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Expression and Characterization of a Novel CD6 Ligand in Cells Derived from Joint and Epithelial Tissues

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CD6 is a T cell surface glycoprotein that plays an important role in interactions of thymocytes with thymic epithelial cells and in mature T cell interactions with selected nonprofessional tissue APCs. We describe a novel CD6 ligand (CD6L) 3A11 Ag that is distinct from the known CD6L (CD166). The 3A11 protein is expressed on cells derived from human thymus, skin, synovium, and cartilage, and its expression is enhanced by IFN-γ. mAbs directed against the 3A11 Ag and CD166 exhibit distinct patterns of binding to a panel of cell lines. Confocal microscopy shows that both CD166 and the 3A11 Ag are expressed at the cell surface, and that these proteins colocalize. The 3A11 Ag has a molecular mass of 130 kDa and is immunoprecipitated using either mAb 3A11 or soluble CD6-Ig fusion protein. mAbs directed against individual CD6L were less potent than was soluble CD6-Ig fusion protein in reducing adhesion of T cells to adherent 3A11-positive epithelial cells in vitro, suggesting that these Abs recognize epitopes on the 3A11 Ag and CD166 that are distinct from CD6 binding sites. Finally, transfection of epithelial cells with CD166-specific small interfering RNAs significantly decreased CD166 expression without alteration in 3A11 Ag levels, and thus confirmed that these two CD6L are distinct. Taken together, our data identifies a novel 130-kDa CD6L that may mediate interactions of synovial and epithelial cells with T lymphocytes.


The CD6 glycoprotein is a type B macrophage scavenger receptor that is expressed primarily on thymocytes and T lymphocytes (1). Its three extracellular cysteine-rich domains are coded by distinct exons (2), and the membrane-proximal portion of CD6 binds the CD6 ligand (CD6L)3 CD166. Anti-CD6 mAbs that bind epitopes of the CD6 extracellular portion, including and distinct from the CD166-binding site, influenced T cell signaling (3–6), suggesting that individual CD6Ls contributed individually or in combination, to CD6-dependent costimulation. In addition, there exist multiple alternatively spliced forms of the CD6 cytoplasmic portion and contain different numbers of potential signaling motifs (7).

CD6L, also known as activated leukocyte cell adhesion molecule (ALCAM), is a 105-kDa Ig superfamily protein that binds to CD6, and is expressed by thymic epithelial cells, activated B and T cells, monocytes (8, 9), melanoma cells (10), and mesenchymal stem cells (11). Initial identification of CD6L as a CD6L was accomplished by demonstrating that a mAb directed against a leukemia cell line, HPB-ALL, partially inhibited binding of soluble CD6-Ig fusion protein (CD6-Ig) to cultured thymic epithelial cells (12). In vitro, this mAb also reduced binding of thymocytes to thymic epithelial cells and homotypic binding of these epithelial cells (9), implying a role for interactions between CD6 and CD6L in thymocyte development. CD166 has been reported to be important in establishment of hemopoiesis (13–15).

Evidence for expression of CD6Ls other than CD166 on a variety of non-T lineage cells was provided by binding studies of CD6+ T cells to epithelial cells in which anti-CD166 mAb either disrupted binding of soluble or cell-bound CD6 incompletely, or not at all (8, 16). In addition, biochemical experiments showed that soluble CD6-Ig precipitated at least three peptides from epithelial cells and synovial fibroblasts (SF) (8, 17). Anti-CD166 mAb and CD6-Ig precipitated a 105-kDa form of CD166 and, in some experiments, also precipitated a 35-kDa peptide of uncertain identity (8, 18). Immunohistochemical studies of human thymus showed that a 35-kDa epithelial Ag is physically adjacent to CD6+ thymocytes, raising the possibility that this peptide may also be a CD6L (19).

To identify novel CD6Ls, we constructed a CD6 fusion protein from the extracellular portion of mouse CD6 (which binds both human and mouse CD6L) and the constant portion of human (h)IgG1 (17). CD6-Ig immunoprecipitated both CD166 and a novel 130-kDa CD6L (3A11 Ag) that could be detected on both epithelial and mesenchymal cells (17). Although this peptide has previously been precipitated using CD6-Ig containing the human extracellular portion of CD6, the 130-kDa peptide was not recognized as a CD6L (20).

