



# Regulation of T Helper Cell Fate by TCR Signal Strength

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T cells are critical in orchestrating protective immune responses to cancer and an array of pathogens. The interaction between a peptide MHC (pMHC) complex on antigen presenting cells (APCs) and T cell receptors (TCRs) on T cells initiates T cell activation, division, and clonal expansion in secondary lymphoid organs. T cells must also integrate multiple T cell-intrinsic and extrinsic signals to acquire the effector functions essential for the defense against invading microbes. In the case of T helper cell differentiation, while innate cytokines have been demonstrated to shape effector CD4<sup>+</sup> T lymphocyte function, the contribution of TCR signaling strength to T helper cell differentiation is less understood. In this review, we summarize the signaling cascades regulated by the strength of TCR stimulation. Various mechanisms in which TCR signal strength controls T helper cell expansion and differentiation are also discussed.

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## INTRODUCTION

CD4<sup>+</sup> T helper (Th) cells play a critical role in mediating protective immunity against bacterial, viral, parasitic and fungal infections by regulating the responses of antibody-producing B cells, cytotoxic CD8<sup>+</sup> T lymphocytes, and macrophages. Naïve CD4<sup>+</sup> T cells are activated when their unique T cell Receptor (TCR) recognizes cognate peptides presented by Major Histocompatibility Complex (MHC) class II molecules (pMHC-II) in the presence of costimulatory molecules on antigen presenting cells (APCs). To ensure that there are sufficient numbers of antigen-specific clones present to combat pathogens at infection sites, activated CD4<sup>+</sup> T cells undergo clonal expansion and acquire effector functions in secondary lymphoid organs (SLOs). Depending on the type of pathogen, CD4<sup>+</sup> T cells can be tailored to become one of several specialized Th subsets defined by their functional attributes (1).

Two prevailing models explain how CD4<sup>+</sup> T cells integrate different signals to determine lineage commitment. The classic “qualitative” model suggests that Th cell responses are shaped dominantly by the cytokines produced by pathogen-exposed innate cells. The second “quantitative” model proposes that the strength of the signal delivered through the TCR regulates the differentiation program of CD4<sup>+</sup> T cells. Multiple T cell-associated factors influence the overall strength of TCR signaling strength. These include the quality of the interactions between MHC and TCR molecules, the amount of antigen, and the degree of costimulation (2). This review will focus mainly on how the potency of signals delivered through the TCR controls the activation, expansion, and differentiation of CD4<sup>+</sup> T cells.

## EXPERIMENTAL APPROACHES USED TO MANIPULATE TCR SIGNAL STRENGTH

Different experimental approaches have been used to study the role of TCR signal strength in controlling T cell responses. One of the most frequently employed methods is to titrate the amount of antigen thereby altering the number of peptide-occupied MHC molecules that engage cognate TCRs (2). Another method is to induce mutations in peptide sequences (Altered Peptide Ligands, APLs), which allows investigators to change the potency of TCR signals by altering antigen-binding affinity to MHC molecules and TCR complexes (2, 3). In addition, by altering pathogen or adjuvant doses, variations in the degree of inflammation modulate the intensity of costimulatory signals delivered through CD28, and hence the overall strength of TCR signals (4). It is important to note that because the TCR itself is not modified, the approaches mentioned above cannot analyze the contribution of T cell-intrinsic variations in TCR signal strength to the T cell response.

One T cell-intrinsic factor that affects the strength of the signal delivered through the TCR is the number of TCR molecules expressed, also known as TCR avidity (5). Manipulation of TCR avidity achieves similar outcomes as changes in antigen dose or increasing the number of peptide-loaded MHC molecules, and is naturally achieved after initial T cell activation in a process known as avidity maturation (6). Alternatively, by introducing mutations into TCR sequences, investigators can change the affinity of the TCR for pMHC complexes (7). This alters the binding kinetics between TCR and pMHC complexes such that high affinity TCRs bind with slower off/dissociation rates ( $K_d$ ) when measured using two-dimensional (2D) kinetic assays. Hence at the level of an individual TCR, slower  $K_d$ 's lead to prolonged pMHC:TCR interactions (2). Alterations in antigen dose and the degree of costimulatory signaling also affect the duration of contact between APCs and T cells (8, 9). Higher antigen levels result in longer interaction or "dwell" times and confer potent, prolonged TCR signals; whereas lower antigen dose stimulation results in shorter interactions and impart protracted, weaker signals (9). However, higher-affinity TCRs do not always facilitate greater dwell times or signal strength (10, 11). It is therefore unclear whether higher affinity TCRs accelerate T cell activation by decreasing the antigen threshold required for activation (12–14), or whether they promote longer interactions to stabilize lineage commitment following T cell activation (9).

