Generation of Immune Responses Against HCV Using Dendritic Cells Containing NS5 Protein-Coated Microparticles

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Running Title: Dendritic cell based immunization against HCV
Abstract

Dendritic cells (DCs) internalize and process antigens as well as activate cellular immune responses. The aim of this study was to determine the capacity of DCs that contained antigen-coated magnetic beads to induce immunity against the nonstructural hepatitis C virus (HCV) antigen 5 (NS5). Splenocytes derived from Flt3 ligand pretreated BALB/c mice were incubated with magnetic beads coated with HCV NS5, LPS and/or anti-CD40, purified and used for immunization. Cellular immunity was measured using cytotoxic T-lymphocyte (CTL) and T-cell proliferation assays, intracellular cytokine staining and a syngeneic tumor challenge using NS5 expressing SP2/0 myeloma cells in vivo.

Splenocytes isolated from animals vaccinated with DCs-beads+NS5+LPS+anti-CD40 secreted elevated levels of IL-2 and IFN-γ in the presence of NS5. The numbers of CD4+IL-2+ cells were increased >5-fold in the group immunized with DC-Beads+NS5+LPS+anti-CD40, paralleled by an enhanced splenocyte proliferative response. Immunization promoted antigen-specific CTL activity 3-fold compared to control mice and reduced significantly the growth of NS5 expressing tumor cells in vivo. Thus, strategies that employ NS5-coated beads induce cellular immune responses in mice, that correlate well with the natural immune responses that occur in individuals who resolve HCV.

Key words: Dendritic cells, hepatitis C virus, vaccine
1. Introduction

An estimated 170 million individuals (3% of the world’s population) are infected with the hepatitis C virus (HCV) leading to cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC). Current standard-of-care therapy for chronically infected HCV patients is the combined administration of pegylated IFN-α and ribavirin (28). A sustained response is seen in approximately 50-60% of individuals (33). Treatment is long-term (6-12 months), costly, and associated with substantial toxicity (42). Clearly, more effective regimens are needed (10).

Clinical studies of the adaptive host immune response in acute HCV infection suggest a rationale for immunization approaches. Clearance of HCV is associated with early, multispecific, strong CD8+ T-cell immunity that is matched by vigorous and sustained CD4+ T-cell proliferation in response to multiple recombinant structural and non-structural viral proteins (13, 18, 47). Activated T-cells secrete pro-inflammatory cytokines (T<sub>H1</sub>-type) such as IFN-γ, coinciding with large reductions in viral load during acute infection (47). HCV infections that are successfully controlled result in durable memory populations (44). This observation is supported by a substantially lower rate of HCV persistence in re-exposed humans with a history of acute resolving HCV (29). Re-challenge experiments in chimpanzees showed that antibody-mediated depletion of CD4+ T-cells resulted in HCV persistence which is in contrast to a marked reduction in duration and peak of viremia found in non-treated animals (4, 20). The importance of CD4+ T-cells is further emphasized by the loss of immune protection against re-exposure to HCV and correlated with low CD4+ T-cell counts, in intravenous drug users, who had recovered from HCV but subsequently acquired HIV infection (29). Whether recovery from acute HCV coincides ultimately with virus eradication is still a matter of debate (34).
HCV-specific antibodies in some individuals 10-20 years after viral clearance indicates that a subgroup of patients achieves complete virus elimination (44).

Persistent HCV infection can be attributed to a number of viral evasion strategies. Rapid spread of HCV in a host outpaces immune response by several weeks (39, 46). Second, HCV is a strong inducer of type I IFN, but appears to render hepatocytes resistant to antiviral activity (45, 46). Third, NK cell function can be inhibited by cross-linking tetraspanin CD81 on the cell surface with the major envelope protein of HCV (HCV-E2), thus blocking NK cell activation, proliferation and cytokine production (12). Finally, persistent HCV infections correlate with the permanent loss of HCV-specific T-cell proliferation during acute HCV, functional exhaustion of an initially vigorous response, and the inability of effector T-cells to migrate into the infected liver (10, 13, 18, 36, 39, 46). Insufficient CD4+ T-cell activity appears to be a key event leading or contributing to chronic HCV. Failure to sustain the CD4+ helper response renders virus-specific CD8+ T-cells inadequate (e.g. loss of cytotoxicity and IFN-γ production) thus contributing to persistent viremia (39).

