ORIGINAL ARTICLE

Longitudinal Follow-up Reveals Peripheral Immunological Changes Upon Tick Bite in α-Gal–Sensitized Individuals

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Abstract

Background: α-Gal syndrome is characterized by specific IgE (sIgE) antibodies to the carbohydrate galactose-α-1,3-galactose (α-Gal) and delayed onset of allergic symptoms after ingestion of mammalian meat. While tick bites are assumed to mediate sensitization, the immune response to tick bites has not yet been investigated.
Objective: To investigate the peripheral immune response to tick bites in humans over time.
Methods: In a longitudinal cohort study, immunological reactions associated with tick bites (*Ixodes* species) were analyzed within 1 day (V1), 2 weeks (V2), 1 month (V3), and 3 months (V4) after the occurrence of a bite. sIgE, sIgG, and sIgG subclass levels, as well as 10 cytokines, were quantified. Deep immune phenotyping was performed using mass cytometry.
Results: A total of 4 controls and 10 patients were bitten by a tick and followed up over 3 months. None of the controls developed sIgE to α-Gal, and sIgE increased in all patients from V1 until V2/V3, as did IL-8 levels. We noted a significant increase in $CD19^+$ B cells and B-cell subpopulations, as well as a decrease in γδ $CD56^+$ T cells in patients between V0 and V1. At V1, frequencies of plasmacytoid dendritic cells (pDCs) and γδ $CD56^+$ T cells were lower in patients than in controls.
Conclusion: Our study provides evidence of significant changes in several immune cell populations in α-Gal–sensitized patients, along with increased levels of IL-8 and sIgE. This is the first exploratory study to investigate longitudinal peripheral immune profiles in patients and controls bitten by ticks.

Key words: α-Gal syndrome. Galactose-α-1,3-galactose. IgE. Immune phenotyping. Mass cytometry. B cell. γδ T cell. Tick bite.

Resumen

Antecedentes: El síndrome α-Gal se caracteriza por la presencia de anticuerpos IgE específicos (sIgE) contra el carbohidrato galactosa-α-1,3-galactosa (α-Gal) y un retraso en la aparición de síntomas alérgicos tras la ingestión de carne de mamífero. Se supone que las picaduras de garrapata median la sensibilización, pero aún no se han investigado las respuestas inmunológicas que ocurren tras las picaduras de garrapata.
Objetivo: Investigar la respuesta inmunológica periférica frente a las picaduras de garrapata en humanos de forma longitudinal a lo largo del tiempo.
Métodos: En un estudio longitudinal de cohortes, se analizaron las reacciones inmunológicas asociadas a las picaduras de garrapatas (Ixodes ssp.) un día (V1), 2 semanas (V2), un mes (V3) y 3 meses (V4) después de la picadura. Se cuantificaron los niveles de sIgE, sIgG y subclases de sIgG, así como 10 citocinas. Se realizó un inmunofenotipo profundo mediante citometría de masas.
Resultados: Un total de 4 controles y 10 pacientes fueron picados por una garrapata y seguidos durante 3 meses. Ninguno de los controles desarrolló sIgE a α-Gal, la sIgE aumentó en todos los pacientes desde V1 hasta V2/V3 que también mostraron niveles aumentados de IL-8. Observamos un aumento significativo de linfocitos B $CD19^+$ y otras subpoblaciones de linfocitos B, así como una disminución de linfocitos T γδ $CD56^+$ en los pacientes entre V0 y V1. En V1, los pacientes mostraron frecuencias más bajas de células dendríticas plasmocitoides (pDC) y células T γδ $CD56^+$ que los controles.
Conclusiones: Nuestro estudio proporciona evidencia de cambios significativos en varias poblaciones de células inmunes en pacientes sensibilizados a α-Gal, junto con el aumento de los niveles de IL-8 y de sIgE. Este es el primer estudio exploratorio que investiga los perfiles inmunitarios periféricos longitudinales de pacientes y controles picados por garrapatas.

Palabras clave: Síndrome α-Gal. Galactosa-α-1,3-galactosa. IgE. Inmunofenotipo. Citometría de masas. Linfocitos B. Linfocitos T γδ. Picadura de garrapata.

Summary box

- **What do we know about this topic?**
  Major shifts in immune cell populations have been observed at the site of a tick bite. However, current understanding of how tick bites change systemic immunological features in humans is very limited.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  Our pilot study shows that *Ixodes* species tick bites induce an increase in α-Gal–specific IgE up to 4 weeks after tick a bite, as well as immediate and significant changes in B-cell and γδ T-cell subpopulation frequencies in α-Gal–sensitized individuals.

Introduction

α-Gal syndrome (AGS) is a tick-borne allergic disease characterized by a delayed allergic reaction upon ingestion of mammalian meat or innards and mediated by specific IgE (sIgE) against α-Gal. Circumstantial evidence, such as the identification of α-Gal in several tick species, the development of sIgE antibodies in α-1,3-galactosyltransferase–deficient (GT-KO) mice, and the boosting of human sIgE responses by tick bites, points to the tick as the main source of sensitization and an important factor for the development of α-Gal allergy [1,2].

Current knowledge of the immune response to tick bites in humans and GT-KO mice remains limited. Tick saliva contains many immunomodulatory components [3-6], including the nonprotein component prostaglandin E2, which has been shown to induce class switching of B cells to IgE-producing B cells in mice [7] and is thought to participate in the induction of IgE targeting α-Gal [8]. Although ticks developed mechanisms to overcome host immunity, host adaptive immune responses are able to counteract these mechanisms upon recurrent infestations, as is the case with acquired tick resistance in some animals, such as guinea pigs [9]. *Amblyomma americanum* tick extract administered subcutaneously triggered the production of tick-specific IgE and IgG1 antibodies in GT-KO and wild-type mice, as well as α-Gal–specific IgE in GT-KO mice [10]. Inflammation at the injection site was more pronounced in GT-KO mice than in wild-type mice. Importantly, only subcutaneous injections were able to induce significant tick-specific IgE, whereas intraperitoneal injections were not. Infestation with *A americanum* nymphs or intradermal injections of *A americanum* salivary gland extract from partially fed adult ticks into GT-KO mice induced α-Gal–specific IgE and IgG1, as well as moderate allergic reactions to oral challenges with cooked pork kidney [11,12]. Differential gene expression in skin biopsies of *A americanum*–infested GT-KO mice showed a shift from an innate immune response on a proinflammatory background (by induction of IL-1β) to primary infestation towards T_H2 differentiation after subsequent infestations [11]. Finally, an increase in circulating and activated basophils and more pronounced skin infiltration by mast cells were observed in GT-KO mice than in control mice upon subcutaneous injections of tick extract [10]. Interestingly, skin-infiltrating basophils were also detected in an α-Gal sensitization model using an autologous protein as the α-Gal carrier [13].

