Plasmablast Frequency and Trafficking Receptor Expression are Altered in Pediatric Ulcerative Colitis

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Abstract

BACKGROUND—The incidence of pediatric ulcerative colitis (UC), a chronic autoinflammatory disease of the colon, is on the rise. Although an increased infiltration of B cells from the peripheral blood into the colon occurs in UC, B cell trafficking is understudied. We hypothesized that the frequency of circulating plasmablasts (PBs) and their trafficking receptor (TR) expression may be indicative of the location and degree of pathology in pediatric UC.

METHODS—We conducted multicolor flow cytometry analyses of circulating $IgA^{+/-}$ PBs and IgA^+ memory B cells (MBCs) in pediatric UC patients with remission, mild, moderate and severe state of disease (n=12), and healthy pediatric (n=2) and adult donors (n=11).

RESULTS—Compared to healthy donors the average frequency of PBs among total peripheral blood lymphocytes is increased 30-fold during severe UC activity, and positively correlates with Pediatric Ulcerative Colitis Activity Index score, C-reactive protein level and erythrocyte sedimentation rate. A greater percent of PBs in severe patients express the gut-homing receptors $\alpha4\beta7$ and CCR10, and the inflammatory homing molecule P-selectin ligand (P-sel lig). The percent of IgA^+ MBCs expressing $\alpha4\beta7$, however, is reduced. Furthermore, expression of the small intestine TR CCR9 is decreased on $\alpha4\beta7^{high}$ PBs, and on $\alpha4\beta7^{high}/CCR10^{high}$ PBs and MBCs in these patients, consistent with preferential cell targeting to the colon.

CONCLUSIONS—Peripheral blood PBs with a colon-homing phenotype ($\alpha4\beta7$/CCR10/P-sel lig) are elevated in children with severe UC. Screening this B cell subset may provide a complementary approach in monitoring disease activity or therapeutic efficacy in pediatric UC.

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All authors have nothing to disclose.

Keywords

Inflammatory Bowel Disease; memory B cell; plasmablast; trafficking receptor; ulcerative colitis

Introduction

Inflammatory Bowel Disease (IBD) is a group of chronic autoinflammatory disorders of the gastrointestinal tract, characterized by lesions in the intestinal mucosa. While the precise etiology of IBD remains unclear, it is apparent that a genetic predisposition coupled with environmental triggers leads to an abnormal immunologic response in the gut[1]. Ulcerative colitis (UC) is an IBD typified by diffuse lesions spread throughout the colon[2]. Although the onset of UC may occur in either childhood or adulthood, pediatric patients often present with a more extensive disease at diagnosis[3]. Furthermore, the incidence of pediatric UC has risen globally[4], as well as in Northern California[5], the location of our study.

Intestinal homeostatic conditions are altered in UC, and antibody-secreting cells (ASCs) are implicated in this immunopathology[6,7]. There is an increased infiltration of immature proliferative plasma cells in ulcer bases[8], and an increased level of IgA and IgG ASCs in the colonic mucosa of active UC patients[9,10]. Furthermore, autoantibody production is detected in UC; IgG ASCs from the peripheral blood and colon of UC patients secrete anti-colon antibodies[11]. In patients with inactive disease, increased numbers of ASCs in the colonic lamina propria is a predictive factor of relapse[12].

While it is clear that B cells contribute to UC, the mechanism controlling their migration to the affected tissue is not fully understood, and is especially understudied in pediatric UC. Under homeostatic conditions B cells circulate through lymphoid tissues to develop and sustain an immune defense. Their recirculation is tightly controlled by the sequential association of adhesion molecules with integrin receptors and chemo-attractant 'trafficking receptors' (TRs) expressed on the B cell surface[13]. Unique combinations of TRs direct circulating B cells to specific tissues[14]. For instance many IgA$^+$ ASCs express TRs that can direct their homing to the intestines, including $\alpha 4\beta 7$, an integrin receptor for the mucosal vascular addressin MAdCAM-1[15] and CCR9 and/or CCR10, chemoattractant receptors for the small intestine chemokine, CCL25 and the common mucosal chemokine CCL28, which is highly expressed in the colon[14,16,17]

Peripheral blood plasmablasts are proliferating, immature precursors of tissue. plasma cells, and thus may be indicative of disease activity in the colon of UC patients. While healthy adults and children maintain low levels of PBs in the peripheral blood[18,19], these levels rise during natural infection[20], after vaccination[21], in bacterial septicemia and liver cirrhosis[22], in children and adults with active systemic lupus erythematosus (SLE)[19,22], and in adults with active IBD[23]. In this study we hypothesize that the percentage and TR phenotype of peripheral blood PBs will reflect the disease severity and location of pathology in pediatric UC. Thus, we compared the frequency of peripheral blood B cell subsets and their associated TR expression in UC patients with different disease activity and healthy donors. Through multicolor flow cytometry analysis we show an increased percent of IgA$^{+/-}$ PBs expressing $\alpha 4\beta 7$, CCR10, and P-selectin binding epitopes in the peripheral blood of children with severe UC. This TR phenotype may be indicative of the mechanism of migration of these cells to the inflamed colon.

Ethical Considerations

This study was performed with IRB approval from Kaiser Permanente (IRB# CN-09AWong-01-H) and San Jose State University (IRB# S0902237). All blood samples used in this study were obtained with informed consent.

Materials and Methods

Experimental design and subjects

Pediatric UC patients were enrolled in this study by the Pediatric Gastroenterologists at Kaiser Permanente in Northern California, use clinical lab tests, endoscopy/colonoscopy, histopathology and radiology findings to establish a diagnosis of UC. Disease activity was assessed using the Pediatric Ulcerative Colitis Activity Index (PUCAI)[24], and patients were grouped according to disease severity for analysis (Table 1). Peripheral blood samples were obtained from the patients during routine blood-work, or prior to anti-TNF-α infusion therapy. A total of twelve pediatric UC patients participated in this study. A second sample was obtained from five patients during different disease severity and/or at a different time point from their first donation, for a total of seventeen peripheral blood samples processed for this study. There were six female and six male patients, ranging from 8-16 years of age (mean of 13). Samples were grouped for analysis according to the patient's disease severity at the time of the blood draw, as outlined in Table 1.

Healthy donors were selected for this study based on lack of IBD disease, as well as any other illness (gastrointestinal, respiratory, etc.) for at least one month prior to blood collection. A total of thirteen different healthy donors (two pediatric and eleven adult) participated in this study—ten female and three male—ranging from 15-47 years of age (mean of 32). One sample per healthy donor was used.

