Expression of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4; CD152) on peripheral blood T lymphocytes in systemic lupus erythematosus children

Mohamed H. Ezzat1, Safaa S. Imam1, Manal M. Yassin2, Ibrahim M. Ibrahim1

Departments of Pediatrics1 and Microbiology and Immunology2
Faculty of Medicine, Ain Shams University, Cairo, Egypt

Background: CTLA-4 (CD152) encodes cytotoxic T lymphocyte-associated antigen-4, a cell-surface molecule providing a negative signal for T-cell activation. CTLA-4/B7 is the most important costimulation-signaling pathway that regulates T cell responses and plays a critical role in maintenance and breakdown of self-tolerance, and hence in susceptibility to autoimmune diseases.

Objective: The aim of this study was to investigate and evaluate the expression of CTLA-4 on peripheral blood T-lymphocytes (PBTL) in children with systemic lupus erythematosus (SLE) in relation to clinical features; disease activity and severity.

Methods: From December 2006 to August 2007, 32 pediatric patients (30 girls, 2 boys) fulfilled at least four of the 1997 revised criteria for the classification of SLE were enrolled in this study. Expression of CTLA-4 on freshly isolated PBTL was assayed by flow cytometry in all SLE patients during activity and remission in addition to 32 age- and sex-matched children serving as controls. Results were expressed as percentage of PBTL cells expressing surface CTLA-4 molecule in comparison to isotype-matched controls. CTLA-4+PBTL % were correlated with some SLE disease activity and severity variables.

Results: CTLA-4 expression on freshly isolated PBTL was significantly higher in SLE patients during disease activity (median = 12; mean ± SD = 10.45 ± 8.3%) than controls (median = 4; mean ± SD = 3.34 ± 3.1%; p < 0.0001). The patients’ values were statistically comparable during quiescence (median = 14; mean ± SD = 15.02 ± 7.1 %; p > 0.05) and activity. Among SLE patients, the median and mean ± SD of CTLA-4+PBTL % of children with lupus nephritis was significantly higher than those without nephritis (15; 13.56 ± 10.4 % versus 10; 9.51 ± 9.1%; p < 0.01). CTLA-4 expression could be related to lupus severity but there was no correlation with disease activity. A positive correlation could be elicited between CTLA-4+PBTL % during lupus activity and the corresponding values during remission. CTLA-4+PBTL % correlated positively with the anti-dsDNA autoantibodies titers, serum creatinine, and 24 hours urinary protein excretion. On the other side, the percentages correlated inversely with the estimated creatinine clearance and serum C3 and C4 levels. CTLA-4 expression did not vary according to therapy.

Conclusion: CTLA-4 surface expression on PBTL in SLE children was up regulated irrespective of lupus activity. The over expression was related to lupus severity and might have a significant role in the pathogenesis of lupus nephritis and cerebritis.

Key words: B7; CD152; costimulatory molecules; CTLA-4; cytotoxic T-lymphocyte; lupus nephritis; systemic lupus erythematosus

INTRODUCTION

The regulation of T cell-mediated immune responses requires a balance between amplification and generation of effector function and subsequent selective termination by clonal deletion. Although apoptosis of previously activated T cells can be induced by signaling of the tumor necrosis factor (TNF) receptor family, these molecules do not appear to regulate T-cell clonal deletion in an antigen-specific fashion (1).

T-cell activation requires at least two signals. The first is the interaction between antigen-specific T-cell receptors (TCRs) and the major histocompatibility complex (MHC). The second is a costimulatory signal that is necessary to achieve complete activation. In the absence of the second signal, responding T cells become anergic (2).

Costimulatory molecules are cell surface glycoproteins that can modulate TCRs signals. The B7-family of costimulatory molecules: B7-1 (CD80) and B7-2 (CD86); the two B7 receptors CD28 and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4; CD152) and the inducible costimulator molecule (ICOS/B7RP-1) provide key second signals that can regulate the activation, inhibition and fine-tuning of T-lymphocyte responses. Although predominantly expressed on professional antigen-presenting cells (APCs), recent evidence shows that the B7 molecules are also expressed on T cells (3).

