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Review

Death, adaptation and regulation: The three pillars of immune tolerance restrict the risk of autoimmune disease caused by molecular mimicry

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Abstract

Extensive cross-reactivity in T cell receptor (TCR) recognition of peptide-MHC (pMHC) complexes seems to be essential to give sufficient immune surveillance against invading pathogens. This carries with it an inherent risk that T cells activated during a response to clear an infection can, perhaps years later, respond to a self pMHC of sufficient similarity. This lies at the heart of the molecular mimicry theory. Here we discuss our studies on the disease-causing potential of altered peptide ligands (APL) based on the sequence of a single autoantigenic epitope, the Ac1–9 peptide of myelin basic protein that induces experimental autoimmune encephalomyelitis in mice. These show that the window of similarity to self for induction of disease by cross-reactive non-self peptides is actually quite restricted. We show that each of the three pillars of immune tolerance (death, anergy/adaptation and regulation) has a role in limiting the risk of molecular mimicry by maintaining a threshold for harm.

Keywords: Autoimmunity; T cell receptor; Molecular mimicry; Multiple sclerosis; Immune tolerance

1. The risk of autoaggression provoked by infection

Autoimmune diseases affect around 5% of Western populations. Why do these diseases develop? Clearly this is a complex question with the answer most likely lying not with nature or nurture alone, but with a combination of pre-disposing genes and environmental triggers. The model for the initial activation of autoaggressive lymphocytes (either T cells or B cells) that has found prominence over the last two decades is based around the molecular mimicry theory proposed by Fujinami and Oldstone in 1989 [1]. Put simply, this theory proposes that an initially useful immune response against a particular epitope derived from an infectious agent can develop into an autoaggressive response through recognition of a similar epitope derived from a self antigen (i.e. the immune response cross-reacts against the foreign and the self antigen). This is an appealing model because it allows a separation of the response against foreign and self in time and space (Fig. 1). Thus the pathogen does not have to show tropism for the organ that is ultimately the target of immune attack. Memory lymphocytes generated in the lymph nodes draining the site of infection could subsequently (perhaps years later) be re-activated by the aberrant presentation of the self mimic by activated antigen presenting cells in a lymph node draining the target organ, or in the organ itself. Although certain infections have been proposed to drive certain autoimmune diseases, it has been notoriously difficult to demonstrate the presence of the infectious agent in all individuals with the disease in question [2,3]. Thus molecular mimicry allows for a ''hit and run'' effect, with the ultimate disease developing long after the relevant infection has been cleared.

T cell receptors (TCRs) recognize antigenic peptide-MHC (pMHC) complexes, therefore it is the shape of this complex that matters not necessarily the primary sequence, if a particular T cell is to respond to a particular antigen. As long as the shape

Abbreviations: AICD, activation-induced cell death; APL, altered peptide ligand; DC, dendritic cell(s); EAE, experimental autoimmune encephalomyelitis; LCMV, lymphocytic choriomeningitis virus; pMHC, peptide-MHC; RIP, rat insulin promoter; TCL, T cell line; TCR, T cell receptor; WT, wild-type.

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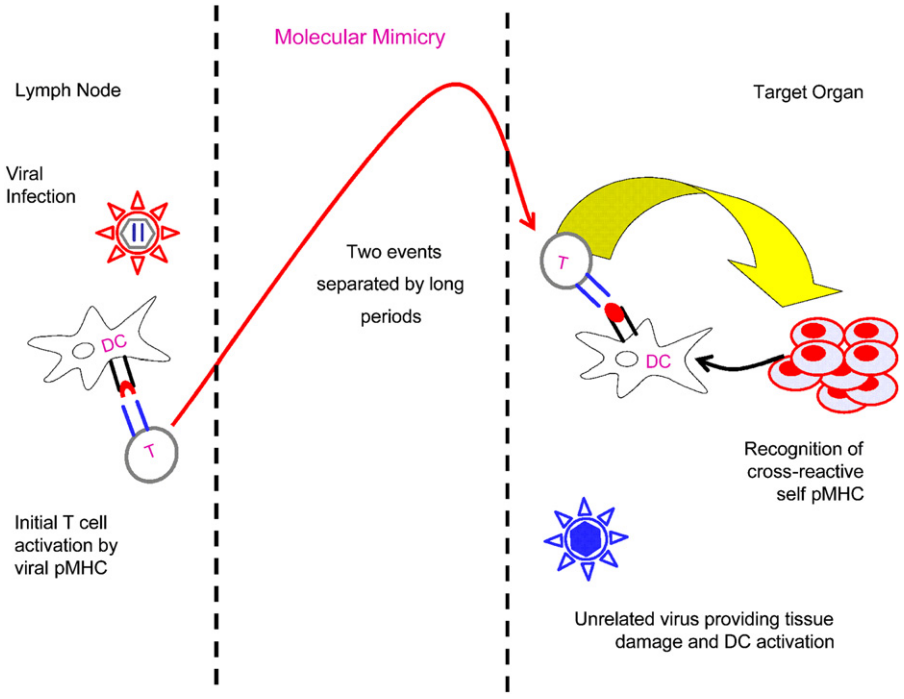


