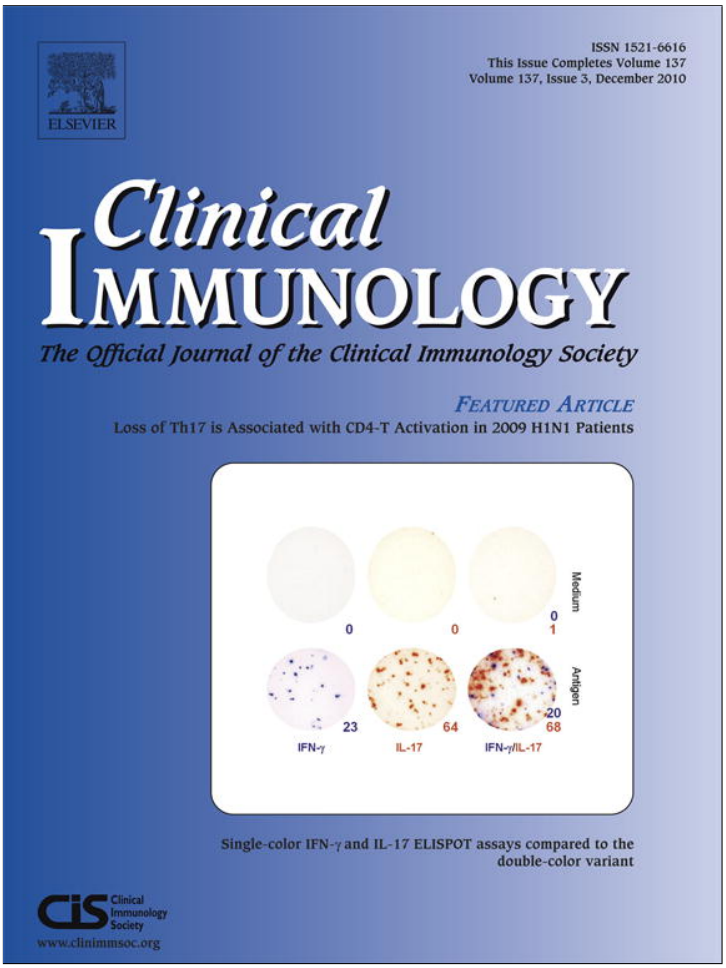

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The simultaneous high expression of Vα24, IFN-γ and FoxP3 characterizes the liver of children with type I autoimmune hepatitis

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Abstract The immunopathogenesis of type I autoimmune hepatitis (AIH-I) might involve the deregulation of different cellular processes. Here, we investigated the liver expression of selected cytokines and genes of regulatory cell populations in children both at diagnosis and during biochemical remission following immunosuppressive treatment (AIH-Ir). We found a higher Vα24, IFN-γ, FoxP3, IL-27p28, IL-12p40 and IL-21 expression at diagnosis as well as a positive correlation between IL-21 and transaminase levels. Interestingly, only IFN-γ and FoxP3 were decreased in AIH-Ir. An "AIH-I phenotype" (high Vα24, IFN-γ and FoxP3 expression at diagnosis) was observed in only 5 out of 22 AIH-Ir patients but not in controls. These results indicate a local deregulation of the innate and adaptive immune responses with an increased transcriptional activity of immunoregulatory cells at diagnosis. In addition, IL-21 is highlighted as a mediator of liver injury. AIH-Ir is characterized by a partial reversal of the deregulated response.

Abbreviations: HLA, human leukocyte antigens; AIH-I, type I autoimmune hepatitis; AIH-Ir, type I autoimmune hepatitis following remission; NKT, natural killer T; iNKT, invariant natural killer T cells; IFN-γ, interferon gamma; LTregs, T lymphocyte regulatory cells; FoxP3, forkhead/winged helix; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IU, international units; U, arbitrary units.

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1. Introduction

Type I autoimmune hepatitis (AIH-I) is a progressive liver disease characterized by the presence of circulating antinuclear and/or anti-smooth muscle autoantibodies, hypergammaglobulinemia, histological evidence of chronic inflammation with interface hepatitis and by its response to immunosuppressive treatment [1–3]. Although great efforts have been made to define the immune mechanisms leading to the breakdown of self-tolerance, the immunopathogenesis of AIH-I in children remains unclear. It may not be the consequence of a single initiating event but of the deregulation of different cellular processes that help maintain the liver immune homeostasis [4]. Particularly, it may involve the local expansion of T cell effectors and natural killer T (NKT) cells and/or the selective recruitment of these populations into the liver. The increased expression of HLA class II molecules observed on the surface of hepatocytes [5,6] and the preponderant $CD4^+$ T cell infiltration of portal spaces [7–10] suggest the participation of $CD4^+$ T helper (Th) effectors in the pathogenesis of AIH-I. Besides the classical Th1 and Th2 subsets, a third effector T cell subset named Th17 has been described. Th17 cells are characterized by the production of interleukin-17A (IL-17A), IL-17F, IL-22, IL-21, IL-6 and tumor necrosis factor α (TNF-α) [11]. Although which are the exact cytokines within the local milieu required for the differentiation and maturation of human Th17 cells is still a matter of controversy, transforming growth factor β (TGF-β), IL-6, IL-21, IL-1β and IL-23 have been proposed to induce the human Th17 cell lineage [12]. IL-23, a heterodimer of the IL-12 family, composed of the p19 and p40 subunits, appears to be essential to maintain and expand Th17 effectors, whereas IL-27, which is composed of the p28 and EBI3 subunits, suppresses Th17 differentiation and IL-17 production and promotes Th1 differentiation [13]. The presence of Th17 cells has also been reported in inflamed human tissues from patients suffering from a variety of inflammatory and autoimmune disorders [14–16]. The functional quartet of $CD4^+$ effectors also includes T lymphocyte regulatory cells (LTregs) constitutively expressing the IL-2 receptor α-chain CD25 and the forkhead/winged helix (FoxP3) master regulatory transcription factor which is highly specific to the $CD4^+CD25^{high}$ Treg lineage [17–19]. $FoxP3^+$ Tregs are hardly detectable in normal livers [20], and it is generally accepted that an impaired Treg number and/or function could lead to liver autoimmunity. For instance, a deficiency in the number and function of peripheral Tregs has been reported to be related to the stage of AIH-I [21–23]. Since it has also been demonstrated that Tregs act through a direct contact with their target cells [22], we decided to assess the presence of this immunoregulatory T cell subpopulation at the local compartment.

