Perfect adaptation of CD8⁺ T cell responses to constant antigen input over a wide range of affinity is overcome by costimulation

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Abstract

Maintaining and limiting T cell responses to constant antigen stimulation is critical to eliminate pathogens and maintain self-tolerance, respectively. Antigen recognition by the T cell receptor (TCR) induces signalling that can activate T cells to produce cytokines and downregulation of the TCR. Precisely how TCR downregulation controls T cell responses to constant antigen stimulation is controversial. In other systems, receptor downregulation can induce perfect adaptation to constant stimulation by a mechanism known as state-dependent inactivation but this relies on complete downregulation of the receptor or the ligand, which is not the case for the TCR. Here, we observed that primary human effector T cells exhibit perfect adaptation in cytokine production to constant antigen stimulation. Perfect adaptation was observed across a wide variation in antigen concentration (2,000-fold) and affinity (100,000-fold) even with partial TCR downregulation. A mechanistic model showed that TCR downregulation produces imperfect adaptation, but when coupled to digital signalling led to perfect adaptation in cytokine production. A prediction of the model is that pMHC-induced TCR signalling continues after adaptation and this is confirmed by showing that, while costimulation cannot prevent adaptation, CD28 and 4-1BB signalling reactivated adapted T cells to produce cytokines in a pMHC-dependent manner. We show that adaptation also applied to 1st generation chimeric antigen receptor (CAR)-T cells but is partially avoided in 2nd generation CARs. These findings highlight the role of even partial TCR downregulation in generating perfect adaptation with implications for costimulation and adoptive T cell therapies.
Introduction

T cell activation is critical to initiate and maintain adaptive immunity. It proceeds by the recognition of peptide major-histocompatibility complex (pMHC) antigens by T cells using their T cell receptors (TCRs). The binding of pMHC to the TCR induces signalling pathways that can activate T cells to directly kill cancerous or infected cells and to secrete a range of cytokines (1). When T cells are confronted with persistent or constant pMHC antigens, maintaining responses to foreign or altered-self pMHC (in chronic infections and cancers (2)) can be just as important as limiting responses to self pMHC (e.g. adaptive tolerance (3)). Like other surface receptors, the TCR is downregulated from the surface of T cells upon recognition of pMHC ligands (4). Precisely how TCR downregulation controls T cell responses to constant pMHC antigen stimulation remains controversial.

In other cellular systems, receptor downregulation is known to induce biological adaptation to constant ligand stimulation (5). Adaptation is defined by the ability of a system to display transient responses that return to baseline when presented with constant input stimulation. The process is known as perfect (or near-perfect) adaptation when the baselines before and after stimulation are similar and is imperfect otherwise. Several mechanisms of adaptation have been reported, including signalling-based feedbacks and feedforwards (6) and receptor-based mechanisms (7–9). In the case of receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and ion channels, the common underlying mechanism has been termed state-dependent inactivation (8). This mechanism relies on receptors becoming inactivated (i.e. no longer able to signal) after sensing the ligand by, for example, receptor downregulation. Perfect adaptation can be observed when all receptors are downregulated (Fig. 1A) or if all the ligand is removed by the downregulation of receptor/ligand complexes (Fig. 1B).

The conditions for perfect adaptation exhibited by other receptors are not readily applicable to the TCR. First, the complete downregulation of the TCR is not commonly observed nor is it required for T cell activation (10–14). Second, the complete removal of the pMHC ligand has not been reported although there are reports that some pMHC can be internalised by the T cell (15). Instead, individual pMHC ligands have been shown to serially engage and downregulate many TCRs (16) and, on the timescale of hours, a single presented pMHC can sustain TCR signalling to induce digital cytokine production (17).

Although TCR downregulation does not appear to meet the criteria for perfect adaptation, it has been suggested to play an important physiological role in limiting T cell responses (18–22). This concept is supported by studies showing that defects in TCR downregulation lead to hyper-responsive T cells with a loss of tolerance to persistent self-antigens resulting in autoimmune phenotypes (23–27) and this is associated with sustained early TCR signalling (28, 29). However, studies where transgenic mice were challenged with peripheral antigens came to inconsistent conclusions, with some investigators reporting near-complete TCR downregulation as the mechanism of tolerance (20, 30–33), while others concluded that TCR downregulation did not play a role in tolerance because overt downregulation was not observed (34–38). Therefore, it would seem that complete TCR downregulation is not necessary for adaptation tolerance (3).

Maintaining T cell responses is critical in adoptive therapies where effector T cells, produced by in vitro expansion, are transferred into cancer patients and migrate into tumour microenvironments with chronic cancer antigens (39). These T cells are often armed with synthetic chimeric antigen receptors (CARs) that re-direct them to tumour cell antigens. Similarly to TCRs, they are downregulated as a function of antigen concentration and initially lower CAR levels render T cells less responsive (40–44). How CAR downregulation shapes T cell responses is poorly understood.

Here, we investigated how constant antigen stimulation regulates responses of primary human effector CD8+ T cells. We observed perfect adaptation in cytokine production, whereby production stops after an initial release. This adaptation was observed across wide variation in pMHC concentration (2,000-fold) and affinity (100,000-fold) even under conditions with only partial TCR downregulation. Using a mechanistic mathematical model, we show that TCR downregulation produces imperfect adaptation but when coupled to a signalling switch lead to
Figure 1: Mechanisms of perfect adaptation based on receptor downregulation. A) Perfect adaptation can be observed if the ligand induces the downregulation of all receptors. This mechanism requires that the re-expression of the receptor on the surface is negligible on the timescale of adaptation. B) Alternatively, perfect adaptation can also be observed with partial receptor downregulation if all ligand is removed by the downregulation of receptor-ligand complexes. This mechanism requires that the receptors are in excess of the ligand (shown) or that receptors are re-expressed on the adaptation timescale (not shown) so that all ligand is removed. C) Receptor output exhibits imperfect adaptation in the model in panel A if the receptor is replenished at the cell surface. In this case, perfect adaptation can be observed if a switch is introduced downstream of the receptor (threshold indicated by dashed horizontal line). In all schematics, the ligand input represents the concentration of ligand available to bind receptor (not including internalised ligand). The mechanism of adaptation by receptor downregulation is a subset of the more general mechanism of state-dependent inactivation (5, 8).