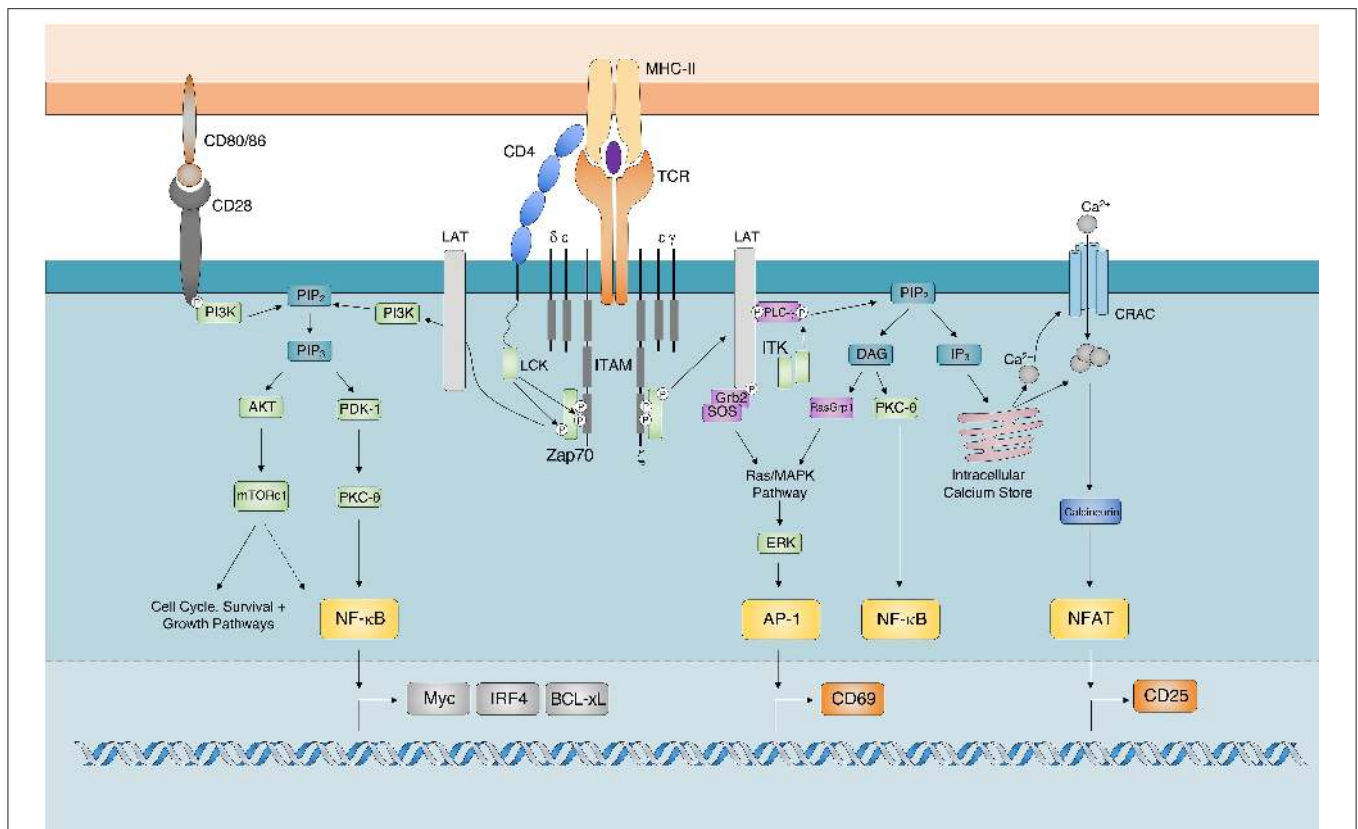
## TCR SIGNALING CASCADE

The TCR complex consists of a variable heterodimer (TCR $\alpha\beta$ ) that binds to antigenic ligands and the invariant signaling component, CD3, which is composed of gamma, delta, epsilon, and zeta subunits. Whilst there is much debate on how TCR signals are initially triggered [reviewed by (15)], it is generally agreed that TCR ligation to agonistic pMHCs results in the aggregation of TCR-CD3 complexes with costimulatory and adhesion proteins such that an immunological synapse is formed (16, 17). The physical force of pMHC:TCR binding

is thought to change the orientation of cytosolic signaling components of the TCR-CD3 complex (18, 19). This change results in the phosphorylation of Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) by Src family Protein Tyrosine Kinase (PTK), such as Lymphocyte-specific protein tyrosine Kinase (LCK) (20, 21). The concurrent exclusion of constitutively active transmembrane tyrosine phosphatases, such as CD45 (that oppose the activity of constitutively active PTKs) away from phosphorylated ITAMs ensures that TCR signals are maintained in a pMHC:TCR-dependent manner (22–24).

Ligation of the TCR, also facilitates the recruitment and docking of the CD4 co-receptor to the pMHC-II complex (25). This positions CD4-associated LCK in locations favorable for ITAM phosphorylation on cytosolic TCR/CD3 complexes (Figure 1). Activated ITAMs serve as docking sites for the Src Homology 2 (SH2) domains of Zeta chain of T cell receptor Associated Protein kinase 70 (Zap70) (26, 27). Importantly, as TCR-CD3 complexes continue to aggregate into microclusters (15), Zap70 undergoes a conformational change associated with enhanced binding affinity and recruitment to ITAMs (28, 29). Zap70 is subsequently activated following its phosphorylation by LCK. Given continued pMHC:TCR-CD3 interactions and receptor clustering, activated Zap70 is released from ITAMs (30) and subsequently phosphorylates signaling scaffold adaptor proteins, such as Linker for Activated T cells (LAT) and SH2 domain containing Leukocyte Protein of 76 kDa (SLP-76) (31). Activation of these adaptor proteins provide SH2- and SH3-binding sites for the initiation of Phosphatidylinositol 3-Kinases (PI3K), Protein Kinase B (AKT) (32), Inducible T cell Kinase (ITK) and Protein Kinase C (PKC) dependent signaling cascades (16, 33). PI3K signaling results in the Phosphoinositide-dependent protein kinase-1 (PDK-1) dependent activation of PKC- $\theta$  for the activation and nuclear translocation of Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) (34–36).