Lymphocyte separation from whole blood

Heparinized peripheral blood samples were obtained via venipuncture from the different donor populations: 10 ml from pediatric UC patients (n=17) and 20 ml from healthy donors (n=13). Blood samples were processed using Ficoll (Histopaque-1077, Sigma-Aldrich, St. Louis, MO) density gradient centrifugation, and the peripheral blood mononuclear cell (PBMC) layer was extracted. PBMCs were washed twice in HBSS without Ca^{++}/Mg^{++}, and were resuspended in staining buffer (1X PBS with 2% FCS and 0.1% sodium azide). Cells were blocked overnight with normal human serum (H4522, Sigma-Aldrich) and normal goat serum (Gibco, Invitrogen by Life Technologies, Carlsbad, CA), and cells were then stained and acquired.

Monoclonal antibodies for flow cytometry phenotypic analysis

Primary conjugated antibodies—mouse anti-human CD38 PE-Cy7 (Clone HB7, BD Biosciences, San Jose, CA), goat F(ab')$_2$ anti-human IgA FITC (Invitrogen), mouse anti-human CD19 Alexa Fluor 700 (Clone HIB19, BD Biosciences), rat anti-human CD49f PE-Cy5 (Clone GoH3, BD Biosciences), and mouse anti-human CD4 V500 (Clone RPA-T4, BD Biosciences).

Primary unconjugated antibodies—mouse anti-human CCR9 (ATCC, Manassas, VA) and anti-human CCR10 (courtesy Dr. Dulce Soler-Ferran, Millennium Pharmaceuticals, Cambridge, MA) were labeled in house using goat F(ab')$_2$ anti-mouse Pacific Blue (Invitrogen) and goat F(ab')$_2$ anti-mouse Qdot655 (Invitrogen) respectively. Mouse anti-human α4β7 (Act-1, ATCC) was PE conjugated by Chromoprobe (St. Louis, MO). Recombinant human P-selectin Fc chimera (R&D Systems, Minneapolis, MN) was labeled

in house with Zenon Alexa Fluor 647 human IgG labeling kit (Invitrogen) as directed. Biotin rat anti-human CLA (cutaneous lymphocyte antigen; Clone HECA-452, BD Biosciences) was labeled in house with Streptavidin Qdot565 (Invitrogen).

Cell staining and flow cytometry analysis—3×10^6 PBMCs per donor sample were stained with the previously mentioned antibodies. First, cells were stained with primary anti-CCR9 antibody, washed, followed by secondary anti-mouse Pacific Blue labeling. After a second wash, cells were blocked with normal mouse serum (NMS, Santa Cruz Biotechnology, Santa Cruz, CA). Next, primary anti-CCR10 antibody was labeled with secondary anti-mouse Qdot655, and was then added to the cells. A bench-top conjugation with P-selectin and Zenon AF647 was performed, then was added to the cells to stain P-selectin ligand (P-sel lig). A cocktail of the primary conjugated antibodies was then applied to the cells. Subsequently, cells were stained with primary anti-CLA Biotin antibody, washed, and then labeled with Streptavidin Qdot565. Then a final wash was performed.

Alongside the donor sample, an isotype control with 3×10^6 PBMCs was prepared for the four primary antibodies labeled in house, using the second stage alone of n-1 (addition of Pacific Blue without CCR9, Qdot655 without CCR10, and Qdot565 without CLA), and an incubation of the cells with 0.5M EDTA (to interrupt the binding of P-sel with P-sel lig). In addition, 10% NMS was added in place of the primary antibodies CCR9, CCR10, and CLA. Apart from these modifications, the isotype control was prepared in the same manner as the donor sample. All samples were acquired on a BD Biosciences LSRII flow cytometer (Butcher lab at Stanford University) using FACSDiva software.

Flow cytometry data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR). TR gates were placed based on the isotype controls, or a bimodal comparison of the positive and negative staining populations. During data analysis, positive staining in the isotype control for CCR9, CCR10, P-sel lig, and CLA were subtracted from the donor sample value to account for non-specific binding.

Statistical analyses

Differences in the percent of $IgA^{+/-}$ PBs and IgA^+ MBCs between the pediatric (n=2) and adult (n=11) healthy donors were analyzed using the Wilcoxon test. In addition, relationships between the demographic variables of the UC patients (age, gender, and drug treatment) and the response variables IgA^+ PBs, IgA^- PBs, and IgA^+ MBCs were investigated. To determine whether there is a relationship between the age of the patient and the response levels, we regressed the responses individually on age and tested for zero slope in regression with a regression F-test. To test whether the gender of the patients has an influence on the responses, we used Wilcoxon's test. To test whether the mean responses of the patients vary by the treatment regimen they are subjected to, we performed an analysis of variance (ANOVA F-test). For this analysis the patients were placed into three groups based on treatment: anti-inflammatory agents, immunomodulators, and biological therapies (therapies detailed in Table 1). If the patient was treated with multiple therapies the patient was placed in a treatment group based on the strongest therapy received (anti-inflammatory agents<immunomodulators<biological therapies). All other statistical analyses were performed using Student's *t*-test or linear regression analysis, as identified in the Results section. A p<0.05 value was considered statistically significant.

Results

Peripheral blood B cell subsets in pediatric UC patients

Circulating plasmablasts (PBs) are in transit to the tissue, where they mature into antibody-secreting cells (ASCs); thus, an increased quantity in the peripheral blood may indicate disease activity in the colon of UC patients. Memory B cells (MBCs) are an immunologic surveillance population that reflect past antigen encounter, and comprise a pool of cells from which PBs specific to previously encountered antigens may be derived. We monitored the percent of IgA$^+$ PBs, IgA$^-$ PBs, and IgA$^+$ MBCs in the peripheral blood, to determine whether these B cell subsets were increased in pediatric UC.

Three B cell populations were gated from the PBMCs (Figure 1A-C): CD38^{high}/IgA$^-$ cells (IgA$^-$ PBs), CD38^{high}/IgA$^+$ cells (IgA$^+$ PBs), and CD38$^{med/low}$/IgA$^+$ cells (IgA$^+$ MBCs)[20]. IgA$^+$ MBCs were further gated to select for CD19 expression. CD4$^+$ cells were gated out to exclude them from analysis. There was no difference in the frequency of IgA$^+$ PBs between pediatric (0.11%) and adult (0.08%) healthy donors (Wilcoxon test, p=0.23), or in the frequency of IgA$^-$ PBs (0.05 and 0.08% respectively, p=0.92) or IgA$^+$MBCs (0.47 and 0.62%, p=0.77) (data not shown). Therefore all healthy donors were combined for statistical comparisons.

