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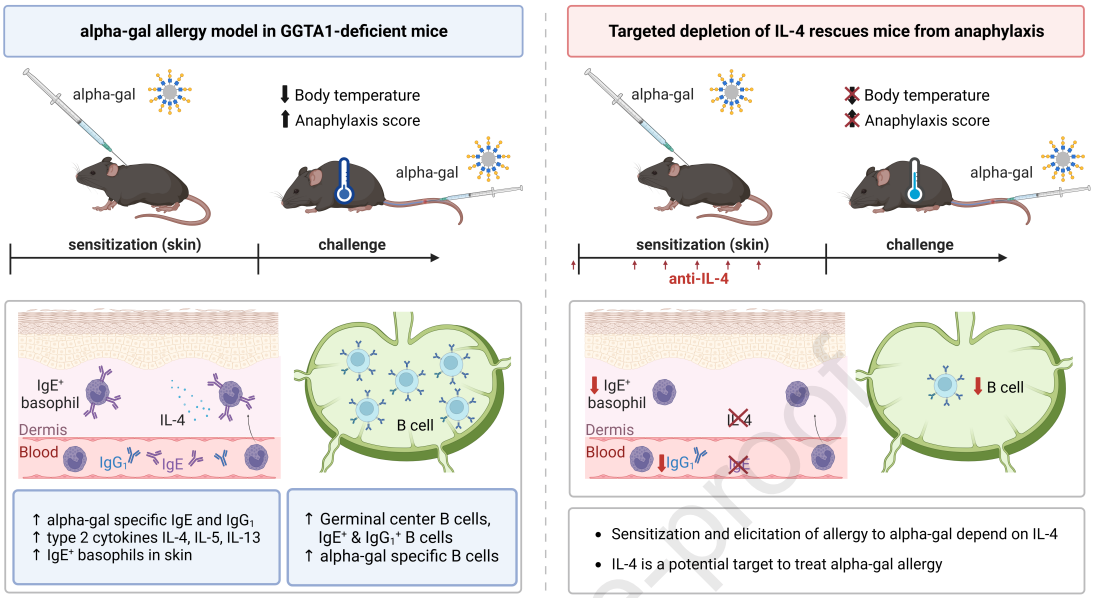

Abbreviations: alpha-gal, Galα1-3Galβ1-4GlcNAc; GGTA1, alpha-galactosyltransferase 1; IL-4, interleukin-4; Ig, immunglobulin

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4782 words

Abstract

Background: Alpha-gal (Galα1-3Galβ1-4GlcNAc) is a carbohydrate with the potential to elicit fatal allergic reactions to mammalian meat and drugs of mammalian origin. This type of allergy is induced by tick bites and therapeutic options for this skin-driven food allergy are limited to the avoidance of the allergen and treatment of symptoms. Thus, a better understanding of the immune mechanisms resulting in sensitization through the skin is crucial, especially in the case of a carbohydrate allergen for which underlying immune responses are poorly understood.

Objective: We aimed to establish a mouse model of alpha-gal allergy for in-depth immunological analyses.

Methods: GGTA1-deficient mice devoid of alpha-gal glycosylations were sensitized with the alpha-gal-carrying self-protein mouse serum albumin via repetitive intracutaneous injections in combination with the adjuvant aluminum hydroxide. The role of basophils and IL-4 in sensitization was investigated by using antibody-mediated depletion.

Results: Alpha-gal-sensitized mice displayed increased levels of alpha-gal-specific IgE and IgG_1 and developed systemic anaphylaxis upon challenge with both alpha-gal-containing glycoproteins and glycolipids. In accordance with alpha-gal-allergic patients, we detected elevated numbers of basophils at the site of sensitization as well as increased numbers of alpha-gal-specific B cells, germinal center B cells and B cells of IgE and IgG_1 isotypes in skin-draining lymph nodes. By depleting IL-4 during sensitization, we demonstrated for the first time that sensitization and elicitation of allergy to alpha-gal and correspondingly to a carbohydrate allergen is dependent on IL-4.

Conclusion: These findings establish IL-4 as a potential target to interfere with alpha-gal allergy elicited by tick bites.

Key messages:

- Repetitive percutaneous sensitization with an alpha-gal-carrying self-protein establishes alpha-gal-specific type 2 immunity, IgE antibodies, and anaphylaxis.
- Sensitization to alpha-gal and corresponding allergic reaction to the oligosaccharide depend on IL-4.
- The described model allows for in-depth analyses of alpha-gal-specific and corresponding carbohydrate-specific immune responses.

Capsule summary: Percutaneous sensitization and anaphylaxis to the carbohydrate alpha-gal depend on IL-4, thus making it a potential therapeutic target in red meat allergy.

Keywords: alpha-gal syndrome, Galα1-3Galβ1-4GlcNAc, GGTA1-deficient mouse model, red meat allergy, food allergy, anaphylaxis, IgE, IL-4

Abbreviations:

alpha-gal (Galα1-3Galβ1-4GlcNAc), MSA (mouse serum albumin), Ig (immunoglobulin), IL (interleukin), BAT (basophil activation test), GGTA1 (alpha-galactosyltransferase 1), alum (aluminum hydroxide), Mcpt1 (mast cell protease 1), sIgE (secreted IgE), FcεRI (Fc ε receptor I, high-affinity IgE receptor), LPS (lipopolysaccharide), BAFF (B cell activating factor).

Introduction

Food allergies affect 5-8% of the population in westernized countries and the incidence is continuing to further increase (1). Among food allergies, there are those in which sensitization is elicited through the skin such as in patients with atopic dermatitis or dysfunctional skin barriers as well as in patients with wheat-dependent exercise-induced anaphylaxis (2, 3) and in patients with allergy to Galα1-3Galβ1-4GlcNAc (alpha-gal), wherein repetitive tick bites elicit IgE to the carbohydrate alpha-gal (4). Therapeutic options are limited to avoidance of the allergen, induction of oral tolerance (as approved for peanut allergy) or treatment of symptoms (5). Thus, for the development of new treatment strategies, more insights into the mechanisms of food allergy initiation are of the utmost importance, especially for skin-driven food allergies and carbohydrate alpha-gal. Alpha-gal is a relatively recently identified food allergen that elicits potentially fatal allergic responses after the ingestion of mammalian meat or innards and the administration of drugs of mammalian origin such as specific therapeutic antibodies (6-12). Interestingly, allergic responses to alpha-gal often develop with a typical and unique delay in the occurrence of symptoms. This is thought to depend on glycolipids and may be related to a slower digestion process in the case of glycolipids compared to glycoproteins (13). Moreover, patients have been described to suffer from more severe allergic reactions after the consumption of fatty meat (14, 15). Alpha-gal or similar epitopes are ubiquitously expressed on many bacteria, fungi and parasites, as well as in all mammals except for old-world primates and humans (16, 17). Herein, during evolution, the expression of the enzyme alpha-galactosyltransferase, which transfers alpha-gal to lipids and proteins, was lost due to a frameshift mutation (18). Consequently, all humans initially develop tolerance to alpha-gal, which is likely mediated by contact with alpha-gal-containing food, as well as microbiota in the gastrointestinal tract, and this effect is associated with alpha-gal-specific antibodies of immunoglobulin (Ig) M and IgG isotypes (17, 19, 20). These antibodies can induce rejections after xenotransplantation, however, they likely offer an evolutionary

benefit against various factors, such as parasite infections (17, 21). In some individuals, repetitive cutaneous contact with alpha-gal results in the induction of alpha-gal-specific IgE antibodies and consequently sensitization (22). In a proportion of these individuals, allergy to red meat and other mammalian-derived food and drugs can develop (23). Currently, it is well accepted that this cutaneous sensitization is mediated by tick bites from several different tick species based on reports from different regions of the world (23-28).

The involvement of ticks in sensitization to alpha-gal has also been suggested by studies using animal models of tick feeding or injection of tick extracts (29-31). In these experiments, the cascade of immune events, as well as the contribution of glycoproteins versus glycolipids, was not examined based on the various proteins and lipids from ticks that were transferred to the skin of mice. Thus, the immune mechanisms induced following exposure to alpha-gal resulting in sensitization and the elicitation of allergic symptoms are still poorly understood, which is partially due to the carbohydrate nature of this allergen.

To perform in-depth analysis of alpha-gal sensitization and allergy, we established a mouse model involving intracutaneous injection of a synthetic alpha-gal-rich protein with alpha-gal bound to the self-protein mouse serum albumin (alpha-gal-MSA). This method allowed us to pinpoint the specific induced responses to alpha-gal. Mice were efficiently sensitized to alpha-gal after cutaneous administration of alpha-gal-MSA as shown by the induction of alpha-gal-specific IgG_1 and IgE antibodies; moreover, systemic anaphylaxis could be elicited upon subsequent challenge with alpha-gal-carrying glycoproteins and glycolipids. Immunological analysis demonstrated basophil infiltration to the sensitization site and enrichment of alpha-gal-specific B cells, germinal center B cells and B cells of IgE and IgG_1 isotypes in skin-draining lymph nodes of sensitized mice. These findings are in accordance with observations that have been described in alpha-gal-allergic patients. With antibody-mediated depletion of interleukin (IL)-4 during sensitization, we proved for the first time that sensitization and

elicitation of allergy to alpha-gal and correspondingly a carbohydrate allergen is dependent on IL-4. These findings establish IL-4 as a potential target to interfere with alpha-gal allergy elicited by tick bites. Furthermore, this model offers a tool for the in-depth analysis of alpha-gal-specific immune responses which will be essential for the understanding of alpha-gal syndrome and for the subsequent development of novel concepts to prevent or treat skin-driven food allergies in the future.