CTLA-4 (CD152), a type I transmembrane glycoprotein with a native molecular weight of 33-37 kDa, is a member of the immunoglobulin supergene family and was initially discovered during a search for molecules involved in T cell cytotoxicity. Of the 186 amino acid residues, 125 are located extracellularly while 37 compose the cytoplasmic domain. CTLA-4 is homologous to CD28 and shares 31% overall amino acid identity, however, its function is different from CD28. CD28 is expressed constitutively on T-cells, whereas CTLA-4 is T-cell-restricted and is expressed transiently and exclusively on activated CD4+ and CD8+ T cells after T-cell activation.
Sjögren's syndrome (9) and rheumatoid arthritis (10). CTLA-4 is the only non-HLA locus. Based on function and data from genome-wide scans, the CTLA-4 locus is the only non-HLA locus. The CTLA-4 gene is highly polymorphic. The CTLA-4 locus is the only non-HLA locus. Based on function and data from genome-wide scans, the gene encoding CTLA-4 has been suggested as a candidate gene for conferring susceptibility to autoimmunity, at least to insulin-dependent diabetes mellitus (IDDM) and systemic lupus erythematosus (SLE) (7). Moreover, CTLA-4 single-nucleotide polymorphisms have been widely studied in connection with genetic susceptibility to various autoimmune diseases (8) namely; Graves' disease, multiple sclerosis, Sjögren's syndrome (9) and rheumatoid arthritis (10). Among these polymorphisms, are the +49GA polymorphism in exon 1 and the CT60A/G polymorphism in the 3’untranslated region (11). There is a burst of papers describing an important role for B7/CTLA-4 interaction in the Th1/Th2 balance. Of clinical interest are observations that CTLA-4 plays a role in bronchial asthma (4) and autoimmune disorders (7-11).

SLE is an autoimmune disorder that includes abnormalities of T-lymphocytes, as well as hyperreactive B-cells that produce autoantibodies. Lupus peripheral blood T-lymphocytes (PBTL) often contain activated T-cells and auto-reactive T-cells that are postulated to be involved in the pathogenesis of SLE. One possible explanation for these abnormal activations in lupus lymphocytes is the lack or modification in the auto-reactive elimination of activated T-cells that respond to foreign antigens and in clonal deletion of auto-reactive T-cells in the periphery (7). The expression of CTLA-4 in relation to the clinical features and activity of juvenile-onset SLE and the function of CTLA-4 were not thoroughly studied. Therefore, we aimed to explore the expression of CTLA-4 on freshly isolated PBTL of SLE children in relation to the clinical and laboratory variables of disease activity and severity. Exploring this pathway may provide clues to novel therapeutic strategies to delete antigen-specific activated T cells in autoimmune diseases.

**SUBJECTS AND METHODS**

**PATIENTS**

This follow-up case-control study was conducted at the Pediatric Allergy and Immunology Unit of Ain Shams University Children’s Hospital during the period from December 2006 to August 2007. All participants provided written informed consent following explanation of the study. The study comprised the following groups:

1-**Systemic Lupus Erythematosus (SLE):**

This group comprised 32 patients with juvenile-onset SLE fulfilled at least four of the 1997 American College of Rheumatology revised criteria for the classification of SLE according to Hochberg (12). They were 30 females (93.75%) and 2 males (6.25%), with a female to male ratio of 15:1. Their ages ranged from 8 to 15 years with a mean age of 11.5 ± 1.04 years. Their duration of illness ranged from 1 to 7 years with a mean duration of 4 ± 0.97 years. Fourteen patients (43.75%) were treated initially with monthly pulse methylprednisolone (500-1000 mg/m²), followed by oral prednisone. Fourteen patients (43.75%) were treated with monthly pulse cyclophosphamide therapy (600 mg/m²/month for 6 months followed by every 3 months therapy for 24 months). Four patients (12.5%) received oral azathioprine therapy (2 mg/kg/day). All children treated with cytotoxic drugs received small doses of oral steroids as well.

2-**Control group:**

Thirty-two clinically healthy children age and sex-matched to SLE patients were enrolled for the purpose of comparison of the laboratory data. They were 29 females (90.6%) and 3 males (9.4%), with a female to male ratio of 9.7:1. Their ages ranged from 6 to 16 years, with a mean age of 12.03 ± 2.01 years. Their duration of illness particular included rheumatic diseases, co-existing chronic inflammation, and active infections.
Assessment of SLE disease activity and severity:
Disease activity was assessed at the time of inclusion in the study by applying scores on the SLE disease activity index 2000 (SLEDAI)\(^\text{(13)}\), physician global assessment (PGA)\(^\text{(14)}\), and measuring the laboratory markers of active inflammation (including ESR, serum levels of C3 and C4, and anti-double-stranded DNA [dsDNA] antibody titers). For convenience, patients with SLEDAI scores of \(\geq 4\) were defined as having active SLE.

