The Pathogen Receptor Liver and Lymph Node Sinusoidal Endotelial Cell C-Type Lectin Is Expressed in Human Kupffer Cells and Regulated by PU.1

Ángeles Domínguez-Soto,[1] Laura Aragoneses-Fenoll,[1] Fernando Gómez-Aguado,[2] María Teresa Corcuera,[2] Joan Clária,[3] Carmelo García-Monzón,[4] Matilde Bustos,[5] and Angel L. Corbí[1]

Human LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin, CLEC4G) is a C-type lectin encoded within the L-SIGN/DC-SIGN/CD23 gene cluster. LSECtin acts as a pathogen attachment factor for *Ebolavirus* and the SARS coronavirus, and its expression can be induced by interleukin-4 on monocytes and macrophages. Although reported as a liver and lymph node sinusoidal endothelial cell-specific molecule, LSECtin could be detected in the MUTZ-3 dendritic-like cell line at the messenger RNA (mRNA) and protein level, and immunohistochemistry analysis on human liver revealed its presence in Kupffer cells coexpressing the myeloid marker CD68. The expression of LSECtin in myeloid cells was further corroborated through the analysis of the proximal regulatory region of the human LSECtin gene, whose activity was maximal in LSECtin+ myeloid cells, and which contains a highly conserved PU.1-binding site. PU.1 transactivated the LSECtin regulatory region in collaboration with hematopoietic-restricted transcription factors (Myb, RUNX3), and was found to bind constitutively to the LSECtin proximal promoter. Moreover, knockdown of PU.1 through the use of small interfering RNA led to a decrease in LSECtin mRNA levels in THP-1 and monocyte-derived dendritic cells, thus confirming the involvement of PU.1 in the myeloid expression of the lectin. *Conclusion:* LSECtin is expressed by liver myeloid cells, and its expression is dependent on the PU.1 transcription factor. (HEPATOLOGY 2009;49:287-296.)

The gene cluster at chromosome 19p13.2 includes the genes encoding for the type II C-type lectins DC-SIGN, L-SIGN, CD23, and LSECtin,[1-4] all of which contain a single carbohydrate-recognition domain followed by a stalk domain, a transmembrane region, and a cytoplasmic tail containing various internalization motifs. DC-SIGN, L-SIGN, and LSECtin function as endocytic receptors and mediate binding and internalization of clinically relevant viral, bacterial, and fungal pathogens.[5,6] CD23 is expressed on myeloid cells and activated B lymphocytes, where it functions as a low affinity receptor for immunoglobulin E and plays a role in limiting the extent of immunoglobulin E–mediated pathologies.[7,8] DC-SIGN is expressed on myeloid dendritic cells (DCs),[1,9] alternatively activated *in vitro* macrophages,[10] interstitial DCs,[11] a subset of CD14+ peripheral blood DCs,[12] and macrophages from various tissues,[13-15] whereas L-SIGN is exclusively expressed on endothelial cells of the liver, lymph nodes, and placenta.[16,17] Although reported to be exclusively expressed on liver and lymph node sinusoidal endothelial cells,[4] LSECtin has been later found to be expressed in *ex vivo* isolated human peripheral blood and thymic DCs, as well as in DCs and alternatively activated macrophages generated *in vitro*.[5] The carbohydrate specificity of LSECtin has been

Abbreviations: DC, dendritic cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-4, interleukin-4; LSECtin, liver and lymph node sinusoidal endothelial cell C-type lectin; MDDC, monocyte-derived dendritic cell; mRNA, messenger RNA; PCR, polymerase chain reaction; PPARγ, peroxisome proliferator-activated receptor gamma; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA.

From [1]Centro de Investigaciones Biológicas, CSIC, Madrid, Spain; [2]Departamento de Anatomía Patológica, Hospital Carlos III, Madrid, Spain; the [3]Department of Biochemistry and Molecular Genetics, Hospital Clínic, Barcelona, Spain; [4]Hospital Universitario Santa Cristina, Madrid, Spain (Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREHD); and [5]Division of Hepatology and Gene Therapy, CIMA, University of Navarra, Pamplona, Spain.

Received April 29, 2008; accepted September 4, 2008.

Supported by the Ministerio de Educación y Ciencia (grants SAF2005-0021 and GEN2003-20649-C06-01/NAC), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (Spanish Network for Research in Infectious Diseases, REIPI RD06/0008/00012 and Red de SIDA RD06/0006/1016), Fundación para la Investigación y Prevención del SIDA en España (FIPSE 36663/07), and Fundación Mutua Madrileña (to A. L. C.), and SAF2006-03191 (to J. C.). A. D. S. was supported by an FPI predoctoral grant (BES2004-4405) from Ministerio de Educación y Ciencia, Spain. CIBEREHD is funded by Instituto de Salud Carlos III.

Address reprint requests to: Angel L. Corbí, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu, 9, Madrid 28040, Spain. E-mail: acorbi@cib.csic.es; fax: (34)-1-5627518.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22678

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

recently determined,[18] and a scavenging function has been proposed because of its ability to recognize glycoproteins with truncated complex and hybrid N-linked glycans terminating in GlcNAcMan.

Kupffer cells constitute more than 50% of resident macrophages in the entire body,[19] account for 15% of all liver cells, and are an integral part of the hepatic sinusoid together with sinusoidal endothelial cells and Ito cells. Kupffer cells exhibit a strong endocytic activity and actively scavenge plasma proteins and potentially hazardous microorganisms from the blood to maintain tissue homeostasis,[20] a function dependent on the large array of scavenger receptors exposed on their cell surface.[21] In fact, Kupffer cells mediate the removal of particulate material from the portal circulation.[22] The presence of LSECtin in myeloid cell subsets[5] prompted us to clarify its cell distribution in liver cells. We report that LSECtin is expressed in human Kupffer cells, where its expression correlates with the presence of the myeloid-restricted CD68 molecule. Moreover, PU.1 binds *in vivo* to the human LSECtin proximal promoter, and PU.1 protein levels determine the extent of LSECtin messenger RNA (mRNA) expression. Therefore, PU.1 contributes to the myeloid expression of LSECtin, which constitutes a novel addition to the arsenal of scavenging molecules expressed by liver Kupffer cells.

Materials and Methods

Cell Culture, Transfection, and Site-Directed Mutagenesis. Monocytes were purified from peripheral blood mononuclear cells via magnetic cell sorting using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), and monocyte-derived dendritic cells (MDDCs) were generated as described.[5,6] The K562 (chronic myelogenous leukemia) and THP-1 (monocytic leukemia) cell lines were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, and 2 mM glutamine (complete medium), at 37°C in a humidified atmosphere with 5% CO_2. MUTZ-3 cells[23-25] were maintained in complete medium supplemented with granulocyte-macrophage colony-stimulating factor (10 ng/mL) and their dendritic differentiation was induced in the presence of 1,000 U/mL interleukin-4 (IL-4) for 5 days.

