



RESEARCH ARTICLE SUMMARY

IMMUNOLOGY

Cellular architecture shapes the naïve T cell response

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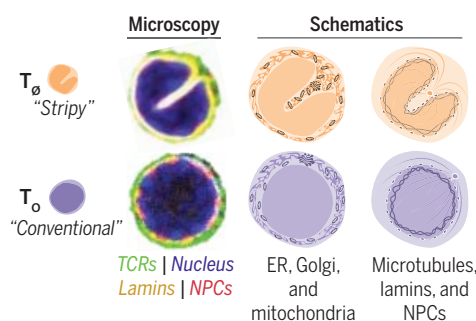
INTRODUCTION: T cells eliminate infected cells and provide long-term protection against reinfection by the same pathogen. Central to this is the detection of foreign antigens by T cell receptors (TCRs). Upon antigen engagement, clonal populations of naïve T cells that express the same TCR differentiate into effector T cells with cytotoxic abilities and memory T cells, which provide long-term immunity. This pattern of diversification is reproducible at the population level, yet individual naïve T cells embark upon diverse trajectories during an antigen response.

RATIONALE: Although the fate of naïve T cells is known to be influenced by external factors, including antigen affinity and availability, clonally identical T cells in controlled antigen environments still exhibit diverse responses and heterogeneous differentiation trajectories.

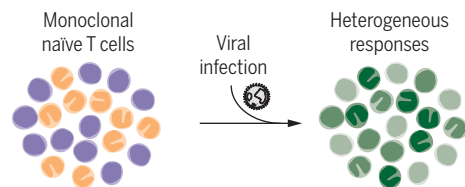
This suggests a potential role for cell-intrinsic predeterminants and cell-to-cell variability in regulating T cell differentiation. An increasingly appreciated source of cellular heterogeneity arises from variations in cell morphology and the spatial organization of subcellular components—i.e., the architecture of a cell. Therefore, we systematically investigated the architectural heterogeneity of primary human and murine T cells and the potential role of this heterogeneity in predetermining the response strength and differentiation trajectory of naïve T cells in response to antigen.

RESULTS: By combining high-throughput fluorescence microscopy with deep learning-based single-cell image analysis, we report that heterogeneity in the cellular architectures of T cells (T_{ARCH}) predetermined their TCR signal strength

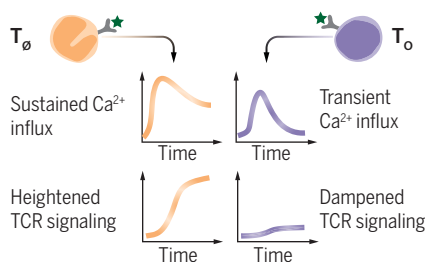
Naïve T cells are architecturally heterogeneous



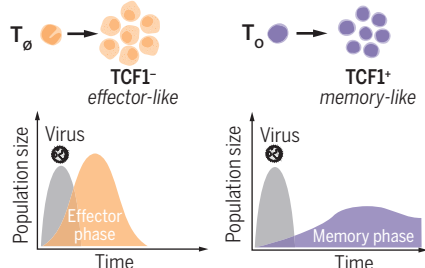
The response heterogeneity of naïve T cells is built into their architecture



Cellular architecture predetermines TCR signal strength



Cellular architecture predetermines T cell fate



T cell architecture shapes the response to antigen. The subcellular architectures of naïve T cells are defined by the presence (T_0) or absence (T_0) of deep NEIs, which spatially colocalize cellular organelles and machinery. In response to antigen, T_0 (stripy) architectures engage in stronger TCR signaling and differentiate into effector-like cells, whereas T_0 (conventional) architectures have dampened TCR signaling and preferentially form memory precursors. ER, endoplasmic reticulum; NPC, nuclear pore complex.

and subsequent differentiation trajectory in response to antigen stimulation. This architectural heterogeneity was defined by three classes: morphologically polarized T cells (T_P) or spherical T cells either without (T_0 ; “conventional”) or with (T_0 ; “stripy”) deep nuclear envelope invaginations (NEIs). These NEIs spatially concentrated cellular machinery, including the endoplasmic reticulum, mitochondria, and nuclear pore complexes. The architectural balance of T cells among these three classes shifted with T cell maturation, activation, and differentiation. Among molecularly defined T cell subsets, architectural profiles were highly reproducible, with 60% of naïve CD8 T cells adopting a T_0 architecture in both humans and mice. Differentiation of naïve CD8 T cells after in vivo viral infection was associated with a decrease in T_0 and an increase in T_P architectures during the effector phase, followed by the reestablishment of a T_0 -high population of viral-specific memory cells 28 days after infection. Furthermore, upon antigenic T cell activation, T_{ARCH} subsets exhibited different molecular and functional responses: T_0 architectures displayed stronger TCR signaling in a mechanism dependent on heightened calcium influx in the first minutes after activation. This was independent of the expression of proteins known to affect TCR signal strength and could be dampened by pharmacological inhibition of store-operated calcium entry as well as through the conversion of T_0 to T_0 architectures. Architecturally resolved ex vivo single-cell fate tracking revealed that T_0 cells initiated cell division earlier compared with T_0 cells and proliferated to form larger colonies of cells that had down-regulated TCF1 and up-regulated tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) cytokine expression, consistent with an effector-like T cell phenotype. By contrast, cells with T_0 architectures were slower to divide upon TCR stimulation and gave rise to TCF1⁺ cells, which suggests that they would preferentially differentiate into T cells with a memory phenotype.

CONCLUSION: Our data suggest that cellular architecture is a predeterminant of early TCR signaling and subsequent cell fate decisions of individual naïve T cells in response to antigen. Understanding cellular architecture as a phenotypic dimension of T cell biology may enable the prediction and therapeutic optimization of T cell responses to protect against infection and combat disease. ■

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Cite this article as B. D. Hale et al., *Science* 384, eadh8967 (2024). DOI: 10.1126/science.adh8967

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RESEARCH ARTICLE

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Cellular architecture shapes the naïve T cell response

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After antigen stimulation, naïve T cells display reproducible population-level responses, which arise from individual T cells pursuing specific differentiation trajectories. However, cell-intrinsic predeterminants controlling these single-cell decisions remain enigmatic. We found that the subcellular architectures of naïve CD8 T cells, defined by the presence (T_O) or absence ($T_\emptyset$) of nuclear envelope invaginations, changed with maturation, activation, and differentiation. Upon T cell receptor (TCR) stimulation, naïve $T_\emptyset$ cells displayed increased expression of the early-response gene *Nr4a1*, dependent upon heightened calcium entry. Subsequently, in vitro differentiation revealed that $T_\emptyset$ cells generated effector-like cells more so compared with T_O cells, which proliferated less and preferentially adopted a memory-precursor phenotype. These data suggest that cellular architecture may be a predeterminant of naïve CD8 T cell fate.

In response to infection and vaccination, antigen-specific T cells reproducibly diversify into a repertoire of functionally disparate communities of effector and memory cells (1, 2). This population-level robustness arises from averaging across variable responses of individual T cells (3–5). Two nonexclusive mechanisms could enable clonally identical T cells to respond heterogeneously during an infection. Cell-extrinsic signals associated with pathogen infection may direct T cells toward certain fates during or after their priming. Additionally, features intrinsic to T cells at the time of activation may predispose them toward a specific response, even in the presence of identical external stimuli. Stochastic intrinsic and extrinsic mechanisms have been proposed, including expression-level variability in T cell co-receptors and metabolic transporters (3, 4, 6–11). Additionally, non-stochastic external cues, such as differences in antigen signal strength, modulate early T cell receptor (TCR) signaling, thereby influencing commitment to either effector or memory lineages (12–14). However, nonstochastic cell-intrinsic mechanisms that predetermine TCR signaling strength and subsequent cell fate decisions remain largely unexplored (15).

Cell morphology and the intracellular organization of organelles—here, collectively referred to as cellular architecture—influence cell signaling and function across a variety of systems (16–20). Among peripheral human T cells, we recently reported a polarized cell morphol-

ogy relating to activation state (21, 22). We therefore set out to investigate the potential connection between T cell architecture (T_{ARCH}) and T cell fate decisions.

Characterizing the architectural heterogeneity of primary T cells

We developed a platform that simultaneously measured single-cell T_{ARCH} , function, and phenotype, using automated fluorescence microscopy and deep learning-based image analysis, and characterized human T_{ARCH} heterogeneity ex vivo from the peripheral blood of 24 healthy human donors (Fig. 1A). T cells were stained for DNA and both surface and intracellular CD3—the TCR signal transducing entity and commonly used T cell marker. Analysis by self-supervised vision transformers (single-cell DINO), which cluster image-based cellular phenotypes in a fully unsupervised manner (23), indicated the presence of three distinct T cell phenotypes (Fig. 1B and fig. S1A), confirmed by visual inspection and feature-based single-cell image analysis (fig. S1, B to E). These could be distinguished as morphologically polarized T cells (T_P), which have been previously linked to T cell activation state in vitro (21, 22); nonpolarized T cells with homogeneous cell surface–distributed CD3 levels (T_O ; pronounced as “conventional”), as typically reported (24); and nonpolarized T cells bearing a linear pattern of CD3 expression proximal to the nucleus ($T_\emptyset$; pronounced as “stripy”), representing a previously uncharacterized T cell phenotype.

To quantify T_O , $T_\emptyset$, and T_P cells, we trained convolutional neural networks (CNNs) (21, 22, 25, 26) to classify single T cells that had been fixed, permeabilized, and stained with 4',6-diamidino-2-phenylindole (DAPI) and anti-CD3 antibodies into their respective T_{ARCH} class (Fig. 1B and fig. S2, A and B),

achieving 96.2% accuracy compared with manual annotation (fig. S2, C and D). CNN-based classification was robust to CD3 intensity shifts caused by variations in staining dilution (fig. S2E). Cleaved caspase-3 and Ki67 immunofluorescence combined with CNN classification confirmed none of the T_{ARCH} subtypes to be either predominantly apoptotic or proliferative (fig. S2, F and G). Furthermore, T_{ARCH} quantification revealed that the linear CD3 expression pattern characterizing $T_\emptyset$ cells was intracellular and that T_O architectures were reduced upon culturing of T cells in vitro for 24 hours (fig. S2, H and I).

To characterize the intracellular differences between the canonical T_O and uncharacterized $T_\emptyset$ architectures, we performed 100X multistack immunofluorescence microscopy of isolated primary human T cells, characterizing a panel of organellar and cellular machinery proteins (Fig. 1, C to E; fig. S3, A to E; Movie 1; and movie S1). This revealed that the intracellular pool of CD3, characteristic of $T_\emptyset$ cells, lay within prominent nuclear envelope invaginations (NEIs). These NEIs also concentrated mitochondria, the endoplasmic reticulum (ER), nuclear pore complexes (NPCs), nuclear lamins, and α -tubulin microtubules. The cortical actin network and the plasma membrane did not extend into the NEIs. Transmission electron micrographs supported these architectural features of $T_\emptyset$ cells, confirming mitochondrial enrichment in close proximity to NEIs (fig. S3F).

Of the markers assessed by light microscopy in fixed cells, the live cell-compatible ER-Bodipy, which stains ER-resident sulphonylurea receptors of adenosine 5'-triphosphate (ATP)-sensitive K^+ channels, visually recapitulated the architectural heterogeneity revealed by surface and intracellular CD3 staining. We therefore trained a separate CNN, achieving 85.9% accuracy in classifying the three architectures on the basis of live-cell staining of nuclear morphology (Hoechst) and ER-Bodipy, which confirmed that the observed architectural heterogeneity was not the result of fixation artifacts (fig. S4, A and B). Thus, the architectural heterogeneity of peripheral human T cells was defined by three phenotypes: polarized T cell morphologies (T_P) versus nonpolarized morphologies either without (T_O) or with ($T_\emptyset$) NEIs that colocalize cytoplasmic machinery.

