

# Analysis of interleukin (IL)-1 $\beta$ IL-1 receptor antagonist, soluble IL-1 receptor type II and IL-1 accessory protein in HCV-associated lymphoproliferative disorders

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**Abstract.** Hepatitis C virus (HCV) causes hepatitis, liver cirrhosis and hepatocellular carcinoma, and may also induce type II mixed cryoglobulinemia syndrome (MC), a disease characterized by clonal B-cell lymphoproliferations that can evolve into non-Hodgkin's lymphoma (NHL). Interleukin-1 (IL-1) is a cytokine that plays an important role in initiating the cascade of events of immunoinflammatory responses through costimulation of T lymphocytes, B-cell proliferation, induction of adhesion molecules and stimulation of the production of other inflammatory cytokines. The role of IL-1 in immunoinflammatory responses is highlighted by the presence of endogenous regulators (IL-1 receptor antagonist, soluble receptors type I and II, human IL-1 accessory protein) that, when secreted into the blood stream may serve as endogenous regulators of IL-1 action. The aim of this study was to evaluate whether abnormalities in the blood levels of IL-1 $\beta$  IL-1 receptor antagonist, soluble IL-1 receptor type II and human IL-1 accessory protein in HCV<sup>+</sup> patients are associated with development of MC and/or NHL. Relative to healthy controls, we observed: i) an increase in the circulating levels of IL-1 $\beta$  in HCV<sup>+</sup> patients simultaneously affected by NHL; ii) increased levels of IL-1 accessory protein in patients singly infected by HCV; iii) increase of IL-1 receptor antagonist in HCV<sup>+</sup> patients and in those affected also by NHL with or without MC; iv) a homogeneous increase of sIL-

1R type II in all the subgroup of patients. These data indicate that an attempt to increased circulating levels of IL-1 inhibitors occurs at different extent in the course of HCV infection as well as in its progression to NHL and/or MC.

## Introduction

Hepatitis C virus (HCV) is hepatotropic and causes hepatitis, liver cirrhosis and hepatocellular carcinoma (1). HCV may also induce autoimmune manifestations such as type II mixed cryoglobulinemia (MC). MC is characterized by cutaneous vasculitis, nephritis, peripheral neuropathy and clonal B-cell lymphoproliferations that can evolve into non-Hodgkin's lymphoma (NHL) (2,3). A systematic review of 66 studies including over 6000 NHL patients, showed a high prevalence of HCV positivity, especially in Italy (19.7% of 2734 cases) and Japan (11.3% of 771 cases) (4).

Although, there is much evidence implicating HCV in lymphoproliferation and lymphoma development, the pathogenetic mechanism underlying B-cell expansion and evolution to overt lymphoma is still unclear. Since HCV is lymphotropic its direct oncogenic role through B-cell infection and deregulation has been proposed but not proven. An additional pathogenetic hypothesis is that specific B-cell clones proliferate as a consequence of the chronic antigenic stimulation sustained by HCV. Indeed, the immunoglobulin variable region gene sequence of tumour B-cells from HCV-positive (HCV<sup>+</sup>) patients is the result of subsequent events of gene rearrangement, somatic mutation and antigenic selection (5).

An involvement of the immune system in the pathogenesis of HCV-associated lymphoproliferative disorders is also likely to occur. Cytokines might be of particular relevance in this context as they play a central role in liver metabolism and in the immune response to viral agents (6) and increased intrahepatic levels of IL-2, IL-6 and IL-8 were demonstrated by RT-PCR in cirrhotic patients (7).

