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Title: Thiopurine use associated with reduced B and natural killer cells in inflammatory bowel disease

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1 What did this study explore?

This study explored lymphocyte subpopulations in the peripheral blood and colonic mucosa of healthy controls and IBD patients with or without thiopurine therapy to determine whether a relative lymphopenia observed with chronic thiopurine use is due to selective depletion of specific lymphocyte populations.

2 How did the authors perform all experiments?

Patients, Specimens, and Clinical Data: 557 healthy controls, 42 IBD patients (31 Crohn's, 10 UC, 1 indeterminate colitis) on azathioprine (n=34) or 6-mercaptopurine (n=8) and 168 IBD patients (105 Crohn's, 61 UC, 2 indeterminate colitis) on no thiopurines provided CBC data in figure 1. Leukocyte subsets in the latter were defined and reported according to the International Council for Standardization in Hematology (ICSH) guidelines (http://icsh.org/guidelines/).

Live, frozen peripheral blood mononuclear cells (PBMC) were obtained from 19 of the above IBD patients (14 Crohn's, 5 UC) on azathioprine (n=16) or 6-mercaptopurine (n=3), and 19 IBD patients (also 14 Crohn's, 5 UC) on no thiopurine medications, but matched in terms of whether or not taking a 5' aminosalicylate agent. Also, PBMC were obtained from 38 healthy controls, age and gender-matched to each of these IBD

patients. None of these blood donors were on glucocorticoids, biopharmaceuticals, or other systemic immunosuppressive agents at the time of phlebotomy.

Live, frozen, colonoscopic biopsies were obtained from 17 IBD patients (13 Crohn's, 4 UC) on azathioprine (n=14) or 6-mercaptopurine (n=3), and 17 IBD patients (also 13 Crohn's, 4 UC) on no thiopurines at the time of colonoscopy. 7 patients in each group were on an anti-TNF biopharmaceutical, and 3 patients in each group were on a glucocorticoid. 6 patients in the thiopurine group and 8 in the no thiopurine group were on a 5' aminosalicylate agent. The colonic mucosa biopsied was deemed by the colonoscopist to be actively inflamed in 6 of the patients on thiopurines and 7 of the patients on no thiopurines. As a control, biopsies from 49 healthy screening colonoscopy recipients were also examined.

Flow Cytometry: Samples were thawed and colon biopsies were digested in a vortex at 37 degrees centigrade for 30 minutes in media containing collagenase and DNAse to liberate single cells. Cells were then filtered, washed, and stained extracellularly with panels of fluorophor-conjugated antibodies. Monoclonal antibodies against CD3 (clone SK7), CD4 (RPA-T4), CD8 (RPA-T8), CD19 (HIB19), CD25 (M-A251), CD27 (L128), CD38 (HIT2), CD49d (9F10), CD56 (NCAM16.2), CTLA4 (BNI3), and Ki67 (B56) were obtained from BD Biosciences (San Jose, CA, USA). Monoclonal antibodies against CD3 (SK7), CD4 (RPA-T4), CD8 (RPA-T8), CD16 (3G10), CD19 (HIB19), CD20 (2H7), CD45RA (HI100), CD49d (9F10), CD56 (HCD56), CD161 (HP-3G10), NKG2D (1D11), IFN gamma (4S.B3), IgD (1A6-2), IgM (MHM-88), TCRva24-Ja18 (6B11), TCRva7.2 (3C10), FOXP3 (236A/E7), and Helios (22F6) were obtained from BioLegend (San Diego, CA, USA). Monoclonal antibodies against CD8 (RPA-T8), NKp46 (9E2), integrin beta 7 (FIB504), gamma delta TCR (B1.1), and IL17A (eBio64DEC17) were obtained from eBiosciences (San Diego, CA, USA). Intracellular staining was performed with a FOXP3 staining kit (eBiosciences). For intracellular cytokine staining (ICCS), cells were first incubated overnight with PMA, ionomycin and brefeldin A. Stained cells were evaluated on a FACSCanto, a Fortessa or, for ICCS, a FACS Calibur (all BD Biosciences) flow cytometer. Data was analyzed with FlowJo (FlowJo, LLC, Ashland,

