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Growth Factors, Cytokines, Cell Cycle Molecules

Identification and Functional Characterization of the Hepatic Stellate Cell CD38 Cell Surface Molecule

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The activation of hepatic stellate cells (HSCs) is a critical event in hepatic fibrosis, because these cells are the main producers of extracellular matrix proteins in the liver and contribute to the modulation of inflammatory responses via the secretion of several cytokines and the expression of adhesion molecules. The goal of the present study was to characterize cell surface proteins that regulate HSC activation. To this end, a panel of monoclonal antibodies (mAbs) was generated. mAb 14.27 recognized a protein of 45 kd that was highly expressed on HSCs. Affinity purification of this protein followed by sequencing revealed that protein to be CD38. We subsequently demonstrated that CD38 was constitutively expressed by HSCs and that its expression increased after in vitro and in vivo activation. mAb 14.27 induced an increase in cytosolic Ca^{2+} levels in HSCs, showing that it functions as an agonistic antibody. Moreover, the effects mediated by the CD38 mAb included induction of the proinflammatory cytokine interleukin-6 and up-regulation of the adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and neural cell adhesion molecule. Collectively, our data suggest that CD38 can act as a regulator of HSC activation and effector functions. *(Am J Pathol 2007, 170:176–187; DOI: 10.2353/ajpath.2007.051212)*

Hepatic stellate cells (HSCs), also known as Ito cells, lipocytes, or fat-storing cells, are nonparenchymal cells that represent 5% of the resident cells in the liver. HSCs are characterized by the presence of intracellular lipid vacuoles containing vitamin A and long dendritic-like cytoplasmic prolongations that wrap the sinusoids. HSCs play a role in several specialized functions in normal liver, including remodeling of the extracellular matrix, storage of retinoids, secretion of a variety of cytokines, and control of the diameter of the sinusoids.[1,2] In the normal liver, most HSCs are in a resting state; however, in response to liver injury, these cells undergo an activation process that induces changes in their structure and function. Functional changes include the expression of cell surface receptors, increased cell proliferation, and the augmentation in synthesis of extracellular matrix (ECM) proteins. In fact, activated HSCs are the primary source of the ECM proteins responsible for liver fibrosis, which can impair normal liver function and ultimately lead to cirrhosis and organ failure.[3–5] Moreover, HSCs can contribute to hepatic inflammation by their ability to secrete and respond to a wide range of cytokines and growth factors.[6,7] Studies conducted in several laboratories have shown the importance of hepatic stellate cells in the pathophysiology of the liver response to injury.[8]

Based on their expression of α-smooth muscle actin and such intermediate filaments as vimentin and desmin, HSCs have been regarded as mesenchymal cells.[9–13] On the other hand, HSCs express glial fibrillary acidic protein (GFAP), nestin, neural cell adhesion molecule (N-CAM) synaptophysin, and neurotrophins consistent with a neural/neuroendocrine origin.[13–17]

Several molecules have been identified on the cell surface of HSCs including growth factor receptors (transferrin receptor, platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor receptors), adhesion molecules of the immunoglobulin superfamily [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and N-CAM-1] and integrins (α1-β1, α2-β1, and α6-β4), tyrosine kinase receptors, seven transmembrane domain receptors (endothelin-1, thrombin, angiotensin-II, and vasopressin receptors), and the extracellular P2Y nucleotide recep-

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tor.[7,18–26] These cell surface molecules are differentially expressed depending on the activation and differentiation stage of the HSCs. Because of their role in the regulation of HSC functions, such as proliferation, migration, ECM protein synthesis, and apoptosis, these molecules represent potential targets for liver disease therapy.[27]

To identify additional cell surface molecules involved in HSC function, we have generated monoclonal antibodies (mAbs) against molecules expressed on the membrane of rat HSCs. This approach yielded a large panel of mAbs, including mAb 14.27. Here, we report that this mAb specifically recognizes rat CD38, a type II transmembrane glycoproteins originally identified as an activation antigen of T and B cells. It is expressed on several leukocytes and early hematopoietic precursor cells. This molecules is also expressed in nonhematopoietic cells, including epithelial cells and astrocytes.[28] CD38 is an ectoenzyme that catalyzes the synthesis of cyclic ADP ribose (a potent second messenger for Ca^{2+} release) and a receptor that initiates transmembrane signaling on engagement with its counterreceptor CD31 or with agonistic mAbs.[29] The effects mediated by CD38 include the production of proinflammatory cytokines, proliferation, and protection from apoptosis in lymphocytes.[30]

In this study, we identified CD38 as a novel membrane molecule of HSCs and characterized its expression in rat HSCs *in vitro* and *in vivo*. In addition, we examined the biological responsiveness of HSCs to CD38 ligation. Thus, the results reported herein indicate that CD38 may be a key regulator of HSC activation and effector functions.

Materials and Methods

Isolation and Culture of HSCs

HSCs were isolated from rat livers as previously described.[31] In brief, livers of rat were perfused with solutions containing collagenase (A type; Boehringer Mannheim, Mannheim, Germany), pronase (Boehringer Mannheim), and DNase (grade II; Boehringer Mannheim). The digested liver was filtered through nylon gauze (100 μm; Becton, Dickinson and Company, San Jose, CA), and parenchymal hepatocytes were removed by centrifugation. The isolated HSCs were cultured with Iscove's modified Dulbecco's medium (Gibco-BRL, Gaithersburg, MD) with 10% fetal calf serum (Gibco-BRL).

Isolation of Liver Cells

Parenchymal Hepatocytes

Hepatocytes were isolated by *in situ* collagenase perfusion through the portal vein according to the method of Seglen with minor modifications.[31] In brief, livers were perfused with Hanks' balanced salt solution without calcium and magnesium and digested with collagenase (A type) (Boehringer Mannheim). The resultant digested liver was filtered through nylon gauze (100 μm) (Becton, Dickinson and Company). Parenchymal hepatocytes were collected in ice-cold Krebs buffer and centrifuged at 50 $\times$ *g* for 3 minutes. The obtained pellet contained the hepatocytes, whereas the supernatants were enriched in nonparenchymal cells. Hepatocytes were washed twice in cold Krebs buffer.

Nonparenchymal Cells

Endothelial and Kupffer cells were isolated as previously described.[32] In brief, supernatants containing nonparenchymal cells (obtained as described above) were centrifuged at 800 $\times$ *g* for 10 minutes at 4°C. The resulting pellet was resuspended in Dulbecco's phosphate-buffered saline (PBS) and then centrifuged at 800 $\times$ *g* for 20 minutes through a 25%/50% Percoll gradient at room temperature. The interface of the gradient containing Kupffer cells and sinusoidal endothelial cells was plated on coverslips in 24-well plates (Costar, Corning, NY) and incubated at 37°C for 30 minutes. Those cells that adhered to the dishes were characterized as Kupffer cells. The nonadherent cells were cultured for 2 days in the presence of rat vascular endothelial growth factor (R&D, Minneapolis, MN).

Monoclonal Antibody Production

mAbs reactive with HSC membrane antigens were generated by the fusion of NS-1 myeloma cells with splenocytes from mice immunized three times with freshly isolated HSCs. Sixteen hybridoma-producing antibodies against cell surface proteins were selected and subcloned by limiting dilution at least three times. Antibody isotypes were determined using a mouse mAb isotyping kit (Boehringer Mannheim). mAbs were purified using the Affi-Gel Protein A MAPS II kit (Bio-Rad, Hercules, CA) from concentrated supernatants obtained from hybridoma cultures in INTEGRA CL 1000 flasks (Integra Biosciences, Chur, Switzerland). mAbs were biotinylated using biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO).

