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# Anti-TNF and thiopurine therapy in pregnant IBD patients does not significantly alter a panel of B-cell and T-cell subsets in 1-year-old infants

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## Abstract

**Objectives:** Infants exposed to combination therapy with anti-tumor necrosis factor (anti-TNF) agents and thiopurines may exhibit increased infections at 1 year of age compared to unexposed infants. We hypothesized that this increased risk of infection is due to abnormal development of the newborn immune system.

**Methods:** We immunophenotyped B-cell and T-cell subsets using multiparameter flow cytometry in 1-year-old infants whose mothers were exposed to therapeutic agents for IBD. We analyzed samples from infants exposed to infliximab (IFX) or adalimumab (ADA) monotherapy (IFX/ADA, $n = 11$), certolizumab pegol (CZP) monotherapy (CZP, $n = 4$), IFX or ADA plus thiopurine combination therapy (IFX/ADA + IM, $n = 4$), and CZP plus thiopurine combination therapy (CZP + IM, $n = 2$).

**Results:** Percentages of B cells, CD4$^{+}$ T helper cells, T regulatory cells ($T_{regs}$), and CD8$^{+}$ cytotoxic T cells, were similar among the groups. Infants exposed to combination therapy (IFX/ADA + IM) exhibited trends toward fewer CD27$^{+}$ B cells, switched memory B cells, plasmablasts, interferon gamma (IFNγ)-producing CD4$^{+}$ and CD8$^{+}$ T cells, and CCR5$^{+}$CD4$^{+}$ T cells, but these did not reach statistical significance.

**Conclusions:** Multiparameter immunophenotyping of major B-cell and T-cell subsets suggests that the adaptive newborn immune system develops largely unaltered after exposure to combination therapy as compared to anti-TNF monotherapy.

## Introduction

Anti-tumor necrosis factor (TNF) agents were the first biologic agents to demonstrate efficacy in achieving and maintaining steroid-free remission in moderate to severe Crohn's Disease (CD) and Ulcerative Colitis (UC). Combination therapy with an anti-TNF agent and an immunomodulator is often used to prevent the development of neutralizing anti-drug antibodies, raise trough levels of the biologic, and maintain remission[1–3]. Achieving and maintaining remission are especially important for pregnant inflammatory bowel disease (IBD) patients. Pregnant IBD patients in general, and specifically those with active disease, are at increased risk for obstetrical complications such as spontaneous abortion, preterm birth, low birth weight, and complications during labor and delivery[4–11]. For pregnant patients with

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moderate to severe disease, achieving remission frequently means treating with an anti-TNF agent or other biologic with or without a concomitant thiopurine such as mercaptopurine (6-MP) or azathioprine (AZA). Anti-TNF therapy is low risk during pregnancy. Thus far, studies looking at anti-TNF monotherapy during pregnancy with Infliximab (IFX), Adalimumab (ADA), and Certolizumab pegol (CZP) have shown no adverse effect on pregnancy outcomes and no increased risk of congenital malformations[12–14]. Although there are some conflicting data regarding the safety of thiopurine immunomodulators during pregnancy, one study looking at 187 patients and another looking at 335 patients treated with thiopurines during pregnancy did not show any increased risk of congenital anomalies or obstetric complications[15,16].

While thiopurines or anti-TNF agents are safe and effective during pregnancy, combination therapy with an anti-TNF agent and an immunomodulator has been associated with an increased risk of common viral and bacterial infections in infants[16,17]. In the Pregnancy in IBD And Neonatal Outcomes (PIANO) registry, a multicenter prospective observational registry of pregnant patients with IBD, 107 infants exposed to thiopurines plus anti-TNF therapy had an increased relative risk (RR) of infection at 9–12 months compared to unexposed infants (relative risk (RR) 1.50, 95% confidence interval (CI) 1.08–2.09)[16]. This finding was replicated in a separate cohort where infants exposed to combination therapy exhibited a 2.7-fold relative risk of infection compared to anti-TNF monotherapy (95% CI 1.09–6.78)[17]. Therefore, while providers may continue combination therapy to achieve or maintain remission during pregnancy, guidelines vary in their recommendations[7,18].

Thiopurine metabolites and some anti-TNF agents are able to cross the placenta[17,19,20]. The neonatal Fc receptor (FcRn) actively transports serum immunoglobulin across the placenta, and therefore IFX and ADA levels are detectable in cord blood at levels greater than maternal concentrations[17,19]. IFX and ADA remain detectable for up to 6–12 months[17,19]. CZP is a monovalent Fab fragment lacking the Fc region required for transplacental transport by the FcRn, therefore it is very low or undetectable in cord blood[19]. IFX and ADA levels in breast milk are very low (<1% of maternal plasma levels), and the small amount transferred through breast milk does not appear to affect the time it takes for these drugs to become undetectable in the infant circulation[17,21–23]. Similarly, thiopurine metabolites are low or undetectable in breast milk and the overall dose to the infant is <1% of therapeutic dosing[24]. Therefore, although infants are exposed to anti-TNF agents and thiopurine metabolites *in utero*, studies suggest that subsequent exposure through breastfeeding is likely negligible.

Since IFX and ADA are typically cleared within 6–12 months after birth, and since only trace amounts of these agents are transferred in breast milk, we hypothesized that the increased risk of infection at 1 year is due to aberrations in the developing newborn immune system. To discover potential differences in infant immunophenotypes, we performed multiparameter flow cytometry on infants at 1 year of age and stratified by drug exposure. For B-cell populations that appeared altered after exposure to combination therapy, we also analyzed WT and TNF-deficient mice to determine if these differences could be explained by TNF-deficiency during development.

## Materials and methods

### Study design, participants, and procedures

The study patients were recruited from the Crohn's and Colitis Foundation PIANO registry, a prospective, observational, multicenter registry of pregnant patients with IBD initiated in 2007[6]. All women in the registry are followed prospectively throughout pregnancy and at delivery. Children born to these mothers are followed every 4 months in the first year of life. The questionnaires and variables for the PIANO registry have been previously described[6]. Pregnant women with IBD and singleton pregnancies who were exposed to an anti-TNF agent, with or without concomitant immunomodulators, anytime from 3 months prior to their last menstrual period to delivery were eligible for this study. For this study, ~2–3 mL of venous blood was drawn from 1-year-old infants into BD Vacutainer™ Plastic Blood Collection Tubes with $K_2EDTA$. Blood was shipped at room temperature to the UCSF Core Immunology Laboratory. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using SepMate Lymphoprep (StemCell Technologies) density centrifugation. PBMCs were removed, washed once with phosphate-buffered saline (PBS) then treated with ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Lonza Walkersville, Inc.) to remove red blood cell contamination. PBMCs were then viably frozen in heat-inactivated fetal bovine serum containing 10% dimethylsulfoxide and maintained at -190°C. PBMC were later thawed, counted and analyzed in a single experiment by multiparameter flow cytometry as described below.

