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Short title: Overexpression of HLA-G in HCV-induced liver fibrosis

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g. List of abbreviations in the order of appearance: HCV: hepatitis C virus, TGF-β: transforming growth factor β, IL: interleukin, ILT Immunoglobulin-like transcript receptors, HIV: human immunodeficiency virus, HCMV: human cytomegalovirus, HBV: hepatitis B virus, IFN: interferon, OSM: oncostatin, MC: mast cell, HSC: hepatic stellate cell, SCF: stem cell factor, HCC: hepatocellular carcinoma

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Abstract:

Background and aims:

Infection by hepatitis C virus is a worldwide health problem. An inadequate Th2 cytokine response promotes the fibrosis-cirrhosis fate. Immune-modulating molecules favoring a Th2 profile, such as HLA-G molecules of the HLA class Ib family, may play a role in chronic hepatitis. HLA-G contributes to the escape of tumors, and their involvement in viral infections has been increasingly described. The aim of this work was to study the expression of HLA-G in the liver, its cellular source and its regulation in cases of chronic C hepatitis.

Methods: HLA-G cells in blocks of liver derived from patients infected with HCV were labeled by immunohistochemistry and enumerated. Double immunofluorescence allowed the identification of the cellular source. HLA-G secretion by a human mast cell line was quantified by ELISA after various stimulations. After treatment with IFN-α, real-time PCR was performed to determine the kinetics of cytokine expression profiles, followed by heat map clustering analysis.

Results: The number of HLA-G + cells was significantly associated with the area of fibrosis. For the first time, we identify the HLA-G+ cells as being mast cells. HLA-G secretion was significantly induced in human mast cells stimulated by IL-10 or interferons of class I. The transcriptome of the secretome of this cell line stimulated by IFN-α revealed that i) the HLA-G gene is upregulated late, ii) T lymphocytes and NK cells are recruited.

Conclusions: These findings suggest an autocrine loop in the genesis of HCV liver fibrosis, based on mast cells expressing HLA-G.

Introduction

Hepatitis C virus (HCV) infections are a worldwide public health problem. Infected patients may develop complications including cirrhosis, liver failure or hepatocellular carcinoma. The dynamics of fibrosis progression to cirrhosis is closely associated with chronic inflammation which is related to the efficiency of the immune response. Cytokines play a central role in the immune response and thus in orienting the fibrosis-cirrhosis fate [1].

In cases of chronic hepatitis, cytokines, including TGFβ and some interleukins such as IL-4 and IL-13, directly induce the production of extracellular matrix proteins and promote fibrosis [2]. Immune escape by HCV and progression of the infection towards chronicity are associated with the development of a Th2 response. These observations suggest that immune-modulating molecules favoring a Th2 cytokine profile play a major role in chronic viral hepatitis. These factors may include HLA-G, a member of the HLA class Ib family, and which has membrane-bound and soluble (sHLA-G) forms which are immunomodulatory molecules. HLA-G was initially described at the fetal-maternal interface of the cytotrophoblast [3, 4]. Its immunomodulatory function has since been described [5] and its involvement in various pathologies, especially in malignancies, has been demonstrated [6]. HLA-G exhibits suppressive properties in various cells by inhibiting (i) the cytolytic function of NK cells and CD8 T cells and also triggering their apoptosis, (ii) the allo response of T4 lymphocytes, (iii) the maturation and function of antigen-presenting cells, (iv) the proliferation and function of B cells and (v) angiogenesis. These functions are the consequence of the interaction between HLA-G and its receptors on immune cells, such as CD8, ILT2, ILT4, KIR2DL4 [7], or CD160 on endothelial cells [8]. HLA-G is also able to induce suppressor/regulatory cells [9, 10]. Its suppressive action is amplified by trogocytosis which allows effector cells to be converted into temporary regulatory cells [11]. HLA-G is involved in many malignant diseases, and participates in the escape of tumors by promoting a

Th2 cytokine environment and by inhibiting immune effector cells. Also, there is increasing evidence of the involvement of HLA-G in viral infections. Increased HLA-G expression, as membrane and/or soluble forms, has been evidenced in various types of viral infection, including those of human immunodeficiency virus (HIV) [12], human cytomegalovirus (HCMV) [13], neurotropic viruses (herpes virus and rabies virus) [14], influenza A virus [15] and hepatitis B virus [16].

However, the expression and role of HLA-G in human chronic hepatitis C have not been thoroughly investigated. We therefore assessed HLA-G expression in liver tissues infected with HCV, and then identified its cellular source and studied its regulation. We show that HLA-G is expressed by some cells in hepatic tissue infected with HCV and that the number of HLA-G-positive liver cells is significantly correlated with the area of fibrosis. In addition, we identified the cell type expressing HLA-G as a subpopulation of mast cells. Finally, we show that HLA-G expression by mast cells is regulated by interferons of class I.

Material and methods

Tissues samples

Liver biopsies from twenty chronically HCV-infected patients were retrospectively selected from the Biological Resource Center (BRC) of Rennes University Hospital. They were classified according to the METAVIR classification evaluating the severity of fibrosis (stage F0 to F4) and the inflammatory activity (A0 to A3). All biopsies were reviewed by an experienced pathologist. The characteristics of the patients are summarized in table 1. Non tumoral areas of the biopsies, away from the origin of the tumor, were used for all analyses.

#	Age	Gender	Metavir F stage	A stage	Etiology	Pathology
1	49	M	F4	A1	HCV	HCC
2	73	F	F4	A1	HCV	HCC
3	72	M	F1	A3	HCV	HCC
4	46	M	F4	A1	HCV + HBV + HIV	HCC
5	56	F	F4	A1	HCV	HCC
6	56	F	F4	A0	HCV	HCC
7	62	M	F4	A1	HCV	HCC
8	47	M	F4	A1	HCV	Transplantation
9	73	F	F4	A1	HCV	HCC
10	84	M	F4	A1	HCV	HCC
11	55	M	F2	A1	HCV	HCC
12	51	F	F4	A1	HCV	HCC
13	83	F	F4	A1	HCV	HCC
14	40	M	F4	A1	HCV + HBV	HCC
15	49	F	F4	A0	HCV + HIV	HCC
16	65	F	F4	A0	HCV	HCC
17	55	M	F4	A1	HCV	HCC
18	75	M	F3	A1	HCV + alcohol	HCC
19	75	M	F4	A2	HCV	No
20	67	M	F4	A1	HCV	HCC

Table 1. Characteristics of the HCV patients.

