Citation: Molecular Therapy — Methods & Clinical Development (2015) **2,** 15006; doi:10.1038/mtm.2015.6

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ARTICLE

Clinical testing of a dendritic cell targeted therapeutic vaccine in patients with chronic hepatitis C virus infection

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The lack of antiviral cellular immune responses in patients with chronic hepatitis C virus (HCV) infection suggests that T-cell vaccines may provide therapeutic benefit. Due to the central role that dendritic cells (DC) play in the activation of T-cell responses, our aim was to carry out a therapeutic vaccination clinical trial in HCV patients using DC. Five patients with chronic HCV infection were vaccinated with three doses of 5×10^6 or 10^7 autologous DC transduced with a recombinant adenovirus encoding NS3 using the adapter protein CFh40L, which facilitates DC transduction and maturation. No significant adverse effects were recorded after vaccination. Treatment caused no changes in serum liver enzymes nor in viral load. Vaccination induced weak but consistent expansion of T-cell responses against NS3 and adenoviral antigens. Patients' DC, as opposed to murine DC or DC from healthy subjects, secreted high IL-10 levels after transduction, inducing the activation of IL-10–producing T cells. IL-10 blockade during vaccine preparation restored its ability to stimulate anti-NS3 Th1 responses. Thus, vaccination with adenovirus-transduced DC is safe and induces weak antiviral immune responses. IL-10 associated with vaccine preparation may be partly responsible for these effects, suggesting that future vaccines should consider concomitant inhibition of this cytokine.

Molecular Therapy — Methods & Clinical Development (2015) **2**, 15006; doi:10.1038/mtm.2015.6; published online 11 March 2015

INTRODUCTION

HCV clearance is associated with strong and multiepitopic antiviral CD4 and CD8 T-cell responses, whereas chronic HCV infection is accompanied by poor and narrow responses, with HCV-specific T cells displaying an exhausted phenotype.[1–3] These results indicate that induction of functional antiviral cellular immune responses may be beneficial for patients with chronic HCV infection.[4] Thus, since the characterization of the antiviral immune response, a great effort has been carried out to develop immunization protocols to be used as therapeutic vaccines.[5] Based on promising preclinical data, a few anti-HCV vaccines have reached clinical trials, but when tested in patients, although induction of immune responses has been detected, these responses and concomitant clinical results have been poorer than expected,[6–10] suggesting that improved protocols are clearly needed.

Dendritic cells (DC) are a heterogeneous population of professional antigen presenting cells which capture and present antigens to T cells for their priming.[11] To accomplish this task, DC need to reach a mature status characterized by upregulation of molecules involved in antigen presentation, T-cell costimulation and immunostimulatory cytokines, which usually occurs after recognition of pathogen-associated molecular patterns or endogenous inflammatory signals. These features have led to the use of DC as vaccines in different settings, mainly in cancer.[12,13] In the case of HCV, administration of *ex vivo* prepared monocyte-derived DC (MoDC) induced T-cell responses of limited efficacy.[8] We have demonstrated that murine DC transduced with a recombinant adenovirus encoding HCV NS3 protein (AdNS3) induces antiviral T-cell responses.[14] These responses are further enhanced when adenoviral transduction is carried out with an adapter protein containing the coxakie-adenovirus receptor fused to the CD40L ectodomain (CFm40L), due to the higher DC transduction and full maturation attained.[15] More importantly, when an equivalent molecule (CFh40L) was used with human MoDC, it induced strong maturation in cells from healthy individuals and HCV patients, leading to a vigorous induction of *in vitro* anti-HCV immune responses in both groups.

With these premises we decided to carry out a pilot phase I/II therapeutic vaccination clinical trial in patients with chronic HCV infection (genotype 1b) based on the administration of autologous MoDC transduced with AdNS3 with the help of the adapter CFh40L. Here, we report data regarding safety of this protocol and its clinical and immunological efficacy in five patients vaccinated with two doses of DC. Moreover, we have characterized the immune response induced by the vaccine and potential mechanisms responsible for the observed results, which would be helpful for the design of future therapeutic vaccines.

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Received 15 January 2015; accepted 21 January 2015

RESULTS

Patient recruitment, vaccine preparation, and characterization

Six patients were initially evaluated. Patient DC02 was excluded because of genotype 1a infection and five patients were finally treated. The first three patients received three doses of 5×10^6 DC while the remaining two patients received three doses of 10^7 DC. Clinical baseline characteristics of patients are shown in Table 1. To prepare the vaccine monocytes from patients were differentiated into immature DC (iDC) and transduced with AdNS3 and CFh40L. In all preparations, >95% of cells expressed CD11c and the MoDC marker CD209 (DC-SIGN), with CD14 values around 2–56%. DC showed a mature phenotype, according to the expression of HLA-DR, CD80, CD86, and CD54 (Supplementary Table S1). Overall, mature DC (mDC) yield was about 10% (range 6.4–16.8%) from initial monocyte input and sufficient numbers of mDC were obtained to produce the three vaccine doses for all patients (Supplementary Table S2). In all cases, DC transduction induced the production of inflammatory cytokines IL-12, TNF-α, and IL-6 (Supplementary Figure S1).

Table 1 Baseline characteristics of patients treated in the study

	DC01	*DC03*	*DC04*	*DC05*	*DC06*
Age	64	55	63	63	50
Sex	F	M	M	F	M
ALT (UI/l)	18	99	43	30	46
Bilirubin (mg/dl)	0.65	0.58	1.27	0.53	0.57
Platelets (10^9/l)	189	255	190	212	276
Creatinin (mg/dl)	0.6	0.9	1.2	0.9	0.8
Liver stiffness	5.1	6	5.9	4.3	5.1

Safety

No serious adverse events related to study procedures or treatment administration were observed, including signs of hematologic, liver, or renal toxicity. A painless indurated erythematous skin lesion was observed in all treated patients at the site of injection, which resolved spontaneously in all cases in 1–3 weeks. No other adverse events related to the study procedure or to treatment were observed and no patients discontinued the study due to adverse events.

