Geraldo A. Passos *Editor*

Thymus Transcriptome and Cell Biology

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Foreword

The importance of the thymus as the primary lymphoid organ responsible for the generation and selection of T lymphocytes is now obvious. Nevertheless, the thymus has long been a mysterious organ. It was not until 1961 that J. F. Miller showed in seminal studies that "the thymus at birth can be essential to life," quickly followed by its role in immunological tolerance by skin grafting experiments in mice. It is always surprising that this key discovery for any immunologist, physician, or biologist did not happen sooner. The skepticism surrounding this discovery, from such eminent immunologists as Burnet, Medawar, or Mitchison, may also be astonishing, Sir Peter Medawar even going so far as to declare in 1963 "we will come to look at the presence of lymphocytes in the thymus as an evolutionary accident with little significance." This controversy remains exemplary and instructive for our ways to get out of accepted dogmas. This was a "golden age" of immunology, the 1960s being particularly remarkable with the discovery of the major histocompatibility complex (MHC), the H-2 system in mice and HLA in humans, by Jean Dausset (1980 Nobel Prize with Baruj Benacerraf and George Snell), Jon van Rood, and many others. These two major discoveries paved the way for the demonstration of thymic selection, a major physiological function of the thymus in shaping the T-cell adaptive immunity. They also provided the basis of our current understanding of how the immune system works. With time, T-lymphocyte subpopulations, T- and B-cell cooperation, mechanisms of allogeneic MHC restriction, T-cell receptor structure and T-cell selection mechanisms, and identification of regulatory T cells have been gradually described. Each of these steps leads us back to thymopoiesis, ranging from the identification of factors required for the entry of hematopoietic progenitors into the T-lymphocyte development program to the factors regulating the expression of "tissue-restricted antigens" within the thymic epithelium. Those are key in establishing the central tolerance, among which *AIRE* and *Fezf2* are the best known, others still being to be described. This has been remarkably studied in murine models, and several chapters of this book are devoted to this central issue.

Although the concepts are very similar, the data concerning human thymopoiesis still need to be further developed. However, they are progressing rapidly thanks to methodological approaches and to large-scale studies. It is now clear

that, contrary to conventional wisdom still present in textbooks, the thymus remains functional in adults. This is important to better understand the parameters that govern the thymic function under physiological conditions, during aging, or in pathologies, especially lymphopenic situations after hematopoietic cell grafts, during HIV disease, or in autoimmunity. We are thus evolving from studies purely focusing on thymocytes to a broader view considering the thymus as an organ in its complexity. Excellent chapters of this book deal with what is called the "cross-talk" between thymocytes and cell populations housed in the thymus whose heterogeneity and complexity are gradually being uncovered. They include of course the different subpopulations of cortical and medullary thymic epithelial cells but also dendritic cells, macrophages, and so-called innate lymphoid cells, important in the process of thymic regeneration, without putting aside endothelial cells, key in the entry of lymphoid progenitors and also in the egress of recently generated naive T cells or "recent thymic emigrants." All this global knowledge will enable to consider future thymic regeneration strategies that will be personalized according to age, gender, and clinical contexts. This is to say the importance of this book and its timely content. The thymus still has a lot to teach!

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Antoine Toubert

Preface

If I had to choose an organ that in humans and mice symbolizes the intersection between immunology, endocrinology, molecular biology, and genetics, this organ would certainly be the thymus gland.

Galen in Greece had anatomically described this organ more than twenty centuries ago but only had its function scientifically attributed in the second half of the twentieth century by Jacques Miller. In his experiments of neonatal thymectomy of mice, Miller observed that the operated pups were suffering from susceptibility to infections with concomitant lymphopenia. These experiments were instrumental in finally assigning to the thymus its function in the immune system.

The fact that the thymus was the last of the major organs of the body to have its function assigned promptly incites exclamation.

Even intersected as said, the main function of the thymus is immunological and strongly associated to the development and positive/negative selection of T cells and induction of central immune tolerance.

One of its major cellular components, the thymocytes, undergoes a maturation process that is dependent on the random recombination of DNA segments (V[D]J recombination) of T-cell receptor (TCR) leading to the generation of the diversity of T-cell clones. The experimental demonstration of V(D)J recombination by Susumu Tonegawa in the mid-1970s, initially involving immunoglobulin gene segments in B cells and later TCR in T cells, has opened a unique perspective, i.e., understanding the molecular basis of diversity of lymphocyte repertoire. For genetics, this represented a great impact, since it was demonstrated that the genome is dynamic and can recombine somatically.

Another very intriguing aspect about the thymus is the functioning of its stroma. Thymic stroma is not merely a connective tissue or a supporting structure. The thymic epithelial cells (TECs), which are part of the stroma, establish a close physical contact with developing thymocytes through TECs-thymocytes adhesion, which is crucial, both for the thymus as a whole and for the thymocytes themselves. This property is termed thymic crosstalk during which the medullary TECs (mTECs) present to the developing thymocytes a vast amount of self-peptides that represent virtually all the organs and tissues of the body.

This showed the intersection of the thymus with the molecular biology of large-scale gene expression or transcriptomics. The mTEC cells have become a very intriguing cell type because they express almost the entire functional genome without losing their characteristics. The meaning of this property is immunological, relative to self-representation and induction of central immune tolerance. Due to the enormous diversity of self-peptide antigens expressed by mTECs, this property was termed *promiscuous gene expression* (PGE), which is controlled by *Aire* and *Fezf2* genes.

The demonstration that the thymic stromal cells express the functional oxytocin hormone made possible an important relation of this organ with endocrinology.

The thymus is also closely associated with human genetics since mutations in *Aire* cause the APECED (APS-1) autoimmune syndrome, which is linked to chromosome 21q22.3, the exact physical location of the *Aire* gene in humans.

