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Immunology

Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation

(germinal center/T cell/CD23)

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ABSTRACT **Herein we describe the molecular characterization of the human leukocyte activation antigen CD100 and identify it as the first semaphorin, to our knowledge, in the immune system. Semaphorins have recently been described as neuronal chemorepellants that direct pioneering neurons during nervous system development. In this study we demonstrate that CD100 induces B cells to aggregate and improves their viability *in vitro*. We show that CD100 modifies CD40-CD40L B-cell signaling by augmenting B-cell aggregation and survival and down-regulating CD23 expression. Thus, these results suggest that semaphorins as exemplified by CD100 also play a functional role in the immune system.**

Previous studies identified CD100 as a 150-kDa cell surface homodimer that is expressed on resting T cells and increases after activation via phytohemagglutinin (1). Antibody crosslinking of the CD100 molecule was shown to provide a proliferative signal to T cells in the presence of submitogenic levels of anti-CD3 or anti-CD2 antibodies, suggesting that this molecule is involved in lymphocyte activation (2). To further elucidate the function of this novel receptor for a costimulatory signal, we cloned the CD100 gene and determined that it is a member of the semaphorin (Sema) gene family of neuronal guidance molecules.

Semas are characterized by a phylogenetically conserved 500 amino acid (aa) Sema domain in their amino terminus. Semas provide a subset of the molecular guidance cues that navigate pioneering axons to their predestined targets during neural development (3–5). *In vitro*, axonal growth processes extend freely in all directions. However, when a Sema source is introduced into the culture, the growth processes are induced to collapse and turn away from the Sema. The *in vivo* biological significance of Semas is evidenced by defective nervous system assembly in *Drosophila* Sema II heterozygous mutants; their defects include inability to fly, drastically reduced life span, and a predisposition for the flies to dramatically raise their flightless wings *ante mortem*. Although Semas have been primarily characterized in the nervous system as chemorepellants, the ligands to which they bind remain unknown. Herein we describe the molecular characterization of CD100 and initiate the study of the function of Semas in the immune system.

MATERIALS AND METHODS

CD100 cDNA Isolation. A cDNA library was constructed in the pCDM8 vector (6) using poly(A)$^+$ RNA from phytohemaglutinin-activated human T cells and size-selected for inserts greater than 3 kb. DEAE-dextran transfected COS cells (6) were incubated with the anti-CD100 mAb BD16 (2). BD16 binding cells were isolated by panning on goat anti-mouse IgG1 (Fisher)-coated plates (7). Plasmid DNA was isolated from adherent cells and transformed into *Escherichia coli* DH10B/P3. Cultures of these bacteria were expanded and used to reintroduce selected plasmid DNA into COS cells by spheroplast fusion. These COS cells were then subjected to a second round of panning as described above. Single-colony DNA preparations from bacteria transformed after the second round were transfected into COS cells.

Transfectants. COS cells were transiently transfected and 3 days later incubated with BD16, BB18 (1), F937G2 (Leukocyte Typing Workshop V), anti-B7-1 mAb B1.1, or isotype-matched mouse-IgG1 (Coulter), followed by goat-anti-mouse IgG1 phycoerythrin (Fisher). Cells were analyzed by flow cytometry. Stable CD100 transfectants were generated by electroporating CHO-K1 and NIH 3T3 fibroblasts with the 4.3-kb CD100 cDNA and/or the drug selection plasmids pGK hygromycin or pSV2 neomycin. Transfectants expressing CD100 were selected twice by cell sorting with anti-CD100 mAb A8 (PharMingen) and cloned. CD40L transfectants have been described (8). All transfectants were tested by ELISA (Boehringer Mannheim) and polymerase chain reaction (Stratagene) methods and found to be free of mycoplasma.

Immunoprecipitation. Cell lysates of ^{125}I-labeled T cells and transfected CHO cells were immunoprecipitated with BB18 or the control antibody, electrophoresed in reducing conditions on an 8.5% SDS/PAGE gel, dried, and autoradiographed for 1 h.

DNA Sequence Analysis. Sequencing was carried out using synthetic oligonucleotide primers and dye-labeled terminator/*Taq* polymerase chemistry and analyzed on an automated fluorescent DNA sequencer (Applied Biosystems/Perkin–Elmer).

