

# Characterization of the Function of Intercellular Adhesion Molecule (ICAM)-3 and Comparison with ICAM-1 and ICAM-2 in Immune Responses

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## Summary

We have characterized the immunobiology of the interaction of intercellular adhesion molecule 3 (ICAM-3; CD50) with its counter-receptor, leukocyte function-associated antigen 1 (LFA-1; CD11a/CD18). Purified ICAM-3 supported LFA-1-dependent adhesion in a temperature- and cation-dependent manner. Activation of cells bearing LFA-1 increased adhesiveness for ICAM-3 in parallel to adhesiveness for ICAM-1. Although CBR-IC3/1 monoclonal antibody (mAb) blocked adhesion of cells to purified LFA-1, when tested alone, neither CBR-IC3/1 nor five novel ICAM-3 mAbs characterized here blocked adhesion of cells to purified ICAM-3 or homotypic adhesion. Two ICAM-3 mAbs, CBR-IC3/1 and CBR-IC3/2, were required to block LFA-1-dependent adhesion to purified ICAM-3- or LFA-1-dependent, ICAM-1-, ICAM-2-independent homotypic adhesion of lymphoid cell lines. Two ICAM-3 mAbs, CBR-IC3/1 and CBR-IC3/6, induced LFA-1-independent aggregation that was temperature and divalent cation dependent and was completely inhibited by ICAM-3 mAb, CBR-IC3/2, recognizing a distinct epitope. Purified ICAM-3 provided a costimulatory signal for proliferation of resting T lymphocytes. mAb to ICAM-3, together with mAbs to ICAM-1 and ICAM-2, inhibited peripheral blood lymphocyte proliferation in response to phytohemagglutinin, allogeneic stimulator cells, and specific antigen. Inhibition was almost complete and to the same level as with mAb to LFA-1, suggesting the most functionally important, and possibly all, of the ligands for LFA-1 have been defined.

Leukocyte cell surface adhesion molecules play an essential role in inflammatory and immune responses (1, 2). These adhesive interactions are mediated in part by leukocyte function-associated antigen-1 (LFA-1)<sup>1</sup> and its ligands, intercellular adhesion molecules (ICAM)-1, ICAM-2, and ICAM-3 (2-5). LFA-1 has been shown to be involved in a wide variety of cell-cell interactions, including T cell-mediated killing, T helper and B lymphocyte responses, natural killing, Ab-dependent cellular cytotoxicity by monocytes and granulocytes, and adherence of leukocytes to endothelial cells, fibroblasts, and epithelial cells (6-8).

The LFA-1 counter-receptors, ICAM-1, ICAM-2, and ICAM-3, have been identified and shown to be type I membrane glycoproteins that are members of the immunoglobulin superfamily (3-5, 9-14). ICAM-1 contains five Ig-like domains (15, 16), whereas ICAM-2 contains only two Ig-like domains (3). The two Ig-like domains of ICAM-2 are 36% identical at the amino acid level to the first two Ig-like

domains of ICAM-1, to which the LFA-1 binding site has been mapped (17, 18).

The third LFA-1 counter-receptor, ICAM-3, was identified by a mAb that inhibited binding of lymphoblastoid cells to purified LFA-1 (5). ICAM-3 is a heavily glycosylated glycoprotein of 124,000 *M<sub>r</sub>* that is well expressed on leukocytes, and absent on endothelium. Cloning of ICAM-3 revealed that it contains five Ig-like domains that are highly homologous to the corresponding domains in ICAM-1 and ICAM-2 (12-14). ICAM-3 shares 52% amino acid identity with ICAM-1, while the first two NH<sub>2</sub>-terminal Ig-like domains of ICAM-3 have 37% amino acid identity to the two Ig-like domains of ICAM-2. Last, ICAM-3 and CD50, a molecule defined by its cell distribution in the Fourth International Leukocyte Workshop, were found to be identical (19).

Despite the homologies between the ICAMs, their patterns of expression suggest specialized roles (5). The finding that adhesion of resting T lymphocytes to purified LFA-1 occurs primarily via ICAM-3 (5), combined with the fact that ICAM-3 is much better expressed than other LFA-1 ligands on monocytes and resting lymphocytes, suggested that ICAM-3 might be important in the initiation of immune responses. ICAM-3 could potentially be important in lymph-

<sup>1</sup> Abbreviations used in this paper: HbsAg, hepatitis B surface antigen; ICAM, intercellular adhesion molecule; LFA-1, leukocyte function-associated antigen 1.

phocyte activation, as well as in adhesion. T cell activation through TCR cross-linking is insufficient by itself, and requires "accessory" signals that are provided by the conjugation of numerous adhesion molecules (20–23). Ligand engagement of LFA-1, through ICAM-1 (24, 25) or ICAM-2 (26), has been shown to act as a costimulus with CD3 mAb cross-linking of the TCR, resulting in cell proliferation and IL-2 secretion.

Here we characterize the immunobiology of the ICAM-3/LFA-1 interaction. Although the previously described ICAM-3 mAb was effective at inhibiting ICAM-3 adhesion to purified LFA-1 (5), it was unable, in combination with ICAM-1 and ICAM-2 mAbs, to inhibit PMA-induced SKW3 aggregation. Further mAbs to ICAM-3 were obtained that completely inhibit homotypic aggregation, and ICAM-3 was purified for use in functional studies. Purified ICAM-3 was found to support LFA-1-dependent adhesion, and also to be capable of providing a costimulatory signal to resting T lymphocytes. ICAM-3 was shown to be important in PBL proliferation in response to PHA, and in allogeneic and antigen specific proliferation, and comparison of inhibition by ICAM-1, -2, and -3 mAbs with LFA-1 mAb does not provide any evidence for a further ICAM.

## Materials and Methods

**Monoclonal Antibodies.** The following previously described murine mAbs to human antigens were used: W6/32 (anti-HLA, A, B, C, IgG2a) (27), G10-2 (anti-CD43, IgG1) (28), G19-1 (anti-CD43, IgG1) (28), TS2/9 (anti-LFA-3, IgG1) (29), TS2/18 (anti-CD2, IgG1) (29), TS1/18 (anti-CD18, IgG1) (29), TS1/22 (anti-CD11a, IgG1) (29), RR1/1 (anti-ICAM-1, IgG1) (9), CBR-IC2/2 (anti-ICAM-2, IgG2a) (4), CBR-IC3/1 (anti-ICAM-3, IgG1) (5), and X63 (nonbinding antibody, IgG1).

