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Syndecan-4 Mediates the Coinhibitory Function of DC-HIL on T Cell Activation

Jin-Sung Chung, Irene Dougherty, Ponciano D. Cruz, Jr., and Kiyoshi Ariizumi

Receptor-ligand interactions between APCs and T cells determine whether stimulation of the latter leads to activation or inhibition. Previously, we showed that dendritic cell-associated heparin sulfate proteoglycan-dependent integrin ligand (DC-HIL) on APC can inhibit T cell activation by binding an unknown ligand expressed on activated T cells. Because DC-HIL binds heparin/heparan sulfate and heparin blocks the inhibitory function of DC-HIL, we hypothesized that a heparin/heparan sulfate proteoglycan on activated T cells is the relevant ligand. Screening assays revealed that syndecan-4 (SD-4) is the sole heparan sulfate proteoglycan immunoprecipitated by DC-HIL from extracts of activated T cells and that blocking SD-4 abrogates binding of DC-HIL to activated T cells. Moreover, cell-bound SD-4 ligated by DC-HIL or cross-linked by anti-SD-4 Ab attenuated anti-CD3 responses, whereas knocked-down SD-4 expression led to enhanced T cell response to APC. Blockade of endogenous SD-4 using specific Ab or soluble SD-4 receptor led to augmented T cell reactions to syngeneic and allogeneic stimulation in vitro and exacerbated contact hypersensitivity responses in vivo. We conclude that SD-4 is the T cell ligand through which DC-HIL mediates its negative coregulatory function. The Journal of Immunology, 2007, 179: 5778–5784.

T cell activation is governed by: 1) a primary signal delivered through interaction of Ag-loaded MHC class molecules on APCs with the TCR on T cells; and 2) accessory signals whose net effect (positive or negative) results from competition between costimulatory and coinhibitory receptors on T cells with corresponding receptors on APC (1). Costimulatory receptors include CD28 and ICOS on T cells (2), and their respective receptors (CD80/B7-1 and CD86/B7-2, and ICOS ligand) on APC (3). Coinhibitory receptors include CTLA-4 on T cells for CD80 and CD86 on APC (4), programmed death-1 (PD-1) for PD-1 ligands 1 and 2 (PD-L1 and PD-L2) (5–7), Tim-3 for Tim-3L (8, 9), and B and T lymphocyte attenuator for herpes virus entry mediator (10–12).

Dendritic cell-associated heparin sulfate proteoglycan-dependent integrin ligand (DC-HIL), a type I transmembrane protein expressed on the surface of APC, including DC, epidermal Langerhans cells, and macrophages (13), has been referred to as gpnmnb and osteoactivin in human and rat studies, respectively (14–16). It is highly glycosylated and contains an Ig-like polycystic kidney disease domain (17) and RGD motif (18) in its extracellular domain. DC-HIL recognizes heparin/heparan sulfate and acts as an integrin receptor through its RGD motif (13). Recently, we showed that DC-HIL binds to activated (but not resting) CD4+ and CD8+ T cells via its Ig-like domain, leading to strong attenuation of primary and secondary T cell responses triggered through the TCR using anti-CD3 Ab (19). In this report, we identify the T cell ligand of DC-HIL to be the heparin/heparan sulfate-bearing proteoglycan (HSPG), syndecan-4 (SD-4).

Materials and Methods

Mice
Female BALB/c and C57BL/6 (5–8 wk old) mice were purchased from Harlan Breeders and BALB/cTac-Tg(DO11.10)-Rag2tm1 (or DO11.10) mice (20) were obtained from Taconic Farms. Following National Institutes of Health guidelines, mice were housed and cared for in the pathogen-free facility and subjected to experimental procedures approved by the Institutional Animal Care Use Center at the University of Texas Southwestern Medical Center (Dallas, TX).

Construction of plasmid vectors
The previously constructed pSTB-DC-HIL-Fc (13), which encodes the extracellular domain of DC-HIL fused to the Fc portion of human IgG1, A plasmid pSTB-SD4-Fc was constructed by replacing the extracellular domain with that of SD-4 obtained by RT-PCR. The V5 epitope sequence (21) was inserted just after the leader sequence of the SD-4-encoding sequence (V5-SD4) and introduced into a lentiviral vector plasmid, pHRSIN-CSGW (GFP)-Ub-Em (22) (gift from Y. Ikeda, Mayo Clinic, Rochester, MN). This recombinant lentivirus coexpresses emerald GFP and V5-SD4. Infectious particles were prepared and their titration was performed according to established protocols (23).

