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Leukocyte-associated immunoglobulin-like receptor-1 is expressed on human megakaryocytes and negatively regulates the maturation of primary megakaryocytic progenitors and cell line

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ABSTRACT

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is an inhibitory collagen receptor which belongs to the immunoglobulin (Ig) superfamily. Although the inhibitory function of LAIR-1 has been extensively described in multiple leukocytes, its role in megakaryocyte (MK) has not been explored so far. Here, we show that LAIR-1 is expressed on human bone marrow $CD34^+CD41a^+$ and $CD41a^+CD42b^+$ cells. LAIR-1 is also detectable in a fraction of human cord blood $CD34^+$ cell-derived MK that has morphological characteristics of immature MK. In megakaryoblastic cell line Dami, the membrane protein expression of LAIR-1 is up-regulated significantly when cells are treated with phorbol ester phorbol 12-myristate 13-acetate (PMA). Furthermore, cross-linking of LAIR-1 in Dami cells with its natural ligand or anti-LAIR-1 antibody leads to the inhibition of cell proliferation and PMA-promoted differentiation when examined by the MK lineage-specific markers (CD41a and CD42b) and polyploidization. In addition, we also observed that cross-linking of LAIR-1 results in decreased MK generation from primary human $CD34^+$ cells cultured in a cytokines cocktail that contains TPO. These results suggest that LAIR-1 is a likely candidate for an early marker of MK differentiation, and provide initial evidence indicating that LAIR-1 serves as a negative regulator of megakaryocytopoiesis.

1. Introduction

Inhibitory receptors that carry immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic tail play an essential role in the regulation of immune mechanisms [1]. Interestingly, platelets also express several Ig–ITIM superfamily members including G6B [2], TREM [3], CEACAM1 [4] and PECAM-1 [5]. While the regulation of platelet activation by these ITIM-bearing receptors has been well described in the literature, only PECAM-1 was demonstrated as being involved with the regulation of megakaryocytopoiesis. PECAM-1 knockout mice exhibit excessive megakaryocytopoiesis accompanied by increased numbers of megakaryocytes (MK) [6,7]. These results indicate that ITIM-bearing receptors may serve as negative regulators in megakaryocytopoiesis by the mechanism of competitive inhibition.

Leukocyte-associated Ig-like receptor-1 (LAIR-1 or CD305) has been recently characterized as a collagen receptor, which belongs to the inhibitory Ig–ITIM superfamily, and is expressed on most of the differentiated peripheral blood leukocytes, but not on platelets and erythrocytes. Upon receptor cross-linking, tyrosines within the ITIMs undergo phosphorylation to recruit phosphatases, SHP-1, SHP-2 and C-terminal Csk, and attenuate activation signals that are initiated by other receptors [8,9]. Collagens are high-affinity ligands for LAIR-1, and their interaction depends on the presence of conserved Gly-Pro-Hyp collagen repeats [10,11]. Furthermore, LAIR-1 is expressed in $CD34^+$ precursor cells [12], and several studies report that LAIR-1 may be involved in the regulation of hematopoietic cell differentiation [13–16]. Although the inhibitory function of LAIR-1 has been extensively described in multiple leukocytes, its role in MK has not been explored so far.

Megakaryocytic maturation is thought to require migration from the osteoblastic niche to the sinusoidal vasculature within the collagen-rich environment of the bone marrow [17]. Given the importance of collagen in regulation of platelet formation [18], it is plausible to hypothesize that LAIR-1 contributes to autoregulatory mechanisms in megakaryocytopoiesis. Therefore, this study aimed to characterize LAIR-1 expression in MK and investigate its role during MK maturation.

Abbreviations: LAIR-1, leukocyte-associated immunoglobulin-like receptor-1; PMA, phorbol ester phorbol 12-myristate 13-acetate; ITIM, immunoreceptor tyrosine-based inhibition motif.

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2. Materials and methods

2.1. Antibodies

PE-conjugated anti-human CD42b (GpIb) monoclonal antibody (mAb), PE-Cy5-conjugated anti-human CD41a (GpII/IIIa complex) mAb, PE-conjugated anti-human CD34 mAb and isotype-matched conjugated mAb were purchased from BD PharMingen (San Diego, CA, USA). FITC-conjugated and un-conjugated mouse anti-human LAIR-1 mAb 9.1C3, mouse anti-*Staphylococcus aureus* exotoxin-D (SED) mAb were as previously described [19].