Transfection of NIH-3T3, Jurkat, K562, and THP-1 cells were performed as described using Superfect (Qiagen, Hilden, Germany) or DEAE-Dextran.[26] In reporter gene experiments, the amount of DNA in each transfection was normalized by using the corresponding insertless expression vector (CMV-0) as a carrier. Each transfection experiment was performed at least three times with different DNA preparations. Transfection efficiencies were normalized via cotransfection with the pCMV-βgal plasmid, and β-galactosidase levels were determined using the Galacto-Light kit (Tropix, Bedford, MA). The LSECtin-based reporter gene constructs pCLEC4G-591, pCLEC4G-296, and pCLEC4G-247 were generated via polymerase chain reaction (PCR) amplification of the -591/+16, −296/+16 and −247/+16 fragments of the LSECtin promoter with oligonucleotides 5′-CCAAGCTTGGAAAACTAAGGCTTCTAGAAGC-3′, 5′-CCAAGCTTGGTGACTAA GCTCCAAAGAGAAG-3′, and 5′-GGGGTACCCGATGCAGGCACCCAGTCC-3′, and the resulting fragments cloned into HindIII and KpnI-digested pXP2 plasmid.[27] Positions within the LSECtin regulatory regions were numbered from the predicted transcriptional start site. The PU.1 expression plasmids for human PU.1, RUNX3, and Myb have been described.[26]

THP-1 cells or MDDCs (2×10^6 cells) were nucleofected with 3 μg of small interfering RNA (siRNA) for PU.1 (*sc*-36330 PU.1 siRNA gene silencer; Santa Cruz Biotechnology, Santa Cruz, CA) or a control siRNA (*sc*-37007 Control siRNA-A, Santa Cruz Biotechnology) using the Cell Line Nucleofector kit V for THP-1 and the Human Dendritic Cell Nucleofector kit for MDDCs (Amaxa, Cologne, Germany). After nucleofection, cells were kept in culture for 24 hours, and one-fifth of the cells were lysed and underwent western blotting for PU.1 detection. Total RNA was isolated from the remaining nucleofected cells and subjected to real-time PCR for the detection of LSECtin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Site-directed mutagenesis was performed on the LSECtin promoter construct pCLEC4G-296Luc using the QuikChange System (Stratagene, La Jolla, CA). For mutation of the PU.1-99 and PU.1-66 elements, the oligonucleotides PU.1-99mutS (5′-CCTGTGATTGGTCCATGGGTCGACGTTGCCAGGAhpATTAAGG-3′), PU.1-99mutAS (5′-CCTTAATTCCTGGCAACGTCGACCCATGGACCAATCACAGG-3′), PU.1-66mutS (5′-GGC CAAGAAAATGGGGTCGACCGACGGGAGTGCGTAGGTCCAGTG -3′), and PU.1-66mutAS (5′-CACTGGACCTACGCACTCCCGTCGGTCGACCCCATTTTCTTGGCC -3′) were used, and the resulting plasmids were termed pCLEC4G-296-99MUT and pCLEC4G-296-66MUT. All DNA constructs and mutations were confirmed by DNA sequencing.

Immunohistochemistry. Immunostaining was performed on formalin-fixed, paraffin-embedded sections

from normal human livers and lymph nodes showing hyperplasia. Paraffin sections were cut at 4 μm thickness and placed onto positively charged capillary gap microscope slides. Deparaffinization in xylene and hydration through graded alcohols was followed by heat-induced epitope retrieval. The slides were pressure-cooked for 3 minutes in 10 mM buffer citrate (pH 6.0) and then left in the buffer for 20 minutes at room temperature. Preparations were incubated with the distinct antibodies for 25 minutes at room temperature. As a secondary antibody, a biotinylated goat anti-rabbit polyclonal (ChemMate detection Kit, DakoCytomation) was applied, followed by horseradish peroxidase–conjugated streptavidin. Finally, the slides were developed in 3,3'-diaminobenzidine (ChemMate Detection Kit, DakoCytomation) and counterstained in hematoxylin. All incubations were performed in a capillary gap principle-based automated immunostainer (TechMate Horizon, DakoCytomation). For each specimen, a negative control was included using preinmune rabbit serum instead of primary antibody. EnVisionTM G/2 Doublestain System (Dako) was used for the simultaneous detection of CD68 and LSECtin, following the manufacturer's recommendations with a mouse monoclonal antibody against CD68 (PG-M1; Dako) and the LSECtin-specific polyclonal antisera ADS1 (against the stalk domain) and ADS4 (against the whole extracellular region).[5,6] Blockade of endogenous peroxidase and alkaline phosphatase activities was accomplished with 0.5% H_2O_2 and enzymatic inhibitors (Dako). After addition of the anti-CD68 antibody (1/100 dilution) for 30 minutes, tissue was incubated with dextran polymer–conjugated horseradish peroxidase–labeled antisera against murine and rabbit immunoglobulins, and CD68-specific staining detected with 3,3'-diaminobenzidine. After using the Doublestain block reagent (Dako), tissue sections were sequentially incubated with the LSECtin-specific antisera (1/500 dilution for 30 minutes) and dextran polymer–conjugated, alkaline phosphatase–labeled antisera against murine and rabbit immunoglobulins. LSECtin staining was visualized with Permanent Red, and samples were later counterstained in hematoxylin.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation assays were performed using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Charlottesville, VA) as described.[26] For PCR detection of the LSECtin promoter, the oligonucleotides 5′-CCAAGCTTGGTGACTAAGCTCCAAAGAGAAG-3′ and 5′-GGGGTACCCGATGCAGGCACCCAGTCC-3′ were used, which together amplify a 312-bp and 263-bp fragment spanning from −296/−247 to +16. Immunoprecipitating antibodies included affinity-purified rabbit polyclonal antibody against human PU.1 (*sc-352*; Santa Cruz Biotechnology) and purified polyclonal rabbit immunoglobulin G as a control.

Quantitative Real-Time Reverse-Transcription PCR. Oligonucleotides for LSECtin and GAPDH genes were designed according to the Roche software for quantitative real-time PCR. Total RNA from MDDCs and THP-1 cells was extracted using the RNAeasy kit (Qiagen) and retrotranscribed and amplified using the Universal Human Probe Roche library (Roche Diagnostics). Assays were made in triplicate, and the results were normalized according to the expression levels of GAPDH. Results were processed with the BioRad iQ5 2.0 software and were expressed relative to the mRNA level of control or untreated samples (relative mRNA level).

Results

Expression of LSECtin in the Human MUTZ-3 Myeloid Cell Line. We have reported the expression of LSECtin in human monocyte-derived macrophages and DCs.[5] To further extend these findings, the presence of the lectin was analyzed in the human CD34+ acute myeloid leukemia cell line MUTZ-3,[23,28,29] which exhibits the capability to differentiate toward DCs.[28] As shown in Fig. 1A, LSECtin mRNA was barely detectable in MUTZ-3 cells grown in the continuous presence of granulocyte-macrophage colony-stimulating factor, but was greatly increased upon IL-4–mediated dendritic differentiation. Quantitative reverse-transcription PCR (RT-PCR) revealed that the level of LSECtin mRNA increased more than 100 times after addition of IL-4 (Fig. 1B). Moreover, LSECtin protein was also detected in IL-4–treated MUTZ-3 cells (Fig. 1C). Therefore, LSECtin is expressed in human myeloid cells with the capability to acquire a DC-like phenotype.