Invariant natural killer T cells (iNKT) expressing the T cell receptor Vα24 and Vβ11 genes are particularly enriched within the human liver and are involved as effectors of the innate response and in the modulation of the adaptive immune response [24]. iNKT have been linked to autoimmune damage both in AIH-I and primary biliary cirrhosis [25–27] as well as in animal models of hepatic autoimmunity [28].

Cytokines may play a key role as essential mediators in the cross-talk between T cells and other immune and non-immune cells in the ongoing pathological processes. Up to date, only limited data are available on their participation during the development of AIH-I in children [27] or during recovery from AIH-I by the current immunosuppressive treatments.

Our aims were to evaluate selected cytokines as well as genes related to regulatory subpopulations (i.e. iNKT and Tregs) in the liver of children with AIH-I and to examine the association between inflammation and the biochemical remission during immunosuppressive treatment.

2. Materials and methods

2.1. Patients

Thirty-four AIH-I patients with positive antinuclear antibodies or smooth muscle antibodies were studied. Twelve patients (10 females: median age, 7.7 years; range, 3–16 years) were studied at disease diagnosis, while 22 (16 females: median age, 12.8 years; range, 7–20 years) were studied during remission following immunosuppressive treatment. The 22 patients studied during treatment were receiving prednisolone (2.5–5 mg daily) with or without azathioprine (1–2 mg/kg/day). The laboratory and histological features of children with AIH-I and AIH-Ir (defined as normal transaminase levels when under immunosuppressive treatment) are shown in Table 1. Slides from all biopsies were examined by two pathologists who scored the liver histology by using previously published histological criteria [29,30]. All patients were negative for HCV RNA by PCR, hepatitis B and A serological markers and for IgM, EBV and CMV. Control liver samples free of infectious diseases were obtained from cadaveric donors aged 7–30 years (median, 16 years) after liver reduction, in virtue of an agreement with the Central Institute of Coordination of Ablations and Implants of Argentina. All the samples coming from donors had normal transaminase values at the time of death.

All patients were informed of the aim of the study and gave their written informed consent. The study was approved by the local Ethics Committee of the Clinical Hospital "José de San Martin," Buenos Aires, Argentina, informed of internationally endorsed standards for the application of the Helsinki Declaration.

2.2. Sample preparation

Liver biopsies were obtained by intrahepatic puncture. After collection, tissue samples were immediately submerged in 1 ml of TRIZOL® reagent (GIBCO BRL, Life Technologies Inc., Grand Island, NY, USA) and stored at −70 °C. Total RNA was isolated from each biopsy according to the protocol provided by the manufacturer. Reverse transcription was carried out by using the SuperScript® First-Strand Synthesis System for reverse transcriptase

Table 1 Laboratory and histological features in children with AIH-I at diagnosis and in remission.

Patients	AST (UI/L) (rf<50)	ALT (UI/L) (rf<50)	ALP (UI/L) (rf<350)	γGT (UI/L) (rf<50)	γ-Glob (rf<1.8)	PT (%) (rf: 90%)	PTT (sec) (rf: 34–50)	HAI	Stage
AIH-I									
1	51	77	365	59	1.01	77	34	na	na
2	na	195	631	97	na	na	na	8	3
3	70	60	340	na	na	na	na	15	4
4	994	689	732	88	5.4	70	56	13	3
5	380	424	1050	62	4	100	33	10	3
6	824	1160	786	182	1.5	100	38	na	na
7	1158	935	486	457	4.3	76	45	13	3
8	71	91	449	109	na	62	31	13	4
9	74	87	540	364	2.76	80	32	15	4
10	50	39	2000	600	2.36	85	27	10	3
11	954	815	1271	296	2.7	71	43	12	3
12	63	73	1714	214	1.84	64	39	na	na
AIH-Ir									
1	23	17	228	42	16.9	85	32	5	3
2	25	12	825	7	1.35	71	29	5	2
3	23	10	367	16	0.96	77	28	5	3
4	19	19	358	12	1.24	87	34	5	3
5	14	25	441	9	1.21	69	36	5	3
6	19	9	164	17	1.59	87	32	5	4
7	36	32	757	42	1.26	80	31	3	3
8	28	30	245	35	1.38	88	24	3	3
9	32	36	344	87	21.6	100	32	2	3
10	31	37	311	64	1.31	73	37	4	3
11	24	17	180	25	na	78	36	7	4
12	25	23	265	39	1.42	67	29	8	4
13	15	14	472	12	14	85	40	3	3
14	25	36	142	57	1.31	80	27	4	3
15	18	12	475	na	1.05	100	40	na	na
16	41	32	439	49	1.94	77	32	8	4
17	18	21	296	15	1.45	80	28	5	3
18	35	48	328	70	1.29	78	31	5	4
19	23	14	211	15	1.24	92	34	9	4
20	52	46	434	58	na	60	36	8	3
21	31	38	299	46	na	78	34	8	4
22	44	50	262	11	1.09	75	35	9	3

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γGT, gammaglutamyl transferase; γ-Glob, gammaglobulin; PT, prothrombin time; PTT, partial prothrombin time; HAI, histological activity index; rf, reference value; na, not available.

