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Where's the Beef? : Understanding Allergic Responses to Red Meat in Alpha-gal Syndrome

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Abstract

Alpha-gal syndrome (AGS) describes a collection of symptoms associated with IgE-mediated hypersensitivity responses to the glycan galactose-alpha-1,3-galactose (alpha-gal). Individuals with AGS develop delayed hypersensitivity reactions, with symptoms occurring more than 2 hours after consuming mammalian ("red") meat and other mammal-derived food products. The mechanisms of pathogenesis driving this paradigm-breaking food allergy are not fully understood. We review the role of tick bites in the development of alpha-gal specific IgE and highlight innate and adaptive immune cells possibly involved in alpha-gal sensitization. We discuss the impact of alpha-gal glycosylation on digestion and metabolism of alpha-gal glycolipids and glycoproteins and the implications for basophil and mast cell activation and mediator release that generate allergic symptoms in AGS.

Introduction

Alpha-gal syndrome (AGS) describes a collection of symptoms associated with type I, IgE-mediated hypersensitivity responses to the glycan galactose-alpha-1,3-galactose. Galactose-alpha-1,3-galactose (alpha-gal) is a carbohydrate found in platyrrhine ("broad-nose" or "New World") monkeys and non-primate mammals (1). These mammals can glycosylate proteins and lipids with alpha-gal because, unlike humans and catarrhine ("down-nosed" or "Old World") monkeys, they possess a functional *GGTA1* gene that encodes for alpha-1,3-galactosyltransferase (2, 3).

Individuals with AGS can experience allergic reactions to food and medications. They develop delayed hypersensitivity reactions after consuming mammal-derived food products (classically mammalian or "red" meat, and in some cases dairy products or gelatin) (4, 5) and immediate hypersensitivity responses to injected pharmaceutical products that contain

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AUTHOR CONTRIBUTIONS

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alpha-gal. In fact, AGS was initially identified following the observation of regional differences within the United States (US) in the frequency of adverse reactions to first-time infusions of the cancer drug cetuximab, a chimeric mouse-human IgG1 monoclonal antibody against epidermal growth factor receptor (6). It was subsequently determined that these reactions were driven by IgE targeted against alpha-gal on the antigen-binding fragment (Fab) of cetuximab (7). Shortly afterward, two seminal reports from the US and Australia described a syndrome of delayed urticaria, angioedema, and/or anaphylaxis after the ingestion of red meat in patients with circulating alpha-gal specific (s)IgE, (8), (9), and recognition of AGS as a food allergy emerged. For the purposes of this review, we focus on AGS as a form of food allergy.

Alpha-gal Syndrome Disrupts Current Paradigms for Food Allergy

AGS cases have been described on every continent except Antarctica (10, 11). In nearly every country with reported AGS cases, bites from hard-bodied ticks have been associated with the development of mammalian meat allergy (11) (9, 12–15). In the US, *Amblyomma americanum* (lone star tick) is the clinically relevant tick species associated with AGS (4, 11). Several other species, including *Ixodes ricinus, Amblyomma sculptum, Ixodes holocylus*, and *Heamaphilis longicornis*, have been linked to AGS in other countries (11) (Table I). A majority of alpha-gal allergic patients report urticaria (≥ 90%) and gastrointestinal symptoms (approximately 70%) after eating mammalian products (16) (17–19). Reports of anaphylaxis (i.e., allergic symptoms comprising at least two organ systems simultaneously) range from 50–65% in most studies (16, 17). In about 20% of AGS cases, the clinical phenotype involves only gastrointestinal symptoms, primarily severe persistent abdominal cramping, diarrhea, and gastroesophageal reflux (20). Like conventional food allergies, AGS can develop during childhood (20, 21), but also arises in older adults who have tolerated mammalian meat for decades (19, 22).

Alpha-gal syndrome as a food allergy upsets our conventional expectations of IgE-mediated food allergies. In contrast to conventional food allergies, IgE antibodies form against the alpha-gal sugar rather than a protein antigen. In addition, the development of IgE antibodies to alpha-gal is associated with tick bites rather than allergen exposure through the gut or via compromised skin epithelium (12). Instead of an immediate hypersensitivity response characteristic of conventional food allergies, allergic symptom onset after meat consumption is often delayed, typically occurring more than 2 hours (often 3–8 hours) after the meal (8) (16, 20). This delay in symptom onset frequently makes it challenging to diagnose AGS (5).

There are conflicting reports regarding an association between AGS and other allergic diseases like allergic rhinitis or food protein allergy. In a study of 261 American children and adults, the frequency of aeroallergen sensitization was comparable between those with and without detectable circulating alpha-gal-sIgE >0.35 IU/ml, but there was a higher frequency of sensitization to wheat and stinging insect venom in AGS subjects (16). By contrast, in two independent cohorts of adult European patients, comprising over 2700 people, sensitization to alpha-gal was associated with atopy, defined as a positive skin prick test to any of several inhalant allergens (23). Over half of a Swedish cohort of AGS patients

were sensitized to aeroallergens, compared to 33% of the general population, and atopy increased the risk of alpha-gal-driven anaphylaxis involving the airways (17).

The discrepancy among studies may be attributed to differences in study design, including participant age, geographic locations, methods used to assess for atopy (serum sIgE levels vs. skin prick testing), and alpha-gal sIgE levels used to define alpha-gal sensitization (>0.1 vs. >0.35 IU/ml). It remains unclear whether individuals with AGS have a higher chance of developing other allergic diseases, but the aforementioned studies demonstrate that pre-existing atopy is not required to develop AGS. Individuals with AGS are not disproportionately represented among those with atopic dermatitis (24). There are no descriptions of spontaneous development of alpha-gal-sIgE or AGS in patients with pre-existing asthma, atopic dermatitis or allergic rhinitis, despite circulating alpha-gal specific IgG in all humans (25). Low levels of alpha-gal sIgE have been reported in association with chronic intestinal helminth infection, but even in the setting of Th2-polarizing helminth infection, AGS does not spontaneously develop (26). Thus, tick bites distinctively seem to break existing tolerance to alpha-gal, although the mechanisms for this are still unknown.

