

Illuminating Innate-Like T-Cell Signaling: From Signaling Architecture to Rational Cancer Immunotherapy

Zixing Zhang

University of California San Diego

Zhengyao Shao

zhs035@health.ucsd.edu

University of California San Diego

Systematic Review

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Abstract

Invariant natural killer T (iNKT), mucosal-associated invariant T (MAIT), and $\gamma\delta$ T cells couple potent effector functions with unconventional antigen and stress recognition, making them promising platforms for cancer immunotherapy. Yet the signaling principles governing their antitumor activity and therapeutic engineering remain poorly defined. In this Review, we examine T-cell receptor (TCR) signaling as a spatiotemporally organized process and highlight how advanced microscopy and live-cell biosensors reveal signaling dynamics and cellular heterogeneity, which is inaccessible to conventional endpoint measurements. We then discuss how iNKT, MAIT, and $\gamma\delta$ T cells retain a conserved T-cell signaling core while integrating distinct lineage-specific receptor, cytokine, metabolic, and stress-associated inputs. This signaling context may determine how synthetic receptors are interpreted, arguing that chimeric antigen receptor (CAR) architecture should be optimized for the intended cellular chassis rather than transferred directly from conventional $\alpha\beta$ T cells. Finally, we envision integrating quantitative signaling fingerprints with synthetic signaling motifs, functional profiling, and machine learning to enable mechanism-guided design of next-generation CAR-iNKT, CAR-MAIT, and CAR- $\gamma\delta$ T-cell therapies.

1. Introduction

Cancer immunotherapy has established T lymphocytes as powerful therapeutic agents, yet durable tumor control remains constrained by antigen heterogeneity and antigen escape, immunosuppressive tumor microenvironments, limited cellular persistence, and progressive functional exhaustion [1–4]. These limitations have stimulated growing interest in lymphocyte populations that combine T-cell effector functions with innate-like modes of recognition. Among them, invariant natural killer T (iNKT) cells, mucosal-associated invariant T (MAIT) cells, and $\gamma\delta$ T cells have emerged as particularly attractive platforms because they recognize non-conventional antigenic or stress-associated signals, can rapidly execute cytotoxic and cytokine responses, and can influence neighboring innate and adaptive immune populations [5–8]. These properties are particularly important for understanding how innate-like T cells interact with the tumor microenvironment and for optimizing their persistence, metabolism, and antitumor function in next-generation cell therapies.

T-cell-based cancer immunotherapy ultimately depends on two linked processes: recognition of a malignant cell and intracellular interpretation of that recognition event [9, 10]. Considerable effort has therefore focused on identifying tumor antigens and engineering synthetic receptors that improve the specificity and efficiency of target recognition [10, 11]. Yet receptor engagement alone does not determine therapeutic outcome. Changes in CAR signaling strength, ITAM organization, or costimulatory architecture can redirect differentiation, metabolism, persistence, and exhaustion even when antigen specificity is unchanged [12–15]. Live-cell studies further show that receptors with similar proximal activation can generate different downstream Ca^{2+} , ERK, and spatial ZAP70 responses, indicating that cellular outcome depends on how signaling is initiated, propagated, spatially organized, and terminated over time [16–18]. This principle is particularly evident in CAR-T cells, where stronger receptor signaling

does not necessarily produce superior therapeutic activity: tonic signaling can accelerate exhaustion, whereas calibrated CD3 ζ ITAM signaling can improve persistence and antitumor function [12, 13].

Understanding these relationships requires methods capable of resolving signaling as a dynamic cellular process [19]. Conventional biochemical approaches such as immunoblotting, phosphoproteomics, and phospho-flow cytometry remain essential for identifying pathway components and phosphorylation events, but most provide snapshots averaged across cells or cellular compartments [20–23]. Microscopy has revealed a fundamentally more organized picture, in which TCR signaling emerges through receptor microclusters, nanoscale membrane organization, and spatially restricted signalosomes [24–27]. Thus, pathway identity alone cannot fully describe T-cell signaling; information is also encoded by where signaling occurs and how it evolves over time. The main text develops this progression from canonical biochemistry to spatially organized signaling in detail.

Genetically encoded biosensors provide a particularly powerful means to access this missing dimension. FRET-based kinase activity reporters have enabled direct visualization of ZAP70 and Lck activity in living T cells, revealing signaling at the immunological synapse and other subcellular regions that cannot be readily distinguished by bulk phosphoprotein measurements [28, 29]. Improved ZAP70 and Fyn reporters have further demonstrated that TCRs and CARs can organize proximal kinase activity differently despite engaging overlapping signaling machinery [16]. Complementary approaches such as kinase translocation reporters provide single-fluorophore measurements of kinase dynamics and have recently revealed that antigen affinity can be encoded through ERK pulse frequency during T-cell activation [30, 31]. Newer cpEGFP- and cpHaloTag-based reporters further expand sensitivity, spectral flexibility, and opportunities for multiplexed imaging [32–34].

Signaling activity can also be followed beyond kinases. Genetically encoded Ca²⁺ indicators have resolved transient, oscillatory, and sustained Ca²⁺ responses during T-cell–APC interactions [35, 36], whereas fluorescent reporters for DAG and PIP₃ reveal the spatial organization of lipid second-messenger signaling [37–40]. Together with live-cell measurements of ERK and NFAT dynamics [41], these tools make it increasingly possible to follow how receptor triggering is transformed through successive levels of kinase activity, second messengers, transcriptional regulation, and metabolic adaptation. Label-free approaches such as autofluorescence lifetime imaging and Raman spectroscopy can further connect these signaling events to broader metabolic and biochemical cell states [42, 43]. The emerging opportunity is therefore not simply to visualize whether a T cell is activated, but to construct a multidimensional signaling trajectory describing activation amplitude, timing, localization, pathway coupling, heterogeneity, and recovery.

These capabilities are particularly relevant to innate-like T cells, including invariant natural killer T (iNKT), mucosal-associated invariant T (MAIT), and $\gamma\delta$ T cells. These populations retain substantial elements of conventional TCR signaling but connect them to unconventional recognition systems [44]. iNKT cells recognize lipid antigens presented by CD1d [45], MAIT cells recognize microbial vitamin-metabolite derivatives presented by MR1 [46, 47], and human V γ 9V δ 2 T cells sense intracellular phosphoantigen

accumulation through BTN3A1/BTN2A1-dependent recognition [48–50]. Their TCR signals are additionally integrated with lineage- and context-specific inputs, including SLAM–SAP–Fyn signaling, inflammatory cytokines, and stress receptors [51–54]. Their distinctive biology may therefore arise less from unique kinase inventories than from how conserved pathways are dynamically combined with unconventional receptor inputs.

Whether innate-like T cells interpret synthetic receptor signals in the same way as conventional $\alpha\beta$ T cells has direct implications for cancer immunotherapy. CAR-engineered iNKT cells have already entered clinical testing [55], whereas CAR-MAIT and CAR- $\gamma\delta$ platforms are advancing preclinically [56, 57]. However, most principles used to design CAR intracellular domains were established in conventional $\alpha\beta$ T cells. Whether the same signaling architecture produces equivalent pathway dynamics in an iNKT, MAIT, or $\gamma\delta$ T-cell chassis remains largely unresolved. Emerging biosensor studies in CAR-T cells already show that receptors with similar proximal activation can produce different downstream Ca^{2+} , ERK, and spatial ZAP70 responses [16–18], suggesting that endpoint cytotoxicity alone may obscure important mechanistic differences.

In this Review, we first examine how advanced microscopy and genetically encoded activity reporters have reshaped understanding of the spatiotemporal architecture of TCR signaling. We then consider how this conserved signaling framework is adapted to the distinct recognition and regulatory environments of iNKT, MAIT, and $\gamma\delta$ T cells. Finally, we discuss how live-cell signaling measurements could be integrated with synthetic signaling motifs, functional screening, and machine learning to define cell-chassis-specific signaling fingerprints for therapeutic design. Rather than seeking the strongest receptor signal, we propose that the more relevant objective may be to identify the dynamic signaling state that best supports cytotoxicity, persistence, metabolic fitness, and repeated tumor-cell killing.

2 Illuminating TCR signaling: live-cell imaging approaches reveal its spatiotemporal activity architecture

2.1 The canonical TCR signaling network

The conventional $\alpha\beta$ TCR provides a useful biochemical framework for understanding both innate-like T-cell signaling and synthetic CAR signaling (Fig. 1). The TCR itself lacks intrinsic enzymatic activity and instead signals through the 10 immunoreceptor tyrosine-based activation motifs (ITAMs) distributed across the CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ cytoplasmic domains [58]. Following peptide–MHC recognition, the Src-family kinase Lck phosphorylates these ITAMs, creating docking sites for the tandem SH2 domains of ZAP70, which is rapidly recruited to the activated receptor complex [59, 60]. Activated ZAP70 then phosphorylates key adaptor proteins, most prominently linker for activation of T cells (LAT), thereby converting receptor-proximal phosphorylation into the assembly of a larger signaling network (Fig. 1) [61, 62]. Phosphorylated LAT acts as a membrane-associated scaffold that recruits PLC γ 1, Grb2, and Gads; Gads in turn bridges LAT to SLP-76, enabling formation of the LAT–Gads–SLP-76 signalosome and recruitment of additional effectors including SOS, Itk, and Vav [63, 64]. Through this multiprotein

signaling hub, a relatively localized receptor-triggering event is amplified and distributed into multiple downstream pathways controlling Ca^{2+} mobilization, Ras–ERK signaling, cytoskeletal remodeling, and transcriptional activation [9, 65].

Downstream of the LAT–SLP-76 signalosome, activated PLC γ 1 hydrolyzes membrane phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate the second messengers inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) [66]. IP₃ engages IP₃ receptors on the endoplasmic reticulum to initiate intracellular Ca^{2+} release, while sustained Ca^{2+} signaling activates the calmodulin–calcineurin pathway, leading to NFAT dephosphorylation and nuclear translocation from cytosol (Fig. 1) [67, 68]. In parallel, membrane-retained DAG recruits RasGRP1 and promotes Ras–RAF–MEK–ERK signaling, thereby contributing to AP-1 activation [69, 70]. DAG-dependent activation of PKC θ provides another branch that is required for efficient TCR/CD28-induced NF- κ B signaling [71, 72]. In parallel, PI3K-generated phosphatidylinositol 3,4,5-trisphosphate (PIP₃) recruits Akt and other PH-domain-containing effectors, linking antigen and costimulatory signals to mTOR-dependent programs controlling biosynthesis, metabolism, proliferation, and differentiation [73, 74]. Thus, TCR signaling rapidly branches from a receptor-proximal phosphorylation cascade into interconnected Ca^{2+} -, MAPK-, NF- κ B-, and metabolic signaling networks (Fig. 1).