Fig. 1. Molecular mimicry. First proposed by Fujinami and Oldstone [1], the molecular mimicry theory proposes that T cells initially activated during the response to infection might bear TCRs capable of cross-reacting with a similar self pMHC complex. Note that the invading pathogen (here depicted as a virus) does not need to infect the target organ. The cross-reactive T cells could enter the memory pool, being activated subsequently to self presented by activated DC during inflammation of the target organ, possibly during infection with an unrelated virus.

is good enough the TCR can bind. Herein lies the essence of the adaptive antigen receptor repertoires—they have the capacity for cross-reactivity with different antigenic shapes that are sufficiently similar to allow stable interaction and lymphocyte signalling leading to activation and effector function. But different shapes will bind better than others. In this discussion, we shall focus on how TCR cross-reactivity might influence the development of an autoaggressive T cell response, and the processes in place to limit this risk.

2. Why are we not all sick?

First, why do we need TCR cross-reactivity? The T cell repertoire is estimated to be of the order of 10^8 [4]. In contrast the universe of potential antigenic peptides that the immune system must at least attempt to deal with has been estimated to be in the order of 10^{12}–10^{15}, depending on the length of the peptide [4,5]. Thus the most extreme scenario proposed that to provide effective immune surveillance, each T cell should be able to recognize up to 10^6 different, but similar, peptides [4]. Thus considerable flexibility in TCR recognition of peptide-MHC (pMHC) complexes seems to be an imperative. If so, the formation of T cells that can cross-react on foreign and self pMHC complexes seems unavoidable. In fact, positive selection of T cells during thymic development is mediated by recognition of self pMHC complexes, so the entire TCR repertoire shows a degree of self-reactivity. If the other feature of thymic education, namely negative selection, worked to delete all developing thymocytes with the potential to respond to self, the peripheral T cell repertoire that would emerge would be severely restricted [6]. Thus negative selection must only be deleting those cells above a particular threshold for self-reactivity (as yet it is not clearly defined what this is).

So we are left with a peripheral T cell repertoire that is inherently self-reactive. How can we ever mount an immune response against an invading pathogen without compromising ourselves through activation of T cells that can respond to our own proteins? The challenge for the immune system is to maintain flexible TCR recognition of antigens without allowing this to happen. So the question is not why do some people develop autoimmune diseases, but why do we not all develop them?

3. The three pillars of immune tolerance

The immune system has potent mechanisms in place to prevent unwanted T cell immune responses through the combination of central and peripheral tolerance. In essence these can be described as three processes: death, anergy/adaptation and regulation.

Death of T cells bearing TCRs with high affinity for self pMHC complexes of course provides the mechanism of negative selection during T cell development in the thymus [6]. Our own data suggest that this can also occur in mature peripheral T cells during an ongoing immune response [6,7]. Another form of T cell death results from exposure to pMHC complexes presented in the periphery by steady state dendritic cells (DC) in

the absence of full costimulation that would be triggered by exposure to pathogen associated molecular patterns during infection [8]. This most likely occurs through a failure to upregulate anti-apoptotic proteins such as Bcl-2 and Bcl-xL and also NF-κB within the T cell [9–11].

Classically, anergy was used to describe in vitro observations that T cell clones stimulated through the TCR in the absence of costimulation were subsequently rendered unresponsive when later challenged with antigen in the presence of costimulation [12]. Such T cells failed to proliferate or to produce their own IL-2, but anergy could be overcome by addition of exogenous IL-2 (i.e., IL-2 receptor signalling was intact). Interestingly, anergic cells retain the capacity to produce effector cytokines such as IFNγ. More recent studies suggest that this classical anergic state may not satisfactorily reflect the development of T cell unresponsiveness (tolerance) in vivo. A new phrase, ''adaptive tolerance'' has been coined to describe a state of generalized unresponsiveness (proliferation, IL-2 production and effector cytokine production all being impaired) as has been found in various models [12]. The difference between anergy and adaptive tolerance is more than mere semantics. Classical anergy is not reversible by removal of antigenic stimulus, i.e. once established it persists without the need for further antigen exposure. In contrast adaptive tolerance, as the name suggests, does require persistent antigen. Thus T cells that have been de-sensitized in vivo by exposure to antigen can regain sensitivity when placed in a host that lacks the antigen [13,14]. These in vivo observations strongly support the tuneable activation threshold model, as proposed by Grossman and Paul, in which a T cell senses antigenic cues from its environment and adjusts its threshold for full activation accordingly [15]. Although experiments using TCR transgenic T cell transfers have clearly highlighted a role for adaptation in the periphery under steady state conditions, this phenomenon has also been seen under conditions that mimic infection (immunization with antigen in complete Freund's adjuvant) [7]. Furthermore, there is evidence that, rather than the ultimate sacrifice of apoptosis, developing thymocytes can also desensitize to some extent in response to a relatively strong antigenic signal [16,17].