We recruited 22 infants and divided them into five groups according to drug exposure between conception and delivery: no exposure to anti-TNF or thiopurines (unexposed, $n = 1$), CZP (CZP monotherapy, $n = 4$), IFX/ADA (IFX or ADA monotherapy, $n = 11$), IFX/ADA + IM (infliximab or adalimumab plus an immunomodulator, $n = 4$), and CZP + IM (CZP plus an immunomodulator, $n = 2$) (Table 1). The one "unexposed" infant and two CZP + IM infants are shown for reference but were

**Table 1 Demographic and basic clinical data for maternal and infant study participants stratified by drug exposure**

| Drug of exposure | Unexposed ($N = 1$) | CZP ($N = 4$) | IFX/ADA ($N = 11$) | CZP+ IM ($N = 2$) | IFX/ADA+ IM (N = 4) | *p*-value |
|---|---|---|---|---|---|---|
| **Mothers** | | | | | | |
| Age, years median (IQR1–IQR3) | 33 | 29 (28–30) | 34 (29–36) | 25,34 | 33 (32-34) | 0.56 |
| *Disease type, n (%)* | | | | | | 0.085 |
| UC | 1 (100%) | 0 | 6 (55%) | 0 | 0 | |
| CD | 0 | 4 (100%) | 5 (45%) | 2 (100%) | 4 (100%) | |
| Disease duration, years median (IQR1–IQR3) | 12 | 11 (10-13) | 8 (5-10) | 7,11 | 9 (2-17) | 0.66 |
| *Disease activity, n (%)* | | | | | | 0.47 |
| None | 1 (100%) | 3 (75%) | 9 (82%) | 2 (100%) | 3 (75%) | |
| Mild | | | 2 (18%) | | | |
| Moderate | | 1 (25%) | | | | |
| Severe | | | | | 1 (25%) | |
| Current smokers, *n* (%) | 0 | 0 | 0 | 0 | 0 | |
| *Disease location, n (%)* | | | | | | *0.03* |
| Ileal CD | | 1 (25%) | 3 (27%) | 1 (50%) | | |
| Colonic CD | | 2 (50%) | | | 2 (50%) | |
| Ileocolonic CD | | | 2 (18%) | 1 (50%) | | |
| Perianal CD | | 1 (25%) | | | 2 (50%) | |
| Ulcerative Proctitis | | | 3 (27%) | | | |
| Left-sided UC | 1 (100%) | | | | | |
| Extensive UC | | | 3 (27%) | | | |
| **Infants** | | | | | | |
| *Gender, n (%)* | | | | | | 0.35 |
| Female | 1 (100%) | 1 (25%) | 5 (45%) | | 3 (75%) | |
| Male | 0 (0%) | 3 (75%) | 6 (55%) | 2 (100%) | 1 (25%) | |
| Gestational age, weeks median (IQR1–IQR3) | 40 | 40 (39-40) | 40 (39-41) | 39,39 | 39 (38-39) | 0.45 |
| Birth weight, lbs median (IQR1–IQR3) | 6.7 | 8.3 (8.0–8.7) | 7.7 (7.2–8.3) | 8.8, 9.1 | 8.1 (7.0–9.2) | 0.53 |
| Length, cm median (IQR1–IQR3) | 18 | 21 (20-22) | 20 (20–21) | 21,21 | 21 (20–21) | 0.56 |
| Congenital anomaly | 0 | 0 | 0 | 0 | 0 | |
| *Infections first 12 months* | | | | | | |
| Any | 2 | 2 | 8 | 1 | 5 | 0.93 |
| Not AOM | 0 | 0 | 5 | 0 | 4 | 0.35 |
| *Mode of delivery* | | | | | | *0.0014* |
| Vaginal | 1 (100%) | 3 (75%) | 11 (100%) | 0 | 1 (25%) | |
| Cesarean | 0 | 1 (25%) | 0 | 2 (100%) | 3 (75%) | |

Median and interquartile ranges (IQR) IQR1–IQR3 are presented for groups with $N \geq 3$. For groups with $N < 3$, individual values are presented. Where appropriate, categorical variables were analyzed by Fisher's exact test and non-normally distributed continuous variables were compared using Kruskal–Wallis one-way ANOVA test (excluding the unexposed mother and infant)

not included in any of the analyses. For each cell subset, our primary analysis was a three-way comparison (CZP versus IFX/ADA versus IFX/ADA + IM). Since acute otitis media (AOM) is common, we reported episodes of infection as "Any" for any infection and "Not AOM". Other common infections included upper respiratory infections including respiratory syncytial virus and pneumonia. The study was approved by the Institutional Review Board at each participating institution.

### Mice

$TNF^{-/-}$ and wild-type C57BL/6 J mice were obtained from Jackson Laboratory and have been previously described. All mice used in this study were housed and bred in a specific pathogen-free facility according to the IACUC guidelines of UCSF.

### Antibodies and reagents

Supplementary Table 1 summarizes the fluorochrome-conjugated antibodies used for human immunophenotyping. The following fluorochrome-conjugated antibodies were used for immunophenotyping mouse splenocytes and lymph node mononuclear cells: Phycoerythrin (PE)-Cy7-conjugated anti-B220 (clone RA3-6B2), allophycocyanin (APC)-conjugated anti-CD138 (clone 281-2), peridinin chlorophyll (PerCP)-Cy5.5-conjugated anti-Gr-1 (clone RB6-8C5), fluorescein isothiocyanate (FITC)-conjugated anti-IgD (clone AMS9.1), and PE-Cy7-conjugated anti-CD95 (clone Jo2) were from BD; PE-conjugated anti-CD93 (clone AA4.1) and PerCP-Cy5.5-conjuagted anti-CD3 (clone 145-2C11) were from eBioscience; Pacific Blue-conjugated anti-I-A/I-E (clone M5/114.15.2), Pacific Blue-conjugated anti-B220 (clone RA3-6B2), Alexa®647-conjugated anti-CD73 (clone TY/11.8), and PE-conjugated anti-CD38 (clone 90) were from Biolegend; and LIVE/DEAD™ Fixable Yellow Dead Cell Stain was from Invitrogen.

