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Therapeutic DNA Vaccination Using *In Vivo* Electroporation Followed by Standard of Care Therapy in Patients With Genotype 1 Chronic Hepatitis C

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Clearance of infections caused by the hepatitis C virus (HCV) correlates with HCV-specific T cell function. We therefore evaluated therapeutic vaccination in 12 patients with chronic HCV infection. Eight patients also underwent a subsequent standard-of-care (SOC) therapy with pegylated interferon (IFN) and ribavirin. The phase I/IIa clinical trial was performed in treatment naive HCV genotype 1 patients, receiving four monthly vaccinations in the deltoid muscles with 167, 500, or 1,500 µg codon-optimized HCV nonstructural (NS) 3/4A-expressing DNA vaccine delivered by *in vivo* electroporation (EP). Enrollment was done with 2 weeks interval between patients for safety reasons. Treatment was safe and well tolerated. The vaccinations significantly improved IFN-γ–producing responses to HCV NS3 during the first 6 weeks of therapy. Five patients experienced 2–10 weeks 0.6–2.4 $\log_{10}$ reduction in serum HCV RNA. Six out of eight patients starting SOC therapy within 1–30 months after the last vaccine dose were cured. This first-in-man therapeutic HCV DNA vaccine study with the vaccine delivered by *in vivo* EP shows transient effects in patients with chronic HCV genotype 1 infection. The interesting result noted after SOC therapy suggests that therapeutic vaccination can be explored in a combination with SOC treatment.

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of severe liver disease and efficiently establishes persistent infections. Data suggest that a specific T cell response is essential for clearance[1,2] and control of HCV.[3–5] Factors correlated to chronicity are a high degree of genetic variability[6,7] and HCV proteins which impair the host response.[8] The specific T cell response is impaired and/or dysfunctional during chronic infection.[9–11] This dysfunction may be caused *via* escape mutations within T cell epitopes if allowed by viral fitness,[12–17] or by a direct effect induced of viral proteins.[18–22] The dysfunction is actively maintained because blocking of regulatory T cells (Tregs) or programmed death receptor-1 ligand *in vitro* restores T cell function.[11,23] The dysfunction may be alleviated by immune-modulating therapies,[24,25] such as therapeutic vaccination.[26] The purpose here is to activate, or reactivate and expand, HCV-specific T cells outside the liver by providing HCV antigens with optimal immunogenic conditions.[27] Since the dysfunction seen in chronic infection involves both $CD4^+$ and $CD8^+$ T cells, a vaccine should activate both of these cell types.[9] $CD8^+$ T cell activation generally requires an endogenous production of antigen to induce a potent human leukocyte antigen (HLA) class I antigen presentation. Hence, $CD8^+$ T cell responses are best activated through genetic immunization. Two major ways exist by which a genetic vaccine can be delivered, either by modified viral vectors, or by a direct injection of plasmid DNA. Recent studies have shown that viral vectors can activate potent immune responses in chimpanzees and humans.[28,29] With respect to DNA vaccines, studies have been rather disappointing.[30] However, new technologies can improve the immunogenicity of plasmid DNA, for example *in vivo* electroporation (EP).[31] With EP, short electrical pulses are administered which cause permeabilization of cellular membranes that increase DNA uptake and vaccine expression, and which also generates a local inflammatory response.[32] This technique has been used in cancer patients,[33] and has been found to raise T cell responses to HCV in chimpanzees.[34] Another question is which antigens should be used. Ideally, the antigen should be highly expressed in infected cells, and represent a well-conserved viral region so that the vaccine-primed T cells will recognize endogenous virus. A recent meta-analysis suggested that the best antigens to use in a protective vaccine would be the structural antigens.[35] Since these are often highly variable and may be less suitable in therapeutic vaccines. The most conserved HCV genes are the core, nonstructural (NS) 3 and NS5B genes,[7] which suggests that these are suitable for inclusion in therapeutic vaccines.

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In the current study, we initiated a small proof-of-concept study to investigate whether a therapeutic HCV vaccination has an impact on the immune response and whether vaccination has any influence on a subsequent standard-of-care (SOC) treatment in patients with chronic HCV infection.

RESULTS

Safety and tolerability of DNA vaccination delivered by *in vivo* EP

All patients tolerated the vaccine injections well and no major side effects were noted. Within a minute after the vaccine injection, EP was performed at the same site. The correct delivery of the EP pulses could easily be discerned by two small muscle twitches in the vaccinated arm. A short-lasting pain was recorded that waned within a few minutes. Subjects receiving the EP procedure described the experience qualitatively as "leaving a small feeling of having been hit". The perceived pain level has been illustrated in **Figure 1**. During and after the EP procedures, patients stayed in the hospital for 2 hours. No major adverse event beside the transient pain was noted. *In vivo* EP was the cause for the transient local pain. The pain was immediate at administration of the electrical pulses. On a relative 10-level pain scale the *in vivo* EP gave scores ranging from 2 to 8 whereas the DNA injection gave a score from 0 to 5 (**Figure 1**; $P < 0.01$, Mann–Whitney U-test). The perceived pain did not increase with repeated doses of the DNA vaccine (**Figure 1**). No significant change in blood chemistry was noted related to the treatment (data not shown). No severe adverse event was seen.