In patients with sIgE to α-Gal and a history of tick bites, local immune responses to a new *Amblyomma testudinarium* tick bite were characterized by skin-infiltrating basophils and a higher ratio of T_H2 cytokine–producing $CD4^+$ cells, whereas patients with no history of bites had lower numbers of infiltrating basophils and a more pronounced T_H1 cytokine profile [14]. Bites by *Ixodes ricinus* ticks elicited strong inflammatory innate immune responses characterized by a dominance of macrophages and dendritic cells, as well as increased mRNA levels of macrophage and neutrophil chemoattractants in the skin lesions of healthy individuals, shifting to an adaptive immune response after 24 hours of attachment [15]. Another study compared cell frequencies at the tick bite site and in autologous healthy skin in healthy individuals and found increased frequencies of neutrophils, B cells, and T cells and decreased frequencies of Langerhans and dermal dendritic cells. Frequencies of basophils, eosinophils, mast cells, macrophages, and plasmacytoid dendritic cells, on the other hand, were comparable to those recorded for healthy skin [16]. A recent study by Tang et al [17], who used single-nucleus RNA sequencing of Ixodes scapularis–induced skin lesions, suggests that some human immune markers align with those found in guinea pigs and that erythema and pruritis might be indicators of acquired resistance in humans with recurrent tick bites. *I ricinus* and *A americanum* bites were shown to increase sIgE levels to α-Gal in most sensitized individuals [18-20]. Patients with AGS show significantly elevated levels of α-Gal–specific IgG and IgG1 [21-23], whereas in healthy individuals and in therapeutic human immunoglobulin preparations, α-Gal IgG2 is the dominant subclass [24-26].

Cellular responses have been studied by in vitro stimulation with adult whole-body *I ricinus* extract [27]. Proliferation of $CD4^+$ T cells and $CD19^+CD23^+$ B cells was observed in AGS patients and, to a lesser extent, in healthy controls, while cytokine profiles shifted towards T_H2 in AGS patients. Recently, discrete B-cell phenotypes, which were enriched for B cells capable of producing α-Gal sIgE upon stimulation, were reported to be more abundant in AGS patients than in controls [28].

Blood immune signatures following allergen challenge have been investigated mostly in respiratory disease [29] but rarely in food allergy [30], whereas peripheral immune responses to stings were characterized in the context of anaphylaxis or when monitoring the success of immunotherapy [31,32]. As data on systemic immune changes following tick bites in humans are limited to healthy individuals and a single sample

collection after a bite [16,17], we aimed to recruit individuals sensitized to α-Gal and nonsensitized controls. After a tick bite, participants were followed for months, and blood humoral and cellular responses were characterized in depth. Our study is the first to investigate immediate and longitudinal immune profiles in patients and controls bitten by *I ricinus* ticks. α-Gal sIgE increased in all sensitized participants, who also showed a constantly higher sIgG1/sIgG2 ratio than controls. Our data provide evidence of longitudinal changes in several immune cell populations in sensitized individuals, as well as marked differences in innate cell frequencies between sensitized persons and controls.

Methods

The methods are described in detail in the online repository.

Patient Recruitment and Blood Sampling

Between May and October 2020, participants were recruited at the Allergy Unit of the Department of Dermatology, Eberhard Karls University, Tübingen, Germany. Patients were defined as being diagnosed with AGS or as α-Gal sIgE–sensitized individuals (1 individual had sIgE below 0.1 kU/L at inclusion but had previously had a sensitized phenotype). Healthy controls were defined as having α-Gal sIgE levels below 0.1 kU/L at study inclusion. The peripheral blood immune parameters, investigated at the Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg were analyzed at baseline (V0) and longitudinally within 1 day (visit V1), 2 weeks (V2), 1 month (V3), and 3 months (V4) after a tick bite. Stereomicroscopic species identification was carried out on available ticks by the Institute of Parasitology, University of Hohenheim, Stuttgart, Germany. At each visit, blood was collected in heparin tubes for the preparation of peripheral blood mononuclear cells (PBMCs) and in citrate tubes for preparation of serum. Serum was separated from coagulated blood by centrifugation, stored at –20°C, and used to determine immunoglobulin and cytokine levels. PBMCs were obtained by separation in SepMate tubes (Stemcell) filled with Histopaque (Sigma-Aldrich) and then frozen in serum-free medium (Bambanker serum-free cell freezing medium, Nippon Genetics) to avoid any bias from α-Gal traces present in the freezing medium containing fetal bovine serum. PBMCs were used for immune cell profiling with CyTOF. Total and specific IgE (sIgE) levels were quantified using ImmunoCAP (Thermo Fisher Scientific). Hemoglobin level, hematocrit, and differential blood count were determined by the central laboratory of the University Hospital of Tübingen using accredited routine methods. The study was approved by the Ethics Committee of the University Medical Faculty in Tübingen (246/2019BO2) and performed according to the standards of the Declaration of Helsinki. Written informed consent was obtained from all study participants.

Tick Extract and Quantification of IgE Against Tick Extract

Protein extract was made from adult *I ricinus* ticks (Insect Services). Enzyme-linked immunosorbent assay (ELISA) was performed to compare specific IgE levels in human sera against tick and deglycosylated tick antigens [33]. Tick extract (TE) was deglycosylated by adding 1 U/5 µg TE of α-galactosidase from green coffee bean (Sigma-Aldrich). Hereafter, the term deglycosylated refers to α-galactosidase–treated TE.

IgG Subtyping and Cytokine Measurements

Anti–α-Gal IgG and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) were assessed in human sera using ELISA, as previously described [34]. Briefly, high-binding 384-well plates were coated with α-Gal 2 µg/mL coupled to human serum albumin (Galα1-3Galβ1-4GlcNAc-HSA [14 atom spacer], Dextra Laboratories). After overnight incubation, nonspecific binding sites were blocked with 1% human serum albumin (Sigma-Aldrich). For purposes of subclass determination, serum was added at a dilution of 1/50 for IgG, IgG1, and IgG2, at 1/20 for IgG3, and at 1/10 for IgG4. IgG, IgG1, IgG2, and IgG3 were detected by incubating sera for 3 hours at room temperature with horizontal shaking at 500 rpm followed by 2 hours of incubation with specific alkaline phosphatase–labeled anti-IgG, anti-IgG1, IgG2, or IgG3 monoclonal antibodies (Southern Biotech). IgG4 were detected by incubating sera overnight at room temperature with horizontal shaking at 500 rpm followed by 2 hours of incubation with specific biotin-labeled monoclonal anti-IgG4 antibody (Southern Biotech) and 1 hour of incubation with alkaline phosphatase–labeled streptavidin (Southern Biotech). After addition of para-nitrophenyl phosphate solution (Sigmafast, Sigma Aldrich), the optical density was read at 405 nm on a Spectramax 384Plus ELISA reader (Molecular Devices).

Standard curves with human IgG, IgG2 (Sigma Aldrich), IgG1, and IgG3 (Southern Biotech) from human serum and recombinant human IgG4 (Biolegend) were obtained by coating the respective immunoglobulins in serial dilutions. The specific monoclonal antibodies were detected as mentioned above, and the concentration of the IgG and IgG subclasses was calculated using SoftMax Pro 7.1 (Molecular Devices) by plotting the standard curve on a 4-parameter logistic regression model (Figure S1).

Cytokine levels in human sera were measured using the U-PLEX custom biomarker electrochemiluminescence assay from Meso Scale Discovery.

Mass Cytometry Data Acquisition

PBMCs were stained using the Maxpar® Deep Immuno-Profiling Assay (Fluidigm/Standard Biotools) and 7 additional antibodies (Table S1). Sample acquisition was performed on a HELIOS mass cytometer (Fluidigm/Standard Biotools).