perfect adaptation in cytokine production. A prediction of the model is that TCR downregulation reduces, but does not abolish, TCR signalling below the threshold for cytokine production. Consistent with this prediction, we show that adapted T cells are reactivated to produce cytokines by ligation of the costimulatory receptors CD28 and 4-1BB and importantly, this reactivation remained pMHC-dependent. Lastly, we show that adaptation is partially avoided in CAR-T cells that incorporate costimulation within the synthetic receptor. Therefore, adaptation can severely limit the production of cytokines in adoptive transfer therapies and have important implications for the design of adaptation-resistant TCR and CAR constructs.
Results

Perfect adaptation of T cell responses to constant antigen over large variation in concentration and affinity

Using a standard adoptive therapy protocol, we generated primary human CD8$^+$ effector T cells expressing the therapeutic affinity-enhanced c58c61 TCR (45), which recognises the NY-ESO-1\textsubscript{157-165} cancer testes antigen peptide bound to HLA-A2 (Fig. 2A, Materials & Methods). In order to allow for constant antigen stimulation and to isolate its effects, T cells were stimulated by recombinant pMHC ligands on plates (46–48). This system has been widely used to isolate the effects of specific ligands and to precisely control the duration of stimulation (49).

T cells stimulated by the high-affinity pMHC antigen (9V, K_D = 70.7 pM) exhibited perfect adaptation with the secretion of TNF-\textalpha stopping after 3 hours (Fig. 2B-C, left column). This adaptation was observed at all antigen concentrations tested. Within this range, high concentrations induced an earlier decline in the rate of TNF-\textalpha secretion starting at 2 hours. This resulted in a bell-shaped dose-response curve, which has been previously observed in this system (46).

Given that this adaptation was observed with a supra-physiological antigen affinity, we could not exclude the possibility that it was an uncharacteristic response to an excessive antigen signal. We therefore repeated the experiment with a physiological affinity pMHC (4A8K, K_D = 7.23 \mu M). Although a higher concentration was required to initiate TNF-\textalpha production, the adaptation phenotype was kinetically identical (Fig. 2B-C, right column). We also observed the adaptation phenotype for IL-2, MIP-1\beta, and IFN-\gamma (Fig. S1-2) with IFN-\gamma adapting on a timescale longer than 3 hours.

The constant level of supernatant cytokine may be established by a balance of uptake with continued secretion or by a stop in secretion. Using single-cell intracellular cytokine staining, we observed that fewer T cells stained positive for TNF-\textalpha beyond 3 hours (Fig. S3) suggesting that production stops, consistent with a recent report (50). Moreover, replacing the media and transferring T cells to new plates does not induce cytokine production (see below; Fig. 4C-D, transfer to pMHC). Collectively, this shows that cytokine production stops in response to constant pMHC ligand stimulation.

Activation-induced cell death may also result in reduced cytokine production but this is unlikely to be the case here. First, we previously confirmed that cell death is minimal in this experimental system (less than 10% of T cells stain positive for Annexin V at 8 hours) (46) and second, adapted T cells can be fully reactivated with co-stimulation (see below; Fig. 4C-D, transfer to pMHC + CD86 or 4-1BBL).

Taken together, perfect adaptation in cytokine production is observed with similar temporal kinetics across a 2,000-fold variation in antigen concentration and a 100,000-fold variation in antigen affinity.

Perfect adaptation cannot be explained by complete TCR or pMHC downregulation

Previously, it has been shown that complete receptor downregulation can produce perfect adaptation provided that the receptor is not replenished (re-expressed) on the surface on the adaptation timescale (Fig. 1A). We therefore examined the surface dynamics of the TCR in our experimental system using flow cytometry. Consistent with previous reports (16, 18, 51, 52), we observed concentration- and affinity-dependent TCR downregulation that reached steady-state within \( \sim 1 \) hour (Fig. 2D). Perfect adaptation in cytokine production was observed at all concentrations and affinities including conditions where the TCR was only partially downregulated (e.g. Fig. 2D, arrow). This partial downregulation was not a result of a fraction of T cells downregulating their TCR (i.e. digital downregulation) because histograms showed the entire population of TCR-transduced T cells reducing their TCR surface expression (i.e. analogue downregulation, see inset of Fig. 2D). Consistent with partial TCR downregulation and with previous reports (18, 53), we observed a small but significant recovery in TCR surface expression on the timescale
Figure 2: Perfect adaptation of T cells to constant pMHC ligand stimulation over large variation in affinity and concentration, and with proportional downregulation of the TCR

A) Primary human CD8+ effector T cells expressing the c58c61 TCR were stimulated using recombinant pMHC immobilised on plates with supernatant cytokine and surface TCR levels measured (see Materials & Methods). B) Cumulative TNF-α over the concentration of 9V (left) or 4A8K (right) pMHC for 1-8 hours. Mean and SD of 3 independent repeats. C) Data in panel B expressed as a rate of TNF-α secretion over time. D) Surface TCR expression measured using pMHC tetramers in flow cytometry (Inset: representative histogram with expression before stimulation shown in grey). Mean and SD of 2 and 5 independent repeats, respectively. E) Recovery of surface TCR was measured by stimulating T cells for 4 hours to induce downregulation (black line) before transferring them to empty plates without pMHC for 4 (blue) or 20 (red) hours before measuring surface TCR levels. The supernatant levels of MIP-1β, IFN-γ, and IL-2 along with raw data prior to averaging is summarised in Fig. S1-2 and single-cell intracellular cytokine staining in Fig. S3.

of ~4 hours. Taken together, perfect adaptation cannot be explained by complete downregulation of the TCR.
It has also been shown that complete removal of the ligand can produce perfect adaptation (Fig. 1B). As already discussed, the efficient removal of all pMHC ligands is not known to take place during T cell activation with previous reports showing that pMHC ligands continually engage TCRs (16, 17). Indeed, the removal of pMHC is unlikely to be the mechanism in this experimental system because transferring T cells after they have adapted to plates newly coated with pMHC did not reanimate them to produce cytokine (see below; Fig. 4C-D, transfer to pMHC).

