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Short communication

Real time detection of peptide–MHC dissociation reveals that improvement of primary MHC-binding residues can have a minimal, or no, effect on stability

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ABSTRACT

The majority of known major histocompatibility complex class I (MHCI)-associated tumor-derived peptide antigens do not contain an optimal motif for MHCI binding. As a result, anchor residue-modified 'heteroclitic' peptides have been widely used in therapeutic cancer vaccination trials in order to enhance immune responsiveness. In general, the improved stability of these heteroclitic complexes has been inferred from their improved immunogenicity but has not been formally assessed. Here, we investigated the binding of 4 HLA A*0201-restricted tumor-derived peptides and their commonly used heteroclitic variants. We utilized a cell surface binding assay and a novel robust method for testing the durability of soluble recombinant pMHCI in real time by surface plasmon resonance. Surprisingly, we show that heteroclitic peptides designed with optimal MHC binding motifs do not always form pMHCs that are substantially more stable than their wildtype progenitors. These findings, combined with our recent discovery that TCRs can distinguish between wildtype peptides and those altered at a primary buried MHC anchor residue, suggest that altered TCR binding may account for a large part of the increased immune response that can be generated by anchor residue-modified ligands. Our results further highlight the fact that heteroclitic peptide-based immune interventions require careful evaluation to ensure that wildtype antigen specificity is maintained *in vivo*.

1. Introduction

The T-cell receptor (TCR) governs T-cell mediated immunity through the recognition of short peptide fragments bound to major histocompatibility complexes (pMHCs) that are expressed on the surface of almost all nucleated cell types. Formation of stable pMHC class I (pMHCI) that can be transported to the cell surface for examination by the TCR depends on the binding affinity between the peptide and MHC. Peptides bind to MHC via interactions between individual residues and binding pockets in a groove on the MHC surface (Bjorkman et al., 1987; Guo et al., 1993). The most favorable residues at primary MHC anchor positions for the most common caucasian human leukocyte antigen (HLA), HLA A*0201, have been determined by elution and sequencing of self-peptides bound to HLA A*0201 at the cell surface (Falk et al., 1991). The current pool of HLA A*0201-restricted tumor epitopes that have been identified often contain sub-optimal primary HLA A*0201 anchor residues and are not thought to form optimally stable pMHCIs. As a result, a number of tumor specific 'heteroclitic' peptides, with modified optimal anchor residues, have been developed that theoretically improve the stability of tumor epitopes (Chen et al., 2000, 2005; Keogh et al., 2001; Parker et al., 1994; Parkhurst et al., 1996; Reche et al., 2004; Rosenberg et al., 2004; Terasawa et al., 2002). These peptides have been designed by introducing 'optimal' MHC anchor-residues at peptide position 2 and the peptide C-terminus based on: (i) peptide–MHC binding algorithms (Keogh et al., 2001) and (ii) intelligent design (Chen et al., 2000; Parkhurst et al., 1996). However, in most cases, the improved stability of these modified pMHCIs has been indirectly inferred due to the heightened T-cell immunogenicity of these molecules. Thus, the improved peptide–MHC affinity of these heteroclitic complexes has not been directly assessed. Here we have developed a surface plasmon resonance (SPR) pMHC stability assay that detects changes in mass at the surface of a gold plated sensor chip. This technology enables the determination of pMHCI half-life by detecting protein density at the sensor chip surface in real time. We have used this assay and a more commonly used cell surface binding assay to compare the stability of four MHCIs in complex with naturally expressed tumor epitopes in parallel with their anchor residue-modified het-

Abbreviations: pMHC, peptide–major histocompatibility complex; MFI, mean fluorescence intensity; FACS, fluorescent activated cell sorting; SPR, surface plasmon resonance; TCR, T-cell receptor.

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eroclitic variants. We show that: (i) pMHCI stability data from our biophysical assay are consistent with data independently generated from a cell-based assay that utilizes transport associated with antigen processing (TAP) deficient T2 cells (Henderson et al., 1992), (ii) altering a peptide in an attempt to improve MHC binding does not always correlate with an increase in stability over the wild-type progenitors, and (iii) commonly used peptide–MHC binding algorithms cannot always predict pMHC stability based on anchor residue-modifications.

2. Materials and methods

2.1. Generation of expression plasmids

The HLA A*0201 α chain, tagged with a biotinylation sequence, and β2m were inserted into separate pGMT7 expression plasmids under the control of the T7 promoter as previously reported (Cole et al., 2009) and the sequences were confirmed by automated DNA sequencing (Lark Technologies, Essex, UK).

2.2. Peptides

Lyophilized peptides were purchased from ProImmune (Oxford, UK). The peptides used in this study comprised: EBNA-$1_{407–415}$ (HPVGEADYF), influenza $M1_{58–66}$ (GILGFVFTL), NY-ESO-$1_{157–165}$ (SLLMWITQC, SLLMWITQ**L**) (Chen et al., 2000), $gp100_{280–288}$ (YLEPGPVA, YLEPGPV**V**) (Parkhurst et al., 1996), HER-1/$neu_{369–377}$ (KIFGSLAFL, K**L**FGSLAF**V**) (Keogh et al., 2001), $PSA_{178–187}$ (VISNDVCAQV, V**L**SNDVCAQV) (Terasawa et al., 2002).