2.2. Cells and cell culture

Bone marrow samples were obtained from three patients with myelodysplastic syndrome (MDS), three patients with immune thrombocytopaenic purpura (ITP), one patient with Hodgkin's disease and one patient with multiple myeloma. Informed consent was obtained before any sample collection. The institutional ethics committee approved this study. Mononuclear cells were freshly isolated by Ficoll–HiPaque (1.077 g/ml) density gradient centrifugation.

Human cord blood was collected after obtaining informed consent from the mothers under guidelines established by the Ethical Committee. $CD34^+$ cells were isolated by a positive selection using an immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The $CD34^+$ cells (8×10^4/ml) were cultured for 4–14 days in StemSpanSFEM serum-free medium (STEMCELL Technologies, USA), which was supplemented with a cytokine cocktail (StemSpan CC220, STEMCELL) consisting of SCF, thrombopoietin (TPO), IL-6 and IL-9 to support the expansion of MK.

Human megakaryocytic leukemic and erythroleukemia cell lines, Dami and HEL, respectively, were maintained in the RPMI 1640 medium with 10% fetal bovine serum. Human megakaryoblastic leukemic cell line Mo7e was maintained in the RPMI 1640 medium with 10% FBS and 100 μg/mL GM-CSF (Peprotech, Rocky Hill, NJ). To induce megakaryocytic differentiation, Dami cells were cultured in serum-free medium containing 6 ng/ml of PMA (Sigma) or 50 ng/ml of TPO (Peprotech, Rocky Hill, NJ).

2.3. Cross-linking of LAIR-1

Flat-bottom plates were coated overnight at 4 °C with collagen (Sigma, USA) or anti-LAIR-1 mAb (10 μg/ml in 0.05 M sodium carbonate buffer, pH9.5). Anti-SED mAb (10 μg/ml) was employed as negative control. After washing and blocking with 1% BSA, LAIR-1-expressing cells were added and incubated at 37 °C in 5% CO_2.

2.4. Cells proliferation assay

Dami cells (2×10^5/ml,) were grown in StemSpanSFEM serum-free medium (STEMCELL Technologies, USA) in 96-well plates (100 μl/well) that were pre-coated with anti-LAIR-1 mAb 9.1C3, collagen or anti-SED control mAb. Proliferation rates were determined using a CCK8-based cell proliferation assay (Dojindo, Kumamoto, Japan) at hours 24, 48 and 72, and then measured at 450 nm with a microplate reader (Labsystem Multi MS, Finland).

2.5. Ploidy analysis

Cells were fixed overnight in 70% ethanol at 4 °C and resuspended in 500 μl PBS containing propidium iodide (100 ng/ml, Sigma, USA) and RNase A (0.2 mg/ml, Sigma, USA) for 30 min at 4 °C. DNA content was analyzed by flow cytometry and the ploidy distribution was determined by setting markers at nadirs between peaks.

2.6. RT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen, USA) and then reverse-transcribed using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Canada), based on manufacturer's instructions. Aliquots of cDNA were then used for PCR using specific primers 5′-ATGTCTCCCCACCCCAC-3′ and 5′-GTGTCTGGCAA CGGCTG-3′. that were designed to identify the full-length *LAIR-1* (861-bp fragment). PCR reaction mixtures contained 1 μl cDNA, 50 pmol of each primer, 22 μl water and 25 μl PCR master mix (Perkin–Elmer Biosystems, Foster City, CA, USA). A 2-min hot start at 95 °C was performed to denature the double-stranded cDNA, and this was followed by 30 cycles of PCR (each cycle comprised: 95 °C for 30 s; 60 °C for 40 s; and 72 °C for 45 s); the reactions were terminated with a 10-min extension at 72 °C. Simultaneously, a 50-bp ladder (Fermentas, Canada) was run in adjacent lanes.

