Systemic TNF-α Reduction by Blocking IgE-mediated Cellular Activation in Inflammatory Bowel Disease

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ABSTRACT

Background: Several cells bearing IgE receptors and IgE circulate in the bloodstream, including basophils. The roles of IgE and IgE-bearing cells have not been well-characterized in inflammatory bowel disease (IBD). Recently, investigators reported that basophils are elevated and may promote inflammatory T cells in IBD. Basophils secrete TNF-α in response to IgE cross-linking suggesting a role in mediating inflammation. We sought to determine the effects of ET523, a drug that reduces IgE-mediated cellular activation, on basophil function and TNF-α secretion in IBD. Anti-IgE cross-linking induced basophil activation and cytokine secretion from cells from Crohn’s disease and ulcerative colitis patients. Cells treated with ET523 reduced anti-IgE mediated cellular activation and TNF-α secretion. Importantly, ET523 reduced unstimulated, basal secretion of TNF-α in IBD. IgE-mediated cellular activation may play a role in systemic TNF-α production in IBD. ET523 appears effective at reducing TNF-α secretion and thus represents a novel agent to treat IBD.

Keywords
Basophils, IgE, TNF-α, sCD23, Inflammatory bowel disease.

Introduction

TNF-α has a pathogenic role in inflammatory bowel disease (IBD) [1]. Uncontrolled release of this highly inflammatory cytokine leads to increased T cell activation, cellular recruitment, and tissue damage [2]. In the last 10 years, biologics aimed at neutralizing soluble TNF-α have transformed the management of IBD and other chronic inflammatory diseases [1]. Several TNF-α targeting drugs have been approved, including infliximab, adalimumab, golimumab, and certolizumab pegol and many others are in development. Nevertheless, up to 40% of recipients exhibit muted or no response to TNF neutralizing agents, which may be due to genetic or physical changes in the patient [3-6]. In addition, blocking TNF-α long term can be associated with adverse effects, such as infection, cancer, or even development of autoimmune diseases. For example, psoriasis has been shown to develop in IBD patients treated with anti-TNF-α agents suggesting the cytokine has an important role in maintaining homeostasis [7,8]. Drugs with different mechanisms of action that better regulate TNF-α would alleviate chronic inflammation in patients. In particular, treatments that inhibit systemic TNF-α secretion would potentially reduce tissue damage and present more options for patients.

Although IBD is primarily a mucosal disease, patients experience systemic inflammatory indicating that cells in the bloodstream contribute to disease [9-11]. Several circulating leukocytes have been shown to secrete TNF-α, including monocytes, neutrophils and eosinophils [12,13]. Degranulation of circulating neutrophils, or ‘spent’ neutrophils, in IBD has been documented and thought to contribute to disease pathogenesis [14]. In addition, basophils have recently been shown to secrete TNF-α in response to IgE cross-linking suggesting a role for these cells in the pathogenesis of IBD [15,16]. Interestingly, basophilia has recently been reported in both ulcerative colitis (UC) and Crohn’s disease (CD) [17]. Basophils are generally rare blood granulocytes, but may be found in lymphoid and non-lymphoid tissues. Similar to mast cells, basophils express the high-affinity receptor for IgE (FceR1), release histamine, inflammatory mediators, and cytokines following FceR1 cross-linking [18]. These features are curious for circulating cells as degranulation of these cells in the...
bloodstream could potentially induce anaphylaxis. Thus, basophils likely evolved to perform specific immunological functions related to cell-bound IgE, but their overall paucity has hindered defining their functional significance in humans.

The roles of IgE and IgE-bearing cells are not well-defined in IBD. Several cells bearing IgE receptors circulate in the bloodstream in addition to basophils, including eosinophils, CD23+ B cells, and monocytes, all of which may circulate pre-loaded with IgE. CD23-bound IgE on monocytes can induce TNF-α secretion as well and may be involved in the pathogenesis of other diseases, such as malaria [19]. Cross-linking of cell-bound IgE on circulating B cells can induce their trafficking to lymph node follicles [20]. Thus, although traditionally considered an allergic mediator, IgE likely plays important roles in IBD. Recently, investigators reported that basophils are not only elevated in the bloodstream of patients with IBD, but also that these cells have the capacity to promote IL-17 and IFNγ production by T cells, though the role of IgE was not defined [21].

We are developing a biologic drug, ET523, which reduces IgE-mediated cellular degranulation by binding free IgE and inhibiting cell-bound IgE from cross-linking [22]. We therefore sought to determine the effect of ET523 in IBD. ET523 appears effective at directly reducing TNF-α secretion by blood cells and may be a potential agent to treat inflammation in patients with IBD. The implications of these findings are discussed in the context of improving treatments for inflammatory bowel disease.