Generation of additional mAbs to ICAM-3 was based on a previously published protocol (30). In brief, 3–12-wk-old BALB/c female mice (Charles River Laboratories, Wilmington, MA) were immunized with purified ICAM-3 in adjuvant (2  $\mu$ g/immunization) three times at 3-wk intervals. 3 d before fusion with the murine myeloma, the mice were injected both intraperitoneally and intravenously with purified ICAM-3. The protocol for fusion and subsequent maintenance of hybridomas was as previously described (31). Initial screening of hybridoma supernatants was performed by ELISA against purified ICAM-3 absorbed onto plastic. Reactive hybridoma supernatants were then tested by indirect immunofluorescence flow cytometry for reactivity to ICAM-3-bearing SKW3 cells and neutrophils. Of 500 hybridomas supernatants, 5 mAbs (CBR-IC3/2, IgG2a; CBR-IC3/3, IgG2a; CBR-IC3/4, IgM; CBR-IC3/5, IgG<sub>a</sub>; CBR-IC3/6, IgE) tested positive in both primary and secondary screens, immunoprecipitated a protein of 124,000 *M<sub>r</sub>* from <sup>125</sup>I-labeled SKW3 lysates, and reacted specifically to COS cell transfectants expressing ICAM-3. Hybridomas were cloned three times by limiting dilution and isotyped by ELISA using affinity-purified antibodies to mouse Igs (Zymed Immunochemicals, San Francisco, CA). All mAbs were used at a saturating concentration of 20  $\mu$ g/ml for adhesion assays, excepting mAb CBR-IC3/6, which was used as a 1:200 dilution of mAb-containing ascites fluid.

**Cell Culture.** The murine myeloma, P3X63Ag8.653, and subsequent hybridomas were maintained as previously described (4). COS cells and human lymphoid cell lines were grown in RPMI

1640 supplemented with 10% FCS, 5 mM glutamine, and 50  $\mu$ g/ml gentamycin at 37°C in the presence of 5% CO<sub>2</sub>.

Isolation of PBMC was as described (4). PBL were enriched by incubating PBMC on tissue culture-treated plastic petri dishes twice for 1 h each and saving the nonadherent cells. For costimulation assays, resting CD3<sup>+</sup> T lymphocytes (95% CD2<sup>+</sup>, 99% CD3<sup>+</sup>) were purified from PBL through two rounds of rigorous immunomagnetic-negative selection using Dynabeads M-450 (Dyna; Robbins Scientific, Mountain View, CA) and mAbs specific for CD14, CD16, CD20, Mac-1, and HLA-DR. The purified T lymphocytes were functionally assessed to be depleted of monocytes by the criterion that there was no proliferative response to optimal concentrations of PHA-M (1:200 dilution; Gibco Laboratories, Grand Island, NY).

**Protein Purification.** LFA-1, ICAM-1, and LFA-3 were purified from JY lysates as previously described (11, 32, 33), while the ICAM-3 purification from SKW3 lysates was adapted from a previously published protocol (12). Proteins were purified from detergent lysates of JY or SKW3 cells by immunoaffinity chromatography using TS2/4 (LFA-1), RR1/1 (ICAM-1), TS2/9 (LFA-3), or CBR-IC3/1 (ICAM-3) mAb-coupled Sepharose. The immunoaffinity column-bound proteins were eluted with either 50 mM triethylamine, pH 11.5, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1% octyl- $\beta$ -D-glucoside (OG) (LFA-1), 50 mM triethylamine, pH 12.5, 150 mM NaCl, 1% OG (ICAM-1), 50 mM glycine, pH 3.0, 150 mM NaCl, 1% OG (LFA-3), or 50 mM glycine, pH 3.8, 150 mM NaCl, 1% OG (ICAM-3). Samples were neutralized, aliquoted, and stored frozen at -70°C for 3–6 mo without loss of activity. Aliquots of the neutralized fractions (10  $\mu$ l) were subjected to SDS-7.5% PAGE (34) under reducing conditions and silver stained (35).

**cDNA and Transfection.** LFA-1 $\alpha$  and  $\beta$  cDNAs in the transient expression vector CDM8 (36, 37) or vector alone (mock) were transfected into COS cells using DEAE-dextran (38). 3 d after transfection, cells were detached with 10 mM EDTA/HBSS, washed three times in 10% FCS/RPMI 1640, and then used for binding to LFA-1-coated plates. As judged by indirect immunofluorescence, 30–50% of LFA-1 $\alpha$ / $\beta$  cDNA transfectants expressed LFA-1 on their cell surface.

**Adhesion Assay.** Purified ligand (LFA-1, LFA-3, ICAM-1, ICAM-3) in 1% OG was diluted in 25 mM Tris HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl<sub>2</sub> (TSM), and adsorbed onto either 96-well plates or 60-mm petri plates as previously described (4, 39). The number of ligand sites per microtiter well was determined using saturating amounts of <sup>125</sup>I-labeled mAb (LFA-1, mAb TS1/22; ICAM-1, mAb RR1/1; ICAM-3, mAb CBR-IC3/1) and calculated assuming monovalent binding of the mAb (40). Site numbers are expressed per square micron; the surface area of a microtiter well and the area of purified protein occupied by a 25- $\mu$ l spot on a petri plate were identical, being 3.85  $\times$  10<sup>7</sup>  $\mu$ m<sup>2</sup>.

Adhesion of cells fluorescently labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6) carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, OR) to 96-well plates coated with purified ligand was performed as previously described (4, 5). For inhibition, wells containing immobilized purified protein were incubated with 20  $\mu$ g/ml of the appropriate purified mAb(s) for 45 min at room temperature, or labeled cells were resuspended in assay buffer (PBS, 5% FCS, 2 mM MgCl<sub>2</sub>), pretreated with 20  $\mu$ g/ml of appropriate purified mAb(s) for 45 min at 4°C, and either 5  $\times$  10<sup>4</sup> (COS transfectants) or 10<sup>5</sup> (cell lines) cells were transferred to each well. After 1 h at 37°C, nonadherent cells were removed with either four 25-gauge (COS cells) or six 23-gauge (cell lines) needle aspirations. Bound cells were quantitated in the 96-well plate using

a Pandex fluorescence concentration analyzer (IDEXX Corp., Westbrook, ME).

Adhesion of cells to ligand-spotted petri plates was performed as previously described (18, 39, 41). For inhibition, petri plates containing immobilized purified protein were incubated with 1 ml of a 20- $\mu\text{g}/\text{ml}$  solution of the appropriate purified mAb(s) for 45 min at room temperature. Alternatively, cells ( $2 \times 10^6$  cells/ml) were resuspended in assay buffer and incubated with appropriate mAbs for 45 min at 4°C. Cells were then added ( $2 \times 10^6$  cells) to a 60-mm petri plate and adhered for 60 min at 37°C. For phorbol ester stimulation, cells were incubated with 50 ng/ml PMA upon addition to the petri plates. Unbound cells were removed by six washes with a transfer pipette. Bound cells were quantitated by visually scoring the number of cells in six microscopic fields ( $\times 100$ ). This number was divided into the input number of cells, which was determined in parallel to obtain percentage of cell binding. Homotypic aggregation did not occur in this assay; it requires 3–5 h to be detectable. Visual inspection confirmed that increased adherence of cells to LFA-1 stimulated by PMA was the result of increased adherence of single cells, rather than to formation of aggregates.

