Multiple ways to cellular immune tolerance

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Both central and peripheral pathways leading to T-cell tolerance were discussed at a recent meeting. The mechanisms that maintain self tolerance, as well as the conditions in which self-reactive T cells launch an autoaggressive attack, were specially emphasized.*

Self-tolerance induction in the cellular immune system involves a multilayered organization in which various self-tolerizing mechanisms are interconnected in series[1]. Insights into the molecular mechanisms underlying the induction and disruption of cellular self tolerance are progressing on several fronts, namely at the level of the interaction of self-peptides and the T-cell receptor (TCR) repertoire, the characterization of the mechanisms of deletion, anergy and suppression, as well as the genetic defects that have to accumulate to allow autoimmune diseases to develop.

Self peptides and self-reactive T cells

Pool sequencing analysis of natural peptides bound to major histocompatibility complex (MHC) molecules reveals that not only MHC class I, but also class II molecules bind peptides in an allele-specific fashion. The peptides binding to MHC class II exhibit allele-specific consensus motifs located between the two ragged N- and C-termini, as well as an allele-independent 'supermotif' probably resulting from specific peptide processing (possibly involving aminopeptidase N) rather than from preferential MHC-binding (K. Falk, Harvard University). In spite of recent progress in predicting peptide binding to MHC molecules, the immunodominance of peptide epitopes of any given antigen still has to be determined empirically. Peptides giving the strongest immune responses upon immunization with a xenoprotein (e.g. hen egg-white lysozyme, HEL) are also the most tolerogenic ones, as shown for mice expressing variable levels of HEL as a neo-self antigen under the control of the ubiquitously expressed HMG-CoA reductase promoter. In normal BALB/c mice most HEL-specific T cells employ anti-$V_\beta 8.2$ gene segments, the dominant rearrangement being $V_\beta 8.2$–$D_\beta 1$–$J_\beta 1.5$. In transgenic mice expressing low HEL serum levels, this dominant rearrangement disappears, being substituted by 'private' repertoires, i.e. T cells specific for the immunodominant HEL peptide 103–117 exhibit V_β–J_β combinations that are not shared by different animals. The surge of such 'private' V_β T-cell repertoires may hamper therapeutic attempts to eliminate oligoclonal self-reactive T lymphocytes for the therapy of autoimmune diseases (J. Kanellopoulos, Pasteur Institute, Paris).

Intrathymic tolerization

During ontogeny, thymocytes come into contact with a variety of peptide–MHC complexes in which certain peptides derived from extrathymic, tissue-specific, proteins may be absent[2]. Interestingly, thymic epithelial cells (TEC) and TEC-derived nurse cells synthesize peptides not expressed in most other thymic cell types, namely members of various peptide hormone families, i.e. the neurohypophysial family (an oxytocin-like self peptide, that is bound to an MHC class I-related neurophysin domain)[3], the tachykinin family (neurokinin A) and the insulin superfamily (insulin-like growth factor 2; IGF-2). Thymic IGF-2, a cross-reactive, dominant epitope of the insulin gene superfamily may be presented in TEC during the first steps of T-cell ontogeny[4]. Thus, at least at this level, TEC exhibit a more promiscuous promoter use than other peripheral tissues. In TEC, peptide mediators are not secreted in granules, as would be the case in endocrine cells, and might tolerize T cells to self-antigens expressed in endocrine organs (V. Geenen, Liège).

Deletion of (neo-)self-reactive T cells is one of the major mechanisms of intrathymic tolerization. This is obviously the case for male mice expressing the α and β TCR transgenes obtained from a male antigen (H-Y)-specific, MHC class I ($H\text{-}2D^b$)-restricted cytolytic $CD4^-8^-$ T-cell clone in conjunction with the correct MHC. In female transgenic mice, $CD4^+CD8^+$ thymocytes exposed to male self-antigen *ex vivo* adopt a $CD4^{dull}CD8^{dull}$ phenotype while undergoing apoptosis, although the exact relationship between deletion and CD4/CD8 down-modulation remains controversial. A relatively broad time window for negative selection of thymocytes is proposed, in which pre-selection thymocytes about to express CD4 or CD8 (e.g. $CD8^+TCR\alpha/\beta^+$ thymocytes from female mice expressing the non-selecting $H\text{-}2^d$ haplotype), as well as cells that already have undergone positive selection ($CD8^+TCR\ \alpha/\beta^{high}$ cells from $H\text{-}2^b$ females) can be deleted. Neither hyperexpression of a *bcl-2* transgene nor the *lpr* mutation in the *Fas* gene perturb male peptide-driven negative selection in the thymus (H. von Boehmer, Basel Institute of Immunology). Apoptotic deletion of thymocytes is also observed when α and β TCR transgenes