Recapitulating successful immune responses and addressing HCV-specific defects in immunity are mandatory in efforts to improve current treatment options. The purported involvement of dendritic cells (DCs) in the impaired immune responses observed in patients with persistent HCV infection make them a principal target for immunomodulatory approaches. Access to a sufficient quantity of mature DCs is a critical issue since only mature DCs are capable of targeting the antigen and inducing cellular immunity rather than tolerance. Previously we outlined a novel method of generating large numbers of DCs in vivo (17). These, in turn, are enriched in vitro by phagocytosis of magnetic beads and separation in a magnetic field. Here we further demonstrate that, by immunizing mice with dendritic cells that contain beads coated with
the nonstructural HCV antigen NS5 and compounds known to induce DC maturation, significant levels of cellular immunity are elicited. The key elements of this approach reside in the combined enrichment, maturation and antigen targeting of DCs in a single step and the generation of immune responses of the type known to promote viral clearance in humans.
2. Materials and Methods

2.1. Mice

Five-to 6-week-old female BALB/c (H-2<sup>d</sup>) mice were obtained from Harlan Sprague Dawley, Inc (Indianapolis, IN) and kept under specific pathogen-free conditions in the animal facility of Rhode Island Hospital. All animal protocols have been reviewed and approved by the Lifespan Animal Care and Use Committee.

2.2 Culture Conditions

DCs were cultured in serum-free HEPES-buffered RPMI 1640 medium (Cambrex Bio Science, Wakersville, MD) supplemented with 2 mM L-glutamine, 1% essential and nonessential amino acids, 5 x 10<sup>-5</sup> M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 U/ml gentamycin (all from Sigma-Aldrich, St. Louis, MO). Splenocytes obtained from immunized animals were cultured in the same medium additionally supplemented with heat-inactivated 10% fetal bovine serum (FBS).

2.3. Coating of magnetic beads with anti-CD40, LPS and HCV NS5

Immunomagnetic beads (Calbiochem, EMD Bioscience, San Diego, CA), 1.3 µm in diameter, were coated using methods previously described (17), and closely following the manufacturer’s protocol. Briefly, beads were suspended in CDI buffer (Sigma-Aldrich, St. Louis, MO) and incubated for 10 min at 50°C, pelleted and washed three times in borate buffer (Fluka / Sigma-Aldrich, St. Louis, MO). After the final wash, borate buffer was added with or without 100 µg of anti-CD40 (3/23, 0.5 mg/ml) (BD Pharmingen, San Jose, CA), 10 µg of LPS (Sigma-Aldrich, St. Louis, MO), and/or a total of 200 µg of NS5 protein (AG712; yeast derived...
recombinant protein; Maine Biotechnology, Portland, ME) as indicated. After incubation for 10 min at 50°C, followed by an over night incubation at 4°C under constant slow agitation, the beads were pelleted in a magnetic field and the supernate was removed by pipetting; the beads were then washed and resuspended in 100 µl PBS. Centrifugation was avoided in all steps to prevent bead aggregation.

According to the manufacturer, approximately 10% of the protein in solution is bound by the beads; therefore, the supernate can be reused for additional coatings. We found most of the beads were eliminated during the DC purification step (see 2.4. Histodenz gradient) and not taken up by phagocytic cells. Since a relatively small fraction of the purified DCs is injected into the animals (1 x 10^6 out of 1.5 x 10^7 purified cells), only minute amounts of antigen and / or anti-CD40 and LPS are finally injected into the animals.

