Grand Central: The role of central tolerance for induction of central nervous system autoimmunity

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Abstract

The contributions of the peripheral adaptive and innate immune systems to central nervous system autoimmunity have been extensively studied. However, the role of thymic selection in these conditions is much less well understood. The thymus is the critical primary lymphoid organ for the generation of T-cells, ensuring that cells with an overt autoreactive specificity are eliminated prior to emigration to the periphery and controlling the generation of thymic regulatory T-cells. Evidence from animal studies highlights thymic T-cell selection as relevant to establish tolerance to self-antigens. However, there is a considerable knowledge gap regarding its role in autoimmune conditions of the human central nervous system. In this review, we critically examine the current body of experimental evidence assessing the contribution of central tolerance to central nervous system autoimmune diseases. An understanding of why dysfunction of either central or peripheral tolerance mechanisms rarely lead to brain or spinal cord inflammation is currently lacking. We examine the potential of *de novo* T-cell formation and central selection as novel therapeutic avenues and highlight avenues for future study important in rendering this process a likely focus of possible future treatments.

Key points

- The thymus is a vital checkpoint in establishing T-cell tolerance
- Evidence from preclinical animal models associates changes to thymic selection in with central nervous system (CNS) autoimmunity
- Studies of human CNS inflammation suggests a potential role for autoreactive T-cells that have escaped thymic negative selection
- The relative contributions of central and peripheral tolerance to CNS disease are understudied
- Thymic selection has been under-appreciated as a potential therapeutic target in CNS autoimmune disease and should be a focus for future research

Central nervous system autoimmunity

Autoimmune disorders affecting the central nervous system (CNS) constitute an important cause of neurological morbidity and mortality with considerable associated economic costs.[1] CNS-specific autoimmune conditions include multiple sclerosis (MS), neuromyelitis optica (NMO), autoimmune encephalitis and stiff person spectrum disorders (SPSD) (**table 1**).[2–7] Many systemic inflammatory disorders can also result in CNS disease either via a direct reaction against the brain or spinal cord parenchyma, or through CNS vasculitis.

[Table 1 around here]

Using MS as an exemplar, the pathogenic mechanisms include aspects of the adaptive and innate immune system, often involving many subpopulations of T-cells (including several types of $CD4^+$ T helper cells such as Th1, Th2 and Th17, $CD8^+$ cytotoxic T-cells, γδ T-cells, T regulatory cells (T_{reg}), and natural killer (NK) T-cells), B-cells (including memory B-cells and plasma cells) and microglia. It is clear from the efficacy of a variety of disease modifying therapies (**box 1**) that the adaptive immune system is key to CNS inflammation in MS, particularly with relation to relapses.[8,9] The discussion of relevant aspects of the innate immune system are beyond the scope of this review and reviewed elsewhere (see [10]).

[Box 1 around here]

For the pathological failure of immune tolerance leading to CNS inflammation, there must have been a failure in the mechanisms that ensure the responsiveness of the adaptive immune system to potentially harmful foreign antigens whilst maintaining a state of tolerance to self-antigens. T-cell tolerance is generally maintained by mechanisms initially operative in the thymus (aka central tolerance induction) and subsequently in peripheral tissues. Together these events shape the repertoire of antigens recognized by the T-cell arm of the adaptive immune system (**box 2**).[11] Similarly, there are several checkpoints that establish B-cell tolerance (**box 3**) and while early B cell checkpoints appear to implicate a limited role for T-cells, autoreactive B-cell clones which have escaped from the bone marrow may be suppressed by T-cells .[12–14]

[Box 2 around here]

[Box 3 around here]

Research has largely focussed on mechanisms that establish and maintain peripheral immunological tolerance, and their role in CNS inflammation.[15] In contrast, less consideration has been given to

appreciate the contribution of the thymus in the pathogenesis of CNS autoimmunity. This review will, therefore, analyse our current cellular, molecular and clinical understanding of central tolerance in CNS autoimmunity, and explain why this is likely to become a future area of research with potentially important therapeutic implications.

Central T-cell tolerance

Thymic epithelial cells (TEC) are derived early during gestation from the endodermal lining of the third pharyngeal pouch. Over time, these cells form an intricate three dimensional scaffold that creates, together with dendritic cells, intrathymic B-cells, macrophages and other stromal cells, an exclusive microenvironment enabling the formation and selection of T cells (**figure 1**).[16,17] Structured into two major anatomical areas, the thymus has an outer region known as the cortex which contains the earlier stages of T cell development, and an inner region known as the medulla where the later stages of thymic T cell differentiation take place. TEC can be further subdivided into cortical (cTEC) and medullary (mTEC) epithelia based on their spatial, morphological transcriptomic and functional characteristics. Positive thymoycte selection is exclusively mediated by cTEC, whereas negative selection can occur both in the cortex and medulla mediated by cTEC and mTEC, respectively, as well as various hematopoietic cells with a capacity for antigen presentation.

[Figure 1 around here]

Negative selection strongly relies on the ability of TECs to express at the population level almost all protein-encoding genes.[18] This capacity is referred to as promiscuous gene expression (PGE) and is thus essential to for probing the antigen-specificity of developing T-cells, which randomly select an antigen-specificity. Engagement of the T-cell antigen receptor (TCR) with its cognate self-antigen/MHC complex will activate the extracellular-signal-regulated kinase (ERK). Differences in ERK strength, kinetics and localization determine the outcome of thymocyte selection whereby a sustained low intensity activation results in positive selection whereas a vigorous and rapid stimulation will lead to negative thymocyte selection.[19,20] Although the molecular mechanisms that control PGE are incompletely understood, the AutoImmune Regulator (AIRE) has been identified to facilitate the expression of approximately 4,000 genes that otherwise would either not be expressed or transcribed only at reduced rates.[18] Individuals with a congenital loss of AIRE function develop the autoimmune polyendocrine syndrome type 1 (APS1), characterised by a combination of Addison's disease, hypoparathyroidism, chronic mucocutaneous candidiasis and other autoimmune manifestations, due to a restricted repertoire of self-antigens presented by TECs, and the consequent failure to eliminate autoreactive T-cells.[21,22] It is however important to note that

negative selection is frequently incomplete even in healthy individuals with normal thymic function. As a result, autoreactive T-cells can be detected in the periphery in healthy individuals but autoimmunity is normally prevented by the dominant effect of regulatory T-cells (T_{reg}).[23,24] Because a large proportion of peripheral T_{reg} are generated in the thymus, their repertoire selection by thymic stromal cells is important to control peripheral T-cell tolerance. Indeed, decreased numbers of both circulating effector T-cells and T_{reg} have been observed in APS1 patients.[25] However, even in the absence of thymic antigen expression, autoimmunity can also be generally prevented through the direct peripheral generation of T_{reg} from effector T-cells.[26]

In addition to mTEC, other cell types have also been implicated in the selection of thymocytes. Intrathymic B-cells expressing AIRE are involved in the presentation of antigens to developing thymocytes, and may also have a role in maintaining the function of mTEC through the production of lymphotoxin.[27,28] APS1 patients have been shown to harbour B-cells producing autoantibodies against multiple autoantigens through a failure of negative T-cell selection including against *pro*-inflammatory cytokines.[29] Thymic dendritic cells are potent antigen presenting cells and can receive antigens from mTEC *via* exosome transfer.[30] Also, dendritic cells within the thymus have been shown to be important for the presentation of some encephalitogenic forms of CNS antigens.[31,32]

Humoral immune responses mainly rely on the function of thymically selected T-cells that licence B cells to produce antibodies.[33] A role for T-cells in neurotoxicity is highlighted by neuropathological studies in autoantibody-mediated forms of autoimmune encephalitis.[34,35] Similarly, T-cells reactive against the autoantigen glutamic acid decarboxylase (GAD) have been isolated from peripheral blood and cerebrospinal fluid (CSF) of patients with stiff person spectrum disorders, associated with GAD65-autoantibodies in around 90% of patients.[36,37] Furthermore, recent experiments have confirmed the importance of T-cell related cytokines (IL-2) and co-stimulatory molecules (CD40-ligand) to generate antigen-specific-antibodies in patients with NMO and NMDAR-antibody encephalitis.[38,39] Also, there is a >90% association of a single HLA-DRB1 allele (*07:01) in patients with LGI1-autoantibodies.[40] Taken together, these findings suggest that autoreactive T-cells, which likely by-passed thymic negative selection, are important contributors to CNS autoantibody-mediated conditions. However, the extremely low incidence of CNS inflammation in conditions, such as APS1, where central selection of T-cells is deficient, suggests that the loss of AIRE-controlled thymic function alone is rarely sufficient to elicit CNS autoimmunity.

