Co-expression of the collagen receptors leukocyte-associated immunoglobulin-like receptor-1 and glycoprotein VI on a subset of megakaryoblasts

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ABSTRACT

Background
The collagen receptor glycoprotein VI generates activating signals through an immunoreceptor tyrosine-based activating motif on the co-associated Fc receptor gamma chain. Leukocyte-associated immunoglobulin-like receptor-1 also ligates collagen but generates inhibitory signals through immunoreceptor tyrosine-based inhibitory motifs. Thus far, the cellular expression of glycoprotein VI and leukocyte-associated immunoglobulin-like receptor-1 appears mutually exclusive.

Design and Methods
Using flow cytometry, we studied expression of collagen receptors on differentiating human megakaryocytes. $CD34^+$ cells were isolated from umbilical cord blood and matured to megakaryocytes *in vitro*. Freshly isolated bone marrow cells were used to study primary megakaryocytes. Upon cell sorting, cytospins were made to examine cytological characteristics of differentiation.

Results
Megakaryocyte maturation is accompanied by up-regulation of glycoprotein VI and down-regulation of leukocyte-associated immunoglobulin-like receptor-1. Interestingly, both in cultures from hematopoietic stem cells and primary cells obtained directly from bone marrow, we identified a subset of morphologically distinct megakaryocytes which co-express glycoprotein VI and leukocyte-associated immunoglobulin-like receptor-1.

Conclusions
This is the first report of a primary cell that co-expresses these collagen receptors with opposite signaling properties. Since megakaryocytes mature in the collagen-rich environment of the bone marrow, these findings may point to a role for leukocyte-associated immunoglobulin-like receptor-1 in the control of megakaryocyte maturation/migration.

Key words: collagen receptors, leukocyte-associated immunoglobulin-like receptor-1, LAIR-1, glycoprotein VI, GPVI, megakaryocyte maturation.

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Introduction

Glycoprotein VI (GPVI) and $\alpha_2\beta_1$ play a crucial role in the platelet response to collagen.[1] Both receptors are expressed when hematopoietic stem cells differentiate into megakaryocytes and are abundantly present on platelets. Unlike GPVI, which can bind collagen directly, $\alpha_2\beta_1$ needs affinity modulation by inside-out signaling through ligated GPVI or other receptors before it can bind collagen effectively. GPVI stimulation on platelets initiates Ca^{2+} mobilization through a mechanism dependent on the tyrosine-kinase Syk, which initiates a downstream signaling cascade leading, via LAT and SLP-76, to activation of multiple effector molecules such as PLCγ_2, small G-proteins, and phosphoinositide-3 kinase.[2,3] GPVI is capable of signal transduction in megakaryocytes too. In these cells, cross-linking via GPVI-specific agonists such as convulxin and collagen-related peptide results in tyrosine phosphorylation of Syk and PLCγ_2, and Ca^{2+} mobilization.[4-6] Megakaryocytes mature in the collagen-rich environment of the bone marrow and platelet formation is preceded by migration from osteoblastic stem cell niches to sinusoids where the platelets are shed into the circulation. The role of collagen receptors in these processes is poorly understood.

A molecule structurally related to GPVI is the inhibitory receptor leukocyte-associated immunoglobulin-like receptor (LAIR)-1.[7,8] The genes encoding LAIR-1 and GPVI are both located on the leukocyte receptor complex on human chromosome 19. The genomic proximity and structural homology between the two receptors suggest that LAIR-1 and GPVI have a common origin. The intracellular tail of GPVI signals via calmodulin[9] and associated Src kinases Fyn and Lyn.[10,11] Furthermore, GPVI has a charged arginine in its transmembrane domain that mediates association with the immunoreceptor tyrosine-based activating motif-containing Fc receptor gamma chain (FcRγ).[12-14] GPVI-associated Fyn and Lyn are crucial for the phosphorylation of the FcRγ immunoreceptor tyrosine-based activating motif.[10] LAIR-1 contains two immunoreceptor tyrosine-based inhibitory motifs in its cytoplasmic tail to impart its inhibitory effect through the phosphatases SHP-1, SHP-2 and the C-terminal Src kinase Csk.[15]

In leukocytes, LAIR-1 plays an important role in dampening immune responses and hence in the maintenance of a balanced immune system.[16,17] We have previously demonstrated that besides effector immune cells, hematopoietic stem cells also express LAIR-1.[18] Furthermore, we have recently shown that collagens are high-affinity ligands for LAIR-1, and that binding of collagen to LAIR-1 results in inhibition of immune cell activation.[8] This is the only inhibitory receptor described so far that binds collagen and the collagen-binding site in LAIR-1 and GPVI overlaps between the two receptors.[19-21] In collaboration with our group, Tomlinson *et al.* showed that when both receptors are ectopically expressed on the same cell, LAIR-1 cross-linking abrogates collagen-induced GPVI-signaling.[22] Co-expression of both receptor types on primary cells would, therefore, potentially affect their responsiveness to collagen. However, at present, GPVI expression and LAIR-1 expression appear mutually exclusive, with GPVI being regarded as a platelet-specific receptor and LAIR-1 being broadly expressed on leukocytes.