Taken together, perfect adaptation by T cells cannot be explained by complete TCR or pMHC downregulation.

**Perfect adaptation by imperfect adaptation at the TCR coupled to digital signalling**

To understand the potential role of TCR downregulation in perfect adaptation, we constructed a mathematical model that coupled the kinetics of TCR downregulation and re-expression to an effective pathway description for cytokine production.

The model included the basal down- and up-regulation of the TCR (Fig. 3A). To accurately model the pMHC induced downregulation of the TCR, we first reproduced the observation that TCR downregulation has an Lck tyrosine signalling-dependent and -independent component (Fig. S4), which has previously been shown to indicate that Lck-dependent TCR signalling can induce downregulation of bystander unbound TCR (13). Therefore, the model included the binding of pMHC to the TCR that can lead to an Lck-dependent modification (e.g. phosphorylation) that induces signalling leading to the downregulation of bystander non-engaged TCR and to an Lck-independent modification that tags the engaged TCR for direct downregulation (e.g. ubiquitination). This model was unable to fit the rapid downregulation kinetics of the TCR unless TCR/pMHC binding was cooperative, which was assumed in previous models of TCR downregulation (52, 53) and is supported by recent observations (54).

We coupled the TCR downregulation reactions to a pathway model for digital cytokine production (Fig. 3A). To explain the temporal delay between TCR downregulation and cytokine production, we included an intermediate node \( Y \) that temporally integrated and sustained signalling from phosphorylated TCR. This mechanism is reasonable because it has been shown that T cells can integrate pMHC signals (55, 56). The production of cytokine \( Z \) relied on the temporal integrator remaining above a threshold value (Fig. 3A, digital switch). The assumption that cytokine production is digital is reasonable because the digital activation of T cells has been extensively documented (57–59), and has been directly observed at the level of cytokine production (17).

We used Approximate Bayesian Computations coupled to Sequential Monte Carlo (ABC-SMC) (60) to directly fit the model to the cytokine data (Fig. 2B), TCR downregulation and re-expression data (Fig. 2D, E), and to data that informed on bystander TCR downregulation (Fig. S4). Given that the experimental data is derived from a population of T cells with some level of heterogeneity, the ABC-SMC method is particularly appropriate because it effectively simulates a population of T cells with potentially different values of the model parameters representing population heterogeneity.

The model produced an excellent fit to the data (Fig. 3B and Fig. S4A, solid lines) indicating that TCR downregulation coupled to digital cytokine production is sufficient to explain perfect adaptation. Importantly, the model reproduced perfect adaptation even with only partial TCR downregulation. By examining the timecourse at a specific concentration (Fig. 3C), it was observed that the concentration of phosphorylated receptors and the temporal integrator exhibited imperfect adaptation, which is consistent with both the receptor and ligand remaining on the surface. Perfect adaptation in cytokine production was observed because once the switch was turned off, as a result of the decay in the temporal integrator, cytokine production stopped.

In summary, and in contrast to perfect adaptation by other receptors, the mathematical model predicts that perfect adaptation is explained here by imperfect adaptation at the TCR by downregulation coupled to a switch in the pathway for cytokine production (Fig. 1C).
Figure 3: A mechanistic mathematical model shows that TCR downregulation coupled to digital cytokine production is sufficient to explain T cell adaptation to constant pMHC ligand stimulation. A) Schematic of an ordinary-differential-equation (ODE) model that includes basal up- and down-regulation of the TCR, cooperative TCR/pMHC binding that induces a modification (tagging) that leads to downregulation of engaged TCR and a modification (phosphorylation) that induces signalling leading to downregulation of non-engaged bystander TCR. The production of cytokine was assumed to depend on a threshold level of Y, which temporally integrated signals from phosphorylated TCRs, and effectively introduced a time delay between TCR downregulation and cytokine production. B) The fit of the mathematical model using ABC-SMC (solid lines with 95% confidence intervals) to the timecourse data in Fig. 2 (see Fig. S5 for parameter distributions). C) Model outputs over time using the fitted parameters for a single condition (teal arrow in panel B). Imperfect adaptation in phosphorylated receptors leads to imperfect adaptation in the temporal integrator, which ultimately falls below the digital threshold required to sustain cytokine production (solid and dashed horizontal black lines in the temporal integrator panel indicate the mean threshold and 95% confidence intervals, respectively).

T cell adaptation to constant pMHC antigen can be overridden by costimulation

The model predicted imperfect adaptation by TCR downregulation so that residual TCR signalling continued after cytokine production had stopped (Fig. 3C). Given that T cells can encounter antigen in vivo with costimulation through other surface receptors, and costimulation is thought to lower the signalling threshold for cytokine production (61, 62), we determined whether costimulation can override adaptation.

We used the mathematical model to predict the outcome of transferring T cells from a first stimulation on the low-affinity antigen to a second stimulation on the same antigen with or without costimulation (Fig. 4A). Note that in these transfer experiments, T cells experience the same concentration of antigen in the first and second stimulation. The effect of costimulation was simulated by lowering the threshold of the temporal integrator required for cytokine production and as expected, this allowed T cells to produce cytokine provided they also continued to receive constant antigen stimulation (Fig. 4B).

