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Original Article

Elevated intestinal IgG-expressing B cell inflammation in coronary artery disease is supported by increased infiltration of $CD4^+CXCR5^+$ T cells

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Abstract: Coronary artery disease (CAD) is currently accounting for millions of death each year. The direct pathogenic process of atherosclerosis can be accelerated by chronic low-grade inflammation. In this study, we examined the intestinal immune system of CAD patients. We first observed that the secreted IgG in CAD patients is significantly higher than that in healthy volunteers but lower than that in inflammatory bowel disease (IBD) patients. Sigmoid colon biopsies from CAD patients contained significantly upregulated frequencies of IgG-expressing B cells, with more $CD27^{++}$ expression, than those from healthy volunteers. The mononuclear cells from CAD patients also secreted significantly higher levels of IgG in vitro than those from healthy volunteers. This upregulation was supported by increased intestinal infiltration of $CD4^+CXCR5^+$ T cells, since depletion of $CD4^+CXCR5^+$ T cells significantly downregulated IgG secretion. Moreover, a subset of CAD patients who presented high intestinal IgG level also presented high LPS levels in the serum. Together, this study provided evidence for dysregulated intestinal immunity in CAD patients, with the potential to influence peripheral immunity.

Keywords: IgG, B cell, Tfh, coronary artery disease

Introduction

Cardiovascular diseases areone of the leading causes of death in many countries, currently accounting for 16.7 million deaths each year with an increasing incidence worldwide [1, 2]. The direct symptoms of cardiovascular disease arise from the deposition of cholesterol and other proteins on the blood vessels in a process termed atherosclerosis, resulting in progressively reduced elasticity of artery walls, constrained blood flow, increased vessel wall lesions, and further complications caused by blockade of rupture of the blood vessels. This process can be accelerated by the feed-forward mechanisms of the immune system [3]. The initial lesions and accumulation of lipids in the artery is accompanied with the infiltration of macrophages, T cells and mast cells, which can be activated by low-density lipoprotein (LDL), oxidized LDL (oxLDL) and other local antigens to initiate a chronic immune response, which in turn can induce more lesions, with apoptotic and necrotic cells acting as new antigens for further immune activation [4-7]. Therefore, studying the involvement of the immune systemis crucial to developing new therapies to cardiovascular diseases, including the most common coronary artery disease (CAD).

The adoption of the western-style diet is partially accounting for the development of CAD. Food intake is shown to modulate metabolic activities as well as immune and inflammatory processes [8]. High-fat, high-processed sugar diet also results in different gut microbiome composition in humanized gnotobiotic mice and humans from low-fat, plant polysaccharide-rich diet [9-11]. Metabolism of L-carnitine, a

Table 1. Demographic information of study participants

Participants	N	Gender M/F	Age median	Age range	BMI median	BMI range	Ethinicity % Chinese
Healthy	20	11/9	55	43-72	23	19.5-25.4	100
CAD	28	17/11	56	44-71	24.1	21.2-26.5	100
IBD	14	7/7	52	46-65	21.6	18.3-23.9	100

common nutrient in red meat, by gut microorganisms canproduce trimethylamine-N-oxide that accelerates cholesterol buildup in the artery [12]. 16S rRNA sequencing of gut samples from patients with atherosclerosis have found correlations between diseasemarkers of atherosclerosis and abundance of certain bacterial phylotypes [13]. Bacterial colonization of the intestinal tract also maintains and modulates mucosal immune responses, which in turn keeps commensals in check while prevents pathogen translocation through secreted immunoglobulin (Ig) [14-16]. In steady state, low-affinity, high avidity IgM and IgA antibodies produced by lamina propria B cells and plasmablasts bind commensals, but do not activate a proinflammatory immune response [17]. During intestinal inflammation, high-affinity IgG-expressing B cells and plasmablasts are upregulated, together with disruptions of the tight junctions and leakage of microbial products, such as lipopolysaccharide (LPS), into the periphery, which contributes to persistent chronic inflammation [18, 19]. The intestinal immune system in cardiovascular diseases and CAD patients, in terms of its activation status and possible role in chronic low-grade inflammation, has not been examined.

Since secreted Ig is crucial for maintaining commensal homeostasis and can be altered during inflammation, we first examined the intestinal Ig secretions in CAD patients. A low-level upregulation of secreted IgG and IgG-expressing B cells/plasmablasts were found in CAD patients, compared to healthy individuals. This upregulation was supported by increased infiltration of $CD4^+CXCR5^+$ T cells, since depletion of $CD4^+CXCR5^+$ T cells significantly downregulated IgG secretion. Moreover, a subset of CAD patients, who presented high intestinal IgG level, also presented high LPS levels in the serum. Together, this study provided evidence for dysregulated intestinal immunity in CAD patients, with the potential to influence peripheral immunity.

