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

Nicole Jeanne Tarlton
San Jose State University

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PLASMABLAST FREQUENCY AND TRAFFICKING RECEPTOR EXPRESSION
ARE ALTERED IN PEDIATRIC ULCERATIVE COLITIS

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Nicole J. Tarlton

May 2012

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The Designated Thesis Committee Approves the Thesis Titled
PLASMABLAST FREQUENCY AND TRAFFICKING RECEPTOR EXPRESSION
ARE ALTERED IN PEDIATRIC ULCERATIVE COLITIS

by

Nicole J. Tarlton

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

SAN JOSÉ STATE UNIVERSITY

May 2012

Dr. Tzvia Abramson, Committee Chair

Department of Biological Sciences

Dr. William J. Murray

Department of Biological Sciences

Dr. Eugene C. Butcher

Department of Pathology, Stanford

ABSTRACT

PLASMABLAST FREQUENCY AND TRAFFICKING RECEPTOR EXPRESSION ARE ALTERED IN PEDIATRIC ULCERATIVE COLITIS

by Nicole J. Tarlton

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. 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 disease (n=12) and healthy pediatric (n=2) and adult donors (n=11). Compared to healthy donors the average frequency of circulating PBs increased 30-fold during severe UC activity and positively correlated with Pediatric Ulcerative Colitis Activity Index score, C-reactive protein level, and erythrocyte sedimentation rate. A greater percent of PBs in severe patients expressed the gut-homing receptors $\alpha 4\beta 7$ and CCR10, and the inflammatory homing molecule P-selectin ligand (P-sel lig). The percent of IgA⁺ MBCs expressing $\alpha 4\beta 7$, however, was reduced. Furthermore, expression of the small intestine TR CCR9 decreased on $\alpha 4\beta 7^{\text{high}}$ PBs and on $\alpha 4\beta 7^{\text{high}}$ /CCR10^{high} PBs and MBCs in these patients, consistent with preferential cell targeting to the colon. In conclusion, peripheral blood PBs with a colon-homing phenotype were 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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This work is the result of collaborative efforts between San José State University, Kaiser Permanente in Santa Clara, and Stanford University. First I would like to thank Dr. Anthony Wong and Dr. Oren Abramson, the pediatric gastroenterologists at Kaiser Permanente, for recruiting patients for this study. In addition, many thanks are due to the patients and healthy donors who participated in this study, without whose contribution this work would not have been possible. Next I would like to thank Caroline Green for getting this project started at San José State University, and for the extensive training she provided me. Many thanks are due to Nicole Lazarus and Lusijah Rott from Stanford University for protocol design, for training and support in the lab—especially with the LSRII flow cytometer—and for critical editing of this manuscript. Also, thank you to Dr. Kenneth Youngman for critically editing this manuscript, and thank you to Dr. Martina Bremer for assistance with statistical analyses.

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PREFACE

This thesis is composed of three chapters. Chapter I provides an overview of the research project, detailing the rationale behind the project design. Chapter II presents the manuscript entitled *Plasmablast Frequency and Trafficking Receptor Expression are Altered in Pediatric Ulcerative Colitis* in journal format according to the guidelines set by the journal *Inflammatory Bowel Diseases*, to which this manuscript was accepted for publication. Chapter III summarizes the conclusions of this work, as well as discusses the future directions for this project.

CHAPTER I
INTRODUCTION

Overview of the Research Project

Inflammatory Bowel Disease (IBD) is a group of chronic autoimmune and inflammatory disorders of the gastrointestinal tract (GI), encompassing Crohn's Disease (CD) and ulcerative colitis (UC). The two diseases are distinguished based on the appearance and location of lesion formation in the GI tract: CD is characterized by discrete penetrating lesions that may be spread all throughout the GI tract, while the lesions in UC are diffuse and superficial, and only occur in the colon. UC was the initial focus of this research because lesion involvement is limited to one organ—the colon—and hence changes in the parameters we measured could more easily be attributed to immunopathology in the colon, vs. in CD where the occurrence of immunopathology in one or more organs would have to be accounted for.

While both children and adults may be affected by UC, pediatric patients were the focus of our study for several reasons: 1) pediatric patients with a recent onset of illness (vs. adults that have had the disease for a longer period of time) would demonstrate fewer confounding effects of comorbidities and long-term medication, 2) fatal $\gamma\delta$ -T cell and plasmablast lymphomas have been reported in pediatric patients with IBD, and 3) pediatric patients with UC are much understudied in comparison to their adult counterparts.