T_{ARCH} heterogeneity characterizes circulating and tissue-resident T cell populations

To gain insight into the relationship between cellular architecture and T cell differentiation, we performed T_{ARCH} profiling of 15 distinct T cell differentiation states from healthy donor peripheral blood mononuclear cells (PBMCs) based on marker expression analysis associated with naïve, memory, and regulatory T cell subsets. For this, we established a

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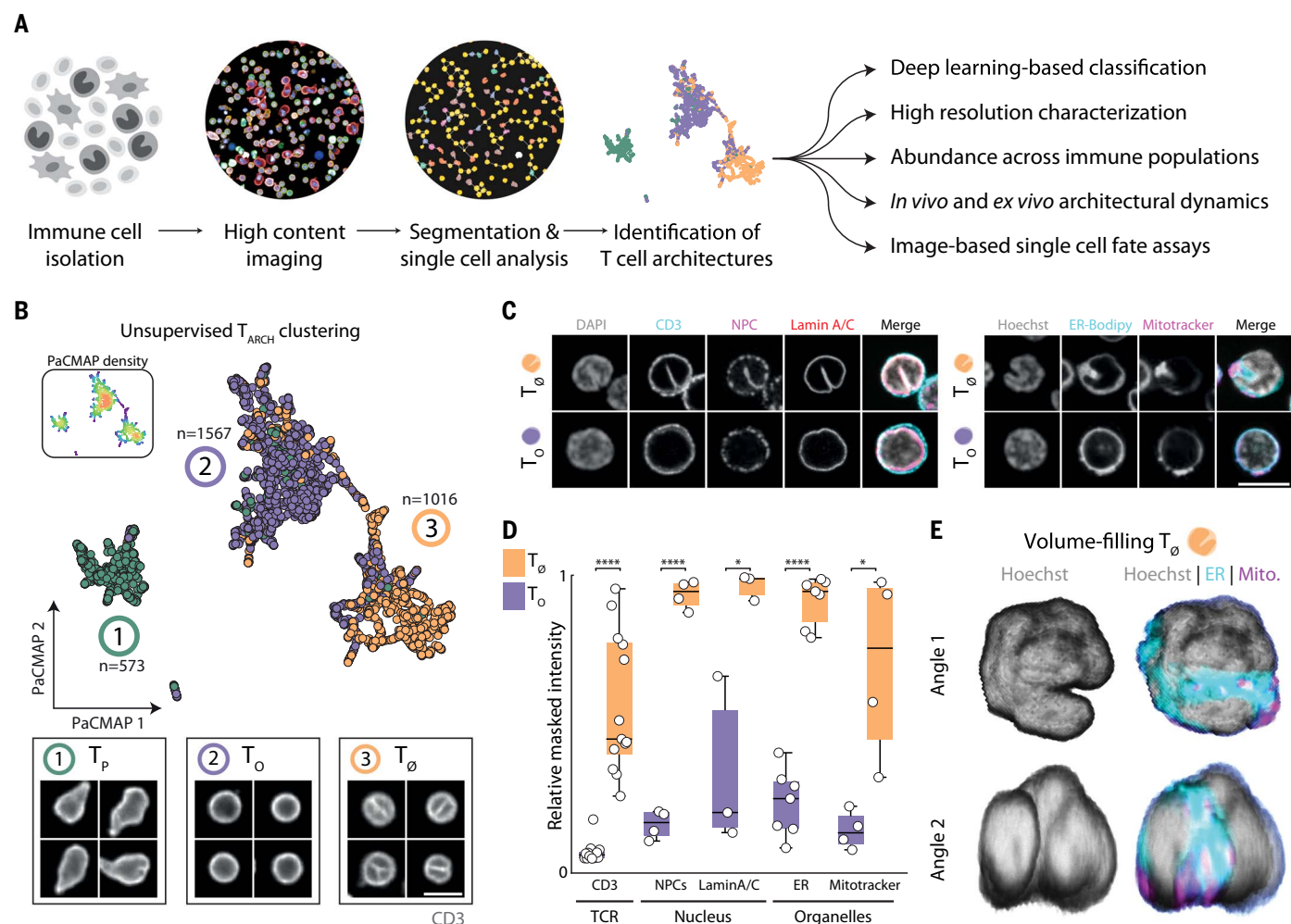


Fig. 1. Discovery and characterization of primary T_{ARCH} heterogeneity.

(A) Workflow for T_{ARCH} discovery and characterization. (B) Unsupervised clustering of human peripheral T cell phenotypes. TopOMetry embedding and subsequent PaCMAP dimensionality reduction of the 384-dimension scDINO CLS-Token feature space, colored by density (insert) and T_{ARCH} (T_P , green; T_O , purple; T_0 , yellow; used consistently throughout all figures). Images show total CD3 staining for representative cells from each cluster. Cells included were $CD3^+$ PBMCs ($n = 24$ healthy human donors). (C) Single z-stack 100X images of fixed (left) and live (right) T_0 and T_O cells derived from PBMCs of a single donor. (D) Relative masked intensity of organelles and

cellular machinery as a function of T_{ARCH} . Cellular intensity was normalized between 0 and 1 before measuring average marker intensity in a subnuclear mask (fig. S3A). Data points indicate single-cell values from one donor, representative of three independent experiments. Boxplot horizontal midlines indicate data medians, boxes span the 25th and 75th percentiles, and whiskers reflect the full range of the nonoutlier data. P values were calculated with two-tailed Student's t tests. ns, nonsignificant; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. (E) Volume-filling image of a live-stained multistack 100X T_0 cell, using the inbuilt volume-filler plugin of ImageJ. Scale bars in (B) and (C), 10 μ m.

workflow combining fluorescence-activated cell sorting (FACS) with automated confocal microscopy and CNN classification (Fig. 2A and fig. S5, A and B). This revealed that the full breadth of architectures was found within each of the molecularly defined T cell subpopulations, with varying abundances that were highly reproducible across the four healthy donors (Fig. 2B, fig. S5C, and fig. S6A). The T_0 architecture was most abundant in naïve CD8 T cells and decreased with differentiation states of higher effector function. By contrast, the polarized T_P architecture, and to a lesser extent the non-polarized T_O architecture, showed the opposite trend.

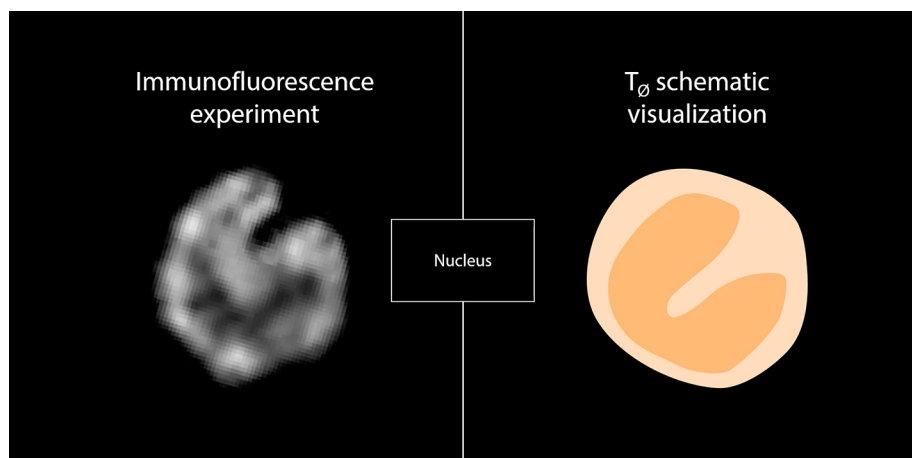
We next addressed whether T_{ARCH} subsets were specific to circulating T cells by quanti-

fying T_{ARCH} in T cells from six tissues derived from wild-type (WT) C57BL/6 mice after confirming that murine circulating T_{ARCH} profiles were similar to those of humans (Fig. 2C and fig. S7, A and B). T_0 cells were most abundant in the blood but were observable in all tissues. We confirmed the presence of tissue-resident splenic T_0 architectures through confocal microscopy of tissue sections (fig. S7C). Thus, T_{ARCH} heterogeneity was correlated with—but not fully explained by—distinct differentiation states of circulating and tissue-resident T cells.

The frequency of T_0 architectures increases during thymic T cell maturation

Given that naïve CD8 T cells in humans and WT mice are heterogeneous with respect to

the specific TCRs expressed by each cell, we postulated that T_{ARCH} subsets may be differentially abundant between distinct T cell clones with different peptide-major histocompatibility complex (MHC) specificities. Therefore, we compared polyclonal naïve CD8 T_{ARCH} profiles with those of transgenic mice where all T cells express the same TCRs. T cells from P14 mice express a TCR with specificity for the H-2D^b-restricted glycoprotein GP₃₃₋₄₁ peptide of lymphocytic choriomeningitis virus (LCMV) and those from OT-I mice express a TCR with specificity for an H-2K^b-restricted ovalbumin (OVA)-derived peptide. Despite their different clonal backgrounds, T_{ARCH} profiles were highly comparable between all three sources of T cells, which suggests that the characteristic



Movie 1. Schematic and immunofluorescence visualization of T_0 architecture. 100X fluorescence images of representative T_0 cells (left). DAPI and Hoechst are shown in grayscale, and additionally stained organelles or proteins reflecting the T_0 architecture are shown in red. A schematic visualization of the T_0 architecture is presented on the right.

T_{ARCH} profile of naïve CD8 T cells appears consistent both across and within TCR clones and peptide-MHC specificities (Fig. 2D).

We next investigated at which developmental stage T_{ARCH} profiles were established. We quantified T_{ARCH} and maturation state of lymphocytes from the thymus of WT C57BL/6 mice through single-cell assessment of CD4 and CD8 (co-)expression, which positively correlates with T cell maturation (27) (Fig. 2, E and F). This revealed differences in cellular architecture associated with the maturation stage. The double-negative CD4[−]CD8[−] pre-T cell population had a high frequency of T_P cells. The frequency of T_O cells increased in double-positive CD4⁺CD8⁺ T cells. In mature CD8 single-positive cells, the majority of cells displayed a T_O architecture (Fig. 2G and fig. S8A).

Most thymic double-positive CD4⁺CD8⁺ T cells commit to apoptosis as a consequence of absent or too-high self-peptide-MHC affinity during development (27). Thus, we hypothesized that the relative increase in T_O architectures in single-positive thymocytes may in part be due to positive selection in response to interaction with self-peptide-MHC complexes, characterized by low-affinity TCR self-peptide-MHC interactions. We assessed the expression of CD5 (28), CXCR3 (9), and baseline TCR signaling through NUR77 expression in mature naïve CD8 T cells (10) because their expression levels correlate with self-peptide-MHC affinity and hence the strength of TCR stimulation during and after thymic maturation. T_{ARCH} profiling revealed positive associations between the frequency of T_O cells and the expression of CD5, CXCR3, and NUR77, which suggested that T_O cells experience stronger self-peptide-MHC TCR signals during and after development (Fig. 2, H to J). We further quantified T_{ARCH} -

associated baseline TCR signaling in isolated double-positive CD4⁺CD8⁺ thymic T cells from P14 *Nr4a1*^{GFP} reporter mice, which express green fluorescent protein (GFP) under the control of the *Nr4a1* promoter, thus reflecting the expression of the early TCR response protein NUR77 (29, 30) (fig. S8, B and C). We observed that CD4⁺CD8⁺ T_O architectures on average exhibited higher baseline NUR77 expression compared with T_O architectures. Thus, this would be consistent with T_O cells having undergone stronger TCR signaling during development, potentially enhancing their relative survival during thymic T cell maturation.

Given the influence of age-dependent thymic involution on T cell phenotype and function (31), we analyzed the naïve CD8 T cell compartment of 16 human donors spanning diverse ages (fig. S9A). Despite a reduction in the overall abundance of naïve CD8 T cells with age (fig. S9B), we observed an increase in T_O cells, which suggests that T_{ARCH} profiles may change during the aging process, potentially as a consequence age-associated alterations in thymic T cell maturation (31) (fig. S9C). Overall, these data indicated that thymic T cell maturation produces an architecturally heterogeneous naïve T cell pool dominated by the T_O architecture, coinciding with their heightened TCR signaling throughout thymic T cell development.

T_{ARCH} is remodeled throughout acute viral infection

The canonical function of naïve CD8 T cells is to proliferate and differentiate into effector and memory phenotypes upon the recognition of cognate foreign antigen. Thus, we followed in vivo T_{ARCH} dynamics and cell fate of virus-specific CD8 T cells throughout an acute LCMV infection. We adoptively transferred recipi-

ent mice (Ly5.2 C57BL/6) with spleen-derived naïve CD8 T cells from Ly5.1 P14 *Tcf7*^{GFP} donor mice expressing GFP under control of the TCF1-encoding *Tcf7* promoter (Fig. 3A). TCF1 expression, and therefore GFP expression by proxy, identifies naïve cells as well as antigen-experienced cells that adopt a central memory phenotype (32). We profiled molecular and T_{ARCH} heterogeneity of the spleen-derived virus-specific CD8 T cell pool during early, effector, and memory phases of the infection by both FACS and imaging (Fig. 3B).

Image-based T_{ARCH} quantification revealed that the architectural landscape of antigen-specific cells was coordinated with T cell phenotype and function in vivo throughout acute infection. This was characterized by fewer T cells exhibiting T_O architectures 3 days after viral challenge, corresponding to loss of the naïve and central memory marker CD62L and preceding the down-regulation of TCF1 expression (Fig. 3, C and D; fig. S10A; and fig. S11A). In parallel, T_P increased from 10% at day 1 to 45% at day 3 after infection, preceding the appearance of the effector marker KLRG1, which peaked together with T_P at day 7 postinfection, and positively correlated with T cell size (fig. S12, A and B). Notably, the initial T_{ARCH} landscape of the LCMV-specific CD8 T cells was largely reestablished during the memory phase, coinciding with increased expression of the memory marker IL7R α , peaking at day 28, and with the reemergence of TCF1 expression. The abundance of T_O cells remained relatively stable—between 10 and 25% of LCMV-specific CD8 T cells—throughout the course of infection, showing a slight reduction at day 7 compared with day 1 postinfection. The early stages of these in vivo architectural changes were further recapitulated in a human setting after in vitro perturbation of PBMC-derived T cells with a large panel of immunomodulators, including microbial compounds, cytokines, and anti-inflammatories (supplementary text and fig. S13, A to H).

To assess the T_{ARCH} association with differentiation state during the in vivo acute infection at the single-cell level, we stratified cells based on the expression of TCF1 and quantified cellular architecture by microscopy. We observed that TCF1[−] cells, making up ~20% of the viral-specific T cell population at 3 days after exposure, were enriched in T_P architectures, confirming the association between morphological polarization and adoption of effector phenotypes at the single-cell level (supplementary text and fig. S14, A to D). Together, these analyses revealed that acute viral infection remodeled the architectural landscape of viral-specific cytotoxic T cell populations, such that naïve and memory phenotypes were dominated by the T_O architecture and effector phenotypes were dominated by morphologically polarized T_P cells.

