Cytokine refacing effect reduces granulocyte macrophage colony-stimulating factor susceptibility to antibody neutralization

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Abstract

Crohn’s Disease (CD) afflicts over half a million Americans with an annual economic impact exceeding $10 billion. Granulocyte macrophage colony-stimulating factor (GM-CSF) can increase patient immune responses against intestinal microbes that promote CD and has been effective for some patients in clinical trials. We have made important progress toward developing GM-CSF variants that could be more effective CD therapeutics by virtue of being less prone to neutralization by the endogenous GM-CSF autoantibodies that are highly expressed in CD patients. Yeast display engineering revealed mutations that increase GM-CSF variant binding affinity by up to ~3-fold toward both GM-CSF receptor alpha and beta subunits in surface plasmon resonance experiments. Increased binding affinity did not reduce GM-CSF half-maximum effective concentration (EC50) values in conventional in vitro human leukocyte proliferation assays. Affinity-enhancing mutations did, however, promote a ‘refacing effect’ that imparted all five evaluated GM-CSF variants with increased in vitro bioactivity in the presence of GM-CSF-neutralizing polyclonal antisera. The most improved variant, H15L/R23L, was 6-fold more active than wild-type GM-CSF. Incorporation of additional known affinity-increasing mutations could augment the refacing effect and concomitant bioactivity improvements described here.

Key words: Alzheimer’s disease, Crohn’s disease, GM-CSF, immunogenicity, irritable bowel syndrome

Introduction

Crohn’s Disease (CD) afflicts more than 500 000 Americans (Floyd et al., 2014) with associated annual treatment costs in excess of $10 billion (Kappelman et al., 2011). Neither drug regimens nor surgery go beyond treating symptoms; CD patients must manage their condition for life (Yamamoto, 2014). Impaired innate and adaptive immune responses to pathogenic microbes within the intestinal mucosa are key contributors to the inflammation that underlies CD, with impaired presentation of microbial antigens by dendritic cells (DCs) being the principal cause of reduced immune activity (Gathungu et al., 2013).

Granulocyte macrophage colony-stimulating factor (GM-CSF) is known to expand DC populations in vivo and the cytokine’s activity is central to maintaining a normal immune response to microbial pathogens in the intestinal mucosa (Sainathan et al., 2008). GM-CSF is unique from most, if not all, cytokines in that it persists at relatively high, i.e. ~150 pM, concentrations in the serum of normal persons but is more than 99% bound by endogenous neutralizing autoantibodies (Uchida et al., 2009). These autoantibodies, which are in serum at concentrations ranging from 3 to 10 nM in normal persons (Han et al., 2009; Uchida et al., 2009; Gathungu et al., 2013), regulate GM-CSF activity, where the half-maximum effective concentration (EC50) for in vitro leukocyte activation by commercial recombinant GM-CSF is in the 5–20 pM range (R&D Systems).

In CD, serum titers of GM-CSF-neutralizing autoantibodies are increased by between 5- and 30-fold, an increase that attenuates GM-CSF activity by reducing the serum concentration of free GM-CSF. Decreased
free GM-CSF concentrations lead to impaired presentation of microbial antigens by DCs in the intestinal mucosa of CD patients relative to normal persons (Han et al., 2009; Gathungu et al., 2013). This phenomenon has motivated the pursuit of intravenous injection of recombinant GM-CSF, commercially known as Sanofi’s Leukine, as a strategy for treating CD. Although GM-CSF injections have improved patient conditions in some cases, results have not been sufficiently strong to motivate broad deployment of GM-CSF as a CD therapeutic (Korzenik et al., 2005; Roth et al., 2011).

There is considerable quantitative support for the hypothesis that the limited efficacy of injected GM-CSF in treating CD is the result of the elevated concentrations of GM-CSF-neutralizing antibodies in patient serum. As noted above, recombinant GM-CSF’s EC50 value for in vitro leukocyte stimulation is 5–20 pM. Several members of a panel of recombinant monoclonal human anti-GM-CSF autoantibodies, characterized in vitro, had half-maximum GM-CSF inhibitory concentrations (IC50s) in the 5–100 pM range (Wang et al., 2013). Given these low IC50 values, the high neutralizing autoantibody concentrations, i.e. 15–100 nM, in CD patient serum (Han et al., 2009; Uchida et al., 2009; Gathungu et al., 2013), and the fact that antibodies can neutralize GM-CSF via binding at multiple epitopes (Wang et al., 2013), it is likely that an appreciable fraction of injected GM-CSF molecules is bound by neutralizing autoantibodies and prevented from interacting with GM-CSF receptors on DC surfaces in vivo.