Cell cultures

The following cell lines were used as a positive control for HLA-G expression: Jeg3 (American Type Culture Collection (ATCC)), and LCL.721.221-G5, a B lymphoblastoid cell line transfected with HLA-G5 (D. Geraghty, Fred Hutchinson Cancer Research Center) as previously described [17]. The human mast cell line used, HMC1.1 (a generous gift from Dr Butterfield, Mayo clinic, Rochester), was established from a patient with mast cell leukemia as previously described [18]. HMC1.1 cells (10^6 cells /ml) were cultured in six-well culture plates in IMDM medium (Iscove Modified Dubelcco Medium, Gibco life technologies, Cergy Pontoise, France) at 37°C with 5% CO_2 for 48 hours. The effects of the presence of the

following cytokines, supplied by Peprotech-Tebu Bio (Neuilly, France), were tested: IL-33 (100ng/ml), IL-31 (50ng/ml), IL-4 (50ng/ml), IL-6 (50ng/ml), TNF-α (10ng/ml), Oncostatin M (OSM) (50ng/ml), IL-1β (10 ng/ml), IL-10 (50ng/ml), TGF-β (5ng/ml), IL-22 (10ng/ml), IFN-α (50ng/ml), IFN-β (50ng/ml), IFN-γ (50ng/ml), IFN-ω (10ng/ml), IFN-λ1 (50ng/ml), and IFN-λ2 (50ng/ml).

Immunohistochemistry

Standard histological staining, including Sirius red staining of collagen accumulation and HES coloration, was performed. Paraffin-embedded sections (4µm thick) were prepared, subjected to an antigen retrieval protocol, and incubated with primary antibody in a Ventana CT 09/021 automated machine (Ventana Medical systems, USA). Primary antibodies and dilutions used were: monoclonal mouse anti-human hepatocyte (clone OCH1E5, Dako, 1:600), mouse monoclonal anti-human HLA-G (Exbio, 4H84, 2 µg/ml), monoclonal mouse anti-human HLA-G (Exbio, MEM-G/1, 1µg/ml or 1:100), rabbit monoclonal anti-human CD3 (Thermo Scientific, SP7, 1:1500), mouse monoclonal anti-human CD163 recognizing liver macrophages (Novocastra, 10 D6, 1:500), polyclonal rabbit anti-human CD117/ckit recognizing myeloid cells (Dako, 1:200), and mouse monoclonal anti-human mast cell tryptase (clone AA1, Dako, 1:1000). Bound primary antibody was revealed with goat biotinylated anti-mouse or anti-rabbit IgG secondary antibody (Vector, ABCYS, Les Ulis, France, 1:700) and then diamino-benzidine (DAB Map Detection Kit, Roche, Meylan, France) and Mayer hematoxylin coloration.

After immunohistochemistry, the image of whole surface of the section was digitized at 20 X magnification using a NanoZoomer Digital Pathology 2.0 RS whole-slide scanner (Hamamatsu, Japan). An appropriate computer script analysis was developed and used for semi automatic enumeration of HLA-G+ cells and MCs or CD3+ T cells. The area of fibrosis

was evaluated by annotation. Fibrosis areas were drawn on the whole section and the surface area was calculated using "simple PCI" software. As direct HLA-G/ human mast cell tryptase double immunofluorescence was not possible because of antibody incompatibility, two double indirect immunofluorescence experiments, sharing CD117 (CD117/HLA-G and CD117/anti human mast cell tryptase) were performed in parallel on serial sections of paraffin-embedded sections from the same liver block. For immunofluorescence detection, fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch, Cy5- affinipure donkey anti-mouse IgG and Cy3-affinipure donkey anti-mouse IgG) were incubated with the sections for 1h at room temperature. Nuclei were counterstained with Hoechst stain (Molecular probe).

Specific Soluble HLA-G Enzyme-Linked Immunosorbent Assay

Soluble HLA-G concentrations in HMC1.1 culture-conditioned media were measured by specific sandwich enzyme-linked immunosorbent assay (ELISA) using MEM-G/9 (Exbio, Prague, Czech Republic; 10 μg/ml) and rabbit anti human beta-2 microglobulin (Dako, Trappes, France) as capture and revelation antibodies, respectively, as previously described [19] with minor modifications: after the second Ab, DAKO envision system HRP was added and tetramethylbenzidine/peroxide (TMB, R&D system) was used as the substrate.

Quantification of chemokine gene expression by real-time quantitative PCR

After various times of stimulation of HMC1.1 cells with IFN-α, total RNA was extracted and purified with the Nucleospin RNA II Kit ^{R} (Macherey-Nagel, Hoerdt, France). The High capacity cDNA archive Kit ^{R} (Applied Biosystems, Cergy Pontoise, France) was used for reverse transcription of aliquots of 3 μg of total extracted RNA following the manufacturer's instructions. Primers having the same melting temperature (Tm) were designed using Primer3 software. A large panel of cytokines and chemokines was tested using a large-scale qPCR

approach as shown in **Fig. 4C.** Real-time PCR for HLA-G was performed in parallel with the same samples and with the HLA-G-positive cell line, Jeg3, using primers designed according the same methodology (**Fig. S1.**). The ΔCT method was used for quantification of the housekeeping genes 18S, GAPDH, and SDHA used for multiple normalization as described previously [20]. To ensure that the primers produced a single and specific PCR amplification product, a dissociation curve was determined during PCR cycles. Dissociation curves were checked and in all cases showed a single dissociation peak; products were also tested by migration on 2% agarose gel to ensure that there was only one single PCR product in each reaction (**Fig. S2.**). The expression level of each gene was adjusted to the level of housekeeping gene mRNAs (18S mRNA, GAPDH mRNA and SDHA mRNA) and is expressed as the mean of the three ratios (mean of the gene to house keeping gene ratios). Gene expression was evaluated as a function of the time of stimulation, so results are expressed as $2^{-\Delta\Delta Ct}$ referred to as the fold induction in relation to the mean Ct at time 0.

qPCR data clustering

The Annotation, Mapping, Expression and Network (AMEN) suite of tools was used for cluster analysis [21]. After a log2 transformation of the qPCR data, genes with the most similar expression profiles were classified into four groups (k=4) using the Partition Around Medoids (PAM) algorithm implemented in AMEN.

