Plasma protein profiling reflects Th1-driven immune dysregulation in common variable immunodeficiency

Jonas Hultberg, MD,a,b Jan Ernerudh, MD, PhD,b Marie Larsson, PhD,a Åsa Nilsdotter-Augustinsson, MD, PhD,c,* and Sofia Nyström, MD, PhD,a,b* Linköping, Sweden

GRAPHICAL ABSTRACT

Plasma protein profiling reflects Th1-driven immune dysregulation in common variable immunodeficiency

CVID (n=29)

HC (n=36)

Immune dysregulation

Data processing

PEA (145 plasma proteins)

CVID-B n=17

CD21low B-cells >20% (6 of 17)

Autoimmunity (5 of 17)

ILD (2 of 17)

CVID-A n=11

CD21low B-cells >20% (8 of 11)

Autoimmunity (7 of 11)

ILD (5 of 11)

HC, healthy controls; CVID, common variable immunodeficiency; PEA, proximity extension assay; ILD, interstitial lung disease

Background: Common variable immunodeficiency (CVID) is a disorder characterized by antibody deficiency. A significant fraction of the patients suffer from immune dysregulation, which leads to increased morbidity and mortality. The pathogenesis of this condition is poorly understood.

Objective: Our aim was to find out whether the plasma protein signature in CVID is associated with clinical characteristics and lymphocyte aberrations.

Methods: A highly sensitive proximity extension assay was used for targeted profiling of 145 plasma proteins in 29 patients with CVID. Phenotyping of peripheral lymphocytes was done by flow cytometry. The findings were correlated with the burden of immune dysregulation.

Results: Unsupervised clustering of plasma protein profiles identified 2 distinct groups of patients with CVID that differed significantly in terms of the degree of complications due to immune dysregulation and in terms of the frequency of activated B- and T-cell subpopulations. Pathway analysis identified IFN-γ and IL-1β as the top enriched upstream regulators associated with higher grade of immune dysregulation.

CVID. Phenotyping of peripheral lymphocytes was done by flow cytometry. The findings were correlated with the burden of immune dysregulation.

Results: Unsupervised clustering of plasma protein profiles identified 2 distinct groups of patients with CVID that differed significantly in terms of the degree of complications due to immune dysregulation and in terms of the frequency of activated B- and T-cell subpopulations. Pathway analysis identified IFN-γ and IL-1β as the top enriched upstream regulators associated with higher grade of immune dysregulation.

From the Division of Molecular Virology, the Department of Clinical Immunology and Transfusions Medicine, and the Department of Infectious Diseases, and Department of Biomedicine and Clinical Sciences, Linköping University, Linköping.

*These authors are senior authors and contributed equally to this work.

Supported by ALF-grants, Region Östergötland, Sweden.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication July 25, 2019; revised January 14, 2020; accepted for publication January 17, 2020.


Corresponding author: Sofia Nyström, MD, PhD, Clinical Immunology and Transfusion Medicine and Department of Biomedicine and Clinical Sciences, University Hospital, S-58185 Linköping, Sweden. E-mail: sofia.c.nyström@liu.se.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections 0091-6749 © 2020 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

https://doi.org/10.1016/j.jaci.2020.01.046

417
dysregulation. In addition, CVID was found to be associated with increased plasma levels of the B-cell–attracting chemokine CXCL13. Conclusion: Clustering based on plasma protein profiles delineated a subgroup of patients with CVID with activated T cells and clinical complications due to immune dysregulation. Thus, data indicate that CVID-associated immune dysregulation is a Th1-mediated inflammatory process driven by the IFN-γ pathway. (J Allergy Clin Immunol 2020;146:417-28.)