Methods

Human samples, alpha-gal-IgE detection using ImmunoCAP system, basophil activation test

Collection of healthy controls' and patients' blood was approved by the ethics committee (419/18 S-KK) and was preceded by information of the patient by a medical doctor including a signed consent. Patient history and other information were obtained by a questionnaire. Alpha-gal-specific IgE titers were determined using the ImmunoCAP system (ThermoFisher Scientific) according to the manufacturer's protocol. For basophil activation test, EDTA blood (S-Monovette, Sarstedt) was incubated with serial dilutions (500, 200, 40, 8, 1.6, 0.32 ng/ml) of alpha-gal-MSA (Galα1-3Galβ1-4GlcNAc-MSA, Dextra Laboratories, UK) or alpha-gal-human serum albumin (HSA) (Bühlmann) and simultaneously stained with antibodies for CCR3, CD203c and CD63 diluted in Flow CAST stimulation buffer (Bühlmann) at 37 °C for 15 min. in a water bath. After lysis of erythrocytes using ACK lysis buffer (Lonza), at least 300 basophils (SSC^{low} $CCR3^{+}$) were analyzed on either a BD FACSCanto™ II or a Beckman Coulter Cytoflex LX flow cytometer. Data was analyzed using FlowJo™ Software (BD Life Sciences) and normalized to the positive control (anti-FcεRI mAb, Bühlmann) by determining the ratio of the percentage of activated ($CD63^{+}$) basophils after stimulation with the alpha-gal-containing allergen to the percentage of activated basophils after stimulation with anti-FcεRI (% CD63+ basophils alpha-gal / anti-FcεRI) multiplied by 100. The cut-off for positivity was set to 15% activated basophils ($CD63^{+}$).

Animals and sensitization protocol

GGTA1-deficient mice were kindly provided by Peter Cowan (Immunology Research Centre St. Vincent's Hospital Melbourne, Australia) and Florian Kreppel (University of Ulm, Germany) and bred under specific opportunistic pathogen free (SOPF) conditions. All animal experiments were performed in accordance with national and institutional guidelines for

animal care and were approved by the Governmental Review Board Oberbayern (Regierung von Oberbayern). 8-14-week old female and male animals were used for experiments. For alpha-gal sensitization, mice were shaved at the back (1x1cm) and a mixture of 25 µg alpha-gal-MSA (Galα1-3Galβ1-4GlcNAc-MSA, Dextra Laboratories, UK; alpha-gal epitope density per molecule: 16-32) in PBS and Alu-Gel-S (alum; Serva), which was specifically designed for adjuvant use in human and veterinary vaccines, at a ratio of 1:1 were intracutaneously injected two times a week over 3 weeks. Controls were injected with PBS and alum. For depletion of basophils, 30µg of anti-CD200R3 antibody (clone Ba13, Biolegend) or isotype control (rat IgG2a isotype control, Biolegend) were intravenously injected one day before the first sensitization and subsequently every 3 days (7 injections in total, last injection at day 19). For depletion of IL-4, 1 mg of anti-IL-4 monoclonal antibody (clone 11B11, BioXCell) or isotype control (rat IgG1 isotype control, BioXCell) in PBS were intraperitoneally injected one day before the first sensitization and subsequently every 3 days starting at day 7 of the sensitization phase (6 injections in total, last injection at day 19).

Allergen challenge

One week after the last sensitization, allergen challenge was performed by intravenous injection of either alpha-gal-MSA (Dextra Laboratories, UK), glycolipids isolated from rabbit red blood cells (micelle size: 30-1000nm)(32) or mouse laminin (Sigma-Aldrich), all 200 µg in PBS. Subsequently, the core body temperature was measured every 7 minutes using a rectal probe (RET-3, World Precision Instruments). Additionally, the behavior and appearance of the mice were continuously monitored using a scoring system published by Li et al. (33) for at least one hour after allergen administration: 1: rubbing of snout / eyes. 2: edema (snout, eyelids), reduced activity, poloerection. 3: enforced breathing, cyanosis, sibilant rhonchus. Mice were sacrificed for organ sampling one day after the allergen challenge.

Results

Generation of a mouse model of alpha-gal allergy allows for in-depth analysis of antigen-specific immune responses

We aimed to establish an animal model of alpha-gal-elicited allergic responses that allows for the investigation of antigen- and corresponding carbohydrate-specific immune responses. To date, existing animal models of alpha-gal allergy in mice have used tick feeding or injections of tick extracts for sensitization (29-31). To guarantee that the elicited immune response is limited to the antigen of interest, the carbohydrate alpha-gal, we decided to use alpha-gal-MSA for sensitization in our new model. Due to the fact that alpha-gal-MSA is a synthetic glycoprotein resulting from coupling alpha-gal to mouse-derived MSA via a short linker, we first confirmed the functional relevance of alpha-gal-MSA in a real-world setting. Thus, we took advantage of our established patient cohort of alpha-gal-allergic patients (Table 1). Sensitization to alpha-gal was confirmed via detection of alpha-gal-specific IgE antibodies in patient (but not healthy control) serum. We applied the basophil activation test (BAT), which is a cellular test allowing to assess the allergenicity of alpha-gal-MSA (gating strategy: Fig.S1A) (34). The activation of patients' basophils by alpha-gal-MSA was comparable to that by alpha-gal coupled to human serum albumin (HSA), which is used in commercial assays to determine the reactivity of basophils to alpha-gal (Fig.1A). As expected, due to the absence of alpha-gal-specific IgE, neither alpha-gal-HSA nor -MSA induced basophil activation in healthy control blood (Fig.S1B). Importantly, alpha-gal-specific B cells could be detected in both patient and healthy individual blood by using biotinylated alpha-gal-MSA for detection (Fig.1B; gating strategy: Fig.S1C), which is in accordance with earlier reports showing that up to 1% of IgG antibodies in human blood are alpha-gal-specific (19). However, alpha-gal-specific B cells of the IgE isotype were a hallmark of alpha-gal-allergic patients, which was as expected and in conjunction with the detection of alpha-gal-IgE

antibodies restricted to patients' blood (Fig.1C). Thus, alpha-gal-MSA carries epitopes that are recognized by human immunoglobulins and B cells of different isotypes, thus demonstrating that it is suited for our further in vivo experiments.

To next establish a mouse model of alpha-gal allergy, we used a mouse line deficient in the enzyme that is responsible for attaching alpha-gal to proteins and lipids, the alpha-galactosyltransferase (GGTA1). Similar to humans, 'natural' alpha-gal-specific antibodies other than IgE and IgG_1 isotypes can be detected in these mice at steady-state (data not shown). We mimicked the tick bites that are responsible for alpha-gal sensitization in humans by using repetitive intracutaneous injections of alpha-gal-MSA together with the adjuvant aluminum hydroxide (alum) (Fig.2A). Efficient sensitization was confirmed via the detection of significantly elevated total serum IgE levels (Fig.2B), as well as alpha-gal-specific IgE (Fig.2C), in sensitized mice compared to control mice. In concordance with observations in alpha-gal-allergic patients, elevated alpha-gal-IgE levels were accompanied by significantly increased alpha-gal-specific IgG_1 levels (Fig.2D; (35)). Sensitized mice exhibited increased serum levels of the type 2 cytokines IL-4, IL-5 and IL-13, thus indicating that cutaneous administration of alpha-gal-MSA with alum results in a skewed type 2 immune response with IL-4 known to be underlying the switch to IgE production in response to protein allergens (Fig.2E). We subsequently challenged the mice via intravenous injection of alpha-gal-MSA. As a read-out for anaphylaxis, the core body temperature of the mice, which is a well-accepted measure for systemic anaphylaxis in mice, was rectally measured for at least one hour after intravenous administration (Fig.S2, Fig.2F; (36)). Furthermore, the mice were constantly monitored and their behavior and appearance were documented (anaphylaxis score, Fig.2G). Alpha-gal-sensitized but not control mice receiving PBS and alum during the prior sensitization period exhibited a significant decrease in body temperature and signs of anaphylaxis, such as reduced activity and edema. These allergic responses were specific to alpha-gal, due to the fact that alpha-gal-MSA-sensitized mice receiving intravenous MSA

failed to develop anaphylaxis (Fig.2F). Moreover, a significant increase in mast cell protease-1 (Mcpt1), which is a protease released by mast cells upon degranulation, was exclusively detected in mice sensitized to alpha-gal-MSA but not in control mice (Fig.2H).