Statistical analyses: Statistical analyses were performed with GraphPad prism software and included the Spearman test and Student's *t* test.

Results

HLA-G expression is correlated with HCV-induced liver fibrosis.

To test for HLA-G in HCV-induced liver fibrosis, we used immunohistochemistry with anti-HLA-G (clone 4H84) to probe serial sections of paraffin-embedded liver. The area of fibrosis was evaluated by sirius red staining and hepatocyte nodules identified by specific anti-hepatocyte labeling (clone OCH1E5) (**Fig. 1.A**). We observed a strong HLA-G staining of numerous cells in fibrosis septa but not in hepatocyte nodules (**Fig. 1.B**). Each HLA-G positive cell had a large and granular cytoplasm. Similar patterns of staining were obtained using another anti-HLA-G antibody (clone MEM-G/1) (**Fig. 1.C**). The HLA-G-positive cell count and the area of fibrosis across the whole surface of the liver section were significantly and positively correlated (Spearman's test, $r = 0.5017$, $p<0.05$, $n=20$) (**Fig. 1.D**). In this series, therapy, and in particular with IFN-α, did not influence this correlation: if the two treated patients in our series were excluded from the analysis, the HLA-G-positive cell count and the area of fibrosis across the whole surface of the liver section were still significantly correlated (Spearman's test, $r = 0.6102$, $p<0.05$, $n=18$).

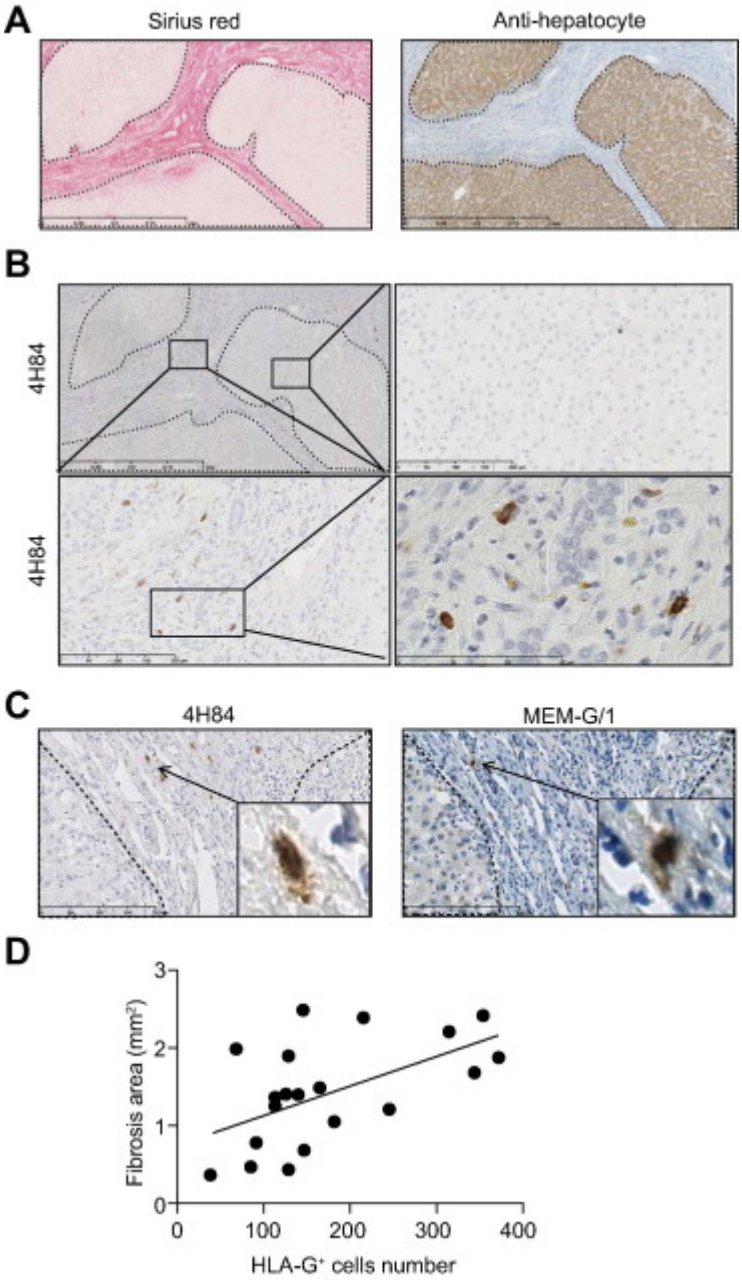

Fig. 1. **Expression of HLA-G in HCV liver fibrosis.** (A) Sirius red staining of collagen accumulation and staining of hepatocyte modules with anti-hepatocyte antibodies in a representative case. (B) 4H84 Labeling is located in fibrosis septa and not in areas composed of hepatocytes. (C) Comparison between 4H84 and MEM-G/1 staining. (D) Correlation between HLA-G+ cell numbers and fibrosis area expressed in mm^2 according to the Spearman test ($r = 0.5017$, $p < 0.05$, $n = 20$).