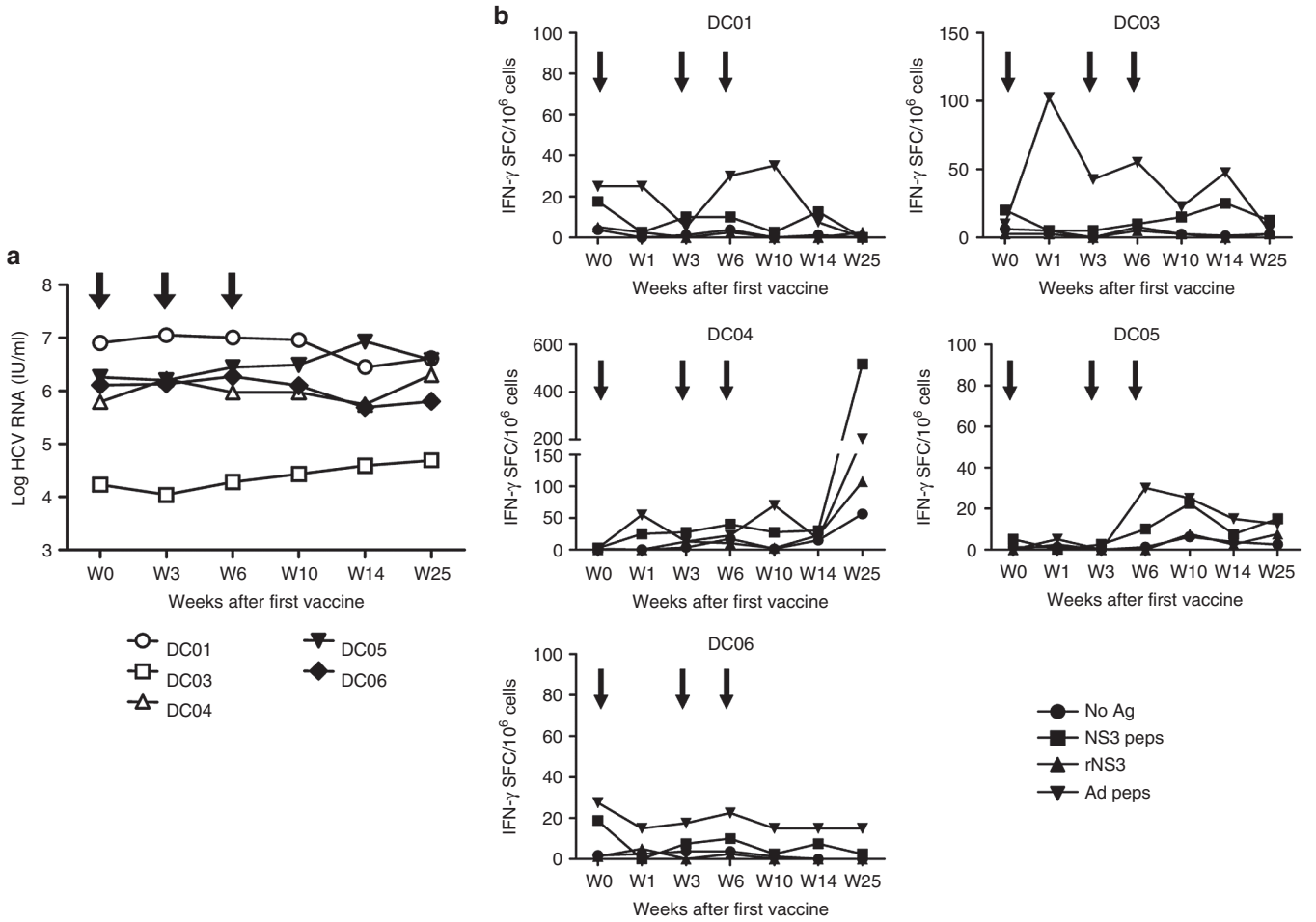

Figure 1 Effect of vaccination on viral load and immune responses. (**a**) Serum viral load at baseline and after vaccination was evaluated in vaccinated patients. (**b**) PBMC from serial samples after vaccination were stimulated *ex vivo* with NS3 protein (rNS3), NS3 peptides, adenoviral peptides or left unstimulated. One day later cells were washed and IFN-γ spot-forming cells developed and counted. Arrows indicate vaccination time points.

Effect of vaccination on viral load and immune responses

No significant change in viral load was observed in any patient (Figure 1a). Analysis of immune responses elicited by the vaccine was first carried out by IFN-γ ELISPOT. Although almost no responses were detected when PBMC were incubated with rNS3 (except in patient DC04 at w25), vaccination induced measurable responses in all patients when using NS3 peptides. However, responses were weak and mainly observable between weeks 3–14 (Figure 1b). In contrast, responses against Ad hexon protein peptides were of greater magnitude, except in patient DC06, who showed no changes with respect basal levels. Immunocompetence of patients was confirmed by strong responses detected upon PHA mitogen stimulation (data not shown).

