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An Antibody to the Sixth Ig-like Domain of VCAM-1 Inhibits Leukocyte Transendothelial Migration without Affecting Adhesion

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VCAM-1 plays a key role in leukocyte trafficking during inflammatory responses. However, molecular mechanisms underlying this function have not been clearly elucidated. In this study, using phage display technology, we developed a rabbit/human chimeric VCAM-1 Ab, termed VCAM-1 domain 6 (VCAM-1-D6), which specifically recognizes aa 511–599 within the sixth Ig-like domain. We report that the VCAM-1-D6 Ab blocked U937 cell transmigration across activated HUVECs but did not alter adhesion of U937 cells to the HUVECs. We also demonstrate that VCAM-1-D6 does not alter TNF-α-stimulated endothelial cell chemokine or cytokine production. Furthermore, through in vivo efficacy testing using a mouse islet allograft model, we demonstrate that VCAM-1-D6 significantly alleviates allograft rejection by blocking leukocyte infiltration to the grafted islets. Taken together, our results suggest that the VCAM-1-D6 Ab may block VCAM-1–mediated inflammation and could be a useful tool in treating inflammatory diseases. The Journal of Immunology, 2012, 189: 000–000.
Materials and Methods

Construction of the Ab library and selection of binders

Four New Zealand white rabbits were immunized and boosted four times with recombinant hVCAM-1 (rhVCAM-1; R&D Systems, Minneapolis, MN) with the approval of the Institutional Animal Care and Use Committee of the Seoul National University Hospital. After the final booster injection, total RNA was prepared from the spleen and the bone marrow. cDNA was reverse transcribed using SuperScript First-Strand cDNA synthesis system (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. A phage-display rabbit/human chimeric Fab Ab library was constructed using pComb3X phagemid vector system as described previously (43). After the library construction, Fab clones were selected from the library through six rounds of biopanning as described previously (2). For each round of biopanning, 2.5 μg recombinant mVCAM-1Fc fusion protein (mVCAM-1–Fc; R&D Systems)-coated magnetic beads (Dynal beads M-270 epoxy; Invitrogen) were used. After the final round of biopanning, individual phage clones displaying Fab were generated from colonies grown on output plates and tested for reactivity to hVCAM-1–Fc and mVCAM-1–Fc by phage enzyme immunoassay as described previously (4).

Preparation of VCAM-1-D6 Fab and IgG

Top10F E. coli cells were transformed with phagemid DNA encoding the VCAM-1–D6 Fab. Bacteria were grown in 1 Luria–Bertani media containing 50 μg/ml carbenicillin at 37°C overnight with constant shaking. The cells were pelleted by centrifugation at 3000 × g for 15 min. Supernatants were collected and concentrated 10 times using the Labscale TFF System (Millipore, Bedford, MA). The VCAM-1–D6 Fab was purified from the concentrated supernatant using hemagglutinin-specific Ab column chromatography as described previously (44, 45). After column chromatography, fractions containing Fab were pooled and analyzed by SDS-PAGE and Coomassie blue staining as described previously (46). The production and purification of VCAM-1–D6 IgG was performed as described previously (47).

ELISA

rhVCAM-1–Fc (R&D Systems) and mVCAM-1–Fc were dissolved in 50 μl PBS and added to the wells of a microtiter plate. The amount of VCAM-1–Fc added to each well was 5, 10, 20, or 50 ng. After incubation overnight at 4°C and washing three times with PBST (PBS containing 0.05% [v/v] Tween 20), the microtiter plate was incubated for 2 h at 37°C with 3% (w/v) BSA in PBS. After washing with PBST, the plate was incubated with 2 μm VCAM-1–D6 Fab or 2 μm of unrelated Fab for 1 h at 37°C, and washed twice with PBST. The plate was incubated with anti-human Fab Ab conjugated to HRP (Sigma, St. Louis, MO) diluted 1000-fold in PBST containing 3% (w/v) BSA in PBS. After washing with PBST, PBST containing 0.3% (v/v) Tween 20, the microtiter plate was incubated with 2 nM VCAM-1–D6 Fab or 2 nM of unrelated Fab for 1 h at 37°C, and washed twice with PBST. The amount of OD was measured at 450 nm using a microtiter plate reader (Labsystems, Barcelona, Spain).