**Methods**

**Human Subjects**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Institutional Review Board of Boston University and Boston Medical Center (BU IRB).

**Flow cytometry on whole blood samples**

Upon informed written consent, blood was drawn into heparinized vacutainers (BD, Franklin Lakes, NJ). 100 µl/tube of heparinized whole blood were incubated with fluorescently-labeled antibodies purchased from BD Biosciences (anti-FcεRI α and anti-CCR3; San Jose, CA) at 4°C for 30 minutes. Red blood cells were lysed with 1X FACS Lysing Buffer (BD Biosciences.).

**Production of ET523**

ET523 was produced in *E. coli* and purified by GenScript (Piscataway, NJ) as previously described [22]. To remove residual LPS, 0.5 ml Pierce High Capacity Endotoxin Removal Spin Column (Catalog #88274; ThermoFisherScientific, Waltham, MA) were used according to the manufacturer’s instructions. Briefly, ET523 was bound to resin for 1 hour at 37°C and eluted with10 mM Tris, 0.4N NaCl, pH 8. Endotoxin levels were assessed using the Charles River Endosafe LAL Endochrome-K kit (Wilmington, MA). ET523 yielded an effective LPS level of 0.005 EU in cell culture experiments.

**Cell Culture**

Because we had relatively low volumes of blood from patients, we were unable to isolate sufficient numbers of basophils for experimentation. Therefore, to determine the effect of IgE-mediated inflammation by circulating cells in IBD, whole blood was diluted at a 1:3 ratio with cell culture media (RPMI, 10% FCS, and pen/strep; all from ThermoFisherScientific) in 1 ml cultures. Anti-IgE antibodies from Sigma Aldrich (St. Louis, Missouri) were screened for the ability to activate cells by cross-linking cell-bound IgE and Sigma #B47352 and Sigma #B47351 were used in our assays (herein anti-IgE). The allergen Derp-1 was used as a more natural IgE cross-linker and basophil activator to compare to the global anti-IgE response (Indoor Technologies, Charlottesville, VA). Xolair (Omalizumab; Genentech), a monoclonal anti-human IgE that does not bind cell-bound IgE, was used as an additional control.

Blood was cultured with anti-IgE, ultrapure *E. coli* LPS (Invivogen Life Technologies, Waltham, MA) with or without ET523 for 18 hours. ET523 was used at concentrations previously shown to have an effect on basophil activation (20 µg/million cells) [22]. 20 ng/ml of recombinant human IL-4 (ebioscience) was used a control in some experiments.

Flow cytometry on cultured whole blood was performed using a modified protocol for fresh blood as described above. Cell-free supernatants were collected and stored at -20°C until use. The remaining cells were collected and spun down to concentrate the cells. The supernatants were discarded and the cells were resuspended in 500 µl of PBS. 100 µl of cells per tube were distributed for staining for flow cytometry following the protocol above for whole blood cells with antibodies (anti-CD63, -CD203c, -FceRI, -CCR3, -CD15) from BD Biosciences.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Fisher Scientific, Hampton, NH) density gradient. 2 x 10^6 cells/ml were stimulated for 18-72 hours with 1 µg/ml of *E. coli* LPS and purified by GenScript the National Disease Research Interchange (Philadelphia, PA).
coli LPS, anti-BCR μ/γ (Jackson ImmunoResearch, ME), 20 ng/ml of recombinant human IL-4 (ebioscience), 20 μg/ml of anti-IgE and 20 μg/ml of ET523.

Tonsils were homogenized with sterile tissue grinders and applied to a cell strainer to obtain a single cell suspension. A Ficoll-Paque gradient was performed to isolate mononuclear cells. 2 x 10^6/ml tonsil mononuclear cells were cultured for 72 hours, 5 days or 12 days. Flow cytometry was performed on PBMCs and tonsil MC for activation and differentiation of IgE-receptor bearing cells, including CD23+ B cells and monocytes/macrophages using antibodies (anti-CD19, -CD14, -CD86, -CXCR5, -CD38, and -IgM), purchased from BD Biosciences. Basophils within PBMCs were assessed using antibodies against CD63, FcεRI, and CD203c (BD Biosciences).

**Measurement of Cytokine Levels**

Cell-free supernatants from whole blood cultures were quantitatively assayed for TNF-α and IL-8 by standard enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN).