PI3K activity also results in the AKT-dependent activation of Mechanistic Target Of Rapamycin (mTOR). Additionally, activated ITK phosphorylates the lipase Phospholipase C gamma (PLC $\gamma$ ), which ultimately cleaves Phosphatidylinositol-4,5-bisphosphate (PIP $_2$ ) in the plasma membrane to generate the secondary messengers inositol-1,4,5-triphosphate (IP $_3$ ) and Diacylglycerol (DAG) (37). The accumulation of ITK promotes the downstream release of calcium ions (Ca $^{2+}$ ) from endoplasmic reticulum (ER) Ca $^{2+}$  stores (38). The release of intracellular calcium, through the effects of calcineurin, activates and promotes the nuclear translocation of Nuclear Factor of Activated T cells (NFAT) (39). The ITK-dependent generation of DAG results in the recruitment of Ras Guanyl-Releasing Protein 1 (GRP1) and PKC $\theta$  for the activation of the Mitogen Activated Protein Kinase (MAPK)/Extracellular signal Regulated Kinase (ERK) and NF- $\kappa$ B pathways, respectively (40). Activation of the MAPK/ERK signaling cascade triggers the formation of the transcription factor Adaptor-related Protein complex 1 (AP-1) (41). Together, the nuclear translocation and transcriptional activity of AP-1, NFAT and NF- $\kappa$ B orchestrate T cell activation.



**FIGURE 1 | TCR Signaling Cascade.** The major signaling components and transcription factors responsible for the transduction of TCR signals following the recognition of cognate pMHC-II. While AKT dependent activation of mTOR signaling regulates cell proliferation, survival, and growth pathways it has also been shown to regulate the expression of Myc, IRF4, and BCL-xL by potentially regulating (indicated by dashed arrow) the activity of NF-κB. AP-1, Activator Protein 1; AKT, Protein Kinase B; BCL-xL, B Cell Lymphoma-extra Large; DAG, Diacylglycerol; CRAC, Calcium Release Activated Channel; ERK, Extracellular signal-Regulated Kinase; GRB2, Growth factor Receptor-Bound protein 2; IP<sub>3</sub>, Inositol-1,4,5-trisphosphate; ITAM, Immunoreceptor Tyrosine-based Activation Motif; IRF4, Interferon Regulatory Factor 4; ITK, Interleukin-2-inducible T cell kinase; mTORc1, Mechanistic Target Of Rapamycin complex 1; NFAT, Nuclear Factor of Activated T cells; NF-κB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; PLC-γ, Phospholipase C-gamma; PDK-1, Phosphoinositide dependent kinase-1; PKC-θ, Protein Kinase C theta; PIP<sub>2</sub>, Phosphatidylinositol-4,5-bisphosphate; RASgrp1, RAS guanyl-releasing protein 1; TCR, T cell Receptor; SOS, Son of Sevenless; Zap70 ζ-chain associated protein kinase of 70 kDa).

## TCR SIGNAL STRENGTH REGULATES THE ACTIVITY OF TCR SIGNALING COMPONENTS

The strength of the input delivered through the TCR is translated into digital/“all or nothing,” or analog/“scaled” alterations to the TCR signaling cascade (42, 43). In this way potent TCR signals regulate the activity of important transcription factors critical in determining the fate of T cells.

### Potent TCR Signals Scale the Activity of Zap70 and PI3K

Strong TCR signals delivered through pMHC:TCR interactions are amplified in accordance to the duration of time ITAMs remain phosphorylated for the subsequent recruitment and activation of Zap70 (9). When compared to lower affinity counterparts, high affinity TCRs bind cognate pMHCs with distinct docking geometries (44) that ultimately result in the

clustering of receptors (29), as well as a mechanical “push” or “pull” of the TCR (19). Whilst the exact mechanism is unclear, the magnitude of this interaction is dependent on TCR/antigen affinity and dictates the type of bond formed between T cells and APCs (45). While low affinity TCRs confer weaker pulling forces that result in the formation of slip-bonds, high TCR affinity induces the formation of catch-bonds (19, 46). Catch-bonds extend the duration of pMHC:TCR interactions and promote the exclusion of CD45 and its intracellular inhibitory phosphates from the immunological synapse (10, 19, 47). Additionally, high affinity pMHC:TCR binding events have recently been shown to enhance the recruitment and retention of CD4 co-receptors in the pMHC:TCR cluster (48) such that CD4-associated Lck can further sustain ITAM activation on cytosolic TCR/CD3 chains. Hence, by tipping the balance of activating PTKs to inhibitory phosphatases in close proximity to TCR/CD3 complexes in favor of activating PTKs, strong TCR signals extend the duration in which ITAMs on TCR/CD3 chains remain phosphorylated. As such, longer pMHC:TCR interactions have been shown to

enhance the recruitment and activation of Zap70 in bulk (49–51) and single (29, 52) cell analyses.