Flares were defined according to Gilkeson and coworkers,\(^\text{(14)}\) as an increase in the PGA of 2 or more. The PGA was graded on a scale of 0-3 and was the physician’s subjective opinion of the disease activity at the time of patient visit, with “0” representing no activity, “1” mild activity, “2” moderate activity and “3” marked activity. Decrease in disease activity was determined as a return of PGA to baseline. Based on selection via the PGA, disease flares occurred in a number of different organ systems including skin, kidney and central nervous system. Lupus flares in this study were nephritis (\(n = 15\); 47%), cerebral vasculitis (\(n = 12\); 37.5%) and others (\(n = 5\); 15.5%) (cutaneous vasculitis, hepatitis, pericarditis, hemolytic anemia, and thrombocytopenic purpura).

Active lupus nephritis was defined according to Gonzalez-Crespo et al.,\(^\text{(15)}\), by one of the following: \(> 5\) red blood cells, \(> 5\) white blood cells, or any cast (red blood casts, granular, tubular or mixed casts) per high power field in the urinary sediment, proteinuria \(> 500\) mg / 24 hours or 3 plus (+++) if quantitation is not performed, serum creatinine \(> 110\) µmol/l, or creatinine clearance < 74.4 ml/min. Patients with stable deterioration, but in remission of renal disease, were included as non-active. Kidney stones, urinary tract infections and other causes of nephritis were excluded. Infection episodes were defined on clinical grounds and confirmed by microbiological results.

Lupus severity was defined according to van-Vollenhoven,\(^\text{(16)}\) as severe lupus (patients requiring doses of prednisone \(>\)10 mg/day and/or patients with severe renal or central nervous system involvement); otherwise patients were considered to have mild to moderate lupus (absence of severe nephritis or severe central nervous system disease). In this study, 24 patients (75%) with active lupus were classified as severe lupus and the remaining 8 patients (25%) as mild to moderate lupus.

METHODS
After full history taking and thorough clinical examination were done, all SLE patients and controls were subjected to the assay of CTLA-4 expression (%) on PBTL. Patients were subjected twice; first during disease activity, then they were followed-up clinically and by ESR until stabilization of their conditions and quiescence of symptoms by treatment (remission), when a follow-up second blood samples were obtained for re-assessment.

1-Clinical assessment: All children were subjected to history taking and clinical examination for revising the diagnosis, disease duration, initial clinical presentation, disease activity, severity and flares, and the concurrent therapy regarding type, dosage and response to therapy (at time of hematological analysis).

2-Laboratory Investigations:
Specimen Collection and Preparation:
- **Blood sampling**: Six ml of venous blood were collected by venipuncture under complete aseptic precautions from each SLE child and divided into 3 parts: first sample (2ml) into 3ml evacuated tubes containing ethylenediamine tetracacetate (EDTA) as an anticoagulant for the assay of plasma creatinine and CTLA-4 assay by flow cytometry; second sample (2ml) on sodium citrate in a ratio of 1:4 for ESR assay, and the third sample (2ml) was left to clot at room temperature for 30 minutes in a clean dry glass tube, centrifuged for 20 minutes at 1600g with separation of sera into sterile aliquots to be stored at -20°C till the time of assay of serum creatinine, anti-dsDNA antibodies, and C3 and C4. Repeated thawing and freezing was avoided. Hemolyzed or lipemic samples were excluded.
- **Urine samples**: 24 hours urine samples were collected in clean dry containers from SLE patients during activity for measuring total 24 hours urinary proteins, urinary creatinine and complete urine analysis.

A. Flow cytometric assay of (CTLA-4; CD152) expression on the surface of peripheral blood T lymphocytes:
This assay was performed to quantitatively determine the percentage of T cells bearing CTLA-4 on their cell surface and qualitatively determine the density of this receptor on T cell surfaces by flow cytometry. CTLA-4 assay was performed using Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL, USA). CTLA-4 monoclonal antibodies (monoclonal anti-human CTLA-4 [CD152]-Fluorescein FITC, Catalog Number: FAB325F), was purchased from R&D Systems, Inc. McKinley Place N.E., Minneapolis Minnesota, MN, USA.
- **Principle of the Test**: Washed cells were incubated with the fluorescein-labeled
monoclonal antibody that binds to the cells expressing human CTLA-4 (CD152). Unbound fluorescein-conjugated antibody was then washed from the cells. Cells expressing the CTLA-4 were fluorescently stained, with the intensity of staining directly proportional to the density of the CTLA-4. Cell surface expression of CTLA-4 was determined by flow cytometric analysis using 488 nm wavelength laser excitation. The surface expression of CTLA-4 was measured on the gated lymphocytes and results were expressed as percentage of cells expressing the predetermined marker in comparison to the isotype matched controls (i.e CTLA-4’ PBTL%).