Northern Blot Analysis. Multiple tissue Northern blots (CLONTECH) were hybridized overnight at 42°C in 50% formamide/6× SSPE (1× SSPE = 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/2× Denhardt's solution/DNA (100 μg/ml) salmon sperm/probe (10^6 cpm/ml) (9). The probe consisted of two *Xho*I fragments encoding the entire CD100 cDNA, which were labeled with [α-^{32}P]dCTP and [α-^{32}P]dATP by random priming (10). After hybridization the filters were washed to a final stringency of 2× standard saline citrate (SSC)/0.1% SDS at 65°C. The filters were used

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Abbreviations: SEMA, semaphorin; H, human, M, mouse.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U60800).

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to expose Kodak XAR-5 film with an intensifier for 1 day at −70°C.

B-Cell Viability and Proliferation Assays. Human B cells were derived from cryopreserved spleen and tonsil as described (8). NIH 3T3 transfectants were harvested, irradiated (96 Gy), plated at a fixed concentration of 2×10^4 total transfectants per well in 96-well plates, and incubated overnight at 37°C. Human splenic B cells were cultured on transfectants in duplicate at 5×10^4 B cells per 100-μl culture in B-cell medium (8). Photographs of cell cultures were taken at a magnification of ×100 at 24, 48, and 72 h. The number of clusters and the size of the clusters was determined from the photographs. B-cell viability was determined for 10^6 B cells plated on 10^5 irradiated transfectants in 24-well plates cultured for 3 days.

Immunocytochemistry. Cryostat sections of lymph node tissue were fixed in acetone for 10 min, washed with PBS, incubated with primary mouse monoclonal antibody for 1 h, washed with PBS, incubated with biotinylated horse anti-mouse antibody (Vector Laboratories) for 30 min, washed with PBS, then incubated with avidin–biotinylated-peroxidase complex (Vector Laboratories) for 40 min, followed by reaction with diaminobenzidine/hydrogen peroxide. Sections were subsequently stained with 2% methyl green. A representative secondary lymphoid follicle was fixed in 10% buffered formaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin/eosin.

RESULTS

Molecular Cloning and Characterization of CD100. We used COS-cell expression cloning (7, 11) to isolate a 4.3-kb cDNA for CD100. COS cells transiently transfected with the CD100 cDNA acquired the ability to bind specifically to three mAbs directed against CD100 but not to control mAbs (Fig. 1*a*). The identity of the protein encoded by the CD100 cDNA was further verified by immunoprecipitation of a 150-kDa protein from both stable CHO-K1 transfectants and activated T cells (Fig. 1*b*).

DNA sequence analysis of the CD100 cDNA revealed a single long open reading frame of 2586 bp that contained five potential initiation codons within the first 150 bases. Initiation was likely to occur at the second ATG that, unlike the first, is in a favorable context for translation initiation (14). Using the 4.3-kb CD100 cDNA as a probe, we rescreened the activated T-cell library and isolated a number of additional CD100 clones including a 7-kb and 4.5-kb cDNA. Sequence analysis of these two clones revealed the identical sequence in the CD100 coding region; these cDNA differed only in having a longer 3′ untranslated region.

A BLAST search of the protein database indicated that the CD100 sequence encodes a novel protein with homology to the Sema gene family. The family of Semas has been categorized into five classes based on whether they are secreted or cell surface molecules and whether the Sema domain is followed by a transmembrane region, an Ig-like domain or a thrombospondin repeat region (15). Of the completely sequenced Semas, human Sema III (H-Sema III) and mouse Sema C (M-Sema C) have the greatest homology to CD100 (Fig. 2*a*). H-Sema III and M-Sema C are secreted molecules in which the Sema domain is followed by a C-2 type Ig-like domain. CD100 shares 39% aa identity with H-Sema III in the Sema domain and 33% identity in the Ig-like domain; the remaining carboxyl terminus of CD100 is unrelated to other Semas. CD100 is a class IV Sema (15) consisting of an amino-terminal signal sequence followed by a Sema domain, an Ig-like domain, a lysine-rich stretch of 104 aa, a hydrophobic transmembrane region, and a cytoplasmic tail of 110 aa (Fig. 2*b*). CD100 contains 15 of the 16 conserved Sema-domain cysteines and 9 potential N-linked glycoslyation sites. There is a consensus site

