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The anti-fibrotic role of mast cells in the liver is mediated by HLA-G and interaction with hepatic stellate cells

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ABSTRACT

Background & aims: We have reported a significant association between HLA-G expression or the number of hepatic mast cells and liver fibrosis. Here, we investigated the role of HLA-G and mast cells in liver fibrosis, focusing, in particular, on interactions between human mast and stellate cells.
Methods: Human mast cells (HMC cell line, CD34-derived mast cells, or tissue-derived mast cells) were co-cultured with purified human hepatic stellate cells (HSCs), and collagen I production by HSCs was evaluated. Mast cells and HSCs were characterized by immunocytochemistry. Various conditions were tested: different times in direct or indirect contact, presence or absence of cytokines, addition or not of HLA-G, and presence or absence of specific protease inhibitors.
Results: The reciprocal interaction between HSCs and mast cells led to the attraction of mast cells to HSCs *in vivo* and *in vitro,* and to a significant decrease in collagen production, at all times of co-culture, following the direct or indirect contact of mast cells with HSCs alone or in the presence of TGF-β, IFN-α or IL-10. We identified the diffusible factors involved in collagen I degradation as mast cell proteases. Moreover, HLA-G expression increased during the co-culture of HSCs and mast cells, with HLA-G acting on both mast cells and HSCs, to enhance collagen I degradation.
Conclusions: Mast cells play a beneficial, anti-fibrotic role in liver fibrosis, via the HLA-G-mediated decrease of collagen I. These findings are consistent with high levels of cross-communication between mast cells and hepatic stellate cells and the role of HLA-G.

1. Introduction

Mast cells were initially identified as effectors of allergy, but they are also innate immune cells involved in acute and chronic inflammation, and in immune processes, in which they act as antigen-presenting cells and anti-infectious agents [1]. Mast cells, a separate group of cells from basophils, are long-lived and characterized by their large, membrane-bound intracellular granules containing an acidic proteoglycan matrix consisting mostly of heparan sulfate [2]. Like dendritic cells, mast cells are among the first cells of the immune system to interact with antigens, toxins, and pathogens, because they are abundant at the interface between the external and internal environments. The capacity of mast cells to interact promptly with the microenvironment and respond by releasing numerous biologically active mediators requires a delicate balance, and the inadequate regulation of mast-cell functions can have deleterious effects [1].

A role for mast cells in liver diseases is emerging [3]. The liver mast cells are mostly associated with the connective tissue, which is located close to the hepatic arteries, veins and bile ducts of the portal tracts in the human liver. In the normal liver, mast cells accumulate in small numbers along the portal tract, becoming more abundant in cases of hepatic injury. Indeed, mast cell numbers increase in patients with cholangiopathies, alcoholic liver injury, fatty liver disease, allograft rejection or liver cancer [3].

Over the last five years, we have investigated the role of HLA-G in liver diseases [4]. HLA-G is a member of the HLA class Ib family

Abbreviations: HSC, hepatic stellate cells; HMC, human mast cells; coll, collagen; α-sma, alpha-smooth muscle actin; SCF, stem cell factor

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expressed as membrane-bound and soluble forms, and it has immunosuppressive properties. Within the liver, microenvironmental factors, such as hypoxia, or various cytokines, including interferons, IL-10, and TGF-β, can increase HLA-G production by various cells, such as tumor cells, cells of the monocyte lineage and mast cells [5]. Furthermore, during viral infections, viral proteins modulate HLA-G expression by interfering with the intracellular trafficking of HLA-G [6]. HLA-G may induce transient local immune suppression, counteracting autoimmune or infection-related inflammatory responses and down-regulating immune responses [7]. HLA-G interacts with immune or endothelial cells and mast cells, via the direct binding of both soluble and membrane-bound HLA-G to inhibitory receptors, such as ILT2, expressed by T and B lymphocytes, NK cells and mast cells, ILT4 on B lymphocytes and dendritic cells, CD8 on T lymphocytes and NK cells, and CD160 on endothelial cells [8–11]. The HLA-G protein is not detected in hepatocytes and bile duct cells from normal livers, but it can be found in tissues and plasma from patients with hepatocellular carcinoma or viral hepatitis, and after liver transplantation [4]. The role of HLA-G in liver cancers and in transplantation has been determined: it is detrimental in cancers, because it inhibits the activities of immune cells, but beneficial in liver grafts. However, the role of HLA-G in liver fibrosis remains largely unclear. Fibrosis results from the excessive deposition of collagen by activated hepatic stellate cells (HSCs). We therefore hypothesized that HSCs and mast cells might interact through the secretion of cytokines, and of the molecule HLA-G. The TGF-β secreted by HSCs is a potent chemoattractant for mast cells [12], and mast cells can produce cytokines involved in hepatic fibrosis, such as IL-33 and IL-13, or in HSC proliferation, such as TNF-α [13].

We previously showed that HLA-G expression and the number of mast cells were significantly associated with the area of the liver displaying fibrosis [5]. In this study, we investigated the *in vitro* interaction of purified human mast cells and HSCs *ex vivo*. We observed a reciprocal interaction between human HSCs and mast cells that led to collagen degradation at various time points, during both brief and prolonged periods of co-culture. We also demonstrated the involvement of mast cell proteases and provide the first evidence of a role for HLA-G in the mechanism of collagen decrease.

2. Materials and methods

2.1. Cell lines

The human mast cell (HMC) line HMC1.1 (generously provided by Dr Butterfield, Mayo Clinic, Rochester) was established from a patient with mast cell leukemia, as previously described [14]. The B-lymphoblastoid cell line LCL721.221-G5 (kindly provided by D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA) was obtained by transfection with the intron 4-containing HLA-G cDNA, as previously described [15]. KG1 and KG1a provided from ATCC are two acute myelogenous leukemia cell lines, KG1a is more immature than KG1.

2.2. Generation of human mast cells from purified CD34-positive cells

The procedure used was adapted from that used by Schmetzer et al. [16]. Briefly, CD34-positive cells were purified from unused samples of peripheral autologous hematopoietic stem cells, with magnetic microbeads (CD34 microbead kit, Miltenyi Biotec, Paris, France), according to the manufacturer's instructions. The methodology is reported in Supplementary Methods document 1.

2.3. Purification of HSCs from liver biopsy specimens

HSCs were isolated from histologically normal regions of partial hepatectomy specimens from patients undergoing hepatic resection for liver metastases. Human samples were obtained from the biological samples processed by the *Centre de Ressources Biologiques (CRB) Santé* of Rennes **BB-0033-00056**. The research protocol conformed to French legal guidelines and fulfilled the requirements of the local institutional ethics committee.

The methodology is reported in Supplementary Methods document 2.