Mammalian glycolipids elicit anaphylaxis in alpha-gal-MSA-sensitized mice

Both glycoproteins and glycolipids have been suggested to play a role in triggering allergic responses to alpha-gal after consumption of mammalian meat and innards (13, 32). However, whether sensitization to alpha-gal epitopes on proteins is sufficient to allow for the development of allergic responses to alpha-gal epitopes on glycolipids has never been shown. Thus, we next investigated the potential of glycolipids to trigger anaphylaxis in our mouse model after cutaneous sensitization with alpha-gal-MSA (Fig.3A). To this end, alpha-gal-carrying glycolipids were isolated from rabbit red blood cells that were previously shown to induce basophil activation in alpha-gal-allergic patients (32). Importantly, we detected significantly elevated levels of alpha-gal-specific IgG_1 antibodies by using alpha-gal-MSA as `catching´ antigens attached to the wells (Fig.3B), as well as by using coupled alpha-gal-carrying glycolipids (Fig.3C). An ELISA detecting IgE specific for alpha-gal on glycolipids could not be performed due to the nonapplicability of the used assay for lipids. This result indicates that alpha-gal-MSA-sensitized animals exhibit IgG_1 antibodies that are specific to the alpha-gal epitope on both proteins and lipids. Consequently, the challenge of mice sensitized to alpha-gal-MSA by using glycolipids efficiently induced anaphylaxis (Fig.3D+E), and induced significantly increased Mcpt1 serum levels (Fig.3F). In addition to glycolipids, we could also elicit anaphylaxis by using other glycoproteins that have been described to carry alpha-gal epitopes, such as laminin (Fig.S3) (37), although laminin administration resulted in less robust anaphylactic responses compared to alpha-gal-MSA or glycolipid challenge. This indicates that alpha-gal epitopes on laminin are either less abundant or differ from those on alpha-gal-MSA. Thus, our model may be suitable for investigating anaphylactic

responses to alpha-gal-carrying proteins and lipids, which are also sources for triggering anaphylaxis in alpha-gal-allergic patients.

Immune profiles induced by cutaneous alpha-gal sensitization resulting in IgE and IgG_1 responses

We next used our model to investigate immune cell subsets induced by intracutaneous exposure to alpha-gal-MSA and alum in the skin (Fig.4A-G) and in skin-draining lymph nodes (Fig.4H-N). We observed increased $CD45.2^+$ leukocyte infiltration in the skin of sensitized mice compared to control mice (Fig.4B, Fig.S4A) with a significant elevation of $CD3^-IgE^+$ cells binding IgE on the surface, likely via FcεRI, being observed (Fig.4C, Fig.S4B). Strikingly, $CD49b^+CD200R3^+$ basophils were significantly enriched (Figure 4D, Fig.S4C) and IgE-coated basophils were almost exclusively detectable in mice sensitized with alpha-gal-MSA (Fig.4E, Fig.S4D), which reflects the situation represented in sensitized humans, wherein basophils have been detected at the site of tick bites (38). Interestingly, IgE-coated basophils were also found in the skin-draining lymph nodes of sensitized mice (Fig.4I, Fig.S4F). In addition to basophils, the leukocyte population in the skin of sensitized mice also contained a substantial amount of $SiglecF^+FcεRI^-$ eosinophils (Fig.4F, Fig.S4E) and mast cells (Fig.4G), which have also been described to infiltrate the site of tick infestation in both animals and humans (39, 40). We focused our subsequent analysis on B cells as the underlying cell type for the humoral immune response to alpha-gal including IgE production by investigating the draining lymph nodes (Fig.4H-N). By using alpha-gal-MSA coupled to biotin as a detection reagent, we observed alpha-gal-specific $CD19^+B220^+$ B cells almost exclusively in the draining lymph nodes of sensitized mice (Fig.4J, Fig.S4G). Importantly, ex vivo stimulation of sorted alpha-gal-specific B cells induced the secretion of alpha-gal-specific antibodies, thus also proving the specificity of the assay (Fig.4K). Moreover, $GL7^+Fas^+$ germinal center B cells (Fig.4L, Fig.S4H) as well as B cells of the IgE and IgG_1

isotypes (Fig.4M+N, Fig.S4I+J) were significantly enriched in mice sensitized with alpha-gal compared to controls. Altogether, this indicates that the systemic IL-4 detected in mice sensitized to alpha-gal (Fig.2E) could be responsible for the switch to alpha-gal-specific IgE production.

The contribution of basophils is not essential for sensitization to alpha-gal.

Due to the fact that basophils were significantly enriched in sensitized skin, we subsequently investigated their role in sensitization to alpha-gal by treating mice with anti-CD200R3 antibody during the course of sensitization to deplete basophils (Fig.5A). Flow cytometry and qPCR confirmed the efficient depletion of basophils in the skin and draining lymph nodes (Fig.5B+C). Levels of total IgE, as well as alpha-gal-specific IgG_1, were not affected by the depletion (Fig.5D+E), and we detected no protection from anaphylaxis in basophil-depleted mice upon allergen challenge (Fig.5F+G).

IgE and anaphylaxis specific to the carbohydrate alpha-gal depend on IL-4

Allergic sensitization to protein allergens resulting in the induction of allergen-specific anaphylactic IgE antibodies requires the type 2 cytokines IL-4 and, to a minor extent, IL-13 (41-43). Based on our results, we wondered whether sensitization with the glycoprotein alpha-gal-MSA and subsequent IgE production to alpha-gal, a carbohydrate, is also dependent on IL-4. Thus, we applied the anti-IL-4 antibody in our model to deplete IL-4 before and during the sensitization phase (Fig.6A). Impressively, the induction of IgE antibodies in response to sensitization was almost completely abolished upon the depletion of IL-4 (Fig.6B). Alpha-gal-specific IgE levels were only detectable in individual mice of the sensitized isotype control group and were under the detection limit for others (data not shown), however, the induction of alpha-gal-specific IgG_1 antibodies was reduced in sensitized mice upon IL-4 depletion (Fig.6C). Furthermore, in skin-draining lymph nodes, the expression of mRNA encoding secreted IgE (*sIgE*) was completely abolished (Fig.6D), thus reflecting the failed induction of

IgE class switching of B cells downstream of the immune cascade within the skin. Indeed, IL-4 expression was also significantly reduced in the draining lymph nodes of mice after IL-4 depletion (Fig.6E), thus indicating a feedforward loop of IL-4 expression that is strongly diminished upon depletion of this cytokine. This was confirmed via ex vivo stimulation of T cells isolated from draining lymph nodes, which showed a complete absence of secreted IL-4 in culture supernatants when isolated from IL-4-depleted mice (Fig.6F). Most importantly, and in concordance with the failed induction of IgE in draining lymph nodes, the proof-of-concept experiment challenging both groups of mice with alpha-gal demonstrated that mice depleted of IL-4 failed to develop anaphylaxis as shown by the absence of any behavioral signs of anaphylaxis and the reduced maximal temperature decrease (Fig.6G+H). IgE^+ cells, including IgE-coated basophils, were also reduced at the site of sensitization in the skin but were not completely abolished, thus indicating that factors other than IL-4 drive infiltration into the skin (Fig.6I+J). Importantly, by investigating skin-draining lymph nodes, we detected significantly reduced numbers of germinal center B cells (Fig.6K), IgE B cells (Fig.6L) and IgG_1 B cells at the control level (Fig.6M), thus demonstrating the crucial role of IL-4 in IgE production specific for the carbohydrate allergen alpha-gal. Thus, as known for IgE induction in response to protein allergens, IgE response to the carbohydrate alpha-gal strictly depends on IL-4.

Discussion

The term alpha-gal syndrome originated following the relatively recent identification of the food allergy to red meat and other mammalian-derived foods, which was paralleled by potential reactivity to drugs of mammalian origin (6, 10, 44). All of these allergic reactions develop based on specific IgE to alpha-gal. Tick bites are well accepted as being the cause of skin-derived sensitization toward alpha-gal; consequently, alpha-gal syndrome is part of the increasing group of food allergies, in which sensitization is elicited through the skin (2, 3). A better understanding of the underlying immune cascade of events in the skin resulting in sensitization and manifestation of food allergy is urgently needed. In this study, we established a mouse model to specifically investigate the role of (i) alpha-gal using an alpha-gal-carrying self-protein to induce alpha-gal-specific IgE and B cells and (ii) basophils and IL-4 in this carbohydrate-specific immune response and allergy. Previous studies in animal models using injections of tick extracts or even tick feeding for sensitization proved the concept of percutaneous sensitization for alpha-gal allergy (29-31). The adjuvant alum is well studied and used in both animal models and humans (45, 46). Interestingly, one study compared tick extracts versus alum as adjuvants in sensitization, and they demonstrated similar increases in allergen-specific IgE antibody levels, as well as T and B cell subsets, in the draining lymph nodes, thus indicating that alum is a suitable adjuvant to study alpha-gal sensitization (29). We improved upon these insights from animal models and patients in that we applied alpha-gal coupled to a murine carrier protein (MSA) by using repetitive intracutaneous injections. Consequently, possible bystander effects by nonself proteins and lipids derived from ticks can be excluded and the outcome of the elicited response can be directly attributed to the carbohydrate alpha-gal. Possible immune effects of the adjuvant alum were controlled via intradermal injections of PBS plus alum in control groups, as well as with the comparison of ‘PBS plus alum’ to ‘untreated’ or to ‘PBS only’ injected skin in several experiments that did not show any differences, thus suggesting that alum mainly

supports the immune response induced by alpha-gal. However, by itself, it only has a minor impact on the immune system in our model.