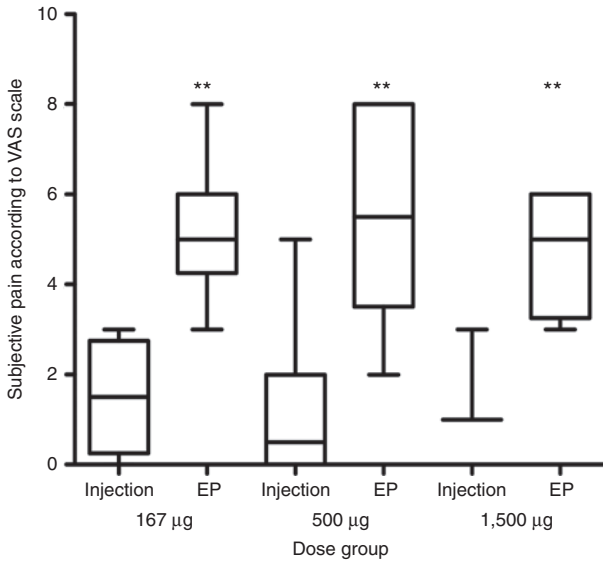

Figure 1 Pain levels recorded immediately after *in vivo* electroporation (EP) given in relation to the pain level related to the DNA injection. Values are summarized from all four treatments and have been given as the mean pain level estimated using the visual analogue scale (VAS). Each box shows the range from the first to third quartiles, and the median divides this large box into two boxes for the second and third quartiles. Also shown are the minimum reported values, from the second quartile box and down, and from the third quartile box up to the maximum reported values. The presence of a statistical difference (e.g., injection compared with EP) has been indicated as follows: $^{**}P < 0.01$ using the Mann–Whitney U-test.

DNA vaccination induces a transient HCV-specific T cell activation

The second endpoint of the study was immunogenicity. This was determined both on the B and T cell level. The levels of anti-NS3 IgG were quantified in all samples. The mean endpoint titers did not change significantly during the vaccinations due to high levels of pre-existing antibodies in all patients (data not shown). However, when analyzing changes in optical densities in the linear slope of the dilution curve (samples should have an optical density at 405 nm of >0.3 to <1.0), an early transient effect of the vaccinations was noted (**Figure 2a**). The first vaccination consistently induced an increase in relative antibody levels detected by a paired comparison of the samples obtained at week 0 and 2 in all patients (**Figure 2a**; $P < 0.05$, Wilcoxon's matched pairs test). The increase was most pronounced in the two lowest dose groups ($P < 0.01$). Three of the six subjects in the two lowest dose groups with an increase in NS3 antibodies had *de novo* T cell activation (labeled with an asterisk (*) in **Figure 2a**). Thus, a limited effect of the vaccination seemingly was observed in the presence of pre-existing antibody levels.

The presence of HCV-specific T cell responses before, during, and after the therapeutic vaccination was determined as the number of interferon (IFN)-γ–producing T cells, or spot-forming cells (SFCs) by ELISpot (**Figures 2–5**), and the level of proliferation as determined by the level of $^{[3]}$H-thymidine incorporation (data not shown). In the ELISpot assay, only the responses to nine peptide pools spanning the whole NS3/4A region were used for the statistical comparison to avoid repeated use of the same epitope and to overcome HLA restriction.

The number of the IFN-γ–producing spots seemed to increase after the two first vaccinations when comparing the number of SFCs at week 0, and the same at weeks 2 and 6 (**Figure 2b**). Proliferative T cell responses to NS3 or NS4 were detected in 8 out of 12 subjects before or after vaccination (data not shown).

In the 167 and 500 µg dose groups, *de novo* ELISpot responses appeared in four subjects, and in the highest dose group, one showed appearance of *de novo* ELISpot responses (**Figures 2c** and **5**). We compared the breadth of the T cell responses to the nine NS3/4A genotype 1a peptide pools by ELISpot 2 weeks after vaccinations with the responses at week 0 (**Figures 2c** and **5**). Before vaccination, a total of 162 peptide pools from 11 of the patients were assayed by ELISpot, and three (2%) were positive (**Figures 2c** and **5**). Altogether, 39 out of a total of 450 peptide pools assayed after vaccination were positive (**Figures 2c** and **5**; $P < 0.0001$, Fisher's exact test). In addition, the frequency of responses to the nine peptide pools significantly increased treatment at week 2 (12 out of 99, $P < 0.05$, Fisher's exact test), week 6 (10 out of 81, $P < 0.05$, Fisher's exact test), and week 36 (9 out of 108, $P < 0.05$, Fisher's exact test), when compared with week 0 (1 out of 81; **Figures 2c** and **5**). The responses were most pronounced in the 167 and 500 µg dose groups. Taken together, this suggests that the vaccination transiently improved T cell activation.

The responses to individual peptide pools have been given for two patients in the 500 µg dose group (**Figure 3**). In both, the activation, or reactivation, of HCV NS3/4A IFN-γ–producing T cells coincided with the suppression of the HCV RNA levels in blood (**Figure 3**).

