We have studied adhesion of eosinophils to various forms of vascular cell adhesion molecule 1 (VCAM-1, CD106), an integrin counter-receptor implicated in eosinophil recruitment to the airway in asthma. Full-length 7d-VCAM-1, with seven immunoglobulin-like modules, contains integrin-binding sites in modules 1 and 4. The alternatively spliced six-module protein, 6d-VCAM-1, lacks module 4. In static assays, unactivated purified human blood eosinophils adhered similarly to recombinant soluble human 6d-VCAM-1 and 7d-VCAM-1 coated onto polystyrene microtiter wells. Further experiments, however, revealed differences in recognition of modules 1 and 4. Antibody blocking indicated that eosinophil adhesion to 6d-VCAM-1 or a VCAM-1 construct containing only modules 1–3, 1–3VCAM-1, is mediated by α4β1 (CD49d/29), whereas adhesion to a construct containing modules 4–7, 4–7VCAM-1, is mediated by both α4β1 and αMβ2 (CD11b/18). Inhibitors of phosphoinositide 3-kinase, which block adhesion of eosinophils mediated by αMβ2, blocked adhesion to 4–7VCAM-1 but had no effect on adhesion to 6d-VCAM-1. Consistent with the antibody and pharmacological blocking experiments, eosinophil leukemic cell lines lacking αMβ2 did not adhere to 4–7VCAM-1 but did adhere to 6d-VCAM-1 or 1–3VCAM-1. Activation of eosinophils by interleukin (IL)-5 enhanced static adhesion to 6d-VCAM-1, 7d-VCAM-1, or 4–7VCAM-1; IL-5-enhanced adhesion to all 3 constructs was blocked by anti-αMβ2. Adhesion of unstimulated eosinophils to 7d-VCAM-1 under flow conditions was inhibited by anti-α4 or anti-αM. IL-5 treatment decreased eosinophil adhesion to 7d-VCAM-1 under flow, and anti-αM had the paradoxical effect of increasing adhesion. These results demonstrate that αMβ2 modulates α4β1-mediated eosinophil adhesion to VCAM-1 under both static and flow conditions.

Vascular cell adhesion molecule 1 (VCAM-1)2 is a multimeric cell-surface glycoprotein up-regulated on human endothelium in response to pro-inflammatory cytokines in the asthmatic lung (1) and has been implicated in recruitment of eosinophils (EOS) from blood to the airway in asthma (2). Heterodimeric integrin receptors interacting with VCAM-1 are important in the rolling, firm adhesion, and movement of EOS (3, 4). These events are regulated by cytokines, which may alter integrin adhesive activity (5, 6). EOS express at least three potential integrin receptors that may be involved in adhesion to VCAM-1: α4β1 (7–9), αMβ2 (7), and α4β7 (8–11).

Considerable information is available about features of human VCAM-1 that are important for adhesion. The loop between β strands C and D in the first immunoglobulin (Ig)-like module is accessible on the protein surface and contains the core integrin-recognition sequence Ille39–Asp-Ser-Pro-Leu63 (IDSPL) (11–15). A second IDSPL site is present in the CD loop in module 4 (12, 14). Based on topology of exons within the mammalian genome and the similarity in amino acid sequence, a three-module unit was likely duplicated to generate VCAM-1 modules 1–3 and 4–6 (16, 17). Modules 1 and 4 of human VCAM-1 share 73% sequence identity and contain two disulfide bonds, which is unusual for Ig-like modules (15, 17).

Alternative splicing of mRNA encoding VCAM-1 generates two protein forms in humans, a variant consisting of seven Ig-like modules, 7d-VCAM-1, containing putative integrin-binding sites in modules 1 and 4, and a variant containing six Ig-like modules, 6d-VCAM-1, which is missing the putative integrin-binding site in module 4 and contains only the site in module 1 (18). Despite the similarities between modules 1 and 4, studies involving lymphocytic and monocytic cell lines demonstrated that interactions with modules 1 and 4 are regulated differently by conditions such as shear stress, divalent cations, and temperature (19–21). Such differences in activities between modules 1 and 4, studies involving lymphocytic and monocytic cell lines demonstrated that interactions with modules 1 and 4 are regulated differently by conditions such as shear stress, divalent cations, and temperature (19–21). Such differences in activities between modules 1 and 4, studies involving lymphocytic and monocytic cell lines demonstrated that interactions with modules 1 and 4 are regulated differently by conditions such as shear stress, divalent cations, and temperature (19–21). Such differences in activities between modules 1 and 4, studies involving lymphocytic and monocytic cell lines demonstrated that interactions with modules 1 and 4 are regulated differently by conditions such as shear stress, divalent cations, and temperature (19–21). Such differences in activities between modules 1 and 4, studies involving lymphocytic and monocytic cell lines demonstrated that interactions with modules 1 and 4 are regulated differently by conditions such as shear stress, divalent cations, and temperature (19–21). Such differences in activities between modules 1 and 4, studies involving lymphocytic and monocytic cell lines demonstrated that interactions with modules 1 and 4 are regulated differently by conditions such as shear stress, divalent cations, and temperature (19–21).
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adhesion of unactivated EOS to module 4 and of IL-5-stimulated EOS to both modules 1 and 4.

EXPERIMENTAL PROCEDURES

EOS Isolation—Human EOS were isolated from peripheral blood by anti-CD16 magnetic bead selection (23, 24). Samples were normal, allergic rhinitic, or allergic asthmatic volunteers. Purity of EOS was greater than 98% as determined by Diff-Quik staining. Viability was at least 99% as assessed by staining with propidium iodide and annexin V-fluorescein isothiocyanate (BD Biosciences). The University of Wisconsin Human Subjects Committee approved the study, and informed consent was obtained before participation of volunteers.