One curiosity that, perhaps, some researchers still do not know: the mouse genome project and science of transcriptomics have in the past benefited greatly from the thymus. To begin massive sequencing of mouse DNA in the early 1990s, researchers in fact made cDNA libraries from total RNA extracted from a mouse thymus. Briefly, thousands of expressed sequence tags (ESTs) were then generated and later positioned along the genome, which in turn was being assembled. The sequenced mouse EST libraries were then used in microarray technology that emerged in 1995 making the science of transcriptomics possible!

As we can see, the thymus is a fascinating organ. It is crucial for the maintenance of immune homeostasis and is the place where the self-non-self distinction occurs. Even so, it is still a neglected organ in immunological research. Immunology is one of the branches of biological sciences that has progressed the most in the last decades, and most of the works are directed to the peripheral effector cells, i.e., B and T cells, NK cells, dendritic cells, macrophages, etc. However, much still has to be better known about thymus maturation, its ontogeny and differentiation, the origin of the TEC cells, and the control of the thymus gene expression in health and autoimmune diseases.

A promising field of research that is still in its early stages is about the control of gene expression of “second” thymus (cervical thymus discovered in 2006) that is present in about 50% of humans and mice.

It is for these reasons that this book was organized, i.e., to review the main aspects of cell biology, gene expression, and clinical intervention of the thymus. It was conceived during the realization of the Third Meeting on Thymus Transcriptome and Cell Biology, held at the University of São Paulo, Ribeirão Preto Medical School, Ribeirão Preto, Brazil, on 21–22 November 2017. Many of the authors of this book participated in this fruitful meeting. The purpose of this book is also to attempt to motivate young scientists to research the thymus.

I am grateful to all the researchers who have dedicated a part of their time to writing their chapters and the Springer Nature Publishing to welcome us and give full support for this book to be published.

Finally, I would like to pay a homage to Dr. Bruno Kyewski (1950–2018) who was an excellent scientist and devoted much of his career at the German Cancer Research Center in Heidelberg, Germany, studying cell biology and promiscuous gene expression in the thymus.

Ribeirão Preto, Brazil
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Geraldo A. Passos

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Chapter 1
History of the Thymus: From a Vestigial Organ to the Programming of Immunological Self-Tolerance

Vincent Geenen and Wilson Savino

Abstract This introductive chapter presents the most important disruptions of concepts concerning the thymus since its discovery in Antique Greece. For centuries, the thymus was considered as a vestigial organ, and its role in T-cell differentiation was proposed only in the 1960s. Most recent studies attribute to the thymus an essential and unique role in programming central immunological self-tolerance. The basic mechanism implicated in this function is the transcription in the thymic epithelium of genes encoding precursors of neuroendocrine-related and tissue-restricted self-peptides. Their processing leads to the presentation of self-antigens by the major histocompatibility complex (MHC) machinery expressed by thymic epithelial and dendritic cells. Already during foetal life, this presentation promotes negative selection of T lymphocytes harbouring a receptor with high affinity for MHC/self-peptide complexes. Mainly after birth, this presentation also drives the generation of regulatory T cells specific for these complexes. Numerous studies, as well as the identification of *Aire* and *Fezf2* genes, have shown that a thymus defect plays a crucial role in the development of autoimmunity. The discovery of the central tolerogenic action of the thymus revolutionized the whole field of immunology, and such knowledge will pave the way for innovative tolerogenic therapies against autoimmunity, the so heavy tribute paid by mankind for the extreme diversity and efficiency of adaptive immunity.

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1.1 Historical Summary from Antique Greece to the Twentieth Century

The name 'thymus' first appeared in Galen's manuscripts (± 160 AD) and was so named because of its morphological analogy with the leaf of *Thymus cunula*. Galen considered the thymus as an excrescence, the function of which was to serve as a cushion between the sternum and basal blood vessels. He also observed that the thymus was larger in young animals and that its volume decreased with age.

Jacopo Berengario de Carpi (1460–1530) was the first anatomist to describe the human thymus by dissecting cadavers at the University of Bologna, one of the great European centres of anatomy at that time, with Padua and Paris. He precisely depicted both vascularisation and innervation of the thymus.

With the help of the students in Titian's school in Venice, the Belgian Andre Vesalius (1514–1564), the great anatomist of the Renaissance period, published in Padua the first anatomical board with a thymus in *De Humani Corporis Fabrica*: it was a small multi-lobed organ just behind the sternum that Vesalius also considered as a cushion protecting blood vessels in the superior mediastinum. Bartolomeo Eustachi (1510–1574), who was the first to describe adrenal glands, drew also the thymus showing its anatomical relationships in the anterior mediastinum. At the same time, the French surgeon Ambroise Paré (1510–1590) mentioned the thymus as a very soft and spongy gland. Felix Plater (1536–1614), a Swiss physician, reported a case of a child death by asphyxia secondary to a tracheal obstruction by an internal mass developed from the thymus.

During the seventeenth century, the English physician Francis Glisson (1599–1677), who first described rickets, speculated that the thymus could produce a fluid devoted to feeding and growth of the baby. William Hensson (1739–1774), an English surgeon known for his discovery of fibrin, published the first textbook on the thymus. He also reported the variations of thymus size during aging, its involution during some acute or chronic diseases, and noted that the thymus was full of the same 'particles' than those present in blood and lymph. He deducted that the thymus only exists at the beginning of life when these particles seem to be most necessary.

In 1832, Astley Cooper (1768–1841) published *The Anatomy of the Thymus Gland* enriched with precise illustrations of this organ. He also described malignant thymoma, a cancer of the thymus. Later, an essay on the physiology of the thymus gland (1845) brought to the English surgeon John Simon (1816–1904) his nomination to the Royal Society of London. Arthur Hill Hassal (1817–1894), physician-chemist, compared the histology of the thymus and other lymphoid organs (spleen, lymph nodes) and described Hassal's corpuscles in the thymic medulla. The Scottish embryologist John Beard (1858–1924) considered that the thymus could be the sources of all lymphocytes in the body. He also suggested an analogy of invasive power between cancer and placental trophoblast, saying that cancer would be an 'irresponsible trophoblast'. In 1902, he published in *The Lancet* a paper entitled 'Embryological aspects and aetiology of carcinoma', which prefigures the current concept of cancer stem cells. He was nominated in 1906 for the Nobel prize of

physiology or medicine with the following argumentation: 'For the discovery of the presence in early vertebrates of a nervous structure that develops and is functional only during first embryological stages, the discovery of the real nature of the thymus gland, and the demonstration of a direct morphological continuation of germinal cells in all vertebrates'. That year however, the Nobel Prize was attributed to Camillo Golgi and Santiago Ramon y Cajal for their work on the cellular structure of the nervous system.