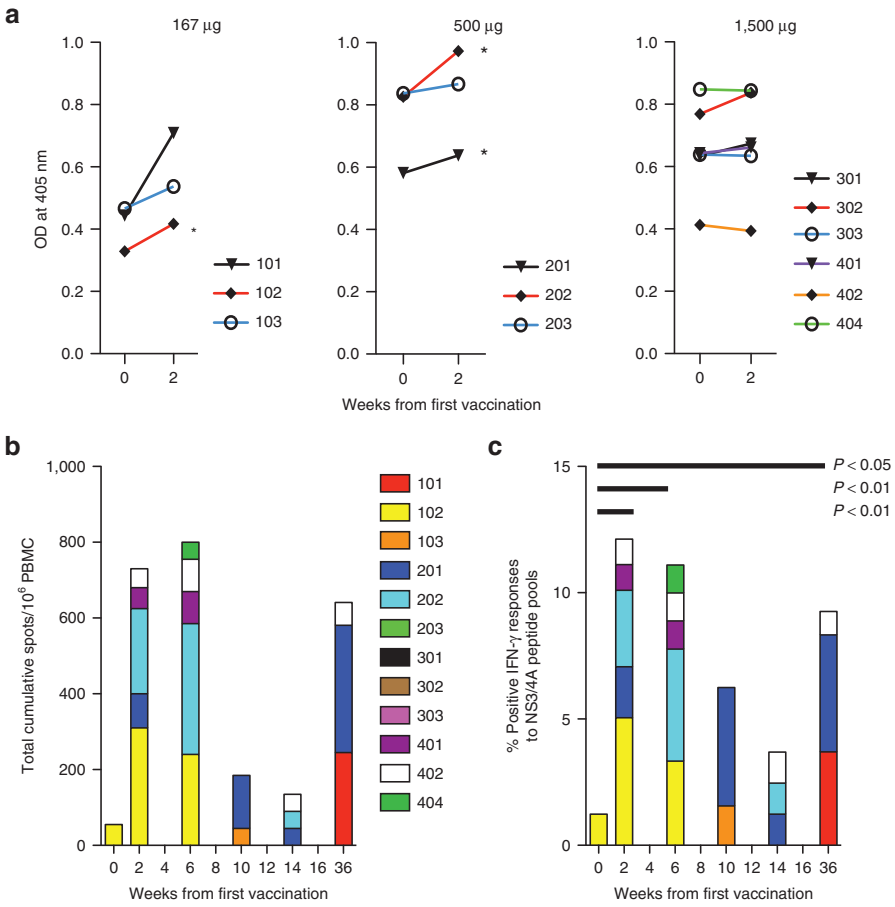

Figure 2 HCV NS3/4A-specific immune responses after vaccination. Immunogenicity of the first two doses of DNA determined as (**a**) increases in relative antibody levels to NS3 or (**b**,**c**) increases in T cell responses to overlapping peptide pools spanning the complete NS3/4A. Antibody levels to NS3 were determined at the linear slope of the dilution curve. Values have been given as the optical density (OD) at 405 nm (as shown in **a**). *Indicate subjects with an increase in NS3 antibodies that also had *de novo* T cell activation. T cell responses were determined using the peptide pools in a standard ELISpot assay where the numbers of IFN-γ–producing cells were measured (as shown in **b** and **c**). Results have been given by adding positive (≥45 SFCs/10^6 PBMC) cumulative SFCs/10^6 PBMC (as in **b**) or the frequency of positive reactions to the nine overlapping peptide pools (as in **c**). In **b**, each color in the staples indicates a specific individual and for this individual the cumulative SFCs/10^6 PBMC to the nine overlapping peptide pools. In **c**, the percent positive IFN-γ responses to the nine overlapping NS3/4A peptide pools are shown for each timepoint. Each color in the staples indicates a specific individual. Values are calculated by multiplying the number of patients analyzed with the number of peptide pools for each timepoint. Statistical comparisons were performed using Wilcoxon's matched pairs test (in **a**) and the Fisher's exact test (in **c**). HCV, hepatitis C virus; IFN, interferon; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell.

T cell responses in relation to the HLA restriction elements of the host

In patient 102, a response to the pool 1326-1410 went from 55 SFCs/10^6 peripheral blood mononuclear cell (PBMC) before vaccination to 100 SFCs/10^6 PBMC at week 2, 90 SFCs/10^6 PBMC at week 6, and <45 SFCs/10^6 PBMC at week 14. This patient carries the HLA-A3 and -B35 alleles (**Table 1**), represented in the $CD8^+$ peptide pool by the NS3-derived A3-restricted epitope LIFCHSKKK at residues 1391-1399, and represented in HLA-B/C $CD8^+$ peptide pool by the NS3-derived B35-restricted epitope HPNIEEVAL at residues 1359-1367. The responses to these two pools at week 0 were <45 and 45 SFCs, at week 2 were 55 and 75 SFCs, at week 6 were <45 and 75 SFCs, at week 10 were <45 and 175 SFCs, and at week 14 were <45 and 75 SFCs/10^6 PBMC, respectively.

Patient 201 had responses to pools 1176-1260, 1326-1410, and 1401-1485 (**Figure 3**). Of these, pool 1326-1410 contains one epitope presented by the patient's HLA-A3 molecule.[36] Patient 202 carrying the HLA-A24 allele had responses to several pools, with the strongest response to the pool spanning 1101-1185 (**Figure 3**). An HLA-A24 epitope spanning residues 1100-1107[37] is present in two overlapping pools to which the patient had reactivity (**Figure 3**).

Patients 401 and 402 were the only patients with responses to the C-terminal peptide pool 1626-1710 containing one HLA-B8–restricted cytotoxic T lymphocyte epitope,[38] and both were the only ones having the HLA-B8 allele.

DNA vaccination has transient effects on serum levels of HCV RNA

Changes in the viral load outside the ±0.5 $\log_{10}$ normal variation was seen as transient changes in eight patients, three had increases and five had reductions in the viral load (**Figures 4** and **6**). Only one patient had an increase in viremia levels within two weeks

