Understanding the Roles of the Immune System in Cow’s Milk Allergy

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1 Introduction

Cow’s milk allergy (CMA) is one of the most commonly reported food allergies in infancy (Prescott et al. 2013). Incidence of CMA peaks during early childhood and tends to recede later, with the reported prevalence to be 0.6–2.5% in pre-schoolers, 0.3% in older children and teens, and less than 0.5% in adults (Fiocchi et al. 2010). A recent study performed in Europe supports these findings by demonstrating the overall incidence of challenge-proven CMA of 0.54% (Schoemaker et al. 2015). The self-perceived prevalence is unfortunately higher: 1–17.5% in pre-schoolers, 1–13.5% in older children and teens, and 1–4% in adults (Fiocchi et al. 2010; Jarvenpaa et al. 2014). This imposes dietary restriction on the CMA-suspected subjects, impairing the quality of life of both children and family, impeding children’s growth and causing unnecessary health care cost (Koletzko et al. 2012). The repetitive exposure to cow’s milk proteins in patients with persistent CMA could result in chronic allergic inflammation accompanied along with anatomical and physiological defects, such as eosinophilic gastroenteropathies (Galli et al. 2008; Leung et al. 2013). Of note is the phenomenon of atopic march, in which CMA patients (particularly the persistent cases) are having substantial risk to suffer from respiratory allergies, such as asthma, in their later life (Sprikkelman et al. 2000; Sampaio et al. 2005). It is therefore important to understand the CMA pathogenesis in order to correctly diagnose and to effectively prevent and manage the disease and its later life
By definition, CMA is a repeated, immune-mediated aberrant reaction to certain proteins within cow’s milk. These proteins, which are harmless food ingredients, comprise of αS1-casein (Bos d 9), β-lactoglobulin (Bos d 5) and others (Table 1). CMA can be mechanismically classified into: 1) the “acute onset” immunoglobulin E (IgE)-mediated; 2) the “delayed onset” non-IgE, cell-mediated; or 3) the mixed type-mediated allergy. The proper CMA diagnosis, thus its management, is complicated by the variation of onsets and clinical manifestations of these different clusters (Fiocchi et al. 2010). Therefore, the reference standard to diagnose CMA is an oral, preferably placebo-controlled and double-blind cow’s milk challenge in suspected subjects after a successful elimination diet (Fiocchi et al. 2011; Savilahti and Savilahti 2013). However, it is resource intensive and not easily to be conducted or interpreted. The oral challenge also carries a substantial risk of anaphylaxis (Fiocchi et al. 2010), thus many clinicians prefer to utilize less hazardous techniques for diagnosing CMA. The IgE-mediated CMA can be diagnosed by performing skin prick test against cow’s milk proteins and measuring cow’s milk-specific IgE levels (Fiocchi et al. 2010). In contrast to the non-IgE-mediated CMA, the IgE-mediated CMA typically persists to school age and seemed to be a risk factor for the atopic march (Saarinen et al. 2005; Schoemaker et al. 2015).

<table>
<thead>
<tr>
<th>Fraction (Proportion)</th>
<th>Allergen Name [Protein]</th>
<th>Size (kDalton)</th>
<th>Concentration (g/L)</th>
<th>Prevalence (patients %)</th>
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<tbody>
<tr>
<td><strong>Casein (80%)</strong></td>
<td>Bos d 9 [αS1-casein]</td>
<td>23.6</td>
<td>12–15</td>
<td>65–100</td>
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<tr>
<td></td>
<td>Bos d 10 [αS2-Casein]</td>
<td>25.2</td>
<td>3–4</td>
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<tr>
<td></td>
<td>Bos d 11 [β-Casein]</td>
<td>24</td>
<td>9–11</td>
<td>35–44</td>
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<td></td>
<td>Bos d 12 [κ-Casein]</td>
<td>19</td>
<td>3–4</td>
<td>35–41</td>
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<tr>
<td><strong>Whey (20%)</strong></td>
<td>Bos d 4 [α-Lactalbumin]</td>
<td>14.2</td>
<td>1–1.5</td>
<td>0–67</td>
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<tr>
<td></td>
<td>Bos d 5 [β-lactoglobulin]</td>
<td>18.3</td>
<td>3–4</td>
<td>13–62</td>
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<tr>
<td></td>
<td>Bos d 6 [Bovine Serum Albumin]</td>
<td>66.3</td>
<td>0.1–0.4</td>
<td>0–76</td>
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<tr>
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<td>Bos d 7 [Immunoglobulins]</td>
<td>160</td>
<td>0.6–10</td>
<td>12–36</td>
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<td></td>
<td>Bos d Lactoferrin [Lactoferrin]</td>
<td>80</td>
<td>0.09</td>
<td>0–35</td>
</tr>
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</table>

Table 1: Main allergens in cow’s milk. This table is adapted from (Hochwallner et al. 2014).

Immune responses consist of both humoral and cellular components. A close and complex interaction occurs between humoral and cellular immunities. While the availability of pro-allergic soluble factors is crucially dependent on immune-cell activities, the allergen-induced cross-linking of surface-bound IgEs provoke pro-allergic immune cells to unleash inflammatory mediators, causing allergy reactions. There is a growing data to address the roles of both humoral and cellular immunities in allergy...
and upon its tolerance induction and maintenance. This chapter focuses on the roles of both immune components in the IgE-mediated CMA in order to enhance our understanding of the CMA immunopathogenesis. Therapeutic strategies for inducing immune tolerance against cow’s milk are also discussed. In addition, mechanistic interactions between CMA and other inflammatory diseases are also proposed and discussed.

2 The Roles of Immune System during CMA Pathogenesis

2.1 Humoral Immunity

Due to the significant overlap between inflammatory mechanisms due to helminth infections and allergy, the humoral aspect of CMA immunopathogenesis has been extensively studied. It is known that helminth infections incite Th2-polarized immune responses, hence elevated IgE levels in order to eliminate the parasites (Fallon and Mangan 2007). IgE is the Ig isotype with the lowest abundance \textit{in vivo} (~50–200 ng/mL of blood in healthy humans compared with ~1–10 mg/mL of blood for other Ig isotypes), the Ig isotype with the shortest serum half-life (~2 days compared with ~20 days for IgG in humans) and the Ig isotype with a tight regulation of its concentration (Dullaers et al. 2012; Wu and Zarrin 2014). IgE binds to its receptors (the high affinity FcεRI and the low-affinity FcεRII/CD23) expressed by immune cells, including mast cells, basophils, dendritic cells, macrophages and B cells (Dullaers et al. 2012; Wu and Zarrin 2014). The facts that IgE half-life is significantly prolonged until weeks or months upon binding to its receptors (Achatz et al. 2010) and that most IgEs are retained in tissues (Dullaers et al. 2012) suggest that IgE plays an important role in local immunity. The \textit{in vivo} production site of IgE is intriguing. While plasma cells (‘Ig-producing B cells’) could secrete IgE into the blood circulation and subsequently IgEs diffuse into the inflamed sites, growing evidences indicate that the IgE synthesis could occur as well at the sites of allergic inflammation in response to persistent allergen exposure (Gould et al. 2006; Wu and Zarrin 2014). With this possibility, it is pertinent to be aware that local IgE production might not be reflected as high levels of circulating allergen-specific IgE (Gould et al. 2006). Of note, the IgE production can occur via two main pathways of Ig class switching: 1) a direct pathway from the IgM to the IgE; or 2) a sequential pathway from IgM to an IgG1 intermediate then to IgE (Wu and Zarrin 2014). It is elusive of whether the direct class switching pathway is more dominant in humans (Wu and Zarrin 2014) or of how to activate a certain pathway of IgE production. It has been suggested that, at least in the murine model, the sequential class switching produces high-affinity IgE antibodies, whereas the direct class switching may generate lower affinity IgE antibodies (Xiong et al. 2012). Of note, among the IgE-mediated CMA patients, the early-phase of CMA clinical manifestations are due to the cross-linking of surface-bound allergen-specific IgEs by allergens that subsequently activate mast cells and basophils to release biologically active substances, such as histamine, interleukin-4 (IL-4), serine proteases, TNF-α and platelet-activating factor (Galli et al. 2008). These factors contribute to the progression of allergic reactions as well as the tissue
It is debatable whether other Ig isotypes also play a pro-allergic role. It is important to remember that any Ig isotype is primarily secreted as a part of physiological immune responses following exposure to any foreign antigen, including food allergen. Hence besides IgE, the existence of other isotype of allergen-specific Ig (e.g., IgM or IgG) perhaps merely indicates an exposition to allergen without any obvious pro-allergic role. Nonetheless, despite one study reported that there was no association between the allergic manifestations and cow’s milk-specific IgG within CMA subjects (Hidvegi et al. 2002), several other studies suggested that CMA subjects had slightly elevated levels of cow’s milk-specific total IgG or IgG subtypes (Ruiter et al. 2007; Scott-Taylor et al. 2010). By referring to the sequential pathway of IgE production via IgG1 as an intermediate (Wu and Zarrin 2014), it is possible that the current existence of cow’s milk-specific IgG1 suggests the subsequent existence of cow’s milk-specific IgE. To rephrase it, allergen-specific IgG1 could be an indicator of present allergen sensitization and thus subsequent allergic reaction (Kukkonen et al. 2011; Orivuori et al. 2014).