Cellular Distribution of LSECtin in Liver Cells. Because LSECtin was originally reported as a liver and lymph node sinusoidal-specific molecule,[4] we decided to determine the identity of LSECtin-expressing cells within hepatic tissue. The LSECtin-specific ADS1 and ADS4 antisera stained cells with a dendritic-like appearance within liver sinusoids, a morphology consistent with Kupffer cells (Fig. 2). Parallel analysis with myeloid cell–specific and endothelial cell–specific markers revealed that anti-CD31 exclusively stained sinusoidal endothelial cells, whereas anti-CD68 and anti-PU.1 antibodies only marked intrasinusoidal Kupffer cells (Fig. 2). Comparison of the staining patterns revealed that LSECtin-specific antisera recognized Kupffer cells as well as other cells lining the hepatic sinusoids (Fig. 2), suggesting that LSECtin can be found in both Kupffer cells and other cells in

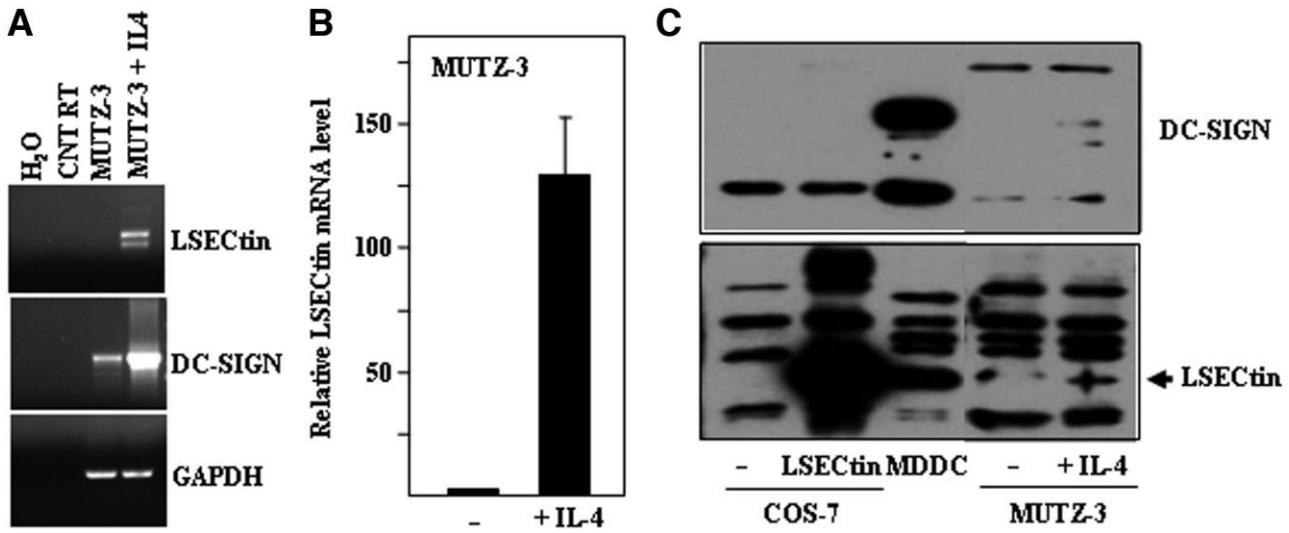

Fig. 1. Expression of LSECtin in the MUTZ-3 cell line. (A) Detection of LSECtin, DC-SIGN, and GAPDH mRNA in untreated and IL-4–treated MUTZ-3 via conventional RT-PCR. The results of control experiments without RNA (H_2O) or without reverse-transcriptase (CNT RT) are shown in each case. (B) Relative levels of LSECtin mRNA in untreated (−) and IL-4–treated MUTZ-3 cells via quantitative RT-PCR, after normalization for the levels of 18S RNA. Determination was performed in triplicate, and the mean and standard deviation is shown. (C) Detection of DC-SIGN and LSECtin protein expression in untreated (−) and IL-4–treated MUTZ-3 cells via western blotting, using a polyclonal antiserum against their corresponding neck regions. For control purposes, the expression of both lectins in MDDCs and untransfected or LSECtin-transfected COS-7 is shown.

the sinusoid wall. The presence of LSECtin on CD68+ macrophages was also suggested upon analysis of human lymph node sections, where LSECtin staining differed from that of anti-CD31 and anti–factor VIII (endothelium-specific) and resembled the tissue staining pattern yielded by a factor XIII–specific antibody (macrophage/DC-specific) (Fig. 3A). Moreover, LSECtin-positive cells were preferentially observed in lymph node areas enriched in CD68+ macrophages (Fig. 3B-C). Double-labeling experiments confirmed the presence of LSECtin in CD68+ cells (Fig. 4), indicating the presence of LSECtin in Kupffer cells. Further support for the presence of LSECtin in Kupffer cells was obtained via RT-PCR on RNA isolated from human hepatocytes, sinusoidal endothelial cells, Ito cells, and Kupffer cells. LSECtin mRNA could be exclusively amplified from Kupffer RNA, which showed considerably higher levels of both CD68 and PU.1 transcripts than the other cell types (Fig. 4B). Therefore, immunohistochemistry on human liver sections and RT-PCR on isolated hepatic cell types revealed that LSECtin is preferentially expressed by myeloid CD68+ PU.1+ Kupffer cells in the human liver.

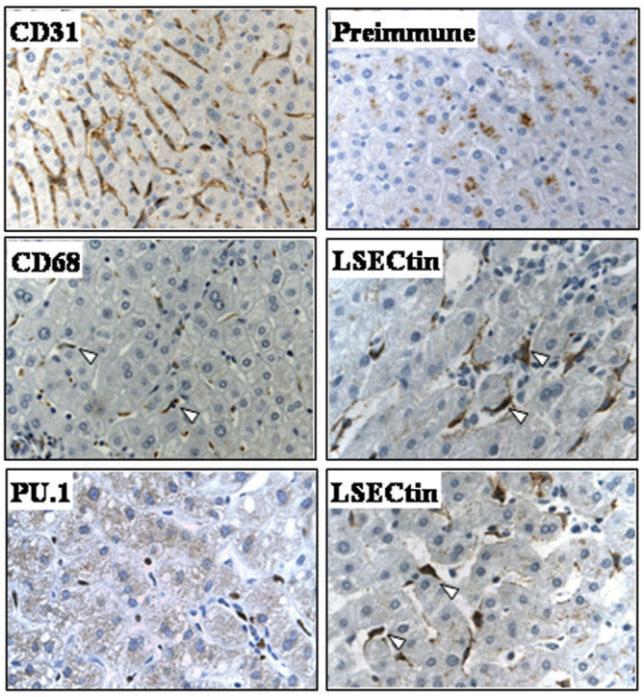

Fig. 2. LSECtin expression in human liver. Immunolocalization of LSECtin, PU.1, CD68, and CD31 on formalin-fixed, paraffin-embedded human liver tissue sections. LSECtin was detected with rabbit polyclonal antisera against its extracellular region (ADS4). The upper right panel shows the staining yielded by a preimmune rabbit antiserum. Arrowheads indicate the position of LSECtin- or CD68-positive cells in their respective panels.