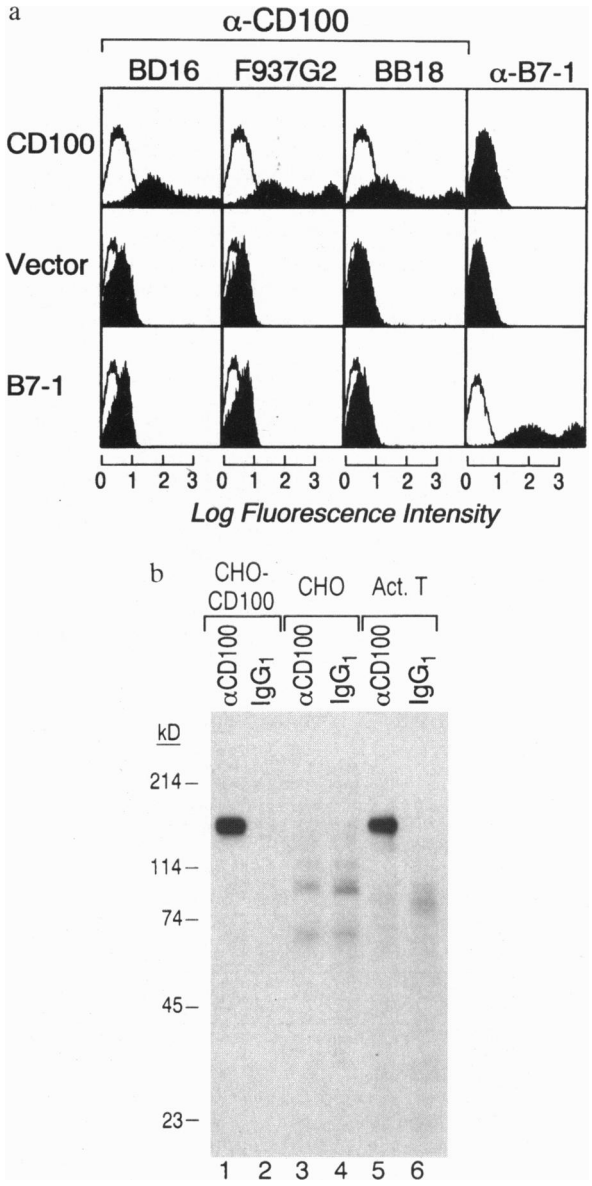
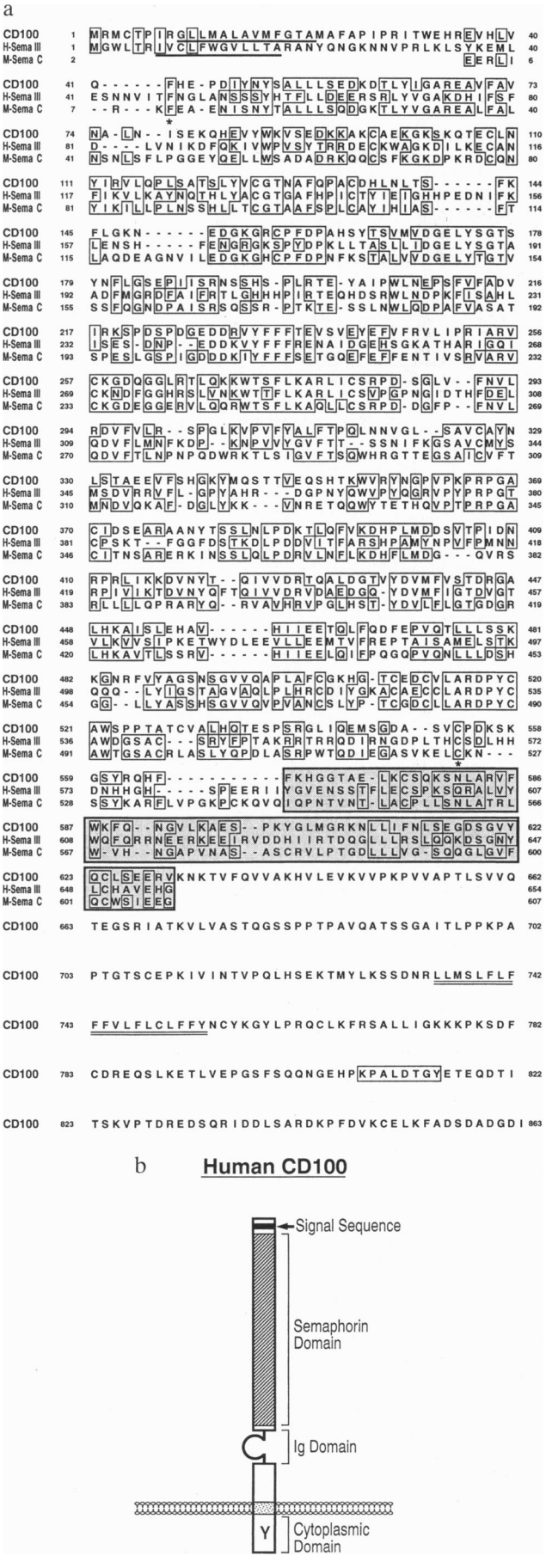