As a more obscure Ig isotype, functionality of secreted IgD is elusive despite it is known as a transmembrane antigen receptor for mature B cells before antigenic stimulation (Chen and Cerutti 2011). Nevertheless, an interesting study using human upper respiratory mucosal samples demonstrated that IgD could bind to basophils and mast cells; subsequently, cross-linking of surface-bound IgDs induced basophil to produce BAFF (a mandatory B cell survival factor) and pro-inflammatory cytokines, including IL-4 and IL-13 (Chen et al. 2009). Taken together, this suggests that IgD may exert a pro-allergic role as well. Nonetheless, further study focusing on CMA subject is definitely required to confirm this hypothesis.

Immunoglobulin-free light chains (Ig-fLCs) are proposed to be a pro-allergic soluble mediator as well. Free κ or λ light chains were shown to be able to induce murine mast-cell degranulation, causing immediate allergic inflammation (Redegeld et al. 2002). Ig-fLC blockade indeed strongly reduced the allergic skin responses in murine models of contact hypersensitivity (van Houwelingen et al. 2007) and CMA (Schouten et al. 2010). Interestingly, one study demonstrated that in its cohort, serum levels of Ig-fLCs in CMA patients were significantly elevated as compared to the ones observed in non-allergic subjects (Schouten et al. 2010), suggesting the pro-allergic role of Ig-fLCs. It is elusive whether in humans, Ig-fLCs exert the pro-allergic role in the presence of allergen-specific IgE (‘complementing the IgE role’) or whether Ig-fLCs could replace allergen-specific IgE in mediating the allergic inflammation (‘substituting IgE in the non-IgE-mediated allergy’). This latter role is possible since it has been reported that the depletion of CD4+CD25+ T cells in whey-allergic mice switched the pathogenesis of allergic inflammation from an IgE-mediated to an Ig-fLC-mediated reactions (van Esch et al. 2010). Of note, elevated levels of Ig-fLCs have been associated with numerous chronic diseases as well, e.g., chronic obstructive pulmonary disease, non-allergic rhinitis with eosinophilia syndrome, rheumatoid arthritis, systemic lupus erythematous, multiple sclerosis and breast cancer (Redegeld and Nijkamp 2003; Powe et al. 2010; Braber et al. 2012; Groot Kormelink et al. 2014), suggesting that this potential
humoral arm of allergic reaction can also mediate other types of chronic inflammatory diseases.

### 2.2 Cellular Immunity

It is important to remember that innate and adaptive immune cells influence each other extensively, which contribute to the occurrence of allergy and its clinical characteristics. Nonetheless, for the sake of simplicity, cellular components of innate and adaptive immunity are discussed separately. A key feature is that most allergens, including cow’s milk proteins, are sampled, processed and presented by antigen-presenting cells (APCs), in particular dendritic cells (DCs), in order to initiate the cascade of cellular and humoral immune reactions leading to allergic inflammation (Figure 1). It is also worthy to mention that type 2 innate lymphoid cells (type 2 ILCs or ILC2s) could respond to the epithelial-derived cytokines in order to initiate or mediate allergic inflammation as well.

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**Figure 1: The intricate cascade of allergic inflammation.** Allergen’s exposure to inflammatory dendritic cells (DCs) allows these cells to process and to present allergen-derived peptides to naïve CD4⁺ T cells. In the early presence of IL-4 (from an unidentified source, probably basophils and mast cells), naïve CD4⁺ T cells differentiate into pro-allergic Th2 cells.
In Figure 1: (1) Activated type 2 innate lymphoid cells (type 2 ILCs) also contribute to the allergy reaction by releasing pro-allergic cytokines, such as IL-4, IL-5 and IL-13. Of note, type 2 ILCs are activated by cytokines IL-25 and IL-33, probably released by mast cells. Concurrently, it appears that there is an impairment of T<sub>Reg</sub>-cell frequency and/or activity, hence no/minimum suppression is exerted on T<sub>H</sub>2-cell activity. Subsequently, T<sub>H</sub>2 cells will drive B cells ('plasma cells'), via cell-contact as well as IL-4, IL-5 and IL-13, to undergo immunoglobulin class switching recombination, in which they eventually produce IgE; (2) Along with the antibody production, plasma cells also secrete significant amount of κ and λ Ig-free light chains (Ig-fLCs). IgE and/or Ig-fLCs will then bind to mast cells and basophils, causing sensitization; (3) Following subsequent exposure to allergen, cross-linking of surface-bound antibodies occurs, causing mast cells and basophils to degranulate and release their biologically active substances, including histamine, IL-4 and IL-5; (4) Released L-4 amplifies the differentiation of T<sub>n</sub>2 and IgE-producing plasma cells, while released IL-5 (also secreted by type 2 ILCs and T<sub>n</sub>2 cells) induces accumulation and activation of eosinophils in the affected tissues. Similarly, histamine activates epithelial or endothelial cells to release eotaxin that also attracts eosinophils into the tissues. Activated eosinophils release active substances, including major basic and eosinophilic cationic proteins that are toxic to the surrounding cells, contributing to further inflammation. Please notice that this figure only enlists major pro-allergic cytokines. This figure is adapted from (Jo et al. 2014).

2.2.1 Innate Components

The principal effector cells upon allergen exposure are subsets of innate immune cells, including tissue mast cells, basophils and eosinophils. Due to their surface expression of high-affinity receptors for IgE (FcεRI) and their ability to secrete mediators of allergic inflammation following IgE cross-linking by the specific allergens, tissue mast cells and basophils play pivotal roles in the IgE-mediated allergic inflammation (Schouten et al. 2010; MacGlashan 2013). These subsets contribute to the early- (immediate) and late-phase reactions of allergy (2–6 hours after exposure) (Galli et al. 2008). In addition, several studies have demonstrated that murine mast cells can be activated by Ig-fLCs to release pro-allergic mediators as well (Redegeld et al. 2002; Schouten et al. 2010).

Routine assessment assays on mast-cell and basophil activation in humans and mice have been developed and extensively used. Mast-cell activity in humans can be indirectly measured by the size of the induced wheal after skin prick test (SPT) with milk extract (Ford et al. 2013). In mice, mast-cell activity can be measured either by the size of cow’s milk protein-induced ear-skin swelling or by the elevation of serum levels of murine mast cell β-chymase or mMCP-1, a specific marker for mucosal mast-cell degranulation (Caughey 2007; Schouten et al. 2008). Basophil activation in humans and mice can be assessed through the measurement of released mediators (e.g., histamine or IL-4) or the up-regulation of degranulation-associated cell-surface proteins, e.g., CD203c or CD63 (MacGlashan 2013). As indicated by the key roles of mast cells and basophils in allergy, wheal diameter as well as expression levels of CD203c and CD63 on milk
protein-activated basophils were indeed much more pronounced in patients with more severe CMA (Ford et al. 2013).