Statistical Analysis

Normalized CyTOF data were manually gated in the FlowJo v10.8.0 software application. The design of the gating strategy enabled identification of 66 cell populations (Figures S2 and S3) [35]. The statistical tests were performed using GraphPad Prism (version 9.3.1). Graphical representations of data were created with GraphPad Prism (version 9.3.1), Biorender, or Tableau Desktop (version 2021.4).

Results

Study Design, Clinical Characteristics, and Baseline Demographics

A total of 51 individuals were recruited, and 25 participants (16 patients and 9 controls) were eventually included in the cohort study. The exclusion criteria were atopic background other than AGS, not attending 1 or several follow-up visits, no or several tick bites, and uncertainties in reported tick bites. During the observation period, 14 study participants (10 patients and 4 controls) were bitten by ticks, and blood samples were collected according to the protocol (Figure 1A). Samples from 6 patients and 5 controls recruited prior to a tick bite (baseline, V0) were used as additional reference samples (Figure S4). Age, sex, allergy history, experience with tick bites and species identification of biting ticks are shown in the Table.

Table. Clinical Characteristics of Study Participants.

Code[a]	Age, y	Sex	αGal-IgE, kU_A/L	Total IgE, kU/L	Clinical allergy (AGS)	Local reaction to bite according to questionnaire	Estimated number of lifetime tick bites	Number of pre-study tick bites (previous 12 mo)	Biting tick species	Biting tick stage
Pat 1	67	F	1.1	242	Yes	Ordinary	6-10	2	*Ixodes ricinus*	Nymph
Pat 2	54	M	1.1	267	Yes	Ordinary	21-30	3	*Ixodes ricinus*	Nymph
Pat 3	62	M	0.46	115	Yes	Hyperergic	6-10	2	*Ixodes ricinus*	Nymph
Pat 4	80	F	3.4	129	Yes	Ordinary	n.d.	0	*Ixodes ricinus*	Nymph
Pat 5	46	F	0.33	570	No	Ordinary	1-5	1	*Ixodes* species	Nymph
Pat 6	19	M	12.4	133	Yes	Hyperergic	31-50	5	*Ixodes ricinus*	Nymph
Pat 7	58	M	<0.1[b]	72.3	No	Ordinary	6-10	2	*Ixodes ricinus*	Female adult
Pat 8	73	F	1.9	7.4	Yes	Hyperergic	6-10	0	ND	ND
Pat 9	51	F	481	1484	Yes	Hyperergic	>100	20	*Ixodes ricinus*	Nymph
Pat 10	59	M	23.2	89.1	Yes	Hyperergic	11-20	1	ND	ND
Pat 11	56	M	19.7	365	Yes	Hyperergic	1-5	0	NA	NA
Pat 12	75	M	>100	820	Yes	Ordinary	1-5	0	NA	NA
Pat 13	80	M	5.2	102	Yes	Hyperergic	1-5	1	NA	NA
Pat 14	75	M	9.6	359	Yes	Hyperergic	21-30	1	NA	NA
Pat 15	63	M	17.1	101	Yes	Hyperergic	21-30	2	NA	NA
Pat 16	65	F	10.4	30.8	Yes	Ordinary	6-10	1	NA	NA
Median Range	**62.5 19-80**		**7.4 0.1-481**	**131.0 7.4-1484**						
Ctrl 1	69	M	<0.1	52	No	Ordinary	1-5	0	NA	NA
Ctrl 2	39	F	<0.1	19.4	No	Ordinary	1-5	0	NA	NA
Ctrl 3	59	M	<0.1	81.9	No	Ordinary	21-30	2	NA	NA
Ctrl 4	57	M	<0.1	ND	No	Ordinary	1-5	0	NA	NA
Ctrl 5	44	M	<0.1	23.6	No	Ordinary	6-10	0	NA	NA
Ctrl 6	54	F	<0.1	34.6	No	Ordinary	31-50	5	*Ixodes* species	Nymph
Ctrl 7	56	F	<0.1	89.8	No	Ordinary	6-10	1	*Ixodes* species	Nymph
Ctrl 8	37	M	<0.1	29.6	No	Ordinary	1-5	1	*Ixodes ricinus*	Nymph
Ctrl 9	60	M	<0.1	18.2	No	Ordinary	21-30	0	*Ixodes* species	female adult
Median Range	**56.0 37-69**		**<0.1**	**32.1 18.2-89.8**						

Abbreviations: AGS, α-Gal syndrome; NA, not applicable; ND, no data.
[a]Participants in italic font are represented at time-point V0 only.
[b]In a study from 2013, a sensitization with α-Gal sIgE of 0.3 kU/L was detected in this individual.