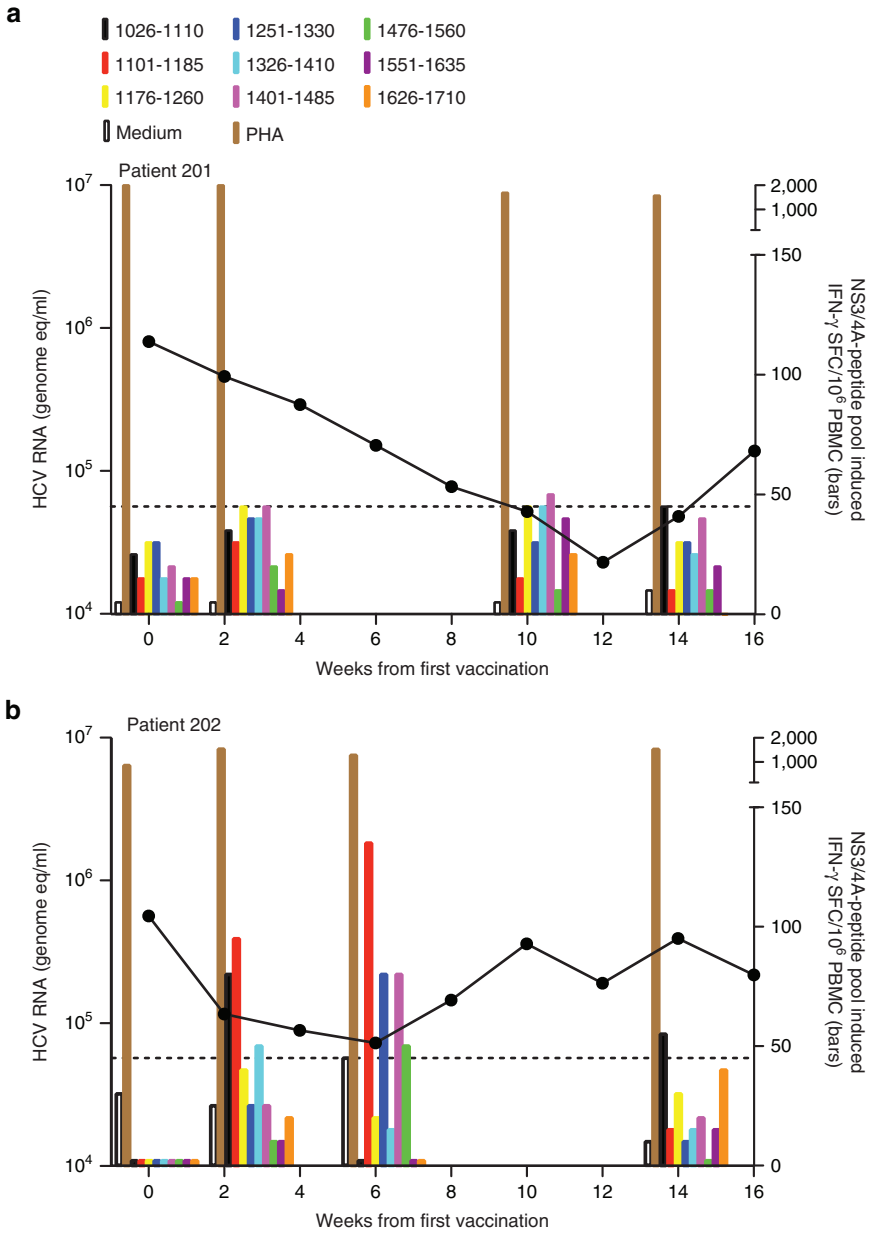

Figure 3 Kinetic analysis of viral load in relation to HCV-specific immune responses. Change in serum levels of HCV RNA and the number of IFN-γ–producing cells to individual peptide pools spanning NS3/4A in patients (**a**) 201 and (**b**) 202 who both received the 500 µg DNA dose. Data has been given as genome equivalents (eq)/ml (line) and IFN-γ–producing SFCs/10^6 PBMC (bars). The dotted line indicates the >45 SFCs/10^6 PBMC cut-off. HCV, hepatitis C virus; IFN, interferon; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SFC, spot-forming cell.

from a vaccination. All five patients with decreases in viremia had these within 2 weeks from a vaccine dose. Reductions in the viral load were only seen in the two highest dose groups. The reductions lasted from 2 to >10 weeks and ranged from 0.6 to 2.4 $\log_{10}$ in the five patients. All the four patients developing an improved T cell response had reductions in the viral load (**Figures 4** and **6**).

Role of the IL-28B genotype and viral genotype

Of the five patients with a transient decline in HCV RNA levels, three had the IL-28B CC genotype (**Table 1**). Of the five patients (numbers 102, 103, 201, 202, and 402) with a transient *de novo* T cell activation, one had the TT,[39] three had the CT, and one had the CC genotype (**Table 1**). Hence, no association between the IL-28B genotype and T cell responses was seen (**Table 1**). Two of these five patients were infected with HCV genotype 1a and three with genotype 1b (**Figures 4** and **5**).

Effects of SOC therapy after vaccination

At baseline, before SOC treatment was commenced, the mean age of the patients was 47 years (range 37–57) and all but two were males (**Figure 6**). The mean HCV RNA level was 662,098 IU/ml (range 8,790–1,700,000 IU/ml). A rapid viral responses was achieved in 5 out of 8 patients (56%), and a complete early viral response and sustained viral response (SVR) in 6 out of 8 patients

Table 1 Baseline demographic characteristics of the 12 patients

Patient ID and dose group	Age/sex	Number of doses	Baseline viral load (IU/ml) and genotype	IL-28B genotype	Effects recorded after vaccination		HLA class I type(s)	
					HCV RNA decline	T cell activation	A	B
167 µg								
101	29/F	4	<15[a] (1b)	CC	No	Yes	11, 24	35, 51
102	55/M	4	14,100 (1b)	CT	No	Yes	3, 19	35, 51
103	45/F	4	1,100,000 (1a)	CT	No	Yes	11, 26	40, 44
500 µg								
201	45/M	4	804,000 (1a)	CC	Yes	Yes	3, 24	7, 27
202	36/F	4	564,000 (1b)	CT	Yes	Yes	1, 24	35, 62
203	49/M	4	790,000 (1a)	CT	No	No	2	7, 44
1,500 µg								
301	37/M	4	1,700,000 (1a)	TT	No	No	1, 2	41, 57
302	46/M	5	19,000 (1)	CC	Yes	No	30, 33	27, 44
303	40/M	4	560,000 (1a)	CC	Yes	No	3, 11	18, 55
401	56/M	4	116,000 (1a)	CT	No	No	1, 24	8, 14
402	61/F	4	242,000 (1b)	TT	Yes	Yes	1, 2	8
404	47/F	5	580,000 (1a)	TT	No	No	3, 25	14, 60

Abbreviations: HCV, hepatitis C virus; HLA, human leukocyte antigen.
[a]Subject HCV RNA positive at screening but <15 IU/ml at start of vaccination (week 0).

(75%). Sustained viral response according to IL-28B genotype was seen in 2 of 2 patients with the CC, 3 of 4 patients with the CT, and 1 of 2 patients with the TT genotype (**Figure 6**).

DISCUSSION

DNA vaccines have not been successful in the past, probably at least due to suboptimal delivery.[30] New delivery techniques including *in vivo* EP possibly will change this for the better.[40] Thus, some key effects of *in vivo* EP are improved DNA uptake, antigen expression, and a local inflammatory response.[32,41] These factors are favorable when DNA vaccination as a concept is moved from small to large animals including humans.

