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Cutaneous Exposure to Clinically Relevant Lone Star Ticks Promotes IgE Production and Hypersensitivity through CD4+ T Cell– and MyD88-Dependent Pathways in Mice

Jessica L. Chandrasekhar,* Kelly M. Cox,*,† William M. Loo,* Hui Qiao,* Kenneth S. Tung,*,‡ and Loren D. Erickson*,†

Tick-borne allergies are a growing public health concern and have been associated with the induction of IgE-mediated food allergy to red meat. However, despite the increasing prevalence of tick bite–induced allergies, the mechanisms by which cutaneous exposure to ticks leads to sensitization and the production of IgE Abs are poorly understood. To address this question, an in vivo approach was used to characterize the IgE response to lone star tick proteins administered through the skin of mice. The results demonstrated that tick sensitization and challenge induced a robust production of IgE Abs and supported a role for IgE-mediated hypersensitivity reactions in sensitized animals following oral administration of meat. The induction of IgE responses was dependent on cognate CD4+ T cell help during both the sensitization phase and challenge phase with cutaneous tick exposure. In addition, IgE production was dependent on B cell–intrinsic MyD88 expression, suggesting an important role for TLR signaling in B cells to induce IgE responses to tick proteins. This model of tick-induced IgE responses could be used to study the factors within tick bites that cause allergies and to investigate how sensitization to food Ags occurs through the skin that leads to IgE production. ***The Journal of Immunology*****, 2019, 203: 000–000.**

Food allergy is an adverse immune response to a given food and is a growing public health concern that can carry a high risk of life-threatening allergic reactions (1–3). It is estimated that an IgE-mediated food allergy affects 2–10% of the general population (4). There is no treatment to prevent or cure food allergies, and thus, for most patients, the only management is avoidance of foods (5). IgE Abs are produced by B cells as a result of Ag exposure through skin, gut, or respiratory tract. Food Ags are then processed by APC and presented to CD4+ T cells that provide help to B cells, leading to the production of specific IgE Abs. Upon re-exposure to the Ag, a more rapid and greater IgE response ensues. In view of the essential role of B cells in IgE-mediated hypersensitivity, interference with their sensitization to foods is considered a potential new therapeutic strategy in food allergy.

Red meat allergy is among a minority of food allergies that pose a serious acute health risk that can induce severe cutaneous, gastrointestinal, and respiratory reactions (6, 7). This novel form of food allergy develops worldwide in adults who have tolerated meat consumption for years and is postulated to result from tick bites through mechanisms that remain unknown (7–15). In the United States, bites from lone star ticks can induce IgE Abs to tick proteins and is the primary tick species associated with a red meat allergy. Allergic reactions in affected individuals following meat consumption are believed to be mediated by IgE Abs specific for galactose-α-1,3-galactose (α-gal), a blood group Ag of nonprimate mammals and therefore present in common dietary meat such as beef, pork, and lamb (16, 17). α-gal is also found within the intestinal tract and saliva of certain tick species (7, 18). Thus, an emerging thought is that exposure to tick bites promotes cutaneous sensitization to tick Ags such as α-gal, leading to the development of IgE-mediated food allergy to meat digested through the oral route. However, the underlying mechanisms by which skin exposure to ticks leads to the development and maintenance of IgE production has not been investigated, and there currently is not a clinically relevant animal model of IgE-mediated hypersensitivity induced by ticks.

In this study, we have developed a new mouse model to study IgE responses to cutaneous tick exposure by administering lone star tick Ags through the skin of mice. We demonstrate that tick sensitization and challenge induce specific IgE and IgG1 Ab production in healthy mice and elicits hypersensitivity reactions in sensitized animals following that oral administration of meat that were more severe in animals that produced α-gal–specific IgE. Upon tick exposure, mice depleted of CD4+ T cells exhibited specific IgG1 but not IgE production compared with controls. The inhibition of T cell help by blocking CD40L (CD154) during either the sensitization phase or the challenge phase revealed that cognate T cell help is required for sensitization and recall to produce specific IgE Ab responses but not for recall to produce specific IgG1. Finally, we show that sensitization to tick exposure requires MyD88 signaling, and this requirement is B cell intrinsic. Collectively, our

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Abbreviations used in this article: alum, adjuvant aluminum hydroxide; α-gal, galactose-α-1,3-galactose; GC, germinal center; GT KO, α-galactosyl transferase$^{-/-}$; HDM, house dust mite; Ighγ1, Ig heavy constant γ 1; KLH, keyhole limpet hemocyanin; MCP-1, mast cell protease-1; NP, 4-hydroxy-3-nitrophenylacetyl; Tfh, T follicular helper.

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findings identify CD4$^+$ T cells and MyD88 signaling pathways as important underlying mechanisms for the induction of IgE responses to cutaneous tick exposure and establish an in vivo model that can be used to investigate how IgE is regulated in tick-borne–induced allergies.

Materials and Methods

Mice

Wild type C57BL/6J, MyD88$^{-/-}$, MyD88^{fl}, Cγ1-cre (all on the C57BL/6J background), and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The α-galactosyl transferase$^{-/-}$ (GT KO) mice were a generous gift from Dr. U. Galili (Rush University, Chicago, IL). All of the mice were maintained at the University of Virginia under specific pathogen-free conditions and used according to the regulations and standard guidelines of the Institutional Animal Care and Use Committee. All experiments were conducted with 8–10-wk-old female mice unless otherwise noted.

Tick extract preparation

Extract from lone star ticks was prepared from larval, pathogen-free *Amblyomma americanum* purchased from the Oklahoma State Tick Rearing Facility (Stillwater, OK), as previously described under endotoxin-free conditions (9, 19). Briefly, ticks were flash frozen, homogenized into powder form, and reconstituted in a borate buffered saline (pH 8) overnight at 4°C in the presence of protease inhibitors. Proteins were extracted by defatting the saline solution with ethyl ether and separated by centrifugation at 3000 rpm × *g* for 5 min. The aqueous layer containing proteins was collected for analysis.

Immunizations

Mice were sensitized by s.c. injections in the flank with 50 μg of tick extract in 100 μl total volume on days 0 and 7. On day 31, tick-sensitized mice were challenged by s.c. injection in the flank with 50 μg of tick extract in 100 μl total volume and analyzed 4 d later. To test the specificity of IgE to tick extract, mice were primed intranasally with 10 μg in 50 μl volume of low-endotoxin house dust mite (HDM) as an irrelevant allergen (Indoor Biotechnologies, Charlottesville, VA) on days 0, 3, and 5. Mice were challenged intranasally on days 10, 12, and 14, and serum Abs were analyzed on day 16. In experiments testing the adjuvant effects of tick extract, mice were s.c. injected with 50 μg of tick extract or the adjuvant aluminum hydroxide (alum) plus 50 μg of the Ag 4-hydroxy-3-nitrophenylacetyl (NP) conjugated to keyhole limpet hemocyanin (KLH) at a ratio of 36 (Biosearch Technologies, Petaluma, CA) in 100 μl volume on days 0, 7, and 31. In experiments testing the effects of α-gal–specific IgE, mice were s.c. injected with 50 μg of tick extract plus 50 μg of α-gal–BSA (Dextra Laboratories, Reading, U.K.) on days 0, 7, and 31 to cutaneously expose mice to a standard known amount. Serum was obtained on day 35 unless otherwise indicated, and Ab titers were measured by ELISA. Single cell suspensions prepared from inguinal lymph nodes were analyzed using flow cytometry. The frequencies of NP-specific germinal center (GC) B cells were analyzed as previously described (20). Briefly, cell suspensions were labeled for flow cytometry at 2.0×10^8 cells/ml in 5% FBS in PBS on ice for 45 min, with predetermined optimal concentrations of the following fluorophore-labeled mAbs: B220- allophycocyanin-Cy7 (RA3-6B2; BD Biosciences, San Jose, CA), GL7-EF450 (GL-7; eBioscience), CD95-BV605 (15A7; BioLegend, San Diego, CA), and NP–allophycocyanin (Biosearch Technologies). The following PE-conjugated mAbs were used as a dump gate: CD4 (RM4-5; BD Biosciences), CD8 (53-6.7; Caltag Laboratories, Burlingame, CA), Ly-6G/Ly-6C (GR-1; BioLegend), and F4/80 (BM8; BioLegend).

Histology

Skin samples for H&E and Toluidine blue staining were obtained on day 35 and fixed in 10% formalin (Thermo Fisher Scientific) for 48 h and then transferred into 70% ethanol. The samples were embedded in paraffin, and 8-μm cross-sections were taken for subsequent staining by the University of Virginia Histology Core. A qualified pathologist at the University of Virginia performed histologic examinations of the stained skin samples. Severity of dermal thickening, muscle fiber atrophy, vasodilation/endotheliitis, leukocyte infiltration within the dermis, and hypodermis were each scored on a scale of 0 to 5 as follows: 0, normal; 1, minimal, focal infiltration; 2, mild infiltration; 3, moderate, multifocal infiltration, no fibrosis; 4, marked infiltration, fibrosis; and 5, diffuse, severe infiltration, fibrosis. Scores for each pathological finding were combined to yield a composite score for each sample. Detection of mast cells in the skin of mice was performed by Toluidine blue staining as previously described (21). In brief, sections of tissues from mice were rehydrated in xylene and xylene plus ethanol and then sequentially in graded percentages of ethanol. Each section was then washed with PBS and stained with 0.1% Toluidine blue dye for 30 min. Dehydration of slides was carried out and mounted. Mast cells were identified by the presence of purple cells and counted by blinded observers for each section (22). The number of mast cells was counted per unit area using a measuring eyepiece. Images were acquired using an Olympus BH2 (BHTU) Polarizing Trinocular microscope and analyzed using the National Institutes of Health ImageJ software (Bethesda, MD).

