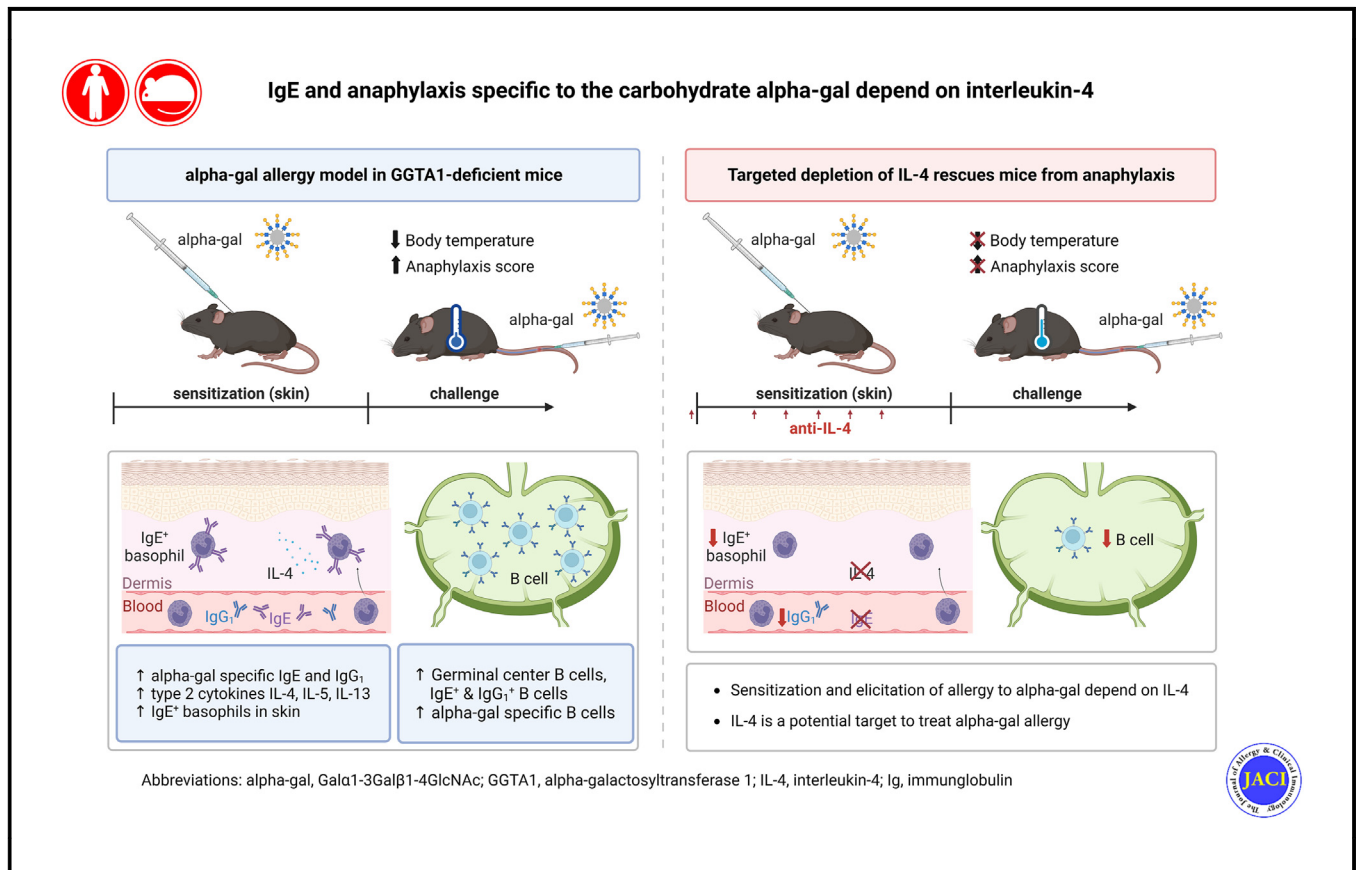


# IgE and anaphylaxis specific to the carbohydrate alpha-gal depend on IL-4

Check for updates

Miriam Hils, PhD, Nils Hoffard, MSc, Caterina Iuliano, MSc, Luisa Kreft, PhD, Neera Chakrapani, PhD, Kyra Swiontek, MSc, et al

## GRAPHICAL ABSTRACT



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

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



Miriam Hils, PhD,<sup>a</sup> Nils Hoffard, MSc,<sup>a</sup> Caterina Iuliano, MSc,<sup>a</sup> Luisa Kreft, PhD,<sup>b</sup> Neera Chakrapani, PhD,<sup>c,d</sup> Kyra Swiontek, MSc,<sup>c</sup> Konrad Fischer, PhD,<sup>e</sup> Bernadette Eberlein, MD,<sup>a</sup> Martin Köberle, PhD,<sup>a</sup> Jörg Fischer, MD,<sup>f,g</sup> Christiane Hilger, PhD,<sup>c</sup> Caspar Ohnmacht, PhD,<sup>b</sup> Susanne Kaesler, PhD,<sup>a</sup> Florian Wölbing, MD,<sup>a</sup> and Tilo Biedermann, MD<sup>a</sup> *Munich, Neuherberg, Freising, Tübingen, and Augsburg, Germany; and Esch-sur-Alzette, Luxembourg*

**Background:** Alpha-gal (Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc) 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 immunologic analyses.

**Methods:** Alpha-galactosyltransferase 1-deficient mice devoid of alpha-gal glycosylations were sensitized with the alpha-gal-carrying self-protein mouse serum albumin by repetitive intracutaneous injections in combination with the adjuvant aluminum hydroxide. The role of basophils and IL-4 in sensitization was investigated by antibody-mediated depletion. **Results:** Alpha-gal-sensitized mice displayed increased levels of alpha-gal-specific IgE and IgG<sub>1</sub> and developed systemic anaphylaxis on 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<sub>1</sub> 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

## Abbreviations used

Alpha-gal: Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc  
Alum: Aluminum hydroxide  
GGTA1: Alpha-galactosyltransferase 1  
HSA: Human serum albumin  
Mcp1: Mast cell protease 1  
MSA: Mouse serum albumin  
qPCR: Real-time quantitative PCR

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. (*J Allergy Clin Immunol* 2024;153:1050-62.)

**Key words:** Alpha-gal syndrome, Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc, GGTA1-deficient mouse model, red meat allergy, food allergy, anaphylaxis, IgE, IL-4

Food allergies affect 5% to 8% of the population in Westernized countries; the incidence continues to increase further.<sup>1</sup> 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<sup>2,3</sup> and in patients with allergy to Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc (alpha-gal), wherein repetitive tick bites elicit IgE to the carbohydrate alpha-gal.<sup>4</sup> Therapeutic options are limited to avoidance of the allergen, induction of oral tolerance (as approved for peanut allergy), or treatment of symptoms.<sup>5</sup> 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.<sup>6-12</sup> 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.<sup>13</sup> Moreover, patients have been described to experience more severe allergic reactions after consuming fatty meat.<sup>14,15</sup> 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.<sup>16,17</sup>

From <sup>a</sup>the Department of Dermatology and Allergy Biederstein, School of Medicine, Technical University of Munich, Munich; <sup>b</sup>the Center of Allergy and Environment (ZAUM) and Institute of Allergy Research, Technical University of Munich, School of Medicine, and Helmholtz Center Munich, Research Center for Environmental Health, Neuherberg; <sup>c</sup>the Department of Infection and Immunity, Luxembourg Institute of Health (LIH), Esch-sur-Alzette; <sup>d</sup>the Faculty of Science, Technology and Medicine, University of Luxembourg, Esch-sur-Alzette; <sup>e</sup>Department of Livestock Biotechnology, School of Life Sciences Weihenstephan, Technical University of Munich, Freising; <sup>f</sup>the Department of Dermatology, Faculty of Medicine, Eberhard Karls University Tübingen, Tübingen; and <sup>g</sup>the Department of Dermatology and Allergology, University Hospital Augsburg, Augsburg.

Received for publication May 15, 2023; revised November 23, 2023; accepted for publication December 1, 2023.

Available online December 21, 2023.

Corresponding author: Tilo Biedermann, MD, Technical University of Munich, Biedersteinerstrasse 29, 80802 Munich, Germany. E-mail: [tilo.biedermann@tum.de](mailto:tilo.biedermann@tum.de).

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749

© 2023 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

<https://doi.org/10.1016/j.jaci.2023.12.003>

**TABLE I.** Characteristics of alpha-gal–allergic patients

| Characteristic                     | No. | %    |
|------------------------------------|-----|------|
| Sex                                |     |      |
| Male                               | 5   | 55.5 |
| Female                             | 4   | 44.5 |
| Age                                |     |      |
| <65 years                          | 7   | 77.7 |
| >65 years                          | 2   | 33.3 |
| Tick-bite history                  |     |      |
| <5 bites                           | 3   | 33.3 |
| ≥5 bites                           | 6   | 66.7 |
| Experienced 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 region of former tick bite.

†Corresponds to alpha-gal–IgE (kUA/L): mild = 0.35-0.7, medium = 0.7-17.5, and severe = 17.5-100.

During evolution, the expression of the enzyme alpha-galactosyltransferase, which transfers alpha-gal to lipids and proteins, was lost as a result of a frameshift mutation.<sup>18</sup> 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 IgM and IgG isotypes.<sup>17,19,20</sup> These antibodies can induce rejections after xenotransplantation; however, they likely offer an evolutionary benefit against various factors, such as parasite infections.<sup>17,21</sup> In some individuals, repetitive cutaneous contact with alpha-gal results in the induction of alpha-gal–specific IgE antibodies and consequently sensitization.<sup>22</sup> In a proportion of these individuals, allergy to red meat and other mammalian-derived food and drugs can develop.<sup>23</sup> Currently, on the basis of reports from different regions of the world, it is well accepted that this cutaneous sensitization is mediated by tick bites from several different tick species.<sup>23-28</sup>

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.<sup>29-31</sup> In these experiments, the cascade of immune events, as well as the contribution of glycoproteins versus glycolipids, was not examined according to the various proteins and lipids from ticks that were transferred to the skin of mice. Thus, the immune mechanisms induced after 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 (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<sub>1</sub> and IgE antibodies; moreover, systemic anaphylaxis could be elicited on subsequent

challenge with alpha-gal–carrying glycoproteins and glycolipids. Immunologic 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<sub>1</sub> 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 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.