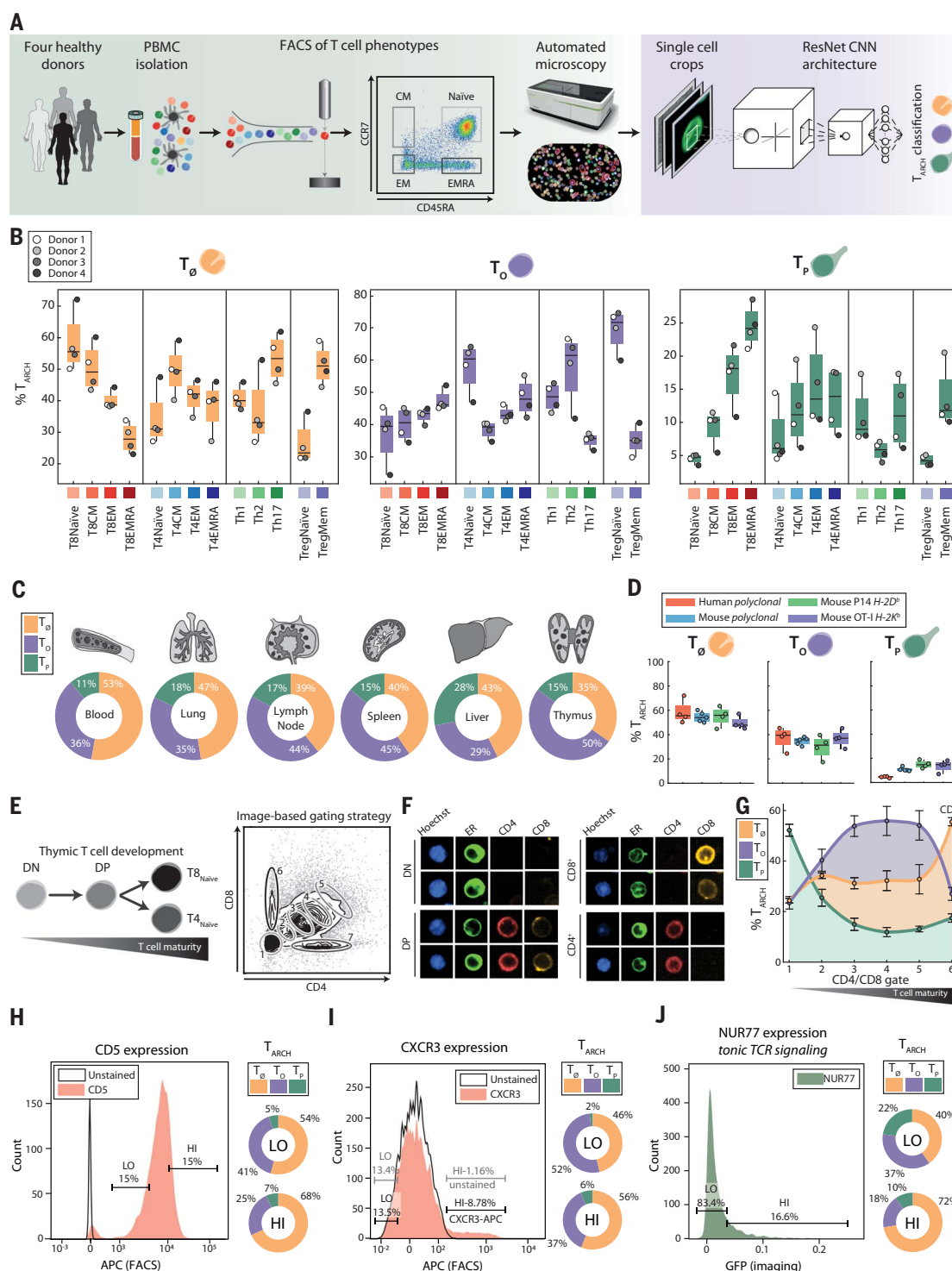


Fig. 2. T_{ARCH} profiles reflect T cell developmental and differentiation state.

(A) Workflow for T_{ARCH} profiling of FACS-sorted T cell populations. (B) Image-based T_{ARCH} quantification of PBMC-derived FACS-sorted T cell populations. Data points indicate average donor T_{ARCH} values of sorted CD3⁺ cells per population ($n = 4$ donors). Boxplot horizontal midlines indicate data medians, boxes span the 25th and 75th percentiles, and whiskers reflect the full range of the nonoutlier data. Data are derived from four independent experiments. (C) Image-based T_{ARCH} quantification of freshly isolated peripheral and tissue-derived T cells from WT C57BL/6 mice. Donut charts indicate average T_{ARCH} abundances of all cells expressing either CD4 or CD8 from three mice. Data are

representative of three independent experiments. (D) Image-based T_{ARCH} quantification of naive CD8 T cells from human blood ($n = 4$), WT C57BL/6 spleen ($n = 5$), P14 H-2D^b spleen ($n = 4$), and OT-I H-2K^b spleen ($n = 4$). Murine naive CD8 T cells were enriched using MACS, whereas human naive CD8 T cells were isolated with FACS. Boxplot horizontal midlines indicate data medians, boxes span the 25th and 75th percentiles, and whiskers reflect the full range of the nonoutlier data. (E) Schema of thymic T cell maturation (left) and associated image-based gating strategy from CD4⁺ and CD8⁺ expression levels (right; data from $n = 3$ WT C57BL/6 mice). DP, double-positive CD4⁺CD8⁺; DN, double-negative CD4⁻CD8⁻. (F) Example images of DN, DP, CD4⁺, and

CD8⁺ single-positive T cells. **(G)** Image-based T_{ARCH} quantification of gated immature T cell states and mature CD8⁺ single-positive T cells, corresponding to the gates indicated in (E). Data indicate average and standard deviation values of T_{ARCH} within each population ($n = 3$ WT C57BL/6 mice). **(H to J)** Expression and T_{ARCH} quantification of naïve CD8 T cells expressing low

or high levels of CD5 (H) and CXCR3 (I) from a WT C57BL/6 mouse, with high and low populations sorted by FACS, and NUR77 (J) from an OT-I *Nr4a1*^{GFP} mouse, with GFP expression quantified through imaging. Data in (H) to (J) are from individual mice, representative of three independent experiments.

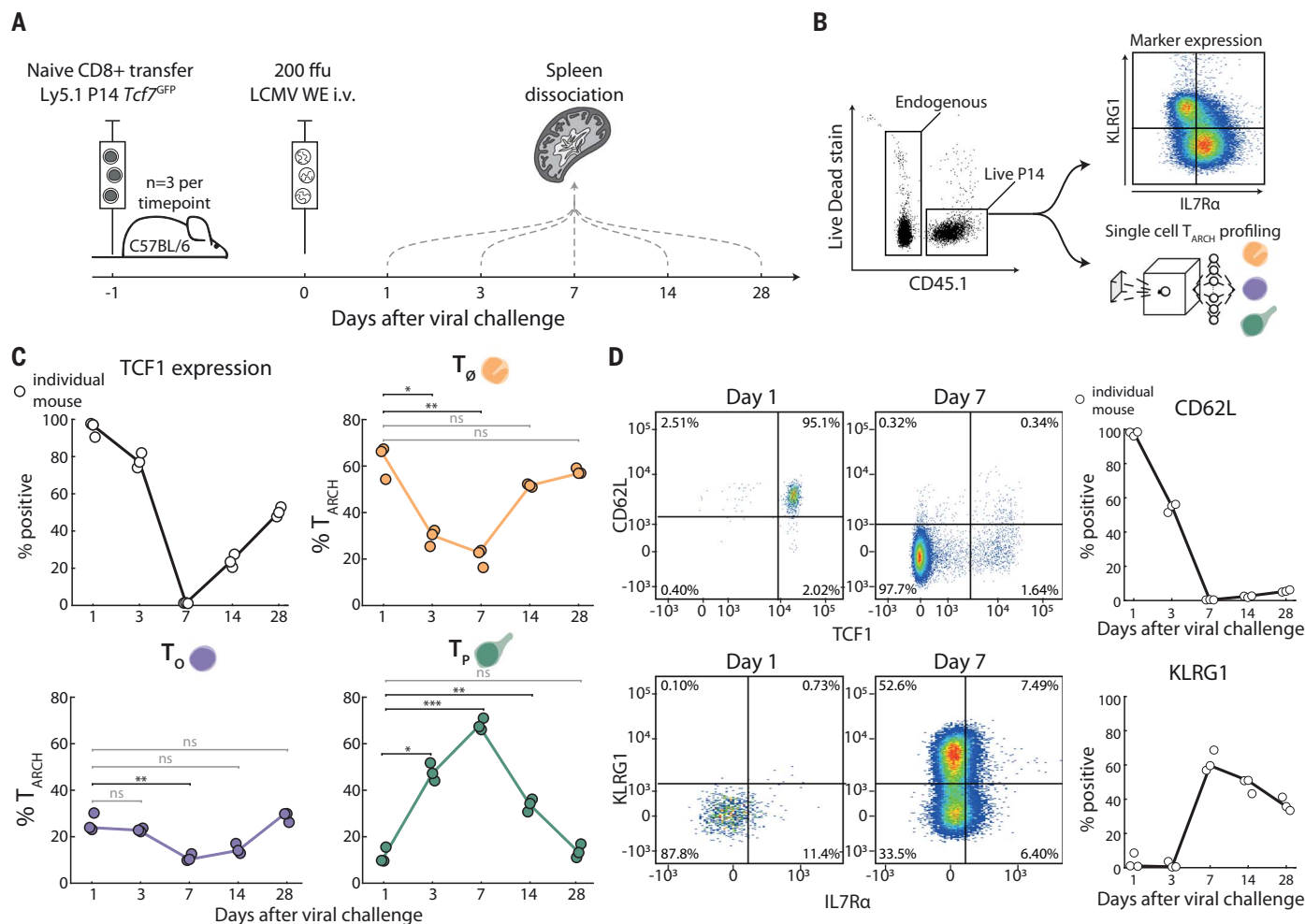


Fig. 3. T_{ARCH} is dynamically coordinated throughout acute viral infection.

(A) Experimental setup for T_{ARCH} and differentiation state profiling throughout an acute viral infection in WT C57BL/6 mice. i.v., intravenously. **(B)** Sorting strategy for live transferred P14 cells before further molecular phenotyping with FACS and image-based T_{ARCH} profiling. **(C)** Image-based TCF1 and T_{ARCH} quantification during an acute viral infection. Each data point represents averages for live transferred P14 cells from individual mice ($n = 3$ per time point). P values were

calculated using two-tailed t tests for each time point against day 1. ns, nonsignificant; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. **(D)** FACS-based quantification of naïve (CD62L, TCF1), memory (CD62L, TCF1, IL7R α), and effector (KLRG1) markers throughout infection. Individual data points indicate values from individual mice ($n = 3$ per time point). FACS plots show day 1 and day 7 expression patterns for CD62L versus TCF1 and KLRG1 versus IL7R α . FACS plots are from a single mouse, representative of three mice per time point.

T_{O} cells are predisposed to heightened TCR signaling

Given that clonally identical naïve CD8 T cells manifest heterogeneous responses to antigen (3, 4), we hypothesized that the architectural state of an individual T cell at the time of antigen stimulation may influence its subsequent antigen response. Therefore, we quantified differences in TCR signaling between T_{ARCH} subsets.

To measure both T_{ARCH} state and single-cell TCR signaling, we isolated naïve CD8 T cells from Ly5.1 OT-I *Nr4a1*^{GFP} reporter mice using

magnetic activated cell sorting (MACS) enrichment (purity > 95%) approaches (fig. S15A). Stimulation of naïve CD8 OT-I *Nr4a1*^{GFP} T cells for 2 hours ex vivo with either OVA peptide-loaded dendritic cells (DCs) or with OVA peptide in the absence of DCs resulted in an up-regulation of NUR77 expression, accompanied by a twofold loss in T_{O} and gain in T_{P} architectures (fig. S16, A and B).

Using this setup, we first performed live-cell time-lapse microscopy of naïve CD8 OT-I *Nr4a1*^{GFP} T cells with antigen-loaded DCs and

stratified NUR77 expression dynamics by initial T_{ARCH} state (supplementary text). This revealed that, although 77% (17 of 22) of the initial T_{O} cells detectably expressed NUR77, only 18% (2 of 11) of the initial T_{O} cells induced NUR77 expression after 5 hours of incubation (Fisher's exact $P < 0.0023$), revealing T_{ARCH} -associated TCR signaling heterogeneity (fig. S17, A and B).