In this paper, we demonstrate that the 3A11 Ag binds to CD6, show that its expression is increased following treatment with IFN-γ, and characterize its expression on a variety of epithelial and mesenchymal cells using a novel mAb. Using confocal microscopy, we demonstrate that 3A11 Ag and CD166 are coexpressed on the cell surface and colocalize with the cytoskeleton. Flow cytometry studies show that specific mAbs to the 3A11 Ag and

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3 Abbreviations used in this paper: CD6L, CD6 ligand; ALCAM, activated leukocyte cell adhesion molecule; CD6-Ig, CD6-Ig fusion protein; SF, synovial fibroblast; h, human; siRNA, small interfering RNA; KC, keratinocyte; RA, rheumatoid arthritis; MFI, mean fluorescence intensity; MtlG1, mouse IgG1.
CD166 do not cross-block each other. Binding studies were performed to compare the ability of soluble CD6-Ig, anti-CD166 mAb, and mAb 3A11 to inhibit binding of cell-cell interactions in vitro. Finally, using small interfering RNA (siRNA) specific to CD166, we demonstrate that the expression of CD166 but not the 3A11 Ag was down-regulated, clearly indicating that the 3A11 Ag is distinct from CD166.

Materials and Methods

**Generation of mAb 3A11**

BALB/c mice were serially immunized i.p. using 10⁶ HBL-100 cells (American Type Culture Collection, Manassas, VA) in accordance with the animal use procedures at the University of Michigan. Following fusion of splenic cells with immortalized mouse myeloma cells, hybridoma supernatants were screened by flow cytometry for reactivity with the immunizing cells and for differential reactivity with resting vs IFN-γ-treated SF. Ig-secreting clones were subcloned by limiting dilution.

**Isolation of human SF and keratinocytes (KC)**

SF were isolated from tissues obtained at joint replacement surgery from patients with rheumatoid arthritis (RA) with approval of the University of Michigan Institutional Review Board and in accordance with the Helsinki guidelines as previously described (17). KCs were isolated from skin punch biopsy specimens from normal healthy volunteers or patients with psoriasis, and were obtained from the Skin Disease Research Center at Case Western Reserve University with the approval of the University Hospitals Institutional Review Board and in accordance with the Helsinki guidelines (21). The epidermis was separated from the dermis by digestion with dispase for 1 h at 37°C or overnight at 4°C (21). Single-cell suspensions were prepared by gentle blunt dissociation of the epidermis followed by filtration using a 70-μm cell strainer (BD Labware, Franklin Lakes NJ). Cells were grown in KC growth medium with supplements (Clonetics, San Diego, CA).

**Cell culture**

The HBL-100 breast carcinoma cell line (American Type Culture Collection) was cultured and passed as described (17). HaCaT KC cells were the kind gift of R. Eckert (Case Western Reserve University) and were grown in 100-mm plastic tissue culture dishes (BD Labware) in a 3:1 mixture of DMEM (BioWhittaker, Walkersville, MD) and Ham’s F12 (Invitrogen Life Technologies, Grand Island, NY), supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 1% nonessential amino acid (BioWhittaker), and 1% Na-pyruvate (Invitrogen Life Technologies). The HaCaT cells were passaged using 0.25% trypsin/1mM EDTA (Invitrogen Life Technologies). In some experiments, HaCaT cells were treated with IFN-γ (250 –1000 IU; R&D Systems, Minneapolis, MN). Culture conditions as previously described for SF (17), thymic epithelial cells and thymic fibroblasts (kind gift of D. Patel, Thurston Arthritis Center, Western Reserve University) were either used immediately or stored in SDS-PAGE buffer. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Invitrogen Life Technologies) for Western blotting. Membranes were treated for 1 h at room temperature with Superblock blocking buffer (Pierce, Rockford, IL) containing 0.5% Tween 20. Nonspecific binding was blocked by incubating fixed cells with 10% horse serum in PBS containing 0.02% Triton X-100 for 1 h. Cells were fixed for 15 min at room temperature with Zinc-formalin (Z-fix) (Anatech, Battle Creek, MI). Cells were permeabilized with 0.1% Triton X-100 for 15 min at 4°C. The proteins were detected using streptavidin-HRP conjugate (Amersham Biosciences, Piscataway, NJ) and visualized using chemiluminescence (ECL+ Plus; Amersham Biosciences, Buckinghamshire, U.K.). For Western blotting the polyvinylidene difluoride membranes were incubated with primary Ab 3A11, followed by HRP-conjugated goat anti-mouse Ab (Cell Signaling Technology, Beverly, MA). Proteins were visualized as described above.

