

# Expression of costimulatory molecules on peripheral blood lymphocytes of patients with systemic lupus erythematosus

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## Abstract

**Objective**—In systemic lupus erythematosus (SLE) autoantibody production is T cell dependent. For a proper T and B cell interaction, signalling of costimulatory molecules on these cells is necessary. The expression of costimulatory molecules on peripheral blood lymphocytes in patients with SLE in conjunction with disease activity was measured to evaluate whether expression of costimulatory molecules in SLE is increased.

**Methods**—Thirteen patients with SLE with active disease, 10 patients with inactive disease, and 14 controls entered the study. In addition, samples from 10 of the 13 patients with active disease could be studied at a moment of inactive disease as well. Isolated peripheral blood lymphocytes were stained for the lymphocyte subset markers CD4, CD8, CD19, their respective activation markers CD25, HLA-DR, CD38, and the costimulatory molecules CD40L, CD28, CD40, CD80, and CD86. Expression was measured by flow cytometry.

**Results**—Peripheral blood lymphocytes of patients with SLE showed signs of increased activation at the moment of active disease. Almost all CD4+ T cells expressed CD28, both in patients and in controls. CD80 expression on CD19+ B cells was low in both groups and did not correlate with disease activity. In contrast, the percentage of CD19+ B cells expressing CD86 was increased in patients with SLE even in patients with inactive disease ( $p=0.04$ ) and correlated with the SLEDAI score ( $p=0.0005$ ) and levels of anti-dsDNA ( $p=0.006$ ). No changes in CD40 or CD40L expression were found in the patients with SLE.

**Conclusion**—In patients with SLE the expression of CD86 on CD19+ B cells is increased and is associated with disease activity, B cell activation, and levels of anti-dsDNA. The increased CD86 expression will render (autoreactive) B cells more susceptible for T cells. This can facilitate autoantibody production and might be a target for immunosuppressive treatments.

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Autoimmune diseases such as systemic lupus erythematosus (SLE) are associated with various immunological abnormalities, such as

increased numbers of activated B cells and autoantibody production. Much of our understanding of the pathogenetic mechanisms involved are derived from animal studies. In lupus prone MRL-lpr/lpr mice the selective B cell proliferation seen is antigen driven and T cell dependent,<sup>1</sup> and treatment of those mice with anti-Thy-1.2 antibodies abrogated IgG anti-dsDNA production.<sup>2</sup> Activation of T cells requires interaction of the T cell receptor with the major histocompatibility complex molecule complexed with the antigen in the presence of additional signalling through costimulatory molecules. Of these, the CD28-CD80/CD86 and CD40-CD40L pathways are well known. The relevance of costimulation in the development of autoimmune disease in animal models is supported by several studies in which costimulation antagonists have been used as an effective approach to treat these diseases.<sup>3,4</sup>

The tight and carefully orchestrated interaction between T and B cells ensures that bystander lymphocytes are not activated. Whenever, for example, a B cell is signalled via CD40 and fails to receive a proper signal through its B cell receptor the cell will die by apoptosis.<sup>5</sup> Adequate stimulation, however, will result in lymphocyte activation and proliferation by a sequence of activating and regulating signals. It can be suggested that an increase in the expression of costimulatory molecules facilitates T and B cell activation and survival, increases autoantibody production, and so may influence the development of autoimmune disease. In line with this hypothesis we measured the expression of costimulatory molecules on peripheral blood lymphocytes in lupus patients and healthy controls in relation to the state of lymphocyte activation to consider the following questions: (a) Is there an increase in expression of costimulatory molecules in patients compared with controls? (b) Are these changes related to the state of lymphocyte activation and levels of antibodies to dsDNA? (c) Is the expression of these molecules in lupus patients dependent on disease activity?