Flow cytometry

HUVECs (Lonza, Baltimore, MD) and human aortic endothelial cells (HAECs; Lonza, Switzerland) were maintained in endothelial cell growth medium-2 in a humidified incubator at 37°C with 5% CO2 according to the manufacturer’s instructions. Mouse vascular endothelial cells (MVECs) were a kind gift of Dr. Saito (Tsunemi University, Tsurumi, Japan) and were maintained in Medium 199 supplemented with 5% (v/v) FBS, 10 μg/ml insulin, 2.4 μg/ml hydrocortisone, and 1% (v/v) penicillin/streptomycin. The endothelial cells were stimulated with 20 ng/ml human (hTNF-α) or mouse TNF-α (Invitrogen) to each well. OD was measured at 450 nm using a microtiter plate reader (Labsystems, Barcelona, Spain).

Immunoblot analysis

SDS-PAGE and immunoblot analysis were performed as described previously (5). The purified proteins were dissolved in a Laemmli sample buffer and loaded into each well of 4–12% Tris-Glycine Gel (Novex NuPAGE; Invitrogen). After electrophoresis, the proteins were transferred to nitro-
cellulose membranes using the wet transfer system (GE Healthcare Life Sciences, Pittsburgh, PA). The membrane was incubated with TTBS (10 mM Tris/Cl, pH 7.5, 150 mM NaCl, and 0.05% [v/v] Tween 20) containing 5% (v/v) skim milk at room temperature for 1 h, followed by incubation with 10 µg/ml VCAM-1-D6 Fab in TTBS containing 5% (v/v) skim milk at room temperature for 2 h. The membrane was washed with TTBS and incubated with anti-human Fab-HRP diluted 1000-fold in TTBS containing 5% (v/v) skim milk at room temperature for 2 h. In a parallel experiment, the membranes were incubated with HRP-conjugated rabbit anti-human Fc (Pierce, Rockford, IL) diluted 5000-fold in TTBS containing 5% (v/v) skim milk at room temperature for 30 min. The membranes were washed with TTBS three times, and protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions.

**Human monocyte isolation**

Heparin-treated blood was collected from healthy volunteers after an informed consent was signed, and in regulation accordance with the ethical guidelines of Seoul National University hospital. PBMCs were isolated by Ficoll density gradient centrifugation (Ficoll-Paque; Amersham, Uppsala, Sweden) and resuspended in DMEM supplemented with 10% FBS, 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µM 2-mercaptoethanol, and 0.25 µg/ml G418 as a selection marker. Clones stably expressing full-length cDNA of hVCAM-1 were ligated into the KpnI or XhoI site of the pcDNA3.1 (+) vector (Invitrogen). After transforming calcium chloride-competent DH5α cells, plasmid DNA was prepared as described previously (38). The full-length cDNA of hVCAM-1 was ligated into the KpnI or XhoI site of the pcDNA3.1 (+) vector (Invitrogen). After transforming calcium chloride-competent DH5α cells, plasmid DNA was prepared as described previously (38). The full-length cDNA of hVCAM-1 was ligated into the KpnI or XhoI site of the pcDNA3.1 (+) vector (Invitrogen). After transforming calcium chloride-competent DH5α cells, plasmid DNA was prepared as described previously (38).

**Transendothelial cell migration assay**

HUVECs (5.0 × 10⁵ cells/well) were added to the upper chambers of a 24-transwell plate with polycarbonate membranes of 8.0-µm diameter pores (Corning, Corning, NY) and incubated overnight in a humidified incubator at 37°C with 5% CO₂. Cells were treated with hTNF-α diluted in endothelial cell growth medium-2 (Lonza, Baltimore, MD). The final concentrations of VCAM-1-D6 Fab, VCAM-1-D6 IgG, or rhVCAM-1 domain 6-16 Fab were adjusted to 10 µg/ml. The islets were added to each well to a final concentration of 10 µg/ml. The plate was incubated for 24 h at 37°C. At a humidified incubator with 5% CO₂. The media were harvested and subjected to the Bio-Plex Suspension Array System for high-throughput multiplex analysis. The assay was performed using a FACS CantoII instrument (BD Biosciences), with excitation at 488 nm and the emission filter set to a 585/30 nm bandpass. In each measurement, 10,000 cells were counted with no gating. Results are expressed as the percentage of CFSE⁺ cell counts over the total cell events.

