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Hepatitis C virus-specific reactivity of CD4+-lymphocytes in children born from HCV-infected women

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***Background/Aims*: T-lymphocyte reactivity against viral antigens may represent the only immunological marker of host contact with a virus. Aim of the present study was to investigate whether vertical exposure to hepatitis C virus (HCV) could activate HCV-specific T-cell responses that may represent a biomarker of previous contact with the virus, and possibly contribute to the low rate of vertical HCV transmission.**

***Methods*: We studied 28 children born from chronically HCV-infected mothers. HCV-specific activation and proliferation of CD4+-lymphocytes and cytokine production were evaluated in cultures of peripheral blood mononuclear cells (PBMCs) stimulated in vitro with HCV-peptides.**

***Results*: HCV-specific CD4+-cell reactivity was observed in 20 out of the 28 children (71%). The proliferation of HCV-specific CD4+-cells was more frequent and vigorous in children than in their mothers. In children, but not in the mothers, activation of CD4+-cells upon stimulation with HCV-peptides was directly correlated with proliferation. Early upon stimulation with HCV-peptides, lymphocytes from children produced lower levels of IL-10 than lymphocytes from the mothers.**

***Conclusions*: Vertical exposure to HCV induces the development of viral-specific CD4+-cell-mediated immune responses, possibly endowed with protective function against infection, which may contribute to the low rate of vertical HCV transmission.**

***Keywords*: HCV; Vertical exposure; T-cell reactivity; Lymphocyte proliferation; Lymphocyte activation; Flow cytometry**

1. Introduction

Exposure to hepatitis C virus (HCV) of children born from HCV-infected mothers can take place in uterus, transplacentally at any time during pregnancy, during labour, or after birth. The rate of mother-to-child transmission of the virus is relatively low, ranging from 4 to 7% of pregnancies in mothers with positive HCV viraemia [1–5]. The mechanisms underlying the low rate of vertical transmission of HCV remain unknown.

Commonly, immune responses against viruses are defined as production of specific antibodies. However, T lymphocytes play a major role in directing effector immune responses to viruses, and following viral exposure the host may develop a T-cell mediated immunity in the absence of humoral responses. Indeed, in a cohort of accidentally exposed adults, HCV-specific helper and cytotoxic T-cell responses were detectable in the blood of most subjects in the absence of HCV-specific

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antibodies [6]. Therefore, T-lymphocyte reactivity against viral antigens may represent the only immunological marker of host contact with the virus, and may endow the host with protective immunity against HCV.

Aim of the present study was to investigate whether, in children born from HCV-infected mothers, vertical exposure activates HCV-specific T-cell responses that may represent a biomarker of previous contact with the virus and possibly contribute to the low rate of vertical HCV transmission. To this purpose, we studied 23 HCV-infected mothers and their 28 children aging 1–4 years. The reactivity of HCV-specific CD4+-cells was evaluated in cultures of peripheral blood mononuclear cells (PBMCs) stimulated in vitro with four individually tested HCV-peptides.

2. Materials and methods

2.1. Study population

Twenty-three women with chronic HCV-infection and their 28 children were included in the study. At delivery, all mothers were positive for anti-HCV antibody and HCV-RNA and negative for HIV. All children underwent clinical examination at birth, every 3 months during the first year of life and every 6 months thereafter, according to a standard laboratory and clinical protocol.

Twenty-five (89%) children did not acquire infection. This was demonstrated by the fact that they lost the passive antibody by 1 year of age, were persistently HCV-RNA negative and showed normal clinical and liver function examinations. Three children were deemed infected with persistent detection of HCV-RNA starting from 3 months of age and persistent anti-HCV antibody beyond 18 months of age. Viral genotypes of the infected children (1b, 2a/2c, 3a) matched those of their mothers.

Immunological evaluation was performed at a single time point on fresh peripheral blood samples simultaneously obtained from children aged 1 to 4 years and from their mothers. Mother and child characteristics at this time are summarized in Table 1. Sixteen healthy women age-matched with the mothers (mean 32 years, range 25–43) were included as controls. Ethics approval was obtained from the local Institutional Review Committee and a signed informed consent was obtained from all participants.

2.2. Virological methods

Anti-HCV antibodies were assayed by a third generation enzyme-linked immunosorbent assay (HCV 3.0 ELISA, Ortho Clinical Diagnostic System, Raritan, NJ). Reactive samples were confirmed using a supplemental recombinant immunoblot assay (RIBA 3.0, Ortho Clinical Diagnostic Systems). HCV-RNA was detected by an in-house RT-PCR assay with nested primers of the 5′ non-coding region of the HCV genome. HCV-genotype was determined using the Innogenetic Line Probe assay (Inno Lipa HCVII, Immunogenetics, Ghent, Belgium).

2.3. HCV-peptides

Thirty-eight peptides spanning the entire HCV-polyprotein were designed, which corresponded to the GenBank published sequences of HCV-1b strain. The peptides were chosen on the basis of their ability to bind to the most common HLA molecules present in the Italian population, as predicted by computer algorithms (SYFPEITHI, http://www.uni-tuebingen.de/uni/kxi; BIMAS, http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html). The peptides were synthesized by solid-phase methods on a 433A automated peptide synthesizer (PE Applied Biosystems, Foster City, CA), as previously described [7]. Peptide purity was verified by reverse-phase high-performance liquid chromatography and electron spray mass spectrometry. The synthetic peptides were lyophilized, reconstituted in DMSO at 50 mg/ml, and diluted in RPMI 1640, as needed. One peptide from the core (C39-63), one from the envelope ($E_1$238–262), and two from the non-structural region ($NS_3$1384–1401, and $NS_3$1406–1415) resulted to be the most immunogenic in preliminary experiments, and were therefore used along this study to analyze HCV-specific T-cell reactivity. A non-antigenic HIV peptide (p23) was used as negative control [8].

