Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients

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SUMMARY

Defective T cell functions, including IL-2 production and proliferation, have been shown in SLE patients. After T cell stimulation (first signal), a costimulatory signal (second signal) is required to achieve complete T cell activation. Main costimulatory signals are provided to T cells by B7 antigens (CD80 and CD86, expressed on antigen-presenting cells (APC)) upon interaction with its receptor, the CD28 molecule expressed on T cells. The aim of this study was to investigate the role of CD28/B7 interactions in the impaired T cell responses of SLE patients. We show that stimulation of T cells with phytohaemagglutinin (PHA) in the presence, but not in the absence, of anti-CD28 MoAb or B7 cells results in tyrosine phosphorylation of specific substrates, transcription of mRNA and production of IL-2 that is indistinguishable in SLE patients and healthy controls. Moreover, proliferation of costimulated T cells from SLE and controls was specifically abrogated by blocking the CD28/B7 interactions by means of addition to the culture of the CTLA4-Ig fusion protein. However, in most patients activated APC failed to up-regulate B7 molecules, giving rise to ineffective costimulatory signalling to T cells. These results indicate that the CD28/B7 costimulatory pathway is defective in SLE patients.

Keywords T cells systemic lupus erythematosus B7/CD28

INTRODUCTION

It is known that peripheral blood T lymphocytes from SLE patients show impaired IL-2 production and proliferation in response to mitogenic stimuli [1–3]. The molecular basis of these abnormalities, the in vivo significance of this defect and its participation in the pathogenesis of the disease are still unresolved. Whether this functional impairment is mainly related to defect(s) intrinsic to T cells, to antigen-presenting cells (APC) or to T–APC cell interactions remains unclear. In this regard, it has been recently shown that the defective response of SLE T cells to nominal antigen is due to impaired APC function [4]. It has also been demonstrated that SLE T cells properly respond to allogeneic APC from healthy donors, whereas SLE APC fail to stimulate normal T cells in autologous and allogeneic mixed lymphocyte reactions [5,6].

All these types of T cell responses are dependent on costimulatory signals provided by APC (reviewed in [7]). Main costimulatory signals are provided by molecules of the B7 family, namely B7-1 (CD80) and B7-2 (CD86) expressed on APC. Both B7-1 and B7-2 interact with their receptors CD28 and CTLA-4, expressed on T cells (reviewed in [8]). Signals delivered via CD28 are a crucial requirement for T cell responses to antigens, superantigens and mitogens [9–15]. Although other receptor–ligand pairs may also contribute to an optimal activation of T cells, the differential feature of the CD28/B7 pair is that their interactions are essential for IL-2 production and T cell responsiveness [16,17].

In a murine model of SLE, in vivo prevention of CD28/B7 binding ameliorates clinical parameters of the disease [18], and recent data in humans support a role for the CD28/B7 pathway in SLE [19]. Therefore, it is conceivable that this signalling mechanism may be prominent in explaining the defects observed in SLE T cells. However, no information is available concerning the role of the CD28/B7 interactions in SLE patients.

Due to this lack of information, we performed experiments to dissect both sides of the CD28/B7 pathway in human SLE. For this purpose, we selected phytohaemagglutinin (PHA) as stimulus, since it is generally accepted that SLE T cells consistently show a reduced ability to produce IL-2 and to proliferate in...
response to this mitogen. Conversely, contradictory data have been obtained when SLE T cells are challenged with anti-CD3 MoAb [20–23].

Thus, under suboptimal culture conditions, we show that stimulation of T cells with PHA in the presence, but not in the absence, of anti-CD28 MoAb or B7 + APC results in tyrosine phosphorylation of specific substrates, transcription of mRNA and production of IL-2, that is indistinguishable in SLE patients and healthy controls. These results indicate that costimulation by autologous monocytes via the CD28 pathway is defective in SLE cells. The analysis of expression of B7 molecules revealed that in most patients, activated APC failed to increase their expression of B7 molecules, giving rise to an ineffective delivery of costimulatory signal(s) to T cells.

To the best of our knowledge, our data provide the first evidence of defective expression of B7 on APC underlying T cell dysfunction in a systemic autoimmune disease.

