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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DOI: 10.1111/all.13794

The contribution made by skin-infiltrating basophils to the development of alpha-gal syndrome

To the Editor,

The galactose-alpha-1,3-galactose (alpha-gal) disaccharide is a typical glycoprotein component in mammals. Nevertheless, over evolutionary time, primates such as humans have lost this because of loss of galactosyltransferase, the enzyme required for its production and large amounts of IgM and IgG, but not IgE, antibodies specific to this molecule are naturally produced in healthy individuals; however, certain patients that have been bitten by ticks that harbor this carbohydrate¹ and may develop alpha-gal syndrome, an immediate-type hypersensitivity to red meat and drugs containing alpha-gal,² which is mediated by anti-alpha-gal IgE antibody. Although we and others have recently reported that a recurrent tick bite history is associated with the development of alpha-gal syndrome,^{3,4} little is known about how tick bites are involved in the development of alpha-gal syndrome. To investigate this in more detail, we stratified the patients visiting our hospital with new tick bites according to their past tick bite histories, where the historical number of bites they had received was added to the new one (1 tick bite [1°], 2 tick bites [2°], or ≥3 tick bites [≥3°]). One hundred and twelve patients with new tick bites, who presented at our hospital, were enrolled in

this study after informed consent was provided. More than 80% of the ticks were identified as *Amblyomma testudinarium* (most are nymphs; a few are adults and no larvae). The study was performed according to the Declaration of Helsinki, and the study protocol was approved by the Shimada Municipal Hospital Ethical Committee.

Because the alpha-gal epitope is structurally related to the blood type B antigen, specific IgE antibody production is reportedly suppressed in patients who have this antigen.⁵ We reassure that the levels of specific IgE antibodies were significantly higher in the A and O blood type patients who had recurrent tick bite histories, compared with only a marginal increase in the B and AB blood type patients (Figure S1 and Table S1); and thus, we excluded the patients with B and AB blood types from any further investigations to delineate clearly the relationships between anti-alpha-gal IgE production and immune cell infiltration. We compared the chronological variations (1-5 days, the early period and ≥6 days after the bite, the late period) in the antibody levels among these three groups (Figure 1A). In the 1° group (N = 32), the IgE-specific antibody levels were constantly below detectable levels (<0.1 U_A/

mL) after the tick bites, except for two cases (Figure 1A, left). In the 2° group (N = 27), the IgE-specific antibody levels tended to increase although it was not statistically significant (Figure 1A). In the ≥3° group (N = 39), the IgE-specific antibody levels were significantly higher in the early (mean, 1.90 U_A/mL) and late period (mean, 14.7 U_A/mL) than those in the 1° group (Figure 1A, right, Mann-Whitney test). We also investigated basophil number in the skin bitten by ticks as previously described.³ In the 1° group, basophil infiltration was around 10 cells/HPF or less during the time course after the bites occurred (Figure 1B). However, we found a significant increase in basophil numbers in the early period in the 2° and ≥3° groups ($P = 0.0065$ and $P = 0.0310$, respectively, Mann-Whitney test), and the cell number tended to decrease in the late period in these groups (Figure 1B) although it was not significant. The cell numbers tended to decrease in the late period in these groups (Figure 1B). As basophils are known to play an important role in orchestrating the Th2-polarized immune response, alterations in basophil kinetics may impact the ability of Th2 cells to infiltrate the bitten skin. Therefore, we next investigated cytokine production in skin-infiltrating T cells from the tick-infested lesions (Data S1). The ratio of the IFN- γ -producing CD4+ T-cell numbers/IL-4-producing CD4+ T-cell numbers (designated here as Th1/Th2) was around 1 in the 1° group, 0.3 in the 2° group, and below 0.1 in the ≥3° group, indicating that the ratios decreased with increasing numbers of tick bites (1° vs ≥3°, $P = 0.0092$. Figure 2A,B, top), as similarly observed in the ratios of IFN-producing CD4+ T-cell numbers/IL-5-producing CD4+ T-cell numbers and IFN-producing CD4+ T-cell numbers/IL-13-producing CD4+ T-cell numbers (Figure 2B middle and bottom). We also preliminarily observed around 25% of IL-3-producing cells among skin-infiltrating CD4+ T cells from the 3° group, but only <5% of those from the 1° group. However, we did not find any differences in IL-17- or IL-22-producing CD4+ T-cell numbers among these groups.