**Costimulation Assays.** Costimulation assays using coimmobilized ligand and anti-CD3 mAb were performed as described previously (24). Preparation of flat-bottomed 96-well tissue culture plates (3596; Costar, Cambridge, MA) with varying concentrations of coimmobilized ICAM-3 and CD3 mAbs (OKT3) was adapted from a previously published protocol (24). Site densities of ICAM-3 were determined as described above; success of CD3 mAb immobilization was determined using  $^{125}\text{I}$ -labeled rabbit anti-mouse IgG (Zymed Labs., Inc., S. San Francisco, CA). Resting T lymphocytes ( $5 \times 10^4$ /well), with appropriate mAbs, were cultured for 3 d in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone Labs., Logan, UT), 5 mM glutamine, and 50  $\mu\text{g}/\text{ml}$  gentamycin. Cells were incubated at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere, and pulsed for 8 h before harvesting on day 3 with 1  $\mu\text{Ci}/\text{well}$  of [ $^3\text{H}$ ]thymidine (3.3 Ci/mM; New England Nuclear, Boston, MA). Incorporation of radioactive label was measured by liquid scintillation counting, and results were expressed as the arithmetic mean counts per minute of triplicate cultures.

**Proliferation Assays.** Proliferation in response to PHA, allogeneic stimulator cells (MLR), or recombinant hepatitis B surface antigen (HBsAg) was as previously described (42, 43). Briefly,  $10^5$  PBL, and appropriate mAbs, were seeded in a volume of 200  $\mu\text{l}$  of RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone Labs.), 5 mM glutamine, and 50  $\mu\text{g}/\text{ml}$  gentamycin into each well of a 96-well flat-bottomed microtiter plate. PBL for HBsAg-dependent proliferation were taken from donors who had been boosted within 2–3 wk. Recombinant HBsAg, free of preservative, was a gift from Dr. C. Alper (Center for Blood Research, Boston, MA). Cells were activated with a 1:200 to 1:1,600 dilution of PHA-M (Gibco Laboratories),  $0.5 \times 10^5$  or  $0.2 \times 10^5$  allogeneic PBMC that were mitomycin C treated (50  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co.) for 20 min at 37°C (MLR), or 0.1–1  $\mu\text{g}/\text{ml}$  recombinant HBsAg (0.02–0.2  $\mu\text{g}$  HBsAg/well). Cells were incubated at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere, and pulsed for 18 h before harvesting (PHA, day 3; MLR, day 5; HBsAg, day 5) with 1  $\mu\text{Ci}/\text{well}$  of [ $^3\text{H}$ ]thymidine (3.3 Ci/mM; New England Nuclear). Incorporation of radioactive label was measured by liquid scintillation counting, and results were expressed as the arithmetic mean counts per minute of triplicate cultures.

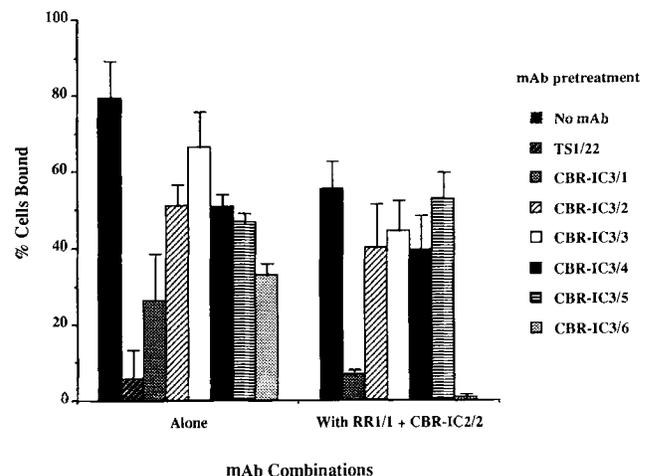
**Aggregation Assay.** Qualitative aggregation assays were carried out as described (44). Briefly, 50  $\mu\text{l}$  of a SKW3 cell suspension ( $2 \times 10^6/\text{ml}$ ) in 10% FCS/RPMI 1640 was preincubated with 20

$\mu\text{g}/\text{ml}$  of purified mAb or 1:200 dilution of ascites for 45 min at room temperature. These cells were then stimulated with 50 ng/ml of PMA and added to a flat-bottomed 96-well microtiter plate well (Becton Dickinson & Co., Lincoln Park, NJ). mAb-induced aggregation was identical, except cells were directly placed in wells in the absence of PMA and allowed to aggregate. Cells were incubated for either 1–2 h (mAb) or 4–6 h (PMA) at 37°C, viewed with an inverted microscope, and scored visually. Photomicrographs of aggregating cells were taken using a Nikon Diaphot-TMD inverted microscope (Nippon Kogaku, Tokyo, Japan) and phase contrast optics.

## Results

**Functional Studies with a Panel of ICAM-3 mAbs.** We previously obtained a single ICAM-3 mAb, CBR-IC3/1 (5). Five further mAbs to human ICAM-3 were obtained using purified ICAM-3 as immunogen, as described in Materials and Methods. Each mAb was tested alone or in conjunction with mAbs to ICAM-1 and ICAM-2 for its ability to inhibit adhesion of SKW3 cells to purified LFA-1 (Fig. 1). In addition to mAb CBR-IC3/1, only mAb CBR-IC3/6 was capable of inhibiting ICAM-3 interactions with purified LFA-1. Blocking with CBR-IC3/1 and CBR-IC3/6 was partial when used alone, and essentially complete when used with mAbs to ICAM-1 and ICAM-2, consistent with expression by SKW3 cells of all three ICAMs (5).

Evidence for LFA-1 counter-receptors that were distinct from ICAM-1 was first obtained with PMA-stimulated homotypic aggregation of the SKW3 T lymphoma cell line, which was inhibited with LFA-1 mAb but not with ICAM-1 mAb (9). Combination of function-blocking ICAM-1 and ICAM-2 mAbs also does not inhibit SKW3 PMA-induced aggrega-



**Figure 1.** Effect of anti-ICAM-3 mAbs on adhesion of SKW3 cells to purified LFA-1. Cells were pretreated with saturating concentrations of one of six ICAM-3 mAbs in the presence or absence of blocking ICAM-1 (RR1/1) and ICAM-2 (CBR-IC2/2) mAbs, allowed to bind to LFA-1-coated microtiter wells for 60 min at 37°C, and then washed six times by aspiration. Alternatively, the absorbed purified LFA-1 was pretreated with LFA-1 $\alpha$  mAb (TS1/22). Site density of LFA-1 as determined by radioimmunoassay was 700 sites/ $\mu\text{m}^2$ . One representative of three is shown and bars show one standard deviation.

tion (4). None of the ICAM-3 mAbs were capable of inhibiting PMA-induced aggregation by themselves or in combination with ICAM-1 and ICAM-2 mAbs (Table 1). Indeed, mAbs CBR-IC3/1 and CBR-IC3/6 actually promoted aggregation (see below). Aggregation was, however, completely inhibited when certain combinations of anti-ICAM-3 mAbs were used in conjunction with ICAM-1 and ICAM-2 mAbs (Table 1). One such example was when CBR-IC3/2 mAb was used in conjunction with CBR-IC3/1 mAb (Table 1). The combination of CBR-IC3/1 and CBR-IC3/2, along with ICAM-1 and ICAM-2 mAbs, was capable of complete inhibition of PMA-induced aggregation of SKW3 cells, to the level seen with LFA-1 mAbs (Table 1). ICAM-3 antisera derived from mice immunized with purified ICAM-3, if used in the presence of ICAM-1 and ICAM-2 mAbs, also completely inhibited PMA-induced SKW3 aggregation (data not shown).