Preclinical evidence for central tolerance in CNS inflammation

The most common animal model in CNS autoimmunity is experimental autoimmune encephalomyelitis (EAE), which uses specific components of myelin as self-antigens, plus adjuvant,

to induce inflammation of the brain and spinal cord.[41] This method has been useful to determine the role of central and peripheral tolerance in inducing CNS inflammation: *Aire*$^{-/-}$ mice have been shown to be more susceptible to EAE at 6 months of age when compared to wild-type animals, whereas the reverse was observed in 2 month old mice.[42,43] The increased susceptibility to succumb to EAE in the absence of AIRE correlated with a reduction in T_{reg} numbers. AIRE deficiency results in a failure to direct thymocytes expressing self-reactive T-cell receptors into the T_{reg} lineage and exerts an important effect on the recirculation of T_{reg} from the periphery back into the thymus.[44,45] These observations underscore the importance of AIRE as its deficiency has a much more pervasive effect on peripheral effector and regulatory T-cell populations than a simple failure of negative selection. However, CNS inflammation is not seen in *Aire*$^{-/-}$ mice without immunological provocation, suggesting that peripheral tolerogenic mechanisms likely compensate for a failure of thymic selection.[46,47] Nonetheless, these findings leave open the question of whether the thymic representation of AIRE-dependent, CNS-specific antigens displays an expression pattern that results in a frequent escape of pathogenic T-cells, a phenomenon that is further enhanced in the absence of AIRE. Bulk transcriptomic data from pooled TEC cannot answer the question as it cannot resolve the proportion of cells in which a particular antigen is expressed and hence determine for individual TEC whether adequate mRNA copy numbers of a specific self-antigen are available for translation. Indeed, this issue can only be resolved by examining gene expression at the level of individual cells. Our single cell transcriptomic data show that many antigens known to be autoantibody or T-cell targets in CNS inflammation are expressed in mTEC at low levels and frequency (**figure 2a**): this pattern of expression is similar to self-antigens that are expressed strongly within other organ systems and that serve as targets of an autoimmune response, such as insulin or thyroperoxidase,. Based on a comparative analysis of transcriptomic data on bulk mTEC samples from wild-type and *Aire*$^{-/-}$ mice, CNS-specific autoantigens are no more likely to be regulated by the presence of AIRE than antigens specifically restricted to other tissues (enrichment in AIRE-enhanced status: 0.95-fold, $p = 0.21$ based on 1,000 permutations of tissue-specific expression). In fact CNS-specific antigens are detected at similar frequencies to other tissue-specific antigens in bulk mTEC samples (proportion of genes expressed: 0.97 *vs.* mean of other tissues of 0.97, $p = 0.51$ based on 1,000 permutations of tissue-specific expression).[18,48] However, a correlation between transcription and peptide presentation in TEC is more difficult to assess given the present methodological constrains for protein analysis at single cell resolution..

[Figure 2 around here]

A functional thymus is certainly necessary to maintain the balance between adaptive effector and tolerogenic mechanisms. Conditional ablation of *Foxn1*, a gene critical for development and

maintenance of thymic epithelial cells, causes both thymic involution and the accumulation of pro-inflammatory T-cells in multiple organs, including the brain.[49] Murine orthologues of two genes implicated in MS by genome-wide association studies were also found to be targets of *Foxn1*: *Map3k14* and *Irf8*.[50] Knock-out of *Map3k14* in TEC resulted in defective γδ T-cell development and alterations in IL-17 secretion.[51] This resulted from a loss of *Rorc* and *Il23r* expression, genes required for IL-17 production in γδ T-cells. Alterations in the γδ T-cell compartment have a plausible connection to MS pathogenesis, as increased numbers of γδ T-cells were associated with disease activity.[52] *Irf8* has been shown to modulate the thymic expression of *Aire* and *CHRNA1*, the major autoantigen in myasthenia gravis, and would therefore alter the expression of AIRE-regulated CNS autoantigens within the thymus, such as *Plp1*.[53,54] Genetic variants in *CLEC16A* confer increased susceptibility to MS; *CLEC16A* has been shown to alter T-cell selection owing to its role in TEC autophagy, a process regulating the MHC-associated antigen presentation of nuclear, lysosomal and mitochondrial peptides.[55–57] In addition *Prss16*, a gene known to be important for TEC function by modulating the proteasomal breakdown of proteins into peptides for presentation to developing thymocytes, has been found to alter the severity of EAE in mice.[58] The major genetic risk variants associated with MS susceptibility are located within the Major Histocompatibility Complex Class II (MHC; particularly the HLA-DRB1*15:01-containing haplotype) but it is difficult to distinguish potential intrathymic effects of specific haplotypes on the quality of thymocyte selection from mechanisms operational in the periphery that influence antigen presentation.[59] Polymorphisms within the MHC associated with autoimmunity are typically postulated to alter T-cell receptor (TCR) - peptide-MHC binding dynamics and allow escape of autoreactive T-cells from negative selection.[60] Furthermore, unique characteristics of the immunological synapses formed between autoreactive TCRs and peptide-MHC can promote failure of thymic selection.[61] Genetic pathways associated with thymic function are generally less well annotated than those associated with peripheral immune effector functions. Therefore variants in other genes presently linked with MS susceptibility may also have an effect on central tolerance beyond their currently understood roles in peripheral and innate immunity.[62]

Thymic loss of *Aquaporin-4* (*Aqp4*) expression, the CNS autoantigen of causative autoantibodies in neuromyelitis optica (NMO), allows the expansion of clonal AQP4-reactive T-cell populations which induce CNS pathology upon adoptive transfer into wild-type mice.[63] Similarly, mice lacking expression of a dominant encephalitogenic splice isoform of proteolipid protein (PLP) in TEC are highly susceptible to EAE, whereas the expression of this PLP isoform in TEC renders mice resistant to EAE (**figure 2b**).[31,64] Conversely, bypassing central tolerance by seeding T-cells reactive to myelin basic protein (MBP) into the periphery of EAE-resistant naïve rats makes these animals susceptible

to experimental CNS inflammation.[65] Since the transferred autoreactive T-cells do not undergo negative selection in the thymus, central tolerance is circumvented. Collectively, these findings suggest that mechanisms maintaining peripheral tolerance are overwhelmed under experimental conditions that promote avoidance of central tolerance through inadequate thymic selection and that the presence of CNS-reactive T cells in the periphery is sufficient to elicit autoimmunity. However, none of these animal models developed spontaneous CNS inflammation in the absence of antigenic priming. Hence, the extent to which these models resemble the immunological situation of human CNS autoimmune diseases remains to be verified.

