Interleukin-6 is associated with steroid resistance and reflects disease activity in severe pediatric ulcerative colitis

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Abstract

Background and aim: Approximately one third of patients with acute severe ulcerative colitis (ASC) will fail intravenous corticosteroids (IVCS). Predicting response to IVCS to initiate early salvage therapy remains challenging. The aim of this study was to evaluate the role of serum inflammatory cytokines in ASC and determine their predictive utility with IVCS treatment failure.

Methods: This preplanned ancillary study, part of the prospective multicenter OSCI study, evaluated pediatric ASC in North America. Serum samples were obtained from 79 children admitted for ASC on the third day of IVCS treatment. Twenty-three (29%) patients required second-line therapy. ELISA-based cytokine arrays were used [TNF-α, IFN-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, and IL-17], selected based on a systematic literature search.

Results: In univariate analysis, only IL-6 was significantly different between responders and non-responders (P = 0.003). The risk for IVCS failure increased by 40% per each pg/mL increase in IL-6.

Abbreviations: ASC, acute severe ulcerative colitis; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; GR, glucocorticoid receptor; IFN, interferon; IL, interleukin; IBD, inflammatory bowel diseases; IQR, interquartile range; IVCS, intravenous corticosteroids; OR, odds ratio; PGA, physician global assessment; PUCAI, Pediatric UC Activity Index; TH, T-helper; TNF, tumor necrosis factor; UC, ulcerative colitis.

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1 Equal contribution.
IL-6 predicts steroid failure in severe UC

1. Introduction

Treatment of acute severe ulcerative colitis (ASC) often requires admission to hospital for use of intravenous corticosteroids (IVCS), which has been the mainstay of treatment for decades. However, as documented in a systematic review of cohort studies, one third of adult patients hospitalized with ASC fail to respond to such therapy, and even more in children. Several measures predict response to IVCS and thus facilitate timely introduction of salvage therapy (i.e., infliximab, cyclosporine, tacrolimus, or colectomy), such as clinical response [reflected in the Pediatric Ulcerative Colitis (UC) Activity Index (PUCAI)], laboratory markers, and changes in the microbiome. However, it remains unclear why some patients respond rapidly to corticosteroids while others do not.

Cytokines are small signaling molecules with critical roles in inflammation. Cytokines first released in response to inflammatory triggers include interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF-α), which activate a second wave of pro-inflammatory transcription factors to produce other cytokines. IL-6 is a central cytokine released mainly from monocytes, macrophages, and T-cells during acute and chronic inflammation. Although this pleiotropic cytokine is related to many biological processes the roles relevant to human inflammatory bowel diseases (IBD) mainly relate to stimulation of additional pro-inflammatory cytokines, proteases, and adhesion molecules, anti-apoptotic effects, and regulation of T-cell differentiation.

Changes in cytokines are well documented in UC and may persist even during disease remission. Studies highlight the complex involvement of cytokines in UC and provide the rationale for understanding the interplay between cytokines and response to steroid therapy in severe UC. We aimed to evaluate the role of inflammatory cytokines in the serum of children with ASC and to determine their association with corticosteroid failure. We hypothesized that specific serum cytokines, measured at the third day of IVCS treatment, can differentiate those who will fail to respond to the medication and require further salvage therapy.

2. Materials and methods

2.1. Study design

This is a preplanned ancillary study performed as part of the large prospective multicenter OSCI study, which evaluated pediatric ASC in North America. Explicit clinical, demographic, and outcome data were prospectively collected at admission, at multiple time-points during the admission, and at discharge of children admitted for IVCS treatment of ASC. The main goal of the OSCI study was to identify clinical and laboratory predictors of non-response to IVCS treatment. Samples of serum, obtained at the third day of corticosteroid therapy were linked to the phenotypic database, and used here to evaluate the association of inflammatory cytokines with steroid resistance. Day 3 of IVCS treatment was chosen to differentiate responders from non-responders as the longer one waits the larger the differences between those who are improving and those left unchanged and since this time provides a balance between the predictive power and timely clinical feasibility.

The work was approved by the appropriate ethical committees related to the institutions in which it was performed and that subjects gave informed consent to the work.

