Modification of P-selectin glycoprotein ligand-1 with a natural killer cell-restricted sulfated lactosamine creates an alternate ligand for L-selectin

Pascale André*, Olivier Spertini†, Sophie Guia*, Pascal Rihet‡, Françoise Dignat-George§¶, Hervé Brailly‖, José Sampol§¶, Paul J. Anderson**, and Eric Vivier*¶††‡‡

*Centre d'Immunologie de Marseille-Luminy, Institut National de la Santé et de la Recherche Médicale/Centre National de la Recherche Scientifique, Case 906, 13288 Marseille Cedex 09, France; †Division of Hematology and the Hematology Central Laboratory, Université de Lausanne, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; ‡Laboratoire de Génétique et Immunologie du Paludisme, Faculté des Sciences de Luminy, Marseille, France; §Laboratoire d'Hématologie et d'Immunologie, Faculté de Pharmacie, Marseille, France; ¶Service d'Hématologie, Hôpital de la Conception, Marseille, France; ‖Immunotech, Marseille, France; **Division of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, MA; and ††Institut Universitaire de France

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Natural killer (NK) cells are components of the innate immune system that can recognize and kill virally infected cells, tumor cells, and allogeneic cells without prior sensitization. NK cells also elaborate cytokines (e.g., interferon-γ and tumor necrosis factor-α) and chemokines (e.g., macrophage inflammatory protein-1α) that promote the acquisition of antigen-specific immunity. NK cell differentiation is accompanied by the cell surface expression of a mucin-like glycoprotein bearing an NK cell-restricted keratan sulfate-related lactosamine carbohydrate, the PEN5 epitope. Here, we report that PEN5 is a post-translational modification of P-selectin glycoprotein ligand-1 (PSGL-1). The PEN5 epitope creates on PSGL-1 a unique binding site for L-selectin, which is independent of PSGL-1 tyrosine sulfation. On the surface of NK cells, the expression of PEN5 is coordinated with the disappearance of L-selectin and the up-regulation of Killer cell Ig-like Receptors (KIR). These results indicate that NK cell differentiation is accompanied by the acquisition of a unique carbohydrate, PEN5, that can serve as part of a combination code to deliver KIR$^+$ NK cells to specific tissues.

Natural killer (NK) cells are lymphocytes of the innate immune system that recognize and induce the lysis of a variety of target cells, including virally infected cells, tumor cells, and allogeneic cells without prior sensitization (1, 2). In addition, NK cells elaborate a variety of regulatory cytokines, including interferon-γ, transforming growth factor-β_1, tumor necrosis factor-α, IL-1β, IL-10, granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor, and CC-chemokines, such as RANTES, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β, which are involved in the elimination of intracellular pathogens *in vivo*, as well as in the generation of antigen-specific immune response (3, 4). A feature of NK cell cytolytic activities and cytokine production is that their initiation depends on the integrity of MHC class I expression on target cells (1, 2, 5). Indeed, NK cells express cell surface receptors such as killer cell Ig-like receptors (KIRs) and CD94/NKG2 heterodimers, which can sense the alteration of MHC class I molecules at the surface of target cells (1, 2, 5).

Detection of diseased cells by NK cells is critically dependent on the ability of NK cell trafficking and homing. In steady-state conditions, NK cells are present mostly in bloodstream and spleen. However, NK cells are capable of extravasation and infiltrate various organs such as lungs and liver at the sites of inflammation, infection and tumor growth (6–8). The egress of leukocytes from the vasculature is initiated by the Ca^{2+}-dependent tethering to endothelial cells or adherent leukocytes that is mediated by interactions between selectins and their carbohydrate ligands (9). Leukocytes express P-selectin glycoprotein ligand-1 (PSGL-1), which mediates adhesion to E- and P-selectins, thereby promoting the initial tethering and rolling of leukocytes along endothelial cells that comprise the high endothelial venules (10). Depending on its post-translational modifications, PSGL-1 can also interact with L-selectin to promote leukocyte–leukocyte interactions that amplify the accumulation of leukocytes to sites of inflammation (11–13). Finally, leukocytes bearing L-selectin can be captured by specific sialylated sulfated oligosaccharides such as sulfated isoforms of sialyl Lewis^x (sLe^x) tetrasaccharides: NeuNAcα2–3Galβ1–4(Fucα1–3)GlcNac. Sulfated isoforms of sLe^x decorate the vascular addressins GlyCAM-1, CD34, MAdCAM-1, Sgp^{200}, and podocalyxin-like protein (14–18). As leukocytes roll along the endothelial cell surface, they are activated to express integrins that promote increased adhesion and spreading, the second phase of leukocyte extravasation (9, 17, 18). Transmigration of firmly adherent leukocytes across endothelial cell junctions requires homotypic adhesions mediated by CD31 (PECAM-1), an Ig superfamily member expressed on both leukocytes and endothelial cells (19).