2.4. Generation and purification of splenic dendritic cells

The DC population was expanded in vivo using methods previously described and outlined in Figure 1 (17, 23, 27). Briefly, the plasmid pUMVC3-hFLex expressing the secreted portion of human Fms-like tyrosine kinase receptor-3 ligand (hFlt3; Vector Core Laboratory, University of Michigan) was injected twice (day 0 and 6) into the tail vein of mice (hydrodynamic gene delivery); the spleens were dissected on day 12. Single cell splenocyte suspensions were mixed with beads and incubated for 3 hrs at 37°C. Cells were harvested and those, which had taken up beads, were separated from the remaining population using magnetic force. Finally, free beads were removed from bead-containing cells by centrifugation on a 20% Histodenz gradient (Sigma-Aldrich, St. Louis, MO).
2.5. Immunization

Groups of 5 mice were immunized with or without DCs as follows: 1) beads coated with LPS+anti-CD40; 2) beads coated with NS5+LPS+anti-CD40; 3) DC containing beads coated with LPS+anti-CD40; 4) DC containing beads coated with NS5 and 5) DC containing beads coated with NS5+LPS+anti-CD40. Unless noted otherwise, animals were inoculated subcutaneously 3-times at 2-week intervals with 50 μl of immunogen in each of two footpads, in the case of DCs, 1 x 10⁶ cells in 100 μl HBSS/immunization. Two weeks after the final immunization, mice were challenged or euthanized and splenocytes collected for subsequent analysis.

2.6. Intracellular cytokine staining (ICCS) and FACS analyses

ICCS and FACS analyses were performed in accordance with methods described (17, 27). Briefly, 1-5 x 10⁵ cells were incubated with excess anti-mouse CD16/32 (clone 93, rat isotype) to block the FC receptor, then stained with 1 μg PE-, FITC-, or PerCP-labeled antibody specific for the following mouse cell surface markers: CD11c (clone N418), CD4 (clone L3T4), CD8α (clone Lyt-2); the recommended isotype controls were included. ICCS was performed with anti-mouse IL-2 (clone JES6-5H4), and the Cytofix/Cytoperm Kit (BD Pharamingen) according to the manufacturer’s instructions. All antibodies were purchased from eBioscience (San Diego, CA) if not otherwise indicated.

2.7. Proliferation assay

Cells (4 x 10⁶) were labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) in Hank’s balanced salt solution (HBSS) by incubation for 15 min at
37°C. Then, the cells were centrifuged, resuspended in pre-warmed medium and incubated for another 30 min to ensure complete modification of the probe. The labeled cells were incubated for 5 days in 6-well plates containing 5 ml culture medium supplemented with 10% FBS and 1µg/ml HCV NS5. The cells were then washed, stained for CD4 or CD8, and evaluated for proliferation by flow cytometry.

2.8. Quantitation of cytokine production

To quantify cytokine production, splenocytes derived from immunized and non-immunized animals were cultured at 5 x 10^5 cells/200 µl/well in 96-well flat-bottom plates at 37°C. Cells were stimulated with 0.1 to 1 µg/ml recombinant HCV NS5 protein. After 24 hours, the supernates were collected and the levels of IFN-γ, IL-4, and IL-2 were quantified using commercial ELISA kits purchased from eBioscience according to manufacturer’s instructions.

2.9. In vitro cytotoxicity assay

CTL activity was assessed in accordance with methods described (17, 27). CTL activities expressed by splenocytes derived from immunized and control mice were determined following in vitro stimulation in which 1 x 10^8 cells in 30 ml of culture medium were incubated with recombinant murine IL-2 (5 U/ml; eBioscience, San Diego, CA) and recombinant HCV NS5 protein (0.3 µg/ml; Biodesign Int., Saco, ME). After three days culture, the cells were harvested and a standard 4-h ^{51}Cr release assay was performed. The ^{51}Cr-labeled syngeneic murine myeloma cells (SP2/0; ATCC, Manassas, VA) stably expressing HCV NS5 (SP-NS5) were used as the targets. All assays were conducted in quadruplicate.
2.10. Tumor challenge model

T-cell activity was assessed in vivo by tumor challenge as outlined previously (16, 27). Stably expressing HCV NS5 cells (SP-NS5) were harvested and washed three times in serum-free medium. The backs of mice were shaved and each animal was inoculated s.c. in the right flank with $1 \times 10^6$ cells resuspended in 100 µl serum free medium. The tumor size was measured daily with a caliper starting on day 7. On day 15, the mice were euthanized; the tumors were dissected and weighed.

