



Complementary targeting of liposomes to IL-1 α and TNF- α activated endothelial cells via the transient expression of VCAM1 and E-selectin

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ABSTRACT

Inflammation is in part defined by the transient upregulation of cell adhesion molecules on the surface of endothelial cells (ECs) in response to cytokines. We hypothesized that liposomes with a complementary surface presentation of antibodies to the pattern of molecules on the EC surface may enhance targeting. We quantified the expression of vascular cell adhesion molecule-1 (VCAM1) and endothelial leukocyte cell adhesion molecule-1 (E-selectin) on ECs upon exposure to either tumor necrosis factor- α (TNF- α) or interleukin-1 α (IL-1 α) as a function of time. Liposomes, composed of 95 mol% dioleoyl phosphatidylcholine (DOPC) and 5 mol% dodecanyl phosphatidylethanolamine (N-dod-PE), were prepared by conjugating different molar ratios of antibodies against VCAM1 (aVCAM1) and E-selectin (aE-selectin). Increased binding was observed when immunoliposomes complemented the presentation of VCAM1:E-selectin expressed on TNF- α activated ECs. The 1:1 aVCAM1:aE-selectin liposomes had maximal binding at both 6 and 24 h on IL-1 α activated ECs due to differences in molecular organization. The results demonstrate that liposomes targeting to inflamed endothelium may be optimized by exploiting the dynamic expression of VCAM1 and E-selectin on the EC surface.

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1. Introduction

The transient expression of cell adhesion molecules on endothelial cells (ECs) presents a targeting opportunity for inflamed endothelium [1–3]. Intercellular cell adhesion molecule-1 (ICAM1), endothelial leukocyte adhesion molecule-1 (E-selectin), and vascular cell adhesion molecule-1 (VCAM1) are upregulated on ECs upon activation with inflammatory cytokines [4–6]. Each of these molecules has been investigated for targeting [7–9]; however, no binding studies have correlated the ratio of antibodies on drug delivery vehicles with the presentation of target molecules on ECs.

Targeting multiple, transiently-upregulated cell adhesion molecules on ECs may amplify binding specificity and cellular uptake of drug delivery vehicles. Microbubble contrast agents conjugated with dual targeting antibodies against VCAM1 and P-selectin bound almost twice as effectively as single-targeting microbubbles [10]. Similarly, the adhesion of microspheres bearing antibodies against ICAM1 (aICAM1) and sialyl Lewis^x, a selectin ligand, was dependent on the concentration of both ligands [11,12]. Our previous reports also demonstrated that the

antibody ratio (aICAM1:aE-selectin) and lipid mobility influenced cellular binding [13].

These observations, however, did not account for the fact that the cell surface is spatiotemporally dynamic. Cell adhesion molecules, such as E-selectin and ICAM1, have been reported to cluster following their engagement in a cytokine or shear-induced response [14]. Clustering of ICAM1 and E-selectin in lipid raft microdomains is required for subsequent cell signaling and gene expression [15,16]. Binding of liposomes, targeted to ICAM1 and E-selectin, was reduced to the non-specific level when lipid raft formation was inhibited by addition of a cholesterol chelation agent, methyl- β -cyclodextrin [17]. These reports clearly demonstrate the importance of molecular organization on the EC surface in liposome targeting.

In addition to clustering, the expression of cell adhesion molecules on ECs changes temporally after exposure to cytokines. Several studies have reported the time-course expression of cell adhesion molecules in response to different inflammatory cytokines. Scholz et al. showed that interleukin-1 β (IL-1 β)-induced VCAM1 and E-selectin expression regressed after 8 h while ICAM1 was maintained for 72 h [18]. With IL-1 α , E-selectin expression peaked at 6 h whereas platelet endothelial cell adhesion molecule-1 (PECAM1) reached a plateau after 12 h [3]. The expression trend for each cell adhesion molecule corresponds to the inflammatory

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response, where they mediate neutrophil rolling, adhesion, arrest, and transmigration [19].

Changes in the endothelial phenotype may be important in the design of drug delivery vehicles that target the endothelium. Targeting of drug delivery vehicles to inflamed ECs may be tailored to the latent stage of the immune response, resulting in consistent delivery of therapeutic agents at the target site (Fig. 1).

