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# The thymus and central tolerance

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T-cell differentiation in the thymus generates a peripheral repertoire of mature T cells that mounts strong responses to foreign antigens but is largely unresponsive to self-antigens. This state of specific immunological tolerance to self-components involves both central and peripheral mechanisms. Here we review the process whereby many T cells with potential reactivity for self-antigens are eliminated in the thymus during early T-cell differentiation. This process of central tolerance (negative selection) reflects apoptosis and is a consequence of immature T cells receiving strong intracellular signalling through T-cell receptor (TCR) recognition of peptides bound to major histocompatibility complex (MHC) molecules. Central tolerance occurs mainly in the medullary region of the thymus and depends upon contact with peptide–MHC complexes expressed on bone-marrow-derived antigen-presenting cells (APCs); whether tolerance also occurs in the cortex is still controversial. Tolerance induction requires a combination of TCR ligation and co-stimulatory signals. Co-stimulation reflects interaction between complementary molecules on T cells and APCs and probably involves multiple molecules acting in consort, which may account for why deletion of individual molecules with known or potential co-stimulatory function has little or no effect on central tolerance. The range of self-antigens that induce central tolerance is considerable and, via low-level expression in the thymus, may also include tissue-specific antigens; central tolerance to these latter antigens, however, is likely to be limited to high-affinity T cells, leaving low-affinity cells to escape. Tolerance to alloantigens and the possibility of using central tolerance to promote acceptance of allografts are discussed.

**Keywords:** thymus; central tolerance; negative selection; positive selection

## 1. INTRODUCTION

Though difficult to define in precise terms, immunological tolerance can be regarded as a state of unresponsiveness to a self- or foreign antigen. As outlined throughout this issue, tolerance in higher vertebrates is usually antigen specific and operates largely at the level of T lymphocytes. Unresponsiveness of T cells can involve several different mechanisms, including clonal deletion (death), induction of a refractory state (anergy), inhibition of function by other cells or their products (immunoregulation) and sequestration of antigen (ignorance) (Kruisbeek *et al*. 1992; Fowlkes & Ramsdell 1993; Nossal 1993; Sprent 1995; Sprent & Webb 1995). T-cell tolerance is induced in two main sites: (i) in the thymus during early T-cell differentiation (central tolerance); and (ii) in the secondary lymphoid tissues after export of mature T cells from the thymus (peripheral tolerance). Here we review the mechanisms controlling induction of central tolerance of T cells in the thymus. To begin, it is important to discuss the formation and differentiation of thymocyte subsets and the pivotal role of major histocompatibility complex (MHC) molecules in tailoring the specificity of mature T cells.

## 2. THE THYMUS AND POSITIVE SELECTION

The specificity of typical T cells expressing αβ T-cell receptor (TCR) molecules is directed to peptides bound to a groove on cell-surface MHC molecules (Marrack *et al*. 1983; Sette *et al*. 1990; Pamer & Cresswell 1998; Benoist & Mathis 1999; Parham 1999); MHC molecules are highly polymorphic and are the main targets for T cells responding to allografts. TCR recognition of peptide–MHC complexes is augmented by CD8 and CD4 molecules on T cells; these co-receptor molecules bind to non-polymorphic sites on MHC class I (MHC I) and MHC class II (MHC II) molecules, respectively, and thereby direct the specificity of mature $CD4^{-}8^{+}$ (CD8) and $CD4^{+}8^{-}$ (CD4) T cells to peptides bound to MHC I and MHC II molecules.

In the thymus, αβ T cells arise from small numbers of proliferating $CD4^{-}8^{-}$ stem cells, which give rise to large numbers of $CD4^{+}8^{+}$ cells (Robey & Fowlkes 1994; Fowlkes & Schweighoffer 1995; Benoist & Mathis 1999). These 'double-positive' (DP) thymocytes form the vast majority of cells found in the cortical region of the thymus. In this site DP thymocytes are in close contact with a dense network of epithelial cells. As discussed below, the MHC molecules on epithelial cells play a decisive role in determining the fate of DP thymocytes.

