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Supplementary Materials for

Alternative ZAP70-p38 signals prime a classical p38 pathway through LAT and SOS to support regulatory T cell differentiation

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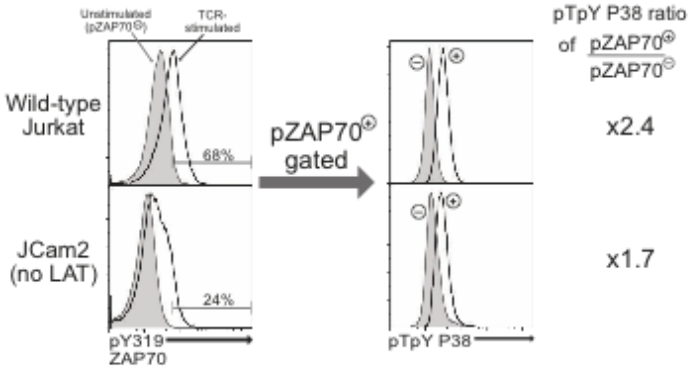
This PDF file includes:

S1A



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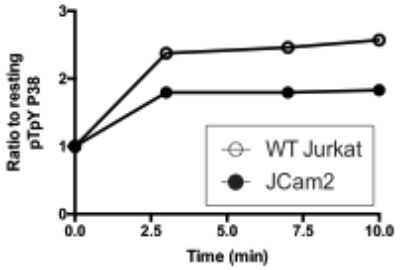

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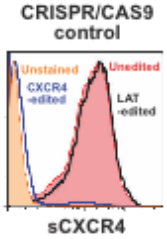

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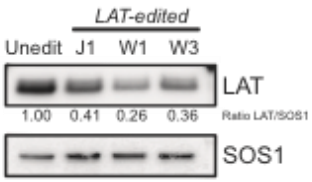

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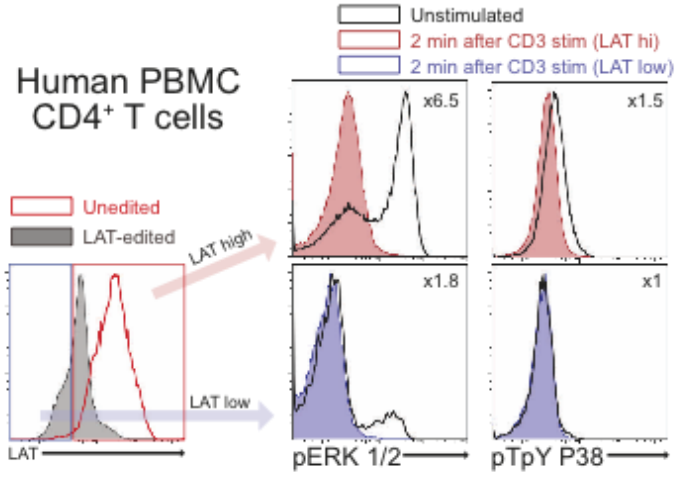
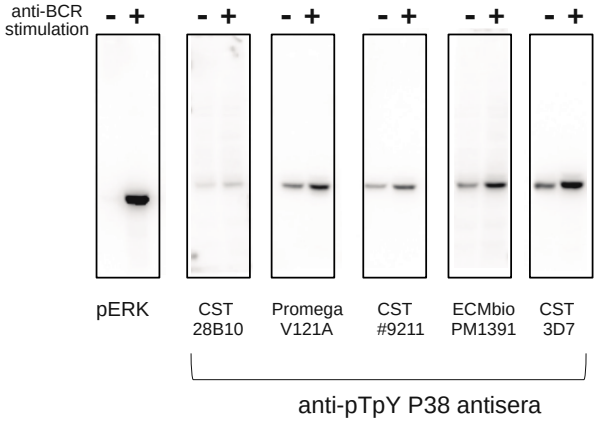


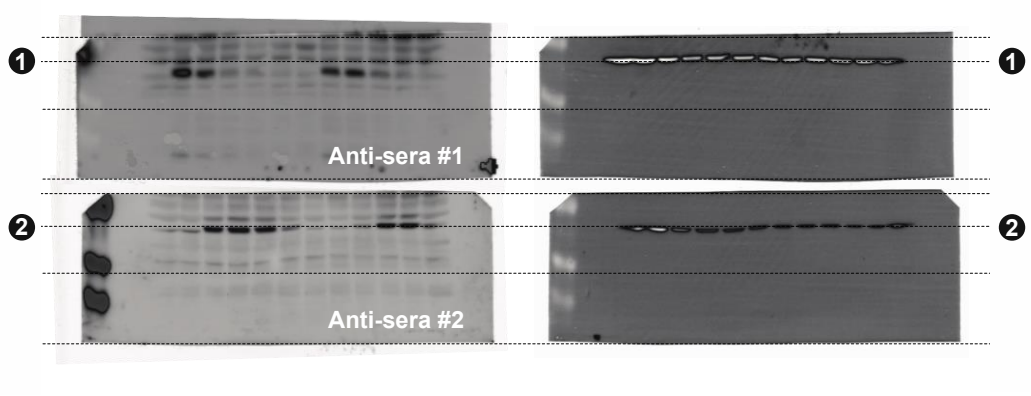
Fig. S1. The role of LAT in p38 activation in Jurkat T cells and human PBMC CD4$^+$ T cells.

