Integrating mechanobiological signaling for improvement of therapeutic CAR-T cells in a platform-based approach

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Abstract:
Chimeric antigen receptor (CAR) T cells have revolutionized cancer immunotherapy. Besides biochemical signaling, mechanical signal transduction plays a key role in T cell activation. To the authors knowledge, the role of the cytoskeleton in CAR-T cell signaling has not been investigated. This study aims to fill this gap by optimizing a multiparametric analysis platform to study CAR-T cell mechanosignaling. The proposed technology combines high resolution imaging with single cell transcriptomics, proteomics and epigenomics to obtain a comprehensive profile of the cellular state. Subsequently, established screening approaches are modified to study novel CAR architectures that take into account mechanosignaling. Identifying mechanoactive costimulatory domains bears potential to improve therapeutic CAR-T cell technologies.

1. Introduction

1.1 CAR-T cell immunotherapy

In recent years, immunotherapy has revolutionized the treatment of cancer (for a review see Hoteit et al[1]). Chimeric antigen receptor (CAR) T cells constitute one of the most promising approaches in this field. This technology is based on genetic modification of patient-derived cytotoxic T cells[2]. The coding sequence for a synthetic CAR is stably integrated in the genome, linking binding of tumor-specific antigens to T cell activation. CARs consist of an extracellular displayed antibody fragment linked to a transmembrane domain and intracellular signaling domains. The antibody-fragment specifically binds a tumor-associated surface antigen.

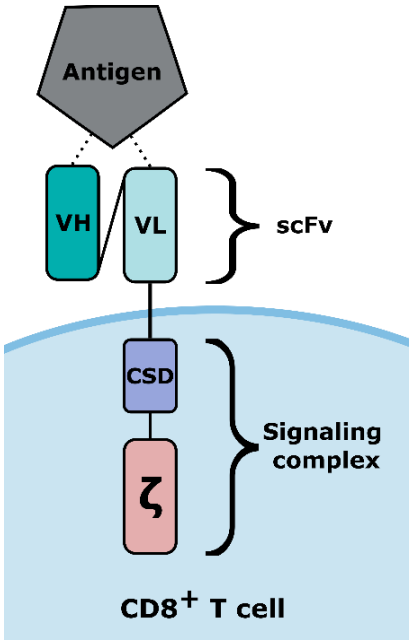

Figure 1: Schematic of a chimeric antigen receptor as discussed in this research proposal. An antigen-specific single chain variable antibody fragment (scFv) is connected to the signaling complex via a transmembrane domain. The extracellular scFv interacts highly specific with an antigen (indicated by dotted lines). Upon antigen binding, the intracellular signaling complex induces T cell activation. This complex consists of CD3 ζ and costimulatory domains (CSDs).

This event triggers cytotoxic T cell activity, resulting in tumor clearance with high specificity. The signaling complex consists of CD3 ζ and further costimulatory domains (CSDs) that modulate T cell activation and cytotoxic activity. The modular structure of this complex allows flexible engineering using state of the art molecular biological tools.

1.2 Mechanobiology of canonical T lymphocytes

The biochemical signaling in T cell activation has been extensively studied in immunological research[3,4]. Recently, the biomechanics involved in this process garnered attention as another important dimension of cellular immunity. T cell interactions with the environment, signaling at the MHC/TCR immunological synapse (IS) and downstream induction of cytotoxicity are mediated by mechanical forces[5–7].

