

ORIGINAL ARTICLE

IL-13R α 2-bearing, type II NKT cells reactive to sulfatide self-antigen populate the mucosa of ulcerative colitis

Ivan J Fuss,¹ Bharat Joshi,² Zhiqiong Yang,¹ Heba Degheidy,² Stefan Fichtner-Feigl,³ Heitor de Souza,⁴ Florian Rieder,⁴ Franco Scaldaferrri,⁴ Anja Schirbel,⁴ Melania Scarpa,⁴ Gail West,⁴ Chuli Yi,¹ Lili Xu,¹ Pamela Leland,² Michael Yao,¹ Peter Mannon,¹ Raj K Puri,² Claudio Fiocchi,⁴ Warren Strober¹

► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/gutjnl-2013-305671>).

¹Mucosal Immunity Section, Laboratory of Host Defenses, NIAID NIH, Bethesda, Maryland, USA

²Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research FDA, Bethesda, Maryland, USA

³Department of Surgery, University of Regensburg, Regensburg, Germany

⁴Department of Pathobiology, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

Correspondence to

Dr Ivan J Fuss, Mucosal Immunity Section, LHD, NIH, 10 Center Dr, CRC Building, Room 5W-3864, Bethesda Maryland, USA; ifuss@niaid.nih.gov

IJF and BJ are co-authors of manuscript.

Received 17 July 2013

Revised 5 January 2014

Accepted 6 January 2014

Published Online First

10 February 2014

ABSTRACT

Objective Previous studies have shown that ulcerative colitis (UC) is associated with the presence of lamina propria non-invariant (Type II) NKT cells producing IL-13 and mediating epithelial cell cytotoxicity. Here we sought to define the antigen(s) stimulating the NKT cells and to quantitate these cells in the UC lamina propria.

Design Detection of Type II NKT cells in UC lamina propria mononuclear cells (LPMC) with lyso-sulfatide loaded tetramer and quantum dot-based flow cytometry and staining. Culture of UC LPMCs with lyso-sulfatide glycolipid to determine sulfatide induction of epithelial cell cytotoxicity, IL-13 production and IL-13R α 2 expression. Blinded quantum dot-based phenotypic analysis to assess UC LPMC expression of IL-13R α 2, CD161 and IL-13.

Results Approximately 36% of UC LPMC were lyso-sulfatide tetramer positive, whereas few, if any, control LPMCs were positive. When tested, the positive cells were also CD3 and IL-13R α 2 positive. Culture of UC LPMC with lyso-sulfatide glycolipid showed that sulfatide stimulates UC LPMC production of IL-13 and induces UC CD161 LPMC-mediated cytotoxicity of activated epithelial cells; additionally, lyso-sulfatide induces enhanced expression of IL-13R α 2. Finally, blinded phenotypic analysis of UC LP MC using multicolour quantum dot-staining technology showed that approximately 60% of the LPMC bear both IL-13R α 2 and CD161 and most of these cells also produce IL-13.

Conclusions These studies show that UC lamina propria is replete with Type II NKT cells responsive to lyso-sulfatide glycolipid and bearing IL-13R α 2. Since lyso-sulfatide is a self-antigen, these data suggest that an autoimmune response is involved in UC pathogenesis.

INTRODUCTION

Previous studies of a murine model of ulcerative colitis (UC), oxazolone-colitis, as well as human UC have provided evidence that these forms of intestinal inflammation are marked by the presence of lamina propria CD4 T cells that by several criteria are Type II (non-invariant) NKT cells. Moreover, these studies showed that upon stimulation, these NKT cells produce IL-13 and exhibit cytotoxicity for epithelial cells that is augmented by IL-13.^{1–3} While these previous studies suggested that NKT cells play an important pathogenic role in UC, they did not

Significance of this study

What is already known on this subject?

- A murine model of ulcerative colitis (UC), oxazolone colitis, as well as human UC, are marked by the presence of CD4 NKT cells; in UC, these cells are Type II NKT cells that are non-invariant.
- Upon activation, the NKT cells secrete large amounts of IL-13, a cytokine that is both cytotoxic for epithelial cells, and that augments NKT cell cytotoxicity for epithelial cells.

What are the new findings?

- The UC lamina propria is populated by a large number of Type II NKT cells recognised by their ability to bind lyso-sulfatide glycolipid-loaded CD1d-tetramer.
- Lyso-sulfatide glycolipid stimulates UC lamina propria NKT cells to produce IL-13, exhibit increased epithelial cell cytotoxicity and display increased levels of IL-13R α 2.
- IL-13R α 2-bearing cells in UC lamina propria constitute approximately 60% of the mononuclear cells present and coexpress IL-13 and CD161.

How might it impact on clinical practice in the foreseeable future?

- The clinical severity of patients with UC might be assessed by quantitation of cells binding lyso-sulfatide-loaded tetramer and/or bearing IL-13R α 2.
- UC could be treated by reducing the levels of lamina propria IL-13R α 2-bearing NKT cell or IL-13 levels.

address two important questions. First, they did not define a glycolipid antigen responsible for TCR stimulation of the NKT cells, and for thus initiating or maintaining the UC inflammatory process. Second, they did not identify an NKT cell surface marker that could be used to quantitatively assess or functionally evaluate these NKT cells in the lamina propria of patients with UC.



CrossMark

To cite: Fuss IJ, Joshi B, Yang Z, *et al.* *Gut* 2014;**63**:1728–1736.

In the present studies we address these unanswered questions. We establish first using CD1d-tetramer binding studies that the lamina propria of UC patients contains a substantial population of CD3 T cells whose TCR binds lyso-sulfatide glycolipid, an endogenous glycolipid antigen previously shown to stimulate Type II NKT cells.⁴⁻⁷ Further evidence that UC NKT cells have TCRs responsive to sulfatide glycolipid came from studies showing that this antigen stimulates UC lamina propria cells to secrete IL-13, and to exhibit enhanced epithelial cell cytotoxicity. Additionally, we show that sulfatide glycolipid antigen upregulates NKT cell expression of IL-13R α 2, a high-affinity IL-13 receptor. Finally, we performed extensive studies of the phenotype of IL-13R α 2-expressing cells, and using this receptor as a UC NKT cell marker obtained evidence that NKT cells make up the majority of cells in the UC lamina propria.

MATERIALS AND METHODS

Patient population

Sixty-four patients (pts) admitted to the Cleveland Clinic (Cleveland, OH) and the NIH Clinical Center contributed colonic tissue for these studies. These included 31 UC patients with severe (26 pts) or moderate (5 pts) disease based on the criteria of Truelove⁸ and 21 Crohn's disease patients with severe (18 pts) or moderate (3 pts) disease based on the criteria of De Dombal⁹; all CD patients had colonic involvement only. Patients with adenocarcinoma of the colon (n=12) who contributed the normal margin zone tissue served as controls. The institutional review boards of the Cleveland Clinic and the National Institute of Allergy and Infectious Disease, NIH approved collection of surgical specimens. Informed consent was obtained from all patients.