The study was designed to investigate whether therapeutic HCV vaccination in patients with chronic HCV infection was safe, had impact on the immune response, and whether it had any effects on the viral load. As an addition, we could also follow some of the patients during a subsequent treatment with pegylated IFN alfa 2a and ribavirin. Although treatment for HCV infection is rapidly changing with the development of new direct antiviral drugs (direct-acting antivirals (DAAs)), the data presented here indicate that therapeutic HCV vaccination has a potential impact on a subsequent SOC treatment. Further studies are needed to address whether a combination of therapeutic vaccination can improve treatment response to the new DAAs developed for HCV treatment. Although DAAs are highly effective, there are still patient groups where an effective therapeutic vaccine may have an influence on the therapy and increase cure rates or by shortening treatment duration. Our study has drawbacks since it was a small clinical trial and with uncontrolled factors that may influence the obtained results. Some effects, however, were noted of the vaccinations concerning the preset criteria. First, the treatment was well tolerated with only a transient pain related to the *in vivo* EP. Second, the patients' T cell responses statistically improved, showing that the regimen seemed to modulate the host T cell response. And finally, a transient effect on the viral replication was noted in some patients.

When eight of our genotype 1 patients received SOC therapy after having had their vaccine doses, six were cured. It is tempting to speculate that the vaccination had a beneficial effect on the response to SOC treatment, but many confounding factors were operating and, hence, no firm conclusions can be drawn. For example, the study was small and many patients had low viral loads, and the time lap from vaccination to SOC treatment was not standardized. Due to these drawbacks, it is important to test whether these observations will stand also in a controlled clinical trial. However, our data add further insight to the previous experience with therapeutic vaccination in chronic HCV infection. A peptide-based vaccine has previously been shown to have some effects on the viral load,[42] whereas a protein-based approach using a HCV NS3 and core fusion protein has been suggested to improve sustained response rates when added to SOC.[43] In a recent study, it was shown that modified vaccinia Ankara-based therapeutic vaccination using a vector expressing HCV NS3/NS4 and NS5B generates results very similar to ours.[29] Thus, our data further support the concept of therapeutic vaccination and its ability to transiently modulate the host response with moderate effects on viral replication, when used as monotherapy.

This is to our knowledge, the first clinical trial utilizing a therapeutic DNA vaccine in patients with chronic hepatitis C delivered by *in vivo* EP, later followed by a SOC therapy with pegylated IFN and ribavirin. The study reached the pre-set study criteria. Hence, therapeutic vaccination delivered with EP was found to be safe, immunogenic, and had transient effects on the viral replication. Importantly, we did note that of the four

