



Novel mechanisms in immune tolerance to allergens during natural allergen exposure and allergen-specific immunotherapy

Willem van de Veen^{1,2}, Oliver F Wirz¹, Anna Globinska^{1,3} and Mübeccel Akdis^{1,2}

Allergen-specific immunotherapy (AIT) has been used for more than 100 years as a clinical tolerance-inducing and immune tolerance-inducing therapy for allergic diseases and represents a potentially curative method of treatment. AIT functions through multiple mechanisms including early desensitization of basophils and mast cells, regulating T-cell and B-cell responses, changing antibody isotypes, and decreasing activation, mediator release and affected tissue migration of eosinophils, basophils, and mast cells. Similar molecular and cellular mechanisms have been observed in subcutaneous AIT, sublingual AIT and peptide immunotherapy as well as natural tolerance to high doses of allergen exposure in beekeepers and cat owners.

Addresses

¹Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos, Switzerland

²Christine Kühne – Center for Allergy Research and Education, Davos, Switzerland

³Department of Immunology, Rheumatology and Allergy, Chair of Clinical Immunology and Microbiology, Medical University of Lodz, Poland

Corresponding author: Akdis, Mübeccel (akdis@siaf.uzh.ch)

Current Opinion in Immunology 2017, 48:74–81

This review comes from a themed issue on **Allergy and hypersensitivity**

Edited by Cezmi

<http://dx.doi.org/10.1016/j.coi.2017.08.012>

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Introduction

The mechanisms of allergen tolerance in humans have been studied through various modes of allergen-specific immunotherapy (AIT) and T cell epitope peptide immunotherapy (PIT) as well as the conditions by which a healthy immune response develops during high dose allergen exposure in cat owners and beekeepers [1–4]. Although the mechanisms are not fully elucidated, they include changes in the characteristics of

mast cells, basophils, eosinophils, allergen-specific memory T and B cell responses and the production of specific antibody isotypes to skew the immune response away from inflammation. Currently, subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) are used in clinical practice while several novel AIT vaccines are undergoing clinical trials [1,2]. Clinical trials employing alternative routes for AIT are being undertaken and provide encouraging results, such as the intralymphatic (ILIT) and epidermal (EIT) immunotherapy. They have been shown to reduce treatment duration, while keeping efficacy and safety in allergic rhinitis [5,6]. Peanut EIT administration is associated with a modest treatment response showing the most efficient clinical responses in younger children. AIT is effective in reducing symptoms of allergic asthma and rhinitis as well as venom-allergy with the possibility of curative change in the course of these diseases [7,8].

The complex physiopathology of allergic diseases is influenced by genetic susceptibility, route of exposure, antigen/allergen dose, time of exposure, allergen/antigen structure, and co-exposure with environmental agents, infections or commensal bacteria. Since the discovery of Th1 and Th2 cell subsets in 1986, an increasing body of evidence has been accumulated demonstrating that there is reciprocal regulation between individual Th cell subsets, such as Th1, Th2, Th9, Th17, and Th22 [9,10]. Treg cells however play a central role in the suppression of effector T cell responses in different diseases [11,12].

The type 2 immune response underlies allergic asthma, atopic dermatitis, chronic rhinosinusitis with nasal polyps and allergic rhinitis. The type 2 immune response involves Th2 cells, group 2 innate lymphoid cells (ILC2), B cells, a small fraction of IL-4-secreting NK cells, IL-4-secreting NK-T cells, basophils, eosinophils and mast cells, and their major cytokines [13]. Follicular helper T (T_{FH}) cells play a critical role in the regulation of IgE production. Exposure to airborne allergens induces IL-4-producing T_{FH} cells that are required for IgE antibody production in mice. T_{FH} and T_H2 cells as two distinct subsets of IL-4-producing CD4⁺ T cells may play additive or synergistic roles open for further research [14].

