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ALTERATIONS IN BLOOD LEUKOCYTES OF G551D-BEARING CYSTIC FIBROSIS PATIENTS UNDERGOING TREATMENT WITH IVACAFTOR

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Abstract

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**Background**—Ivacaftor improves clinical outcome by potentiation of mutant G551D CFTR. Due to the presence of CFTR in monocytes and polymorphonuclear neutrophils (PMNs), we hypothesized that ivacaftor may impact leukocyte activation.

**Methods**—We examined blood leukocytes from G551D CF subjects prior to and at one and six months after receiving ivacaftor. Blood leukocytes from ivacaftor-naïve G551D, F508del, and healthy controls were also treated with ivacaftor *ex vivo* to assess mutation-specific effects.

**Results**—Compared to healthy controls, G551D CF subjects had significantly higher expression of active CD11b on PMNs and of CD63 on monocytes, which were normalized by *in vivo* ivacaftor treatment. *Ex vivo* exposure to ivacaftor of blood cells from G551D, but not F508del and healthy subjects, resulted in changes in CXCR2 and CD16 expression on PMNs.

**Conclusions**—*In vivo* and *ex vivo* exposure of G551D CF leukocytes to ivacaftor resulted in an altered activation profile, suggesting mutation-specific leukocyte modulation.

**Keywords**
CFTR; G551D; ivacaftor; VX-770; leukocyte; blood

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive condition caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1). CFTR is an epithelial anion transporter which maintains salt, fluid, redox and pH balance (2), as well as membrane recycling (3) in a variety of organs. As a result of deranged CFTR function, multiple epithelial organs are affected including the airways, pancreas, intestine, and vas deferens (4). More recently the protean manifestations of CFTR have been realized, including its expression in peripheral leukocytes and other non-epithelial tissues, which have also been postulated to contribute to disease pathogenesis (5, 6).

CF lung disease is characterized by an increased and dysregulated inflammatory response (7). Recruitment and activation of polymorphonuclear neutrophils (PMNs) are particularly prominent in CF airways (8). Monocyte/macrophage dysfunction is also believed to contribute to disease pathogenesis (9). As a result, CF airways are characterized by protease activation, which contributes to subsequent airway remodeling, ultimately leading to bronchiectasis (10). The ongoing cycle of inflammation and remodeling is central to the progression of CF lung disease and is a major contributor to morbidity and mortality.

Recently, the CFTR potentiator ivacaftor (formerly known as VX-770) was approved for the treatment of CF caused by the G551D gating mutation (but not for the most common folding and gating mutation, F508del) (11). In a recent series of pivotal clinical trials, ivacaftor treatment was found to be highly efficacious towards ameliorating CF lung disease. Marked improvements in spirometry, pulmonary exacerbation frequency, and clinical symptoms were observed, while elevated sweat chloride, a marker of CFTR function, significantly improved, approaching the normal range (12, 13). Following approval, CF patients with the G551D mutation were monitored in the G551D Observational (GOAL) Study. Clinical results in this larger population documented similar improvements as those observed in
previous placebo-controlled studies (14). In addition, following ivacaftor treatment, mucociliary clearance, gastrointestinal pH, and Pseudomonas aeruginosa infection all improved, suggesting a robust effect across various organs and disease symptoms, including an indicator of pulmonary immunity.

While changes in inflammatory markers of expectorated sputum were not apparent in the GOAL study at 6 months, prior trials demonstrated improved C-reactive protein (CRP) following 6 months of ivacaftor administration, suggesting a beneficial effect on the CF inflammatory phenotype (11). While this could be due to the restoration of proper CFTR function in the airways, the impact (direct or otherwise) of ivacaftor on CF inflammatory pathways has not yet been examined. Based on these findings, we hypothesized that leukocyte function might be improved in G551D CF patients treated with ivacaftor. The aim of the present study is to characterize the activation state of peripheral blood leukocytes in G551D CF patients pre/post ivacaftor initiation, and after ex vivo exposure to ivacaftor of blood cells from G551D CF patients compared to F508del CF patients and healthy control subjects.