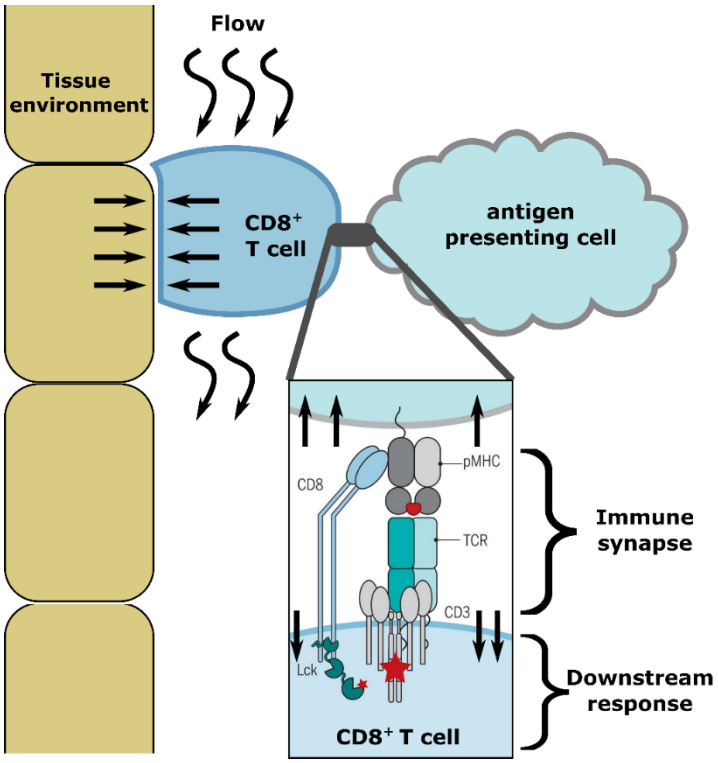

Figure 2: Schematic of the canonical immunological synapse. Forces are indicated by arrows. Environmental interactions include compression, adherence to the tissue environment and shear forces exerted by lymphatic and blood circulation. Catch-bonds in the MHC/TCR synapse increase specificity of the interaction. Downstream responses are partially mediated by cytoskeletal rearrangements in response to the extracellular signal. Figure adapted from Zareie et al[30].

These effects can be categorized in three layers:

- Surface interactions: T lymphocytes endogenously interact with environments of varying mechanical properties (lymphoid organs, lymphatic and blood circulation, tissue-specific microenvironments). Immunological responses have been shown to depend on cellular deformation and the stiffness of the surrounding environment[8,9]. Further the topological spacing of receptors on the cellular surface and the distribution of the different IS subunits depend on mechanical membrane properties[10,11].

- Mechanical forces in the immune junction: IS formation itself strongly depends on mechanical behavior of bonds between the involved proteins. Catch-bonds in the TCR tighten under mechanical tension and have been proposed to increase specificity of the interaction between antigen-presenting cell and T cell. This could function as additional layer of specificity to prevent peptide-independent MHC/TCR-interaction[12–14]. Force activated ion channels (mainly Piezo 1) have been observed to play a crucial role in the induction of downstream effects[13,14].

- Intracellular mechanosignaling: Upon MHC-peptide recognition, actin polymerization and rearrangement are triggered in the cell[15,16]. These processes are assumed to modulate gene expression, as cytoskeletal forces are known to be relayed to the nucleus by the LINC complex[17]. Additionally, cytoskeletal remodeling in the nucleus upon T lymphocyte activation has been observed by Tsopoulidis and collegues[18]. Mediated by a wide variety of transcription factors and modifying enzymes, this signal induces transcriptional and epigenetic changes. These

have been shown to be crucial to activate cytotoxic responses[5,16]. Further, cytotoxic vesicles are anchored and transported on microtubules. Actin/Microtubule crosstalk was therefore discussed to play a role in the T cell effector function[9,19].

1.3 Obtaining insight into CAR-T cell Mechanobiology

It is well established that mechanotransduction plays an important role at different steps of T cell activation. To the authors knowledge, no such investigations have been conducted for CAR systems.

The presented project investigates the mechanosignaling in CAR-T cells upon antigen recognition. Cytoskeletal rearrangements will be studied using microscopic techniques to better understand differences to classical T cell responses. Subsequently, effects on gene expression will be analyzed on the single cell level by implementing a high-throughput screening platform. Droplet-microfluidics allow to integrate transcriptomics with protein detection and epigenetic parameters. Studying these different dimensions provides insight into the mechanisms of CAR-T cell activation that will be correlated with the biomechanical observations. Based on the obtained findings, CAR structures are modified to optimize physical signal transduction for therapeutic application. Different CSDs will be investigated in a screening approach. To maintain a mechanical environment close to the biological context, cells will be cultured in stiffness-tunable hydrogel**.**