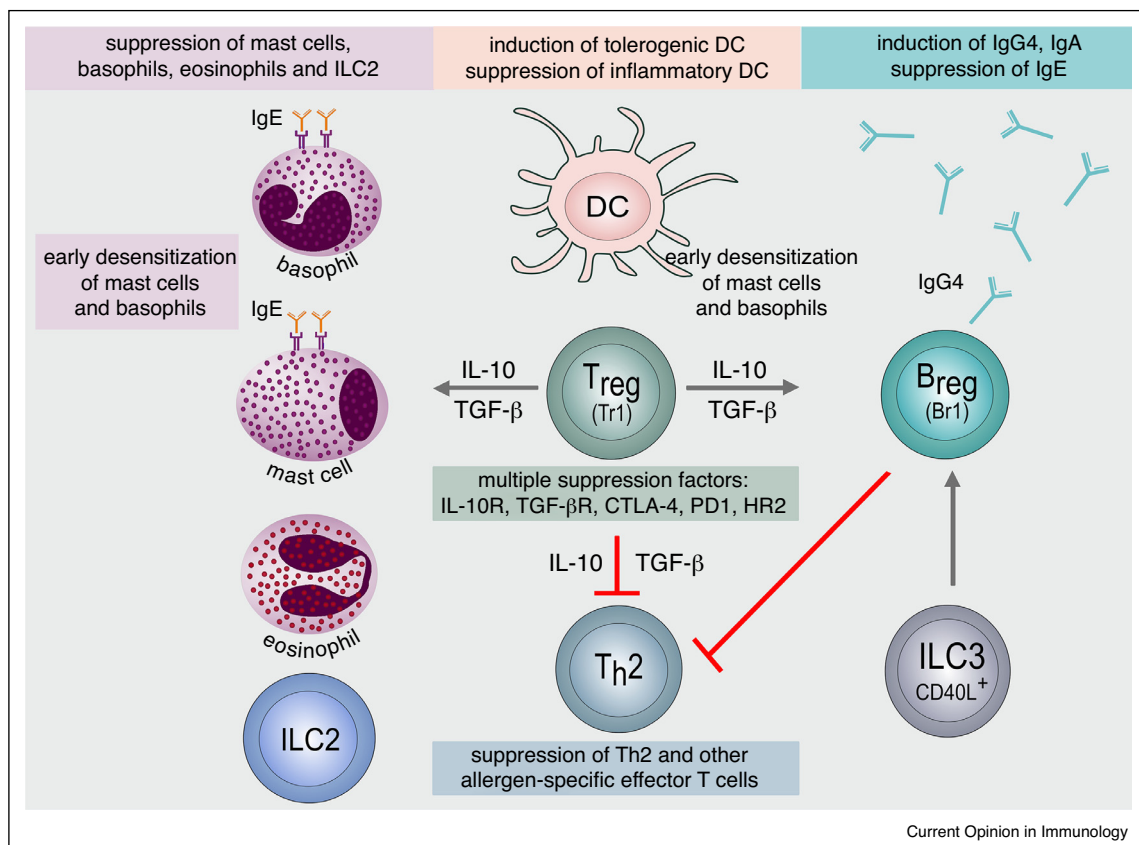
Studies on endotypes of allergic disease suggest an AIT responsive endotype

AIT is not successful in all patients, and an important unmet need is the identification and validation of biomarkers that are predictive of clinical response. Some clinical trials may have been unsuccessful in the past, because they were performed without proper stratification of patients into subgroups of 'endotypes' [15,16]. An endotype is defined by a distinct pathophysiological mechanism as a subtype of a disease condition. Whereas observable characteristic of a disease without any implication of a mechanism define phenotype of a disease [17]. The definition of an AIT responsive endotype of allergic diseases and relevant biomarkers is urgently needed for patient selection and, possibly, also for the selection of the type of vaccine or route of application.

Mechanisms of allergen tolerance

Four groups of cellular and molecular events take place during AIT (Figure 1) and multiple cell types in the blood and affected organs show these changes (Table 1). It should be noted that there is significant variation between donors and protocols. The first group of events is characterized by reduced mast cell and basophil activity and degranulation, and a decreased tendency for systemic anaphylaxis. The second group of events comprises generation of allergen-specific Treg and Breg cells and suppression of allergen-specific effector T cell subsets. The third group of events is regulation of antibody isotypes demonstrating early and continuous increase in specific IgG4 and late decrease or no change in specific IgE. The fourth group of events takes place after several months with decreases in mast cells and eosinophils and release of

