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Intrathymic Selection: New Insight into Tumor Immunology

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Abstract. Central tolerance to self-antigens is formed in the thymus where deletion of clones with high affinity to "self" takes place. Expression of peripheral antigens in the thymus has been implicated in T cell tolerance and autoimmunity. During the last years, it has been shown that medullary thymic epithelial cells (mTECs) are the unique cell type expressing a diverse range of tissue-specific antigens. Promiscuous gene expression is a cell autonomous property of thymic epithelial cells and is maintained during the entire period of thymic T cell output. The array of promiscuously expressed self-antigens was random and included well-known targets for cancer immunotherapy, such as α-fetoprotein, P1A, tyrosinase, and gp100. Gene expression in normal tissues may result in tolerance of high-avidity cytotoxic T lymphocyte (CTL), leaving behind low-avidity CTL that cannot provide effective immunity against tumors expressing the relevant target antigens. Thus, it may be evident that tumor vaccines that targeted the tumor-associated antigens should be inefficient due to the loss of high-avidity T cell clones capable to be stimulated. Stauss with colleagues have described a strategy to circumvent immunological tolerance that can be used to generate high-avidity CTL against self-proteins, including human tumor-associated antigens. In this strategy, the allorestricted repertoire of T cells from allogenic donor is used as a source of T cell clones with high avidity to tumor antigens of recipient for adoptive immunotherapy. Then, the T cell receptor (TCR) genes isolated from antigen-specific T cells can be exploited as generic therapeutic molecules for antigen-specific immunotherapy.

1. Background

The T lymphocyte repertoire is formed in the thymus as a result of random rearrangement of germinal sequences of the T cell receptor (TCR) gene fragments and other processes that bring about the diversity of TCRs. Immunologists used to consider the traits of the immune system in the context of its coevolution with pathogenic microorganisms. Apparently, the most important problem for the immune system is to avoid the transplantation conflict within the organism. Indeed, the receptors of adaptive immunity "see" self-antigens much more frequently than pathogenic bacteria. In any case, an existence of potentially dangerous system in the organism could be not less important factor for the evolution of the immune system than pathogenic microorganisms.

Therefore, major histocompatibility comple (MHC)-restricted recognition could develop not as much in the struggle with pathogenic microorganisms but in inhibiting the reactions with "self." On this way, restriction of immune reactions by several types of recognized molecules would be helpful, because other biological macromolecules are rescued from danger. MHC molecules have allowed focusing these reactions on short peptides containing amino acid substitutions not presented in the responding organism. On the other hand, they have allowed an efficient formation of specific central tolerance to self not involving in this process a huge diversity of other protein molecules. Thus, thymic selection can be considered as a "first and last" life-long immune reaction of preselected repertoire to self-transplantation antigens. This process solves two problems: (i) to delete autoimmune and cross-reactive (promiscuous) clones that react with self transplantation antigens and (ii) to keep the repertoire of specificities to all conceivable pathogens as broad as possible. The first problem is resolved by negative selection in the thymus and deletion of autoimmune and cross-reactive clones. The second task is accomplished via preservation of a portion of T cell repertoire through a weak degenerated interaction with self-transplantation antigens. Since the repertoire is specific to the whole spectrum of the species' MHC molecules, the remaining portion of the repertoire would contain the clones that might be "autoimmune" or "cross-reactive" in different MHC environment (Zerrahn et al. 1997; Logunova et al. 2005). This provides explanation for frequent cross-reactivity of clones raised in the allogeneic response and provides the reason for degenerated mode of recognition of foreign antigens by TCR. Definitely, the degenerative fashion of recognition is necessary for preserving a broad spectrum of specificity of the repertoire to yet non-encountered pathogens.

