Role of Regulatory T-cells in Oral Tolerance and Immunotherapy

Mark Farrugia and Byron Baron*
Centre for Molecular Medicine and Biobanking, Faculty of Medicine and Surgery, University of Malta, Msida, MSD2080, Malta

Abstract
Food allergies encompass a range of disorders ranging from being an inconvenience to even causing fatalities, mainly due to anaphylaxis. A large number of individuals are affected and this presents great health and economic implications. However as yet, apart from dietary avoidance, effective treatment strategies are practically non-existent. The immune environment related to allergen-tolerance is highly complex and the role of regulatory T-cells in allergen-specific tolerance, their interaction with other cells in inflamed tissues, and their role in antibody regulation have been demonstrated in several studies. Regulatory T-cells are able to control acquired immunity and achieve oral tolerance to food allergens. Immunotherapy for food allergies focuses on desensitisation by increasing the allergen reactivity threshold. So far, the only long-term curative treatment used effectively is allergen-specific immunotherapy which involves the administration of increasing doses of the causative allergen, such that a state of allergen-specific immune tolerance is induced over the course of the treatment. This review covers various forms of allergen-specific immunotherapy, focusing on the role of regulatory T-cells in such therapies, and includes a number of small studies providing ideas for future work in the area.

Keywords: Regulatory T-cells; Immunotherapy; Immune tolerance; Oral tolerance

Abbreviations: AIT: Allergen-specific Immunotherapy; APCs: Antigen-Presenting Cells; CCR: C-C Chemokine Receptor; CSR: Class Switch Recombination; CD: Cluster of Differentiation; CTLA-4: Cytotoxic T-Lymphocyte-Associated Protein 4; DCs: Dendritic Cells; EPIT: Epicutaneous Immunotherapy; FPIES: Food-Protein-Induced Enterocolitis Syndrome; SPT: Skin Prick Test; OFC: Oral Food Challenge; ELISAs: Enzyme-Linked Immunosorbent Assays; FOXP3: Forkhead Box P3; GITR: Glucocorticoid-Induced Tumour Necrosis Factor Receptor-Related Protein; HKLM: Heat-Killed Listeria monocytogenes; HR1/2: Histamine Receptor 1/2; Ig: Immunoglobulin; iNKT: Inducible T regulatory 1; IFN-y: Interferon-Gamma; IL-10: Interleukin-10; ITAM: Natural T regulatory 1; NO: Nitric Oxide; OIT: Oral Immunotherapy; PBMC: Peripheral Blood Mononuclear Cell; PD-1: Programmed Cell Death Protein-1; Breg: Regulatory B-cells; Treg: Regulatory T-cells; SCIT: Subcutaneous Immunotherapy; SLIT: Sublingual Immunotherapy; T cells: T-Helper Cells; TGF-β: Transforming Growth Factor-β; IL22: Type 2 Innate Lymphoid Cells

Introduction
Food allergies are defined as an immunologically-mediated adverse reaction to particular foods, most commonly milk (mostly in children), eggs, nuts (including peanuts, walnuts and brazil nuts), wheat and other grains with gluten (including barley and oats), fish and shellfish (mostly in adults). Depending on the type of allergy and the severity, a range of disorders may arise including Immunoglobulin E (IgE)-mediated anaphylaxis, food-protein-induced enterocolitis syndrome (FPIES), and other gastrointestinal disorders such as vomiting, reflux, abdominal pains, diarrhea or constipation [1]. Food allergies are quite widespread and affect approximately 5% of adults and 8% of young children in countries with a Western lifestyle [2,3]. Food allergy is one of the most common causes of anaphylaxis that may lead to fatalities. Studies in the United States and United Kingdom showed that the number of hospitalisations for food-induced anaphylaxis has increased more than 3-fold in the past decade [4]. The health and economic effect of food allergies is not only reflected in the cost of healthcare expenditures, but also in the effect it has on the workplace, food industry, food regulatory agencies, and, most importantly, patients and their families, whose lives are affected daily.