**Measurement of HUVEC-secreted cytokines and chemokines**

HUVECs (3.0 × 10⁵ cells) were added to each well of a 6-well plate and treated with 20 ng/ml hTNF-α for 24 h at 37°C inside a humidified incubator with 5% CO₂. The VCAM-1-D6 IgG or the mouse anti-hVCAM-1 Ab 51-10C9 was added to each well to a final concentration of 10 µg/ml. The plate was incubated for 24 h at 37°C inside a humidified incubator with 5% CO₂. The media were harvested and subjected to the Bio-Plex Multiplex Chemokine and Cytokine assay (Bio-Rad Laboratories, Hercules, CA) or an IL-8 ELISA (Invitrogen) with a dynamic range between 15.6 pg/ml and 1 ng/ml, following the manufacturers’ recommendations.

**Mice**

Female C57BL/6 and BALB/c inbred mice, aged 8–10 wk, were purchased from Charles River Laboratories (Wilmington, MA) and maintained in the Seoul National University specific pathogen-free animal facility. These mice were bred at the Biological Services Unit of the Seoul National University according to the Institutional Animal Care and Use Committee guidelines.

**Induction of diabetes mellitus and islet transplantation**

Diabetes mellitus was induced in mice (8–10 wk old) by i.p. administration of 250 mg/kg streptozotocin (STZ; Sigma) freshly dissolved in citrate buffer (pH 4.5). Blood glucose in nonfasting conditions was measured from the snipped tail by a portable glucometer (Lifescan, Milpitas, CA). Two consecutive nonfasting blood glucose readings of >250 mg/dl were obtained from whole blood. Seven days after STZ administration, mice were anesthetized with isoflurane. For islet transplantation, the left kidney was exposed and 500 islet equivalents were delivered beneath the kidney capsule using PE-50 polyethylene tube (Becton Dickinson, Franklin Lakes, NJ). Mice were treated with either VCAM-1-D6 IgG or control IgG (0.1 mg, i.p. injection on days 1, 0, 1, 2, 3, 4, 5, 6, and 7. Day 0 is defined as the time of islet transplantation.