Table 1
Summary of mother and child characteristics

Variable	Mothers ($n=23$)	Children ($n=28$)
Patient demographics		
Male (%)	NA	17 (61)
Age, mean (range)[a]	33 years (27–41)	28 months (12–51)
Laboratory results		
Positive HCV antibodies, *n* (%)	23 (100)	3 (11)
Positive HCV RNA, *n* (%)	23 (100)	2 (7)
Genotype 1a, *n* (%)	6 (26)	0 (0)
Genotype 1b, *n* (%)	3 (13)	1 (4)
Genotype 1a/1b, *n* (%)	2 (9)	0 (0)
Genotype 2a/2c, *n* (%)	4 (17)	1 (4)
Genotype 3a, *n* (%)	8 (35)	1 (4)
ALT <40 U/l, *n*	18	26
ALT >40 U/l, *n* (range)	5 (45–70)	3 (50–62)
Method of delivery		
Vaginal, *n* (%)	NA	25 (89)
Cesarean, *n* (%)	NA	3 (11)
Neonatal feeding		
Breast, *n* (%)	NA	19 (68)
Bottle, *n* (%)	NA	9 (32)

NA, not applicable; ALT, alanine aminotransferase.
[a] Age and laboratory results referred to the time of immunological evaluation.

2.4. In vitro assays for CD4+-cell reactivity

Peripheral blood mononuclear cells (PBMCs), isolated from fresh blood by Ficoll density gradient centrifugation (Cedarlane, Hornby, Canada) were cultured in RPMI 1640 supplemented with 10% human AB serum at 2×10^5/200 μl/well in 96-well U-bottomed plates (Corning, Corning, NY). To assess immune-competence the responses induced by a polyclonal mitogen (PHA) were evaluated. PBMCs were cultured without stimulation, or were stimulated with PHA (M form, Sigma Chemicals Co., St Louis, MO) or with the selected HCV-peptides at 10 μg/ml. For inhibition experiments, PBMCs were preincubated with various amounts of neutralizing mAb against IL-10 (clone JES3-9D7, PharMingen, San Diego, CA) for 30 min, and the inhibitor was left in culture for the entire stimulation period. Cultures were performed in duplicates. In all cases, 5-bromo-2′-deoxyuridine (BrdU, 20 μM; Sigma Chemicals) was added to each well during the last 6 h of culture, and lymphocyte proliferation was assessed by flow cytometry as BrdU incorporation by $CD4^+$ lymphocytes, as described elsewhere [9,10]. Briefly, supernatants were stored at −20 °C for further analyses and cells were double-stained with anti-CD4 and -CD25 mAbs (Caltag, Burlingame, CA). Following cell fixation, permeabilization and partial DNA denaturation, cells were directly stained with anti-BrdU mAb (Becton Dickinson, San Jose, CA). Cells were collected and analyzed using a FACScan (Becton Dickinson) within 24 h. The degree of HCV-specific CD4+-cell proliferation was expressed as proliferation index (PI), calculated from the fraction of CD25+/BrdU+ CD4+ cells found with antigen divided by that found without antigen. A representative analysis is shown in Fig. 1. A significant PI in response to the HCV-peptides was defined as being >2.75, which was the cut-off value greater than the mean PI plus three times the standard deviation obtained in a group of 16 healthy HCV-seronegative controls.

The degree of CD4+-cell activation was evaluated in the same samples and expressed as activation index (AI), calculated from the fraction of CD25+ CD4+ cells found with antigen divided by that found without