Hypersensitivity responses to meat consumption

Naive and immune mice were orally gavaged with 50 μg of α-gal–containing beef thyroglobulin (Sigma-Aldrich, St. Louis, MO) in 100 μl of UltraPure Distilled Water (Invitrogen, Carlsbad, CA) on day 35. Approximately 200 μl of blood was collected and combined with an equal volume of heparin on day 34, prior to gavage as a baseline control, and on day 35 at 60 and 90 min following oral gavage. Heparinized blood was either treated with ammonium chloride–Tris to lyse RBCs and basophil activation was assessed by flow cytometry or stimulated to release histamine from basophils in whole blood using a modified protocol as previously described (23). Briefly, heparinized blood was centrifuged at 600 × *g* for 10 min at room temperature. Plasma was removed, and cells were washed with a buffer of RPMI 1640 that contained 1 mM L-glutamine and 5% human AB serum. Equal parts of washed blood and buffer that contained no stimulant or 100 ng/ml of either cetuximab or deglycosylated cetuximab were incubated in a 96-well v-shaped plate for 90 min at 37°C. The plate was centrifuged at 1500 rpm × *g* for 5 min, the supernatants were collected, and the histamine levels were measured by ELISA. In addition to heparinized blood, serum was also obtained on day 35 at 90 min following oral gavage and analyzed for Ab and mast cell protease-1 (MCP-1) titers by ELISA. Finally, the body temperature of mice was measured using a digital thermometer with a rectal probe (YSI, Yellow Springs, OH) prior to oral gavage and at 15, 30, 45, and 60 min following gavage.

CD4$^+$ T cell depletion

Mice received s.c. injections with 50 μg lone star tick extract on days 0, 7, and 31. Mice administered tick extract were divided into two groups with one receiving i.p. injections of 250 μg anti-CD4 mAb (GK1.5 clone; Bio X Cell, West Lebanon, NH) and the other receiving 250 μg control rat IgG on days 27, 29, and 31. On day 35, serum was isolated for ELISA analysis, and inguinal lymph node single cell suspensions were evaluated by flow cytometry.

MR1 treatment

Mice sensitized and challenged with tick extract were divided into three groups. The first group received i.p. injections of 500 μg of the CD40L (CD154) mAb (MR1 clone; Bio X Cell) on day 5 and 250 μg MR1 on days 7, 9, 11, and 13 following the initial s.c. injection of tick extract. The second group received i.p. injections of 500 μg MR1 on day 29 and 250 μg MR1 on days 31 and 33 before and after the third s.c. injection of tick extract. The third group received i.p. injections of 500 μg control hamster IgG on days 5 and 29 and 250 μg hamster IgG on days 7, 9, 11, 13, 31, and 33.

TLR screen

TLR ligand screening was performed by InvivoGen (San Diego, CA). Briefly, HEK293 cells engineered to express a single TLR and a SEAP reporter plasmid that is induced upon activation of NF-κB and AP-1 were incubated with tick extract. TLR activation was assessed by SEAP expression and measured as absorbance at OD_{650} nm. Cells incubated with known TLR ligands served as the positive control, and unstimulated cells served as the negative control.

ELISAs

Total serum IgE, tick-specific IgE and IgG1 titers, and NP hapten-specific Ab titers were determined by ELISA. For total serum titers, Costar high binding plates (Corning, Corning, NY) were coated with 5 μg/ml unlabeled anti-mouse IgE (SouthernBiotech, Birmingham, AL). For Ag-specific ELISAs, high binding plates were coated with tick extract at a concentration of 5 μg/ml or NP conjugated to BSA at a ratio of 32 or 4 (Biosearch Technologies) at a concentration of 5 μg/ml, as previously described (24).

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Serum was diluted 1:100 for IgE and 1:10,000 for IgG1 and serially titrated in 3-fold increments. HRP-labeled anti-mouse IgE or IgG1 (SouthernBiotech, applied according to manufacturer's instructions) served as the detection Ab, and the assay was developed using tetramethylbenzidine (BD Pharmingen) with 2N H_2SO_4 used as the stop solution. To measure α-gal–specific Ab titers, plates were coated with 5 μg/ml of cetuximab, a chimeric mouse-human IgG1 mAb against epidermal growth factor receptor that contains α-gal residues on the Fab portion of the cetuximab H chain that are recognized by IgE specific for α-gal (25). Binding to α-gal was confirmed by testing cetuximab that was deglycosylated to remove α-gal residues by preincubating cetuximab with α-1→ (3, 6)-Galactosidase for 1 h at 37°C, according to manufacturer's instructions (Sigma-Aldrich). No binding of the monoclonal anti–α-gal Ab M86 (Enzo Life Sciences, Farmingdale, NY) confirmed deglycosylation of α-gal. No binding of M86 to BSA alone was also confirmed. Serum was diluted 1:10,000 for IgG1 and serially titrated in 3-fold increments. To avoid competitive binding to α-gal epitopes by α-gal–specific IgG Abs, which may be more than 100-fold excess to IgE and thus interfere with detection of α-gal–specific IgE, serum samples were depleted of IgG using a modified protocol (26) with a Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) and then tested for IgE Ab to α-gal at a 1:10 dilution and serially titrated in 3-fold increments. Total IgE Ab titers were quantified through a standard curve obtained using unlabeled IgE (SouthernBiotech), whereas OD values were compared for Ag-specific assays. All samples were assessed in duplicate. Mouse MCP-1 serum levels were measured by ELISA according to the manufacturer's instructions (BioLegend). Histamine levels in basophil culture supernatants were determined using a Histamine ELISA Kit according to the manufacturer's instructions (Enzo Life Sciences). All plates were read at 450 nm using a BioTek Plate Reader.

Flow cytometry

Single cell suspensions from pooled inguinal lymph nodes of each mouse were treated with erythrocyte lysis buffer (20 mM Tris/HCl and 155 mM NH_4Cl [pH = 7.2]) and washed with 5% FBS in PBS. Cell counts were determined using a Neubauer hemocytometer. Cells were stained with fluorescently labeled mAbs as previously described (27). The following Abs were used: B220-allophycocyanin-Cy7 (RA3-6B2; BD Biosciences), CD4-FITC (RM4-5; BD Biosciences), CD95-biotin (15A7; eBioscience), streptavadin-PE (BioLegend), GL7-EF450 (GL-7; eBioscience), PD-1–PECy7 (RMP1-30; BioLegend), CXCR5- allophycocyanin (L138D7; BioLegend), FcεRI-FITC (MAR-1; BioLegend), CD200R-PE (OX-108; BioLegend), cKit- allophycocyanin (2B8; eBioscience), CD45- allophycocyanin (104; eBioscience) CD49b-PerCPCy5.5 (DX5; BioLegend), phosphorylated p38-PECy7 (pT180/pY182; BD Biosciences), and CD41-BV421 (WMReg30; BD Biosciences). Cell viability was determined using LIVE/DEAD Aqua (Invitrogen), and doublets were excluded based on forward scatter and pulse width. Samples were acquired on a CyAn ADP LX (Beckman Coulter, Brea, CA) or CytoFLEX (Beckman Coulter) and analyzed using FlowJo software version 10.1r7 (Tree Star, Ashland, OR). Gates were determined using fluorescence minus one staining controls.

Statistical analyses

All analyses were performed using Prism 8.0 software (GraphPad Software, San Diego, CA). Statistical significance comparing two experimental groups was determined using unpaired Student *t* test with Welch correction or Mann–Whitney *U* test. Data are presented as mean ± SEM, unless otherwise stated. The threshold for significance was $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Cutaneous exposure to tick extract induces an IgE response in mice

To determine whether skin exposure to lone star ticks induces a systemic IgE response in mice, protein extract prepared from *A. americanum* larvae was administered s.c. to C57BL/6 mice. This approach was taken because larvae aggressively bite humans (28–30) and would expose the dermis and blood to tick proteins. Mice were sensitized with tick extract on days 0 and 7 and then challenged with tick extract on day 31 (Fig. 1A). The immune response to tick extract challenge was assessed on day 35. Results demonstrated that total IgE levels in serum of immunized mice increased significantly compared with naive controls (Fig. 1B). Analysis of serum IgE titers over the course of tick immunization indicated that sensitization induced a small but not statistically significant increase in IgE after 1 wk (day 7), which increased significantly after 2 wk (day 14) following the second immunization (Fig. 1C). Levels of IgE were found reduced by day 31 nearly to the levels measured in naive mice. Upon challenge, serum IgE rapidly increased within 4 d (day 35), surpassing the levels observed following the second immunization (Fig. 1C). IgE Abs specific for *A. americanum* proteins correlate strongly with IgE Abs to α-gal in human subjects with a history of tick bites (9). Thus, we further assessed the specificity of IgE by ELISA and found that mice immunized with tick extract mounted a significant tick protein–specific IgE response compared with naive controls (Fig. 1D). Tick-specific IgE was not detected from the serum of mice immunized with the irrelevant HDM allergen, thereby confirming the specificity of the assay. BALB/C mice sensitized and challenged with tick extract also showed greater total and tick-specific IgE serum levels compared with naive controls, demonstrating that the IgE response induced by cutaneous tick exposure is not strain dependent (Supplemental Fig. 1A, 1B).

