All-*trans*-Retinoic Acid Induces Integrin-Independent B-Cell Adhesion to ADAM Disintegrin Domains[†]

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ABSTRACT: Cell adhesion is an integral aspect of immunity facilitating extravasation of immune cells during homing and activation. All-*trans*-Retinoic acid (*t*-RA) regulates leukocyte differentiation, proliferation, and transmigration. However, the role of *t*-RA in immune cell adhesion is poorly defined. In this study, we evaluated the impact of *t*-RA and its metabolism on B and T cell adhesion. Specifically, we address the impact of *t*-RA on the adhesive properties of the human mature B and T cell lines RPMI 8866, Daudi and Jurkats. The effect of *t*-RA exposure on cell adhesion to vascular cell adhesion molecule-1 (VCAM-1), a well-established integrin counter receptor involved in immunity, and to nonconventional ADAM integrin ligands was assessed. We show for the first time that *t*-RA potently induces B cell adhesion in an integrin-independent manner to both VCAM-1 and select ADAM disintegrin domains. Using retinoid extraction and reverse-phase HPLC analysis, we identify the retinoid that is functionally responsible for this augmented adhesion. We also provide evidence that this novel *t*-RA adhesive response is not prototypical of lymphocytes since both Daudi and Jurkats do not alter their adhesive properties upon *t*-RA treatment. Further, the *t*-RA metabolic profiles between these lineages is distinct with 9-*cis*-retinoic acid being exclusively detected in Jurkat media. This study is the first to demonstrate that *t*-RA directly induces B cell adhesion in an integrin-independent manner and is not contingent upon *t*-RA metabolism.

Vitamin A (retinol) and its analogues, retinoids, are essential for many critical life processes, including establishment and maintenance of immunity (*1*). In 1928, Green and Mellanby referred to vitamin A as an "anti-infective agent" (*2, 3*). The literature is sated with the influences of vitamin A on the growth and function of immune cells in culture systems (*4–9*). Retinoids profoundly affect immune function by regulating differentiation, proliferation, and transmigration of leukocytes (*7–10*).

Retinoic acid, a biologically active metabolite of vitamin A, is specifically responsible for lymphocyte differentiation and proliferation. All-*trans*-Retinoic acid (*t*-RA[1]) and 9-*cis*-retinoic acid (9-*cis*-RA) are potent activators of retinoic acid receptors (RAR α, *β*, and *γ*) and retinoid X receptor (RXR α, *β*, and *γ*) (*11–15*). Retinoids act as ligand-dependent transcription factors with *t*-RA activating RARs, while 9-*cis*-RA serves as a pan-agonist for RARs and RXRs. These receptors bind as heterodimers or homodimers to specific retinoid response elements, RARE and RXRE, in the promoter of target genes (*16*). However, RXR forms homodimers or heterodimers with other ligand-dependent members of the nuclear receptor family including thyroid hormone, vitamin D receptors, and more recently perioxisome proliferating activator receptor gamma (PPAR*γ*) (*17*).

Retinoid availability in cells dictates which heterodimer/homodimer partners will activate transcription of retinoid responsive genes. The cytochrome P450 (CYP) family of monooxygenases are involved in the biotransformation of variety of endogenous and exogenous compounds including *t*-RA and 9-*cis*-RA. The predominant pathway is oxidation at the 4-position of the cyclohexenyl ring to form 4-hydroxy- and 4-oxo-retinoic acid or 4-hydroxy- and 4-oxo-9-*cis*-retinoic acid (*18, 19*). Several human CYP isoforms are capable of metabolizing *t*-RA including CYP2C8, CYP3A4, CYP2C9, and more recently CYP26 (*20–22*).

Recently, *t*-RA has attracted considerable attention for its role in modulating cellular processes including establishment and maintenance of immunity. For example, *t*-RA impacts adhesion to molecules such as vascular cell adhesion molecule-1 (VCAM-1) by modulating integrin expression levels (*23*). Integrins are a well-characterized superfamily of adhesion receptors that play a central role in immunity (*24–26*). For example, integrin recognition of the VCAM-1 counter receptor is essential in arresting peripheral blood leukocytes to the endothelial vessel wall during an immune response (*27*). The importance of *t*-RA in immunity is further underscored by recent reports that *t*-RA imprints integrin expression in certain immune cell subsets and regulates the

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[1] Abbreviations: *t*-RA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9-*cis*-RA, 9-*cis*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; PPAR*γ*, perioxisome proliferating activator receptor gamma; ADAM, a disintegrin and metalloprotease; VCAM-1, vascular cell adhesion molecule-1; BSA, bovine serum albumin; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; AU, absorbance units; PSGL-1, P-selectin glycoprotein ligand-1; SD, standard deviation.

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expression of selected ADAM (a disintegrin and metalloprotease) family members localized to immune cell types (*28, 29*). ADAMs, a novel class of integrin ligand, derive their name from a unique molecular architecture that is composed of both proteolytic and adhesive domains (*30, 31*). Of the over 20 human members identified, at least 7 members have expression, substrate specificity, or integrin ligand properties indicative of immune function. For example, ADAM28 is expressed by lymphocytes, is recognized by a the α4/α9 integrin group associated with leukocyte mobility, and is up-regulated at the mRNA level in a monocytic cell line upon *t*-RA treatment (*29, 32–34*).