Pathogenesis of Conventional Food Allergy: Sensitization and Effector Phases

The drivers for the development of food allergies to conventional protein allergens have been under active investigation for over 2 decades. For a non-food-allergic individual to generate and maintain tolerance to dietary antigens, the intestinal immune system (intestinal epithelial barrier, phagocytic innate immune cells, tolerogenic antigen presenting cells (APCs), and regulatory and effector lymphocytes), integrates signals from intestinal luminal food antigens with signals from the gut microbiota and transmits these to the systemic immune system to promote oral tolerance (27). In food-allergic individuals, however, a dysregulated epithelial microenvironment causes APCs to process and present the food antigen to naïve CD4+ T cells in a manner that skews the T cells to a type 2 / Th2-biased phenotype, characterized by production of cytokines such as IL-4, IL-5, and IL-13. CD4+ Th2 cells subsequently interact with B cells that have also taken up food antigen for presentation to these T cells. These T cells and their secreted type 2 cytokines push B cells to class switch their immunoglobulin isotypes to antigen-specific IgE. Secreted IgE eventually binds to the IgE receptor FcεRI on migrating basophils and tissue resident mast cells, thus sensitizing the host to that food allergen (28). During the effector phase, the host is re-exposed to the food allergen, which binds to food-specific IgE on the surface of mast cells and basophils, crosslinks the IgE-FcεRI complexes, and drives mast cell and basophil activation and degranulation. Degranulation causes the release of pre-formed and newly synthesized mediators such as histamine, leukotrienes, and prostaglandins, which generate the symptoms associated with allergic responses (29).

Sensitization Phase in Alpha-gal Syndrome

Galactose-alpha-1,3-galactose is immunogenic

Antibodies specific to alpha-gal are already present in the human body, due to the presence of so-called “natural” antibodies generated in a T cell-independent manner in response to host microbial communities (25, 30). These existing antibodies are typically IgM, IgA, or IgG isotypes, with IgG isotypes being the most abundant (25, 31) (30). These alpha-gal specific antibodies help the immune system distinguish self from non-self and can drive acute organ rejection of xenogenic organ transplants (32, 33). They also increase the efficiency with which the host immune system neutralizes and resists alpha-gal expressing pathogens (3, 34). The presence of alpha-gal on viruses improves the ability of naïve human sera lacking specific viral immunity to neutralize these viruses (35). Alpha-gal-specific antibodies also provide protection against parasites that express alpha-gal, such as the malaria-causing *Plasmodium* (34), and shape the quality of microbes colonizing our intestines by selecting for less pathogenic microbial colonizers (3).

While alpha-gal specific IgG, IgA, and IgM facilitate immune responses against pathogens, the generation of alpha-gal sIgE (allergic sensitization to alpha-gal), heralds a potentially detrimental allergic immune response (36). Moreover, the presence of alpha-gal sIgE in red meat allergic patients has been associated with a difference in circulating alpha-gal sIgG subclasses. Investigators studying independent cohorts of patients with AGS in Europe, the US, Kenya and Ecuador have reported that circulating alpha-gal sIgG4 is undetectable or significantly reduced in individuals with AGS compared to non-allergic controls (37) (26, 38), while alpha-gal sIgG1 and IgG3 levels are higher (38). The coexistence of alpha-gal sIgE, sIgG1, and sIgG3 in individuals with AGS is intriguing since neither IgG subclass has been associated with IgE-blocking activity that might prevent the development of food allergy. By contrast, the presence or development of food-specific IgG4 has been associated with a tolerance to food antigen (39).

Tick-mediated development of alpha-gal-specific IgE

In the US, significant overlap was observed between the geographic distribution of known cases of AGS and cases of Rocky Mountain spotted fever (RMSF) and ehrlichiosis (40, 41). The causative species of these diseases (*Rickettsia* and *Ehrlichia*, respectively) are transmitted by *A. americanum* (41). This led investigators to hypothesize that in US, bites from the lone star tick triggered sensitization to alpha-gal (8, 12). Additional reports have supported the association of AGS with tick bite (9, 13–15) with high titer alpha-gal sIgE linked to 2 or more bites in close temporal succession (12, 14). Titers decline in those who avoid repeat tick bites (42), implicating ticks as sensitizing agents.

Th2 and type I immediate hypersensitivity reactions, including anaphylaxis, to tick bites have been described (43–45), making ticks and tick saliva plausible sensitizing agents. Basophils, eosinophils, and to a lesser extent, tissue resident mast cells, infiltrate the bite site soon after tick bites occur (14, 46, 47). Allergic responses to tick bites promote the development of acquired resistance to future tick infestation, interfering with the transmission of numerous tick-borne pathogens in several mammalian hosts (43). It has

also been hypothesized that the allergic response helps rid epithelial surfaces of these multicellular ectoparasites (48).

Initially, investigators hypothesized that adult ticks transmitted mammal-derived alpha-gal antigens to human hosts during a blood meal (11). Subsequent reports described individuals developing alpha-gal sIgE after bites from larval stage (“seed”) ticks that had never had a blood meal from a mammalian host, suggesting that alpha-gal is an endogenous tick component (41, 49). Indeed, alpha-gal has been found in the saliva, salivary glands, hemolymph, and gastrointestinal tracts of ticks, independent of whether they have consumed a blood meal (41, 50). Galactosyltransferase genes encoding for the enzyme necessary for alpha-gal production are present in ticks (11), and alpha-gal expression is upregulated in tick saliva the longer ticks feed on blood from humans (41) and other mammals (51). Some have proposed that upregulation of alpha-gal in tick saliva serves as a form of molecular mimicry deployed to avoid triggering a massive clearing immune response by the mammalian host (51).