(S1A) Flow cytometry analysis of pZAP70(Tyr^{319}) and pp38(Thr^{180}Tyr^{182}) in wild-type Jurkat cells and LAT-deficient JCam2 cells at rest (filled) or at 3 min after TCR activation (open).
(S1B) Activation of p38 after TCR stimulation was compared among cells that activated ZAP70 comparably (pZAP70$^+$ gate), and the fold increase relative to unstimulated cells is indicated (right).
(S1C) Flow cytometry analysis of cell surface CXCR4 (sCXCR4) abundance on human CD4+ T cells after CRISPR/CAS9 RNP electroporation. The efficiency of editing was determined in unedited (Red filled), LAT-edited (Black open), CXCR4-edited (Blue open) and unstained control (Orange filled histogram) cells. Data are representative of two independent experiments.
(S1D) Western blot analysis of LAT abundance in lysates from unedited human CD4+ T cells or after CRISPR/CAS9 RNP electroporation with LAT-specific CRISPR RNAs (crRNA's). Blots are representative of two independent experiments and band intensity values are given relative to the SOS1 control.
(S1E) The activation of ERK1/2 and p38 were determined in LAT hi and LAT low cells after TCR stimulation and insets indicate the fold increase after activation (open histogram) relative to unstimulated cells (shaded histogram).

S2A


anti-BCR stimulation
- + - + - + - + - + - +
pERK
CST 28B10
Promega V121A
CST #9211
ECMbio PM1391
CST 3D7
anti-pTpY P38 antisera

S2B


pY323 P38 probed
1
2
Anti-sera #1
Anti-sera #2

S2C

Total P38 probed
1
2

Fig. S2 Experimental validation of anti-phospho p38 antisera for pTpY p38 and pY323 p38 detection.

(**S2A**) To confirm inducible P38 phosphorylation, lysates were prepared from wild-type DT40 B cells before or after 5 min stimulation with anti-BCR (clone M4), followed by SDS-PAGE and Western blot transfer. Replicate membranes were probed in parallel with several pTpY P38 anti-sera from various manufacturers. Cell stimulation was confirmed by parallel probing of pERK2 (Cell Signaling Tech, Cat#9101). Tested antibodies are obtained from Cell Signaling Tech. (clone 28B10, Cat#9216; Cat# 9211; clone 3D7, Cat#9215), Promega (Cat# V121A) and ECM Bioscience (Cat# PM1391).

(**S2B & C**) Two reagents for pY323 P38 detection were compared and validated using identical lysate samples prepared from $CD4^+$ T blasts as in **Fig. 2F**: anti-sera #1 (ECM Bioscience, Cat#PP3411) and anti-sera #2 (Fisher Scientific/Invitrogen, Cat#PA5-12868). (C) The specificity of pY323 P38-probing reagents was confirmed by back-blotting each membrane with total P38 antibody. For accurate confirmation, developed images were overlayed with bright-field image visualizing membrane boundaries and molecular weight marker bands. Dashed lines mark the upper and lower edges of each membrane, as well as internal molecular size marker bands. Notably, the prominent induced bands from anti-sera #1 does not match the observed mobility of total P38 bands (symbol ❶), while bands from anti-sera #2 overlaps with total P38 signal (symbol ❷). Based this analysis, we conclude that anti-sera #1 detects inducible but non-specific signal, while anti-sera #2 probes stimulation-induced signal matching mobility of P38 bands.