As a part of the original demonstration that syngeneic anticancer immunity is possible, it has been shown that, among sarcomas induced in mice by a hydrocarbon, each tumor, when transplanted, could arouse an inhibitory immune reaction against itself (Prehn and Main 1957). However, it also became clear that each tumor was antigenically unique, even if each had been induced by identical means in one and the same animal; although cross-reactions were reported, these were the exceptions. Since it is probable that the immunogenicity was caused by the mutations induced by the carcinogen, obviously these chemicals produced different spectra of mutations in each tumor with very little overlap. Consequently, one had to conclude that any of a vast array of possible mutations could be found in phenotypically similar cancers, a not impossible idea. However, it was also clear that none of the carcinogen-induced mutations, at least among those identified by their resulting antigenicity, could be considered essential or causative for the induction of the cancer (Prehn 2005). Moreover, the vast majority of tumor antigens were identified as non-mutated "self" proteins. The identification of many tumor-associated epitopes as non-mutated "self" antigens led to the hypothesis that the induction of large numbers of self/tumor antigen-specific T cells would be prevented because of central and peripheral tolerance (Rosenberg et al. 2005).

2. Tumor-Specific and Tumor-Associated Antigens as the Consequences of Genetic and Epigenetic Alterations

Accumulating evidence shows that tumor formation is accompanied by both genetic and epigenetic alterations of the genome (Hahn and Weinberg 2002; Felsher 2003; Egger et al. 2004). Correspondingly, cancers in mouse and man express multiple tumor-specific as well as tumor-associated antigens encoded by mutant and normal cellular genes.

Most chemical or physical carcinogens are mutagens. Therefore, it is generally assumed that tumor-specific antigens on tumors induced by these carcinogens are products of mutated genes, possibly single genes with "hot spots" for mutations. Some tumor-specific antigens are retained during tumor progression possibly because they are essential for survival of the malignant phenotype. Since 1995, the genetic origins of several T cell-recognized unique antigens from murine and human cancers have been identified, and in every case, the antigen was caused by a somatic mutation (i.e., by a genetic change absent from autologous normal DNA) and thus found to be truly tumor specific (Monach et al. 1995; Coulie et al. 1995; Wolfel et al. 1995; Robbins et al. 1996; Brandle et al. 1996; Dubey et al. 1997).

Unlike genetic changes, epigenetic changes do not alter the primary DNA sequence and are therefore reversible. Examples of epigenetic modifications are the methylation of DNA and histones, the acetylation/deacetylation of histones, and the packing of chromatin into euchromatic and heterochromatic regions (Li 2002). Epigenetic modifications play an important role during normal development by regulating gene expression through stable activation or silencing of differentiation-associated genes. Similarly, epigenetic changes can promote cell proliferation, inhibit apoptosis, and induce angiogenesis during tumorigenesis by activating oncogenes and silencing tumor suppressor genes (Felsher 2003). Treatment of tumor cells with methylation- and histone-modifying drugs can inhibit malignancy, and this inhibition correlates with the reactivation of important tumor suppressor loci (Egger et al. 2004).

Despite differences in their tissue of origin, in many tumors, certain tumor-associated proteins are highly expressed. Many studies have been focused on the possibility of utilizing antigenic components of these proteins as a focus for T cell immunotherapy of cancer. The advantage of targeting such commonly expressed proteins is founded on the fact that such therapy could be of value in eliminating many different tumor types. A potential barrier in the identification of T cell epitopes derived from these proteins and presented by tumor cells is that these proteins are also expressed at low levels in normal tissues, and therefore, self-tolerance may eliminate T cells capable of recognizing these epitopes with high avidity (Sherman et al. 1998).

3. Does Immunological Surveillance Make Tumor-Specific Antigens Undetectable?

The idea of immunological surveillance against cancer has existed for nearly 100 years. However, the importance of the cellular immune defense in the detection and removal of incipient or existing tumors is still a hotly debated subject. In order to select a relevant immunotherapeutic strategy in cancer treatment, a fundamental understanding of the basic immunological conditions under which tumor is developed and exists is a prerequisite. Therefore, several murine models were set up that would enable to confirm or decline the theory of immunological surveillance.