Preparation of lamina propria mononuclear cells

Lamina propria mononuclear cells (LPMC) were isolated from freshly obtained surgical specimens from IBD and non-IBD control patients as previously described.¹ For determination of ex vivo LPMC cytokine production for IL-13, IFN- γ or IL-4, LPMC were cultured as previously described¹ and as determined in the online supplementary information.

Real-time PCR

Aliquots of LPMC cells obtained from UC, Crohn's disease and control individuals were subjected to RNA extraction using RNeasy tissue kit (Qiagen Valencia Calif). A total of 100 ng template RNA was reversed transcribed with Superscript III RT-PCR kit (Invitrogen, Carlsbad, California). Primer sequences for IL-13R α 2, IL-13R α 1, CD161, IL-17A and β -actin are as listed in online supplementary information.

Protein synthesis inhibition assay

A protein synthesis inhibition assay (as described in online supplemental information) was employed to secondarily assay the surface expression of IL-13R α 2 on LPMC.^{10 11}

Quantum dot immunostaining of LPMC cells

LPMC cells from UC, CD or control individuals were subjected to in situ histochemical (IHC) staining using a modified quantum dot (Q dot) technique as described in online supplementary information.¹²

Detection of sulfatide glycolipid-loaded CD1d-tetramer-binding cells

LPMC were stained with unloaded or loaded tetramer, and underwent flow cytometric and Q-dot staining analysis as indicated in online supplemental information.

Statistical analysis

Statistical differences were assessed using the Student t test and Bonferroni correction analysis for multiple parameter correlation. All values of $p < 0.05$ were considered statistically significant.

RESULTS

LPMC of UC is populated by NKT cells that bind lyso-sulfatide-loaded tetramer

As noted above, inflamed UC tissue contains Type II (non-invariant) NKT cells that do not express V α 24 and do not respond to α -galactosylceramide.¹ We therefore considered the possibility that these cells have TCRs that recognise one or more of the sulfatide family of glycolipids, that is, self-glycolipids that have recently been shown to stimulate Type II NKT cells.⁴⁻⁷ To examine this possibility, we generated sulfatide-loaded CD1d tetramers using individual sulfatide isoforms previously shown to have NKT cell stimulatory activity:^{14 15} ceramide-galactoside-3-sulfate (ceramide sulfatide) and sphingosine-1-galactoside-3-sulfate (lyso-sulfatide). We then incubated LPMC purified from UC and control lamina propria tissue with sulfatide-loaded tetramers, and subjected the cells to a highly sensitive Q-dot-staining technique that maximises detection of stained cells in dispersed cell populations (see Methods and online supplementary information).¹² Finally, we enumerated tetramer-positive cells by blinded counting of stained cells in dispersed cell populations using both visual observation and computer analysis; alternatively, we enumerated tetramer-positive cells by flow cytometry. We found that LPMCs from UC patients contained a population of lyso-sulfatide tetramer-positive cells that was not only approximately fourfold to fivefold greater than in LPMCs from Crohn's disease or normal control patients (figure 1A), but also characterised by far greater tetramer staining intensity (figure 1B). Indeed, while the low-level staining characterising Crohn's disease and normal control LPMC was observed by examination of slides by light microscopy, it was too faint for photographic display. Further studies indicated that these lyso-sulfatide positive cells in UC LPMC were indeed T cells, as they were concomitantly positive for the T cell marker CD3 (figure 1C). Similar results were obtained when tetramer staining was analysed by flow cytometry (figure 1D). It should be noted that cells in UC LPMC did not exhibit significant binding of ceramide sulfatide-loaded tetramers (see online supplementary figure 1), possibly reflecting the fact that they have TCRs that bind this form of sulfatide with an affinity below that necessary to detect tetramer binding using the technique presently available.

Sulfatide glycolipids induce UC NKT cell IL-13 production

In a second approach to evaluating if UC NKT cells recognise a sulfatide glycolipid, we determined if the latter stimulates NKT cells in UC LPMC to produce IL-13, a cytokine previously shown to be produced by UC NKT cells.¹ In this case, we stimulated the cells with lyso-sulfatide and ceramide sulfatide as well as an additional sulfatide family member also shown to stimulate NKT cells, N-tetra-sulfatide (N-tetracosanoyl-sphingosyl- β -D-galactoside-3-sulfate).^{14 15} We found that lyso-sulfatide and ceramide sulfatide stimulated UC LPMC to produce IL-13 (74 pg and 31 pg/mL, respectively), whereas, N-tetra-sulfatide had no stimulatory effect (figure 2A).

Important negative results obtained in these studies included the fact that none of the sulfatides induced increased secretion of IFN- γ (figure 2: the mean IFN- γ secretion of 32.7 pg/mL by cells