As an important first step of evaluation, we confirmed the functional relevance of our antigen of choice, alpha-gal-MSA, by using our established cohort of alpha-gal-allergic patients. Indeed, alpha-gal-MSA efficiently activated basophils in alpha-gal-allergic patients; importantly, it also allowed for the detection of alpha-gal-specific B cells. Thus, although the linked alpha-gal epitopes in alpha-gal-MSA are reduced in number and complexity compared to carbohydrate structures that are present in mammalian meat, alpha-gal-MSA allows for efficient binding (i) to IgE bound to FcεRI on human basophils and (ii) to human B cell receptors, including those of the IgE isotype. By using a self-protein as a protein backbone for alpha-gal in our model, it was highly unlikely that allergic reactions could occur in response to MSA alone in alpha-gal-MSA-sensitized mice. However, due to the structure of alpha-gal-MSA consisting of the trisaccharide Galα1-3Galβ1-4GlcNAc coupled to the carrier protein MSA by a short linker, it was important to exclude the elicitation of anaphylaxis by the carrier itself. Indeed, the challenge of alpha-gal-MSA-sensitized mice with MSA did not result in any signs of anaphylaxis. Interestingly, intradermal injections of uncoupled alpha-gal failed to induce alpha-gal-specific IgE or IgG_1, thus indicating that, similar to haptens and bacterial polysaccharides (47, 48), a carrier protein is also relevant for type 1 sensitization to a carbohydrate antigen.

In alpha-gal-allergic patients, allergic symptoms upon oral ingestion of alpha-gal sources such as red meat often develop with a typical delay, in contrast to the immediate reactions that are triggered by intravenous administration, such as that of cetuximab. This phenomenon of delay in patients is thought to depend on glycolipids (13). Indeed, the role of glycolipids versus glycoproteins in regard to alpha-gal epitopes and the elicitation of sensitization and allergic symptoms is a matter of debate. For the first time, we demonstrate that sensitization induced

by alpha-gal on a carrier protein is sufficient to elicit allergic symptoms with alpha-gal-carrying glycolipids. In addition, alpha-gal-MSA-sensitized mice also developed allergic symptoms upon challenge with the natural protein laminin, albeit with a response that was generally milder than the responses elicited with alpha-gal-MSA. Laminins are glycoproteins and components of the extracellular matrix, and we hypothesized that the alpha-gal present on laminin (i) is less abundant and/or (ii) exists as differently accessible epitopes, with only parts of them allowing for the binding of IgE that is developed in response to alpha-gal-MSA. Further research in this aspect is ongoing. However, these observations indicate that the relatively simple alpha-gal epitopes that are present on alpha-gal-MSA can induce a humoral immune response, which correspondingly allows glycolipids and -proteins that contain more complex carbohydrate structures (32) to trigger anaphylactic responses by crosslinking alpha-gal-specific IgE on mast cells and basophils. Thus, our model allows for the investigation of various alpha-gal sources including glycoproteins and glycolipids.

Thus far, we have focused our analyses on the immunological mechanisms resulting in sensitization to alpha-gal by using the elicitation of anaphylaxis mainly as a readout for sensitization efficiency. We chose intravenous administrations of alpha-gal for the challenge to standardize the readout for effective sensitization and to avoid an impact of digestion and intestinal uptake. This reflects the anaphylaxis in patients caused by alpha-gal-containing intravenous therapeutics, such as cetuximab exhibiting complete penetrance, whereas delayed anaphylaxis triggered by red meat varies considerably and may depend on cofactors (9). Thus, this scenario represents a limitation of this study; however, we plan to include oral challenges in future analyses focusing more on the elicitation of anaphylaxis, especially in regard to the delayed responses to alpha-gal upon oral ingestions. Herein, the oral administration of glycolipids is of particular interest to better understand their potential role in retarding allergic symptoms during digestion.

Most interestingly, we demonstrated several similarities when comparing findings in our mouse model to findings observed in alpha-gal-allergic patients, thus further strengthening the value of our new model. (i) Immune cell phenotyping of skin revealed an immune cell infiltrate enriched in basophils, eosinophils and mast cells at the site of sensitization in our mouse model. Strikingly, basophils, eosinophils and mast cells have been reported to infiltrate the site of tick reinfestation in various animal models including guinea pigs and mice as well as in humans (39, 40). Basophils have even been shown to be critically involved in acquired resistance to tick feeding (49, 50); most interestingly, basophils are enriched at the site of the tick bite in alpha-gal-allergic patients (38). The depletion of basophils during the sensitization phase did not impact the subsequent anaphylactic response, thus indicating that basophil contribution is not critical for sensitization and/or elicitation of anaphylaxis to alpha-gal, but they may act as an initial source of IL-4 during the early sensitization phase. (ii) Repetitive cutaneous injection of alpha-gal-MSA induced alpha-gal-specific IgG_1 and IgE. In humans, repetitive tick bites induce alpha-gal-specific IgE which is accompanied by elevated levels of alpha-gal-specific IgG_1 (23, 24, 27, 35, 51, 52). Although it is well accepted that mouse IgG_1 corresponds to human IgG_4 and IgG_4 is linked to tolerance, e.g. induced in allergen immunotherapy (AIT), by potentially competing with IgE for allergen binding, it is difficult to define direct homolog between human and murine Ig isotypes (53, 54). IL-4 and IL-13 induce class switching to IgG_1 and IgE in mice and to IgG_1, IgG_4 and IgE in humans. Thus, both IgG_1 and IgG_4 in humans can be clearly linked to allergic type 2 responses. Indeed, other studies involving mouse models of food allergy induced by proteins have shown similar results to our study, in that murine IgG_1 was induced together with IgE (55, 56). Moreover, although the transfer of total IgG from allergic mice to recipients dampened the allergic response, IgG_1 alone did not cause this effect (56). Thus, murine IgG_1 does not share the tolerance-favoring attributes of human IgG_4 in allergic inflammation. (iii) Most but not all sensitized mice developed anaphylaxis upon allergen challenge, which is similar to the situation in humans,

wherein only approximately 8% of individuals with detectable alpha-gal-IgE (> 0.35 kU/ml) who are thus classified as being sensitized can develop allergic symptoms in response to alpha-gal exposure.

Due to the fact that carbohydrate-specific immune responses have thus far been sparsely investigated in general, our model not only allows for the investigation of alpha-gal allergy but is also ideally suited for the detailed analysis of carbohydrate-specific humoral and cellular immune responses. This understanding is also highly relevant beyond allergy research due to the manifold contributions of carbohydrates to immune regulation, cancer immune control and vaccine development (57-59).

Most importantly, we showed that IL-4 is indispensable for efficient IgE sensitization and subsequent elicitation of anaphylaxis to alpha-gal. For protein antigens, it is well accepted that the induction of IgE antibodies requires IL-4 as the key effector cytokine (41, 43). However, for a carbohydrate allergen such as alpha-gal, this effect is still not known. By depleting IL-4 before and during sensitization, we showed that, similar to what is known for protein allergens, IgE induction was completely abolished, and IgG_1 levels were strongly reduced in the absence of IL-4. IL-4 secretion by T cells was completely abolished in IL-4-depleted mice accompanied by the complete absence of IgE induction, thus indicating that the IL-4 needed for class switching to IgE is mainly produced by T cells. The first analyses on T cells including ex vivo restimulation in our model did not demonstrate any significant differences (data not shown); however, detailed analyses of alpha-gal-specific T cells are planned, which will likely require modifications of the protocols generated for analysis of protein-specific T cells. The requirement of IL-4 for alpha-gal sensitization is highly relevant for patient care. It is known that the avoidance of tick bites reduces alpha-gal-specific IgE, whereas new bites can boost IgE levels along with stronger allergic responses (23, 24). Dupilumab, which is a monoclonal antibody targeting the IL-4 receptor α subunit and approved for several atopic

diseases associated with type 2 immune responses (such as atopic dermatitis and asthma), has also been investigated as being a potential treatment option for other allergic diseases including IgE-mediated food allergy (60). Based on our findings and the peculiar setting of exclusive percutaneous sensitization/booster in alpha-gal allergy, the blockage of IL-4 signaling may even reduce disease activity when directly applied after (new) tick bites. Even though this is a hypothesis and needs confirmation by depleting IL-4 after efficient sensitization and monitoring treatment efficacy via subsequent alpha-gal rechallenge, in selected patients/individuals, adding dupilumab or the appropriate JAK inhibitors as a type of emergency treatment following tick bites could be beneficial.

In conclusion, we developed a mouse model of alpha-gal allergy, which allowed for in-depth analysis of alpha-gal-triggered anaphylactic responses to various alpha-gal sources as well as alpha-gal- and thus carbohydrate-specific immune responses in general. Indeed, our first analyses prove the crucial role of IL-4 in the development of type 2 immunity and IgE to alpha-gal following percutaneous exposure and therefore, we propose IL-4 signaling as a potential therapeutic target.