Key words: CVID, immunodeficiency, lymphoproliferation, immune dysregulation, proximity extension immunoassay, plasma proteomics

Common variable immunodeficiency (CVID) is a heterogeneous disorder characterized by hypogammaglobulinemia and poor vaccine responses.1-3 A number of genetic autosomal variants that predispose for B-cell failure have been identified in familial CVID.4 However, the vast majority of CVID cases are sporadic and match a polygenic model.5,6 Immunoglobulin replacement therapy reduces the number of infections in CVID, but a significant fraction of the patients with CVID develop noninfectious complications that are the main drivers of morbidity and mortality.7 Noninfectious complications involve immune dysregulation, including organ-specific autoimmunity, polyclonal lymphoid infiltration of nonlymphoid tissue, and lymphoid hyperplasia.8 Patients with low numbers of class-switched memory B cells and abnormalities in T cells, including low frequencies of naive CD4 T cells and an immunosenescent T-cell phenotype, are at higher risk of complications related to immune dysregulation.11-15 Another potential predictor of immune dysregulation in CVID is increased frequency of B cells with low expression of CD21 (CD21-low), a distinct population of activated B cells with reduced effector functions.14,15 The population closely resembles CD11c+ B cells, an atypical B-cell population that is maintained under conditions of chronic antigen stimulation characterized by aberrant type 1 and type 2 interferon responses, such as autoimmunity and persistent viral infections.16

Recent evidence suggests that interferon activation is involved in CVID-related immune dysregulation, as shown by whole blood transcriptional profiling and increased serum levels of IFN-γ.17,18 It has also been reported that immune dysregulation and the expansion of CD21-low B cells are associated with a Th1-biased CD4 T-cell compartment in CVID.15 Deeper insight into the molecular mechanisms and putative role of interferons in CVID-associated immune dysregulation can pave the way for new treatment strategies to mitigate CVID-related immune dysregulation.

Proteomics is a promising technique to provide new diagnostic and prognostic information as well as insights into new disease mechanisms in cancer, inflammatory disease, and cardiovascular disease.20-23 Prior work in CVID has used conventional immunoassays for the measurement of restricted numbers of biomarkers in plasma. The proteomic approach can be useful in the identification of pathways involved in CVID with and without immune dysregulation; it can also provide information about the underlying molecular pathogenesis and provide markers for disease progression.

To characterize potential systemic inflammatory risk signatures in CVID we assessed a large number of established and exploratory immune-related biomarkers by using targeted proteomics. Unbiased cluster analysis of 145 immune-related proteins in plasma, assessed by highly sensitive multiplex proximity extension assay (PEA) technology,24,25 identified a distinct group of patients with CVID with immune dysregulation, low frequencies of naive CD4 T cells, and increased frequencies of CD21-low B cells. Pathway analysis of plasma proteins showed that immune dysregulation was primarily associated with IFN-γ and IL-1β. Plasma protein profiling can provide mechanistic information on the pathophysiology underlying immune dysregulation in CVID and can also provide sensitive biomarkers and guidance on tailored treatment strategies.

METHODS

Patients

The study protocol was approved by the regional ethics committee in Linköping (2017/214-32). All participants gave and signed consent before participation in this study. Before enrollment all patients were reevaluated for fulfilling the diagnostic criteria of CVID. In all, 29 patients with CVID (19 males and 10 females aged 18-77 years [median age 53 years]) were enrolled in the study. Clinical data were collected from their medical records and covered the time of the study start to 10 years back. An evaluation document, based on division into distinct clinical phenotypes, was used to summarize the medical records of each patient. In brief, patients were assessed for the presence of 3 noninfectious complications: autoimmunity, gastroenteropathy, and lymphoproliferation (see Table E1 in this article’s Online Repository at www.jacionline.org). Each item of complications was scored 0 to 4; hence, the sum formed a disease severity score between 0 and 12. Patients were also given an infection score between 0 and 4 based on the number of suspected infections per year after initiation of IgG substitution therapy, as documented in the medical records of the patients. Information about B-cell subsets was retrieved from the clinical records of the patients. CVID was classified according to the Freiburg criteria based on the proportions of B-cell subsets of peripheral blood lymphocytes.26 Blood donors (27 males and 9 females) aged 18 to 67 years (median age 50 years) served as healthy controls. Blood samples were obtained in EDTA tubes from patients and healthy controls. One blood sample was used for plasma isolation and another was used for immediate lymphocyte immunophenotyping. All samples were collected in a standardized manner by experienced staff using the same technique (Vacutainer [BD Biosciences, Franklin Lakes, NJ]) and tubes. Samples were kept at room temperature up to 5 hours before plasma was separated.