In order to test whether CD28 costimulation could override adaptation, we stimulated T cells with the physiological...
Figure 4: Adapted T cells can be reactivated by CD28 or 4-1BB costimulation. A) Schematic of the experiment showing that T cells were first stimulated for 8 hours before being transferred for a second stimulation for 16 hours with either antigen alone, costimulation alone, or antigen and costimulation. B) Predicted cytokine production by the mathematical model where costimulation is assumed to lower the threshold for digital cytokine production. C) Representative TNF-α production when providing costimulation to CD28 by recombinant biotinylated CD86 (inset is averaged E_{max} values with standard deviations from 4 experiments). D) Representative TNF-α production when providing costimulation to 4-1BB by recombinant biotinylated trimeric 4-1BBL (inset is averaged E_{max} values with standard deviations from 3 experiments). Additional cytokines are shown in Fig. S6-7). Statistical significance was determined by ordinary one-way ANOVA corrected for multiple comparisons by Dunnett’s test.

low-affinity 4A8K pMHC (first stimulation) before transferring them to the same titration of this pMHC with or without recombinant CD86, which is the ligand for CD28 (second stimulation). Consistent with the adaptation phenotype, there was a dramatic reduction in TNF-α production in the second stimulation without CD86 but when CD86 was present, strong cytokine production was observed (Fig. 4C). Importantly, T cells transferred to empty wells without pMHC or to wells coated with only CD86 produced no cytokines.

In addition to CD28, the costimulatory receptor 4-1BB is also known to play an important role in the activation of CD8⁺ T cells. We repeated the experiments with the recombinant ligand to 4-1BB showing that this TNFR is also able to override adaptation but as with CD28, it critically relied on TCR/pMHC interactions (Fig. 4D).

Previous work on T cell anergy has described unresponsive T cell states that are induced when T cells are activated...
in the absence of CD28 costimulation. We therefore tested whether CD28 costimulation can prevent T cell adap-
tation. We repeated the CD28 costimulation transfer experiments but now transferred T cells that were stimulated
with both pMHC and CD86 in the first stimulation to a second stimulation that included pMHC with or without
CD86. We observed a dramatic reduction in cytokine production in the second stimulation irrespective of whether
CD86 was included in the first stimulation (Fig. S7), suggesting that CD28 costimulation cannot prevent adaptation
to constant antigen.

Taken together, these results indicate that perfect adaptation in cytokine production induced by constant pMHC
antigen stimulation does not lead to perfect adaptation in TCR signalling because extrinsic costimulation through
CD28 and 4-1BB can induce adapted T cells to produce TNF-α in a pMHC-dependent manner. This phenotype
was also observed for other cytokines (Fig. S6-7).

Adapted T cells can be reactivated by a higher-affinity ligand

The mechanism of adaptation provided by the model is not an unresponsive cellular state but rather a context-
dependent dynamic state that is induced to the specific combination of antigen concentration and affinity expe-
rienced by the T cell. We therefore hypothesised that increasing the antigen strength would allow T cells to be
reactivated. We used the model to predict the outcome of transferring T cells from a titration of the low-affinity
antigen (first stimulation) to either the same titration of the low-affinity antigen or the same titration of the high-
affinity antigen (second stimulation) (Fig. 5A). The model predicted that cytokine production would be minimal
on transfer to the low-affinity antigen (i.e. adaptation) but would be substantial on transfer to the higher-affinity
antigen with further TCR downregulation (Fig. 5B).

The model predictions were tested by stimulating T cells with the low-affinity pMHC before transferring them to
the same titration of either the same pMHC or the high-affinity pMHC (Fig. 5C). Consistent with the adaptation
phenotype, TNF-α was barely detected after the second stimulation when transferred to the same pMHC (Fig. 5C,
compare light blue to dark blue lines). However, a strong increase in TNF-α was observed in the second stimulation
when transferring to the higher-affinity pMHC (Fig. 5C, compare dark blue to orange lines). Importantly, we ob-
serve adaptation and rescue at concentrations that induced only partial TCR downregulation (Fig. 5C, highlighted
concentration and insets).

We note that beyond the highlighted concentration, the rescue following transfer to the high-affinity pMHC was
progressively reduced at higher concentrations (Fig. 5C, compare red and orange curves at 3 largest concentra-
tions). This likely reflects the lower amount of surface TCR induced by high concentrations of the low-affinity
pMHC in the first stimulation. Although the model qualitatively reproduced this phenotype, quantitatively it
showed near complete rescue at these concentrations (Fig. 5B) and this reflects the fact that this prediction is
an extrapolation based on fitting the timecourse data where surface TCR were initially constant. Indeed, the model
could explain the entire set of transfer experiments if it was directly fitted to the data using ABC-SMC (not shown).
It was computationally not practical to simultaneously fit the timecourse and transfer experiments.

In summary, T cells that adapt to constant antigen stimulation can be made responsive by presenting them with a
higher-affinity antigen at the same concentration provided that they have not completely downregulated their TCR.
We note that presenting T cells with a lower-affinity antigen did not lead to rescue and that these phenotypes are
observed with both TNF-α and IFN-γ (Fig. S8).