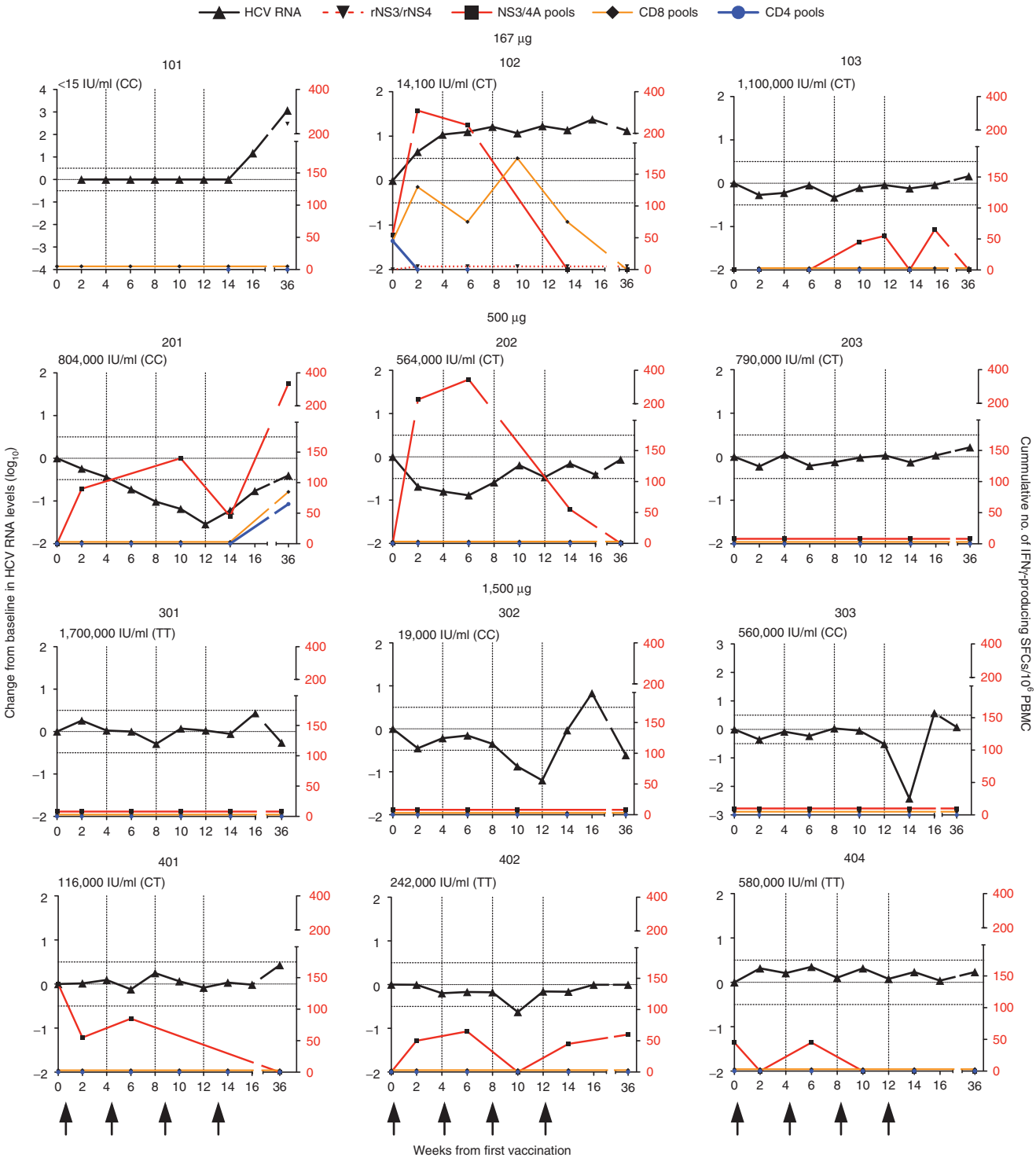

Figure 4 Summary of changes in the viral load (black line), and ELISpot responses to recombinant NS3/NS4 (rNS3/rNS4; red dotted line), nine overlapping peptide pools spanning NS3/4A (red) or peptide pools with known CD8 epitopes (orange line). The figure indicates the viral load at baseline. The hepatitis C virus (HCV) RNA levels have been given as change relative to baseline. The ELISpot data has been given as the cumulated SFCs/10^6 PBMC. Numbers 101, 102, 103, 201, 202, 203, 301, 302, 303, 401, 402, and 404 indicate individual subject number in the different dose groups. Also the baseline HCV RNA levels and the IL-28B genotype (CC, CT, and TT) has been given. PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell.

subjects who mounted an early T cell response to the vaccine, three simultaneously or later, displayed decreases in the serum levels of HCV RNA. This implies that vaccine induced, or reactivated, HCV-specific T cell responses, which possibly induced an antiviral effect. However, this immunological effect was only transient. Moreover, it is of interest that the main improvements in specific T cell responses appeared after the first and second vaccination, and that the responses were not improved further

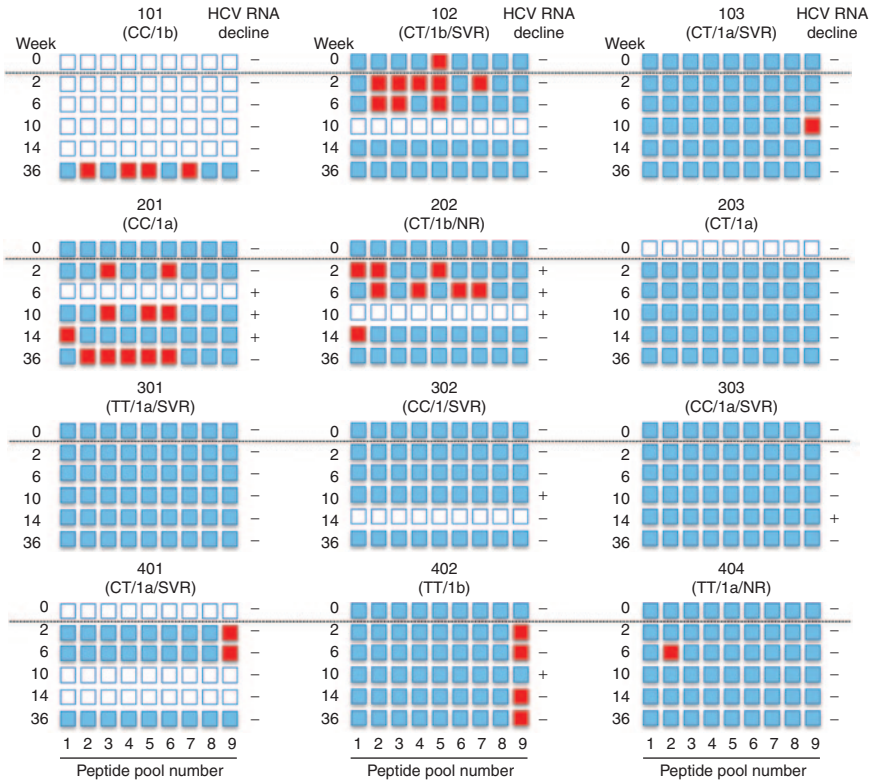

Figure 5 Overview of IFN-γ ELISpot responses to the nine peptide pools spanning the NS3/4A proteins in the 12 vaccinated patients. An open box indicates that the sample was not tested, a blue box indicates that the sample was tested with a negative result, and a red box indicates that the result was positive (≥45 SFCs/10^6 PBMCs). Numbers 101, 102, 103, 201, 202, 203, 301, 302, 303, 401, 402, and 404 indicate individual subject number in the different dose groups. Also the IL-28B genotype (CC, CT, and TT), HCV genotype, and treatment outcome has been given. Week 0, 2, 6, 10, 14, and 36 indicate timepoint for withdrawal of blood sample. HCV, hepatitis C virus; IFN, interferon; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell; SVR, sustained viral response.

by additional vaccinations. Surprisingly, at week 36, again stronger T cell responses were detected in two patients. The reasons for the failed specific T cell activation after the third and fourth vaccination are not clear. However, one may speculate that the vaccine currently has a suboptimal immunogenicity and therefore the primed responses are unable to permanently break the inhibition of the HCV-specific responses. Hence, repeated vaccination may therefore have no beneficial effects. Thus, similar studies using a vaccine with improved immunogenicity merits further investigation. In addition, it is also likely that the strong regulatory effects present in the chronic infection are difficult to overcome with the used vaccine alone. Furthermore, four vaccine doses with this vaccine alone were not sufficient to achieve sustained virological response by itself in patients with chronic HCV. However, when combined with pegylated IFN and ribavirin treatment or in the future with DAAs,[44] therapeutic vaccination possibly will have an influence on the response rates and clearance of HCV. In our small study, 75% of the vaccinated patients, all infected by HCV genotype 1, were cured with the subsequent SOC treatment, which is an encouraging result. However, with the development of highly effective HCV-targeted drugs, the role for a therapeutic vaccine is likely to be small. On the other hand, it is possible that some patient groups in need of alternative treatment options, may still benefit from therapeutic vaccines. Such groups could include patients resistant to DAAs, patients not eligible for DAA treatment, patients exposed to HCV repeatedly (e.g., injection drug users at risk of HCV reinfection), and patients in resource-poor environments. To be able to cure 100% of HCV-infected patients, it is not unlikely that successful HCV treatment for selected patients will consist of several DAAs used in combination with immune modulatory measures including therapeutic vaccines.