2.12. Statistical analysis

Results were analyzed using the SigmaStat 3.0 statistics program (Jandel Scientific, San Rafael, CA). Individual means were compared using a non-paired Student’s $t$ test. When comparing more than two groups, a one-way ANOVA was performed followed by a Tukey test to determine which groups differed significantly ($p<0.05$).
3. Results

3.1. Generation, enrichment and targeting of NS5 antigen to DCs

In agreement with our previous findings, hydrodynamic delivery of the hFlt3L expressing plasmid pUMVC3-hFLex increased the total number of splenocytes ten-fold to an approximate $4 \times 10^8$ cells per spleen (17, 27). Twenty-five per cent of splenocytes were CD11c+, representing a 100-fold expansion of the murine DC population. After dissecting the spleens of Flt3L-treated mice, the total splenocyte population was incubated with coated magnetic beads for 3 hrs. Successful coating of beads with recombinant NS5 was verified in advance by binding of anti-NS5 antibodies to the beads. An enriched DC population was obtained by passing the cells over a magnetic column and separating bead-containing cells from the remaining population. This procedure increased the CD11c+ cells from 25% (after Flt3L treatment) to greater than 80% of the total population (Fig. 1).

The majority of the remaining bead-containing, CD11c negative cells have been previously characterized based on the expression of CD11b, CD4, CD8 and CD45R/B220 (17). The CD11c negative cell population stained brightly positive for CD11b / Mac-1 and expressed low levels of CD4, CD8 and CD45R characteristic of a macrophage population.

3.2. Cytokine secretion by the total splenocyte population

A series of experiments was undertaken to evaluate the potential of antigen coated beads in stimulating anti-viral responses \textit{in vitro} and \textit{in vivo}. Since a T$_{H1}$ response is strongly associated with resolution of HCV, we examined the ability of splenocytes derived from immunized animals to secrete IFN-γ and IL-2; IL-4 secretion was quantified as a measure of T$_{H2}$ type responses. Relative to all other groups, splenocytes derived from mice immunized with
DCs containing NS5, LPS and anti-CD40 coated beads produced elevated concentrations of IL-2 when cultured in the presence of recombinant NS5 (Fig. 2A). IL-2 production was increased even in cultures that contained as little as 0.1 µg/ml NS5. In contrast, IL-4 production was relatively low and not significantly different between groups regardless of culture conditions. The production of IL-2 and IFN-γ was maximal in splenocyte cultures derived from mice immunized twice with DCs containing NS5+LPS+anti-CD40-coated beads; additional immunizations did not promote greater cytokine production (Fig. 2B). A single immunization, however, elicited a splenocyte population capable of producing IL-2 in specific response to antigen (Fig. 2C).

3.3. Intracellular Cytokine Staining

ICCS was performed to determine the cells source(s) of IL-2. Previously we have shown that the CD4+ and CD8+ population derived from animals immunized with DC-Beads+NS5+anti-CD40+LPS displayed a significant (4-5 fold) increase in number of IFN-γ-producing cells in contrast to all the control groups (17). Here we observed a somewhat different pattern when staining for IL-2. Only the CD4+ population obtained from animals immunized with DC-Beads+NS5+anti-CD40+LPS displayed a marked increase in number of IL-2 secreting cells (Fig. 3). None of the other groups had a substantial number of CD4+IL-2+ cells, nor did any of the groups have a sizable number of IL-2-producing, CD8+ cells.