Figure 1



Immune tolerance to allergens in healthy immune response and successful SIT. During successful AIT, decreases in mast cell and basophil activity, degranulation and tendency for systemic anaphylaxis take place within the first hours after the first injection as an early desensitization effect. Allergen-specific Treg (Tr1) and Breg (Br1) cells that particularly produce IL-10 are generated. The release of TGF-β and IL-10 by Treg cells may contribute to the suppression of mast cells, basophils, eosinophils, and ILCs. In addition, these cytokines inhibit Th2 cells and other allergen-specific effector T cells, induce IgG4 and IgA, and suppress IgE. Multiple suppressor factors play roles in these events, including CTLA-4, PD1, and HR2. After a few months, IgE-to-IgG4 ratio decreases substantially and decreased numbers of tissue mast cells and eosinophils and their mediators occurs. CD40L⁺ ILC3 may be involved in induction of immature translational Breg cells that produce IL-10.

Table 1**Changes in cellular responses related to the development of tolerance to allergens**

Cell type	Change in response of tolerant compared to allergic state	Reference
T cells	Decreased proliferation in response to allergen	[31,34,80]
	Increased frequency of allergen-specific IL-10-producing Tr1 cells and upregulation of suppressive molecules such as CTLA4, HR2, and PD-1	[31,34,37–39]
	Reduced frequency of Th2 cells	[34,80–82]
	Reduced numbers of tissue infiltrating T cells during LPR	[70,83]
B cells	Increased frequency of allergen-specific Br1 cells	[35]
	Upregulation of CCR5 on allergen-specific B cells	[42*]
	Reduced (specific) IgE production	[44*,60–62]
	Increased specific IgG4 and IgA production	[63–66]
Dendritic cells	Increased pDC (in tonsils)	[25]
	Development of a tolerogenic phenotype characterized by expression of IL-10, TGF- β , IL-27, and C1q	[27,28]
Eosinophils	Reduction in tissue infiltrating eosinophils	[84]
Basophils	Reduced degranulation upon Fc ϵ RI crosslinking. Possibly mediated through upregulation of H2R	[19]
	Reduced DAO release upon stimulation with relevant allergen	[20]
Innate lymphoid cells	Inhibition of seasonal increases in ILC2 cells after AIT	[55]

their mediators in the affected tissues. These changes are accompanied with decrease in type I skin test reactivity.

Early desensitization effect on mast cells and basophils

Within minutes to hours after the first injection of AIT, decreased mast cell and basophil degranulation and systemic anaphylaxis can be observed, even though all the treated individuals have high quantities of specific IgE. This effect occurs when intact allergens are used with an IgE cross-linking three-dimensional structure. Several mechanisms have been proposed and they seem similar to the one observed when these two cell types are rapidly desensitized in anaphylactic reactions to drugs [18]. The so-called desensitization effect of venom immunotherapy (VIT) was linked to rapid upregulation of histamine receptor 2 (H2R) within the first 6 hours of the build-up phase of VIT was linked to the desensitization effect.

Fc ϵ RI-cross-linking-induced activation and mediator release of basophils, including histamine and sulfidoleukotrienes, as well as cytokine production *in vitro* were strongly suppressed via triggering of H2R and increased intracellular cAMP [19]. In agreement with these findings, immunotherapy inhibits basophil histamine release. Diamine oxidase (DAO) was used as a novel functional readout of basophil histamine release after immunotherapy, suggesting a surrogate biomarker for monitoring tolerance and efficacy after immunotherapy [20].

In contrast to specific IgE, specific IgG4 has tolerance inducing roles that have been demonstrated. Specific IgE development, Th2 responses, and anaphylactic reactions have been prevented by administration of specific IgG to food allergy-prone mice. IgG and Fc γ RIIB suppress the adaptive allergic responses via effects on mast cell function and are favoring the expansion of Foxp3⁺ Treg cells [21*]. A mechanism for this effect was proposed in a study, in which Fel d 1-specific IgG antibodies were shown to interact with Fc γ RIIB, which induces internalization of IgE and inhibits mast cell activation [22]. Three murine regimens of allergen-specific immunotherapy were studied in ovalbumin (OVA)-sensitized mice, such as rapid, intermediate, or gradual protocols. Suppression of IgE-mediated mast cell activation was a common feature of all desensitization schedules, whereas prolonged desensitization schedules were required for induction of immunoregulatory mechanisms [23].