## Subjects and methods

### PATIENTS AND BLOOD SAMPLES

Outpatients eligible for this study fulfilled at least four American College of Rheumatology criteria for SLE,<sup>6</sup> were non-smokers, and had either active or inactive disease. Patients with active disease had to fulfil predefined criteria.<sup>7</sup> Inactive disease was defined as the persistent absence of clinical disease activity for at least four months while patients were without, or

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**Table 1** Expression of activation markers and costimulatory molecules on peripheral blood lymphocytes. Results are given as mean (SD)

	Control	Inactive	Active
CD4/CD25+ (%)	4.0 (1.4)	4.4 (3.2)	8.5 (6.3)*
CD8/HLA-DR+ (%)	5.6 (2.5)	8.1 (5.3)	10.6 (8.3)
CD19/CD38+ (%)	79.7 (8.7)	84.3 (7.7)	86.5 (9.8)*
CD4/CD28+ (%)	95.3 (3.8)	93.6 (7.9)	95.0 (3.5)
CD19/CD40+ (%)	85.0 (9.3)	81.3 (11.2)	81.9 (12.4)
CD19/CD80+ (%)	1.7 (0.9)	3.1 (5.7)	4.4 (5.8)
CD19/CD86+ (%)	2.9 (1.4)	6.9 (7.2)*	12.5 (8.9)***

\* $p < 0.05$ , \*\*\* $p < 0.001$  compared with controls. Percentages are expressed as proportion of total CD4+, CD8+, and CD19+ subpopulations from healthy controls (n=10), patients with inactive disease (n=20), and patients with active disease (n=13).

receiving a constant dose of, immunomodulating drugs. To evaluate changes due to disease activity, re-evaluation of patients with initially active disease took place at a moment of inactive disease as well. The SLEDAI score was calculated for each patient.<sup>8</sup> Healthy volunteers, matched for age and sex, were included as controls.

Blood samples for anti-dsDNA detection were drawn in EDTA and anti-dsDNA were detected by the Farr assay using <sup>125</sup>I labelled recombinant dsDNA (Diagnostic Products Corporation, Los Angeles, USA) as described.<sup>7</sup>

#### CELL ISOLATION AND STAINING PROCEDURES

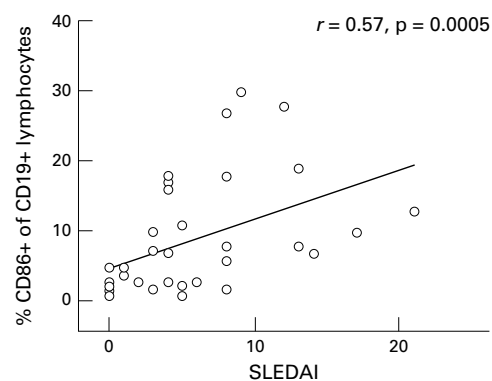
Peripheral blood mononuclear cells were isolated from heparinised blood by lymphoprep density gradient centrifugation. For surface staining the following phycoerythrin (PE) or fluorescein isothiocyanate (FITC) and allophycocyanine (APC) conjugated monoclonal antibodies were used: CD4 (APC), CD19 (APC), CD25 (FITC/PE), CD38 (FITC/PE), CD40 (PE), CD40L (PE), CD80 (PE), CD86 (PE), and HLA-DR (FITC/PE) (Becton Dickinson, Mountain View, CA); CD8 (APC) (Pharmin-gen, Hamburg, Germany). After washing with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), 10<sup>6</sup> cells were stained by adding labelled monoclonal antibodies in the relevant combinations, with subsequent incubation for 45 minutes on ice in the dark. Samples were washed with PBS/BSA. Three colour immunofluorescence analysis was performed on a Coulter Epics-Elite equipped with a gated amplifier (Coulter Electronics, Mijdrecht, The Netherlands). Cells were gated for lymphocyte characteristics using both forward and sideward scatter. For final analysis only those samples were included in which more than 500 cells of the respective subsets were counted.

#### STATISTICS

Differences between groups were evaluated by Student's *t* test or the Mann-Whitney U test when appropriate. Paired samples were analysed separately using the Wilcoxon rank sum test. Spearman's rank correlation was applied for detecting correlations between different study parameters. A *p* value less than 0.05 was considered significant.