Immunoprecipitation

HSCs cells contained in a 75-cm^2 flask were washed with PBS and surface labeled with 40 μl of Sulfo-NHS-Biotin (50 mg/ml; Sigma) for 25 minutes at 4°C. Biotin was removed, and cells were incubated for 10 minutes with RPMI (Invitrogen, Carlsbad, CA). Cells were washed twice with PBS and lysed in 1 ml of buffer containing 1% Nonidet P-40 and protease inhibitors. Immunoprecipitations were performed with 5 μg of mAb 14.27 as previously described.[33]

Protein Purification and Identification by Mass Spectrometry

Isolated HSCs were grown to confluence, washed twice with PBS, and lysed in 1 ml of buffer containing 1%

Nonidet P-40 and protease inhibitors. mAb 14.27 was covalently coupled to CNBr-activated Sepharose 4B (Amersham, Uppsala, Sweden) and used to affinity-purify the unknown cell surface antigen from HSC lysates. Unbound protein was then washed off with PBS containing 0.5 mol/L NaCl and 1% NP-40. Protein was eluted with 0.1% trifluoroacetic acid (Merck, Darmstadt, Germany). Elutes were vacuum-dried to remove trifluoroacetic acid and resuspended in sodium dodecyl sulfate (SDS) loading buffer (200 mmol/L Tris-HCL, pH 6.8, 30% glycerol, 6% SDS, and 0.06% bromphenol blue). The affinity chromatography-purified protein was resolved on SDS-polyacrylamide gel electrophoresis under nonreducing conditions and stained with silver (Merck). The protein band was excised from the gel with a scalpel, reduced with 10 mmol/L dithiothreitol, and alkylated with 55 mmol/L iodoacetamide (Sigma). The proteins were digested in gel with trypsin (Promega, Madison, WI) following standard procedures.[34,35] The protein was identified by peptide-mass fingerprinting using matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectroscopy. This was performed using a Voyager DE-PRO (Applied Biosystems, Warrington, UK) instrument in the reflectron mode. Spectra were externally mass calibrated using a standard peptide mixture. For the analysis, 0.5 μl of peptide extract and 0.5 μl of matrix (α-cyano-4-hydroxycinnamic acid, 5 mg/ml) were loaded in the MALDI plate. The software PROTEIN PROSPECTOR version 3.4.1 (UCSF Mass Spectrometry Facility, University of California, San Francisco, CA) was used for protein identification from the peptide-mass fingerprint. The Swiss-Prot (European Bioinformatics Institute, Heidelberg, Germany, updated 20 February 2002) database was used for the search.

Rat CD38 Cloning and Transfection

RNA was isolated from HSCs with Trizol (Sigma). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the SuperScript First-Strand kit (Invitrogen). The following specific primers for rat CD38 were used: 5′-ATGGCCAACTATGAATTTTCCCA-3′ sense and 5′-TGAGATCCAAGTCCTTCACACATTAAG-3′ antisense. The PCR product was cloned using the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen) and subsequently sequenced using the Big Dye Terminator v3.1 Cyclase Sequencing kit (Applied Biosystems). COS-7 cells (1×10^6) were seeded into 100-mm dishes and, 24 hours later, transfected with 15 μg of rat CD38 or HLy9 cDNA using LipofectAMINE (Invitrogen) following the manufacturer's instructions. Cells were incubated for 24 hours and stained with biotinylated CD38.14.27 or biotinlylated HLy9.1.84 mAbs followed by Streptavidin-PE (Becton, Dickinson and Company). Fluorescence was analyzed using a FACSCalibur (Becton, Dickinson and Company) flow cytometer equipped with CellQuest software. Fluorescence intensity was plotted on a log scale. A minimum of 5000 cells was acquired for each sample. The negative control was set at 5 mean fluorescence intensity.

Western Blot Analysis of CD38 in Cultured HSCs, Transfected COS Cells, and Liver Tissue

Samples from cultured HSCs, transfected COS cells, and rat liver were prepared in lysis buffer containing 1% Nonidet P-40 and the protease inhibitors leupeptin, pepstatin A, aprotinin, and phenylmethylsulfonyl fluoride. Aliquots of 25 or 100 μg of protein were run on a 12% sodium dodecyl sulfate-polyacrylamide gel in nonreduced conditions and transferred to a nitrocellulose membrane. The blots were subsequently blocked for 2 hours with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk and proved with 2 μg of mAb CD38.14.27 for 2 hours at room temperature, followed by an incubation with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Sigma) for 30 minutes at room temperature. After 30 minutes, immunodetection was performed using the ECL blotting detection system (Pierce, Rockford, IL).

Hepatic Fibrosis Model

CCl_4 cirrhosis was induced in male Wistar rats as previously described.[36] The animals were kept in an environmentally controlled animal facility at Institut d'Investigacions Biomediques August Pi i Sunyer. All experiments were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals at the Hospital Clínic and Institut d'Investigacions Biomediques August Pi i Sunyer.

Immunocytochemistry

Isolated HSCs, hepatocytes, Kupffer cells, and sinusoidal endothelial cells and liver tissue sections of wild-type, cirrhotic, and lipopolysaccharide (LPS)-treated rats were fixed with 4% paraformaldehyde and incubated for 45 minutes at room temperature with blocking buffer (PBS with 2% BSA) (Sigma). In some experiments, the tissues or cells were also permeabilized with 0.1% Triton. After blocking with PBS (2% BSA), tissue sections and cells were stained with primary and secondary antibodies for 1 hour at room temperature. Washes with PBS were performed between incubations. Finally, nuclei were visualized by DNA staining with Hoechst reagent (10^{-3} mg/ml). Samples were mounted using Fluoromount-G (Southern Biotechnology, Birmingham, AL). Fluorescence images were acquired using a confocal spectral microscope (TCS SL; Leica, Heidelberg, Germany).

The following primary antibodies were used: mouse mAb CD38.14.27 (2 μg/ml), rabbit anti-GFAP polyclonal antibody (1:400; Chemicon, Temecula, CA) (1:500), rabbit anti-asialoglycoprotein receptor (anti-ASGPR) polyclonal antibody (1:400; provided by Dr. C. Enrich, University of Barcelona), and mouse ED-2 and RECA (1:100; Serotec, Oxford, UK). The secondary antibodies used were Cy3-conjugated anti-mouse IgG and Cy2-conjugated anti-rabbit IgG (1:500; Jackson Immuno Research, West Grove, PA).

Intracellular Ca^{2+} Determination

Ca^{2+} mobilization induced by the mAb CD38.14.27 was determined with Fluo-4 acetoxymethyl ester as previously described.[37] Intracellular Ca^{2+} levels were measured in individual cells using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) with argon and HeNe lasers attached to a Leica DMIRE2 inverted microscope equipped with an incubation system with temperature and CO_2 controls. Images were taken at 3-second intervals for 20 minutes. Changes in the intracellular calcium concentration are given as the relative change in the fluorescence ratio F/F_0 of Fluo4-AM, where F is the fluorescence intensity at any time and F_0 is the baseline fluorescence intensity. Image treatment and movie assembly were performed using the Image Processing Leica Confocal Software. Measurements of Ca^{2+} were performed at baseline and after administration of mAb CD38.14.27 at a dose of 6 μg/ml or an isotype control mAb. Ionomycin (10 μmol/L) was used as a positive control.

