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Research paper

A novel assay for detection of hepatitis C virus-specific effector CD4$^+$ T cells via co-expression of CD25 and CD134

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ABSTRACT

Hepatitis C virus (HCV)-specific CD4$^+$ effector T cell responses are likely to play a key role in the immunopathogenesis of HCV infection by promoting viral clearance and maintaining control of viraemia. As the precursor frequency of HCV-specific CD4$^+$ T cells in peripheral blood is low, favoured assay systems such as intracellular cytokine (ICC) or tetramer staining have limited utility for *ex vivo* analyses. Accordingly, the traditional lymphocyte proliferation assay (LPA) remains the gold standard, despite detecting responses in only a minority of infected subjects. Recently, we reported development and validation of a novel whole blood CD4$^+$ effector T cell assay based on *ex vivo* antigen stimulation followed by co-expression of CD25 and CD134 on CD4$^+$ T cells. Here we report adaptation of this assay to assessment of HCV-specific responses in cryopreserved peripheral blood mononuclear cells using standardised antigens, including peptide pools, viral supernatants and recombinant viral proteins. The assay allowed detection of HCV-specific CD4 responses in donors with both resolved and chronic infection. Responses were highly correlated with those revealed by LPA. Application of this assay will further define the role of CD4$^+$ T cells in the immunopathogenesis of HCV infection.

1. Introduction

CD4$^+$ T cell responses are believed to be critical in determining the outcome of primary hepatitis C virus (HCV) infection (Aberle et al., 2006; Day et al., 2002; Diepolder et al., 1995, 1996; Folgori et al., 2006; Grakoui et al., 2003; Kaplan et al., 2007; Shata et al., 2002). In this context, poorly controlled early viraemia generally predicts ultimate viral persistence that may be explained, at least in part, by the failure of some individuals to generate potent cell mediated immune responses, including both CD4$^+$ and CD8$^+$ T cells (Cooper et al., 1999; Diepolder et al., 1995; Folgori et al., 2006; Gerlach et al., 1999, Missale et al., 1996; Shoukry et al., 2003; Thimme et al., 2001). The importance of CD4$^+$ T cell responses in particular has been highlighted in studies investigating HCV persistence in humans (Gerlach et al., 1999; Smyk-Pearson

Abbreviations: aa, amino acid;Ag, antigen;AP, autologous plasma;APC, allophycocyanin;ANOVA, analysis of variance;BSA, bovine serum albumin; CFSE, carboxyfluorescein diacetate succinimidyl ester;CM, complete media; CMV, cytomegalovirus;DMSO, dimethyl-sulfoxide;ELISpot, enzyme-linked immunospot;FACS, fluorescence-activated cell sorting;FITC, fluorescein-isothiocyanate; Flu, influenza virus;h, hours;HCV, hepatitis C virus;HCVcc, hepatitis C virus cell culture;HI-ABS, heat inactivated AB serum;HI-FCS, heat inactivated foetal calf serum;HITS, hepatitis C incidence and transmission study;HIV, human immunodeficiency virus;HSV, herpes simplex virus;ICC, intracellular cytokine;IFN-γ, interferon-gamma;IL-2Rα, interleukin-2 receptor alpha;JFH-1, Japanese fulminant hepatitis-1;LPA, lymphocyte proliferation assay;min, minutes;MTb, *Mycobacterium tuberculosis*;PBMC, peripheral blood mononuclear cells;PBS, phosphate buffered saline;PE, phycoerythrin;PEG, polyethylene glycol;PerCP, peridinin chlorophyll;PF, proliferating fraction;PFA, paraformaldehyde;PHA, phytohaemagglutinin;TT, tetanus toxoid;UNSW, University of New South Wales;UV, ultra-violet;v/v, volume/volume.

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et al., 2008) and also in chimpanzee models of infection (Grakoui et al., 2003; Shata et al., 2002). These studies report associations between strong, broadly reactive and sustained HCV-specific $CD4^+$ T cell responses and resolved infection. By contrast, failure to generate or sustain these responses leads to viral persistence suggesting that HCV antigen-specific $CD4^+$ T cell help is essential for development of anti-viral immunity.

