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Gut Microbial Metabolites Fuel Host Antibody Responses

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SUMMARY

Antibody production is a metabolically demanding process that is regulated by gut microbiota, but the microbial products supporting B cell responses remain incompletely identified. We report that short-chain fatty acids (SCFAs), produced by gut microbiota as fermentation products of dietary fiber, support host antibody responses. In B cells, SCFAs increase acetyl-CoA and regulate metabolic sensors to increase oxidative phosphorylation, glycolysis and fatty acid synthesis, which produce energy and building blocks supporting antibody production. In parallel, SCFAs control gene expression to express molecules necessary for plasma B cell differentiation. Mice with low SCFA production due to reduced dietary fiber consumption or microbial insufficiency are defective in homeostatic and pathogen-specific antibody responses, resulting in greater pathogen susceptibility. However, SCFA or dietary fiber intake restores this immune deficiency. This B cell-helping function of SCFAs is detected from the intestines to systemic tissues and conserved among mouse and human B cells, highlighting its importance.

Abstract

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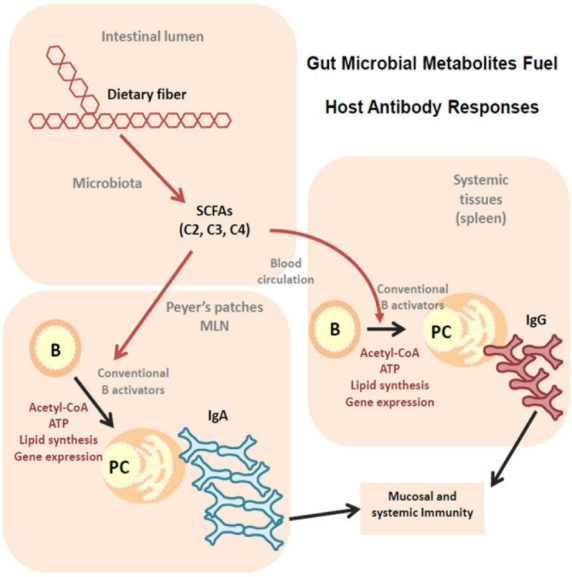
AUTHOR CONTRIBUTIONS MK and YQ performed experiments and analyzed data. JP performed the commensal bacterial analysis and glucose uptake assay for T cells. YQ originally carried out in vitro experiments to determine SCFA effects on B cells, and MK finished the study in vitro and in vivo and prepared most of the figures. CK conceived the study and obtained funding, and analyzed data and drafted the manuscript with MK.



Keywords

Short chain fatty acids; antibody; B cells; metabolism

INTRODUCTION

Host and gut microbial flora functionally interact in the gut for mutual benefits (Brown et al., 2013; Tremaroli and Backhed, 2012). A mounting body of evidence indicates that gut microbiota profoundly influences the host immune system (Alexander et al., 2014; Belkaid and Hand, 2014). For example, germ-free mice are variably impaired in epithelial integrity, gut-associated lymphoid tissue development, innate and adaptive immunity, and immune tolerance (Round and Mazmanian, 2009). One of the defective components of the adaptive immunity in GF mice is mucosal IgA production (Moreau et al., 1978). The gut microbiota also supports systemic antibody responses (Zeng et al., 2016).

Numerous microbial products, including proteins, polysaccharides, and molecules that activate pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and NOD-like receptors (NLR), stimulate the mucosal immune system (Abreu, 2010). This activation induces the production of cytokines, retinoic acid (RA) and other B cell-activating molecules by various cell types (Gutzeit et al., 2014; Kim, 2013; Mora et al., 2006). It is generally accepted that TLR ligands support antibody responses (Buchta and Bishop, 2014; Pone et al., 2012; Zeng et al., 2016). The gut microbiota produces additional factors that potentially support antibody responses. In this regard, the roles of microbial metabolites in regulation of host antibody responses remain unclear.

There is a positive correlation between dietary fiber (DF) intake and intestinal IgA levels (Kudoh et al., 1998; Lim et al., 1997). This implies that either DF or their metabolites have the potential to support B cell responses in the gut. Short chain fatty acid (SCFAs) are the major microbial metabolites of DF (Macfarlane and Macfarlane, 2003). The most

predominant SCFAs in the gut are acetate (C2), propionate (C3), and butyrate (C4) (Cummings et al., 1987). SCFAs support epithelial cell integrity, innate immune functions, and the generation of regulatory and effector T cells (Arpaia et al., 2013; Furusawa et al., 2013; Kim et al., 2013; Park et al., 2015; Sakata, 1987; Schauber et al., 2003). The total luminal SCFA concentration in humans reaches ~130 mmol/Kg in the colon and ~15 mmol/Kg in the small intestine (SI) (Cummings et al., 1987). SCFAs exert their functions through several different mechanisms, including activation of cell surface receptors (GPR41, GPR43 and GPR109A), histone deacetylase (HDAC) inhibition, and metabolic integration (Ganapathy et al., 2013; Kim et al., 2014; Licciardi et al., 2011). The role of SCFAs in regulation of antibody responses has not been established to date.

We found that SCFAs function as commensal-derived stimulators of host antibody responses. Mice that are low in SCFA production due to low DF consumption or microbial insufficiency are defective in homeostatic and pathogen-specific antibody responses but SCFA or DF intake restores the immune deficiency. SCFAs accelerate cellular metabolism and regulate gene expression to promote B cell differentiation into antibody-producing cells. The results shed light on the important roles of microbial metabolites in regulating host antibody responses.

RESULTS

DF and SCFAs Increase Both Intestinal IgA and Systemic IgG Responses

To determine the impact of SCFA-producing DF on antibody responses, we fed mice with special diets containing high (HFD, 15%), medium (MFD, 5%) and low (LFD, 0%) levels of mixed DF (pectin and inulin), which created different levels of SCFAs in the gut lumen and blood circulation (Figure S1A). The frequencies of IgA^+ B cells in the small and large intestines were significantly decreased in LFD- but increased in HFD-fed mice compared to MFD-fed mice (Figure 1A). We treated mice with a minimally effective dose of mixed antibiotics (meAbx), which effectively decreased gut microbiota and SCFA production but did not affect B cell survival and IgA expression in vitro (Figures S1B–E). The meAbx treatment eliminated the DF effect on intestinal IgA^+B220^- plasma cells (PCs, Figure 1A). Interestingly, the numbers of IgA^+ GC-B cells in the Peyer's patches (PPs) were also increased by DF, whereas numbers of IgM^+ cells were slightly decreased by DF (Figure 1B). Microbiota suppression by the meAbx treatment abolished the positive effect of DF on IgA^+ GC B cells in PPs. Similar increases in IgA^+ cell numbers were observed in the MLN and spleen by DF (Figure 1C), which indicates potentially systemic effects of DF and their metabolites in regulating B cell responses. Furthermore, the levels of secreted IgA in luminal (cecal) and serum levels of IgG were increased by DF (Figure 1D). IgA^+ $Blimp1^+$ PCs or IgA expression was increased by DF in intestinal tissues (Figures S1F, S1G). Consistently, the frequencies of IgA-coated bacteria were increased in the colon of DF-fed mice (Figure 1E).