Plasma protein profiling by proximity extension assay

Plasma samples were collected, centrifuged, and stored at −80°C until further processing. Aliquots were analyzed with the Proseek multiplex assay (Olink Proteomics, Uppsala, Sweden), a PEA technology.24,25 Two panels
plasma protein profiling was carried out by using a 2-tailed t test in Microsoft Excel. The P value for each protein was adjusted for multiple comparisons by using the corrected P value ($P_{corr}$) obtained by the Benjamini-Hochberg procedure. Ingenuity Pathway Analysis (IPA) (Qia-gen, Hilden Germany) of canonic pathway enrichment and upstream regulators was performed by using the differences in mean plasma protein levels that were considered statistically different between groups; that is, mean protein levels that differed by at least a log2 value of 0.3 but no more than a negative log2 value of –0.3 between groups in combination with a $P_{corr}$ value less than .01 between patients with CVID and healthy controls (Fig 2, A and B). The mean plasma levels of these proteins were 2.8 to 4 times higher ($\log_2 = 1.5–2.0$) in the CVID group than in the controls. Chemokine C-X-C motif ligand 13 (CXCL13), also known as B-cell lymphocyte chemoattractant factor, was the soluble factor with the highest levels in patients with CVID compared with in the controls (Fig 1, A). In addition, CXCL9 and CXCL10 were upregulated in CVID compared with in the controls (Fig 1, A). The top upregulated membrane-bound proteins in patients with CVID compared with in the controls included 2 members of the tumor necrosis factor receptor superfamily (TNFRSF), the immune checkpoint primary death protein 1 (PDCD1), and the cytotoxic and regulatory T-cell molecule (CRTAM) (Fig 1, B). Enrichment analysis by IPA revealed significant overlap of the differently expressed plasma proteins in patients with CVID with proteins associated with several canonic pathways. The 3 pathways with the highest probability to be affected in patients with CVID when compared with the controls were communication between innate and adaptive immune cells ($P < 10^{-13}$), the Treg1 pathway ($P < 10^{-11}$), and the Th1 and Th2 activation pathways ($P < 10^{-12}$) (see Fig E2 in this article’s Online Repository at www.jacionline.org). Further IPA analysis predicted IL-36B as the top upstream regulator of CVID plasma protein profiles ($P < 10^{-16}$) based on increased levels of the following proteins: IL-6, CXCL10, chemokine C-C motif ligand CCL20, IL-18, CD83, CXCL8, CXCL1, and CD40, proteins that are all regulated by IL-36B (see Table E5 in this article’s Online Repository at www.jacionline.org).

The plasma protein profiles identified a distinct group of patients with CVID

Unsupervised hierarchic clustering analysis identified 1 group of patients with CVID (11 of 29) that clustered separately from the other study subjects (Fig 2, A) (ie, CVID group A). Of the remaining patients, 17 who intermingled with the controls to some extent were classified as CVID group B. One patient was not allocated to any of the groups (Fig 2, A). Characterization of CVID group A and CVID group B showed that the frequency of atypical B cells, with low expression of CD21, was significantly higher in CVID group A (Table I). The presence of noninfectious complications—lymphadenopathy, enteropathy, splenomegaly, or lymphocytic infiltration with or without autoimmunity—was significantly higher in group A than in group B, but there was no difference in the frequency of documented infections between groups (Fig 2, B). Only 4 of 28 patients had infection only, and they all clustered in group B. The number of patients classified as CVID Freiburg class 1a, which indicates an increased risk of noninfectious complications, was significantly higher in CVID group A (Fig 2, C).
Histopathologic signs of interstitial lung disease were also more common in CVID group A (Table I). Accordingly, plasma protein pattern profiling can identify patients with CVID with a severe clinical phenotype characterized by immune dysregulation. Female-to-male ratio, age, and a positive family history of primary immunodeficiency were equally distributed between groups (Table I). There was no significant difference in the presence of gastroenteropathy between groups. However, IPA indicated enrichment of plasma proteins linked to hepatic fibrosis and hepatic stellate activation pathways (P value $10^{-11}$) in CVID group A versus in CVID group B. There was overlap between the top enriched pathways in CVID group A and those in CVID group B and between the pathways in patients with CVID and those in healthy controls (see Fig E2 in this article’s Online Repository at www.jacionline.org). Four patients in CVID group B had a high disease score. A PCA loadings plot showed that decreased levels of CXCL5 had strong influence on the separation of these patients from CVID group A patients in addition to decreased levels of CXCR3 ligands (see Fig E3 in this article’s Online Repository at www.jacionline.org).
The CVID plasma protein profiles are associated with increased T-cell activation