Two mAbs to ICAM-3, CBR-IC3/1 and CBR-IC3/6, promoted SKW3 homotypic aggregation (Fig. 2, B and C). Induction of aggregation occurred within 1–2 h in both PMA-stimulated and unstimulated SKW3 cells. Aggregation of SKW3 cells in the absence of PMA is very noticeable, as these cells normally do not display any spontaneous aggregation (Fig. 2 A). None of the four mAbs to ICAM-3, nor Fab fragments of mAb CBR-IC3/1, induced aggregation. The presence of mAb CBR-IC3/2 prevented both CBR-IC3/1- and CBR-IC3/6-induced aggregation (Fig. 2 D, and data not shown). It is interesting that the two mAbs that blocked binding to purified LFA-1 were the same mAbs that stimulated aggregation. It seemed unlikely that Fc receptors were involved, since CBR-IC3/1 and CBR-IC3/6 are IgG1 and IgE, respectively. Furthermore, aggregation was absent at 4°C or when performed in the presence of 5 mM EDTA (data not shown), seemingly suggestive of an integrin interaction rather than simple crossbridging effects of the mAb. A variety of mAbs to other adhesion structures, including VLA ( $\alpha_{1-6}$ ), CD29 ( $\beta_1$ ), LFA-1, Mac-1, p150,95, CD18 ( $\beta_2$ ), CD2, LFA-3,  $\beta_4$ , and  $\beta_7$ , had no effect on this adhesion

(data not shown). ICAM-3 mAb-induced aggregation was not restricted to SKW3 cells, as it occurred with a variety of cells, including the CD18-deficient cell line SLA (data not shown).

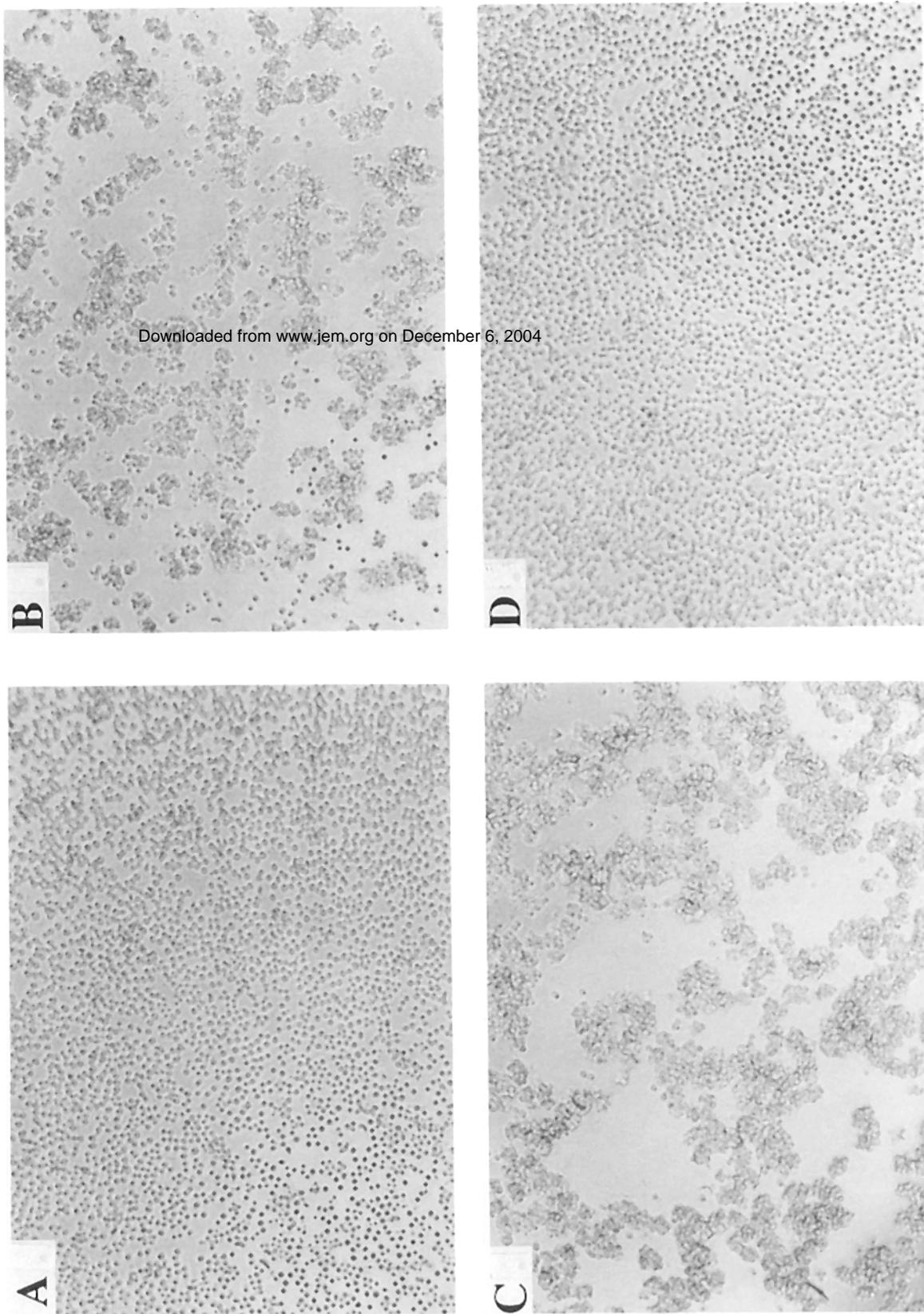
**Adhesion to Purified ICAM-3.** To better elucidate ICAM-3 function as an LFA-1 ligand, we compared LFA-1-dependent binding to purified ICAM-1 and ICAM-3. PMA-stimulated SKW3 cells bound to purified ICAM-1 and ICAM-3 in a LFA-1-specific manner (Fig. 3). No morphological differences were observed in cells binding to either purified ICAM (data not shown). Adhesion was completely inhibited by LFA-1 $\alpha$  and CD18 mAbs (Fig. 3). Adhesion to purified ICAM-3 was not blocked with either CBR-IC3/1 or CBR-IC3/2 mAbs alone, but only when the two were used together (Fig. 3 A). Contrary to a report of CD43 interaction with ICAM-1 (45), SKW3 adhesion to purified ICAM-1 and ICAM-3 was not only completely LFA-1 specific, but also was not inhibited with CD43 mAbs (Fig. 3 B). Specificity of the interaction was further proved through adhesion assays using cDNA transfectants (Fig. 4). COS cells transiently transfected with either cDNA for both  $\alpha$  and  $\beta$  chains of LFA-1 (CD11a, CD18) or only transfected with  $\alpha$  chain cDNA (mock) were allowed to adhere to purified ICAM-1 and ICAM-3 (Fig. 4). Mock transfectants did not adhere to either purified protein, while transfectants expressing LFA-1 bound to ICAM-1 and ICAM-3 in a LFA-1 mAb-inhibitable manner. Similarly, SLA, a cell line derived from a LFA-1-deficient patient (46), did not adhere to either ICAM-1 or ICAM-3 (data not shown).