2.4. Co-culture of a human mast cell line or primary human mast cells with HSCs

Primary HSCs in subconfluent monolayers were treated with trypsin and incubated in 24-well tissue culture plates at a density of 2×10^4 cells/ml in DMEM for 48 h. The HMC cell line, CD34-derived mast cells or tissue-derived mast cells were then added, to give an HSC/HMC or mast cell ratio of 1:2.25 for short-term cultures (3.5 h) and at a ratio of 1:0.5 for other conditions.

The difference of the ratio is explained by the low quantity of HMC at 3H30 (10 000) and the high quantity of HMC cells at longer time because of their capacity of proliferation.

We also assessed the effects of various cytokines supplied by Peprotech-Tebu Bio (Neuilly, France) on HSC:HMC co-cultures: IL-10 (50 ng/ml), TGF-β (5 ng/ml), and IFN-α (50 ng/ml).

We assessed the effects of a specific tryptase inhibitor APC 366 (Santa Cruz biotechnologies, Clinisciences, Nanterre, France) or a specific chymotryptase inhibitor, chymostatin (Enzo Life Sciences, Lyon, France) (in short-term HSC:HMC (1:2.25) cocultures, after the prior incubation of HMCs for 30 min with the inhibitor or the solvent.

2.5. Immunohistochemistry

Cells on Cytospin or flexiPERM slides were fixed in 4% formol. Paraffin-embedded sections (4 μm thick) were prepared, subjected to antigen retrieval, and incubated with primary antibody in a Ventana CT 09/021 machine (Ventana Medical systems, USA). Further details about the method used are reported in Supplementary Methods document 3.

2.6. Western blotting

The methodology is reported in Supplementary Methods document 4.

2.7. Purification of soluble HLA-G protein

HLA-G5 was obtained as previously described [17]. The method is reported in Supplementary Methods document 5. Commercial recombinant purified HLA-G is provided from Origen (Clinisciences, Nanterre, France).

2.8. Statistical analyses

Statistical analyses, including Student's *t* tests, were performed with GraphPad Prism software.

3. Results

3.1. Mast cells are located within or close to zones of fibrosis and inflammation in liver biopsy specimens

We previously demonstrated the existence of a significant association between the number of mast cells expressing HLA-G and the number of areas of liver fibrosis [5]. We therefore sought to determine the locations of mast cells, vascular endothelial cells, collagen (Coll) and alpha-smooth muscle actin (α-SMA), a biomarker of activated HSCs, on biopsy specimens. Using quadruple immunofluorescence staining on a paraffin-embedded liver section from a patient with HCV virus-induced liver fibrosis, we showed that the mast cells were located in the connective tissue of a hepatocyte lobule (Fig. 1A1), close to sites

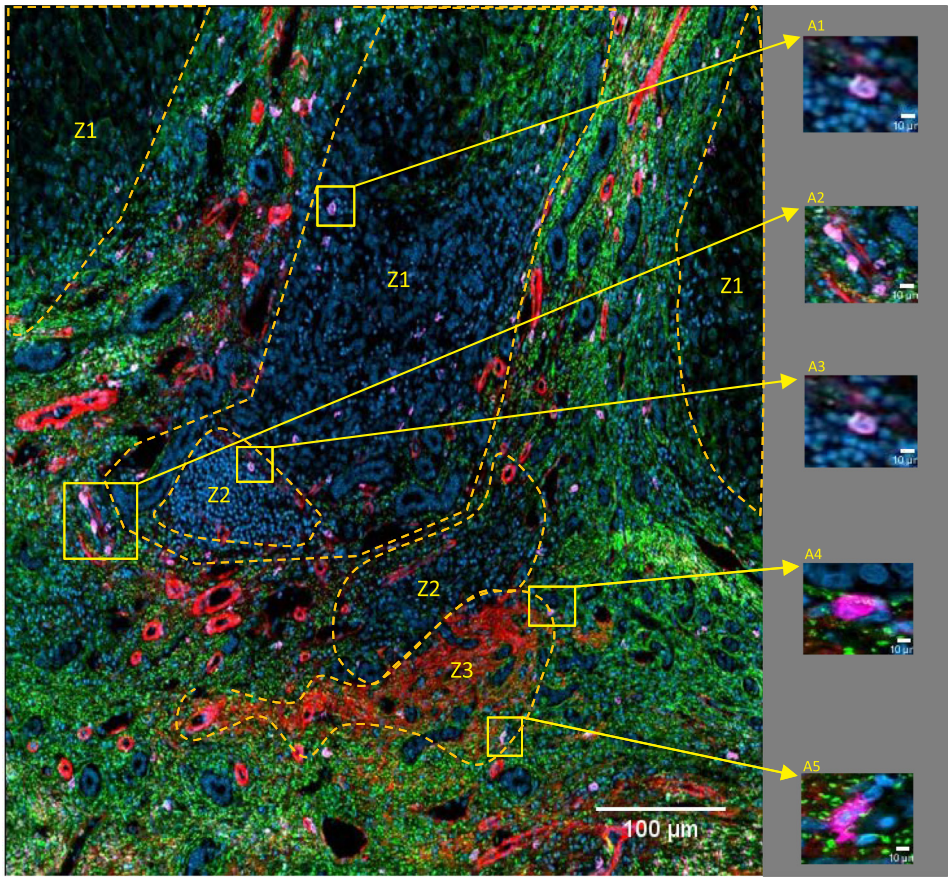


Fig. 1. Mast cells (MCs) are located in or close to zones of liver fibrosis. (A1) Immunohistochemistry with quadruple immunofluorescence staining was performed on paraffin-embedded liver sections from patients suffering from HCV-induced liver fibrosis ($n = 3$). The nuclei are stained blue with Hoechst 33348, the collagen I spans are shown in green and α-SMA-positive areas are red, mast cells labeled with CD117 are pink. In a first time, the three pictures respectively obtained after nuclear labelling, collagen I and α -sma staining are merged. Then the tissue slide was treated by antigen retrieval method to eluate the staining. Then a step with three new labeling with Hoechst, α -sma and CD117 was performed, a second merge of these three staining was realized and for calibrating the two merges, the same labelling with Hoechst and and anti- α sma was performed. Z1 corresponds to hepatocyte lobules, Z2 to inflammatory infiltrates, and Z3 to activated hepatic stellate cell-rich zones. Mast cells, shown in the insets, are located in connective tissue in Z1 (A1), in Z2 within or close to an inflammatory infiltrate (A2, A3), in the α-SMA-positive zone (A2, A5) or in collagen spans (A4) in Z3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of inflammation (Fig. 1A3), within or close to collagen spans (Fig. 1A4 and A5), and in the vicinity of α-SMA (Fig. 1A2 and A4), corresponding to regions close to but separate from collagen spans. Mast cells appeared to be more differentiated in Z3 (A4, A5) if located close to α-SMA than if present in isolation in connective tissue in Z1 (A1).