To link T_{ARCH} and NUR77 expression dynamics, we next performed single-cell time-lapse imaging by automated microscopy of

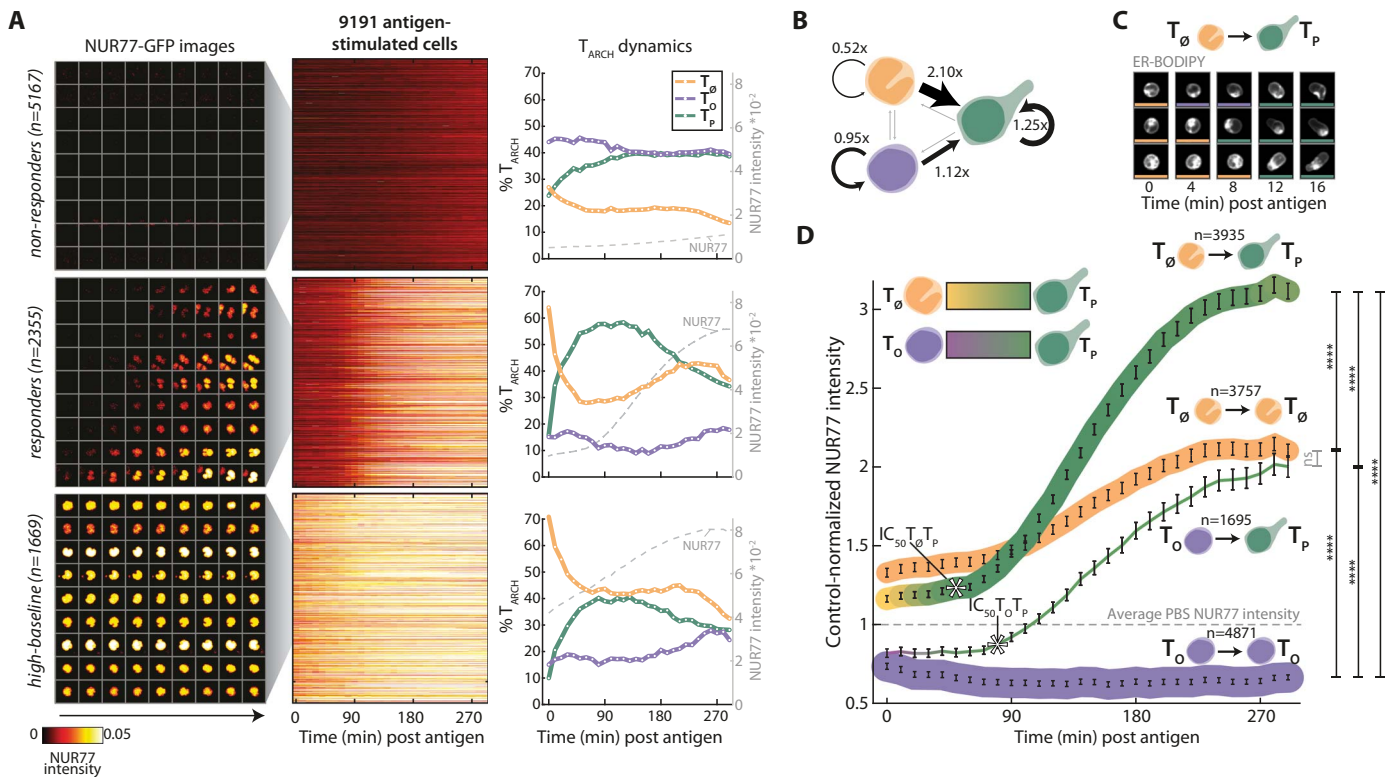


Fig. 4. T_0 architectures heighten TCR signaling in response to antigen.

(A) Clustering of single-cell NUR77 trajectories from OVA-stimulated naïve CD8 OT-I $Nr4a1^{GFP}$ T cells, showing representative pseudocolored NUR77 intensity images (left), NUR77 intensities per single cell over time (middle), and T_{ARCH} composition per cluster over time (right). Cell trajectories were grouped through spectral clustering ($k = 3$) of binarized NUR77 expression values (threshold of 0.02), grouping cells into nonresponders (top), responders (middle), and high-baseline (bottom) clusters. (Right) T_{ARCH} composition plots further show the average NUR77 intensity (light gray dashed line), corresponding to the (light gray) y axis on the right. Cells ($n = 9191$) shown were incubated with 1 $\mu\text{g}/\text{ml}$ OVA and tracked every 10 min for 5 hours. Data from one experiment, representative of three, are shown. (B) Fold-change in the single-cell T_{ARCH} trajectories (as categorized in fig. S20A) in OVA- versus PBS-stimulated naïve CD8 OT-I $Nr4a1^{GFP}$ T cells. Data are representative of three independent repeats. (C) Representative images of

naïve CD8 OT-I T cells transitioning from T_0 to T_P architectures, imaged every 4 min after the addition of 1 $\mu\text{g}/\text{ml}$ OVA. Horizontal bars reflect CNN-based classification per frame. (D) Average NUR77 throughout OVA stimulation of naïve CD8 OT-I $Nr4a1^{GFP}$ T cells, stratified by T_{ARCH} trajectory (as categorized in fig. S20A). Plots comprise data from three separate experiments. Single-cell NUR77 intensity values represent fold-changes to the average NUR77 cellular intensity in PBS (dashed gray line) per experiment (y axis). T_{ARCH} -transitioning trajectories ($T_0 \rightarrow T_P$ and $T_0 \rightarrow T_0$) are colored by average abundance of indicated T_{ARCH} at each time point (see legend), and the asterisks indicate the first time point where 50% of cells are T_P (IC_{50}). Line thickness represents the relative abundance of each trajectory. P values indicate one-way ANOVAs and post hoc Bonferroni multiple correction in NUR77 intensity at the end of the time course between T_{ARCH} trajectories. ns, nonsignificant; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. Error bars represent standard errors.

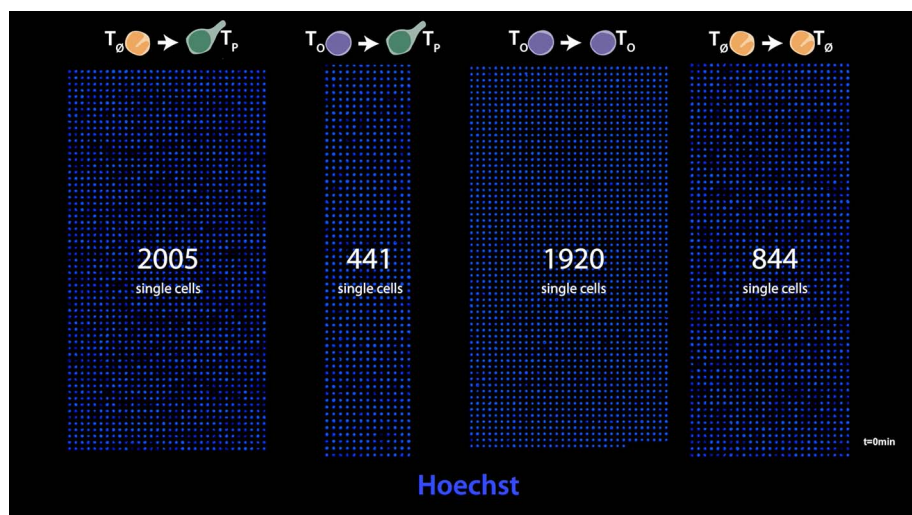
unstimulated and OVA-stimulated naïve CD8 OT-I $Nr4a1^{GFP}$ T cells over 5 hours. CNN-based T_{ARCH} classification, using a live cell-compatible ER dye and single-cell tracking, was used to quantify the dynamical relationship between NUR77 expression and T_{ARCH} state (supplementary text; fig. S18, A to C; and fig. S19, A to C).

We performed unsupervised clustering of discretized NUR77 expression of 9191 tracked cells after antigen exposure. This revealed three characteristic responses: T cells with NUR77 expression that remained at background levels (nonresponders; $n = 5167$; 56%); those with NUR77 expression that increased above background (responders; $n = 2355$; 26%); and those with detectable (low-level) NUR77 expression at the start of the time course (high-baseline; $n = 1669$; 18%) (Fig. 4A), the fraction of which was in line with previous studies (30). Re-

sponders showed a strong increase of antigen-induced NUR77 expression 90 min after stimulation, plateauing at 240 min, whereas the high-baseline T cells showed an immediate yet more linear increase in OVA-induced NUR77 expression. Nonresponding T cells were predominantly T_0 and showed limited architectural changes over time. By contrast, the majority of high-baseline and responder cells were initially T_0 and displayed a rapid loss of T_0 and an increase in T_P architectures upon stimulation. For responders, this T_{ARCH} switch from T_0 to T_P occurred before increasing NUR77 expression (Fig. 4A).

We next classified 95% of the architectural T cell responses based on their T_{ARCH} across the 5-hour time course into five characteristic profiles (fig. S20A): trajectories converting from $T_0 \rightarrow T_P$ (22%) or $T_0 \rightarrow T_0$ (9%) contrasting with

T cells maintaining their T_0 (21%), T_P (14%), or T_0 (27%) architecture (fig. S20, B and C). Comparison of the response trajectories against a phosphate-buffered saline (PBS) control revealed that antigen stimulation specifically led to a doubling in $T_0 \rightarrow T_P$ trajectories while halving the fraction of static T_0 cells, such that the majority of T_P -destined cells originated from initial T_0 cells (Fig. 4, B and C, and fig. S20D). Additionally, the 22% of T cells that followed the $T_0 \rightarrow T_P$ trajectory showed variable temporal dynamics, with rapid conversion to T_P characterizing most of these cells (Fig. 4D and fig. S21A). Stratification of NUR77 expression by T_{ARCH} trajectory class revealed that cells following a $T_0 \rightarrow T_P$ conversion showed by far the highest increase in subsequent NUR77 expression (Fig. 4D and Movie 2), with the morphological polarization



Movie 2. T_{ARCH} determines early cell-to-cell antigen response heterogeneity. Visualization of 5210 single-cell images of naive CD8 OT-I $Nr4a1^{\text{GFP}}$ cells over time upon antigen exposure, as presented in Fig. 4D. Cells were stratified according to the four main T_{ARCH} trajectories. Nuclear masks of each cell are colored by their NUR77 intensity. Data are from a single experiment, representative of three independent experiments.

largely preceding NUR77 up-regulation. Inversely, the 9% of cells that converted from T_0 to T_P showed a dampened NUR77 response, and static T_0 cells showed on average no NUR77 response, even in the presence of cell-cell contacts at the beginning of the time course experiment (fig. S22, A and B). These T_{ARCH} -associated signaling differences were maintained when culturing cells on plates coated with antigen-loaded MHC complexes, ensuring that the lack of signaling of T_0 cells was not due to differences in antigen exposure (fig. S22C). To validate these findings in a polyclonal human setting, we stimulated human PBMC-derived T cells with staphylococcal enterotoxin B (SEB) and measured phosphorylation of proteins in the mitogen-activated protein kinase (MAPK), nuclear factor κB (NF- κB), and mammalian target of rapamycin (mTOR) pathways (supplementary text). We observed heightened protein phosphorylation in T_P and T_0 compared with T_0 architectures, which confirmed reduced TCR signal transduction in T cells lacking NEIs (fig. S23, A to D).

Given the known role of antigen affinity in modulating TCR signaling strength and T cell fate decisions (12, 33–35), we evaluated how different antigen affinity affects T_{ARCH} -associated NUR77 expression. To this end, we stimulated naive CD8 OT-I $Nr4a1^{\text{GFP}}$ T cells with either the previously used high-affinity OVA_{N4} peptide or a low-affinity OVA_{I4} peptide (34, 36). Whereas OVA_{I4} induced NUR77 to a lower level compared with OVA_{N4} (fig. S24A), T_{ARCH} -associated NUR77 expression heterogeneity was maintained, whereby initial T_0 architectures induced stronger responses compared with initial T_0 architectures (fig. S24, B and C). Notably, com-

paring the T_{ARCH} stratified responses between OVA_{N4} and OVA_{I4} revealed that T_0 architectures manifested increased NUR77 expression in high- versus low-affinity stimulations, whereas T_0 NUR77 responses remained similar between antigens. Thus, specifically T_0 cells modulated their TCR signal strength in response to stronger external stimuli. Moreover, initial T_{ARCH} predicted the early antigen response in both low- and high-affinity TCR stimulations.

Cellular architecture shapes the naive T cell response independently of known molecular regulators

We set out to evaluate whether cellular architecture acted independently from known regulators of early T cell signaling response in naive CD8 T cells. Particularly, CD5, CXCR3, and NUR77 (as a proxy for low-level baseline TCR activation, called tonic signaling) have previously been reported to correlate with enhanced antigen responsiveness (9, 10, 37), and naive CD8 T cells that highly expressed these proteins were enriched in the T_0 architecture (Fig. 2, H to J). We therefore quantified NUR77 expression levels and T_{ARCH} trajectories of CD5^{HI} , CD5^{LO} , CXCR3^{HI} , CXCR3^{LO} , NUR77^{HI} , and NUR77^{LO} naive CD8 T cell populations and quantified their T_{ARCH} trajectories and TCR signal strength by imaging after antigen stimulation. Although higher levels of all three proteins, particularly CD5 and NUR77, were associated with increased antigen-induced NUR77 expression, T_{ARCH} -dependent responses were maintained across all populations, with initial T_0 architectures displaying the strongest responses independent of CD5, CXCR3, and NUR77 levels (fig. S25, A to C).

To investigate a direct effect of T_{ARCH} on antigen response, we disrupted the T_{ARCH} profile of naive CD8 P14 $Nr4a1^{\text{GFP}}$ before antigen stimulation. To do so, we cultured the cells in complete media without TCR stimulation for 24 hours, resulting in a population shift from 60% T_0 to 20% T_0 without a loss of CD5 and CXCR3 expression (fig. S25, C and D). Antigen stimulation of the T_0 -low cultured population of T cells resulted in a 38% decrease in NUR77 expression after 5 hours compared with the T_0 -high population response (fig. S25E). Stratification of NUR77 expression by initial T_{ARCH} revealed that, at the single-cell level, T_0 NUR77 levels were almost identical between conditions. Consistently, 90% of the population-level response difference could be predicted by the initial T_0 -high T_{ARCH} profile and the average single-cell responses per T_{ARCH} in the T_0 -low population (fig. S25, F and G). Thus, the architectural heterogeneity present in monoclonal naive CD8 T cells influenced the population-level response to antigen independent of the expression levels of antigen response-associated proteins.

Heightened T_0 antigen responses are dependent upon store-operated calcium entry

We next explored whether TCR signaling events occurring upstream of NUR77 expression associated with T_{ARCH} . One of the earliest events after TCR activation is calcium ion (Ca^{2+}) influx into the cytosol, occurring within minutes of TCR engagement (38). We performed live-cell time-lapse calcium imaging of naive CD8 T cells from Ly5.1 OT-I mice in response to antigen exposure, using the Ca^{2+} dye Fluo-4. Tracking of 259 individual cells revealed peak Ca^{2+} levels at 4 min after OVA stimulation (Fig. 5A). Stratifying the average Ca^{2+} responses by initial T_{ARCH} revealed that all T_{ARCH} subsets induced a strong Ca^{2+} influx upon stimulation (Fig. 5B and fig. S26, A to C). Compared with T_0 cells, the T_0 cells showed a 50% increase in peak intracellular Ca^{2+} concentrations and displayed more sustained Ca^{2+} levels after antigen recognition (Fig. 5, B to D).

