The Journal of Immunology

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J Immunol 2021; 206:245-247; ;
doi: 10.4049/jimmunol.2001285
http://www.jimmunol.org/content/206/2/245

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Pillars of Immunology

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Conceiving the Inconceivable: The Function of Aire in Immune Tolerance to Peripheral Tissue-Restricted Antigens in the Thymus

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The pursuit of the understanding of mechanisms underlying immune tolerance has led to some of the most important breakthroughs in immunology. The discovery by Mathis, Benoist, and colleagues (1) of an essential function for the autoimmune regulator (*Aire*) gene in establishing tolerance to tissue-restricted self-antigens during T cell differentiation in the thymus rightfully belongs to this foundational body of work. The landmark 2002 paper by Anderson et al. (1) reporting this finding is featured as a *Pillars of Immunology* article in this review compilation on stromal immunology (https://science.sciencemag.org/content/298/5597/1395).

Discrimination between "self" and "nonself" is a core principle of immunity aimed at protection of the integrity and sovereignty of living organisms found in all three domains of life. In vertebrates, the emergence of the adaptive immune system, based on somatic generation of clonally distributed receptors as the result of random gene rearrangement afforded efficient protection against pathogens at a cost of destructive and potentially life-threatening self-reactivity.

The concept that immunological tolerance could be actively acquired was first established by seminal experiments by Medawar, Billingham, and Brent (2–4). Building on Ray Owen's (5) earlier observation of erythropoietic stem cell chimerism in dizygotic cattle, they showed that inoculation of mice with donor foreign Ags, either in utero (2, 3) or during a critical neonatal developmental window (4), led to long-term acceptance of transplanted skin grafts from the same donor strain in adulthood. Thus, tolerance, akin to immunity to pathogens through vaccination, could be acquired. Similarly inspired by Owen's (5) experiments, Burnet (6) had noted that the immune system must discriminate self from nonself. In his theory of clonal selection, separately posited by Talmage (7), a cellular basis for immune recognition of Ag was proposed, whereby each Ag activates a specific lymphocyte with specificity for that Ag. Following Burnet's (6) theory, immune tolerance would represent elimination of immune cell clones reactive to self.

At the time, the cellular "unit" proposed by Burnet (6) and Talmage (7), was thought to be an Ab-producing cell. It was not until a decade later that the role of the thymus, described by Medawar as an "evolutionary accident," would come to be appreciated with the existence of a specialized population of thymus-derived lymphocytes. Seminal studies by Miller, Cooper, and Good (8, 9) in the 1960s revealed the critical role of the thymus as the generative lymphoid organ in mice and chicken. In Miller's (8, 10) studies, mice that underwent a thymectomy in the neonatal period were unable to reject allogeneic skin grafts. Transplanting a neonatal thymus restored their ability to reject the foreign skin graft; however, if mice were grafted with an allogeneic thymus graft, the recipient mice exhibited tolerance to donor histocompatibility Ags. Miller (11) proposed that tolerance might be learned in the thymus and that immunological tolerance was equivalent to an "immunological thymectomy" with negative selection of a thymic population of self-reactive immune cells.

Although these discoveries opened the flood gates to exploration of the differentiation and function of T lymphocytes, addressing the question of their selection and establishment of immune tolerance in the thymus required the ability to track expression of defined self-antigens and the T cells capable of recognizing them. This goal became feasible with advances brought by genetic approaches, including genetic engineering, over 20 years later. Marrack and Kappler (12) were the first to show that developing T cells specific for self-peptides bound by MHC class II I-E, subsequently identified as Mls Ags (viral superantigens) encoded by endogenous mammary tumor viruses, undergo negative selection within the thymus, in line with Burnet's (6) original hypothesis. Further studies confirmed thymic deletion of additional T cell clones reactive to *Mls*-encoded Ags (13, 14). The advent of mouse transgenesis enabled von Boehmer (15) to provide a definitive proof of clonal deletion of developing T cells specific for bona fide self-antigens in the thymus by demonstrating that $CD4^+CD8^+$ thymocytes carrying a single TCR with known specificity to the male Ag H-Y undergo elimination specifically in male but not female mice. These observations, however, raised the question as to how immune tolerance is being

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This work was supported by the Wellcome Trust (WT201483/Z/16/Z) and a National Cancer Institute Cancer Center Support Grant P30 (CA008748). A.Y.R. is a Howard Hughes Medical Institute investigator. C.C.B. is a Wellcome Trust Clinical Research Fellow.