In this report, we characterized the expression of VCAM1 and E-selectin as a function of time in response to IL-1 α and TNF- α . Liposomes were modified with different molar ratios of aVCAM1:aE-selectin to evaluate optimum binding to activated ECs as a function of time. Drug delivery vehicles designed to target inflamed endothelium may benefit from understanding the temporal dynamics of molecules expressed on the EC surface.

2. Experimental methods

2.1. Materials

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-dodecanyl (N-dod-PE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Human recombinant interleukin-1 α (IL-1 α), mouse anti-human VCAM1 (aVCAM1), mouse anti-human E-selectin (aE-selectin) monoclonal antibodies (mAbs), and IgG₁ isotype mAb were purchased from R&D Systems (Minneapolis, MN). N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), human recombinant tumor necrosis factor- α (TNF- α), rhodamine-B isothiocyanate-conjugated dextran (10 kDa MW), ammonium molybdate, ascorbic acid, anhydrous dimethyl sulfoxide (DMSO), and ethanol (EtOH) were purchased from Sigma (St. Louis, MO). Formaldehyde was obtained from EMD Chemicals, Inc. (Gibbstown, NJ). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (FITC)-conjugated mouse anti-rat, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated rabbit anti-sheep secondary antibodies were obtained from Abcam (Cambridge, MA). Sheep aE-selectin polyclonal antibodies were purchased from Leinco Technologies (St. Louis, MO). Dulbecco's phosphate buffered saline (PBS), 0.25% trypsin/2.6 mM EDTA, and Hoechst 33258 were obtained from Invitrogen (Carlsbad, CA).

2.2. Liposome preparation

Unilamellar liposomes were prepared by the extrusion method as previously described [13]. A mixture of DOPC:N-dod-PE (95:5 mol%) in chloroform was dried in a rotary evaporator under reduced pressure. The lipids were then dissolved in a mixture of DMSO:EtOH (7:3 v/v). Lipid mixtures (0.3 mL) were injected in 3 mL PBS (pH 7.4) with or without rhodamine-conjugated dextran (1 mg/mL) while being agitated at 650 rpm with a stir bar to yield 50 nm lipid. The multilamellar vesicles were subjected to 10 freeze–thaw cycles (utilizing liquid nitrogen) prior to extrusion. Large unilamellar vesicles were prepared by utilizing a LIPEX™ extruder (Northern Lipids, Burnaby, Canada). Vesicles were extruded 10 times through

a 200 nm polycarbonate membrane (Whatman Nucleopore 25 mm track-etched membranes, GE Healthcare Biosciences, Piscataway, NJ). Dextran-encapsulated liposomes were dialyzed against PBS using a Slide-A-Lyzer dialysis cassette (MWCO 20 kDa, Pierce Biotechnology, Inc., Rockford, IL) overnight at room temperature (RT). Liposome size was measured by dynamic light scattering on a ZetaPALS analyzer (Brookhaven Instruments, Corp., Holtsville, NY) in PBS (pH 7.4).

The concentration of lipid in solution was determined by a phosphate assay as previously described [13]. Briefly, a diluted liposome sample was ashed with 0.2 mL sulfuric acid (10% v/v) at 200 °C for 1 h, followed by addition of 50 μ l hydrogen peroxide (30% v/v) and further heating at 200 °C for 40 min. After the sample was cooled down to RT, 480 mL deionized water and 0.5 mL of color reagent (0.5% w/v ammonium molybdate, 2% w/v ascorbic acid) were added to each sample followed by heating at 45 °C for 20 min. The samples were read at 820 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA). A calibration curve was prepared with known phosphate quantities.

2.3. Preparation of antibody-labeled liposomes and microspheres

Mouse aVCAM1 and mouse aE-selectin mAbs were conjugated to liposomes via the N-dod-PE anchor. EDC (2 mg) and NHS (3 mg) were added to 1 μ mol lipid (liposomes) in PBS (pH 7.4) and incubated for 6 h at RT. Excess EDC and NHS were removed using the Zeba Spin desalting column (MWCO 7 kDa, Pierce Biotechnology, Inc.). IgG₁ isotype (100 mol%, unspecific liposome) or mixtures of aVCAM1:aE-selectin (1:0, 1:1, 1:4, 1:8, 1:20, and 0:1 mol:mol ratios) were added to EDC-modified liposomes at a molar ratio of 1:1000 antibody:phospholipids and incubated overnight at RT. Free antibodies were separated using a Sepharose CL-4B column (Fractionation range: 60–20,000 kDa, Sigma).

