Identification of a Novel Inducible Cell-surface Ligand of CD5 on Activated Lymphocytes

By Luigi Biancone,* Michael A. Bowen,† Alice Lim,* Alejandro Aruffo,‡ Giuseppe Andres,* and Ivan Stamenkovic*

Summary

CD5 is a 67-kD glycoprotein that is expressed on most T lymphocytes and on a subset of mature B cells. Although its physiologic function is unknown, several lines of evidence suggest that CD5 may play a role in the regulation of T cell activation and in T cell-antigen presenting cell interactions. Using a CD5-immunoglobulin fusion protein (CD5Rg, for receptorglobulin), we have uncovered a new CD5 ligand (CD5L) expressed on the surface of activated splenocytes. Stimulation of murine splenocytes with anti-CD3 and anti-CD28 antibodies induces transient expression of CD5L on B lymphocytes that lasts for ~72 h. Binding of CD5Rg to activated splenocytes is trypsin-resistant and independent of divalent cations. However, it is pronase sensitive and dependent on N-linked glycosylation of CD5, since treatment of CD5Rg with PNGaseF or N-glycanase completely abrogates its ability to bind activated splenocytes. In addition to splenocytes, CD5L is expressed on activated murine T cell clones. Immunoprecipitation, antibody, and recombinant protein blocking studies indicate that CD5L is distinct from CD72, which has been proposed to be a CD5 ligand. To determine whether CD5-CD5L interaction might play a role in vivo, we tested the effect of CD5Rg in a murine model of antibody-mediated membranous glomerulonephritis. Injection of CD5Rg was found to abrogate development of the disease. Taken together, our results help identify a novel ligand of CD5 and propose a role for CD5 in the regulation of immune responses.
CD5Rg recognizes a novel, transiently expressed ligand on activated splenocytes, and that it can prevent antibody-mediated glomerulonephritis in vivo.

Materials and Methods

Development and Production of Soluble Recombinant Fusion Proteins. Soluble receptor globulins were developed by genetic fusion of sequences that encode the extracellular region of human CD5 to genomic DNA sequences that contain exons that encode the hinge, CH2, and CH3 domains of human IgG, as described (21). The plasmid containing sequences encoding CD5Rg were introduced into CHO cells by electroporation at 250 V/960 μF in 4-mm cuvettes using a gene pulser (Bio Rad Laboratories, Richmond, CA). Serum-free supernatants were collected 5–7 d after transfection, and soluble fusion proteins were purified on protein A-Sepharose (PAS) as previously described (21). A soluble CD8–Hlg fusion protein previously shown to be nonreactive with murine tissues (22) was prepared using the same approach, and served as a control. Purified soluble fusion proteins were subjected to SDS/10% PAGE under reducing conditions and were analyzed after the staining of gels with Coomassie blue. Soluble CD72 was prepared and produced in COS cells as described (23).

Splenocyte Preparation and Cell Lines. For splenocyte preparation, BALB/c mouse spleens were sheared through a 90-μm stainless steel sieve, and cells were pelleted by centrifugation. Erythrocytes were osmotically lysed by incubation with 2 ml sterile distilled water for 3 s followed by rapid addition of 50 ml PBS. After three washes in PBS, splenocytes were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 5% heat-inactivated FBS (GIBCO BRL).

Four T cell clones were used: the I-Ek-restricted, conalbumin-specific Th2 clone D10.G4.1 and pigeon cytochrome C-specific MHC class II-restricted Th1 clone F1-28, specific for bovine myelin basic protein (Sigma) was used at 20 μg/ml. Conalbumin, cytochrome C (Sigma) provided by the vendor. Untreated and PNGaseF-treated CD5Rg were subjected to SDS/10% PAGE, the gels were fixed and dried, and the precipitates were eluted by boiling. Precipitated proteins were subjected to SDS/10% PAGE under reducing conditions and were analyzed after the staining of gels with Coomassie blue.