**Immunohistochemistry**

The kidney with the islet graft was removed and embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA). Frozen sections were obtained on a cryostat (CM1850; Leica, Bannockburn, IL) at 5-µm thickness and fixed in acetone for 10 min at 4°C. Sections were washed three times with PBS, followed by incubation in an endogenous peroxide-blocking solution for 5 min at room temperature. Nonspecific staining was prevented by treating the sections with 1% (v/v) PBS in PBS for 30 min at room temperature. The primary Ab was used in a guinea pig anti-insulin (1:20 dilution; 0.7 mg/ml). Dako, Carpinteria, CA), rat anti-C4D (1:50 dilution; 0.62 mg/ml, RM4-5; BD Biosciences, San Jose, CA), and rat anti-RANTES Ab (1:20 dilution; 100 µg/ml; R&D Systems). The kidney was stained with primary Ab conjugated to fluorescein isothiocyanate (FITC; Sigma). The kidney sections were treated with 20 ng/ml hTNF-α for 24 h at 37°C inside a humidified incubator with 5% CO₂. The kidney with the islet graft was removed and embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA). Frozen sections were obtained on a cryostat (CM1850; Leica, Bannockburn, IL) at 5-µm thickness and fixed in acetone for 10 min at 4°C. Sections were washed three times with PBS, followed by incubation in an endogenous peroxide-blocking solution for 5 min at room temperature. Nonspecific staining was prevented by treating the sections with 1% (v/v) PBS in PBS for 30 min at room temperature. The primary Ab was used in a guinea pig anti-insulin (1:20 dilution; 0.7 mg/ml). Dako, Carpinteria, CA), rat anti-C4D (1:50 dilution; 0.62 mg/ml, RM4-5; BD Biosciences, San Jose, CA), and rat anti-RANTES Ab (1:20 dilution; 100 µg/ml; R&D Systems). The kidney was stained with primary Ab conjugated to fluorescein isothiocyanate (FITC; Sigma). The kidney sections were treated with 20 ng/ml hTNF-α for 24 h at 37°C inside a humidified incubator with 5% CO₂. The kidney with the islet graft was removed and embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA). Frozen sections were obtained on a cryostat (CM1850; Leica, Bannockburn, IL) at 5-µm thickness and fixed in acetone for 10 min at 4°C. Sections were washed three times with PBS, followed by incubation in an endogenous peroxide-blocking solution for 5 min at room temperature. Nonspecific staining was prevented by treating the sections with 1% (v/v) PBS in PBS for 30 min at room temperature. The primary Ab was used in a guinea pig anti-insulin (1:20 dilution; 0.7 mg/ml). Dako, Carpinteria, CA), rat anti-C4D (1:50 dilution; 0.62 mg/ml, RM4-5; BD Biosciences, San Jose, CA), and rat anti-RANTES Ab (1:20 dilution; 100 µg/ml; R&D Systems). The kidney was stained with primary Ab conjugated to fluorescein isothiocyanate (FITC; Sigma). The kidney sections were treated with 20 ng/ml hTNF-α for 24 h at 37°C inside a humidified incubator with 5% CO₂. The kidney with the islet graft was removed and embedded in OCT compound (Tissue-Tek, Sakura, Torrance, CA). Frozen sections were obtained on a cryostat (CM1850; Leica, Bannockburn, IL) at 5-µm thickness and fixed in acetone for 10 min at 4°C. Sections were washed three times with PBS, followed by incubation in an endogenous peroxide-blocking solution for 5 min at room temperature. Nonspecific staining was prevented by treating the sections with 1% (v/v) PBS in PBS for 30 min at room temperature. The primary Ab was used in a guinea pig anti-insulin (1:20 dilution; 0.7 mg/ml). Dako, Carpinteria, CA), rat anti-C4D (1:50 dilution; 0.62 mg/ml, RM4-5; BD Biosciences, San Jose, CA), and rat anti-RANTES Ab (1:20 dilution; 100 µg/ml; R&D Systems).
Results

An Ab isolated from a rabbit/human chimeric Fab library reacts with hVCAM-1 and mVCAM-1

Rabbits were immunized and boosted four times with purified rhVCAM-1. Total RNA was prepared from spleen and bone marrow, and subjected to cDNA synthesis. Using this cDNA, we generated a rabbit/human chimeric Fab library containing rabbit variable regions and human constant regions with a complexity of $5.7 \times 10^{9}$. After six rounds of biopanning on immobilized mVCAM-1–Fc, clones were randomly selected, rescued by infection of helper phage, and tested for their reactivity to hVCAM-1 and mVCAM-1. Ab clones reactive to both hVCAM-1 and mVCAM-1 were selected for further study.

After expression in E. coli and subsequent purification, the Fab purity (>90%) was confirmed by SDS-PAGE and Coomassie blue staining (data not shown). Using an ELISA, we determined that 0.02 nM VCAM-1-D6 Fab bound to both hVCAM-1– and mVCAM-1–coated ELISA plates (Fig. 1A). In a Western blot, the VCAM-1-D6 Fab detected 5–50 ng rhVCAM-1 and rmVCAM-1 (Fig. 1B).

In real-time interaction analysis using VCAM-1–coated chips (Table I), the $K_D$ constant for VCAM-1-D6 Fab interaction with hVCAM-1 was $1.35 \pm 0.02 \times 10^{-8}$. For the interaction with mVCAM-1, the $K_D$ constant was 4.78 $\pm 0.06 \times 10^{-10}$. When using the IgG form of the VCAM-1-D6 Ab, the $K_D$ constant for interaction with hVCAM-1 and mVCAM-1 was 3.06 $\pm 0.04 \times 10^{-10}$ and $7.31 \pm 0.42 \times 10^{-11}$, respectively (Table I). These data indicate a strong affinity for VCAM-1-D6 Fab and IgG with either hVCAM-1 or mVCAM-1. The reactivity of VCAM-1-D6 Fab to endogenous VCAM-1 expressed on human and mouse endothelial cells, and its localization pattern were investigated using flow cytometry and immunocytochemistry (Fig. 1C, 1D). VCAM-1-D6 Fab reacted to VCAM-1 on HAECS, HUVECs, and MVECs stimulated by hTNF-α or mTNF-α, but not to unstimulated cells.