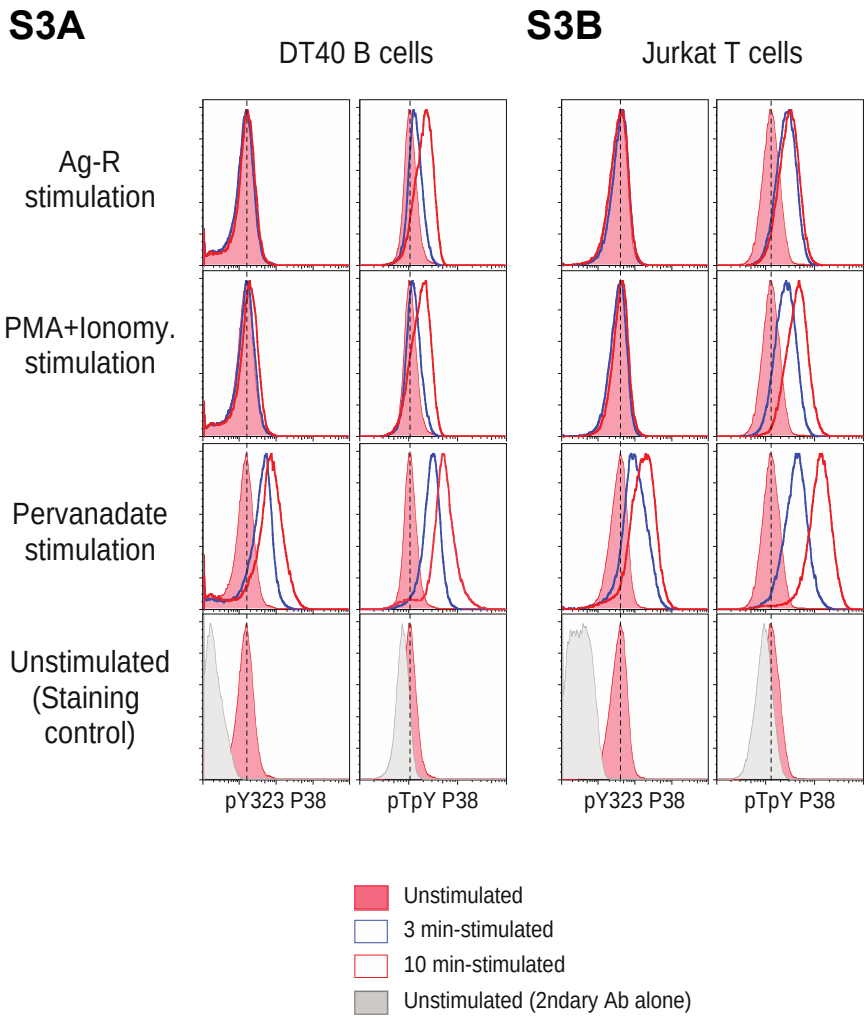

S3A
DT40 B cells
S3B
Jurkat T cells
Ag-R stimulation
PMA+Ionomy. stimulation
Pervanadate stimulation
Unstimulated (Staining control)
pY323 P38
pTpY P38
pY323 P38
pTpY P38
Unstimulated
3 min-stimulated
10 min-stimulated
Unstimulated (2ndary Ab alone)

Fig. S3 Testing anti-pY323 p38 antibody for pFLOW assay.

Aforementioned anti-pY323 antibody (Fisher Scientific/Invitrogen, Cat#PA5-12868) was further tested for application in pFLOW assay using DT40 B cells (A) or Jurkat T cells (B). DT40 B cells represent cells lacking alternate pathway through ZAP70-pY323 P38 when stimulated through antigen receptor (anti-BCR IgM, clone M4). Similarly, stimulation with PMA and ionomycin combination bypasses ZAP70-pY323 P38 connection, excluding alternate pathway even in Jurkat T cells. Activated pervanadate stimulation super-induces proteome-wide tyrosine phosphorylation and serves as a positive control for antibody staining. To note, tested anti-pY323 P38 reagent fails to detect inducible signal after TCR stimulation in Jurkat cells. Therefore, we conclude this reagent is not suitable for pFLOW application.