Megakaryocytes differentiate from hematopoietic stem cells in the bone marrow, primarily under the control of thrombopoietin.[23] Hematopoietic stem cells initially develop into megakaryocyte progenitors (CFU-MEG). Further transition from progenitor cells to mature megakaryocytes is divided into four stages. The first stage of megakaryocytopoiesis is represented by megakaryoblasts, which have a low cytoplasmic/nuclear ratio, compact nucleus, basophilic cytoplasmic staining and small cell size. Successive stages are represented by promegakaryocytes, granular megakaryocytes and, finally, mature megakaryocytes. During differentiation the nucleus becomes highly lobulated, the size of the cell and its cytoplasmic mass increase, and the cytoplasmic staining becomes eosinophilic.[24] These cells form proplatelet projections shedding several thousands of platelets per cell.[23] In addition to cytological characteristics, the expression of surface receptors can be used as markers for differentiation. Expression of CD34 decreases, and CD41/CD61 expression is induced, followed by expression of CD42b.[25] Upon further maturation, GPVI and $\alpha_2\beta_1$ are induced[4] making these proteins markers for the late stages of maturation.

In the present study we identified a subset of megakaryoblasts co-expressing an activating and inhibiting collagen receptor. This property may mark a separate stage in human megakaryocytopoiesis with possibly important consequences for the maturation/differentiation of megakaryocytes.

Design and Methods

Antibodies and reagents

Fetal calf serum was from Bodinco (Alkmaar, the Netherlands). Horse serum, L-glutamine, RPMI 1640, Iscove's modified Dulbecco's medium and Fischer's medium pH 7.0 were from Gibco (Breda, the Netherlands). Bovine serum albumin was from Sigma. The Hy101 anti-GPVI monoclonal antibody was kindly provided by Prof. Kahn, University of Pennsylvania. Anti-human FcγRI and FcγRIII monoclonal antibodies (clone 10.1 and 3G8) were from Biolegend, and anti-human FcγRII (clone 6C4) was from eBiosciences. CLB-MB15 anti-CD42b-biotin (mIgG1) monoclonal antibody was purchased from Sanquin (Amsterdam, the Netherlands). The Hy101 anti-GPVI (mIgG1) monoclonal antibody was labeled with fluorescein isothiocyanate (FITC; Molecular Probes). Y2/51 anti-CD61 FITC (mIgG1) monoclonal antibody was from Dako. AK-7 anti-CD49b FITC (mIgG1) (to stain the α subunit of $\alpha_2\beta_1$) was from Biolegend. Anti-CD11b FITC was from Immunotech. Goat anti-mouse allophycocyanin (APC) was from Southern Biotech. 8A8 anti-LAIR biotin (mIgG1) was produced in-house. DX26 anti-LAIR phycoerythrin (PE) (mIgG1), RUU-PL7F12 anti-CD61 PerCP (mIgG1), streptavidin-PerCP, MphiP9 anti-CD14 APC Cy7 (mIgG2b), RPA2.10 anti-CD2 FITC (mIgG1), UCTH1 anti-CD3 FITC (mIgG1), RPA-T4 anti-CD4 FITC (mIgG1), M-T701 anti-CD7 FITC (mIgG1), RPA-T8 anti-CD8 FITC (mIgG1), M5E2 anti-CD14 FITC (mIgG2a), HIB19 anti-CD19 FITC (mIgG1), 2H7 anti-CD20 FITC (mIgG2b), GA-R2 anti-CD235a FITC (mIgG2b), 8G12 anti-CD34 PE-Cy7 (mIgG1), HIT2 anti-CD38 APC (mIgG1), 7G3 anti-CD123 PE (mIgG2a), HI100 anti-CD45RA PE Cy5 (mIgG2b), mouse isotype control monoclonal antibodies IgG1 biotin, IgG1 FITC, IgG2a FITC, IgG2b FITC, IgG1 PE-Cy7, IgG1 APC, IgG2a PE, IgG2b PE-Cy5 and streptavidin-APC-Cy7 were purchased from BD Biosciences. A CD34 progenitor cell isolation kit based on magnetic-activated cell sorting was from Miltenyi Biotech (Bergisch Gladbach, Germany). Stem cell factor and thrombopoietin were from Peprotech (Rocky Hill, NJ, USA). Giemsa stain was from

Sigma, whereas the May Grünwald stain was from Merck Chemicals.

Cell lines

Three megakaryoblastic cell lines were analyzed. MEG-01 cells were cultured in RPMI 1640 supplemented with 20% fetal calf serum. DAMI cells were cultured in Iscove's modified Dulbecco's medium containing HEPES, supplemented with 10% horse serum. CHRF-288-11 (henceforth referred to as CHRF) cells were cultured in Fischer's medium pH 7.0 supplemented with 20% horse serum. Cell lines were analyzed by flow cytometry using DX26 anti-LAIR-1 PE, anti-GPVI FITC and anti-CD49b FITC. Dead cells were excluded by gating on the basis of forward and side scatter.

Platelet isolation

Freshly drawn venous blood was collected with informed consent from healthy donors into 0.1 volume 130 mmol/L trisodium citrate 3. The blood was centrifuged (15 min, 200 × g, 22 °C) and the platelets resuspended in Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na_2HPO_4, 1 mmol/L $MgSO_4$, 10 mmol/L Hepes, 5 mmol/L D-glucose, pH 6.5). Prostaglandin I_2 was added to a final concentration of 10 ng/mL and after centrifugation cells were resuspended in Hepes-Tyrode buffer (pH 7.2). The platelet count was adjusted to 2.25×10^{11} cells/L and suspensions were left at room temperature for 30 min to ensure a resting state. Fc receptors on platelets were blocked using anti-human FcγRI, anti-human FcγRII and anti-human FcγRIII monoclonal antibodies, and cells were analyzed by flow cytometry using DX26 anti-LAIR-1 PE, anti-GPVI FITC and anti-CD49b FITC.

