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+ T, 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

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a breakdown of self-tolerance leading to autoantibody production and formation of immune complexes that deposit in multiple organs causing inflammation and tissue damage[1]. Tumor necrosis factor-α (TNF-α), a pleiotropic cytokine that has both immunoregulatory and proinflammatory effects[2,3], is increased in SLE and correlates with disease activity[4,5,6]. Increased TNF-α levels have also been demonstrated in the renal tissue of patients with SLE and are associated with active renal disease[7,8,9]. In murine lupus, administration of TNF-α has been shown to exacerbate renal inflammation and damage[10], whereas TNF-α-deficient mice demonstrate enhanced autoimmunity[11]. Similar findings have been observed in humans, where TNF-α blockade leads to increased levels of autoantibody production, but improvement in inflammatory arthritis and nephritis[12].

TNF-α mediates its biologic effects through 2 receptors, TNF-α receptor type I (TNFR1) and TNF-α receptor type II (TNFR2). Ligand-mediated trimerization of these receptors leads to recruitment of intracellular adapters that activate

---

From the Arthritis Centre of Excellence and Toronto Western Hospital Research Institute, Divisions of Genetics and Development and Population Health, Toronto Western Hospital; Departments of Medicine and Immunology, University of Toronto, Toronto; Department of Nephrology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, Peoples Republic of China; and Centre for Prognosis Studies in the Rheumatic Diseases, Toronto Western Hospital, Toronto, Ontario, Canada.

Supported by a New Emerging Team grant from the Canadian Institutes of Health Research (CIHR; to J. Wither and P. Fortin). Dr. Wither is the recipient of salary support from the Arthritis Centre of Excellence. Dr. Fortin is a Distinguished Senior Research Investigator of The Arthritis Society and receives support as Director of Clinical Research of the Arthritis Centre of Excellence. Dr. Zhu is the recipient of a China Scholarship Council Award. Dr. Yang is the recipient of Chinese National Natural Science Research Grant no. 30572290.

L-J. Zhu, MD, PhD, Arthritis Centre of Excellence and Toronto Western Hospital Research Institute; Department of Rheumatology, The Second Affiliated Hospital, Sun Yat-Sen University; C. Landolt-Marticorena, MD, PhD, FRCPC; T. Li, MSc; D.D. Gladman, MD, FRCPC; M.B. Urowitz, MD, FRCPC; P.R. Fortin, MD, MPH, FRCPC, Arthritis Centre of Excellence and Toronto Western Hospital Research Institute, Toronto Western Hospital; Department of Medicine, University of Toronto; J. Wither, MD, PhD, FRCPC, Arthritis Centre of Excellence and Toronto Western Hospital Research Institute, Toronto Western Hospital; Departments of Medicine and Immunology, University of Toronto; X. Yang, MD, PhD; X-Q. Yu, MD, PhD, Department of Nephrology, First Affiliated Hospital, Sun Yat-Sen University.

*Address correspondence to Dr. J.E. Wither, Toronto Western Hospital, 1E420, 399 Bathurst Street, Toronto, ON, M5T 2S8, Canada.
E-mail: jwither@uhnres.utoronto.ca*

Accepted for publication March 10, 2010.

multiple signal transduction pathways[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-κB (NF-κB) and c-Jun N-terminal kinase, respectively. Trimerization of TNFR2 also results in activation of NF-κB 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[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[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-α signaling in SLE patients favoring cellular activation, as opposed to proapoptotic pathways.

MATERIALS AND METHODS

Subjects. Forty-five patients who satisfied ≥ 4 revised 1997 American College of Rheumatology classification criteria for SLE[18] were recruited from the University of Toronto Lupus Clinic. Disease activity was measured using the SLE Disease Activity Index 2000 (SLEDAI-2K)[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.