In France, Jona Salkind showed in his PhD thesis a first and complete comparative study on the histology of the thymus, from fish to human. In this work he even reported experiments with thymectomy in adult fish but did not find any significant changes in this animal (Salkind 1915).

1.2 The Thymus at the Crossroad Between Endocrinology and Immunology

At the beginning of the twentieth century, the thymus was still some 'enigma'; it could be an epithelial gland infiltrated by many small lymphocytes at the second month of the embryonic life in humans. These thymic lymphocytes (thymocytes) actively divide and thus, contrary to the epithelial framework, are very sensitive to the X-rays identified in 1895 by Wilhelm Röntgen. The scientific community then considered that the thymus was a vestigial and transitory organ, which prematurely declined and ceased to function very early in life. However, already in 1890, the German anatomist Wilhelm Waldeyer (1836–1921) had noticed that, even in elderly people, islets of thymic tissue could be observed in adipose thymus. Jan-August Hammar (Sweden, 1861–1946) confirmed these findings and detailed that, although the maximal development of the thymus is reached at puberty, normal thymic tissue persists until advanced age. He also showed that animal castration before puberty maintains an important volume of the thymus and that an involution of this organ accompanies pregnancy, undernourishment, as well as some infectious diseases Hammar (1921). Inversely, thymus hyperplasia is associated with autoimmune Graves' thyroid disease, Addison's adrenal deficiency, myasthenia and acromegaly. Consequently, the thymus was considered as another glandular component of the endocrine system. Actually, it was directly linked to the hypothalamus-pituitary-adrenal (HPA) axis by the Hungarian investigator Hans Selye who showed in 1946 that stress conditions, stimulating the HPA axis, simultaneously caused thymic atrophy (Selye 1946). Such findings led him to create the concept of an HPA-thymus axis.

Despite brilliant works made before 1950, the immunological function of the thymus and thymic lymphocytes remained completely unknown: indeed, similar to what had been reported for fish, adult mice that were thymectomized did not present any immunological problems. However, Jacques Miller (Australia, born in 1931) made a key observation, showing that thymectomy in mice, performed immediately

after birth, provokes their premature death: the presence of a thymus at birth would thus be essential for survival. Further studies by Miller showed that mice thymectomized at the first day of life (and not later than 1 week) are very susceptible to infections. He observed an important lymphopenia in blood, spleen and lymph nodes of these animals, which were also unable to reject a foreign skin graft, an essential immune response. In 1961, he concluded that the thymus is the organ responsible for the development of immunocompetent cells that constitute a specific cell population, thymus-dependent (T) lymphocytes (Miller 1961, 1964). Not without irony, he will write later that the success of his experiments was due to the fact that mice had been bred in a non germ-free environment. Despite the rightness of Miller's experiments and conclusions, Sir Peter Medawar still wrote in 1963: '*We shall come to regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance*' (Medawar 1963).

In the following of studies conducted by Donald Metcalf (1929–2014), in respect to the identification of hematopoietic growth factors, Jacques Miller advanced the hypothesis of one or several soluble thymic factors that would be responsible for driving T-cell differentiation (Osoba and Miller 1963). Innumerable studies will try to characterize such factor(s) but it will be never possible to establish the reality of such thymus-specific growth factor(s) and to apply the endocrine model to the communication between epithelial cells and lymphocytes in the thymus. The demonstration of a crucial role of the thymus during embryonic and foetal life, as well as the absence of any pathogenicity resulting from thymectomy a few days after birth, reinforced the idea that the thymus is, if not a vestigial organ, at least an organ that quickly becomes useless, this being verified in human clinics: the Di George congenital syndrome, which is the most common form of genetic micro-deletion, associates, besides other defects, the absence or hypoplasia of the thymus and a severe immunodeficiency. On the other hand, children who have been thymectomized during surgical correction of a congenital cardiac defect do not present any patent immune deficiency further in life. This latter question, however, would deserve to be further investigated in careful longitudinal studies.[1]

1.3 Interactions Between the Immune and Neuroendocrine Systems

Pioneer work published in 1970s by the Argentinean researchers (working in Switzerland) Hugo Besedovsky and Adriana del Rey revealed that neonatal thymectomy also promoted hypoplasia in the development of secondary reproductive organs (Besedovsky and Sorkin 1974; Besedovsky et al. 1985).

[1] In parallel to the launching of the FP6 European Integrated Project *Euro-Thymaide*, the European Commission published a directive prohibiting total thyroidectomy in children needing corrective cardiac surgery.

Using experimental psychology approaches, and reproducing old studies carried out during the 1950s at the Pavlov Institute (Saint Petersburg, Russia), the psychologist Robert Ader, showed early in the 1980s that typical Pavlovian reflex could be seen in the immune response. For such discoveries, he is presently considered the father of psychoneuroimmunology (Ader 1983).

Later, in October 1983, the Cardiologic Princess Lilian Foundation organized in Brussels the international symposium *Neural Modulation of Immunity* (Guillemin et al. 1985), chaired by the neuroendocrinologist Roger Guillemin, Nobel Prize of physiology or medicine in 1977, and two eminent immunologists, Melvin Cohn and Theodor Melnechuk. Invited speakers of this symposium revealed a completely new field of research, immune-(neuro)endocrinology, as well as several original approaches for a better understanding of integrated physiology.

One year later, Herbert Novera Spector (1919–2017) organized the First International Workshop on Neuroimmunomodulation, at the National Institutes of Health (Bethesda, USA) devoted to the same subject. In particular, during this symposium, there was a nice discussion on the putative production of endogenous opioids, including met-enkephalin, by the human thymic epithelium.