Assay for Interleukin (IL)-6 Production

HSCs were cultured in 24-well plates for either 3 or 6 days, and cytokine production was induced by several concentrations of CD38.14.27 (1.5 to 6 μg/ml) or a polyclonal antibody reactive with rat CD38 (6 μg/ml) in the presence or absence of phorbol 12-myristate 13-acetate (PMA) (5 ng/ml). Supernatants were collected after 18 hours and used to measure IL-6. The enzyme-linked immune specific assay (BD Biosciences, San Diego, CA) was performed following the manufacturer's instructions.

Analysis of Adhesion Molecule Expression Using Laser-Scanning Cytometry

CD38-induced up-regulation of the adhesion molecules I-CAM, V-CAM, and N-CAM on cultured HSCs was assessed by laser-scanning cytometry (LSC). HSCs were plated on coverslips in 24-well plates (Costar, Corning, NY) and cultured for either 3 or 6 days. Cells were cultured overnight in the presence of CD38.14.27 (6 μg/ml) or an isotype control mAb (6 μg/ml). Cells were washed and fixed with 4% paraformaldehyde for 15 minutes at room temperature and blocked as described above. Cells were incubated with mAbs against I-CAM (1:100), V-CAM (1:100; Pharmingen), N-CAM (1:100; Sigma), or an irrelevant control mAb and then with a secondary fluorescein isothiocyanate-conjugated antibody (1:200 dilution; Caltag, Burlingame, CA). LSC analysis was performed using a laser-scanning cytometer (CompuCyte, Cambridge, MA) with analysis by WinCyte 2.1 PC-based software. For analysis, instrument scan areas were set to include at least 2500 cells per coverslip. The slides were scanned with a $\times$20 objective lens using an argon laser set at 5 mW to excite the fluorochromes while the filters used were 530/30 nm for fluorescein isothiocyanate and 625/28 nm for propidium iodide. The primary contouring parameter used to detect and quantify cells centered on the red fluorescence emitted by the propidium iodide as previously described.[38] Green surface fluorescence of each HSC cell was sampled by defining a perimeter around the nucleus wherein green fluorescence was quantified. The dimensions of this perimeter were set by adding a defined number of pixels to the threshold contour so that overlap of adjacent cells was avoided.

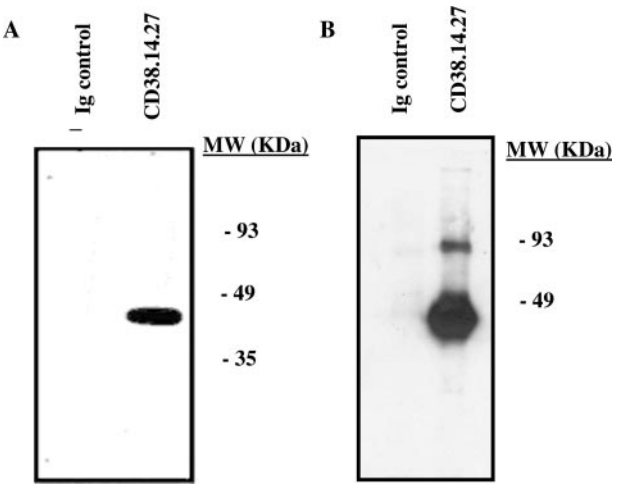

Figure 1. Immunoprecipitation of the cell surface protein recognized by mAb 14.27. Detergent lysates of surface-labeled HSCs were immunoprecipitated with 14.27 mAb or with an irrelevant mAb. The immunoprecipitated material was analyzed under nonreducing conditions on a 12% SDS-polyacrylamide gel. Two independent immunoprecipitations are shown at two different exposure times (**A**, short exposure time; **B**, long exposure time). Molecular masses (in kilodaltons) were determined by the migration of a protein standard.

Results

Production of mAbs Against HSCs Cell Surface Molecules

We generated a panel of 16 mAbs that recognized antigens expressed on the cell surface of HSCs. mAb 14.27 was chosen for further analysis because of its apparent restricted pattern of reactivity with HSCs and its ability to immunoprecipitate a clear band.

Characterization of the Protein Recognized by mAb 14.27

mAb 14.27 immunoprecipitated a single band of 45 kd in reducing and nonreducing conditions from a lysate of cultured HSCs (Figure 1A; data not shown). In films with longer exposures, an extra band of approximately 90 kd, which represented about 10% of the precipitate, could be observed (Figure 1B), suggesting the presence of a homodimeric form. A strong band of 45 kd was detected in Western blots of HSCs lysates (Figure 2A). A fainter band of the same molecular mass was observed in a Western blot of whole-liver lysates (Figure 2B).

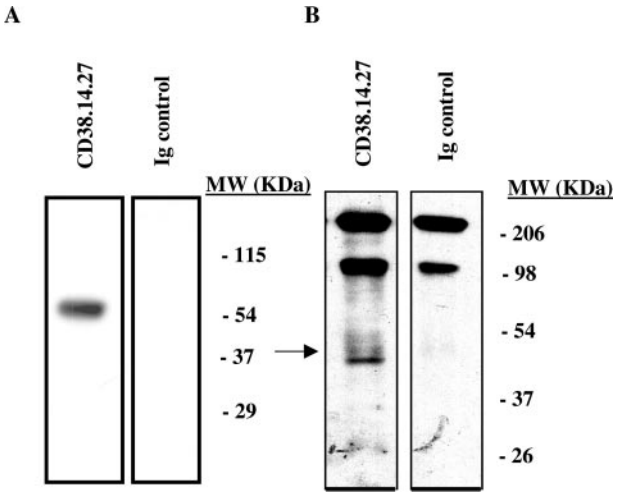

Figure 2. Western blot analysis of the protein recognized by mAb 14.27. Detergent lysates of HSCs (25 μg) (**A**) or liver tissue (100 μg) (**B**) were analyzed by Western blotting (12% SDS-polyacrylamide gel) using an anti-CD38 mAb (14.27). Molecular masses (in kilodaltons) were determined by the migration of a protein standard.

Identification of the Protein Recognized by mAb 14.27

Affinity purification of the protein recognized by mAb 14.27 from HSC lysates yielded a single band of 45 kd, as observed by silver staining of a SDS-polyacrylamide gel electrophoresis gel. This protein band was analyzed by mass spectrometry (MALDI-TOF). All of the analyzed tryptic peptides obtained from this band matched rat CD38 (accession no. Q64244), covering 33% of the protein sequence (Figure 3).

To confirm the identity of the recognized protein, rat CD38 cDNA was isolated and transiently transfected in COS-7 cells. CD38 expressed in COS-7 cells was specifically recognized by mAb 14.27 using flow cytometry and immunochemistry (Figure 4). Furthermore, Western blot analysis showed that this mAb also recognized a single band of approximately 45 kd from the COS-7 transfected cells (Figure 4C). All of these data demonstrate that this mAb recognizes rat CD38; we renamed the mAb as CD38.14.27.