Pediatric UC patients with severe disease had a greater percent of IgA$^+$ PBs among total peripheral blood lymphocytes (2.48%) than healthy controls (0.09%, p=0.02), as well as patients with mild (0.26%, p=0.02) and moderate (0.16%, p=0.02) disease activity (Figure 1D). While the percent of IgA$^-$ PBs was greater in severe UC patients (2.54%) compared to other groups, this difference was not statistically significant (Figure 1E). However, when a severe patient who had 7.6% IgA$^-$ PBs (a high outlier) was removed from the analysis, the percent of IgA$^-$ PBs in severe patients (1.5%) was greater than that in healthy donors (0.07%, p=0.03), mild (0.23%, p=0.048) and moderate (0.18%, p=0.04) patients. In contrast to the PBs, the percent of IgA$^+$ MBCs was similar between healthy and UC patient groups (average range from 0.5-1.2%, p>0.05 for all comparisons) (Figure 1F).

Within the UC patients, there was no correlation between age of the patient and percent of IgA$^+$ PBs (regression F-test, p=0.93), IgA$^-$ PBs (p=0.67), or IgA$^+$ MBCs (p=0.31), or between the gender of the patient and the percent of IgA$^+$ PBs (Wilcoxon test, p=0.35), IgA$^-$ PBs (p=0.92), or IgA$^+$ MBCs (p=0.92) (data not shown). Additionally, no significant difference was observed in the mean percent of IgA$^+$ PBs between the patients being treated with anti-inflammatory agents, immunomodulators, or biological therapies (Anova F-test, p=0.62), or in the percent of IgA$^-$ PBs (p=0.72) and IgA$^+$ MBCs (p=0.56) (data not shown).

Correlation between circulating plasmablasts and clinical parameters in pediatric UC patients

Inflammatory activity in pediatric UC patients is closely monitored using clinical lab tests such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). In addition, the PUCAI scoring system is an assessment tool that provides a way to quantify UC disease severity based on reported symptoms.

Both CRP and ESR values positively correlate with PUCAI score in pediatric UC patients (Figure 2) (R=0.72 and R=0.71 respectively). The percent of peripheral blood IgA$^+$ PBs, and to a lesser degree IgA$^-$ PBs, correlate with PUCAI score at a comparable level to these established clinical tests (R=0.67 and R=0.54 respectively). In addition, the percent of IgA$^+$ PBs in circulation correlates very well with CRP (R=0.89) and with ESR (R=0.74), while the percent of IgA$^-$ PBs correlates well, but to a lesser extent (with CRP R=0.87, and with ESR R=0.62). Overall, the frequency of IgA$^+$ PBs in the peripheral blood correlated well,

and better, than the IgA⁻ PBs with each of the tested parameters (data not shown for IgA⁻ PBs or ESR).

Single trafficking receptor analysis of plasmablasts

Previous studies indicate that expression of the TR ligands MAdCAM-1 and CCL28, and the vascular receptor P-selectin, is increased in the inflamed colon tissue of UC patients[25,26,27]. Thus, we determined whether circulating PBs in UC patients demonstrate an altered expression of the corresponding TRs, $\alpha 4\beta 7$ and CCR10, and P-sel lig respectively. In addition CCR9 (a TR with particular relevance to small intestine homing but not colon homing[16,28]) was examined. Since extraintestinal manifestations may occur in patients with IBD, we also measured the expression of the skin homing receptor CLA[29], a carbohydrate ligand for vascular E-selectin, and the integrin $\alpha 6$ chain (i.e. CD49f), whose expression is associated with homing to the skin, lung, and other extraintestinal sites[30,31], IgA$^{+/-}$ PBs were identified as shown in Figure 1, then TR expression was examined using the gating strategy in Figure 3A.

Severe UC patients displayed a greater percent of $\alpha 4\beta 7^{high}$ IgA$^+$ PBs (69% of IgA$^+$ PBs) in circulation than healthy donors (43%, p=0.002) and moderate patients (46%, p=0.001) (Figure 3B). Similarly, the percent of CCR10^{high} IgA$^+$ PBs was increased in severe UC patients (56% of IgA$^+$ PBs) compared to healthy donors (12%, p=0.0001), and patients with mild (28%, p=0.02) and moderate (26%, p=0.003) disease. Furthermore, P-sel lig^{high} IgA$^+$ PBs were more frequent in severe UC patients (29% of IgA$^+$ PBs) compared to healthy donors (18%, p=0.03) and patients with mild disease (15%, p=0.02). The IgA⁻ PBs demonstrated similar trends to the IgA$^+$ PBs for all three TRs (Figure 3C).

On average UC patients had fewer CCR9^{high} PBs than HDs, and UC patients with moderate disease demonstrated significantly less CCR9^{high} IgA$^+$ and IgA⁻ PBs (9 and 4% respectively) than healthy donors (18 and 13%, p=0.04 and p=0.048 respectively). The percent of CD49f^{high} and CLA^{high} PBs was mainly similar between groups.

Trafficking receptor expression on mucosal and non-mucosal plasmablasts

The combinatorial expression of TRs drives cell homing to the intestines and other sites[13]. Since we observed an increase in PBs that express CCR10 and P-sel lig in severe UC, we further investigated whether this correlated with $\alpha 4\beta 7$ expression, and whether this TR expression occurred primarily on the mucosal ($\alpha 4\beta 7^{high}$) or non-mucosal ($\alpha 4\beta 7^{low}$) homing cells. IgA$^+$ and IgA⁻ PBs from Figure 1 were segregated into $\alpha 4\beta 7^{high}$ and $\alpha 4\beta 7^{low}$ populations, then analyzed for the expression of other TRs as shown in Figure 4A.

The percent of mucosal IgA$^+$ PBs with CCR10^{high} and P-sel lig^{high} expression was greater in severe UC patients (59% and 28% respectively) than in healthy controls (15%, p=0.0002, and 9%, p=0.001 respectively), and patients with mild (30%, p=0.03, and 13%, p=0.0099) and moderate disease (29%, p=0.004, and 13%, p=0.01) (Figure 4B). Although the percent of CCR10^{high} cells was similar in the $\alpha 4\beta 7^{high}$ and $\alpha 4\beta 7^{low}$ IgA$^+$ PB subsets, P-sel lig had a greater percentage of expression in the non-mucosal cells. Similar expression patterns for CCR10 and P-sel lig were observed in the mucosal and non-mucosal IgA⁻ PBs (Figure 4C).

The frequency of mucosal IgA$^+$ PBs with CCR9^{high} expression was decreased in moderate (16%, p=0.02) and severe (15%, p=0.002) UC patients compared to healthy donors (36%); similar trends were observed in the IgA⁻ PBs. The percent of CCR9^{high} non-mucosal PBs was low in all groups, and on average less than 5% of non-mucosal PBs demonstrated CCR9^{high} expression. The frequency of mucosal IgA$^{+/-}$ PBs with CD49f^{high} expression was similar for healthy and UC patient groups (average range between 12-20%). However,

$\alpha4\beta7^{low}$ non-mucosal PBs with CD49f ^{high} expression were consistently increased (average range between 27-32%) compared to their $\alpha4\beta7^{high}$ mucosal homing counterparts.