Analysis of hematopoietic progenitor cells

Umbilical cord blood was collected during normal full-term deliveries and used within 48 h. All mothers gave written informed consent before labor and delivery. $CD34^+$ progenitor cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation followed by magnetic activated cell sorting purification of $CD34^+$ cells. Samples were stained and washed in phosphate-buffered saline supplemented with 5% fetal calf serum. Live cells were gated on the basis of forward and side scatter. Lineage-positive cells were detected with a mixture of anti-CD2 FITC, anti-CD3 FITC, anti-CD4 FITC, anti-CD7 FITC, anti-CD8 FITC, anti-CD11b FITC, anti-CD14 FITC, anti-CD19 FITC, anti-CD20 FITC and anti-CD235a FITC. A mixture of FITC-labeled mouse isotype control monoclonal antibodies (IgG1, IgG2a and IgG2b) was used to make the distinction between lineage-positive and lineage-negative cells. Lineage negative (LIN^-) cells were gated and expression of the progenitor markers was detected using anti-CD34 PE-Cy7, anti-CD38 APC, anti-CD123 PE and anti-CD45RA PE-Cy5 by flow cytometry. To ensure specificity of the progenitor staining, a separate staining was performed in which the panel of lineage markers and CD34 and CD38 was combined with isotype control monoclonal antibodies for CD123 (IgG2a PE) and CD45RA (IgG2b PE-Cy5), LIN^- cells were gated, and quadrants were set based on isotype controls. To examine LAIR-1 expression on hematopoietic stem cells and progenitor cells, stains with both the lineage markers and the progenitor markers CD34, CD38, CD123 and CD45RA were combined with either a mouse IgG1 biotin isotype control monoclonal antibody or with 8A8 anti-LAIR biotin. SA-APC-Cy7 was used as a secondary antibody to detect LAIR-1 expression. Protocols were approved by the ethics committee of the University Medical Center, Utrecht.

Analysis of megakaryocytic cells in bone marrow samples

Bone marrow cells were obtained from healthy donors. All donors gave written informed consent. Erythrocytes were lysed from the total population of cells using pH 7.4 ammonium chloride shock buffer. Cells were incubated and washed in phosphate-buffered saline supplemented with 1% bovine serum albumin and 5 mM EDTA. Cells were first stained with anti-GPVI, followed by a secondary step with goat anti-mouse APC. The third step was anti-CD42b biotin, and the fourth step was streptavidin-PerCP, anti-CD14 APC-Cy7, anti-CD61 FITC and DX26 anti-LAIR-1 PE. Samples were analyzed using a BD LSRII flow cytometer, or anti-CD14 APC Cy7, anti-CD61 PerCP and DX26 anti-LAIR-1 PE were used to sort megakaryocytic cells with a BD FACSAria flow cytometer to obtain isolated megakaryocyte subsets. Live cells were gated on the basis of forward and side scatter, $CD14^-$ cells were gated for CD61 and CD42b. $CD61^+CD42b^+$ cells were analyzed for expression of GPVI and LAIR-1. Isotype control staining for CD61, CD42b, LAIR-1 and GPVI (mIgG1 FITC, mIgG1 biotin followed by streptavidin-PerCP, mIgG1 PE and mIgG1 followed by goat anti-mouse APC, respectively) was used to set quadrants depicting positive and negative stains. Protocols were approved by the ethics committee of the University Medical Center, Utrecht.

In vitro *culture of megakaryocytes from* $CD34^+$ *cells*

$CD34^+$ cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation followed by magnetic activated cell sorting purification of $CD34^+$ cells. The purity of the isolated population, based on CD34 expression, was determined by flow cytometry and always exceeded 90%. Isolated $CD34^+$ cells were seeded at a density of 3×10^5 cells/mL. To induce megakaryocyte development, 50 ng/mL stem cell factor and 20 ng/mL thrombopoietin were added to the culture media on days 0 and 3 of subculture. From day 7 onwards, cells were seeded at a density of 5×10^5 cells/mL and only thrombopoietin was added to the culture. Culture media consisted of Iscove's modified Dulbecco's medium supplemented with 1% L-glutamine, 0.1 mM bovine serum albumin-absorbed cholesterol, 0.5% bovine serum albumin, 10 μg/mL insulin, 200 μg/mL iron-saturated transferrin, 50 μM β-mercaptoethanol and antibiotics (adapted from Den Dekker *et al.*[26]). On days 0, 3, 7, 10 and 14, cells were analyzed by flow cytometry after staining with anti-CD34 PE-cy7, anti-CD61 PerCP, CD42b biotin, GPVI FITC, and DX26 anti-LAIR PE, or the same staining with a PE isotype control instead of anti-LAIR. Streptavidin-APC was used as a second step to detect CD42b expression. Another staining was performed with only anti-CD49b FITC. Live cells were gated on the basis of forward and side scatter. Quadrants depicting positive and negative staining were set based on isotype control stains for CD61, CD42b, GPVI, CD49b and LAIR-1.

Preparation of cytospins

Cytospins were made from sorted bone marrow samples and from cells obtained from *in vitro* megakaryocytopoiesis. From 10,000 to 100,000 cells were collected and centrifuged on glass coverslips. Samples were fixed in 100% methanol, and stained with Giemsa and May-Grünwald.