DF can generate more than SCFAs and, therefore, the observed DF effect is not necessarily due to SCFAs only. To determine the effect of SCFAs on antibody responses, mice on LFD (or regular diet) were orally administered with C3 in drinking water. C3 administration significantly increased C3 levels in the small and large intestinal tissues and blood at levels

comparable to the C3 levels created by HFD (Figure S2A). It also increased the luminal concentration of C3 with insignificant increases of C2 and C4 (Figure S2B). C3 increased the numbers of IgA^+ B cells in the small and large intestines (Figure 2A). While the absolute levels were lower than those of non-meAbx treated mice, the positive effect of C3 on IgA expression was also observed when the gut microbiota was suppressed with meAbx (Figure 2A). C3 or a SCFA mixture increased the numbers of IgA-secreting lamina propria (LP) B cells in LFD-fed mice (Figure 2B). The impact on IgA^+ GC B cells in PP of regular chow-fed mice was negligible but it was clear when mice were treated with meAbx to suppress normal SCFA production (Figure 2C). Interestingly, the numbers of $CXCR5^+$ follicular T helper (Tfh) cells were increased as well upon C3 administration (Figures 2D, S2C). C3 and HFD enhanced GC formation, activation-induced cytidine deaminase (AID) expression, and the generation of IgA switch circles in PPs (Figure 2E), which are consistent with elevated GC and Ig class switch recombination (CSR) activity in inductive tissues.

We observed that administration of C3 or a SCFA mixture increased IgA expression or levels of secreted IgA in various compartments of the intestine as well as the levels of IgA and IgG in the blood circulation (Figures S2D–G). Moreover, the administration of C3 or a SCFA mixture increased the proportion of IgA-coated gut bacteria (Figure 2F). C3 and DF altered gut microbiota but their effects were not identical. Both DF and SCFAs decreased Firmicutes but were different in regulating other bacterial groups (Figure S2H). We performed mouse rotation through old cages every 2 days for 4–5 weeks to equilibrate gut microbiota, but the positive effect of DF on IgA^+ B cells was not affected by the cage rotation (not shown). Overall, the results indicate that SCFAs boost antibody responses in vivo.

SCFAs Directly Regulate B cells and Skew Gene Expression for Antibody Production

We, next, studied if SCFAs directly affect the differentiation of B cells into PCs in vitro. All of the major SCFAs, such C2, C3, and C4, enhanced the generation of IgA-expressing B cells (Figure 3A). In appropriate cytokine conditions, SCFAs also enhanced the differentiation of naïve B cells into B cells expressing Ig isotypes such as IgG1, IgG2a, IgG2b, and IgG3 (Figure 3B). The positive effect of SCFAs on B cells was also observed when B cells were activated with anti-CD40 (Figure S3A). This positive effect was not due to the change in Na^+ ion levels (Figure S3B). The expression of genes associated with PC differentiation, including the *Aicda*, *Xbp1*, *Irf*4, *Prdm1*, and *Sdc1* genes, was enhanced by SCFAs (Figure S3C). The generation of post-switch transcripts (PST) for the expression of IgG3, IgG1, IgG2b, IgG2a, and IgA was highly increased by SCFAs (Figure S3D). Thus, SCFAs can directly act on B cells undergoing activation to promote their differentiation into PCs that produce class-switched antibodies.

SCFAs are HDAC inhibitors and, therefore, have the potential to regulate gene expression and cell signaling through regulation of protein acetylation (Davie, 2003). SCFAs displayed dose-dependent short (2 h) and long-term (2 d) suppressive effects on HDAC activity in B cells in vitro (Figure 3C). Also, the overall HDAC activity in B cells was decreased in PP and intestinal LP of the mice treated with C3 or DF (Figure S3E). Trichostatin A (TSA), a class I/II HDAC inhibitor, partially mimicked the SCFA function in making IgA^+ plasma

cells, whereas histone acetyl transferase (HAT) inhibitors (garcinol and anacardic acid) suppressed the SCFA activity (Figure 3D). A chromatin immunoprecipitation study revealed increased histone acetylation in the regulatory region of the *Aicda* gene and IgG3, IgG1, and Iga class switch regions of the *Igh* gene in SCFA-treated B cells (Figure 3E). SCFAs, at the physiologically relevant doses used in this study according to the serum or tissue concentrations of SCFAs (Figure S2A) (Furusawa et al., 2013), did not affect cell death (Figure S3F). However at high concentrations (~1 mM), C4 induced cell death and decreased B cell proliferation and IgA expression (Figure S3G).

Because of the HDAC inhibitor activity of SCFAs, we performed a microarray transcriptome analysis for the B cells activated in the presence or absence of C2. We found that the expression of Ig (IgGs, IgA, Igj, Igk, and Igl) and other genes, including *Prdm1* (the Blimp-1 gene), *Aicda* (activation-induced cytidine deaminase), *Xbp-1* (X-box binding protein 1), *Ada* (adenosine deaminase), *CD69*, and *Irak3*, were up-regulated by C2 (Figures 3F and S4). These genes are linked to B cell differentiation and/or IgA and IgG production (Aldrich et al., 2003; Muramatsu et al., 2000; Turner et al., 1994). We also found that genes involved in B cell differentiation and function, such as Hepatitis A virus cellular receptor 1 (HAVcr-1)/T-cell immunoglobulin and mucin domain 1 (TIM-1), microphthalmia-associated transcription factor (*Mitf*), CD27, and LAPTM5 (Lin et al., 2004; Ouchida et al., 2010; Xiao et al., 2015; Xiao et al., 2004), were down-regulated by C2 (Figures 3F and S4). Thus, SCFAs change the expression of a number of genes related to B cell differentiation. Also decreased by C2 was the expression of some genes involved in phosphoprotein, acetylation and cellular macromolecule catabolic processes (Figure 3F).

Because SCFA receptors such as GPR41 and GPR43 mediate certain functions of SCFAs, we examined B cell responses in GPR41 or GPR43-deficient mice. We found no detectable difference in numbers of IgA^+ PCs between WT and the SCFA receptor-deficient mice (not shown). In this regard, B cells do not express *GPR41* or *GPR43* at significant levels (Kim et al., 2013).

