Supplementary Information (SI) Appendix

Febrile temperature change modulates CD4 T cell differentiation via a TRPV channel-regulated Notch-dependent pathway

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Materials and methods (detailed information)

Mice

All mouse strains used (from the Jackson Laboratory, Bar Harbor, ME, USA) were maintained in the Small Animal Facility of the National Institute of Immunology. Mice of either sex aged 6-10 weeks were used for all the experiments. The mouse strains used were C57BL/6 (B6), IL4-/- (H-2b), OTII (H-2b), and Notch1-/- (kind gift of Prof. Apurva Sarin, INSTEM, Bangalore, India). OTII mice have transgenic TCRs restricted to H-2Ab and specific for an ovalbumin (Ova) peptide (amino acids 323–339 (Ova2), Invitrogen, USA). Mice were euthanised by cervical dislocation in all the experiments. For each set of experiment between 3–6 mice were used per group.

All mice were maintained and used in accordance with the guidelines and with the prior approval of the duly constituted 'Institutional Animal Ethics Committee' (IAEC) of the National Institute of Immunology. All methods were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) authorised for this purpose.

Mouse immunisation and re-stimulation assays

Three groups of BALB/c mice (n=3) were immunised subcutaneously with ovalbumin (OVA) in alum (100 µg/mouse). One group received daily sulprostone (2 µg/mouse) or capsaicin (200 µg/mouse) intraperitoneally, while the third group received vehicle alone. On day 7, draining lymph nodes (dLNs) were harvested and lymph node cells were stimulated *in vitro* within 96-well plates (3x10^5 cells/well) with OVA (300 µg/ml), and levels of IL13 and IFNg in the culture supernatants were assayed by ELISA 72 h later.

Human peripheral blood mononuclear cells (PBMCs)

PBMCs were separated by Ficoll-Hypaque (Ficoll-Paque, GE Healthcare) density gradient centrifugation from venous blood collected from healthy young adult volunteers of either sex (age range 23-30 years) after consent.

All human experiments were carried out in accordance with the guidelines and with the prior approval of the duly constituted 'Institutional Human Ethics Committee' (IHEC) of the National Institute of Immunology, New Delhi, and the 'IISER Ethics Committee for Human Research' (IECHR) of the Indian Institute of Science Education and Research (IISER), Pune. All protocols were approved by the IHEC, NII, or by the IECHR, IISER Pune, as appropriate for this purpose.

Reagents

RPMI 1640 medium (Biological Industries, Rehovot, Israel) containing 10% FCS, antibiotics, sodium pyruvate and non-essential amino acids (all from Sigma Aldrich) was used for all cultures. Various fluorochrome- or biotin-coupled antibodies to detect mouse CD4, CD25, CD44, CD62L, IFNg, IL4, IL5, IL13, Tbet, GATA-3, TCRValpha2, and human CD4, CD45RO, and CD25 along with appropriate isotype controls (BD Biosciences, eBioscience, BioLegend, Cell Signaling Technology) were used. Secreted IL4, IL13 and IFNg from mouse and human cell cultures were detected

using kits based enzyme-linked immunosorbent assays (eBiosciences). Cultures were supplemented with recombinant human IL2 (Roche) where necessary. Brefeldin, phorbol myristate acetate (PMA) and ionomycin (Sigma Aldrich) were used as indicated. For human and mouse T cell activation, in vitro culture-grade anti-CD3 and anti-CD28 (eBiosciences) were used. In cultures, recombinant cytokine IL12 and anti-mouse IL-12 antibodies (all from eBiosciences) were used where indicated. Various inhibitors were also used along with the priming culture after being optimised for culture conditions. Capsaicin was used (2 μM; Sigma Aldrich) as indicated. Recominant double-knot earth tiger tarantula toxin (DkTx) was generated as described (1) and used at 300 nM after titration as a TRPV1 agonist. Other inhibitors used (Sigma Aldrich) were: Ruthenium Red (pan-TRPV inhibition; 300 nM), AB14772 (TRPV1 inhibition; 300 nM), SB452533 (TRPV1 inhibition; 300 nM), SKF96365 (TRPV2 inhibition; 300 nM), and RN1734 (TRPV4 inhibition; 300 nM). The gamma-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was also used as appropriate (1 μM; Merck). HDMAPP (1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate, 1 μM, Sigma) was used to specifically activate human γ/δ T cells.

Flow cytometric purification of naive CD4 T cells

Mice were euthanised by cervical dislocation and spleens and lymph nodes were dissected. Erythrocyte lysis using Gey's solution was used where necessary. Mouse CD4 T cells were sorted as naive (CD4+CD44-CD62L+CD25-) to include only conventional T cells and exclude both effector/memory and Treg cells.

Human CD4 T cells were sorted from PBMCs of independent donors as naive (CD4+CD45RO-CD25-).

For sorting, cells were suspended in complete RPMI1640 medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% FBS, 2 mM L-glutamine, 1.35 g/l sodium bicarbonate, 5 mM HEPES, 50 μM β-mercaptoethanol, 100 μg/ml streptomycin and 100 IU penicillin and stained with the relevant antibody combinations and incubated at 4°C for 20 min. The cells were then washed, re-suspended at a density of 30 million cells/ml in complete RPMI and filtered through a 40-micron size sieve. This was followed by analysis and sorting under sterile conditions (BD FACSAria III; BD Biosciences, CA). Sorted populations showed >95% purity. All sorted cells were rested for 2-3 h at 37ºC before use.

Priming and/or re-stimulation of CD4 T cells in vitro

These assays were done as previously described (2). Naive CD4 T cells from various strains of mice, as specified for each experiment, were activated without any polarising milieu, by optimal concentrations of plate-coated anti-CD3 and anti-CD28 (3 μg/ml each) for 72 h at temperatures mentioned for each experiment. Cells were then harvested, washed, rested in IL2 (5 U/ml) for 24 h at 37ºC, and used for subsequent assays. The recall assay temperature was also 37ºC. The in vitro priming protocol for naive T cells was also optimised with titrations of days for priming and for resting. IL2 concentrations were titrated to maximise cell survival and live cell yields without influencing Th1/Th2 cytokine balances. Rest period titration was done to achieve return of cells to baseline in terms of cytokine secretion, so that stimulation-induced production could be unambiguously followed. For detection of

intracellular cytokines and transcription factors (TFs), cells were treated with phorbol myristate acetate (50 ng/ml) and Ionomycin (500 ng/ml) (P+I) for 6 h and Brefeldin A (5 ng/ml) for the last 3 h before permeabilisation and staining with anti-cytokine and anti-TF antibodies. For analysing secreted cytokines, cells were re-stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 24 h, and cytokines were assayed in culture supernatants.

Human naive CD4 T cells were similarly primed, using 50 IU/ml IL2 during the rest period.

For experiments to test γ/δ T cells, an earlier published protocol (3, 4) was used with further optimisation. PBMCs were incubated with IL2 and HDMAPP (1 μM) in 24-well plates. Cells were supplemented with IL2 every third day, subcultured on day 6 and harvested on day 12 and processed for qRT-PCR.

For priming in vitro cultures in the presence of recombinant IL12 or in presence of anti-IL12 antibody, mouse naïve CD4 T cells were activated with plate-coated anti-CD3 and anti-CD28 as above for 72 h in presence of recombinant murine IL12 (10 ng/ml) or anti-mouse IL12 (10 μg/ml).