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

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-active 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-CD4 (RPA-T4), anti-CD8 (RPA-T8), and anti-CD14 (MϕP9); PerCP-Cy5.5-conjugated IgG2a (MOPC-173); and PE-Cy™5-conjugated anti-CD19 (HIB19) and anti-CD45RO (UCHL1). Additional antibodies were purchased from the following sources: allophycocyanin-conjugated anti-CD38 (IB6) and PE-conjugated IgG2a (S43.10), Miltenyi Biotec, Auburn, CA, USA; PE-conjugated anti-TNFR1 (16803.1), anti-TNFR2 (22235), and anti-TRADD (313203), and FITC-conjugated anti-TNFR2 (22235), 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 F(ab')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[20,21,22,23,24,25,26,27,28,29,30]. 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, ß-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

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

Cell Population	Surface Markers	Reference
CD4+ T cell subsets		
Naive CD4+ T cells	CD4+CD45RA+CD45RO–	20–22
Memory CD4+ T cells	CD4+CD45RA–CD45RO+	20–22
CD8+ T cell subsets		
Naive CD8+ T cells	CD8+CD45RA+CD45RO–	21, 22
Memory CD8+ T cells	CD8+CD45RA–CD45RO+	21, 22
CD19+ B cell subsets		
Memory CD19+ B cells	CD19+CD27+CD38–/+	23, 24
Mature naive CD19+ B cells	CD19+CD27–CD38–/+	25–27
Immature transitional cells	CD19+CD27–CD38++	28
Pre-germinal center	CD19+CD27+CD38++	29
Monocytes		
"Classical"	CD14+CD64+	30

ß-actin. TaqMan Gene Expression Primers were as follows: TNF-α (Hs99999043_m1), TNFR1 (Hs00236902_m1), TNFR2 (Hs00961752_m1), TRADD (Hs00182558_m1), RIP (Hs01041866_m1), TRAF2 (Hs01060310_m1), and TaqMan Pre-Developed Assay Reagents Human ACTB (ß-actin).

Measurement of serum TNF-α levels. Serum TNF-α and soluble TNFR2 (sTNFR2) were measured using human TNF-α and sTNFR2 ELISA detection kits (R&D Systems) according to the manufacturer's instructions.

Cell culture. Freshly isolated PBMC from lupus patients or controls (1×10^6 cells/well in 24-well plates) were cultured for 24 or 48 h in media alone (RPMI-1640 with additives) or together with recombinant human TNF-α (from 5 ng/ml to 1 μg/ml; R&D Systems). At the end of the culture period cells were harvested, washed, and stained for flow cytometry.

Statistical analysis. Data are expressed as the mean ± SD. The Mann-Whitney nonparametric test was used for comparisons between patients and controls. Correlations between the experimental and clinical variables were determined by linear regression analysis using Spearman's rank correlation coefficients.

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)[4,5,6]. We have previously shown that mRNA levels of several TNF signaling adapters were altered in PBMC of individuals with SLE[17]. 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 observed[17]. 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.

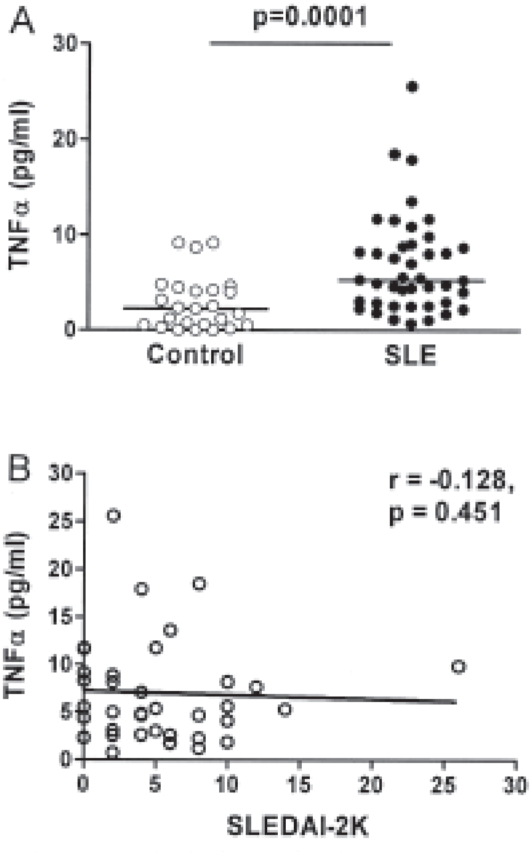


Figure 1. Elevated serum levels of TNF-α from lupus patients. A. Scatterplot showing serum TNF-α levels in SLE patients (n = 45) and healthy controls (n = 25), measured by ELISA. Horizontal lines show the median for each population. P value calculated using the Mann-Whitney U test. B. Linear regression plot showing the lack of correlation between serum TNF-α levels and the SLEDAI-2K. P and r values calculated using Spearman's rank correlation coefficients.