Results

Expression of collagen receptors on platelets and megakaryocytic cell lines

Expression of the collagen receptors LAIR-1, GPVI and $\alpha_2\beta_1$ was studied in the megakaryocytic cell lines MEG-01,[27] DAMI[28] and CHRF[29] by flow cytometric analysis and compared with that of platelets as a reference to end-stage

receptor expression on mature megakaryocytes (Figure 1). These cell lines are thought to show similarities with megakaryocytes at different developmental stages, with MEG-01 representing an early stage, DAMI an intermediate stage and CHRF a late stage of normal megakaryocytopoiesis.[30] In line with this arbitrary classification, the expression of GPVI and $\alpha_2\beta_1$ was low in MEG-01, increased in DAMI cells, and high in CHRF. Conversely, LAIR-1 expression was absent in MEG-01, high in DAMI and intermediate in CHRF. Thus, the DAMI and CHRF cell lines co-express GPVI and LAIR-1. This is the first identification of a cell type that co-expresses an activating and an inhibitory receptor for collagen. Importantly, GPVI and $\alpha_2\beta_1$ were highly expressed by platelets but LAIR-1 expression was absent.

Leukocyte-associated immunoglobulin-like receptor-1 is expressed on hematopoietic stem cells and on multipotent progenitor cells

We have previously reported that LAIR-1 is highly expressed by hematopoietic stem cells.[18] To investigate its expression during further differentiation, we determined LAIR-1 expression on subsets of hematopoietic multipotent progenitor cells. To this end, we gated LIN^- cells and analyzed these cells for expression of progenitor markers (Figure 2). Quadrants were defined based on isotype control stains. True hematopoietic stem cells were defined as $CD34^+CD38^-$ cells, megakaryocyte/erythrocyte progenitors (MEP) as $CD34^+CD38^+CD123^-CD45RA^-$ cells, common myeloid progenitors (CMP) as $CD34^+CD38^+CD123^+CD45RA^-$ cells and granulocyte/ macrophage progenitors (GMP) as $CD34^+CD38^+CD123^+CD45RA^+$ cells.[31] Detailed analysis revealed that all subsets expressed LAIR-1. Thus, hematopoietic stem cells as well as hematopoietic progenitor cell subsets have considerable expression of LAIR-1.

Expression of leukocyte-associated immunoglobulin-like receptor-1 and glycoprotein VI is differentially regulated during in vitro *megakaryocytopoiesis*

Since we observed co-expression of GPVI and LAIR-1 in DAMI and CHRF megakaryocytic cell lines, we investigated whether *ex vivo*-generated megakaryocytes also showed this property. $CD34^+$ cells were cultured with thrombopoietin and stem cell factor and cells were collected after 0, 3, 7, 10 and 14 days of culture. Cytospins were stained with May-Grünwald Giemsa reagent and lobulation of the nucleus, nuclear/cytoplasmic ratio, cytoplasmic staining and cell size were examined. Cytospins showed the transition of progenitor cells through stage I, II and III of normal megakaryocytopoiesis during the 14-day culture (Figure 3A). The size of the cells and nuclei increased significantly as did the quantity of cytoplasm, however lobulation of the nucleus was less pronounced. Flow cytometric analysis showed that LAIR-1 was expressed on all progenitor cells, whereas only a small proportion of cells expressed LAIR-1 later in differentiation (Figure 3B-C). Similarly, CD34 was expressed on all progenitor populations and lost during differentiation. Concomitantly, expression of CD61, CD42b and GPVI, absent in progenitor cells, gradually increased during culture with CD61 and CD42b expression induced after 3 days on a small population of cells. GPVI and $\alpha_2\beta_1$ expression was detected from day 7 onwards. In line with the observations in the cell lines, we found a subset of megakaryocytes which co-expressed LAIR-1 and GPVI. This population amounted to approximately 20% of all $GPVI^+$ cells at day 7 of culture

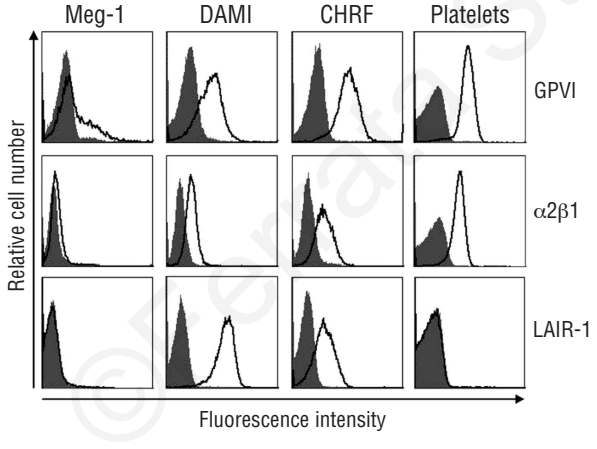

Figure 1. Megakaryoblastic cell lines show differential expression of GPVI, $\alpha_2\beta_1$ and LAIR-1. The expression of the collagen receptors LAIR-1, GPVI and $\alpha_2\beta_1$ was studied on platelets and on the megakaryocytic cell lines MEG-01, DAMI and CHRF. Freshly drawn venous blood was centrifuged and platelets were resuspended in Hepes-Tyrode buffer pH 6.5. Prostaglandin I_2 was added and, after centrifugation, cells were resuspended in Hepes-Tyrode buffer (pH 7.2). The platelet count was adjusted to $2.25x10^{11}$ cells/L and suspensions were left at room temperature for 30 min to ensure a resting state. Platelets and cell lines were stained with anti-LAIR, anti-GPVI, anti-CD49b (for $\alpha_2\beta_1$) or isotype control monoclonal antibodies and analyzed by flow cytometry. For the cell lines, live cells were gated on the basis of forward and side scatter. The gray histograms represent isotype control stains; the open histograms represent LAIR-1, GPVI and $\alpha_2\beta_1$ stains. The results are representative of three independent experiments (in a total of three donors for the platelet staining).