FIG. 1. Surface expression of human CD100 in transfected cells. (*a*) COS cells were transiently transfected with CD100 4.3-kb cDNA (*Top*), pCDM8 vector alone (*Middle*), and B7-1 cDNA (*Bottom*). After 72 h, cells were stained with antibodies against CD100-BD16 (2), BB18 (1), and F937G2 (13), and an isotype matched B7-1 antibody, B1.1. Binding of the mAbs was detected by indirect immunofluorescence using goat anti-mouse IgG1 coupled to phycoerythrin and analyzed by flow cytometry. Binding of control IgG1 and the indicated mAbs are shown by unshaded and shaded histograms, respectively. (*b*) CD100 mAb BB18 immunoprecipitated a 150-kDa protein from activated T cells and CHO-K1 cells transfected with CD100 cDNA. CHO cells were stably transfected with CD100 cDNA or vector alone. The positions of the molecular weight markers are indicated to the left of the gel. Lanes 1 and 2 are lysates from CHO CD100 immunoprecipitated with BB18 or the control antibody. Lanes 3 and 4 correspond to lysates from mock-transfected CHO immunoprecipitated with BB18 or the control antibody. Lanes 5 and 6 correspond to lysates from activated T cells immunoprecipitated with BB18 or the control antibody.

for tyrosine phosphorylation, KPALTGY at aa 813 in the cytoplasmic tail and multiple consensus sites for serine phosphorylation.

CD100 Is Broadly and Differentially Expressed. Northern blot analysis of CD100 mRNA expression indicated that CD100 is broadly expressed in hematopoietic and nonhematopoietic tissues, suggesting that CD100 may be functional in



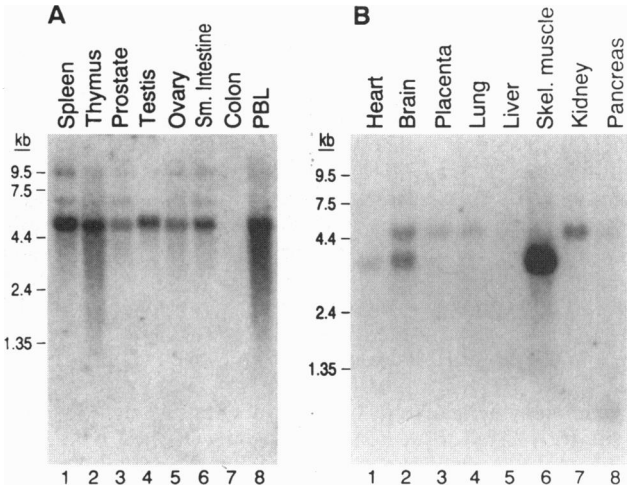


FIG. 3. CD100 mRNA expression. Expression of CD100 in multiple human tissue Northern blots (CLONTECH) containing (A) spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood lymphocytes and (B) heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas RNA. Each lane contains 2 μg of poly(A)$^+$ RNA and was probed with the 4.3-kb CD100 cDNA insert. The positions of the molecular weigth markers are indicated on the left of the gel in kb.

multiple systems (Fig. 3). CD100 transcripts were strongly expressed in skeletal muscle, peripheral blood lymphocytes, spleen, and thymus and also expressed at lower levels in testes, brain, kidney, small intestine, prostate, heart, placenta, lung, and pancreas but not in colon or liver. In most of these tissues, the major transcript was 4.5 kb with larger transcripts of 7 and 10 kb also present. Heart and muscle expressed only a 3.8-kb transcript. The 3.8-kb transcript was also expressed in the brain along with the other three transcripts.