### Flow cytometry

Infant PBMC samples were analyzed by the UCSF Core Immunology Laboratory in batch experiments using cryopreserved PBMCs. PBMCs were plated in a 96-well v-bottom plate and stained for 30 min at 4°C, then washed once with FACS buffer (PBS containing 0.5% bovine serum albumin and 1 mM Ethylenediaminetetraacetic Acid). Cells were then fixed in 0.5% formaldehyde and data was acquired on a customized 4-laser BD LSR II Flow cytometer (BD Biosciences), with ≥50,000 lymphocytes collected for each sample. CPT beads (BD Bioscience) were used for instrument set up. $CD19^+$ B cells, $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells were defined after standard lymphocyte, singlet, and dead cell exclusion gates were applied to the data. FMO controls were used to define positive gates for expression of IgD, IgM, CD38 and HLA-DR on B cells, CCR7, CCR5, CD38, CD57, HLA-DR, and $PD^{-}1$ on $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell populations and CD127, CD25, and FoxP3 on $T_{regs}$. To assess T-cell functional responses after thawing, PBMCs were initially rested in complete Roswell Park Memorial Institute (RPMI) medium containing 10% heat-inactivated fetal bovine serum and 10 ug/mL of DNase I (Sigma-Aldrich) for 2 h at 37 °C in a 5% $CO_2$ incubator. PBMCs were then counted using Guava ViaCount Reagent on a BD Accuri flow cytometer. One million PBMCs were plated per well into a 96-well U-bottom plate and either cultured in complete RPMI alone (unstimulated control) or stimulated using plate-bound anti-CD3 (clone UCHT1) plus soluble anti-CD28. PBMCs were stimulated overnight at 37 °C in a 5% $CO_2$ incubator in the presence of brefeldin A and monensin to inhibit cytokine secretion. Approximately 16 h after stimulation, PBMC were harvested, washed once in FACS buffer and stained for cell surface markers at room temperature for 15 min in a 96-well V-bottom plate. Cells were then washed twice, fixed and permeabilized with BD Cytofix/Cytoperm buffer for 20 min at 4 °C. Cells were washed twice with the BD Perm/Wash Buffer prior to intracellular staining for cytokines at 4 °C for 30 min. Cells were washed twice more with BD Perm/Wash Buffer and fixed a final time in 1% formaldehyde and data was acquired on a customized 4-laser BD LSR II Flow cytometer (BD Biosciences). A FMO control was used to define the positive gate for expression of TNF. Mouse splenocytes and mesenteric lymph nodes were analyzed on an LSR II flow cytometer. Mouse memory B cells were defined as $B220^+IgD^-CD38^+CD95^+CD73^+$ and plasmablasts were defined as $B220^{lo}CD138^+CD93^+$I-A/I-E$^+$[25–27]. For all cell populations, the frequency of each population is reported as a percentage of total parent B cells, $CD4^+$ T cells, or $CD8^+$ T cells. All flow cytometry data were compensated and analyzed in FlowJo V9 or V10 (TreeStar).

### Statistical analysis

Demographic and clinical characteristics were summarized by descriptive statistics. Categorical variables were analyzed by Fisher's exact test to determine if there was statistical association between the categorical variable and the groups (excluding the unexposed mother and infant). Non-normally distributed continuous variables were summarized using median and interquartile ranges and were compared using Kruskal–Wallis one-way ANOVA test (excluding the unexposed mother and infant). For the primary three-way analysis comparing CZP versus IFX/ADA versus IFX/ADA + IM, cell population frequencies for each subset were compared using Kruskal–Wallis one-way ANOVA test. Multiple comparisons were then adjusted by controlling the False Discovery Rate (FDR) <10% as previously described by

**Table 2 Frequency (%) of infant B-cell populations stratified by drug exposure**

| Cell population | Unexposed ($N = 1$) | CZP ($N = 4$) Median (IQR) | IFX/ADA ($N = 11$) Median (IQR) | IFX/ADA+ IM ($N = 4$) Median (IQR) | CZP+ IM ($N = 2$) Range | CZP vs IFX/ADA vs IFX/ADA+ IM *p*-value | CZP vs IFX/ADA vs IFX/ADA+ IM *q*-value |
|---|---|---|---|---|---|---|---|
| B cells | 10.2 | 11.33 (7.8–13.8) | 16.60 (11.8–19.9) | 10.46 (6.6–14.6) | (13.3, 16.0) | 0.14 | 0.69 |
| CD21$^+$ B cells | 91.5 | 91.05 (88.5–92.4) | 92.00 (88.2–93.0) | 89.25 (84.9–91.4) | (88.2, 91.5) | 0.52 | 0.88 |
| CD27$^+$ B cells | 2.59 | 3.96 (2.9–5.7) | 2.92 (2.2–4.5) | 1.79 (1.7–2.0) | (1.1, 4.8) | 0.04 | 0.55 |
| CD38$^+$ B cells | 96.9 | 96.35 (94.5–97.1) | 94.70 (92.7–96.2) | 92.45 (90.8–94.1) | (95.9, 97.8) | 0.54 | 0.88 |
| HLA-DR$^+$ B cells | 99.6 | 98.90 (98.2–99.1) | 98.70 (97.9–99.4) | 97.85 (95.0–98.3) | (98.1, 98.7) | 0.40 | 0.88 |
| IgD$^+$ B cells | 94.6 | 92.15 (86.7–93.0) | 91.90 (91.0–92.9) | 91.50 (86.3–93.5) | (91.8, 94.5) | 0.99 | 0.99 |
| IgM$^+$ B cells | 96.4 | 96.55 (90.8–97.0) | 95.00 (94.8–96.9) | 93.30 (88.6–98.0) | (93.4, 96.1) | 0.98 | 0.99 |
| IgD$^-$CD27$^+$ switched memory | 1.41 | 2.31 (1.8–3.9) | 2.06 (1.0–2.4) | 1.08 (1.0–1.2) | (0.27, 2.6) | 0.10 | 0.65 |
| IgD$^+$CD27$^+$ non-switched memory | 1.24 | 1.36 (1.3–1.6) | 1.56 (0.9–1.9) | 0.75 (0.6–0.9) | (0.56, 1.9) | 0.16 | 0.69 |
| IgD$^+$CD27$^-$ naive B cells | 93.4 | 90.50 (84.7–91.9) | 90.50 (89.6–91.8) | 90.75 (85.7–92.6) | (89.9, 93.9) | 0.96 | 0.99 |
| IgD$^-$CD27$^-$ memory effector | 3.94 | 5.57 (4.9–9.7) | 6.21 (5.4–7.4) | 7.56 (5.4–12.8) | (5.3, 5.6) | 0.70 | 0.91 |
| CD38$^{hi}$CD27$^+$ plasmablasts | 0.29 | 0.51 (0.4–0.6) | 0.26 (0.2–0.5) | 0.18 (0.13–0.23) | (0.11, 0.34) | 0.04 | 0.55 |

Median and Interquartile Ranges (IQR) 1–3 are presented for groups with $N \geq 3$. For groups with $N < 3$, individual values are shown
*CD* cluster of differentiation, *HLA* human leukocyte antigen, *IgD* immunoglobulin D, *IgM* immunoglobulin M