Given the above considerations, we engineered GM-CSF variants with increased binding affinity toward both of the cytokine’s two receptor subunits, i.e. CD116 and CD131. We posited that increased receptor binding affinity, which reduces EC50 values for in vitro leukocyte proliferation in the context of GM-CSF/human transferrin (Trf) fusion proteins (Heinzelman and Priebe, 2015), could have the same effect in decreasing EC50 values for standalone GM-CSF. Such decreased EC50s would allow these cytokines to more effectively stimulate DCs when in the presence of high concentrations of neutralizing autoantibodies. Further motivating pursuit of high affinity GM-CSF variants as CD therapeutics is the possibility of increased bioactivity due to a ‘refacing effect.’ This refacing effect would arise from GM-CSF mutations that increase receptor binding affinity also altering the epitopes recognized by autoantibodies and thus elevating IC50 values for autoantibody-mediated neutralization. The plausibility of such an effect is supported by SPR experiments in which monoclonal GM-CSF-neutralizing antibodies had reduced binding affinities toward SPR sensor chip-immobilized recombinant GM-CSF point mutants (Wang et al., 2013). In this work, we have shown that it is possible to leverage a refacing effect to increase GM-CSF bioactivity in the presence of GM-CSF-neutralizing antibodies.

Materials and methods

Expression and purification of recombinant GM-CSF

Codon optimized wild-type and variant GM-CSF genes were expressed in Escherichia coli using a proprietary expression vector and purified as previously described (Doherty et al., 2005). Duplicate 400 ml expression cultures were prepared for both wild-type and variant GM-CSFs. Purified proteins were stored in 20 mM Bis-Tris, ~2 M sodium acetate, pH 7.2 and sterile filtered using a 0.2 µm polyethersulfone syringe filter (Pall Life Sciences). Protein concentrations were determined by measuring absorbance at 280 nm and using an extinction coefficient of 0.981.

Surface plasmon resonance measurement of GM-CSF binding to CD116 and CD131

Recombinant CD116 and CD131 (Life Technologies) were immobilized on sensor chips using amine conjugation chemistry. Kinetic binding affinity assays were performed using a SensiQ Pioneer SPR instrument (SensiQ Technologies). The sensor chip was exposed to GM-CSF using a SensiQ One Step gradient mode (40 µl/min flow rate) injection with a final GM-CSF concentration of 100 nM for CD116 binding experiments and 1 µM for CD131 binding experiments. All injections were performed in duplicate. Prior to injection, GM-CSF samples were exchanged into phosphate buffered saline (PBS), pH 7.4 to avoid buffer effects. Binding assays were conducted in PBS, pH 7.4 with 0.005% Tween-20. Binding parameters were determined using the SensiQ Qdat data analysis software package.

GM-CSF in vitro bioactivity assays

TF-1 human leukocytes (ATCC product CRL-2003) were cultured in RPMI1640 media, ATCC formulation, supplemented with 10% fetal calf serum at 37°C under 5% CO2. Wild-type or variant GM-CSF was added to cells in 96-well plates at a density of 5000 cells/well in 100 µl of media. After 3 days of incubation, 20 µl of MTS Cell Titer AqueousOne viability indicator (Promega) was added to each well and absorbance values at 492 nm read after 4 h of color development in a 37°C, 5% CO2 incubator. Antibody neutralization assays were carried out as above with the following modifications: goat anti-human GM-CSF polyclonal antibody (R&D Systems product AF-213-NA) was added to all wells at a concentration of 5 nM and 96-well plates were incubated for 4 days prior to MTS metabolic indicator dye addition.

Size exclusion chromatography analysis of GM-CSF

Fifteen micrograms of wild-type and mutant GM-CSFs were run through a Superdex 200 Increase 10/300 GL size exclusion column (GE Healthcare) using an AKTA Pure FPLC system (GE Healthcare). The column was calibrated with a Biorad Gel Filtration standard (Cat# 151-1902) to verify GM-CSF molecular weights. Elution was carried out in MES buffer with 150 mM NaCl, pH 6.0, in isocratic mode at a flow rate of 0.5 ml/min.