Methods

Study participants

This study included age- and gender-matched healthy volunteers, CAD disease patients, and active inflammatory bowel disease (IBD) patients. Participants did not present evidence of other serious illnesses, such as chronic HBV, HCV or HIV infection, diabetes, neoplastic diseases, or autoimmune diseases. They were also excluded if they previously had heart failure, acute infections and/or surgery within the last 2 months. All CAD and IBD patients were newly diagnosed and were not taking disease-modifying medications at the time of sample collection. CAD were diagnosed with electrocardiogram and coronary angiography using previously established standards by the American Heart Association [20]. IBD, including ulcerative colitis (UC) and Crohn's disease (CD), were diagnosed using standard criteria with serial biopsies confirming active disease [21]. Demographic information was summarized in **Table 1**. The use of human samples was approved by Ethics boards of Changhai Hospital and Changzheng Hospital. Written informed consent was obtained from all subjects.

Measurement of Ig concentration at the intestinal mucosa

Intestinal mucosal Ig was obtained using a previously published protocol [22]. Briefly, colonic mucosa of each study participant was washed with 100 ml 0.9% saline, with great care taken to aspirate fluid encountered during insertion. The wash fluid was then aliquotedinto 10 ml 1 mm PMSF and 0.2 ml 500 mm EDTA to inhibit proteolysis. The mucosal washings were pushed though a 40 μm cell strainer (Fisher Scientific) to remove debris and then mixed with polyethylene glycol (PEG) to a final concentration of 15% PEG and centrifuged at 6000 g for 10 min at 4°C to obtain a pellet that contained mucosal Igs, which was resuspended in 2 ml 15% PEG. This procedure was repeated once. The final pellet was resuspended in 1 ml PBS

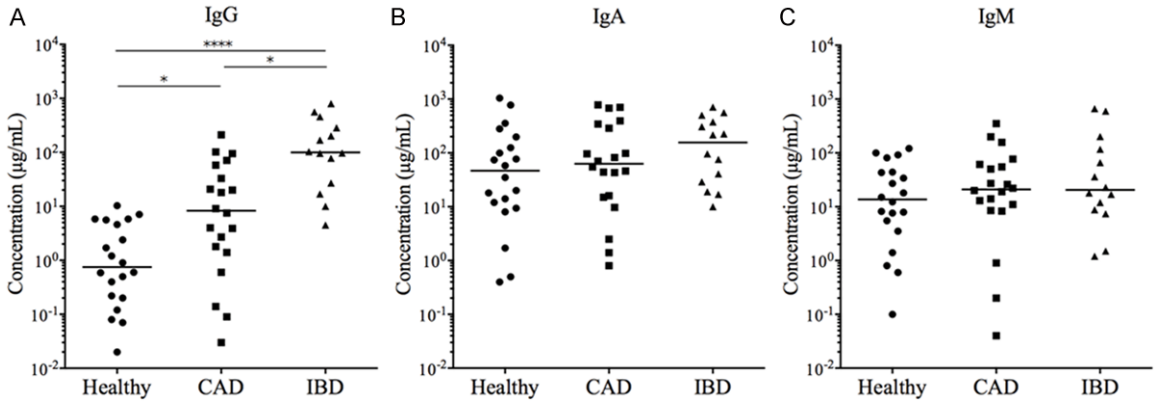

Figure 1. Intestinal Ig concentrations in healthy, CAD and IBD patients. The (A) IgG, (B) IgA, and (C) IgM concentrations in colonoscopic washing samples obtained from 20 healthy, 20 CAD and 14 active IBD subjects were measured in ELISA. Solid bars indicate the median values. Kruskal-Wallis one-way ANOVA followed by Dunn's test. *P < 0.05. ****P < 0.0001.

for Ig measurement by ELISA, using commercial human IgG, IgA and IgM ELISA kits from eBioscience. In cases where the Ig concentration was above the upper detection limit, the samples were diluted for ELISA measurement and the readings were multiplied with the dilution factor to obtain the Ig concentration.