3.4. Cell proliferation in response to stimulation with recombinant NS5

A CFSE proliferation assay with additional staining of CD4 and CD8 was performed to identify the cell types responsive to HCV NS5 immunization. A 2-3 fold increase in the number
of proliferating CD4$^+$ cells was observed among splenocytes derived from mice immunized with DCs containing beads coated with NS5 only (Fig. 4). A much larger number of proliferating CD4$^+$ cells was observed in the group immunized with DCs-NS5+anti-CD40+LPS, i.e., a 6-10 fold increase in proliferating CD4$^+$ cells compared to animals immunized with beads alone or with DCs containing beads+LPS+anti-CD40, but not NS5. Interestingly, animals immunized only with beads coated with NS5+LPS+anti-CD40 (i.e., no DCs) displayed a weak, 2-fold increase in proliferating CD8$^+$ cells. More proliferating CD8$^+$ cells were detected in animals immunized with DCs containing NS5-beads (4-fold). The strongest proliferative response, however, was seen in the group immunized with DC-Beads+NS5+anti-CD40+LPS; 6-fold more CD8$^+$ cells displayed a strong shift towards weak CFSE staining.

3.5. CTL response

CTLs play a critical role in eliminating virus-infected host cells. Standard $^{51}$Cr-release assays were performed to determine the capacity of bead-containing DCs to elicit CTL activity. After a single immunization, slightly greater CTL activity was expressed by splenocytes obtained from the group inoculated with DCs-Beads+NS5+anti-CD40+LPS (Fig. 5). Immunizing a second and third time significantly increased the CTL activity exhibited by splenocytes derived from this group, relative to the other groups. Indeed, following the second immunization, splenocytes obtained from mice immunized with DC-Beads+NS5+anti-CD40+LPS displayed $\geq$2-fold more CTL activity. Notably, splenocytes derived from mice immunized with DCs containing beads coated with NS5 alone did not exhibit more CTL activity than did splenocytes obtained from animals immunized with DCs containing non-NS5-coated beads (i.e., DCs-Beads+anti-CD40+LPS group). Immunization with DCs induced a certain level of non-specific
cytolytic activity. Greater SP2/0-NS5 tumor cell lysis was found in co-cultures that contained splenocytes derived from mice immunized with DCs containing beads coated with LPS and anti-CD40 (no NS5) relative to mice immunized with Beads+LPS+anti-CD40.

3.6. Tumor challenge

To determine the efficacy of DC-based immunization in vivo, mice were challenged with the same stable, SP2/0-NS5-expressing tumor cell line used to assess CTL activity in vitro. Tumor growth was compared in animals vaccinated with DCs containing beads coated with LPS and anti-CD40 with or without NS5, and in animals immunized with only beads+NS5+LPS+anti-CD40, i.e., no DCs (Fig. 6). After 15 days observation, the animals were euthanized; the tumors were dissected and weighed. Mice immunized with beads alone or with DC-Beads+LPS+anti-CD40 displayed fast tumor growth. Tumor weight paralleled the rate of tumor growth observed, with an average weight of 0.5 g/animal in the two control groups (DCs+beads+LPS+anti-CD40 and NS5+beads+LPS+anti-CD40). By comparison, the tumors dissected from animals immunized with DCs containing beads coated with NS5+LPS+anti-CD40 were less than half the size (≤0.2 g/mouse).
4. Discussion

Robust memory CD4\(^+\) and CD8\(^+\) T-lymphocyte activities characterize host immune responses leading to resolution of HCV infection (44). Strategies to enhance immunity should recapitulate these naturally occurring immune responses. In this regard, we and others have investigated the ability of DNA based immunization to evoke sustained cellular immune responses against HCV antigens (6, 15, 16, 32). Even though significant CTL activity was observed \textit{in vitro} and \textit{in vivo}, success was limited by a lack of memory and an overall weak CD4\(^+\) T-cell response (16, 27). Notably, sustained CD4\(^+\) T-cell responses appear to be a key factor in control of HCV infection; HCV persistence, on the other hand, is related to impaired CD4\(^+\) T-cell activity (39). The latter might be explained by a reduction in DC activity. Indeed, DCs obtained from chronically-infected HCV individuals display decreased stimulatory capacity possibly due to a reduction in their ability to produce IL-12 and IFN-\(\gamma\) (3, 25). In addition, DCs from persistent HCV positive individuals failed to mature in response to conventional stimuli, evidenced by diminished co-stimulatory molecule expression and antigen uptake (2). The underlying cause(s) for these changes in DC function can be attributed to the ability of HCV to infect DCs (3, 19) and/or the effects of HCV proteins on DC function (14, 37, 38), though the latter concept is controversial. Moreover, the microenvironment regulates DC maturation and function; T-cells are required (40). The CD4\(^+\) T-cells associated with chronic HCV infection produce anti-inflammatory cytokines such as IL-4 and IL-10 (48, 49). A significant number of HCV-specific CD8\(^+\) cells are negative for the CD38 and CD69 activation markers (22).