The stimulation of uterine contractions and galactokinesis of thymus extracts had already been reported at the beginning of the twentieth century by English authors (Ott and Scott 1909). Today, we know that such action is specific of the neurohypophysial peptide oxytocin but this first non-steroid hormone was then unknown since Vincent du Vigneaud will characterize it, only in 1953 in New York (du Vigneaud et al. 1953).

The hypothesis of an oxytocin synthesis in the thymus immediately raised and, indeed, specific radioimmunoassays revealed important quantities of oxytocin and neurophysin[2] in the human thymus. In this organ, coexistence in equimolar concentrations of those peptides already argued for a local synthesis. Human thymus extracts were then sent to Françoise Acézat, a great specialist at that time of biological assays of oxytocin, who observed that such extracts were able to contract myometrium of female rats. Furthermore, quantification of their biological activity was in close concordance with data obtained by radioimmunoassay (Geenen et al. 1986). Oxytocin-synthetizing cells in the thymus are cortical and medullary thymic epithelial cells (TECs), but not thymocytes (Geenen et al. 1987). Oxytocin is also synthetized in the epithelial component of thymic 'nurse' cells (TNCs), but not in T cells engulfed within those cellular complexes (Geenen et al. 1988). Thus, TNCs became a remarkable example of an intimate association between to cell populations derived from the distinct neuroendocrine and immune systems (Geenen et al. 1987). Specific neurohypophysial receptors are expressed by distinct thymic T-cell subsets and, after binding to these receptors, oxytocin promotes phosphorylation of tyrosine-kinases implicated in focal adhesion (Martens et al. 1998; Hansenne et al. 2004) (Fig. 1.1).

[2] Neurophysin is a 10-kDa transport and binding protein of oxytocin, which is encoded by the same gene (pro-oxyphysin).

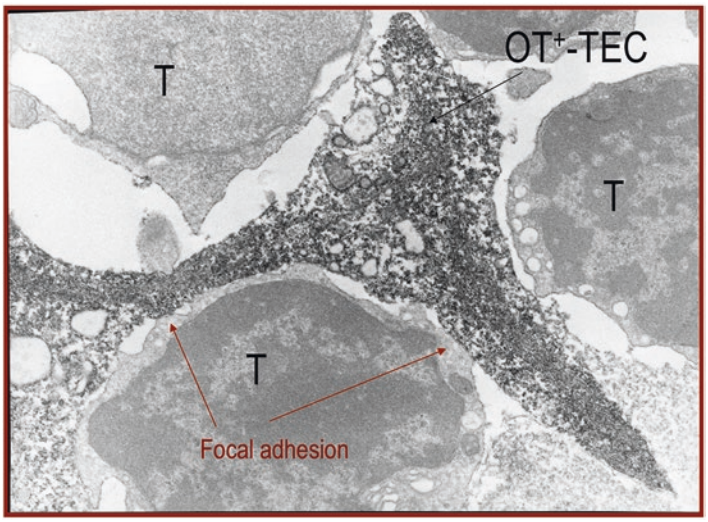

Fig. 1.1 The thymus microenvironment. Extension of a murine TEC (labelled with anti-oxytocin (OT) antibody) is surrounded by thymic T cells (T). No classical secretory granules could be observed in TEC cytoplasm, contrary to the situation in hypothalamic nerve endings of the posterior pituitary. A series of focal adhesion points between TEC and T cells are visualized and correspond to immunological 'synapses' between the two types of cells

In 1986, an international symposium of Neuroimmunomodulation was organized at the University of Liège under the chairmanship of Joseph Wybran and Jean-Jacques Vanderhaeghen, respectively professor of immunology and neuropathology at the Free University of Brussels (ULB). Starting in 1988, interactions between the neuroendocrine and immune systems were thoroughly investigated in the excellence research network *Neuroimmunomodulation* (1988–1992) under the auspices of the European Science Foundation (ESF).

A specific journal, also entitled *Neuroimmunomodulation*, was launched by Karger Editors in 1994, having as first editors the late brilliant researchers Samuel M. McCann (1925–2006) and James M. Lipton (1938–2016). This journal actually publishes for two times special issues devoted to the thymus, namely: Neuroendocrine Control of The Thymus (1999) and Neuroendocrine Immunology of The Thymus (2011).

1.4 Immunological Self-Tolerance

As soon as 1900, the father of immunology, Paul Ehrlich (1854–1915), proposed the formula '*horror autoxicus*' to claim the impossibility that one organism could be aggressed in normal conditions by its own cells in charge for its defence. Ehrlich thought then that either structures or mechanisms should exist to avoid autotoxicity,

and this should be of the highest importance for individual health and species survival (Ehrlich 1900). In the continuation of his revolutionary theory of clonal selection, the virologist and immunologist Frank Macfarlane Burnet (1899–1985) introduced the term '*tolerance*' to characterize one of the cardinal properties of the adaptive immune system with diversity, specificity and memory. During a conference at the University of London in 1962, he declared: '*If, as I think, the thymus is the site where occur proliferation of lymphocytes in clones with precise immunological functions, we have also to consider another function: elimination or inhibition of clones with reactivity to self*'. Burnet also named 'forbidden' clones lymphocytes having escaped clonal negative selection (Burnet and Mackay 1962).

In 1976, Susumu Tonegawa provided the scientific basis with the explanation for the extreme diversity of the adaptive immune response against infectious pathogens through the elucidation of the molecular mechanisms responsible for the random recombination of gene segments encoding variable domains of the immunoglobulin B cell receptor for antigen (BCR) (Tonegawa 1976). In 1984, Tak Mak, Mark Davis and other authors showed that an analogous mechanism takes place in the thymus for the generation of TCR diversity, which recognizes the MHC/antigen complex (Malissen et al. 1984; Toyonaga et al. 1984; Davis et al. 1984). There is a close homology between TCR and BCR, and a more distant one with MHC, which suggests mechanisms of replication and diversification from ancestral genes throughout evolution.