3.2. Cell characterization and functionality of the HSC/mast cell co-culture

We reproduced the proximity between mast cells and HSCs observed *in vivo,* and the potential communication between these cell types, by establishing an *in vitro* model of HSC/mast cell co-culture. Clonal human mast cells from the HMC cell line were first co-cultured with HSCs. Using immunohistochemistry (Suppl. Fig. 1A1 and 1B1) and western blotting (Suppl. Fig. 1A2), we checked that the purified HSCs expressed collagen I, α-SMA, fibronectin, and laminin, as expected, and that they were functional, producing collagen I in response to TGF-β induction (Suppl. Fig. 1A3). Finally, we checked that the HMC cell line expressed the typical markers of mast cells: CD117, tryptase and chymase (Suppl. Fig. 1B1). We also showed that HSCs continued to proliferate for 168 h, demonstrating that decreases in collagen I levels were not due to the death of HSCs (Suppl. Fig. 1C). The co-culture of purified human HSC and HMCs led to the rapid fixation of HMCs, after just 6 h, and the continuing attachment of HMCs to HSCs, after 168 h (Fig. 2A1 and A2). The capacity of HMC to adhere to HSC cells seems to be specific in contrast to other cell lines such as KG1 or KG1a as shown in Suppl. Fig. 2 A, B, C, D. As HSCs were the only cells in the culture capably of producing collagen I, we investigated whether collagen production was modulated by co-culture with HMCs, by densitometry analyses of the collagen I bands on western blots. HMC co-culture with HSCs at a HSC/HMC ratio of 1:2.25 for 3.5 h (Fig. 2B) resulted in significantly lower type I collagen levels ($p < 0.0001$, $n = 11$). The extension of co-culture to 168 h (7 days), with a HSC/HMC ratio of 1:0.5 revealed that the decrease in collagen levels was most pronounced after 24 h of co-culture, remaining significant at 72 h, and that collagen I levels remained low after 168 h of culture, although it had risen slightly during the course of culture. Similar significant decreases in collagen levels were observed following both direct or indirect contact, with a microporous membrane used top separate HMCs from HSCs in conditions of indirect contact ($p < 0.05$, $n = 6$) (Fig. 2C). We investigated whether exogenous cytokines could modulate the decrease in collagen levels, by incubating HSC/HMC co-cultures with TGF-β, IFN-α or IL-10. We found that TGFβ induced collagen production in HSCs cultured alone, as expected, but collagen I degradation continued when HSC/HMC co-cultures were exposed to this cytokine (Fig. 2D1). More surprisingly, IFNα (Fig. 2D2) and IL-10 (Fig. 2D3) had similar effects.

3.3. Human CD34-positive cell-derived mast cells have similar properties to the cells of the HMC cell line in co-culture with HSCs

We investigated whether primary mast cells yielded similar results, using human CD34-positive cell-derived mast cells co-cultured with HSC cells. As expected, human CD34-positive cell-derived mast cells expressed CD117, tryptase and chymotryptase (Fig. 3A and Suppl. Fig. 1B2). The co-culture of human CD34-positive cell-derived mast cells with HSCs resulted in significantly lower levels of collagen I in the short term (3.5 h) and at 24 h. The decrease in collagen I levels was similar in conditions of direct contact ($p < 0.01$; $n = 5$) or indirect contact, in which a microporous membrane separated human CD34-positive cell-derived mast cells from HSCs ($p < 0.01$; $n = 4$), or medium conditioned by incubation with mast cells with 3.5 h from HSCs ($p < 0.01$, $n = 5$) (Fig. 3B and C). These findings strongly suggest that diffusible factors are involved in collagen I degradation.