Food allergies are generally diagnosed by means of a skin prick test (SPT) or blood tests, although the oral food challenge (OFC), or elimination diet may also be used as highly specific diagnostic tests. Both the SPTs and blood tests measure the presence of IgE, the antibody that triggers food allergy symptoms. SPTs are the preferred method of testing because they are inexpensive, produce results in under an hour, and can be performed in the clinic. On the other hand, blood tests consisting of enzyme-linked immunosorbent assays (ELISAs) have a higher cost and do not provide immediate results but are much more accurate and quantitative.

The only validated remedy for food allergies is the identification and elimination of the foods responsible [5], and the use of self-injectable epinephrine to reverse the acute and severe allergic reaction [1]. However, it is not easy to totally avoid contact with food allergens as these tend to be used in most food manufacturing processes. Therefore, developing effective treatment strategies outside of dietary avoidance of antigens has been a high priority for research teams in recent years.

Oral Tolerance
Food allergies can be induced by both IgE-mediated and non-IgE-mediated pathways. In the case of IgE-mediated food allergies, food-specific IgE antibodies are produced after exposure to particular food allergens that then bind to the Fc receptors on mast cells, basophils, and macrophages [6]. Mediators from the activated cells are released and result in local or systemic symptoms immediately. These mediators may attract other cells, like eosinophils and lymphocytes, to prolong the inflammation [7].

*Corresponding author: Byron Baron, Centre for Molecular Medicine and Biobanking, Faculty of Medicine and Surgery, University of Malta, Msida, MSD2080, Malta, Tel: 00356 21639913; E-mail: byron.baron@um.edu.mt

Received January 28, 2016; Accepted February 09, 2016; Published February 16, 2016


Copyright: © 2016 Farrugia M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Exposure to a food antigen also generates a regulatory T-cell (T\textsubscript{reg}) response that can suppress allergic sensitisation to that food allergen. The adoptive transfer of T\textsubscript{reg} cells was shown to prevent or cure several T-cell-mediated diseases, including allergy, asthmatic lung inflammation, and autoimmune diseases by restoring immune tolerance to allergens, self-antigens, or alloantigens in animal models, and this has demonstrated the pivotal role of T\textsubscript{reg} cells in inducing and maintaining immune tolerance [8]. Hadis et al. [9] showed that oral tolerance could suppress experimental food allergy through the development of antigen-specific forkhead box P3 positive (FOXP3\textsuperscript{+}) T-cells in mice. In humans, antigen-specific cluster of differentiation (CD) 25\textsuperscript{+} FOXP3\textsuperscript{+} T\textsubscript{regs} are associated with the onset of clinical tolerance to milk [10]. Tolerance is initiated by dendritic cells (DCs) residing in the gastrointestinal lamina propria. CD103\textsuperscript{+} DCs capture antigens in the lamina propria, migrate, and initiate oral tolerance in the draining lymph node by activating antigen-specific T\textsubscript{reg} cells that then migrate back to the lamina propria.

**Subsets of T\textsubscript{regs}**

There are at least five subsets of T\textsubscript{reg} identified so far. This makes T\textsubscript{reg} one of the most complicated and diverse T-cell groups. More novel subsets of T\textsubscript{reg} will surely be discovered. All subsets discovered so far are derived from naïve T-cells and develop under different conditions. For the purpose of this review we shall only focus on those subsets that are directly linked to the food allergy sensitisation scenario.

The largest subset of T\textsubscript{reg} is the so-called natural T\textsubscript{reg} (nT\textsubscript{reg}). These are CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+} cells which secrete Interleukin-10 (IL-10) and Transforming Growth Factor-β (TGF-β). These T\textsubscript{reg} originate from the thymus in response to self-antigens [11]. Two mechanisms have been invoked to describe the function of these T\textsubscript{reg}, a contact-dependent mechanism in which membrane-bound TGF-β blocks T-cell proliferation and a contact-independent mechanism involving soluble TGF-β and IL-10 [12]. These nT\textsubscript{reg} have a lot of roles in allergen-specific immune reactions (Figure 1). These include the suppression of dendritic cells (these support the generation of effector T-cells), the inhibition of the production of allergen-specific IgE, the inhibition of both function and migration of effector T\textsubscript{h}1, T\textsubscript{h}2, and T\textsubscript{h}17 cells, the induction of IgG4 secretion and also the suppression of mast cells, basophils, and eosinophils [13]. It is important to keep in mind that IgG4 represents a non-inflammatory Ig isotype that does not activate complement (a system that mediates the specific antibody response) and is thought to block the activation of the more severe IgE [14].