In conclusion, the concept of therapeutic DNA vaccine delivered by *in vivo* EP to treat chronic HCV is safe and should be further investigated in combination with other compounds with different modes of action, and/or by further improving the immunogenicity.

MATERIALS AND METHODS

Human subjects. This trial was approved by the Regional Ethical Council in Stockholm, Sweden and by the Swedish Medical Products Agency. The Declaration of Helsinki protocols were followed and all patients gave their written informed consent. The trial has been registered at http://www.clinicaltrials.gov (http://clinicaltrials.gov/ct2/show/NCT00563173). Twelve patients with verified chronic HCV genotype 1 infections of a duration of >6 months were included in the study. Background data of the patients, five females and seven males, with a mean age of 46 years (range 29–60 years), have been given in **Table 1**. All were treatment naive and four had genotype 1b, seven had genotype 1a, and one had genotype 1 unspecified (**Table 1**). The mode of HCV acquisition was transfusions in two, intravenous drug use in four, and sporadic including sexual transmission in six. All but one

a

Patient	DNA dose (µg)	IL-28B gt	HCV RNA levels (IU/ml) before start of vaccinations	Transient effects during vaccinations		HCV RNA levels (IU/ml) before start of SOC	HCV RNA response during SOC therapy			
				T cell activation	HCV RNA decline		w4	w12	EOT	24 fu
102	167	CT	14100	+	–	293,000	–	–	–	–
103	167	CT	1,100,000	+	–	503,000	+	–	–	–
202	500	CT	564,000	+	+	440,000	+	+	NA	NA
301	1,500	TT	1,700,000	–	–	1,200,000	+	–	–	–
302*	1,500	CC	19,000	–	+	8,790	–	–	–	–
303	1,500	CC	560,000	–	+	1,700,000	–	–	–	–
401	1,500	CT	116,000	–	–	547,000	+	–	–	–
404*	1,500	TT	580,000	–	–	605,000	+	+	+	+

b

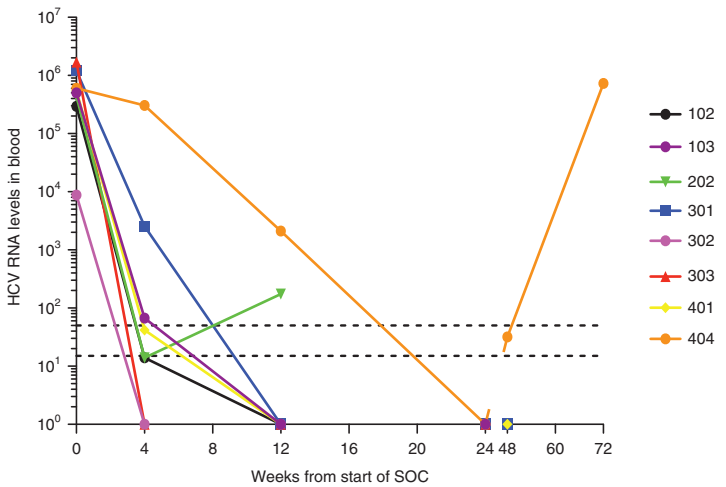

Figure 6 Kinetics of serum HCV RNA levels after vaccination and SOC therapy. (**a**) Summary of the effect of an SOC therapy in 8 out of the 12 patients. The two patients receiving a fifth booster dose of the vaccine has been indicated by an asterisk (*). EOT, indicate end of treatment response; fu, indicate follow-up; and NA, indicate not available. (**b**) The kinetics of hepatitis C virus (HCV) RNA levels in blood has been given during SOC and follow-up has been given as the IU/ml. The dotted lines indicate the 50 and 15 IU/ml (sensitivity limit of the quantitative assay) levels. Numbers 102, 103, 202, 301, 302, 303, 401, and 404 indicate individual subject number in the different dose groups. gt, genotype; HCV, hepatitis C virus; SOC, standard-of-care.

patient had an evaluation of their liver injury by transient elastography for staging of liver fibrosis by measuring the liver stiffness.[45] The median liver stiffness was 7.8 kPa (range 3.8–25.7 kPa) indicating a generally mild fibrosis in the majority of patients. At baseline, the median HCV RNA level was 562,000 IU/ml (range 14–1,700,000 IU/ml). The typing for the IL-28B rs12979860 single-nucleotide polymorphism genotype[39] was determined using a PCR using the primers GCCTGTCGTGTACTGAACCA (forward) and GCGCGGAGTGCAATTCAAC (reverse), and probes FAM-CTGG TTACGCCTTC-MGB and VIC-TGGTTCGCGCCTTC-MGB. HLA typing was performed by PCR.

SOC therapy. After a mean interval of 15 months (range 1–30) from last vaccination, 8 out of 12 patients were given treatment with pegylated IFN alfa 2a 180 µg q.w. + ribavirin 1,000/1,200 mg q.d. (SOC). Two patients who received a booster dose started treatment within 1–2 months after the booster dose. Treatment duration was intended to be 24 weeks if rapid viral response was achieved (HCV RNA <50 IU/ml by Roche Amplicor Monitor; Roche, Basel, Switzerland) and 48 weeks if no rapid viral response was achieved but HCV RNA became undetectable at week 24 and a 2 $\log_{10}$ drop was seen from baseline at week 12. No immunological analysis was performed on patients undergoing SOC treatment.

Vaccine and vaccine delivery. The DNA vaccine (GenBank accession number AR820945.1, http://www.ncbi.nlm.nih.gov/genbank, ChronVac-C; ChronTech Pharma AB, Huddinge, Sweden) encodes a codon-optimized HCV NS3/4A genotype 1a gene with expression controlled by the cytomegalovirus immediate early promoter.[46] The vaccine was produced under good manufacturing practice at Vecura (Huddinge, Sweden). Preclinical testing suggested that the regimen was safe,[32] and a similar vaccine was found immunogenic in macaques.[47] A volume of 0.5 ml of 0.9% sodium chloride containing the DNA was injected in the deltoid muscle (alternating left and right) using a 27-G needle at a depth of 1.2 cm. The injection site was marked with a surgical pen before injection and then sterilized by swiping with an alcohol pad. Immediately after the

injection a four-electrode array (Medpulser DDS; Inovio, Blue Bell, PA) was inserted around the site of injection and EP was administered as two 60-ms pulses of 106 V/cm. The 1.5-cm long electrodes were mounted in a square pattern with 0.5 cm between each.