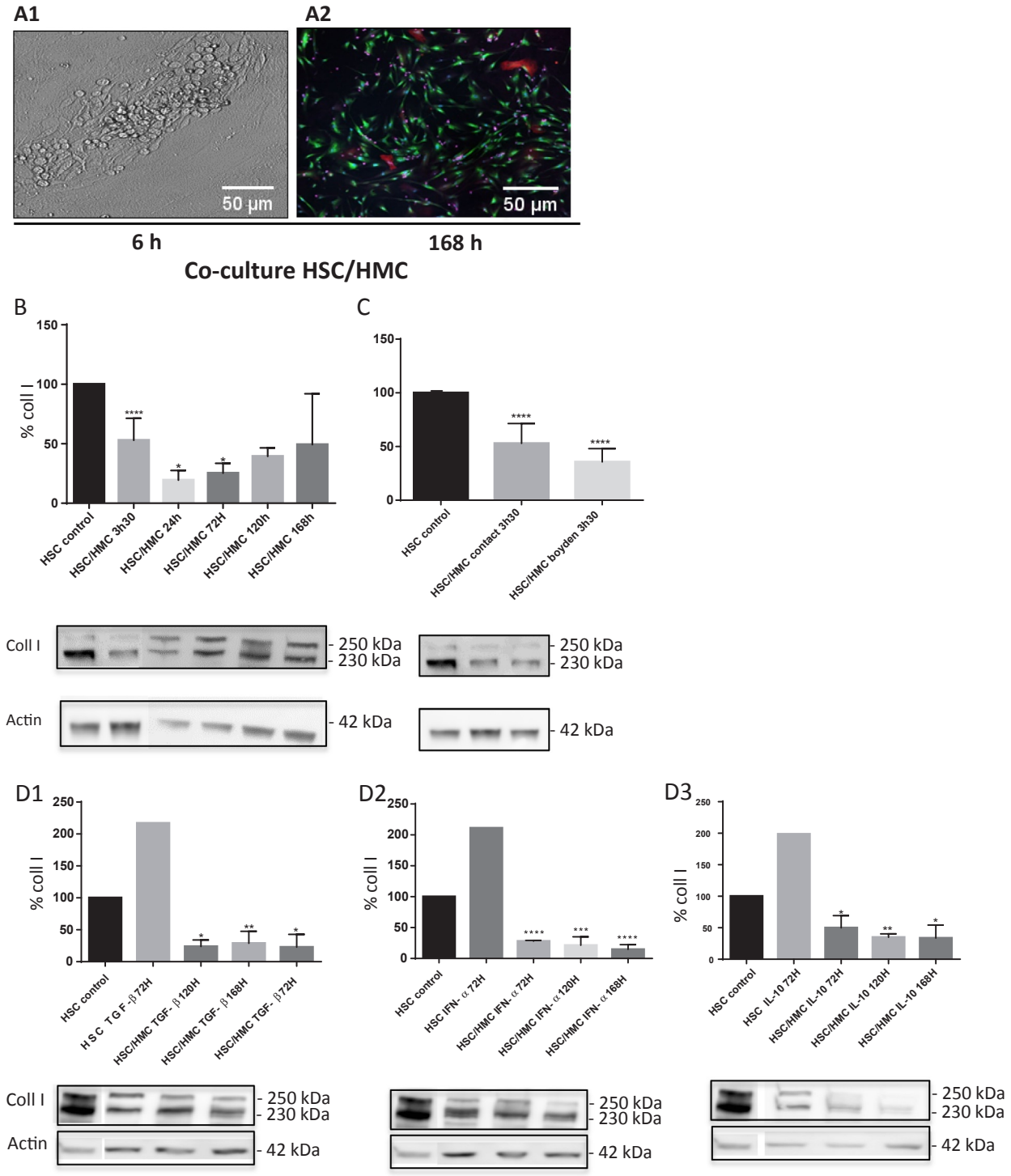


Fig. 2. Human mast cells co-cultured with human primary hepatic stellate cells. (A1) In co-culture, clusters of HMCs rapidly adhere to HSCs, as seen here at 6 h. (A2) The binding of HMCs to HSCs is also observed at 168 h. Mast cells, cell nuclei, collagen I and α-SMA are shown in pink, blue, green and red, respectively. (B) The collagen I (coll I) produced by HSCs, expressed as a percentage of that produced by HSCs alone (20 000 cells), was determined by the western blotting of co-cultures with HMCs at various time points: 3.5 h, 24 h, 72 h, 120 h and 168 h. At 3.5 h, the HSC/HMC ratio is 1:2.25, whereas, at the other time points it was 1:0.5. (C) The collagen I (coll I) produced by HSCs, expressed as a percentage of that for HSCs alone after densitometry of the band intensity, was evaluated by western blotting at 3.5 h during co-culture with HMC in conditions of direct contact ($n = 11$) or with a Boyden chamber separating HMCs and HSCs ($n = 6$). Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown. (D) The collagen I (coll I) produced by HSCs expressed as a percentage of that produced by HSCs alone after densitometry of the band intensity, was evaluated by western blotting during co-culture with HMCs, at various time points, in the presence or absence of 5 ng/ml TGF-β (D1), 50 ng/ml IFN-α (D2) and 50 ng/ml IL-10 (D3) ($n = 3$). Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown under the quantification by densitometry. The data shown are the means ± SD and the *p*-values for Student's *t* tests indicating the significance of differences between groups ($^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$; $^{****}P < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

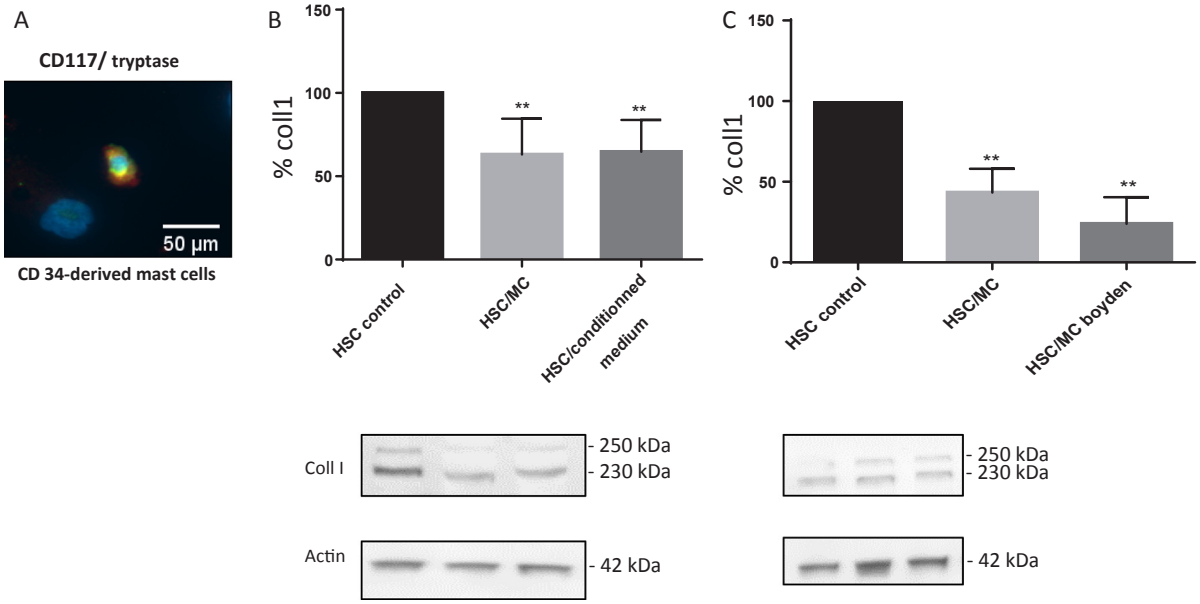


Fig. 3. Human CD34-positive cell-derived mast cells (MCs) have properties identical to those of HMC cells in co-culture with HSCs. (A) CD117/tryptase double-staining on a flexiPERM slide of CD34-positive cell-derived mast cells (MC) with HSCs. Nuclei stained with Hoechst stain are shown in blue, and labeling for CD117 and tryptase is shown in green and red, respectively. (B) The collagen I (coll I) produced by HSCs expressed as a percentage of that for HSCs alone was evaluated by western blotting at 3.5 h in co-cultures with CD34-positive cell-derived MCs in direct contact ($n = 5$) or during culture with mast cell-conditioned medium ($n = 5$). Results of Western Blot analysis of the different conditions with coll I (1/500) and actin (1/5000) under reducing conditions are shown under the quantification by densitometry (C) The collagen I (coll I) produced by HSCs, expressed as a percentage of that for HSCs alone, was evaluated by western blotting after 24 h of co-culture with HMCs in direct contact conditions ($n = 5$) or with a Boyden chamber separating the CD34-positive cell-derived mast cells from the HSCs ($n = 4$). Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown under the quantification by densitometry. The data shown are the means ± SD, and *p*-values for Student's *t* test indicate the significance of differences between groups ($^{**}P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Mast cell proteases degrade collagen I

For identification of the diffusible factors involved in collagen I degradation, we first incubated cells with inhibitors of the proteases known to be present in the granules of HMCs/mast cells, such as tryptase and chymotryptase. HMC cells were first incubated for 30 min with either APC (100 µM) or chymostatin (50 µg/ml). The pretreated HMC cells were then incubated with HSCs for 3.5 h. Comparison with an amount of solvent equivalent to that in which the protease inhibitors were dissolved showed that the inhibitors caused a significant partial reversion of the decrease in collagen I levels induced by APC or chymostatin (Fig. 4).

