Research Article

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Viral Hepatitis

A heterogeneous hierarchy of co-regulatory receptors regulates exhaustion of HCV-specific CD8 T cells in patients with chronic hepatitis C

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Background & Aims: The functionality of virus-specific T cells is regulated by a sophisticated network of an expanding repertoire of co-regulatory receptors, which could be harnessed for immunotherapeutic applications. However, targeting particular pathways during persistent virus infections has resulted in variable outcomes. The extent to which T cell exhaustion can be reversed, by targeting multiple co-regulatory pathways, still remains not fully investigated.

Methods: We analysed the phenotype and *in vitro* functionality of HCV-specific CD8$^+$ T cells expressing PD-1, CTLA-4, TIM-3 or 2B4 either alone or in various combinations and compared expression levels to those of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) specific T cells in peripheral blood mononuclear cells (PBMCs) from the same cohort of patients with chronic hepatitis C (CHC) infection.

Results: Blockade and/or crosslinking of distinct co-regulatory pathways in exhausted HCV-specific CD8$^+$ T cells resulted in rather diverse and individualized T cell responses, irrespective of the type and number of receptors targeted. Overall, *in vitro* manipulations of these pathways yielded three response possibilities: (i) total non-response (ii) good single blockade response and (iii) good dual/multiple blockade response, with each comprising approximately one-third of the patients tested. The diversity of the *in vitro* responsiveness of HCV-specific T cells was reflected by an enormous *ex vivo* phenotypic heterogeneity. Despite this broad heterogeneity, HCV-specific CD8$^+$ T cells differed from EBV- and CMV-specific T cells in particular by TIM-3 expression, which also correlated with liver disease activity and viral load.

Conclusions: HCV-specific CD8$^+$ T cell functionality, upon co-regulatory receptor manipulations, was characterized by an individual pattern of responses in patients with CHC, suggesting that treatment approaches, targeting these receptors, should consider inter-individual differences and be personalized.

Keywords: Chronic hepatitis C; Co-regulatory receptors; T cell exhaustion; HCV; CMV; EBV; PD-1; CTLA-4; TIM-3; 2B4.

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Abbreviations: HCV, hepatitis C virus; PD-1, programmed death protein 1; CTLA-4, cytotoxic T lymphocyte–associated antigen 4; TIM-3, T cell immunoglobulin and mucin domain-containing protein 3; CMV, cytomegalovirus; EBV, Epstein-Barr virus; PBMC, peripheral blood mononuclear cell; CHC, chronic hepatitis C; HLA, human leukocyte antigen; IFNγ, interferon-gamma; TNF, tumor necrosis factor; IHL, intra-hepatic lymphocytes.

Introduction

Functional exhaustion of virus-specific T cells is a key contributing factor to the establishment of viral persistence. In patients with chronic hepatitis C (CHC) in particular, cytotoxic T lymphocyte responses that are crucial for the elimination of virus-infected hepatocytes are functionally impaired in both the liver and peripheral blood [1,2]. Reversing T cell exhaustion could provide a promising therapeutic approach for restoring an effective natural immunological control over persistent viral infections. To achieve this, however, there is the need to fully understand the factors contributing to T cell exhaustion.

Recent results in patients with CHC show an upregulation of the co-regulatory molecules programmed death protein 1 (PD-1), cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) and natural killer cell receptor 2B4 (CD244), which have all been associated with the exhaustion of HCV-specific CD8$^+$ T cells [2–5]. Importantly, *in vitro* and *in vivo* antibody-mediated blockade and/or stimulation of any of these receptor signalling in unison restored antigen-driven proliferation and other effector functions of HCV-specific CD8$^+$ T cells [6]. In spite of these promising observations, several studies in both the lymphocytic choriomeningitis virus (LCMV) mouse model of persistent virus

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infection and in patients with CHC have suggested that interruption of a single co-regulatory pathway alone may be insufficient for immune reconstitution [7,8]. In line with these findings, the first clinical trials that have capitalised on anti-PD-1 or anti-CTLA-4 antibodies as immunotherapeutic interventions against persistent HCV infection in both humans and chimpanzees revealed an individualized response pattern to co-regulatory receptor blockades [9–11]. This further indicated that a more hierarchical co-regulation of multiple co-regulatory pathways defines exhausted HCV-specific $CD8^+$ T cell function. Although a simultaneous blockade of dual co-regulatory pathways has been suggested to improve HCV-specific $CD8^+$ T cell responses, we [4,12] and others [13,14] have demonstrated various co-regulatory receptor combinations whose modulations rather resulted in more broadly variable outcomes of both synergy and antagony in different persistent virus infection settings. Therefore, in aid of designing more effective therapeutic strategies, the question, which combinations of co-regulatory receptor blockades can synergize the most optimal benefit, becomes all the more crucial. Equally important is the question whether targeting multiple co-regulatory receptors is ultimately required for the immune restoration of exhausted virus-specific $CD8^+$ T cells as a whole [6,15,16].

