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T Cell Resistance: On the Mechanisms of T Cell Non-activation

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ABSTRACT

Immunological tolerance is a fundamental arm of any functioning immune system. Not only does tolerance mitigate collateral damage from host immune responses, but in doing so permits a robust response sufficient to clear infection as necessary. Yet, despite occupying such a cornerstone, research aiming to unravel the intricacies of tolerance induction is mired by interchangeable and often misused terminologies, with markers and mechanistic pathways that beg the question of redundancy. In this review we aim to define these boarders by providing new perspectives to long-standing theories of tolerance. Given the central role of T cells in enforcing immune cascades, in this review we choose to explore immunological tolerance through the perspective of T cell 'resistance to activation,' to delineate the contexts in which one tolerance mechanism has evolved over the other. By clarifying the important biological markers and cellular players underpinning T cell resistance to activation, we aim to encourage more purposeful and directed research into tolerance and, more-over, potential therapeutic strategies in autoimmune diseases and cancer. The tolerance field is in much need of reclassification and consideration, and in this review, we hope to open that conversation.

Keywords: T cells; Immune Tolerance; Immunotherapy; T-Cell Exhaustion; Senescence; Clonal Anergy

INTRODUCTION

Randomised recombination events that generate TCRs represent a double-edged sword in immunity: whilst they endow the immune response with remarkable flexibility and robustness, they also potentiate debilitating autoimmune diseases. Despite being vetted in the thymus to reduce autoimmune threat, autoreactive T cells remain in the repertoire and such is their nature that, if chronically activated, they can severely damage the host. Thus, tolerance mechanisms that intercept or cease effector functionality of rogue T cell clones are a backbone for a functioning immune system. However, as the field progresses, it is becoming increasingly clear that tolerance is not clear cut for good reason: tolerance mechanisms can and do work together in physiological contexts, and even within these tolerance programmes there are regulatory parameters in place, whether it be cellular (e.g. Tregs, dendritic cells [DCs]), spatial (Ag restriction to thymus and tissue) or simply a game of

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Abbreviations
AICD, activation-induced cell death; APC, Ag presenting cell; Bcl-2, B-cell lymphoma-2; CAR, chimeric Ag receptor; DC, dendritic cell; DISC, death-inducing signal complex; DR, death receptor; EAE, experimental autoimmune encephalomyelitis; GRAIL, gene related to anergy in lymphocytes; HINT1, histidine triad nucleotide binding protein 1; Hsp70, heat shock protein 70; ICAM1, intercellular adhesion molecule 1; IL-7R, IL-7 receptor; IRF-4, interferon regulatory factor 4; IS, immunological synapse; KIR, killer cell immunoglobulin-like receptor; LAG-3, lymphocyte-activation gene 3; LAT, linker for activation of T cells; LFA1, lymphocyte function-associated Ag-1; mTEC, medullary thymic epithelial cell; NO, nitric oxide; NOS, nitric oxide synthase 2; nTreg, natural Treg; PLCγ, phospholipase Cγ; PKC-θ, protein kinase C-θ; pTreg, periphery Treg; RA, rheumatoid arthritis; RIPK, receptor-interacting protein kinases; SLE, Systemic lupus erythematosus; SV, synaptic vesicles; TAM, tumour-associated macrophage; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIL, tumour-infiltrating lymphocyte; TNFR, TNF receptor; tolDC, tolerogenic dendritic cell; ZAP70, zeta-chain-associated protein kinase 70.

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numbers (moderate instead of severe impairment of proliferation) to ensure a robust immune responses can be mounted to pathogen or tumours if required.

Thymic selection provides an early opportunity to observe this paradigm in action. Typically, autoreactive cells are culled as they develop, however it is imperfect and inevitably necessitates peripheral interventions. Evidence suggests 25%–40% of T cells reactive to a single self-epitope escape central tolerance (1). Yet, Yu et al. (2) argue this may be by evolutionary design: a perfectly efficient culling of auto-reactive cells leaves too many 'holes' in the T cell repertoire, lending itself to invasion by opportunistic pathogens. The phenomena of molecular mimicry as a precedent for some autoimmune diseases may provide contextual evidence for this (2). Instead, the risk is offset by mechanisms of peripheral deletion, ignorance, exhaustion and anergy, functioning under the peripheral tolerance umbrella to supress self-destructive T cell activity, even at the expense of chronic infection and cancer progression.

Unsurprisingly, there is considerable overlap in how these states of tolerance are described, and at time warrants reconsideration of how we discuss 'tolerance.' Some tolerance mechanisms share tantalisingly similar phenotypes, making it hard to differentiate them and begging the question of why the immune system evolved several mechanisms that achieve similar results. However, certain contexts reveal the value of one mechanism over another, or how certain combinations can help to offset risks. In addition, the response capacity of T cells can also be influenced by mechanisms beyond classical peripheral tolerance, for example by senescence associated with ageing. There are instances wherein nomenclature can only go so far to describe the shared cellular state across diseases, such as the use of exhaustion to described T cells within chronic infection models, cancer and autoimmune disease, wherein it is still unclear if their cellular programming is indeed the same (3,4). Finally, semantically, we would not consider HIV-1 under a 'tolerance' framework, however its employment of peptide evolution shaped by host immune selection could be considered akin to ignorance, and is not wholly unique (5).

Though it is ever important to refine definitions of tolerance states and mechanisms, we suggest also taking a step back to consider the wider shared feature of 'resistance to activation'. In doing so, we propose how this framework might garner new perspectives as it has done already in improving some therapies. Kondo et al. (6) were able to enforce sharper specificity of chimeric Ag receptor (CAR) T cell responses towards tumour, by coupling the classical activation 'resistance' mechanism of endogenous TCR self-discrimination towards weak Ag alongside the standard CAR-construct. In a similar vein, inclusion of a stimulatory step is widely adopted in tolerogenic DCs (tolDCs) protocols, and logically makes sense in order to ensure utilisation of mature DC characteristics—e.g. migration and peptide-MHC (pMHC) presentation—that allow engagement with T cells. In this context tolDCs are not passive and are partially resistant to activation/maturation.

In this review, we will explore the concept of peripheral T cell tolerance or more broadly T cell resistance to activation. First, we will examine the different nature of external signals driving T cell resistance. Next, we will emphasise the molecular and cellular origins of T cell resistance in the context of T cell (non-)activation, and we will discuss the types of immune cells considered professional inducers of T cell resistance. Finally, we will review the therapeutic potential of the mechanisms of T cell resistance.

EXTERNAL SIGNALS MODULATING PERIPHERAL T CELL ACTIVATION AND DIFFERENTIATION

Types of peripheral T cells

T cell progenitors migrate from the bone marrow to the thymus, where they are subjected to a complex process of maturation and selection for weak recognition of self pMHC, before being released to the periphery as naive T cells. Each naive T cell has a unique TCR that can recognize a degenerate set of peptides on the same pMHC or, in some cases, non-self pMHC (alloreactivity). Once they encounter the relevant Ag presenting cell (APC) or target cell with one of the possible cognate pMHC, the activation process begins. Effector T cells (CD4+, CD8+, Treg cells) are short-lived populations that originate from the expansion and differentiation of activated T cells. Effector T cells carry out specific activities in response to antigenic stimulation and play a key role in steering the immune responses to execute immune functions. Effector T cells can promote, redirect or curtail different types of immune responses. Memory T cells are a long-lived population that survive the contraction phase of the immune response and retain the Ag-specificity. Together, memory T cells and naive T cells prepare the immune system to encounter both previously seen and novel foreign Ags.

Types of extracellular signals integrated during T cell activation

Efficient T cell activation, expansion and differentiation are dependent on exposure to, at least, four types of signals: Ag recognition by the TCR (signal 1), activation of co-stimulatory receptors (signal 2), cytokines (signal 3) and synaptic vesicles (SV; signal 4) (**Fig. 1**). The timing, strength and identity of such signals will determine the fate of the lymphocyte.

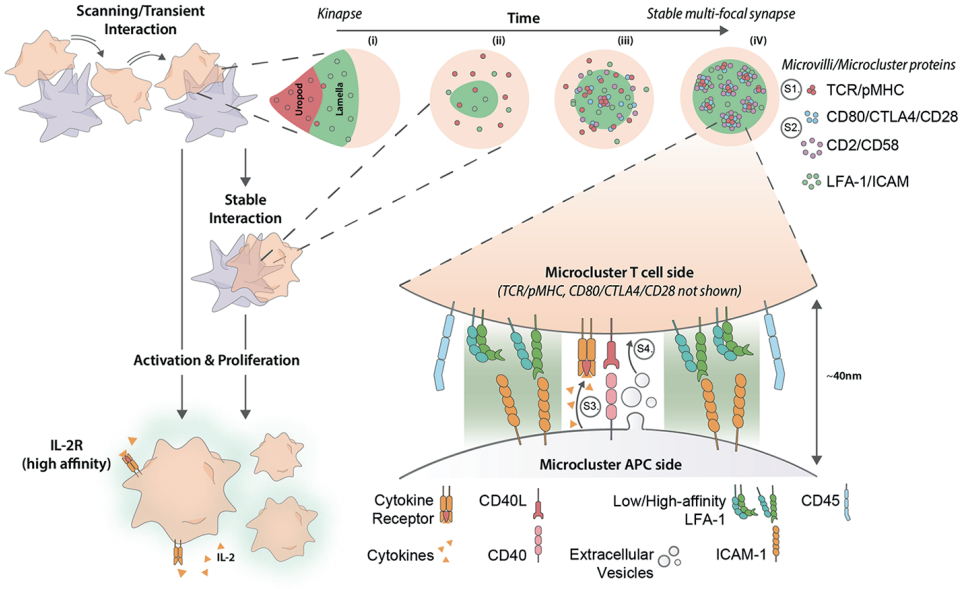

Figure 1. When a T cell (orange) contacts an APC (purple) or target cell, the contact progresses from an early kinapse to a stable immunological synapse. During this contact, distinct types of internal and external signals are integrated to coordinate the process of T cell activation and subsequent differentiation. When a specific antigen is recognized by the TCR S1), the activation is initiated and signalling microclusters, containing the TCR and other co-activator molecules (S2), are immediately formed. The architecture of a multifocal synapse (shown here) is less well characterised than a monofocal synapse wherein microclusters migrate to the centre of the contact. The cytokine milieu (S3) during and after the contact will affect the differentiation of the T cell into effector or memory cell, for example. Release of synaptic vesicles will follow (S4) to mediate the horizontal transfer of biological material between the two cells involved.
S1, signal 1; S2, signal 2; S3, signal 3; S4, signal 4.

