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Identification of mediators of T cell receptor signaling via screening of chemical inhibitor libraries

All changes to the main text have been highlighted in red. Email addresses of all authors have been added to the manuscript. The protocol is updated with more details and the tenses used have been rectified. Figures 2 and 3 have been modified, with new data used to prepare the plots and graphs. The dataset used for Figure 3 is now entirely new and no longer overlaps with previously published data. Lastly, the journal titles are no longer abbreviated.

**Reviewer #1:**

1. The assay is using the whole thymocytes population. This is problematic for several reasons. First, thymocytes are so heterogeneous. This means that the tested compounds could be inhibiting CD4-CD8- DN (ETP, DN1, DN2 and DN3; 3-5% of total thymocytes), CD4+CD8+ DP (80-85% of total thymocytes), and/or CD4+/CD8+ SP thymocytes (5-10% of total thymocytes). Even the DP thymocytes have different subpopulations (DP1, DP2 and DP3; please refer to Sinai M. Sci Signal 2010; Rafei et al. Blood 2013), which are at different developmental stages and display different responsiveness to TCR stimulation. How come peripheral T cells were not used for the screen as reported by Fouda A et al. JoVE 2017? This would be much easier.

It is indeed interesting to use the fluorescent reporter cells that were used by Fouda et al. However, our strategy is to exploit the association of cell death with strong TCR stimulation, thereby allowing the use of caspase-3 activation as a meaningful readout for TCR stimulation. The principle behind stimulation-induced cell death also functions as a self-correcting readout, controlling for inhibitor-induced cell death. Furthermore, thymocytes are more sensitive to TCR stimulation than peripheral T cells (Davey et al., J Exp Med, 1998). Essentially, assays carried out on thymocytes and lymphocytes are complementary, and any putative TCR signal inhibitors should be tested on both populations (Chen et al., Sci Rep, 2018). We agree that the thymocyte population comprises many different populations, but our analysis focused only on the bulk population of the double positive thymocytes, and we did not include the double negative thymocytes, which do not have TCR and would not respond to strong TCR stimulation. We have included the possibility of carrying out more in-depth analysis of thymocyte subsets by adding more cell surface markers in the manuscript.

1. The author should acknowledge that such HTS could not be performed using human cells as human thymocytes cannot be isolated from healthy or diseased individuals. This is a major limitation for the pharmaceutical industry.

We agree with the reviewer that our assay is not suitable for carrying out multiple high throughput screens for human thymocytes due to the difficulty in obtaining sufficient human thymocyte numbers, and we have included this limitation in the manuscript. However, if necessary, human thymus samples can be obtained from pediatric cardiac biopsies (Varas et al., J Immunol, 2000; Mitchell et al., Immunol Res, 2016; Verstichel et al., Sci Immunol, 2017) or from fetuses (Yamaguchi et al., Int Immunol, 1999; Farley et al., Development, 2013). Nonetheless, as TCR signaling pathways and the sequences of many signaling proteins are conserved between mice and humans, our thymocyte assay can identify TCR signaling pathways components that affect both mouse thymocyte and primary T cell activation, as well as human T cell activation (Chen et al., submitted). The thymocyte assay provides a useful preliminary screening strategy, and any results obtained with this assay with mouse thymocytes can then be verified using primary human lymphocytes.

1. A quick experiment should be conducted on peripheral T cells to confirm the screening data obtained using thymocytes as this is the major objective of the study. Are some of the identified inhibitors capable of inducing peripheral T-cell death?

We agree with the suggestion that additional assays should be carried out in peripheral T cells. We have verified the effect of some of the inhibitors on peripheral mouse and human T cell activation, using several different readouts of T cell activation, for example CD69 and CD25 (Chen et al., Sci Rep, 2018). We did not assess cell death in peripheral T cells as cell death is a physiologically relevant functional outcome of strong TCR stimulation in thymocytes, but not in peripheral T cells. With the exception of several special cases such as T cell exhaustion and a few others, the TCR signal strength that induces thymocyte death induces proliferation in peripheral T cells.

1. Thymocytes and T cells do not respond in a similar fashion to TCR stimulation. The major problem is the TCR threshold. For instance, DP thymocytes express a higher level of miR181a, which is known to inhibit over 40 phosphatases therefore enabling lower TCR thresholds for ZAP70 and ERK activation in response to TCR stimulation (Li QJ et al. Cell 2007; Li G et al. Nat. Med 2012). In other words, a weak or medium range kinase inhibitor displaying an effect on thymocytes might not be functional against peripheral T cells as a higher threshold is needed for their activation.