Typical cortical DP cells express low levels of αβ TCR molecules and are programmed to die within three to four days unless rescued by a TCR signal (Sprent & Webb 1987; Benoist & Mathis 1989, 1999; Sprent 1993; Fink & Bevan 1995; Sebzda *et al*. 1999). Such signalling reflects TCR contact with specific peptide–MHC complexes on cortical epithelial cells; the peptides concerned are generated through breakdown and processing of various intracellular proteins, thus causing MHC molecules on

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epithelial (and other) cells to display a wide variety of self-peptides (Germain 1993; Cresswell 1994; Pamer & Cresswell 1998; Parham 1999; Rock & Goldberg 1999). Of the large numbers of DP cells in the cortex, only a small proportion of these cells (1–5%) have significant binding specificity for the various self-peptide–MHC complexes expressed on adjacent epithelial cells. DP cells binding these complexes receive a low-level TCR signal that allows the cells to survive, upregulate TCR expression and differentiate into mature 'single-positive' (SP) CD8 and CD4 cells. This process of positive selection is aided and fine-tuned by the CD8 and CD4 molecules on DP cells. Thus, for DP cells recognizing peptide–MHC I molecules, co-recognition of MHC I molecules by CD8 causes the cells to retain CD8 expression but down-regulate CD4 expression, thus generating $CD4^{-}8^{+}$ SP cells. Conversely, recognition of peptide–MHC II complexes causes DP cells to retain CD4 but lose CD8, thereby forming $CD4^{+}8^{-}$ cells. A point to emphasize is that the vast majority of DP cells (over 95%) have negligible affinity for thymic peptide–MHC complexes. These cells fail to undergo TCR signalling and, in the absence of this protective signal, the cells succumb to apoptosis ('death by neglect').

## 3. THE PURPOSE OF POSITIVE SELECTION

In understanding thymic selection, it is important to point out that DP cells die by two quite separate mechanisms. As mentioned above, DP cells are programmed to die via apoptosis unless the cells receive a protective signal via TCR–peptide–MHC interaction. If this TCR signal is too intense, however, the cells are instructed to undergo an active, TCR-mediated form of apoptosis. As discussed later, this type of apoptosis occurs during negative selection and reflects contact with strong (agonist) peptides. By contrast, the typical peptides inducing positive selection are relatively weak (Sprent & Webb 1987; Benoist & Mathis 1989, 1999; Sprent 1993; Robey & Fowlkes 1994; Fink & Bevan 1995; Fowlkes & Schweighoffer 1995; Sebzda *et al.* 1999). These antagonist peptides cause significant but low-level TCR signalling, the intensity of signalling being sufficient to rescue the cells from death by neglect but inadequate to induce TCR-mediated apoptosis.

Most of the peptides inducing positive selection are not unique to epithelial cells and are probably expressed throughout the body. Hence immature thymocytes undergoing positive selection to particular self-peptides in the thymus will eventually re-encounter these same peptides in the peripheral lymphoid tissues. Continuous contact with positively selecting peptides, first in the thymus and then in the periphery, is of interest for two reasons.

(i) A key feature of typical naive T cells is that, despite continuous contact with self-peptides in the periphery, the cells remain in a resting state and display no signs of activation unless the cells encounter a foreign antigen (Kruisbeek *et al.* 1992; Fowlkes & Ramsdell 1993; Nossal 1993; Sprent & Tough 1994). The implication therefore is that the peptides inducing positive selection in the thymus are ignored when T cells achieve full maturity and contact these peptides after entering the post-thymic environment. Yet at the level of immature thymocytes the peptides trigger T cells to differentiate and also to express activation markers such as CD69 (Sprent & Webb 1987; Benoist & Mathis 1989, 1999; Sprent 1993; Robey & Fowlkes 1994; Fink & Bevan 1995; Fowlkes & Schweighoffer 1995; Sebzda *et al.* 1999). So, T cells seem to be able to 'see' the peptides in the thymus but not in the periphery. *A priori*, the simplest explanation for this paradox is that maturation of T cells is associated with a process of TCR desensitization where the cells can no longer recognize the original selecting peptides but retain reactivity for foreign peptides (most of which are strong peptides). There is some evidence for such TCR desensitization (Kruisbeek *et al.* 1992; Fowlkes & Ramsdell 1993; Kishimoto *et al.* 1996) but the molecular mechanisms involved are still unclear.

