Persistent Activation of the Tumor Necrosis Factor System in a Subgroup of Patients With Common Variable Immunodeficiency—Possible Immunologic and Clinical Consequences

By Pål Aukrust, Egil Lien, Anne K. Kristoffersen, Fredrik Müller, Charlotte J. Haug, Terje Espevik, and Stig T. Freland

In patients with common variable immunodeficiency (CVI), we have previously defined a subgroup of patients (CVIHypm) characterized by decreased numbers of CD4+ lymphocytes in peripheral blood, splenomegaly, and persistent immune activation in vivo, particularly of monocytes/macrophages. To further characterize this hyperactivity, parameters of activation of the tumor necrosis factor (TNF) system (TNFα and soluble TNF receptors [sTNFRs]) were measured in 24 patients with CVI and 20 healthy controls. Patients with CVI had significantly higher serum levels of TNFα and both types of sTNFRs, with the highest levels in the CVIHypm subgroup. In vitro, peripheral blood mononuclear cells (PBMC) and purified monocytes from CVIHypm patients spontaneously released significantly higher levels, and, after lipopolysaccharide (LPS) stimulation, significantly lower levels of TNFα and soluble p75-TNF than cells from both other CVI patients and healthy controls. CVIHypm patients also had significantly higher TNFα: sTNFRs ratios in both serum and in unstimulated PBMC supernatants. The present study demonstrates persistent in vivo activation of the TNF system in CVI, particularly in the CVIHypm subgroup. This activation may contribute to the pathogenesis of both clinical and immunologic manifestations in CVI.

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Table 1. Clinical and Immunologic Characteristics of the Study Group

<table>
<thead>
<tr>
<th></th>
<th>CVI</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td>Range</td>
<td>21-74</td>
<td>26-68</td>
</tr>
<tr>
<td>Males/females</td>
<td>9 (38%)/15 (62%)</td>
<td>9 (45%)/11 (55%)</td>
</tr>
<tr>
<td>CD2+ lymphocytes ($\times 10^{6}$/L)</td>
<td>850 (680-1,160)</td>
<td>1,019 (730-1,190)</td>
</tr>
<tr>
<td>CD4+ lymphocytes ($\times 10^{6}$/L)</td>
<td>350 (220-500)</td>
<td>400 (409-740)</td>
</tr>
<tr>
<td>CD8+ lymphocytes ($\times 10^{6}$/L)</td>
<td>10 (270-700)</td>
<td>360 (210-450)</td>
</tr>
<tr>
<td>CD19+ lymphocytes ($\times 10^{6}$/L)</td>
<td>110 (40-190)</td>
<td>150 (120-180)</td>
</tr>
<tr>
<td>Monocytes ($\times 10^{6}$/L)</td>
<td>290 (200-350)</td>
<td>270 (230-310)</td>
</tr>
<tr>
<td>Neopterin (nmol/L)</td>
<td>35.4 (14.1-43.4)</td>
<td>9 (0.6-10)</td>
</tr>
</tbody>
</table>

Data are given as medians and 25th to 75th percentiles if not otherwise stated.

 Was collected and stored at −70°C in multiple aliquots until analysis. Serum samples for cytokine analysis were frozen and thawed only once.

Cells. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Isopaque-Ficoll gradient centrifugation within 2 hours after blood sampling, as described elsewhere. Mononuclear cells were resuspended in RPMI 1640 (Gibco, Paisley, UK) with 2 mM/L-L-glutamine supplemented with gentamicin (40 μg/mL) and 10% heat-inactivated pooled human AB+ serum (culture medium). The fractions of monocytes (CD14+) and T lymphocytes (CD2+) were determined by immunomagnetic quantification. Isolation of monocytes was performed by plastic adherence as previously described. The endotoxin level of the culture medium was less than 10 pg/mL (limulus amoebocyte lysate test).

