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Unpredicted phenotypes of two mutants of the TcR DMF5

Fitsum G. Tadesse [a], Nadia Mensali [a,c], Lars-Egil Fallang [a,1], Even Walseng [a,2], Weiwen Yang [a,c], Johanna Olweus [a,c], Sébastien Wälchli [a,b,*]

[a] Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway
[b] Department of Cellular Therapy, Oslo University Hospital Radiumhospitalet, Oslo, Norway
[c] K.G. Jebsen Center for Cancer Immunotherapy and K.G. Jebsen Center for Inflammation Research, Institute for Clinical Medicine, University of Oslo, Norway

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ABSTRACT

When a T-cell Receptor (TcR) interacts with its cognate peptide-MHC (pMHC), it triggers activation of a signaling cascade that results in the elicitation of a T cell effector function. Different models have been proposed to understand which parameters are needed to obtain an optimal activation of the signaling. It was speculated that improving the binding of a TcR could bring a stronger pMHC recognition, hence a stronger stimulation of the T cell. However, it was recently shown that an increase in affinity does not seem to be sufficient to guarantee improved functionality. A combination of factors is necessary to place the modified TcR in an optimal functional window. We here compared the binding parameters of two mutants of the melanoma antigen peptide MART-1_{27-35} specific TcR DMF5. The first mutant was previously isolated by others in a screen for improved TcR. It was reported to have an increased CD8-independent activity. We confirmed these data and showed that the enhancement was neither due to change in half life ($t_{1/2}$) nor Kd of the pMHC-TcR complex. The second mutant was designed based on a previous report claiming that a particular polymorphic residue in the TRAV12-2 chain was stabilizing the TcR. We created a DMF5 mutant for this residue and showed that, unexpectedly, this TcR had acquired a reduced overall activity although the TcR-pMHC complex was more stable when compared to the TcR wild type complex (increased $t_{1/2}$). In addition, the soluble TcR form of this mutant bound target cells less efficiently. From this we concluded that kinetic parameters do not always predict the superior functionality of mutant TcRs.

1. Introduction

Activation of T cells is a tightly regulated process. Among several molecules that participate in the activation of T cells, the T-cell Receptor (TcR) is the main component since it provides the specific target recognition. TcRs engage with peptides presented in the context of MHC (pMHC) by antigen presenting cells (van der Merwe and Dushek). The signal that is required to activate T cells is to a large extent determined by the binding parameters of TcR-pMHC interaction (Holmberg et al., 2003). From all the models proposed, it seems that the kinetic requirements vary with the TcR and the pMHC, and a universal picture is unlikely to emerge (Bridgeman et al., 2012). However, recent reports tend to agree that each TcR-pMHC would have their ideal time and affinity window (Kalergis et al., 2001; Stone et al., 2009; Aleksic et al., 2010), and some outsiders have been isolated as specific and potent without following the prediction coming from biophysical parameters (Zhong et al., 2013).

The development of high affinity TcRs represents an attractive strategy in cancer immunotherapy since naturally generated TcRs against tumor associated antigens are usually of low affinity (Aleksic et al., 2012). This is likely due to the negative selection of high affinity self reactive TcRs in the thymus and periphery (von Boehmer et al., 2003). Therefore, the development of TcRs with combined improved affinity and avidity lying within physiological boundaries should be favored. Indeed, increased affinity out of the physiological range may lead to non-specific cross-reactivity and inhibition of the serial engagement of the TcR (Holler et al., 2003; Irving et al., 2012; Hebeisen et al., 2013). However, if these high affinity TcRs cannot be used in T-cell immunotherapy, they can still be valuable tools as soluble TcR (sTcR) since they have the ability to detect low amount of pMHC (Molloy et al., 2005; Liddy et al., 2012; McCormack et al., 2012).

In the present paper, we investigated the TcR-pMHC interaction parameters of two TcR mutants of the well characterized MART1_{27-35} peptide (MART1p) specific TcR DMF5. In a previous study, Robbins and colleagues (2008) reported the screening of T cells expressing different full length DMF5 mutants. One of these variants carrying the T54A mutation on the CDR2β of TRBV6-4 (IMGT annotation, (Giudicelli et al., 2006)) induced an enhanced level of IFNγ release in response to melanoma cell-lines when expressed in $CD4^+$ T cells, suggesting that DMF5 T54A had a decreased CD8-dependency. In another

* Corresponding author.
E-mail address: sebastw@rr-research.no (S. Wälchli).
[1] Present address: Biogenidec Oslo, Norway.
[2] Present address: Department of Cancer Biology, The Scripps Research Institute, Scripps Florida, Jupiter, FL 33458, USA.