Identification of B cells and T cells in colon biopsies

Biopsies were taken from the sigmoid colon 25 to 30 cm from the anal verge and were immediately placed in RPMI-1640 media containing penicillin, and GlutaMAX (ThermoFisher Scientific). Sigmoid colon mucosal mononuclear cells were isolated by two sequential Collagenase II digestions at 0.5 and 1.0 mg/ml Clostridiopeptidase A (Sigma-Aldrich), respectively, for 45 min each at 37°C with shaking. Mucosal cells were passed through a 100 μm cell strainer and centrifuged with the standard Ficoll procedure to obtain mononuclear cells. Anti-human CD3, CD4, CD19, CD27, CXCR5, IgA, and IgG (BioLegend) were used in different combinations for surface-staining of mucosal mononuclear cells. The stained samples were acquired in BD FACS LSR II and analyzed in FlowJo.

De novo IgG production

Whole mucosal mononuclear cells or $CD4^+$ $CXCR5^+$ T cell-depleted mononuclear cells were cultured at 2×10^5 cells per 200 μL complete media directly ex vivo without stimulation, or treated with heat-killed *Staphylococcus aureus*-Cowan (EMD Millipore) and CpG (Invivogen) (1 μg/ml each). For $CD4^+CXCR5^+$ T cell-depletion, $CD4^+$ T cells were isolated from gut mononuclear cells using Human CD4 Positive Selection Kit (Stemcell), and were then stained with PE-conjugated anti-human CXCR5 (BioLegend). The $CD4^+CXCR5^+$ cells were depleted from total $CD4^+$ T cells through PE positive selection (Stemcell). The remaining $CD4^+CXCR5^-$ T cells were then added back into the gut mononuclear cells proportionally, such that the potential loss of $CD4^+CXCR5^-$ T cells during the washing steps were compensated. After 12 h incubation in 37°C 5% CO_2, 150 μl supernatant were carefully removed after centrifugation. IgG concentration was then measured by ELISA. For some experiments, additional 150 μl media, without or with SAC+CpG, were added for 2 additional days and removed for IgG measurement, for a total of three rounds (6.5 days in total from the initial culturing date).

Serum LPS

Serum samples from all participants were diluted to 20% with HyClone endotoxin-free water (GE Healthcare) and heated to 70°C for 10 min to inactivate serum proteins. The LPS levels were then detected with commercially available Limulus Amebocyte assay (Cambrex) following the manufacturer's protocol, in triplicates for each sample.

Statistics

Lines and error bars represent mean ± SD unless otherwise specified. Data normality was