SCFAs Increase Cellular acetyl-CoA Level, Mitochondrial Respiration, and Lipid Droplets in B cells

B cell differentiation into PCs and production of antibodies are metabolically demanding. It has been established that SCFAs are converted into acetyl-CoA by acetyl/propionyl/butyryl-CoA synthetases in cells and integrated into the mitochondrial Krebs cycle or fat synthesis (Bergman, 1990). We examined the effect of SCFAs on B cell metabolism. The levels of acetyl-CoA and mitochondrial mass in B cells were significantly increased after SCFA treatment (Figures 4A, 4B, S5A). Moreover, the expression of *Atp5g1* (a subunit of mitochondrial ATP synthase) and *Ucp3* (a mitochondrial uncoupling protein 3) genes was increased in SCFA-treated B cells (Figure S5B). The B cells cultured with SCFAs were generally bigger than control B cells and had higher Ki-67 expression compared to control B cells (Figure S5C). Moreover, SCFAs enhanced the mitochondrial ATP production and maximal respiration rates in B cells (Figure 4C). Acetyl-CoA is used in the Krebs cycle to produce ATP (Bergman, 1990). In this regard, SCFAs increased the ATP/ adenosine diphosphate (ADP) ratio in B cells (Figure 4D).

Acetyl-CoA, which can be derived from SCFAs, is the main substrate in fatty acid synthesis (FA, more specifically palmitic acid) (Bloch and Vance, 1977). Because FA synthesis is important for plasma B cell differentiation (Dufort et al., 2014; Fagone et al., 2007) and palmitic acid stimulates antibody production by B cells (Kunisawa et al., 2014), we examined lipid content in B cells treated with SCFAs. The lipid content of B cells was increased by SCFAs with elevated numbers of cellular lipid droplets (Figures 4E, S5D). Acetyl-CoA carboxylase (ACC) catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, and fatty acid synthase (FAS) extends the length of fatty acids to make palmitic acid (Girard et al., 1994). Inhibitors of ACC or FAS effectively abolished the effect of SCFAs on IgA expression (Figure 4F). Overall, SCFAs increased the cellular metabolism and biogenesis necessary for B cell differentiation and Ig production (Figure 4G).

SCFAs Boost mTOR activation and Glycolytic Activity in B cells

ATP production consumes adenosine monophosphate (AMP), which is a major agonist to activate 5' AMP-activated protein kinase (AMPK). AMPK is a key sensor for cellular energy status and a negative regulator of mTOR activation through phosphorylation of TSC2 and Raptor (Figure 5A). In line with this, AMP levels were decreased in SCFA-treated B cells along with decreased phosphorylation of AMPK but increased phosphorylation of rS6, which is a downstream effector molecule for the mTOR pathway (Figures 5B, 5C). A similar regulatory effect of DF or C3 on AMPK and mTOR was observed in vivo in colonic LP (Figure S5E). SCFAs enhanced glucose uptake by B cells under various activation conditions employing anti-CD40 and LPS (Figure 5D), and this SCFA effect was abolished by 2-Deoxy-D-glucose (2-DG), an inhibitor of glycolysis. We measured extracellular acidification rate (ECAR) with a real-time metabolic analyzer employing glucose, oligomycin, and 2-DG. We found that both glycolysis and glycolytic capacity of B cells were increased by SCFAs (Figures 5E, S5F). The SCFA effect on B cells was suppressed when B cells were forced to use galactose as the energy source for mitochondrial oxidative phosphorylation in the absence of glucose-dependent glycolysis (Figure 5F). In support of the role of AMPK-mTOR pathway in B cells, AMPK activators (AICAR and metformin) or an mTOR inhibitor (rapamycin) effectively suppressed the SCFA-induced activation of rS6, IgA secretion, and B cell proliferation (Figures 5G–I, S5G). Thus, SCFAs boost cellular glycolytic activity in B cells, in part, through the regulation of AMPK and mTOR.

We also examined the effect of SCFAs on the metabolism of other cell types, such as T helper cells and dendritic cells (DCs), which regulate B cells and antibody responses. SCFAs increased glucose uptake and enhanced the generation of $CXCR5^{+}PD\text{-}1^{+}$ Tfh cells in vitro (Figures S6A, S6B). SCFAs had positive effects on maximal respiration, glycolysis, and glycolytic capacity of $CD4^{+}$ T cells (Figure S6C). However, the effect on bone marrow-derived DCs was not conclusive (Figure S6D). We failed to detect any effect of SCFA on MLN DCs (Figure S6E). Only the SCFA treatment during B-DC co-culture, but not the SCFA treatment on DCs before culture, improved antibody production, implying the importance of B cells as the major cell targets of SCFAs.

SCFAs Boost Antibody Production by Human B cells

A question of interest is if SCFAs also regulate human B cells. SCFAs increased the differentiation of naïve human B cells to $CD20^{-}CD38^{+}$ plasma B cells and boosted the secretion of IgA and IgG in culture (Figures 6A and B). The expression of *Aicda* gene was increased by SCFAs (Figure 6C). The cellular ATP/ADP ratio was increased by SCFAs (Figure 6D). SCFAs decreased AMPK activity but increased mTOR activity in human B cells (Figure 6E). In a manner similar to mouse B cells, SCFAs enhanced the fat content of human B cells (Figure 6F). Thus, SCFAs also promote the generation of human plasma B cells.

SCFAs Promote Pathogen-Specific Antibody Responses

We examined if DF and SCFAs boost antibody responses to pathogens. For this, *Citrobacter rodentium*, a mouse attaching and effacing pathogen, was employed. LFD-fed mice were more susceptible to *C. rodentium* than MFD or HFD-fed mice were (Figures 7A, S7A, and S7B). DF enhanced GC formation, the levels of IgA and IgG specific for *C. rodentium*-OVA, and numbers of plasma B cells in the gut and spleen (Figures 7B, 7C, 7D, S7C–E). In both the large and small intestinal LP of infected mice, IgA^{+} B cells were increased by DF. C3 administration increased the host resistance and IgA response to *C. rodentium* infection in LFD- or regular rodent chow-fed mice (Figures 7C, S7F). However, C3 had no additional protective effect on HFD-fed mice (Figures 7C, 7E, 7F), which were already high in C3 levels (Figure S1A). Thus, SCFAs and DF promote pathogen-specific antibody responses.

DISCUSSION

The current paradigm for the function of gut microbiota in supporting antibody responses is mediated through TLR-activating bacterial DNA, proteins, and cell wall components, which directly and/or indirectly promote antibody responses (Kirkland et al., 2012; Teng et al., 2016). In contrast, the roles of microbial metabolites in regulating B cell responses remain unclear. Our findings indicate that SCFAs are a class of microbiota-derived factors that profoundly affect host antibody responses.