IL-1, with the two isoforms IL-1 $\alpha$  and IL-1 $\beta$  is a cytokine belonging to the innate immune system that plays an important

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role in initiating the cascade of events of immunoinflammatory responses. By binding to specific high-affinity cell surface receptor type II in synergy with the interleukin receptor accessory protein (reviewed in ref. 8), IL-1 shows pleiotropic effects that include costimulation of T lymphocytes, B-cell proliferation, growth of fibroblasts, induction of adhesion molecules, stimulation of production of other cytokines and inflammatory mediators, growth-inhibitory and cytotoxic effect for several cell lines (9). The importance of IL-1 in the physiology of the immune system is highlighted by the presence of multiple endogenous regulators such as the IL-1 receptor antagonist (ra), the interleukin receptor (IL-1R) type I and type II and the IL-1 accessory protein (Acp) that, when released into the bloodstream, may serve as naturally occurring inhibitors of IL-1 (10). Previous studies support the hypothesis that an imbalance between IL-1 and these naturally occurring inhibitors either in the general circulation or at the level of the organ targeted from the immunoinflammatory response may regulate the development and the natural course of chronic inflammatory diseases, such as inflammatory bowel diseases (11), in rheumatoid arthritis (10,12), multiple sclerosis (13,14) and systemic lupus erythematosus (15). Genetic polymorphism for the IL-1ra gene has also been reported for some autoimmune diseases (16-19).

An imbalance of IL-1 $\beta$  and IL-1ra has also been found to occur in liver tissues from HCV related chronic hepatitis (20) and genetic polymorphism for both IL1 $\beta$  and IL-1ra may increase the risk of HCV<sup>+</sup> patients to develop cirrhosis (21). However, it has not yet been studied whether a dysregulated balance between IL-1 and its naturally occurring inhibitors may also occur during the lymphoma development in the context of HCV infection. Hence, we studied the levels of IL-1 $\beta$ , soluble IL-1ra, IL-1 AcP and IL-1R type II in serum samples from HCV<sup>+</sup> patients who were also affected, either singly or in combination, by MC and B-cell NHL.

## Patients and methods

**Patients.** Serum samples were collected from 114 subjects, 20 HCV<sup>+</sup> patients with B-cell NHL (8 female and 12 male, mean age 61 $\pm$ 9 years), 15 HCV<sup>+</sup> patients with both MC and B-cell NHL (7 female and 8 male, mean age 63 $\pm$ 5 years), 20 HCV<sup>+</sup> patients with MC (15 female and 5 male, mean age 60 $\pm$ 8 years), 14 HCV<sup>+</sup> patients, (9 female and 5 male, mean age 52 $\pm$ 9 years) and 45 sex and age-matched healthy donors (25 females). The healthy controls did not suffer from infectious, allergic or autoimmune diseases nor had taken immunomodulatory drugs during the last 6 months before samples collection.

All patients were seronegative for human immunodeficiency virus (HIV). Informed consent was obtained from all patients, and peripheral blood collection was approved by Institutional Review Board. Fasting blood samples were obtained between 8 and 10 am to avoid possible circadian variations in the levels of the analyses. For all patients the sera were obtained at disease diagnosis and stored at -80°C until analysis.

HCV infection was screened by searching for serum antibodies against HCV by the enzyme-linked immunosorbent assay (HCV 3.0; Ortho Diagnostic Systems, Raritan, NJ) and the recombinant-based immunoblot assay (Chiron RIBA; Ortho

Diagnostic Systems). Serum HCV RNA determination and genotype characterization were performed according to previously described procedures (22). All consecutive B-cell NHL cases were classified according to the '2001 World Health Organization (WHO) classification of tumors of Haematopoietic and Lymphoid Tissues' (23). MC was diagnosed according to already described criteria (24). In particular, these patients had detectable serum cryoglobulins for more than 6 months and at least 2 of the following symptoms and signs consistent with purpura, asthenia, arthralgia, renal involvement, and peripheral neuropathy were investigated, as well as serum cryoglobulins, C3 and C4, and rheumatoid factor levels.

## Cytokine analysis

**IL-1 $\beta$ .** Serum levels of IL-1 $\beta$  were measured by solid-phase ELISA purchased by R&D Systems (Minneapolis, MN). The assay was performed according to the manufacturer's instruction. The limit of the sensitivity of the assay was 2 pg/ml.