t-RA influences adhesion to established integrin counter receptors and matrix components in multiple cell lineages (*23, 35, 36*). However, it remains to be determined if *t*-RA also influences cell recognition of other nonconventional substrates such as ADAMs. The contribution of *t*-RA metabolism and the specific retinoid responsible for regulating cell adhesion has not been reported. Here we report for the first time that *t*-RA potently induces RPMI 8866 B-cell adhesion to various ligands, including ADAMs, independent of retinoid metabolism. Previous reports demonstrate a direct correlation with integrin expression levels and cell adhesive properties upon *t*-RA exposure. However, direct evidence that integrin function is necessary for *t*-RA mediated adhesion has been limited. We present evidence that *t*-RA mediates cell specific adhesion through a novel integrin-independent mechanism.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Chinese hamster ovary cell derived recombinant human VCAM-1 was purchased from R&D systems (Minneapolis, MN). The inhibitory anti-α4 antibody clone P1H4 was purchased from Chemicon International (Temecula, CA). The anti-PSGL-1 H300 polyclonal antibody and purified preimmune IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The SuperScript III Cells Direct cDNA Synthesis System was purchased from Invitrogen Corporation (Carlsbad, CA). The colorimetric phosphatase substrate, all-*trans*-retinoic acid, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid were purchased from Sigma (St. Louis, MO). 4-Oxo-retinoic acid was kindly provided by Hoffman-La Roche (Nutley, NJ). Because of retinoid photosensitivity, all experiments were performed under dim light. Samples and reference compounds were stored at −20 °C or +4 °C. Retinoids were dissolved at the desired concentration in ethanol. Other reagents used in the extraction process, analysis, or standard preparation were Optima grade hexane, methanol, HPLC grade water, and Tracemetal grade acetic acid.

Cell Culture. The mature human B-cell line RPMI 8866 was the generous gift of Dr. John Wilkins (University of Manitoba, Manitoba, Canada). Jurkat human T-lymphoblastoma cells and Daudi B-cells were obtained from ATCC (Manassas, VA). Cell lines were maintained in a 5% CO_2 environment with RPMI 1640 media supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 10% (v/v) fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin.

Reverse Transcriptase PCR Profiling of RPMI 8866 Cells. RPMI 8866 cells were cultured for 72 h in the presence of 1 μM *t*-RA or an equimolar concentration of ethanol (vehicle). Cells were manually counted and prepared for RNA extraction and reverse transcriptase priming. A total of 10,000 cells were utilized for each cDNA synthesis reaction. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Human GAPDH primers were utilized as a diagnostic to discriminate between genomic and cDNA amplification products as well as to control for cDNA template amounts. GAPDH control primers used were as previously published with the cDNA amplification product being 229 bp and a genomic template yielding a 330 bp product (*37*). RAR primers were as previously described (*38*). Samples were amplified with *Taq* polymerase (Thermo Scientific, Waltham, MA), and parameters were as follows: initial denaturatioin at 94 °C for 1 min with subsequent 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s.

Recombinant Fc-Fusion Proteins. Methods for the production and purification of Fc-fusion proteins were described previously (*33, 34, 39*). Briefly, DNA constructs were generated by PCR overlap extension to anneal the 5′ GP67 insect secretion signal and the 3′ human IgG3 Fc affinity tag onto regions encoding for the entire disintegrin domains of human ADAM7 (Tyr^{397}−Gly^{497}), ADAM28 (Thr^{407}−Gly^{500}), ADAM12 (Asn^{414}−Gly^{519}), or the fist two ectodomains of VCAM-1 (Gln^{23}− Leu^{217}). ADAM disintegrin domain constructs terminated within 5 residues of the disintegrin-cysteine rich domain boundary. The IgG3 Fc fusion-tag consisted of residues C-terminal of Ala^{130} to exclude the hinge region thereby preventing dimerization of the purified ligands. PCR products were cloned into the TOPO pIB/V5-His vector (Invitrogen, Carlsbad, CA). After sequence verification, High 5 insect cells were transfected with DNA constructs and selected for expression by eukaryotic blasticidin resistance. Conditioned media was harvested, polyethylene glycol concentrated, and applied to a protein-G affinity resin. After extensive washing, bound proteins were eluted with 100 mM citric acid pH 3.0 into a reservoir of 1 M Tris at pH 9.0 for immediate neutralization of the eluant.

Static Cell Adhesion Assay. Assays were adapted from established techniques (*40*). Briefly, recombinant proteins were immobilized at the desired concentration on Immulon-2 HB microtiter wells (Thermo Scientific, Waltham, MA) in a total volume of 100 μL of 0.1 M $NaHCO_3$. For maximal coating, plates were incubated overnight at 4 °C. Nonspecific adhesion was minimized by blocking wells with 2% (w/v) bovine serum albumin (BSA) in 0.1 M $NaHCO_3$ at room temperature for 1 h. Cells were cultured for the designated times (24 or 72 h) in the presence of 1 μM *t*-RA or an equimolar concentration of ethanol. Before addition to wells, cells were washed twice in HEPES-Tyrodes buffer, manually enumerated, and resuspended to the desired concentration. Cells were added to wells (2×10^5/well) in HEPES-Tyrodes with or without 1 mM $MnCl_2$. When appropriate, inhibitors were added simultaneously to cells in HEPES-Tyrodes. The monoclonal antibody P1H4 was used at a final concentration of 10 μg/mL, the polyclonal H300 and control IgG were added at a final concentration of 20 μg/mL, and EDTA was used at 3 mM. Cells were incubated with ligands in the presence or absence of inhibitors for 1 h at 37 °C in 5% CO_2. After three consecutive washes with HEPES-Tyrodes, wells were analyzed for bound cells by determining the relative cellular acid phosphatase activity within each well. Phosphatase assay buffer (1% v/v Triton X-100, 50 mM sodium acetate at pH 5.0 and 6 mg/mL *p*-nitrophenyl

phosphate) was added to wells, and wells were incubated with the colorimetric substrate for 30 min at 37 °C. Color was disclosed by addition of 50 μL/well of 1 N NaOH. Absorbance values were obtained at 405 nm with a plate reader. Specific numbers of adherent cells/well were obtained by correlating experimental absorbance values to a standard curve. Adhesion values obtained with wells coated exclusively with BSA were considered as background values for each experimental condition and were subtracted before reporting final values.

