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Research paper

Effect of peptide pools on effector functions of antigen-specific CD8+ T cells

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ABSTRACT

Peptide pools are routinely used to study antigen specific T cell responses, both in epitope discovery as well as immune monitoring. However, optimal assay conditions such as concentration of peptides or the best possible number of peptides per pool have not been defined. Thus, we examined whether different peptide concentrations or varying number of peptides per pool influence effector functions of antigen-specific human T-cells. PBMC isolated from HLA-A2-positive individuals with known responses to frequently recognised dominant CD8+ T cell epitopes derived from four different viruses (influenza virus, CMV, EBV, or HCV) were studied. PBMC were cultured with one of these HLA-A2 restricted peptides and varying concentrations of overlapping peptide pools derived from unrelated viruses specific for the hepatitis D and E viruses, the subjects have not been exposed to. Importantly, unrelated peptide pools inhibited the proliferation of IV-M1$_{58}$, CMVpp65$_{495–503}$, EBV-BMLF$_{1259–267}$ and HCV NS3$_{1073}$-specific CD8 T-cells in a dose dependent manner. Similarly, an increase in the number of peptides per pool also impaired antigen specific CD8+ T cell proliferation. In contrast, secretion of cytokines such as IL-2, IL-10, IFN-gamma, TNF-alpha or IP-10 as well as cytotoxicity was not affected by these unrelated peptide pools. The inhibition of proliferation could be restored by blocking PD-1/PDL-1 interaction and was not dependent on DMSO when DMSO concentration was ≤0.5%.

Thus, peptide-specific CD8 T-cell proliferation but not cytokine production may be largely underestimated when using a peptide pool which warrants caution in immunomonitoring during clinical trials and in epitope discovery studies.

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1. Introduction

Monitoring of human T cell responses during immunotherapies as well as epitope discovery studies should be robust and unbiased. In the best possible setting, (i) responses against all class I and class II peptides are detected, (ii) the assay is sensitive enough to pick apparently weak responses while maintaining a high specificity, (iii) different effector functions such as production of various cytokines, cytotoxicity and proliferation are investigated, (iv) T cell responses reflect the situation *in vivo* and are not biased by assay conditions, (v) the number of cells required is limited enabling frequent testing over time. This scenario, however, has not been achieved yet and thus compromises have to be made depending on the primary question to be answered in the respective experimental setting.

Overlapping peptides spanning the whole genome of pathogens and tumor antigens have been used for investigating

Abbreviations: CBA, cytokine bead array; CFSE, 5, 6-carboxyfluorescein diacetate succinimidyl ester; CMV, cytomegalo virus; EBV, Epstein–Barr virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HDV, hepatitis D virus; HLA, human leukocyte antigen; ICS, intracellular cytokine staining; IV, Influenza virus; PBMC, peripheral blood mononuclear cells; PD-1, programmed death1.

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T cell responses in epitope discovery (Walton et al., 2006; Wertheimer et al., 2003), vaccine development (Rothman et al., 1998; Smyk-Pearson et al., 2006; Reap et al., 2007) and cancer research (Durso et al., 2007; McCarthy et al., 2006). This approach is more sensitive than simply using viral or tumor specific proteins (Maecker et al., 2001) and also appropriate for a comprehensive analysis of CD4 and CD8 subset responses without any bias towards HLA haplotype (Maecker et al., 2001; Wertheimer et al., 2003). However, special care is required while using large panels of overlapping peptide pools as the concentration, number of peptides per pool, length of the peptides and range of overlap may greatly influence the outcome of T cell responses. There is no systematic published study addressing how variations in the number of peptides or overall peptide concentration can influence effector functions of a particular epitope specific T cell. These studies are highly desirable in view of the fact that the number of peptides per pool used by investigators has been varying widely ranging from 10 (Aggarwal et al., 2007) to 50 (Pillai et al., 2007) or even 123 peptides per pool (Jones et al., 2006). Also the final concentration of each peptide in a pool varied from 1 µg/ml (Lauer et al., 2005) to 200 µg/ml (Addo et al., 2003), so are the length of peptides and the range of overlap. In our own experience when studying virus specific T-cell responses during clearance of chronic HCV infection in the presence of acute Delta hepatitis (Deterding et al., 2007), we observed that the percentage of proliferating virus-specific T cells may vary with the number of peptides used per pool. Therefore, it is highly required to have standard optimal conditions in investigating T cell responses.