It was observed many years ago that DF intake potentiates IgA responses in rats (Kudoh et al., 1998). Our study now identifies a group of B cell-stimulating DF metabolites (i.e. SCFAs), which are produced by gut microbiota. While SCFAs are derived from DF, DF and SCFAs are not identical in their effects because DF can generate more than SCFAs and may also affect gut microbiota differently than SCFAs (Lee and Hase, 2014). Despite such differences, our results determine that SCFAs closely mimic the positive function of DF in antibody responses. The data that SCFA administration together with high levels of DF did not further enhance antibody responses indicate that SCFAs are the major DF metabolites that boost antibody responses.

Luminal SCFAs are absorbed into the blood circulation and, therefore, SCFAs have the potential to influence B cell responses in non-gut tissues as well. Indeed, systemic blood IgG and IgA levels are elevated by high DF intake or oral SCFA administration. HFD or SCFA feeding increased SCFA levels in the blood circulation, and this accounts for the increased

antibody responses in MLN and spleen. Antibody responses in SI LP and PP were increased as well by DF and SCFAs, which is consistent with the increased SCFA levels in intestinal tissues and blood. Thus, our results establish a significant role of SCFAs in regulating both mucosal and systemic antibody responses.

SCFAs effectively increase cellular metabolism in B cells. This function provides energy and building blocks to support B cell activation, differentiation and antibody production. B cells utilize both glycolysis and mitochondrial energy production for plasma cell differentiation (Caro-Maldonado et al., 2014). In line with this, we found that SCFAs increase both mitochondrial energy production and glycolysis. SCFAs are converted into acetyl-CoA, which is metabolized to produce energy and synthesize fatty acids. We observed elevated levels of cellular acetyl-CoA and lipid droplets, which promote B cell differentiation and antibody production. mTOR activation increases glycolytic activity in general (Masui et al., 2013; Shi et al., 2011). The increased glycolysis in SCFA-treated B cells is in line with the enhanced mTOR activity. AMPK activation suppresses the mTOR pathway (Green et al., 2011; Limon and Fruman, 2012). Therefore, the heightened mTOR activity in SCFA-treated B cells is explained, in turn, by suppressed AMPK activity following AMP depletion by SCFAs.

The impact of SCFAs on cellular metabolism is supported also by the function of SCFAs in regulating gene expression. Our microarray data indicate that key genes involved in B cell differentiation is regulated by C2. The most prominent gene group increased by SCFAs is the immunoglobulin gene family. SCFAs also increase the expression of *Xbp1*, *Irf4* and *Aicda* genes, all of which are required for successful plasma B cell differentiation and Ig class switch recombination. SCFAs are effective HDAC inhibitors, and the histone proteins on *Aicda* and Ig heavy chain genes became hyper-acetylated in the presence of SCFAs. Moreover, SCFAs induced the expression of Ig switch circle transcripts. Many genes involved in cellular metabolism are also regulated by SCFAs.

SCFAs have the potential to activate non-B cells to boost antibody responses. Cells that are known to be regulated by SCFAs include T cells, phagocytes, and epithelial cells (Kim et al., 2013; Le Poul et al., 2003; Park et al., 2015; Smith et al., 2013). We found that SCFAs promote the generation of Tfh cells. SCFAs increased cellular metabolism in activated T cells and increased the generation of Tfh cells in vitro and in vivo. SCFAs increase colonic $FoxP3^+$ Tregs (Furusawa et al., 2013; Smith et al., 2013). While Tregs, particularly follicular Tregs, suppress B cell responses (Chung et al., 2011; Lim et al., 2004; Linterman et al., 2011), some Tregs can convert to Tfh cells in PP (Tsuji et al., 2009). While not examined in this study, this could be another pathway for SCFAs to influence the intestinal antibody response. C2, C3 and C4 activate cell surface receptors such as GPR41 and GPR43 but T and B cells hardly express these receptors (Kim et al., 2013). We found no detectable role for GPR41 and GPR43 in regulation of B cell responses. While our data do not completely rule out the effect of SCFAs on DCs, we found no convincing evidence that SCFAs regulate B cells through DCs.

To create physiologically relevant levels of SCFAs, we used C3 at 80 mM or a SCFA mixture (a combined concentration of C2, C3, and C4 at 120 mM) in drinking water, which

is close to the total luminal concentration of SCFAs (~124 mM/Kg) in the human colon (Bergman, 1990). The total concentrations of SCFAs in blood and colon tissues are significantly lower (Bergman, 1990; Furusawa et al., 2013). We used biologically active concentrations of SCFAs (C2 at 10 mM; C3 at 1 mM; C4 at 0.1 mM) in vitro. While SCFAs at these concentrations do not affect cell survival, SCFAs can kill B cells and decrease antibody production at higher concentrations (e.g. C4 at 1 mM). Indeed, others observed that C4 or other HDAC inhibitors at such high concentrations can suppress B cells or kill T cells in vitro (White et al., 2014; Zimmerman et al., 2012). Thus, we don't rule out the possibility that SCFAs, at supraphysiological concentrations, may even have negative effects on B cells.

We demonstrated in this report that SCFAs effectively support host antibody responses. SCFAs regulate gene expression and energy metabolism in mouse and human B cells to facilitate the production of class switched antibodies. SCFAs support B cell responses in the steady state and during infection. Our results identify a class of B cell-stimulating microbial factors. Compared to TLR ligands, which work through receptor-mediated activation, SCFAs work through metabolic regulation and gene expression to facilitate PC differentiation and antibody production. Because the levels of SCFAs can be regulated through prebiotics and SCFA administration, the outcomes have high application potentials in modulating host immunity. The results have significant ramifications in regulating humoral immune responses in the steady state as well as during infection and inflammatory conditions.

EXPERIMENTAL PROCEDURES

Mice and in vivo studies

All animal experiments were approved by the Purdue Animal Care and Use Committee. C57BL/6 mice (Harlan) were maintained at Purdue for at least 24 months before use. Mice were maintained on a regular rodent chow (Harlan 2018S Global 18% Protein Rodent Diet). The special diets containing different amounts of DF (0, 5 and 15% of pectin and inulin at 1:1 ratio) were custom-ordered from Harlan Teklad (Indianapolis, IN). These diets are called low fiber diet (LFD), medium fiber diet (MFD) or high fiber diet (HFD). Mice were weaned at 3 weeks of age and then fed with the special diets or drinking water containing C3 (80 mM, pH 7.4) or a SCFA mixture (C2: 70 mM; C3: 30 mM; C4: 20 mM, pH 7.4) for 4 weeks before start of the experiments. Mice were on the same diet or drinking water until the end of experiments. When indicated, mice were treated with a minimally effective dose of antibiotics mixture (meAbx: ampicillin, neomycin, and metronidazole at 40 mg/L and vancomycin at 20 mg/L) with or without C3 for 4-6 weeks. For infection with *C. rodentium*, please see Supplemental Information.