3.5. Functional effect of HLA-G on collagen I degradation

As we had previously shown that one third of the mast cells in patients with liver fibrosis express HLA-G [5], we investigated whether the HMCs expressed HLA-G during HMC/HSC co-culture. HLA-G was found to be expressed after long periods of co-culture (Fig. 5A), as in CD34-derived mast cells or mast cells purified from the bone marrow of patients with mastocytosis (Fig. 5B). We investigated the effect of recombinant HLA-G on HSC/HMC co-cultures, by incubating the complete soluble form of HLA-G, at a concentration of 1 µg/ml, with HSC/HMC co-cultures for 72 h. We found that the addition of exogenous recombinant HLA-G to the HSC/HMC co-culture resulted in significantly lower collagen I levels, about half those observed in the absence of HLA-G ($p < 0.01$; $n = 6$) (Fig. 5C1). We searched for the cellular target of HLA-G, by assessing its effects on HSCs alone. A significant effect was also observed with HSCs alone (Student's *t* test $p < 0.001$, $n = 6$) (Fig. 5C2).

We performed a dose-response experiment on HSC cells by adding a commercial purified recombinant HLA-G at 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml during 72 h (n = 3). The production of coll I decreased proportionally and significantly with the HLA-G concentration of 1, 0.5 and 0.25 µg/ml (Fig. 5D).

4. Discussion

In a previous study, we demonstrated the existence of an association between the number of mast cells and liver fibrosis and showed that a third of hepatic mast cells expressed HLA-G in patients with hepatitis C virus-induced fibrosis [5]. In this study, we localized the mast cells within fibrotic liver. Mast cells are present within the portal tracts and sinusoids of normal livers [18], but were found to be located within or near zones rich in collagen or activated hepatic stellate cells in fibrotic tissues. Mast cells involved in inflammatory responses release pro-inflammatory molecules, such as cytokines and chemokines, in addition to heparin, proteases and histamine [19,20], and would be able to communicate with cells in fibrotic tissues. In particular, the interactions between mast cells and hepatic stellate cells considered to make a major contribution to the excessive production of extracellular matrix in liver fibrosis [21] could potentially affect the outcome of liver fibrosis in the pathophysiological processes of liver diseases. We tested this hypothesis by establishing an *in vitro* model consisting of human HSCs obtained by primary culture from livers after surgery co-cultured with the human mast cell line HMC1.1, or with human mast cells derived from CD34-positive cells. This model is, to our knowledge, the first such model based on primary liver cells. Similar models have already been reported for human skin and human and mouse lung cells [22–24].

Human mast cells rapidly bound to HSCs in co-culture *in vitro,* suggesting that these two cell types have a high affinity for each other. This attraction may be due to the stem cell factor (SCF) released by HSCs, which regulates the migration and maturation of mast cell precursors originating from pluripotent hematopoietic stem cells in bone marrow [25]. Extracellular matrix molecules and the c-kit receptor have also been implicated in this attachment [26]. Surprisingly, the

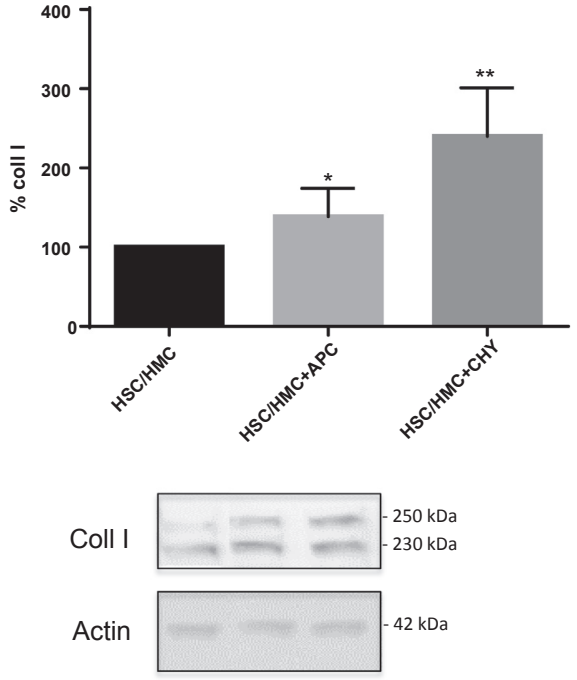


Fig. 4. Effect of specific inhibitors of mast cell proteases (APC and chymostatin) on the amount of collagen I produced in HSC/HMC co-cultures. The collagen I (coll I) produced by HSCs expressed as a percentage of that produced by HSCs alone was evaluated by western blotting at 3.5 h during co-culture with HMCs in the presence or absence of 100 μM APC ($n = 6$) and 50 μg/ml chymostatin ($n = 5$), specific inhibitors of respectively tryptase and chymotryptase, respectively. Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown under the quantification by densitometry. The data shown are the means ± SD, and the *p*-values for Student's *t* test indicate the significance of differences between groups ($^{*}P < 0.05$; $^{**}P < 0.01$).

interaction between mast cells and HSCs, in both long- and short-term co-culture led to significant collagen I degradation. We checked that the decrease in collagen I levels during HSC/HMC co-culture was not due to HSC cell death, by counting the numbers of HSCs at various time points during co-culture (Suppl. Fig. 1C). The number of HSCs was greater at 168 h than at co-culture initiation. A profibrotic effect of mast cells on fibroblasts due to their secretion of TGF-β has been reported in several studies. TGF-β is known to activate the synthesis of collagen I by HSCs, as confirmed by our experiments on HSCs alone and with other cytokines, including IL-10. We assessed the physiological relevance of the data we obtained with the HMC1.1 cell line, by repeating experiments with mast cells derived from CD34-positive human progenitor cells. Similar results were obtained.