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Signal 1: Ag

Recognition of the relevant Ag through the pMHC-TCR interaction in F-actin-dependent protrusions or microclusters initiates T cell activation (7,8). Such interaction is unique and narrows the activation process towards a specific clone of T cell. The TCR is a heterodimer (αβ) that lacks an intracellular signalling domain. It associates with signal transduction subunits CD3ε, γ, δ, and CD247 (also referred to as ζ-chain), which contain intracellular tails bearing include Tyr-based activation motif and other signalling motifs that initiate multiple signalling pathways in response to Ag recognition (9). The specific MHC-TCR interaction is possible in the presence of different types of peptides, with a broad range of affinities. Depending on the strength of the pMHC-TCR interaction, the T cell output can differ.

Signal 2: co-stimulatory receptors

Co-stimulatory receptors are a structurally diverse group of molecules that share the ability to positively modulate TCR signalling and to promote the activation and expansion of the T cell in a manner that is dependent upon concurrent Ag recognition. Co-stimulatory receptors are activated by ligand recognition, which can be another surface molecule on the APC/target cell in *trans* or a ligand expressed on the T cell that can engage the receptor in *cis* (10,11). Down-stream signalling events emerging from co-stimulatory receptors largely overlap with those from the TCR (12) and are often dependent on the expression of the TCR (13). Co-stimulatory signals can be overcome by co-inhibitory signals, which can compete for the ligand and/or counteract the intracellular signalling events (14). Both co-stimulatory and co-inhibitory receptors are strategically reorganised within protrusions/microclusters and the distal supramolecular activation clusters within the immunological synapse (IS) interface to boost or attenuate TCR signalling, respectively (15). The original concept was that signal 1, in the absence of signal 2, can induce a permanent state of anergy, and even death, in the T cell (16,17). Integrin lymphocyte function-associated Ag-1 (LFA1) interaction with intercellular adhesion molecule 1 (ICAM1) can also induce co-stimulatory signals. Even though LFA1-ICAM1 interaction is spatially segregated from the TCR-pMHC interaction, LFA1 function is stimulated by the TCR, and integrin signalling is highly correlated with TCR signalling (8). However, analysis of steady state DCs that induced anergy or deletion of T cells reveals that they present intermediate levels of co-stimulatory ligands like CD86 and ICAM1, suggesting a more complex picture (18).

Signal 3: cytokines

Combination of signal 1 (Ag) and signal 2 (co-stimulation) are enough to initiate the clonal expansion of the relevant T cell subset. However, T cells require the presence of additional cytokines (signal 3) to properly orchestrate their differentiation and to achieve optimal effector or memory functions (19). These signals can include soluble ligands, like IL-2, or membrane anchored or trans-presented cytokines, like 4-1BB and IL-15. Signal 3 output depends on the composition of the cytokine milieu during T cell expansion and on the timing of exposure. For example, strong signal 3 before the signal 1 and 2, can lead to a reversible state of unresponsiveness to the Ag (16), while absence of signal 3 can irreversibly impair the effector function of primed cells (20). Beyond the temporal sequence of signal delivery, the spatial relationship between signals can also influence T cell outcomes. For example, simultaneous presentation of IL-2 and Ag at the IS—both spatially and temporally—can markedly enhance T cell responses to both signals. This highlights that the spatial context, in addition to the timing, of signal presentation is crucial for optimizing T cell activation and function (21).

Signal 4: SV

Recently, a fourth signal has been proposed. During the immune synapse, there is a bidirectional release of extracellular vesicles, known as SV, which transfer information that can modulate and mediate the function of the T cell. SV delivered by the T cell towards the synaptic cleft have a unique molecular composition that includes specific immune receptors, miRNA and RNA-associated proteins (22), mediating the horizontal transfer of biological material between neighbouring T cells or T cells and APC/target cells.

In addition to these four types of signals, factors regulating T cell metabolism also assume a fundamental role in influencing T cell activation, representing a significant aspect rather than the conventional notion of being another T cell activation signal. Hongbo Chi's proposition regarding the involvement of metabolism in T cell activation has gained substantial recognition and support in recent years (23). It is now widely acknowledged that metabolic reprogramming is crucial following the initial signals of Ag recognition, co-stimulation, and cytokine signalling. Metabolic cues are instrumental in shaping the trajectory of T cell responses. It plays a crucial role in determining the fate of T cells, whether they undergo activation, exhaustion, senescence, or other functional states (24).

INTRINSIC MECHANISMS OF T CELL RESISTANCE

We introduce the concept of T cell resistance to activation to define a heterogeneous group of responses observed in T cells that oppose (or resist) Ag-induced activation. We will discuss different types of resistance mechanisms based on their molecular and cellular origins and their (patho-)physiological implications. We will focus first on intrinsic mechanisms within the T cell, but we acknowledge up front that these operate in tandem with extrinsic processes that we will discuss in section 3.

Ignorance

T cell ignorance is one of the means of peripheral T cell tolerance dependent upon resistance to activation, where T cells do not appear to notice or be affected in any way by the relevant autoAgs and is more likely to occur when there is no thymic expression of the Ag.

Naive T cell sensitivity to self pMHC is attenuated following thymic development. For example, differentiation from single positive thymocyte to naive T cell is accompanied by down-regulation of miR-181a, which results in upregulation of a multiple negative regulators of signalling that reduce the naive T cell's sensitivity to pMHC (25). Non-response of naive T cells in response to self-pMHC is further favoured by the following factors: low Ag expression, low affinity between TCR and pMHC and low affinity between peptide and MHC. When ignorance is operative, naive autoreactive T cells ignore islet Ags and recirculate without causing damage unless activated by an external stimulus. However, the discriminatory power of the TCR is imperfect and T cells can respond to very low affinity pMHC, which explains why self-Ags can sometimes trigger autoimmune reactions (26). Additionally, a subset of naive T cells, such as those expressing high levels of CD5 or low levels of Ly6C, has been shown to retain higher self-reactivity in the periphery (27,28). These cells, upon stimulation, are more prone to Foxp3 expression and Treg differentiation (28,29), suggesting an alternative tolerance mechanism beyond ignorance. Thus, while ignorance typically prevents activation by self-Ags, these examples show that self-reactive naive T cells may take on a regulatory role, contributing to peripheral tolerance through Treg induction.

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Studies have shown that the failure of T cell ignorance leads to multiple autoimmune diseases like type 1 diabetes and systemic lupus erythematosus (SLE) (30). However, few studies in the late 90s report that immunological ignorance aids in tumour evasion due to the failure of immune surveillance against cancer. Linette et al. (31), 2019 show that immunological ignorance of clonal neoantigens leads to ineffective T cell immunity against melanoma.

Interestingly, Kurts et al. (32), 1999 showed that the states of T cell tolerance and ignorance were determined by the concentration of the Ag. In fact, the concept of ignorance was derived from the existence of rare diseases where physical damages to an organ result in changed Ag availability and an autoimmune state (30). Ignorance might engender a unique state in naive T cells, reminiscent of the concepts proposed by ElTanbouly and Noelle (17). Throughout the early naive T cell phase, quiescence and ignorance actively contribute to maintaining tolerance. In circumstances where co-stimulation is lacking, anergy assumes a central role, rendering T cells unresponsive. As T cells progress to the effector stage through successful stimulation, exhaustion and senescence step in, mitigating excessive inflammation and preventing potential immunopathological consequences.

Quiescence

T cell quiescence is a reversible state of indolence in which the T cell is not proliferating but maintains the potential to quickly enter the cell cycle. It is defined by small cell size with limited cytoplasmic volume, low rates of cell metabolism, transcription, and translation activities (33). A majority of the lifetime of peripheral T lymphocytes is spent in quiescence. Quiescence allows cells to remain non-dividing for long periods of time while also enacting mechanisms to defend themselves from injury (34).