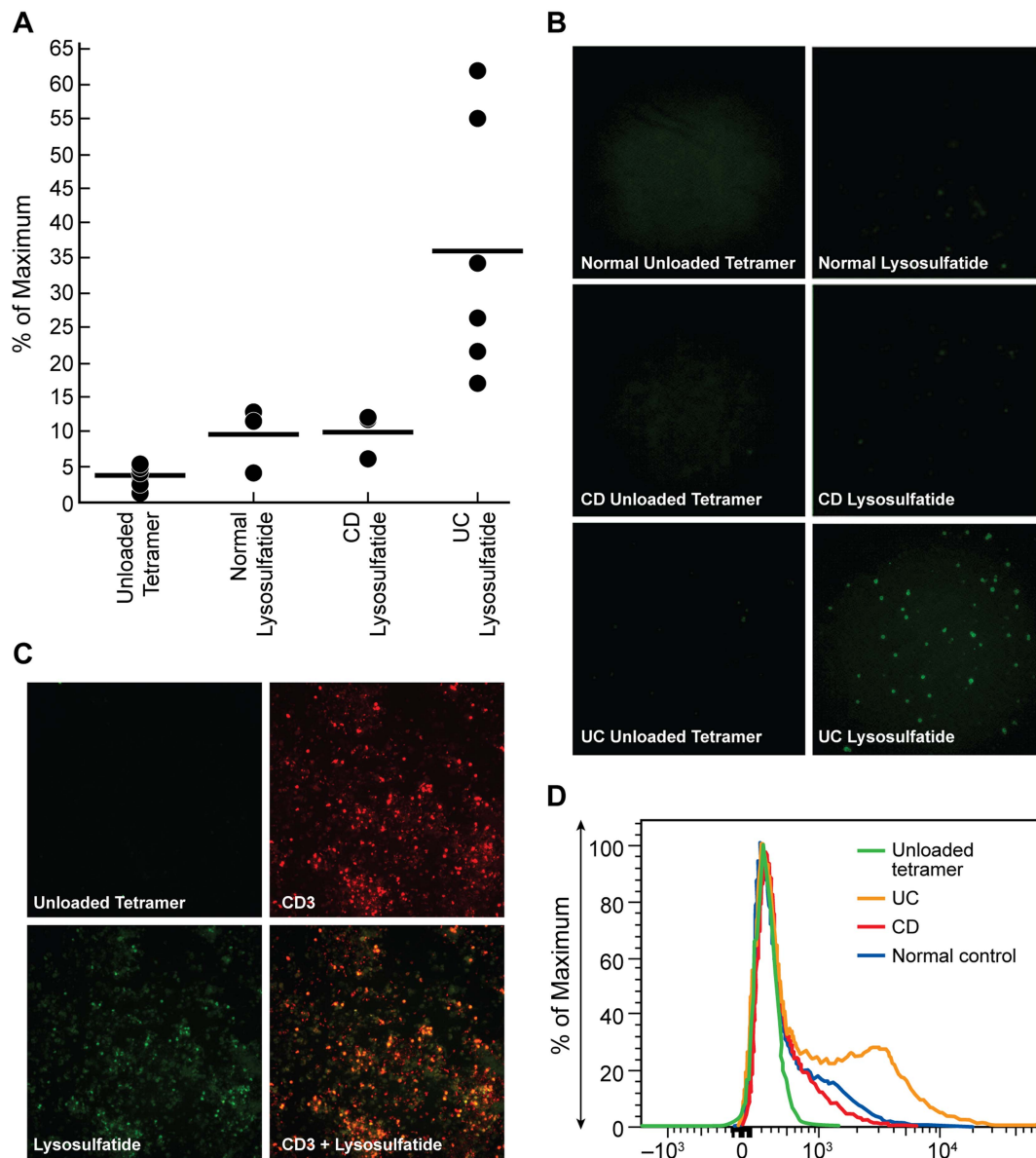


Figure 1 (A) Purified LPMCs were isolated from UC (n=6), CD (n=3) and control patient populations (n=3), and subjected to Q-dot staining analysis to detect binding of lyso-sulfatide tetramer. Percent expression of lyso-sulfatide tetramer binding is shown. Percent expression represents lyso-sulfatide binding for each patient sample minus expression of binding for unloaded tetramer of each patient sample. All datasets from each patient were performed in triplicate. For staining percentage comparison, $p < 0.01$ ulcerative colitis versus Crohn's disease, or normal controls and $p < 0.0001$ for staining intensity with no statistical difference for Crohn's disease versus normal controls for percentage staining ($p > 0.9$), or staining intensity ($p > 0.4$) (B) Purified LPMCs were isolated from UC (n=6), CD disease (n=3) and control patients (n=3) and subjected to Q-dot staining to detect binding of lyso-sulfatide tetramer. One representative staining experiment is shown. (C) Purified LPMCs were isolated from UC patient population (n=2), and subjected to Q-dot staining to detect binding of lyso-sulfatide tetramer and CD3, range 52–58% Lyso-sulfatide⁺/CD3. (D) Purified LPMCs were isolated from UC (n=6), CD (n=3) and control patient populations (n=3) were subjected to Q-dot staining for flow cytometry analysis to detect binding of lyso-sulfatide tetramer. Mean MFI for normal control 886, Crohn's disease 726, Ulcerative colitis 2076.

stimulated with lyso-sulfatide was not different than that of unstimulated cells. Additionally, LPMC from Crohn's disease patients were not stimulated by any of the sulfatides tested to produce measurable amounts of IL-13 or IFN- γ (figure 2A and B: mean production of 9 and 210 pg/mL, respectively, by cells stimulated with lyso-sulfatide similar to that of unstimulated cells. Finally, none of the sulfatides induced either UC or CD LPMC to produce measurable amounts of IL-4 (data not shown).

In another series of studies involving additional UC patients, we determined whether induction of IL-13 by lyso-sulfatide was inhibited by the presence of anti-CD1d. We found that addition of optimal amounts of anti-CD1d to cultures of LPMC led to a

statistically significant decrease in IL-13 secretion (range 41–63%; $p < 0.04$). By contrast, addition of anti-MHC class II had no effect on the level of lyso-sulfatide-induced IL-13 secretion (data not shown). These results indicate that lyso-sulfatide stimulation was acting, at least in part, via CD1d-mediated sulfatide presentation to NKT cells.

Lyso-sulfatide stimulation of UC LPMC enhances CD161 T cell cytotoxicity of epithelial cells

In other studies also addressing the capacity of UC LPMC to recognise and respond to a sulfatide glycolipid, we determined whether the most active sulfatide in the IL-13 secretion study,

