Impaired CD40L signaling is a cause of defective IL-12 and TNF-α production in Sézary syndrome: circumvention by hexameric soluble CD40L

Lars E. French, Bertrand Huard, Maria Wysocka, Ryan Shane, Emmanuel Contassot, Jean-François Arrighi, Vincent Piguet, Silvio Calderara, and Alain H. Rook

Sézary syndrome (SzS) is an advanced form of cutaneous T-cell lymphoma characterized by peripheral blood involvement, impaired cell-mediated immunity, and T-helper 1 (TH1) cytokine production. To understand the mechanism of these defects, we studied the expression and function of CD40L in peripheral blood mononuclear cells (PBMCs) of patients with SzS. We found that PBMCs of patients with SzS have a defect in interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α) production upon anti-CD3 stimulation and that tumor CD4+ T lymphocytes have a specific defect in CD40L induction after anti-CD3 ligation in vitro. This defect may explain the poor IL-12 production, because IL-12 production by anti-CD3–stimulated PBMCs was dependent on CD40L in healthy donors. The observed defect in tumor cell CD40L expression appears to be due to inappropriate T-cell signaling upon CD3 ligation, because expression of other T-cell activation antigens such as CD25, and to a lesser extent CD69, are also impaired on tumor cells. Importantly however, the inability of SzS PBMCs to appropriately produce IL-12 and TNF-α could be restored by recombinant hexameric CD40L. Taken together, our results demonstrate that impaired IL-12 and TNF-α production in SzS is associated with defective CD4+ T lymphocyte CD40L induction and indicate that CD40L may have therapeutic potential in SzS. (Blood. 2005;105:219-225)

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Patients, materials, and methods

Patients, peripheral blood mononuclear cells (PBMCs), and dendritic cell preparation

All 5 patients participating in this study were diagnosed with Sézary syndrome (SzS), the leukemic form of CTCL, on the basis of clinical, histopathologic, and immunohistologic criteria (Table 1). Patients’ circulating malignant T cells were analyzed on 1-μm sections of formalin-fixed peripheral blood buffy coats by the detection of mononuclear cells with cerebroform nuclear morphology. Additionally, for this report, patients with SzS were divided into 3 groups based on tumor load. Those with 5% to 20% circulating Sézary cells were defined as having low tumor burden; those with more than 20% to 50% circulating Sézary cells were defined as having medium tumor burden; and those with more than 50% circulating Sézary cells were defined as having high tumor burden. White blood cell...
counts of patients with SzS selected for these studies ranged from 3.5 × 10^6 to 5 × 10^6 cells per milliliter. All patients with SzS underwent identical treatment consisting of the use of extracorporeal photopheresis approximately every 4 weeks. None of the patients studied had been treated with chemotherapy, radiation, or other immunosuppressive drugs. As controls, blood samples from age-matched healthy donors were used. Donations of blood by patients or healthy volunteers in this study conformed to institutional review board (IRB)–approved protocol, and informed consents were obtained.

PBMCs were prepared as described previously. Briefly, venous blood was collected into heparinized syringes using uniform standards for both patients with SzS and healthy volunteers. The blood was then diluted 2-fold with Dulbecco phosphate-buffered saline (DPBS; BioWhittaker, Walkersville, MD), pH 7.2, layered over Ficoll-Hypaque (Amersham, Uppsala, Sweden), and centrifuged at 500g for 30 minutes at room temperature. The interface containing the mononuclear cell fraction was collected, and cells were washed twice with PBS. Cells were used immediately after purification.

Cell cultures were set up at a final concentration of 1 × 10^6 cells per milliliter in RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 mM L-glutamine, and 100 U/mL penicillin-streptomycin.