**Soluble IL-1ra.** The soluble form of IL-1 receptor antagonist was measured by solid-phase ELISA purchased by R&D (Minneapolis, MN). The assay was performed according to the manufacturer's instruction. The limit of sensitivity of the assay was 20 pg/ml.

**Soluble IL-1 receptor type II.** The circulating levels of the soluble IL-1 receptor type II were measured by a solid-phase ELISA the reagents of which were kindly provided by AMGEN (Thousand Oaks, CA). The assay was performed according to the manufacturer's instructions. Briefly, Nunc maxisorb 96-well plates (Nunc, DK) were coated in 0.05 M citrate buffer with 2  $\mu$ g/ml of anti-hIL-1R II mAb at 4°C. Standards or samples were incubated for 2 h at RT. After washing the plates 6 times with PBS/Tween 0.05%, 5  $\mu$ g/ml of 1R II mAb Ab-peroxidase was incubated for 1 h at RT. As substrate 100  $\mu$ l of TMB (Sigma, St. Louis, MO) was used. The limit of the sensitivity of the assay was <25 pg/ml.

**IL-1 AcP.** The circulating levels of the soluble IL-1 AcP were measured by a solid-phase ELISA the reagents of which were kindly provided by AMGEN. The assay was performed according to the manufacturer's instructions. Briefly, Nunc maxisorb 96-well plates (Nunc, DK) were coated overnight in PBS with 1  $\mu$ g/ml of anti-HuIL-1R AcP mAb (clone M355) at 4°C and then saturated for 1 h at RT with PBS/0.005% Tween/ 0.01% casein with 0.5 mg/ml purified rat IgG. Standards and samples were incubated for 1 h at RT. After washing the plates 6 times with PBS/Tween 0.05%, biotinylated anti HuIL-1R AcP Ab (clone 60), diluted 1:1000, was incubated for 1 h at RT. Following six washings streptavidin peroxidase (Sigma, St. Louis, MO) was added for 30 min at RT. As substrate 100  $\mu$ l of TMB (Sigma, St. Louis, MO) was used. The limit of the sensitivity of the assay was 5 pg/ml.

**Statistical analysis.** Data are presented as mean values  $\pm$  standard deviation (SD). Statistical analysis was performed by using one-way ANOVA. A p-value lower than 0.05 was considered significant. When blood levels of analyses were below the assay sensitivity, a theoretical value was assigned.



	Healthy donors (n=45) Mean $\pm$ SD	HCV <sup>+</sup> patients (n=14) Mean $\pm$ SD	HCV <sup>+</sup> MC (n=20) Mean $\pm$ SD	HCV <sup>+</sup> NHL with MC (n=15) Mean $\pm$ SD	HCV <sup>+</sup> NHL (n=20) Mean $\pm$ SD
IL-1 $\beta$ (pg/ml)	5 $\pm$ 3	7 $\pm$ 8	4 $\pm$ 2	25 $\pm$ 79	70 $\pm$ 204 <sup>a</sup>
IL-1ra (pg/ml)	113 $\pm$ 114	298 $\pm$ 401 <sup>a</sup>	127 $\pm$ 162	200 $\pm$ 176 <sup>a</sup>	393 $\pm$ 753 <sup>a</sup>
IL-1R II (pg/ml)	2670 $\pm$ 1235	6025 $\pm$ 1973 <sup>a</sup>	5512 $\pm$ 1245 <sup>a</sup>	6536 $\pm$ 4267 <sup>a</sup>	6658 $\pm$ 2598 <sup>a</sup>
IL-1 hAcP (pg/ml)	1053 $\pm$ 1223	1892 $\pm$ 887 <sup>a</sup>	1391 $\pm$ 562	1007 $\pm$ 615	1097 $\pm$ 625

<sup>a</sup>p<0.05; SD, standard deviation; MC, type II mixed cryoglobulinemia syndrome; NHL, non-Hodgkin's lymphoma.