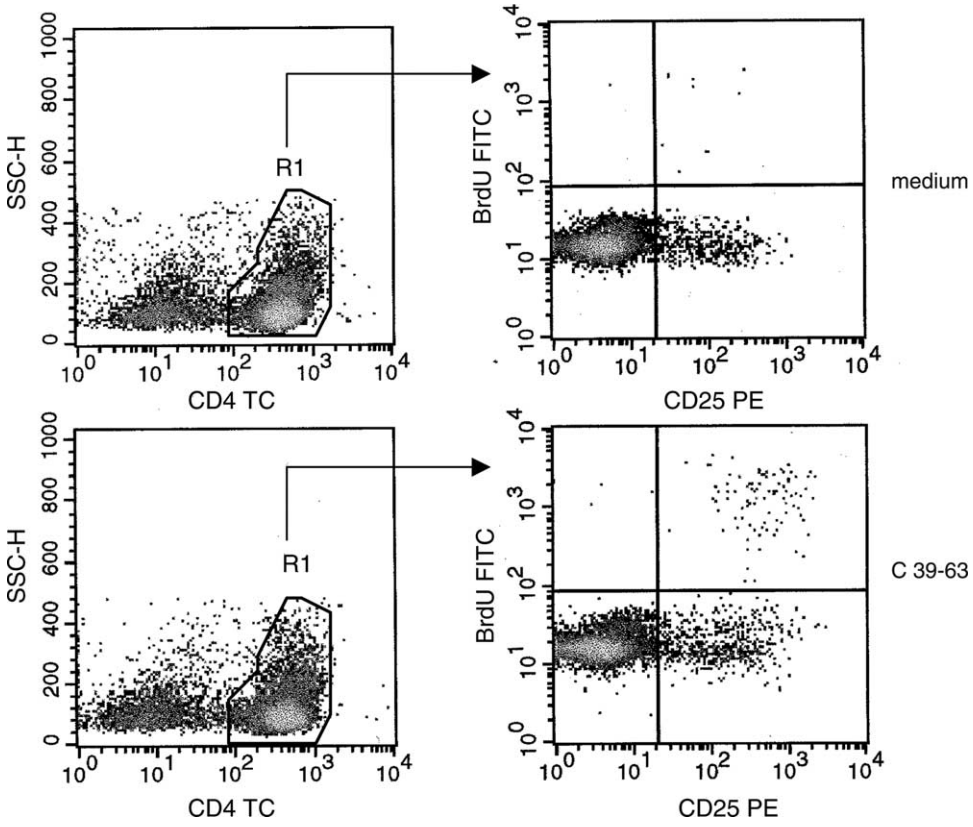

Fig. 1. Representative flow cytometric analysis of CD4+-lymphocyte activation and proliferation upon stimulation of PBMCs with HCV-peptides. PBMCs were cultured in the absence or presence of HCV-peptides for 6 days, and BrdU incorporation was allowed during the last 6 h. Cells were then double stained with TC-conjugated anti-CD4 and PE-conjugated anti-CD25 mAbs. After cell fixation, permeabilization and partial DNA denaturation, cells were stained with FITC-conjugated anti-BrdU mAb. 3-Colour flow cytometry was performed within 24 h. Gated on CD4+-lymphocytes (R1), cell activation was assessed as surface expression of CD25, and proliferation was assessed as incorporation of BrdU by CD25+ cells. The degree of HCV-specific activation was expressed as activation index (AI), calculated from the fraction of CD25+ CD4+ cells found with antigen divided by that found without antigen. The degree of HCV-specific proliferation was expressed as proliferation index (PI), calculated from the fraction of CD25+/BrdU+ CD4+ cells found with antigen divided by that found without antigen.

antigen. The cut-off value obtained in the controls that defined a positive AI response was 1.79.

Samples with intra-assay coefficient of variation >10% were excluded from the analysis; inter-assay variability, analyzed on three mothers and their children at two time points over a period of 2 months, was approximately 10%.

2.5. Cytokine measurements

After secretion in the culture supernatants, cytokines may undergo partial consumption by PBMCs. Hence, their levels were evaluated at both day 2 and at the ending of the culture (day 6). The release of IL-2, IL-10 and IFN-γ in the supernatants was measured by specific sandwich enzyme-linked immunosorbent assay (ELISA), by use of commercially available pairs of monoclonal antibodies (Endogen, Woburn, MA), according to the manufacturer's instructions.

2.6. Statistical analysis

All statistical analyses assumed a 2-sided significance level of 0.05. The Fisher exact test and the Mann–Whitney *U* test were used for comparisons between mothers and children. The paired Wilcoxon test was used to analyze inhibition experiments. The Spearman rank test was used to describe correlations. Data analyses were performed with Openstat3 software.

3. Results

3.1. CD4+-cell proliferative responses

Proliferative response to PHA did not differ between controls (PI, mean ± SEM: 769.39 ± 72.57), mothers (771.75 ± 76.25) and children (610.11 ± 56.14), indicating that both the HCV chronically infected mothers and their children were immune-competent.

The analysis of the HCV-specific CD4+-cell responses revealed that lymphocytes from children born from HCV-infected mothers were reactive to viral peptides. In these children, the proportion of subjects with an antigen-specific CD4+-cell proliferative response (i.e. with PI greater than the positive cut-off value of 2.75) to at least one HCV-peptide was 71%, compared with 52% in the mothers and 0% in healthy controls. Fig. 2(A) shows the frequency of subjects with positive proliferation to individual HCV-peptides in the three groups. The proportion of responders among children was significantly higher than among healthy controls upon stimulation with any peptide. Also, the frequency of children who responded to the peptide from the

