Not just a protein machine: how ribosomes regulate immune response

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Chapter 3

Ribosomal protein target identification: the beginning of an unexpected journey

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The goal of **chapter 3** was to identify RP candidates with potential effects on $CD8^+$ T cell recognition. This goal was defined based on a paper published at the beginning of this project in 2019, showing that RPs do play a role in immune response to tumors. In this study, Wei et al. discovered a subset of RPs that regulate peptide generation and/or MHC-I levels for immunosurveillance, without affecting translation in general [1]. Specifically, they focussed on three RP candidates, eL6 (RPL6), eL28 (RPL28) and eS28 (RPS28) and found that KD of eL6 decreased peptide presentation, while KD of eL28 and eS28 increased it. Further, KD of eS28 resulted in increased T cell killing of a melanoma cell line (Mel624). Until 2019, this was the only published paper that provided experimental evidence for the role of RPs in tumor immune surveillance and evasion. This study provided a great starting point for the RP selection process and was the reason we included eL6, eL28 and eS28 in our panel.

In 2017 and 2020, Campos et al. have described P1 and P2 as crucial RPs needed to translate viral mRNAs encoding transmembrane-domains (TMDs) [2, 3]. Based on these findings, we hypothesized that the preferential translation of TMD-containing mRNAs by P1-containing ribosomes could substantially affect the translation of APP components, such as HLA or TAP. This could disproportionately affect the translation of APP members crucial to mount immune responses, which could potentially affect T cell recognition of target cells. While the P-stalk proteins have long been considered as not essential for translation [4], it is known that they dynamically associate with the ribosome [5], making them particularly interesting to study in the context of ribosome specialization. Over the following years, additional evidence on the role of P-stalk proteins in immune response was published. In 2023, Siodmak and colleagues found that *Arabidopsis thaliana* deficient in P-stalk proteins (P0, P1, P2) show decreased immune function against bacterial infections [6] and that this was the result of altered P-stalk phosphorylation and dissociation from actively translating ribosomes. Another study by Filipek and others published in 2024 supported this idea, and showed the biological relevance of P-stalk phosphorylation for optimized protein translation [7]. Based on this compelling evidence, we decided to include P1 in our RP panel.

In 2017, Patel et al. showed that uL14 (*RPL23*) overexpression in melanoma cells resulted in resistance to T cell killing, suggesting that this could be an important RP in tumor immunosurveillance [8]. This study was particularly interesting to us, because uL14 was previously known for its role in p53-mediated apoptosis of tumors [9-11], but never described in the context of immune response. However, this study did not assess whether the role of uL14 was ribosome-associated or the result of extra-ribosomal functions. To dissect this, we included uL14 as the last RP in our panel.

While the mentioned papers provided valuable insights into the role of various RPs in immune response, it remains unclear how these RPs regulate immune response mechanistically. Therefore, we selected a set of 5 RP candidates (eL6, eL28, eS28, P1, uL14) and another RP, uS15, that has not yet been described in this context, and explored them further. The goal of the following preliminary experiments was to understand whether depletion of either of these RPs would cause alterations in HLA I levels and/or $CD8^+$ T cell recognition of melanoma cells.

To investigate this, we used lentiviral shRNA technology to knock down (KD) each of these RPs and a scrambled control (shScr) in the M026 melanoma cell line. Successful shRNA transduction was measured by flow cytometry using RFP expression (%) gated on live tumor cells. This resulted in a transduction efficiency of between 85-96% across RP targets and the scrambled control (Fig. 1A).

As it has been shown previously that RP KD can alter HLA I surface expression [1] we assessed whether this was the case for our RP candidates. To test this, we measured pan class I surface expression in M026 cells upon RP KD by flow cytometry. While we observed a significant increase in pan class I surface levels in shL6, shL28 and shS28 tumor cells compared to the scrambled and shS15 control, we did not observe a significant change in shP1 and shL14 cells (Fig. 1B).

