The CD5 ectodomain interacts with conserved fungal cell wall components and protects from zymosan-induced septic shock-like syndrome

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The CD5 lymphocyte surface receptor is a group B member of the ancient and highly conserved scavenger receptor cysteine-rich superfamily. CD5 is expressed on mature T and B1a cells, where it is known to modulate lymphocyte activation and/or differentiation processes. Recently, the interaction of a few group B SRCR members (CD6, Spα, and DMBT1) with conserved microbial structures has been reported. Protein binding assays presented herein indicate that the CD5 ectodomain binds to and aggregates fungal cells (*Schizosaccharomyces pombe*, *Candida albicans*, and *Cryptococcus neoformans*) but not to Gram-negative (*Escherichia coli*) or Gram-positive (*Staphylococcus aureus*) bacteria. Accordingly, the CD5 ectodomain binds to zymosan but not to purified bacterial cell wall constituents (LPS, lipotheicoic acid, or peptidoglycan), and such binding is specifically competed by β-glucan but not by mannan. The K_d of the rshCD5/(1→3)-β-D-glucan phosphate interaction is 3.7 ± 0.2 nM as calculated from tryptophan fluorescence data analysis of free and bound rshCD5. Moreover, zymosan binds to membrane-bound CD5, and this induces both MAPK activation and cytokine release. In vivo validation of the fungal binding properties of the CD5 ectodomain is deduced from its protective effect in a mouse model of zymosan-induced septic shock-like syndrome. In conclusion, the present results indicate that the CD5 lymphocyte receptor may sense the presence of conserved fungal components [namely, (1→3)-β-D-glucans] and support the therapeutic potential of soluble CD5 forms in fungal sepsis.

β-glucans | fungal sepsis | lymphocytes | scavenger receptor

Pathogen recognition by the innate immune system relies on a limited number of fixed germline-encoded receptors, which have evolved to identify conserved microbial structures not shared by the host and essential for their survival, the so-called "pathogen-associated molecular patterns" (PAMPs) (1, 2). Examples of PAMPs are LPS from Gram-negative bacteria, lipotheichoic acid (LTA) and peptidoglycan (PGN) from Gram-positive bacteria, lipoarabinomannan from mycobacteria, and mannan from fungi. Several structurally and functionally diverse classes of pattern-recognition receptors (PRRs) exist that induce various host defense pathways. Protein domains involved in pattern recognition include, among others, the C-type lectin domain from dendritic cell (DC) lectins, the leucine-rich repeat from Toll-like receptors (TLRs), and the scavenger receptor cysteine-rich (SRCR) domains (2). The latter was described on cloning of mouse type I class A macrophage scavenger receptor (SR-AI) (3). Sequence comparison with several other proteins, such as the sea urchin speract receptor, human and mouse CD5, and complement factor I, revealed the existence of a conserved, 100-aa-long motif characteristic of a new superfamily (SF) of protein receptors, named SRCR. This family is currently composed of more than 30 different cell-surface and/or secreted proteins with representatives in most animal phyla, from low invertebrates to mammals (4). The SRCR-SF members are divided into 2 groups: Group A contains SRCR domains composed of 6 cysteines and encoded by 2 exons, whereas group B is composed of 8 cysteines and is encoded by a single exon. Recent structural data indicate, however, that both group A and B SRCR domains share a similar scaffold (a central core formed by 2 antiparallel β-sheets cradling 1 α-helix), with the main differences being observed at the connecting loops (5). This situation recalls that of the few other successful protein modules of the immune system from which evolution has settled and built a myriad of different proteins (e.g., Ig domain). The versatility of these conserved domains lies in the fact that key residues stabilizing the domain structure are conserved throughout evolution, whereas others can evolve freely (especially those at the external loops), giving rise to great functional diversity (6). Accordingly, there is not a unifying function reported for the SRCR domains despite their high degree of structural and phylogenetic conservation. Some of them have been involved in protein-protein interactions, with interaction of the CD6 lymphocyte receptor with CD166/ALCAM (7) and that of the CD163/M130 macrophage receptor with the hemoglobin-haptoglobin complex being the most well studied examples (8). A few members of both group A (i.e., SR-AI/II, MARCO, SCARA5) and group B (i.e., DMBT1, Spα, CD6) are known to interact with microbial surfaces. These interactions were initially mapped outside the SRCR domains (9), but recent evidence demonstrates the direct involvement of SRCR domains (10–13). However, whether pathogen scavenging is a general property shared by all or only a selected group of SRCR-SF members still remains to be analyzed.