While the etiology and mechanism of UC is not fully understood, immunologic abnormalities play a role in this disease. An increased recruitment of lymphocytes from the peripheral blood into the colon is one such abnormality, ultimately leading to inflammation and GI tissue damage. The recruitment of circulating lymphocytes, i.e. B

and T cells, is tightly controlled by a multi-step process involving trafficking receptors (TRs) expressed on the lymphocyte surface, that act in combination to govern these immune cells' migration to the target organ. Focusing on B cells—a lymphocyte subset highly implicated in autoimmune/autoinflammatory disorders such as UC—we hypothesized that the percent of circulating plasmablasts (an activated B cell population) would be increased during disease activity in pediatric UC and would demonstrate an altered pattern of TR expression indicative of cell homing to the colon.

We used multicolor flow cytometry analysis to examine the combinatorial expression of TRs on the B cell surface. Our approach is novel in that we examine 10+ surface molecules simultaneously using only one tube of cells. In addition, our protocol was designed to require only one tube of peripheral blood from the pediatric donors, making participation in this study minimally invasive to the patients.

Our research was designed to address the following issues: 1) the methods to monitor the progression of disease and the immune response to therapeutics are typically invasive, and 2) there remains a need to more effectively follow changes in the immune system—which may occur as a natural progression of the disease itself or may result from disease treatment—that may lead to severe side effects, such as lymphomas. We have found that pediatric UC patients with severe disease have an increased frequency of plasmablasts in the peripheral blood that express $\alpha 4\beta 7$, CCR10, and P-selectin ligand—TRs which, in combination, are indicative of cell homing to the colon. Screening this B cell subset may provide a complementary approach in monitoring disease activity or

therapeutic efficacy, and may provide a target for therapeutics in the treatment of pediatric UC.

CHAPTER II

PLASMABLAST FREQUENCY AND TRAFFICKING RECEPTOR EXPRESSION
ARE ALTERED IN PEDIATRIC ULCERATIVE COLITIS

This is the pre-peer-reviewed version of the following article, “Tarlton, N. J., Green, C. M., Lazarus, N. H., Rott, L., Wong, A. P., Abramson, O. N., Bremer, M., Butcher, E. C. and Abramson, T. (2012), Plasmablast frequency and trafficking receptor expression are altered in pediatric ulcerative colitis. *Inflamm Bowel Dis.* doi: 10.1002/ibd.22962,” which has been published online for early view prior to inclusion in the journal at <http://onlinelibrary.wiley.com/doi/10.1002/ibd.22962/full>.

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

Nicole J Tarlton¹, M.S., Caroline M Green¹, M.S., Nicole H Lazarus^{2,3}, Ph.D., Lusijah Rott^{2,3}, M.S., Anthony P Wong⁴, M.D., Oren N Abramson⁴, M.D., Martina Bremer⁵, Ph.D., Eugene C Butcher^{*2,3}, M.D., and Tzvia Abramson^{*1}, Ph.D.

¹San Jose State University, Department of Biology, One Washington Square, San Jose, CA 95192; ²Laboratory of Immunology and Vascular Biology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305; ³Veterans Affairs Palo Alto Health Care System, Palo Alto, California 94304; ⁴Kaiser Permanente Santa Clara, 710 Lawrence Express Way, Santa Clara, CA 95051; ⁵San Jose State University, Department of Mathematics, One Washington Square, San Jose, CA 95192

* Co-senior authors for this study; both directed this study.

Disclosures: All authors have nothing to disclose.

Correspondent Footnote: Tzvia Abramson, Department of Biological Sciences, One Washington Square, San Jose, CA 95192 (Phone: 408-924-4872; Fax: 408-924-4840; E-mail: tzvia.abramson@sjsu.edu).

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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 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 increased 30-fold during severe UC activity and positively correlated with Pediatric Ulcerative Colitis Activity Index score, C-reactive protein level, and erythrocyte sedimentation rate. A greater percent of PBs in severe patients expressed the gut-homing receptors $\alpha 4\beta 7$ and CCR10, and the inflammatory homing molecule P-selectin ligand (P-sel lig). The percent of IgA⁺ MBCs expressing $\alpha 4\beta 7$, however, was reduced. Furthermore, expression of the small intestine TR CCR9 decreased on $\alpha 4\beta 7^{\text{high}}$ PBs and on $\alpha 4\beta 7^{\text{high}}/\text{CCR10}^{\text{high}}$ PBs and MBCs in these patients, consistent with preferential cell targeting to the colon. **Conclusions:** Peripheral blood PBs with a colon-homing phenotype ($\alpha 4\beta 7/\text{CCR10}/\text{P-sel lig}$) were 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.

Key words: Inflammatory Bowel Disease; memory B cell; plasmablast; trafficking receptor; ulcerative colitis.