T_{reg} constitute another population required for the normal function of the adaptive immune system. As discussed earlier, these cells develop either in the thymus as part of normal thymopoiesis or in the periphery from effector T-cells in response to host and environmental cues. Therefore thymus-derived T_{reg} and T_{reg} differentiating in the periphery complement central selection of effector T-cells.[66] The loss of T_{reg} function through mutations in *FOXP3* results in the X-linked (IPEX) syndrome marked by multi-organ autoimmunity.[67] The dynamic interplay between these different effector mechanisms maintaining tolerance to CNS antigens is highlighted by observations demonstrating thymic T_{reg} output to be critical for the recovery from EAE because thymectomy effectively prevents spontaneous resolution of clinically apparent EAE.[68] Indeed, a novel role for T_{reg} in promoting remyelination in mouse models of demyelinating disease highlights the importance of this population of T-cells in CNS inflammation.[69] Despite these significant experimental observations, CNS involvement in IPEX patients has so far only been associated with blood pressure dysregulation.[70] However, T_{reg} interactions with B-cells constitute a critical process in modulating peripheral B-cell tolerance (**box 3**) and this may contribute to autoantibody-mediated CNS diseases. Also, there is evidence of a failure in peripheral B-cell tolerance in MS but, in contrast to type 1 diabetes mellitus and rheumatoid arthritis, central B-cell selection appears to be unaffected.[71]

The seeming dominance of central tolerance in certain models of EAE leaves the principal question unanswered: why does AIRE deficiency not result in spontaneous CNS inflammation? A possible explanation could be that the CNS constitutes an immune privileged site partly due to the presence of a tight blood brain barrier.[72] In the context of T cell-mediated CNS inflammation, transmigration across the blood brain barrier is a key initial step in the pathogenesis of CNS autoimmunity.[73–75] With the break-down of the blood brain barrier secondary to several insults, including ischaemic stroke, expansion of CNS-reactive, interferon-γ producing $CD4^+$ T-cells can occur, although the cells' presence within the brain parenchyma is not associated with apparent functional impairment.[76] *In vivo* tracing demonstrated that T-cell surveillance of the leptomeninges was common to both CNS-reactive and non-CNS-reactive T-cells, suggesting that crossing of the blood-brain barrier occurs

frequently and is a physiological process important for immune homeostasis and ongoing immunological surveillance.[77] A constitutive, intact network of T-cells within the CNS, including a resting population of T-cells expressing TCRs that recognise components of myelin, has indeed been shown to be important in cognition and recovery from brain injury (reviewed in [78,79]). A further facet of CNS immune surveillance is the recent identification of a distinct lymphatic system within the brain, which drains into the deep cervical lymph nodes.[80–82] This conduit is functionally important as antigens injected into the brain parenchyma or present in the CSF appear within the deep cervical lymph nodes.[83–85] A second, albeit more controversial explanation as to why CNS inflammation rarely occurs centres on the notion that antibodies directed against pro-inflammatory cytokines present in APS1 patients result in a decreased propensity to prompt an inflammatory response.[29] A third explanation proposes that peripheral mechanisms achieve immunological tolerance through deleting or at least functionally silencing autoreactive clones that have escaped thymic negative selection.[26] In this context, it should be remembered that most EAE models do not result in spontaneous CNS inflammation but require antigenic priming. It may, however, be the combination of a relatively inaccessible target organ and its anti-inflammatory state that creates a condition whereby the CNS is less prone to inflammation despite the presence of pathogenic T-cell clones that have escaped stringent thymic TCR repertoire selection.

Taken together the data from animal models provide evidence that central selection may well be aberrant in CNS autoimmunity but that peripheral tolerogenic mechanisms are likely in place to compensate most of the time for this failing. Moreover, additional environmental factors, such as is seen in NMO where a *Clostridium perfrigens* peptide mimics the antigenic peptide of AQP4, could be required to break peripheral tolerance and allow autoreactive T-cells to be primed and activated.[86]

Central tolerance in human CNS autoimmunity

There are important caveats to extrapolating findings derived from animal models of EAE to human CNS autoimmune diseases. As mentioned previously, EAE does not occur spontaneously and its pathology is driven in parallel by innate and adaptive immune processes.[41] Also, mechanisms that maintain the peripheral T-cell compartment differ between rodents and humans;[87] in mice, robust thymic function assures throughout the animal's life a generation of naïve T-cells, whereas in humans thymic involution begins in the second year of life, requiring the peripheral T-cell population to be primarily maintained by homeostatic proliferation within an already largely established T-cell compartment.

Linking a loss of thymic tolerance in humans to CNS autoimmunity has been fraught by several factors including the relative inaccessibility of thymic tissue for clinical studies and the early

senescence of the organ. Clinical interest in the role of the thymus in CNS autoimmunity may also have waned after a trial of thymectomy in MS.[88] This study could not identify clinical benefits for patients with MS but rather observed a trend towards worsening of the disease in individuals with progressive MS. However, the small number of patients enrolled in this trial and the invasiveness of the procedure limits the interpretation of the trial findings.

One indirect measure of thymic activity readily available using peripheral blood is the quantification of T-cell receptor excision circles (TREC). TREC are circular, non-replicating fragments of DNA produced by developing thymocytes during rearrangement of TCR chain *loci* (reviewed in [89]). Since TREC are only generated during thymocyte maturation, the number of TREC (usually quantified per million peripheral T-cells) is correlated with the amount of recent thymic emigrants that have seeded to the periphery but have not yet undergone significant homeostatic expansion.

Several studies have compared TREC in patients with MS to healthy controls, with the intent to better understand the relationship in MS between thymopoiesis and peripheral clonal T cell expansion (**figure 3**).[90–101] A remarkably consistent reduction of TREC levels was noted in different lymphocyte subsets of MS patients when compared to healthy controls, which was interpreted to suggest premature thymic senescence. However, reduced TREC levels do not necessarily indicate a reduction in thymic export of naïve T cells, because this molecular marker will also decrease as a consequence of peripheral T-cell proliferation resulting in a dilution of TREC.[102] Most TREC studies of peripheral blood drawn from MS patients had only analysed unseparated T-cells which likely obscured the results as a decrease in TREC frequencies is most obvious and best detailed in naïve T-cells following either homeostatic expansion or antigen-experienced cell proliferation. Newer investigations in paediatric MS patients and their age-matched, healthy controls demonstrated however that the frequency of $CD31^+$ T-cells, which signify recent thymic emigrants, was reduced in the patients, thus strongly suggesting a defect in thymopoiesis to be present early in the disease course.[103] Similarly, in adult-onset MS, variants in *IL7RA* (IL-7 is an early lymphocyte survival factor) associated with disease susceptibility are correlated with the number of recent thymic emigrant T-cells.[104]

[Figure 3 around here]

Peripheral T-cells from MS patients show in comparison to healthy individuals an increased cell proliferation paralleled by a heightened release of IL-17 and IFNγ in response to CNS antigens, arguing for the presence of autoreactive T-cells.[105] Clonal populations of T-cells reactive against CNS autoantigens have also been reported in NMO and neuropsychiatric systemic lupus erythematosus.[86,106] The presence of such clonal populations can be proven by locating specific

rearrangements of the TCR within the T-cell compartment of patients with MS. Sequencing across the TCRβ chain was attempted to identify expansion of particular TCR clonotypes. Comparing these sequences between MS patients and healthy controls identified T-cells with identical TCRβ chains, particularly in the CSF, and their detection correlated with CNS autoimmunity.[107,108] Clonally expanded T-cell populations could also be isolated from MS brain lesions and, over the course of 18 years, also from the CSF and blood.[109] These studies demonstrate that CNS-reactive T-cells had likely escaped thymic negative selection and contributed to the peripheral T-cell pool, where specific TCRβ clonotypes were detected at a significant frequency. However, these and similar analyses provide only limited information because sequencing T-cell populations in lieu of single T-cells cannot determine the precise TCRα and TCRβ pairing and precludes ascertaining the size of individual T-cell clones.