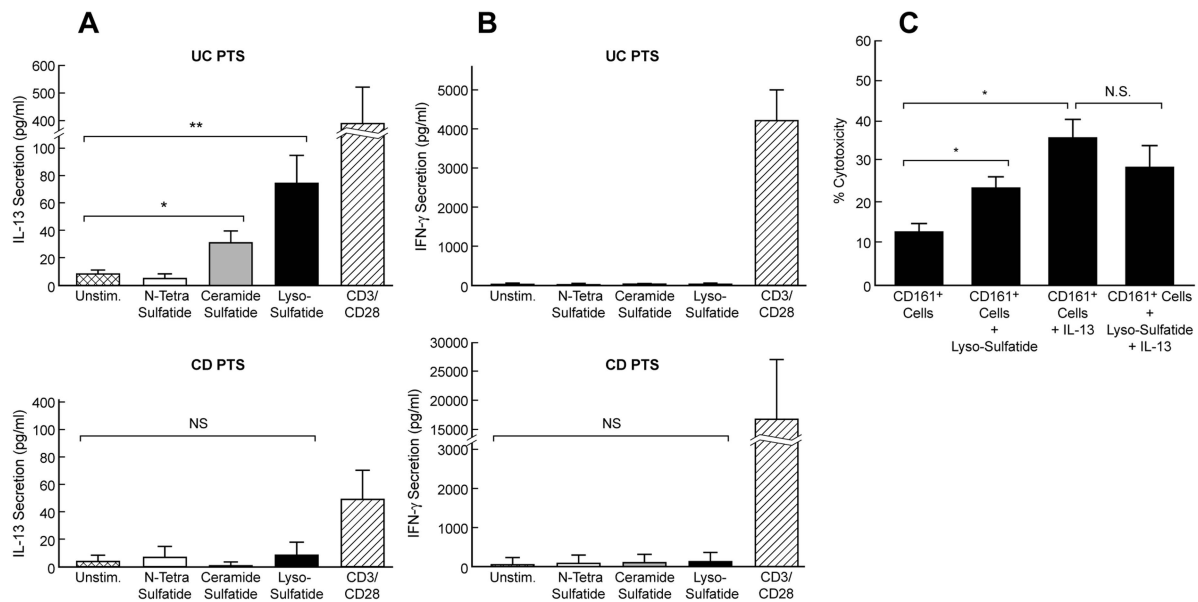


Figure 2 Cytokine secretion by UC (upper panels of A & B) and CD (lower panels of A & B) LPMCs. (A) IL-13 and (B) IFN- γ secretion obtained from inflamed UC (n=3) and inflamed CD (n=3) tissues induced by either indicated sulfatide glycolipids or CD3/CD28 as previously indicated.^{4–7} Error bars represent 1 SD. * $p \leq 0.05$ Ceramide sulfatide versus unstim, ** $p \leq 0.05$ Lyso-sulfatide vs unstim, NS; Not statistically significant (C) Cytotoxicity of CD161 T cells. HT-29 epithelial cells were pre-stimulated with LPS to induce increased expression of CD1d (data not shown). Purified UC lamina propria CD4 CD161 T cells were cultured with target HT-29 cells at a ratio of 10 : 1 and in the presence or absence of lyso-sulfatide and/or IL-13, and the percentage of cytotoxicity was measured (n=3). Error bars represent 1SD. * $p \leq 0.05$, N.S.; Not statistically significant $p \geq 0.1$.

lyso-sulfatide, induces cytotoxicity of LPS-stimulated HT-29 epithelial cells, that is, epithelial cells expressing surface CD1d (see Methods). In this case, we evaluated the cytotoxic potential of purified CD4 CD161 LP cells rather than whole LPMCs, since, in a prior study, we have shown that this cell subpopulation mediates epithelial cell cytotoxicity.¹ We found that CD4 CD161 LP cells obtained from lamina propria of UC patients exhibited considerable baseline cytotoxic activity (mean 12.4%) in the absence of any added sulfatide (figure 2C). It is likely that this was due to the fact that normal epithelial cells display sulfatide glycolipids on their cell surface and, therefore, can stimulate cytotoxic activity by CD161 T cells in the absence of added sulfatide glycolipid¹⁶; thus, the true baseline may be better represented by that exhibited by purified CD4 CD161 LP cells obtained from CD patients (2.9%) which do not respond to the sulfatide glycolipids studied here (data not shown). Thus, addition of lyso-sulfatide led to a significant further enhancement of cytotoxicity when compared to the UC cell baseline.

Lyso-sulfatide stimulation of UC LPMC induces upregulation of IL-13R α 2

In previous studies, we observed that IL-13 augmented UC NKT cell cytotoxicity of an epithelial target cells.¹ This implied that UC NKT cells bear an IL-13 receptor and prompted us to determine if lyso-sulfatide stimulation of UC LPMC induces such receptor expression. In initial studies, we assessed IL-13R α 1 expression in LPMCs employing a Q-dot-based in situ histochemical staining technique. We found that IL-13R α 1 was barely detectable in UC LPMC (+1 low-staining intensity) and stained cells comprised a relatively low fraction of UC LPMC that was not augmented by lyso-sulfatide stimulation (34 \pm 5.3% prelyso-sulfatide, and 35 \pm 4.1% postlyso-sulfatide stimulation) (figure 3A). This result was corroborated by the fact that UC LPMC displayed no detectable IL-13R α 1 receptor mRNA

expression prelyso-sulfatide and postlyso-sulfatide stimulation (data not shown).

A very different result, however, was found applying the same approaches to assessment of UC LPMC IL-13R α 2 expression. In this case, the LPMCs displayed a much higher baseline percentage of detected stained cells bearing IL-13R α 2 than LPMCs from CD or control patients, as well as greatly increased levels of IL-13R α 2 mRNA (figure 3B and see online supplementary figure 2). Additionally, quantitative assessment of staining data using both computer analysis and visual observation disclosed that the percentage of IL-13R α 2⁺ cells in UC LPMC populations increased from a baseline value of 61 \pm 5.6% to a lyso-sulfatide-stimulated value of 79 \pm 7.8% ($p < 0.05$) (figure 3C); thus, lyso-sulfatide stimulation of LPMC induced a substantial increase in the number of cells bearing this receptor, and augmented the intensity of IL-13R α 2 staining on individual cells.

In complementary studies to verify the above effect of lyso-sulfatide on UC LPMC IL-13R α 2 expression, we measured changes in lyso-sulfatide-induced IL-13R α 2 expression on individual cells in a cell lysis assay dependent on targeting cell surface IL-13R α 2 with increasing concentrations of IL-13-pseudomonas-exotoxin (IL-13-PE), an agent that reacts specifically with and then induces cytotoxicity of IL-13R α 2-bearing cells. In this assay, cell lysis by IL-13-PE pre-exposure and postexposure of cells to lyso-sulfatide is assessed by changes in the capacity of unlysed cells to incorporate leucine (a measure of protein synthesis); additionally, since cell lysis by IL-13-PE is dependent on the density of IL-13R α 2 expression on individual cells, the rate of decrease in leucine incorporation with increasing IL-13PE concentration is a measure of lyso-sulfatide-induced IL-13R α 2 expression in individual cells.^{10 11}

These cell-lysis studies showed that the rate of leucine incorporation decrease by UC LPMC was enhanced postlyso-sulfatide exposure compared to prelyso-sulfatide exposure (see online

