Binding of DC-HIL to Dermatophytic Fungi Induces Tyrosine Phosphorylation and Potentiates Antigen Presenting Cell Function

Jin-Sung Chung, Tatsuo Yudate, Mizuki Tomihari, Hideo Akiyoshi, Ponciano D. Cruz, Jr. and Kiyoshi Ariizumi

*J Immunol* 2009; 183:5190-5198; Prepublished online 30 September 2009;
doi: 10.4049/jimmunol.0901319
http://www.jimmunol.org/content/183/8/5190

**References** This article cites 45 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/183/8/5190.full#ref-list-1

**Why The *JI***? Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Binding of DC-HIL to Dermatophytic Fungi Induces Tyrosine Phosphorylation and Potentiates Antigen Presenting Cell Function

Jin-Sung Chung,* Tatsuo Yudate,† Mizuki Tomihari,‡ Hideo Akiyoshi,*§ Ponciano D. Cruz, Jr.,* and Kiyoshi Ariizumi2*

APCs express receptors recognizing microbes and regulating immune responses by binding to corresponding ligands on immune cells. Having discovered a novel inhibitory pathway triggered by ligation of DC-HIL on APC to a heparin/heparan sulfate-like saccharide of syndecan-4 on activated T cells, we postulated DC-HIL can recognize microbial pathogens in a similar manner. We showed soluble recombinant DC-HIL to bind the dermatophytes Trichophyton rubrum and Microsporum audouinii, but not several bacteria nor Candida albicans. Dermatophyte binding was inhibited completely by the addition of heparin. Because DC-HIL contains an ITAM-like intracellular sequence, we questioned whether its binding to dermatophytes can induce tyrosine phosphorylation in dendritic cells (DC). Culturing DC with T. rubrum (but not with C. albicans pseudohyphae) induced phosphorylation of DC-HIL, but not when the tyrosine residue of the ITAM-like sequence was mutated to phenylalanine. To examine the functional significance of such signaling on DC, we cross-linked DC-HIL with mAb (surrogate ligand), which not only induced tyrosine phosphorylation but also up-regulated expression of 23 genes among 662 genes analyzed by gene-array, including genes for profilin-1, myristoylated alanine rich protein kinase C substrate like-1, C/EBP, LOX-1, IL-1β, and TNF-α. This cross-linking also up-regulated expression of the activation markers CD80/CD86 and heightened APC capacity of DC to activate syngeneic T cells. Our findings support a dual role for DC-HIL: inhibition of adaptive immunity following ligation of syndecan-4 on activated T cells and induction of innate immunity against dermatophytic fungi. The Journal of Immunology, 2009, 183: 5190–5198.
found DC-HIL to bind the dermatophytes Trichophyton rubrum and Microsporum audouini. Coculture of DC with *T. rubrum* induces phosphorylation of the tyrosine residue in the ITAM-like intracellular sequence of DC-HIL at a level markedly greater than that induced by ligation to the T cell ligand SD-4. This phosphorylation up-regulated expression of genes responsible for DC maturation and augmentation of APC function. Thus, DC-HIL is a PRR for dermatophytic fungi that can heighten APC properties, while also negatively regulating T cell activation.

**Materials and Methods**

**Mice**

Female BALB/c and C57BL/6 (5- to 8-wk-old) mice were purchased from Harlan Breeders, and OT-I and OT-II (5- to 8-wk-old) mice obtained from Taconic Farms. Following National Institutes of Health guidelines, these animals were housed and cared for in the pathogen-free facility of the Institutional Animal Care Use Center of The University of Texas Southwestern Medical Center.

**Fc-fused recombinant protein**

Fc-fused proteins (DC-HIL-Fc, SD-4-Fc, Dectin-2-Fc, and Fc alone) were produced in COS-1 cells and purified as described previously (15, 19).

**Antibodies**

Rat mAb generated against CD4 (L3T4), CD8, I-A/I-E (2G9), CD11b (M1/70), CD11c (N418), CD11d (PDC-Ack-1), and hamster mAb against CD14 (M1/69), CD80 (16A-10A1) were purchased from eBioscience. Secondary Abs were purchased from Jackson ImmunoResearch Laboratories.