Adaptation by CAR-T cells to constant antigen can be overridden by costimulatory domains

Given that CAR-T cells experience chronic antigen stimulation in vivo, we analysed their adaptation phenotype.
To do this, we utilised the previously described T1 CAR (63) fused to the cytoplasmic tail of the ζ-chain (1st
Figure 5: T cells adapted to a low-affinity antigen can be reactivated with a higher-affinity antigen. A) Schematic of the experiment showing that T cells were first stimulated for 5 hours on the low-affinity antigen before being transferred for a second stimulation of 5 hours with the same or different pMHC antigen (at the same antigen concentration). B) Predicted TCR surface expression (top) and cytokine production (bottom) for the transfer experiment by the mathematical model. C) TCR surface expression (top) and TNF-α production (bottom) with a detailed comparison performed at the indicated concentration (right). Consistent with the adaptation phenotype, a first stimulation with the low-affinity 4A8K pMHC (light blue) leads to reduced cytokine production in a second stimulation on the same pMHC (dark blue). However, transferring T cells to the high-affinity 9V pMHC leads to more TCR downregulation and consequently more cytokine production (orange). Data are mean and standard deviation of 3 independent experiments with statistical significance determined by ordinary one-way ANOVA corrected for multiple comparisons by Dunnett’s test. Individual experiments, additional cytokines, and additional conditions (including transfers from high-affinity to low-affinity) can be found in Fig. S8).

generation CAR) that also recognises the NY-ESO-1_{157–165} peptide on HLA-A2 in a similar orientation to the TCR (K_D= 4 nM (64)). These CAR-T cells were first stimulated with a titration of 9V pMHC before being transferred for a second stimulation on the same titration of 9V (Fig. 6A).

We observed reduced cytokine production by CAR-T cells that experienced the antigen in the first stimulation compared to CAR-T cells that were directly placed on the second stimulation (Fig. 6B, purple and orange lines, respectively). Given that costimulation can override adaptation, we repeated the experiments with a 2nd generation CAR containing the CD28 costimulatory domain finding that these CAR-T cells were able to partially avoid adaptation (Fig. 6C). Compared to cytokine production in the 1st stimulation (100%), the production of TNF-α and IL-2 were reduced in the second stimulation to 26% (p<0.0002) and 2.1% (p=0.002) in the 1st Generation CAR but were only reduced to 58.7% (p=0.001) and 79% (p=0.0031) in the 2nd generation CAR (Fig. 6D-E). We note that overall cytokine production was higher in 2nd generation CARs (Fig. 6B-D) even though this receptor was consistently expressed at lower levels (Fig. S9). Taken together, the adaptation phenotype observed with the TCR
Figure 6: Adaptation is partially avoided in 2nd but not 1st generation CAR-T cells. A) Schematic of the experiment. T cells expressing the T1 CAR that recognises the 9V pMHC antigen were transferred to the same titration of antigen. B-C) Representative TNF-α and IL-2 production over antigen concentration from CAR-T cells expressing the B) the 1st generation variant containing only the ⍺-chain and C) the 2nd generation variant containing the cytoplasmic tail of CD28 fused to the ⍺-chain. D) Averaged E_{max} values and standard deviation for 3 independent experiments. E) Fold reduction of E_{max} between the first and second stimulation for the 1st and 2nd generation CARs highlighting that 2nd generation CARs are more resistant to adaptation induced by constant antigen. Expression profile of both CARs and antigen-induced CAR downregulation is shown in Fig. S9. Statistical significance was determined by ordinary one-way ANOVA corrected for multiple comparisons by Dunnett’s test.

can also be observed with a 1st generation CAR that can be partially overridden by a 2nd generation CAR that includes costimulation.

Discussion

Using a reductionist system that allows for the controlled constant stimulation of T cells with pMHC antigens, we observed that primary human CD8^+ T cells do not maintain cytokine production but instead exhibit perfect adaptation. This adaptation was observed across a 100,000-fold variation in affinity with identical temporal kinetics.
Although adaptation could not be prevented by costimulation, it can be overridden by it.

**Mechanism of adaptation.** Mechanisms of biological adaptation have been extensively studied (5–8) and the mechanism invoked here is based on state-dependent inactivation, which contributes to RTK, GPCR, and ion channel desensitization (5). Perfect adaptation by state-dependent inactivation requires that all receptors become downregulated (Fig. 1A) or that all ligand is downregulated (Fig. 1B). Here, perfect adaptation in cytokine production is observed with only partial TCR downregulation (Fig. 2) and moreover, the downregulation of the pMHC ligand is unlikely to contribute because T cells remain adapted after transfer to new plates (e.g. Fig. 5; low-affinity pMHC to low-affinity pMHC). Therefore, the kinetics of TCR/pMHC binding and downregulation produce conditions for imperfect adaptation at the TCR (Fig. 1C).

Perfect adaptation in cytokine production is achieved by coupling this imperfect adaptation mechanism to an intracellular digital switch (Fig. 1C). This mechanism predicts that TCR signalling continues after adaptation, which is supported by the observation that extrinsic CD28 or 4-1BB costimulation can reactivate adapted T cells in a pMHC-dependent manner (Fig. 4). Given that the TCR is part of a larger group of immune receptors known as non-catalytic tyrosine-phosphorylated receptors (NTRs) that share many features (65), the present mechanism may be more widely applicable.

**Relation to previous models.** A number of molecular models have been formulated to explain different features of T cell activation, including receptor downregulation by cooperative TCR/pMHC binding (52, 53), antigen discrimination by kinetic proofreading (57, 66–69) and digital activation by positive feedback (57, 58). In the present work, we focused on the phenotype of adaptation in cytokine production. Given that an entire signalling pathway exists between our input (pMHC ligand) and output (cytokine production), the model we invoked coupled these previously reported mechanisms into an effective phenotypic pathway model (70).

Previously, we argued that the bell-shaped dose-response curve for high-affinity pMHC can be explained by an incoherent feedforward loop and not by TCR downregulation (46). The key assumption underlying those conclusions were that the rate of cytokine production was in the steady-state, which is the case for Jurkat T cell lines (46), but the detailed analysis in the present work has revealed that, without costimulation, it is not the case for primary T cells. The bell-shaped dose-response curve generated by the kinetic model in the present study (Fig. 3B) is based on faster TCR downregulation at higher pMHC concentrations so that cytokine production stops at an earlier time point.