To determine the antibody density of aVCAM1 and aE-selectin on liposomes, 2-micron borosilicate beads (Duke Scientific, Palo Alto, CA), large enough to be detected by flow cytometry, were coated with a lipid bilayer. Microbeads and PC:N-dod-PE (95:5) liposomes were agitated in PBS for 6 h. Microbeads coated with PC:N-dod-PE bilayers were washed 3 times with PBS through centrifugation–suspension cycles to separate free liposomes. In order to simultaneously label both aVCAM1 and aE-selectin, sheep aE-selectin antibodies were conjugated on microbeads instead of mouse aE-selectin antibodies. Conjugation of different ratios of aVCAM1:aE-selectin and control IgG (non-specific binding) to microbeads was performed as described above using EDC/NHS chemistry. After free primary antibodies were separated, microbeads were conjugated with FITC-conjugated goat anti-mouse (aVCAM1 binding) and TRITC-conjugated rabbit anti-sheep (aE-selectin binding) secondary antibodies (10 μ l, 10 ng/mL) overnight at 4 °C. The secondary antibodies were separated using suspension–spin cycles. The density of aE-selectin and aVCAM1 conjugated to microbeads was determined with reference to Quantum™ Simply Cellular® anti-mouse IgG microbeads (Bangs Laboratory, Inc., Fishers, IN), which have defined numbers of antibody binding sites per bead. The microbeads were stained with FITC-conjugated mouse anti-rat secondary antibodies using the protocol provided by the manufacturer.

2.4. Cell culture

Human umbilical vein endothelial cells (ECs) were grown in endothelial growth medium-2 (EGM-2) with supplements as described by the distributor (Lonza,

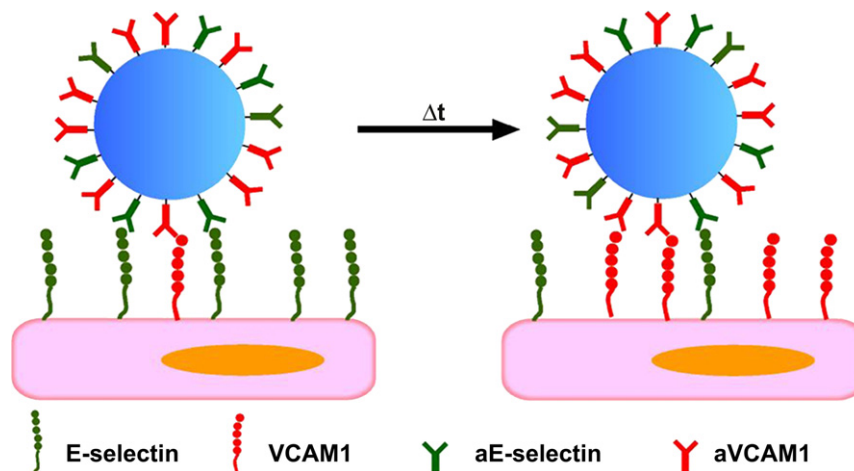


Fig. 1. Schematic of immunoliposomal drug delivery vehicles targeting the transient expression of VCAM1 and E-selectin on ECs. VCAM1 and E-selectin are transiently expressed on ECs after activation with inflammatory cytokines TNF- α and IL-1 α . E-selectin expression is instantaneous while VCAM1 is progressively expressed with time. Optimum binding of immunoliposomes can be continuously enhanced by complementing the antibody surface presentation of aVCAM1 and aE-selectin to the VCAM1 and E-selectin expression on ECs over time (Δt).

Table 1
Antibody density and zeta potential of 95:5 DOPC:N-dod-PE immunoliposomes. All liposomes were assumed to have a surface area of $0.126 \mu\text{m}^2$.

