Characterization of Murine Intercellular Adhesion Molecule-2[1]

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Rat mAbs were raised against murine intercellular adhesion molecule-2 (ICAM-2). Immune precipitation and purification reveal that the murine ICAM-2 glycoprotein is 55 kDa and is similar in size to human ICAM-2. ICAM-2 is expressed on a variety of leukocyte cell lines, including T and B lymphoma, mastocytoma, and macrophage lines. ICAM-2 is well expressed on endothelioma cell lines, and in contrast to ICAM-1, expression is not increased by inflammatory cytokines. One of the mAb to ICAM-2 partially or completely inhibits binding of cells expressing LFA-1 to purified ICAM-2, and binding of cells expressing ICAM-2 to purified LFA-1. The findings in the mouse are congruent with those in the human, suggesting functional conservation of ICAM-2 across species. *The Journal of Immunology,* **1996, 156: 4909–4914.**

Intercellular adhesion molecule-2 (ICAM-2, CD102)[4] is one of three ligands for the integrin LFA-1 (CD11a/CD18) (1–6). As a member of the Ig superfamily, ICAM-2 has two Ig-like domains with 34% amino acid identity to the first two N-terminal Ig-like domains of ICAM-1 (7). In humans, it has been shown that ICAM-2 expression is restricted to vascular endothelium and to leukocyte subpopulations, including lymphocytes and monocytes (2, 7, 8). The expression of ICAM-2 on endothelial cells is constitutive and is higher than the basal expression of ICAM-1. In contrast to ICAM-2, ICAM-1 has a much wider tissue distribution, and its expression is strongly induced by inflammatory mediators such as LPS and the cytokines TNF-α, IL-1β, and IFN-γ (1, 2, 9–11). The third ligand for LFA-1, ICAM-3 (CD50), is constitutively expressed on leukocytes only (4, 5). Many LFA-1-mediated functions have been attributed to LFA-1/ICAM interaction, such as T cell-mediated killing, Ag presentation, T helper and B lymphocyte responses, and infiltration of lymphocytes, monocytes, and granulocytes during inflammation (1, 12). A distinct function for ICAM-2 has yet to be defined. Based on the constitutive expression of ICAM-2 that is restricted to endothelium and leukocytes, it has been postulated that ICAM-2 may play an important role in lymphocyte recirculation (2).

Many cell adhesion molecules involved in leukocyte function are conserved across species. Some, such as CD2, differ in tissue distribution and ligand recognition between mouse and human (13, 14). By contrast, LFA-1 and ICAM-1 are well conserved across species in tissue distribution and function (1, 12, 15–17). ICAM-2 has been described in the mouse by molecular cloning, but little is known about it at the protein or functional level (18). Here, we report rat mAbs to murine ICAM-2 and characterize the ICAM-2 glycoprotein and its surface expression and function.

Materials and Methods

Cell lines

Mouse B lymphoma cell line A20 (19) and CH-1 (20) were cultured in DMEM supplemented with 10% FCS and 0.1 mM β-ME. The YAC-1 (21) cell line, the T lymphoma lines BW5147 (22) and EL-4 (23), and the macrophage cell line P388D1 (24) were maintained in complete RPMI 1640 medium containing 10% FCS. The mouse neuroblastoma line Neuro-2a was obtained from Dr. Emmanuel Katsanis (University of Minnesota, Minneapolis, MN). The two endothelioma cell lines, sEnd.1 and eEnd.2, were kind gifts from Dr. Dietmar Vestweber (Max Plank Institute for Immunology, Freiburg, Germany) (25, 26). Neuro-2a and endothelioma cell lines and the mastocytoma cell line P815 were cultured in DMEM/10% FCS.

COS cell transfection

Mouse and human ICAM-2 cDNAs were cloned into mammalian expression vectors Ap^{r}M8 (18) and pCDM8 (7), respectively, and transfected into COS cells using DEAE-dextran (27). COS cells were also transfected with vector alone (mock). Transfectants were detached with 10 mM HBSS/EDTA 3 days after transfection and were washed three times with RPMI 1640/5% FCS before use.

