Published in final edited form as:

Immunol Lett. 2017 December ; 192: 61–71. doi:10.1016/j.imlet.2017.10.005.

Density dependent re-tuning of autoreactive T cells alleviates their pathogenicity in a lymphopenic environment

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Summary

Peripheral T cell tolerance is challenging to induce in partially lymphopenic hosts and this is relevant for clinical situations involving transplant tolerance. While the shortage of regulatory cells is thought to be one reason for this, T cell-intrinsic tolerance processes such as anergy are also poorly triggered in such hosts. In order to understand the latter, we used a T cell deficient mouse model system where adoptively transferred autoreactive T cells are significantly tolerized in a cell intrinsic fashion, without differentiation to regulatory T cells. Intriguingly these T cells often retain sufficient effector functions to trigger autoimmune pathology. Here we find that the high population density of the autoreactive T cells that accumulated in such a host limits the progression of the cell-intrinsic tolerance process in T cells. Accordingly, reducing the cell density during a second transfer allowed T cells to further tune down their responsiveness to antigenic stimulation. The retuning of T cells was reflected by a loss of the T cell's abilities to proliferate, produces cytokines or help B cells. We further suggest, based on altering the levels of chronic antigen using miniosmotic pumps, that the effects of cell-density on T cell re-tuning may reflect the effective changes in the antigen dose perceived by individual T cells. This could proportionally elicit more negative feedback downstream of the TCR. Consistent with this, the retuned T cells showed signaling defects both proximal and distal to the TCR. Therefore, similar to the

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immunogenic activation of T cells, cell-intrinsic T cell tolerance may also involve a quantitative and progressive process of tuning down its antigen-responsiveness. The progress of such tuning seems to be stabilized at multiple intermediate stages by factors such as cell density, rather than just absolute antigen levels.

Introduction

Studies into the mechanisms of immunological tolerance have led to the development of many clinical strategies for treating autoimmune disorders or facilitating transplant tolerance. [1–6]. The clinical successes of such strategies are sometimes hampered by factors that were not originally anticipated in the model systems used for their discovery. One such variable is the state of the lymphoid niches in many patients who are the intended recipients of these therapeutic interventions [7–9]. The use of immunosuppressive drugs or radiation exposure during autoimmune disorders or transplantation can leave patients with various degrees of lymphopenia for extended periods of time. Unfortunately, many clinical tolerance regimens are ineffective under these conditions – either due to the paucity of regulatory T cells and/or the overtly aggressive behavior of effector T cells under lymphopenic conditions [10–12]. While the former can be compensated adoptive transfer of T-regs, there has been no significant progress towards a definition of strategies controlling the latter.

Aggressive T cell activation can be controlled in a cell intrinsic fashion. The earliest evidence for this is a phenomenon known as T cell anergy – a form of biochemical silencing wherein the cell remains viable, but with greatly reduced ability to respond pathogenically to its cognate antigen [13, 14]. In many cases, *in vivo* equivalents of T cell anergy can be reversed by removal of antigen and tuned to the strength of stimulation [15]. Similarly other states where T cells “turn off” to chronic viral or self-antigens, such as exhaustion have also been described as progressive and quantitative states [16]. Overall many of these states of T cell “tolerance” share some aspects of reduced sensitivity to antigen (see [17] for a more detailed discussion). This led us and others to suggest that peripheral T cells employ a form of tuning – a mechanism originally envisioned by Grossman and Paul in which T cells faced with chronic stimulation adjust their activation thresholds such that the ambient levels of self-antigen can no longer activate them [18]. Arguably, such a mechanism could also have an evolutionary advantage over clonal deletion, as it retains a larger repertoire of T cells in the peripheral compartment rather than eliminate every self-reactive receptor [2]. But leaving these cells alive in the immune compartment carries with it the risk of autoimmunity, if their anergy is somehow disrupted.

In fact, one of the factors thought to disrupt such a tolerance is lymphopenia [9, 19, 20]. However, we have previously shown that even under lymphopenic conditions, the core biochemical signatures of the anergic state (blunted TCR signaling and IL-2 secretion) were in fact preserved [21]. However, the tolerant T cells do show enhanced differentiation and survival in a lymphopenic environment [21, 22]. This survival is not a cell intrinsic property, but reflects a lack of competition from specific neighboring T cells [22]. From a clinical standpoint, the development of immunopathology by such amplification mechanisms, in

spite of T cells having tuned down their intrinsic ability to respond to antigen, remains a severe hurdle. An important question is whether a degree of tolerance sufficient to eliminate all effector functions can indeed be achieved – purely by intrinsically tuning down the T cell – i.e. without transferring in a trans-acting dominant regulatory cell or competitor. This would allow us to fine tune therapeutic strategies in transplantation (such as costimulatory blockade etc.) under existing clinical protocols.

In this report, we therefore concentrate on strategies to eliminate this residual disease even in a lymphopenic context [21]. We were able to do so with a laboratory protocol involving a second transfer of the T cells – a process that has previously been shown to trigger a re-tuning of the cells, resulting in a deeper state of tolerance [23]. Since this occurs in the continued absence of other regulatory T cells, in a lymphopenic host, this is the first demonstration of the exclusive sufficiency of a cell-autonomous process for peripheral tolerance, *in vivo*. In order to understand how the second transfer eliminates autoimmune pathology we examined the cellular and molecular mechanisms facilitating re-tuning of the T cells.

We find that the re-tuning occurs in response to the change in T cell density and is influenced by the activation history of the T cell. Chronic but not acute changes in antigen concentration could re-tune the T cell suggesting that acute infections are unlikely to change this basic state. Finally we also map the broad molecular networks underlying the deeper state which (after future investigations) might suggest potential therapeutic targets for achieving stable cell-intrinsic tolerance even under lymphopenic conditions.