Involvement of PU.1 in the Myeloid Expression of LSECtin. The DC-SIGN lectin is expressed in human myeloid cells, and its expression is regulated by PU.1,[26] a member of the Ets family of transcription factors. The expression of LSECtin in myeloid cells[5] and the similar pattern of staining yielded by anti-LSECtin and anti-PU.1 antibodies on human liver tissue (Fig. 2) suggest that PU.1 could participate in the restricted expression of LSECtin. This hypothesis was supported by the presence of three potential Ets-binding sequences (TTCCTTCCTTCC) at position −66 within the LSECtin gene proximal regulatory region (Fig. 5A). In fact, alignment of the

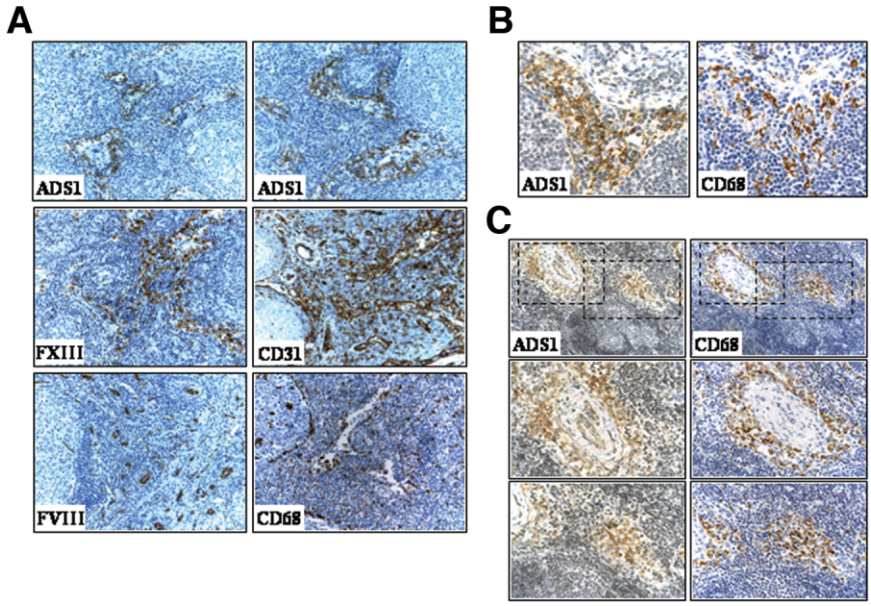

Fig. 3. LSECtin expression in human lymph nodes. (A) Immunolocalization of LSECtin, factor XIII (FXIII) CD68, CD31, and factor VIII (FVIII) on formalin-fixed, paraffin-embedded human lymph node tissue sections (magnification ×20). LSECtin was detected with rabbit polyclonal antisera against the neck domain (ADS1, upper panels). (B,C) Detection of LSECtin-positive cells in human lymph node tissue section areas enriched in CD68+ cells. (C) The areas marked by boxes are shown at a higher magnification in the lower panels (magnification ×40).

corresponding proximal regulatory regions of the human, murine, and rat LSECtin genes evidenced that this putative Ets-binding sequence is highly conserved (Fig. 5A). Evaluation of the activity of the LSECtin gene proximal regulatory region revealed that three distinct constructs exhibit promoter activity well above the promoterless pXP2 plamid (Fig. 5B). More importantly, the activity of the three constructs was significantly higher in cells of myeloid origin and with the ability to express LSECtin (THP-1) than in erythroleukemic (K562) and T lymphoid cells (Jurkat), which do not express LSECtin (Fig. 5B). Therefore, the proximal regulatory region of the LSECtin promoter preferentially functions within a myeloid context, providing a molecular explanation for the presence of the lectin in normal and leukemic myeloid cells. The relevance of the −66 putative Ets-binding element in LSECtin promoter activity was evaluated after mutation of the three putative Ets cognate sequences in

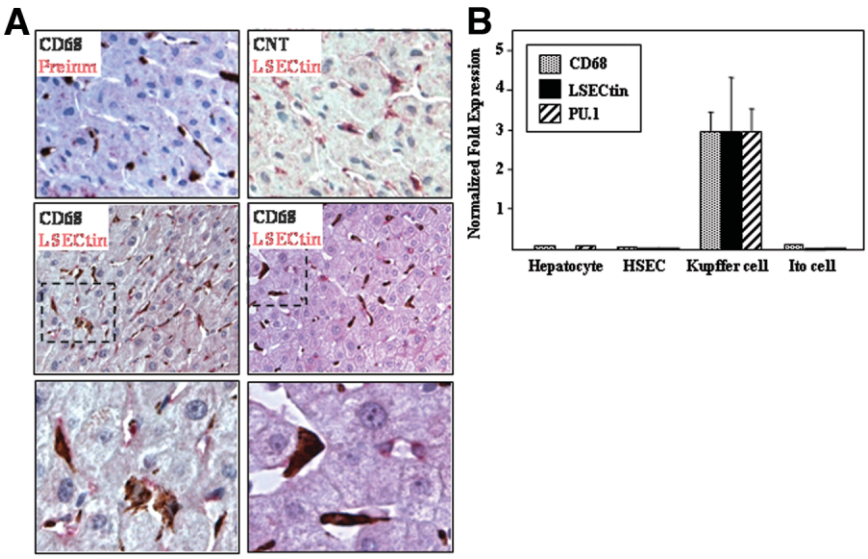

Fig. 4. Coexpression of LSECtin and the CD68 myeloid-specific marker in human liver tissue sections. (A) Simultaneous immunolocalization of LSECtin (red) and CD68 (brown) on formalin-fixed, paraffin-embedded human liver tissue sections (magnification ×40). LSECtin was detected with rabbit polyclonal antisera against the extracellular region (ADS4), and CD68 was detected with the PG-M1 monoclonal antibody. Two different preparations are shown (middle panels). The areas marked by boxes are shown in the lower panels at higher magnification (magnification ×100). The staining yielded by each individual antibody in the presence of the corresponding negative controls is shown in the upper panels. (B) Relative levels of PU.1, CD68, and LSECtin mRNA in human hepatocytes, sinusoidal endothelial cells (HSEC), Kupffer cells, and Ito cells via quantitative RT-PCR after normalization for the levels of GAPDH RNA. Determination was performed in triplicate, and the mean and standard deviation is shown.