Cell stimulation. For evaluation of TNFα and sTNFR concentrations in cell supernatants, PBMC (3 × 10^6/mL) or monocytes (3 × 10^6/mL) were incubated in flat-bottomed, 96-well trays (Costar, Cambridge, MA; PBMC) or 24-well trays (Costar; monocytes) with or without stimulants (lipopolysaccharide from Escherichia coli O26:B6 [LPS; final concentration, 10 ng/mL; Sigma Chemical, St Louis, MO] and staphylococcal enterotoxin B [SEB; final concentration, 10 ng/mL; Sigma]). After various time periods, cell-free supernatants were harvested and stored at −70°C in several aliquots until analysis.

Enzyme immunoassays (EIAs) for detection of sTNFRs and TNFα. The sTNFRs, p55 and p75, were analyzed by immunoassays described by Liabakk et al. Immunoplates were coated with the monoclonal antibodies IV4E and 3H5, recognizing non–TNF-binding sites of p55 and p75 TNFR, respectively. Reombinami human p55 and p75 TNFRs (provided by Dr H. Loetscher, F. Hoffmann-La Roche, Basel, Switzerland) served as standards. TNFα concentration was quantified by EL4 (Medgenix, Fleurus, Belgium) according to previously described methods. Immunoassays were washed and autoradiographed using Hyperfilm (Amersham) or Super Rapid Screens (Eastman Kodak, Rochester, NY), and the relative sensitivity of the autoradiographs was quantified using scanning densitometry (Pharmacia LKB, Uppsala, Sweden). When comparing mRNA levels in patients and controls, a quantitative estimate of the TNFα and sTNFRs mRNA levels was attempted by comparing with the mRNA level of the GADPH gene.

RESULTS

Serum levels of TNFα and sTNFRs in patients with CVI and in controls. As illustrated in Fig 1, patients with CVI had significantly elevated serum levels of TNFα and sTNFRs compared with healthy controls. Although levels of both types of sTNFRs were raised, the ratio between levels of soluble p75 and p55 receptors was also significantly elevated in patients with CVI (3.11 [2.49 to 3.36] vs 1.47 [1.12 to 1.76], P < .001; patients with CVI and controls, respectively). TNFα bioactivity was not detected in any serum samples from either patients or controls.

Serum levels of TNFα and sTNFRs in patients with CVI in relation to clinical and immunologic manifestations. We have previously defined a subgroup of patients with CVI (CVIhyper) characterized by persistent immune activation in vivo, low numbers of CD4+ lymphocytes in peripheral blood, and splenomegaly. In the present study, patients were classified as CVIhyper if they had CD4+ lymphocyte counts less than 400 × 10^6/L, serum neopterin levels greater than the mean + 4 SD of controls (23 nmol/L), and splenomegaly. These characteristics were constant when patients were tested and evaluated at least three times over a minimum of 2 years. As shown in Fig 2, the 11 CVIhyper patients had significantly raised serum levels of TNFα and both types of sTNFRs compared with other patients with CVI.

The ratio between TNFα and sTNFRs may provide an estimate of the molar balance in serum between TNFα molecules and sTNFRs. In molecular terms, this ratio was defined as TNFα (pmol/L)/[p55 + p75](pmol/L) × 100, assuming a molecular mass of (17 × 3) kD and 30 kD for TNFα (trimer) and both types of sTNFRs, respectively. We found that CVIhyper patients had significantly higher ratios of TNFα to sTNFRs, reflecting an approximate 400-fold molar excess of sTNFRs in the CVIhyper group compared with an approximate 1,000-fold molar excess among the other patients with CVI (Fig 2).

Bacterial products such as endotoxins are known to be
potent inducers of TNFα. However, patients with CVI and chronic rhinosinusitis or bronchiectasis had not significantly higher serum levels of TNFα and sTNFRs than other patients with CVI (data not shown). Moreover, we found no significant association between occurrence of autoimmunity, chronic HCV infection, or NILH and raised serum levels of TNFα and sTNFRs. However, the two patients with necrocaseating granulomata were among the five patients with CVI with highest levels of these TNF parameters (data not shown).