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 cells[29,31,32,33,34]. Since expression of TNF-related signaling molecules varies in different cellular populations[35,36], 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[31].

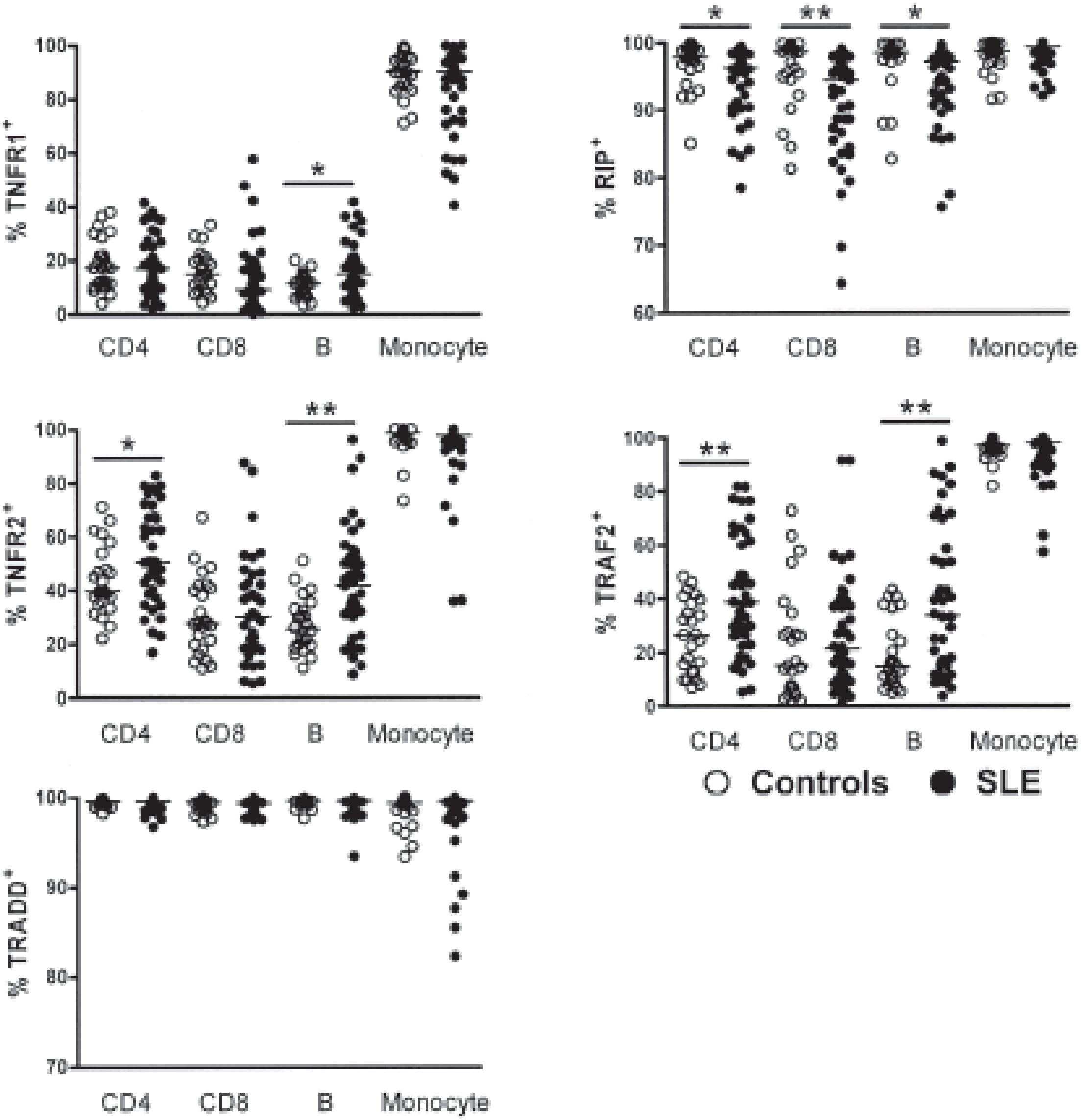


Figure 2. Expression of various TNF-related signaling molecules in peripheral blood mononuclear cells (PBMC) from healthy controls (n = 25) and SLE patients (n = 45). Freshly isolated PBMC were analyzed by 4-color flow cytometry. Scatterplots show the percentage of cells staining positively for the indicated signaling molecules in CD4+ T, CD8+ T, B, and monocyte populations. Horizontal lines show the median for each population. P values for the difference between controls and SLE patients calculated using the Mann-Whitney U test; $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

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 $r \geq 0.420$, $p \leq 0.008$), 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 sent 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 naive 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 FSClo (resting) and FSChi (activated) cell populations in the SLE patient compared to control. Similar results were obtained for TNFR1 and TRAF2.