The third pillar of immune tolerance is provided by the activity of regulatory T cells. Although these cells come in various guises, depending on the precise experimental approach, the population that has come to the fore in recent years are the so-called ''natural'' CD4+ CD25+ Treg cells that express the forkhead box P3 (Foxp3) transcription factor [18]. This discussion will focus on these cells because, unlike other regulatory populations such as Th3 cells or Tr1 cells that can be induced experimentally [19,20], they appear spontaneously within the peripheral T cell repertoire and are generated in the thymus [18]. Mice or humans that lack a functional foxp3 gene succumb to multi-organ inflammatory diseases indicating that Tregs have a key function in preventing spontaneous immune activation [21,22]. The paradigm that has developed is that those developing thymocytes bearing TCRs with high affinity for self pMHC complexes, but below the threshold for death by negative selection, have a propensity to differentiate into Tregs [23]. Although this is based on only limited experimental evidence [24] and the mechanisms for this conversion are not defined, it would make sense in that once in the periphery those cells with the greatest capacity to respond to self would be Tregs giving them a selective advantage over their potentially autoaggressive counterparts bearing self-reactive TCRs of lower affinity. The observation that those CD4+ T cells that are spontaneously activated (presumably by self antigens) in foxp3-deficient mice have similar TCR gene-usage to the foxp3+ cells of normal mice supports this argument [25]. As well as controlling the spontaneous activation of potentially autoaggressive T cells under steady state conditions, Tregs can certainly play a role in limiting immune priming under inflammatory conditions and immunopathology in the target organ, since in either setting priming or pathology is exacerbated in mice that have been depleted of Tregs [26–28].

Thus the three pillars of immune tolerance can act separately or in concert during thymic development and in the periphery, upon exposure to self pMHC complexes either under steady-state or inflammatory conditions, to limit the chances of mounting an autoaggressive T cell response. The fact that these processes can impose limits on T cell responses during inflammation is particularly pertinent to a consideration of molecular mimicry because the initial T cell activation in response to viral or bacterial infection would of course be under such conditions, with presentation of the foreign pMHC complexes by fully activated DC.

4. How strong is the case for molecular mimicry?

Early experiments from the Oldstone lab and from Ohashi et al. tested the ability of infection with a virus containing a defined T cell epitope to lead to autoimmune disease in an organ that also expressed the virus-derived antigen [29,30]. Antigens (glycoprotein or nucleoprotein) from lymphocytic choriomeningitis virus (LCMV) were transgenically expressed in pancreatic β cells under the control of the rat insulin promoter (RIP). Oldstone's group found that infection of these mice with LCMV led rapidly to islet destruction and autoimmune diabetes [29]. In Ohashi's study, the RIP-LCMV mice also expressed a transgenic TCR that recognized the LCMV antigen. Even though these mice therefore had a T cell repertoire focussed on an antigen expressed in the β cells they did not develop spontaneous diabetes (they were functionally tolerant). Again, infection with LCMV broke this tolerance, precipitating diabetes [30]. The important feature of these two elegant studies that should be stressed is that they did not address molecular mimicry, but molecular identity (the foreign antigen and the transgenic self antigen were the same).

The likelihood that a T cell will encounter a foreign pMHC complex that provides precisely the same topography for TCR recognition as a self pMHC complex is extremely low. Studies using altered peptide ligands (APL) reveal that although an individual T cell clone can respond to several APL, even subtle differences in amino acid side chains at particular residues can have profound effects on the outcome of the TCR–pMHC interaction, either quantitatively or qualitatively, in terms of

how the T cell responds [31,32]. It is known that perhaps only two or three residues of the peptide protrude from the MHC peptide binding cleft and are available of TCR binding. It is possible that these key TCR contact residues could be shared by a foreign and a self antigen, but differences in other residues of the peptides can subtly modify the conformation of the peptide MHC complex again providing a different topography for TCR interaction.

Thus to really study models of molecular mimicry it is essential that the foreign and self antigens differ in sequence. Studies using the RIP-LCMV model have revealed that the ability to induce diabetes is readily lost when infection is with a variant LCMV that has alterations in the key T cell epitope that lead to a lower affinity of TCR–pMHC interaction [33,34].