(ii) At face value positive selection is a conspicuously wasteful process: of the total numbers of thymocytes generated, more than 95% are destined for rapid destruction. This begs the question of the raison d'être of positive selection. Until recently positive selection has been viewed as a manifestation of MHC polymorphism. In this respect, studies on T cells arising in MHC-different bone-marrow chimeras and thymus-grafted mice, plus more recent studies with TCR transgenic mice raised on different MHC backgrounds, have indicated that T cells display 'self-MHC restriction' (Sprent & Webb 1987; Von Boehmer 1990). Thus, when confronted with a particular foreign peptide, T cells respond preferentially to this peptide bound to self-MHC molecules (the MHC molecules encountered during positive selection in the thymus) rather than to foreign MHC molecules. This line of reasoning implies that TCR recognition of self-MHC molecules, though covert, is sufficient to somehow augment TCR contact with foreign peptides bound to these same self-MHC molecules. Accordingly, positive selection skews the T-cell repertoire to covert recognition of self-MHC molecules, thereby enhancing responsiveness to foreign peptides. A problem with this line of reasoning is that it is difficult (though not impossible) to explain why T cells display strong reactivity to MHC alloantigens, i.e. to foreign MHC molecules displaying a multitude of foreign peptides.

Very recently, a quite different explanation for positive selection has emerged (Boursalian & Bottomly 1999; Ernst *et al.* 1999; Freitas & Rocha 1999; Goldrath & Bevan 1999; Murali-Krishna *et al.* 1999; Viret *et al.* 1999). This theory hinges on two observations. First, adoptive transfer experiments have shown that naive T cells transferred to MHC-deficient hosts or to hosts lacking the peptides that induced positive selection causes the cells to disappear gradually. Second, the unresponsiveness of naive T cells to self-MHC molecules plus bound self-peptides can be overcome by reducing overall T-cell numbers below a certain threshold. In this situation naive T cells begin to proliferate and undergo considerable clonal expansion. Significantly, this proliferative response to self-peptides seems to be directed specifically

to the peptides that led to initial positive selection of the cells in the thymus. In view of these findings, the chief purpose of positive selection may be simply to keep mature T cells alive. The argument here is that all cells, including T cells, are destined to die by default unless rescued by life-sustaining signals from other cells or their products (Raff 1992; Boursalian & Bottomly 1999; Freitas & Rocha 1999; Murali-Krishna *et al*. 1999). For mature T cells such signalling is provided by continuous low-level ligation of the TCR molecules, i.e. through contact with peptide–MHC complexes. The purpose of positive selection is to generate mature T cells that have significant specificity for these self-ligands. Under normal steady-state conditions the level of TCR stimulation induced by these ligands is sufficient to keep T cells alive but not to induce overt T-cell activation. For reasons that are still unclear, lowering total T-cell numbers enhances TCR signalling and causes T cells to proliferate. Such homeostatic expansion of T cells could be an important mechanism for expanding the T-cell pool in patients receiving chemotherapy or irradiation and perhaps also in old age, where input of new T cells from the thymus is limited.

## 4. THE THYMUS AND NEGATIVE SELECTION

As discussed above, most of the self-peptides controlling positive selection are relatively weak and poorly immunogenic for mature T cells. These peptides are thus unlikely targets for inducing overt, destructive responses to self-antigens (autoimmune disease). However a spectrum of other MHC-associated self-peptides has the potential to bind strongly to the TCR. Hence if T cells specific for these peptides were allowed to exit from the thymus, there would be a high risk that the cells would attack self-components and thereby induce autoimmune disease. Based on this line of reasoning it has long been argued that T cells with high affinity for self-antigens are deleted in the thymus. This process of negative selection (central tolerance) is now well documented.

## 5. MODELS FOR NEGATIVE SELECTION

The notion that T cells undergo central tolerance in the thymus stemmed from early studies on skin allograft tolerance developing in dizygotic twin calves (Owen 1945) and neonatal mice injected with MHC-different lymphoid cells (Billingham *et al*. 1953). These studies were carried out prior to the discovery that T cells arise in the thymus (Miller 1961). Hence the precise site of tolerance induction was unclear. However, clear evidence that the thymus controls transplantation tolerance came from the observation that T cells arising in T-depleted neonatally thymectomized strain A mice reconstituted with an MHC-different strain B thymus failed to reject strain B skin grafts but rejected third-party strain C grafts (Miller 1962).