Previous studies showed that IgE production in secondary responses required $CD4^+$ T cell help (31, 32). Thus, we evaluated the development of GC responses in the draining inguinal lymph nodes and nondraining spleens of mice. Challenge with tick extract induced a small but significant increase in both GC B cells (Fig. 1E, 1F) and T follicular helper (Tfh) cells (Fig 1G, 1H) in the inguinal lymph nodes. In contrast, no induction of either GC B cells or Tfh cells were observed in the spleen (data not shown), consistent with the route of s.c. exposure to Ag. Taken together, these data indicate that s.c. sensitization and challenge of healthy mice with lone star tick extract induces a strong IgE response.

Meat-allergic patients experience local reactions to tick bites (7, 9, 33). To determine whether cutaneous exposure to tick extract similarly induced local inflammatory responses in mice, histopathological examination of skin sections taken at the site of immunization was performed. Skin sections from the site of tick extract injections showed the following four findings: dermal thickening; vasculitis and muscle fiber atrophy; mixed inflammatory cell infiltration within the dermis and hypodermis, including granulocytes, lymphocytes, and plasma cells, some with Russell bodies; and inflammation of the vasculature (Fig. 2A–E). In the skin of mice injected with PBS, no such symptoms were evident. These findings were separately evaluated in a blinded manner using a scoring system, and the combined pathology scores were quantified. Results demonstrated a greater pathology score in skin sections of mice immunized with tick extract compared with naive, PBS-treated controls (Fig. 2F). In addition, greater numbers of mast cells were found within the skin of mice sensitized and challenged with tick extract compared with those from naive animals (Fig. 2G). These findings establish the capacity of cutaneous tick exposure to induce local inflammation.

Skin inflammation is strongly linked with food allergy (34–36). The heightened effect of environmental peanut allergen exposure in children with skin inflammation suggests that sensitization to allergens occurs through the skin that can lead to disease at other anatomic sites (37, 38). Therefore, we assessed whether the development of an IgE response was dependent on cutaneous tick exposure or could also be induced through other routes of immunization. Mice sensitized and challenged i.p. with tick extract failed to produce significantly increased levels of total and tick-specific IgE compared with those immunized s.c. (Supplemental Fig. 2A, 2B). These results indicate that the cutaneous route of exposure plays an important role in inducing an IgE response to tick extract and is in keeping with the evidence in humans that sensitization to α-gal requires skin exposure.

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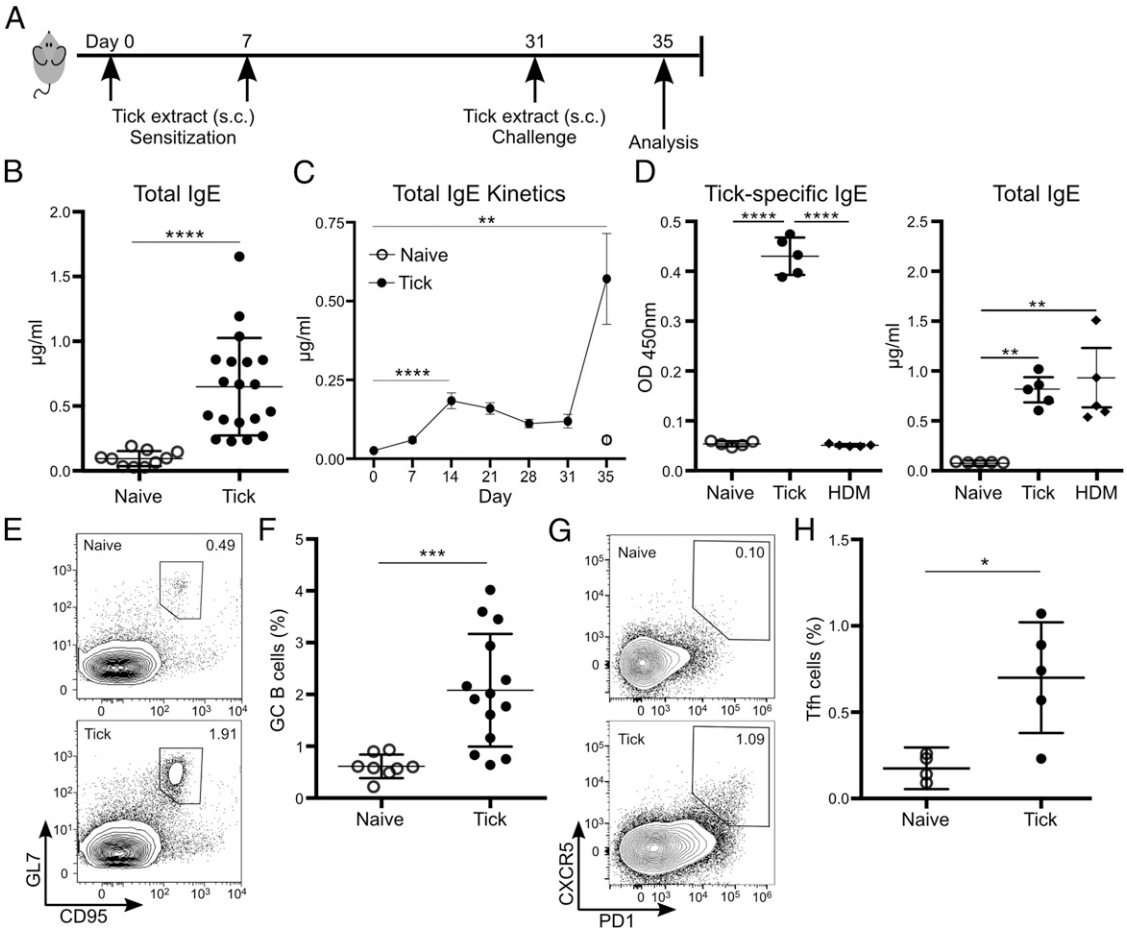

FIGURE 1. Cutaneous tick exposure induces an IgE response. (**A**) Mice were injected s.c. with tick extract on days 0, 7, and 31 and analyzed on day 35. (**B** and **C**) Serum levels of total IgE and (**D**) tick-specific IgE were assessed by ELISA. Serum from mice immunized with the irrelevant HDM allergen was used to confirm specificity of the assay. (**E** and **F**) Percentages of GC B cells ($B220^+CD95^+GL7^+$) and (**G** and **H**) Tfh cells ($CD4^+PD\text{-}1^+CXCR5^+$) in inguinal lymph nodes of mice were quantified by flow cytometry. Representative contour plots depict the frequencies of (E) GC B cells and (G) Tfh cells. Each symbol represents an individual mouse, and error bars show the mean ± SEM. One of two experiments with similar results is shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Tick-immunized mice exhibit a hypersensitivity response following oral exposure to beef

Mice synthesize α-gal epitopes and therefore naturally do not produce α-gal IgG Abs because of immune tolerance (16, 39). To determine whether s.c. exposure to tick extract was capable of inducing an α-gal–specific IgE response and its effect on hypersensitivity, we analyzed wild type animals and mice deficient in the GT KO (40) immunized with tick extract in the presence of a known amount of α-gal and orally gavaged with beef thyroglobulin (Fig. 3A). Levels of total IgE and tick-specific IgE and IgG1 were induced both in wild type and GT KO mice after tick immunization, and these Ab levels did not significantly change following oral gavage (Fig. 3B–D). α-gal–specific IgG1 was not detected in wild type mice as expected (Fig. 3E). In contrast, α-gal–specific IgG1 was present in GT KO mice and was not substantially increased in immune animals compared with naive controls (Supplemental Fig. 3A). That α-gal–specific IgG1 is found in naive GT KO mice is consistent with it being produced throughout life as a result of continuous antigenic stimulation by carbohydrate Ags on gastrointestinal bacteria of the normal flora, which is in keeping with immunocompetent humans that produce abundant natural IgG Abs specific to α-gal (41). α-gal–specific IgE was also not detected in wild type mice but was induced in GT KO that were immunized with tick extract (Fig. 3F). The loss of binding to deglycosylated cetuximab confirmed the α-gal specificity of Ab levels, which were not substantially changed following oral gavage. Together, these findings establish that wild type mice produce tick-specific IgE Abs but lack the ability to produce IgE specific for α-gal, in addition to α-gal–specific IgG, in this experimental model. After immunization, GT KO mice produce both tick-specific and α-gal–specific IgE Abs.