Extraction Procedure. Cells were treated with an equimolar concentration of ethanol or 1 μM *t*-RA for 24 or 72 h. Cells and media were harvested and stored at −20 °C until extraction. Vehicle and *t*-RA cell media were thawed at room temperature. Ten milliliters of ethanol was added, and the solution was mixed. The samples were acidified using 2 N HCl, and 10 mL of hexane was immediately added. The samples were mixed and stored on ice for 20 min. The upper layer was removed, and hexane was added and removed twice. The hexane fractions were combined and evaporated to dryness. The residual sample was stored at −20 °C until HPLC analysis (*41*). Vehicle and *t*-RA cells were treated as previously described. Cell and media samples were pooled. For all metabolism experiments, *t*-RA was added to cell-free media and allowed to incubate for either 24 or 72 h. The sample was processed as previously described. These samples served as a control for oxidation/isomerization processes that may occur in the extraction process. Additionally, control cell-free medium was spiked with *t*-RA, 9-*cis*-RA, 13-*cis*-RA, or 4-oxo-RA standards to verify the extraction procedure and to identify retinoid artifacts. *t*-RA, 13-*cis*-RA, 9-*cis*-RA, and 4-oxo-RA were analyzed by HPLC for purity prior to use in cell culture or retinoid artifact identification.

HPLC Analysis. Reverse-phase HPLC analysis was performed using a Waters model 6000A delivery system, a 6 port Rheodyne sample injector, and a Waters Millennium Chromatography Manager. The latter consists of a pump control module, a 996 photodiode array detector, and the Millennium^{32} chromatography software. Analytical separations were carried out on a stainless steel (23.5 cm × 0.47 cm) Whatman Partisil 5 ODS-3 5 μm particle column. The HPLC gradient consisted initially of 50% methanol: 50% 0.01 M acetic acid which was employed for 10 min followed by a 60 min linear gradient up to 100% methanol which was used to elute for 20 min. Flow rate was 1.00 mL/min. The retinoid absorption spectrum was recorded between 200 and 450 nm. Chromatograms were monitored at 350 nm.

RESULTS

t-RA Exposure Enhances B-Cell Adhesion to the Disintegrin-like Domain of Human ADAM7. Retinoids govern integral immunological events. Of critical importance is the ability of lymphocytes to physically interact with the endothelial vessel wall through integrin-dependent adhesion to facilitate extravasation. Although *t*-RA alters adhesion properties of various cell lineages, the influence of *t*-RA on human B-cell adhesion has not been investigated. The mature human B-cell line RPMI 8866 was utilized as a model to assess the impact of *t*-RA or *t*-RA metabolism on B-cell adhesion. RAR α, β, and γ were detected in both *t*-RA treated and untreated cells at the mRNA level (Figure 1A). The presence of all three RARs suggests that RPMI 8866 cells are capable of *t*-RA receptor mediated events.

We have previously described a recombinant fusion protein encompassing only the disintegrin domain of human ADAM7 (ADAM7 Dis-Fc) that supports α4β7 integrin-mediated adhesion of RPMI 8866 cells (*34*). A prototypical integrin ligand property of ADAM disintegrin domains is the requirement of exogenous activation of the receptor by Mn^{2+} or activating monoclonal antibodies for recognition. We employed ADAM7 Dis-Fc to evaluate if *t*-RA exposure promotes RPMI 8866 cell adhesion in the absence of exogenous integrin activation. As shown in Figure 1B, a 72 h treatment with 1 μM *t*-RA confers RPMI 8866 cell adhesion to ADAM7 Dis-Fc in a dose-dependent and saturable manner. Saturation curves between *t*-RA treated cells lacking exogenous integrin activation and vehicle treated cells containing 1 mM Mn^{2+} were comparable. In addition, when no Mn^{2+} was present, *t*-RA treated cells generated substantially higher levels of adhesion compared to cells treated with vehicle alone. Similar results were obtained for human ADAM28 Dis-Fc, a lymphocyte expressed ADAM recognized by the same integrin subsets as ADAM7 (data not shown). To address if the contribution of *t*-RA to the augmented adhesion was time-dependent, we restricted the exposure of RPMI 8866 cells to *t*-RA from 72 to 24 h. Statistically higher levels of adhesion were observed for cells cultured with 1 μm *t*-RA for 24 h on the ADAM7 Dis-Fc substrate as compared to nonactivated vehicle controls (Figure 1C). *t*-RA treatment had no effect on RPMI 8866 cell adhesion to human ADAM12 Dis-Fc, a nonimmune ADAM ligand. These data establish that *t*-RA regulates B-cell adhesion to select ADAM disintegrin domains.