Dendritic cells (DCs) were derived from human cord blood CD34+ cells amplified in a primary culture with FLT3-ligand, thrombopoietin, and stem cell factor as previously described. Briefly, DC precursors recovered from primary culture were washed, counted, and seeded at 2 × 10^6/mL in 24-well plates containing 1 mL DC medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 10 mM HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid], 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin). Cells were induced for 3 days with granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/mL) and IL-4 (20 ng/mL) (Strathmann Biotec, Hamburg, Germany) in the presence of 50 µM 2-mercaptoethanol (Sigma, St Louis, MO). After 3 days, cells were refed with 0.5 mL fresh DC medium, cytokines were renewed, and cells were further cultured for 3 days. Maturation factors such as tumor necrosis factor-α (TNF-α) (100 ng/mL), lipopolysaccharide (LPS) (20 ng/mL), or ACRP30-CD40L (megaCD40L, 100 ng/mL) as well as control molecules such as adipocyte complement–related protein of 30K (ACRP30) were added for the last 48 hours. Flow cytometric analysis of DC surface CD1a, CD83, and HLA-DR expression was performed after 6 days of DC induction.

Antibodies and reagents

Fluorescein-isothiocyanate (FITC)–conjugated monoclonal antibodies (mAbs) specific for human CD40L (clone TRAP1, mlgG1), CD40, CD80, and CD86 were purchased from BD Biosciences Pharmingen (San Jose, CA). Phycoerythrin (PE)–labeled mAbs specific for CD3 (clone HIT3a, mIgG2a) used for T-cell activation and unlabeled mAbs specific for CD4 were purchased from BD Biosciences Pharmingen. Unlabeled mAbs specific for CD3 (clone HIT3a, mlgG2a) used for T-cell activation and unlabeled antagonistic mAbs specific for CD40L (TRAP1) used for CD40L blockade were purchased from BD Biosciences Pharmingen. Neutralizing Abs to IL-4, IL-10, and tumor growth factor-β1 (TGF-β1) were purchased from R&D Systems (Minneapolis, MN). Phorbol myristate acetate (PMA) (50 ng/mL) and CD3 activation and ELISA quantification of secreted cytokines

Anti-CD3 was dissolved in carbonate buffer (pH 9.6) at a concentration of 1 µg/mL and aliquoted into 24-well tissue culture plates at 250 µL per well. After a 2-hour incubation at 37°C, the plates were washed twice in sterile PBS. Cell suspensions were then added. When necessary, human IFN-γ was used at 1000 U/mL, SAC (Calbiochem) at 1:10 000 (wt/vol), and LPS from Escherichia coli (Sigma) at 1 µg/mL. For fluorescence-activated cell sorter (FACS) analysis, SzS or healthy-donor PBMCs were incubated at 37°C for 6 hours, after which cells were collected and analyzed. For quantification of cytokine secretion into the media, PBMCs were similarly incubated but for 48 hours, supernatants collected, centrifuged twice to pellet contaminant cells, and frozen at −20°C until analysis. IFN-γ, IL-12, and TNF-α concentrations were determined by specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer’s instructions.

Generation of soluble hexameric CD40L

Cloning, expression, and purification of recombinant proteins were performed essentially as described previously. Expression constructs for ACRP30 and ACRP30-CD40L (megaCD40L) were generated according to standard molecular biology protocols and cloned in the PCR-3 vector (Invitrogen, Leek, The Netherlands). The constructs encode the signal peptide of hemagglutinin (MAHYLYLFFAVGRG), the Flag sequence (DYKDDDDK), a linker (GGGQVLQ), the collagen domain of huACRP30 (amino acids [aa] 16 to 108), and the C-terminal portion of human CD40L (huCD40L; aa 116 to 219). Recombinant proteins were also expressed from stable HEK-293 clones and were purified by affinity on M2-agarose.

Immunoscore analysis of the Vβ repertoire

Total RNAs from CD4+ CD7+ cells were extracted with Qiagen RNeasy Kit upon manufacturer’s instructions (QIagen, Basel, Switzerland) and converted to cDNA by standard methods using reverse transcriptase and an oligo(dT) primer (Invitrogen). The cDNAs were amplified in nonsaturating polymerase chain reaction (PCR) conditions (30 cycles) with a panel of previously validated 5’ sense primers specific for 22 Vβ subfamilies (Proligo, Paris, France) and one 3’ anti sense primer specific for the corresponding C gene segment. Amplification products were run in a 2% agarose gel stained with ethidium bromide and visualized under a UV lamp.

