Optimizing T cell/Antigen Presenting Cell coculture conditions for analysis of signaling from the T cell receptor and Major Histocompatibility Complex

by
Tobias Robert Hildebrandt
BSc Computational and Applied Mathematical Sciences
with a concentration in Biology, Plymouth State University 2021

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Date: 04/25/23

Dr. Arthur Salomon, Advisor

Date: 04/25/23

Dr. Diana Horrigan, Reader

Date: 04/25/23

Dr. John Marshall, Reader

Approved by the Graduate Council

Date:_____

Dr. Thomas A. Lewis, Interim Dean of the Graduate School, Prof. of Religious Studies

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Abstract

Abstract of Optimizing T cell/Antigen Presenting Cell coculture conditions for analysis of signaling from the T cell receptor and Major Histocompatibility Complex, by Tobias Robert Hildebrandt, Degree ScM., Brown University, May 2023.

T-cell receptor ligation of peptides displayed on the Major Histocompatibility Complex (MHC) of Antigen Presenting Cells (APC) initiate signaling events. Several studies reported canonical signaling events upon engagement of the T-cell receptor complex, yet MHC reverse signaling remains understudied. Here, we describe an optimized approach to permit the study of proximal signaling in live T-cells and APCs simultaneously via coculture of $CD8^+$ Jurkat T-cells expressing an OT-1 receptor capable of ligating Ovalbumin with a B-cell X T-cell hybrid expressing an empty MHC capable of binding to Ovalbumin in solution. A protein of the MAPK/ERK pathway was used as readout for early signaling. It was shown that only in the presence of Ovalbumin, T-cells with an OT-1 receptor and cells with an empty MHC Complex can fully activate T-cell signaling. Methods such as the one described in this body of work permits the simultaneous study of proximal signaling events in live APCs and T-cells, allowing for the characterization of reverse MHC signaling in different cell types. This method also permits the study of signaling events unique to cell therapies, including signaling in Chimeric Antigen Receptor T-cells (CAR-T), in an *in vitro* model that closely resembles the human's body biology.

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1 Introduction

The immune system has evolved as a multicellular defense against infections from ever changing pathogenic viruses, bacteria, single- and multicellular eukaryotes (Reddick and Alto, 2014). Other functions of the immune system include responding to toxins and allergens (Lautscham et al., 2003) as well as suppressing carcinogenesis (Adam et al., 2003). The immune response can be split into an innate response and an adaptive response. Innate responses start immediately upon infection, and are functionally identical irrespective of how often an antigen, a foreign molecule, was encountered before. In contrast, the adaptive immune system is a slow and highly specific response to particular and often recurring antigens, eventually resulting in immunological memory. Repeated exposure to antigens leads to a faster adaptive immune response. Central to innate and adaptive immunity are T-cells, a subset of lymphocytes which mature in the thymus. There are two major types of T-cells: cytotoxic T-cells, capable of lysing infected cells; and helper T-cells, which coordinate other immune cells during an immune response. The activity of T-cells depends on the specific interactions between cell surface receptors on the T-cells and those expressed on the infected cells or other immune cells.

The specific interactions between Antigen Presenting Cells (APCs) and T-cells take place at the immune synapse, an intercellular space that is densely populated with adhesion and signaling molecules (Wan et al., 2008). The APC is responsible for presenting an epitope, a short peptide chain, through specialized transmembrane protein complexes termed Major Histocompatibility complexes (MHCs). There are two MHC classes: Class I MHCs (MHC-I) and Class II MHCs (MHC-II). Class I MHCs are found in all cells with the exception of erythrocytes (Dersh et al., 2019) and they present epitopes from intracellular pathogens. Professional APCs, including B-cells, macrophages, and dendritic cells express Class II MHCs and are responsible for presenting extracellular antigens. Overall, both classes are structurally similar (Murphy and Weaver, 2018). The membrane-proximal region of MHC resembles immunoglobulin domains while the membrane-distal regions form a cleft in which the peptides are bound.The combined recognition of MHC and peptide by the T-cell and its coreceptor is called MHC restriction. During a two-step selection process double positive thymocytes ($CD4^+/CD8^+$) with problematic binding properties are eliminated and the majority of remaining cells differentiate into two classes of T-cells: (1) $CD8^+$ (cytotoxic) T-cells, which are restricted to interactions with peptides bound to MHC-I and (2) $CD4^+$, T-helper cells, that only interact with peptides bound to MHC-II. Both types of T-cells use the same type of peptides to make their T-Cell receptors (TCRs). The TCR complex is formed by combining the TCR$\alpha\beta$ heterodimers with their highly variable

peptide binding region as well as the invariable CD3$\delta\epsilon$, CD3$\gamma\epsilon$ heterodimers and the TCRζ-chain homodimer. An effective T-cell response is only initiated when the T-Cell receptor matches the MHC-peptide complex and the coreceptor binds to the MHC-peptide complex. Unsuitable responses to this interaction can lead to autoimmunity or severe infections (Parkin and Cohen, 2001).

