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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