Next, we investigated the effect of RP KD on $CD8^+$ T cell recognition of M026 tumor cells. To do this, we made use of a well-established co-culture system [8] consisting of donor $CD8^+$ T cells retrovirally transduced with a TCR of choice and RP KD M026 tumor cells. $CD8^+$ T cell activation was assessed using IFNγ levels measured by flow cytometry. These data showed that amongst the tested RPs, loss of P1 and uL14 in tumor cells had the most striking effect on T cell recognition, and these cells were significantly less recognized by both MART-1 and NY-ESO-1 specific $CD8^+$ T cells compared to the control (Fig. 1C, D).

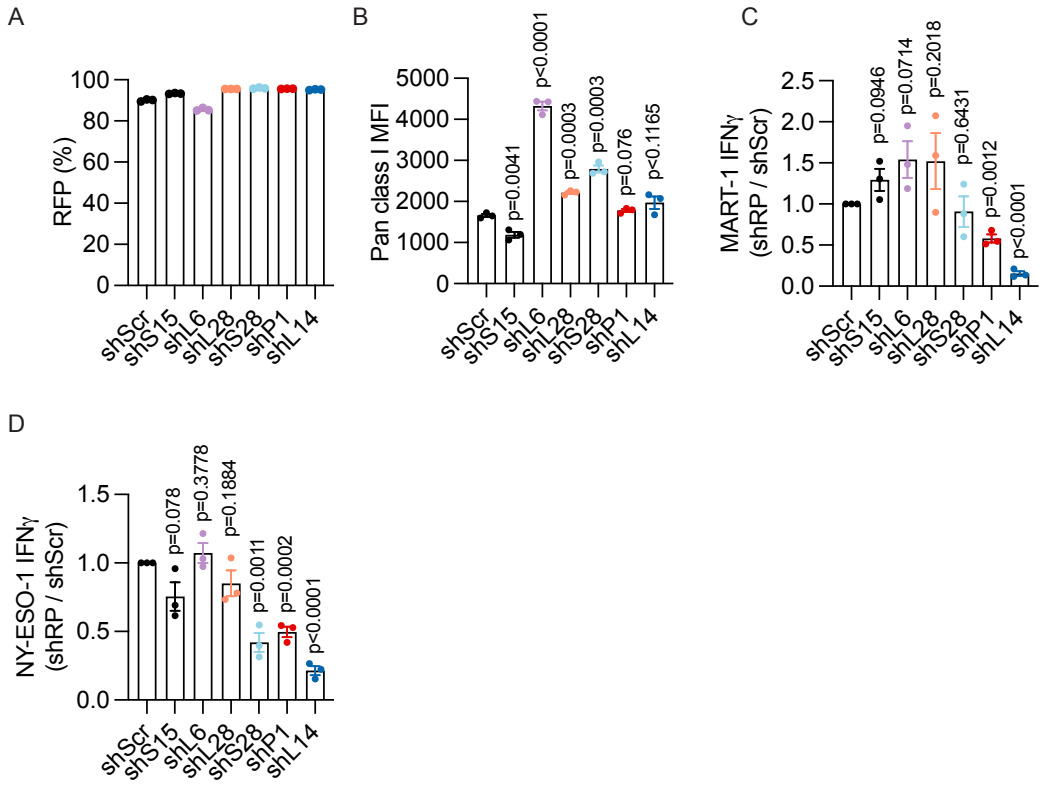

Figure 1. Preliminary experiments for RP target selection in M026 cells.

(**A**) Transduction efficiency was measured by flow cytometry using RFP (%) expression. n=3 independent experiments represented as mean ± SEM.

(**B**) Pan class I levels were measured by flow cytometry using the MFI. n=3 independent experiments represented as mean ± SEM and p values were determined using a two-tailed t test.

(**C**) MART-1-specific $CD8^+$ T cell recognition was assessed by flow cytometry using IFNγ levels. n=3 independent experiments represented as mean ± SEM and p values relative to shCtrl were determined using a two-tailed t-test.