Though clearly implicating the thymus in tolerance, these early studies failed to establish whether tolerance involved clonal deletion or some other mechanism. The first clear evidence for clonal deletion came from the observation that murine T cells express Vβ-specific reactivity to mammary tumour virus (Mtv) superantigens (Sags) and that mouse strains expressing particular endogenous Sags show Vβ-specific 'holes' in the T-cell repertoire, both in the thymus and the periphery (Marrack & Kappler 1990; Herman *et al*. 1991; Hodes & Abe 1992). Proof that clonal deletion also applies to T cells specific for conventional antigens, i.e. for MHC-associated peptides, came from the advent of techniques for detecting antigen-specific T cells with TCR clonotype-specific monoclonal antibodies (mAbs) (Kappler *et al*. 1987; Bill *et al*. 1989; Von Boehmer 1990).

## 6. SITES OF NEGATIVE SELECTION AND THE ROLE OF ANTIGEN-PRESENTING CELLS

The discovery that T cells can undergo negative selection in the thymus has focused attention on which particular cell types act as antigen-presenting cells (APCs) for tolerance induction. Since peptide–MHC complexes are expressed throughout the thymus, any cell type expressing these complexes would seem to have the potential to induce tolerance. Here, particular attention has been given to the role of thymic epithelial cells (TECs) and bone-marrow (BM)-derived APC.

Various models, including studies on tolerance developing in BM chimeras, have provided strong evidence that efficient induction of tolerance in the thymus requires the presence of BM-derived APCs (Matzinger & Guerder 1989; Nossal 1993; Sprent *et al*. 1993; Sprent 1995; Sprent & Webb 1995). Dendritic cells (DCs) are the most efficient type of APC (Steinman 1999) and, in the thymus, DCs are expressed almost exclusively in the medulla. DCs express high levels of MHC I and II molecules and there is general agreement that these cells play a key role in tolerance induction. Whether other APCs, such as macrophages and B cells, contribute to tolerance is unclear. Macrophages are scattered throughout the thymus, including the cortex, but, at least for CD4 cells, the low levels of MHC II molecules on macrophages makes it unlikely that these cells play more than a minor role in tolerance induction.

Whether TECs are involved in tolerance induction has long been controversial (Sprent 1995). Here, it is important to point out that TECs in the cortex and medulla are phenotypically distinct. For medullary TECs, a number of studies suggest that these cells do make a significant contribution to tolerance, although full tolerance requires the presence of DCs (Matzinger & Guerder 1989; Webb & Sprent 1990; Burkly *et al*. 1993; Degermann *et al*. 1994). Whether cortical TECs can participate in tolerance induction is less clear. Nevertheless, the bulk of evidence suggests that cortical TECs are much less tolerogenic than medullary TECs. This topic raises the question of the anatomical site of negative selection.

Many workers view the cortex as a principal site for negative selection. In favour of this idea, for some TCR transgenic lines intrathymic contact with antigen, e.g. the male antigen in the HY-specific line, causes marked atrophy of the cortex (Von Boehmer 1990; Fowlkes & Ramsdell 1993; Sprent 1993, 1995). Although these data are clearly consistent with negative selection occurring in the cortex, it should be pointed out that αβ TCR expression in transgenic mice is usually higher than normal at the level of DP thymocytes and, in contrast to

normal mice, can also be apparent at the level of $CD4^-8^-$ cells. Hence, it is not clear in this model whether negative selection occurs at the level of cortical DP cells rather than at the $CD4^-8^-$ precursor stage. Hence, extrapolating from these data on transgenic thymocytes to physiological negative selection in the normal thymus is difficult. Further evidence for tolerance in the cortex is provided by the finding that injecting TCR transgenic mice with specific peptide can cause massive destruction of cortical thymocytes (Murphy *et al.* 1990). However, a problem with this approach is that injecting TCR transgenic mice with peptide can cause stimulation of peripheral T cells (Jondal *et al.* 1993; Martin & Bevan 1997). Via release of toxic cytokines and production of corticosteroids, stimulation of peripheral T cells can then lead to 'by-stander' death of cortical thymocytes. In view of these and other concerns there is still no irrefutable evidence that negative selection occurs in the cortex. In fact, evidence against negative selection in the cortex is provided by the finding that, for CD4 cells, transgenic mice expressing MHC II molecules solely on cortical TECs display only positive selection and not negative selection (Laufer *et al.* 1999).