Mediators released upon mast-cell degranulation, particularly histamine, could stimulate endothelial or epithelial cells to release a potent eosinophil chemoattractant, i.e., eotaxin (Menzies-Gow et al. 2004). This causes the infiltration of eosinophils, along with basophils, into inflamed tissues (Menzies-Gow et al. 2002). In addition, mast cells, type 2 ILCs and Tn2 cells also release IL-5 that attracts eosinophils, prolongs their survival, increases adhesion to endothelial cells and enhances their effector function (Galli et al. 2008; Takatsu and Nakajima 2008; Diefenbach et al. 2014). The tissue-infiltrating eosinophils subsequently release highly basic and cytotoxic granule proteins, including major basic protein and eosinophil cationic protein, which are toxic to epithelial and endothelial cells (Gleich et al. 1979; Kita 2013), contributing to the late-phase reaction of allergy (Galli et al. 2008). This tissue inflammation eventually results in eosinophilic gastroenteropathies (Hogan et al. 2001).

It is worthy to briefly mention the pro-allergic roles of type 2 ILCs as well. These are a recently identified subset of innate lymphocytes that do not express rearranged receptors and play important roles in innate immunity and tissue remodelling. Three subsets of ILCs are identified currently: type 1 (ILC1s), type 2 (ILC2s) and type 3 ILCs (ILC3s) (Diefenbach et al. 2014; Hazenberg and Spits 2014). Type 2 ILCs are activated by epithelial-derived cytokines IL-25 and IL-33 in order to produce Tn2-cytokines, such as IL-4, IL-5 and IL-13 (Moro et al. 2010; Neill et al. 2010; Price et al. 2010; Saenz et al. 2010). Due the production of these cytokines, type 2 ILCs are highly suggested to play important roles in immune responses against helminth infections as well as allergens (Barlow and McKenzie 2014). Of note, activated mast cells, among other cells, could produce IL-25 (Ikeda et al. 2003) and IL-33 (Hsu et al. 2010), suggesting the very intricate cross-talks among cellular components during allergic inflammation. Importantly, it has been reported that ILC2s existed in human skin and were enriched in patients with atopic dermatitis. These cells were activated by IL-25 and IL-33, but could be inhibited by the E-cadherin ligation on human ILC2s (Salimi et al. 2013). Following this notion, it is also possible that type 2 ILCs play an important role in initiating or even mediating CMA immunopathogenesis.

Roles of other innate immune subsets including neutrophils, monocytes, NK, γδ and NK T cells during allergic reaction to cow’s milk are elusive yet. Despite accumulation of neutrophils, γδ and NK T cells in the chronically inflamed digestive tissues of CMA patients (Turunen et al. 2004; Semeniuk et al. 2009; Jonouchi et al. 2014), actual roles of these innate cells within allergic reaction are unknown. It is possible that these cells are accumulated in the inflamed sites due to the high levels of inflammatory chemokines during chronic allergic reaction. These cells can be indirectly activated by the circulating inflammatory cytokines as well, further contributing to the chronic inflammatory reactions (Galli et al. 2008). Nonetheless, several murine studies suggest that these cells could play key roles during allergy. Neutrophils have been proposed to be important in both sensitization and induction of allergic skin inflammatory reactions as well as mediating alternative mechanisms of anaphylactic reaction (Mocsai 2013; Orivuori et al. 2014). In addition, murine tissue γδ and invariant NK T cells were
suggested to exert regulatory roles to suppress food allergy (Bol-Schoenmakers et al. 2011; Schouten et al. 2012). Human studies are definitely required to clarify these murine findings. The different cellular elements between human and murine immune systems further complicate the extrapolation of murine data to the human setting. For example, several studies (Treiner and Lantz 2006; Tang et al. 2013) have demonstrated that while CD1d-restricted invariant NK T cells are abundant in mice but low in human, MR1-restricted mucosal-associated invariant T (MAIT) cells are instead abundant in human but low in mice. Pertaining to CMA, it will be more relevant to study a particular immune cell subset that is enriched in humans, such as MAIT instead of NK T cells.

As a part of professional APCs, DCs are crucial in order to sample, process and display antigens to naïve T cells, either to initiate immune responses or to induce immune tolerance (Steinman et al. 2003). Pertaining to food-derived antigens, DCs in the intestine and associated lymphoid tissues are of particular interest, partly due to facts that these cells can pick-up antigens directly from the intestinal lumen or antigens that have been transported across the intestinal epithelial cells (IECs) (Coombes and Powrie 2008). Importantly in the healthy gastrointestinal tract, commensal bacteria and their products modulate intestinal DCs to be hyporesponsive or tolerant via interaction with the pattern recognition receptors of DCs (Coombes and Powrie 2008; de Kivit et al. 2014). In addition, non-inflamed healthy IECs are also able to suppress activity of inflammatory DCs while inducing tolerogenic DCs (de Kivit et al. 2014). Taken together, an interaction between gut microbiota, IECs and intestinal DCs under homeostatic conditions contributes to immune tolerance in the healthy gastrointestinal tract. Of particular interest is the existence of tolerogenic CD103+ DCs in murine intestines and mesenteric lymph nodes because they were able to convert naïve CD4+ T into FOXP3+ T_{\text{Regulatory}} (T_{\text{Reg}}) cells via TGF-β and retinoic acid (Coombes et al. 2007). T_{\text{Reg}} cells are proposed able to suppress allergic sensitization. A recent study shows a functional homology between murine CD103+ DCs and human CD141^high DCs in cross-presenting antigens to CD8+ T cells (Haniffa et al. 2012), hence eliciting a query of whether human intestinal CD141^high DCs can also serve as tolerogenic DCs. Notably in a murine model of peanut allergy, oral sensitization with peanut extract was accompanied by a shift in intestinal DC subsets, i.e., less tolerogenic CD103+ DCs but more inflammatory CD11b+ DCs (Smit et al. 2011). DC-recognition of allergens can be mediated by their C-type lectin receptors, such as DC-SIGN and mannose receptor (Salazar and Ghaemmaghami 2013). Subsequently in the early presence of IL-4 (potentially released by activated proallergic innate immune cells, including basophils and mast cells), allergen-presenting DCs polarize naïve CD4+ T cells into Th2 cells, which in turn differentiate Ig-producing B cells (‘plasma cells’) to produce IgE (Galli et al. 2008). Of note, the important role of DCs for mediating allergic reaction against cow’s milk proteins is supported by a finding from the adoptive transfer study of DCs from cow’s milk-allergic mice into naïve recipients. This DC transfer induced spontaneous production of cow’s milk-specific IgE in the naïve mice in the absence of antigen challenge (Chambers et al. 2004). In summary, inflammatory DCs initiate the allergic reaction by sampling and processing cow’s milk allergens, then presenting allergen-derived peptides to CD4+ T cells to become Th2 cells. Type 2 ILCs could also be activated by specific cytokines to
release Th2-cytokines during allergy. These events are followed by the IgE class switching and the activation of pro-allergic effectors including tissue mast cells, basophils and eosinophils.