It was suggested that TCR/pMHC ligation initiates signaling events (see Figure 1) by leading to conformational changes within the T-cell receptor complex resulting in exposed cytoplasmic sites ready for phosphorylation (Chakraborty and Weiss, 2014). These phosphorylation sites are found on CD3δ, CD3ϵ, CD3γ and TCRζ and they are named Immunoreceptor Tyrosine-based Activation Motifs (ITAMs). Their conserved order is repeated twice and has a consensus sequence of YxxL/Ix_{6-12}YxxL/I (Xu et al., 2008). Two Src family tyrosine kinases Lymphocyte-specific protein tyrosine kinase (LCK) and FYN that phosphorylate the two tyrosine residues on the ITAMs by their association with CD4/CD8 coreceptor and CD3, respectively (Abbas et al., 2014). Phosphorylated ITAMS recruit ZAP70, a Syk family protein tyrosine kinase, and function as its docking site. Bound ZAP70 is phosphorylated from nearby LCK, leading to conformational changes of ZAP70 resulting in its activation. Activated ZAP70 then phosphorylates multiple tyrosine residues on the membrane bound adaptor protein Linker for activation of T-cells (LAT), leading to the formation of a large signaling complex. The phosphorylated adaptor LAT binds GRB-2 and this complex then recruits Son of Sevenless (SOS). SOS is a GTP/GDP exchange factor capable of catalyzing GDP to GTP on RAS. GTP-bound RAS initiates a three step kinase cascade by activating RAF, RAF then phosphorylates MEK-1 and MEK-1 ultimately phosphorylates ERK-1/2. All these steps lead to the regulation of transcription. Defects in any of these interactions are known to cause immune related disorders, including ZAP70 mutations in SCID, a severe immunodeficiency (Walkovich and Vander Lugt, 2021).

The TCR-pMHC interaction is a two sided interaction and does therefore not only lead to signaling events in the T-cell but also in the APC. However, compared with TCR signaling knowledge about MHC-I reversed signaling research is still in its infancy. Almost all cell types have MHC-I molecules but signaling studies were conducted for only a few cell types. Among them were immune cells, like B-cells (Baba and Kurosaki, 2011), NK cells (Jewett and Bonavida, 2000), T-cells (Woodle et al., 1997) and tumor cells (Woodle et al., 1997), MHC-I ligation may cause signal transduction and cell regulatory effects leading to programmed cell death (PCD) / apoptosis. Other recent studies suggests that MHC-I reverse signaling takes part in controlling the response of the immune system to pathogens and malignancies (Muntjewerff et al., 2020). An example from 2012 showed that MHC-I reverse signaling attenuated the inflammatory response via the Fps-SHP-2 pathway preventing sepsis in mice (Wan et al., 2008).

Cell signaling studies do not usually use two different kind of cells. In most instances soluble antibodies are used that directly stimulate the receptor of interest. Other signaling studies do rely on a second cell type but in this instances the second cell type is almost always fixed. The method used in this study overcomes limitations associated with the two previous methods by more closely resembling natural biological processes and the signaling events that result from them. While similar studies observed signaling events over longer time periods (Lo et al., 2018), this is to the best of my knowledge the first study using this method to observe signaling over a time period of minutes.

In this paper parameter were optimized to study events related to signaling on the TCR and pMHC side. This can be used to study global signaling events in biological relevant systems.

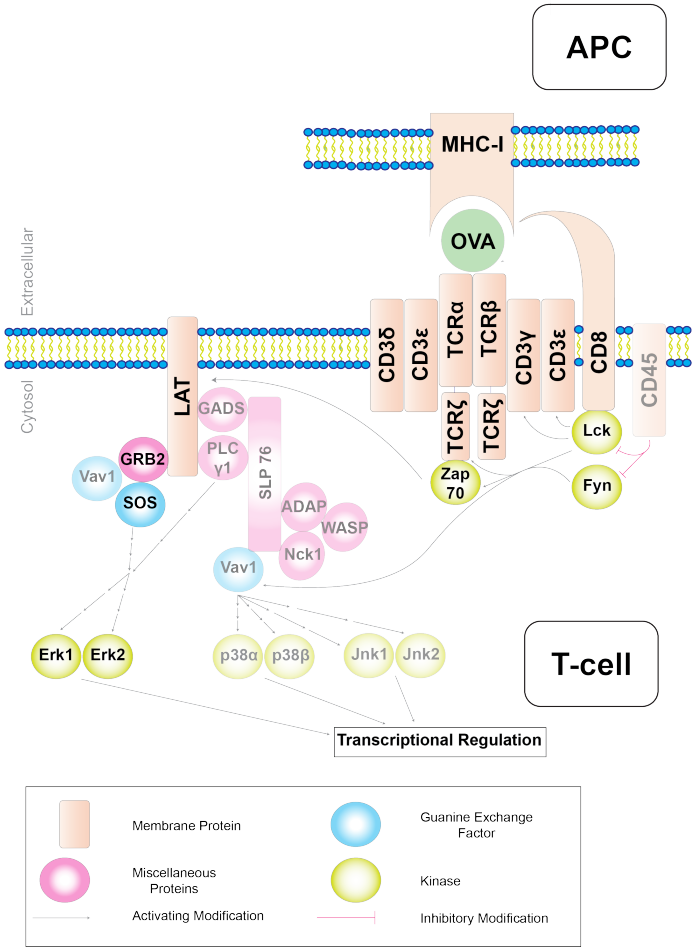

Figure 1: Schematic representation of T-cell receptor (TCR) ligation and early T-cell signaling events. Rich-colored proteins play an important part in activating the MAPK/ERK signaling pathway. Light-colored proteins highlight some alternative targets for observing early TCR signaling events.