(**D**) NY-ESO-1-specific $CD8^+$ T cell recognition was assessed by flow cytometry using IFNγ levels. n=3 independent experiments represented as mean ± SEM and p values relative to shCtrl were determined using a two-tailed t-test.

Chapter 3 marks the beginning of an unexpected journey, and we have uncovered a much broader role of RPs in immune response than has been previously considered. Based on the preliminary results presented in this chapter, **chapter 4** and **5** investigate the regulatory role of P1 and uL14 in this process.

Methods

Cell culture

M026 tumor cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml Penicillin- 100ug/ml Streptomycin (Life Technologies 15140-122).

Lentivirus shRNAs

shRNA targeting sequences were cloned into a DECIPHER pRSI9-U6- (sh)-UbiC-TagRFP-2A-Puro [1]. shRNA targeting sequences were selected based on previous literature [1] and the Broad Institute RNAi consortium (https://portals.broadinstitute.org/gpp/public/). The shRNA target sequences used for RP target selection in this chapter were TCCTTCACAGATCGGTGTAAT (shS15), GTATTCCCGATCTGCCATGTA (shL6), CCGCAATTCCTTCCGCTACAA (shL28), CGATCCATCATCCGCAATGTA (shS28), GTCACGGAGGATAAGATCAAT (shP1), CGGTAGGAGCTGTAATCAATT (shL14). For lentiviral packaging, we used HEK293T cells (Sigma-Aldrich, 12022001) and a second generation packaging system (pMDL RRE and psPAX2). M026 cells were infected with shRNA lentivirus containing supernatant at a MOI=0.5 for 96 hours. Subsequently, transduction efficiency was determined by flow cytometry using RFP (%) expression.

Pan class I surface staining

After harvesting, M026 tumor cells were washed in PBS and stained for 30 minutes on ice with BV605™ anti human HLA-A, B, C (clone W6/32, BioLegend 311432). The LIVE/DEAD Fixable IR Dead Cell Stain Kit (Thermo Fisher L10199) was used to measure cell viability by flow cytometry. Data were acquired with the BD FACSymphony™ flow cytometer (BD Biosciences) and analyzed with the FlowJo version 10.8.1 software (FlowJo LLC).

Generation of MART-1 and NY-ESO-1 TCR CD8⁺ T cells

The isolation of TCR transduced $CD8^+$ T cells from PBMCs has been described in detail previously [12]. Briefly, PBMC-derived $CD8^+$ T cells were isolated using the Miltenyi $CD8^+$ T Cell Isolation Kit, human (Miltenyi Biotec 130-096-495). $CD8^+$ T cells were activated with Dynabeads Human T-Activator CD3/CD28 (Miltenyi Biotec 111.31D) for 48 hours at 37°C and mixed with MART-1 or NY-ESO-1 TCR-containing retrovirus. The mix was added to a retronectin-coated (Takara T100A) non-tissue culture 24-well plate and spun for 1.5 hours at 2500 rpm at 10°C. After harvesting TCR-transduced $CD8^+$ T cells, they were maintained in T cell medium containing RPMI supplemented with 100 U/ml Penicillin- 100ug/ml Streptomycin and 10% human serum (Sigma-Aldrich), IL-7 (ImmunoTools 11340073, 5ng/ml), IL-15

(ImmunoTools 11340153, 5ng/ml) for 7 days. Subsequently, TCR transduction efficiency was determined by flow cytometry using the PE a-mouse TCRβ antibody (clone H57-597, BD Biosciences 553172) and anti-PE MicroBeads (Miltenyi Biotec 130-048-801). Lastly, irradiated feeder PBMCs were used to rapidly expand TCR-transduced $CD8^+$ T cells for 5 days in medium containing 60U/ml IL-2 (Proleukin, Novartis) and Ultra-LEAF™ Purified anti-human CD3 Antibody (clone OKT3, BioLegend 317326, 1:2000). Following rapid expansion, T cells were expanded in medium lacking the Ultra-LEAF™ Purified anti-human CD3 Antibody for 2 weeks and used in experiments or cryopreserved until needed.

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