The cellular expression of $\alpha4\beta7$ and CLA are usually mutually exclusive, as these TRs mediate lymphocyte homing to different sites. Therefore the percent of mucosalPBs that were CLA^{high} was very low—on average less than 6% of cells (CLA data not shown). The frequency of CLA^{high} cells among non-mucosal PBs was also low—on average less than 10% of cells.

Trafficking receptor expression on memory B cells in pediatric UC patients

MBCs act as an immunological surveillance population, and their presence reflects past antigen encounter. Since UC is a chronic disease, we predicted that TR expression on peripheral blood MBCs may be indicative of the location of past disease activity in UC patients. We compared the TR expression of IgA$^+$ MBCs to their activated counterparts, the IgA$^+$ PBs, and also compared IgA$^+$ MBC TR expression between donor groups.

The differential TR expression on IgA$^+$ PBs and MBCs is visually demonstrated in overlay dot plots of the two B cell populations from all severe UC patients (Figure 5A). Co-expression of $\alpha4\beta7$ and CCR10 or P-sel lig was consistently higher on PBs than MBCs. However, the co-expression of $\alpha4\beta7$ and CCR9 or CD49f was more similar between the cell subsets.

Unlike the PBs, the frequency of CCR10^{high}, P-sel lig^{high}, CCR9^{high}, and CD49f^{high} IgA$^+$ MBCs was similar in healthy donors and UC patients (p>0.05 for all comparisons; data not shown). However in contrast to the $\alpha4\beta7^{high}$ PBs, the frequency of $\alpha4\beta7^{high}$ IgA$^+$ MBCs was decreased in severe UC patients (35%) compared to healthy donors (51%, p=0.004) (Figure 5B).

CCR9 expression on B cell subsets co-expressing $\alpha4\beta7$ and CCR10

$\alpha4\beta7$ and CCR10 expression without CCR9 is expected to favor cell homing to the colon, as MAdCAM-1 and CCL28 are highly expressed in the colon, whereas CCR9 and its ligand CCL25 are major determinants of lymphocyte recruitment to the small intestine[14]. Therefore we examined $\alpha4\beta7^{high}$/CCR10^{high} B cell subsets for CCR9 expression.

In general the proportion of $\alpha4\beta7^{high}$/CCR10^{high} PBs and MBCs expressing CCR9 decreased as the severity of UC disease intensified (Figure 6). The frequency of $\alpha4\beta7^{high}$/CCR10^{high} IgA$^+$ and IgA$^-$ PBs with CCR9 expression was decreased in the moderate (23%, p=0.04, and 8%, p=0.03 respectively) and severe (17%, p=0.009, and 12%, p=0.04) UC disease activity groups compared to healthy donors (43 and 33%). In addition, the percent of $\alpha4\beta7^{high}$/CCR10^{high} IgA$^+$ MBCs expressing CCR9 in severe UC (30%) was decreased compared to healthy controls (50%, p=0.02).

Discussion

B lymphocytes contribute to the immunopathology of UC[6,7], however the mechanisms controlling migration to the affected tissue in this disease is understudied. We examined peripheral blood B cell subsets in UC patients using the rationale that their frequency and migratory potential, reflected by their TR expression, would be altered and therefore indicative of B cell activity in the colon. The purpose of this study was to characterize TR expression on B cell subsets, specifically PBs, elicited in UC. We focused our study on pediatric patients with recent onset of illness, and hence with fewer confounding effects of comorbidities and long-term medication.

Here we show that the average frequency of both IgA$^+$ and IgA$^-$ PBs are elevated in children with severe UC as compared to healthy donors and patients with mild and moderate disease activity. Similar findings have recently been reported by Hosomi et. al[23] in adult UC patients with active disease. In contrast, the percent of peripheral blood IgA$^+$ MBCs was unaltered in patients at all disease activity levels compared to healthy individuals; a feature that has been demonstrated in other chronic autoimmune disorders, such as pediatric SLE[19]. Therefore the frequency of peripheral blood PBs, but not MBCs, is a good indicator of disease severity and inflammation in UC.

As the UC disease activity increased in severity, we observed a dramatic increase in the percent of IgA$^+$ and IgA$^-$ PBs with α4β7^{high} and CCR10^{high} or P-sel lig^{high} co-expression, supporting the hypothesis that these TRs play a role in PB migration to the colon in UC. Of note, Hosomi et al.[23] did not observe an increase in CCR10 expressing PBs (defined as CD19$^+$/CD20$^-$ cells) in adult UC. Since all activity levels were combined in their study, perhaps a rise in CCR10 expressing PBs was masked by UC patients with milder disease. Another possibility is that the relevance of CCR10 may be different in adult UC vs. pediatric UC, and this TR may have greater significance during the initial onset or early in disease.

P-sel lig, along with its corresponding receptor P-selectin, is upregulated in immune cells and on the vascular endothelium in response to inflammation[32]. Here we observed an increased frequency of both α4β7^{low} and α4β7^{high} PBs expressing P-sel lig in severe UC, further indicating the high levels of colon inflammation at this stage of disease, and perhaps reflecting the massive migration of cells expressing P-sel lig into the inflamed colon.

There was a trend in the mild and moderate activity groups for an increased percent of α4β7^{high} PBs but not CCR10^{high} and P-sel lig^{high} cells; only patients with severe disease activity displayed a higher frequency of CCR10^{high} and P-sel lig^{high} PBs. It is possible that UC therapeutics decrease certain TR responses, i.e. CCR10 and P-sel lig upregulation on cells. Alternatively, high CCR10 and high P-sel lig expression may be imprinted on PBs only at advanced stages of disease, where intensified exposure to colon antigens has occurred due to extensive inflammation and tissue damage. Thus, a rise in the frequency of peripheral blood PBs imprinted with high CCR10 and high P-sel lig expression may reflect a more acute manifestation of UC, than does a rise in PBs with high α4β7 only.

The frequency of IgA$^+$ MBCs expressing α4β7 at high levels decreased as UC disease activity intensified, and severe patients had a lower proportion of these cells compared to healthy donors. α4β7^{high} MBCs may redistribute from the blood to the intestines in response to inflammation, or may be re-activated in patients during a flare in disease activity, and thus become depleted from the circulating α4β7 MBC pool.