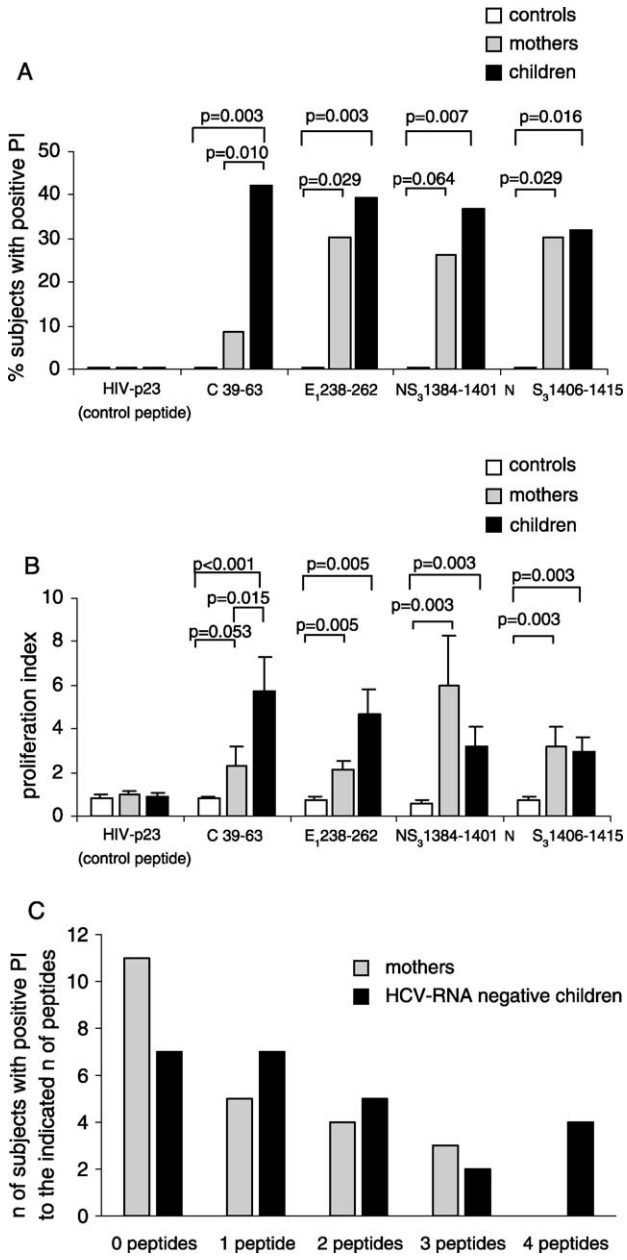

Fig. 2. Presence of significant CD4+-cell proliferative response to HCV-peptides in children vertically exposed to HCV. (A) Percentage of subjects, in the groups of controls (white bars), mothers (gray bars) and children (black bars), with a significant proliferation index (PI) to the indicated HCV-peptides. A significant PI was defined as being >2.75, which was the cut-off value calculated from the group of healthy HCV-seronegative controls (see Section 2). *P* values calculated using the Fisher exact test. (B) Intensity of CD4+-cell proliferation to the indicated HCV-peptides in the three groups. Mean ±SEM from 16 controls (white bars), 23 mothers (gray bars), and 28 children (black bars). *P* values calculated using the Mann–Whitney *U* test. (C) Number of subjects, in the groups of mothers (gray bars) and HCV-RNA negative children (black bars), with a significant PI to the indicated number of peptides.

core region was significantly higher than that of mothers (42.3 vs. 8.7%, $P=0.01$). As shown in Fig. 2(B), similar results were observed when the intensity of the proliferative responses, expressed as PI, was evaluated. Children's PI were significantly higher compared with healthy controls in response to all the individual peptides. The intensity of proliferation in response to the peptide derived from HCV-core region was significantly lower in the mothers than that observed in children (PI, mean ± SEM: 2.28 ± 0.95 vs. 5.7 ± 1.59, $P=0.015$). The intensity of the proliferative response to the other peptides did not significantly differ between mothers and children. Subjects unresponsive to any of the four peptides tested, as well as subjects with CD4+-cells able to respond to multiple peptides (Fig. 2(C)), were observed in both the mothers and the children groups. However, the number of unresponsive subjects was higher among mothers (11 of 23) than among the HCV-RNA negative children (7 of 25); and only within the children there were subjects whose CD4+-lymphocytes responded to all the peptides tested. One of the three HCV-RNA positive children was unresponsive, and two responded to a single peptide. No correlation was observed between CD4+-cell proliferation to any HCV-peptide and HCV-genotype, duration of maternal infection, mode of delivery, or type of neonatal feeding. The analysis of HCV-specific proliferative responses in the four families with multiple children generally showed concordance in CD4+-cell reactivity between siblings, although lymphocyte proliferation could be directed against different viral antigens (Fig. 3).

Inhibition experiments were performed to investigate the possible role of IL-10 in conditioning the low proliferative response observed in the mothers. As shown in Fig. 4, neutralizing anti-IL-10 mAb induced a dose-dependent increase in the percentage of proliferating CD4+-lymphocytes, that resulted in a significant increase of PI (PI to HCV-core, mean ± SEM: 3.72 ± 0.85 vs. 0.50 ± 0.11, $P=0.034$; PI to HCV-envelope: 5.11 ± 1.87 vs. 2.17 ± 0.80, $P=0.034$).

3.2. HCV-specific CD4+-cell activation

The intensity of CD4+-activation in response to HCV-peptides, measured as surface expression of CD25 and expressed as AI, did not differ between mothers and children (Fig. 5(A)). However, in the children proliferation and activation of CD4+-cells were directly correlated upon stimulation with all the viral peptides; on the contrary, the same direct correlation was not observed in the mothers, where high AI were not generally coupled with high PI (Fig. 5(B)).