### 2.2.2 Adaptive Components

CD4+ T cells serve an important role as the master regulator of adaptive immune responses. CD4+ T cells crucially influence the outcome of inflammatory reactions via their plasticity to differentiate into at least pro-inflammatory Th1-Th2-Th17 or anti-inflammatory T<sub>Reg</sub> cells (Zhu et al. 2010), either resulting as a resolved reaction or a persistent inflammation. While Th1 and Th17 cells are physiologically important to eliminate intracellular and extracellular pathogens respectively, Th2 cells are important for eradicating helminth infections. Th2 cells also contribute to the pathogenesis of allergy through secretion of Th2-cytokines (Galli et al. 2008; Zhu et al. 2010). CMA patients indeed exhibited cow’s milk protein-specific Th2-polarized immune responses in their peripheral blood, i.e., high levels of IL-4, IL-5 and IL-13, with low production of Th1-cytokine IFN-γ (Andre et al. 1996; Campbell et al. 1998; Schade et al. 2000; Tiemessen et al. 2004; Vocca et al. 2011; Michaud et al. 2014). This Th2-cytokine profile was importantly also displayed by duodenum-infiltrating T cells derived from CMA patients upon stimulation with cow’s milk proteins (Beyer et al. 2002). In addition, cow’s milk-specific Th2-immune responses were observed in murine models of CMA as well (Li et al. 1999; Adel-Patient et al. 2005). Of note, partly due to many potential allergens within cow’s milk, it is still elusive whether there is any difference of T-cell epitopes recognized by CMA patients who developed tolerance and by the ones who developed persistent allergy (Schade et al. 2003). Taken together, CMA partially occurs due to persistent uncontrolled Th2-polarized immune responses.

It is important to elucidate whether there is any regulatory mechanism exists against allergy. One possible mechanism is partly attributed to the suppressive role of T<sub>Reg</sub> cells. These cells can be further classified as thymus-derived, peripherally derived, or in vitro-induced T<sub>Reg</sub> cells (Abbas et al. 2013). However in order to simplify the nomenclature used in this review, various T<sub>Reg</sub> cells are grouped as one entity. Their suppressive functions can occur through either secretion of inhibitory cytokines (e.g., IL-10 and TGF-β), cytosis, metabolic disruption, or attenuation of DC maturation and/or functionality (Vignali et al. 2008). It has been demonstrated that a fine balance between T<sub>Reg</sub> and pro-allergic Th2 cells, including cell frequency and functionality, determines the development of allergy (Akdis et al. 2004). Noteworthy, a supporting evidence of a T<sub>Reg</sub> cell suppressive role in allergy came from clinical studies of patients with IPEX (‘Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked’) syndrome caused by a deletion in a noncoding region of the FOXP3 gene, the central gene for T<sub>Reg</sub> differentiation (Bennett et al. 2001). These patients had defects in T<sub>Reg</sub> frequency as well as functionality and more importantly exhibited severe food allergic phenotype particularly against cow’s milk proteins (Torgerson et al. 2007). IPEX patients also suffer from autoimmune diabetes and/or thyroiditis (Cheng and Anderson 2012), indicating that the impairment of T<sub>Reg</sub> cells loosens the brakes for many types of
inflammation to occur. Indeed, CMA patients who had a higher frequency of circulating cow’s milk protein-specific T_{Reg} cells exhibited a milder symptom and a favourable prognosis (Shreffler et al. 2009). In addition, lower frequencies of TGF-β-producing T cells were observed in the duodenal mucosa of children with food allergy as compared to non-allergic subjects (Perez-Machado et al. 2003; Westerholm-Ormio et al. 2010). To summarize, defects in T_{Reg}-cell frequency and functionality partly contribute to CMA pathogenesis.

Of note, several studies demonstrated the existence of regulatory B (B_{Reg}) cells that were associated with the suppression of excessive inflammation in mice and humans (Mauri and Bosma 2012). This subset of lymphocytes potentially supports the immunological tolerance via the production of anti-inflammatory cytokines IL-10, IL-35 and TGF-β (Rosser and Mauri 2015). Furthermore, B_{Reg} cells are required for the development and maintenance of T_{Reg} cells as well (Sun et al. 2008; Carter et al. 2011; Tadmor et al. 2011; Flores-Borja et al. 2013), thus can also indirectly regulate the immune responses. It is therefore of interest to elucidate B_{Reg}-cell role within CMA patients. However, it is difficult to study this subset of cells due the rarity in its frequency, the heterogeneity of its phenotypes and the lack of a unique transcription factor unlike T_{Reg} cells (Rosser and Mauri 2015; Tedder 2015). The last notion can be partially interpreted that B_{Reg} cells are not a lineage-specific B cell, but rather are expanded in response to inflammation (Rosser and Mauri 2015). In addition, B_{Reg} cells can differentiate into the Ig-producing plasma cells after the resolution of inflammatory responses (Maseda et al. 2012; Rosser and Mauri 2015), aggravating the difficulty to study this subset of cells. Nonetheless, a few in vitro studies suggested the presence of B_{Reg} cells in milk-tolerant subjects based on the IL-10 or TGF-β production upon casein stimulation (Lee et al. 2010; Noh et al. 2010; Lee et al. 2011). Whether these cells actively mediate immune tolerance against cow’s milk allergens in vivo, it remains inconclusive.

Despite exogenous antigens, including cow’s milk proteins, can be cross-presented by DCs to initiate CD8+ T-cell responses (Joffre et al. 2012), it is elusive whether CD8+ T cells play any role within CMA immunopathogenesis. It was even reported that upon unspecific stimulation, there was a significant difference in the frequency of IFN-γ-expressing CD4+, but not of CD8+ T cells, between CMA infants and healthy controls (Osterlund and Suomalainen 2002). On contrary, it is obvious that differentiated Ig-producing B cells (‘plasma cells’) serve an important pathogenic role during allergy. The presence of IL-4 and IL-13 released by Tn2 cells promote immunoglobulin class switching recombination, inducing plasma cells to secrete IgE (Galli et al. 2008). Taken together, CMA pathogenesis is attributed to the pro-allergic activity of cellular components of innate (DCs, tissue mast cells, basophils, eosinophils and type 2 ILCs) and adaptive immunity (Tn2 and IgE-producing plasma cells along with the impaired T_{Reg}-cell activity), as summarized in Table 2.
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<tr>
<th>Type</th>
<th>Cell</th>
<th>Role in Allergy</th>
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<tr>
<td>Tissue Mast cells</td>
<td>Key effectors during allergy. Upon Ig-E cross-linking (perhaps Ig-fLCs as well) with allergen, 3 classes of biologically active product are secreted (Caughey 2007):</td>
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<td></td>
<td>(1) Pre-stored cytoplasmic granules:</td>
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<td></td>
<td></td>
<td>a) biogenic amines (e.g., histamine);</td>
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<td>b) serglycin proteoglycans (e.g., heparin and chondroitin sulphate);</td>
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<td></td>
<td>c) serine proteases (tryptases, chymases and carboxypeptidase-ses); and</td>
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<td></td>
<td>d) some cytokines (e.g., TNF-α and VEGFA).</td>
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<td>(2) Lipid-derived mediators (prostaglandins, leukotriene B4, cysteinyl leukotrienes and platelet-activating factors).</td>
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<td>(3) Newly synthesized factors (cytokines, chemokines and growth factors).</td>
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<tr>
<td>Innate Cells</td>
<td>Basophils</td>
<td>Key effectors during allergy.</td>
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<td>Similar to mast cells, upon cross-linkage of IgE, 3 type of mediators can be released (MacGlashan 2013):</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Preformed, immediately released (e.g., histamine);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) Newly synthesized, immediately released (phospholipid meta-bolites including leukotriene C4); and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) Newly synthesized, slowly released (cytokines including IL-4).</td>
</tr>
<tr>
<td></td>
<td>Eosinophils</td>
<td>Key effectors during allergy.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Upon activation with cytokine (e.g., IL-5), highly basic and cyto-toxic granule proteins are secreted (Kita 2013):</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Major basic protein/MBP and MBP2;</td>
</tr>
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<td></td>
<td></td>
<td>(2) Eosinophilic cationic protein/ECP;</td>
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<td></td>
<td></td>
<td>(3) Eosinophilic peroxidase/EPX; and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4) Eosinophil-derived neurotoxin/EDN.</td>
</tr>
<tr>
<td></td>
<td>Initiators of Th2-cell response during allergy (Galli et al. 2008).</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inflammatory DCs uptake and process allergens, subsequently presenting allergen-derived peptides to naïve CD4+ T cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In the presence of IL-4, DCs polarizing naïve CD4+ T becomes Th2 cells.</td>
</tr>
</tbody>
</table>
Type 2 Innate Lymphoid Cells (ILC2s) | Probably act as the initial drivers of allergic inflammation by secreting Th2-cytokines (Barlow and McKenzie 2014; Diefenbach et al. 2014; Hazenberg and Spits 2014).
---|---
Other innate cells (Neutrophils, NK, MAIT and γδ T cells) | Unknown roles.