2.3. Cell line

The mutant LCL × T-lymphoblastoid hybrid cell line, 174 × CEM.T2 (referred to as T2 cells) is a mutant antigen-presentation cell line (Henderson et al., 1992). These cells express stable human leukocyte antigen (HLA) class I molecules on their surface in the presence of exogenous peptides. Cells were maintained in Gibco RPMI 1640 (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% heat inactivated foetal calf serum (FCS) (Invitrogen), 2 mM L-glutamine (Invitrogen), penicillin 50 Units/ml (Invitrogen) and streptomycin 50 μg/ml (Invitrogen).

2.4. T2-binding assay

The T2-binding assay was performed as previously described (Bell et al., 2009). Briefly, T2 cells were washed in AIM-V media (Invitrogen), and concentrated to 10^6 cells/ml. The peptides were prepared in AIM-V media and serially diluted providing concentrations of 200 μM, 100 μM, 20 μM and 2 μM. The cells were mixed 1:1 with each peptide dilution to give a final volume of 200 μL and final peptide concentrations of 100 μM, 50 μM, 10 μM and 1 μM. A HLA A*0201 binding peptide, GILGFVFTL, and a non-HLA A*0201-restricted peptide, HPVGEADYF (HLA-B*3501), were included as positive and negative controls respectively. The assay was subjected to 10 min incubation at 37 °C 5% CO_2 before incubation at room temperature overnight. Cells were then incubated for 2 h at 37 °C and stained with mouse anti-human HLA A*0201:RPE (Serotec, Oxford, UK). The cells were washed twice with PBS and analyzed using a BD FACS Canto II (BD Biosciences Immunocytometry Systems, San Jose, CA, USA), collecting 30,000 events in gate P1. Average MFI of the HLA A*0201:RPE antibody staining was used to measure the strength of binding in the T2 cell peptide–MHCI based assay. The MFI reading from the FACS Diva software was analyzed in GraphPadPrism using a log (agonist) vs. response non-linear curve to generate the logEC50 results.

Table 1
Stability of wild-type, versus anchor residue-modified, peptide-MHCIs.

Peptide	[1]LogEC50 (M)	[2]K_{off} (s^{-1})	[3]Half-life (h)
KIFGSLAFL	−3.885	9.5×10^6	20.1
K**L**FGSLAF**V**	−3.389	1.5×10^5	12.8
SLLMWITQC	−3.532	2.2×10^5	8.9
SLLMWITQ**L**	−4.365	1.9×10^5	10.1
VISNDVCAQV	−4.301	1.1×10^5	16.9
V**L**SNDVCAQV	−4.622	1.07×10^5	17.8
YLEPGPVA	−3.587	3.3×10^5	5.8
YLEPGPV**V**	−4.588	2.3×10^5	8.5

[1]LogEC50 (M) for the T2-binding assays were generated using multiple concentrations (100 μM, 50 μM, 10 μM and 1 μM) for each peptide to calculate the half maximal response for each peptide–MHC interaction. The SPR biophysical stability data were analyzed by using Langmuir dissociation analysis[2] which was then converted to half-life in hours[3].
Heteroclitic modifications are shown in bold and underlined.

2.5. pMHCI refolding and biotinylation

pMHCI was refolded and biotinylated as previously reported (Cole et al., 2007).

2.6. Real time SPR pMHC stability assay

The binding analysis was performed using a BIAcore 3000™ (GE Healthcare, UK) equipped with a CM5 sensor chip as previously reported (Cole et al., 2008). For SPR-based pMHCI stability assays, ~500 RUs of biotinylated pMHCI were immobilized to streptavidin, which was chemically linked to the chip surface. A flow rate of 10 μl/min was established for 15,000 s. The biophysical results were analyzed by using Langmuir dissociation analysis which was then converted to half-life in seconds. The biophysical binding data from the BIAcore 3000™ was analyzed in the BIAevaluation software™ (GE Healthcare).

3. Results

3.1. Analysis of pMHC stability using TAP deficient T2 cells

The binding of 4 different HLA A*0201-restricted tumor-associated peptides was compared to their commonly used heteroclitic variants using a cell surface pMHCI stabilization assay. This assay made use of T2 cells that are deficient in the transporter associated with antigen processing (TAP) and that therefore lack the ability to transport peptide fragments into the endoplasmic reticulum to form stable peptide–HLA A*0201 complexes (Henderson et al., 1992). As a result, peptide–HLA A*0201 molecules expressed on the cell surface of T2 cells are generally of low stability. Incubation of T2 cells with exogenous peptide that binds to HLA A*0201 stabilizes the molecule so that surface pMHC expression levels can be quantitatively assessed (Bell et al., 2009). No peptide and the HLA B*3501-restricted EBNA-$1_{407–415}$ (HPVGEADYF) peptide were used as negative controls for binding, and the HLA A*0201 restricted influenza $M1_{58–66}$ (GILGFVFTL) peptide was used as a positive control for binding. Flow cytometric based analysis, using anti-human HLA A*0201:RPE antibody, revealed that the wildtype antigen, HLA A*0201-KIFGSLAFL, (logEC50 of −3.885 M), was more stable than the heteroclitic variant HLA A*0201-K**L**FGSLAF**V** (logEC50 of −3.389 M) that was designed to improve MHC binding (Fig. 1, Table 1). However, the wildtype pMHCIs, HLA A*0201-SLLMWITQC, HLA A*0201-YLEPGPVA and HLA A*0201-VISNDVCAQV were less stable than their heteroclitic counterparts (Fig. 1, Table 1), albeit by a relatively small degree. These data show that: (i) some heteroclitic peptides do not form more stable pMHCIs than their wildtype progenitors; and (ii) the difference in the stability between some heteroclitic vs. natural pMHCIs, particularly VISNDVCAQV and