2 Materials and Methods

2.1 Cell lines

Three cell lines were used in this experiment. Two of them were immortalized T lymphocyte, Jurkat, cell lines. They were the wild type JE6 cell line (American Tissue Culture Collection, Manassas, VA) and the J.Lck-.muOT-1.hCD8.hLckFL cell line (Dr. Arthur Weiss, UCSF, San Francisco, CA), which will be called from now on J.OT1. T2-Kb (Dr. Arthur Weiss, UCSF, San Francisco, CA), a T-cell X B-cell hybrid (Lautscham et al., 2003), with an empty class I MHC receptor, was the third cell line.

2.2 Cell culture

All cell lines were cultured in RPMI-1640 media containing 10% heat inactivated, sterile-filtered Fetal Bovine Serum (FBS), 2mM $_{\text{L}}$-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin under standard conditions (5% CO_2, 37°C) and maintained in log phase.

2.3 General coculture procedure

The cells were counted, the exact number of cells harvested, and then centrifuged at 500g for 5 minutes. After centrifugation the media was aspirated, and the cells were resuspended in Dulbecco's phosphate-buffered saline (DPBS). The in DPBS resuspended cells were then aliquoted into microcentrifuge tubes. Cell were allowed to rest at 37°C for 40 minutes to reduce basal cell activation before coculture. Cell-to-cell contact was initiated by centrifugation at 500g for 30 seconds and then kept at 37°C. Basal state samples (denoted '0 minute' samples) were centrifuged at 500g separately for 30 seconds and lysed immediately. All other coculture samples were lysed after the specified time by 1:1 dilution in 8M Urea lysis buffer containing a phosphatase inhibitor cocktail (8M Urea, 20mM HEPES, 1mM Sodium orthovanadate, 2.5mM Sodium pyrophosphate, 1mM β-glycerophosphate) and vortexing. Samples were then transferred to ice for at least 15 minutes, sonicated at 49% amplitude for 30 seconds, and cleared by centrifugation for 10 minutes at 24,000g before diluting in Lamelli Western sample buffer (125mM Tris pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue)

2.4 Negative control experiments

2.4.1 T2Kb resting time determination

To determine the optimal resting time for the T2Kb cell line five resting time points (0, 10, 20, 30, and 40 minutes) were tested. Every time point had two conditions: lysing and first spinning down and then lysing. Each condition had $2e^7$ T2Kb cells. The result of this experiment informed all following experiments about the optimal resting time.

2.4.2 Determining the effect of centrifugation on basal Erk1/2 phosphorylation in JE6, JOT1, and T2Kb

The lyse times were set for this and all following experiments at 0, 2, 5, and 10 minutes. Both Jurkat cell lines had $5e^6$ cells per replicate and the T2Kb cell line used $2e^7$ cells per replicate.

2.4.3 Testing the effect of T2Kb:JOT1 and T2Kb:JE6 coculture conditions in the absence of Ovalbumin on ERK1/2 activation

In this experiment $2e^7$ T2Kb and $5e^6$ JOT1/JE6 cells were used per replicate. The T2Kb cell line was rested for 40 minutes.

2.4.4 The effects of a missing OT-1 TCR on ERK1/2 activation were tested in a T2Kb:JE6 coculture with Ovalbumin

This parameter change tells us the effect of a TCR that is not specialized in recognizing Ovalbumin in the presence of Ovalbumin (Lot #32307, Rockland Immunochemicals, Pottstown, PA) on ERK1/2 signals. JE6 cells do not harbor a TCR specific to OT-1/MHC and therefore are not expected to signal upon coculture. Each T2Kb and JE6 replicate had $2e^7$ and $5e^6$ cells, respectively. The T2Kb cells were centrifuged at 500g for 5 minutes and after the initial spinning down, the cells were resuspended in RPMI-1640 media containing Ovalbumin (1 μm) for 180 minutes. This incubation was followed by aspirating the Ovalbumin containing media off and resuspending them in DPBS. The remaining steps were as described in the general procedure.

2.5 Parameter optimization experiments

2.5.1 Different Ovalbumin incubation times in a T2Kb:JOT1 coculture experiment and its effect on ERK1/2 signaling

Cell ratio between T2Kb and JOT1 cells and the Ovalbumin concentration stayed fixed in this experiment and the one independent variable in this setup is the Ovalbumin incubation time. After the initial spinning down of 500g for 5 minutes the cells were split into three equal amounts and then resuspended in RPMI-1640 media containing 1 μm Ovalbumin. The incubation times for this experiment were 30, 90, and 180 minutes. This incubation was followed by aspirating the Ovalbumin containing media and resuspending cells in DPBS. The remaining steps were as described in the general procedure. The result of this experiment informed all following experiments about the optimal Ovalbumin incubation time.