In the immunocytochemical study, the localization pattern of VCAM-1 obtained using VCAM-1-D6 Fab was similar to that obtained with the mouse anti–hVCAM-1 Ab 51-10C9 that was used as a positive control.

The VCAM-1-D6 Ab specifically recognizes the sixth Ig-like domain of VCAM-1

To identify the epitope region for the VCAM-1-D6 Fab, we constructed expression vectors for six truncated forms of the extracellular domain of hVCAM-1 as Fc fusion proteins (Fig. 2A). These constructs were transfected into HEK293F cells, and the Fc fusion proteins were purified by protein A column chromatography. An equal amount of each fusion protein was subjected to immunoblot analysis. The VCAM-1-D6 Fab detected hVCAM-1-WT (1–698)–Fc and hVCAM-1-C1 (1–599)–Fc, whereas it did not bind to hVCAM-1-C2 (1–510)–Fc, suggesting hVCAM-1 aa 511–599 as an epitope for VCAM-1-D6. The HRP-conjugated human Fc Ab reacted to all of the Fc fusion proteins (Fig. 2B).

To further confirm that hVCAM-1 aa sequence 511–599 includes the epitope of the VCAM-1-D6 Fab, we prepared a Fc fusion protein containing these residues (hVCAM-1-D6 [511–599]–Fc;...
The VCAM-1-D6 Ab specifically recognizes the sixth Ig-like domain of hVCAM-1 (aa 511–599)–Fc (data not shown). From these data, we concluded that the VCAM-1-D6 Fab specifically recognizes a C-terminal region (aa 511–599) of hVCAM-1, which represents the sixth Ig-like domain of hVCAM-1 (aa 511–595).

The VCAM-1-D6 Ab significantly inhibits U937 cell and human monocyte transmigration across activated endothelial cells without inhibiting U937 cell or human monocyte adhesion

To investigate whether the VCAM-1-D6 Ab regulates leukocyte transmigration across activated endothelial cells, we performed transendothelial cell migration assays with HUVECs and U937 human monocytic cells. HUVECs were added to the upper chamber of transwells, allowed to grow to confluence, and then treated with hTNF-α for 24 h to induce VCAM-1 expression. After adding U937 cells to the upper chambers, the chemottractant human SDF-1α was added to the lower chambers. After 24 h, the U937 cells that migrated through the HUVEC monolayer to the lower chamber were collected and counted. When VCAM-1-D6 Fab or VCAM-1-D6 IgG was added to the upper chamber, the number of migrating U937 cells decreased significantly in a concentration-dependent manner. We also found that both the VCAM-1-D6 Fab and IgG almost completely inhibited the migration at 10 μg/ml (Fig. 3A, 3B). To check whether rhVCAM-1 domain 6 is directly involved in leukocyte transendothelial migration, we performed a transmigration assay with rhVCAM-1 domain 6 (aa 511–599)–Fc and control IgG. rhVCAM-1 domain 6–Fc significantly inhibited the transmigration of U937 cells across activated HUVECs in a concentration-dependent manner (Fig. 3C). We also performed the transmigration assay with monocytes isolated from human peripheral blood. VCAM-1-D6 Fab or VCAM-1-D6 IgG and rhVCAM-1-domain 6–Fc inhibited the transmigration of human monocytes across activated HUVECs (Fig. 3D).