Abbreviations used in this thesis: ASC, antibody secreting cell; CLA, cutaneous lymphocyte antigen; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HD, healthy donor; IBD, Inflammatory Bowel Disease; MBC, memory B cell; NMS, normal mouse serum; 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.

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 gut¹. Ulcerative colitis (UC) is an IBD typified by diffuse lesions spread throughout the colon². Although the onset of UC may occur in either childhood or adulthood, pediatric patients often present with a more extensive disease at diagnosis³. Furthermore, the incidence of pediatric UC has risen globally⁴, as well as in Northern California⁵, 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 bases⁸ 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 antibodies¹¹. In patients with inactive disease, increased numbers of ASCs in the colonic lamina propria is a predictive factor of relapse¹².

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 surface¹³. Unique combinations of TRs direct circulating B cells to specific tissues¹⁴. 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-1¹⁵, 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 infection²⁰, after vaccination²¹, in bacterial septicemia and liver cirrhosis²², in children and adults with active systemic lupus erythematosus (SLE)^{19,22}, and in adults with active IBD²³. 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 José 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 pediatric gastroenterologists at Kaiser Permanente in Northern California, who used 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. Twelve pediatric UC patients

participated in this study. A second sample was obtained from five patients during a different severity of disease and/or at a different time point than their first donation, for a total of seventeen peripheral blood patient 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**.

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
^ξ 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
^ξ 6	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
10	50	Moderate	15	F	13	0.2	*5ASA, *Steroids, and **6MP before ***Remicade
[†] 11	60	Moderate	9	M	40	0.8	***Humira
[†] 12	65	Severe	9	M	39	0.8	***Humira
[§] 13	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
[§] 16	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, [§], [†], ^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.

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 $\text{Ca}^{++}/\text{Mg}^{++}$, 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 $\alpha 4\beta 7$ (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 the Zenon Alexa Fluor 647 human IgG labeling kit (Invitrogen). 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

PBMCs (3×10^6 per donor sample) were stained with the previously mentioned antibodies. First, cells were stained with primary anti-CCR9 antibody, washed, and labeled with secondary anti-mouse Pacific Blue. 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, and the conjugate 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 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 (in the Butcher lab at Stanford University) using FACSDiva software.

Flow cytometry data were 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 was subtracted from the donor sample value to account for non-specific binding.

Statistical analysis

Differences in the percent of $\text{IgA}^{+/-}$ 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) UC 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**).