The precursor frequency of HCV specific T cells in the blood of infected individuals is very low (Von Hahn et al., 2007; Rehermann et al., 1996; Rosen et al., 2002) making it difficult to detect these cells *ex vivo* using conventional assay systems, such as the lymphocyte proliferation assay (LPA) (Rehermann and Naumov, 2007). In addition, cytokine-based detection assays such as the interferon (IFN)-γ ELISpot assay detects only a limited subset of effector cells and does not allow phenotypic analysis of responding cells, whereas HCV-responses are generally below the limit of detection of intra-cellular cytokine (ICC) staining. In this study we have investigated the utility of a novel assay system developed recently and validated for several recall antigens (Zaunders et al., 2009), and now adapted to detect HCV specific $CD4^+$ T cells. This assay is based on *ex vivo* stimulation with antigens followed by flow cytometric detection of both CD25 (interleukin-2 receptor alpha, IL-2Rα) and CD134 (OX40) to identify antigen-specific $CD4^+$ T cells. After T cell stimulation by antigen there is an up-regulation of both CD25 and CD134 over 24–48 h, with the optimal readout determined to be 44 h (Zaunders et al., 2009). The assay (termed here the OX40 assay) readily detects responses to common recall antigens such as cytomegalovirus (CMV), tetanus toxoid (TT), influenza virus (Flu), herpes simplex virus (HSV) and *Mycobacterium tuberculosis* (MTB). It is highly sensitive, evidenced by the ability to detect human immunodeficiency virus (HIV) $CD4^+$ T cell responses in late stage HIV infection at significantly higher rates compared with ICC or LPA (Zaunders et al., 2009).

We report adaptation of this assay system for analysis of peripheral blood mononuclear cells (PBMCs), including cryopreserved PBMCs, in HCV infection. HCV-specific responses were highly correlated with carboxyfluorescein (CFSE)-based LPA, and the assay successfully detected HCV specific $CD4^+$ T cell responses in HCV infected donors with both resolved and chronic infection using a range of HCV antigens, including pools of overlapping HCV peptides, a viral supernatant from the HCV cell culture (HCVcc) system, JFH-1 (UV irradiated), and recombinant viral proteins.

2. Materials and methods

2.1. Subjects

Peripheral blood samples were obtained from uninfected healthy laboratory staff volunteers, and subjects enrolled in the Hepatitis C Incidence and Transmission Study (HITS), a prospective cohort study of Australian prison inmates (Dolan et al., 2010; Teutsch et al., 2010). All subjects provided written informed consent. The protocol was approved by the institutional review boards of Justice Health and Department of Corrective Services.

2.2. PBMC isolation, cryopreservation, culture medium, antibodies and reagents

Peripheral blood mononuclear cells were isolated from sodium heparin anti-coagulated whole blood using Ficoll–Hypaque density gradient centrifugation. The cells were washed twice in sterile phosphate-buffered saline (PBS) and resuspended at 5×10^6/mL in complete medium (CM) comprised of RPMI-1640 (Gibco BRL, USA) supplemented with 10% (v/v) heat inactivated human AB serum (HI-ABS; Sigma Aldrich, Australia), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Remaining cells were cryopreserved in 10% dimethyl-sulfoxide (DMSO)/90% autologous plasma (v/v). The following anti-human monoclonal antibodies from BD Biosciences (Australia) were used in flow cytometry: CD3-fluorescein isothiocyanate (FITC); CD134-phycoerythrin (PE); CD4-peridinin chlorophyll (PerCP) and CD25-allophycocyanin (APC). PE and APC conjugated mouse IgG1a antibodies were used in combination with CD3-FITC and CD4-PerCP as isotype controls (BD Biosciences, Australia). The following antigens/mitogens were used for *in vitro* stimulation: cytomegalovirus (CMV, 1/250 dilution, DKSH, Australia), tetanus toxoid (TT, 1 μg/mL, DKSH, Australia), influenza A (Flu, 1/250, DKSH, Australia), Phytohaemagglutinin (PHA, 5 μg/mL, Sigma Aldrich, Australia), HCV peptides (1 μg/mL; see Section 2.6.1 for details, BEI Resources), UV irradiated HCV JFH-1 (1/250; see Section 2.6.2), and HCV NS5B recombinant protein (10 μg/mL; see Section 2.6.3).

2.3. Whole blood culture compared to PBMC culture

The whole blood assay has previously been described in detail (Zaunders et al., 2009). Briefly, sodium heparin-anticoagulated whole blood (0.25 mL) was mixed with (0.25 mL) complete media containing CMV, Flu, or PHA. Cell cultures were incubated at 37 °C for 44 h in a humidified atmosphere of 5% CO_2 in sterile 24-well tissue culture plates.

Peripheral blood mononuclear cells were isolated as described in Section 2.2 and cultured at 5×10^5 cells/well (200 μL/well) in sterile 96-well tissue culture plates using the same culture conditions and antigens as for whole blood.

2.4. Fresh PBMCs compared to cryopreserved PBMCs

Freshly isolated PBMCs were set up in assays with the recall antigens CMV and Flu as well as negative (unstimulated media alone) and positive (PHA) control stimuli. Peripheral blood mononuclear cells from the same sample were cryopreserved as described in Section 2.2 and later set up in culture to compare with fresh PBMCs. Healthy control donor PBMCs were used for this study ($n=7$).

2.5. Culture conditions

Varied culture conditions were used to assess background levels of stimulation in healthy control donors ($n=5$). In particular, autologous plasma (AP), heat inactivated human AB serum (HI-ABS) and heat inactivated foetal calf serum (HI-FCS) were compared in culture using both fresh and frozen PBMCs.