Thymic function may also be modulated by several disease-relevant risk factors. For example, MS has been linked to female gender, vitamin D deficiency, smoking, high body mass index and Epstein-Barr virus (EBV) infection.[110–112] Oestrogen exposure has been associated with early thymic atrophy through loss of early thymocyte progenitors and, consequent to a reduction in thymic cross-talk, a decrease in AIRE expression.[113–115] Conversely AIRE expression is increased following exposure to androgens and correlates in mice with a resultant reduction in susceptibility to EAE.[116] Vitamin D deficiency has been related to a decreased thymic volume *in utero* but not to changes in TREC levels in later life.[117,118] This association may not be unexpected since vitamin D impacts on the growth and differentiation of endoderm-derived epithelia, likely already *in utero*, which is in keeping with the observation that insufficient maternal 25(OH)D during pregnancy may increase the risk of MS in the offspring.[119,120] Similarly, thymic output, as measured by TREC, is altered by month of birth; given the association between maternal vitamin D status and seasonality, strongly arguing for a link between vitamin D sufficiency and thymopoiesis.[121,122] Smoking, another risk factor for MS, has no known impact on thymic output, albeit maternal smoking has been linked to reduced fetal thymic size.[123] In adulthood, a higher body mass index was associated with premature fatty degeneration of the thymus.[124] Finally, EBV infection, which is likely to be necessary for the subsequent development of MS, has been shown to increase the expression of CCL17, which both controls the balance between different subsets of thymic T_{reg} secreting distinct patterns of chemokines and, within the CNS, promotes CNS inflammation through the generation of Th17 cells.[125,126] This provides tentative evidence that infection of the thymus with EBV, which may occur in myasthenia gravis (although this is currently controversial), could provide a link between EBV and CNS autoimmunity.[127,128] Understanding the environmental impact on central tolerance remains, however, limited as the evidence for such a link is still mostly indirect. Further research should therefore focus on how the

interaction between particular genetic backgrounds and environmental exposures can alter thymic function.

If the loss of central tolerogenic mechanisms are important in the pathogenesis of CNS autoimmunity, then it would be logical that successful treatments of these conditions could be associated with in alterations in central tolerance. Only indirect measures of the loss in central selection have been assessed in MS, using measures of thymic output including TREC numbers and the appearance of $CD31^+$ recent thymic T-cell emigrants. The effect of MS treatments on TREC numbers either overall in the entire lymphocyte compartment, or for the compartments of $CD4^+$ and $CD8^+$ T-cells is contradictory (**figure 4**).[94,96,99–101,129,130] However, recovery of the peripheral T-cell pool after the transient, early reduction in TREC seen after autologous haematopoietic stem cell transplant is associated with the appearance of $CD31^+$ recent thymic emigrants, suggesting that modulation of thymopoiesis is likely to be important even in established CNS autoimmune diseases.[130] However, detailed functional phenotyping of recent thymic emigrant T-cells in this clinical situation has not yet been performed, so it remains undetermined which if any mechanism links post-treatment thymopoiesis to a reduction in disease activity.

[Figure 4 around here]

Further evidence for a role of the thymus in CNS autoimmunity has been drawn from patients with interruption of normal thymus function either due to the development of a thymoma or following thymectomy. Thymoma, in which there is most commonly an expansion of cortical thymic tissue, was frequently associated with different types of autoimmune encephalitis and led to the use of the acronym TAPE (thymoma associated paraneoplastic encephalitis).[131] Similarly SPSD and anti-glycine receptor antibody related disorders have been associated with thymoma.[132] In some cases, thymectomy could lead to clinical improvement, suggesting its direct association with an ongoing production of antigen-specific T-cells.[131,133,134] However, large-scale trials of thymectomy in these conditions have not yet been done, and evidence for its therapeutic effectiveness remains at present only observational. In the case of myasthenia gravis, there is an appreciable risk of subsequently developing NMO, particularly following thymectomy.[135] This risk appears to be independent of the production of AQP4 antibodies, as these were present in patients prior to thymectomy, suggesting that the ablation of thymopoesis results in the manifestation of CNS autoimmunity, possibly to a failure of T-cell negative selection or inadequate production of T_{reg}. Furthermore, NMO develops at a median age of approximately 40 years, demonstrating that intervening in thymic function even in mid-adulthood can have clear therapeutic effects. This observation supports the concept that manipulating central tolerance could be a highly effective, novel therapeutic modality for CNS autoimmune diseases. Without a more detailed understanding of how thymic function alters over

time in health and disease, there remain many unknowns regarding how and when to intervene in the process of central tolerance.

Future therapeutic implications

Interference with thymic function offers a unique therapeutic opportunity. Thymectomy is an established safe and effective treatment in patients with myasthenia gravis.[136] However, this therapy will likely be considerably less straightforward in cases of CNS autoimmunity because, unlike in myasthenia gravis, gross abnormalities of the thymus, such as thymoma or thymus hyperplasia, are much less frequent (**figure 5**).

[Figure 5 around here]

An alternative strategy could be to manipulate central tolerance in order to eliminate pathogenic T-cells and promote the production of CNS-specific T_{reg}. Novel methods of generating thymic epithelium from human stem cells may permit the bioengineering of thymus tissue either *in situ* or using appropriate scaffolds *ex vivo*.[137–140] The success of this approach would also allow manipulation of the genetic profile of grafted autologous progenitors to ensure that their TEC progeny expresses known or candidate antigens and therefore gain the competence to remove effectively those thymocytes by negative selection that have a pathogenic potential, or, alternatively, foster the development of new T_{reg} that promote antigen-specific tolerance. This strategy has already been successfully employed in a preclinical model where transplanting thymic epithelial cell precursors genetically engineered to express an encephalitogenic protein rendered mice resistant to EAE through the generation of antigen-specific T_{reg}.[141] The less prominent role of thymopoiesis in the maintenance of human compared with rodent peripheral T-cell pool is expected to make this approach more challenging.[87] However, the detection of recent thymic emigrants concomittant with a re-establishment of peripheral T-cells in cytoablated patients rescued with either autologous or allogeneic haematopoietic stem cells provides ample evidence that the potential for significant thymopoiesis remains intact in adulthood at practically all stages, albeit with diminishing efficacy with age. Further evidence that manipulating thymopoiesis in adult patients can modulate the effect of disease modifying therapies came from the use of anti-thymocyte globulin in association with haematopoietic stem cell ablation for MS. The addition of anti-thymocyte globulin to haematopoietic stem cell ablation was associated with a lower frequency of post-transplant immune dysfunction, suggesting that removing the pool of developing thymocytes is an important clinical consideration, although the fact that chemotherapy and anti-thymocyte globulin also depletes peripheral T-cells complicates the interpretation of this feature of the treatment.[142] These important

findings clearly signify that modulation of thymopoiesis could indeed represent a viable treatment modality in CNS autoimmunity.[130]

Understanding thymocyte development both in health and disease may identify intrinsic and environmental biological factors that could be targeted, for example early in childhood, to reduce the risk of autoimmunity later in life. It is clear that nutritional factors such as obesity, zinc and vitamin D, sex hormones, and the processes associated with aging are important determinants of the size and composition of the peripheral T-cell pool.[143–147,113,148] Importantly, these metabolic, gender- and age-related influences identify molecular pathways that could also be attractive targets for modulating thymopoiesis in high risk patients, for example those with a strong family history of autoimmunity.

Interrogating the TCR clonality of recent thymic emigrants also has the potential to infer disease-relevant changes in central selection. Importantly, new analytical tools are now available to precisely define the composition of the TCR repertoire and predict their ligands.[149] TCR binding is determined by interactions between alpha and beta TCR chains. Single cell sequencing has made it possible to obtain paired TCR sequence information from individual T-cells.[150] The detection of persisting pathogenic T-cell clones despite treatment of CNS autoimmune disease offers a powerful diagnostic tool to predict with some certainty treatment failure likely to result in relapse and may eventually help to identify novel antigens that either initiate or maintain the autoimmune response.

Unfortunately our knowledge of thymic biology and, specifically, methods of modelling human thymopoiesis *in vitro* remains limited and thus it is for now largely untested how practical these potential therapeutic measures targeting central tolerance may be in pre-clinical disease models and subsequently in the clinical context. Future research should therefore focus on efforts to bridge this restrictive knowledge gap.