**Statistics**

We compared the groups and controls using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison posttest. Differences between groups were considered to be significant at a P value of <0.05. Chi-square was used to test the relationships between demographic and immunological data. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

**Results**

**Basophil levels in IBD**

The percentages of basophils as a function of whole blood leukocytes in CD, UC, and healthy blood samples was measured by flow cytometry. Figure 1A illustrates the electronic separation of blood leukocytes by forward and side scatter (FSC/SSC) with distinct populations of lymphocytes, neutrophils, monocytes, and eosinophils. Basophils normally account for 0.5% to 1% of circulating leukocytes, which are located within the lymphocyte gate. FcɛRI+ basophils and eosinophils express relatively high levels of CCR3 [24,25]. We found that CCR3 was a superior marker to use to calculate basophil percentages as cell-bound IgE can interfere with detecting FcɛRI α. Figures 1 B & C depicts our gating strategy for measuring basophils in whole blood samples from two different subjects. Thus, basophils and eosinophils are distinguished from other cells by high CCR3 expression and from each other by FSC/SSC profiles.

The mean percentage of basophils was higher in UC compared to CD patients (P=0.02; Figure 1D). Within UC, there was a positive correlation between the percentage of basophils and disease activity (Figure 1E; n=15), but not within CD. There was no association between increased or decreased basophil percentages with medication usage for any group (not shown.). In addition, there was no correlation between eosinophil and basophil percentages (not shown.).

**Whole blood culture assay to measure IgE-mediated responses**

We utilized a whole blood culture assay to assess the effect of IgE cross-linking on release of inflammatory mediators known to be pathogenic in IBD. Binding of the activating anti-IgE antibody to cell-bound IgE was confirmed by measurement of IgE on the cell surface by flow cytometry following 6 hours of culture. The anti-IgE cross-linking antibody (shown is Sigma #B47352) blocks the ability to detect surface bound IgE (Figure 2A). Xolair does not bind to cell-bound IgE and thus did not interfere with the ability to detect surface IgE (Figure 2A). Anti-IgE induced TNF-α secretion by whole blood cells from healthy subjects demonstrating the antibody was activating (Figure 2B).
able to bind to cell-bound IgE and activate cells. Omalizumab and *E. coli* LPS do not affect detection of surface IgE. Representative of two separate experiments.

**Anti-IgE cross-linking induces TNF-α secretion in IBD**

To determine the effect of IgE activation in IBD, whole blood samples from CD and UC patients were cultured for 18 hours and supernatants were tested for their level of TNF-α. Figures 3 A & B demonstrate that even without culture stimulation, basal secretion of TNF-α was high (see levels in Media). As anti-TNF-α biologics interfere with the ability to measure the cytokine in samples from blood, only blood from patients who were not on this treatment are shown. Anti-IgE induced TNF-α secretion from cells from patients with CD, but we had too few patients to measure the effect in UC (Figure 3A).

**Figure 3: Basal and IgE-mediated cytokine secretion is reduced by ET523 in IBD.**

A. In patients not currently treated with anti-TNF-fN biologics, TNF-fN is measured in supernatants from untreated wells of cultured whole blood. ET523 treatment reduces basal levels, as well as anti-IgE induced, TNF-fN secretion (n=8). B. Basal levels of TNF-fN and the reduction by ET523 in three UC patients not currently on anti-TNF-fN biologics are shown. C. Basal secretion of IL-8 is high in CD patient blood cultures and is reduced by ET523. However, anti-IgE did not increase IL-8 levels and ET523 did not significantly reduce IL-8 secretion (n=7). D. Spontaneous production of IL-8 is lower by blood cultures from UC patients compared to CD, and ET523 did not reduce level. Anti-IgE induced increased IL-8 production in UC, which was significantly reduced by ET523 (n=6). *P<0.05; **P<0.001.

**ET523 reduces basal levels and anti-IgE mediated secretion of TNF-α and IL-8**

ET523 inhibited anti-IgE-mediated production of TNF-α in CD (Figure 3A). We also tested the effect of ET523 on another inflammatory cytokine found to be elevated in IBD, namely IL-8 [23,26,27]. As previously reported, cells from CD patients spontaneously secrete IL-8 (Figure 3C) [23]. Treatment with ET523 dramatically reduced basal IL-8 production (Figure 3C). However, anti-IgE did not significantly upregulate IL-8 in CD and the level was not affected by ET523 (Figure 3C). In contrast, basal production of IL-8 was low and not affected by ET523 in UC (Figure 3D). Further, anti-IgE increased IL-8 secretion, which was reduced by concurrent ET523 treatment (Figure 3D). These results highlight the differences in responses to *E. coli* LPS between CD and UC with blood cells from CD subjects demonstrating a muted response reminiscent of tolerance (Figure 3 C&D) [9]. Surprisingly, ET523 reduced TNF-α and IL-8 secretion in response to *E. coli* LPS as well (Figures 3 A&C.)