Cell Lines—The human AML14.3D10 and EoL-3 eosinophilic leukemic cell lines were obtained and cultured as described (25). Jurkat T lymphocytic leukemic cells were provided by Laura Kiessling (University of Wisconsin-Madison, Madison, WI) and grown in Roswell Park Memorial Institute medium (RPMI) 1640 with 10% FBS, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and subcultured by dilution to 1 × 10^5/ml twice a week.

Expression of VCAM-1 Constructs—Total RNA from Chinese hamster ovary (CHO) cells expressing full-length human 6d- or 7d-VCAM-1 (26, 27), a kind gift from Dr. Roy Lobb of Biogen, was purified and reverse-transcribed with the primer sequence 5′-GTAACGACTATGCAGAGCCCGGGTTTAAAATCGAGACACCCACCCAG-3′ to generate cDNA encoding extracellular 6d-VCAM-1 or 7d-VCAM-1 lacking the cytosolic tail and transmembrane region (Fig. 1A). The PCR products encoding 6d-VCAM-1 and 7d-VCAM-1 were 1771 and 2041 bp, respectively, and contained an Xmal site at the 5′ end and an SpeI site at the 3′ end. PCR fragments were digested with Xmal and SpeI restriction enzymes and cloned in-frame into the pAcGP67.coco plasmid (pCOCO) (28). The pCOCO plasmid containing the DNA insert encoding extracellular 7d-VCAM-1 was used as a template to generate the 4–7VCAM-1, 1–3VCAM-1, and 1–2VCAM-1 constructs (Fig. 1A). The 4–7VCAM-1 construct was PCR-amplified with the following primer sequences: forward primer, 5′-GTCTCCCGGGT TTAAAAATCGAGACACCCACCCAG-3′ and reverse primer, 5′-GCAGACTTTGTTGAGATCTCCCCTGG-3′. The PCR products encoding 4–7VCAM-1, 1–3VCAM-1, and 1–2VCAM-1 constructs were monomeric. For the proteins with a tendency to multimerize, small amounts of nickel-nitrilotriacetic acid beads were added to protein solutions to preferentially bind and remove multimers, leaving monomers behind in solution. Recombinant soluble human 6d-VCAM-1, 7d-VCAM-1, and 4–7VCAM-1 proteins were expressed in SF9 insect cells because protein yield was better in comparison to the yield in High 5 cells. Yields of pure monomers were between 5 and 20 mg/liter concentrations of conditioned medium.

Other Materials—Several of the mAbs have been described previously (25). Other mAbs included: anti-α-d 2171 and 240I (both mouse IgG1) (7, 29), a gift from ICOS (Bothell, WA); anti-β1 4B4 (mouse IgG1), purchased from Coulter (Miami, FL); anti-β1 MAR 4 (mouse IgG1) and the HUTS-21 (mouse IgG1) and 9EG7 (mouse IgG1) activation-specific mAbs against β1, purchased from BD Biosciences; anti-αM (CD11b) 2LPM19c, purchased from Biomedia (Foster City, CA); N29 activation-specific mAb against β1 (mouse IgG1), purchased from Chemicon (Temecula, CA). mAb 2A6.21 (mouse IgG1) was generated against recombinantly expressed 7d-VCAM-1 by Dr. Mary Ann Accavitti at the Hybridoma Core Facility, University Alabama-Birmingham and demonstrated to react with the LVPRGAAG sequence in the C-terminal purification tag of the VCAM-1 constructs. Peroxidase-conjugated goat anti-mouse IgG (H+L) and alkaline phosphatase-conjugated donkey anti-mouse IgG (H+L) were from Jackson ImmunoResearch Laboratories (West Grove, PA). Wortmannin was purchased from Sigma-Aldrich, and LY294002 was purchased from Calbiochem.

Cell Adhesion under Static Conditions—Polystyrene 96-well non-tissue culture-treated plates (BD Biosciences) were coated in triplicate with 100 μl of protein solution in Tris-buffered saline (TBS), pH 8.0, per well for 3 h at 37 °C, decanted, and then blocked for 20 min at 37 °C with heat-inactivated FBS for EOS or BSA rendered free of fatty acids for cell lines. FBS was heat-inactivated by heating to 56 °C for 30 min, mixed every 10 min, and then rapidly cooled in an ice-water bath. For inhibition experiments, mAbs or phosphoinositide 3-kinase (PI3K) inhibitors were added to cells at a final concentration of 10 μg/ml
mAbs, 100 nM wortmannin, or 10 μM LY294002. Cells were incubated with mAbs for 5 min at 22 °C or with PI3K inhibitors for 15 min at 37 °C before the addition to wells. EOS were suspended in HBSS, 0.2% BSA at 1 × 10^5 cells/100 μl, and cell lines were suspended in RPMI 1640, 0.2% BSA at 2 × 10^5 cells/100 μl. The Ca^{2+} and Mg^{2+} concentration of HBSS were 1.2 and 0.9 mM and of RPMI were 0.4 and 0.4 mM, respectively. Cells were added to wells in volumes of 100 μl/well for 1 h at 37 °C, after which the wells were washed 3 times with TBS, pH 8.0. For cell lines, wells were fixed by the addition of 100 μl of 4% paraformaldehyde in TBS for 10 min, stained by the addition of 1% bromphenol blue in 1% acetic acid for 5 min, and washed 3 times with 1% acetic acid, and color was developed by the addition of 100 μl of 10 mM Tris-HCl, pH 10.0. Adherence of EOS was quantified by an assay of EOS peroxidase. Briefly, 100 μl of HBSS was added to wells with 100 μl of a solution containing 0.1% Triton-X100, 1 mM o-phenylenediamine dihydrochloride (Sigma-Aldrich), 1 mM H_2O_2, and 55 mM Tris, pH 8.0 (30). After 30 min of color development at 22 °C, 50 μl of 4 M H_2SO_4 was added to quench the reaction. Absorbances for EOS and cell lines were read at 490 and 595 nm, respectively, in an EL microplate reader (Bio-Tek Instruments, Winooski, VT). Percentages of adherent EOS were calculated by dividing the absorbance per experimental well by the absorbance from uncoated wells containing 100% of input EOS and multiplying by 100. Percentages of adherent cell lines were calculated by reference to a parallel experiment in which adherent cells were assayed by both the bromphenol blue method and the CellTiter-Glo luminescent cell viability reagent (Promega, Madison, WI), which can be used to measure both adherent and total cells. Absorbances and signal values were within the linear range of the detector as determined by standard curves for all three methods. Mean absorbances, S.D., or S.E. are indicated and are from experiments performed on at least three separate occasions. For inhibition studies, values are given as percentages of the absorbances obtained in the presence of respective isotype control mAbs.