To determine if s.c. exposure to tick extract and α-gal was capable of inducing a hypersensitivity response after consumption of red meat, we first measured the activation of circulating basophils in the peripheral blood of mice sensitized and challenged with tick extract following oral gavage with beef thyroglobulin. Frequencies of basophils were substantially increased following oral gavage in immunized mice compared with naive controls (Fig. 4A). Increased frequencies of basophils were detected at 60 and 90 min after gavage, with greater frequencies of circulating basophils found in GT KO mice compared with wild type mice. GT KO mice immunized with tick extract also exhibited increased frequencies of basophils that expressed CD200R and CD41, markers that are upregulated upon basophil activation (42–44), 60 and 90 min following oral gavage with beef thyroglobulin compared with naive controls and wild type mice (Fig. 4A). No differences in the frequencies of basophils expressing phosphorylated

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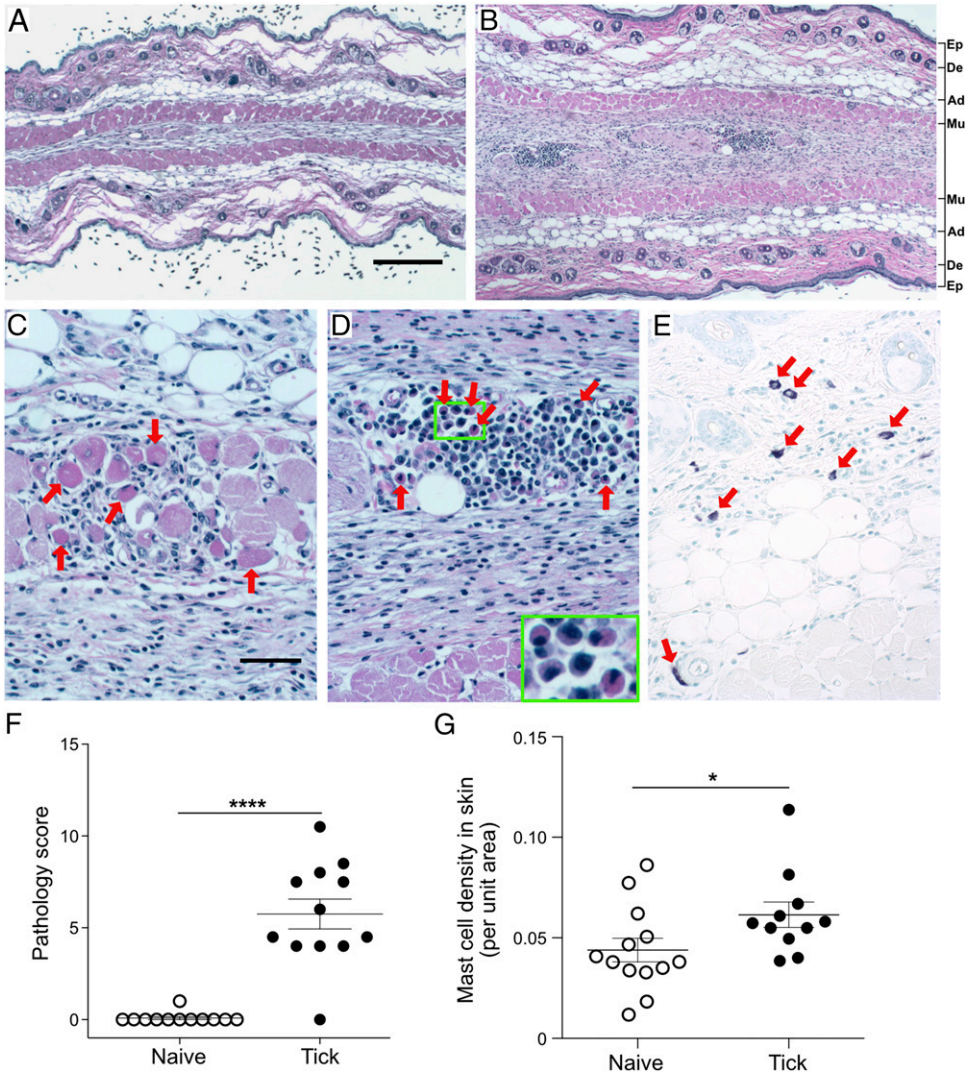

FIGURE 2. s.c. immunization with tick extract induces a local inflammatory response. (**A**–**E**) Skin samples were obtained from the site of tick extract injection on day 35 and then analyzed by H&E and Toluidine blue staining. Representative skin sections of three to five sections per animal from (A) naive and (B) tick-immunized mice showed increased cellular infiltration and enlarged dermis from immune mice. Two butterflied layers of skin per mouse are shown. Original magnification ×40. Scale bar, 100 μm. (C–E) Representative skin sections from mice immunized with tick extract showed (C) atrophic muscle fibers (red arrows); (D) lymphocyte infiltration with clusters of plasma cells, some containing the Russell bodies (red arrows; inset); and (E) mast cells. Original magnification ×200. Scale bar, 20 μm. (**F**) Skin pathology was scored based on dermal thickening, vasculitis, and leukocyte infiltration within the dermis and hypodermis. (**G**) Mast cell infiltration in skin sections was assessed by Toluidine blue staining and normalized to relative units. Each symbol represents an individual mouse, and error bars show the mean ± SEM. Data are representative of two independent experiments. *$p < 0.05$, ****$p < 0.0001$. μ, muscle; Ad, adipose; De, dermis; Ep, epidermis.

p38, a marker of human basophil activation indicative of degranulation (45–47), were observed in gavaged wild type animals but were significantly increased in GT KO mice. Stimulation of blood basophils with cetuximab but not deglycosylated cetuximab induced the release of histamine from basophils of GT KO mice but not wild type mice that were immunized with tick extract (Fig. 4B). Last, serum mouse MCP-1 was found at greater levels in GT KO mice compared with wild type mice that were immunized with tick extract following oral gavage (Fig. 4C). These data demonstrate that sensitization to tick Ags through the skin can contribute to a hypersensitivity response following meat consumption but mildly, whereas sensitization to α-gal leads to a more severe hypersensitivity reaction. Interestingly, histopathological examination of skin sections taken at the site of immunization demonstrated a greater pathology score in GT KO mice compared with wild type mice, suggesting more acute cutaneous inflammation (Fig. 2F, Supplemental Fig. 3B). Reduced body temperature, a sign of a systemic anaphylactic reaction, was not evident in mice regardless of strain (Supplemental Fig. 3C).

T cell help is required for the IgE response to cutaneous tick exposure

Having established that cutaneous tick exposure induced Tfh and GC B cell frequencies in the skin draining lymph nodes (Fig. 1E–H), we assessed the requirement of helper T cells for the IgE response. Sensitized mice were treated with the $CD4^+$ T cell–depleting mAb GK1.5 before tick challenge, and serum Ab titers were then analyzed on day 35 (Fig. 5A). This regimen efficiently depleted $CD4^+$ T cells (Fig. 5B). Treatment with GK1.5 significantly reduced total and tick-specific IgE levels compared with the isotype-treated controls (Fig. 5C–E). We also measured tick-specific IgG1 levels because class switching to IgE is dependent on Th2 cell–associated cytokines (48), and elevated levels of IgG1 to allergens often associate with IgE production including patients

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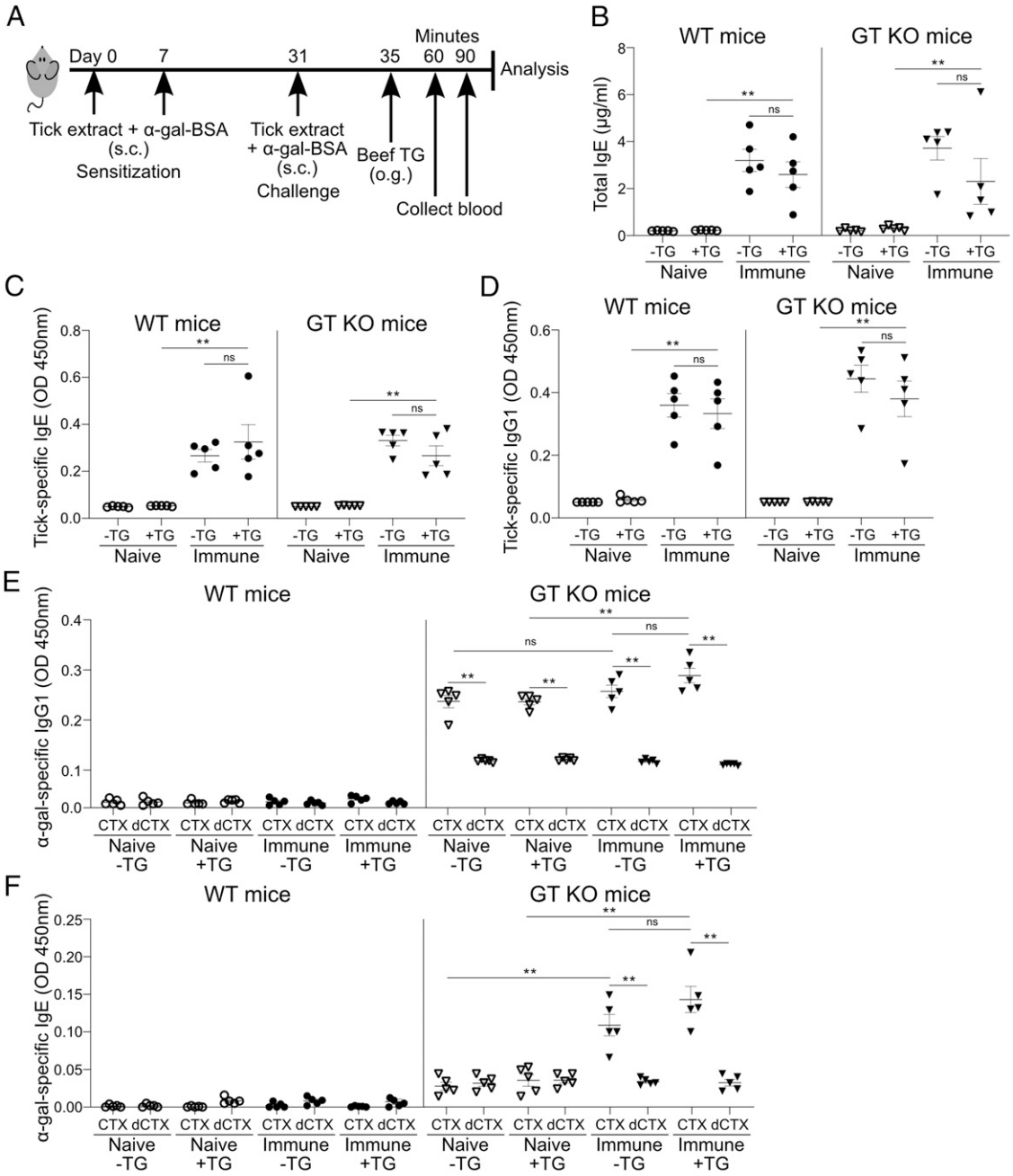

FIGURE 3. Mice deficient in α-gal expression produce α-gal–specific Abs following cutaneous exposure to tick extract. (**A**) Wild type (WT) and α-gal–deficient (GT KO) mice were injected s.c. with tick extract and α-gal on days 0, 7, and 31 and oral gavaged with beef thyroglobulin on day 35. (**B**–**D**) Serum levels of total IgE, tick-specific IgE, and tick-specific IgG1 from naive or immune mice at 90 min after gavage were measured by ELISA. Sera was tested in animals that were orally gavaged with beef thyroglobulin (+TG) or not (−TG). (**E** and **F**) Serum levels of α-gal–specific IgG1 and IgE from mice at 90 min after gavage were measured by ELISA using cetuximab (CTX) or deglycosylated cetuximab (dCTX) for the presence and absence of α-gal epitopes, respectively. Each symbol represents an individual mouse, and error bars show the mean ± SEM. Data are representative of two independent experiments, each with five mice per group. $^{**}p < 0.01$.

with red meat allergy (49, 50). Results demonstrated that the tick-specific IgE response is characterized by elevated tick-specific IgG1, which is incompletely blunted in mice treated with GK1.5 (Fig. 5F). These findings demonstrated that the IgE, and to a lesser extent the IgG1, recall response to cutaneous tick exposure is dependent on $CD4^+$ T cells.