Billions of BCR and TCR combinations result in the fantastic lottery acting in the generation of their diversity and a majority of these combinations are able to recognize molecular structures of the host (the '*self*'). In normal conditions however, the adaptive immune system does not aggress self and, for long, immunology has been defined as the science of self-nonself discrimination, almost evoking some philosophical question. Burnet was readily speaking about immunology as the science of self and nonself; however, lymphocytes are not intelligent cells able to discriminate between self and nonself. In 1987 and 1988, the research groups of Nicole Le Douarin (Nogent-sur-Marne, France) (Ohki et al. 1987), John Kappler and Philippa Marrack (Denver, USA) (Kappler et al. 1987), Hugh Robson Macdonald (Epalinges, Switzerland) (MacDonald et al. 1988), and Harald von Boehmer (Basel, Switzerland) (Kisielow et al. 1988) scientifically demonstrated the theory of thymic clonal selection proposed by Burnet so many years before. The study published in Nature by the group of the Basel Institute for Immunology (Kisielow et al. 1988) was particularly elegant after generating transgenic mice with lymphocytes expressing a unique TCR specific of the HY antigen. The thymus of male mice was extremely poor in living thymocytes contrary to the female thymus fulfilled with thymocytes. This could only be explained by the deletion of transgenic lymphocytes due to the presence of the HY antigen in the male thymus, a situation of course impossible in the female thymus. So, the thymus appeared first as a cemetery for early T cells expressing a TCR specific of self-antigens.

It is interesting to note that the various interactions leading to death versus survival fate of developing thymocytes occur simultaneously during the highly organised and the oriented migration throughout the thymic lobules. During the

1990s key contributions were provided stating that both soluble and insoluble moieties (respectively chemokines and extracellular matrix) are involved in migration of developing T-cells into the thymus (Savino et al. 1993, 2002). According to one hypothesis, cell migration itself into the thymus would result from several simultaneous interactions in a sort of multivectorial migration, each interaction being one individual vector (Mendez-da-Cruz et al. 2008). We presently think that thymocytes, in a given moment of their differentiation, can be simultaneously exposed to 30 or more cell migration-related molecular interactions. The future will tell us more about this particular issue.

By the same time, scientific community was facing a fundamental problem of basic cell biology. Oxytocin is the basis for the model of hormonal secretion by neurons (neurosecretion). However, primary cultures of human TECs, even after diverse stimuli, did not secrete any oxytocin, which was in contradiction not only with the scientific truth established for oxytocin, but also with works showing oxytocin synthesis in TECs from different species and expression of specific oxytocin receptor by thymic T cells. Furthermore, Martin Wiemann published a paper that confirmed by electronic microscopy the presence of oxytocin in TEC cytosol but not in classical secretory granules issued from the Golgi apparatus (Fig. 1.1) (Wiemann and Ehret 1993). A crucial problem thus raised: if oxytocin is not secreted by TECs, how can it bind to specific receptors expressed by thymocytes? Could this be some 'illegitimate' transcription of the oxytocin gene in thymus epithelium? All hypotheses, even that of a new gene member of the neurohypophysial family, have been investigated then ruled out. Several observations however led to understand the signification of oxytocin synthesis in the thymus. In 1990, the Australian expert in adrenal physiology,

John Funder, proposed a new model of cell-to-cell signalling, the cryptocrine communication (from the Greek word *cryptos*, hidden). According to Funder, who has worked before with Burnet on the expression of steroid receptors by TECs, two examples of cryptocrine communication exist in the body: on the one hand, in the testis, between nursing Sertoli cells and differentiating spermatozoids and, on the other hand, in the thymus, between TECs/TNCs, and developing T lymphocytes (Funder 1990). At the same time, Hans-Georg Rammensee (Max-Planck Institute, Tubingen, Germany) deciphered the biochemical mechanisms that were responsible of antigen presentation by MHC proteins (Rammensee et al. 1993). On this basis was advanced the hypothesis that thymic oxytocin could behave not as the classical secreted neurohormone but rather as the self-peptide of the neurohypophysial hormone family that would be presented to T cells during their education to self-tolerance in the thymus. This hypothesis was shown to be true after different experiments (Geenen et al. 1993a, Martens et al. 1996a). Oxytocin is indeed presented by MHC proteins at the outer surface of the TEC plasma membrane and this presentation is responsible for programming immunological self-tolerance to neurohypophysial functions assumed by oxytocin and vasopressin. Contrary to vasopressin, the antidiuretic hormone, oxytocin is highly expressed in the thymus and, consequently, tolerance to oxytocin is stronger than tolerance to vasopressin. This also explained why, in our hands, rabbits immunized with oxytocin reacted so