(RT)-PCR Kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) using oligodT primers.

2.3. Quantitative polymerase chain reaction (qPCR)

mRNA levels of IL-23p19, IL-12p35, IL-12p40, IL-27p28, IFN-γ, TGF-β, IL-6, IL-21, IL-17A, IL-17F, Vα24 and FoxP3 were determined by real-time PCR using a LightCycler® instrument (Roche Applied Science, Mannheim, Germany). Reactions were performed in a volume of 20 μl using either the FastStart SYBR Green I MasterMix (Roche Applied Science) or FastStart DNA Master HybProbe (Roche Applied Science). Then, 1 μl of thermolabile Uracil DNA glycosylase (UDG) (Roche Applied Science) was added to each reaction to prevent carry-over contamination. Gene primer sets are described in Table 2. Molecules showing no detectable levels were given an arbitrary unit (U) of 0.01, 0.001 or 0.0001 as indicated.

2.4. Statistical analysis

Non-parametric statistical analyses of the mRNA expression levels between groups (AIH-I, AIH-Ir and controls) were performed using the Kruskal–Wallis one-way

Table 2 Genes, primer sets, PCR products (bp, base pairs), temperature of annealing, and source of the primers used for quantitative PCR.

Molecule	Primer sequences	bp	T° of annealing	Primers source
β-actin	fw: 5′-ATGGGTCAGAAGGATTCCTATGTG-3′ rv:5′-CTTCATGAGGTAGTCAGTCAGGTC-3′	359	60	[31]
IL-23p19	fw: 5′-AGCAGCTCAAGGATGGCACTCAG-3′ rv:5′-CCCCAAATTTCCCTTCCCATCTA-3′	206	60	[32]
IL-27p28	fw: 5′-GCGGAATCTCACCTGCCA-3′ rv:5′-GGAAACATCAGGGAGCTGCTC-3′	69	64	[33]
IL-12 p35	fw: 5′-TGTCACCGAGAAGCTGATGT-3′ rv:5′-GAGGTTTCTGGCCAAACTGA-3′	278	68	[34]
IL-12p40	Commercial primers and taqman probes			
IL-17A	fw: 5′-GACCTCATTGGTGTCACTGC-3′ rv: 5′-GAGATTCCAAGGTGAGGTGG-3′	214	62	
IL-17F	fw: 5′-GGGCTTGCCTTTCTGAGTG-3′ rv:5′-TGGGGTCCCAAGTGACAG-3′	211	63	
Il-6	Commercial primers and taqman probes			
Il-21	Commercial primers and taqman probes			
IFN-γ	fw: 5′-TGGAAAGAGGAGAGTGACAG-3′ rv: 5′-ATTCATGTCTTCCTTGATGG-3′	129	60	[35]
TGF-β	fw: 5′-GGACACCAACTATTGCTTCAG-3′ rv:5′-TCCAGGCTCCAAATGTAGG-3′			
Vα24	fw: 5′-CTGGAGGGAAAGAACTGC-3′ rv:5′-TGTCAGGGAAACAGGACC-3′	105	65	[36]
FoxP3	fw: 5′-CAGCACATTCCCAGAGTTCCTC-3′ rv:5′-GCGTGTGAACCAGTGGTAGATC-3′	154	60	[37]

Fw: forward, rv: reverse, bp: base pairs.

analysis of variance test and the Mann–Whitney *U* test with a Bonferroni correction factor of 3. Results are expressed as the median value and the interquartile range. Analysis of the non-parametric correlation was performed using the Spearman's rank correlation test. The statistical analysis was performed using the software