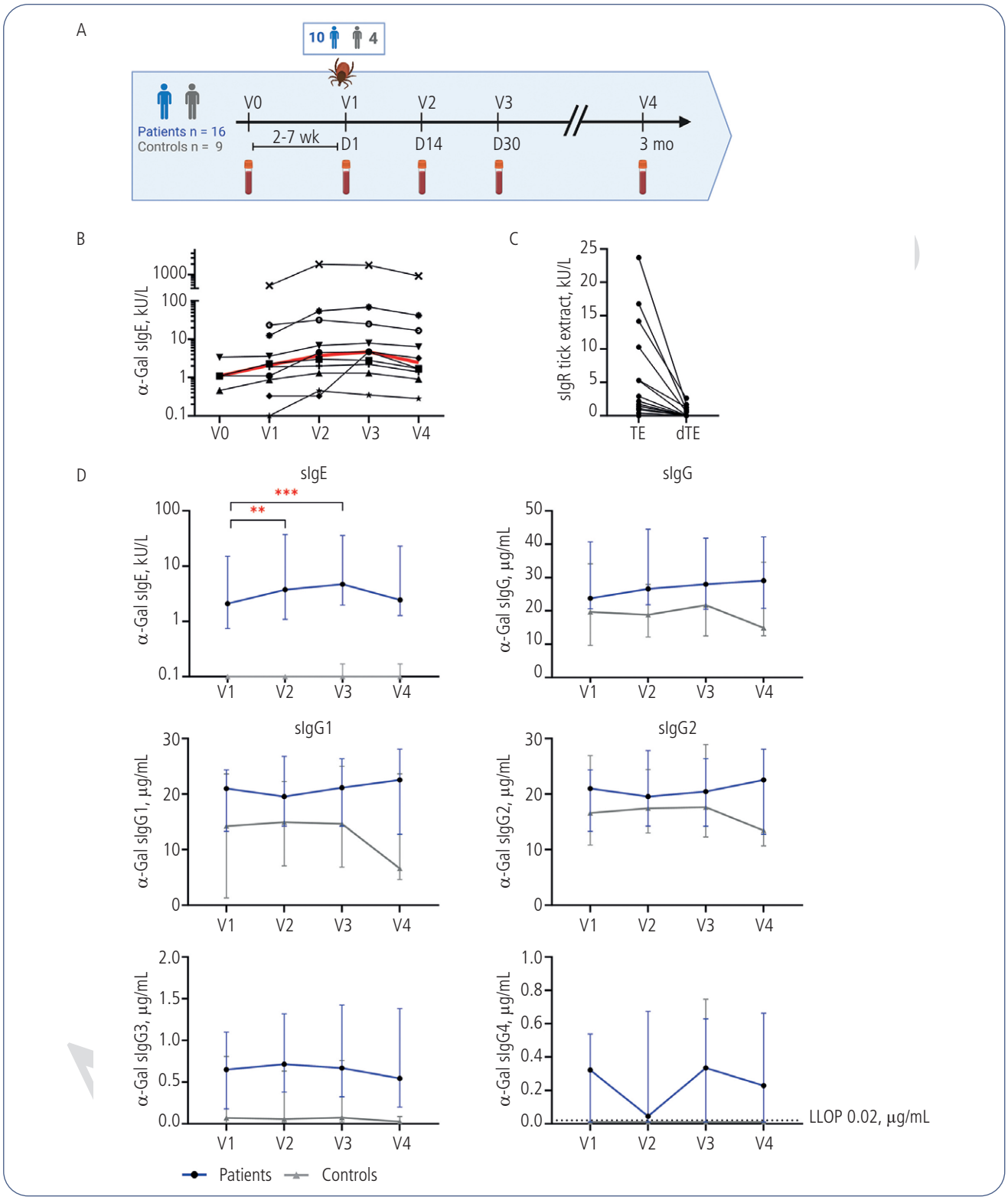

Figure 1. Study design and longitudinal profiles of anti-α-Gal immunoglobulins. A, The study comprised 16 patients and 9 controls. Ten patients and 4 controls were bitten by a tick and followed over 3 months (V1 to V4). Six patients and 5 controls were included as V0 reference samples. B, Serum α-Gal sIgE of 10 patients were measured over time and expressed in kUA/L. The red line represents the median. C, Serum sIgE of 16 patients, expressed in kUA/L, were measured for adult Ixodes ricinus tick extract (TE) and for deglycosylated tick extract (dTE). D, Serum α-Gal sIgG, sIgG subclasses, and sIgE of patients (blue line) and controls (gray line) were measured over time and expressed as median (IQR) of kU/L for sIgE and µg/mL for IgG and IgG subclasses. Sera were diluted 1/50 for IgG, IgG1, and IgG2, 1/20 for IgG3, and 1/10 for IgG4. Levels that were below the limit of detection were set to the value of 0.01 µg/mL. Adjusted *P* values ** <.01 and *** <.001 (Friedman test with a Dunn correction performed on the patient group are indicated with a red star).

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Tick Bites Induce an Increase in sIgE in α-Gal and Moderately Influence sIgG Levels in Patients and Controls

Serum IgE and IgG anti-α-Gal–specific antibody responses were monitored in patients and controls over 3 months. None of the controls developed an IgE response to α-Gal, whereas α-Gal sIgE increased in all patients from V1 to V2 or V3 and decreased slightly at V4, with a median fold change of 3.1 (range, 1.2-14.2) (Figure 1B, 1D). Patient IgE bound primarily to the α-Gal epitope. IgE binding to tick extract was almost completely abolished by deglycosylating the extract (median reduction, 100%; range 82%-100%) (Figure 1C).

In patients, α-Gal sIgG levels, as well as sIgG1, sIgG2, and sIgG3 levels, remained relatively constant over the 3-month period after the tick bite. Levels of sIgG4 seemed to vary erratically, although considering the very low scale, it is important to note that 64% (46/72) of the samples tested were below the lower limit of detection (LLOD, 0.02 µg/mL). The main finding was that α-Gal sIgG4 levels were barely detectable, consistent with our previous observation [34].

Median levels of α-Gal sIgG antibodies and of the 4 subclasses tended to be lower in the control group, although they remained quite stable until V3. A decrease in sIgG, sIgG1, and sIgG2 levels 3 months after the tick bite (V4) marks the main differences with the patient group. The lower

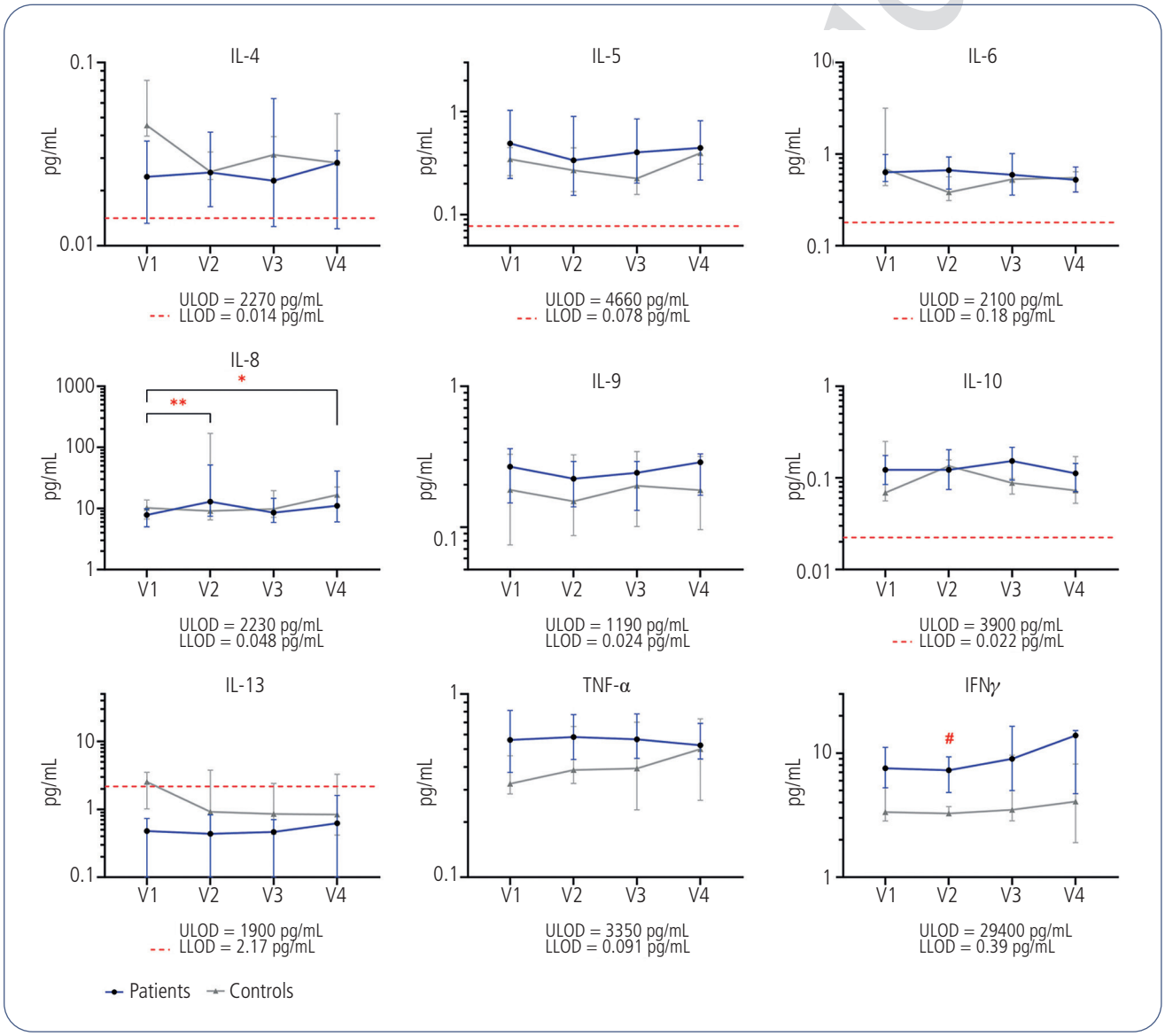

Figure 2. Analysis of serum cytokines show differences in T_H1 cytokines between patients and controls. Serum levels of 9 cytokines were measured by electrochemiluminescence assay for patients (blue line) and controls (gray line), expressed as median (IQR) in pg/mL. Adjusted *P* value * <.05 and **<.01 (Friedman test with a Dunn correction performed on the patient group). Adjusted *P* value # <.05 for multiple Mann-Whitney tests between control and patient groups. ULOD indicates upper limit of detection; LLOD, lower limit of detection (red dotted line).