Generation of DO11.10-expressing V5-SD4
A T cell hybridoma DO11.10 line (provided by J. Kappler and P. Marrack, National Jewish Medical and Research Center, Denver, CO) was infected with V5-SD4 lentiviruses at a multiplicity of infection of 10. Two days after infection, GFP-positive cells were enriched three times by flow cytometric sorting.

Production of Fc-fused recombinant proteins
Fc-fusion proteins (DC-HIL-Fc and SD4-Fc) were produced by COS-1 cells and purified as described previously (13). Purity of final preparations was high, as judged by a single band in SDS-PAGE/Coomassie blue staining and immunoblotting with goat anti-human IgG Ab or a specific Ab.

Isolation of T cells and binding of DC-HIL
Following manufacturer’s recommendations, CD4+ and CD8+ T cells were purified from mouse spleen using CD4+ and CD8+ T cell isolation
kinds (Milenyi Biotec), respectively. For binding of DC-HIL to T cells, purified CD4+ T cells (1 × 10⁶) were activated by culturing with immobilized anti-CD3 Ab (1 or 3 μg/ml) for 3 days. Activated T cells were treated with 5 μg/ml Fc blocker (BD Pharmingen) on ice for 30 min to block Fc-binding activity on activated T cells and incubated with 10 μg/ml DC-HIL-Fc or control Ig plus 2.5 μg/ml FITC-anti-human IgG (both from Jackson ImmunoResearch Laboratories). For experiments examining ability of heparin to block binding of DC-HIL-Fc to T cells, DC-HIL-Fc or control Ig (10 μg/ml) was pretreated with heparin for 30 min at room temperature and then incubated with 5 × 10⁵-activated Fc-blocked T cells. For experiments examining specificity of SD-4 binding to DC-HIL, DC-HIL-Fc (5 μg/ml) was pretreated with SD-Fc at indicated concentrations for 30 min at room temperature before binding to activated/Fc-blocked T cells (5 × 10⁶). Alternatively, activated T cells were pretreated with anti-SD-4 Ab (KY/8.2; eBioscience) or control IgG for 30 min on ice before binding.

**T cell proliferation assays**

To assay effects of heparin on DC-HIL-mediated inhibition, DC-HIL-Fc or control Ig (5 μg/ml) was incubated with heparin at increasing concentrations at room temperature for 30 min, followed by coating ELISA wells (in triplicate) that were precoated with anti-CD3 Ab (0.01–0.3 μg/ml). Purified CD4+ T cells (2 × 10⁶/well) were added to the coated wells and cultured for 2 days. After pulsing with [³H]Thymidine (1 μCi/well) for the last 20–22 h of the culture period, cells were harvested and evaluated for [³H]radioactivity. Culture supernatant was stored at −85°C until needed for IL-2 assay using a mouse IL-2 ELISA kit (BD Pharmingen).

Effects of anti-SD-4 Ab on T cell activation were examined as follows: CD4+ T cells (2 × 10⁶/well) were treated with biotinylated anti-CD3 Ab at varying concentrations plus biotinylated anti-SD-4 Ab or biotinylated control IgG (10 μg/ml) on ice for 30 min. After adding anti-biotin microbeads (1 μl; Miltenyi Biotec), treated T cells were incubated in the 96-well plate and examined for proliferation as described above. For V5-SD4-DD10.10 T cells, (3 × 10⁵/well) were cultured in DC-HIL-Fc (or anti-SD-4 Ab) and anti-CD3 Ab, followed by IL-2 assay.

MLR was performed as described previously (19), in the continuous presence of Fc fusion protein or control Ig. After 3 days in culture, [³H]Thymidine incorporation (pulsing for 20 h) was measured.

**Expression of SD**

Expression of SD was determined by RT-PCR (for mRNA) and flow cytometry (for surface expression). Total RNA was extracted from freshly isolated (or resting) CD4+ or CD8+ T cells or from T cells activated 3 days after treatment with immobilized anti-CD3 Ab, and then subjected to RT-PCR analysis using primers for syndecans and β-actin as described previously (21). Primers for SD-1: 5'-CCCTCCTCCGCAAATGTGCGCT-3’ (5’ primer) and 5'-CCCTCCTCCGCAAATGTGCGCTG-3’ (3’ primer); primers for SD-2: 5'-CGGGGGCCGGAGGAAGA-3’ and 5'-TT TTTGGGGAAAGCAGCTACTA-3'; primers for SD-3: 5'-CTTTGGACACACAAGGGCCGACCACC-3’ and 5'-GGCCACACACCCCAAGCCTTT-3’; and primers for SD-4: 5’-CCCTCCGCGACGAGTACC-3’ and 5’-AACGCCGCCACCACACAC-3’. We also examined mRNA expression of all members of the glypican family using primers reported previously (25).