Perturbation of the CD4 T_{H} subset is associated with an increased risk of noninfectious complications in CVID.\textsuperscript{29} We therefore assessed the extended phenotype of peripheral T cells in patients with CVID and controls. The number of CD4 T cells was decreased in CVID group B, which was characterized by the milder phenotype, compared with in healthy controls. In both CVID groups there was a decrease in the frequencies of naive CD4 T cells compared with in controls, and the frequency was significantly lower in CVID group A than in CVID group B (Fig 3, A). This is in line with previous reports that low numbers of naive CD4 T cells are associated with noninfectious complications in CVID.\textsuperscript{12} The frequency of effector memory CD4 T cells was increased in CVID group A compared with in CVID group B and in healthy controls (Fig 3, A). In addition, the frequency of CD4 T cells expressing the activation marker human leukocyte antigen–antigen D–related (HLA-DR) was higher in CVID group A than in CVID group B (Fig 3, B). Increased levels of CD4 T cells expressing the immune checkpoint PDCD1 were found in both CVID groups, but the levels of PDCD1{\textsuperscript{+}} CD4 T cells were higher in CVID group A than in CVID group B (Fig 3, B). Plasma levels of PDCD1 correlated (r = 0.57; P = .001) with the frequency of PDCD1{\textsuperscript{+}} CD4{\textsuperscript{+}} T cells (see Fig E4 in this article’s Online Repository at www.jacionline.org). The levels of central memory (CD45RA{\textsuperscript{−}} and CD62L{\textsuperscript{+}}) and terminally differentiated RA{\textsuperscript{+}} (CD45RA{\textsuperscript{−}} and CD62L{\textsuperscript{−}}; terminally differentiated RA{\textsuperscript{+}}) CD4 T cells did not differ between groups.

The number of CD8 T cells was higher in CVID group A than in CVID group B and in the healthy controls (Fig 3, C). There was also a decrease in the frequencies of naive CD8 T cells in CVID group A and CVID group B compared with in the controls. CD8 T cells in CVID group A (ie, patients characterized by a more severe clinical phenotype) had a higher expression of the activation marker HLA-DR than did those in CVID group B and healthy controls (Fig 3, D). Increased levels of CD8 T cells with expression of PDCD1 was restricted to CVID group B when compared with the controls (Fig 3, D). There was no difference in the levels

---

### Table

<table>
<thead>
<tr>
<th>Freiburg class</th>
<th>CVID-A</th>
<th>CVID-B</th>
<th>P-value\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>N=8</td>
<td>N=4</td>
<td>0.010</td>
</tr>
<tr>
<td>1b</td>
<td>N=1</td>
<td>N=5</td>
<td>ns</td>
</tr>
<tr>
<td>2</td>
<td>N=2</td>
<td>N=8</td>
<td>ns</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Chi-square test. ns, Not significant.
of the CD8 memory T-cell subsets (central memory, effector memory, and terminally differentiated RA⁺) between groups.

Noninfectious complications are associated with a plasma protein profile under the influence of IFN-γ

Plasma proteins were considered upregulated, with a fold change of 1.3 or more (linearized value) and significantly different if \( P_{corr} \) was less than .05 when CVID group A was compared with CVID group B (see Table E6 in this article’s Online Repository at www.jacionline.org). In total, 84 proteins were upregulated and no proteins were downregulated in CVID group A, when compared with CVID group B. The plasma profiles of the patients in CVID group A were dominated by increased levels of 3 CXCR3 IFN-γ-inducible ligands: CXCL9, CXCL10 and CXCL11 (Table II). The top upregulated CVID group A-associated membrane proteins include Fc receptor-like protein (FcRL) 6, TNFRSF4, and TNFRSF9 (Table II). As an unbiased approach, we next used the IPA to analyze the upstream regulators associated with the upregulated proteins, 10 were unique for IFN-γ, 7 were unique for IL-1b, and 6 for NF-kB. Of the 38 proteins, 10 were unique for IFN-γ, 7 were unique for IL-1b, and 4 were unique for NF-kB, and 9 proteins were shared by all upstream regulators (Fig 4, B). CXCL9, CXCL10, and CXCL11 were shared between all 3 regulators. Next, a PCA was performed for factors regulated by IFN-γ, including for all patients in CVID group A and CVID group B and the healthy controls. Dimensional reduction of protein levels under the regulation of IFN-γ showed a clear separation of CVID group A and CVID group B when the data were projected to PC1 and PC2 (Fig 4, D). The relative plasma levels of the markers are shown in heatmaps (Fig 4, D). IFN-γ was initially detected in less than 15% of the analyzed samples and was therefore not included in the pathway analysis. However, a PEA assay with improved sensitivity for IFN-γ showed significantly increased plasma levels in patients in CVID group A compared with in patients in CVID group B and healthy controls (Fig 4, C).