RPMI 8866 B-Cell Adhesion to VCAM-1 Is Induced by t-RA Treatment. Since *t*-RA promotes RPMI 8866 cell adhesion to ADAM disintegrin domains, we tested if *t*-RA would alter adhesion to VCAM-1. Unlike ADAM disintegrin function, the role of VCAM-1 as an integrin counter receptor for recruitment of leukocytes from the blood flow is well-defined (*27*). A soluble, recombinant protein spanning the first two domains of human VCAM-1 (sVCAM-Fc, Gln^{23}−Leu^{217}) was expressed and purified from High 5 insect cell media. Treatment of RPMI 8866 cells with 1 μM of *t*-RA for 24 or 72 h resulted in a statistically higher number of cells bound per well compared to unactivated vehicle control cells on 5 μg/mL of sVCAM-Fc (Figure 2A). To rule out nonspecific artifacts of our eukaryotic protein expression system, commercially available VCAM-1 lacking an affinity tag was utilized in the adhesion assay. As shown in Figure 2B, the results obtained with the commercially purchased VCAM-1 confirm those reported for sVCAM-Fc. The number of adherent cells per well of *t*-RA treated RPMI 8866 cells was statistically higher than vehicle treated cells lacking integrin activation. As with ADAM7 Dis-Fc in Figure 1B, *t*-RA treatment resulted in adhesion levels similar to those observed upon inclusion of 1 mM Mn^{2+} when wells were coated with commercial VCAM-1 (Figure 2B).

t-RA Promotes B-Cell Adhesion through a Novel Adhesion Mechanism. *t*-RA has been reported to modulate cell adhesion to integrin ligands including fibronectin and VCAM-1 (*23, 35, 36*). To elucidate if the robust B-cell adhesion observed with *t*-RA treatment was specifically

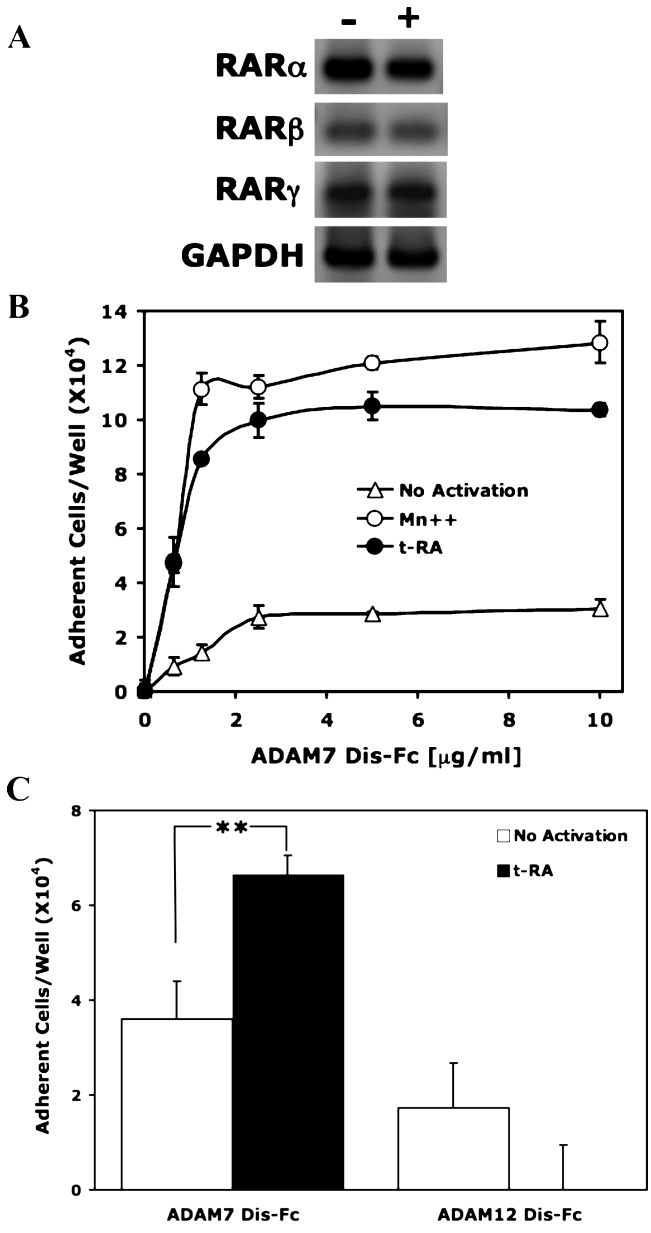

FIGURE 1: *t*-RA treatment promotes RPMI 8866 B-cell adhesion to the human ADAM7 disintegrin domain. (A) Receptor profiling of the mature human B cell line RPMI 8866. mRNA from RPMI 8866 cells cultured in the presence of vehicle (−) or 1 μM *t*-RA (+) for 72 h was analyzed via reverse transcriptase PCR for RAR signals. GAPDH was simultaneously amplified to verify consistent cDNA template amounts across samples and to rule out genomic contribution as described in Experimental Procedures. Shown is a representative experiment from three separate RNA extractions and amplifications. No bands indicative of genomic contamination were evident. (B) Microtiter wells were coated with various concentrations of recombinant ADAM7 Dis-Fc protein. RPMI 8866 cells cultured for 72 h in the presence of vehicle (△) or 1 μm *t*-RA (●) were added to wells (2×10^5 cells/well) in Hepes-Tyrodes buffer containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Vehicle treated cells in Hepes-Tyrodes containing 1 mM $MnCl_2$ (○) were utilized as a positive control for adhesion. (C) RPMI 8866 cells cultured for 24 h with vehicle (white bars) or 1 μM *t*-RA (black bars) were added to wells as previously described. Cells were incubated with 5 μg/mL of human ADAM7 or human ADAM12 Dis-Fc recombinant proteins. The number of adherent cells/well was quantified as outlined in Experimental Procedures. ** signifies statistically higher levels of adhesion in the *t*-RA treatment versus vehicle (Student's *t* test, $p < 0.05$). Adherent cells/well = $(\text{adherent cells})_{\text{recombinant protein}} - (\text{adherent cells})_{\text{BSA}}$. Results shown are the average ± SD of triplicate determinations.