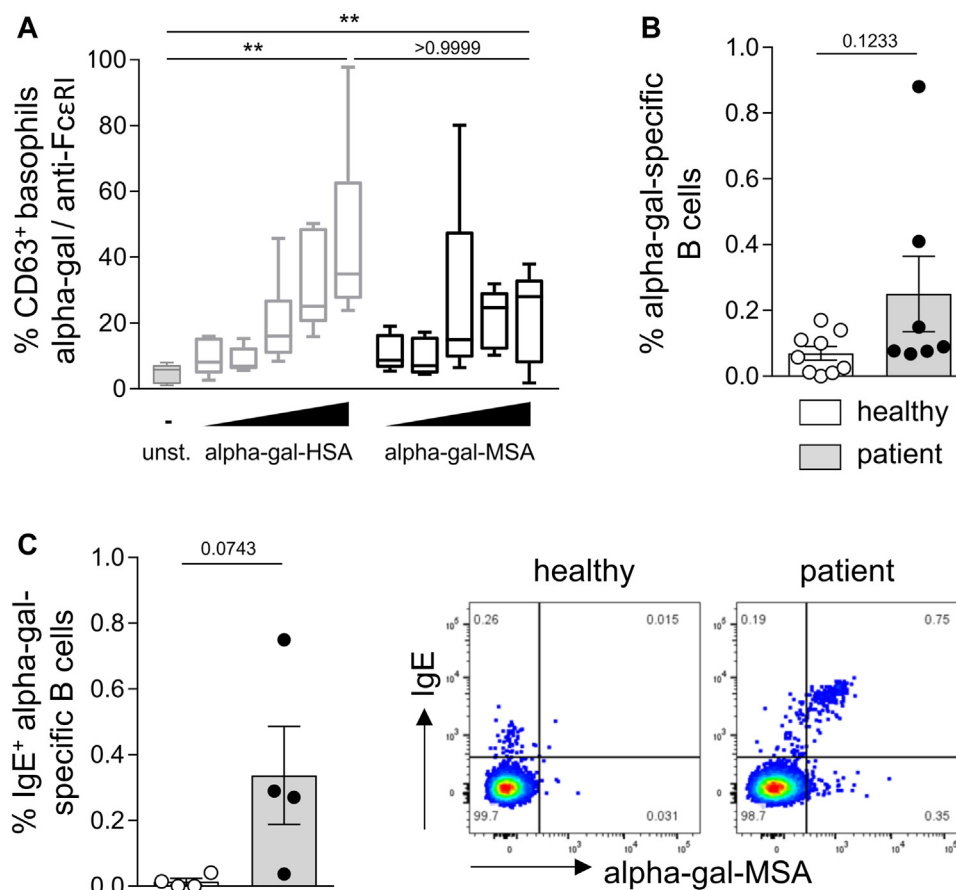
## METHODS

### Human samples, alpha-gal–IgE detection, and basophil activation test

Collection of healthy controls' and patients' blood was approved by the ethics committee (419/18 S-KK) and was preceded by a medical doctor's obtaining patient information, including signed consent. Patient history and other information were obtained by a questionnaire. Alpha-gal–specific IgE titers were determined by the ImmunoCAP system (Thermo Fisher Scientific) according to the manufacturer's protocol. For basophil activation testing, EDTA blood (S-Monovette, Sarstedt) was incubated with serial dilutions (500, 200, 40, 8, 1.6, and 0.32 ng/mL) of alpha-gal–MSA (Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc–MSA, Dextra Laboratories) 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 minutes in a water bath. After lysis of erythrocytes with ACK lysis buffer (Lonza), at least 300 basophils (SSC<sup>low</sup>CCR3<sup>+</sup>) were analyzed on either a BD FACSCanto II or a Beckman Coulter Cytoflex LX flow cytometer. Data were analyzed by FlowJo software (Becton Dickinson) and normalized to the positive control (anti-Fc $\epsilon$ RI mAb, Bühlmann) by determining the ratio of the percentage of activated (CD63<sup>+</sup>) basophils after stimulation with the alpha-gal–containing allergen to the percentage of activated basophils after stimulation with anti-Fc $\epsilon$ RI (percentage CD63<sup>+</sup> basophils alpha-gal/anti-Fc $\epsilon$ RI) multiplied by 100. The cutoff for positivity was set to 15% activated basophils (CD63<sup>+</sup>).

### Animals and sensitization protocol

Alpha-galactosyltransferase 1 (GGTA1)-deficient mice were kindly provided by Peter Cowan (Immunology Research Centre, St Vincent's Hospital, Melbourne, Australia) and Florian Kreppel (University of Ulm, Ulm, Germany) and bred under specific opportunistic pathogen-free conditions. All animal experiments were performed in accordance with national and institutional guidelines for animal care and were approved by Governmental Review Board Oberbayern (Regierung von Oberbayern). Eight- to 14-week old female and male animals were used for experiments. For alpha-gal sensitization, mice were shaved at the back (1 × 1 cm) and a mixture of 25  $\mu$ g alpha-gal–MSA



**FIG 1.** Alpha-gal-MSA elicits basophil activation in alpha-gal-allergic patients and allows for 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. **(B and 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<sup>+</sup> alpha-gal-specific B cells by using biotinylated alpha-gal-MSA as antigen in blood of 1 healthy donor (*left*) and 1 patient (*right*) are shown at *right*. Shown are **(A)** box plots with whiskers from minimum to maximum of 6 allergic patients and **(B and C)** means with SEMs; each data point represents 1 individual. Data were analyzed by FlowJo and GraphPad Prism. Statistical analysis was performed by **(A)** 1-way ANOVA followed by Bonferroni correction or **(B and C)** Student *t* test. \*\**P* < .01.

(Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc-MSA, Dextra Laboratories; alpha-gal epitope density per molecule, 16-32) in PBS and Alu-Gel-S (aluminum hydroxide [alum]; Serva), which was specifically designed for adjuvant use in human and veterinary vaccines, at a ratio of 1:1 were intracutaneously injected 2 times a week over 3 weeks. Controls were injected with PBS and alum. For depletion of basophils, 30  $\mu$ g of anti-CD200R3 antibody (clone Ba13, BioLegend) or isotype control (rat IgG<sub>2a</sub> isotype control, BioLegend) were intravenously injected 1 day before the first sensitization and subsequently every 3 days (7 injections in total, with the 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 IgG<sub>1</sub> isotype control, BioXcell) in PBS were intraperitoneally injected 1 day before the first sensitization and subsequently every 3 days starting at day 7 of the sensitization phase (6 injections in total, with the last injection at day 19).

### Allergen challenge

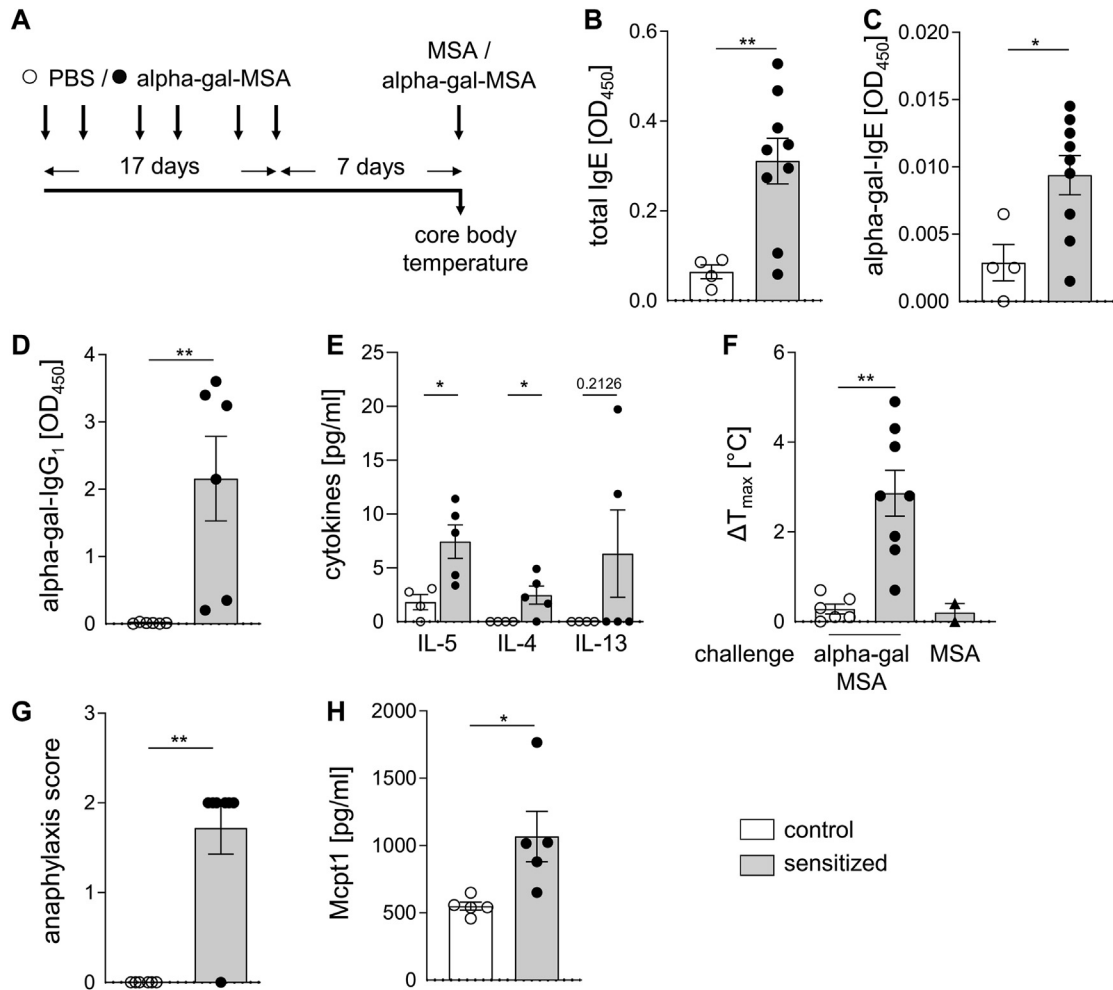
One week after the last sensitization, allergen challenge was performed by intravenous injection of either alpha-gal-MSA