Results

Choosing candidate high affinity GM-CSF mutants for expression as standalone cytokines

Yeast surface display has been used to identify GM-CSF positions P12, H15 and R23 as being among a larger set of residues that are key determinants of GM-CSF receptor subunit binding affinity (Heinzelman and Priebe, 2015). SPR studies showed that mutants H15/V/R23L, H15L/R23V, P12D/H15L/R23L and P12V/H15F/R23L all possess increased binding affinity toward both immobilized CD116 and CD131 when these variant GM-CSFs are expressed as fusions to the N-terminus of Trf. All four GM-CSF/Trf mutants have lower than wild-type EC50 values in cultured leukocyte proliferation assays. In the studies reported here, the two most bioactive GM-CSF variants, i.e. H15L/R23V and P12D/H15L/R23L, have been expressed as standalone cytokines. Motivated by our yeast display GM-CSF site saturation mutant library screens having suggested that H15L and R23L mutations increase GM-CSF binding affinity toward both immobilized CD116 and CD131, we have also expressed H135L, R23L and H15L/R23L GM-CSF mutants as standalone cytokines.

Expression and purification of recombinant GM-CSF

Wild-type and variant GM-CSFs were expressed in E. coli and refolded from inclusion bodies as described (Doherty et al., 2005). SDS-PAGE
Table I. Yields of purified wild-type and variant GM-CSFs for single culturing and purification trials

<table>
<thead>
<tr>
<th>Clone</th>
<th>Yield (mg protein/l culture)</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>26</td>
</tr>
<tr>
<td>H15L</td>
<td>11</td>
</tr>
<tr>
<td>R23L</td>
<td>15</td>
</tr>
<tr>
<td>H15L/R23L</td>
<td>11</td>
</tr>
<tr>
<td>H15L/R23V</td>
<td>15</td>
</tr>
<tr>
<td>P12D/H15L/R23L</td>
<td>5</td>
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</table>

Clone Yield (mg protein/l culture)

Protein concentrations determined by measuring absorbance at 280 nm.

and size exclusion chromatography analysis (Supplementary Figs S1 and S2) of purified proteins shows that wild-type and variant GM-CSFs exist predominantly as a conformationally homogeneous monomeric isoform with a molecular weight near the anticipated 14.5 kDa. Post-purification yields for all but one of the GM-CSF variants exceed 10 mg/l of culture (Table I).

Surface plasmon resonance determination of CD116 and CD131 binding parameters

Four of the five GM-CSF variants possess the desired increase in CD116 binding affinity. Equilibrium binding dissociation constant ($K_D$) decreases for these four variants are between 2- and 3-fold (Table II). The H15L/R23V variant’s $K_D$ value is increased by ~35% relative to wild type. In all cases, differences in binding affinity among wild-type and variant GM-CSFs are primarily the result of variation in binding dissociation rate constant ($k_{off}$) values. Our observed $K_D$ of ~8.4 nM for soluble wild-type GM-CSF binding to immobilized CD116 is within an order of magnitude of the reported value of 100 ± 13 nM (Ishino et al., 2008). The similarity of these $K_D$ values suggests that our CD116 SPR assay is free of binding artifacts and/or confounding steric phenomena.

We are not aware of prior SPR measurements of GM-CSF CD131 binding to which our CD116 binding SPR assay results can be compared. There are, however, reported $K_D$ values for GM-CSF’s respective interactions with CD116 and CD131 on the surface of cultured TF-1 leukocytes (Hoang et al., 1993). These assays report that GM-CSF’s binding affinity toward CD116 is ~1000-fold greater than toward CD131. This result is commensurate with the differences in CD116 and CD131 $K_D$ values measured in our SPR experiments (Table II) and implies that our CD131 SPR assay is free of binding artifacts and/or confounding steric phenomena.