To investigate the relation between expression of HLA-G and inflammatory activity, the number of CD3 T cells detected over the entire surface of the liver section was counted. The number of HLA-G positive cells and that of CD3 T lymphocytes were positively and significantly correlated (Spearman‘s test, $r = 0.7273$, $p<0.05$) **(Fig. S.1.).**

HLA-G positive cells in HCV-induced liver fibrosis are mast cells.

To identify the cell type expressing HLA-G, we used anti-CD3, anti-CD163, anti-CD117, anti-mast cell tryptase (clone AA1) and anti-HLA-G (clone 4H84) antibodies to visualize T lymphocytes, liver macrophages (Kupffer cells), myeloid cells and mast cells (**Fig. 2.B**). Topographically and cytologically, we excluded T lymphocytes, macrophages and Kupffer cells as HLA-G positive cells. In contrast, cells presenting morphological features similar to those positive for HLA-G labeling stained positive for anti-CD117, a marker of mast cells. To test whether HLA-G is expressed by mast cells in liver fibrosis, we used anti-HLA-G and anti-mast cell (clone AA1) antibodies on serial liver sections from 20 patients. Consistent with the results with anti-CD117, the anti-mast cell antibody stained cells presenting morphological features similar to those showing HLA-G labeling, located in fibrotic areas. Mast cell counts and HLA-G-positive cell counts were significantly correlated (Spearman test: r= 0.7083, p =0.0005, n= 20) (**Fig. 2.C**), with 15 ± 7 HLA-G positive cells and 51 ± 34 mast cells per 1 mm^2 of fibrotic area.

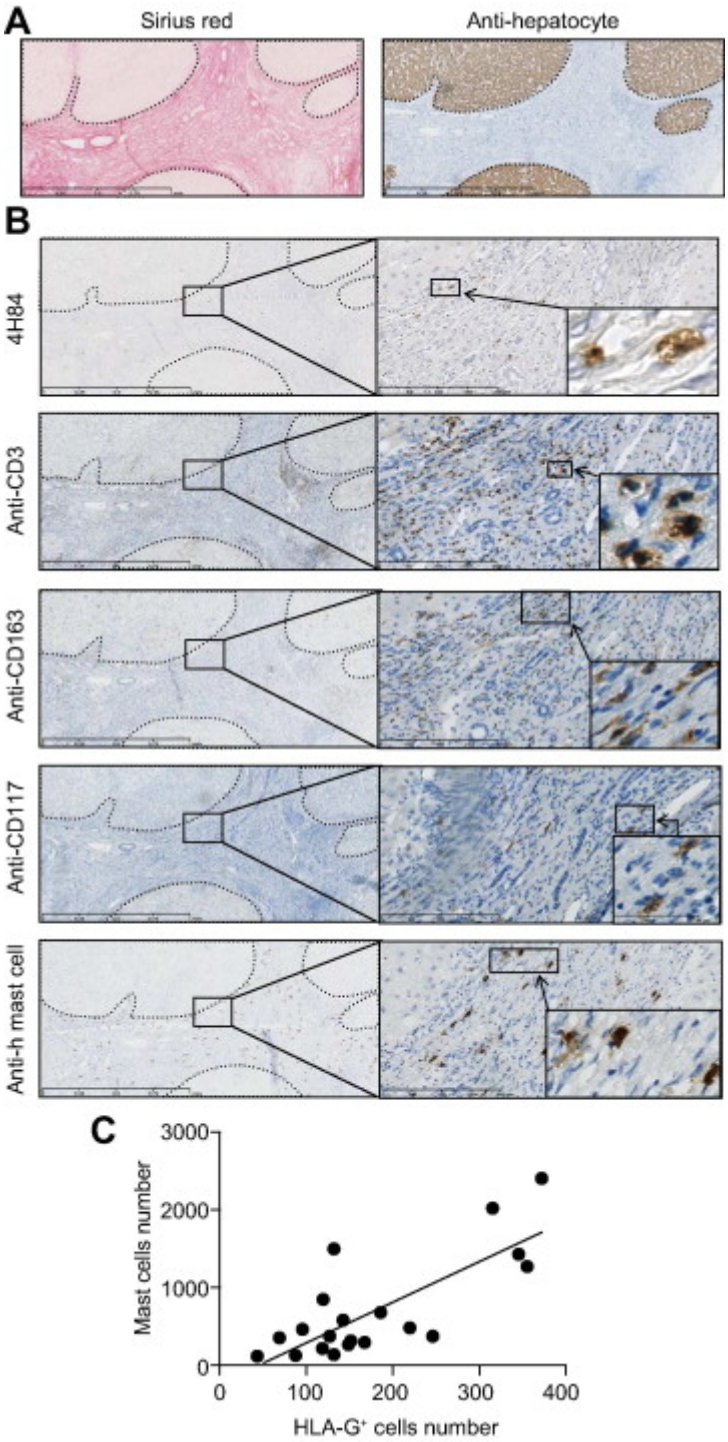

Fig. 2. **Pattern of HLA-G expression with morphological characteristics in human HCV fibrosis liver.** (A) Representative Sirius red staining of collagen accumulation and staining of hepatocyte modules with anti-hepatocyte Ab. (B) Representative immunohistochemical analysis of HLA-G, CD3, CD163, CD117, and human mast cells in fibrosis and hepatocyte areas. (C) Positive correlation between the number of HLA-G+ cells and the number of mast cell according Spearman test ($r = 0.7083$, $p < 0.01$, $n = 20$).

The commercially available antibodies are not compatible for use in straightforward double immunostaining experiments. Therefore, to confirm that the cells expressing HLA-G in liver fibrosis are hepatic mast cells, we performed two double indirect immunofluorescence tests with one shared antibody, anti-CD117, on pairs of serial sections **(Fig. 3.B).** The CD117-positive cells expressed both HLA-G and tryptase. This demonstrates that a subpopulation of hepatic human mast cells is HLA-G positive (defined as HLA-G+CD117+tryptase+) (**Fig. 3. B1 and B2**).

