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Abbreviations: IPEX: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome · **T1D**: type 1 diabetes

The effector functions of regulatory T cells in immune regulation

Regulatory T cells and immune computation

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The role of Treg in immune regulation is the topic of this Viewpoint series in the *European Journal of Immunology* (EJI); the question to be discussed in this section is the effector function of T_{reg} in immune regulation. In this manuscript, we take on the following three postulates outlined by Rolf Zinkernagel on the role of T_{reg} in the control of immunity. First, the immune response is regulated primarily by the antigen and not by T_{reg}. Second, immune non-responsiveness results from the deletion of specific receptor-bearing T cells. Third, there is no definitive proof of the existence of specialized Treg that know what is needed for an equilibrated immune response. Herein, we discuss data demonstrating the existence of specialized T_{reg} and therefore arguing against the validity of the first two postulates. However, based on the reactive nature of the immune system, we agree with Rolf's third postulate in that T_{reg} cannot know ahead of time an ideal set-point for immune homeostasis.

Rolf Zinkernagel and T_{reg}

This issue honors Rolf Zinkernagel, who is now winding up his service as Chairman of the EJI for the past seven years. We would like to refer to Rolf Zinkernagel's published ideas about Treg as a framework for our discussion in this publication honoring him. Rolf Zinkernagel has published a "*credo*" outlining 20 rules governing immune reactivity [1] and, more recently, a commentary listing a number of recommendations for immunologists [2]; we have extracted from these two papers three teachings relating to T_{reg} and immune regulation. According to Rolf Zinkernagel:

(1) The immune response is regulated primarily by contact of immune cells with antigen according to the site of the encounter, the dynamics of antigen concentration and the persistence of antigen over time [1, 2]; immune regulation does not need special T_{reg}.
(2) Immune non-responsiveness, another aspect of regulation, results from the deletion of specific receptor-bearing T cells or from a state termed immune ignorance [2]. Thus, there is no need for T_{reg} to account for non-responsiveness.
(3) Finally, "a special type of Treg that 'knows' in foresight what is needed for an equilibrated immune response has not been convincingly shown" [2].

We shall use these points to guide our discussion.

First of all, what type of agent would qualify as an effector of immune regulation?

Effectors of immune regulation

To avoid semantic misunderstanding, we define a regulating factor to be an agent or process that influences the response of a system to a given stimulus.

The term immune regulator cell therefore can be applied to any cell that influences the magnitude of an immune response, its quality (the sum and integration of all the molecular and cellular elements comprising the response), its dynamics, its anatomical site, or the effects of the response on the immune system itself – memory, tolerance, repertoires, cell numbers, *etc.* T_{reg}, which have been shown to down-regulate certain immune response phenotypes [3], can certainly be termed effectors of immune regulation. Just note for now that up-regulators, too, are regulators.

Regulation by antigen

The idea that the immune response is regulated exclusively by contact with specific antigen has a long history: It is one of the fundamental ideas behind the clonal selection theory of adaptive immunity as originally proposed by Burnet [4]. The clonal selection view of the immune reaction was compatible with a worldview that sees the evolution of the adaptive immune system as an ongoing response to the threat to survival posed by infectious agents [1, 2, 5]. An immune response is

triggered by contact with foreign antigens associated with a foreign invader; the response to the invader-associated antigens gets rid of both the invader and its foreign antigens, and the immune response, for lack of stimulation, returns to an inactive state – turns off [5].

A problem with this elegantly simple view of the role of antigen in regulating the immune system is its simplicity; the contact with antigen is important, but the biological expression of any immune response clearly is not determined by contact with antigen alone. The phenotype of the immune response is the outcome of a long and complex list of regulatory factors beyond antigens: adjuvants, APC, diverse types of T cells (including T_{reg}) and B cells with positive and negative effects, the requirement for multi-cellular interactions in generating immune responses, the diverse roles of various cytokines and chemokines, and so on and so forth. The simple idea of immune regulation by antigen alone belies the complexity of immune reality [6]. Rolf Zinkernagel adds some complexity to the antigen stimulus by calling attention to the importance of the site of antigen contact, the dynamics of antigen concentration and antigen persistence [1]. Making more demands on the antigen, however, does not explain the need for the great complexity of cells and molecules confronting immunologists. Regulation-by-antigen may seem logical [1, 5], but the idea is deficient in explanatory power and can even encourage misunderstandings [6].