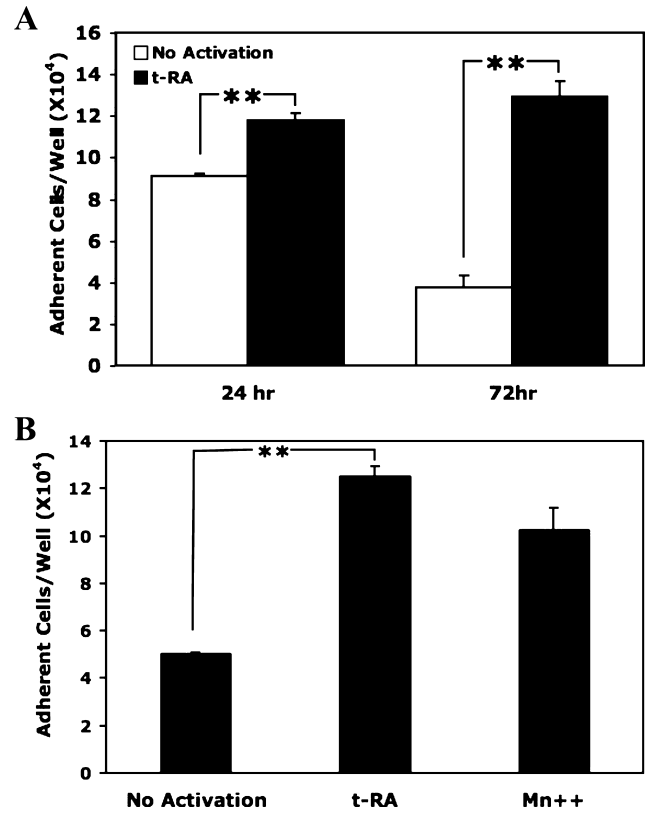

FIGURE 2: *t*-RA augments RPMI 8866 cell adhesion to VCAM-1. (A) RPMI 8866 cells cultured for 24 or 72 h in the presence of vehicle (white bars) or 1 μm *t*-RA (black bars) were added to wells coated with 5 μg/mL of recombinant sVCAM-Fc. Cells (2×10^5 cells/well) were suspended in Hepes-Tyrodes buffer containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. (B) The cells from a 72 h time point described in panel A were added to wells coated with 1 μg/mL of commercially available VCAM-1. ** signifies statistically higher levels of adhesion in the *t*-RA treatment versus vehicle (Student's *t* test, $p < 0.01$). Adherent cells/well = $(\text{adherent cells})_{\text{recombinant protein}} - (\text{adherent cells})_{\text{BSA}}$. Results shown are the mean adhesion ± SD of a representative experiment performed in triplicate.

attributable to integrin function, adhesion assays were repeated in the presence of various integrin inhibitors. Although the RPMI 8866 cell line is an $\alpha4\beta7$ high expressing cell line, these cells also express detectable cell surface levels of the $\beta1$ subunit allowing for potential $\alpha4\beta1$ expression (*34*). ADAM7 is an established $\alpha4\beta1/\alpha4\beta7$ ligand so the $\alpha4$ function blocking monoclonal antibody P1H4 was selected to simultaneously inhibit these two ADAM7 disintegrin receptors. As previously observed, inclusion of 5 μg/mL of P1H4 resulted in greater than 80% inhibition when activated RPMI 8866 cells were added to wells coated with ADAM7 Dis-Fc (Figure 3). In contrast, inclusion of P1H4 with RPMI 8866 cells treated with 1 μM *t*-RA for 72 h did not reduce adhesion to an appreciable level. To rule out the contribution of other integrin subunits, RPMI 8866 cells were added to wells in adhesion buffer containing 3 mM of the cation chelator EDTA. While EDTA resulted in greater than 50% inhibition with cells containing Mn^{2+}, nominal inhibition was observed for *t*-RA treated cells. Similar inhibition results were obtained with sVCAM-Fc (data not shown). These results demonstrate for the first time that *t*-RA is capable of promoting integrin-independent cell adhesion.

Recently, the ADAM28 disintegrin domain has been reported to interact with the decamer repeats of P-selectin

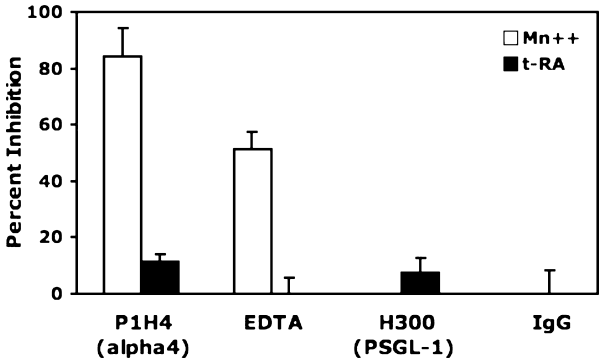

FIGURE 3: *t*-RA mediated B-cell adhesion occurs through a novel integrin-independent mechanism. Microtiter wells were coated with 5 μg/mL of ADAM7 Dis-Fc. RPMI 8866 cells cultured for 72 h with vehicle were added to wells in Hepes-Tyrodes buffer containing 1 mM $MnCl_2$ (white bars) or cells cultured for 72 h with 1 μM *t*-RA were added to wells in Hepes-Tyrodes buffer lacking 1 mM $MnCl_2$ (black bars). Adhesion assays were performed as previously described. For inhibition studies, cells were incubated with ADAM7 Dis-Fc in the presence of either 5 μg/mL of function-blocking P1H4, 3 mM EDTA, or polyclonal antibody 20 μg/mL. $MnCl_2$ condition was not conducted for the H300 or control IgG experiment. Percent inhibition = $\{1 - [(\text{adherent cells})_{\text{inhibitor}} - (\text{adherent cells})_{\text{BSA}}]/[(\text{adherent cells})_{\text{no inhibitor}} - (\text{adherent cells})_{\text{BSA}}]\} \times 100$. Data displayed are the average inhibition values $\pm$ SD from representative experiments done in triplicate.

glycoprotein ligand-1 (PSGL-1) (*42*). This seminal work is the first to characterize molecular recognition of an ADAM disintegrin domain by a receptor other than an integrin heterodimer. To determine if PSGL-1 is responsible for the *t*-RA dependent cell adhesion, we utilized the polyclonal H300 antibody shown to disrupt PSGL-1/ADAM28 interaction. Coincubation of H300 with *t*-RA treated RPMI 8866 cells and immobilized ADAM7 or ADAM28 Dis-Fc did not result in statistically different inhibition values compared to control preimmune IgG (Figure 3). These data conclusively discount integrin or PSGL-1 contribution to *t*-RA mediated RPMI 8866 cell adhesion. Further studies will be needed to delineate the exact mechanism by which *t*-RA promotes cell adhesion.