To test whether the activity of LFA-1 for ICAM-3 could be regulated by PMA, as has been previously demonstrated for ICAM-1, cells treated with or without PMA were assayed for binding to purified ICAM-3 (Fig. 5). Binding to ICAM-3 was enhanced fivefold by PMA stimulation, while binding to ICAM-1 was also enhanced, as previously reported (32, 47). Adhesion to purified ICAM-1 and ICAM-3 was specific as it was inhibited by the pretreatment of cells with an LFA-1 mAb, TS1/22. PMA treatment of SKW3 cells also resulted in a threefold increase in binding to purified LFA-3

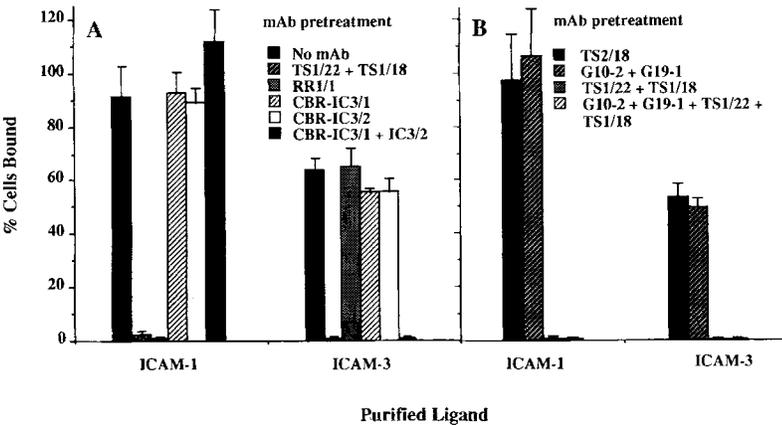
**Table 1.** Effect of ICAM-3 mAbs on PMA-induced SKW3 Aggregation in the Presence of ICAM-1 and ICAM-2 mAbs

mAb	CBR-IC3/1	CBR-IC3/2	CBR-IC3/3	CBR-IC3/4	CBR-IC3/5	CBR-IC3/6
CBR-IC3/1	3	0	0	0	0	5
CBR-IC3/2		3	3	1	3	0
CBR-IC3/3			3	3	3	1
CBR-IC3/4				2	3	2
CBR-IC3/5					3	1
CBR-IC3/6						5

SKW3 cells were preincubated at room temperature for 45 min with one or combinations of any two ICAM-3 mAbs, in the presence of ICAM-1 (RR1/1) and ICAM-2 (CBR-IC2/2) mAbs. Cells were stimulated with 50 ng/ml PMA, and aggregation was scored visually after 5 h. 0, no cells clustered; 1, <10% of cells aggregating; 2, 10–50% of cells aggregating; 3, 50–100% of cells aggregating; 4, nearly 100% of cells in loose aggregates; 5, 100% of cells in very compact aggregates. Cells aggregated in the absence of mAbs (score 4), and in the presence of only ICAM-1 and ICAM-2 mAbs (score 3). Aggregation was not inhibited by ICAM-3 mAbs alone (score 4); LFA-1 mAbs completely inhibited aggregation (score 0). One of two representative experiments is shown.



**Figure 2.** Aggregation of SKW3 cells is induced by ICAM-3 mAbs in the absence of PMA. Photomicrographs of SKW3 cells aggregating in the presence of: (A) no mAb; (B) mAb CBR-IC3/1; (C) mAb CBR-IC3/6; (D) mAbs CBR-IC3/1 and CBR-IC3/2.



**Figure 3.** Binding of PMA-stimulated SKW3 cells to purified ICAM-1 and ICAM-3. (A) SKW3 cells ( $2 \times 10^6$  cells/ml) were incubated in the presence of either no mAb or mAbs TS1/22 and TS1/18 (anti-LFA-1 $\alpha$  and anti-CD18) or, alternatively, the absorbed purified ICAM-1 and ICAM-3 was pretreated with either mAb RR1/1 (anti-ICAM-1), mAb CBR-IC3/1 (anti-ICAM-3), mAb CBR-IC3/2 (anti-ICAM-3), or mAbs CBR-IC3/1 and CBR-IC3/2 (anti-ICAM-3). (B) SKW3 cells ( $2 \times 10^6$  cells/ml) were incubated in the presence of control mAb TS2/18 (anti-CD2), mAbs TS1/22 and TS1/18 (anti-LFA-1 $\alpha$  and anti-CD18), mAbs G10-2 and G19-1 (anti-CD43), or mAbs TS1/22, TS1/18, G10-2, and G19-1 (anti-LFA-1 $\alpha$ , anti-CD18, and anti-CD43). SKW3 ( $2 \times 10^6$  cells) were then added to an equal volume of assay buffer containing 100 ng/ml PMA in 60-mm petri dishes coated with spots of purified ICAM-1 and ICAM-3, and incubated for 60 min at 37°C. Unbound cells were removed by six washes with a transfer pipette. The ICAM-1 and ICAM-3 site densities were determined by radioimmunoassay to be 1,400 sites/ $\mu\text{m}^2$  each. One of three (A) or two (B) representative experiments is shown and bars show 1 SD.

(Fig. 5). Adhesion to LFA-3 was completely inhibited with the LFA-3 mAb, TS2/18 (data not shown). These results confirmed an earlier report that described increased adhesiveness of CD2 for LFA-3 in response to phorbol ester stimulation (48).

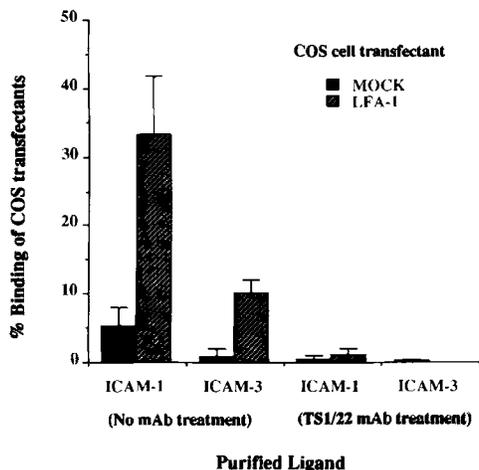
SKW3 cells bound to both purified ICAM-1 and ICAM-3 in a site density-dependent manner (Fig. 6). The level of binding of unactivated cells to purified ICAM-1 was higher than that previously published (32); this was due to the lower stringency washing conditions used in this adhesion assay.

ICAM-1/LFA-1 interactions were previously shown to be both temperature and cation dependent (11). Adhesion of LFA-1-bearing cells to purified ICAM-1 and ICAM-3 was cation

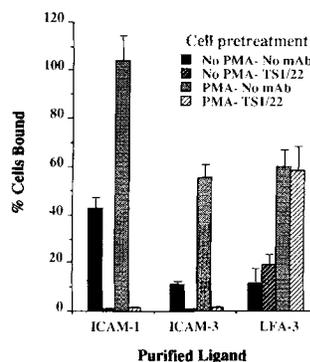
dependent (Fig. 7 A). Removal of  $\text{Mg}^{2+}$  resulted in loss of binding to both purified ICAM-1 and ICAM-3. Maximal adhesion of LFA-1-bearing cells to purified ICAM-1 and ICAM-3 was seen at 37°C, with decreasing binding at lower temperatures (Fig. 7 B). No binding was seen to either purified ICAM-1 or ICAM-3 at 4°C, and only minimal binding was seen to ICAM-1 at 16°C.