Additional functions in CD4 and CD8 T cells (expression of IFN-γ, TNF-α and IL-2 and the degranulation marker CD107) were analyzed by flow cytometry after a 5-hour *ex vivo* stimulation with NS3 peptides. CD4 and CD8 responses were detected in all patients at different time-points (Figure 2a) and were usually consistent, as shown by simultaneous production of several cytokines. In order to measure T-cell responses that were below the detection level of direct *ex vivo* tests, PBMC were stimulated with NS3 peptides and expanded with IL-2 for 10 days before stimulation with the relevant peptides for 5 hours. Specific responses primed by the vaccine were again observed at different time-points for all patients and in many cases they were simultaneously positive for several markers (Figure 2b). Equivalent experiments carried out in three patients measuring responses against adenoviral peptides also showed clear expansion of vector-specific T cells (except CD8 T cells in DC06) (Supplementary Figure S2), confirming results obtained in ELISPOT assays.

Vaccine does not expand exhausted T cells

Since patient DC01 was HLA-A2$^+$, we could analyze vaccination effect on the number and phenotype of NS3-specific T cells by using HLA-A2/peptide dextramers. We detected very low basal levels of NS3(1073–1081)-specific CD8 T cells, which did not change following vaccination. By contrast, NS3(1406–1415)-specific T cells were easily detected, and their number increased at weeks 3 and 10 after vaccination (Figure 3a). HCV-specific T cells in chronically infected patients are characterized by an exhausted phenotype[16–19] expressing inhibitory molecules PD-1 and Tim3. To understand the poor

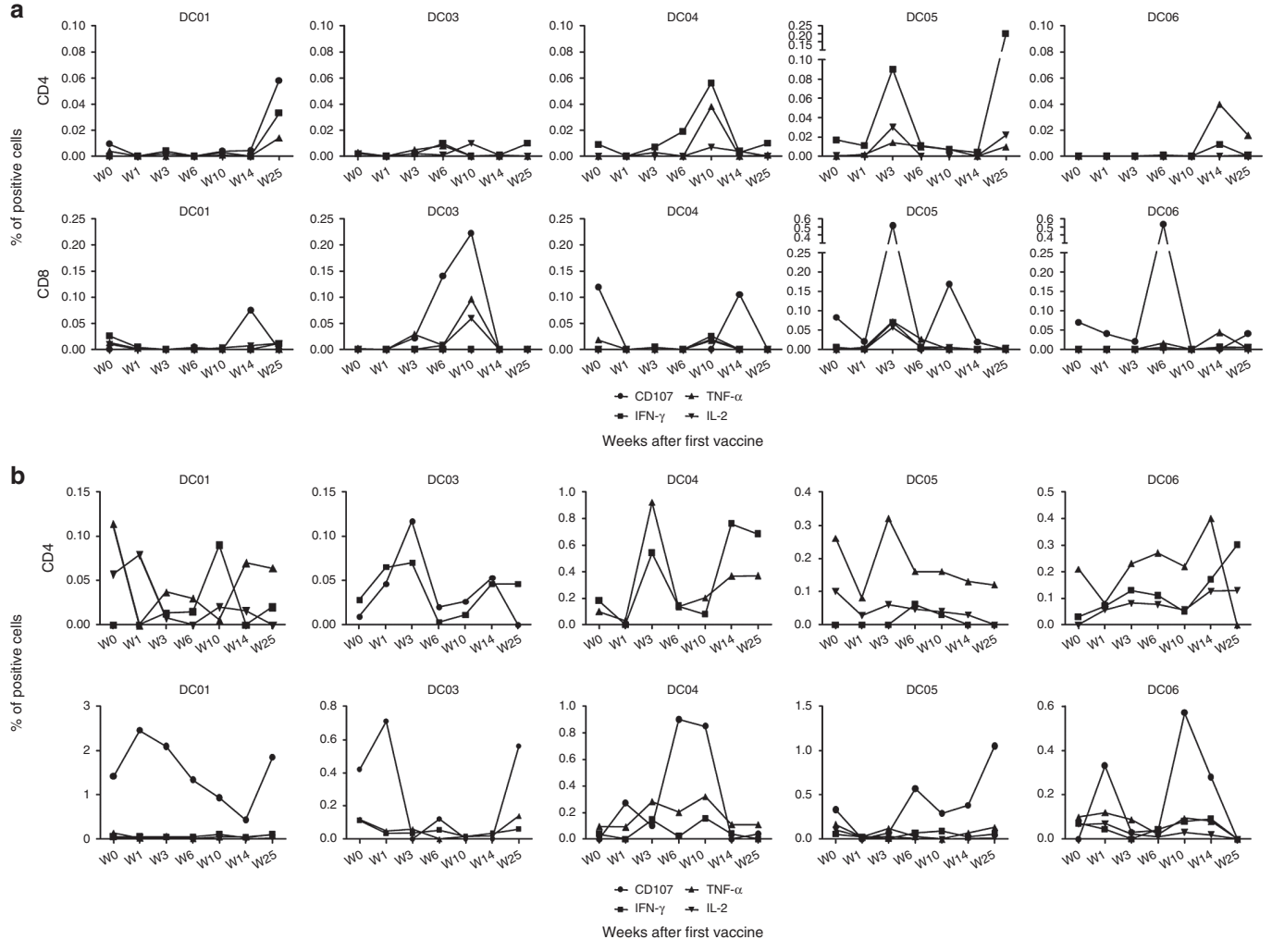

Figure 2 Anti-NS3 T-cell responses in vaccinated patients: cytokine production by CD4 and CD8 T cells. (**a**) PBMC from serial samples after vaccination were stimulated *ex vivo* with NS3 pooled peptides for 5 hours and intracellular production of IFN-γ, TNF-α and IL-2 as well as CD107 expression were analyzed by flow cytometry in CD4 and CD8 T cells. (**b**) PBMC samples were stimulated as in **a** and expanded with IL-2 for 10 days. Then, resulting T cells were restimulated with or without NS3 peptides for 5 hours and cytokine-producing T cells were measured as above. Results correspond to the difference between peptide-stimulated values minus unstimulated values.