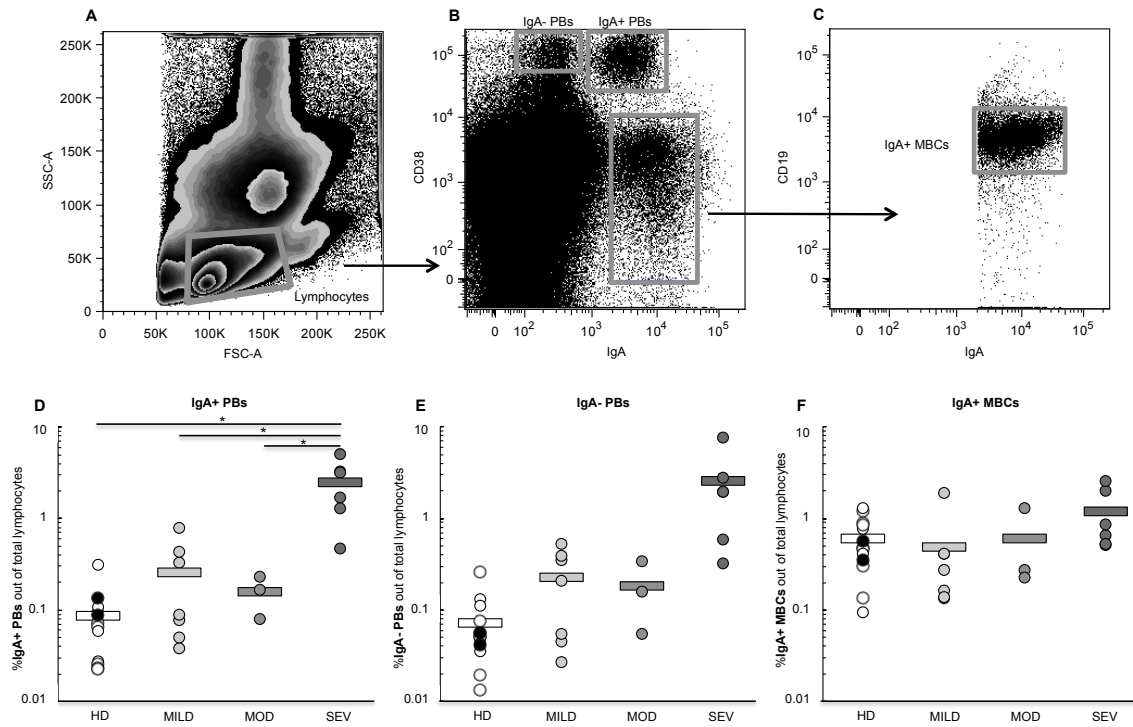


FIGURE 1. Representative flow cytometry plots of B cell subset gating strategy. From left to right: (A) lymphocytes are gated, (B) three B cell subsets are identified—the IgA⁻ plasmablasts (PBs), the IgA⁺ PBs, and the IgA⁺ memory B cells (MBCs), then (C) the IgA⁺ MBCs are further gated for CD19 expression. 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.

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).

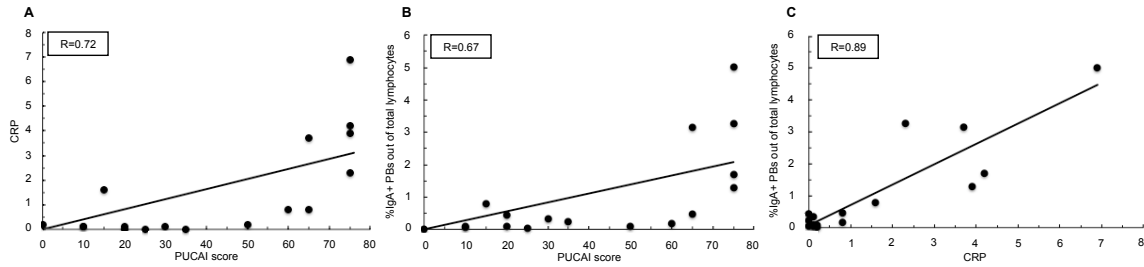


FIGURE 2. Linear regression analyses in pediatric patients with UC. (A) Correlation between serum C-reactive protein (CRP) levels and PUCAI score. (B) Correlation between percent of IgA⁺ plasmablasts (PBs) out of total peripheral blood lymphocytes and Pediatric Ulcerative Colitis Activity Index (PUCAI) score. (C) Correlation between percent of IgA⁺ PBs out of total peripheral blood lymphocytes and CRP.

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 demonstrated 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 CLA²⁹, 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 $\alpha 4\beta 7^{\text{high}}$ 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