PATIENTS AND METHODS

Patients and controls

Twenty-four female patients aged 25–42 years, with established SLE followed at the Rheumatology Unit of Hospital Universitario Reina Sofia (Córdoba, Spain), were studied. All patients met the American College of Rheumatology criteria for SLE [24]. At the time of the study 11 patients were classified as having active disease according to published criteria [25]. Fourteen patients were taking prednisone to control systemic symptoms (7.5–20 mg/day). This group included five patients with asthma and four with vasculitis.

MoAbs

MoAbs used in this study were: PE-conjugated mouse anti-human CD3, CD4, CD8, CD14 and CD19 molecules, purchased from Becton Dickinson (San Jose, CA); FITC-conjugated and unconjugated mouse anti-human anti-CD28 were obtained from Janssen Research Institute (Seattle, WA). Anti-CD80 MoAb BB-1 (5 μg/ml) was purchased from Pharmingen (San Diego, CA). Goat F(ab')2 anti-mouse immunoglobulins (GAM) and goat F(ab')2 anti-human immunoglobulins were purchased from Tago Inc. (Burlingame, CA).

Flow cytometric analysis

Cells were washed and resuspended in a solution consisting of PBS, 1% bovine serum albumin (BSA) and 0-01% NaN3 with saturating amounts of MoAbs directed against cell surface antigens or isotype-matched irrelevant controls. After 30 min at 4°C cells were washed, incubated as above with GAM-FITC second antibody if needed, washed again and analysed by flow cytometry (FACSort; Becton Dickinson). Monocytes were distinguished from cocultured cells by electronic gating on cells stained with CD14-PE. The expression of all other cell surface markers was determined by staining with FITC-conjugated MoAbs.

Cell isolation

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway). In some experiments, adherent cells were removed by incubating the PBMC in 100-mm plastic Petri dishes (Costar, Cambridge, MA) at 37°C for 60 min. The cells were passed over a nylon-wool column to deplete residual B cells and macrophages. This resulted in a population of T cells (NWT) that was typically >90% CD3+ <1% CD19+ cells <1% CD14+ cells, as determined by flow cytometry.

T cell proliferation and IL-2 production assays in response to anti-CD28 MoAb

For T cell proliferation experiments, 5 × 10^6/well of either PBMC or purified T cells (100 μl/well) were stimulated with PHA (1 μg/ml) (ICN Biomedicals, Cleveland, OH) in 96-well flat-bottomed culture trays (Costar). Anti-CD28 MoAb (1 μg/ml) was used alone or with PHA at the concentrations indicated above during 48 h, pulsed with 0.5 μCi/well of [3H]-thymidine ([3H]-TdR; Amersham, Aylesbury, UK) for 16 h and then harvested for liquid scintillation counting.

T cell culture supernatants for IL-2 determination were collected after 24 h of incubation in the presence or absence of relevant stimuli, and IL-2 was immediately determined by means of an immunoenzymatic assay (Biokine; T Cell Sciences, Cambridge, MA) with a sensitivity limit calculated to be 80 pg/ml. Cytosolic tyrosine-kinase (PTK) involvement in CD28-mediated IL-2 secretion and proliferation was investigated in some cultures by pretreating the cells during 12 h with the specific PTK inhibitor herbimycin A (1 μM) (Kamiya Biomedical Co, Thousand Oaks, CA). To test the effect of cyclosporin A (CsA; Sandoz, Basel, Switzerland) on CD28-dependent T cell proliferation, cells were stimulated in the presence of 1 μg/ml CsA. As a CsA-sensitive control, we used T cells costimulated with a combination of PHA plus phorbol ester (PMA; Sigma, St Louis, MO). PMA was used at 10 ng/ml.

T cell proliferation and IL-2 production assays in response to B7+ cells

In order to check whether CD28 antigen expressed on SLE T cells is able to interact properly with its ligands, in some cultures B7+ Raji and B7+ Daudi cell lines were used as accessory cells (5 × 10^5/well). Raji and Daudi cells were treated with 50 μg/ml Mitomycin C (Sigma) for 90 min and washed twice before use. After this treatment, cells had a 1H-TdR uptake <200 cpm/min. For blocking experiments, excess (1 μg/ml) of CTLA-4-Ig hybrid molecule was added and kept throughout the culture.