Cells must precisely govern their entry into quiescence, maintenance of this phase and their exit from quiescence to ensure a reversible state of arrest and thus necessitate the activation of key cell-cycle regulators, which in turn respond to extracellular and intracellular signals as well as inputs from upstream factors (34). Quiescence is promoted by cyclin-dependent kinase inhibitors, and quiescent cells often have high quantities of these proteins. As a result, a cell's decision to enter or exit quiescence is influenced by a number of cell cycle and transcriptional factors. In 2018, Newton et al. (35) showed that FOXO1 has a critical role in regulating specialized lymphocyte functions and maintaining T cell quiescence. In this study, by sustaining FOXO1 activity beyond normal cell activation, the authors observed disruptions in the homeostasis of CD4 conventional and regulatory T cells. While continuous FOXO1 activity led to increased activation of Akt kinase and an intrinsic proliferative advantage, it also resulted in decreased survival and cell division under competitive conditions or limited growth-factor availability (35). In 2014, Miller et al. (36) revealed that the survival of quiescent T cells is, in part, reliant on the interaction between the soluble factor IL-7, produced by various stromal cells, and the IL-7 receptor (IL-7R) present on the surface of T cells. This study demonstrated that naive T cells possess a basal nuclear level of the NF-κB transcription factor, which played a pivotal role in maintaining IL-7R expression, ensuring their survival (36).

The active maintenance of a quiescent state in naive T lymphocytes increases their survival and persistence. During quiescence exit, T cells rapidly acquire biomass which necessitates an increase in amino acid, lipid, and cholesterol biosynthesis and uptake from their surrounding via glucose and amino acid transporters in order to allow for increased protein and membrane synthesis (37,38). Ag and co-stimulatory receptor engagement cause T cells to exit quiescence, permitting clonal proliferation and functional differentiation, both of which

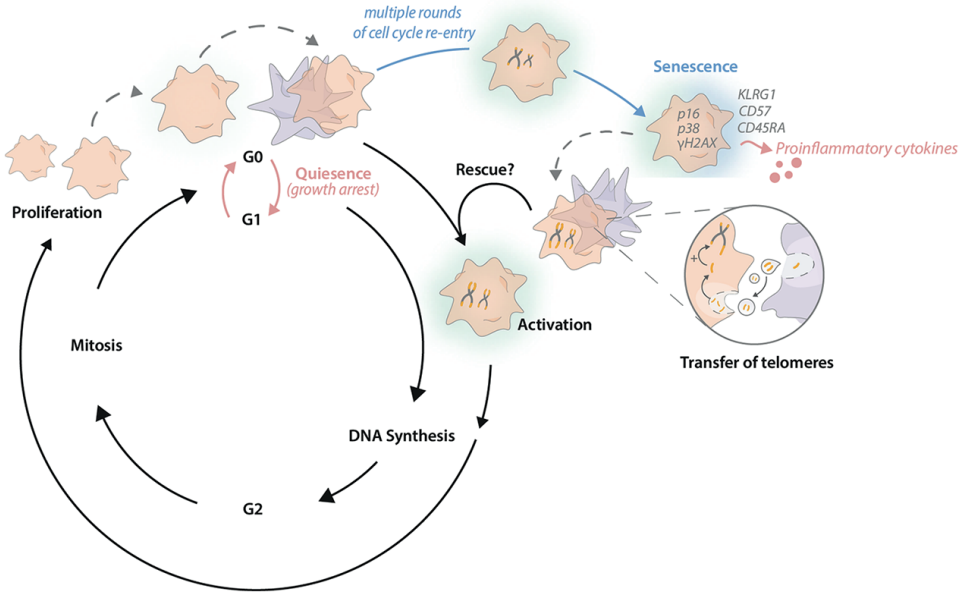

Figure 2. Cartoon representing the cell cycle phases and a T cell (orange) exiting quiescence after being activated by an effective contact with an APC (purple). T cells will enter the cell cycle each time they recognize an antigen in order to expand and respond. T cells of old individuals will, after multiple rounds of cell-cycle entry, naturally enter a permanent state of senescence that will impair their activation potential. Shortening of telomeres has been suggested to drive senescence. APCs can transfer telomeres within vesicles across the immunological synapse to rescue a T cell from senescence.

are necessary for generating an appropriate immune response (**Fig. 2**). Extensive alteration of cellular morphology and metabolism is connected with the prompt exit from quiescence, which occurs before activation-induced proliferation (39).

Adoptive T cell and checkpoint blockade therapies are progressively gaining traction as effective cancer therapies. The efficacy of these therapies could be improved by altering the quiescence exit hallmarks. The anticancer responses of adoptively transplanted CD8+ T cells are improved by enforcing phosphoenolpyruvate production or oxidative phosphorylation, suggesting that altering the metabolic signatures of quiescence exit can improve cancer adoptive T cell treatments. Checkpoint blockade medicines targeting PD-1 and CTLA-4 can also improve endogenous T cell accumulation and activity in the tumour microenvironment (40).

Anergy

Anergy is commonly defined within two frameworks, either: *in vitro*, sometimes referred to as 'clonal anergy,' wherein anergy accompanies some retained effector cellular function; or that induced *in vivo*, termed 'adaptive tolerance,' and associated with a complete loss of effector functionality (41). In the absence of co-stimulation, clonal anergic T cells *in vitro* can be rescued from their refractory state, or anergy staved off altogether with exogenous IL-2. But this is not possible *in vivo* (42). Equally, cells anergised *in vivo* can be rescued and slowly regain effector function in the absence of persistent Ag exposure, otherwise not observed *in vitro* (43). Thus, continual Ag exposure appears necessary for regulating anergic states *in vivo* (43).

Much research has identified candidate transcriptional inducers and mediators of anergy, and their activating pathways. More challenging has been identifying any consistent surface molecules involved. Expression of ligands commonly associated with anergic pathways, such as PD-L1, do not strictly define tolerogenic capacity, neither is lack of co-stimulation a pre-

requisite (44,45). These suggest other parameters, independent of ligand specificity, might equally regulate anergy induction.

For example, nuclear export of the transcription factor NFAT has been reported to be slower than its transcription partner AP-1 (46). Whilst NFAT is heavily implicated in anergy, it preferentially forms high-affinity complexes with AP-1 upon sufficient signalling to trigger typical effector genes (47). Thus, in the absence of sufficient signalling, it is possible that the residual pool of NFAT begins to self-associate into low affinity homodimers that target so called 'anergy associated genes' (47). Therefore, parameters such as cell-cell contact duration alongside synapse maintenance and organisation etc. could also affect NFAT accumulation, particularly if considered within threshold or 'summing' models of T cell activation (48). Such parameters might better dictate activation or anergy, and crucially emphasise less the necessity of a cell to express certain receptors to be characterised as activating or tolerogenic (44).

Research already suggests the DC maturation state encourages formation of a stable synapses and thus parameters such as contact duration and stability between a T cell and APC may predilect activation over tolerance induction (49,50). Interestingly, when primary human naïve and memory CD8 and CD4 T cells were observed interacting with a 2D stimulatory surface only human CD8+ memory T cells preferred formation of stable synapses. All other subsets formed motile, asymmetric contacts termed kinapses, which, nonetheless led to equal duration of contact between T cells and Ag presenting cites (51). This supports a consensus that prolonged contact tends towards T cell activation, regardless of contact phenotype, i.e. synapse vs. kinapse, provided there is sufficient recruitment of signalling machinery (52).

Kinapses have also been associated with anergic states. Murine CD4+ T cell blasts that had been treated with ionomycin to induce a NFAT dependent anergy programme formed kinapses, whereas non-anergized counterparts formed stable synapses over the same time frame. Kinapses can be a signature of a T cell's ongoing search for pMHC to engage the TCR, or in the case of anergic T cells, the impact of degrading signalling proteins that are required to sustain TCR signalling and a stable synapse or productive kinapse. In ionomycin induced anergy, a number of key signalling proteins are targeted for degradation by E3 ubiquitin ligases, including phospholipase Cγ (PLCγ), linker for activation of T cells (LAT), protein kinase C-θ (PKC-θ), and zeta-chain-associated protein kinase 70 (ZAP70) (53). PLCγ, LAT, and ZAP70 are all required for cytoplasmic Ca^{2+} increases associated with stable synapse formation. Equally, lipid rafts that coalesce and localise these signalling molecules in the central SMAC appear dysfunctional in orally tolerised cells (54). Expression of E3 ubiquitin ligases such as c-Cbl and Cbl-b have also been observed at the c-SMAC of some anergic cells and thus may also maintain their hyporesponsive state (55). The interpretation of the kinapse mode of interaction must be considered in a functional context as kinapses can be part of a mitogenic/activatory programme, or anergy.

A passive model of anergy induction could be under steady-state conditions wherein anergy is induced by many transient unstable interactions with homeostatic/spontaneously matured immature DCs that present low levels of innocuous Ag (56,57). It would be interesting to explore what contact tolDCs—DCs which are semi-mature and 'professionally' tolerogenic—form with T cells. Given their semi-mature state, they may *actively* promote instability, perhaps through PD-L1 mediated inhibition of co-stimulation and productive TCR signalling. Equally, as they present lower levels of MHC, they may encourage more durable, stable

contacts through other means such as using their own integrins to hold the T cells and sufficiently deliver partial signals leading to anergy. So far few or no studies have investigated the tolDC synapse, most likely because of the complexity of analysing T-DC synapses in general and that there is a lack of consensus on how to generate tolDC *ex vivo* (see tolDC section for more information).