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Figure Legends:

Figure 1: Alpha-gal-MSA elicits basophil activation in alpha-gal-allergic patients and allows for the detection of antigen-specific B cells. (A) Dose-dependent in vitro basophil activation in patients with alpha-gal allergy using increasing concentrations of alpha-gal-HSA or alpha-gal-MSA for stimulation. Unst.: unstimulated control, HSA: human serum albumin, MSA: mouse serum albumin. (B+C) Alpha-gal-specific B cells in whole blood from healthy controls (white) or alpha-gal-allergic patients (gray). Exemplary plots of flow cytometric detection of IgE^+ alpha-gal-specific B cells by using biotinylated alpha-gal-MSA as antigen in the blood of one healthy donor (healthy, left) and one patient (patient, right) are shown on the right. Shown are (A) box plots with whiskers from minimum to maximum of 6 allergic patients or (B+C) the mean with SEM; each data point represents one individual. Data were analyzed by using FlowJo and GraphPad Prism Software. Statistical analysis was performed by using (A) one-way ANOVA followed by Bonferroni correction or (B+C) Student's t test. **: $p<0.01$.

Figure 2: Mouse model of red meat allergy. (A) Schematic view of the sensitization protocol. GGTA1-ko mice were repetitively injected with alpha-gal-MSA (black circles / gray bars) or vehicle (white circle / white bars) in combination with the adjuvant alum and subsequently challenged via intravenous injections of alpha-gal-MSA or MSA as a control. Serum levels of (B) total IgE, alpha-gal-specific(C) IgE and (D) IgG_1 antibodies. (E) Serum cytokine levels of IL-5, IL-4 and IL-13. (F) Maximal decrease in core body temperature within 1 hour after intravenous injection of alpha-gal-MSA or MSA as a control. (G) Anaphylaxis score of mice upon challenge with alpha-gal-MSA. (H) Serum levels of mouse mast cell protease-1 (Mcpt1). Data are representative of at least 10 individual experiments; (E) was obtained from one experiment with 4 control and 5 sensitized animals or (F) was obtained from two experiments with 6 control and 10 sensitized animals, and each dot

represents one single mouse. Data were analyzed by using Microsoft Excel and GraphPad Prism. Shown are the mean with SEM. **: $p<0.01$, *: $p<0.05$.

Figure 3: Alpha-gal-carrying glycolipids elicit anaphylaxis in mice sensitized with alpha-gal-MSA. (A) Schematic view of the sensitization protocol as described in Fig. 2A but with intravenous injection of glycolipids extracted from rabbit red blood cells for allergen challenge instead of alpha-gal-MSA. Serum levels of (B) alpha-gal-MSA-specific IgG_1 and (C) glycolipid-specific IgG_1. (D) Maximal decrease in core body temperature and (E) anaphylaxis score within 1 hour after intravenous injection of glycolipids. (F) Mouse mast cell protease-1 (Mcpt1) levels. The presented data were obtained from two independent experiments (B+C) with 6 control and 8 sensitized mice or are representative of two independent experiments (D-F) with 3-4 mice per group. Data were analyzed by using Microsoft Excel and GraphPad Prism. Shown are the mean with SEM. **: $p<0.01$, *: $p<0.05$.

Figure 4: Skin basophils and IgE^+ B cell responses in skin-draining lymph nodes are enriched in sensitized mice. Single cell suspensions of murine skin biopsies (A-F) or draining lymph nodes (H-N) from control (PBS, white) and sensitized (alpha-gal-MSA, black) mice were analyzed via flow cytometry. (A) Gating strategy for skin to identify (B) total leukocytes, (C) IgE^+ cells (D) basophils, (E) IgE-coated basophils and (F) eosinophils. (G) Representative example of toluidine blue-stained sections of sensitized skin (left; scale bar: 100 µm), magnification thereof (middle; scale bar: 50 µm) and mast cell quantification (right). (H) Gating strategy for lymph nodes to identify (I) IgE-coated basophils, (J) antigen-specific B cells, (L) germinal center B cells and (M) IgE- or (N) IgG_1-specific B cells. Data are representative of at least 5 individual experiments or were obtained from one experiment (G) with 4 control and 8 sensitized mice, and each dot represents one single mouse. (K) Alpha-gal-specific (blue) and control (green) B cells from skin-draining lymph nodes of sensitized mice were stimulated ex vivo and alpha-gal-specific IgG_1 antibodies in culture

supernatants were determined. Data were analyzed by using FlowJo and GraphPad Prism Software. Shown are the mean with SEM. ****: $p<0.0001$, ***: $p<0.001$, **: $p<0.01$, *: $p<0.05$.

Figure 5: Basophil contribution is not essential for sensitization to alpha-gal. (A) Schematic view of the protocol. GGTA1-ko mice were intravenously injected with an anti-CD200R3 antibody (blue) or isotype control (black) before and throughout the sensitization with alpha-gal-MSA (filled circles) or vehicle (open circles) plus alum. Antibody injections are depicted by dashed arrows. (B) Total number of IgE-coated basophils in the skin as determined by flow cytometry. (C) Relative gene expression of *Ccr3* in skin-draining lymph nodes. Serum titers of (D) total IgE antibodies and (E) alpha-gal-specific IgG_1. (F) Anaphylaxis score and (G) maximal decrease in core body temperature of mice within 1 hour after intravenous injection. Data were obtained from one experiment with 3-4 mice per group. Shown are the mean with SEM. ***:$p<0.001$, *: $p<0.05$.

Figure 6: IL-4 is essential for efficient sensitization and elicitation of anaphylaxis to alpha-gal. (A) Schematic view of the protocol. Intraperitoneal injection of GGTA1-ko mice with anti-IL-4 antibody (blue) or isotype control (black) throughout the sensitization with alpha-gal (filled circles) or vehicle (open circles) plus alum. Antibody injections are depicted by dashed arrows. Serum titers of (B) total IgE and (C) alpha-gal-specific IgG_1. Relative gene expression levels of (D) secreted IgE (*sIgE*) and (E) *Il4* in skin-draining lymph nodes. (F) IL-4 levels in culture supernatants of restimulated draining lymph node cells. (G) Anaphylaxis score and (H) maximal decrease in core body temperature within 1 hour after intravenous injection of alpha-gal-MSA. Single cell suspensions from (I+J) skin and (K-M) draining lymph nodes were analyzed via flow cytometry. Total cell numbers of (I) IgE^+ cells (J) IgE-coated basophils, (K) germinal center B cells and B cells of the (L) IgE and (M) IgG_1 isotypes were determined, as outlined in Figure 4. Data were obtained from (B-E) two independent

709 experiments with 4 control and 7 sensitized mice or (F-M) are representative of two
710 experiments with 3-5 mice per group. Shown are the mean with SEM. ****: $p<0.0001$, ***:
711 $p<0.001$, **: $p<0.01$, *: $p<0.05$.