(Dextra Laboratories), glycolipids isolated from rabbit red blood cells (micelle size, 30-1000 nm),<sup>32</sup> or mouse laminin (Sigma-Aldrich), all 200  $\mu$ g in PBS. Subsequently, the core body temperature was measured every 7 minutes with 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<sup>33</sup> for at least 1 hour after allergen administration, as follows: 1, rubbing of snout and/or eyes; 2, edema (snout, eyelids), reduced activity, and piloerection; and 3, enforced breathing, cyanosis, and sibilant rhonchus. Mice were humanely killed for organ sampling 1 day after 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,

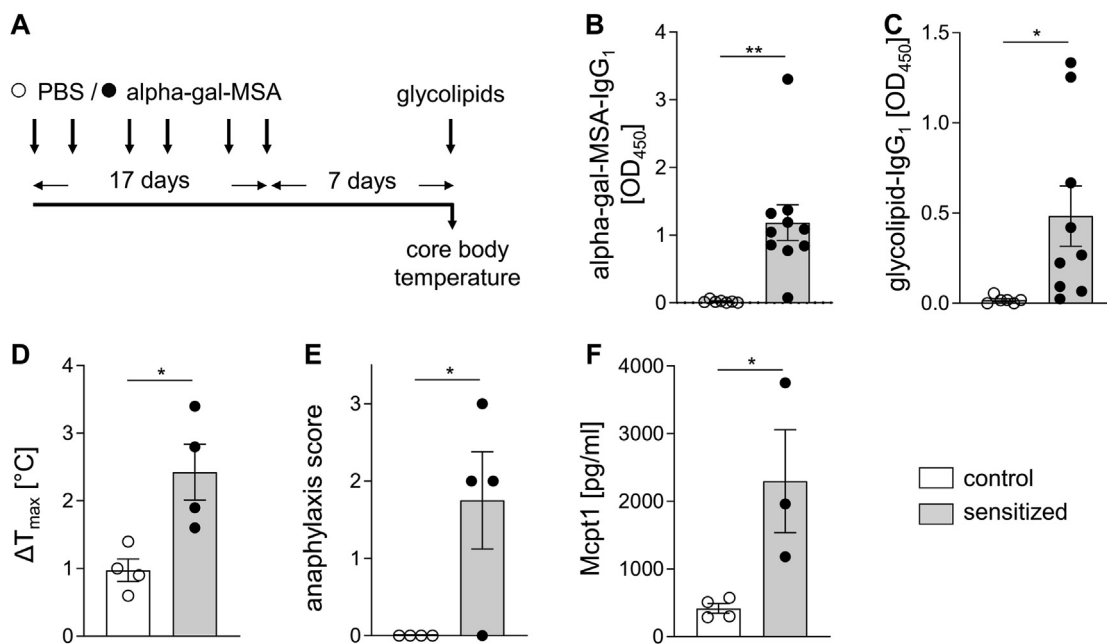


**FIG 2.** Mouse model of red meat allergy. **(A)** Schematic view of sensitization protocol. GGTA1-knockout mice were repetitively injected with alpha-gal-MSA (black circle, gray bars) or vehicle (white circle, white bars) in combination with adjuvant alum and subsequently challenged by intravenous injections of alpha-gal-MSA or MSA as control. Serum levels of **(B)** total IgE and alpha-gal-specific **(C)** IgE and **(D)** IgG<sub>1</sub> 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 control. **(G)** Anaphylaxis score of mice on challenge with alpha-gal-MSA. **(H)** Serum levels of mouse Mcpt1. Data are representative of at least 10 individual experiments; data for **(E)** were obtained from 1 experiment with 4 control and 5 sensitized animals or **(F)** were obtained from 2 experiments with 6 control and 10 sensitized animals; each dot represents 1 mouse. Data were analyzed by Microsoft Excel and GraphPad Prism. Data are shown as means with SEMs. \*\**P* < .01, \**P* < .05.

existing animal models of alpha-gal allergy in mice have used tick feeding or injections of tick extracts for sensitization.<sup>29-31</sup> 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. Because 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 I). Sensitization to alpha-gal was confirmed by detection of alpha-gal-specific IgE antibodies in patient (but not healthy control) serum. We applied the basophil activation test, which is a cellular test allowing to assess the allergenicity of alpha-gal-MSA. The gating strategy is shown in Fig E1, A, which is available in this article's Online Repository available at [www.jacionline.org](http://www.jacionline.org).<sup>34</sup> The activation of

patients' basophils by alpha-gal-MSA was comparable to that by alpha-gal coupled to HSA, which is used in commercial assays to determine the reactivity of basophils to alpha-gal (Fig 1, A). As expected, as a result of the absence of alpha-gal-specific IgE, neither alpha-gal-HSA nor -MSA induced basophil activation in healthy control blood (Fig E1, B). 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 1, B; the gating strategy is provided in Fig E1, C), which is in accordance with earlier reports showing that up to 1% of IgG antibodies in human blood are alpha-gal specific.<sup>19</sup> 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 1, C). Thus, alpha-gal-MSA carries epitopes that are recognized by human immunoglobulins and B cells of different





**FIG 3.** Alpha-gal-carrying glycolipids elicit anaphylaxis in mice sensitized with alpha-gal-MSA. **(A)** Schematic view of sensitization protocol as described in Fig 2, A, 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<sub>1</sub> and **(C)** glycolipid-specific IgG<sub>1</sub>. **(D)** Maximal decrease in core body temperature and **(E)** anaphylaxis score within 1 hour after intravenous injection of glycolipids. **(F)** Mouse Mcpt1 levels. Presented data were obtained from 2 independent experiments (*B* and *C*) with 6 control and 8 sensitized mice or are representative of 2 independent experiments (*D-F*) with 3 or 4 mice per group. Data were analyzed by Microsoft Excel and GraphPad Prism. Data are shown as means with SEMs. \*\**P* < .01, \**P* < .05.

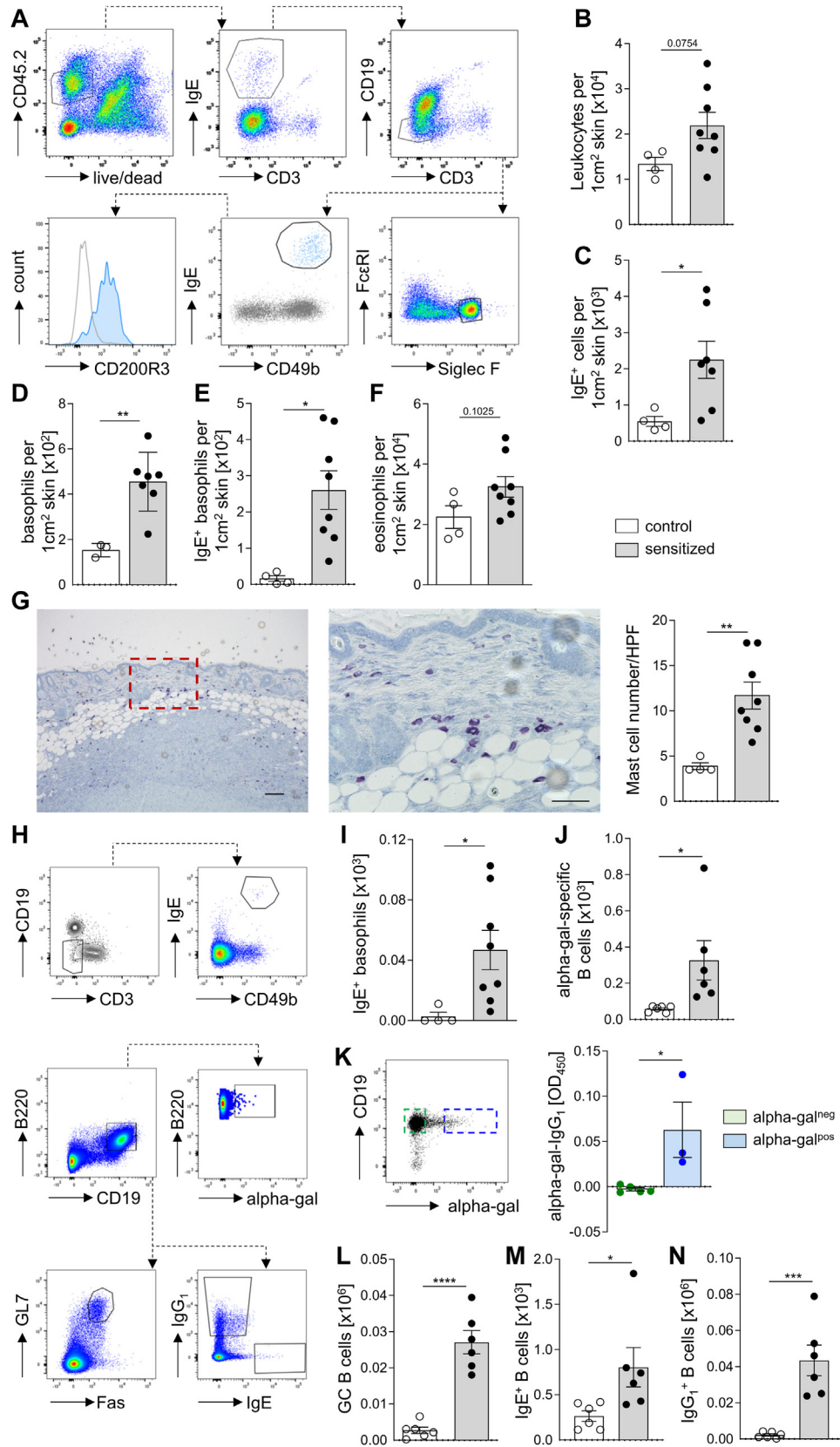
isotypes, demonstrating that it is suited for our further *in vivo* experiments.