Tick mouth parts, including barbed telescoping chelicerae, penetrate the epidermis, securing the tick to the skin (52). This structure and the tubular, blood-drawing hypostome induce trauma to the skin. The potential introduction of pathogenic bacteria present in tick gut and saliva (53–55) could disrupt skin microbiota. These cumulative hits to the epithelial barrier may trigger the production of alarmins IL-33, IL-25, and TSLP by skin epithelial cells, generating a microenvironment that drives Th2/type 2 immune responses (27, 56) (Figure 1). Yet, individuals with atopic dermatitis do not spontaneously develop AGS, despite skin barrier disruption, exposure to alpha-gal in skin microbiota, and epigenetic DNA modifications in skin epithelium and lymphocytes that promote alarmin and type 2 cytokine release (57, 58). This suggests that the physical breach and interference with skin microbiota induced by tick bites, coupled with alpha-gal in tick saliva secreted into the host, may trigger distinct epigenetic programs in epithelial cells or resident cutaneous immune cells that favor alpha-gal sIgE production.

Inflammatory factors or adjuvants in clinically-relevant ticks may also promote production of alpha-gal sIgE. Chandresekhar et al. demonstrated that a synthetic alpha-gal glycoprotein (alpha-gal bovine serum albumin) injected subcutaneously with tick whole body extract (TWBE) from the lone star tick induced polyclonal, tick-specific, and alpha-gal sIgE responses in alpha-galactosyltransferase deficient (AGKO) mice that cannot produce alpha-gal (59). Lone star TWBE had adjuvant effects similar to the classic adjuvant alum, when sensitizing mice to tick-independent antigens. The adjuvant effects of TWBE depended on signaling through MyD88, an adaptor molecule downstream of the toll-like receptors (TLRs), including TLR2, 4, 5, and 9, through which TWBE had a robust stimulatory effect (59).

We developed a mouse model showing that subcutaneous injection of tick salivary gland extract (TSGE) from sheep blood-fed lone star ticks, independent of an exogenous alpha-gal glycoprotein or glycolipid, induced alpha-gal sIgE in AGKO mice. Mice sensitized with TSGE developed 190-fold higher levels of total IgE compared to controls and alpha-gal sIgE went from undetectable to 158.4 pg/ml. In addition, AGKO mice sensitized with lone star

TSGE demonstrated a drop in core body temperature among other allergic signs following oral challenge with pork kidney, a well-established source of alpha-gal glycoproteins, as well as pork fat (60). Surprisingly, circulating alpha-gal sIgE has been found in dogs naturally infested with ticks, even though as non-primate mammals, they possess functional galactosyltransferase enzymes critical for synthesizing endogenous alpha-gal. Hodzic et al. found that 50% of the banked serum samples from clinically healthy, naturally tick-infested dogs had detectable alpha-gal sIgE whose levels correlated with tick salivary gland protein sIgM and sIgE levels (61). These animal models of alpha-gal sensitization and allergy, coupled with observational studies of humans, support the idea that tick bites from clinically-relevant species can induce alpha-gal sIgE, predisposing to the development of AGS (15).

Cellular Players involved in Sensitization to Alpha-gal

Dendritic Cells.—DCs are important players in the initiation of immune responses to food. The presence of alpha-gal on an antigen impacts DC function. Immature monocyte-derived DCs take up proteins glycosylated with alpha-gal more readily than proteins lacking alpha-gal and degrade alpha-gal glycoproteins at a slower rate (62). This may have implications for the efficiency and rate of DC-mediated processing and presentation of alpha-gal antigens to T cells. Sensitization to alpha-gal starts with a cutaneous insult, and notably, tick saliva from the clinically-relevant *Ixodes ricinus* species has been shown to hamper maturation and migration of skin DCs to draining lymph nodes, disrupting DC ability to promote Th1 or Th17 responses, and favoring Th2 responses (63). The role of cutaneous CD301b+ DCs in the AGS sensitization phase will be critical to address since cutaneous CD301b+ DCs have been shown to migrate to draining lymph nodes, in a chemokine receptor CCR7 and CCR8-dependent manner, to initiate CD4+ Th2 cell differentiation after cutaneous allergen exposure (64).

Basophils and Mast Cells.—DCs do not secrete the IL-4 needed to direct naive CD4+ T cells towards a type 2/Th2 response (65). This opens the door for alternative immune cells, such as basophils, to serve as APCs and sources of IL-4 that can promote Th2 differentiation. Basophils produce IL-4 after TLR stimulation (66) and express the co-stimulatory molecules CD40, CD56, and CD86 (65, 67). They can acquire peptide-MHC-II complexes used for antigen presentation from DCs (65). They also express endogenous MHC-II and have been shown to endocytose, process and present peptide antigens to naïve CD4+ T cells *in vitro* (67). In addition, basophils were shown to be essential for Th2 differentiation *in vitro* and *in vivo* in a mouse model of subcutaneous immunization with the papaya cysteine protease papain (67). Basophils can also produce TSLP (65), which, along with other alarmins classically produced by disrupted epithelial barriers, creates a tissue microenvironment that favors Th2/type 2 immune responses (56). Human mast cell lines and primary mast cells isolated from eosinophilic allergic polyps are also capable of producing cytokines important for antibody isotype switching, independent of IgE-FcεRI signaling. Mast cells secreted B-cell activating factor (BAFF), IL-4 and IL-21, a cytokine classically associated with T follicular helper cells that regulate B cell class switching (68). Triggered by tick-associated adjuvants and host alarmins, these mast cell-derived cytokines

could stimulate B-cell class switching into alpha-gal specific IgE+ B-cells that eventually differentiate into IgE-secreting plasmablasts.