The lymphocyte receptors CD5 and CD6 are group B members of the SRCR-SF (4) that share important similarities at both the structural and functional levels. They are encoded by contiguous genes thought to derive from duplication of a common ancestral gene (14) and are expressed on thymocytes, mature peripheral T cells, and B1a cells; the latter is a small subset of mature B cells responsible for the production of polyreactive natural antibodies,

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which is expanded in certain autoimmune and B-cell lymphoproliferative disorders (15). The extracellular regions of both CD5 and CD6 are exclusively composed of 3 consecutive group B SRCR domains, which show extensive amino acid sequence identity (5). The main differences between CD5 and CD6 are found at their cytoplasmic regions, which are devoid of intrinsic catalytic activity but contain several structural motifs compatible with a function in signal transduction (4). CD5 and CD6 are physically associated with the antigen-specific complex present on T and B cells (16, 17) and colocalize with it at the center of the immunological synapse (17, 18). Therefore, CD5 and CD6 are well positioned to modulate, either positively or negatively, the activation and differentiation signals generated by the antigen-specific receptor through still incompletely understood and complex signaling pathways (4, 7, 19, 20). This is likely achieved through engagement of their ectodomains by different cell surface counterreceptors. Although it is well established that CD6 binds to CD166/ALCAM (7), a *bona fide* CD5 ligand has yet to be identified (4).

The bacterial binding capabilities of the CD5 and CD6 ectodomains, both known to exist as soluble forms circulating in serum (21, 22), have been explored recently. The reported data indicate that soluble and membrane forms of CD6, but not of CD5, bind to Gram-negative and Gram-positive bacteria through recognition of LPS and LTA, respectively (13). The present report has extended these studies to fungal structures. Data provided herein indicate that the CD5 ectodomain is well suited for the recognition of conserved components on fungal cell surfaces, namely β-glucans.

Results

The Ectodomain of Human CD5 Binds to Fungal Cells. To study the microbial binding properties of the extracellular regions of CD5 and CD6 further (13), direct protein binding assays to fungi were performed. To this, biotin-labeled, affinity-purified, recombinant soluble proteins encompassing the 3 SRCR ectodomains of human CD5 (rshCD5-b) or human CD6 (rshCD6-b) were incubated with whole fungal cells, and bound protein was then revealed by Western blotting. The rshCD5 and rshCD6 proteins have previously been shown to be indistinguishable (in apparent molecular mass, antibody reactivity, and cell binding properties) from equivalent circulating forms present in normal human serum (13, 23). Fig. 1*A* shows that rshCD5-b binds to both the saprophytic (*S. pombe*) and pathogenic (*C. albicans*, *Cryptococcus neoformans*) fungal cells tested, whereas rshCD6-b binds only to the saprophytic fungal cells. The binding of rshCD5-b was dose dependent and saturable and was greatly facilitated by Ca^{2+} (Fig. 1*B*). According to previously reported data (13), either little or no binding to Gram-negative (*E. coli*) or Gram-positive (*S. aureus*) bacteria was observed for rshCD5-b (Fig. 1*C*). This indicates that the extracellular region of CD5 is well suited for recognition of fungal but not bacterial cell wall structures.

To identify which of the 3 SRCR domains of CD5 was involved in fungal interaction, further cell binding assays were performed by using culture supernatants from HEK 293-EBNA transfectants expressing individual soluble CD5 domains. As illustrated in Fig. 1*D*, all 3 individual SRCR domains of CD5 retained the ability to interact with fungal cell surfaces. This suggests that a conserved structural motif shared by all 3 SRCR domains is responsible for fungal scavenging.

Induction of Fungal Cell Aggregation by the CD5 Ectodomain. Whether the presence of multiple binding sites on the CD5 ectodomain would lead to fungal aggregation was further investigated by incubating FITC-labeled *C. albicans* cells with different concentrations of soluble unlabeled proteins (BSA, rshCD5, and rshCD6). As shown in Fig. 1*E*, rshCD5 induced dose-dependent fungal aggregation, whereas neither rshCD6 nor BSA did. The same results were also observed when *C. neoformans* was assayed (data not shown). The rshCD5-induced fungal cell aggregation was significantly reduced in the presence of excess amounts of either zymosan or β-glucan but not mannan (Fig. 1*E Bottom*). This suggests that binding to and aggregation of fungal cells by rshCD5 is likely mediated through recognition of β-glucan, a structural component of fungal cell walls.