To next establish a mouse model of alpha-gal allergy, we used a mouse line deficient in the enzyme responsible for attaching alpha-gal to proteins and lipids: GGTA1. Similar to humans, “natural” alpha-gal-specific antibodies other than IgE and IgG<sub>1</sub> isotypes can be detected in these mice at steady state (data not shown). We mimicked the tick bites responsible for alpha-gal sensitization in humans by repetitive intracutaneous injections of alpha-gal-MSA together with the adjuvant alum (Fig 2, A). Efficient sensitization was confirmed by detecting significantly elevated total serum IgE levels (Fig 2, B), as well as alpha-gal-specific IgE (Fig 2, C), 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<sub>1</sub> levels (Fig 2, D).<sup>35</sup> 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 2, E). We subsequently challenged the mice by intravenous injection of alpha-gal-MSA. As a readout 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 1 hour after intravenous administration (Fig 2, F, and see Fig E2 in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)).<sup>36</sup> Furthermore, the mice were constantly monitored and their behavior and appearance documented (Fig 2, G). 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, because alpha-gal-MSA-sensitized mice receiving intravenous MSA failed to develop anaphylaxis (Fig 2, F). Moreover, a significant increase in mast cell protease 1 (Mcpt1), which is a protease released by mast cells on degranulation, was exclusively detected in mice sensitized to alpha-gal-MSA but not in control mice (Fig 2, H).

### 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.<sup>13,32</sup> 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 3, A). 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.<sup>32</sup> Importantly, we detected significantly elevated levels of alpha-gal-specific IgG<sub>1</sub> antibodies by using alpha-gal-MSA as “catching” antigens attached to the wells (Fig 3, B), as well as by using coupled alpha-gal-carrying glycolipids (Fig 3, C). An ELISA detecting IgE specific for alpha-gal on glycolipids could not be performed because of the nonapplicability of the assay



**FIG 4.** Skin basophils and IgE<sup>+</sup> B-cell responses in skin-draining lymph nodes are enriched in sensitized mice. Single-cell suspensions of murine skin biopsy samples (A-F) or draining lymph nodes (H-N) from control (PBS, white) and sensitized (alpha-gal-MSA, black) mice were analyzed by flow cytometry. (A) Gating strategy for skin to identify (B) total leukocytes, (C) IgE<sup>+</sup> cells, (D) basophils, (E) IgE-coated basophils,

for lipids. This result indicates that alpha-gal-MSA-sensitized animals exhibit IgG<sub>1</sub> 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 glycolipids efficiently induced anaphylaxis (Fig 3, D and E) and induced significantly increased Mcpt1 serum levels (Fig 3, F). 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 (see Fig E3 in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)),<sup>37</sup> 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<sub>1</sub> 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 4, A-G) and in skin-draining lymph nodes (Fig 4, H-N). We observed increased CD45.2<sup>+</sup> leukocyte infiltration in the skin of sensitized mice compared to control mice (Fig 4, B, and see Fig E4, A, in the Online Repository available at [www.jacionline.org](http://www.jacionline.org)), with a significant elevation of CD3<sup>-</sup>IgE<sup>+</sup> cells binding IgE on the surface, likely via FcεRI, being observed (Fig 4, C, and Fig E4, B). Strikingly, CD49b<sup>+</sup>CD200R3<sup>+</sup> basophils were significantly enriched (Fig 4, D, and Fig E4, C), and IgE-coated basophils were almost exclusively detectable in mice sensitized with alpha-gal-MSA (Fig 4, E, and Fig E4, D), which reflects the situation represented in sensitized humans, wherein basophils have been detected at the site of tick bites.<sup>38</sup> Interestingly, IgE-coated basophils were also found in the skin-draining lymph nodes of sensitized mice (Fig 4, I, and Fig E4, F). In addition to basophils, the leukocyte population in the skin of sensitized mice also contained a substantial amount of SiglecF<sup>+</sup>FcεRI<sup>-</sup> eosinophils (Fig 4, F, and Fig E4, E) and mast cells (Fig 4, G), which have also been described to infiltrate the site of tick infestation in both animals and humans.<sup>39,40</sup> 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 4, H-N). By using alpha-gal-MSA coupled to biotin as a detection reagent, we observed alpha-gal-specific CD19<sup>+</sup>B220<sup>+</sup> B cells almost exclusively in the draining lymph nodes of sensitized mice (Fig 4, J, and Fig E4, G). 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 4, K). Moreover, GL7<sup>+</sup>Fas<sup>+</sup> germinal center B

cells (Fig 4, L, and Fig E4, H) as well as B cells of the IgE and IgG<sub>1</sub> isotypes (Fig 4, M and N, and Fig E4, I and J) were significantly enriched in mice sensitized with alpha-gal compared to controls. Taken together, these data indicate that the systemic IL-4 detected in mice sensitized to alpha-gal (Fig 2, E) could be responsible for the switch to alpha-gal-specific IgE production.

### Contribution of basophils is not essential for sensitization to alpha-gal

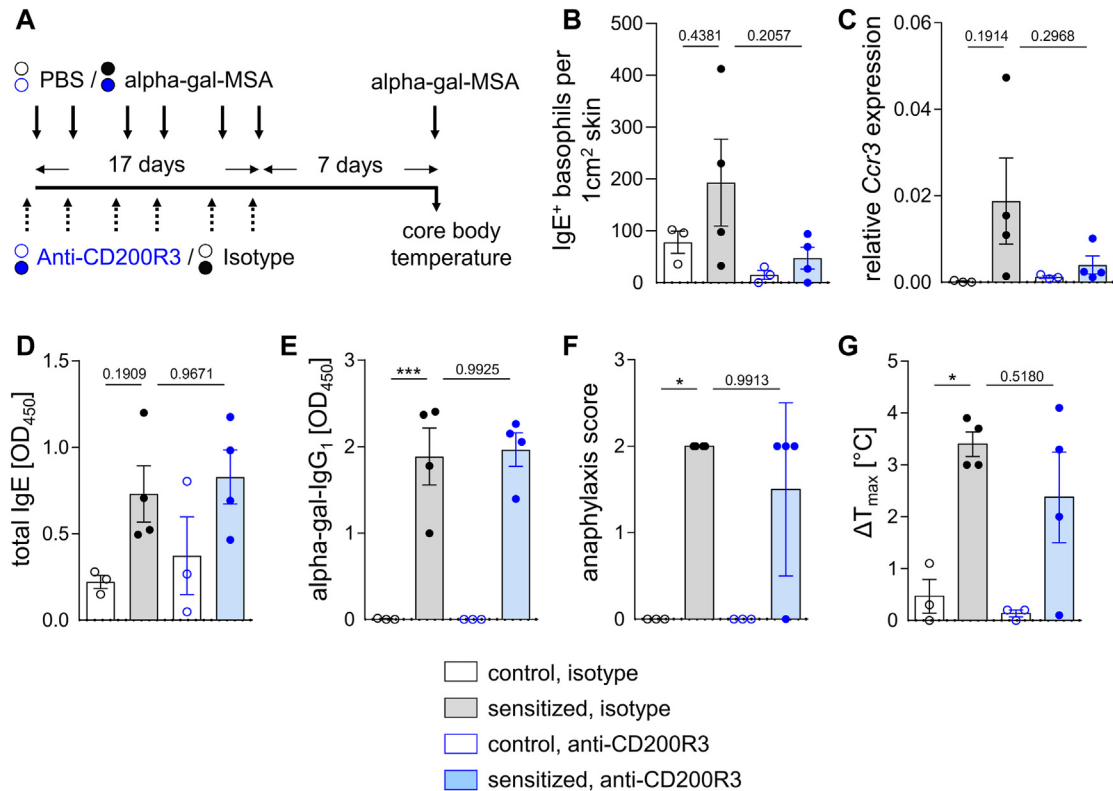
Because basophils were significantly enriched in sensitized skin, we subsequently investigated their role in sensitization to alpha-gal by treating mice with anti-CD200R3 antibody during the course of sensitization to deplete basophils (Fig 5, A). Flow cytometry and real-time quantitative PCR (qPCR) confirmed the efficient depletion of basophils in the skin and draining lymph nodes (Fig 5, B and C). Levels of total IgE, as well as alpha-gal-specific IgG<sub>1</sub>, were not affected by the depletion (Fig 5, D and E), and we detected no protection from anaphylaxis in basophil-depleted mice on allergen challenge (Fig 5, F and G).

### IgE and anaphylaxis specific to 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.<sup>41-43</sup> On the basis of 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 6, A). Impressively, the induction of IgE antibodies in response to sensitization was almost completely abolished on the depletion of IL-4 (Fig 6, B). 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<sub>1</sub> antibodies was reduced in sensitized mice on IL-4 depletion (Fig 6, C). Furthermore, in skin-draining lymph nodes, the expression of mRNA encoding secreted IgE was completely abolished (Fig 6, D), 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 6, E), thus indicating a feedforward loop of IL-4 expression that is strongly diminished on depletion of this cytokine. This was confirmed by *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 6, F). Most importantly, and in concordance with the failed induction of IgE in draining lymph nodes, the proof-of-concept experiment challenging both groups of

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<sub>1</sub>-specific B cells. Data are representative of at least 5 individual experiments or were obtained from 1 experiment (G) with 4 control and 8 sensitized mice; each dot represents 1 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<sub>1</sub> antibodies in culture supernatants were determined. Data were analyzed by FlowJo and GraphPad Prism. Data are shown as means with SEMs. \*\*\*\**P* < .0001, \*\*\**P* < .001, \*\**P* < .01, \**P* < .05.