MANYEFSQVSEDRPGCRLTRKAQIGLGV
GLLLLVALVVVVVIVLWPRSPLVWKGKPT
TKHFADIILGRCLIYTQILRPEMRDQDCKK
ILSTFKRGFISKNPCNITNEDYAPLVKLVT
QTIPCNKTLFWSKSKHLAHQYTWIQGKM
FTLEDTLLGYIADDLRWCGDPSTSDMNY
DSCPHWSENCPNNPVAVFWNVISQKFA
EDACGVVQVMLNGSLSEPFYRNSTFGS
VEVFNLDPNKVHKLQAWVMHDIKGTSSN
ACSSPSINELKSIVNKRNMIFACQDNYRP
VRFLQCVKNPEHPSCRLNV

Figure 3. Amino acid sequence of rat CD38. CD38 was identified by peptide-mass fingerprinting using MALDI-TOF mass spectroscopy. The tryptic peptides obtained from the 45-kd band matched the rat CD38 amino acid sequence (UniProtKB accession no. Q64244) and covered 33% of the protein. Darks lines indicate amino acids predicted by mass spectrometry.

CD38 Expression in Isolated HSCs

mAb CD38.14.27 strongly stained the cell surface of recently isolated HSCs (Figure 5A). All isolated $CD38^+$ cells strongly co-expressed the cytoplasmic HSC marker, GFAP (Figure 5, A, B, and G). Most of these cells displayed numerous autofluorescent vitamin A-containing vacuoles located in the cytoplasm, characteristic of the quiescent phenotype (Figure 5, G–J). The reactivity with mAb CD38.14.27 was maintained in long-term cultures in which HSCs started to show a myofibroblast-like morphology, characterized by cell enlargement and a reduction in the number of intracellular vacuoles (data not shown).

CD38 Expression in the Liver

Immunohistochemistry with mAb CD38.14.27 on normal rat liver sections showed a strong and discontinuous staining of cells located along the sinusoids (Figure 6). The observed expression pattern was compatible with the location of the HSCs in the Disse space in close contact with hepatocytes and sinusoidal endothelial cells. An identical staining pattern was observed in samples obtained from rats treated with LPS (Figure 6). The staining patterns of the HSC maker GFAP and CD38 were very similar, indicating that most of the cells in liver co-express these two proteins (Figure 6). Interestingly, a significant increase in CD38 levels was observed in the sinusoidal and fibrotic septa areas of tissue sections from CCl_4-intoxicated cirrhotic rats (Figure 6). Moreover, flow cytometric analysis of three independent experiments showed a significant increase in CD38 expression in HSCs isolated from cirrhotic rats (mean fluorescence intensity, 98 $\pm$ 10) compared with control rats (mean fluorescence intensity, 48 $\pm$ 5). A representative histogram is shown in Figure 7.

CD38 Expression in Hepatocytes, Endothelial Cells, and Kupffer Cells

In tissue sections, we were unable to detect a significant staining of hepatocytes and the sinusoidal endothelium with mAb CD38.14.27 (Figure 6). No reactivity was observed in central and the portal tract endothelium (Figure 8). Moreover, hepatocytes ($ASGP\text{-}R^+$) and sinusoidal endothelial cells ($RECA^+$) isolated from normal liver did not express significant amounts of CD38 (Figures 8 and 9). In contrast, approximately 55% of Kupffer ($ED2^+$) cells expressed CD38 on their surface (Figure 9).

Ca^{2+} Mobilization in HSCs by CD38 Ligation

Previous reports showed that the ligation of CD38 with agonistic CD38 antibodies induced a rise in intracellular Ca^{2+} levels from the intracellular stores in hematopoietic

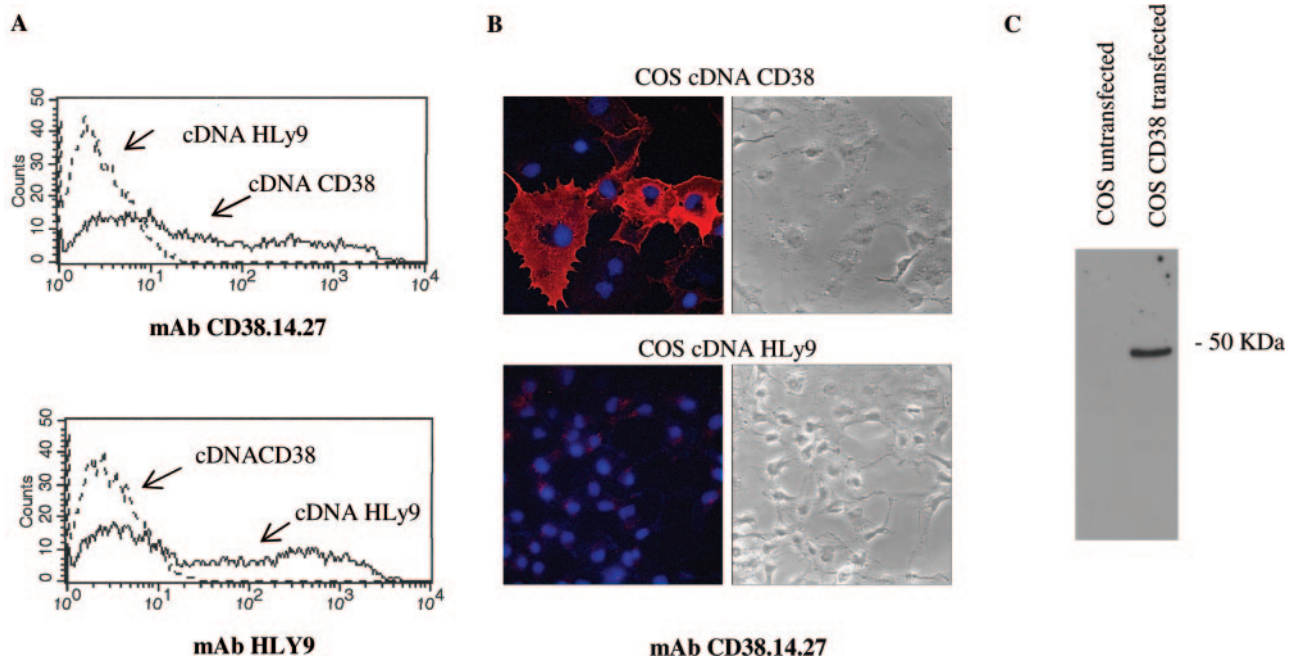

Figure 4. Reactivity of 14.27 mAb with CD38 cDNA-transfected COS cells. **A:** COS cells transfected with rat CD38 cDNA (solid lines) or human HLy9 cDNA (dotted lines) were stained with 14.27 and analyzed by flow cytometry (**top**). As a positive control, COS cells transfected with rat CD38 cDNA (dotted lines) or human HLy9 cDNA (solid lines) were stained with HLY9 mAb (**bottom**). The fluorescence intensity is shown over a 3-decade log scale. **B:** COS cells transfected cells with rat CD38 cDNA (**top**) or human HLy9 cDNA (**bottom**) were washed with PBS, fixed with paraformaldehyde, and incubated with 14.27 mAb. Cells were washed and incubated with a Cy-3-conjugated secondary antibody (red). Nuclei were stained with Hoechst reagent (blue). Fluorescence images were acquired using a confocal spectral microscope with an original magnification of ×63. **C:** Detergent lysates of COS untransfected and COS CD38-transfected cells were analyzed by Western blotting (12% SDS-polyacrylamide gel) using anti-CD38 mAb (14.27). Molecular mass (in kilodaltons) was determined by the migration of a protein standard.

cells. We investigated whether CD38 binding by mAb CD38.14.27 could mediate similar effects in HSCs. An increase in Ca^{2+} levels after ligation with the mAb CD38.14.27 was observed, whereas no effect was detected using an isotype-matched irrelevant mAb as a control (Figure 10).