At different time points after activation of CD4+ or CD8+ T cells with immobilized anti-CD3 Ab, T cells were pretreated with Fc blocker and stained with biotinylated anti-SD-4 Ab or biotinylated isotypic control IgG (5 μg/ml) plus PE-streptavidin. Cells were also stained with Ab raised against SD-1 (eBioscience), SD-2, and SD-3 (Santa Cruz Biotechnology). Finally, PE-labeling intensity was measured by FACS.

**Immunoblotting and immunoprecipitation**

Whole cell extracts were prepared from resting or activated T cells (21) and subjected to SDS-PAGE/Western blotting using anti-SD-1 or anti-SD-4 Ab (each 5 μg/ml) as described previously (21). For immunoprecipitation, the protein extracts were incubated with DC-HIL-Fc or control Ig (5 μg/ml) for 3 h at 4°C. Resulting immunocomplexes were precipitated with protein A-agarose (50 μl of 50% slurry) overnight at 4°C, and washed extensively with PBS. The complexes were dissociated by boiling and then analyzed for CD4+ T cells (1 × 10⁶) using protocols recommended by the Mouse T Cell Nucleofector kit (Amazaxa). Immediately after transfection, cells were cultured in ELISA wells precoated with anti-CD3 Ab (2 μg/ml) and cells were harvested the next day to evaluate GFP expression by FACS. Under the same conditions, CD4+ T cells (1 × 10⁶) isolated from DO11.10-transgenic mice were transfected separately with SC-siRNA and Sf-siRNA (2 μg) and protein expression of SD-4 and β-actin was assayed by Western blotting (anti-β-actin Ab; Abcam).

Efficiency of gene delivery into freshly isolated T cells was assayed using the Amaxa Nucleofector System. We transfected a FITC-labeled control siRNA (2 μg, purchased from Qiagen) into BALB/c CD4+ T cells (1 × 10⁶) using protocols recommended by the Mouse T Cell Nucleofector kit (Amaxa). Immediately after transfection, cells were cultured in ELISA wells precoated with anti-CD3 Ab (2 μg/ml) and cells were harvested the next day to evaluate GFP expression by FACS. Under the same conditions, CD4+ T cells (1 × 10⁶) isolated from DO11.10-transgenic mice were transfected separately with SC-siRNA and Sf-siRNA (2 μg). After culturing for 2 days with immobilized anti-CD3 Ab, expression of SD-4 or PD-1 was measured by FACS.

**Ag-presentation assay**

Bone marrow (BM)-derived DC were prepared as described previously (26). BM-DC harvested from day 6 of culture of BALB/c BM cells in the presence of 10 ng/ml GM-CSF (PeproTech) were seeded on 96-well plate (5 × 10⁴/well) and pulsed with OVA, 323–339 peptide (2 μg/ml) (27) synthesized by the Protein Chemistry Technology Center (University of Texas Southwestern Medical Center). After 6 h of Ag pulsing, DC were mixed with CD4+ T cells (1 × 10⁵/well) purified from spleen of unprimed DO11.10-transgenic mice, and cultured in the presence of control Ig or SD-Fc. In some experiments, DC were cocultured with T cells transfected with siRNA. After coculturing for 2 days, cells and supernatant were harvested separately. Cells were stained with FITC-anti-CD4 and PE-anti-CD69 Ab (both obtained from eBioscience) and determined by FACS for frequency of CD69+ cells in CD4+ T cells. The supernatant was assayed by ELISA for IL-2 production.

**Contact hypersensitivity assays**

BALB/c mice (n = 5) were sensitized and challenged with oxazolone (Ox) as described previously (19, 28). Different panels of mice were injected i.p. with PBS, anti-SD-4 Ab, or rat isotypic control IgG2a (one infection, 5 mg/kg each) 3 h before sensitization or challenge. In some experiments, mice were injected i.p. with PBS, SD4-Fc, or control Ig (10 mg/kg) on days 5 and 6 after sensitization. The Student t test was used to determine statistically significant differences in ear swelling responses.