2. Methods

2.1 Study subjects

The GOAL study has been described elsewhere (ClinicalTrials.gov: NCT01521338). Briefly, CF patients age 6 and older carrying at least one copy of the G551D mutation and no recent or sustained exposure to ivacaftor were eligible. Patients must have been clinically stable upon enrollment, with no symptoms of CF respiratory exacerbation or alteration in chronic therapies within 4 weeks. Studies were conducted at Visit 1 (pre-ivacaftor and within 4 weeks of study drug initiation), Visit 3 (1 month post-ivacaftor), and Visit 5 (6 months post-ivacaftor); samples from visits 2 and 4 were not collected from any patients for this report. Patients underwent study procedures mandated in the GOAL protocol including spirometry, sweat chloride analysis, patient reported outcomes, and collection of blood, urine, and expectorated sputum (if available) for biomarker analysis. In addition, patients participating in this report underwent an additional blood draw at visits 1, 3, and 5 for flow cytometric analysis. Demographics for the five CF patients included in this ancillary study and healthy controls are reported in Table 1. All study subjects provided written informed consent. The Institutional Review Board of the University of Alabama at Birmingham approved all studies reported here (F111202001 and F070813009).

For experiments involving the ex vivo treatment of blood with ivacaftor, peripheral blood samples were collected from CF patients not enrolled in the GOAL study and from healthy, adult volunteers. The median age of the G551D group was 6.5 years (range of 2 – 18) with 75% Caucasian and 75% male distribution. This group had a median FEV1 (percent predicted) of 95% (range of 89–95%) and a median FVC (percent predicted) of 93% (range of 89–95%). One subject in this group did not undergo spirometry testing. Among the F508del subjects, the median age was 8 years (range of 5 – 15), with 100% Caucasian and 66% male distribution. The F508del group had a median FEV1 of 89% (range of 85–94%) and a median FVC of 110% (range of 81–112%). All controls were Caucasian males with a median age of 29 (range of 23 to 39).
2.2 Peripheral blood collection, processing, and stimulation

Peripheral blood was collected by venipuncture into K2EDTA vacutainers (BD Biosciences) and maintained on ice until preparation for stimulation and analysis. Processing was begun within 45 minutes of blood being drawn. Samples were centrifuged at 400xg for 10 minutes at 4°C with minimal breaking to separate the cells from platelet-rich plasma. After removal of the platelet-rich plasma, blood cells were resuspended in a volume of PBS containing 2.5 mM EDTA equal to that of the removed plasma. This plasma removal step was necessary to prevent artifacts due to effects of downstream ex vivo stimulation of free platelets. For ex vivo stimulation studies, platelet-depleted blood was treated with 1.2 µg/mL phorbol-12-myristate-13-acetate (PMA, Fisher) for 15 minutes at 37°C. For ex vivo Ivacaftor treatment studies, platelet-depleted whole blood was incubated with 100 mM ivacaftor for 2 hours at 37°C.

2.3 Flow cytometric analysis of blood leukocytes

Whole blood staining, acquisition of flow cytometric data, and analysis of this data were performed as previously described (15). The gating strategy used for data acquisition is depicted in Supplemental Figure 1. Single live cells were selected using sequential gating with FSc-A vs. FSc-H, and negative Live/Dead Yellow staining (Life Technologies). Additionally, leukocyte-platelet aggregates were excluded using an anti-CD41 antibody (clone HIP8, BioLegend) labeled with Pacific Orange (labeling kit from Life Technologies). Monocytes were gated based on their distinctive FSc and SS profile, and CD63 expression. PMNs were gated based on their distinctive FSc and SS profile, and CD16 expression. Other antibodies used during analysis include antibodies against surface CD16 (clone 3G8), CD63 (clone H5C6), activated CD11b (clone CRBM1/5), and CXCR2 (clone 5E8), all from BioLegend. Intracellular caspase-1 was detected using a cell-permeable fluorescent probe, FLICA Green, which binds to the active enzyme (ImmunoChemistry Technologies). After staining, all samples were fixed using Phosflow Lyse/Fix Buffer (BD Biosciences). A LSRII flow cytometer (BD Biosciences) was used for data acquisition, and this data was analyzed using Flowjo (Treestar). To ensure that data was acquired in standardized ways between experiments, the cytometer was calibrated using 6-peak Rainbow Calibration Particles (Spherotech) each time before analyzing patient samples, with fixed target median fluorescence intensities (MFI) in each measurement channel. Autocompensation was performed in Flowjo using data from AbC anti-mouse capture beads (Life Technologies) and ArC reactive beads (Life Technologies) stained with single colors and Live/Dead yellow, respectively. Values from flow cytometric analyses are reported as MFI.