IL-6 Secretion of HSCs by CD38 Ligation

Analysis of cytokine release was used to provide additional confirmation of the ability of CD38 to transduce biologically relevant signals in HSCs. The secretion of cytokines into the culture medium revealed the suc-

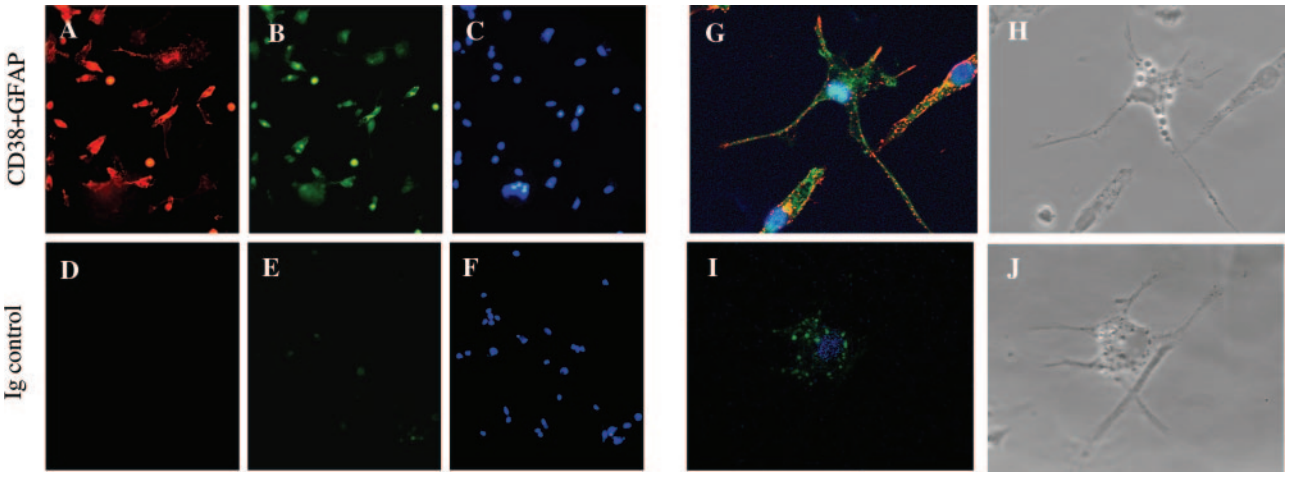

Figure 5. Expression of CD38 and the HSC-specific marker GFAP on cultured HSCs. HSCs were maintained for 3 days in culture. Cells were washed with PBS, fixed with paraformaldehyde, and permeabilized. Double immunostaining was performed. Cells were incubated with CD38.14.27 mAb and a rabbit anti-GFAP polyclonal antibody (**A–C**, **G**, and **H**, **top**) or with Ig controls (**D–F**, **I**, and **J**, **bottom**). Cells were washed and incubated with an anti-mouse Cy-3-conjugated secondary antibody (red) and an anti-rabbit Cy-2-conjugated secondary antibody (green). **I:** Vitamin A autofluorescent droplets were visualized in the negative control. **C** and **F:** Nuclei were stained with Hoechst reagent (blue). **H** and **J:** Phase contrast microscopic view. Fluorescence images were acquired using a fluorescence microscope with an original magnification of ×40 (**A–F**) or using a confocal spectral microscope with an original magnification of ×63 (**G–J**).

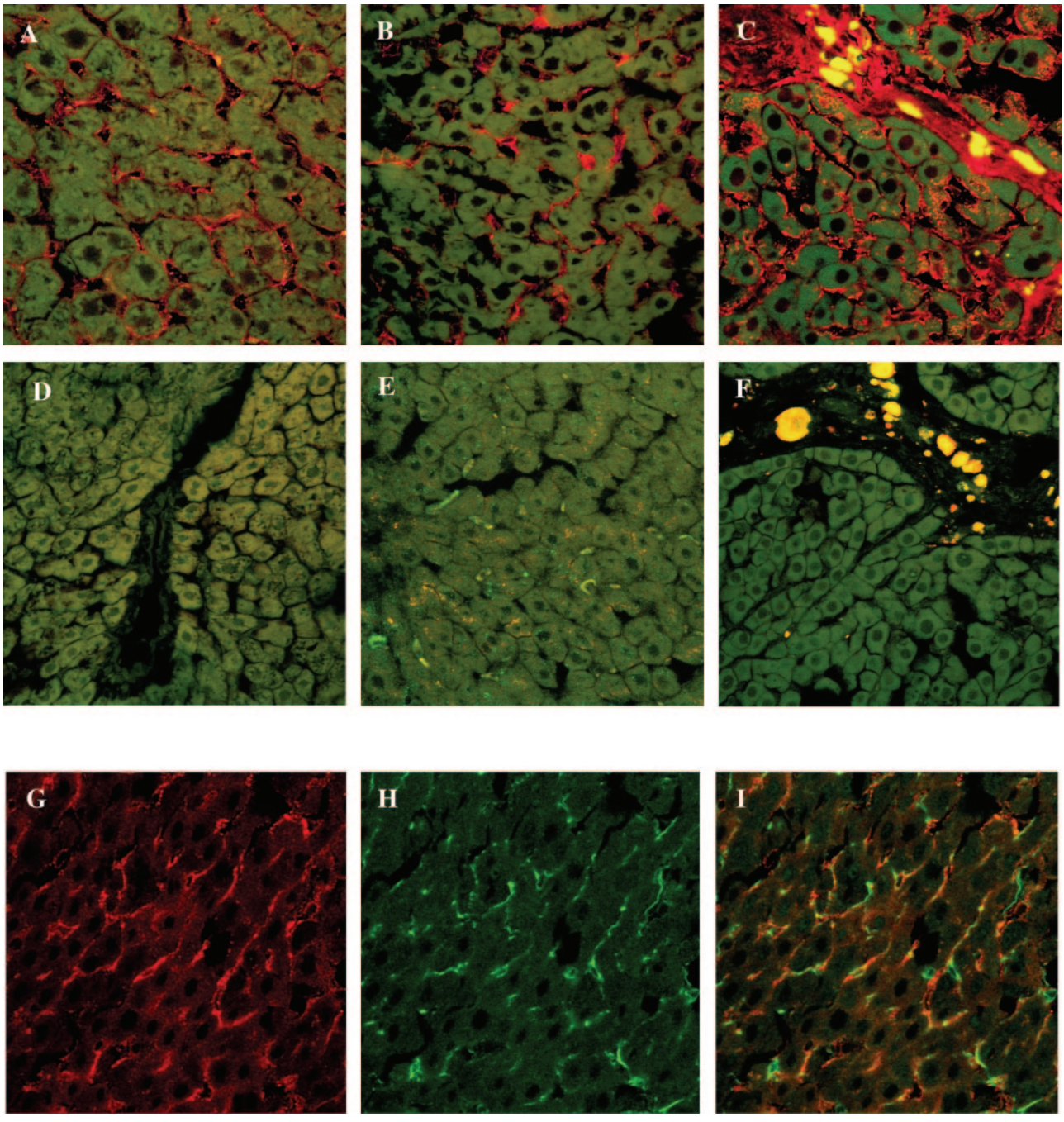

Figure 6. Immunolocalization of CD38 in rat liver sections. **A–F:** Liver sections of control rats (**A** and **D**), rats pretreated with LPS (550 μg/rat) (**B** and **E**) and cirrhotic rats with an advanced stage of fibrosis displaying prominent scars (**C** and **D**) were stained with an antibody against CD38 (CD38.14.27) (**A–C**) or with an Ig control (**D–F**). Liver sections were washed and incubated with an anti-mouse Cy-3-conjugated secondary antibody (red). Hepatocyte autofluorescence from hepatocytes is seen in green. **G–I:** Double-immunostaining was performed. Liver sections of control rats were incubated with mAb CD38.14.27 (**G**) and a rabbit anti-GFAP polyclonal antibody (**H**). Merged image is seen in **I**. Liver sections were washed and incubated with an anti-mouse Cy-3-conjugated secondary antibody (red) and an anti-rabbit Cy-2-conjugated secondary antibody (green).