**Costimulation via ICAM-3.** Purified ICAM-3 coimmobilized with CD3 mAb resulted in a strong proliferative signal (Fig. 8 A). This costimulatory activity was present at several concentrations of CD3 mAb and several ICAM-3 site densities, whereas CD3 mAb or purified ICAM-3 alone did not result in any proliferative signal. This proliferative response was completely inhibited in the presence of appropriate LFA-1 $\alpha$ , LFA-1 $\beta$ , and ICAM-3 mAbs (Fig. 8 B). The requirement for TCR cross-linking in delivering a costimulatory signal is demonstrated by the inability of soluble CD3 mAb and immobilized ICAM-3 to result in a proliferative response (Fig. 8 B). Purified ICAM-1 coimmobilized with CD3 mAb also resulted in a significant proliferative response, consistent with previous results (24, 25), while coimmobilization of purified LFA-1 and CD3 mAbs had no effect (data not shown).

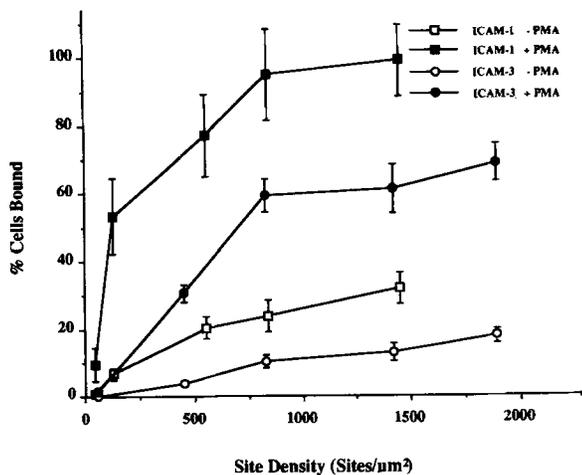
**Role of ICAMs in Immune Responses.** To test the hypothesis that ICAM-3 plays an important role in the initiation



**Figure 4.** LFA-1-expressing COS cell transfectants adhere specifically to purified ICAM-1 and ICAM-3. Mock or LFA-1-transfected COS cells ( $8 \times 10^5$  cells/ml) were incubated in the presence of either no mAb or mAb TS1/22 (anti-LFA-1 $\alpha$ ). COS transfectants ( $8 \times 10^5$  cells) were then allowed to bind in the presence of PMA to spots of purified ICAM-1 and ICAM-3, exactly as in Fig. 3. One of three representative experiments is shown and bars show 1 SD.



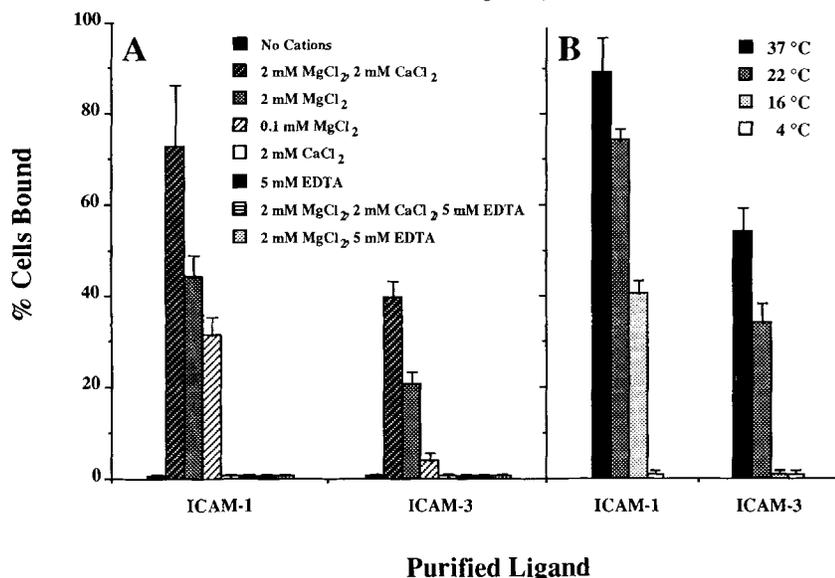
**Figure 5.** PMA-induced binding to purified LFA-3, ICAM-1, and ICAM-3. SKW3 cells ( $2 \times 10^6$  cells/ml) were incubated in the presence of either no mAb or mAb TS1/22 (anti-LFA-1 $\alpha$ ), and allowed to bind in the presence or absence of 50 ng/ml PMA to spots of purified ICAM-1 and ICAM-3, exactly as described in Fig. 3. One of three representative experiments is shown and bars show 1 SD.



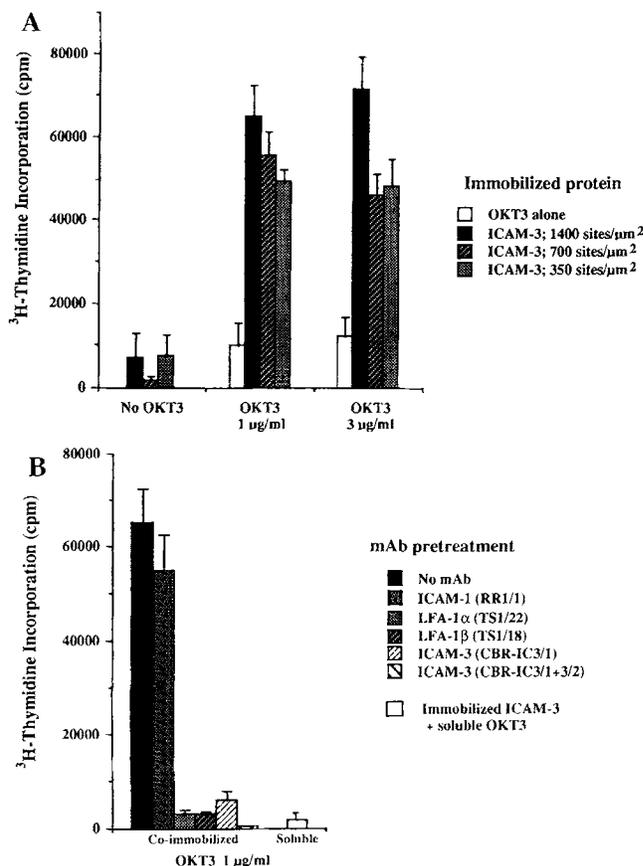
**Figure 6.** Effect of PMA stimulation on SKW3 binding to different densities of ICAM-1 and ICAM-3. SKW3 cells were added to an equal volume of either binding buffer alone or binding buffer containing 100 ng/ml PMA, and allowed to bind to petri dishes coated with spots of purified ICAM-1 and ICAM-3, as described in Fig. 3 legend. Site densities were determined by radioimmunoassay. One of three representative experiments is shown and bars show 1 SD.

of immune responses, the relative importance of each ICAM, separately and in various combinations, was tested in a variety of immunological assays (Fig. 9). Proliferation to PHA (Fig. 9 A) was inhibited by LFA-1 mAb TS1/22, as previously described (42). ICAM-1 mAb showed little effect, whereas ICAM-2 and ICAM-3 mAbs, when used alone, each significantly inhibited proliferation. Treatment of cells with mAbs to two of the ICAM molecules resulted in further increased inhibition of proliferation in response to PHA. mAbs to all three ICAM molecules reduced the PHA-proliferative response to levels equivalent to when no PHA was added.