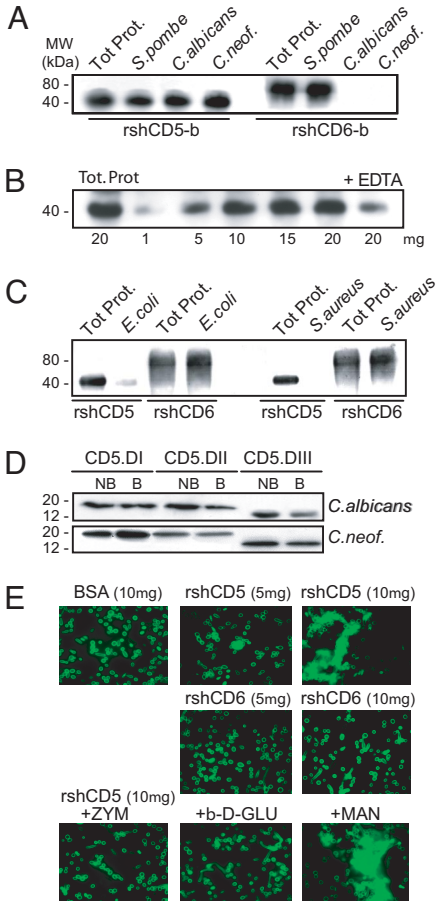

Fig. 1. Interaction of the CD5 ectodomain with whole fungal cells. (*A*) Biotin-labeled rshCD5 (rshCD5-b) or rshCD6 (rshCD6-b) (15 μg) was incubated with 10^6 *S. pombe*, *C. albicans*, or *C. neoformans* cells. Cell-bound protein was solubilized and run on SDS-PAGE. Detection of total and bound biotin-labeled proteins was performed by Western blot analysis by using HRP-streptavidin (SAv). (*B*) Binding of different amounts (1–20 μg) of rshCD5-b to *C. albicans* was analyzed as in *A*. The binding of 20 μg of rshCD5-b in presence of 5 mM EDTA is also shown. (*C*) Binding of a fixed amount (15 μg) of rshCD5-b or rshCD6-b to 10^8 *E. coli* or *S. aureus* bacteria is shown. (*D*) Culture supernatants from HEK 293-EBNA transfectants expressing individual ectodomains (DI, DII, or DIII) of rshCD5 were incubated with 10^6 *C. albicans* or *C. neoformans* overnight at 4 °C. Unbound protein was washed off and precipitated with 10% (weight/vol) trichloroacetic acid. Unbound precipitated (NB) and cell-bound (B) proteins were analyzed by Western blot with a rabbit polyclonal anti-CD5 antiserum plus HRP-labeled sheep anti-rabbit Ig antiserum. (*E*) Fungal cell aggregation was assayed by incubating FITC-labeled *C. albicans* (10^6) overnight at 4 °C with 5–10 μg of BSA, rshCD5, or rshCD6 in the presence or absence of 20 μg of zymosan (ZYM), β-D-glucan (β-D-GLU), or mannan (MAN) as a competitor. Cells were then transferred onto glass slides and visualized by fluorescence microscopy. (Magnification: ×400.) Tot prot., total protein.

The CD5 Ectodomain Binds Directly to Conserved Components of Fungal but Not Bacterial Cell Walls. The direct binding of the CD5 ectodomain to purified microbial cell wall components was further analyzed by ELISA. In agreement with data shown in Fig. 1, dose-dependent binding of rshCD5-b to plates coated with zymosan but not with LPS, LTA, or PGN was observed (Fig. 2*A*). As expected (13), rshCD6-b bound to LPS-, LTA-, PGN-, or zymosan-

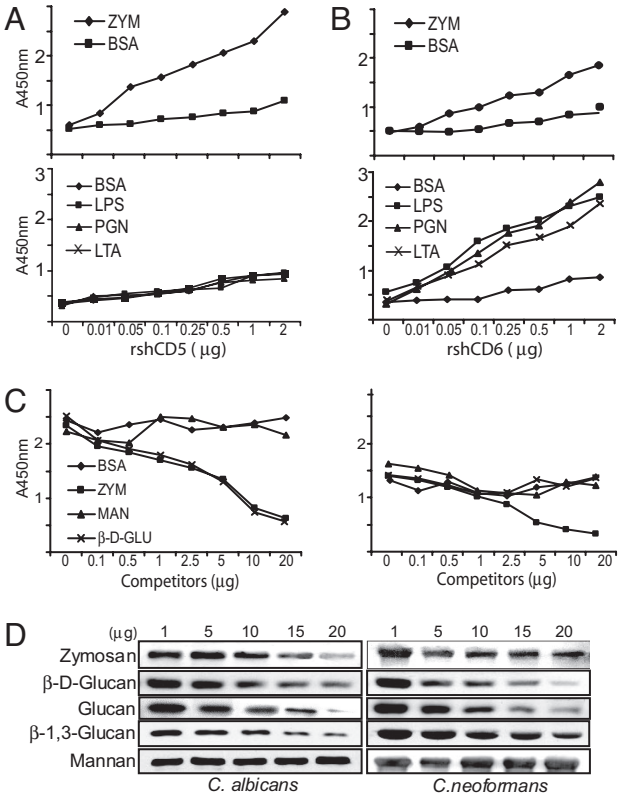

Fig. 2. The CD5 ectodomain binds to zymosan through recognition of β-D-glucans. (*A*) ELISA plates coated with BSA, zymosan (ZYM), LPS, PGN, or LTA were incubated with increasing amounts (0.01–2 μg) of rshCD5-b. Bound protein was detected with HRP-streptavidin (SAv). (*B*) Binding of rshCD6-b to BSA-, ZYM-, LPS-, PGN-, or LTA-coated ELISA plates was analyzed as in *A*. (*C*) Binding of rshCD5 to ZYM is competed by β-D-glucans. A fixed amount (2 μg) of rshCD5-b (*Left*) and rshCD6-b (*Right*) was incubated with ZYM-coated ELISA plates in the presence or absence of increasing amounts (0.01–20 μg) of unlabeled competitors (β-D-glucans, zymosan, mannan, or BSA). Bound protein was detected with HRP-SAv. (*D*) Binding of rshCD5 to whole fungal cells is competed by β-D-glucans. A fixed amount (15 μg) of rshCD5-b was incubated with 10^8 *C. albicans* or *C. neoformans* cell suspensions in the presence of increasing amounts (1–20 μg) of unlabeled competitors: Zymosan (*S. cerevisiae*), β-D-glucan (barley), β-1,3-glucan (*E. gracilis*), glucan (*S. cerevisiae*), and mannan (*S. cerevisiae*). Bound rshCD5-b was detected by Western blot analysis using HRP-SAv.