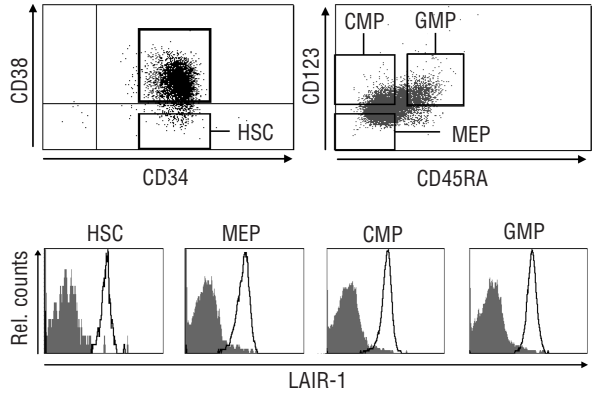

Figure 2. LAIR-1 is expressed on hematopoietic stem cells (HSC) and on all lineages of multipotent progenitor cells. HSC and progenitor cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation. Samples were stained and washed in phosphate-buffered saline supplemented with 5% fetal calf serum. Live cells were gated on the basis of forward and side scatter. Cells negative for lineage markers CD2, CD3, CD4, CD7, CD8, CD11b, CD14, CD19, CD20 and CD235a were analyzed for expression of progenitor cell markers (top panels). Quadrants were set on the basis of isotype control stains. LAIR-1 was analyzed in progenitor subsets (bottom panels). The gray histograms represent isotype control staining; the open histograms represent LAIR-1 staining. MEP stands for megakaryocyte/erythrocyte progenitor; CMP for common myeloid progenitor and GMP for granulocyte/ macrophage progenitor. The results are representative of two independent experiments (in a total of four donors).

(Figure 3B bottom panel). LAIR-1 expression on GPVI$^+$ cells was completely lost during further maturation (Figure 3B). Since the progenitor marker CD34 is down-regulated concurrently with LAIR-1 in the total cell population (Figure 3C), we determined whether both markers were co-expressed on megakaryocytes, as expression of CD34 is indicative of the differentiation state of the cells. We analyzed CD34 expression on CD42b$^+$CD61$^+$GPVI$^+$LAIR-1$^+$ cells from day 7 of *in vitro* culture and found that about one-third of the LAIR-1$^+$ megakaryocytes had lost CD34 expression, whereas 65% of the population was positive for both markers (Figure 3D). To further characterize the maturation status of LAIR-1-expressing cells, LAIR-1$^+$GPVI$^+$ and LAIR-1$^-$GPVI$^+$ cells were sorted on day 7 of culture and cytospins were made and stained for morphological analyses. With regard to nuclear/cytoplasmic ratio and cytoplasmic staining, LAIR-1$^+$GPVI$^+$ cells consisted of CFU-MEG and megakaryoblasts and were consequently more immature than LAIR-1$^-$GPVI$^+$ cells, which consisted predominantly of promegakaryocytes (Figure 3E-F). Thus, a subset of GPVI$^+$ megakaryoblasts from all donors expressed LAIR-1 during an early stage of *in vitro* culture.

Leukocyte-associated immunoglobulin-like receptor-1 and glycoprotein VI are co-expressed by megakaryoblasts in vivo

Since *in vitro* maturation of megakaryocytes may differ from *in vivo* maturation, we investigated whether megakaryocytes freshly isolated from bone marrow also contained a subpopulation which co-expressed LAIR-1 and GPVI. Expression of these receptors was determined in the CD14$^-$CD42b$^+$CD61$^+$ population, with quadrants set based on isotype controls. Nearly all cells positive for CD61 were also positive for CD42b and GPVI. Notably, all donors examined had a large population of LAIR-1 and GPVI co-expressing megakaryocytes amounting to about 50% of GPVI-expressing cells (Figure 4A-B). In addition, two out of four donors examined also showed, besides the population of GPVI$^+$LAIR-1$^+$, a population of GPVI$^+$LAIR-1^{high} cells (Figure 4A). This population amounted to approximately 6% of all the GPVI$^+$ cells. We next determined whether LAIR-1-expressing cells co-express CD34, as seen in the *in vitro* culture. CD14$^-$CD61$^+$CD42b$^+$GPVI$^+$LAIR-1$^+$ cells were analyzed for the expression of CD34. Surprisingly, the percentage of CD34$^+$ cells was much

