Functional Characterization of a Transmembrane Adaptor Protein in T Cell Activation and Immune Synapse Formation

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This project is a reduced version form the confidential original one.

It is based on the results obtained from my internship in the group led by Janna Saarela from the Norwegian Center of Molecular Biosciences and Medicine (NCMBM), under the direct supervision of Francisca Hinrichsen. This study has been tutored by Iris Ginés Mir.

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Institution Details

The Norwegian Center of Molecular Biosciences and Medicine (NCMBM) is a leading research institution dedicated to advancing knowledge in molecular biology and medical sciences. The center is part of the University of Oslo, located in Oslo, Norway.

NCMBM integrates data science, molecular biology, biochemistry, and medicine to enhance our understanding of health and disease. Through interdisciplinary collaboration, the center conducts cutting-edge research focused on fundamental life science processes, promoting scientific excellence, translational impact, and international cooperation. NCMBM actively participates in a partnership with the European Molecular Biology Laboratory (EMBL), pursuing global collaboration and the exchange of knowledge.

Abstract

Inborn Errors of Immunity (IEIs) are genetic disorders that disrupt the normal functioning of the immune system, often impairing critical processes such as T-cell activation. Effective T-cell activation relies on the precise coordination of signalling cascades, many of which involve transmembrane adaptor proteins.

This study aims to identify the effects of the transmembrane adaptor protein deficiency in T-cell activation in humans using cells from a patient with a total loss-of-function genetic variation. To achieve this objective, immunocytochemistry and western blot techniques were employed to compare the patient's cellular profile with that of healthy controls.

The deficiency was studied through the dynamics of T-cell receptor microclusters, focusing on their formation, vesicle-mediated transport, and recruitment of proteins involved in the activation signalling cascade.

Keywords

Immune synapse

Inborn Errors of Immunity (IEIs)

T-cell activation

TCR microclusters

1. Introduction

Inborn errors of immunity (IEIs) are defined as monogenic disorders of the immune system predisposing to immunodeficiency, allergy, autoimmunity, autoinflammation, bone marrow failure, and in some cases to neoplasms. IEIs were typically classified as rare diseases, but lately, they have been discovered at an increasing rate. IEIs' identification has also helped to define more clearly their associated clinical phenotypes. The prevalence of IEIs is estimated to be between 1 in 1000 and 1 in 5000 individuals (Tangye et al., 2020). The spectrum of IEIs is notably diverse, encompassing 485 genetic defects described, which are categorized in 10 tables depending on the phenotypes (Tangye et al., 2022).

IEIs have a broad spectrum of clinical manifestations. They usually start with an increased susceptibility to infections, but also often present immune dysregulation and malignancy. Recent discoveries in the field of IEI have enabled the better characterization of their non-infectious manifestations, specifically immune dysregulation. Immune dysregulation, encompassing autoimmune or autoinflammatory manifestations, is defined as at least one of the following alteration: chronic lymphoproliferation (hepatomegaly, splenomegaly, lymphadenopathy); autoimmunity (cytopenia, hepatitis, autoimmune arthritis and other rheumatologic diseases, thyroid disease, vitiligo, alopecia, diabetes, celiac disease); granuloma; inflammatory bowel disease (IBD) or enteropathy; severe allergies; or autoinflammatory symptoms (persistent fever, rashes, elevated inflammatory markers) (Thalhammer et al., 2021).

Therefore, a significant component of IEI pathogenesis is attributed to dysregulated T-cell function. T-cell receptor (TCR) signalling is an essential process for the immune system's activity, being related to T-cell development, activation, proliferation, and immune tolerance (Figure 1). The main factor responsible for T-cell activation is the TCR, which is composed of two different chains (alpha and beta), but for its activation, the protein family Cluster of Differentiation 3 (CD3) (which comprises CD3 delta, epsilon, and gamma), and the zeta TCR chain are necessary. The CD3 family is a group of proteins associated with the TCR, and whose function is related to TCR location on the T-cell surface and the TCR signalling. The TCR complex is usually composed of the TCR chains alpha and beta, and two sets of CD3 epsilon, CD3 delta, and TCR zeta chains. All these sets are located on both sides of the TCR.