2.4 Data analysis

Statistical analysis was performed using JMP10 and SPSS (SAS Institute). Non-parametric statistics were used because of the small number of subjects in our study. Within-group, paired analyses comparing pre- to post-treatment responses (for both in vivo and ex vivo assays) were by the Wilcoxon signed rank test (two-tailed). Comparisons of responses between G551D CF, F508del CF and healthy control groups were by the Kruskal-Wallis test (two-tailed). P-values ≤0.05 were considered statistically significant.
3. Results

3.1 GOAL patient demographics

This study enrolled 5 CF patients with at least one G551D allele, each of whom participated in the GOAL study. In addition, we enrolled 5 healthy controls for comparison with the GOAL study patients. As shown in Table 1, all five G551D patients responded to ivacaftor treatment with a significant improvement in sweat chloride. However, not all showed a significant improvement in lung function, based on forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC), mirroring the results of the larger, multi-center GOAL study.

3.2 Cell surface markers of activation are reduced on blood leukocytes in patients after \textit{in vivo} treatment with ivacaftor

To examine the phenotype of leukocytes from CF patients with the G551D mutation, blood was stained for flow cytometric analysis before ivacaftor treatment and at one and six months post drug treatment. Included in the staining cocktails were markers of priming/activation including CD63 [a marker of degranulation (16)], the activation-specific epitope of CD11b [no cross-reactivity with native CD11b molecules on non-activated PMNs and monocytes (17)], and intracellular activity of the inflammasome-associated enzyme caspase-1 (18), which leads to production of the potent pro-inflammatory cytokine interleukin-1β. The baseline levels of these markers before patients began treatment with ivacaftor were higher than levels found in age, sex, and race-matched healthy controls, and these levels decreased over the course of ivacaftor treatment. Two of these markers (activated CD11b on PMNs and CD63 on monocytes) were significantly higher at baseline in the CF patients compared to controls. However, after treatment, no markers were significantly different from healthy controls. These observations suggest that ivacaftor treatment in these patients results in a decrease, toward normalization, of the activation status of blood leukocytes \textit{in vivo}.

3.3 \textit{In vivo} ivacaftor treatment dampens blood leukocyte response to \textit{ex vivo} PMA stimulation

We next examined leukocyte activation \textit{ex vivo} after stimulation with PMA, an activator of protein kinase C (19). Both PMNs and monocytes had smaller PMA-induced changes in activated CD11b (increases upon PMA stimulation) and CXCR2 (decreases upon PMA stimulation) post-treatment with ivacaftor, as compared to pre-treatment (Figure 2). Even before ivacaftor treatment, leukocytes from GOAL patients did not respond to PMA to the same extent as those from healthy control subjects. This may be in part due to the fact that the cells were in a more activated state before PMA stimulation (see Figure 1), and, therefore, may have had less of a capacity to change upon stimulation. Although the questions brought about by these observations are beyond the scope of this study, a decrease in the inflammatory response of blood leukocytes in patients undergoing chronic inflammation might provide an advantage to overall health. These results suggest that ivacaftor treatment decreases the responsiveness of blood PMNs and monocytes to PMA stimulation based primarily on surface CD11b and CXCR2 modulation.
3.4 Ex vivo ivacaftor treatment results in changes in surface CXCR2 and CD16 expression on G551D PMNs