Equally important are the mechanisms that set signaling thresholds, terminate individual pathways, and reshape information as it propagates through the network. CD45 illustrates this dual regulatory logic: by opposing Csk-dependent inhibitory phosphorylation, it maintains a regulatable pool of active Lck, yet it can also suppress TCR ζ -chain phosphorylation generated by weak or antigen-independent stimuli, thereby functioning as a signaling gatekeeper [75]. Antigen discrimination is further sharpened by competing feedback loops. Weak ligands preferentially recruit the phosphatase SHP-1, promoting Lck inactivation and receptor desensitization, whereas stronger ligands activate an ERK-dependent positive-feedback circuit that limits SHP-1 recruitment and sustains productive signaling [76]. Lipid second messengers are similarly regulated: DGK α and DGK ζ convert DAG to phosphatidic acid, thereby restricting DAG-dependent RasGRP1 and PKC θ signaling; manipulation of these enzymes correspondingly alters Ras–ERK activation and T-cell responsiveness [77–79].

CD45, SHP-1, ERK feedback, and DAG metabolism regulate TCR signaling at different points in the pathway. Together, they set the threshold for activation and shape how long and where signals persist. The response therefore depends on the balance between amplification and attenuation across interconnected pathways, as well as on which molecules are activated.

2.2 From biochemical pathways to spatially compartmentalized TCR signaling

Conventional biochemical approaches, including immunoblotting, phospho-flow cytometry, and biochemical pulldown assays, remain the gold standard for defining pathway components and quantifying phosphorylation or protein interactions. However, these methods generally average signals across large cell populations or entire cells and therefore provide limited information about where and

when signaling events occur. Microscopy complements these approaches by resolving signaling with spatial and temporal precision at the single-cell and, increasingly, single-molecule level. Early three-dimensional imaging of T cell–antigen-presenting-cell interfaces revealed the formation of spatially segregated supramolecular activation clusters at the immunological synapse [80]. Subsequent live-cell imaging showed that signaling begins before formation of a mature central synapse: newly generated peripheral TCR microclusters recruit ZAP70 and SLP-76, coincide with tyrosine phosphorylation and Ca^{2+} signaling, and continue to form as activation proceeds [24].

Super-resolution microscopy further revealed that this organization extends below the diffraction limit. High-speed photoactivated localization microscopy (PALM) demonstrated that, in resting T cells, TCR and LAT occupy distinct nanoscale membrane “protein islands” that concatenate following activation [25]. Two-color PALM subsequently resolved the nanoscale architecture of downstream signaling complexes, showing that ZAP70 colocalizes closely with TCR ζ , whereas LAT forms nanoscale clusters with SLP-76 preferentially positioned at their periphery [81]. Super-resolution imaging in intact lymph nodes further showed that TCRs are organized into nanometer-scale protein islands within larger membrane territories and that in vivo activation causes these territories to contract and form stable microclusters [82].

Nanoscale organization is functionally linked to TCR signal initiation. Single-molecule localization microscopy showed that only sufficiently dense TCR–CD3 nanoclusters were efficiently phosphorylated and associated with downstream ZAP70 and LAT, while cluster density varied with both peptide–MHC concentration and TCR affinity [27]. These observations suggest that receptor clustering itself contributes to antigen discrimination and signal amplification rather than simply representing a morphological consequence of activation.

Single-molecule imaging has also clarified where amplification occurs within the pathway. Tracking individual agonist peptide–MHC:TCR complexes together with ZAP70 recruitment revealed an approximately 1:1 relationship between productive receptor engagement and proximal ZAP70 triggering, suggesting that substantial amplification occurs downstream of the initial receptor event [83]. LAT-centered signaling assemblies provide one mechanism for this amplification: multivalent interactions among LAT-associated proteins can drive phase-separated signaling condensates that enrich signaling components and enhance downstream output [26].

Imaging studies place receptor nanoclusters, kinase recruitment, adaptor organization, and signalosome formation at distinct locations and times. These assemblies link antigen recognition to downstream transcriptional and metabolic responses. Whether innate-like T cells differ from conventional $\alpha\beta$ T cells in receptor organization, signalosome assembly, integration with parallel receptors, or downstream decoding remains an open question.

2.3 Fluorescent kinase activity reporters resolve TCR signaling dynamics in living cells

The development of genetically encoded kinase activity reporters (KARs) has enabled kinase signaling to be monitored directly in living cells with temporal and, depending on reporter architecture, subcellular resolution. In contrast to biochemical measurements that quantify phosphorylation at selected endpoints in cell lysates or fixed samples, KARs continuously translate kinase-dependent phosphorylation into an optical readout, allowing signaling amplitude, kinetics, localization, and cell-to-cell heterogeneity to be followed in real time. Over decades of bioengineering efforts, KARs now comprise several distinct design strategies to accommodate spatiotemporal signaling dissection requirement in immunology.

The classical KAR design is based on Förster resonance energy transfer (FRET) (Fig. 2A). In a typical unimolecular FRET reporter, a kinase-responsive substrate sequence and a phosphoamino-acid-binding domain are sandwiched between donor and acceptor fluorescent proteins. Kinase-dependent phosphorylation induces a conformational rearrangement that alters FRET efficiency, thereby converting kinase activity into a ratiometric optical readout. Because these reporters can also be targeted to defined subcellular compartments, they are particularly useful for resolving the spatial organization of kinase activity.

An early example of FRET-based KARs in T cells was ROZA (Reporter of ZAP-70 Activity), a ZAP70-dependent FRET biosensor incorporating a LAT-derived phosphorylation sequence. ROZA enabled real-time visualization of the kinetics and subcellular distribution of ZAP70-dependent activity following TCR engagement (Table 1) [28]. Unexpectedly, ZAP70-dependent signaling was detected not only at the immunological synapse but also at the opposite cellular pole, termed the “antisynapse,” demonstrating that proximal TCR signaling is not necessarily confined to the visible T-cell–target-cell interface [28].

Table 1
Fluorescent reporters for spatiotemporal analysis of TCR signaling.

Signaling level	Target / process	Reporter / approach mentioned	Sensor principle	References
Proximal kinase	Lck	ZapLck	FRET-based kinase activity reporter	[29]
Proximal kinase	Fyn	High-throughput-engineered Fyn FRET biosensor	Optimized phosphorylation-dependent FRET reporter	[16]
Proximal kinase	ZAP70	ROZA	LAT-derived ZAP70 substrate incorporated into a unimolecular FRET reporter	[28]
Proximal kinase	ZAP70	ROZA-XL	Optimized second-generation ROZA FRET reporter	[84–86]
Proximal kinase	ZAP70	High-throughput-engineered ZAP70 FRET biosensor	Sequence-optimized phosphorylation-responsive FRET reporter	[16]
Second messenger	Ca ²⁺	mCameleon	FRET-based genetically encoded Ca ²⁺ indicator using CaM–M13 interaction	[35]
Second messenger	Ca ²⁺	Yellow Cameleon 3.60 (YC3.60)	FRET-based GECI	[36]
Second messenger	Ca ²⁺	TN-XXL / Twitch-family sensors	Genetically encoded FRET Ca ²⁺ indicators	[87, 88]
Second messenger	DAG	GFP–C1-domain translocation reporters derived from RasGRP1, PKC θ , and other DAG-binding proteins	DAG-binding C1 domain translocates to DAG-enriched membranes	[37]
Second messenger	DAG	Daglas	FRET-based genetically encoded DAG biosensor	[38]
Second messenger	PIP ₃ / 3'-phosphoinositides	GFP–Akt-PH	PH-domain translocation reporter	[39]

Signaling level	Target / process	Reporter / approach mentioned	Sensor principle	References
Second messenger	PIP ₃	GFP–PH-domain reporter(s)	PH-domain lipid-binding translocation	[89]
Second messenger	PIP ₃	GFP–Grp1PH	Grp1 PH-domain PIP ₃ translocation probe	[40]
Downstream kinase	ERK	ERK-KTR	Kinase translocation reporter containing ERK substrate plus engineered NLS/NES	[30, 31]
Downstream kinase	JNK	JNK-KTR	Kinase translocation reporter	[30]
Downstream kinase	p38	p38-KTR	Kinase translocation reporter	[30]
Downstream kinase	PKA	ExRai-AKAR	cpEGFP-based single-fluorophore excitation-ratiometric KAR	[32]
Downstream kinase / platform	Multiple kinases	cpEGFP-based KARs	Circularly permuted EGFP embedded in phosphorylation-responsive molecular switch	[32]
Downstream kinase / platform	Multiple kinases	cpHaloTag-based KARs	Phosphorylation-responsive circularly permuted HaloTag coupled to synthetic dye	[33, 34]
Cellular metabolic state	NAD(P)H / FAD metabolism	Autofluorescence FLIM	Endogenous fluorescence lifetime measurement	[42]
Biochemical cell state	Global molecular composition	Raman spectroscopy	Label-free molecular vibrational spectroscopy	[43, 90]
Receptor organization / morphology	TCR/CD8 membrane organization and scanning	Lattice light-sheet microscopy	Rapid volumetric fluorescence imaging with low phototoxicity	[91]

The original ROZA reporter was subsequently optimized into a second-generation sensor, ROZA-XL, which displayed an approximately three- to fourfold greater dynamic range and a more robust baseline FRET signal in Jurkat T cells, enabling more sensitive single-cell measurements of TCR-induced ZAP70 activity (Table 1) [84]. ROZA-XL was later used to show that CCR7 stimulation itself can recruit and activate ZAP70, whereas combined CCR7 and TCR stimulation enhances and prolongs ZAP70 kinase activity, revealing direct signaling crosstalk between chemokine and antigen receptors [85]. ROZA-XL was also used to show that cyclophilin A associates with ZAP70 and negatively regulates its catalytic activity during early TCR/CD3 signaling [86]. These applications identify distinct receptor inputs and molecular interactions that shape proximal ZAP70 activity.

FRET biosensors have subsequently been extended to Src-family kinases. The ZapLck biosensor enabled visualization of dynamic Lck activity and demonstrated a substantial basal pool of preactivated Lck in the experimental T-cell system, while also revealing changes in Lck interaction and mobility following receptor aggregation (Table 1) [29]. Such probes are particularly valuable because conventional phospho-specific measurements cannot easily distinguish spatially separated pools of kinase activity.

A further methodological advance combined high-throughput FRET screening with sequencing to engineer improved Fyn and ZAP70 biosensors [16]. These sensors were applied to both TCR and CAR signaling and revealed a highly organized subcellular pattern of ZAP70 activity following TCR engagement that was not reproduced in the same manner following CAR activation. The ZAP70 reporter was also used to interrogate how CAR ITAM organization influenced ZAP70 activation and CAR function [16]. This study provides particularly strong proof of principle for the central argument of this review: synthetic receptors that ultimately use many of the same signaling proteins can nevertheless organize those signals differently in space and time.