cessful delivery of signals to the nucleus and subsequent implementation of genetic programs controlling protein synthesis. We found that HSCs cultured in the presence of the mAb CD38.14.27 alone or together with a low doses of PMA (5 ng/ml), induced IL-6 secretion into the culture medium (Figure 11A). The induction of IL-6 by CD38.14.27 was dose-dependent (Figure 11A). This effect of CD38.14.27 could be observed in HSCs cultured for 4 or 7 days (Figure 11; data not shown). We could also observe that an anti-rat CD38 polyclonal antibody was also able to significantly induce HSCs to secrete IL-6 (Figure 11B), although the secreted amount was lower than that observed after treatment with the mAb.

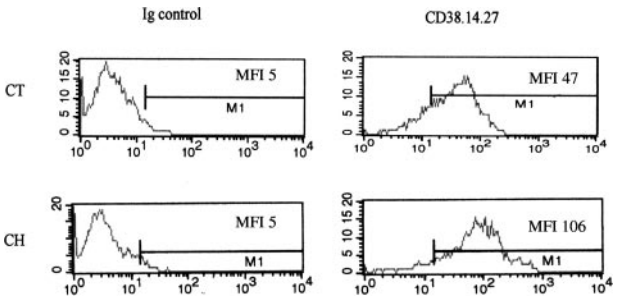

Figure 7. Expression of CD38 on activated HSCs. HSCs were freshly isolated from control (**top**) and cirrhotic (**bottom**) rats. $CD38^+$ cells were gated, and the intensity of CD38 expression was assessed by flow cytometry (FACS) using CD38.14.27 mAb. Fluorescence intensity is shown over a 3-decade log scale.

Expression of HSC Adhesion Molecules by CD38 Ligation

The expression of the adhesion molecules ICAM-1, VCAM-1, and NCAM was analyzed in cultured HSCs after CD38 ligation. HSCs bind very strongly to plastic or glass, especially during the first weeks of *in vitro* culture. Excessive trypsinization or other treatments to detach the cells for flow cytometry analysis seriously affect the expression of cells surface molecules. Thus, to address the effects of CD38 cross-linking on the expression of adhesion molecules, we used LSC. This novel technique is a slide-based fluorescence analytical method analogous to flow cytometry. Extensive quantification of cellular or nuclear events is possible using LSC analysis. In contrast to flow cytometry, the position of each fluorescent event is recorded as it is scanned on the slide, and electronic bitmap images of the scan are created.[38] HSCs cultured for 4 days expressed significant levels of ICAM-1, VCAM-1, and NCAM. CD38 ligation did induce a slight increase in the expression of VCAM-1 and NCAM-1 in these cells (Figure 12). In contrast, CD38 ligation induced a dramatic up-regulation of VCAM-1 and NCAM expression in HSCs cultured for 7 days (Figure 12). A less pronounced increase could also be detected in ICAM-1 expression in these cells (Figure 12). These data indicate that CD38 ligation has a differential effect on HSCs, depending on its activation/differentiation stage.

Discussion

This study identifies CD38 as a major cell surface molecule expressed on HSCs. Although the vast body of data on CD38 are derived from studies performed with hematopoietic cells, little is known about the function, regulation, and expression of CD38 in other cell types.[28] The identification and characterization of a novel mAb to rat CD38 from a panel of antibodies directed to cell surface molecules expressed on HSCs allowed us, for the first time, to show the biochemical characteristics, expression, and functional significance of this molecule in HSCs. Immunoprecipitation of HSC CD38 showed that this protein has a molecular mass of 45 kd. The difference found between the molecular mass predicted by the rat CD38 sequence (34.43 kd) and the observed (45 kd) mass may be attributed to the extensive glycosylation of CD38 previously reported.[39] Our results also suggest that part of CD38 may be found as homodimers. This has also been reported for CD38 in mouse B cells and suggests that CD38 homodimers may play an important role in stabilizing the molecule on the plasma membrane.[40]

Here, we show that CD38 regulates not only HSC activation through the induction of the proinflammatory

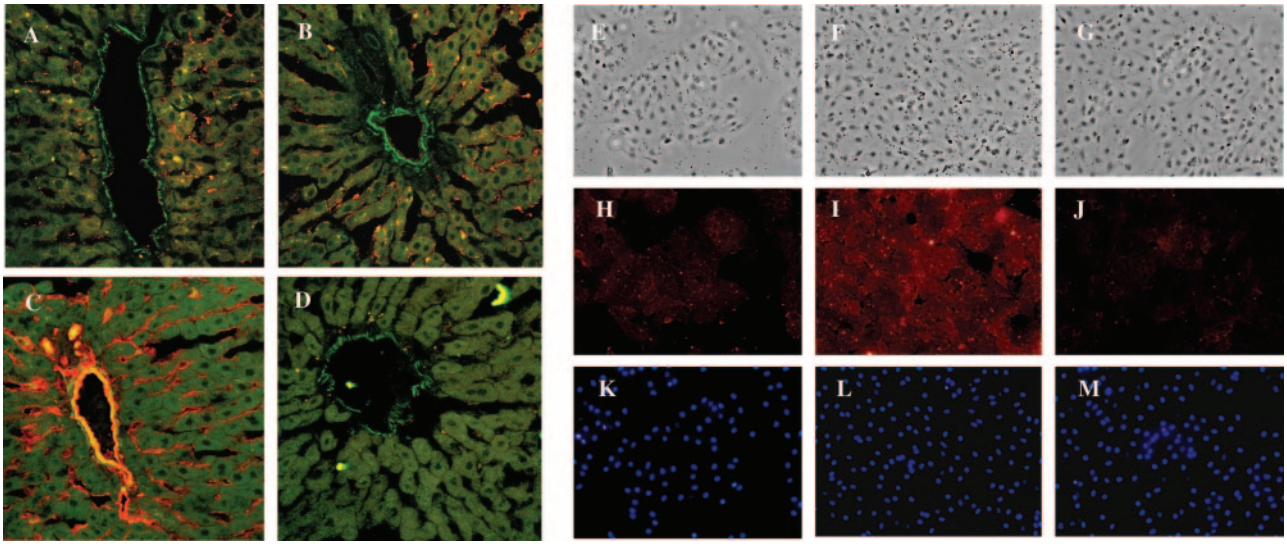

Figure 8. CD38 expression in endothelial cells in the liver. Rat liver sections from central venule (**A**) and portal areas (**B**) were stained with the antibody CD38.14.27 (**A** and **B**), RECA (pan-endothelial marker) (**C**) or with an Ig control (**D**). Sections were washed and incubated with an anti-mouse Cy-3-conjugated secondary antibody (red). Hepatocyte autofluorescence from hepatocytes and vessels shown in green. **E–M:** Sinusoidal endothelial cells were isolated from rat livers and cultured for 2 days. Cells were washed with PBS, fixed with paraformaldehyde, and single immunostaining was performed. Cells were incubated with CD38.14.27 mAb (**E**, **H**, and **K**), RECA (**F**, **I**, and **L**), or Ig controls (**G**, **J**, and **M**). **H** and **J:** Cells were washed and incubated with an anti-mouse Cy-3-conjugated secondary antibody (red). Corresponding phase-contrast images are shown (**E–G**) and corresponding nuclei are seen by Hoechst staining (**K–M**). Fluorescence images were acquired using a confocal spectral microscope with an original magnification of ×40 (**A–D**) or using a fluorescence microscope with and original magnification of ×20 (**E–M**).