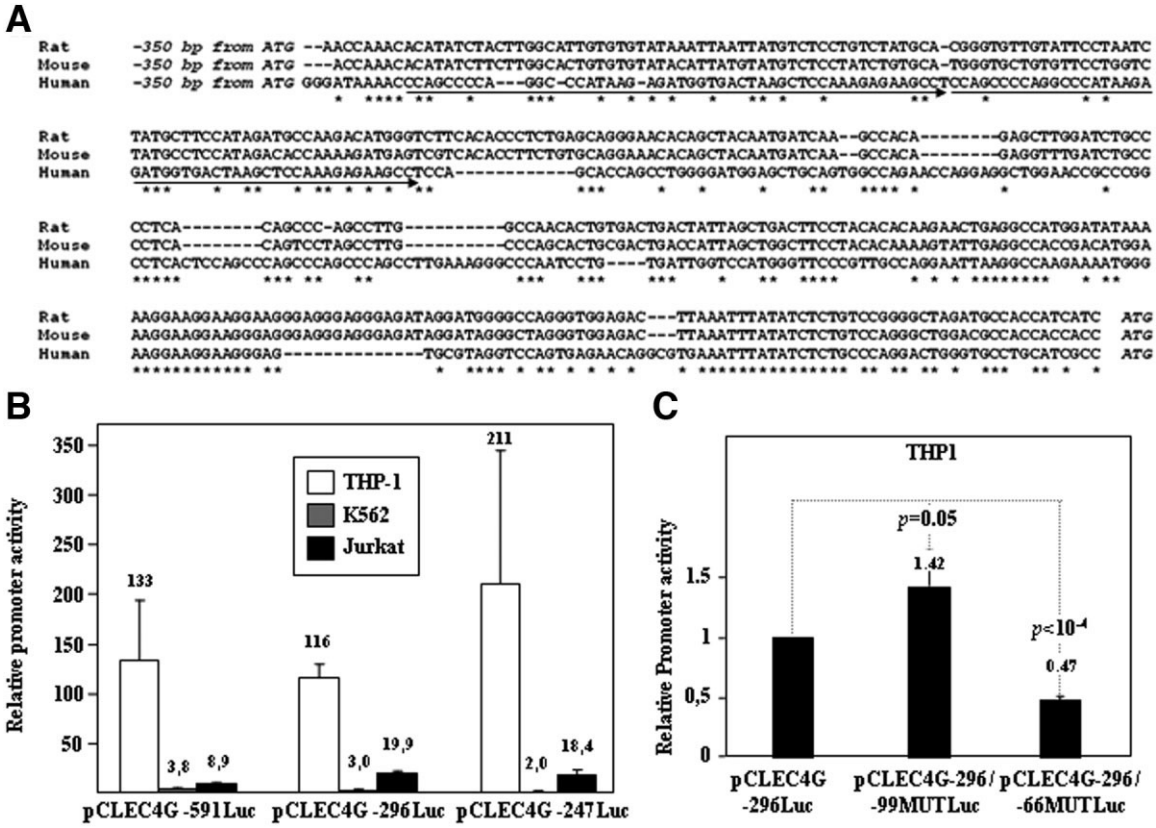

Fig. 5. Structural and functional analysis of the LSECtin gene proximal regulatory region. (A) Sequence alignment of the proximal regulatory regions (from −350 to the translation initiation site) of the human, murine, and rat LSECtin genes. Identities are shown by asterisks below the sequences. The position of the 48-nucleotide direct repeats within the human LSECtin promoter is indicated by arrows below the sequence. (B) THP-1, K562, and Jurkat cells were transfected with the indicated reporter plasmids, and luciferase activity was determined after 24 hours. Promoter activity is expressed relative to the activity produced by the promoterless pXP2 plasmid in each cell type and after normalization for transfection efficiency. Data represent the mean ± SD of four independent experiments using two different DNA preparations. (C) THP-1 cells were transfected with the indicated reporter plasmids, and luciferase activity was determined after 24 hours. Promoter activity is expressed relative to the activity produced by the wild-type pCLEC4G −296Luc construct after normalization for transfection efficiency. Data represent the mean ± SD of four independent experiments.

the context of the pCLEC4G −296Luc construct. As shown in Fig. 5C, pCLEC4G-296/-66MUTLuc construct exhibited significantly lower activity in the THP-1 myeloid cell line than the wild-type pCLEC4G −296Luc construct ($P < 10^{-4}$), while mutation of the putative Ets-binding site at −99 did not affect LSECtin proximal promoter activity (Fig. 5C). These results indicate that the preferential activity of the LSECtin gene regulatory region in myeloid cells is partly dependent on the integrity of the sequence around −66, which includes three potential binding sites for Ets family members.

Next, because PU.1 is a myeloid-restricted Ets transcription factor, we evaluated its ability to modulate the function of the LSECtin proximal promoter. In agreement with its preferential activity in myeloid cells, both Myb and RUNX3 transactivated the LSECtin regulatory region in a nonhematopoietic cellular context (Fig. 6A). More importantly, PU.1 overexpression enhanced the LSECtin promoter activity in the presence of RUNX3 (Fig. 6A), although it reduced the transactivation ability of c-Myb (Fig. 6A). Therefore, PU.1 positively modulates the LSECtin promoter activity in the presence of transcription factors which, like RUNX3, are preferentially expressed in hematopoietic cells. The positive regulatory action of PU.1 on the LSECtin promoter was mainly exerted via the −66 Ets element, because its mutation reduced RUNX3/PU.1 transactivation by more than 50% (Fig. 6B). Considering the above results, we decided to determine whether the LSECtin regulatory region was actually occupied by PU.1 *in vivo*. To that end, genomic DNA from LSECtin+ MDDCs was subjected to chromatin immunoprecipitation with an anti-PU.1 polyclonal antiserum. The LSECtin promoter was readily amplified in the anti-PU.1–precipitated DNA, whereas no amplification was detected in the DNA brought down by a control antibody or in the absence of antibody (Fig. 6C). The presence of two distinct bands derived from the LSECtin promoter (Fig. 6C) is explained by the presence