However, FRET represents only one strategy for converting kinase activity into a measurable optical signal. Kinase translocation reporters (KTRs) provide an alternative strategy for monitoring kinase activity through phosphorylation-dependent changes in nucleocytoplasmic localization (Fig. 2B). KTRs combine a kinase-responsive substrate with engineered nuclear localization and export sequences, such that phosphorylation shifts the reporter from the nucleus to the cytoplasm. Because each KTR requires only a single fluorescent protein, this architecture is particularly attractive for multiplexed measurements. The original KTR platform developed by Regot and colleagues enabled simultaneous measurement of ERK, JNK, and p38 activities in individual living cells (Table 1) [30]. More recently, Dunlock and Regot applied real-time ERK activity imaging to antigen-stimulated T cells and demonstrated that TCR signaling is encoded through pulsatile ERK dynamics [31]. Intermediate-affinity antigens generated ERK pulses of different frequencies, and cumulative ERK activity correlated with T-cell activation. Mechanistically, LCK activity regulated ERK pulse frequency, whereas MEK primarily controlled pulse amplitude, demonstrating that distinct levels of the TCR–MAPK cascade independently encode temporal features of the signaling response [31].

A complementary approach of modern KAR design uses single fluorescent proteins or self-labeling enzyme tags whose optical properties are directly altered by phosphorylation-dependent conformational changes (Fig. 2C). Excitation-ratiometric reporters based on circularly permuted EGFP (cpEGFP), exemplified by ExRai-AKAR, position cpEGFP within a phosphorylation-responsive molecular switch (Table 1) [32]. Phosphorylation changes the excitation properties of the fluorescent protein, generating a ratiometric signal without requiring a donor–acceptor FRET pair. This architecture has been adapted to reporters for several kinases and offers high sensitivity while preserving spectral space for multiplexed measurements [32]. More recently, chemigenetic reporters based on circularly permuted HaloTag (cpHaloTag) have further expanded the available spectral and photophysical properties of KARs (Fig. 2D) [33, 34]. In these sensors, phosphorylation-dependent conformational changes modulate the fluorescence of a synthetic HaloTag-bound dye (Table 1). The use of bright and photostable synthetic fluorophores can facilitate far-red imaging, multiplexing, and potentially super-resolution analysis, providing a complementary strategy to fluorescent-protein-based reporters.

To date, these newer conformational cpEGFP- and cpHaloTag-based KAR designs have not, to our knowledge, been systematically adapted to proximal TCR signaling or extensively applied in primary immune cells. Their high sensitivity, compact spectral footprint, and compatibility with multiplexed imaging nevertheless make them particularly attractive for immunological applications. Future development of single-fluorophore and chemigenetic reporters for TCR-associated kinases such as Lck, Fyn, and ZAP70, together with existing reporters for downstream pathways such as ERK and Akt, could enable simultaneous visualization of multiple signaling nodes within the same T cell. Such approaches may reveal previously inaccessible relationships between signaling amplitude, spatial organization, pathway coupling, and cellular fate, and be especially informative for iNKT, MAIT, and $\gamma\delta$ T cells, in which unconventional TCR recognition is integrated with cytokine, costimulatory, and stress-receptor signaling [52–54]. Defining these dynamic signaling patterns could reveal whether innate-like T cells possess characteristic signaling fingerprints distinct from conventional $\alpha\beta$ T cells and, ultimately, provide a mechanistic foundation for the rational design of cancer immunotherapies with optimal TCR signaling responses.

2.4 Beyond kinases: visualizing second messengers and transcriptional dynamics

TCR signaling is propagated not only through sequential kinase activation but also through rapidly changing second messengers that connect receptor-proximal phosphorylation to downstream effector programs. Among these, Ca^{2+} , diacylglycerol (DAG), and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) are particularly important because they regulate complementary branches of T-cell activation and can be monitored dynamically in living cells using genetically encoded or fluorescent translocation-based reporters.

Following TCR engagement, PLC γ 1 hydrolyzes membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate IP_3 and DAG. IP_3 initiates release of Ca^{2+} from intracellular stores, followed by store-operated

Ca²⁺ entry, ultimately coupling TCR activation to calcineurin–NFAT signaling. Intracellular Ca²⁺ dynamics can be visualized using genetically encoded calcium indicators (GECIs) (Table 1). A particularly informative example in T cells is the Cameleon family of FRET-based reporters, in which Ca²⁺-dependent binding of calmodulin to an M13 peptide induces a conformational change that alters FRET between fluorescent proteins. Le Borgne and colleagues generated an inducible knock-in mouse expressing a modified Cameleon (mCameleon) and directly visualized transient, oscillatory, and sustained Ca²⁺ responses during naïve T-cell interactions with antigen-presenting cells in vitro and in lymphoid tissue (Table 1) [35]. A complementary conditional mouse model expressing Yellow Cameleon 3.60 (YC3.60) in CD4-lineage T cells enabled intravital analysis of T-cell Ca²⁺ signaling and revealed enhanced Ca²⁺ flux in an autoimmune-prone setting [36]. Related FRET-based Ca²⁺ reporters from the TN-XXL/Twitch family have also been used to track antigen-specific T-cell activation and migration in lymph nodes and the central nervous system in vivo (Table 1) [87, 88]. These studies resolved Ca²⁺ responses during T-cell interactions in vitro and in tissues, across time and locations that are difficult to access with conventional Ca²⁺ dyes.

The second product of PLC γ 1 activity, DAG, remains within cellular membranes and recruits C1-domain-containing signaling proteins including RasGRP1 and PKC θ , thereby coupling TCR activation to Ras–ERK and NF- κ B pathways. DAG can be visualized using several genetically encoded reporter strategies. In living Jurkat T cells, GFP-tagged C1 domains derived from RasGRP1, PKC θ , and other DAG-binding proteins were used as translocation reporters to reveal distinct intracellular DAG pools following TCR stimulation [37]. Because DAG binding drives redistribution of these probes, local lipid accumulation is inferred from changes in fluorescence localization rather than from a conformational optical response. Complementing this approach, Sato and colleagues developed Daglas, a FRET-based DAG biosensor that can be targeted to defined membrane compartments and directly reports local DAG dynamics through changes in FRET efficiency [38]. Daglas revealed that DAG concentrations are dynamically regulated not only at the plasma membrane but also at endomembranes and the mitochondrial outer membrane, highlighting the spatial compartmentalization of lipid second-messenger signaling.

A second major lipid-signaling branch in TCR signaling is generated by PI3K, which converts PIP₂ into PIP₃. PIP₃ recruits proteins containing appropriate pleckstrin-homology (PH) domains and links receptor activation to Akt signaling, cytoskeletal remodeling, metabolism, proliferation, and survival. PIP₃ has been visualized directly in T cells using PH-domain-based fluorescent translocation probes. Harriague and Bismuth expressed a GFP fusion containing the Akt/PKB PH domain (GFP–Akt-PH) in T cells; because the PH domain binds 3'-phosphoinositides, antigen-induced PIP₃ production caused redistribution of the fluorescent probe from the cytosol to the plasma membrane. Live cell imaging demonstrated sustained PIP₃ generation following T cell–APC conjugation, including accumulation at the immunological synapse, and showed that PIP₃ accumulation preceded the intracellular Ca²⁺ increase [39]. A contemporaneous study using GFP–PH-domain probes likewise demonstrated that PIP₃ signaling can remain dynamically elevated for hours and occurs both within the T cell–APC contact zone and at the antipodal pole [89].

More spatially resolved imaging has subsequently linked PIP_3 dynamics directly to cytotoxic function. In antigen-stimulated cytotoxic T lymphocytes, a PIP_3 reporter consisting of the Grp1 PH domain fused to GFP (GFP-Grp1PH) revealed an annular pattern of PIP_3 accumulation surrounding the immunological synapse [40]. This lipid distribution recruited Dock2 and organized Rac-dependent actin polymerization, and perturbation of the pathway markedly altered T-cell cytotoxicity [40]. Fluorescent lipid-binding domains provide a technical advantage to visualize not only whether PI3K signaling occurs, but also where lipid signaling is positioned relative to the cytoskeletal machinery that executes target-cell killing.

The importance of TCR signaling dynamics becomes even more apparent at the level of transcriptional decoding. Information generated at the receptor is progressively amplified through multiple regulatory layers, including kinase cascades, LAT-centered signaling assemblies, second-messenger generation, and the coordinated activation of transcription factors such as NFAT, AP-1, and NF- κ B [9, 23, 26]. These pathways do not operate independently; their relative amplitude, duration, and temporal overlap determine how upstream TCR inputs are converted into distinct transcriptional programs. Simultaneous live-cell measurements of ERK and NFAT demonstrated that T cells encode information about peptide-MHC stimulation over unexpectedly long timescales [41]. Although different antigen conditions can produce broadly similar early pathway activation, ERK and NFAT signaling subsequently diverges, with later pulsatile dynamics carrying information about antigen affinity and dose [41]. Apparently similar early receptor activation can be progressively reshaped through signaling amplification, feedback regulation, transcription-factor cooperation, and chromatin-level control, ultimately producing markedly different cellular states and functional outcomes.

For tumor immunology, this distinction between upstream signaling propagation and downstream functional signaling output is particularly important. Most studies of engineered lymphocytes quantify CD69 expression, cytokine production, phosphoproteins, or cytotoxicity at isolated endpoints. Yet therapeutic cells undergo repeated cycles of tumor recognition, signaling, killing, disengagement, and reactivation. Combining kinase activity reporters with Ca^{2+} sensors and DAG- and PIP_3 -sensitive lipid probes could therefore reveal how proximal phosphorylation is converted into downstream second-messenger dynamics during each encounter. The kinetics of signal initiation, pathway coupling, spatial redistribution, termination, and resetting may ultimately be as important for therapeutic fitness as the maximal activity reached by any individual signaling component.

2.5 Advanced optical imaging connects T-cell signaling to cellular state and organization

Beyond direct measurements of kinase and second-messenger activity, advanced optical approaches can capture how signaling is translated into broader cellular states. Autofluorescence lifetime imaging microscopy (FLIM) provides one such example by exploiting endogenous metabolic cofactors as label-free reporters [42]. Walsh and colleagues showed that NAD(P)H- and FAD-associated lifetime features could distinguish quiescent from activated human T cells and further resolve activation state together

with CD4/CD8 identity [42]. Although FLIM does not directly report proximal TCR signaling, it provides a functional readout of the metabolic reprogramming that emerges downstream of activation.

This concept of non-invasive imaging can be extended beyond metabolism. Raman spectroscopy interrogates intrinsic molecular vibrational signatures and therefore provides a complementary label-free view of biochemical cell state. Single-cell Raman profiling distinguished T from B cells and separated naïve from anti-CD3/CD28-activated T cells [43]. Raman spectra also evolved during T-cell differentiation over several days [90], suggesting that optical fingerprints can capture not only acute activation but also longer-term remodeling of immune-cell identity.

At a different scale, advanced microscopy can reveal how these molecular states are organized in space during immune surveillance. Lattice light-sheet microscopy enables rapid three-dimensional imaging of cell-surface dynamics with minimal phototoxicity. In CD8⁺ T cells, this approach revealed dynamic microvillar scanning and preorganized TCR- and CD8-containing membrane patches that converge during antigen recognition [91]. These observations highlight how receptor organization and cell morphology add another layer of regulation that cannot be inferred from bulk biochemical measurements alone.