The impaired function of DCs during chronic HCV infections contrasts the ability of naïve DCs to induce a strong T-cell response. This observation and the failure of current vaccine strategies to induce a vigorous HCV antigen-specific CD4\(^+\) T-cell response have brought recent
attention to the role of DCs in this regard. Targeting DCs is an approach capable of interrupting the vicious cycle of impaired DC function that causes insufficient T-cell priming and, in turn, the generation of anergic T-cells not capable of stimulating DC maturation.

Insufficient DC maturation is addressed herein by the ingestion of beads coated with LPS (a toll like receptor 4 (TLR4) ligand) and antibodies specific for CD40, a member of the TNF receptor family. Thus, two receptor families involved in DC maturation are engaged. TLR4 and CD40 are integral membrane proteins (5, 7), which likely interact with LPS and anti-CD40 bound to beads prior to ingestion. Accordingly, beads coated with both LPS and anti-CD40 induced phenotypic maturation of the CD11c+ population, as we have recently documented by elevated expression of maturation markers and increased secretion of IFN-γ, IL-2, TNF-α and IL-12 (17). Mature, IL-12 secreting DCs polarize CD4+ T-cells towards a Th1 type response (9).

The ingestion of coated microbeads not only stimulated DC maturation, but served as a mechanism for antigen delivery. In the present study, beads were coated with the entire recombinant HCV NS5 protein, rather than known immunogenic peptides to avoid the possible exclusion of important epitopes (52). Cells that ingested magnetic microbeads were readily separated from the remaining population; ≥80% of the bead-positive cells derived from hFlt3L-treated-animals were CD11c+DCs (Fig. 1).

Adoptive transfer of DCs containing beads coated with LPS, anti-CD40 and recombinant HCV NS5 (DCs-beads+NS5+LPS+anti-CD40) induced an antigen-specific cellular immune response that was evident in vitro and in vivo. Splenocytes derived from animals vaccinated with DCs-beads+NS5+LPS+anti-CD40 secreted elevated levels of IL-2 and IFN-γ in the presence of NS5 (Fig. 2). Maturation of DCs in the presence of LPS and anti-CD40 appears to be critical; cytokine production by splenocytes derived from animals inoculated with DCs containing beads...
coated with NS5 only was sharply diminished (Fig. 2). Interestingly, the CD4+ T cell population was identified as a main source of cytokine production and proliferative response, when immunized with DCs-beads+NS5+LPS+anti-CD40 (Fig. 3 & 4). These findings suggest that additional maturation signals are required to elicit a DC population capable of inducing a vigorous T\(_\text{H}1\)-type CD4+ T cell response. In the absence of these factors, the cellular immune response was weak. The importance of CD4+ T cell for an effective anti-HCV response is evidenced by patient studies (18) and underscored by a recent chimpanzee study (30) that demonstrated a temporal relationship between the recurrence of HCV viremia after apparent viral clearance and the loss of a detectable CD4+ T cell response. Importantly, a vigorous CD8+ T cell response is linked to the presence of HCV-specific CD4+ T cell activity. Recovery of CD8+ T cell effector function and a significant decrease in HCV viremia occurred at the time when CD4+ T-cells became detectable (47). Other animal models support the critical role of CD4+ T cells in the immune response to viral infections. CD8+ T cell-dependent control of lymphocytic choriomeningitis virus (LMCV) and herpesvirus infections, for example, is progressively lost in the absence of CD4+ T cells (8, 24). Notably, a recent patient study by Kaplan et al. (26) showed that sustained HCV clearance depended upon a CD4 T-cell response that included both antigen-specific proliferation and IFN-\(\gamma\) production. Viremia was only transiently controlled by either HCV-specific CD4 T-cell proliferation or IFN-\(\gamma\) production alone. By contrast, the resolution of HCV infection did not correlate with the HCV-specific response of CD8 T-cells or the production of virus-specific antibody.