2.7. Flow cytometry and confocal laser scanning microscopy analysis

Cells were stained with different combinations of mAb including FITC anti-human LAIR-1 mAb, PE-Cy5 anti-CD41a mAb, PE anti-CD42b mAb and PE anti-CD34 mAb. After incubation for 20 min at 4 °C, cells were analyzed by flow cytometry (EPICS XL, Beckman Coulter, USA) and confocal laser scanning microscopy (LEICA TCS SPE, Germany). To study the intracellular expression of LAIR-1, Dami cells were fixed and permeabilized in buffer (BD PharMingen, USA) and stained with FITC anti-human LAIR-1 mAb and PE-Cy5 anti-CD41a mAb for 30 min at room temperature. The cell nucleus was stained by Hoechst 33258 (Amresco, USA). Isotype controls were used in each experiment.

2.8. Statistical analysis

Values are expressed as means ± SEM, and unpaired *t*-tests were used for comparisons. Statistical significance was defined as values with $P < 0.05$.

3. Results

3.1. LAIR-1 is expressed on human bone marrow and cord blood derived MK

In order to investigate the role of LAIR-1 in megakaryocytic maturation, we first analyzed the expression of LAIR-1 in human primary MK using flow cytometry. LAIR-1 was expressed on a fraction of $CD34^+CD41a^+$ and $CD41a^+CD42b^+$ of bone marrow mononuclear cells. The average percentage of $CD34^+CD41a^+LAIR\text{-}1^+$ and $CD41a^+CD42b^+$ $LAIR\text{-}1^+$ cells was 74.38 ± 3.15% and 45.35 ± 2.80% for 3 patients with MDS; 72.38 ± 3.79% and 53.37 ± 3.46% for 3 patients with IPD; 83.33% and 31.09% for a patient with HD and 80.00% and 36.07% for a patient with multiple myeloma. Fig. 1A present selected results for patients with MDS. In freshly isolated $CD34^+$ cells, the percentages of $CD34^+LAIR\text{-}1^+$ cells and $CD34^+CD41a^+LAIR\text{-}1^+$ cells were approximately 50% and 7%, respectively (Fig. 1B). When these $CD34^+$ cells were induced to megakaryocytic differentiation from days 0 to 14, the expression of LAIR-1 on $CD41a^+$ and $CD42b^+$ cells increased from days 0 to 10 and was followed by a decrease at Day 14. On day 10, about 27% of $LAIR\text{-}1^+$ cells were present in the $CD41a^+CD42b^+$ cells (Fig. 1C). Confocal microscopic study demonstrated that LAIR-1 is expressed in $CD41a^+$ cells that have morphological characteristics of immature MK (Fig. 1D).

3.2. PMA up-regulated the surface expression of LAIR-1 in Dami cells

Expression of LAIR-1 on Dami, Mo7e and HEL cells was investigated by RT-PCR and flow cytometry. RT-PCR results indicate a