Another type of T\textsubscript{reg} is inducible T\textsubscript{reg} (iT\textsubscript{reg}), and these are peripherally-induced T\textsubscript{reg}, not produced in the thymus. Naïve CD4\textsuperscript{+} T-cells in the periphery are induced to express the FOXP3 transcription factor in response to foreign antigens [11] and these cells develop a suppressive function similar to nT\textsubscript{reg} [15], including the production of IL-10 and TGF-β.

There are also CD4\textsuperscript{+} T-cells that although do not express the FOXP3 gene (which was until recently used as a definitive marker for T\textsubscript{reg}) still
secrete IL-10 and suppress effector functions of T helper cells (T\(^h\) cells). These cells therefore still qualify as regulatory cells and are known as Tr1 T\(_{reg}\) [16]. Tr1 cells suppress effector T-cell responses by multiple mechanisms that depend on IL-10, TGF-β, programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [17], and histamine receptor 2 (HR2) [17,18].

The differential regulation of T\(_1\) and T\(_2\) cells by histamine is due to the presence of different receptors, as human CD4\(^+\) T\(_1\) cells predominantly express histamine receptor 1 (HR1), while CD4\(^+\) T\(_2\) cells predominantly express histamine receptor 2 (HR2) [19]. Histamine induces the production of IL-10 by DCs [20] and T\(_2\) cells [18,21] as well as enhances the suppressive activity of TGF-β on T-cells [22], mediated through HR2, suppressing IL-4 and IL-13 production and T-cell proliferation [18,19] This suggest that HR2 acts as a critical receptor in the peripheral tolerance to allergens.

It is also reported that in human peripheral blood and lymphoid tissue (but not in the thymus) there also exist CD4\(^+\)FOXP3\(^+\) T-cells that also express the CCR6 gene (which is translated into the C-C chemokine receptor type 6 protein) and are also able to produce IL-17 upon activation [23]. The CCR6\(^+\) IL-17-producing FOXP3\(^+\) T\(_{reg}\) strongly inhibit the proliferation of CD4\(^+\) effector T-cells. A recent report however shows that IL-17-producing FOXP3\(^+\) T\(_{reg}\) are a new crossover immune cell population that could be converted from T\(_{reg}\) to T\(_{17}\) cells, and thus associated with a decreased suppressive function of T lymphocytes [24].

Recently another subset of T\(_{reg}\) has been discovered that are induced by Nitric Oxide (NO), and fittingly these are called NO-T\(_{reg}\). NO-T\(_{reg}\) are distinct from other T\(_{reg}\) subsets in that apart from lacking the expression of FOXP3, they also express Glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR) and are CD27\(^+\), thus having a T-helper phenotype while still maintaining suppressive properties against effector cells, mainly through the production of IL-10 [25]. IL-10 is the only detectable cytokine produced by NO-T\(_{reg}\).

It has been repeatedly shown that all subsets of T\(_{reg}\) coexist and overlap in many immune tolerance-related situations in humans, including allergies. T\(_{reg}\) share major non-lymphoid tissue trafficking receptors, such as C-C chemokine receptor type 4 (CCR4), CCR5, CCR6, CXCR3, and CXCR6, with T\(_{17}\) cells [26]. This implies that these T-cells migrate to and within lymphoid tissue. The mechanisms used by T\(_{reg}\) cells to suppress a large number of target immune cell types can be broadly divided into two: those that target T-cells (by means of suppressor cytokines, IL-2 consumption, and granzyme/perforin-induced cell death pathways) and those that target antigen-presenting cells (APCs) (by inhibiting antigen presentation or down-modulating the expression of CD80 and CD86) [27].