For priming TCR-transgenic OTII naive CD4 T cells, Ova2 peptide and syngeneic bone marrow-derived dendritic cells (BMDCs) prepared as described below were used as alternative to anti-CD3 and anti-CD28 as above, both for priming and for re-stimulation.

RNA-seq analysis of activated CD4 T cells

Naive CD4 T cells were purified as above from C57BL/6 mice, and activated with plate-bound anti-CD3+anti-CD28 as above at 37ºC or 39ºC for 3 h. RNA was isolated using trizol from the starting unstimulated T cells, as well as T cells activated at 37ºC or at 39ºC, and subjected to library preparation and sequencing on the Illumina platform (Macrogen, Seoul, S. Korea). Reads were subjected to quality control using the FastQC package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) after adapter trimming. Reads were mapped to the mouse genome downloaded from Ensembl (5) using HISAT2 (6)**.** The number of reads mapped to each gene was calculated using the htseq-count tool (7). DEseq2 (8) was used to perform differential expression analysis. GSEA (9) and g:profiler tools (10) were used for enrichment analyses.

Generation of BMDCs and LPS-activated B cell blasts

Bone marrow cells from mouse femora were harvested and cultured with GM-CSF (10 ng/ml) at a cell density of 0.5 million/ml at 37ºC. Fresh medium was supplemented on alternate days after removing spent medium from the cultures. BMDCs were obtained at the end of 7 days as clusters of non-adherent rounded cells. These BMDCs were either used in T cell priming assays as above, or were stimulated with LPS and supernatants tested for IL-12 secretion by kit-based ELISA.

Spleen cells were stimulated with LPS (10 μg/ml) for 60 h, live cells isolated, confirmed to be >90% B cells, and used as APCs where indicated.

ELISA assays for cytokines

As described previously (2), secreted cytokines were detected by commercially available cytokine detection kits (eBiosciences) as mentioned above, essentially following recommended protocols. A reference standard run in parallel was used to calculate the cytokine concentrations.

Flow cytometric staining and data analysis

Staining and data analysis were done as described previously (2). Cells were stained with combinations of the following antibodies: mouse CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), CD25 (PC61.5), IFNg, IL-4, IL-5, IL-13, T-bet, GATA-3, TCRValpha2, and human CD4, CD45RO, and CD25 (eBioscience, ThermoFisher Scientific, Carlsbad, CA). Surface staining was performed in staining buffer (phosphate-buffered saline (PBS)+1% foetal bovine serum (FBS)+0.05% NaN3) at 4°C for 20 min followed by two washes with staining buffer. For intracellular proteins, cells were first fixed and permeabilised using Transcription Factor Staining Buffer Set (eBioscience, ThermoFisher Scientific, Carlsbad, CA) and then stained with appropriate combinations of antibodies. Flow cytometric data collection was done (FACSVerse; BD Biosciences) and data analysed with FlowJo software (Treestar, Ashland, OR).

qRT-PCR assays

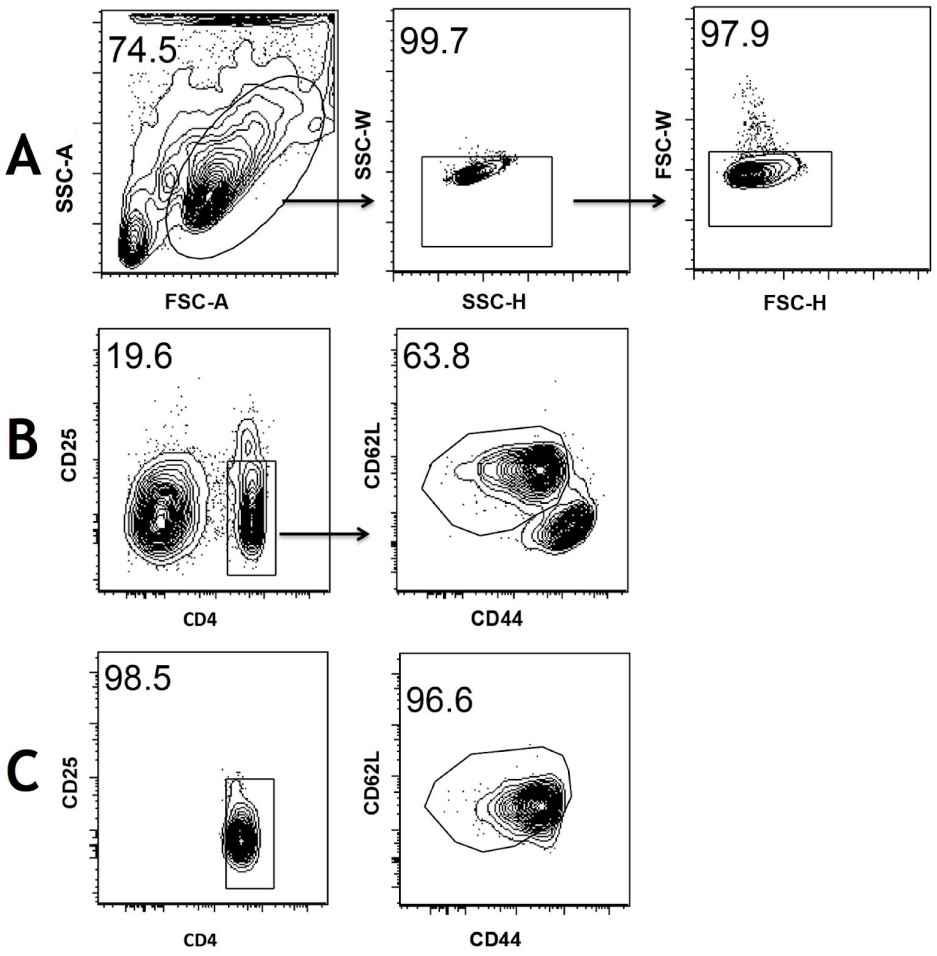
These assays were done as described previously (2). RNA was extracted from ~2-3 million T cells using Trizol (Invitrogen) following recommended procedures. Using 1 µg RNA, cDNA synthesis was done by reverse transcription kit (Promega). The primers used (SI Appendix,, Table S2) were custom-synthesized (Sigma) and validated before use for qRT-PCR (2). The primer sequences for *Hes1* and *Hey1* were from published reports (11). Ribosomal protein *l7* or *gapdh* were used as housekeeping genes (2).

Sorted NCD4 cells from C57BL/6 mice were stimulated, in triplicates, for 3h or 6h with plate coated anti-CD3 (3ug/ml) and anti-CD28 (3ug/ml) at 37°C and 39°C. After each time point, cells were harvested and RNA extracted with TRIzol reagent. cDNA was synthesized using Verso cDNA synthesis kit with oligo-dT primers. cDNA was amplified in duplicates with specific primers (Table) using Power SYBR Green PCR master mix and an ABI Prism 7000 cycler. Data represented as ΔCt (normalised to GAPDH) or fold change of mRNA calculated using the ΔΔCt method (normalised to the 37°C unstimulated control). The qRT-PCR reactions were performed in triplicate each time (Eppendorf Mastercycler or Bio-Rad CFX96) using pre-optimized conditions for a total of 30-40 cycles. Ct values obtained were normalised with L7/GAPDH (mouse)/18S (human) values and ΔΔCt values were calculated (12) and plotted to show enhancement in signal above unstimulated T cell values.