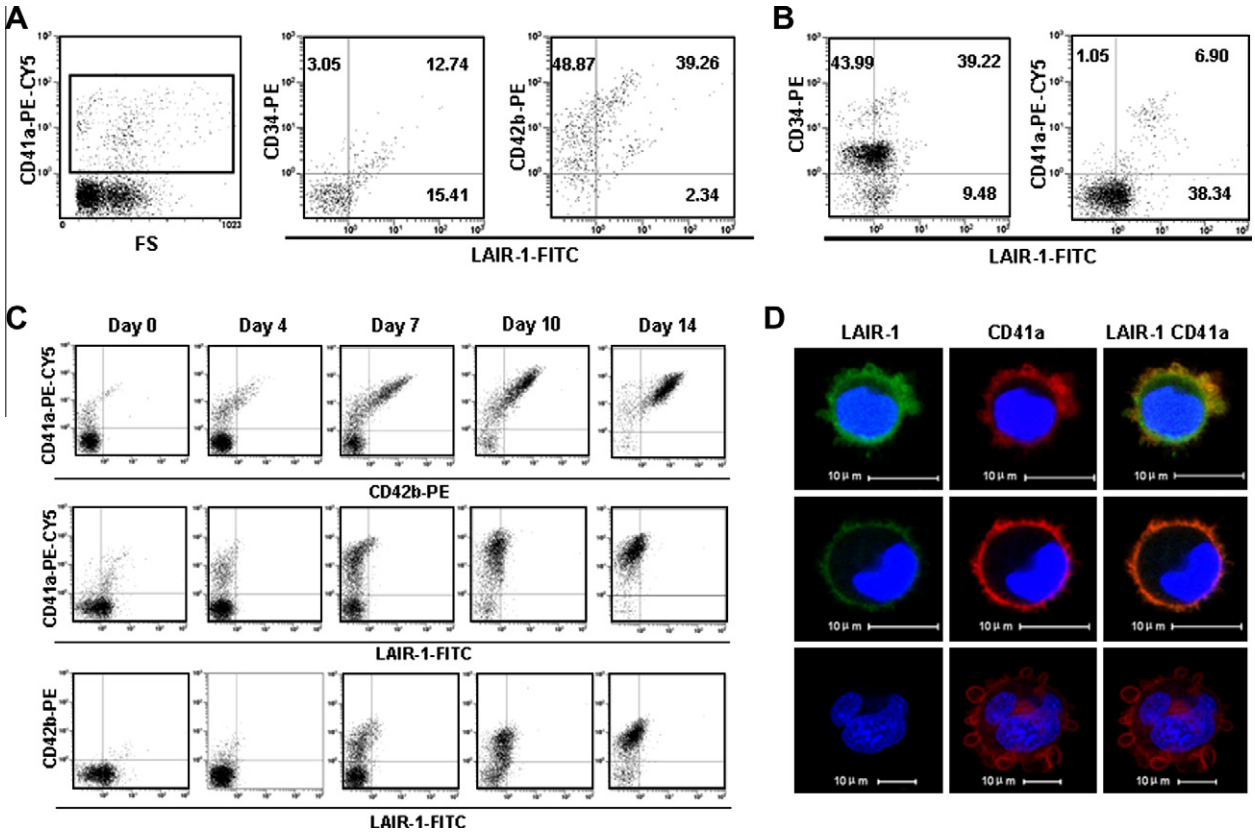

Fig. 1. Surface expression of LAIR-1 on bone marrow and cord blood cells derived MK. (A) Human bone marrow CD41a$^+$ fraction was gated (left) for analysis the co-expression of LAIR-1, CD34, CD41a (middle),or LAIR-1, CD41a and CD42b (right) using flow cytometry. Results are representative of three patients with MDS. (B) Flow cytometry analysis of LAIR-1 expression on fresh CD34$^+$ cells isolated from cord blood (left); co-expression of LAIR-1 and CD41a in CD34$^+$ fraction (right). (C) Purified cord blood CD34$^+$ cells were cultured in serum-free medium supplemented with a cytokine cocktail to support the expansion of MK. Expression of LAIR-1, CD41a and CD42b was analyzed on days 0, 4, 7, 10 and 14 of culture. Results are representative of three experiments. (D) Confocal microscopic analyses of cell-surface expression of LAIR-1 and CD41a. Representative cells were cultured for 10 days and stained with the FITC-conjugated anti-LAIR-1 mAb (green) and PE-CY5-conjugated anti-CD41a mAb (red). Cell nucleus was stained with Hoechst 33258 (blue). An overlay (yellow) shows the colocalization of LAIR-1 and CD41a. Scale bars indicate 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

higher level of mRNA for LAIR-1 was present in Dami cells as compared with the other two cell lines (Fig. 2A). Flow cytometry studies revealed surface protein expression of LAIR-1 on all the three cell lines was low (Fig. 2B). As the results of LAIR-1 expression on Dami cells were not consistent between the RT-PCR and flow cytometry assays, cells were then fixed and permeabilized. Flow cytometry and confocal microscopy analysis revealed a high level of intracellular expression of LAIR-1 (Fig. 2C and D). Because PMA and TPO are known to enhance megakaryocytic features in Dami cells [20], we next attempted to determine whether the surface expression of LAIR-1 could be enhanced during Dami cell differentiation. After stimulating with PMA (6 ng/ml), the surface expression of CD41a, CD42b and LAIR-1 was markedly increased. Following administration of PMA for 3, 6 and 9 days, respective positive LAIR-1 expression rates of 29.88 ± 1.53%, 49.52 ± 1.86% and 49.49 ± 2.31% were obtained (Fig. 2D and E). By contrast to the results for PMA, stimulation with TPO (50 ng/ml) has no evident effect on LAIR-1 expression (data not shown).