Cell isolation and culture

Spleen total B cells were isolated by depleting cells expressing CD3ε (145-2C11), CD4 (RM4), CD11b (M170), Ter-119 (Ter119) and CD43 (1B11), using biotin-labeled antibodies and anti-biotin beads (Miltenyi Biotec) (purity > 95% based on CD19 expression). The total B cells were further processed to deplete cells expressing IgG (Poly4053), IgA (RMA-1) or CD11c (N418) (purity > 95% based on $CD19^{+}IgD^{+}$ expression) as previously described

(Pone et al., 2012). Total B cells were also isolated using anti-CD19 and magnetic beads to assess HDAC activity, signaling, and transcriptome analysis. B cells were cultured at 5×10^5 cells/ml in various activation conditions: LPS (5 μg/ml; Sigma-Aldrich) and IL-4 (2.5 ng/ml) for IgG1; LPS and IFN-γ (20 ng/ml) for IgG2a; LPS and TGFβ1 (2 ng/ml) for IgG2b; LPS alone for IgG3; LPS or anti-CD40 (20 μg/ml, FGK45; Enzo life Science), TGFβ1 (1 ng/ml), IL-5 (5 ng/ml), IL-6 (5 ng/ml), and RA (10 nM) for IgA-inducing conditions. C2 (1, 5, 10 mM), C3 (0.1, 0.5, 1 mM), C4 (0.1, 0.25, 0.5, 1 mM), Trichostatin A (TSA, 1 nM), garcinol (2 μM), anacardic acid (2 μM), rapamycin (25 nM), 2-DG (0.5 mM), AICAR (0.5 mM), C75 (2 μg/ml), TOFA (1 μg/ml) and/or metformin (1 mM) were added to culture. For the microarray of SCFA-treated B cells, $CD19^+$ B cells were stimulated with anti-IgM and mature BM-DCs as described in Supplemental Information. Cells were collected on day 1, 2, or 3 to assess cell metabolism, HDAC activity, chromatin immunoprecipitation (ChIP), and qRT-PCR analysis. Cells were cultured for 6 days to assess surface Ig expression or for 6-7 days to measure secreted Igs with ELISA. For isolation and culture of T cells, mouse intestinal LP cells, DCs and human B cells, please see Supplemental Information.

Flow cytometry

Freshly isolated B cells from spleen, intestinal LP and PP, and B cells were blocked with anti-CD16/CD32 (clone 93) and stained with antibodies to mouse B220 (RA3-6B2), CD138 (281-2), T-and B-cell activation antigen (GL-7), CD95 (15A7), CD4 (RM4-5), CXCR5 (2G8), PD-1 (29F.1A12), IgM (RMM-1), IgA (C10-3), IgG1 (A85-1), IgG2a (RMG2a-62), IgG2b (R12-3), and/or IgG3 (RMG3-1). For intracellular Ki-67 staining, cells were fixed, permeabilized and stained with anti-Ki-67 (16A8) or Blimp-1 (5E7) using the FoxP3 staining buffer (eBioscience). For cell proliferation, 3-day cultured cells were stained with the EdU flow cytometry assay kit (Life Technology). For assessing cell death, cells were cultured for 3 days before they were stained with Annexin V and propidium iodide (PI). For assessment of mTOR and AMPK activation, cultured B cells for indicated time periods were stained with antibodies to phosphorylated rS6 (Ser235/236; D57.2.2E) and phosphorylated AMPKα (172; 40H9) (all from Cell Signaling Technology). Human B cells were stained with antibodies to human CD20 (2H7) and CD38 (HIT2). Unless indicated otherwise, the antibodies were from BioLegend, eBioscience or BD Biosciences. The numbers of IgA-coated and IgA-free bacteria were assessed as described previously (Chu et al., 2014). Please see Supplemental Information for flow cytometry of fecal bacteria.

Real-time metabolic analysis, glucose uptake, and metabolite measurements

For oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), an XF24 Extracellular Flux analyzer (Seahorse Bioscience, North Billerica, MA) was used. Splenic B cells and T cells were cultured with SCFAs for 2 days. For BM-DCs, BM cells were cultured with GM-CSF (20 ng/ml) for 6 days, then with GM-CSF with or without SCFAs for additional 3 days before they are activated with LPS (1 μg/ml) for 24 h. Culture cells (1.4×10^6 lymphocytes or 0.5×10^6 BMDCs) were plated onto Cell-Tak™ (BD Biosciences)-coated (22.4 μg/ml) XF24 (V7) polystyrene culture plates. The real-time metabolic analysis was performed at 37°C. Each measurement cycle was consisted of 3 minutes of mixing and 3 minutes of acquisition. Metabolic inhibitors (75 μL) were added to each injection port. Three baseline measurements were taken prior to the addition of any compounds, and 3

response measurements were taken after the addition of each compound. For the information on measuring glucose uptake, ATP/ADP ratio, and levels of AMP, Acetyl-CoA and SCFAs, please see Supplemental Information.

Confocal microscopy

Frozen sections (8 μm) of ileum, proximal colon, PPs, and spleen were fixed in cold acetone and stained with biotin-conjugated antibody to AID (m-AID2, eBioscience) followed by Streptavidin-PE, fluorochrome-conjugated antibodies to Collagen IV (Cat# BP975, Acris Antibodies), IgA, CD3ε, IgD (11-26c.2a), and/or GL-7. Fluorescence images were collected with a SP5 II laser scanning microscope system (Leica). For imaging mitochondrial mass, sorted B cells were cultured with SCFAs in the IgA-inducing condition for 3 days and stained with MitoTracker Green FM (200 nM, Invitrogen) and Tetramethylrhodamine (TRITC, 5 μg/ml) for 30 min at 37°C. For confocal imaging of cellular lipid droplets, B cells were cultured with indicated SCFAs in the IgA-inducing condition for 3 days, fixed, and stained with BODIPY (10 μM, Invitrogen) and DRAQ5 (1 μM) (Gold et al., 2012). Stained cells were transferred onto slide glasses for immediate confocal imaging. Numbers of lipid droplets in B cells were counted in 3D with IMARIS software (Bitplane).

Statistical analysis

Student's t-test (paired, two-tailed) was used to determine the significance of differences between two groups. P values < 0.05 were considered significant. Repeated measures ANOVA was used to determine differences between two sets of weight change data (SAS, version 9.2, SAS Institute Inc. Cary, NC). All error bars indicate standard error of the mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Short-chain fatty acids (SCFAs) produced by gut microbiota promote antibody responses.
- SCFAs activate B cell metabolism for production of energy and building blocks.
- SCFAs control gene expression for plasma B cell differentiation.
- SCFAs boost antibody responses during infection, decreasing susceptibility to pathogens.