2.2. Patients

All patients with available serum at the time of the array analysis were included. To determine the association of cytokines with steroid failure, patients were categorized as steroid-responsive or steroid-refractory depending on the need for second line therapy (anti-TNF therapy, calcineurin inhibitors, or colectomy) prior to hospital discharge. Because physician factors may influence decision to institute second-line therapy, patients were further categorized into more homogenous extreme groups. Stringent-failure, defined as a combination of at least moderate disease activity on the fifth day of steroid therapy (PUCAI > 65) and the need for second line therapy by discharge and stringent-response was considered with no more than mild disease activity (PUCAI < 35) on the fifth steroid day and discharge without the need for second line therapy. While PUCAI > 65 does not include laboratory measures of severity, it has been validated as a measure of severity and predictor of response.

2.3. Serum cytokine profiling

Serum was separated immediately after collection and shipped to the central study laboratory within 24 h, where it was stored in −80 °C for subsequent analysis. A total of 12 relevant cytokines were determined using a human Th1/Th2 multiplex cytokine array panel [for TNF-α, interferon (IFN)-γ,
IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, and IL-13] and separate individual ELISA kits for IL-6 and IL-17, according to the manufacturer’s instructions (Meso Scale Discovery, Gaithersburg, MD). Briefly, 0.25 mL of serum was placed in duplicates in wells containing spots of capture antibodies specific to each cytokine. After washing 3 times with PBS with Tween (0.05%), detection antibodies were incubated for 2 h, washed, and then the read buffer was added followed by analysis using a luminescence reader. Results are expressed in pg/mL, calculated by standard curves included in each plate.

2.4. Statistical analysis

For the exploratory analysis of Table 1, data were presented as means (± standard deviation), or medians [interquartile range (IQR)] and compared using Wilcoxon rank sum test as appropriate for the non-normally distribution of the cytokine levels. Categorical variables were compared using chi-square or Fisher exact tests, as appropriate. Adjusted odds ratios (OR; with corresponding 95% confidence intervals (CI)] were calculated from multivariate logistic regression models, controlling for possible confounding variables, including disease duration and disease severity (PUCAI; sample size did not enable inclusion of additional factors). Goodness of fit was established using the Hosmer and Lameshow test.

Factor analysis was performed to explore the relationship between various cytokines. Principal component analysis was used as the extraction method. The number of retained components was dictated by using the criteria of eigenvalue of 1 and by examining the scree plot, after varimix rotation.

To correlate the cytokine levels to disease activity, a matrix of Spearman’s correlation was constructed using all 12 cytokines and the following constructs determined concurrently at day 3 of steroid treatment: PUCAI score,18 See index,19 Lindgren index,20 physician global assessment of disease severity (PGA, determined on a 100 mm visual analogue scale), and blood tests [C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), hemoglobin, and albumin]. Only correlations of >0.3 were considered a-priori as positive correlations.

A two-sided alpha level of 0.05 was used to declare significance. Analyses were performed using SPSS for windows V15.0, and SAS V9.1.

3. Results

3.1. Serum IL-6 is associated with failure to respond to steroids

Of the 79 patients included in the study, 23 were considered corticosteroid-failures requiring second line therapy by discharge (Table 1). Although characteristics of patients were mostly similar between groups, more IVCS-responders presented with a first attack (59% vs. 26%), which could also explain the trend for higher previous steroid exposure in the failure group (56% vs. 21%). In univariate analyses comparing levels of all 12 tested cytokines, only levels of IL-6 were different between responders and non-responders to IVCS (Table 2). Using unadjusted logistic regression, an increase of serum IL-6 value by 1 pg/mL was calculated to be associated with 40% increased risk for corticosteroid failure [odds ratio (OR)=1.4; 95% confidence interval (CI): 1.1–2.0], where we applied the alternative analyses of stringent corticosteroid response (n=37) vs. stringent failure (n=22), the difference in serum IL-6 levels was even larger [0.52 pg/mL (IQR 0.24–0.79) in the responders vs. 1.37 pg/mL (0.59–3.0) in the failures; P=0.003, lower than the Bonferroni corrected threshold of P=0.0042 for multiple testing]; IL-8 was marginally different [31.2 pg/mL (19.4–53) vs. 48.3 pg/mL (26.7–111); P=0.045]. All other comparisons remained insignificant.