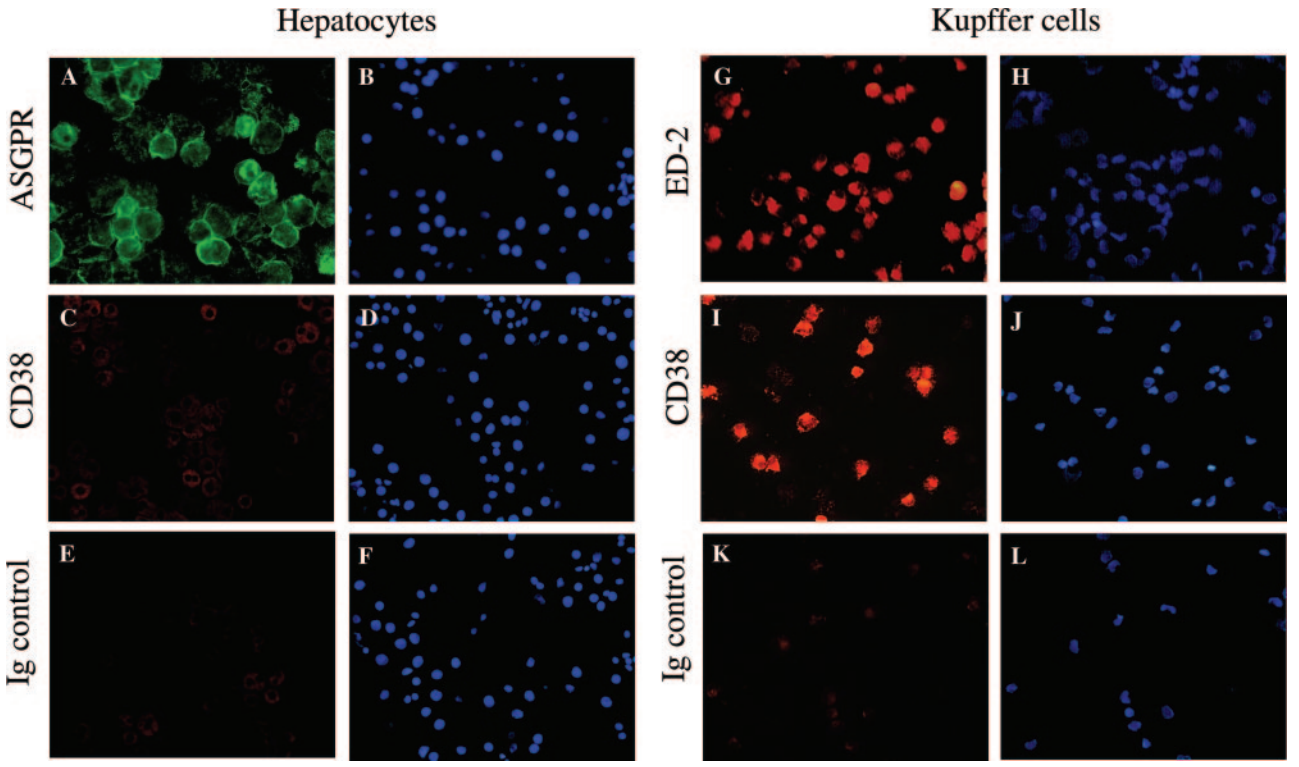

Figure 9. CD38 expression in hepatocytes and Kupffer cells. **A–F:** Hepatocytes. Freshly hepatocytes were isolated and cytospun followed by single immunostaining. Hepatocytes were incubated with rabbit ASGPR polyclonal antibody (**A** and **B**), CD38.14.27 mAb (**C** and **D**), and Ig control (**E** and **F**). Cells were then washed and incubated with an anti-rabbit Cy-2-conjugated secondary antibody (green) (**A** and **B**) or an anti-mouse Cy-3-conjugated secondary antibody (red) (**C–F**). Corresponding nuclei are shown by Hoechst staining (blue). Ig control incubated with anti-rabbit Cy-2-conjugated secondary antibody (data not shown). **G–L:** Kupffer cells. Fresh Kupffer cells were isolated, and single immunostaining was performed. Kupffer cells were incubated with ED-2 mAb (**G** and **H**), mAb CD38.14.27 (**I** and **J**), and Ig control (**K** and **L**). **G** and **L:** Cells were then washed and incubated with an anti-mouse Cy-3-conjugated secondary antibody (red). Corresponding nuclei are shown by Hoechst staining (blue). Fluorescence images were acquired using a fluorescence microscope with an original magnification of ×40.

cytokine IL-6 but also the up-regulation of the expression of several adhesion molecules. The expression of CD38 in hematopoietic cells has a peculiar distribution. It is strongly expressed on lymphocyte precursors, decreases once the cells differentiate (approximately 5% of freshly isolated T cells are $CD38^+$), and is then up-regulated again on mature lymphocytes and plasma cells.[41–43] In the liver, CD38 was preferentially detected on the cell surface of hepatic stellate cells, as well as on a subset of Kupffer cells. CD38 was constitutively expressed in resting HSCs. Under chronic liver disease conditions, or when cultured on plastic, HSCs become activated, proliferate, and produce extracellular matrix proteins that are responsible for the bulk of abnormal matrix depositions in liver cirrhosis.[4,44,45] Cultivated

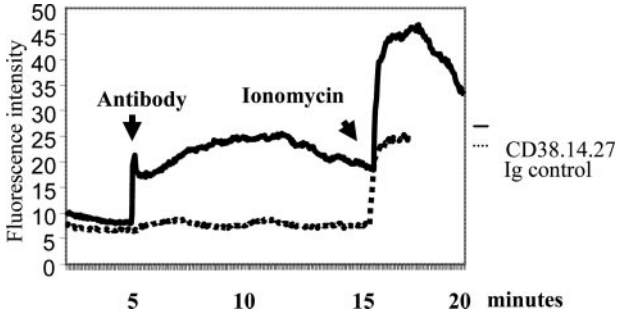

Figure 10. Ca^{2+} mobilization in HSCs by CD38 ligation. HSCs cultured for 6 days were loaded with the fluorescent indicator Fluo-4/AM. Cells were incubated with mAb CD38.14.27 (6 μg/ml) and immediately analyzed using an inverted confocal microscope. The image was analyzed using software Image Processing Leica Confocal Software. The same experiment was performed with an isotype-matched mAb (IgG_{2b}).