3.3. HCV-specific production of cytokines

The production of IL-2 and IFN-γ did not differ significantly between mothers and children, although the overall production of both cytokines upon stimulation with HCV-peptides tended to be lower in the mothers than in their children (at day 6: IL-2 mean ± SEM 25.75 ± 15.47 pg/ml vs. 33.59 ± 15.97; IFN-γ 84.42 ± 26.60 vs. 178.13 ± 61.55, P = ns). However, the overall production of

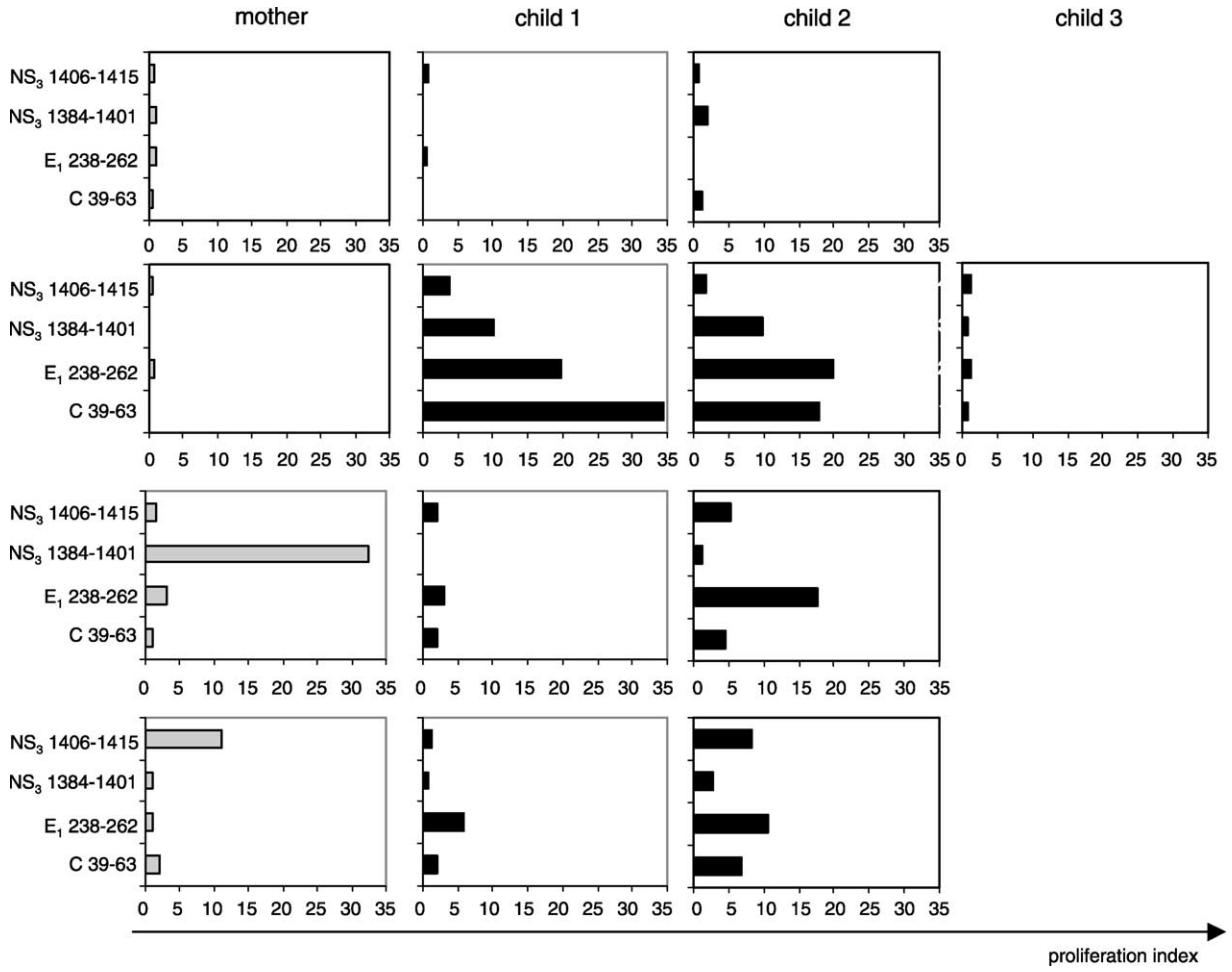

Fig. 3. Analysis of HCV-specific proliferative responses in four families with more than one child. In most cases, concordance in CD4+-cell reactivity between siblings was observed. Proliferation index (PI) to HCV-peptides from a mother (gray bars) and her multiple children (black bars) is shown.

the immunosuppressive cytokine IL-10 upon stimulation with HCV-peptides was significantly higher in mothers than in children, both at day 2 (mothers 31.14 ± 4.63 pg/ml vs. children 9.02 ± 2.73, $P = 0.003$) and at day 6 (31.88 ± 4.67 vs. 20.53 ± 3.24, $P = 0.046$). Fig. 6(A) and (B) show IL-10 production in response to the different viral peptides after 2 and 6 days of culture, respectively. As shown in Fig. 6(C) the levels of IL-10 produced at day 2 were inversely correlated with the intensity of the proliferative responses in the mothers, but not in the children.

4. Discussion

In this study, we evaluated HCV infection and HCV-specific CD4+-cell reactivity in a cohort of children born from HCV infected mothers. Our results clearly indicate that, despite the absence of anti-HCV antibodies, the majority of children (71%) born from HCV-infected women have circulating CD4+-lymphocytes reacting against HCV-specific antigens. This observation deserves several considerations. First of all, as previously demonstrated in adults exposed to HCV and to other viral infections [6,11], it seems to confirm that the evaluation of T-cell reactivity may represent a useful tool in revealing previous exposures of the host to a viral agent. Hence, T-cell reactivity is able to detect a component of the immune response that is not revealed when considering antibody seroconversion solely. Second, the finding of a high rate of HCV immunization in our children seems to indicate that vertical exposure to HCV could be quantitatively more frequent than what revealed by the rates of vertical infection, defined as detection of anti-HCV antibodies. Third, the activation of HCV-specific CD4+-cell responses upon exposure to the virus may depend on factors related to the virus, to the modality of viral exposure and to the host.