Rat monoclonal antibody to mouse ICAM-2

Transfected COS cells expressing murine ICAM-2 ($5–10 \times 10^6$ cells) were used to immunize i.p. 2- to 4-mo-old female Lewis rats (Charles River Laboratories, Wilmington, MA). Secondary immunizations were performed using 2×10^7 BW5147 thymoma cells that express high levels of ICAM-2. Three days before the fusion, the rats were injected i.v. with 5×10^6 BW5147 cells. Fusion of rat spleen cells with murine myeloma P3X63Ag8.653 was conducted as previously described (28). ICAM-2-specific hybridomas were cloned three times by limiting dilution. Four rat mAbs to murine ICAM-2, mIC2/1.2.3 or 1B12 (IgG2b), mIC2/2.2.3 or 1F3 (IgG2a), mIC2/3.2.3 or 3B1 (IgG2b), and mIC2/4.2.3 or 3C4 (IgG2a) were isotyped by ELISA using affinity-purified Abs to rat Igs (Zymed Immunochemicals, San Francisco, CA). Rat mAbs were used as supernatants or after purification by protein G fast flow Sepharose chromatography (Pharmacia, Piscataway, NJ). The mAb mIC2/4 (3C4) is available from PharMingen (San Diego, CA).

Other antibodies and reagents

The following mAbs were used: TS1/22 (anti-human LFA-1 α-subunit, IgG1) (29), M17/4 (anti-mouse LFA-1, IgG2a) (30, 31), CBR-IC2/2 (anti-human ICAM-2, IgG2a) (2), YN1/1.7 (anti-mouse ICAM-1, IgG2a) (32), and 584 (anti-mouse VCAM-1, IgG2a) (33). Rat serum IgG was obtained from Zymed Immunochemicals. X63 is a nonbinding myeloma protein

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[4] Abbreviations used in this paper: ICAM-2, intercellular adhesion molecule-2; TSM, 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2 mM $MgCl_2$.

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(IgG1). Purified mouse TNF-α was obtained from Genzyme (Boston, MA). Human LFA-1 was purified from JY lysates (2).

Flow cytometry

Detached COS cell transfectants or other cell lines (5×10^5) were incubated with primary Abs at 4°C for 30 min, washed three times with PBS/0.5% BSA, and then incubated with 100 μl of a 1/20 dilution of FITC-labeled goat anti-mouse Ig (Zymed Immunochemicals) or mouse anti-rat κ-chain from AMAC (Westbrook, ME). After 30-min incubation, the cells were washed three times with PBS/0.5% BSA and fixed in 1% paraformaldehyde/PBS. Samples were analyzed using an Epics V (Coulter Diagnostics, Hialeah, FL).

Immunoprecipitation

Cell surface labeling with ^{125}I was performed as previously described (2) using Iodogen (Pierce Chemical Co., Rockford, IL). Cells were lysed for 45 min at 4°C in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton-X-100, 1 mM iodoacetamide, 1 mM PMSF, 0.24 trypsin inhibitory units/ml aprotinin, and 0.025% azide. After centrifugation, the cell lysates were precleared with Pansorbin (Calbiochem, La Jolla, CA) or IgG-coupled Sepharose overnight at 4°C. The lysates were then precipitated with mAb mIC2/4 or CBR-IC2/2 conjugated to Sepharose 4B (10 mg/ml). Rat IgG-Sepharose 4B was used as a control.

Purification of murine ICAM-2

Murine ICAM-2 was purified from BW5147 cells in a fashion similar to that described for ICAM-1 purification (16). Briefly, the BW5147 plasma membrane fraction was solubilized with 1% Triton X-100, 10 mM Tris (pH 7.5), 1 mM EDTA, 145 mM NaCl containing 2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin and combined with lysate from cell surface biotinylated (34) BW5147 cells. ICAM-2 was purified by immunoaffinity chromatography using the mIC2/4 Ab coupled to Affi-Gel 10 beads (Bio-Rad, Richmond, CA; 2 mg Ab/ml beads). These beads were incubated with the solubilized membranes for 8 h with constant agitation at 4°C and washed with 400 ml of 1% Triton X-100, 10 mM Tris (pH 7.5), 1 mM EDTA, and 145 mM NaCl, followed by 100 ml of the same buffer containing only 0.1% Triton X-100 at 4°C. Bound protein was eluted with 100 mM glycine-HCl (pH 2.9), Triton X-100, and 145 mM NaCl, and the pH was neutralized. Aliquots from each collected fraction were subjected to 10% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The filter was then probed with streptavidin-horseradish peroxidase as previously described (34). Fractions containing a 50- to 55-kDa protein were pooled and concentrated. Purity and yield were assessed by SDS-PAGE and silver staining (35).