Adoptive transfer of DCs-beads+NS5+LPS+anti-CD40 induced CTL activity \(\textit{in vitro}\) (Fig. 5) and anti-tumor immunity \(\textit{in vivo}\) (Fig. 6); activities required for immunity against HCV. The observation that DCs internalizing recombinant NS5 protein are capable of inducing CD8+ T
cell responses, underscores the ability of DCs to cross present antigens, previously described by several investigators (1, 21, 35). Albeit statistically significant, the CTL response and anti-tumor immunity demonstrated here show the need for improvement. Recent studies employing microparticles for DC based immunization suggest a number of possible strategies. These include: the use of biodegradable beads (31); encapsulating or coating the beads with DC maturation compounds such as CpG motifs (41), GMCSF (50), and mono-phospho-lipid A (MPLA) (11); and targeting DCs in vivo by coating the beads with DC-specific receptor antibodies (i.e., anti-DEC 205) (43).

In conclusion, an orchestrated cell-mediate immune response to viral antigens that includes proliferation and T\textsubscript{H}1 cytokine secretion by CD4\textsuperscript{+} T-cells is required to resolve acute HCV infections and to prevent viral persistence. The documented link between DC impairment and chronic HCV infection emphasizes targeting antigen to DCs specifically as a natural cell based adjuvant against HCV. Although these studies are performed in an experimental animal model system, applicability of this immunization technique to a human setting may be achievable through the employment of biodegradable, superparamagnetic beads and the use of compounds, that optimize maturation of human DCs, e.g. poly-(I:C), CpG motifs, and R-848 (51). The correlation between the type of cellular immune responses induced by DCs containing coated beads and the natural immune response of individuals who resolve HCV illustrates the potential of this approach.
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Fig. 1. *In vivo* hFlt3L treatment and *in vitro* enrichment yields an 80% CD11c+ cell population. (A) CD11c+ cells constitute ~25% of the total splenocyte population isolated from hFlt3L-treated mice. (B) CD11c+ cells comprise 80% of the enriched, magnetic bead-containing population.

Fig. 2. Mice immunized with DC-beads+NS5+anti-CD40+LPS exhibit a Th1-type cytokine response. (A / C) Splenocytes obtained from mice immunized two times were cultured with recombinant HCV NS5 at the concentration listed. Culture supernates were collected after 48 hrs; IFN-γ, IL-2 and IL-4 concentrations were quantified by ELISA. (B) Splenocytes derived from animals after each immunization were cultured with 1.0 µg/ml HCV NS5. Data represent the means ± SD derived from 4 animals treated comparably in a single experiment. Experiments were repeated three times with similar results. *P<0.05; **P<0.005, compared to the other groups incubated with the same concentration of recombinant HCV NS5.

Fig. 3. The CD4+ T-cell population derived from animals immunized with DC-Beads+NS5+anti-CD40+LPS secrete IL-2. The total splenocyte population obtained from mice immunized as indicated were incubated in the presence of 1 µg/ml HCV NS5 for 24 hrs; GolgiPlug (BD Pharmingen) was added during the final 6 hrs of culture. Intracellular cytokine staining of splenocytes was performed for IL-2. The phenotype of the cells was determined by staining for CD4+ and CD8+ positive cells. The plots are representative of at least two similar experiments with consistent results.
Fig. 4. CD4⁺ and CD8⁺ T-cells derived from mice immunized with DC-NS5+anti-CD40+LPS display a vigorous proliferative response to HCV-NS5. Proliferation was estimated from the decreased fluorescence intensity exhibited by splenocytes stained with CFSE and then cultured for five days in the presence of HCV NS5 (0.5 ug/ml). The phenotype of the proliferating cells was determined by staining for CD4⁺ and CD8⁺ positive cells. The plots are representative of two or more comparable experiments.