In addition, strong TCR signals enhance the activity of phosphorylated Zap70 (28). Importantly, when low potency TCR signals are delivered, in addition to phosphorylating ITAMs, LCK phosphorylates Src Homology region 2 domain-containing Phosphatase-1 (SHP-1). During low potency TCR stimulation, phosphorylated SHP-1 inactivates the kinase activity of LCK (53) and Zap70 (54) which results in the attenuation of TCR signals. In contrast to this, strong TCR signals result in a greater proportion of cells expressing phosphorylated ERK which itself phosphorylates LCK and prevents the binding of SHP-1 (53). These results suggest that strong TCR signals resulting from longer pMHC:TCR interactions augment the quantity, quality and duration of Zap70s catalytic action, extending the duration in which downstream signaling cascades remain active.

Through its ability to scale the activity of Zap70, sustained interactions between pMHC:TCR complexes promote the recruitment of PI3K to the adaptor protein LAT (32, 55). Activated PI3K phosphorylates PIP<sub>2</sub> for the generation of the secondary messenger Phosphatidylinositol (3,4,5) triphosphate (PIP<sub>3</sub>). Scaled increases in PIP<sub>3</sub> concentrations regulate both the recruitment of AKT and the activation of its serine/threonine kinase activity (56). AKT indirectly activates mTOR, whose kinase activity is critical for preparing activated T cells for the bioenergetic demands of clonal expansion. Whilst it is well-established that TCR signaling can activate mTOR in a PI3K/AKT-dependent manner (57), TCR-dependent activation of mTOR is considered weak and transient (58). Nevertheless, initial mTOR expression is scaled in an antigen dose-dependent manner (58, 59), suggesting that whilst transient, TCR signals can directly scale the expression of mTOR. In addition, CD28 ligation leads to the phosphorylation of its cytoplasmic tail and the subsequent recruitment of PI3K (60, 61) for the activation of AKT (62) and mTOR (57, 63). As such, PI3K/AKT can be posited as a rheostat that modulates the amplitude of mTOR expression in accordance to the strength of signals delivered through costimulatory molecules.

## Dynamic Regulation of NF- $\kappa$ B, NFAT, and ERK by TCR Signal Strength

The activation and nuclear translocation of NF- $\kappa$ B in CD4<sup>+</sup> T lymphocytes regulates cell survival by controlling the expression of Interleukin (IL) 2 (IL-2) and anti-apoptotic proteins (64–67). TCR/ITK and CD28/PI3K signaling pathways can independently activate PKC- $\theta$  for the downstream activation and nuclear translocation of NF- $\kappa$ B (35) (**Figure 1**). Once a threshold TCR signal has been achieved, TCR and CD28 signaling results in the digital activation and nuclear translocation of NF- $\kappa$ B (68). Despite this, TCR signal strength has been shown to scale the expression of NF- $\kappa$ B-dependent targets, such as pro-survival molecule B-Cell Lymphoma-extra large (BCL-xL) (69) and the transcription factor IRF4 (70, 71). This suggests that whilst TCR signal strength does not regulate the amplitude of

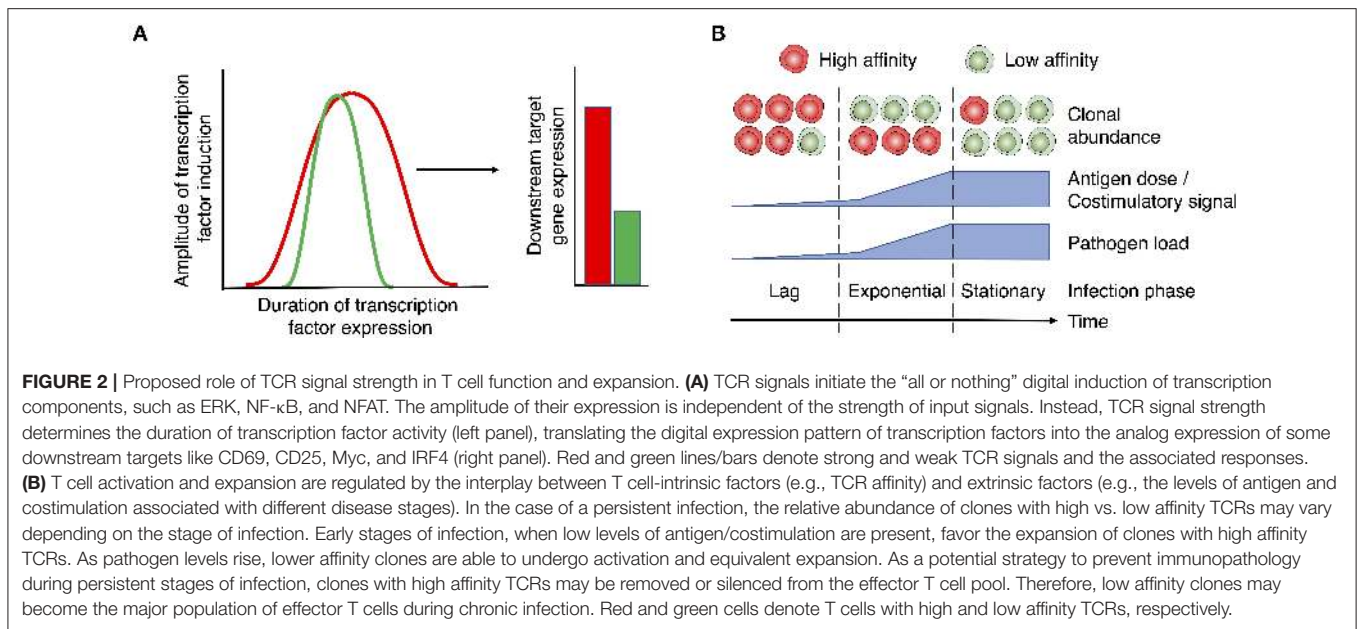
NF- $\kappa$ B expression, it may control its transcriptional activity. Alternatively, there is evidence to suggest that the TCR signal strength-sensitive PI3K/AKT/mTOR pathway can modulate NF- $\kappa$ B activity (**Figure 1**). AKT is proposed to regulate the duration of NF- $\kappa$ B nuclear translocation (72, 73), as well as the range of genes it targets for transcription (74). Albeit in a cancer cell line, the ability of AKT to control NF- $\kappa$ B activation was shown to be reliant on mTOR (75). As mTOR is known to regulate the antigen affinity-driven expression of IRF4 in CD8<sup>+</sup> T cells (76), these findings further suggest that the PI3K/AKT/mTOR signaling cascade may translate analog TCR inputs into analog NF- $\kappa$ B transcriptional outputs.