coated plates in a dose-dependent manner (Fig. 2*B*). Interestingly, the absorbance values obtained for the interaction of zymosan with rshCD6-b were always lower than those obtained with rshCD5 (Fig. 2 *A* and *B*). This reinforces the suitability of the CD5 ectodomain for scavenging fungal but not bacterial cell wall constituents, compared with the CD6 ectodomain.

ELISA competition assays were next performed to determine which fungal wall component was responsible for the interaction with CD5. To this, the binding of a fixed amount (2 μg) of rshCD5-b to plastic-bound zymosan was competed with increasing concentrations of β-glucan, mannan, or zymosan. In accordance with results shown in Fig. 1, β-glucan and zymosan, but not mannan, were able to compete the binding of rshCD5-b to zymosan in a dose-dependent manner (Fig. 2*C*). By contrast, the binding of rshCD6-b was only competed by zymosan (Fig. 2*C*).

The ability of different β-glucan–containing preparations to compete the binding of CD5 to fungal cell wall structures was further analyzed. The binding of a fixed amount of rshCD5-b (15 μg) to whole fungal cells was competed by increasing concentrations of β-glucan purified from barley, (1→3)-β-glucan from *Euglena gracilis*, and glucan from *Saccharomyces cerevisiae* as well as with zymosan or mannan (both from *S. cerevisiae*) used as positive and negative controls, respectively. As illustrated in Fig. 2*D*, all glucan preparations competed the binding of rshCD5-b to both *C. albicans* and *C. neoformans* in a dose-dependent manner. This suggests that the interaction of the CD5 ectodomain with fungi is likely mediated through recognition of β-glucan, a highly conserved and abundant constituent of fungal cell walls.

The interaction of CD5 with β-glucan was further analyzed by monitoring the changes in tryptophan fluorescence emission intensity of rshCD5 on excitation at 295 nm before and after addition of increasing amounts of (1→3)-β-D-glucan phosphate. The spectrum of fluorescence emission of rshCD5 is characterized by an emission maximum at 335 nm [supporting information (SI) Fig. S1]. A glucan concentration-dependent increase of fluorescence intensity was observed for rshCD5 (Fig. S1*A Left*) but not for human serum albumin (Fig. S1*B*), reaching saturation at molar ratio of 1:1 (Fig. S1*A Right*). The apparent K_d was calculated and was 3.7 ± 0.2 nM.

Zymosan Binds to Membrane-Bound CD5 and Induces CD5-Mediated Activation of MAPK Cascade. The interaction of membrane-bound CD5 with fungal cell wall constituents was next analyzed. To this, the binding of increasing amounts of FITC-labeled zymosan to 2G5, a Jurkat T-cell derivative selected for deficient expression of both CD5 and CD6 receptors (24), was investigated. As shown by Fig. 3*A*, fluorescence intensity of 2G5 cells transfected with CD5 (2G5-CD5.WT) was higher than that of untransfected cells. Competition binding experiments, as illustrated in Fig. 3*B*, show that staining of 2G5-CD5.WT cells with a fixed amount of FITC-labeled zymosan (15 μg) was competed by increasing concentrations of unlabeled β-glucan and zymosan, but not mannan, in a dose-dependent manner. These results suggest that CD5-expressing cells could sense the presence of conserved fungal cell wall constituents. Further evidence for the latter was obtained from activation of the MAPK signaling cascade in stable 2G5 transfectants expressing similar surface levels of either WT (2G5-CD5.WT) or cytoplasmic tail-less (2G5-CD5.K384^{stop}) forms of CD5 (25) (Fig. S2*A*). As shown in Fig. 3*C*, exposure to zymosan (40 μg/mL) induced time-dependent phosphorylation of both MEK and ERK1/2 in 2G5-CD5.WT cells but not in 2G5 cells. Zymosan-induced phosphorylation of both MEK and ERK1/2 was not observed in 2G5-CD5.K384^{stop} transfectants (Fig. 3*C*), which lack the most C-terminal 88 amino acids of CD5 (25). Similarly, abrogation of both MEK and ERK1/2 phosphorylation was observed in 2G5-CD5.WT cells exposed to zymosan in the presence of rshCD5 (Fig. S3). This indicates that activation of MAPK by zymosan in 2G5 cells depends on the expression of CD5 as well as on the integrity of its cytoplasmic domain.