Plasma CXCL10 is a potential biomarker of immune dysregulation in CVID

The correlation of CXCL9, CXCL10, and CXCL11 plasma levels with naive CD4 T cells, PDCD1⁺ CD4 T cells, and HLA-DR⁺ CD8 T cells was tested in patients with CVID (Fig 5, A). Plasma levels of CXCL9 correlated negatively with naive CD4 T cells and positively with proportions of PDCD1⁺ of CD4 T cells and proportions of HLA-DR⁺ of CD8 T cells (Fig 5, A). CXCL10 and CXCL11 also showed significant positive correlations (\( r > 0.50 \) and \( P < .01 \)) with the proportions of PDCD1⁺ of CD4 T cells and HLA-DR⁺ of CD8 T cells (Fig 5, A). In addition, CXCL10 correlated with the frequencies of CD21-low B cells (Fig 5, B). To investigate the performance of CXCL9, CXCL10, and CXCL11 in classifying patients as belonging to CVID group A or CVID group B, we constructed receiver operating characteristic curves. Plasma levels of CXCL10 performed well in the classification of CVID group A and CVID group B, with an AUC of 0.93 (95% CI = 0.762-1.0) (Fig 5, C). Sensitivity was 90% and specificity was 90% at a cutoff of more than 9.7 normalized protein expression units (Fig 5, D), which equals an analytic level of approximately 1 ng/mL (IMMUNO-ONCOLOGY, v.3101; www.olin.com/downloads). CXCL9 and CXCL11, both with an AUC greater than 0.90, performed less well and resulted in misclassification of 2 patients in CVID group A (data not shown). CXCL10 showed a significant correlation with the immune dysregulation disease score (\( r = 0.52; P = .004 \); Spearman correlation), and
FIG 3. The plasma protein signature reflects increased T-cell activation. Phenotype of peripheral blood lymphocytes in patients in CVID group A (CVID-A), patients in CVID group B (CVID-B), and healthy controls (HCs). Panels show CD3⁺CD4⁺ T cells (A), CD3⁺CD8⁺ T cells (C), and the proportions of naive (CD45RA⁺ and CD62L⁺), central memory (CD45RA⁻ and CD62L⁺), effector memory (CD45RA⁻ and CD62L⁻), and terminally differentiated RA⁺ (TEMRA) (CD45RA⁺ and CD62L⁻) subsets. Proportions of HLA-DR⁺ and PDCD1⁺ CD4 T cells (B) and CD8⁺ T cells (D) in the 3 groups. Error bars represent the 95% CI. *P < .05; **P < .01; ***P < .001; 1-way ANOVA and Tukey posttest). HC, Healthy control; ns, not significant.
Table II. Top 21 upregulated plasma proteins in patients in CVID group A compared with in patients in CVID group B