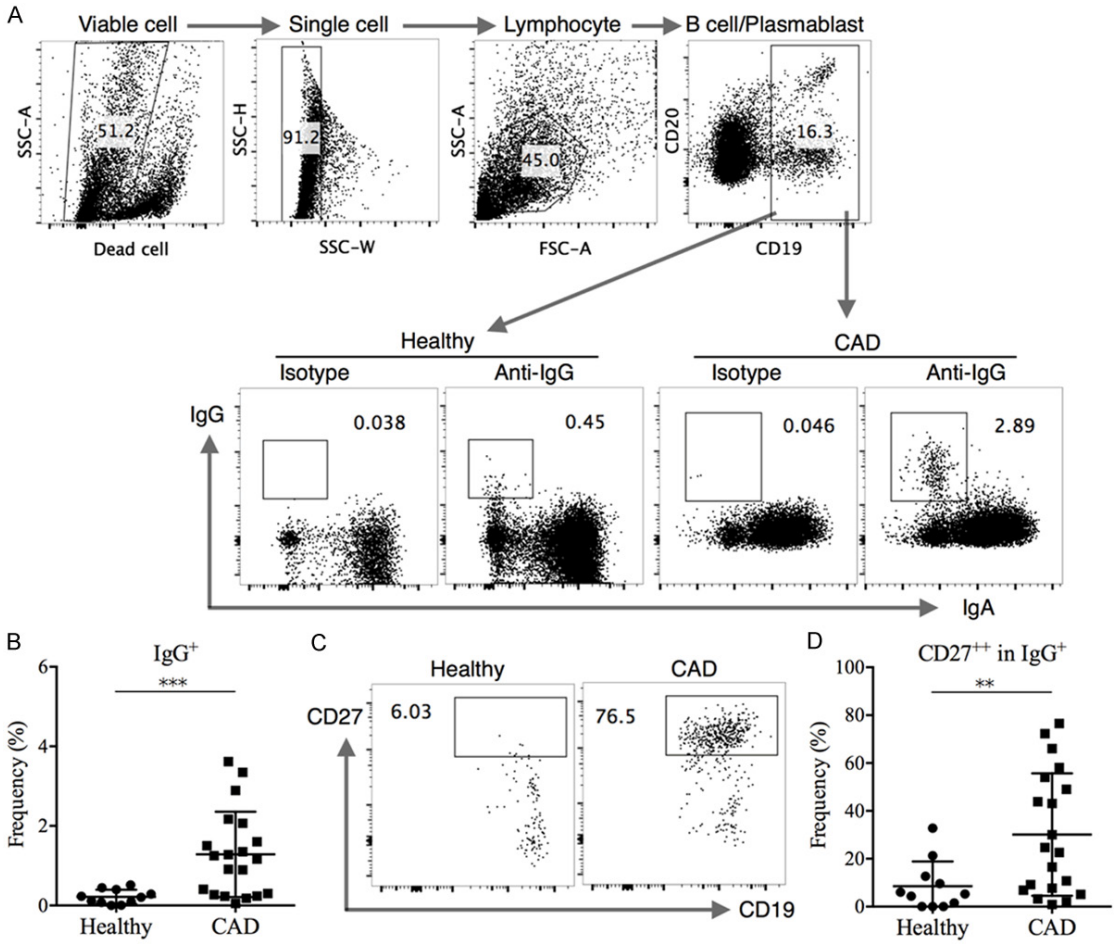

Figure 2. Characteristics of intestinal IgG-expressing cells in healthy and CAD subjects. Mononuclear cells isolated from fresh sigmoid colon biopsies of 11 healthy donors and 20 CAD patients were surface-stained with dead cell stain and anti-human monoclonal antibodies and were examined by FACS. (A) Representative schematic of viable B cell/plasmablast identification in colon biopsies. The IgG-expressing B cells/plasmablasts in healthy and CAD individuals were then identified with negative standards established by using an isotype control of the anti-human IgG monoclonal antibody (Clone HP6017). (B) The frequencies of IgG-expressing B cells/plasmablasts in healthy and CAD individuals. (C) Representative gating of $CD27^{++}$ cells in one healthy donor and one CAD subject. The panels were pre-gated on viable IgG^{+} B cells/plasmablasts as shown in (A). (D) The frequencies of $CD27^{++}$ cells in IgG^{+} B cells/plasmablasts in all healthy and CAD individuals. Unpaired *t* test with Welch's correction. **P < 0.01. ***P < 0.001.

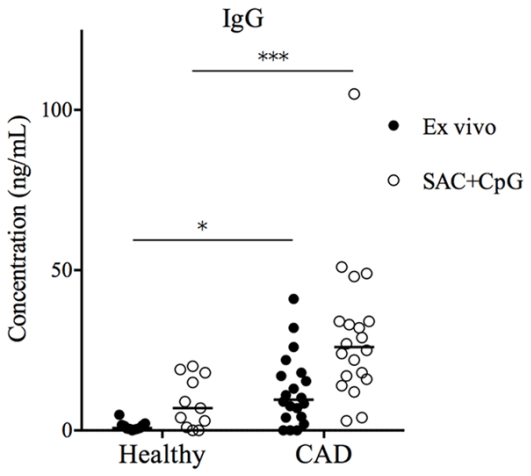

Figure 3. De novo IgG expression by intestinal B cells/plasmablasts in healthy and CADsubjects. Mononuclear cells isolated from fresh sigmoid colon biopsies of 11 healthy donors and 20 CAD patients were cultured at 2×10^{5} cells per 200 μL complete media directly ex vivo without stimulation, or treated with SAC and CpG (1 μg/ml each). After 12 h, the IgG concentration in the supernatant was measured by ELISA. Solid bars indicate the median values. Two-way ANOVA followed by Sidak's test. *P < 0.05. ***P < 0.001.

tested by D'Agostino-Pearson test, the result of which was used to choose parametric or non-parametric tests. All tests were done in Prism 6.

