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

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Abstract

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 antibodies 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 robust production of IgE antibodies and supported a role for IgE-mediated hypersensitivity reactions in sensitized animals following oral administration of meat. 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 antigens occurs through the skin that leads to IgE production.

Introduction

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 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 antibodies are produced by B cells as a result of antigen exposure through skin, gut, or respiratory tract. Food antigens are then processed by antigen presenting cells and presented to CD4⁺ T cells that provide help to B cells leading to the

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production of specific IgE antibodies. Upon re-exposure to the antigen, 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 antibodies to tick proteins and is the primary tick species associated with red meat allergy. Allergic reactions in affected individuals following meat consumption are believed to be mediated by IgE antibodies specific for galactose- α -1,3-galactose (α -gal), a blood group antigen 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 antigens 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.

Here, we have developed a new mouse model to study IgE responses to cutaneous tick exposure by administering lone star tick antigens through the skin of mice. We demonstrate that tick sensitization and challenge induce specific IgE and IgG1 antibody production in healthy mice, and elicits hypersensitivity reactions in sensitized animals following 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 to controls. Inhibition of T cell help by blocking CD40 ligand (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 antibody 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 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

WT C57BL/6J, MyD88^{-/-}, MyD88^{fl}, C γ 1-cre mice (all on the C57BL/6J background), and BALB/c mice, were obtained from The Jackson Laboratory (Bar Harbor, ME). The α -galactosyl transferase^{-/-} mice (GT KO) were a generous gift from Dr. Uri 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-week 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 8.0, 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 for 5 minutes. 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 days 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 antibodies 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 antigen, 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 alpha-gal specific IgE, mice were s.c. injected with 50 µg of tick extract plus 50 µg of alpha-gal-BSA (Dextra Labs, Reading, United Kingdom) 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 antibody 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 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 minutes, with predetermined optimal concentrations of the following fluorophore-labeled mAbs: B220-APCCy7 (RA3-6B2; BD Biosciences, San Jose, CA), GL7-EF450 (GL-7; eBioscience), CD95-BV605 (15A7; BioLegend, San Diego, CA), and NP-APC (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 hematoxylin-and-eosin (H&E) and toluidine blue staining were obtained on day 35 and fixed in 10% formalin (Fisher Scientific) for 48 hours 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; 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 re-hydrated in xylene, 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 minutes. 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 NIH Image J software (Bethesda, MD).

Hypersensitivity Responses to Meat Consumption

Naïve and immune mice were orally gavaged with 50 µg of alpha-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 minutes following oral gavage. Heparinized blood was either treated with ammonium chloride-Tris to lyse red blood cells 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 600xg for 10 minutes 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 minutes at 37°C. The plate was centrifuged at 1,500 rpm for 5 minutes and the supernatants were collected and histamine levels were measured by ELISA. In addition to heparinized blood, serum was also obtained on day 35 at 90 minutes following oral gavage and analyzed for antibody and 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 minutes 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 CD40 ligand (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, measured as absorbance at OD₆₅₀ 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 antibody 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 (Southern Biotech, Birmingham, AL). For antigen-specific ELISAs, high binding plates were coated with tick extract at a concentration of 5 µg/ml, or NP conjugated to Bovine Serum Albumin (BSA) at a ratio of 32 or 4 (Biosearch Technologies) at a concentration of 5 µg/ml, as previously described (24). Serum was diluted 1:100 for IgE and 1:10,000 for IgG1, and serially titrated in three-fold increments. HRP-labeled anti-mouse IgE or IgG1 (Southern Biotech, applied according to manufacturer's instructions) served as the detection antibody and the assay was developed using TMB (BD Pharmingen), with 2N H₂SO₄ used as the stop solution. To measure alpha-gal-specific antibody titers, plates were coated with 5 µg/ml of cetuximab, a chimeric mouse-human IgG1 mAb against epidermal growth factor receptor that contains alpha-gal residues on the Fab portion of the cetuximab heavy chain that are recognized by IgE specific for alpha-gal (25). Binding to alpha-gal was confirmed by testing cetuximab that was deglycosylated to remove alpha-gal residues by pre-incubating cetuximab with α-1→(3,6)-Galactosidase for 1 hour at 37°C, according to manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO). No binding of the monoclonal anti-alpha-gal antibody M86 (Enzo Life Sciences, Farmingdale, NY) confirmed deglycosylation of alpha-gal. No binding of M86 to BSA alone was also confirmed. Serum was diluted 1:10,000 for IgG1 and serially titrated in three-fold increments. To avoid competitive binding to alpha-gal epitopes by alpha-gal-specific IgG antibodies, which may be more than 100-fold excess to IgE and thus interfere with detection of alpha-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 antibody to alpha-gal at a 1:10 dilution, serially titrated in three-fold increments. Total IgE antibody titers were quantified through a standard curve obtained using unlabeled IgE (Southern Biotech), while optical density (OD) values were

compared for antigen-specific assays. All samples were assessed in duplicate. Mouse mast cell protease-1 (MCP-1) serum levels were measured by ELISA according to the manufacturer's instructions (BioLegend, San Diego, CA). 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, 155 mM NH₄Cl, 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 antibodies were used: B220-APCCy7 (RA3-6B2; BD Biosciences, San Jose, CA), CD4-FITC (RM4-5; BD Biosciences), CD95-Biotin (15A7; eBioscience), Streptavidin-PE (BioLegend, San Diego, CA), GL7-EF450 (GL-7; eBioscience), PD-1-PECy7 (RMP1-30; BioLegend), CXCR5-APC (L138D7; BioLegend), FcεRI-FITC (MAR-1; BioLegend), CD200R-PE (OX-108; BioLegend), cKit-APC (2B8; eBioscience), CD45-APC (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, Carlsbad, CA) 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 (Treestar, Ashland, OR). Gates were determined using fluorescence-minus-one staining controls.