Metabolism of all-trans-Retinoic Acid by RPMI 8866 Cells. The literature is replete with studies of retinoid induced proliferation, differentiation, and induction of retinoid responsive genes which are critical in lymphocyte signaling. However, little is known about the metabolism of all-*trans*-retinoic acid within these cell types. We have shown that retinoids regulate human B-cell adhesion. To identify the retinoid responsible for eliciting this effect, metabolism studies were conducted. The mature human B-cell line RPMI 8866 was cultured in *t*-RA for 72 or 24 h. Media volumes were comparable with vehicle and *t*-RA treated cells averaging 28.4 and 28.3 mL, respectively ($n = 6$). Equal amounts of cells (1×10^6) were extracted from vehicle or *t*-RA cultured cells.

The HPLC profiles of 72 h vehicle and *t*-RA media extracts are shown in Figure 4A. Two compounds were detected. The parent compound *t*-RA was identified with a retention time of 63.98 min and maximum UV absorbance of 354.4 nm. The second peak detected was confirmed as 13-*cis*-RA with a retention time of 62.18 min and a maximum UV absorbance of 356.8 nm. Retinoid artifacts may be produced from interactions with the biological media

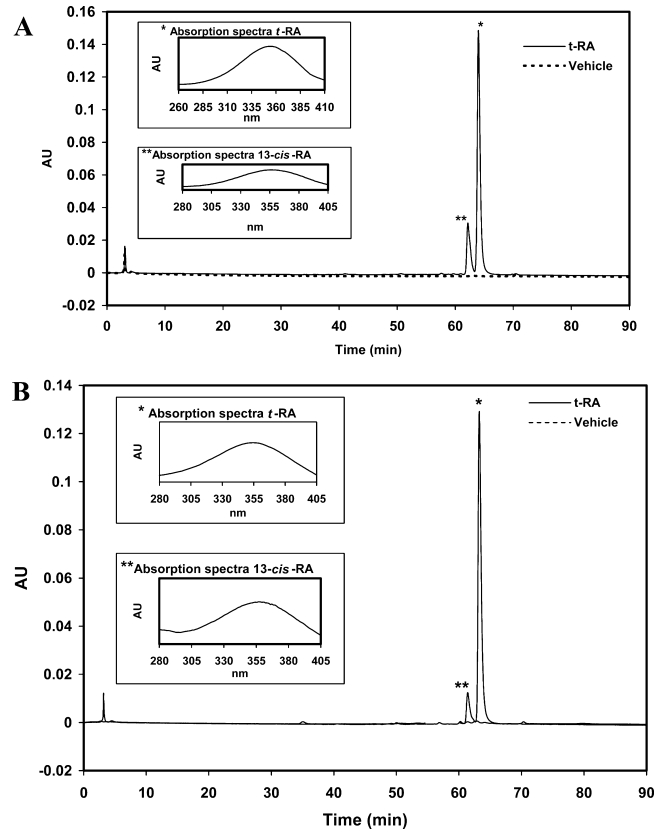

FIGURE 4: All-*trans*-Retinoic acid metabolism in RPMI 8866 cells. (A) RPMI 8866 cells cultured for 72 h in the presence of vehicle (ethanol) or 1 μM *t*-RA. Cells and media were collected, pooled, and extracted as previously described. Chromatograms are shown with vehicle treated media extract (dashed line) and *t*-RA treated media (solid line). The parent compound, *t*-RA, was detected at 63.98 min, and the absorption spectrum is shown in the inset (*). 13-*cis*-RA was detected at 62.18 min, and the absorption spectrum is shown in the inset (**). (B) RPMI 8866 cells cultured for 24 h in the presence of vehicle (ethanol) or 1 μM *t*-RA. Cells and media were collected, pooled, and extracted as previously described. Chromatograms are shown with vehicle treated media extract (dashed line) and *t*-RA treated media (solid line). The parent compound, *t*-RA, was detected at 63.25 min, and the absorption spectrum is shown in the inset (*). 13-*cis*-RA was detected at 61.40 min, and the absorption spectrum is shown in the inset (**). The presence of 13-*cis*-RA is suggestive of an artifact attributed to the extraction procedure.

or in response to heat or natural/fluorescent lighting since retinoids are sensitive to oxidation and/or isomerization. For all metabolism experiments, *t*-RA was incubated for 72 h in cell-free media and then extracted. In those samples, both *t*-RA and 13-*cis*-RA were detected (data not shown). Cell-free medium was spiked with a *t*-RA standard, and the extraction and HPLC analysis were repeated. In those samples, both *t*-RA and 13-*cis*-RA were detected (data not shown). This data suggests that 13-*cis*-RA is an artifact of the extraction procedure and not a metabolic product of cells. Other studies with carotenoids have found that the addition of acid during the extraction procedure has promoted isomerization (*43*). This could be the case for retinoids as well. CYP-dependent metabolites of *t*-RA were not detected. To confirm that polar metabolites of *t*-RA could be extracted and detected by our method, cell-free medium was spiked with 4-oxo-RA and this compound was detected with a retention time of 50.02 min and a maximum absorbance of 362 nm. To evaluate intracellular *t*-RA metabolism, cells

were extracted and HPLC analysis was performed. Metabolites of *t*-RA were not detected in the cell extracts.