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Uniprot-id</th>
<th>CVID group A and CVID group B</th>
<th>Pcorr</th>
<th>Location of protein</th>
<th>Upstream Regulator</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL9</td>
<td>Q07325</td>
<td>2.09</td>
<td>1.2E-04</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Q14625</td>
<td>2.09</td>
<td>4.2E-05</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>CXCL10</td>
<td>P02778</td>
<td>1.86</td>
<td>1.4E-04</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>CXCL5</td>
<td>P42830</td>
<td>1.63</td>
<td>4.6E-02</td>
<td>Extracellular</td>
<td>IL-1β, NF-κB</td>
</tr>
<tr>
<td>CCL19</td>
<td>Q99731</td>
<td>1.61</td>
<td>6.5E-04</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>EGF</td>
<td>P01133</td>
<td>1.55</td>
<td>4.7E-03</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>GZMH</td>
<td>P20718</td>
<td>1.54</td>
<td>3.1E-03</td>
<td>Cytoplasm</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>CD40LG</td>
<td>P29965</td>
<td>1.50</td>
<td>4.4E-03</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>DAPP1</td>
<td>Q9UN19</td>
<td>1.49</td>
<td>1.1E-02</td>
<td>Cytoplasm</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>FCRL6</td>
<td>Q6DN72</td>
<td>1.45</td>
<td>6.3E-04</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>PDCD1</td>
<td>Q15116</td>
<td>1.43</td>
<td>4.7E-03</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>TNFRSF4</td>
<td>P43489</td>
<td>1.43</td>
<td>6.6E-04</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>IL2A</td>
<td>P29459</td>
<td>1.41</td>
<td>1.7E-03</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>EIF4G1</td>
<td>Q04637</td>
<td>1.41</td>
<td>5.6E-04</td>
<td>Cytoplasm</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>CXCL13</td>
<td>O43927</td>
<td>1.40</td>
<td>2.1E-02</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>KRAB1</td>
<td>Q13241</td>
<td>1.39</td>
<td>5.4E-05</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>LILRB4</td>
<td>Q9NHJ6</td>
<td>1.39</td>
<td>4.3E-05</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>CCL3</td>
<td>P10147</td>
<td>1.38</td>
<td>2.0E-05</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>TNFRSF9</td>
<td>Q07011</td>
<td>1.35</td>
<td>6.3E-04</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>SH2B3</td>
<td>Q9UQQ2</td>
<td>1.33</td>
<td>1.1E-02</td>
<td>Membrane</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
<tr>
<td>IL18</td>
<td>Q14116</td>
<td>1.28</td>
<td>9.7E-04</td>
<td>Extracellular</td>
<td>IFN-γ, IL-1β, NF-κB</td>
</tr>
</tbody>
</table>

Pcorr: P value corrected for multiple comparisons.
Difference between CVID group A and CVID group B is the difference in mean plasma protein levels between CVID group A and CVID group B expressed as normalized protein expression in log2. Upstream regulator indicates whether the protein is regulated by IFN-γ, IL-1β, and/or NF-κB.

there was no correlation of CXCL10 with the infection score (r = 0.13) (data not shown).

**DISCUSSION**

Although CVID is characterized by low IgG levels and recurrent infections, as many as 40% to 70% of the patients have noninfectious inflammatory and autoimmune complications that are major drivers of morbidity and mortality. Although previous studies have described a limited set of potential biomarkers in the blood of patients with CVID, this study is the first to investigate a broad array of immune-related proteins in plasma of patients with CVID compared with in plasma healthy controls. The plasma protein profile in patients with CVID was profoundly different from that in healthy controls, with 72 dysregulated immune-related proteins of the 145 detected. Increased plasma levels of IFN-γ and a distinctive plasma protein signature that included elevated levels of 24 proteins regulated by IFN-γ was strongly linked to CVID-associated immune dysregulation.

Unbiased clustering of plasma protein profiles separated the patients into 2 distinct groups, of which 1 group was characterized by noninfectious complications related to immune dysregulation as well as aberrant B and T-cell phenotypes that are associated with increased risk of CVID-related immune dysregulation. Thus, the plasma protein profiling succeeded in distinguishing a group of patients with CVID with more severe immune dysregulation from the other patients. There is a need to identify novel noninvasive biomarkers that can be used to predict the risk for CVID-related immune dysregulation in patients. We found that plasma CXCL10 correlated with the frequencies of CD21-low B cells, PDCD1 expression of CD4 T cells, and activated CD8 T cells; hence, plasma CXCL10 is a potential biomarker for noninfectious complications in CVID. Importantly, there was no correlation between CXCL10 and the infection score; hence, it is unlikely that chronic infections drive the CXCL10 aberration. However, further investigations are required in larger cohorts to establish a set of biomarkers that can direct specific immunomodulatory therapy to align with the principles of precision medicine.