UTX-103 rabbit anti-mouse DC-HIL mAb was generated as follows: Rabbids were immunized four times with 0.5 mg of DC-HIL-Fc (fused with human IgG-Fc) 3 wk apart. A week after the third immunization, sera were collected and the titer of anti-mouse DC-HIL evaluated by ELISA using DC-HIL-mFc (fused with mouse IgG-Fc) to eliminate anti-human IgG Ab. Rabbit spleen cells were fused with a rabbit myeloma cell line (Epitomites). One clone (UTX-103 mAb IgG1) was purified from the culture supernatant using protein A-agarose and toxofree buffers. Compared with our previous IE4 rat anti-DC-HIL mAb (19), this mAb has much higher affinity for native DC-HIL protein. Purified UTX-103 mAb and control IgG preparations used for cross-linking experiments were tested for endotoxin contamination using a LAL Chromagenic Endpoint Assay (HyCult Biotechnology; Cedarlane Laboratories). Endotoxin levels were <0.5 EU/ml.

**Preparation of APC**

Epidermal cells were isolated from ear skin of BALB/c mice using trypsin (Mediatech); LC were enriched by centrifugation over Histopaque (1.083, Sigma-Aldrich) (21). Bone marrow (BM) DC were harvested from day 6 culture of BM cells with GM-CSF (22). Other DC subpopulations were procured from the spleen cells of naive mice. Macrophages were harvested from the peritoneal cavity of mice stimulated with thioglycollate (21).

**Flow cytometry**

Freshly isolated or cultured leukocytes were assayed for surface and intracellular expression by flow cytometry. For surface expression, cells (1 × 10⁶) were incubated with UTX-103 rabbit anti-DC-HIL mAb or the isotypic control IgG1 (each 10 μg/ml). After washing, cells were labeled with fluorescent secondary Ab (PE-anti-rabbit IgG, 1 μg/ml). For intracellular staining, cells (1 × 10⁶) were fixed with 4% paraformaldehyde at room temperature for 30 min. After washing with Dulbecco’s PBS, cells were treated with permeabilization buffer (0.5% Saponin, 0.5% BSA in DPBS) for 10 min, and then incubated with UTX-103 mAb (10 μg/ml) in the permeabilization buffer for 30 min, followed by incubation with the secondary Ab (1 μg/ml). Fluorescence intensity of stained cells was analyzed by FACS Calibur (BD Biosciences).

**Binding assays for microbe**

* Candida albicans (ATCC 10231) and the dermatophytes Trichophyton rubrum (ATCC 14001) and Microsporum audouini (ATCC 10008) were purchased from the American Type Culture Collection and grown in medium recommended by the American Type Culture Collection. *C. albicans* yeast was transformed into pseudohyphae by culturing freshly prepared yeasts at 37°C for 90 min in HBBS containing 1.25 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES (pH 7.7), and 10% heat-inactivated FCS (15). Aliquots of pseudohyphae (4 × 10⁷) were washed with DPBS and incubated with staining buffer (0.1% BSA, 2 mM CaCl₂ in DPBS) containing 20 μg/ml Fc proteins on ice for 1 h. After extensive washing with buffer, cells were resuspended in 2.5 μg/ml FITC-anti-human IgG Ab on ice for 30 min. For immunostaining of dermatophyte fungi, single colonies of fungi were grown on Sabouroud’s agar plates, harvested and suspended in DPBS. After washing with DPBS and with water, small aliquots were spotted onto a slide glass, air dried, and stained with Fc proteins as before. Binding of Fc proteins to microbes was examined by confocal microscopy. In inhibition experiments, 20 μg/ml DC-HIL-Fc was preincubated with indicated concentrations of hemiphenylamine using Quick Change Site-Directed Mutagenesis and Pho polymerase (Stratagene) performed according to the manufacturer’s recommendations for the following oligonucleotide pairs: Y523F 5'-GGTACCATCTTGGTCTGAAAAACAAACGAGGCGC-3' (5' primer, which italic letters show mutations) and 5'-CGCCATTGGTTTTCATACACACCGCCTCCAGGCAATTGACAACGTG-3' and 5'-CATGTTCATCTTTGAGAATCGTTCGCTGTTAGAACCTGTGTTTACCTG-3'. Two days after transfection, cells were cross-linked by UTX-103 mAb plus secondary Ab, and then immunoprecipitated as described above.

**Tyrosine mutation**

Either or both tyrosine residues (amino acids 523 and 529) in the intracellular domain was replaced with phenylalanine using Quick Change Site-Directed Mutagenesis and Pho polymerase (Stratagene) performed according to the manufacturer’s recommendations for the following oligonucleotide pairs: Y523F 5'-GGTACCATCTTGGTCTGAAAAACAAACGAGGCGC-3' (5' primer, which italic letters show mutations) and 5'-CGCCATTGGTTTTCATACACACCGCCTCCAGGCAATTGACAACGTG-3'. Two days after transfection, cells were cross-linked by UTX-103 mAb plus secondary Ab, and then immunoprecipitated as described above.