**Flow cytometry**

Cell staining was performed as previously described (17). Analysis of labeled cells was performed using an EPICS XL-MCL instrument (Beckman Coulter, Miami, FL) equipped with a 488-nm argon-ion laser operating at 15 mW of power and System II (version 3.0) acquisition software for data collection (23). Live cells were gated on the basis of forward- and side-scatter characteristics. At least 10,000 gated events were acquired per sample in a list mode file. Off-line data analysis was performed using WinList PC, version 5.0, software (Verity Software House, Topsham, ME).

**Cross-blocking studies**

HaCaT cells were used for cross-blocking studies. Cells were incubated separately with either unlabelled mAb 3A11 or anti-CD166 mAb followed by incubation either with FITC-conjugated 3A11 mAb or anti-CD166 mAb. To quantitate basal level of 3A11 Ag and CD166 expression, cells were directly incubated with FITC-conjugated 3A11 and CD166 mAbs, and then analyzed by flow cytometry as described above.

**Immunoprecipitation and Western blotting**

Confluent adherent cells were biotinylated using E-Z Link sulfo-NHS-LC-biotin (Pierce, Rockford IL) as previously described (17). SFs were lysed and immunoprecipitated as described (17). Epithelial cells were lysed using 1% Nonidet P-40 (Calbiochem-Novabiochem, IEMD, San Diego, CA), 0.1% SDS (Bio-Rad, Hercules, CA), 0.1% deoxycholic acid (sodium salt) (Sigma-Aldrich, St. Louis, MO), and Complete Protease Inhibitor (1 tablet/ml) (Roche Diagnostics, Mannheim Germany). Cell lysates used for immunoprecipitation were preincubated with protein (A+G) agarose alone (Invitrogen Life Technologies) or with MsG1 followed by protein (A+G) agarose. Immunoprecipitation was performed overnight at 4°C using either mAbs: 3A11, anti-ALCAM, or MsG1 control; or fusion proteins: mouse CD6-Ig or h-Selectin-hlg (17). Ag-Ag complexes were immobilized using protein (A+G) agarose. For Western blots, cell lysates were either used immediately or stored in SDS-PAGE buffer. Lysis buffer for Western blot contained 1% detergent and Complete Protease Inhibitor in PBS (BioWhittaker) with 0.1 mM Ca²⁺ and 1 mM Mg²⁺ (pH 7.4). Protein concentration was determined using the detergent-compatible Bio-Rad protein assay system according to the supplier’s instructions, and 20 μg of protein/lane was loaded for SDS-PAGE/Western blot experiments. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Invitrogen Life Technologies) for Western blotting. Membranes were treated for 1 h at room temperature with Superblock blocking buffer (Pierce, Rockford IL) containing 0.05% Tween 20. For analysis of biotinylated immunoprecipitates, the membranes were washed three times in TTBS (20 mM Tris, 500 mM NaCl, 0.1% Tween 20 (pH 7.5)), and the proteins were detected using streptavidin-HRP conjugate (Amersham Biosciences, Piscataway, NJ) and visualized using chemiluminescence (ECL+ Plus; Amersham Biosciences, Buckinghamshire, U.K.). For Western blotting the polyvinylidene difluoride membranes were incubated with primary Ab 3A11, followed by HRP-conjugated goat anti-mouse Ab (Cell Signaling Technology, Beverly, MA). Proteins were visualized as described above. To verify specificity of mAb 3A11, control blots were developed using either second Ab alone or anti-CD9 mAb (Ancell).

**Confocal microscopy**

HaCaT cells (3 × 10⁶) were grown on glass coverslip inserts in 60-mm tissue culture dishes (MatTek, Ashland, MA) and analyzed while subconfluent. Where indicated, cells were treated with IFN-γ for 48 h. Cells were fixed for 15 min at room temperature with Zinc-formalin (Z-fix) (Anatech, Battle Creek, MI). Cells were permeabilized with 0.1% Triton X-100 for 90 s. Nonspecific binding was blocked by incubating fixed cells with 10% horse serum in PBS containing 0.02% Triton X-100 for 1 h. Cells were stained with primary Ab for 1–2 h at room temperature followed by fluorescein-labeled secondary Ab for 1 h. After washing, coverslips were allowed to air dry. Mounting medium containing anti-fade Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL) was added, and images were analyzed using a Leica confocal microscope (TCS SP2 with AOBS; Leica, Mannheim Germany).