3.3. Up-regulation of LAIR-1 expression inhibits maturation of Dami cells

To assess whether increased surface expression of LAIR-1 has an effect on the PMA-induced megakaryocytic maturation, Dami cells was treated with PMA (6 ng/ml) for 4 days to up-regulate LAIR-1 expression and then followed by cross-linking of LAIR-1 with its natural ligand collagen or LAIR-1 specific mAb 9.1C3. By such a measure, a decreased cell proliferation was observed when LAIR-1 was cross-linked with 9.1C3 or collagen as compared with SED mAb control (Fig. 3A).To determine the effect of LAIR-1 on PMA-promoted polyploidization of Dami cells, the DNA content was analyzed by flow cytometry after 3 days of exposure to 9.1C3 and collagen. We observed a higher percentage of cells with ploidy of 4N in the presence of 9.1C3 and collagen (43.46 ± 1.63% and 42.43 ± 1.75%) and a lower percentage with 8N (20.70 ± 1.19% and 22.71 ± 1.27%) when compared with the SED control (4N = 38.47 ± 1.37%, 8N = 25.32 ± 1.68%). However, only a lower percentage of cells with 16 N (8.18 ± 0.42%) was observed when cells were cultured in the presence of collagen as compared with SED control (10.82 ± 0.63%) (Fig. 3B and C). Next we tested the effect of LAIR-1 on the expression of MK lineage-specific markers CD41a and CD42b. After cross-linking of LAIR-1 for 3 days, the expression levels of CD41a were lower in cells cultured either in the presence of collagen or 9.1C3 as compared with SED mAb. The results of CD42b expression were similar to that of CD41a, but a statistical significance was only observed in cells cultured in the presence of 9.1C3 (Fig. 3D). Above all, cross-linking of LAIR-1 that is up-regulated in Dami cells leads to an inhibition of PMA-promoted differentiation.

3.4. LAIR-1 inhibits the generation of MK from cord blood CD34$^+$ cells

To examine the role of LAIR-1 in primary MK differentiation, cord blood CD34$^+$ cells were cultured in StemSpanSFEM serum-free