To evaluate further the contribution of T cell help in driving GC B cell responses and Ab production induced by tick immunization, we treated mice with the CD154 mAb (MR1) to block GC B cell formation either when mice were sensitized or challenged with tick extract (Fig. 5G, 5H). This approach allowed us to separately test the importance of GC B cells in allergen sensitization and recall responses. Results demonstrated that treatment with MR1 during either tick sensitization or challenge prevented total IgE and tick-specific IgE compared with the isotype-treated controls (Fig. 5I–K). In contrast, mice treated with MR1 during tick sensitization but not the challenge prevented tick-specific IgG1 production despite the lack of GC B cells, suggesting that extrafollicular B cell activation may contribute to the IgG1 response. Overall, these data suggest that GC responses to cutaneous tick exposure are required for allergen sensitization and for recall IgE Ab production.

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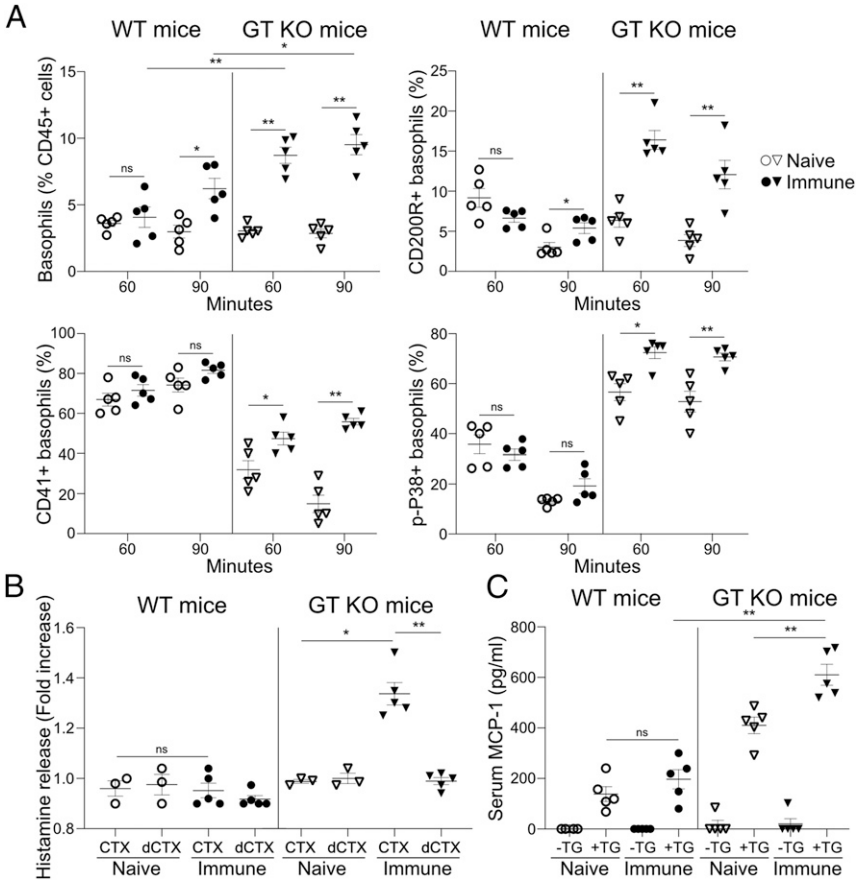

FIGURE 4. Mice deficient in α-gal expression cutaneously exposed to tick extract exhibit a more severe hypersensitivity response following oral exposure to beef than wild type mice. Mice were immunized with tick extract and oral gavaged with beef thyroglobulin as described in Fig. 3. (**A**) Frequencies and activation of circulating basophils in peripheral blood from naive and immune mice at 60 and 90 min after oral gavage were evaluated by flow cytometry. (**B**) Heparinized blood from naive and immune mice before oral gavage was incubated with either cetuximab (CTX) or deglycosylated cetuximab (dCTX), and basophil release of histamine was measured by ELISA. (**C**) Serum levels of MCP-1 were measured by ELISA. Each symbol represents an individual mouse, and error bars show the mean ± SEM. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$. ns, not significant.

Tick extract has an adjuvant effect

In addition to allergen exposure, sensitization usually requires the presence of other factors that may function as adjuvants. These include experimental adjuvants such as bacterial toxins, bacteria colonization of lesional skin, or damage to the skin barrier (51–53). The rapid increase in IgE titers in red meat allergy patients following tick bites suggests that, in addition to allergen, ticks contain inflammatory factors that drive IgE production to previously tolerated Ags (7, 9, 11). To investigate whether tick extract has an adjuvant effect in our mouse model, we sensitized and challenged mice with the exogenous T cell–dependent NP–KLH Ag in the presence of tick extract or in the vaccine adjuvant alum that provokes an Ab response characterized by a predominance of IgG1 and IgE and measured GC responses and NP-specific Ab production (Fig. 6A). Results demonstrated that total IgE levels were similarly increased in mice that received NP Ag in tick extract compared with those that were immunized s.c. with NP Ag in alum (Fig. 6B). Mice immunized with NP–KLH Ag in tick extract or in alum also exhibited significantly increased NP-specific IgE (Fig. 6C), which included high affinity Abs (Fig. 6D). These data were consistent with increased frequencies of Tfh cells, total GC B cells, and NP-specific GC B cells found in mice immunized with NP Ag in tick extract or in alum (Fig. 6E–G). The frequency of NP-specific GC B cells was greater in mice immunized with NP Ag in tick extract compared with those immunized with alum. To our knowledge, this is the first report to specifically compare tick extract and alum as adjuvants in driving recall immune responses through the cutaneous route. Tick-specific IgE and IgG1 Abs were produced only in animals that were immunized with tick extract (Fig. 6H, 6I). Taken together, these data demonstrate that tick extract has an adjuvant effect.

MyD88 is required for the induction of an IgE response to tick extract, and this requirement is B cell intrinsic

To determine how tick extract might be providing an adjuvant effect in our mouse model, we assessed the ability of tick extract alone to activate TLR through a commercial TLR screen (see *Materials and Methods* for more information). The screen indicated that tick extract had a robust stimulatory effect through TLR2, TLR4, TLR5, and to a lesser extent, TLR9 (Supplemental Fig. 4). Because all of the TLRs identified by the screen signal through MyD88, we investigated whether signaling through MyD88 was important for the IgE response to cutaneous tick exposure. MyD88$^{-/-}$ and wild type mice were sensitized and challenged with tick extract using the same immunization strategy shown in Fig. 1A. Results demonstrated that total IgE as well as tick-specific IgE and IgG1 production was attenuated in the absence of MyD88 (Fig. 7A–C).

Several allergens have been reported to contain TLR ligands and activate MyD88 signaling in innate and adaptive immune cells, including B cells (54–58). Using mice expressing Cre recombinase from the endogenous Ig heavy constant γ 1 (Ighγ1) locus (59) crossed with MyD88$^{fl/fl}$ mice (60), we generated conditional knockout mice that delete MyD88 in IgG1^{+} B cells to study the B cell–intrinsic function of MyD88 to control IgE responses. Attenuated levels of total IgE and tick-specific IgE and IgG1 Abs were found when we performed the same experiment using the Ighγ1^{Cre}MyD88$^{fl/fl}$ mouse strain as with germline MyD88$^{-/-}$ mice (Fig. 7D–F). These data demonstrate a B cell–intrinsic role for MyD88 signaling to induce IgE responses following cutaneous tick exposure and, moreover, suggests that IgE class switch recombination occurs through an IgG1 intermediate B cell.