Antibodies. FITC-conjugated rat anti-mouse CD8a was from Biosource (Camarillo, CA); FITC rat anti-mouse CD4 and rat anti-mouse Thy 1.2, PE-conjugated rat anti-mouse B220, purified hamster anti-mouse CD28, hamster anti-mouse CD3 (145-2C11), rat anti-mouse CD72, rat anti-mouse CD32, and CD16 (FcγRII and III receptors) and hamster anti-mouse CD48 were from Pharmingen (San Diego, CA). Rat anti-mouse CD2 (12-15) was a kind gift from Dr. P. Altevogt (Heidelberg, Germany). FITC-conjugated goat anti-rabbit IgG, rabbit anti-mouse IgG, and rabbit anti-rabbit C3 were purchased from Cappel. The goat anti-mouse IgG was absorbed with rabbit IgG and its specificity was confirmed by the absence of binding to kidneys of the mice killed 1 d after injection of rabbit anti-rat glomerular basement membrane antiserum (20), while staining with FITC goat Anti-rabbit IgG showed marked linear deposition of rabbit IgG on the glomerular basement membrane.

Flow Cytometry. FACS® (Becton Dickinson & Co., Mountain View, CA) analysis was performed on both splenocytes and cell lines. To avoid nonspecific Fc receptor-dependent binding, cells were preincubated for 1 h at 4°C with 100 μg/ml purified mouse IgG (Cappel) in PBS, washed, and stained with the appropriate reagents. The receptorglobulins were used at 5–10 μg/ml. For two-color analysis, splenocytes were first incubated with the receptor globulins for 1 h at 4°C, washed, incubated with affinity-purified, FITC-conjugated goat anti-human IgG (Cappel) preabsorbed with mouse, rat, and hamster serum, washed, and incubated with PE-conjugated mAbs recognizing distinct lymphoid lineages.

Immunoprecipitation. For metabolic labeling, cells were washed and incubated with methionine-free RPMI 1640 (GIBCO BRL) supplemented with 5% dialyzed, heat-inactivated FBS (Sigma) and appropriate stimulating antibodies (anti-CD3 and anti-CD28, anti-CD3 and anti-CD48, or anti-CD3 alone), and labeled with 250 μCi/ml [35S]methionine (Amersham International, Amersham, UK) for 12 h. Cells were then washed in PBS and lysed at 4°C for 1 h in a lysis buffer containing 1% Triton X-100 (Sigma), 10 μg/ml leupeptin (Sigma), 100 U/ml aprotinin (Sigma), and 10 mM PMSE (GIBCO BRL, Bethesda, MD). Nuclei were removed by centrifugation, and lysates were preclarified by a 1-h incubation with PAS CL4B beads (Pharmacia). Lysates were then incubated with the soluble receptorglobulin for 1 h at 4°C. PAS CL4B beads were subsequently added, and the incubation continued for another hour. PAS beads were washed, and immunoprecipitates were eluted by boiling. Precipitated proteins were subjected to SDS/10% PAGE, the gels were fixed and dried, and the proteins were analyzed after exposure of gel for autoradiography.

Induction of Membranous Glomerulonephritis and Treatment with CD5Rg. 8–9-wk-old C57Bl/10 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Membranous glomerulonephritis was induced in three groups of six animals each, as previously described (20). Briefly, all animals were mononephrectomized under sterile conditions and allowed to recover for at least 3 wk. On day 0 of the experiment, all animals were immunized with a single tail vein injection of rabbit anti-mouse tubular basement membrane IgG (RbAMBb), prepared as previously described (20). From days 0–40, the three groups of mice were injected every other day with 50 μg/ml of CD5Rg or CD8Rg in 150 μl of PBS or with 150 μl of PBS alone. All animals received the first 10 injections intravenously and the subsequent injections intraperitoneally, and all were killed on day 40. Immunofluorescence and electron microscopy studies of kidney sections, ELISA for detection of mouse anti-rabbit IgG in the sera, FACS® analysis of splenocyte populations, PBL counts, and proteinuria measurements were performed as previously described (20).
Fluorescence intensity

Figure 1. Binding of CD5Rg to mouse splenocytes. FACS® analysis of (A) binding of control receptor globulin CD8Rg to unstimulated splenocytes (a comparable FACS® profile was obtained using activated splenocytes, data not shown); (B) binding of CD5Rg to unstimulated splenocytes and to splenocytes stimulated for 24 h with anti-CD3 mAb alone (C) or anti-CD3 plus anti-CD28 mAbs (D).