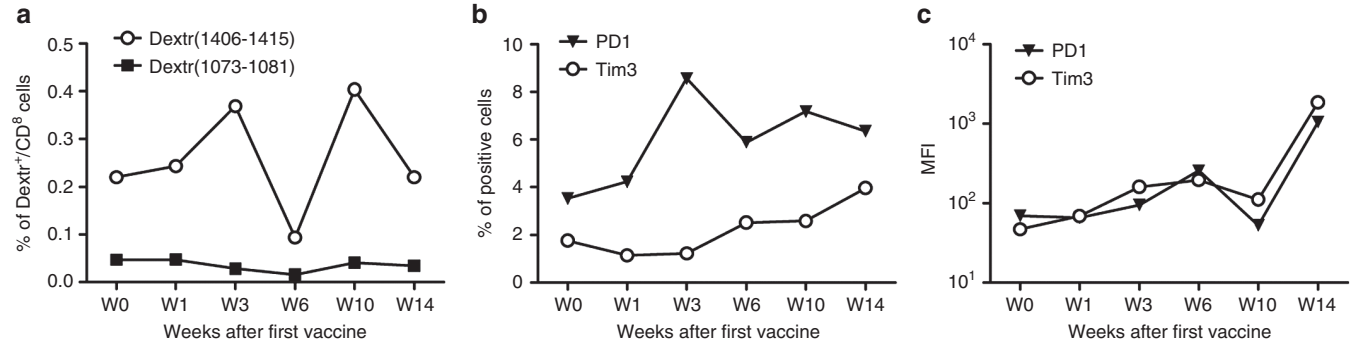

Figure 3 Characterization of HLA-A2 dextramer-positive NS3-specific CD8 T cells. (**a**) NS3-specific CD8 T cells were enumerated in serial PBMC samples from patient DC01 (HLA-A2+) by using HLA-A2 dextramers containing NS3 CD8 epitopes (1073–1081) and (1406–1415). Percentage of dextramer(1406–1415)$^+$ cells expressing PD-1 and Tim-3 (**b**) as well as fluorescence intensity (MFI) (**c**) were also determined by flow cytometry.

responses expanded by the vaccine, we analyzed their expression in HCV-specific T cells. In this patient, less than 4% of NS3(1406–1415) Dextramer$^+$ cells expressed PD-1 or Tim3 at baseline and vaccination caused a very modest rise of these values (Figure 3b). However, the intensity of surface expression of PD-1 and Tim3 on the positive cells clearly increased at the end of follow-up (Figure 3c).

The DC vaccine produces immunosuppressive cytokines TGF-β and IL-10 and induces IL-10–secreting T cells

The poor immunogenicity of the vaccine observed in HCV patients, as opposed to preclinical results observed in the murine model,[15] prompted us to study immunomodulatory molecules potentially expressed by DC transduced with AdNS3 plus CFh40L. TGF-β was detected in supernatants from mDC, with similar levels in cells from HCV patients and healthy subjects (Figure 4a). In contrast, IL-10 (a cytokine not detectable in the study using murine DC)[15] was abundantly produced by DC from HCV patients, with values significantly higher than those observed with cells from healthy individuals (Figure 4b). Due to the immunomodulatory role that IL-10 plays on antigen presenting cells as activators of T cells, we analyzed factors responsible for its induction after DC transduction. IL-10 was mainly induced by CFh40L, since AdNS3 alone did not induce IL-10, while its secretion was highly stimulated when AdNS3 was combined with CFh40L (Figure 4c). Since IL-10 secreted by DC may induce IL-10–producing T cells,[20] we analyzed in four patients IL-10 production by T cells after stimulation with NS3 or Ad peptides and expansion with IL-2. Vaccination increased NS3-specific IL-10$^+$ CD4 and CD8 T cells, mainly in patients DC03 and DC04 (Figure 3c), those exhibiting the highest IL-10 values produced by DC. In remaining patients, IL-10$^+$ cells barely exceeded basal values. Regarding, anti-Ad responses, they followed a similar profile of IL-10–producing cells, except for patient DC05, who had high basal anti-Ad IL-10$^+$ cells, confirming the capacity of the vaccine to induce IL-10$^+$ T cells (Figure 5).

IL-10 blockade during DC maturation promotes activation of Th1 NS3-specific cells

We had demonstrated that IL-10 blockade during DC stimulation improved their functional properties.[21] To study the relevance of IL-10 in our DC vaccine protocol, we blocked IL-10 in DC from patients after transduction and during the maturation phase. Significantly higher IL-12 levels were found after IL-10 blockade (Figure 6a), associated to increased upregulation of maturation markers (Supplementary Figure S3). Regarding T-cell priming