Thymocytes are indeed more sensitive to TCR stimulation than peripheral T cells. Therefore, it is more difficult to inhibit thymocyte activation than that of peripheral T cells, and our thymocyte assay therefore has the potential to identify the effect of a weak or medium range kinase inhibitor that could have been missed in screens for lymphocyte activity. So far, most of the inhibitors that reduced TCR signal in thymocytes were also effective in inhibiting peripheral T cell activation (Chen et al., Sci Rep, 2018).

1. The chances of identifying a druggable inhibitor of kinase for T cells using this approach might be low as it would have a negative impact on thymocyte development. This should be discussed more in detail as it is in my opinion a major limitation and could have a negative impact on the TCR repertoire and immunocompetency.

Our screening strategy employs an *in vitro* assay that does not assess thymocyte development in its entirety, which would require either an *in vitro* FTOC system or *in vivo* studies, but focuses on using caspase-3 activation and CD69 upregulation as readouts for TCR signaling in thymocytes. Therefore, the outcome of our assay is independent of any negative impact on thymocyte development that could be apparent if FTOC or *in vivo* experiments were used. Moreover, our analysis so far has identified several kinase inhibitors of TCR signaling (Chen et al., Sci Rep, 2018; Chen et al., submitted). In general, any inhibitor of T cell signaling could have a detrimental effect on thymocyte development, which is an effect independent on the cell type used for the screening assays.

Regarding the effect of inhibitors targeting key kinases of TCR signaling, including kinases with key roles not limited to T cells, a blockade of signal transduction could have repercussions on embryonic development and/or T cell maturation. It is possible to work on such key kinases using conditional or inducible KO models (Wex et al., Eur J Immunol, 2011; Berman-Booty et al., Vet Pathology, 2018). Moreover, not all kinases have only kinase-dependent functions. In such cases, kinase-dead mutant models can be used for studying the kinase of interest (Deakin et al., PLoS One, 2014).

1. The cell number used per well is too high! Is it because of the detection levels needed for the flow assays?

Based on our preliminary data, cell numbers used in this assay can be scaled up or down, particularly when adapting it for high-throughput screens. Furthermore, if stimuli in the weak or intermediate range were used, where response is not uniform and responding population is comparatively small, having more events per data paint may be needed to achieve robust readouts. Another point pertains to thymocytes being smaller than the usual cell types that companies base their estimates on for cultures.

1. Please note that Dexamethasone kills DP thymocytes with no effect on DN or SP thymocytes. The authors should consider a chemotherapeutic agent capable of inducing the cell death of all thymocyte populations. Otherwise, the effects observed might be only on DP thymocytes, which are already sensitive to cell death.

In our assay, Dexamethasone is used as a control for gating the population that has activated caspase 3. If it truly only affects DP thymocytes, it would only work to our advantage, since our analysis is based only on the DP thymocytes.

1. Please refer to the figure panels in the protocol as it may help the reader to follow while reading.

We apologize for the lack of clarity. We have added more references to the figures in the protocol.

**Reviewer #2:**

1. A better description of the cell washing system and perhaps its alternatives would greatly increase the impact of the manuscript.

We have added more details to the manuscript to provide a clearer picture of the pros and cons of the washing system.

1. Some technical details that could be addressed in the manuscript:

* The use of 10% charcoal stripped fetal bovine serum as an additive to RPMI will increase the viability of thymocytes especially if the time between dissection and experiment is long.
* Gently tweezing the thymus with two forceps and letting thymocytes flow out of the thymic epithelium generates much healthier thymocytes than mashing with syringes. These modifications may not affect thymocyte viability in routine experiments but in extreme conditions, such as long experiments or storage conditions, they may confound the experimental results because of handling associated apoptosis. Gentle handling will also decrease experimental variability due to novice dissectors applying too much pressure with the syringe. It should be noted that careful antibody titration rather than fixed dilution of 1:100 or 1:200 will decrease lot-to-lot differences in staining.

We are grateful for all the suggestions to improve the protocol. We have incorporated these suggestions to the protocol.

1. In Fig.2., it is important to show a CD4 vs CD8 plot and the gate taken for the cells being analyzed in this figure. Stimulated cells will downregulate CD8 gene expression, so the size of the DP gate is important where novice users may exclude signaled cells by using a tight DP gate.

We thank the reviewer for pointing this out. We agree that the gating strategy could be tricky to someone without prior experience and have added the requested plots and included a note of warning regarding the gating process in the protocol.

1. Also, in this figure on the right, it is not clear why there is such a big difference between cells exposed to DMSO and those that are not.

We apologize for the lack of clarity in the figure. We believe the issue was due to having collected too few cells during sample acquisition. We have repeated the experiment and collected more events for a more accurate representation of the effects due to the different treatments.

1. Plotting results as dot plots would be more consistent (with Fig. 1 and 4) and easier to understand than the averaged contour plotting method used here. The same applies to Fig 3.

We used the averaged contour plots due to the low event counts. As requested, we have changed the plots to the dot plots.