Conclusions and Future Directions

Preclinical models of CNS autoimmune disease have demonstrated a potential role for the thymus in susceptibility and severity of inflammation. Research into central tolerogenic mechanisms in human CNS autoimmunity has lagged behind studies into peripheral and humoral immunological mechanisms. However, there are data that implicate central tolerance not only in the pathogenesis of CNS autoimmunity, particularly MS, but also in disease response to treatments. Many of these data are alas indirect measures of thymic function making it difficult to separate unambiguously central tolerance induction from peripheral tolerogenic mechanisms. Before any treatments aimed at modulating central selection can become viable therapeutic options, research efforts will need to

fill in some of the major gaps in our knowledge of thymic function in health and disease. We detail in **box 4** some of these areas, the controversies within them and strategies to resolve some of these issues. Better understanding of processes underlying central tolerance could open up novel avenues of interventions with far fewer side effects than the current untargeted immunomodulatory treatments.

Boxes

Box 1 Effects of MS disease modifying therapies on the adaptive immune system[8,9]

Interferon beta: alters balance between pro- and anti-inflammatory cytokines in multiple lymphocyte subsets

Glatiramer acetate: pseudo-random Myelin Basic Protein-based amino acid polymer causing alterations in Th2 polarisation

Dimethyl fumarate: alterations in Th2 polarisation

Teriflunomide: dihydroorotate dehydrogenase inhibitor preventing proliferation of autoreactive T-cells

Fingolimod: antagonist of sphingosine 1-phosphate receptors trapping lymphocytes in lymphoid tissues

Mitoxantrone: type 2 topoisomerase inhibitor preventing proliferation of autoreactive T-cells

Natalizumb: anti-α_4 integrin blocking lymphocyte extravasation

Alemtuzumab: anti-CD52 depleting mature lymphocytes

Box 2 Mechanisms of central and peripheral tolerance

As part of T cell development, central tolerance is instructed in the thymus by two sequential, albeit distinct mechanisms.[151] The process of positive selection chooses developing T cells (thymocytes) expressing a T cell antigen receptor (TCR) that displays sufficient affinity for its cognate peptide/MHC complex expressed on the surface of thymic epithelial cells (TECs). Thymocytes that fail to fulfil this criterion are prohibited from further maturation and die. Surviving thymoyctes are next subjected to negative selection whereby TECs and other thymus-resident antigen presenting cells ensure that thymocytes with high affinity TCR for self-antigens will undergo programmed cell death and thus are removed from the pool of the emerging population of naïve T cells to be exported to peripheral (lymphoid) tissues. In parallel, the process of clonal diversion guarantees that T cells with intermediate-to-high affinity TCRs reactive to self-antigens expressed by TECs differentiate into T_{reg} able to restrain auto-reactive T cell responses.[152–154] Hence, central (i.e. thymic) selection establishes a repertoire of T-cells that recognise antigens in the context of an individual's MHC haplotype but typically are unable to elicit an immune response directed against self-antigens. Because the processes of clonal deletion and diversion are not uniformly effective, the thymus generates and also exports naïve T-cells that recognise self-antigens with high affinity. A downstream process of peripheral tolerance ensures, however, that these potentially harmful lymphocytes are prevented from causing autoimmune disease. This charge is realized by multiple different mechanisms, including the deletion of self-reactive T-cells encountering self-antigens outside of the thymus, the induction of functional unresponsiveness upon antigen recognition under unfavorable conditions (a state referred to as anergy) and the suppression of T-cell reactivity via the control of T_{reg}.[151]

Box 3 Mechanisms of B-cell tolerance

The initial random generation of antibody specificities requires stringent selection processes that establish tolerance to self-antigens in B-cells.[12] Over 70% of early, immature B-cells within the bone marrow show binding to cytoplasmic or nuclear autoantigens, compared with approximately 40% of new emigrant B-cells and fewer than 20% of mature naïve B-cells.[13] This suggests both central and peripheral tolerance mechanisms affect B-cell selection.[14] Central selection of B-cells is dependent on B-cell receptor signalling and appears to be independent of T-cells.[155] Autoreactive B-cells undergo immunoglobulin gene rearrangement to render them no longer autoreactive through the process of central selection.[156] Conversely, peripheral B-cell selection is critically reliant on CD40-mediated signalling pathways and is absolutely dependent on T-cell interactions, specifically interactions between B-cells and T_{reg}.[157] IL-10-producing B-cells (regulatory B-cells) are also able to suppress peripheral T-cells responses.[158] Therefore, thymopoiesis and the generation of T_{reg} modulate peripheral B-cell tolerance, while regulatory B-cells influence the peripheral reactivity of T-cells.

Box 4 Controversies in central tolerance in CNS autoimmunity and proposed research directions

1. Resistance of the CNS to autoimmunity - *Observations:* Either with deficits in central selection (AIRE deficiency) or peripheral tolerance (FOXP3 deficiency), CNS inflammation is rarely seen. *Future research direction:* Adaptive and innate immune cells isolated from the CNS should be probed by comprehensive functional phenotyping to understand mechanisms underlying the resistance of the CNS to inflammation stemming from failure of either central or peripheral tolerance, or any other mechanism.
2. Modelling *in vitro* thymopoiesis - *Observations:* Existing models of *in vitro* thymopoiesis and TCR repertoire selection are incomplete, limiting insight into human thymus function. *Future research direction*: Research efforts should focus on establishment of stem cell-derived thymic models that reliably recapitulate the *in vivo* function of thymic epithelium and other stromal cells.
3. Changes in thymic biology with age - *Observations:* Many autoimmune diseases show peaks of incidence at specific ages that typically coincide with thymic senescence. Thymopoiesis is enhanced after autologous haematopoietic stem cell transplantation in adults and actively contributes anew to the peripheral T cell pool. *Future research direction:* Research should focus on changes in antigen presentation and thymopoiesis as a function of age, and analysis of the function of recent thymic emigrant T-cells after haematopoietic stem cell transplantation to treat CNS autoimmune diseases.
4. Thymic expression of CNS antigens - *Observations:* At the RNA level, most CNS antigens are detected in the thymus but the prevalence of CNS autoimmune diseases suggests that immunological tolerance is incomplete. The correlation between RNA and protein expression levels are low for many transcripts, and how this corresponds to peptide presentation is unclear. *Future research direction:* Assessment of the thymic peptidome through emerging high throughput assays should clarify the proportion of CNS antigens that are presented to developing thymocytes.
5. Environmental factors modulating thymic functions - *Observations:* Indirect evidence suggests that multiple environmental exposures alter aspects of thymic biology. Future *research direction:* Large-scale epidemiological studies using reliable measures of thymic function should assess the contribution of environmental factors to thymopoiesis and hence the potential for co-opting these for novel treatment strategies and targets.
6. Harnessing thymic selection for therapy - *Observations:* T_{reg} generated against CNS antigens are effective in suppressing CNS inflammation in mouse models. Whether similar strategies could work in human patients with CNS autoimmunity is unknown. *Future research direction:*

Efforts should concentrate on methods of efficiently generating T_{reg} specific for CNS antigens and thereafter trials should study potential ways of using these to ameliorate CNS inflammation in human subjects.

Figures

Figure 1 Mechanisms of thymic central tolerance. Schematic of positive and negative selection of thymocytes. Non-TEC cells, including dendritic cells and macrophages, also present antigen to developing thymocytes in the cortex and medulla. Conv T-cell = conventional T-cell ($CD4^+$ and $CD8^+$ T-cells).

Figure 2 CNS antigen expression in thymus. (**a**) A scatter plot of mean expression *vs.* the proportion of mTEC with detectable expression of each gene. Red, highlighted transcripts are known CNS antigens. Tissue specific genes were derived from Pan *et al.* and TEC expression was obtained from our data.[18,48] For comparison, housekeeping genes, including *Actb*, are highlighted in blue.[159] Common protein names that differ from the gene symbol, are indicated in parentheses. (**b**) Thymic expression of an encephalitogenic form of myelin protein reduces susceptibility to experimental autoimmune encephalomyelitis.[31,64] Peripheral injection of myelin-reactive T-cells bypasses central selection and renders previously resistant mice strains susceptible to experimental autoimmune encephalomyelitis.[65]

Figure 3 Measuring T-cell receptor excision circles in MS. Log_2 ratios of T-cell receptor excision circle abundances between patients with MS and healthy controls are shown in forest plots derived from [90–101]. Red points indicate meta-analysed values inferred from a random effects model weighted by inverse variance. Standard errors were estimated from data presented in each study. RRMS = relapsing remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS, Treg = regulatory T-cells, PBMC = peripheral blood mononuclear cells, $CD45RA^+$ = naïve T-cells.