Although metabolites were not detected at 72 h, metabolite production and accumulation may occur at an earlier time point. Since a 24 h *t*-RA exposure generated higher levels of adhesion in RPMI 8866 cells, we assessed *t*-RA metabolism. The HPLC profiles of 24 h vehicle and *t*-RA media extracts are shown in Figure 4B. Qualitatively similar retinoids were observed in 24 h media extracts as compared to 72 h media extracts. Two compounds were identified. The first was the parent compound *t*-RA with a retention time of 63.25 min and maximum UV absorbance of 354.4 nm. The second was confirmed as 13-*cis*-RA with a retention time of 61.40 min and a maximum UV absorbance of 356.8 nm. As discussed ear1ier, 13-*cis*-RA appears to be an artifact of the extraction and not a metabolite of *t*-RA. CYP-dependent metabolites of *t*-RA were not detected. To evaluate intracellular *t*-RA metabolism, cells were extracted and HPLC analysis was performed. Metabolites of *t*-RA were not detected in the 24 h cell extracts. Our data establishes that *t*-RA is not metabolized in RPMI 8866 cells within a 24 or 72 h incubation. This data conclusively establishes that *t*-RA is responsible for mediating cell adhesion.

t-RA Induced Cell Adhesion Is Cell Specific. To evaluate if *t*-RA impacts the adhesion of other immune lineages, we analyzed the adhesion trends of the human T-cell line Jurkat when cultured with *t*-RA. No detectable changes were observed with Jurkat adhesion to ADAM7 Dis-Fc, sVCAM-Fc, or ADAM28 Dis-Fc when cells were passaged in the presence of 1 μM *t*-RA for 72 h (Figure 5A, data not shown). Vehicle treated Jurkat cells exogenously activated with Mn^{2+} were fully capable of integrin-mediated adhesion. These data demonstrate that the effect of *t*-RA on cell adhesion is not prototypical of all lymphocyte lineages but may be localized to specific cell subsets.

Metabolism of all-trans-Retinoic Acid by Jurkat Cells. The adhesion properties between Jurkats and RMPI 8866 cells are clearly different in the presence of *t*-RA. We evaluated the *t*-RA metabolic profile of Jurkat cells cultured in 1 μM *t*-RA for 72 h. The HPLC profiles of 72 h vehicle and *t*-RA media extracts are shown in Figure 5B. The parent compound was detected at 63.48 min with a maximum UV absorbance of 354.4 nm. 13-*cis*-Retinoic acid was detected at 61.63 min and a maximum UV absorbance of 356.8 nm and suggested to be an artifact of the extraction procedure. A third peak was identified as 9-*cis*-retinoic acid with a retention time of 60.59 min and a maximum UV absorbance of 347.3 nm. CYP-dependent metabolites were not detected in media or cells.

Since *t*-RA did not induce Jurkat T-cell adhesion, we tested if *t*-RA would influence the adhesion of a human B-cell line other than the RPMI 8866 subset. Using Daudi cells, an early mature human B-cell line, we establish that *t*-RA does not promote cell adhesion with all B-cell types. Daudi cells cultured in the presence of 1 μM *t*-RA for 72 h resulted in adhesion levels comparable to unactivated vehicle control cells when added to wells coated with 5 μg/mL of the ADAM7 Dis-Fc substrate (data not shown). The metabolic profile of Daudi cells treated with 1 μM *t*-RA for 72 h mimicked results obtained with the RPMI 8866 cells (data not shown).