**Peripheral blood T cells preparation:** Whole blood was collected in tubes containing EDTA as the anti-coagulant. Spleen cells were first mechanically disaggregated into a single cell suspension. Contaminating serum components were removed by washing the cells three times in an isotonic phosphate buffer (supplemented with 0.5% BSA) by centrifugation at 500 x g for 5 minutes. 50 µL of packed cells were then transferred to a 5 mL tube for staining with the monoclonal antibodies. Blood cells required lysis of RBC following the staining procedure.

**Sample Staining:** Cells used for staining with the antibody were first Fc-blocked by treatment with 1 µg of mouse IgG/10^5 cells for 15 minutes at room temperature. Excess blocking IgG from this reaction was not washed. 25 µL of the Fc-blocked cells (1 x 10^5 cells) or 50 µL of packed whole blood was transferred to a 5 mL tube. 10 µL of fluorescein-conjugated anti-human CTLA-4 reagent was added and incubated for 30-45 minutes at 2-8°C. Following this incubation, un-reacted anti-CTLA-4 reagent was removed by washing the cells twice in 200-400 µL of PBS buffer (the washing step described above). Noteworthy, the whole blood required a RBC lysis step at this point using a commercially available lysing reagent (R&D Systems’ Human Erythrocyte Lysing Kit, Cat. WL1000). Finally, the cells were resuspended in 200-400 µL of PBS buffer for final flow cytometric analysis. As a control for analysis, cells in a separate tube were treated with fluorescein-labeled mouse IgG2B antibody.

**B. Routine laboratory investigations done to SLE patients only for diagnosis of SLE activity and severity:**

1. Erythrocyte sedimentation rate (ESR; in mm/hour) 1st hour by Westergren method.
2. Quantitative measurement of serum anti-double stranded-DNA antibodies by EIA (anti-dsDNA Kit, Ortho Diagnostic, Raritan, NJ, USA), (Farr assay).
3. Complement 3 and 4 (C3 & C4) using turbidimetry on Turbitimer (Turbiquant C3 and C4; Behringwerke Diagnostics GmbH, Marburg, Germany).
4. Complete urine analysis.
5. Serum and urinary creatinine (Cr) using a modified rate Jaffe method on Synchron CX7 autoanalyzer (Beckman Instruments, Brea, California USA). Calculation of creatinine clearance was done from the following equation: clearance = urinary creatinine (mg/dl) x urinary volume (cc) x body surface area (m²) divided by (plasma creatinine x 1.73).

**Statistical Analyses:** All statistical analyses were carried out using SPSS software for Windows system (version 11.5; SPSS Inc, Chicago, IL). Data were expressed as mean, standard deviation (SD), median, and interquartiles range (IQR) (25th and 75th percentiles). For non-parametric data, Mann-Whitney test (Z-value) was used to compare between two groups, whereas, comparison between the same groups in two repeated measurements was done via Wilcoxon-signed rank test. Kruskall-Wallis test (H value) was used for comparison between more than two groups. As regards parametric data, ANOVA test (F-value) was used for comparison between more than two groups and the student’s “t” test of significance to compare between two groups. The correlation coefficient (r) was also used to interrelate the numeric variables. A P-value of less than 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

Table (1) shows that the CTLA-4’PBTL % in all SLE patients during disease activity ranged between 2 and 19 % (median = 12, mean ± SD = 10.45 ± 8.3%). These values were statistically comparable to the corresponding values of the same patients during quiescence, which ranged from 3 to 17 % (median = 14, mean ± SD = 15.02 ± 7.1 %) (Z = 0.6; p > 0.05). The healthy children had lower CTLA-4’PBTL % (range = 1 to 7%, median = 4, mean ± SD = 3.34 ± 3.1 %) as compared to the patients’ data whether during activity (Z = 5; p < 0.0001) or quiescence (Z = 5.6; p < 0.0001).

On comparing the three groups of lupus flares (nephritis, cerebritis, and other flares) together
using the Kruskall-Wallis test, the result revealed that the highest CTLA-4 expression was found in SLE children with renal flare (H value = 4; p < 0.001) (Fig. 1). The median and mean ± SD of CTLA-4+PBTL % in SLE children with renal flare (the nephritis positive group; n =15) were significantly higher in comparison to those without renal involvement (nephritis negative group; n = 17) (15; 13.56 ± 10.4 % versus 10; 9.51 ± 9.1%; Z = 1.2; p < 0.01). In addition, the percentage in both groups was significantly higher in comparison to the percentage of controls (p < 0.001) (Fig. 2).