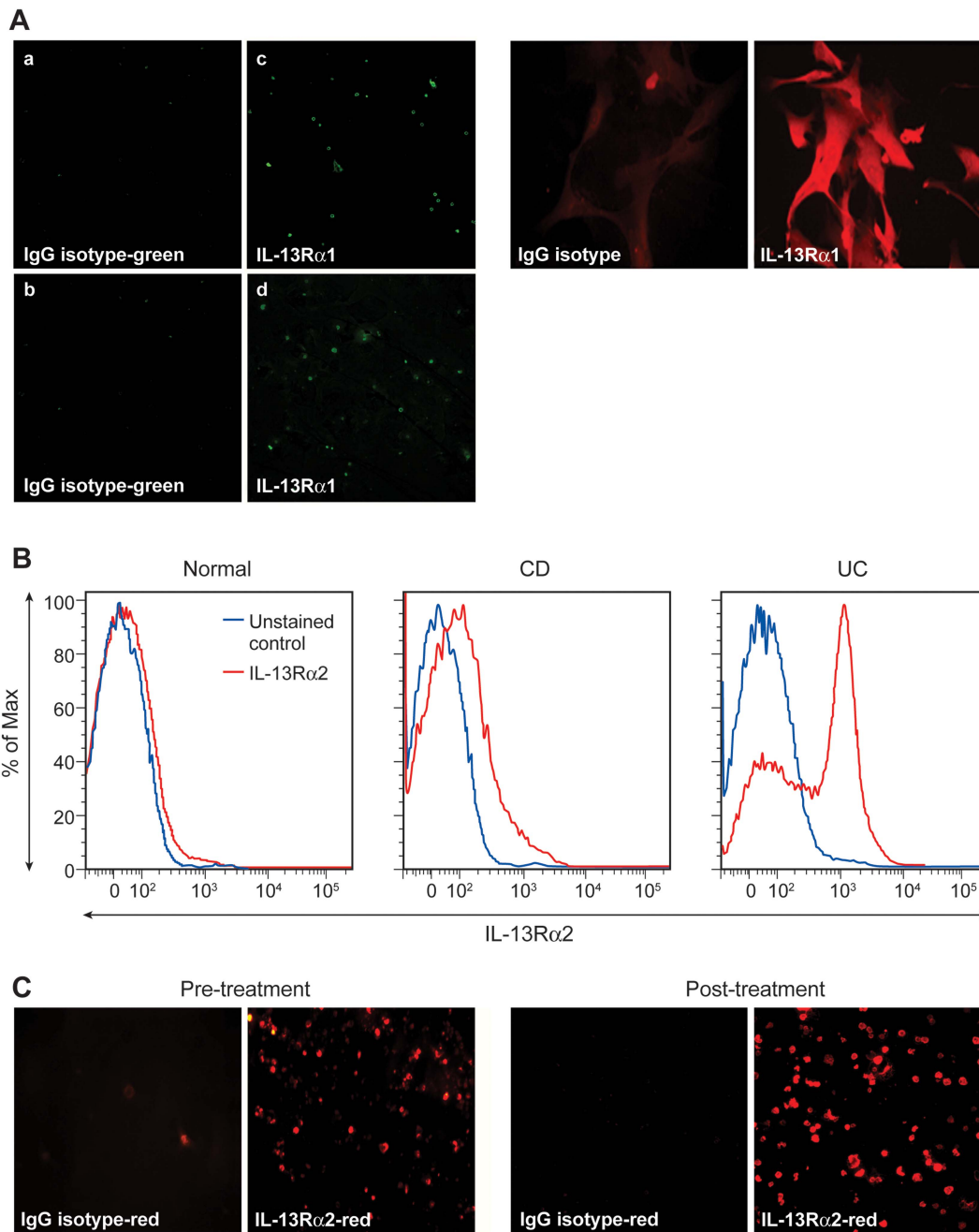


Figure 3 UC LPMC IL-13R α 1 and IL-13R α 2 expression. (A) Left panel: UC LPMC cells stimulated in vitro with lyso-sulfatide and subjected to Q-dot staining for IL-13R α 1. One representative Q-dot staining of UC LPMC depicting IL-13R α 1 expression, pretreatment (top left panel) and post-treatment with lyso-sulfatide (bottom left panel) is shown. Right panel: Glioblastoma cell line (U251) which has a high expression of IL-13R α 1 served as a positive control. (B) Purified LPMC were obtained from UC, CD and control patient populations and subjected to Q-dot staining FACS analysis for IL-13R α 2. One representative histogram from each group depicting % expression of IL-13R α 2 is shown. Range of expression for each group: UC 48–65% (n=5), CD 4.0–8.3% (n=3), controls 1.8–4.0% (n=3). (C) UC LPMC cells stimulated in vitro with lyso-sulfatide and subjected to Q-dot staining for IL-13R α 2. One representative Q-dot staining of UC LPMC depicting IL-13R α 2 expressing cells is shown (n=3).

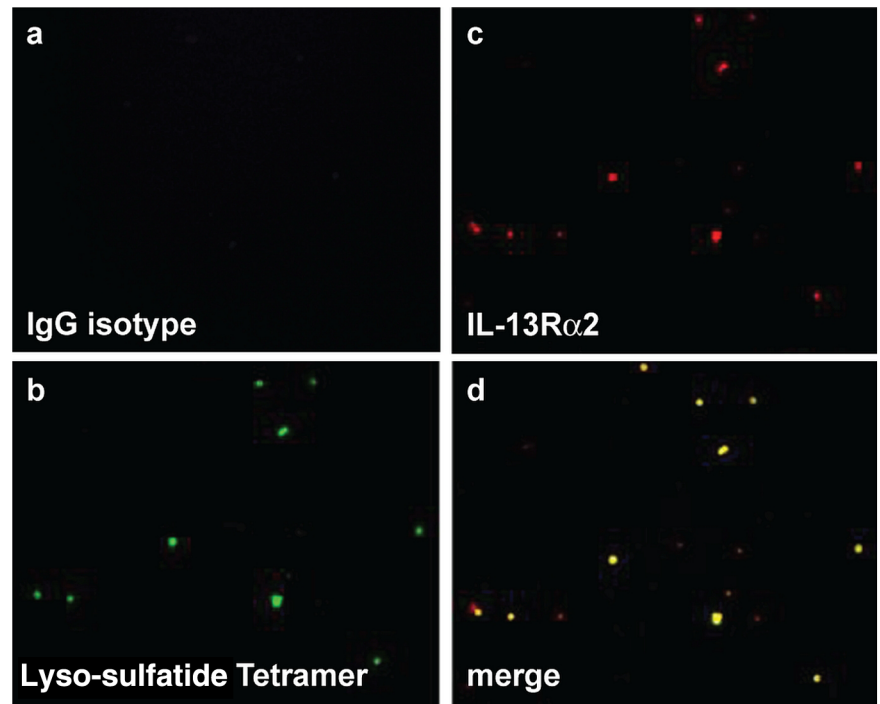
supplementary figure 3). Thus, while approximately 10 ng/mL of IL-13-PE was required to achieve a 50% decrease in protein synthesis in cells, prelyso-sulfatide exposure, only 1 ng/mL of IL-13-PE was necessary for the same decrease postlyso-sulfatide exposure. These results are consistent with those obtained with the Q-dot staining studies in showing that lyso-sulfatide induces an increased number of IL-13R α 2-expressing cells; however, they go a step further in showing that lyso-sulfatide also induces an increased concentration of IL-13R α 2 on individual cells. Of

note, no change in leucine incorporation was observed in studies of CD and normal control LP cells indicating that these cells exhibit little, if any, IL-13R α 2 expression.

Expression of IL-13R α 2 on lamina propria cells in patients with ulcerative colitis

Given the above relationship of IL-13R α 2+ cells to lyso-sulfatide stimulation, we next determined the expression of this receptor on lyso-sulfatide-loaded tetramer-positive UC LPMCs

Figure 4 Purified LPMCs isolated from ulcerative colitis patients (n=5) were subjected to Q-dot staining to detect IL-13R α 2 expression and binding of lyso-sulfatide tetramer. Expression of cells demonstrating dual IL-13R α 2 staining and binding of lyso-sulfatide tetramer was 36 \pm 6% (with a staining intensity of +3) while that of Crohn's disease (n=3) was 4 \pm 1% (data not shown) with a staining intensity of < +1) (p<0.008). Dataset from each patient was performed in triplicate.



using dual Q-dot staining. We found that, indeed, virtually all tetramer-positive cells coexpressed IL-13R α 2 (figure 4), whereas by contrast, these dual-staining cells were absent in CD LPMC populations. This result suggested that IL-13R α 2 could be used as a marker of NKT cells in UC lamina propria and prompted us to conduct studies of the phenotype and frequency of IL-13R α 2-positive cells in the UC lamina propria. In initial semiquantitative staining studies of LPMC, we found that IL-13R α 2-bearing cells were numerous in these populations and frequently coexpress IL-13 and CD161 (figure 5A). Moreover, the IL-13R α 2-bearing cells did not coexpress IL-4 or IFN- γ

(figure 5B), and did not coexpress CD14, the latter ruling out expression by inflammatory macrophages (data not shown).