Assays to study cytokine secretion directly *ex vivo* have been developed during the last 25 years. However, both ELISpot assays and flow-cytometry-based intracellular cytokine stainings may be problematic if the frequency of antigen-specific T cells is low, when cryo-preserved cells are used and when the number of PBMC available is limited. Thus, many other standard methods to study antigen specific T-cell responses are based on in-vitro expansion of T cells. The functionality of expanded antigen specific T cells can be assessed by different methods such as direct enumeration of antigen-specific cells using peptide/MHC complexes or labelling cells with fluorescent dyes, intracellular cytokine staining, or by assays determining cytotoxicity (Rehermann and Naoumov, 2007). Proliferation in response to antigenic stimulation has been used as readout in immunology for several decades and proven to be of high sensitivity, reproducibility and easy performance (Agrawal et al., 1998; Lauer et al., 2002; Malhotra et al., 2007). The classical radioactive 3[H]-thymidine incorporation assay for assessing T cell proliferation is nowadays frequently being replaced in several laboratories by the flow-cytometry based CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) assay (Kumar et al., 2006; Hilchey and Bernstein, 2007; Lee et al., 2007; Pillai et al., 2007;). This assay has been documented to be more sensitive, especially in cases of persistent viral infections which makes it possible to study proliferation of T cells at single cell level, to differentiate between CD4 and CD8 T cell responses and to monitor the actual number of divisions that a given cell population has undergone (Lyons, 2000; Lee et al., 2007; Ganusov et al., 2007).

In the present study, we have investigated the potential factors, such as DMSO concentration, overall peptide concentration per pool or number of peptides per pool that may influence *in vitro* cytokine production, binding of peptide/MHC complexes and proliferation of peptide-specific CD8+ T cells. In order to investigate this we used PBMCs from individuals with known definite responses to dominant MHC-class I-restricted epitopes and co-cultured these with completely unrelated peptide pools. We decided to use pools of peptides derived from the hepatitis E and D viruses since those viruses show an extremely low prevalence in our country. Thus study subjects were not exposed to these viruses previously and the presence of HEV or HDV specific memory T cells is very unlikely. Our results clearly indicate that the peptide concentration greatly influences T cell proliferation. In contrast, the production of pro-inflammatory cytokines such as interferon gamma and TNF-alpha is not affected. Moreover, blocking PD-1/PDL-1 interaction using anti-PDL-1 could restore the inhibition of antigen specific CD8 T cells proliferation.

2. Methods

2.1. Study subjects

Nineteen healthy, HLA-A2 positive subjects with no previous exposure to HEV and HDV viruses were included and screened by pentamer/ tetramer staining for the presence of Influenza virus (IV) matrix peptide-$M1_{58}$ (GILGFVFTL), Cytomegalovirus (CMV) protein $pp65_{495-503}$ (NLVPMVATV), Epstein–Barr virus (EBV)-$BMLF1_{259-267}$ (GLCTLVAML) and Hepatitis C virus (HCV) $NS3_{1073}$ (CINGVCWTV) specific T cells. Seven individuals had Influenza virus specific (0.67±0.3% of CD8+ T cells), five individuals had Cytomegalovirus specific (0.46±0.27% of CD8+ T cells), five individuals had Epstein–Barr virus specific (0.36± 0.16% of CD8+ T cells) and two individuals had Hepatitis C virus specific (0.066±0.063% of CD8+ T cells) T cells. The two individuals with HCV specific responses were healthy volunteers who expanded HCV-$NS3_{1073}$-specific CD8+ T cells after 1 week of in vitro stimulation. One of the PD-1/PD-L1 blocking experiments was performed with PBMC from a patient with acute hepatitis C showing strong HCV-NS3-1073-specific CD8+ T cell responses already directly ex vivo (3.4% of tetramer positive cells).

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from blood using standard Ficoll–Hypaque density gradient centrifugation (BIOCHROM, Berlin,

Fig. 1. Effect of different DMSO concentrations on T cell proliferation. Effect of DMSO concentration on spontaneous T cell proliferation was tested by analysing T cell proliferation in the presence of different DMSO concentrations using the CFSE method. (a). At lower concentrations (less than 1%) DMSO had no effect on total T cell proliferation. (b). Antigen specific CD8 T cell proliferation in the presence of different concentrations of DMSO was also tested by stimulating IV-$M1_{58}$, CMV $pp65_{495-503}$ or EBV-$BMLF1_{259-267}$ reactive cells with IV-$M1_{58}$, CMV $pp65_{495-503}$ or EBV-$BMLF1_{259-267}$ peptide alone or in combination with different concentrations of DMSO after CFSE staining. Lower concentrations of DMSO (up to 0.5%) have no effect on proliferating antigen specific CD8 T cells. Six out of eight subjects had no effect on proliferating CD8 T cells up to a DMSO concentration of 0.5%. (c). Representative figure showing proliferation of antigen specific CD8 T cells was not affected by DMSO concentration up to 0.5%. (d). Staining using multimeric peptide-MHC complexes revealed that binding of pentamer could not be altered using DMSO concentration up to 0.5%.

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