CD4+ Th2 cells.—Repetitive tick infestations have been shown to generate a Th2 cytokine profile in mice (69) and increase the frequency of CD4+ Th2 cells compared to Th1 cells in humans (14). Tick saliva contains factors, including prostaglandins, sphingomyelinase, and cysteine protease inhibitors, that appear to promote type 2/Th2 responses (70, 71). CD4+ T cells stimulated with tick saliva from *Ixodes scapularis* produce significantly more IL-4 and IL-13 than IFN-gamma, the canonical type 1 cytokine (72, 73). Chandresekhar et al showed that CD4+ T cells were essential for the generation of a Th2-skewed antibody profile following subcutaneous injection with *A. Americanum* TWBE. Elevations in total IgE and tick-specific IgE and IgG1 were abrogated in mice whose CD4+ T cells were depleted prior to tick exposure (59). In addition, we recently showed that the transcriptional immune profiles in circulating peripheral blood mononuclear cells (PBMCs) from alpha-gal-sensitized human subjects were distinct from controls without detectable alpha-gal sIgE. There was increased expression of genes associated with antigen presentation, MHC-II surface expression, and cytokines and chemokines associated with itch and allergic dermatitis, including *IL-13RA*, which encodes the receptor for the Th2 cytokine IL-13) (19). These findings suggest that the generation of alpha-gal-sIgE may depend in part on MHC-II mediated antigen presentation to CD4+ Th2 cells, although to date, this has not been formally demonstrated.

Unconventional and semi-variant T cells.—Classically, CD4+ Th2 cells recognize peptide-, and not glycan, MHC-II complexes. Thus, unconventional T cells with the ability to recognize exogenous glycolipid antigen (gamma-delta T cells, invariant natural killer T (iNKT) cells, and mucosal-associated invariant T (MAIT) cells (74)) may factor into the generation of alpha-gal sIgE. The most evidence that exists supports a potential role for iNKT cells, which recognize glycolipid antigens in the context of the MHC-I-like molecule CD1d. In peach allergy, for example, the Pru p 3 lipid-ligand acts as an adjuvant, promoting sensitization to the allergen Pru p 3, through a CD1d-mediated interaction (75). iNKT cell activation and type 2 cytokine skewing has also been demonstrated in cow's milk allergy (76) and eosinophilic esophagitis (77). Both mouse and human iNKT cells can recognize the alpha-gal glycolipid isoglobotrihexosylceramide (iGb3) presented in the context of CD1d (78). Alpha-gal sIgM and sIgG produced by human B cells depend in part on B cells interfacing with iNKT cells via CD1d (79). In addition, we showed that the median frequency of circulating iNKT cells expressing the early activation marker CD69 was 2.5-fold higher in alpha-gal sensitized individuals (with no reported recent tick bite; no mammalian meat ingestion) than in control participants seronegative for alpha-gal sIgE. We identified a weak positive linear correlation between the frequency of circulating CD69+, activated iNKT cells and alpha-gal sIgE levels (19). Activated iNKT cells also accumulate in the spleen and liver of AGKO mice sensitized with TSGE and challenged orally with alpha-gal rich pork kidney (O.I. Iweala, S.K. Choudhary, and S. P. Commins, unpublished data). Studies exploring the potential role for iNKT cells in alpha-gal allergy are in their infancy, but these findings suggest that CD1d-restricted, activated, unconventional iNKT cells may promote AGS.

B cells.—As the cells ultimately responsible for producing IgE, there is considerable interest in delineating the role for B cells in the development of mammalian meat allergy. B-cell intrinsic MyD88 expression was critical for tick-specific IgE production after sensitization with TWBE in a mouse model of lone star tick-induced IgE responses (59). This highlighted a role for TLR signaling through MyD88 in B cells in the generation of immune hypersensitivity responses to an AGS-associated tick. There is also evidence that circulating B cells in alpha-gal sensitized, mammalian meat-allergic individuals are distinct from B cells in non-allergic controls. When we examined bulk PBMCs using multiplex gene transcription array, we found differential expression of genes associated with B cell function in patients sensitized and allergic to alpha-gal compared to controls. These included upregulation of BCL-6, a transcription factor expressed both in germinal center B cells and in T follicular helper cells, and CD70, a ligand of the B cell marker CD27 that can regulate IgG synthesis and enhance B cell-mediated IgE production (19). Cox and colleagues also identified distinct B cell immunophenotypes linked to alpha-gal sIgE production in patients with AGS. CD27+ memory B cells from AGS participants expressed lower IgM but higher amounts of IgD, and the chemokine receptors CXCR4 and CCR6 compared to non-allergic controls. The memory B cells were also enriched for IgE-secreting B cells (80).

The observation that alpha-gal specific IgE can wane fairly rapidly over time if patients avoid ingesting red meat or getting tick bites (42), has led some to postulate that alpha-gal sIgE is produced by circulating plasmablasts rather than longer-lived, bone marrow resident plasma cells (5, 73). Preliminary studies suggest that although very rare, circulating IgE+, alpha-gal+ CD27 high, CD38 high, CD138- B cell plasmablasts are detectable in mammalian meat allergic individuals (81, 82). The transcriptional signatures and functional phenotypes of these cells require additional investigation, but the early evidence suggests that a specialized B cell subset with an unconventional memory phenotype drives alpha-gal sIgE production. This alpha-gal sIgE+ unconventional B cell population may include marginal zone (MZ) B cells, found in spleen (mice and human), and lymph nodes and intestinal Peyer’s patches (human (83)). They can mount polyclonal antibody responses to T-cell dependent protein and T-independent carbohydrate antigens. MZ-like, class-switched IgG+ B cells with clonally-restricted B cell receptors (BCRs) are also detectable in human skin. These heterogeneous CD22+CD27- mature and CD22+CD27+ memory B cells accumulate in the skin 3 to 7 days after intradermal injection with varicella zoster virus or candida antigen (84). Thus, tick bites may effectively act like intradermal injections of alpha-gal and tick factors. Alpha-gal and tick factors could activate and expand MZ-like, skin-resident, alpha-gal specific B cells by simultaneously engaging BCRs and B cell pattern recognition receptors. Activated B cells surrounded by basophil and mast cell-derived IL-4 and other cytokines could subsequently class switch to IgE (Figure 1).

