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Regulatory T cells in the establishment and maintenance of self-tolerance: role of the thymic epithelium

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ABSTRACT The thymus constitutes the microenvironment for T lymphocyte differentiation and acquisition of self-tolerance. Aiming to specify the contributions of the two essential parts of the thymus, namely hemopoietic and epithelial, we have devised experimental models in birds and mice. Chimeric thymuses, xenogeneic in birds and allogeneic in mice, were constructed early in development. In both models we could demonstrate a critical role of the epithelial component of the thymic stroma in induction and maintenance of self-tolerance. These experiments showed that suppression mechanisms are also implicated in these events, strongly suggesting the existence of regulatory T cells in both models. Before these experiments the control of self-tolerance was usually attributed to suppressive cells. However, as the cell phenotypes were not identified, the role of these cells was disregarded. Numerous studies since our investigations argue in favour of regulatory mechanisms. The work we initiated several years ago represents a contribution to our understanding of the two linked and opposite aspects of immune-responded control, namely self-tolerance and autoimmunity.

KEY WORDS: *embryo, thymus, graft, self-recognition, regulatory T-cell*

Introduction

T cell tolerance to self is acquired primarily within the thymus. As is well established, production of T cells in the thymus is controlled by a combination of positive and negative selection. Positive selection results from moderate affinity between T cells expressing the T Cell receptor (TCR) that recognize the Major Histocompatibility Complex (MHC) molecules and associated self-peptides. Negative selection leads to the elimination or apoptosis of T cells expressing TCR that bind the MHC-self peptide complex with too high affinity (von Boehmer, 1994 and for reviews, Sprent and Webb, 1995; Naji, 1996).

Clonal deletion of self-reactive T cells in the thymus was experimentally demonstrated by means of monoclonal antibodies (Mabs) directed against a defined variable region of the TCRβ chain (Kappler *et al.,* 1987) and by means of appropriately designed transgenic mice (Kisielow *et al.,* 1988; Pircher *et al.,* 1989). At the same time analysis of the minor lymphocyte stimulatory (Mls) system provided insights into general aspects of T-cell recognition and repertoire generation (reviews in Abe and Hodes, 1989; Festenstein *et al.,* 1989).

Transgenic mice also allowed the demonstration of "anergy", a non-deletional mechanism, which inactivates post-thymic autoreactive T cell clones (review in Fowlkes and Ramsdell, 1993).

On the other hand, the well established existence of autoreactive T cells in normal healthy individuals and the frequent occurrence of autoimmune diseases indicate that clonal deletion does not completely purge the repertoire of T cells able to respond to self-antigens. Besides these two mechanisms, designated as"recessive" resulting in the elimination and inactivation of autoreactive T cells, a "dominant" process involving regulatory T cells has been demonstrated in several experimental models (see for reviews: Le Douarin N. *et al.,* 1996; Modigliani *et al.,* 1996; Sakaguchi, 2000; Annacker *et al.,* 2001; Bach *et al.,* 2003; Wood and Sakaguchi, 2003).

In the 1980ies, while the crucial role of the thymus in self-tolerance induction was recognized, implication of the epithelial component of this organ was still controversial. Removing the thymic rudiment prior to hemopoietic cell (HC) colonization, both in avian and mouse embryos, has allowed us to definitively demonstrate the capacity of the thymic epithelium to induce tissular tolerance.

Abbreviations used in this paper: MHC, major histocompatibility complex; Mls, minor lymphocyte stimulatory; TCR, T-cell receptor.

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In the present report we will review our studies based on the construction of chimeric thymus in which the thymic epithelial component was of one type and HC of another (Fig. 1 A, B).

Avian chimeras

The quail-chick model has yielded a precise picture of thymus development, in which three precisely-timed waves of colonization by HC during incubation was established precisely (Dieterlen-Lièvre and Le Douarin, 2004).

