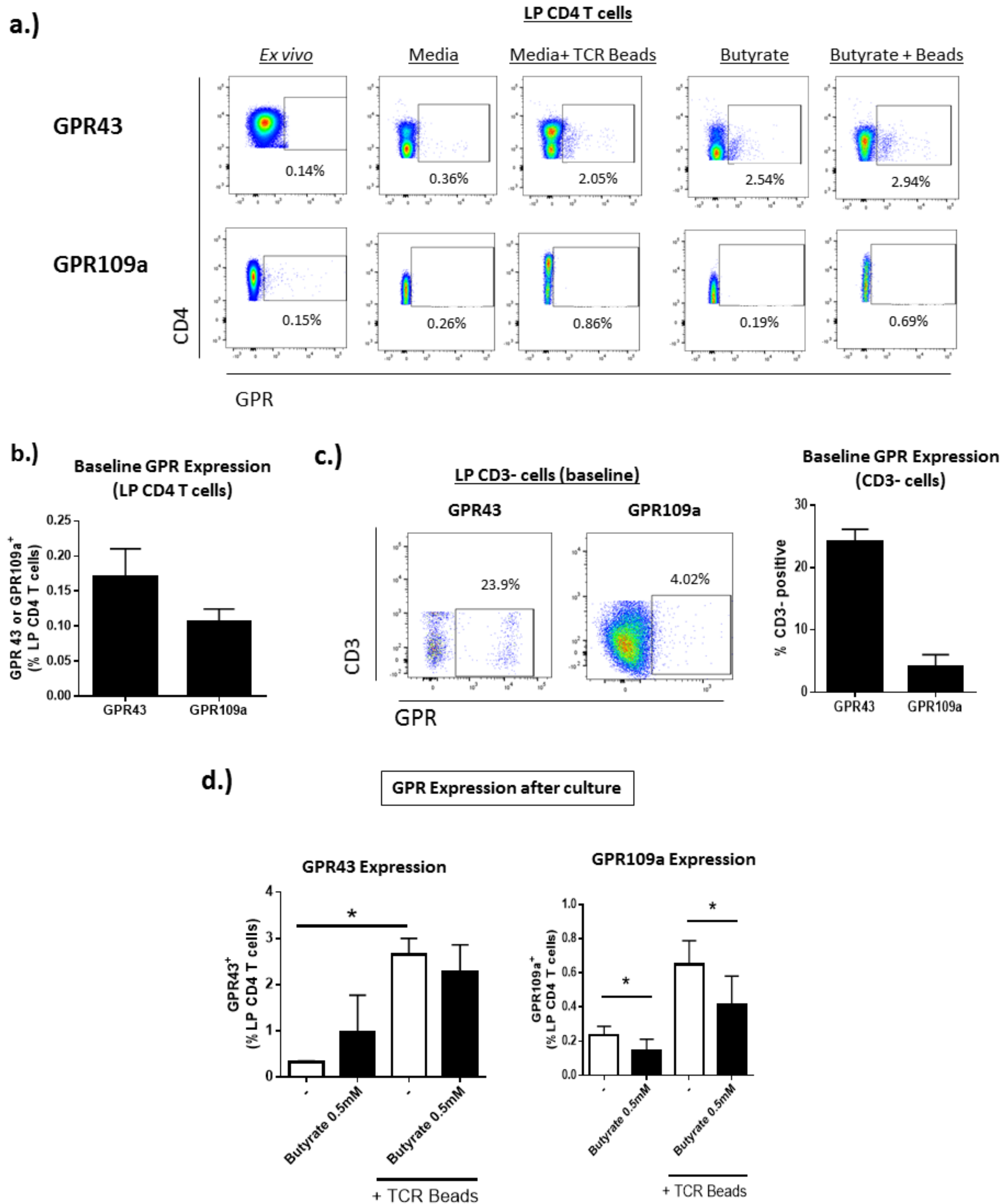

Supplemental Figure 7. LP CD4 T cells express low levels of the butyrate receptors GPR43 and GPR109a. Expression of GPR43 and GRP109a were determined at baseline (*ex vivo*) and after 4 days of *in vitro* culture with or without TCR-stimulatory beads in the presence of absence of butyrate (0.5mM) using multi-color flow cytometry. A) Example flow stain profiles for GPR43 and GPR109a on LP CD4 T cells *ex vivo* and after *in vitro* culture. CD4 T cells were identified within viable, CD3+ single lymphocytes. Isotypes were used for staining controls. B) Percentages of LP CD4 T cells expressing GPR43 or GPR19a ex vivo (N=3) with isotype values removed. C) Example flow stain profiles for GPR43 and GPR109a expressed by $CD3^-$ cells *ex vivo* and summary data for the percentages of $CD3^-$ cells expressing GPR43 or GPR109a (N=3) with isotype control values subtracted. D) Percentages of LP CD4 T cells expressing GRP43 or GPR109a following stimulation with or without TCR-stimulatory beads in the presence or absence of butyrate with isotype control values subtracted. Bars represent mean+SEM values. Statistical significance was determined using paired t-tests. *P<0.05.