S4

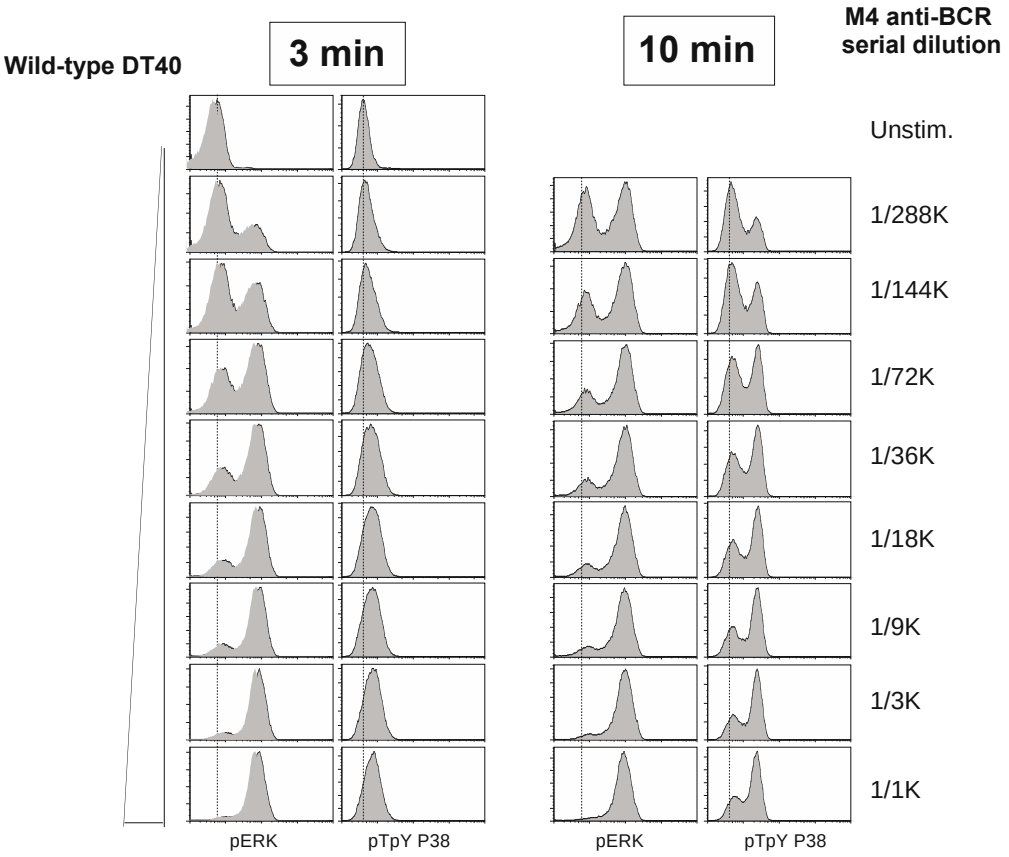
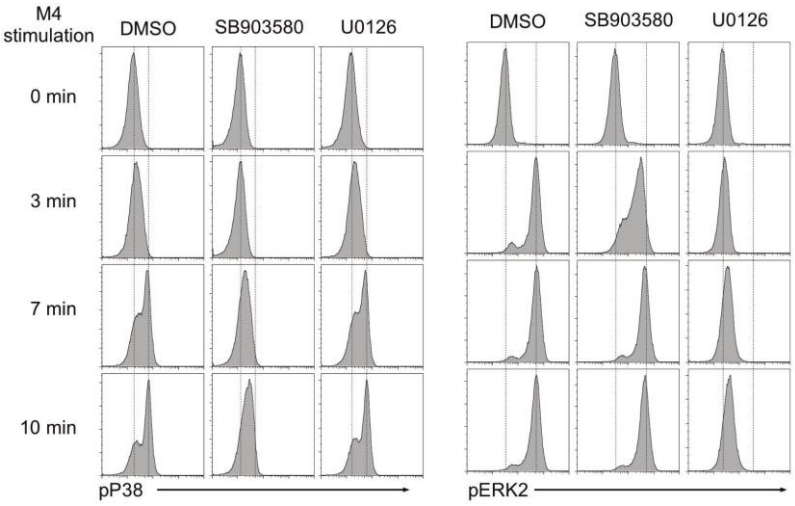

Wild-type DT40
3 min
10 min
M4 anti-BCR serial dilution
Unstim.
1/288K
1/144K
1/72K
1/36K
1/18K
1/9K
1/3K
1/1K
pERK
pTpY P38
pERK
pTpY P38

Fig. S4. Full activation of p38 through the classical pathway is thresholded and time delayed regardless of stimulus strength.

Stimulation of wild-type DT40 B cells with an increasing dosage of anti-BCR (clone M4) IgM antibodies for 3 and 10 min. Measurement of p38 or ERK2 phosphorylation by pFLOW. Dashed lines indicate the level of pERK or pP38 of unstimulated cells. Activation of both ERK and P38 in response to BCR stimulation shows bimodal pattern. To note, increase in time duration and stimuli strength alters the fraction of cells fully activating pERK (the right population in the bimodal histogram), while the appearance of bimodal status in pP38 assay is time-delayed regardless of M4 concentration.