Figure 4 Measuring T-cell receptor excision circles in MS treatments. A forest plot of individual studies derived from references [94,96,99–101,129,130]. IFNβ = interferon-beta treatment, HSCT = haematopoietic stem cell transplant, PBMC = peripheral blood mononuclear cells.

Figure 5 Potential therapeutic options in central tolerance. (**a**) Environmental or intrinsic factors could be modified in order to promote a thymic microenvironment less conducive to the generation of CNS autoreactive T-cells. (**b**) The generation of thymic organoids from host iPSC (induced pluripotent stem cells) could be used to drive the expression of CNS antigens in iPSC-derived mTEC, thus apoptosing thymocytes that might develop into autoreactive T-cells and generate CNS antigen-specific T_{reg} able to suppress peripheral autoreactivity. (**c**) Sequencing the TCR (T-cell receptor) pool before and after immunosuppressive therapy could predict treatment failure by identifying the re-emergence of CNS autoreactive T-cells and relating this to the risk of relapse.

Tables

Table 1 Summary of the most common autoimmune conditions specifically targeting CNS antigens

Condition	Main antigens	Cellular pathogenesis	Clinical manifestations
Multiple sclerosis	Uncertain but likely myelin components	Combined B- and T-cell inflammation plus innate immunity	Relapsing remitting inflammatory disease affecting multiple parts of the CNS; progressive neurological deficits associated with inflammation in primary or secondary progressive MS
Neuromyelitis optica	AQP4, MOG	Primarily autoantibody mediated	Mono- or polyphasic inflammatory disease, mainly restricted to the spinal cord or optic nerves
Autoimmune encephalitis	Multiple (NMDAR, LGI1, CASPR2, AMPAR, $GABA_{A/B}R$ and others)	Primarily autoantibody mediated	Clinical syndrome depends on antibody target; frequently typified by subacute encephalopathy and seizures
Stiff person spectrum disorder	GAD, GlyR	Combined B-cell and T-cell involvement +/- GAD antibodies	Muscle rigidity and spasms
Rasmussen's encephalitis	Unknown	T-cell inflammation plus innate immunity	Progressive hemiplegia, drug-resistant focal epilepsy and cognitive decline
Cerebellitis	GAD, CASPR2, Yo	Varying components for each subtype	Subacute onset of ataxia plus other neurological features
Bickerstaff encephalitis	Gangliosides (GQ1b)	Primarily autoantibody mediated	Brainstem neurological deficits
Combined central and peripheral demyelination	Neurofascin	Primarily autoantibody mediated	Focal CNS neurological deficits plus global weakness and areflexia

AQP4 = Aquaporin-4; MOG = Myelin Oligodendrocyte Glycoprotein; NMDAR = N-methyl-D-aspartate Receptor; LGI1 = Leucine Rich Glioma Inactivated 1; CASPR2 = Contactin-associated protein; AMPAR = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor; GAD = Glutamic Acid Decarboxylase; GlyR = Glycine Receptor; $GABA_{A/B}R$ = gamma-aminobutyric acid (A or B) receptor.

Glossary

Auto-antigen: a peptide that leads to T-cell cross-reactivity with host tissues

Autoimmune polyendocrine syndrome type 1 (APS1): multi-system autoimmune disorder characterised by Addison disease, hypoparathyroidism or chronic mucocutaneous candidiasis caused by mutations in *AIRE*

Central tolerance: selection of thymocytes within the thymus

Negative selection: apoptosis of thymocytes bearing T-cell receptor pairings that bind strongly to peptide-MHC complexes derived from endogenous proteins

Peripheral tolerance: the activity of regulatory T-cells and innate cells that dampens down inflammation when occurring in the incorrect context

Positive selection: apoptosis of thymocytes bearing T-cell receptor pairings that are unable to bind productively to peptide-MHC complexes

T-cell receptor excision circles (TREC): circular pieces of DNA excised in thymocytes during the recombination of T-cell receptor genes