## Results

Table I indicates mean serum levels of IL-1 $\beta$ , soluble (s)IL-1ra, IL-1AcP and sIL-1R type II analyzed in the different subsets of patients and the healthy donors. The mean serum level of each cytokine from each group of patients was compared with that from the healthy subjects. The mean serum level of IL-1 $\beta$  was higher among the group of HCV<sup>+</sup> patients with B-cell NHL than healthy donors (p<0.03); however, only a trend was observed among the HCV<sup>+</sup> patients with both B-cell NHL and MC syndrome (p<0.07). The mean serum level of sIL-1R type II was higher among all groups of patients than healthy donors (p<0.0001). There were no significant differences in the magnitude of the increase among the different groups of patients. Relative to healthy controls, the mean serum level of IL-1ra was higher among HCV<sup>+</sup> patients, HCV<sup>+</sup> patients with B-cell NHL and HCV<sup>+</sup> patients with both B-cell NHL and MC syndrome, respectively (p<0.007; p<0.01; p<0.03). The mean serum level of IL-1 AcP was significantly higher only among HCV<sup>+</sup> patients compared to controls (p<0.02).

## Discussion

Increasing evidence indicates that cytokines play an important role in liver metabolism and in the immune response to viral agents (6) and elevated intrahepatic levels of IL-2, IL-6 and IL-8 were demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) in patients with cirrhosis (7). IL-1 $\beta$  has been suggested to be involved in liver physiology and pathophysiology of the liver (25).

In the context of B-cell proliferation, it has been shown that HCV plays a pathogenic role in inducing B-NHL (2). The release of both cytokines and soluble mediators, HCV-induced, causes the chronic antigenic B-cell stimulation that has been proposed as an important pathogenic mechanism contributing to neoplastic transformation.

The lymphotropic action of HCV is consistent with studies in severe combined immunodeficiency (SCID) mice (26) showing the persistence and low-rate multiplication of HCV infection in human mononuclear cells.

Because B lymphocytes are responsive to the stimulatory action of IL-1 we have aimed to ascertain in this study whether imbalances in the circulating levels of IL-1 system including IL-1 $\beta$ , sIL-1R type II, IL-1AcP and circulating IL-1ra could occur in HCV<sup>+</sup> patients with or without concurrent affection from NHL and/or MC and whether selective abnormalities could characterize any group of these patients.

The only selective results obtained from measuring these different analytes were the augmented serum levels of IL-1 $\beta$  observed in HCV<sup>+</sup> patients simultaneously affected by NHL and the increase of IL-1AcP that only occurred in the group of patients infected by HCV without evidence of lymphoproliferative disorders. No additional clear-cut data could be obtained from the analysis of our results as a homogeneous increase in the blood levels of sIL-1R type II observed in all the subgroup of patients and a significant increase of circulating IL-1ra in singly infected HCV<sup>+</sup> patients as well as in those simultaneously suffering from NHL with or without MC. Interestingly the increase in the circulating levels of IL-1ra, that has been reported in HCV<sup>+</sup> patients (27), was not observed in HCV<sup>+</sup> patients with MC.

Taken together, the above data suggest that the overall elevation in at least one of the analytes from our sample series may reflect an ongoing immunoinflammatory response in which IL-1 is involved. In addition, the elevation of one or more endogenous IL-1 antagonists in our subset of patients indicates an attempt at mounting anti-inflammatory responses to, possibly, IL-1 driven immunoinflammatory events. That an upregulated production of IL-1 $\beta$  might play a role in lymphomagenesis in HCV<sup>+</sup> patients seems consistent with the augmented blood levels of this cytokine found in HCV-associated NHL patients. Although, the increased blood levels of IL-1 $\beta$  in these patients could represent a secondary epiphenomenon devoid of pathogenic relevance, further studies are warranted to understand whether an abnormal synthesis of IL-1 $\beta$  plays a role in development of NHL during HCV infection. Proving a pathogenic role of IL-1 in lymphomagenesis in the context of HCV infection could open novel therapeutic approaches for prevention of NHL that are aimed at negating the action of endogenous IL-1 with specific inhibitors such as the IL-1ra that has already shown clinical efficacy in patients with rheumatoid arthritis (28).

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