Immunoblot analysis of protein tyrosine phosphorylation

The analysis of proteins phosphorylated in tyrosine residues was

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performed as previously described [26]. NWT cells (2 × 10^7) from each group were incubated with the anti-CD28 MoAb 9.3 (1 μg/ml) and cross-linked with GAM IgG (40 μg/ml) during 5 min. Stimulation was terminated by the addition of ice-cold 10 × lysis buffer, yielding a final concentration of 0.5% Triton X-100, 50 mM Tris-base, 300 mM NaCl, 1 mM Na_2VO_3, 5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 2 μM PMSF pH 7.6. After lysis on ice, nuclei were pelleted and the postnuclear supernatants subjected to SDS–PAGE on a 7.5% gel and electroblotted onto polyvinylidene difluoride membranes (ICN, Costa Mesa, CA). Transferred tyrosine-phosphorylated proteins were identified using the horseradish peroxidase (HRP)-conjugated PY20 antibody (ICN).

RNA analysis
PBMC (10^7) from SLE or healthy donors were stimulated with PHA alone or in combination with anti-CD28 MoAb, at the concentration indicated above. Total RNA from 10^7 cells from each group was extracted as previously described [27]. Total RNA (10 μg) was electrophoresed on a 1% agarose, 2% formaldehyde gel, and capillary transferred onto nylon membranes (Appligene, Heidelberg, Germany). The RNA was cross-linked to the membrane by UV irradiation and the blots were prehybridized for 3 h at 65°C in 5% SDS, 100 mM NaCl, 50 mM Pipes, and 1 mM EDTA, and then hybridized for 12 h in a new aliquot of the same solution, containing an α-32P random primed probe (Promega, Madison, WI). The IL-2 cDNA probe is a Pst-1 fragment from a pGEM plasmid obtained from American Type Culture Collection (ATCC, Rockville, MD). The hybridized membranes were washed three times for 5 min each at 65°C in 0.1% SDS, 2× SSC, and three times for 5 min each at 65°C in 0.1% SDS, 1× SSC. Autoradiographs were exposed at ~70°C with enhancer screens.

Statistical analysis
Data were described as mean ± s.e.m. Calculation of P values for the difference between groups was done by Wilcoxon rank sum.

RESULTS
We addressed in SLE patients the functional integrity of T cell

Fig. 1. Proliferation and IL-2 response of peripheral blood mononuclear cells (PBMC) from SLE patients and healthy controls. (a) Dose–response curve to phytohaemagglutinin (PHA) of normal (○) and SLE cells (●). (b) Time-course production of IL-2 after PBMC stimulation with 1 μg/ml PHA. (c,d) Effect of CD28-mediated costimulation on PBMC proliferation (c) and IL-2 production (d). PBMC (5 × 10^4/well) from SLE patients (●) (n = 11), non-SLE steroid-treated (□) (n = 9) or healthy individuals (■) (n = 7), were stimulated in the presence of PHA (1 μg/ml) alone or in combination with anti-CD28 MoAb (1 μg/ml). Cells were incubated during 48 h, pulsed with 3H-TdR and proliferation determined 16 h later. IL-2-containing supernatants were measured by ELISA at indicated times (b) or after 24 h (d). Results are expressed as mean ± s.e.m. S.e.m. values in a and b were always <20% of the mean values. In each experiment at least one healthy control was included.
PHA and anti-CD28 MoAb resulted in a vigorous transcription of stimulation (Fig. 2, lane 2). However, the simultaneous presence of negligible steady-state IL-2 mRNA of SLE T cells after PHA degradation due to mRNA instability. This analysis revealed a consequence of a diminished IL-2 mRNA transcription, or a rapid deficient IL-2 production by SLE T cells in response to PHA is a (Fig. 1c,d).