Statistical analysis

To determine the statistical significance, levels of cytokines were compared between healthy donors and patients with SzS using the unpaired Student t test. Level of significance assumed in these comparisons was a P value of less than .05.

Results

Impaired IL-12 and TNF-α production by APCs from patients with SzS

In our study of the immune function in patients with SzS, we determined the capacity of PBMCs from patients with SzS to produce IL-12 and TNF-α when stimulated with plate-bound anti-CD3. To this end we cultured PBMCs from patients with SzS and healthy donors with anti-CD3 and measured IL-12 and TNF-α secretion into the culture media by ELISA after 48 hours. As shown in Figure 1A, PBMCs of patients with SzS produced significantly reduced levels of both IL-12p70 and TNF-α as compared with healthy donors. In patients with SzS, PBMC IL-12 and TNF-α levels were more than 6-fold reduced, as they produced a mean of 0.5 ± 1 pg/mL IL-12p70 and 1350 ± 1100 pg/mL TNF-α as compared with a mean of 21 ± 10 pg/mL IL-12p70 and 8925 ± 1456 pg/mL TNF-α in healthy donors (P = .025 and .0002 for IL-12 and TNF-α, respectively). This reduced production could not solely be attributed to an intrinsic inability to synthesize these cytokines, because in patients with SzS, LPS/IFN-γ (Figure 1B,D) and SAC/IFN-γ (not shown) were able to induce significant IL-12 and TNF-α production.
The production of IL-12p70 after 24 hours is shown.

In our assay for anti-CD3 stimulation of total PBMCs, production of IL-12 is thought to be due to induction of APC stimulatory molecules on T cells upon anti-CD3 stimulation. TNF-α production in this assay could occur by this indirect effect but also by anti-CD3–stimulated T cells themselves. Because CD40 signaling in APCs is known to induce IL-12, CD40L is likely to be one of the costimulatory molecules involved in our assay. To determine if blockade of CD40L signaling could reproduce the same defect in IL-12 production in an identical experimental setting, we performed the same experiment with PBMCs from healthy donors (n = 2) in the presence of an antagonistic antibody to CD40L. As shown in Figure 1E, inhibiting CD40L–CD40 interaction abrogated the capacity of PBMCs from healthy donors to produce IL-12 upon CD3 stimulation. Deficient CD40L signaling by CD3-stimulated T cells of patients with SzS could thus possibly explain the inability of PBMCs from patients with SzS to produce cytokines such as IL-12 that are essential for the generation of TH1 responses.

**Defective induction of CD40L expression on SzS tumor cells**

Previous studies have shown that T-cell activation leads to rapid surface expression of CD40L, thus enabling T cells following CD40L–CD40 interaction to stimulate APCs to produce cytokines.26-29 We next analyzed induction of CD40L upon CD3 ligation of PBMCs from patients with SzS. To discriminate between the tumor and nontumor CD4+ T-cell populations, we took advantage of the loss of the CD7 marker reported on CD4+ tumor cells from some patients with SzS.30 In healthy donors, most of the CD4+ T cells expressed CD7 (Schanberg et al31 and Figure 2A, left panel). As previously reported, several of the patients with SzS studied here harbored a CD4+ T-cell tumor population with a marked loss of CD7 expression, as evidenced by an abnormally increased CD4+CD7− cell population in total PBMCs (Figure 2A, middle and right panels; Table 1). We analyzed induction of CD40L expression upon anti-CD3 stimulation on tumor T cells. Figure 2B shows that stimulated tumor CD4+CD7− T cells did not express CD40L on their surface (Figure 2A, middle and right panels) compared with stimulated CD4+CD7+ T cells from healthy donors (left panel). This absence of CD40L induction upon anti-CD3 stimulation was observed in all the patients with a CD4+CD7− tumor (n = 3, Table 1) and 1 patient with a CD4+CD7+ tumor phenotype (Table 1). One patient with a CD4+CD7+ tumor and a high burden (S3) also had a complete absence in CD40L induction on tumor cells (Table 1). On the contrary, the CD4+CD7− T cells from healthy donors (n = 12) expressed CD40L upon anti-CD3 stimulation (data not shown; mean of 10% ± 7% CD40L+ cells). Altogether, this demonstrates that in patients with SzS there is a marked deficit in the ability for CD40L induction on tumor T cells.