**Table 3 Frequency (%) of infant CD4$^+$ T-cell populations stratified by drug exposure**

| Cell population | Unexposed ($N = 1$) | CZP ($N = 4$) Median (IQR) | IFX/ADA ($N = 11$) Median (IQR) | IFX/ADA+ IM ($N = 4$) Median (IQR) | CZP+ IM ($N = 2$) Range | CZP vs IFX/ADA vs IFX/ADA+ IM *p*-value | CZP vs IFX/ADA vs IFX/ADA+ IM *q*-value |
|---|---|---|---|---|---|---|---|
| CD4$^+$ T cells | 75.7 | 72.25 (70.5–73.2) | 67.20 (64.2–74.4) | 72.00 (69.7–73.2) | (66.1, 84.3) | 0.97 | 0.99 |
| CD4+CCR5$^+$ T cells | 0.13 | 0.43 (0.3–0.6) | 0.18 (0.1–0.4) | 0.07 (0.06–0.08) | (0.12, 0.31) | 0.06 | 0.55 |
| CD4+CD57$^+$ T cells | 0.12 | 1.30 (0.8–1.9) | 0.22 (0.2–0.7) | 0.51 (0.24–1.0) | (0.16, 0.18) | 0.21 | 0.74 |
| CD4+PD1$^+$ T cells | 1.05 | 2.80 (1.9–3.2) | 1.95 (1.2–2.4) | 0.77 (0.6–0.95) | (1.4, 6.8) | 0.10 | 0.65 |
| CD4+CD38$^+$HLA-DR$^+$ T cells | 1.27 | 2.98 (2.1–4.1) | 1.93 (1.7–3.1) | 2.59 (1.8–3.3) | (0.62, 2.3) | 0.69 | 0.91 |
| CD4$^+$CCR7$^+$CD45RA$^-$ CM | 16.9 | 18.45 (14.7–22.6) | 22.00 (16.2–23.4) | 19.40 (19.3–19.4) | (21.3, 27.9) | 0.81 | 0.99 |
| CD4$^+$CCR7$^+$CD45RA$^+$ naive T cells | 78.2 | 76.35 (70.5–82.2) | 73.00 (71.0–81.8) | 77.85 (76.7–79.0) | (70.7, 71.3) | 0.86 | 0.99 |
| CD4$^+$CCR7$^-$CD45RA$^+$TEMRA | 1.55 | 1.62 (1.2–2.2) | 1.40 (0.9–2.6) | 1.10 (0.6–1.7) | (0.33, 2.33) | 0.51 | 0.88 |
| CD4$^+$CCR7$^-$CD45RA$^-$ EM | 3.36 | 3.02 (1.7–4.5) | 2.46 (1.4–3.8) | 1.70 (1.1–2.2) | (1.0, 5.1) | 0.30 | 0.84 |
| CD4$^+$Foxp3$^+$CD25$^+$CD127$^-$ Tregs | 5.91 | 6.29 (4.9–7.5) | 5.96 (4.5–6.4) | 5.29 (4.7–5.6) | (4.5, 6.2) | 0.60 | 0.90 |
| CD4$^+$CD107a$^+$ T cells | 1.01 | 1.48 (0.8–2.2) | 1.10 (0.8–1.5) | 0.92 (0.7–1.2) | (0.51, 0.81) | 0.69 | 0.91 |
| CD4$^+$IFNg$^+$T cells | 0.2 | 0.23 (0.1–0.5) | 0.17 (0.1–0.28) | 0.05 (0.03–0.07) | (0.03, 0.14) | 0.21 | 0.74 |
| CD4$^+$IL-2$^+$ T cells | 2.66 | 1.86 (1.5–2.3) | 1.86 (1.3–3.0) | 1.33 (0.77–1.49) | (1.2, 2.6) | 0.42 | 0.88 |
| CD4$^+$ TNF$^+$ T cells | 2.51 | 1.87 (1.7–2.1) | 1.85 (1.7–3.2) | 1.13 (0.8–1.2) | (5.0, 8.8) | 0.12 | 0.65 |

Median and Interquartile Ranges (IQR) 1–3 are presented for groups with $N \geq 3$. For groups with $N < 3$, individual values are shown
*CCR5* C-C chemokine receptor type 5, *CCR7* C-C chemokine receptor type 7, *EM* effector memory, *Foxp3* forkhead box P3, *IL-2* interleukin-2, *PD1* programmed cell death protein 1, *TEMRA* T-cell effector memory RA cells, *TNF* tumor necrosis factor

**Table 4 Frequency (%) of infant CD8$^{+}$ T-cell populations stratified by drug exposure**

| | Unexposed ($N = 1$) | CZP ($N = 4$) | IFX/ADA ($N = 11$) | IFX/ADA+ IM ($N = 4$) | CZP+ IM ($N = 2$) | CZP vs IFX/ADA vs IFX/ADA+ IM | |
|---|---|---|---|---|---|---|---|
| Cell population | | Median (IQR) | Median (IQR) | Median (IQR) | Range | P-value | q-value |
| CD8$^{+}$ T cells | 20 | 21.15 (20.2–23.7) | 26.30 (19.6–28.2) | 22.85 (20.9–25.7) | (12.7, 27.9) | 0.94 | 0.99 |
| CD8$^{+}$CCR5$^{+}$ T cells | 1.08 | 1.48 (0.9–2.0) | 0.87 (0.6–1.3) | 0.62 (0.43–0.75) | (0.5, 0.72) | 0.35 | 0.88 |
| CD8$^{+}$CD57$^{+}$ T cells | 3.58 | 4.91 (2.9–8.0) | 2.06 (1.4–12.8) | 2.44 (1.6–3.3) | (0.67, 0.92) | 0.70 | 0.91 |
| CD8$^{+}$PD1$^{+}$ T cells | 1.16 | 2.11 (1.5–2.8) | 2.19 (1.6–2.5) | 1.37 (0.9–1.9) | (2.2, 3.8) | 0.51 | 0.88 |
| CD8$^{+}$CD38$^{+}$HLA-DR$^{+}$ T cells | 2.26 | 6.80 (4.3–9.0) | 3.79 (2.9–7.0) | 3.44 (2.7–4.2) | (1.4, 3.4) | 0.24 | 0.78 |
| CD8$^{+}$CCR7$^{+}$CD45RA$^{-}$ CM cells | 10.2 | 9.78 (8.3–11.8) | 11.90 (9.5–16.0) | 14.15 (10.7–18.6) | (6.7, 13.3) | 0.47 | 0.88 |
| CD8$^{+}$CCR7$^{+}$CD45RA$^{+}$ naive | 79.9 | 84.70 (79.7–85.9) | 80.60 (68.9–84.0) | 78.00 (76.3–81.1) | (81.2, 85.8) | 0.78 | 0.98 |
| CD8$^{+}$CCR7$^{-}$CD45RA$^{+}$ TEMRA | 4.3 | 3.46 (2.1–4.9) | 2.74 (1.7–8.9) | 1.67 (1.2–2.4) | (1.6, 2.2) | 0.28 | 0.84 |
| CD8$^{+}$CCR7$^{-}$CD45RA$^{-}$ EM | 5.6 | 3.49 (2.8–6.0) | 3.67 (2.0–7.4) | 3.44 (2.7–4.4) | (3.8, 5.3) | 0.95 | 0.99 |
| CD8$^{+}$CD107a$^{+}$ T cells | 1.36 | 2.43 (1.0–3.8) | 1.10 (0.2–2.0) | 0.47 (0.5–1.7) | (0.00, 1.0) | 0.58 | 0.90 |
| CD8$^{+}$IFNg$^{+}$ T cells | 0.47 | 0.42 (0.3–1.5) | 0.62 (0.3–1.7) | 0.11 (0.06–0.16) | (0.06, 0.13) | 0.06 | 0.55 |
| CD8$^{+}$IL-2$^{+}$ T cells | 0.13 | 0.19 (0.07–0.34) | 0.29 (0.2–0.4) | 0.11 (0.06–0.22) | (0.04, 0.08) | 0.44 | 0.88 |
| CD8$^{+}$TNF$^{+}$ T cells | 0.29 | 0.17 (0.08–0.8) | 0.27 (0.09–0.9) | 0.08 (0.06–0.1) | (0.09, 0.50) | 0.41 | 0.88 |