The Effector Phase in Alpha-Gal Syndrome

Intestinal Absorption of Alpha-gal and Delayed Allergic Reactions to Mammalian Meat in AGS

One of the notable characteristics of AGS is the delay between eating red meat and symptoms of an allergic reaction (4, 5). Our mouse model of AGS has also recapitulated

this delay in allergic responses to mammal fat compared to conventional food-protein allergens (60). A potential explanation is that alpha-gal molecules are somehow delayed in reaching the bloodstream (4, 85, 86). This could be due to how AGS patients process alpha-gal in its glycolipid and glycoprotein forms. When Steinke et al. conducted metabolic profiling of AGS and non-alpha-gal-allergic controls, they showed significant differences in metabolic pathways for lipids, proteins, and carbohydrates between the groups (87). Even before oral pork challenge, investigators found that participants with detectable serum alpha-gal sIgE differed significantly in lipid and fatty acid metabolism pathways compared to non-AGS controls, although the reason for this remains unclear. Following oral pork challenge, the differences in lipid and fatty acid metabolism between AGS and control participants increased; lipid blood levels did not increase above baseline in AGS participants for several hours, compared to 2 hours for controls (87). Additionally, an *in vitro* simulation of beef digestion and intestinal transport demonstrated that only alpha-gal in glycolipid, not glycoprotein, form could effectively cross a simulated intestinal epithelial barrier composed of Caco-2 cells (85). Alpha-gal glycolipids were incorporated into chylomicrons in order to traffic through enterocytes (85). Lipids are metabolized more slowly than proteins (85), which, coupled with the alterations to AGS patients' metabolism (87), likely contribute to the delay in allergic reaction (Figure 2).

However, another report assessing digestion and transport of alpha-gal containing compounds across a Caco-2 cell monolayer had contrasting results: mammalian proteins glycosylated with alpha-gal could pass through the mock intestinal epithelium. However, significantly smaller amounts of alpha-gal glycoproteins were detected than proteins without alpha-gal (88). Differences in the form and concentration of alpha-gal used could explain these conflicting results. Nevertheless, both studies demonstrated that alpha-gal glycoproteins are hindered in moving across the intestinal epithelium. In addition, glycosylating a mammalian protein with alpha-gal increased both its resistance to digestion *in vitro* and the amount detected in the endosomal fraction of the Caco-2 lysates, even after 24 hours of incubation (88). These results suggest that delays in the digestion and transport of alpha-gal glycoproteins across the intestinal epithelium may also contribute to delayed allergic responses to alpha-gal (Figure 2).

Alpha-gal Glycoproteins and Glycolipids Activate Allergy Effector Cells

In the effector phase of IgE-mediated food allergies, antigen crosslinks IgE bound to FcεRI on the surface of mast cells and basophils, triggering degranulation and the release of inflammatory mediators. The roles of mast cells, basophils and the signaling molecules downstream of alpha-gal sIgE-FcεRI complexes in these cells during the effector phase of AGS are not entirely clear.

Basophils.—Basophils circulate in the bloodstream, making them more accessible to retrieve from human study subjects than tissue-dwelling mast cells. Given their central role in the immune responses to ticks associated with the development of AGS, basophil responses to alpha-gal stimulation have been widely studied. In alpha-gal-allergic participants undergoing open mammalian meat challenge, basophils upregulated the activation marker CD63, with maximal expression 4 hours after meat ingestion. This also

correlated with the appearance of clinical symptoms (89). Basolateral media of Caco-2 cells containing chylomicrons with alpha-gal glycolipid also activated basophils from an alpha-gal-allergic subject (85). Using an indirect basophil activation test, we were able to show that alpha-gal glycoproteins and glycolipids activated basophils sensitized with plasma from alpha-gal allergic individuals. Omalizumab, a monoclonal antibody against IgE, impaired alpha-gal-mediated basophil activation (86), indicating that activation is IgE-dependent. These reports highlight a unique role for a glycan – both in glycolipid and glycoprotein forms -- activating allergy effector cells through surface-bound IgE in the context of food allergy.

Basophil activation tests have also been studied as a confirmatory test for the diagnosis of AGS. For example, basophils from alpha-gal sIgE seropositive red meat allergic patients stimulated with alpha-gal-rich cetuximab upregulated expression of cell surface activation markers. This mirrored positive percutaneous and intradermal test results in these patients using cetuximab (90). The basophil activation test has also been explored as a surrogate for clinically-observed oral red meat challenges in AGS patients, which are currently the gold standard to distinguish between alpha-gal sensitized and alpha-gal / mammalian meat- allergic individuals (91). Taken together, these reports illustrate that basophils sensitized with alpha-gal sIgE are activated following exposure to alpha-gal, likely playing a significant role in driving allergic reactions in AGS.

Mast cells.—While the function of basophils in AGS has become clearer, there is less known about the role of mast cells in AGS reactions following mammalian meat ingestion. In conventional food protein allergy, percutaneous skin prick testing using the extracts of the allergenic food serves as one *in vivo* test of mast cell function in humans. When cutaneous mast cells sensitized with food-specific IgE contact food proteins scratched onto the skin, the food protein triggers mast cell degranulation, histamine release, and a corresponding wheal and flare response on the skin at the scratch site. Skin prick testing using standardized mammalian meat extracts frequently results in negative or very small wheals in patients with AGS (8). However, intradermal testing, a more sensitive *in vivo* test of allergen-induced mast cell activation that takes advantage of the higher density of mast cells in the dermis compared to the epidermis, is positive in AGS patients (8). Notably, intradermal skin testing for food allergy is considered high risk for inducing anaphylaxis and only performed under research protocols. In the case of AGS, intradermal testing provided initial evidence demonstrating that cutaneous mast cells in AGS patients could be activated by alpha-gal glycoproteins (8).

Serum tryptase serves as a marker for mast cell degranulation in humans but is not consistently elevated during severe allergic reactions to food protein (92). Similarly, only 30% of AGS participants with allergic symptoms after oral red meat challenge experienced elevations in serum tryptase, peaking 4 hours after meat ingestion (89). Although it was not statistically significantly different, in lone star TSGE-sensitized mice challenged with pork kidney homogenate, there was a 4-fold increase in levels of mouse mast cell protease (MMCP)-1, a serum marker for mast cell degranulation in mice, compared to controls (60). The need for both human and animal studies linking mast cell activation to clinical AGS symptoms remains.