Both GM-CSF single mutants have CD131 $K_D$ values that are comparable with wild type (Table II). Conversely, all three combinatorial variants have markedly increased binding affinity, with up to 3-fold decreases in $K_D$ values being driven by reduced $k_{off}$ values (Table II). The $k_{off}$ values for wild-type and variant GM-CSFs binding to CD131 are ~10 000 times greater than those measured for binding to CD116 (Fig. 1, Supplementary Figs S3 and Table II), resulting in CD131 $K_D$ values that are increased by several hundred-fold relative to CD116 $K_D$s for these cytokines.

There are intramolecular interactions among residues comprising GM-CSF helices A, C and D (Hansen et al., 2008; Supplementary Fig. S4). Furthermore, both CD116 and CD131 contain amino acids with side chains that are proximal to GM-CSF helix A while CD131 possesses residues proximal to GM-CSF helix C and CD116 features residues local to GM-CSF helix D. Our observation that the H15L/R23L and P12D/H15L/R23L substitution combinations impact both CD116 and CD131 binding affinity (Table II) is highly plausible given the above described network of amino acid interactions among GM-CSF, CD116 and CD131.

Conventional in vitro TF-1 leukocyte proliferation assay EC50 measurement

The in vitro TF-1 leukocyte proliferation assay EC50 values for four of the five variant GM-CSFs are mildly increased relative to the wild-type cytokine EC50 (Table II). The EC50 value for the fifth variant, H15L/R23L, is equivalent to the wild-type value.

The absence of correlation between GM-CSF receptor subunit binding affinity and bioactivity could be explained by the fact that reductions in variant GM-CSF $K_D$ values are the result of decreased $k_{off}$s rather than increased $k_{on}$s (Table II). GM-CSF endocytosis rates do not appear to have been specifically measured. Given, however, that both granulocyte colony-stimulating factor (G-CSF) and GM-CSF form multimeric complexes with their respective receptors on the cell surface one can take G-CSF endocytosis rates as being representative of those for GM-CSF.

G-CSF is typically endocytosed within 30 min of binding to its cognate receptor on the cell surface (Kuwabara et al., 1996; Sarkar et al., 2002). Our $k_{off}$ value, measured by SPR (Table II), for GM-CSF binding to CD116 indicates that the half-time for GM-CSF dissociation from cell surface CD116 exceeds 1 h. A comparison of these respective endocytosis and receptor dissociation timescales suggests that dissociation from cell surface receptors does not limit GM-CSF bioactivity and supports the hypothesis that decreased $K_D$ values do not lead to reduced TF-1 leukocyte proliferation assay EC50 values because none of the GM-CSF variants possess increased $k_{on}$s.

GM-CSF neutralization assays

Addition of commercial goat polyclonal GM-CSF-neutralizing antibodies to TF-1 cell proliferation assay media at a concentration of 5 nM substantially reduces or abolishes GM-CSF-mediated proliferation of cells incubated with GM-CSF at concentrations at or below ~670 pM (Fig. 2). These results are commensurate with the reported finding that adding 1–10 nM of GM-CSF-neutralizing antibodies...
isolated from human intravenous immunoglobulin preparations to leukocyte culture media containing ∼700 pM GM-CSF markedly reduces GM-CSF bioactivity (Uchida et al., 2009). The addition of 5 nM neutralizing antibody to assay media increases EC50 values for both wild-type and variant GM-CSFs (Fig. 2). The extremely rapid, e.g. almost step function, decrease in TF-1 cell proliferation with decreasing GM-CSF concentration in the 500–1000 pM range prevents accurate calculation of EC50 values.

The difficulty in determining EC50 in antibody neutralization assays motivates the use of cell viability at GM-CSF concentrations supporting cell proliferation as a metric for GM-CSF susceptibility to antibody neutralization. Absorbance values for cells incubated with ∼670 pM GM-CSF are particularly interesting; assayed GM-CSF concentrations, i.e. ∼1000 and ∼1500 pM, above this level cause cells to proliferate at the upper assay detection limit whereas concentrations at or below ∼450 pM do not support cell proliferation.

All five GM-CSF variants support higher viable cell counts than the wild-type cytokine when added to TF-1 cell culture media at ∼670 pM in the presence of neutralizing antibody (Fig. 3). Incubation with the most active variant, H15L/R23L, increases cell proliferation ∼6-fold relative to wild-type GM-CSF (Fig. 3). As shown in Table III, all of the GM-CSF variants have bioactivity that is equivalent to or lower than wild-type GM-CSF in conventional cell proliferation assays. As such, the neutralization assay bioactivity increases for these variants relative to wild type suggest that the mutations carried by these high affinity clones reduce their susceptibility to neutralization by anti-GM-CSF antibodies (Fig. 4).