Exhaustion

Exhausted T cells are a heterogeneous population that shows attenuated (but not absent) function in the context of persistent Ag exposure. This prevents excessive tissue damage and immunopathology in response to chronic viral infections and cancer. However, mechanisms leading to T cell exhaustion can be exploited by viral-infected cells or cancer cells to escape immune responses (58).

Exhausted T cells survive the contraction phase and are thought to originate from a progenitor exhausted population, with self-renewal capacity (T-cell factor; TCF^+), that is transcriptionally and epigenetically different from the effector and memory populations. Progenitor exhausted T cells progressively lose proliferative and re-activation potential, to become a terminally differentiated exhausted population (TCF^-). Effector function is not completely lost but reduced. Terminally differentiated exhausted populations co-express effector and inhibitory receptors, such as granzyme B and PD-1, respectively (58).

Mechanisms mediating exhaustion are not fully understood but involve the integration of different cellular and molecular inputs (**Fig. 3**). Repeated TCR activation by chronic Ag exposure is a key aspect for the initiation of the exhausted programme and TCR-dependent pathways, like NFAT, are involved in the exhausted phenotype (59,60). Interestingly, forming a signalling axis with NFAT, the NR4A subfamily of orphan nuclear receptors have been implicated in T cell exhaustion and anergy by impeding AP-1 signalling – thus perhaps

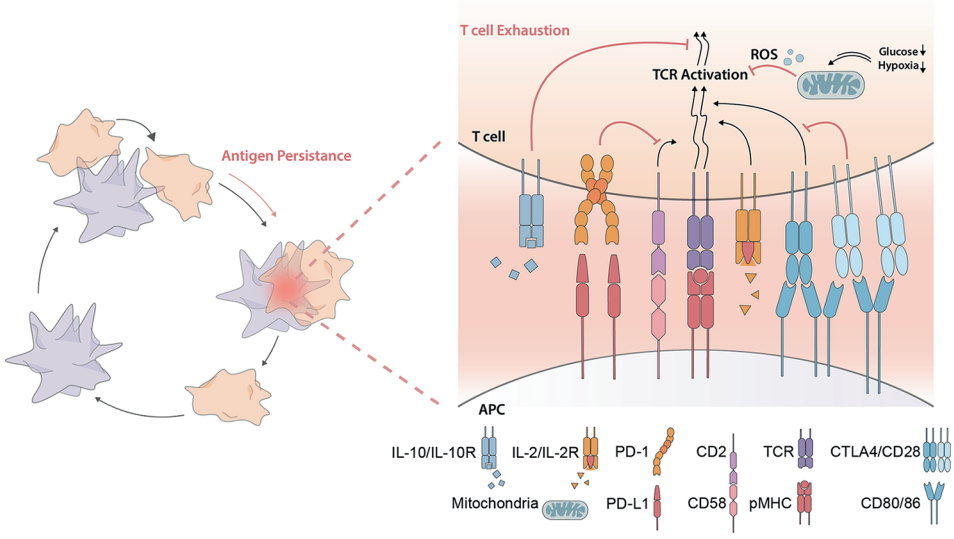

Figure 3. Cartoon representing the molecular mechanisms involved in exhaustion of T cells after repeated exposure to an antigen. In the context of antigen persistence, several co-inhibitory receptors, like PD-1 or CTLA4, counteract TCR signalling pathways. Other mechanisms driving T cell exhaustion involve the cytokine milieu. For example, while IL-2 can potentiate TCR signalling to reverse exhaustion, IL-10 impairs it. Exhaustion is also intimately linked to the metabolic state of the T cell. Hypoxia or low glucose availability, usually related to the tumour microenvironment, can dramatically impair mitochondrial function and therefore, T cell activation.

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promoting NFAT dimerization (see Anergy section) – and aiding expression of inhibitory receptors such as PD-1, T-cell immunoglobulin and mucin-domain containing-3 (TIM3) and c-Cbl. Expression of the NR4A subfamily receptors are immediate following TCR mediated signals, suggesting the possibility of an early negative feedback that is intrinsic to all TCR signalling events setting the stage for anergy in the absence of costimuation, or exhaustion programmes in the face of chronic TCR signalling (61).

Activation of co-inhibitory receptors, concomitantly to TCR activation, also have a central role in driving exhaustion. PD-1 forms a non-covalent dimer on T cells and is highly expressed in exhausted T cells (62). PD-1 is activated by ligand binding with PD-L1 or PD-L2, which also can form non-covalent dimers through the transmembrane domains. Phosphorylation of the PD-1 intracellular tail leads to the recruitment and activation of phosphatases that counteract the TCR downstream signalling, thereby suppressing T cell activation and function (63). Similarly, other co-inhibitory receptors are highly expressed in exhausted T cells, like CTLA-4, lymphocyte-activation gene 3 (LAG-3), and the TIM3 (64).

CTLA-4 and PD-1 pathways are non-redundant checkpoints for T cell activation, and their blockade has been at the frontline of current anti-cancer therapies (65). CTLA-4 out-competes with CD28 for the binding to CD80, preventing the CD28-dependent co-stimulation required for a proper T cell activation and leading the cell to a state of anergy. CTLA-4 can also prevent the formation of a stable contact between the T cell and the APC (66) or directly inhibit TCR-dependent pathways (67).

The cytokine milieu is also an important player driving T cell exhaustion. For example, IL-10 produced during chronic infections promotes exhaustion, and simultaneous blockade of IL-10 and PD-1 improves reversion of exhaustion (68). On the contrary, IL-2 treatment can improve virus control by CD8+ T cells, and combination of IL-2 treatment with PD-1 blockade strongly reversed exhaustion during viral infections (69).

TCR-dependent pathways can also be integrated with metabolic pathways to induce the exhausted phenotype (70). For example, repeated activation of the TCR under hypoxic conditions (like the tumour microenvironment) inhibits mitochondrial adaptations to hypoxia, causing a dramatic increase in the ROS production that rapidly promotes exhaustion of $CD8^+$ (71,72). The simultaneous deprivation of oxygen and glucose in the tumour microenvironment is also a key inducer of mitochondrial dysfunction and ROS production that can lead to exhaustion of cytotoxic lymphocytes. In addition, PD-1 signalling can also favour the accumulation of depolarized mitochondria, enhancing the process (70).

Senescence

Ageing is a natural biological process that occurs due to the accumulation of a wide variety of molecular and cellular damage over time and results in the decline of physiological functions. The immune system also undergoes age-related changes called immunosenescence, which results in the deterioration of the immune response. Immunosenescence affects both innate and adaptive immunity and is one of the major reasons for increased susceptibility of aged individuals to infections and diseases (73,74).

T cells near the end of their lifespan become senescent i.e., undergo cell-cycle arrest while staying viable and metabolically active (**Fig. 2**). Senescent T cells have also been described as those cells which do not proliferate in response to TCR stimulation, produce inflammatory

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cytokines and increase in number during ageing (75,76). Immunosenescence is marked by a gradual decline in the efficiency of the adaptive immune system, particularly in B and T cells, might contribute to inflammaging. As individuals age, their reliance on the innate immune system increases, which can trigger a chronic low-grade inflammatory response. This shift in the balance of the immune system dynamics can give rise to persistent, low-level inflammation. Additionally, the chronic and mild inflammation associated with inflammaging can have detrimental effects on the immune system. It can lead to the exhaustion of immune cells, a decrease in their functionality and an increased production of proinflammatory cytokines. This inflammatory environment can expedite the aging of immune cells, thereby exacerbating immunosenescence (77,78). Also, studies have shown that senescent T cells acquire characteristics of NK cells (79,80).

Many cellular mechanisms have been shown to trigger T cell senescence. Replicative senescence is a process that occurs as a result of many rounds of replication leading to shortened telomeric length and subsequent senescent state to prevent its potential progression toward malignancy (76). Premature senescence is a telomere-independent mechanism, which is induced by external factors like cellular stress (81). Involution of thymus has been described as one of the causes of immunosenescence. The functional tissues of the thymus get replaced with fat soon after birth (82). Subsequently, the output of newly generated naive T cells is greatly reduced in the elderly contributing to immunosenescence.

T cell surface markers are known to act as guides throughout the differentiation journey of T cells. Likewise, some of the surface markers act as hallmarks of senescent T cells. Senescent T cells lose the cell surface stimulatory molecules such as CD27 and CD28 while they acquire killer cell lectin like receptor G1 and CD57 (83). These cells also express senescence related molecules such as Atm, phosphorylated histone H2AX, the cyclin inhibitor p16, sestrins, and p38 mitogen-activated protein kinase (84). High level of expression of senescence related β-galactosidase has been observed in senescent T cells (85). Re-expression of CD45RA can occur in senescent T cells (76). Although these cells have lost their proliferative capacity, they have potent cytotoxic activity (79,84).