**Adhesion assays**

HaCaT cells, grown on Thermosonax Plastic Coverslips (Nunc, Rochester, NY) were treated with PMA (1 ng/ml) and IFN-γ (250 IU/ml) for 2 days before use in the assays. Hut-78 cells were labeled with 51-Ion-chromate (ICN Biomedical, Costa Mesa, CA) as previously described (16). Adhesion assays were performed by preincubating either Hut-78 or HaCaT cells with saturating concentrations of mAbs or fusion protein, and then allowing cells to adhere to each other for 45 min at 37°C (16). Adhesion was quantitated by directly measuring adherent cells on coverslips using a gamma counter and comparing the counts per minute to a standardized titration curve (16). The titration curves were generated using Microsoft (Redmond, WA) Excel. SEMs were determined using the same programs. Statistical analysis was performed using ANOVA models.

**RNA interference**

A mixture of four siRNAs (SMARTpool siRNA) specific for CD166 was purchased from Dharmacon (Lafayette, CO). HaCaT cells were cultured in serum-free KC medium (KC growth medium) (Invitrogen Life Technologies) supplemented with 2.5 ng/ml epidermal growth factor and 25 μg/ml bovine pituitary extract. Cells were seeded at a density of 0.25 × 10⁶ per
60-mm plate before transfection and used when between 40 and 70% confluent. Cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions. Complexes were replaced with serum-free medium 6 h later and allowed to culture for 24–72 h. Thereafter, cells were harvested and analyzed as indicated in the figures.

**Results**

**Identification of a new CD6 surface ligand on human epitheloid and fibroblastic cells**

We have previously demonstrated that cell surface CD6 can mediate binding of the human T cell line Hut-78 to IFN-γ-activated human KCs in vitro (16). A component of the adhesion of human thymocytes to cultured thymic epithelium is mediated by CD6, and also in part, by the known CD6L, CD166 (8). Previously, we did not detect CD166-dependent binding of T cells to KCs, despite prominent KC surface expression of CD166, which suggested to us the existence of at least one additional functional CD6L (16). These data, and biochemical studies showing that soluble CD6 binds to at least three peptides (8, 17), lead us to hypothesize that CD6L(s) are expressed on the surface of epitheloid and/or fibroblastic tissue APCs.

We generated mAbs against CD6L-expressing HBL-100 cells as described in Materials and Methods. Our prior studies indicated that candidate CD6L(s) would be expressed both on epithelial cells and on RA SF, and surface expression of such a CD6L might be increased with IFN-γ in culture. One mAb, termed mAb 3A11, reacted with a cell surface protein that met these criteria; this led us to hypothesize that mAb 3A11 recognized a novel CD6L.

To determine whether the surface Ag recognized by mAb 3A11 (hereafter referred to as the “3A11 Ag”) had a unique pattern of reactivity, we stained a variety of cell types with either mAb 3A11 (Fig. 1, left) or anti-CD166 mAb (right). Both the 3A11 Ag and CD166 were expressed on cells of epithelial and mesenchymal origin, but levels of expression of these two proteins were distinct. Cross-blocking studies showed that neither mAb 3A11 nor anti-CD166 substantially inhibited binding of the other mAb to HaCaT cells (Fig. 2). Taken together, the data suggest that mAb 3A11 and anti-CD166 mAb recognize different cell surface Ags, but did not entirely exclude the possibility that these Abs could recognize non-cross blocking epitopes of the same protein that might be variably expressed.

**3A11 Ag surface expression on RA SF and on epithelial cells and is increased by treatment with IFN-γ**

Because CD6-dependent binding of T cells to KCs was augmented by IFN-γ, when searching for novel CD6L, we selected mAbs that recognized cell surface proteins that were increased following culture with IFN-γ. When RA SF cells were cultured with IFN-γ, 3A11 Ag but not CD166 surface expression was increased (Fig. 3a). 3A11 Ag surface expression was transiently increased by stimulation with IFN-γ on HBL-100 cells at both 24 and 48 h (Fig. 3b). By 72 h, 3A11 Ag expression returned to a level similar to that of cells cultured in medium alone (data not shown). This experiment was performed in quadruplicate, and a representative experiment is shown (Table I). The results were normalized by subtracting the mean fluorescence intensity (MFI) values of the negative control from that of 3A11 Ag or CD166 and then averaged. The MFIs of 3A11 were 20.64 ± 7.5 (untreated cells), 26.63 ± 7.28 (cells treated with IFN-γ for 24 h), and 30.79 ± 7.5 (cells treated with IFN-γ for 48 h). A statistically significant increase in 3A11 Ag surface expression on RA SF and on epithelial cells and is increased by treatment with IFN-γ.
expression was observed at both 24-h \( (p = 0.017) \) and 48-h \( (p = 0.028) \) IFN-\( \gamma \) treatment. The MFIs for CD166 at the 0 h \( (20.37 \pm 7.87) \) vs 24 and 48 h, respectively \( (19.7 \pm 6.56 \ (p = 0.329) \) and \( 19.25 \pm 6.36 \ (p = 0.268) \)) were not significantly different. Expression of CD54, which is known to mediate T cell-KC binding, is increased by IFN-\( \gamma \) at both the 24- and the 48-h time points (Table I).