CD100 Expression in the Lymph Node. Analysis of the *in vivo* distribution of CD100 in lymphoid organs was carried out by immunoperoxidase staining of lymphoid follicles in the lymph node, spleen, and tonsil. CD100 expression was distinctly localized to the interfollicular areas and the germinal center (GC) and virtually absent in the mantle zone (MZ) (Fig. 4*a*). The CD100 staining was broader than that observed with CD3, a pan T-cell marker that predominantly stained cells in the interfollicular areas and few cells in the GC or MZ (Fig. 4*b*). The CD100 expression pattern also differed from that observed with the B-cell-restricted molecule CD20, which stained most of the cells in both the GC and the MZ (Fig. 4*c*). The CD100 expression observed was consistent with cell surface staining. These results confirm the findings that CD100 is expressed on activated T and B cells.

CD100 Induces B Cells to Aggregate and Improves Their Viability *in Vitro*. In the lymphoid follicle, activated T cells express molecules such as CD40L that are required for mediating activation, survival, and developmental signals to B cells (16). We initiated our analysis of CD100 immunological function by determining whether CD100 was able to mediate a signal to B cells. We compared splenic B cells cultured in the

FIG. 2. Predicted sequence and structure of CD100. (*a*) Predicted full-length amino acid sequence of CD100 compared with the Sema and Ig domains of H-Sema III and M-Sema C. The sequences were aligned using CLUSTAL and regions of identity boxed by SEQVU (The Garvan Institute, Sydney, Australia). The putative signal sequence is underlined; Sema domains are delimited by *; shaded box indicates the Ig-like domain; transmembrane region is double-underlined; putative tyrosine phosphorylation site KPALTGY is in the thin clear box. The nucleotide sequence of CD100 was determined for both strands of the original 4.3-kb clone. (*b*) Schematic structure of CD100.

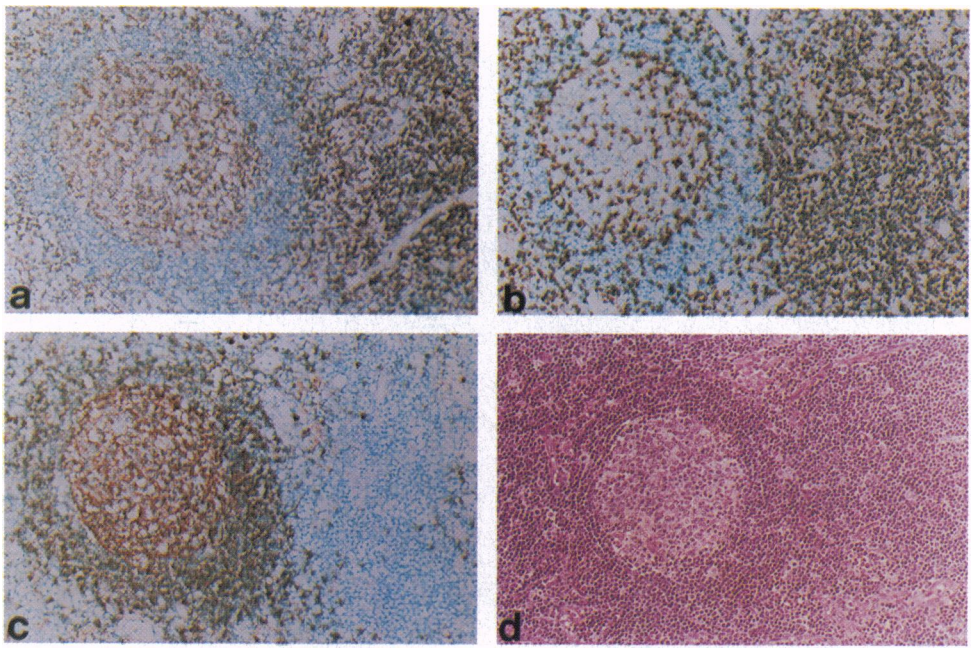


FIG. 4. Immunoreactivity in serial sections of a reactive lymph node. (*a*) CD100 mAb. (*b*) CD3, a pan-T cell marker. (*c*) CD20, a pan-B cell marker. (*d*) Representative germinal center and mantle zone of a secondary lymphoid follicle with adjacent interfollicular T-cell zone, from a reactive lymph node fixed in 10% buffered formaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin/eosin. (Original magnification: ×100.)