Store-operated calcium entry (SOCE) is a critical mechanism mediating sufficient intracellular Ca^{2+} concentrations required for T cell activation. In SOCE, the depletion of ER Ca^{2+} stores triggers cytosolic influx of extracellular Ca^{2+} by coupling the ER-resident stromal interaction molecules (STIM1 and STIM2) with plasma membrane Ca^{2+} release-activated Ca^{2+} (CRAC) channels, predominantly ORAI1 (39–42). To assess whether SOCE differs between T_{ARCH} , we performed time-lapse imaging of unperturbed and Fluo-4-loaded human CD8 T cells (fig. S27A). After calibration, we triggered ER Ca^{2+} store release with thapsigargin in the absence of extracellular Ca^{2+} , which led to equal increases in Fluo-4 intensity between T_0 and

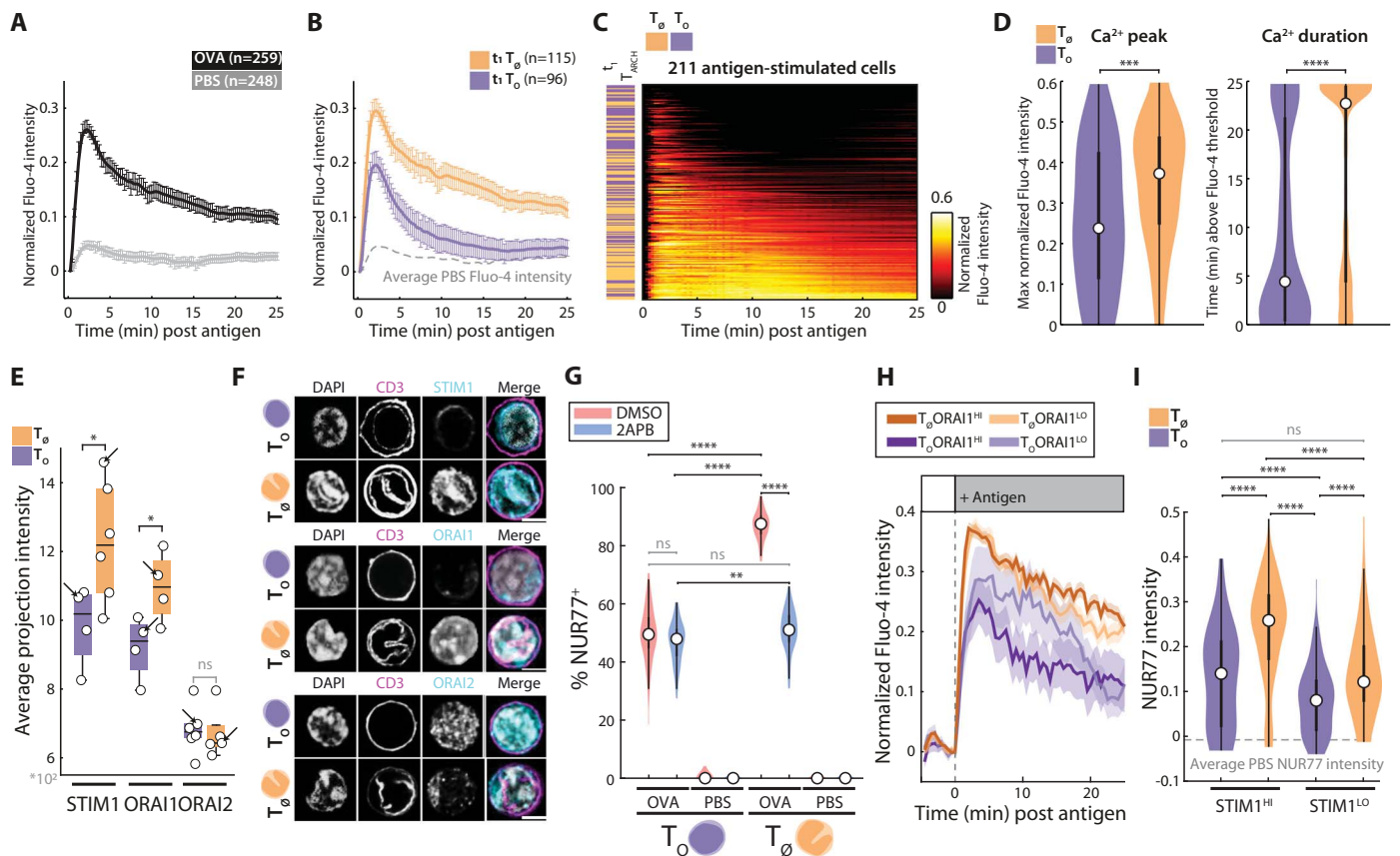


Fig. 5. Increased SOCE underlies heightened T_O antigen responses.

(A) Average single-cell Fluo-4 intensities (y axis) over time (x axis) of naïve CD8 OT-I T cells, normalized to starting Fluo-4 intensity for 1 μ g/ml OVA-stimulated ($n = 259$) and PBS control ($n = 248$) cells. Cells were imaged every 12 s for 25 min. Error bars represent standard errors. (B) Data from OVA-incubated cells [as in (A)] stratified by initial T_O ($n = 115$) and T_O ($n = 96$) architectures. Error bars represent standard errors. (C) Single-cell trajectories of all OVA-incubated naïve CD8 OT-I T cells shown in (B), sorted by average Fluo-4 intensity (see legend) across the whole time course (x axis). Initial T_{ARCH} is color-coded (left). (D) Violin plots showing the maximum Fluo-4 intensity per cell (left) and time with Fluo-4 intensity above 0.1 per cell (right), stratified by initial T_O and T_O architectures [as in (C)]. Violin plot outlines indicate local data density, inner white circles indicate data median, thick black vertical lines indicate the 25% to 75% percentile, and thin lines indicate the data range. Data in (A) to (D) are representative of two independent experiments. (E) Boxplots of STIM1, ORAI1, and ORAI2 protein abundance in T_O and T_O architectures across cells. Data represent average projection intensities of multistack 100X immunofluorescence microscopy images. Highlighted data points indicate cells shown in (F). Boxplot horizontal midlines indicate data medians, boxes span the 25th and 75th percentiles, and whiskers reflect the full range of the nonoutlier data. P values in

(D) and (E) were calculated with two-tailed Student's t tests. (F) Representative images of STIM1, ORAI1, and ORAI2 in T_O and T_O architectures. Single z-stacks are shown for CD3 and DAPI, and average intensity projections across 50 500-nm z-stacks are shown for STIM1, ORAI1 and ORAI2. Scale bars, 5 μ m. (G) Violin plots representing the average percentage of naïve CD8 $Nr4a1^{GFP}$ cells expressing NUR77 after 5-hour incubation with either 1 μ g/ml OVA or PBS in the presence or absence of 10 μ M 2-APB across 81 image frames. Populations are additionally stratified by their T_{ARCH} at the end of the time course. Data are representative of two independent experiments. (H) Average single-cell Fluo-4 intensities (y axis) over time (x axis) of FACS-sorted ORAI1^H and ORAI1^{L0} naïve CD8 P14 T cells incubated with 1 μ g/ml GP₃₃₋₄₁. Data are stratified by initial T_{ARCH} , normalized to starting Fluo-4 intensity. Cells were imaged every 30 s for 5 min at steady state and 25 min after addition of antigen. Shaded error bars represent standard errors. (I) Violin plots showing single-cell NUR77 intensities after TCR activation of naïve CD8 P14 $Nr4a1^{GFP}$ T cells with 1 μ g/ml GP₃₃₋₄₁ for 5 hours. Cells are stratified by their high and low STIM1 expression and their T_{ARCH} at the end of the time course. Data in (H) and (I) are representative of two independent experiments. P values in (G) and (I) indicate one-way ANOVA and post hoc Bonferroni multiple correction. ns, nonsignificant; $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

T_O (fig. S27B). Upon subsequent addition of extracellular Ca^{2+} , Ca^{2+} influx was, however, greater in T_O compared with T_O (fig. S27C). We quantified STIM1, ORAI1, and ORAI2 levels in human T cells by high-resolution confocal microscopy, which revealed increased expression levels of the positive SOCE regulators STIM1 and ORAI1 in T_O compared with T_O , with particularly STIM1 localization concentrated within NEIs (Fig. 5, E and F). By contrast, ORAI2, a proposed negative regulator of SOCE-mediated

calcium influx (43), showed equal expression levels between architectures.

To establish a causal link between SOCE and T_{ARCH} -induced Ca^{2+} influx heterogeneity, we activated naïve CD8 P14 $Nr4a1^{GFP}$ T cells in the presence or absence of the SOCE inhibitor, 2-APB (44). At the population level, 2-APB treatment reduced NUR77 expression by ~50% (fig. S27D). Subsequent stratification by T_{ARCH} revealed that 2-APB specifically affected T_O architectures, reducing their NUR77 expres-

sion to the level of that of T_O architectures in the absence of 2-APB. By contrast, T_O responses were not affected by 2-APB treatment. Thus, the heightened response to antigen of the T_O architecture was critically dependent on SOCE (Fig. 5G).

We next investigated whether T_{ARCH} -associated antigen response differences were due to increased expression levels of ORAI1 and STIM1, whose expression was higher in cells with T_O architectures. We compared Ca^{2+} influx and

NUR77 expression between T_O and T_O architectures in FACS-sorted populations of cells either high or low in ORAI1. At the population level, we observed that ORAI1^{HI} and ORAI1^{LO} cells did not display differential Ca^{2+} influx or NUR77 expression after antigen stimulation (fig. S27, E to G). Rather, within both ORAI1^{HI} and ORAI1^{LO} populations, initial T_O architectures displayed sustained Ca^{2+} signaling and heightened NUR77 expression compared with T_O architectures (Fig. 5H and fig. S27G). STIM1 is not expressed on the cell surface, rendering live-cell sorting of STIM1^{HI} and STIM1^{LO} naïve CD8 T cell populations impractical. We therefore related end-point T_{ARCH} and NUR77 expression to intracellular STIM1 IF by microscopy. Although STIM1 levels correlated with NUR77 expression (fig. S27, H to J), T_O architectures displayed heightened NUR77 intensities within both STIM1^{HI} and STIM1^{LO} populations (Fig. 5I). In contrast to ORAI1, which displayed similar spatial distributions between architectures, STIM1 was spatially enriched within the NEIs characteristic of the T_O architecture. Thus, the different activation potential between T_O and T_O architectures may have been explained in part by their spatial colocalization of STIM1 and other subcellular machinery, resulting in increased SOCE-mediated Ca^{2+} influx and NUR77 expression after TCR activation.

T_{ARCH} shapes the differentiation trajectory of naïve CD8 T cells

Because TCR signaling strength is known to influence the differentiation trajectory of naïve CD8 T cells (12, 13, 34), we measured T cell differentiation in relation to T_{ARCH} before activation. To investigate whether T_{ARCH} contributes to the formation of effector and memory populations, we performed ex vivo image-based single-cell fate tracking, which has recently been used to measure memory versus effector differentiation ex vivo (34). To this end, we isolated single naïve CD8 T cells from $Tcf7^{GFP}$ mice into individual wells and tracked their colony formation and phenotypes after activation by plate-bound α -CD3 and α -CD28 antibodies (Fig. 6A).

We identified 124 wells that contained single T cells and manually annotated their initial T_{ARCH} , revealing a comparable abundance of T_O and T_O cells compared with the expected T_{ARCH} profile (fig. S28A). Of these, 42 cells formed colonies of at least five cells after 7 days (Fig. 6B). Quantification of the abundance of cells in each colony at day 7 revealed that initial T_O architectures had undergone more proliferation, resulting in larger colonies (Fig. 6C and fig. S28B). Furthermore, 72% of T_O architectures had undergone their first cell division within 36 hours compared with only 40% of T_O architectures (Fig. 6D). Quantification of the entire population of cells

across all wells at day 7 revealed that 95% were derived from T_O architectures (Fig. 6E). The largest three T_O -derived colonies accounted for 56% of all cells, whereas the three largest T_O -derived colonies contributed <4% of the entire cell population at day 7 (Fig. 6F).

We next investigated the phenotypes of the expanded colonies by evaluating TCF1 expression. Our data recapitulated the known negative correlation between colony size and percentage of cells expressing TCF1 (34) (Fig. 6G). Stratification of colony TCF1 expression by initial T_{ARCH} revealed a strong association, whereby 84% of TCF1⁺ effector precursors were T_O -derived, whereas 100% of the TCF1⁺ memory precursors were T_O -derived (Fig. 6, H to K, and fig. S28C). Consistently, SEB-activated human T_O and T_P architectures expressed higher levels of the effector cytokines interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) after 48 hours compared with T_O architectures (fig. S29, A and B).

Together, these data illustrated that T_O architectures divided faster in response to antigen stimulation and down-regulated TCF1, which is associated with differentiation into effector cells. By contrast, T_O architectures were slower to initiate the first cell division, proliferated less, and were predisposed to form TCF1⁺ prememory cells.