5. Modelling molecular mimicry using CNS autoimmune disease

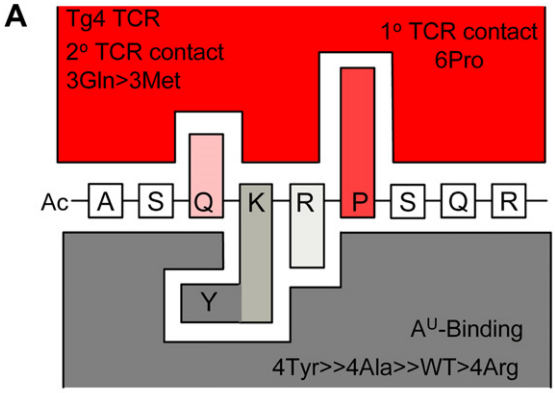
Molecular mimicry has been proposed to play a role in the aetiology of multiple sclerosis (MS), with support for this coming from reports that T cell clones initially derived from MS patients on their ability to respond to an ''immunodominant'' epitope of the CNS autoantigen myelin basic protein (MBP) could cross-react against peptides derived from various bacterial and viral species. However, it should be noted that no microbial peptide could stimulate all clones and that not all clones showed responsiveness to a microbial peptide [35]. Of course it is impossible to definitively show that molecular mimicry following infection can account for a human autoimmune disease, so in context of MS investigators rely on its animal model, experimental autoimmune encephalomyelitis (EAE) [36]. Although an imperfect model of MS, EAE in mice has some clear advantages in that it can be induced in various strains by immunization with a variety of well-defined peptide epitopes in complete Freund's adjuvant, leading to the activation of CNS-aggressive CD4+ T cells. For several of these encephalitogenic peptides, the key amino acids that act either as TCR contacts or MHC binding residues are clearly defined.

For information on the ability of cross-reactive antigens recognized in an infectious setting to cause encephalomyelitis we direct the reader to a series of elegant studies by Miller et al. [37,38]. These have used infection with Theiler's virus that has been genetically modified to express microbial antigens known to stimulate T cells recognizing myelin proteolipid protein. The EAE model that we have been studying for several years uses the acetylated N-terminal nonamer of MBP (Ac1–9) in mice expressing the A^u MHC class II molecule. The influence of various APL with substitutions at either MHC or TCR contact residues have been analysed in terms of T cell activation in vitro and encephalitogenic potential in vivo. So far these studies have not directly assessed the influence of microbially derived epitopes (infection models have not been developed). Nevertheless, our findings do have relevance for the scope for autoimmune disease provoked by the initial activation of T cells by peptides that are similar to the self antigen.

The molecular requirements for the binding of the Ac1–9 peptide to the A^u molecule and the subsequent recognition of this molecule by encephalitogenic T cells have been provided by a series of experiments by Wraith et al. and are summarized in Fig. 2a. The key residues for binding to MHC are 4Lys and 5Arg, of which position 4 appears most critical [39]. Strikingly, the wild type (WT) peptide has a very low binding affinity for MHC because the Lys residue normally at position 4 fits poorly into the large hydrophobic P6 pocket of the A^u binding cleft [40,41]. Thus APL with substitutions at position 4 that fit this pocket well show stronger MHC binding [42]. Most notably, the Ac1–9(4Tyr) APL has been found to bind A^u with >100,000-fold higher affinity than WT Ac1–9. In contrast, the 4Arg APL displays even poorer binding than the WT peptide [42]. These differences in MHC binding convert directly into antigenic potential in vitro. Thus the WT peptide will stimulate Ac1–9-reactive T cells in the nanomolar range. The 4Tyr APL is active at very much lower (femtomolar) concentrations (i.e. has ''superagonist'' activity) [7], whereas the 4Arg APL is only stimulatory in the micromolar range (i.e. is a ''sub-agonist'') [43].

The key residues for TCR recognition of Ac1–9 are positions 3Gln and 6Pro. Various analyses using APL with substitutions at either of these residues have been performed using Ac1–9-reactive T cell clones, polyclonal T cells lines and naive or activated TCR transgenic T cells. Studies in the Wraith laboratory using the 1934.4 T cell hybridoma [44] and the Tg4 mouse that is transgenic for the 1934.4 TCR [45] have shown that position 6 is the primary TCR contact residue (no substitution of the Pro that is normally found at this position is permitted if TCR stimulation is to occur). In contrast, position 3 is the secondary TCR contact residue with certain APL providing sub-agonist stimulation. Thus the WT > 3Met > 3Thr ~ 3-Phe ~ 3Tyr hierarchy for T cell stimulation has been established [44,46]. It is worth noting that analyses of polyclonal T cell lines and another transgenic mouse bearing a different Ac1–9-reactive TCR have revealed that there is some flexibility possible in recognition of position 6 [46], but because our studies have predominantly used T cells from the Tg4 TCR transgenic mouse, the above information is relevant to this discussion.