Zymosan Induces CD5-Mediated Cytokine Release. To explore the biological outcome of the interaction of membrane-bound CD5 with fungal cell wall constituents further, subsequent cytokine release was analyzed. Unfortunately, stimulation of both 2G5 and 2G5-CD5.WT cells did not result in significant cytokine release at different time points (data not shown). This unresponsiveness was observed following exposure to high concentrations of zymosan but also after exposure to potent T-cell specific stimuli, such as combinations of anti-CD3 and anti-CD28 mAb, thus indicating the likely existence of a blockade of cytokine release in 2G5 cells. In light of these observations, the membrane form of CD5 was expressed in a nonlymphoid mammalian cell system, the HEK 293 cells. Transient expression of WT (CD5.WT) and cytoplasmic tail-truncated (CD5.K384^{stop}) forms of CD5 was achieved on both the parental 293 cells and 293 cell transfectants stably expressing TLR2, a well-known receptor for zymosan (Fig. S2 *B* and *C*). Cells were then subjected to zymosan exposure (40 μg/mL) for 24 h, and IL-8 concentration was measured. As shown in Fig. 3*D*, significant IL-8 release was observed for 293 cells expressing CD5.WT compared with either untransfected cells or cells expressing the cyto-

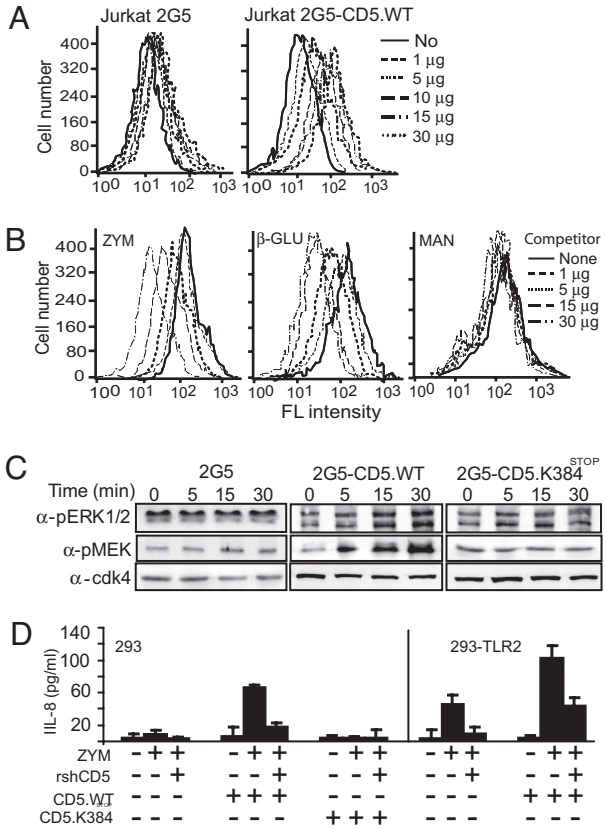

Fig. 3. Zymosan (ZYM) binds to membrane-bound CD5 and induces downstream signaling events. (*A*) Increasing amounts (1–30 μg) of FITC-labeled ZYM were incubated with 2G5 cells either untransfected (*Left*) or transfected (*Right*) to express the WT membrane CD5 receptor (2G5-CD5.WT). Fluorescence intensity of stained cells was analyzed by flow cytometry. (*B*) 2G5-CD5.WT transfectants were stained with a fixed amount (15 μg) of FITC-labeled ZYM in the presence or absence of increasing amounts (1–30 μg) of ZYM, β-D-glucan (β-GLU), and mannan (MAN). Fluorescence intensity was analyzed by flow cytometry. (*C*) 2G5 cells (2×10^6) either untransfected or stably expressing WT (2G5-CD5.WT) or cytoplasmic tail-truncated (2G5-CD5-K384^{STOP}) CD5 surface molecules were pulsed for 0, 5, 15, and 30 min with 40 μg/mL ZYM at 37 °C. Subsequently, cell solubilizates were analyzed by Western blot with anti-pERK1/2, anti-pMEK, and anti-cdk4 antisera plus HRP-labeled sheep anti-rabbit or anti-mouse Ig antisera. (*D*) HEK 293 cells either stably expressing TLR2 or not were transfected in transient for expression of WT (CD5.WT) or cytoplasmic tail-truncated (CD5.K384^{STOP}) membrane CD5 forms. Cells were then pulsed for 24 h with 20 μg/mL ZYM in the presence or absence of rshCD5 (25 μg) and assayed for IL-8 secretion by ELISA.

plasmic tail-less CD5.K384^{stop}. That release was significantly reduced by the presence of rshCD5. The IL-8 levels detected for 293-CD5.WT were similar to those observed for 293-TLR2 transfectants, which were used as a positive control. Moreover, coexpression of CD5.WT and TLR2 did result in additive effects on IL-8 release following zymosan exposure. Once again, the presence of rshCD5 significantly reduced the zymosan-induced IL-8 release in both 293-TLR2 and 293-TLR2+CD5.WT transfectants. Taken together, the results indicate that the membrane form of CD5 senses the presence of fungal cell wall constituents, thereby initiating an independent signaling cascade resulting in cytokine release.