Neither serum elevations of mast cell mediators, nor the percutaneous and intradermal skin prick testing, nor the mouse model of AGS formally prove that alpha-gal induced mast cell activation is IgE-dependent. Nor do they prove that signaling pathways downstream of alpha-gal sIgE-FcεRI complexes are the same as pathways activated by conventional protein allergens. Additional studies formally investigating this are needed. We have found that primary human mast cells derived from skin mast cell progenitors or adipose tissue stem cells (93) and sensitized with alpha-gal sIgE seropositive plasma can be activated by alpha-gal glycoproteins (Iweala and Kepley, unpublished results). Interestingly, skin-derived, but not lung-derived, mast cells reacted to alpha-gal *in vitro* (Iweala and Kepley, unpublished results). This may underlie the finding that over 90% of AGS patients describe cutaneous symptoms following red meat ingestion, nearly three times higher than those who report respiratory symptoms (16, 19, 94).

Alpha-gal glycolipids can activate appropriately sensitized basophils (85, 86), but it remains unclear whether alpha-gal glycolipid can activate mast cells. Mast cells have receptors for low density lipoprotein (LDL) (95). In mouse models, LDL, high-density lipoprotein, and very low-density lipoprotein induce mast cell activation (96). Since dietary alpha-gal is found in lipoprotein particles (85, 97), it is possible that alpha-gal glycolipid in these particles could activate human mast cells in AGS. Human mast cell lines (98), primary human mast cell culture (93), and emerging technology to make human monoclonal specific IgE (99) stand as promising tools in the quest to determine whether alpha-gal glycans can activate human mast cells.

Emerging Therapies for Alpha-gal syndrome

There is currently no cure for AGS. Individuals with AGS are simply counseled to avoid red meat, mammalian products, and in some cases dairy (4, 5). There is no one-size fits all for these avoidance diets, as tolerance to mammalian products varies from patient to patient (4). Allergies to conventional food-protein allergens, like peanut, tree nuts, and shellfish can develop in both childhood and adulthood and almost never resolve spontaneously (28). By contrast, alpha-gal sIgE has been shown to wane with time in children and some adults who avoid repeat tick bites from ticks associated with AGS development (42). This means that in some individuals, AGS can spontaneously resolve, allowing the reincorporation of mammalian meat and food products into the diet (4). More studies are required to establish how long alpha-gal sIgE persists and whether this correlates with the absence of bound alpha-gal sIgE on basophils and mast cells. In addition, there are currently no available biomarkers to predict persistence or waning of alpha-gal sIgE.

Avoidance can be difficult for AGS patients, since many foods, cosmetics, and medications contain alpha-gal in the form of mammal-derived ingredients. Severe allergic symptoms and anaphylaxis following accidental alpha-gal ingestion are treated with intramuscular epinephrine (4). For patients who continue to report mild allergic symptoms even after strict alpha-gal avoidance, oral antihistamines and mast cell stabilizers like cromolyn are used to treat and to prevent symptoms associated with unknown, accidental alpha-gal ingestion (5). Therapies for food allergy, alpha-gal syndrome included, are in their infancy. There is only one FDA-approved therapy for the treatment of food allergy, oral immunotherapy

(OIT) specific for peanut allergy (29). OIT involves feeding an allergic individual increasing doses of food allergen until a treatment dose is reached and administered daily; it has been shown to alter cellular and antibody responses to food allergen (39). Although a case report detailed successful desensitization to alpha-gal in a child using dose escalation of beef, followed by daily beef ingestion (100), there are currently no FDA-approved, standardized OIT protocols to treat alpha-gal syndrome. Anecdotal, clinical observations suggest that AGS is more likely to resolve in patients who tolerate moderate amounts of dairy in their diet (4, 5). Informed by this observation, a small, nonrandomized, non-placebo controlled, unblinded pilot study involving 7 participants with AGS evaluated the safety of using cow's milk containing 6mg alpha-gal over 36 months as daily OIT to treat AGS (https://clinicaltrials.gov/ct2/show/results/NCT02350660). No serious adverse events were reported, but it is unclear how much mammalian meat participants tolerated on challenge, as the study was terminated early due to funding. These findings suggest that OIT to treat alpha-gal syndrome will likely be tolerated, but additional studies are clearly required to assess the utility of alpha-gal OIT in managing AGS.

Antigen-independent therapies to treat food allergies are attractive to patients with multiple food allergies or who cannot tolerate side effects associated with OIT, including allergic reactions (29). Biologics, including monoclonal antibodies omalizumab and dupilumab are under active investigation as adjuncts to OIT or independent food allergy treatments in their own right (27). Omalizumab in particular binds the Fc portion of IgE, preventing it from binding to FCεRI on mast cells and basophils and hampering alpha-gal-induced activation of basophils sensitized with alpha-gal plasma *in vitro* (86). A small prospective study of 14 AGS patients, started on omalizumab to treat chronic urticaria that persisted even after appropriate alpha-gal avoidance diets, showed a decline in frequency and severity of participants' urticaria and symptom improvement in those reporting accidental or intentional ingestion of mammalian products (Commins, unpublished results). This suggests that omalizumab may serve as a therapeutic option for AGS patients with persistent allergic symptoms despite alpha-gal avoidance diets.

Conclusion

Red meat allergy in alpha-gal syndrome disrupts our current understanding of the mechanisms of pathogenesis driving food allergy. In food protein allergy, protein allergens crosslink specific IgE-FcεRI complexes on sensitized mast cells and basophils typically after minimal digestion or processing. By contrast, current evidence suggests that after prolonged digestion and metabolism of alpha-gal glycoproteins and additional packaging of alpha-gal glycolipids into chylomicrons and other lipid particles, these multimeric forms of alpha-gal crosslink alpha-gal sIgE-FcεRI complexes. This may explain the delay in allergic symptom onset.