| Adaptive Cells | CD4+ Th2 cells | Drivers of allergic inflammation. Through cell-contact and cytokines (IL-4 and IL-13), Th2 cells promote immunoglobulin class-switch recombination in B cells to drive the IgE production (Galli et al. 2008).
---|---|---
| CD4+ Treg cells | Suppressors of allergic inflammation, via (Akdis 2012): (1) Suppression of tissue mast cells, basophils and eosinophils; (2) Suppression of inflammatory DCs and induction of tolerogenic DCs; (3) Suppression of allergen-specific Th2 cells; and (4) Early induction of IgG4 and late decrease in IgE.
| Ig-producing B cells (Plasma cells) | Co-drivers of allergic inflammation along with Th2 cells by secret-ing IgE and Ig-fLCs (Galli et al. 2008).
| Other B- and T-cell subsets | Unknown roles.

Table 2: The roles of cellular immunity in allergy. This table is adapted from (Jo et al. 2014).

3 The Roles of Immune System upon Tolerance to Cow’s Milk Proteins

The majority of infants with CMA spontaneously develop clinical tolerance to cow’s milk proteins or outgrow the allergic disorder to cow’s milk proteins by school age (Savilahti and Savilahti 2013). However, it has been shown that infants with the IgE-mediated CMA recovered later than those with the non-IgE-mediated allergy (Host et al. 2002; Vanto et al. 2004; Schoemaker et al. 2015). Hence, the dire consequences due to the dietary restriction before these children outgrow their allergic reactions, the tissue damage due to chronic allergic inflammation, as well as the potential atopic march in later life serve as an important reminder that CMA need to be properly diagnosed and managed early on. A proper understanding of immunity upon tolerance induction to cow’s milk is crucial for diagnosing and managing CMA effectively.
3.1 Humoral Ommunity

A few studies had demonstrated an association among the IgE-mediated CMA subjects who later develop tolerance with the reduction of allergen-specific IgE and with the increment of allergen-specific IgG4 levels (Ruiter et al. 2007; Savilahti and Savilahti 2013). This alteration is apparently mediated by IL-10 secreted by T\textsubscript{Reg} cells (Akdis 2012). IgG4, which is normally found at very low concentrations, is unique because it is the only IgG subtype unable to form any immune complex as well unable to activate complement (Tao et al. 1993; van der Neut Kolfschoten et al. 2007; Aalberse et al. 2009; Jackson et al. 2014). It is postulated that IgG4 acts as a blocking antibody via competition with allergens for binding to IgE on the Fc\varepsilon receptors (James et al. 2011) and as an anti-inflammatory factor due to its dynamic Fab arm exchange resulting as a bi-specific antibody with a substantially decreased capacity for cross-linking (van der Neut Kolfschoten et al. 2007). Therefore it is considered to have an anti-inflammation (hence anti-allergic) role. However, it is prudent to be cautious on generalizing anti-allergic role of IgG4 since not all allergy studies observed a correlation between elevated levels of IgG4 and anti-allergic effects (Williams et al. 2012). It remains possible that since IL-10 exerts anti-inflammatory role and since IL-10 promotes class switching of IgG1 to IgG4 instead to IgE, IgG4 does not directly induce immune tolerance (Williams et al. 2012; Jackson et al. 2014).

Several groups have also studied role of antigen-specific IgA (either in blood or as a secretory form) upon tolerance induction. An association was reported between cow’s milk avoidance by breast-feeding mothers with low levels of cow’s milk-specific IgA in their breast milk and in their infants’ sera and with the development of CMA, suggesting high levels of cow’s milk specific-IgA, in particular secretory IgA (such as in the breast milk), might have a protective effect against cow’s milk allergens (Jarvinen et al. 2014). In addition, several recent studies suggesting an association of elevated levels of cow’s milk-specific IgA with the development of tolerance against cow’s milk (Savilahti et al. 2010; Jarvinen et al. 2014; Savilahti et al. 2014). Whether the IgA-induced immune tolerance is achieved by simply removing food allergens before they are absorbed by the gastrointestinal tract (Silbart and Keren 1989) or by a more intricate mechanism (Corthesy 2013), it remains to be elucidated.

Infants who received non-digestible carbohydrates (‘prebiotics’) during the first six months of age had a lower incidence of atopic dermatitis, which was linearly associated with lower levels of Ig-fLCs (Schouten et al. 2011). Hence this suggests that the decreasing levels of Ig-fLCs might be associated with an anti-allergic role. Taken together, the decreasing levels of allergen-specific specific IgE (probably Ig-fLCs too) as well as the increasing levels of allergen-specific IgG4 and/or allergen-specific secretory IgA partly contribute to the development of immune tolerance (Table 3).

3.2 Cellular Immunity

With regard to the cellular immunity, the tolerance mechanism is essentially contributed by two primary mechanisms: 1) the suppression of pro-allergic innate effectors and type 2 ILCs; and 2) the up-regulation of T\textsubscript{Reg}-cell regulatory activity.
Isotype | Role in Allergy
--- | ---
Allergen-specific IgE | **Key effector** during allergy. Cross-linkage of surface-bound IgEs induce tissue mast cells and basophils to degranulate and release allergic mediators (Galli et al. 2008).

Allergen-specific IgG | Not well defined for allergen-specific IgG1-IgG2-IgG3. Allergen-specific IgG4 probably exerts an **anti-allergic** role (van der Neut Kolfschoten et al. 2007).

Allergen-specific IgM | Unknown role.

Allergen-specific IgA | Not well defined; secretory IgA probably exerts an **anti-allergic** role (Jarvinen et al. 2014; Savilahti et al. 2014).

Allergen-specific IgD | Not well defined; probably exerts a **pro-allergic** role (Chen and Cerutti 2011).

Ig-fLCs | Probably exert a **pro-allergic** role (Redegeld et al. 2002).

Table 3: The roles of secreted immunoglobulins and immunoglobulin-free light chains (Ig-fLCs) in allergy.

Arguably, the latter mechanism is the principal way to induce and maintain tolerance to allergens because it partially affects the former mechanism in the disease progression. Moreover, activity of functional T_{Reg} cells could contribute to T-cell anergy, i.e., a tolerance mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive (Schwartz 2003). Immunologic findings of spontaneously and treatment-induced tolerance against cow’s milk allergens are discussed together because both approaches, arguably, follow similar immune mechanisms.

3.2.1 Inhibition of Pro-allergic Innate Effector Cells and Type 2 ILCs

During tolerance development, pro-allergic innate effectors could undergo rapid desensitization against allergens, causing them to be less likely to release inflammatory factors (Akdis 2012). One probable mechanism is due to the presence of allergen-specific IgG4, as mentioned briefly above. It has been demonstrated that basophils from CMA children who developed clinical tolerance were significantly less responsive to the allergen (Wanich et al. 2009; Ford et al. 2013). Interestingly, the reduced responsiveness of basophils was partially due to an inhibitory factor present in serum probably allergen-specific IgG4 (Wanich et al. 2009). Furthermore, it is also known that the secreted anti-inflammatory IL-10 cytokine reduced the release of pro-inflammatory cytokines by mast cells (Marshall et al. 1996) and suppressed activity of eosinophils (Takanaski et al. 1994). Of note, due to the ability to respond to epithelial-derived cytokines IL-25 and IL-33, type 2 ILCs could secrete Th2-cytokines to mediate allergic reactions (Barlow and McKenzie 2014). Thus, it is possible that the immune tolerance
against allergens is partly contributed by the activation blockade of type 2 ILCs. Supporting evidences for this notion indeed come from a few studies which demonstrated the existence of natural factors that inhibited the activation of type 2 ILCs, such as lipoxin A4 (Barnig et al. 2013) or soluble excretory/secretory products of parasitic nematode Heligmosomoides polygyrus, e.g., IL-1β (Zaiss et al. 2013; McSorley et al. 2014). Taken together, these findings suggest that tolerance to cow’s milk allergens is associated with suppressed activities of innate type 2 ILCs and pro-allergic innate effectors.