**Cell Adhesion under Flow Conditions**—The assay for adhesion under flow conditions was based on our experience with platelets (31) and the experience of others with EOS (32–35). We used a parallel plate flow chamber (Glycotech, Rockville, MD). A silicone rubber gasket with a thickness of 0.254 mm and a flow path width of 2.5 mm was placed on polystyrene 3.5-cm-diameter tissue culture Petri dishes (BD Biosciences) previously coated with VCAM-1 constructs at 220 nm. Coating of dishes was done with 1 ml of protein solution in TBS, pH 8.0, for 2 h at 37 °C, then dishes were washed with TBS and blocked with heat-inactivated FBS for 15 min. The inlet of the flow chamber was connected to a buffer reservoir by silicone tubing, and the outlet was connected with a peristaltic pump to draw fluid through the flow chamber. The chamber was pre-perfused with prewarmed TBS. EOS were suspended in HBSS, 0.2% BSA at 2 × 10^6 cells/ml (35) and incubated with or without IL-5 (50 pg/ml final concentration) and with mAbs (10 μg/ml) for 5 min at 22 °C before perfusion and placed in a water bath at 37 °C, and the inlet was connected to the EOS suspension. Perfusion was performed at a shear rate of 150 s^{-1}, a rate previously used for purified EOS (34). This rate corresponds to a shear force of 0.15 newtons·m^{-2} (1.5 dyn/cm^2), assuming a viscosity value of 0.001 newton·sec·m^{-2} (0.01 poise or dyn·sec/cm^2) (36). This value is within the range of shear force (1–30 dyn/cm^2) considered physiological for post-capillary venules, a relevant location for leukocyte extravasation (32, 33, 37). After perfusion for 2 min, the dish was taken out and washed 3 times with TBS. Perfusions were performed within 4 h of EOS purification, and variables were tested in a randomized order that was different among donors. Adherence of EOS was quantified by the EOS peroxidase assay as described above, except that 1 ml of HBSS and 1 ml of substrate solution were added to each dish. After 30 min of color development, 200 μl of this mixture was transferred to a well in a 96-well plate, and 50 μl of 4 M H_2SO_4 was added to quench the reaction. Absorbance was read as above.

**Enzyme-linked Immunosorbent Assay**—Wells were coated in triplicate with 100 μl of VCAM-1 constructs in TBS, pH 8.0, on polystyrene 96-well non-tissue culture-treated plates (BD Biosciences) for 3 h at 37 °C. Subsequent steps were performed in phosphate-buffered saline with 1% BSA. Wells were decanted, blocked with 100 μl of 1% BSA for 15 min at 37 °C, washed, and then incubated for 1 h at 22 °C in 100 μl of 1 μg/ml mAb 1G11B1 or with mAb 2A6.21 diluted 1:50,000 from cell culture supernatant. Wells were washed twice, and 100 μl of alkaline phosphatase-conjugated donkey anti-mouse diluted 1:5000 was added for 1 h at 22 °C. Wells were washed 3 times followed by the addition of 100 μl of 1 mg/ml Sigma 104 phosphatase substrate (Sigma-Aldrich) in TBS, pH 9.0. Absorbances were read at 409 nm in the EL microplate reader.

**Flow Cytometric Analysis of Integrin Expression**—EOS (4–5 × 10^5) in 0.2 ml of HBSS, 0.2% BSA or cell lines (4–5 × 10^5) in RPMI, 0.2% BSA were incubated with or without 50 pg/ml IL-5 (R&D systems, Minneapolis, MN), 1 mM MnCl_2 (Sigma-Aldrich), or 100 nM PMA (Sigma-Aldrich) at 37 °C for 20 min along with primary mAbs. Cells were chilled on ice for 5 min, pelleted, washed with 0.25 ml of HBSS or RPMI, resuspended in 0.25 ml of HBSS or RPMI, and then incubated with secondary mAb for 30 min at 4 °C in the dark. Primary mAbs were incubated in final concentrations of 2.5 μg/ml, and secondary mAb fluorescein isothiocyanate-conjugated goat anti-mouse IgG was incubated at 20 μg/ml. After secondary mAb incubation, EOS or cell lines were pelleted, fixed by resuspension in 1% paraformaldehyde, 67.5 mM sodium cacodylate, 113 mM NaCl, pH 7.2, stored at 4 °C in the dark, washed with HBSS or RPMI, and analyzed. Fluorescence measurements were collected on a FACS Calibur (BD Biosciences; available through the Flow Cytometry Facility, Comprehensive Cancer Center, University of Wisconsin, Madison). Data were collected from 10,000 cells per condition using Cellquest software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Fixed cells were gated based on forward and side scatter. The specific geometric mean channel fluorescence (gmCF) is reported. The specific gmCF was obtained by subtracting the gmCF of the isotype control from the gmCF of the experimental sample according to the following equation, where gmF is geometric mean fluorescence: gmCF = [1024 × (log gmF of experimental sample)] − [1024 × (log gmF of isotype control sample)]. A gmCF value equal to 110 is equivalent to a ratio of the gmF experimental sample to gmF isotype control.
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trol sample equal to 1.3. Subunits or activation states with values less than 110 were considered either not expressed or not recognized by mAbs.