Bites from select hard-bodied ticks, including *Amblyomma americanum* in the US induce elevations in alpha-gal specific and total IgE levels. Sensitization to alpha-gal likely involves several of the same innate and adaptive immune cells classically associated with allergic sensitization in food protein allergy, including DCs, CD4+ Th2 cells, and CD27+ memory B cells. However, there may also be roles for cells not commonly associated with allergic

sensitization to protein allergen, including basophils, iNKT cells, unconventional marginal zone B-cells, and IgE+ plasmablasts. Questions remain regarding the mechanisms of pathogenesis behind alpha-gal syndrome. Unravelling these immunologic mechanisms will enhance and expand our understanding of food allergy overall.

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Abbreviations:

AGKO	alpha-gal knockout
alpha-gal	galactose-alpha-1,3-galactose
AGS	alpha-gal syndrome
DC	dendritic cell
iNKT	invariant natural killer T cell
LDL	low density lipoprotein
PBMC	peripheral blood mononuclear cell
sIgE	specific IgE
Th2	T helper 2
TLR	toll-like receptor
TSGE	tick salivary gland extract
TSLP	thymic stromal lymphopoeitin
TWBE	tick whole body extract

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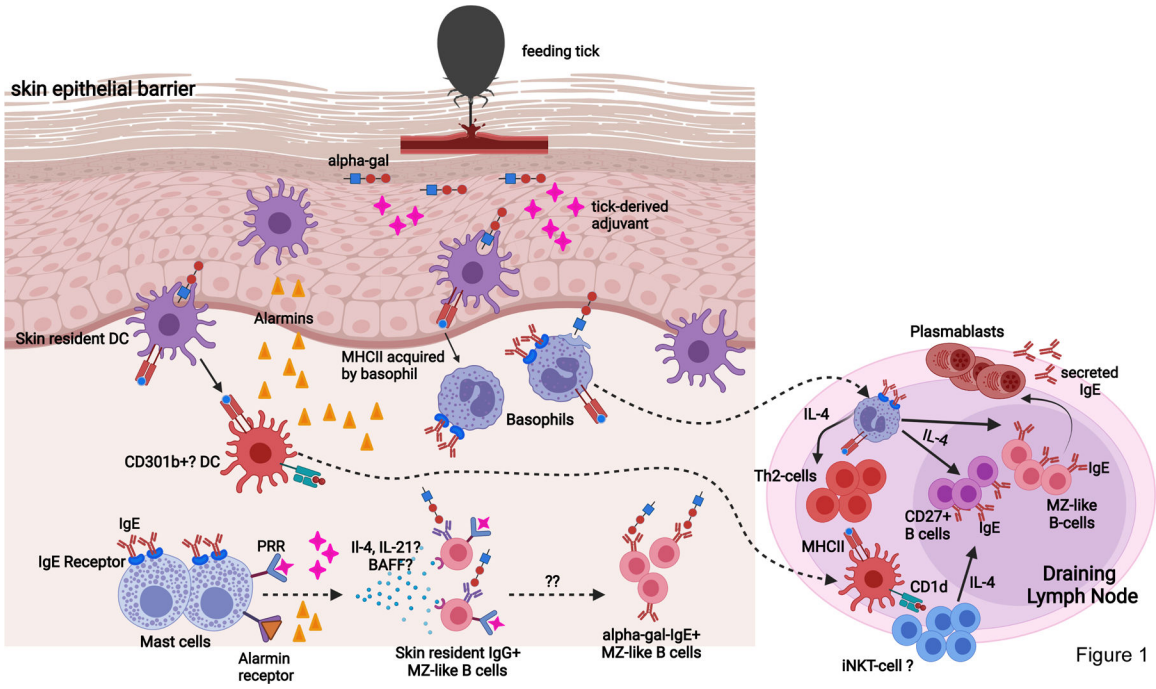

Figure 1.

Sensitization Phase in Alpha-gal Syndrome. During feeding, tick mouth parts induce physical trauma to the skin epithelial barrier while introducing alpha-gal, potentially pathogenic bacteria, and adjuvants present in tick saliva. This may trigger the production of alarmins like IL-33, IL-25, and TSLP by skin epithelial cells, generating a microenvironment that favors the generation of Th2/type 2 immune responses. Skin-resident and migrating dendritic cells (DCs) take up alpha-gal glycoproteins and glycolipids. Migrating DCs, possibly expressing CD301b+, traffic to draining lymph nodes to present peptide antigen to naïve CD4+ T cells. DCs may also present alpha-gal glycolipid complexed with CD1d to IL-4-producing iNKT cells. IL-4-secreting basophils may acquire peptide-MHC-II complexes from DCs or express endogenous peptide-MHC-II complexes, which they present to CD4+ T cells inducing Th2 responses. Tick saliva adjuvants binding to pattern recognition receptors (PRR) may induce basophil and mast cell-derived IL-4, IL-21, and B-cell activation factor (BAFF) which can promote class switching of clonally-restricted, skin resident, IgG+ marginal zone (MZ)-like B cells (in pink) that recognize alpha-gal and accumulate in skin. MZ-like and other heterogeneous CD27+ alpha-gal specific B cell populations (purple) accumulate in draining lymph nodes and basophil-derived IL-4 may promote class switching to alpha-gal specific IgE+ B-cells that eventually differentiate into IgE-secreting plasmablasts.