Median and interquartile ranges (IQR) 1–3 are presented for groups with $N \geq 3$. For groups with $N < 3$, individual values are shown

Benjamini and Hochberg[28]. The *q*-value is the FDR-adjusted p-value. $q < 0.1$ was used as the threshold for statistical significance. Given the sample size, analysis of 39 parameters, q-value 0.1, we had 80% power to detect an effect size of 2. All B cell, CD4$^{+}$ T cell, and CD8$^{+}$ T-cell subsets in Tables 2–4 were analyzed simultaneously, but displayed separately for convenience. Spearman Principal Component Analysis (PCA) was performed using XLSTAT. All other statistical analyses were performed using Prism 7.

## Results

### Study population

The study population included 22 women and their infants from the PIANO registry who consented to an infant blood draw at 1 year of age. Baseline demographic and clinical data are shown in Table 1 for both maternal and infant study participants, stratified by drug exposure. Infants exposed to CZP monotherapy provided a "negative control" or "reference" population since CZP is not actively transported across the placenta, it is not present at biologically meaningful levels in breast milk, and CZP exposure is not associated with an increased risk of infection in infants[16]. The maternal participants in the CZP and IFX/ADA+ IM groups all had CD, while 6/11 (55%) of participants in the IFX or ADA group had UC. The maternal participants had a median disease duration of 9 years, with a range from 1 to 22 years. There was no statistically significant difference in disease severity at baseline, and most of the maternal participants (18, 82%) were in clinical remission during pregnancy. The baseline demographic and clinical characteristics of the infants are also shown in Table 1. None of the infants were preterm, although there were 4 early term infants born between 37 and 38 weeks. There were no statistically significant differences in gestational age, birth weight, or length (Table 1). There were no congenital anomalies. There was a statistically significant difference in mode of delivery. C-sections were more frequent in infants exposed to combination therapy, partly due to active perianal disease in some of the mothers. As described above, in the PIANO registry as a whole there was a statistically significant increase in infections at 1 year of age among infants exposed to combination therapy as compared to unexposed infants (Relative Risk (RR) 1.50, CI 1.08–2.09). We did not observe a statistically significant difference in this sub-study in the number of infections in the infants when stratified by exposure, likely due to the small sample size (Table 1). With regards to vaccination adherence, 17 infants received the recommended vaccinations except for rotavirus, which is not recommended for babies exposed to anti-TNF in utero, and one infant did not receive the Hepatitis B vaccine. Vaccination history was not available for 5 of the infants. No mothers received prednisone

during pregnancy, and only two mothers in the IFX/ADA group were treated with a prednisone taper during the infant's first year of life. Additionally, 17 of 19 infants were breastfed. Data on breastfeeding were not available for 3 infants, one of which was in the IFX/ADA+ IM group.

## Globally preserved T-cell and B-cell immune profiles among infants with different drug exposures

To generate a more detailed assessment of the adaptive immune system in the infants, we collected blood at 1 year of age and performed multiparameter flow cytometry on B and T lymphocytes. Viability of lymphocytes after freeze/thaw was excellent, ranging from 87 to 99% with a median viability of 97%. Gating strategies are depicted in Supplementary Figure 1 & 2. We analyzed 39 immune cell subsets in total (Tables 2, 3, 4). Although the frequencies were analyzed together, they are reported separately as 12 B-cell subsets (Table 2), fourteen $CD4^+$ T-cell subsets (Table 3), and thirteen $CD8^+$ T-cell subsets (Table 4). As shown in Tables 2–4, the B and T-cell immune profiles for most subsets did not vary significantly among the different drug exposures. Principal component analysis revealed largely overlapping datasets, consistent with overall similarity of the immune profiles despite different drug exposures (Fig. 1a). Similarly, hierarchical clustering did not clearly cluster infants exposed to combination therapy separate from anti-TNF monotherapy (data not shown). Looking at individual subsets, overall frequencies of total $CD19^+$ B cells, $CD4^+$ T cells, and $CD8^+$ T cells were not significantly altered based on drug exposure (Fig. 1b-e). $CD4^+CD25^+Foxp3^+CD127^-$ $T_{regs}$ are an important subset of $CD4^+$ T cells that suppress T-cell responses to antigen[29], yet development of $T_{regs}$ did not appear to vary significantly by drug exposure (Fig. 1e). The frequencies of $CCR7^+CD45RA^+$ naïve, $CCR7^-CD45RA^+$ T effector memory RA (TEMRA), $CCR7^+CD45RA^-$ central memory (CM), and $CCR7^-CD45RA^-$ effector memory (EM) $CD4^+$ and $CD8^+$ T cells did not vary significantly among the groups (Tables 3, 4). Moreover, markers of terminally differentiated T cells (i.e., PD-1 and CD57) were similar among the groups (Tables 3, 4). Similarly, percentages of naïve, switched memory, non-switched memory, and memory effector B cells were preserved regardless of therapeutic exposure (Table 2). Overall, lymphocyte subsets appeared to be relatively preserved among infants exposed to anti-TNF monotherapy and combination therapy with anti-TNF agents and immunomodulators.

