Molecular Immunology 48 (2011) 714–719

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Short communication

CLEC5A (*MDL-1*) is a novel PU.1 transcriptional target during myeloid differentiation

Jasmin Batliner[a], Maria Michela Mancarelli[b], Mathias Jenal[a,1], Venkateshwar A. Reddy[c], Martin F. Fey[a,d], Bruce E. Torbett[b], Mario P. Tschan[a,d,*]

[a] *Experimental Oncology/Hematology, Department of Clinical Research, University of Bern, Bern, Switzerland*
[b] *Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, United States*
[c] *Cancer Stem Cell Program, Novartis Institute of Functional Genomics, San Diego, CA, United States*
[d] *Department of Medical Oncology, Inselspital, Bern University Hospital, Bern, Switzerland*

ARTICLE INFO

Article history:
Received 3 August 2010
Received in revised form 21 October 2010
Accepted 23 October 2010
Available online 20 November 2010

Keywords:
CLEC5A
PU.1
Myeloid differentiation
Innate immunity

ABSTRACT

C-type lectin domain family 5, member A (*CLEC5A*), also known as myeloid DNAX activation protein 12 (DAP12)-associating lectin-1 (*MDL-1*), is a cell surface receptor strongly associated with the activation and differentiation of myeloid cells. CLEC5A associates with its adaptor protein DAP12 to activate a signaling cascade resulting in activation of downstream kinases in inflammatory responses. Currently, little is known about the transcriptional regulation of *CLEC5A*. We identified *CLEC5A* as one of the most highly induced genes in a microarray gene profiling experiment of *PU.1* restored myeloid PU.1-null cells. We further report that *CLEC5A* expression is significantly reduced in several myeloid differentiation models upon *PU.1* inhibition during monocyte/macrophage or granulocyte differentiation. In addition, *CLEC5A* mRNA expression was significantly lower in primary acute myeloid leukemia (AML) patient samples than in macrophages and granulocytes from healthy donors. Moreover, we found activation of a *CLEC5A* promoter reporter by PU.1 as well as *in vivo* binding of PU.1 to the *CLEC5A* promoter. Our findings indicate that *CLEC5A* expression in monocyte/macrophage and granulocytes is regulated by PU.1.

1. Introduction

CLEC5A is a cell surface receptor involved in the activation of myeloid cells. It associates non-covalently with the adaptor and signaling molecule DAP12 (also called KARAP and TYROBP) and was the first DAP12-associating receptor described in myeloid cells (Bakker et al., 1999). Signaling via this complex constitutes a significant activation pathway in myeloid cells and has an important role in immune defense. DAP12 is expressed on several immune cells, contains an immunotyrosine-based activation motif (ITAM) and associates with more than 20 different cell surface activating receptors to regulate immune responses. Upon ligand binding by a DAP12-associated receptor, the DAP12 cytoplasmic ITAM is phosphorylated by Src kinases and interacts with the Syk cytoplasmic tyrosine kinase, initiating a cascade of events that lead to macrophage activation (Campbell and Colonna, 1999; Lanier and Bakker, 2000; Lanier et al., 1998). Furthermore, recent studies have demonstrated that CLEC5A is a receptor for Dengue virus on the surface of macrophages. CLEC5A interacts directly with the Dengue virion, thereby causing phosphorylation of DAP12 and triggering a signaling cascade, which results in the release of proinflammatory cytokines (Chen et al., 2008).

In myeloid development *CLEC5A* expression is associated with mature stages of myeloid differentiation (Gingras et al., 2002). Furthermore, *CLEC5A* is expressed constitutively at very low levels but is highly induced in activated macrophages during infections (Aoki et al., 2004, 2009; Bakker et al., 1999). In addition, CLEC5A is also implicated in osteoclastogenesis and associates with DAP10 in osteoclasts and bone marrow-derived macrophages. This association seems to be dependent on the presence of DAP12, and signaling through this trimolecular complex stimulates osteoclastogenesis and bone remodeling (Inui et al., 2009). *CLEC5A* expression is also induced upon neutrophil differentiation and activation of mouse 32Dcl3 myeloid cells (Aoki et al., 2009). In general, *CLEC5A* has an important function in innate immunity due to its role in macrophage and neutrophil differentiation as well as activation.

The *CLEC5A* gene is located on human chromosome 7q33 and murine chromosome 6B2, two loci that have not been associated with any disease relation to date. The molecular mechanisms that are responsible for the transcriptional regulation of the *CLEC5A*

* Corresponding author at: Experimental Oncology/Hematology, Department of Clinical Research, University of Bern, Murtenstrasse 35, MEM E829, Bern CH-3010, Switzerland. Tel.: +41 31 632 8780.
E-mail address: mtschan@dkf.unibe.ch (M.P. Tschan).
[1] Present address: The Netherlands Cancer Institute, Amsterdam, The Netherlands.

0161-5890/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2010.10.016

gene remain mostly unidentified. We discovered strong induction of *CLEC5A* in myeloid PU.1 knockout cells where PU.1 had been restored and provide evidence that *CLEC5A* is a direct PU.1 target gene in AML cells. Our results suggest that PU.1 is a major regulator of *CLEC5A* expression during myeloid differentiation.

2. Materials and methods

2.1. Cell lines, primary patient samples and culture conditions

The human acute myeloid leukemia (AML) cell lines HL60, HT93, U937 and THP1 were maintained in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich, Buchs, Switzerland) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. For differentiation experiments, cells were treated with 1 μM all-*trans* retinoic acid (ATRA; Sigma–Aldrich, Buchs), 10^{-7} M vitamin D_3 (1,25-$(OH)_2D_3$) or 10 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich) for days indicated.