Despite the recent advances in the identification of the dynamic balance between activating and inhibitory signals that control NK cell activation programs, the mechanisms that govern NK cell trafficking are still poorly characterized. Peripheral blood NK cells are equipped with a panel of cell surface molecules that have been documented to participate in the binding of NK cells to endothelial cells, such as lymphocyte function-associated antigen-1, very late antigen 1, CD44, CD2, and CD31 (19, 20). Selectin ligands also have been described on NK cells, such as sialy stage-specific embryonic antigen 1 and an uncharacterized sLe^x-bearing receptor, which may serve as E-selectin ligand (21, 22). Although PSGL-1 is expressed on freshly isolated NK cells, only a minor population of NK cells bind P-selectin-Ig (22, 23). Mature CD56^{dim}CD16$^+$ NK cells expressing little or no L-selectin can also leave the vasculature and accumulate in peripheral tissues (24, 25).

We show here that PEN5, a sulfated lactosamine epitope selectively expressed on mature CD56^{dim}CD16$^+$ NK cells, is a carbohydrate decoration of PSGL-1. The PEN5 decoration confers on PSGL-1 the ability to bind to L-selectin, suggesting that PEN5/PSGL-1 may serves as a NK-homing/trafficking receptor.

Abbreviations: NK, natural killer; PSGL-1, P-selectin glycoprotein ligand-1; KIR, Killer cell Ig-like Receptor; CLA, cutaneous lymphocyte-associated antigen; sLe^x, sialyl Lewis^x.

‡‡To whom reprint requests should be addressed. E-mail: vivier@ciml.univ-mrs.fr.

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Materials and Methods

Cell Preparation. Peripheral blood mononuclear cells were isolated by density gradient separation (Ficoll-Paque Plus; Pharmacia) from the peripheral blood of healthy volunteers. Purified NK cells were prepared from peripheral blood mononuclear cells by negative selection using mAb directed against CD3, CD4, CD5, CD14, and CD20 (Immunotech, Luminy, France) and goat anti-mouse IgG-conjugated magnetic beads (PerSeptive Biosystems) (26). Negatively selected cells were >80% $CD56^{+}CD16^{+}$ and <10% $CD3^{-}$ and <5% $CD19^{-}$. In some experiments, highly purified NK cells (>96% $CD56^{+}CD16^{+}$) were obtained by cell sorting using a FACStar flow cytometer (Becton Dickinson) after a first round of magnetic separation and staining with phycoerythrine (PE)-conjugated goat anti-mouse IgG antibodies (PE-GAM; Jackson ImmunoResearch). The U937 cell line (American Type Culture Collection) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine (GIBCO/BRL).

Enzymatic Treatments. Cells (10^7/ml) were incubated in RPMI 1640 medium in the presence of either 0.1 unit/ml endo-β-galactosidase (keratanase; Boehringer Mannheim) or 50 μg/ml *O*-sialoglycoprotein endopeptidase (Cedarlane Laboratories), 0.1 unit/ml *Vibrio cholerae* neuraminidase (Calbiochem), 0.4 unit/ml hyaluronidase (Sigma), or 1 μg/ml mocarhagin (kindly given by Gray Shaw, Genetics Institute, Boston) for 30–60 min at 37°C, washed, and then analyzed for expression of PEN5, PSGL-1, and CD56 by flow cytometry or infused into the flow chamber for adhesion assays. Mock treatment was performed under similar conditions in the absence of enzyme.

Flow Adhesion Assay. Recombinant human selectins were purchased from R & D Systems. Recombinant selectins were diluted in PBS (5 μg/ml for E-selectin, 25 μg/ml for L-selectin, and 10 μg/ml for P-selectin) and absorbed on 35-mm polystyrene dish overnight at 4°C. Plates were blocked with 2% human serum albumin (Sigma) for at least 30 min at room temperature before use. In some experiments, NK cells (10^7/ml) were preincubated at 4°C for 20 min with 10 μg/ml blocking anti-PSGL-1 mAb (PL1, mouse IgG1; Immunotech), 20 μg/ml anti-CD56 mAb (3B5, mouse IgM; Immunotech), 20 μg/ml anti-PEN5 mAb (5H10, mouse IgM; Immunotech), or a 1:2 dilution of 5H10 supernatant or 1:2 dilution of a mixture of anti-KIRs mAb supernatant (mouse IgM to CD158a and CD158b, XA141 and Y249 respectively; kind gift from A. Moretta, University of Georgia). NK cells were then diluted (10^6/ml). In other experiments, the substrates were preincubated for 10 min with one blocking mAb: anti-L-selectin mAb (Lam1-3), anti-E-selectin mAb (7A9), or anti-P-selectin mAb (WASP). Cell interactions with selectins were studied in a parallel plate flow chamber (GlycoTech, Rockville, MA). The chamber was mounted on the stage of an inverted microscope. Cell suspensions (10^6/ml) in RPMI 1640/1% FCS, were perfused through the chamber with an automated syringe pump (Harvard Apparatus). Interacting cells in the fields were quantitated by analysis of videotaped images (5-min perfusion period) (27).