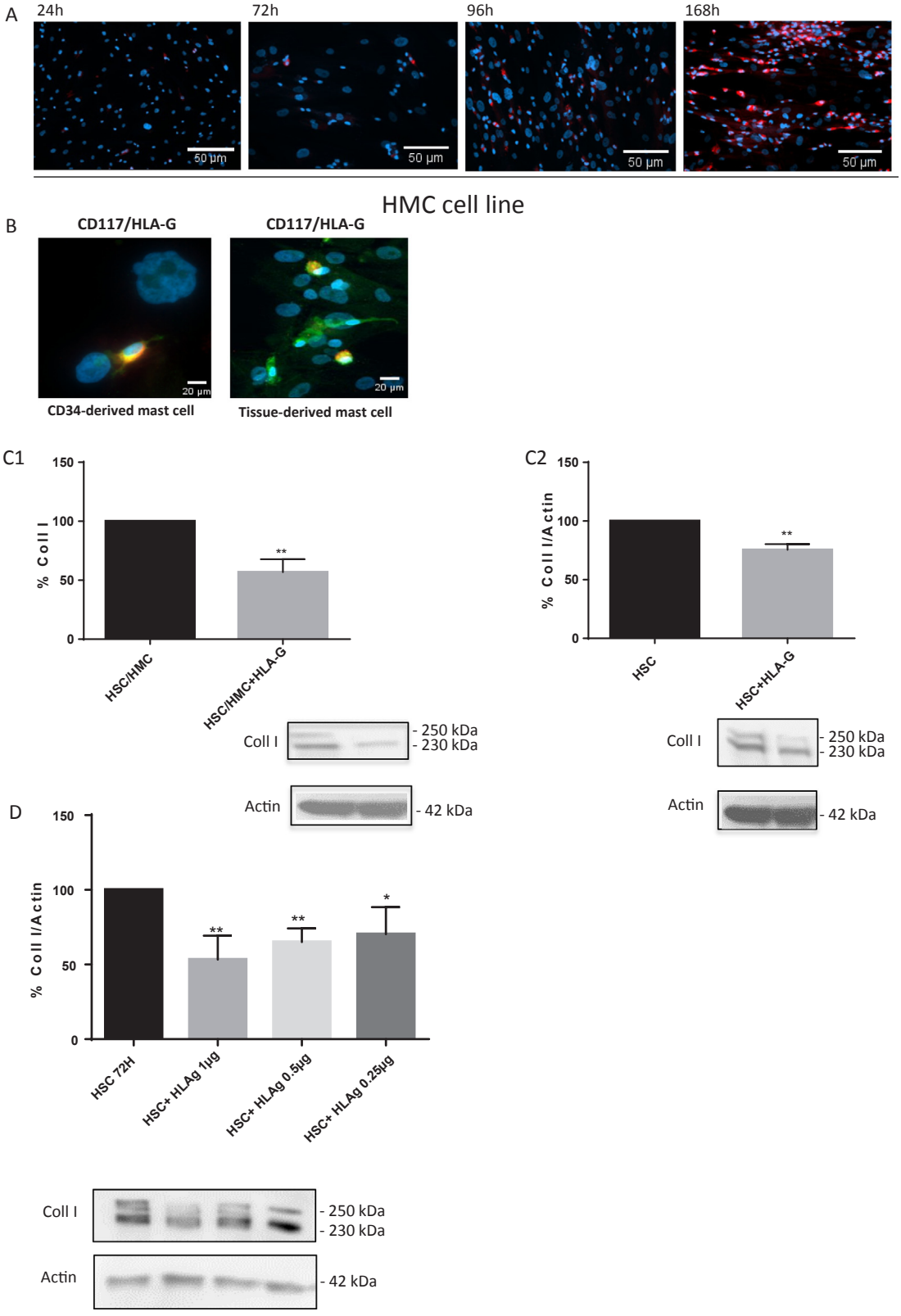
The differences between our results and those of previous studies suggest that reports of a profibrotic role for mast cells [23,24,27] may reflect differences in technical approach, or particular features of the liver microenvironment, including its tolerogenic nature. The cytokines and immune cells responsible for the specific features of the liver may, therefore, prime mast cells and HSCs, potentially accounting for the difference in the responses observed for the liver and for other tissues, such as skin or lung tissues. Consistent with our findings, a study in mast cell-deficient rodents reported that mast cells did not promote liver fibrosis [28], and another study described the initiation, by tryptase, of a cascade of metalloproteinases responsible for matrix degradation [29].

We then evaluated the molecular mechanisms underlying collagen I degradation. We assumed that soluble factors were involved, and we therefore evaluated the effect of inhibitors of the main proteases of mast cell secretory granules: tryptase and chymase. In liver fibrosis, the mast cells produce both tryptase and chymase (data not shown). We incubated co-cultures of HSCs and HMCs separately with APC 366 and chymostatin, which inhibit tryptase and chymase, respectively, and we observed a partial but significant decrease in collagen I degradation relative to the control [30]. This finding implies that mast cell proteases contribute to the anti-fibrotic action of mast cells. The tryptase inhibitor APC 366 has been shown to reduce hepatic fibrosis scores and collagen content in rats [31].

As we previously showed that one third of liver mast cell express HLA-G, and as HLA-G expression by mast cells is increased by microenvironmental cytokines, such as IFN-α and IL-10, we assessed HLA-G levels during co-culture with HSCs. We found that co-culture with HSCs strongly increased HLA-G expression by HMCs at late time points, and that HSCs displayed no HLA-G expression. Moreover, for confirmation of these results in normal primary cells rather than a tumor cell line, we repeated these experiments but replacing the HMC cell line with mast cells derived from CD34-positive cells and mast cells purified from the bone marrow of a patient suffering from mastocytosis. Similar results were obtained. We then assessed the effect of the soluble HLA-G protein in a HSC/HMC co-culture. We found that this protein significantly decreased collagen I levels. This finding sheds light on the role of the HLA-G expressed by mast cells in liver fibrosis. HLA-G or mast cells have a beneficial effect on liver fibrosis, and this conclusion is supported by the increase in HLA-G secretion by mast cells following stimulation with class I interferons, particularly interferons α, β and ω, which have antiviral properties.

It was previously shown that murine and human NK cells are selectively able to kill via NKP46 or NKG2D, early activated or senescence-activated HSCs cells expressing high levels of NK cell-activating ligands[32] therefore inhibiting liver fibrosis [33], in contrast to quiescent or fully activated HSCs. Moreover NK cells secrete IFNγ that has an anti fibrotic effect. The anti fibrotic action is exerted by a subset of NK cells characterized by the phenotype CD56 bright, CXCR3 + [34]. The interaction between NK cells and HSC is mediated by TLR9 [35]. In this context we suggest the involvement of HLA-G in the process of fibrogenesis. Indeed, HLA-G can be expressed by mast cells at basal state and its expression increases with the interaction HSC/mast cell (Fig. 5A, B). Activation of HSCs leads to production of MMPs that can degrade collagen. Indeed, we observed a partial restoration of Coll 1 decrease when batimastat, a pan metalloprotease inhibitor, is added to HSC cells. In contrast, no effect is observed in co-culture HSC/HMC, suggesting either the quantity of MMPs produced is too important to be inhibited or proteases such as serine proteases (trypsin like, elastase like, chymotrypsine like…) that are insensitive to inhibition by batimastat, are produced (Suppl. Fig. 2 E and F). Moreover, the MMP2 that is largely produced by HSC (data not shown) generates soluble HLA-G by cell surface proteolytic shedding [36]. The regulation via HLA-G is very complex because it is well known that HLA-G inhibits the cytotoxicity of NK cells [37] and their secretion of IFN gamma [38] therefore increasing fibrosis. In addition of this indirect action on fibrosis via NK cells, we show that HLA-G acts directly on HSC cells by decreasing their production of collagen I, therefore decreasing fibrosis. These direct and indirect properties respectively anti fibrotic and pro fibrotic of HLA-G show the complexity of the regulation of fibrogenesis resulting from the interaction HSC-Mast cells.

Mast cells expressing the HLA-G receptor, ILT2, are the most likely target of HLA-G. We therefore also assessed the effect of HLA-G on HSCs alone. We found that this molecule significantly decreased collagen I production by these cells, although the observed decrease was larger when the two types of cells (HMCs and HSCs) were cultured together. Thus, HMCs and HSCs act in synergy to exert their anti-fibrotic effects. HLA-G1 has been reported to decrease collagen I via decrease of TGF-β production significantly in LX2 cells, an immortal line of human HSCs [39]. In addition to its known immunosuppressive properties, a dimer form of HLA-G has been shown to have long-term effects [40] in mice with collagen-induced arthritis mice, and such effects could be



(caption on next page)

accounted for by the anti-inflammatory effects described in humans [41].