We found a reduced frequency of PBs with CCR9^{high} expression in pediatric UC, and moderate and severe patients had fewer PBs co-expressing α4β7 and CCR9. There is no small intestine involvement in UC, a disease that solely affects the colon. Therefore no upregulation in CCR9, which preferentially targets lymphocytes to the small intestine, was expected. In severe UC patients there was also a decrease in the percent of α4β7^{high}/CCR10^{high} PBs and MBCs that express CCR9, which indicates these patients have increased levels of PBs that may preferentially migrate to the colon vs. the small intestine[33].

Extraintestinal manifestations may occur in pediatric IBD, and some patients develop pathologies of the skin, liver and other complications[34,35]. The proportion of cells that expressed CLA, a trafficking molecule associated with skin homing, was low on both PBs and MBCs. However, only one of the pediatric UC patients in this study developed a skin pathology, which may explain the low frequency of cells expressing this molecule.

While the role of the integrin receptor CD49f (aka α6) in UC is not well documented, B cells in the colon of UC patients, but not in the colon of Crohn's or non-IBD patients, were found to express this receptor[36]. In our study the frequency of B cells expressing CD49f was unaltered in UC, and the expression of the receptor was more prominent on $\alpha4\beta7^{low}$ cells, supporting the idea that CD49f is not associated with gut-homing lymphocytes[30]. Our results indicate that CD49f plays a minimal role in B cell trafficking in pediatric UC.

In conclusion, our study demonstrates an increase in the frequency of IgA$^+$ and IgA$^-$ PBs in the peripheral blood of severe UC patients. PB frequency positively correlates with UC disease severity and inflammation, indicating circulating PB subsets as a good indicator of disease activity in pediatric UC. In addition, circulating IgA$^{+/-}$ PBs with $\alpha4\beta7^{high}$/CCR10^{high}/P-sel lig^{high} expression are elevated in pediatric UC patients with severe disease. In combination these trafficking receptors likely facilitate PB homing to the inflamed colonic tissue. Thus, this cell subset may provide a therapeutic target for the treatment of UC, and the surveillance of circulating PBs and their associated TR expression may potentially be used as a non-invasive, complementary approach to monitor disease activity or therapeutic efficacy in children with this disease.

Acknowledgments

Abbreviations used in this manuscript

ASC	antibody secreting cell
CRP	C-reactive protein
ESR	erythrocyte sedimentation rate
HD	healthy donor
IBD	Inflammatory Bowel Disease
MBC	memory B cell
PB	plasmablast
PBMC	peripheral blood mononuclear cell
P-sel lig	P-selectin ligand
PUCAI	Pediatric Ulcerative Colitis Activity Index
SLE	systemic lupus erythematosus
TR	trafficking receptor
UC	ulcerative colitis

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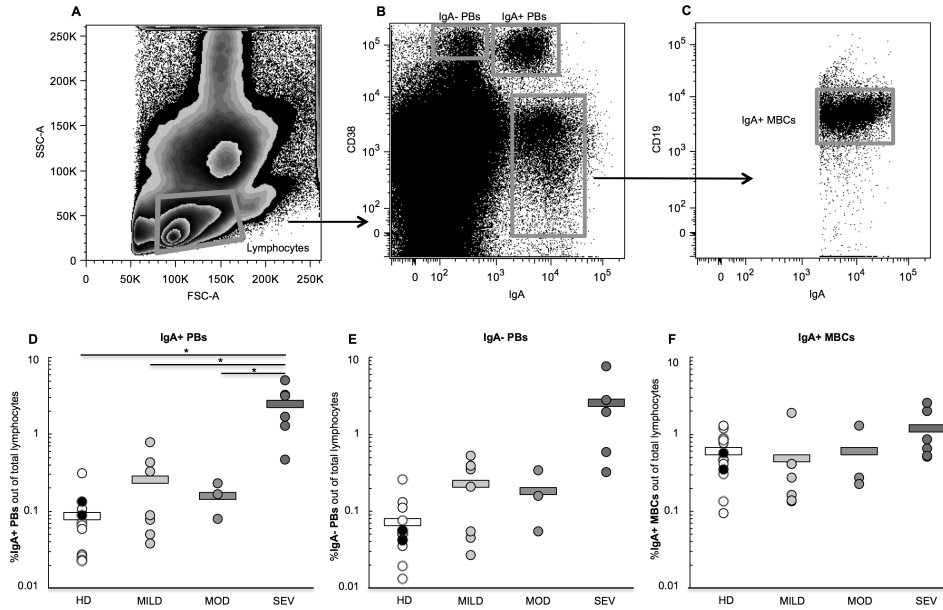**Figure 1.**
Representative flow cytometry plots of B cell subset gating strategy. From left to right: (A) lymphocytes are gated, (B) three B cell subsets are identified— the IgA⁻plasmablasts (PBs), the IgA⁺ PBs, and the IgA⁺ memory B cells (MBCs), then (C) the IgA⁺ MBCs are further gated for CD19 expression. The percent of (D) IgA⁺ PBs, (E) IgA⁻ PBs, and (F) IgA⁺ MBCs out of total peripheral blood lymphocytes was determined for all healthy donor and pediatric UC patient blood samples. Samples were grouped according to type of donor; groups here are healthy donors (HD) (black dots are pediatric HD, and white dots are adult HD), and pediatric patients with mild UC (MILD), moderate UC (MOD), and severe UC (SEV) disease activity. Circles represent individual samples and bars represent the group's average. Student's *t*-test was performed between all UC patient groups vs. healthy donors, and between the severe group vs. the mild and moderate UC groups. *p<0.05; no asterisk indicates p>0.05. Significance (p<0.05) is present in (E) when the severe patient with the highest percent of IgA⁻ PBs (a high outlier) is removed from the analysis.
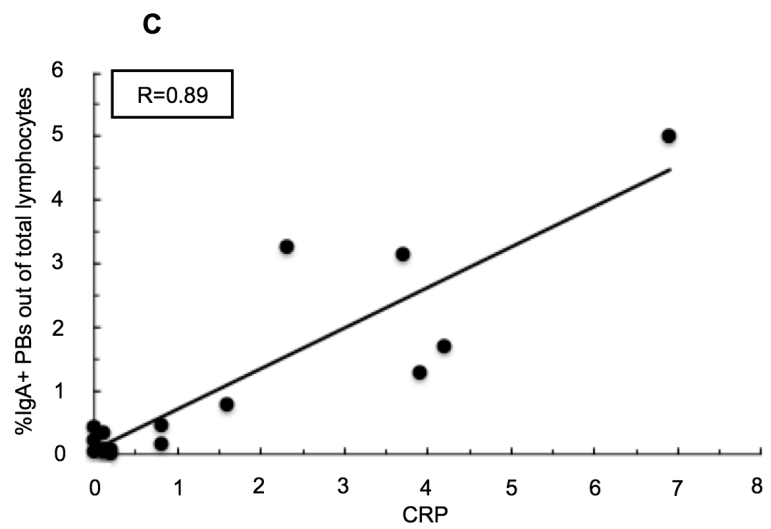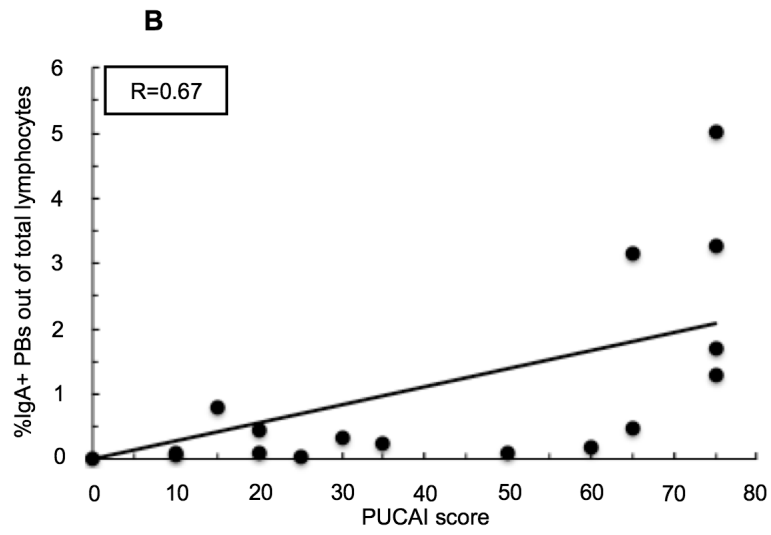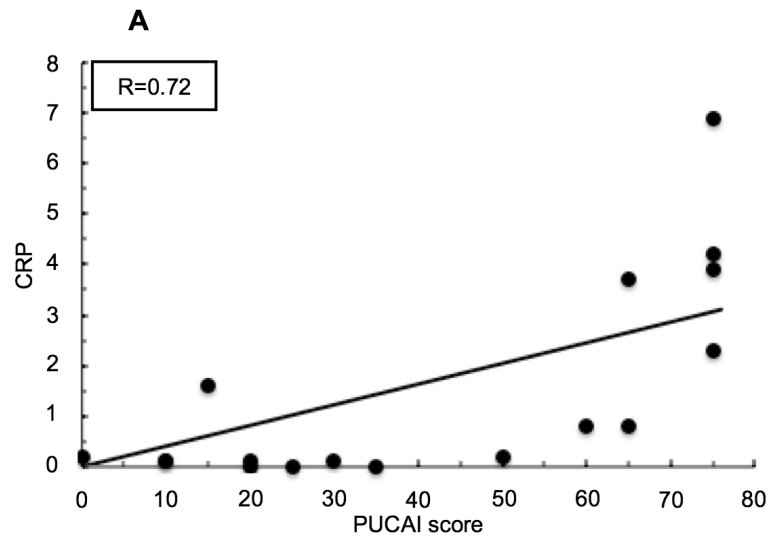
A