The subgroup of patients with CVID with noninfectious complications showed prominent findings of T-cell abnormalities, with reduced numbers of naïve T cells, increased T-cell activation, and increased numbers of CD4 effector memory cells. These findings indicate that impaired T-cell function is a factor that strongly contributes to clustering and that aberrant T-cell function is a driving force in immune dysregulation. This is also supported by the increased expression of the immune checkpoint PDCD1 in CD4 T cells. Increased CD4 expression of immune checkpoint PDCD1 has previously been reported in CVID-associated autoimmunity and as a result of increased bacterial translocation in CVID, and it is consistent with chronic T-cell activation. Plasma levels of PDCD1 correlated with the proportions of PDCD1+ CD4 T cells, a finding in support of the idea that plasma protein levels reflect the phenotype of circulating cells. The CVID group with immune dysregulation also showed increased plasma levels of FcRL6, which also provides an immunosuppressive signal that directly represses natural killer cell cytotoxicity and effector T-cell activity. Notably, FcRL6 expression is increased on cytolytic T cells in HIV infection, and increased expression of FcRL6 was recently associated with acute Epstein-Barr virus infection. Thus, increased levels of both PDCD1 and FcRL6 are in line with T-cell dysregulation. On the other hand, levels of molecules that augment cellular immune responses, such as TNFRSF9 (4-1BB), which is expressed by activated natural killer cells and CD8 T cells, and TNFRSF4 (OX40), which is mainly expressed by activated CD4 T cells, were increased in plasma of the group with immune dysregulation CVID. These findings are consistent with...
Noninfectious complications are associated with a plasma protein profile under the influence of IFN-γ. A, Top 3 upstream regulators of the plasma protein profiles in CVID group A (CVID-A) when compared with CVID group B (CVID-B). Pathway analysis predicted a high likelihood of activation, indicated by high \( z \) scores and low \( P \) values, based on the number of upregulated targets in group CVID-A. B, The overlap of the targets between the 3 regulators. C, Plasma levels of IFN-γ. D, PCA and heatmaps of normalized protein expression (NPX) of plasma of proteins regulated by IFN-γ in group CVID-A and group CVID-B and the mean NPX of healthy controls (HCs).
FIG 5. Plasma levels of CXCR3 ligands correlated with T-cell phenotypes. **A**, Scatter plots of plasma protein correlation with different T-cell phenotypes. **B**, Receiver operating curve analysis of plasma CXCL10 for classification of patients with CVID into CVID group A (CVID-A) and CVID group B (CVID-B). Sensitivity and specificity at the optimal cutoff is shown. **C**, Box plot of CXCL10 plasma levels showing the 95% CI and medians. The mean is indicated by a diamond, and the optimal cutoff is indicated by a red line.
an activated phenotype, as supported by increased HLA-DR expression and increased numbers of effector memory T cells. Knowledge regarding the relation between soluble membrane molecules and cellular expression or cellular turnover is sparse. Soluble ligands and receptors may also interfere with immune cell cross-talk and cell signaling pathways. Taken together, our data indicate that the unique plasma protein profile reflects the presence of T-cell activation and a complex T-cell dysfunctionality that are more pronounced in patients with CVID with immune dysregulation.

When integrating plasma protein profiles for pathway analysis the CVID group with immune dysregulation displayed a profile consistent with T~g~1~1 deviation because the top 3 upregulated proteins in this group were CXCL9, CXCL10, and CXCL11, all of which are ligands of the CXCR3 receptor and regulated by IFN-γ. Increased levels of CXCL9, CXCL10, and CXCL11, in combination with increased plasma IFN-γ levels in patients in CVID group A, support the notion that CVID-related immune dysregulation is under strong influence of IFN-γ, a cytokine that functions as a stimulator and modulator of T~g~1 immune responses. This is consistent with previous reports in which CVID-related immune dysregulation has been associated with increased IFN-γ production of lymph node CD4 T cells and peripheral blood CD4 T cells on stimulation. Also, increased serum IFN-γ levels were reported in CVID with noninfectious complications as a result of the expansion of inflammatory type 3 innate lymphoid cells. CD21-low B cells resemble autoreactive CD11c~1 B cells associated with systemic lupus erythematosus, and chronic viral infections have been reported to express the IFN-γ inducible transcription factor Tbet and CXCR3. IFN-γ was recently reported to augment the development of autoreactive CD11c~1 B cells, a pathophysiologic link that has also been proposed in CVID. We show correlations of CXCL10 with aberrant T-cell populations in CVID as well as with CD21-low B cells and suggest that the CXCR3 axis is an attractive target for specific treatment to mitigate CVID-related immune dysregulation. MDX-1100, a fully human anti-CXCL10 mAb that was well tolerated and demonstrated clinical efficacy in Phase II clinical trials of rheumatoid arthritis, is a drug that may prove effective in the treatment of CVID-related immune dysregulation by counteracting T~g~1-driven T-cell activation and by preventing the expansion of CD21-low B cells.