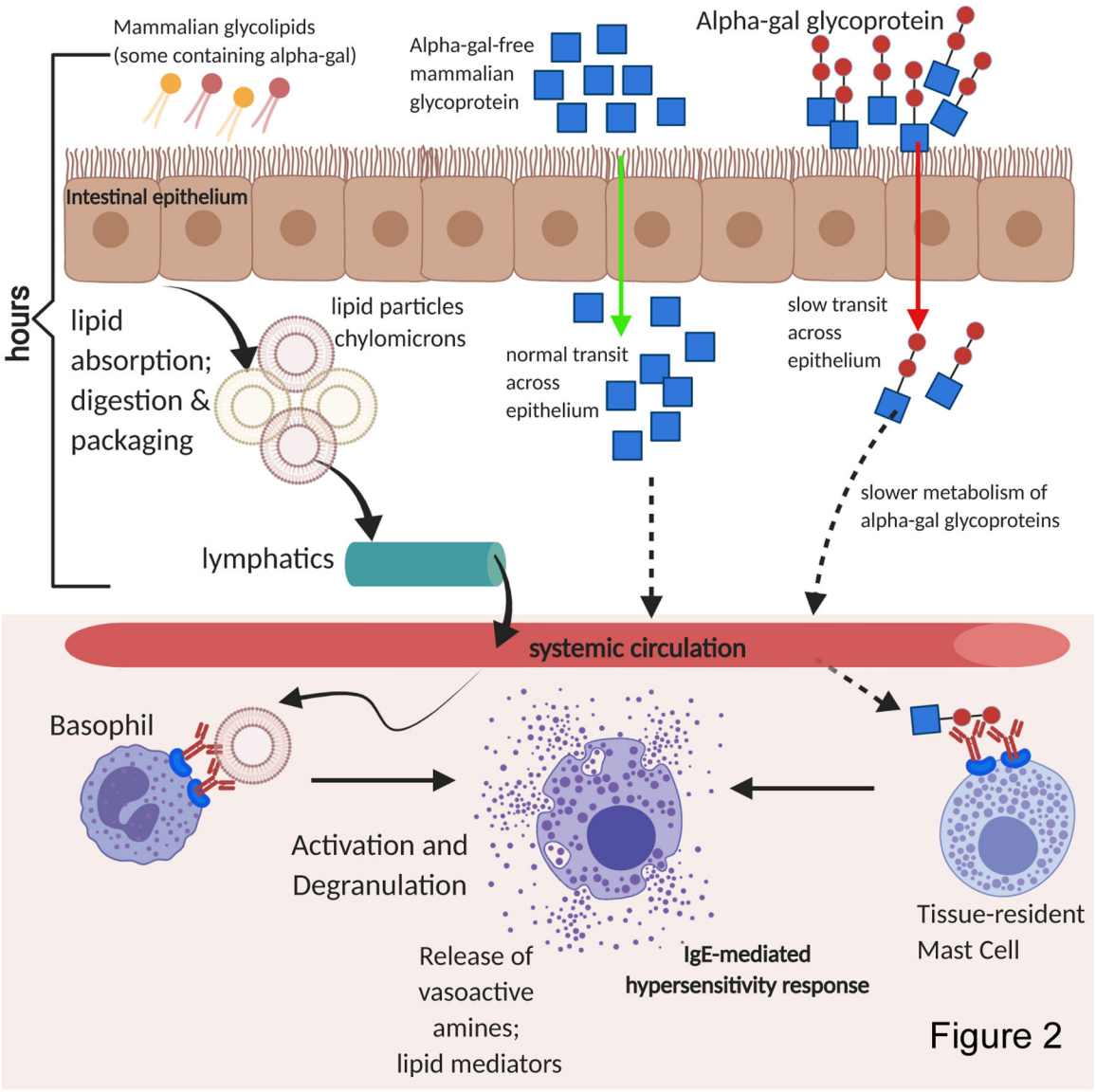

Figure 2.

Slow transit and metabolic processing of alpha-gal glycolipids and glycoproteins by the intestinal epithelium may contribute to delayed allergic responses to alpha-gal. Alpha-gal glycoproteins are hindered in moving across the intestinal epithelium and glycosylating a mammalian protein with alpha-gal may increase its resistance to digestion and trafficking time through the cellular endosomal compartments. After prolonged digestion and metabolism of alpha-gal glycoproteins and additional packaging of alpha-gal glycolipids into chylomicrons or other lipid particles, multimeric alpha-gal glycans (alpha-gal glycolipid or glycoprotein) crosslink alpha-gal sIgE-FcεRI complexes on circulating basophils and tissue resident mast-cells. The subsequent activation and degranulation generate vasoactive amines, lipid mediators and cytokines that drive the IgE-mediated hypersensitivity response.

Table I.

Global Reach of Alpha-gal Syndrome

Continent	Country	Associated Tick	Alpha-Gal Detected in Tick Saliva	References
Africa	Côte d'Ivoire	*Amblyomma variegatum?*	Unknown	Kaloga et al. (101); Reviewed in Cabezas-Cruz (11)
	South Africa			Mabelane et al. (20)
	Zimbabwe			Reviewed in van Nunen (102).
Asia	Korea	*Haemaphysalis longicornis* *Amblyomma testudinarium* *Ixodes niponnensis?*	Yes Unknown Unknown	Chinuki et al. (103) Hashizume et al. (14) Reviewed in van Nunen (45)
	Japan	*Amblyomma testudinarium* *Haemaphysalis longicornis*	Unknown Yes	Hashizume et al. (14) Chinuki et al. (103)
Australia		*Ixodes holocyclus* *Ixodes (Endopalpiger) australiensis*	Unknown Unknown	Reviewed in van Nunen(102) Kwak et al. (104)
Europe	France			Reviewed in van Nunen (102)
	Germany	*Ixodes ricinus*	Yes	Fischer et al. (50) Reviewed in van Nunen (102)
	Italy	*Ixodes ricinus*	Yes	Reviewed in van Nunen (45)
	Netherlands			Reviewed in Cabezas-Cruz et al. (11)
	Norway	*Ixodes ricinus*	Yes	Reviewed in Cabezas-Cruz et al. (11)
	Spain	*Rhipicephalus* spp.?	Yes (*Rhipicephalus microplus*)	Nunez et al. (105); Villar et al. (106)
	Sweden	*Ixodes ricinus*	Yes	Hamsten et al.(107); Hamsten et al.(108)
	Switzerland	*Ixodes ricinus*	Yes	Michel et al. (90)
	United Kingdom			Harper et al. (109)
North America	Costa Rica	*Amblyomma cajennense*	unknown	Reviewed in van Nunen (102)Cabezas-Cruz et al. (11)
	Panama	*Amblyomma cajennense*	unknown	Reviewed in van Nunen (45)and Cabezas-Cruz et al. (11)
	USA	*Amblyomma americanum*	Yes	Commins et al. (12); Crispell et al. (41)
South America	Brazil	*Amblyomma sculptum*	Yes	Araujo et al. (110)