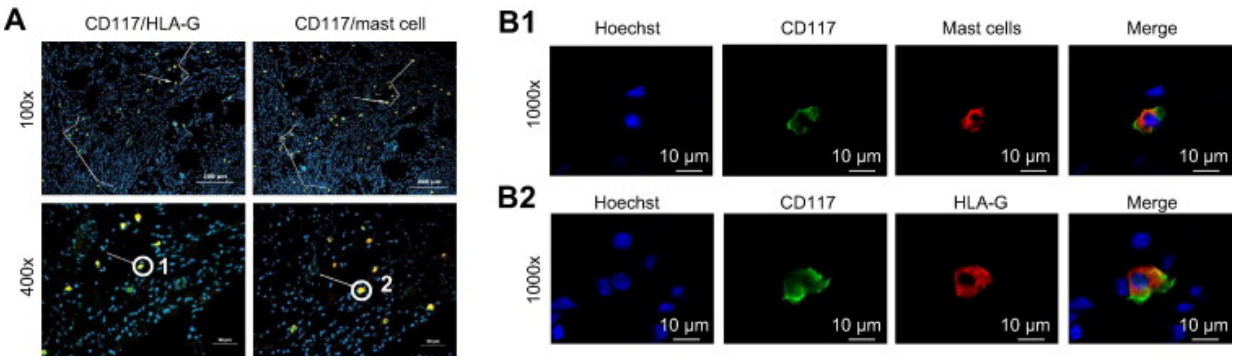

Fig. 3. **HLA-G+ liver cells identified as mast cells by double immunofluorescence.** (A) Double staining for CD117/HLA-G and CD117/human-mast cell tryptase was performed on pairs of serial sections from paraffin-embedded liver blocks. The same double-positive cell stained yellow, indicated by an arrow, is first identified within a region of double-positive stained cells (at magnification 100× and 400×) with CD117/HLA-G (labeled 1) and CD117/human mast cell tryptase (labeled 2). (B) B1 and B2 at magnification 1000×. Nuclei were stained blue with Hoechst 33348; CD117 was identified by green fluorescence and HLA-G or mast cells by red fluorescence; CD117 colocalized with HLA-G and CD117 also colocalized with h-mast cells. Thus, HLA-G co-localized with h-mast cells.

Soluble HLA-G secretion is regulated by type I interferons in mast cells

To assess the regulation of HLA-G secretion by mast cells, we used the human mast cell line HMC1.1 and cultures of these cells were induced with various cytokines known to be associated with the immunopathology of HCV. After 48 hours of stimulation, conditioned medium was tested for secreted HLA-G by specific ELISA. IL-33, IL-31, IL-4, IL-6, TNFα, OSM, IL-1β, TGFβ and IL-22 did not increase HLA-G secretion (**Fig. 4.A**). IL-10 induced a significant increase of the HLA-G concentration in the medium (7.8 ng/ml versus 4.0 ng/ml in unstimulated controls, n=5; Student's *t* test: $p<0.05$). Various interferons were tested, and the

class I interferons IFN-α, IFN-β and IFN-ω induced a significant increase of HLA-G secretion (11.7 ng/ml for IFN-α and IFN-β, and 15.3 ng/ml for IFN-ω versus 3.0 ng/ml for controls) whereas the class II interferon IFN-γ and class III interferons IFN-λ1 and IFN-λ2 had no significant effects (**Fig. 4.B**).