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Abbreviations used in this article: *Aire*, autoimmune regulator; APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; mTEC, thymic medullary epithelial cell; TRA, time-restricted Ag.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.2001285

established for Ags whose expression is controlled in a tissue-specific or developmental stage-restricted manner. It was assumed that autoreactive T cells specific for such Ags would undergo functional inactivation or deletion in the periphery, as it was inconceivable that developing thymocytes would be able to "see" tissue- or development time-restricted Ags (TRAs) within the thymus.

This "common sense" view was challenged by Bruno Kyewski (16, 17), who in an elegant series of studies revealed the "inconceivable" thymic expression of transgene-encoded and naturally occurring TRAs, including C-reactive protein and proteolipid protein. This ectopic display of TRAs in the thymus led to the deletion of thymocytes expressing TCRs of corresponding specificities. Using laborious techniques of thymic epithelial cell fractionation, Kyewski (16, 17) showed that TRA expression was restricted to thymic medullary epithelial cells (mTECs). Further contemporaneous studies confirmed promiscuous thymic expression of a host of TRAs with variations in intrathymic expression correlating with autoimmune disease susceptibility (18, 19).

One major question remained: how was ectopic expression of TRAs regulated in mTECs? The clue came from studies of a clinical "experiment of nature": an autosomal recessive autoimmune disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; or APS1), first described in the 1940s. APECED presents early in childhood with a spectrum of autoimmune-mediated clinical manifestations. A major advance in the understanding of the disease came in 1997 when two groups elucidated the genetic basis for APECED by using positional cloning strategies to identify the underlying mutations in a novel gene, subsequently named Aire (autoimmune regulator) (20, 21). A mouse homolog was soon identified (22), and Aire was shown to be highly expressed within the thymus (23), specifically mTECs, suggesting a tantalizing link between two parallel lines of investigation.

The work of Diane Mathis, Christophe Benoist, and colleagues (1) provided the critical and final piece of the puzzle by conclusively demonstrating the essential role for Aire protein in TRA expression by mTECs. They showed that, akin to APECED patients, Aire-deficient mice developed age-dependent autoimmunity, with progressive involvement of specific tissues. Through a series of bone marrow transfer and thymic transplant experiments, Aire expression within radio-resistant thymic stromal cells was shown to be critical for prevention of autoimmunity. Furthermore, gene expression analysis of Aire-deficient versus wild-type mTECs confirmed the authors' hypothesis that Aire specifically promotes ectopic transcriptional activity in mTECs, resulting in the induction of TRA-encoding genes, whose expression is normally restricted to peripheral tissues. In this way, in the authors' own words, Aire creates an "immunological shadow" of the peripheral self in mTECs. These TRAs can either be presented directly to developing T cells or indirectly as a result of ingestion by thymic dendritic cells. Presentation of TRAs leads to deletion of self-reactive T cells, the failure of which results in autoimmunity. The latter was also proven by Anderson et al. (1), demonstrating the onset of a multiorgan autoimmune disease in lymphopenic hosts upon adoptive transfer of T cells educated in the absence of Aire, similar to that observed in Aire-deficient mice.

Since the discovery of its role in immune tolerance to TRAs, Aire continues to intrigue. First, immunostaining and more recently single-cell RNA sequencing studies have highlighted the stochastic nature of TRA expression in individual Aire-expressing mTECs (24), yet the biochemical mechanistic basis of Aire targeting of TRA-encoding genes remains to be fully elucidated, with several, nonmutually exclusive modes of action proposed. In addition, several studies have suggested that AIRE regulates differentiation and Ag presentation functions of mTECs as well as their architectural organization of the medulla. Furthermore, Aire expression has also been reported in peripheral APCs (25), raising the question of a role for Aire in peripheral immune tolerance. Whether Aire in peripheral APCs mediates TRA expression or promotes immune tolerance by other means remains a matter of debate.

In closing, the seminal paper (1) by the Mathis and Benoist laboratory answered a major open question in immunology by establishing the mechanistic basis for immune tolerance to tissue-restricted Ags acquired in the thymus. While capping and bridging several lines of prior scientific inquiry, this study continues to serve as a stepping-stone for ongoing and future exploration of the molecular and cellular mechanisms underlying discrimination between self and nonself in the adaptive immune system.

Disclosures

The authors have no financial conflicts of interest.

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