Ratio aVCAM1: aE-selectin	aVCAM1 (molecules/liposome)	aE-selectin (molecules/liposome)	Zeta potential (mV)
1:0	842 ± 47	-	1.1 ± 0.6
1:1	571 ± 30	488 ± 41	-0.1 ± 2.3
1:4	215 ± 19	621 ± 64	0.9 ± 0.6
1:8	91 ± 7	623 ± 54	1.3 ± 0.7
0:1	-	849 ± 89	0.5 ± 3.3

Allendale, NJ). Cells were maintained at 37°C in a humidified incubator with 5% CO_2 and used for experiments at passages 2–5. Growth medium was changed the day after cell seeding and every other day thereafter.

2.5. VCAM1 and E-selectin immunostaining

ECs (2×10^5 cells) were seeded on $18 \times 18 \text{mm}^2$ coverslips in 6-well plates with 2 mL media (VWR, West Chester, PA) and cultured overnight at 37°C . Cells were

incubated with IL- 1α at 5 ng/mL or TNF- α at 20 ng/mL in fresh EGM-2 media the next day for 6 h or 24 h. After medium was removed, cells were rinsed with PBS three times and fixed with 4% formaldehyde in PBS at RT for 15 min followed by washing with PBS. Samples were blocked with 1% BSA in PBS (1% BSA) at 4°C for 1 h. Samples were then stained with aVCAM1 and aE-selectin mAbs (10 ng/mL in 1% BSA) at 4°C for 2 h and rinsed with PBS. Samples were incubated with FITC-conjugated goat anti-mouse (1:300), TRITC-conjugated rabbit anti-sheep secondary mAbs (1:300) and Hoescht 33258 (1:1000) at 4°C for 1 h followed by washing with PBS. Samples were then mounted on microscope slides ($3'' \times 1''$, VWR) with VECTASHIELD® mounting medium (Vector Laboratories, Burlingame, CA), and sealed. Samples were examined under an Axiovert 200 inverted fluorescent microscope (Carl Zeiss, Thornwood, NY) equipped with a Hamamatsu CCD camera (Bridgewater, NJ). Digital images were captured with AxioVision digital image processing software (Zeiss).

2.6. VCAM1 and E-selectin expression

VCAM1 and E-selectin expression by ECs was evaluated by flow cytometry after 1, 6, 12, and 24 h incubations with either IL- 1α (5 ng/mL) or TNF- α (20 ng/mL). Quantification of the density of molecules on the surface was determined using