Adhesion to LFA-1

Transfected COS cells and lymphoma cell lines were harvested and washed with RPMI 1640/5% FCS and labeled with 15 μg/ml of 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (Molecular Probes, Eugene, OR) for 30 min at 37°C with gentle rocking. Cells were washed three times with RPMI 1640/5% FCS, counted, and resuspended in assay medium (RPMI 1640 containing 10% FCS, 10 mM HEPES, and 2 mM $MgCl_2$). Adhesion of 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein-labeled COS transfectants or lymphoma cell lines to purified LFA-1 was performed as previously described (18, 36). Purified LFA-1 in 1% octyl β-D-glucopyranoside was diluted 1/10 or 1/20 in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 2 mM $MgCl_2$ (TSM), and 50 μl was absorbed onto 96-well polystyrene microtiter plates (Linbro-Titertek, Flow Laboratories, McLean, VA) overnight at 4°C. Nonspecific binding sites were blocked by TSM containing 1% BSA for 1 h at room temperature before the assay. COS transfectants (5×10^4) and lymphoma cells (2×10^5) were incubated with LFA-1 coated on plastic for 60 min at 37°C. For inhibition, LFA-1-coated plates were preincubated with TS1/22 (anti-LFA-1), or labeled cells were preincubated with mAbs to ICAM-1 or ICAM-2, at 4°C for 30 min. Nonadherent cells were removed by multiple washes with aspiration through a 26-gauge needle. The fluorescence of the total input cells and the bound cells was determined by a Pandex fluorescence concentration analyzer (Baxter Healthcare Corp., Mundelein, IL).

Isolation and activation of splenic T cells

Splenic T cells from BALB/c mice were isolated using nylon wool (37), and incubated in RPMI 1640 + 10% FCS containing 50 ng/ml PMA for 20 min at 37°C/5% CO_2. The activated T cells were washed, labeled with Calcein AM (Molecular Probes, Eugene Or) according to the manufacturer's protocol, and used for adhesion assays.

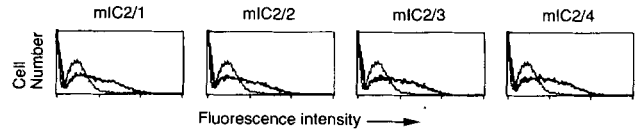

FIGURE 1. Immunofluorescence flow cytometry with ICAM-2 mAbs. COS cells transfected with murine ICAM-2 cDNA were labeled with rat mAb to ICAM-2 (thick line) or nonbinding control mAb X63 (thin line).

Adhesion to ICAM-2

Purified murine ICAM-2 was covalently coupled to microwells as previously described (16). Purified recombinant soluble murine ICAM-1 (38) was also coupled to microwells and used as a control. Splenic T cells were PMA activated, labeled with calcein AM, and added (10^5 cells/well) to wells in a final volume of 100 μl of HBSS and 5% FCS. Blocking Abs (2 μg/ml unless otherwise stated) were added 15 min before the addition of cells to the wells. Upon addition of the cells, the plates were centrifuged at $10 \times g$ (300 RPM) for 1 min, and incubated for 8 min at 37°C. Unbound cells were removed by washing the wells five times with prewarmed HBSS and 5% FCS, and fluorescence was measured by a CytoFluor 2300 (Millipore).

Cell:cell adhesion

L cells were transfected (39) with murine ICAM-1 (16) or ICAM-2 cDNA (40) and grown as monolayers in microwells. Adhesion of PMA-activated splenic T cells to the monolayers was determined in the same way as described for adhesion to purified ICAM-2.

Results

Monoclonal antibodies to murine ICAM-2

Lewis rats were immunized with COS cells transfected with murine ICAM-2 cDNA and boosted with murine BW5147 lymphoma cells previously shown to express large amounts of ICAM-2 mRNA (18). Supernatants from hybridomas were screened for staining of transfected COS cells. Four rat mAb, designated mIC2/1 (B12), mIC2/2 (1F3), mIC2/3 (3B1), and mIC2/4 (3C4), were produced that bound specifically to COS cells transfected with murine ICAM-2 (Fig. 1), but not mock-transfected COS cells (data not shown).