Discussion

Collectively, our data support cellular architecture as a phenotypic dimension of T cells that may predetermine TCR signal intensity and differentiation trajectory within a population of naïve CD8 T cells. We identified three predominant T_{ARCH} subsets, including a polarized T cell morphology (T_P) and nonpolarized morphologies defined by either the presence (T_O ; stripy) or absence (T_O ; conventional) of NEIs. This architectural heterogeneity was observed in humans and mice, both ex vivo and in situ. The relative abundance of different T_{ARCH} within a population of clonal T cells was regulated in response to extrinsic stimuli during activation and differentiation and shaped both early TCR signaling heterogeneity and effector and prememory differentiation of naïve CD8 T cells.

We report a strong link between T_{ARCH} and the subsequent response to cognate antigen exposure. Upon TCR stimulation of monoclonal naïve CD8 T cells, the T_O subpopulation rapidly lost its characteristic NEIs and adopted a T_P morphology, engaging in enhanced SOCE and TCR signaling-induced gene expression. This increased signaling of T_O cells preceded increased cell proliferation and effector differentiation. By contrast, the T_O subpopulation showed reduced TCR signaling associated with preferential formation of smaller colonies with prememory phenotypes.

Early T_{ARCH} -dependent response differences were not explained fully by expression-level

differences in either key SOCE machinery or other proteins previously reported to influence naïve T cell response dynamics, including CD5, CXCR3, and baseline NUR77 expression reflecting self-peptide-driven tonic signaling (9, 28). Thus, our data indicated the presence of other architecture-associated factors contributing to the observed antigen response differences. These could include as-yet unidentified critical regulatory proteins with mechanisms that act independently of cellular architecture. Alternatively, these factors may act in a manner that is dependent on the differential spatial organization of proteins, complexes, and organelles. For example, the enhanced calcium signaling occurring in T_O cells within minutes of initial TCR stimulation could stem from preexisting spatial enrichment of ER-resident key SOCE machinery, including STIM1. Additionally, more efficient early recruitment of adapter proteins Zap70 and SLP76 to the TCR complex and nuclear import of transcription factors could further contribute to the enhanced propensity of T_O cells toward effector differentiation. It is very well possible that the architecture-dependent functional disparities result from a combination of such mechanisms.

Our data revealed that, during thymic T cell development, a T_O -high population of naïve T cells emerged from immature CD4⁺CD8⁺ double-positive populations high in T_O architectures. This was potentially influenced by T_O architectures manifesting because of stronger TCR signals to self-antigen resulting in their positive selection, as supported by the high CD5 expression of mature T_O architectures. However, given that T_{ARCH} profiles were largely consistent between P14 and OT-I mouse models, whose CD5 levels differ (28), further layers of regulation in addition to TCR signal strength are likely to play a role in T_{ARCH} establishment during development. In the periphery, homeostasis of the T_O architecture could be influenced by self-peptide-dependent tonic signaling, as suggested by their higher levels of baseline NUR77 expression. Finally, the molecular mechanisms and cellular contexts through which NEIs develop within individual T cells remain to be elucidated. Because some thymic CD4⁺CD8⁺ double-negative cells also displayed T_O architectures, the formation of NEIs in some pre-T cells may take place in the bone marrow before T lymphocyte fate commitment (45). A limitation of this study is that current technologies prevent the ability to examine the fate of T_O or T_O in vivo, which would allow full evaluation of how cellular architecture contributes to fate decisions, as indicated by our in vitro TCR stimulation studies. Developments in image-based cell sorting (46, 47) could provide a means to isolate architecturally pure T cell subsets, enabling the molecular profiling of T_{ARCH} as well as in vivo

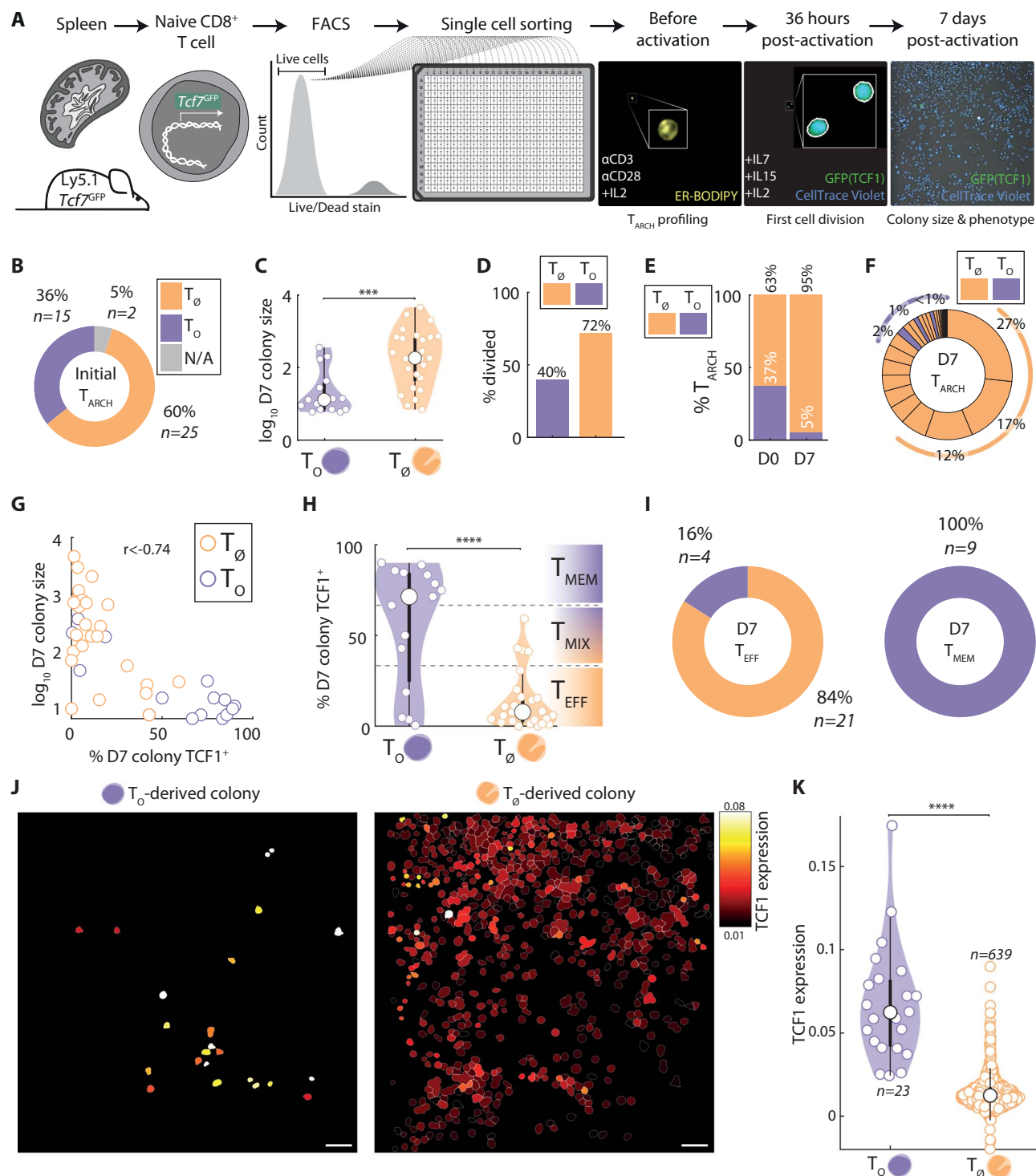


Fig. 6. T_{ARCH} shapes the differentiation trajectory of naïve CD8 T cells.

(A) Workflow for the investigation of T_{ARCH}-associated differentiation trajectories. Individual ER-Bodipy and CellTrace Violet-stained naïve CD8 Tcf7^{GFP} T cells were FACS-sorted into α-CD3- and α-CD28-coated 384-well plates, imaged, and incubated with additional cytokine supplementation. Live cells were further imaged 36 hours and 7 days after activation. (B) Donut plot illustrating the T_{ARCH} profile of cells immediately after FACS sorting. Data comprise cells for which colonies of more than five cells were identified after 7 days. N/A indicates cells where T_{ARCH} could not be assigned because of technical reasons. N values indicate the number of single cells assigned to each T_{ARCH} subset. (C) Violin

plots of the log₁₀-transformed number of cells identified per colony 7 days after activation, stratified by initial T_{ARCH}. Data points indicate individual colonies at day 7 (D7). (D) Bar plots of the percentage of cells that had divided after 36 hours, stratified by initial T_{ARCH}. (E) Bar plots comparing the T_{ARCH} profile of all cells at day 0 (D0), and the initial T_{ARCH} corresponding to all cells at D7. (F) Colony sizes (as percentage of all cells across all colonies at D7) per colony, colored by initial T_{ARCH}. The largest three colonies per initial T_{ARCH} are annotated. (G) Scatter plot of log₁₀-transformed colony size and percentage of cells expressing TCF1 per colony, 7 days after activation. Pearson correlation indicated (r). Data points indicate individual colonies at D7. (H) Violin plots

reflecting the percentage of cells expressing TCF1 per colony, stratified by initial T_{ARCH} . Each colony is annotated as memory (T_{MEM} ; $\geq 66\%$ TCF1⁺), mixed (T_{MIX} ; $>33\%$ and $<66\%$ TCF1⁺), or effector (T_{EFF} ; $\leq 33\%$ TCF1⁺). Data points indicate individual colonies at D7. (I) Donut plots displaying the percentages of the T_{EFF} and T_{MEM} colonies derived from T_0 and T_0 architectures. N values indicate the number of colonies at D7. (J) Pseudocolored images representing

T_0 - and T_0 -derived cells 7 days after activation. Cells are outlined according to their nuclear segmentation and colored by mean cellular TCF1 intensity. Scale bars, 20 μm . (K) Violin plots of TCF1 expression of the cells in (J). Data points indicate single-cell TCF1 intensity. P value represents a two-tailed Student's t test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. Data are from a single experiment, representative of two independent experiments.

transfer studies that may further elucidate their development, maintenance, and function.

We provide a cell-intrinsic mechanism explaining the heterogeneity observed in the early naïve T cell response, independent of the baseline expression of CD5, CXCR3, and NUR77 (9, 28). Given that early TCR signaling strength is known to affect T cell fate trajectory (12, 35, 48), it is likely that initial T_{ARCH} -dependent TCR signaling heterogeneity plays a critical role for the observed T cell fate decisions, thereby providing the organism a means to regulate its overall immune response and prevent simultaneous and homogeneous activation of the entire antigen-specific T cell pool. Furthermore, given that we observed architectural heterogeneity among all populations of circulating CD4 and CD8 T cells, it is likely that this regulatory concept shapes the antigenic responses in additional contexts. For example, the replenishment of the T_0 architecture within the memory T cell population observed in both human peripheral memory phenotypes as well as within the monoclonal memory T cell pool after acute viral infection in mice raises the possibility that T_{ARCH} also contributes to response heterogeneity upon reinfection. Additionally, because naïve CD8 T cells of older donors are known to preferentially differentiate into effector cells at the expense of memory cells, the observed increased T_0 abundance may contribute to this functional age-related shift in T cell function.

Together, our results reveal an important role for cellular architecture in regulating T cell function. By combining automated microscopy with deep learning, we provide a morphological framework that can be used to investigate, predict, and control the response of individual T cells upon antigen encounter.

Materials and methods

Human blood

Buffy coats and whole blood were obtained from coded healthy donors provided by the Blutspende Zürich, under a study protocol approved by the Cantonal Ethics Committee, Zürich (KEK Zürich, BASEC-Nr 2019-01579). Informed, signed consent was obtained from all blood donors. Detailed donor information can be found in table S2.

Human PBMC and T cell enrichment

Peripheral blood was diluted 1:1 PBS (Gibco; ThermoFisher Scientific, Waltham, MA), before

addition of Lymphoprep (Stemcell Technologies, Vancouver, Canada) enabling purification of PBMCs by density centrifugation at 800g for 30 min. After isolation, PBMCs were washed twice with PBS and cell counts were determined with automated cell counting (Countess II, ThermoFisher Scientific). All cells were resuspended in RPMI-1640 media supplemented with either 10% fetal bovine serum (Gibco) or 10% human serum (Chemie Brunschwig AG, Basel, Switzerland) and seeded into clear-bottom PhenoPlate 384-well plates (Revvity, Waltham, MA) corresponding to ~20,000 cells per well. When required, perturbations were added to 384-well plates before cell seeding manually, or using an Echo liquid handler (Labcyte, San Jose, CA). Cells were subsequently incubated at 37°C with 5% CO₂ between 1 and 48 hours, as indicated. For single-cell cytokine staining, 5 $\mu\text{g}/\text{ml}$ Brefeldin A (Biolegend, San Diego, CA) was added 2 hours before fixation to prevent cytokine secretion and improve staining.

When required, human T cells were enriched from PBMCs using a column-based MACS Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) or Pan CD8 T cell Isolation Kit, according to the manufacturer's specifications.

FACS of human and murine T cell subpopulations

MACS-enriched human T cells or MACS-enriched CD8 P14 $Nr4a1^{\text{GFP}}$ were resuspended to $\sim 1 \times 10^8$ cells/ml in PBS supplemented with 5% fetal bovine serum (FBS) before staining with fluorescently tagged antibodies (table S3) in the dark on ice for 30 min. For unconjugated ORAI1 staining, 1/50 ORAI1 was added and stained at room temperature, before washing and resuspending to the initial cell concentration before staining with a conjugated secondary antibody as described above. Stained T cells were washed with PBS before resuspension at a final concentration of $\sim 1 \times 10^7$ cells/ml in 5% FBS. Cell sorting was performed with either an Aria Fusion flow cytometer (BD Biosciences) or a BD FACSAriaTM (BD Biosciences) at 4°C. Sorting of human subpopulations achieved an average purification of 96.8% validated by fluorescence imaging (table S4). FACS data were analyzed using FlowJo software (FlowJo Enterprise, version 10.0.8, BD Biosciences). Purified human T cells were collected in 10% FBS in RPMI and concentrated to 400,000 cells/ml before plating. Cells were plated in at

least triplicate wells in 384 well plates and incubated for 30 min at 37°C with 5% CO₂ to allow cells to settle at the bottom of each well. Purified murine T cells were collected in phenol red-free RPMI-1640 supplemented with Glutamine (Bioconcept), 1% (v/v) penicillin-streptomycin, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, 0.1 mM nonessential (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine) amino acids, 20 mM HEPES (all from Gibco) before further staining and live-cell imaging.