Materials and Methods

Mice models and adoptive transfer

All mice experiments were performed in compliance with the guidelines of their Institutional Animal Care and Use Committee (IACUC). Mice used in these experiments were generated by breeding at the NIAID contract facilities, Taconic farms (Germantown, NY), or at the NIAID shared animal facilities (Bethesda, MD) and maintained under SPF conditions. The B10.A,RAG2^{-/-},5C.C7 TCR transgenic and the B10.A,PCC⁺,CD3e^{-/-} models have been previously described [21, 23]. Adoptive transfer experiments involved the sub-orbital injection of the required number of T cells (1 million, if not specified) using unpurified single cell suspensions from the lymph nodes of the former, which are composed of >90% naïve CD4⁺ TCR transgenic cells. In retransfer experiments, T cells were enriched by negative selection (see below) before injection. In a typical adoptive transfer experiment, the expansion of T cells *in vivo* was quantitated at multiple time points by euthanizing the recipients. All of the visible lymph nodes and the spleen was isolated from the mice and single cell suspensions of cells counted to figure out total cellularity. Following this, an aliquot (typically 10–20 million total cells) were then stained for Flow cytometry (see below) to identify the fraction of 5C.C7 donor derived T cells in the recipient. This fraction was multiplied with the total cellularity in order to count the total T cells recovered at each time point.

Purification and *in vitro* assays

T cells were enriched *ex vivo* using a negative selection protocol that has been described previously [15]. Briefly, single cell suspensions of spleen and lymph nodes were bound to a cocktail of antibodies targeting non T cells (B220, CD11b, NK1.1, CD8a and Class II) and then to anti-mouse or anti-rat coated Dynabeads (Life Technologies, CA). This fraction was separated using magnetic beads and the enriched T cells used for downstream assays. Occasionally T cells were sorted using a flow cytometer (BD ARIA II or BD FACS Vantage, at the NIAID Flow Cytometry core facility, by Carol Henry or Cal Eigsti) after the above enrichment process. In this case the T cells were labeled with CD4 (GK 1.5 PE or APC, BD Biosciences, CA) and TCR V β 3 (KJ-25 PE, BD Biosciences, CA). For *in vitro* measurements of cytokines, 10,000 T cells were stimulated with 10 μ M MCC 81-104 peptide (Anaspec, CA) in the presence of 250,000 irradiated splenocytes from B10.A,CD3e^{-/-} mice. Culture supernatants were isolated at 60 hours and assayed for cytokines.

Measurement of cytokines and Immunoglobulins

The culture supernatants were assayed for cytokines (IL-2, IFN γ) by ELISA using Quantikine ELISA kits (R&D, MN). Serum Ig levels were measured as previously described [21]. Briefly, plates coated with anti-kappa or anti-lambda antibodies (Caltag/BD biosciences, CA) were blocked with BSA and bound to dilutions of serum. Specific antibodies to murine Ig isotypes, coupled to HRP were then used to detect bound antibodies.

Intracellular staining for signaling molecules

1–2 million T cells purified as described above were stimulated *in vitro*. T cells were labeled on ice with 10 μ g/ml biotinylated anti-CD3 (2C11, BD Biosciences, CA) and anti-CD4 (GK1.5, BD Biosciences, CA) and washed in cold 1xPBS. The antibody coated cells (in 100 μ l) were added to a pre-warmed solution of 5 μ g/ml Streptavidin (Jackson Immuno, ME) in 1xPBS at 37°C. At various time points, the reactions were stopped by directly adding excess of 4%PFA (buffered in 1xPBS). The fixed cells were permeabilised with 0.1% Triton X-100 and stained with antibodies to detect specific signaling intermediates as described in the text (all from BD Biosciences, CA).

Analysis of Flow Cytometry

Flow Cytometry data was analyzed with the FlowJo software (Treestar). For CFSE data, T cell proliferation was quantitated using the proliferation plugin in FlowJo. In order to estimate the proliferative potential of T cells, the effective precursor of each generation of T cells was calculated by dividing the number of T cells in each peak of cell division by (2^{^(generation number)}) as described in [24]

Implantation of mini-osmotic pumps

For the implantation of mini-osmotic pumps (Alzet, CA) mice were anesthetized by intra-peritoneal injections of 0.25 % Avertin (0.4–0.5 ml). The hair about the ventral abdominal skin was shaved, the skin disinfected with 3 alternating scrubs of Betadine and alcohol-based disinfectants, and a vertical midline incision about 5 mm long was made through the skin, peritoneal and muscle layers. Alzet model 1002 osmotic pumps certified for mouse

implants were inserted through the incision and placed in the peritoneal cavity. The peritoneum was sutured with a sterile absorbable suture, and the skin closed with a surgical clip. A mild topical analgesic was applied just before skin closure. Surgical clips were removed 5–7 days after the surgery.

Microarray analysis

T cells from 1R or 2R mice were enriched by negative selection and FACS sorted to greater than 99% purity as described above. Sorted T cells were suspended in Trizol reagent (Invitrogen/Life Technologies, CA) followed by homogenization by spinning through a Qiashredder column (Qiagen, MD). 0.2 volumes of chloroform was added to the lysate and the aqueous phase separated out after centrifugation. An equal volume of 70% Ethanol was added to this phase and the suspension passed through a RNeasy mini column (Qiagen). Bound RNA was eluted and quantitated. 50ng of total RNA was then amplified using the Ovation RNA amplification system v2 (Nugen, CA), purified with DNA Clean & Concentrator -25 (Zymo Research), labeled with Cy3 using the Agilent ULS protocol. A standard reference RNA used in the laboratory derived from pooled mouse T cells was similarly labeled using the Cy5 dye. The two probes were mixed and hybridized to Agilent Mouse V2 chips at the NIAID Microarray core facility (Qin Su and Tim Myers). Data extracted from the Genepix software was subjected to quality control on the mAdb platform (NIAID). Eight 2R arrays and five 1R arrays were selected for further analysis, based on the QC. Subsequent analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team.

Online Supporting Information

The extended list of significant genes derived from the microarray analysis (Supporting Information Table 1) and further network models (Supporting Information Figure 2a and 2b) are provided as online Supporting Information.