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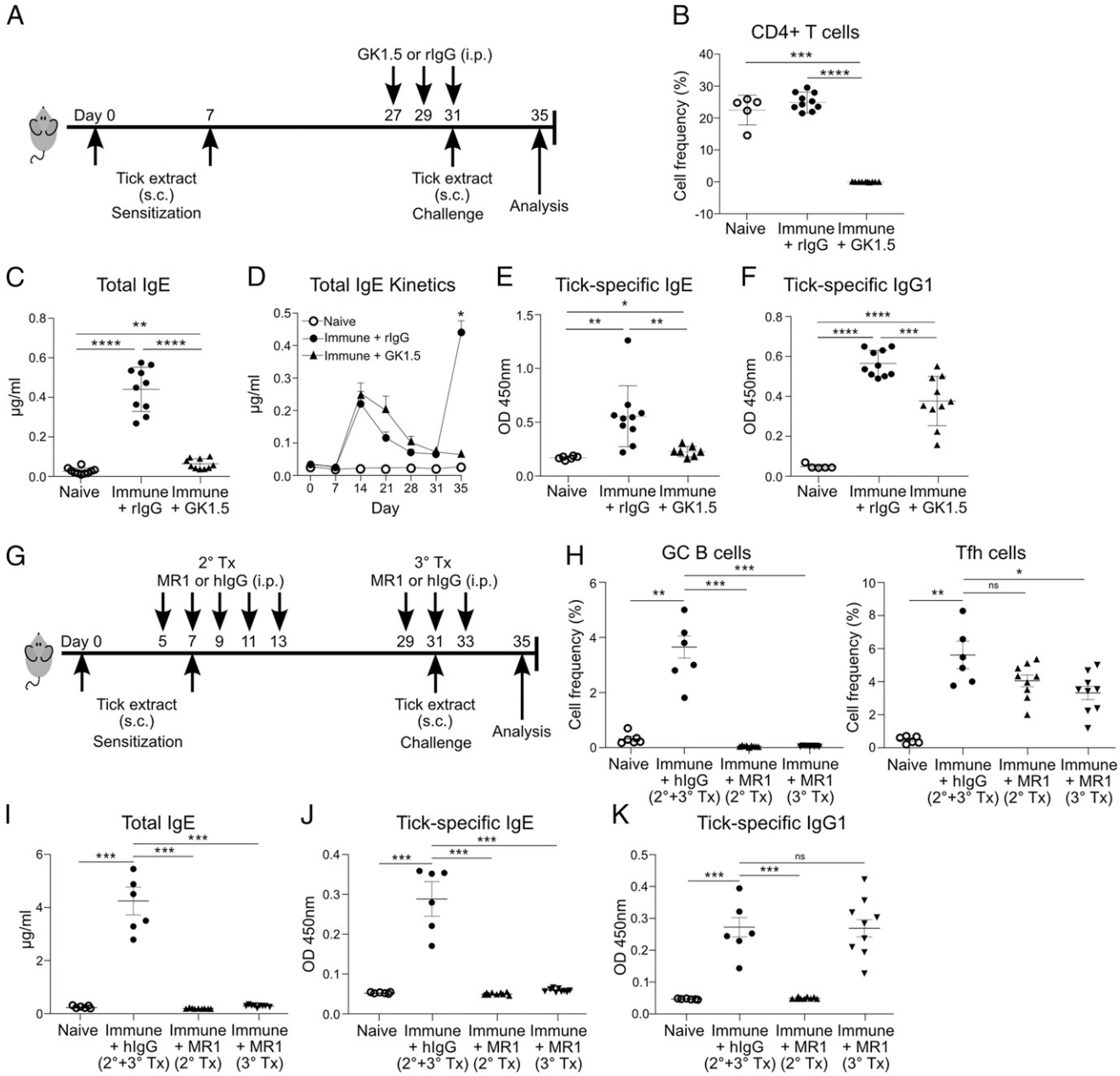

FIGURE 5. T cell help is required for the induction of IgE response to tick extract. (**A**) Mice were injected s.c. with tick extract on days 0, 7, and 31. GK1.5 or isotype control mAb was administered i.p. on days 27, 29, and 31, and mice were analyzed on day 35. (**B**) Depletion efficiency of $CD4^+$ T cells in the inguinal lymph nodes was assessed by flow cytometry. (**C** and **D**) Serum levels of total IgE and tick-specific (**E**) IgE and (**F**) IgG1 were assessed by ELISA. (**G**) Mice were injected s.c. with tick extract on days 0, 7, and 31 and administered MR1 or isotype control mAb i.p. either on days 5, 7, 9, 11, and 13 or on days 29, 31, and 33. (**H**) Reduced GC B cells and Tfh cells in the inguinal lymph nodes were demonstrated by flow cytometry. (**I**) Serum levels of total IgE, tick-specific (**J**) IgE, and (**K**) tick-specific IgG1 were assessed by ELISA. Each symbol represents an individual mouse, and error bars show the mean ± SEM. One of two experiments with similar results is shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. ns, not significant.

Discussion

The mechanisms that lead to sensitization to food allergens and the production of IgE are poorly understood. The objective of this study was to develop and characterize a mouse model of allergic sensitization to cutaneous tick exposure that potentially could be used to identify mechanisms controlling the induction of IgE Abs associated with tick-borne allergic diseases. s.c. immunization with proteins isolated from lone star ticks induced IgE Ab production, including tick-specific IgE as well as tick-specific IgG1 Abs. Our model is unique in that wild type mice and α-gal–deficient mice were used, which were both sensitized to tick extract and produced robust total and tick-specific IgE. Moreover, only α-gal–deficient mice produced α-gal–specific IgE and, following oral consumption of meat, generated a more severe hypersensitivity response than wild type mice, consistent with cutaneous inflammatory lesions at the site of tick bites and basophil activation found in patients with red meat allergy after meat consumption (61). We were unable to demonstrate acute systemic anaphylaxis when meat was administered orally to the sensitized animals. This could be because of differences between mice and humans or environmental factors. Even so, in humans, the frequency of meat-allergic patients that experience anaphylaxis is rare (62). It remains to be determined which meat-allergic patients will experience anaphylaxis because there does not appear to be a correlation between

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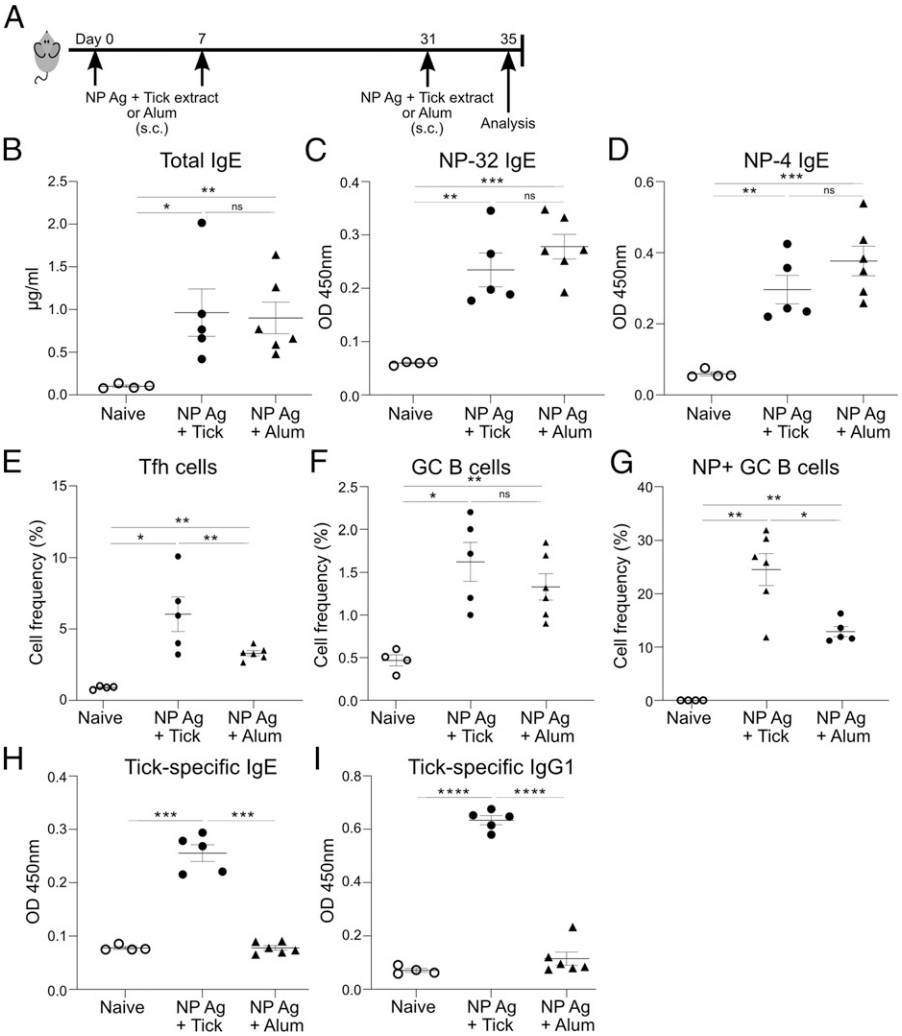

FIGURE 6. Tick extract functions as an adjuvant. (**A**) Mice were injected s.c. with NP Ag in the presence of tick extract or in the presence of alum on days 0, 7, and 31, and Ab titers and GC responses were analyzed on day 35. (**B**–**D**) Serum levels of total IgE, total NP-specific IgE, and high affinity NP-specific IgE were assessed by ELISA. (**E**–**G**) Frequencies of Tfh cells, total GC B cells, and NP-specific GC B cells ($B220^+CD95^+GL7^+NP^+$) in inguinal lymph nodes were quantified by flow cytometry. (**H** and **I**) Serum levels of tick-specific IgE and IgG1 were measured by ELISA. Each symbol represents an individual mouse, and error bars show the mean ± SEM. One of three experiments with similar results is shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

anaphylaxis and α-gal–specific IgE titers. Our study also supported a s.c. route was needed for an IgE Ab response to tick immunization compared with an i.p. route and suggested a critical role for T cell help within the skin draining lymph nodes in this process. Depletion of $CD4^+$ T cells during the challenge phase prevented IgE production, whereas tick-specific IgG1 was induced but at lower levels. Inactivation of cognate T cell help to B cells by blockade of CD40L–CD40 interactions during either the sensitization or challenge phase prevented IgE production in immunized mice, whereas blockade during the sensitization but not the challenge phase inhibited tick-specific IgG1 production. Together, these results suggest an important role for cognate T cell help in driving both sensitization and recall Ab responses to tick proteins. Moreover, MyD88 signaling was found to be required for a robust IgE response to immunization with tick extract, and this requirement was B cell intrinsic. These studies support a useful model to further investigate the immunologic signals driving IgE production in tick-borne allergic responses.