In recent studies in mice infested with ectoparasites, basophils were found to infiltrate the lesions of a second infestation more densely and more rapidly than those during the first infestation.⁶ Fc receptor activation in basophils contributes to the acquired immunity required to dislodge ectoparasites via secretion of histamine, a soluble repellent factor,⁷ and by IL-4 and IL-13, which trigger local inflammation.⁶ Concurrently, basophils contribute to Th2 polarization by acting as both a provider of early IL-4 and an antigen-presenting cell with or without dendritic cell coordination.⁸ These raise the possibility that repeated tick bites trigger a vicious cycle of Th2 polarization by basophils, which results in enough anti-alpha-gal IgE antibody production to induce alpha-gal syndrome. Although it has a limitation, our finding also supports a scenario where skin-infiltrating basophils are rapidly activated by cross-linking to Fc receptors with IgE antibody and promptly leaving the skin to migrate to the lymph nodes. In conclusion, repeated tick bites promote rapid basophil recruitment and attract Th2 cells to the skin to provide a cytokine milieu that enhances IgE production specific to tick-derived antigens, thereby facilitating effective acquired immunity to ticks on the one hand and development of alpha-gal syndrome on the other. Our findings shed light on the collaborative association of basophils and T cells in terms of clarifying the mechanisms involved in allergic disorders.

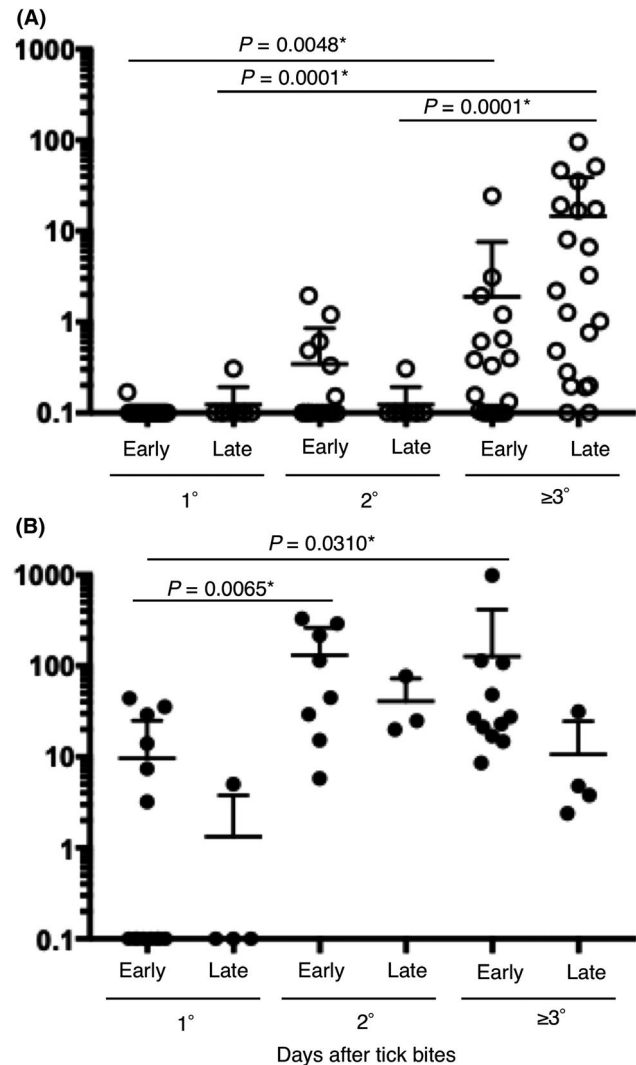


FIGURE 1 A, Chronological variations in anti-alpha-gal IgE antibody levels (U_A/mL) in the patients with various tick bite histories (1°–≥3°). B, Chronological variations in basophil numbers (cells/HPF) in the bitten skin from the patients with various tick bite histories (1°–≥3°). *Mann-Whitney test

FUNDING INFORMATION

This work was partly supported by Health and Labor Sciences Research Grants (Research on Intractable Diseases) from the Ministry of Health, Labor and Welfare of Japan.