B

C

Figure 2.
Linear regression analyses in pediatric patients with UC. (A) Correlation between serum C-reactive protein (CRP) levels and PUCAI score. (B) Correlation between percent of IgA[+] plasmablasts (PBs) out of total peripheral blood lymphocytes and Pediatric Ulcerative Colitis Activity Index (PUCAI) score. (C) Correlation between percent of IgA[+] PBs out of total peripheral blood lymphocytes and CRP.
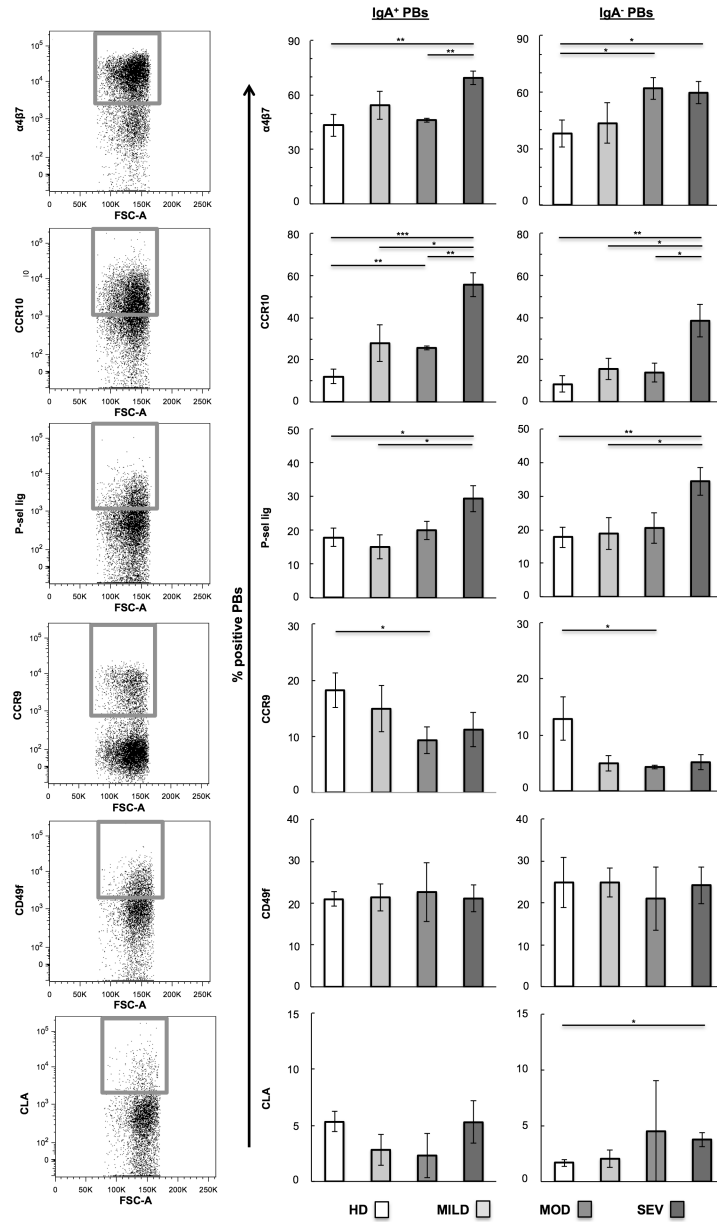
Figure 3.
Single trafficking receptor (TR) analysis on IgA$^{+/-}$ plasmablasts (PBs). (A) Representative flow cytometry plots of individual TR expression on PBs. Gates represent high-TR-expressing cells. Samples were grouped according to type of donor: healthy donors (HD) and pediatric patients with mild UC (MILD), moderate UC (MOD), and severe UC (SEV) disease activity. Average percent of (B) IgA$^+$ PBs and (C) IgA$^-$ PBs with TR^{high} expression of α4β7, CCR10, P-selectin ligand (P-sel lig), CCR9, CD49f, or CLA. Student's *t*-test was performed between all UC patient groups vs. healthy, and between the severe group vs. the mild and moderate UC groups. *p<0.05, **p<0.01, ***p<0.001; no asterisk indicates p>0.05.
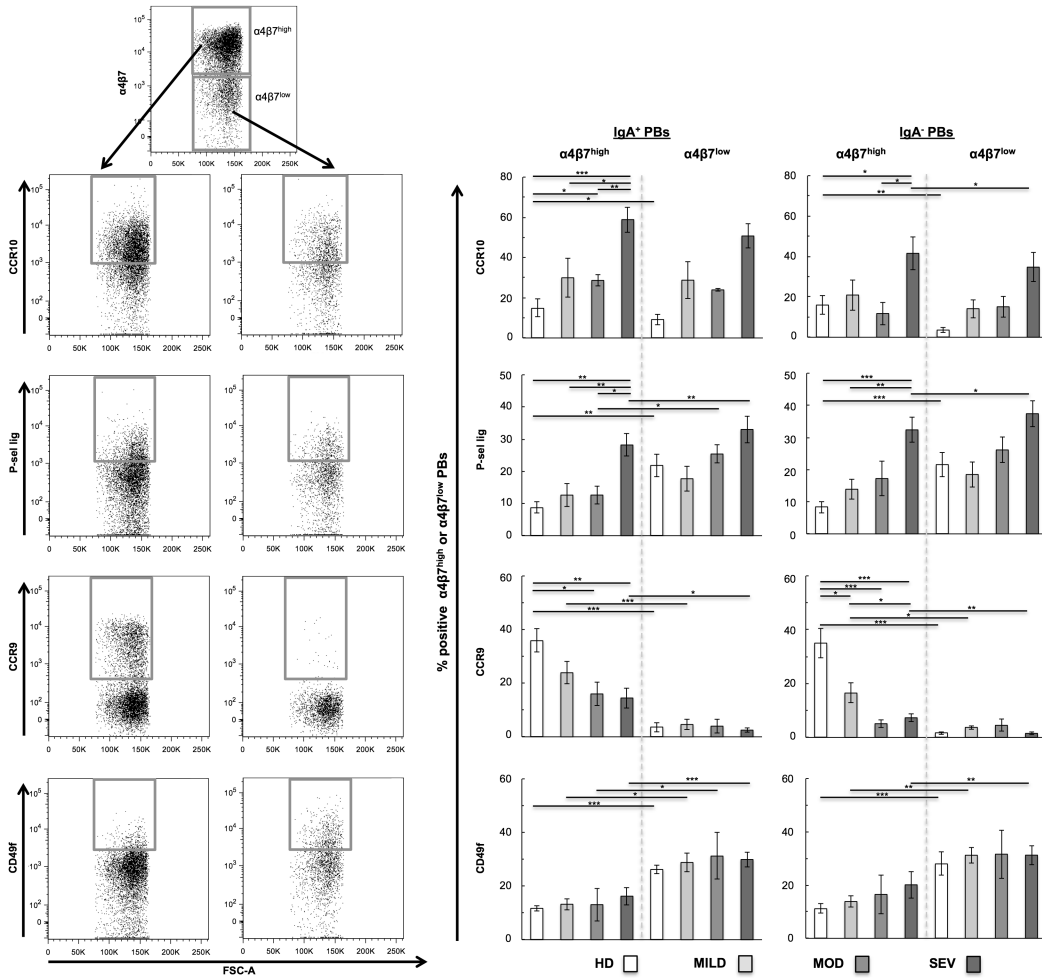
Figure 4.
Trafficking receptor (TR) analysis on mucosal ($\alpha4\beta7^{high}$) and non-mucosal ($\alpha4\beta7^{low}$) IgA$^{+/-}$ plasmablasts (PBs). (A) Representative flow cytometry plots for TR gating strategy on PBs. From top to bottom: IgA$^{+/-}$ PBs are gated for $\alpha4\beta7^{high}$ expression (upper gate) and $\alpha4\beta7^{low}$ expression (lower gate), then individual TR expression is examined on each of these subsets. Gates represent cells with TR^{high} expression of CCR10, P-selectin ligand, CCR9, or CD49f. Bar graphs display the average percent of $\alpha4\beta7^{high}$ and $\alpha4\beta7^{low}$ (B) IgA$^+$ PBs and (C) IgA$^-$ PBs with TR^{high} expression. Samples were grouped according to type of donor: healthy donors (HD) and pediatric patients with mild UC (MILD), moderate UC (MOD), and severe UC (SEV) disease activity. Statistical analyses for $\alpha4\beta7^{high}$ cells were performed between all UC patient groups vs. healthy, and between the severe group vs. the mild and moderate UC groups. In addition, statistical analyses were performed between $\alpha4\beta7^{high}$ and $\alpha4\beta7^{low}$ cells within groups using Student's t-test. *p<0.05, **p<0.01, ***p<0.001; no asterisk indicates p>0.05.
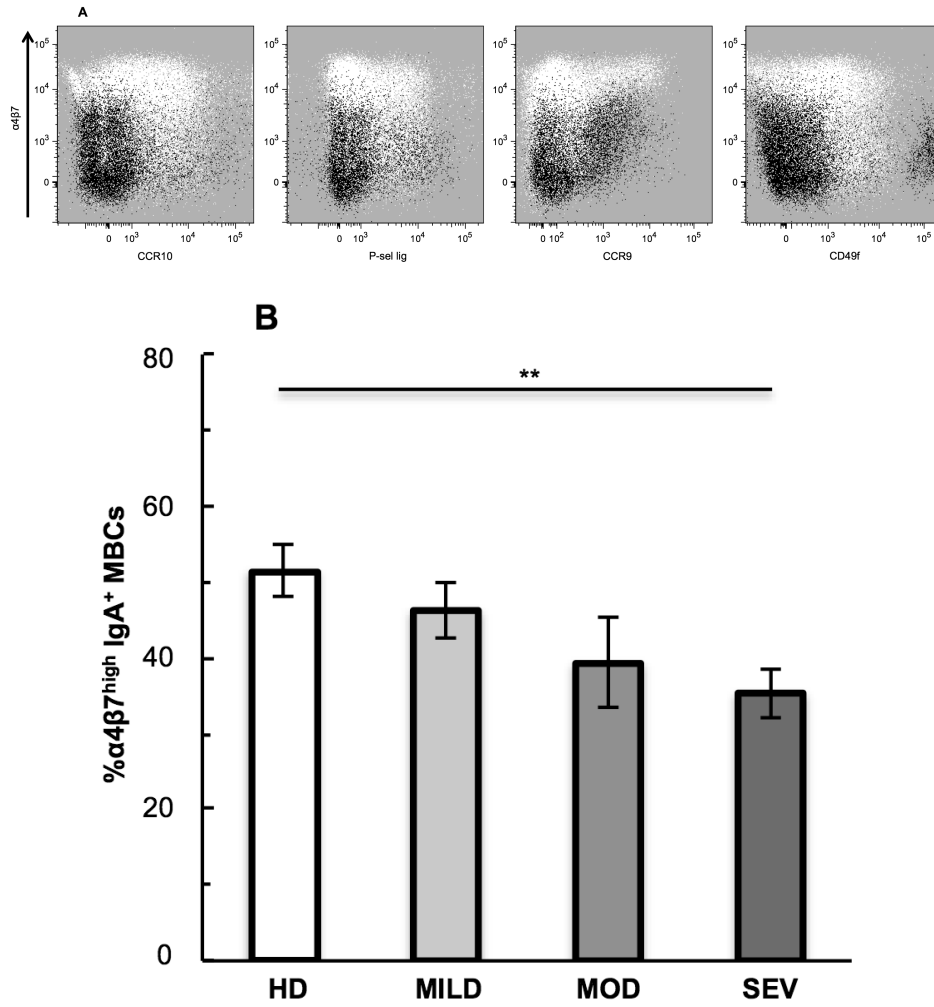
Figure 5.
Comparison of IgA$^+$ plasmablasts (PBs) and IgA$^+$ memory B cells (MBCs). (A) Flow cytometry dot plot overlays of trafficking receptor (TR) co-expression of α4β7 with CCR10, P-sel lig, CCR9, or CD49f on IgA$^+$ PBs (white dots) and IgA$^+$ MBCs (black dots) from all severe UC patients combined. (B) Single TR expression on IgA$^+$ MBCs, showing the average percent of MBCs with α4β7^{high} expression in each donor group. Student's t-test was performed between all UC patient groups vs. healthy, and between the severe group vs. the mild and moderate UC groups. **p<0.01; no asterisk indicates p>0.05.
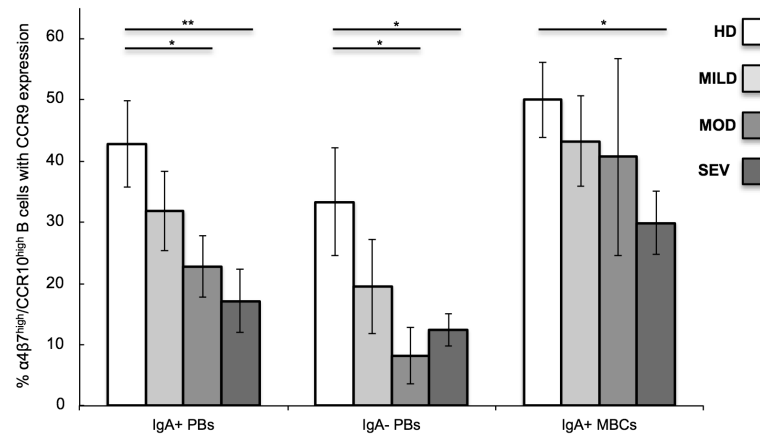
Figure 6.
CCR9 expression on $\alpha4\beta7^{high}$/CCR10^{high} IgA$^{+/-}$ plasmablasts (PBs), and IgA$^+$ memory B cells (MBCs). Samples were grouped according to type of donor: healthy donors (HD) and pediatric patients with mild UC (MILD), moderate UC (MOD), and severe UC (SEV) disease activity. Statistical analyses within each B cell subset were performed between healthy donors and UC patient disease activity groups using Student's *t*-test. *p<0.05; no asterisk indicates p>0.05.