The effect of anti-IgE cross-linking on basophil activation

Whole blood cultures contain a mixed-cell population with several cells bearing surface IgE. For example, anti-IgE treated cultures contain CD15+ activated neutrophils [28], which increase surface CD63. Activated neutrophils may thus account for some of cytokine secretion observed (Figure 4A). Although reduction of inflammatory cytokine from any cellular source is likely important for IBD, we sought to comprehensively define the effect of anti-IgE and ET523 treatment on basophil activation.

We utilized whole blood from healthy subjects cultured with anti-IgE to better characterize the effect in vitro. Figure 4B depicts the increase of CD63 on CCR3+FcrRI+ basophils in response to anti-IgE. As anti-IgE induces a global, highly potent effect on basophils bearing IgE, we also tested the effect of Derp1, a natural allergen, on basophil activation for comparison. Derp1 had a similar effect on CD63 detection following stimulation (Figure 4 B&C). In contrast, *E. coli* LPS did not affect levels of detectable CD63 (Figure 4D). Anti-IgE also increased FcRI expression on CCR3+ basophils (Figure 4D). The effect was similar with Derp1, but to a lesser degree (Figure 4E). In contrast, *E. coli* LPS did not change the expression of FcRI (Figure 4E). Basophils from healthy subjects within isolated PBMCs also increased CD203c, CD63, and FcRI levels upon anti-IgE cross-linking (not shown).

**Figure 4: Development of the whole blood cell assay to specifically assess basophil responses.**

A. Neutrophils in whole blood cultures treated with anti-IgE demonstrated activation as measured by increase in CD63 surface levels. B. Specific basophil activation measures were assessed in whole blood cultures. CCR3+FcrRI+ basophil activation as measured by CD63 detection was assessed by flow cytometry following anti-IgE
ET523 reduces basophil cellular activation in IBD

To test the effect of anti-IgE on basophils in IBD, blood from CD and UC patients was cultured in the presence of anti-IgE. Basal levels of activation were higher in UC compared to CD samples (Figures 5 A & B). Basophil activation was increased as measured by CD63 and FcrrI in both CD (Figure 5A) and UC (Figure 5B). However, Derp1 treatment did not induce a significant effect on these markers (not shown.). ET523 greatly reduced anti-IgE mediated basophil activation in both CD (Figure 5A) and UC as well reduced basal basophil activation levels in UC (Figure 5B). Shown are levels as a function of whole blood leukocytes to demonstrate the magnitude of potential activation in the bloodstream.

ET523 does not activate lymphocytes or monocytes

Soluble CD23, which is the basis of ET523, in its trimeric form, has been shown to stimulate memory B cell production of IgE and monocyte secretion of TNF-α. Although ET523 lacks the stalk region of CD23 and thus the ability to homo-dimerize [22], we tested the effect of ET523 on mononuclear cellular activation. PBMCs were isolated from blood from the three groups and cultured for 72 hours. Figure 6A demonstrates that while the positive control stimuli activated B cells, ET523 did not have an effect (shown are CD86 levels on CD19+ B cells). In addition, monocytes were not activated in the presence of ET523 (not shown).

We next tested the effect of ET523 on mucosal immune responses. Tonsil mononuclear cells were cultured in the presence of ET523 for up to 12 days. ET523 did not affect mucosal B cell activation or differentiation at any time point tested (Figure 6B; shown is day 12). Similarly, tonsil macrophages were not activated by ET523 (not shown).

Discussion

Reducing TNF-α levels remains an important goal in decreasing disease activity in IBD. Multiple cellular sources and triggers of TNF-α secretion likely contribute to pathology; thus, expanding...
targets of anti-TNF-α drugs may lead to better disease control. In this report, we demonstrate that anti-IgE cross-linking induced the release of TNF-α from circulating cells. Basophils are a major source of cell-bound IgE in the bloodstream and both IgE and LPS activated basophils in vitro. Basophils are thought to be primarily circulating granulocytes, but their functional significance remains poorly understood [18]. Basophils were reported to be elevated in the bloodstream and also accumulated in inflamed mucosal tissues from CD and UC patients [17]. This finding was specific to basophils and not mucosal mast cells, suggesting that recruited basophils have an additional, explicit role in the tissues that differs from mast cells. High expression of CCR3 on basophils suggests functional significance in the tissues, particularly in the gastrointestinal tract [24]. We found a positive association between the concentration of basophils in the blood and disease activity in UC and a higher overall basal activation level of basophils in both UC and CD suggesting that basophils are affected and/or play a role in systemic and mucosal disease.