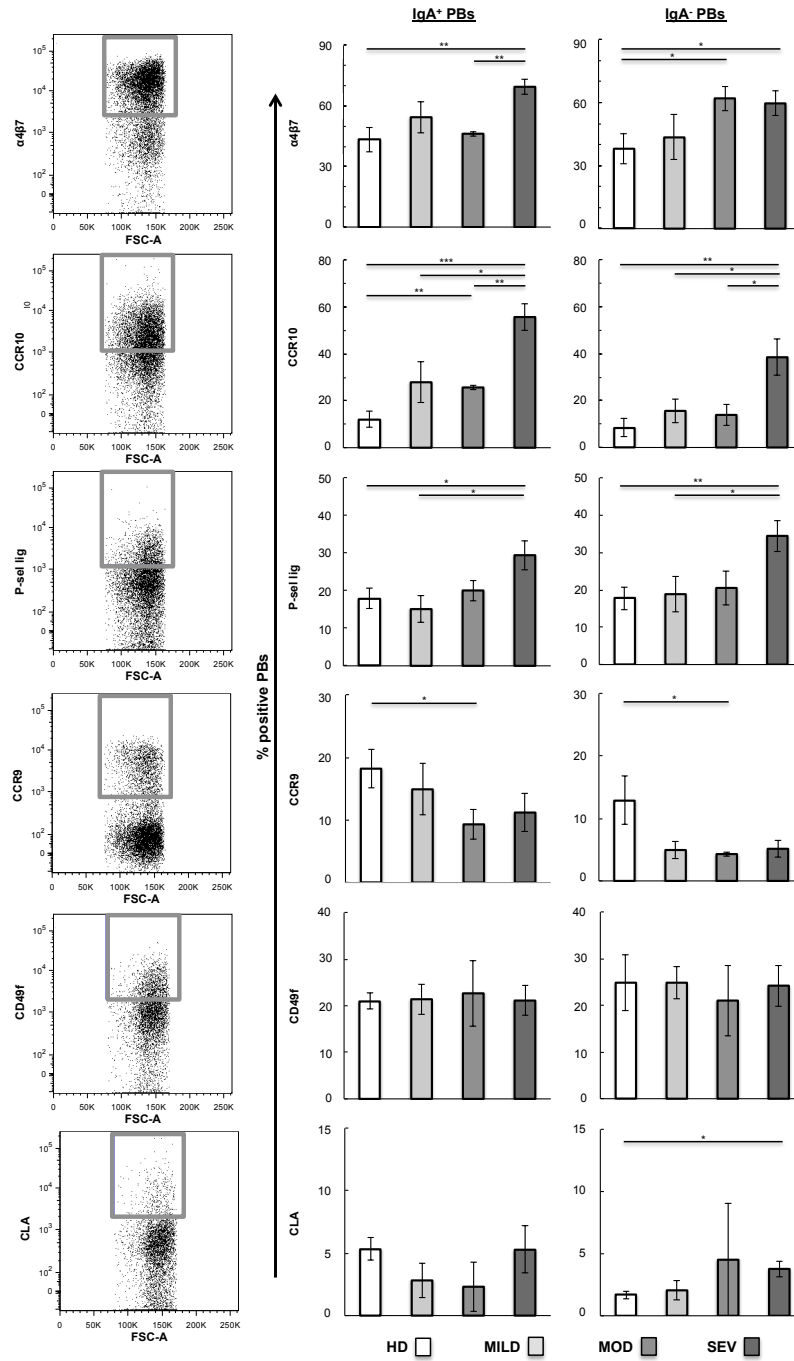


FIGURE 3. Single trafficking receptor (TR) analysis on IgA^{+/−} plasmablasts (PBs). (A) Representative flow cytometry dot plots of individual TR expression on PBs. Gates represent high-TR-expressing cells. Average percent of (B) IgA⁺ PBs and (C) IgA[−] PBs with TR^{high} expression of $\alpha 4\beta 7$, 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.

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 sites¹³. 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 ($\alpha 4\beta 7^{\text{high}}$) or non-mucosal ($\alpha 4\beta 7^{\text{low}}$) homing cells. IgA⁺ and IgA⁻ PBs from **Figure 1** were segregated into $\alpha 4\beta 7^{\text{high}}$ and $\alpha 4\beta 7^{\text{low}}$ 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 $\alpha 4\beta 7^{\text{high}}$ and $\alpha 4\beta 7^{\text{low}}$ 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, $\alpha 4\beta 7^{\text{low}}$ non-mucosal PBs with CD49f^{high} expression were consistently increased (average range between 27-32%) compared to their $\alpha 4\beta 7^{\text{high}}$ mucosal homing counterparts.

The cellular expression of $\alpha 4\beta 7$ and CLA are usually mutually exclusive, as these TRs mediate lymphocyte homing to different sites. Therefore the percent of mucosal PBs 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.