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derived from two H-2K^b specific T-cell clones, KB.5C20 or BM3.3, are exposed *in vitro* to fibroblasts transfected with H-2K^b. Deletion can be inhibited by addition of anti-CD8 antibody in the case of KB.5C20, not BM3.3. In both cases, deletion is accompanied by expression of the activation markers CD25 and CD44 (A-M. Schmitt-Verhulst, Marseille-Luminy). As to the mechanism of apoptosis induction, CD4$^+$CD8$^+$ thymocytes appear to be particularly vulnerable to the induction of endonucleolysis by agents leading to an increase in intracellular calcium (TCR/CD3 crosslinking, glucocorticoids) or an activation of protein kinase A (through use of prostaglandin E_2, forskolin or dibutyryl cAMP). *In vivo*, anti-CD3 and 5'-(N-ethyl)-carboxamido-adenosine (NECA, an adenosine deaminase-resistant adenosine analogue that binds A2 type receptors and thereby activates adenylic cyclase and causes cAMP formation) both induce the deletion of CD4$^+$CD8$^+$ thymocytes in an agonistic fashion (M. Jondal, Karolinska Institute, Stockholm). The glucocorticoid receptor antagonist RU-38486 impedes the deletion of CD4$^+$CD8$^+$ thymocytes caused by administration of NECA, anti-CD3[5,6] or the V_β8-specific superantigen staphyloccal enterotoxin B (SEB) (G. Kroemer, Madrid). Thus, endogenous glucocorticoids are obligatory cofactors for clonal deletion and may determine the threshold of TCR-mediated signalling that leads to this form of apoptosis.

Post-thymic clonal deletion and anergy induction

Apoptosis and deletion of splenic $V_\beta8^+$ T cells exposed *in vivo* to SEB during a 12- to 18-hour-period is also prevented by simultaneous treatment either with RU-38486 (Ref. 7), retinol acetate or the immunostimulatory mediator linomide. None of these manipulations abolishes SEB-induced anergy. Application of pertussis toxin several days after SEB does not interfere with the SEB-driven elimination of $V_\beta8^+$ splenocytes, but completely abolishes anergy. Thus, external agents can determine whether a peripheral T cell will become anergic or undergo apoptosis, allowing for a clear dissociation of both processes (G. Kroemer, Madrid). Irreversible clonal deletion can also be induced *in vivo* in peripheral $V_\beta8^+$ T cells exposed to immune cells from animals expressing an endogenous retrovirus-encoded (Mtv-7) (Ref. 8) product, possibly as a type II membrane protein (G. Waanders, Ludwig Institute, Lausanne branch), as well as in peripheral T cells carrying the transgenic H-Y specific TCR. In female, thymectomized α/β TCR transgenic animals immunized with male cells, deletion of clonotype$^+$ CD8$^+$ cells is detected. If interleukin 2 (IL-2) is transduced into male lipopolysaccharide blasts that are used in the transfer, deletion is partially prevented[9]. Thus, it can be speculated that the absence of T-cell help[10] (in this case, absence of IL-2) favors clonal deletion. Moreover, the functional depletion of CD4$^+$ T cells by anti-CD4 (Ref. 11) might facilitate tolerance induction among CD8$^+$ T cells (H. von Boehmer).

The state of anergy induced in female α/β transgene-expressing splenocytes by transfer to male athymic nude recipients is reversible *in vivo* upon 'parking' of cells in an antigen-free environment[12]. In H-2^b mice, expressing the anti-H-2^b α/β TCR transgenes from the clones KB.5C20 (CD8-dependent) or BM3.3 (CD8-independent), important differences in the mechanism of clonal anergy can be detected. Peripheral T cells that escape clonal deletion in the thymus are CD8$^-$ in both models, although the density of TCR expression is higher in the CD8-dependent than in the CD8-independent model. For the activation of their cytolytic potential *in vitro*, peripheral T cells expressing the BM3.3 α/β TCR on an H-2K^b background require exogenous IL-2, i.e. they become helper-dependent due to a failure in endogenous IL-2 secretion. In contrast, in the KB.5C20 model, IL-2 alone is not sufficient to overcome anergy and, in addition, the H-2K^b self-antigen must be overexpressed on target cells. Thus, in the presence of IL-2, T cells tolerized to normal levels of H-2K^b will differentiate into CTL in response to supranormal expression of the H-2K^b antigen. In contrast, H-2K^b specific T cells that have been tolerized in the context of transgene-enforced overexpression of H-2K^b cannot be rescued *in vitro*. These data suggest the importance of antigen dose, as well as TCR affinity (and fine-specificity?), in determining the type of anergy induced in self-reactive peripheral T cells. It is suggested that tolerance induction functions in a minimalistic way, in which the level of self-antigen expression (e.g. H-2K^b) determines the type of tolerance (deletion and different levels of anergy) that is just sufficient for precluding self-antigen-driven activation *in vivo*. This 'fine tuning' has the advantage of maintaining a T-cell repertoire as large as possible (A-M. Schmitt-Verhulst).