Immunofluorescence Staining and Flow Cytometry Analysis. For antibody staining, 0.2–0.5 × 10^6 cells were incubated with 5 μg/ml of biotinylated 5H10 (anti-PEN5), 10 μg/ml PL1 (anti-PSGL-1), KPL1 (mouse IgG1 mAb to PSGL-1; kind gift from G. Kansas, Northwestern University Medical School), or HECA-452 (rat IgM mAb to the cutaneous lymphocyte-associated antigen; CLA) followed by FITC-conjugated streptavidine or PE-GAM. For double or tri-color staining, PC5-conjugated anti-CD56 mAb, and PE-conjugated anti-L-selectin or anti-CD16, anti-NKG2A, or anti-KIRs mAb (a mixture composed of anti-CD158a, anti-CD158b, anti-p70, and anti-p50.3 mAb; Immunotech) were added after blocking with 10% mouse serum. Flow cytometry analysis was performed on a FACScalibur (Becton Dickinson) using CELLQUEST software (Becton Dickinson). Results are representative of multiple independent observations for each data set.

Immunoblotting and Immunoprecipitations. Cells were lysed in 1% Nonidet P-40 lysis buffer; postnuclear supernatants were prepared as described elsewhere (26) and immunoprecipitated with anti-PSGL-1 mAb (PL1 and PL2) or with control mouse IgG1 or with control mouse IgM or with anti-PEN5 mAb (5H10). Immunoprecipitates were treated with 10% 2-ME for 72 hr at 4°C and then subjected to SDS/PAGE. After transfer to Immobilon-P (Millipore), blots were developed using anti-PSGL-1 mAb or anti-PEN5 mAb, and revealed using peroxydase-conjugated rabbit anti-mouse Ig (for anti-PSGL-1 blots) or rabbit anti-mouse Ig plus peroxydase-conjugated goat anti-rabbit Ig (for anti-PEN5 blots) followed by chemiluminescence (Renaissance, NEN).

Statistical Analysis. All data are shown as mean values ± SEM. Statistical differences between experimental groups were eval-

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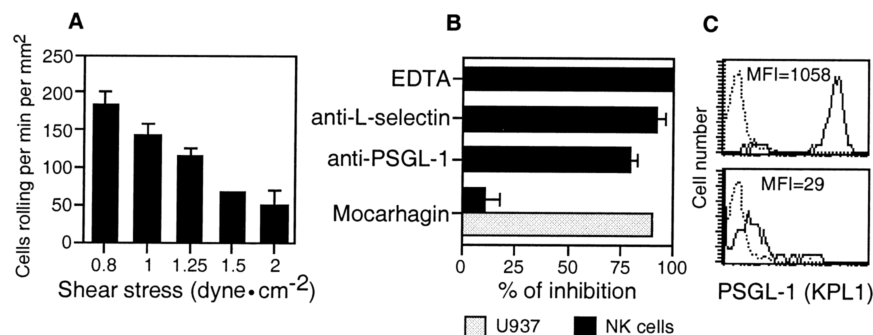

Fig. 1. Tethering and rolling of NK cells on L-selectin under flow conditions. (*A*) Freshly isolated NK cells were perfused at the indicated shear stress (dyne/cm^2) over immobilized L-selectin for a 5-min period. Interacting cells were determined by analysis of videotapes in which two fields (0.14 mm^2 per field) on a video monitor were counted throughout the flow period. Values represent mean ± SEM of results from three experiments. (*B*) Cells were perfused at 1.25 dyne/cm^2 over immobilized L-selectin either in the continued presence of 5 mM EDTA, after preincubation of the substrate with a blocking anti-L-selectin mAb (LAM1-3), after preincubation of NK cells with a blocking anti-PSGL-1 mAb (PL1), or after treatment of NK cells (solid bars) or U937 (shaded bars) with mocarhagin. The percentage of inhibition for each condition is calculated relative to the untreated control. Data represent mean ± SEM of results from three experiments. (*C*) Flow cytometric analysis of KPL1 mAb (continuous line; isotype-matched control mAb, dotted line) binding to NK cells incubated for 30 min in the absence (*Upper*) or presence (*Lower*) of mocarhagin (mean fluorescence intensity, MFI; *x* axis, fluorescence, 4-decade log scale).