exception of a correlation for TNFR2, p = 0.005). For RIP, the expression levels in B and CD8+ T cells correlated with each other (r = 0.626, $p < 0.001$) but not with those in the CD4+ T cell populations. Within each lymphoid cell subset, the expression levels in the various subpopulations strongly correlated with those in the subset as a whole and each other, suggesting that the mechanisms leading to altered expression of TNF-α-related signaling molecules within the different lymphoid populations are similar.

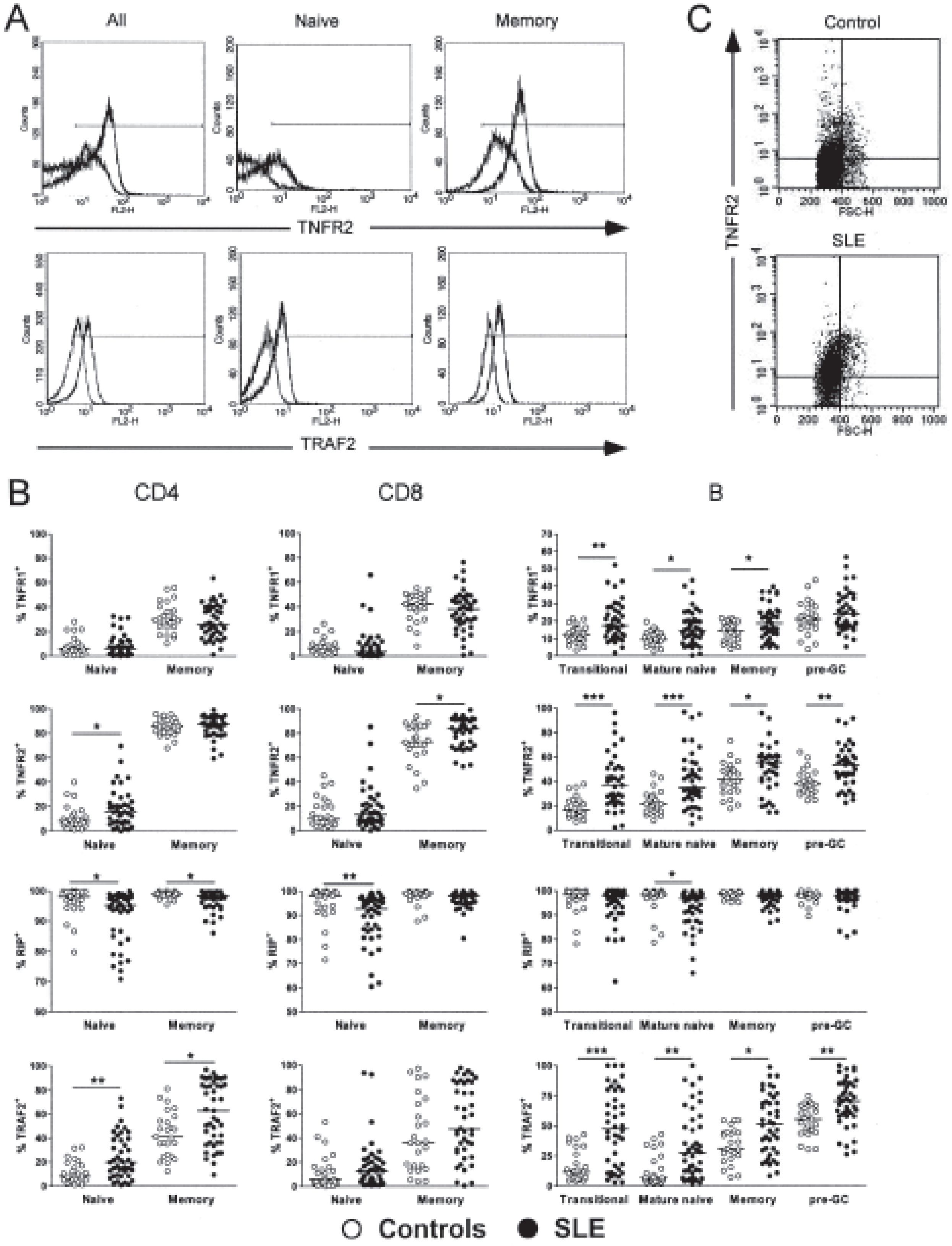
To determine whether the same mechanism was driving altered expression of the TNFR1, TNFR2, RIP, and TRAF2, the association between the levels of these molecules within each lymphoid subset was examined. For B, CD4+ T, and CD8+ T cells, expression of TNFR1 and TNFR2 was positively correlated with each other but not with RIP or TRAF2 (all $r \geq 0.668$, $p \leq 0.001$ for TNFR1 and TNFR2). Similar findings were observed for monocytes for TNFR1 and TNFR2 (r = 0.463, p = 0.003). However the levels of TNFR2 and RIP (r = 0.464, p = 0.002) and RIP and TRAF2 (r = 0.4, p = 0.008) were also positively correlated in this population. Thus, it is likely that the mechanisms driving the altered expression of TNFR1 and TNFR2 in lupus differ, at least in part, from those leading to altered expression of RIP and TRAF2.