The addition of soluble anti-CD28 MoAb to PHA-stimulated cells completely restored the above defects. Indeed, proliferation and IL-2 production levels reached by PHA-stimulated T cells in the presence of the anti-CD28 MoAb 9.3 were equal to control groups. The restoration of defects is specifically mediated by CD28, since the addition of an isotype-matched irrelevant antibody does not modify the PHA response (Fig. 1c,d).

Northern blot analysis was carried out to determine if the deficient IL-2 production by SLE T cells in response to PHA is a consequence of a diminished IL-2 mRNA transcription, or a rapid degradation due to mRNA instability. This analysis revealed a negligible steady-state IL-2 mRNA of SLE T cells after PHA stimulation (Fig. 2, lane 2). However, the simultaneous presence of PHA and anti-CD28 MoAb resulted in a vigorous transcription of the IL-2 gene in SLE T cells that was indistinguishable from that of normal cells (Fig. 2, lanes 3 and 4). Preliminary data indicate that pretreatment of SLE PBMC with actinomycin D did not increase IL-2 mRNA level upon stimulation with PHA (data not shown).

Two distinctive features of the CD28/B7 signalling pathway were further explored. Namely, that CD28-mediated activation of T cells requires PTK activity [28,29] and that it is resistant to CsA [30,31]. Data obtained after exploring PTK activity are shown in Fig. 3. Occupancy of the CD28 receptor with a specific MoAb induced tyrosine-phosphorylation of two main substrates of approximate relative masses of 75 kD and 100 kD. The tyrosine phosphorylation pattern obtained in SLE T cells was indistinguishable from that of normal cells (Fig. 4, lanes 3 and 4). These phosphorylated substrates have a prominent role in the activation mechanisms leading to cellular proliferation and IL-2 production, since treatment of both normal and SLE T cells with the specific PTK inhibitor herbimycin A abrogates CD28-mediated proliferation and cytokine production (Table 1). In contrast, proliferation and IL-2 production of both SLE and normal T cells after CD28-receptor occupancy were not inhibited by CsA (Table 1). Neither herbimycin A nor CsA treatment at the dose used affected cellular viability (data not shown).

The examination of the functional expression of B7-1 and B7-2 molecules in SLE APC revealed conspicuous alterations in most patients (12 out of 19). The fusion protein CTLA4-Ig is a hybrid molecule that binds with high avidity to both B7-1 and B7-2 molecules. Resting SLE monocytes had a minute expression of CD28 ligands, as revealed by staining with CTLA4-Ig in flow cytometry analysis (Fig. 4, upper panel). The abnormalities in the expression of B7 molecules are even more notorious after activation of the SLE monocytes in the experimental conditions used to trigger cellular proliferation of PBMC. Thus, incubation of normal PBMC with PHA during 48 h resulted in a vigorous induction of CD28 ligands recognized by CTLA4-Ig in monocytes, whereas only a slight shift in the cytometry profile was observed in SLE

![Fig. 2. Northern blot analysis of IL-2 gene expression in normal and SLE peripheral blood mononuclear cells (PBMC). Cells were stimulated with phytohaemagglutinin (PHA) alone (lanes 1 and 2), or PHA plus anti-CD28 MoAb (lanes 3 and 4) for 20 h before isolation of total RNA. Lower panel shows ethidium bromide staining of ribosomal bands to verify equal loading of RNA. A representative experiment out of three performed is displayed.](image1)

![Fig. 3. Triggering of the CD28 receptor induces tyrosine phosphorylation in T cells from SLE patients and controls. Nylon wool-purified T (NWT) cells (2 x 10^7) from healthy or SLE patients were treated with soluble (lanes 1 and 2) or goat anti-mouse antibody (GAM)-crosslinked (lanes 3 and 4) anti-CD28 MoAb (1 μg/ml). A representative experiment out of three performed is shown.](image2)
surface phenotype of these cell lines differed significantly from one another only in the expression of B7 molecules (Fig. 5, inset). Normal and SLE T cells strongly proliferated to PHA when cultured in the presence of the B7+ Raji cells, whereas Daudi cells were unable to sustain a comparable proliferative reaction in either control or SLE purified T cells. The specificity of the CD28–B7 interactions underlying the proliferation of T cells to PHA in the presence of the B7+ Raji cells was also examined. Addition to the cultures of CTLA4-Ig displaced the binding of B7 to CD28 by virtue of its greater affinity. It can readily be observed that CTLA4-Ig blocked the proliferative response to PHA in the presence of Raji cells (Fig. 5), whereas this proliferation was not disturbed by the presence of an isotype-matched irrelevant antibody (not shown).