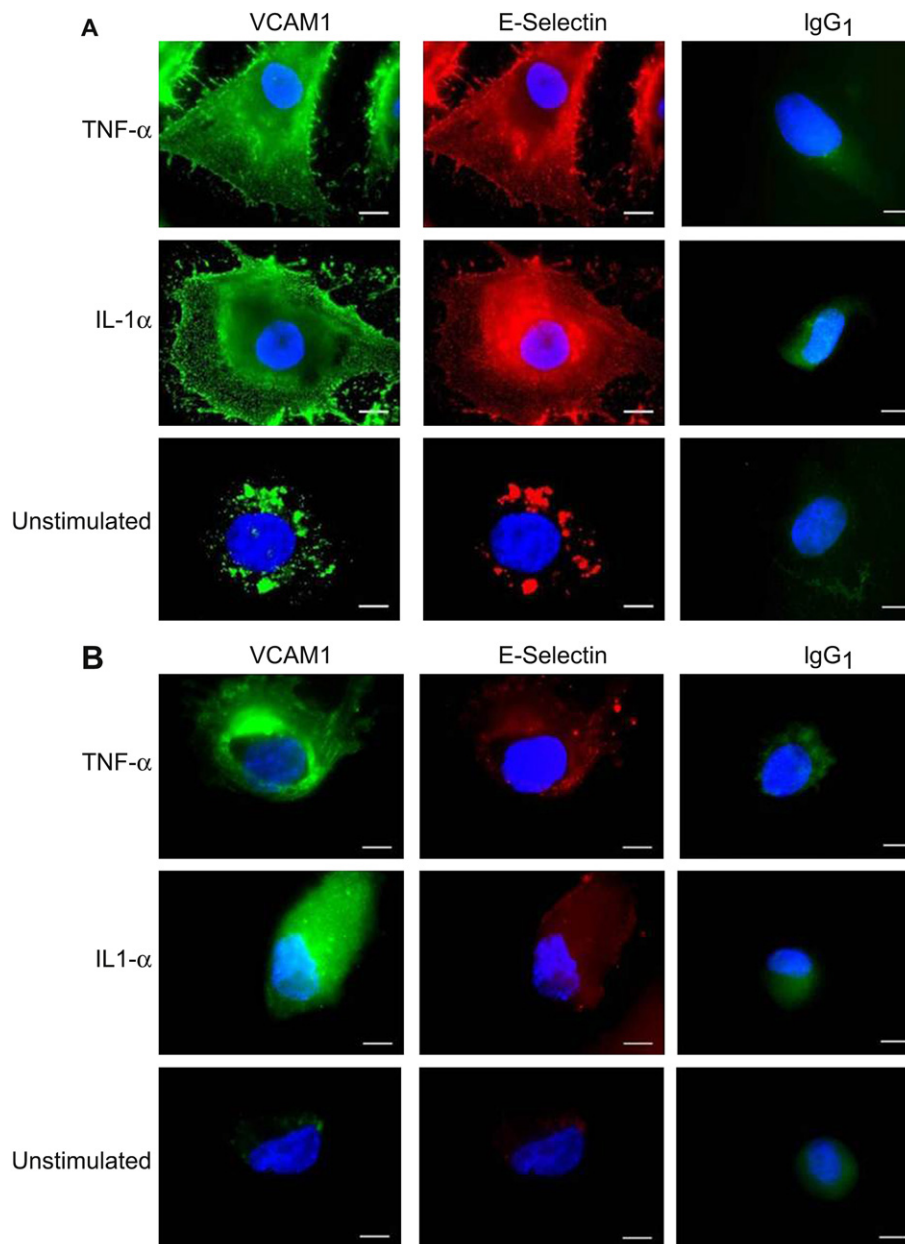


Fig. 2. VCAM1, E-selectin, and IgG₁ isotype control immunostaining for TNF- α and IL- 1α -stimulated and unstimulated ECs. ECs were activated with TNF- α and IL- 1α for 6 h (A) and 24 h (B), then fixed, and immunostained with aVCAM1 and aE-selectin. After activation with either TNF- α or IL- 1α , ECs transiently expressed VCAM1 and E-selectin. Scale bar, 10 μm .

Quantum™ Simply Cellular® microbeads. After ECs were activated with IL-1 α or TNF- α , 10^6 cells were collected from a 6-well plate, spun down, and resuspended with ice cold 1% BSA. ECs were rinsed 3 times through suspension–spin cycles and blocked with 1% BSA for 30 min in an ice bath. ECs were incubated with mouse anti-human VCAM1 and sheep anti-human E-selectin mAbs (1 mL, 10 ng/mL) for 30 min in an ice bath. After rinsing with 1% BSA 3 times to remove free mAbs, ECs were stained with FITC-conjugated goat anti-mouse and TRITC-conjugated rabbit anti-sheep secondary antibodies for 30 min in an ice bath. ECs were finally rinsed with 1% BSA three times, resuspended in PBS, and analyzed by flow cytometry.

2.7. Liposome uptake by ECs

Liposome binding by ECs was analyzed using flow cytometry as previously described [20,21]. ECs were seeded in 6-well plates (3×10^5 cells/well) and allowed to adhere overnight. After activation with IL-1 α or TNF- α for 6 h or 24 h, ECs were incubated overnight at 37 °C with: (1) dextran-loaded non-specific (IgG₁) liposomes; and (2) dextran-loaded liposomes conjugated with different ratios of aVCAM1:aE-selectin mAbs. The concentration used was 1 μ mol lipid/ 10^6 cells.

Treated ECs were washed with PBS, harvested using trypsin/EDTA solution, and collected in a polystyrene culture tube (BD, Franklin Lakes, NJ). Cells were washed with PBS three times. Liposomes were not detected by fluorescence in the trypsin solution used to remove ECs [17]. Binding data were acquired using an LSRII flow cytometry (BD Immunocytometry Systems, San Jose, CA) and analyzed with WEASEL software developed by WEHI (Parkville, Australia). The fold-over isotype value was calculated by dividing the mean fluorescence intensity for liposomes conjugated with aVCAM1:aE-selectin by that of the isotype-conjugated liposomes. Significant differences in liposome uptake were evaluated using a 2-way ANOVA analysis. A *p* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Characterization of liposomes

Liposomes were prepared from 95 mol% DOPC and 5 mol% N-dod-PE. The diameters of unconjugated DOPC:N-dod-PE liposomes were 205 ± 5 nm, as determined from dynamic light scattering. After conjugation with aVCAM1 and aE-selectin, liposome diameters increased to 223 ± 3 nm; the diameters were similar for all liposome formulations. The antibody density and aVCAM1:aE-selectin ratio were determined using standardized microbeads (Table 1). The average antibody density for 1:0 aVCAM1:aE-selectin ratio was 6703 ± 379 molecules/ μ m² or 842 ± 47 molecules/liposome; this is comparable to other reports (7000 molecules/ μ m² [26], 4100 molecules/ μ m² [27]).