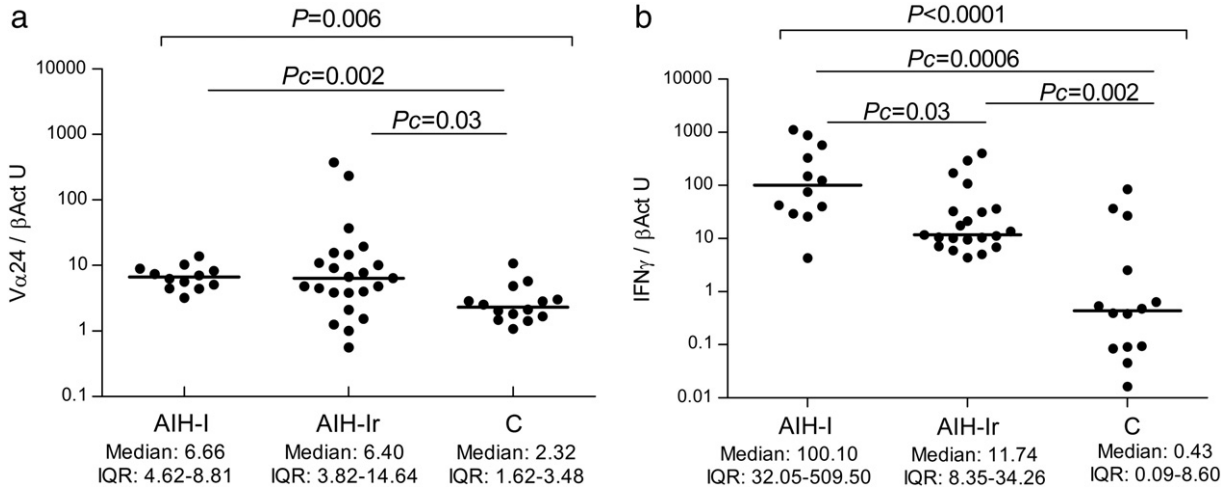

Figure 1 Hepatic mRNA expression of Vα24 (a) and IFN-γ (b) in arbitrary units (U). mRNA levels of Vα24 (a molecular marker for iNKT cells) were increased in both groups of patients compared with controls. The expression of IFN-γ was increased in patients at diagnosis compared with controls. Although the expression of IFN-γ in treated AIH patients was significantly lower than in patients at disease presentation, it still remained higher than controls. Kruskal–Wallis test, $P=0.006$ and $P<0.0001$. Horizontal bars indicate median values. P_c: two-tailed Mann–Whitney *U* test with a Bonferroni correction. IQR, interquartile range. AIH-I, pediatric patients with autoimmune hepatitis at diagnosis ($n=12$); AIH-Ir, pediatric patients with autoimmune hepatitis during remission ($n=22$); C, controls ($n=14$).

program GraphPad Prism 4. The level of significance was fixed at $P<0.05$.

3. Results

3.1. Increased Vα24 and IFN-γ mRNA expression in patients with AIH-I

The expression of Vα24 mRNA was increased in both groups of patients compared with controls (AIH-I: 6.66 U, AIH-Ir: 6.40 U and C: 2.32 U) (Fig. 1a). Besides, no differences were found in the expression of Vα24 mRNA between patients at diagnosis and patients during remission.

The analysis of IFN-γ mRNA showed a marked increase in both groups of patients compared with controls (AIH-I: 100.10 U, AIH-Ir: 11.74 U and C: 0.43 U). For this transcript, the relative expression at the time of AIH-I diagnosis was significantly higher than that at remission. Although the median expression of IFN-γ decreased during the treatment, it did not reach the median control values (Fig. 1b).

3.2. Cytokine expression in AIH-I patients

3.2.1. Increased expression of IL-27p28 and IL-12p40 mRNA subunits in the liver of AIH-I patients

The expression of the IL-27p28 subunit was higher in patients than in controls (AIH-I: 4.209 U, AIH-Ir: 16.320 U and C: 1.879 U). IL-27p28 mRNA levels were also significantly higher during remission than at the time of disease diagnosis (Fig. 2a). Although mRNA expression of the IL-12p40 subunit remained below detectable levels in some samples among the AIH-I patients, a similar median value was obtained both at diagnosis and during remission (AIH-I: 0.800 U and AIH-Ir: 0.903 U). These values were increased as compared with the control group (0.010 U) (Fig. 2b). No significant differences were found in the expression of the IL-23p19 and IL-12p35 subunits (Figs. 2c and d) of all the groups studied.

3.2.2. Absence of the main Th17 effector cytokines in the liver of 214 AIH-I patients

In order to investigate whether the Th17 lineage can be associated with the development of AIH-I, we evaluated a