**Immunoblotting and tyrosine phosphorylation assay**

Whole cell extracts were prepared from untreated or activated BM-DC (cultured for 2 days with 1 μg/ml LPS) and assayed for protein concentration (23). An aliquot (0.1 μg/lane) was applied to 4–15% SDS-PAGE, followed by immunoblotting using UTX-103 mAb and control IgG (each 1 μg/ml) (23). Tyrosine phosphorylation of DC-HIL on DC, BM-DC were treated with three different stimuli: BM-DC (3 × 10⁶ cells/ml complete medium) were cultured with indicated amounts (as dried weight) of *T. rubrum* or *C. albicans* hyphae; BM-DC (5 × 10⁶ cells in 500 μl of DPBS) were also incubated with UTX-103 mAb or control rabbit IgG (10 μg/ml) on ice for 30 min, followed by cross-linking with 100 μg/ml goat anti-rabbit IgG, and BM-DC (5 × 10⁶ cells) were cultured in culture dish precoated with SD-4-Fc or control Ig (20 μg/ml). At indicated time periods at 37°C, treated DC were lysed using 500 μl of 2X lysis buffer (15). DC-HIL protein was immunoprecipitated by incubation at 4°C for 3 h with 2–5 μl of UTX-103 mAb and overnight incubation with protein-A agarose (50 μl of 50% slurry). The immune-complexes were dissociated by boiling and then analyzed for expression of phosphotyrosin by immunoblotting using biotinylated anti-phosphotyrosine (0.5 μg/ml) (4G10, Upstate Biotechnology) and HRP-streptavidin (1/10,000 dilution). The blotted membranes were also stripped and reanalyzed using IE4 rat anti-DC-HIL mAb (1 μg/ml) (23) and HRP-anti-rat IgG (1/10,000 dilution). To examine tyrosine phosphorylation of DC-HIL mutants, COS-1 cells were seeded at a density of 5 × 10⁶ cells/dish and transfected with a plasmid vector encoding wild-type or tyrosine mutants of DC-HIL (2 μg) using Fugene 6 (19). Two days after transfection, cells were cross-linked by UTX-103 mAb plus secondary Ab, and then immunoprecipitated as described above.

**Expression analysis**

Gene expression by cross-linked BM-DC was analyzed using oligonucleotide probe-blotted gene arrays (Dendritic and APC and Autoimmunity and Inflammation) according to the manufacturer’s recommendations (SuperArray Biosciences). In brief, BM-DC were harvested and cultured in 96-well plate (1 × 10⁵ cells/well) precoated with UTX-103 mAb or control IgG (20 μg/ml). After 6 h of culture at 37°C, total RNA was isolated from treated cells (1 × 10⁶) using ArrayGrade total RNA isolation kit (SuperArray) and ArrayGrade cDNA probes were prepared using the TrueLabeling-AMP 2.0 kit (SuperArray) and hybridized with gene arrays; hybridization signals were then detected using chemiluminescent detection kit (SuperArray). Image acquisition and analysis of data were performed by ImageQuant 400 (Amersham Biosciences). Expression of some genes up-regulated by the cross-linking was reanalyzed by real-time PCR following the manufacturer’s recommendations (LightCycler FastStart DNA MasterPlus SYBR Green I Roche). Primers for oxidized low-density lipoprotein receptor (Oir-1, GenBank: AY075791) include: 5'-CGCGGAGAAGTGACGAGAA-3' (5' primer) and 5'-AGAACG

The Journal of Immunology 5191

Downloaded from jimmunol.org by guest on May 15, 2022
GOAGGTGTGTATGG-3' (3' primer); myristoylated alanine rich protein kinase C substrate like-1 (Marchkl-1, GenBank: NM_009851), 5'-CAGACCCCCATCAT-3' and 5'-CTGGCCTGCTGCTGCTTCC-3'. For production of TNF-α and IL-1β, BM-DC (1 × 10^6 cells) were incubated similarly with immobilized UTX-103 mAb or control IgG for indicated time periods. BM-DC (2 × 10^6 cells) were treated with 10 μg/ml dried T. rubrum or Candida hyphae at 37°C for 30 min, immediately after which cells were washed extensively and cultured for 2 days. Culture supernatant was recovered and cytokines measured by ELISA kits (eBioscience).