2.5.2 Signaling effects in an experiment with changing cell ratios in Ovalbumin incubated T2Kb cells together with JOT1 cells in coculture

The Ovalbumin incubation time for the T2Kb cells stayed at 90 minutes throughout this experiment and the Ovalbumin concentration was fixed at 1μm. Three different T2Kb cell numbers ($2e^7$, $1.25e^7$, and $5e^6$ T2Kb) were combined with $5e^6$ JOT1 cells in this experiment to observe the effect of different cell ratios on signaling. After the initial spinning down of 500g for 5 minutes the cells were resuspended in RPMI-1640 media containing 1 μm Ovalbumin. The T2Kb incubation time for this experiment was 90 minutes. This was followed by aspirating the Ovalbumin containing media off and resuspending cells in DPBS. When coculture was initiated $2e^7$, $1.25e^7$, and $5e^6$ T2Kb cells were paired with $5e^6$ JE6. The remaining steps were as described in the general procedure. The result of this experiment informed the final experiment about the optimal cell ratio.

2.5.3 Effects of changing Ovalbumin concentrations in T2Kb:JOT1 coculture experiments

In this third experiment the T2Kb cells were incubated for 90 minutes and each replicate used 2e7 T2Kb cells and $5e^6$ JOT1 cells while three different Ovalbumin concentrations were tested for their effect on signaling. After the initial spinning down of 500g for 5 minutes, the cells were split in three equal amounts and resuspended in RPMI-1640 media containing various concentrations of Ovalbumin: 0.1, 0.01, and 0.001 μm, respectively. This 90 minute incubation was followed by spinnning down the cells for 5 minutes at 500g and aspirating the Ovalbumin containing media off and resuspending the cells in DPBS. The remaining steps

were as described in the general procedure.

2.6 Western Blot

Proteins were separated on a 10% SDS-PAGE gel, transferred to a PVDF Immobilion membrane (MilliporeSigma, Burlington, MA), and blocked using SuperBlock Blocking Buffer (ThermoFisher Scientific, Waltham, MA). Then the membrane was incubated with α-pERK1/2 (T202/Y204) and α-ERK1/2 (Catalog numbers 9101 and 9107, Cell Signaling, Danvers, MA) primary antibodies. The membrane was then incubated with donkey-α-mouse IgG and goat-α-rabbit IgG (Catalog numbers 926-68072 and 926-32211, LI-COR, Lincoln, NE) secondary antibodies and scanned on a Odyssey CLx Imaging System (LI-COR, Lincoln, NE). Fluorescence intensity of each lane for the 700nm and 800nm channels were measured and quantified using FIJI (Schindelin et al., 2012). FIJI and Adobe Illustrator were used to prepare the images.

3 Results and Discussion

3.1 Missing components of the TCR/Peptide/MHC complex lead to incomplete ERK1/2 Activation

The JOT1 cells express the CD8 coreceptor and have a transgenic T-cell receptor (OT-1) capable of binding to Ovalbumin$_{257-264}$ (SIINFEKL) peptide. T2Kb are APCs with an empty class I MHC capable of presenting Ovalbumin$_{257-264}$ (SIINFEKL) peptide when pulsed *in vitro*. Wildtype JE6 with its low Ovalbumin affinity was included as a negative control for JOT1. Phosphorylated extracellular signal-regulated Kinase (ERKs) was used as a read out for T-cell receptor activation. ERK1/2 was an attractive, but not the only possible, target for TCR activation readouts. It was an attractive target because ERK1/2 antibodies are readily available, well established and of high quality. This readout was used for all experiments in the project. It was hypothesized that ERK1/2 activation as a result of TCR signaling would only take place when the following components Ovalbumin, an APC with a MHC-I receptor capable of presenting Ovalbumin and a T-cell with a TCR capable of ligating Ovalbumin (JOT1) plus a CD8 coreceptor are present during the procedure.

Previously it was shown that cell-to-cell contact due to centrifugation can activate signaling pathways (Orlova et al., 2008). After an initial spin down at 500g for 5 minutes the T2Kb cells were exposed to different resting times and treatments. This was done to determine how long it takes for the T2Kb cells to