The Soluble CD5 Ectodomain Protects from Zymosan-Induced Septic Shock-Like Syndrome in Mice. In vivo validation on the interaction of the CD5 ectodomain with fungal constituents was obtained from the mouse model of septic shock-like syndrome induced by zymosan (26). In this model, administration of a single dose of zymosan (500 mg/kg, i.p.) causes both acute peritonitis and multiple organ injury within 18 h as well as increased mortality over a period of 12 days. As shown in Fig. 4, administration of a single i.p. dose (25 μg) of rshCD5 1 h before zymosan challenge induced a significant reduction in toxicity score, peritoneal leukocyte count, IL-6 and IL-1β serum levels, and liver myeloperoxidase (MPO) activity at 18 h. Mouse survival was significantly increased (45% vs. 15%) for animals pretreated with rshCD5 compared with untreated controls. The effects of rshCD5 on zymosan-induced septic shock-like syndrome were shown to be dose dependent (Fig. S4). Contrary to rshCD5, the infusion of rshCD6 (25 μg, i.p.), before zymosan challenge, resulted in a nonsignificant reduction of the inflammation parameters analyzed (Fig. S5). Taken together, these data indicate that pretreatment of mice with rshCD5 prevents systemic inflammation induced by zymosan and unveils the therapeutic potential of rshCD5 for fungal septic shock. Accordingly, the anti-inflammatory effect of rshCD5 was also evident when treatment was delayed for 1 or 3 h after zymosan administration (Fig. S6).

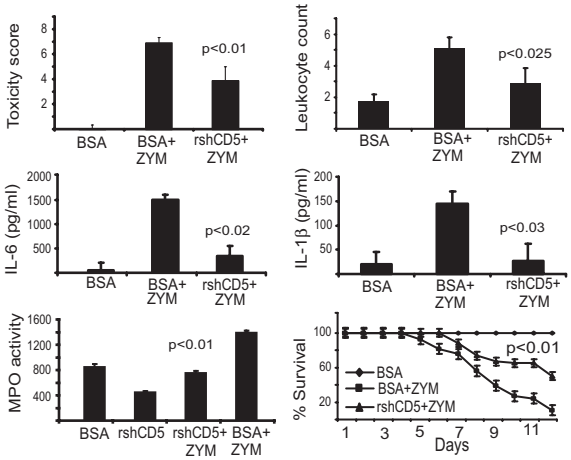

Fig. 4. rshCD5 protects from septic shock-like syndrome induced by zymosan in mice. rshCD5 pretreatment protects from zymosan-induced sepsis. The toxicity score, peritoneal total leukocyte count (10^3 cells/mm^3), IL-6 and IL-1β serum levels, and liver MPO activity (mU/mg protein) of CD1 mice pretreated with BSA or rshCD5 (25 μg, i.p.) 1 h before infusion of zymosan (500 mg/kg, i.p.) were assessed at 18 h. The survival of mice from the same groups was monitored for 12 days.

Discussion

Fungal infections initiate mammalian immune responses through engagement of fungal-surface polysaccharides by a variety of cell-bound and/or soluble receptors, including mannose receptor, complement receptor 3 (CR3, CD11b/CD18), TLRs (TLR2, TLR4), collectins (SP-A, SP-D), pentraxin-3, and C-type lectins (dectin-1) (27). The present report shows that CD5, a lymphoid member of the SRCR-SF, should be added to the list of receptors able to sense the presence of fungal cell wall components. The soluble CD5 ectodomain not only binds to but aggregates several fungal species (saprophytic or pathogenic), and this is achieved through recognition of β-glucans, a major component of fungal cell walls. The relevance of this finding is highlighted by the fact that soluble CD5 prevents septic shock-like syndrome induced by the β-glucan-rich particle zymosan in mice.

β-glucans are important cell wall components of mushrooms, seaweeds, yeast, and pathogenic fungi. Individual β-glucans are heterogeneous in terms of molecular weight, number of branches, and helical construction, but they basically consist of a backbone of polymerized β-(1→3)-linked β-D-glucopyranosyl units and

β(1→6)-linked side chains. β-glucans can be recognized by a limited number of receptors, including CR3, lactosylceramide, scavenger receptors, and dectin-1 (27). The latter is considered the major β-glucan receptor on myeloid cells and is responsible for most biological effects of β-glucans, such as TNF-α cytokine production by macrophages. Dectin-1 is a C-type lectin-like receptor highly expressed on neutrophils and inflammatory macrophages and, at lower levels, on resident macrophages, dendritic cells (DCs), B cells and, some T cells, although the functional significance of the latter remains to be explored (28).