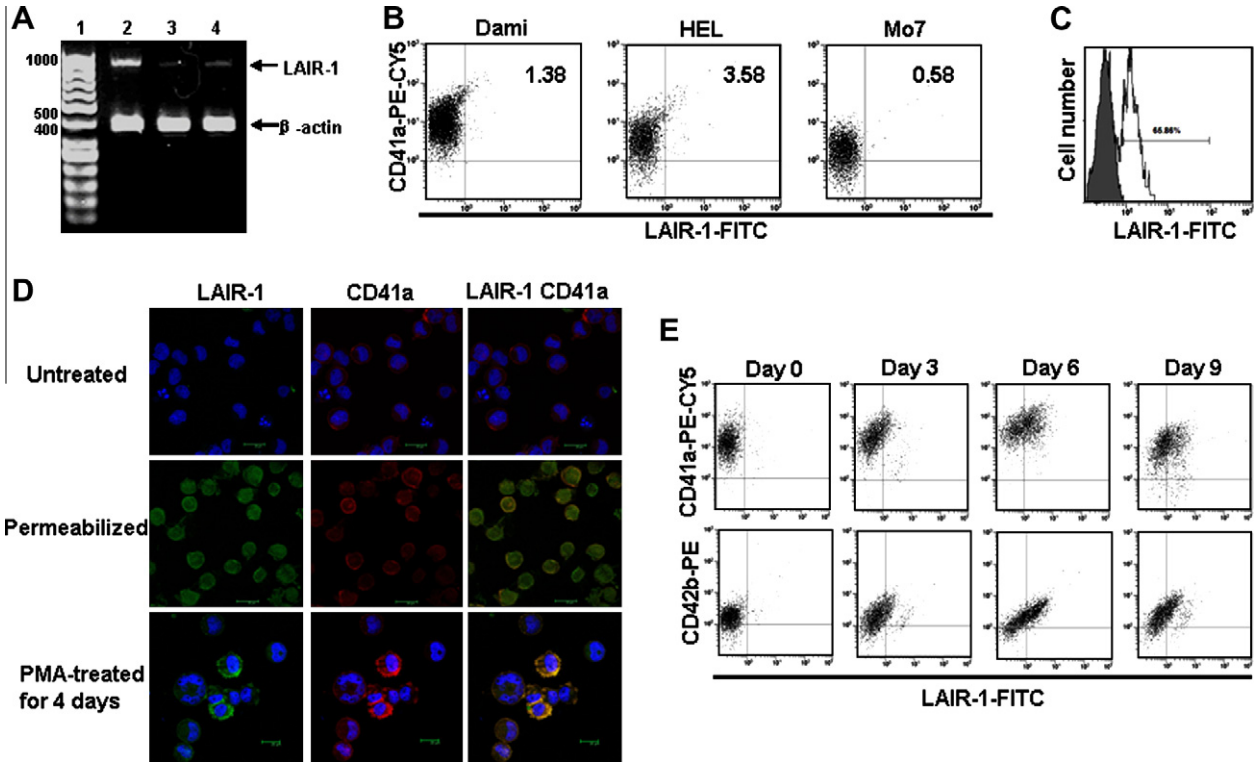

Fig. 2. The expression of LAIR-1 on megakaryocytic cell lines. (A) Presence of LAIR-1 was assessed by RT-PCR analysis. Lane 1: 1-kb marker; lane 2: Dami cells; lane 3: Mo7e cells; lane 4: HEL cells. (B) Cell-surface expression of LAIR-1 was assessed by flow cytometry. (C) Dami cells were permeabilized and stained with FITC-conjugated anti-LAIR-1 mAb. Intracellular expression of LAIR-1 was analyzed by flow cytometry. (D) Confocal microscopic analysis of the cellular localization of LAIR-1 on Dami cells. Cells were stained with the FITC-conjugated anti-LAIR-1 mAb (green) and PE-CY5-conjugated anti-CD41a mAb (red).The cell nucleus stain and intracellular stain of LAIR-1 were performed as described above. An overlay (yellow) shows the colocalization of LAIR-1 and CD41a. Scale bars indicate 20 μm. (E) Dami cells were stimulated by PMA and cultured for 9 days. Surface expression of LAIR-1, CD41a and CD42b during the differentiation was analyzed by flow cytometry. Results are representative of five experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