Strong TCR signals have historically been shown to amplify the accumulation of intracellular calcium (14, 77, 78). It is believed that strong TCR signals increase the number activated ITKs recruited to facilitate the IP<sub>3</sub>-dependent accumulation of intracellular Ca<sup>2+</sup> (38, 79, 80). The magnitude of Ca<sup>2+</sup> release into the cytosol has traditionally been associated with increased levels of activated, nuclear NFATc (81, 82). Despite this, single cell analyses have revealed analog increases in intercellular calcium translate into digital, “all or nothing” NFATc1 or NFATc2 expression patterns in the nuclei of CD4<sup>+</sup> T cells (83–88).

Similarly, the MAPK/ERK signaling cascade exhibits a digital signal response and hence is not scaled by TCR signaling (42, 89, 90). The digital expression of NFAT and ERK in response to analog TCR/ITK input is thought to be a result of positive feedback circuits that reinforce NFAT (91, 92) and ERK (89) expression once a threshold of activation has been reached. NFAT and ERK/AP-1 predominantly drive the expression of surface markers associated with CD4<sup>+</sup> T cell activation, such as CD25 (93, 94) and CD69, respectively (89, 95). Importantly, recent single cell analyses indicates that TCR signal strength scales their expression (84, 85, 96). How then does the digital expression of NFAT/ERK translate into analog downstream gene/protein expression? In the case of NFAT, the extent of initial pMHC:TCR interactions has been shown to modulate the duration of its activity in the nucleus, even after TCR signals have ceased (97). Therefore, similar to the regulation of NF- $\kappa$ B activity discussed above, whilst TCR signal strength may not directly control the magnitude of NFAT or ERK expression, it may extend the duration in which these transcription factors remain active, which subsequently leads to greater expression of target genes and their functional outputs (**Figure 2A**). Future studies need to delineate how TCR signal strength influences the magnitude or duration of downstream signals to achieve functional differences.

## THE POTENCY OF TCR SIGNALS DICTATE THE MAGNITUDE OF T CELL EXPANSION

Control of microbial infection requires the rapid generation of large numbers of effector Th cells. While naïve CD4<sup>+</sup> T cells acquire the ability to produce IL-2 following activation by a single agonistic pMHC-II complex (52, 98), enduring Th



cell proliferation appears to require stronger TCR stimulation (99) with an estimated 50–400 pMHC-II<sub>s</sub> being required (100, 101). The ability for strong TCR signals to promote the expansion of cytotoxic and Th cells is well-established (102). For example, CD8<sup>+</sup> T cells primed by high affinity antigens *in vivo* underwent greater expansion when compared to those stimulated by low affinity antigens (103–105). Moreover, CD4<sup>+</sup> T lymphocytes with high affinity/avidity TCRs (106–113) or those that were stimulated with high affinity antigens or doses (71, 114–118) generally underwent greater expansion *in vivo*. In general, TCR signaling can scale the magnitude of T cell expansion by coordinating the tightly linked metabolic and cell cycle pathways. These programs determine the extent of T cell expansion by regulating the time taken for individual cells to enter and then progress through the cell cycle, the rate of subsequent proliferation and the cells proliferative capacity (119).

Stimulation with high concentrations of antigen or enhanced cross-linking of CD3 accelerates the progression of naïve Th cells from senescence ( $G_0$ ) into the Gap phase 1 ( $G_1$ ) of the cell cycle (120), reducing the time it takes for CD4<sup>+</sup> T cells to start dividing *in vitro* (121, 122). Whilst not studied in CD4<sup>+</sup> T cells, high antigen affinity and doses have been shown to reduce the time to first division in CD8<sup>+</sup> T cells (123). In addition to reducing the time taken for Th cells to undergo their first division (124), potent CD28 ligation enhances the subsequent rate of proliferation in CD4<sup>+</sup> (120) and CD8<sup>+</sup> T cells (125) *in vitro*. Although IL-2 signaling is proposed to regulate the subsequent rate of cell division in CD8<sup>+</sup> T cells, the contribution of IL-2 to the rate of Th cell proliferation is unclear, with negligible (126) and supportive (121) roles being reported. Hence, the mechanism by which strong TCR signals accelerate Th cell progression through the first and subsequent cell cycles remains incompletely understood.