S5A


M4 stimulation
DMSO
SB903580
U0126
DMSO
SB903580
U0126
0 min
3 min
7 min
10 min
pP38
pERK2

S5B

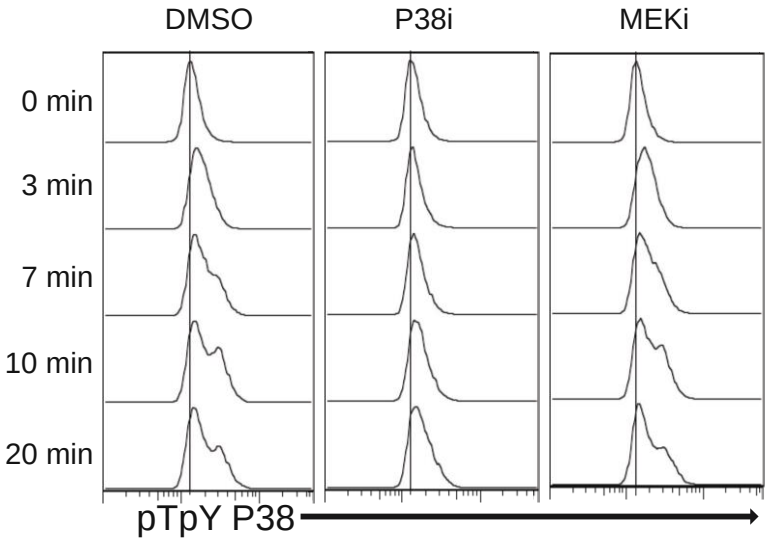
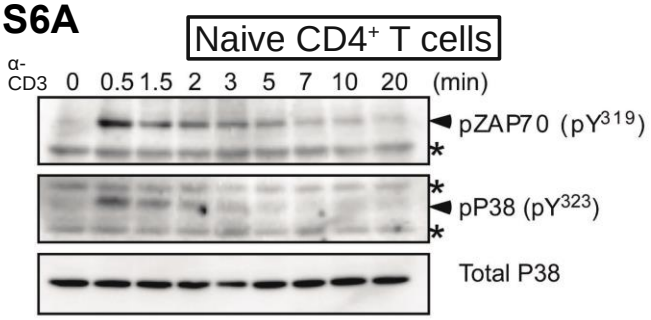

DMSO
P38i
MEKi
0 min
3 min
7 min
10 min
20 min
pTpY P38

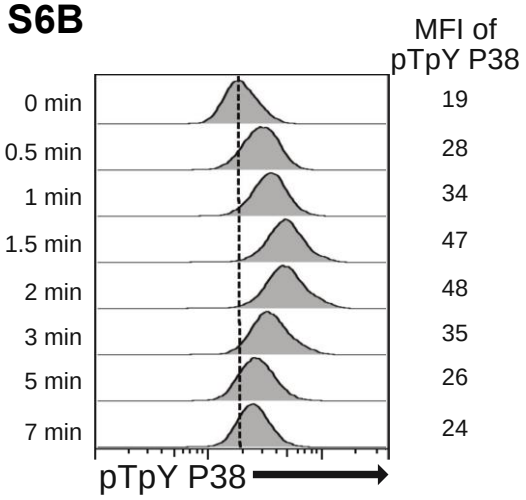
Fig. S5. Impact of p38 or MEK1 inhibition on BCR-induced ERK activation.

(S5A) Wild-type DT40 B cells are treated with DMSO (control), P38i (SB203580) or MEKi (U0126) and were stimulated with anti-BCR for pERK measurement at the indicated time. Dashed lines indicate the resting and fully activated pERK signal level.
(S5B) Flow cytometry analysis of p38 activation DT40 B cells treated with DMSO, P38i (SB203580) or MEKi (U0126) and stimulated with antibody against mouse BCR for the indicated times.

S6A


Naive CD4+ T cells
α-CD3 0 0.5 1.5 2 3 5 7 10 20 (min)
pZAP70 (pY319)
pP38 (pY323)
Total P38

S6B


MFI of pTpY P38
0 min 19
0.5 min 28
1 min 34
1.5 min 47
2 min 48
3 min 35
5 min 26
7 min 24
pTpY P38

S6C

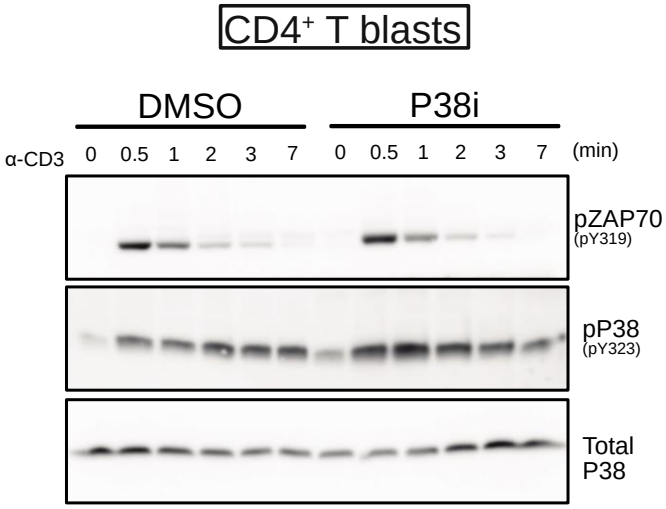
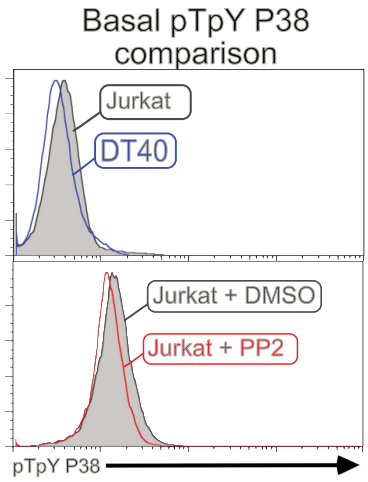