Senescent T cells have been implicated in chronic viral infections, autoimmune disorders and cancer (76). Evidence suggests that malignant tumours evade immune system by using T cell senescence as one of the strategies. Thus, the role of signalling network of senescent T cells in tumour microenvironment has paved way for its use as prognostic biomarkers in several cancers (86). Evidence also suggest that senescent T cells are involved in the pathogenesis of various inflammatory conditions, cardiovascular diseases such as atherosclerosis, acute coronary syndrome and essential hypertension (87). Also, the possibility of preventing senescence in tumour-specific T cells makes these senescent T cells potential therapeutic targets. In fact, it has been recently shown that APC can transfer telomeres to the T cell during the IS, favouring the lifespan of the Ag-specific T cells (88). This represents a new telomerase-independent mechanism to prevent T cell senescence in the context of normal immune responses through a process initiated by terminally differentiated APCs. Hence, further exploration of characteristics and roles of senescent T cells can help in using these cells as potential therapeutic targets in many diseases.

Cell death

Cell death is a key regulator of immune homeostasis, allowing processes such as the controlled termination of adaptive immune responses once the pathogen is cleared or

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the pruning of the T cell repertoires by negative selection during development. There are different mechanisms that can lead to a controlled T cell death. These include extrinsic and intrinsic apoptosis, necroptosis and ferroptosis, among others.

Apoptosis is executed by the serial activation of several caspases, a type of cysteine-aspartic proteases, which cleave cellular components and irreversibly cause cell death. This process can be initiated intrinsically by the cell itself or extrinsically by a surrounding cell.

Intrinsic apoptosis involves the formation of pores at the outer mitochondrial membrane, which allow the release of cytochrome C into the cytosol. This induces the assembly of the apoptosome and initiates the cascade of caspase activation. Perforation of the mitochondrial membrane is regulated by the B-cell lymphoma-2 (Bcl-2) protein family. Members of the Bcl-2 family can be classified in three groups: the pro-apoptotic Bax and Bak members (i), which form the pores that permeabilize the outer mitochondrial membrane; the pro-survival Bcl-2 members (ii), which inhibit the pro-apoptotic members of the family by direct interaction; and the BH3-only proteins (iii), which sense the state of the cell and initiate apoptosis by directly activating Bax and Bak or by repressing the pro-survival Bcl-2 members (89). Initiation of the intrinsic apoptotic pathway is regulated by the balance between pro- and anti-apoptotic Bcl-2 proteins expressed in the T cell. For example, during negative selection in the thymus, too strong TCR signalling increases expression of Bim, a pro-apoptotic BH3-only protein (90). Bim expression is also crucial for the apoptosis of activated CD8+ T cells after an acute or chronic viral infection (91,92) or during cytokine withdrawal-induced cell death (93). On the contrary, homeostatic signals, like IL-7, can upregulate members of the pro-survival Bcl-2 family, thus protecting from apoptosis (94,95).

Extrinsic apoptosis is initiated by the activation of a death receptor (DR) by ligand recognition. DRs are members of the TNF receptor (TNFR) superfamily that contain a cytoplasmic death domain. When the corresponding ligand binds to the DR, adaptors and procaspases are recruited to the cytosolic tails of the DR to form the death-inducing signal complex (DISC). Assembly of DISC initiates the cascade of caspase activation. The extrinsic apoptotic pathway is important during activation-induced cell death (AICD), a process that leads to apoptosis following TCR activation. AICD allows for an efficient termination of the immune response, but it is also important for the removal of autoreactive T cells, which are activated without proper co-stimulatory signals. For this reason, DRs, like FAS or TNFR, have been linked to various autoimmune disorders (96).

Apoptosis is a non-immunogenic mode of cell death as tissue cells, including macrophages, can phagocytose the corpses of apoptotic cells without activating innate immunity, consistent with it being a normal developmental and homeostatic process.

Necroptosis is a type of regulated necrosis that occurs under caspase-inhibitory conditions and is dependent on the activity of receptor-interacting protein kinases (RIPK). In T cells, necroptosis can be triggered by activation of DRs when caspase 8, the initiator caspase of the extrinsic apoptotic pathway, is absent or inhibited. In this context, RIPK can aggregate to form the necrosome, which phosphorylates and activates mixed lineage kinase domain-like protein, the main executor of necroptosis that eventually leads to the disruption of the plasma membrane through gasdermin-mediated pores (97,98). Necroptosis is considered immunogenic in that signals released lead to DC maturation and amplification of the immune response. However, T cell necroptosis is also linked to the pathology of some viral infections, like HIV (99).

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Ferroptosis, is a type of programmed cell death induced by iron-dependent lipid peroxidation that causes an irreversible damage to cellular membranes, including the plasma membrane. Iron is at the core of many metabolic processes involved in ROS production. Ferroptosis is observed in resting T cells, making it a mechanism of TCR-independent cell death (100). Due to membrane damage and release of damage associated molecular patterns, ferroptosis is immunogenic.

EXTRINSIC MEDIATORS OF T CELL RESISTANCE

T cells can intrinsically promote their resistance to activation as part of their physiological (non-)response to the Ag. However, these intrinsic processes are calibrated to work with APCs and Tregs, that set levels for signals 1–4. The main APC for T cells are DCs, which exist in different states including operationally defined tolDC. In this section we will also discuss other immune cells involved in the control of T cell resistance to activation.

TolDCs

DCs bridge innate and adaptive responses through their ability to capture and present Ags to T cells, driving their differentiation for a tailored immune response. Naturally, this places DCs in a powerful position to induce T cell resistance to control the immune response.

Previous dogma characterised immature DCs as tolerogenic and mature DCs as immunogenic. In this context, maturation accompanies upregulation of MHC, co-stimulatory receptors, cytokines, and extracellular vesicles that can overcome T cell resistance to activation (101). However, immature DCs might better be seen as non-stimulatory as they lack surface MHC II expression needed for any CD4+ T cell functional engagement. Experimentally, immature DC are inherently susceptible to maturation stimuli that are difficult to control in cell culture conditions, such that immature DC are a transient state *in vitro* that rapidly became contaminated with semi-mature and mature DC over the course of experiments with T cells. In fact, T cells produce CD40L which leads to DC maturation. Over the past decade, active approaches to generate stable and expertly tolDCs have been developed *ex vivo* as a potential therapeutic for autoimmune disease (102). But while anti-inflammatory and tolDC signatures have been identified from bulk and scRNA seq analysis *in vivo* (103,104), these are provisional, and many aspects are poorly characterised. Though regulatory DC subsets have been identified *in vivo*, their ontogeny is unknown (105). Indeed, physiologically, whether tolDCs even represent a stable lineage, as opposed to a transient maturation state, is far from certain (106).

A leading challenge to understanding what constitutes a tolDC is consolidating any functional signature from the myriad of protocols now published to generate tolDC *in vitro and ex vivo* (107). Many tolDC phenotypes have been described, often exhibiting capacity for one or few tolerance mechanisms. Nonetheless, recent attempts revealed elements of a transcriptomic signature and confirm an active transcriptional programme that differentiates them from immature and mature DCs (108).

This still leaves much up to speculation, but there is some consensus on what could constitute a tolDC. Ag-specific tolerance mediated by DCs can be abrogated upon stimulation with anti-CD40 Ab, confirming their semi-mature status as a useful identifier and even crucial to their functionality (109). A higher ratio of inhibitory to co-stimulatory receptors (e.g high PD-1 and CTLA-4) is also expected, as well as a similar imbalance towards soluble inhibitors such as

indoleamine-pyrrole 2,3-dioxygenase and IL-10 (109). TolDCs are also thought to delete cells from the repertoire through expression of FASL and/or TNF-related apoptosis-inducing ligand, and both are attractive targets to enhance tolDC generation *ex vivo* (110).

Though therapy with tolDCs is promising, the methodology to administrate tolDCs generated *ex vivo* raises significant challenges regarding their stability and migratory capacity (see T cell resistance as a target for immunotherapy section). This has prompted strategies to stabilise or induce tolDCs *in situ*. Early pioneering animal studies into tolerance targeted the DEC-205 receptor of DCs with Ag-conjugated Abs. In the absence of adjuvants, targeting DCs this way established peripheral tolerance, likely mediated by IL-10 producing Ag-specific Treg populations (111). These early studies suggested the capacity to generate tolDCs *in situ* which then propagate tolerance through their close relationship with Tregs (see Tregs section). More recent efforts in the clinical field have utilised bio-degradable nanoparticles that encapsulate Ag and an immunomodulatory agent, such as rapamycin, already validated to generate tolDCs *ex vivo* (112). Notably, the concomitant delivery of Ag and rapamycin within a particle appears crucial, supporting a central role of APCs, most likely DCs, which phagocytose the particle to undergo tolerogenic re-programming. Again, tolDCs were thought to propagate FOXP3+Ag-specific Tregs to establish a tolerance that was durable against inflammatory stimuli (112,113).

Tregs

As discussed, much evidence supports the heavy interlinking functionality of tolDCs and Treg activity, as both induce differentiation of the other in some models and contexts, and Tregs are potent mediators of immune suppression and tolerance in their own right (114,115). However, much like the current state of tolDC research, diverse suppressive mechanisms and lack of consistent surface markers have hampered further characterisation of Tregs beyond the initial classical markers defined in the early 2000s.