The expression of CTLA-4 on PBTL cells was significantly higher in children enrolled with severe lupus (median and mean ± SD = 15 & 14.8 ± 11 %) as compared to those with mild to moderate lupus (7 & 7 ± 5.3 %; Z = 3; p < 0.001) (Fig. 3).

Within the study group, patients with active SLE disease had significantly higher SLEDAI scores, anti-dsDNA levels and daily prednisone dose than did patients with inactive SLE disease. The mean anti-dsDNA antibody serum levels and SLEDAI scores of patients with SLE were 30.56 ± 12.78 IU/ml (range: 15-50) and 15.35 ± 6.43 (range: 5-49) respectively. SLEDAI scores in patients with SLE was significantly correlated with serum anti-dsDNA levels (r = 0.66; p < 0.01).

Table (2) shows that during disease activity CTLA-4+PBTL percentage correlated positively with the serum creatinine (range = 0.3-2.6; mean ± SD = 1.2 ± 0.91 mg/dl; r = 0.53; p < 0.05) and 24 hours urinary protein excretion (range = 0.1-4.5; mean ± SD = 1.8 ± 0.72 gm/day; r = 0.67; p < 0.001). In addition, CTLA-4+PBTL% correlated positively with the anti-dsDNA autoantibodies titers (range= 15-50; mean = 30.56 ± 12.78 IU/ml; r = 0.83, p < 0.001). On the other side, CTLA-4+PBTL % correlated inversely with the estimated creatinine clearance (range = 10-129; mean ± SD = 57.48 ± 24.51 ml/min; r = -0.91; p < 0.0001) and serum C3 (range = 12-65; mean ± SD = 34.30 ± 9.46 mg/dl; r = -0.63; p < 0.01) and C4 levels (range = 5-26; mean ± SD = 12.85 ± 4.51; r = -0.52; p < 0.01).

A positive correlation could be elicited between the CTLA-4+PBTL% during lupus activity and the corresponding values during remission meaning that the higher the level got during exacerbations the higher it remained after remission (r = 0.83; p < 0.001). Moreover, CTLA-4+PBTL% could be related to the duration of illness (r = 0.65; p < 0.01).

CTLA-4+PBTL percentage could not be related to the total leukocyte count during both disease activity and remission (p > 0.05 for both); Could not be correlated to age, ESR, or SLEDAI score during or after subsidence of exacerbations. Moreover, there was no significant difference between males and females as regards CTLA-4+PBTL % expression neither in the studied patients nor in controls (p > 0.05 in both).

CTLA-4+PBTL expression did not vary according to therapy. The median and mean ± SD of CTLA-4+PBTL % in SLE children treated with monthly pulse methylprednisolone (17; 16.11 ± 14.8 % during activity and 15; 12.01 ± 11.2 % during remission) and those treated with cytotoxic drugs (16; 14.33 ± 13.1 % during activity and 14; 12.01 ± 11.23 % during remission) were statistically comparable both during activity (Z = 0.62; p > 0.05) and remission (Z = 0.81; p > 0.05).

Table (1): Percentage of peripheral blood T lymphocytes (PBTL) expressing cytotoxic T lymphocyte-associated antigen-4 (CTLA-4; CD152) in children with systemic lupus erythematosus (SLE) in comparison to healthy controls

<table>
<thead>
<tr>
<th>CTLA-4+ PBTL %</th>
<th>SLE (n = 32)</th>
<th>Control group (n = 32)</th>
<th>SLE (activity versus controls)</th>
<th>SLE (remission versus controls)</th>
<th>SLE (activity versus remission)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>2-19</td>
<td>3-17</td>
<td>1-7</td>
<td>Z = 5</td>
<td>Z = 5.6</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>10.45 ± 8.3</td>
<td>15.02 ± 7.1</td>
<td>3.34 ± 3.1</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Median</td>
<td>12</td>
<td>14</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interquartile range (IQR)</td>
<td>8</td>
<td>9</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mann–Whitney test was used in the comparisons
Figure (1): Box-plot summary of PBTL % expressing (CTLA-4; CD152) in SLE according to lupus flare. Kruskall-Wallis test was used in the comparisons. Black squares represent the median and the boxes encompass the interquartiles (25th and 75th percentiles). The ranges are marked as maximum and minimum.