Once the TCR alpha and TCR beta interact with the major histocompatibility complex (MHC), recognizing the peptide being presented, they promote conformational changes in the associated CD3 chains. This change recruits the leukocyte-specific tyrosine kinase (LCK) and another kinase from SRC family, Fyn. These kinases phosphorylate CD3 molecules and the TCR zeta chain on their C-terminal immunoreceptor tyrosine-based activating motifs (ITAMs). The phosphorylations on CD3 chains provoke the recruitment and activation of the Syk family kinase zeta-activated protein 70 kDa (ZAP70) via Src-homology-2 (SH2)-domain interactions. ZAP70 phosphorylates the linker for activation of T-cells (LAT). This membrane-associated protein is used as a scaffold, recruiting different proteins to become a multiprotein complex called the LAT signalosome. LAT signalosome is responsible for downstream signalling as the activation of MAPK (Mitogen-Activated Protein Kinase), NFkB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), and mTOR (mammalian Target of Rapamycin) pathways, rearrangements in the cytoskeleton, or changes in transcription or translation (Hwang et al., 2020).

Once LAT is activated by ZAP70, it can recruit another scaffolding protein, known as SH2-domain containing leukocyte protein of 76 kDa (Slp-76), which is also phosphorylated by ZAP70. Both phosphorylated proteins form a complex where Interleukin-2 inducible tyrosine kinase (ITK) interacts. The ITK interaction with LAT-S1p-76 enables ITK to activate by autophosphorylation. ITK is responsible for phospholipase C gamma 1 (PLCγ1) phosphorylation and activation (Lee et al., 2023).

As PLCγ1 is activated, it catalyses the hydrolysis of 1-phosphatidyl-1D-myo-inositol 4,5-bisphosphate (PI(4,5)P2) into diacylglycerol (DAG) and 1D-myo-inositol 1,4,5-trisphosphate (IP3). This reaction has crucial importance for T-cell activation as it is a source of DAG and IP3. IP3 allows the release of calcium ions from the endoplasmic reticulum, the calcium efflux activates calmodulin (CaM), which in turn activates Calcineurin (CaN). CaN is a phosphatase that acts on the nuclear factor of activated NFAT. Once NFAT has lost its phosphorylation, it can be translocated to the nucleus, where it exerts its function (Shah et al., 2021).

Meanwhile, the lipid DAG acts as a second messenger, activating lots of downstream proteins, such as protein kinase C (PKC) and RAS guanyl nucleotide-releasing protein (RasGRP). On one hand, PKC is involved in the activation of the NF-kB pathway. On the other hand, RasGRP initiates the MAPK pathway. Those two pathways, as well as NFAT,

are responsible for changes in gene expression. DAG is also important as a lipidic membrane constituent, especially at immune synapses, where it has been demonstrated to be essential throughout the PKC recruitment leading to centromere polarization toward the contact site (Quann et al., 2009).