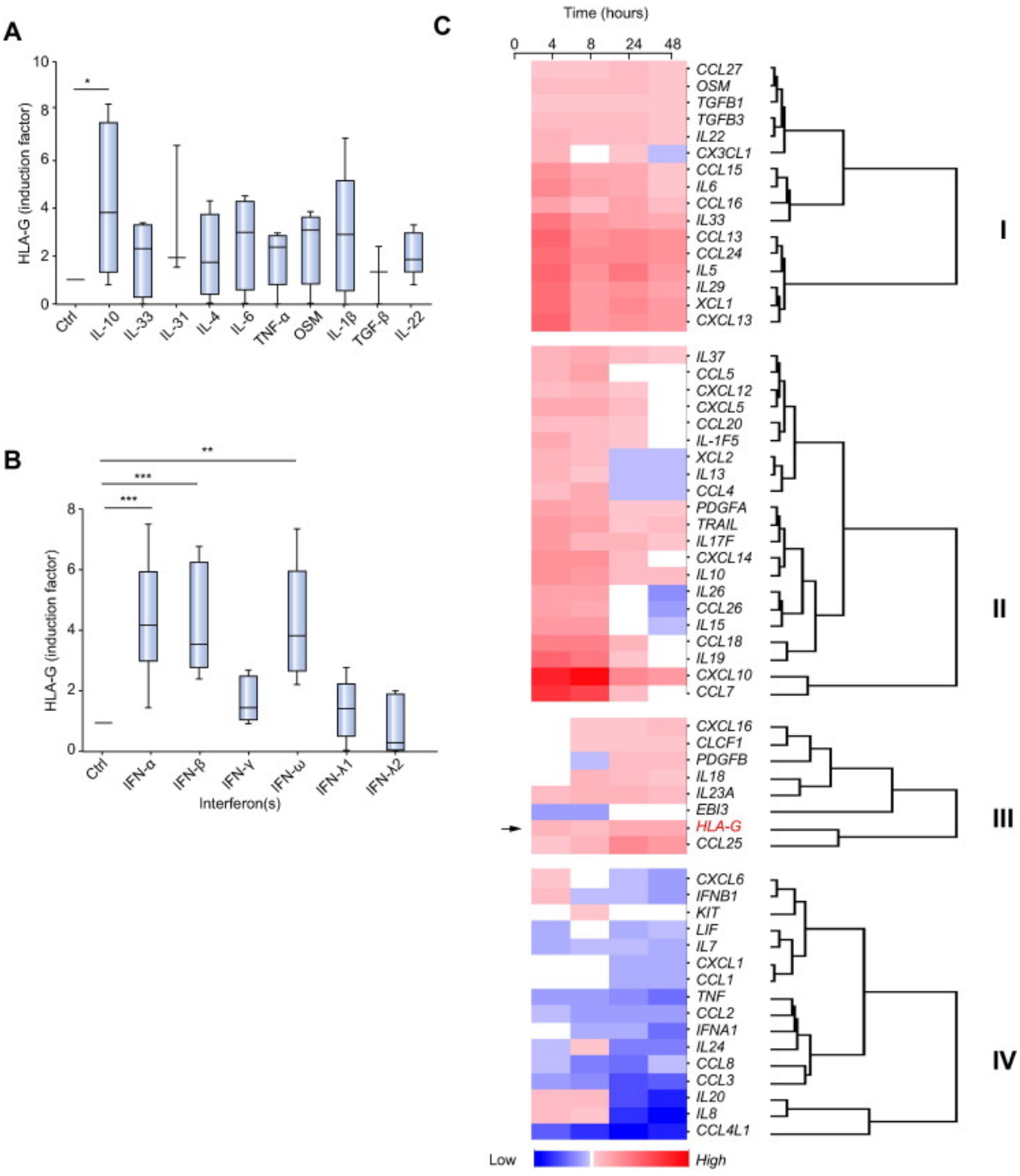

Fig. 4. **Regulation of HLA-G expression by the human mast cell line HMC1.1.** The control (Ctrl) corresponding to the cell line before any stimulation is arbitrarily assigned a value of 1. (A) Stimulation of HMC1.1 by various cytokines: IL-10, IL-33, IL-31, IL-4, IL-6, TNF-α, OSM, IL-1β,

IL-22. Results from at least three independent experiments are presented. Significant expression was noted (p <0.05). (B) Stimulation of HMC1.1 by various interferons of the three classes: type I interferons IFN-α,β,ω, the type II interferon IFN-γ, and the type III interferons IFN-λ1 and IFN-λ2. Significant expression was noted thus: p <0.001, p <0.0001. (C) Cluster analysis of cytokine gene expression by HMC1.1 after various times of stimulation with IFN-α (4 h, 8 h, 24 h, and 48 h) relative to the baseline value (0 h) before addition of IFN-α. Panel C shows a false-color heatmap of genes (y-axis) having an altered expression pattern after IFN-α stimulation at the time points given (x-axis). Genes are grouped into four clusters (termed I, II, III, and IV) according to their relative expression levels at different time points. Dendrogram plots based on the hierarchical clustering of each individual group are displayed to the right. Briefly, groups I, II, and III and include genes upregulated after IFN-α stimulation but differing according to the time of increase, whereas group IV represents genes downregulated by IFN-α stimulation. The color scale bar to the bottom of the heatmap indicates $\log_2$-ratio (blue = down-regulation, white = no change in expression, red = up-regulation).

To determine whether this regulation by IFN-α involved only soluble forms or membrane-bound forms, HLA-G expression on the surface of HMC1.1 cells was determined by flow cytometry with the antibody 87G, a specific conformational anti-HLA-G antibody. This expression was determined after 0, 4, 8, 24, and 48 hours of IFN- α stimulation, and intracellular expression was studied at the same timepoints by immunocytochemistry and western blotting (**Fig. S.3.**). We found strong cell surface expression of HLA-G associated with intracellular expression by HMC1.1 cells at baseline (hour 0), which was confirmed by western blot analysis. Cell surface expression as assessed by flow cytometry did not significantly increase with stimulation with IFN- α, whether scored as % positivity or MFI (mean of MFI ratio =6, range 5.7 to 6.8). Immunohistochemical observation revealed that the extent of labeling of the HMC1.1 cells was very diverse: some cells were strongly labeled, some partially labeled and some only weakly labeled (**Fig. S.3**).

In toxicity and proliferation assays, there were no significant differences in the numbers of HMC1.1 cells between the IFN-α-stimulated and control conditions (**Fig. S. 4**). Furthermore, the response of HMC1.1 cells to IFN-α treatment was dose- and time-dependent (**Fig. S.4.).**

To study the profile of cytokines expressed by mast cells concomitantly with HLA-G during IFN-α stimulation, the transcripts of 81 cytokine genes were assayed by real-time PCR after 4, 8, 24 and 48 hours of stimulation by IFN-α. Cluster analysis identified four groups of genes

in addition to the group of 19 genes not responsive to IFN-α (IL-1α, IL-1β, IL-2, IL-3, IL-17; IL-17B, IL-17D, IL21, IL-12p35, IL-12p40, IL-31, IFN-γ, CCL17, CCL19, CCL21, CCL22, CXCL1, CXCL2 and CXCL5) (data not shown). The first group, termed cluster I, included 16 genes that were up-regulated by IFN-α in a continuous manner; OSM, IL-33, TGF-β and IL-6 belonged to this cluster I (**Fig. 4.C**). The second group (cluster II) included 21 genes up-regulated at 4 and 8 hours but with a decreased expression from 24 hours (**Fig. 4C**). The third group (cluster III) contained eight genes, including HLA-G, that were up-regulated by IFN-α at late time points (**Fig. 4.C**). Finally, the fourth group (cluster IV) included 16 genes that were down-regulated during IFN-α stimulation (**Fig. 4.C**). Thus, nine cytokines (CCL25, CXCL10, XCL1, CCL24, CXCL13, IL-29, IL-33, IL-5, CCL13) were up-regulated by IFN-α concomitantly with HLA-G in the mast cell line HMC1.1 (Table 2).

	Induction	**Receptor**	**Target cells**
CCL25	6.85	CCR9	T lymphocytes
CXCL10	7.38	CXCR3	T lymphocytes, NK cells
XCL1	7.65	XCR1	?
CCL24	9.23	CCR3	T lymphocytes, NK cells
CXCL13	7.81	CXCR5	B lymphocytes
IL-29/IFN-λ2	5.17	IFN-λR	Host defense against virus
IL-33	3.28	IL-33R	T helper, mast cells
IL-5	7.85	IL-5R	B cells
CCL13	9.72	CCR2	monocytes

Table 2. Chemokine and cytokine genes induced in mast cells by IFN-α stimulation, and their receptors and cell targets.