Figure (2): Box-plot summary of PBTL % expressing (CTLA-4; CD152) in SLE according to the presence of nephritis. Mann–Whitney test was used in the comparisons.

Figure (3): Box-plot summary PBTL % expressing (CTLA-4; CD152) in SLE according to disease severity. Mann–Whitney test was used in the comparisons.
Table (2): Correlations between the percentage of PBTL expressing (CTLA-4; CD152) and studied parameters

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>CTLA-4⁺ PBTL %</th>
<th>r</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine “mg/dl”</td>
<td></td>
<td>0.53</td>
<td>&lt; 0.05 *</td>
</tr>
<tr>
<td>24 hours urinary protein excretion (gm/day)</td>
<td></td>
<td>0.67</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Serum anti-ds DNA antibodies (iu/ml)</td>
<td></td>
<td>0.83</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>-</td>
<td>0.91</td>
<td>&lt; 0.0001 *</td>
</tr>
<tr>
<td>Serum C3 (mg/dl)</td>
<td>-</td>
<td>0.63</td>
<td>&lt; 0.01 *</td>
</tr>
<tr>
<td>Serum C4 (mg/dl)</td>
<td>-</td>
<td>0.52</td>
<td>&lt; 0.01 *</td>
</tr>
</tbody>
</table>

* Significant

**DISCUSSION**

Recent advances in the understanding of T cell activation have led to new therapeutic approaches in the treatment of immunological disorders. Dendritic cells are believed to play an essential role in regulating the balance between immunogenic and tolerogenic responses to antigens by controlling T cell differentiation and activation via costimulatory and coinhibitory signals. One attractive target of intervention has been the blockade of T cell costimulatory pathways, which result in more selective effects on only those T cells that have encountered specific antigen. In fact, in some instances, costimulatory pathway antagonists can induce antigen-specific tolerance that prevents the progression of autoimmune diseases. The expression of CTLA-4 in both normal children and children with SLE has not been extensively studied. Therefore, we aimed to assay the frequency of freshly isolated PBTL expressing CTLA-4 in pediatric patients with SLE in comparison to a group of healthy age and sex-matched controls, in anticipation that the findings could help in understanding the possible role of CTLA-4 in the pathogenesis of pediatric SLE.

In this work the surface expression of CTLA-4 in SLE children was significantly higher than controls irrespective of lupus activity. This result is consistent with other adult studies and confirmed that CTLA-4 expression on PBTL cells in SLE patients was intact at the mRNA and at the protein levels and that intracellular expression in T-cells was significantly greater in SLE patients than controls. Worth mentioning that, the only study conducted in children was that of Lee et al., who reported increased expression of CTLA-4 mRNA in CD4⁺ T cells in children with active SLE compared with children with inactive disease and controls.

The difference in CTLA-4 expression between SLE children and controls could not be explained by age or sex distribution since matched controls were used. In addition, no significant difference was found between males and females as regards the frequency of expression neither in the studied patients nor in the controls. Noteworthy, CTLA-4⁺PBTL% could not be related to the total leukocyte count during both disease activity and remission. This indicates that CTLA-4 over expression did not simply reflect a raised leukocyte population.

Little is known about the functional involvement of CTLA-4 in SLE, however, CTLA-4 plays two pivotal roles in immune regulation and autoimmunity: the transduction of negative control to activated T cells and autoreactivity, and the activation of the suppressive function of CD4⁺CD25⁺ regulatory T cells (Tregs). CTLA-4 function is critical in maintaining self-tolerance and preventing organ-specific autoimmunity. T-cell proliferation is inhibited by B7-1 in a CTLA-4-dependent manner.