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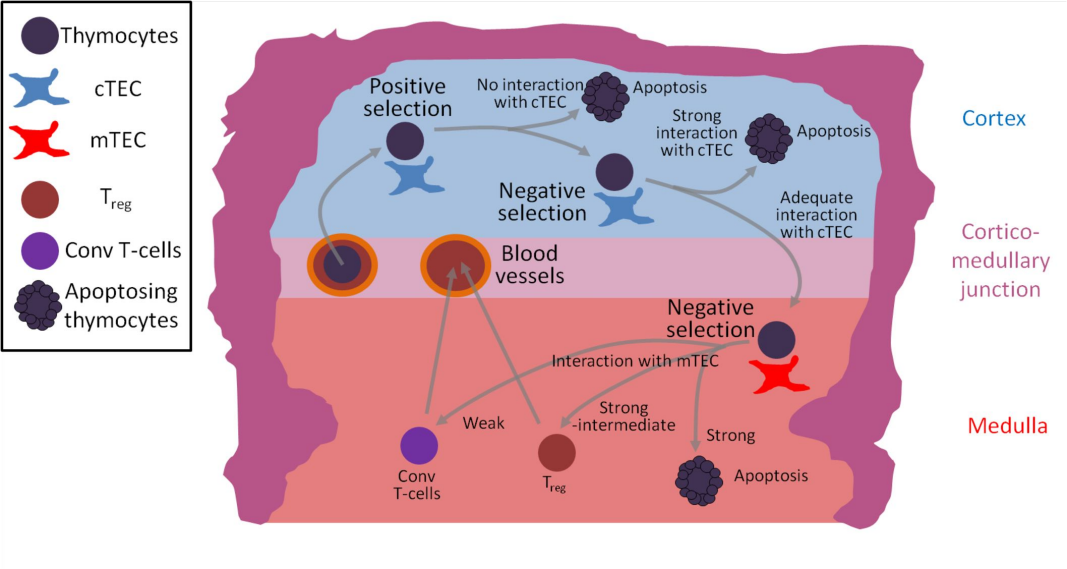
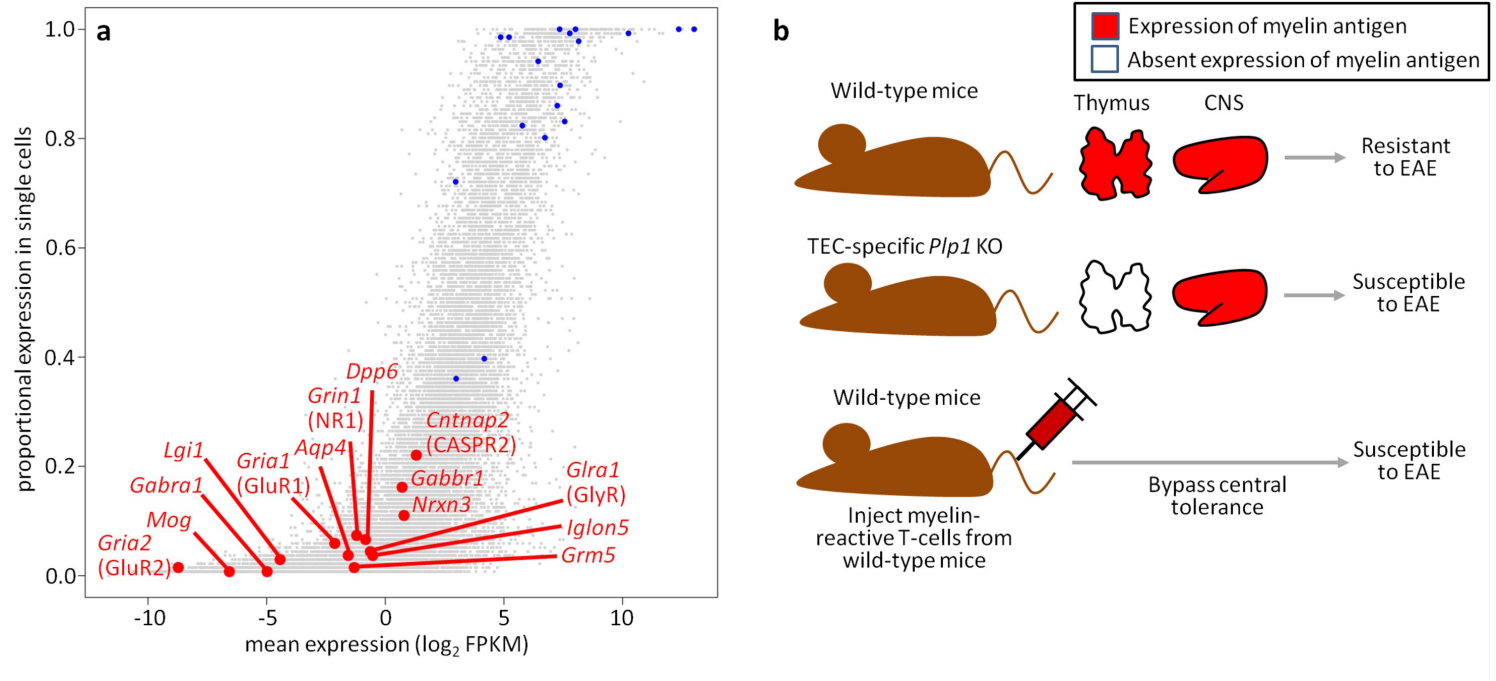
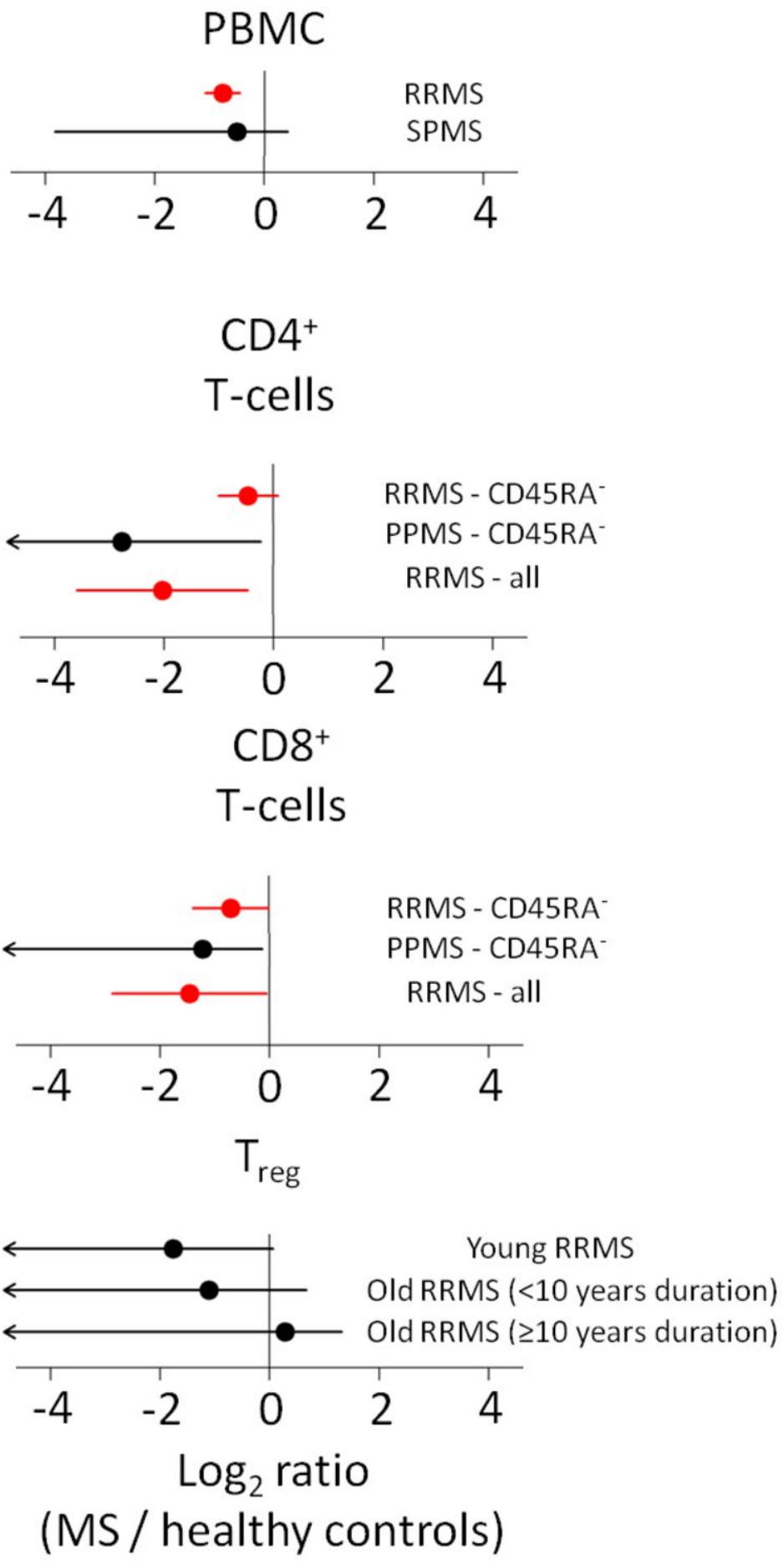
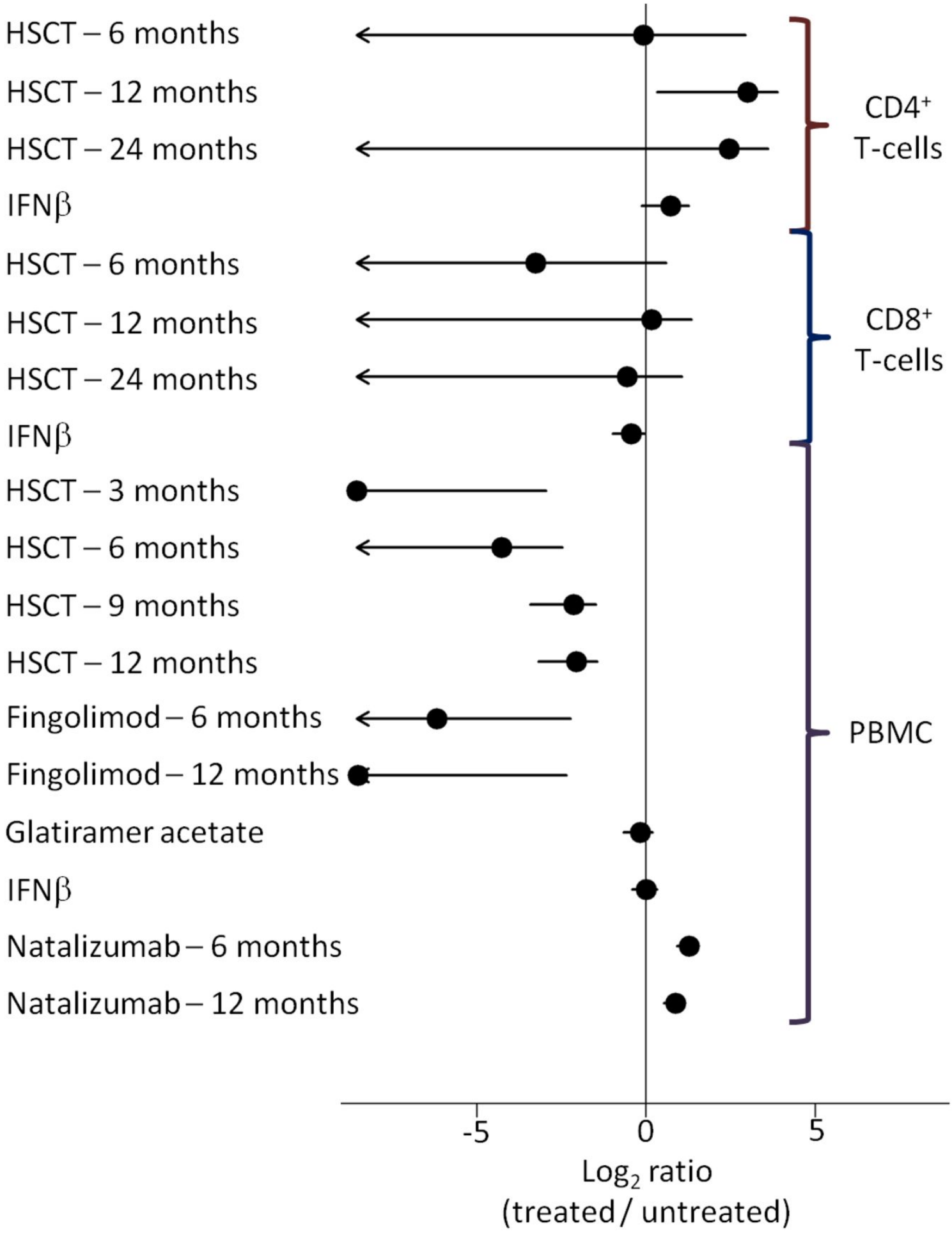
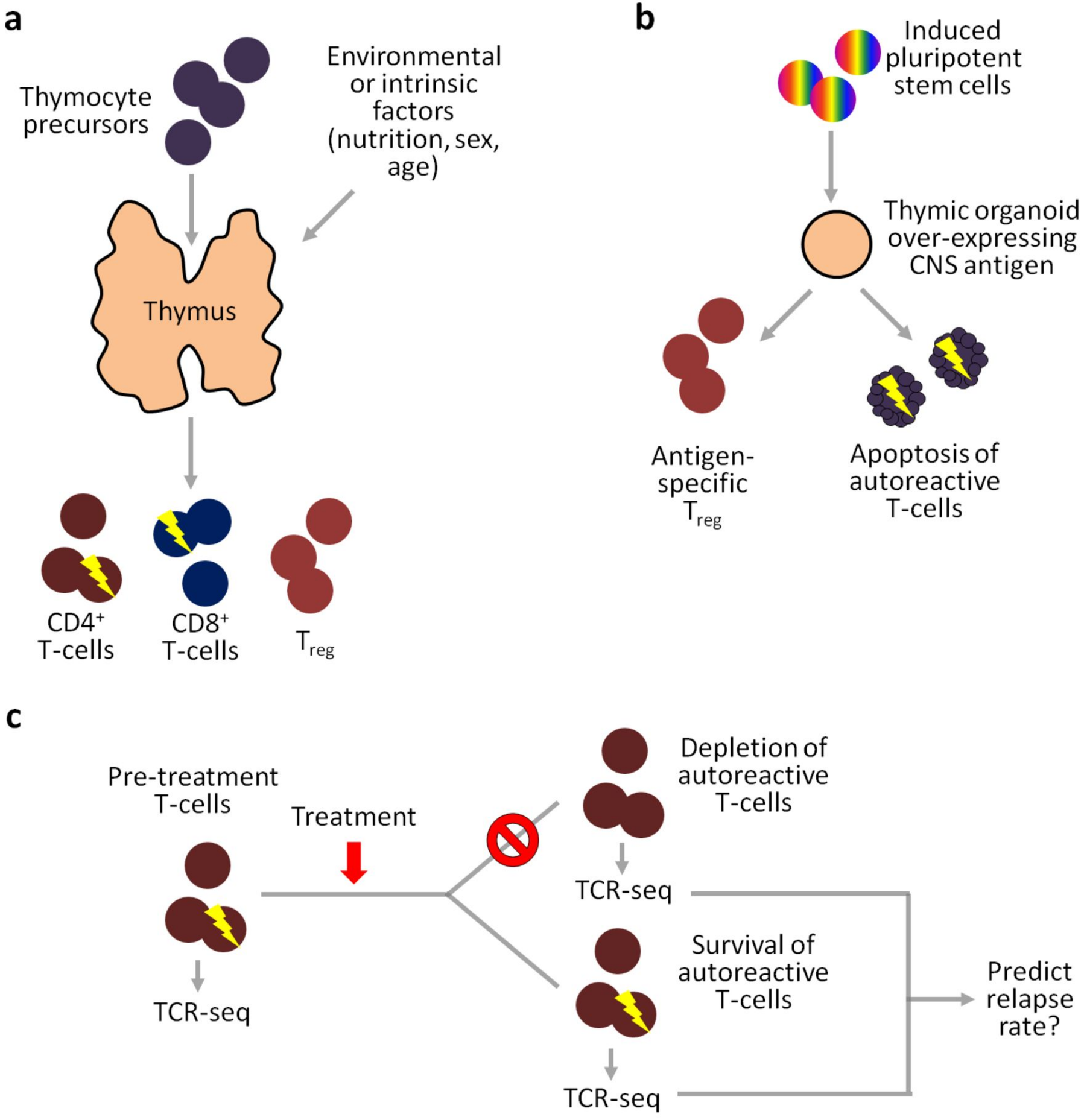