To investigate a possible role of the thymic epithelial component in inducing tolerance, pre-colonized quail thymic rudiments were implanted *in situ* into thymectomized chick hosts which also received a quail limb bud from the same donor. The operations were performed at 4.5 days of incubation (E4.5), i.e. before the stage of thymic colonization by HC. Because the operation is tricky, some embryos turn out to be only partially thymectomized. In such chimeras either a definitive state of tolerance or a significant delay in the onset of wing rejection was observed in most animals kept alive between 2 and 16 months post hatching. In some cases, rejection was chronic and reversible whereas in non-thymus engrafted hosts rejection was acute (Okhi *et al.,* 1987).

Chimerism analysis of the thymus grafted in these chimeras was carried out with species-specific anti-class II Mabs. Epithelial cells in the cortex were of donor type whereas medullary dendritic cells were, like lymphoid cells, of recipient type thus of hematopoietic origin. In order to induce tolerance, thymic epithelial tissue of donor origin in tolerant birds had to amount to more than one third of the total thymic tissue in the chimera (Okhi *et al.,* 1988). Tolerance to other somatic tissues than wing could also be induced (Belo *et al.,* 1989). While chimeric bursas of Fabricius where rejected after hatching (Belo *et al.,* 1985; Corbel *et al.,* 1987), isotopic grafts of the thymus epitheliomesenchymal rudiment from the same quail donor as that of the bursal rudiment induced permanent acceptance of the bursa, which was invaded by chick hemopoietic progenitor cells (HPC) giving rise to lymphocytes and dendritic cells (Belo *et al.,* 1989) (Fig. 1A).

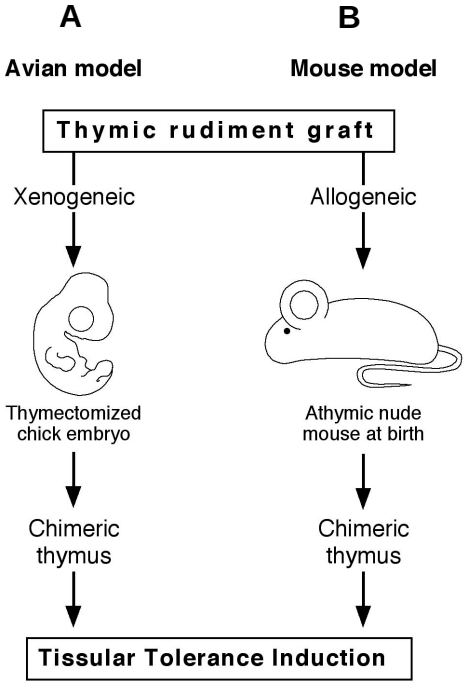

Fig. 1. Construction of chimeric thymuses (A) *in birds; chicken thymic anlagen were removed at E4.5, i.e. before the stage of HPC colonization and replaced by thymic anlagen from quail embryos. Quail wing or bursal buds grafted at the same time were definitively accepted.* **(B)** *In mice: athymic nude mice were grafted at birth with thymic rudiments removed from euthymic, allogeneic embryos at E10 (i.e. before the colonization by HPC). Tissular grafts from the haplotype of the donor were definitively accepted.*

Mouse thymic chimeras

Early embryonic allogeneic chimeras were performed in the mouse (Salaün *et al.,* 1986). In this species the thymus arises from the third pharyngeal pouches. (Metcalf and Moore, 1971; Dieterlen-Lièvre and Le Douarin, 2004). The first HPC enter the thymic anlagen at 11 days of gestation. Fontaine-Pérus *et al.* (1981) showed further that the thymus in the third pharyngeal pouch from E10 embryos, cultured *in vitro*, remains entirely epithelial.

In the mouse like in birds, it is possible to dissect the early uncolonized thymic rudiment (Jotereau *et al.,* 1987). We grafted E10 pouches into allogeneic nude neonates (Fig. 1B). Several months later, each of these mice was engrafted with tail skin from host, thymic donor and third party haplotypes. Third party skin was rejected whereas syngeneic and donor type skins were accepted. Thus, the fully allogeneic thymic epithelium has the capacity to induce tolerance to skin grafts belonging to its MHC haplotype. Ectopic heart grafts of donor type were also accepted. However, in a majority of the cases, cells from spleen and lymph node of these *in vivo* tolerant mice were reactive *in vitro* against thymic donor type cells (Salaün *et al.,* 1990; Thomas-Vaslin *et al.,* 1995).