Results

1. Re-tuning eliminates the pathogenicity of autoreactive T cells in lymphopenic hosts

The adoptive transfer of the 5C.C7 TCR transgenic T cells, specific for a peptide (81–104) from Pigeon Cytochrome C (PCC) has been previously used by us and others to model the fate of naïve CD4 T cells to activation in vivo [15, 23, 25]. Importantly, in recipient mice that are also transgenic of the PCC antigen, these T cells undergo chronic stimulation. We have previously shown that 5C.C7 T cells are overly aggressive in PCC expressing that are also genetically lymphopenic (CD3e^{-/-}) and trigger an autoimmune pathology marked by swelling in the limbs and digits (Fig. 1a – top panel) as well as the loss of joint flexibility [21]. This pathology requires PCC antigen as well as the lymphopenia, as we have previously shown [25]. We examined if the 5C.C7 T cells recovered from such a transfer recipient would still be pathogenic in a fresh PCC+CD3e^{-/-} host. Interestingly, even 6 months after this second transfer, we could not detect any clinical symptoms in the new host (2R - Fig. 1a). While the transfer of naïve T cells induced scorable pathology, the limbs of re-transfer recipients were not different from control mice even at 8 weeks (Fig. 1b). A further transfer of the 2R-derived T cells to another fresh host (3R - Fig. 1b) also failed to

trigger pathology anew. We have previously shown that the manifestation of pathological symptoms due to naïve 5C.C7 T cell transfer correlated with the development of class switched anti-self-antibodies [21]. In contrast, there was no significant increase in serum levels of switched Igs in recipients with a second or third successive transfer of T cells (Fig. 1c). Therefore, even in a lymphopenic environment, the re-transfer of self-reactive tolerant T cells abrogates any residual helper activity and pathogenicity.

Since the rest of the experiments in this report involves the use of successive transfers of the 5C.C7 T cells through new PCC+CD3e^{-/-} hosts, we will define a terminology that will be used here. The first host is always referred to as the 1R host and the second and third hosts as 2R and 3R respectively. Therefore when Naïve 5C.C7 T cells are injected into the PCC +CD3e^{-/-} host for the first time, the recipient becomes 1R and the T cells that are resident in this host become 1R (or 1R-derived) T cells. Importantly, the proliferation that is measured during the engraftment of naïve T cells into this host reflects the proliferative ability of the naïve T cell – as it goes into a state that is identified as a 1R state.

At the cellular level, the transfer of 1R-derived T cells to a new PCC+CD3e^{-/-} host (setting up a 2R recipient and 2R-derived state of T cells) is already known to induce a re-tuning of the T cells resulting in further losses in its ability to proliferate, secrete IL-2 etc. This was previously called a deeper state of tolerance [23]. We now show that, in fact, this progression continues quantitatively down through to the 3R state. In the primary host (1R), the naïve T cells go through a robust period of expansion followed by a stable plateau phase that maintains up to 20 million T cells in the animal (closed circles, Fig. 1d). At the plateau phase, the T cells are significantly tolerant to antigenic re-stimulation – with a >90% loss of cytokine production ability, relative to acutely stimulated 5C.C7 T cells (Fig. 1f). Re-transfer of these T cells to a fresh host (2R) results in a significantly reduced burst and a lower plateau (Fig. 1d open squares). These T cells show a further 3–4 fold reduction in cytokine production relative to the primary tolerant T cells (Fig. 1f). Interestingly, an additional transfer of the 2R-derived T cells to a new PCC+,CD3e^{-/-} host (3R - Fig. 1d filled triangles) yielded no further increase in the number of the T cells (Fig. 1d) or significant proliferation (Fig. 1e). An analysis of the CFSE profiles of these cells (Fig. 1f) shows that ~80% of the 2R-derived cells which went into a new PCC+CD3e^{-/-} host (as they progress to a 3R state) remain undivided up to 4 days. This is in contrast to the 1R-derived cells, of which >90% of the cells had divided by this time in their new (2R) host. There was also no significant differences in cell death as marked by Annexin V expression in the 1R and 2R T cells (data not shown). In order to broadly estimate any cell loss in the transfer of these cells, we used the T cell numbers and CFSE profiles to back calculate the precursor equivalents in each transfer remaining after 4 days of transfer (Fig. 1g). Since the day 1 cells have not divided, the total T cells recovered at this time indicate the amount of cells that seeded the nodes and spleen of the recipient mice. At day 4, we then calculated (dividing the cell numbers in each peak of CFSE by (2ⁿ—where n is the generation number) how many of the original cells we can account for at this time. As shown in Fig 1g, no significant difference was observed in the day1 to day 4 recovery between each group, suggesting that disproportionate cell death is not involved here. It should be noted that any subtle differences in cell death – especially those limited to very highly divided cells – will be missed in this form of analysis.

The few cells that could be recovered from the 3R host, show a profound blockade in antigen responsiveness (Fig. 1h). Control groups that were acutely challenged with antigen however retained significant ability to secrete cytokines. Since the 3R state yielded very few cells for further study and the 2R host was already devoid of immunopathology we focused on characterizing the latter. Similar to the 1R-derived T cells, there was no significant increase in the induction of Foxp3⁺ T cells in the 2R-derived host either (Fig. 1j) – illustrating the possible lack of dominant inhibitory processes in this model system.

Taken together, these data suggest that in the context of the 2nd transfer, a largely cell intrinsic and further loss of T cell responsiveness to antigen occurs. The absence of pathology in the 2R hosts therefore suggests that this (recessive) tolerance mechanism when allowed to progress fully is perhaps sufficient to prevent pathology even in a lymphopenic environment. Understanding the cellular and molecular mechanisms regulating the re-tuning process could therefore have profound implications for translating this finding to clinical contexts where lymphopenia has confounded tolerance regimens. We therefore used the 5C.C7 model to characterize the 2R state further.