The initial studies have shown that the incidence of spontaneous tumors in immunodeficient *nude* mice was similar to that reported for the thymus-bearing background strain arguing against the thymus dependency of the putative immunological surveillance mechanisms (Pelleitier and Montplaisir 1975; Sharkey and Fogh 1979). Possibly, many spontaneous tumors are induced by oncogenic viruses requiring the host immune system for propagation. For example, MMTV utilizes cells of the immune system in its infection pathway. Therefore, subsequent carcinogenesis was highly dependent on T cells (Golovkina et al. 1992; Pobezinskaya et al. 2004). It has been argued that the apparent general lack of tumor immunogenicity may be an artifact caused by immune selection for non-immunogenic tumor variants. Perhaps most tumors, according to the immunosurveillance hypothesis, are really highly immunogenic and what we see is actually a small surviving, relatively non-immunogenic, highly selected subpopulation. This popular concept can account for the paucity of tumor immunogenicity.

First, cloned cell lines of chemically induced murine fibrosarcomas maintained in tissue culture usually fail to grow when transplanted to normal syngeneic mice. They grow, however, in various categories of T cell-deficient mice, and after such passages grow readily in normal mice. Both cultured and mouse-passaged lines possess strong tumor transplantation antigens (Woodruff and Hodson 1985).

Second, methylcholanthrene-induced tumors originating from the immunodeficient *nude* mice turned out to be far more immunogenic than tumors from normal mice, resulting in a high rejection rate after transplantation back to normal histocompatible congenic mice. *Nude* mice developed tumors most quickly and with the highest incidence, leading to the conclusion that in this model the immune system constituted a "tumor-suppressive factor" delaying and sometimes abrogating tumor growth, that is, performing immune surveillance. Cytotoxic $CD8^+$ T cells were found to be indispensable for this rejection, leading to the conclusion that the cytotoxic T cells perform immune selection in normal mice, eliminating immunogenic tumor cell variants in the incipient tumor (Svane et al. 1996; Svane et al. 1999).

Third, the rejection of murine UV-induced skin cancers by normal mice is a striking example of powerful immune surveillance of the normal host against malignant cells. UV-induced regressor tumors grew progressively and killed mice that were depleted of $CD8^+$ T cells. Depletion of $CD4^+$ T cells had no effect, suggesting that $CD8^+$ but not $CD4^+$ T cells were required for this immune surveillance. There was no correlation between the ability of a tumor to grow progressively in a normal immunocompetent host and the level of constitutive class I expression or the level of expression induced in vitro by γ-interferon (Ward et al. 1990).

Thus, tumor antigenicity can be detected during carcinogenesis in immunodeficient animals but gradually lost in normal ones. An important point here is that immunological surveillance provides a borderline between immunity and tolerance in response to tumor cells.

4. Gaze into Cells: MHC-Binding Motifs

To "see" mutant or inappropriately expressed proteins in transformed cells, the immune system needs to "look" into these cells. This capability is provided by expression of MHC class I molecules associated with endogenous peptides. The mechanism of how antigen presenting cell(s) (APC) determines the restriction of effector T cells was uncovered by the Rammensee's group. As the author mentioned in his review on MHC-binding motifs in antigens, immunology owes two students, Olaf Rotzschke and Kirsten Folk, who were interested in the structure of peptides that interact with class I MHC molecules. To isolate these molecules, proteins of cell membranes were adsorbed on an immune affine column followed by the acidic elution and dissociation of MHC/β_2-microglobulin/peptide complexes. The Edman sequencing of isolated peptides showed invariant amino acid residues near the C and N ends. Most importantly, the peptides bound by different allelic forms of MHC class I molecules had similar length but different allele-specific motifs (Falk et al. 1990, 1991a; Rotzschke, et al. 1990). The authors realized that they possess a powerful method for identification of the allele-specific sequences among a huge variety of peptides that could be derived from one antigenic protein (Falk et al. 1991b).