One potential mechanism by which TCR signal strength controls Th cell proliferation is by scaling the activity of mTOR. It is well-established that progression from  $G_0$  is the result of the PI3K/mTOR complex 1 (mTORc1) dependent expression of cyclins and cyclin dependent kinases (CDKs) (127–130). Additionally, mTORc1-dependent upregulation of the Large neutral Amino acid Transporter (LAT1, CD98) and Glucose Transporter 1 (GLUT-1) facilitates nutrient uptake and sustains T cell growth during the  $G_1$  phase (57). Interestingly, although reduced, continuous T cell proliferation can occur when mTORc1 signaling is low or absent (63, 130–132). As activated T cells continuously proliferate by entering the cycle from the  $G_1$  phase (133), these findings suggest that a major function of mTOR may be to initiate cell cycle entry and regulate the timing of initial T cell division in a TCR signal strength-dependent manner.

The number of times a T cell divides before it senesces is also known as the proliferative capacity or Division Destiny (DD) (125). Potent CD28 stimulation, antigen affinity or antigen doses have separately been shown to enhance the proliferative capacity of CD8<sup>+</sup> T cells (123, 125). Recent findings have suggested that the amplitude of Myc expression prior to a T cell entering their first division enables them to undergo a greater number of divisions (134, 135). Given the short half-life of Myc, the greater the nuclear concentration of Myc, the longer it takes for Myc levels to fall below a threshold that can promote cell division. In this way, Myc acts as a timer for the DD of a cell. Interestingly, changes in the strength of CD28 signaling, but not antigen affinity or dose, scale the amplitude of Myc expression in CD8<sup>+</sup> T cells (134, 135). As both PI3K and NF- $\kappa$ B have both been shown to regulate the expression of Myc (136, 137), these data may suggest that TCR and CD28 signals differentially scale the expression of cell division related

transcription programs. Supporting this are observations that increased antigen affinity and dose enhance the amplitude and the transcriptional specificity of IRF4 in CD4<sup>+</sup> T cells (71, 138), whereas increases in CD28 stimulation have a minor impact on IRF4 expression (139). The amplitude of IRF4 expression is known to control the magnitude and duration of CD8<sup>+</sup> T cell expansion by regulating aerobic glycolysis (140), inhibiting repressors of the cell cycle and by preventing the expression of the pro-apoptotic protein Bim (76). As TCR signals also promote IRF4 expression in an mTOR- and NF- $\kappa$ B-dependent manner (70, 76), these data suggest that whilst the TCR and CD28 signal through overlapping pathways (i.e., PI3K, NF- $\kappa$ B), they differentially regulate the cumulative strength of this signal and the resulting proliferative capacity of T cells, possibly by controlling distinct sets of transcription factors (such as Myc and IRF4).

The presence of Th cells with high affinity TCRs may be critical in mobilizing T cell responses to invading pathogens during early stages of infection when pMHC-II and tissue inflammation levels are low. As Th cells with high affinity TCRs possess a lower antigen activation threshold, they may enter the cell cycle quicker than their low affinity counterparts, such that they are preferentially recruited into the activated T cell pool. This accelerated response provides an early burst of effectors that may be critical for preventing the dissemination of infection (**Figure 2B**). However, if antigen levels rise or persist, as is the case with persistent infections, T cell clones with lower affinity TCRs may come to dominate the effector cell pool. Given greater antigenic and inflammatory signals, high affinity effectors may succumb to Activation-Induced Cell Death (AICD) or exhaustion (141–143). Moreover, high affinity effectors may be functionally silenced from the immune response to prevent potential immunopathology through the downregulation of the TCR (144). Therefore, during the persistent phase of an infection, lower affinity clones are less likely to be out-competed by their high affinity counterparts (145, 146) and are able to undergo activation and expansion (**Figure 2B**). This dynamic may account for recent observations that lower affinity/avidity clones make up a large and underappreciated fraction of responding Th cell populations during late stages of infection (144, 146–152).

Given that the overall strength of the TCR signal is a sum of TCR affinity, pMHC-II densities and costimulatory signals, which are in part dictated by the intensity of infection, TCR signaling can be highly dynamic as pathogen loads vary during different stages of infection. This allows T cells with a spectrum of affinities to be effectively stimulated and sufficient numbers of effector T cells to be generated to contain invading pathogens regardless of the stage of infection. Taken together, it is likely that TCR affinity, antigen potency, and antigen dose play dynamic and potentially distinct roles in the regulation of Th cell expansion. This has been proposed by other studies, albeit in models lacking inflammation (153, 154). Future studies will need to formally investigate these hypotheses by dissecting the interplay between changes in TCR affinity, the timing of clonal contraction and by examining how changes in pathogen levels affect this dynamic across different tissues.

## TCR SIGNALING STRENGTH AND CD4<sup>+</sup> T CELL DIFFERENTIATION

The role of TCR signal strength in CD4<sup>+</sup> T cell differentiation has traditionally been determined by investigating the ability of naïve Th cells to commit to one effector lineage over the other in response to stimulation with different model antigens. Early *in vitro* investigations into the role of TCR signal strength have generally reported that stimulation with high antigen doses favored IFN- $\gamma$  over IL-4 production in TCR transgenic T cell cultures (155–160). However, high dose antigen stimulation has also been shown to promote IL-4 production *in vitro* (161–165). This discrepancy might be explained by variations in mouse strains used between studies. For example, TCR transgenic cells with a B10.A genetic background favored IFN- $\gamma$  production (155, 159), whereas those on a BALB/c background skewed to IL-4 production (161, 163). Intriguingly, the same TCR transgenic T cells from the BALB/c mice that predominantly produced IL-4 in response to high dose antigen stimulation (161, 163–165) have been shown to favor IFN- $\gamma$  production in other studies (156, 157), suggesting that the outcome of T cell differentiation is influenced by *in vitro* culture conditions.