Statistics. Statistical analysis, when applicable, was performed using Statview IV software (Abacus Concepts, Berkeley, CA) on a Macintosh SE computer (Apple Computer, Inc., Cupertino, CA). Differences between groups were compared by one-way analysis of variance and unpaired Student's t test.

Results

CD5Rg Recognizes a Ligand on Activated Murine Splenocytes To test the ability of CD5 to recognize putative cell-surface ligands, resting and activated murine splenocytes were incubated with CD5Rg, and specific binding was assessed by FACS® analysis. Binding of CD5Rg to resting and anti-CD3 antibody-activated splenocytes was minimal or absent (Fig. 1). However, CD5Rg bound splenocytes triggered with a combination of anti-CD3 and anti-CD28 antibodies at a concentration of 5 μg/ml each. Binding of CD5Rg to splenocytes was observed from 12 to 72 h after anti-CD3/CD28 stimulation, but was not detectable thereafter. Similar CD5Rg binding was observed to splenocytes costimulated with anti-CD3 and anti-CD48 antibodies (data not shown). Interestingly, two-color FACS® analysis revealed that CD5Rg binding was confined to the B220+ cell population (Fig. 2).

Binding assays were performed after preincubation of the splenocytes with 100 μg/ml of purified mouse IgG and with 100 μg/ml of anti-CD16 and anti-CD32 Fc receptor-blocking mAbs to minimize Fc receptor-dependent binding. Soluble human CD8Rg, Fas-Rg, and CD44Rg (22), used as negative controls, failed to bind activated splenocytes, while mouse CTLA4-Rg (25), used as a positive control, bound splenocytes as expected (data not shown). CD5Rg binding was not significantly altered by the presence of 10 mM EDTA or by pretreatment of splenocytes with trypsin (Table 1). In contrast, treatment of splenocytes with pronase totally abrogated CD5Rg binding, as did treatment of CD5Rg with PNGase-F or N-glycanase, which cleave N-linked glycans (Table 1).

Activated Splenocyte CD5L is Distinct from CD72. The T and B cell-surface receptor CD72, which is constitutively expressed on splenocytes, has been proposed to be a ligand of CD5 (14, 15). To determine whether the observed binding of CD5Rg to activated splenocytes is mediated by CD72, possibly altered by activation-associated posttranslational modifications, activated splenocytes were pretreated with the anti-CD72 mAb clone 10-1.D.2, which is reported to prevent CD5-CD72 interaction, at a saturating concentration (100 μg/0.5 × 10^6 cells/ml). Neither the anti-CD72 mAb nor preincubation of the minimal concentration of CD5Rg required to detect binding (5 μg/ml) with excess recombinant CD72–CD8 fusion protein (23) had any effect on CD5Rg binding to activated splenocytes (Fig. 3).

Binding of CD5Rg to Murine T Helper Clones. To further assess CD5 ligand expression, AE-7 (Th1), CDC25 (Th2), FI-28 (Th1), and D10.G4.1 (Th2) murine culture-propa-
Figure 2. B cells express CD5L after splenocyte stimulation with anti-CD3 and anti-CD28 mAbs. Two-color FACS analysis of splenocytes costimulated for 24 h with anti-CD3 plus anti-CD28 mAb and stained for B220 expression (vertical axes) and for CD8Rg (A) and CD15Rg (B) binding (horizontal axes).

Table 1. Characterization of CD5Rg Binding to Splenocytes and the AE.7 T Cell Clone

| Treatment       | Splenocytes | AE.7 |
|-----------------|-------------|------|
| CD8Rg           | --          | --   |
| CD5Rg           | +           | +    |
| CD5Rg + EDTA    | ++          | ++   |
| CD5Rg + Trypsin | +           | +    |
| CD5Rg + Pronase | --          | --   |
| CD5Rg + PNGase-F| --          | --   |
| CD5Rg + N-glycanase| --  | --   |

Mean fluorescence intensity (MFI) as a reflection of receptor/globulin binding is graded as follows: (+) MFI <15, weak binding; (++) 15 >MFI <100, intermediate binding; (+++) MFI >100, strong binding.

CD5Rg Inhibits the Development of Murine Membranous Glomerulonephritis, an Immune Complex-mediated Disease that Requires T–B Cell Interaction.