3.2. Cytokine clustering

Factor analysis is used to describe dependency of variables on each other, which enables grouping of observed variables according to common patterns. Four components were retained based on an eigenvalue over 1, but since the necessity of the fourth component was marginal in the screen plot we present also a three-component model (Table 3). Interestingly, IL-6 and IL-17 clustered in a single factor in both

Table 1 Baseline characteristics of the 79 children included in the analysis.

<table>
<thead>
<tr>
<th></th>
<th>IVCS response (n=56)</th>
<th>IVCS failure (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>28 (50%)</td>
<td>16 (70%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>13.1±3.8</td>
<td>14.9±2</td>
</tr>
<tr>
<td>Range (years)</td>
<td>4–20</td>
<td>10–18</td>
</tr>
<tr>
<td>Disease duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First attack</td>
<td>33 (59%)</td>
<td>6 (26%)</td>
</tr>
<tr>
<td>Exacerbation</td>
<td>23 (41%)</td>
<td>17 (74%)</td>
</tr>
<tr>
<td>Disease duration prior</td>
<td>7.4 (3–29)</td>
<td>9.7 (4–31)</td>
</tr>
<tr>
<td>to exacerbation (months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease extent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left sided</td>
<td>7 (13%)</td>
<td>3 (13%)</td>
</tr>
<tr>
<td>Extensive/pancolitis b</td>
<td>49 (87%)</td>
<td>20 (87%)</td>
</tr>
<tr>
<td>Steroid dose (mg/kg/day)</td>
<td>1±0.31</td>
<td>0.9±0.28</td>
</tr>
<tr>
<td>PUCAI at admission</td>
<td>71±12</td>
<td>74±11</td>
</tr>
<tr>
<td># moderate d</td>
<td>8 (14%)</td>
<td>4 (17%)</td>
</tr>
<tr>
<td># severe d</td>
<td>48 (86%)</td>
<td>19 (83%)</td>
</tr>
<tr>
<td>% Weight loss</td>
<td>5.7 (1.4–7.9)</td>
<td>4.4 (1.6–7.7)</td>
</tr>
<tr>
<td>Days of bloody diarrhea</td>
<td>16 (8–30)</td>
<td>20 (12–65)</td>
</tr>
<tr>
<td>Steroids during the</td>
<td>19 (21%)</td>
<td>13 (56%)</td>
</tr>
<tr>
<td>previous year</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Count (%), medians (interquartile range) or mean±SD are presented as appropriate for the data distribution.

All values are non-significantly different between the two groups except for rates of first attack (P=0.013), IVCS, intravenous corticosteroids and PUCAI, Pediatric Ulcerative Colitis Activity Index.

a Within one month of admission.
b According to the Paris classification.42
c All patients, but one who was treated with hydrocortisone, received methylprednisolone.
d As previously defined.43
e At the start of intravenous corticosteroid therapy.
models while other factors generally followed the expected T\textsubscript{H}1/T\textsubscript{H}2 grouping.

### 3.3. IL-6 levels is confounded by disease activity but not duration

Corticosteroid-responders and non-responders in our study were not balanced by disease duration, as there were more patients with new-onset of UC in the responders group. However, in a multivariable model adjusted for disease duration, IL-6 was strongly associated with response to corticosteroids, while disease duration was not [OR of IL-6 = 1.5 (95% CI 1.1, 2.0), and 1.02 (0.996, 1.04), respectively] eliminating any potential confounding effect. These results were similar when analyzing only the 59 patients with stringent definitions of response (data not shown).

To explore whether the association of IL-6 with corticosteroid response merely reflects a more severe disease state, a multivariate logistic regression model was constructed, adjusted for the PUCAI score calculated concurrently at the third treatment day. In that model, IL-6 lost its association with corticosteroids response while the PUCAI assumed all the significance (P=0.32 for IL-6; P<0.001 for PUCAI). This might indicate that IL-6 is only a surrogate marker for disease severity.\textsuperscript{1} To further explore this notion, we then correlated all cytokines with other constructs of disease activity. Only IL-6, IL-17, and IL-1\textsubscript{β} had correlations of >0.3 (in absolute values) with at least one of the constructs, of which IL-6 showed the most striking correlations with almost all severity measures (Table 4).

