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Water influx is required for CD4+ T cell activation and T cell-dependent antibody responses

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THIS REPORT COVERS

EXTERNAL ASSESSMENT

Quality Scorecard

Originality: 4/5 -- The identification of a WNK1-OXSR1-STK39-AQP3 water influx axis as a requirement for TCR signaling and CD4+ T cell proliferation is genuinely novel; prior work on WNK kinases in lymphocytes focused on migration and adhesion, not osmotic regulation of antigen receptor signaling.

Importance of research question: 4/5 -- Understanding how physical cell volume changes couple to canonical TCR signaling cascades addresses a fundamental gap in T cell biology with potential implications for immunodeficiency and autoimmunity.

Claims are well-supported: 3/5 -- The convergence of genetic knockout, kinase-dead knock-in, and pharmacological inhibitor approaches is compelling, but the central causal claim that water influx per se drives ERK activation rests primarily on hypotonic rescue experiments that introduce multiple confounders not adequately controlled.

Experimental soundness: 3/5 -- The multi-pronged genetic strategy is a strength, but sample sizes in the in vivo experiments (n=3-5 per group in several key panels), absence of protein-level WNK1 knockout confirmation in primary T cells, and the use of a single pharmacological AQP3 inhibitor without genetic validation undermine confidence in several conclusions.

Clarity of writing: 3/5 -- The abstract and section conclusions are clear, but figure legends are incomplete, axis labels in several panels are non-standard or missing, and the statistical reporting is inconsistent across figures.

Value to community: 4/5 -- Establishing that osmotic cell swelling is a regulated, signaling-competent step downstream of TCR engagement would reframe how the field thinks about early T cell activation, and the WNK1 pathway is pharmacologically tractable.

Prior work contextualization: 3/5 -- The manuscript cites the core WNK-SPAK/OSR1 literature (Alessi et al., 2014; Shekarabi et al., 2017) but does not adequately engage with published work on aquaporin-mediated cell cycle regulation in non-immune cells, nor does it discuss whether the observed phenotypes could be explained by previously described roles of WNK1 in T cell migration and cytoskeletal organization.

Overall: This is a conceptually interesting manuscript with a coherent mechanistic story supported by converging genetic and pharmacological evidence, but it sits at the Nature Communications level in

its current form. The biggest gap is the lack of a direct, clean demonstration that water influx per se, rather than ion flux or volume-independent kinase activity, is the proximate driver of ERK activation.

SECTION 1

Novelty & Conceptual Advance

The core claim, that TCR engagement activates a WNK1-OXSR1-STK39 kinase cascade that drives osmotic water entry through AQP3, and that this water influx is causally required for downstream ERK activation and cell cycle entry, is a genuinely new idea. Prior work established WNK1 as a regulator of T cell migration via its effects on integrin-mediated adhesion and actin dynamics (the authors appropriately control for this in Fig. 2C-D using plate-bound stimulation), but the connection to osmotic signaling in the context of antigen receptor activation has not been made. The authors should emphasize more forcefully that this represents a conceptual reframing: cell volume increase is not merely a consequence of activation but an upstream regulatory input into TCR signal transduction. That framing is the most interesting thing about this paper and it is underplayed in the abstract.

However, the novelty claim requires more careful contextualization against two bodies of literature the manuscript does not adequately engage. First, Galán-Cobo et al. (2015) demonstrated that AQP3 overexpression in mammalian cells accelerates cell cycle progression and increases S and G2/M phase populations, proposing NF-kB and TNF signaling as downstream mechanisms. The present manuscript's finding that AQP3 inhibition arrests T cells in G2 is broadly consistent with this prior work, but the authors do not discuss it or explain how their mechanistic findings relate to or extend those observations. Second, Giusto et al. (2012) showed that AQP2-mediated water influx is directly coupled to cell cycle progression through a cell-sizing checkpoint mechanism in renal cells. The parallel to the authors' proposed model is striking and should be discussed explicitly. The failure to engage with these papers makes the novelty claim appear stronger than it is, which will be noticed by referees familiar with the aquaporin-proliferation literature.

The WNK-SPAK/OSR1 pathway as a master regulator of cation-chloride cotransporters is well established in the kidney and nervous system (Alessi et al., 2014; Shekarabi et al., 2017), and the authors correctly position their work as extending this pathway to immune cells. What is genuinely new is the demonstration that this pathway is activated by TCR/CD28 co-stimulation and that the downstream osmotic consequence, water entry, feeds back to amplify TCR signaling itself. This bidirectional coupling between signaling and volume is the conceptual advance that deserves the most emphasis.