presence of the CD100 molecule provided by stable CD100 transfectants (t-CD100) to B cells cocultured with control mock transfectants (t-mock). After 3 days of coculture of splenic B cells with t-mock, no significant macroscopic changes in the B-cell culture were observed (Fig. 5 *a* and *c*). However, on coculture with t-CD100, B-cell aggregates formed as early as 6 h (Fig. 5 *b* and *c*). The number of aggregates was maximal at 24 h. In addition, the number of viable B cells was significantly increased in the presence of t-CD100 compared with the t-mock control ($P < 0.05$, Fig. 6*a*), suggesting that CD100 enhanced the viability of B cells. Splenic B cells sorted for $CD38^+/IgD^-$ formed similar aggregates in response to CD100 but $CD38^-/IgD^+$ B cells did not (data not shown). CD100 did not upregulate the activation antigens CD80 (B7-1), CD86 (B7-2), CD58, CD54, or CD11a (LFA-1) or induce the B cells to proliferate as determined by [^{3}H]thymidine incorporation (data not shown). Of note, B cells from other sources including tonsillar B cells, follicular lymphoma, and acute lymphoblastic leukemias were similarly induced to aggregate when cocultured in the presence of t-CD100 (unpublished data).

CD100 Augments CD40L-Induced B-Cell Aggregation and Cell Survival. B-cell signals such as those mediated by crosslinking the B-cell antigen receptor or by cytokines require CD40 crosslinking to sensitize the B cells and thus amplify the effect of the signal (17). CD40L delivers a contact-dependent activation signal to B cells via CD40 that results in B-cell aggregation, proliferation, increased survival, and differentiation (18, 19). To determine whether crosslinking CD40 amplifies the effects of CD100 on B cells, we combined CD40L transfectants (t-CD40L) with equal numbers of either CD100 or mock transfectants. The total number of transfectants was kept constant at 2×10^4 cells. CD40L transfectants induced B cells to aggregate as described (20) (Fig. 5 *a* and *b*). The combination of CD100 and CD40L signaling gave rise to B-cell aggregates that were much larger than those observed with either transfectant alone or with the control (Fig. 5*b*). Other ratios of transfectants were also examined (75% t-CD100/25% t-CD40L and 25% t-CD100/75% t-CD40L) and these consistently induced larger clusters than observed with the equivalent t-mock/t-CD40L control.

As was shown, CD40L signaling increases the viability of B cells (21). When CD100 was present in combination with CD40L, the viability of the B cells was significantly enhanced compared with CD40L alone ($P < 0.05$; Fig. 6*a*). The rate of proliferation, as determined by [^{3}H]thymidine incorporation was not affected by the presence of CD100 (data not shown).

CD100 Down-Regulates the Induction of CD23 Expression by CD40L. When we examined the effect of CD100 on the CD40L-mediated induction of B-cell activation markers, we found that the levels of CD80, CD86, CD58, CD54, and CD11a (LFA-1) expression were not affected by the presence of CD100 transfectants in the coculture (data not shown). However, the expression of CD23, the low-affinity IgE receptor, a B-cell activation and differentiation marker that is induced by CD40L, was markedly reduced in the presence of CD100 (Fig. 6*b*). To determine whether this was a result of CD100 mediating a signal that resulted in blockade of CD40L-induced CD23 expression or whether CD23 was expressed and then removed, we sequentially incubated the B cells with t-CD40L for 3 days, thereby inducing CD23, and then incubated them with either t-CD100 or control transfectants for an 3 additional days. B cells incubated with CD40L followed by t-mock continued to express CD23. In contrast, on B cells cultured sequentially with CD40L and then CD100, the CD23 expression was down-regulated after being induced (data not shown).

DISCUSSION

This paper describes the molecular cloning of the novel lymphocyte activation antigen CD100. CD100 is homologous to the semaphorin family of neuronal transmembrane and secreted chemorepellants. CD100 is a transmembrane semaphorin that contains a sema domain followed by an immunoglobulin-like domain, a hydrophobic transmembrane domain, and a cytoplasmic tail that encodes a site for tyrosine phosphorylation and multiple sites for serine/threonine phosphorylation, consistent with evidence showing the association of CD100 with a kinase (12, 22).