These findings show that naked thymic epithelium is able to attract allogeneic precursors, restore T-cell function in nude mice and induce *in vivo* tolerance to tissue grafts of its haplotype: however *in vivo* tolerance is not always correlated with *in vitro* tolerance. The dichotomy between *in vivo* and *in vitro* responses suggests that complete clonal deletion is not required to induce physiological tolerance to tissue grafts.

It is well established that the main mechanism imposing tolerance upon self-reactive T-cells occurs intrathymically. This mechanism involves deletion of cells bearing T-cells receptors with a high enough avidity for the antigens encountered within the thymus. The association between some Vβ families of T cell receptors and reactivities to endogenous superantigens (Mls) has contributed much of the experimental support for clonal deletion as well as positive selection operating on Mls-reactive Vβ TCR (Mac Donald *et al.,* 1988; Bill and Palmer, 1989; Benoist and Mathis, 1989). In order to elucidate the mechanisms involved in the induction of tissue tolerance, we constructed chimeras in which nude mice from non-deleting strains were reconstituted with thymic epithelium or colonized thymuses from embryos of deleting strains.

Restored mice were tolerant to recipient and donor skin grafts but rejected third party skin graft. The percentage of Mls-reactive Vβ T-cells in such mice was similar to this percentage of T-cells

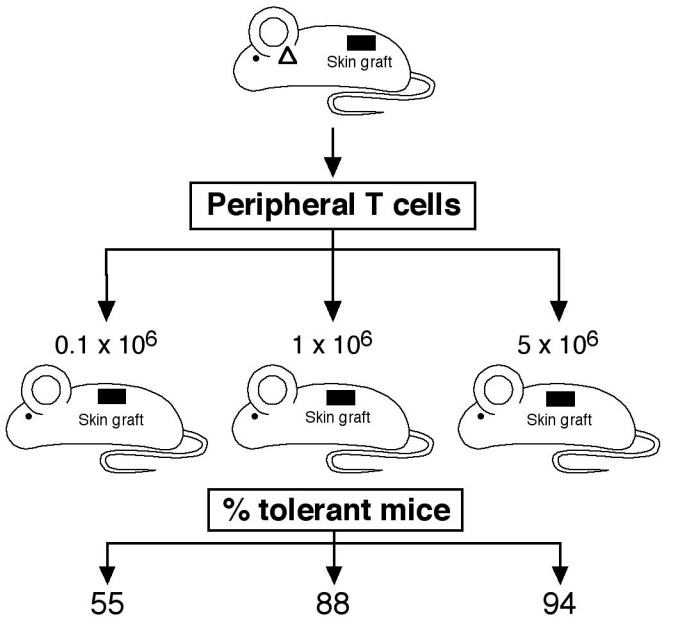

Fig. 2. Transfer of tolerance. *Nude mice reconstituted by allogeneic thymic epithelium are tolerant to skin graft of the donor haplotype. Different numbers of peripheral T cells from these mice were injected to naive nude mice. Tolerance to skin graft of the haplotype of the thymic epithelium depends of the number of the injected T cells.*

from nude mice engrafted with syngeneic thymic anlagen (Bandeira *et al.,* 1992; Salaün *et al.,* 1992).

These results have shown that neither thymic epithelial cells nor the progeny of the first wave of HPC express or functionally present the studied superantigens and that tolerance to skin grafts and superantigens T-cell deletion are unrelated phenomena.

Peripheral tolerance

Since many antigens, such as tissue-specific antigens, are not represented in the thymus, tolerance must develop in the post-thymic environment. Peripheral tolerance induction has been demonstrated mainly by means of transgenic mice constructed so that a selected antigen is expressed in extrathymic tissues (Allison *et al.,* 1988; Morahan *et al.,* 1989a-b, 1991; Hammerling *et al.,* 1991; Schonrich *et al.,* 1991).

In these cases, anergy is responsible for tolerance induction. However, in the periphery, as well as inside the thymus, clonal deletion may occur (Webb *et al.,* 1990).

