Expression of the leukotriene B4 receptor, LTB4R, is perturbed in the blood of patients with Crohn's Disease.

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A significant portion of patients with the inflammatory bowel disease Crohn's Disease (1) fail first-line treatment which includes the 5-aminosalicylate drug mesalamine and steroids (2, 3). Both of these drugs are thought to have physiologically pleiotropic functions but it is still not understood how these drugs exert their therapeutic function in CD (4,5). Deciphering their mechanism of action can be facilitated by a basic understanding of the relevant therapeutic targets in patients with Crohn's Disease, and can assist in determining which patients will benefit from first-line treatment with mesalamine versus top-down treatment with biologics (6) which are more beneficial but also more costly and associated with an increased risk of development of non-Hodgkin's and hepatosplenic T-cell lymphomas (7, 8, 9). Transcriptome analysis to understand how gene expression differs in the tissues of patients with Crohn's Disease can facilitate this understanding. We found, using two published datasets (10, 11), that the leukotriene B4 receptor, LTB4R (12, 13), was among the genes whose expression was most different when comparing blood from patients with Crohn's Disease with blood from healthy, non-affected subjects. Interestingly, while LTB4R was also a differentially expressed gene in CD4⁺ CD25⁺ regulatory T-cells from patients with Crohn's Disease, in T_{reg} it was expressed at significantly lower levels compared to non-affected subjects as opposed to whole blood where it was expressed at significantly higher levels. This is the first report documenting differential expression of LTB4R in the hematopoietic tissues of patients with Crohn's Disease.

Keywords: LTB4R, leukotriene B4, eicosanoids, Crohn's Disease, differential gene expression analysis.

Medications used to treat Crohn's Disease include the sulfasalazine-derivative mesalamine (14), corticosteroids (15), antibiotics including metronidazole (16) and ciprofloxacin (17), immunosuppressive drugs azathioprine (18) and 6-mercaptopurine (19), as well a newer class of biologic monoclonal antibodies including the tumor necrosis alpha antibody infliximab (20) and the alpha4beta1 (VLA-4) / alpha4beta7 integrin antibodies natalizumab and vedolizumab (21, 22). While biologic therapies are the most effective class of drugs used for Crohn's Disease, their cost and associated risks of development of non-Hodgkin's lymphoma limit their broad usage in this patient population (23). Historically, gastroenterologists have first used mesalamine and steroids, then azathioprine and mercaptopurine, and finally infliximab and natalizumab only when prior treatment options have failed (3). Some patients will fail treatment with mesalamine and mercaptopurine, become dependent on corticosteroids (24, 25) which are associated with significant metabolic skeletal risks and growth failure in pediatric populations (26), and only respond to biologic therapies. In these populations, top-down treatment with biologics first (6) can prevent dependency on steroids, prevent growth failure and spare loss of the gastrointestinal tract associated with surgery when faced with loss of response to medical treatments. Unfortunately, at this time is virtually impossible to predict using specific gene expression signatures which patients will require top-down treatment and whose disease course dictates a cost benefit analysis that weighs in favor of treatment with biologic therapies first. This is due in part to a poor understanding of the basic mechanisms by which mesalamine and corticosteroids achieve their therapeutic function in patients with Crohn's Disease (4, 5). Understanding which pathways are targeted by mesalamine and corticosteroids, and importantly, which of these pathways are relevant in the Crohn's Disease patient population, can help stratify patients into groups that will benefit from less efficacious but more tolerable mesalamine versus patient groups that will become steroid-dependent, require surgical intervention and will eventually require treatment with biologics in any case.

Understanding gene expression at a global level, as it occurs in patients with Crohn's Disease and how it compares to the non-diseased population can provide critical insights into the ways gene expression programs are perturbed in Crohn's Disease. Importantly, deciphering how gene expression differs in the tissues of patients with Crohn's Disease can provide answers to what pathways are targeted by mesalamine and steroids, as they were not designed towards specific targets as infliximab and natalizumab were. Towards this end, we performed global differential gene expression analysis using published transcriptome data from the blood of patients with Crohn's Disease (10, 11). We found that the receptor for the B4 leukotriene, LTB4R (12, 13, 27), was among the genes whose expression was quantitatively most different when comparing whole blood from patients with Crohn's Disease with that of non-affected subjects (10). Analysis of a separate published dataset (11) revealed that LTB4R was also a differentially expressed gene when comparing CD4+ CD25+ regulatory T-cells from patients with Crohn's Disease with Treg from non-affected subjects. Leukotrienes are targeted by both mesalamine and corticosteroids (28-31). LTB4R may be a therapeutic target of interest in patients with Crohn's Disease.