The relevance of the former two factors is suggested by our observation of a general concordance in CD4+-cell reactivity between siblings, who were probably exposed to the same virus and with similar modalities. The influence of host-related factors is suggested by the finding that, within pairs of siblings with HCV-specific CD4+-cell reactivity, responses were sometimes directed against different viral antigens. Also, in the only case analysing three siblings, two

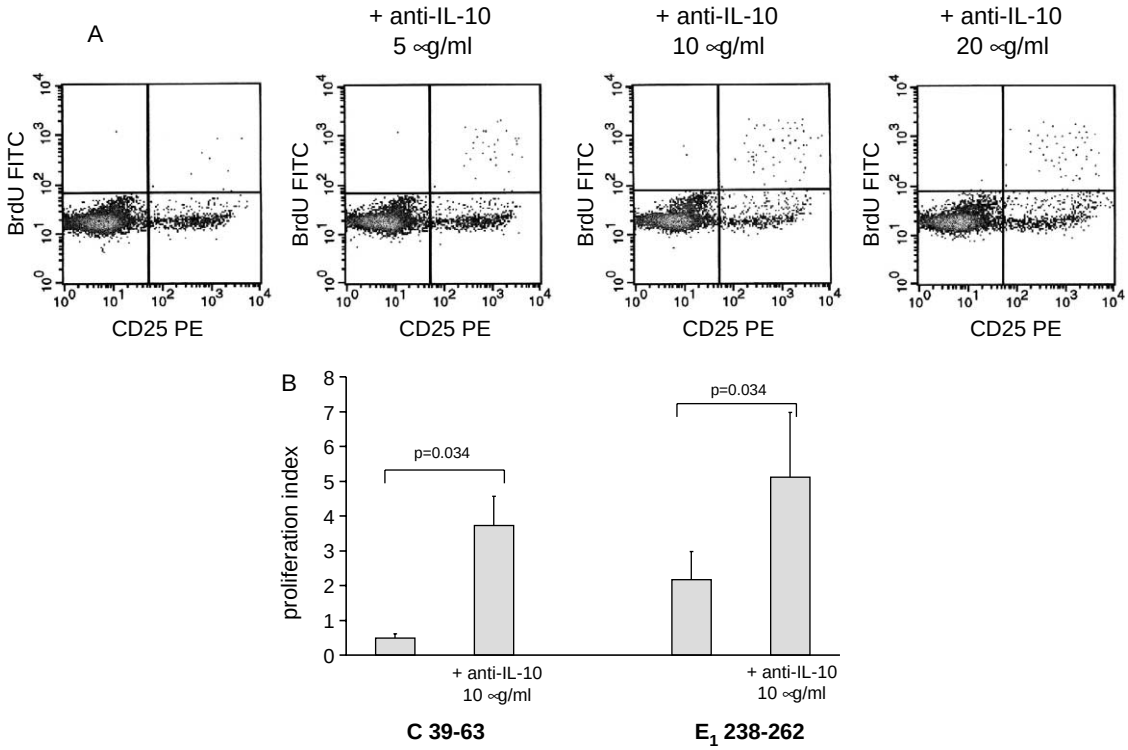

Fig. 4. Contribution of IL-10 in the low proliferative responses of CD4+-lymphocytes from chronically HCV-infected mothers. PBMCs from mothers were preincubated with neutralizing mAb against IL-10 for 30 min, and the inhibitor was left in culture for the entire stimulation period. At the end of the culture, the cells were prepared and analyzed as described in Fig. 1. (A) Anti-IL-10 mAb induced a dose-dependent increase in the percentage of proliferating CD4+-lymphocytes, reaching plateau at 10 μg/ml. One representative analysis of two independent experiments is shown. (B) Proliferation index (PI) to the indicated HCV-peptides was significantly increased by 10 μg/ml anti-IL-10 mAb. Mean±SEM from four mothers. *P* values calculated using the paired Wilcoxon test.

children presented positive HCV-specific proliferative responses while the third showed no such response. The role of factors that are known to influence the vertical transmission of HCV, such as maternal intravenous drug use and HCV viral load [1,3,5,12,13], was impossible to evaluate because only one of 23 mothers reported a history of drug use, and quantification of HCV viraemia during pregnancy and at delivery was unavailable in most cases. Other factors that do not affect vertical transmission, such as HCV genotype, duration of maternal infection, mode of delivery and type of neonatal feeding [2,3,12–15], did not seem to influence the development of HCV-specific CD4+-cell reactivity in our children.

Moreover, our results suggest that the HCV-specific immune responses observed in children, who did not show any marker of HCV replication in most cases, are qualitatively different from those observed in their chronically HCV-infected mothers. In fact, we observed that in the children the proliferative responses, particularly those against peptides derived from the HCV core region, were more frequent and vigorous, and the activation of CD4+-cells was directly correlated with proliferation upon stimulation with any peptide. On the contrary, in the mothers the proliferative responses to HCV-peptides were less frequent, restricted to a lower number of peptides, and the proliferation to the peptide derived from the core protein was less intense. These findings are in accordance with those reported in several cohorts of adults in different clinical stages of HCV infection, demonstrating impaired HCV-specific T-cell reactivity in chronically infected patients, and highlighting the protective role of T-cell responses to HCV structural proteins against the development of chronic hepatitis [16–18]. Notably, none of the three children with vertical HCV infection presented lymphocyte proliferation in response to peptides from core or envelope. In our study, the lower proliferation of CD4+-lymphocytes from the mothers was not accompanied by lower activation of these cells. Therefore, CD4+-cells from the mothers appeared to respond to HCV antigens with incomplete activation, characterized by normal levels of IL-2 secretion and CD25 (IL-2 receptor) expression, but low levels of proliferation. Incomplete activation of HCV-specific T-cells in chronically infected patients had been previously described by other Authors and ascribed to abnormal priming of CD4+T-cells specific to structural proteins by dendritic cells infected by HCV [19,20]. We suggest that production of high levels of IL-10 early upon stimulation with HCV-antigens may represent an additional mechanism underlying the incomplete activation of HCV-specific lymphocytes in chronically