CD80 (B7-1) and CD86 (B7-2) interact with both CD28 and CTLA-4, which are produced in different kinetics and locate at different compartments in T cells. CD28 is expressed constitutively at a significant level on the T cell surface, and its expression is enhanced after T cell activation. Engagement of CD28 enhances T cell activation. In contrast, CTLA-4 is expressed at low levels in resting T cells and resides primarily within the cytoplasm. Recent intensive analysis of the CD28-CTLA-4/B7 pathway has revealed unexpected means in which this pathway may be involved in the maintenance and breakdown of self-tolerance. Two hypotheses have been proposed. First, optimal costimulatory activity of B7 requires its interaction with both CD28 and CTLA-4. Alternatively, it was suggested that CD28 and CTLA-4 play opposite roles in T cell activation; CD28/B7 interaction promotes T cell activation, and augment T cell proliferative response and effector function such as cytokine production, whereas B7/CTLA-4 inhibits this response.
The inability to generate effective down-regulatory networks of immune hyperactivity is one pathogenesis in patients with SLE. Owing to the inhibitory role of the CTLA-4 molecule in the immune response and the frequently observed hyperactivities of both B and T lymphocytes in SLE, it is reasonable to speculate defective surface expression of CTLA-4 molecule in SLE, however, increased surface expression of CTLA-4 in SLE children was found. This implies at least that T cells from SLE children possess the ability to express CTLA-4 molecule after T-cell activation, and that CTLA-4 function might be defective in spite of surface expression. Activation of T cells, a pathogenic key in SLE, would certainly result in both increased CTLA-4 gene expression and trafficking of CTLA-4 protein to the cell surface, increasing its surface expression. Another possibility is that the defect may reside in CTLA-4 mRNA that was not sought in this study.

Hirashima et al., (19) reported that the level of surface expression of CTLA-4 on T cells was quite modest with no significant differences of its expression between controls and SLE patients; while intracellular CTLA-4 expression in T-cells was significantly greater in SLE patients than in controls. In addition, surface and intracellular expression of CTLA-4 was up regulated after activation and the kinetics of cell surface CTLA-4 expression paralleled the kinetics of intracellular expression. Therefore, CTLA-4 expression is not impaired in SLE patients. Worthy of note it is a functional defect with failure to generate its down-regulatory effect. Whether this defect is a primary inherent feature of autoimmunity or a consequence of the immune dysfunction in SLE is to be elucidated.

Two additional possibilities, though not investigated in this study, might be considered as the reason why CTLA-4 function was defective in spite of intact expression in SLE.

The first possibility is polymorphisms of the human gene. The chromosome 2q33 region, where the CTLA-4 and CD28 genes are located, is one of the potential susceptibility loci for human SLE (22-24). CTLA-4 polymorphisms in SLE Japanese and Slovak populations patients have been reported (25). CTLA-4 +49A:CT60G haplotype polymorphisms might have affected the CTLA-4-mRNA stability and subsequent CTLA-4 expression (26). A recent study has identified several haplotype blocks across the extended CD28-CTLA-4-ICOS region, with SLE associations observed in the distal 3' flanking region of CTLA-4 on a haplotype that includes variants in the promoter of ICOS. Therefore, the balance of evidence supports a genetic association between this region and SLE, although the precise nature, definition and boundaries of the haplotypes involved remain to be fully defined (27). On the contrary, other studies (5, 28) reported that these polymorphisms are unlikely to affect CTLA-4 function. Therefore, studying the well-known and other undetectable CTLA-4 polymorphisms involved in CTLA-4 function and expression in SLE is recommended.

The second possibility is that other molecules that are associated with CTLA-4 may be responsible for the impairment of CTLA-4 function. Recent studies have suggested that functional defects of SLE T-cells lie in the early signaling pathways between TCRs and protein kinase C (5). CTLA-4 is known to be associated with the TCR complex ζ chain, and CTLA-4-TCR ζ interaction inhibits TCR signal transduction after T-cell activation. It has been reported that TCR ζ chain expression in PBTL from SLE patients is decreased compared with the controls. In addition, Src family tyrosine kinases such as Fyn and Lck regulate cell-surface expression of CTLA-4 through tyrosine phosphorylation of CTLA-4. If CTLA-4 is not phosphorylated, surface CTLA-4 rapidly internalizes intracellularly. These reports raise the possibility that the expression and functions of CTLA-4 themselves are not defective, but the early signaling pathways associated with CTLA-4 molecules including TCR complex ζ chain and Src family tyrosine kinases are impaired in SLE patients (19).

Lastly, some studies (18, 28) had identified a native soluble CTLA-4 in humans. The presence of functional capability has been demonstrated in vitro through its inhibitory actions in mixed leukocyte response, even though it has not yet been clearly defined. Soluble CTLA-4 level in SLE patients has been evaluated and reported in comparison to that of controls, but correlation to its activity was not confirmed or identified. In the present study, soluble CTLA-4 level was not evaluated. Evaluation of the soluble CTLA-4 expression and its correlation to the variables of SLE disease is recommended.

Therefore , it seems that CTLA-4 surface expression is not impaired in SLE children, but there is a possibility of decreased inhibitory effect of CTLA-4 involved in the pathogenesis of SLE. Although this preliminary data are in line with previous reports (17-20), the results should be interpreted with great caution, because of the limited number of subjects involved in this study. The reason of defective CTLA-4 function in spite of intact surface expression is to be elucidated in future studies.