Thus the use of these defined APL in the Ac1–9 system allows us to provide potentially autoaggressive T cells with different levels of TCR stimulation. So what is the capacity of these APL to trigger an autoaggressive T cell response leading to EAE? Clearly immunization with the WT peptide induces EAE. Based on the in vitro data, the sub-agonist APL should be less effective whereas the superagonists should give greater disease. However, from a series of studies, the picture that emerges is that the sub-agonists are indeed inefficient at inducing EAE but, surprisingly, the superagonists are also poorly encephalitogenic. This loss of disease-causing capacity increases in-line with the strength of the superagonists, such that the strongest superagonist (4Tyr) gives the lowest level of disease (Fig. 2b). So in effect we have a ''Goldilocks''



B

	Activity in vitro	EAE-induction in vivo
4Tyr	fM	<10%
4Ala	pM	~30%
WT	nM	100%
3Met	nM	~20%
4Arg	μM	<10%

Fig. 2. Characteristics of MBP Ac1–9 interactions with the A^u class II molecule and the Tg4 T cell receptor. (A) The WT Ac1–9 peptide creates a very unstable interaction with the A^u class II molecule because the Lys at position 4 interacts unfavourably with the large hydrophobic p6 pocket of A^u. Alterations at this residue produce APL with greatly increased binding affinities: 4Ala binds 1000-fold better, whereas 4Tyr binds >100,000-fold better. Positions 3 and 6 interact with the Tg4 TCR. No alterations from the WT 6Pro are allowed if T cell activation is to occur (i.e. position 6 is the primary TCR contact residue). Position 3 is the secondary contact residue. The 3Met, 3Thr, 3Phe and 3Tyr APL can all activate Tg4 cells, but higher concentrations of peptide are needed than the WT peptide (i.e., these APL are sub-agonists). (B) Summary of the ability of the various APL to either induce T cell activation in vitro, or to induce EAE in vivo, relative to the WT peptide. The WT peptide stimulates T cells that were raised against it at around 1–10 nM. The 4Ala and 4Tyr APL are superagonists, stimulating in the pM and fM ranges respectively. 3Met and 4Arg are sub-agonists, needing around 3-fold and 1000-fold higher doses respectively than the WT peptide to activate Tg4 cells. As the difference in the stimulatory capacity of APL increases (either stronger or weaker than the WT peptide), so the ability to induce EAE upon immunization of non-TCR transgenic mice decreases.

phenomenon: if the strength of antigenic signal received is too cold (sub-agonists) or too hot (superagonists) the T cells do not become fully autoaggressive, but when the signal is "just right" (as is provided by the WT peptide) disease develops. So what mechanisms control the potentially autoaggressive T cells when the signal is too hot or too cold? Our recent studies have pointed to roles for each of the three pillars of immune tolerance.

6. When the signal is too hot, death and adaptation control autoaggressive potential

The early experiments using superagonist APL (in these experiments the 4Ala APL was used) produced two important findings: (a) when mice were co-immunized with the WT peptide and the APL, the APL effect (i.e. lack of disease) was dominant [39]; (b) protection from EAE could not be transferred to naive mice using splenocytes from mice that had been immunized with the APL [47]. One possible explanation was that the APL simply did not activate T cells in vivo (responses to WT peptide were found to be low or absent in APL-immunized mice). The fact that giving the APL at the same time as the WT peptide did have a beneficial effect indicated that the T cells were aware of the APL's presence The second finding, that protection was not transferable, argues against the development of a suppressive/regulatory population of cells that controls a distinct autoaggressive cohort. Rather, it points to an intrinsic effect in the autoaggressive cells themselves.

We re-addressed the question of whether immunization with the superagonist APL could effectively prime for a T cell response. We generated T cell lines (TCL) from APL-immunized non-transgenic mice. Importantly, instead of trying to restimulate the T cells with the WT peptide, we used the same APL that the mice were originally primed against (i.e. we had cells that had only ever seen an individual APL). By this approach we could readily produce TCL (so APL-immunization did prime for T cell responses) [7]. Analysis of these different TCL revealed two things. First, each TCL responded to the APL it was raised against in the nanomolar range (i.e. at the same dose that TCL raised against WT peptide responded to WT peptide). Thus there was a selective pressure for CD4+ T cells to respond to their immunizing antigen at a pre-determined threshold (the nanomolar range). The second observation was that there was an increasing loss in the capacity of APL-induced T cells to respond to the WT Ac1–9 peptide. Thus TCL raised against the strongest APL (4Tyr) would only respond in vitro to the WT peptide at concentrations above 10 μM. Thus we concluded that the reason that immunization with the 4Tyr APL does not induce EAE is not because it does not prime for T cell responses, but because the T cells it elicits are so insensitive to the WT Ac1–9 that they cannot respond to it when presented at physiological levels in the CNS. Immunization with the 4Ala APL primes T cells that respond to WT peptide at around 1 μM and can induce modest levels of EAE. We therefore have proposed a "threshold for harm" of 1 μM, with only T cells that recognize self antigens at concentrations below this being autoaggressive [6]. T cells that respond only to higher concentrations of self antigen are autoreactive, but not autoaggressive and so can be permitted [6,48,49].