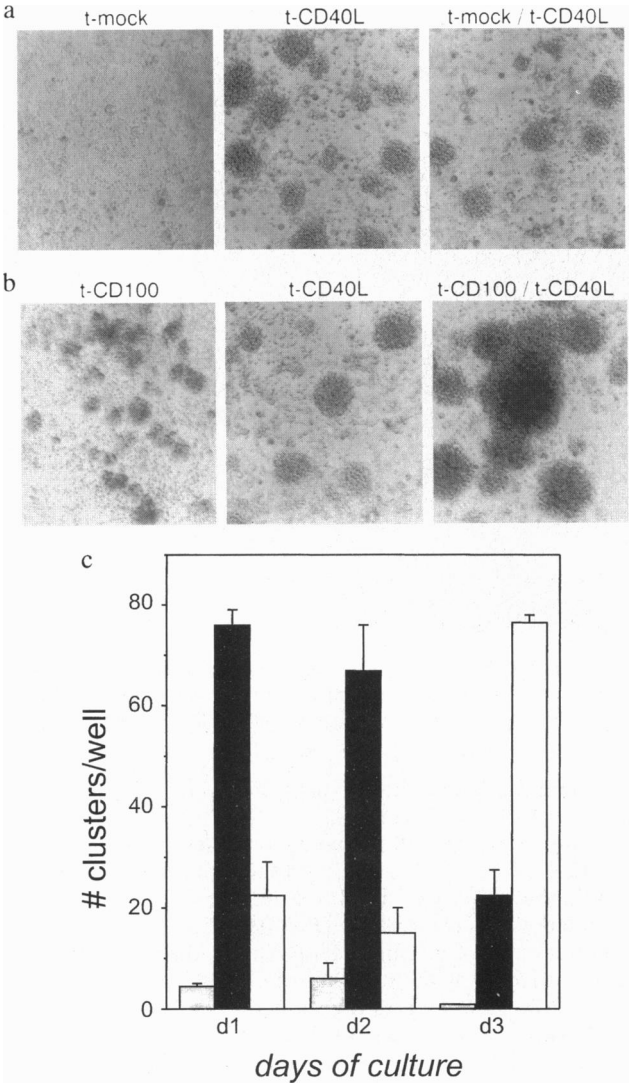


FIG. 5. CD100 transfectants stimulate B-cell aggregation. Aggregates of human splenic B cells cultured on transfectants: t-mock, t-CD40L, and t-mock/t-CD40L (50% t-mock + 50% t-CD40L) (*a*) and t-CD100, t-CD40L, and t-CD100/t-CD40L (50% t-CD100 + 50% t-CD40L) (*b*). Photographs of cell cultures were taken at 72 h. (×100.) (*c*) The number of B-cell aggregates after coculture on transfectants t-mock (shaded bars), t-CD100 (solid bars), and t-CD40L (open bars) per 96-well plate well enumerated on days 1–3.