CD4+ T blasts
DMSO
P38i
α-CD3 0 0.5 1 2 3 7 0 0.5 1 2 3 7 (min)
pZAP70 (pY319)
pP38 (pY323)
Total P38

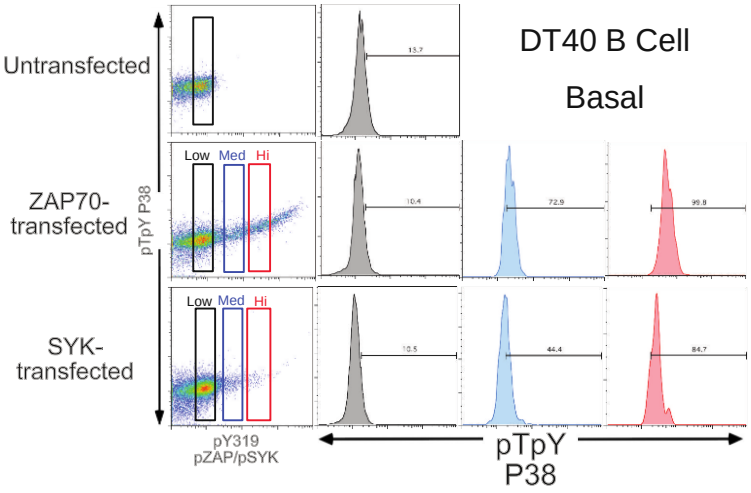
Fig. S6. p38 activation kinetics in primary T cells.

(S6A) Western blot analysis of pZAP70 and pp38 in lysates from primary naïve $CD4^+$ T cells stimulated with antibodies against CD3ε.
(S6B) Flow cytometry analysis of p38 activation of $CD4^+$ T blast cells stimulated with antibodies against CD3ε.
(S6C) Western blot analysis of phosphorylation of the indicated proteins in lysates from $CD4^+$ T cell blasts treated with DMSO or P38i and stimulated with antibodies against CD3ε **.**

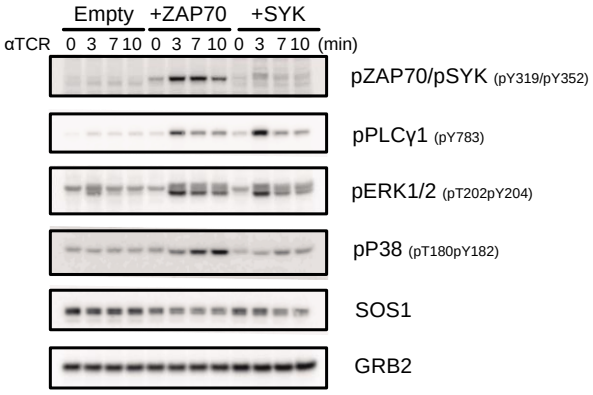
S7A



S7B



S7C



S7D

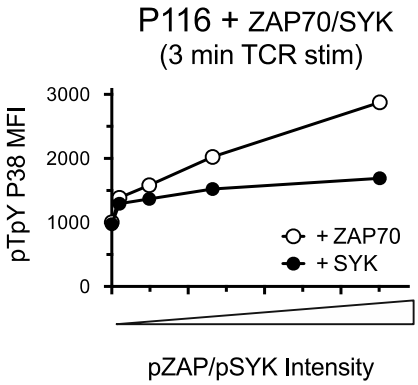
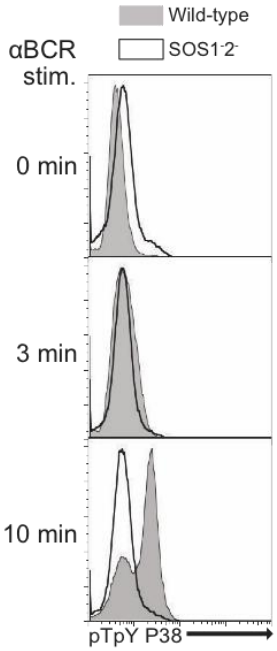


