Altered Expression of TNF-α Signaling Pathway Proteins in Systemic Lupus Erythematosus

LANG-JING ZHU, CAROLINA LANDOLT-MARTICORENA, TIMOTHY LI, XIAO YANG, XUE-QING YU, DAFNA D. GLADMAN, MURRAY B. UROWITZ, PAUL R. FORTIN, and JOAN E. WITHER

ABSTRACT. Objective. To investigate the expression of tumor necrosis factor receptors (TNFR1 and TNFR2) and adapter proteins (TRADD, RIP, and TRAF2) in peripheral blood mononuclear cell (PBMC) subsets from patients with systemic lupus erythematosus (SLE).

Methods. PBMC were isolated from 45 SLE patients and 25 controls, and stained with labeled antibodies that enabled identification of various T cell, B cell, and monocyte subpopulations. Expression of TNF-related signaling molecules was measured by staining with labeled antibodies either directly or following fixation and permeabilization. Apoptosis was quantified using an anti-active caspase 3 antibody. RNA expression of TNF-related signaling molecules was assessed by quantitative RT-PCR and serum levels of TNF-α by ELISA.

Results. SLE patients had increased levels of TNFR1, TNFR2, and TRAF2, together with decreased levels of RIP, on various B, CD4+, and CD8+ T cell subsets as compared to controls. This altered expression was seen in both naive and memory subpopulations, and reflected altered staining of the whole population rather than a subset of cells that were activated. The levels of these molecules were not significantly correlated with serum TNF-α levels or their RNA expression in whole peripheral blood. TNFR1 and TNFR2 expression was negatively correlated with disease activity. There was no association between the proportion of apoptotic cells in any of the subpopulations and serum TNF-α levels or expression of TNF-related signaling molecules.

Conclusion. Patients with SLE had altered expression of TNF-related signaling molecules, suggesting that there may be an imbalance in TNF-α signaling favoring cellular activation as opposed to proapoptotic pathways. (First Release June 1 2010; J Rheumatol 2010;37:1658–66; doi:10.3899/jrheum.091123)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS TUMOR NECROSIS FACTOR SIGNALING
multiple signal transduction pathways\textsuperscript{13,14,15}. In the case of TNFR1, TRADD acts as a scaffold protein for recruitment of FADD, RIP, and TRAF2. Recruitment of FADD leads to activation of the caspase 8 pathway, resulting in apoptosis, whereas RIP and TRAF2 play an important role in activation of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) and c-Jun N-terminal kinase, respectively. Trimerization of TNFR2 also results in activation of NF-\(\kappa\)B and JNK pathways, which is mediated by the adapters TRAF1 and TRAF2. Both types of receptors are expressed on immune cells; however, their levels vary with cell type and activation status. Notably, variations in the levels of these cell-surface receptors and their downstream adapters can result in altered cellular function\textsuperscript{16}.

In a previous study, we found that the mRNA expression of several TNF signaling adapters including TRADD, FADD, RIP-1, and TRAF2 was decreased in the peripheral blood mononuclear cells (PBMC) of patients with SLE, and this was negatively correlated with disease activity\textsuperscript{17}. These findings raised the possibility that TNF signaling is abnormal in patients with SLE. In this study we used flow cytometry to examine the protein expression of these molecules in various lymphocyte populations. We show that SLE patients have increased cell-surface levels of TNFR1 and TNFR2, and increased intracellular levels of TRAF2 on their lymphocytes. The findings suggest that there may be an imbalance in TNF-\(\alpha\) signaling in SLE patients favoring cellular activation, as opposed to proapoptotic pathways.

**MATERIALS AND METHODS**

**Subjects.** Forty-five patients who satisfied \textgreater\textasciitilde 4 revised 1997 American College of Rheumatology classification criteria for SLE\textsuperscript{18} were recruited from the University of Toronto Lupus Clinic. Disease activity was measured using the SLE Disease Activity Index 2000 (SLEDAI-2K)\textsuperscript{19}. Control blood samples (\(n=25\)) were obtained from healthy donors who had no family history of SLE. The clinical characteristics of the subjects examined are shown in Table 1.

