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Immunization against hepatitis C virus with a fusion protein containing the extra domain A from fibronectin and the hepatitis C virus NS3 protein☆

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***Background/Aims*: Vaccination strategies able to induce strong T-cell responses might contribute to eradicate hepatitis C virus (HCV) infection. We previously demonstrated that fusion of an antigen to the extra domain A from fibronectin (EDA) targets the antigen to TLR4-expressing dendritic cells (DC) and improves its immunogenicity. Here, we studied if fusion of EDA with the non-structural HCV protein NS3 might constitute an effective immunogen against HCV.**

***Methods*: Recombinant NS3 and the fusion protein EDA-NS3 were produced and purified from *E. coli*, and tested *in vitro* for their capacity to activate maturation of DC and to favour antigen presentation. HHD transgenic mice expressing the human HLA-A2 molecule were immunized with recombinant proteins in the absence or presence of poly(I:C) and anti-CD40 agonistic antibodies and responses elicited by vaccination were tested *in vitro*, and *in vivo*, by their capacity to downregulate intrahepatic expression of HCV-NS3 RNA.**

***Results*: EDA-NS3, but not NS3 alone, upregulated the expression of maturation markers, as well as Delta-like 1 and Delta-like 4 Notch ligands in DC and induced the production of IL-12. Mice immunized with EDA-NS3 had strong and long lasting NS3-specific CD4+ and CD8+ T-cell responses and, in combination with poly(I:C) and anti-CD40, downregulated intrahepatic expression of HCV-NS3 RNA.**

***Conclusions*: Recombinant EDA-NS3 may be considered for the development of vaccines against HCV infection.**

***Keywords*: Hepatitis C; EDA; Adjuvant; Dendritic cell; Vaccine**

1. Introduction

There is no effective vaccine against hepatitis C virus (HCV), a pathogen which is thought to infect 170 million people worldwide. HCV causes a high rate of chronic infection, which can lead to liver cirrhosis and hepatocellular carcinoma. The outcome of HCV infection appears to be modulated by a complex interplay of immunological and virological factors, and both antiviral humoral and cellular immune responses seem to be required to control the infection. Thus, rapid induction of neutralizing antibodies after viral

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Abbreviations: HCV, hepatitis C virus; EDA, extra domain A from fibronectin; DC, dendritic cell; Poly(I:C), polyinosinic-polycytidylic acid; BMDC, bone marrow derived dendritic cell; DLL1 and DLL4, Delta-like 1 and Delta-like 4 Notch ligands.

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infection may have an impact on viral clearance, and although HCV may continuously escape from this response, broadly neutralizing antibodies may have an impact on the protection against heterologous viral infection (reviewed in [1]). On the other hand, HCV chronic infection is associated with a low or absent T-cell response against viral antigens, and in contrast, those individuals who clear viral infection display potent and wide antiviral CD4 and CD8 T-cell responses (reviewed in [2]). In particular, immune responses against non-structural protein NS3 from HCV have been associated with viral clearance [3]. Thus, induction of a vigorous anti-HCV T-cell response directed against NS3 is considered an important feature of a candidate HCV vaccine.

Dendritic cells (DCs) are the professional antigen presenting cells (APCs) of the immune system. They orchestrate a repertoire of immune responses from tolerance to self-antigens to resistance to infectious pathogens depending on their maturation status (reviewed in [4]). Thus, it is generally accepted that efficient activation of T-cell immune responses are dependent on DC maturation triggered by a combination of stimuli derived from microbial products or inflammatory signals. For these reasons, several vaccination strategies incorporate ligands for pattern recognition receptors (PRRs) [5–8], agonistic antibodies against costimulatory molecules [9,10] or cytokines [11] able to trigger maturation of DC. We have previously reported [6] that fusion of an antigen with the extra domain A from fibronectin (EDA) leads to antigen targeting to TLR4-expressing DC, enhancing cross-presentation and immunogenicity. To test if EDA-NS3 might behave as an immunogen capable of eliciting robust anti-HCV responses, we prepared this fusion protein and tested its capacity to activate DC maturation *in vitro* and its immunogenicity *in vivo*. We also studied the effect of combining EDA-NS3 and other DC stimulators, specifically polyinosinic-polycytidylic acid (poly(I:C)), a TLR3 ligand [12] and anti-CD40, an antibody mimicking the effect of CD40L present on activated T-cells. Our results suggest that EDA-NS3 combined with these adjuvants may be considered for the development of a vaccine against HCV infection.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice, 6–8 weeks old, from Harlan (Barcelona, Spain) and HHD mice, transgenic for human HLA-A2.1 and beta-2 microglobulin molecules [13], kindly provided by Dr. F. Lemonnier (Institute Pasteur, Paris, France), were housed in appropriate animal care facilities during the experimental period and handled following the international guidelines required for experimentation with animals.