Ag-presentation assay
BM-DC were cultured in 96-well plate precoated with UTX-103 mAb or control IgG (20 μg/ml) for 1 day. Treated DC were harvested and reseded on 96-well plate at a different cell density and pulsed for 6 h with MHC class II-restricted OVA_{123–139} and MHC class I-OVA_{257–264} peptide (each 2 μg/ml) synthesized by the Protein Chemistry Technology Center at The University of Texas Southwestern Medical Center. After pulsing, DC were cocultured with the constant number of CD4+ or CD8+ T cells (1 × 10^6/ well) purified from spleen of unprimed OT-II or OT-I transgenic mice, respectively, using T cell isolation kits (Miltenyi Biotec). Two days after coculture, culture supernatant was recovered and IL-2 and IFN-γ production was assayed by ELISA kits (eBioscience).

Results
Expression of DC-HIL by APC
Using a newly developed UTX-103 rabbit anti-DC-HIL mAb with markedly higher affinity to native DC-HIL than our previously generated 1E4 rat anti-DC-HIL mAb (19), we reexamined surface expression of DC-HIL on different APC subsets by flow-cytomeric analysis (Fig. 1). Epidermal LC were identified as I-A/I-E+ epidermal cells, almost all of which expressed DC-HIL constitutively at high levels on the surface and intracellularly (Fig. 1A). By contrast, DC-HIL was not expressed by I-A/I-E+ epidermal cells. CD11c+ BM-DC also expressed DC-HIL constitutively on their surface. LPS stimulation up-regulated DC-HIL surface expression by some (but not all) CD11c+ DC (Fig. 1B). This up-regulation was confirmed by immunoblotting of protein extracts from BM-DC using UTX-103 mAb that immunostained two bands (95 and 125 KDa) (Fig. 1C). In spleen, there are at least three distinct DC subsets (CD11c+/CD4+ lymphoid DC, CD11c-/CD8+ myeloid DC, and CD11c-/PDCA-1+ plasmacytoid DC) (1), all of which also expressed DC-HIL constitutively on the surface, albeit at lower levels (Fig. 1D).

DC-HIL expression by these DC subsets appeared invariant of in vivo stimulation (data not shown). Finally, peritoneal macrophages from mice treated with thioglycolate also expressed surface and intracellular DC-HIL at high levels (Fig. 1E). These results indicate that DC-HIL is expressed by a wide variety of APC subsets.

DC-HIL binds to dermatophyte cell wall
Many C-type lectin receptors (e.g., mannose receptor, DC-SIGN, dectin-1, and dectin-2) bind to saccharides ligands expressed by microbes (13). Similarly, we showed DC-HIL to bind a heparin/ heparan sulfate (HS)-like saccharide of SD-4 on activated T cells (20). We thus posited that DC-HIL may also recognize microbial pathogens through a similar sugar moiety. To address this issue, we performed binding assays using fluorescent-labeled DC-HIL-Fc, dectin-2-Fc (as control), orFc alone. Neither Staphylococcus aureus, group A streptococci, Pseudomonas aeruginosa, nor Escherichia coli bound to DC-HIL (data not shown). We then examined binding to Candida albicans pseudo-hyphae consisting of round yeast and filamentous hyphae (Fig. 2A). Using dectin-2-Fc as a positive control because it is known to bind hyphal (but not yeast) components (15) (Fig. 2A), we observed that neither DC-HIL-Fc nor Fc alone bind to Candidal pseudo-hyphae. We then examined binding to dermatophytes (Fig. 2, B and C). DC-HIL-Fc bound to Trichophyton rubrum with high affinity and to Microsporum audouinii at lower affinity. Binding of DC-HIL to T. rubrum was blocked completely by addition of heparin (2 μg/ml) (Fig. 2D), which we showed previously to inhibit DC-HIL binding to activated T cells (20). To sort the fungal ligands of DC-HIL, T. rubrum fungi were pretreated withN-glycosidase that removes saccharide residues from glycoproteins (Fig. 2E), or DC-HIL-Fc was preincubated with fungal saccharides, including chitin, galactomannan, β-glucan, and mannann before binding assays (Fig. 2, F and G). N-glycosidase treatment inhibited binding of DC-HIL-Fc to T. rubrum almost completely. None of the saccharide inhibitors showed complete inhibition as was observed with heparin: Chitin and galactomannan were moderate inhibitors and others (β-glucan and mannan) weak inhibitors. These results indicate that DC-HIL can bind dermatophytes, suggesting that the fungal ligands of DC-HIL may be saccharides structurally related to HS, chitin, and/or galactomannan.