3.2.2. Up-regulation of TReg-cell Activity

Up-regulated TReg cells can attenuate allergic responses through 1) suppression of mast cells, basophils and eosinophils; 2) suppression of inflammatory DCs and induction of tolerogenic DCs; 3) suppression of allergen-specific Th2 cells, hence contributing to T-cell anergy; and 4) early induction of IgG4 and late reduction of IgE production (Akdis 2012). All of these mechanisms can be mediated through secretion of IL-10 and TGF-β or through cell contact-dependent suppression (Akdis 2012). By treating a CMA murine model with various kind of treatments, including dietary long-chain n-3 polyunsaturated fatty acids, prebiotics, Bifidobacterium breve M-16V strain (‘probiotics’), synbiotics (‘prebiotics + probiotics’), or cow’s milk protein-derived peptides, a linear correlation of TReg-cell frequency and activity with the CMA suppression were observed (Schouten et al. 2009; Schouten et al. 2012; Meulenbroek et al. 2013; van den Elsen et al. 2013). Furthermore, several human studies also demonstrated the association between the increment of frequency and in vitro suppressive capacity of TReg cells with the clinical tolerance in children who outgrown CMA (Karlsson et al. 2004; Shreffler et al. 2009). Collectively, these findings indicate that the up-regulation of TReg-cell functionality would partly contribute to the tolerance to cow’s milk allergens.

4 Therapeutic Strategies to Induce Immune Tolerance

As mentioned, there is no established method to cure CMA to date. The only available approach to date is to apply strict avoidance of cow’s milk proteins, either by 1) perform maternal elimination diet of cow’s milk for breast-fed infants; 2) use partial or extensively hydrolysed milk formula for formula-fed children less than 2 years of age; or 3) switch to a milk-free diet for children more than 2 years of age (Fiocchi et al. 2010). This strict dietary avoidance should be accompanied with ready access to self-injectable epinephrine (Muraro et al. 2014). Drawbacks of this approach include impairment of the quality of life of both child and family, restricted children’s growth and higher health care cost (Koletzko et al. 2012). Therefore, novel therapeutic strategies are currently being developed for preventing and curing allergy in general and food allergy in particular. Hereby we review two promising approaches (Table 4).
Biological Immune Response Modifiers | Allergen-Specific Immunotherapy
--- | ---
Targeting (Akdis 2012): (a) IgE or its receptor, or (b) pro-allergic cytokines. | Consisting the whole antigen or a part of it, e.g., peptide (Kostadinova et al. 2013).

Administered via (Akdis 2012; Narisety and Keet 2012): 1) Skin: (a) Subcutaneous immunotherapy (SCIT), or (b) Epicutaneous immunotherapy (EPIT). 2) Gastrointestinal tract: (a) Sublingual immunotherapy (SLIT), or (b) Oral immunotherapy (OIT).

Table 4: Therapeutic strategies for treating the allergic inflammation.

### 4.1 Biological Immune Response Modifiers

The first strategy is to develop biological immune response modifiers to suppress pathological immune responses (Akdis 2012). The immune modifiers are developed to suppress pro-allergic soluble mediators, including IgE and cytokines (e.g., IL-4, IL-5, IL-13, IL-25 or IL-33). IgE, the pro-allergic Ig isotype, is the most obvious target for this approach. Several humanized monoclonal antibodies (mAbs) targeting the Fc portion of IgE have been developed and tested (Akdis 2012). One of the anti-IgE mAbs, omalizumab, has been suggested to be effective in a few clinical trials on patients with poorly controlled, moderate to severe respiratory allergic reactions (Casale et al. 2001; Lin et al. 2004; Busse et al. 2011). Omalizumab acts by decreasing the free IgE levels in serum and the expression of FcεRI on various immune cells (Lin et al. 2004). However, the clinical application of anti-IgE mAbs is very limited due to a few reasons: 1) high doses of anti-IgE mAbs are required to successfully neutralize IgE; 2) low cost effectiveness of using anti-IgE mAbs; and 3) unexpected efficacy without any reasonable explanation in some cases (Oba and Salzman 2004; Akdis 2012). Therefore an alternative approach is developed to replace mAbs, i.e., by using designed ankyrin repeat proteins (‘DARPins’). DARPins represent a promising scaffold due to high specificity and low concentration binding to the respected target. A few studies have demonstrated that DARPins can effectively bind to the Fc portion of IgE as well as the alpha chain of FcεRI, hence preventing the release of pro-inflammatory mediators (Eggel et al. 2009; Baumann et al. 2010). These exciting findings indicate that the DARPins technology is promising to be utilized to create allergy-attenuating candidate molecules.

Immune modifiers are also developed to target pro-allergic cytokines because these cytokines coordinate the inflammatory processes upon allergy. Cytokine inhibitors have been developed and tested for, at least, IL-4, IL-5, IL-13 or TNF-α. However, the low efficacy (due to the intricate cytokine network, hence targeting a single cytokine only may not induce any significant improvement) and/or the
unfavourable risk-benefit ratio of using cytokine inhibitors become a big hurdle in order to bring forward this therapeutic option to the clinical settings (Akdis 2012).

4.2 Allergen-Specific Immunotherapy (Allergen SIT)

The ultimate goal in allergology is to discover a long-term cure for allergic diseases. This goal potentially can be achieved by using allergen SIT, since its primary mode of action is to induce desensitization and subsequently immune tolerance to the allergens, by administering repeated, increased doses of the allergens (Akdis 2012). Desensitization means an increment in threshold of exposed/ingested harmless antigen needed to cause allergic symptoms (Jones et al. 2009). Immune tolerance refers to the state of immunologic hyporesponsiveness to harmless antigen (Kostadinova et al. 2013). Despite allergen SIT actually has been performed for several allergic reactions in the clinic, the current strategy of administering allergen SIT has limited efficacy, high side effects, low patient adherence and high cost due to long duration of the treatment (Akdis 2012; Kostadinova et al. 2013). This indicates that an improved/novel strategy is crucially required.

With regard to food allergy, allergen SIT requires thorough evaluation in two aspects. The first aspect is to properly choose the most optimum route of administering allergen SIT. The classical way to administer allergen SIT is through the subcutaneous route (SCIT), i.e., injecting soluble allergen under the skin. It is employed frequently for respiratory and bee venom allergies due to its safety and effectiveness (Kostadinova et al. 2013). The only food allergen that has been tested via SCIT thus far is peanut allergen. Unfortunately, despite the promising efficacy rate of SCIT, the rate of systemic adverse reactions, including anaphylactic shock, was high when using peanut allergen (Oppenheimer et al. 1992; Nelson et al. 1997). Hence this finding discourages the SCIT utilization in its current form for treating food allergy (Kostadinova et al. 2013). A modified method for SCIT is the epicutaneous immunotherapy (EPIT), i.e., administering soluble allergen via skin patch into the stratum corneum, the outer layer of the skin. Due to the non-invasive nature of EPIT, it is possibly a safer method for treating food allergy (Kostadinova et al. 2013). Interestingly, a pilot study of using EPIT to treat the IgE-mediated CMA-diagnosed children in France demonstrated that this method is safe and acceptable (Dupont et al. 2010). Further clinical trials will definitely be required in order to determine the efficacy of EPIT in treating CMA, similar to the ones that have been initiated for peanut allergy (Senti et al. 2014).