For TNFR2, RIP, and TRAF2 there were a number of patient outliers with values above (for TNFR2 and TRAF2) or below (for RIP) those seen for normal controls. Notably, there was no significant difference in prednisone dose or the proportion of patients on specific drug treatments between individuals with these outlier values and those within the range seen for normal controls. There was also no correlation between the levels of RIP or TRAF2 in any of the lymphoid subsets and disease activity as measured by the SLEDAI. Expression of TNFR1 in CD4+ (including both the naive and memory subpopulations) and CD8+ T cells was negatively correlated with the SLEDAI-2K (r = –0.594 and –0.423, $p < 0.0001$ and $p < 0.011$, respectively, for CD4+ and CD8+ T cells). Similar findings were observed for TNFR2 in CD4+ and CD8+ T cell subsets (r = –0.359 and –0.386, p = 0.037 and p = 0.026, respectively).

As the levels of serum sTNFR2 are elevated in SLE and have been shown to correlate with disease activity[4,6], we questioned whether the negative correlation between cell-surface TNFR2 and disease activity reflected the increased cleavage of this molecule with active disease. To address this question we measured the levels of serum sTNFR2 in the SLE and control populations. As reported[6], the levels of sTNFR2 were significantly increased in SLE patients compared to controls (sTNFR2 in SLE = 414.3 ± 158.3 pg/ml; in controls = 163.4 ± 40.8 pg/ml; $p < 0.0001$); however, no correlation was observed between the levels of sTNFR2 and disease activity or cell-surface TNFR2 in the SLE patients. Thus, the negative correlation between cell-surface TNFR2 and disease activity 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[16]. Since SLE patients have increased numbers of apoptotic cells[37,38], 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,