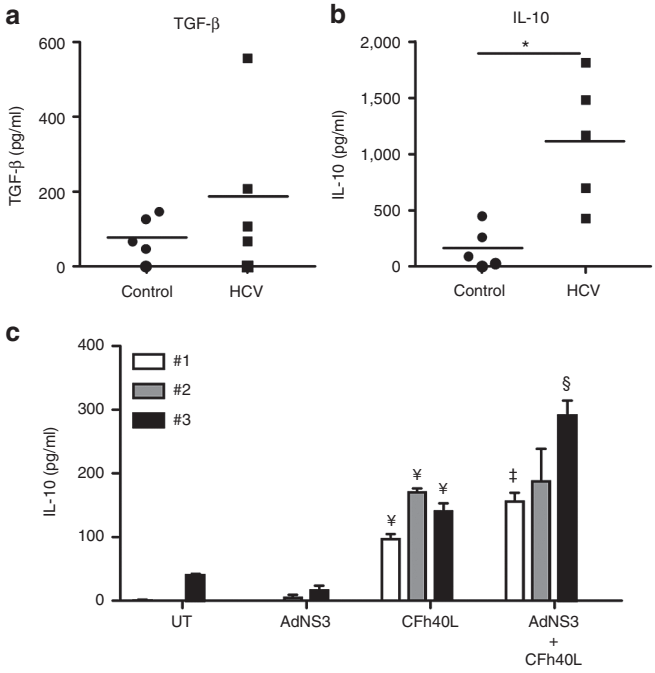

Figure 4 Transduction of DC using CFh40L induces immunosuppressive cytokine IL-10. DC from HCV patients and healthy controls were transduced with AdNS3/CFh40L and (**a**) TGF-β and (**b**) IL-10 secreted to their supernatants were measured 1 day later. *$P < 0.05$ (**c**) Immature DC from three individuals were treated with AdNS3, CFh40L, both or left untreated (UT) and cultured for 24 hours. Then supernatants were harvested and IL-10 content measured by ELISA. Data correspond to mean + SEM ($n = 3$) $^{¥}P < 0.05$ CFh40L versus UT or AdNS3; $^{‡}P < 0.05$ AdNS3+CFh40L versus CFh40L $^{§}P < 0.05$ AdNS3+CFh40L versus CFh40L, according to nonparametric Mann–Whitney test.

ability, DC-mediated stimulation of T cells at baseline showed that IL-10 blockade increased the number of NS3-specific CD4 T cells producing IFN-γ or IL-2 (Figure 6b). In the case of CD8 T cells, IFN-γ, TNF-α, IL-2, and CD107-producing cells also augmented after IL-10 blockade. Interestingly, IL-10$^+$ CD4 and CD8 cells remained unchanged or even decreased after blockade. By contrast, IL-10 blockade stimulated poorer responses against Ad (Supplementary Figure S4). Phenotypic analyses of antigen-specific (IFN-γ$^+$) CD8 cells responding after IL-10 blockade showed that most NS3-specific cells were naive T cells, whereas effector memory responses predominated within adenovirus-specific T cells (Supplementary Figure S5).

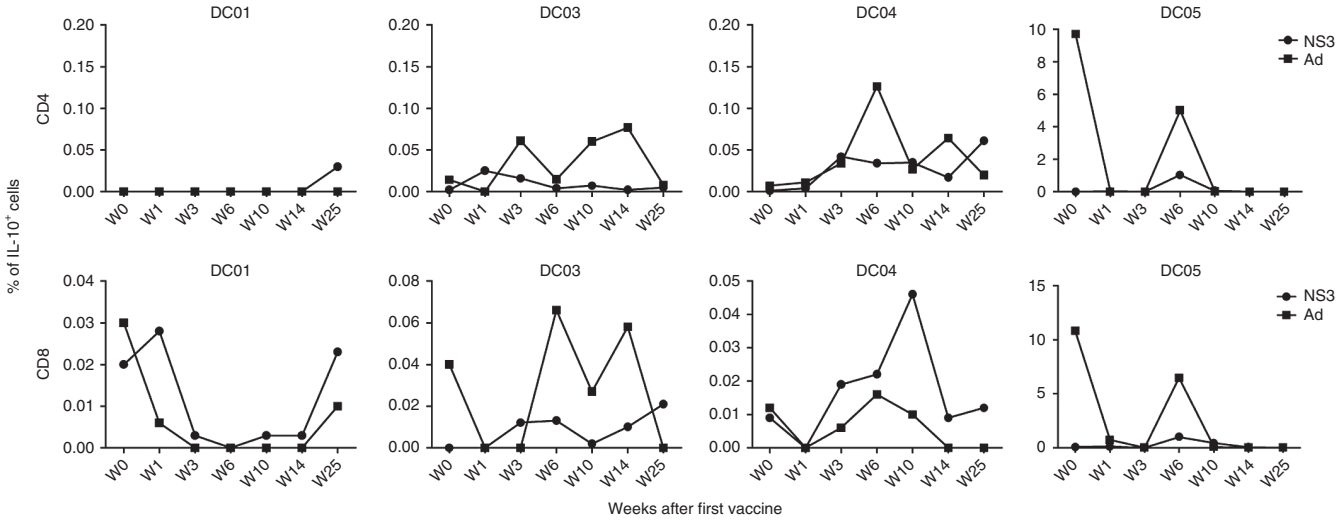

Figure 5 Vaccination with DC transduced with AdNS3/CFh40L induces IL-10–producing T cells. PBMC from serial samples obtained after vaccination were incubated with NS3 or Ad peptides, expanded with IL-2 for 10 days, stimulated again for 5 hours with corresponding peptides and intracellular production of IL-10 was analyzed by flow cytometry in CD4 and CD8 T cells. Results correspond to the difference between peptide-stimulated values minus unstimulated values.