Isolation of ICAM-2

A broad band of 46 to 65 kDa was immunoprecipitated with mAb mIC2/4 from ^{125}I-labeled cell lysates of the BW5147 T lymphoma cell line and the P815 mastocytoma cell line (Fig. 2*A*). This band comigrated with human ICAM-2 immunoprecipitated from transfected COS cells.

ICAM-2 purified by mAb affinity chromatography from BW5147 was a homogeneous protein, as shown by silver staining (Fig. 2*B*). Approximately 5 μg was obtained per liter of cultured BW5147 cells.

Expression of ICAM-2 on cell lines

Expression of ICAM-2 on cell lines was examined by immunofluorescence flow cytometry (Fig. 3). All lymphoma cell lines examined expressed amounts of ICAM-2 greater than or equal to those of ICAM-1. The macrophage cell line P388D1 and the mastocytoma cell line P815 expressed less ICAM-2 than ICAM-1. Weak expression of ICAM-2, but not ICAM-1, was detected on the neuroblastoma cell line Neuro-2a. The fibroblast cell line NIH-3T3 was negative for ICAM-2. The relative levels of expression of ICAM-2 on various cell lines detected by flow cytometry corresponded to those detected by Northern blotting (18).

High levels of ICAM-2 were expressed on the mouse endothelioma cell lines, sEnd.1 and eEnd.2 (Fig. 4). ICAM-2 expression was unaffected by stimulation with TNF-α. ICAM-1

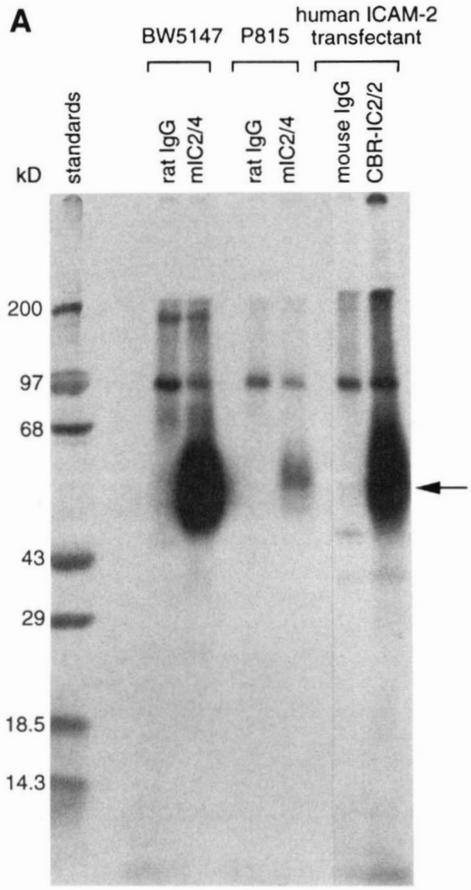

FIGURE 2. Immunopurification of ICAM-2. *A*, ^{125}I surface-labeled BW5147 and P815 cells were immunoprecipitated with either control rat IgG or rat anti-mouse ICAM-2 (mIC2/4), and surface-labeled human ICAM-2-transfected COS cells were immunoprecipitated with control mouse IgG or mouse anti-human ICAM-2 mAb (CBR-IC2/2) and subjected to SDS-PAGE and autoradiography. *B*, Murine ICAM-2 immunoaffinity purified from BW5147 cells was subjected to nonreducing SDS-10% PAGE and silver staining.

was expressed basally at lower levels than ICAM-2 and was increased by stimulation with TNF-α. VCAM-1 was induced by TNF-α on eEnd.2, but not sEnd.1, cells.