Since strong TCR signals as a result of high antigen dose stimulation promote IL-4 over IFN- $\gamma$  expression under some circumstances (161, 163–165), a bi-phasic Th2 differentiation model has been proposed by Nakayama and Yamashita (166). This model hypothesizes that naïve CD4<sup>+</sup> T cells can differentiate into IL-4-expressing cells in the presence of both very low- and high-levels of cognate antigen. Although activation of ERK by strong TCR signals reduces IL-4 expression in peptide-activated Th cells (159, 167), ERK signaling has been shown to promote IL-4 expression in CD4<sup>+</sup> T cells stimulated with TCR cross-linking antibodies (166). These findings suggest that ERK could play a dual role in TCR signal strength-dependent Th2 differentiation.

On the other hand, strong TCR signaling as a result of stimulation with high affinity APLs has generally favored the generation of IFN- $\gamma$  over IL-4 producing effectors *in vitro* (81, 158, 167–170) and *in vivo* (171). Furthermore, when two TCR transgenic T cell lines recognizing the same antigen were compared *in vitro*, CD4<sup>+</sup> T cells with weak TCR signaling due to a mutation in the TCR alpha chain were shown to favor IL-4 induction (172). Studies that have attempted to uncover the mechanism that results in enhanced Th1 differentiation *in vitro* have suggested that strong TCR signals prevent default Th2 programs rather than actively driving Th1 polarization. Here, strong TCR signals are believed to prevent the early expression of IL-4, and its autocrine signaling that results in the expression of GATA Binding Protein 3 (GATA3) for Th2 differentiation (1, 159), by enhancing the nuclear translocation of NFATp (81) and altering the DNA binding activity of AP-1 (167). Some studies have demonstrated that potent costimulation or stimulation with high antigen doses can actively promote the generation of IFN- $\gamma$ -expressing effectors by regulating the ability of CD4<sup>+</sup> T cells to respond to the Th1 polarizing cytokine IL-12 (4, 173). IL-12 is known to promote Th1 differentiation and IFN- $\gamma$  expression by promoting the expression of T-box

binding transcription factor (T-bet), the master regulator of Th1 polarization (1). While these studies associated high antigen dose stimulation with enhanced IL-12 Receptor Beta 2 (IL-12R $\beta$ 2) chain expression, the mechanism linking the two events remains to be identified.

There is also evidence to suggest that strong TCR signaling may indirectly promote Th1 differentiation by altering the function of APCs. Here, stimulation of CD4<sup>+</sup> T cells with high affinity antigens has been shown to enhance the production of IL-12 from co-cultured APCs by enhancing the expression of CD40L on developing Th cells *in vitro* (157, 174, 175). In summary, when comparing Th1 and Th2 differentiation *in vitro*, the majority of studies indicate that strong TCR signals seem to favor the generation of Th1 effectors by directly preventing the early expression of IL-4 and GATA3, as well as by promoting the expression of the IL-12R $\beta$ 2. Importantly, T-bet is known to drive IFN- $\gamma$  expression and prevent Th2 differentiation by sequestering GATA3 (176–178). Hence it is likely that strong TCR signals further favor the generation of Th1 over Th2 cells by promoting IL-12-dependent T-bet expression for the subsequent sequestration of GATA3. Future studies will need to formally investigate how potent TCR signals regulate the crosstalk between T-bet and GATA3 for the generation of Th1 and Th2 effectors, respectively.

Interestingly, early *in vivo* studies have demonstrated that high antigen dose stimulation promotes the generation of IL-4 producing effectors (179–182). Whilst this appears to support the *in vitro* findings underlying the signal strength model proposed by Nakayama and Yamashita (166), the apparent Th2 phenotype was also accompanied with greater levels of class-switched antibodies, which are now recognized to be a result of IL-4 producing follicular T helper cells (Tfh) that are difficult to stably generate *in vitro* (183–185). Hence these studies provided the initial indication that high antigen doses promoted the generation of Tfh cells *in vivo* and may help build on findings that indicate that medium-strong TCR signaling can promote the generation of IL-4 expressing effectors *in vitro*. This has since been confirmed in recent *in vivo* studies where high antigen dose stimulation resulted in longer dwell times, and favored the generation of Tfh cells over Th1 effectors (11, 115, 186–188). As IL-2 signaling is known to suppress the expression of BCL-6, the master regulator of Tfh differentiation (189), it was proposed that high antigen dose stimulation favored Tfh cell generation by reducing IL-2 signaling (188).