2.2. Cell lines

T2 cells were used as target cells in chromium release assays with CTL from HHD mice. THP1 cells (American Type Culture Collection, Manassas, VA) were used for *in vitro* assays of monocyte activation by EDA-containing fusion proteins. They were grown in complete medium (CM) (RPMI 1640 containing 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine and 50 μM 2-mercaptoethanol (Life Technologies, Paisley, UK).

2.3. Peptides

HLA-A2-restricted peptides p1073 (CVNGVCWTV) and p1038 (GLLGCIITSL) [14] from HCV NS3 protein were synthesized manually in a multiple peptide synthesizer using Fmoc-chemistry. Their purity was always above 90% as determined by HPLC.

2.4. Recombinant proteins

Plasmid pET20b-EDA, expressing the extra domain A from fibronectin, [6] was used for the construction of the expression plasmid pET20b-EDA-NS3, to express a fusion protein containing the first 195 amino acids from HCV-NS3 protein (corresponding to the serine protease domain of NS3 [15]) linked to the C-terminus of EDA and carrying six histidines at the carboxy-terminus. Plasmid pET45b-NS3 expressing the 195 first amino acids from HCV-NS3 protein linked to a six histidine tag at the N-terminus was constructed using conventional cloning techniques. EDA-NS3 was purified from inclusion bodies by affinity chromatography (Histrap, GE Healthcare, Uppsala, Sweden) followed by ion exchange chromatography (DEAE-Sepharose column, GE Healthcare). NS3 was purified also from inclusion bodies by a semi-preparative isoelectrofocussing followed by affinity chromatography. Resulting proteins were refolded in a sepharose G25 column using a urea gradient size-exclusion chromatography and purified from endotoxins by using Endotrap columns (Profos Ag, Regensburg, Germany) until endotoxin levels were below 0.1 EU/mg protein (tested by Quantitative Chromogenic Limulus Amebocyte Lysate assay (Cambrex, Walkersville, MD, USA)). Purified recombinant proteins were analyzed by SDS–PAGE and stained with Coomassie blue (Bio-Safe Coomassie reagent, Bio-Rad, Hercules, CA).

2.5. Plasmids

Plasmids pShuttle-HTLP-EDA-NS3 (pEDA-NS3) and pShuttle-HTLP-NS3 (pNS3) expressing secretable versions of EDA-NS3 and NS3 were constructed by PCR using plasmid pET20b-EDA-NS3 as a template and specific primers to introduce at the N-terminus of the protein a sequence coding for the human tyrosinase leader peptide (HTLP). Resulting PCR products were cloned in pShuttle plasmid (Clontech, BD Biosciences).

2.6. In vitro analysis of THP-1 monocyte cell line activation

THP-1 cells were plated at 10^6 cells/well and cultured with EDA-NS3 (2 μM), NS3 (2 μM), LPS (1 μg/mL) or culture medium for 15 h. In some cases EDA-NS3 was previously digested with proteinase-K using agarose-proteinase K beads according to manufacturer's instructions (Sigma, St. Louis). Human TNF-α released to the medium by THP-1 cells in response to the stimuli was quantified using a commercial ELISA assay (BD-Pharmingen).

2.7. In vitro analysis of DC stimulation

Bone marrow DC (BMDC) were generated from C57Bl6 mice femur marrow cell cultures as previously described [6]. Non-adherent DC were harvested at day 6 and cultured in the presence or absence