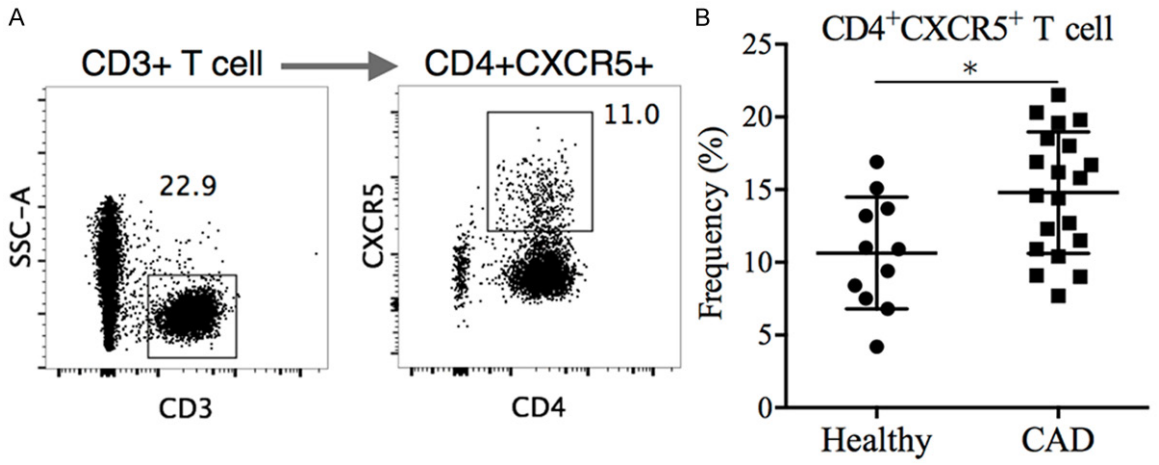

Figure 4. Intestinal infiltration of $CD4^+CXCR5^+$ T cells in healthy and CAD subjects. Mononuclear cells isolated from fresh sigmoid colon biopsies were surface-stained with dead cell stain and anti-human monoclonal antibodies and then examined by FACS. A. Schematic of $CD4^+CXCR5^+$ T cell identification. The first panel was pre-gated on viable lymphocytes as shown in **Figure 2A**. B. The frequencies of $CD4^+CXCR5^+$ T cells in all healthy and CAD individuals. Unpaired *t* test with Welch's correction. *P < 0.05.

Results

Overrepresentation of IgG in gut from CAD patients

At steady state, the majority of gut antibodies are of the IgA isotype, seconded by IgM, while IgG, the main antibody in the peripheral blood with high hypermutation rate and high antigen-specificity, is rarely present [17, 23]. Elevated IgG has been thought of as a characteristic of intestinal inflammation [22]. To examine the intestinal mucosa-secreted antibody concentrationof healthy and CAD patients, colonoscopic washing samples were obtained from all subjects, on which antibody ELISA was performed.We observed that CAD subjects had a significant upregulation of IgGcompared to healthy subjects (**Figure 1A**). The concentration of IgG in CAD is still significantly lower than that in patients with active inflammatory bowel diseases (IBD). No significant differences in IgA and IgM between the healthy, CAD, and IBD groups were observed (**Figure 1B** and **1C**). The B cells and plasmablasts from sigmoidcolon biopsies were also examined. We found that the frequencies of IgG^+ B cells in CAD patients were significantly higher compared to healthy individuals and significantly lower compared to active IBD patients (**Figure 2B**). Moreover, although IgG^+ B cells can be found in the colon biopsies of healthy individuals, only a few of them presented the $CD27^{++}$ plasmablast phenotype, while in the colon biopsies of CAD individuals, a significantly higher frequencies of IgG^+ B cells presented the $CD27^{++}$ phenotype (**Figure 2D**). The de novo antibody production of colonic B cells cultured in vitro for 12 h was also examined. We found that colonic B cells from CAD patients secreted significantly higher levels of IgG compared to those from healthy individuals, both directly ex vivo and with bacterial antigen stimulation (heat-killed *Staphylococcusaureus* Cowan [SAC] and CpG) (**Figure 3**). Together, these data demonstrated an intestinal low-grade inflammation in CAD patients compared to healthy individuals, characterized by the overrepresentation of secreted IgG and IgG-expressing B cells/plasmablasts.

Increased intestinal infiltration of $CD4^+CXCR5^+$ T cells supported B cell IgG production

Follicular helper T cells (Tfh), characterized by $CD4^+CXCR5^+PD\text{-}1^+ICOS^+Bcl6^+$ expression and IL-4, IL-21 and CXCL13 secretion upon activation, are the specialized providers of B cell help [24, 25]. Tfh cells in the germinal center arecritical for supporting B cell survival, proliferation, and plasma cell differentiation after B cell activation. Their peripheral blood counterpart $CD4^+CXCR5^+$ T cells, but not $CD4^+CXCR5^-$ T cells, can potently support naive B cell antibody production and Ig class-switching recombination [26-28]. Here, we found that the intestinal infiltrations of $CD4^+CXCR5^+$ T cells in CAD subjects is significantly upregulated, compared to that in healthy subjects (**Figure 4B**).