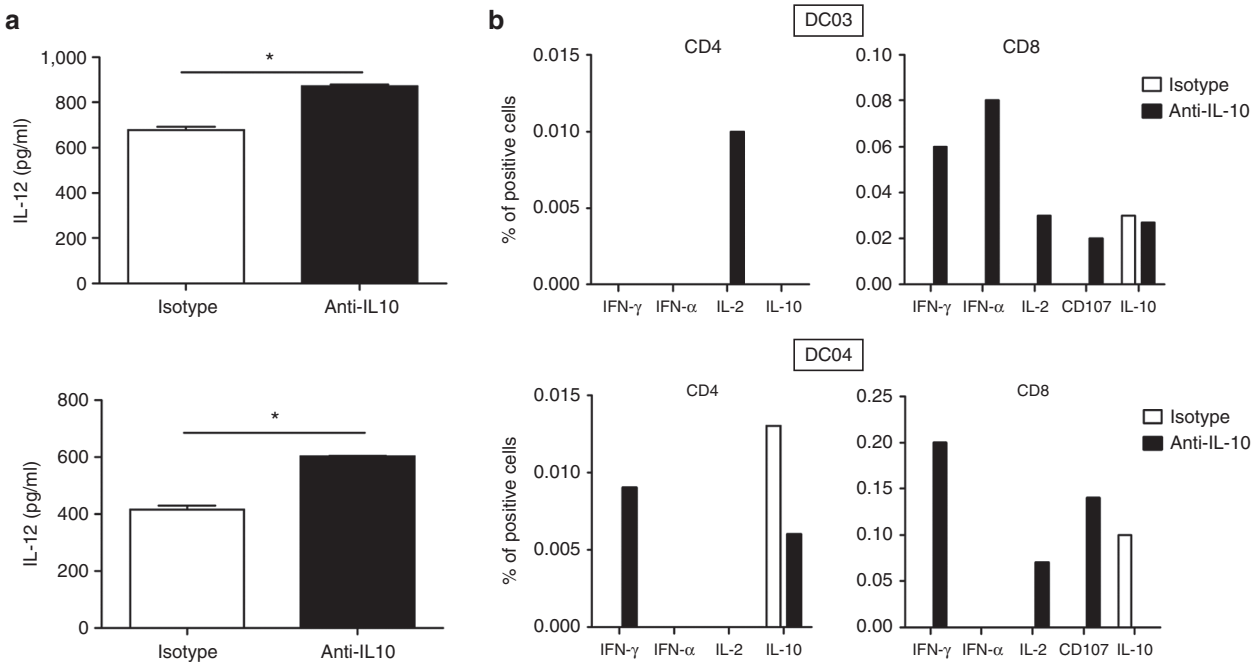

Figure 6 IL-10 blockade during DC maturation promotes activation of Th1 NS3-specific cells. Immediately after transduction, DC from HCV patients were cultured in the presence of anti-IL-10 or isotype control antibodies. Next day, (**a**) supernatants were harvested and IL-12 content was measured. Data correspond to mean + SEM ($n = 4$). *$P < 0.05$, according to nonparametric Mann-Whitney test. (**b**) DC were used to stimulate autologous T cells and 10 days after stimulation and expansion with IL-2, resulting T cells were restimulated with NS3 peptides and cytokine production in CD4 and CD8 T cells was measured by flow cytometry. Results correspond to the difference between peptide-stimulated values minus unstimulated values.

DISCUSSION

The fact that robust antiviral T-cell responses are detected in HCV patients who clear the virus suggests that therapeutic vaccines could be useful in chronic HCV infection.[22] According to this, we carried out a therapeutic vaccination pilot clinical trial in HCV patients, administering autologous MoDC transduced with a recombinant adenovirus encoding NS3 by using the chimeric molecule CFh40L. As reported,[15] MoDC prepared from HCV patients after transduction with CFh40L/AdNS3 showed phenotypic and functional features of mature DC. Transduction of murine DC using a similar strategy resulted in strong maturation and release of proinflammatory cytokines, with concomitant induction of Th1 responses. Due to these properties described in the murine model, which were confirmed with human MoDC from healthy individuals and HCV patients, and since one of the primary endpoints of the study was to analyze vaccine safety, we designed an escalating approach, with three dose levels, starting from 5×10^6, and later increasing to 10^7 and 2×10^7 DC. The first group was at a dose below that reported by Gowans *et al.*[8] in their DC vaccination clinical trial, whereas high-dose groups were decided based

on feasibility of the protocol, according to cell availability and yield of mDC from initial monocyte input calculated in preclinical experiments with human cells. The 2×10^7 dose group was not finally included, since the trial was prematurely stopped because of new direct antiviral drugs. In the two groups tested vaccine was safe, without serious adverse effects, in association with stable levels of biochemical markers, as occurred with viral load, a secondary end-point of the study. A similar safety profile has been reported for vaccination of HCV patients with a DC/lipopeptide vaccine,[8] indicating safety of DC vaccination strategies in HCV patients. Another secondary aim was the analysis of the immune response induced by the vaccine. Consistent with the lack of antiviral effect, CD4 and CD8 T-cell responses against NS3 induced by the vaccine were of low magnitude, as demonstrated using different *ex vivo* assays, such as ELISPOT or detection by flow cytometry of cytokines and the degranulation marker CD107. Antiviral immune responses were also evaluated by using *in vitro* culture assays and expansion with IL-2, and activation of discrete CD4 and CD8 T cells responses at defined time-points was again observed. The profile of responses detected using both assays shows some discrepancies, as described in other HCV vaccination clinical trial,[23] presumably due to the different functions analyzed, direct cytokine production in 5-hour *ex vivo* assays versus proliferative capacity and cytokine production in IL-2–expanded cultures, but confirms the activation of low magnitude responses after immunization. In addition, in agreement with other HCV vaccination clinical studies using viral vectors,[10] responses against NS3 were lower than those induced against adenoviral antigens (vector used for DC transduction). Interestingly, there is a lack of correspondence between kinetics and peak of anti-NS3 and anti-Ad immune response in the patients tested, presumably due to differential properties of NS3- and Ad-specific T cells. The exhausted phenotype described for HCV-specific T cells, as opposed to cells recognizing unrelated viral peptides, or the presence of naive/memory phenotypes in these populations may explain the different kinetics of responses against both types of antigens. Finally, the magnitude of responses induced by the vaccine is in contrast to those obtained in the murine model when using a similar strategy,[15] but in agreement with results reported in therapeutic vaccination in HCV patients, where responses induced against HCV are usually poor[6,9,10] when compared to those induced in healthy individuals.[24,25] The inability to induce strong anti-HCV immune responses by therapeutic vaccines despite the use of different approaches (peptides, viral vectors, DC) suggests that intrinsic properties of T cells in HCV patients, such as T-cell exhaustion,[17,19] may be involved in the lack of proper T-cell activation. Indeed, although we could only monitor expansion and phenotype of NS3-specific T cells by using HLA dextramers in a single patient, we observed that those CD8 T cells with the exhausted PD-1$^+$Tim3$^+$ phenotype were poorly expanded by the vaccine, confirming the low responsiveness of these cells. Moreover, a high PD-L1 expression was detected in mDC (Supplementary Figure S6), which would prevent activation of PD-1$^+$ exhausted T cells. Another factor potentially implicated in these results is the type of patients included in the study. All vaccinated individuals are patients who previously failed Interferon/Ribavirin-based treatment. Thus, although combination with antiviral treatment might help to decrease viral load, facilitating thus vaccine effect, resistance to IFN therapy by these patients, together with immunomodulatory properties of IFN treatment, which include loss of T cells,[26] led us to avoid this combination. All together, these results suggest that low anti-HCV responses induced by our vaccine as well as those reported in other therapeutic vaccination clinical trials may partly depend on intrinsic properties of T cells, explaining thus the lack of relevant clinical and virological effects.