**Histological examination of skin and analysis of draining lymph node (LN) cells**

Two days after painting Ox on ears of Ox-sensitized mice treated with anti-SD-4 Ab, ear skin and cervical LN (proximal to sensitized ear) were procured (19). Ear skin was embedded in paraffin, thin-sectioned, and stained with H&E (Sigma-Aldrich). Histological examination was conducted under light microscopy at a magnification of ×10. LN cells were counted and examined for spontaneous proliferation and frequency of CD4+, CD8+ T cells, B cells, and CD69+ cells. For proliferation, LN cells (5 × 10⁵/well) from untreated or treated mice were cultured without stimulation for 3 days and pulsed with [³H]Thymidine (1 μCi/well) for 20 h. For frequencies of T and B cells, LN cells (5 × 10⁵) were stained with PE-anti-CD69 Ab (eBioscience) in the presence or absence of Ab directed against T cell or anti-B cell surface markers (CD4, CD8, and B220) (2.5 μg/ml each) and examined by FACS for surface expression of CD69. LN cells were also doubly stained with PE-anti-SD-4 Ab (or PE-anti-PD-1 Ab) and FITC-anti-CD4 or FITC-anti-CD8 Ab (all from eBioscience).

**Results**

**Heparin antagonizes DC-HIL function**

Heparin scavenging of CD-HIL binds to heparin/heparan-sulfated polysaccharides (13), we considered a role for these polysaccharides in the interaction between DC-HIL and activated T cells. Pretreatment of DC-HIL-Fc with heparin blocked binding of this soluble receptor to activated T cells in a dose-dependent fashion.
FIGURE 1. Among HSPG, SD-4 is likely a ligand of DC-HIL. A, Heparin blocks binding of DC-HIL to T cells. Activated CD4\(^+\) T cells were incubated with control Ig (shaded histograms) or DC-HIL-Fc (unshaded histograms) (10 µg/ml) in the absence (none) or presence of heparin at indicated concentrations. After labeling cell-bound DC-HIL-Fc with PE-anti-human IgG Ab, fluorescence intensity on cells was examined by FACS. B, Heparin blocks the inhibitory function of DC-HIL. Splenic CD4\(^+\) T cells were treated with immobilized anti-CD3 Ab (0.3 µg/ml) and control Ig (○) or DC-HIL-Fc (5 µg/ml, ●) pretreated with varying doses of heparin (in triplicate). T cell activation was measured by \(\text{[}^{3}\text{H}\text{]}\)thymidine incorporation. C, mRNA expression of syndecans and \(\beta\)-actin determined by RT-PCR. D, Surface expression of SD on CD4\(^+\) T cells. Resting (day 0) or activated CD4\(^+\) T cells (day 3 after stimulation) were determined by FACS for surface expression of SD-1, SD-3, and SD-4. E, Surface expression of SD-4 by CD8\(^+\) T cells. Resting (day 0) and activated CD8\(^+\) T cells (similarly treated as CD4\(^+\) T cells) were examined for surface expression of SD-4 by FACS. F, Protein expression of SD-1 and SD-4. (Fig. 1A). To assess effects on the inhibitory function of immobilized DC-HIL, T cells were cultured in microwells precoated with anti-CD3 Ab and with control Ig or DC-HIL-Fc pretreated with heparin. Pretreatment with heparin abrogated the ability of DC-HIL to inhibit T cell activation triggered by anti-CD3 Ab (Fig. 1B). Heparin alone had no effect on anti-CD3 Ab response. Similar results were noted using heparan sulfate (data not shown). The ability of exogenous heparin to block binding of DC-HIL to T cells and to antagonize its inhibitory function indicates a role for heparin/heparan sulfate in these processes.

SD-4 on activated T cells is a ligand of DC-HIL

Because the SD and glypican families of transmembrane heparin/HSPG are major sources of cell surface-heparan sulfate (29), we questioned whether these molecules are involved in binding of DC-HIL to T cells. We first examined expression by resting vs activated CD4\(^+\) or CD8\(^+\) T cells of all known syndecans and glypicans. At the mRNA level, all four known syndecans were expressed by resting and activated T cells at differing levels, but only SD-4 expression was up-regulated by T cell activation (Fig. 1C). None of the six known glypicans was expressed by T cells (data not shown). At the surface protein level, SD-1 and SD-3 were not expressed but their mRNAs were detected by RT-PCR (Fig. 1C). Again, SD-4 was the sole HSPG whose expression was up-regulated by T cell activation (Fig. 1D). SD-4 expression on CD8\(^+\) T cells was also induced by activation, albeit to a lesser degree than observed with CD4\(^+\) T cells (Fig. 1E). Activated T cells produced SD-4 proteins at greater levels than resting T cells (Fig. 1F); de novo protein synthesis (rather than just movement of the protein to the cell surface) is likely to account for this activation-inducible expression of SD-4. The expression profile of SD-4 was consistent with that of DC-HIL binding.