Ligation of DC-HIL leads to tyrosine phosphorylation of its ITAM-like motif
Because DC-HIL has an ITAM-like signaling motif (YxxI), we questioned whether binding of DC-HIL to T. rubrum transduces tyrosine phosphorylation of this protein in DC (Fig. 3A). BM-DC were cocultured with varying doses of C. albicans hyphae or T. rubrum (as dried weight), and tyrosine phosphorylation on DC-HIL was assayed by immunoprecipitation and blotting using anti-p-tyrosine Ab. Tyrosine phosphorylation of DC-HIL was induced in DC following coculture with T. rubrum, but not with C. albicans hyphae even at the highest dose tested, consistent with selective binding by DC-HIL. Such phosphorylation was also detected in BM-DC after cross-linking of DC-HIL with UTX-103 mAb (but not with control IgG) (Fig. 3B). However, the level of phosphorylation induced by the mAb was considerably less than by T. rubrum. We then questioned whether the T cell ligand SD-4 can induce tyrosine phosphorylation (Fig. 3C). Treatment of BM-DC with immobilized SD-4-Fc (but not control Ig) induced phosphorylation, albeit at a weaker level compared with the two previous stimulators.

DC-HIL contains two tyrosine residues in its intracellular domain: at aa 523 proximal to the transmembrane domain and aa 529 in the YxxI sequence corresponding to the ITAM-like motif (note that a typical ITAM has two tandem-repeats of YxxI/L) (24). To determine which tyrosine residue is responsible for phosphorylation, point-mutation analysis was performed (Fig. 3D), in which either or both Tyr 523 or 529 was (or were) mutated to phenylalanine (designated Y523F, Y529F, or Y523F/Y529F). Mutants and wild-type DC-HIL were transfected separately into COS-1 cells and assayed for tyrosine phosphorylation (Fig. 3E). Surface expression of mutant DC-HIL on COS-1 cells was similar to those of wild-type DC-HIL (data not shown). Wild-type DC-HIL was tyrosine phosphorylated as early as 10 min in COS-1 cells crosslinked with UTX-103 mAb (but not with control IgG). DC-HIL bearing Y523F mutation was phosphorylated at a similar level, whereas Y529F mutant and doubly mutated DC-HIL failed to undergo phosphorylation (Fig. 3E), thereby identifying the former (tyrosine on aa 529 in the YxxI motif) as the relevant moiety.

Cross-linking of DC-HIL with UTX-103 mAb up-regulates particular genes in DC
Because phosphorylated YxxL (even just one unit) can induce gene expression (25), we examined changes in DC gene expression profile following stimulation of DC-HIL (Fig. 4). For this study, we chose UTX-103 mAb as a surrogate ligand for DC-HIL because cell wall extracts from T. rubrum are toxic to BM-DC (>2 h coculture with extract kills most DC). BM-DC...
FIGURE 1. DC-HIL expression by DC and macrophages. Different APC subpopulations were labeled fluorescently with UTX-103 anti-DC-HIL mAb (or control IgG) and marker Ab. Surface or intracellular expression of DC-HIL was analyzed by flow cytometry. A, Epidermal LC were identified by I-A/I-E expression in epidermal cell suspension. Frequency (%) of positive cells is shown on dot-blot. B and C, BM-DC were left untreated or activated with LPS and identified as CD11c⁺ cells. These cells were also assayed for protein expression in whole cell extracts by immunoblotting using UTX-103 mAb (αDC-HIL). Closed arrowheads indicate two major protein species (125 and 95 KDa) of DC-HIL. D, Three different DC subpopulations with phenotypes of CD11c⁺/CD4⁺, CD11c⁺/CD8⁺, or CD11c⁺/PDCA-1⁺ DC were sorted from spleen cells of naïve BALB/c mice. DC-HIL expression is shown in histograms. E, Peritoneal macrophages were distinguished by high expression of CD11b from peritoneal cells of mice stimulated with thioglycolate. A second experiment showed similar results.
were cross-linked with UTX-103 mAb or control IgG, followed 6 h later by isolation of mRNA, and then gene expression analysis using two different Oligo GEArray Dendritic and APC and Autoimmune and Inflammatory microarrays, on which 260 and 440 gene-specific oligonucleotide probes were blotted, respectively. (These were a total of 662 genes because the two arrays have overlapping gene probes.) Gene expression in DC treated with UTX-103 mAb was expressed relative to that of the housekeeping gene GAPDH (Fig. 4, A and C) and compared with that of DC treated with control IgG (evaluated by fold difference). Genes up-regulated more than 2-fold greater than control are listed (Fig. 4, B and D), including profilin-I (an actin-binding protein involved in turnover and restructuring of the actin cytoskeleton) (26), Marcksl-1 (myristoylated alanine-rich C-kinase substrate involved in regulating cell shape, motility, secretion, transmembrane transport, and cycling) (27), CCAAT/enhancer binding protein (C/EBP or Cebpb) involved in LC commitment (28), and a lectin-like receptor for oxidatively modified low-density lipoprotein (LOX-1 or Orl-1) (29, 30). Our analyses showed 23 genes to be up-regulated following cross-linking with UTX-103 mAb, corresponding to 3% of 662 genes tested. Not one gene examined was down-regulated <2-fold. Because endotoxin content in preparations of UTX-103 mAb and control IgG were similar (<0.5 EU/ml), we think it can be excluded as a cause of the changes.