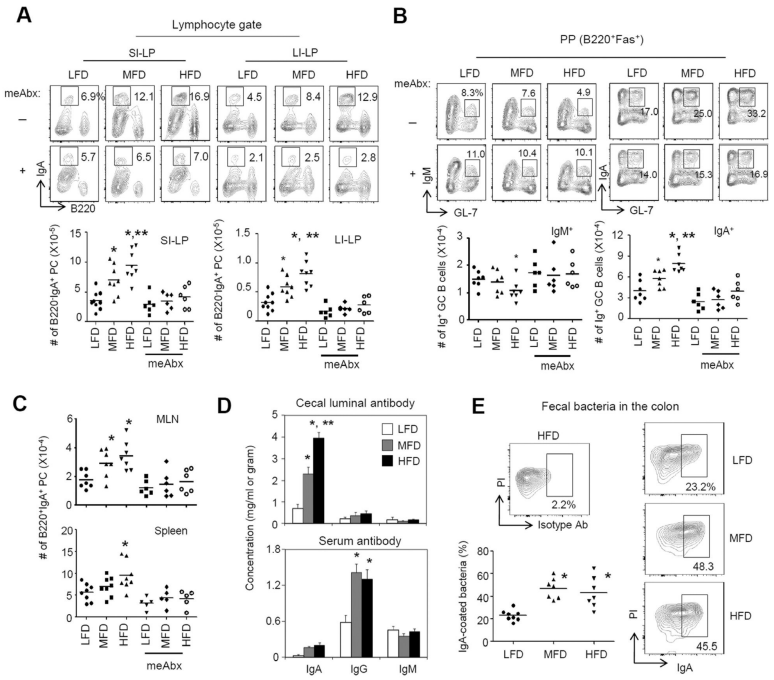

Figure 1. Dietary Fiber Increased IgA and IgG Production

(A) DF increased numbers of IgA^+ PCs in the intestinal LP. (B) DF increased numbers of IgA^+ GC B cells ($B220^+GL\text{-}7^+Fas^+$) in PP. (C) DF increased numbers of IgA^+ cells ($B220^+IgA^+$) in MLN and spleen. Mice were fed different levels of DF with or without oral administration with a minimally effective dose of antibiotics (meAbx) for 4-6 weeks. (D) DF increased Ig levels in the serum and cecal contents. (E) DF increased the frequency of IgA-coated bacteria in the colon. The data were from three independent experiments (n=6–11). Error bars indicate SEM. Significant differences from control or LFD* or MFD** groups. See also Figures S1A–G.

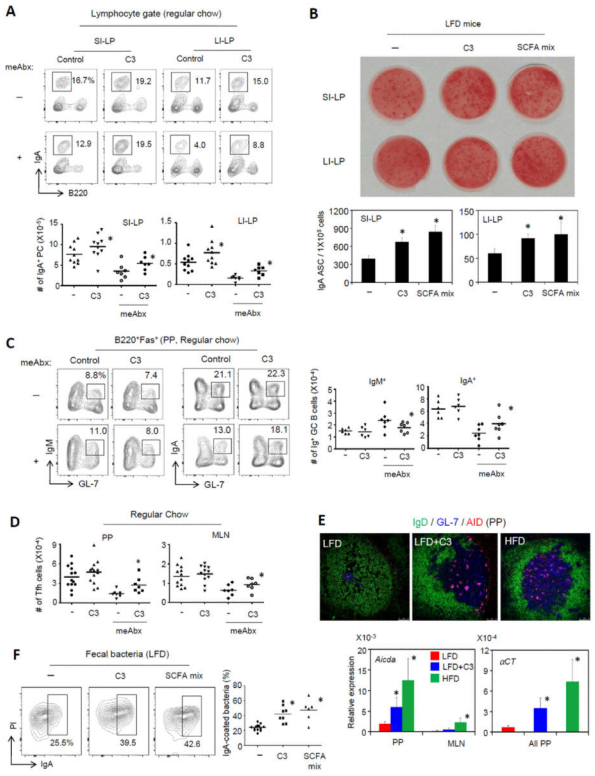

Figure 2. SCFAs Increase Intestinal Antibody Responses

(A) C3 increased the frequencies of IgA^+ PCs in intestinal LP of regular chow-fed mice. (B) C3 alone (80mM) or a SCFA mixture (C2: 70mM; C3: 30mM; and C4: 20mM) increased IgA-secreting intestinal LP cells (ASCs) in LFD-fed mice. ASCs were identified with an ELISPOT assay. (C) C3 increased numbers of IgA^+ GC B cells in PP. (D) C3 increased numbers of Tfh cells ($CD4^+CXCR5^+PD\text{-}1^+$) in PP and MLN. (E) C3 or HFD treatment promoted AID expression and Ig clsass switch in PP. AID expression in PP was examined by immunohistochemistry. Expressions of *Aicda* and αCT mRNA in cells of indicated organs were examined by qRT-PCR. (F) C3 or SCFA feeding increased the frequency of IgA-coated fecal bacteria in the colon of LFD mice. The average frequency of isotype antibody-coated bacteria in SCFA-fed mice was ~2%. Mice were fed with indicated diet or water for 5–6 weeks. The data were from 2-3 experiments (n=6–13). Error bars indicate SEM. *Significant differences from control or LFD groups. See also Figures S2A–F.

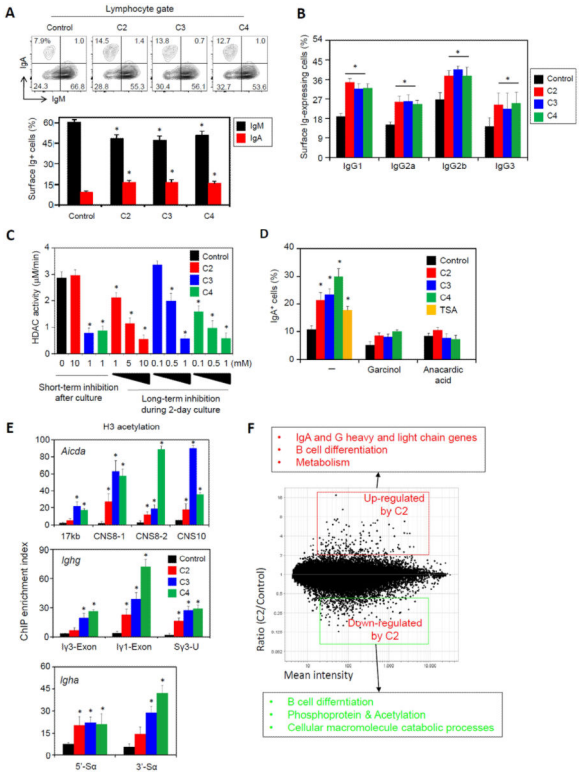

Figure 3. Effects of SCFAs on in vitro B cell Differentiation, HDAC Activity, and Gene Expression