The effect of the VCAM-1-D6 Fab on leukocyte adhesion to VCAM-1 was examined using hVCAM-1– or mock-transfected HEK293 cells, as well as HUVECs and HAEcs induced to express VCAM-1 by treatment with hTNF-α. Cell surface expression in hVCAM-1–transfected HEK293 cells was confirmed by flow cytometric analysis (Fig. 4A). For adhesion assays, the cells were grown to confluence on six-well dishes. U937 cells were labeled with CFSE and then added to each well in the absence or presence of 50 μg/ml VCAM-1-D6 Fab, control Fab, or the mouse anti-hVCAM-1 Ab 51-10C9. After 1 h, U937 cells attached to hVCAM-1-overexpressing HEK-293 cells, HUVECs, or HAEcs were

**TABLE I.** Affinity constants (K<sub>a</sub>, K<sub>d</sub>) evaluated by BIAcore for VCAM-1-D6 Fab and IgG/hVCAM-1 and mVCAM-1 interactions

<table>
<thead>
<tr>
<th>Immobilized Molecule</th>
<th>Injected Molecule</th>
<th>K&lt;sub&gt;a&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;S&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (S&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<tr>
<td>hVCAM-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>Anti–VCAM-1 Fab</td>
<td>1.35 ± 0.02 × 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>1.31 ± 0.02 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.76 ± 0.03 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>mVCAM-1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>Anti–VCAM-1 Fab</td>
<td>4.78 ± 0.06 × 10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>5.04 ± 0.01 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.41 ± 0.12 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>hVCAM-1&lt;sup&gt;Δ6&lt;/sup&gt;</td>
<td>Anti–VCAM-1 IgG</td>
<td>3.06 ± 0.04 × 10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>1.03 ± 0.002 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.16 ± 0.006 × 10&lt;sup&gt;-10&lt;/sup&gt;</td>
</tr>
<tr>
<td>hVCAM-1&lt;sup&gt;Δ6&lt;/sup&gt;</td>
<td>Anti–VCAM-1 IgG</td>
<td>7.31 ± 0.42 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>1.18 ± 0.003 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>8.64 ± 0.84 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
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<sup>a</sup>hVCAM-1 and mVCAM-1 mean Fc fusion proteins.

K<sub>a</sub>: Dissociation equilibrium constant calculated based on K<sub>a</sub>/K<sub>d</sub>.
detached by trypsinization and counted using flow cytometry. The adhesion blocking Ab for domain 1 of VCAM-1, mouse anti–hVCAM-1 Ab 51-10C9, significantly inhibited U937 adhesion to hVCAM-1–overexpressing HEK 293 cells, HUVECs, or HAECs, whereas VCAM-1-D6 Fab and rhVCAM-1 domain 6-Fc did not affect U937 cell attachment to the VCAM-1–overexpressing cells (Fig. 4B–D). VCAM-1-D6 IgG does not activate endothelial cells

We used a fluorescence multiplex immunoassay to investigate whether VCAM-1-D6 IgG activates cells via VCAM-1 cross-linking. HUVECs were treated with 10 μg/ml VCAM-1-D6 IgG or the mouse anti–hVCAM-1 Ab 51-10C9 (as a positive control) (23, 52) for 24 h. In parallel experiments, HUVECs were pre-treated with 20 ng/ml hTNF-α for 24 h before incubation with the VCAM-1 Abs. The endothelial cell culture supernatant was examined for two chemokines (GM-CSF and IL-8), as well as nine cytokines (IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-γ, and TNF-α). The agonistic mouse anti–hVCAM-1 Ab 51-10C9 significantly enhanced TNF-α–stimulated endothelial cell IL-8 production (Fig. 5) but did not affect production of the other cytokines (data not shown). In contrast, the VCAM-1-D6 Ab did not enhance IL-8 production (Fig. 5), nor did it enhance any of the other cytokines or chemokines tested (data not shown).