To verify our results, we examined mRNA expression of Marcksl-1 and Orl-1, using real-time PCR. Both genes were chosen because they were highly up-regulated in each microarray (Fig. 5, A and B). Profilin-I and Cebpb (the highest up-regulated genes) were not examined in this manner because we were unable to find relevant primers capable of producing dose-dependent amplification. After BM-DC were cross-linked with UTX-103 mAb or control IgG, mRNA expression was analyzed and expression levels calculated as fold-increases. Consistent with results of microarray-gene expression analysis, Orl-1 and Marcksl-1 genes were up-regulated (12 and 4.5-fold, respectively, greater than controls) 6 h after cross-linking. Their up-regulation was transient because a return to baseline expression levels was noted 2 days after stimulation.
We also examined protein expression of TNF-α and IL-1β by ELISA (Figs. 5C and D). Without stimulation, BM-DC did not secrete detectable levels of either cytokine, and control IgG treatment induced very low levels of expression. By contrast, treatment with UTX-103 mAb led to markedly elevated levels of TNF-α and IL-1β secretion that lasted at least 2 days. We then questioned

FIGURE 3. Ligation of DC-HIL induces tyrosine phosphorylation of an ITAM-like motif in the intracellular domain. A, BM-DC were cocultured with/without 0.1 or 1 mg/ml (dried weight) \textit{C. albicans} pseudohyphae or \textit{T. rubrum}, 15 min after of which whole cell extracts were prepared. DC-HIL protein was then immunoprecipitated, followed by immunoblotting using anti-p-tyrosine or anti-DC-HIL mAb (phosphorylation assay). B and C, At indicated time point after cross-linking of DC-HIL on BM-DC with UTX-103 mAb (B) or culturing BM-DC with immobilized SD-4-Fc (C) or control IgG (Ctrl), phosphorylation levels on DC-HIL in treated cells was examined as before. D, Amino acid structures of DC-HIL with no mutations (WT), a mutation of Y523F or Y529F, or double mutations (DM) are aligned and schematically shown, consisting of, from the N terminus, the extracellular domain (ECD), transmembrane (TM), and intracellular domain (ICD) in which location of tyrosine residues at a 523 and 529 is indicted by a triangle and circle, respectively. Closed and open symbols represent no mutation and replacement with phenylalanine, respectively. At the right, results of tyrosine phosphorylation assays are shown: + and − indicate significant phosphorylation levels detected and no phosphorylation. E, Tyrosine phosphorylation assays using WT and mutants. Two days after transfection of COS-1 cells with WT or mutant DC-HIL gene, cells were treated similarly with UTX-103 mAb or control IgG for 10 min and the level of tyrosine phosphorylation assayed. A second experiment showed similar results.

FIGURE 4. Gene expression analysis of BM-DC stimulated by cross-linking of DC-HIL. Six hours after cross-linking with UTX-103 mAb (αDC-HIL) or control IgG (Ctrl IgG), cDNA probe was prepared from RNA of treated BM-DC and hybridized to oligo-microarrays Dendritic and APC (A) and Autoimmune and Inflammatory (C). Hybridization images (A and C) on the array are shown. Gene location on the array matrix is shown by alphabetic and numerical numbers according to designation by Super Array Inc (Refer to Gene Table at http://www.sabiosciences.com/genetable). B and D, After computer-assisted expression analysis, genes whose expression levels were more than 2-fold greater than in control IgG-treated DC are listed with the matrix location code (shown in the parentheses), among of which genes marked with an asterisk are further analyzed using real-time PCR or ELISA (Fig. 5). These data are registered as the accession number of GSE17699 at Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/).
IgG-treated DC. These results are representative of at least two independent experiments.

whether T. rubrum (or Candida hyphae) stimulates DC to produce these proinflammatory cytokines (Fig. 5, E and F). BM-DC were stimulated with/without dried T. rubrum or Candida hyphae and measured for secretion of these cytokines. DC treated with T. rubrum produced TNF-α and IL-1β cytokines at a level similar or higher than by DC stimulated with cross-linking of DC-HIL. T. rubrum was a stronger stimulator than Candida.