Here, we studied whether different co-regulatory molecules have overlapping functions or whether they contribute independently to T cell dysfunction during persistent HCV infection. Additionally, we addressed the central question whether T cell exhaustion can be fully reversed by blocking multiple co-regulatory pathways. To do this, we assessed the *in vitro* functionality of HCV-specific $CD8^+$ T cells, compared to CMV- and EBV-specific T cells, after targeting PD-L1, CTLA-4, TIM-3, and 2B4 in various combinations in patients with CHC. Overall, the results from our cohort point to a more heterogeneous response to co-regulatory receptor blockade by HCV-specific $CD8^+$ T cells with no clearly dominant pathway(s).

Materials and methods

Study subjects and samples

Heparinized peripheral whole blood samples were collected from 128 patients with persistent hepatitis C virus mono-infection and compensated liver disease. All patients were HBV and HIV seronegative. A total of 51 HLA-A2+ patients were selected for further experiments, including 10 with liver cirrhosis. Only patients with genotype 1 infection were selected for subsequent *in vitro* functional studies (n = 28). Accordingly, all HCV peptides and multimers were based on the genotype 1 sequences. All patients were not under any antiviral therapy at the time of sample collection. Patient characteristics are summarized in Table 1.

All patients were recruited from cases presenting to the hepatitis outpatient clinic of the Hannover Medical School (Germany), after giving their written informed consent. Sample collection procedures and experiments were performed under protocols reviewed and approved by the local ethics committee.

For a detailed description of the Materials and methods and the statistical analyses employed see the Supplementary Materials and methods section.

Table 1. HLA-A2 positive patients with chronic hepatitis C (study participants).

Parameter	Chronic HCV patient cohort
Number of HLA-A2 positive patients	51
Male sex - no. (%)	34 (67)
Age - yr	
Median	51
Range	22-80
HCV RNA - (IU/ml)	
Median	$1.5x10^6$
Range	$4.4x10^4$-$9.9x10^6$
≥$8x10^5$ IU/ml - no./total no. (%)	28/46 (61)
Unknown	5/51 (10)
Alanine aminotransferase - (U/L)	
Median	78.5
Range	10-270
*Above median - no./total no. (%)	25/48 (52)
Unknown	3/51 (6)
Aspartate aminotransferase - (U/L)	
Median	55
Range	22-279
*Above median - no./total no. (%)	26/49 (53)
Unknown	2/51 (4)
HCV genotype - no. (%)	
1	34 (67)
2 or 3	12 (23)
4	5 (10)
Liver cirrhosis - no./total no. (%)	
Yes	10/48 (21)
Unknown	3/51 (6)
Compensated liver disease - no./total no. (%)	
Yes	47/49 (96)
Unclear	2/51 (4)

Results

HCV-specific $CD8^+$ T cells are detectable in the majority of patients with chronic hepatitis C

We first screened a total of 128 patients with CHC and selected only HLA-$A2^+$ individuals for further studies; n = 54 (42%), (Supplementary Fig. 1). To measure virus-specific $CD8^+$ T cells, PBMCs from HLA-$A2^+$ patients were stained with 8 different HCV-multimers, one CMV- and one EBV-specific multimer *ex vivo*. Out of 54 HLA-$A2^+$ patients, 3 tested negative for all three virus-specific multimer stainings. Notwithstanding, 41 patients showed detectable *ex vivo* multimer+ $CD8^+$ T cells to at least one HCV-specific multimer, resulting in a total of 92 HCV-positive stainings. Similarly, 27 and 32 patients were *ex vivo* positive for CMV- and EBV-specific multimers, respectively. Overall, most patients, showing detectable HCV-multimer+ $CD8^+$ T cells *ex vivo*, were also positive for CMV and/or EBV (i.e. HCV+CMV, n = 2; HCV+EBV, n = 6; CMV+EBV, n = 3, and HCV+CMV+EBV, n = 19). Despite these considerable overlaps, some patients were positive exclusively for only one of these multimers (HCV = 14, CMV = 4, and EBV = 3). Out of the 41 patients that were HCV-multimer positive, we selected 28 genotype 1-infected (representing 32 HCV-positive stainings) for subsequent *in vitro* co-regulatory receptor blocking and/or crosslinking experiments. As controls, 13 CMV- and 14 EBV-specific, randomly selected cell lines were subjected to similar *in vitro* experiments (Supplementary Fig. 1).

Functionally exhausted HCV-specific $CD8^+$ T cells are not universally revived by blockade of single or dual co-regulatory molecules

In vitro stimulation of PBMCs from our CHC patient-cohort with virus-peptides confirmed the previous findings [1,2] that HCV-,