Non-responsiveness by deletion or ignorance

Zinkernagel's second teaching about regulation follows from his first teaching: if we fail to detect an immune response to a specific antigen that has entered the system, then either there are no clones of lymphocytes with receptors for that antigen (deletion) or there are such clones of lymphocytes but they do not get to see the antigen (ignorance). Deletion (negative selection) and ignorance may play some role in immune down-regulation. Indeed, pioneering depletion experiments carried out by Sakaguchi and colleagues [7] demonstrated that depletion of $CD4^+$ $CD25^+$ T cells results in the induction of autoimmune disorders that usually target immune-privileged organs such as the eye, ovaries or testis. Thus, depletion and ignorance are important mechanisms for immune well-being, but they certainly are not the only regulatory factors, or even the most important factors, in down-regulating or suppressing immune effector responses. Active regulation by regulatory T cells is needed for immune homeostasis. Following the seminal experiments by Sakaguchi and coworkers, Treg and their effects on the immune response are well documented in an extensive literature – the term 'regulatory T cell' elicits over 23 000 original articles and over 3000 reviews in the PubMed online database. The existence of T_{reg} as subjects of concrete experimentation cannot be denied.

Immune equilibrium

Zinkernagel's third point, in our opinion, is a most insightful and thought-provoking observation that cannot be easily dismissed: No T_{reg} has been found that knows ahead of time what kind of regulation is needed for immune equilibrium [1, 2]. After all, standard regulators should know their endpoint, the objective of their regulation. Temperature regulation, for example, needs a thermostat with a known set-point – about 21°C for your hotel room, precisely 37°C for your core body temperature. Regulation of body homeostasis by the lungs, kidneys, heart, or endocrine system works by set-points for O_2 and CO_2, fluid volume, osmolarity, blood pressure, hormonal needs, and so forth. Rolf here makes a telling point; if T_{reg} really are an important factor in immune homeostasis, then such T_{reg} would have to know what the immune system is aiming at – T_{reg} would have to know the physiological set-point of the healthy immune system. Since no one has come up with a T_{reg} that manifests such "foresight", then, as Rolf points out, there is no reason to propose that T_{reg} have evolved to regulate immune responses. One may accept the fact that certain types of T cells, upon contact with an immunogenic stimulus, secrete cytokines like TGF-β or IL-10 that down-regulate certain immune response phenotypes; but why call them T_{reg}? If these T cells cannot be demonstrated to aim at a predetermined immune set-point, how can they possibly regulate immune homeostasis? Imagine your room or your body heated or cooled by a thermostat that does not know its ideal set-point?

Unless, of course, regulation of the immune system differs fundamentally from the regulation of body homeostasis in that it does not depend on predetermined set-points [8].

We have not asked Rolf Zinkernagel about his views on this point, but we think we could suggest a plausible response: The aim of the immune system is to rid the body of foreign invaders and their foreign antigens [1, 2, 5]; thus, immune regulation does not need a predetermined set-point; it only has to get rid of the foreign invader – no foreign antigen, no activity. From this point of view, the ideal state of the immune system, its set-point, is quiescence. The adaptive immune system, in an "ideal" world free of invading pathogens, would have nothing to do, apparently. This state of affairs exists only in theory; the body, of course, is continuously confronted with invaders and their foreign molecules, and so in practice the immune system can never rest; it is always busy fighting pathogens. Indeed, the immune system needs this basal activity to continuously reset itself, as shown by the role of commensal flora [9] and immune experience [10] in the development of immune regulation.