(A) SCFAs increased B cell differentiation to IgA-expressing cells. (B) SCFAs increased B cell differentiation to IgG-expressing cells. B cells were cultured for 6 days in Ig isotype-specific conditions: LPS and IL-4 for IgG1; LPS and IFN-γ for IgG2a; LPS and TGFβ1 for IgG2b; LPS alone for IgG3; LPS, TGFβ1, IL-5, IL-6 and RA for IgA-inducing conditions. (C) SCFAs inhibit HDAC activity in B cells. B cells were examined for HDAC activity after a 2-day culture with SCFAs (long term suppression) or first cultured for 2 days without SCFAs but measured after 2 h incubation with SCFAs. (D) HDAC or HAT inhibitors (TSA as an HDAC inhibitor; garcinol and anacardic acid for HAT inhibitors) reciprocally regulate IgA responses. (E) SCFAs induced histone acetylation on the *Aicda* gene and the switch regions of the Ig heavy chain genes. A ChIP assay to assess H3 acetylation was performed for the conserved regulatory sequences of the *Aicda* gene and the switch regions of Ig genes. (F) C2 regulates gene expression in B cells. A microarray study was performed for B cells cultured in the presence and absence of C2 for 5 days. The functional gene groups regulated by C2 were identified with the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7. Average data from two array experiments are shown. Spleen $CD19^{+}$ IgA^{-} IgG^{-} (A, B, D) or total (C, E, F) B cells were used. The data were from 3–4 experiments and combined data with SEM (n=3-6) are shown. *Significant differences from control groups. See also Figures S3 and S4.

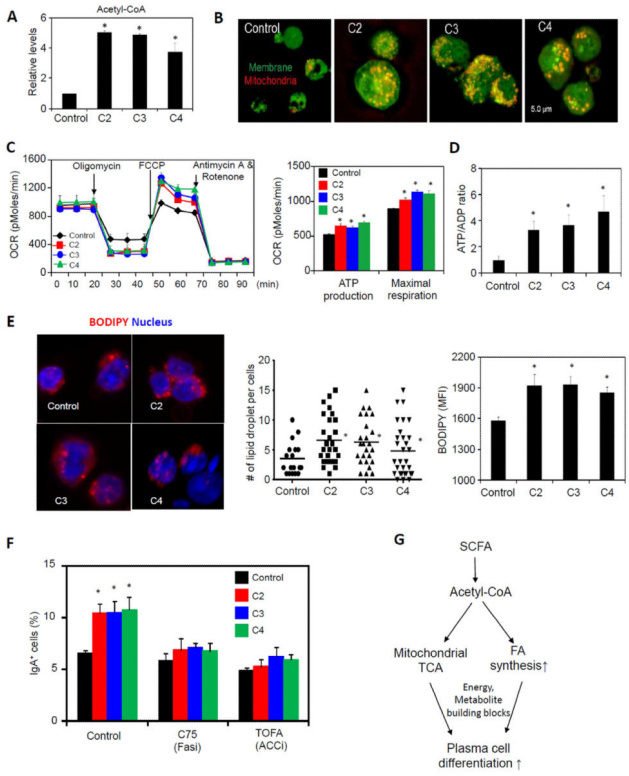

Figure 4. SCFAs Increase Mitochondrial Energy Production and Fatty Acid Content in B cells

(A) SCFAs increased cellular acetyl-CoA levels in B cells. (B) SCFAs increased mitochondrial mass in B cells. B cells were stained with MitoTracker Green. (C) SCFAs increased mitochondrial respiration in B cells. Mitochondrial activity based on oxygen consumption rate (OCR) was measured with a Seahorse real-time metabolic analyzer (Oligo: Oligomycin; RO/AA: rotenone/antimycin A). (D) SCFAs increased cellular ATP/ADP ratio. (E) SCFAs increased lipid droplets in B cells. Shown are confoal images of lipid droplets stained with BODIPY and numbers of lipid droplets along with BODIPY fluorescent intensity (MFI) in SCFA-treated B cells. (F) Effects of fatty acid synthesis inhibitors (C75 for fatty acid synthase and TOFA for Acetyl-CoA carboxylase) on SCFA-induced IgA expression. (G) A summary diagram for the observed function of SCFAs in regulation of acetyl-CoA metabolism in B cells. Spleen total $CD19^{+}$ (A–D) or IgA^{-} IgG^{-} (E, F) B cells were cultured for 2-3 (A–E) or 6 (F) days in an IgA-inducing condition (LPS, TGFβ1, IL-5, IL-6 and RA) for all experiments. The data were from 3–5 in vitro experiments, and combined data with SEM (n=3-6) are shown. Error bars indicate SEM. *Significant differences ($P < 0.05$) from control groups. See also Figures S5A–D.

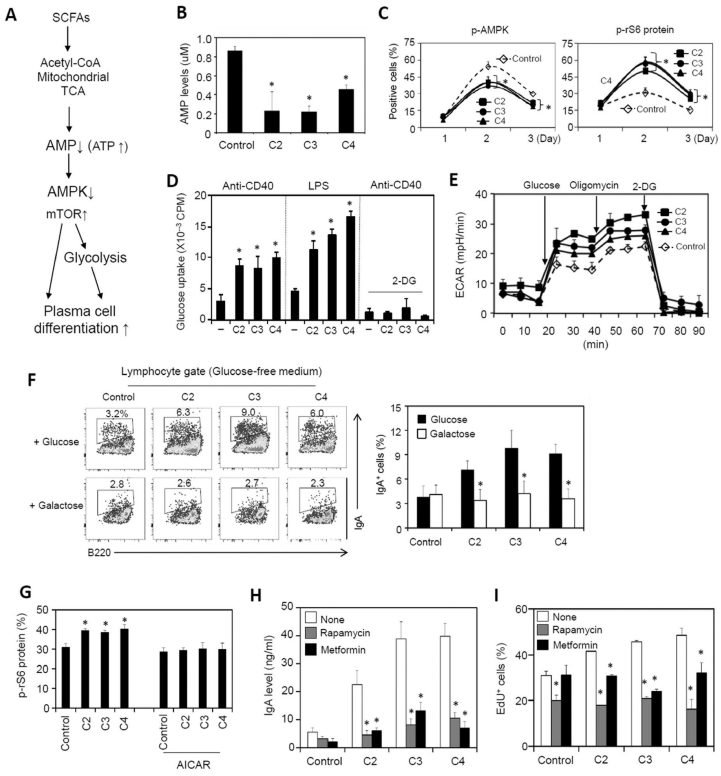

Figure 5. SCFAs Increase mTOR Activiation and Glycolysis to Support Antibody Production