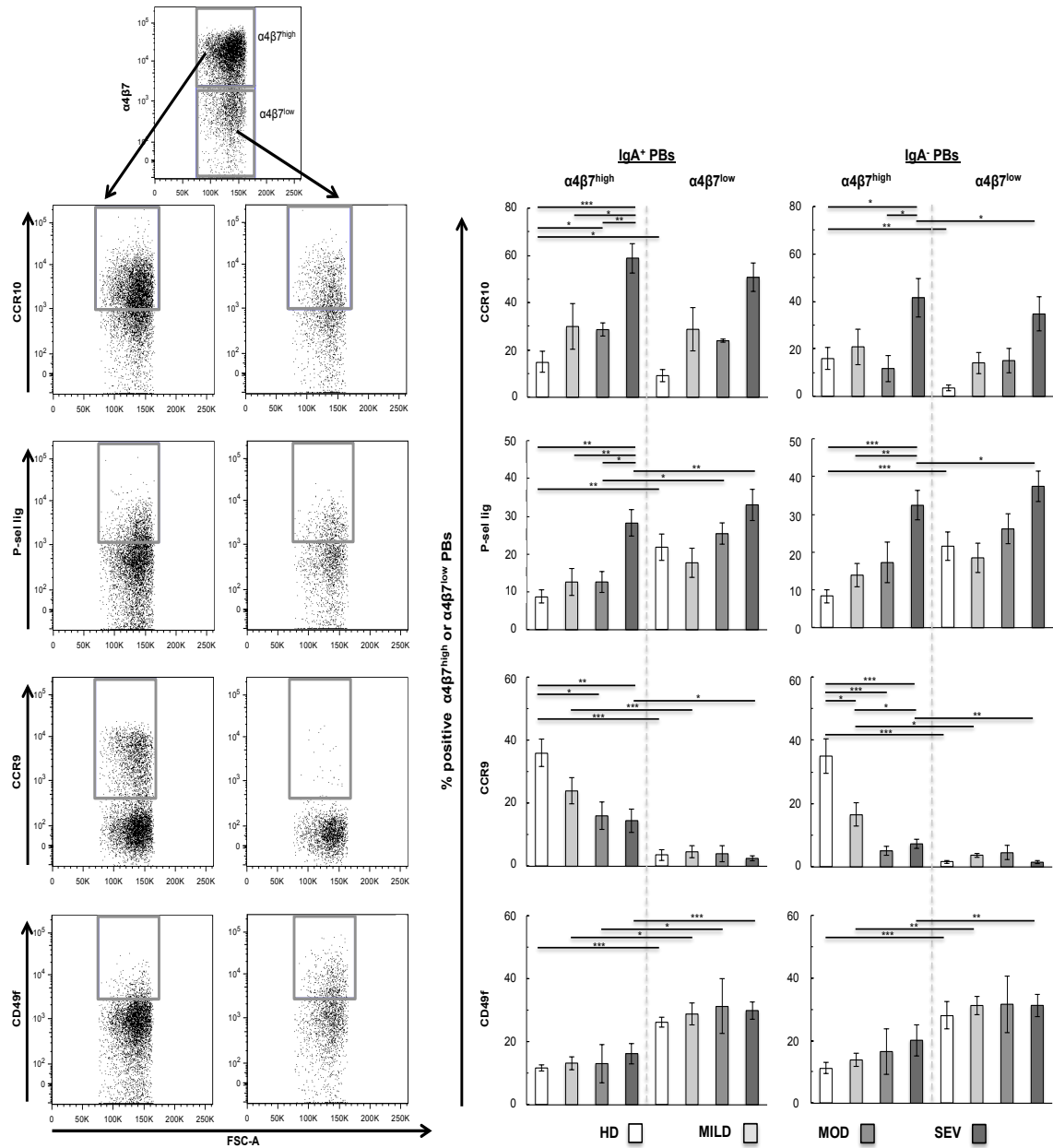


FIGURE 4. Trafficking receptor (TR) analysis on mucosal ($\alpha 4\beta 7^{\text{high}}$) and non-mucosal ($\alpha 4\beta 7^{\text{low}}$) IgA⁺ plasmablasts (PBs). (A) Representative flow cytometry plots for TR gating strategy on PBs. From top to bottom: IgA⁺ PBs are gated for $\alpha 4\beta 7^{\text{high}}$ expression (upper gate) and $\alpha 4\beta 7^{\text{low}}$ 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 $\alpha 4\beta 7^{\text{high}}$ and $\alpha 4\beta 7^{\text{low}}$ (B) IgA⁺ PBs and (C) IgA⁻ PBs with TR^{high} expression. Statistical analyses for $\alpha 4\beta 7^{\text{high}}$ 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 $\alpha 4\beta 7^{\text{high}}$ and $\alpha 4\beta 7^{\text{low}}$ cells within groups using Student's *t*-test. **p*<0.05, ***p*<0.01, ****p*<0.001; no asterisk indicates *p*>0.05.

Trafficking receptor expression on memory B cells

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 $\alpha 4\beta 7$ and CCR10 or P-sel lig was consistently higher on PBs than MBCs. However, the co-expression of $\alpha 4\beta 7$ and CCR9 or CD49f was more similar between the cell subsets.