FLIM, Raman spectroscopy, and high-speed volumetric imaging provide complementary views of immune cells: metabolic state, biochemical composition, and the spatial organization of receptors and cell–cell interactions. Used alongside activity reporters, they may help relate signaling dynamics to later changes in cell state.

3 Distinct signaling logic of innate-like T cells: from unconventional recognition to antitumor function

3.1 A shared signaling core with distinct input architectures

Although iNKT, MAIT, and $\gamma\delta$ T cells exhibit markedly different recognition biology from conventional CD4⁺ and CD8⁺ T cells, current evidence does not support the existence of an entirely separate intracellular signaling network in each lineage. Rather, these cells retain much of the canonical TCR signaling machinery while connecting it to distinct antigen-recognition systems and parallel activating inputs. iNKT TCRs recognize glycolipid–CD1d complexes [45], whereas MAIT TCRs recognize microbial metabolite antigens presented by MR1 [46, 92]. Human V γ 9V δ 2 T cells employ an even more unconventional recognition system in which intracellular phosphoantigens are sensed through BTN3A1 and translated into TCR recognition involving BTN2A1 [48–50]. Their signaling distinctiveness therefore arises less from a unique set of kinases than from how conserved TCR machinery is engaged and combined with lineage-specific receptor inputs.

These additional inputs give each lineage more than one route to activation. iNKT cells can be activated through IL-12/IL-18 even without direct TCR engagement [93, 94], while MAIT cells integrate MR1-dependent TCR signals with IL-12/IL-18-driven activation [53, 95]. V γ 9V δ 2 T cells can combine TCR recognition with stress-receptor signals such as NKG2D [54]. Signaling requirements also vary by developmental stage and subset: TCR signal strength influences iNKT lineage differentiation [96], whereas Syk–PI3K signaling has a specific role in murine $\gamma\delta$ T17 development [97]. The contribution of any pathway must therefore be considered in the context of the lineage, subset, and activation state being studied.

3.2 iNKT cells: TCR–SLAM integration and lineage-specific signaling

iNKT cells recognize lipid antigens presented by the non-polymorphic molecule CD1d through a highly restricted TCR repertoire and can respond rapidly through both TCR-dependent and cytokine-mediated pathways [98, 99]. Their signaling therefore builds on the conventional TCR network but is shaped by lineage-specific developmental and costimulatory inputs.

During thymic development, iNKT precursors display stronger and more sustained TCR-induced Egr1/Egr2 expression than conventional T-cell precursors [100]. Egr2 directly binds and activates the Zbtb16 promoter, inducing PLZF, the lineage-defining transcription factor that establishes the innate-like effector program of iNKT cells. In parallel, Egr2 promotes Il2rb expression, encoding the IL-2/IL-15 receptor β -chain (CD122), thereby increasing responsiveness to IL-15, which supports later iNKT maturation and survival [100, 101]. TCR signal strength further influences iNKT subset differentiation. Stronger signaling sustains Egr2 expression and PLZF induction, favoring iNKT2 and iNKT17 development, whereas reduced ZAP70-dependent signaling diminishes these programs [96]. Consistently, iNKT2-specific regulatory elements are enriched for NFAT- and Egr-binding motifs and become less accessible when TCR signaling is weakened, linking quantitative TCR input to chromatin remodeling and lineage fate.

A distinctive feature of iNKT signaling is its integration with the SLAM–SAP–Fyn axis. During thymic development, homotypic interactions between SLAMF1 and SLAMF6 on neighboring thymocytes provide signals required for efficient iNKT lineage expansion and differentiation [102]. Their cytoplasmic ITSM motifs recruit the adaptor SAP, which couples SLAM-family receptors to the Src-family kinase Fyn, thereby providing a signaling module that operates alongside TCR signaling [51]. This pathway remains functionally relevant after development: in mature mouse and human iNKT cells, disruption of SAP or Fyn impairs TCR-induced cytotoxicity, target-cell conjugation, and microtubule-organizing-center polarization toward the immunological synapse [103]. The SLAM–SAP–Fyn axis helps translate TCR recognition into an efficiently organized lytic response rather than merely adding another proximal phosphorylation signal.

iNKT cells also integrate inflammatory cytokine signals, particularly IL-12 and IL-18, which can activate iNKT cells independently of direct TCR engagement and drive robust IFN- γ production and effector

function [93, 104]. The route and history of activation can in turn shape subsequent cellular state: α -galactosylceramide-mediated TCR stimulation and IL-12/IL-18-driven activation produce distinct expansion kinetics and phenotypic programs [52], while repeated α -galactosylceramide stimulation can induce prolonged hyporesponsiveness associated with sustained PD-1 expression [105]. Thus, iNKT behavior reflects the integrated strength, duration, and combination of TCR, SLAM–SAP–Fyn, and cytokine signaling, rather than the output of any single pathway [51, 52, 103], providing a rationale for directly visualizing these signaling dynamics in mature therapeutic iNKT cells.

3.3 MAIT cells: signal integration at the interface of antigen and inflammation

MAIT cells provide a particularly clear example of an innate-like T cell functioning as a multimodal signal integrator. Their semi-invariant TCR recognizes microbial vitamin B₂-derived metabolites presented by the monomorphic MHC class I-related molecule MR1 [46]. The most potent MAIT-cell antigens, 5-OP-RU and 5-OE-RU, originate from the riboflavin (vitamin B₂) biosynthetic pathway used by many bacteria and yeasts but absent in mammals [46, 106]. The microbial intermediate 5-A-RU reacts non-enzymatically with the glycolytic by-products methylglyoxal or glyoxal, which can be produced by either microbial or host metabolism, to generate 5-OP-RU or 5-OE-RU, respectively [47]. These highly unstable intermediates are stabilized upon binding within MR1 and form potent ligands for MAIT TCR recognition [47]. Unlike conventional peptide-reactive T cells, MAIT cells couple a largely conserved TCR signaling apparatus to direct surveillance of microbial metabolism.

MAIT-cell function is not determined by TCR engagement alone. IL-12 and IL-18 can activate human MAIT cells independently of the TCR, providing a mechanism for responses to inflammatory environments even when cognate MR1 antigen is absent [95]. Direct comparison of activation routes showed that TCR stimulation induces a rapid and broad program involving cytokines, chemokines, cytotoxic molecules, transcription factors, and costimulatory receptors, whereas IL-12/IL-18-driven activation develops more slowly and produces a more restricted response [53]. More recent single-cell analysis further resolved these pathways: NFATC1, EGR1, and NFkB1 were preferentially associated with the TCR-driven trajectory, whereas STAT4, STAT1, and IRF1 were more prominent during cytokine-driven activation [107]. These findings provide a mechanistic basis for how MAIT cells can translate distinct upstream inputs into different effector programs.

These two activation modes: MR1–TCR-dependent antigen recognition and IL-12/IL-18-driven cytokine activation, can cooperate rather than functioning independently. MAIT cells show relatively high responsiveness to inflammatory signals, while simultaneous TCR and inflammatory stimulation synergistically enhances IFN- γ and granzyme B responses [108]. Multi-omics profiling similarly showed that anti-CD3/CD28 and IL-12/IL-18 stimulation generate distinct molecular states, whereas their combination produces a more complete activation program resembling bacterial stimulation and induces feedback regulators including TWIST1 and LAG3 [109]. Other inflammatory signals can further tune this response; for example, type I interferons directly enhance IFN- γ and granzyme B production

during TCR-mediated MAIT activation [110]. MAIT-cell function is better understood as the product of integrated MR1–TCR and inflammatory signaling, rather than as the output of a single TCR-driven pathway.

Tissue adaptation can alter the baseline state upon which subsequent TCR and cytokine signals act. These two activation routes themselves drive overlapping but distinct transcriptional programs, with TCR engagement preferentially inducing genes associated with tissue repair and homeostasis [111]. This distinction becomes particularly relevant in tissue-resident MAIT cells, where local transcriptional and metabolic adaptation can further modify how these signals are interpreted [112, 113]. Human liver MAIT cells are metabolically and transcriptionally distinct from circulating MAIT cells and respond more polyfunctionally to cytokine stimulation; TCR stimulation of liver MAIT cells similarly enriches a tissue-repair program, whereas resting liver MAIT cells show enhanced IL-12 signaling and fatty-acid metabolism [112]. Even similar TCR inputs can be decoded differently depending on the pre-existing tissue-conditioned state of the cell.

This adaptive plasticity may help explain the apparently opposing roles of MAIT cells in cancer. In mouse tumor models, MAIT cells promoted tumor growth and metastasis through a mechanism requiring tumor MR1 and involving suppression of NK-cell activity, partially through IL-17A [114]. Conversely, deliberate MAIT activation with antigen enhanced antitumor immunity in other models and strengthened NK-cell responses; activated human MAIT cells similarly enhanced the function of NK cells isolated from patient tumors [115]. Rather than being contradictory, these studies suggest that activation state determines whether MAIT-mediated immune regulation becomes tumor-promoting or tumor-restraining.

MAIT-cell function depends on the timing and combination of TCR, cytokine, costimulatory, and tissue-derived inputs. Measuring proximal TCR activity, ERK, Ca^{2+} –NFAT, PI3K–AKT, and metabolism under defined combinations of MR1 antigen and inflammatory cytokines could help distinguish signaling patterns associated with cytotoxic, tissue-repair, immunoregulatory, and dysfunctional states.

3.4 $\gamma\delta$ T cells: unconventional TCR recognition coupled to metabolic and stress sensing

$\gamma\delta$ T cells require particularly careful interpretation because they comprise multiple biologically distinct populations with different TCR repertoires, tissue distributions, and recognition mechanisms [116, 117]. Findings from one $\gamma\delta$ subset, developmental stage, or species therefore should not be generalized to the entire lineage. Nevertheless, $\gamma\delta$ T cells provide a striking example of how a broadly conserved T-cell signaling backbone can be coupled to highly unconventional modes of target recognition [118, 119].

Early studies demonstrated that even the composition of the $\gamma\delta$ TCR complex can differ from that of conventional $\alpha\beta$ T cells. In mice, most ex vivo $\gamma\delta$ TCR complexes examined lacked CD3 δ and generated greater Ca^{2+} mobilization, ERK activation, and proliferation than $\alpha\beta$ TCRs under matched experimental conditions [120]. These observations established that receptor architecture can influence signaling

potential, although they do not imply that every mature human $\gamma\delta$ T-cell subset intrinsically signals more strongly than conventional $\alpha\beta$ T cells.

Human V γ 9V δ 2 T cells provide a particularly distinctive example because their TCR detects changes in intracellular isoprenoid metabolism rather than conventional peptide–MHC complexes [121, 122]. Microbial HMBPP, generated through the non-mevalonate/MEP isoprenoid pathway, is a highly potent phosphoantigen, whereas the host-derived metabolite IPP can accumulate when the mevalonate pathway is dysregulated in transformed cells or pharmacologically perturbed [122]. Metabolic disturbance within a target cell can ultimately be translated into $\gamma\delta$ TCR activation. Earlier signaling studies already suggested that this mode of recognition is kinetically unusual: phosphoantigen stimulation produced early Lck activation but comparatively delayed and sustained downstream signaling relative to anti-CD3 crosslinking [122].