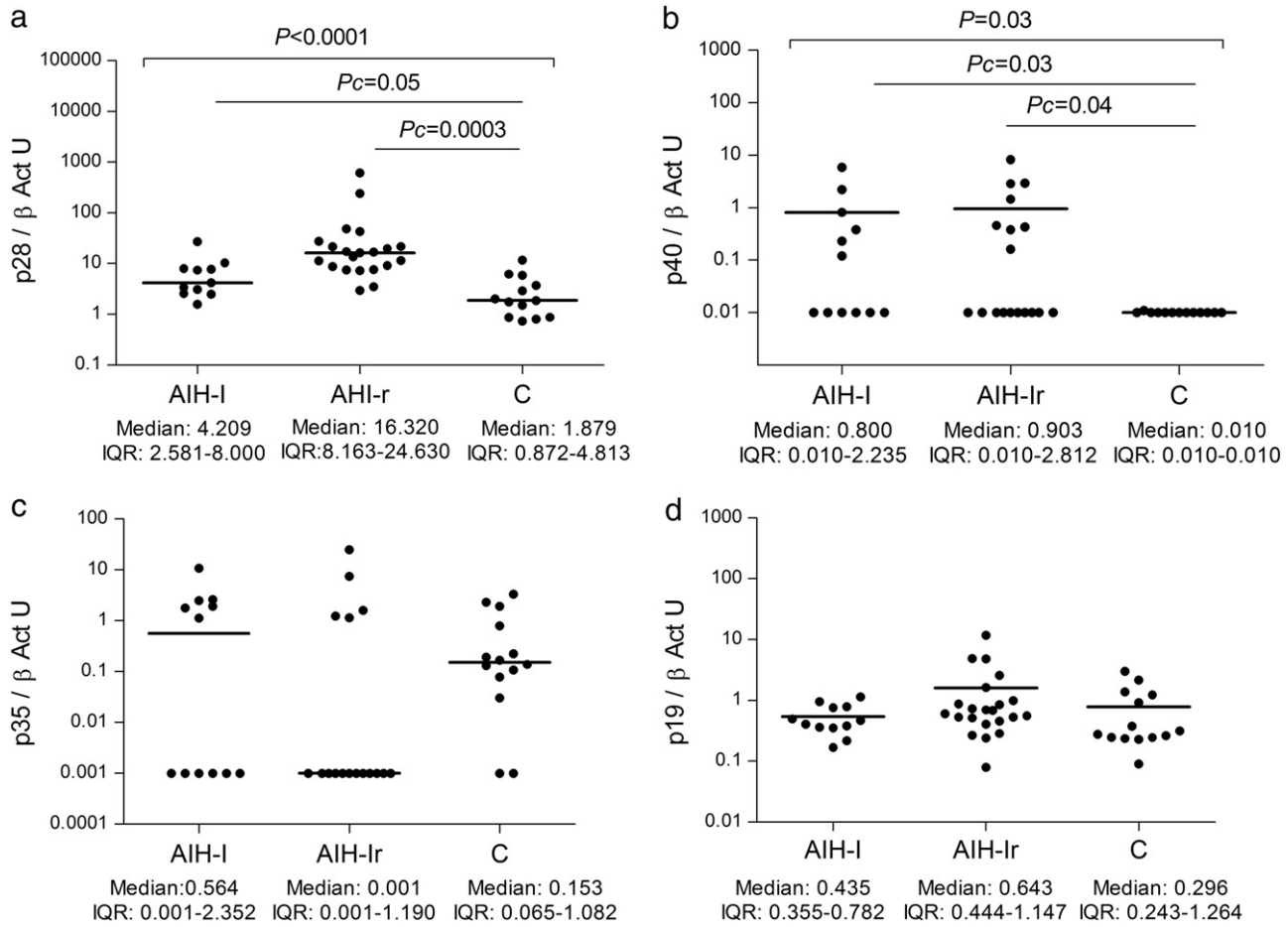

Figure 2 Hepatic mRNA expression in arbitrary units (U) of key cytokines involved in Th1 response. mRNA levels of interleukin (IL-27p28 (a) and IL-12p40 (b) were increased in both groups of patients, while IL-12p35 (c) and IL-23p19 (d) mRNA levels were not changed. Kruskal–Wallis test, $P<0.0001$ and $P=0.03$. Horizontal bars indicate median values. P_c: two-tailed Mann–Whitney U test with a Bonferroni correction. IQR, interquartile range. AIH-I, pediatric patients with autoimmune hepatitis at diagnosis ($n=12$); AIH-Ir, pediatric patients with autoimmune hepatitis during remission ($n=22$); C, controls ($n=14$).

panel of cytokines characteristic of Th17 response. To this end, TGF-β, IL-6, IL-21, IL-17A and IL-17F mRNA levels were studied. For TGF-β mRNA, a small dispersion of values, with no differences between patients (AIH-I: 1.0040 U and AIH-Ir: 0.7287 U) and controls (0.7965 U), was observed (Fig. 3a). In contrast, both groups of patients showed lower IL-6 mRNA levels than controls (AIH-I: 0.4118 U, AIH-Ir: 0.1011 U and C: 4.7770 U) (Fig. 3b). The IL-21 mRNA levels observed were higher in patients at diagnosis (12.2100 U) than in patients at biochemical remission (0.0001 U) or controls (0.0001 U) (Fig. 3c). The mRNA expression from the IL-17A and IL-17F genes remained below detectable levels in most samples

