Response to Reviewers:

We thank the reviewers for their thorough evaluation of our manuscript, and have addressed their concerns as follows:

Reviewer #00049331:

"In this study authors aimed to determine if and how thiopurine use is associated with depletion of specific lymphocyte populations. Authors demonstrated that a relative lymphopenia associated with thiopurine use is attributable to decreased NK and B cells, rather than T cell depletion. This is a good study and it gives us a well information in terms of thiopurine mechanism. However studies design should be checked and rearranged. Aim of study should be pay attributed and placed either in abstract and in introduction. "

We placed the phrase "The aim of this retrospective observational cohort study was to identify which lymphocyte populations are specifically depleted by thiopurine use in vivo" in the abstract and "our aim was to determine if and how thiopurine use is associated with depletion of specific lymphocyte populations" in the last paragraph of the introduction.

"Result informations that comes from this study should be removed in introduction. After correction, this study is acceptable."

The text "In the peripheral blood, we found more FOXP3+ Helios+ Tregs and fewer MAIT and iNKT cells in IBD patients than controls, but thiopurine use correlated with no changes in any T cell subpopulations. Thiopurine use was instead significantly associated with reduced B and NK cells, and particularly CD38+ transitional B cells, but not memory B cells or plasmablasts" has been removed from the introduction.

Reviewer #01022086:

“The authors have performed a flow cytometric analysis to quantify the immune subsets in healthy controls versus IBD patients, either treated or not with thiopurines. The data show that T cells are not affected, whereas B and NK cells are reduced upon thiopurine use. This is a descriptive manuscript; it does not give any mechanistic insight in the effect of thiopurines on B and NK cells. Major: 1) Fig. 1A should include the results of healthy controls. In analogy with all the other figures, it would also be best to show the ‘No Thiopurine’ before the ‘Thiopurine’ results. “

Figure 1a has been modified to include data from healthy controls, and reverse the positioning of IBD cohorts, as recommended.

“Fig. 1C should also show the Treg cell numbers per ml. These Treg cell numbers will probably not be different between healthy controls and IBD patients. “

Figure 1c has been modified to include an additional graphic, reporting Treg numbers per volume of blood, in addition to reporting them as a percentage of CD4 T cells.

“2) The NK cell data should be completed by also showing CD56bright and CD56dim cell numbers, in combination with CD16 expression. “

Figure 3 has been expanded to show data on CD16+ and CD161+ NK cells as figures 3c and 3d, and on $CD56^{high}$ (or bright) and $CD56^{low}$ (or dim) populations as figures 3e and 3f. We added to the text the phrase “CD56 was expressed bimodally in NK cells, with a small but distinct CD56high population among CD16- cells discernable from the majority of NK cells, which are CD56low. Thiopurine use was associated with a decreased number of the latter cells (figure 3e), but not significantly with any change in CD56high cells (figure 3f)”.

Due to the addition of these extra figures making figure 3 too large, what was previously figures 3c-f has been converted to figure 4a-d, and the prior figure 4 is now figure 5, with associated changes in the figure legends.

3) It is surprising and unexpected that less than 20% of the CD4+FOXP3+Helios+ cells express CD25 (supplemental fig 2).

The labels for supplemental figure 2c were modified to now say "CD25^{high}" instead of "CD25+", as this modification more accurately reflects the stringency with which CD25 expression was ascertained.

"Minor: 1) Grammatical errors: ? Abstract, line 4: 'their ability control IBD at lower doses': include 'to' (control) ? p. 9, line 1: 'our data suggest': suggests ? Fig. 1 legend: 'Thiopurine use associated with lymphopenia': include 'is' (associated) ? Supplemental figure 1 legend: 'TCRva24/ja18 …. TCRva7.2': replace 'a' with 'α' "

These minor grammatical errors have all been corrected in the text.

"2) It should be indicated how lymphocytes, monocytes and granulocytes were defined. "

The phrase "Leukocyte subsets in the latter were defined and reported according to the International Council for Standardization in Hematology (ICSH) guidelines (http://icsh.org/guidelines/)" has been added to the text in the materials and methods section.

"3) Which are the CD3- CD19- cells that are not CD56+ NK cells?"

This is not known, but we speculate these rare cells could be plasmacytoid dendritic cells, innate lymphoid cells, or other rare populations not evaluated in this study. Alternatively, they could be monocytes small and agranular enough to fit into the lymphocyte gate on forward and side scatter. Conversely, platelet clumps and other debris large enough to fit into the lymphocyte gate would be negative for the lineage markers evaluated in this study.

Reviewer #00055041:

“The paper is interesting. The manuscript would benefit from inclusion of introducing/bridging sentences between the individual parts of the "Results" that explain the logical order and rationale for the experiments”

The phrase “Finding no difference in T cells between patients on or off thiopurines, we next turned our attention to CD3- lymphocytes” was added at the start of the Results section titled “Thiopurine use is associated with fewer circulating NK and B cells”, and the phrase “To determine if our findings in the peripheral blood were reflected at the site of IBD activity, we evaluated lymphocyte populations in the intestinal mucosa” was added at the start of the Results section titled “Thiopurine use is associated with decreased B, but not T or NK cell, frequency in the intestinal mucosa”.

“In the Discussion, the Authors should highlight the possible clinical significance of their findings”

The phrases “Thus a potential clinical significance of our findings concerning B cells in thiopurine recipients is that they may help identify a peripheral biomarker for successful thiopurine-mediated ADA prophylaxis, or even identify drug-naïve IBD patients at low enough risk of ADA to obviate the need for thiopurine prophylaxis”, and “our findings may explain why thiopurine recipients are at increased risk of certain types of cancer, particularly lymphoma mediated by Epstein-Barr virus(30), or cervical cancer mediated by the human papilloma virus(31)”, and “Future studies associating them with clinical outcomes, such as ADA formation, may provide useful biomarkers to guide therapy for IBD patients. Furthermore, exploring the mechanism by which such changes occur may elucidate more selective means with which to tailor therapy while reducing the risk of off-target toxicities currently associated with thiopurine medications” were added to the discussion section.

In addition to the peer reviewers above, an internal biostatistician review, as requested by the editors, recommended some reanalysis of the data, particularly that

the parametric t-tests we used be replaced by non-parametric Mann-Whitney evaluations, due to flow cytometry data not being of a Gaussian distribution. This changed our p-values slightly, and actually strengthened our main conclusions, but occasionally made some of the more marginal findings more or less significant, as is now reflected in the text.

We believe these changes should address all of the issues raised by the reviewers and thank them for having thus strengthened our manuscript.

-James Lord, MD, PhD