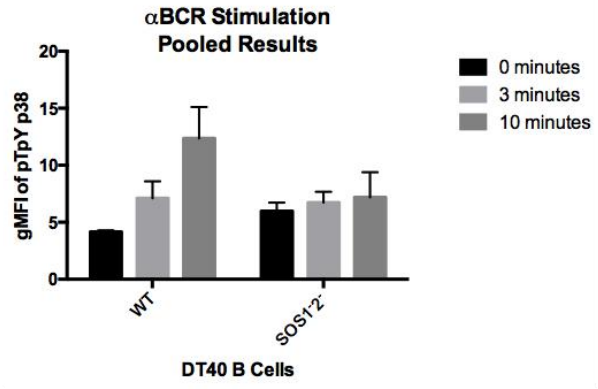
Fig. S7. Zap70 expression facilitates p38 activation.

(S7A) Flow cytometry analysis of p38 activation Jurkat and DT40 B cells (upper) or Jurkat cells treated with DMSO or the Src kinase inhibitor PP2.
(S7B) Flow cytometry analysis of pZAP70 and pp38 in DT40 B cells transfected with expression constructs for human ZAP70 or SYK kinases. Three sub-populations were arbitrarily defined as low (black), med (blue) and high (red) for pZAP70 or pSYK (left), and histograms indicate pp38 of the indicated sub-population with MFI values inset.
(S7C) Western blot analysis of the indicated proteins in lysates from ZAP70-deficient Jurkat T cells (P116) transfected with ZAP70 or SYK expression constructs and stimulated with antibody against CD3ε for the indicated times.
(S7D) Flow cytometry analysis of pZAP70 and pp38 in ZAP70-deficient Jurkat T cells (P116) transfected with ZAP70 or SYK expression constructs and stimulated with antibody against CD3ε for the indicated times. Mean Fluorescent Intensity (MSI) of pp38 was plotted as a function of pZAP/pSYK intensity.

S8A



S8B



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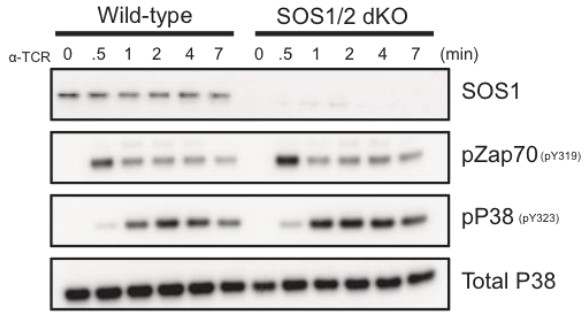

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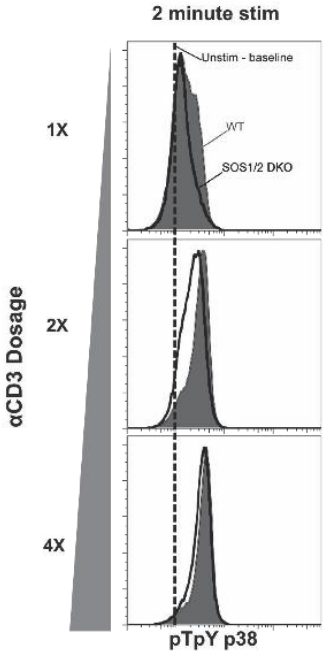
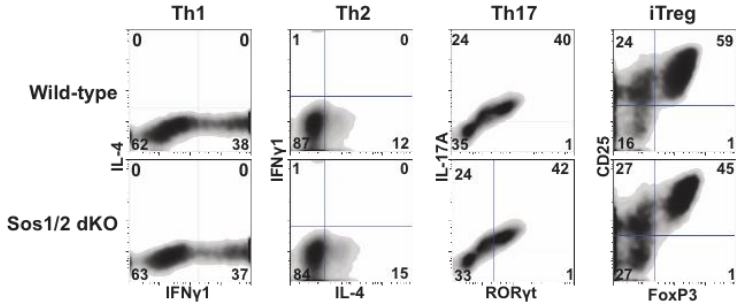