The fact that these motifs have been formed by "anchoring" amino acid residues necessary for high-affinity binding of the peptide with respective MHC molecule also indicated that APC expressing different MHC haplotypes can present various peptides of the same antigen (Rammensee et al. 1993; 1995). For example, the nucleoprotein of influenza virus contains the epitope that binds with H-2K^d in positions AA147–155 (T**Y**QRTRAL**V**) and with H-2D^b in positions AA366–374 (MTEM**N**ENS**A**). For human MHC molecules, the epitope for binding with HLA-A2 is within AA85–94 (K**L**GEFYNQ**M**), and with HLA-A3 within AA265–273 (I**L**RGSVAH**K**), with HLA-B8 within AA380–388 (IAWY**R**SR**L**E), and with HLA-B27 in AA383–391 (S**R**YWAIRT**R**) (underlined are anchoring, i.e., motif forming, residues). The highest binding affinity of influenza virus nucleoprotein-derived peptides with H-2D^b is achieved if the peptide has the canonical motif (Cerundolo et al. 1991). To see whether the expression of certain MHC molecule influenced the efficacy of peptide epitope presentation, the amounts of correctly processed K^b-restricted epitope of the minor histocompatibility antigen H-4b in H-2K^b-positive and-negative cells were estimated. The difference was 3000-fold, indicating an instructive role of MHC molecules in peptide-processing machinery (Wallny et al. 1992a). The mechanisms of peptide/MHC molecule association allowed predicting the structure of T cell peptide epitopes including tumor antigens (Rotzschke et al. 1991; Wallny et al. 1992b). Furthermore, these studies set rational molecular basis for an association between autoimmune diseases and certain MHC haplotypes (Vartdal et al. 1996; Kalbus et al. 2001; Munz et al. 2002). Finally, the ability of individual

allelic products of MHC molecules to bind particular peptides of the pathogen directly links MHC with genetically determined immune response to pathogens.

The experience gained in the studies of MHC-binding motifs in MHC-associated peptides is currently used for constructing combinatorial peptide libraries. These tools make possible the positional screening for high-affinity ligands and cross-reacting ones for TCRs. Furthermore, making MHC tetramers can help to directly visualize the antigen-specific T lymphocytes (Wilson et al. 2004; Xu and Screaton 2002). There are a number of on-line services making possible the search for T cell epitopes among different protein sequences. One of them, RANKPEP service is a powerful mean for search of potential T cell epitopes presented by allelic forms of mouse and human MHC classes I and II molecules, taking into account protein degradation by proteasome (Reche et al. 2004). Obviously, the analysis of a specific protein reveals restricted number of epitopes capable to be presented by the MHC molecules of an individual. It assumes that some mutations in cancer-related genes can be invisible to the immune system making possible tumor escape from immunological surveillance.

5. Aire and Thymic Selection

Aire (autoimmune regulator), the gene responsible for the clinical disorder autoimmune polyendocrinopathy syndrome type I, has recently been identified as an important mediator of central tolerance. Structural characteristics and biochemical data suggest that Aire might play a direct role in transcription and function as an ubiquitin ligase. Aire up-regulates the transcription of certain organ-specific self-antigens in medullary thymic epithelial cells (mTECs) and has a role in the negative selection of organ-specific thymocytes (Su and Anderson 2004). Aire promotes the tolerance of thymocytes by inducing the expression of a battery of peripheral-tissue antigens in mTECs. The mechanism whereby Aire exerts its tolerance-promoting function is not primarily positive selection of regulatory T cells but rather negative selection of T effector cells. Surprisingly, supplementing its influence on the transcription of genes encoding peripheral-tissue antigens, Aire somehow enhances the antigen-presentation capability of mTECs. Thus, this transcriptional control element promotes central tolerance both by furnishing a specific thymic stromal cell type with a repertoire of self-antigens and by better arming such cells to present these antigens to differentiating thymocytes. In Aire's absence, autoimmunity and ultimately overt autoimmune disease develops (Anderson et al. 2005).

At certain stages of male gametogenesis, a broad range of genes is expressed. The underlying mechanism and the biological significance of this phenomenon remain elusive. Derbinsky et al. inquired whether the spectrum of ectopically expressed genes is distinct or shared between mTECs and testis. They analyzed gene expression in cDNA libraries prepared either from whole testis or highly enriched immature gametocytes. Of 19 tissue-specific genes expressed in the thymus, 14 were also expressed in the testis. The transcription factors Whn and Aire, both of which have been ascribed specific roles in thymus biology, were also found in male gametocytes. The authors suggested that this mechanism may facilitate tolerance induction

to self-antigens that would otherwise be temporally or spatially secluded from the immune system.