To determine whether DC-HIL binds directly to SD-4 on activated T cells, we extracted proteins from activated CD4\(^+\) T cells, immunoprecipitated these with DC-HIL-Fc or control Ig, and analyzed precipitants by Western blotting using anti-SD-4 Ab (Fig. 2A). A single band of SD-4 (45 kDa) was detected in DC-HIL-bound (but not control Ig-bound) immunoprecipitates. We then questioned whether SD-4 is the sole ligand of DC-HIL on activated T cells. Pretreatment of DC-HIL-Fc with SD4-Fc (soluble receptor fused with Fc portion of IgG) or pretreatment of activated T cells with anti-SD-4 Ab abrogated binding of DC-HIL to T cells (Fig. 2, B and C); control Ig had no effect. Finally, transgene expression of SD-4 in DO11.10 T cells (lacking native SD-4 expression) conferred on these cells the ability to bind DC-HIL (Fig. 2, D and E), and this binding was also blocked completely by heparin. These results indicate that SD-4 is the major (if not sole) ligand of DC-HIL on activated T cells.

Engagement of SD-4 leads to inhibition of T cell activation

We examined the function of SD-4 on T cells again using transfectected DO11.10 T cells. V5-SD4-expressing DO11.10 T cells (V5-SD4-DO) or those expressing GFP (GFP-DO) were treated with immobilized anti-CD3 Ab in the presence/absence of DC-HIL-Fc, followed by measurement of IL-2 production (Fig. 3A). DC-HIL-Fc inhibited anti-CD3-induced IL-2 production by V5-SD4-DO cells in a dose-dependent fashion, but did not inhibit IL-2 production by GFP-DO cells. In the absence of DC-HIL, both cell whole cell protein extracts (7 x 10^6 cells equivalent/lane) prepared from resting and activated CD4\(^+\) T cells were subjected to immunoblotting using Ab to SD-1 or SD-4. Arrows indicate molecular weights of SD-1 and SD-4. All data are representative of at least two independent experiments.
with DC-HIL-Fc. 

were pretreated with anti-SD-4 Ab or isotypic IgG before binding assays (or rat) IgG and surface labeling measured by FACS. Frequency (%) of GFP and V5-SD4 were stained with anti-V5 Ab, anti-SD-4 Ab, or mouse DO11.10 T cells infected with a lentiviral vector encoding both emerald control Ig at varying concentrations before binding to activated CD4+ T cells. Binding was determined by FACS. C. Pretreatment of T cells with anti-SD-4 Ab blocks binding of DC-HIL to T cells. Activated CD4+ T cells were pretreated with anti-SD-4 Ab or isotypic IgG before binding assays with DC-HIL-Fc. D. Transgene expression of V5-SD4 by DO11.10 T cells. DO11.10 T cells infected with a lentiviral vector encoding both emerald GFP and V5-SD4 were stained with anti-V5 Ab, anti-SD-4 Ab, or mouse (or rat) IgG and surface labeling measured by FACS. Frequency (%) of GFP+/V5+ or GFP+/SD4+ cells is shown in the dot-blots. E, Binding of DC-HIL-Fc to DO11.10 T cells. DO11.10 parental cells or those with expression of V5-SD4 were incubated with control Ig (shaded histograms) or DC-HIL-Fc (open histograms), followed by FACS. Binding assay was also performed in the presence of heparin (2 μg/ml). All data shown are representative of three independent experiments.

lines showed similar anti-CD3 responses (Fig. 3A), indicating that expression of V5-SD4 had no effect on the T cell response. Cross-linking of SD-4 on T cells also led to inhibited IL-2 production, albeit to a lesser degree (Fig. 3B).

We also examined the inhibitory function of SD-4 on CD4+ T cells (Fig. 3C). CD4+ T cells were activated by cross-linking of CD3 (using increasing doses of anti-CD3 Ab) and SD-4 (constant dose of anti-SD-4 Ab). T cells treated with anti-SD-4 Ab (but not isotypic IgG control) exhibited very low responses to anti-CD3 stimulation at each dose tested (Fig. 3C). Cross-linking of SD-4 produced outcomes mimicking the inhibitory function of DC-HIL (19).