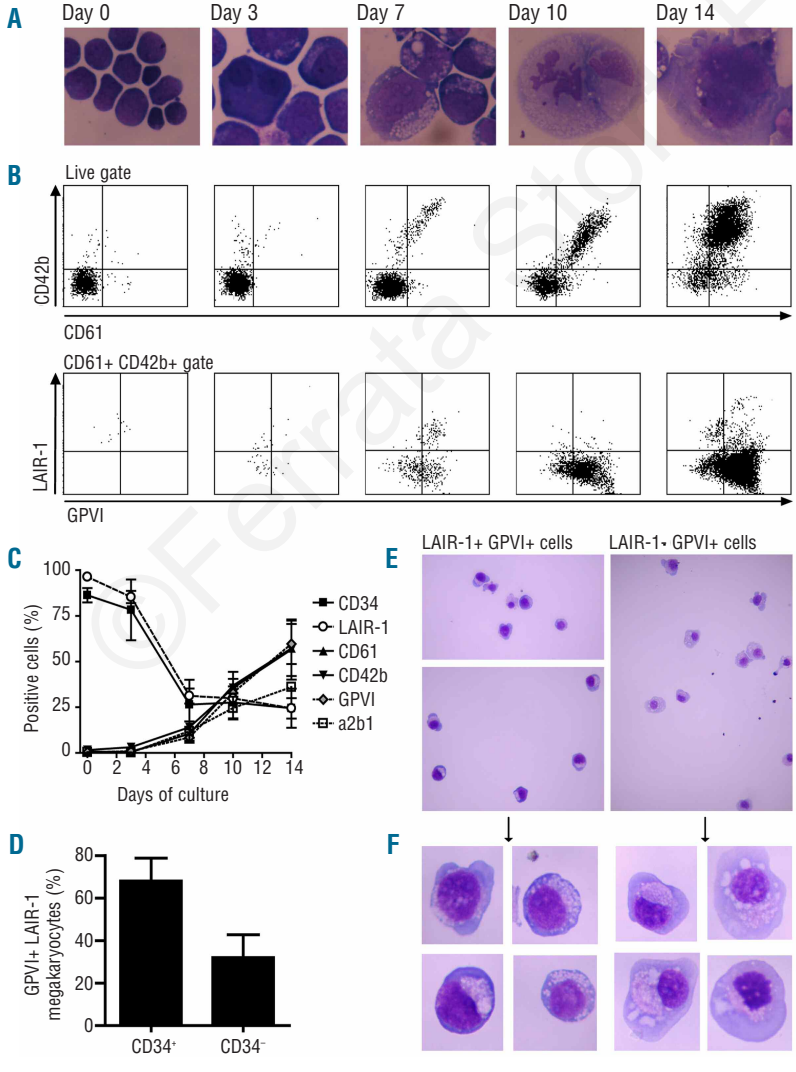

Figure 3. Expression of LAIR-1 and GPVI is differentially regulated during *in vitro* megakaryocytopoiesis. Umbilical cord blood was collected following normal full-term deliveries. Hematopoietic stem and progenitor cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation followed by magnetic-activated cell sorter purification of CD34$^+$ cells. (A) Cytospins were made on days 0, 3, 7, 10 and 14 of *in vitro* megakaryocytopoiesis, fixed in methanol and stained with Giemsa and May-Grünwald. Representative cytospins are shown. Pictures were taken with 1000x magnification. (B) On days 0, 3, 7, 10 and 14 of *in vitro* megakaryocytopoiesis, live cells were gated on the basis of forward and side scatter and analyzed for expression of CD42b and CD61 by flow cytometry. In the lower panels, cells co-expressing CD42b and CD61 were gated to specifically examine GPVI and LAIR-1 expression on megakaryoblasts. Quadrants were set on the basis of isotype stains. (C) as in (B), live cells were gated on the basis of forward and side scatter and analyzed for expression of CD34, GPVI, CD42b, CD61, $\alpha_2\beta_1$ and LAIR-1 by flow cytometry. The percentages of receptor-positive cells are averaged for three donors. Error bars represent SEM. (D) On day 7 of *in vitro* megakaryocytopoiesis, live cells were gated on the basis of forward and side scatter and CD42b$^+$ CD61$^+$ GPVI$^+$ LAIR-1$^+$ cells were analyzed for expression of CD34. The specificity of the staining was confirmed by the use of isotype control monoclonal antibodies. The percentage of CD34$^+$ cells is averaged for three donors. Error bars represent SEM. (E) LAIR-1$^+$ GPVI$^+$ and LAIR-1$^-$ GPVI$^+$ cells were sorted on day 7 of *in vitro* culture and cytospins were made as described in (A). Representative pictures from one of four donors are shown. Pictures were taken with 400x magnification. (F) As in (E), the same pictures were taken with 1000x magnification to show individual cells in greater detail.

lower in bone marrow than during *in vitro* culture (Figure 4C). To further determine the maturation stage of these megakaryocytes, CD61$^+$ cells were sorted according to high, intermediate or absent LAIR-1 expression (Figure 4D) and cytospins were made and stained for morphological analyses. LAIR-1^{high} cells were the most immature population, consisting of megakaryoblasts and CFU-MEG. LAIR-1^{dim} were intermediate-stage cells, consisting of megakaryoblasts and a few promegakaryocytes. Cells that did not express LAIR-1 were the most mature cells, consisting of promegakaryocytes and granular megakaryocytes (Figure 4E). These findings demonstrate that LAIR-1 expression is down-regulated during *in vivo* differentiation of CD61$^+$ megakaryocytes.