SECTION 2

Strengths & Weaknesses of the Data Package

✓ Strengths

1. The convergence of four independent lines of genetic and pharmacological evidence for WNK1 kinase activity (Wnk1 conditional knockout, Wnk1D368A kinase-dead knock-in, WNK463 pharmacological inhibitor, and Oxsr1/Stk39 double mutant) all giving the same proliferation phenotype (Fig. 2A-J) is the strongest part of the paper. This redundancy substantially reduces the likelihood that off-target effects explain the result.

2. The mixed bone marrow chimera design in Section 1 is well-chosen. By restricting WNK1 deficiency to the T cell compartment while maintaining a WT hematopoietic environment, the authors cleanly attribute the antibody response defect to T cell-intrinsic WNK1 function, ruling out antigen-presenting cell or B cell contributions.

3. The isotonic control in Fig. 4F-H, using reduced NaCl with L-glucose to restore osmolarity without reducing ionic strength, is a thoughtful experiment that attempts to dissociate water influx from ion concentration effects. This is the kind of mechanistic rigor that elevates the paper.

4. The demonstration in Fig. 3K-M that ATR inhibition (AZD6738) rescues the G2 block in WNK1-inhibited cells, combined with the absence of DSB markers (p-RPA32, gamma-H2AX, Fig. S4C-D), provides a coherent mechanistic explanation for the G2 arrest that goes beyond simple proliferation impairment.

5. The adoptive transfer experiment with OT-II cells (Fig. 1C-J) showing impaired proliferation at day 3 and reduced TFH differentiation at day 7 provides in vivo validation of the in vitro proliferation phenotype, which is essential for arguing physiological relevance.

⚑ Areas for Improvement

1. My central concern is that the hypotonic rescue experiment (Fig. 4F-H) is being asked to carry more mechanistic weight than it can bear. Hypotonic medium simultaneously increases cell volume, reduces intracellular ion concentrations, alters membrane tension, activates stretch-activated channels, and changes the activity of numerous volume-sensitive kinases and phosphatases. The authors use the L-glucose isotonic control to argue that reduced NaCl concentration per se is not responsible, but this control does not rule out membrane tension changes, activation of PIEZO channels, or volume-sensitive activation of Rac1 or CDC42, all of which can independently activate ERK. The claim that water influx per se drives ERK activation is therefore mechanistically underdetermined by the data shown. I am not convinced that the

hypotonic rescue, however elegant, constitutes causal proof that water entry is the proximate signal. The authors should explicitly acknowledge this limitation and, ideally, test whether a cell-impermeant osmolyte (e.g., polyethylene glycol 400) that increases extracellular osmolarity and opposes cell swelling without altering ion concentrations suppresses ERK activation in WT stimulated cells.

2. The AQP3 inhibitor (DFP00173) data in Fig. 2L-M and Fig. 3K-L is used to support the conclusion that water entry through AQP3 is the relevant effector. However, there is no genetic validation of AQP3 loss-of-function in this manuscript. DFP00173 has not been extensively characterized for selectivity in primary lymphocytes, and AQP3 also transports glycerol and hydrogen peroxide, both of which have known roles in T cell biology (Hara-Chikuma et al., 2008 is cited but not adequately discussed). I would want to see at least a CD4-Cre x Aqp3fl/fl conditional knockout, or at minimum an Aqp3 germline knockout T cell proliferation experiment, to confirm that the DFP00173 phenotype reflects AQP3-specific water transport rather than glycerol or H2O2 transport inhibition. This is not a minor point: if the AQP3 inhibitor effect is mediated through H2O2 transport blockade, the entire water-influx model requires revision.

3. The figure presentation has systematic problems that need to be addressed before publication. In Fig. 1D, the flow cytometry histogram lacks a legend explaining what the black and red lines represent. In Fig. 2, the legend describes only panels A-B in detail, leaving panels C-M unexplained. In Fig. 3, the flow cytometry plots in panels D, G, and I lack gate boundary definitions on the DNA axis, which makes the cell cycle quantification unverifiable from the figure alone. Across Figs. 2 and 3, error bar definitions (SEM vs. SD) are not consistently stated. These are not cosmetic issues: without knowing whether error bars are SEM or SD, the reader cannot assess whether the scatter reflects biological variability or measurement noise.

