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Enhanced Recruitment of Th2 and CLA-Negative Lymphocytes by the S128R Polymorphism of E-Selectin

Ravi M. Rao, Dorian O. Haskard, and R. Clive Landis

E-selectin is a cytokine-inducible endothelial cell adhesion molecule that binds a restricted population of T lymphocytes consisting of Th1 memory cells bearing the cutaneous lymphocyte Ag (CLA). A serine to arginine (S128R) polymorphism in E-selectin has been reported at increased frequency in patients with systemic lupus erythematosus and atherosclerosis. Here we tested the hypothesis that the S128R substitution may contribute to increased vascular disease by altering the number and/or phenotype of lymphocytes interacting with E-selectin under shear flow. We observed that CHO cell monolayers transfected with S128R recruited significantly greater numbers of unfractonated lymphocytes than monolayers expressing an equivalent density of wild-type (WT) E-selectin. Depletion of the CLA+ subpopulation or generation of CLA- lymphoblasts abolished rolling and arrest on WT E-selectin, but left a residual population that interacted with S128R. Generation of Th subsets revealed preferential interaction of Th0 and Th2, but not Th1, cells with S128R compared with WT. However, only T cells of a memory phenotype interacted with S128R, since neither monolayer supported rolling of CD45RA+ cells. Our results demonstrate that the S128R polymorphism extends the range of lymphocytes recruited by E-selectin, which may provide a mechanistic link between this polymorphism and vascular inflammatory disease. The Journal of Immunology, 2002, 169: 5860–5865.

Materials and Methods

Abs and reagents

The mAb SPLAT-1 (anti-E-selectin) was provided by Dr. M. Robinson (Celltech-Chiron Bioscience, Slough, U.K.), and HECA452 (anti-CLA) was purchased from American Type Culture Collection (Manassas, VA). Anti-PSGL-1 (PL-1 and PL-2) Abs and directly conjugated FITC-anti-CD45RO and R-PE-anti-CD45RA Abs were purchased from Serotec (Oxford, U.K.). Anti-rat Ig-conjugated microbeads and anti-CD45RA-conjugated microbeads were purchased from Miltenyi Biotec (Bisley, U.K.). Recombinant human IL-2 was purchased from Roche.
Lymphocytes were isolated from healthy volunteers by density sedimentation over Ficoll (Nycomed Amersham, Little Chalfont, U.K.), followed by panning on petri dishes to remove adherent cells. Following panning, monocyte contamination was <2%, as shown by FACS analysis. Lymphocytes were >98% viable, as assessed by trypan blue exclusion. For removal of CLA\(^+\) cells, lymphocytes were labeled with HECA-452 before magnetic depletion using anti-rat Ig-conjugated microbeads (7). CD45RO\(^+\) and CD45RA\(^+\) populations were generated by magnetic depletion using anti-CD45RA- and anti-CD45RO-conjugated microbeads, respectively. The purity of undepleted, positively and negatively selected cells was assessed by flow cytometric analysis using appropriate, directly conjugated (FITC and R-PE) Abs. To generate CLA\(^+\) cells, lymphocytes were resuspended at 3 \times 10^6 cells/ml in either RPMI 1640 supplemented with 5% AB\(^-\) serum, or XVIVO 15 complete medium (BioWhittaker, Wokingham, U.K.). Lymphocytes were stimulated for 72 h in the presence of 2 \mu g/ml PHA-P (7), after which they were expanded in the presence of 2 ng/ml IL-2, with splitting and supplementation of fresh IL-2 every 48 h. Expression of CLA and PSGL-1 was monitored daily from day 6 onward, and cells were typically used in the parallel plate chamber between days 9–12. To cell subsets were generated by expansion of PHA-P lymphoblasts in the presence of 2 ng/ml IL-2 (Th0 cells), 2 ng/ml IL-2 plus 2 ng/ml IL-12 (Th1 cells), or 2 ng/ml IL-2 plus 10 ng/ml IL-4 (Th2 cells) as previously described (14) Expression of WT E-selectin was always equal to or greater than S128R, and at least three independent experiments, taking from at least 10 days of culture.