**Relation to unresponsive T cell states.** T cells are known to enter unresponsive states upon recognition of persistent self- and viral-antigens (2, 3). While the underlying mechanisms that induce and maintain these states are debated, we note that the phenotype we describe here is context-dependent so that T cells respond immediately to a stronger antigen or with costimulation. This suggests that they are unlikely to be in a distinct signalling or epigenetic state, as is the case for exhausted T cells (2). Interestingly, their reactivation is dependent on CD28 costimulation (Fig. 4C) which has recently been reported to be the case for exhausted T cells (71). Although similarities can be identified, by studying effector T cells generated by in vitro-expansion, we are inherently limited in making direct comparisons with in vivo unresponsive states described in mice (20, 31, 33–38, 72–74). We highlight that the mechanism of adaptation that we report can take place with only minor TCR downregulation, which may help reconcile previous reports arguing either that TCR downregulation can explain tolerance (20, 30–33) or that TCR downregulation did not play a role in tolerising T cells because overt downregulation was not observed (34–38).

**Function of adaptation in T cells.** Adaptation is a critically important and widely implemented process in biology. In the case of T cells, continuous tuning in the periphery has long been appreciated to be an important process to prevent autoimmunity without deleting self-reactive T cells (75). Unlike other receptors, perfect adaptation in T cells is achieved by imperfect adaptation at the TCR. This has the important consequence that adapted T cells are rendered dependent on extrinsic costimulation yet remain dependent on pMHC. In the specific example of effector T cells, whose killing capacity is thought to be less dependent on costimulation, perfect adaptation in...
cytokine production may be an important mechanism to ensure that their ability to initiate or maintain inflammation continues to be extrinsically regulated by making them dependent on costimulation.

**Implications for adoptive therapy.** The effector T cells we have used were generated using a protocol for adoptive therapy with TCRs or CARs. Consistent with the present work, it has recently been observed that CAR-T cells exposed to chronic antigen become unresponsive but can respond to a higher antigen dose, which correlates with CAR expression (43). The ability of 2nd generation CARs to partially avoid adaptation may explain why they generate much more potent and persistent anti-tumour responses *in vivo* (76–79) even though their *in vitro* killing capacity is comparable to 1st generation receptors (77–80). Our results suggest that receptor downregulation renders effector T cells dependent on costimulation for cytokine production, which is in line with the finding that mouse T cells with impaired TCR downregulation lose their dependence on costimulation (25, 27). The optimisation of TCRs and CARs has focused on affinity, surface levels, and signalling potency, but the engineering for optimal downregulation has yet to be explored.
Materials & Methods

**Protein production.** pMHCs were refolded in vitro from the extracellular residues 1-287 of the HLA-A*02:01 α-chain, β2-microglobulin and NY-ESO-1_{157–165} peptide variants as described previously (46). CHO cell lines permanently expressing the extracellular part of human CD86 (amino acids 6-247) with a His-tag for purification and a BirA biotinylation site were kindly provided by Simon Davis (Oxford, UK). Cells were cultured in GS selection medium and passed every 3-4 days. After 4-5 passages from thawing a new vial, cells from 2 confluent T175 flasks were transferred into a cell factory and incubated for 5-7 days after which the medium was replaced. The supernatant was harvested after another three weeks, sterile filtered and dialysed over night. The His-tagged CD86 was purified on a Nickel-NTA Agarose column. 4-1BB Ligand expression constructs were a kind gift from Harald Wajant (Wuerzburg, Germany) and contained a Flag-tag for purification and a tenascin-C trimerisation domain. We added a BirA biotinylation site. The protein was produced by transient transfection of HEK 293T cells with XtremeGene™ HP Transfection reagent (Roche) according to the manufacturer’s instructions and purified following a published protocol (81), with the exception of the elution step where we used acid elution with 0.1 M glycine-HCl at pH 3.5. The pMHC or costimulatory ligand was then biotinylated in vitro by BirA enzyme according to the manufacturer’s instructions (Avidity) and purified using size-exclusion chromatography.

**Production of lentivirus for transduction.** HEK 293T cells were seeded into 6-well plates 24 h before transfection to achieve 50–80% confluency on the day of transfection. Cells were cotransfected with the respective third-generation lentiviral transfer vectors and packaging plasmids using Roche XtremeGene™ 9 (0.8µg lentiviral expression plasmid, 0.95 µg pRSV-rev, 0.37 µg pVSV-G, 0.95 µg pGAG). The supernatant was harvested and filtered through a 0.45 µm cellulose acetate filter 24-36h later. The 1G4 TCR used for this project was initially isolated from a melanoma patient (82). The affinity maturation to the c58/c61 variant used herein (referred to as 1G4Hi) was carried out by Adaptimmune Ltd. The TCR and all CARs in this study have been used in a standard third-generation lentiviral vector with the EF1α promoter. The CAR constructs that bind the NY-ESO-1_{157–165} HLA-A2 pMHC complex (64, 83) were a kind gift from Christoph Renner (Zurich, Switzerland). The high-affinity version T1 was used for this project. All CAR constructs contained the scFv binding domain, a 2 Ig domain spacer derived from an IgG antibody Fc part and the CD28 transmembrane domain. We modified the different CARs to contain the CD3ζ signalling domain alone or in combination with the CD28 signalling domain.