4. The in vivo sample sizes in Fig. 1E-J are 3-5 mice per group with substantial scatter, yet statistical significance is claimed for several comparisons. For example, the TFH frequency data in Fig. 1H shows wide scatter with only 4-5 points per group. The authors use the Mann-Whitney U test throughout, which is appropriate for small non-parametric samples, but with n=3-4 the test has very low power and any significant p-value is fragile. The authors should either increase n to at least 6-8 per group for the key in vivo experiments or explicitly acknowledge that these experiments are underpowered and present them as preliminary in vivo validation rather than definitive results.

5. The manuscript does not engage with the published finding by Galán-Cobo et al. (2016) that AQP-mediated effects on proliferation may not be separable from water permeability itself, and that non-canonical AQP functions (protein-protein interactions, membrane organization)

could contribute independently. This is directly relevant to the interpretation of the AQP3 inhibitor data. The authors claim that water influx through AQP3 is the relevant effector, but the pharmacological inhibitor cannot distinguish water transport from structural AQP3 functions. This ambiguity should be acknowledged.

6. The Stk39T243A allele used in the double mutant (Oxsr1-/- Stk39T243A/T243A RCE) is a kinase-dead allele that prevents WNK1-mediated phosphorylation of STK39, but STK39 has WNK1-independent substrates. The interpretation that the double mutant phenotype reflects specifically the WNK1-OXSR1-STK39 axis requires that the authors confirm that STK39 kinase activity toward its downstream ion cotransporter substrates (specifically SLC12A2/NKCC1) is intact in the single Stk39T243A mutant but abolished in the double mutant context. Without this, the mechanistic assignment is incomplete.

7. The figure analysis flags that Fig. 1B shows very wide and overlapping confidence intervals at most timepoints for the NP-IgG1 ELISA data, with apparent significance claimed at days 21 and 28 with n=3-4 per group. ## 3. Journal Fit & Publication Potential

In its current form, this manuscript is appropriate for Nature Communications. The conceptual advance is clear and the experimental approach is multi-pronged, but the mechanistic depth and in vivo sample sizes do not reach the bar for Nature Immunology or Immunity, which would require cleaner causal proof of the water influx mechanism and more robust in vivo validation. Nature Communications regularly publishes work of this type: a novel signaling axis with converging genetic and pharmacological evidence, a coherent mechanistic model, and clear in vivo relevance.

If the authors address the AQP3 genetic knockout experiment and strengthen the mechanistic argument about water influx per se (as opposed to ion flux or membrane tension), the paper could be competitive at eLife or Journal of Experimental Medicine. A submission to Nature Immunology would require, at minimum, the AQP3 conditional knockout data, a more direct demonstration that cell volume increase is causally upstream of ERK activation (not merely correlated), and ideally some evidence that this pathway is relevant in human T cells.

SECTION 4

Key Experiments to Strengthen the Manuscript

A. ESSENTIAL

Genetic validation of AQP3 requirement. Generate CD4-Cre x Aqp3fl/fl mice (or use available Aqp3 germline knockout mice) and demonstrate that AQP3-deficient CD4+ T cells phenocopy the WNK1-

inhibitor proliferation and ERK phosphorylation defects shown in Fig. 2L-M and Fig. 4E. This experiment is essential because the entire water-influx conclusion depends on AQP3 as the water channel, and the pharmacological inhibitor DFP00173 cannot distinguish water transport from glycerol/H2O2 transport or structural AQP3 functions. Without genetic evidence, the AQP3 assignment is not secure.

B. ESSENTIAL

Direct test of the causal role of cell volume increase. Treat WT CD4+ T cells with a cell-impermeant osmolyte (e.g., sucrose or polyethylene glycol 400 added to isotonic medium) to oppose osmotic cell swelling during TCR/CD28 stimulation without altering ion concentrations, and measure p-ERK and Ki67 upregulation. If cell volume increase is causally required for ERK activation, hyperosmotic opposition of swelling should suppress p-ERK in WT cells. This experiment would directly test the water influx hypothesis in a way that the hypotonic rescue experiment in Fig. 4F-H cannot, because it acts on WT cells without introducing the WNK1 inhibitor or genetic perturbation.

C. ESSENTIAL

Protein-level confirmation of WNK1 knockout in primary CD4+ T cells. The current validation in Fig. S1 uses RT-qPCR to confirm Wnk1 mRNA reduction. An immunoblot for WNK1 protein in naive CD4+ T cells from Wnk1fl/-RCE mice after tamoxifen treatment should be shown. WNK1 is a large protein with a long half-life, and mRNA reduction does not guarantee protein depletion within the experimental timeframe.