Statistical methodology

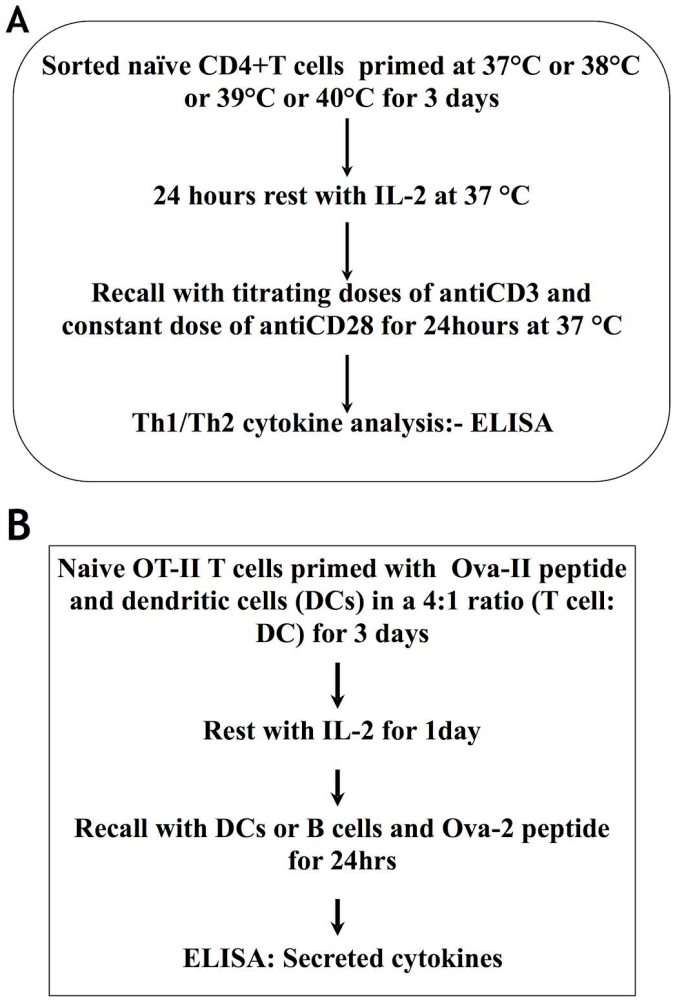
For statistical analysis, Student's 't' test or two-way ANOVA test were used as indicated. Values of $p < 0.05$ were considered statistically significant.

Figure S1



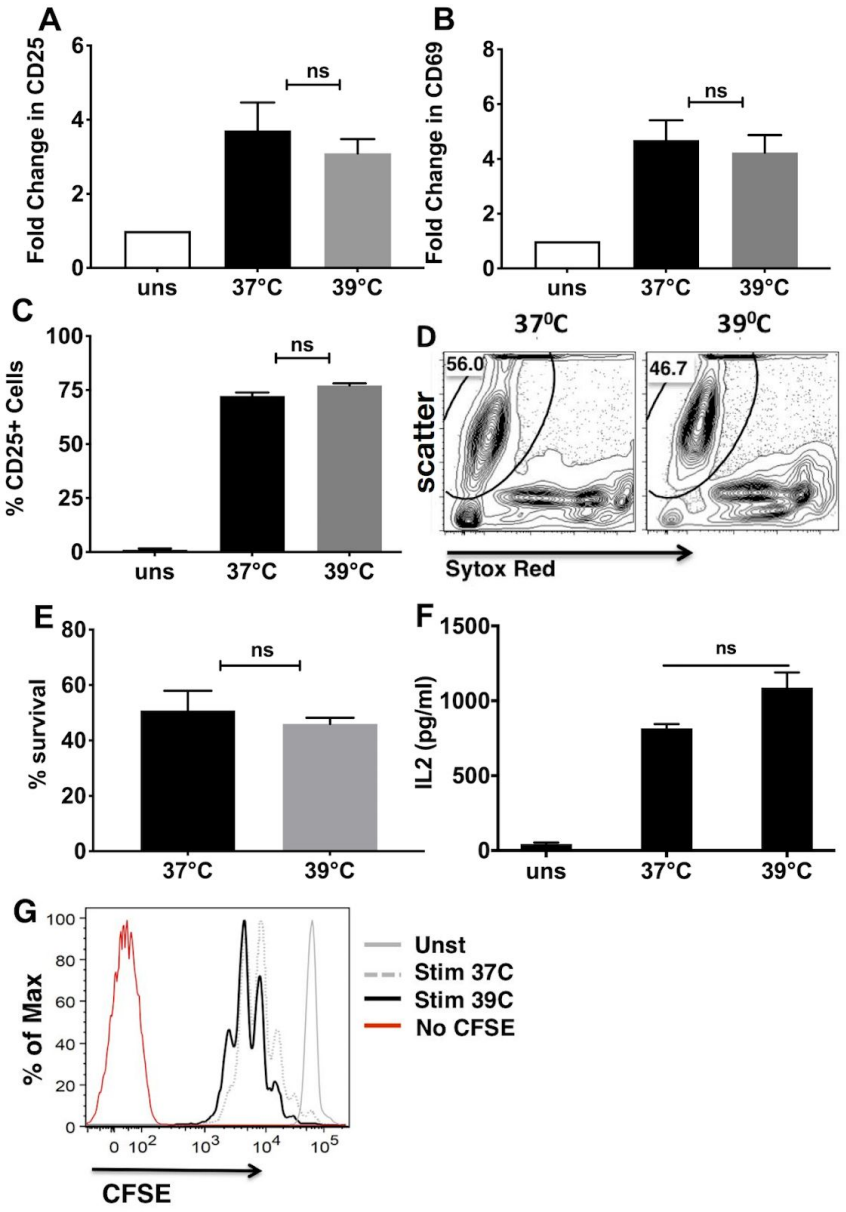
Sort profile for NCD4 T cells
A. Pre-sort profile showing scatter gating and doublet discrimination.
B. Pre-sort profile showing gating on CD4 and CD25 to eliminate activated cells and Treg cells (left panel), and then the CD44lowCD62Lhigh population as naïve cells.
C. Post-sort profile of naïve CD4 T cells with >95% purity.

Figure S2



A. Schematic representation of the experimental protocol for 3-day priming of sorted mouse or human naive CD4 T cells, followed by a day of rest prior to re-stimulation. B. Schematic representation of the experimental protocol for 3-day priming of sorted naive OT-II CD4 T cells with OvaII peptide, dendritic cells (or LPS-activated B cells as APCs), followed by a day of rest before re-stimulation.

Figure S3



Activation at fever temperatures does not change early activation events or survival in responding NCD4 T cells

A-B. Fold-increase in mRNA levels above unstimulated NCD4 (uns) 6 h post-activation with plate-bound anti-CD3+anti-CD28 for *cd25* [A] and *cd69* [B]. Mean ± s.e., n=3.