**Table 1.** Demographic and clinical variables of the SLE patients and control subjects.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients, (n=45)</th>
<th>Controls, (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range, yrs</td>
<td>19–68</td>
<td>22–48</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>34.9 (13.0)</td>
<td>33.9 (8.2)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>40 (88.8)</td>
<td>21 (84.0)</td>
</tr>
<tr>
<td>Disease duration, yrs</td>
<td>12.4 (10.9)</td>
<td>NA</td>
</tr>
<tr>
<td>SLEDAI-2K, mean (SD)</td>
<td>5.3 (5.1)</td>
<td>NA</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisone, mean (SD)</td>
<td>10.5 (10.7)</td>
<td>NA</td>
</tr>
<tr>
<td>Antimalarials, n (%)</td>
<td>29 (66.3)</td>
<td>NA</td>
</tr>
<tr>
<td>Any immunosuppressive, n (%)</td>
<td>25 (55.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Azathioprine, n (%)</td>
<td>11 (24.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Mycophenolate mofetil, n (%)</td>
<td>10 (22.2)</td>
<td>NA</td>
</tr>
<tr>
<td>Methotrexate, n (%)</td>
<td>5 (11.1)</td>
<td>NA</td>
</tr>
<tr>
<td>Cyclosporine, n (%)</td>
<td>3 (6.7)</td>
<td>NA</td>
</tr>
<tr>
<td>Tacrolimus, n (%)</td>
<td>1 (2.2)</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: not applicable; SLEDAI: SLE Disease Activity Index.

**Flow cytometry staining and analysis.** PBMC were isolated from heparinized blood by Ficoll density gradient centrifugation and treated with Gey’s solution to remove red blood cells. One-half million cells were stained with various combinations of conjugated monoclonal antibodies (mAb), fixed and permeabilized using Cytofix/Cytoperm (BD BioScience, Franklin Lakes, NJ, USA), and then stained with phycoerythrin (PE)-conjugated antibodies specific for intracellular molecules. Flow cytometry was performed using a FACSCalibur instrument (BD BioScience) and the results analyzed using CellQuest software, with at least 50,000 lymphoid events acquired per sample. The following mAb were purchased from BD Pharmingen: PE-conjugated IgG2b (27–35), IgG1 (MOPC-21) and anti-asepatic caspase 3; FITC-conjugated IgG1 (MOPC-21), anti-CD27 (M-T271), anti-CD45RA (HI100), and anti-CD64 (10.1); allophycocyanin-conjugated mouse IgG2b (2-35), anti-CD8 (RPA-T8), and anti-CD14 (M4P9); PerCP-Cy5.5-conjugated IgG2a (MOPC-173); and PE-Cy7-conjugated anti-CD19 (HB19) and anti-CD45RO (UCHL1). Additional antibodies were purchased from the following sources: allophycocyanin-conjugated anti-CD38 (I6) and PE-conjugated IgG2a (SA43.10), Miltenyi Biotec, Auburn, CA, USA; PE-conjugated anti-TNFFR1 (16803.1), anti-TNFFR2 (22335), and anti-TRADD (313203), and FITC-conjugated anti-TNFFR2 (22335), R&D Systems, Minneapolis, MN, USA; PE-conjugated anti-TRAF2, Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit anti-RIP antibody, Cell Signaling Technology, Boston, MA, USA; rabbit IgG and PE-conjugated goat Fab’/2 anti-rabbit IgG, Invitrogen, Carlsbad, CA, USA.

For analysis, cells were first gated on the lymphocyte population based on forward and side scatter characteristics. The combinations of stains used to identify the lymphocyte subpopulations are shown in Table 2. CD27++CD38+++ plasma cells were excluded from the analysis of pregerminal center (GC) and memory cells by gating. The proportion of cells that stained positively for each TNF-related signaling molecule was determined by comparison with isotype controls, with the percentage of background staining with isotype controls being subtracted.