Besides the cutaneous route, allergen SIT can be administered via the enteral route, including sublingual immunotherapy (SLIT) and oral immunotherapy (OIT). For SLIT, concentrated liquid allergen extract is administered under the tongue for 2 minutes and subsequently discarded or swallowed. For OIT, the powder form or mixture of culprit food with a vehicle (e.g., apple sauce) is directly ingested (Narisety and Keet 2012; Kostadinova et al. 2013). Due to the difference of used forms, allergen quantity in the OIT can be increased from milligram amounts to several grams, while the one in the SLIT can only be increased from microgram amounts to maximum milligram amounts. Nonetheless, SLIT has other advantages, i.e., allowing the food
proteins to bypass gastric digestion and potentially enhancing tolerance induction because the oral mucosa supposedly contains many tolerogenic APCs but fewer effector cells responsible for the allergic reactions (Narisety and Keet 2012). The safety profile of SLIT for treating CMA has been tested in a few clinical studies. Indeed, it has been shown that the SLIT for CMA has an encouraging safety profile (de Boissieu and Dupont 2006; Keet et al. 2012). However, it has been demonstrated that for desensitizing CMA-diagnosed patients, the efficacy of SLIT was much lower than the one belongs to OIT (Keet et al. 2012). Thus far, OIT is the most actively studied form of allergen SIT for primary food allergies including CMA, partially due to the ability to use allergen at high doses reaching the actual amount of ingested food (Narisety and Keet 2012). Several clinical studies performed in Europe and North America demonstrated that OIT with cow’s milk could induce desensitization, as reflected by ability to consume larger amount of cow’s milk without exhibiting any adverse effect, reduction of sensitivity on skin prick test, reduction of cow’s milk-specific IgE levels and/or elevation of cow’s milk-specific IgG4 levels (Patriarca et al. 2003; Meglio et al. 2004; Staden et al. 2007; Longo et al. 2008; Skripak et al. 2008; Narisety et al. 2009; Pajno et al. 2010; Martorell et al. 2011; Salmivesi et al. 2013; Savilahti et al. 2014). The subsequent question is of course whether OIT could induce immune tolerance for long term or even permanently. Interestingly, a few clinical studies have demonstrated the OIT efficacy to induce oral tolerance against cow’s milk for a long period, even for more than 3 years of follow-up (Staden et al. 2007; Martorell et al. 2011; Salmivesi et al. 2013). It is important to notice that OIT has been tested in children with severe cases of CMA as well, resulting as 36% of tested subjects were able to tolerate at least 150 mL of cow’s milk (Longo et al. 2008). Taken together, cow’s milk OIT is potential to induce desensitization and immune tolerance that can persist in the long term. However, higher dose of cow’s milk allergen used in OIT (than SLIT) is also associated with a higher risk to develop adverse effects (Keet et al. 2012). Therefore, it is mandatory to exert cautions while administering OIT in its current form.

The second aspect of evaluation is related to how to minimize the high rates of adverse effects in OIT by reducing the size and structure of administered food protein. A way to do this is by using peptides (‘peptide immunotherapy’), as they consist T-cell epitopes (hence, potentially retain their therapeutic benefit) but less likely to induce side effects due to peptides’ inability to cross-link surface-bound IgE on effector immune cells. Theoretically, since the distance between two FcεRI molecules is ranging from 8 to 24 nm, using peptides shorter than 30 amino acids should not cross-link surface-bound IgEs, but shall retain T-cell epitopes (Knipping et al. 2012). This strategy of peptide immunotherapy has been more actively studied for respiratory allergies, in particular of allergy to cat and bee venom (Larche 2005; Moldaver and Larche 2011). Several clinical studies focusing on both allergens had been performed. Indeed, allergen-specific hyporesponsiveness can be induced via down-regulation of T-cell allergic responses (skewing to the regulatory phenotype) as well as of pro-allergic cytokine (e.g., IL-4 and IL-13) and IgE production (Larche 2005; Verhoef et al. 2005; Moldaver and Larche 2011). Furthermore, exposure of tolerogenic peptide induces early apoptotic deletion in naïve CD4+ T cells (Kearney et al. 1994; Hochweller and Anderton 2005; Hochweller et al.
It was also demonstrated that the peptide immunotherapy could reduce allergic responses if the allergy was driven by effector memory CD4+ T (TEM), but not by central memory T (TCM) cells (Mackenzie et al. 2014). Interestingly, a study demonstrated that following at least two rounds of peptide exposure, reactivated memory CD4+ T cells failed to survive (marked by low expression of the antiapoptotic molecule Bcl2 and high expression of activated caspase molecules), hence fewer pro-allergic T cells existed to sustain subsequent hyper-sensitivity response (David et al. 2014). Furthermore, the peptide immunotherapy is associated with a phenomenon called ‘linked epitope suppression’, i.e., a treatment with selected epitopes form a single allergen can suppress responses to other epitopes from the same allergen, probably through the action of IL-10 (Campbell et al. 2009). Of note, the peptide immunotherapy was generally well tolerated, although a few adverse effects were observed in some studies, including delayed symptoms of asthma or erythema with palm pruritus, hence it is still of concern (Larche 2005; Moldaver and Larche 2011). Taken together, these findings support the concept to utilize selected peptides in order to prevent inflammatory responses against an allergen; however, the clinical efficacy of the peptide immunotherapy potentially will be varied among different allergies and individuals.

Pertaining to CMA, peptide immunotherapy has been mainly investigated in murine models. Several groups demonstrated that prior treatment with αS1-casein- or β-lactoglobulin-derived peptide(s) rendered mice to be immune tolerant to cow’s milk protein (Hirahara et al. 1995; Pecquet et al. 2000). In a recent study, particular β-lactoglobulin-derived tolerogenic peptides were identified, in which these peptides were able to reduce acute allergic response in a murine model of CMA. The allergic reduction, as manifested as a reduction in ear swelling, was importantly associated with decreasing levels of cow’s milk-specific IgE and increasing frequencies of TReg cells (Meulenbroek et al. 2013). Collectively, this alternative approach is promising to prevent or treat CMA, although further clinical studies are required to assess its clinical efficacy and safety.

5 Possible Mechanistic Interaction between CMA and Other Inflammatory Reactions

The allergic reactions are characterised by the Th2-polarized immune responses, by the activation of pro-allergic innate effectors and by the impairment of TReg cells. As mentioned above, CD4+ T cells are heterogeneous due to their ability to differentiate into Th1, Th2, Th9, Th17, Th22, T follicular helper (Tfh) or TReg cells, hallmarked by different lineage-specifying transcription factors and different signature cytokines (O'Shea and Paul 2010). For example, Th1 cells express T-bet and secrete IFN-γ, Th2 cells express GATA3 and secrete IL-4, Th17 cells express RORγt and secrete IL-17, while TReg cells express Foxp3 and secrete IL-10 and TGF-β. It was originally proposed that each subset of CD4+ T cells permanently retain their differentiated identity, resulting as non-overlapping distinct subsets (O'Shea and Paul 2010). However, it is clear now that the differentiation process is dynamic instead, particularly during chronic inflammation in
vivo (Hirahara et al. 2013). This allows a particular differentiated subset of CD4+ T cells to secrete signature cytokines that belong to other subsets or even to further convert into another subset. For example, it has been shown that T cells derived from chronic allergic asthma patients co-expressed and co-produced both Th2- and Th17-transcription factors and cytokines (Wang et al. 2010). In addition, atopic dermatitis patients predominantly displayed Th2-immune responses with a Th17 component at the acute phase of the disease, which often converted into Th1-immune responses at the chronic stage (Oyoshi et al. 2009). Thus, it incites a speculation of whether Th2-polarized immune responses in CMA could also exhibit or even convert to Th1- or Th17-immune responses in minority group of patients who never outgrow their CMA.