Note that, in getting rid of pathogens, the immune response to the foreign has to cause as little harm as possible to the rest of the normal body. As Rolf has written [1], "immune protection always includes immunopathology". According to this view, inflammation – immune activity – is intrinsically harmful and a necessary burden on the organism. Thus, the immune system's ongoing confrontation with the foreign requires mechanisms that ensure a minimum of inflammation. T cells that produce effector molecules like TGF-β or IL-10 just might reduce self-immunopathology. But, as Rolf has said, why call them T_{reg}? These T cells do not regulate; foreign antigen regulates.

Immune attention to the self

An experimental challenge to Zinkernagel's notion of immune regulation by foreign antigen alone is the body of evidence that the immune system also attends actively to the self [11]. It is now clear that the immune system maintains health not only by its cells and molecules that attack foreign invaders; immune molecules and cells are key factors in healing wounds, organizing the structure of connective tissue, growing (angiogenesis) or destroying blood vessels, triggering regeneration of certain organs, activating the apoptosis of aged, sick or dangerous cells, degrading accumulations of abnormal molecules, disposing of waste, and other vital activities [11]. These beneficial activities are the outcome of what has been called physiological inflammation; from this point of view, balanced inflammatory responses maintain the body [11]. These findings should make it clear that inflammation is not a necessary burden, as proposed by Zinkernagel [1, 2], but a necessary benefactor. Healthy inflammation is physiological. Like other physiological processes, inflammation demands proper regulation – a lapse in its regulation can lead to recurrent or chronic inflammatory and autoimmune diseases [11].

One might argue that most of these ongoing health maintenance functions are performed by the innate arm rather than by the adaptive arm of the immune system, but humans and other vertebrates are naturally outfitted from birth with adaptive antibodies and T cells that respond to self antigens [11, 12]. Indeed, positive selection of developing T cells [13] and B cells [14] by self antigens seems to be critical in the development of the adaptive immune repertoire. Natural autoimmunity appears to perform a variety of positive functions [11]; autoimmune T cells, for example, have been shown experimentally to protect organs like the brain from secondary degeneration following trauma [15]. Thus, adaptive autoimmunity exists and is functional. In other words, adaptive immune repertoires include cells reactive to self epitopes [11, 12]. In this state of affairs, how can the entry of antigen function act as the primary immune regulator? Foreign antigens may come and go, but self antigens are with us all the time. Natural autoimmunity invites us to further clarify the issues we raised above: We need to consider the ideal set-point of the immune system and the effector functions of T_{reg} in immune regulation. Let us take a look at the plasticity of T_{reg}.

T_{reg} conversion

Although T_{reg} were first described as differentiating in the newborn thymus [16], it is now clear that T_{reg} develop in the periphery from non-T_{reg} T cells, a process termed conversion [17]. T_{reg} conversion appears to be dependent on the integration of many signals leading to a complex signature of gene transcription molecules that regulate the T_{reg} phenotype [18]. We may conclude that T_{reg} are a particular state of T cell differentiation triggered in response to a variety of immune signals and antigen activations. Coming back to Rolf's first postulate, the dose and manner of antigen administration play a central role in the generation of T_{reg}: targeting of an antigen to dendritic cells with DEC205-specific antibodies [19] or long-term administration of ultra-low antigen doses [20] are just two examples of how controlled antigen administration can induce specific T_{reg}. What other signals, besides those triggered by antigen, can influence T_{reg} conversion and function?

Immune system regulation of T_{reg}

The expression of the T_{reg} phenotype appears to be influenced by a variety of agents originating from the immune system itself: Competition between T effectors and T_{reg} can determine the numbers of T_{reg} developing within the immune system [21]. Indeed, T cells expressing Th1, Th2 or Th17 phenotypes can inhibit the development or the activity of T_{reg} [22, 23]. Moreover, T_{reg} are themselves indirectly regulated by the effector cells they regulate. T_{reg} express high levels of the IL-2 receptor αchain (CD25), and IL-2 is indeed required for the peripheral fitness and survival of T_{reg} [24]. Notably, T_{reg} do not produce IL-2, and therefore rely on the IL-2 supplied by activated effector T cells [25], thus providing a feedback loop for the regulation of T_{reg}.