Intriguingly, when the responses of two TCR transgenic populations that recognize the same antigen with different binding affinities were recently compared *in vivo*, a greater proportion of high affinity TCR-bearing Th cells were found to undergo Th1 commitment (108). Moreover, stimulation with high affinity antigens have also been shown to favor the generation of Th1 effectors (115, 190). It is thought that high affinity TCR interactions drive STAT5-dependent Th1 differentiation (191) by upregulating and maintaining the expression of the high affinity IL-2 receptor, CD25 (108, 192). Although high affinity TCR interactions generally favor Th1 differentiation, high affinity TCRs have been shown to promote the generation of Tfh cells (107) or play a more capricious role

(11). As has been suggested previously (115), these data again suggest that antigen dose and TCR affinity play distinct roles in determining the fate of Th cells. Therefore, while high antigen dose stimulation favors the generation of Tfh cells *in vivo*; the function of TCR affinity in determining Th differentiation may be secondary to T cell-extrinsic factors such as innate cytokines. Hence, the role of TCR affinity in lineage differentiation may vary depending on the experimental model employed.

The strength of TCR signaling has also been suggested to regulate Th17 differentiation. Under Th17-polarizing conditions *in vitro*, high antigen dose and persistent TCR stimulation has been shown to promote IL-17 expression (193, 194). Furthermore, when TCR signals are attenuated through the deletion of ITK, CD4<sup>+</sup> T lymphocytes were more prone to differentiate into Forkhead box P3 (Foxp3) expressing regulatory Th cells (Tregs) over IL-17 producing cells (195). However, low levels of CD28 costimulation or antigen stimulation, as well as exposure to low potency antigens, have each been shown to favor the generation of IL-17 over IFN- $\gamma$  (196, 197) or IL-4 expressing effector CD4<sup>+</sup> T cells (198). Therefore, the role of TCR signal strength in Th17 differentiation remains unclear.

Distinct mechanisms for how TCR signal strength modulates Th17 polarization were proposed in these studies. TCR dependent AKT signaling was shown to be associated with both enhanced (195) and reduced (197) Th17 differentiation. Moreover, antigen dose dependent scaling of CD40L expression on activated Th cells was shown to promote IL-6 production by APCs for the enhanced generation of Th17 cells *in vitro* (193). Regardless, the generation of IL-17 expressing effectors was shown to be dependent on exposure to the correct cytokine milieu (193–198). This suggests that when compared to polarizing cytokines, TCR signal strength may play a secondary, context-dependent role in directing Th17 differentiation. In addition to studying the role of TCR signal strength in relevant Th17-mediated disease models, future studies will need to investigate how TCR signal strength regulates the expression of the Th17 master regulator of transcription, RAR-related Orphan Receptor gamma t (ROR $\gamma$ t).

A growing number of studies indicate that TCR signal strength does not dictate T cell effector function at the individual T cell level (11, 98, 199–202). Rather, potent TCR signals are proposed to regulate the number of T cells recruited into the effector cell pool and the ratio of effector phenotypes throughout the T cell response. Therefore, TCR signal strength may regulate the overall magnitude and function of T cell responses at a population level. It is possible that antigen doses and TCR affinity promote different “selection” or “survival” strategies to shape Th cell responses at a population level. For example, high antigen dose stimulation generally seems to favor Tfh over Th1 populations *in vivo*. As Th1 cells are susceptible to AICD following high antigen dose stimulation (142, 143), the induction of BCL-6 in these conditions might confer a selection-advantage by promoting Th cell survival (203) and resistance to AICD, as has been proposed by Keck et al. (115). In support of this, BCL-6 has been reported to regulate the survival of multiple cell types (204, 205), and is known to be

predictive of the long-term survival of T cells into the memory phase (191, 206, 207).

High TCR affinity generally seems to promote commitment to Th1 over Tfh and Th17 lineages. As IL-12 has long-been proposed as a polarizing agent for the selective survival and expansion of Th1 populations (208, 209), it is possible that TCR affinity regulates the ratio of Th cell effectors at the population level by controlling the receptivity to IL-12. In this way the selective expansion of high affinity Th1 populations may mask the the generation of lower affinity Th2 populations (210). Discrepancies regarding the role for TCR signal strength in CD4<sup>+</sup> T cell differentiation may result from differences in how T cell responses are analyzed between different studies (at the population vs. individual cell level). Furthermore, analyses of Th cell responses at a single time-point or in a single location is likely to lead to confounding observations. Future studies need to take an unbiased approach to studying the role of TCR signal strength by tracking the T cell response across multiple time-points, tissues and at both the individual and population levels.

## CONCLUDING REMARKS

T cells must integrate distinct signals to coordinate their proliferation and differentiation. A deeper understanding of the molecular mechanisms underpinning various signaling pathways not only sheds new insights into the biology of TCR signal transduction, but also paves the way for manipulating T cell function for immunotherapy. Future studies will need to delineate the model-dependent, relative contribution of TCR signaling strength and polarizing cytokines to CD4<sup>+</sup> T cell differentiation. As is the case with the regulation of the IL-12R

and commitment to Th1 differentiation, van Panhuys et al. (9) has proposed that TCR signal strength may dominantly control Th cell polarization by regulating the expression of cytokine receptors important for the polarization of other Th subsets. Whilst this is an attractive hypothesis, it may only apply to the generation of some but not all Th cell subsets. For example, strong TCR signaling has been shown to both enhance and suppress the expression of the IL-4 receptor or its transcript (165, 211–213). Furthermore, it would be critical to investigate how TCR signal strength spatiotemporally regulates the T helper cell response. Different lymphoid and non-lymphoid sites vary in their cellular composition and environmental factors. Therefore, it is likely that T cell turnover and differentiation vary across different stages of infection and in different locations.

## AUTHOR CONTRIBUTIONS

NB and CF wrote and edited this manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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