The generation of CHO cell clones expressing S128R and WT E-selectin has been previously described (23). To identify a pair of precisely matched clones, >50 WT and >150 S128R stable transfectants were screened. This yielded two clones, IB9 and 15F2, respectively, that expressed nearly identical levels of E-selectin (23). Each clone was then further subcloned, and up to six subclones of each were maintained in continuous culture to allow exactly matched pairs to be picked for each experiment. The expression of WT E-selectin was always equal to or greater than S128R expression. CHO cells were grown to confluence in 9-cm² Nunc Slide Flasks (Nalge-Nunc International, Roskilde, Denmark) and mounted in a parallel plate flow chamber (channel height, 0.15 cm). Untransfected CHO cells were used as a negative control. Lymphocytes in the perfusate were labeled with 1 \mu g/ml Calcein-AM (Molecular Probes, Eugene, OR) and resuspended at 0.3 \times 10^6 cells/ml in HBBS containing 2% FCS (viscosity, 0.007 Poise) before perfusion at 37°C over CHO cell monolayers at a shear stress of 1.5 dynes/cm². Where specified, CHO cells were preincubated for 30 min at 37°C with either 50 \mu g/ml Spleen (anti-E-selectin) Ab or control Ig. Experiments were visualized using an inverted Diaphot 300 fluorescence microscope (Nikon, Melville, NY) connected to a JVC TK-C1360B color video camera and recorded on a Panasonic AG-6730 S-VHS video recorder (Microscope Service & Sales, Egham, U.K.). Following an initial period of perfusion (2 min) to allow the cells to adhere, lymphocytes were stimulated for 72 h in the presence of 2 \mu g/ml PHA-P (Microscope Service & Sales, NY) connected to a JVC TK-C1360B color video camera and recorded on using an inverted Diaphot 300

The S128R polymorphism of E-selectin allows interactions with CLA\(^+\) lymphocytes

To determine whether increased recruitment was due to the capacity of S128R E-selectin to interact with CLA\(^+\) lymphocytes, CLA\(^+\) cells were depleted using mAb HECA-452-conjugated magnetic beads. CLA expression varied between donors (15–40% of untransfected lymphocytes), but depletion with mAb HECA 452 consistently reduced this to <5% of total CLA\(^+\) cells regardless of starting CLA levels (Fig. 1, C and D), Depletion of CLA\(^+\) cells led to almost complete abolition of lymphocyte recruitment to WT E-selectin, whereas a significant residual population of CLA\(^+\) lymphocytes interacted with and arrested on S128R monolayers (Fig. 1, A and B).

![Image](https://example.com/figure1.png)

**FIGURE 1.** The S128R polymorphism of E-selectin enhances interaction with CLA\(^+\) lymphocytes. Lymphocytes were isolated from volunteer donors and depleted of HECA452\(^+\) cells by magnetic bead selection. Fractions of untransfected and unfractonated lymphocytes were perfused over CHO cell monolayers expressing equivalent densities of WT or S128R E-selectin at 1.5 dynes/cm². The number of interacting cells (rolling plus adherent) was calculated as described in Materials and Methods. A, There was a significant increase in the number of lymphocytes interacting with S128R compared with WT monolayers both before and after HECA452 depletion (*, p < 0.05). All interactions were abolished in the presence of the anti-E-selectin Ab SPLAT-1. B, Although no differences in mean rolling velocities were observed when comparing unfractonated lymphocytes perfused over WT or S128R monolayers, there was a significant increase in the number of cells that arrested on S128R monolayers compared with WT both before and after HECA 452 depletion (*, p < 0.05). No cells arrested in the presence of the E-selectin Ab, SPLAT-1. C, Flow cytometric histograms depict the percentage of cells staining positively with HECA452 Ab before and after magnetic bead depletion. Typically, 15–40% of undepleted lymphocytes were HECA452\(^+\). D, Following depletion, <5% were HECA452\(^-\).

### Results

Unfractonated lymphocytes demonstrated significantly increased interactions when perfused at 1.5 dynes/cm² over CHO cell monolayers bearing S128R compared with equivalent levels of WT E-selectin (Fig. 1A, *, p < 0.05). The rolling velocity on WT vs S128R was similar (12.36 ± 0.71 vs 10.03 ± 1.16 \mu m/s). All interactions on both S128R and WT transfectants were abrogated in the presence of the anti-E-selectin Ab, SPLAT-1 (Fig. 1, A and B, *), and untransfected monolayers did not support any lymphocyte interaction (data not shown), thus confirming the requirement for E-selectin in the system. The differences in accumulation between S128R and WT were greatest at 1.5 dynes/cm², since few interactions of CLA\(^+\) cells were observed at shear stresses >2 dynes/cm² (not shown), consistent with other reports (15).
Parallel experiments were carried out using cultured CLA<sup>+</sup> and CLA<sup>-</sup> lymphoblasts, prepared by in vitro culture in serum-free or serum-supplemented medium, as previously described (7). Thus, T cells expanded under serum-free conditions (XVIVO 15 medium) have been shown to express elevated levels of FT VII, leading, in turn, to post-translational modification of PSGL-1 and expression of CLA (24). We confirmed that lymphocytes cultured in XVIVO 15 exhibited both PSGL-1 and CLA expression, whereas cells cultured in RPMI 1640 (enriched with 5% AB<sup>+</sup> serum) expressed PSGL-1, but no detectable CLA epitope (Fig. 2, A and B). CLA<sup>-</sup> and CLA<sup>-</sup> T lymphoblasts thus prepared were perfused over WT or S128R monolayers, and the total number of interactions was quantified as before. No difference in the number of CLA<sup>-</sup> cells interacting with either WT or S128R monolayers was observed (Fig. 2C, □), and again there was no significant difference in rolling velocity (13.35 ± 0.63 vs 10.97 ± 1.09 μm/s). Comparatively few CLA<sup>-</sup> T lymphoblasts interacted with WT E-selectin, but significantly more CLA<sup>-</sup> cells were recruited to S128R (Fig. 2C, ■; p < 0.01). As with unfractionated lymphocytes, there was no difference in the mean rolling velocity between CLA<sup>+</sup> and CLA<sup>-</sup> T cells rolling on WT or S128R monolayers (15.68 ± 1.47 vs 12.84 ± 0.86 μm/s for CLA<sup>-</sup> cells). All interactions of lymphoblasts with WT or S128R E-selectin were abolised in the presence of the anti-E-selectin Ab, SPLAT-1 (data not shown).