The present report shows that CD5 binds to zymosan, a polysaccharide particle from the cell wall of *S. cerevisiae*, which is the most commonly used β-glucan-containing experimental agent, although it also contains mannans, other glucans, and chitins. Interestingly, the binding of CD5 to zymosan and to different fungal specimens (*C. albicans* and *C. neoformans*) is competed by β-glucans prepared from different sources (barley, *S. cerevisiae*, and *E. gracilis*) but not mannan. One of the β-glucans used was β-1–3-glucan from *E. gracilis*, which is composed of a linear chain of glucose units without any branching. The apparent K_d of the interaction of rshCD5 with soluble (1→3)-β-D-glucan phosphate was assessed and was 3.7 ± 0.2 nM, which is within the range of binding affinities (2.6 mM–2.2 pM) reported for the interaction of dectin-1 with (1→3)-β-D-glucan from different sources (29).

The fact that the soluble ectodomain of CD5 not only binds but also aggregates fungal cells is of relevance, because aggregation is a common strategy used by components of the innate immune system to prevent pathogen dissemination and to facilitate pathogen clearance by phagocytes. Fungal aggregation is also indicative of the multivalency of the CD5 ectodomain regarding fungal recognition. Indeed, each SRCR domain of CD5 retains the ability to bind to whole fungal cells. Thus, it is reasonable to speculate on the existence of a shared structural motif that is responsible for scavenging fungal cell wall components. Previous studies have shown the presence of 2 bacterial recognition motifs located on similar protein loops of the SRCR domains of MARCO (RXR) and DMBT1 (VEVLXXXXW) (10, 11). Available data do not implicate these 2 motifs in fungal recognition, and they are not fully conserved among the 3 SRCR domains of CD5. Thus, other yet unknown structural motifs may be responsible for the interaction of the SRCR domains of CD5 with fungi.

This study also shows that zymosan, through binding to membrane-bound CD5, induces relevant signaling and biological effects, such as activation of the MAPK cascade and IL-8 cytokine release. These 2 zymosan-induced phenomena are dependent on the integrity of the cytoplasmic tail of CD5. This confirms that CD5 is a surface receptor transducing signaling events and likely modulating cell activation and/or differentiation processes (19). Moreover, the zymosan-induced cytokine release was observed after expression of CD5 in nonlymphoid cells (HEK 293), and this would imply that different cell types may gain responsiveness to fungal cell wall components through acquisition of CD5 expression. CD5 is considered a lymphoid-specific receptor constitutively expressed on mature T and B1a lymphocytes (15), whose expression can be up-regulated under certain circumstances, such as chronic autoantigen stimulation in vivo of conventional B2 cells (30) or differentiation to cells with regulatory function (Tregs) (31). Moreover, extralymphoid expression of CD5 has been reported on endothelial cells (32), macrophages (33, 34), and peripheral blood and vaginal DCs (35, 36). The ultimate reason for such expression is unknown, but it is tempting to speculate its possible involvement in pathogen detection. This is well exemplified for B1a cells, which are cells at the interphase of the innate and adaptive responses. B1a cells are responsible for the production of polyreactive natural antibodies recognizing either autoantigens or microbial components (15). Detection of anti-β-glucan antibodies in normal human and mouse sera has been previously reported (37, 38). Moreover, the protective properties of $CD5^+$ B cells in a rat vaginal candidiasis model have also been shown (39).

The binding of the CD5 ectodomain to fungal wall components is underscored by the beneficial effects observed in the mouse model of septic shock-like syndrome induced by zymosan. Pretreatment with a single i.p. dose of rshCD5 induced significant reductions in toxicity score, serum levels of IL-6 and IL-1β, peritoneal and liver leukocyte infiltrates, and survival rate of mice challenged with a high dose of zymosan. Moreover, the advantageous effects of rshCD5 in zymosan-induced septic shock-like syndrome were still evident when its administration was delayed for 1 or 3 h after zymosan challenge. This suggests that rshCD5 should be considered as a potential therapeutic agent in fungal sepsis. The mechanistic basis for this protective effect in vivo may rely on the neutralizing capability of zymosan particles by the soluble ectodomain of CD5. In fact, the addition of rshCD5 inhibited in vitro phenomena directly triggered by zymosan (e.g., MAPK activation, IL-8 release). Moreover, rshCD5 also interfered with the IFN-γ release by peripheral T lymphocytes stimulated with plastic-bound anti-CD3 mAb either alone or in combination with zymosan (Fig. S7). This is in agreement with the previously shown inhibitory effect of rshCD5 on CD3-mediated T-cell proliferative responses (18) and indicates that additional anti-inflammatory mechanisms may also account for the protective properties of rshCD5 in fungal sepsis.