Because IL-1β induces DC maturation and acquisition of strong immunostimulatory capacity (31), we next examined the effect of cross-linking of DC-HIL on DC expression of activation/maturation markers. BM-DC were cross-linked with UTX-103 mAb and then cultured for 2 days. Surface expression of CD80 and CD86 was examined by flow cytometry (Fig. 6, A and B). Treatment with UTX-103 mAb markedly increased expression of CD80 (47 vs 356 mean fluorescent intensity, MFI) and CD86 (18 vs 49). We then examined T cell-stimulatory capacity by UTX-103 mAb-treated DC (Fig. 6, C–E). After cross-linking and pulsing DC with MHC class I- or II-restricted OVA peptide, increasing numbers of these cells were cocultured with a constant number of CD4⁺ or CD8⁺ T cells isolated from OT-II or OT-I transgenic mice, respectively. Activation of T cells was measured by IL-2 production for CD4⁺ T cells and IFN-γ for CD8⁺ T cells (Fig. 6D). UTX-103 mAb-stimulated DC produced IFN-γ (mean 105 cells/well) of splenic CD4⁺ T cells isolated before stimulation (Fig. 6E). Altogether, cross-linking of DC-HIL up-regulated DC gene expression, resulting in DC maturation and augmented T cell-stimulatory capacity.

FIGURE 5. Expression of up-regulated genes by DC following stimulation with anti-DC-HIL mAb or T. rubrum. At varying time points after cross-linking of DC-HIL on BM-DC with mAb or control IgG, RNA or culture supernatant were isolated/recovered, and then mRNA for Orl-1 and Marksl-1 genes or protein expression of TNF-α and IL-1β was measured by real-time PCR (A and B) or ELISA (C and D). mRNA expression levels are normalized by expression of GAPDH and expressed as fold-increase over values in control IgG-treated DC. E and F. BM-DC also were treated with/without dried T. rubrum or Candida pseudohyphae (10 μg/ml) and cultured for 2 days. Secretion of TNF-α (E) or IL-1β (F) was measured by ELISA. Statistical significance of presented data is p < 0.001 (Student’s t test) compared with controls. Data shown are representative of at least two independent experiments.

FIGURE 6. DC-HIL-stimulated DC exhibit augmented T cell-stimulatory capacity. Two days after cross-linking with UTX-103 mAb (UTX-treated) or control IgG (IgG-treated), BM-DC were harvested and examined by flow cytometry for expression of CD80 (A) and CD86 (B) activation markers. Staining of DC treated with UTX-103 mAb or control IgG are shown by histograms with solid or dashed lines, respectively. Expression levels on untreated DC (just before stimulation) are also shown. Histogram filled in gray represents staining of untreated DC with isotypic control IgG (for marker Ab). Mean fluorescent intensity for CD80 (or for CD86) was 47 (or 18) for control IgG-treated and 356 (or 49) for UTX-103 mAb-treated DC. C–E. These treated DC were also assayed for APC capacity: Increasing numbers of DC were pulsed with OVA peptides and cocultured with a constant number (1 × 10⁶ cells/well) of splenic CD4⁺ T cells from OT-II transgenic mice (C) or CD8⁺ T cells from OT-I mice (D and E). Activation of T cells was measured by production levels of IL-2 (C and D) and IFN-γ (E) (mean ± SD, n = 3). Statistical analysis (Student’s t test) for all data (C–E) shows p < 0.0001 compared with values by IgG-treated DC. These results are representative of at least two independent experiments.
Discussion
While infecting skin, hair, and nails, dermatophytic fungi obtain nutrients from keratinized material (32). Although these fungi usually do not invade living tissue, their metabolic by-products cause inflammation, which is especially severe in immunosuppressed patients (33). An early defense mechanism against dermatophytic infection may be mediated by epidermal LC (skin-resident DC) that reside close to the usual primary site of infection. Having shown that DC-HIL acts as a PRR for dermatophytes and that epidermal LC express DC-HIL constitutively at high levels (human LC also express DC-HIL at high levels; Ref. 34), we posit that DC-HIL exerts antifungal immunity via innate immune recognition and potentiation of LC function. Concurrently, DC-HIL may suppress cutaneous inflammation by attenuating activity of T cells that home to skin. Thus, the net effect of these positive and negative regulations exerted by DC-HIL may determine, at least in part, the outcome of antidermatophyte immunity.