Discussion

HLA-G was initially described at the maternal-fetal interface [4]. It was subsequently found to contribute to tumor escape due to its tolerogenic properties involving the inhibition of various immune-competent cells such as NK cells, T lymphocytes, B lymphocytes and dendritic cells [6]. Various recent evidence shows that HLA-G is induced by viral infection.

Blood HLA-G titers are high in most cases of HIV [12], HCMV [22], HBV [16] and HCV [23] infection, and in the majority of viral infections, HLA-G is expressed by blood cells, notably monocytes and/or T lymphocytes [15].

Here, we show that in human livers infected by HCV, HLA-G is associated with the progression of fibrosis; the number of HLA-G-positive cells correlated significantly with the area of fibrosis on tissue sections. By immunohistochemical analysis, we identified the cell type expressing HLA-G in liver samples from HCV-positive patients. In contrast with other reports of analyses of tumors and viral infections, we found that the monocytic lineage, i.e. monocytes [24], dendritic cells [25] and T lymphocytes [26, 27], in HCV-induced liver fibrosis does not express HLA-G. Also, we did not find that hepatocytes are specifically stained (**Fig. 1** and **Fig. 2**), as previously reported by Donadi et al [28], possibly due to the lower concentration of antibody 4H84 used. Morphological observations (the large and granular cytoplasm) and immunostaining approaches with anti CD117 and anti-mast cell tryptase, indicate that cells expressing HLA-G in HCV-induced liver fibrosis are mast cells. Also, the number of HLA-G positive mast cells correlated with the area of fibrosis. It was recently shown that the number of mast cells increases during the course of chronic HCV-related disease [29], [30]. Mast cells play a role in acute and chronic inflammation promoting fibrosis in heart [31], lung [32] and kidney [33] and in the secretion of histamine, heparin and IL-4 able to enhance fibroblast proliferation and collagen deposition [34]. Mast cells also inhibit degradation of the extra cellular matrix by producing tissue inhibitors of metalloproteinase [29]. Mast cells have traditionally been linked to immunoglobulin E-hypersensitivity, asthma and host defense against parasites [35]. As is the case in almost all tissues, in HCV-induced liver fibrosis, the hepatic mast cells may serve as sentinels for immune responses and directly promote the activation of the liver fibrosis through the proliferation of hepatic stellate cells (HSC) [34]. HSC can produce TGF-β which is a potent

mast cell chemoattractant [36] [37] and this may explain the presence of mast cells in liver fibrosis. Other cytokines, such as IL-4 and IL-33, involved in liver fibrosis may also explain the chemoattractant and activation of mast cells in liver [51, 52].

We used the HMC1.1 cell line to study the regulation of HLA-G by 16 cytokines. We identified IL-10 as a potential inducer of HLA-G in mast cells. This is consistent with previous reports of induction of HLA-G expression in trophoblasts and monocytes [38] and in some tumoral pathologies [39]. Furthermore, it was recently shown that murine mast cells can secrete IL-10 [40]. IL-10 is an immunoregulatory cytokine that plays a key role in progression to chronic HCV infection [41]. Interferons of classes II and III had no effect on the secretion of HLA-G by mast cells. By contrast, class I IFNs, including IFNα, β and ω, induced secretion of HLA-G. There is an apparent discrepancy between the increased production of the soluble form and absence of change in the abundance of the membrane-bound form after IFN-α treatment as evaluated by flow cytometry. This could be a consequence of the susceptibility of HLA-G1 to cleavage by metalloproteinases resulting in soluble forms detected by ELISA but not on the cell surface by flow cytometry. Indeed, IFN-α may favor proteolytic cleavage by acting on the cleavage site. IFN α and β are known to induce HLA class I expression in monocytes and many tumoral cells by binding with the interferon-stimulated response element (IRSE) motif [42]. IFN-α and β, and class III IFNs are induced by HCV and constitute the main anti-HCV viral response of the host. Thus, during the evolution of HCV-induced liver fibrosis, IFN-α produced in response to HCV attack may induce HLA-G in mast cells in areas of fibrosis.

To investigate the cytokine microenvironment, we measured cytokine and chemokine transcripts expressed concomitantly with that of HLA-G after IFNα induction. CCL25, CXCL10, XCL1, CCL24, CXCL13, IL-29, IL-33, IL-5 and CCL13 were up-regulated by IFN-α concomitantly with HLA-G, suggesting that these cytokines and chemokines are

present in or around areas of fibrosis. Using various investigative approaches (immunohistochemistry, quantitative PCR and DNA microarrays), the production of most of these cytokines has already been found to be associated with HCV liver fibrosis [1, 43] and the expression of some of them by mast cells has been reported [44-47]. The expression of these nine cytokines concomitantly with HLA-G defines leukocyte recruitment involving T lymphocytes, NK cells and to a lesser degree B lymphocytes and monocytes (table 2); all these cell types are known to infiltrate HCV-induced liver fibrosis. Furthermore, the human mast cell line expresses an HLA-G receptor, ILT2 (**Fig. S3**), suggesting an autocrine loop. This type of regulation with concomitant HLA-G and ILT4 expression has been suggested in dendritic cells or monocytes [5]. HLA-G can control the expression of it own receptors on antigen presenting cells (APC), T cells and NK cells [48]. Similarly, it was shown that HLA-G acts in an autocrine regulatory feedback loop in HIV infection: it is secreted by dendritic cells and regulates the functional characteristics of these cells via interaction with its receptor, ILT4 [49].