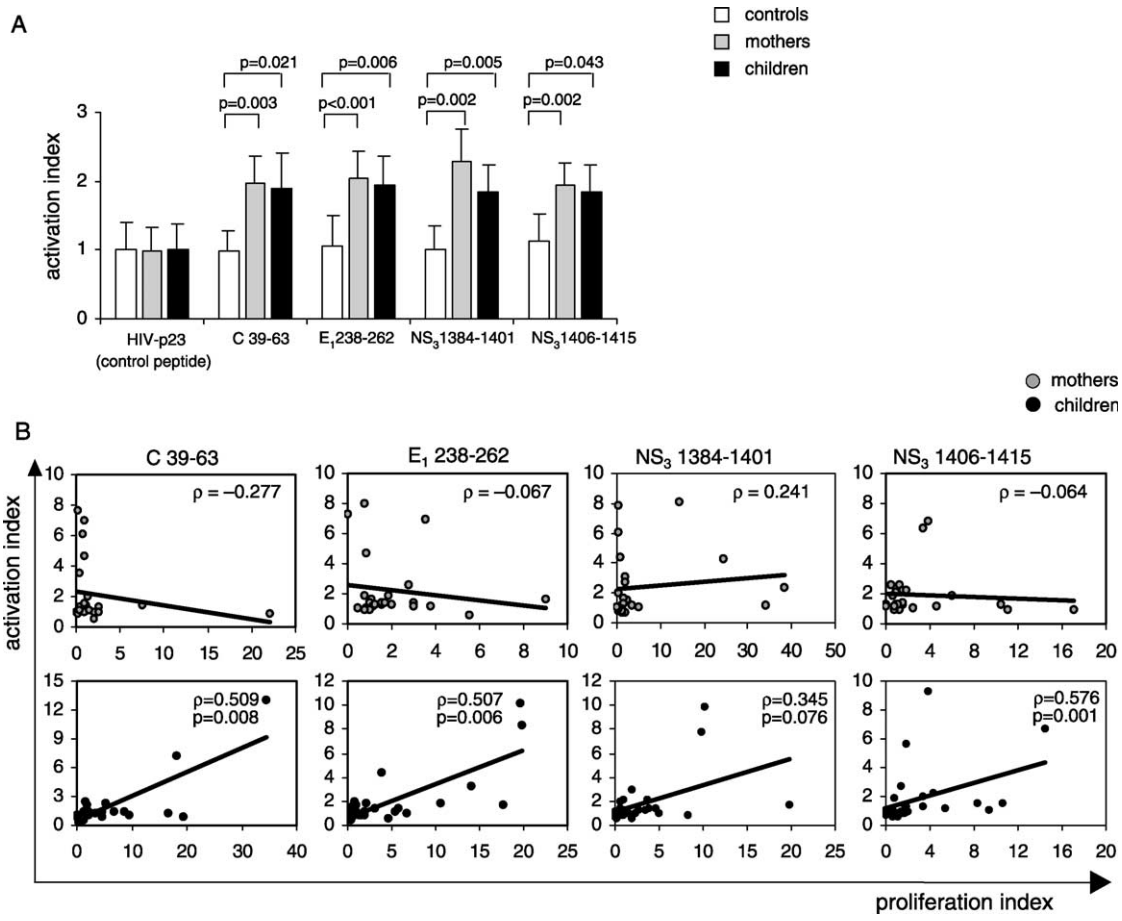

Fig. 5. (A) CD4+-lymphocyte activation in response to stimulation with individual HCV-peptides. The intensity of cell activation, measured as surface expression of CD25 on CD4+-cells and expressed as activation index (AI), did not differ between mothers and children; both mothers and children had significantly higher AI than controls. Mean ± SEM from 16 controls (white bars), 23 mothers (gray bars), and 28 children (black bars). *P* values calculated using the Mann–Whitney *U* test. (B) Correlation between AI and proliferation index (PI) upon stimulation with individual HCV-peptides. While in the children AI and PI were directly correlated in response to all the viral peptides, in the mothers high AI was not generally coupled with high PI. *ρ* and *P* values calculated using the Spearman rank test.

infected individuals. In fact, IL-10 is a suppressive cytokine that hampers the activation of immune responses at various levels, with a suggested role in preventing eradication of chronic infection by intracellular pathogens [21]. Several observations in our study seem to support a role of IL-10 in incomplete activation. First, we observed that the production of IL-10 in the supernatants of PBMCs stimulated with HCV-peptides was markedly higher in the mothers than in the children, especially in the first days of culture. Second, we found that IL-10 production and CD4+-cell proliferation were inversely correlated in the mothers. Third and most relevant, we demonstrated that neutralization of IL-10 during stimulation of PBMCs with HCV-peptides was able to restore CD4+-lymphocyte proliferation.