Statistics—Results were analyzed by one-way analysis of variance (ANOVA) with Dunnnett’s or Tukey-Kramer multiple comparison post test on GraphPad Prism software or by two-tailed t test where appropriate.

RESULTS

To learn how EOS interact with modules 1 and 4, we expressed constructs containing the two modules either in combination or separately (Fig. 1A). Soluble recombinant human 6d-VCAM-1, 7d-VCAM-1, 4–7VCAM-1, 1–3VCAM-1, and 1–2VCAM-1 constructs migrated with expected molecular masses of ~66, 76, 45, 34, and 24 kDa, respectively, on non-reduced SDS-PAGE gels (Fig. 1B). The 1–3VCAM-1 construct migrated as a doublet, which is likely the result of differential N-glycosylation of two sites in module 3. A similar difference in apparent molecular weight was also detected previously in immunoprecipitations of soluble 1–3VCAM-1 from transiently transfected COS cells (38).

The coating efficiencies of VCAM-1 constructs on polystyrene microtiter wells were determined by direct enzyme-linked immunosorbent assay with two mAbs that recognize different epitopes in recombinant soluble VCAM-1; mAb 1.G11B1 binds an epitope in modules 1–3, and mAb 2A6.21 recognizes an epitope in the C-terminal sequence purification tag introduced into recombinant proteins by the pCOCO expression system based on Western blotting and enzyme-linked immunosorbent assays involving VCAM-1 constructs. The 6d-VCAM-1, 7d-VCAM-1, and 4–7VCAM-1 constructs were equally efficient in coating polystyrene, whereas 1–3VCAM-1 coated ~3-fold less efficiently on a molar basis, and there was little or no detectable coating of 1–2VCAM-1 (not shown). To achieve equal coatings of 1–3VCAM-1, we calculated the amount required to match the signal generated from the coating of other VCAM-1 constructs. These calculations were then verified by subsequent enzyme-linked immunosorbent assays (not shown). When layers of VCAM-1 coated on glass coverslips were stained and examined by immunofluorescence microscopy, the VCAM-1 layers appeared bright and homogeneous (not shown).

In comparative static adhesion assays, EOS adhered to 6d-VCAM-1 and 7d-VCAM-1 with identical dependence on the coating concentrations of the two proteins, and the two proteins supported equal maximum numbers of adherent EOS (Fig. 2A). Approximately 50% of purified EOS adhered at the highest coating concentrations. The mAbs recognizing α4 (HP2/1) and β1 (mAb 13) subunits inhibited adhesion of unstimulated EOS to 6d-VCAM-1 (Fig. 2B) and 7d-VCAM-1 (Fig. 2C). Inhibitory mAbs that recognize subunits of other potential VCAM-1 integrin receptors, including αD (217I and 240I), β2 (TSI/18), or β7 (Fib 504), did not inhibit adhesion of unstimulated EOS to 6d-VCAM-1 or 7d-VCAM-1. Indeed, these mAbs stimulated adhesion. Although there was no significant inhibition of mean adhesion of unstimulated EOS to 6d-VCAM-1 or 7d-VCAM-1 by mAb 2LPM19c recognizing the αM subunit (Fig. 2C), there was variability in blocking the adhesion to 7d-VCAM-1 (Fig. 2C) such that adhesion of unstimulated EOS from two of five donors to 7d-VCAM-1 was partly inhibited by mAb 2LPM19c. There were no occasions, however, in which anti-αM partially blocked adhesion to 6d-VCAM-1. The 1–3VCAM-1 construct supported adhesion of EOS; such adhesion was inhibited by mAbs to α4 and β1.
FIGURE 2. Adhesion of EOS to recombinant soluble 6d-VCAM-1 and 7d-VCAM-1. A, the 6d- and 7d-splice forms were coated onto polystyrene 96-well microtiter plates and assayed in cell adhesion of EOS incubated with or without 50 pg/ml IL-5 on 6d-VCAM-1 or 7d-VCAM-1. B–E, antibody blocking of adhesion of unstimulated EOS to 6d-VCAM-1 (B) or 7d-VCAM-1 (C) and of EOS incubated with 50 pg/ml IL-5 to 6d-VCAM-1 (D) or 7d-VCAM-1 (E). Results are the mean and S.E. of adhesions performed in triplicate from five separate donors. p < 0.001 (*) and p < 0.05 ($) comparing adhesion in the presence of the inhibitory mAb to adhesion in the presence of the isotype control mAb; § indicates variable inhibition of two of five donors; *, p < 0.001 represents a stimulation of adhesion in comparison to the adhesion in the presence of the isotype control mAb; one-way ANOVA with Dunnett's post-test.
although maximum adhesion was lower in comparison to the concentration to the left (Fig. 2). Instead, antibodies to 7d-VCAM-1, and the mAb to 6d-VCAM-1, did not inhibit adhesion of IL-5-treated EOS to 6d-VCAM-1 or 7d-VCAM-1. There was a coating-dependent increase in the static adhesion of EOS to 6d-VCAM-1 and 7d-VCAM-1 were assayed in adhesion of EOS incubated with or without 50 pg/ml IL-5. Antibody blocking of unstimulated EOS (Fig. 2A), the 4–7VCAM-1 construct was coated onto polystyrene 96-well microtiter plates and assayed in adhesion of EOS incubated with or without 50 pg/ml IL-5, a concentration within the physiological range measured in the serum of human asthmatics (42–44), exhibited an ~1.5-fold enhancement in maximum adhesion to both 6d-VCAM-1 and 7d-VCAM-1 are equally efficient in supporting adhesion of unstimulated EOS; α4β1 is the major integrin on unstimulated EOS that mediates adhesion to 6d-VCAM-1, 7d-VCAM-1, and 1–3VCAM-1, and αMβ2 may be involved in the adhesion of unstimulated EOS from some donors to 7d-VCAM-1.