Clinical trial design. The pre-specified primary, secondary, and tertiary aims of the clinical trials were safety, immunogenicity, and effects on the serum levels of HCV RNA, respectively. The number of patients per group was set to three for reproducibility and the possibility to perform the trial at a single clinical site to minimize the operator variability at EP. The patients were divided into three dose groups, 167 μg ($n = 3$), 500 μg ($n = 3$), and 1,500 μg ($n = 6$) of DNA. Each patient received four monthly vaccinations with the same dose. Since this was a first-in-man study, with this a DNA vaccine against HCV delivered by *in vivo* EP, 2 weeks were allowed between the enrollments of each patient to monitor safety. After passing screening, patients were admitted to hospital for 8 hours after the first vaccination and then phoned back at 24 hours. The local reaction at the site of vaccination was recorded during the first week. Venous blood was sampled before each vaccination, 6 hours after, and then every second week until treatment week 16. A final sample was taken 24 weeks after last vaccination, which was performed at week 12. These samples were tested for blood biochemistry, hematology, and HCV RNA. Samples were tested by a qualitative (COBAS AmpliPrep/COBAS AMPLICOR HCV Test, v2.0; Roche Diagnostics, Pleasanton, CA) and a quantitative assay (sensitivity 15 IU/ml, COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Diagnostics) kit for HCV RNA. PBMCs for analysis of immune responses were isolated by Ficoll-Paque gradient density centrifugation at week 0, 2 weeks after each vaccination, and at week 39. PBMC were aliquoted and frozen in liquid nitrogen until tested.

Immunological analysis. All T cell assays were performed on frozen PBMC at ImmuSystems (Munich, Germany) as described previously.[48] All samples were tested for proliferation by ^{3}H-thymidine incorporation and for IFNγ production by ELISpot.[48] Peptides corresponding to known HLA class I epitopes and to the complete NS3/4A sequence of the vaccine were generated by standard techniques.[49] The following peptide pools were generated: 1: CD4 peptide pool (SPVFSDNSSPPAVPQSYQVA, AQGYKVLVLNPSVAATMG, GRHLIFCHSKKKCD, TTVRLRAYMNTPGLPVCQDH, ENLPYLVAYQ ATVCARAQ, SGKPAIIPDREVLYREFDEM), 2: CD8 peptide pool, HLA-A2 (CINGVCWTV, YLVTRHADV, LLCPAGHAV, TGSPITYSTY, KLVALGVNAV, YLVAYQATV, VLAALAAYCL), 3: CD8 peptide pool, HLA-A non-A2 (ATDALMTGF (A1), TMGFGAYMSK (A11), GAYMSKAHGI (A11), TLTHPVTK (A11), AYAQQTRGL (A24), MYTNVDQD (A24), TYSTYGKFL (A24), MGFGAYMSK (A3), LIFCHSKKK (A3)), 4: CD8 peptide pool, HLA-B and HLA-C (HPNIEEVAL (B35), TPAETTVRL (B35), IPDREVLY (B35), CHSTDATSIL (B38), HSKKKCDEL (B8), ELAAKLVAL (B8), LIRLKPTL (B8), EVTLTHPVTKYIMTCMSA (B8), AYAAQGYKVL (C3)), 5: nine peptide pools containing 15 overlapping peptides of HCV NS3 and NS4A (pool $1_{1026\text{-}1110}$, pool $2_{1101\text{-}1185}$, pool $3_{1176\text{-}1260}$, pool $4_{1251\text{-}1335}$, pool $5_{1326\text{-}1410}$, pool $6_{1401\text{-}1485}$, pool $7_{1476\text{-}1560}$, pool $8_{1551\text{-}1635}$, and pool $9_{1626\text{-}1710}$).

In the proliferation assay, PBMCs (5×10^4/well) were incubated in 96-well U-bottom plates (TPP, Trasadingen, Switzerland) in triplicates for 5 days in the presence of different concentrations (3, 1, and 0.3 μg/ml each) of HCV proteins, NS3 and NS4 (Mikrogen, Neuried, Germany), tetanus toxoid (10 μg/ml; Chiron Behring, Marburg, Germany) and phytohemagglutinin (PHA, 1 μg/ml; Remel, Lenexa, KS). The stimulation index was calculated as the ratio of counts per minute obtained in the presence of antigen to that obtained without antigen. A stimulation index of >3 was considered significant.

The human IFN-γ ELISpot (Mabtech AB, Nacka Strand, Sweden; #3420-2APT-10) was performed as recommended by the manufacturer using 2.5×10^5 PBMC/well for antigen stimulation and 0.25×10^5 PBMC/well for stimulation with PHA. Plates were incubated for 48 hours in the presence of NS3 (1 μg/ml), NS4 (1 μg/ml), thirteen different peptide pools (10 μg/ml of each peptide), tetanus toxoid (10 μg/ml), and PHA (1 μg/ml). Cut-offs with ≥9 delta spots (counted spots − control spots) and an ELISpot-stimulation index (counted spots/control spots) of 2 were used in the study to define significant positive antigen responses as determined in validation experiments with healthy individuals. The positive PHA control was >150 SFCs/10^6 PBMC in all samples included in the analysis proving the viability of the cells.

Statistical analysis. Statistical comparisons were performed using the GraphPad InStat 3 for Macintosh (version 3.0b; GraphPad Software, San Diego, CA) and Microsoft Excel 2008 for Macintosh (version 12.2.8; Microsoft, Redmond, WA). Nonparametric data were compared using the Fisher's exact test, the Mann–Whitney U-test, and Wilcoxon's matched pairs test (InStat 3).

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