The present report also shows the ability of the CD6 ectodomain to recognize certain fungal structures of still unknown nature. This is not surprising, because high homology exists between the CD5 and CD6 lymphocyte receptors. However, important differences are found regarding bacterial recognition between the two molecules, and this also seems to be the case regarding fungal recognition. Compared with CD5, the soluble CD6 ectodomain binds to a lesser spectrum (saprophytic but not pathogenic) of fungal species, it binds to fungal cell wall structures distinct from the highly conserved β-glucans and mannans, it binds to zymosan with lower avidity as deduced from ELISAs, and it does not induce significant protection against zymosan-induced septic shock-like syndrome. Taken together, this indicates that the ectodomains of CD5 and CD6 are complementary regarding pathogen recognition: whereas CD6 is well suited for bacterial (both Gram-negative and Gram-positive) detection, CD5 is better adapted for fungal binding. This implies that, by coexpressing CD5 and CD6, lymphocytes could increase their sensing potential for microbial products in the extracellular milieu. This agrees with accumulating evidence showing the expression of different PRRs on lymphocytes (28, 40). Although the functional consequences of PRR ligation by microbial components on lymphocytes is a matter of current study, available evidence indicates that lymphocytes respond directly to microbial products through PRRs and that this modulates their functions (40–44). Recently, a model of TLR-mediated control of Treg function during pathogen challenge has been proposed in which TLR2 ligands rapidly increase the host's adaptive immunity by expanding effectors and also by attenuating suppressive activity of Tregs (42, 45); in the process, Tregs also expand and recover their suppressive activity when the infection has subsided, in time to limit potential autoimmunity from overactivated effectors (43, 46). Intriguingly, CD5 has been found up-regulated on Tregs (31) as well on anergic T (47, 48) and B (30) cells. Whether simultaneous binding of CD5 (and/or CD6) to microbial products collaborates in the TLR-mediated control of adaptive immune responses needs further investigation.

In conclusion, the present study unveils the unprecedented fungal binding properties of lymphoid members of the SRCR-SF, namely, CD5 and, to a lesser extent, CD6. Whether this is a general property shared by other members of the SRCR-SF still remains to be explored. Moreover, the present results add further evidence to the notion that the SRCR domains may have emerged as protein modules of the innate immune system and have evolved to built-in different structural motifs for recognition of different PAMPs.

Finally, the present results also support the therapeutic potential of the CD5 ectodomain for the intervention of septic shock syndrome or other inflammatory disorders of fungal origin.

Materials and Methods

Constructions. A detailed description of the cloning and expression procedures of constructs coding for soluble (rshCD5, rshCD6, rshCD5.DI, rshCD5.DII, and rshCD5.DIII) and membrane-bound (CD5.WT and CD5.K384^{STOP}) proteins can be found in *SI Appendix*.

Cells. The source and the growth conditions of the cell lines and transfectants used in this study (2G5, HEK 293-EBNA, HEK 293, and HEK 293-TLR2) are detailed in *SI Appendix*.

Purification and Biotin Labeling of Recombinant Proteins. Individual ectodomains of CD5 were used as unfractionated serum-free culture supernatants, whereas rshCD5 and rshCD6 were affinity-purified as described in *SI Appendix*. Protein biotinylation was performed with EZ-Link PEO-maleimide–activated biotin (Pierce/Perbio Science) following the manufacturer's instructions.

Bacterial and Fungal Binding Studies. The bacterial and fungal cells used were clinical isolates. Cells were suspended to a final density of 10^{10} bacteria or 10^8 fungi per milliliter, and binding to recombinant proteins was analyzed in the presence or absence of competitors as described in *SI Appendix*. ELISA binding assays were performed on 96-well plates (Nunc) coated with 20 μg of LPS, LTA, PGN, or zymosan (all from Sigma) and then incubated with biotin-labeled BSA, rshCD5, or rshCD6 in the presence or absence of cold competitors as detailed in *SI Appendix*. Fluorescence assays to determine the binding characteristics of rshCD5 to soluble (1→3)-β-D-glucan phosphate were done as described in *SI Appendix*.

Fungal Aggregation Assays. Aggregation of FITC-labeled fungal cells (10^6) in the presence or absence of competitors was performed as described in *SI Appendix*.

Flow Cytometry Analysis. 2G5 cells (2×10^5) either untransfected or stably expressing CD5.WT were stained with different amounts of FITC-labeled zymosan in the presence or absence of increasing amounts of cold competitors as detailed in *SI Appendix*.

Cytokine Assays. Culture supernatants of HEK transfectants pulsed with 20 μg/mL zymosan for 24 h were assayed for IL-8 by ELISA (BD OptEIA, Human IL-8 ELISA Set; BD Biosciences) following the manufacturer's instructions. ELISA for mouse IL-6 and IL-1β serum level determination was performed according to the manufacturer's protocols (Quantikine Immunoassay; R&D Systems).

MAP Kinase Assays. Cell lysate samples from 24-h serum-starved cells (2×10^7) stimulated with zymosan (40 μg/mL) were assayed for ERK1/2 and MEK phosphorylation as described in *SI Appendix*.

Zymosan-Induced Septic Shock-Like Syndrome. Male CD1 mice were subjected to zymosan-induced septic shock-like syndrome as previously described (26). A single i.p. dose of rshCD5, rshCD6, or BSA was given before or after zymosan challenge (500 mg/kg). For further details, see *SI Appendix*.

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