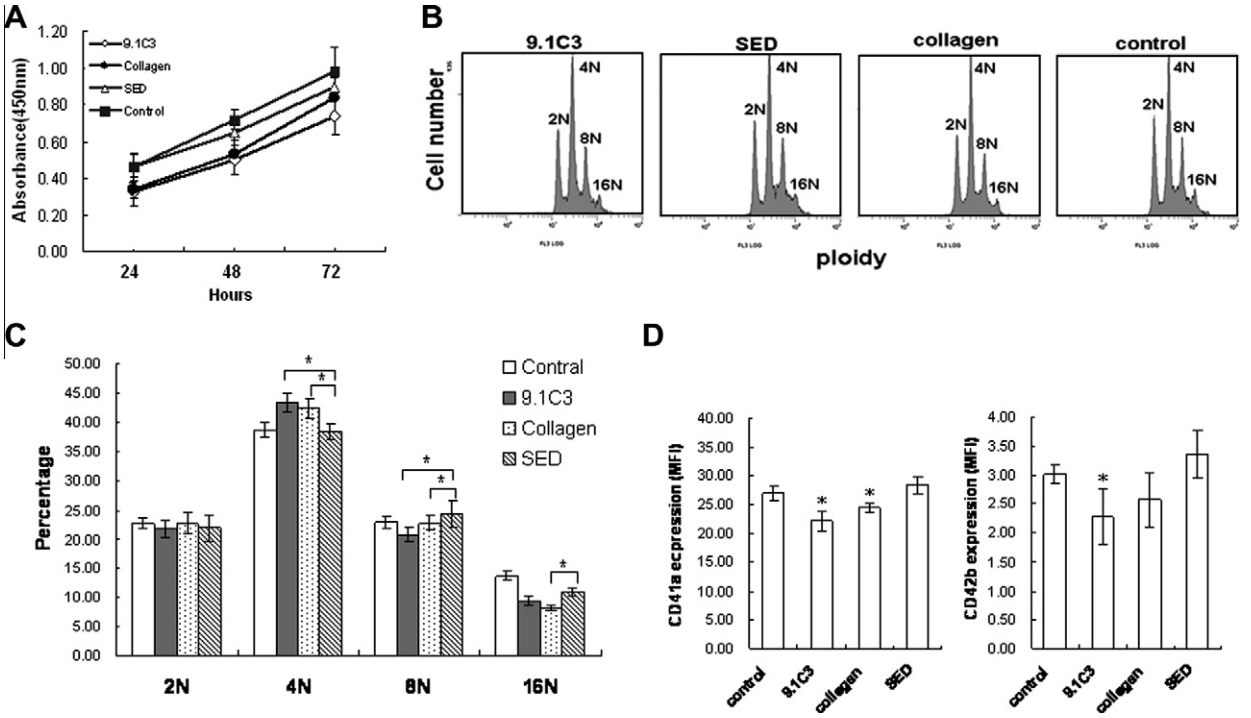

Fig. 3. Effects of LAIR-1 cross-linking on PMA-promoted megakaryocytic maturation of Dami cells. (A) Cell proliferation decreased when LAIR-1 was cross-linked with 9.1C3 or collagen at 24 and 48 h of culture as compared with SED mAb control ($P < 0.05$). Data expressed as the mean ± SEM of three experiments are shown. (B) Representative data from three experiments showing ploidy of Dami cells. (C) Ploidy distribution Dami cells are expressed as the mean ± SEM of three experiments. $^{*}P < 0.05$. (D) CD41a and CD42b expression were analyzed via flow cytometry as mean fluorescent intensity (MFI). Data are expressed as the mean ± SEM of three experiments. $^{*}P < 0.05$.

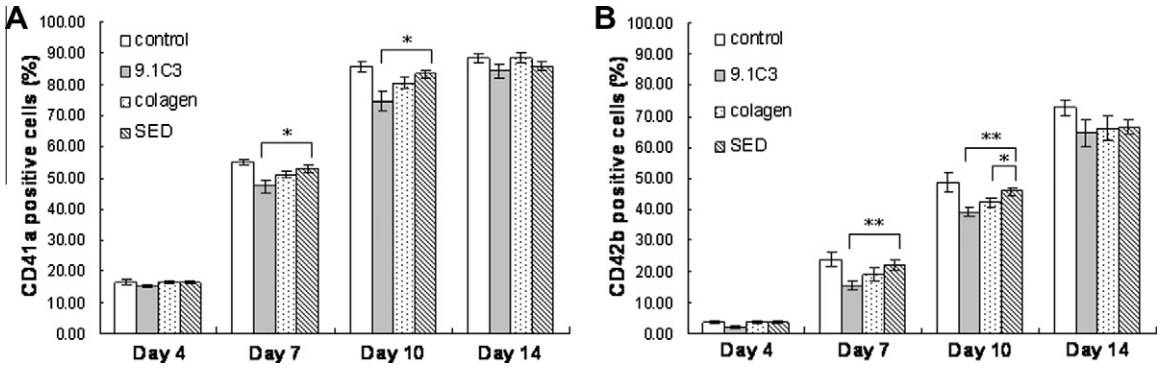

Fig. 4. Effects of LAIR-1 cross-linking on cytokine-induced megakaryocytic differentiation of cord blood CD34$^+$ cells. Cell culture was performed as described in Fig. 1. CD34$^+$ cells were cultured in the absence or presence of 9.1C3, anti-SED mAb and collagen. Flow cytometry was used to study the expression of CD41a and CD42b on the surface of the cells. Results are expressed as percentages of positive cells. Data are representative of three experiments (mean ± SD). $^*P < 0.05$; $^{**}P < 0.01$.

medium supplement with cytokines promoting MK development in the presence of anti-LAIR-1 specific mAb 9.1C3 or collagen. Expression of MK lineage-specific markers was determined at days 4, 7, 10 and 14 after cross-linking of LAIR-1. Consistent with the data of Dami cell, after exposure of CD34$^+$ cells to 9.1C3 mAb we observed a decrease in the percentages of CD41a and CD42b-positive cells as compared with anti-SED control mAb. By contrast to the results for 9.1C3, only a decrease of CD42b-positive cells was observed in the presence of collagen (Fig. 4A and B). Overall, these findings suggest that the MK generation from human cord blood CD34$^+$ is down-regulated by cross-linking of LAIR-1.