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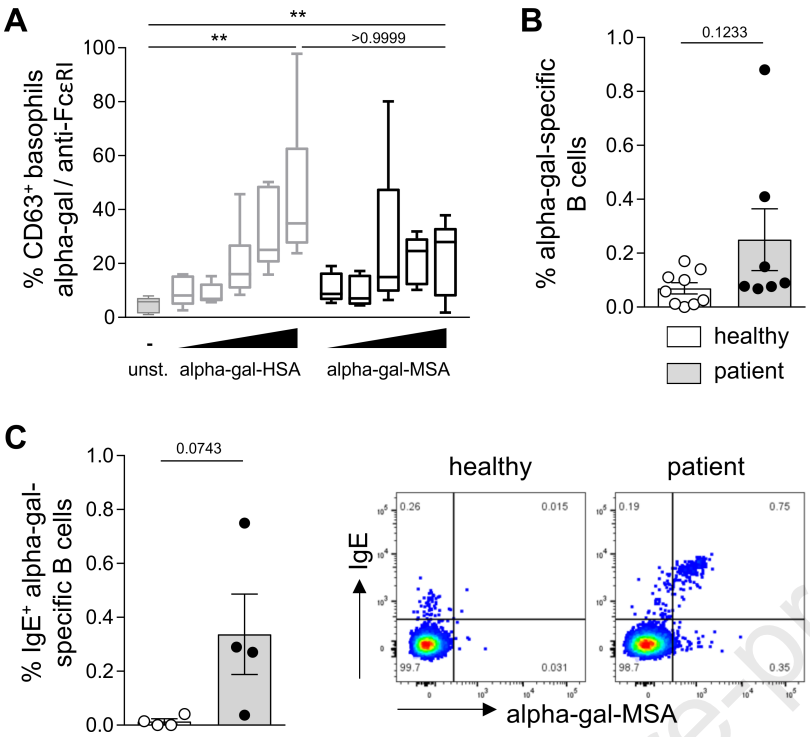
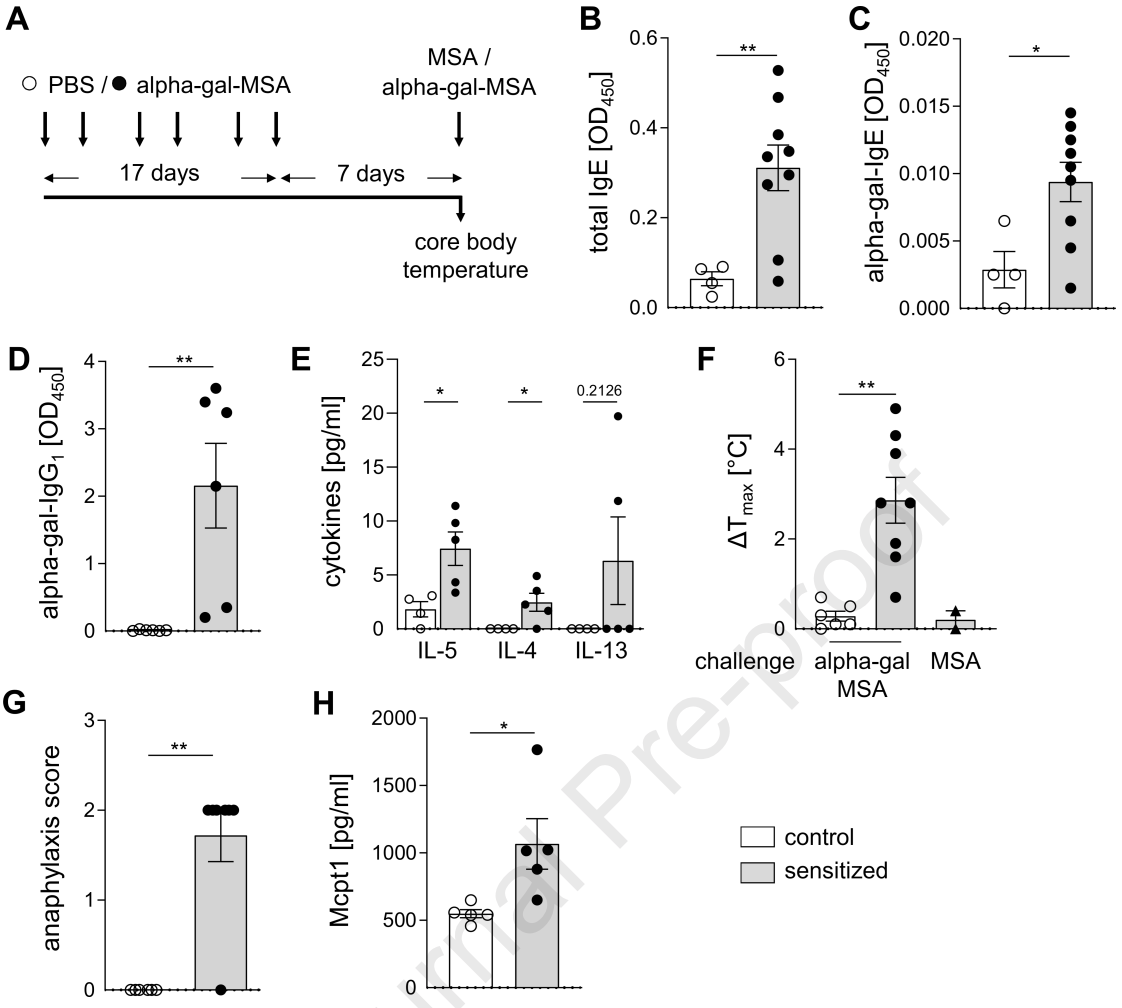
Table 1: Characteristics of alpha-gal-allergic patients.

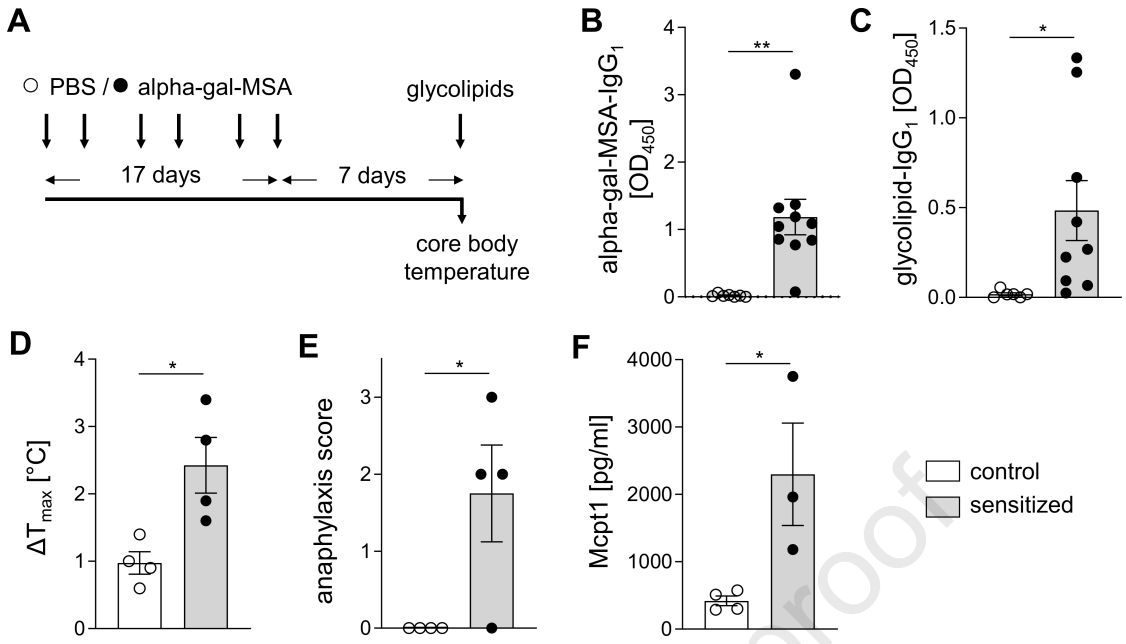
Characteristics	Number of patients (n)	%
Gender		
Male	5	55.5
Female	4	44.5
Age (years)		
<65	7	77.7
>65	2	33.3
Tick bite history		
<5	3	33.3
≥5	6	66.7
Reaction to tick bite*		
yes	6	66.7
no	3	33.3
Residential habitat		
rural	6	66.6
urban	3	33.3
ImmunoCAP score**		
mild	0	0
medium	8	88.8
severe	1	11.1

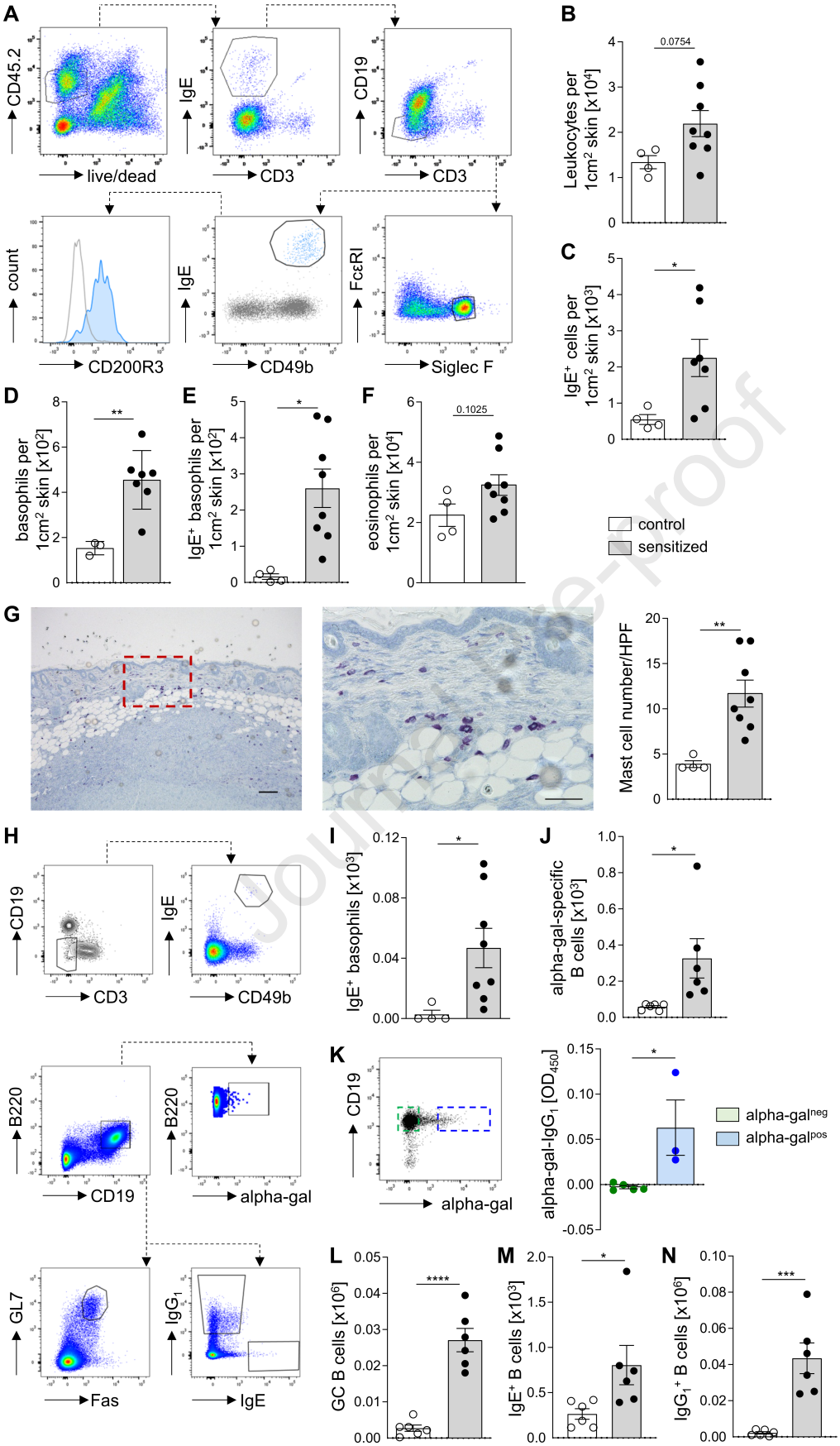
*allergic reaction (reddening, edema, pruritus) in a region of a former tick bite