Thus, different mechanisms seem to underlie peripheral tolerance induction; they might depend on the site where the antigen is expressed, the amount of antigen (Auphan *et al.,* 1992) and the cell type bearing the antigen.

Besides the transgenic model, exclusively locally expressed foreign antigens (i.e., not in the thymus), when introduced into the embryo, can induce tolerance to adult skin grafts of a same haplotype during the entire life span of the animal. Experiments designed according to this scheme had previously indicated the existence of peripheral tolerance inducing mechanisms in birds (Corbel *et al.,* 1990; Martin *et al.,* 1991). In the chick model, allogeneic limb buds were transplanted from embryos (B4 or B12 MHC haplotype) into (B15xB21) F1 chick recipients. They were studied from birth onwards for periods ranging from 5 to 11 months posthatching. A lasting state of tolerance to the grafted wing was observed in most animals between 2 and 4 months. Moreover, adult skin grafts from a donor with the same MHC haplotype as the wing were also tolerated. Tolerance did not extend to the MLR (Mixed Lymphocyte Reaction) since proliferative responses of blood T cells from chimeras against wing donor MHC haplotype stimulator T cells were comparable to those elicited by T cells of control ungrafted chicks (Corbel *et al.,* 1990). In allogeneic bursal chimeras, a tolerant state was induced also, but it varied according to the haplotype combination and was short-lived compared with that induced by grafing in wings (Martin *et al.,* 1994).

Regulatory T cells

Our work both in birds and in mice demonstrates that thymic epithelium grafts induce tolerance, not only to the thymic epithelium itself, but also to peripheral tissues although effector cells, are present in uncompletely thymectomized chicken chimeras tolerate the grafted quail thymus and since some cells in grafted mice are reactive *in vitro* against donor type cells.

We have shown that peripheral spleen and lymph node T cells from nude mice restored with allogeneic thymic epithelium, when injected into naive nude recipients, are able to restore these mice and to induce tissue graft tolerance to donor type skin (Fig.2).

Transfer of tolerance however depends on the number of injected T cells: the fraction of tolerant recipients increases with increasing numbers of transferred cells, suggesting suppressive

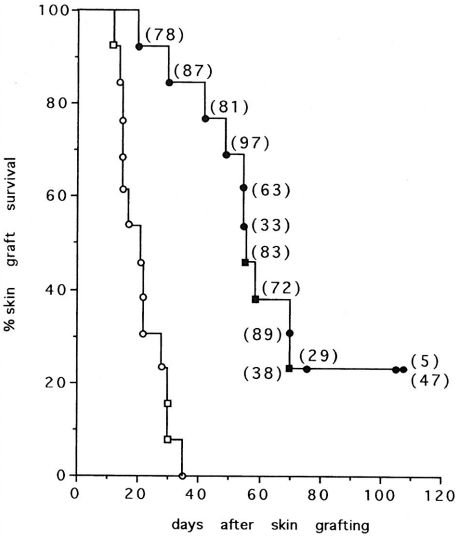

Fig. 3. Survival of skin grafts. *C57BL/6 naive nude recipient mice were restored by the i.v injection of peripheral cells from normal C57BL/6.Thy1.1 mice and from C57BL/6 Thy1.2 nude mice grafted with BALB/c thymic epithelium. The percentages of chimerism in each mouse is indicated in brackets as: % chimerism of Thy1.1 T cells = % Thy1.1 / (% Thy1.1 + % Thy1.2). Open symbols: third party C3H skin graft; closed symbols: BALB/c skin graft; squares: skin grafting before cell transfer; circles: skin grafting after cell transfer. C57BL/6 skin graft persist in all cases. From* C.R. Acad. Sci. III, *1996, 319, 401, with permission.*

mechanisms mediated by regulatory T cells. The phenotype of T cells able to transfer tolerance was shown to be $CD4^+$ (Modigliani *et al.,* 1995).

In order to study whether T cells from chimeras are able to regulate activity of effectors from normal mice, we have devised a model in which naive nude C57BL/6 were reconstituted with a cell mixture of peripheral T cells from C57BL/6 Thy1.2 nude mice grafted with BALB/c thymic epithelium and from normal C57BL/6 Thy1.1 (Fig. 3).