2. Methods

2.1 Microscopical study of cytoskeletal rearrangements upon CAR-T cell activation

To gain insight into the cytoskeletal responses upon activation, CAR-T cells are compared to primary T lymphocytes. For this project, clinically approved CAR-T cells (see reference *20* for a continuously updated list) are cocultured with complementary tumor cells. Imaging based techniques provide insights into cytoskeletal rearrangements. To evaluate and optimize the model system, actin-structures are imaged via Spinning disk confocal microscopy. Comparing the actin response can indicate important deviations from the canonical mechanisms. F-actin structures are labeled using phalloidin-fluorophore conjugates (e.g. Alexa Fluor 488™ Phalloidin[21]). After optimization of culture and analysis, higher resolution 3D images of the cytoskeleton are obtained. Following the description of Fritzsche, Fernandes and colleagues, cytoskeletal structures are imaged using super-resolution stimulated emission depletion microscopy and lattice light-sheet microscopy[22].

2.2 Single cell resolution study of CAR-T cell signaling in high throughput

Structural information obtained in *2.1* is correlated with induced downstream signals. To achieve this, a high throughput screening platform is set up. A single cell suspension is generated using 10x genomics droplet microfluidics. The commercially available system is used to generate single cell transcriptomics data, indicating intracellular responses upon activation. It is combined with CITE-seq[23] to label characteristic proteins with oligonucleotide barcode-tagged antibodies. The antigen-specific barcodes are read out in the same assay to investigate an additional dimension of the T cell response. Parallel readout of epigenetic markers is also implemented. Information about chromatin-accessibility can be read out via single cell ATAC-seq[24]. Alternatively, single cell Cut&Tag[25] can be used to investigate specific chromatin modifications. Integrating transcriptomic changes, chromatin state and protein levels allows to produce a complex profile of single cells at different timepoints after receptor activation. Correlating these changes with the microscopical observations allows to generate more detailed hypotheses, defining candidate domains for subsequent experiments.

2.3 Screening of novel CAR domains to identify CSDs with favorable mechanical properties

Using the obtained information, the CAR structure is adapted to mimic the mechanical response of canonical T cells. Alternatively, optimization of mechanosignaling can be used to finetune the cytotoxic activity for specific therapeutic applications. A promising approach is the incorporation of additional CSDs. Domains modulating physical signal transduction are investigated using genome engineering approaches. The speedingCARs platform is used to screen variants in high throughput[26]. A library of shuffled CARs is generated by combining different CSDs while maintaining antigen-binding. Using CRISPR Cas9, these molecules are expressed in primary human T cells that are cocultured with tumor cells. In a pooled screen, single cell transcriptomics is used to read out the receptor sequences and the gene expression profile, which is used as phenotypic proxy to study activation.

The speedingCARs approach is modified by culturing cells in a hydrogel microenvironment with defined mechanical properties. Alginate scaffolds and polyacrylamide hydrogels have been successfully used to fine-tune 3D mechanical microenvironments[27–29]. Antigens and additional cytokines can be incorporated in this approach to simulate tumor environments[29].

The fourth generation of CAR-T cells provides guidance for promising domain candidates. These systems use CSDs that elevate cytokine-expression. Cytokines are often linked to mechanosignaling (e.g. IL-12 and PD1). At the time being, these possible mechanical links between effectors and activity have not been studied in CAR-T cells. Further promising mechanisms to be modulated by CSD candidates include calcineurin signaling, LFA-1 activation and kinase signaling (Lck/ZAP70, PI3K).

3. Aims

3.1 Implementing a high throughput, multiparameter screening platform

This study aims to correlate microscopic imaging of the cytoskeleton of CAR-T cells with transcriptomic data. Further, proteomic and epigenetic markers are included in the analysis. For this, a multiparametric screening platform based on microscopy and microfluidics is set up and optimized. With this, extensive profiles of cellular states are obtained at different timepoints after T cell activation.

3.2 Identifying differences between CAR and canonical T cell mechanobiology

A high amount of data is generated by the proposed platform. The analysis correlates different studied dimensions, providing comprehensive insights into the activation-induced intracellular effects.