Being enriched in BM-derived APCs, the medulla is a logical site for negative selection. Moreover, entry of soluble antigens into the thymus from the bloodstream is much more prominent in the medulla than the cortex (Sprent 1995). Nevertheless, it should be borne in mind that the medulla lacks DP cells and is populated almost entirely by the progeny of these cells, i.e. CD4 and CD8 SP cells. Are these cells susceptible to tolerance induction? In considering this question, it should be emphasized that exposing mature T cells to antigen typically induces an overt immune response associated with extensive proliferation and differentiation into effector cells; the progeny of these cells are often eventually eliminated *en masse*, but this process of 'activation-induced cell death' (AICD) occurs late in the immune response and is preceded by proliferation (Kawabe & Ochi 1991; Sprent & Webb 1995; Leonardo *et al.* 1999; Pinkoski & Green 1999). Hence, the form of clonal elimination of T cells seen at the end of many typical immune responses in the peripheral lymphoid tissues seems to be quite distinct from the rapid-onset apoptosis that characterizes negative selection in the thymus. Since nearly all medullary T cells are CD4 and CD8 cells rather than DP cells, it would seem to follow that negative selection cannot occur in the medulla. However, it is now apparent that most medullary SP cells are only partly mature. In fact, unlike mature peripheral T cells, a high proportion (*ca.* 60%) of medullary SP cells resemble DP cells in expressing the heat-stable antigen (HSA) marker (Kishimoto & Sprent 1997). As discussed in the next section, these 'semi-mature' $HSA^+$ medullary SP cells are highly sensitive to negative selection.

Leaving aside the tolerance susceptibility of T-cell subsets, the only direct test for determining the site of negative selection is to define where cells die when thymocytes confront antigen *in situ*. Here, convincing evidence for negative selection in the medulla has come from studies on transgenic mice undergoing Vβ (Vβ5)-specific tolerance to endogenous Sags. In this situation, staining the thymus of Vβ5 transgenic mice for apoptotic cells using the TUNEL technique has shown that apoptosis is prominent in the medulla but inconspicuous in the cortex (Surh & Sprent 1994).

Collectively, the above data suggest that negative selection occurs largely in the medulla, although some degree of negative selection in the cortex cannot be excluded. It should be mentioned that some workers support a two-site model for negative selection (Punt *et al.* 1997): thymocytes receive a 'lethal hit' for negative selection in the cortex but do not undergo apoptosis until the cells migrate to the medulla and make contact with APCs. This interesting idea is difficult to test experimentally *in vivo*.

## 7. TOLERANCE SUSCEPTIBILITY OF THYMOCYTE SUBSETS

Studies on the effects of exposing purified subsets of thymocytes to TCR ligation *in vitro* have provided useful information on the relative sensitivity of thymocyte subsets to negative selection.

In the case of cortical DP cells, it is clear that exposing these cells to TCR ligation, i.e. by culturing cells with anti-TCR mAbs cross-linked on plastic, fails to kill the cells (Page *et al.* 1993; Punt *et al.* 1994; Kishimoto & Sprent 1997). However, marked apoptosis of DP cells occurs when the cells are cultured with a mixture of anti-TCR and anti-CD28 mAbs (both presented in cross-linked form). In this situation, ligation of CD28 molecules provides a powerful co-stimulatory signal that synergizes with TCR signals and induces onset of apoptosis. This is an interesting contrast to TCR-stimulated mature T cells where the co-stimulatory effects of CD28 ligation (or interaction with the natural ligands for CD28, B7-1 and/or B7-2, on APCs) induces T cells to proliferate rather than to die. These data indicate that DP cells are clearly susceptible to negative selection and are cited in favour of the notion that negative selection occurs in the cortex. However, extrapolating from studies on the effects of TCR−CD28 ligation *in vitro* to the normal thymus *in vivo* is not easy because B7 expression in the thymus is very low in the cortex but high on APCs in the medulla (Degermann *et al.* 1994). Hence, if negative selection occurs at the level of DP cells in the cortex, how do the cells receive an obligatory co-stimulatory signal? Since CD28 expression on T cells is not essential for negative selection (see below), an obvious possibility is that negative selection of DP cells is controlled by other co-stimulatory molecules, the ligands for these molecules being expressed on cortical TECs. The problem with this notion is that testing the effects of mAb ligation of a wide variety of different molecules on DP cells has shown that ligation of only one molecule, CD28, provides co-stimulation for TCR-mediated apoptosis (Punt *et al.* 1997; Kishimoto & Sprent 1999). In the light of this finding, negative selection of DP cells either does not occur or is delayed until the cells migrate to the cortex and meet B7 on APCs.