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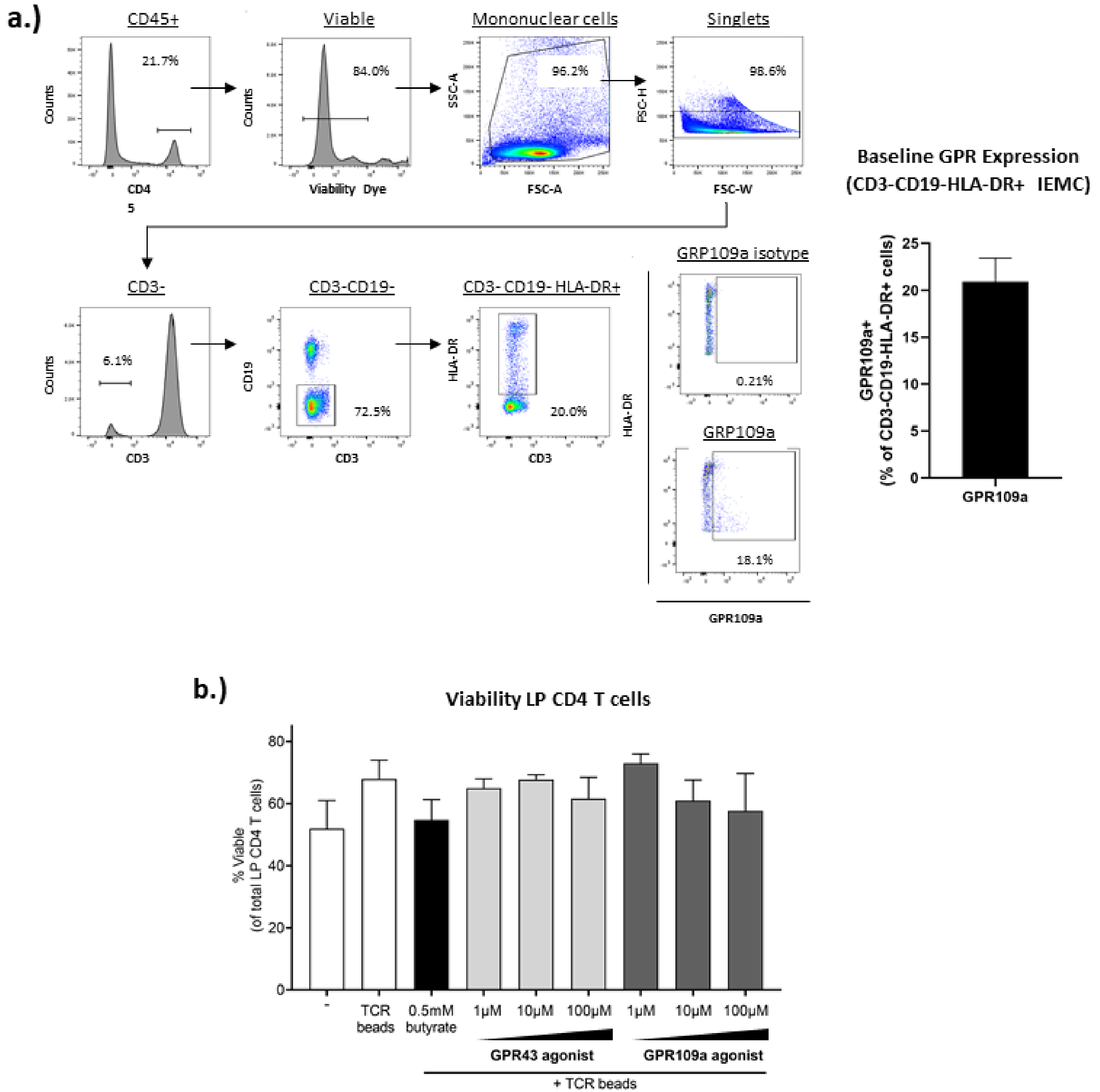

Supplemental Figure 8. GPR109a expression, viability of GPR43 and GPR109a agonists and confirmation of functional impact of GPR agonists. A) Expression of GRP109a on myeloid cells located within the epithelial layer of jejunal tissue was determined at baseline (*ex vivo*) using multi-color flow cytometry. Representative flow stain profiles for GPR109a on myeloid cells within total mononuclear cells (CD45+) of the epithelial layer. Live cells were gated using a viability exclusion dye (viability dye^{-}) with total mononuclear cells identified based on size (Forward Scatter Area; FSC-A) and granularity (Side Scatter Area (SSC-A) and doublets excluded (Forward Scatter Width (FSC-W versus Forward Scatter Height, FSC-H). Myeloid cells were identified as CD3-CD19-HLA-DR+ cells within CD45+ viable, single mononuclear cells. GPR109a-specific staining was established using an isotype control. Graph illustrates percentages of myeloid cells expressing GPR109a *ex vivo* (N=3) with isotype values removed (net). B) CFSE-labeled purified LP CD4 T cells were exposed to TCR beads in the presence of butyrate (0.5mM) or varying doses (1μm, 10μm, 100μm) of GPR agonists for 4 days and viability determined by Trypan Blue on a TC-120 cell counter (n=4 except GPR agonists at 1μm and 100μm where n=3).