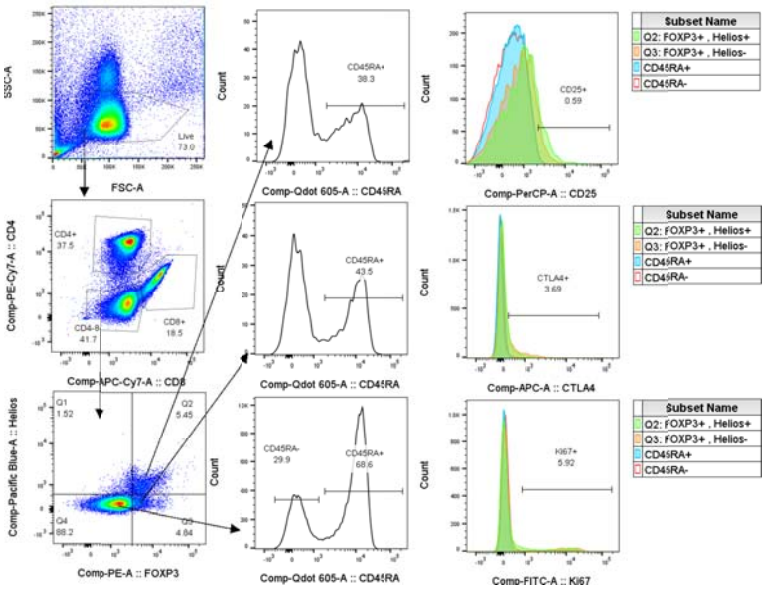
OR) Excel (Microsoft, Inc, Redmond, WA), and GraphPad Prism (Graph Pad Software, Inc., La Jolla, CA) software.

3 How did the authors process all experimental data?

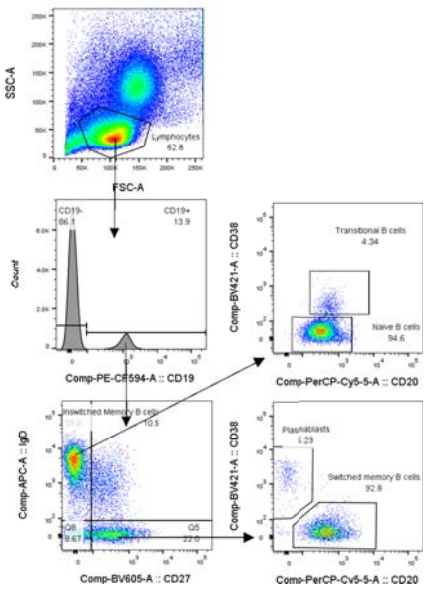
CBC data in Figure 1a was obtained from a clinical diagnostic lab and analyzed by JDL with GraphPad Prism software to produce graphics and statistical analyses, the latter with an unpaired Student's two-way t test for each comparison shown.

Flow cytometry data for all other figures was acquired by DMS on flow cytometers listed above, with multicolor compensation in FACSCanto and Fortess instruments calculated via FACSDiva software (BD Biosciences), using single-color beads. FCS files were analyzed by JDL with FlowJo software. Gating templates were applied to sample data as depicted below, using batch functions for consistency.

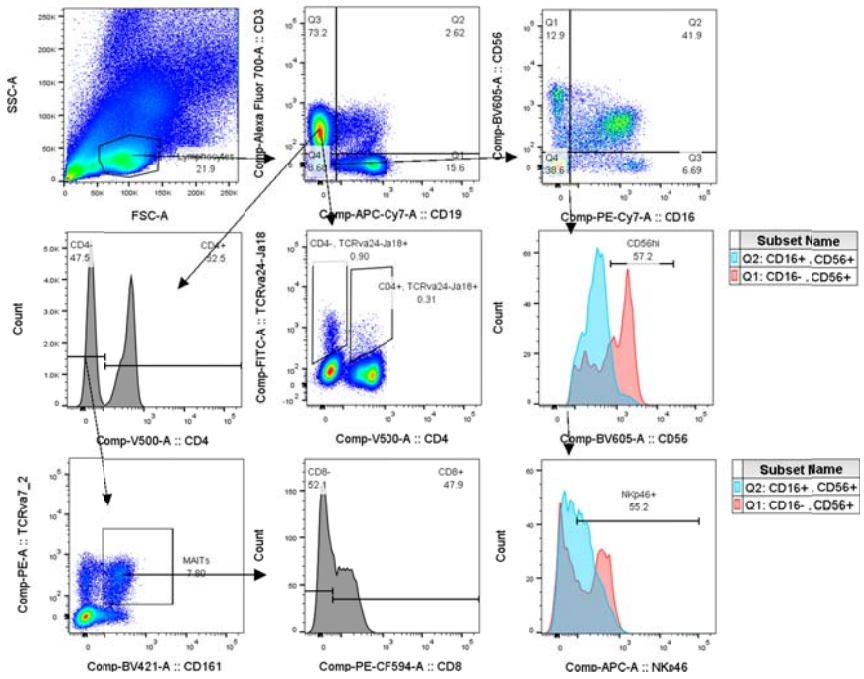
T cell panel:



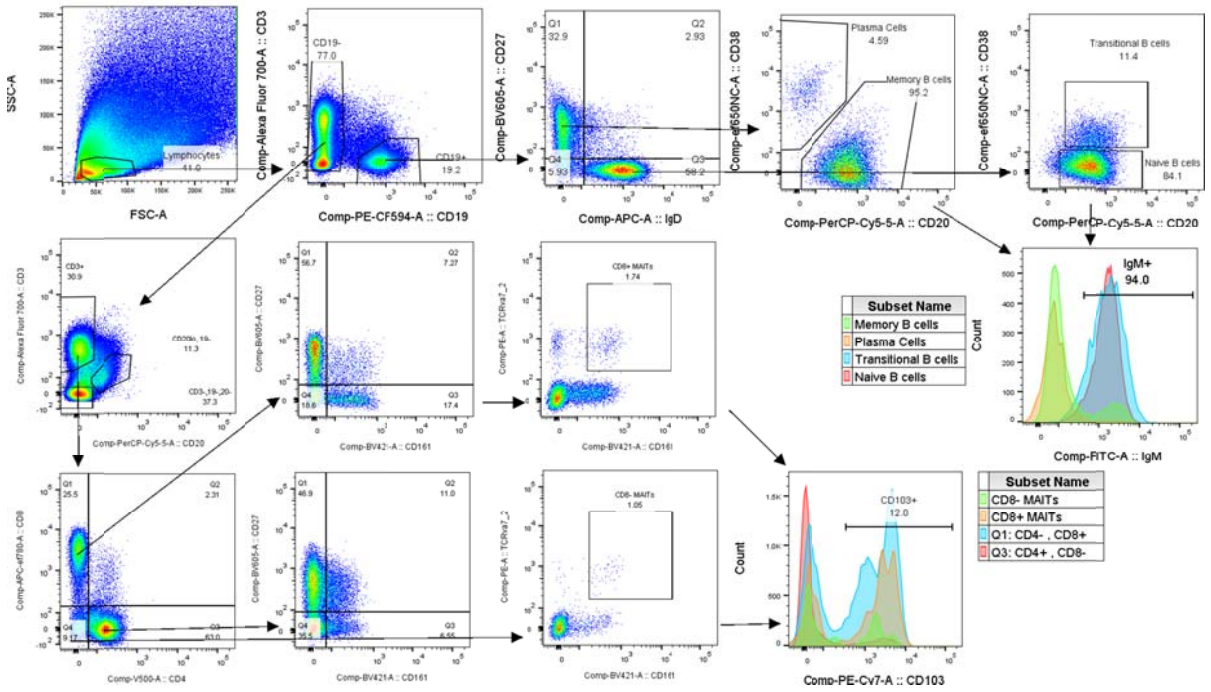
B cell panel:



NK/MAIT/iNKT cell panel:



Biopsy panel B, T cells:



Data was exported to Microsoft Excel for organization and labeling, and then imported into GraphPad Prism for graphics and statistical analyses, the latter with an unpaired, two-way, non-parametric (Mann-Whitney) test for each comparison shown.

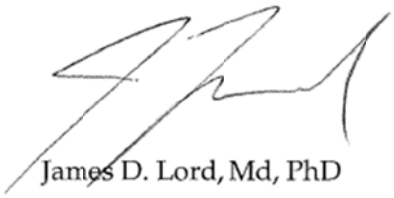
4 How did the authors deal with the pre-study hypothesis?

As this was not a clinical trial, the pre-study hypothesis was simply that thiopurine use selective diminished specific lymphocyte populations more than others, rather than globally reducing all populations equally. Being agnostic to what those specific populations might be, this was necessarily a discovery project, to determine which of many possible lymphocyte populations were depleted selectively in thiopurine recipients, and therefore could not be constrained to a single primary testable hypothesis, or a limited number of secondary hypotheses, amenable to correction for multiple comparisons. Having said that, the most striking finding of these studies, namely the depletion of transitional B cells from the peripheral blood, was validated in the colonic mucosa, using an independent cohort of IBD patients on or off thiopurines.

5 What are the novel findings of this study?

In contract to *in vitro* data suggesting that thiopurines are selectively toxic to T cells, our results showed no depletion of T cells, or even rare subpopulations thereof, associated with thiopurine use in IBD. Instead, we found thiopurine recipients to have reduced NK and B cell populations in the periphery, with a striking elimination of transitional B cells that was also seen in the colonic mucosa. By linking thiopurine use to B and NK cell depletion, our findings suggest mechanisms by which these therapeutics may reduce anti-drug antibodies and increase risk of virally-mediated neoplasia, respectively, in clinical practice.

Sincerely,



James D. Lord, Md, PhD