These experiments have shown that the transfer of peripheral T cells from chimeras, together with syngeneic T cells from normal mice, induces a significant delay in the rejection of skin graft of the thymic haplotype (BALB/c). This delay depends on the ratio of the two types of injected cells.

Thus, regulatory T cells selected on an allogeneic thymic epithelium are able to control the effector activity of peripheral T cells derived from normal mice (Thomas-Vaslin *et al.,* 1996). All these results are in favour of "dominant" control mechanism where a population of T cells regulates the activation of self-reactive T cells.

Role of the thymic epithelium in autoimmunity

Evidence for the importance of the thymus in autoimmune disease development has been clearly demonstrated by the thymectomy experiments performed by Sakaguchi in mice. Thymectomy between days 3 and 5 after birth leads to the development of autoimmune diseases (Asano *et al.,* 1996; Sakaguchi *et al.,* 1985). Transfer of $CD4^+$ peripheral T cells from normal animals to recipients with autoimmune manifestations can prevent these diseases (review in Seddon and Mason, 2000). In autoimmune diseases, the target organs are heavily infiltrated with T cells. This is particularly the case in insulinodependent diabetes, both in human and in Non Obese Diabetic mice (NOD).

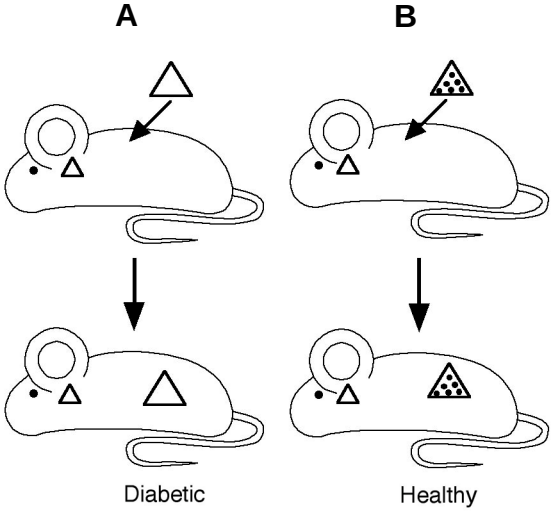

Fig. 4. Protective effect of ectopic thymus injected with pancreatic islets in NOD mice. *NOD female mice were grafted by an ectopic new-born NOD thymus* **(A)** *or by an ectopic new born NOD thymus containing allogeneic pancreatic islets* **(B)**. *Frequence of the disease in A is similar as this frequence observed in non-operated NOD female mice. On the contrary, appearance of the disease is significantly decreased in (B).*

In NOD mice, the disease is primarily mediated by CD4 and CD8 T cells (Bendelac *et al.,* 1987; Wong *et al.,* 1996). To investigate the role of T cell repertoire selection in the immunopathenogenesis of the disease in the NOD strain, we have restored nude mice by grafting thymic rudiment from NOD embryos (Thomas-Vaslin *et al.,* 1997). Autoimmune disease development was monitored in these chimeras, after sacrifice. The pancreas and the salivary glands were heavily infiltrated with $CD4^+$ and $CD8^+$ infiltrating T cells.

These results demonstrate that non diabetic nude T cells selected in a chimeric thymus with a NOD embryo thymic epithelium are able to mediate insulitis and sialitis in nude mice. Thus, NOD thymic epithelium alone can select an autoimmune T cell repertoire, suggesting a defect of the thymic epithelium in selecting an appropriate repertoire of regulatory T cells. By using transgenic mice where major histocompatibility complex class II $I\text{-}A^b$ expression is limited to thymic cortical epithelium Bensinger and coll. (2001) have demonstrated that development of regulatory $CD4^+CD25^+$ T cells is dependent on MHC class II-positive thymic cortical epithelium.

Regulatory T cells, "key controllers of immunologic self-tolerance" (Sakaguchi *et al.,* 2000) are released from the thymus as demonstrated by their functional defect in neonatally thymectomized mice (Sakaguchi, 1985).