In six CVIHyper patients, six other patients with CVI, and six controls, analyses of TNFα and sTNFRs were performed in serum samples taken at a median period of 9 months (range, 4 to 18 months) before the study, confirming persistently raised levels of TNFα and sTNFRs in patients with CVI. In addition, levels of these TNF parameters were again significantly higher among patients in the CVIHyper group compared with other patients with CVI (data not shown). Furthermore, in these six CVIHyper patients, serum levels of TNFα were measured four times during a period of 2 years, and they always had concentrations above the mean + 5 SD of healthy controls (data not shown).

In vitro release of TNFα and sTNFRs from PBMC. To further study the activation of the TNF system in patients with CVI, PBMC from six CVIHyper patients, seven other patients with CVI, and six healthy controls were stimulated with LPS, and supernatants from stimulated and unstimulated PBMC were harvested after 5, 20, and 72 hours and assayed for quantification of TNFα and sTNFR levels. Among PBMC, monocytes are both the target for LPS and appear in vitro to be the major source of TNFα and soluble p75-TNFR release. In the present study, we, therefore, expressed TNFα and sTNFR levels in supernatants as concentration per 10^6 monocytes. As illustrated in Fig 3, CVIHyper patients had significantly higher levels of spontaneous TNFα release compared with levels in both other CVI patients and in healthy controls. Spontaneous in vitro release after only 5 hours might reflect the in vivo situation, and indeed, among patients with CVI, TNFα levels in supernatants at this time point were significantly correlated with TNFα levels in serum (r = .70, P < .01). In contrast with spontaneous release, CVIHyper patients had decreased maximum levels of LPS-stimulated TNFα release compared with both other patients with CVI and healthy controls (Fig 3). Furthermore, among patients with CVI, there was an inverse correlation between serum levels of TNFα and maximum levels of LPS-stimulated TNFα release (r = .67, P < .01). The significantly decreased spontaneous and significantly increased spontaneous release compared with other patients with CVI and controls. Furthermore, soluble p75-TNFR was the predominant sTNFR in PBMC from CVIHyper patients was confirmed when TNFα bioactivity was analyzed in supernatants from three CVIHyper patients and three controls (data not shown).

In both patients and controls, we observed a gradual increase in LPS-stimulated as well as spontaneous release of sTNFRs, with maximum concentrations for both types of sTNFRs after 72 hours (data not shown). The maximum sTNFR levels are shown in Table 2, demonstrating significantly higher unstimulated and significantly lower LPS-stimulated soluble p75-TNFR levels in CVIHyper patients compared with other patients with CVI and controls. Furthermore, soluble p75-TNFR was the predominant sTNFR in PBMC supernatants in both patients with CVI and controls (Table 2), and the ratio between soluble p75 and p55 receptors in PBMC supernatants was considerably higher than in serum in both groups (data not shown).

To investigate if the decrease in stimulated release of TNFα and soluble p75-TNFR from PBMC in the CVIHyper group was restricted to stimulation with LPS, PBMC from all patients and controls were also stimulated with SEB. We observed similar findings as for LPS response, with preferential release of soluble p75 compared with soluble p55-TNFR and significantly decreased release of both TNFα and soluble p75-TNFR (expressed both as concentration per 10^6 PBMC and per 10^5 monocytes) in the CVIHyper group (data not shown). Thus, the impaired in vitro release in the CVIHyper group seems not to be restricted to the LPS stimulatory pathway.

In vitro release of TNFα and sTNFRs from isolated monocytes. In a separate experiment, spontaneous and LPS-stimulated TNFα and sTNFR release was measured in isolated monocytes from five controls and five CVIHyper patients
TNFα IN COMMON VARIABLE IMMUNODEFICIENCY

Fig 2. Serum levels of soluble p55-TNFR (A), soluble p75-TNFR (B), and TNFα (C), and the TNFα:sTNFRs ratio expressed as TNFα (trimer)/pmol/L/(soluble p55 + p75-TNFRs)/pmol/L × 100 (D) in two different subgroups of CVI patients. CVIHyp (n = 11), patients with CD4+ lymphocyte counts less than 400 x 10⁶/L, serum neopterin levels greater than 23.0 nmol/L, and splenomegaly (see Methods); CVINorm (n = 13), the rest of the CVI group. Bars represent median values.