Besides problems inherent to T cells, we also checked vaccine-associated factors potentially linked to these low responses. Immunosuppressive cytokines TGF-β and IL-10 were produced by the DC vaccine. Although both cytokines possess well-described immunosuppressive effects on T-cell priming, we focused subsequent analyses on IL-10, because IL-10 levels were significantly higher in MoDC from HCV patients than in healthy controls, suggesting that patients' monocytes (the source of our DC vaccine) are somehow "pre-conditioned" and more prone for IL-10 production. IL-10 overproduction has been reported in monocytes[27–29] from HCV patients, as well as induced by viral proteins[30,31] and particles,[32] which may explain the properties of the vaccine. This enhanced IL-10 production was mainly due to CFh40L, since AdNS3 alone did not induce IL-10 over background. It has been reported that CD40/CD40L interaction is associated with IL-10 induction in DC,[33,34] results which contrast with those observed in our murine model, where as opposed to IL-12, IL-10 was not induced, highlighting the importance of validating these strategies with human cells. Our results regarding production of IL-10 after CD40L stimulation are similar to those showing that DC transduction with an adenovirus encoding CD40L induces a mature DC phenotype with simultaneous IL-12 and IL-10 production.[20] Since stimulation with these DC may lead to IL-10–producing T cells, we analyzed IL-10 secretion after antigen stimulation. For both NS3- and adenovirus-specific T cells, we found that IL-10 was induced in some patients, mainly in those with the highest IL-10 production by DC. Interestingly, in an *in vitro* situation resembling patients at the time of vaccination, IL-10 neutralization during transduction of patients' DC led to higher IL-12 levels, and concomitant NS3-specific Th1 responses emerged, as we previously showed in a murine model.[21] To our surprise, adenovirus-specific T-cell responses decreased after IL-10 blockade. Conflicting data have been reported regarding the role of IL-10 and T-cell responses, differing between CD4, CD8 T cells and even between effector and memory responses. While some authors describe that IL-10 restricts effector and memory responses,[35,36] others report no effect,[37] a selective effect only in CD4 T cells[38] or even a beneficial effect for CD8 T cells.[39,40] Predominant presence of naive cells in HCV-specific lymphocytes versus adenovirus-specific memory cells might explain these results. From a practical point of view, it is clear that IL-10 blockade is beneficial to promote anti-HCV Th1 responses induced by the vaccine in these patients.

Nowadays, the landscape of HCV therapy has changed dramatically with the introduction of DAA which resolve the disease in more than 90% of patients. The economic cost of this treatment is presently very elevated but its efficacy supersedes other therapeutic options, like therapeutic vaccination, a procedure which on the other hand has demonstrated limited efficacy in the present study as well as in other previous reports, as mentioned above.

In summary, we have carried out a therapeutic vaccination pilot clinical trial in HCV patients using autologous DC, which was safe and without detectable virological effects, presumably due to the insufficient immune response induced by the vaccine. Improvement of vaccine properties by blockade of endogenous immunosuppressive factors, as well as combination with therapies aimed at enhancing functional properties of exhausted T cells (*e.g.*, inhibition of

CTLA4, PD-1/PD-L1), as we have previously described in HCV/HCC patients,[41] might be a promising strategy not only for HCV but for other therapeutic vaccines.

MATERIALS AND METHODS

Study design and patients

This was a phase 1–2, open-label, dose-escalation clinical trial that recruited patients with chronic hepatitis C (genotype 1b) that had previously failed Interferon/Ribavirin-based treatment (including null response, breakthrough, relapse or intolerance). Co-infection with HBV or other HCV genotypes, liver stiffness >17 kPa, hepatocellular carcinoma and any clinical condition potentially impairing immune response (autoimmune diseases requiring immunosuppressive treatment, liver transplantation, HIV infection, etc.) were considered exclusion criteria. Patients with intense biochemical activity (ALT >7 ULN) or poor liver function (total bilirubin >1.5 mg/dl) at baseline were not considered eligible. Primary end-points were assessment of treatment safety and tolerability. Secondary end-points included treatment effect on viral load and immune response. All patients provided informed written consent and the study protocol conforms to the ethical guidelines of the Declaration of Helsinki and was approved by the Navarra Clinical Research Ethics Committee and by the Spanish Agency of Medicines and Medical Devices (AEMPS). The study was registered in the European Clinical Trials Database as 2010-024043-32. Blood samples used to prepare DC from healthy HCV-negative individuals were obtained from the Navarra Blood and Tissue Bank (Navarrabiomed Biobank, Navarra Health Department).