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study, Aggen et al. (2010) reported a polymorphic variant, S49, located upstream of the CDR2α loop of TRAV12-2 that increased thermal stability and surface expression of 868 TcR when expressed as a single chain TcR (scTcR). Since DMF5, like 868, is composed of a TRAV12-2 chain containing the Phe_{49} allele, we generated a thermostable form of DMF5, referred to as F49S. We first verified that DMF5 T54A and F49S mutants were still functional and able to selectively recognize their target. We then characterized selected TcR-pMHC binding parameters of these two variants and identified their effect on the functionality of transduced T cells and as soluble TcRs. The T54A mutation did not dramatically affect the binding properties of DMF5 when compared to the wild type (WT). However, when tested in a CD8 independent system the T54A mutant revealed increased potency. On the other hand, the F49S mutation was found to slightly decrease the dissociation constant (K_d) and increase the $t_{1/2}$ of the TcR-pMHC complex. When F49S was tested in a functional assay, its activity was decreased compared to DMF5 WT. Surprisingly, the binding pattern of sTcR F49S was also decreased despite a favorable increase in $t_{1/2}$. From these data we discuss the difficulty to predict the potency of TcR-pMHC binding.

2. Materials and methods

2.1. Antibodies, peptides, multimers, cell lines, and cell culture, retrovirus preparation and transduction

The following antibodies, peptides and multimers were used: anti-HLA-A2 Alexa Fluor 647 (clone BB7.2, labeled in-house), anti-CD3 Alexa Fluor 647 (clone OKT-3, labeled in-house), anti-CD8 Pacific Blue (BD Biosciences, Erembodegem, Belgium), MART-1 $peptide_{26-35}$ (ELAGIGILTV), CD20 $peptide_{188-196}$ (SLFLGILSV) and EBV $peptide_{280-288}$ (GLCTLVAML) were from ProImmune Ltd. (Oxford, UK), pMHC multimer-PE were prepared in-house following previously described method (Toebes et al., 2009).

Hek-Phoenix (Hek-P, our collection) were grown in DMEM (PAA, Paschung, Austria) supplemented with 10% HyClone FCS (HyClone, Logan, UT, USA) and 1% antibiotic-antimicotic (penicillin/streptomycin, p/s, PAA). T2 (our collection), J76 (a kind gift from M. Heemskerk, Leiden University Medical Center, (Heemskerk et al., 2003)), and SupT1 (gift from M. Pule, University College London, UK) were maintained in RPMI-1640 (PAA), supplemented with 10% FCS and p/s.

Viral particles were produced as previously described (Walchli et al., 2011), briefly, Hek-P cells were transfected using Fugene-6 (Roche, Basel, Switzerland) with retroviral packaging vectors and the expression vector. After 24 h of incubation at 37 °C, medium was replaced with DMEM 1% FCS and cells were incubated at 32 °C. Supernatants were harvested after 24 and 48 h.

Spinoculation of J76 was performed with 1 Volume of retroviral supernatant in a 12-well culture non-treated plate (Nunc A/S, Roskilde, Denmark) pre-coated with retronectin (20 μg/mL, Takara Bio. Inc., Shiga, Japan). After two days, cells were harvested with PBS-EDTA (0.5 mM). PBMCs isolated from healthy donors were cultured and activated in X-vivo 20 media (Lonza Group Ltd., Basel, Switzerland) supplemented with 5% human serum (HS) and 100 U/ml IL2 (R&D Systems, Abingdon, United Kingdom) for 48 h in a 24 well plate precoated with anti-CD3 (OKT-3) and anti-CD28 antibodies (BD Biosciences). After two days of culture PBMCs were harvested and transduced twice with retroviral supernatant as J76. After 2 days PBMCs were harvested and cultured in X-vivo 20, 5% HS, 100 U/ml IL-2 and 2 ng/ml IL-15. On day 7 post-transduction, PBMCs were used for experiments.