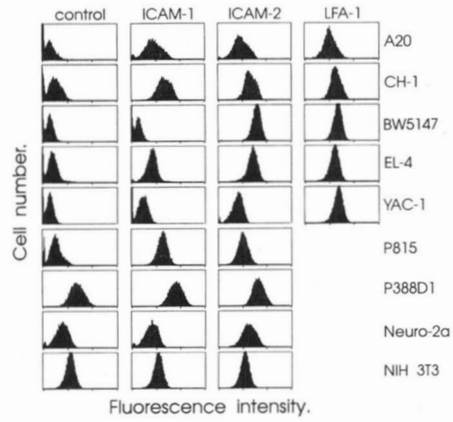

FIGURE 3. Expression of ICAM-1 and ICAM-2 on murine cell lines. Cells were stained with either nonbinding control Ab X63 or mAb supernatants YN1/1 (anti-ICAM-1), mIC2/1 (anti-ICAM-2), or M17/4.4 (anti-LFA-1) and subjected to immunofluorescence flow cytometry.

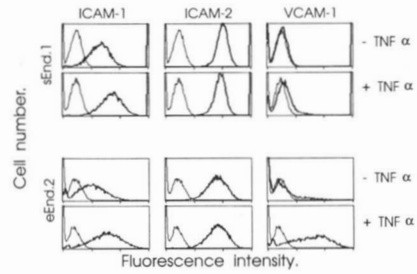

FIGURE 4. Expression of ICAM-1, ICAM-2, and VCAM-1 on murine endothelioma cells. The sEnd.1 and eEnd.2 endotheliomas were stimulated with or without TNF-α (400 U/ml) for 16 h; labeled with nonbinding Ab X63 (control), YN1/1 (anti-ICAM-1), mIC2/4 (anti-ICAM-2), and 548 (anti-VCAM-1); and subjected to immunofluorescence flow cytometry.

Monoclonal antibody mIC2/4 blocks binding of mouse ICAM-2 to human LFA-1

Binding of transfected COS cells or lymphoma cells to purified human LFA-1 was tested for inhibition by mAbs. COS cell transfectants expressing murine ICAM-2 were able to bind to human LFA-1 on plastic, and binding was completely abolished in the presence of mAb against human LFA-1 (Fig. 5*A*). Among the four rat anti-mouse ICAM-2 Abs, mIC2/4 completely inhibited binding of ICAM-2-transfected COS cells, whereas the other three Abs partially inhibited binding.

Two lymphoma cell lines that differed in the levels of expression of ICAM-1 and ICAM-2 were examined for binding to purified human LFA-1. BW5147 cells that express ICAM-2 and not ICAM-1 (Fig. 3) efficiently bound to LFA-1 (Fig. 5*B*). Binding was unaffected by mAb to ICAM-1 and was partially or almost completely blocked by different mAb to ICAM-2. YAC-1 cells that express ICAM-2 and lesser amounts of ICAM-1 bound well to purified LFA-1 (Fig. 5*B*). Binding was little affected by mAb to ICAM-1, partially blocked by mAb to ICAM-2, and almost completely blocked by combination of mAb to ICAM-1 and ICAM-2. Among the four mAb to ICAM-2, mIC2/4 gave the strongest inhibition of binding to LFA-1 for all three cell types assayed.

Purified murine ICAM-2 binds to cell surface murine LFA-1

PMA-activated murine splenic T cells adhered to purified murine ICAM-2 on plastic (Fig. 6). They also adhered to purified murine ICAM-1. Approximately 54 and 36% of cells bound