C. Frequency of CD25+ cells 24 h post-activation at temperatures shown. Mean ± s.e., n=3.

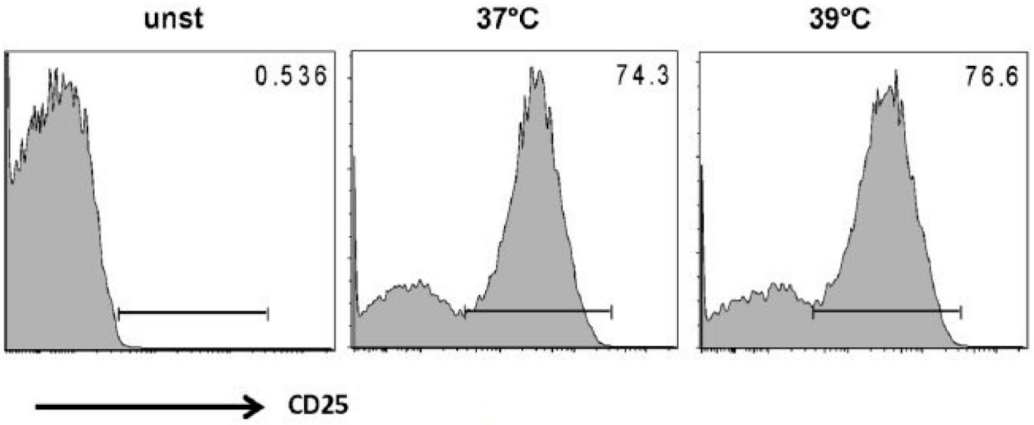
D. Representative plot to show Sytox Red-negative (i.e. viable) cell frequencies when NCD4 cells were primed for 3 days with plate-bound anti-CD3+anti-CD28.

E. Cumulative data to show frequency of surviving cells after 3 days of priming. Mean ± s.e., n=3.

F. IL2 concentrations in CD4 T cell culture supernatants 24 h post activation with anti-CD3+anti-CD28 at temperatures shown.

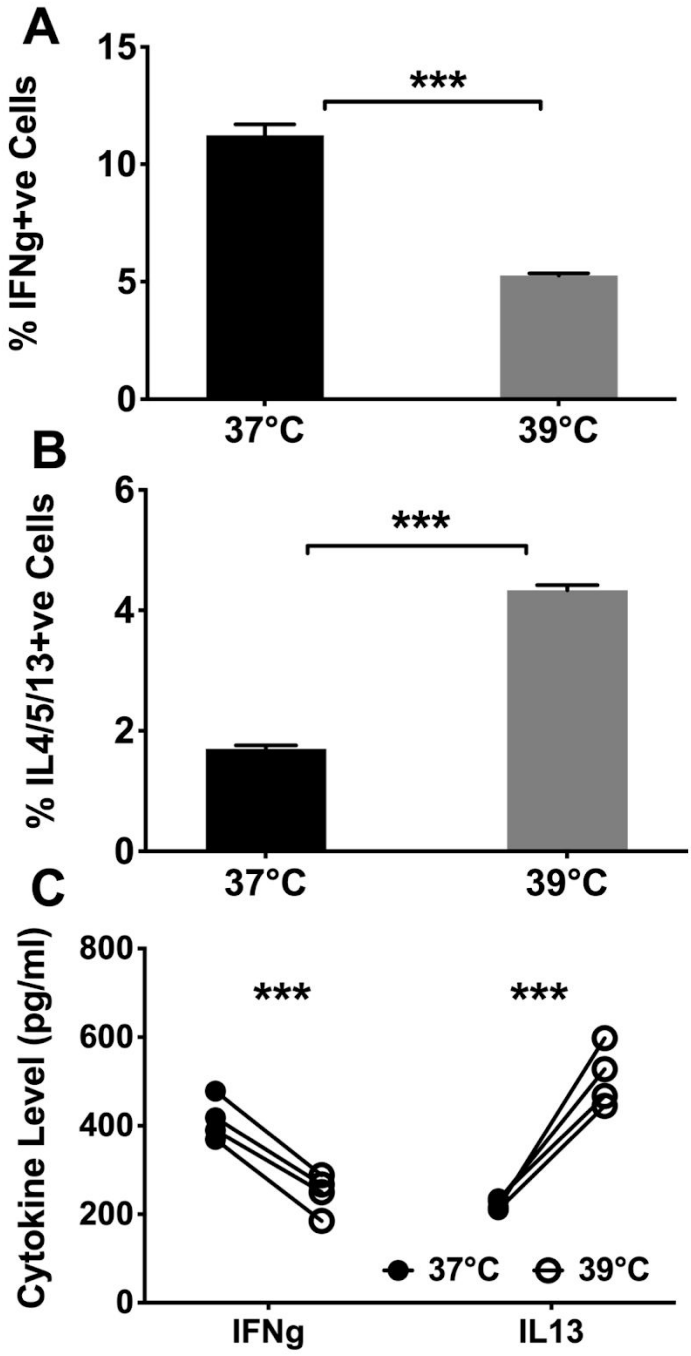
G. A representative plot of CFSE dilution profile of live-gated naïve (Unst) and anti-CD3+anti-CD28-primed CD4 T cells (Stim) cultured at 37°C or 39°C for 60 h.

Figure S4



Representative CD25 staining profile of NCD4 T cells activated with plate-bound anti-CD3+anti-CD28 at 37ºC or 39ºC for 24 h.

Figure S5



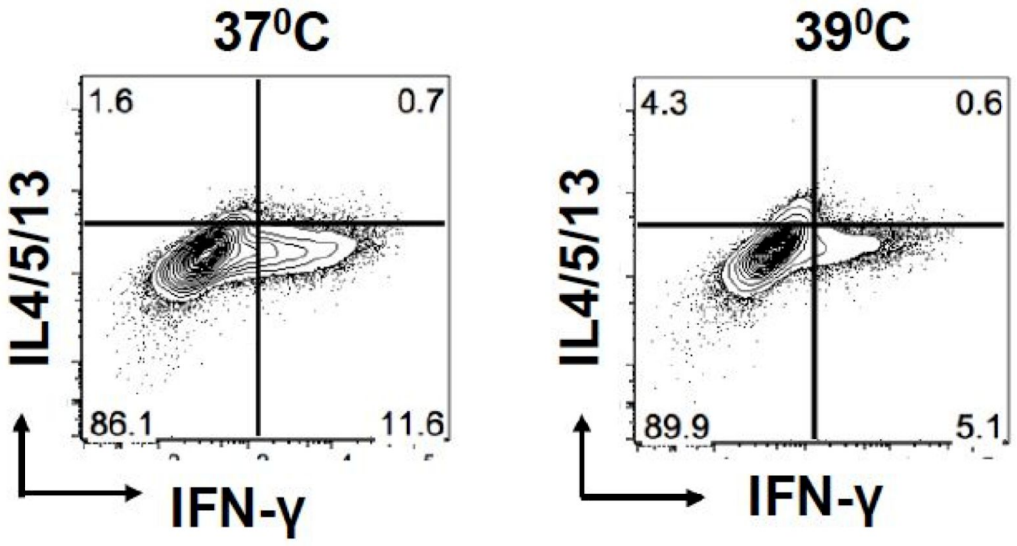
Both mouse and human NCD4 cells activated at fever temperature show Th2-skewed programming

A-B. Frequencies of IFNg [A] and IL4/5/13 [B] producing cells in mouse NCD4 T cells activated for 3 days with anti-CD3+anti-CD28, rested for 24 h, re-stimulated and stained post-permeabilisation. Mean ± s.e., n=3.