2. Tuning is not reset by acute perturbations or division history

The process of re-transferring the primary tolerant 5C.C7 T cells to fresh PCC⁺,CD3e^{-/-} hosts introduces several changes in the system, any of which could be triggering the re-tuning. First of all, the re-transfer is comparable to a renewed stimulation of the T cells as the grafted T cells encounter a fresh bolus of antigen presentation in the new host. Secondly, as a consequence of the fresh re-stimulation, the T cells undergo several additional rounds of cell division. Furthermore, in contrast to the primary transfer, where the T cells being transferred are in the naïve state, the subsequent ones use antigen-experienced T cells. We therefore asked if systematically tweaking any of these parameters would allow the induction of a deep state in the primary host itself (without the need for a re-transfer).

First, we asked if an acute re-challenge of antigen in the primary host results in T cell re-tuning. A single bolus of PCC peptide (1 mg) was injected ip, 28 days after the primary transfer of 5C.C7 T cells to a PCC⁺,CD3e^{-/-} host. The peptide also triggered a proliferative burst that was quite transient and resulted in a 2–3 fold expansion of the T cells (Fig. 2a). At various days after the transfer, we then examined the level of tuning in the T cells by measuring IL-2 production in isolated and re-stimulated T cells (Fig. 2b). Although there was a slight increase in IL-2 production by the T cells at the early time points after the second challenge (blue squares, Fig. 2b), by 2 weeks it was restored to levels similar to that routinely seen in the primary tolerant T cells (Fig. 2b). This level of tuning was then robustly stable for up to 60 days. Peptide re-challenged or unchallenged 1R-derived T cells also proliferated equivalently on an *in vivo* retransfer (Fig. 2c) suggesting that an acute re-stimulation was insufficient to trigger stable re-tuning.

Next, we examined if the level of tuning was influenced by the extent of cell division undergone by the T cells. Lymphopenic hosts have previously been shown to allow extended T cell proliferation – as lower numbers of transferred cells try to fill up the niche in the periphery. We first examined if we could induce a greater expansion in the naïve T cells, by simply transferring lower numbers of T cells to the PCC⁺,CD3e^{-/-} host. Indeed, transferring

log dilutions of T cells resulted in greater expansion of the transferred cells as each cohort tended to reach the same steady state plateau density (Fig. 2d). This allowed us a model to examine the consequence of a 1000 fold range of T cell expansion on tuning. Interestingly, after reaching the stable plateau levels, the level of tuning (as measured by IL-2 production upon re-stimulation *in vitro* (Fig. 2e) or *in vivo* proliferation on an acute retransfer to a fresh PCC+,CD3e^{-/-} host (Fig. 2f)) was not significantly different, no matter how much the original naïve T cells had expanded.

Finally we examined if the previous activation status of the 5C.C7 T cells could influence the subsequent level of tuning achieved. Primed (but non-tolerant) T cells were generated in antigen-deficient CD3e^{-/-} hosts as previously described [15]. Briefly, 5C.C7 T cells transferred to CD3e^{-/-} hosts were challenged with an intra-peritoneal injection of PCC-peptide mixed with LPS, to generate a typical pre-activated population. Cells isolated from these mice were transferred to PCC+,CD3e^{-/-} hosts. Adoptive transfer of the pre-primed T cells resulted in a rapid expansion initially (Fig. 2g). However, they peaked lower than naïve T cells and subsequently stabilized at a plateau that was 5 fold lower than the 1R levels and not significantly higher than that of the 2R host. Interestingly, in their plateau phase, the tolerant T cells resulting from a previously primed population had downregulated IL-2 production almost as deeply as the 2R T cells (Fig. 2h). They also down regulated IFN γ secretion, to a similar degree, although the absolute amount was higher than those made by the 2R T cells (Fig. 2i). Therefore the previous activation state of the T cells clearly contributed significantly to the eventual level of tuning. These data also suggest that quite counter-intuitively, a previously primed “memory” phenotype T cell is more strongly tolerisable than a naïve one.

These results seemed paradoxical, since the 1R T cells are in fact constantly engaging with cognate antigen in the PCC+ mice; yet this “re-stimulation” of the cells does not push them into a deeper state. As discussed above, even the secondary challenge of these “previously activated” 1R T cells with a bolus of antigen sufficient to drive a further proliferative burst, does not re-tune the cells.

3. A changed T cell density triggers re-tuning, despite identical antigen expression

A potential explanation for the critical necessity for an adoptive transfer of 1R T cells to a fresh PCC+,CD3e^{-/-} host in order for a re-tuning process to take place might stem from a quantitative variable inherent in the adoptive transfer. In typical adoptive transfer experiments, only 10–20% of the transferred T cells successfully seed the peripheral lymphoid organs with most others presumably being scavenged in the lung and liver. In the 1R host, the 5C.C7 T cells reach a plateau level between 15–20 million T cells. When we inject 1 million of these cells are injected to a fresh host, considering the seeding efficiency, this would result in a 100–200 fold reduction in the density of the 1R T cells. We therefore examined if this change in T cell density was a critical variable in re-tuning the T cells. In order to test this, we transferred 120 million purified 1R T cells to a fresh PCC+,CD3e^{-/-} host and interrogated the responsiveness of the cells 39 days later (Fig. 3a). Relative to the 2R state resulting from the transfer of a million 1R T cells, those stemming from a 120 million cell transfer maintained IL-2 expression at levels similar to that of a 1R – suggesting

that they had not undergone significant re-tuning (Fig. 3a). In a titration of T cells from 100 million to 0.1 million 1R T cells injected into fresh PCC⁺, CD3 $\epsilon^{-/-}$ mice, the complete re-tuning was observed only when the T cell density was reduced to a million T cells – i.e. by injecting 10 million or less 1R T cells (Fig. 3b & 3c). This striking result demonstrates the critical role of T cell density in re-tuning peripheral responsiveness to a self-antigen.