**mAb 3A11 and CD166 precipitated distinct peptides**

The flow cytometry data suggested that mAb 3A11 recognized a surface protein other than CD166 (Figs. 1–3). Therefore, we wanted to demonstrate that mAb 3A11 and anti-CD166 each reacted with peptides that are distinct from each other. RA SF cells

**FIGURE 2.** Cross-blocking studies using mAb 3A11 and CD166. HaCaT cells were preincubated with mAb 3A11 or CD166 as described in Materials and Methods. As shown, anti-CD166 mAb does not cross-block binding of mAb 3A11-FITC (left). Minimal cross-blocking of anti-CD166-FITC mAb is observed when cells are preincubated with mAb 3A11 (right). Incubation of cells with unconjugated mAb 3A11 or anti-CD166 mAb reduces binding of FITC-conjugated 3A11 mAb and anti-CD166 mAb, respectively. Background staining (using MsIgG1) is also shown.

**FIGURE 3.** IFN-\( \gamma \) augments expression of the 3A11 surface Ag on RA SFs and on cultured epithelial cells. Surface expression of novel (3A11 Ag) and known (CD166) CD6Ls were measured by flow cytometry. Results are expressed as single-color histograms of cell number (y-axis) vs units of fluorescence expressed on a four-decade logarithmic scale \( (a \text{ and } b) \) or as a table of MFI for a representative experiment. 

\( a \), RA SFs were cultured with IFN-\( \gamma \) for 48 h. By 48 h, expression of the 3A11 Ag was increased \( (\text{left}) \), whereas surface CD166 expression was not \( (\text{right}) \). Background staining (using MsIgG1) was similar for untreated and IFN-\( \gamma \)-treated cells. 

\( b \), HBL-100 epithelial cells were cultured with medium alone or with IFN-\( \gamma \) for 24–48 h. Shown is expression of the 3A11 Ag and CD166 following culture with medium alone for 24 or 48 h. Following culture with IFN-\( \gamma \), expression of the 3A11 Ag is increased at 24 and 48 h, whereas expression of CD166 is not.
were surface-labeled using sulfo-NHS-LC-biotin, and then lysed using buffer containing 1% Nonidet P-40. Immunoprecipitation was performed using mAbs 3A11 or anti-CD166 mAb, with MsIgG1 as a control (Figs. 4 and 5) or using soluble fusion protein CD6-Ig (Figs. 4 and 5) with selectin-Ig as a control (Fig. 5); peptide-containing complexes were immobilized with protein A- and/or protein G-agarose beads (Figs. 4 and 5). Shown in Fig. 4 are the results of a representative immunoprecipitation experiment using RA SF lysates. Biotinylated RA SF peptides were dissociated from the solid phase by boiling, and electrophoresed under reducing conditions. mAb 3A11 precipitated a peptide of 130 kDa (Fig. 4, lane 2), whereas anti-CD166 mAb precipitated a 105-kDa peptide (lane 3). Soluble CD6-Ig precipitated several proteins, including proteins with molecular weights of 130 and 105 kDa (Fig. 4, lane 2). The protein bands at 50/55 kDa (Fig. 4, lanes 1 and 4) and 30 kDa (lanes 2 and 4) have not been definitively identified and characterized. Those bands also seen in the MsIgG1 lane are likely not of particular importance to these studies, because the control reagent contains Abs of unknown and unrelated specificity.

Evidence that the 3A11 Ag is a CD6L

Our hypothesis that 3A11 Ag is a CD6L is supported by immunoprecipitation experiments using soluble CD6-Ig (Fig. 4). To demonstrate that CD6-Ig and mAb 3A11 recognize the same peptide, we performed serial immunoprecipitations using RA SF lysates. Biotinylated RA SF lysates were preclarified and then depleted of CD6Ls using soluble CD6-Ig, control fusion protein (selectin-Ig), or Ab control (MsIgG1), and protein G-agarose beads for immobilization in the solid phase. The preclarified lysates were incubated with mAb 3A11, and those immunoprecipitates were recovered as described above. The data show that preclarifying the lysates with soluble CD6-Ig but not with either control fusion protein (selectin-Ig) or control Ig (MsIgG1) resulted in depletion of the 130-kDa peptide (Fig. 5). These data confirm that CD6-Ig and mAb 3A11 bind to an identical protein.