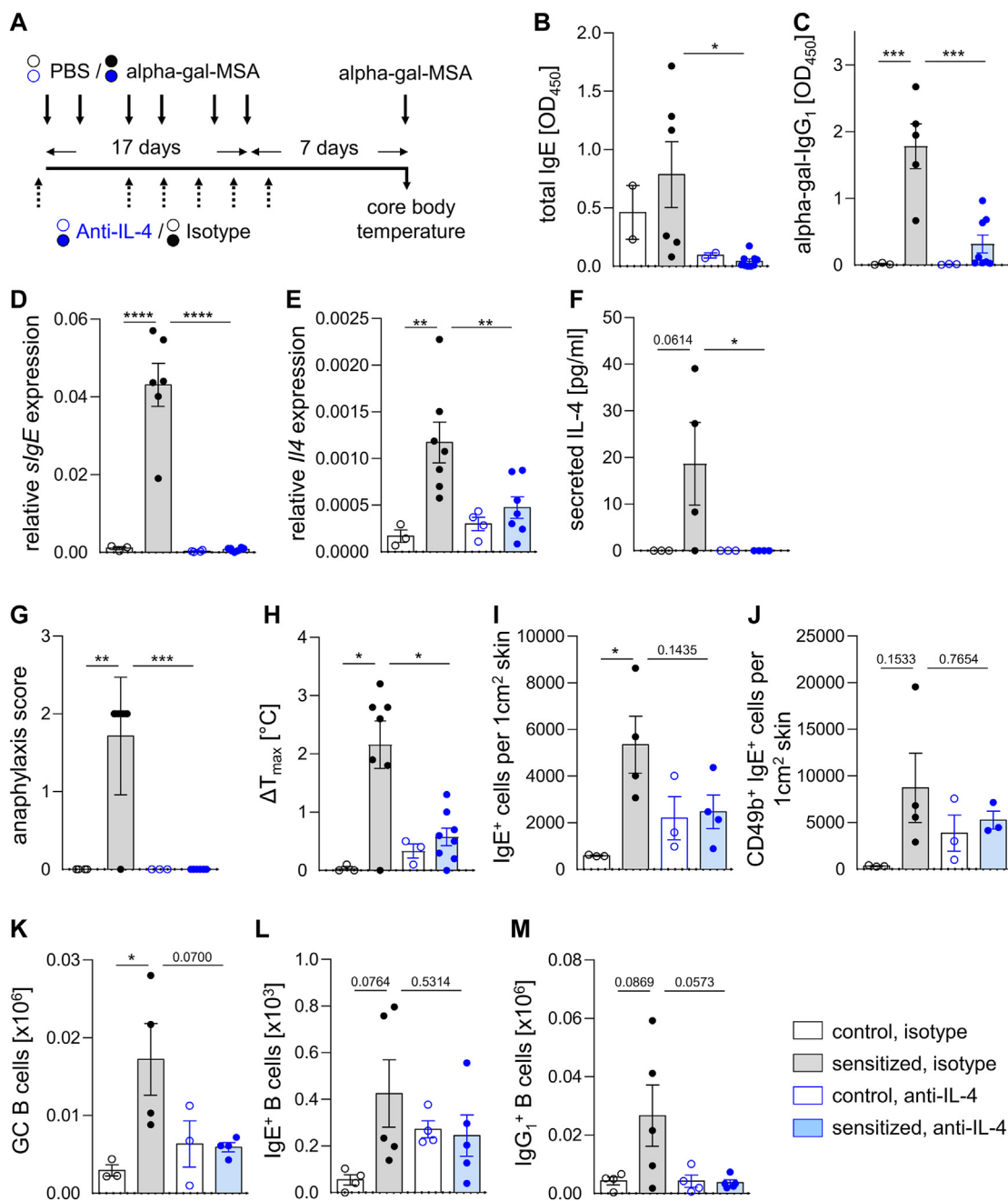


mice with alpha-gal demonstrated that mice depleted of IL-4 failed to develop anaphylaxis, as shown by the absence of any behavioral signs of anaphylaxis and the reduced maximal temperature decrease (Fig 6, G and H). IgE<sup>+</sup> cells, including IgE-coated basophils, were also reduced at the site of sensitization in the skin but were not completely abolished, thus indicating that factors other than IL-4 drive infiltration into the skin (Fig 6, I and J). Importantly, by investigating skin-draining lymph nodes, we detected significantly reduced numbers of germinal center B cells (Fig 6, K), IgE B cells (Fig 6, L) and IgG<sub>1</sub> B cells at the control level (Fig 6, M), 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 after the relatively recent identification of food allergy to red meat and other mammalian-derived foods, which was paralleled by potential reactivity to drugs of mammalian origin.<sup>6,10,44</sup> All of these allergic reactions develop on the basis of 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.<sup>2,3</sup> 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, first, alpha-gal using an alpha-gal-carrying self-protein to induce alpha-gal-specific IgE and B cells, and second, basophils and IL-4 in this carbohydrate-specific immune response and allergy. Previous studies in animal models that used injections of tick extracts or even tick feeding for sensitization proved the concept of percutaneous sensitization for alpha-gal allergy.<sup>29-31</sup> The adjuvant alum is well studied and used in both animal models and humans.<sup>45,46</sup> Interestingly, one study compared tick extracts versus alum as adjuvants in sensitization; it 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.<sup>29</sup> We improved on these insights from animal models and patients in that we applied alpha-gal coupled to a murine carrier protein (MSA) by 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



**FIG 6.** IL-4 is essential for efficient sensitization and elicitation of anaphylaxis to alpha-gal. **(A)** Schematic view of protocol. Intraperitoneal injection of GGTA1-knockout mice with anti-IL-4 antibody (blue) or isotype control (black) throughout sensitization with alpha-gal (solid 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<sub>1</sub>. 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)** and **(J)** skin and **(K-M)** draining lymph nodes were analyzed by flow cytometry. Total cell numbers of **(I)** IgE<sup>+</sup> cells, **(J)** IgE-coated basophils, **(K)** germinal center B cells, and B cells of **(L)** IgE and **(M)** IgG<sub>1</sub> isotypes were determined, as outlined in Fig 4. Data were obtained from 2 independent experiments with 4 control and 7 sensitized mice or **(F-M)** are representative of 2 experiments with 3 to 5 mice per group. Data are shown as means with SEMs. \*\*\*\**P* < .0001, \*\*\**P* < .001, \*\**P* < .01, \**P* < .05.

directly attributed to the carbohydrate alpha-gal. Possible immune effects of the adjuvant alum were controlled by intradermal injections of PBS plus alum in control groups, as well as with the comparison of PBS plus alum to untreated or 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 to IgE bound to FcεRI on human basophils and 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, as a result of 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<sub>1</sub>, thus indicating that, similar to haptens and bacterial polysaccharides,<sup>47,48</sup> a carrier protein is also relevant for type 1 sensitization to a carbohydrate antigen.

In alpha-gal-allergic patients, allergic symptoms on 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.<sup>13</sup> 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 on 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 is less abundant and/or 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 on 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 glycoproteins that contain more complex carbohydrate structures<sup>32</sup> to trigger anaphylactic responses by cross-linking 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 immunologic 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.<sup>9</sup> 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 on 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. First, 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.<sup>39,40</sup> Basophils have even been shown to be critically involved in acquired resistance to tick feeding;<sup>49,50</sup> most interestingly, basophils are enriched at the site of the tick bite in alpha-gal-allergic patients.<sup>38</sup> The depletion of basophils during the sensitization phase did not affect 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. Second, repetitive cutaneous injection of alpha-gal-MSA induced alpha-gal-specific IgG<sub>1</sub> and IgE. In humans, repetitive tick bites induce alpha-gal-specific IgE that is accompanied by elevated levels of alpha-gal-specific IgG<sub>1</sub>.<sup>23,24,27,35,51,52</sup> Although it is well accepted that mouse IgG<sub>1</sub> corresponds to human IgG<sub>4</sub> and that IgG<sub>4</sub> is linked to tolerance—for example, induced in allergen immunotherapy by potentially competing with IgE for allergen binding—it is difficult to define direct homology between human and murine immunoglobulin isotypes.<sup>53,54</sup> IL-4 and IL-13 induce class switching to IgG<sub>1</sub> and IgE in mice and to IgG<sub>1</sub>, IgG<sub>4</sub>, and IgE in humans. Thus, both IgG<sub>1</sub> and IgG<sub>4</sub> 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<sub>1</sub> was induced together with IgE.<sup>55,56</sup> Moreover, although the transfer of total IgG from allergic mice to recipients dampened the allergic response, IgG<sub>1</sub> alone did not cause this effect.<sup>56</sup> Thus, murine IgG<sub>1</sub> does not share the tolerance-favoring attributes of human IgG<sub>4</sub> in allergic inflammation. Third, most, but not all, sensitized mice developed anaphylaxis on 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.

Because 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 because of the manifold contributions of carbohydrates to immune regulation, cancer immune control, and vaccine development.<sup>57-59</sup>

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.<sup>41,43</sup> 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<sub>1</sub> 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.<sup>23,24</sup> Dupilumab, which is a monoclonal antibody targeting the IL-4 receptor  $\alpha$  subunit and which is 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.<sup>60</sup> On the bases of 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 by subsequent alpha-gal rechallenge, in selected patients or individuals, adding dupilumab or the appropriate Janus kinase inhibitors as a type of emergency treatment after 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-specific, 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 after percutaneous exposure. We therefore propose IL-4 signaling as a potential therapeutic target.

## DISCLOSURE STATEMENT

Supported by DFG grants BI 696/12-1, CRC1371 (project P06), CRC1335 (project P17), and RTG 2668; and by the Luxembourg National Research Fund (FNR) (project C17/BM/11656090). C.O. is supported by grants from the European Research Council (ERC starting grant, project 716718) and the DFG (grant OH 282/1-2 within FOR2599), project 490846870-TRR355/1 (project A05), and project 395357507-CRC1371 (project P07).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

We particularly thank Peter Cowan (Immunology Research Centre, St Vincent's Hospital Melbourne, Australia) and Florian Kreppel (University of Ulm, Germany) for the GGTA1-deficient mice. We gratefully acknowledge Sabrina Engel and Desiree Argiriou for excellent technical assistance and Valentina Faihs, Sonja Mathes, Teresa Nau, Christine Schönmann, and Martin Vitus for recruitment of alpha-gal-allergic patients. We thank Franziska

Martin and Doris Langer for determination of alpha-gal-specific IgE levels by ImmunoCAP assay. The graphical abstract was created with BioRender.com.