Comparing obtained profiles between CAR-T cells and canonical T cells indicates differences in cytoskeletal signaling. It is expected that the synthetic CAR produces different downstream effects than the more complex canonical IS. This might be caused by absence of signaling domains or the structural difference in the receptor-ligand complex. As CAR mechanobiology is currently not well understood, obtained information are expected to be valuable for further optimization of mechanical signaling in therapeutic applications. Here, cytoskeletal differences are correlated with altered downstream effects to guide optimization of immunotherapies.

3.3 Implications for improving therapeutic CARs

From linking cytoskeletal changes and expression profiles, potential engineering approaches to improve CAR-T cell signaling are inferred. Based on these findings, a library of CAR-T cells with shuffled mechanoactive CSDs is screened to identify candidates with desired traits. Different combinations are expected to generate non-linear changes in activation, therefore a comprehensive screening is required. The identified candidates will be further optimized for use in CAR-T cell therapies. Once established, the described system can be used to investigate candidates identified in future studies of T cell mechanobiology.

4. Discussion

T lymphocytes evolved to respond to a wide variety of targets in different biological contexts. The semi-synthetic CAR-T cells currently have a much narrower range of uses. This allows optimization for a desired application – namely the highly specific killing of cancer cells. To pursue this goal, detailed knowledge about the underlying mechanisms is necessary. While the involved biochemical signaling has been studied, the biomechanical dimension of this process remains mostly unclear. This study aims to fill this gap.

As external signaling, intracellular signal propagation and induced responses are tightly linked, it is necessary to combine different technologies to read out the phenotypic states. In this study, a platform is optimized to generate multidimensional data on the single cell level. By comparing cellular responses, factors influencing mechanosignaling are identified. For further evaluation, observations are used to generate a group of novel CAR T cells, designed with consideration of mechanobiological signaling. These are screened for cytotoxic activity, generating promising candidates for therapeutic applications.

This study mainly focusses on the mechanical signaling, therefore investigated candidates should be critically evaluated for immunological off-targets and other possible side-effects. This analysis is deemed to be out of the scope of this project. Future investigations of T cell mechanosignaling might lead to new candidate domains that can be screened with the presented approach.

In conclusion, this project aims to establish a platform to investigate the mechanobiology of CAR-T cells using state of the art imaging and sequencing technologies. Findings are implemented by screening a library of CARs with improved mechanosignaling properties. By optimization of mechanobiological signaling, a major gap in CAR design is addressed, potentially improving cancer immunotherapies.

References

1. Hoteit, M. *et al.* Cancer immunotherapy: A comprehensive appraisal of its modes of application (Review). *Oncology Letters* **22**, 1–18 (2021).

2. Zhao, L. & Cao, Y. J. Engineered T Cell Therapy for Cancer in the Clinic. *Frontiers in Immunology* **10**, 2250 (2019).

3. Courtney, A. H., Lo, W. L. & Weiss, A. TCR Signaling: Mechanisms of Initiation and Propagation. *Trends in Biochemical Sciences* **43**, 108–123 (2018).

4. Mørch, A. M., Bálint, Š., Santos, A. M., Davis, S. J. & Dustin, M. L. Coreceptors and TCR Signaling – the Strong and the Weak of It. *Frontiers in Cell and Developmental Biology* **8**, 1147 (2020).

5. Ma, Z. & Finkel, T. H. T cell receptor triggering by force. *Trends in Immunology* **31**, 1–6 (2010).

6. Kumari, S., Curado, S., Mayya, V. & Dustin, M. L. T cell antigen receptor activation and actin cytoskeleton remodeling. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1838**, 546–556 (2014).

7. Colin-York, H., Barbieri, L., Pfannenstill, V., Korobchevskaya, K. & Fritzsche, M. Mechanobiological control of T-cell activation. *Biophysical Journal* **121**, 319a (2022).

8. Colin-York, H. *et al.* Cytoskeletal Control of Antigen-Dependent T Cell Activation. *CellReports* **26**, 3369-3379.e5 (2019).

9. Jin, W. *et al.* T cell activation and immune synapse organization respond to the microscale mechanics of structured surfaces. *Proc Natl Acad Sci U S A* **116**, 19835–19840 (2019).