2.2. IL-2 and CD107a/b assays

If indicated, cells were loaded O/N with the indicated amount of peptide in OPTI-MEM (Life technologies™, New York, NY, USA) at 37 °C. Hundred thousand presenting cells were incubated with the same amount of effector cells (transduced J76) for 20 h in X-vivo 22 medium containing 1 nM phorbol myristic acetate (PMA, Roche Ltd., Germany). Supernatants were harvested and IL-2 ELISA was performed following manufacturer's procedure (R&D Systems). The optical density was read in a Sunrise™ Microplate reader (Tecan Group Ldt., Männedorf, Switzerland).

Measurement of degranulation (CD107a/b) in transduced T cells was performed as described in Stronen et al. (2009). Briefly, 0.5×10^5 DMF5 transduced PBMCs were mixed with 1×10^5 target cells in a medium containing 10 μM monensin (Sigma-Aldrich, St. Louis, MO, USA), 10 μg/mL Brefeldin A (Sigma-Aldrich) and 10 μg/mL of anti-CD107a and anti-CD107b antibodies conjugated with Alexa Fluor-647 and incubated for 5 h at 37 °C. Finally, cells were stained with anti-CD8 Pacific Blue antibody to separate T cells from presenting cells and analyzed by flow cytometry using an LSRII instrument (BD Biosciences).

2.3. DNA constructs

HLA-A2 construct was from Walchli et al. (2011) and HLA-A2 mutants for the residues DTKA (HLA-A2-DTKA, CD8 binding site (Salter et al., 1990)) were generated using site directed mutagenesis on HLA-A2 with the following primers: 5′-GAGGACCAGACCCAGAAGGCGGAG CTCGTGGAGAC-3′ and 5′-GTCTCCACGAGCTCCGCCTTCTGGGTCTGGTC CTC-3′.

The DMF5 variants, both soluble and full length forms, were generated by site directed mutagenesis on WT DMF5 using the following primers: 5′-CAAATACTGCAGGTGCCACTGGCAAAGGAGAAG-3′ and 5′-CTTCTCCTTTGCCAGTGGCACCTGCAGTATTTG-3′ for T54A and 5′-GAGT TGATAATGTCCATATACTCCAATGG-3′ and 5′-CCATTGGAGTATATGGAC ATTATCAACTC-3′ for F49S.

Single chain trimers were ordered as a synthetic ORF from Eurofins MWG Operon (Ebersberg, Germany) following the design of Yu et al. (2002), sequence can be sent on request. All constructs were sequence-verified after cloning (Eurofins MWG).

2.4. Binding analysis

Hundred thousand cells were resuspended in a solution containing the indicated antibody or multimer-PE for 20 min at room temperature in the dark. Cells were washed twice with flow buffer (PBS, 2% FCS, 0.5 mM EDTA) both before and after staining, and analyzed using an LSR II flow cytometer (BD Biosciences). For soluble TcR analysis 0.34 μM of biotinylated soluble TcR tetramerized with SA-PE was used to stain target cells expressing single chain trimer (HLA-A2-β2 microglobulin-MART-1 peptide) or MART-1 peptide pulsed-T2 cells for 20 min at room temperature in the dark, and analyzed by flow cytometry. Figures were prepared using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.5. Tetramer binding and decay assays

Tetramer binding at equilibrium was analyzed by staining 2×10^5 TcR expressing cells with six serial dilutions of the MART-1 tetramer (PE) and a saturating amount of anti-CD3. After 2 h of incubation at 4 °C in the dark, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry. Cells were washed twice with flow buffer, before and after staining. The K_d value of the tetramer-TcR binding was identified using a Scatchard plot, as depicted in Holmberg et al. (2003) and Savage et al. (1999). The amount of bound tetramers as reflected by the GMF divided by the amount of unbound tetramers (represented by the amount used for staining) was plotted against the GMF itself. The K_d value was calculated as the negative inverse of the slope. Briefly, 2×10^5 cells were stained with sub-saturating amounts of tetramers (PE) and anti-CD3 for 1 h at 4 °C and washed twice with flow buffer to remove unbound tetramers. Cells were incubated with saturating amount (100 μg/mL) of anti-HLA-A2 antibody to prevent rebinding of already dissociated tetramers. At the respective time point cells were