C. IFNg and IL13 levels from human NCD4 cells activated at indicated temperatures for 3 days, rested for 1 day and restimulated with 3 µg/ml of anti-CD3. Mean ± s.e., n=4 independent donors.

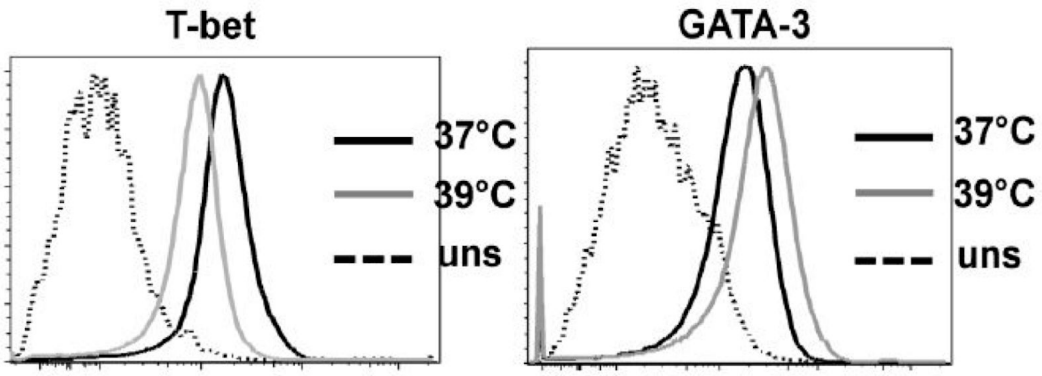
***, $p<0.0001$.

Figure S6



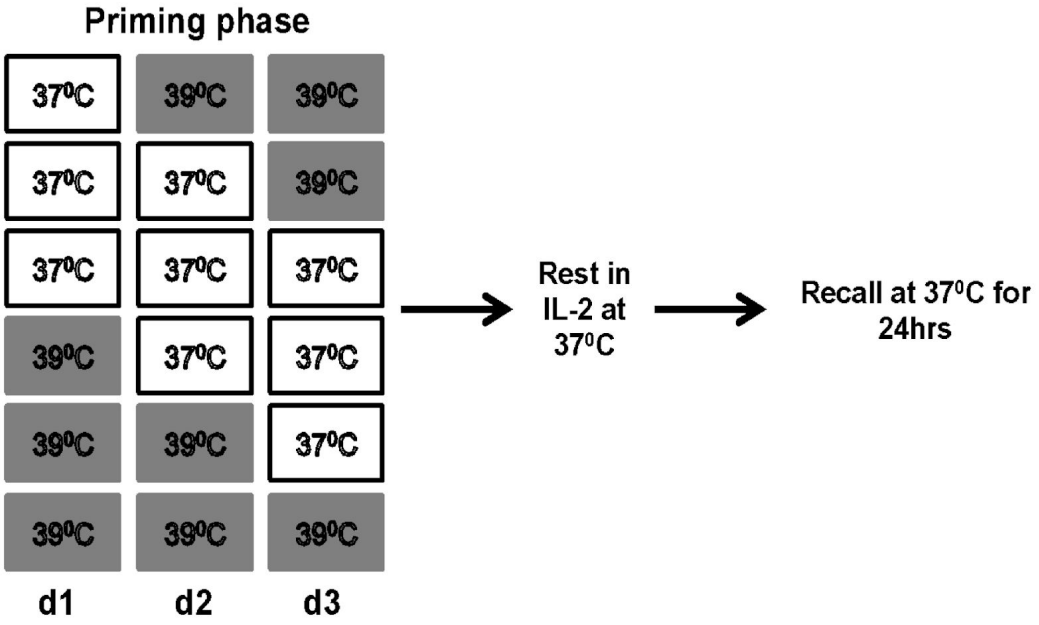
Representative staining profile of NCD4 T cells primed for 3 days at 37ºC or 39ºC, rested for 24 h, and re-stimulated with PMA and ionomycin in the presence of brefeldin A. Staining for intracellular IFNg versus IL4/5/13 after permeabilisation is shown.

Figure S7



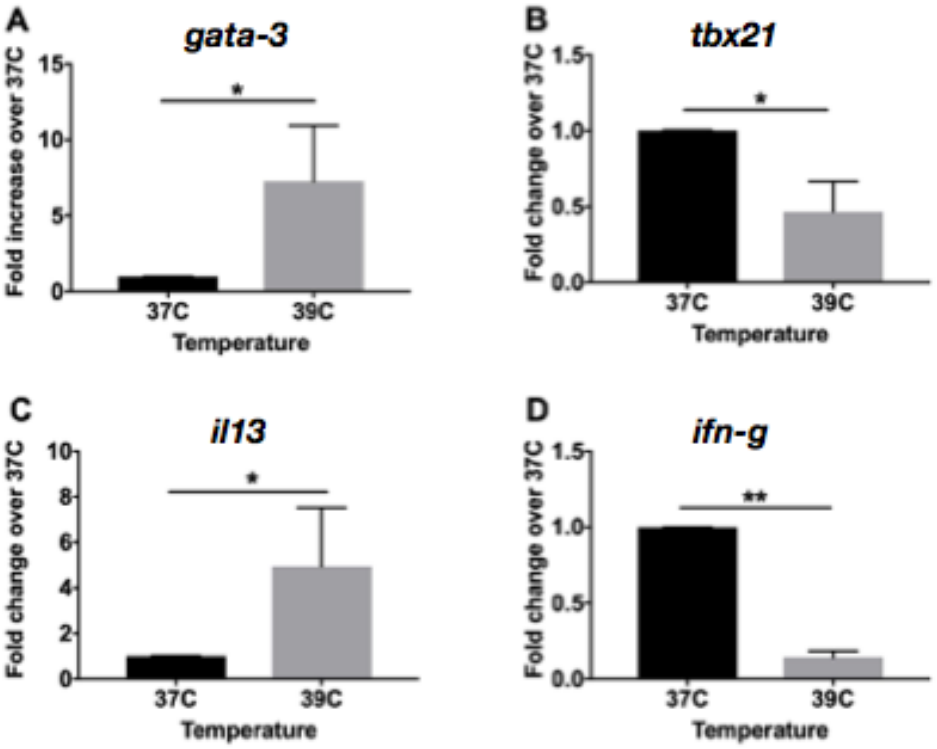
Representative histogram overlays for T-bet and GATA-3 expression in CD4 T cells primed at 37°C and 39°C for 3 days. Dead cells were first gated out using fixable violet live/dead stain. Dotted lines show expression in naïve cells [uns] and solid lines show expression in primed cells.