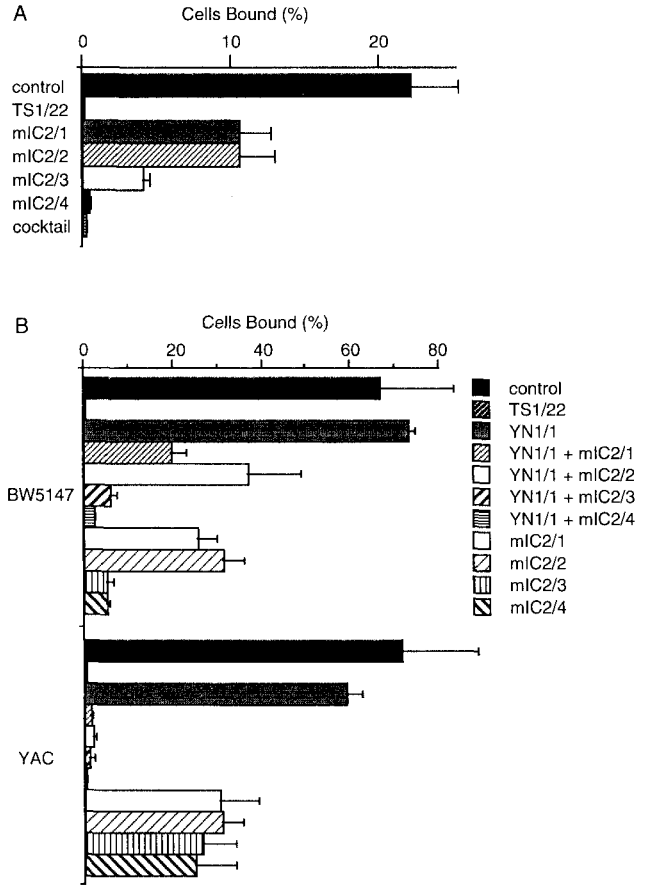

FIGURE 5. Inhibition of binding of cells to purified human LFA-1. *A*, COS cells transfected with murine ICAM-2 cDNA were assayed for binding to purified human LFA-1 adsorbed to microtiter wells in the presence of mAb to human LFA-1 (TS1/22), murine ICAM-2, a cocktail of all four ICAM-2 mAb, or control myeloma (X63). *B*, Binding of murine lymphoma cells BW5147 and YAC-1 to purified human LFA-1. Lymphoma cells were assayed for binding to purified LFA-1 adsorbed to microtiter wells in the presence of 50 ng/ml PMA and the indicated mAb as described in *A*. One representative experiment of three is shown, and error bars indicate 1 SD.

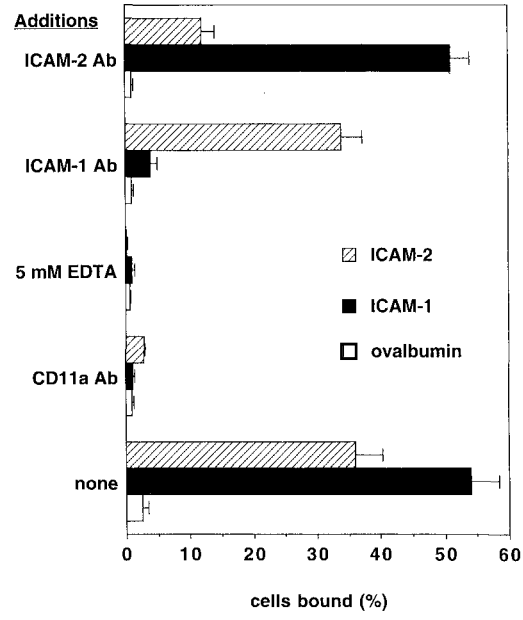

FIGURE 6. Adhesion of murine splenic T cells to purified ICAM-1 and ICAM-2. ICAM-2, ICAM-1, and OVA were immobilized on microculture wells. To each well, 10^5 PMA-activated splenic T cells labeled with calcein AM were added. Blocking Abs were present at 2 μg/ml, except for ICAM-2 Ab, which was present at 4 μg/ml. Unbound cells were removed by washing five times with HBSS and 5% FCS. Results are expressed as a mean of triplicate wells ± SEM.

ICAM-1 and ICAM-2, respectively. Cell adhesion to ICAM-2 was inhibited by mAb to ICAM-2, but not by mAb to ICAM-1, whereas cell adhesion to ICAM-1 was inhibited by mAb to ICAM-1, but not by mAb to ICAM-2. The binding to ICAM-2 was further decreased to 7% (80% inhibition) when the ICAM-2 Ab concentration was increased from 4 to 15 μg/ml (data not shown). Anti-LFA-1 (CD11a) mAb inhibited cell adhesion to both proteins.

ICAM-2 antibody blocks cell:cell contact

L cells transfected with murine ICAM-1 or ICAM-2 cDNA expressed these molecules on the surface (Fig. 7*A*). PMA-activated splenic T cells readily adhered to monolayers of the transfected L cells expressing ICAM-1 or ICAM-2 (Fig. 7*B*). Although the level of ICAM-2 on the transfected L cells was higher than that of ICAM-1, T cells adhered to ICAM-1 transfectants (85% of input cells) more efficiently than to ICAM-2 transfectants (60%). The adhesion of T cells to the transfected L cells was effectively inhibited by mAb to LFA-1. Anti-ICAM-2 mAb also inhibited the adhesion of T cells to ICAM-2-transfected L cells, whereas the adhesion to ICAM-1-transfected L cells was effectively inhibited by mAb to ICAM-1.