Soluble SD-4 enhances T cell activation

We next compared effects of SD4-Fc and DC-HIL-Fc on activation of alloreactive T cells (MLR), in which added Fc-fusion proteins were used to block endogenous binding of DC-HIL on APC with SD-4 on T cells (Fig. 3D). Addition of SD4-Fc to the MLR led to enhanced T cell activation (2-fold higher than control) in a dose-dependent fashion, as shown previously using DC-HIL-Fc (19). We also added soluble SD4-Fc to a syngeneic APC assay, in which OVA peptide-pulsed BM-DC were cocultured with splenic CD4+ T cells isolated from DO11.10-transgenic mice (Fig. 3E). SD4-Fc enhanced T cell activation triggered by DC, whereas control Ig had little effect. Because SD4-Fc acted as an antagonist (i.e., it can bind to DC-HIL on DC, but is unable to induce tyrosine phosphorylation of DC-HIL in DC) (data not shown), we interpret our results to mean that SD-4 is a negative regulator of allogeneic and syngeneic T cell responses.

T cells with knocked-down SD-4 display enhanced responses to DC

To better study the role of SD-4 in regulating T cell activation, we knocked-down SD-4 expression on splenic CD4+ T cells and examined their response to Ag presentation by DC. We first determined the efficacy of siRNA’s ability to block SD-4 expression in COS-1 cells cotransfected with the SD-4 gene and SD-4-targeting SC-siRNA or shuffled control siRNA (Sf-siRNA), using Western blotting for protein expression of the SD-4 transgene or endogenous β-actin (as control) (Fig. 4A). SC-siRNA knocked-down SD-4 protein expression almost completely (control siRNA had no effect); specificity was supported by unchanged β-actin expression. We then transfected both siRNA into CD4+ T cells freshly isolated from DO11.10-transgenic mice (Fig. 4B), which resulted in SD-4 knock-down, which were transferred to MLR, which resulted in an inhibition of T cell activation. The inhibition was abrogated when T cells were transfected with the SC-siRNA designed to block SD-4. We then showed that the decline in SD-4 expression was associated with enhanced T cell activation triggered by DC (Fig. 4C). We next examined the effects of SD-4 knock-down on T cell activation in vivo, using MLR as the model system. Both SC-siRNA and SC-siRNA/Fc were administered to mice, which were subsequently transferred into an MLR. The mice were killed 3 days post-transplantation, and the splenic CD4+ T cells were isolated and stained with anti-V5 Ab, anti-SD-4 Ab, or mouse DO11.10 T cells infected with a lentiviral vector encoding both emerald GFP and V5-SD4 were stained with anti-V5 Ab, anti-SD-4 Ab, or mouse DO11.10 T cells infected with a lentiviral vector encoding both emerald GFP and V5-SD4 were stained with anti-V5 Ab, anti-SD-4 Ab, or mouse DO11.10 T cells infected with a lentiviral vector encoding both emerald GFP and V5-SD4 were stained with anti-V5 Ab, anti-SD-4 Ab, or mouse DO11.10 T cells infected with a lentiviral vector encoding both emerald GFP and V5-SD4 were stained with anti-V5 Ab, anti-SD-4 Ab, or mouse (or rat) IgG and surface labeling measured by FACS. Frequency (%) of GFP+/V5+ or GFP+/SD4+ cells is shown in the dot-blots. E, Binding of DC-HIL-Fc to DO11.10 T cells. DO11.10 parental cells or those with expression of V5-SD4 were incubated with control Ig (shaded histograms) or DC-HIL-Fc (open histograms), followed by FACS. Binding assay was also performed in the presence of heparin (2 μg/ml). All data shown are representative of three independent experiments.

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in 60% delivery of siRNA into cells (data not shown). Transfection of SC-siRNA knocked-down SD-4 expression induced by activation (51% reduced to 11%); control siRNA had no effect. Specificity of SC-siRNA was supported by no effect on PD-1 expression by activated T cells as a control. T cells were then evaluated for response to activation by DC pulsed with OVA peptide. A day after coculture with Ag-pulsed DC, T cells transfected with SC-siRNA produced 2-fold higher levels of IL-2 compared with that produced by control T cells (untreated vs pulsed alone vs transfected with control siRNA) (Fig. 4C). Similar results were observed after 2 days of coculture. These results indicate that downregulated SD-4 expression enhances T cell responses to activation signals delivered by DC.