(A) A potential impact of SCFAs on AMP levels, mTOR activation, and glycolysis in B cells. (B) SCFAs decreased cellular AMP levels in B cells. (C) Changes of mTOR (p-rS6 protein) and AMPK (p-AMPK) activity in SCFA-treated B cells. (D) SCFA increased [3-3H]-glucose uptake. (E) SCFAs increased glycolysis in B cells undergoing activation. Extracellular acidification rate (ECAR) was measured with a Seahorse real-time metabolic analyzer. (F) Forced oxidative phosphorylation with galactose abolished the SCFA effect on B cells. B cells were cultured with indicated SCFAs in glucose-free medium in the presence of glucose or galactose (10 mM). (G) AMPK activation abolished the SCFA effect on mTOR activation. (H) Rapamycin and metformin abolished the SCFA effect on IgA production. (I) Rapamycin and metformin decreased SCFA-induced B cell proliferation. Unless indicated otherwise, B cells were cultured for 2-3 days in panel B, D, E, G and I, and 5–7 days in panel F and H in an IgA-inducing condition (LPS, TGFβ1, IL-5, IL-6 and RA). Glu: glucose; Oligo:oligomycin; 2-DG: 2-deoxy-D-glucose. Spleen $CD19^+$ IgA^- IgG^- (B, G) or total (C–F, H, I) B cells were used. The data were from 4-6 in vitro experiments and combined data with SEM (n=4-6) are shown. Error bars indicate SEM. *Significant differences ($P < 0.05$) from control groups. See also Figures S5E–G.

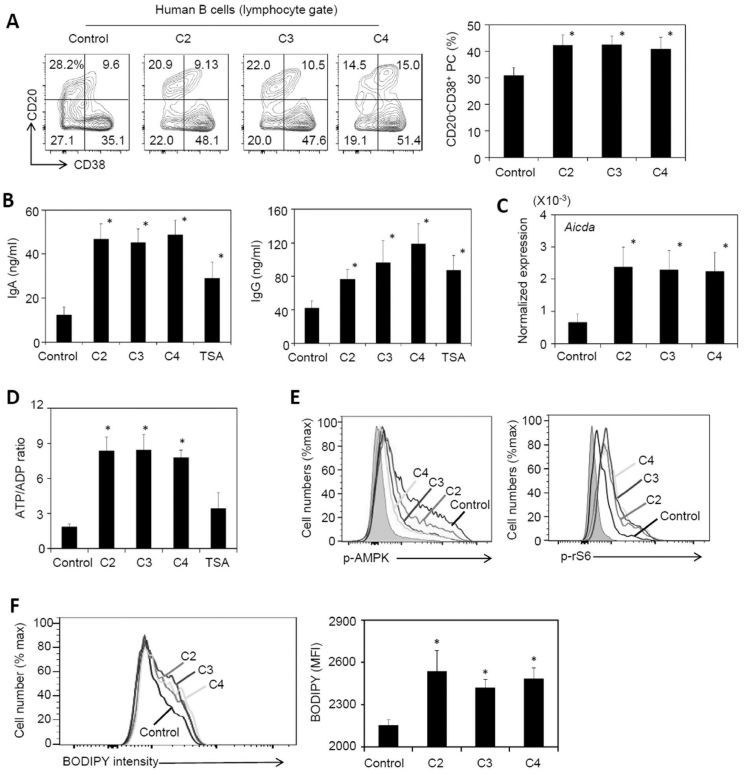

Figure 6. SCFAs Increase Ig Production by Human B cells

(A) SCFAs increased the numbers of $CD20^{-}CD38^{+}$ human plasma B cells. (B) SCFAs increased IgA and IgG production. (C) SCFAs induced the expression of the human *Aicda* gene. (D) SCFAs increased the cellular ATP/ADP ratio. (E) SCFAs suppressed AMPK and enhanced mTOR activation in B cells. (F) SCFAs increased lipid content in B cells. B cells were cultured for 3 (C–F) or 6-7 (A, B) days with anti-CD40 and the cytokines described in the supplemental information. Tonsil naïve (A, F) or total (B–E) B cells were used. The data were from 3-6 in vitro experiments and combined data with SEM (n=3-6) are shown. *Significant differences ($P < 0.05$) from control groups.

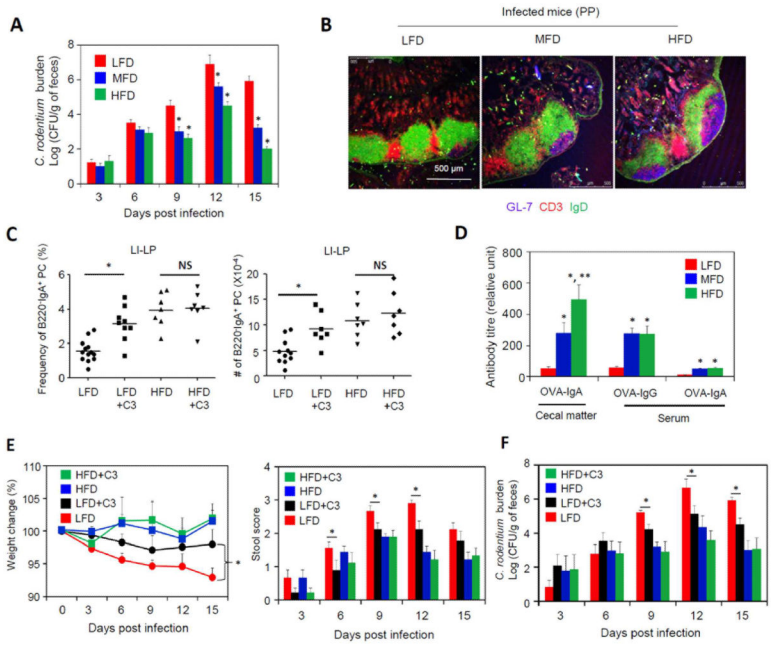

Figure 7. SCFAs Increase Antibody Responses During Infection

(A) DF enhanced the clearance of *C. rodentium*. (B) DF enhanced GC formation in PP following infection. (C) DF increased IgA^+ B cells in intestinal LP. A total lymphocyte gate was used for flow cytometry. (D) DF increased OVA-specific Ig responses to *C. rodentium-OVA*. Luminal and serum OVA-specific IgG and IgA levels were measured by ELISA. (E) C3 administration enhanced anti-*C. rodentium* immunity in LFD, but not HFD, mice. C3 effects on weight change and stool consistency score were measured. (F) Impact of C3 and/or DF on *C. rodentium* burden. Mice on LFD or HFD diet were fed regular or C3 drinking water (80 mM) for 4 weeks and infected with *C. rodentium-OVA*. The data were from 2–4 in vivo experiments (n=7–13). Significant differences from LFD* or MFD** groups. See also Figure S7.