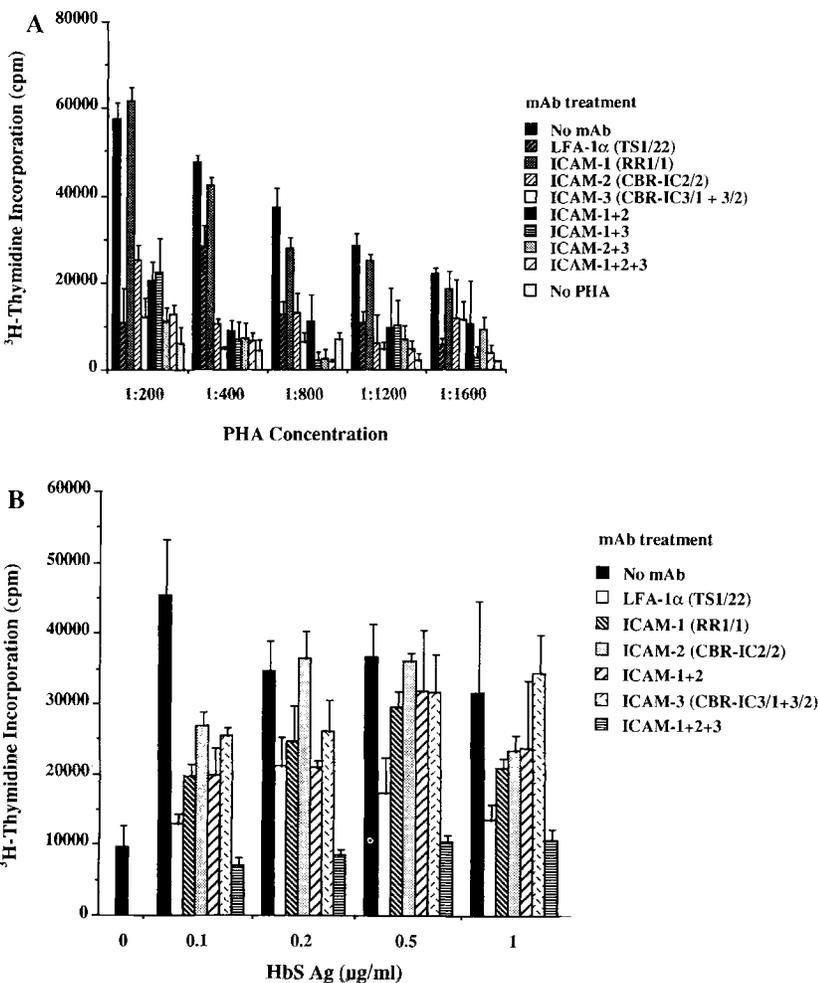
As previously reported (42), anti-LFA-1 and anti-CD18 mAbs efficiently inhibited proliferation in the MLR. The combination of mAbs to all three ICAMs completely reduced



**Figure 7.** Effect of divalent cations and temperature on PMA-stimulated SKW3 binding to ICAM-1 and ICAM-3. (A) SKW3 cells were washed once and resuspended ( $2 \times 10^6$  cells/ml) in either (A) Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS containing the indicated concentrations of MgCl<sub>2</sub>, CaCl<sub>2</sub>, or EDTA, or (B) assay buffer (PBS, 5% FCS, 2 mM MgCl<sub>2</sub>). SKW3 ( $2 \times 10^6$  cells) were then added to 60-mm petri dishes coated with spots of purified ICAM-1 and ICAM-3, and incubated for 60 min at either (A) 37°C or (B) the indicated temperature. Binding was measured as described in Fig. 3 legend. One of two (A) or three (B) representative experiments is shown and bars show 1 SD.



**Figure 8.** Costimulatory effect of immobilized purified ICAM-3 and CD3 mAb. (A) Concentration dependence of costimulation. Various concentrations of purified ICAM-3 and CD3 mAbs (OKT3) were coimmobilized before the addition of  $5 \times 10^4$  resting T lymphocytes. (B) Costimulation is mediated by purified ICAM-3 through LFA-1 and requires coimmobilized CD3 mAb. Purified ICAM-3 (1,400 sites/μm<sup>2</sup>) and CD3 mAb (1 mg/ml OKT3) were either coimmobilized or CD3 mAb was only added in soluble form. Blocking mAbs were added at day 0 at a concentration of 20 μg/ml. Proliferation responses in these cultures were measured by [<sup>3</sup>H]thymidine incorporation on day 3. One of three representative experiments is shown and bars show 1 SD.



**Figure 9.** Inhibition of (A) PHA response and (B) Ag-specific proliferation by LFA-1, CD18, ICAM-1, ICAM-2, and ICAM-3 mAbs. PHA response and Ag-specific response of freshly separated PBL were as described in Materials and Methods. In all assays mAbs were added at the start of the cell incubations at final concentrations of 20  $\mu\text{g}/\text{ml}$ . mAbs used were to LFA-1 (TS1/22), CD18 (TS1/18), ICAM-1 (RR1/1), ICAM-2 (CBR-IC2/2), and ICAM-3 (CBR-IC3/1 and CBR-IC3/2). One of either five (A) or three (B) representative experiments is shown and bars show 1 SD.

proliferation to the levels seen with either stimulators or responders alone.

Last, all three ICAM mAbs were tested for their ability to inhibit HBsAg-dependent proliferation of PBLs from vaccinated, responding individuals (Fig. 9 B). Proliferation was inhibited by LFA-1 mAb to levels seen in unstimulated PBLs. ICAM-1, -2, and -3 mAbs showed slight inhibition when used separately, however, a combination of mAbs to all three ICAMs reduced proliferation to levels seen in unstimulated PBL.