In this study the percentage of PBTL expressing CTLA-4 in SLE patients with glomerulonephritis was significantly higher than
those without nephritis. Moreover, on comparing the three groups of lupus flares (nephritis, cerebritis, and other flares) together, the highest expression was found in SLE children with nephritis. During disease activity, the percentage of PBTL expressing CTLA-4 correlated positively with the serum creatinine and 24 hours urinary protein excretion and correlated inversely with the estimated creatinine clearance and serum C3 and C4 levels.

So, CTLA-4 might be implicated in the pathogenesis of lupus nephritis. In this regard impaired CTL-4 function was reported in SLE patient with nephritis, and the CTLA-4 locus showed maximal linkage to nephritis development in murine SLE model.

In this work, the percentage of PBTL expressing CTLA-4 in SLE patients with cerebritis was significantly higher than the group with the miscellaneous flares. The role of costimulation in autoimmune demyelination and multiple sclerosis was previously approved. Noteworthy, in murine relapsing experimental autoimmune encephalomyelitis, CTLA-4 expression was up regulated during acute disease activity, peaked at remission, and persisted during relapses.

The expression of CTLA-4 on PBTL was significantly higher in children enrolled with severe lupus as compared to those with mild to moderate lupus. This is a reasonable finding since severity is related to certain clinical phenotypes in lupus; nephritis and cerebritis, indicating CTLA-4 over expression with increased severity.

In this study, the percentage of PBTL expressing CTLA-4 during activity and remission was statistically comparable. Moreover, CTLA-4 PBTL expression could not be correlated to ESR, or SLEDAI score. This data is consistent with other reports, and indicates that CTLA-4 expression was not related to lupus activity. Reinforcing this finding is the significant positive correlation between the CTLA-4 PBTL% and the duration of illness on one hand, and the correlation between the % during lupus activity and the corresponding values during remission on the other hand meaning that the higher the level got during exacerbations the higher it remained after remission. This could be due to the ongoing process of sub-clinical inflammation during clinical remission meaning that hyper-activated helper T-cells that cause polyclonal B-cell secretion of pathogenic autoantibodies and formation of immune complexes is still present in patients with seemingly stable conditions. Since CTLA-4 plays an essential role in the prevention of uncontrolled T-cell activation, it seems that there is a defective T cell suppressive function required for immune control of active SLE.

The finding that, CTLA-4 PBTL% could be related to anti-dsDNA autoantibodies titers in this work is in agreement with the report of Lee et al. and implies that a breakdown of tolerance as a result of impaired CTLA-4 function might contribute to the polyclonal B-cell expansion, excessive pathogenic autoantibody formation, and formation of immune complexes seen in active SLE.

CTLA-4 expression could not be related to age, weight centiles, height centiles, during or after subsidence of exacerbations. Moreover, CTLA-4 expression did not vary according to therapy. Our results were in agreement with that of Hirashima et al.

Blocking CTLA-4-CD28/B7 costimulatory pathway was investigated as a novel therapeutic approach in autoimmune diseases. Various combinations of recombinant human and recombinant mouse CTLA-4 proteins with human and mouse immunoglobulins have been constructed. Several studies suggest that blocking the CTLA-4:B7 pathway using B7 antagonists such as anti-CD80 and/or -CD86 mAbs or CTLA-4-immunoglobulin in vivo, interferes with disease progression in well-characterized animal models of experimental allergic encephalomyelitis, SLE, and rheumatoid arthritis. However, this treatment does not result in complete tolerance. In addition, the timing of intervention is crucial to the effect. Generally, early treatment prevents or reduces the severity of disease, whereas treatment at or after clinical onset has little effect, although some divergences are obvious.

From the present study, it could be concluded that the increased CTLA-4 surface expression on PBTL in children with SLE, irrespective of lupus activity, provides an evidence about the implication of CTLA-4 in the pathogenesis of the disease. The significant elevation of CTLA-4 PBTL% in patients with nephritis & cerebritis suggest a key role of CTLA-4 in such phenotypes' and so, it might be considered as a marker of them. Finally, it seems that the generation of effective down-regulatory networks of immune hyperactivity in SLE was defective in spite of intact CTLA-4 surface expression. Therefore, future studies are recommended to elucidate the reasons of defective CTLA-4 function which might hold promise for manipulation of CTLA-4 as a novel approach for therapeutic immunomodulation in pediatric lupus.

REFERENCES