How these intracellular phosphoantigens are converted into a TCR-recognizable surface signal is now understood to involve the coordinated actions of BTN3A1 and BTN2A1 [49, 50, 122]. Phosphoantigens bind the intracellular B30.2 domain of BTN3A1, initiating an “inside-out” signaling process required for V γ 9V δ 2 activation [48, 123]. BTN2A1 acts as an essential partner that directly engages germline-encoded regions of the V γ 9 TCR and cooperates with BTN3A1 to form the recognition interface [49, 50]. More recent structural studies showed that phosphoantigens can function as molecular “glues” promoting intracellular BTN3A1–BTN2A1 association, thereby coupling intracellular metabolite sensing to extracellular TCR engagement [124].

This recognition system therefore differs fundamentally from classical peptide–MHC recognition: the $\gamma\delta$ TCR does not simply bind the phosphoantigen itself at the cell surface. Instead, changes in intracellular metabolite abundance are sensed by butyrophilins and converted into a cell-surface configuration recognized by the V γ 9V δ 2 TCR, which provides a direct mechanistic link between infection- or transformation-associated metabolism and immune surveillance [48, 124].

$\gamma\delta$ T-cell activation is further shaped by stress-sensing receptors. NKG2D, for example, can provide potent costimulatory signals and, under some conditions, directly activate human V γ 9V δ 2 T cells, inducing cytotoxic granule release and TNF production in response to stress-induced ligands such as MICA [54]. $\gamma\delta$ T-cell tumor recognition can therefore integrate TCR-dependent metabolic sensing with NK-like recognition of cellular stress, potentially broadening target recognition beyond any single antigenic pathway [125].

Distinct proximal kinase requirements have also been identified, although in a more restricted developmental context. During murine thymic differentiation of IL-17-producing $\gamma\delta$ T cells, Syk, rather than ZAP70, was required to efficiently activate PI3K–AKT signaling and generate the $\gamma\delta$ T17 lineage; ZAP70 could not functionally substitute for Syk in this setting [97]. This provides strong evidence that $\gamma\delta$ TCR signaling can acquire lineage-specific proximal wiring, but the finding specifically concerns murine $\gamma\delta$ T17 development and should not be extrapolated to mature human antitumor $\gamma\delta$ T cells.

$\gamma\delta$ T cells retain a shared T-cell signaling backbone, but the inputs vary by subset and context: TCR architecture, butyrophilin-mediated metabolic sensing, stress receptors, and, in selected developmental programs, proximal kinase usage. Live-cell studies in defined mature therapeutic $\gamma\delta$ T-cell populations are needed to determine how these inputs combine over time.

3.5 From signaling architecture to tumor killing, amplification, and exhaustion

Collectively, these aforementioned studies indicate that the functional output of innate-like T cells is shaped not only by which signaling pathways are engaged, but also by how strongly, how long, and where within the cell those signals are generated. Signal amplitude is particularly important because stronger receptor engagement does not necessarily translate into superior therapeutic activity. T-cell signaling networks contain both amplification and negative-feedback mechanisms that sharpen antigen discrimination and constrain inappropriate activation [9, 76]. When stimulation becomes excessive or persistent, these same signaling networks can drive altered differentiation or dysfunctional states, including NFAT-associated exhaustion and tonic-signaling-induced CAR-T dysfunction [12, 126]. This principle is well established in engineered $\alpha\beta$ T cells, where tonic CAR signaling can promote exhaustion and calibrated reduction of CD3 ζ ITAM signaling can improve persistence and antitumor activity [12, 13].

The duration of signaling further influences how receptor engagement is decoded into transcriptional programs and long-term functional states. iNKT and MAIT studies show that TCR-dependent and cytokine-driven activation can generate distinct subsequent phenotypes and transcriptional states [52, 53]. In conventional CD8⁺ T cells, persistent NFAT activity in the absence of balanced AP-1 cooperation can induce transcriptional programs associated with anergy and exhaustion [126]. Although this mechanism has not been established as a universal pathway in iNKT, MAIT, or $\gamma\delta$ T cells, it provides a useful framework for considering how repeated or sustained stimulation may progressively reshape innate-like T-cell function.

Where signaling occurs within the cell is also an important determinant of how receptor engagement is amplified and translated into downstream responses. TCR microclusters, LAT-centered signaling assemblies, and synaptic versus extrasynaptic kinase activity demonstrate that receptor signaling is highly compartmentalized [24, 26]. Moreover, live-cell biosensor studies have shown that TCRs and CARs can generate distinct spatial patterns of ZAP70 activity despite using overlapping proximal signaling machinery [16]. Such observations suggest that signaling topology may influence downstream output by controlling local kinase–substrate encounters, adaptor assembly, and exposure to positive or negative regulators.

The amplitude, duration, spatial organization, and convergence of TCR-associated signals provide a mechanistic basis for how relatively limited receptor-proximal events are amplified into large functional responses. Single-molecule measurements indicate an approximately 1:1 relationship between productive pMHC:TCR engagement and ZAP70 recruitment, arguing that substantial signal amplification occurs downstream of the initial triggering event rather than through repeated amplification at the

receptor itself [83]. Downstream amplification can emerge at several levels: LAT-centered assemblies concentrate kinases and adaptor proteins while excluding phosphatases [26], kinase cascades and second messengers propagate the signal across multiple pathways, and transcriptional networks integrate these inputs over longer timescales [26]. In innate-like T cells, this amplification is further shaped by convergence with non-TCR inputs. For example, inflammatory cytokines can synergize with MAIT TCR signaling to enhance effector responses [108], while iNKT and $\gamma\delta$ T cells similarly integrate cytokine- or stress-receptor signals with TCR activation [52, 54].

We propose that the combination of recognition inputs generates a signaling trajectory defined by pathway amplitude, duration, location, and convergence. Transcriptional and metabolic responses to that trajectory may help determine whether an innate-like T cell becomes cytotoxic, immune-amplifying, regulatory, or dysfunctional. These states may also depend on the signaling history created by repeated tumor encounters, inflammatory cues, and tissue-specific inputs. Direct measurements across these contexts are needed to test why similar receptor engagement can lead to different antitumor outcomes.

4 From signaling fingerprints to synthetic design: activity imaging-guided engineering of innate-like T-cell therapies

4.1 The optimal CAR signal is not necessarily the strongest CAR signal

CAR engineering provides an unusually tractable system in which receptor signaling architecture can be deliberately manipulated and linked to therapeutic phenotype. Evidence from conventional $\alpha\beta$ CAR-T cells increasingly argues against a simple “stronger is better” model. Antigen-independent CAR clustering, for example, can drive tonic CD3 ζ phosphorylation and premature exhaustion; in one extensively characterized system, CD28 costimulation augmented this phenotype, whereas 4-1BB reduced the consequences of persistent tonic signaling [12]. Conversely, very weak tonic signaling can also compromise CAR-T-cell fitness, and tuning surface charge within the antigen-binding domain toward an intermediate level improved persistence and function [127]. Both insufficient and excessive signaling can be detrimental, suggesting that therapeutic performance depends on signal calibration rather than signal maximization.

The same principle is evident at the level of CD3 ζ ITAM organization. Reducing the three functional ITAMs of a CD28–CD3 ζ CAR to a single appropriately positioned ITAM generated a more balanced effector–memory state and improved therapeutic activity in the tested models [13]. Replacement of a single residue within the CD28 intracellular domain reduced differentiation and exhaustion while improving CAR-T-cell persistence and antitumor durability [15]. Costimulatory domains also impose distinct metabolic programs, with CD28 favoring glycolytic effector differentiation and 4-1BB promoting mitochondrial biogenesis and central-memory characteristics [14]. These examples show that CAR sequence affects how activation is routed into differentiation, metabolism, and persistence, as well as the magnitude of the initial signal.

4.2 Biosensor-enabled activity imaging directly connect CAR architecture to signaling dynamics

The functional consequences of CAR modification have traditionally been evaluated through endpoint phenotypes such as cytokine production, proliferation, differentiation, persistence, and tumor control [12, 13]. Live-cell biosensors provide a complementary mechanistic layer by directly resolving how changes in CAR architecture alter the kinetics and spatial organization of intracellular signaling before these downstream phenotypes emerge [16, 18]. FRET imaging of TSHR-specific CAR-T cells, for example, demonstrated rapid ZAP70 and ERK activation following target-cell engagement and revealed differences in downstream ERK signaling between CD28- and 4-1BB-containing CARs [17]. CD28- and 4-1BB-containing CARs generated similar proximal ZAP70 activation in this system, yet CD28 CARs produced somewhat greater ERK activity [17], indicating that differences between costimulatory domains can emerge downstream even when proximal kinase activation appears similar.

An even clearer example comes from comparison of conventional CD3 ζ and single-ITAM 1XX CARs [18]. Despite having fewer functional ITAMs, 1XX CARs generated broadly similar ZAP70 activation but greater Ca²⁺ elevation and more rapid ERK activation, together with enhanced persistence and antitumor activity in the tested models [18]. Reducing receptor-proximal signaling capacity does not necessarily attenuate the entire signaling network proportionally; instead, it can rebalance signaling between downstream branches. Consistent with this concept, FRET-Seq-derived biosensors revealed that TCR and CAR engagement generate different spatial patterns of ZAP70 activity despite using overlapping proximal machinery [16]. These findings argue that CAR optimization should consider the kinetics, branching, and spatial organization of signaling rather than relying on a single measure of proximal activation.

4.3 CAR signaling domains should be optimized within the innate-like T-cell chassis

The importance of signaling architecture becomes even greater when CARs developed in conventional $\alpha\beta$ T cells are transferred into iNKT, MAIT, or $\gamma\delta$ T cells. Because these lineages differ in endogenous receptor inputs, adaptor usage, transcriptional state, and metabolic programming, the same CAR signaling domain may not be interpreted identically by each cellular chassis.

CAR-iNKT studies provide an early indication that costimulatory domains cannot be ranked independently of the cellular context in which they operate. In GD2-CAR iNKT cells, 4-1BB signaling altered polarization, whereas incorporating both CD28 and 4-1BB improved *in vivo* persistence in the tested system [128]. Subsequent optimization, however, showed that 4-1BB-containing CARs were more susceptible to activation-induced cell death and expanded less efficiently than CD28-based constructs, while provision of IL-15 to CD28 CAR-iNKT cells enhanced persistence, serial killing, and tumor control [129]. Rather than establishing a universal advantage of CD28 or 4-1BB, these studies suggest that the

functional consequence of a costimulatory module emerges from its interaction with CAR architecture, cytokine support, and the intrinsic signaling program of the iNKT cell.