**T cell isolation and culture.** Up to 50 ml peripheral blood were collected by a trained phlebotomist from healthy volunteer donors after informed consent had been taken. This project has been approved by the Medical Sciences Inter-Divisional Research Ethics Committee of the University of Oxford (R51997/RE001) and all samples were anonymised in compliance with the Data Protection Act. Alternatively, leukocyte cones were purchased from National Health Services Blood and Transplant service. Only HLA-A2+ peripheral blood or leukocyte cones were used due to the cross-reactivity of the high-affinity receptors used in this project which leads to fratricide of HLA-A2+ T cells (63, 64, 84). CD8+ T cells were isolated directly from blood using the CD8+ T Cell Enrichment Cocktail (StemCell Technologies) and density gradient centrifugation according to the manufacturer’s instructions. The isolated CD8+ T cells were washed and resuspended at a concentration of 1 × 10^6 cells per ml in completely reconstituted RPMI supplemented with 50 units/ml IL-2 and 1 × 10^6 CD3/CD28-coated Human T-Activator Dynabeads (Life Technologies) per ml. The next day, 1 × 10^6 T cells were transduced with the 2.5 ml virus-containing supernatant from one well of HEK cells supplemented with 50 units/ml of IL-2. The medium was replaced with fresh medium containing 50 units/ml IL-2 every 2–3 d. CD3/CD28-coated beads were removed on day 5 after lentiviral transduction and the cells were used for experiments on days 10-14.

**T cell stimulation.** T cells were stimulated with titrations of plate-immobilised pMHC ligands with or without co-immobilised ligands for accessory receptors. Ligands were diluted to the working concentrations in sterile PBS. 50 µl serially two-fold diluted pMHC were added to each well of Streptavidin-coated 96-well plates (15500, Thermo Fisher). After a minimum 45 min incubation, the plates were washed once with sterile PBS. Where accessory receptor ligands were used, those were similarly diluted and added to the plate for a second incubation of 45-90
In experiments with small molecule inhibitors, the T cells were incubated with the inhibitor at 37 °C for 20-30 min prior to the start of the stimulation. The inhibitors were left in the medium for the whole duration of the stimulation. All control conditions were incubated with DMSO at a 1:1000 dilution so that the DMSO concentration was the same for inhibitor and non-inhibitor samples. PP2 was used at a 20 µM concentration. After washing the stimulation plate with PBS, 7.5 x 10⁴ T cells were added in 200 µl complete RPMI without IL-2 to each stimulation condition. The plates were spun at 9 x g for 2 min to settle down the cells and then incubated at 37 °C with 5 % CO₂. For transfer experiments, the T cells were pipetted from the stimulation plate into a V-bottom plate and pelleted after the first round of stimulation. The supernatant was stored at -20 °C for later cytokine ELISAs, the cells were resuspended in 200 µl fresh R10 medium and – depending on the experiment – either rested for some time or transferred to another stimulation plate with a new set of conditions. The cells were then again settled down by centrifuging at 9 x g before incubation.

Flow cytometry. Flow cytometry was used to assess receptor expression after TCR and CAR transductions, and to quantify receptor downregulation at the end of stimulation experiments. After stimulation, T cells were pelleted in a V-bottom plate and resuspended in 40 µl PBS with 2% BSA and fluorescent 9V pMHC tetramers that were produced with Streptavidin-PE (AbD Serotec) and used at a predetermined dilution (1:100-1:1000). The staining was incubated for 20-60 min after which the cells were pelleted, resuspended in 70-100 µl PBS and analysed on a FACScalibur™ or LSRFortessa X-20 (BD) flow cytometer. Flow cytometry data was analysed with Flowjo V10.0.

ELISA. Supernatants from stimulation experiments were stored at -20 °C. Cytokine concentrations were measured by ELISAs according to the manufacturer’s instructions in Nunc MaxiSorp™ flat-bottom plates (Invitrogen) using Uncoated ELISA Kits (Invitrogen) for TNF-α, IFN-γ, MIP-1β, and IL-2.

Data analysis. The fraction of T cells expressing the transduced TCR and the amount of supernatant cytokine exhibited variation between independent repeats. Therefore, averaging the data produced curves that were not representative of individual repeats.

To average data for model fitting (see below), the maximum surface TCR levels and cytokine levels were normalised to 1 in each repeat before averaging. In the case of the TCR, we also subtracted the gMFI of the TCR-negative cell population, which resulted in TCR surface expression near 0 for high concentrations of the high-affinity pMHC (Fig. 2D). The averaged data and individual repeats can be found in Fig. S2-1.

To average data for statistical analysis, the maximum amount of cytokine produced across different pMHC concentrations (Eₘₐₓ) were expressed as a fold-change to pMHC alone before averaging. Given that the dose-response curves often exhibited a bell-shape, it was not possible to use a standard Hill function to estimate Eₘₐₓ. Instead, we used lsqcurvefit in Matlab (Mathworks, MA) to fit a function that was the difference of two Hill curves in order to generate a smooth spline through the data from which the maximum value of cytokine was estimated. This procedure was used to extract Eₘₐₓ values in Fig. 4, 6, S6, S7. In a limited number of cases, individual outlier values were excluded prior to data fitting but are still shown as data points in respective figures.

Statistical analysis. Ordinary one-way ANOVA corrected for multiple comparisons by Dunnett’s test was performed on experimental data to determine statistical significance levels (Fig. 4C,D, Fig. 5C, Fig. 6D,E, Fig. S7). Statistical significance for surface TCR recovery (Fig. 2E) was performed by using an F-test for the null hypothesis that a single Hill curve (with the same parameters) can explain the data. GraphPad Prism was used for all statistical analyses.