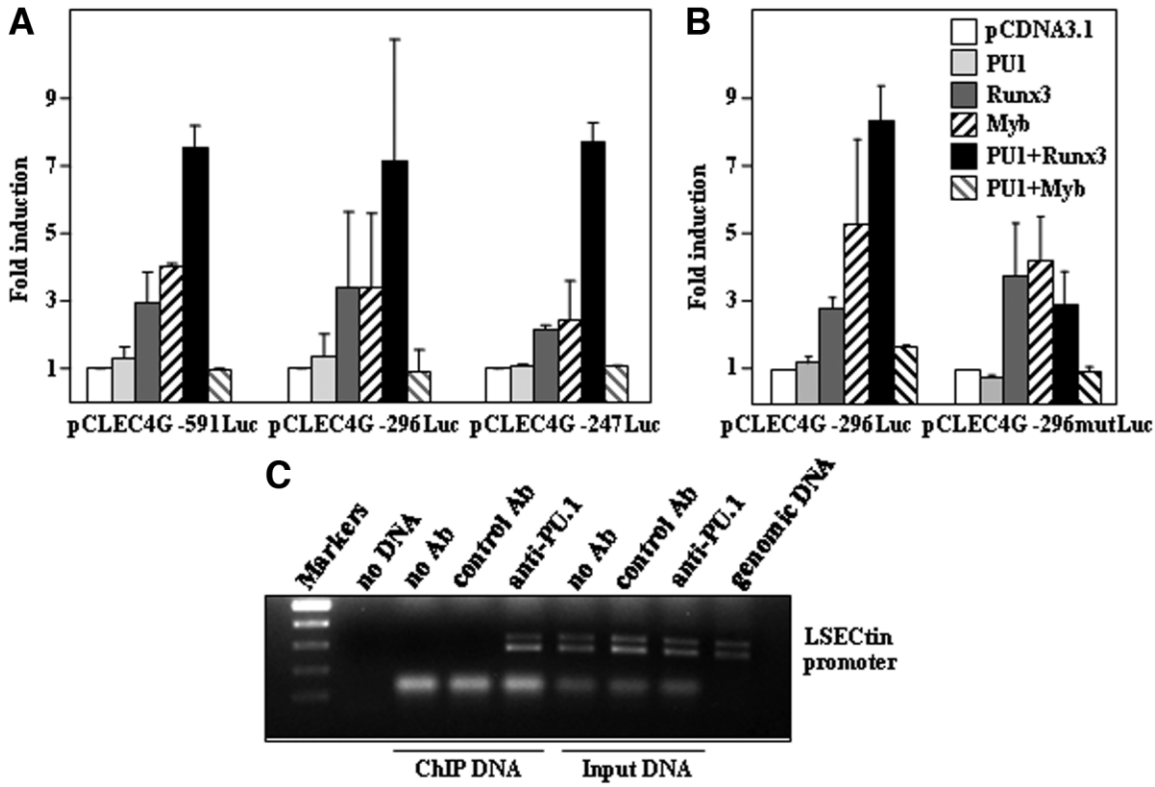

Fig. 6. PU.1 binds *in vivo* and modulates the activity of the LSECtin promoter *in vitro*. (A,B) NIH-3T3 cells were transfected with the indicated LSECtin promoter-based reporter plasmids in the presence of an empty expression vector (pCDNA3.1) or expression vectors for RUNX3, Myb, or PU.1, either alone or in combination. For each individual reporter construct, fold induction represents the luciferase activity yielded by each expression vector combination relative to the activity produced by an identical amount of empty CMV-0 plasmid. In all cases, total DNA was kept constant (1.5 μg) by adding CMV-0 plasmid DNA, and luciferase activity was determined after 24 hours. Data represent the mean $\pm$ standard deviation of three independent experiments using two different DNA preparations. (C) *In vivo* occupancy of the LSECtin proximal promoter by PU.1. Shown are chromatin immunoprecipitations on immature monocyte-derived DCs using an affinity-purified polyclonal antiserum specific for PU.1, a nonspecific affinity-purified antiserum (control Ab), or no antibody (no Ab). Immunoprecipitated chromatin was analyzed via PCR using a pair of LSECtin promoter-specific primers that together amplify 312-bp and 263-bp fragments spanning from $-296/-247$ to $+16$, since the forward primer anneals to a 48-nucleotide direct repeat. Input DNA lanes represent the PCR analysis performed on DNA from a 1:20 dilution of the starting sonicated lysate. Additional controls include amplification in the absence of DNA (no DNA) or amplification of human genomic DNA (genomic DNA).

of a direct repeat within the LSECtin proximal regulatory region (Fig. 5A). Therefore, LSECtin expression correlates with that of the PU.1 transcription factor, which binds to and enhances the activity of the LSECtin proximal regulatory region in LSECtin-expressing cells of myeloid origin.

Finally, the influence of PU.1 on LSECtin mRNA expression level was assessed by a knockdown approach on LSECtin-expressing cells. Nucleofection of a PU-1–specific siRNA in THP-1 cells, which reduced PU.1 levels by more than 50% (Fig. 7A), led to down-modulation of the LSECtin mRNA levels as determined via quantitative RT-PCR (Fig. 7A). Moreover, nucleofection of the PU-1–specific siRNA also reduced the steady-state level of LSECtin mRNA in MDDCs (Fig. 7B). Therefore, decreasing PU.1 expression had a direct impact on the LSECtin RNA levels in both cell types, thus confirming the involvement of PU.1 in LSECtin gene expression.

Discussion

We herein provide evidence that the LSECtin pathogen-attachment lectin, originally described as a liver/lymph node sinusoidal-specific molecule,[4] is expressed in cells of myeloid origin within the liver, and that the PU.1 transcription factor contributes to its restricted expression. The myeloid expression of LSECtin allows the definition of a chromosome 19 cluster of lectin-encoding genes (CD23, DC-SIGN, and LSECtin) which mediate antigen capture for subsequent presentation during immune responses. The presence of DC-SIGN[30,31] and LSECtin (this study) on human Kupffer cells indicates that at least two members of this gene cluster are actively involved in scavenging and antigen capture by liver myeloid cells, and might therefore contribute to the establishment of the peripheral tolerance. As in the case of DEC-205[32] and DC-SIGN,[33] the generation of LSEC-

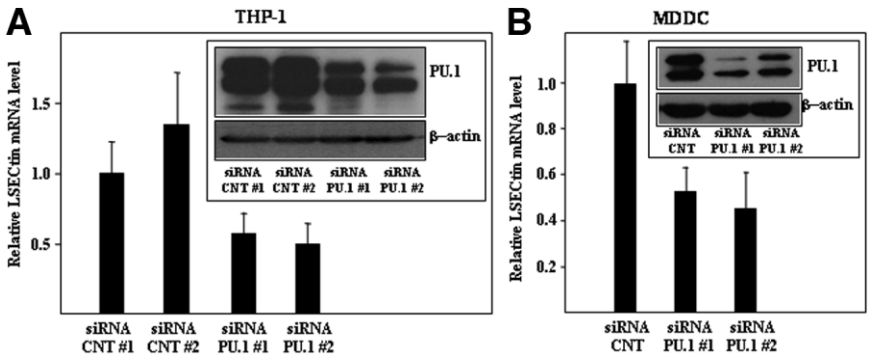

Fig. 7. Knockdown on PU.1 results in diminished LSECtin mRNA levels. (A) THP-1 cells or (B) MDDCs were nucleofected with either siRNA for PU.1 (siRNA PU.1) or a control siRNA (siRNA CNT). After 24 hours, total RNA was isolated and LSECtin mRNA was measured via quantitative RT-PCR. Each experiment was performed in duplicate, and both experiments are shown. Results are expressed as the relative LSECtin mRNA level, which indicates the level of LSECtin mRNA in each sample relative to its level in control siRNA-nucleofected cells. To confirm siRNA efficiency in each individual experiment, one-third of the cells were lysed and underwent western blotting (inserts), using a polyclonal antiserum against human PU.1 and a β-actin–specific monoclonal antibody for loading control purposes.

tin-specific reagent will be a very useful tool to evaluate its potential role as a tolerance-promoting capturing receptor. On the other hand, the pattern of expression of DC-SIGN and LSECtin, together with the presence of DC-SIGNR on liver sinusoidal endothelial cells,[3,17] provides support for a role of this family of C-type lectins in the scavenging function of the liver. Such a function can be inferred from a large list of pathogenic and endogenous ligands of DC-SIGN[33] and by the restricted sugar specificity of LSECtin.[18]

Kupffer cells constitute more than 80% of the tissue macrophages present in the body and are the first macrophage population exposed to material derived from the gastrointestinal tract.[19] The removal of bacteria-derived products and microbial debris by Kupffer cells is mediated through a large array of scavenging molecules on their cell surface, and LSECtin could be an additional molecule engaged in their clearance function. The ability of LSECtin to interact with virally encoded molecules and glycoproteins with truncated complex and hybrid N-linked glycans[18] suggests its role as a scavenging molecule. Moreover, biochemical studies using recombinant LSECtin indicates its ability to specifically interact with serum proteins (data not shown). On the other hand, and by analogy with DC-SIGN,[34] LSECtin could be also implicated in cell–cell adhesion. If so, LSECtin might participate in either attachment of Kupffer cells to liver sinusoidal cells or in Kupffer cell interactions with Ito cells, a process that has proven relevant during liver tissue injury and repair.[35,36]