The change in inflammatory activation and PMA response of blood leukocytes in G551D CF patients after ivacaftor treatment in vivo may be due to a direct effect of ivacaftor on leukocytes or due to a decrease in airway inflammation and its systemic manifestations (i.e. an indirect effect of ivacaftor). To begin to address this question, we examined the effect of direct exposure of leukocytes from G551D CF patients to ivacaftor ex vivo. We found that short-term ex vivo treatment of blood with ivacaftor resulted in the emergence of a subpopulation of PMNs in G551D CF patients, but not in F508del CF patients and healthy controls (Figure 3). This subpopulation is characterized by altered surface CXCR2 and CD16 expression. Of note, surface CD63 levels on the subpopulation do not change, suggesting that these cells are not in a state of activation. This subpopulation of PMNs (CXCR2intCD16int) may be generated by a direct effect of ivacaftor on G551D CFTR in PMNs. One possible explanation is that G551D CFTR induction by ivacaftor may affect membrane recycling, which, on the one hand, is known to be regulated by CFTR (3), and, on the other hand, has been previously linked to changes in surface CXCR2 and CD16 expression in PMNs (20, 21). It is important to note that this CXCR2intCD16int PMN subset was not observed in patients' blood and that CD63, activated CD11b, and Caspase-1 levels were not significantly altered in treated cells (Figure 3, and data not shown), suggesting that ivacaftor effects on leukocytes differ between in vivo and ex vivo treatments, possibly due to discrepancies in effective drug concentration, duration of exposure, and timing of sample collection and analysis.

4. Discussion

Innate immunity and inflammation play a critical role in the progression of CF airway disease, with a notable impact on airway remodeling and loss of lung function. Our studies highlight changes in the baseline activation status of monocytes and PMNs in CF subjects with the G551D mutation over a 6 month period of treatment with the G551D-specific potentiator, ivacaftor. In addition to these significant baseline changes, we also observed that PMNs collected from patients treated with ivacaftor exhibited less (albeit not significantly) PMA-induced activation. While these results are limited due to the small cohort of ivacaftor-treated G551D CF patients enrolled in this study, they contribute to mounting evidence of changes in leukocyte activation after treatment in vivo with a CFTR potentiator.

Our ex vivo findings are particularly interesting as they demonstrate that direct treatment with ivacaftor of PMNs from CF patients with the G551D mutation (in contrast to F508del homozygous CF patients or healthy controls) leads to changes in CXCR2 and CD16 expression. We hypothesize that these observations reflect changes in membrane recycling in response to correction of CFTR function (3) but not in response to cellular activation, as surface CD63 and activated CD11b as well as intracellular caspase-1 activity did not change upon ex vivo ivacaftor treatment. As we observed cells treated with ivacaftor ex vivo two hours after treatment and cells from patients treated with ivacaftor 1 month and 6 months after treatment, we cannot rule out that the effects observed ex vivo are not also observed in vivo, but these effects may be very short-lived and, therefore, were not observed in this
study. These results highlight a potential mutation-specific biological effect of ivacaftor on leukocyte responsiveness.

A recent study demonstrated that blood neutrophils from CF patients have abnormal ion contents and activation profiles, linked to the presence of dysfunctional CFTR, since the use of CFTR modulators altered these phenotypes (22). In particular, this study showed that PMNs from CF patients exhibited impaired release of secondary and tertiary granules, and that this impairment was no longer present in PMNs from patients with the G551D mutation who were undergoing treatment with ivacaftor. These results, combined with those from our work here, suggest that CFTR potentiators may impact leukocyte function. Since CFTR is found in a variety of cell types including circulating erythrocytes (23–26), other cells known to exhibit low levels of CFTR expression may undergo alterations in cell function in patients treated with ivacaftor. A role for CFTR as a regulator of non-epithelial cell abnormalities that significantly impact disease pathogenesis has been previously hypothesized (27).