VCAM-1-D6 IgG protects against grafted islet rejection by blocking leukocyte infiltration

To investigate the effect of the VCAM-1-D6 Ab on inflammation in vivo, we used an MHC-mismatched mouse islet allograft model. Diabetes was induced by STZ and the experimental animals received VCAM-1-D6 IgG, whereas control animals received an irrelevant IgG. Abs were injected into mice a total of nine times (0.1 mg/injection) before islet transplantation on day 7 (Fig. 6A). Blood glucose levels were monitored twice per week. Median graft survival in the control IgG group was 28.2 ± 8.6 d. In contrast, animals treated with the VCAM-1-D6 Ab showed no evidence of graft rejection at the time of their sacrifice, 110 d posttransplantation (Fig. 6B, 6C). In addition, the islet grafts from control and VCAM-1-D6 IgG-treated animals were histologically examined. The islet tissue from the control animals demonstrated clear evidence of immune rejection with the loss of insulin-positive cells (Fig. 7A), as well as significant mononuclear leukocytic infiltrate into the graft (Fig. 7B, 7C). In contrast, the islet grafts from the VCAM-1-D6 Ab-treated mice were intact with many insulin-positive β cells (Fig. 7D). Interestingly, the long-term functioning grafts (>110 d) from the VCAM-1-D6 IgG-treated mice showed persistent peri-islet mononuclear cellular infiltration of both
Discussion

In this study, we demonstrate that VCAM-1-D6 binds to the sixth Ig-like domain of VCAM-1, a domain that does not mediate leukocyte binding. However, VCAM-1-D6 binding to this VCAM-1 domain blocks leukocyte transendothelial migration. Furthermore, VCAM-1-D6 IgG does not alter TNF-α-stimulated endothelial cell chemokine or cytokine production. In addition, VCAM-1-D6 IgG inhibited the recruitment of leukocytes to transplanted islets and blocked rejection of islet transplants.

Leukocyte α4β1 integrin binding to domains 1 and 4 of VCAM-1 on the endothelium plays a critical role in recruiting leukocytes during the initiation and progression of inflammation. Abs that block this interaction have anti-inflammatory properties. Natalizumab, a humanized α4β1 integrin Ab that inhibits the interaction between the integrin and domains 1 and 4 of VCAM1, was approved for treatment of multiple sclerosis (39). In animal experiments, the rat anti-mVCAM-1 Abs MK1.9 and MK2.7 were shown to successfully inhibit the progression of inflammatory processes. MK1.9 blocks Ramos cell binding to murine endothelium lines (40) and also increased the graft survival rates of C57BL/6 mice that received tail skin grafts from B10.BR mice (53). In an experimental autoimmune encephalomyelitis model, MK1.9 reduced disease severity (54). Another VCAM-1 Ab, MK2.7, allowed long-term islet allograft survival of >100 d with a survival rate of 75% of the islet grafts (55). In a mouse model of Crohn’s disease, MK2.7 treatment yielded a 70% resolution of the acute inflammation (56). Furthermore, MK2.7 also reduced the development of arthritis in...
a collagen-induced arthritis model (57) and inhibited inflammatory cell infiltration into the skin in a keratin-14 IL-4 transgenic mouse atopic dermatitis model (58). The mouse anti–hVCAM-1 Abs 4B9, BBIG-V1, 1G11, and P3H12 block binding of lymphoma cell lines to VCAM-1–expressing endothelial cells (52, 59, 60). However, these Abs are species specific. A desirable property for a VCAM-1 Ab for therapeutic development is its reactivity to both hVCAM-1 and animal VCAM-1. The VCAM-1-D6 Ab described in this study has broad reactivity to hVCAM-1, mVCAM-1, and pig VCAM-1 (Fig. 1A and data not shown). This broad species reactivity allows evaluation of this Ab in various animal disease models, as well as in human disease interventions.

The epitopes of VCAM-1 neutralizing Abs have been reported to be either domain 1 or 4, the domains involved in α4β1 integrin binding (23, 24). The MK2.7 Ab is reactive to VCAM-1 domains 1 and 4 (61). The epitope for the mouse anti–hVCAM-1 Abs, 51-10C9 and 4B9, is localized to domain 1 (23, 24).