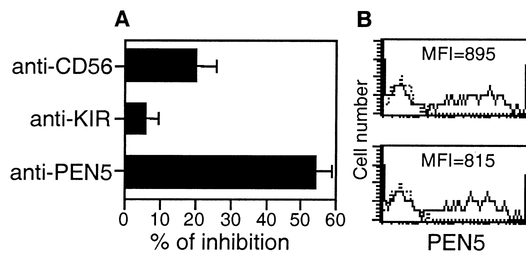

Fig. 2. Anti-PEN5 mAbs inhibit NK cell adhesion to L-selectin. (*A*) NK cells were incubated with either anti-CD56 mAb (3B5) or anti-PEN5 mAb or with 1:2 dilution of 5H10 supernatant or 1:2 dilution of anti-KIRs supernatant; then, NK cells were diluted and the assay was performed as described in Fig. 1*B*. The percentage of inhibition for each condition is calculated relative to untreated controls. Values represent mean ± SEM of results from four experiments. (*B*) Flow cytometric analysis of PEN5 expression (continuous line; isotype-matched control mAb, dotted line) on NK cells incubated for 30 min in the absence (*Upper*) or presence (*Lower*) of mocarhagin treatment.

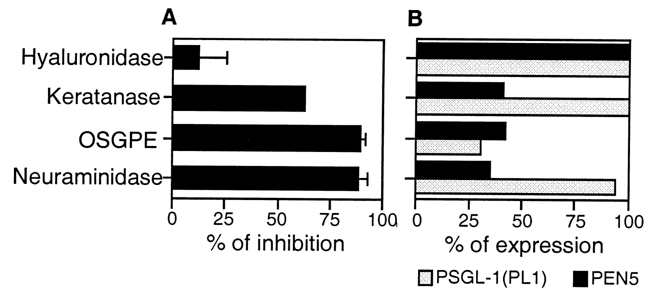

Fig. 3. Sensitivity of NK cells binding to L-selectin and of the PEN5 and PL-1 epitopes to glycosidase treatment. Cells were incubated for 30–60 min at 37°C in the absence or presence of the indicated enzymes, then washed, and either tested for adhesion (*A*) or analyzed by indirect immunofluorescence and flow cytometry for their reactivity with the PL1 (anti-PSGL-1; gray bars) and 5H10 (anti-PEN5; black bars) mAbs (*B*). Hyaluronidase was used as a negative control enzyme. The percentages of inhibition for each condition (*A*) and of surface expression for each antigen (*B*) are calculated relative to untreated controls. Values represent mean ± SEM of results from three experiments. Binding of anti-CD56 mAb to enzyme-treated NK cells was not affected (data not shown).

uated by Student's *t* test by using paired comparisons; *P* values < 0.05 were considered significant.

Results

Tethering and Rolling of NK Cells on L-Selectin. We quantitated the ability of human peripheral blood NK cells to tether and roll on immobilized recombinant L-selectin in a parallel plate flow chamber. L-selectin supports tethering and rolling of purified NK cells under flow conditions, and the ability of NK cells to interact with L-selectin depends on the level of wall shear stress (Fig. 1*A*). NK cell tethering and rolling on L-selectin is abolished in the presence of EDTA, consistent with the Ca^{2+} requirement for the interaction between L-selectin and its ligands (Fig. 1*B*) (28). As expected, treatment of the adsorbed substrate with LAM1-3 (29), a blocking mAb that binds to L-selectin, also impairs NK cell interactions with L-selectin (Fig. 1*B*). Treatment of NK cells with PL1, a blocking mAb that binds to the N-terminal residues L49 to M62 of PSGL-1, also prevents NK cell tethering and rolling on L-selectin (Fig. 1*B*) (10, 12). Surprisingly, mocarhagin, a metalloprotease that cleaves the N-terminal selectin-binding domain of PSGL-1 at residue Y51 (30), fails to inhibit the tethering and rolling of NK cells on L-selectin (Fig. 1*B*). Enzymatic activity was verified by showing that mocarhagin-treated NK cells no longer express the KPL1 epitope comprised between residues Y46 and Y51 of PSGL-1 (Fig. 1*C*) and by the ability of mocarhagin to eliminate the tethering and rolling of U937 cells on L-selectin (Fig. 1*B*). These results prompted us to postulate that a mocarhagin-resistant decoration of PSGL-1 might create a NK cell-specific binding site for L-selectin.