A
All
Naive
Memory
Counts
FL2-H
TNFR2
TRAF2
C
Control
SLE
TNFR2
FSC-H
B
CD4
CD8
B
% TNFR1+
% TNFR2+
% RIP+
% TRAF2+
Naive
Memory
Transitional
Mature naive
Memory
pre-GC
*
**
***
○ Controls
● SLE

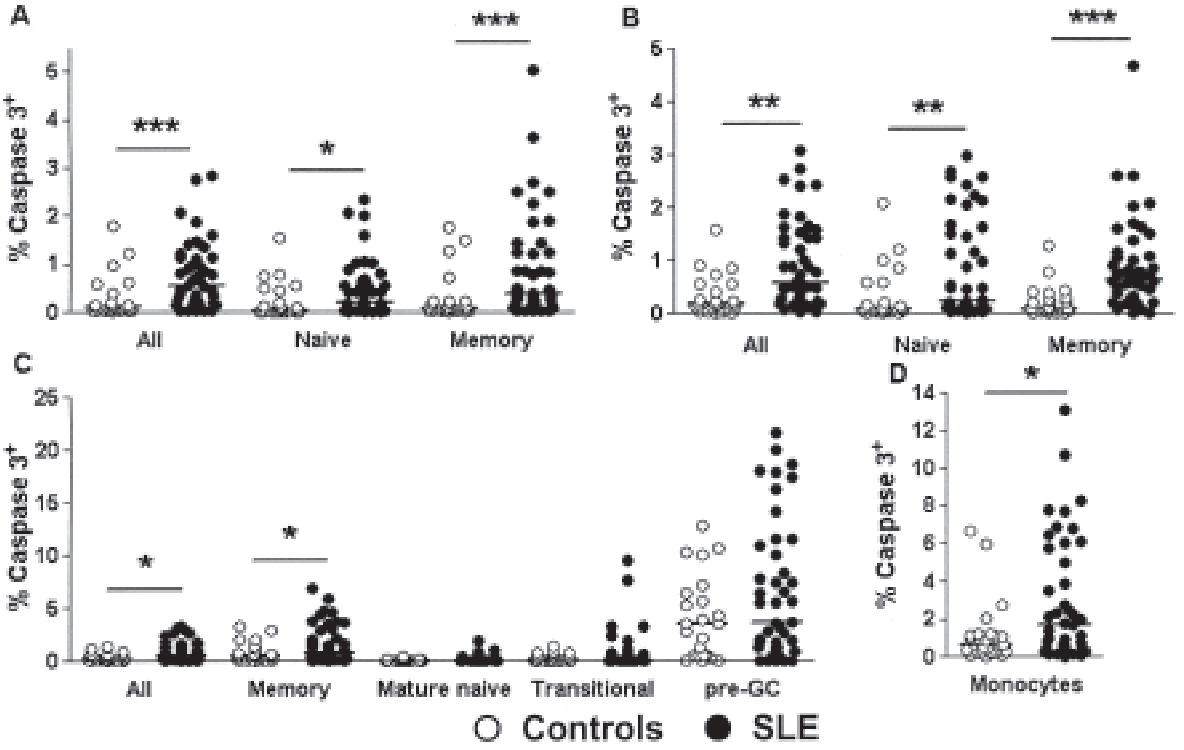


Figure 4. Increased activation of caspase 3 in several distinct peripheral blood T cell and B cell subsets in lupus patients. Freshly isolated peripheral blood mononuclear cells (PBMC) were analyzed by 4-color flow cytometry. Scatterplots show the percentage of cells staining positively for the cleaved activated form of caspase 3 as an indicator of apoptosis. Results are shown for subpopulations within the (A) CD4+ T; (B) CD8+ T; (C) B cell; and (D) monocyte population cell subsets (gated as indicated in Table 2): healthy controls (n = 25) and SLE patients (n = 45). Horizontal lines show the median for each population examined. P values were calculated using the Mann-Whitney U test; $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

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 PBMC[17] 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 1ß (IL-1ß), 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-1ß, 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 TNFAIP3, 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[37]. 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