#### Results

Ten patients (two male, eight female) with inactive SLE entered the study. Their mean (SD) age was 48.5 (1.8) years. Of 13 different



**Figure 1** Correlation between the percentage of CD86 expressing B (CD19+) cells and disease activity as measured by the SLEDAI score analysed for all samples taken (n=33).

patients (1 male, 12 female) aged 39.2 (16.7) years, blood samples could be drawn at the moment of active disease. In addition, from 10 of these 13 patients with active disease, samples could be analysed at a moment of inactive disease as well. This resulted in a total of 20 patients evaluated at the moment of inactive disease.

Lymphocytes of patients with SLE showed signs of increased activation at the moment of active disease. The percentage of CD4+ lymphocytes expressing CD25 was higher ( $p = 0.03$ ) in patients with active disease than in controls (table 1). Furthermore, the percentage of activated (CD38+) B lymphocytes was raised in patients with active disease ( $p = 0.04$  *v* controls). Expression of costimulatory molecules was measured on CD4+ T cells and on CD19+ B cells. Almost all CD4+ T cells expressed CD28 on their surface, which was comparable between controls and patients with SLE (table 1). CD40L was hardly detectable on CD4+ T cells of controls and patients (data not shown). Regarding B cells, the percentage of cells expressing CD40 was comparable between controls and patients. The percentage of CD19+ B cells expressing CD80 was low in controls as well as patients. A correlation between CD80 expression and the disease activity of patients with SLE was not found. In contrast, CD86 expression differed between the groups investigated. Compared with controls the percentage of CD19+ B cells expressing CD86 was increased in patients with SLE with inactive disease ( $p = 0.04$ ) and increased even more in patients with active disease ( $p = 0.0003$  *v* controls). Furthermore, CD86 expression correlated with the SLEDAI score (fig 1) and levels of antibodies to dsDNA ( $r = 0.47$ ,  $p = 0.006$ ). To analyse the relation between CD86 expression and cell activation we concentrated on activated—that is, CD38+ B cells. Indeed, the percentage of B cells positive for both markers, though low, was substantially increased among patients with active disease ( $p = 0.006$  *v* controls).

For all costimulatory molecules paired samples from patients with active and inactive disease were analysed separately. This analysis

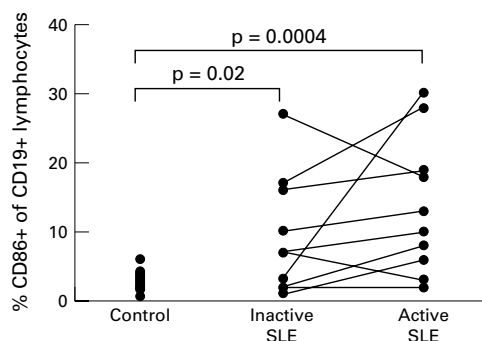


Figure 2 Changes in CD86 expression in paired observations ( $n=10$ ) of patients measured at the moment of active and inactive disease, respectively. Results for CD86 expression on B cells of controls ( $n=13$ ) are also shown.

gave similar results, showing increased expression of CD86 but not of CD80 or the other costimulatory molecules measured (fig 2).

### Discussion

In this study we show that CD86 expression on CD19+ B cells from patients with SLE is increased in patients with inactive disease and increases further in conjunction with disease activity and B cell activation. Such a relation could not be shown for CD80 expression. No changes were found in the expression of the CD80/CD86 counter receptor CD28 on CD4+ T cells. Furthermore, expression of CD40 on CD19+ B cells and CD40L on CD4+ T cells was comparable between patients and healthy controls.