### 4. Discussion

Although the outcome of pediatric UC has improved with the introduction of new therapies such as anti-TNF antibodies,\textsuperscript{21} one of the main challenges clinicians face is selecting the most appropriate timing of introducing these interventions. In the case of hospitalized children with ASC, better prediction of response to IVCS could prevent unnecessary surgery while reducing the risk of postponing surgery when it is really needed.

Using a well-defined cohort of children hospitalized with IVCS therapy for ASC and utilizing several analyses and outcomes, we found that increased serum IL-6 levels predicted lack of response to IVCS and the need for second-line therapy or colectomy. This association was further supported by using a more stringent definition of treatment failure, likely since IL-6 was found to be correlated with a higher PUCAI, which was used to define the stringent criteria. Our results also show that increased IL-6 is linked to elevated IL-17 and IL-1\textsubscript{β} and that IL-6 correlated well with multiple clinical markers of disease activity. Although increased IL-6 levels in mucosal samples of patients with UC and specifically in those non-responsive to corticosteroid therapy, has been described,\textsuperscript{22,23} using serum levels is much more practical in the clinical setting and could potentially be used for prediction. The reasons why some patients respond poorly to therapy could be related to a variety of causes. Our observation that IL-6 is associated with corticosteroid failure could shed some light on potential mechanisms of steroid resistance. However, the association of IL-6 with corticosteroids failure is linked by multivariable analysis to disease severity, as measured by PUCAI. Although this suggests that IL-6 may only serves as a marker of severity, it also points to a specific role for this cytokine in mediating refractory disease course.

IL-6 is a potent and pleiotropic cytokine involved in regulating inflammation at many levels in immune-mediated diseases, including IBD.\textsuperscript{14,15} Interestingly, genetic polymorphisms in the IL-6 gene, specifically IL-6-174GG, which is linked to increased IL-6 activity, was suggested by some studies to correlate with susceptibility to UC,\textsuperscript{24} Acute phase effects of IL-6, including stimulation of CRP production, could also explain the observed association between CRP and UC activity.\textsuperscript{10,25} However, how this relates to failure to respond to corticosteroids remains unclear.

### Table 3  Factor analysis of serum cytokines in severe ulcerative colitis.

<table>
<thead>
<tr>
<th>Factor model</th>
<th>Cytokines included in group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four component model a</td>
<td>1. IFN-γ (0.66), IL-1\textsubscript{β} (0.75), IL-2 (0.94), IL-4 (0.82), IL-8 (0.69), 2. IL-12 (0.98), IL-13 (0.94), IL-5 (0.94), 3. IL-6 (0.65), IL-17 (0.76), 4. IL-10 (0.90), TNF-\textalpha (0.25)</td>
</tr>
<tr>
<td>Three component model b</td>
<td>1. IFN-γ (0.65), IL-1\textsubscript{β} (0.77), IL-2 (0.94), IL-4 (0.82), IL-8 (0.70), TNF-\textalpha (0.25), 2. IL-10 (0.25), IL-12 (0.97), IL-13 (0.94), IL-5 (0.93), 3. IL-6 (0.73), IL-17 (0.69)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the loading factor.

a IL-1\textsubscript{β} loaded also on component 4 (0.41); TNF-\textalpha loaded also on component 1 (0.22), and IL-6 loaded also on component 4 (0.41).

b IL-1\textsubscript{β} loaded also on component 3 (0.45).
In general, the association between failure to respond to corticosteroids and elevated IL-6 could represent a few scenarios of cause and effect. First, reduced bioactivity or bioavailability to corticosteroids for other reasons could lead to indirect increase in IL-6, since IL-6 has been shown, at least in vitro, to be inhibited by corticosteroids. On the other hand, there could be a direct inhibitory effect of IL-6 on response to steroids. This is supported by an association between polymorphisms in the IL-6 gene (IL-6 174GG genotype, as mentioned above) and resistance to corticosteroids in children with nephrotic syndrome and the steroid-sparing ability of anti-IL-6 antibodies in children with juvenile rheumatoid arthritis. A third possibility relates to Src kinases, which are involved in the activity of both steroids and IL-6. While most effects of glucocorticoids are usually attributed to transcriptional regulation, they also cause dissociation of a protein complex containing the T-cell receptor with the Src kinases Lck and Fyn acting as an inositol 1,4,5-trisphosphate [IP(3)] receptor regulator. On the other hand, IL-6 induces Fyn activity while another Src kinase, Hck, regulates IL-6 production in response to TLR4. These complex interactions may help explain why IL-6 is elevated in steroid-refractory patients (for example, if Src kinases are less inhibited in refractory patients they may further stimulate IL-6 production) but this has not been specifically explored in the setting of intestinal inflammation.