Further analysis identified activation of IFN-γ, IL-1β, TNF, NF-κB, and RelA as top upstream regulators of plasma protein profiles. Collectively, these regulators control pathways that converge in canonic NF-κB activation. Dysregulation of these pathways is predicted to mainly affect cell-cell interactions and cellular movement. Significant functional annotations include activation of leukocytes, leukocyte migration, inflammatory response, and activation of lymphocytes. Interestingly NFκB2 and NFKB1 haploinsufficiency have been associated with CVID-like disease.

In addition to distinguishing the subgroups of patients with CVID, the plasma protein profiling also revealed aberrant expression that differed between the whole CVID group and the controls. A novel finding was the increased level of the B-cell–attracting chemokine CXCL13 in patients with CVID. CXCL13 is the ligand of CXCR5 and controls the formation of germinal centers. CXCL13 is mainly produced by follicular dendritic cells and follicular T~g~1 cells in the lymphoid tissue and has been suggested to be a plasma biomarker of germinal center activity. Increased plasma levels of CXCL13 may reflect aberrant germinal center formation that has been reported in CVID. Increased levels of IL-12A, CXCL9, and PDCD1 in patients with CVID are consistent with previous findings. Pathway analysis predicted IL-36B to be the top upstream regulator of CVID plasma profiles. IL-36B is a poorly understood immune-activating cytokine belonging to the IL-1 family that has been suggested to play an important role in host defense by counteracting microbial immune evasion.

Our study has some limitations. The first is the restricted number of patients included, especially with regard to the heterogeneity of the clinical manifestations of CVID. On the other hand, the number was sufficient for an unbiased approach of proteomic analysis to reveal the distinct findings that were obtained by the protein profiling. The restricted number of patients was a weakness of the evaluation of potential plasma biomarkers because it was not feasible to split the observations into training and test sets. However, we show that a single marker can perform well in predicting immune dysregulation. Second, a targeted approach was used for the proteomic studies, which does not give a complete picture of the plasma protein profiles. On the other hand, the PEA combines high sensitivity (by PCR amplification) and high specificity (by dual antibodies against different epitopes), which are needed for safe detection of low-abundant proteins, which is more difficult with mass spectrometry techniques. Finally, this is an explorative study, and an alternative study design is required to dissect the mechanistic role of each pathway in the development of CVID-related immune dysregulation.

To our knowledge, this study represents the first study of CVID based on plasma proteomics. The unbiased use of the plasma protein profiles demonstrates novel proteins and associated pathways that likely contribute to the development of CVID-related immune dysregulation. The plasma protein profiles associated with immune dysregulation are under strong influence of IFN-γ signaling, with increased levels of T~g~1 chemokines. Increased numbers of CD8 T cells and increased T-cell activation can also be explained by IFN-γ–driven inflammation. Given the heterogeneity of the clinical manifestations of CVID, plasma proteomics provide a tool for distinguishing patients with CVID, who are at risk of increased morbidity, and it could also provide information of perturbed pathways available for specific immunotherapy.

We would like to acknowledge the support of the Clinical Biomarker Facility at SciLifeLab Sweden for providing assistance in protein analyses. The study was financially supported by ALF Grants, Region Östergötland.

Clinical implications: Profiling of plasma protein identifies biomarkers associated with immune dysregulation in CVID that may help in delineating the underlying molecular mechanisms and pave the way for tailored treatment strategies.

REFERENCES