Discussion

Mutations that increase GM-CSF binding to CD116 and CD131 in SPR experiments give rise to a ‘refacing effect’ that imparts GM-CSF mutants with increased ability to activate intracellular signaling in the presence of neutralizing antibodies. Given GM-CSF autoantibodies’ role CD pathology, this refacing effect could prove enabling in engineering GM-CSF variants as CD therapeutics.

The mutants characterized here illustrate the potential to develop a second generation of ‘completely refaced’ GM-CSF variants with even further reduced antibody neutralization susceptibilities. With respect
to a strategy for complete refacing, we note that different neutralizing GM-CSF autoantibodies bind to distinct GM-CSF epitopes and that only a fraction of the anti-GM-CSF antibodies in human serum reduce GM-CSF bioactivity; many anti-GM-CSF antibodies bind to GM-CSF epitopes that are not involved in receptor binding (Wang et al., 2013).

The epitopes recognized by GM-CSF-neutralizing antibodies contain residues lying within GM-CSF helices A, C and D (Wang et al., 2013). These helices comprise the same GM-CSF surfaces that the GM-CSF/CD116/CD131 co-crystal structure shows are in contact with CD116 and CD131. These structural observations suggest that completely refaced GM-CSF quadruple mutants could be created by making one helix C residue substitution and one helix D residue substitution in the background of the H15L/R23L double mutant. Recombinant mutant protein expression yield generally decreases as the number of amino acid substitutions within a given mutant increases. As such, decreased antibody neutralization susceptibility for H15L/R23L mutant progeny that carries respective single helix C and D mutations is the ideal scenario for achieving complete GM-CSF refacing.

GM-CSF residue positions within helices C and D that can be mutated to achieve increases in both CD116 and CD131 binding affinity (Heinzelman and Priebe, 2015) have been identified (Fig. 4). Our observation that affinity-increasing helix A mutations have a small or no effect upon bioactivity in the absence of neutralizing antibodies suggests that introducing mutations at the above noted helix C and D positions will not unduly reduce GM-CSF’s ability to activate intracellular signaling cascades. Although lower than for wild-type GM-CSF, mutant GM-CSF yields (Table I) are substantial and comparable with yields reported for other cases of purifying human cytokines that have been expressed in E.coli (Bishop et al., 2001; Thomson et al., 2012).

![Fig. 4 Structure of GM-CSF denoting residues that can be mutated to increase receptor subunit binding affinity. Helix A residues P12, H15 and R23 appear in red. Helix C residues K72 and K74 appear in cyan. Helix D residues D112, L115 and P118 appear in magenta. Figure created based on PDB entry 2GMF using Pymol.](image)

![Table III. EC50 values for wild-type and variant GM-CSFs](image)

<table>
<thead>
<tr>
<th>Clone</th>
<th>EC50 (pM)</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>130 ± 20</td>
</tr>
<tr>
<td>H15L</td>
<td>180 ± 10</td>
</tr>
<tr>
<td>R23L</td>
<td>170 ± 10</td>
</tr>
<tr>
<td>H15L/R23L</td>
<td>120 ± 20</td>
</tr>
<tr>
<td>H15L/R23V</td>
<td>170 ± 10</td>
</tr>
<tr>
<td>P12D/H15L/R23L</td>
<td>190 ± 10</td>
</tr>
</tbody>
</table>

Values are averages of four or more trials with error bars denoting standard deviations.
background of H15L/R23L will reduce yields for the resulting quad-
rule mutants to levels that prevent production of adequate protein for
animal or pilot clinical studies.

The data presented in this work and the above discussion show
that leveraging the refacing effect to make GM-CSF an effective CD
therapeutic is a tractable objective. We are eager to build upon these
results in seeking to realize this goal.

**Supplementary data**

Supplementary data are available at PEDS online.

**Funding**

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**Conflict of Interest statement.** P.H. is pursuing patent applications per-
taining to GM-CSF positions that were shown in this work to enable
increases in receptor subunit binding affinity and reduce susceptibility
to antibody neutralization.

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