levels of α-Gal sIgG, sIgG1, sIgG2, and sIgG3 are in line with a recent study by Chakrapani et al [34], namely, lower sIgG1, sIgG2, and sIgG3 levels in tick-bitten, nonsensitized forestry employees than in AGS patients.

We directly compared α-Gal sIgG subclass levels in individual patients and controls (Figure S5). Subclasses of sIgG1 and sIgG2 were the most abundant, followed by lower levels of sIgG3 and barely detectable sIgG4 levels. However, tick bites seem to induce an increase in α-Gal sIgG1, sIgG2, and sIgG3 antibody levels only in some patients (Patients 5, 7, and 9), whereas most patients (Patients 2, 3, 4, 6, 8, and 10) presented a relatively stable sIgG profile despite an increase in sIgE. The same types of profiles were seen in controls, where increasing levels of sIgG1 and sIgG2 were observed in Controls 6 and 8, and stable or even decreasing levels of sIgG were observed in Controls 7 and 9. Strikingly, patients had a higher percentage of sIgG1 than sIgG2 (Figure S6A) and a higher ratio of sIgG1/sIgG2 (median ratio 1.2; range 0.5-7.8) than controls (median ratio 0.8; range 0.005-1.2) (Figure S6B), indicating that tick bites favor the production of sIgG1 in sensitized individuals, whereas the natural predominance of α-Gal sIgG2 over IgG1 is maintained in nonsensitized tick-bitten controls.

Globally, patients showed higher levels of sIgG and sIgG1, sIgG2, and sIgG3 than controls, although the percentage of sIgG2 was higher in controls. Immunoglobulin levels remained constant over the 3-month observation period in patients and increased in some participants upon a tick bite. sIgE levels increased in all patients, with a slight decline at 3 months. None of the controls developed sIgE to α-Gal.

Tick Bites Induce a Proinflammatory T_H1 Cytokine Response in α-Gal–Sensitized Patients

Serum cytokine levels were measured at different time-points but did not reveal major statistically relevant changes

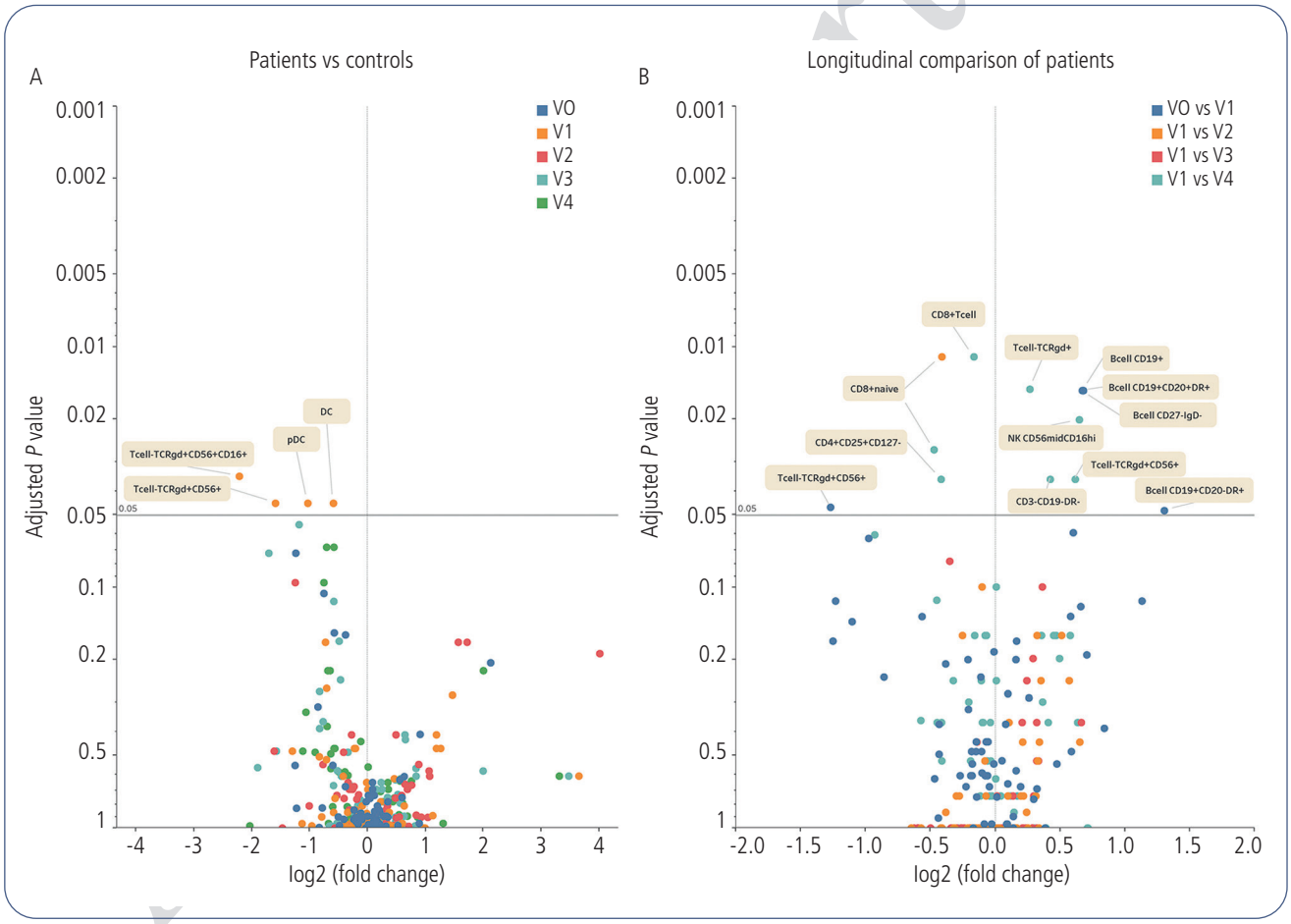

Figure 3. Tick bites induce changes in immune cell frequencies in patients and controls. Peripheral blood mononuclear cells were analyzed by mass cytometry and cell frequencies were determined by manual gating, comparing changes between patients and control cell populations at each visit (A) and comparing changes in patient cell populations over time (B).
A, The volcano plot shows the fold change in cell frequencies against adjusted *P* values from multiple Mann-Whitney tests with a Holm-Šídák correction, with patient and control groups compared at the corresponding time-points. The control group is the reference for the fold change calculation. The familywise α threshold is set at 0.05, and *P* values <.05 are considered statistically significant.
B, The fold change in patient cell frequencies is plotted against *P* values from the Mann-Whitney test (time-point V0 vs V1). Adjusted *P* values for the Friedman test with the Dunn correction (V1 to V2, V3, V4). Time-point V0 vs time-point V1 is the reference for the fold change calculation. The α threshold is set at 0.05, and *P* values <.05 are considered statistically significant.