Discussion

We have developed mAb to murine ICAM-2 and used them to characterize the structure, function, and distribution of ICAM-2. Murine ICAM-2 migrates as a broad band of 46 to 65 kDa in SDS-PAGE, suggesting glycosylation heterogeneity. Human ICAM-2 exhibits a similar molecular mass and degree of size heterogeneity (2, 8). The deduced amino acid sequence of murine ICAM-2 predicts a polypeptide backbone of 28 kDa and five potential N-linked glycosylation sites (18). The average size of 55 kDa for ICAM-2 in SDS-PAGE suggests that approximately half of the molecular mass is carbohydrate.

ICAM-2 is well expressed on murine lymphoid cells, strongly expressed on endothelial cells, weakly expressed on myeloid cells, and absent on fibroblasts. ICAM-1 expression, but not ICAM-2 expression, was increased by stimulation of endothelial cells with TNF. These findings are in agreement with those for mRNA expression (18) and show that ICAM-2 has a similar tissue distribution in the mouse and the human (2).

The effects of the Abs against murine ICAM-2 on adhesive interactions with LFA-1 were examined in several different types of adhesion assays. ICAM-2-transfected COS cells bound to purified human LFA-1, and this interaction was inhibited by mAb against murine ICAM-2. Abs to ICAM-2 completely inhibited BW5147 cell adhesion to purified LFA-1. Abs to both ICAM-1 and ICAM-2 were required to inhibit adhesion of YAC lymphoma cells to LFA-1, showing that both ICAM-1 and ICAM-2 contributed. No evidence for a third LFA-1 ligand equivalent to ICAM-3 was obtained in the mouse.

Converse experiments examined binding of cells to purified murine ICAM-2. PMA-activated splenic T cells bound to purified ICAM-2, and the binding was inhibited by mAbs to LFA-1 or ICAM-2. Furthermore, splenic T cells adhered to ICAM-2$^+$ L cells, and this was also inhibited by mAbs to LFA-1 or ICAM-2.