3.2. VCAM1 and E-selectin changes temporally

Inflammatory cytokines were used to upregulate the expression of VCAM1 and E-selectin on ECs as observed by immunostaining at 6 and 24 h (Fig. 2). The surface expression of VCAM1 and E-selectin on TNF- α and IL-1 α -activated ECs was quantified as a function of time (Fig. 3). The expression of VCAM1 and E-selectin was affected by the duration of exposure, but not cytokine type (no statistical difference, *p* > 0.05). The density of E-selectin peaked at 6 h by ten-fold and nine-fold relative to unactivated ECs for both TNF- α and IL-1 α , respectively. VCAM1 increased by three- and two-fold relative to expression on unactivated ECs after 24 h of exposure to TNF- α and IL-1 α , respectively. The surface expression of VCAM1 and E-selectin on activated ECs was used to define a complementary antibody surface presentation on liposomes.

3.3. Complementary liposomes increase cellular binding

Liposomes with different ratios of aVCAM1:aE-selectin were incubated with cytokine-activated ECs to observe the sensitivity of EC surface expression to liposome antibody presentation. ECs are known to transiently express VCAM1 and E-selectin in response to cytokines; this reproducible gene expression profile makes ECs a good model system for studying how changes in the EC surface presentation affects liposome targeting [22]. EC surface expression

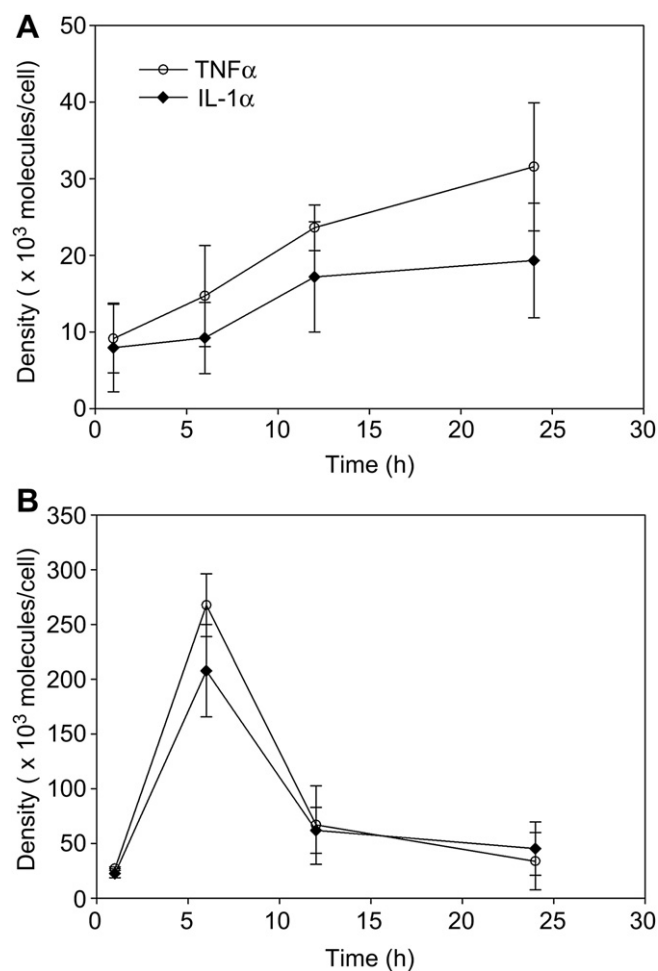


Fig. 3. Time-course expression of VCAM1 and E-selectin on TNF- α and IL-1 α activated ECs. ECs were activated with either TNF- α or IL-1 α for various durations. Expression of VCAM1 (A) and E-selectin (B) was determined using flow cytometry as a function of time. VCAM1 expression increased with time for up to 24 h while E-selectin peaked at 6 h for both TNF- α and IL-1 α .

of VCAM1 and E-selectin was similar for IL-1 α and TNF- α , approximately 1:20 and 1:1 VCAM1:E-selectin at 6 and 24 h, respectively (Fig. 3). EC binding of dextran-encapsulating liposomes was evaluated by flow cytometry as a function of the aVCAM1:aE-selectin ratio (1:0, 1:1, 1:4, 1:8, and 0:1) and activation time (6 and 24 h) (Fig. 4A and B).