We next studied whether normal CD4+ T cells (nontumor cells) from patients with SzS are also defective for CD40L induction. We performed our analysis on CD4+CD7+ T cells. Patients S2 and S3, whose tumors are CD4+CD7−, were excluded. To confirm that we were analyzing a nontumor population, polyclonality was assessed by the immunoscope technique. Table 2 shows that the Vβ repertoire of the CD4+CD7+ population of 2 selected patients with SzS is as polyclonal as the normal counterpart from a healthy donor, indicating its nontumor characteristic. Figure 3A shows that a fraction (14%) of normal CD4+CD7+ T cells from patient S1 expressed CD40L upon anti-CD3 stimulation. When 3 patients with SzS with a detectable population of nontumoral CD4+CD7+ cells were analyzed, we observed that the mean percentage of CD40L+ cells was not significantly different on the normal CD4+CD7+ T cells of patients with SzS (14% ± 13%) as compared with those of healthy donors, which ranged from 5% to 46% (n = 13) (Figure 3B). This suggests that the remaining normal CD4+ T cells in patients with SzS are capable of expressing CD40L upon stimulation, in contrast to their tumor counterpart.

**Figure 1. Impaired IL-12p70 and TNF-α production in patients with SzS.** Total PBMCs from healthy donors and patients with SzS were activated with anti-CD3 (A,C) or a mixture of LPS (1 μg/mL) and IFN-γ (500 IU/mL) (B,D). After 24 hours, supernatants were harvested and assayed for IL-12p70 (A,B) and TNF-α (C,D) production. (E) Total PBMCs from a healthy donor were activated as in panel A in the presence of a blocking mAb against CD40L (10 μg/mL) or an isotype-control mAb. The production of IL-12p70 after 24 hours is shown.

**Figure 2. Impaired CD40L induction on SzS tumor cells.** (A) PBMCs from healthy donors and patients with SzS were double-stained for CD4 and CD7 expression. Representative dot plots are shown for a healthy donor (left panel), patients S1 (middle panel), and S5 (right panel), which both have a CD4+CD7− tumor. The percentage of labeled cells for each quadrant is indicated in the upper right quadrant. (B) PBMCs were activated with anti-CD3 as in Figure 1. After 6 hours, cells were immunostained for CD40L and analyzed by flow cytometry. Fluorescence histograms are shown for unstimulated (thin lines) and anti-CD3–stimulated (thick lines) gated CD4+CD7− cells.
Healthy donor surface of T cells,33 CD4 gating could not be applied for PMA-cytometry. Because PMA is known to down-regulate CD4 from the cinn (PI) and then analyzed for expression of CD40L by flow patients with SzS (n completely abolished in the SzS tumor cells.

T-cell receptor (TCR)/CD3 signaling was severely impaired but not potently induce T-cell CD40L expression. 32 Total PBMCs from patients with SzS, the significant expansion of tumor CD7

Table 2. Representation of 22 Vβ subfamilies expressed on CD4+CD7+ T cells

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observed in healthy-donor PBMCs. This shows that the deficiency in IL-12 and TNF-α production observed in patients with SzS can be restored in vitro by exposure of PBMCs to an active form of soluble human CD40L (megaCD40L).