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over time (Figure 2), except for the proinflammatory chemokine IL-8, which had increased 2 weeks after the tick bite (median fold change, 1.6; range, 1.0-10.5) and remained elevated at the 3-month visit in patients (median fold change, 1.4; range, 0.95-13.0). IL-2 (data not shown) and IL-13 levels were below the LLOD. Levels of IFN-γ increased over time and were higher in patients than in controls, the difference being significant only at V2. Although this finding is based on a small sample and must be confirmed with larger data sets, surprisingly, some proinflammatory T_H1 cytokine levels seem to be higher in AGS patients than in nonallergic controls.

Tick Bites Induce Variations in Immune Cell Profiles

The analysis of the main leukocyte frequencies from the complete blood count revealed no significant differences between patients and controls at any time-points (Figure S7).

Deep immunophenotyping by mass cytometry, however, showed marked differences between patients and controls and over time. In total, 67 out of 70 PBMC samples were included in the final immunological analysis (48 patient and 19 control samples distributed over 5 time-points) (Figure S4). Three samples did not fulfill the inclusion criteria (defined as cell viability over 50% or live $CD45^+$ cell count more than 50 000) and were excluded from the analysis.

The frequencies of 66 cell populations, identified by our gating strategy (Figure S3) and expressed as a percentage of $CD45^+$ live cells, were compared at each time-point between patient and control groups. Lower frequencies of dendritic cells (DCs) and the subgroup of plasmacytoid DCs (pDCs) were observed in the patient group than in the control group at time-point V1 (Figure 3A). A similar decreasing trend in the frequencies of circulating DCs in patients was observed for the other time-points (Figure 4) and is not a result of the influence of the circadian rhythm on myeloid cell frequencies in peripheral blood (Figure S8). Although the frequency of pDCs was lower in the afternoon samples in both groups, the difference between patients and controls was significant at both times of the day. As higher frequencies of circulating DCs have been found in peanut-allergic adolescents than in non–food-allergic controls [36], a decreased DC/pDC frequency may be a particular signature of α-Gal allergy in our specific patient population.

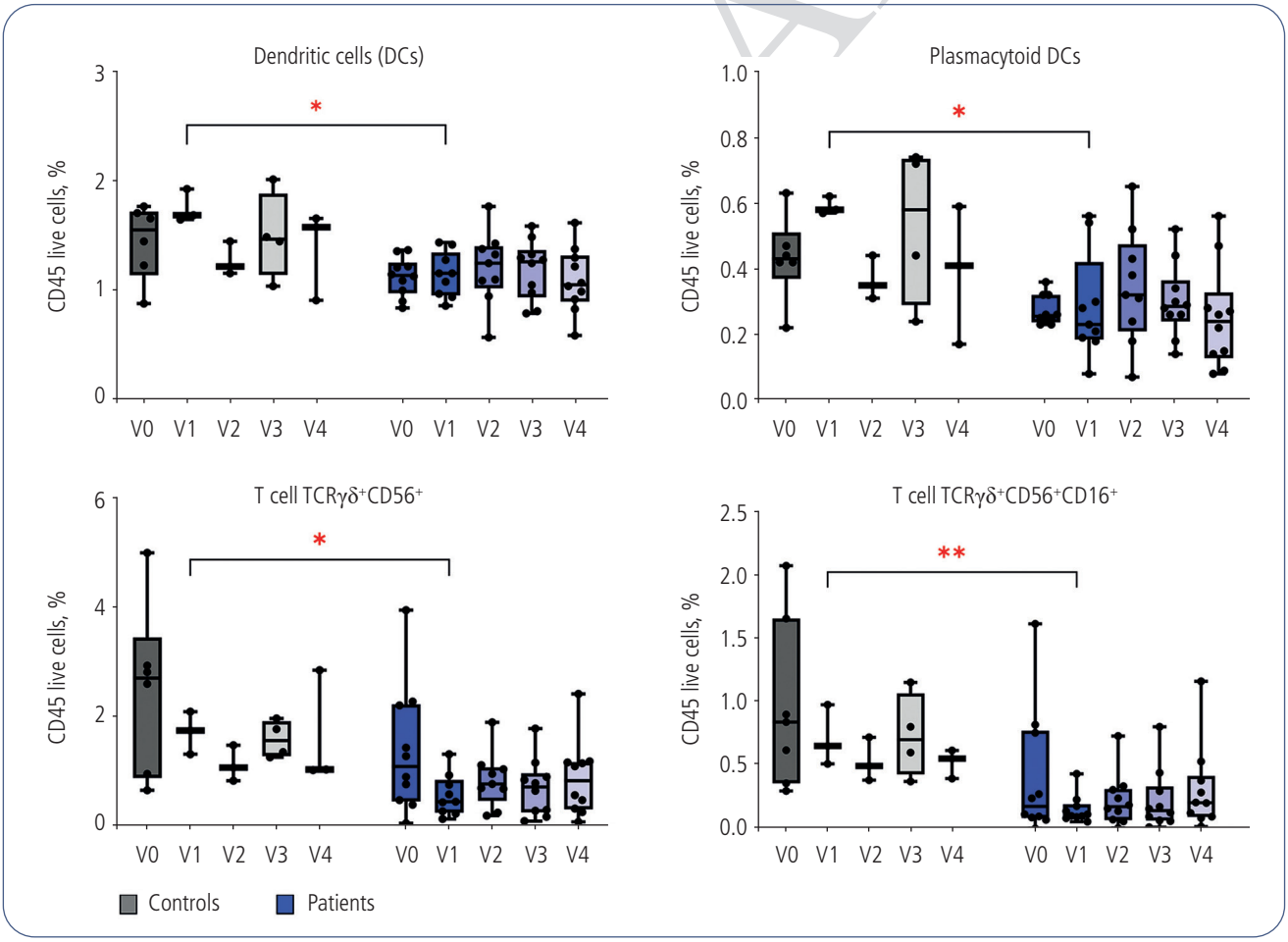

Figure 4. Cell population frequencies differ between controls and patients. Cell population frequencies in the control group (gray boxes) and the patient group (blue boxes) are expressed as percent of live $CD45^+$ cells (box delimited by interquartile range with median line and whiskers from minimal to maximal value). The control group is compared to the patient group at each corresponding time-point. Adjusted *P* values * <.05 and ** <.01 (multiple Mann-Whitney tests with a Holm-Šídák correction).