This principle has now gained increasing clinical relevance. In a phase I study of GD2-CAR.15 iNKT cells in relapsed or refractory neuroblastoma, objective responses were observed, and a higher frequency of CD62L⁺ cells in the infused product was associated with greater subsequent CAR-iNKT expansion [55]. Because CD62L marks a less differentiated iNKT population with greater proliferative potential, this association suggests that therapeutic efficacy depends not only on the strength of acute CAR-mediated killing but also on whether receptor and cytokine signaling preserve a cellular state capable of expansion and persistence. The same study implicated BTG1 in iNKT hyporesponsiveness, further emphasizing that intracellular cell-state programs can constrain the output of an otherwise active CAR [55].

Evidence in MAIT and $\gamma\delta$ T cells remains more limited, but similarly suggests that CAR signals are interpreted within a lineage-specific cellular context. Anti-CD19 and anti-HER2 CAR-MAIT cells efficiently killed antigen-positive targets while retaining MR1-dependent recognition, yet produced a cytokine profile distinct from conventional CAR-CD8⁺ T cells, including lower IFN- γ production in the reported system [56]. This suggests that CAR engagement does not replace the underlying MAIT program but is integrated with it, allowing synthetic and native recognition pathways to coexist within the same cell [56]. More recently, BCMA CAR- $\gamma\delta$ T cells showed antigen-induced activation comparable to CAR- $\alpha\beta$ T cells but exhibited reduced proliferation and less durable cytotoxicity, while intracellular-domain composition substantially influenced their performance [57]. Given the distinct recognition and effector biology of $\gamma\delta$ T cells [130], these findings support the view that similar CAR inputs can be translated into different downstream functional programs depending on the cellular chassis.

A 2026 phosphoproteomic comparison provides more direct evidence that CAR signals can be processed differently by $\gamma\delta$ and $\alpha\beta$ T cells [131]. CAR- $\gamma\delta$ cells displayed extensive differences in phosphorylation networks, reduced metabolic capacity, and weaker AP-1 activation; engineering an additional costimulatory receptor enhanced AP-1 activity and persistence [131]. Because this study is currently a preprint, these findings should be considered preliminary, but they provide a mechanistic rationale for chassis-specific CAR signaling design rather than simply transferring architectures optimized in $\alpha\beta$ T cells.

4.4 From intact costimulatory domains to programmable signaling motifs

Most CAR designs treat intracellular signaling domains such as CD28, 4-1BB, ICOS, OX40, and CD3 ζ as intact modules, even though these domains engage distinct downstream signaling partners [10, 132]. At the biochemical level, however, these receptor tails are composed of smaller sequence motifs that recruit specific signaling proteins. For example, phosphorylation of the YMXM/YMNM motif in CD28 creates a docking site for the p85 regulatory subunit of PI3K [133, 134], whereas the corresponding YMFM motif in ICOS preferentially recruits PI3K but not Grb2 [135]. Members of the TNFR superfamily use different motif logic: 4-1BB and OX40 recruit TRAF-family adaptors through their cytoplasmic tails,

thereby engaging pathways including NF- κ B [136–138]. In parallel, phosphorylation of CD3 ζ ITAMs creates high-affinity docking sites for the tandem SH2 domains of ZAP70, coupling receptor triggering to the proximal TCR kinase cascade [139, 140]. Receptor tails can therefore be viewed as collections of phosphorylation, kinase-, adaptor-, and effector-recruitment instructions, whose identity, number, and position determine how downstream signaling is assembled [141, 142].

This modular view opens the possibility of moving beyond naturally occurring costimulatory domains by recombining individual signaling motifs to selectively tune pathway recruitment and downstream cellular state. Daniels and colleagues constructed approximately 2,300 CAR costimulatory domains from combinations of 13 signaling motifs and showed that motif identity, combination, and position generated markedly different T-cell phenotypes [142]. Neural-network analysis extracted design rules from this library and identified non-natural combinations of TRAF- and PLC γ 1-recruiting motifs that promoted both cytotoxicity and stem-like features. Adding PLC γ 1-recruiting motifs improved 4-1BB-like CARs but provided little benefit in the CD28 context, consistent with PLC γ 1 signaling being complementary to TRAF signaling but more redundant with pathways already engaged by CD28 [142]. Synthetic receptor design can move from selecting intact costimulatory domains toward programming specific signaling interactions and pathway combinations.

The next challenge is to connect these synthetic motifs to the signaling dynamics that generate therapeutic phenotype. Current motif libraries are largely optimized using terminal outputs such as cytotoxicity, proliferation, or memory state, but these endpoints do not reveal how a given motif reshapes signaling between receptor engagement and cell fate. Incorporating intermediate measurements of kinase activity, Ca²⁺, ERK, and transcription-factor dynamics could reveal whether a favorable motif acts by altering signal strength, duration, pathway coupling, or signal termination. Comparing these responses across iNKT, MAIT, and $\gamma\delta$ T cells could then determine which signaling rules are broadly transferable and which require chassis-specific optimization.

4.5 Defining the dynamic signaling fingerprint of an effective therapeutic cell

A logical extension of this framework is to characterize engineered receptors not only by their sequence or terminal phenotype, but by the dynamic signaling state that they generate during tumor recognition. A multiplexed biosensor panel could resolve proximal Lck/Fyn and ZAP70/Syk activity together with Ca²⁺, ERK, NFAT, PI3K–AKT, and metabolic responses, thereby capturing how receptor architecture is translated into coordinated pathway activity. Measurements during the first target-cell encounter, repeated rounds of tumor recognition, and post-killing disengagement would be particularly informative, because receptors that produce similar endpoint phenotypes may differ substantially in how rapidly they activate, how long signaling persists, and how efficiently they reset between encounters.

A dynamic signaling fingerprint could include activation latency, peak amplitude, signal duration, pulse frequency, spatial polarization, cell-to-cell heterogeneity, and return-to-baseline kinetics. Comparing these measurements with serial killing, proliferation, differentiation, metabolic fitness, persistence, and

exhaustion-associated phenotypes could link receptor architecture and intracellular signaling to therapeutic function. Such comparisons could identify signaling features associated with a receptor's functional advantage.

One particularly relevant but poorly defined parameter is signal resetting. CAR-T cells can sequentially kill multiple tumor targets, requiring repeated cycles of synapse formation, cytotoxicity, disengagement, migration, and reactivation [143]. A receptor that generates strong activation during the first encounter but fails to terminate efficiently could therefore be disadvantaged during subsequent encounters or progressively accumulate maladaptive signaling. Directly measuring the rate and completeness with which kinase, Ca^{2+} , ERK, and NFAT signals return toward baseline after target disengagement may reveal whether efficient resetting contributes to sustained serial killing.

Drawing on conventional $\alpha\beta$ CAR-T literature, one testable model is that effective signaling combines sufficient but transient activation with efficient downstream propagation and recovery, rather than maximizing every pathway. Excessive tonic signaling promotes dysfunction [12, 127], whereas calibrated ITAM signaling can improve persistence [13]; persistent, unbalanced NFAT activity has likewise been linked to dysfunctional transcriptional programs in conventional T cells [126]. Whether analogous dynamic signatures predict function in CAR-iNKT, CAR-MAIT, or CAR- $\gamma\delta$ cells remains unknown and should therefore be treated as a hypothesis for experimental testing rather than an established design rule.

The signaling pattern associated with effective function may depend on the therapeutic cell. iNKT cells integrate TCR signaling with SAP–Fyn and cytokine pathways, MAIT cells combine MR1–TCR and inflammatory inputs, and V γ 9V δ 2 cells integrate butyrophilin-dependent metabolic recognition with stress-receptor signaling. CAR designs may therefore need to generate different signaling dynamics in each lineage.

4.6 Toward an activity imaging- and AI-guided closed-loop platform for CAR optimization

Machine learning provides a natural framework for navigating this multidimensional design space [144]. Synthetic signaling-domain libraries have already shown that neural networks can learn relationships between motif composition and T-cell phenotype [142]. Complementary approaches are beginning to address tonic signaling at the antigen-binding domain itself. CAR-Toner, for example, uses a protein-language model to predict and optimize positively charged surface patches associated with CAR clustering and tonic signaling [145], building on experimental evidence that tuning these charge features can alter CAR-T-cell fitness [127].

A logical extension of this framework is to incorporate dynamic signaling measurements as an intermediate mechanistic layer linking receptor architecture to therapeutic outcome. In this model, receptor sequence and motif composition would be related to cell-chassis-specific signaling dynamics, summarized as a quantitative signaling fingerprint, and then connected to cellular phenotype and in vivo

performance. These relationships could subsequently inform predictive modeling and rational design of next-generation receptors (Fig. 3).

The key advantage of such a framework is mechanistic interpretability. Rather than learning only that one CAR sequence produces greater cytotoxicity than another, a model could identify whether a particular motif combination changes ZAP70 activation, ERK propagation, Ca^{2+} -NFAT persistence, metabolic adaptation, or signal resetting, and then relate those intermediate states to serial killing and persistence. This would allow subsequent receptor designs to be modified on the basis of a defined signaling defect rather than selected solely through empirical screening (Fig. 3).

Dynamic measurements could be combined with phosphoproteomics, single-cell transcriptomics, chromatin accessibility, metabolomics, and spatial profiling. Omics approaches provide molecular breadth; live-cell biosensors add temporal and spatial information that is largely lost at discrete endpoints. Models developed separately in CAR-iNKT, CAR-MAIT, and CAR- $\gamma\delta$ T cells could then test which relationships between receptor architecture, signaling, and function are shared across lineages and which depend on the cell type.

5 Outstanding questions and limitations

An immediate limitation lies in the performance and validation of the biosensors themselves. For kinase activity reporters, greater sensitivity and dynamic range will be important for resolving low-amplitude or highly localized TCR signaling; indeed, systematic optimization of substrate sequences can substantially improve Fyn and ZAP70 reporter performance [16, 146]. Equally important is substrate selectivity, because kinase activity reporters function as surrogate substrates and related kinases can have overlapping sequence preferences. Reporter substrates should therefore be systematically evaluated against relevant kinase families and validated using kinase perturbation and phosphorylation-site controls to ensure that the measured signal faithfully reflects the intended kinase [146, 147]. For second-messenger sensors, a different constraint is response kinetics. Ca^{2+} and lipid-derived messengers can change on very rapid timescales, making fast sensor association and dissociation kinetics essential for accurately capturing signal onset, peak amplitude, oscillations, and return to baseline [148, 149]. Improving these properties will therefore be essential before quantitative signaling fingerprints can be interpreted as faithful representations of endogenous T-cell signaling.

Despite rapid progress, several limitations currently constrain how confidently signaling principles can be transferred from conventional T cells to innate-like T-cell therapies. Most mechanistic live-cell imaging studies of proximal TCR and CAR signaling have been performed in conventional $\alpha\beta$ T cells, whereas comparable measurements in mature human iNKT, MAIT, and $\gamma\delta$ T cells remain relatively sparse [16, 18]. Consequently, some of the signaling relationships proposed here necessarily combine lineage-specific functional observations with mechanistic frameworks established in $\alpha\beta$ T cells. These comparisons are useful for generating hypotheses, but should not be interpreted as evidence that identical signaling dynamics operate in every innate-like T-cell lineage.