Fig. 5. Splenocytes derived from mice vaccinated with DC-Beads+NS5+anti-CD40+LPS exhibit elevated antigen-specific CTL activity. Groups of 4 mice were inoculated s.c. three times at a 2-week interval with beads, or DCs containing beads, coated with the factors indicated. Splenocytes obtained from mice 1 week after each inoculation were cultured for 3 days in the presence of 0.5 µg/ml recombinant NS5. Subsequently, the cells were co-cultured with ⁵¹Cr-labeled, NS5-expressing SP 2/0 myeloma target cells. Values are the mean ± SD of specific cytotoxicity calculated from quadruplicate wells in a single experiment representative of 2 similar experiments. CTL activity was assessed after each immunization at an effector to target cell ratio of 30:1. *P < 0.001 compared to the other groups.

Fig. 6. Vaccination with DCs containing beads coated with NS5, LPS and anti-CD40 induces NS5-specific immunity in vivo. Groups of animals were inoculated 3 times at 2-week intervals with beads+NS5+LPS+anti-CD40, DCs that contained beads+LPS+anti-CD40 or DCs that contained beads+NS5+LPS+anti-CD40. Vaccinated animals were administered subcutaneously 1 x 10⁶ myeloma cells stably expressing HCV NS5 two weeks after the last inoculation. Mice vaccinated with DC-beads+NS5+LPS+anti-CD40 showed significantly less NS5-specific tumor cell...
growth than the other two groups (*P < 0.01) (A). On day 15 of growth, the tumors were dissected, weighed and compared. Data are the means ± SEM weights of tumors obtained from 8 mice in each group (B). *P < 0.01 compared to the other two groups.
Hydrodynamic gene delivery of pUMVC3-hFLex (hFLt3L) twice (day 0, day 6) in the tail vain of Balb/c mice

Co-cultivation of magnetic beads with the total splenocyte population over a period of 3 hrs

Isolation of beads containing cells through passage over a column placed in a magnetic field

Sacrifice and isolation of total splenocyte population at day 11

Separation of free beads from beads containing cell with a Histodenz (Sigma) gradient

in vitro purification duration 6 hrs

in vivo treatment duration 11 days

Figure 1

in vivo treatment duration 11 days

Hydrodynamic gene delivery of pUMVC3-hFLex (hFLt3L) twice (day 0, day 6) in the tail vain of Balb/c mice
Figure 2 (A)
**Figure 2 (B)**

**IL-2**

- **beads+LPS+anti-CD40**
- **NS5-beads**
- **DC + LPS+CD40 beads**
- **DC + NS5-beads**
- **DC + LPS-CD40-NS5-beads**

1st Immunization

2nd Immunization

3rd Immunization

**IFN-γ**

- **beads+LPS+anti-CD40**
- **NS5-beads**
- **DC + LPS+CD40 beads**
- **DC + NS5-beads**
- **DC + LPS-CD40-NS5-beads**

1st Immunization

2nd Immunization

3rd Immunization

2 (C)

**IL-2**

- **Beads+LPS+anti-CD40**
- **Beads+NS5+LPS+anti-CD40**
- **DC-Beads+ LPS+anti-CD40**
- **DC-Beads+NS5**
- **DC-Beads+NS5+LPS+anti-CD40**

0.0 0.1 0.3 1.0 NS5 (µg/ml)

pg/ml

0 5 10 15 20 25

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Figure 3
Figure 4
Figure 5
Figure 6

The diagram on the left shows the change in tumor weight (in mg) over time (in days) for different groups of treatments: NS5+beads+LPS+anti-CD40, DCs-beads+LPS+anti-CD40, and DC-NS5+beads+LPS+anti-CD40.

The diagram on the right compares the tumor weight (in mg) between the NS5+beads+LPS+anti-CD40 group and the DCs-beads+LPS+anti-CD40 group, with statistical significance indicated by asterisks.