## $CD27^+$ B cells, $IgD^-CD27^+$ switched memory B cells, and plasmablasts in infants exposed to combination therapy

None of the B-cell subsets reached statistical significance after adjusting for multiple comparisons, suggesting that B-cell development in infants at 1 year is largely unaltered by these maternal immunosuppressive regimens. While there were no statistically significant differences, three B-cell subsets exhibited trends toward reduction with exposure to combination therapy including plasmablasts, $CD27^+$ B cells, and $IgD^-CD27^+$ switched memory B cells (Fig. 2a–d). The $CD27^+CD38^{hi}$ plasmablast frequency appeared reduced in the IFX/ADA + IM exposure group as compared to the CZP or the IFX/ADA monotherapy groups, but this was not significant after correcting for multiple comparisons ($p = 0.037$, $q = 0.55$; Table 2, Fig. 2b). The median frequency of $CD27^+$ B cells in the combination therapy group was approximately half of the CZP monotherapy group, although again not statistically significant ($p = 0.04$, $q = 0.55$; Table 1 and Fig. 2c). Similarly, the switched memory $IgD^-CD27^+$ B-cell subset exhibited a trend toward reduction with combination IFX/ADA+ IM exposure as compared to the CZP and IFX/ADA groups ($p = 0.099$, $q = 0.65$; Table 1, Fig. 2d). For all of these B-cell subsets, the trends were most apparent between the CZP and the IFX/ADA+ IM groups, while the IFX/ADA group exhibited intermediate values.

The possibility of reductions in switched memory B cells and plasmablasts in infants exposed to IFX/ADA with an immunomodulator suggested that it was not an effect of TNF neutralization alone. In mice, TNF is required for splenic primary B-cell follicles, although immunoglobulin class switching can occur in the absence of TNF[30]. Therefore, we asked if TNF-deficient ($TNF^{-/-}$) mice would exhibit a similar pattern of reduced memory B cells and plasmablasts as compared to wild-type (WT). Although mouse memory B cells and plasmablasts are defined by different surface markers than human memory B cells and plasmablasts, we analyzed the corresponding populations in WT and $TNF^{-/-}$ mice[25–27]. Murine memory B cells and plasmablasts were similar in the spleen and mesenteric lymph nodes (MLNs) of unimmunized WT and $TNF^{-/-}$ mice (Supplementary Fig 3). These data are consistent with the human data in that TNF neutralization alone does not reduce baseline memory B cells and plasmablast frequencies.

## $CD4^+IFN\gamma^+$, $CD8^+IFN\gamma^+$, and $CCR5^+CD4^+$ T cells in infants exposed to combination therapy

With regard to alterations in T cell-mediated immunity, we observed a trend toward lower frequencies of $CD4^+IFN\gamma^+$, $CD8^+IFN\gamma^+$, and $CCR5^+CD4^+$ T cells in infants exposed to combination therapy, though these did not reach statistical significance (Tables 3, 4 and Fig. 2e–g). $CD8^+IFN\gamma^+$ cells appeared slightly reduced in infants exposed to combination IFX/ADA + IM therapy as compared to CZP and IFX/ADA monotherapy

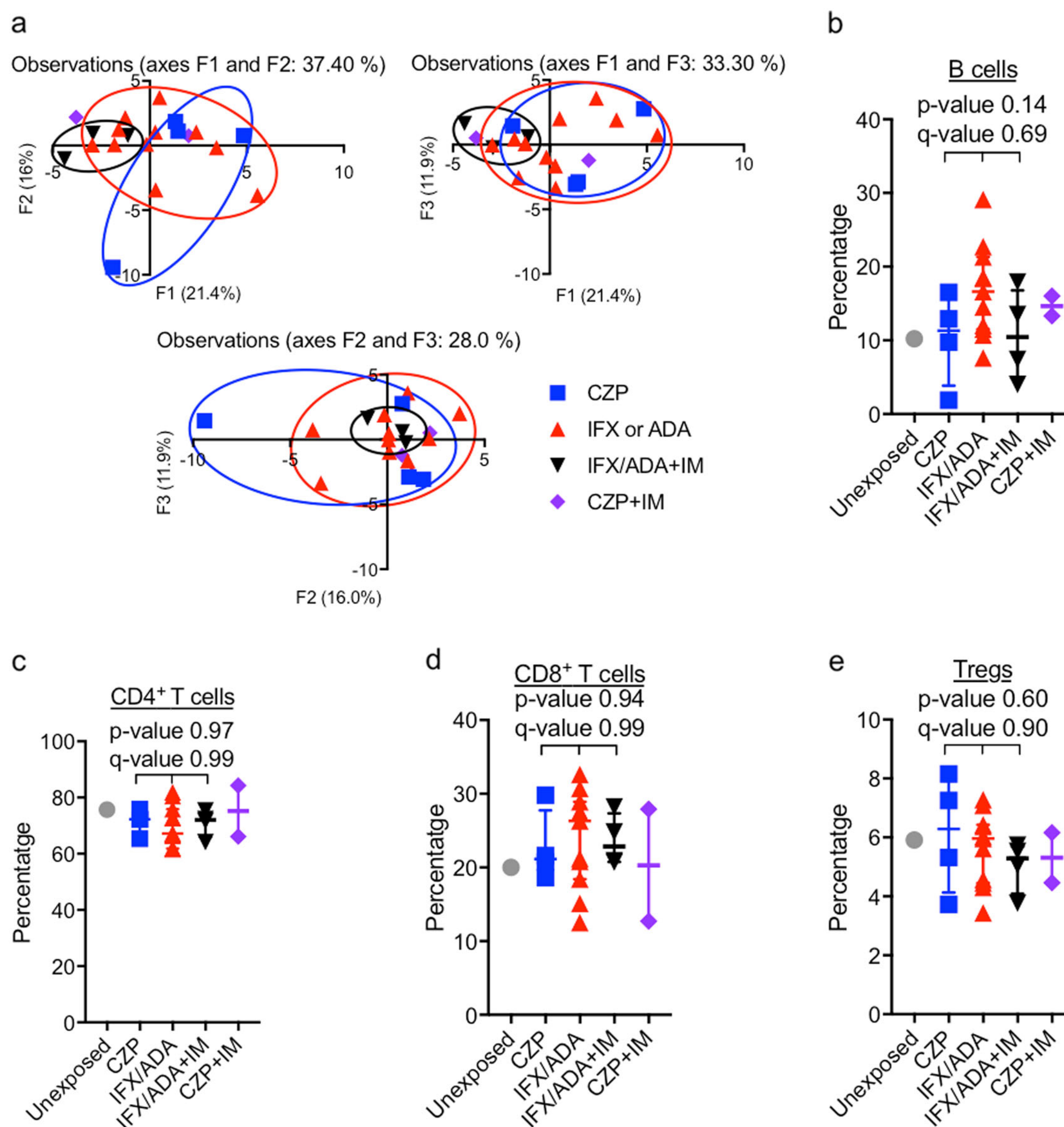


**Fig. 1 Infants exposed to anti-TNF monotherapy or combination therapy with an anti-TNF agent and a thiopurine immunomodulator exhibited similar immune profiles. a** Spearman's PCA of immunophenotyping and study subjects by drug exposure. Principal components F1, F2, and F3 are shown. Each individual is represented by one data point. The percentage of the variance explained by each principal component is shown on the axis. **b**–**e** Median, interquartile ranges, and ranges of frequencies of each cell subset, expressed as a percent of the parent population, are shown. The *p*-value and *q*-value displayed in **b**–**e** were calculated in the three-group analysis (CZP vs IFX/ADA vs IFX/ADA+ IM)