The data in the present manuscript may help resolving the issue of the cellular distribution of LSECtin in liver tissue. The immunochemical colocalization of CD68 and LSECtin in Kupffer cells is compatible with the PU.1 dependency of the LSECtin expression, since PU.1 is expressed by Kupffer cells,[37] which are characterized by the expression of the PU.1-dependent CD68 gene.[38,39] However, immunohistochemistry also revealed the existence of LSECtin-expressing cells that were devoid of CD68. Therefore, and based on data from previous reports, it is possible that LSECtin can be expressed by myeloid (Kupffer) and nonmyeloid (sinusoidal endothelial) cells. A similar conclusion has been reached for a number of scavenger and lectin receptors expressed on human lymph node sinuses.[40] Along this line, the mannose receptor, whose expression in myeloid cells is PU.1-dependent,[41] is expressed on both liver sinusoidal endothelial cells and Kupffer cells,[42] and its absence from myeloid cells appears to trigger a compensatory enhancement of its expression on liver sinusoidal cells.[43] Therefore, the expression of LSECtin on two distinct liver cell types might be indicative that its sugar-binding specificity and internalization capability contributes to both arms of the innate response: scavenging and antigen-presentation.

Regarding the regulation of LSECtin expression, the inducibility of CD23, DC-SIGN, and LSECtin by IL-4[5,7,9] suggests the presence of a common mechanism for their expression in myeloid cells. We have previously demonstrated that the activity of the DC-SIGN promoter is regulated by the myeloid-specific transcription factor PU.1, whose level of expression is critically dependent on the presence of IL-4.[26] The present study demonstrates that PU.1 is also involved in the expression of LSECtin, since (1) PU.1 is constitutively bound to the LSECtin proximal promoter *in vivo*; (2) PU.1 potentiates the activity of the LSECtin regulatory region; and (3) PU.1 down-modulation translates into diminished levels of LSECtin mRNA in leukemic and primary LSECtin+ cells. Although induced by IL-4,[7] the participation of PU.1 in the expression of CD23 has not been reported,

but it is tempting to hypothesize that PU.1 might also regulate CD23 expression, especially considering their overlapping patterns of expression. If this is the case, PU.1 would become an essential component for the expression of the three antigen-capturing and pathogen-attachment lectins encoded within the chromosome 19p13.2 gene cluster.

However, although PU.1 appears as a requirement for DC-SIGN and LSECtin expression, it is not sufficient for their expression, because several PU.1-expressing myeloid cell types are devoid of both lectins (e.g., neutrophils). It is therefore possible that a threshold level of PU.1 is required for both lectins to be expressed, and that IL-4 might induce their expression primarily through an increase in PU.1 expression. Alternatively, IL-4 might activate a signaling pathway/transcription factor that ultimately synergizes with PU.1 for the induction of both lectins. An obvious potential candidate would be STAT6, which functionally collaborates with PU.1, appears to bind to the CD23 gene regulatory region, and whose transcriptional activity is induced by IL-4.[44] However, no obvious STAT6-binding sequences are located within the LSECtin proximal regulatory region, and STAT6 does not influence the activity of the DC-SIGN promoter in spite of the presence of three elements with STAT6-binding ability *in vitro* (data not shown). A second potential factor that might participate in the IL-4 inducibility of LSECtin expression is peroxisome proliferator-activated receptor gamma (PPARγ), whose activity and expression is controlled by IL-4.[45] In fact, PPARγ expression is currently considered as a hallmark for the alternative activation of macrophages,[46,47] which results in the induction of both LSECtin[5] and DC-SIGN.[10] However, the synthetic PPARγ ligand GW7845, or the PPAR antagonist inhibitor GW9662, did not modify the basal or inducible expression of either LSECtin or DC-SIGN (data not shown), thus arguing against a role for PPARγ in the control of the expression of both lectins.

In conclusion, we report that the pathogen attachment C-type lectin receptor LSECtin is expressed in Kupffer cells, and that its expression is controlled by the PU.1 transcription factor. Unlike DC-SIGN or DC-SIGNR, an obvious murine orthologue of LSECtin gene (*Clec4g*) has been mapped between the DC-SIGN–related *cd209a* and *cd23* murine genes. Given its recognition specificity and internalization capability, LSECtin constitutes an additional scavenger molecule present on liver myeloid cells whose specific range of endogenous and pathogenic ligands needs to be determined. The conservation of the LSECtin gene in other mammals suggests that it might play a relevant and nonredundant function and will allow the generation of animal models in which both issues can be addressed.

References

1. Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 2000;100:575-585.
2. Soilleux EJ, Barten R, Trowsdale J. DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13. J Immunol 2000;165:2937-2942.
3. Bashirova AA, Geijtenbeek TB, van Duijnhoven GC, van Vliet SJ, Eilering JB, Martin MP, et al. A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. J Exp Med 2001;193:671-678.
4. Liu W, Tang L, Zhang G, Wei H, Cui Y, Guo L, et al. Characterization of a novel C-type lectin-like gene, LSECtin: demonstration of carbohydrate binding and expression in sinusoidal endothelial cells of liver and lymph node. J Biol Chem 2004;279:18748-18758.
5. Dominguez-Soto A, Aragoneses-Fenoll L, Martin-Gayo E, Martinez-Prats L, Colmenares M, Naranjo-Gomez M, et al. The DC-SIGN-related lectin LSECtin mediates antigen capture and pathogen binding by human myeloid cells. Blood 2007;109:5337-5345.
6. Gramberg T, Hofmann H, Moller P, Lalor PF, Marzi A, Geier M, et al. LSECtin interacts with filovirus glycoproteins and the spike protein of SARS coronavirus. Virology 2005;340:224-236.
7. Kijimoto-Ochiai S. CD23 (the low-affinity IgE receptor) as a C-type lectin: a multidomain and multifunctional molecule. Cell Mol Life Sci 2002;59:648-664.
8. Yu P, Kosco-Vilbois M, Richards M, Kohler G, Lamers MC. Negative feedback regulation of IgE synthesis by murine CD23. Nature 1994;369:753-756.
9. Relloso M, Puig-Kroger A, Pello OM, Rodriguez-Fernandez JL, de la Rosa G, Longo N, et al. DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. J Immunol 2002;168:2634-2643.
10. Puig-Kroger A, Serrano-Gomez D, Caparros E, Dominguez-Soto A, Relloso M, Colmenares M, et al. Regulated expression of the pathogen receptor dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin in THP-1 human leukemic cells, monocytes, and macrophages. J Biol Chem 2004;279:25680-25688.
11. Soilleux EJ, Morris LS, Leslie G, Chehimi J, Luo Q, Levroney E, et al. Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. J Leukoc Biol 2002;71:445-457.
12. Engering A, Van Vliet SJ, Geijtenbeek TB, Van Kooyk Y. Subset of DC-SIGN(+) dendritic cells in human blood transmits HIV-1 to T lymphocytes. Blood 2002;100:1780-1786.
13. van Lent PL, Figdor CG, Barrera P, van Ginkel K, Sloetjes A, van den Berg WB, et al. Expression of the dendritic cell-associated C-type lectin DC-SIGN by inflammatory matrix metalloproteinase-producing macrophages in rheumatoid arthritis synovium and interaction with intercellular adhesion molecule 3-positive T cells. Arthritis Rheum 2003;48:360-369.
14. Engering A, van Vliet SJ, Hebeda K, Jackson DG, Prevo R, Singh SK, et al. Dynamic populations of dendritic cell-specific ICAM-3 grabbing nonintegrin-positive immature dendritic cells and liver/lymph node-specific ICAM-3 grabbing nonintegrin-positive endothelial cells in the outer zones of the paracortex of human lymph nodes. Am J Pathol 2004;164:1587-1595.
15. Granelli-Piperno A, Pritsker A, Pack M, Shimeliovich I, Arrighi JF, Park CG, et al. Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin/CD209 is abundant on macrophages in the normal human lymph node and is not required for dendritic cell stimulation of the mixed leukocyte reaction. J Immunol 2005;175:4265-4273.