A related issue is the distinction between developmental signaling and mature effector signaling. Strong and sustained TCR–Egr–PLZF signaling clearly shapes iNKT development [96, 100], while Syk–PI3K signaling has a specific requirement during murine $\gamma\delta$ T17 differentiation [97]. These studies establish that signaling strength and pathway usage can determine lineage fate, but they do not necessarily predict how mature human iNKT or $\gamma\delta$ T cells process native-TCR or CAR stimulation in tumors. Direct comparison of developmental, resting mature, activated, and repeatedly stimulated states will therefore be important for distinguishing lineage-establishing pathways from those that control therapeutic function.

The experimental context also strongly influences the signaling state being measured. Antibody-mediated CD3 crosslinking, supported lipid bilayers, antigen-presenting cells, and tumor-cell conjugates differ in ligand mobility, receptor density, mechanical coupling, synapse geometry, and duration of cell–cell contact. Live-cell imaging has already shown that TCR signaling is dynamically reorganized through microclusters and nanoscale receptor assemblies rather than occurring uniformly across the membrane [24, 25, 27]. Signaling kinetics obtained from one stimulation format should therefore not automatically be treated as fixed physiological properties of a given receptor.

Biosensor measurements themselves introduce additional constraints. Genetically encoded reporters can perturb the system if expressed at excessive levels, while most current reporters visualize only a limited number of pathways simultaneously. FRET-based approaches also consume substantial spectral bandwidth, although single-fluorophore KTR, cpEGFP, and chemigenetic sensor architectures may increasingly alleviate this limitation [30, 32, 33]. Conversely, label-free approaches such as NAD(P)H/FAD FLIM provide valuable information about metabolic state but do not identify which upstream receptor or signaling pathway produced that state [42]. A major methodological challenge is therefore to increase multiplexing without sacrificing temporal resolution or substantially perturbing endogenous signaling.

Most importantly, an informative signaling fingerprint must ultimately predict *in vivo* therapeutic behavior. Tumor cells are encountered within environments shaped by antigen heterogeneity, hypoxia, nutrient limitation, suppressive myeloid populations, stromal interactions, and repeated target recognition, all of which may alter signaling trajectories that appear favorable under simplified *in vitro* conditions. Measurements obtained during a single tumor-cell encounter should therefore be extended to repeated killing, tissue infiltration, persistence, biodistribution, and toxicity. The most useful signaling features will be those that remain associated with therapeutic performance across these increasingly complex settings.

Finally, predictive modeling will be limited by the quality and diversity of the underlying data. Neural-network approaches have already demonstrated that CAR signaling-motif composition can be related to cellular phenotype [142], and sequence-based models can predict features associated with tonic CAR signaling [145]. However, small datasets derived from limited donors, receptor architectures, or experimental conditions may capture batch-specific correlations rather than general biological rules. Future platforms will therefore require standardized perturbations, longitudinal measurements, multiple

donors and cell states, independent validation, and, most importantly, prospective testing of model-predicted receptor designs.

Before signaling fingerprints can guide the design of iNKT-, MAIT-, and $\gamma\delta$ T-cell therapies, experiments must establish which dynamic features are causal, reproducible, and predictive across donors and physiological contexts.

6 Conclusions

A central theme emerging from this Review is that the signaling distinctiveness of iNKT, MAIT, and $\gamma\delta$ T cells does not generally arise from possession of completely different intracellular pathways. Instead, these lineages largely retain the canonical T-cell signaling framework but connect it to different recognition systems, accessory receptors, developmental programs, and tissue-derived inputs. iNKT cells combine TCR signaling with SLAM–SAP–Fyn and cytokine pathways; MAIT cells integrate MR1-dependent antigen recognition with inflammatory cytokines and tissue adaptation; and V γ 9V δ 2 cells translate alterations in intracellular isoprenoid metabolism into TCR recognition through BTN3A1 and BTN2A1 while also responding to cellular stress [53, 54, 103, 112, 124]. The relevant question is therefore less whether these cells possess a unique kinase and more how a shared signaling network is configured and dynamically integrated within each lineage.

This distinction becomes apparent when TCR signaling is considered as a dynamic process rather than a static pathway diagram. Microscopy has shown that receptor triggering is organized through microclusters, nanoscale receptor domains, and LAT-centered signaling assemblies [24–26]. At the same time, kinase and second-messenger reporters reveal substantial temporal structure, including spatially separated ZAP70 activity, sustained or pulsatile ERK responses, and oscillatory Ca²⁺ signals [28, 31, 35, 41]. These observations indicate that signaling information can be encoded through several interdependent dimensions: magnitude, persistence, localization, pathway coupling, and signal termination, rather than through phosphorylation magnitude alone.

Differences in signaling amplitude, duration, localization, and pathway integration may help explain why the same innate-like T-cell lineage can adopt markedly different functional states. MAIT cells, for example, can generate different molecular programs following TCR-dependent versus IL-12/IL-18-dependent activation, while simultaneous stimulation produces yet another integrated response [53, 108, 109]. iNKT cells likewise acquire different subsequent phenotypes depending on whether activation occurs through the TCR or inflammatory cytokines [52]. These findings suggest that cytotoxic, immunoregulatory, tissue-repair, or dysfunctional phenotypes need not represent fixed properties of a lineage. Instead, they may emerge from different signaling trajectories imposed on a shared cellular program. The same reasoning may explain why repeated or persistent signaling can ultimately produce outcomes very different from those generated by acute activation, as illustrated by NFAT-driven dysfunctional programs in conventional T cells [126].

This concept has direct implications for synthetic receptor engineering (Fig. 3). CAR design has historically been organized around modular receptor components, including an antigen-binding domain, hinge/transmembrane region, one or more costimulatory domains, and a CD3 ζ signaling module [10, 11]. Increasing evidence, however, shows that relatively subtle changes within these modules can substantially alter downstream signaling and cell fate. Repositioning or reducing individual CD3 ζ ITAMs can redirect differentiation and therapeutic potency [13], while modification of a single residue within the CD28 cytoplasmic domain can reduce exhaustion and improve CAR-T-cell persistence and antitumor durability [15]. Tonic signaling, ITAM number and position, individual residues within CD28, and costimulatory-domain identity can alter differentiation, metabolism, persistence, and exhaustion [12–15]. Live-cell measurements show that these effects cannot always be predicted from proximal activation alone: CARs with similar ZAP70 activity can generate different Ca²⁺ or ERK responses, and TCRs and CARs can organize ZAP70 activity differently in space [16–18]. The therapeutic objective should therefore not be to maximize one signaling node, but to generate a coordinated signaling trajectory appropriate for the desired cellular state.

Innate-like T cells add an additional layer to this design problem because CAR signaling is superimposed on lineage-specific endogenous networks, including SAP–Fyn signaling in iNKT cells, TCR–cytokine integration in MAIT cells, and stress-receptor signaling in $\gamma\delta$ T cells [53, 54, 103]. CAR-iNKT studies already show that the effects of CD28, 4-1BB, and IL-15 depend strongly on cellular context [128, 129], while CAR-MAIT and CAR- $\gamma\delta$ studies suggest that apparently comparable synthetic receptor engagement can be translated into different cytokine, proliferative, and cytotoxic programs [56, 57]. Accordingly, there may be no universally optimal CAR signaling architecture. An intracellular domain optimized in an $\alpha\beta$ T cell may therefore be suboptimal in a cell whose native signaling network is simultaneously shaped by SAP–Fyn signaling in iNKT cells, MR1–TCR and cytokine integration in MAIT cells, or butyrophilin- and stress-receptor-dependent pathways in $\gamma\delta$ T cells [48, 49, 53, 54, 103]. Cellular chassis should therefore be considered a design variable rather than merely a vehicle for receptor expression.

The emerging ability to construct signaling domains from individual biochemical motifs provides a route toward testing this idea directly. Synthetic combinations of TRAF-, PLC γ 1-, and other signaling motifs can generate phenotypes not readily achieved by intact natural costimulatory domains, and machine-learning models can already extract relationships between motif composition and cellular phenotype [142]. The next conceptual step is to place a mechanistically interpretable layer between sequence and phenotype. Rather than learning only that one receptor sequence performs better than another, models could incorporate dynamic signaling fingerprints describing ZAP70 activation, Ca²⁺ and ERK propagation, NFAT persistence, metabolic adaptation, spatial polarization, and signal resetting. Such an approach could shift receptor optimization from empirical screening toward mechanism-guided design by revealing which signaling features underlie therapeutic performance.

Realizing this framework will require advances in both measurement technology and experimental design. Kinase reporters will need improved dynamic range, sensitivity, and rigorously validated substrate selectivity, whereas second-messenger reporters must respond sufficiently rapidly to

reproduce fast endogenous kinetics. Multiplexing remains limited, and signaling patterns measured under antibody crosslinking or simplified in vitro stimulation may not reproduce those generated within a tumor microenvironment. These limitations are particularly important because direct activity imaging in mature human iNKT, MAIT, and $\gamma\delta$ T cells remains sparse. The signaling fingerprints proposed here should therefore be regarded as testable mechanistic hypotheses, not yet as established engineering rules.

Live-cell activity imaging, synthetic receptor engineering, single-cell and spatial omics, and machine learning could be combined to test how receptor architecture affects signaling, cellular state, and therapeutic performance. Comparisons across iNKT, MAIT, and $\gamma\delta$ T cells would show which signaling relationships hold across lineages and which require a different receptor design. The critical next step is to test whether these measured dynamics predict sustained antitumor function more reliably than terminal phenotypes alone.

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Ethics approval and consent to participate

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Author Contribution

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Data availability

No datasets were generated or analyzed during the current study.