Several questions remain regarding the mechanisms required to support an IgE response following cutaneous tick exposure. First, further work is needed to explore the phenotypic composition of B cells that have a propensity for allergic sensitization as well as the molecular pathways by which B cells differentiate from naive to IgE-producing cells. Recent studies using IgE reporter mice support two differentiation pathways for the generation of IgE-producing B cells. The first is that IgE-secreting plasma cells can develop from the responding progeny of GC-derived IgE^+ B cells and IgE^+ memory B cells that are directly derived from IgM^+ naive B cells (63). The second is that IgE plasma cells develop from sequential class switching of the responding progeny of GC-derived $IgG1^+$ B cell intermediates and from $IgG1^+$ memory B cells after Ag exposure (64, 65). Discrete subsets of $IgG1^+$ memory B cells based on CD80 and CD73 expression levels recently have been shown to contribute distinctively to the affinity and pathogenicity of IgE (66). However, the immunophenotypes of these putative $IgG1^+$ B cell intermediates and memory B cells and their function in allergy are unknown. Characterization of these cell types in our model might help elucidate the contribution of sequential class switching to IgE during recall responses to tick proteins and the roles of cognate and noncognate T cell help in this process.

Second, our findings suggest that the production of tick-specific IgE is mediated largely through TLRs expressed in B cells and, in particular, that MyD88 signaling in $IgG1^+$ B cells is important for driving sequential class switch recombination to IgE. Although initially shown to be involved in Th1 cell responses (67), more recent work has demonstrated that MyD88 signaling plays a role in Th2 cell–mediated responses as well (55, 68, 69). Interestingly, work using an airway allergy model demonstrated that B cell–specific loss of MyD88 resulted in a significantly reduced production of both IgE and IgG1 (55), suggesting that MyD88 expression in B cells is important for sensitization to respiratory allergens. Previous work from animal models has demonstrated that allergic sensitization can be induced by topical allergen

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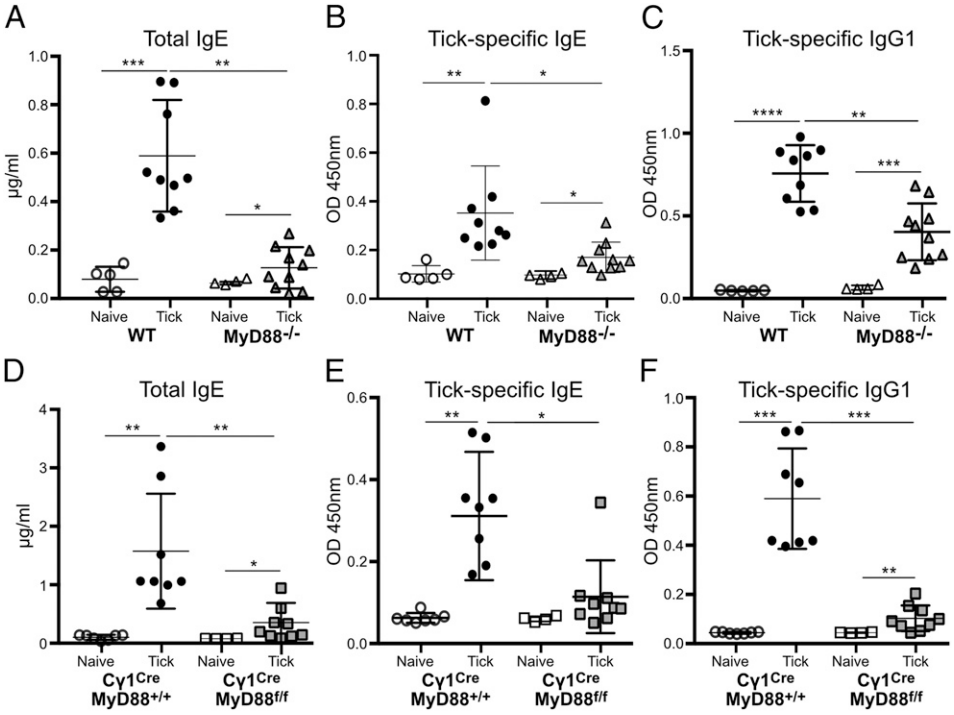

FIGURE 7. B cell–intrinsic expression of MyD88 is required for the induction of a robust IgE response to tick extract. (**A**–**C**) Wild type (WT) and MyD88$^{-/-}$ mice were injected s.c. with tick extract or PBS (naive) on days 0, 7, and 31, and then Ab titers were analyzed on day 35. (A) Serum levels of total IgE, (B) tick-specific IgE, and (C) tick-specific IgG1 were assessed by ELISA. (**D**–**F**) Ighγ1^{Cre}MyD88$^{fl/fl}$ conditional knockout mice and Ighγ1^{Cre}MyD88$^{+/+}$ littermate controls were injected s.c. with tick extract as described above. (D) Serum levels of total IgE, (E) tick-specific IgE, and (F) tick-specific IgG1 were quantified by ELISA. Each symbol represents an individual mouse, and error bars show the mean ± SEM. Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

exposure. However, these models show that additional factors beyond exposure are necessary to induce sensitization, including adjuvant or damage to the skin (70–72). Our finding that tick extract had an adjuvant effect and induced IgE Abs specific for NP hapten points to a possible role of TLR ligands within the tick extract to mediate IgE responses. Several allergens have been reported to contain TLR ligands and activate MyD88 signaling in innate and adaptive immune cells (54–58). For example, TLR2, TLR4, and TLR9 have modulatory effects on the mucosal immune system and have been shown to help shape IgE production (73–75). However, little is known about the role of TLRs in skin sensitization and food allergy, and it might be expected that multiple TLRs are involved. Our observation that a commercial screen identified that several TLR ligands are present in lone star ticks suggests that microbes contained within ticks, or the ectoparasitic tick itself, are sources of TLR ligands and might be important for sensitization to tick proteins.

TLRs are not the only immune receptors affected by genetic disruptions of MyD88 signaling. The IL-1 receptor family (including IL-1R, IL-18R, and IL-33R) also signals through MyD88 (76). IL-1β has been recently identified to play a role in the pathophysiology of allergic disorders (77). Given that our screen focused on the presence of TLR ligands in tick extract, it is possible that IL-1β and IL-18 play an important role in stimulating B cells cooperatively for IgE production. Furthermore, we cannot rule out the possibility of the involvement of MyD88-independent signals in enhancing IgE production in this experimental model. Although both germline MyD88$^{-/-}$ mice and Cγ1^{Cre}MyD88$^{f/f}$ mice had significantly defective Ab responses to tick extract immunization, Ab titers were higher than naive animals. The toll–IL receptor domain containing adaptor-inducing IFN-β molecule can promote MyD88-independent NF-κB activation in TLR4 signaling pathways and is essential for LPS-driven activation of the Cε locus and class switching to IgE (78, 79), which may contribute to IgE induced by tick extract. Further study of the MyD88-dependent and -independent signaling pathways induced by cutaneous exposure to tick extract may provide insights into the molecular mechanisms that trigger Th2 cell–associated immune responses and improve our capacity to design new, safe, and effective vaccine adjuvants.

Finally, an important question in the study of food allergy is how an immune response to Ag exposure through the skin leads to a loss of tolerance to food Ags in the gut. Sensitization of α-gal–deficient mice by s.c. injections of saliva obtained from the *A. sculptum* tick species has been shown to produce IgE Abs against α-gal (80). In contrast, no detectable levels of IgE against α-gal were observed in α-gal–deficient mice when sensitized to virus-like particles containing α-gal epitopes. This suggests that *A. sculptum* saliva might have an adjuvant effect that is needed to induce IgE against α-gal despite α-gal not present in these mice, similar to humans. Our findings that α-gal–deficient mice sensitized to lone star tick extract and α-gal drives the production of α-gal–specific IgE and a greater hypersensitivity response following meat consumption compared with wild type mice suggest a role for α-gal–specific B cells in the gut. Further work will be required to assess how GALT responds to food Ags and its relationship with cutaneous immune responses such as the potential trafficking of Ag-specific lymphocytes from the skin draining lymphoid tissue to the gut. Increasing our understanding of the mechanisms underlying tick-borne allergic diseases is critical for identifying new targets to treat and prevent allergies.

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Disclosures

The authors have no financial conflicts of interest.