T_{reg} function is also influenced by the antibody repertoire, as shown in studies using intravenous immunoglobulins [26]. Indeed, regulatory T cells can be induced by intravenous [27] or oral [28] administration of antibodies to CD3. Thus, we can conclude that the conversion, numbers and functions of T_{reg} are responsive to the state of the immune system itself.

Tissue regulation of T_{reg}

The plasticity of T_{reg} is highlighted by the influence on the conversion and function of T_{reg} by factors originating from body tissues: retinoic acid, for example, regulates the growth and differentiation of T_{reg}, and also imprints T_{reg} to home to gut tissue [29]. UV light up-regulates the expression by skin keratinocytes of the ligand to receptor activator of NFκB (RANK), which then interacts with $RANK^+$ Langerhans cells to promote the proliferation of T_{reg} [30]. Physiological levels of hormones such as estrogens can induce T_{reg} [31]. The state of neurons in the central nervous system can induce the conversion into T_{reg} of encephalitogenic effector T cells, and thereby suppress autoimmune encephalomyelitis [32]. Tumor cells are notorious for generating signals that up-regulate T_{reg}, leading to evasion by the tumor of immune surveillance [33]. Thus, signals originating from body tissues can determine T_{reg} and T_{reg} function.

HSP60 can innately activate T_{reg}

The 60kDa heat shock protein (HSP60) acts as a molecular chaperone inside cells, but self HSP60 also acts as a powerful regulatory signal to the immune system as a ligand for both adaptive [34] and innate [35] immune receptors. The expression of HSP60 apparently functions as a biomarker for the immune system that can alert immune cells to the state of body cells – including their states of physical and molecular stress [36]. Information about the expression and amounts of HSP60 is used by the immune system to orchestrate the initiation, progression and termination of the inflammatory process; HSP60 is a key factor in the physiological dialogue between the body

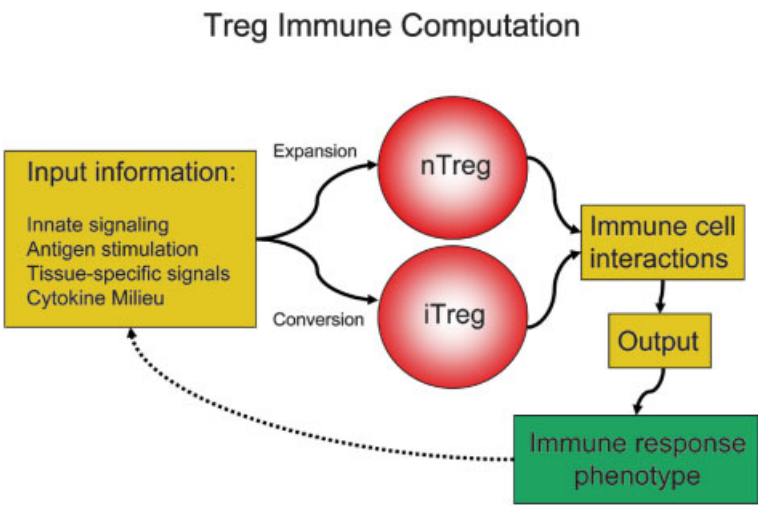

Figure 1. Treg immune computation. Computation is the conversion by a system of input signals into output signals according to organized rules. The input signals into the Treg compartment are innate signaling, antigen stimulation, tissue-specific signals and cytokine milieu. This input information activates and expands Treg developed in the thymus (natural Treg, nTreg) or generated in the periphery by conversion (induced Treg, iTreg). The activated Treg interact with various types of immune cells (both innate and adaptive) and generate an organized output – the immune response phenotype. The immune response feeds back on the input information to regulate the numbers and behaviors of the Treg cells. In this way, Treg function as important elements in the dynamic and mutual adjustment of the state of the immune system to the state of the body.

and the immune system [36]. Most telling is the recent observation that HSP60 and one of its defined peptides at relatively low concentrations can signal T_{reg} *via* TLR2 to enhance their down-regulation of T effectors [37]. Here we have an example of the integration of a tissue state biomarker (HSP60) with an innate receptor (TLR2) on an adaptive immune cell (peripheral T_{reg}), in which the outcome of this signaling is to adjust the level of T_{reg} action on T effectors and other immune system cells.