### T\(_{reg}\) Action in the Allergy Scenario

The suppressive actions of T\(_{reg}\) on other immune cells, including effector T-cells, B-cells, DCs and mast cells, may shed light on the complex nature of how T\(_{reg}\) are able to control acquired immunity and achieve oral tolerance to food allergens. Figure 2 summarizes the effects of T\(_{reg}\) on other immune cells involved in allergen-immunity.

The T\(_{reg}\) suppressive nature seems to focus a lot on affecting the activity of other effector T-cells derived from CD4\(^+\) cells, mainly the T\(_h\) cells. T\(_h\) cells are thought to differentiate into three main subsets, which are T\(_1\), T\(_2\) and T\(_17\) effector cells. T\(_1\) are Interferon-gamma (IFN-γ) T-cells, T\(_2\) cells produce IL-4 and IL-5, while T\(_17\) secrete IL-17 [28]. These three T\(_h\) cell types are all responsible in invoking an immune response resulting in an allergic reaction, and are kept in check by T\(_{reg}\) in healthy non-allergic individuals. TGF-β-producing T\(_{reg}\) inhibit T\(_1\) cell differentiation and instead promote T\(_17\) cell differentiation [29]. However, NO-T\(_{reg}\) inhibit T\(_17\) cell production but not T\(_1\) cell differentiation and function [25]. T\(_{reg}\) can also selectively inhibit IFN-γ synthesis [30]. It is important to note however that the nature of T\(_{reg}\) and their effects on other T-cells in vivo is still under debate and several experimental factors might play a role in different results.

FOXP3\(^+\) T-cells have been known to also affect B-cell function. They have been discovered to exist in the T-B area borders and within germinal centres in secondary lymphoid organs (the areas where B-cells interact with T\(_h\) cells and undergo Ig production) [31]. It was also shown that T\(_{reg}\) can suppress B-cells without needing to suppress

---

**Figure 2:** Schematic representing the response caused by an allergen, inducing the production of T\(_{reg}\), which in turn alter numerous components of the immune system. These include the suppression of inflammatory dendritic cells, T\(_1\), T\(_2\) (mast cells, basophils, and eosinophils), and T\(_17\) cells as well as allergen-specific IgE through B\(_{reg}\), with the concomitant induction of IgG, IgA and IL-10 secreting dendritic cells.
The development of food allergies tends to be the result of a deregulation of immune tolerance, which can develop against any immune-activating substance, and is known to be mediated by multiple mechanisms. It is generally characterised by an altered allergen-specific memory T- and B-cell response [42-45], consequent to the induction of a type 2 immune response that includes T<sub>2</sub> cells and type 2 innate lymphoid cells (ILC2s), together with the production of allergen-specific IgE antibodies and increased eosinophil numbers in the affected tissues and sometimes in peripheral blood [46].

Generally in healthy individuals, T-cells do not show any proliferative response to allergens in peripheral blood mononuclear cell (PBMC) cultures. This can be due to a low frequency of specific T-cells because of a lifetime lack of exposure. Moreover, if a detectable allergen-specific T-cell response is mounted in a non-allergic individual, active suppression against allergens takes place in cultures by Tr1 cells or CD4<sup>+</sup>FOXP3<sup>+</sup> T<sub>reg</sub> cells [17,18,47,48].

The prevention of sensitisation to new antigens [49] and prevention of progression to a more severe allergic state, are the major clinical implications of immune tolerance. The main aim of immunotherapy for food allergies is desensitisation by increasing the allergen reactivity threshold in subjects receiving the immunotherapy and retention of this increased reactivity threshold after the therapy has been discontinued. So far, the only long-term curative treatment used effectively is allergen-specific immunotherapy (AIT), which involves the administration of increasing doses of the causative allergen, such that a state of allergen-specific immune tolerance is induced over the course of AIT treatment.