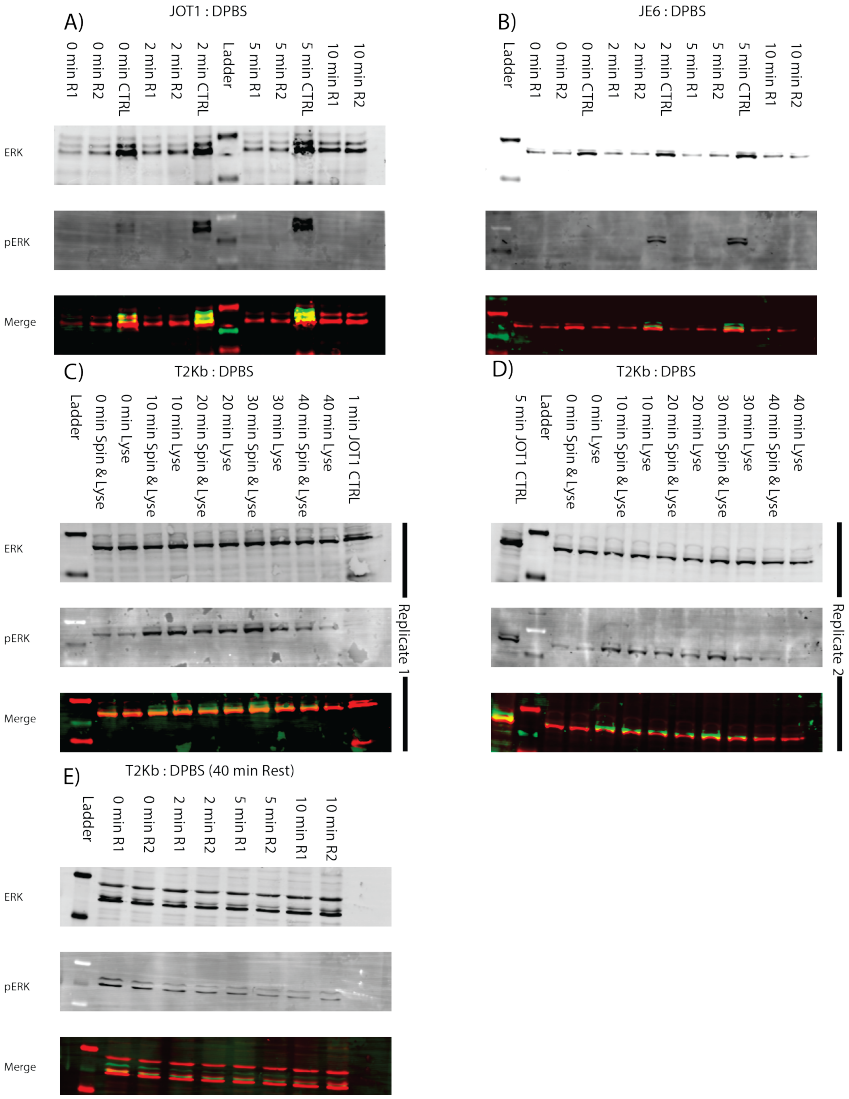

Figure 2: The Western Blots (A-E) show the results from the procedural control experiments. A) No ERK phosphorylation was observed when JOT1 was by itself during the experiment, while the loading controls showed strong signaling at the 2 and 5 minute time points. B) ERK1/2 activation was missing for all time points of the treatment groups (Loading Control showed ERK phosphorylation. C and D) shows replicate 1 and replicate 2 of the same T2Kb resting time experiment. Each time point had two treatments (lysis after a spin for 30s at 500g and cell lysis directly). ERK1/2 activation goes back to basal signaling after 40 minutes. E) is the T2Kb cell line after a 40 resting time. Only basal pERK signaling is observed during the entire time course.

go back to basal state after agitation (see Figure 2 C and D for results). It was not prudent to quantify most of the Western Blots due to persistent background flourescence, artifacts on the membrane, and loading issues combined with a low number of replicates. A qualitative analysis of the Western Blot reveals peak

ERK activation after 10 minutes and it stays elevated for another 20 minutes. At the 40 minute time point signaling is back to basal in both replicates. In conclusion, the 40 minute resting time was used in all following experiments.

The next three negative control experiments included the two Jurkat cell lines and the T2Kb cell lines by themselves. Figure 2 A and B show the two Jurkat cell lines JOT1 and JE6 by themselves (MHC is vacant and unable to stimulate TCR signaling and ERK activation). These experiments were conducted to establish baseline signaling for the three cell lines. The JOT1 ERK blot suggests uneven loading across the four time points for both replicates. Phosphorylation activity is not visible across them. Wildtype JE6 did not show any pERK activity either. Figure 2 E showed the repeated T2Kb experiment using the 0, 2, 5, and 10 minute time points and only minimal pERK activity was observed.

The purpose of the next two negative control experiments was to observe ERK activation in a coculture experiment that included APC and Jurkats but no Ovalbumin. It was hypothesized that in the absence of Ovalbumin only minimal signaling is observed. The Western Blots of the T2Kb with JOT1 and T2Kb with JE6 coculture experiements showed (Figure 3 A and B) when compared to the loading control samples minimal ERK activation. Both experiments were at the end of the 10 minute time course back to basal. The results supported the hypothesis.

The final negative control coculture experiment was conducted using the Ovalbumin incubated APCs with JE6. This was done to observe signaling were all components for effective signaling are present with the exception of a high affinity TCR receptor capable of effectively recognizing Ovalbumin. It was hypothesized that we would see some signaling because the JE6 receptor will show some affinity towards Ovalbumin. A clear downwards trend in pERK over the time course was observed in this JE6 with T2Kb coculture experiment (Figure 3 C). Signaling was constantly low when compared with the loading control.

3.2 ERK activation across all incubation times

Figure 4 A and B reveals for both replicates in the ERK plot uneven loading of the wells. ERK activation is found across all three incubation times. Uneven loading, high background, and artefacts on the membrane made a reliable quantification impossible. A 90 minute Ovalbumin incubation period was chosen for the next two experiments.