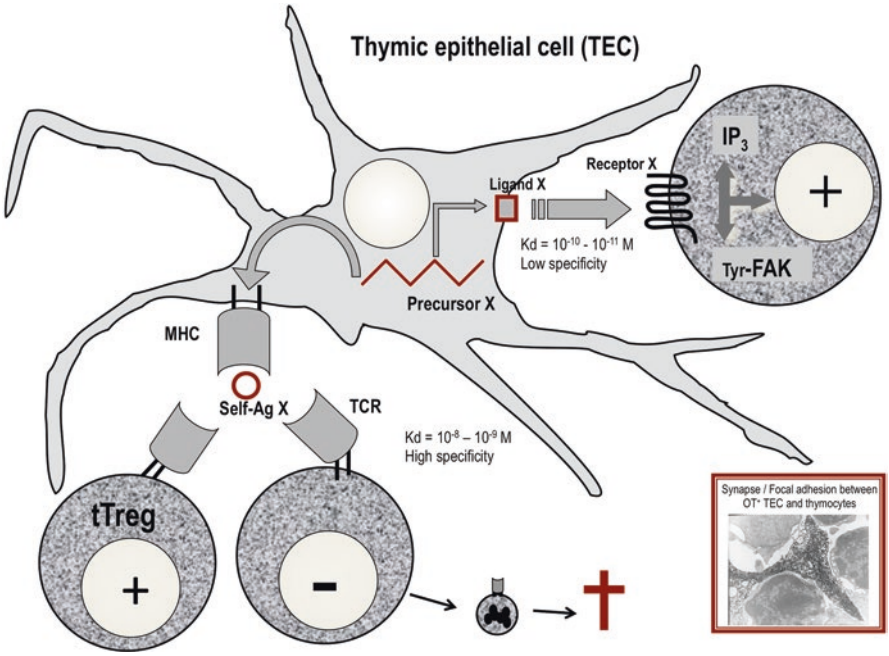

Fig. 1.2 The different roles of neuroendocrine precursors in T-cell differentiation. Following its transcription under Aire (or Fezf2) control in TEC nucleus, a neuroendocrine precursor X is processed according two distinct pathways. On the one hand, it is the source of a cryptocrine ligand X that is able to bind to a neuroendocrine receptor expressed by thymic T cells, to mobilize second messengers such as IP3, and to phosphorylate focal adhesion-related kinases (such as $p125^{Fak}$ and $p130^{Cas}$) for thymic oxytocin. This constitutes a positive accessory signal during T-cell development. On the other hand, the same precursor X is also processed as a self-antigen/peptide X that is presented by MHC proteins of TEC or thymic dendritic cells. During foetal life, self-presentation induces the negative selection of T-cells, which are randomly bearing a TCR specific of this CMH/self-Ag X. Mainly after birth, self-presentation also promotes Treg cells specific of the same complex

weakly than after immunization with vasopressin. Tolerance to oxytocin is so high that it is extremely difficult to break contrary to tolerance to vasopressin.

On the basis of these studies, a theoretical model was proposed, which transposes at the molecular level the multiple roles of the thymus in T cell differentiation, both in thymopoiesis and in the programming of central self-tolerance (Martens et al. 1996b). This model relies on the two types of behaviour that oxytocin is able to display in the thymus (Fig. 1.2). As a cryptocrine signal targeted at the TEC outer surface, oxytocin can bind to a specific neuroendocrine receptor expressed by early T cells after their migration into the thymus. This binding promotes mobilization of second messengers (inositol triphosphate, IP3) within thymocytes, as well as phosphorylation of focal adhesion-related kinases. By this way, thymic oxytocin could be able to stimulate the formation of immunological '*synapses*' between TECs and T cells, a structure playing a major role in T-cell development. In the same time,

thymic oxytocin also behaves as the self-peptide of the neurohypophysial family, which is presented by thymic MHC proteins and is responsible for programming central tolerance to neurohypophysial functions. Oxytocin presentation by TECs may induce either negative selection of T cells expressing a TCR specific of MHC/oxytocin, or generation thymic Treg cells with the same specificity. Thereafter, this model has been successfully applied to other dominant members of different neuroendocrine families synthetized in TECs such as neurokinin A for the tachykinins, neuropeptide Y (Ericsson et al. 1990), neurotensin (Vanneste et al. 1997), and insulin-like growth factor 2 (IGF-2) for the insulin family (Geenen et al. 1993b, Geenen and Lefebvre 1998) (Fig. 1.2).

The biochemical nature of neuroendocrine self may therefore be defined according to the following principles:

1. One dominant member per neuroendocrine gene family is expressed in TECs from different species.
2. Because of close homology, this dominant gene/protein in the thymus mediates cross-tolerance to all members of the family.
3. Most importantly, thymic neuroendocrine precursors are not processed according to the model of neurosecretion. They are actually processed as self-peptides that are presented by MHC proteins expressed by TECs and thymic dendritic cells.

Through this new paradigm of 'neuroendocrine self-peptides', very specific of the thymus, an integrated and harmonious was ensured between the neuroendocrine and immune systems when the genes RAG1 and RAG2 activating recombination of BCR and TCR appeared in cartilaginous fishes some 450 million years ago (Geenen 2012).

Thereafter, the elegant studies performed by Bruno Kyewski and colleagues showed that TECs, essentially in thymic medulla, are also the site for the promiscuous expression of genes encoding many tissue-specific antigens (Derbinski et al. 2001). Their publication in *Annual Reviews of Immunology* has definitively installed the central role of the thymus in central self-tolerance (Kyewski and Klein 2006). However, contrary to neuroendocrine self-peptides, most of tissue-restricted antigens do not exert any accessory signalling during T-cell development in the thymus.

In 1972, Richard Gershon (1932–1983) identified in Yale immunosuppressive cells regulating immunocompetent lymphocytes (Gershon et al. 1972). Quite ironically, he evoked this suppression as the 'second law of thymodynamics'. After his premature death, his studies were followed by Shimon Sakagushi who, in 1995, identified another thymus-dependent major tolerogenic mechanism (Sakagushi et al. 1995). Mainly after birth, the thymus is indeed the source of a new population of regulatory T (tTreg) cells that are able to inhibit in periphery self-reactive T cells having escaped central thymic negative selection. The generation/selection of tTreg cells also depends on presentation of self-peptides by thymic MHC proteins. How the same mechanism of MHC-mediated self-peptide presentation promotes two so distinct T cell fates (negative selection and tTreg cell generation) is still a matter of scientific discussion.

The programming of an intrathymic tolerance to neuroendocrine proteins was an absolute necessity for general homeostasis. As so many studies have shown, hormones and neuropeptides exert a tight control upon immune and inflammatory responses. They bind to specific cognate receptors that are expressed by different types of immune cells and thereby modulate their activity. If tolerance to these neuroendocrine signals and receptors were not firmly installed, the risk for developing autoimmune reactions toward these molecules would be very high and would compromise species survival (Geenen and Chrousos 2004).