Today, CD4+ Tregs are frequently characterised by expression of CD25 (IL-2Rα) and a reliance on IL-2, which maintains their regulatory state, and intracellular expression of FOXP3 which drives their induction and correlates negatively with CD127. Thus, they are often defined as $CD3^{+}CD4^{+}CD25^{hi}Foxp3^{+}CD127^{lo}$. Further surface markers to differentiate Tregs induced in the periphery (pTregs) or in the thymus (natural or nTregs) during negative selection are not yet known, however expression of Helios (116) across species and neuropilin-1 in mice (117) may identify the former. FOXP3 stability may also differentiate subtypes. pTregs exhibit unstable expression of FOXP3, and reports suggest proinflammatory autoimmune conditions may trigger loss of FOXP3 and trans-differentiation into pathogenic Th-17-like cells termed exFOXP3 Th-17 (118). Strong methylation of the Conserved Noncoding Sequence 2 (CNS2, also termed Treg-specific demethylated region or TSDR) enhancer locus of FOXP3 may confer this instability in pTregs, whereas the CNS2 locus is demethylated in nTregs and ensures stable FOXP3 expression (119). In fact, whilst FOXP3 expression is necessary, it appears not sufficient to define a stable Treg lineage, and instead hypomethylation patterns within the FOXP3 locus are a more stringent characteristic (120). Overall, it is generally believed that nTregs predominately confer tolerance against restricted autoantigen, but can do so in both the thymus and periphery, whilst pTregs generally recognise foreign Ag (121).

The suppressive action of Tregs appears integrated with their heterogeneity. In mice, for each CD4+ effector population there are reports of a 'sister'—Treg population that expresses FOXP3 alongside effector transcription factors (122). Tregs expressing T-bet, that otherwise

defines the Th-1 lineage, selectively suppress these effector cells (123). Similarly, IFN regulatory factor 4 (IRF-4) expression, whilst essential for Th-2 generation, is expressed highly in Tregs with enhanced suppression of Th-2 cells (124), and there are similar reports of Treg expression of retinoic acid-related orphan receptor gamma t and STAT3 for Th-17 (125) and BCL-6 and T-follicular helper (126,127) cell suppression. Together, this paradigm suggests some Tregs adapt to their cytokine milieu to acquire specific suppressive functionality, responding to IFN-γ to express T-bet for example, ensuring suppression of a Th-1 response. Such targeted suppression is likely through tailored mechanisms: Tbet+ Tregs express T-cell immunoreceptor with Ig and ITIM domains (TIGIT) which in turn induces DCs via CD155 to express IL-10 and downregulate IL-12 (128). Similarly, IRF-4 induces expression of RBJ transcription factors alongside inducible T-cell costimulator and CTLA-4, all implicated in Th-2 specific suppression (129).

Concurrent expression of FOXP3 with effector transcription factors appears paradoxical yet may be integral to their functionality. Interestingly, human Tregs exposed to only strong—not weak—TCR and APC signals undergo transient loss of regulatory capacity and instead contribute to an IL-17-driven inflammatory response in co-cultures (130). If applicable *in vivo*, this has been suggested to ensure a sufficient inflammatory response before contraction (130). Of note, however, Tregs in arthritic mice have been reported to lose FOXP3 expression and differentiate into a Th17-like phenotype, thus potentially exacerbating disease. Indeed, the pTreg and Th17 lineage are inherently connected by TGF-β mediation and as discussed, FOXP3 expression is considered unstable in pTreg populations (131).

Similar to tolDCs, Tregs express an arsenal of co-inhibitory receptors including CTLA4 (132), PD-1 (133), LAG-3 (134), herpesvirus entry mediator (135), and TIGIT (128) which sustain and enhance their many and varied suppressive activities. Characterising the Treg synapse has been challenging because systems by which to study the synapse vary substantially across studies. Unstable cell-cell synapses have been reported in Ag-specific systems between human Tregs and cognate APCs (136) and mouse Tregs with DCs (137). However elsewhere, anti-CD3 and ICAM-1 loaded bilayers confer stable synapses with human Tregs with a notable dislocation of PKC-θ away from the contact, which is otherwise a key component of the signalling machinery in effector T cell synapses (138).

Interestingly, within lymph nodes, Tregs appear to form stable aggregates around DCs, greatly inhibiting their contact with naive cells (139,140). Such Treg synapses exhibit constitutive recruitment of CTLA-4, but not CD28, compared with other cell types (141), which in turn outcompetes CD28 binding from naive cells further limiting their activation (140). Treg can also use CTLA-4 to down-regulate CD80/86 on APCs through a capture mechanism of trans-endocytosis, also employed to downregulate cognate-pMHC-II in a CTLA-4 independent manner (142,143). As a result, DCs with reduced CD80/86 expression form weaker and fewer contacts with non-Treg cells (144). Indeed, anti-CTLA-4 Ab *in vivo* abrogates Treg contacts with DC, permitting formation of stable DC-effector cell contact that induce proliferation (145). Such 'hyper-stable' Treg-DC synapses have been reported elsewhere, wherein Tregs appear to sequester fascin-1, an actin bundling protein of the cytoskeleton, to their immediate contact with DCs (146). DCs periodically regulate components of their actin cytoskeleton to optimally contact and activate many T cells within a given time (147). In this instance, sequestration of fascin-1 to the Treg contact appears to restrict LFA-1 mobility leading to a hyper-stable Treg-DC synapse, but an in-ability of DCs to adhere sufficiently to new target cells, leading to reduced priming capacity of the Treg engaged DC (146).

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Finally, as touched upon earlier, clustering and recruitment of PKC-θ and other kinases, crucial for IL-2 expression and T cell activation, are also greatly diminished from the cSMAC of Treg synapses and become sequestered once CD4+ T cells acquire the regulatory phenotype (148). In this context, PKC-θ accumulates on the distal pole of the IS by binding to the cytoskeletal protein vimentin (149). Interestingly, inhibiting PKC-θ or disrupting the vimentin intermediate filament networks promotes Treg suppressive capacity (150). However, PKC-θ deficiency in mouse Tregs (149), or deletion of downstream signalling components, such as CARMA1 (151), impairs differentiation and stability. Thus, it appears localisation of PKC-θ to the distal pole acts as a break for Treg activity (149), emphasising the influence of IS organisation on cell functionality.

CD8+ regulatory subsets have also been reported, though have received far less attention. What is understood is often extrapolated from research pertaining the MHC Ib Qa-1/HLA-E restricted CD8+ regulatory subset (152), which has been aided considerably by mouse models and investigations into germinal centre tolerance (153) and experimental autoimmune encephalomyelitis (EAE) (154). The more recently identified classically restricted MHC-I CD8+ Tregs in the context of EAE has opened the field further (155).

Similar to peripheral CD4+ Tregs, Helios maintains CD8+ Treg identity though the subset is best identified by triad expression of CD44+CD122+Ly49+ (156), and lack of FOXP3. Interestingly, Helios may play a more crucial role in the suppressive activity of CD8+ Tregs. Ablation of CD8+ specific Helios results in mass organ infiltration of immune cells, and Helios deficiency in CD8+ Tregs immune cells promotes lupus-like disease in mice and SLE in humans (157). Here, the entwinement of Tregs and tolDCs is further demonstrated as tolDCs—compared to their mature and immature counterparts—have been reported to greatly upregulate helios expression in CD8+ Tregs of healthy mice. Most interestingly, tolDC mediated upregulation was impaired in the LPR SLE mouse model (158). The cytolytic and cytotoxic abilities uniquely differentiates CD8+ Tregs from CD4+ Tregs. Naturally this relies on the intimate contact achieved through organisation of an IS allowing for the targeted release of membrane lysing proteins such as perforin (159). Evidence for this as primary suppression mechanism of CD8+ Tregs is supported by reports that $Prf1^{-/-}$ negative mice are incapable of supressing T_{FH} in $Rag2^{-/-}$ mice (160) and proliferation of Ag-activated CD4+ T cells in EAE model (161). Equally, as mentioned, expression of Ly49 identifies CD8+ Tregs and is homologous to the inhibitory killer cell immunoglobulin-like receptors (KIRs) on human NK cells. CD8+KIR+Tregs—not CD8+KIR− Tregs—are capable of supressing CD4+ T cell expansion, and could be abrogated by separation via membrane insert, demonstrating again that such suppression was cell-contact dependant and likely operates through formation of an IS (162).

Macrophages

Macrophages, because of their excellent scavenging function, play an important role in Ag presentation, and therefore, in T cell tolerance. In 1993, Miyazaki et al. (163) demonstrated the function of macrophages in Ag presentation and T cell tolerance in class II, I-E restricted fashion *in vivo*, by producing transgenic mice expressing class II MHC I-E molecules only on macrophages. It was seen that Ag presentation and T cell priming were impaired in these I-E restricted mice. With respect to T cell tolerance, I-E reactive T cells were anergised, but not clonally deleted. These results clearly demonstrate that macrophages by themselves are defective in efficient I-E restricted Ag presentation, so that T cells exposed to Ags expressed on macrophages are led to anergy.