**RNA isolation and real-time polymerase chain reaction (PCR).** Total RNA was isolated from blood archived in PAXgene tubes utilizing the PAXgene Blood RNA Kit (Qiagen, Basel, Switzerland) with modifications to improve RNA yield and quality including addition of RNase inhibitor, off-column DNase I digestion, and final ethanol precipitation. A first-strand complementary DNA was produced using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR amplifications were performed with TaqMan Universal PCR Master Mix on an ABI Prism 7900 HT sequence detector system according to the manufacturer’s instructions (Applied Biosystems). PCR amplification of the housekeeping gene, \(\beta\)-actin, was done for each sample as a control for sample loading. Normalization and quantification of the PCR signals was performed by comparing the cycle threshold value of the gene of interest, in duplicate, with the mean cycle threshold value of \(\beta\)-actin.

**Table 2.** Cell-surface markers used to identify different T cell and B cell subpopulations.

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Surface Markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cell subsets</td>
<td>CD4+CD45RA+CD45RO-</td>
<td>20–22</td>
</tr>
<tr>
<td>Memory CD4+ T cells</td>
<td>CD4+CD45RA-CD45RO+</td>
<td>20–22</td>
</tr>
<tr>
<td>CD8+ T cell subsets</td>
<td>CD8+CD45RA+CD45RO-</td>
<td>21, 22</td>
</tr>
<tr>
<td>Memory CD8+ T cells</td>
<td>CD8+CD45RA-CD45RO+</td>
<td>21, 22</td>
</tr>
<tr>
<td>CD19+ B cell subsets</td>
<td>CD19+CD27+CD38+/+</td>
<td>23, 24</td>
</tr>
<tr>
<td>Memory CD19+ B cells</td>
<td>CD19+CD27-CD38+/+</td>
<td>25–27</td>
</tr>
<tr>
<td>Mature naive CD19+ B cells</td>
<td>CD19+CD27-CD38+/+</td>
<td>28</td>
</tr>
<tr>
<td>Immature transitional cells</td>
<td>CD19+CD27-CD38++</td>
<td>29</td>
</tr>
<tr>
<td>Pre-germinal center</td>
<td>CD19+CD27+CD38++</td>
<td>29</td>
</tr>
<tr>
<td>Monocytes</td>
<td>CD4+CD68+</td>
<td>30</td>
</tr>
</tbody>
</table>

Zhu, et al: TNF-\(\alpha\)-related signaling molecules in SLE

**Personal non-commercial use only. The Journal of Rheumatology Copyright © 2010. All rights reserved.**
however, there was no correlation with disease activity. Consistent with previous reports, SLE patients had a non-parametric test was used for comparisons between patients and controls.

RESULTS

Altered levels of TNF-α, TNFRs, and TNF signaling adapters in SLE. Consistent with previous reports, SLE patients had elevated serum levels of TNF-α as compared to controls; however, there was no correlation with disease activity (Figure 1)⁴⁻⁶. We have previously shown that mRNA levels of several TNF signaling adapters were altered in PBMC of individuals with SLE¹⁷. To determine whether these changes were also seen in whole peripheral blood, RNA was prepared from blood collected in PAXgene tubes. In contrast to our previous findings, mRNA expression of TNFR1, TNFR2, TRADD, RIP, and TRAF2 did not differ significantly between SLE patients and controls. It is likely that this difference reflected the nature of our patient population since the majority of our patients had an SLE Disease Activity Index (SLEDAI) < 10, where differences in expression of these molecules were not observed¹⁷. TNF-α mRNA expression in whole peripheral blood cells was also not significantly different between SLE patients and controls, and did not correlate with the serum levels of TNF-α.