Clinical protocol

After inclusion, patients underwent a standard leukapheresis without previous hematopoietic growth factor administration. The procedure was stopped after 240 minutes or sooner, depending on patient tolerance. Peripheral veins were used for vascular access. Vaccines, prepared according to the protocol described below, were administered by intradermal injection in the thigh at weeks 0, 3 and 6. Two dose levels of 5×10^6 DC and 10^7 DC were tested. Although the trial was initially designed to test a third dose level of 20×10^6 DCs, the emergence of the newest direct antiviral drugs had a strong negative impact on recruitment and the trial was prematurely stopped. Monitoring of clinical, biochemical, virological and/or immunological variables was carried out in the first 3 days after vaccine administration and at weeks 1, 2, 3, 6, 10, 14, and 24. Liver US and transient elastography were performed at inclusion and end of follow-up.

Vaccine preparation

DC were generated from CD14+ monocytes. PBMC were purified from apheresis products and monocytes selected by using the CliniMACS CD14 System (Miltenyi Biotec, Madrid, Spain). For the first dose, an aliquot of monocytes (2×10^6 cells/ml) was differentiated in culture bags (Miltenyi Biotec) in AIM-V medium (Life Technologies) supplemented with 10^3 U/ml of GM-CSF (Leukine, Genzyme, Cambridge, MA) and IL-4 (R&D Systems, Minneapolis, MN), whereas remaining cells were frozen for boosting doses. Three days later cytokine-containing fresh medium was added. On day 7, iDC were harvested, and an aliquot of cells was used for phenotypic and viability analysis. Remaining cells were transduced with GMP-grade AdNS3 and CFh40L adaptor protein (both from Vector Production Facility, University of Alabama at Birmingham) as described.[15] Briefly, CFh40L (0,6 μg/10^6 cells) and Ad-NS3 (MOI = 15) were incubated in PBS (50 μl/10^6 cells) for 30 minutes at 37 °C and 5% CO_2. This mix was then added to immature DC, previously adjusted to 10^7 cells/ml in AIM-V medium, and further incubated for 1 hour. Cells were then diluted and placed in culture bags at 2×10^6 cells/ml for additional 24 hours. Next day, mDC were harvested and resuspended in PBS for vaccination. For second and third doses, monocytes were thawed and processed as above. In those cases involving IL-10 blockade for *in vitro* experiments, immediately after transduction, DC were cultured with anti-IL-10 blocking or isotype control antibodies (20 μg/ml; R&D Systems). DC purity (according to the expression of DC markers CD11c and CD209 and monocyte marker CD14), as well as their phenotypic maturation (as determined by upregulation of HLA-DR, CD80, CD86, and CD54 molecules) was assessed by flow cytometry using antibodies described in Supplementary Material. Sterility and viability (based on trypan blue staining) were assayed before vaccine administration.

ELISPOT

IFN-γ–producing cells were enumerated by ELISPOT according to manufacturer instructions (BD-Biosciences, Franklin Lakes, NJ) as described,[41] by incubating fresh PBMC with or without antigens (recombinant NS3, 1 μg/ml; 20-mer NS3 peptide pool, 1 μg/ml each peptide;[15] adenoviral peptide pool, 1 μg/ml (Peptivator AdV5 Hexon, Miltenyi)) or PHA (1 μg/ml).

Analysis of T-cell responses by flow cytometry

PBMC (2×10^6 cells) were stimulated with NS3 or adenoviral peptide pools (1 μg/ml) in complete medium (RPMI 1640 supplemented with 10% FBS and 1% antibiotics; CM) containing IL-2 (50 U/ml) in the presence of GolgiStop and Golgi Plug (BD-Biosciences) with anti-CD107 antibodies. Five hours later cells were stained with anti-CD3, CD4, CD8 antibodies, treated with Fix/Perm (BD-Biosciences) and subsequently stained with antibodies against IFN-γ, TNF-α, IL-2, IL-10 (antibodies and gating strategy are described in Supplementary Material). For expansion studies PBMC (10^6 cells/well) were stimulated with peptides or DC (ratio 10:1) and IL-2 (100 U/ml) added on day 5. At day 10 cells were harvested and submitted to 5-hour stimulation as above. In experiments determining number and phenotype of NS3-specific cells, PBMC were stained with anti-CD3, CD8, and HLA-A2 dextramers (Immudex, Copenhagen Denmark) containing NS3 peptides (1073–1081) or (1406–1415). In all cases, cells were acquired using a FACSCanto II flow cytometer (FACSdiva) and analyzed using FlowJo version 10 (Treestar, Ashland, OR).

ELISA

Determination of cytokines in DC culture supernatants was carried out by ELISA according to manufacturer (BD-Biosciences).

Statistical analysis

In the clinical trial, descriptive statistics of safety and virological outcomes were used. Since each patient served as his own control, changes of clinical, biochemical, virological and immunological variables have been compared at baseline and at different time points of the follow-up with nonparametric test for repeated measures (Friedman test) using SPSS software version 20. For some immunological analyses, nonparametric Mann–Whitney tests from GraphPad Prism software were used. A P value <0.05 was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors thank T Strong for her help in AdNS3 and CFh40L production, members of the Cell Therapy Unit for their collaboration in vaccine preparation and Carmen Fuertes, Verónica Aróstegui and Virginia Villar for their valuable assistance. This study was supported by grants from the Ministerio de Ciencia e Innovación (SAF2010-15074) to P.S., from Ministerio de Sanidad y Política Social (TRA-105 and EC10-327) to J.P.

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