Regulatory DCs in allergen tolerance

Immature or partially mature DCs play a role in peripheral tolerance [24,25]. Maturing pDCs isolated from human tonsils have the ability to generate Treg cells [25]. The role of TLR4 and TLR8, but not TLR7 and TLR9 stimulations in breaking of allergen tolerance supports the contribution of pDCs to tolerance induction [26], which is in line with the findings supporting a key role for tonsillar pDCs in the induction and maintenance of peripheral tolerance to food and inhalant allergens. As an additional role for regulatory dendritic cells, retinoic acid-skewed dendritic cells display a tolerogenic phenotype, expressing IL-10, TGF- β , IL-27, and aldehyde dehydrogenase 1A2. IL-10 and TGF- β together drive their suppression of T_H2 cell proliferation [27]. The complement subunit C1q supports the differentiation of IL-10-secreting CD4⁺ T cells with a suppressive activity by monocyte-derived regulatory dendritic cells. C1q expression was upregulated in peripheral blood mononuclear cells of allergic patients during successful AIT [28]. In some studies these tolerance inducing DCs upregulate a suppressive ligand. A recently developed vaccine targeting dendritic cells by coupling allergoids to nonoxidized mannan enhanced allergen uptake and induced functional Treg cells through upregulation of programmed death ligand 1 (PD-L1) [29**].

T regulatory cells in allergen tolerance

Peripheral T cell tolerance to allergens is essential for a healthy immune response to high dose allergen exposure and successful treatment of allergic disorders [30]. Multiple factors contribute to the tolerant state of allergen-specific T cells such as IL-10, TGF- β , CTLA-4, PD-1, and HR2 [31–34]. IL-10 is secreted by activated and antigen-specific Treg and Breg cell populations and increases during AIT and natural allergen exposure [34–38]. Purification of allergen-specific CD4⁺ T cells that predominantly produced IFN- γ , IL-4, and IL-10 and represent Th1, Th2, and Tr1-like cells, respectively demonstrated that healthy and allergic individuals exhibit all three subsets, but in different proportions. In healthy individuals, IL-10-secreting Tr1 or IL-10-Treg cells are the dominant subset against common environmental allergens, whereas in allergic individuals allergen-specific IL-4-secreting T cells (Th2-like) exist in high frequency [34]. Therefore, a change in the dominant subset may lead to either the development of allergy or recovery. In a mouse model of chronic allergic asthma, thymic and peripherally induced Ag-specific Treg cells cooperate in AIT to ameliorate allergic asthma via IL-10 [39]. There is still a lack of consensus regarding the differences between an anergic cell and a tolerant cell is and the definitions of these cell subsets in humans need to be clarified. AIT induces allergen-specific CD4⁺ T cells to shift toward an ‘anergic’ Th2 T-cell phenotype that is missing in pretreatment patients and healthy controls. These findings show association with the induction, expansion, and maintenance of immunotherapy-specific memory and naive T-cell phenotypes even after withdrawal of AIT [40^{*}].

B regulatory cells

Similar to T helper cells, B cells can be classified into functional subsets according to the cytokines and some of the surface markers that they express. Regulatory B cells (Bregs), control excessive inflammatory responses particularly through secretion of IL-10, which inhibits proinflammatory cytokines and supports regulatory T cell differentiation [41]. As observed in Tr1 cells, recently highly purified human IL-10-secreting Breg cells (Br1) were identified [35]. Bee venom major allergen phospholipase A2 (PLA)-specific B cells were isolated from beekeepers with tolerance to bee venom antigens and allergic patients before and after AIT. High surface expression of CD25 and CD71 and low CD73 were characterizing these human IL-10⁺ Br1 cells, which potently suppressed antigen-specific CD4⁺ T-cell proliferation [42^{*}]. This indicates that T and B cell subsets, which are becoming predominant during AIT and natural antigen exposure, represent the Tr1 or IL-10-Treg cells and Br1 or IL-10-Breg cells in humans. Besides, it has been recently shown that PLA-specific B cells upregulate CCR5 expression [43], a marker expressed by Tregs that is needed for migration to inflamed tissues, in both healthy beekeepers