At early times, E-selectin is highly expressed in activated ECs; thus, liposomes presenting more aE-selectin exhibit greater binding. Liposome binding increased from 1.7-fold to 3.7-fold relative to IgG-presenting liposomes, when the aVCAM1:aE-selectin ratio was reduced from 1:0 to 1:8 on TNF- α activated ECs, respectively. At later times, the increase in VCAM1 density on TNF- α activated ECs resulted in greater binding of liposomes with a comparable presentation of aVCAM1:aE-selectin. Liposome binding at 24 h peaked at 1:1 aVCAM1:aE-selectin; this was 2.3-fold greater than 1:0 and 0:1 aVCAM1:aE-selectin liposome formulations and ~4-fold greater than the IgG conjugated liposome control. Cellular binding at all non-optimal ratios were significantly lower than the 1:1 aVCAM1:aE-selectin liposomes (*p* < 0.001).

For TNF- α , maximal binding was achieved when liposomes complemented the expression of VCAM1 and E-selectin on the EC surface; peak binding was observed at 1:8 and 1:1 aVCAM1:aE-selectin at 6 and 24 h, respectively (Fig. 4). This was analogous to

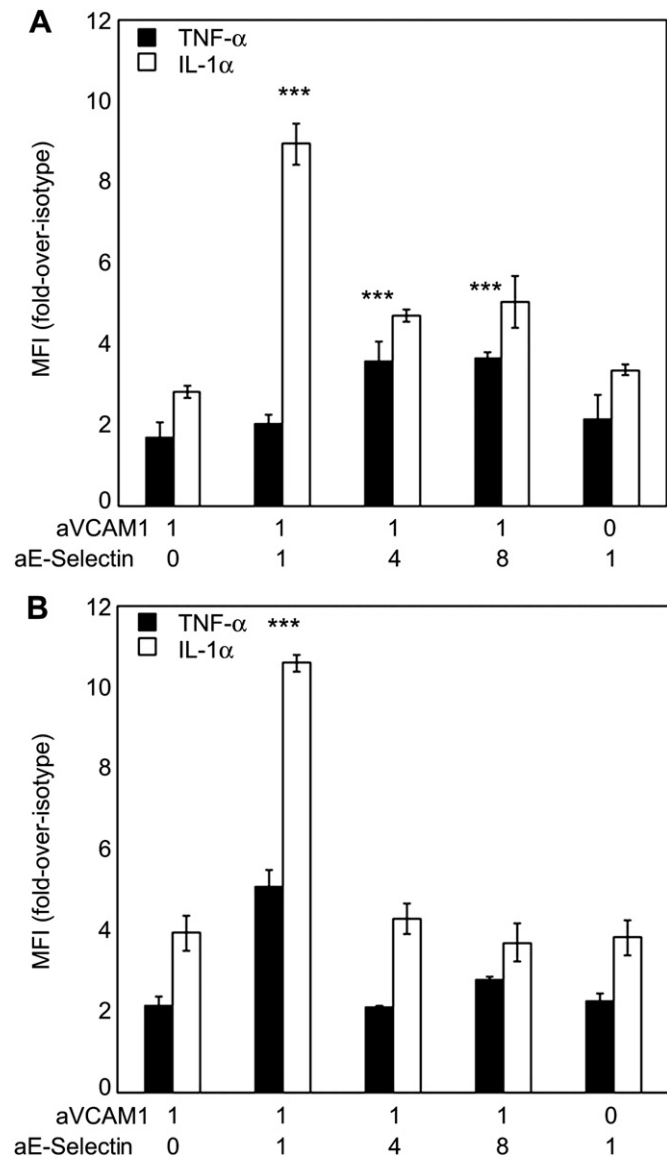


Fig. 4. Uptake of immunoliposomes conjugated with various ratios of aVCAM1 and aE-selectin by ECs. ECs activated with TNF- α and IL-1 α were treated with rhodamine-labeled dextran encapsulated in DOPC:N-dod-PE (95:5 mol:mol) for either 6 h (A) or 24 h (B). Flow cytometry was used to determine the cellular uptake of DOPC immunoliposomes conjugated with various ratios of aVCAM1:aE-selectin. Results are presented relative to uptake by IgG₁-labeled liposomes (In (A) statistical significance was observed between TNF- α 1:4 and 1:8 aVCAM1:aE-selectin groups vs. all other TNF- α conditions and between IL-1 α 1:1 aVCAM1:aE-selectin vs. all other IL-1 α conditions. In (B) statistical significance was observed between TNF- α 1:1 aVCAM1:aE-selectin group vs. all other TNF- α conditions and between IL-1 α 1:1 aVCAM1:aE-selectin vs. all other IL-1 α conditions. *** $p < 0.001$ as measured by 2-way ANOVA).

the ratio of VCAM1:E-selectin expressed on the EC surface (Fig. 3). No significant difference in binding was observed between the 1:20 and 0:1 aVCAM1:aE-selectin liposome formulation except for ECs activated for 24 h with TNF- α ($p < 0.05$) (Supplementary Fig. 1A and B). Complementary liposomes had significantly higher binding than the 1:0 and 0:1 aVCAM1:aE-selectin liposomes, confirming a cooperative interaction between colocalized VCAM1 and E-selectin [17].

Both TNF- α and IL-1 α activated ECs exhibited a ~4-fold increase in binding at 6 h for the 1:8 aVCAM1:aE-selectin liposomes. However, the 1:1 aVCAM1:aE-selectin liposomes exhibited maximal binding on IL-1 α activated ECs at both 6 and 24 h. The