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Figures

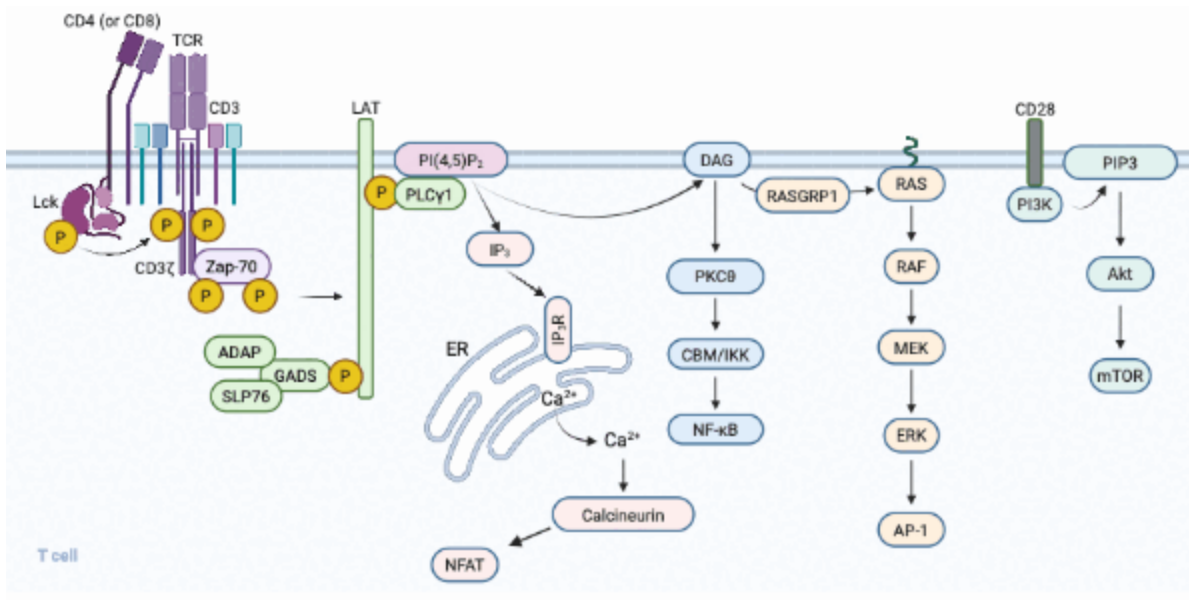


Figure 1

Canonical T-cell receptor signaling network and major downstream branches.

TCR engagement promotes Lck-dependent phosphorylation of CD3 ITAMs and recruitment of ZAP-70, which phosphorylates LAT and drives assembly of the LAT–Gads–SLP-76 signalosome. Activated PLCγ1 hydrolyzes PI(4,5)P₂ into IP₃ and DAG. IP₃ induces ER Ca²⁺ release and calcineurin–NFAT signaling, whereas DAG activates PKCθ–NF-κB and RasGRP1–Ras–RAF–MEK–ERK–AP-1 pathways. In parallel, CD28–PI3K signaling generates PIP₃ to activate Akt–mTOR. Together, these branches illustrate how proximal TCR signaling is distributed into Ca²⁺, MAPK, NF-κB, and metabolic programs that can be resolved by live-cell imaging.

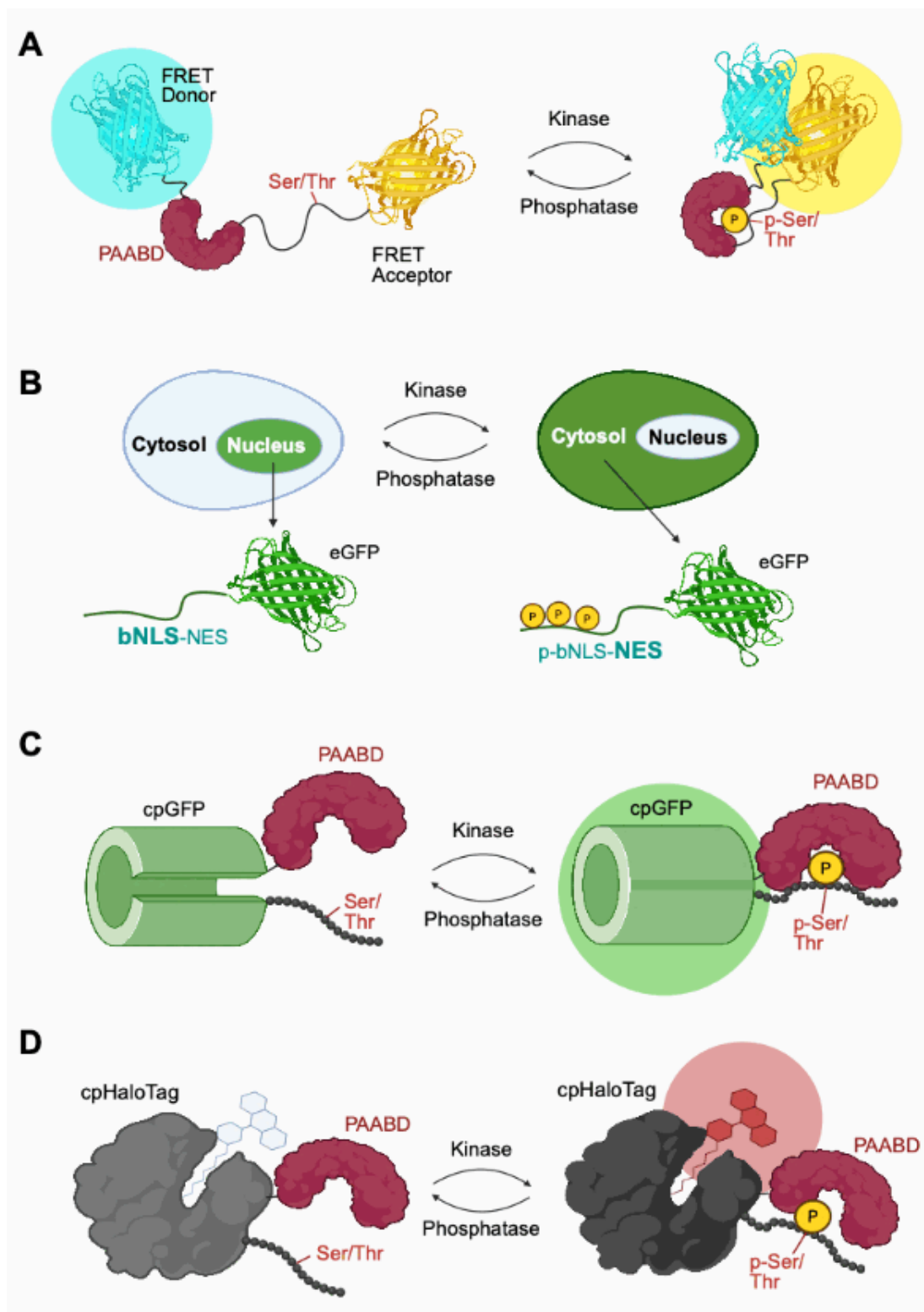


Figure 2

Design strategies of genetically encoded kinase activity reporters.

(A) FRET-based KARs contain a kinase-responsive Ser/Thr substrate and a phosphoamino-acid-binding domain (PAABD) positioned between donor and acceptor fluorescent proteins. Phosphorylation induces intramolecular binding and a conformational change that alters FRET efficiency. (B) Kinase translocation

reporters (KTRs) couple phosphorylation to changes in nucleocytoplasmic localization through a kinase-responsive module containing a bipartite nuclear localization signal (bNLS) and nuclear export signal (NES). Phosphorylation weakens nuclear import and/or promotes export, shifting the fluorescent reporter from the nucleus to the cytosol; dephosphorylation reverses this redistribution. **(C)** cpGFP-based KARs use phosphorylation-dependent intramolecular binding to alter the conformation and optical properties of circularly permuted GFP. **(D)** cpHaloTag-based chemigenetic KARs similarly translate phosphorylation-dependent conformational changes into altered fluorescence of a HaloTag-bound synthetic dye. In each design, phosphatase activity reverses the phosphorylation-dependent response, enabling dynamic monitoring of kinase activity in living cells.

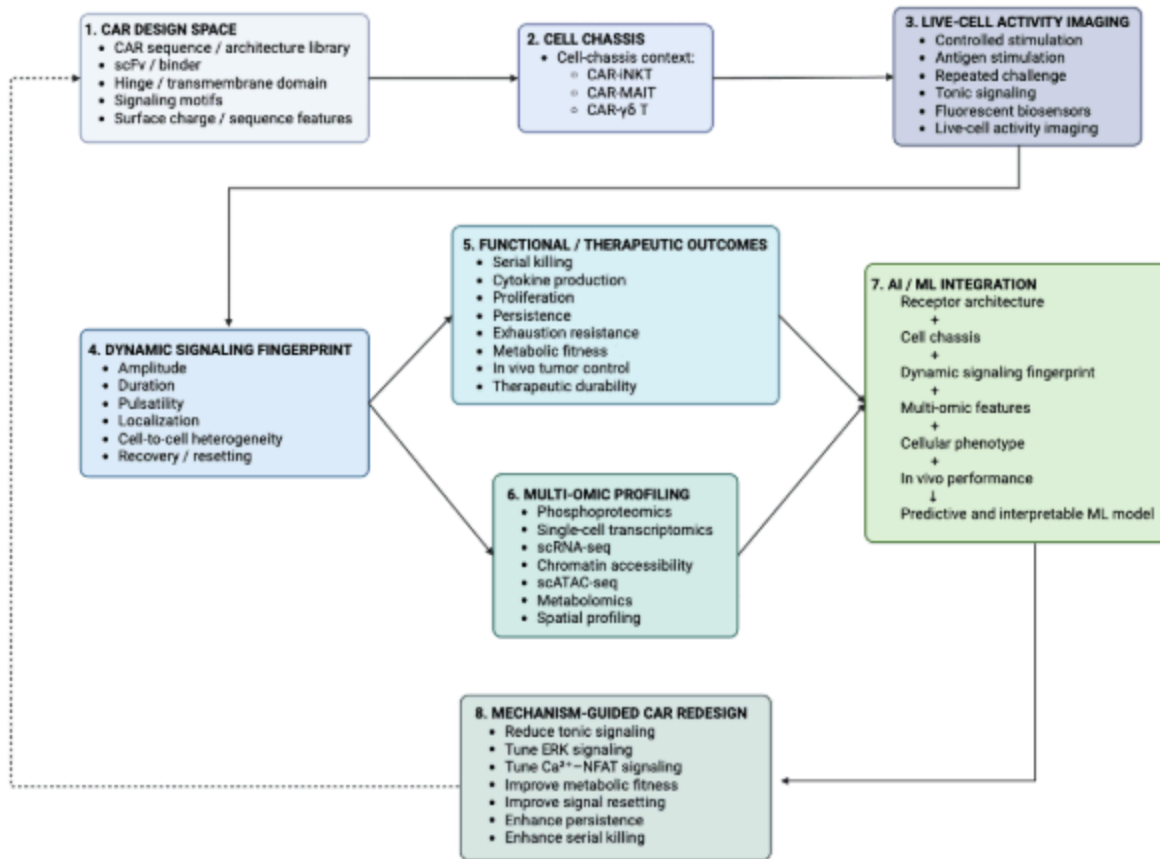


Figure 3

Activity imaging- and AI-guided closed-loop framework for CAR optimization.

CAR sequence and architecture are first evaluated across distinct immune-cell chassis, including CAR-iNKT, CAR-MAIT, and CAR- $\gamma\delta$ T cells. Controlled stimulation and live-cell biosensor imaging are then used to extract dynamic signaling fingerprints defined by features such as amplitude, duration, pulsatility, localization, heterogeneity, and signal resetting. These signaling features are integrated with functional and therapeutic outcomes together with multi-omic profiling to train predictive and interpretable machine-learning models. AI and ML model-derived relationships between receptor architecture, cell chassis, signaling dynamics, molecular state, and therapeutic performance can then guide mechanism-

based CAR redesign. Iterative repetition of this design–measure–learn–redesign cycle provides a conceptual closed-loop strategy for rational optimization of next-generation CARs.