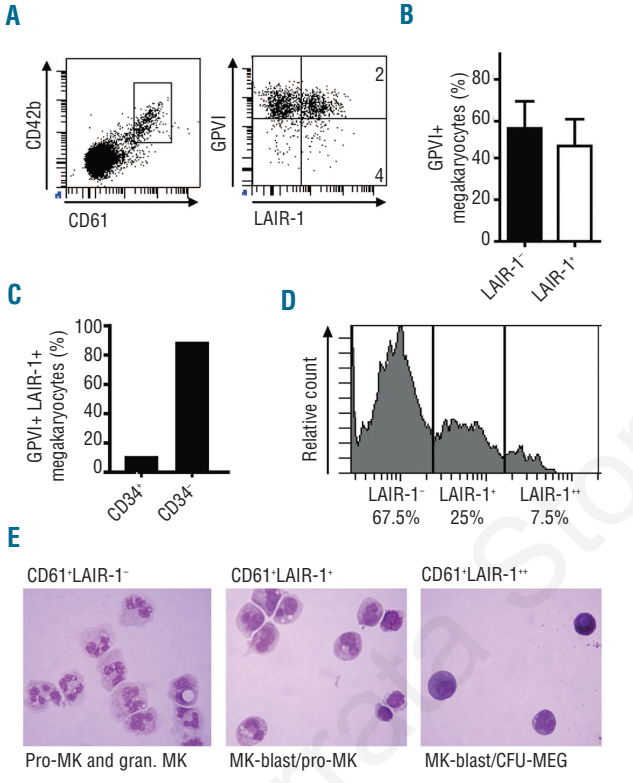

Figure 4. A subset of megakaryoblasts co-expresses GPVI and LAIR-1 *in vivo*. Bone marrow cells were obtained from healthy donors. Erythrocytes were lysed from the population using pH7.4 ammonium chloride shock buffer. Phosphate-buffered saline supplemented with 1% bovine serum albumin and 5 mM EDTA was used for staining and washing of bone marrow cells. (A) Primary live megakaryocytes in human bone marrow were gated on the basis of forward and side scatter, and CD14$^-$ CD42b$^+$ CD61$^+$ megakaryocytes were gated (left panel). All gated cells were GPVI$^+$, and approximately 50% of cells co-expressed LAIR-1 (right panel). Quadrants were set on the basis of isotype stainings. (B) Cells were analyzed as described in (A). The percentages of LAIR-1$^+$ cells are averaged for three donors. Quadrants were set on the basis of isotype staining. (C) Live bone marrow cells were gated on the basis of forward and side scatter and CD14$^-$ CD42b$^+$ CD61$^+$ GPVI$^+$ LAIR-1$^+$ cells were analyzed for expression of CD34. The specificity of the staining was confirmed by the use of isotype control monoclonal antibodies. The percentage of CD34$^+$ cells is shown for a representative donor (n=2). (D) CD14$^-$ CD61$^+$ megakaryocytes were sorted on the basis of LAIR-1 expression. (E) Cytospins were made of sorted cells from (D), fixed in methanol and stained with Giemsa and May-Grünwald. LAIR-1$^-$ cells had the most mature phenotype and consisted of promegakaryocytes (pro-MK) and granular megakaryocytes (MK), whereas the LAIR-1^{high} cells were the most immature cells and consisted of megakaryoblasts (MK-blasts) and CFU-MEG. Pictures were taken with 400x magnification. Data shown are representative of at least three different donors analyzed in independent experiments.

Discussion

In this study we examined the expression of collagen receptors (LAIR-1, GPVI, $\alpha_2\beta_1$) at different stages of megakaryocyte maturation using a combination of cytological characteristics and surface-marker expression of CD34, CD61 and CD42b. During megakaryocyte maturation, the inhibitory collagen receptor LAIR-I is down-regulated while the activating collagen receptor GPVI is up-regulated. An intermediate subset of cells isolated from the bone marrow co-express these collagen receptors with opposite functions (Figure 5).[25]

Analysis of cytospins of sorted CD61$^+$LAIR-1^{high}, CD61$^+$LAIR-1^{dim} and CD61$^+$LAIR-1$^-$ megakaryocytes revealed that LAIR-1^{high} cells had the most immature phenotype and consisted of CFU-MEG and megakaryoblasts. LAIR-1^{dim} cells consisted of megakaryoblasts and promegakaryocytes. During *in vitro* megakaryocytopoiesis, LAIR-1 expression was only found in an early phase of culture and disappeared after 10 days from the CD61$^+$CD42b$^+$GPVI$^+$ population. Part of the GPVI$^+$LAIR-1$^+$ population had lost CD34 expression both *in vivo* and *in vitro*, indicating that LAIR-1 expression is maintained for a longer period during differentiation. Thus, LAIR-1 is a novel marker for megakaryocytopoiesis and is expressed by megakaryoblasts and promegakaryocytes.

Cells co-expressing both activating and inhibitory collagen-receptors might represent an important intermediate in megakaryocyte maturation since they are present in a significant number. About 50% of the megakaryocytes isolated from bone marrow co-expressed GPVI and LAIR-1, and 20-40% of cells were GPVI$^+$LAIR$^+$ after 7 days of *in vitro* culture. Discrepancies between percentages of GPVI$^+$LAIR-1$^+$ cells may be partly explained by the fact that we started with isolated stem and progenitor cells and