Anti-PEN5 mAbs Inhibit NK Cell Tethering and Rolling on L-Selectin. We previously reported the selective expression on NK cells of PEN5, a keratan sulfate-like carbohydrate epitope of a still undefined sialomucin glycoprotein (26). Anti-PEN5 mAb were tested for their ability to block the tethering and rolling of NK cells under flow conditions. Anti-PEN5 mAb inhibit the number of NK cells interacting with L-selectin by 54 ± 3.5% ($P < 0.0001$, $n = 15$) (Fig. 2*A*). Isotype-matched mAb reactive with NK cell surface molecules CD56 or KIRs (CD158a and CD158b) do not significantly inhibit interactions between NK cells and L-selectin (Fig. 2*A*). Importantly, the cell surface expression of PEN5 is resistant to mocarhagin treatment (Fig. 2*B*). In contrast, keratanase, an enzyme that cleaves sulfated lactosamine disaccharides, both eliminates the PEN5 epitope while preserving the PL1 epitope, and inhibits interaction between NK cell and L-selectin by 63 ± 0.2% ($n = 4$) (Fig. 3 *A* and *B*). Similarly, treatment of NK cells with either *O*-sialoglycoprotein endopeptidase or neuraminidase coordinately inhibits NK cell adhesion to L-selectin and the cell surface expression of PEN5 (Fig. 3 *A* and *B*). As expected, PL-1 expression is only affected by *O*-sialoglycoprotein endopeptidase treatment of NK cells (Fig. 3*B*). Therefore, masking or removal of the PEN5 epitope strictly correlates with the ability of NK cells to interact with L-selectin under flow conditions.

The PEN5 Epitope Is a Carbohydrate Decoration of PSGL-1. PEN5 is selectively expressed on a subset of terminally differentiated $CD56^{dim}$ NK cells, which comprise 65 ± 4% ($n = 23$) total NK cells (Fig. 4*A*). Both $PEN5^+$ and $PEN5^-$ NK cells express similar levels of PSGL-1 (Fig. 4*B*). These results confirm that PSGL-1 is expressed on all NK cells (23) and support the possibility that the PEN5 epitope is an NK cell-restricted carbohydrate modification of PSGL-1 acquired during NK cell maturation. This hypothesis was tested by separating anti-PSGL-1 and anti-PEN5 immunoprecipitates prepared from NK cell lysates on SDS-polyacrylamide gels and immunoblotting using anti-PSGL-1 mAb or anti-PEN5 mAb. Anti-PSGL-1 immunoblotting of anti-PSGL-1 immunoprecipitates reveal two polydispersed glycoproteins (≈220–240 kDa and ≈110–140 kDa) corresponding to monomers and dimers of PSGL-1 (31) (Fig. 5, lane 1). As described earlier, the dimeric form of PSGL-1 is highly resistant to reduction induced by β-mercaptoethanol (31). As previously reported (26), anti-PEN5 mAb immunoprecipitate and recog-

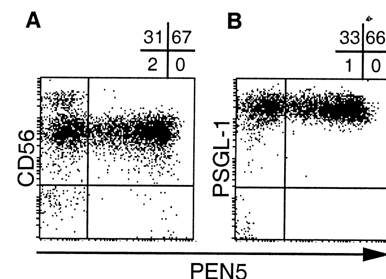

Fig. 4. Cell surface expression of PEN5 and PSGL-1 on NK cells. Flow cytometric analysis of highly purified NK cells using anti-PEN5, anti-CD56 (*A*) and anti-PSGL-1 (PL1) (*B*) mAb. Horizontal and vertical lines delineating positive and negative staining were set with appropriate negative control mAb. The percentage of cells in each quadrant is indicated at the top of each scatter profile.

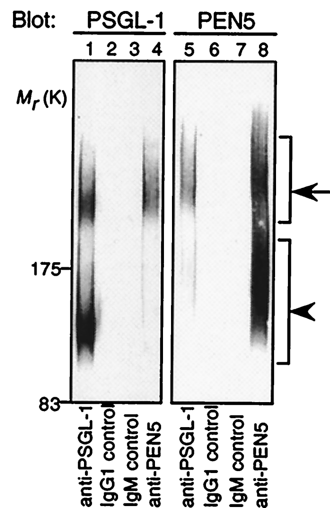

Fig. 5. Biochemical analysis of PSGL-1 and PEN5 on NK cells. NK cell lysates were immunoprecipitated with anti-PSGL-1 mAb (lanes 1 and 5), with control mouse IgG1 (lanes 2 and 6), with control mouse IgM (lanes 3 and 7), or with anti-PEN5 mAb (lanes 4 and 8). Immunoprecipitates were subjected to SDS/PAGE and immunoblotted with anti-PSGL-1 mAb (*Left*) or anti-PEN5 mAb (*Right*). The anti-PSGL-1 and anti-PEN5 mAb specifically identified two polydispersed bands of apparent M_r 220–240 kDa (arrow) and 110–140 kDa (arrowhead). Consistent with previous report (31), the relative enhancement of the upper band relative to the lower band is reproducible and is likely the consequence of a higher efficiency of the dimeric PSGL-1 to be included in the immunoprecipitates.