To determine whether the observed CD5–CD5L interaction might play a role in T–B cell interaction in vivo, we administered CD5Rg to mice in which development of antibody-mediated membranous glomerulonephritis had been induced.

The murine antibody-mediated model of membranous glomerulonephritis was first described by Assmann et al. (19), and its dependence on T–B cell interaction has been clearly established (20). MGN was induced in mononephrectomized C57Bl/10 mice according to a previously developed strategy (20). On day 0 of the experiment, all animals were immunized with a single intravenous injection of 7.5 mg RbAMBB, which among other antigens, recognizes dipeptidyl peptidase IV (DPPIV) on the surface of glomerular podocytes. Binding of RbAMBB to DPPIV on podocytes results in local shedding of immune complexes which, in the absence of an autologous immune response, are eliminated by local clearing mechanisms.

Receptor globulin

Figure 2. B cells express CD5L after splenocyte stimulation with anti-CD3 and anti-CD28 mAbs. Two-color FACS analysis of splenocytes costimulated for 24 h with anti-CD3 plus anti-CD28 mAb and stained for B220 expression (vertical axes) and for CD8Rg (A) and CD15Rg (B) binding (horizontal axes).

gated T helper cell clones were tested for CD5Rg binding. Each of the clones was stimulated with irradiated APCs and antigen and cultured in IL-2. CD5Rg was observed to bind to all four cell lines 10–12 d after stimulation (Fig. 4). The cells maintained the ability to bind CD5Rg for 3–4 d, but lost it thereafter (data not shown). The features of CD5Rg binding to the T cells were assessed on the AE-7 clone (Fig. 5 and data not shown).

CD5Rg Immunoprecipitates a 35–37-kD CD5L on Activated Splenocytes and T Cell Clones. To characterize the putative CD5L, radiolabeled lysates from anti-CD3/CD28 antibody-activated splenocytes were subjected to immunoprecipitation by CD5Rg. A single 35–37-kD protein was observed to be immunoprecipitated by CD5Rg (Fig. 6 A). Expression of this protein was consistent with activated splenocyte reactivity with CD5Rg. Immunoprecipitation of the protein was obtained only from lysates of day 1 to 2 anti-CD3/CD28 antibody–activated splenocytes. CD5Rg failed to precipitate the 35–37-kD polypeptide or any other protein species from the lysates of resting splenocytes and of cells that had been activated more than 3 d previously (Fig. 6 A). PNGaseF treatment of CD5Rg abrogated its ability to immunoprecipitate the 35–37-kD polypeptide from activated splenocyte lysates (Fig. 6 B), and CHO cell-derived CD44Rg displayed no reactivity with activated splenocytes (data not shown). Taken together, these observations support the notion that appropriate glycosylation of CD5, and not the Ig portion of the fusion protein, is required for CD5L recognition. Immunoprecipitation of T cell clone-derived lysates by CD5Rg revealed a similar 35–37-kD species (data not shown).

CD5Rg Inhibits the Development of Murine Membranous Glomerulonephritis, an Immune Complex-mediated Disease that Requires T–B Cell Interaction. To determine whether the observed CD5–CD5L interaction might play a role in T–B cell interaction in vivo, we administered CD5Rg to mice in which development of antibody-mediated membranous glomerulonephritis had been induced.
Figure 3. Binding of CD5Rg to splenocytes is CD72 independent. Staining of activated splenocytes was performed with control CD8Rg (A), with CD5Rg (B), with CD5Rg after preabsorption of the cells with anti-CD72 mAb (C), or with CD5Rg after preabsorption of the receptor globulin with soluble CD72-CD8 fusion protein (D).

Figure 4. Activated murine T cell clones bind CD5Rg. Murine T cell clones were stimulated for 12 d as described in Materials and Methods and incubated with PBS (solid line), CD8Rg (dotted line), or CD5Rg (dashed line), followed by an FITC-labeled goat anti-human IgG.
Fluorescence intensity

Figure 5. Binding of CD5Rg to the AE.7 T cell clone is CD72 independent. Staining of activated AE.7 cells was performed with control CD8Rg (solid line) and with CD5Rg (A, dotted line). CD5Rg after preabsorption of the cells with anti-CD72 mAb (B, dotted line), or CD5Rg after preabsorption of the receptorglobulin with soluble CD72-CD8 fusion protein (C, dotted line).