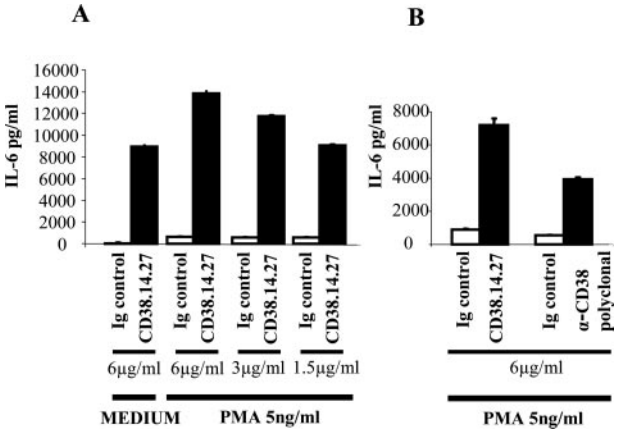

Figure 11. Secretion of IL-6 by HSCs followed by CD38 ligation. **A:** HSCs were incubated in the presence or absence of PMA (5 ng/ml) and with mAb CD38.14.27 at different concentrations (6, 3, and 1.5 μg/ml). The same concentrations of an isotype-matched mAb (IgG_{2b}) were used as controls. **B:** HSCs were incubated in the presence of PMA (5 ng/ml) and 6 μg/ml mAb CD38.14.27 or a polyclonal anti-CD38 antibody. The same concentrations of an isotype-matched antibody (IgG_{2b} or goat IgG) were used as controls.

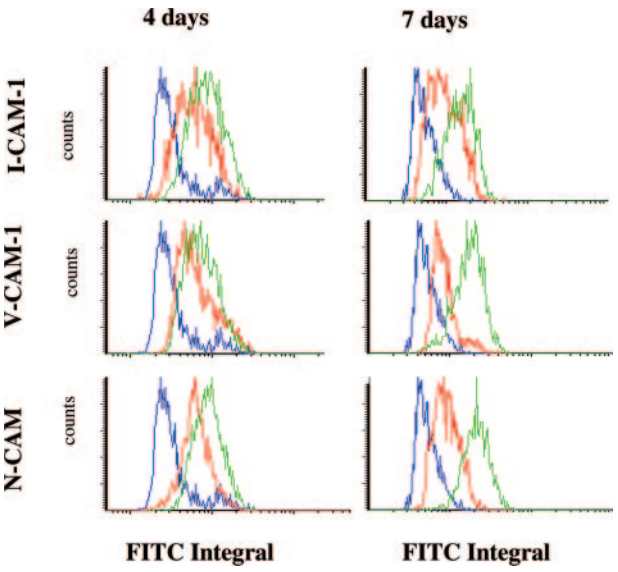

Figure 12. Expression of ICAM-1, VCAM-1, and NCAM in HSCs followed by CD38 ligation. HSCs cultured for 3 or 6 days were incubated for 12 hours with mAb CD38.14.27 or with an isotype-matched mAb (IgG_{2b}) as a control. Expression of these adhesion molecules was assessed by LSC cytometry using the primary and secondary antibodies described in Materials and Methods. Lines in the histogram: negative control (blue), isotype-matched control activation (red), and CD38 ligation (green).

HSCs that presented a vitamin A-rich phenotype and expressed the HSCs marker GFAP expressed high levels of CD38 on the cell surface.

Moreover, increased expression could also be observed in the livers of rats with carbon tetrachloride-induced cirrhosis by immunohistochemical studies. This is a significant result, because activated HSCs in culture do not necessarily reflect the *in vivo* condition of the injured liver.[46] Several structural genes have been shown to be markedly up-regulated in activated HSCs, including the adhesion molecule ICAM-1, αB-crystalline, and several genes involved in actin rearrangement.[7,47,48]

The ability to increase intracellular Ca^{2+} levels in lymphocytes using selected agonistic mAbs has been well characterized.[28] Moreover, the effects induced by agonistic CD38 mAbs include the production of proinflammatory and regulatory cytokine by monocytes, dendritic cells, NK cells, and T lymphocytes; the proliferation of T lymphocytes; the protection of B lymphocytes from apoptosis; the tyrosine phosphorylation of selected substrates; and the activation of nuclear factors.[29,30,49–53] Ligation of CD38 with the novel mAb generated in this study (CD38.14.27) increased intracellular Ca^{2+} levels, thereby showing its agonistic capacity. This allowed us to analyze CD38-mediated signaling affects such as cytokine production by HSCs. Here, we show that CD38 ligation induces IL-6 secretion of cultured HSCs. This induction was dose dependent and independent of the day of culture. IL-6 secretion was observed after CD38 ligation without the addition of any extra stimulus. However, increased levels were observed when subinducing doses of phorbol ester were added to the cultures. This result was also observed when using a polyclonal antiserum against rat CD38, although the effect was lower than that observed with the mAb. IL-6 may provide an autocrine pathway that helps perpetuate the activated phenotype of these cells, thereby stimulating HSC proliferation.[54] We hypothesize that the capacity of HSCs to produce IL-6 after CD38 engagement may represent an important mechanism by which these cells participate in the local regulation of inflammatory responses in the liver. Because the expression of CD38 is increased in the HSCs of cirrhotic animals, this may determine the production of higher IL-6 levels. IL-6 also has a paracrine effect on hepatocytes, inducing the production of acute phase proteins, which play an important role in innate immunity against injury.[55]

In this study, we also demonstrate that CD38 ligation induced increased expression of the adhesion molecules VCAM-1, N-CAM, and ICAM-1. In contrast to the induction of IL-6 secretion, this effect was significantly stronger in HSCs cultured for 7 days compared with cells undergoing fewer days in culture, thus indicating that CD38 signaling preferentially affects the expression of these molecules on cells having an activated/differentiated phenotype. The increased expression of these adhesion molecules may have a direct role in the transmigration of leukocytes from the hepatic sinusoid to sites of tissue damage during inflammatory responses in the liver.[7] Infiltration by leukocytes and monocytes/macrophages into the inflamed liver is an important component of tissue injury. It has been shown that blockade of macrophage infiltration inhibits the activation of HSCs and leads to suppression of liver cirrhosis.[56] Interestingly, the expression of IL-6 and the adhesion molecules ICAM-1 and VCAM is regulated by the transcription factor nuclear factor κB (NF-κB).[55] NF-κB has been shown to be persistently activated in activated HSCs and to mediate the production of IL-6 and ICAM-1 in these cells.[57] Moreover, it has been recently demonstrated that not only can NF-κB activation be induced by CD38 signaling, but it also is required for CD38-mediated signal transduction.[58]

Taken together, these data suggest that CD38, via interaction with its natural ligand CD31, can induce the propagation of signals promoting HSC activation, which in turn determines the degree of the inflammatory response after liver injury. It has been shown that sinusoidal endothelial cells lack the expression of CD31. However, during the process of sinusoidal capillarization that occurs in liver cirrhosis, CD31 is strongly up-regulated.[59] Therefore, we hypothesize that the interaction of HSC CD38 with overexpressed CD31 in the sinusoids could be an important key in mediating HSC activation and effectors functions. It is well established that all monocytes and granulocytes express CD31.[60] Infiltrating leukocytes entering the liver could also be a source of CD31, inducing the ligation of HSC CD38, at early phases of the inflammatory processes before the up-regulation of endothelial CD31 occurs.

In conclusion, our data show that CD38 is a regulator of HSC activation and effector functions, playing an important role in the inflammatory response mediated by these cells. Thus, understanding the molecular basis of CD38-mediated signaling in HSCs may lead to novel therapeutic strategies for treating liver cirrhosis.

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