The concept of using AIT for food allergy has long been tested through several studies. Current drug development and therapeutic strategies exploit T<sub>reg</sub> control of allergen-specific immune responses to induce a tolerant state in peripheral T-cells, showing potential for preventive therapies and cures for allergic diseases. The aim is to generate allergen-specific T<sub>reg</sub> cells and suppress the proliferative and cytokine responses against the major allergen [50]. The basis of AIT, similar to treatment with glucocorticoids or b2-agonists, promotes the numbers and activity of IL-10-secreting Tr1-like cells [24,51,52]. The signaling of T<sub>reg</sub> cells in AIT is initiated by the production of IL-10 and TGF-β by the antigen-specific Tr1 cells [29,53,54]. However, since these cells are CD4<sup>+</sup> and CD25<sup>+</sup>, it is still unclear whether these are inducible Tr1 cells upregulating CD25 or naturally occurring CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> cells that produce suppressive cytokines [55]. It has been shown that circulating CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> cells and IL-10- and TGF-β-secreting Tr1 cells represent overlapping populations in adults. Moreover, it has been shown that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells from atopic donors are less effective at suppressing the proliferation of CD4<sup>+</sup> CD25<sup>+</sup> T-cells after allergen AIT [29,32]. As a consequence, it has been suggested that upregulation of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells plays a role in allergen AIT.

In the 1980s, subcutaneous immunotherapy (SCIT) was tested for peanut allergy. SCIT is a mode of therapy that has been proven effective and safe for the treatment of allergies to environmental factors and insect stings [56,57]. These trials had shown the efficacy of SCIT against peanut allergy, however there was an unacceptably high rate of severe allergic reactions [58]. Other routes of immunotherapy have therefore been investigated, although no such methods are yet ready for routine clinical practice [59].

Oral Immunotherapy (OIT) is the most studied immunotherapy treatment so far and is the method which shows the greatest efficacy for the treatment of food allergy. This method invokes an immune response to antigens that are delivered orally, and the subject gradually gains immune tolerance mediated by regulatory T-cells. The presumed mechanism of action for OIT is that of the activation of gut mucosal
dendritic cells, which in turn affect the allergic response through immunomodulation of tissue and circulating effector cells [60]. This immunomodulation is done via the interaction of T<sub>reg</sub> with effector T-cells, as previously explained. Other mechanisms which are presumed to be important include the modulation of IgE responses, polyclonal increases in specific IgG4 levels [61], as well as suppression of basophils through an IgE receptor pathway [62].

Although at the moment OIT is the most researched of any immunotherapy treatments for food allergy, yet to date there is still limited evidence of long-term follow-up of subjects after OIT, with mixed results on the achievement of long-term tolerance. In 2013, it was demonstrated for the first time that sustained unresponsiveness developed in half of the subjects with peanut allergy able to complete treatment after years of OIT, and it was still not entirely proved that this was due to OIT [63]. Another report issued in 2013 assessing the long-term follow-up of cow's milk immunotherapy concludes that long-term outcomes after cow’s milk immunotherapy are mixed, with some patients losing desensitisation over time and not more than a third of the subjects in these studies tolerating a full servings of cow’s milk without symptoms [64]. The conclusion of these reports and similar investigations agree that only a small fraction of those starting treatment achieve long-term tolerance, and therefore the need to develop better therapies for treatment of food allergies is evident.

Even though OIT seems to be the most promising regarding its efficacy, safety is a major concern with the oral route since it is also the route that normally leads to food-induced allergic reactions. Sublingual immunotherapy (SLIT) and epicutaneous immunotherapy (EPIT) were thus proposed as alternative routes that could have a significantly better safety profile yet still retain the ability to induce tolerance.

The APCs present in the sublingual environment induce T<sub>reg</sub> similar to those of the intestinal tract [65], whilst the limited antigen dose applied through this route improves the safety of these trials [66]. This improvement in safety comes at the price of being less effective than OIT, although some groups report promising efficacy with SLIT for treatment of peanut allergy [67,68]. The suggested mechanism of how SLIT affects the T<sub>reg</sub> cells and subsequently modulates the T<sub>1</sub>/T<sub>2</sub> balance is that of allergen interaction with protophagocytic Langerhans cells in the oral mucosa, and these in turn lead to downregulation of the allergic response [69] (Figure 2).