and patients undergoing AIT after venom exposure [42^{*}]. The IL-10 overexpressing human Breg cells demonstrated a prominent immunoregulatory profile consisting of upregulated IL-2 receptor α chain (CD25), PD-L1, suppressor of cytokine signaling 3 (SOCS3), glycoprotein A repetitions predominant (GARP) [44^{*}]. Their cytokine secretion profile was characterized by a significant reduction in proinflammatory TNF- α , IL-8, and macrophage inflammatory protein 1 α and increased anti-inflammatory IL-1 receptor antagonist and vascular endothelial growth factor in addition to IL-10. IL-10-overexpressing B cells secreted less IgE and potently suppressed proinflammatory cytokines in PBMCs, maturation of monocyte-derived dendritic cells (rendering their profile to regulatory phenotype) [44^{*}]. Mesenteric IL-10-producing CD5⁺ regulatory B cells suppress cow’s milk casein-induced allergic responses in mice via Foxp3(+) regulatory T cells and could potentially act as a regulator of food tolerance [45]. The adoptive transfer of CD9(+) B cells normalized airway inflammation and lung function by inhibiting Th2-driven and Th17-driven inflammation in an IL-10-dependent manner, restoring a favorable immunological balance in lung tissues of mice in asthma model [46].

Role of ILCs in allergy and tolerance

Populations of lymphoid cells that lack rearranged antigen receptors and markers for myeloid and lymphoid lineages that show similarities to Th1, Th2, and Th17/22 type of immune responses have been identified. These cells are defined as innate lymphoid cells (ILC) type 1, 2, and 3 cells. ILC1 mainly produce IFN- γ , ILC2 produce IL-5 and IL-13 [47], and ILC3 produce IL-17 and IL-22 [48]. ILCs control the mucosal environment through close interaction with epithelial cells and other tissue cells, production of cytokines and induction of chemokines that recruit suitable cell populations to initiate and promote distinct types of immune response development and tissue inflammation [49^{*}]. These cells can be detected in several body fluids and tissues such as, sputum, peripheral blood, nasal polyps, esophagus for their characterization in allergy and asthma patients [47,50]. ILC2 activated by epithelial cell-derived cytokines produce high levels of IL-13 and IL-5 enhancing mucus production and eosinophilia [51] and can promote IgE production [52^{*}]. Several recent studies have demonstrated a role for ILCs in exacerbation of allergic disease and their regulation during AIT [53,54]. Seasonal increases in peripheral ILC2s are inhibited by subcutaneous grass pollen immunotherapy [55]. Low doses of allergen were sufficient to prime ILC2s and Th2 cells, but did not induce antibody responses. Apparently, for natural sensitization cumulative exposure to small amounts of allergen that induces both Th2 and antibody responses is required [56].

Recently, a mutually beneficial relationship between ILC3s and B cells was demonstrated. ILC3s and Breg cells were in close connection with each other in human

palatine tonsils. CD40L⁺ILC3s provided innate B cell help, and were involved in an innate immunoregulatory mechanism by the induction of immature transitional Breg cell differentiation. This mechanism may contribute to the maintenance of the immune tolerance that may become insufficient in allergic diseases [57].

Specific-IgE and IgG4 regulation during AIT

Increases in serum allergen-specific IgE levels in response to natural allergen exposure, which can be observed in pollen-allergic patients during pollen seasons, is prevented by AIT [58,59]. Similarly, AIT often induces a transient increase in serum specific IgE in the first weeks and months followed by gradual decrease over years of continued treatment [60–62]. This late decrease in serum IgE is relatively small and poorly correlates with clinical improvement after AIT and cannot account for the diminished responsiveness to specific allergen due to AIT.