EOS incubated with IL-5, a cytokine found in the blood and airway of asthmatics (39), exhibit enhanced adhesion to human endothelial cells (40) and diverse ligands, including albumin (7, 8, 41). To learn whether IL-5 stimulates static adhesion to the soluble VCAM-1 splice forms, EOS were incubated with IL-5 and studied in cell adhesion assays. EOS incubated with 50 pg/ml IL-5, a concentration within the physiological range.

The adhesion of unstimulated EOS to 6d-VCAM-1 or 7d-VCAM-1 is consistent with the finding that exposure of EOS to IL-5 induces an increase in reactivity of the CBRM1/5 conformation-sensitive mAb recognizing the I domain of αM (45, 46).

To investigate whether the differences in integrin usage in static adhesion of EOS to 6d-VCAM-1 and 7d-VCAM-1 were due to module 4, we assayed static adhesion of EOS to 4–7VCAM-1. There was a coating-dependent increase in the adhesion of unstimulated EOS to 4–7VCAM-1 (Fig. 3A), although maximum adhesion was lower in comparison to the adhesion of unstimulated EOS to 6d-VCAM-1 or 7d-VCAM-1 (compare Fig. 2A to 3A). Antibody blocking indicated that unstimulated EOS adhere to 4–7VCAM-1 via contributions from α4β1 and αMβ2, since mAbs recognizing α4, β1, αM, and β2 subunits all inhibited adhesion by ~50% (Fig. 3B). Consistent with the antibody blocking results, incubation of EOS with the structurally unrelated PI3K inhibitors wortmannin or LY294002, which inhibit αMβ2-dependent but not α4β1-dependent adhesion of EOS (41), blocked adhesion of unstimulated EOS to 4–7VCAM-1 (Fig. 4A) but did not block adhesion to 6d-VCAM-1 (Fig. 4B). Incubation with IL-5 caused an ~2-fold increase in maximum adhesion of EOS to 4–7VCAM-1 (Fig. 3A). Adhesion after IL-5 treatment was inhibited by mAbs recognizing the αM or β2 subunit and not inhibited by mAbs recognizing the α4 or β1 subunit (Fig. 3C). Incubation of IL-5-treated EOS with wortmannin or LY294002 inhibited adhesion less well to 4–7VCAM-1 compared with without IL-5 (compare Figs. 4, A and C), and there was no inhibition of adhesion to 6d-VCAM-1 after IL-5 stimulation of EOS (Fig. 4D). The adhesion of unstimulated EOS to module 4 of 4–7VCAM-1 is, therefore, different from the adhesion to constructs containing module 1, 2, 3, or 4 in that unstimulated EOS adhere less efficiently to module 4 in comparison to module 1, and adhesion to module 4 involves the use of two integrins, α4β1 and αMβ2, whereas adhesion to module 1 involves only α4β1. Adhesion of EOS to module 4 is preferentially enhanced by incubation with IL-5. Such enhanced adhesion in response to IL-5 is resistant to inhibition of PI3K.

As described in the introduction, EOS express putative VCAM-1 receptors other than α4β1 and αMβ2, namely α4β7 and αDβ2 (7–10). Antibodies to β7, αD, and β2 enhanced static adhesion to VCAM-1 by unstimulated EOS (Fig. 2), which complicates the interpretation of antibody blocking experiments. We, therefore, studied the AML14.3D10 and EoL-3 eosinophilic cell lines and Jurkat T lymphocytic cell line, which
express simpler repertoires of VCAM-1-interacting integrins in comparison to EOS (Table 1). The αD subunit was not detected on EoL-3 cells, and β7 subunit was undetectable on AML14.3D10 cells (Table 1). Neither αD nor β7 was detected on Jurkat cells (Table 1). Finally, the αM subunit was not detected on AML14.3D10, EoL-3, or Jurkat cells (Fig. 5). These flow cytometric results are consistent with the findings that the promoter of the αM gene is functionally inactive when transfected into Jurkat cells (47), and αM is expressed on only the most differentiated granulocytes (47).

AML14.3D10 (Fig. 6A) or EoL-3 cells (Fig. 6B) did not adhere to 4–7VCAM-1 but did adhere to constructs containing module 1, including 6d-VCAM-1 (Fig. 6A and B), 7d-VCAM-1 (Fig. 6A and B), and 1–3VCAM-1 (not shown). Similarly, Jurkat cells adhered to 6d-VCAM-1 (Fig. 6C), 7d-VCAM-1 (Fig. 6C), and 1–3VCAM-1 (not shown). Antibody blocking indicated that the adhesion of eosinophilic leukemic and Jurkat cells to 7d-VCAM-1 (Fig. 7) is mediated by α4β1 since mAbs recognizing α4 and β1 subunits completely blocked the adhesion of AML14.3D10 (Fig. 7A), EoL-3 (Fig. 7B), and Jurkat cells (Fig. 7C). Antibodies recognizing β2 or β7 did not inhibit adhesion of cell lines to 7d-VCAM-1. These results, therefore, corroborate the antibody and PI3K blocking results for EOS, which indicate that αMβ2 is importantly involved in adhesion of EOS to module 4, whereas α4β1 principally mediates adhesion to module 1.

Even though the eosinophilic cell lines lacking αMβ2 expression did not adhere to 4–7VCAM-1, adhesion of EOS to 4–7VCAM-1 was partially blocked by mAbs to α4 and β1 (Fig. 3B), indicating that α4β1 contributes to adhesion of EOS to module 4. We, therefore, investigated if adhesion to 4–7VCAM-1 could be induced in the cell lines by activators of α4β1. We related adhesion of cell lines to activation states of β1 by probing with three conformation-sensitive mAbs: N29, HUTS-21, and 9EG7. N29 recognizes an activation-induced epitope in the N-terminal region of the plexins, semaphorins, and integrins (PSI) domain (48), HUTS-21 recognizes an activation-induced epitope in the hybrid domain (49), and 9EG7 recognizes an activation-induced epitope in the epidermal growth factor domains of the “leg” (50, 51). The locations of these epitopes in various structures assumed by αVβ3 or αIIbβ3 suggest that the antibodies recognize increasingly activated forms in the order N29 < HUTS-21 < 9EG7 (52, 53).