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Research on T cell anergy also indicates that the anergy of T cells against superantigens is incomplete only in the presence of macrophages. Only certain cells which have a high affinity for superantigens may be anergised by macrophages. This suggests that the T cells interact with the superantigens associated with macrophages and led to tolerance. This also indicates that the defective clonal deletion of T cells is not due to the impaired presentation of the endogenous superantigens by macrophages solely because of the strict tissue distribution of the superantigens. It was not clear if the superantigens presented by macrophages are produced by macrophages or picked up by the cells. But it was evident that macrophages fail to stimulate normal T cells. There were two possible reasons for this scenario. First, is that low Ag concentration on cell surface leads to defective presentation of Ag and to anergy induction. Second, is the potential differences in accessory molecules. It has been proposed that for the high avidity T cell-APC interaction, APCs not only have to present Ag-MHC complexes but also need to deliver appropriate 'second signals' through certain accessory molecules. Although the precise function of APC second signals is still obscure, a consensus is emerging that T cell recognition of Ag in the absence of these signals tends to cause anergy, rather than deletion in the thymus or stimulation in the periphery. It is possible in macrophages that the accessory molecules on the cell surface might be different from those on other APCs capable of induction of clonal deletion, thus resulting in a lack of delivery of the appropriate second signals.

Interestingly, several works have also shown that nitric oxide (NO) generated by macrophages and fibroblasts when treated with IFN-γ, lead to the inhibition of T cell activation. A study by Yamazaki et al. (164) uncovered that anti-PD-L1 Abs inhibit naive CD4+ T cell proliferation but boost the production of IL-2 and IFN-γ when macrophages are involved. The inhibition of T cell proliferation by anti-PD-L1 Abs is primarily due to increased IFN-γ production, with a subsequent rise in NO production by macrophages (164). In 2011, Lukacs-Kornek et al. (165) also revealed that fibroblastic reticular cells and lymphatic endothelial cells in lymphoid organs also inhibit activated T cell proliferation through a tightly regulated process dependent on NO synthase 2 (NOS2). The expression of NOS2 and NO production was triggered by a combination of IFN-γ, TNF, and direct contact with activated T cells (165).

In 2020, Diskin et al. (166) revealed that in cancer, the expression of PD-L1 on T cells was regulated by tumour Ags and sterile inflammatory signals. PD-L1-expressing T cells promoted tumour tolerance through three mechanisms: 1) PD-L1 binding induced STAT3-dependent 'back-signalling' in CD4+ T cells, leading to reduced activation, diminished Th1-polarization, and the promotion of Th17-differentiation. PD-L1 signalling also induced an anergic state in CD8+ T cells, which is equally suppressive as PD-1 signalling; 2) PD-L1+ T cells restrain effector T cells through the conventional PD-L1/PD-1 axis, accelerating tumorigenesis, even in the absence of endogenous PD-L1; 3) PD-L1+ T cells interacted with PD-1+ macrophages, triggering an alternative M2-like programming that severely impairs adaptive antitumour immunity. In summary, their work showed that PD-L1+ T cells exhibited diverse tolerogenic effects on tumour immunity (166).

In 2022, A study from Kersten et al. (167) demonstrated an extensive connection between the behaviour of tumour-associated macrophages (TAMs) and exhausted T cells within the tumour microenvironment. Depletion of TAMs *in vivo* is shown to diminish exhaustion programs in tumour-infiltrating CD8+ T cells, rejuvenating their capacity to function as effectors. The study revealed that TAMs and CD8+ T cells engage in prolonged, Ag-specific interactions that, paradoxically, do not activate T cells but instead prepare them for

exhaustion, particularly under hypoxic conditions (167). In 2022, another study in murine breast cancer model by Nixon et al. (168) showed that TAMs induce CTL exhaustion within the tumour. The authors demonstrated that deletion of IRF8 specific to TAMs prevents CTL exhaustion and hampers tumour growth. This phenomenon was also observed in immune-infiltrated renal cell carcinoma patients, highlighting the role of IRF8 in CTL exhaustion promoted by TAMs across various cancer types (168).

NKs

NK cells (natural killer cells) are an integral part of the innate immune system and have been shown to be involved in immunoregulation of autoimmune diseases. In 2019, Galazka et al. (169), studied the role of gene related to anergy in lymphocytes (GRAIL) induction in maintenance of T cell anergy. The study showed that NK cells in EAE mice can induce anergy in CD4+ T cells upon histidine triad nucleotide binding protein 1 (HINT1)/heat shock protein 70 (Hsp70) treatment rather than inducing necrosis or apoptosis. The authors showed that HINT1/Hsp70 treatment generated regulatory NK cells expressing GRAIL, indispensable for their inhibitory function. It was seen that GRAIL expression was downregulated by specific siRNA and GRAIL overexpression was induced by pcDNA-GRAIL transfection. Though in general GRAIL was reported to target p53 degradation mediating p53-dependent cell cycle arrest and apoptosis, in NK cells upon HINT1/Hsp70 treatment they seem to play a key role in T cell anergy. The deletion of GRAIL in CD4+ T cells reversed inhibitory effects on T cell proliferation induced by PLP139–151 autoantigen. Therefore, it was proposed that NK cells induce T cell anergy upon HINT1/Hsp70 treatment through GRAIL expression (169).

In 2022, A study by Lindsay et al. (170) revealed that NK cells play a significant role in enhancing the expression of homing receptor ligands on tumour vasculature and controlled the development of anergic T cells, leading to improved tumour control. The work demonstrated that when NK cells were depleted, there was a notable increase in the population of intratumoural T cells exhibiting an anergic phenotype. This anergic T cell development in the lymph nodes draining the tumour correlated with heightened TCR signalling but reduced proliferation and functional activity of effector cells. NK cells have been shown to regulate T cell anergy, through the secretion of IFN-γ and expression of homing receptor ligands (170).

TISSUE-RESTRICTED Ags AND T CELL RESISTANCE

With such an arsenal of mechanisms to impede activation of autoreactive T cells, an outstanding question is how and why one form of tolerance is induced over another. Some autoimmune mouse models suggest the organ/tissue localisation—aka. the restriction—of Ag is one deciding parameter. This has only come to light from careful consideration of transgenic mouse model studies of polyclonal T cell repertoires, rather than monoclonal systems which inaccurately report on more physiological contexts (121). Previously, numbers of deleted cells were over-estimated for ubiquitous Ag non-exclusive to the thymus, where in fact it is possible many of these autoreactive cells actually survive deletion. Instead, polyclonal studies suggest a finer 'tuned' model of tolerance that is sometimes impermanent or otherwise restrained and resonates with our use of 'resistance' nomenclature.

Studies by Malhotra et al. (171) and Legoux et al. (121) coupled 'self'-Ag to promotors which across studies crucially showcased different degrees of tissue restriction and thus expression

patterns. Malhotra et al. (171) employed two pancreatic promotors *Ins1* and *Ins2* in their study, but observed partial deletion of T cells when Ag was coupled to only *Ins2* which was cited to be minimally expressed by medullary thymic epithelial cells (mTECs) in the thymus as well as pancreatic beta-cells. Legoux et al. (121) however only studied a pancreatic promotor exhibiting no overt thymic expression, and thus observed no deletion. The two pancreas-exclusive promotor models studied by Malhotra et al. (171) and Legoux et al. (121) both exhibited ignorance induction, with expansion of these cells highly comparable to those from WT mouse upon immunisation.

Interestingly, both studies observed thymic nTreg involvement under different promotor locations: Malhotra et al. (171) for Ag coupled to the 'promiscuously' expressed *Ins2* pancreatic cell promotor; Legoux et al. (121) for promotors coupled exclusively to the lung (*CC10*) or intestine (*Vil)*. Again, the presence of thymic expression for *Ins2* appeared to localise the Treg tolerance to the thymus. When expression was thymically excluded in the case of *CC10* and *Vil,* nTregs were induced in similar levels to WT mice, but tolerance was likely primarily mediated by their expansion in the periphery upon Ag exposure, showcasing again how environment can shape resistance to activation (121). Interestingly, this peripheral nTreg expansion did not induce durable tolerance, as upon secondary Ag challenge robust expansion of auto-reactive CD4+ T cells was observed. *Legoux et. al* emphasise the greater durability of deletional tolerance that contrasts the short-lived, and thus flexible Treg tolerance that could allow for cross-reactive anti-tumour/pathogen responses. Here, a resistance to activation is highlighted again, as such cells could treat cancers. Another interesting speculation by Legoux et al. (121) was the size and environment of the pancreas, which only saw ignorance, compared to the lung and intestines which observed solely Treg induced tolerance and likely provide more efficient and rich sampling environments for APCs.

By developing a transgenic mouse incapable of expressing a melanocyte protein, Trp2 ($Dct^{-/-}$), Truckenbrod et al. (172) demonstrated tolerance induction which simply impairs proliferative capacity, but otherwise leaves autoreactive cells phenotypically similar to non-tolerised cells specific for the same Ag (i.e. no anergy or exhaustion markers). It is unclear where this tolerance mechanism occurred in this study or the extent of Ag expression in the thymus, though this was suggested to be minimal and likely not through mTECs. Adoptive transfer of Trp2-specific CD8+ T cells between models revealed tolerance was unlikely induced nor maintained peripherally, suggesting a permanent rewiring in the thymus unlike that reported in the studies previously discussed (121,171,172). The premature plateauing of expansion was ascribed to lower expression of CD25 and inability to differentiate into highly proliferative cells, meaning they were unable to elicit any deleterious levels of tissue damage akin to vitiligo, but were still functional (172). Thus, Truckenbrod et al. (172) emphasised that tolerance was not binary, an important parameter which sits centre to our proposal for the use of 'resistance.' These studies showcase the many layers of sometimes subtle, but significant braking mediating tolerance. Multiple mechanisms can contribute together, regulated by many extrinsic parts on cellular and spatial levels to achieve activation resistance outputs, such as longevity and extent of suppression, that better protect against overt pathogenic stimulation.