In this study, we showed that an Ab reactive to VCAM-1 domain 6 inhibited the transmigration. Because rhVCAM-1 domain 6 (aa 51–599) Fc fusion also inhibited the transmigration of U937 cells and human monocytes across HUVECs, it is more likely that the domain 6 directly interacts with another VCAM-1 molecule or with tetraspanins CD9, CD81, CD 151 in tetraspanin-enriched microdomains to initiate downstream signaling (62, 63). This may regulate transendothelial migration because VCAM-1 cross-linking activates downstream signaling molecules in endothelial cells that are required for leukocyte transendothelial migration (64). For example, treatment of human and mouse endothelial cells with anti–mVCAM-1–coated beads (clone MV-CAM.A) increased the intracellular level of reactive oxygen species, tyrosine phosphatase activity, and protein kinase Cα activity, all of which are required for leukocyte migration (49). VCAM-1 cross-linking with the VCAM-1 Ab (1G11) induced the activation of Rac1, a small GTP binding protein, reactive oxygen species production, and p38 MAPK activation (65). VCAM-1 activation by beads coated with the mouse anti–hVCAM-1 Ab 51-10C9 stimulates NADPH oxidase, metalloproteinase-2, and metalloproteinase-9 activity (42). In this study, the agonistic mouse anti–hVCAM-1 Ab 51-10C9 enhanced TNF-α–stimulated endothelial cell production of IL-8. However, an anti–VCAM-1 domain 6 Ab did not alter TNF-α–stimulated production of chemokines or cytokines. Nevertheless, the VCAM-1-D6 Ab has the unique function of blocking leukocyte transendothelial migration without affecting leukocyte adhesion. In vivo intravital microscopy studies demonstrate bound leukocytes that do not migrate are often released and continue with the blood flow (41).

**FIGURE 6.** The VCAM-1-D6 Ab maintains long-term graft survival in allogeneic islet transplantation assay. (A) Schematic depiction of Ab treatment protocol. (B) Islets harvested from BALB/c mice were transplanted into the subcapsular space of kidney. Blood glucose levels were monitored twice per week from control IgG (○) or VCAM-1-D6 IgG (□)-treated groups. (C) Treatment with the VCAM-1-D6 Ab induces prolonged graft survival in C57BL/6 recipients (mean survival day > 110 d, n = 5). Control IgG treatment group was hyperglycemic after a brief normoglycemia period (mean survival day = 28.2 d, n = 5). Graft survival is presented as Kaplan–Meier survival curves.

**FIGURE 7.** The VCAM-1-D6 Ab significantly promotes islet cell survival by blocking leukocyte migration inside the grafted islets. The kidneys of control animals were isolated at the time of rejection and kidneys from VCAM-1-D6 Ab-treated mice were isolated 110 d after transplantation. Histological comparison of islet grafts from the two groups was performed using immunohistochemical staining. (A and D) Islet cell staining using an insulin Ab. (B and E) CD4 T cell staining using a CD4 Ab. (C and F) Macrophage staining using the F4/80 Ab. Images are representative of three independent experiments. Arrows indicate the stained cells. Original magnification ×100 (A–D), ×200 (E, F).
In this study, the in vivo efficacy of the VCAM-1-D6 Ab was demonstrated in a mouse islet allotransplantation model. VCAM-1 plays a critical role in recruitment of leukocytes to allografts (66–68). VCAM-1 is overexpressed in grafted islets through inflammatory cytokines such as IFN-γ, TNF-α, and IL-1 (14, 68). The mVCAM-1–specific Ab MK2.7 blocks adhesion to domain 1 and protects against mouse islet allograft rejection by blocking leukocyte adhesion to endothelium. In contrast, we found that VCAM-1-D6 IgG did not block adhesion but did block leukocyte transendothelial migration. In addition, it successfully inhibited islet graft transplant rejection for at least 110 d after transplantation. Immunohistochemical studies also indicated an inhibition of CD4+ T cell and macrophage infiltration toward the grafted islets. This infiltration of CD4+ T cells and macrophages in the peri-islet space without invasion into the graft site was previously observed when immunological tolerance was achieved (69). Thus, we have identified a unique function for VCAM-1-D6 Abs in blocking islet graft transplant rejection and blocking leukocyte recruitment.

The unique property of the VCAM-1-D6 Ab to not block leukocyte adhesion may provide some advantages. It is reported that in lymphoid tissues, the interaction of lymphocyte VLA-4 with vascular cell adhesion molecule-1 (VCAM-1) in activated vascular endothelium. Am. J. Pathol. 138: 815–820.


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