Cell fixation, permeabilization, and antibody staining

After incubation, PBMCs or T cells were fixed and permeabilized for 20 min at room temperature with 1% (w/v) formaldehyde, 0.05% (v/v) triton X-100 or 0.5% (w/v) formaldehyde, 0.05% (v/v) Triton X-100, 10 mM sodium(met)aperiodate, and 75 mM L-lysine monohydrochloride (all from Sigma-Aldrich, St. Louis, MO) before blocking at 4°C with PBS supplemented with 5%.

A combination of primary conjugated, primary unconjugated and secondary conjugated antibodies were used in this study (table S3). When required, primary unconjugated antibodies were diluted in 5% FBS and used to stain fixed and permeabilized cells overnight at 4°C, which were subsequently washed twice with PBS before secondary antibody staining. Conjugated primary, secondary antibodies, and phalloidin were diluted in PBS containing 10 μM DAPI (Sigma-Aldrich) and added to blocked cells in the dark at room temperature for 1 hour before aspiration. Cell solutions were aspirated, and PBS added to cells before confocal imaging.

Cell lines

DC lines (DC2.4, Merk) were cultured in RPMI-1640 supplemented with Glutamine (Bioconcept), 1% (v/v) penicillin-streptomycin, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, 0.1 mM nonessential (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine) amino acids, 20 mM HEPES (all from Gibco). Cells from passage two were used in this study.

Mice

Six- to 10-week-old male or female mice were used for the experiments performed in this study. WT Ly5.2 C57BL/6 mice were obtained

from the ETH Phenomics Center or from Janvier Labs. Ly5.1 C57BL/6J, Ly5.1 P14 mice (CD8 T cells with a transgenic TCR with specificity for the glycoprotein GP₃₃₋₄₁ epitope of lymphocytic choriomeningitis virus (LCMV) in the context of H-2D^b) (49), Ly5.1 OT-I (CD8 T cells with a transgenic TCR with specificity for the OVA₂₅₇₋₂₆₄ SIINFEKL peptide) (50), *Nr4a1*^{GFP} mice expressing GFP under the control of the NUR77 promoter (51), and *Tcf7*^{GFP} reporter mice (expressing GFP under the control of the *Tcf7* locus) (52) were housed and bred under specific pathogen-free conditions in animal facilities at ETH Zurich, Hönggerberg. P14 *Tcf7*^{GFP}, P14 *Nr4a1*^{GFP}, and OT-I *Nr4a1*^{GFP} mice were obtained by crossing *Tcf7*^{GFP} and *Nr4a1*^{GFP} mice to either P14 or OT-I mice.

All animal experiments were conducted in accordance with the Swiss federal regulations and were approved by the cantonal veterinary office of Zurich (animal experimental permissions: 115/2017, 022/2020). Specifically, animals were housed in individually ventilated cages (500-cm² floor space) each with between two and five mice per cage and fed on a normal chow diet. Mice were housed in a specific pathogen-free environment with day and night cycles of 12 hours (06:00 to 18:00) with an ambient temperature of 20° to 22°C and an air humidity of 40%. Cages were enriched with tissues, shredded paper and cotton for nest building, a plastic house, loft, and tunnel. The tunnel was further used for animal handling to avoid lifting by the tail. Mice were euthanized with a gradual increase in CO₂ using a GasDocUnit (medres Medical Research, Cologne, Germany).

Murine organ isolation and digestion

Blood, lymph nodes, spleen, thymus, and lung analyzed in Fig. 2, C to G, and figs. S7 and S8 were obtained from PBS-perfused mice. Spleen, thymus, and lymph nodes were smashed through 70-μm strainers (BD Biosciences) using a syringe plunger to prepare single-cell suspensions. Lungs were cut into smaller pieces and further incubated in RPMI-1640 (BioConcept) containing 2 mM L-Glutamine (BioConcept), 2% penicillin-streptomycin (Sigma-Aldrich), 1x Non-Essential Amino Acids (Sigma-Aldrich), 1 mM Sodium Pyruvate (Gibco), 10% fetal bovine serum (Omnilab), 25 mM HEPES (Gibco), 50 μM β-Mercaptoethanol (Gibco), and 2.4 mg/ml collagenase type I (Gibco) and 0.2 mg/ml DNase I (Roche Diagnostics) for 40 min at 37°C. Blood-derived mononuclear cells were obtained by gradient centrifugation over 30% Percoll (Sigma-Aldrich). Erythrocytes were removed by ACK lysis buffer treatment at room temperature for 5 min.

Naïve CD8 T cells were isolated from mouse spleens after spleen cell suspension as described above, using the EasySep™ Mouse Naïve CD8 T cell Isolation Kit (Stemcell Technologies),

following manufacturer's instructions. Naïve CD8 T cells were enriched to >95% of all cells.

Adoptive transfer

For adoptive transfer, 2×10^6 (for day 1 and day 3 after infection analysis) or 10,000 sorted naïve CD8 T cells were intravenously injected into naïve young CD45.2 C57BL/6 recipient mice.

Virus, viral peptides, and infections

LCMV strain WE was kindly provided by R. M. Zinkernagel (University Hospital Zurich), propagated on baby hamster kidney 21 cells (53), and viral titers were determined as previously described (54). Acute LCMV infections were conducted by injecting 200 ffu of the LCMV WE strain intravenously into the tail vein of recipient mice. OVA_{N4} (SIINFEKL), OVA_{I4} (SIILFEKL), and GP₃₃₋₄₁ (KAVYNFATC) peptides were obtained from EMC Microcollections GmbH.

Acute infection flow cytometry phenotyping and sorting

Identification of viable cells was done by fixable near-infrared (IR) dead cell staining (Life Technologies). Surface staining was conducted at 4°C for 30 min. After staining, cell suspensions were washed and stored in PBS containing 2% FBS (Omnilab) and 5 mM of EDTA (Sigma-Aldrich) for acquisition. Multiparameter flow cytometry analysis was performed on a FACS Canto™ (BD Biosciences) cell analyzer and FACS was performed using a BD FACS Aria™ (BD Biosciences) cell sorter with FACS Diva software. Data were analyzed using FlowJo software (FlowJo Enterprise, version 10.0.8, BD Biosciences).

For T_{ARCH} quantification of antigen-specific CD8 T cells throughout the infection, live CD45.1 cells were sorted for subsequent architectural profiling.

Live-cell staining

Freshly isolated tissue or blood-derived murine and human T cells were filtered through a 70-μm strainer and concentrated at 1×10^6 cells/ml in phenol red-free RPMI-1640 supplemented with Glutamine (Bioconcept), 1% (v/v) penicillin-streptomycin, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, 0.1 mM nonessential (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine) amino acids, and 20 mM HEPES (all from Gibco) before staining. Live cells were stained for 30 min at 37°C and 5% CO₂ with 2 μM ER-Bodipy green or red (Thermo Fisher Scientific) and 5 ng/ml Hoechst (VWR International GmbH) for nuclear staining. For CD4 and CD8 staining of live cells, antibodies were added to a final concentration of 1/400 during incubation. To stain dead cells that were used to train CNNs to identify dead cell morphological signatures (fig. S19, A to C), 0.4 μg/ml

propidium iodide (PI) was also supplemented during the staining incubation. Subsequently, stained cell suspensions were carefully washed with PBS, before resuspension in complete media and further supplemented with 20 U/ml interleukin-2 (IL-2) (PeproTech).

High-resolution fluorescence microscopy

MACS-enriched T cells derived from PBMCs of a single healthy human donor were fixed and permeabilized before antibody staining, or live stained with organelle markers, as described above, using μ-Slide VI imaging slides (Ibidi). Multichannel fluorescence imaging was performed using a Nipkow spinning disk microscope (Visitron) coupled with a W1-T2 Confocal Scanner Unit (Yokogawa) and a 100x 1.4 CFI Plan Apo Oil objective. Images comprised 47 500-nm z-stacks, which were processed using ImageJ (55) to create 3D cell models presented in Movie 1, movie S1, and Fig. 1E (using the in-built ImageJ Volume Viewer plugin).

Invaginated organelle and machinery intensities were calculated using MATLAB R2022b. Nuclei from individual z-stacks best reflecting the T_{ARCH} of each cell were segmented, and pixel intensities for each marker rescaled between 0 and 1 within the nuclear masks. Masks were subsequently shrunk to enable quantification in invaginated marker enrichment without peripheral membrane stain, and either average normalized marker intensities, or pixel correlations with CD3 or ER, were calculated. Using ImageJ, average projections of STIM1, ORAI1, and ORAI2 intensity across 50 500-nm z-stacks were calculated to determine their abundance (Fig. 5, E and F).

In situ imaging of spleen sections

PBS-perfused spleens were fixed in BD Cytofix/Cytoperm (BD Biosciences) and diluted in 1X PBS for 6 to 7 hours at 4°C. After a short wash in 1X PBS, organs were cryoprotected in 30% sucrose in 1X PBS overnight at 4°C, followed by an embedding in OCT compound and snap-freezing with liquid nitrogen. Frozen tissue sections 5- to 7-μm thick were generated using a cryostat-microtome (ThermoFisher Scientific). Tissue sections were washed with 1X PBS, blocked in 1X PBS containing 1% BSA and 0.1% Triton X-100 for 1 hour at room temperature. Fluorochrome-conjugated antibody stainings were executed for 1 hour at room temperature in a humid chamber. Slides were washed in 1X PBS and mounted in Fluoromount G (ThermoFisher Scientific). Image acquisition was performed using a Leica Stellaris 5 confocal microscope with a magnification of 63X (pixel size, 0.1810 μm × 0.1810 μm).

Transmission electron microscopy (TEM)

MACS-enriched T cells derived from PBMCs of a single healthy human donor were fixed

in 0.5% glutaraldehyde (EM grade; Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany) and 2% formaldehyde (EM grade; Polysciences) in 0.15 M cacodylate buffer and centrifuged at 3000 rpm (Beckman Microfuge B). The cell pellet was mixed with freshly prepared low gelling temperature agarose (4%; Carl Roth GmbH, Karlsruhe, Germany). After gelling on ice, pieces of 0.5 mm³ were cut from the gelled block and transferred into cacodylate buffer for washing. Samples were subsequently postfixed with 2% osmium tetroxide (Polysciences Europe GmbH, Hirschberg an der Bergstrasse, Germany) in the same buffer and 1% aqueous uranyl acetate (Polysciences). Between staining steps, samples were washed with cacodylate buffer (after OsO₄ staining) and double distilled water. Then, the samples were dehydrated in increasing concentrations of ethanol, and infiltrated with Spurr resin (Spurr low viscosity embedding kit, Polysciences). All steps were performed in a BioWave Pro+ Tissue Processor (Ted Pella Inc., Redding CA, USA). The samples were left in the last 100% Spurr wash for 2 hours at RT on the shaker, then transferred into fresh Spurr resin and polymerized at 60°C for 3 days.

Thin sections of 50 nm were obtained with a diamond knife (Diatome Ltd., Switzerland) on a Leica UC7 ultramicrotome (Leica Microsystems, Heerbrugg, Switzerland), placed on Formvar and carbon coated TEM grids (Quantifoil, Großlobichau, Germany), and stained with 2% uranyl acetate and Reynold's lead citrate. Stained sections were then visualized using a Morgagni 268 TEM at 100 kV (FEI Company, Eindhoven, Netherlands). Image mosaics were recorded to cover an entire cell. These were stitched using the TrakEM2 plugin in Fiji (55).

Automated microscopy of primary cells

Automated fluorescence microscopy was performed with an Opera Phenix automated spinning-disk confocal microscope (PerkinElmer). Human-derived antibody-stained T cells were imaged with a 20x 0.4NA air objective or a 20x 0.8NA air objective with 5x5 nonoverlapping images capturing the entirety of each well. Murine and human live T cells were imaged at 40x magnification, with 9x9 nonoverlapping images capturing the entirety of each well. Images were captured sequentially for each channel: Brightfield, DAPI (435 to 480 nm), enhanced GFP (EGFP) (500 to 550 nm), phycoerythrin (PE) (570 to 630 nm), and allophycocyanin (APC) (650 to 760 nm). Finally, the acquired raw .tiff images were automatically transferred from the microscope for subsequent analysis.

Live-cell time-lapse imaging

All noncalcium time-lapse imaging experiments used live cells in phenol red-free RPMI-1640

supplemented with Glutamine (Bioconcept), 1% (v/v) penicillin–streptomycin, 1 mM sodium pyruvate, 50 μ M beta-mercaptoethanol, 0.1 mM nonessential (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine) amino acids, 20 mM HEPES, and 20 U/ml IL-2. Images were acquired with an Opera Phenix automated spinning-disk confocal microscope using a 40x air objective, as described above. Cell viability was controlled through staining with PI, or morphological CNN-based classification of dead cells.