T cells raised against WT Ac1–9 have the capacity to respond to the 4Tyr in vitro at femtomolar concentrations. These cells should have a selective advantage upon 4Tyr immunization in vivo, so why do they not dominate the response to 4Tyr? The answer is because they are removed from the repertoire. Use of Ac1–9(4Tyr)-A^u multimers allowed us to indirectly assess the affinity of the TCRs displayed by our different TCL [7]. Those generated in response to the WT peptide were clearly heterogeneous populations of T cells displaying a range of TCR affinities from low to high. In contrast, TCL raised against the 4Tyr APL only expressed low affinity TCRs. Importantly, levels of TCR expression and CD4 expression did not vary between the TCL, indicating that the loss in sensitivity to the 4Tyr APL

was due predominantly to a deletion of T cells bearing high and medium affinity TCRs. The Tg4 TCR transgenic T cells express a moderate affinity TCR and these cells showed a higher rate of apoptosis when taken from a host mouse shortly after immunization with 4Tyr compared with WT Ac1–9. Thus superagonist APL did not induce EAE because Ac1–9-reactive cells bearing TCRs with sufficient affinity to confer autoaggressive potential were being deleted by activation induced cell death; in essence, negative selection was happening during a peripheral immune response to antigen [7].

The Tg4 TCR transgenic cells express a moderate affinity TCR, just above the threshold for deletion in response to 4Tyr [7]. This allowed us to assess how T cells with a TCR of fixed affinity would integrate signals of differing strengths. In vitro analysis of the response of these cells revealed that their activation-induced cell death (AICD) in response to 4Tyr required expression of both Fas and FasL by the T cells themselves [50]. We therefore established an adoptive transfer system using host mice that would only develop EAE in response to immunization with the WT Ac1–9 peptide if they had first been seeded with naive Tg4 T cells. As expected these mice did not develop EAE after immunization with the 4Tyr APL. We then refined the model to compare the effects of transfer of Tg4 cells that either could, or could not express Fas; the prediction being that Fas-deficient cells would now induce full-blown EAE in response to 4Tyr, because they would not undergo Fas-dependent AICD. The result was that, under these conditions, mice could develop more EAE in response to 4Tyr, but it was still considerably less severe disease than seen using the WT peptide [50]. This suggested that a further mechanism was also acting to inhibit disease in response to the superagonist. A clue to what this was again came from in vitro analysis of the Tg4 response.

In fact, not all Tg4 cells died immediately in response to 4Tyr. Those that survived showed clear reductions in their sensitivity to both 4Tyr and WT Ac1–9. This seemed to be a completely quantitative effect in that culture with 3-log lower doses of 4Tyr produced cells with the "normal" response patterns. Thus the Tg4 were able to undergo sensory adaptation in vitro that was determined by the strength of signal received through their TCR. The adaptation was not explained by changes in expression of TCR or CD4, but did correlate with increased expression of CD5 in the desensitized cells. Returning to the in vivo model, adoptive transfer of Tg4 cells that were traceable due to expression of CD45.1 revealed that a significant population of cells was maintained after 4Tyr immunization, but these cells also had elevated levels of CD5 expression and reduced sensitivity to culture with WT Ac1–9. Strikingly these adapted cells (from mice that were resistant to EAE) only responded the WT peptide at concentrations of 1 μM and above; thus the "threshold for harm" had not been breached [50]. At this point we should re-iterate that, in our hands, the effects of superagonists on Tg4 responses appear to be totally quantitative in nature. We see sensory adaptation of proliferation and effector cytokine (IFNγ) production. We do not see a shift to a Th2 phenotype as has been proposed by others [51].

How do the above observations relate to the risk of autoaggression via molecular mimicry as a result of infection? Autoaggressive cells would need to respond to self antigen with sensitivity above the threshold for harm. These cells will be more sensitive to the foreign antigen encountered during infection; i.e. the foreign antigen will be a superagonist. The initial activation of potentially autoaggressive cells with the superagonist will lead to death of autoaggressive cells with high affinity TCRs and/or the sensory adaptation (desensitization) of T cells with moderate affinity TCRs. The net effect, at the population level, will be to shift the self reactive repertoire below the threshold for harm, so autoimmune disease will not develop. Thus initial exposure would prevent those cells that remain from being triggered even by a stimulus equivalent to the self antigen (because they have adapted). On this point, our more recent data indicate that immunization with 4Tyr provides a relatively large antigen experienced cohort, but that these mice are protected from the EAE that should develop upon subsequent immunization with WT Ac1–9 (SDP, unpublished).