However, the mouse immune response to rabbit immunoglobulins results in the binding of mouse antibodies to rabbit Ig and local deposition and in fixation of the immune complexes.

Mice in the experimental groups were injected on alternate days, from day 0 to day 40, with 50 μg CD5Rg or CD8Rg in 150 μl PBS or with 150 μl PBS alone. All of the mice received the first 10 injections intravenously and the subsequent injections intraperitoneally. All animals were killed on day 40, and renal tissue was subjected to examination by fluorescence and electron microscopy.

Mice injected with RbAMBB and treated with PBS or CD8Rg developed diffuse granular deposits of rabbit and mouse IgG in glomerular peripheral capillary walls (Fig. 7). Granular deposits of rabbit IgG, with small amounts of mouse IgG, were also present along the basement membranes of proximal tubules and in Bowman's capsule. Electron microscopy revealed small dense deposits in the subepithelial part of the glomerular basement membranes and "spikes" similar to those seen in human MGN (data not shown). Mesangial deposits were comparable to those observed in untreated mice. Antibodies to rabbit IgG became detectable in the serum of these mice on day 7 and reached a peak on day 21 (Fig. 8). In mice immunized with RbAMBB and treated with CD5Rg, tissue examination by immunofluorescence and electron microscopy revealed normal glomerular peripheral capillary walls (Fig. 7 and data not shown). Some residual granular deposits of rabbit IgG were found in the basement membranes of proximal tubules and in Bowman's capsule, consistent with previous observations (20). Staining for mouse IgG and C3 in the mesangium did not differ appreciably from that seen in control mice (data not shown). During the first 3 wk after immunization, mouse anti-rabbit IgG antibody levels were significantly lower than in PBS or CD8Rg-treated animals (Fig. 8). Receptorglobulin treatment did not result in mod-
Figure 7. Immunofluorescence microscopy of glomeruli from a C57Bl/10 mouse injected with RbAMBB and treated for 40 d with CD8Rg (a and c) or with CD5Rg (b and d). (a) Granular deposits of mouse IgG in the peripheral glomerular capillary walls; (b) Absence of detectable mouse IgG in glomeruli of animals treated with CD5Rg. (c) Diffuse, granular deposits of rabbit IgG in the peripheral glomerular capillary walls; coarse deposits are also present in the mesangium. (d) Absence of rabbit IgG deposits in glomerular capillary walls; some residual IgG is present in the tubules and the Bowman's capsule. a–d, ×400.

Figure 8. Circulating antibodies to Rb IgG detected by ELISA. At days 7 and 21, the titers of antibodies of CD5Rg-treated mice were significantly lower than those in PBS- or CD8Rg-treated mice (*P < 0.05). Naive (untreated) mice had an average titer of 0.082 ± 0.020, which was considered as background in the assay: PBS, □, CD8Rg; □, CD5Rg.

Discussion

In the present work, we provide evidence of the existence of a cell-surface CD5 ligand (CD5L) that is distinct from CD72. Soluble CD5Rg is shown to bind CD5L in splenic B cells 1–3 d after stimulation of splenocytes with a combination of anti-CD3 and anti-CD28 antibodies. It recognizes CD5L on Th1 and Th2 clones 10–12 d after stimulation with antigen in the presence of APC and IL-2. Our present observations do not distinguish between de novo expression of CD5L upon activation and activation-dependent induction of posttranslational modifications of a resident cell-surface receptor that transiently enables its interaction with CD5. However, it appears clear that CD5–CD5L interaction requires activation of CD5L–expressing cells. This is reminiscent of interactions between some of the counterreceptors that underlie key T–B cell costimulatory mechanisms, including B7-1/B7-2–CD28/CTLA-4 and CD40–CD40L, where the costimulatory signal is exchanged between a newly expressed ligand and a constitutively expressed receptor. Interestingly, B220+ splenocytes expressed CD5L after triggering CD3 and CD28, both of which are restricted to T cells. This observation suggests that CD5L expression on B cells requires T cell–derived signals. The putative signals appear to be distinct from CD40L and IL-4, based on the observation that blocking anti-CD40L and anti–IL-4 receptor mAbs did not alter CD5L expression (data not shown). A similar observation...
has recently been made on T cell activation–dependent expression of CTLA-4 on B cells (27).