The evidence by which cytokines can influence the glucocorticoid pathway goes beyond IL-6. IL-2 and IL-4, reduce the affinity of glucocorticoid receptor (GR)α to steroids by inducing phosphorylation of GRα through p38-MAPK. TNF-α, IL-8, and IL-1β have also been shown to induce GRα expression while IL-10 acts the opposite way, increasing steroid sensitivity. Indeed, a defect in IL-10 secretion has been noticed in asthma patients resistant to steroid therapy, and the colonic levels of cytokines (IL-6, IL-8, and TNF-α) were higher in UC-refractory compared with responsive disease. However, an alternative explanation for the association between IL-6 and glucocorticoid refractoriness needs to be considered. Since disease severity and refractoriness are generally linked (although without good scientific evidence), it is possible that increased inflammation in refractory patients, for which IL-6 is a marker, overwhelms the effect of steroid therapy, leading to failure to respond.

### 4.1. IL-6 and resistance to corticosteroids — potential mechanisms of interference

In our study, IL-6 loaded primarily with IL-17 but also with TNF-α. It is not known why TNF-α did not load on the same component as IL-6 and IL-17 when only three components were forced. It can be speculated that the complex interaction of TNF-α with other cytokines dilutes its impact on one specific pathway. To support this hypothesis, TNF-α did not load highly on any component.

Mucosal samples from UC patients have shown increases in IL-6, IL-8, and TNF-α. In our study, IL-6 loaded primarily with IL-17 but also with TNF-α. It is not known why TNF-α did not load on the same component as IL-6 and IL-17 when only three components were forced. It can be speculated that the complex interaction of TNF-α with other cytokines dilutes its impact on one specific pathway. To support this hypothesis, TNF-α did not load highly on any component.

UC represents common phenotypes for likely distinct pathogenic processes that lead to tissue damage and similar symptoms. Since corticosteroids are the mainstay of treatment for ASC, biological markers that could shed light onto disease pathogenesis in individual patients and predict who will respond to therapy are useful. Our findings highlight IL-6, potentially with IL-17, as such marker that could impact management and predict response to therapy by serving as a marker for disease activity in children with severe UC. Moreover, the results of our study may provide mechanistic insights and warrant further study to determine if clinical trials on the effectiveness of monoclonal antibodies against IL-6, IL-6 receptor, or IL-23 p40 are feasible in ASC.

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Author contribution

EW designed the study, acquired and analyzed data, and wrote the manuscript; DRM collected patient samples and data, critically reviewed the manuscript; JH collected patient samples and data, critically reviewed the manuscript; AMG collected patient samples and data, critically reviewed the manuscript; WVC collected patient samples and data, critically reviewed the manuscript; NL collected patient samples and data, critically reviewed the manuscript; AMM assisted in designing the study, critically reviewed the manuscript; AMG assisted in designing the study, obtained funding, collected patient samples and data, and critically reviewed the manuscript; DT designed the study, collected patient samples and data, analyzed data, wrote the manuscript.

Conflict of interest

EW has served on advisory board for Janssen; DRM has served on advisory board and received unrestricted education grant from Janssen; JH received research support, consultation fee, and speaking bureaus from Janssen; ARO has served on advisory board for Janssen and Abbott and received unrestricted education grant from Janssen; JM has served as a consultant for Janssen; WC received research support from Abbott Laboratories; NL and AMM have no pertinent disclosures; AMG declares receiving research support, consultation fees, and speaker fees from Janssen and MSD; DT declares receiving research support, consultation fee and speaking bureaus from MSD and Janssen.

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