For the component of SP thymocytes found in the medulla, about one-third of these cells are $HSA^-$ and closely resemble mature peripheral T cells in being strongly resistant to negative selection. Thus, as for peripheral T cells, exposing $HSA^-$ medullary SP cells to combined TCR−CD28 ligation induces proliferation rather than apoptosis. The situation with $HSA^+$ medullary

SP cells is different. Thus, as with cortical DP cells, TCR–CD28 ligation of $HSA^+$ medullary cells induces rapid onset of apoptosis without entry into cell cycle (Kishimoto *et al.* 1998).

The above findings indicate that two distinct populations of thymocytes, cortical DP cells and $HSA^+$ medullary SP cells, are sensitive to an artificial form of negative selection *in vitro*. A key issue is whether these data are relevant to negative selection *in vivo*. This question has been assessed by injecting normal or transgenic mice with antigen and then examining deletion of antigen-specific cells at the level of various thymocyte subsets (Kishimoto *et al.* 1998; Kishimoto & Sprent 1999). To limit by-stander death of thymocytes through stimulation of peripheral T cells (see above), these studies were performed on neonatal mice; unlike adult mice, neonatal mice have low levels of corticosteroids and only minimal numbers of mature T cells. The notable finding in these studies is that deletion of antigen-specific T cells at one to two days after antigen injection is largely restricted to $HSA^+$ medullary SP cells (Kishimoto *et al.* 1998; Kishimoto & Sprent 1999); deletion of DP cells is minimal.

## 8. CO-STIMULATORY MOLECULES FOR NEGATIVE SELECTION

As mentioned above, ligation of only one known molecule on DP cells, CD28, has been found to provide co-stimulation for TCR-mediated apoptosis *in vitro*. Yet most studies on $CD28^{-/-}$ mice have failed to show that these mice display an obvious defect in negative selection (Walunas *et al.* 1996). Likewise, a variety of other mice lacking individual molecules with known or potential co-stimulatory function show an apparently normal pattern of negative selection (Dautigny *et al.* 1999). These studies do not exclude the possibility that other, as yet unknown, molecules control negative selection. However, another perhaps more likely possibility is that co-stimulation for negative selection is not controlled by a single molecule but by multiple molecules acting in consort (Kishimoto & Sprent 1999; Page 1999).

The notion that several different molecules control negative selection clearly implies that co-stimulation-induced apoptosis of thymocytes *in vitro* is not unique to CD28 ligation. On this point it is of interest that the apparently exclusive role of CD28 in providing co-stimulation for death of DP thymocytes *in vitro* does not apply to $HSA^+$ medullary SP cells. For these latter cells, ligation of two other molecules, CD5 and CD43, provides co-stimulation for TCR-mediated apoptosis (Kishimoto & Sprent 1999). These findings are consistent with a redundancy model where multiple molecules control negative selection. A corollary of this model is that inactivation of one of these molecules, e.g. by mutation, would have little effect: the other molecules would fill in functionally and negative selection would remain intact. The observation that gene knockout mice lacking individual co-stimulatory molecules display near-normal negative selection is consistent with this view.