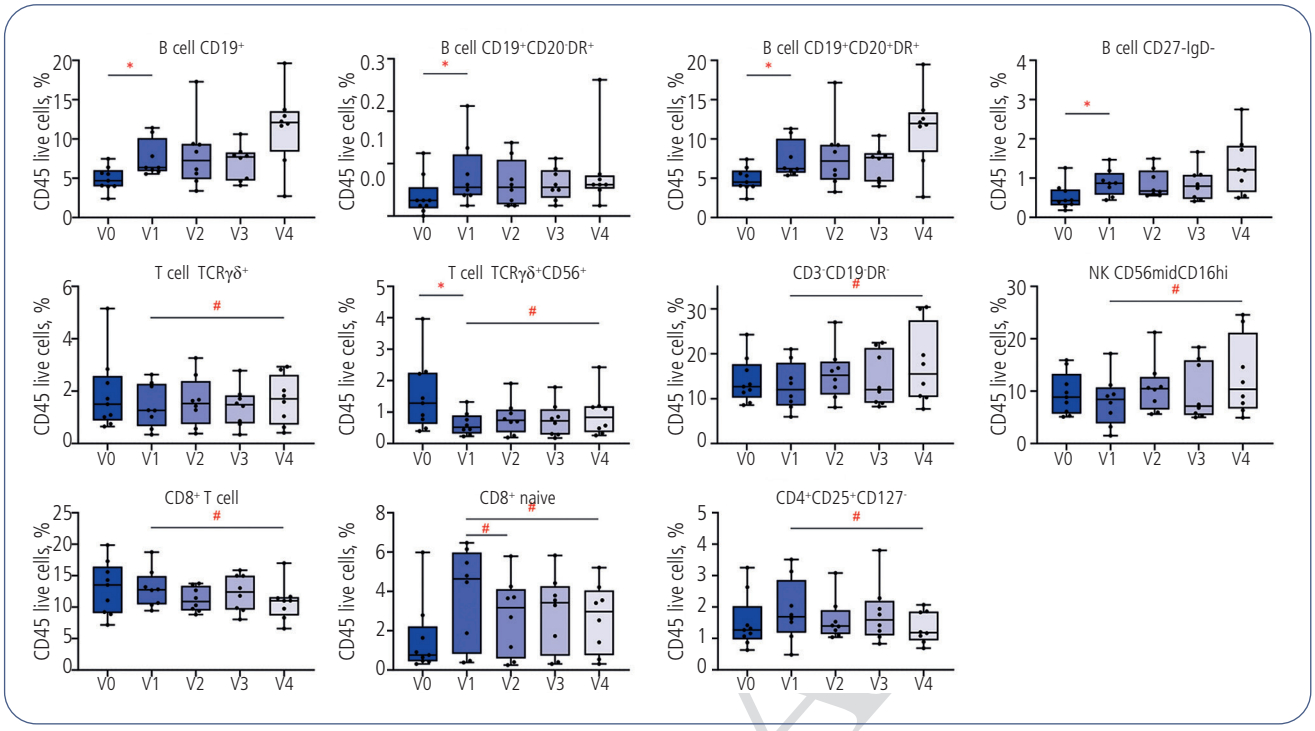

Figure 5. Longitudinal immunophenotyping reveals changes in patient cell population frequencies. Cell population frequencies of the patient group (blue boxes) are expressed as percent of live CD45$^+$ cells (box delimited by IQR with median line and whiskers from minimum to maximum value). Adjusted *P* values * <.05 (Mann-Whitney test between time-points V0 and V1); *P* values # <.05 (Friedman test with a Dunn correction).

Patients also had lower frequencies of T-cell receptor γδ T cells carrying the CD56 cell marker (TCRγδ$^+$CD56$^+$ and TCRγδ$^+$CD56$^+$CD16$^+$) than controls at the time of tick bite (V1) (Figures 3A and 4), with a decrease in the TCRγδ$^+$CD56$^+$CD16$^+$ cell population in patients at V1 compared to baseline (V0) (Figures 3B and 5).

In the patient group, we observed increased frequencies of several B-cell subsets from baseline V0 to V1, the day after tick bite (Figures 3B and 5). This increase was seen in the parent population of CD19$^+$ B cells, in CD19$^+$CD20$^+$HLADR$^+$ B cells, in the subgroup of CD27$^-$IgD$^-$ B cells, and in CD19$^+$CD20$^-$ HLADR$^+$ plasma cells.

The long-term effects of tick bites are increased frequencies of natural killer (NK) cells (CD56^{mid}CD16^{hi}) and their parent population (CD3$^-$CD19$^-$HLADR$^-$), as well as increased γδ T cells and TCRγδ$^+$CD56$^+$ T cells over a 3-month period (Figures 3B and 5). In contrast, the frequencies of CD8 and CD8-naïve T cells, as well as the parent population of CD4 regulatory T cells (CD4$^+$CD25$^+$CD127$^-$), had decreased at V4, 3 months after a tick bite.

Tick bites induced both short-term and long-term effects in the patient cohort analyzed. Immediate changes are reflected by increased B-cell frequencies at V1, whereas NK cells and γδ T cells increase over time, and naïve CD8 T cells and CD4 regulatory T cells decrease.

Discussion

Tick bites have been recognized as the main source of sensitization and a major risk factor for the development of α-Gal syndrome. Current knowledge on the immune response to α-Gal in relation to tick bites is limited to GT-KO mouse models and the characterization of cells infiltrating human skin at the tick bite location [10,11,14,15]. Serum sIgE to α-Gal was shown to increase after a tick bite [18-20], although longitudinal data on systemic changes upon a tick bite are lacking.

Our study is the first to follow patients and controls for up to 3 months after a tick bite. We longitudinally analyzed sIgE, sIgG, and sIgG subclass responses, serum cytokine levels, major blood cell counts, and the frequencies of 66 cell populations by mass cytometry. sIgE increased in all patients during the first 2-4 weeks after the bite and decreased slightly at the 3-month visit. The binding of sIgE to tick extract was abolished almost entirely by deglycosylating the extract, confirming that most sIgE was directed against the α-Gal epitope. Residual IgE reactivity detected in some patients might be directed against tick proteins or other sugar moieties, although this hypothesis requires further investigation. All participants except one, for whom no data were available, had experienced tick bites before the study and had high sIgG at baseline that remained constant for up to 3 months after the bite. The range of the sIgG measured in our study (9-174 μg/mL) is in line with a recent evaluation of sIgG in AGS patients by the ImmunoCAP system (25-190 μg/mL) [37]. We found that the ratio of sIgG1 to sIgG2 indicated a higher abundance of sIgG1 in patients, whereas the ratio was more balanced in controls, in line with previous findings that α-Gal sIgG2 are the dominant subclass in healthy individuals [24-26]. It is important to note that sIgG4 antibodies were either absent or detectable only at very low levels, confirming a recent observation in tick-exposed forestry

workers and AGS patients [34]. Compared to specific IgG4 levels in allergies to other foods such as fish [23] and apple [21], the nearly undetectable α-Gal–specific IgG4 levels can be considered one of the characteristics of α-Gal syndrome. These findings also contrast with those from studies on sting-exposed beekeepers, revealing an increase in venom-specific IgG4 levels correlated with the number of bee stings [38,39] or higher values in tolerant than in allergic beekeepers [40]. Thus, the lack of sIgG4 seems to be related to the carbohydrate nature of the antigen rather than to the mode of sensitization via the skin.