In subsequent and more quantitative studies, we subjected LPMC to staining studies in which cell counts were established by blinded computer analysis and visual assessment of stained cells. The results, depicted in table 1, show that IL-13R α 2⁺ cells comprised 70.3 \pm 9.4% of total dispersed lamina propria cells, a percentage not significantly different from that of CD161 cells, 67 \pm 7.6%; (p>0.5) moreover, these markers were generally present on the same cells since IL-13R α 2⁺/CD161 double-positive cells comprised 58.7 \pm 9.2% of the total LPMC

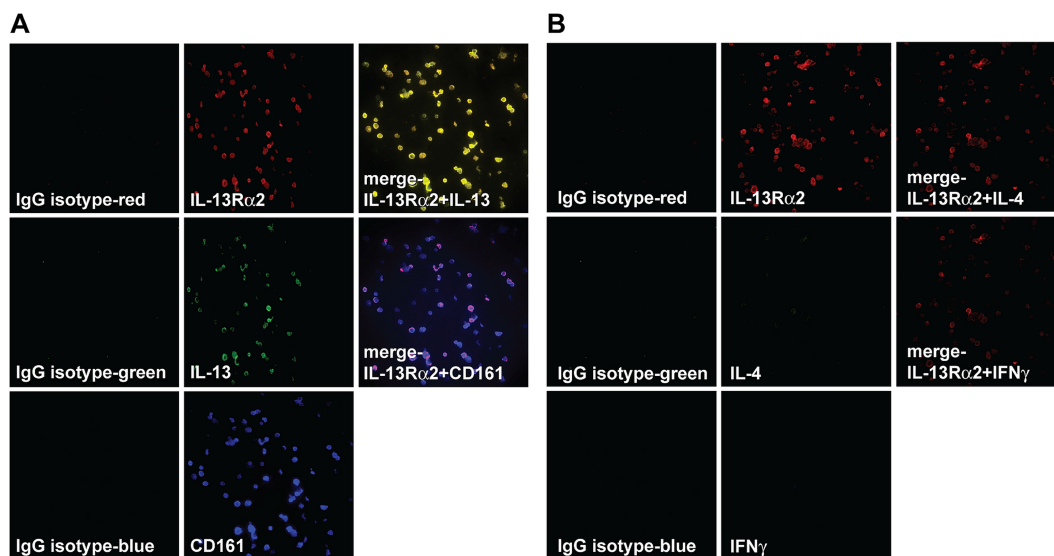


Figure 5 (A) Q-dot staining of UC LPMC reveals CD161 cells exhibit increased expression of IL-13R α 2 and IL-13. Purified LPMC obtained from inflamed UC tissue were subjected to Q-dot staining and analysed for expression of IL-13R α 2, IL-13, CD161 and for coexpression of these markers. One representative Q-dot staining of UC LPMC is shown (n=7). (B) Purified LPMC obtained from inflamed UC tissue were subjected to Q-dot staining and analysed for expression of IL-13R α 2, IL-4, IFN- γ and for coexpression of these markers. Q-dot study of UC LPMC shown is representative of seven such studies of individual patients. One representative Q-dot staining of UC LPMC is shown (n=7).

Inflammatory bowel disease

Table 1 Analysis of lamina propria cells by Q-dot staining

Mean staining intensity Mean±SD (% positive cells) (range)	IL-13Rα2	CD161	IL-13	IL-4	IFN-γ	IL-13Rα2+CD161	IL-13Rα2+IL-13
Ulcerative colitis (n=7)	3.3+ 70.3±9.4 ^a (58–84)	3.4+ 67±7.6 ^b (54–78)	3+ 65.7±5.9 ^c (58–74)	1+ 8±1.9 (6–10)	1+ 7.7±1.8 (6–10)	3+ 58.7±9.2 ^d (45–68)	2.9+ 58.4±10.7 ^e (39–70)
Crohn's disease (n=6)	<1+ 9.2±3.1 (6–14)	<1+ 7.8±2.0 (6–10)	<1+ 9.5±4.0 (6–16)	2+ 38±5.0 (30–44)	2.5+ 56.5±6.1 (51–64)	<1+ 5.5±1.5 (4–8)	<1+ 6±1.4 (4–8)
Normal (n=4)	<<1+ 7±2.2 (5–10)	<<1+ 6.3±1.3 (5–8)	<<1+ 6.3±1.3 (5–8)	1.25+ 15±2.6 (4–28)	<<1+ 6±1.4 (5–8)	<<1+to 1+ 3.3±1 (2–4)	<<1+ 5.8±1.7 (4–8)

Statistical analysis: a vs b: $p \geq 0.5$; a vs c: $p \geq 0.3$; b vs c: $p \geq 0.5$; b vs d: $p \geq 0.07$; c vs e: $p \geq 0.1$.

dispersed cells ($p > 0.07$). Additionally, IL-13⁺ cells comprised 65.7±5.9% of the total cells, again not significantly different from the percentage of IL-13Rα2⁺ or CD161 cells ($p > 0.3$, $p > 0.5$ respectively) and most of these cells also bore IL-13Rα2 since the percentage of IL-13Rα2⁺/IL-13⁺ cells was 58.4±10.7%, a percentage equivalent to that of IL-13-bearing cells ($p > 0.1$) allowing for the fact that the detection of double positive cells was not as efficient as the detection of single positive cells. Finally, about 45–50% of the LPMC in UC were CD4 T cells, most of which were present in the IL-13Rα2⁺CD161 population; additionally, in the one patient studied, 6% of LPMC were lightly stained CD8 T cells. Overall, these studies provide definitive evidence that a large proportion of UC lamina propria cells bear markers associated with NKT cells, IL-13Rα2 and CD161, most, if not all of which secrete IL-13.