Both eosinophilic cell lines, like purified blood EOS, reacted with N29 (Table 1). EoL-3 cells, unlike purified blood EOS, also reacted with HUTS-21 and 9EG7, and AML14.3D10 cells did not react with either mAb unless stimulated with Mn2+ or PMA (Table 1). Incubation of eosinophilic cell lines with Mn2+ or PMA enhanced α4β1-mediated adhesion of AML14.3D10 (Fig. 6A) or EoL-3 (Fig. 6B)
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### TABLE 1

Expression and activation states of integrins of EOS and cell lines

Results are the mean and S.D. of experiments from at least four different donors for EOS or three different experiments for cell lines. gMCF, geometric mean channel fluorescence, detected with fluorescein isothiocyanate-conjugated secondary mAb; data are shown as the mean specific gMCF in assays performed in HBSS buffer for EOS or RPMI for cell lines. Cells were coincubated at 37 °C with the primary mAb for 20 min in the presence or absence of 50 pg/ml IL-5, 1 mM Mn2⁺, or 100 nM PMA. Epitopes exhibiting a specific gMCF less than 110 are counted as not recognized. Note that in addition to the aforementioned subunits, the following subunits were also expressed by EOS or cell lines: EOS, α6; AML14.3D10, α5; EoL-3, α5, α6 (very low, variable); Jurkat, α5.

| Specific gMCF | Activated β1 subunit | Total β1 | Total α4 | Total αD | Total β7 |
|---------------|----------------------|----------|----------|----------|----------|
|               | N29                  | HUTS-21  | 9EG7     | MAR4     | 9F10     | 2041     | Fib 504 |
| EOS           |                      |          |          |          |          |          |          |
| Buffer        | 470 ± 150            | <110     | <110     | 560 ± 180| 570 ± 180| 180 ± 60 | 400 ± 90 |
| IL-5          | 410 ± 200            | <110     | <110     | 660 ± 120| 610 ± 270|          |          |
| Mn2⁺          | 460 ± 80             | <110     | <110     | 510 ± 110| 520 ± 150|          |          |
| AML14.3D10    |                      |          |          |          |          |          |          |
| Buffer        | 890 ± 20             | <110     | <110     | 930 ± 40 | 830 ± 70 | 160 ± 110| <110     |
| Mn2⁺          | 890 ± 30             | 450 ± 110| 410 ± 90 | 950 ± 40 | 830 ± 40 |          |          |
| PMA           | 840 ± 40             | 400 ± 10⁸| 380 ± 4⁷ | 930 ± 10 | 830 ± 30 |          |          |
| EoL-3         |                      |          |          |          |          |          |          |
| Buffer        | 1210 ± 10            | 470 ± 140| 450 ± 150| 1320 ± 20| 1310 ± 20| <110     | 620 ± 140|
| Mn2⁺          | 1200 ± 30            | 850 ± 10⁰| 620 ± 16⁰| 1310 ± 50| 1310 ± 10|          |          |
| PMA           | 1170 ± 80            | 810 ± 13⁰| 770 ± 16⁰| 1290 ± 80| 1230 ± 120|          |          |
| Jurkat        |                      |          |          |          |          |          |          |
| Buffer        | 1160 ± 190           | 1200 ± 140| 1150 ± 170| 1660 ± 210| 1640 ± 240| <110     | <110     |
| Mn2⁺          | 1300 ± 190           | 1450 ± 180| 1230 ± 210| 1610 ± 260| 1600 ± 280|          |          |
| PMA           | 1190 ± 200           | 1340 ± 190| 1230 ± 220| 1600 ± 240| 1600 ± 240|          |          |

*Reactivity is significantly different from incubation in RPMI buffer, p < 0.05.

*Reactivity is significantly different from incubation in RPMI buffer, p < 0.01.

[FIGURE 5. Expression of αMβ2 on EOS and cell lines. A–D, flow cytometric histograms of total αM (dotted line) and total β2 (bold line) subunit expression on EOS (A), AML14.3D10 (B), EoL-3 (C), and Jurkat cells (D). The histogram generated from incubation of cells with the isotype control mAb is also shown (normal line).]

To test the generality of results of the static adhesion assays, we studied adhesion of EOS to insolubilized VCAM-1 in a parallel plate chamber using EOS peroxidase as a readout. In the absence of IL-5, EOS adhered similarly to 6d-VCAM-1 and 7d-VCAM-1 concomitant with ~6- and 2-fold increases in reactivities of mAb HUTS-21 with AML14.3D10 cells and EoL-3 cells, respectively (Table 1). Even after stimulation with Mn²⁺ or PMA, however, AML14.3D10 cells failed to adhere to 4–7VCAM-1 (Fig. 6A), and there was only slight adherence of EoL-3 cells to the highest coating concentrations (Fig. 6B). Jurkat cells reacted strongly with mAbs HUTS-21 and 9EG7 (Table 1), adhered to 4–7VCAM-1, and were stimulated to adhere further by Mn²⁺ or PMA (Fig. 6C). The adhesion of unstimulated and stimulated Jurkat cells to 4–7VCAM-1 was mediated solely by α4β1 based on antibody blocking assays (not shown). Thus, in the absence of αMβ2 expression, α4β1-mediated adhesion to module 4 apparently requires a highly active form of α4β1 that is not present on either unstimulated or IL-5-stimulated EOS (Table 1).