Concerning sTNFRs, both unstimulated and LPS-stimulated monocytes almost entirely released soluble p75-TNFR in concentrations suggesting that monocytes are the most important source of soluble p75-TNFR in PBMC (data not shown). CVIHyp patients tended to have higher spontaneous and lower stimulated soluble p75-TNFR release than controls (data not shown).

To further elucidate the balance between TNFα and TNFRs, we analyzed mRNA levels for TNFα and both types of TNFRs in unstimulated and LPS-stimulated monocytes in four CVIHyp patients and four controls. Two significant findings were revealed. First, in both CVIHyp patients (Fig 4) and controls (data not shown), there was a decrease in LPS-stimulated mRNA levels for p55-TNFR compared with unstimulated levels after 3 hours, before a slight increase was observed after 12 hours. Second, we could not detect any significant differences between patients and controls concerning mRNA levels for either TNFα or TNFRs (data not shown).

The balance between TNFα and sTNFRs in PBMC supernatants. As shown in Fig 5, CVIHyp patients had significantly higher TNFα-to-sTNFR ratios than both other CVI patients and controls in supernatants from unstimulated cells, while they had significantly lower ratios after LPS stimulation. In both patients and controls, the maximum levels of this ratio were significantly correlated with maximum levels of TNFα bioactivity when eval-
uated in three CVI\hyp patients and three controls ($r = .87, P < .03$ and $r = .82, P < .03$, unstimulated and LPS stimulated, respectively). Furthermore, as shown in Figs 5 and 2, the balance between TNFa and sTNFRs is markedly different in serum and in PBMC supernatants. While CVI patients had a 400- to 1,000-fold molar excess of sTNFRs in serum, unstimulated supernatants (5 hours) from CVI\hyp patients had an approximate two-fold molar excess of TNFa, and 5 hours after LPS stimulation, there was an approximate 40-fold (controls) or 10-fold (CVI\hyp) molar excess of TNFa.

**DISCUSSION**

The present study demonstrates that patients with CVI had significantly and persistently elevated serum levels of TNFa and both types of sTNFRs compared with healthy controls. In addition, spontaneous in vitro release of TNFa from PBMC and monocytes was significantly higher in patients with CVI than in controls. These findings strongly indicate a persistent activation of the TNF system in patients with CVI, and this activation was particularly pronounced in a subgroup of patients with CVI previously described by us$^{17,19}$ and characterized in vivo by chronic immune activation, low numbers of CD4+ lymphocytes, and splenomegaly.

We$^{19}$ and others$^{30}$ have previously not been able to detect raised serum levels of TNFa in patients with CVI by other EIAs. This most probably reflects methodologic differences, and we have recently suggested that the EIA used in the present study may better detect TNFa in complex with sTNFRs than other EIAs.$^{24}$

In contrast with the increased spontaneous TNFa production, we observed a significant decrease in stimulated TNFa release among CVI\hyp patients both in PBMC and in purified monocytes. A similar dissociation between circulating TNFa levels and in vitro release from mononuclear cells has previously been reported in septicemia$^{31}$ and in human immunodeficiency virus type 1 (HIV-1) infection.$^{32,33}$ It

![Graph](image-url)
TNFα in Common Variable Immunodeficiency

Fig 5. The balance between TNFα and sTNFRs in supernatants from unstimulated (A) and LPS (from E coli)-stimulated (B) PBMC (3 × 10⁶/mL) after 5, 24, and 72 hours. PBMC were taken from healthy controls (n = 6; □) and two different subgroups of patients with CVI. The definitions of CVI hyper (n = 6; Ⅶ) and CVI norm (n = 7; Ⅶ) are given in the legend to Fig 2. TNFα:sTNFRs ratio is expressed as TNFα (pmol/L)/sTNFRs (pmol/L) × 10⁷. Data are given as medians and 25th to 75th percentiles. *P < .02 versus controls and CVI norm.