The S128R polymorphism of E-selectin enhanced recruitment of Th2 and Th0 cells

A number of previous reports have demonstrated that E-selectin preferentially recruits Th1 compared with Th2 or Th0 cells (14, 15, 25, 26). To assess whether the S128R polymorphism could extend the range of Th cells recognized, Th1, Th2, or Th0 lymphoblasts were generated in culture as previously described (14). As expected, expression of the CLA epitope was promoted (42%) under Th1 culture conditions (IL-2 and IL-12), but was down-regulated (≤10%) under Th2 (IL-2 and IL-4) or Th0 (IL-2) conditions (Fig. 3, A–C). Th1 lymphoblasts interacted well with both WT and S128R E-selectin monolayers (Fig. 3D, ■), but there was no significant difference between either monolayer and no significant difference in rolling velocity (17.78 ± 1.89 vs 13.87 ± 1.61 μm/s). Th2 and Th0 cells, on the other hand, interacted poorly on WT monolayers (Fig. 3D, □ and △), but showed significantly enhanced interaction on S128R (p < 0.05 and < 0.01 for Th2 and Th0, respectively) at the same rolling velocities as Th1 cells (Th2, 13.59 ± 3.43 μm/s; Th0, 12.71 ± 1.89 mm/s). All Th2 interactions on S128R were abrogated in the presence of the anti-E-selectin Ab, SPLAT-1 (Fig. 3D).

The S128R polymorphism of E-selectin does not enhance recruitment of CD45RA<sup>+</sup> lymphocytes

Previous work has indicated that E-selectin mediates rolling of a subset of CD45RO<sup>+</sup> (memory) cells, but not CD45RA<sup>+</sup> (naive) cells (8). To address whether the S128R polymorphism alters this paradigm, populations of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> cells were isolated by positive magnetic selection. Compared with unfractionated lymphocytes, which were 37% CLA<sup>-</sup>, CD45RO<sup>+</sup> cells were 58% CLA<sup>-</sup>, and CD45RA<sup>+</sup> cells were 14% CLA<sup>-</sup>. When assessed in the parallel plate flow chamber, CD45RO<sup>+</sup> cells interacted well with both WT and S128R monolayers, but significantly more cells interacted with S128R (Fig. 4C, ■; p < 0.01). In contrast, CD45RA<sup>+</sup> cells exhibited fewer interactions on WT E-selectin, and this was not enhanced on S128R monolayers (Fig. 4C, □). The rolling velocity of CD45RO<sup>-</sup>-enriched cells (15.3 ± 4.46 μm/s on WT; 11.82 ± 1.45 μm/s on S128R) was similar to that of unfractionated lymphocytes (Fig. 1B).

Discussion

In our previous studies we demonstrated that the S128R polymorphism of E-selectin conferred a gain-of-function phenotype, leading to neuraminidase-resistant tethering of myeloid cells under flow conditions (23). In this study we have extended our characterization of the S128R polymorphism to examine whether lymphocyte recruitment may be altered, in both number and specificity.

We have shown that S128R E-selectin significantly enhances the rolling and arrest of unfractionated and CLA-depleted lymphocytes. This suggests either that non-Th1 subsets or B cells (27) were additionally recruited to S128R. To better define the T cell specificity of S128R interaction, we generated T lymphoblasts under defined culture conditions and found that Th0 and Th2 lymphoblasts were preferentially recruited to S128R compared with
WT E-selectin. However, no difference was detected in the recruitment of CD45RA+ lymphocytes between WT and S128R. This is therefore the first study to demonstrate that an adhesion molecule polymorphism can alter the specificity of leukocytes recruited under shear flow. Since all interactions were abolished in the presence of an anti-E-selectin Ab, this ruled out any contribution from other rolling mechanisms, such as very late Ag-4/VCAM-1 (28–30). Rolling interactions were not fully blocked with anti-PSGL-1 Ab (PL-1) on either WT or S128R (data not shown). This is in keeping with recent observations that other WT E-selectin ligands exist on T cells (31, 32) and furthermore suggests that the augmented interaction on S128R is independent of PSGL-1. In contrast to the recently described 95-kDa WT E-selectin ligand identified in T cell lysates (32), the S128R monolayers interacted with cells that did not express CLA.