Figure S8



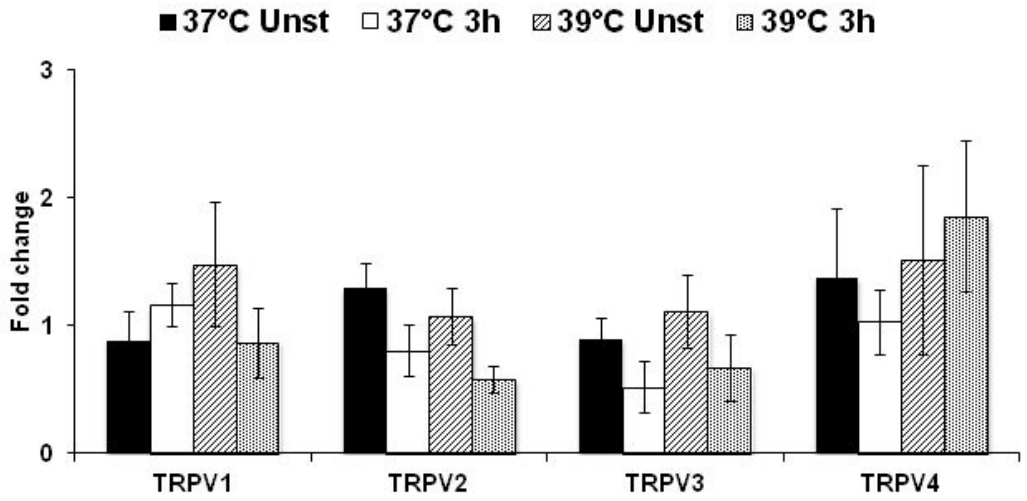
Schematic representation of the experimental protocol to vary temperatures during the priming of NCD4T cells with plate-bound anti-CD3+anti-CD28. Three-day priming was followed by a day of rest and subsequent recall at 37ºC.

Figure S9



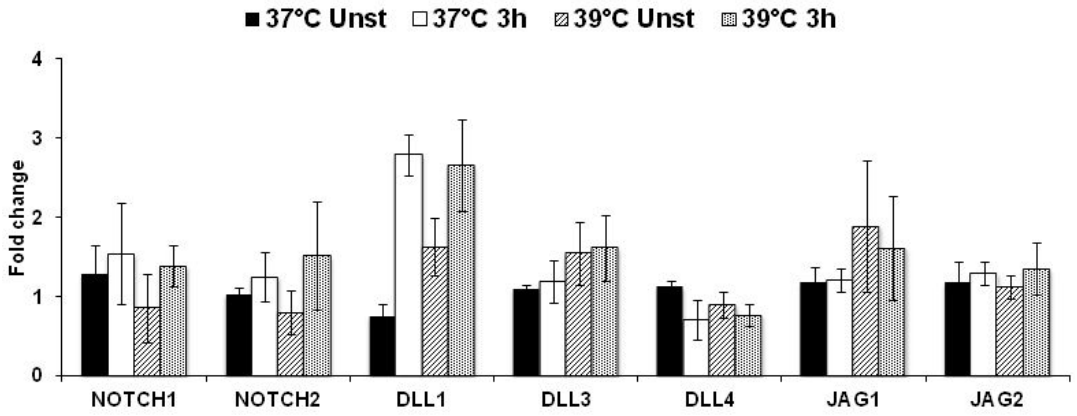
Fold-change in the mRNA levels of *GATA3* [A], *TBX21* [B], *IL13* [C], and *IFNG* [D], when human gamma-delta T cells were expanded and differentiated by activation with HDMAPP and IL2 for 12 days at 37ºC or 39ºC. Data normalised to 37C-stimulated values. Mean + s.e., n=3, *p<0.05, **p<0.01.

Figure S10



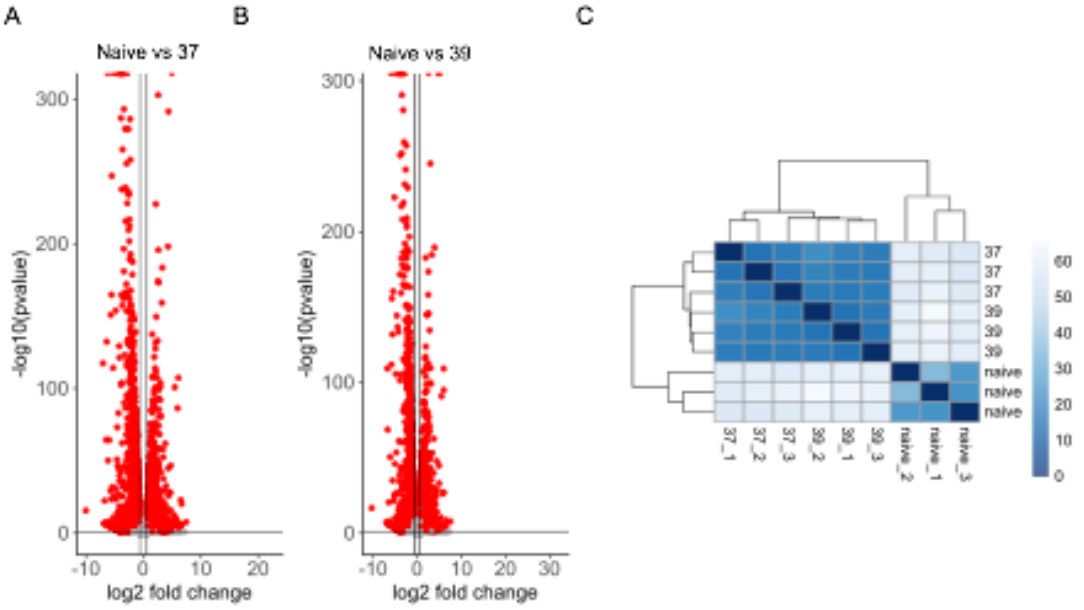
Fold-changes in the mRNA levels of *Trpv1*, *Trpv2*, *Trpv3* and *Trpv4* when purified naive mouse CD4 T cells were activated for 3 h at 37ºC or 39ºC with plate-bound anti-CD3+anti-CD28 mAbs. Data normalised to 37ºC-stimulated values. Mean + s.e., n=3.