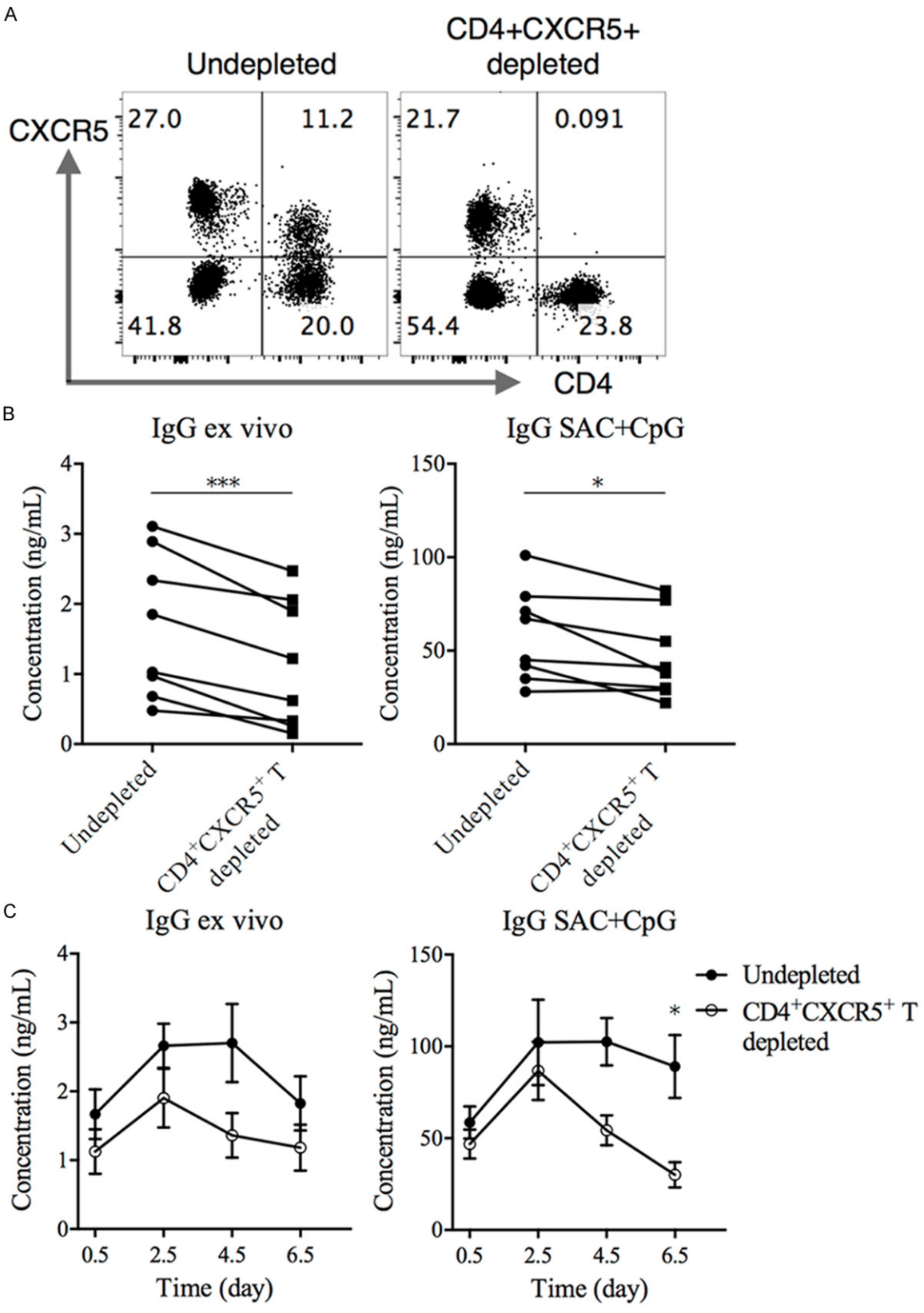

Figure 5. Support of IgG expression by intestinal $CD4^+CXCR5^+$ T cells in CAD patients. After isolation of mononuclear cells from colon biopsies from 8 additional CAD individuals, $CD4^+$ T cells were first positively selected from gut mononuclear cells by paramagnetic beads, and were then stained with PE-conjugated anti-human CXCR5 anti-

body. The $CD4^+CXCR5^+$ cells were depleted from total $CD4^+$ T cells through PE positive selection. The remaining $CD4^+CXCR5^-$ T cells were then added back into the gut mononuclear cells proportionally, such that the potential loss of $CD4^+CXCR5^-$ T cells during the washing steps were compensated. A. The efficacy of $CD4^+CXCR5^+$ T cell-depletion using this procedure. Panels shown were pre-gated on viable lymphocytes as shown in **Figure 2A**. B. Expression of IgG in the supernatant, by undepleted or $CD4^+CXCR5^+$ T cell-depleted intestinal mononuclear cell cultures (2×10^5 cells per 200 µl complete media), after 12 h incubation either directly ex vivo or with SAC+CpG (1 µg/ml each). Paired *t* test. *P < 0.05. ***P < 0.001. C. Expression of IgG in the supernatant by undepleted or $CD4^+CXCR5^+$ T cell-depleted intestinal mononuclear cell cultures (2×10^5 cells per 200 µl complete media) over a period of 6.5 days following biopsy. The supernatant (without or with SAC+CpG) was harvested and replaced every 2 days after the first 12 hours. The IgG concentration was measured in each harvest of supernatant. Mean ± SEM. RM two-way ANOVA followed by Sidak's test. *P < 0.05.