In conclusion, our data show clearly that HLA-G is expressed by mast cells in fibrotic areas of liver from patients infected with HCV, and that IFN-α produced in response to HCV infection up-regulates the secretion of HLA-G. However, the function of HLA-G during HCV infection remains to be described. HLA-G may promote viral escape from the immune system, as in cancers, by inhibiting both adaptive and innate immunity, such that HCV-infected cells would be protected and viral progression favored. Another possibility is that, like in septic shock, HLA-G expression reflects an appropriate and efficient response to inflammatory processes occurring during viral infection. Indeed, HLA-G may have different roles according to the stage of infection: deleterious at the beginning of the chronic process of hepatitis, and protective during the stage of established fibrosis. The effects need to be interpreted in

relation with those of the mast cells, the cells expressing HLA-G. Further work is needed to elucidate the respective roles of HLA-G and mast cells in the genesis and the evolution of liver fibrosis.

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Table 1: The characteristics of the HCV patients

#	Age	Gender	Metavir F stage	Metavir A stage	Etiology	Pathology
1	49	M	F4	A1	HCV	HCC
2	73	F	F4	A1	HCV	HCC
3	72	M	F1	A3	HCV	HCC
4	46	M	F4	A1	HCV + HBV + HIV	HCC
5	56	F	F4	A1	HCV	HCC
6	56	F	F4	A0	HCV	HCC
7	62	M	F4	A1	HCV	HCC
8	47	M	F4	A1	HCV	Transplantation
9	73	F	F4	A1	HCV	HCC
10	84	M	F4	A1	HCV	HCC
11	55	M	F2	A1	HCV	HCC
12	51	F	F4	A1	HCV	HCC
13	83	F	F4	A1	HCV	HCC
14	40	M	F4	A1	HCV + HBV	HCC
15	49	F	F4	A0	HCV + HIV	HCC
16	65	F	F4	A0	HCV	HCC
17	55	M	F4	A1	HCV	HCC
18	75	M	F3	A1	HCV + alcohol	HCC
19	75	M	F4	A2	HCV	No
20	67	M	F4	A1	HCV	HCC

Table 2: Chemokine and cytokine genes induced in mast cells by IFN-α stimulation, and their receptors and cell targets.

	Induction	Receptor	Target cells
CCL25	6.85	CCR9	T lymphocytes
CXCL10	7.38	CXCR3	T lymphocytes, NK cells
XCL1	7.65	XCR1	?
CCL24	9.23	CCR3	T lymphocytes, NK cells
CXCL13	7.81	CXCR5	B lymphocytes
IL-29/IFN-λ2	5.17	IFN-λR	Host defense against virus
IL-33	3.28	IL-33R	T helper, mast cells
IL-5	7.85	IL-5R	B cells
CCL13	9.72	CCR2	monocytes

Legends

Figure 1. Expression of HLA-G in HCV liver fibrosis. (A) Sirius red staining of collagen accumulation and staining of hepatocyte modules with anti-hepatocyte antibodies in a representative case. (B) 4H84 labeling is located in fibrosis septa and not in areas composed of hepatocytes. (C) Comparison between 4H84 and MEM-G/1 staining. (D) Correlation between HLA-G+ cell numbers and fibrosis area expressed in mm^2 according to the Spearman test ($r=0.5017$, $p<0.05$, $n=20$).

Figure 2. Pattern of HLA-G expression with morphological characteristics in human HCV fibrosis liver (A) Representative Sirius red staining of collagen accumulation and staining of hepatocyte modules with anti-hepatocyte Ab. (B**)** Representative immunohistochemical analysis of HLA-G, CD3, CD163, CD117, and human mast cells in fibrosis and hepatocyte areas. (C) Positive correlation between the number of HLA-G+ cells and the number of mast cell according Spearman test ($r=0.7083$, $p<0.01$, $n=20$).

Figure 3. HLA-G+ liver cells identified as mast cells by double immunofluorescence. (A) Double staining for CD117/HLA-G and CD117/human-mast cell tryptase was performed on pairs of serial sections from paraffin-embedded liver blocks. The same double-positive cell stained yellow, indicated by an arrow, is first identified within a region of double-positive stained cells (at magnification 100 x and 400x) with CD117/HLA-G (labeled 1) and CD117/human mast cell tryptase (labeled 2), (B) B1 and B2 at magnification 1000. Nuclei were stained blue with Hoechst 33348; CD117 was identified by green fluorescence and HLA-G or mast cells by red fluorescence; CD117 colocalized with HLA-G and CD117 also colocalized with h-mast cells. Thus, HLA-G co-localized with h-mast cells.

Figure 4. Regulation of HLA-G expression by the human mast cell line HMC1.1

The control (CTRL) corresponding to the cell line before any stimulation is arbitrarily assigned a value of 1. (A) Stimulation of HMC1.1 by various cytokines: IL-10, IL-33, IL-31, IL-4, IL-6, TNF−α, OSM, IL-1β, IL-22. Results from at least three independent experiments are presented. * Significant expression was noted ($p<0.05$). (B) Stimulation of HMC1.1 by various interferons of the three classes: type I interferons IFN−α, β, ω, the type II interferon

IFN-γ, and the type III interferons IFN- λ1 and IFN-λ 2. Significant expression was noted thus: $p<0.05$ = *, $p<0.001$ = **, $p<0.0001$=***. (C) Cluster analysis of cytokine gene expression by HMC1.1 after various times of stimulation with IFN-α (4h, 8h, 24h, and 48) relative to the baseline value (0h) before addition of IFN-α. Panel C shows a false-color heatmap of genes (y-axis) having an altered expression pattern after IFN-α stimulation at the time points given (x-axis). Genes are grouped into four clusters (termed I, II, III and IV) according to their relative expression levels at different time points. Dendrogram plots based on the hierarchical clustering of each individual group are displayed to the right. Briefly, groups I, II and III include genes upregulated after IFN-α stimulation but differing according to the time of increase, whereas group IV represents genes downregulated by IFN-α stimulation. The color scale bar to the bottom of the heatmap indicates log2-ratio (blue=down-regulation, white=no change in expression, red=up-regulation).