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and 7d-VCAM-1 (not shown). The adhesion was specific compared with the FBS blocker (Fig. 8 and not shown). After IL-5 treatment, adhesion under flow to 7d-VCAM-1 was low (Fig. 8A) and not greater than to FBS alone (Fig. 8B). Anti-α4 inhibited adhesion of unstimulated EOS to 7d-VCAM-1 (Fig. 8B). mAb 2LPM19c against αM also inhibited adhesion of unstimulated EOS to 7d-VCAM-1 (Fig. 8A and B). With IL-5-treated EOS, in contrast, there was increased adhesion to 7d-VCAM-1 upon treatment with 2LPM19c, resulting in significant specific adhesion compared with FBS (Fig. 8B), at a level similar to that in the absence of IL-5 and antibody (Fig. 8A).

**DISCUSSION**

The present study has revealed that VCAM-1 modules 1 and 4 have unique functions in static adhesion of human EOS. Adhesion of unstimulated purified blood EOS to constructs containing module 1 was robust and mediated by α4β1, whereas there was less adhesion of unstimulated EOS to 4–7VCAM-1 that contained module 4. Adhesion to module 4, surprisingly, was mediated by a combination of α4β1 and αMβ2. The enhanced adhesion after IL-5 treatment was sensitive to blocking with anti-αMβ2. The conclusion that module 4 is preferentially engaged by αMβ2 is strengthened by studies involving inhibitors of PI3K that preferentially interfere with αMβ2-mediated adhesion of EOS (41) and by eosinophilic cell lines that do not express αMβ2. Thus, eosinophilic cell lines adhered poorly to 4–7VCAM-1 even when the activity of α4β1 was up-regulated by incubation with Mn²⁺ or PMA and adhesion of unstimulated EOS to 4–7VCAM-1, but not 6d-VCAM-1, was diminished in the presence of the PI3K inhibitors, wortmannin, or LY294002. Jurkat cells, which constitutively express a highly active form of β1 as assayed by reactivity with activation-specific antibodies, adhered to 4–7VCAM-1 by α4β1 even in the absence of stimulation.
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Tandem duplication of modules of VCAM-1, therefore, has resulted in two binding sites that interact with distinct allosteric populations of α4β1 and αMβ2 on EOS. The conclusion that VCAM-1 contains distinct sites of integrin recognition is consistent with deletion, mutagenesis, and antibody blocking studies. Indeed, although COS cells expressing 7d-VCAM-1 missing module 1 support adhesion of Ramos cells, a construct both missing module 1 and containing a mutation of glutamine or leucine to serine or alanine in the QIDSPL motif of module 4 fails to support adhesion (12). Transfected CHO cells expressing a form of 7d-VCAM-1 containing only modules 1–3 or 4–7 support adhesion of B lymphoma cells, and deletion of both modules 1 and 4 completely abrogates the adhesion (19). The 4B9 mAb recognizing module 1 of 7d-VCAM-1 does not completely block adhesion of Ramos cells to IL-1-stimulated HUVEC monolayers, whereas coincubation of monolayers with GH12 mAb recognizing module 4 in combination with 4B9 completely abolishes such adhesion (21).

Our studies indicate that α4β1 in a minimally activated state on EOS recognizes module 1. Module 4 is recognized by α4β1 in conjunction with αMβ2. Activation of EOS by IL-5 causes αMβ2 to assume a dominant role in recognition of module 4 and a significant role in recognition of module 1. Studies of lymphocytic and monocytic cells are in accord with these conclusions. The 38-β1 B lymphoma cell line expressing α4β1 requires a 2-fold greater Mn2+ concentration to achieve half-maximal attachment to CHO monolayers expressing human modules 4–6 compared with modules 1–3 (19). Adhesion of the U937 human myelomonocytic cell line to module 1 occurs at 37 °C, whereas adhesion to module 4 occurs at 37 °C but not 4 °C (20). Taken together, these results show that α4β1-mediated adhesion to module 4 requires a different, presumably higher activation state, whereas adhesion to module 1 is constitutive and less dependent on activation. These results are consistent with our present findings for EOS and the eosinophilic and T lymphocytic cell lines in that activation by IL-5, Mn2+, or PMA results in enhanced adhesion to module 4, whereas adhesion to module 1 takes place without activation.

Adhesion of EOS to VCAM-1 constructs under flow conditions demonstrated interesting differences from adhesion under static conditions. One difference was that IL-5 did not increase adhesion to 7d-VCAM-1, but rather there was low adhesion to 7d-VCAM-1 under flow. A second difference was that whereas anti-αM mAb inhibited adhesion of unactivated EOS to 7d-VCAM-1 under flow, adhesion of IL-5-treated EOS to 7d-VCAM-1 was increased, restoring adhesion to the original level of unstimulated EOS in the absence of antibody. The simplest explanation for the results is that in its resting state αMβ2 together with α4β1, presumably through cooperation between the two integrins, contributes to arrest, whereas the IL-5-induced conformation of αMβ2 inhibits arrest. Thus, the constitutive state of αMβ2, which is susceptible to PI3K inhibition, is sufficient for it to be involved in adhesion to VCAM-1 under flow. The IL-5-induced conformation of αMβ2, which is refractory to PI3K inhibition, down-regulates adhesion to 7d-VCAM-1.

VCAM-1 is a member of cell surface integrin counter-receptors of the Ig superfamily (54), including ICAM-1, ICAM-2, ICAM-3, and MAdCAM-1 (mucosal addressin cell adhesion molecule 1). Each of these proteins contains putative integrin binding sequences in the linker between β strands C and D that is at the position of the IDSPL sequence in modules 1 and 4 of human VCAM-1 (55). Integrins αMβ2 (56) and αLβ2 (57) contain an I domain in the α subunit. Through the I domain, αLβ2 recognizes the IETPL, LETSL, or LETSL binding sequences in module 1 of ICAM-1, ICAM-2, or ICAM-3, respectively (12, 58–62). In contrast, α4β1 or α4β7, which lack an I-domain in α4 but have the I-like domain in β1 or β7 (54), ligate the IDSPL sequence in VCAM-1 or IDTSL sequence in MAdCAM-1.
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Mucosal addressin cell adhesion molecule 1 (mucosal addressin cell adhesion molecule 1) (55, 63). Thus, β2 integrins prefer glutamate at the second position of the ligand sequence, whereas α4 integrins prefer aspartate. Interestingly, αMβ2 has been shown to engage an aspartate-containing DQR sequence in module 3 of ICAM-1 (64), consistent with the possibility that the aspartate-containing sequence in module 4 of VCAM-1 engages αMβ2. αMβ2 also interacts with many ligands that are unrelated to each other, VCAM-1, or the ICAMs (65, 66).