Methods

We used GSE94648 (10) and GSE23996 (11) for this analysis in conjunction with GEO2R. GSE94648 (10) was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology with n=22 whole blood from controls and n=50 whole blood from patients with Crohn's Disease. GSE23996 (11) was generated using the HCI Human TReg Chip with n=6 CD patients and n=11 healthy controls. The Benjamin and Hochberg method of p-value adjustment was used for ranking of differential expression but raw p-values were used for assessment of statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used.

A statistical test was performed to evaluate the significance of difference between mRNA expression levels of LTB4R in the blood of patients with Crohn's disease as compared

to healthy controls, and between monocyte-derived macrophages from CD patients and from control subjects using a two-tailed, unpaired t-test with Welch's correction. Only p-values less than 0.05 were considered statistically significant.

Results

We mined a transcriptome dataset generated using the blood of patients with Crohn's Disease (10) as well as a dataset containing mRNA gene expression data (11) generated using regulatory T-cells isolated from the blood of patients with Crohn's Disease, and performed differential gene expression analysis to determine the genes whose expression was quantitatively most different when compared to whole blood or regulatory T-cells from non-affected control subjects, respectively.

The receptor for leukotriene B4, LTB4R/BLT1, is among the most differentially expressed genes in the whole blood of patients with Crohn's Disease.

We identified the high-affinity receptor for leukotriene B4, LTB4R, also known as BLT1 as a differentially expressed gene in the blood of patients with Crohn's Disease. When ranking all of the genes expressed in whole blood based on change in expression between CD patient blood and blood from non-affected subjects, a transcript generated from the LTB4R gene ranked 244 out of 19674 transcripts. Differential expression of LTB4R was statistically significant (Table 1; $p=7.85E-05$).

LTB4R is expressed at significantly higher levels in the blood of patients with Crohn's Disease when compared to blood from non-affected control subjects.

We extracted exact mRNA expression values for the LTB4R gene from the blood of patients with Crohn's Disease and from blood from non-affected control subjects. LTB4R was expressed at significantly higher levels in the blood of patients with Crohn's Disease as compared to blood from non-affected subjects (Figure 1; $p<0.0001$).

LTB4R is a differentially expressed gene in regulatory T-cells from patients with Crohn's Disease.

LTB4R was also identified as a differentially expressed gene in sorted CD4+ CD25+ regulatory T-cells from patients with Crohn's Disease (Table 2). When ranking all of the genes whose expression was measured on a chip specifically designed for T_{reg} based on change in expression between T_{reg} from patients with CD and T_{reg} from non-affected subjects, a transcript generated from the LTB4R gene ranked 212 out of 397 transcripts. Differential expression of LTB4R in T_{reg} was statistically significant (Table 2; $p= 3.54E-02$).

LTB4R is expressed at significantly lower levels in regulatory T-cells from patients with Crohn's Disease when compared to regulatory T-cells from control subjects.

We extracted exact mRNA expression values for the LTB4R gene from regulatory T-cells from patients with Crohn's Disease and from T_{reg} from non-affected control subjects. While LTB4R was expressed at higher levels in whole blood from patients with Crohn's Disease, LTB4R was expressed at significantly lower levels in the T_{reg} from patients with Crohn's Disease as compared to blood from non-affected subjects (Figure 2; $p=0.0202$).

Thus, we identified LTB4R as one of the genes whose expression changed the most in the blood of patients with Crohn's Disease when compared to the blood of non-affected patients, and found that it was expressed at significantly higher levels in CD patient blood. We also found that LTB4R was a differentially expressed gene in sorted T_{reg} , but expressed at significantly lower levels in CD patient T_{reg} .

Discussion

In this study we mined transcriptome data from patients with Crohn's Disease (10) and identified the high affinity receptor for leukotriene B4, LTB4R/BLT1 as one of the most differentially expressed genes in the whole blood of patients with Crohn's Disease. Interestingly, while we also identified LTB4R as differentially expressed in regulatory T-cells from patients with Crohn's Disease, in T_{reg} it was expressed at significantly lower levels than in

1 non-affected subjects while in whole blood from patients with Crohn's Disease it was
2 expressed at significantly higher levels compared to non-affected subjects.