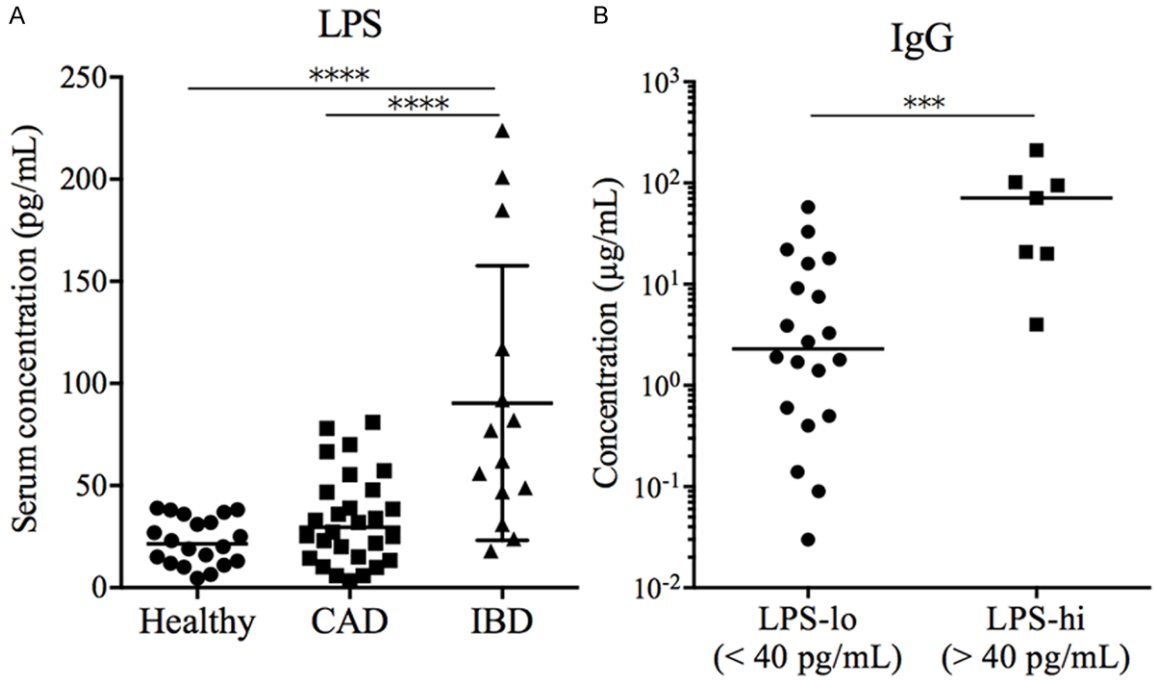

Figure 6. Serum LPS levels in healthy and CAD subjects. A. The serum LPS concentration in 20 healthy, 28 CAD and 14 IBD subjects. Unpaired *t* test with Welch's correction. B. The IgG concentration in colonoscopic washing samples obtained from all 28 CAD subjects, grouped by serum LPS concentration into LPS-lo (< 40 pg/ml) and LPS-hi (> 40 pg/ml) groups. Mann-Whitney test. ***P < 0.001.

To examine whether this increased infiltration of $CD4^+CXCR5^+$ T cells in colon biopsies supported IgG overrepresentation in the gut mucosa of CAD patients, we recruited eight additional CAD patients and performed $CD4^+CXCR5^+$ T cell-depletion experiments using colon biopsy samples. Briefly, $CD4^+$ T cells were first positively selected from gut mononuclear cells by paramagnetic beads. $CXCR5^+$ cells were then stained with phycoerythrin (PE)-conjugated anti-human CXCR5 antibody and depleted from total $CD4^+$ T cell culture through anti-PE positive selection. The remaining $CD4^+CXCR5^-$ T cells were then added back into the gut mononuclear cells proportionally, such that the potential loss of $CD4^+CXCR5^-$ T cells during the washing steps were compensated. As a result, the $CD4^+CXCR5^+$ T cells were depleted but B cells, which were $CXCR5^{++}$, were preserved (**Figure 5A**). The undepleted and $CD4^+CXCR5^+$ T cell-depleted colonic mononuclear cultures were then examined for their ability to secrete IgG. After 12 hours, the undepleted cultures contained significantly higher IgG level in the supernatant than $CD4^+CXCR5^+$ T cells, both directly ex vivo without stimulation and with stimulation by SAC+CpG (**Figure 5B**). The latter was surprising since SAC and CpG were considered T cell-independent stimulants. The differences in IgG secretion between the undepleted and the $CD4^+CXCR5^+$ T cell-depleted cultures increased over time (**Figure 5C**).