Delineating the nature of a 70-kDa band that is immunoblotted by mAb 3A11

Western blot experiments using mAb 3A11 detected a 70-kDa band that was not seen in immunoprecipitation experiments (Figs. 4 and 5). To determine whether the 70-kDa band was a closely associated or cross-reactive peptide but not the 3A11 Ag itself, and whether it was an intracellular protein rather than a surface protein, we used CD6-positive T cell line Hut-78 (lacks surface 3A11 Ag) and HaCaT cells as the source of 3A11 Ag negative and positive cell lines, respectively. Western blotting showed that mAb 3A11 detected a 130-kDa band from HaCaT cell lysates only (Fig. 6), whereas the 70-kDa band was detected in both HaCaT and Hut78 cell lysates (Fig. 6) leading us to conclude that the 70-kDa band represents a peptide distinct from the 3A11 Ag, and that it is localized in intracellular compartments rather than on the cell surface.

Role of the 3A11 Ag in adhesion of T cells to epithelial cells

We performed adhesion assays designed to determine whether the 3A11 Ag mediates adhesion of CD6+ T cells to HaCaT in vitro.
Adhesion of Hut-78 T cells that had previously been reported to be maximal following treatment of HaCaT with both the phorbol ester PMA and IFN-γ (24). To test the hypothesis that 3A11 Ag and CD166 mediated binding of CD6+ T cells to HaCaT, we preincubated HaCaT with mAbs directed against CD6L or soluble CD6L-ig (or control MsIgG1 or hIg). CD6-positive Hut-78 cells were adherent to HaCaT with mAbs directed against CD6L or soluble CD6L-ig (or control MsIgG1 or hIg). CD6-positive Hut-78 cells were adhered to CD6L-ig activated HaCaT cells for 45 min. In our experiments, T cell/CD6L-ig adhesion was significantly inhibited by CD6L-ig compared with control hIg (Table II). Although there was a trend toward inhibition of adhesion by mAbs directed against either 3A11 Ag or CD166, this did not reach statistical significance.

To determine whether the 3A11 Ag and CD166 colocalize on the cell surface, we used confocal microscopy, and determined the cellular expression of each of the CD6L (Fig. 7, a and b).

The 3A11 Ag was localized to the cell surface membrane both in HaCaT cells that were activated using IFN-γ (Fig. 7a) and in those cultured in medium alone (d). The 3A11 Ag was distributed diffusely on the cell surface in collections of cells that were subconfluent (Fig. 7, a and d). 3A11 was prominent in small fingerprint-like projections on cells found at the periphery of cell clusters, suggesting that it may play a role in cell attachment and spreading (Fig. 7, a and d). CD166 was localized to the cell surface membrane, also appeared to be involved in cell spreading and attachment, and was prominent in lower-power images, in which cells that appeared to be growing toward each other had filaggrin-like projections (data not shown). Single-color staining for CD166 showed similar cellular localization patterns in untreated and in IFN-γ-treated HaCaT cells (Fig. 7b and data not shown).

To determine whether the 3A11 Ag and CD166 colocalize, HaCaT cells were double stained and then imaged sequentially (Fig. 7, a–c). Images were overlaid; staining with green indicated expression of the 3A11 Ag, staining with red indicated expression of CD166 expression, and staining that appeared yellow indicated coexpression of the 3A11 Ag and CD166. The data confirm that colocalization of the two CD6L occurs in IFN-γ-treated cells (Fig. 7c).

Indirect evidence suggested that CD166 is associated with the actin cytoskeleton (27). To investigate whether one or both CD6L-associated with the actin cytoskeleton, HaCaT cells were double-stained with Ab directed against either the 3A11 Ag (Fig. 7e) or CD166 (f) and fluorescently labeled phalloidin (stains F-actin). In IFN-γ-treated HaCaT cells, the actin cytoskeleton is more prominent than in untreated HaCaT cells (data not shown) with prominent colocalization of both CD6L and actin in IFN-γ-treated cells (Fig. 7, e and f).

Use of siRNA demonstrates that the 3A11 Ag and CD166 are distinct proteins.