7. Treg cells limit the ability of sub-agonist ligands to provoke disease

Central tolerance should remove those cells that have TCRs with excessively high affinity for self [52]. This would then provide the above scenario in which the self-reactive T cells in question respond better to the superagonist foreign antigen than they do to the self antigen. What happens if the reverse is true, i.e. the foreign antigen is a sub-agonist? Now a weak stimulation upon infection could trigger cells that subsequently respond better to self. Further, it may be that adaptation actually allows cells to increase their sensitivity to the foreign antigen, with the knock-on effect that their sensitivity to self rises above the threshold for harm. Our studies of the MBP Ac1–9 model point to a role for Tregs in limiting the risk of autoimmune disease in such a scenario [53]. The 3Met APL, with a substitution at the secondary TCR contact residue for Tg4 cells, is a sub-agonist in vitro, requiring ~3-fold higher concentrations than are needed of the WT Ac1–9 to stimulate Tg4 cells [44,53]. However, this relatively slight loss in antigenic potency has a major effect on the ability of the 3Met APL to induce disease, with little or no EAE seen in non-transgenic mice [44]. As described above, the paradigm has arisen that Tregs are generated in the thymus from precursors with the highest affinity self-reactive TCRs that avoid negative selection [23], which would make sense as our most self-reactive peripheral T cells would have concentrated regulatory function. The prediction that would account for the failure of 3Met to induce EAE in a mouse with a complete peripheral T cell repertoire (i.e. not TCR transgenic) would therefore be that this repertoire incorporates Tregs with high affinity TCRs and therefore a selective advantage to respond upon immunization with the sub-agonist, preventing activation of potentially autoaggressive T cells. To test this we depleted mice of Tregs using anti-CD25 shortly before immunization with the 3Met

APL and found that these mice had significantly increased EAE [53].

These results verified the concept of Tregs having a major role in maintaining the threshold for autoaggression. How they do this is still not fully understood. In our system, the effect most likely occurs at the point of initial T cell activation in the lymph node draining the site of immunization. A recent report using two-photon intravital imaging studied the interaction of naive Ac1–9-reactive TCR transgenic cells (note these were not Tg4 cells) with Ac1–9-loaded DC in lymph nodes under conditions in which Tregs were present or absent [54]. These concluded that the responder T cells made more meaningful interactions with the DC when Tregs were absent, perhaps allowing for full activation. Notably, polyclonal Treg populations were used suggesting that there was no necessity for Ac1–9-reactive Tregs. An alternative interpretation could be that polyclonal Tregs might include cells bearing higher affinity TCRs than used by the TCR transgenic cells.

Thus far our data do not fit a model in which T cells with moderate affinity TCRs can be fully activated by sub-agonists when released from Treg control. We failed to detect the activation of transferred Tg4 cells in draining lymph nodes after immunization with 3Met in non-transgenic host mice, even after depletion of Tregs (in contrast, WT Ac1–9 clearly triggered the activation of these transferred cells). Moreover, Tg4 cells did not accumulate significantly in the CNS of Treg depleted mice primed with 3Met [53]. An explanation for this unexpected result may lie in the fact that our initial studies using the Ac1–9(4Tyr)-A^u multimers showed that Tg4 cells use a moderate affinity TCR, but that there is clearly a repertoire in WT mice that uses TCRs of higher affinity [7]. Of note, immunization of WT mice with another sub-agonist APL (4Arg) was found to selectively expand T cells with high affinity TCRs [43]. These cells were hyper-responsive to WT Ac1–9. Even so, mice immunized with this peptide did not develop full-blown EAE. At the time we interpreted this to be because, although these cells were present, their numbers were too few to cause sufficient damage in the CNS. An alternative (which might account for the great difficulty we had in generating in vitro TCL against the 4Arg peptide) would be that these cells with higher affinity TCRs were already Tregs. With hindsight, it was a significant omission that we did not assess these cells for foxp3-expression.

In summary our use of the MBP Ac1–9 peptide, and its well-defined interaction with the A^u class II molecule and the TCR has allowed us to show that all three of the pillars