Increased serum LPS in a subset of CAD patients

Microbial translocation can occur in patients with sustained intestinal inflammation and disruption at the tight junctions and mucosal barriers [18, 29]. This can result in elevated serum LPS level, which could in turn maintain chronic immune activation in the peripheral immune system [30]. Since evidence of low-grade inflammation in the intestinal mucosa of CAD patients was observed, we then measured the serum LPS levels in healthy and CAD patients. Both groups had significantly lower serum LPS level than that in IBD patients (**Figure 6A**). No significant differences existed between healthy and CAD groups, although a few CAD patients presented elevated serum LPS. Since no healthy individuals had > 40 pg/ml LPS in serum, we used 40 pg/ml as an arbitrary cut-off to categorize the CAD patients into LPS-lo and LPS-hi groups. We found that the LPS-hi group had significantly higher IgG in colonoscopic washings than the LPS-lo group (**Figure 6B**), suggesting an association between IgG inflammation in the gut and LPS translocation.

Discussion

This study provided evidence for the existence of low-level intestinal inflammation in CAD patients. We first observed that the secreted IgG in CAD patients is significantly higher than that in healthy volunteers but lower than that in IBD patients. Sigmoid colon biopsies from CAD patients contained significantly upregulated frequencies of IgG-expressing B cells than those from healthy volunteers. Moreover, the IgG-expressing B cells from CAD subjects contained significantly higher frequencies of $CD27^{++}$ cells, characteristic of plasmablast differentiation. The mononuclear cells from CAD patients also secreted significantly higher levels of IgG in vitro than those from healthy volunteers. We also found that CAD patients had upregulated $CD4^+CXCR5^+$ T cell infiltration in the gut, which supported IgG secretion in vitro. Overall, these data demonstrated a previously unknown dysregulation of intestinal immunity in CAD patients.

In undepleted vs. $CD4^+CXCR5^+$ T cell-depleted experiments, we found that the presence of $CD4^+CXCR5^+$ T cells increased IgG secretion when the cultures were stimulated with SAC and CpG, which was surprising since SAC and CpG could directly activate B cells and increase Ig production through T cell-independent stimulation of Toll-like receptors (TLRs) [31, 32]. It is possible that the presence of $CD4^+CXCR5^+$ T cells improved mucosal B cell survival in vitro, since prolong culturing increased the IgG difference between the undepleted cultures and the $CD4^+CXCR5^+$ T cell-depleted cultures, due to a decline in IgG production in the $CD4^+CXCR5^+$ T cell-depleted cultures over time. In germinal center reactions, Tfh cells can provide survival signals to B cells via multiple pathways, including CD40L, IL-4, IL-21 and BAFF [25]. Intestinal T helper 17 (Th17) and Tfh cells are required for normal IgA secretion [33-35]. Whether the gut-infiltrating $CD4^+CXCR5^+$ T cells supported IgG secretion in vivo, and what mechanisms are in place that favored IgGupregulation, require further studies.

In healthy uninflamed gut, multiple barriers, such as physical blockade by tight junctions and mucus, as well as IgA and IgM binding to the commensals, exist to prevent the infiltration of microbial metabolites into the peripheral blood system. These mechanisms can be disrupted during acute intestinal inflammations, allowing LPS into the serum. Here, we observed that CAD patients with higher serum LPS also had higher intestinal IgG production. It is unlikely that IgG caused LPS translocation in these patients, but rather, both intestinal IgG and serum LPS could suggest an ongoing disruption in the mucosal barriers due to intestinal inflammation. Whether serum LPS contributes to the maintenance of chronic low-grade inflammation in CAD requires further investigations. We did not examine the bacterial composition in this study, but it has previously been demonstrated that western-style diet, with high fat and high processed sugar content, could shift the microbial composition and change microbial gene expression, with potential proinflammatory implications [9, 10, 15, 36]. This diet profile also increases the risk of CAD. It is possible that the IgG overrepresentation and LPS infiltration observed here was a result of diet-induced changes in the microbiota. If so, this represents an additional mechanism by which western-style diet contributes to CAD pathogenesis.

Acknowledgements

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Disclosure of conflict of interest

None.

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