**DISCUSSION**

We have examined functional interactions between T cells and APC leading to cell activation in SLE patients. The aim was to determine if an improper triggering of the cell activation mechanism that results after binding of the CD28 molecule with its natural ligands can be held accountable for the proliferation and IL-2 production defects observed in SLE T cells. We chose a system based on PHA stimulation for two reasons. First, we found in preliminary experiments a reproducible defective proliferative response to PHA and IL-2 production in SLE T cells that was consistent with previously published observations [1,2]. Second, controversial data have been reported regarding the response of SLE T cells to anti-CD3 MoAbs [20–23]. This is due to the use of varying anti-CD3 MoAbs of different isotypes, epitope specificity and culture conditions. We have found that the proliferative capacity of SLE T cells in response to PHA is fully restored when costimulatory signals are provided after coupling the CD28 receptor.

The defective proliferation after PHA stimulation in SLE cells is mirrored in their IL-2 production, directly correlating with the low levels of IL-2 mRNA detected by Northern analysis. A similar situation can be found in animal models of SLE [32,33]. In normal T lymphocytes, second signals delivered to the cell after the occupancy of the CD28 receptor result in a significant induction

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**Table 1.** Effect of treatment with cyclosporin A (CsA) or herbinycin A on proliferation and IL-2 production of normal and SLE T cells costimulated with phytohaemagglutinin (PHA) and anti-CD28 MoAb

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation (ct/min)</th>
<th>IL-2 production (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SLE</td>
</tr>
<tr>
<td>PHA alone</td>
<td>1145 ± 405</td>
<td>1022 ± 210</td>
</tr>
<tr>
<td>PHA + CsA</td>
<td>1289 ± 543</td>
<td>1455 ± 187</td>
</tr>
<tr>
<td>PHA + αCD28</td>
<td>33 518 ± 2129</td>
<td>31 312 ± 2745</td>
</tr>
<tr>
<td>PHA + αCD28 + CsA</td>
<td>30 456 ± 1826</td>
<td>28 345 ± 2543</td>
</tr>
<tr>
<td>PHA + αCD28 + Herb A</td>
<td>1234 ± 234</td>
<td>1654 ± 123</td>
</tr>
</tbody>
</table>

Proliferation and IL-2 production of normal and SLE nylon wool-purified T (NWT) cells costimulated via CD28 are resistant to treatment with CsA but not herbinycin A. NWT cells (5 x 10^6/well) were activated with PHA and αCD28 MoAb in the presence of 1 μg/ml CsA (added at time 0) or 1 μM of herbinycin A (added at time ~12 h). Proliferation and IL-2 production were measured as in Fig. 1. As a CsA-sensitive control, NWT cells from four healthy subjects were costimulated with PHA and PMA with the following results (ct/min): PHA + PMA (10 ng/ml), 28 878 ± 657; PHA + PMA + CsA, 1984 ± 325.
of the transcription of the IL-2 gene that is accompanied by a greater stability of its mRNA [9,10]. The combination of both mechanisms gives rise to an increase in cellular proliferation and IL-2 secretion by T cells. The fact that the behaviour of SLE T lymphocytes after CD28 occupancy is comparable to that of normal cells suggests that the biochemical cascade of the T cell side of the CD28/B7 cell activation pathway is functionally intact in SLE patients. This is further supported by the normal pattern of protein tyrosine-phosphorylated substrates that can be observed in SLE T cells following CD28 stimulation. It is known that some PTKs are physically linked to the CD28 molecule, playing an important role in this T cell activation mechanism [34]. We have also shown the functional importance of these tyrosine-phosphorylated proteins. Thus, the treatment of both normal and SLE T cells with the specific PTK inhibitor herbimycin A results in complete abrogation of the CD28-costimulated cell proliferation. As expected [31], this proliferative response was not affected by treatment with CsA. Taken together, our data strongly suggest that the restoration of T cell-associated defects observed in SLE T cells is specifically mediated by the triggering of the CD28 molecule.