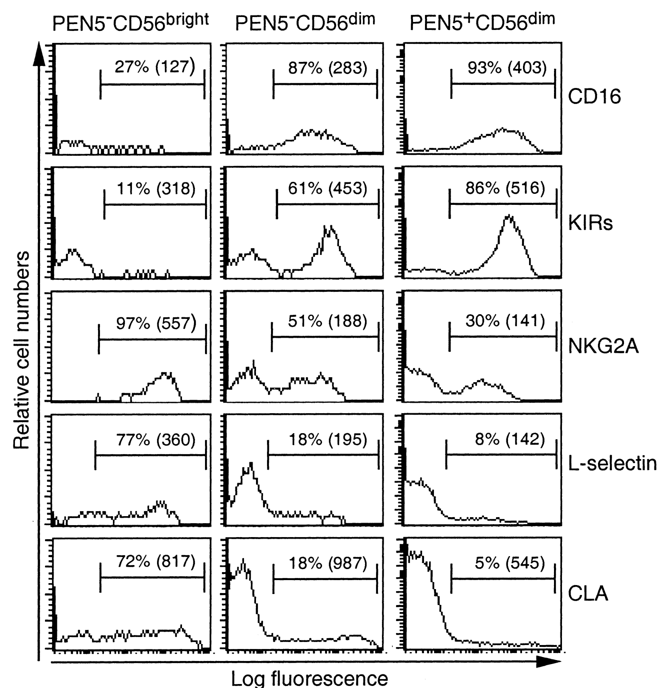

Fig. 6. Coordinated cell surface expression of PEN5 and KIRs on NK cell subsets. Purified NK cells were stained with anti-PEN5, anti-CD56, and anti-L-selectin, anti-CD16, anti-NKG2A, or anti-KIRs mAbs and analyzed by flow cytometry. $PEN5^-CD56^{bright}$, $PEN5^-CD56^{dim}$, and $PEN5^+CD56^{dim}$ were analyzed for CD16, KIRs, NKG2A, L-selectin, and CLA expression. Vertical cursor was set with appropriate negative control mAb. The proportions and the mean fluorescence intensity (MFI) of positive cells are indicated in each histograms.

nize in immunoblots, two polydispersed bands with apparent M_r comparable with the monomeric and dimeric forms of PSGL-1 (Fig. 5, lane 8). Remarkably, anti-PEN5 immunoprecipitates include a ≈220- to 240-kDa glycoprotein reactive with anti-PSGL-1 mAb (Fig. 5, lane 4), and anti-PSGL-1 immunoprecipitates contain anti-PEN5 mAb-reactive glycoproteins (Fig. 5, lane 5). Control immunoprecipitates lack these anti-PSGL-1 and anti-PEN5 mAb reactive glycoproteins (Fig. 5, lanes 2, 3, 6, and 7). These results, taken together with previous data (26), indicate that PEN5 is a sulfated polylactosamine epitope found on a subpopulation of PSGL-1 molecules expressed on NK cells.

Reciprocal Expression of PEN5 and L-Selectin on NK Cell Subsets. Human peripheral blood NK cells can be divided into two distinct subsets based on the expression of CD56. Approximately 90% of NK cells are $CD56^{dim}$ $CD16^+$, whereas $CD56^{bright}$ NK cells are $CD16^{dim/-}$ (Fig. 6). $CD56^{dim}$ $CD16^+$ NK cells have the attributes of terminally differentiated NK cells because they exert potent cytolytic function *in vitro* and their proliferative potential is low. In contrast, $CD56^{bright}CD16^{dim/-}$ NK cells exert poor cytolytic activities and proliferate in response to cytokines such as interleukin-2, *in vitro* (32). Although no definitive evidence has been reported regarding to the ontogenic relationship between $CD56^{dim}$ $CD16^+$ and $CD56^{bright}CD16^{dim/-}$ NK cells, it is likely that the latter represents an immature pool of NK cells. The expression of L-selectin on NK cells has been described to be restricted to the $CD56^{bright}CD16^{dim/-}$ NK cell subset (25). We therefore investigated by flow cytometry whether the cell surface expression of PEN5 and its L-selectin ligand defines reciprocal nonoverlapping NK subsets. Anti-PEN5 and anti-CD56 staining allowed to define three distinct NK cell subsets (Figs. 4*A* and 6): $PEN5^-CD56^{bright}$, $PEN5^-CD56^{dim}$, and $PEN5^+CD56^{dim}$. L-selectin is preferentially expressed on $PEN5^-CD56^{bright}$ NK cells and barely detectable on $PEN5^+CD56^{dim}$ NK cells. Consistent with the mature phenotype of the $PEN5^+CD56^{dim}$ NK subset and the absence of KIR expression on NK cell precursors (1, 2), the cell surface expression of KIRs gradually enhances from the $PEN5^-CD56^{bright}$ to the $PEN5^+CD56^{dim}$ NK cell subset. In contrast, the lectin dimers CD94/NKG2A are expressed at the $PEN5^-CD56^{bright}$ stage of NK cell differentiation, and their expression gradually diminishes with the progression toward the $PEN5^+CD56^{dim}$ NK cell compartment. The CLA, a carbohydrate modification of PSGL-1, recognized by the HECA-452 mAb is a marker of cells that infiltrate tissues (31, 33). Remarkably, the cell surface expression of PEN5 and CLA is mutually exclusive (Fig. 6). Therefore, these results confirm the developmentally regulated expression of PEN5 during NK cell maturation, and define two reciprocal nonoverlapping NK cell subsets based on the coordinated expression of PEN5, CD56, CD16, KIRs, and CLA: $PEN5^-CD56^{bright}$ $CD16^-$ KIR^- CLA^+ L-$selectin^+$ NK cells and $PEN5^+$ $CD56^{dim}$ $CD16^+$ KIR^+ CLA^- L-$selectin^-$ NK cells. These results further suggest that the trafficking properties of KIR^+ and KIR^- NK cells are different.