Blockade of endogenous SD-4 augments contact hypersensitivity (CH)

To evaluate SD-4 function in vivo, we used CH, an experimental model of delayed-type, T cell-mediated, skin inflammation (30). Mice were sensitized by topical application of Ox on abdominal skin and challenged by painting ear skin with Ox (Fig. 5); CH was measured by ear swelling from days 0 to 5. Mice were given an i.p. injection of anti-SD-4 Ab (vs PBS vs control IgG) 3 h before

**FIGURE 4.** T cells knocked-down for SD-4 respond more strongly to DC. A, Efficacy of siRNA. Whole cell extracts were prepared from COS-1 cells untransfected or cotransfected with a SD-4 gene and SD-4-targeted SC-siRNA or Sf-siRNA oligonucleotide (control) and determined by immunoblotting for protein expression of SD-4 (left) or β-actin (right). B, Knockdown of SD-4 expression in T cells. Splenic CD4+ T cells freshly isolated from D011.10-transgenic mice were untreated, pulsed alone, or pulsed with SC-siRNA or Sf-siRNA using the Amaxa system and cultured with immobilized anti-CD3 Ab. After culturing for 2 days, surface expression of SD-4 or PD-1 was measured by FACS. C, OVA-Ag presentation. CD4+ T cells transfected with siRNA were cocultured for 1 or 2 days with BM-DC (from wild-type mice) pulsed with the OVA peptide. IL-2 production was measured. Data shown are representative of three independent experiments.

**FIGURE 5.** Infusion of anti-SD-4 or SD4-Fc in mice enhances elicitation of contact hypersensitivity. Sensitization of BALB/c mice (n = 5) with Ox for CH (A–C): on day 0, mice were sensitized by painting 2% Ox on abdominal skin. On day 6, CH was elicited in sensitized mice by painting 1% Ox or solvent control to right or left ears, respectively (challenge). Ear thickness was measured daily from day 1 through day 5 following challenge. Mice were also given i.p. injection of PBS, control IgG, or anti-SD-4 Ab 3 h before the sensitization (A) or challenge (B). Some panels of mice were i.p. injected with PBS, control Ig, or SD4-Fc before challenge (C). Daily change in ear thickness (×10^-3 inch) after challenge was plotted for each panel. Statistical significance is denoted by * (p < 0.001) as compared with ear thickness treated with control Ig. D, Histological examination of ear skin. Mice treated with Ox and PBS, control IgG, or anti-SD-4 Ab were sacrificed 2 days after challenge; ear skin biopsies were stained with H&E and examined histologically (×10 magnification). In an independent experiment, LN cells prepared from BALB/c mice treated similarly were examined: (E) LN cells counted, and (F) their spontaneous activation measured by [3H]thymidine incorporation of LN cells cultured for 3 days without stimuli. G and H, Frequency of leukocytes: LN cells were stained with FITC-Ab against CD4, CD8, or B220 alone (G) or doubly stained with PE-anti-CD69 (H), and then analyzed by FACS. CD69 expression (H) is shown in LN cells stained positively with the surface marker Ab. Data shown are representative of three independent experiments (A–D) and two experiments (E–H).
sensitization (Fig. 5A) or challenge (Fig. 5B). Infusion of anti-
SD-4 Ab before sensitization had little effect on ear swelling (Fig.
5A). By contrast, mice treated with anti-SD-4 Ab before challenge
developed twice greater ear swelling compared with controls at
each time point tested after challenge (Fig. 5B); this enhancement
lasted at least 5 days after challenge. Similar enhancement was
seen following infusion of SD-4-Fc at challenge (Fig. 5C). These
time-dependent effects were consistent with previous outcomes us-
ing DC-HIL-Fc (19). We interpret these results to mean that SD-4
is expressed optimally on fully activated T cells (after challenge)
but not yet on early activated T cells (during sensitization).

Histological examination confirmed enhanced ear swelling and
larger numbers of infiltrating leukocytes in mice treated with Ox
and anti-SD-4 Ab (Fig. 5D). We next examined the phenotype of
draining LN in mice similarly treated with Ox and anti-SD-4 Ab 2
days after challenge including: cell numbers (Fig. 5E); spontaneous
activation measured by [3H]thymidine incorporation for pro-
liferative capacity in vitro (Fig. 5F); and frequency of leukocyte
subpopulations (Fig. 5G) and of CD69+ cells measured by FACS
(Fig. 5H). In mice treated with anti-SD-4 Ab (vs control IgG), LN
size was almost three times bigger and spontaneously proliferating
LN cells 3-fold greater; there were more CD4+ T cells (40 vs
32%), CD8+ T cells (17 vs 15%), and B220+ B cells (33 vs 24%);
and the frequency of CD69+ cells in all three leukocyte populations
increased. In sum, the ability of anti-SD-4 Ab (or SD4-Fc) to recapitulate
DC-HIL function (inhibition of T cell activation in vitro while augmenting
MLR, Ag presentation, and CH responses) strongly
support SD-4 as the ligand through which DC-HIL mediates its
inhibitory signal in T cells.