Mathematical model. The mathematical model is the result of an iterative process of testing different model architectures based on information from the literature. The reason for including specific reactions is described in detail in the results section. The system of non-linear coupled ordinary-differential-equations (ODEs) representing
the model (Fig. 3A) is formulated as follows,

\[ \frac{dL}{dt} = -k_{on}(LR)^{n_b} + (k_{off} + k_{basal})(C_0 + C_{act} + C_{tag} + C_{act+tag}) + k_{int}(C_{tag} + C_{act+tag}) \]

\[ \frac{dR}{dt} = k_{syn} - k_{basal}R - k_{off}(C_0 + C_{act} + C_{tag} + C_{act+tag}) - k_{by}R^{n_{by}}(C_{act} + C_{act+tag})^{n_{by}} \]

\[ \frac{dC_0}{dt} = k_{on}(LR)^{n_b} - (k_{off} + k_p + k_t)C_0 - k_{basal}C_0 \]

\[ \frac{dC_{act}}{dt} = k_pC_0 - (k_{off} + k_t)C_{act} - k_{basal}C_{act} \]

\[ \frac{dC_{tag}}{dt} = k_tC_0 - (k_{off} + k_p + k_{int})C_{tag} - k_{basal}C_{tag} \]

\[ \frac{dC_{act+tag}}{dt} = k_tC_{act} + k_pc_{tag} - (k_{off} + k_{int})C_{act+tag} - k_{basal}C_{act+tag} \]

\[ \frac{dY}{dt} = k_{fy}(C_{act} + C_{act+tag}) - k_{fy}Y \]

\[ \frac{dZ}{dt} = k_{prod}H[t - k_{delay}]H[Y - p_t] \]

where \( L \) is the concentration of free pMHC, \( R \) is the concentration of free TCR, \( C_0 \) is the TCR/pMHC complex that is unmodified, \( C_{tag} \) is the complex modified to be tagged for downregulation, \( C_{act} \) is the complex modified so that it can activate signalling (e.g. phosphorylated), \( C_{tag+act} \) is the complex modified by both tagging and activation, \( Y \) is the temporal integrator, and \( Z \) is the cumulative amount of cytokine (i.e. it exhibits no degradation). All reactions are standard 0th, 1st, or 2nd-order with the exception of 1) TCR/pMHC binding, which is described with order \( n_b \), 2) Bystander TCR downregulation, which is described with order \( n_{by} \), and 3) the digital activation of \( Z \), which is described by the multiplication of two heaviside-step functions; the first represents the digital switch that requires the value of \( Y \) to remain above the threshold value of \( p_t \) to maintain cytokine production and the second is included to account for the observation that the rate of cytokine production increases initially (i.e. the amount of cytokine produced in the first hour is less than in the second hour, which is likely associated with a transcriptional/translational delay). By including a 1st order reaction before downregulation \( (k_t) \) and activation \( (k_p) \) the model includes kinetic proofreading on both processes.

**Fitting model to data using ABC-SMC.** A Matlab (Mathworks, MA) implementation of a previously published algorithm for Approximate Bayesian Computation coupled to Sequential Monte Carlo (ABC-SMC) was developed and used throughout for data fitting (60). The mathematical model was evaluated using the Matlab (Mathworks, MA) function `ode23s`.

To simplify the fitting, we first used a simplified model that only included basal up and down-regulation of the receptor to directly fit the TCR recovery kinetics (Fig. 2E) using ABC-SMC (not shown). This provided estimates for \( k_{syn} = 3.1 \times 10^{-4} \mu m^{-2}s^{-1} \) and \( k_{basal} = 3.1 \times 10^{-6} s^{-1} \) assuming a steady-state concentration of TCR of 100 \( \mu m^{-2} \), which were similar to previously reported values (53). Using this fitted synthesis rate, it is estimated that it would take \( \sim3.5 \) days to observe full recovery of the TCR at the surface (i.e. upregulate \( \sim30,000 \) TCRs).

With \( k_{syn} \) and \( k_{basal} \) fixed, the remaining model parameters were fitted simultaneously to 1) the cumulative TNF-\( \alpha \) timecourse and the TCR downregulation timecourse for both low- and high-affinity pMHC (Fig. 2B,D) and 2) TCR downregulation after 4 hours of stimulation with or without inhibition of Lck (Fig. S4). The distance measure used was the sum-squared-residual (SSR), which was the normalised cytokine level in the data and \( Z \) in the model and the normalised surface TCR in the data and the sum of the surface receptor in the model \((R + C_0 + C_{tag} + C_{act} + C_{tag+act})\). A total of 408 experimental data points were fitted to the model. All model parameters were fitted with the exception of \( k_{syn} \) and \( k_{basal} \) simultaneously to the data (Fig. S5). The initial conditions were \( L(t = 0) = k_{prop}L_T \mu m^{-2}, R(t = 0) = 100 \mu m^{-2}, \) and all other states were set to 0. To convert the amount of pMHC in the experimental data in ng/well \((L_T)\) to a concentration in molecules/\( \mu m^2 \), a constant of proportionality was also fitted \((k_{prop})\) which multiplied the initial concentration of pMHC in the model.
A population of 10,000 particles were initialised with uniform priors on the parameters in log-space and propagated through 20 iterations at which point the distance measure was no longer decreasing. The final population of 10,000 particles, each of which had a different set of model parameters (Fig. S5), was used to display the quality of the fit and to make model predictions. To do this, the model was simulated 10,000 times for each particle with the mean and 95% confidence interval from these simulations being displayed (Fig. 3B-C). Although the ODE model represents the reactions within a single cell, and hence the dose-response curve for a single cell would exhibit a perfect switch (with an infinite Hill number), the population averaged dose-response curves from the model include particles (i.e. cells) with different parameter values accounting for population heterogeneity. It is noted that the posterior distribution of only a subset of the model parameters exhibited a tight distribution, suggesting that only a limited number of parameters could be uniquely identified (Fig. S5). Nonetheless, we were still able to make predictions using the model by simulating different experimental conditions for all particles in the final population (see Fig. 4B and Fig. 5B).
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Author contributions. NT, PK, JN, and SG performed experiments; NT, PK, and OD performed the mathematical modelling; NT, PK, JN, SG, and OD analysed data; NT, PK, and OD designed the research and wrote the paper; NT and PK contributed equally. All authors discussed the results and commented on the paper.

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