Such spatially secluded antigens as cancer/testis (CT) antigens, of which more than 40 have now been identified, are encoded by genes normally expressed in the human germ line but are also expressed in melanoma and carcinomas of the bladder, lung, and liver. These immunogenic proteins are being vigorously pursued as targets for therapeutic cancer vaccines (Van Der Bruggen, Zhang, Chaux, Stroobant, Panichelli, Schultz, Chapiro, Van Den Eynde, Brasseur, and Boon 2002; Simpson, Caballero, Jungbluth, Chen, and Old 2005). It is very important that the array of promiscuously expressed self-antigens in mTECs includes well-known targets for cancer immunotherapy, such as α-fetoprotein, tyrosinase, and gp100. Therefore, intrathymic selection makes the immune system tolerant to tumor-associated antigens. Gene expression in normal tissues may result in tolerance of high-avidity CTL, leaving behind low-avidity CTL that cannot provide effective immunity against tumors expressing the relevant target antigens. Evidently, any antitumor vaccine targeted to tumor-associated antigens should be inefficient due to the loss of high-avidity T cell clones capable to be stimulated.

6. Transduction with Allorestricted TCR as a Mean to Overcome Central Tolerance

Normally negative selection ablates high-avidity T cell clones that can react with self antigens of individual 1 in the context of self MHC molecules (H-2^x-P1). But the clones specific to H-2^x-P1 can be presented in allogeneic individual 2 because negative selection in this organism deletes the clones specific to tumor-associated antigens in the context of "another self" (i.e., allogeneic to P1) MHC (H-2^y-P2). Therefore, allorestricted recognition can supposedly provide the basis for obtaining clones of individual 2 specific to the combination of MHC molecule with H-2^x-P1 peptide of allogeneic recipient for adoptive immunotherapy (Figure 1).

One example of successful use of allorestricted recognition of tumor-associated antigens is the work of Elena Sadovnikova and Hans Stauss who obtained allorestricted CTL clones of H-2^d mice. These clones were specific to the complex of H-2K^b plus mdm-2-derived peptide; it is noteworthy that mdm-2 is frequently overexpressed in tumor cells. In culture, these clones selectively reacted with tumor versus normal cells and killed melanoma and lymphoma cells but not normal H-2K^b- expressing dendritic cells. In vivo, the allorestricted clones caused retardation of growth of melanoma and lymphoma in syngeneic (H-2^b) recipients (Sadovnikova and Stauss 1996). The same authors attempted to obtain allorestricted clones specific to a cyclin D1 peptide in the context of human HLA-A2. The clones lysed cyclin D1 overexpressing breast carcinoma cells but not Epstein–Barr-transformed lymphoblastoid cells (Sadovnikova et al. 1998). Allorestricted recognition became an efficient means of breaking tolerance to tumor-associated antigens and to get responses to leukemia-associated markers such as WT1, CD68, and CD45 (Gao et al. 2000; Sadovnikova et al. 2002; Amrolia et al. 2003).