1.5 Thymus and the Failure of Tolerance: Autoimmunity

The demonstration of the essential role of the thymus in self-tolerance raised the intuitive question about the potential role of a thymus dysfunction in the pathogenesis of autoimmune diseases. To answer this question, the transcription of insulin-related genes was investigated in the thymus of BioBreeding (BB) rats, which are with non-obese diabetic (NOD) mice a classical animal model of human type 1 diabetes (T1D). While insulin and IGF-1 gene expression was detected in the thymus of all BB rats examined, IGF-2 gene transcription was deficient in the majority of thymuses of diabetes-prone BB rats (BB-DP). This thymus deficiency in BB-DP rats might explain the characteristic lymphopenia of these animals, lymphopenia that also affects the population of Treg cells (Kecha-Kamoun et al. 2001).

Identification of the AutoImmune REgulator gene (*AIRE*/*Aire*) by a German-Finnish consortium in 1997 played a definitive role in the acceptation by the scientific community of the concept that a defect in thymus-dependent self-tolerance is a crucial event in the pathophysiology of many autoimmune diseases. *AIRE* mutations are responsible for a very rare congenital poly-autoimmune syndrome that associates hypoparathyroidism (with severe hypocalcemia), adrenal insufficiency (Addison's disease), as well as recurrent mucosal infections by *Candida albicans* (APECED or APS-1 syndrome) (The Finnish-German Consortium 1997). Further studies conducted in the laboratory of Diane Mathis and Christophe Benoist at Harvard University showed in 1992 that *Aire* expression is maximal in medullary TECs, and that $Aire^{-/-}$ mice develop several autoimmune processes in peripheral tissues (Anderson et al. 2002). Expression of many genes was diminished in the thymus of these transgenic mice, including genes coding for oxytocin, neuropeptide Y, insulin and IGF-2. Thus, the AIRE factor controls intrathymic transcription of many tissue-specific genes; *AIRE* mutations and *Aire* ablation lead to a marked decrease in the intrathymic transcription of these genes, which is associated with the development of autoimmune responses. So, it became evident that AIRE constitutes a major rampart against autoimmunity and thymus-dependant central self-tolerance became the principal research topic in laboratories interested in the pathophysiology of autoimmunity. More recently, it has been shown that the gene encoding fasciculation and elongation protein zeta family zinc finger 2 (*FEZF2*/*Fezf2*) protein

also controls intrathymic transcription of self-peptides, the majority of which is not regulated by AIRE. Interestingly, *Fezf2* is also a transcription factor implicated in some developmental processes of the central nervous system (Takaba et al. 2015).

Although they do not develop autoimmune diabetes, *Igf2*$^{-/-}$ mice exhibit a tolerance to insulin that is markedly decreased in comparison with WT mice (Hansenne et al. 2006). IGF-2 expression seems thus necessary for establishment of a full tolerance to insulin, confirming that the close homology between IGF-2 and insulin mediates cross-tolerance between two members of the same family.

Congenital absence or acquired breakdown of central tolerance to the insulin family is condition necessary but not sufficient to the development of T1D. In homozygote twins, T1D concordance is only 30–40%, which confirms the importance of genetic factors in T1D susceptibility but particularly the influence of environmental factors such as infections with Coxsackie enteroviruses. Several data argue that a Coxsackie virus B4 (CV-B4) infection could induce a thymus dysfunction and disturb the programming of tolerance to the insulin family. CV-B4 was shown to be able to infect the epithelial and lymphoid compartments of human and murine thymus (Brilot et al. 2002). In human fetal thymic organ cultures, this infection induced an important decrease in thymocyte number, as well an increase in MHC expression by TECs and double-positive CD4+CD8+ thymocytes (Brilot et al. 2004). Also, CV-B4 infection of a medullary TEC line provoked a marked and specific decrease in IGF-2 expression (Jaïdane et al. 2012).

With regard to autoimmune thyroiditis, which is the most frequent autoimmune disease, the major thyroid antigens (thyroglobulin, thyroperoxydase, thyrotropin receptor/TSHR) are expressed in TECs in normal conditions. As observed first by Jan-August Hammar, thymus hyperplasia was repeatedly observed in Graves' hyperthyroidism and this hyperplasia progressively vanished along the treatment of this disease (Paschke and Geenen 1995; Murakami et al. 1996). A defective central tolerance linked to the level of intrathymic expression of TSHR has also been incriminated in the pathogenesis of Graves' disease (Colobran et al. 2011).

Moreover, the advent of tolerance as a cardinal property of the immune system contributed to a fundamental discrimination between what is antigenic, *i.e.* able to activate an immune response (immunogenic antigen), and what is tolerogenic (self-peptide/antigen) (Fig. 1.3).

1.6 Towards a Novel Type of Vaccination Against T1D?

IGF-2 is the dominant member of the insulin family expressed in the thymus and IGF-2 possesses tolerogenic properties (Yang et al. 2014; Geng et al. 2014). A thymus dysfunction drives the development of the diabetogenic autoimmune response and CV-B4 infection could be implicated as an environmental factor in T1D pathogenesis. Insulin is the primary antigen targeted in T1D and its high immunogenicity could be linked to the very low level of its transcription in medullary TEC subsets. In the same perspective, GAD65 is also well known as an important T1D antigen, but it is very