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Table 1

List of pediatric UC patients and sample information

Sample	PUCAI score (0-85)	£PUCAI disease activity group	Age (years)	Gender (M/F)	ESR	CRP	Treatment
1	0	¥Remission	12	M	9	0.2	**Methotrexate
£2	10	Mild	16	M	3	0.1	***Remicade
3	10	Mild	9	F	33	0.1	**Imuran, *5ASA
4	15	Mild	16	M	51	1.6	***Humira
§5	20	Mild	8	M	13	0	*Steroids
ξ6	20	Mild	16	M	8	0.1	***Remicade
7	25	Mild	15	F	35	0	***Remicade, **Imuran
8	30	Mild	15	F	24	0.1	*Steroids
9	35	Moderate	15	F	31	0	*5ASA *5ASA, *Steroids, and **6MP before
10	50	Moderate	15	F	13	0.2	***Remicade
†11	60	Moderate	9	M	40	0.8	***Humira
†12	65	Severe	9	M	39	0.8	***Humira
δ13	65	Severe	15	M	29	3.7	***Remicade
§14	75	Severe	8	M	87	2.3	*Steroids
‡15	75	Severe	15	F	48	4.2	***Remicade
δ16	75	Severe	15	M	85	6.9	***Remicade
‡17	75	Severe	15	F	71	3.9	***Remicade

£Activity group is based on PUCAI user guide (score range from 0-85): Remission, <10; Mild, 10-34; Moderate, 35-64; Severe, 65.

¥The remission group was excluded from most analyses due to the small sample size, which precluded the use of statistical analyses on this patient as a group.

*Anti-inflammatory agents: 5ASA and steroids.

**Immunomodulators: Imuran, Methotrexate, and 6MP.

***
Biological therapies: anti-TNF-α monoclonal antibodies, Humira and Remicade.

ξ
Samples with the same symbol were obtained from the same patient. *M* male, *F* female, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *5ASA* 5-aminosalicylic acid, *6MP* 6-mercaptopurine.

§
Samples with the same symbol were obtained from the same patient. *M* male, *F* female, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *5ASA* 5-aminosalicylic acid, *6MP* 6-mercaptopurine.

†
Samples with the same symbol were obtained from the same patient. *M* male, *F* female, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *5ASA* 5-aminosalicylic acid, *6MP* 6-mercaptopurine.

δ
Samples with the same symbol were obtained from the same patient. *M* male, *F* female, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *5ASA* 5-aminosalicylic acid, *6MP* 6-mercaptopurine.

‡
Samples with the same symbol were obtained from the same patient. *M* male, *F* female, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *5ASA* 5-aminosalicylic acid, *6MP* 6-mercaptopurine.