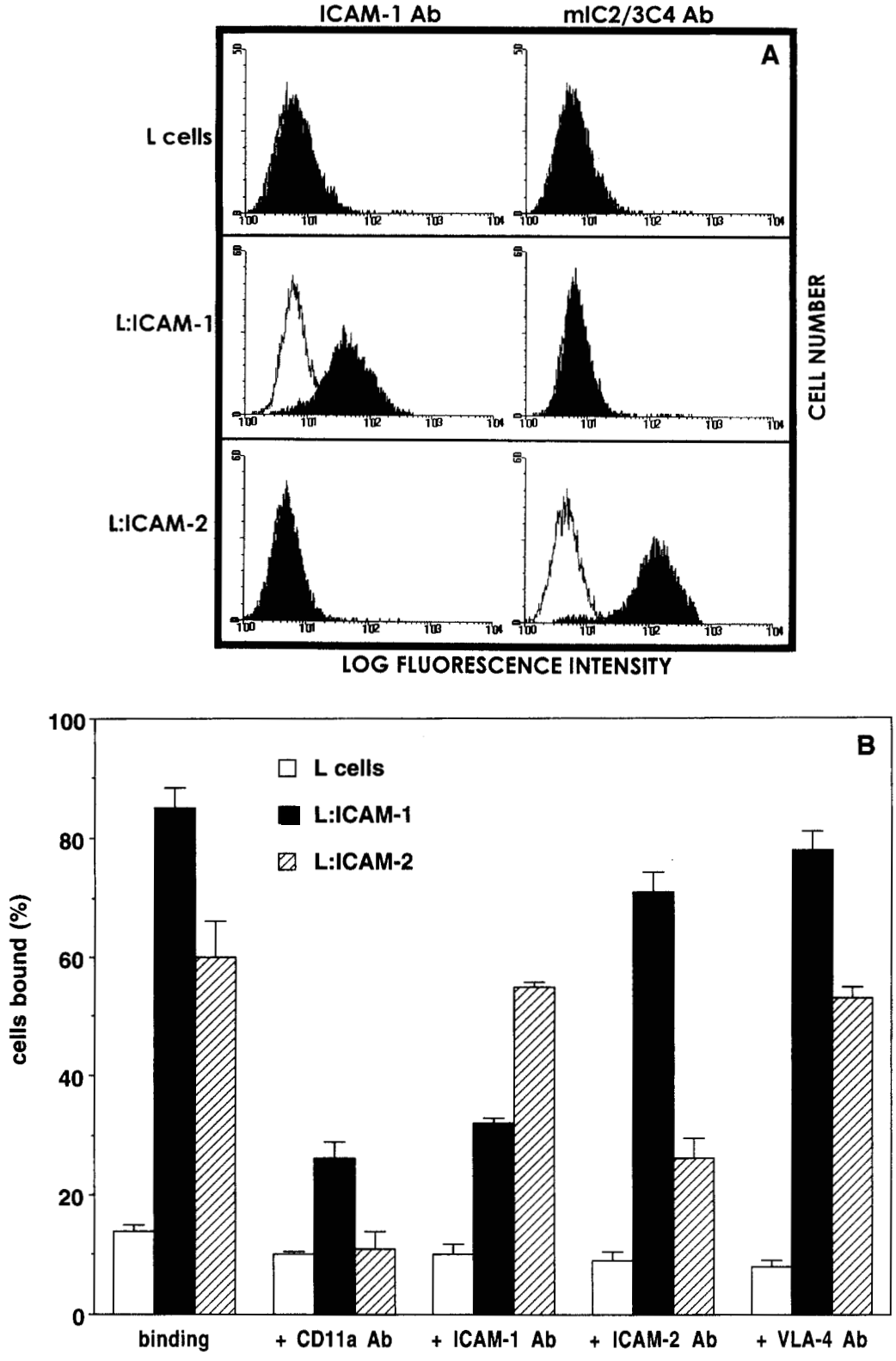

FIGURE 7. Adhesion of splenic T cells to L cells transfected with ICAM-2. *A*, L cells, untransfected or transfected with ICAM-1 (L:ICAM-1) or ICAM-2 (L:ICAM-2) cDNA were subjected to flow cytometry using the appropriate Abs and F $(ab')_2$ goat anti-rat IgG conjugated to FITC. ICAM-1 and ICAM-2 expressions are shown by the dark histogram, and secondary Ab alone is shown by the outlined histogram. *B*, L cells expressing ICAM-1 or ICAM-2 cDNA were grown in 96-well plates. PMA-activated splenic T cells labeled with calcein AM were added to each well containing a subconfluent monolayer of L cells, and binding was measured as described in Figure 6.

Binding of T cells to ICAM-2-transfected L cells was consistently lower than to ICAM-1-transfected L cells, although the level of ICAM-2 on the transfected cells was higher than that of ICAM-1. These results suggest that cell adhesion mediated by ICAM-2 and LFA-1 is not as efficient as that mediated by ICAM-1 and LFA-1. Nevertheless, leukocytes expressing activated LFA-1 are able to

adhere to ICAM-2$^+$ cells in the absence of ICAM-1 expression. Some cells, including unstimulated endothelial cells, express relatively high levels of ICAM-2 but low levels of ICAM-1. Leukocytes expressing activated LFA-1 may adhere to these cells by binding to ICAM-2.

Although the binding of ICAM-2 to LFA-1 has been well established in vitro, the functional significance of this cell adhesion pathway in vivo is still unknown. It is of interest that inflammatory responses of ICAM-1-deficient mice are only partially defective (41, 42). Whether ICAM-2 is compensating for the ICAM-1 deficiency is unknown. The mAb to murine ICAM-2 reported here can inhibit LFA-1:ICAM-2 binding and may be a useful tool to elucidate the functional role of ICAM-2 in immune and inflammatory responses in vivo. Of further interest is whether ICAM-3 exists in the mouse. We obtained no functional evidence for ICAM-3. Cross-hybridization with human ICAM-3 cDNA has also failed to yield genetic evidence for murine ICAM-3 (L. Klickstein and T. Springer, unpublished observations).

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