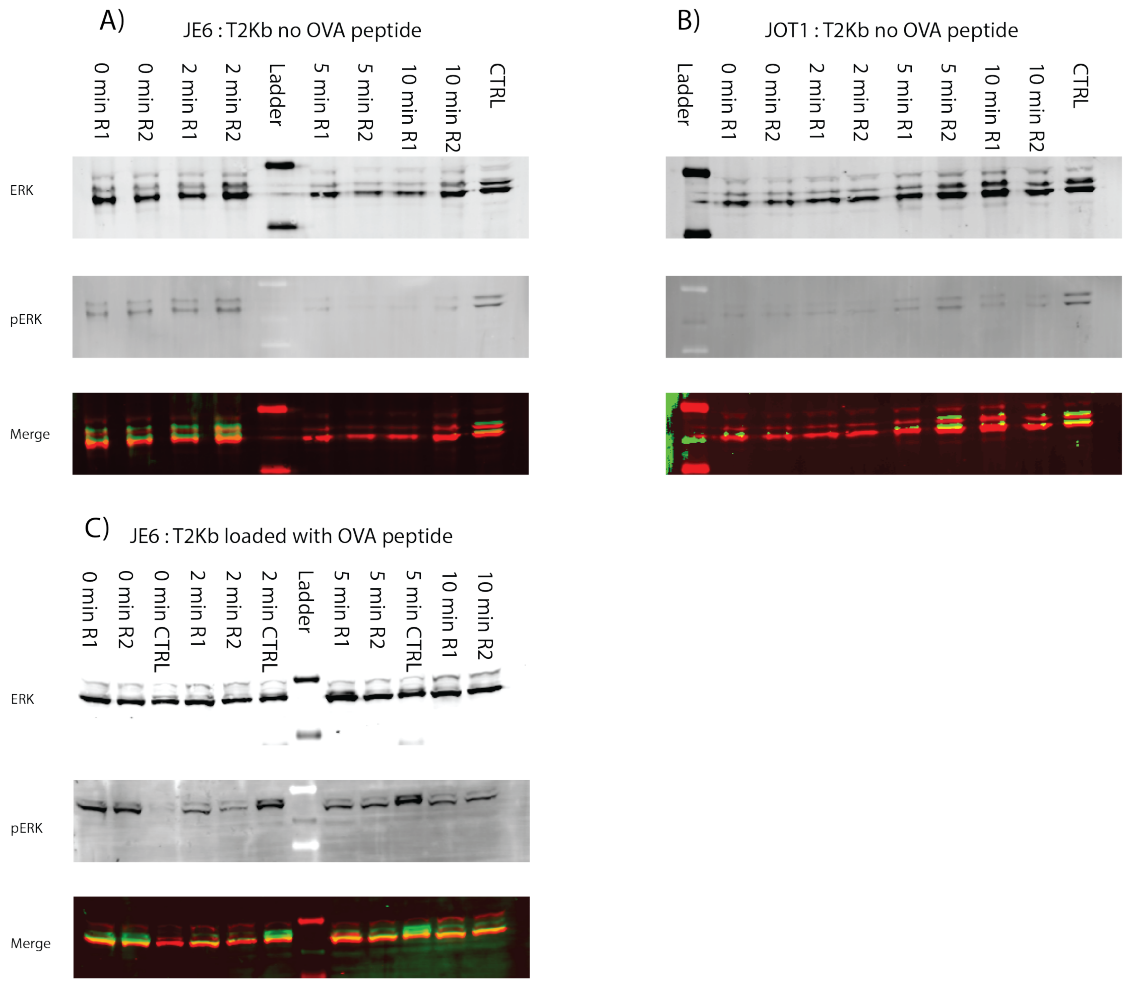

Figure 3: The two Jurkat cell lines were tested together with T2Kb cells in the coculture experiments A and B. The JE6:T2Kb and the JOT1:T2Kb coculture experiments were conducted without Ovalbumin. Both experiments show only minimal ERK1 and ERK2 phosphorylation across all time points. Figure C shows the JE6:T2Kb experiment. The JE6 cells were incubated for 180 minutes with Ovalbumin, and the cell ratio was kept at 1:4. Some pERK signaling was observed, but it went back to basal after 10 minutes. This ERK activation might have resulted from some affinity of JE6 towards Ovalbumin.

3.3 A T2Kb 1:4 JOT1 ratio produces the highest amount of pERK

It was hypothesized in this experiment that a higher total cell count would lead to increased ERK phosphorylation. The two replicates were generated in two different experiments and this explains in part the vastly different pERK levels between the replicates. Additionally, a comparison is further complicated by the fact that the 1:1 ratio experiment has only about 40% of the cells when compared with the 1:4 ratio experiment. However, both replicates (Figure 5 A and B) seem to show the highest amount of pERK in the 1:4 ratio groups. This was the condition picked for the final experiment.