In conclusion, this study presents evidence for alterations in leukocyte activation observed in G551D CF subjects treated with the CFTR potentiator ivacaftor. In addition, we provide limited data suggesting a direct, mutation-specific modulation of PMN activation by ivacaftor. These findings suggest the potential of such leukocyte phenotypic outcomes as useful parameters to be examined in future clinical trials utilizing CFTR modulators and suggest a role for CFTR towards modulating response to host defense via leukocyte interactions.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

- **FEV1** forced expiratory volume in 1 second
- **FVC** forced vital capacity
- **PMN** polymorphonuclear neutrophil
- **PMA** phorbol-12-myristate-13-acetate
- **MFI** median fluorescence intensity

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the cystic fibrosis transmembrane conductance regulator gene in cells of non-epithelial origin.

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expression and function are detectable in blood monocytes: development of a new blood test for

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Highlights

- Neutrophils and monocytes from G551D-bearing CF patients have increased levels of markers of activation, and these levels decrease over the course of ivacaftor treatment
- Treatment of G551D-bearing CF patients with ivacaftor resulted in neutrophils and monocytes with a diminished response to PMA
- Ex vivo exposure to ivacaftor of blood cells from G551D-bearing CF patients resulted in changes in CXCR2 and CD16 expression on PMNs
Figure 1. Surface marker expression and intracellular caspase-1 activity in peripheral blood leukocytes

Flow cytometry was used to analyze the activity of caspase-1 and surface expression of CD63 and activated CD11b on PMNs and monocytes. Cells from patients were analyzed at enrollment in the study (V1), one month post enrollment (V3), and six months post enrollment (V5). Additionally, cells from healthy controls (HC) were also analyzed. For these plots, samples from the same patient are connected by a dotted line, and a solid horizontal line was drawn at the mean for each group. An asterisk (*) denotes a significant difference compared to the healthy control group by Kruskal-Wallis with Dunns Multiple Comparisons test.
Figure 2. Changes in surface marker expression on peripheral blood leukocytes stimulated with PMA

Flow cytometry was used to analyze the surface expression of activated CD11b and CXCR2 in PMA-stimulated PMNs and monocytes. For these plots, the line represents the mean, and a dotted line is used to connect values from the same patient. The mean change in activated CD11b on PMNs for the healthy control (HC) group is 21.5 fold.
Figure 3. Effects of *ex vivo* ivacaftor treatment on peripheral blood PMNs
A: Histograms of surface CD16 (top row), CXCR2 (middle row), and CD63 (bottom row) expression on PMNs treated with either Ivacaftor (black line) or vehicle (DMSO, shaded gray) from representative G551D, F508del and healthy control subjects. B: Box plots illustrating the changes in CD16 and CXCR2 expression in PMNs by Ivacaftor treatment in the three groups. C: Box plots illustrating the difference in CD16$^{\text{int}}$ and CXCR2$^{\text{int}}$ subsets induced among blood PMNs by Ivacaftor treatment. CD16$^{\text{int}}$ and CXCR2$^{\text{int}}$ subsets were defined by gates set below the lower 99% percentile in matched DMSO-treated PMNs. The G551D group showed a significant difference compared to other groups, as tested using the Kruskal-Wallis method (for CD16, p = 0.0376 and for CXCR2, p = 0.0302). The whiskers in all plots represent the minimum and maximum values.
Table 1

Subject Demographics

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<th>GOAL Study Subjects</th>
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<td>Sex</td>
<td>% Male</td>
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<tr>
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<tr>
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<tr>
<td>Genotype</td>
<td>Genotype (%)</td>
<td>G551D/F508del (80), G551D/N1303K (20)</td>
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<td>∆FEV1 (% change)</td>
<td>Mean (SD); Median (Range)</td>
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<tr>
<td>∆FVC (% change)</td>
<td>Mean (SD); Median (Range)</td>
<td>2.58 (7.81); 0.2 (−7.4 – 13.7)</td>
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<tr>
<td>∆Sweat Chloride</td>
<td>Mean (SD); Median (Range)</td>
<td>−51.9 (17.37); −57 (−75 – −31.5)</td>
<td></td>
</tr>
</tbody>
</table>

Healthy Controls

| Sex                 | % Male   | 40       |          |
| Age                 | Mean (SD); Median (Range) | 25.6 (4.98); 24 (22 – 34) |          |
| Race                | % Caucasian | 100     |          |