S8E



S8F

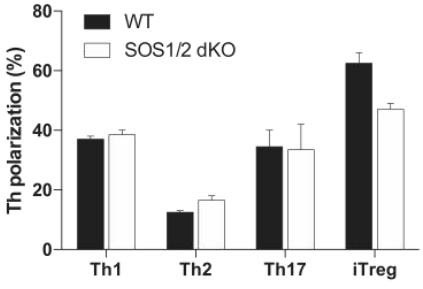
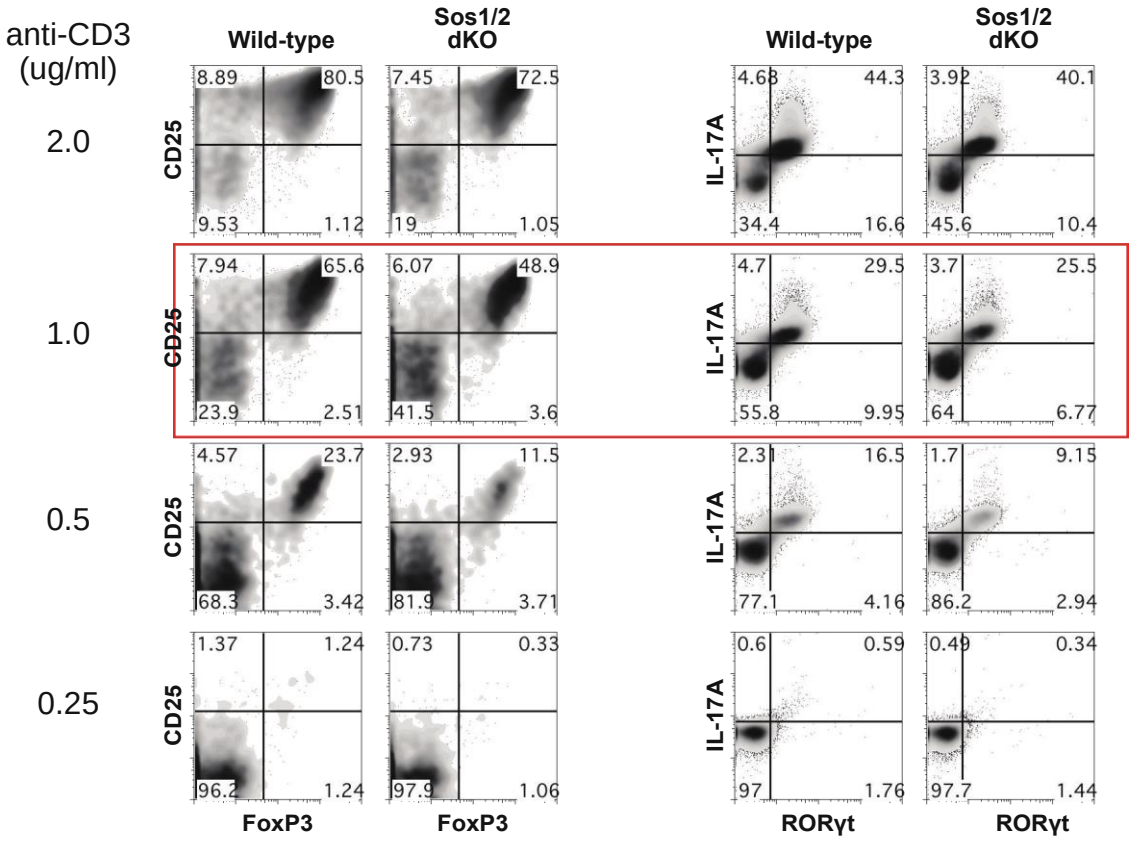


Fig. S8. The role of SOS in p38 activation and iT_{reg} formation.

(S8A) Flow cytometry analysis of pp38 in wild-type (filled) and SOS1/2$^{-/-}$ (open) DT40 B cells stimulated with antibody against the BCR for the indicated times.
(S8B) Mean and standard error of the independent experiments in figures 5A and S8A.
(S8C) Flow cytometry analysis of pp38 in wild-type and SOS1/2 dKO naïve CD4$^+$ T cells stimulated with antibody against CD3ε for the indicated times.
(S8D) Western blot analysis of the indicated proteins in lysates from wild-type or Sos1/2 dKO naïve CD4$^+$ T cells with antibody against CD3ε for the indicated times.
(S8E) Flow cytometry analysis of the indicated cytokine and transcription factor expression in wild-type (upper) and Sos1/2 dKO (lower) naïve T cells cultured under T_H1, T_H2, T_H17 or iT_{reg} polarizing conditions.
(S8F) Mean and standard error of the independent experiments in Figures 5E-G and S8E.

S9A iTreg (TGFb)

S9B Th17 (TGFb+IL-6)



(Figure S6)

Fig. S9. Sos1/2 double deficiency has more substantial impact on iT_{reg} than T_H17 cell differentiation.

FACS sorted naïve resting $CD4^+$ T cells were cultured with varying dosage of immobilized anti-CD3 antibody supplemented with 2 μg/ml soluble anti-CD28 in the presence of TGFβ alone or TGFβ + IL-6 inducing iT_{reg} or T_h17 cell differentiation respectively. Cells were cultured for 4 days for iT_{reg} ($CD25^{hi}FoxP3^+$) and T_h17 ($IL\text{-}17A^+ROR\gamma t^+$). Plots in the RED rectangle are shown examples in **Fig. 5G**.