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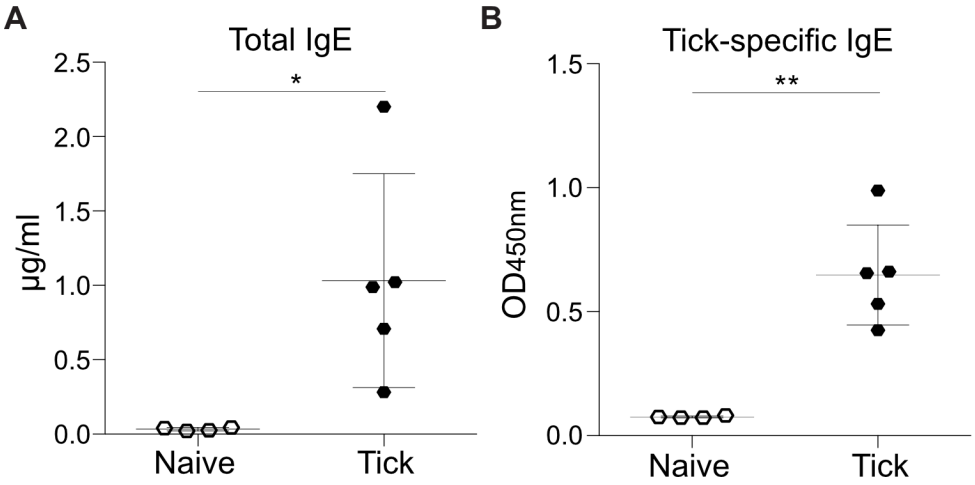
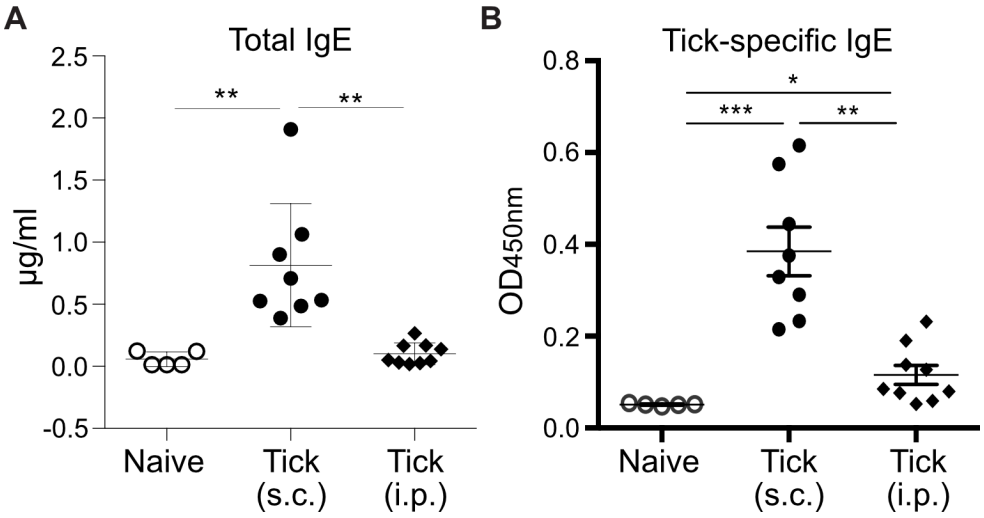
Figure S1



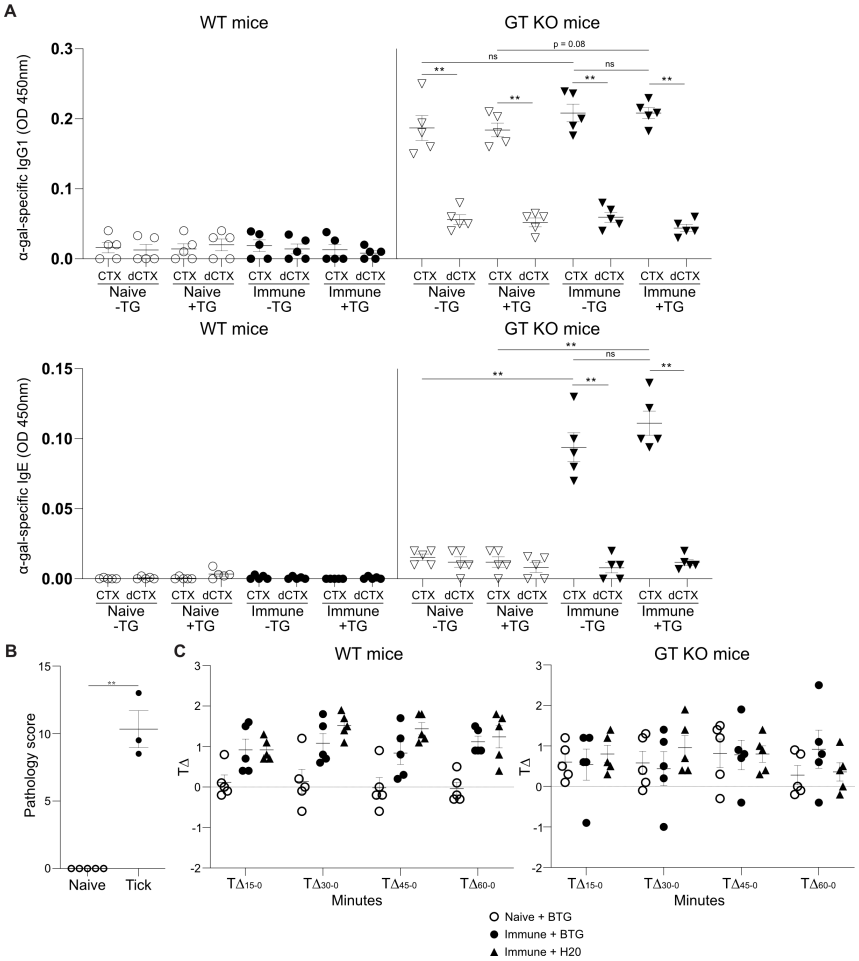
SUPPLEMENTAL FIGURE S1. The IgE response to tick extract is not strain specific. *A* and *B*, Balb/C mice were immunized subcutaneously with tick extract on days 0, 7 and 31, and analyzed on day 35 for serum levels of total IgE and tick-specific IgE by ELISA.

Figure S2



SUPPLEMENTAL FIGURE S2. Subcutaneous but not intraperitoneal immunization with tick extract induces an IgE response. *A* and *B*, Mice were sensitized and challenged s.c. or i.p. with tick extract on days 0, 7 and 31, and analyzed on day 35. Serum levels of total IgE and tick-specific IgE were measured by ELISA. Each symbol represents an individual mouse and error bars show the mean ± SEM. *$p < 0.05$, **$p < 0.01$., ***$p < 0.001$.

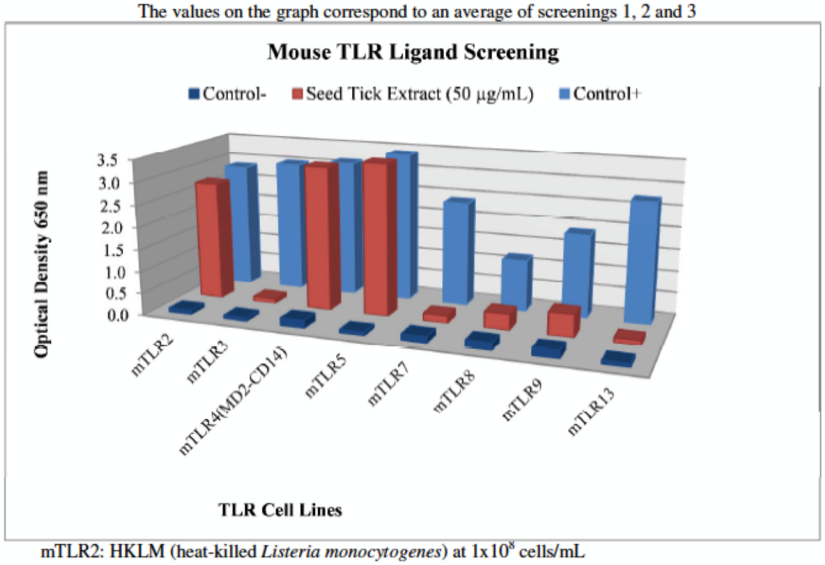
Figure S3



SUPPLEMENTAL FIGURE S3. Subcutaneous immunization with tick extract induces alpha-gal IgE and a local inflammatory response in mice deficient in alpha-gal expression, but does not lead to a systemic anaphylactic reaction. *A,* Serum levels of alpha-gal-specific IgG1 and IgE from mice at 90 min after oral gavage with beef thyroglobulin (+TG), or not (-TG), were measured by ELISA using cetuximab (CTX) or deglycosylated cetuximab (dCTX) for the presence and absence of alpha-gal epitopes, respectively. *B*, GT KO mice were sensitized and challenged s.c. with tick extract on days 0, 7 and 31, and skin sections at the injection site were analyzed on day 35. Skin pathology was scored based on dermal thickening, muscular necrosis, leukocyte infiltration, and fibrosis. *C*, Body temperature of WT and GT KO mice was measured using a digital thermometer with a rectal probe at 15, 30, 45, and 60 min following oral gavage. Each symbol represents an individual mouse and error bars show the mean ± SEM. **$p < 0.01$.

Figure S4

The values on the graph correspond to an average of screenings 1, 2 and 3



mTLR2: HKLM (heat-killed *Listeria monocytogenes*) at $1x10^8$ cells/mL
mTLR3: Poly(I:C) HMW at 1 µg/mL
mTLR4: *E. coli* K12 LPS at 100 ng/mL
mTLR5: *S. typhimurium* flagellin at 100 ng/mL
mTLR7: CL097 at 1 µg/mL
mTLR8: CL075 at 10 µg/mL + Poly(dT) at 10 µM
mTLR9: CpG ODN1826 100 ng/mL
mTLR13: ORN Sa19 at 2 µg/mL

SUPPLEMENTAL FIGURE S4. Tick extract predominantly contains ligands for mouse TLR2, TLR4 and TLR5, and to a lesser extent TLR8 and TLR9. A commercial screen by InvivoGen using the indicated TLR reporter cell lines was performed.