($p = 0.056$, $q = 0.55$; Fig. 2e). $CD4^{+}IFN\gamma^{+}$ cells showed a similar trend ($p = 0.21$, $q = 0.74$; Fig. 2f). IL-2 and TNF were similar in the $CD4^{+}$ and $CD8^{+}$ T cells across the different groups (Tables 3, 4). CD107a or lysosomal-associated membrane protein-1 (LAMP-1), a marker of degranulation, was also similar in stimulated T cells independent of drug exposure (Tables 3, 4). Finally, we also observed a trend toward reduction in $CD4^{+}CCR5^{+}$ cells in infants compared to anti-TNF monotherapy ($p = 0.055$, $q = 0.55$; Fig. 2g), although this again did not reach statistical significance. Overall, exposure to combination therapy did not significantly alter the frequencies of $CD4^{+}$ and $CD8^{+}$ T-cell subsets, although some subsets exhibited trends which could be consistent with an increased susceptibility to infection.

## Discussion

In summary, infants exposed to combination therapy with anti-TNF agents and thiopurines have been shown to have an increased risk of infection during the first year of life, suggesting an underlying difference in immunity. We used multiparameter flow cytometry as a discovery tool to

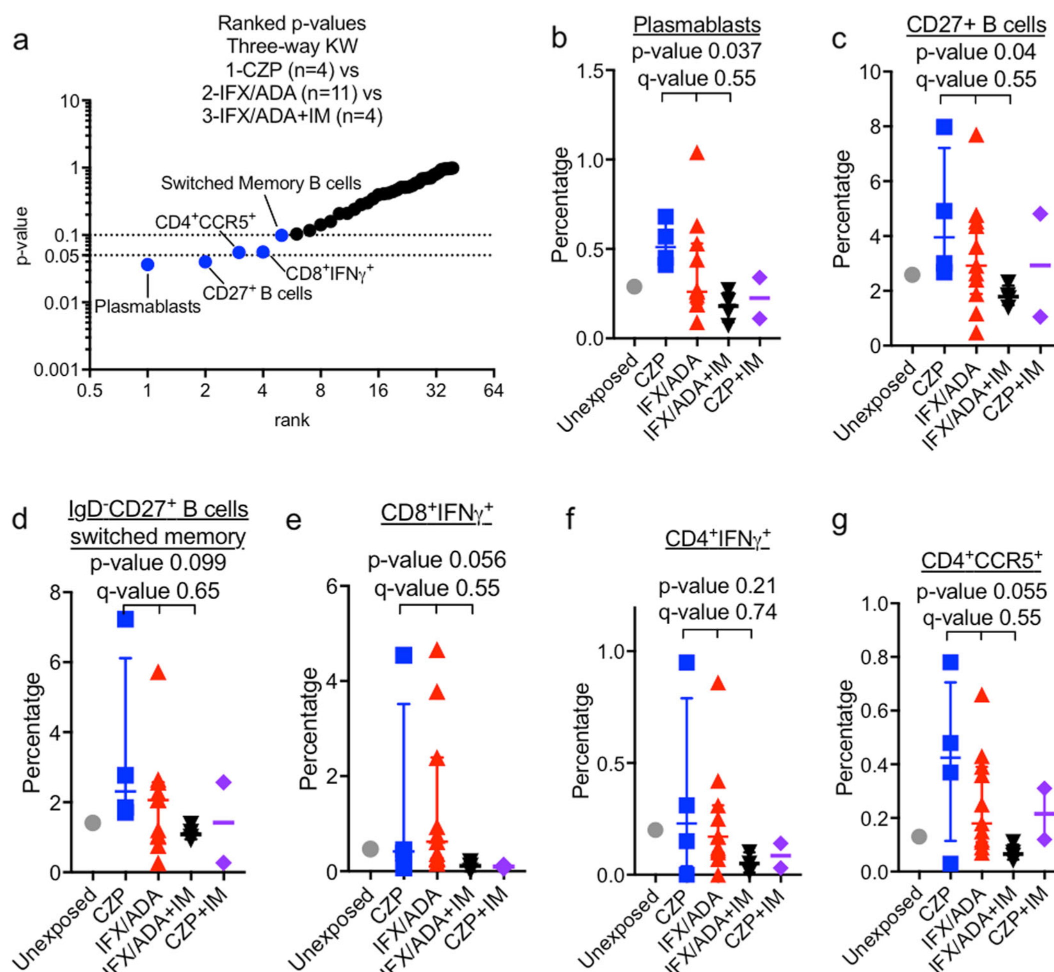


**Fig. 2 Infants exposed to combination therapy with an anti-TNF agent and an immunomodulator exhibited a trend toward reduced CD27+ B cells, switched memory B cells, plasmablasts, IFNγ-producing CD4+ and CD8+ T cells, and CD4+CCR5+ T cells which did not reach statistical significance.** (**a**) Ranked p-values for cell subsets in the three-group analysis comparing CZP vs IFX/ADA vs IFX/ADA+IM. (**b**-**g**) Median, interquartile ranges, and ranges of frequencies of each cell subset, expressed as a percent of the parent population, are shown. The p-value and q-value displayed in (**b**-**g**) were calculated in the three-group analysis. KW-Kruskal Wallis

identify changes in the frequency of B and T lymphocyte subsets. On a global level, these data should be reassuring to providers and pregnant mothers with IBD that major B and T-cell subsets, including $T_{regs}$, are relatively unperturbed at 12 months of age after exposure to these treatment regimens.

Given the large number of comparisons and relatively small sample size, after adjusting for multiple comparisons, reductions in B-cell subsets did not meet statistical significance. However, several cell subsets demonstrated intriguing trends toward reduction after exposure to combination therapy. Reductions in $CD27^{+}$ B cells, switched memory B cells, and plasmablasts in infants exposed to combination therapy as compared to CZP or IFX/ADA monotherapy could contribute to an increased susceptibility to infection. CD27 is a co-stimulatory TNF receptor superfamily member that plays critical roles in survival, function, and differentiation of T, B, NK, and plasma cells[31,32]. In humans, mutations in CD27 have been shown to cause a monogenic common variable immunodeficiency (CVID) syndrome characterized by recurrent infections, hypogammaglobulinemia, and impaired T cell-dependent B-cell activation[31–34]. In fact, plasmablasts are also reduced in CVID patients as compared to normal controls, and the levels of plasmablasts are significantly correlated with the presence of switched memory B cells[32]. Importantly, anti-TNF agents can alter B-cell populations in adult patients. A recent study of Rheumatoid Arthritis patients exposed to anti-TNF therapy revealed reductions in $IgD^{-}CD27^{-}$ memory B-cell frequency compared to pre-treatment levels, while some individuals exhibited reductions in plasmablasts or

switched memory B-cell subsets[35]. Overall, the trend toward reductions in $CD27^+$ B cells, switched memory B cells, and plasmablasts in infants exposed to combination therapy are consistent with compromised humoral immunity and could partially explain an increased risk of infection in these infants.