We used flow cytometry to further examine expression of TNF-related signaling molecules in PBMC. PBMC were stained with labeled antibodies to identify distinct cell subsets together with antibodies specific for TNFR1, TNFR2, or the TNF signaling adapters TRADD, RIP, and TRAF2. In SLE patients, there was altered expression of all of the TNF-related signaling molecules examined except TRADD (Figure 2). This altered expression was not seen in all PBMC populations. For example, similar high levels of these molecules were seen in the monocytes of SLE patients and controls. Notably, there was no correlation between the mRNA levels of the various signaling molecules and cell surface or intracellular protein expression, suggesting that posttranslational mechanisms play an important role in the regulation of the levels of these molecules.

Altered expression of TNF-α-related signaling molecules is seen in several B and T cell subpopulations of SLE patients. SLE patients have altered proportions of B and T cell subpopulations, such as memory cells²⁹⁻³₄. Since expression of TNF-related signaling molecules varies in different cellular populations³⁵⁻³⁶, it is possible that the altered proportion of cells expressing these molecules in SLE reflects expansion of these cell subsets. To investigate this possibility, PBMC were stained with labeled antibodies to enable identification of various B and T cell subpopulations (Table 2).

As shown in Figure 3A and 3B, the levels of TNFR1, TNFR2, RIP, and TRAF2 were higher in memory/effector T cell and memory and pre-GC B cell subpopulations of both SLE patients and controls. Within each subpopulation, the differences in proportions of positively-staining cells between SLE patients and controls reflected variations in the levels of expression of these molecules rather than the presence of distinct positive and negative cell populations (Figure 3A). In SLE patients, with the exception of TNFR1, which was increased only in B cell subsets, similar trends to altered proportions of cells expressing each TNF-related signaling protein were seen in all cell subsets examined, which achieved statistical significance for many of these cell subsets (Figure 3B).

We previously noted an increased proportion of activated B cells within the naive B cell population of SLE patients.Π
Since antigen-experienced cell populations, such as memory and pre-GC cells, have higher levels of TNFR1, TNFR2, and TRAF2, we questioned whether the increased proportions of cells expressing these molecules were due to contamination of the “naive” B and T cell populations with activated cells. To address this possibility, we examined whether the increased levels of expression were restricted to activated cells, as measured by forward scatter (FSC). FSChi (activated) cells were not seen in the naive T cell population of SLE patients or controls, but were readily apparent in the naive B cell population of SLE patients. Although activated B cells (FSChi) had a higher level of TNFR2 than those that were resting (FSClo), the level of TNFR2 in the resting population was still increased in lupus patients as compared to controls (Figure 3C). Thus, the altered expression of several TNF-α-related signaling molecules observed in SLE is seen in multiple B and T cell subpopulations and is not due to increased activation within these subsets.

Altered expression of TNFR2, RIP, and TRAF2 in lupus patients does not correlate with disease activity or TNF-α levels. In general, for TNFR1, TNFR2, and TRAF2 the levels of expression in CD4+ T, CD8+ T, and B cell subsets were strongly correlated with each other (all r ≥ 0.420, p ≤ 0.008), but not with those in the monocyte population (with the
Figure 3. Expression of TNFR1, TNFR2, RIP, and TRAF2 on several distinct peripheral blood T cell and B cell subsets in lupus patients. Freshly isolated peripheral blood mononuclear cells (PBMC) were analyzed by 4-color flow cytometry. A. Histograms gated on all, naive (CD45RA+CD45RO–), and memory (CD45RA–CD45RO+) CD4+ T lymphocytes. Results for a representative control (shown in gray) have been overlaid on a representative SLE patient (shown in black). Markers indicate levels above those of the isotype control. The top row shows cells that have been stained with anti-TNFR2 and the bottom anti-TRAF2. Similar shifts in staining were seen for TNFR1 and RIP, and for other cell populations examined. B. Scatterplots show percentage of cells staining positively for the indicated signaling molecules in CD4+ T, CD8+ T, and B cell subpopulations: healthy controls (n = 25), SLE patients (n = 45). Populations have been gated as indicated in Table 2. Horizontal lines show the median for each population. P values calculated using the Mann-Whitney U test; *p < 0.05, **p < 0.005, ***p < 0.0005. C. Dot plots gated on the naïve B cell populations of a representative control and SLE patient. Levels of TNFR2 have been plotted against forward scatter (FSC), as an indicator of cell activation. Note increased expression of both FSCclO (resting) and FSCchi (activated) cell populations in the SLE patient compared to control. Similar results were obtained for TNFR1 and TRAF2.