## Discussion

In this study, we have functionally characterized the ICAM-3/LFA-1 interaction and investigated the relative roles of the ICAMs in immunological responses. Purification of ICAM-3 was achieved in a manner similar to that used in purifying ICAM-1. Alongside purified ICAM-3, cells were also tested for binding to purified ICAM-1. Lymphoid cells adherent to both purified ICAM-1 and ICAM-3 had a spread morphology, indicative of cytoskeletal interactions (7, 49). This binding to purified ICAMs was completely inhibitable with anti-CD18 mAb, indicating that for both ICAM-1 and

ICAM-3, no additional ligands existed on lymphoid cells. Similarly, a leukocyte adhesion deficiency (LAD) patient-derived cell line, SLA, did not interact with either purified ICAM-1 or ICAM-3. These cells expressed levels of CD43 comparable to those of LFA-1; thus, we could not confirm ICAM-1 interaction with CD43 (45). Additionally, CD43 mAbs, including those used in the previous study, had no effect on binding of lymphoid cells to either purified ICAM-1 or ICAM-3. This is in agreement with previous studies in which mAbs to CD11a, CD11b, or CD18, or inherited deficiency of leukocyte integrins, have been found to abolish interactions of lymphoid cells and neutrophils, which express CD43, with ICAM-1 (11, 39, 50–52). The interactions of cells with purified ICAM-3 was cation and temperature dependent, similar to that described for purified ICAM-1 (11). The only difference was the lower overall binding of LFA-1-bearing cells to purified ICAM-3 than ICAM-1 at comparable site densities. This suggests that ICAM-3 has lower affinity for LFA-1 than ICAM-1, consistent with results on the relative contributions of ICAM-1 and ICAM-3 as LFA-1 ligands compared with their level of expression on different cell lines (5).

Binding of cells to both purified ICAM-1 and ICAM-3

was increased upon activation of LFA-1 adhesiveness. This result confirmed studies previously performed for ICAM-1 (32, 47) and extend them to include ICAM-3. Thus, the binding site on LFA-1 for ICAM-1 and ICAM-3 is sufficiently similar to demonstrate low and high avidity states. Using purified LFA-3, we also confirmed the increased adhesiveness of CD2 in response to phorbol esters (48).

The CBR-IC3/1 mAb blocked binding of SKW3 cells to purified LFA-1, but not LFA-1-dependent PMA-induced cell aggregation, or binding of LFA-1-bearing cells to purified ICAM-3. To fully characterize the role of ICAM-3 in immunological responses, additional ICAM-3 mAbs were generated. No single ICAM-3 was capable of completely inhibiting, in conjunction with mAbs to ICAM-1 and ICAM-2, SKW3 aggregation or the binding of SKW3 cells to purified ICAM-3. However, a combination of two mAbs, CBR-IC3/1 and CBR-IC3/2, was capable of completely inhibiting these pathways of adhesion. The epitopes recognized by CBR-IC3/1 and CBR-IC3/2 mAbs were determined by radioimmunoassay to be distinct, and found to map to the first and second NH<sub>2</sub>-terminal Ig-like domains of ICAM-3, respectively. As the LFA-1 binding site was found to localize solely to the NH<sub>2</sub>-terminal Ig-like domain (Klickstein, L., A. de Fougères, and T. Springer, manuscript in preparation), it appears that neither CBR-IC3/1 nor CBR-IC3/2 binds directly in the LFA-1 binding site, and a combination of two mAbs is required to sterically hinder binding to a nearby site. It appears that CBR-IC3/1 binds close to the LFA-1 binding site, however, because it inhibits in assays in which LFA-1 is immobilized on a substrate, although not in assays in which LFA-1 is present on a cell surface.

Two NH<sub>2</sub>-terminal Ig-like domain-specific ICAM-3 mAbs (CBR-IC3/1 and CBR-IC3/6) clearly stimulate ICAM-3<sup>+</sup> cells to aggregate in the absence of PMA. Other ICAM-3 mAbs, including some also recognizing the first NH<sub>2</sub>-terminal Ig-like domain, did not induce aggregation (de Fougères, A., and T. Springer, unpublished results). This aggregation was cation and temperature dependent, and was inhibited only by mAbs recognizing a different ICAM-3 epitope, one example being mAb CBR-IC3/2. Certain  $\alpha_4$  and  $\beta_1$  mAbs are capable of inducing homotypic aggregation of lymphoid cells in a similar manner (53–55). As with  $\alpha_4$  and  $\beta_1$  mAb-induced aggregation, Fab fragments of mAb CBR-IC3/1 were unable to induce aggregation. Last, the CBR-IC3/1 mAb-induced aggregation was not reversible with subsequent, as opposed to simultaneous, incubation with mAb CBR-IC3/2 (de Fougères, A., and T. Springer, unpublished results). Whether this aggregation represents evidence for additional ICAM-3 ligand(s), signaling through the ICAM-3 molecule resulting in aggregation, or agglutination that synergizes with or stabilizes integrin-mediated homotypic aggregation remains to be determined.

Purified ICAM-3, when coimmobilized with CD3 mAb, provided a strong costimulatory signal for T cell proliferation. The ability of ICAM-1 (24, 25), ICAM-2 (26), and now ICAM-3, when ligated with LFA-1 to provide a costimulatory signal, indicates not only that the site recognized on LFA-1 is likely very similar, but also that the role of these molecules in immune responses may be dependent on their relative levels of expression on APC. Given the high level of ICAM-3, and to a lesser extent ICAM-2, expressed on monocytes and B lymphocytes (5), these molecules may support the activation of T cells. Their presence on APC may act as a means to deliver a costimulatory signal to TCR-engaged T lymphocytes, which might be especially important on ICAM-1<sup>-</sup> or ICAM-1<sup>low</sup> APC before expression of ICAM-1 can be upregulated.

The relative contribution of ICAM-1, ICAM-2, and ICAM-3 was examined in several functional assays. In all three cases (PHA stimulation, MLR, and antigen-specific presentation), inhibition was achieved with LFA-1 mAb, as previously reported (42). mAbs to ICAM-1, ICAM-2, and ICAM-3, when used separately, resulted in partial inhibition. Consistent with these results are reports that ICAM-1 mAbs (8, 56, 57) and CD50 (ICAM-3) mAbs (58) are capable of partially inhibiting immune responses. Complete inhibition was achieved with a combination of mAbs to all three ICAM molecules, suggesting that ICAM-1, ICAM-2, and ICAM-3 are the only LFA-1 ligands important in antigen-specific lymphocyte responses. The importance of the LFA-1/ICAM interaction in the initial phase of an immune response is underscored by the ability of LFA-1, ICAM-1, and CD50 (ICAM-3) mAbs to inhibit immune responses, and their inability to do so if added subsequent to cell–cell conjugation (8, 58).

ICAM-1 was first identified in 1986 (9), followed by ICAM-2 in 1989 (3), and ICAM-3 in 1992 (5), based on evidence that previously identified ICAMs could not account for all of the ligand activity for LFA-1. Our results underscore the high degree of functional similarity and redundancy in the LFA-1 counter-receptors. The three LFA-1 counter-receptors differ dramatically in inducibility and tissue distribution, suggesting specialization for different cell interactions. Our study on ICAM-3, and previous ones on ICAM-1 (24, 25) and ICAM-2 (26), show they induce similar costimuli in LFA-1-bearing cells; however, signals induced by the ICAMs on the cells on which they are expressed may vary. The ability of a combination of mAbs to ICAM-1, ICAM-2, and ICAM-3 to inhibit LFA-1-dependent adhesion of lymphoid cell lines, such as SKW3, and three different T cell functions to the same extent as mAb to LFA-1, suggests that the most functionally important, and possibly all, of the ICAMs expressed on lymphocytes have now been defined.

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