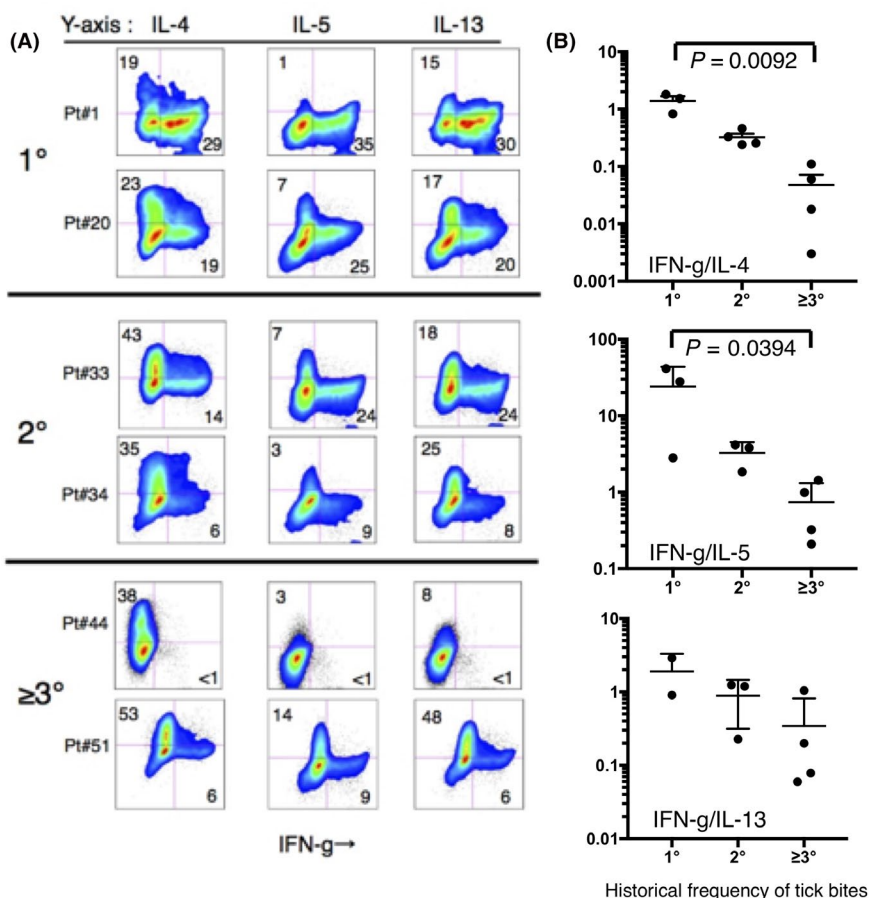
CONFLICTS OF INTEREST

The anti-galactose- α 1,3-galactose IgE antibody measurements for the study participants were partly performed by Phadia Co., Tokyo, under a gratuitous contract as a collaborative project.

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FIGURE 2 Cytokine production in skin-infiltrating T cells from tick bitten skin. A, IL-4, IL-5, IL-13 (y-axis), and IFN- γ (x-axis) production in CD4 $^{+}$ T cells infiltrating the primary lesions (1 $^{\circ}$), the 2nd lesions (2 $^{\circ}$), and the $\geq$ 3rd lesions ($\geq$ 3 $^{\circ}$). Numbers indicate the percentages of the positive cells. Representative data. B, IFN- γ -producing cell/IL-4 $^{-}$, IL-5 $^{-}$, and IL-13-producing cell ratio for the CD4 $^{+}$ populations in bitten skin. *Mann-Whitney test [Colorfigure can be viewed at wileyonlinelibrary.com]