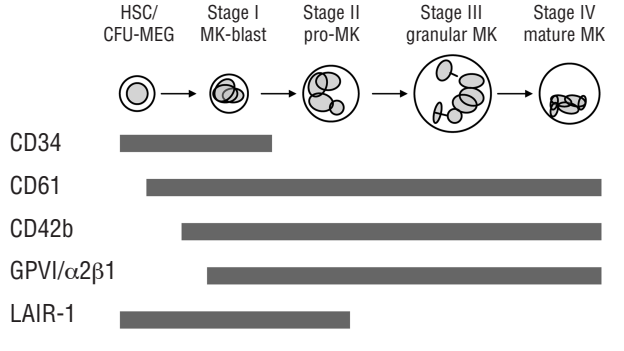

Figure 5. Expression of cell surface markers during megakaryocytopoiesis. The transition from hematopoietic stem cell (HSC) to mature megakaryocyte is divided into four distinct stages. Cells develop into multi-potent megakaryocyte progenitors (CFU-MEG), before differentiating into megakaryoblasts (MK-blast, stage I) with a low cytoplasmic/nuclear ratio, compact nucleus and small cell size. Successive stages are represented by promegakaryocytes (pro-MK), granular megakaryocytes (MK) and finally mature megakaryocytes. During differentiation the nucleus becomes highly lobulated and cell size and cytoplasmic mass increase. Expression of the progenitor cell marker CD34 is lost in an early phase of differentiation. CD61 expression is induced first in CFU-MEG. CD42b is a later marker for differentiation, preceded by CD61. Upon further maturation, GPVI and $\alpha_2\beta_1$ are induced. LAIR-1 is expressed early in megakaryocytopoiesis and on HSC and progenitor cells. A population of stage I and stage II megakaryocytes co-expresses GPVI and LAIR-1.

differentiated cells in phase for the *in vitro* culture, whereas during *in vivo* differentiation cells are not synchronized. Furthermore, the presence and dose of thrombopoietin and other cytokines may differ between *in vitro* and *in vivo* conditions of maturation. These factors may also be an explanation for the difference in percentages of LAIR-1$^+$CD34$^+$ cells in bone marrow and in *in vitro* culture. Alternatively, the difference in the number of GPVI$^+$LAIR-1$^+$ cells might be caused by differences in distribution. In bone marrow, mature megakaryocytes migrate to the capillary-rich vascular niche where they shed platelets. Collection of bone marrow samples might favor sampling of cells from the osteoblastic environment. Indeed, cytospin analysis from bone marrow megakaryocytes revealed the presence of stage II and stage III cells, but not mature megakaryocytes (Figure 4).

Some megakaryocytes derived from *in vitro* culture seem to develop from LAIR-1$^-$GPVI$^-$ to LAIR-1$^-$GPVI$^+$ instead of from LAIR-1$^+$GPVI$^-$ via LAIR-1$^+$GPVI$^+$ to LAIR-1$^-$GPVI$^+$ (Figure 3). Most likely this is due to the fact that hematopoietic stem cells differentiate along multiple, partially asynchronous routes.[32,33] It remains to be determined whether this alternative differentiation route is also followed *in vivo*.

In line with the concept that MEG-01, DAMI and CHRF cells represent megakaryocytes at increasing stages of maturation it would be predicted that MEG-01 cells express more LAIR-1 than DAMI cells and this was clearly not observed. Initially, the classification was based on expression of GPIIb-IIIa and GPIb[30,34] and the expression of GPVI and $\alpha_2\beta_1$ reported here supports this early definition. Morphological criteria, such as relative absence of α-granules and demarcation membranes, suggest that both MEG-01 and DAMI represent early megakaryoblasts[27,28] and this property together with LAIR-I expression would define DAMI cells as being less mature than MEG-01 cells. The onset of megakaryocyte protein expression in megakaryocytic cell lines obtained from monoclonal leukemic progenitor cells that have differentiated via partially asynchronous routes may differ. How cells that co-express LAIR-I and GPVI respond to collagen in terms of Ca^{2+} mobilization or secretion of granule contents remains a subject for further studies.

The importance of collagen receptors in megakaryocyte maturation, motility and platelet shedding is poorly understood. Differentiating megakaryocytes reside in the bone marrow niche, which expresses collagen abundantly. Unlike LAIR-1 and GPVI, which can bind collagen directly, $\alpha_2\beta_1$ needs affinity modulation by inside-out signaling through ligated GPVI or other receptors before binding collagen effectively. Sabri *et al.* demonstrated that primary megakaryocytes depend on both GPVI and $\alpha_2\beta_1$ ligation for optimal formation of actin stress fibers[35] and, therefore, migration.[36] The latter, however, was not affected by expression of constitutively active $\alpha_2\beta_1$.[37] Alternatively, collagen signaling might lead to inhibition of platelet formation. Megakaryocytes adhering to collagen by $\alpha_2\beta_1$ ligation produce fewer proplatelets than do control cells.[35]

One could speculate that GPVI and $\alpha_2\beta_1$ signaling induces migration of megakaryocytes, which is inhibited by LAIR-1 signaling on immature cells. Upon maturation, LAIR-1 expression is lost, and megakaryocytes migrate to the capillary-rich vascular niche. In this collagen-low environment, GPVI and $\alpha_2\beta_1$ signaling ceases and proplatelet formation and platelet release occur.

In conclusion, LAIR-1 is differentially expressed during megakaryocytopoiesis and is a novel marker for classifying different stages of megakaryocyte development. The activating and inhibitory collagen receptors GPVI and LAIR-1 are simultaneously expressed on a subset of megakaryoblasts and promegakaryocytes. This property might reveal a role for LAIR-1 in increasing the threshold of collagen-activation through GPVI and $\alpha_2\beta_1$ in developing megakaryoblasts.

Authorship and Disclosures

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