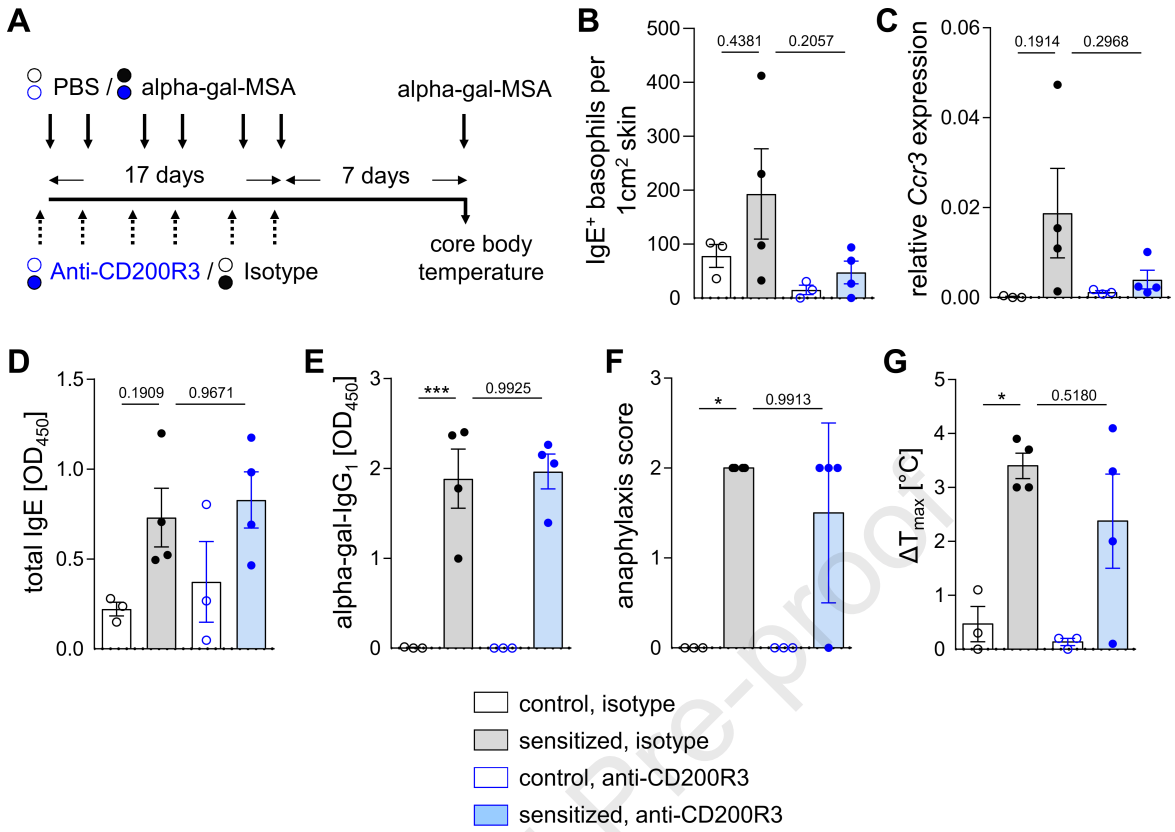
**corresponds to alpha-gal-IgE [kUA/l]: mild=0.35-0.7, medium=0.7-17.5, severe=17.5-100

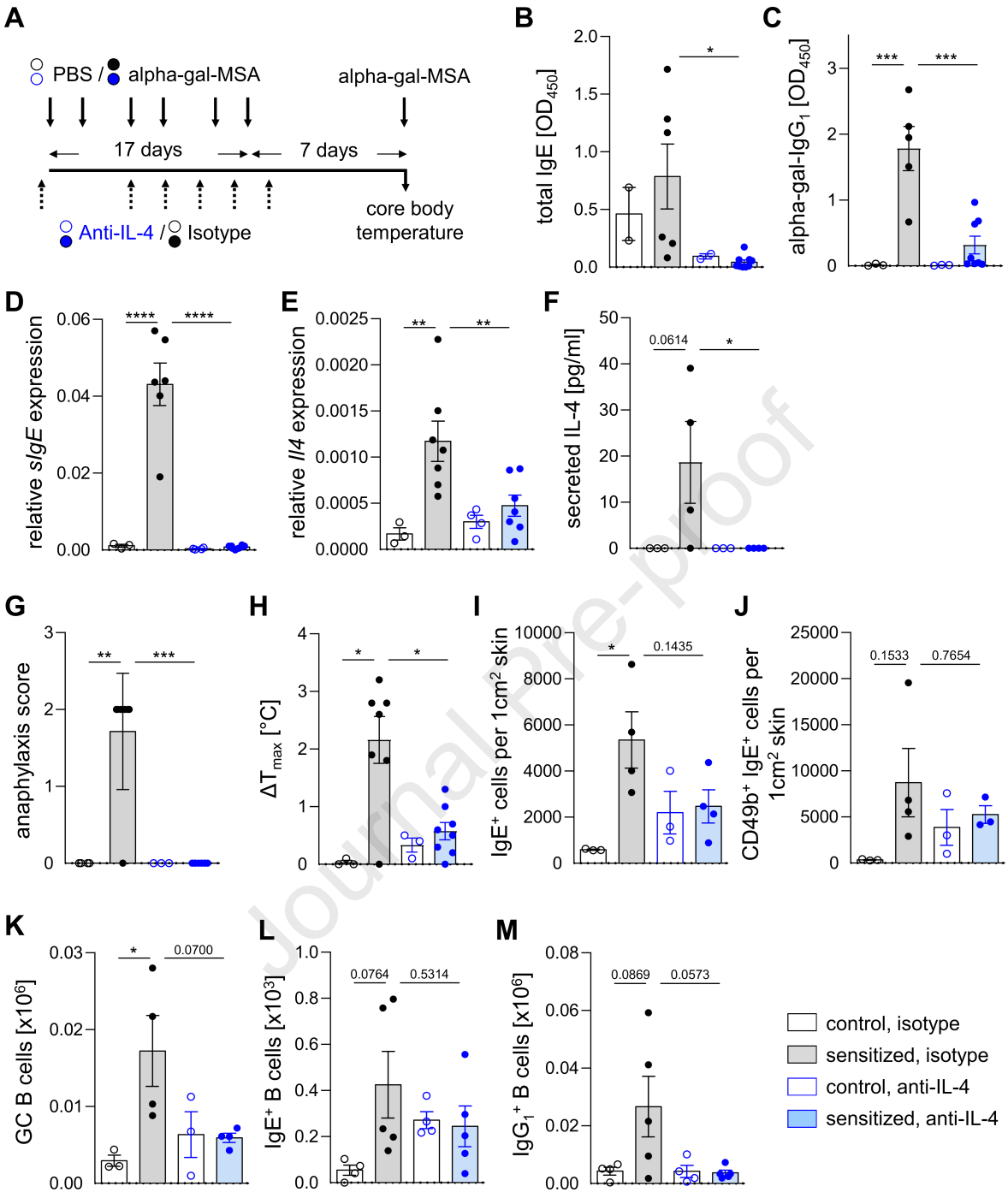







A
CD45.2
live/dead
IgE
CD3
CD19
CD3
count
CD200R3
IgE
CD49b
FcεRI
Siglec F
B
Leukocytes per 1cm² skin [x10⁴]
0.0754
C
IgE⁺ cells per 1cm² skin [x10³]
control
sensitized
D
basophils per 1cm² skin [x10²]
E
IgE⁺ basophils per 1cm² skin [x10²]
F
eosinophils per 1cm² skin [x10⁴]
0.1025
G
Mast cell number/HPF
H
CD19
CD3
IgE
CD49b
B220
CD19
B220
alpha-gal
GL7
Fas
IgG1
IgE
I
IgE⁺ basophils [x10³]
J
alpha-gal-specific B cells [x10³]
K
CD19
alpha-gal
alpha-gal-IgG1 [OD450]
alpha-galneg
alpha-galpos
L
GC B cells [x10⁶]
M
IgE⁺ B cells [x10³]
N
IgG1⁺ B cells [x10⁶]





IgE and anaphylaxis specific to the carbohydrate alpha-gal depend on interleukin-4

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Repository Material

ELISA assays

Total IgE (mouse IgE ELISA Set, BD Biosciences) and Mcpt1 (Invitrogen) ELISAs were performed according to the manufacture's protocol. For detection of alpha-gal specific IgE, Thermo Scientific™ Nunc MicroWell 96-Well plates were coated with purified anti-IgE antibodies (Biolegend, clone RME-1) in sodium carbonate coating buffer overnight at 4 °C. After blocking and washing, undiluted murine serum was added and incubated overnight at 4 °C. Alpha-gal specific antibodies were subsequently detected using biotinylated alpha-gal-MSA (alpha-gal-MSA (Dextra Laboratories) biotinylated using a protein biotin labeling kit (Roche) according to the manufacturer's protocol) which was detected using streptavidin coupled to HRP (Biolegend) and TMB substrate reagent (BD Biosciences) after overnight incubation at 4 °C. Alpha-gal specific IgG_1 was detected as follows: plates were coated with alpha-gal-MSA or glycolipids in sodium carbonate coating buffer overnight at 4 °C. After blocking and incubation with murine serum (diluted 1:50 in blocking buffer), IgG_1 was detected using a biotinylated anti-IgG_1 antibody (Biolegend, clone RMG1-1), streptavidin coupled to HRP and TMB substrate reagent as indicated above.