The NOD model: immunosuppression, complex genetics and role of the TCR repertoire

A role for immunosuppression is suggested by detailed studies of the pathogenesis of insulin-dependent diabetes mellitus in non-obese diabetic (NOD) mice. One member of the heat shock protein family (hsp65) has been identified as an important autoantigen involved in diabetogenesis. The C9 cell line (that recognizes the p277 epitope of hsp65) is capable of inducing diabetes upon adoptive transfer into young NOD and non-NOD recipients, but prevents diabetes when injected upon inactivation by γ-irradiation ('T-cell vaccination'). C9 exemplifies an autoimmune response that determines the natural history of diabetogenesis. Spontaneously arising anti-hsp65 T-cell responses and anti-hsp antibodies precede the onset of diabetes in NOD mice, whereas the injection of hsp65 emulsified in incomplete Freund's adjuvant or p277 fused to ovalbumin induces a transient disease peak in young NOD mice. Immunization with the p277 carrier also causes insulitis and diabetes in normal C57BL/6 and SJL mice. On the contrary, spontaneous anti-C9 responses decline in NOD mice concomitant to the development of the disease. Vaccination with the C9 clone or a peptide derived from the CDR3 region of the C9 TCR β-chain engineered into the flagellin gene, both prevent the development

of diabetes and elicit anti-clonotypic T cells which can confer protection from disease after adoptive transfer (D. Elias, Weizmann Institute, Rehovot).

Immunogenetic studies performed in backcrosses of the NOD mouse with diabetes-resistant strains have unravelled a minimum of ten insulin-dependent diabetes susceptibility loci (*Idd*). This illustrates that diabetes is a multigenic disease and that numerous defects have to accumulate to allow for the disruption of all self-tolerizing mechanisms and/or for the execution of a full-blown autoaggressive response. Interestingly, *Idd-3* maps near to a mutation in the 3' coding region of the IL-2 gene. *Idd-10* which is associated with an increase in Mac1$^+$ cells reacting with anti-IgG2a antibodies in NOD mice, is linked to a deletion of four base pairs in the gene encoding the high affinity Fc receptor for IgG2a (*Fcgr1*). This deletion affects the boundary between the transmembrane and the cytoplasmic domains[13]. Thus, deficient FcγR1-mediated signalling could determine part of the NOD phenotype (J-B. Prins, Oxford University).

Transgene-directed expression of a CD4-restricted islet-reactive α/β TCR (clone BDC 2.5, not reactive with hsp, which employs $V_\beta 4$ and $V_\alpha 1$ gene elements) in an NOD background leads to the accumulation of non-anergic, self-reactive T cells in the periphery that cause rampant, aggressive insulitis, accelerate the manifestation of diabetes and increase the penetrance of the disease. Expression of an Eα transgene in I-Eα$^-$ NOD mice does not modulate the disease when the α/β TCR is also present. Thus, cells expressing the diabetogenic TCR can evade all of the recognized forms of tolerance induction. In contrast, NOD mice expressing only a $V_\beta 4$ TCR β chain never develop diabetes[14]. The inability to delete thymocytes expressing the transgene-encoded self-reactive α/β TCR is also observed in backcrosses of NOD mice with other inbred strains, thus suggesting that an *Idd*-determined defect in clonal deletion cannot be responsible for this phenomenon (D. Mathis, Strasbourg). These results invalidate the opposing suggestion that the nature of the TCR is of no relevance for the NOD model, which was based on the previously reported finding that expression of a $V_\beta 8.2$ transgene, alone or in conjunction with a TCR-α transgene, presumably not involved in the induction of diabetes, does not modulate the disease[15]. Two ontogenetic 'checkpoints' may determine the capacity of NOD mice expressing a self-reactive α/β TCR, as well as of wild-type NOD controls, to develop insulitis (from 2–3 weeks of age) and diabetes (from 4–5 months), respectively[14]. The genetic factors determining the intrinsic capacity to proceed to target organ infiltration and destruction, respectively, remain to be elucidated.

Conclusion

This workshop has illustrated the long way that remains to go before we understand the precise mechanisms leading to the state of physiological immune tolerance or to its breakdown. Nevertheless, it is clear that a hierarchy exists in the mechanism of self-tolerance induction (deletion and various levels of anergy), as well as in the compartmentalization of tolerogenic antigen distribution (central *versus* peripheral), antigen density and the antigenic molecular make-up (immunodominant *versus* subdominant or cryptic self-peptides). These fundamental characteristics will have to be taken into account in the future design of immune interventions that prevent autoimmune disease development.

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