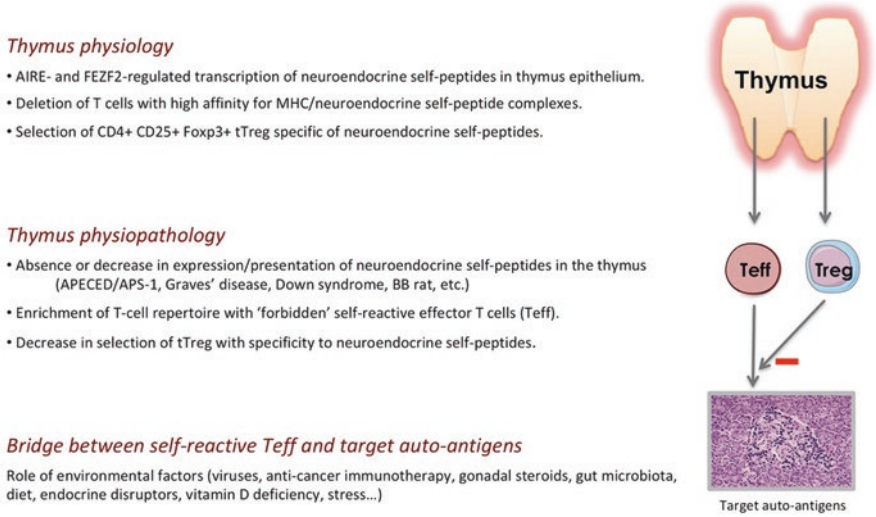

Fig. 1.3 The central role of the thymus in the programming of self-tolerance en in the development of autoimmunity. In normal conditions, under the control of *Aire* and *Fezf2*, TECs express numerous genes related to neuroendocrine families or encoding tissue-restricted antigens. MHC presentation of these self-antigens induces both negative selection of self-reactive T cells and generation of Treg cells with the same specificity. This dual mechanism is responsible for the programming of central self-tolerance to neuroendocrine functions. In some pathological conditions, the decrease in intrathymic expression and presentation of the self-antigens leads to continuous generation in blood of self-reactive 'forbidden' T cells (Teff), as well as to a decrease in differentiation of thymic self-reactive Treg cells. This is a condition, necessary but not sufficient, for the development of an autoimmune response against target tissue antigens. For the clinical development of an autoimmune disease, environmental factors are also requested

weakly expressed in the thymus contrary to the isoform GAD67. On the basis of the fundamental tolerogenic properties of the thymus, IGF-2 and GAD67 in particular, a novel type of vaccination, '*negative self-vaccination*' is under current development (Geenen et al. 2010). In opposition to the classical 'positive' immunogenic vaccination, the final objective of negative self-vaccination would be to reprogram self-tolerance through elimination of anti-insulin and anti-GAD65 forbidden clones and recruitment of related Treg cells. This vaccination would be used both for preventing T1D and curing this disease with transplantation of novel insulin-secreting β cells and suppression of the autoimmune diabetogenic memory (Fig. 1.4).

1.7 Conclusion: The Prominent Role of the Thymus in Evolution

The role of the thymus may be also evaluated from a global evolutionary point of view (Geenen et al. 2013). The thymus appeared as a single organ in the same time or shortly after the immunological 'bing-bang', *i.e.* the emergence of the adaptive

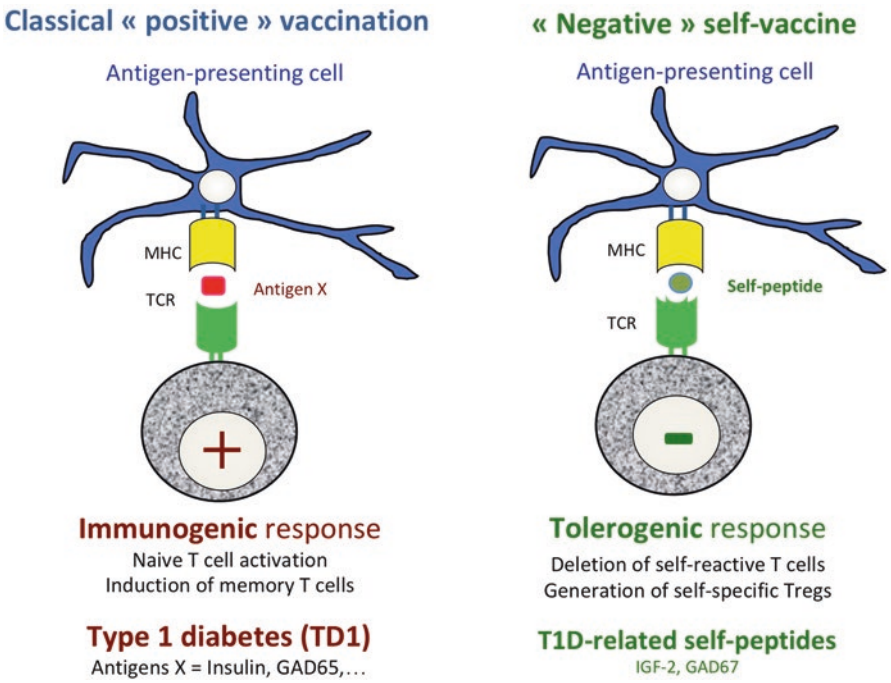

Fig. 1.4 Classical 'positive' vaccination as opposed to 'negative' self-vaccination. Classical vaccination essentially relies on the immunogenic response (naïve T cell activation and induction of memory immune cells) elicited by administration of antigen(s) representative of pathogens. Type 1 diabetes pathogenesis also includes an immunogenic response targeting T1D antigens such as insulin and GAD65. The novel type of 'negative' self-vaccination proposes to use thymus self-peptides for promoting a tolerogenic response (deletion of self-reactive T cells and generation of self-reactive Treg cells). For T1D prevention and cure, corresponding thymus self-peptides are IGF-2 and GAD67, respectively

immune response depending on RAG1 and RAG2 genes in cartilaginous fishes (rays and sharks) some 450 million years ago. Thymoid formations dispersed in the branchial apparatus of lamprey have preceded the advent of a unique thymus. They already expressed *Foxn4* (forkhead box N4), the paralog of *Foxn1*, the transcription factor specific of TEC differentiation (Bajoghli et al. 2011; Boehm 2011). The same study also provided evidence for a functional analogy between variable lymphocyte receptor (VLR) in thymoids and TCR recombination in the thymus, thus opening the question of the occurrence of autoimmune-like phenomena in jawless vertebrates. Thymus emergence allowed the orchestration of central self-tolerance, which was a rampart absolutely needed to counter the high risk of autotoxicity inherent to the tremendous lottery of the new adaptive system. A defect in thymus dependant self-tolerance, either genetic (*AIRE* and *FEZF2* mutations) or acquired (*i.e.* after CV-B4 infection) is a condition, necessary but not sufficient, for the development of many organ-specific autoimmune diseases (Fig. 1.5).