SD-4 is a transmembrane protein heavily laden with HS chains consisting of alternating disaccharide residues (glucuronaric acid and iduronic acid with glucosamine). It is expressed constitutively by B cells but not by naive T cells (its expression can be induced by activation; Ref. 20). Despite its expression profile, DC-HIL binds to activated T cells, but not to B cells (our unpublished data). Because the binding is abrogated by heparin or by heparinase treatment of activated T cells, DC-HIL is likely to recognize the structure of HS chain expressed on T cells. Our results suggest that nonself DC-HIL ligands are expressed by T. rubrum and M. audouinii. Unlike TLR ligands, expression of these ligands may be restricted to only some fungi because DC-HIL does not bind bacteria and C. albicans. The cell wall of dermatophytes is made up primarily of chitin, mannan, and galactomannan, none of which structurally resemble HS (35). In fact, chitin and galactomannan did not strongly inhibit DC-HIL binding as heparin did, and mannan was a weak inhibitor. We thus postulate that the putative ligand(s) on dermatophytes are saccharides structurally resembling HS on T cells.

Our findings indicate that the membrane-proximal YxxI sequence of DC-HIL is the functional tyrosine-based signal motif. It is not a typical ITAM because two ITAM units have been shown to be required for signal transduction (36). ITAM was identified originally by mutation analysis of Ag receptors containing multiple activation motifs comprising two YxxL/I sequences with defined spacing between them (24). ITAM is phosphorylated by Src family kinases and the resultant phosphorylated ITAMs are recognized by two Src homology 2 domains of Syk kinases, that enable transduction of signals (37). Thus, the space between two ITAMs is important for recognition by Syk kinases. However, only one ITAM unit was demonstrated to be sufficient to initiate Syk-mediated signal transduction (e.g., dectin-1, which has one YxxL motif; Ref. 9, 14, 25). In fact, ligation of dectin-1 by β-glucan (or zymosan) can induce expression of a number of genes necessary for innate immunity against yeast pathogens, cooperatively with TLR (8, 38). Like dectin-1, we speculate that the YxxI motif in DC-HIL is capable of inducing Syk-mediated signaling responsible for potentiating DC function.

Several ligand/receptor pairs controlling T cell activation are involved in reciprocal signaling between APC and T cells. Although the impact of these pairs has been well studied for T cells, relatively less is known about effects on APC. Programmed cell death-1 ligands (PD-L1 and PD-L2/B7-DC) on APC negatively regulate T cell activation by interacting with PD-1 on T cells (39, 40). Because PD-L1 and PD-L2 have short cytoplasmic tails lacking known motifs for signal transduction, these ligands are thought to be incapable of transducing signals following binding to PD-1. PD-L2 possesses a four amino acid-long intracellular domain and recent studies have shown that cross-linking PD-L2 directly potentiates DC function by enhancing DC presentation of Ag-loaded MHC molecules, promoting DC survival and increasing secretion of IL-12 (41, 42). Possibly, PD-L2 associates with a coreceptor (that contains intracellular signaling motifs) through two charged amino acids in its transmembrane domain. To our knowledge, PD-L1-induced signaling has not been formally reported. Herpesvirus entry mediator on APC is a ligand for coregulatory receptors BTLA, CD160, and LIGHT on T cells. Herpesvirus entry mediator is a member of the TNF receptor family that can recruit several members of the TNFR-associated factor family, enabling activation of NF-κB and Jun N-terminal kinase, eventually leading to augmented immune responses (43). These coinhibitory ligands including DC-HIL deliver negative signals to T cells when engaged to their corresponding T cell receptors, and conversely they can transduce positive signals within APC.

Such reciprocal signaling may confer balance in the activation of APC and T cells. Immature DC such as epidermal LC constitutively express coinhibitory ligands at levels higher than costimulatory ligands (44). This is true for DC-HIL, which is expressed highly on epidermal LC. Such DC are less potent activators of naive T cells than mature DC (45), but may down-regulate the status of recently activated T cells because they express high levels of coinhibitory receptors. The DC-HIL ligand SD-4 also is expressed on activated (but not resting) T cells. Shortly after engaging with activated T cells, signals induced by coinhibitory ligands may drive DC to undergo maturation, enabling them to become highly potent APC in activating naive T cells. Infection by T. rubrum may release fungal products containing the DC-HIL ligands that modulate this DC-HIL/SD-4 pathway.

In sum, our results document direct binding of DC-HIL to the cell wall of dermatophytes to transduce a signal potentiating DC function, indicating that DC-HIL is a PRR for these fungi. Thus, DC-HIL can regulate immune responses in a dual manner: previously we showed it to be an inhibitor of adaptive immunity following ligation of SD-4 on activated T cells, and now we present evidence that it can induce innate immunity against dermatophytes.

Acknowledgments
We thank Irene Dougherty for technical expertise and Susan Milberger for administrative assistance.

Disclosures
The authors have no financial conflict of interest.

References

Downloaded from http://www.jimmunol.org/ by guest on May 15, 2022

The Journal of Immunology