We provide here the first demonstration of the anti-fibrotic effect of HLA-G and mast cells in the human liver. We propose the following model to explain the role of mast cells in the pathogenesis of liver fibrosis. After persistent hepatic injury, regardless of the cause of the injury, the process of regeneration is associated with an inflammatory response attracting mast cells and other cell types. Mast cells activated by danger signals, such as the SCF secreted by HSCs, in turn produce various mediators of inflammation, which activate other leukocytes and

Fig. 5. Expression and role of HLA-G during the co-culture of HMCs/MCs with HSCs. (A) HLA-G labeling during HMC/HSC co-culture (ratio: 1:0.5) at various time points: 0 h, 72 h, 96 h, 192 h as assessed by immunofluorescence. Nuclei stained with Hoechst stain are shown in blue, and HLA-G stained with the 4H84 antibody is shown in red. (B) Expression of HLA-G by CD34-positive cell-derived mast cells or -bone marrow derived mast cells purified from patients suffering from medullary mastocytosis co-cultured with HSC cells (ratio: 1:1), as shown by triple immunofluorescence staining on flexiPERM slides. Some CD34-positive or tissue-derived MCs express CD117 and HLA-G (yellow). (C1) The collagen I (coll I) produced by HSCs, expressed as a percentage of that produced by HSCs alone, was evaluated by western blotting at 72 h in HMC/HSC co-cultures (1:1) in the presence or absence of 1 μg/ml purified protein HLA-G ($n = 6$). Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown under the quantification by densitometry. (C2) The collagen I (coll I) produced by HSCs, expressed as a percentage of that produced by HSCs alone, was evaluated by western blotting after 72 h of culture of HSCs alone, in the presence or absence of 1 μg/ml purified HLA-G protein ($n = 6$). The densitometry represented the ratio Coll1/actin found after densitometry of the bands obtained after Western blotting with actin or coll 1. Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown under the quantification by densitometry. The data shown are the means ± SD, and the *p*-values for Student's *t* test indicate the significance of differences between groups ($^{**}P < 0.01$). (D) HLA-G inhibits significantly Collagen I production in a dose dependent manner. A dose response was performed using purified recombinant protein HLA-G provided from Origen at 1; 0.5; 0.25 μg/ml. The collagen I (coll I) produced by HSCs, expressed as a percentage of that produced by HSCs alone, was evaluated by western blotting at 72 h in HSC cultures in the presence or absence of 1; 0.5 and 0.25 μg/ml purified protein HLA-G ($n = 3$). The quantity of Coll I was evaluated by the ratio coll1/actin, calculated from values obtained by densitometry of the bands of actin and coll I. Results of Western Blot analysis of the different conditions with coll I (1/500) and actin under reducing conditions are shown under the quantification by densitometry. The data shown are the means ± SD, and the *p*-values for Student's *t* test indicate the significance of differences between groups ($^{*}P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HSCs. The mast cells are attracted by HSCs, to which they adhere. This interaction increases HLA-G expression and secretion by mast cells. HLA-G acts in an autocrine/paracrine manner, by interacting with its receptor, ILT2, expressed on HSC or mast cells, and also with HSC cells, thereby decreasing collagen production by HSCs. The specific proteases present in mast cells, tryptase and chymase, may also partly account for this decrease in collagen I levels, but they play a more limited role than HLA-G.

5. Conclusions

Both HLA-G and mast cells exert immunomodulatory functions [42]. The immunosuppressive functions of mast cells, including their antifibrotic effects in particular, may be mediated by HLA-G in the environmental conditions prevailing in cases of established fibrosis [4], at least partly through its paracrine and autocrine interactions with its receptor, ILT2, on mast cells.

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Conflict of interest

The authors have no financial or commercial conflict of interest to declare.

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Appendix A. Supplementary material

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References

[1] E.Z. da Silva, M.C. Jamur, C. Oliver, Mast cell function: a new vision of an old cell, J. Histochem. Cytochem.: official journal of the Histochemistry Society 62 (10) (2014) 698–738.
[2] S.S. Craig, A.M. Irani, D.D. Metcalfe, L.B. Schwartz, Ultrastructural localization of heparin to human mast cells of the MCTC and MCT types by labeling with antithrombin III-gold, Lab. Invest.; a journal of technical methods and pathology 69 (5) (1993) 552–561.
[3] V. Jarido, L. Kennedy, L. Hargrove, et al., The emerging role of mast cells in liver disease, Am. J. Physiol. Gastrointest. Liver Physiol. 313 (2) (2017) G89–G101.
[4] L. Amiot, N. Vu, M. Samson, Biology of the immunomodulatory molecule HLA-G in human liver diseases, J. Hepatol. 62 (6) (2015) 1430–1437.
[5] L. Amiot, N. Vu, M. Rauch, et al., Expression of HLA-G by mast cells is associated with hepatitis C virus-induced liver fibrosis, J Hepatol. 60 (2) (2014) 245–252.
[6] L. Amiot, N. Vu, M. Samson, Immunomodulatory properties of HLA-G in infectious diseases, J. Immunol. Res. 2014 (2014) 298569.
[7] J. LeMaoult, J. Caumartin, M. Daouya, et al., Immune regulation by pretenders: cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells, Blood 109 (5) (2007) 2040–2048.
[8] M. Colonna, F. Navarro, T. Bellon, et al., A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells, J. Exp. Med. 186 (11) (1997) 1809–1818.
[9] M. Colonna, J. Samaridis, M. Cella, et al., Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules, J. Immunol. 160 (7) (1998) 3096–3100.
[10] P. Le Bouteiller, P. Fons, J.P. Herault, et al., Soluble HLA-G and control of angiogenesis, J. Reprod. Immunol. 76 (1–2) (2007) 17–22.
[11] J.J. Perez-Villar, I. Melero, F. Navarro, et al., The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell-mediated recognition of cells expressing HLA-G1, J. Immunol. 158 (12) (1997) 5736–5743.
[12] M.D. Gaca, J.A. Pickering, M.J. Arthur, R.C. Benyon, Human and rat hepatic stellate cells produce stem cell factor: a possible mechanism for mast cell recruitment in liver fibrosis, J. Hepatol. 30 (5) (1999) 850–858.
[13] R.C. Benyon, Mast cells in the liver, Clin. Exp. Allergy 29 (2) (1999) 155–158.
[14] J.H. Butterfield, D. Weiler, G. Dewald, G.J. Gleich, Establishment of an immature mast cell line from a patient with mast cell leukemia, Leuk Res. 12 (4) (1988) 345–355.
[15] T. Fujii, A. Ishitani, D.E. Geraghty, A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4, J. Immunol. 153 (12) (1994) 5516–5524.
[16] O. Schmetzer, P. Valentin, A. Smorodchenko, et al., A novel method to generate and culture human mast cells: peripheral CD34+ stem cell-derived mast cells (PSCMCs), J. Immunol. Methods 413 (2014) 62–68.
[17] G. Le Friec, B. Laupeze, O. Fardel, et al., Soluble HLA-G inhibits human dendritic cell-triggered allogeneic T-cell proliferation without altering dendritic differentiation and maturation processes, Hum. Immunol. 64 (8) (2003) 752–761.
[18] D.J. Farrell, J.E. Hines, A.F. Walls, P.J. Kelly, M.K. Bennett, A.D. Burt, Intrahepatic mast cells in chronic liver diseases, Hepatology 22 (4 Pt 1) (1995) 1175–1181.
[19] H. Virk, G. Arthur, P. Bradding, Mast cells and their activation in lung disease, Trans. Res.: the journal of laboratory and clinical medicine 174 (2016) 60–76.
[20] N. Landolina, R.S. Gangwar, F. Levi-Schaffer, Mast cells' integrated actions with eosinophils and fibroblasts in allergic inflammation: implications for therapy, Adv. Immunol. 125 (2015) 41–85.
[21] L. Atzori, G. Poli, A. Perra, Hepatic stellate cell: a star cell in the liver, Int. J. Biochem. Cell Biol. 41 (8–9) (2009) 1639–1642.
[22] M. Abel, H. Vliagoftis, Mast cell-fibroblast interactions induce matrix metalloproteinase-9 release from fibroblasts: role for IgE-mediated mast cell activation, J. Immunol. 180 (5) (2008) 3543–3550.
[23] M. Wygrecka, B.K. Dahal, D. Kosanovic, et al., Mast cells and fibroblasts work in concert to aggravate pulmonary fibrosis: role of transmembrane SCF and the PAR-