The IDSPL and (I/L)ETSL integrin recognition motifs in VCAM-1 and the ICAMs, respectively, are homologous except for the P/S change in the fourth position (12). In crystal structures of the I domains and the I-like domains, ligand binding involves coordination of a metal ion that is buried in the integrin headpiece to the ligand glutamate or aspartate (46, 52, 67–69). The ligand orients its glutamate or aspartate in a position that facilitates recognition by specific integrins, making extensive use of hydrophobic, hydrophilic, ionic, and hydrogen bonding interactions for docking to the integrin (54). The α7 helix at the C terminus of the αM I domain undergoes a 10-Å swing in response to crystallization in Mg²⁺ versus Mn²⁺ (46, 69), and IL-5 exposes the epitope within the I domain of αM recognized by mAb CBRM1/5 (45). Thus, conformational changes within αMβ2 may better orient the integrin for engagement of aspartate residues in VCAM-1.

α4β1 of unstimulated EOS partly supported static adhesion to 4–7VCAM-1, whereas α4β1 of the eosinophilic cell lines did not support static adhesion even though α4β1 was expressed at higher levels and activation states on the eosinophilic cell lines in comparison to EOS. Thus, one must postulate that EOS have mechanisms to acquire recognition of module 4 by α4β1 that are different from the activation mechanisms involving Mn²⁺ or PMA. We previously reported that β1 of EOS adherent to 7d-VCAM-1, but not of eosinophilic cell lines, localizes to podosomes (25), structures that are important in migration and proteolysis of matrix proteins (70). The difference in adhesive behavior of α4β1 between EOS and cell lines may, therefore, be related to differential activation of signaling pathways that lead to podosome formation. In particular, engagement of module 4 by αMβ2 on EOS may alter the adhesive activity of α4β1 by integrin trans-regulation or cross-talk (71). T cells expressing a form of αLβ2 with a deleted I-domain display enhanced constitutive β1 integrin activity (72). Ligation of VCAM-1 by α4β1 on T cells enhances αLβ2-mediated adhesion (73) and migration (71), indicating that αLβ2 and α4β1 are able to synergize and mutually regulate the activity of one another. αMβ2 on EOS may similarly trans-regulate α4β1 and render α4β1 competent to engage module 4 of VCAM-1. α4β1 on eosinophilic cell lines, which do not express αMβ2, is not trans-regulated by αMβ2 and is, therefore, unable to ligate module 4. Trans-regulation may also explain the puzzling finding of stimulation of static adhesion of EOS to 6d-VCAM-1 and 7d-VCAM-1 after incubation with mAbs recognizing β2, β7, or αD subunits (Fig. 2, B and C), i.e. engagement of β2 or β7 integrins by antibodies may trans-regulate α4β1. Similarly, a possible explanation to the increased adhesion of IL-5-treated EOS to 7d-VCAM-1 under flow after preincubation with anti-αM is that the mAb, when interacting with the IL-5-induced conformation of αMβ2, enhances αMβ2-mediated trans-regulation and stimulates α4β1-mediated adhesion. In the case of the β1 subunit, a 12-amino acid sequence in the β1 headpiece is recognized by both inhibitory and stimulatory mAbs (74), indicating that there is a fine line between inhibition and activation of integrins.

α4β1 supports rolling and firm adhesion of EOS on VCAM-1 (7–9, 75), and α4β1 and αMβ2 mediate transmigration of EOS (22, 76). EOS purified from bronchoalveolar lavage fluid in antigen-challenged human subjects exhibit enhanced αMβ2 activity and increased migration compared with blood EOS (8, 77). Such EOS recruited to the asthmatic airway exhibit increased αMβ2 expression compared with EOS in blood (78–80). Activation of αMβ2 (45, 77) and formation of podosomes of EOS (25), structures believed important in cell migration (70), are induced by exposure to IL-5, which is up-regulated in the blood and airway of human asthmatics (42, 81–83). In addition, the chemokines RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemotactic protein-3, and complement activation factor-5 expose an activation-sensitive epitope of αMβ2 (84) and promote migration of blood EOS (84–87).

A multistep scenario linking the enhanced αMβ2 phenotype of bronchoalveolar lavage EOS and recruitment of EOS to the asthmatic lung is that minimally stimulated EOS first roll as a result of α4β1 engagement of module 1 of VCAM-1-expressing endothelium and then arrest involving both α4β1 and αMβ2, presumably interacting with both modules 1 and 4. Exposure of circulating EOS to IL-5 and allosteric alteration of αMβ2 may disrupt the orchestrated interplay between αMβ2 and α4β1, with the paradoxical effect of not facilitating arrest under flow but rather down-regulating it.

A scenario in which α4β1 on circulating EOS is primarily responsible for arrest on VCAM-1 and extravasation into lung is supported by the findings that α4β1 activation state on EOS in blood, as assessed by reactivity with the activation-sensitive anti-β1 mAb N29, varies in asthmatic patients, and correlates with decreased lung function in an inhaled corticosteroid withdrawal study and with a measurement of airway inflammation (exhaled nitric oxide) after steroid withdrawal (88). We propose that activation of αMβ2 in response to IL-5 is important in subsequent phases in recruitment of already arrested EOS, possibly for de-adhesion from VCAM-1 to allow transmigration, and for transmigration itself through endothelium and tissue through interaction with various extracellular matrix ligands (77).

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