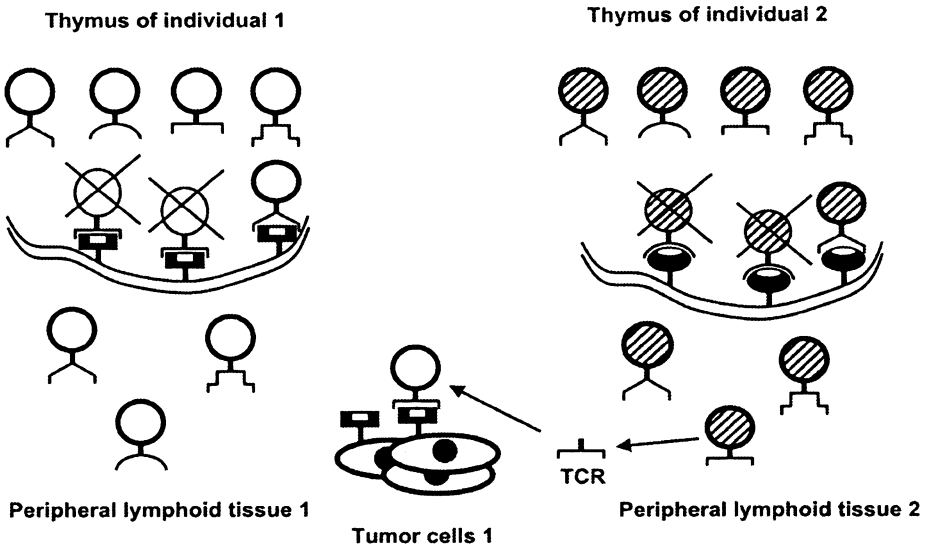

FIGURE 1. Negative selection ablates high-avidity clones specific to tumor-associated antigens of individual 1. This process makes antitumor immune response and vaccination of individual 1 inefficient. Clones with required specificity may be isolated from allogeneic individual 2 for subsequent cloning of T cell receptors(TCRs) and retroviral transduction of T lymphocytes of individual 1.

Then, the TCR genes isolated from antigen-specific T cells can be exploited as generic therapeutic molecules for antigen-specific immunotherapy. Retroviral TCR gene transfer into patient T cells can readily produce populations of antigen-specific lymphocytes after a single round of polyclonal T cell stimulation. The TCR gene-modified lymphocytes are functionally competent in vitro and can have therapeutic efficacy in murine models in vivo. The TCR gene expression is stable, and the modified lymphocytes can develop into the memory T cells. Introduction of TCR genes into $CD8^+$ and $CD4^+$ lymphocytes provides an opportunity to use the same TCR specificity to produce antigen-specific killer and helper T lymphocytes. Thus, TCR gene therapy provides an attractive strategy to develop antigen-specific immunotherapy with autologous lymphocytes as a generic treatment option (Morris et al. 2005; Xue et al. 2005).

The ultimate goal of cancer immunotherapy is to utilize the immune system to eliminate malignant cells. Recently published research has mainly focused on the generation of effective antigen-specific T cell responses because of the general belief that T cell immunity is essential in controlling tumor growth and protection against viral infections. However, isolation of antigen-specific T cells for therapeutic application is a laborious task. Therefore, strategies were developed to genetically transfer tumor-specific immune-receptors into patients' T cells. To this end, the chimeric receptors were constructed that comprise the antibody fragments specific for tumor

associated antigens linked to genes encoding signaling domains of TCR or Fc receptor. T cells with such chimeric antibody receptors recapitulate the immune-specific responses mediated by the receptor (Willemsen et al. 2003).

It is noteworthy that murine TCRs are highly functional when expressed in human lymphocytes. Recently published work compared human and mouse TCR function and expression to delineate the molecular basis for the apparent superior biological activity of murine receptors in human T lymphocytes. To this end, authors created hybrid TCRs where they swapped the original constant regions with either human or mouse ones. Murine or "murinized" receptors were overexpressed on the surface of human lymphocytes compared with their human/humanized counterparts and were able to mediate higher levels of cytokine secretion when cocultured with peptide-pulsed antigen-presenting cells. Preferential pairing of murine constant regions and improved CD3 stability seemed to be responsible for these observations. This approach allowed circumventing the natural low avidity of class I MHC TCR in $CD4^+$ cells by introducing the murinized TCR into $CD4^+$ lymphocytes, giving them the ability to recognize melanoma. These findings have implications for human TCR gene therapy (Sommermeyer et al. 2006).

In another work, the TCR gene transfer was investigated as a convenient method to produce antigen-specific T cells for adoptive therapy. The authors focused on the expression of two TCRs in T cells, which could impair their function or cause unwanted effects of mixed TCR heterodimers. With five different TCRs and four different T cells, either mouse or human, they have shown that some TCRs were strong—in terms of cell surface expression—and replaced weak TCRs on the cell surface, resulting in exchange of antigen specificity. Two strong TCRs were coexpressed. The mouse TCR replaced human TCR on human T cells. Even though it is still poorly understood why some TCR$\alpha\beta$ combinations are preferentially expressed on T cells, the data suggest that T cells with exclusive tumor reactivity can potentially be generated by T cell engineering (Cohen et al. 2006).

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