Figure S11



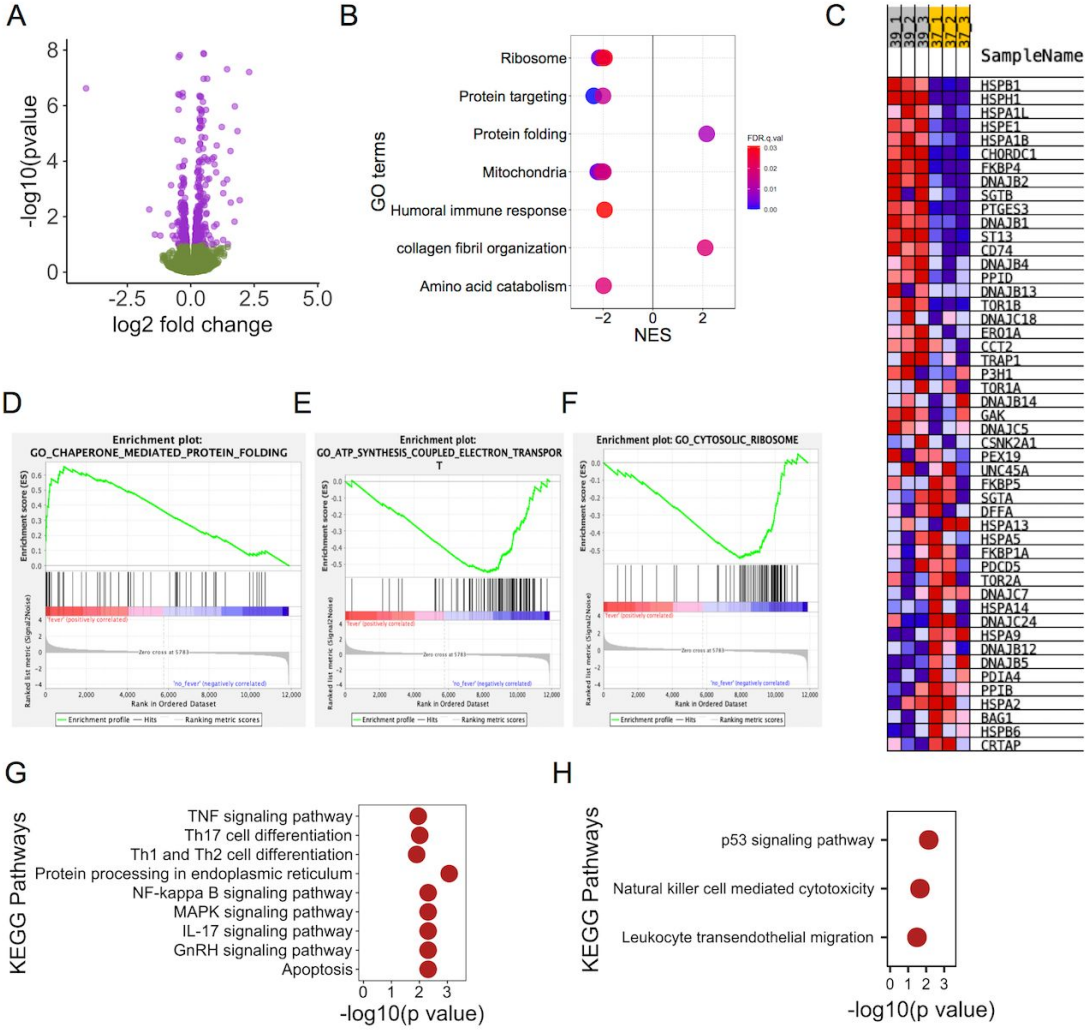
Fold-changes in the mRNA levels of Notch and Notch ligand genes when purified naive mouse CD4 T cells were activated for 3 h at 37ºC or 39ºC with plate-bound anti-CD3+anti-CD28 mAbs. Data normalised to 37ºC-stimulated values. Mean + s.e., n=3.

Figure S12



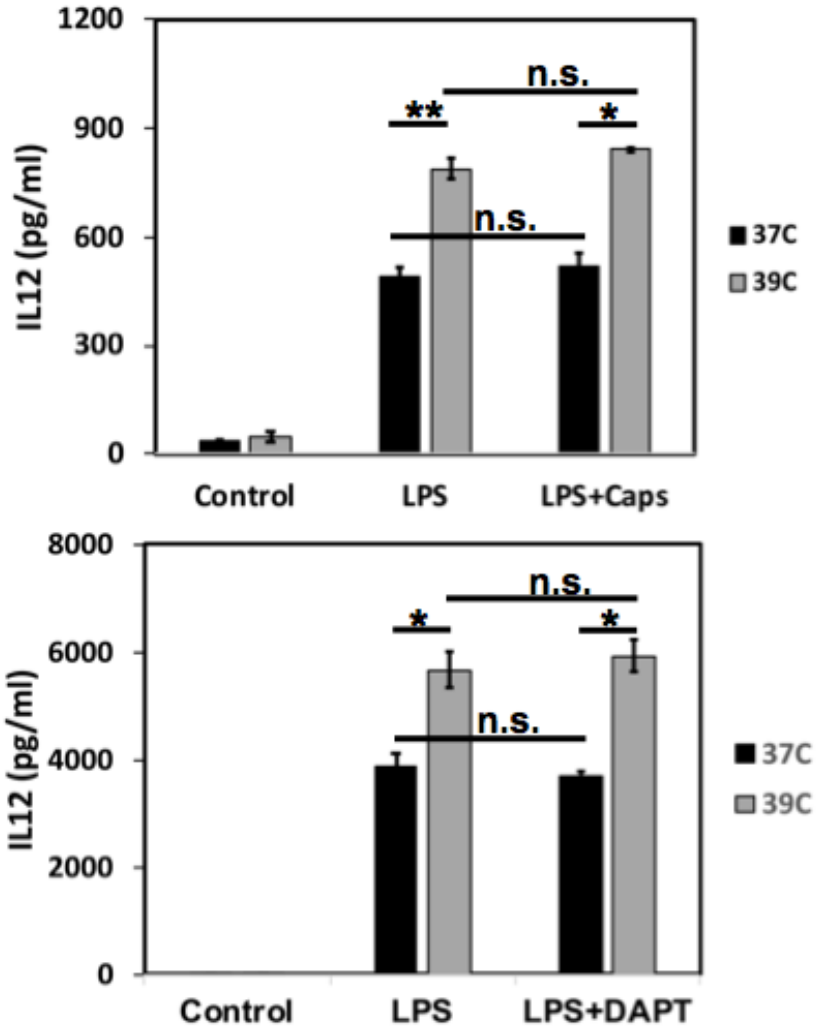
Protein folding, protein synthesis and mitochondrial ATP generation were affected by changes in temperature.
A-B) Volcano plots highlighting the genes that were differentially expressed between unstimulated and stimulated at 37ºC (A) or 39ºC (B).
C) Heatmap showing the clustering of all samples in the RNA-seq analysis.

Figure S13



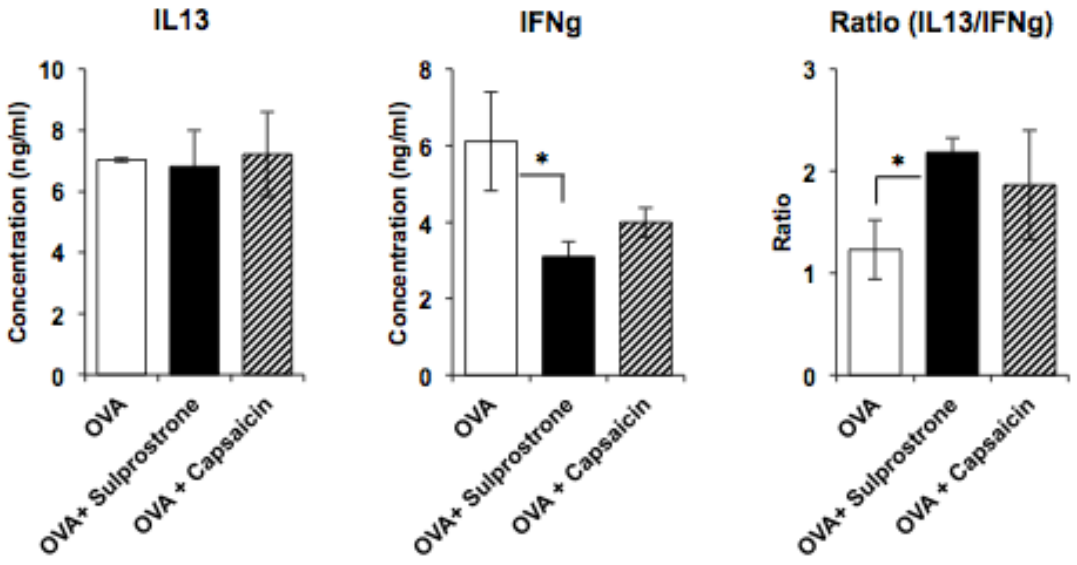
Expression of genes involved in protein folding, protein synthesis and mitochondrial ATP generation is differentially modified at fever temperature in activated T cells
A. Volcano plot highlighting (purple) the mRNAs that are differentially expressed in T cells activated at 37ºC or at 39ºC. A total of 445 genes are differentially expressed.
B. Plot showing the normalised enrichment score for gene sets associated with some gene ontology terms. Positive values (right hand side) shows enrichment in cells activated at 39ºC and negative values (left hand side) shows enrichment in cells activated at 37ºC.
C. Heatmap showing expression levels of members of the chaperone-mediated protein folding gene set in cells activated at 37ºC or at 39ºC.
D-F. Enrichment score plots for specific gene sets, namely, chaperone-mediated protein folding (D), ATP synthesis-coupled electron transport (E), and cytosolic ribosome (F).
G-H. KEGG pathway analysis on genes preferentially upregulated at 39ºC (G) or at 370C (H). The x axis shows -10 log(p value) and the y axis shows pathway names.