The nature of the ligands involved in the recruitment of CLA− and Th2 lymphocytes to S128R E-selectin remains unknown. Unfortunately, we were not able to examine the sialic acid requirement for the enhanced interaction with S128R E-selectin, since even the briefest treatment of lymphocytes with neuraminidase (<30 min) resulted in gross morphological change and significant cell death. Our previous study, however, demonstrated that myeloid cell interactions with S128R were fucose-independent and neuraminidase-insensitive (23). Since neither CLA− nor Th2 lymphoblasts express sufficient FucT-VII to generate adequate functional ligands for E-selectin binding (12, 14, 15), it is likely that augmented lymphocyte recruitment by S128R does not require fucosylation, although further studies would be required to prove this.

In our experimental model we consistently observed that lymphocytes not only rolled, but also arrested, on E-selectin CHO cell transfectant monolayers in the absence of any additional stimulus. Our previous studies have shown that arrest of HL60 cells is β2 integrin dependent, presumably mediated through binding to hamster ICAM-1 (23). Whether contact with E-selectin is sufficient to stimulate β2 integrin adhesion, as has been reported for neutrophils (33–35), remains an open question, as does the possibility that enhanced interaction with S128R may generate further signals, leading to up-regulated β2 integrin function. Even if no additional signals are generated, our previous studies with β2 integrin-transfected K562 cells have shown that neuraminidase-insensitive tethering on S128R are of sufficient strength and duration to be converted into firm adhesion (23), a principle that has already been established for T cells in the conversion of very late Ag-4/VCAM tethers to static adhesion in the absence of an obligate rolling step (29).

The chemokine receptors expressed by lymphocyte subsets are thought to play a key role in the regional homing of lymphocytes (36). For example, CLA+ lymphocytes recruited to skin also express the chemokine receptors, CCR4 (9), which binds thymus- and activation-regulated chemokine and plays a role in converting rolling into static interactions, and CCR10 (10), which binds cutaneous T cell-attracting chemokine, and plays a role in attracting CLA+ T cells to the epidermis (37). The ability of S128R E-selectin to bind CLA+ lymphocytes raises the possibility that CLA+ cells may become inappropriately exposed to regional chemokines, thus disturbing their normal homing patterns. To our knowledge, no clinical studies to date have examined the role of this polymorphism in the recruitment of T cell subsets to the affected tissues of patients with either atherosclerosis or SLE. There is some evidence that the presence of Th2 cells may worsen disease pathology in SLE (38), particularly in the chronic stages of murine lupus models (39). Furthermore, a Th1/Th2 switch is observed in severe hypercholesterolemia in the apolipoprotein E−/− mouse (40). The recruitment of CLA− or Th2 cells to skin may also impact on the pathology of diseases such as psoriasis or T cell lymphoma, which are characterized by CLA+ Th1 cell infiltration. Thus, the recruitment of Th2 cells may allow local synthesis of IL-4 and IL-5, leading to recruitment of eosinophils or, possibly,
regulatory CD25+ T cells. In the absence of further clinical information, it remains to be seen what impact alterations in T cell subset recruitment would have on disease expression or response to treatment in S128R-bearing individuals.

The S128R polymorphism lies in the EGF domain of E-selectin within a sequence of three amino acids (Ser-126-Cys-Ser) that is conserved among all selectins and across species. The cysteine at 127 forms part of a cysteine-rich repeat sequence described in a number of molecules containing an EGF-like domain and is thought to be structurally important due to its ability to form disulfide bonds (41, 42). Although the principal ligand contact points of the selectins lie within the lectin domain (43, 44), domain swaps between L- and P-selectins have suggested that the EGF domain can modulate the binding properties of the lectin domain to surface-immobilized ligand without affecting the equilibrium binding properties toward soluble ligand (45, 46). Thus, substitution of an uncharged serine with a positively charged arginine at residue 128 may influence E-selectin function either directly, by binding novel ligands, or indirectly, by inducing a conformational change in the lectin-EGF domains.

In summary, we have described important functional consequences of the S128R polymorphism in E-selectin that could result in imbalanced lymphocyte recruitment during inflammation and tissue-specific homing. These observations provide a possible mechanistic link between the expression of the S128R polymorphism and increased incidence of atherosclerosis and autoimmune disease.

References