10. Pageon, S. v. *et al.* Functional role of T-cell receptor nanoclusters in signal initiation and antigen discrimination. *Proc Natl Acad Sci U S A* **113**, E5454–E5463 (2016).

11. Davis, S. J. & van der Merwe, P. A. The kinetic-segregation model: TCR triggering and beyond. *Nature Immunology 2006 7:8* **7**, 803–809 (2006).

12. Liu, B., Chen, W., Evavold, B. D. & Zhu, C. Accumulation of dynamic catch bonds between TCR and agonist peptide-MHC triggers T cell signaling. *Cell* **157**, 357–368 (2014).

13. Hong, J. *et al.* A TCR mechanotransduction signaling loop induces negative selection in the thymus. *Nature Immunology 2018 19:12* **19**, 1379–1390 (2018).

14. Zhu, C., Chen, W., Lou, J., Rittase, W. & Li, K. Mechanosensing through immunoreceptors. *Nature Immunology 2019 20:10* **20**, 1269–1278 (2019).

15. Yi, J., Wu, X. S., Crites, T. & Hammer, J. A. Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat T cells. *Molecular Biology of the Cell* **23**, 834–852 (2012).

16. Basu, R. *et al.* Cytotoxic T Cells Use Mechanical Force to Potentiate Target Cell Killing. *Cell* **165**, 100–110 (2016).

17. Alam, S. G. *et al.* The nucleus is an intracellular propagator of tensile forces in NIH 3T3 fibroblasts. *Journal of Cell Science* **128**, 1901–1911 (2015).

18. Tsopoulidis, N. *et al.* T cell receptor–triggered nuclear actin network formation drives CD4+ T cell effector functions. *Science Immunology* **4**, (2019).

19. Hui, K. L. & Upadhyaya, A. Dynamic microtubules regulate cellular contractility during T-cell activation. *Proc Natl Acad Sci U S A* **114**, E4175–E4183 (2017).

20. CAR T Cells: Engineering Immune Cells to Treat Cancer - NCI (Date accessed: 26/06/2022). https://www.cancer.gov/about-cancer/treatment/research/car-t-cells.

21. Invitrogen: Alexa Fluor™ 488 Phalloidin (Date accessed: 27/06/2022). https://www.thermofisher.com/order/catalog/product/de/de/A12379.

22. Fritzsche, M. *et al.* Cytoskeletal actin dynamics shape a ramifying actin network underpinning immunological synapse formation. *Science Advances* **3**, (2017).

23. Stoeckius, M. *et al.* Simultaneous epitope and transcriptome measurement in single cells. *Nature Methods 2017 14:9* **14**, 865–868 (2017).

24. Product Sheet: Profiling chromatin accessibility at single cell resolution; 10x Genomics | Chromium | Single Cell Assay for Transposase Accessible Chromatin (Date accessed: 26/06/2022). https://www.10xgenomics.com/products/single-cell-atac.

25. Bartosovic, M., Kabbe, M. & Castelo-Branco, G. Single-cell CUT&Tag profiles histone modifications and transcription factors in complex tissues. *Nature Biotechnology 2021 39:7* **39**, 825–835 (2021).

26. di Roberto, R. B. *et al.* speedingCARs: accelerating the engineering of CAR T cells by signaling domain shuffling and single-cell sequencing. *bioRxiv* 2021.08.23.457342 (2021) doi:10.1101/2021.08.23.457342.

27. Kandow, C. E., Georges, P. C., Janmey, P. A. & Beningo, K. A. Polyacrylamide Hydrogels for Cell Mechanics: Steps Toward Optimization and Alternative Uses. *Methods in Cell Biology* **83**, 29–46 (2007).

28. Cameron, A. P. *et al.* Biophysical properties of hydrogels for mimicking tumor extracellular matrix. *Biomaterials Advances* **136**, 212782 (2022).

29. Vahala, D. & Choi, Y. S. Modelling the Tumor Microenvironment: Recapitulating Nano- and Micro-Scale Properties that Regulate Tumor Progression. *Frontiers in Cell and Developmental Biology* **0**, 1222 (2022).

30. Zareie, P. *et al.* Canonical T cell receptor docking on peptide–MHC is essential for T cell signaling. *Science (1979)* **372**, (2021).