Based on the observations that deglycosylation of CD5Rg abrogates interaction with CD5L, and that COS cell derived CD5Rg does not recognize CD5L on activated splenocytes (data not shown), it seems likely that CD5–CD5L is either a lectin type interaction, or that glycosylation of CD5 is critical in providing the appropriate conformation to the ligand-binding site. The importance of lectin type interactions in leukocyte–endothelial adhesion, mediated by the selectin family of receptors (28), and in the adhesion of cells to extracellular matrix glycosaminoglycans (GAG), mediated by CD44 (21) and other GAG-binding cell-surface receptors, is well established. However, recent work has shown that lectins participate in leukocyte cell–cell interactions as well, and that a subclass of immunoglobulin receptors, including the B cell receptor CD22 (29), the macrophage receptor sialoadhesin (30), and the myeloid cell antigen CD33 (31), behave primarily as sialic acid-binding lectins. CD22 has recently been proposed to behave as a costimulatory molecule for both B and T cell activation (32, 33). Thus, CD5–CD5L may possibly extend the number of leukocyte receptor interactions that display lectin activity. Alternatively, CD5–CD5L interaction may require specific glycosyl chain–dependent CD5 conformation. It has been demonstrated that attachment of a single N-linked oligosaccharide chain to the extracellular domain of CD2 is critical in conferring a ligand-binding configuration (34), and recent results indicate that the same holds true for CD22 (35). Additional work will be required to determine the nature of CD5 glycosylation in T and B cells, and whether the activation state of lymphocytes alters the composition of CD5-associated glycans in a manner that influences its ability to recognize ligand.

The CD5 ligand identified in the present study appears to be distinct from CD72. First, binding of CD5Rg to resting splenocytes is not observed, despite constitutive CD72 expression on T and B cells. Second, CD5Rg binds to activated T cell clones that do not express detectable CD72 (data not shown). Third, a soluble CD72 fusion protein as well as an anti-CD72 mAb that has been suggested to block CD5–CD72 interaction failed to inhibit CD5Rg binding to activated splenocytes and T cell clones. Finally, CD5Rg was observed to immunoprecipitate a 35–37-kD protein from lysates of activated splenocytes and T cell clones, which provides a candidate for CD5L. However, immunoprecipitation of the 35–37-kD protein by CD5Rg does not prove that this species is itself CD5L, since our data do not exclude the possibility that it may be associated with another molecule that serves as the actual ligand. Elucidation of the identity of CD5L will require further investigation.

The observation that CD5 and its ligands are both present in T and B cells supports a role for CD5–CD5L interaction in T–B cell costimulation during a T cell–dependent immune response. Experimental MGN provides a suitable model to study a classic T cell–dependent, antibody-mediated immune response to rabbit IgG (19, 20). In this model, antibody–antigen complexes are deposited in glomeruli, leading to the development of membranous glomerulonephritis with well-defined morphologic lesions (19, 20). The disease does not develop in athymic mice and is prevented by blocking the CD40–CD40L pathway of T–B cell costimulation (20). Our present results show that treatment with CD5Rg significantly reduces the levels of circulating mouse anti–rabbit IgG antibodies, indicative of the autologous immune response, and prevents the deposition of immune complexes. The inhibition of the autologous immune response was sufficient to allow clearance of immune complexes by glomerular visceral epithelial and possibly mesangial cells, thereby preventing accumulation of immune deposits in the peripheral glomerular capillary walls.

Taken together, our results demonstrate the existence of an inducible CD5 ligand that is distinct from CD72, and provide evidence that CD5–CD5L interaction may play a significant role in T–B cell costimulation in vivo. These observations suggest that CD5–CD5L provides a new pathway in lymphocyte activation whose role relative to that of CD28/CTLA-4–B7.1/B7.2 and CD40–CD40L can now be explored.

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Address correspondence and reprint requests to Ivan Stamenkovic, M.D., Pathology Research, Massachusetts General Hospital, 149 13th Street, Charlestown Navy Yard, Boston, MA 02129.

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