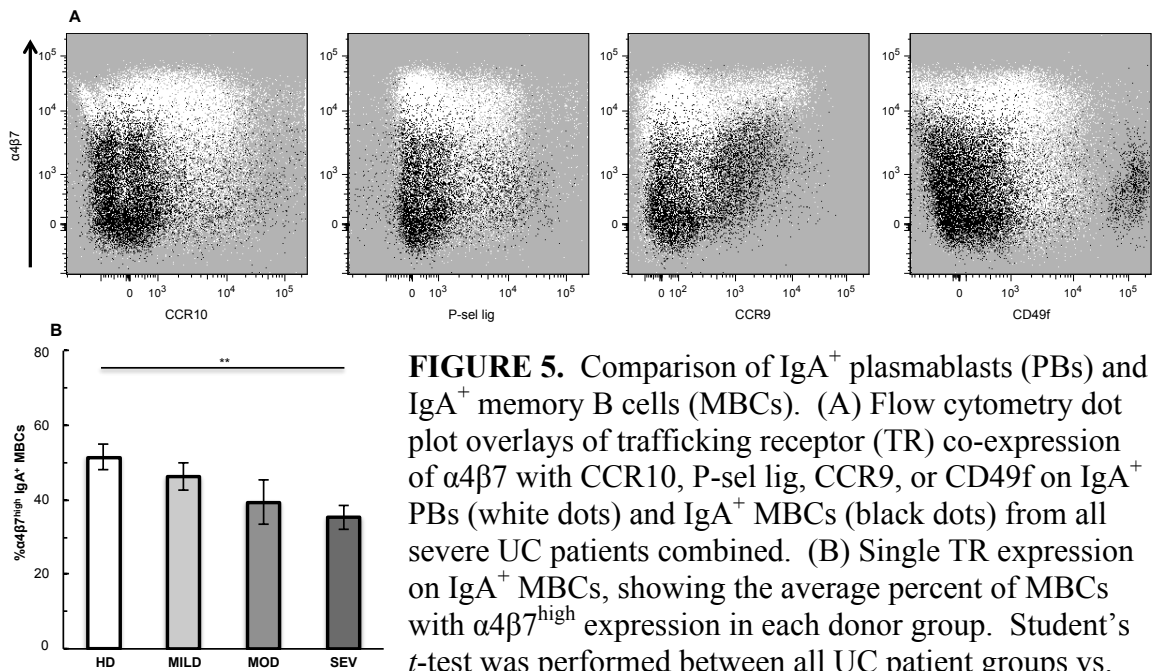


FIGURE 5. Comparison of IgA⁺ plasmablasts (PBs) and IgA⁺ memory B cells (MBCs). (A) Flow cytometry dot plot overlays of trafficking receptor (TR) co-expression of $\alpha 4\beta 7$ with CCR10, P-sel lig, CCR9, or CD49f on IgA⁺ PBs (white dots) and IgA⁺ MBCs (black dots) from all severe UC patients combined. (B) Single TR expression on IgA⁺ MBCs, showing the average percent of MBCs with $\alpha 4\beta 7^{\text{high}}$ 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.

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 $\alpha 4\beta 7^{\text{high}}$ PBs, the frequency of $\alpha 4\beta 7^{\text{high}}$ 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 $\alpha 4\beta 7$ and CCR10

$\alpha 4\beta 7$ 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 intestine¹⁴. Therefore we examined $\alpha 4\beta 7^{\text{high}}$ /CCR10^{high} B cell subsets for CCR9 expression.

In general the proportion of $\alpha 4\beta 7^{\text{high}}$ /CCR10^{high} PBs and MBCs expressing CCR9 decreased as the severity of UC disease intensified (**Figure 6**). The frequency of $\alpha 4\beta 7^{\text{high}}$ /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 $\alpha 4\beta 7^{\text{high}}$ /CCR10^{high} IgA⁺ MBCs expressing CCR9 in severe UC (30%) was decreased compared to healthy controls (50%, p=0.02).

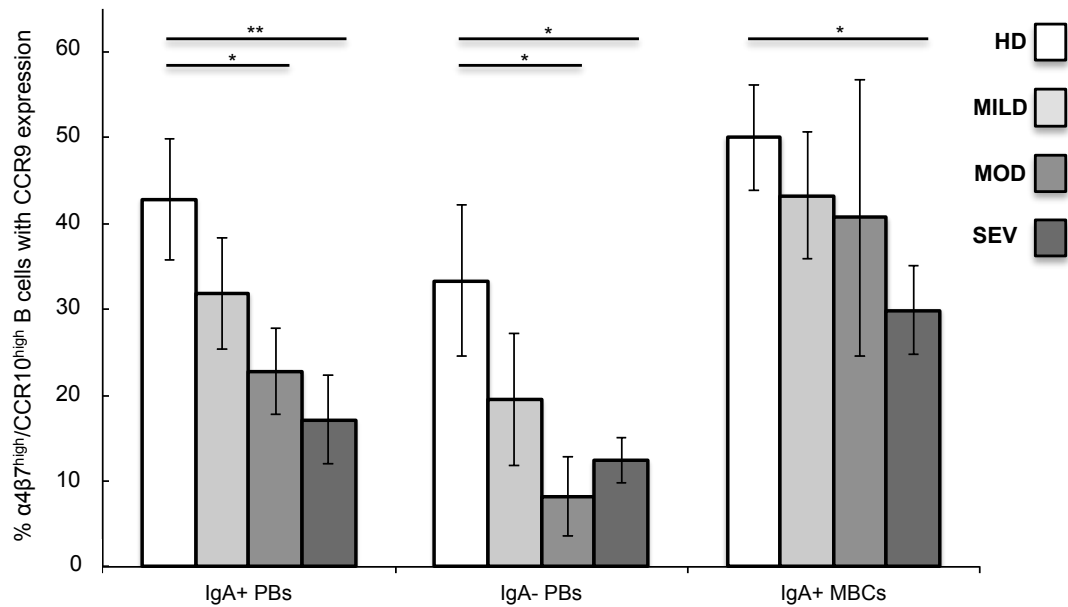


FIGURE 6. CCR9 expression on $\alpha 4\beta 7^{\text{high}}/\text{CCR}10^{\text{high}}$ IgA^{+/−} plasmablasts (PBs), and IgA⁺ memory B cells (MBCs). Samples were grouped according to type of donor: healthy donors (HD) and pediatric patients with mild UC (MILD), moderate UC (MOD), and severe UC (SEV) 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.