The success of a peripheral tolerance mechanism based on T cell tuning hinges on the cell's ability to calibrate activation thresholds in precise correspondence to the level of peripheral antigen. The fact that the 5C.C7 T cells change the level of tolerance in mice with identical antigen expression – simply by changing T cell density – would, at first glance, seem to challenge this principle. However, a change in cell density has another consequence. Since T cells can compete for antigen during an immune response, a higher density of T cells could effectively lower the stimulus available for each T cell – prompting it to tune proportional to the apparent dose rather than the real one. One prediction from this model would be that if one were able to alter the level of antigen presentation in the 1R host itself, the T cells should re-tune. The failure of an acute stimulus to do so, could be because of the need for an extended duration of stimulation for stable tuning to accumulate. We therefore examined if chronically altering antigen presentation by implanting mini-osmotic pumps would alter the level of tuning in the 1R host. Indeed, 12 days after pump implantation, peripheral T cells in the 1R host had further down-regulated IL-2 production by another 3 fold (Fig. 3d). Taken together, these data suggest that the antigen activated and hypo-responsive 1R T cells undergo a further re-tuning in response to a stronger, persistent antigenic stimulus, resulting from the reduced T cell density in the fresh host. Furthermore a molecular understanding of the processes regulating the re-tuning process, should allow us to tailor tolerance induction in the clinical contexts of lymphopenia to eliminate any residual immunopathology.

4. Re-tuning involves deeper blocks in TCR signaling pathways

The level of tuning achieved in the 1R state is characterized by a marked decrease in the competency of TCR proximal signaling machinery in the tolerant T cells, starting with a reduced phosphorylation of Zap70 [26]. We therefore compared the robustness of these signaling modules in 1R and 2R T cells. Although in many cases, differences were obvious between naive and 1R T cells and 2R T cells were occasionally less capable of activating kinases such as Zap70 (Fig. 4a), the differences between 1R and 2R T cells were too subtle to be statistically significant over multiple experiments (Fig. 4b). In addition, we have previously reported pharmacological evidence for the nature of the signaling impairments in the 1R state [27]. Since stimulation with the combination of PMA, Ionomycin and anti-CD28 could overcome most of the unresponsiveness in the 1R T cells, it is most likely that the predominant biochemical negative feedback in these T cells operates proximal to the TCR prior to the Ca²⁺ flux and PKC activation. Interestingly, 2R T cells stimulated with these drugs failed to significantly recover IL-2 production (Fig. 4c) suggesting a more “deep” set of biochemical impairments in this state.

We therefore analyzed the transcriptome of T cells in the 1R and 2R states in order to decipher the molecular networks underlying the secondary tuning. A total of 596 transcripts were significantly different ($P < 0.001$) in a class comparison between the two groups

(Supporting Information 2). Interestingly many of the usual suspects, expected to act alone or in concert are not significantly differentially expressed between the 1R and 2R cells (Fig. 4d). Although subtle changes in molecules such as PD1 and LAG3 may perhaps in combination account for the phenotype we observe, preliminary experiments with PD1-knockout 5C.C7 T cells do not support this idea (data not shown). Clearly further work is required to understand the molecular mechanisms better. A handicap in this regard is also the incomplete understanding of the 1R state, so far.

In order to obtain a broad cellular picture of the action of these genes, using the microarray data (which is now publicly available) we computed the network relationships within the significantly different genes and merged the networks with an enrichment score > 35 (Fig. 5). Based on the cellular localization of the gene products and their functional relatedness, the merged network can be classified into 4 discrete functional groups. It should be noted that the network contains genes that are upregulated (Fig. 5, red shades) or downregulated (Fig. 5, green shades) in the 2R T cells as well as those closely associated with either category above – but not actually found in the microarray gene list (Fig. 5, not colored). Also, a more comprehensive version of the network, which takes into consideration indirect relationships between these proteins as well, is shown in Supporting Information Fig. 2a.

The first group revealed by this analysis, located at the cell surface includes molecules such as CD3, CD28, Notch etc., which are associated with the TCR and participate in downstream signaling. The second group of genes includes structural components such as Spectrin, Scinderin, Stk35, Dynein, Rock1, etc. which are known to regulate the actin cytoskeleton [28, 29]. The lower expression of this set of genes in the 2R state suggests impairment in optimal cytoskeletal rearrangement in these cells – which is critical for strong and sustained TCR signaling. In fact, an impairment of a proper immunological synapse formation in the 1R cells has already been implicated in their relative insensitivity to antigen [30]. Furthermore, an additional group of cytoplasmic proteins suggests a range of signaling modulations (Fig. 5, group #3). These include members of the MAPK family as well as components of the NF- κ B cascade. Although these are brought to fore as a result of the changes in expression levels of some molecules involved, often signaling changes are not accompanied by changes in the transcripts for proteins involved in the cascades themselves. For example, the PI3K and PLC γ are revealed in this analysis, although their expression is not significantly different between the groups, based on their known interactions. Also implicated by association is the NF κ B complex itself, in the nucleus.

The most interesting group of genes are present in the nuclear groups found in this analysis. The first of these (Group #4) involves a fascinating array of molecules involved in chromatin remodeling and gene expression. These include genes recently implicated in epigenetic regulation in T cells such as SATB1 [31] or transcriptional regulators like LKLF. In addition, molecules such as Topo2B and Dynactin are known to be modulators of chromatin compaction [32, 33]. This group is especially significant – since the failure of PMA +Ionomycin to rescue the functionality of 2R T cells implicates distal and nuclear elements as potential players at this level of tolerance (Fig. 4c). The necessity to stably remodel the chromatin complexes in order to silence the T cell more effectively may also explain the slower kinetics of onset of the 2R state and the need for prolonged exposure to antigen.