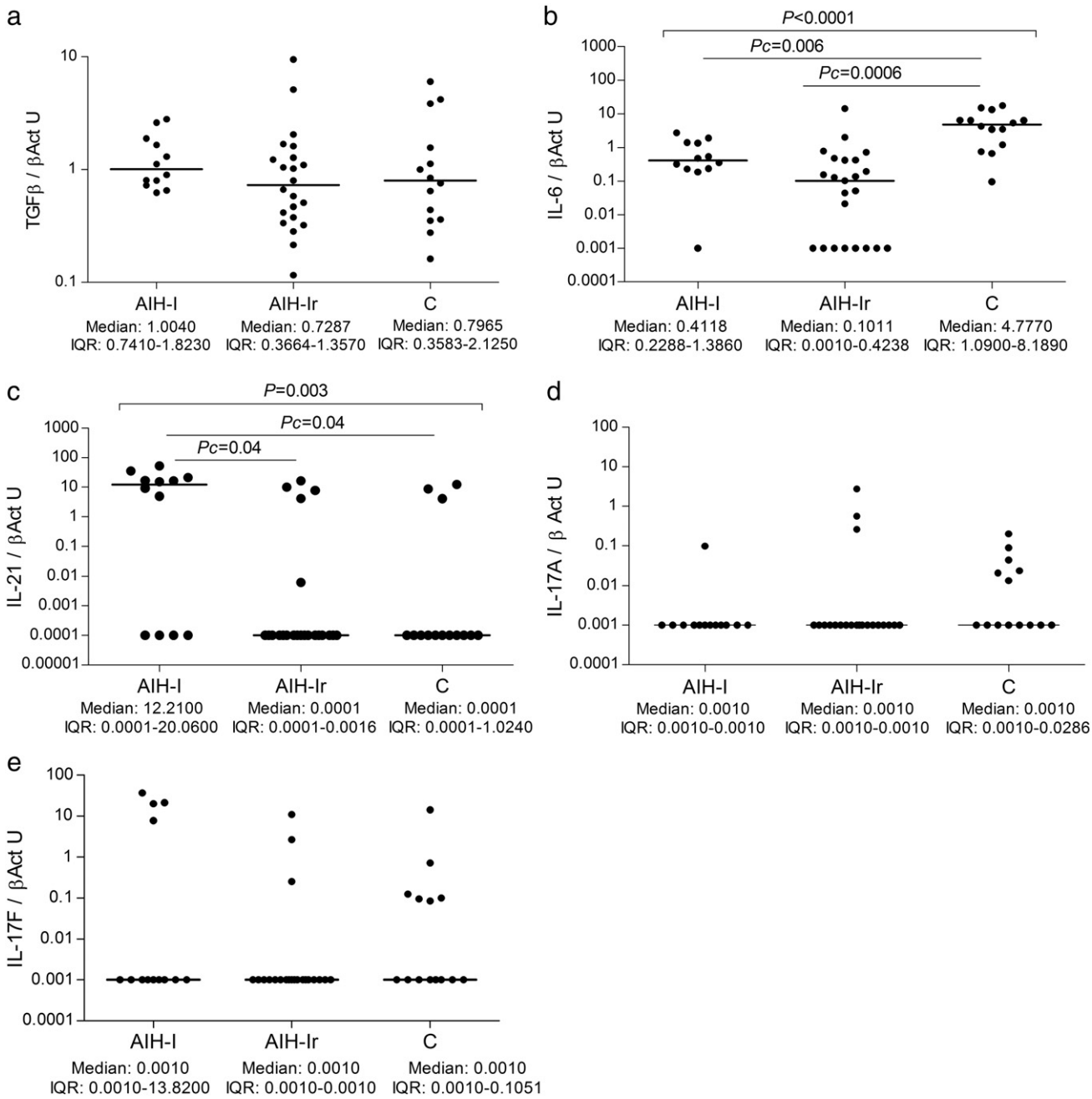

Figure 3 Hepatic mRNA expression in arbitrary units (U) of key cytokines involved in Th17 response. Similar mRNA levels of TGF-β were found in all study groups (a). The expression of IL-6 in both groups of patients with AIH-I was significantly lower than in controls (b). The expression of IL-21 in AIH-I patients was significantly higher than that in patients during remission and controls (c). No differences were found in IL-17A and IL-17F mRNA expression between the studied groups. Kruskal–Wallis test, $P<0.0001$ and $P=0.003$. Horizontal bars indicate median values. P_c: two-tailed Mann–Whitney U test with a Bonferroni correction. IQR, interquartile range. AIH-I, pediatric patients with autoimmune hepatitis at diagnosis ($n=12$); AIH-Ir, pediatric patients with autoimmune hepatitis during remission ($n=22$); C, controls ($n=14$).

from all the groups studied (Figs. 3d and e). Interestingly, in patients at diagnosis, there was a direct correlation between IL-21 and the transaminase levels (AST: $r=0.7$, $P=0.007$; ALT: $r=0.7$, $P=0.007$).

3.3. High FoxP3 mRNA expression in the liver of AIH-I patients

Next, we investigated whether a decrease in Tregs is involved in the emergence of autoimmune hepatitis. Therefore, we analyzed FoxP3 mRNA levels in patients and controls. The expression of this molecule was higher in patients than in controls (AIH-I: 5.83 U, AIH-Ir: 0.81 U and C: 0.03 U). Within AIH-Ir patients, a decreased median expression that did not reach the control values was found (Fig. 4). Furthermore, in patients at diagnosis, there was a direct correlation between FoxP3 expression and the transaminase levels (AST: $r=0.8$, $P=0.004$; ALT: $r=0.7$, $P=0.005$).

3.4. The analysis of the transcriptional status at the individual level defined an expression pattern in AIH-I patients

Finally, a heat map was constructed by using the entire values obtained in order to appreciate the transcriptional status at the individual level (Fig. 5). Its visual examination allowed us to describe three different expression patterns characterized by the simultaneous high expression of the following genes: *(i)* Vα24, IFN-γ and FoxP3; *(ii)* Vα24, IFN-γ, FoxP3 and IL-21; and *(iii)* Vα24, IFN-γ, FoxP3, IL-21 and the p40 subunit. The first pattern was observed in 12/12 AIH-I patients. The second pattern *(ii)*, defined by the high expression of IL-21 together with the transcripts included in *(i)*, was observed in 8/12 AIH-I patients, whereas the third pattern *(iii)*, defined by the high expression of p40 (IL-12) subunit transcripts together with the transcripts included in *(ii)*, was observed in 6/12 AIH-I patients evaluated. Conversely, the *(i)* pattern was observed in 5/22 AIH-Ir patients, whereas the *(iii)* pattern was expressed only in 1 patient. Compared to (*iii*), an expected opposed expression pattern characterized by the simultaneous low levels of Vα24, IFN-γ, FoxP3, IL-21 and p40 (IL-12) subunit transcripts was found in liver samples from most of the control individuals. Furthermore, none of the control samples exhibited the typical expression pattern observed in liver biopsies from all of the AIH-I patients. The analysis of p40 (IL-12), p35 (IL-12) and p19 (IL-23) subunits performed at individual patient level revealed the presence of IL-12 and/or IL-23 heterodimeric cytokines in all the AIH-I samples.