Thymocytes
cTEC
mTEC
T_{reg}
Conv T-cells
Apoptosing thymocytes
Positive selection
No interaction with cTEC
Apoptosis
Strong interaction with cTEC
Apoptosis
Negative selection
Adequate interaction with cTEC
Blood vessels
Negative selection
Interaction with mTEC
Weak
Strong -intermediate
Strong
Conv T-cells
T_{reg}
Apoptosis
Cortex
Cortico-medullary junction
Medulla


a
b
proportional expression in single cells
mean expression (log2 FPKM)
1.0
0.8
0.6
0.4
0.2
0.0
-10
-5
0
5
10
Dpp6
Grin1
(NR1)
Cntnap2
(CASPR2)
Lgi1
Gria1
(GluR1)
Aqp4
Gabbr1
Glra1
(GlyR)
Gabra1
Nrxn3
Mog
Iglon5
Gria2
(GluR2)
Grm5
Expression of myelin antigen
Absent expression of myelin antigen
Wild-type mice
Thymus
CNS
Resistant
to EAE
TEC-specific Plp1 KO
Susceptible
to EAE
Wild-type mice
Susceptible
to EAE
Bypass central
tolerance
Inject myelin-
reactive T-cells from
wild-type mice


PBMC
RRMS
SPMS
-4
-2
0
2
4
CD4+
T-cells
RRMS - CD45RA-
PPMS - CD45RA-
RRMS - all
-4
-2
0
2
4
CD8+
T-cells
RRMS - CD45RA-
PPMS - CD45RA-
RRMS - all
-4
-2
0
2
4
Treg
Young RRMS
Old RRMS (<10 years duration)
Old RRMS (≥10 years duration)
-4
-2
0
2
4
Log2 ratio
(MS / healthy controls)


HSCT – 6 months
HSCT – 12 months
HSCT – 24 months
IFNβ
HSCT – 6 months
HSCT – 12 months
HSCT – 24 months
IFNβ
HSCT – 3 months
HSCT – 6 months
HSCT – 9 months
HSCT – 12 months
Fingolimod – 6 months
Fingolimod – 12 months
Glatiramer acetate
IFNβ
Natalizumab – 6 months
Natalizumab – 12 months
CD4+ T-cells
CD8+ T-cells
PBMC
-5
0
5
Log2 ratio
(treated / untreated)


a
Thymocyte precursors
Environmental or intrinsic factors (nutrition, sex, age)
Thymus
CD4+ T-cells
CD8+ T-cells
Treg
b
Induced pluripotent stem cells
Thymic organoid over-expressing CNS antigen
Antigen-specific Treg
Apoptosis of autoreactive T-cells
c
Pre-treatment T-cells
Treatment
TCR-seq
Depletion of autoreactive T-cells
TCR-seq
Survival of autoreactive T-cells
TCR-seq
Predict relapse rate?