## 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.

## REFERENCES

1. Gupta RS. Anaphylaxis in the young adult population. *Am J Med* 2014;127(1 suppl):S17-24.
2. Brockow K, Reidenbach K, Kugler C, Biedermann T. Wheat-dependent exercise-induced anaphylaxis caused by percutaneous sensitization to hydrolysed wheat protein in cosmetics. *Contact Dermatitis* 2022;87:296-7.
3. Brough HA, Liu AH, Sicherer S, Makinson K, Douiri A, Brown SJ, et al. Atopic dermatitis increases the effect of exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy. *J Allergy Clin Immunol* 2015;135:164-70.
4. Hils M, Wolbing F, Hilger C, Fischer J, Hoffard N, Biedermann T. The history of carbohydrates in type I allergy. *Front Immunol* 2020;11:586924.
5. Sicherer SH, Warren CM, Dant C, Gupta RS, Nadeau KC. Food allergy from infancy through adulthood. *J Allergy Clin Immunol Pract* 2020;8:1854-64.
6. Chung CH, Mirakhur B, Chan E, Le QT, Berlin J, Morse M, et al. Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N Engl J Med* 2008;358:1109-17.
7. Commins SP, Satinover SM, Hosen J, Mozena J, Borish L, Lewis BD, et al. Delayed anaphylaxis, angioedema, or urticaria after consumption of red meat in patients with IgE antibodies specific for galactose-alpha-1,3-galactose. *J Allergy Clin Immunol* 2009;123:426-33.
8. Eberlein B, Mehlich J, Reidenbach K, Pilz C, Hilger C, Darsow U, et al. Negative oral provocation test with porcine pancreatic enzyme plus cofactors despite confirmed alpha-gal syndrome. *J Investig Allergol Clin Immunol* 2020;30:468-9.
9. Fischer J, Hebsaker J, Caponetto P, Platts-Mills TA, Biedermann T. Galactose-alpha-1,3-galactose sensitization is a prerequisite for pork-kidney allergy and cofactor-related mammalian meat anaphylaxis. *J Allergy Clin Immunol* 2014;134:755-9.e1.
10. Hilger C, Fischer J, Swiontek H, Hentges F, Lehnert C, Eberlein B, et al. Two galactose-alpha-1,3-galactose carrying peptidases from pork kidney mediate anaphylactogenic responses in delayed meat allergy. *Allergy* 2016;71:711-9.
11. Morisset M, Richard C, Astier C, Jacquenet S, Croizier A, Beaudouin E, et al. Anaphylaxis to pork kidney is related to IgE antibodies specific for galactose-alpha-1,3-galactose. *Allergy* 2012;67:699-704.
12. Roenneberg S, Bohner A, Brockow K, Arnold A, Darsow U, Eberlein B, et al. Alpha-gal—a new clue for anaphylaxis in mastocytosis. *J Allergy Clin Immunol Pract* 2016;4:531-2.
13. Wilson JM, Platts-Mills TAE. The oligosaccharide galactose- $\alpha$ -1,3-galactose and the  $\alpha$ -gal syndrome: insights from an epitope that is causal in immunoglobulin E-mediated immediate and delayed anaphylaxis. *EMJ Allergy Immunol* 2018;3:89-98.
14. Platts-Mills TAE, Li RC, Keshavarz B, Smith AR, Wilson JM. Diagnosis and management of patients with the alpha-gal syndrome. *J Allergy Clin Immunol Pract* 2020;8:15-23.e1.
15. Steinke JW, Pochan SL, James HR, Platts-Mills TAE, Commins SP. Altered metabolic profile in patients with IgE to galactose-alpha-1,3-galactose following *in vivo* food challenge. *J Allergy Clin Immunol* 2016;138:1465-7.e8.
16. Avila JL, Rojas M, Galili U. Immunogenic gal alpha 1-3Gal carbohydrate epitopes are present on pathogenic American trypanosoma and leishmania. *J Immunol* 1989;142:2828-34.
17. Yilmaz B, Portugal S, Tran TM, Gozzelino R, Ramos S, Gomes J, et al. Gut microbiota elicits a protective immune response against malaria transmission. *Cell* 2014;159:1277-89.



18. Galili U. Significance of the evolutionary alpha1,3-galactosyltransferase (*GGTA1*) gene inactivation in preventing extinction of apes and Old World monkeys. *J Mol Evol* 2015;80:1-9.
19. Galili U, Anaraki F, Thall A, Hill-Black C, Radic M. One percent of human circulating B lymphocytes are capable of producing the natural anti-gal antibody. *Blood* 1993;82:2485-93.
20. Galili U, Mandrell RE, Hamadeh RM, Shohet SB, Griffiss JM. Interaction between human natural anti-alpha-galactosyl immunoglobulin G and bacteria of the human flora. *Infect Immun* 1988;56:1730-7.
21. Stone KR, Ayala G, Goldstein J, Hurst R, Walgenbach A, Galili U. Porcine cartilage transplants in the cynomolgus monkey. III. Transplantation of alpha-galactosidase-treated porcine cartilage. *Transplantation* 1998;65:1577-83.
22. Biedermann T, Fischer J, Yazdi A. Mammalian meat allergy: a diagnostic challenge. *Allergo J Int* 2015;24:81-3.
23. Fischer J, Lupberger E, Hebsaker J, Blumenstock G, Aichinger E, Yazdi AS, et al. Prevalence of type I sensitization to alpha-gal in forest service employees and hunters. *Allergy* 2017;72:1540-7.
24. Commins SP, James HR, Kelly LA, Pochan SL, Workman LJ, Perzanowski MS, et al. The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose-alpha-1,3-galactose. *J Allergy Clin Immunol* 2011;127:1286-93.e6.
25. Gonzalez-Quintela A, Dam Laursen AS, Vidal C, Skaaby T, Gude F, Linneberg A. IgE antibodies to alpha-gal in the general adult population: relationship with tick bites, atopy, and cat ownership. *Clin Exp Allergy* 2014;44:1061-8.
26. Mateo Borrega MB, Garcia B, Larramendi CH, Azofra J, Gonzalez Mancebo E, Alvarado MI, et al. IgE-mediated sensitization to galactose-alpha-1,3-galactose (alpha-gal) in urticaria and anaphylaxis in Spain: geographical variations and risk factors. *J Investig Allergol Clin Immunol* 2019;29:436-43.
27. Mitchell CL, Lin FC, Vaughn M, Apperson CS, Meshnick SR, Commins SP. Association between lone star tick bites and increased alpha-gal sensitization: evidence from a prospective cohort of outdoor workers. *Parasit Vectors* 2020;13:470.
28. Van Nunen SA, O'Connor KS, Clarke LR, Boyle RX, Fernando SL. An association between tick bite reactions and red meat allergy in humans. *Med J Aust* 2009;190:510-1.
29. Chandrasekhar JL, Cox KM, Loo WM, Qiao H, Tung KS, Erickson LD. Cutaneous exposure to clinically relevant lone star ticks promotes ige production and hypersensitivity through CD4<sup>+</sup> T cell- and MyD88-dependent pathways in mice. *J Immunol* 2019;203:813-24.
30. Choudhary SK, Karim S, Iweala OI, Choudhary S, Crispell G, Sharma SR, et al. Tick salivary gland extract induces alpha-gal syndrome in alpha-gal deficient mice. *Immun Inflamm Dis* 2021;9:984-90.
31. Maldonado-Ruiz LP, Boorgula GD, Kim D, Fleming SD, Park Y. Tick intrastadial feeding and its role on IgE production in the murine model of alpha-gal syndrome: the tick "transmission" hypothesis. *Front Immunol* 2022;13:844262.
32. Chakrapani N, Fischer J, Swiontek K, Codreanu-Morel F, Hannachi F, Morisset M, et al. Alpha-gal present on both glycolipids and glycoproteins contributes to immune response in meat-allergic patients. *J Allergy Clin Immunol* 2022;150:396-405.e11.
33. Li XM, Srivastava K, Grishin A, Huang CK, Schofield B, Burks W, et al. Persistent protective effect of heat-killed *Escherichia coli* producing "engineered," recombinant peanut proteins in a murine model of peanut allergy. *J Allergy Clin Immunol* 2003;112:159-67.
34. Mehlich J, Fischer J, Hilger C, Swiontek K, Morisset M, Codreanu-Morel F, et al. The basophil activation test differentiates between patients with alpha-gal syndrome and asymptomatic alpha-gal sensitization. *J Allergy Clin Immunol* 2019;143:182-9.
35. Rispens T, Derksen NI, Commins SP, Platts-Mills TA, Aalberse RC. IgE production to alpha-gal is accompanied by elevated levels of specific IgG<sub>1</sub> antibodies and low amounts of IgE to blood group B. *PLoS One* 2013;8:e55566.
36. Kanagaratham C, Sallis BF, Fiebiger E. Experimental models for studying food allergy. *Cell Mol Gastroenterol Hepatol* 2018;6:356-69.e1.
37. Takahashi H, Chinuki Y, Tanaka A, Morita E. Laminin gamma-1 and collagen alpha-1 (VI) chain are galactose-alpha-1,3-galactose-bound allergens in beef. *Allergy* 2014;69:199-207.
38. Kageyama R, Fujiyama T, Satoh T, Keneko Y, Kitano S, Tokura Y, et al. The contribution made by skin-infiltrating basophils to the development of alpha-gal syndrome. *Allergy* 2019;74:1805-7.
39. Anderson JM, Moore IN, Nagata BM, Ribeiro JMC, Valenzuela JG, Sonenshine DE. Ticks, *Ixodes scapularis*, feed repeatedly on white-footed mice despite strong inflammatory response: an expanding paradigm for understanding tick-host interactions. *Front Immunol* 2017;8:1784.
40. Krause PJ, Grant-Kels JM, Tahan SR, Dardick KR, Alarcon-Chaidez F, Bouchard K, et al. Dermatologic changes induced by repeated *Ixodes scapularis* bites and implications for prevention of tick-borne infection. *Vector Borne Zoonotic Dis* 2009;9:603-10.
41. Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* 2003;3:721-32.
42. Gowthaman U, Chen JS, Zhang B, Flynn WF, Lu Y, Song W, et al. Identification of a T follicular helper cell subset that drives anaphylactic IgE. *Science* 2019;365(6456):eaaw6433.
43. Kobayashi T, Iijima K, Dent AL, Kita H. Follicular helper T cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol* 2017;139:300-13.e7.
44. Apostolovic D, Tran TA, Hamsten C, Starkhammar M, Cirkovic Velickovic T, van Hage M. Immunoproteomics of processed beef proteins reveal novel galactose-alpha-1,3-galactose-containing allergens. *Allergy* 2014;69:1308-15.
45. Exley C, Siesjö P, Eriksson H. The immunobiology of aluminium adjuvants: how do they really work? *Trends Immunol* 2010;31:103-9.
46. He P, Zou Y, Hu Z. Advances in aluminum hydroxide-based adjuvant research and its mechanism. *Hum Vaccin Immunother* 2015;11:477-88.
47. Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. *Nat Med* 2011;17:1602-9.
48. Erkes DA, Selvan SR. Hapten-induced contact hypersensitivity, autoimmune reactions, and tumor regression: plausibility of mediating antitumor immunity. *J Immunol Res* 2014;2014:175265.
49. Wada T, Ishiwata K, Koseki H, Ishikura T, Ugajin T, Ohnuma N, et al. Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. *J Clin Invest* 2010;120:2867-75.
50. Yoshikawa S, Miyake K, Kamiya A, Karasuyama H. The role of basophils in acquired protective immunity to tick infestation. *Parasite Immunol* 2021;43:e12804.
51. Commins SP, Platts-Mills TA. Tick bites and red meat allergy. *Curr Opin Allergy Clin Immunol* 2013;13:354-9.
52. Hashizume H, Fujiyama T, Umayahara T, Kageyama R, Walls AF, Satoh T. Repeated *Amblyomma testudinarium* tick bites are associated with increased galactose-alpha-1,3-galactose carbohydrate IgE antibody levels: a retrospective cohort study in a single institution. *J Am Acad Dermatol* 2018;78:1135-41.e3.
53. Collins AM. IgG subclass co-expression brings harmony to the quartet model of murine IgG function. *Immunol Cell Biol* 2016;94:949-54.
54. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 2014;5:520.
55. Udoye CC, Rau CN, Freye SM, Almeida LN, Vera-Cruz S, Othmer K, et al. B-cell receptor physical properties affect relative IgG1 and IgE responses in mouse egg allergy. *Mucosal Immunol* 2022;15:1375-88.
56. Yamashita H, Hayashi T, Saneyasu K, Matsuhara H, Matsui T, Tanaka H, et al. Immune suppression of food allergy by maternal IgG in murine models. *Allergol Int* 2018;67:506-14.
57. Comstock LE, Kasper DL. Bacterial glycans: key mediators of diverse host immune responses. *Cell* 2006;126:847-50.
58. Macauley MS, Crocker PR, Paulson JC. Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol* 2014;14:653-66.
59. Taniguchi N, Kizuka Y. Glycans and cancer: role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. *Adv Cancer Res* 2015;126:11-51.
60. Licari A, Castagnoli R, Marseglia A, Olivero F, Votto M, Ciprandi G, et al. Dupilumab to treat type 2 inflammatory diseases in children and adolescents. *Paediatr Drugs* 2020;22:295-310.