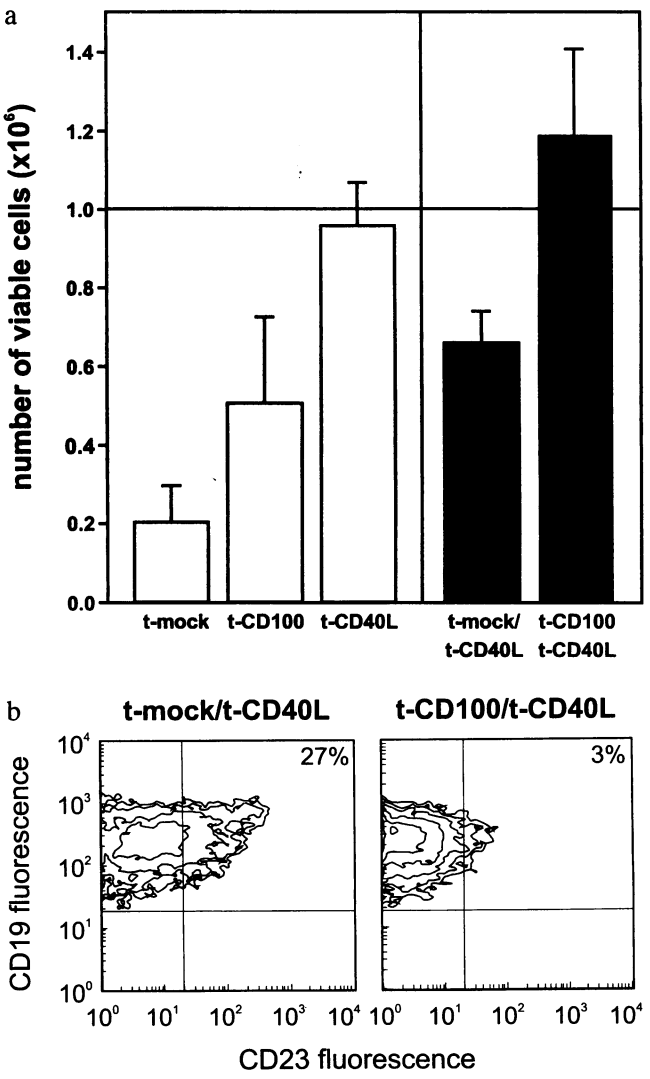


FIG. 6. (*a*) Cell survival of human B cells after CD40L- and/or CD100-induced signaling. Cocultures of B cells and transfectants were set up as described in Fig. 5. Viable B cells were counted by trypan blue exclusion analysis after 72 h in coculture with the indicated transfectants (*x* axis). Data shown are the average of six experiments. The horizontal line indicates the number of B cells added at the initiation of the culture (10^6 cells). (*b*) Effect of CD100 signaling on CD40L-induced CD23 expression. Two-color flow cytometric analysis of human splenic B cells was performed after 72 h of coculture with t-mock/t-CD40L cells or t-CD100/t-CD40L as described in Fig. 5. Cells were stained with CD19 conjugated to phycoerythrin (*y* axis) and CD23 conjugated to fluorescein isothiocyanate (*x* axis) (Coulter). Nonviable cells were excluded by propidium iodide counterstaining prior to analysis. The percentage of $CD23^+$ $CD19^+$ cells is indicated in the upper right corner of each contour histogram. Data shown are representative of five experiments.

CD100 was originally defined as a T cell activation antigen. Previous studies have shown that CD100 is expressed on B and T lymphocytes, natural killer cells, granulocytes, and monocytes but not on red blood cells or platelets (1). The low levels of CD100 on resting T cells were shown to decrease initially after activation with PHA and then gradually increase to high levels (2). Further T cell studies demonstrated that in the presence of sub-mitogenic levels of anti-CD3 or CD2 mAbs, anti-CD100 mAb provided a proliferative signal (1, 2) and that CD100 associated with the tyrosine phosphatase CD45 (23). Thus CD100 may signal in a bipolar way, being able to signal into a cell via its associations with a kinase and CD45 phosphatase and as shown in this paper, being able to deliver a signal to other cells via engagements of its counter-receptor.

We show herein that CD100 mRNA is broadly expressed in hematopoietic as well as nonhematopoietic tissues. Four different transcripts of approximately 10, 7, 4.5, and 3.8 kb were observed to be variably expressed. The predominant transcript is 4.5 kb. The broad expression of CD100, like other molecules in the immune system such as CD58 and CD95, does not preclude that this molecule has an important immunological function.

To further elucidate the immunological function of CD100, we used CD100 transfectants to mimic CD100-bearing activated T cells and determined what effect this molecule had on human B cells. We found that CD100 induced splenic and tonsillar B cells to aggregate as well as enhanced their *in vitro* survival. Given that CD100 is uniquely expressed in lymphoid follicles, CD100-induced aggregation may be important in the physiological context of the formation of the germinal center, a tight aggregate of mostly B and some T cells.

Activated T cells also express CD40L, a molecule that induces B-cell activation and aggregation. To understand the

role of CD100 in the context of this strong signal, we examined the effect of culturing B cells in the presence of both CD100 and CD40L. We found that CD40L-mediated B-cell aggregation and survival was significantly increased in the presence of CD100. Furthermore, we observed that CD23, which is induced by CD40L, was not expressed in the presence of CD100. CD23 the low-affinity IgE receptor, has been reported to be autoproteolytic and function as an adhesion molecule (24). Interestingly, CD23 proteolysis has been shown to induce B-cell aggregation and soluble CD23 has also been shown to function as a cytokine. Our data are consistent with the hypothesis that CD100 affects CD40L-mediated B-cell aggregation and survival by enhancing the proteolysis of CD23, resulting in enhanced B-cell aggregation and *in vitro* viability (25).

The formation of germinal centers in lymphoid follicles requires CD40L–CD40 interaction. The germinal center is the location where the multiple signals that govern B-cell differentiation and survival collaborate in an exquisitely regulated temporal and spatial dynamic. Despite the biological importance of CD40L, little is known about other cell–cell contact-mediated signals that act in concert with or downstream of CD40L. One would expect that B cells exiting the periarteriolar sheath and traversing activated T-cell-rich and, therefore, CD100-rich interfollicular areas will receive both a CD40L and a CD100 signal. To our knowledge, CD100 is the first cell surface molecule that *in vitro* can down-regulate the induction of CD23 after CD40 crosslinking. Furthermore, since CD23 is expressed on centroblasts but not on centrocytes, the down-regulation of CD23 associated with CD100 may be related to this developmental B-cell transition.

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