Gene interference by dsRNA has been described, and it appears to be important as a silencing mechanism for developmentally important genes. Mammalian interfering RNAs have been described (28, 29), and has been used experimentally, in vitro and in vivo, to reduce specific mRNA and thus protein expression (30). We hypothesized that the 3A11 Ag was distinct from CD166, then CD166 expression will be down-regulated in cells treated with siRNA specific for CD166 (CD166-siRNA), whereas the expression of the 3A11 Ag would be unaffected. To test this hypothesis, HaCaT cells were transfected with SMARTPool siRNA specific for CD166 (CD166-siRNA), laminin, or with unrelated nonspecific siRNA. The data showed a time-dependent decrease in CD166 expression that was evident at 24, 48, and 72 h (data not shown). Maximal depression of CD166 protein occurred at 36 h, which is consistent with other published data using siRNA to decrease expression of a variety of proteins (30). The percentage of cells affected by transfection of siRNA differed depending on the expression of the cells at the time of transfection; cells transfected with CD166-siRNA at low confluence, were uniformly CD166low. When cells were similarly transfected at higher confluence, a subpopulation remained CD166low, suggesting that the degree of cellular confluency affects transfection efficiency (data not shown).

The effect of CD166-siRNA on CD166 protein expression was similar when cells were transfected with a range of concentrations of duplexes (25–100 nM, Fig. 8a, and data not shown). Dose dependency was observed at concentrations of duplexes between 0 and 25 nM (data not shown). As predicted, 3A11 Ag expression (Fig. 8b), and expression of the unrelated protein, CD98 (data not shown), was unaffected by transfection with CD166-siRNA. When the time course of CD166 expression was examined, transfected cells were uniformly CD166low, suggesting that the degree of cellular confluency affects transfection efficiency (data not shown).

As predicted, 3A11 Ag expression (Fig. 8b), and expression of the unrelated protein, CD98 (data not shown), was unaffected by transfection with CD166-siRNA. When the time course of CD166 expression was examined, transfected cells were uniformly CD166low, suggesting that the degree of cellular confluency affects transfection efficiency (data not shown).
shown), were not affected by transfection with CD166-siRNA duplexes. Furthermore, there was no effect on CD166, 3A11 Ag, or CD98 expression following transfection of HaCaT cells with either nonspecific SMARTPool siRNA duplexes or with laminin-specific siRNA duplexes. Taken together, the data clearly show that the 3A11 Ag is distinct from CD166.

**Discussion**

In this report, we demonstrate the existence of a novel ligand for CD6 that is distinct from CD166, the known CD6L. The CD6L CD166 is found on the surface of epithelial cells, SFs, activated macrophages, and mesenchymal stem cells (9–11, 17, 31). CD166 mediates homotypic binding of cells, is strongly expressed in RA synovium and in melanoma metastases, and has recently been reported to play a role in neoangiogenesis (13–15). We had previously hypothesized that individual CD6Ls might exist at the cell surface as part of a CD6-binding complex. Confocal microscopy showed that the 3A11 Ag and CD166 are localized in association with each other (Fig. 7). Activation of HaCaT cells with IFN-γ increased the coexpression of CD6L at the cell surface and induced appearance of prominent F-actin filaments (Fig. 7, e and f). The 3A11 Ag appeared to be associated with the actin cytoskeleton even in unactivated HaCaT cells (data not shown). It is conceivable but not proven that multiple CD6L that associate in a CD6L complex can engage multiple epitopes of CD6 (4), and that differences in the amounts of these ligands may be one way of varying the quantity or quality of signal transduced from CD6L+ cells to CD6+ T cells. CD6L binding to T cells occurs, but which CD6L have the capacity to costimulate T cells has not been discerned. Our studies that showed CD6L associated with the actin cytoskeleton also raise the possibility that reverse signaling (CD6 to CD6L) might occur when CD6+ T cells encounter tissue-specific CD6L.

To further demonstrate that the 3A11 Ag is a novel protein, and not simply an epitope of CD166, we used RNA interference to
reduce CD166 expression (Fig. 8). siRNAs, and related micro-RNAs, have been shown to interfere with mRNA transcription and translation, respectively (34). For our purposes, whether or not the CD166-specific SMARTPool RNA duplexes inhibited CD166 transcription or also affected translation was unimportant, because our goal was to show that CD166 but not 3A11 Ag expression was decreased. The siRNA experiments indicated that the mRNAs coding for CD166 and the 3A11 Ag were different from each other. The flat dose-response curve we observed for CD166 over a range of 25–100 nM siRNA duplexes, combined with the absence of effect on CD166 levels of either nonspecific duplexes (Fig. 8) and laminin-specific siRNA (data not shown), suggests that the CD166-specific siRNA duplexes were highly effective in partially or almost completely silencing the expression of only CD166 and not the 3A11 Ag.