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 showed that the average frequency of both IgA⁺ and IgA⁻ PBs were 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 SLE¹⁹. 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 $\alpha 4\beta 7^{\text{high}}$ 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 is different in adult UC vs. pediatric UC, and this TR has 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 inflammation³². In this study we observed an increased frequency of both $\alpha 4\beta 7^{\text{low}}$ and $\alpha 4\beta 7^{\text{high}}$ 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 $\alpha 4\beta 7^{\text{high}}$ 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 $\alpha 4\beta 7$ only.

The frequency of IgA⁺ MBCs expressing $\alpha 4\beta 7$ at high levels decreased as UC disease activity intensified, and severe patients had a lower proportion of these cells compared to healthy donors. $\alpha 4\beta 7^{\text{high}}$ 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 $\alpha 4\beta 7$ 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 $\alpha 4\beta 7$ 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

$\alpha 4\beta 7^{\text{high}}/\text{CCR}10^{\text{high}}$ 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 intestine³³.

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 (i.e. $\alpha 6$) 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 receptor³⁶. In our study the frequency of B cells expressing CD49f was unaltered in UC, and the expression of the receptor was more prominent on $\alpha 4\beta 7^{\text{low}}$ cells, supporting the idea that CD49f is not associated with gut-homing lymphocytes³⁰. 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 $\alpha 4\beta 7^{\text{high}}/\text{CCR}10^{\text{high}}/\text{P-sel lig}^{\text{high}}$ 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.

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CHAPTER III

CONCLUSION

Conclusions

Ulcerative colitis (UC) is a serious and life altering disorder for which an effective therapeutic regimen can be difficult to establish. In this work we utilized multicolor flow cytometry analysis to determine the relationship between the frequency of circulating plasmablasts (PBs) and their associated trafficking receptors (TRs) with the severity of disease in the colon in pediatric UC patients. We show here that the percent of peripheral blood PBs with $\alpha 4\beta 7^{\text{high}}$ /CCR10^{high}/P-sel lig^{high} expression are indicative of the severity of disease and location of pathology in pediatric UC.

Our work suggests that circulating PB subsets may provide a useful parameter to monitor in the evaluation of pediatric UC patients' progression of disease and response to treatment. Our complementary approach would provide both a relatively non-invasive and specific method for monitoring the immune system in these patients. The PB subsets and TRs we have indicated here may also provide a therapeutic target for the treatment of UC.

Future Directions

To further investigate the significance of PBs and their associated TR expression in Inflammatory Bowel Disease (IBD), we plan to expand our research in the following directions:

1. We will expand the panel of TRs examined on PBs to include additional trafficking molecules with relevance in inflammation—for instance the chemokine receptors CXCR3 and CXCR4.
2. We will examine circulating PB subsets in pediatric Crohn's Disease (CD)

patients. The study of CD will contribute to a greater understanding of TR expression in IBD because patients with CD can experience lesion pathology all throughout the gastrointestinal (GI) tract. For instance, patients may experience lesions in one or many locations, i.e. the large bowel, the small bowel, the upper gastrointestinal tract, or a combination of these. We will compare the TR phenotype of PBs in UC to those in CD patients that demonstrate only colon involvement. We predict that this comparison will have either of two outcomes: TR expression may be the same since the location of tissue involvement is identical in both circumstances, or different TRs may be involved in CD since the appearance of lesions is distinct from that in UC.

3. Although not an IBD, we plan to investigate TR expression on PBs in celiac disease—a GI disorder characterized by localized inflammation/lesions in the small bowel due to gluten protein intolerance. We will compare the TR phenotype of PBs in celiac disease to those in CD patients with small bowel involvement, again to determine if TR expression is the same if the location of tissue involvement is identical, or if different TRs are involved in the two diseases because the appearance of lesions is distinct.

Ultimately, through the comparison of TR expression on PBs in UC, CD, and celiac disease, we aim to develop (or would like to determine if it is possible to develop) a multicolor flow cytometry analysis of TRs that would enable us to distinguish between disease activity and location of pathology in these three inflammatory disorders.

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