$CD4^+$ and $CD8^+$ subsets were largely similar, regardless of treatment. Production of IL-2 and TNF appeared largely preserved. IFNγ production exhibited a trend toward suppression in infants exposed to combination therapy. Interestingly, IFNγ responses to bacterial endotoxin in $TNF^{-/-}$ mice are reportedly normal[36], suggesting that TNF neutralization alone would not reduce $IFN\gamma^+$ cells. With regard to potential reductions in the $CD4^+CCR5^+$ population, CCR5 plays a critical role in inflammatory responses[37]. CCR5 deficiency has been reported to increase susceptibility to certain neurotropic viruses and fungal pathogens[38]. Here we did not observe any single subset with dramatic differences after exposure to combination therapy. However, it could be that multiple subtle simultaneous aberrations in both the T-cell and B-cell compartments could cooperatively impair the overall adaptive immune response and lead to an increased risk of infection in infants exposed to combination therapy.

Our data reinforce a recent analysis of vaccine responses in a subset of PIANO cohort infants[39]. Infants exposed to biologic agents exhibited similar protective antibody titers to both tetanus toxin and *Haemophilus influezae* (HiB) vaccines as infants without exposure[39]. Notably, exposure to combination therapy with an immunomodulator also did not affect the percentage of infants with adequate titers to HiB or tetanus. Furthermore, the concentration of the biologic in the infant or in cord blood did not affect the serologic response to vaccines. Adequate titers to both tetanus and HiB vaccines implies that T cell-mediated and B cell-mediated immune responses are functionally intact in these infants.

A recent study looking at seven infants born to mothers exposed to anti-TNF agents and eight healthy control infants showed decreased $T_{reg}$ frequency at birth, as well as more immature B and $CD4^+$ T-cell phenotypes, and a muted response to mycobacterial stimuli[40]. There are similarities between the findings of our study and the study by Esteve-Solé et al.[40], as both studies show that at 12 months there are essentially normal frequencies of naïve and memory $CD4^+$ T cells, $CD8^+$ T cells, and B cells, with modest differences among various adaptive immune subsets. Interestingly, they found lower levels of $T_{regs}$ at birth and at 12 months, while we observed normal frequencies of $T_{regs}$ at 12 months. That study differs from ours in several important ways. First, our study includes more infants exposed to combination therapy with immunomodulators, since that is the group of infants that appear to have the highest risk of infection during the first year of life. Exposure to anti-TNF monotherapy does not seem to increase the risk of infection in exposed infants[16,17]. Our study also focuses on infants at 12 months of age, since that is around the time that the increased risk of infection is most apparent. In the first 6 months of life, infants are protected by transferred maternal immunity and their own immune dysfunction may not be detectable until afterwards. Our dataset includes infants exposed to CZP monotherapy as a rigorous reference population. CZP-exposed infants are born to mothers with IBD, and there is anti-TNF in the maternal circulation, yet the infants themselves are not exposed to the biologic in utero or during breastfeeding. Taken together, these studies provide the first immunophenotyping analyses of adaptive immune development in infants exposed to biologic agents, with or without concomitant thiopurines.

There are several important limitations to our study. The sample size is small due to the difficulty of recruiting to a study involving an infant blood draw without tangible results to the mother. We also do not have a group of infants exposed to immunomodulators alone. Infants exposed to thiopurine monotherapy have not been previously reported to have an increased risk of infection, so we would hypothesize that those infants would not exhibit reductions in any of the populations we analyzed. Finally, C-sections were more common in infants exposed to combination therapy, so this could account for some of the trends we observed in those infants. Delivery by C-section alters the microbiome, which could in turn alter infant immune development[41]. Our sample size is too small to control for the effect of C-sections, but it is something that could be examined in future studies. Despite these limitations, analysis of these rare and difficult to obtain samples has generated clear hypotheses to test in future studies.

Future directions include replicating these results, as well as extending immune profiling to include additional components of the immune system. This study focused on B and T-cell subsets and the adaptive immune response. The innate immune response would also be important to evaluate. For example, monocytes, dendritic cells, NK cells, and neutrophils would all be of interest. Additionally, including Ki67 and CD45RA in our $T_{reg}$ staining panel would allow us to better characterize $T_{reg}$ activation status in these infant samples[42]. In order to assess all of these populations, we could use mass cytometry, which has the ability to profile over 40 markers simultaneously with single-cell resolution[43]. Using that approach, even with a small amount of initial sample, we could profile cells of the innate immune system in addition to lymphocytes. Additionally, it could be that biologic therapy in the pregnant IBD patient alters the maternal

and infant microbiome, which in turn alters the immune system and predisposes to infection. Simultaneously profiling the immune system and the microbiome could be informative. Lastly, incorporating functional analyses on immune subsets could uncover more subtle defects.

In conclusion, this analysis adds to a growing body of literature regarding the effect on infants after treating pregnant IBD patients with anti-TNF agents, as monotherapy or in combination with immunomodulators. Although we highlight trends toward subtle differences in B and T-cell subsets, none of the 39 that we analyzed are statistically significant, and the infant adaptive immune system appears to develop largely intact regardless of drug exposure.

## Study Highlights

### What is current knowledge

- Anti-TNF agents and thiopurines are low risk and effective during pregnancy and breastfeeding, but infants exposed to combination therapy may exhibit an increased risk of infection

### What is new here

- Overall frequencies of B cells, $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells, $T_{regs}$, and various subsets are similar among infants exposed to anti-TNF monotherapy versus combination therapy

### Translational impact

- Normal infant immune development will help counsel pregnant IBD patients regarding the favorable risk/benefit profile of continuing combination therapy during pregnancy

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## Conflict of interest

**Guarantor of the article:** Michael G. Kattah.

**Specific author contributions:** U.M. conceived of the study and recruited patients. J.M.M. designed and performed the human flow cytometry experiments. M.G.K. analyzed and interpreted the clinical and flow cytometry data, and performed all statistical analyses. RPM recruited patients and provided critical review of the manuscript. TB aided in experimental design, design of human flow cytometry panels, and provided critical review of the manuscript. M.G.K., M.I.W., and A.M. performed the mouse experiments. M.G.K. and U.M. wrote the manuscript.

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