In immunoprecipitation experiments, the molecular mass of the 3A11 Ag was \( \sim 130 \) kDa (Fig. 4). In contrast to mAb 3A11, anti-CD166 mAb precipitated a 105-kDa peptide that was consistent with the reported molecular mass for CD166 (Fig. 4). The molecular mass of the 3A11 Ag also appears to be 130 kDa in Western blotting experiments performed under reducing and nonreducing conditions (data not shown). In using mAb 3A11 for Western blotting, we also observed reactivity with a 70-kDa peptide. The 70-kDa peptide was also detected in lysates from Hut-78 cells, which did not express surface 3A11 Ag (Fig. 6), and it was observed under reducing and nonreducing conditions. We consider it more likely that the 70-kDa peptide cross-reacts with mAb 3A11, but that it is not 3A11 Ag per se compared with the attractive possibilities that it is a precursor or degradation product of the 3A11 Ag. The presence of detergent in the cell lysates interfered with the ability of mAb 3A11 to detect the target peptide, suggesting that intercalation of these detergents interferes with epitope recognition and that the 3A11 Ag has lipophilic residues (35).

Studies using soluble CD6-Igs demonstrated that CD166 was a CD6L (8). We too used soluble CD6-Ig to demonstrate that the 3A11 Ag was a CD6L, and demonstrated that when CD6L were specifically depleted from cell lysates using soluble CD6-Ig, the 130-kDa peptide precipitated by mAb 3A11 was absent (Fig. 5). In these experiments, we used a CD6 fusion protein that contains the mouse form of the extracellular domain of CD6. Mouse and human CD6 bind mouse and human CD166 cross-species (36). Our data show that mouse CD6 also binds the CD6L, 3A11 Ag cross-species. This implies the existence of a mouse homolog of the 3A11 Ag that is likely to bind to mouse CD6.

In vitro, our adhesion assays showed that presence of soluble CD6-Lg reduced baseline binding of CD6\(^{+}\) human T cells to IFN-\(\gamma\)-activated HaCaT cells by 25% (\( p = 0.05 \)) (Table II). The HaCaT activation protocol used in the current assay and the time point chosen at which to measure adhesion (45 min instead of 30 min) had been optimized to detect CD6L dependence. When Abs directed against either the 3A11 Ag or CD166 were used, there was a trend suggesting that adhesion was inhibited, but these results did not reach statistical significance. In our prior assays, we did not detect CD6L-dependent binding (16), and this is explained by data suggesting that none of the anti-CD166 Abs available to us recognized the CD6-binding site contained in what is known as CD166 domain 1 (D1) (25, 26). The observation that we detected CD6L dependence in our adhesion assays when we used soluble CD6 fusion protein, but that it was difficult to detect when we used Abs against individual CD6L led to our conclusion that neither the 3A11 Ab nor anti-CD166 mAb bind precisely the same epitope of the respective CD6L, as cellular CD6 does (25, 37, 38).

Our studies of thymic selection and mature T cell responses (20, 33) imply a role for CD6L and CD6 in T cell development (23) and in autoreactivity (33, 39). In vitro, CD6\(^{+}\) T cells proliferate less well than CD6\(^{-}\) T cells to stimulation by allo-APCs despite equivalent proliferation to stimulation by exogenous Ags (40), suggesting that CD6-dependent signaling occurs. Indirect evidence that CD6 functions as a costimulatory molecule for mature T cells in skin and synovium includes data showing that transplanting bone marrow depleted of CD6\(^{+}\) T cells reduces the incidence of graft-vs-host disease (manifestations of which include inflammatory skin and joint disease) (41–45). It is likely that the overall functional avidity of interactions between T cells and tissue APCs via CD6/CD6L are low in isolation, but could nonetheless help to regulate T cell responsiveness. We hypothesize that CD6-dependent stimuli, induced by binding of CD6L, augment the overall...
functional avidity of T cells for MHC/alloantigen complexes and in some circumstances for MHC/autoantigen. Further identification of the molecular structure and the relevant interactions of CD6 and 3A11 should allow for greater understanding of these issues and perhaps new therapeutic strategies in the future.

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