## METHODS

### Enzyme-linked immunosorbent assays

Total IgE (mouse IgE ELISA Set, Becton Dickinson) and Mcpt1 (Invitrogen; Thermo Fisher Scientific) ELISAs were performed according to the manufacturer's protocol. For detection of alpha-gal-specific IgE, Thermo Fisher 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 with 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 by streptavidin coupled to horseradish peroxidase (BioLegend) and TMB substrate reagent (Becton Dickinson) after overnight incubation at 4°C. Alpha-gal-specific IgG<sub>1</sub> 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<sub>1</sub> was detected by a biotinylated anti-IgG<sub>1</sub> antibody (BioLegend, clone RMG1-1), streptavidin coupled to horseradish peroxidase, and TMB substrate reagent as indicated above.

### Preparation of single-cell suspensions and flow cytometry

Skin-draining lymph nodes (axillary, inguinal) were dissociated in fluorescence-activated cell sorting (FACS) buffer (PBS + 2% fetal calf serum) with a 70 μmol cell strainer (EASYstrainer, Greiner Bio-One) and subsequently used for flow cytometric analysis. For isolation of skin immune cells, a 1 × 1 cm piece was excised from the site of sensitization and digested overnight at 4°C with 2.5 mg/mL Dispase II (Sigma-Aldrich). Next, dermis and epidermis were separated with forceps and digested in 0.25 mg/mL Liberase TL (Roche) for 75 minutes at 37°C, followed by dissociation of the digested skin piece through a 100 μmol cell strainer (EASYstrainer) 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 with mouse CD16/32 Tru stain fcX (BioLegend), dead cells were stained with Live/Dead Fixable Aqua dead cell stain kit (Invitrogen) in PBS for 20 minutes at 4°C. Subsequently, surface antigens were stained with antibodies specific for the indicated markers in FACS buffer for 20 minutes at 4°C. Murine tissues were treated with 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; Thermo Fisher Scientific, clone 1D3), B220 (BioLegend, clone RA3-6B2), CD95/Fas (BioLegend, clone SA367H8), GL7 (BioLegend, clone GL7), and IgG<sub>1</sub> (BioLegend, clone RMG1-1); for human blood, CCR3 (BioLegend, clone 5.e8), CD203c (BioLegend, clone NP4D6), CD63 (BioLegend, clone H5C6), CD19 (BioLegend, clone HIB19), and 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 minutes after surface staining, followed by staining with fluorochrome-coupled streptavidin (BioLegend) or an anti-biotin antibody (Miltenyi Biotec, clone Bio3-18E7) for 20 minutes at 4°C. Cells were

analyzed with either a BD FACSCanto II or a Beckman Coulter Cytoflex LX flow cytometer. Flow cytometry-based cell sorting was performed on a BD FACS Aria Fusion. Data were analyzed by FlowJo (Becton Dickinson). 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 by using total cell counts of single-cell suspensions as determined by a Neubauer counting chamber or an automated cell counter (Cell-Drop BF, DeNovix).

### Histology

Histology sections from paraffin-embedded tissues were stained with toluidine. Sections were analyzed with a Keyence BZ-X810 All-in-One fluorescence microscope at 10× and 50× magnification. Images were processed by BZ-X800 Analyzer v1.1.2.4 software (Keyence).

### RNA isolation, cDNA synthesis, and qPCR

RNA from skin-draining lymph nodes was isolated by TRIzol-chloroform extraction according to the manufacturer's protocol (TRIzol reagent from Thermo Fisher Scientific). cDNA was synthesized with the TaqMan Gold RT-PCR kit and Oligo(dT)18 primers according to the manufacturer's protocol (Thermo Fisher Scientific). cDNA was subsequently used for qPCR analysis using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and the following primer pairs: *GAPDH* forward: ACCCAGAAGACTGTGGATGG, *GAPDH* reverse: CACATTGGGGGTAGGAACAC, secreted IgE primer pair as published by He et al,<sup>E1</sup> *mIL4* forward: GACGGCACA GAGCTATTGATG, *mIL4* reverse: ACCTTGGAAGCCCTA CAGACG. Gene expression data were normalized to expression of the housekeeping gene *Gapdh*.

### Ex vivo T-cell stimulation and bead-based cytokine assay

A total of  $0.5 \times 10^6$  skin-draining lymph node cells were stimulated with 2.5 μL CD3/CD28 activator beads (Thermo Fisher Scientific) per 96-well plate in complete RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, penicillin, streptomycin, HEPES, nonessential amino acids, and β-mercaptoethanol. Supernatants were collected after 24 hours 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 and 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 by flow cytometry-based cell sorting (living CD3<sup>-</sup>CD19<sup>+</sup>Alpha-gal-MSA<sup>+/-</sup>) on a BD FACS Aria Fusion device. A total of 1000 to 2000 B cells per 96-well plate, sorted from a pool of 3 or 4 mice each, were cultured *ex vivo* in complete RPMI 1640 medium supplemented with 1 μg/mL lipopolysaccharide from *Salmonella minnesota* R595 (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 (BioLegend). Culture supernatants were collected

after 6 days, and alpha-gal–specific IgG<sub>1</sub> antibodies were determined by ELISA as described.

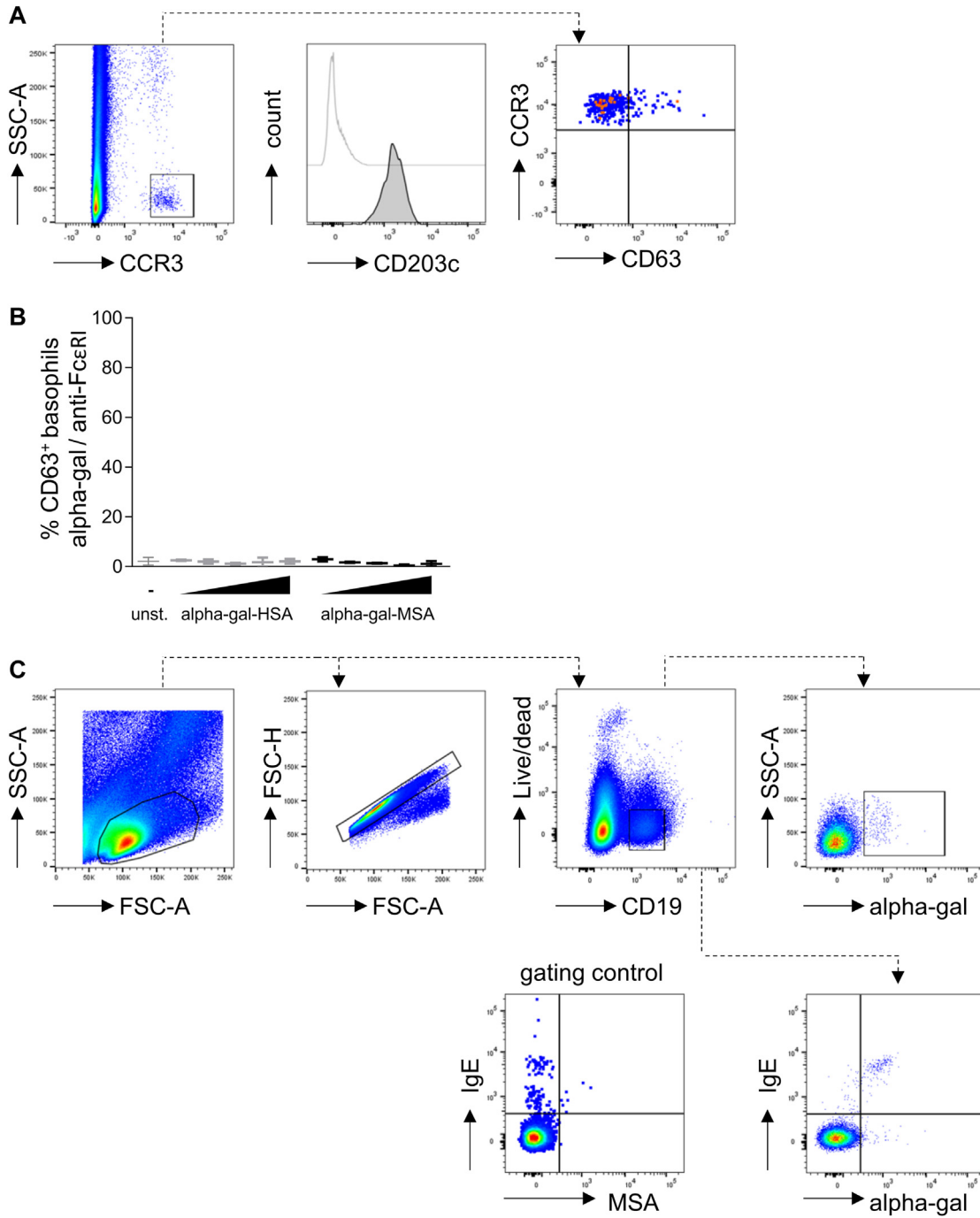
### Statistical analyses

Statistical analyses were performed by GraphPad Prism. Statistical significance for 2 groups was performed by unpaired 2-tailed Student *t* test, except for anaphylaxis scores, which were analyzed by Mann-Whitney test. For 3 or more groups, statistical