The consensus of food allergy occurs due to the imbalance between Th2- and TReg-polarized immune responses indeed incites a speculation of whether inflammation of CMA affects other type of inflammatory reactions (infection as well as other chronic inflammatory non-communicable diseases or NCDs) and vice versa. The published data does not allow a definite conclusion to be constructed; nonetheless it provides some hints that permit various speculations. First, despite there is no prospective study following children with food allergy to determine whether they have a lower predilection to suffer from helminth infection, CMA infants with the elevated Th2-polarized immune responses should be more protected against helminths. A supporting finding came from a population study in Cameroon who demonstrated that subjects with elevated IL-5 and IL-13 cytokines indeed had reduced reinfection rates with Ascaris lumbricoides and Trichiuris trichiura (Jackson et al. 2004), supporting the importance of Th2-immune responses against helminths. On the other hand, helminth infections is associated with Th2-polarized immune responses, hence theoretically it could increase the susceptibility of infected hosts to develop allergic reactions. However, it appears not to be the case. A study on infants living in areas endemic for helminth infections suggested that despite potent Th2-responses were observed early in life, it did not translate into a higher SPT reactivity to various allergens at 4 years of age (Djuardi et al. 2013). A supporting finding came from a study on mice chronically infected with Heligmosomoides polygyrus bakeri suggested that the helmints induced IL-1β secretion in small intestines that acts to suppress the production of IL-25 and IL-33, thus resulting in suboptimal Th2-immune responses and hence chronic helminth infection (Zaiss et al. 2013). Helminth infections could also induce activation of TReg cells, resulting in IL-10 and IgG4 production, hence attenuating the Th2-immune responses (Fallon and Mangan 2007). A murine study indeed demonstrated that infection with intestinal helmints (Heligmosomoides polygyrus) prior to the sensitization and challenge with peanut extract per oral indeed significantly reduced peanut-specific IgE levels and diminished systemic anaphylactic symptoms via IL-10 production (Bashir et al. 2002). More importantly, several clinical studies demonstrated associations between human infections with Schistosoma haematobium or Schistosoma mansoni and lowered allergic responses, probably through the action of IL-10 (Araujo et al. 2000; van den Biggelaar et al. 2000; Medeiros et al. 2003). Therefore, prior exposures to helmints might reduce allergy incidence to cow’s milk proteins.

Second, the activation of Th1-polarized immune responses is required for the
control and elimination of intracellular pathogens. Due to the suppression of IFN-γ gene transcription by IL-4, i.e., Th2 cytokine suppresses Th1-functionality (Nakamura et al. 1997), it is plausible to assume that allergic infants might be more susceptible to be infected with intracellular pathogens. Interestingly, a prospective birth cohort study in the Netherlands, PIAMA (n=4,146), demonstrated an association between children having risk factors for allergy (i.e., having allergic parents and attending child care or having older siblings) with a higher risk of suffering low respiratory tract infections in the first year of life (Koopman et al. 2001). However, it is elusive whether CMA infants are more susceptible than healthy infants to develop infections in the gastrointestinal tract, respiratory tract or skin. Next, gastrointestinal infection with intracellular pathogens can cause enteral inflammation along with the disruption of the healthy intestinal flora. This perturbs homeostasis among host immunity, host gut microbiota and gut antigens, which may represent the critical determinant in the development of food allergy, including CMA (Macdonald and Monteleone 2005). Indeed, there is a case report demonstrating a Japanese infant who developed CMA associated with enterotoxigenic Escherichia coli and methicillin-resistant Staphylococcus aureus infections (Omata et al. 2008). In addition, another study of Japanese newborns who underwent small intestine surgery and received antibiotics due to symptoms resembling postoperative infection showed that 9 out of 30 subjects subsequently developed CMA (Ezaki et al. 2012). Importantly, within a subset of patients who received prophylactic probiotics, most of the patients (~98%) did not suffer from CMA (Ezaki et al. 2012), suggesting that restoration and maintenance of gastrointestinal immune tolerance is imperative in order to prevent food allergy. Arguably, gastrointestinal infections that incite enteral inflammation may represent an important risk factor to develop CMA.

Third, consistent with the fact that allergy is the most common and earliest-onset of inflammatory NCDs (Prescott 2013), it is important to understand how the immune mechanisms underlying food allergy interact with the ones constituting other NCDs, including other types of allergy, metabolic diseases, autoimmunity and cancer. It is noteworthy to mention that there are common risk factors for most NCDs, i.e., diet patterns, microbial patterns, behaviour and environmental pollutants (Prescott 2013). These common risks may initiate similar alterations within the immune system to cause many NCDs, arguably through the impairment of T_{Reg} cells. T_{Reg}-cell defect causes uncontrolled inflammation (Vignali et al. 2008), contributing to the pathogenesis of many NCDs (Prescott 2013). Indeed, the reduction of T_{Reg} cells has been linked to the dysregulated inflammation of other NCDs, such as obesity and insulin resistance (Priceman et al. 2013). Furthermore, a prospective mother-child study conducted in Germany, LINA (n=629), demonstrated a clear correlation between history of maternal exposure to tobacco smoke, lower T_{Reg}-cell frequencies in maternal and cord blood, as well as a higher risk for those children to develop atopic dermatitis within the first 3 years of life (Herberth et al. 2013). It is arguably that the common defect of immune regulation may cause several NCDs to occur concurrently, though the responsible mechanism still needs to be confirmed. Nonetheless, it is of interest to quote a recent data from the National Health and Nutrition Examination Survey, demonstrating that US children and adolescents who were obese indeed had higher levels of total IgE and
C-reactive protein levels as well as higher incidences of food allergy (Visness et al. 2009). Taken together, similar impairment in the immune mechanism that causes inflammation may mediate occurrence of many NCDs.

As allergic inflammation still remains a big mystery of the immune system, it is worthy to view allergy from a different perspective in order to understand it better. The current belief is that allergy is an adverse effect of an aberrant or misdirected immune response that evolved to provide immunity to macroparasites, e.g., helminths (Fallon and Mangan 2007; Galli et al. 2008). However, this view does not reconcile issues of 1) why many allergens do not have any relationship with helminth antigens as well as why many allergens do not share any common characteristic (e.g., peanut and penicillin); and 2) why many allergic responses occur very fast despite helminths are slowly replicating relatively to bacteria or viruses (Palm et al. 2012). In order to reconcile this issue, Medzhitov’s group proposed that allergic inflammation actually provide an important defence mechanism against various noxious environmental factors, including 1) macroparasites; 2) noxious xenobiotics; 3) venoms and haematophagous fluids; and 4) environmental irritants (Palm et al. 2012). The induced allergic reactions reduce exposure and promote expulsion of noxious factors as well as ensure avoidance of unfavourable environments (Palm et al. 2012). Despite it is an interesting concept, this hypothesis could not explain why certain innocuous antigens, such as cow’s milk or peanut, induce allergic inflammation and why the induced allergic inflammation only occurs in certain groups of people. It will be extremely important to analyse allergic reactions as well as specific allergens from various perspectives in order to elucidate our understanding of allergic inflammation.

6 Conclusion

Immune system, comprising both the innate and adaptive components, mediates allergic reactions as well as immune tolerance toward cow’s milk proteins. The CMA pathogenesis is mediated by the activation of pro-allergic innate (inflammatory DCs, tissue mast cells, basophils, eosinophils and type 2 ILCs) and adaptive effectors (TH2 and IgE-producing plasma cells), by the suppression of TReg cells as well by the production of cow’s milk-specific IgE. The immune tolerance against cow’s milk proteins is contributed by the activation of TReg cells and by the suppression of TH2-polarized immune responses as well as pro-allergic effectors. Possible therapeutic strategies to induce immune tolerance against CMA have been discussed, including the usage of the biological immune response modifiers or the allergen-specific immunotherapy. One of the promising methods of the allergen-specific immunotherapy is the utilization of peptide immunotherapy. Finally, due to the significant overlapping between the inflammatory reaction of CMA and of infection or other NCDs, the proper management of CMA may positively contribute to a better control of systemic inflammation.
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