1. Manson JJ, Isenberg DA. The pathogenesis of systemic lupus erythematosus. Neth J Med 2003;61:343-6.
2. Aringer M, Smolen JS. The role of tumor necrosis factor-alpha in systemic lupus erythematosus. Arthritis Res Ther 2008;10:202.
3. O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. Nat Rev Immunol 2002;2:37-45.
4. Aderka D, Wysenbeek A, Engelmann H, Cope AP, Brennan F, Molad Y, et al. Correlation between serum levels of soluble tumor necrosis factor receptor and disease activity in systemic lupus erythematosus. Arthritis Rheum 1993;36:1111-20.
5. Maury CP, Teppo AM. Tumor necrosis factor in the serum of patients with systemic lupus erythematosus. Arthritis Rheum 1989;32:146-50.
6. Studnicka-Benke A, Steiner G, Petera P, Smolen JS. Tumour necrosis factor alpha and its soluble receptors parallel clinical disease and autoimmune activity in systemic lupus erythematosus. Br J Rheumatol 1996;35:1067-74.
7. Herrera-Esparza R, Barbosa-Cisneros O, Villalobos-Hurtado R, Valos-Diaz E. Renal expression of IL-6 and TNF alpha genes in lupus nephritis. Lupus 1998;7:154-8.
8. Malide D, Russo P, Bendayan M. Presence of tumor necrosis factor alpha and interleukin-6 in renal mesangial cells of lupus nephritis patients. Hum Pathol 1995;26:558-64.
9. Takemura T, Yoshioka K, Murakami K, Akano N, Okada M, Aya N, et al. Cellular localization of inflammatory cytokines in human glomerulonephritis. Virchows Arch 1994;424:459-64.
10. Brennan DC, Yui MA, Wuthrich RP, Kelley VE. Tumor necrosis factor and IL-1 in New Zealand Black/White mice. Enhanced gene expression and acceleration of renal injury. J Immunol 1989;143:3470-5.
11. Kontoyiannis D, Kollias G. Accelerated autoimmunity and lupus nephritis in NZB mice with an engineered heterozygous deficiency in tumor necrosis factor. Eur J Immunol 2000;30:2038-47.
12. Aringer M, Graninger WB, Steiner G, Smolen JS. Safety and efficacy of tumor necrosis factor alpha blockade in systemic lupus erythematosus: an open-label study. Arthritis Rheum 2004; 50:3161-9.
13. Lee NK, Lee SY. Modulation of life and death by the tumor necrosis factor receptor-associated factors (TRAFs). J Biochem Mol Biol 2002;35:61-6.
14. Li H, Lin X. Positive and negative signaling components involved in TNF alpha-induced NF-kappa B activation. Cytokine 2008; 41:1-8.
15. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001; 104:487-501.
16. Aggarwal S, Gollapudi S, Gupta S. Increased TNF-alpha-induced apoptosis in lymphocytes from aged humans: changes in TNF-alpha receptor expression and activation of caspases. J Immunol 1999;162:2154-61.
17. Zhu L, Yang X, Chen W, Li X, Ji Y, Mao H, et al. Decreased expressions of the TNF-alpha signaling adapters in peripheral blood

mononuclear cells (PBMCs) are correlated with disease activity in patients with systemic lupus erythematosus. Clin Rheumatol 2007;26:1481-9.

18. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997;40:1725.
19. Gladman DD, Ibanez D, Urowitz MB. Systemic Lupus Erythematosus Disease Activity Index 2000. J Rheumatol 2002;29:288-91.
20. Bell EB, Sparshott SM, Bunce C. CD4+ T-cell memory, CD45R subsets and the persistence of antigen — a unifying concept. Immunol Today 1998;19:60-4.
21. Mackall CL, Hakim FT, Gress RE. T-cell regeneration: all repertoires are not created equal. Immunol Today 1997;18:245-51.
22. Pilarski LM, Gillitzer R, Zola H, Shortman K, Scollay R. Definition of the thymic generative lineage by selective expression of high molecular weight isoforms of CD45 (T200). Eur J Immunol 1989;19:589-97.
23. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 1998;188:1679-89.
24. Tangye SG, Liu YJ, Aversa G, Phillips JH, de Vries JE. Identification of functional human splenic memory B cells by expression of CD148 and CD27. J Exp Med 1998;188:1691-703.
25. Bohnhorst JO, Bjorgan MB, Thoen JE, Natvig JB, Thompson KM. Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren’s syndrome. J Immunol 2001;167:3610-8.
26. Hutloff A, Buchner K, Reiter K, Baelde HJ, Odendahl M, Jacobi A, et al. Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. Arthritis Rheum 2004;50:3211-20.
27. Pascual V, Liu YJ, Magalski A, de Bouteiller O, Banchereau J, Capra JD. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med 1994;180:329-39.
28. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood 2005;105:4390-8.
29. Arce E, Jackson DG, Gill MA, Bennett LB, Banchereau J, Pascual V. Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus. J Immunol 2001;167:2361-9.
30. Tacke F, Randolph GJ. Migratory fate and differentiation of blood monocyte subsets. Immunobiology 2006;211:609-18.
31. Chang NH, McKenzie T, Bonventi G, Landolt-Marticorena C, Fortin PR, Gladman D, et al. Expanded population of activated antigen-engaged cells within the naive B cell compartment of patients with systemic lupus erythematosus. J Immunol 2008;180:1276-84.
32. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, et al. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. J Immunol 2000;165:5970-9.
33. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol 2004;113:161-71.
34. Wither J, Cai YC, Lim S, McKenzie T, Roslin N, Claudio JO, et al. Reduced proportions of natural killer T cells are present in the relatives of lupus patients and are associated with autoimmunity. Arthritis Res Ther 2008;10:R108.
35. Erikstein BK, Smeland EB, Blomhoff HK, Funderud S, Prydz K, Lesslauer W, et al. Independent regulation of 55-kDa and 75-kDa tumor necrosis factor receptors during activation of human peripheral blood B lymphocytes. Eur J Immunol 1991;21:1033-7.
36. Ware CF, Crowe PD, Vanarsdale TL, Andrews JL, Grayson MH, Jerzy R, et al. Tumor necrosis factor (TNF) receptor expression in T lymphocytes. Differential regulation of the type I TNF receptor during activation of resting and effector T cells. J Immunol 1991;147:4229-38.
37. Aringer M, Feierl E, Steiner G, Stummvoll GH, Hofler E, Steiner CW, et al. Increased bioactive TNF in human systemic lupus erythematosus: associations with cell death. Lupus 2002;11:102-8.
38. Emlen W, Niebur J, Kadera R. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. J Immunol 1994;152:3685-92.
39. Dett CA, Gatanaga M, Ininns EK, Cappuccini F, Yamamoto RS, Granger GA, et al. Enhancement of lymphokine-activated T killer cell tumor necrosis factor receptor mRNA transcription, tumor necrosis factor receptor membrane expression, and tumor necrosis factor/lymphotoxin release by IL-1 beta, IL-4, and IL-6 in vitro. J Immunol 1991;146:1522-6.
40. Reddy J, Chastagner P, Fiette L, Liu X, Theze J. IL-2-induced tumor necrosis factor (TNF)-beta expression: further analysis in the IL-2 knockout model, and comparison with TNF-alpha, lymphotoxin-beta, TNFR1 and TNFR2 modulation. Int Immunol 2001;13:135-47.
41. Bell JH, Herrera AH, Li Y, Walcheck B. Role of ADAM17 in the ectodomain shedding of TNF-alpha and its receptors by neutrophils and macrophages. J Leukoc Biol 2007;82:173-6.
42. Wertz IE, O’Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, et al. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappa B signalling. Nature 2004;430:694-9.
43. Boussiotis VA, Nadler LM, Strominger JL, Goldfeld AE. Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. Proc Natl Acad Sci USA 1994;91:7007-11.
44. Scheurich P, Thoma B, Ucer U, Pfizenmaier K. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-alpha: induction of TNF receptors on human T cells and TNF-alpha-mediated enhancement of T cell responses. J Immunol 1987;138:1786-90.
45. Yokota S, Geppert TD, Lipsky PE. Enhancement of antigen- and mitogen-induced human T lymphocyte proliferation by tumor necrosis factor-alpha. J Immunol 1988;140:531-6.