Preparation of single cell suspensions and flow cytometry

Skin draining lymph nodes (axillary, inguinal) were dissociated in FACS buffer (PBS + 2 % FCS) using a 70 µM cell strainer (EASYstrainer, Greiner Bio-one) and subsequently used for

flow cytometric analysis. For isolation of skin immune cells, a 1x1 cm piece was excised from the site of sensitization and digested overnight at 4 °C using 2.5 mg/ml dispase II (Sigma-Aldrich). Next, dermis and epidermis were separated using forceps and digested in 0.25 mg/ml liberase TL (Roche) for 75 min at 37 °C, followed by dissociation of the digested skin piece through a 100 µM cell strainer (EASYstrainer, Greiner Bio-one) and FACS buffer for washing. Single cell suspensions of skin and skin draining lymph nodes were then used for flow cytometric analysis. After blocking Fc receptors using mouse CD16/32 Tru stain fcX (Biolegend), dead cells were stained using Live/Dead™ Fixable Aqua dead cell stain kit (Invitrogen) in PBS for 20 min at 4 °C. Subsequently, surface antigens were stained with antibodies specific for the indicated markers in FACS buffer for 20 min at 4 °C. Murine tissues: CD45.2 (Biolegend, clone 104), CD3 (Biolegend, clone 145-2C11), IgE (Biolegend, clone RME-1), CD49b (Biolegend, clone DX5), CD200R3 (Biolegend, clone Ba13), Siglec F (Biolegend, clone S17007L), CD19 (eBioscience, clone 1D3), B220 (Biolegend, clone RA3-6B2), CD95/Fas (Biolegend, clone SA367H8), GL7 (Biolegend, clone GL7), IgG_1 (Biolegend, clone RMG1-1). Human blood: CCR3 (Biolegend, clone 5.e8), CD203c (Biolegend, clone NP4D6), CD63 (Biolegend, clone H5C6), CD19 (Biolegend, clone HIB19), IgE (Miltenyi Biotec, clone MB10-5C4). For staining of murine and human alpha-gal specific B cells, single cell suspensions were incubated with biotinylated alpha-gal-MSA (Dextra laboratories) for 30 min after surface staining followed by staining with fluorochrome-coupled streptavidin (Biolegend) or an anti-biotin antibody (Milteyi Biotec, clone Bio3-18E7) for 20 min at 4 °C. Cells were analyzed on either a BD FACSCanto™ II or a Beckman Coulter Cytoflex LX flow cytometer. Flow cytometry-based cell sorting was performed on a BD FACSAria™ Fusion. Data was analyzed using FlowJo™ Software (BD Life Sciences. All flow cytometric analyses included exclusion of debris (FSC-A vs. SSC-A) and doublets (FSC-A vs. FSC-H). Total cell numbers from skin were normalized to the weight of the excised skin piece. Total cell numbers of populations in draining lymph nodes were calculated

using total cell counts of single cell suspensions as determined using a Neubauer counting chamber or an automated cell counter (CellDrop BF, DeNovix).

Histology

Histology sections from paraffin-embedded tissues were toluidine stained. Sections were analyzed using a Keyence BZ-X810 All-in-one fluorescence microscope (Keyence Corporation) with 10x and 50x magnification. Images were processed using BZ-X800 Analyzer software, version 1.1.2.4 (Keyence Corporation).

RNA isolation, cDNA synthesis, qPCR

RNA from skin draining lymph nodes was isolated using Trizol-Chloroform extraction according to the manufacturer's protocol (TRIzol reagent from ThermoFisher Scientific). cDNA was synthesized using the TaqMan Gold RT-PCR kit and Oligo(dT)18 primers according to the manufacture's protocol (ThermoFisher Scientific). cDNA was subsequently used for qPCR analysis using PowerUp SYBR Green Master Mix (ThermoFisher Scientific) and the following primer pairs: *GAPDH* fd: ACCCAGAAGACTGTGGATGG, *GAPDH* rev: CACATTGGGGGTAGGAACAC, *sIgE* primer pair as published by He et al. (29), *mIL4* fd: GACGGCACAGAGCTATTGATG, *mIL4* rev: ACCTTGGAAGCCCTACAGACG. Gene expression data was normalized to expression of the housekeeping gene, Gapdh.

Ex vivo T cell stimulation, bead-based cytokine assay

0.5*10^6 skin draining lymph node cells were stimulated with 2.5 µl CD3/CD28 activator beads (Thermofisher) per 96well in complete RPMI medium supplemented with 10 % fetal calf serum, L-glutamine, penicillin, streptomycin, HEPES, non-essential amino acids and beta-mercaptoethanol. Supernatants were harvested after 24 h and stored at -20 °C until the bead-based cytokine assay was performed according to the manufacturer's protocol (LEGENDplex™ MU Th Cytokine Panel (12-plex)).

Isolation of alpha-gal specific B cells, ex vivo B cell stimulation

Single cell suspensions of skin-draining lymph nodes were prepared as described. Subsequently, alpha-gal specific and control B cells were isolated using flow cytometry-based cell sorting (living $CD3^-$ $CD19^+$ alpha-gal $MSA^{+/-}$) on a BD FACSAria™ Fusion. 1000-2000 B cells per 96 well, sorted from a pool of 3-4 mice each, were cultured ex vivo in complete RPMI medium supplemented with 1 µg/ml lipopolysaccharide from *Salmonella minnesota* R595 (LPS, Alexis Biochemicals), 2 µg/ml anti-CD40 antibody (clone 1C10, Biolegend), 10 ng/ml IL-4 (Peprotech) and 100 ng/ml B cell activating factor (BAFF, Biolegend). Culture supernatants were harvested after 6 days and alpha-gal specific IgG_1 antibodies were determined by ELISA as described.

Statistical analyses

Statistical analyses were performed using GraphPad Prism. Statistical significance for two groups was performed using an unpaired two-tailed Student's t-test, except for anaphylaxis scores which were analyzed using Mann-Whitney test. For three or more groups, statistical significance was determined using an ANOVA with Bonferroni's multiple comparison test or Dunn's multiple comparison test for analysis of anaphylaxis scores. Results are shown as mean ± standard error of the mean (SEM). P values smaller than 0.05 were considered significant.

Repository Figure Legends

S1 (A) Gating strategy to define activated basophils after stimulation with different concentrations of antigen. (B) Dose-dependent in vitro basophil activation in healthy controls using increasing concentrations of alpha-gal-HSA or alpha-gal-MSA for stimulation. Unst.: unstimulated control, HSA: human serum albumin, MSA: mouse serum albumin. (C) Gating strategy to define alpha-gal-specific B cell subsets in human blood. Alpha-gal-specific B cells were detected using biotinylated alpha-gal-MSA, a MSA-biotin stained sample was used as gating control.

S2 Core body temperature was determined every 7 minutes in mice sensitized to alpha-gal-MSA and challenged with alpha-gal-MSA (black symbols) or MSA (white symbols) as control. Each symbol represents a single mouse.

S3 (A) Schematic view of the sensitization protocol. Alpha-gal-deficient GGTA1-ko mice were repetitively injected with alpha-gal-MSA (black circles / light gray bars) or vehicle (white circle / white bars) in combination with the adjuvant alum into the back skin. One week after the last sensitization, mice were challenged via intravenous injection of laminin, followed by measurement of the core body temperature, scoring of behavior as well as organ sampling for subsequent analyses. (B) Serum levels of laminin-specific IgE antibodies as determined by ELISA. (C) Maximal decrease in core body temperature and (D) anaphylaxis score of mice within 1 hour after intravenous injection of laminin. Data presented are the pool of 3 individual experiments, each dot represents one single mouse. Data were analyzed using Microsoft Excel and GraphPad Prism. Shown are the mean with SEM. Statistical analysis was performed using Student‘s t-test or Mann-Whitney test for anaphylaxis scores (D).

S4 Relative abundances of cell populations in skin (A-E) and skin-draining lymph node (F-J) single cell suspensions. Populations were defined as outlined in Figure 4.