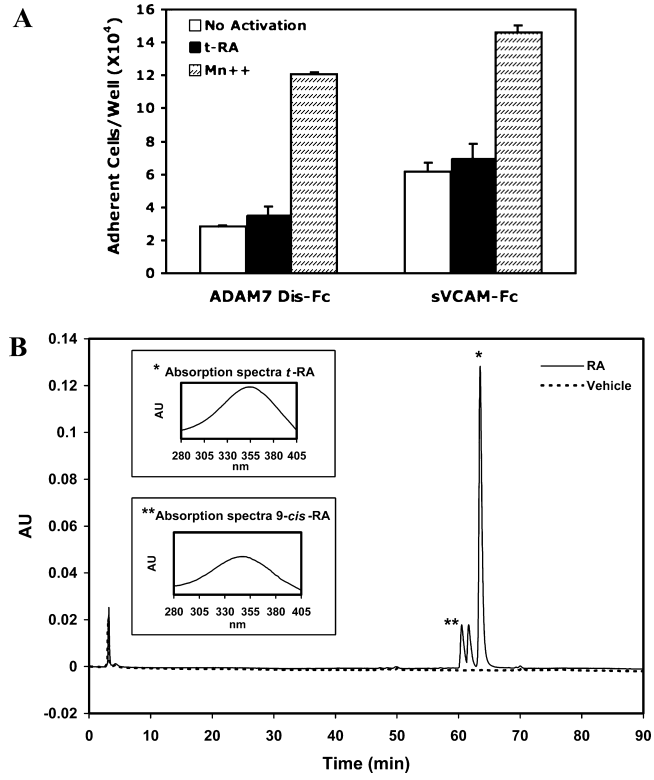

FIGURE 5: Jurkat T-cell adhesion to the ADAM7 disintegrin domain is not altered upon *t*-RA exposure. (A) Jurkat cells cultured for 72 h in the presence of vehicle or 1 μM *t*-RA were assessed for adhesion to immobilized ADAM7 Dis-Fc at 5 μg/mL. Vehicle treated cells were added to wells in Hepes-Tyrodes buffer lacking (white bars) or containing 1 mM $MnCl_2$ (hatched bars). Cells exposed to *t*-RA were added to wells in Hepes-Tyrodes with no $MnCl_2$ (black bars). Adherent cells/well = $(\text{adherent cells})_{\text{recombinant protein}} - (\text{adherent cells})_{\text{BSA}}$. Results shown are the mean adhesion ± SD of a representative experiment performed in triplicate. (B) Jurkat cells cultured for 72 h in the presence of vehicle (ethanol) or 1 μM *t*-RA. Cells and media were collected, pooled, and extracted as previously described. Chromatograms are shown with vehicle treated media extract (dashed line) and *t*-RA treated media (solid line). The parent compound, *t*-RA was detected at 63.48 min and the absorption spectrum is shown in the inset (*). 13-*cis*-RA was detected at 61.63 min. 13-*cis*-RA appears to be an artifact of the extraction procedure. 9-*cis*-RA was detected at 60.59 min, and the absorption spectrum is shown in the inset (**).

DISCUSSION

The current study demonstrates that *t*-RA potently induces RPMI 8866 B-cell adhesion independent of established adhesion receptors and cellular metabolism of *t*-RA. Since *t*-RA is a strong activator of RARs, the mature B-cell line RPMI 8866 was utilized to evaluate the impact of *t*-RA on B-cell adhesion due to its unique integrin repertoire and expression of the three major RARs within this cell lineage (Figure 1A). Integrin-dependent adhesion of RPMI 8866 cells to VCAM-1 and select ADAM family members has been previously reported (*34, 44*). A hallmark of ADAM integrin ligands is that integrin recognition is dependent upon the activation state of the receptor. We evaluated if *t*-RA promotes cell adhesion to these physiologically relevant substrates in the absence of exogenous integrin activation. We provide the first evidence that cell adhesion to select ADAM family members and VCAM-1 is increased by *t*-RA exposure, and the extent of B-cell adhesion attained with

t-RA exposure was reminiscent of levels achieved upon integrin activation.

t-RA has been shown to both augment and dampen cell adhesion, which directly correlates with expression of distinct integrin subunits at the protein level (*23, 35, 36*). In contrast to previous work, adhesion of *t*-RA treated cells was not diminished in the presence of multiple integrin inhibitors. This establishes that *t*-RA mediated adhesion of RPMI 8866 cells is integrin-independent. PSGL-1 recognition of the ADAM28 disintegrin domain facilitates transient rolling and cell adhesion to endothelial vessel surfaces during inflammation (*42*). However, we were unable to prevent *t*-RA mediated cell adhesion to ADAM7 or ADAM28 with a polyclonal antibody previously shown to disrupt PSGL-1/ADAM28 interaction. These data collectively suggest that a novel *t*-RA inducible adhesion receptor or complex exists for both ADAMs and VCAM-1.

Retinoic acid efficacy dictates heterodimer/homodimer retinoid receptor pairing and is attenuated by its metabolism. Since our results demonstrate that retinoids dictate adhesive properties of RPMI 8866 cells, it is physiologically important to address how RPMI 8866 cells metabolize *t*-RA. Recently, studies have provided significant insight into the role of retinoids in adhesion; however, these studies do not address *t*-RA metabolism and its contribution to cell adhesion. In the current study, we attribute increased cell adhesion directly to *t*-RA. This is the first study to characterize *t*-RA metabolism and its functional consequence in RPMI 8866 cells.

The effect of *t*-RA on the adhesion properties of the RPMI 8866 cells was also cell specific. In contrast to the RPMI 8866 B-cells, no detectable difference in adhesion was observed for Jurkat T-cells or Daudi, a distinct B-cell line, when exposed to *t*-RA. Divergent adhesive properties may be attributed to cell specific expression of retinoid receptors and metabolism of *t*-RA. Previous investigations profiled RAR expression within Jurkat cells and detected signals for RARα and γ, but not β (*45*). This is distinct from our characterization of RPMI 8866 cells in which all three receptors were present. This different expression pattern may account for the ability of RPMI 8866 cells to respond to *t*-RA with respect to adhesion. Additionally, the *t*-RA metabolic profiles are different between these two cell types. Within RPMI 8866 cells, *t*-RA is not metabolized. However, in comparison, Jurkat cells produce both *t*-RA and 9-*cis*-RA. Studies have shown that *t*-RA is biologically isomerized to 9-*cis*-RA, and our control experiments illustrate that 9-*cis*-RA detected within Jurkat media is not an artifact (*46, 47*). Unlike *t*-RA, 9-*cis*-RA is an agonist for RXR, which is a promiscuous receptor for other members of the steroid nuclear receptor family, including PPARγ, a receptor implicated in immune function (*48*). The presence of 9-*cis*-RA within cells dictates heterodimer/homodimer pairing plausibly influencing pathways that regulate adhesive properties. This potential difference in receptor pairing may culminate in the distinct cell specific response to *t*-RA reported here.

This study is the first to report that *t*-RA directly augments B-cell adhesion to VCAM-1 and to particular ADAM family members. Enhanced adhesion of *t*-RA treated cells was not dependent upon previously established receptors for VCAM-1 and ADAMs. Further studies are needed to elucidate the mechanism by which *t*-RA promotes RPMI 8866 B-cell adhesion. Mounting an effective immune response and normal homing of leukocytes require extensively governed adhesive events. The results presented here shed light on the role of *t*-RA and its metabolism in mediating immune cell adhesion.

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