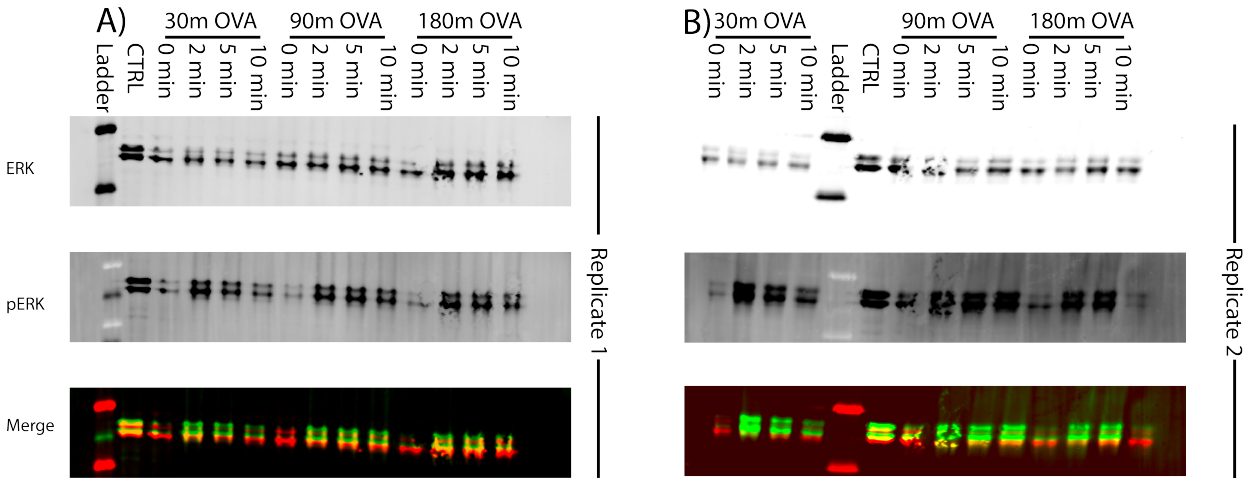

Figure 4: The Ovalbumin incubation was changed in this coculture experiment. There were a 30min, a 90min, and 180min incubation time groups. The JOT1:T2Kb cell ratio was fixed at 1:4, and Ovalbumin concentration was for all three treatment groups 1μM. Figures A and B show ERK1 and ERK2 activation for all three different incubation times. Quantification was not possible due to the artifacts and high background signals on the membrane. After a qualitative analysis a 90-minute incubation time point was picked.

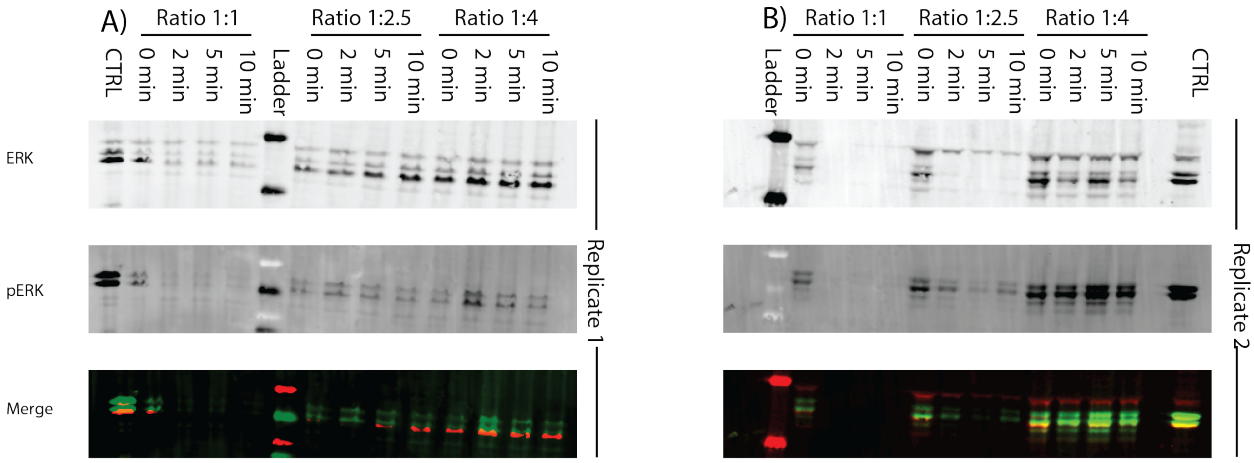

Figure 5: Three different JOT1:T2Kb ratios (1:1, 1:2.5, 1:4) were tested in this coculture experiment. The controlled variables were the incubation time of 90 minutes and the Ovalbumin concentration of 1μM. Replicate 1 (A) and Replicate 2 (B) of this experiment show almost no ERK for the ratio 1:1 in (A) and (B) and also at 1:2.5 in (B). The lack of an ERK signal could mean one of two things. Either there was a problem while loading the gel lanes or this is a consequence of the reduced cell count in these treatment groups. That said significant pERK activation was only observed in the 1:4 treatment group.

3.4 Minimal Ovalbumin concentration achieves ERK activation

Three different (0.01 μM, 0.1 μM, and 1μM) Ovalbumin concentrations were chosen for this experiment. This was done to find the minimal Ovalbumin concentration that achieves meaningful ERK activation.

In Figure 6 A and B pERK activity is seen across all Ovalbumin concentrations. Uneven loading (see ERK in 6 A) makes an exact interpretation of the result difficult. However, it seems that even the lowest concentration achieves ERK activation.