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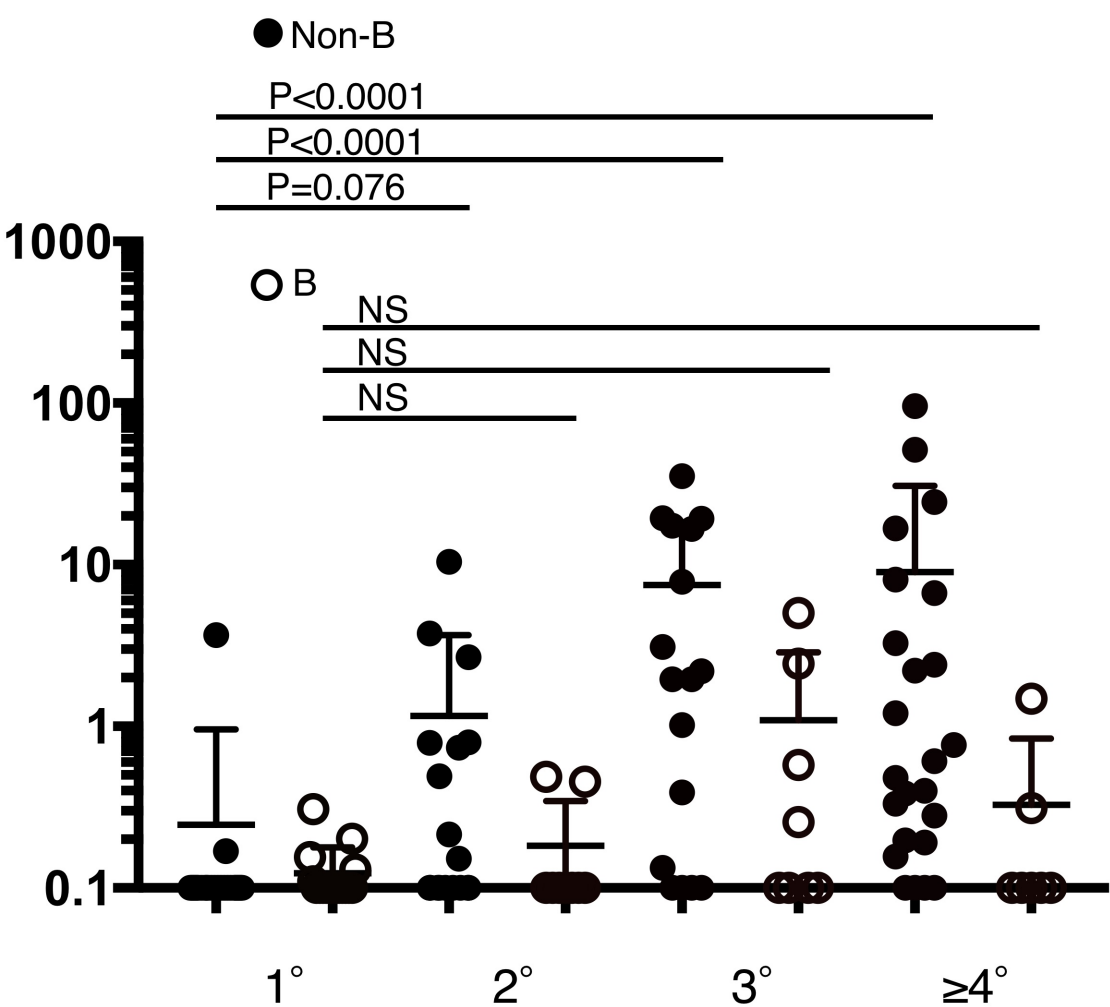


Table S1 Prevalence of positive anti-alpha gal IgE antibody among different blood types.

Bite frequency*	A/O (%)	B/AB (%)	P value**
1	1/25 (4.0)	1/17 (5.9)	>0.9999
2	7/18 (38.9)	2/9 (22.2)	0.6673
≥3	27/41 (65.9)	4/15 (26.7)	0.0145

*, historical frequency of tick bites (times). **, Fisher's exact test.

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Online supplementary

Fig. S1. Anti-alpha gal IgE antibody levels (U_A/ml) in the patients with various tick-bite histories (from 1^o- $\geq 4^o$ tick bites) who were blood type A or O (non-B) and AB or B (B).

Materials and Methods

Reagents and antibodies

Fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against the interferon- γ (IFN- γ) cytokine, and interleukin (IL)-3, IL-4, IL-5, IL-13, IL-17 and IL-22 were purchased from BD PharMingen (San Diego, CA, USA). The basophil-specific monoclonal antibody (BB1, anti-basogranulin Ab) we used was kindly provided by Prof. AF Wall (Southampton General Hospital, UK). Cells were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with L-glutamine, sodium pyruvate, 2-mercaptoethanol, nonessential amino acids (Life Technologies) and 10% pooled human AB blood-type serum. For T lymphocyte expansion, the medium was supplemented with human recombinant IL-2 (R&D Systems) and/or anti-CD3/CD28 Ab-conjugated microbeads (T-cell Expander; Dynal, Copenhagen, Denmark), as previously described⁹.

Histological study

Formalin-fixed, paraffin-embedded sections were treated with antigen-retrieve solution after deparaffinization and then stained with BB1, as previously described⁹. The number

of infiltrating basophils was enumerated in 5 high-power fields randomly selected in each of the lesions.

Cytokine production in skin-infiltrating T cells

Skin-infiltrating T cells were propagated from the skin specimens using our previously established method. Cytokine production was investigated by intracytoplasmic cytokine staining of the cells after stimulation with phorbol myristate acetate (PMA) as previously described¹.

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