Discussion

Our findings demonstrate that T cells from patients with SzS have an important defect in expressing CD40L after CD3 ligation. This deficiency is restricted to the clonal tumor T-cell population. Furthermore, our data suggest that the reduced ability of PBMCs from patients with SzS to produce IL-12 and TNF-α after exposure to CD3 is due to the inappropriate induction of CD40L expression on tumor CD4+ T cells and the reduced number of normal CD4+ T cells because: (1) a similar defect in cytokine production is reproduced when PBMCs from healthy donors are stimulated by anti-CD3 in the presence of CD40L-blocking antibodies; and (2) production of IL-12 and TNF-α can be virtually completely restored in this setting in the presence of active soluble hexameric CD40L. It is thus likely that the abnormalities in TH1 cytokine and IL-12 production that characterize SzS are in part attributable to deficient CD40L signaling.

Several mechanisms could potentially account for the diminished CD40L expression in patients with SzS. Because certain TH2-derived cytokines such as IL-10 inhibit antigen-dependent IL-12 secretion by DCs, we considered the possibility that they may also affect CD40L expression by CD4+ T cells, we considered the possibility that they may also affect CD40L expression by CD4+ T cells, and the reduced number of normal CD4+ T cells because: (1) a similar defect in cytokine production is reproduced when PBMCs from healthy donors are stimulated by anti-CD3 in the presence of CD40L-blocking antibodies; and (2) production of IL-12 and TNF-α can be virtually completely restored in this setting in the presence of active soluble hexameric CD40L. It is thus likely that the abnormalities in TH1 cytokine and IL-12 production that characterize SzS are in part attributable to deficient CD40L signaling.

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Some of these mechanisms may account for the diminished CD40L expression and in IL-12 and TNF-α production observed in patients with SzS can be restored in vitro by exposure of PBMCs to an active form of soluble human CD40L (megaCD40L).
peripheral APCs in patients with SzS are able to produce IL-12 and PBMCs are stimulated with anti-CD3. These results imply that they fail to produce significant levels of these cytokines when their PBMCs are stimulated with bacterial products such as LPS or SAC in conjunction with IFN-γ. The role of CD40L-CD40 interaction in TH1 development and cell-mediated immunity thus provides a basis for the finding that patients with SzS present diverse T-cell defects. One of the most profound of these defects is the inability of patients with SzS to produce TH1 cytokines as well as IL-12.

In the present study we have demonstrated that although patients with SzS have the capacity to produce IL-12 and TNF-α when their PBMCs are stimulated with bacterial products such as LPS or SAC in conjunction with IFN-γ or with soluble CD40L, they fail to produce significant levels of these cytokines when their PBMCs are stimulated with anti-CD3. These results imply that peripheral APCs in patients with SzS are able to produce IL-12 and TNF-α as a consequence of direct stimulation but manifest defects when such cytokine production is to be elicited by antigen stimulation. This is most likely due to the fact that most CD4+ T cells in the peripheral blood of such patients are tumor cells and consequently unable to transmit TCR/CD3 signals and express CD40L. As such, this study not only provides an explanation for some of the immune abnormalities associated with SzS but also raises the possibility that the poor TH1 T-cell responses and antitumor immunity in patients with SzS may be ameliorated by treatment with recombinant CD40L.

Acknowledgments

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References


Figure 5. Recombinant megaCD40L induces IL-12p70 and TNF production in patients with SzS. (A) Dendritic cells derived from CD34+ stem cells of healthy donors were stimulated with the indicated reagents. MegaCD40L (ACRP30-CD40L) and ACRP30 were used at 100 ng/mL. After 18 hours, CD83 surface expression was monitored by immunostaining and flow cytometry analysis. Fluorescence histograms are shown. (B-C) Total PBMCs from a healthy donor and 2 patients with SzS were stimulated with the indicated reagents. IFN-γ was used at 500 IU/mL, LPS at 1 μg/mL, and megaCD40L at 100 ng/mL. After 24 hours, supernatants were harvested and assayed for IL-12p70 (B) and TNF-α (C) production.


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