Intrathymic transplantation of pancreatic islet allografts was initially investigated by Posselt *et al.* (1990) in diabetic rats. The data in rats and subsequently in NOD mice (Mayo *et al.,* 1994; Gerling *et al.,* 1992; Cetkovic-Cvrlje *et al.,* 1997) were usually interpreted as the results of negative selection of T cell precursors. We have investigated whether regulatory T cells could be selected by intrathymic islet graft. NOD mice were grafted with newborn NOD thymuses into which allogeneic pancreatic islets had been injected (Fig. 4): 70% of the controls developed the disease as compared with only 24% of the mice engrafted with NOD thymuses + allogeneic pancreatic islets (Salaün *et al.,* 2002).

This protective effect of the pancreatic islet containing in the ectopic thymus is in favour of a role of regulatory T cells.

Conclusions

The first aim of our work, both in birds and in mice, was to determine the respective roles of the two thymic components, hemopoietic and epithelial cells, in T cell differentiation. Appropriate quail-chick chimeras had disclosed the contribution of the three embryonic germ layers, endoderm, mesoderm and ectoderm, to the ontogeny of the thymus. The timing of thymus colonization by hemopoietic cells was precisely defined in birds, then in mice (Jotereau et Le Douarin, 1982; Jotereau *et al.,* 1987). Removing the thymic rudiment before hemopoietic colonization has allowed the *in vivo* construction of chimeric thymuses in which epithelium is of one species or, strain and hemopoietic cells of another. Such chimeric thymuses afforded the opportunity to reveal the role of thymic epithelium in self tolerance induction and to study mechanisms implicated in this event, namely deletion and suppression. In both avian and murine models we have observed tissular tolerance despite the persistence of autoreactive T cells. Our experiments of tolerance transfer by injection of peripheral T cells point out a crucial role of regulatory T lymphocytes in the maintenance of self tolerance (Modigliani *et al.,* 1995a, b). Suppressive mechanisms in transplantation tolerance were previously suggested by Kindred

(1971) and by Dorsch and Roser (1977). Since these results, the presence in the periphery of T cells, able to prevent autoaggressive immune reactions, has been established (see for review: Annacker *et al.,* 2001; Wood and Sakaguchi, 2003). Our experimental reconstitution of nude mice with NOD thymic rudiments show further that abnormal thymic selection of T cells could induce autoimmune manifestations in non-autoimmune strains (Thomas-Vaslin *et al.,* 1997). Moreover, these results have indicated that a thymic epithelium graft is sufficient to induce autoimmunity in the host.

Thymic generation of T cells able to regulate the activation of autoreactive T lymphocytes is now well established. The thymic stroma has an important role in this process (our work and Jordan *et al.,* 2001). Failure of this mechanism can lead to the development of autoimmunity (see for review Seddon and Mason, 2000). In NOD mice, improvement obtained by grafting supplementary thymuses injected with pancreatic islets suggests that the disease in this strain is due to an inefficient generation of regulatory T cells (Salaün *et al.,* 2002).

Despite cumulative evidence for the crucial role of regulatory T cells in self tolerance, the ontogeny, phenotype and mode of action of these cells remain incompletely known. Different subsets of $CD4^+$ regulatory T cells were found; $CD4^+$ $CD25^+$, $CD4^+$ $CD25^+$ $CD45RB^{low}$ and $CD4^+$ $CD25^+$ $CD62L^+$ (see for reviews Annacker *et al.,* 2001; Bach, 2003), showing the diversity of regulatory T cells. Recently, genetic defects in Foxp3 which encodes a forkhead-winged-helix transcription factor were described in some inflammatory diseases both in man and in mouse (Bennett *et al.,* 2001). Hori and coll. (2003) have shown that Foxp3 is expressed at high levels in thymic and peripheric $CD25^+CD4^+$ regulatory T cells. Foxp3 can be considered as a key gene for the development of regulatory T cells (Fontenot *et al.,* 2005).

A better knowledge of how these cells are selected could help understanding the immune function both in normal development and in immunopathogenic processes.

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