In SLE, dsDNA antibody production is T cell dependent. Studies in lupus prone mice indicate that costimulatory molecules play an essential part in the interaction between B and T cells. Extrapolating these data to lupus patients, it can be speculated that aberrant expression of costimulatory molecules provides a condition in which autoantibody production is facilitated, possibly because autoreactive memory B cells and T cells can expand in the absence of adequate apoptotic signalling.<sup>9</sup>

CD86 interacts with CD28 on the T cell. This interaction regulates the activation induced apoptosis in thymocytes. In vivo, stimulation of CD28 prevents apoptosis of thymocytes in mice.<sup>10</sup> Therefore, increased expression of CD86 might promote lymphocyte survival intervening with the elimination of autoreactive lymphocytes. Furthermore, it may render B cells more susceptible for T cell help. These changes may facilitate autoantibody production. Indeed, in murine lupus the production of anti-dsDNA seems to be dependent on CD86 costimulation. Injection of CTLA-4 immunoglobulin in NZB/W F1 mice, which neutralises both CD80 and CD86, blocked the production of dsDNA antibodies and prolonged life.<sup>3</sup> In MRL-*lpr/lpr* mice treatment with anti-CD86 alone inhibited anti-dsDNA expression while treatment with anti-CD80 did not change anti-dsDNA expression.<sup>4</sup> Furthermore, it was shown that the presence of costimulatory molecules influenced the course of the disease. CD86 deficient MRL-*lpr/lpr* mice had significantly milder glomerulonephritis than wild-type mice.<sup>4</sup>

Aberrant CD86 expression on B lymphocytes has been reported in SLE. Folzenlogen *et al* analysed CD80 and CD86 expression on B cells of 13 patients with inactive SLE and found CD86 increased in patients compared with controls.<sup>11</sup> We confirm these data and show that during disease activity CD86 is further up regulated. In addition, our data suggest a discrepancy in the expression of CD80 and CD86. Costimulatory signals of CD80 and CD86 lead to differential polarisation of T helper cell responses. There is evidence that CD80 preferentially acts as a costimulus for the generation of Th1 cells, whereas CD86 costimulates and induces Th2 cells.<sup>12</sup> Our data, showing a difference in the expression of CD80 and CD86, are in line with the concept of SLE being a predominantly Th2 mediated disease.

In T cells, ligation of CD28 stimulates membrane expression of another accessory molecule, CD40 ligand (CD40L).<sup>10</sup> CD40L is necessary for T cell help for B cells as shown by defective antigen-specific T cell responses in CD40L deficient mice.<sup>13</sup> For example, CD40L influences apoptosis when ligation with its counter receptor CD40 takes place. Ligation of CD40 by CD40L can block B cell apoptosis induced by antigen receptor cross linking.<sup>14</sup> In line with the CD28-CD80/CD86 pathway, aberrant expression of CD40 or CD40L may be a relevant factor in the development of autoimmune disease. Indeed, in murine lupus, treatment with anti-CD40L reduced the level of antibodies to dsDNA, and delayed the development of nephritis.<sup>9</sup> Furthermore, a dysregulation of CD40L expression in human lupus has been described.<sup>15</sup> We did not find changes in CD40L expression on CD4+ T lymphocytes in patients with SLE. CD40L expression was low in patients with inactive disease and controls and, unlike the results of others, did not increase during active disease. In experiments in which we stimulated isolated peripheral blood mononuclear cells with anti-CD3, we detected proper increase of CD40L expression in conjunction with T cell activation, excluding the possibility that our CD40L monoclonal antibody was inappropriate (data not shown). Also, CD40 expression on B cells, being high in lupus patients irrespective of disease activity, did not differ from that in healthy controls.

In conclusion, our data clearly show that in patients with SLE, even at the moment of inactive disease, the expression of CD86 on CD19+ B cells is increased, and is associated with B cell activation and levels of antibodies to dsDNA. Whether aberrant expression of CD86 in patients with SLE is constitutive or merely results from increased lymphocyte activation needs further investigation. Nevertheless, our data provide increasing evidence that manipulating costimulatory pathways in lupus may be a potentially beneficial therapeutic strategy.

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