Successful granulocyte or monocyte differentiation was evidenced by FACS analysis of CD11b and CD14 (BD Pharmingen), respectively. Successful macrophage differentiation was assessed morphologically. Isolation and differentiation of primary myeloid cells was done as described (Tschan et al., 2001, 2003). Protocols and the use of all human samples were approved by the Cantonal Ethical Committee at the Inselspital.

2.2. Microarray analysis

Chip assays and analysis of the myeloid 503 PU.1 null cell line and PU.1 restored cells were utilized as described (Jenal et al., 2010).

2.3. Human CLEC5A promoter reporter assay

The *CLEC5A* promoter region was PCR amplified from genomic DNA of HL60 AML cells using the GC-RICH PCR system (Roche Diagnostics, Rotkreuz, Switzerland) and cloned into pCR-XL vector using the TOPO XL cloning kit (Invitrogen). The *CLEC5A* KpnI/HindIII promoter fragment was further subcloned into the pGL4-basic luciferase vector (Promega, Madison, WI, USA) using standard cloning techniques. PU.1 binding site mutations were introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

For reporter assays, 293T cells were transfected in triplicate with 100 ng reporter, 300 ng effectors and 10 ng of pRL-TK plasmid per 24-well using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were lysed 24 h after transfection and luciferase activity was measured using the Dual-Luciferase Reporter Plasmid System (Promega, Madison, WI). Results, expressed relative to a value of 1.0 for cells transfected with empty vector, are the means of two replications, and error bars represent standard deviations.

2.4. Chromatin immunoprecipitation assay (ChIP)

U937 cells were collected and ChIP assay performed as described (Weinbert et al., 2006). Antibodies used were anti-PU.1, anti-C/EBPA (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-RNA pol II (Active Motif, Carlsbad, CA) antibodies. The following primers were used to amplify the *CLEC5A* genomic region containing the proximal PU.1 binding site by SYBR® green based quantitative PCR: Fw 5′-GGAAGTCTGCTCTTGCCACCACTag-3′ and Rev 5′-CTGCCTTGGTAGCATCCCCAAG-3′. Results were normalized to an IgG control and are given as % input chromatin.

2.5. TaqMan Low Density Arrays (LDAs) and quantitative real-time RT-PCR (RQ-PCR)

RQ-PCR for LDAs and the 96-well format were performed using the ABI 7900HT Fast Real-time PCR System or the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland), respectively. Taqman® Gene Expression Assays for *HMBS*, *ABL1*, *PU.1* and *CLEC5A* preloaded on LDAs were Hs00609297_m1, Hs00245445_m1, Hs00231368_m1, and Hs00183780_m1 (Applied Biosystems), respectively. For the 96-well format, we used the *CLEC5A* Taqman® Gene Expression Assay Hs00183780_m1. Primers and probes for *PU.1*, *ABL1* and *HMBS* as well as data analysis have been described (Tschan et al., 2008).

2.6. Lentiviral knockdown constructs

pLKO.1 lentiviral vectors expressing short hairpin RNAs targeting PU.1 (shPU.1_256: NM_003120.1-256s1c1/TRCN0000020536 and shPU.1_928: NM_003120.1-928s1c1/TRCN0000020538) and the non-targeting control shRNA vector (SHC002) were purchased from Sigma–Aldrich. Lentivirus production and transduction were done as described (Tschan et al., 2003).

2.7. Western blot analysis

Immunoblotting and protein extraction have been described previously (Andrews and Faller, 1991; Radziwill et al., 2003). Primary antibodies used were anti-PU.1 (Cell Signaling) and anti-GAPDH (MAB374; Millipore). Secondary antibodies used were donkey anti-rabbit and sheep anti-mouse horseradish peroxidase-conjugated IgG (Amersham, Zurich, Switzerland).

2.8. Statistical analysis

Nonparametric Mann–Whitney *U* tests were applied to compare the difference between two groups using the program GraphPad Prism 4. *p*-Values <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Silencing PU.1 significantly impairs CLEC5A induction during monocyte/macrophage differentiation

To identify novel PU.1-regulated genes, we introduced *PU.1* into murine 503 PU.1-null myeloid cells using lentiviral gene transfer. We subsequently assessed gene expression profiles of PU.1 restored versus 503 PU.1-null myeloid cells. Several known PU.1 target genes were upregulated in the PU.1 rescued 503 cell line thereby confirming successful restoration of PU.1 (Jenal et al., 2010). *CLEC5A* was among the most highly induced genes in PU.1-restored 503 cells. To validate this finding we decreased PU.1 expression in HL60 AML cells, which can be differentiated into monocytes by vitamin D_3 ($VitD_3$) treatment. HL60 cells were stably transduced with non-targeting shRNA (SHC002) or with two different short hairpin RNAs targeting PU.1. Both shRNA constructs significantly reduced PU.1 expression in the two knockdown cell lines (Fig. 1A, right panel and Supplementary Fig. 1). We observed significantly reduced *CLEC5A* mRNA expression levels in $VitD_3$-differentiated HL60 cells from 11.5-fold in parental cells to 2.3- and 2-fold in the two PU.1 knockdown cell lines, respectively (Fig. 1A). We further assessed *CLEC5A* protein surface expression by flow cytometry. During monocytic differentiation of HL60 cells the induction of CLEC5A in the two knockdown cell lines was significantly impaired compared to control cells (CD11b MFI in control cells 422, compared to 110 and 140 in the two PU.1 knockdown lines, respectively). In parallel, CD14