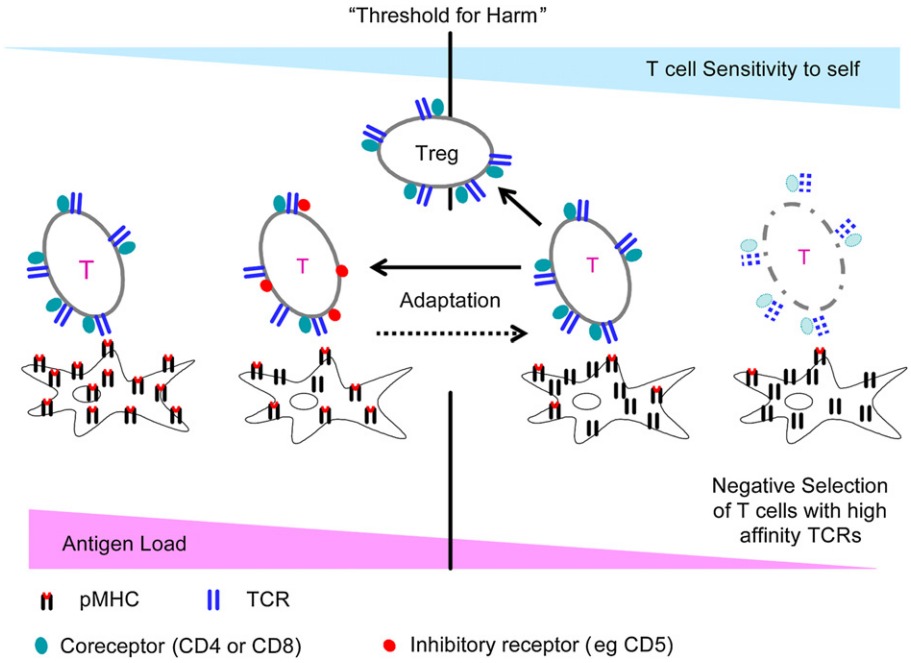


Fig. 3. The three pillars of immune tolerance maintain the threshold for harm. The sensitivity with which a particular T cell will respond to a self antigen (i.e. the number of pMHC complexes needed to be displayed in order to fully activate the cell) is determined primarily by the affinity with which the TCR binds the pMHC complex. Sensitivity is refined by (a) the level of TCR expression, (b) the level of expression of coreceptors (CD4 or CD8) or other surface receptors that enhance signalling; (c) the level of expression of inhibitory surface receptors (e.g. CD5); (d) the relative incorporation of TCR-proximal signalling molecules that either enhance or inhibit T cell activation (not shown here). Only those cells whose sensitivity is above the threshold for harm (i.e. those activated by low levels of self pMHC complexes) are likely to become autoaggressive. Those cells with high affinity TCRs are deleted via negative selection either during thymic maturation, or in response to superagonist foreign antigens during a peripheral immune response. Those cells with moderate affinity TCRs can adapt in response to superagonist stimulation to move below the threshold for harm. This may be due to decreased expression of TCR and/or coreceptor, increased expression of inhibitory receptors, or alterations in their TCR-signalling machinery. Note that cells that are desensitized in this way (as part of a functional anti-pathogen response for example) may be efficiently expanded and enter the memory pool. There is then potential for these cells to re-sensitize to self over time creating a possible risk of autoaggression. An alternative fate for developing thymocytes bearing TCRs of relatively high affinity for self pMHC is to differentiate into foxp3+ Tregs. These cells would then have a selective advantage to respond to foreign sub-agonists during peripheral responses, thereby preventing the activation of their potentially autoaggressive counterparts of lower sensitivity.

of immune tolerance can exert their effects to restrict the range of TCR stimulation that will ultimately lead to autoaggression (the ''Goldilocks model'', Fig. 3). Superagonist stimulation leads to sensory adaptation that expands/maintains an autoreactive repertoire with a sensitivity that is set below the threshold for harm. In extreme cases, if the superagonist ''hit'' is too strong the T cell has no alternative but to die. If the stimulus is too cool (sub-agonist), autoaggression will not develop because of the dominant effects of highly sensitive Tregs. Strong signals are therefore sensed intrinsically by the potentially autoaggressive T cell, whereas weak signals are suppressed by the action of other cells. This three-edged control serves to limit the scope for infection leading to autoaggression via molecular mimicry to situations when the self and cross-reactive foreign antigens give TCR signals of almost identical strengths.

8. Final thoughts

Why should the immune system rely on these complex safety measures to limit autoaggression? As we discussed earlier, the majority if not the entire peripheral T cell repertoire displays a degree of self-reactivity. If tolerance was absolute, working through deletion of self reactive cells, we would not have a T cell repertoire. Therefore the best approach is to keep the ultimate sanction of death of the T cell (either in the thymus or in the periphery) to an absolute minimum. This is why adaptation is such an attractive option as it allows for the maintenance of the most diverse T cell repertoire, but one that is kept below the threshold for harm. Although the adapted phenotype correlates with elevated expression of CD5, as has been observed in other studies on sensory adaptation [55], we have yet to show a causative effect. Increased CD5 may simply act as a marker for adapted cells, rather than being the key driver in adaptation.

The negative side to adaptation is that, by definition, it is reversible [12, 15] and herein lies an inherent risk. A superagonist foreign antigen could expand a substantial population of adapted memory T cells (these may have had an important role of clearance of the infection). If, in the absence of the original foreign antigen, these cells can regain sensitivity to self over time and then be stimulated by another foreign antigen that more closely resembles self (or indeed by the self antigen itself) autoaggression could result. Thus a system based on adaptation in response to superagonist infection maintaining the self-reactive repertoire below the threshold for harm would actually be reliant on a reasonably constant exposure to infection to prevent some cells drifting back above the threshold for harm. We constantly hear of the increase in autoimmune and allergic diseases in industrialized countries being a consequence of reduced immune regulation because of lower infection rates (i.e. the hygiene hypothesis) [56]. The model outlined above could equally account for the observed correlation.

Finally the model of our most self-reactive T cells exiting the thymus as pre-formed Tregs is appealing for limiting the activation of other T cells by ''weakly'' cross-reactive foreign antigens. If this is so, there is an imperative that the Treg program should be absolutely fixed in these cells. If they possess the capacity to be converted in the periphery to an aggressive, pro-inflammatory function, they would be the most dangerous cells that we would possess. But then again, they should commit suicide in response to any superagonist antigen.

Additional discussion on other aspects of loss of tolerance are discussed elsewhere in this special issue [57–70].

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