The analysis of serum cytokine levels showed a significant increase in IL-8 in patients at 2 weeks and 3 months after the bite. There was no difference between patients and controls at baseline. Previous studies observed a strong local immune response to *I ricinus* tick bites in human skin. These were characterized by elevated mRNA levels of macrophage and neutrophil chemoattractants, including CXCL8 (IL-8) [15]. TNF-α and IFN-γ were not elevated. Treatment of a human skin keratinocyte cell line with exosomes from tick saliva induced up-regulation of IL-8 and down-regulation of CXCL12, thus impairing the repair process [41]. Kageyama et al [14] noted a T_H2 cytokine profile secreted by CD4 T cells from individuals bitten by *A testudinarium* and who reported a history of previous tick bites. Apostolovic et al [27] measured cytokine levels upon in vitro stimulation of PBMCs from patients with α-Gal syndrome and controls with *I ricinus* tick extract. Analysis of patient PBMCs revealed increased numbers of cells secreting cytokines with a T_H2 profile. These numbers decreased only moderately upon deglycosylation of the extract, suggesting that α-Gal was not the T_H2-inducing molecule. There was no difference for the T_H1 cytokine IFN-γ or for the anti-inflammatory cytokine IL-10 between patients and controls. In our study, we observed a significant increase in the inflammatory chemokine IL-8 and a trend toward higher levels of IFN-γ in the serum of patients than in that of controls. These higher levels of IFN-γ in patients and the increasing trend over time are in line with the increasing frequencies of NK cells and γδ T cells as major IFN-γ–secreting cell types [42,43]. In contrast to a recently described mouse model, where, using an α-Gal carrier protein, the authors observed an increase in serum IL-4 and IL-5 after sensitization to α-Gal [13], we saw no change in serum levels of T_H2 cytokines following a tick bite. A possible explanation for this apparent incoherence might lie in the time difference and type of sampling. Whereas most studies found T_H2 responses mainly in the skin up to several days after a tick bite or after in vitro stimulation of PBMCs [14,15,27], we analyzed peripheral blood samples within the first 24 hours and over a longer period of up to 3 months. Furthermore, a recent study recruiting healthy individuals with tick bites revealed a simultaneous reduction in IL-4–producing (type 2) and IFN-γ–producing (type 1) T cells or innate lymphoid cells in tick bite–affected skin as well as in peripheral blood, pointing to a complex interplay between local and systemic innate and adaptive immune responses to a tick bite [16]. The results obtained in human studies are difficult to compare because of marked variability related either to the tick (tick species and developmental stage, duration of attachment) or to human participants (healthy or α-Gal sensitized, number of recent tick bites, time of sample collection after tick removal, local reaction at the site of the bite).

Deep immunophenotyping by mass cytometry revealed lower frequencies of γδ T cells ($TCR\gamma\delta^+CD56^+$ and $TCR\gamma\delta^+CD56^+CD16^+$) directly after the tick bite in patients than in controls, suggesting a reduced potential of cellular cytotoxicity in patients. Both CD16 and CD56 are expressed in a subpopulation of γδ T cells and associated with a cytotoxic phenotype [44,45]. Interestingly, an overall increase in CD8 T-cell numbers, and frequencies of tissue-resident memory T cells and γδ T cells were found in tick bite lesions of healthy individuals compared to autologous healthy skin [16]. Taken together, γδ T cells and other cell types seem to be affected by tick bites, and their implication in sensitization to α-Gal should be investigated in greater depth. Furthermore, in our study, patients had lower frequencies of DCs overall and, more specifically, pDCs. It has been shown that depletion of pDCs induces allergic airway sensitization [46] and inflammation in a mouse asthma model [47]. Infants with a subsequent asthma diagnosis have lower pDC counts than healthy children [48]. DCs are also predominant in early-stage tick bite lesions [15], and pDCs rapidly infiltrate damaged skin and promote wound repair [49,50]. We could thus speculate that a lower abundance of circulating pDCs in patients may reflect a lower abundance of pDCs in damaged skin upon a tick bite, favoring allergic sensitization mechanisms similar to those observed in lung tissue. The levels of type 1 IFN production by these skin-infiltrating pDCs, which is essential for preventing a T_H2 response, remain to be analyzed in future studies [51].

The most prominent longitudinal changes in patients were an increase in the B-cell populations from baseline to V1, including naïve B cells, IgM memory cells, switched memory B cells, $CD27^-IgD^-$ B cells ($CD19^+CD20^+HLADR^+$), and plasma cells ($CD19^+CD20^-HLADR^+$). For the first time, we demonstrate that tick bites induce rapid expansion of B cells followed by the production of sIgG and sIgE antibodies. Interestingly, $CD27^-IgD^-$ B cells have been reported to contain IgE^+CD27^- memory B cells in the blood of food-allergic children [52] and to play a role in the production of sIgE to α-Gal [28]. Single-cell transcriptomic analyses of B cells from a cat-allergic patient revealed the existence of a $CD27^-IgD^-IgE^+$ B-cell subset [53]. This subset of memory B cells has been poorly studied to date, although its implications in autoimmune and inflammatory disorders, as well as in infectious diseases and cancer, have been highlighted [54,55]. Future studies might reveal the role of $CD27^-IgD^-$ memory B cells in the context of α-Gal syndrome.

Initially falling after a tick bite, frequencies of circulating γδ T cells and $TCR\gamma\delta^+CD56^+$ T cells recovered slowly and constantly over a 3-month period. Similarly, NK cells (CD56midCD16hi) increased constantly up to 3 months after a tick bite. These findings may reflect the fast recruitment of innate cells to the site of inflammation and the importance of continuous innate immune surveillance for infection at epithelial barriers [56,57]. Conversely, the higher numbers of circulating naïve CD8 and CD4 regulatory T cells observed at V1 and their decrease over time could be interpreted as their egress from lymph nodes, transitioning to the epithelial site of inflammation in response to vector-borne immunomodulatory

agents [5,58] and leveling out over time. However, these long-term changes could also be considered a result of the time span between sampling time-points. Time-point V1 was at the end of spring/beginning of summer; time-point V4 was at the end of summer/beginning of autumn. Changes in the immune cell populations that were observed in this time interval might merely follow the natural circannual fluctuations of lymphocytes, which begin to decrease at the beginning of autumn [59]. Differences between V0 and V1/V2/V3 and between patients and controls at each time-point are less likely to be affected by such seasonal variations.

The longitudinal design of our study is both a strength and a weakness. For the first time, we describe humoral and cellular immune responses following a tick bite in α-Gal–sensitized patients and controls at defined time-points. However, as the study design relied on natural exposure, the number of participants experiencing a tick bite was limited to only 4 controls and 10 patients who were monitored over time. As several time-points were missing for the controls, a paired longitudinal analysis was only possible for the patient group. While this study was designed to be explorative and observational, future studies should strive to gain a deeper insight into cell activation and function. Ideally, those investigations should be complemented by the analysis of skin biopsies.

In conclusion, our study is the first to monitor immunological changes in peripheral blood in α-Gal–sensitized patients after a tick bite. The most important findings are the increase in α-Gal–specific IgE levels, as well as an early increase in B-cell subset frequencies, paralleled by an early decrease in cytotoxic γδ T cells in response to a tick bite. Patients had lower frequencies of pDCs and cytotoxic γδ T cells than controls, especially after a tick bite. The findings of the present explorative study pave the way for future investigations on the key factors at play in sensitization to α-Gal.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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