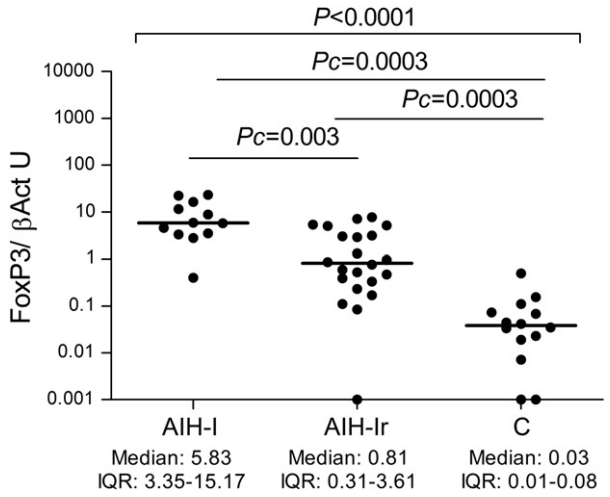

Figure 4 FoxP3 mRNA expression in liver biopsies from patients with AIH-I at disease presentation during remission and controls. The expression of FoxP3 in both groups of patients was significantly higher than in controls. However, FoxP3 mRNA levels in patients during remission were lower than in AIH-I. Kruskal–Wallis test, $P<0.0001$. Horizontal bars indicate median values. P_c: two-tailed Mann–Whitney U test with a Bonferroni correction. IQR, interquartile range. AIH-I, pediatric patients with autoimmune hepatitis at diagnosis ($n=12$); AIH-Ir, pediatric patients with autoimmune hepatitis during remission ($n=22$); C, controls ($n=14$).

4. Discussion

Upon stimulation, dendritic cells secrete IL-12p70, IL-23 or IL-27 and when one of these cytokine signals becomes dominant, it determines the type of immunity it develops, i.e., it determines whether the immune response is skewed toward Th1 or Th17 differentiation. When cytokine expression was studied at the mRNA level, we found an increased expression of IFN-γ in AIH-I patients. Although our global gene expression profile data were inadequate to identify the main local source of IFN-γ, they reinforced the idea that the emergence of AIH-I may not be the consequence of a single initiating event but of the deregulation of different cellular processes. Hence, the increased expression of Vα24 mRNA in samples from patients at diagnosis, the potential cytokine secretion profiles previously described for iNKT cells [39] and our previous results obtained by means of immunostaining of liver biopsy sections from patients with AIH-I by using a Vα24-specific antibody [27] suggest that those cells may be one of the local sources of IFN-γ. The increased expression of Vα24 mRNA in samples from patients at diagnosis reinforces our previous findings pointing to the influx of iNKT cells into the liver [27]. Besides iNKT cells, the presence of additional sources of IFN-γ ($CD8^+$, NK and/or Th1 cells) might be inferred, given that transcript levels for this cytokine decreased during immunosuppressive treatment, whereas comparable high levels of Vα24 mRNA remained (Fig. 1). We have previously demonstrated that although $CD45^+$ and $CD3^+$ cells localize to the central areas of portal tracts and liver parenchyma both in control livers and AIH-I samples, the latter shows a clear predominance of those positive subpopulations. Non-significant differences concerning NK cell markers (i.e. CD56 and CD57) were found between AIH-I and controls [10]. In addition, our results revealed only circumstantial signs of Th17 effectors' functions as deduced from the limited production of IL-17A and IL-17F transcripts. Together with Th17 cells, iNKT cells are a source of IL-21. Since a seemingly limited presence of a Th17 subpopulation and an amplified expression of $V\alpha24^+$ transcripts were detected, our present results suggest that iNKT cells may be the main source for IL-21 in the livers of patients with AIH-I.

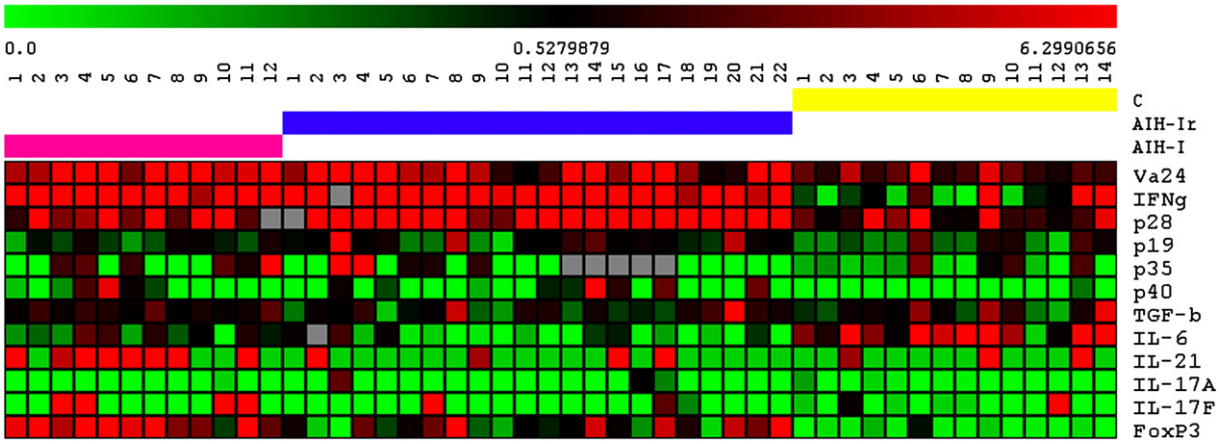

Figure 5 Heat map graph for each cytokine in individual patient samples. The simultaneous high expression of Vα24, IFN-γ and FoxP3 was described in the liver of children with active disease. This pattern was also found in some AIH-Ir patients. Heat maps were constructed using the FiRe Macro v2.2 for Microsoft Excel from the University of Fribourg (Switzerland) available at http://www.unifr.ch/plantbio/FiRe/main.html [38] and represent the relative expression ratio between the gene of interest and β-actin in each patient. Two-color gradients were used: red for high levels of gene expression and green for low levels of gene expression. Black plots represent midpoint values meanwhile gray plots are samples that were not evaluated. AIH-I, pediatric patients with autoimmune hepatitis at diagnosis ($n=12$); AIH-Ir, pediatric patients with autoimmune hepatitis during remission ($n=22$); C, controls ($n=14$).