4. Discussion

Megakaryocytopoiesis occurs within a complex bone marrow microenvironment wherein a diverse array of cell-surface receptors and signaling proteins act in concert to modulate lineage differentiation [21]. Therefore, an appropriate regulation of positive and negative signaling is critical for maintaining normal megakaryocytopoiesis. Several factors are known to inhibit MK development, including transforming growth factor-β1, PF4, pituitary adenylate cyclase-activating peptide (PACAP) and vasointestinal peptide (VIP) [18]. In the present study, we demonstrate that collagen receptor LAIR-1 serves to negatively regulate megakaryocytic maturation. This conclusion is based on an analysis of aggregate findings from experiments performed in this study and includes premises that: (1) LAIR-1 is expressed from an early stage of human primary MK; (2) upon receptor cross-linking, LAIR-1 negatively regulates cytokine-mediated differentiation of MK derived from cord blood CD34$^+$ cells; and (3) the up-regulation of LAIR-1 expression in the megakaryoblastic cell line Dami was associated with a negative regulation of cell proliferation and differentiation.

These findings further raise the question of why LAIR-1 would be expressed at these early stages of MK differentiation. One possible explanation is that such a process could prevent the excessive activation of immature MK that results following exposure to surrounding collagens. The main collagen receptors expressed by MK are the integrin α2β1 and glycoprotein VI (GPVI)-FcRγ-chain complex, which has an immunoreceptor tyrosine-containing activation motif (ITAM). The expression and activation of these collagen receptors are tightly regulated during megakaryocytopoiesis [22,23]. It has been shown previously that GPVI is expressed early during megakaryocytic differentiation but allows MK adherence to collagen only at later stages of differentiation [24]. Thus, LAIR-1 may play a predominant role during this early stage of megakaryocytic differentiation.

Based upon recent studies in B-cell chronic lymphocytic leukemia (CLL), deficiency in LAIR-1-mediated ITIM-dependent signal appears to eliminate one of the molecular mechanisms controlling B-cell activation and proliferation [25].Of interest was the finding that, despite a higher level of LAIR-1 mRNA in the megakaryoblastic Dami cell line, the cell-surface expression of LAIR-1 was low. Furthermore, the membrane protein expression of LAIR-1 was up-regulated significantly after stimulation with PMA. Intracellular staining and confocal microscopy studies confirmed that LAIR-1 was principally localized in the cytoplasm of untreated cells. These findings suggest a perturbed program of LAIR-1 trafficking in Dami cells. Based on these results, the Dami cell line was used as a model system to assess the effects of LAIR-1 on megakaryocytic differentiation. Cross-linking of LAIR-1 that is overexpressed in Dami cells leads to an inhibition of PMA-promoted ploidy increase, CD41a and CD42b expression and cell proliferation. Interestingly, the inhibitory effects of LAIR-1 in the presence of anti-LAIR-1 specific antibody appear to be stronger than those in the presence of the collagen ligand. A possible explanation for these observations is that the ITAM-bearing GPVI, which is the major signaling receptor for collagen [24] and is co-expressed with LAIR-1 in megakaryoblasts [26], may attenuate signals mediated by the ITIM-bearing LAIR-1.

Multiple thrombopoietic cytokines are involved in the regulation of MK terminal differentiation. TPO is the most potent cytokine for stimulation, proliferation and maturation of MK progenitor cells [18]. To ensure an appropriate and controlled cellular response, signal transduction of these cytokines is actively counterbalanced by several families of inhibitory gene products, including the SH2-containing phosphatases (SHP), the protein inhibitors of activated STATs (PIAS) and the suppressors of cytokine signaling (SOCS) [27]. In this study, we also observed that the cross-linking of LAIR-1 results in decreased MK generation from primary human CD34$^+$ cells cultured in a cytokine cocktail that contains TPO. It is known that ligand engagement by inhibitory receptors results in ITIM phosphorylation by Src kinases [28] and the Src family kinase lyn has been confirmed as an important negative regulator of TPO signaling with regard to megakaryocyte proliferation and differentiation [29,30]. Thus, LAIR-1 may be involved in the regulation of thrombopoietic cytokine signaling. Taken together, although additional research will be required to confirm the mechanism by which LAIR-1 is involved in megakaryocytopoiesis, we speculate that the cross-talk between LAIR-1 and other thrombopoietic elements may contribute to a complex network that regulates the differentiation of MK.

Conflict of interest

None declared.

Acknowledgments

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