The final group of molecules revealed by the analysis also includes transcription factors and components of the transcriptional machinery. A separate analysis (Supporting Information Fig. 2c) focusing on transcription factor enrichment within the dataset suggests the involvement of four major factors in this process – PPARG, HOXA9, HOXA10 and FOS. While FOS is known to be a major regulator of T cell activation and has been implicated in peripheral tolerance [34, 35], the other factors are relatively unknown in this context. The HoxA9 and A10 genes are adjacent members of the homeobox domain gene group related to *Abdominal-b* in mammals and are involved in differentiation and development of hematopoietic lineages [36]. Although they share many interesting targets, it is notable that the set of genes enriched in our analysis are not all overlapping. In fact the HOXA9 targets are mostly composed of the chromatin modeling and transcriptional regulators discussed above, while the HOXA10 targets include many of the cytoskeletal regulators and signaling modulators. This interesting synergy might suggest a very intriguing new combinatorial activity for these factors in T cell tolerance. Finally PPARG (a member of the retinoic acid receptor R-X-R family) targets are also enriched in the analysis. These are largely genes involved in metabolism and nutrient uptake – which are depressed in the 2R T cells. Taken together, these data describe a picture of the deeper state of tolerance in which multiple cellular functions – signaling, cytoskeletal rearrangement, chromatin remodeling and transcription, are repressed in order to robustly restrain the self-reactive T cells.

Discussion

Peripheral T cells that encounter their cognate antigen in an immunogenic context are thought to progress through a continuum of activation and differentiation events, rather than transition into discrete binary states [37]. In fact, T cells with phenotypes that are intermediate to classically defined benchmark states (such as naïve/effector/memory or Th1/Th2 etc.) are also becoming increasingly relevant in specific clinical scenarios [38]. Nevertheless, in the context of self-tolerance, the fate of T cells that encounter a peripheral self-antigen, is often described as a binary state – possibly reflecting the influence of early studies on clonal deletion and clonal inactivation as digital states of tolerance [39–41]. We and others have argued that cellular tolerance can also span a range of responsiveness to antigen and that immunopathology can result from an insufficient progression of the tolerance process [21, 42]. One instance where such an incomplete tolerance is often observed is under conditions of lymphopenia [8, 9]. Under the assumption that cellular tolerance is digital, the lymphopenic environment has therefore been claimed to “break” tolerance [11, 19, 20]. Our demonstration that the progression of T cell hypo-responsiveness to a deeper state is sufficient to eliminate this residual pathology is therefore not only (potentially) clinically relevant but also biologically revealing. It supports the argument that cell intrinsic tolerance is a tunable quantitative trait - affected by factors such as T cell density, in addition to the strength of stimulation. It suggests potential remedies for promoting the progression of tolerance further, especially under lymphopenic conditions. Of course, the cellular strategies we employed here – i.e., density reduction and osmotic pump implantation, while quite valuable towards demonstrating the concept, may only be directly translatable to a rather limited set of clinical scenarios. But several other options can now be considered, in the light of this particular perspective.

The simplest of these options relate to modulating the stimulus itself. In our model, chronically increasing the antigenic stimulation (using mini-osmotic pumps) to overcome the inhibitory effect of cell density leads to a level of T cell tuning that is more suited for preventing immunopathology. By extension, in clinical contexts, increasing the strength of the tolerising regimen appropriately would likely be effective – a concept that is neither difficult to implement nor alien to the field [43]. In the case of therapies involving immunomodulatory agents, this can be easily tailored to the context of lymphopenia. However, in cellular strategies such as donor-specific transfusion or hematopoietic microchimerism, the titration is likely to require more careful optimization. The molecular data we present should also be helpful in the design of novel therapeutics that are potentially combinatorial in nature and target the multiple nodes of tunability observed in this model. Additional studies will be required to shortlist and develop these strategies further.

Another interesting aspect of the study is the fact that previously activated (“memory” phenotype) T cells can be tolerised to a greater degree than naïve T cells. Although counter-intuitive, this is in fact consistent with the idea that cell-intrinsic tolerance is achieved by tuning the T cell’s response to the amount of ambient stimulation [44, 45]. Since memory T cells have assembled signaling machinery to respond to antigen better [46], they are likely to receive a stronger stimulus from the self-antigen and therefore induce a greater level of tolerance. Alternately, some of the changes in signaling that a previously activated T cell has undergone (upregulation of certain negative regulators such as PD1, SHP1 or the down-regulation of some aspects of TCR signaling) might allow the tuning machinery to act more potently [47, 48]. It is also interesting to note that despite tuning down their responsiveness to antigen more profoundly, the tolerant T cells generated from previously activated T cells (1PR) still made more amounts of the effector cytokine IFN- γ compared to those generated from naïve T cells (1R). The decrease in IL-2 production as a result of tuning down a pre-activated T cell is about 30 fold (2R levels), while tuning down a naïve T cell effects a ~10 fold reduction (1R levels). The effector cytokine production in the former is also depressed about 30 fold; however since the pre-activated T cell had already differentiated significantly to make a much larger amount of IFN γ before the retransfer, the subsequently tuned T cell still ends up making this cytokine at a level comparable to the 1R level and not the 2R one. Of course, if only the absolute amounts of the cytokine were measured, this conclusion would be difficult to arrive at. And in fact, the fact that they do make more absolute amounts of effector cytokines may also make them more pathogenic. This is an important distinction when extrapolating tolerance at the single T cell level to systemic peripheral tolerance. Depending on the initial state of the T cell, identical operation of the tolerance process can lead to very different outcomes – based on the parameters that are being measured.

Acknowledgments

We thank the NIAID Flow Cytometry core (Cal Eigsti and Carol Henry) for FACS sorts, the Genomics core (Tim Myers and Qin Su) for hybridization and Pascal Chappert for discussions. This research was supported by NIH R01-AI110719 and NIH R21-AI126184 awarded to NJS.

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Highlights

1. T cell intrinsic tolerance – without Tregs OR deletion - can prevent immunopathology
2. Clonal density of self-reactive T cells limits attainment of “complete” tolerance
3. High clonal density reduces effective antigen perception by T cells.
4. Antigen experienced T cells can be directly tolerised to a deeper state.
5. Molecular pathways affected by progressive tolerance are profiled.