This study did not investigate the cellular sources of IL-10 and of the other investigated cytokines. However, other Authors have demonstrated that the main source of cytokines, in the same culture model used in our study, are CD4+-T-cells [22, 23]. Therefore, our finding of different cytokine levels in the culture supernatants from mothers and children is likely to reflect a qualitative difference in CD4+-cell responses between chronically infected and healthy virus-exposed subjects. In this respect, we observed not only that the production of IL-10 was markedly higher in the mothers than in the children, but also that their IFN-γ production tended to be lower. These results are in accordance with those reported by other Authors, describing higher levels of IL-10 and lower levels of IFN-γ in HCV viraemic patients in comparison to individuals with serological viral clearance [22,24]. The lower levels of IFN-γ in the cultures from mothers should be considered with the notion that, through the production of IFN-γ, CD4+-lymphocytes mainly promote cellular immune responses and cytotoxic T-lymphocyte activity that, in chronic HCV infection, mediate control of viral replication. These considerations do not exclude the possibility that immune cells other than CD4+-lymphocytes may be affected in their ability to produce cytokines in subjects with chronic HCV-infection. In fact, supporting a key role of IL-10 in impairing CD4-cell

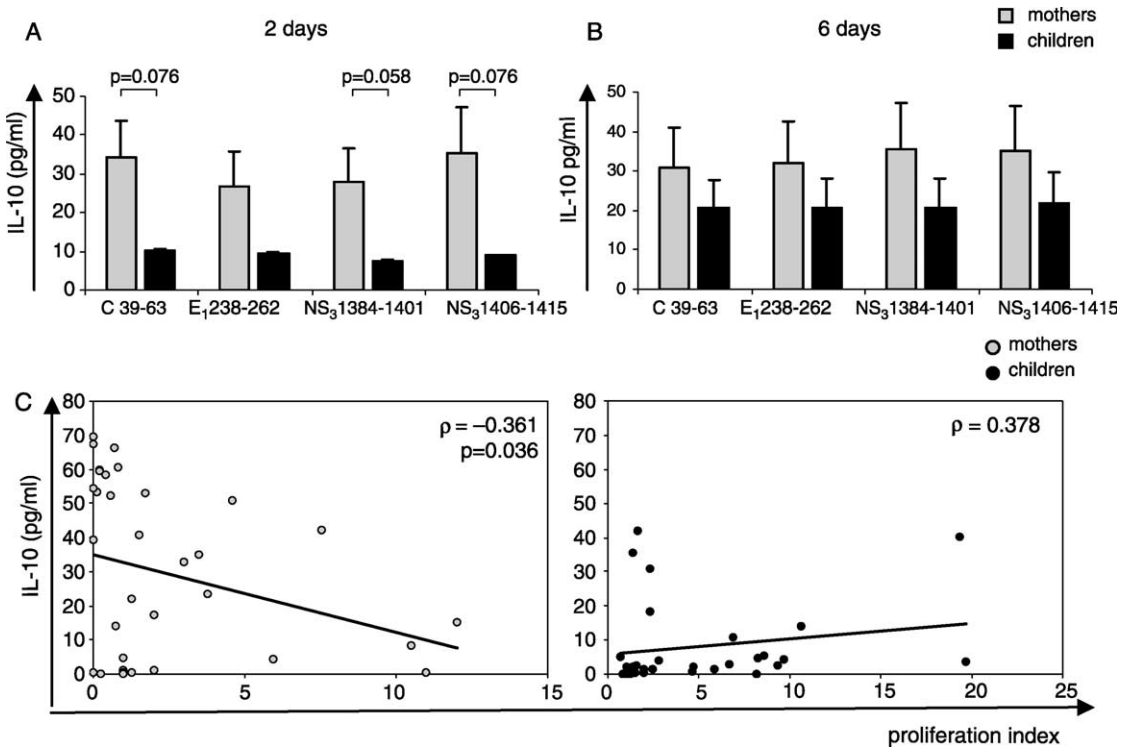

Fig. 6. In vitro production of IL-10 upon stimulation of PBMCs with individual HCV-peptides for (A) 2 days, or (B) 6 days. Mean ± SEM from nine mothers (gray bars) compared with nine children (black bars). *P* values calculated using the Mann–Whitney *U* test. (C) Correlation between IL-10 production at 2 days and proliferation index (PI), upon stimulation with individual HCV-peptides. Within the group of mothers, but not in the group of children, the levels of IL-10 produced at day 2 were inversely correlated with the intensity of the proliferative responses. *ρ* and *P* values calculated using the Spearman rank test.

reactivity, in a different setting of HCV-infected adults, we similarly observed that the production of IL-10 by dendritic cells was higher from chronically infected subjects than from subjects who serologically cleared the virus, and that IL-10 produced by dendritic cells was inversely correlated with HCV-specific T-cell proliferation (manuscript in preparation).

In conclusion, the present study provides evidence that the majority of children born from HCV-infected women develop antigen-specific CD4+-cell immune responses, which are likely to have a protective role against infection. It is possible that the modality of viral exposure, possibly contact with the virus in presence of maternal anti-HCV antibodies, could prevent successful infection in most of these children. Further studies planned to characterize CD4+-cell reactivity at different time-points from birth would be of value to better define the mechanisms underlying this phenomenon.

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