Selectivity of Interaction of PEN5-Decorated PSGL-1 with Selectins. As PSGL-1 can bind to L-, E-, and P-selectin when appropriately modified, we further tested whether the PEN5 glycoform of PSGL-1 could also serve as a ligand for E-and P-selectins. NK cell attachment and rolling on adsorbed recombinant soluble E- and P-selectins was assayed in a parallel plate flow chamber. As previously described (22), NK cells tether and roll on both selectins: 15 ± 1 and 12 ± 1 NK cells rolling per field were detected at a shear stress of 1.5 dyne/cm^2 (1 dyne = 10 μN) on P- and E-selectins, respectively (n = 12). Anti-PEN5 mAb or mocarhagin treatments of NK cells do not impair the ability of NK cell to interact with E-selectin (Fig. 7). Treatment of NK cells with anti-PEN5 mAb, but not with isotype-matched control mAb

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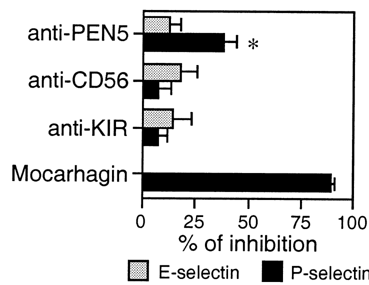

Fig. 7. Tethering and rolling of NK cells on P- and E- selectin under flow conditions. NK cells were incubated with the indicated mAb or with mocarhagin, as described in Fig. 2*A*; then, NK cells were diluted and perfused at 1.5 dyne/cm^2 through the chamber. Interactions of NK cells with P- (solid bars) or E-selectin (shaded bars) were abolished in the presence of 5 mM EDTA in the perfusing medium (data not shown) or when the substrates were preincubated for 10 min with 10 μg/ml of anti-P-selectin or anti- E-selectin (data not shown). No inhibition of rolling on E-selectin was observed when NK cells were incubated with mocarhagin. The accumulated numbers of rolling cells were determined by analysis of videotapes in which at least six fields randomly chosen were counted throughout the last 2 min of the flow period. The percentage of rolling NK cells for each condition is calculated relative to untreated controls. Data represent mean ± SEM of results from four experiments. *, $P < 0.05$.

(anti-CD56, anti-CD158a, anti-CD158b), leads to a weak but significant inhibition of NK cell interaction with P-selectin ($P = 0.04$, $n = 15$) (Fig. 7). In marked contrast to the results obtained with L-selectin (Fig. 1*B*), mocarhagin treatment of NK cells inhibits their tethering and rolling on P-selectin (89.3 ± 1.9%, $n = 3$; Fig. 7). Therefore, PEN5/PSGL-1 cannot mediate NK cell adhesion to E-selectin and does not serve as a major NK cell ligand for P-selectin. Furthermore, these results do not rule out the possibility that inhibition of NK cell interaction with P-selectin by anti-PEN5 mAb is merely the consequence of steric hindrance.