Concomitant studies of Crohn's disease LPMC disclosed that high-level expression of IL-13Rα2 and CD161 was a unique feature of UC cells. Thus, both IL-13Rα2 and CD161 was detected on only 5–10% of cells, and the intensity of staining of these positive cells was quite low (see online supplementary figure 4). Likewise, only 6–16% of the cells exhibited IL-13 staining, and the intensity of staining of these positive cells was low. Thus, UC LPMCs contained a significantly higher percentage of cells than Crohn's disease or control LPMCs of cells positive for IL-13, IL-13Rα2, CD161, IL-13Rα2/CD161 and IL-13/IL-13Rα2 ($p < 0.007$ Bonferroni correction for multiple parameter analysis), whereas CD and control LPMCs exhibited no difference in these parameters ($p > 0.1$). Additionally, while UC lamina propria cells revealed only low-level intensity and insignificant staining for IL-4 and IFN-γ, CD lamina propria cell populations exhibited strong IFN-γ staining and some low-intensity IL-4 staining. With regard to the latter, it should be noted the IL-4 staining was not accompanied by detectable IL-4 secretion either in the present studies or in previous studies.¹⁷ IL-17A mRNA production in UC LPMCs was slightly greater than that of normal control, but decreased in comparison with that of CD LPMC (data not shown). Finally, whereas a substantial subpopulation of UC LPMC CD161 cells also expressed IL-5, virtually little of these cells expressed IL-17 (see online supplementary figure 5).

Secretion of IL-13 by UC LP cells following treatment with IL-13-PE

Taking a more functional approach to relating IL-13Rα2 expression to NKT cells in UC, we measured IL-13 production by LPMC before and after culture with IL-13-PE using the latter to selectively eliminate IL-13Rα2-bearing cells. We found that prior exposure of LPMC from UC patients to IL-13-PE led to a

significant decrease in the amount of IL-13Rα2⁺ cells (see online supplementary figure 6A). Additionally, LPMC from UC patients pre-exposed to IL-13-PE, and then activated with anti-CD3/anti-CD28 in vitro caused a mean 68% reduction in IL-13 secretion but no change in IFN-γ secretion (see online supplementary figure 6B and data not shown, respectively). This reduction was similar in magnitude to the reduction in the number of IL-13Rα2⁺ cells, suggesting that most, if not all, of IL-13 was, in fact, being produced by receptor-positive cells. Similar results were obtained when cytotoxicity was used as a read-out rather than IL-13 production in that again, the reduction in cytotoxicity was similar to the reduction in the number of CD161 cells (see online supplementary figure 6C and D). While these results require verification with studies of additional patients, they provide additional evidence that IL-13Rα2⁺ is a marker of LP NKT cells in UC patients.

DISCUSSION

In previous studies, we obtained several kinds of evidence suggesting that UC was associated with the presence of lamina propria non-invariant (Type II) NKT cells. Most importantly, we showed that in parallel with invariant NKT cells in the lamina propria of mice with oxazolone colitis (oxa-colitis) that produce IL-13 upon stimulation with α-GalCer, non-invariant CD161 cells in the UC lamina propria produce IL-13 when stimulated with CD1d-expressing APCs.^{1 2} Additionally, we showed that the UC lamina propria contains T cells that are cytotoxic for LPS-stimulated epithelial cells, and that this cytotoxicity is augmented by the presence of IL-13.¹ In the present study, we expand on these findings by showing that a substantial population of LPMC from UC patients bind sulfatide glycolipid-loaded CD1d tetramer and, thus, bear TCRs that react to a glycolipid previously shown to interact with non-invariant NKT cells.⁴⁻⁷ Additionally, we show that UC LPMC can be stimulated by sulfatide glycolipid to produce IL-13 and to manifest cytotoxicity for LPS-stimulated epithelial cells that is substantially inhibited by the presence of anti-CD1d antibody. These new data not only provide strong new support for the idea that non-invariant NKT cells do, in fact, populate the LP of UC patients but may establish the identity of the TCR-reactive antigen capable of stimulating these cells.

Staining of UC lamina propria cells with labelled lyso-sulfatide-loaded tetramers indicated that a very substantial fraction of these cell, approximately 36%, were, in fact, Type II NKT cells. However, this could be an underestimate of the percentage of NKT cells in UC lamina propria because lyso-sulfatide might bind to the TCR of UC NKT cells with an affinity that is suboptimal when compared to that of an as-yet

unidentified endogenous stimulatory sulfatide actually present in the UC lamina propria. As an alternative approach to NKT cell quantitation in UC, we explored the possibility that IL-13R α 2 present on LPMCs could be a surrogate marker of the NKT cell subpopulation. Two observations suggested that was likely to be the case. First, stimulation of UC LPMC with sulfatide upregulated the number of cells bearing IL-13R α 2, and also increased the level of expression of this receptor on individual cells. Second, dual staining of LPMC to identify tetramer-positive cells and IL-13R α 2-positive cells revealed that virtually all the tetramer-positive cells were also IL-13R α 2-positive.

In further studies along these lines, we evaluated the number and phenotype of IL-13R α 2-bearing cells in UC lamina propria using a sensitive and unbiased *in situ* staining technique coupled with flow cytometry. This revealed that the majority of UC LPMCs bear IL-13R α 2, whereas only a small percentage of Crohn's disease, or control LPMCs, bear this receptor, and then at a low expression level. Additionally, the receptor-bearing cells also bear the NKT cell marker, CD161, and most are IL-13-producing cells. These latter findings provide additional support for the view that IL-13R α 2 is a valid marker of NKT cells in UC since CD161 is a known marker of NKT cells and IL-13 production, as noted previously in studies of oxazolone colitis and UC patients.^{1 2} Additional support for the idea that IL-13R α 2 is an NKT cell marker in UC LP came from studies showing that IL-13-PE-mediated depletion of UC lamina propria cells led to a greatly reduced level of IL-13 production and cytotoxic activity, both of which were proportional to the extent of depletion. Overall, these phenotypic studies of lamina propria cell populations provide strong evidence that IL-13R α 2⁺ IL-13⁺CD161 T cells make up the majority of cells in the inflamed UC mucosa.

The fact that UC NKT cells express IL-13R α 2, but little or no IL-13R α 1, was a somewhat unexpected characteristic of these cells although it was presaged by a previous study showing that this receptor is expressed in the lamina propria of oxazolone-colitis in western blot studies that did not identify the cellular origin of the IL-13R α 2.^{18 19} Of interest, in this previous study, down-regulation of IL-13R α 2 expression was shown to be associated with a reduced level of colitis. This correlates with the fact that sulfatide glycolipid induces NKT cell IL-13 production, and the latter, in turn, stimulates NKT cell cytotoxic function.