3 LTB₄ is produced by the combinatorial actions of phospholipase A₂, 5-lipoxygenase (5-
4 LO) and leukotriene A₄ hydrolase (32). The high affinity receptor for leukotriene B₄, LTB₄R
5 (BLT₁), is important for T-lymphocyte homing and recruitment to inflamed tissues, specifically
6 for sticking (adhesion) but not rolling phase of extravasation, wherein leukocytes exit the
7 vasculature and enter the tissues (12, 13). One study has documented enhanced production
8 of LTB₄ in the colonic mucosa of patients with Crohn's Disease (33) while another study
9 documented a small decrease in expression of LTB₄R in the polymorphonuclear neutrophils of
10 patients with Crohn's Disease (34). Mice deficient in the low-affinity receptor for LTB₄, BLT₂,
11 display increased sensitivity to dextran sodium sulfate (DSS) colitis demonstrating a protective
12 role of BLT₂ against murine colitis (35).

14 Leukotriene B₄ itself is a metabolite of arachidonic acid and a chemoattractant for
15 leukocytes consistent with the role of LTB₄R in T-lymphocyte homing and recruitment (36). The
16 eicosanoid PGE₂ is produced at significantly higher levels in the inflamed colonic mucosa of
17 patients with Crohn's Disease (37). Leukotrienes are critical in the pathogenesis of asthma and
18 in alveolar macrophages, the eicosanoids leukotriene B₄ and prostaglandin E₂ have opposing
19 functions as LTB₄ counteracts the stimulatory function of PGE₂ on cyclic AMP production and
20 the inhibitory action of PGE₂ on phagocytosis (38). Leukotrienes are relevant to the
21 pathogenesis of inflammatory diseases and are targeted at least in part by mesalamine and
22 corticosteroids, both used for the treatment of Crohn's Disease. We found that the leukotriene
23 B₄ receptor was among the genes whose expression was most different between the blood of
24 patients with Crohn's Disease and that of non-affected subjects. In the anti-inflammatory
25 CD4⁺ CD25⁺ regulatory T-cell subset, we intriguingly found that LTB₄R was expressed at
26 significantly lower levels than in non-affected subjects, as opposed to whole blood where we
27 found that LTB₄R was expressed at significantly higher levels. The role of LTB₄R in
28 maintenance and induction of the inflammatory state in the gut and peripheral circulation of

patients with Crohn's Disease through crosstalk with other members of eicosanoid family of lipid mediators of inflammation (39) such as PGE2 requires evaluation.

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Rank	ID	p-value	t	B	Gene	Gene name
244	1241_at	7.85E-05	4.1852682	1.423383	LTB4R	leukotriene B4 receptor

Table 1: The receptor for leukotriene B4, LTB4R/BLT1, is among the most differentially expressed genes in the whole blood of patients with Crohn's Disease.

The rank of differential expression out of 19674 transcripts, probe ID, p-value (of global differential expression), t, a moderated t-statistic, and B, the log-odds of differential expression between the two groups compared are represented in this chart (obtained from GEO2R).

Figure 1: LTB4R is expressed at significantly higher levels in the blood of patients with Crohn's Disease when compared to blood from non-affected control subjects.

The mRNA expression values of LTB4R in the blood of non-affected patients (n=22) and in the blood of patients with Crohn's disease (n=50) is displayed with the mean mRNA expression value in each group marked and p-value listed (left), and as a violin plot with the median value, 25th and 75th quartiles marked (right).

Rank	ID	p-value	t	B	Gene	Gene name
212	6050659/ NG_001336.2/ NM_000752	3.54E-02	-2.18679	-4.3755	LTB4R	leukotriene B4 receptor

Table 2: LTB4R is a differentially expressed gene in regulatory T-cells from patients with Crohn's Disease.

The rank of differential expression out of 397 transcripts, probe/transcript IDs, p-value (of differential expression), t, a moderated t-statistic, and B, the log-odds of differential expression between the two groups compared are represented in this chart (obtained from GEO2R).

Figure 2: LTB4R is expressed at significantly lower levels in regulatory T-cells from patients with Crohn's Disease when compared to regulatory T-cells from subjects.

The mRNA expression values of LTB4R in the regulatory T of non-affected patients (n=11) and in the blood of patients with Crohn's disease (n=6) is displayed with the mean mRNA expression value in each group marked and p-value listed (left), and as a violin plot with the median value, 25th and 75th quartiles marked (right).