inconsistency in binding may be a result of the organization of the VCAM1 and E-selectin on the surface. At 6 h, E-selectin is observed predominantly in the perinuclear region in IL-1 α activated ECs, which does not consistently overlap with VCAM1 (Fig. 2A). Although the average ratios of VCAM1:E-selectin are similar between TNF- α and IL-1 α , the organization of the molecules may differ upon assembly within lipid rafts. We have previously reported that the organization of cell adhesion molecules within lipid rafts is critical for achieving cooperative binding of liposomes to activated ECs [17].

EC surface expression is used to regulate leukocyte-EC interactions [2,23]. E-selectin, whose expression is restricted on activated ECs, mediates the homing and rolling of leukocytes during the inflammatory response [24]. VCAM1 is virtually absent on normal endothelium; its expression is more widely expressed by cytokine-activated endothelial cells [28,29]. Recently, VCAM1 expression has been shown to cluster primarily on the apical surface of ECs [22,25]. The upregulation of VCAM1 and E-selectin is not enough to target inflamed ECs. The organization of the cell adhesion molecules is important in regulating liposome binding and cell–cell interactions.

Our results suggest that liposomes, designed to be complementary to the activated EC surface at a particular time, may be optimized to deliver agents at specific stages of inflammation. Liposome binding is dependent on the density and organization expressed of molecules on ECs, which is regulated by the exposure time and type of cytokine.

In this report, we have demonstrated that liposomes with a complementary surface presentation to ECs resulted in increased binding relative to liposomes presenting either aVCAM1 or aE-selectin alone. Transiently-upregulated cell adhesion molecules may be exploited to design drug delivery vehicles that will have sustained binding to activated ECs.

4. Conclusions

Liposomes were targeted to IL-1 α and TNF- α activated ECs via cell adhesion molecules, VCAM1 and E-selectin, expressed transiently after cytokine stimulation. Maximal binding was achieved for TNF- α activated ECs when the ratio of aVCAM1:aE-selectin on the liposome surface was complementary to the VCAM1:E-selectin expression. However, the 1:1 aVCAM1:aE-selectin liposomes had maximal binding on ECs activated with IL-1 α at both 6 and 24 h. The density and organization of cell adhesion molecules on activated ECs is important for cooperative binding. Efficient targeting may be achieved by understanding the transient expression, relative density, and organization of target EC surface molecules.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.08.093.

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