Figure 1. A re-tuning of auto-reactive T cells eliminates immunopathology

a. Hind limbs of PCC⁺, CD3^e-mice 64 days after receiving naïve 5C.C7 T cells (1R, top panels) or a re-transfer of 1R T cells (2R, bottom panels).

b. Secondary transfers of T cells abrogate pathology. The clinical scores of mice that received naïve 5C.C7 T cells (called the 1R host), or mice that received 5C.C7 T cells that were re-purified from a 1R animal (the recipient now called a 2R host) or mice that received cells re-purified from a 2R animal (the recipient now called a 3R host). Scoring was done on examination of mice 8 weeks after each T cell transfer. The differences between the 1R hosts with either the 2R ($P=0.003$ in a Two-tailed t Test) or 3R ($P=0.0033$) hosts are highly significant while the scores of the 2R and 3R hosts are not significantly different between themselves ($P=0.76$ in a two-tailed T test).

c. Total IgG1, IgG2a and IgG2a levels in serum of the recipients of each successive transfer (as in 1b) measured 8 weeks after T cell infusion, using isotype specific ELISA. The levels of each immunoglobulin isotype in the 1R group was significantly ($P<0.01$ in multiple 1way

ANOVA comparisons) different from those in the 2R and 3R sera samples. However, neither 2R nor 3R sera samples were significantly different from each other ($P=0.625$ in a Two-tailed non parametric t test with Mann-Whitney correction).

d. The number of 5C.C7 T cells recovered at various days after transfer in the case of 1R hosts (open red squares), 2R hosts (open blue squares) and 3R hosts (open black squares). It should be noted that this is essentially measuring the behavior of naive T cells (upon their first transfer into the 1R host), 1R T cells (as they enter a fresh PCC+, CD3 ϵ -host to become a 2R host) and 2R T cells (as they proliferate in a new PCC+, CD3 ϵ -host to become a 3R host. Data shown are from three independent mice for each group, i.e. $n=3$ per time point. The average cell recovery and standard deviations are plotted at each time point

e. CFSE dilution profiles from a representative experiment at 1 day (black), 4 days (red) and 7 days (blue) after transfers in the 1R (top panel), 2R (middle panel) or 3R (bottom panel) are shown. While the black peak indicates the position of undivided T cells on Day 1, it should be noted that passive loss of CFSE alters the location of the undivided peak in successive days. For instance, the bulk of the 3R cells remain undivided over the experiment, although a shift in the actual intensity of the CFSE-highest T cell population can be observed over the 3 time points

f. Data from five independent mice ($n=5$) at the day 4 time point as in (g) are quantitated. The numbers of T cells that remain undivided are calculated by multiplying the fraction of cells in the CFSE-highest peak with the actual number of T cells recovered from each mice. Importantly, no undivided T cells were detected in the 1R host at this time point. There were significantly more undivided T cells in the 3R host compared to the 1R ($P=0.0004$ in a two-tailed t test) or 2R ($P=0.0007$). Given that the 1R hosts did not tend to have undivided T cells, the undivided cells the 2R hosts were significantly different as well ($P=0.0094$).

g. In order to estimate the survival of the transferred T cells between days 1 and 4, we plotted the recovery of cells on day 1 (left box-and-whisker plots in each of the column-pairs) when the transferred cells were all undivided (CFSE-high). We then back-calculated the effective precursor frequency of the cells on day 4 (as described in methods) to estimate what the effective cell number would be if no division had occurred. The lack of any significant difference between the day 1 and day 4 plots suggests that there was no differential cell death in these groups up to this time point.

h. IL-2 secretion over 48 hours of in vitro stimulation is progressively lost in successive transfers to PCC+,CD3 ϵ -hosts (1R, 2R and 3R) while successive transfer and acute re-challenge of primed T cells in PCC-ve,CD3 ϵ -hosts (1M, 2M and 3M) retains responsiveness.

i. Expression of activation markers (CD44 and CD62L) shows the progressive differentiation of 5C.C7 T cells from naive (no transfer yet; left panel) to 1R (cells recovered 7 days after transfer from 1st host; middle panel) and 2R (cells recovered 7 days after transfer from 2nd host; right panel) stages. Plots are gated on TCR-V β 3+, CD4+, 7AAD-ve cells and representative of three mice in each group.

j. Neither the T cells from a 1R host (left plot), nor 2R (right plot) T cells show significant expression of Treg markers. Plots are gated on TCR-V β 3+, CD4+, 7AAD-ve cells and representative of three independent experiments at days 4, 7 and 65 after transfer.

Figure 2. Role of T cell activation state on the re-tuning of tolerant T cells

a. T cells in a stable plateau phase of a 1R host challenged on Day 28 with PCC acutely (blue lines) showed a transient expansion while unchallenged 1R T cells maintained a stable plateau (red lines). Data are from n=3 to 4 mice per time point for each group over the time course shown.

b. T cells isolated from 1R hosts, various days after acute challenge (now called 1Rp cells - blue lines with blue open squares; n=3 mice per time point) produced similar amounts of IL-2 upon *in vitro* re-stimulation, as those from unchallenged 1R (red lines with red open squares; n=3 mice per time point). Both were ~90% lower than naïve T cell secretion (black line, with black closed square). The IL-2 production from naïve T cells (black line) is provided here for comparison. Note that each naïve 5C.C7 T cell data point is from cells freshly isolated from a 5C.C7 TCR transgenic mouse and stimulated *ex vivo* in similar conditions as the 1R and 1Rp cells at each time point –before the supernatants were used for IL-2 ELISA.

c. *In vivo* proliferation of Naïve 5CC7 T cells (N, top panel), T cells recovered from an 1R host with no additional peptide challenge (1R, middle panel) or T cells recovered from an 1R host that was acutely challenged 14 days ago with an additional bolus of peptide (1Rp, bottom panel). In order to assess their proliferation, a million of each of these cells were CFSE labelled and transferred to fresh PCC+,CD3e-hosts. Cells were recovered from these hosts 1 day (Black lines in each plot) or 4 days (blue lines) after this transfer. Plots are representative of 5 mice each from two separate experiments. Preliminary analysis of CFSE

dilution as discussed in methods do not reveal any differences between 1R and 1Rp cells in this assay (data not shown).