16. Mukhtar M, Harley S, Chen P, BouHamdan M, Patel C, Acheampong E, et al. Primary isolated human brain microvascular endothelial cells express diverse HIV/SIV-associated chemokine coreceptors and DC-SIGN and L-SIGN. Virology 2002;297:78-88.
17. Pohlmann S, Soilleux EJ, Baribaud F, Leslie GJ, Morris LS, Trowsdale J, et al. DC-SIGNR, a DC-SIGN homologue expressed in endothelial cells, binds to human and simian immunodeficiency viruses and activates infection in trans. Proc Natl Acad Sci U S A 2001;98:2670-2675.
18. Powlesland AS, Fisch T, Taylor ME, Smith DF, Tissot B, Dell A, et al. A novel mechanism for LSECtin binding to Ebola virus surface glycoprotein through truncated glycans. J Biol Chem 2007.
19. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. Liver Int 2006;26:1175-1186.
20. Naito M, Hasegawa G, Ebe Y, Yamamoto T. Differentiation and function of Kupffer cells. Med Electron Microsc 2004;37:16-28.
21. Laskin DL, Weinberger B, Laskin JD. Functional heterogeneity in liver and lung macrophages. J Leukoc Biol 2001;70:163-170.
22. Kolios G, Valatas V, Kouroumalis E. Role of Kupffer cells in the pathogenesis of liver disease. World J Gastroenterol 2006;12:7413-7420.
23. Quentmeier H, Duschl A, Hu ZB, Schnarr B, Zaborski M, Drexler HG. MUTZ-3, a monocytic model cell line for interleukin-4 and lipopolysaccharide studies. Immunology 1996;89:606-612.
24. Masterson AJ, Sombroek CC, De Gruijl TD, Graus YM, van der Vliet HJ, Lougheed SM, et al. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. Blood 2002;100:701-703.
25. Larsson K, Lindstedt M, Borrebaeck CA. Functional and transcriptional profiling of MUTZ-3, a myeloid cell line acting as a model for dendritic cells. Immunology 2006;117:156-166.
26. Dominguez-Soto A, Puig-Kroger A, Vega MA, Corbi AL. PU.1 regulates the tissue-specific expression of dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin. J Biol Chem 2005;280: 33123-33131.
27. Nordeen SK. Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 1988;6:454-458.
28. Santegoets SJ, Masterson AJ, van der Sluis PC, Lougheed SM, Fluitsma DM, van den Eertwegh AJ, et al. A CD34(+) human cell line model of myeloid dendritic cell differentiation: evidence for a CD14(+)CD11b(+) Langerhans cell precursor. J Leukoc Biol 2006;80:1337-1344.
29. Santegoets SJ, Schreurs MW, Masterson AJ, Liu YP, Goletz S, Baumeister H, et al. In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. Cancer Immunol Immunother 2006;55:1480-1490.
30. Schwartz AJ, Alvarez X, Lackner AA. Distribution and immunophenotype of DC-SIGN-expressing cells in SIV-infected and uninfected macaques. AIDS Res Hum Retroviruses 2002;18:1021-1029.
31. Pereira CF, Torensma R, Hebeda K, Kretz-Rommel A, Faas SJ, Figdor CG, et al. In vivo targeting of DC-SIGN-positive antigen-presenting cells in a nonhuman primate model. J Immunother 2007;30:705-714.
32. Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, et al. In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 2004;199:815-824.
33. Tacken PJ, de Vries IJ, Gijzen K, Joosten B, Wu D, Rother RP, et al. Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. Blood 2005;106:1278-1285.
34. Ludwig IS, Geijtenbeek TB, van Kooyk Y. Two way communication between neutrophils and dendritic cells. Curr Opin Pharmacol 2006;6:408-413.
35. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 2005;115:56-65.
36. Friedman SL. Mac the knife? Macrophages—the double-edged sword of hepatic fibrosis. J Clin Invest 2005;115:29-32.
37. Rai RM, Loffreda S, Karp CL, Yang SQ, Lin HZ, Diehl AM. Kupffer cell depletion abolishes induction of interleukin-10 and permits sustained overexpression of tumor necrosis factor alpha messenger RNA in the regenerating rat liver. HEPATOLOGY 1997;25:889-895.
38. Li AC, Guidez FR, Collier JG, Glass CK. The macrosialin promoter directs high levels of transcriptional activity in macrophages dependent on combinatorial interactions between PU.1 and c-Jun. J Biol Chem 1998;273: 5389-5399.
39. O'Reilly D, Quinn CM, El-Shanawany T, Gordon S, Greaves DR. Multiple Ets factors and interferon regulatory factor-4 modulate CD68 expression in a cell type-specific manner. J Biol Chem 2003;278: 21909-21919.
40. Martens JH, Kzhyshkowska J, Falkowski-Hansen M, Schledzewski K, Gratchev A, Mansmann U, et al. Differential expression of a gene signature for scavenger/lectin receptors by endothelial cells and macrophages in human lymph node sinuses, the primary sites of regional metastasis. J Pathol 2006;208:574-589.
41. Eichbaum Q, Heney D, Raveh D, Chung M, Davidson M, Epstein J, et al. Murine macrophage mannose receptor promoter is regulated by the transcription factors PU.1 and SP1. Blood 1997;90:4135-4143.
42. Lee SJ, Evers S, Roeder D, Parlow AF, Risteli J, Risteli L, et al. Mannose receptor-mediated regulation of serum glycoprotein homeostasis. Science 2002;295:1898-1901.
43. Linehan SA, Weber R, McKercher S, Ripley RM, Gordon S, Martin P. Enhanced expression of the mannose receptor by endothelial cells of the liver and spleen microvascular beds in the macrophage-deficient PU.1 null mouse. Histochem Cell Biol 2005;123:365-376.
44. Hebenstreit D, Wirnsberger G, Horejs-Hoeck J, Duschl A. Signaling mechanisms, interaction partners, and target genes of STAT6. Cytokine Growth Factor Rev 2006;17:173-188.
45. Huang JT, Welch JS, Ricote M, Binder CJ, Willson TM, Kelly C, et al. Interleukin-4-dependent production of PPAR-gamma ligands in macrophages by 12/15-lipoxygenase. Nature 1999;400:378-382.
46. Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S, et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007;6:137-143.
47. Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, et al. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. Nature 2007;447: 1116-1120.