Discussion

We described earlier the structural properties of PEN5, an NK cell-restricted glycoprotein expressed on the surface of peripheral blood and tissue-infiltrating NK cells (24, 26). PEN5 is selectively expressed on terminally differentiated NK cells, which are characterized by their high cytolytic activity and their poor proliferative potential (26). Anti-PEN5 mAb recognizes a highly glycosylated sialomucin bearing lactosamine carbohydrates that are immunochemically related to keratan sulfate, a component of glycosaminoglycans (26). We report here that NK cell differentiation is accompanied by the modification of PSGL-1 with the PEN5 sulfated lactosamine that creates an alternate binding site for L-selectin. The extended rod structure of PEN5 at the surface of NK cells is consistent with its function as a selectin ligand (24).

The post-translational modifications of PSGL-1 that determine its relative affinity for individual selectins have been extensively investigated (28). On neutrophils and monocytes, the binding of selectins requires the addition of sialylated, fucosylated core 2 O-glycans to residue T57 at the N terminus of PSGL-1 (34–36). On these cells, the sulfation of residues Y46, Y48 and Y51 is also mandatory to the binding to L- and P-selectins. Therefore, the PSGL-1-binding site for L- and P-selectins consists of sialylated, fucosylated lactosamine carbohydrates and one or more adjacent sulfate esters. These same components are also required for the binding of L-selectin to the vascular addressins (14, 37). However, in marked contrast to the selectin-binding site on PSGL-1, the essential sulfate esters are attached to the sixth position of the GlcNAc residue of the addressin lactosamine carbohydrates (28). Although the precise structure of the PEN5 epitope has not been determined, it is related to keratan sulfate, a known L-selectin ligand (38). Keratan sulfates are lactosamines (Galβ1–4GlcNAc) in which the sixth position of the Gal residue, the GlcNAc residue, or both are modified by sulfation. The presence of a 6-O-sulfate at the GlcNAc position of PEN5 is consistent with (*i*) the susceptibility of PEN5 to digestion by keratanase (26), an enzyme which attacks sites comprised of an unsulfated Gal unit adjacent to a 6-sulfated GlcNAc (completely unsulfated disaccharides cannot serve as substrates for this enzyme); (*ii*) the requirement of 6′-sulfo-sLe^x and 6-sulfo-sLe^x moieties for GlyCAM-1 binding to L-selectin (14); and (*iii*) the demonstration that 6-O-sulfate on GlcNAc residues potentiates binding to L-selectin, whereas 6-O-sulfate on Gal residues inhibits binding to L-selectin (39). It is thus possible that the attachment of sulfate esters to the sixth position of GlcNAc residues in PEN5 fulfills the same function as tyrosine sulfation at the N terminus of PSGL-1.

Post-translational modifications of PSGL-1 resulting in the acquisition of selectin-binding sites have been previously shown to be induced by cellular differentiation and/or activation (31, 40). In particular, the ability of functionally distinct populations of T cells to migrate to sites of inflammation in the skin is regulated by posttranslational modifications of PSGL-1 (31), emphasizing the vast structural and functional plasticity of PSGL-1. NK cells are specialized lymphocytes, which through their cytolytic function and their skewed cytokine repertoire, operate at diverse stages of innate as well as adaptive immune responses (1, 2, 4). The selectivity of PEN5 expression on NK cells, and its absence on classical antigen-specific T and B lymphocytes, suggest that specific NK cell functions depend on the acquisition of this developmentally regulated L-selectin ligand. Therefore, the control of NK cell interactions via PEN5 may have important implications for the induction and maintenance of immunity to tumor and infectious diseases. In particular, CD56^{bright}KIR$^-$ NK cells are L-selectin$^+$ whereas CD56^{dim}KIR$^+$ are L-selectin$^-$ (Fig. 6). It thus remains to be examined whether the reciprocal expression of PEN5 and its L-selectin ligand might be involved in an interaction between PEN5$^-$CD56^{bright}KIR$^-$ L-selectin$^+$ NK cells and PEN5$^+$CD56^{dim}KIR$^+$ L-selectin$^-$ NK cells, which might control the maturation and/or effector function of the NK cell compartments. By analogy to the ability of rolling neutrophils to capture free flowing neutrophils in a PSGL-1:L-selectin-dependent manner (11), the PEN5 epitope might also allow NK cells to interact with other L-selectin$^+$ leukocytes (e.g., neutrophils, monocytes, or T/B lymphocytes) attached to inflamed endothelium to amplify the immune response to an inflammatory stimulus. Mouse anti-PEN5 mAb do not react with splenic mouse NK cells, hampering the *in vivo* analysis of PEN5 contribution to NK cell biology. However, elucidation of the NK cell-borne glycosyltransferase machinery may allow the generation of null mutant mice for PEN5 biosynthesis pathways. Such mutant mice represent unique tools to further document the role of PEN5 in the migration of NK cells *in vivo*. In particular, these analysis may establish the circumstances during which PEN5 serves as a NK trafficking receptor.

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