In the case of peripheral T cells undergoing AICD, the elimination of effector cells is largely under the control of Fas, which transduces apoptotic signals to T cells following interaction with FasL (Green *et al.* 1992; Nagata & Golstein 1995; Leonardo *et al.* 1999; Pinkoski & Green 1999). Fas also plays a role in negative selection, but only under defined conditions, namely when the concentration of antigen is very high (Kishimoto & Sprent 1997; Kishimoto *et al.* 1998). With low concentrations of antigen, negative selection is Fas-independent. When the concentration of antigen is raised to a high level, however, the Fas-independent pathway of apoptosis fails and negative selection becomes heavily dependent on Fas. This conclusion stems from the observation that exposing $HSA^+$ medullary SP cells to strong TCR–CD28 ligation *in vitro* induces apoptosis of normal cells but not Fas-deficient *lpr/lpr* cells (Kishimoto & Sprent 1997). Likewise, injecting mice with a high concentration of antigen eliminates $HSA^+$ medullary SP cells only in normal and not *lpr/lpr* mice (Kishimoto *et al.* 1998). With lower concentrations of antigen, by contrast, negative selection is as efficient in *lpr/lpr* mice as in normal mice.

The explanation for this paradox is that strong co-stimulation-dependent stimulation of $HSA^+$ medullary cells causes increased expression of anti-apoptotic molecules, thereby preventing the cells from dying; however, the protective function of the anti-apoptotic molecules does not apply to Fas-mediated death, with the result that with strong T-cell stimulation negative selection proceeds normally, provided that the cells retain Fas expression. Negative selection via Fas is thus viewed as a backup pathway that becomes important only when the Fas-independent pathway fails, i.e. when the concentration of the antigen concerned is high.

## 9. RELATIVE IMPORTANCE OF CENTRAL AND PERIPHERAL TOLERANCE

Even though central tolerance is well documented experimentally, it is often argued that T-cell deletion in the thymus is never complete and that autoreactive cells will inevitably escape to the periphery: to prevent these cells from attacking self-components and inducing autoimmune disease, peripheral mechanisms of tolerance are essential and, indeed, could be more important than central tolerance.

In considering this issue it should be emphasized that in practice negative selection is a highly efficient process. This point is illustrated by the finding that, in TCR transgenic mice, intrathymic exposure to specific antigen expressed constitutively deletes virtually all thymocytes (Von Boehmer 1990; Fowlkes & Ramsdell 1993; Sprent 1995; Sprent & Webb 1995). A few T cells may escape to the periphery by decreasing TCR or CD4–CD8 expression, but these cells are poorly responsive to antigen. The key observation is that, even though close to 100% of the TCR transgenic cells in the thymus are potentially autoreactive, these cells are eliminated *en masse* in the thymus and the mice fail to show signs of autoimmune disease.

Despite the efficiency of negative selection, this process can only occur when the concentration of antigen displayed in the thymus is above a certain threshold. Here, the affinity of the TCR for antigen is crucial. Thus, for high-affinity T cells, exposure to even very low concentrations of antigen is sufficient to eliminate these cells. With low-affinity T cells, however, deleting these cells may require quite high concentrations of antigen. Hence,

with low concentrations of antigen, low-affinity T cells with potential reactivity to the antigen will escape into the periphery. Provided that the concentration of antigen in the periphery is no higher than in the thymus, however, the exported low-affinity T cells will not be activated and pose no danger for autoimmune disease. But what happens when the concentration of antigen is higher in the periphery than in the thymus? For ubiquitous self-antigens or circulating antigens in the blood, this situation is unlikely to arise. For tissue-specific antigens, e.g. antigens expressed selectively in the brain or the pancreas, the concentration of antigen in the tissue concerned is presumed to be much higher than in the thymus. How then does the immune system avoid attacking organs harbouring tissue-specific antigens?

The prevailing explanation here is that tolerance to tissue-specific antigens is solely dependent upon various peripheral mechanisms (Kruisbeek *et al.* 1992; Fowlkes & Ramsdell 1993; Nossal 1993; Sprent & Webb 1995). A popular view is that naive autoreactive T cells remain within the confines of the lymphoid system (lymphoid tissues, blood and lymph) and fail to make contact with tissue-specific antigens, thereby leading to a state of T-cell ignorance. If ignorance is overcome and specific T cells do encounter tissue-specific antigens, it is then argued that the ensuing immune response is abortive, either because of rapid onset of immunoregulation or because, in the absence of adjuvant, the expression of co-stimulatory molecules on APCs is too low to induce an effective immune response. The problem with this line of reasoning is that entrusting tolerance to tissue-specific antigens entirely to peripheral mechanisms would seem highly dangerous. Thus, the not-uncommon combination of trauma (which overcomes ignorance) and infection (which leads to upregulation of co-stimulatory molecules on APCs) would be expected to be associated with a high incidence of florid autoimmune disease. But this is rarely the case. In fact, even in situations where mice are injected with purified tissue-specific antigens together with a powerful adjuvant, only a small proportion of mouse strains develops autoimmune disease.