does not appear to arise solely from increased cleavage of this molecule.

Incubation with TNF-α does not result in altered expression of TNF-α-related signaling molecules. Although there was no correlation between serum TNF-α levels and the altered expression of TNF-α-related signaling molecules in SLE patients, we investigated whether the altered levels of these molecules might arise in response to TNF-α signaling. To assess this possibility, freshly isolated PBMC from lupus patients and healthy controls were incubated with media alone or recombinant human TNF-α at various concentrations (from 5 ng/ml to 1 µg/ml) for 24 or 48 hours, and we examined expression of the various TNF-α-related signaling molecules by flow cytometry. No differences were seen between cells incubated in media alone and those with TNF-α, suggesting that the observed changes in lupus patients are not induced by TNF-α (data not shown).

Lack of correlation between TNF-α signaling abnormalities and increased caspase 3 activation in lupus patients. Altered expression of TNF-α-related signaling molecules can tilt the balance between pro- and antiapoptotic pathways. Since SLE patients have increased numbers of apoptotic cells, we examined whether the proportion of these cells correlated with differences in TNF signaling. Apoptotic cells in freshly isolated peripheral blood were detected by staining for intracellular expression of the cleaved activated form of caspase 3. Increased proportions of cells expressing activated caspase 3 were seen within all the PBMC subsets of SLE patients (Figure 4). This trend was also seen for each of the subpopulations within the various lymphoid subsets, but achieved statistical significance only for the naive CD4+ and CD8+ T cell subpopulations and memory T and B cell subpopulations. Although there was a strong positive correlation between the proportions of cells expressing activated caspase 3 in the various subpopulations of each lymphoid subset, there was no correlation between the proportions in the B and T subsets,
suggesting that the immune mechanisms leading to apoptosis in B and T cells differ. There was also no correlation between the proportion of cells expressing active caspase 3 in any of the lymphocyte subsets and the SLEDAI, serum levels of TNF-α, or expression of the TNF-related signaling molecules in lupus patients. Thus, the increased levels of apoptosis in lupus patients do not arise solely from TNF signaling.

DISCUSSION
We examined the expression of several TNF-related signaling molecules in SLE patients. We show that there are increased levels of TNFR1, TNFR2, and TRAF2, together with decreased levels of RIP, on the B and/or T cells of SLE patients as compared to controls. These findings contrast with those from examination of mRNA expression of corresponding molecules in PBMC17 and whole blood (reported here). Notably, there was no correlation between the levels of blood mRNA and protein expression, suggesting that there is substantial posttranscriptional regulation of these molecules. In addition, variation in the proportions of various cell populations rather than differences in the levels of TNF-related signaling molecule within each population may be contributing to this lack of correlation.