Collectively, our results suggest that a local process of Th1 polarization might be synergistically sustained by IL-12 and IL-23, as well as by the early effects of IL-27. An increased expression of IL-27 in AIH-I might be associated not only with Th1 differentiation enhancement but also with the IL-27-driven inhibition of Th17 responses [40]. Interestingly, although AIH-Ir patients achieved normal transaminase levels after the immunosuppressive treatment, they expressed high levels of IFN-γ. It is noteworthy that, at remission, the median expression values of IFN-γ decreased as compared with patients with active disease. Furthermore, as a consequence of the immunosuppressive treatment, an unexpectedly increased expression of IL-27p28 was observed in samples from AIH-Ir vs. AIH-I and controls. This might be due to a generalized down-regulatory effect of IL-27 on both Th1 and Th17 cells reported to be mediated by an enhancement of suppressor Tr1 cells [41]. Although the inflammation according to the ALT levels seems to be sufficiently treated, the cytokine expression observed suggests that the range of hepatic lesions in AIH-I may be wider than suspected on the basis of histological examination, as was found in intestinal biopsies from patients with Crohn's disease [42,43]. Although the present study was not centered on the previously reported imbalance between Tregs and Th17 cells [44,45], limited signs for the presence of characteristic cytokines of the Th17 lineage together with a regular abundance of FoxP3 transcripts were found in liver biopsies from patients at diagnosis.

At the peripheral compartment, a lower *in vitro* ability of LTregs from patients with AIH-I to expand explains the relative reduction in the number of LTregs previously demonstrated [21]. The significant increase in FoxP3 transcripts herein described suggests that this relative reduction in the number of LTregs might be the consequence of their active recruitment into the liver. Besides, in view of the direct correlation between FoxP3 expression and the transaminase levels found in patients with AIH-I, we can infer that LTregs may be actively recruited to the liver in order to suppress proinflammatory immune responses. However, the extent to which the relative reduction in the number of peripheral LTregs accurately reflects liver Tregs proportions is still not clear. For instance, a decrease in the peripheral blood LTregs has been previously described in correlation with a high expression of mucosal FoxP3 in patients with inflammatory bowel disease [46]. In line with the previously suggested impaired function of peripheral LTregs in AIH-I [23], we cannot discard that the presence of some local factors impairs the successful suppressive function of Tregs [47–49]. On the basis of previous findings in human immune inflammatory diseases [50], IL-21, a member of the γC family of cytokines [51], can increase T cell-activated responses. Thus, the increased levels of IL-21 mRNA observed in AIH-I may counteract the suppressive activities of Tregs. The potential ability of IL-21 to subvert immunological protection by Tregs within the liver and therefore to predispose to an autoimmune attack deserves further investigation. In addition, the noteworthy correlation between the expression of IL-21 and transaminase levels observed in patients with AIH-I at diagnosis allows us to suggest that IL-21 may contribute to the maintenance of inflammation and liver injury.

In the current study, we described a conserved pattern of transcription given by the simultaneous high expression of Vα24, IFN-γ and FoxP3 in the liver of children with active disease denominated "AIH-I phenotype." A high expression of IL-21 was also observed in 66% of those samples, whereas the simultaneous expression of the IL-12p40 subunit was detected in only 50% of them. Conversely, a "control phenotype" described in 65% of liver samples from control individuals was characterized by a lower expression of Vα24, IFN-γ and FoxP3 in the absence of IL-21 and IL-12p40 transcripts. The remarkably increased expression of IL-6 mRNA found in control samples was not an unexpected finding since IL-6, together with IL-1, TNF-α, acute phase proteins and complement, is released as part of the early proinflammatory response that follows the shock and the organ ischemia usually associated with the traumatic death of liver donors [52,53].

Among the AIH-I patients evaluated during biochemical remission, less than 25% of the samples showed the "AIH-I phenotype," whereas 1 out of 22 samples was characterized by the "control phenotype." In summary, AIH-I in children involves a local deregulation of both the innate and the adaptive branches of the immune response. An increased iNKT related expression and a putatively non-functional subpopulation of Tregs are involved in the evident failure to control the inflammatory response underlying the emergence of AIH-I. Despite a biased modulation of IFN-γ, IL-27p28 subunit and FoxP3 genes described during AIH-I biochemical remission, the immunosuppressive treatments were not able to reverse the so-called "AIH-I phenotype" towards the typical hepatic milieu regularly found in the control livers. In the same way, a partial reversal on immunosuppressive treatment concerning both the number and functional characteristics of LTregs has been previously demonstrated [23]. Follow-up studies are necessary to elucidate the mechanisms of the immune response that remain active despite the treatment and whether those pathways could be amenable for a further therapeutic intervention.

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