The finding that UC is characterised by the presence of a lamina propria self-antigen (sulfatide)-responsive Type II NKT cell population invites a re-examination of the underlying pathogenesis of this disease. One important possibility that needs to be considered on the basis of this new information is that the key pathologic event occurring in UC consists of an abnormality in the way epithelial cells process and display a self-glycolipid (sulfatide) antigens perhaps in response to a 'stress' signal from the luminal microbiota. Support for this possibility comes from a recent report that an epithelial cell-specific mutation of XBP1, an intracellular protein critical to the initiation of the endoplasmic reticulum stress response of secretory cells, is associated with being hyper-responsive to an innate ligand and results in spontaneous intestinal inflammation or increased susceptibility to induced colitis.²⁰ On this basis, one might speculate that while a proximal cause of UC is an NKT cell-mediated autoimmune-like response, the more basic cause of this disease lies in a defect in the way epithelial cells respond to the endogenous microbiome. This latter possibility finds support in a recent study by Olszak *et al*, which showed that neonatal germ-free mice exhibit increased LPs NKT cell infiltration and heightened oxazolone colitis, a mouse model of UC.²¹

Finally, the findings reported here showing that UC is characterised by the presence of NKT cells bearing IL-13R α 2 suggest that this receptor could be used as a marker for UC and at the same time, would provide insight into disease severity. Additionally, since NKT cells which produce IL-13 may be the main effector cell driving UC inflammation, this receptor could also be targeted by potential UC therapeutic agents. The latter presently being investigated with therapeutic agents targeting IL-13.

Acknowledgements This manuscript is dedicated to the continued memory of my father, Abe Fuss; may others never know the words IBD.

Contributors IJF and BJ: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. ZY, HD, CY, LX, PL, MY: acquisition of data; analysis and interpretation of data. SF-F, HdS, FR, FS, AS, MS: obtained technical or material support. PM: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content. RKP: study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; study supervision. CF: analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; obtained technical or material support; study supervision. WS: study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis; study supervision.

Competing interests This work was supported by the Intramural Research Program of NIAID, National Institutes of Health.

Ethics approval The institutional review boards of the Cleveland Clinic and the National Institute of Allergy and Infectious Disease, NIH-approved collection of surgical specimens. Informed consent was obtained from all patients.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All data included within the manuscript. No further unpublished data exists.

REFERENCES

- 1 Fuss IJ, Heller F, Boirivant M, *et al*. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* 2004;113:1490–97.
- 2 Heller F, Fuss IJ, Nieuwenhuis EE, *et al*. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. *Immunity* 2002;17:629–38.
- 3 Heller F, Florian P, Bojarski C, *et al*. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 2005;129:550–64.
- 4 Shamshev A, Gober HJ, Donda A, *et al*. Presentation of the same glycolipid by different CD1 molecules. *J Exp Med* 2002;195:1013–21.
- 5 Jahng A, Maricic I, Aguilera C, *et al*. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* 2004;199:947–57.
- 6 Zajonc DM, Maricic I, Wu D, *et al*. Structural basis for CD1d presentation of a sulfatide derived from myelin and its implications for autoimmunity. *J Exp Med* 2005;202:1517–26.
- 7 Wu D, Xing GW, Poles MA, *et al*. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc Natl Acad Sci USA* 2005;102:1351–56.
- 8 Truelove S, Witts LF. Cortisone in ulcerative colitis: a final report on a therapeutic trial. *Br Med J* 1955;2:1041–46.
- 9 De Dombal FT, Burton IL, Clamp SE, *et al*. Short term course and prognosis of Crohn's disease. *Gut* 1974;15:435–9.
- 10 Shimamura T, Fujisawa T, Husain SR, *et al*. Interleukin 13 mediates signal transduction through interleukin 13 receptor α 2 in pancreatic ductal adenocarcinoma: role of IL-13 Pseudomonas exotoxin in pancreatic cancer surgery. *Clin Cancer Res* 2010;16:577–86.
- 11 Debinski W, Miner R, Leland P, *et al*. Receptor for interleukin (IL) 13 does not interact with IL4 but receptor for IL4 interacts with IL13 on human glioma cells. *J Biol Chem* 1996;271:22428–33.
- 12 Joshi BH, Puri RA, Leland P, *et al*. Identification of interleukin-13 receptor α 2 chain overexpression *in situ* in high-grade diffusely infiltrative pediatric brainstem glioma. *Neuro Oncol* 2008;10:265–74.
- 13 Liu Y, Goff RD, Zhou D, *et al*. A modified α -galactosyl ceramide for staining and stimulating natural killer T cells. *J Immunological Methods* 2006;312:34–9 (see supplement material online).

Inflammatory bowel disease

- 14 Roy KC, Maricic I, Khurana A, *et al.* Involvement of secretory and endosomal compartments in presentation of an exogenous self-glycolipid to type II NKT cells. *J Immunol* 2008;180:2942–50.
- 15 Blomqvist M, Rhost S, Teneberg S, *et al.* Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. *Eur J Immunol* 2009;39:1726–35.
- 16 Jansson L, Tobias J, Jarefjäll C, *et al.* Sulfatide recognition by colonization factor antigen CS6 from enterotoxigenic *Escherichia coli*. *PLoS One* 2011;4:e4487.
- 17 Fuss IJ, Neurath M, Boirivant M, *et al.* Disparate CD4⁺ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN- γ , whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 1996;157:1261–70.
- 18 Fichtner-Feigl S, Young CA, Kitani A, *et al.* IL-13 signaling via IL-13R α 2 induces major downstream fibrogenic factors mediating fibrosis in chronic TNBS colitis. *Gastroenterology* 2008;135:2003–13.
- 19 Fichtner-Feigl S, Strober W, Kawakami K, *et al.* IL-13 signaling through the IL-13 α 2 receptor is involved in induction of TGF- β 1 production and fibrosis. *Nat Med* 2006;12:99–106.
- 20 Kaser A, Lee AH, Franke A, *et al.* XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* 2008;134:743–56.
- 21 Olszak T, An D, Zeissig S, *et al.* Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* 2012;336:489–93.



IL-13R α 2-bearing, type II NKT cells reactive to sulfatide self-antigen populate the mucosa of ulcerative colitis

Ivan J Fuss, Bharat Joshi, Zhiqiong Yang, Heba Degheidy, Stefan Fichtner-Feigl, Heitor de Souza, Florian Rieder, Franco Scaldaferrri, Anja Schirbel, Melania Scarpa, Gail West, Chuli Yi, Lili Xu, Pamela Leland, Michael Yao, Peter Mannon, Raj K Puri, Claudio Fiocchi and Warren Strober

Gut 2014 63: 1728-1736 originally published online February 10, 2014
doi: 10.1136/gutjnl-2013-305671

Updated information and services can be found at:
<http://gut.bmj.com/content/63/11/1728>

These include:

Supplementary Material

Supplementary material can be found at:
<http://gut.bmj.com/content/suppl/2014/02/10/gutjnl-2013-305671.DC1>

References

This article cites 21 articles, 11 of which you can access for free at:
<http://gut.bmj.com/content/63/11/1728#BIBL>

Email alerting service

Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections

Articles on similar topics can be found in the following collections
[Ulcerative colitis](#) (1113)

Notes

To request permissions go to:
<http://group.bmj.com/group/rights-licensing/permissions>

To order reprints go to:
<http://journals.bmj.com/cgi/reprintform>

To subscribe to BMJ go to:
<http://group.bmj.com/subscribe/>