What is leading to the altered expression of TNF-α-related signaling molecules in SLE patients? In SLE patients and controls, antigen-experienced populations had elevated levels of TNFR1, TNFR2, TRAF, and RIP as compared to naive cells; however, it is unlikely that the elevated levels of TNFR1, TNFR2, and TRAF2 in the naive populations of SLE patients result from antigen engagement because the levels of these molecules are increased in the population as a whole, rather than in a subset of cells, as would be expected with antigen engagement. A variety of cytokines increase TNFR1 and/or TNFR2 expression, including interleukin 1β (IL-1β), IL-2, IL-4, IL-6, interferon-γ (IFN-γ), and TNF-α.36,39,40 Thus, the elevated levels of TNFR1 and TNFR2 could reflect the presence of increased levels of these cytokines, several of which have been reported to be increased in SLE. It is unlikely that the increased levels of TNF-α are leading to this altered expression since there was no correlation between serum TNF-α and the levels of TNFR1 or TNFR2. Further, we were unable to demonstrate altered expression of TNF-α-related signaling molecules following culture of PBMC with TNF-α in vitro. A preliminary study of a small number of patient samples also showed no obvious correlation between the levels of IL-1β, IL-2, IL-6, or IFN-γ and the levels of these molecules;
however, further studies examining additional patients and a broader array of proinflammatory factors will be necessary before definitively excluding this possibility. The cell-surface levels of TNFR1 and TNFR2 are also modified by cell-surface shedding, which is mediated predominantly by the metalloprotease, a disintegrin and metalloproteinase 17\(^{41}\). Although decreased shedding could lead to increased levels of cell-surface TNFR, in SLE patients the levels of soluble TNFR1 and TNFR2 are increased, and correlate with disease activity\(^4,6,37\), suggesting that there is increased shedding of these receptors that is driven by proinflammatory factors associated with disease activity. While we did not measure the serum levels of soluble TNFR1, sTNFR2 was elevated in our patient population. Although the observation that cell-surface levels of TNFR1 and TNFR2 are inversely correlated with disease activity might be consistent with increased shedding in active disease, we found no correlation between the levels of sTNFR2 and cell-surface TNFR2. Thus, it is likely that the etiology of the relative reduction in TNFR1 and TNFR2 with active disease is multifactorial.

In addition to production, the levels of RIP are regulated by proteasomal degradation\(^42\). This process in governed by TNFαIP3, which is upregulated following TNF and Toll-like receptor stimulation, resulting in increased K48 ubiquitination of RIP targeting it for degradation. Thus, the reduced levels of RIP in the lymphocytes of some SLE patients could be a consequence of TNF-α signaling. Consistent with this possibility, there was a trend to negative association between the levels of RIP and serum TNF-α levels.

Regardless of the mechanisms leading to altered expression of TNF-α-related signaling molecules in SLE, it is likely that these changes are functionally relevant, since variations in the levels of TNF receptors and TRAF2 have been shown to be associated with altered function of lymphocytes. For example, in aged individuals increased expression of TNFR1 and TRADD, together with decreased expression of TNFR2 and TRAF2, was associated with increased TNF-α-induced apoptosis of their lymphocytes\(^{16}\). The presence of increased levels of TNFR1 and TNFR2 together with TRAF2 in SLE suggests that the antiapoptotic, as opposed to the proapoptotic, effects of TNF may be augmented. Since TNF is a growth factor for B lymphocytes\(^{43}\) and short-term stimulation of activated T lymphocytes with TNF also augments T cell activation, proliferation, and production of IFN-γ\(^{44,45}\), it is likely that this contributes to the expansion of antigen-engaged self-reactive lymphocytes in patients with SLE.

Our results do not support a direct link between TNF signaling and the increased apoptosis that is seen in the PBMC of SLE patients, and contrast with the findings of Aringer, et al, who found an association between levels of sTNFR2, which they argued was a surrogate for TNF-α levels, and the proportion of dead cells in SLE\(^{57}\). It is unlikely that this difference arises from the different measures of TNF-α levels, because comparison of the levels of sTNFR2 with the proportion of active caspase 3+ cells in the various cell populations in our study failed to reveal a correlation (data not shown). An important difference between our study and the study of Aringer is that we measured apoptosis in cells immediately \(ex vivo\), whereas Aringer and colleagues measured apoptosis after 24 h culture of PBMC in serum-free media. Thus the increased apoptosis observed by that group may reflect the absence of TNF or other growth factors rather than apoptosis induced by TNF signaling.

REFERENCES