Our observations suggest that impaired responses detected in SLE T cells do not reflect an intrinsic defect in these cells, bringing the need to explore in detail both arms of the CD28-mediated cell activation mechanism. Therefore, we examined the APC end of the CD28/B7 signalling pathway. We have clearly shown a greatly diminished expression of CD28 counter-receptors on monocytes. This abnormal expression is more striking following PHA stimulation of PBMC for 48 h, the same conditions used in proliferation experiments. It is evidently a defect in the up-regulation of B7 gene products in SLE monocytes, since in sharp contrast with normal cells no shift in the CTLA4-Ig fluorescence profile can be detected at the end of the culture. During that time a number of cytokines, including interferon-gamma (IFN-\(\gamma\)) and IL-10, are released to the medium. The cause(s) underlying defective up-regulation of B7 expression in SLE APC remains unclear, but alterations in cytokine production by SLE T cells have to be considered [35–37]. On one hand, it has been reported that IL-10 exerts its immunosuppressive effect by inhibiting B7 expression on normal APC [38,39], and abnormally high production of IL-10 has been shown in murine models of SLE [40]. On the other hand, T cells from SLE patients have a diminished ability to produce IFN-\(\gamma\) following in vitro stimulation with mitogens [41], and it is known that IFN-\(\gamma\) strongly induces the expression of B7 molecules in normal monocytes [42].

Recently, it has been documented that normal alveolar macrophages deficiently present antigen to T cells due to defective expression of B7 cell surface molecules, and that IFN-\(\gamma\) is unable to up-regulate its expression [43]. Thus, a functional consequence of impaired up-regulation of B7 molecules in SLE might be the coupling of an insufficient number of CD28 molecules per T cell, thereby preventing the efficient delivery of the second signal needed for achieving cell proliferation.

Since the cell activation defects observed in SLE T cells could apparently be attributed to defective expression of B7 molecules on APC, it was of importance to assess the ability of the CD28 expressed on SLE T lymphocytes to interact properly with its ligands. This is relevant, because the result of the antibody–receptor binding is not necessarily a faithful reflection of the interactions occurring between the same receptor and its natural ligands.

We have shown that SLE T lymphocytes strongly proliferate in response to PHA if the B7 antigen is introduced in the culture. The proliferation observed in the presence of the B7\(^+\) Raji cells is probably due to occupancy by this antigen of the CD28 receptor expressed on SLE T cells, since almost no proliferation in normal and SLE T cells is detected in the presence of the B7\(^+\) Daudi cells. Moreover, we have demonstrated that proliferation of purified normal and SLE T cells is prevented by CTLA4-Ig, further supporting the notion that the response observed in the presence of Raji is a direct and specific consequence of the engagement of the CD28 receptor by its ligand. These are conditions that resemble physiological situations, and the normal response of SLE T lymphocytes indicates that their CD28 molecule can efficiently interact with its ligand if adequately provided. Both Raji and Daudi are B cell lines of similar origin whose only known phenotypic differences lies in the expression of HLA class I and B7 antigens, being Daudi-negative for both. There is no evidence for the participation of class I in these types of cell–cell interactions, and although we cannot formally rule out the involvement of these antigens in the effect we observed, such a possibility is considered extremely unlikely.

In conclusion, the data reported here provide evidence that the CD28-mediated signalling pathway is functionally active in SLE T cells. In contrast, accessory cells from some SLE patients have a defect in the regulation and expression of CD28/CTLA-4 ligands which could be responsible for a deficient delivery of costimulatory signals to T cells. However, it remains to be elucidated whether this is a primary defect of APC that could be involved in the pathogenesis of the disease, or otherwise represents an in vivo mechanism directed to anergize peripheral autoreactive T cells. If the latter possibility is correct, prevention of CD28/B7 interactions would be of greater potential therapeutic interest [44], at least during episodes of exacerbation of the disease.
REFERENCES