Data from several studies indicated that increases in specific IgG4 levels accompanied clinical improvement, but it was never demonstrated as a biomarker [63–67]. This research was particularly handicapped because IgG4 does not exist in mice. IgG4 plays a blocking antibody role *in vitro*, suggesting that the allergen can be captured, before reaching the mast cell-bound and basophil-bound IgE, and thus preventing their activation. Allergen-induced release of inflammatory mediators from basophils and mast cells, IgE-facilitated allergen presentation to T cells, and allergen-induced boost of memory IgE production during high dose allergen exposure during pollen season have been linked to blocking IgG type antibodies. In a study using well defined recombinant allergen mixtures, all treated subjects developed strong allergen specific IgG1 and IgG4 antibody responses [68]. Some patients were not showing IgE and IgG4 against Phl p 5 at the start of AIT-demonstrating that they were not initially sensitized to Phl p 5. After receiving 5 recombinant allergen containing AIT that includes Phl p 5, they developed strong IgG4 antibody responses to that allergen without IgE supporting the protective immune tolerance inducing role of AIT. In a recent study intralymphatic immunotherapy (ILIT) with modular allergen transport (MAT) linked to Fel d 1 elicited high levels of total IgG that were maintained for at least 12 months, whereas IgE levels remained constant [69]. Interestingly, a strong increase in IgG4 and some increase in IgG2 were observed during the study, however Fel d 1-specific IgG1 and IgG3 were not stimulated by MAT-Fel d 1 ILIT.

IL-10 is increasingly secreted from Tr1 and Br1 cells during AIT, and may counter-regulate antigen-specific IgE and IgG4 antibody synthesis [31–33,35]. Recently, it was demonstrated that IgG4 production was selectively upregulated in human Br1 cells. B cells specific for the major bee venom allergen phospholipase A2 isolated from

AIT-receiving patients and nonallergic beekeepers showed increased expression of IL-10 and IgG4. IL-10 was demonstrated to upregulate IgG4 and suppress IgE in several *in vitro* models [33]. Therefore, multiple roles of IL-10 in immune tolerance include skew of allergen-specific Ab isotype response from an IgE-dominated to an IgG4-dominated phenotype [31].

Suppression of mast cells, basophils, eosinophils, and mediators

Long-term AIT is associated with significant reduction of the immediate response to allergen provocation and the late phase reaction (LPR) in the nasal and bronchial mucosa or the skin. The mechanism of LPR is different from mast cell-mediated immediate reaction and involves the recruitment, activation and persistence of eosinophils and activation of T cells at the sites of allergen exposure. The immunopathologic changes in the mucosal tissues of subjects chronically exposed to inhalant allergens resemble those seen during the LPR. The effect of AIT on the LPR seems to be relevant to its clinical efficacy, because it mimics the pathologic condition of chronic allergic inflammation associated with increased bronchial and nasal hyperresponsiveness [70]. Bronchial, nasal, and conjunctival hyperreactivity and underlying mucosal inflammation decrease after AIT and correlate with clinical improvement [71,72].

AIT regulates the thresholds for basophil and mast cell activation and decreases IgE-mediated histamine release [73,74]. Chemotactic factors for eosinophils and neutrophils and decreased plasma levels of eosinophil cationic protein (ECP) correlate with decreased bronchial hyperreactivity and clinical improvement in birch pollen AIT [71]. Mast cells and basophils require T cell cytokines for survival, priming and activity, which are not anymore provided by suppressed Th2 cells. These functions are also directly or indirectly suppressed by activated Treg cells [75,76] and many cytokines and mediators of these cells have been shown to be inhibited by IL-10 [77–79].

Conclusion

Immune tolerance to allergens is essential to develop a healthy immune response to allergens in highly exposed individuals during AIT and natural exposure. Multiple mechanisms and receptors play a role in allergen tolerance, such as IL-10, TGF- β , HR2, PD-1, and CTLA-4. Recently, a role for innate lymphoid cells has been demonstrated in allergen tolerance. It is highly plausible that development of biomarkers to select AIT-responsive individuals will be essential for designing future therapies. Similar cellular and molecular mechanisms are apparently utilized in AIT-based prevention of allergic disease. The development of advanced vaccines with less side effects, usage of novel adjuvants with immune tolerance inducing capacity as well as discovery of reliable

biomarkers to select responder patients will be defining the future.

Conflict of interest

WV, OW, AG, and MA declare they have no competing interests.

Acknowledgements

The authors' laboratories are supported by Swiss National Science Foundation Grant No. 320030-159870, Sean Parker Asthma and Allergy Center, Stanford University, USA.

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Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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