d. In order to evaluate the density dependent effect on T cell retuning, different numbers of naïve T cells were transferred to new PCC⁺,CD3^e-hosts – effectively recreating a 1R host but with different levels of input naïve T cells which would have to proliferate differentially to reach a similar plateau. The proliferative expansion of 50 million Naïve T cells (open red triangles; *note that the size of each triangle symbol on this graph matches with the dilution of the input cells*), 5 million Naïve T cells (open blue triangles), 0.5 million Naïve T cells (open brown triangles), 0.05 million Naïve T cells (open orange triangles) or 0.005 million Naïve T cells (gray closed triangles) in a fresh PCC⁺,CD3^e-host. Given the scope and time point of these experiments, the data in each group represents cells recovered from one mouse per time point per group. Repeats of this experiment with different combinations of the above are shown in supplementary figure 1.

e. The IL-2 production from the T cells recovered from (d) at each time point (now equivalent to 1R state T cells) upon stimulation with fresh splenocytes presenting 10uM peptide, is compared to that of naïve T cells (black squares) stimulated under similar conditions for 48 hours (n values same as d).

f. The in vivo proliferative potential of the cells recovered 22 days after transfer as in (d) was examined by injecting them into fresh PCC⁺,CD3^e-hosts. Typically a million cells are injected – and the recovery of T cells 60 hours (2.5 days) and 84 hours (3.5 days) after transfer was enumerated. The data is presented as a fold expansion relative to the recovery on day 1. Each datapoint is an average of recovery from two mice (n=2 per time point, per group); however repeat experiments are presented in Supplementary figure 1a.

g. Expansion of naïve 5C.C7 T cells (open black squares; setting up a 1R host), previously primed (open red triangles; essentially setting up a 1R host – but with previously activated T cells as the input – referred to as PR hosts hitherto) or 1R T cells (closed blue triangles; setting up a 2R host as in Figure 1, in fresh PCC⁺,CD3^e- hosts. (n=3 mice per time point per group)

h. T cells recovered from 4–5 mice per groups (at 14 or 38 days) were independently stimulated ex vivo with fresh splenocytes and 10uM cognate peptide. The production of IL-2 (h) was measured in the culture supernatants from the cells that were either naïve (N), Primed (P) or recovered from 1R, 2R or PR hosts

i. IFN- γ production measured in the same cultures as in (h).

Figure 3. Density dependent re-tuning of tolerant T cells

a. The ability of T cells recovered 39 days after transfer from a 1R host (open red squares) to make IL-2 after ex vivo stimulation with APCs and cognate peptide is compared to that of naïve 5C.C7 T cells (closed black squares) and cells from a standard 2R host (made by transferring in 1 million 1R T cells to a PCC+,CD3e- host, open green inverted triangles) at the same time. In order to evaluate the role of cell density in the re-tuning to a 2R state, 125 million cells from a 1R host were transferred to a fresh PCC+,CD3e- hosts (blue squares) in parallel. It was necessary to purify 1R T cells from about 15 mice to generate a sufficient cell number for the last time point. Representative data from one animal each (as recipient) is shown; replicates are in (b) and (c).

b. Similar to (a), IL-2 production (at the 10uM dose) from T cells recovered 28 or 40 days later from 2R hosts that were created with different numbers of input 1R cells are shown (closed blue symbols and blue line; n=3–5 mice per point combining 2 separate experiments). The IL-2 is plotted as a percentage of that made by naïve T cells (i.e. relative to the black line in (a) for example)). Also plotted is the relative IL-2 made by 1R T cells (open red square; n=4 mice) generated by transferring naïve T cells to PCC+,CD3e- hosts.

c. The IFN- γ production from the same culture supernatants as in b – normalized to the relative amounts made by 1R T cells (since naïve CD4 T cells do not typically secrete significant levels of this cytokine)

d. IL-2 production during the in vitro re-stimulation of Naïve (N), 1R or 2R T cells are compared those from 1R hosts with a mini-osmotic pump constantly outputting PCC peptide for 14 days (1R+P). n= 4 mice per point as shown.

Figure 4. Molecular alterations underlying the 2R state

- T cells from 1R and 2R cells were stimulated ex vivo with antibodies to CD3 and the levels of TCR signals transduced measured by intracellular staining for the tyrosine phosphorylation of Zap70 at the Y292 position by flow cytometry. Levels of intracellular pZap70-Y292 at baseline – i.e. zero minutes (orange histogram), 0.5 minute (green), 1 minute (blue) and 5 minutes (red) after stimulation of Naïve, Primed, T cells from 1R or 2R indicated hosts are shown. The plots are a representative histogram for replicates summarized in (b).
- The ratio of peak vs baseline intensities of pZap70-Y292 staining calculated from a Mean Fluorescence Intensity measurement using FlowJo software from multiple experiments as in (a)
- In order to evaluate if a TCR proximal signaling deficit is involved, T cells were stimulated via TCR (using APCs pulsed with cognate peptide – left 3 columns) or using a drug cocktail that bypasses some of the proximal mediators of TCR signaling (right 3 columns). IL-2 secretion after in vitro stimulation of indicated T cell states measures by ELISA are shown.
- The relative expression levels (normalized to a common RNA reference in the microarray experiment) of key negative regulators of T cell activation are shown from comparing T cells from the 1R and 2R group. The microarray analysis does not identify a significant relationship between usual suspects and the re-tuning process, although this requires future analysis.

Figure 5. A birds-eye view of the gene expression changes in the 2R state

A connectivity map of genes enriched in the microarray comparison between FACS sorted 1R and 2R T cells. The top four networks inferred using the Ingenuity platform were merged, orphans deleted and the topology reformatted to show predicted cellular localization of the proteins. Genes in red are upregulated in 2R and those in green are downregulated. The mean ratios are also shown below each gene symbol. In addition, the network also shows additional proteins (not directly detected as different in the arrays) which have experimentally demonstrated interactions to those elements found in the analysis (not colored) as well as protein families that are involved in the pathways shown in the analysis (double ring circles). The arrows show the interactions and experimentally observed

directionalities; however the distance and weight are only scaled for clear representation and not to any biologically relevant criteria.

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