2/PKC-alpha/Raf-1/p44/42 signaling pathway, Am. J. Pathol. 182 (6) (2013) 2094–2108.
[24] Q. Zhang, A.P. Kelly, L. Wang, et al., Green tea extract and (-)-epigallocatechin-3-gallate inhibit mast cell-stimulated type I collagen expression in keloid fibroblasts via blocking PI-3K/AkT signaling pathways, J. Invest. Dermatol. 126 (12) (2006) 2607–2613.
[25] F. Grizzi, G. Di Caro, L. Laghi, et al., Mast cells and the liver aging process, Immun. Age.: I & A 10 (1) (2013) 9.
[26] Y. Koma, A. Ito, K. Watabe, et al., Distinct role for c-kit receptor tyrosine kinase and SgIGSF adhesion molecule in attachment of mast cells to fibroblasts, Lab. Invest.; a journal of technical methods and pathology 85 (3) (2005) 426–435.
[27] A. Trautmann, G. Krohne, E.B. Brocker, C.E. Klein, Human mast cells augment fibroblast proliferation by heterotypic cell-cell adhesion and action of IL-4, J. Immunol. 160 (10) (1998) 5053–5057.
[28] A. Sugihara, T. Tsujimura, Y. Fujita, Y. Nakata, N. Terada, Evaluation of role of mast cells in the development of liver fibrosis using mast cell-deficient rats and mice, J. Hepatol. 30 (5) (1999) 859–867.
[29] B.L. Gruber, M.J. Marchese, K. Suzuki, et al., Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation, J. Clin. Invest. 84 (5) (1989) 1657–1662.
[30] S.H. He, P. Chen, H.Q. Chen, Modulation of enzymatic activity of human mast cell tryptase and chymase by protease inhibitors, Acta Pharmacol. Sin. 24 (9) (2003) 923–929.
[31] J. Lu, B. Chen, S. Li, Q. Sun, Tryptase inhibitor APC 366 prevents hepatic fibrosis by inhibiting collagen synthesis induced by tryptase/protease-activated receptor 2 interactions in hepatic stellate cells, Int. Immunopharmacol. 20 (2) (2014) 352–357.
[32] C. Gur, S. Doron, S. Kfir-Erenfeld, et al., NKp46-mediated killing of human and mouse hepatic stellate cells attenuates liver fibrosis, Gut 61 (6) (2012) 885–893.
[33] B. Gao, S. Radaeva, Natural killer and natural killer T cells in liver fibrosis, Biochim. Biophys. Acta 1832 (7) (2013) 1061–1069.
[34] M. Eisenhardt, A. Glassner, B. Kramer, et al., The CXCR3(+)CD56Bright phenotype characterizes a distinct NK cell subset with anti-fibrotic potential that shows dysregulated activity in hepatitis C, PLoS ONE 7 (7) (2012) e38846.
[35] L. Abu-Tair, J.H. Axelrod, S. Doron, et al., Natural killer cell-dependent anti-fibrotic pathway in liver injury via Toll-like receptor-9, PLoS ONE 8 (12) (2013) e82571.
[36] R. Rizzo, A. Trentini, D. Bortolotti, et al., Matrix metalloproteinase-2 (MMP-2) generates soluble HLA-G1 by cell surface proteolytic shedding, Mol. Cell Biochem. 381 (1–2) (2013) 243–255.
[37] E.D. Carosella, I. Khalil-Daher, J. Dausset, N. Rouas-Freiss, HLA-G mediates protection from natural killer cytolysis: implications in immune tolerance, Transplant Proc. 31 (1–2) (1999) 1192–1193.
[38] F. Gros, F. Cabillic, O. Toutirais, A.L. Maux, Y. Sebti, L. Amiot, Soluble HLA-G molecules impair natural killer/dendritic cell crosstalk via inhibition of dendritic cells, Eur. J. Immunol. 38 (3) (2008) 742–749.
[39] A. Hodge, D. Lourensz, V. Vaghjiani, et al., Soluble factors derived from human amniotic epithelial cells suppress collagen production in human hepatic stellate cells, Cytotherapy 16 (8) (2014) 1132–1144.
[40] K. Kuroki, K. Hirose, Y. Okabe, et al., The long-term immunosuppressive effects of disulfide-linked HLA-G dimer in mice with collagen-induced arthritis, Hum. Immunol. 74 (4) (2013) 433–438.
[41] A. Takahashi, K. Kuroki, Y. Okabe, et al., The immunosuppressive effect of domain-deleted dimer of HLA-G2 isoform in collagen-induced arthritis mice, Hum. Immunol. 77 (9) (2016) 754–759.
[42] S.J. Galli, M. Grimbaldeston, M. Tsai, Immunomodulatory mast cells: negative, as well as positive, regulators of immunity, Nat. Rev. Immunol. 8 (6) (2008) 478–486.