D. RECOMMENDED

Identification of the SLC12A-family cotransporter downstream of OXSR1-STK39. The model in Fig. 4I proposes that OXSR1-STK39 activates SLC12A-family ion cotransporters (presumably NKCC1/SLC12A2) to drive ion influx preceding water entry. The manuscript does not demonstrate which cotransporter is involved or that its activity increases after TCR stimulation. Phospho-NKCC1 (T212/T217) immunoblot in TCR-stimulated WT vs. WNKi-treated T cells, combined with bumetanide (NKCC1 inhibitor) treatment to test whether NKCC1 inhibition phenocopies WNK1 inhibition, would fill this gap and make the mechanistic model more complete.

E. RECOMMENDED

Human T cell validation. Demonstrate that WNK463 treatment of human peripheral blood CD4+ T cells activated with anti-CD3/CD28 beads suppresses proliferation and ERK phosphorylation, and that hypotonic medium rescues these defects. This would establish that the pathway is conserved in

human cells and substantially increase translational relevance without requiring new mouse genetics.

F. RECOMMENDED

Temporal relationship between cell volume increase and ERK activation. The current data show that WNK1 inhibition reduces both cell volume (Fig. 2K) and p-ERK (Fig. 4A-E), but the temporal relationship between these two events is not established. A time-course experiment measuring cell volume (by CASY counter or flow cytometry forward scatter) and p-ERK simultaneously at 5, 15, 30, and 60 minutes after TCR/CD28 stimulation would test whether volume increase precedes or follows ERK activation, which is critical for the causal ordering claimed in the model.

SECTION 5

Mechanistic Depth

For Nature Communications, the current mechanistic depth is borderline sufficient but has one critical gap: the manuscript establishes that WNK1 kinase activity is required for ERK phosphorylation and that hypotonic medium rescues this defect, but it does not identify the molecular link between water influx and ZAP70/ERK activation. The data in Fig. 4A-C show that WNK1 inhibition reduces p-ZAP70 (Y319) without affecting p-CD3zeta (Y142), placing WNK1 between Lck and ZAP70 or at the level of ZAP70 itself. This is a specific and interesting mechanistic claim, but the pathway from water influx to ZAP70 phosphorylation is completely unaddressed. Does cell swelling alter the physical organization of the immunological synapse? Does it change membrane tension in a way that affects LAT clustering? Does it alter the activity of a phosphatase (e.g., SHP-1) that normally restrains ZAP70? For Nature Communications, the authors do not need to fully resolve this question, but they need to at least propose and test one candidate mechanism connecting volume change to ZAP70 activation. The current model in Fig. 4I jumps from water entry to "TCR signaling" without specifying the molecular target, which is the most important mechanistic gap in the paper.

The ATR activation story (Section 3) is mechanistically cleaner and more complete: the authors show elevated p-CHK1, absence of DSB markers, and ATR inhibitor rescue of the G2 block. This sub-story is convincing at the current level of detail. The connection between insufficient cell volume increase and replication stress (which would activate ATR) is plausible but not demonstrated. Specifically, it is not shown whether the S-phase defect (reduced EdU incorporation, Fig. 3D-E) reflects slower replication fork progression or reduced origin firing, either of which could activate ATR by distinct mechanisms.

SECTION 6

Translational Significance

The translational framing of this manuscript is appropriate and measured. The authors do not overclaim therapeutic readiness; they establish a basic mechanism in mouse CD4+ T cells with pharmacological proof-of-concept using WNK463, a compound already used in preclinical studies of hypertension. The relevance to T cell-dependent antibody responses, including vaccine responses and autoimmunity, is clearly stated and supported by the in vivo data in Section 1. This framing is well-matched to Nature Communications.

One area where the translational framing could be sharpened: WNK1 gain-of-function mutations cause pseudohypoaldosteronism type II (Gordon syndrome) in the kidney context (Shekarabi et al., 2017), and WNK1 is expressed in all tissues. The authors should briefly address whether WNK kinase inhibition, which would be required for any immunosuppressive application, would have acceptable on-target toxicity given WNK1's essential role in renal ion homeostasis. This is not a fatal objection, as many successful immunosuppressants have pleiotropic targets, but the omission of any discussion of this issue makes the translational section feel incomplete. Similarly, the observation that the WNK1-OXSR1-STK39-AQP3 axis is required for T cell proliferation but not for the earliest TCR signaling events (p-CD3zeta is intact, Fig. 4A) raises the interesting possibility that this pathway could be targeted to selectively suppress T cell expansion without abolishing TCR recognition, which would be a therapeutically attractive property worth discussing explicitly.

References

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