Activation of the TNF system may be reflected in elevated serum levels of sTNFRs persisting longer in circulation than TNFα itself.12,39 In accordance with recent reports,40,41 we found that both monocytes and PBMC primarily released soluble p75-TNFR both unstimulated and after activation with LPS and SEB, suggesting that the increased circulating levels of soluble p75-TNFR found in CVI reflect increased release from PBMC and, in particular, from monocytes. However, the relative concentration of soluble p75 compared with soluble p55-TNFR was higher in cell supernatants in comparison with those concentrations found in peripheral blood in both patients with CVI and controls, suggesting that other cellular sources such as endothelial cells42 may be important contributors to the circulating levels of soluble p55-TNFRs.

Although only a modest increase in soluble p55-TNFR levels are found after LPS stimulation of monocytes, down-regulation of membrane p55-TNFR is reported after stimulation of these cells.43 This may reflect internalization of the receptor rather than shedding.42 In the present study, and not previously reported for mononuclear phagocytes, we also observed a decrease in steady-state mRNA levels for this receptor after LPS stimulation of monocytes from both patients with CVI and controls, possibly resulting in decreased synthesis.

The molar balance between TNFα and sTNFRs seems to be an important parameter for the activation of the TNF system.13 We found that CVI hyper patients had significantly higher TNFα:sTNFRs ratios both in serum and in unstimulated PBMC supernatants and significantly lower ratios in PBMC supernatants after LPS stimulation than other patients with CVI and controls. Interestingly, in PBMC supernatants, this ratio was significantly correlated with levels of TNFα bioactivity. The significance of these findings is uncertain, and the role of sTNFRs in immunologic reactions is not clear. It seems that the systemic TNFα effect depends on the relative concentrations of TNFα and sTNFRs in circulation.44 However, it appears that sTNFRs may not only have neutralizing but also enhancing effects on TNFα activity. It seems that enhancing effects of sTNFRs on TNFα activity are seen in the presence of high TNFα-to-sTNFRs ratios, and inhibitory effects are observed with marked molar excess of sTNFRs.45,46 Interestingly, we found that while the TNFα-to-sTNFRs ratio in serum was low, suggesting an inhibitory effect of sTNFRs, this ratio was high in PMBC supernatants from both patients with CVI and controls, suggesting a possible enhancing effect of sTNFRs in the cellular microenvironment.

The enhanced and persistent activation of the TNF system seen in CVI may have both clinical and immunologic consequences. Indeed, persistent TNFα activation has been implicated in the pathogenesis of a number of disorders seen with increased frequency in CVI,15 eg, autoimmune disorders,3 granuloma formation,16 and chronic diarrhea and malabsorption in the absence of any known pathogen.4

TNFα is known to enhance B- and T-cell proliferation and B-cell differentiation after short-time exposure.1 However, prolonged TNFα exposure in vivo and in vitro seems to impair lymphocyte proliferation response,48,49 T cell-50 and
natural killer cell-mediated cytotoxicity, and T-cell interleukin-2 production, possibly by modulating intracellular redox status. Interestingly, there are also reports of suppressive effects of TNFα exposure on mitogen-induced IgG and IgM production from PBMC.

In addition to possible immunosuppressive effects on B and T cells, the persistent TNFα activation in vivo in CVI, which was associated with impaired TNFα response when further stimulated in vitro, might contribute to depressed microbicidal defense if a similar refractory state is induced in monocytic cells in vivo.

The present results suggest that sustained TNFα activity partly reflecting monocyte hyperactivity is an important characteristic of the CVIHyper subgroup. These phenomena may reflect a fundamental immunologic dysfunction in CVI, but they may also be secondary to chronic infections, for example, though we could not relate the activation of the TNF system in CVI to ongoing bacterial infections. Interestingly, however, chronic viral infection has been suggested to be implicated in the pathogenesis of CVI. Regardless of the cause of this persistent activation of the TNF system, biologic effects of TNFα may contribute to the pathogenesis both of clinical and immunologic manifestations in subgroups of CVI. It is, therefore, conceivable that therapeutic modulation of the TNF system might be worth exploring in CVI.

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