Expression of SD-4 on LN T cells

The time-dependent effect of anti-SD-4 Ab in mice (Fig. 5) led us
to examine the kinetics of SD-4 expression by T cells during de-
velopment of CH (Fig. 6). We also compared SD-4+ vs PD-1+ T
cells in LN from control mice vs those treated with Ox at different
time points after hapten challenge. In Ox-sensitized mice, SD-4+ T
cells were not detected in LN before challenge. A day after
challenge, both CD4+ and CD8+ T cells expressed SD-4 at low
frequency, that increased dramatically 2 days after challenge
(6.6% in CD4+ cells and 2.5% in CD8+ cells). This was followed
by a gradual decrease to 4.2% in CD4+ and 0.6% in CD8+ cells
5 days after challenge. The frequency of PD-1+ T cells also in-
creased after hapten challenge, peaking at day 3. The expression of
SD-4 in LN T cells after challenge (but not after sensitization)
likely accounts for the ability of anti-SD-4 Ab or soluble SD4-Fc
to augment CH responses when injected during elicitation (but not
sensitization) (Fig. 5, A–C).

Discussion

Our results demonstrate that SD-4 is the ligand through which
DC-HIL inhibits T cell activation. Among known coinhibitory re-
ceptors, SD-4 resembles PD-1 in that expression requires TCR
activation and appears during a later phase of T cell activation
(e.g., elicitation of CH). Unlike all known coinhibitory receptors
including PD-1 that bind their counterreceptors via protein-protein
interaction, binding of SD-4 to DC-HIL appears to require involve-
ment of heparin/heparan sulfate residues.

Because binding of DC-HIL to activated T cells involves its
Ig-like polycystic kidney disease domain (19) and because DC-
HIL does not bind to CD44, another HSPG expressed on resting
and activated T cells (data not shown), we hypothesize that DC-
HIL/SD-4 binding requires simultaneous recognition of heparin/
heparan sulfate and of a peptide epitope of SD-4. This circum-
stance may resemble selectins, which bind the carbohydrate
moiety, sialyl-Lewis x, and a peptide sequence on the backbone of
its ligands (31).

Because endothelial cells and B cells constitutively express
SD-4 (29, 32), it is possible such cells are also stimulated by DC-
HIL. We have shown DC-HIL to bind endothelial cells in an he-
parin-dependent fashion (13). By contrast, B cells do not bind
DC-HIL (data not shown), reflecting a discordance between ex-
pression and binding activity that may be due to diverse heparan
sulfate structures expressed by disparate cells, in turn correspon-
ding to different binding activities (33, 34). DC-HIL may recognize
a unique heparan sulfate structure on SD-4 synthesized by acti-
vated T cells, similar to T cell-specific glycosylation again exhib-
ited by the selectin ligands (31, 35).

The enhancing effect of anti-SD-4 Ab or soluble rSD4-Fc on CH
indicates interference with endogenous binding of DC-HIL to
SD-4. A good question is whether such enhancement is due en-
tirely to blockade of DC-HIL/SD-4 binding because SD-4 partic-
ipates in leukocyte rolling and migration (36). If SD-4 antagonists
block the latter processes, our experiments should have produced
down-regulated CH instead. That our outcomes were the reverse
indicate that the primary target of our interventions is endogenous
binding of DC-HIL to SD-4, and thus modulators of DC-HIL
and/or SD-4 may be used to treat T cell-mediated diseases.

B and T lymphocyte attenuator, CTLA-4, and PD-1 possess a
typical ITIM, an ITIM-like motif, and an immunoreceptor ty-
rosine-based switch motif, respectively (37), that can activate
the tyrosine phosphatases, SHP-1 and SHP-2, responsible for mediat-
ing negative T cell effector function (11, 38). By contrast, SD-4
does not contain any of these inhibitory motifs. Rather, ligated
SD-4 is known to induce serine and tyrosine autophosphorylation,
which may regulate intracellular interactions of SD-4 with other cell
surface receptors (39–41). Although we have no direct evi-
dence connecting these events to the SHP-1/SHP-2 pathway in T
cells, activated SD-4 has been shown to complex with other intra-
cellular proteins like syntenin that can bind directly to the intra-
cellular domain of CD148 (42, 43), a membrane protein tyrosine
phosphatase η known to inhibit CD3-mediated T cell activation
(44). Moreover, SD-4 can regulate activation of protein kinase Cε
(PKCe), that in turn can modulate phosphorylation of CD148 (42).
Finally, we speculate that ligated SD-4 on T cells partners with
CD148 to activate tyrosine phosphatases, which can lead to neu-
tralization of TCR-induced activation signals.
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Disclosures

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References