T_{reg} never rest

The above observations can help us respond to Rolf Zinkernagel's skepticism regarding the function of T_{reg} in immune homeostasis. T_{reg}, in contrast to other physiologic regulators, do not need to know the ideal set-point of the system; the immune system, we propose, operates without a set-point [8]. The immune system, like the brain, is a reactive system [38]; both systems continuously operate to adjust themselves to the ever-changing state of the body. The live brain has no set-point at which it rests; a brain at rest (in homeostasis) is a dead brain. Likewise, we see the immune system as continuously active in adjusting the inflammatory response to the changing states of body cells and tissues [11, 36]. Physiological inflammation usually goes unnoticed; physiological inflammation is subclinical. Nevertheless, healing every cut, bruise, broken bone or blood vessel, apoptosis of abnormal cells, removal of accumulated waste molecules, processes of angiogenesis and regeneration, all need highly regulated inflammatory processes — both innate and adaptive — for their proper repair and maintenance. Inflammation is not a burden, but is essential for health. Immune rejection of infectious agents, which also involves inflammation, is only one aspect of immune body maintenance. The immune system, the conductor and virtuoso of inflammatory harmony, never rests on any set-point. Like the brain, the immune system just reacts to the states of self and the environment. Obviously, the type of immune reaction has to suit the state of the body; infections have to be cured, wounds have to be healed, scars have to be fashioned, and so forth and on. Reactive regulation, even without a set endpoint, has to be carefully regulated [11]. Note, however, that the regulation of a reactive system is much more complex than is the regulation of a simple feedback system such as a thermostat. Reactive regulation is more like a form of computation.

T_{reg} and immune computation

Elsewhere we have described ongoing immune reactivity as a form of immune computation in which the state of the body is transformed into a suitable state of the immune response at any particular time and site [36]. In immune computation, molecular signals from an infection or injury, for example, serve as the input; the output is a dynamic immune response suitable to the evolving situation. The immune system thus can be likened to a biological Turing Machine in which the state of body cells and tissues is the input information and the state of the immune response is the computed output information [36]. It should be clear that T_{reg} are a major element in immune computation; both the development and function of T_{reg} is very much influenced, as we have cited, by the state of the immune system and by signals emanating from the body – hormones, growth and differentiation factors, tissue-specific signals, and markers of cellular differentiation.

T_{reg} contribute to immune computation at several levels: The conversion of T cells into T_{reg} involves the integration of diverse information, both innate and adaptive, from the tissues and the immune system; at this level, T_{reg} assemble and concentrate input signals into the immune system. Once they differentiate into functioning cells, T_{reg} recognize antigens and respond to innate signals (HSP60 *via* TLR-2, for example) to express TGF-β or IL-10 and other molecules that down-regulate or influence the behavior of effector T cells, DC, and other immune elements; at this level, T_{reg} contribute to the output signals of immune computation (Fig. 1). From this point of view, T_{reg} are not merely the minus or negative agents of a simple binary effector-regulator balance; T_{reg} are complex versatile elements in the ongoing management of inflammation and body maintenance (Fig. 1).

Closing remarks

If indeed the immune system is a reactive system that functions to compute the state of the body [36], then it should be clear that T_{reg} are not the only immune regulators; all of the elements of the immune system, innate and adaptive, share in the task of sensing the needs of the body and converting that input into an appropriate immune response output. From the computational perspective, even T effectors can be viewed as regulators, and T_{reg} can be viewed as effectors, too. In this matter, Rolf Zinkernagel, at least to our mind, is right [1, 2]: T_{reg} cannot know ahead of time an ideal set-point for immune homeostasis; no such information is needed.

Conflict of interest:
The authors declare no financial or commercial conflict of interest.

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