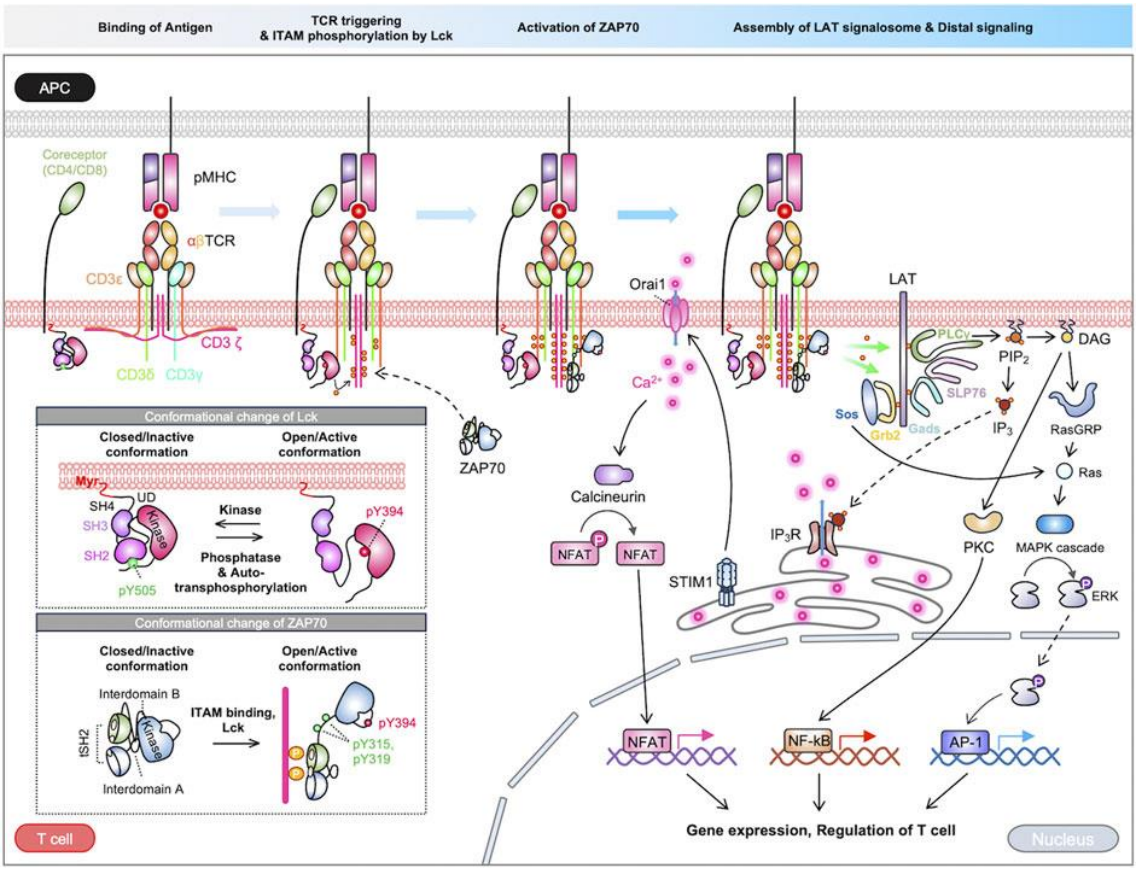

Figure 1. Overview of TCR signalling pathways*. Obtained from: (Lee et al., 2023)*

It should be mentioned that TCR activation depends not only on TCR-MHC peptide interaction. It has been extensively described that T-cell activation usually requires the ligation to other molecules, called co-stimulatory proteins. The absence of co-stimulation can lead to cell death or an anergic state in which the T-cell will not respond to antigen stimulation and will not be able to be stimulated again. Weak TCR signals are amplified strongly by Cluster of Differentiation 28 (CD28) engagement, which is one of the most important co-stimulatory receptors, thereby resulting in cell proliferation and differentiation. However, only CD28 engagement results in the expression of a few genes transiently, with no biological consequences (Xia et al., 2018).

T-cell activation not only involves signalling pathways, but also conformational changes. TCR triggering has been demonstrated to be related to the cytoskeleton rearrangements

and the formation of the immune synapse (IS). IS is described as a specialized supramolecular structure formed at the T-cell interface with the antigen-presenting cell (APC) in response to TCR-MHC interaction. This structure also requires the accumulation of coreceptors, adhesion molecules such as intercellular adhesion molecule 1 (iCAM-1), and signalling and cytoskeletal components in the contact area (Dustin, 2014). IS mature conformation presents a particular structure known as "bull's eye" architecture, which is composed of different domains that are disposed concentrically, named supramolecular activation clusters (SMACs). Three SMACs are distinguished depending on their location: central (cSMAC), peripheral (pSMAC), and distal (dSMAC) (Monks et al., 1998). cSMAC is mainly composed of TCR and its associated proteins, such as CD28. Surrounding cSMAC, pSMAC is a region enriched in integrins such as lymphocyte function-associated antigen (LFA-1), which interacts with iCAM-1. In this domain, there are also cytoskeleton-associated proteins. The distal SMAC is enriched in F-actin, proteins excluded from the center due to steric hindrance or to their ability to negatively affect the signalling, such as the transmembrane tyrosine phosphatase Cluster of Differentiation 45 (CD45) (Johnson et al., 2000), which is involved in both, LCK activation and TCR inactivation (Courtney et al., 2019).