The findings observed in SLIT seem to be similar to those seen with injection AIT. Increases in serum allergen-specific IgG4 levels [70,71], decreases in allergen-stimulated T-cell proliferation [72], induction of IL-10 in T-cells [72-74], suppression of T<sub>2</sub> cells [73], and decreased eosinophilia and eosinophil migration in response to allergen challenge [75,76] have been reported. However, a significant number of studies have not detected any immunologic changes [70,77]. In a recent study after 4 weeks of SLIT, higher frequencies of circulating CD4<sup>+</sup>CD25<sup>+</sup> cells were detected together with increased FOXP3 and IL-10 and reduced IL-4 and IFN-γ mRNA expression compared with expression seen before SLIT [74]. Proliferation to all 3 antigens was markedly reduced but increased significantly after depletion of CD25<sup>+</sup> cells or addition of anti-IL–10 antibodies. Neither TGF-b levels nor cell-cell contact–mediated suppression of CD4<sup>+</sup>CD25<sup>+</sup> cells changed during the course of SLIT.

EPIT compares to the other methods described in that its delivery of allergen is done to the skin surface through the application of an allergen-containing patch. This activates the skin Langerhans cells, and these then migrate to lymph nodes and eventually downregulate the effector cell responses by activating IT<sub>reg</sub> [78,79]. These induced T<sub>reg</sub> are thought to be able to disperse to a wider range of target areas in subjects when compared to OIT and SLIT. Pre-clinical studies in mice have shown that EPIT leads to the suppression of allergic inflammation in the lung and also in the gastrointestinal tract, with reduction of IgE, enhancement of IgG, and suppression of T<sub>2</sub> effectors responses [80]. In mice, it was observed that the application of the antigen to non-damaged skin led to cutaneous dendritic cells to acquire the antigen and promote the development of T<sub>reg</sub> [81].

Future Work

So far, most immunotherapy trials which have been done, focus on the use of allergens-mediated immunotherapy mechanisms. Although they are promising, their safety profile makes it difficult for more rigorous and widespread testing. Using recombinant technology, food allergens can not only be produced in large quantities with standard quality, but the IgE-binding epitopes of such recombinant proteins can be further modified. Food antigens have already been modified by adding sugar structures that allowed binding to the receptor SIGNR1 on gastrointestinal DCs, and this enhanced tolerance through induction of IL-10-producing T<sub>reg</sub> [82], presenting a potential future approach for immunotherapy. Other modifications may include for example site-directed mutagenesis to reduce the allergenic power of these proteins.

In addition to humoral immunity, allergen-specific T-cells, especially T<sub>reg</sub> themselves, also play an important role in allergy and are another therapeutic target. Synthetic peptide-based vaccines have been developed and clinically evaluated [83-86]. Mixtures of short peptides demonstrated downregulation of systemic T<sub>1</sub> and T<sub>2</sub> cell responses to allergen [83], together with concomitant induction of IL-10 production [86]. Studies of immunotherapy using synthetic peptides containing immuno-dominant T-cell epitopes from an allergen have shown that this can induce T-cell non-responsiveness [87]. In these studies it was shown that upon the exposure of the epitope, IL-4 and secretion of IgE and IgG1 was reduced, while CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells increased, coupled with an increase in IL-10 [88]. Further studies should investigate whether T<sub>reg</sub> can be used therapeutically once sensitisation has already been achieved.

There are also ideas of using DNA vaccines and other forms of gene therapy for allergies, but at the moment they are still in their infancy and none seem to be targeting T<sub>reg</sub> specifically yet [89]. However, there have been other novel approaches which are intended to modulate the T<sub>reg</sub> in immunotherapy, including the introduction of adjuvants such as heat-killed Listeria monocytogenes (HKLM), CpG motifs, and mannoside used with modified allergens during immunotherapy, which were showed to enhance the type 1 helper T-cells and/or regulatory T-cell responses [90]. There was also a recent study on airway allergic inflammation that involved the use of recombinant DNA immunotherapy to introduce bacterial HSP in the subject, and this led to an increase in T<sub>reg</sub> activity [91]. Such studies, although not directly tested in the food allergy scenario, might shed some light on new approaches of how to stimulate further the T<sub>reg</sub> activity in a non-allergen-related manner.

References