significance was determined by ANOVA with Bonferroni multiple comparison test or Dunn multiple comparison test for analysis of anaphylaxis scores. Results are shown as means  $\pm$  SEMs. *P* < .05 was considered significant.

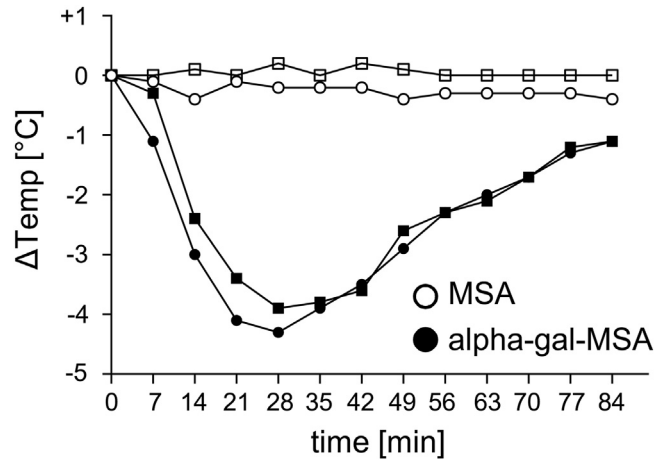
### REFERENCES

- E1. He JS, Meyer-Hermann M, Xiangying D, Zuan LY, Jones LA, Ramakrishna L, et al. The distinctive germinal center phase of IgE+ B lymphocytes limits their contribution to the classical memory response. *J Exp Med* 2013;210:2755-71.

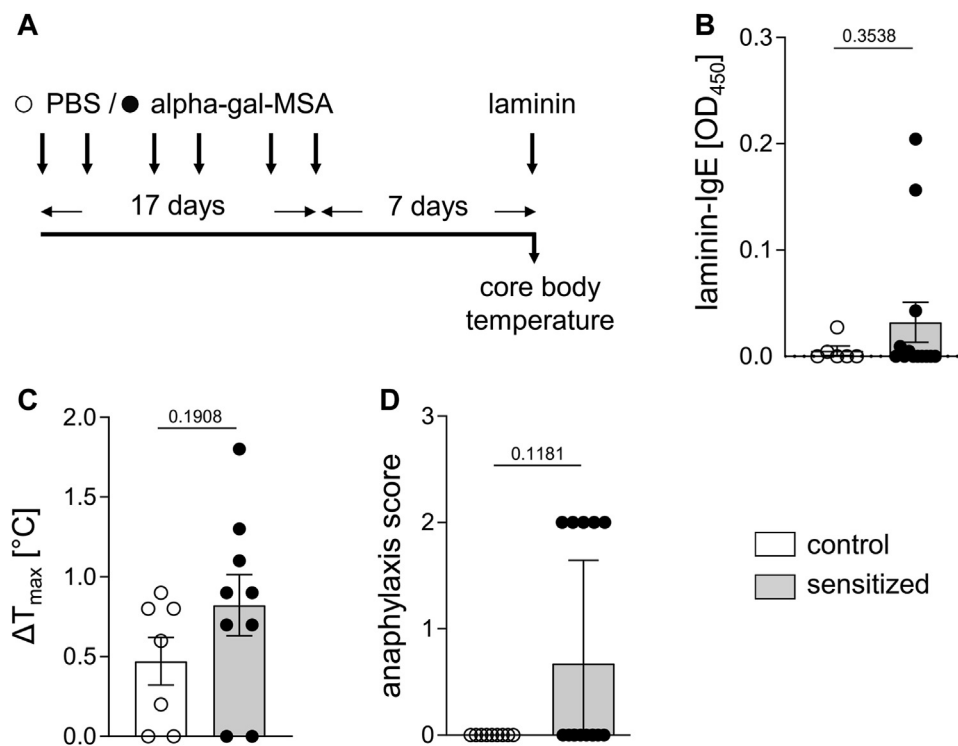


**FIG E1.** (A) Gating strategy to define activated basophils after stimulation with different concentrations of antigen. (B) Dose-dependent *in vitro* basophil activation in healthy controls with increasing concentrations of alpha-gal-HSA or alpha-gal-MSA for stimulation. *Unst.*, Unstimulated control. (C) Gating strategy to define alpha-gal-specific B-cell subsets in human blood. Alpha-gal-specific B cells were detected by biotinylated alpha-gal-MSA; MSA-biotin-stained sample was used as gating control.

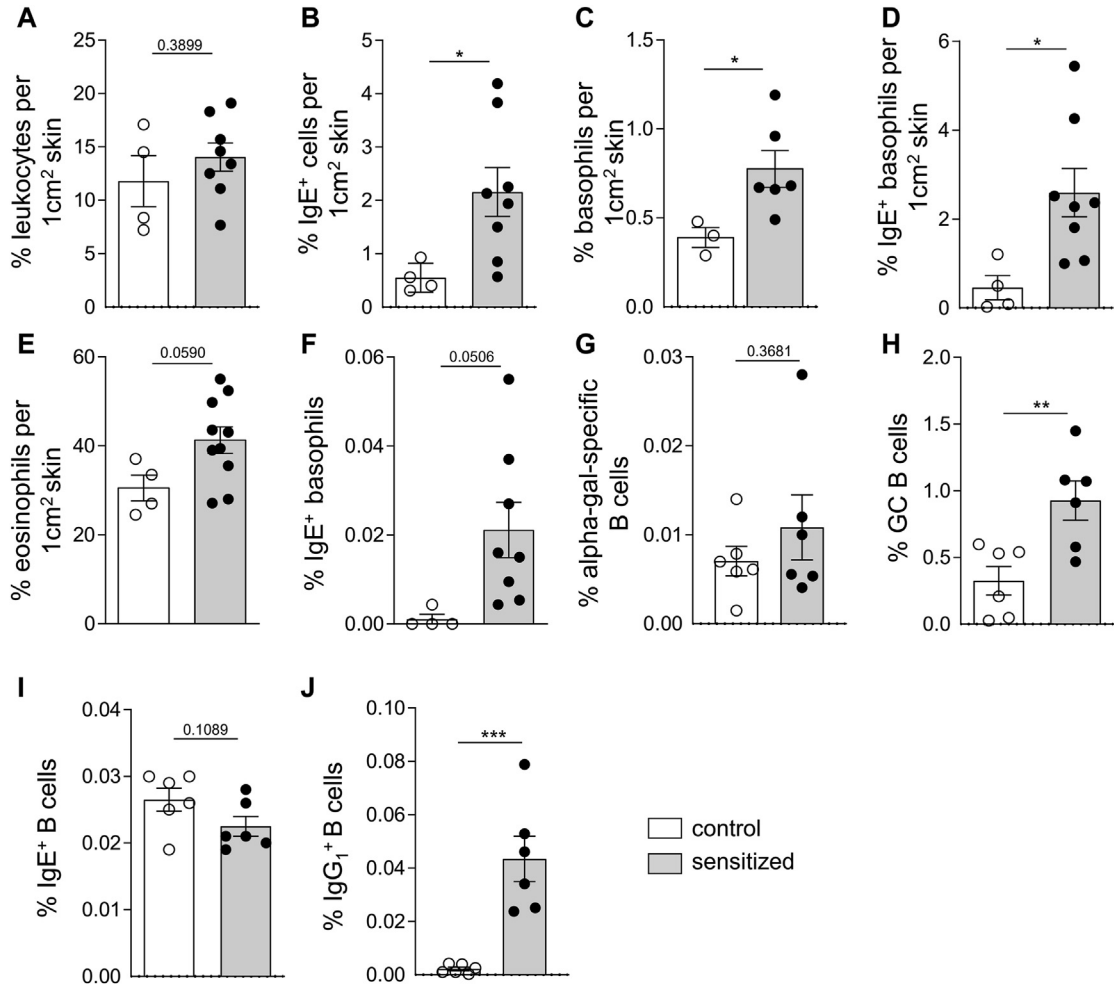




**FIG E2.** Core body temperature was determined every 7 minutes in mice sensitized to alpha-gal-MSA and then challenged with alpha-gal-MSA (black symbols) or MSA (white symbols) as control. Each symbol represents 1 mouse.



**FIG E3.** (A) Schematic view of sensitization protocol. Alpha-gal-deficient GGTA1-knockout mice were repetitively injected with alpha-gal-MSA (black circles, light gray bars) or vehicle (white circle, white bars) in combination with adjuvant alum into back skin. One week after last sensitization, mice were challenged by intravenous injection of laminin, followed by measurement of core body temperature, scoring of behavior, and 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 pool of 3 individual experiments; each dot represents 1 mouse. Data were analyzed by Microsoft Excel and GraphPad Prism. Shown are means with SEMs. Statistical analysis was performed by Student *t* test or Mann-Whitney test for anaphylaxis scores (D).



**FIG E4.** 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 Fig 4.