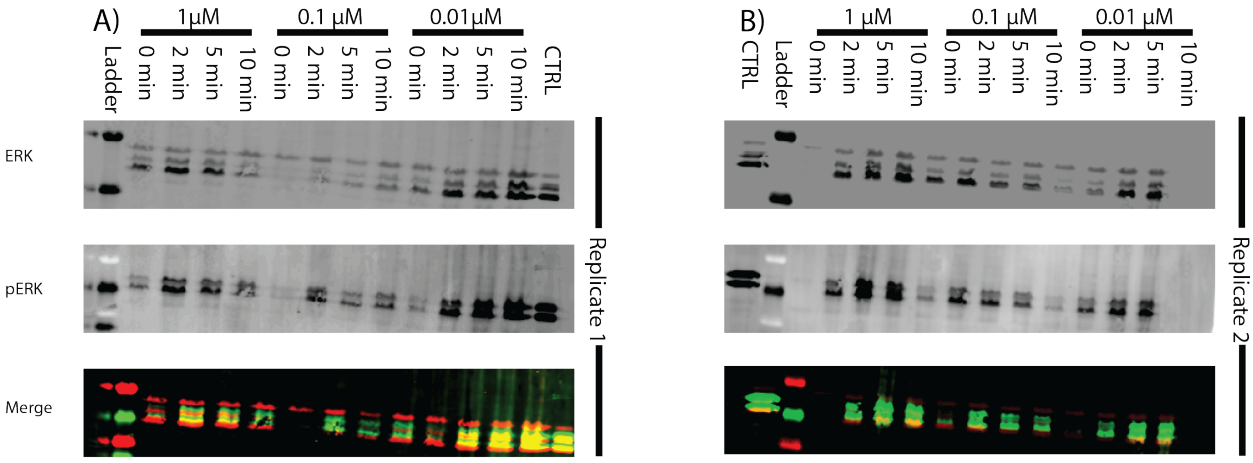

Figure 6: T2Kb cells were incubated with three (0.01μM, 0.1 μM and 1μM) different Ovalbumin concentrations for 90 minutes. The JOT1:T2Kb cell ratio was 1:4 in this experiment. Western Blots (A and B) show both replicates of this JOT1:T2Kb coculture experiment. This being said, blot A and blot B stem from the same experiment but they were conducted at different times. The lighter colors in the ERK blot (A) suggest that the lanes were loaded unevenly. This and the fact that they are not true replicates make an accurate interpretation of the results difficult. However, it seems even the lowest concentration produced a significant elevation of phospho-ERK in both blots.

4 Discussion

While quantitative analysis was not possible due to persistent artefactual issues, a qualitative analysis of the procedural controls revealed that missing components to the TCR/Peptide/MHC complex leads to suppressed phospho-ERK1/2 signaling. Substantial phopho-ERK1/2 activation was only observed when a $CD8^+$ T-cell with a mutated OT-1 TCR and Ovalbumin bound to MHC was present. Erk activation was achieved across all tested Ovalbumin concentrations, suggesting that the amount used in our protocols can be lowered. A similar result was found for Ovalbumin incubation times. Based on the results of the Ovalbumin incubated T2Kb with JOT1 incubation time coculture experiment, it seems acceptable to reduce the Ovalbumin incubation time from 180 minutes to 90 minutes. The data also suggests that the T2Kb:JOT1 cell ratio is an important parameter as ERK activation was only observed is the T2Kb:JOT1 in the 1:4 ratio. However, this study revealed another important result: It is now possible to study short term signaling

events in two distinct cell lines at the same time. The results from this method will encapsulate all cell-to-cell interactions and is not limited to one receptor. This is a clear advantage of this method over fixing cells or using antibodies to directly stimulate a receptor. The implications of this study are therefore twofold: Firstly, these positive findings allow for resource-sparing and therefore less expensive proteomics experiments, an often cost-prohibitive technique. Secondly, most T-cell signaling studies either used fixed cells (Zaru et al., 2002) or antibodies (Bisikirska et al., 2005) to stimulate the TCR-complex but the method described here allows to study signaling events between live T-cells and APCs that more closely resemble the interactions in the human body. Crucially, the method described in this paper allows us to observe signaling events in the minutes after the immune synapse was formed. Methods already developed like SILAC phosphoproteomics (Griffith et al., 2022) enables the analysis of signaling in APC and T-cells simultaneously. This would require to transduce understudied cell types with an OT-1 presenting MHC-I. A recent (Muntjewerff et al., 2020) review paper on reverse MHC-I signaling mentions only a few cell types that were studied: four different type of immune cells, epithelial and endothelial cells as well as smooth muscle cells and some tumor cells. Studying cell-to-cell interactions that way might illuminate new potentially drugable pathways for example in tumors or autoimmune disease. As an example, type 1 Diabetes is a disease in which T-cells are not self-tolerant and destroy pancreatic beta cells resulting in this autoimmune disease (Burrack et al., 2017). A better understanding of all the signalling pathways that lead to the destruction of beta cells might reveal a novel drugable target. In addition to studying class I MHC reverse signaling, methods that closely resemble the interactions in the human body can potentially be used to improve cell-based therapies. In 2017 the first two Chimeric Antigen Receptor (CAR) T-cell therapies were approved by the FDA for the treatment of B-cell abnormalities like certain leukemias and lymphomas (Brower, 2017). CARs use parts of the T-cell receptor signalosome for their anti-tumor activity and this approach achieved remarkable results in hematological cancers (Brentjens et al., 2013). While it is true that parts of the T-cell receptor signalosome are used, many aspects of CAR signaling are unique and a deeper understanding about differences and similarities between canonical TCR and CAR signals (Salter et al., 2021) would allow for a more rational engineered and improved CAR design (Lindner et al., 2020). Improved CAR constructs have the potential to move this promising new therapy out of the hematological cancer niche market into the broader application of solid tumors.

Building on these positive results, if quantitative results are required it seems advisable to repeat these experiments with more replicates allowing then for a rigorous statistical analysis. It would be of great interest to me to participate in a proteomics experiments using this method to illuminate and better understand

CAR-T signaling in the hope to improve that cell based therapy.

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