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T CELL RESISTANCE AS A TARGET FOR IMMUNOTHERAPY

Mechanisms of T cell resistance are widely targeted to either boost or reduce immune responses in the context of cancer or autoimmunity, respectively. In this section, we will discuss current approaches in immunotherapy, as well as promising and less explored targets, to manipulate T cell resistance towards activation.

Fighting T cell exhaustion is at the core of current cancer immunotherapy efforts, with more than 5,000 ongoing clinical trials worldwide targeting PD-1/PD-L1 pathways, with a growing body of combination therapies; including chemotherapy agents, anti-angiogenic therapeutics or other immune modulators, such as anti-CTLA4, anti-LAG-3, or IL-2 variants (173). The current challenge of targeting exhausted T cells is to direct these therapies to precursors of terminally exhausted T cells, in which the exhaustion programme is still reversible.

Another T cell resistance mechanism currently under intense study is anergy. Understanding what drives anergy has prognostic and therapeutic value, especially in patients with autoimmune disorders or transplant recipients in the context of graft-versus-host disease. Approved regimens for treatment of autoimmune conditions often fail to induce long-lasting remission and given their broad specificity can carry a wealth of side effects that increase susceptibility to opportunistic infections and even cancers (174). Harnessing anergy itself is thus an attractive target, and much therapeutic research is particularly focused tolDCs and Tregs given their capacity to reinstate alloantigen-specific tolerance locally and without compromising protective immunity (175).

Clinical trials for both Tregs (176,177) and tolDCs (178,179) are well underway, and have so far demonstrated both cellular therapies are safe and have potential, but are not without their shared challenges in relation to delivery and efficacy. Though defective Treg behaviour and/or diminished numbers appear core to many autoimmune diseases (180,181), a minority of research has challenged this (182,183) and suggest the local environment and potential aberrant state of host effector cells as equally important considerations for investigating Treg based therapies. In any case, addressing the imbalance between tolerance and inflammation is a clear objective. As previously mentioned, introduction into an inflammatory environment has the potential to convert Tregs to an effector memory phenotype, and tolDCs into their immunogenic 'fully mature' counterpart and thus may exacerbate disease in each case (184,185). Indeed, this could explain early reports that the longevity of transplant tolerance is improved following depletion of the host T cell repertoire (186). The phenomenon of bystander suppression might not require such longevity, however, as allografted Tregs and tolDCs need only to persist long enough to establish the autonomous state of 'infectious tolerance' through induction of the other regulatory cell types from host precursors (159). Other considerations arise from the various methodologies used to isolate Tregs and tolDCs which likely explain the many inconsistencies in their fields (187). Better characterisation of tolDC biology would also improve therapy as migration of tolDC to lymph nodes or specific tissues has been a significant challenge (79,185). Much like Tregs, the benefit of tolDCs lies in their potential for establishing Ag-specific tolerance. However, knowledge on what particular auto-Ags drive some auto-immune diseases are limited, and an incorrect choice could be deleterious. In this context, rheumatoid arthritis (RA) is linked to citrullinated peptides generated through posttranslational modification of arginine residues in proteins. Recent studies show that HLA-DR-bound citrullinated peptides are not necessarily arthritis-initiating neo-Ags; rather, they appear to induce varying degrees of immune tolerance, which may prevent the development of RA in the majority of individuals (188).

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In other cases, T cell resistance is not the central target of the therapeutic approach, but an unwanted side-effect of some therapeutic strategies, for example, cell death may impact on T cell resistance. Anti-neoplastic drugs are designed to kill (cancer cells), and therefore their main side-effect is the unwanted collateral death of other healthy cells, and T cells are no exception. T cell death associated to cancer treatment leaves the patient not only physically weakened, but also immunologically unprotected from surviving cancer cells. Current immunotherapies specifically directed to kill cancer cells greatly ameliorate such side-effects. However, premature T cell death is still a problem to overcome, largely caused by the upregulation of pro-apoptotic molecules in the tumour microenvironment (189-191) and during infections. For this reason, protecting tumour-infiltrating lymphocytes (TILs) from death pathways is of primary interest. In fact, TILs death, in addition to low immunogenicity, may contribute to the “cold” tumours, which are resistant to immunotherapy. Current efforts aim to prevent cell death specifically for TILs. For example, low persistence of infused CAR T cells (192) can be improved by overexpressing anti-apoptotic BCL-2 in the CAR T cells, which increases their persistence and therapeutic efficacy (193).

Another resistance mechanism associated to CAR T cell failure is ignorance. CAR T cell therapy is a revolutionary new pillar in cancer treatment. Although treatment with CAR T cells has produced remarkable clinical responses with certain subsets of B cell leukaemia or lymphoma, many challenges limit the therapeutic efficacy of CAR T cells in solid tumours and other haematological malignancies. Since 2017, six CAR T cell therapies have been approved by the Food and Drug Administration. Currently available CAR T cell therapies are customized for each individual patient. However, the failure of CAR T cells in curbing tumours remains evident due to the reduced sensitivity of these receptors to the antigenic load on the cancers. This points to the intrinsic mechanism of T cell ignorance, where the affinity of CAR to Ag or Ag abundance are too low to elicit T cell activation. Thereby studying T cell ignorance paves ways to develop more sensitive CAR T cell therapy where the cells can be made sensitive to even low levels of antigenic loads on the tumour.

However, it is also important to consider that constitutive tonic CAR signalling can lead to T cell exhaustion, which represents another key barrier to CAR T cell efficacy. Under these conditions, transient periods of "rest" or ignorance may actually help prevent exhaustion and promote anti-tumour responses by allowing the T cells to recover and regain functionality (194). Thus, while ignorance can inhibit CAR T cell activation, it may also serve a protective function by reducing exhaustion in the context of tonic signalling, ultimately enhancing therapeutic efficacy.

Even though TCR are more sensitive than CARs immunological ignorance is still a cancer-enabling feature of the TCR mediated oligo-clonal T cell response to melanoma neoantigens (31). Ignorance of clonal neoantigens is at the basis for ineffective T cell immunity to melanoma and supports the concept that therapeutic vaccination, as an adjunct to checkpoint inhibitor treatment, increases the breadth and diversity of neoantigen-specific CD8+ T cells.

Finally, some mechanisms of T cell resistance to activation are just emerging as promising future targets for immunotherapy, such as the challenge of reversing T cell senescence to achieve immune system rejuvenation and more effective vaccine responses or cancer immunotherapies. Recent literature suggests that inducing T cell senescence is a key strategy used by malignant tumours to evade immune surveillance (195,196). This immune evasion

strategy might be responsible for advanced cancer as the accumulation of senescent T cells possibly lowers the response rate to chemo(radio)therapy and immunotherapy (86). Apart from their role in cancer progression, immunosenescence has other widespread effects and impact multiple aspects including ageing, chronic viral infections, and autoimmune disorders where Ag stimulation persists (197,198). Further, owing to the immunological relevance of senescence in T cells, several ongoing studies aim to delay senescence by developing strategies to maintain telomere length and prolong telomerase activity in specific populations of T cells (199). Understanding telomerase activity and ageing in T cells will help in the development of effective immunotherapies targeting senescence in T cells. Potential immunotherapeutic approaches include the replacement, reprogramming, and restoration of the immune system, as well as modulation of signalling in tumour sites and shifting immunosuppressive microenvironments to become more effector like microenvironments (200).

CONCLUDING REMARKS

In conclusion, the intricate exploration of diverse T cell resistance mechanisms not only reveals the complex challenges within the immune landscape but also unveils promising avenues for therapeutic interventions across a spectrum of diseases. Anergy, a pivotal mechanism under intense study, holds both prognostic and therapeutic value, particularly in the realms of autoimmune disorders and graft-versus-host disease for transplant recipients. Harnessing anergy, especially through cellular therapies involving tolDCs and Tregs, emerges as an attractive target, albeit with challenges that demand ongoing exploration.

The delicate balance between tolerance and inflammation remains a focal point, with Tregs and tolDCs playing critical roles in establishing Ag-specific tolerance. Overcoming challenges related to their function, numbers, and the intricate interplay with the local environment becomes vital for successful therapeutic outcomes. The evolving landscape of T cell resistance also unravels unintended consequences, such as T cell death induced by anti-neoplastic drugs, prompting innovative strategies to protect TILs and enhance the efficacy of immunotherapies.

T cell ignorance surfaces prominently in the context of CAR T cell therapy, where strategies to overcome reduced sensitivity of receptors to tumour Ags are essential for improving therapeutic outcomes. This principle extends to therapeutic vaccination strategies aimed at augmenting the diversity of neoantigen-specific CD8+ T cells. Additionally, emerging mechanisms like T cell senescence offer promising targets for future immunotherapy, with ongoing studies exploring strategies to delay senescence and rejuvenate the immune system.

As the field progresses, the multifaceted exploration of T cell resistance mechanisms promises transformative breakthroughs in immunotherapy and healthcare. From refining cellular therapies to mitigating unintended consequences, the journey into the intricate world of T cell resistance opens new horizons for enhancing immune responses and addressing dysfunctional states across various disease contexts. This comprehensive understanding sets the stage for future advancements that may revolutionize the landscape of immunotherapy and improve patient outcomes.

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