Figure S14



Dendritic cells were generated from mouse bone marrow using GM-CSF. The DCs were stimulated with LPS for 72 h in the presence or absence of 1 μM capsaicin or 1 μM DAPT, either at 37ºC or at 39ºC as shown. IL12 levels in 72-h culture supernatants were assayed by ELISA. Statistical analysis was done using 2-way ANOVA from data from 3 independent experiments. *p<0.05, **p<0.005, n.s. not significant.

Figure S15



Exposure to pyrogenic stimuli in vivo during immunisation leads to a Th2 shift in the resultant CD4 T cell response.

IL13 and IFNg levels in culture supernatants during recall responses from draining lymph nodes of mice subcutaneously immunised seven days earlier with Ova (100 µg/mouse). Lymph node cells were restimulated with Ova (300 µg/ml) and culture supernatants harvested 3 days later for assay. IL13:IFNg ratios are also shown. Unstimulated cultures for all groups showed no detectable cytokine levels (detection limits for IL13 >10 pg/ml and for IFNg >50 pg/ml). Data represent 3 mice in each group. Student's 't' test was used to calculate p values.

Table S1

The table is provided at:

https://github.com/MolGenLab/Umar_etal_2020_supdata

Table S2

Primers used for qRT-PCR assays

Gene	**Mouse Primer Sequence (5' - 3')**
Notch1 (F)	GCTGCCTCTTTGATGGCTTCGA
Notch1 (R)	CACATTCGGCACTGTTACAGCC
Notch2 (F)	CCACCTGCAATGACTTCATCGG
Notch2 (R)	TCGATGCAGGTGCCTCCATTCT
Dll1 (F)	GCTGGAAGTAGATGAGTGTGCTC
Dll1 (R)	CACAGACCTTGCCATAGAAGCC
Dll3 (F)	CCAGCACTGGATGCCTTTTACC
Dll3 (R)	ACCTCACATCGAAGCCCGTAGA
Dll4 (F)	GGGTCCAGTTATGCCTGCGAAT
Dll4 (R)	TTCGGCTTGGACCTCTGTTCAG
Jag1 (F)	TGCGTGGTCAATGGAGACTCCT
Jag1 (R)	TCGCACCGATACCAGTTGTCTC
Jag2 (F)	CGCTGCTATGACCTGGTCAATG
Jag2 (R)	TGTAGGCGTCACACTGGAACTC
Trpv1 (F)	CATCTTCACCACGGCTGCTTAC
Trpv1 (R)	CAGACAGGATCTCTCCAGTGAC
Trpv2 (F)	CTGTCAACAGCGTTGCCACTGA
Trpv2 (R)	TTGGTGCCAACTTTCAGCAGCC
Trpv3 (F)	ATCCTGCTGAGGAGTGGCAACT
Trpv3 (R)	TTGATCTCGCGGCTGAGGATGT
Trpv4 (F)	TCACCGCCTACTATCAGCCACT
Trpv4 (R)	GAACAGGACTCCTGTGAAGAGC
tbx21 (F)	AGAACTTTGAGTCCATGTACG
tbx21 (R)	TAACTGTGTTCCCGAGGTG
gata3 (F)	ACCGGGTTCGGATGTAAG
gata3 (R)	GACAGTTCGCGCGCAGGATGT
hes1 (F)	GTGGGTCCTAACGCAGTGTC
hes1 (R)	ACAAAGGCGCAATCCAATATG
hey1 (F)	TGAATCCAGATGACCAGCTACTGT
hey1 (R)	TACTTTCAGACTCCGATCGCTTAC
L7 (F)	AGCTCATCTATGAGAAGGC
L7 (R)	AAGACGAAGGAGCTGCAGAAC
Gapdh (F)	ATGGCCTTCC GTGTTCCTA

Gapdh (R)	TGAAGTCGCAGGAGACAACCT
Gene	**Human Primer Sequence (5’ - 3’)**
tbx21 (F)	GTGACTGCCTACCAGAATGCC
tbx21 (R)	GCTGGTGTCAACAGATGTGTA
gata3 (F)	AACTGTCAGACCACCACAACCACAC
gata3 (R)	GGATGCCTTCCTTCTTCATAGTCAGG
Ifnγ (F)	GCATCGTTTTGGGTTCTCTTG
Ifnγ (R)	AGTTCCATTATCCGCTACATCTG
IL13 (F)	CAGTGCCATCGAGAAGACCC
IL13 (R)	GGATATTCAGCCAGCTTCCC
18S (F)	GGAGAGGGAGCCTGAGAAAC
18S (R)	CCTCCAATGGATCCTCGTTA

References

1. D. Sarkar, Y. Singh, J. Kalia, Protein–Lipid Interfaces Can Drive the Functions of Membrane-Embedded Protein–Protein Complexes. *ACS Chem. Biol.* **13**, 2689–2698 (2018).
2. A. Das, *et al.*, Effector/memory CD4 T cells making either Th1 or Th2 cytokines commonly co-express T-bet and GATA-3. *PLoS One* **12**, 1–25 (2017).
3. N. Atre, L. Thomas, R. Mistry, K. Pathak, S. Chiplunkar, Role of nitric oxide in heat shock protein induced apoptosis of gammadeltaT cells. *Int. J. cancer* **119**, 1368–1376 (2006).
4. M. Moulin, *et al.*, Vgamma9Vdelta2 T cell activation by strongly agonistic nucleotidic phosphoantigens. *Cell. Mol. Life Sci.* **74**, 4353–4367 (2017).
5. D. R. Zerbino, *et al.*, Ensembl 2018. *Nucleic Acids Res.* **46**, D754–D761 (2018).
6. D. Kim, B. Langmead, S. L. Salzberg, HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360 (2015).
7. S. Anders, P. T. Pyl, W. Huber, HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
8. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
9. A. Subramanian, *et al.*, Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* **102**, 15545–15550 (2005).
10. J. Reimand, M. Kull, H. Peterson, J. Hansen, J. Vilo, g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res.* **35**, W193-200 (2007).
11. M. Moriyama, *et al.*, Notch signaling via Hes1 transcription factor maintains survival of melanoblasts and melanocyte stem cells. *J. Cell Biol.* **173**, 333–339 (2006).
12. K. J. Livak, Analysis of relative gene expres-KJ and Schmittgen TD: Analysis of relative gene expres-TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)) method. *Methods.* **25**, 40 (2001).