For T cell DC coculture time-lapse experiments (fig. S17 and movie S2), DCs were incubated in the presence or absence of 1 μ g/ml OVA peptide for 1 hour at 37°C, to enable MHC class I loading and presentation, before washing with PBS. Naïve CD8 OT-I *Nr4a1*^{GFP} cells were subsequently added to OVA-loaded DCs and centrifuged for 1 min at 100g to collect the cells at the bottom of the wells. T cell–DC cocultures were immediately imaged as described above, imaging each individual frame every 5 min over the course of a 5-hour time course. Live-cell time-lapse imaging of purified CD5^{LO}, CD5^{HI}, CXCR3^{LO}, CXCR3^{HI}, and naïve CD8 OT-I *Nr4a1*^{GFP} and P14 *Nr4a1*^{GFP} cells in the presence or absence of OVA, L4-OVA (for OT-I), or GP₃₃₋₄₁ (for P14) was assisted by coating imaging wells overnight at 4°C with 5 μ g/ml anti-CD43 and anti-CD44 to reduce movement of T cells, or 10 μ g/ml peptide-loaded peptide-MHC complexes to provide TCR stimuli independent of cell-cell contacts. Plates were shortly centrifuged for 1 min at 100g to collect the cells at the bottom of the wells. The antigen solution was diluted to 1 μ g/ml in the relevant well, and the control with PBS. Imaging was immediately started after the addition of antigen, and each individual frame was performed every 10 min over the course of a 5-hour time course.

Calcium imaging

Primary murine or human T cells were loaded with 1 μ M Fluo-4 AM and 0.02% Pluronic F127 for 30 min at 37°C and 5% CO₂ in conjunction with Hoechst and ER-Bodipy staining. After washing and resuspension in PBS, cells were left in the dark at room temperature for 30 min enabling dye desertification. Images were acquired with an Opera Phenix automated spinning-disk confocal microscope using a 40x air objective, as described above. For live-cell time-lapse calcium imaging of ORAI^{LO}, ORAI^{HI} and bulk naïve CD8 P14 and OT-I *Nr4a1*^{GFP} cells in response to antigen (Fig. 5, A to D, G, and H; fig. S26; and fig. S27, D to F), imaging was begun immediately after 1 μ g/ml GP₃₃₋₄₁ or OVA, respectively, or PBS addition. Three frames of antigen and control conditions were imaged every 12 s over the course of 25 min. For the SOCE assay (fig. S27, A to C), enriched human CD8 T cells from a single

donor were imaged identically as described above, for 3 min before stimulation, for 10 min after 5 μ M thapsigargin addition, and for a further 10 min after addition of 2 mM CaCl₂. The acquired raw .tiff images from all time course experiments were transferred from the microscope for subsequent analysis.

Single-cell isolation and expansion

Naïve CD8 T cells from a *Tcf7*^{GFP} mouse were isolated and kept in phenol red-free RPMI-1640 supplemented with Glutamine (Bioconcept), 1% (v/v) penicillin–streptomycin, 1 mM sodium pyruvate, 50 μ M beta-mercaptoethanol, 0.1 mM nonessential (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine) amino acids, and 20 mM HEPES. Cells were then stained with 1 μ M ER-Bodipy, 1 μ M CellTrace Violet, and 1 μ M Live/Dead far-red before FACS-sorting of individual live T cells into separate wells of a 384-well plate, coated with 10 μ g/ml α -CD3 and 10 μ g/ml α -CD28 and containing T cell media as described above. Cells were briefly centrifuged at 100g for 1 min and immediately imaged with automated microscopy, enabling T_{ARCH} identification of each cell before, or shortly after binding the plate-bound antibodies. After imaging, cells were supplemented with 10% FBS and 50 U/ml IL-2 and incubated at 37°C and 5% CO₂. Approximately 36 hours after incubation, cells were briefly imaged with automated microscopy to quantify the number of cells that had undergone cell division, before being further incubated and supplemented with 10 ng/ml IL-7 and 5 ng/ml IL-15. After 7 days, cells were stained with 5 ng/ml Hoechst to identify nuclei where CellTrace Violet had become diffuse, and subsequently imaged to quantify TCF1 expression and cell number per colony, related back to the initial T_{ARCH} state.

Image processing and cell segmentation

Images acquired from the Opera Phenix were subject to nuclear segmentation and single-cell analysis using CellProfiler version 2 (56). Nuclear segmentation was performed through DAPI or Hoechst intensity thresholding. Cell outlines, used for quantification of cellular protein intensity levels, were approximated by an expansion around the nucleus. Furthermore, a larger expansion from cell nuclei captured local cellular background intensity for which raw fluorescent intensities could be normalized (57). Subsequent image analysis was performed with MATLAB R2019a–R2022b. Cell types were derived by CNN classification (21) or by gating local background corrected intensity measurements.

Single-cell DINO

To profile T_{ARCH} heterogeneity, we used a dataset of 29,771 randomly selected single-cell crops of CD4 and CD8 T cells derived from 59 healthy

human donors and trained self-supervised vision transformers with the DINO method (self-distillation with no labels) (23, 58). We used the single-cell DINO (scDINO) workflow and exclusively trained on CD3 single-channel images using a ViT architecture featuring six attention heads and a patch size of 16 (ViT-S/16). We conducted scDINO training of the ViTs with a batch size of 240 for 355 epochs, using 8 GPUs. All other hyperparameters were in line with vanilla DINO training and the standard configuration of the scDINO snakemake workflow, the code for which is publicly available at <https://github.com/JacobHanimann/scDINO>.

For downstream analyses, we used the teacher ViT to investigate the phenotypic heterogeneity of a subset of 3156 crops of noninteracting primary CD4 and CD8 T cells derived from 24 healthy donors, which were rescaled from 50x50 to 224x224. The resulting 384-dimensional scDINO feature space used for subsequent clustering was derived from the CLS-Token output of the teacher ViT. Specifically, we used TopOMetry (59), a phenotype discovery tool which extracts information on cellular phenotypes via the approximation of the Laplace-Beltrami Operator (LBO), combined with PaCMAP (60) to visualize the T_{ARCH} phenotype space.

CNNs

Four CNNs were implemented in this work (table S5), all of which were trained using the R2019a-2022b MATLAB Neural Network Toolbox. Cells for each of the CNNs were manually curated into their respective architectural classes following enrichment of phenotypes through feature-based stratification. $T_{NET\alpha}$ was trained to classify fixed and permeabilized human T cells as T_O , T_{CO} , or T_P cells via DAPI, Brightfield, and CD3 48 pixel $\times$ 48 pixel (14.4 μ m $\times$ 14.4 μ m) single-cell crops imaged at 20X. The training set comprised 12253 T_O , 14396 T_{CO} , and 8437 T_P cells, derived from primary human samples. Testing was performed on 1535 T_O , 1795 T_{CO} , and 1229 T_P cells (~10% of the training set). An additional network trained and tested with the same images ($T_{NET\alpha100}$), but whose sizes were rescaled to 100 pixel $\times$ 100 pixels to enable convenient transfer learning of subsequent CNNs imaged at 40X, achieved 96.2% accuracy (table S5). $T_{NET\beta}$ was trained to classify live human and murine T cells as T_O , T_{CO} , or T_P cells via Hoechst, Brightfield, and ER-Bodipy 100 pixel $\times$ 100 pixel (15 μ m $\times$ 15 μ m) single-cell crops. The training set comprised 8349 T_O , 5726 T_{CO} , and 3634 T_P cells, derived from primary human and murine samples. Testing was performed on 2088 T_O , 1432 T_{CO} , and 909 T_P cells. $T_{NET\gamma}$ was trained with the same labeled data as $T_{NET\beta}$, with the addition of 6044 PI^+ cells, used as dead cell ground truths, as well as 3084 manually classified dead cells based on the morphological signature of PI^+ cells. Testing was performed on the same

dataset as for $T_{NET\beta}$, with the addition of 2285 dead cells, of which 1512 were PI^+ . Further validation of the CNN-based morphological detection of dead cells is presented in fig. S19, A to C.

TCF1 expression classifier was trained to classify live murine T cells derived from the infection time course data as either $TCF1^+$ or $TCF1^-$ via Hoechst, Brightfield, and ER-Bodipy 100 pixel $\times$ 100 pixel (15 μ m $\times$ 15 μ m) single-cell crops. The training set comprised 3437 $TCF1^+$ and 3105 $TCF1^-$ cells, from two of the three mice profiled per time point throughout the acute viral infection (Fig. 3). Testing was performed on 2383 $TCF1^+$ and 2077 $TCF1^-$ cells from a mouse profiled at each time point that were not used for CNN training.

Each network used a 39-layer CNN based on an adapted ResNet architecture (fig. S2B) (61). For $T_{NET\alpha}$, $T_{NET\alpha100}$, and the TCF1 expression classifier, weights and biases of each layer were randomly initialized immediately before training, and the adaptive learning rate optimization "ADAM" was implemented. For the $T_{NET\beta}$ and $T_{NET\gamma}$ CNNs, weights and biases from $T_{NET\alpha100}$ were maintained to enable transfer learning, with the exception of the final fully connected layer ($fc3$) and the output class layer, whose size was increased to four for the 4-class CNN $T_{NET\gamma}$. Networks were trained for 20 epochs with an initial learning rate of 0.001, which was dropped every 5 epochs with a factor of 0.1. For transfer learning networks ($T_{NET\beta}$ and $T_{NET\gamma}$), learning rate factor and the bias learn rate factor was changed from 1 to 10 for the last convolutional layer. Mini batch size of 512 images with L2 regularization of 0.001 were applied. In each training iteration, images were randomly rotated by 45-degrees and mirrored vertically or horizontally per iteration to limit orientation biases toward cellular features. Each network was trained five times to assess training robustness, and the most accurate network selected for downstream analysis. These CNNs were used to classify individual T cell crops into their output classes.

Drug screening

PBMCs from 13 healthy donors were incubated on drug plates created using the Echo liquid handler for 24 hours at 37°C with 5% CO_2 . Drug concentrations are indicated in table S1.

Perturbation annotations presented in fig. S13, C to F, were manually assigned based on their clinical and biological function (table S1). To uncover perturbation annotations that altered T_{ARCH} , we performed hypergeometric cumulative enrichment tests. The probabilities of finding at least n perturbations from annotation X , taking into account the total number of perturbations, the total number of significant perturbations which alter T_{ARCH} ,

and the total number of perturbations in annotation X , were calculated.

Single-cell tracking algorithm

k-NN-based single-cell tracking was performed using MATLAB R2022b. Cell tracking trajectories were calculated by finding the nearest neighbor between time point t and $t-1$ for each cell, within a radius of 7.5 μ m. Due to slight camera shifts which uniformly adjust the relative position of all cells in an image, a general translation vector of all cells was calculated between t and $t-1$, whereby unidirectional movement was subtracted from the $t-1$ cell positions before neighbor identification. Unmatched cells at $t-1$ were attempted to be matched at $t-2$ within a maximum radius of 15 μ m. Cells that were either not tracked for at least two consecutive time points or morphologically classified as dead for four consecutive time points were removed from analysis. For the retracked cells, NUR77 intensity values at the missing time points were inferred by averaging values between $t_{+1,2}$ and $t_{-1,2}$.

Morphological trajectory definition

T_{ARCH} trajectories were classified using the following criteria: First, cells presenting T_{ARCH} transitions were stratified based on their initial state (T_O or T_{CO}) along with displaying at least four consecutive time points classified as T_P . The remaining static T_{ARCH} trajectories were stratified when 16 of the 30 time points were labeled as either T_O , T_{CO} , or T_P .

Statistical analyses

All significance values were calculated on the basis of a one-way analysis of variance (ANOVA) and post hoc Bonferroni multiple correction unless otherwise stated. False discovery rate (FDR)-adjusted P values were calculated using the procedure described by Storey (62). Boxplot whiskers indicate the full range of nonoutlier data. Data were classified as outliers when greater than $q3 + w \times (q3 - q1)$ or less than $q1 - w \times (q3 - q1)$, where w is the multiplier whisker and $q1$ and $q3$ are the 25th and 75th percentiles of the sample data, respectively.

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ACKNOWLEDGMENTS

We thank the anonymous blood donors from the Blutspende Zürich for contributions to this study. We further acknowledge support from ScopeM light and electron microscopy core facilities; the ETH FACS core facility; as well as S. Bühler, J. L. Gutierrez, N. Zamboni, I. Sandu, and all members of the Snijder laboratory for discussion and additional experimental support. **Funding:** This work was supported by the Swiss National Science Foundation (project nos. PP00P3_163961 and PP00P3_194809 to B.S.). The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication. **Author contributions:** Conceptualization: B.D.H., Y.S., and B.S. Methodology: B.D.H., Y.S., D.Gu., J.M., J.H., and M.M. Investigation: B.D.H., Y.S., F.G., D.S., D.Gu., Y.F., S.L., and N.S.Z. Visualization: B.D.H., Y.S., D.Gu., J.H., and B.S. Funding acquisition: A.O. and B.S. Project administration: A.O. and B.S. Supervision: A.O. and B.S. Writing – original draft: B.D.H., Y.S., F.G., D.Gu., A.O., and B.S. Writing – review & editing: B.D.H., Y.S., F.G., D.S., D.Gu., J.M., Y.F., S.L., J.H., M.M., O.L., D.Go., B.M.F., A.O., and B.S. **Competing interests:** B.S. was a scientific cofounder of Allicyte GmbH, which has been acquired by Exscientia. B.S. is a shareholder of Exscientia and is a coinventor on US patent application 15/514,045 relevant to the study. B.S. declares research funding from Roche and speaker fees from Novartis, GSK, and AbbVie. All other authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the supplementary materials. Curated datasets for CNN training (63) and single-cell tracking code and example data (64) are deposited. **License information:** Copyright © 2024 the authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original

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Submitted 4 April 2023; resubmitted 13 October 2023
Accepted 16 April 2024
[10.1126/science.adh8967](https://doi.org/10.1126/science.adh8967)