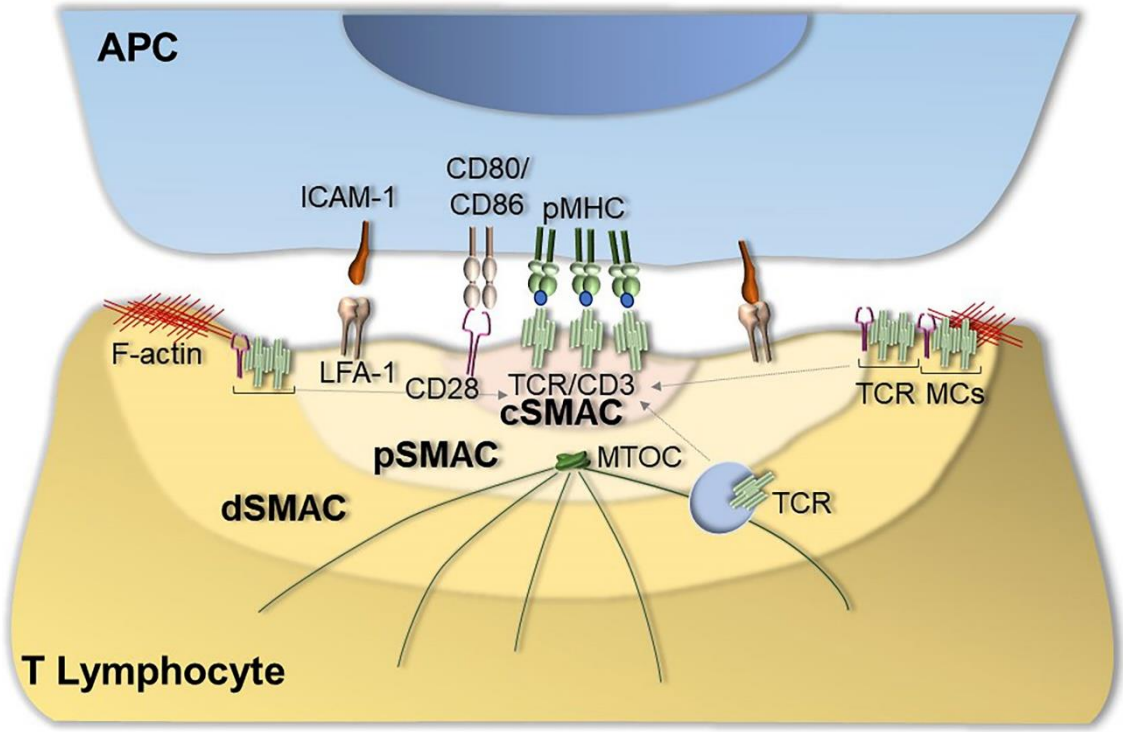

Figure 2. Representation of the immune synapse structure. *Obtained from:* (Capitani & Baldari, 2022)

IS formation is initiated and maintained by TCR microclusters, which are sub-micron TCR clusters that form in an F-actin-dependent manner. TCR microclusters are formed continuously in the periphery of IS and transported to the cSMAC (Varma et al., 2006). Nevertheless, TCR is not only localized at the plasma membrane, but it can also be found associated with recycling endosomes. Delivery to the synaptic membrane of this intracellular TCR pool is essential to replenish the plasma membrane pool as TCRs are internalized at the cSMAC, sustaining the signalling for the extended period necessary for T-cell activation (Das et al., 2004). This process depends on the centrosome polarization, which also allows multivesicular bodies to move toward the area under the contact point between the T-cell and the APC. These multivesicular bodies are related to protein recycling and could be used as exocytic vesicles at the IS (Martín-Cófreces et al., 2013).

As has been previously described, TCR signalling is an extensive pathway involving numerous molecules. For this reason, it should be carefully controlled by a precise network of many components that interact in a regulated manner. Transmembrane adaptor proteins (TRAPs) are a group of specialized proteins that act as a platform for signalling molecules with SH2-domains. These signalling hubs organize and transmit signals from the cell surface receptors into the downstream intracellular environment. LAT is the most well-characterized TRAP. LAT is phosphorylated by ZAP70 upon TCR engagement and can be considered a master switch of T-cell activation (Finco et al., 1998). LAT deficiency manifests as severe combined immunodeficiency (Bacchelli et al., 2017).

In this study, the effect of a TRAP deficiency in humans was investigated by the identification of a splice-donor variant, leading to the first known total loss-of-function of this protein seen in humans. This variation offers a unique opportunity to discover the function of this protein without genetic engineering techniques, and in human cells.

2. Hypothesis and objectives

Confidential

3. Materials and methods

Confidential

4. Results

Confidential

5. Discussion

Confidential

6. Conclusion

Confidential

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8. Self-assessment

The development of this project has been a significant challenge, not only from a scientific perspective but also on a personal level. This project is based on the results obtained during an internship at the Norwegian Center of Molecular Biosciences and Medicine (NCMBM) in Oslo, Norway. This hands-on experience has provided me with a very different professional environment compared to what I was used to. At NCMBM, I was introduced to a new kind of working dynamic, more focused on collaborative teamwork, regular scientific meetings, and knowledge exchange.

On the other hand, although I had studied immunology as part of my academic background, I had never worked with primary or immunological cells before. While I had previously performed techniques such as immunocytochemistry and western blotting, I had never applied them using a stimulatory attachment layer. Confocal microscopy, on the other hand, was completely new to me. At NCMBM, I had the opportunity to personally acquire the images used in this study, and I was responsible for all the image analysis. I even developed a methodology using ImageJ to detect the presence of proteins in TCR microclusters.

On a personal level, this project has pushed me far outside my comfort zone. I had to adapt not only to a new country and work culture but also to the challenge of carrying out an independent scientific project. During my undergraduate studies, I became familiar with searching for scientific literature, following protocols, writing up results, and drawing conclusions. However, I had limited experience in designing experiments from scratch and critically discussing them in light of existing scientific knowledge.

In summary, this project has allowed me to better understand how a research group works and experience a completely new scientific environment. I acquired practical knowledge in techniques such as stimulation kinetics and confocal microscopy. Most importantly, this experience has helped me grow both professionally and personally, and it has strengthened my motivation to continue pursuing a career in science.