ET523 is a biologic under development to reduce IgE-mediated diseases [22]. ET523 is a modified form of sCD23, an IgE binding protein, with increased affinity for IgE possessing additional anti-inflammatory properties [22]. We published that ET523 binds free IgE and reduces basophil activation in vitro in the presence of anti-IgE cross-linking antibody [22]. ET523 functions similarly to Omalizumab (Xolair), a monoclonal antibody that binds the Fc region of IgE [30], but has an additional mechanism of reducing the effect of cellular activation mediated by IgE-crosslinking [22]. CD23 and FceRI have been shown to bind IgE in different regions, which may account for the ability of ET523 to inhibit activation through cell-bound IgE cross-linking [31]. Here, we demonstrate that ET523 reduced basal levels of basophil activation and global TNF-α secretion in IBD. Thus, ET523 may act as a more natural suppressor of systemic inflammation and a potential drug to control inflammation in IBD in addition to bona fide IgE-mediated diseases, such as allergy.

Surprisingly, ET523 also reduced the effect of E. coli LPS on some basophil activation markers. This suggests that IgE may be LPS-specific on the surface of basophils in IBD. However, we were unable to detect LPS-specific IgE in the serum of IBD patients, most likely from technical difficulties with an LPS-specific ELISA or from lack of sufficient IgE in IBD. A previous report demonstrated that E. coli LPS enhanced CD63 surface expression and histamine release from basophils of atopic subjects in combination with antigen stimulation, likely through TLR4 [32]. Similarly, stimulation through FceRI and TLR4 or TLR9 synergistically upregulated several chemokines and cytokines by basophils, including IL-4, CXCL8, and CCL5 [18]. Exogenous and endogenous TLR ligands are elevated in the bloodstream of IBD patients, which likely contribute to basophil activation either through antigen specific IgE or TLRs [9,33-35].

Overall, the role of IgE in IBD has not been well-defined. Most studies have not found an increase in IgE levels in IBD, and IgE activity or specificity did not correlate with eosinophil degranulation in one report [36]. However, some patients with UC demonstrate concurrent food allergies and UC-specific disease was reduced by immunotherapy [37]. In this clinical study, reduction of allergic inflammation was associated with lower IgE levels. Asthmatic patients treated by administration of Omalizumab with improved clinical outcomes have reduced basophil numbers suggesting that IgE regulates basophil homeostasis [38]. Functionally, basophils were found to augment inflammatory T cell responses by increasing IL-17 and IFN-γ production. This process involved basophil-derived histamine and H2 and H4 receptors, though the roles of IgE and basophils from IBD patients were not tested directly [21].

IgE has many important functions in immunity [39]. For example, CD23+ B cells may use IgE as a mechanism to augment antigen-specific responses and traffic into follicles [20,40-42]. CD23-bound IgE on monocytes is cross-linked to release TNF-α and other mediators [43]. In malaria, which is not considered an allergic disease, Plasmodium falciparum-specific IgE bound by CD23 on monocyte induces release of TNF-α and nitric oxide (NO)(19). Immune complexes containing antigen, IgE, and sCD23 trimers can engage CD21 on antigen presenting cells and IgE+ memory B cells amplifying responses and IgE secretion [16,44,45]. In contrast, sCD23 monomers, which are similar in structure and function as ET523, have been shown to block the effect of larger dimeric isoforms and reduce inflammatory responses [45,46]. We demonstrate that ET523 does not activate other IgE-bearing cells including CD23+ B cells or monocytes. The ability of ET523 to reduce TNF-α secretion specifically from monocyte/macrophages is under investigation. Our data largely suggest that ET523 may have multiple beneficial roles and the role of IgE in regulating basophil concentrations and other functions should be explored further in IBD [47].

In conclusion, ET523 may be an effective and a more natural biologic drug to reduce inflammatory mediators in IBD and other chronic inflammatory diseases [22]. We show the utility of using ET523 to reduce systemic TNF-α secretion and basophil activation upon IgE cross-linking. In addition, the drug appears to not affect other aspects of the immune system.

Compliance with Ethical Standards
The authors have no conflicts of interest. As described in the Methods, all subjects agreed to participate in the study through informed consent.

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