One explanation for this puzzle is that tissue-specific antigens are only partly sequestered from the immune system. Thus, low levels of these antigens may reach the thymus and delete high-affinity cells: low-affinity T cells escape to the periphery but respond poorly to tissue-specific antigens and/or are easily inhibited by immune-regulatory mechanisms.

Some support for this model has come from the finding that a number of different antigens that had previously been thought to be tissue-specific are synthesized in the thymus, especially in the medulla (Hanahan 1998; Heath *et al.* 1998; Klein *et al.* 2000). Although it is still unclear whether synthesis of 'tissue-specific' antigens in the thymus is the exception or the rule, it is also possible that these antigens leak into the bloodstream in soluble form and reach the thymus in low but significant concentrations. In either situation, one can envisage that presentation of low concentrations of tissue-specific antigens in the thymus is sufficient to delete high-affinity T cells, leaving only less dangerous, low-affinity cells to escape to the periphery. The point to emphasize is that, contrary to current dogma, tolerance to tissue-specific antigens could be partly, perhaps largely, dependent on central tolerance.

## 10. CENTRAL TOLERANCE AND THE PROBLEM OF ALLOGRAFT REJECTION

As discussed elsewhere in this issue, the various approaches used to prevent allograft rejection centre largely on inducing non-antigen-specific immunosuppression (Turka 1998; Hancock 1999). Although these approaches are generally highly effective in the short term, allografts may eventually undergo rejection; moreover, long-term immunosuppression predisposes to infection. In view of these problems, there is increasing interest in developing techniques for inducing specific tolerance to allografts. Discussing methods for inducing peripheral tolerance to allografts is beyond the scope of this article. But what about central tolerance? This topic is discussed in depth in the article by Sykes & Sachs (this issue) but merits brief comment here.

Based largely on studies in rodents, inducing central tolerance to allografts requires a combination of (i) elimination of pre-existing non-tolerant T cells, and (ii) induction of a state of thymic chimerism where newly formed T cells encounter BM-derived APCs expressing the alloantigens concerned (Sprent *et al.* 1992). This situation arises after allogeneic BM transplantation. Thus, if strain A mice are irradiated and reconstituted with strain B BM cells (depleted of mature T cells), the host strain A thymus will be repopulated with strain B APCs, with the result that newly formed strain B T cells will be tolerant to B and accept B allografts permanently without any need for immunosuppression. A similar situation occurs when strain A mice are reconstituted with a mixture of strain A and B BM cells (Singer *et al.* 1981; Sharabi & Sachs 1989; Sharabi *et al.* 1990). Here, thymic chimerism with strain B APCs induces B-specific tolerance of newly formed T cells of both donor and host origin. In this situation, the host A T cells are clonally depleted of B reactivity and will tolerate B allografts indefinitely. However, the point to emphasize is that, because the thymus continues to produce new T cells throughout life (though in much reduced numbers in old age), thymic chimerism has to be both significant and permanent. Since most APCs have a short life span, inducing permanent thymic chimerism requires joint chimerism in the bone marrow.

The great advantage of inducing clonal deletion via mixed thymic chimerism is that allografts are accepted permanently and long-term immunosuppression is avoided. However, the chief problem with this approach is that initial induction of chimerism is often difficult, especially in primates, because of rejection by the host. At least in rodents, such rejection can be overcome by using high doses of irradiation and/or other techniques for inducing rigorous depletion of lymphoid cells. Whether this also applies in primates and man is still uncertain.

The side-effects of the conditioning regimens used to establish BM chimerism are often severe. For this reason, the approach of using central tolerance to induce long-term allograft acceptance has aroused scepticism, especially in view of the 'reasonable' success of more conservative methods. However, future studies may well

lead to significant breakthroughs, making it possible to achieve BM chimerism without the need for initial severe immunosuppression. Inducing allograft acceptance via central tolerance could then become a clinical reality.

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