Statistical Analyses

All analyses were performed using Prism 8.0 software (GraphPad Software Inc., San Diego, CA). Statistical significance comparing two experimental groups was determined using unpaired Student t-test with Welch's 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 since 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 to naïve 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 one week (day 7), which increased significantly after two weeks (day 14) following the second immunization (Fig.

1C). Levels of IgE were found reduced by day 31 nearly to the levels measured in naïve mice. Upon challenge, serum IgE rapidly increased within 4 days (day 35), surpassing the levels observed following the second immunization (Fig. 1C). IgE antibodies specific for *A. americanum* proteins correlate strongly with IgE antibodies to alpha-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 to naïve controls (Fig. 1D). Tick-specific IgE was not detected from serum of mice immunized with the irrelevant house dust mite (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 to naïve controls, demonstrating that the IgE response induced by cutaneous tick exposure is not strain dependent (Fig. S1, A and B).

Previous studies showed that IgE production in secondary responses required CD4⁺ T cell help (31, 32). Thus, we evaluated the development of germinal center (GC) responses in the draining inguinal lymph nodes and non-draining spleens of mice. Challenge with tick extract induced a small but significant increase in both GC B cells (Fig. 1, E and F) and T follicular helper (Tfh) cells (Fig. 1, G and H) 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 subcutaneous exposure to antigen. Taken together, these data indicate that subcutaneous 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. 2, A – E). In 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 to naïve, PBS-treated controls (Fig. 2, F). In addition, greater numbers of mast cells were found within the skin of mice sensitized and challenged with tick extract compared to those from naïve animals (Fig. 2, G). 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 intraperitoneally with tick extract failed to produce significantly increased levels of total and tick-specific IgE compared to those

immunized subcutaneously (Fig. S2, A and B). 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 alpha-gal requires skin exposure.

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

Mice synthesize alpha-gal epitopes and therefore naturally do not produce alpha-gal IgG antibodies because of immune tolerance (16, 39). To determine whether subcutaneous exposure to tick extract was capable of inducing an alpha-gal-specific IgE response and its effect on hypersensitivity, we analyzed wild type animals and mice deficient in the alpha-galactosyl transferase (GT KO) (40), immunized with tick extract in the presence of a known amount of alpha-gal, and orally gavaged with beef thyroglobulin (Fig. 3, A). 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 antibody levels did not significantly change following oral gavage (Fig. 3, B–D). Alpha-gal-specific IgG1 was not detected in wild type mice as expected (Fig. 3, E). In contrast, alpha-gal-specific IgG1 was present in GT KO mice and was not substantially increased in immune animals compared to naïve controls (Fig. S3, A). That alpha-gal-specific IgG1 is found in naïve GT KO mice is consistent with it being produced throughout life as a result of continuous antigenic stimulation by carbohydrate antigens on gastrointestinal bacteria of the normal flora, which is in keeping with immunocompetent humans that produce abundant natural IgG antibodies specific to alpha-gal (41). Alpha-gal-specific IgE was also not detected in wild type mice but was induced in GT KO that were immunized with tick extract (Fig. 3, F). The loss of binding to deglycosylated cetuximab confirmed the alpha-gal specificity of antibody levels, which were not substantially changed following oral gavage. Together, these findings establish that wild type mice produce tick-specific IgE antibodies but lack the ability to produce IgE specific for alpha-gal, in addition to alpha-gal-specific IgG, in this experimental model. After immunization, GT KO mice produce both tick-specific and alpha-gal-specific IgE antibodies.

To determine if subcutaneous exposure to tick extract and alpha-gal was capable of inducing a hypersensitivity response after consumption of red meat, we first measured the activation of circulating basophils in 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 to naïve controls. (Fig. 4, A). Increased frequencies of basophils were detected at 60 and 90 minutes after gavage, with greater frequencies of circulating basophils found in GT KO mice compared to 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 minutes following oral gavage with beef thyroglobulin compared to naïve controls and wild type mice (Fig. 4, A). No differences in the frequencies of basophils expressing phosphorylated 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. 4, B). Lastly, serum mouse MCP-1 was found at greater levels in GT KO mice compared to wild type mice that were immunized with tick extract, following oral gavage (Fig. 4, C). These data demonstrate that sensitization to tick antigens through the skin can contribute to a hypersensitivity response following meat consumption, but mildly, whereas sensitization to alpha-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 to wild type mice, suggesting more acute cutaneous inflammation (Fig. S3, B; Fig. 2, F). Reduced body temperature, a sign of a systemic anaphylactic reaction, was not evident in mice regardless of strain (Fig. S3, C).

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. 1, E–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 antibody titers were then analyzed on day 35 (Fig. 5, A). This regimen efficiently depleted CD4⁺ T cells (Fig. 5, B). Treatment with GK1.5 significantly reduced total and tick-specific IgE levels compared to the isotype-treated controls (Fig. 5, C–E). We also measured tick-specific IgG1 levels since class switching to IgE is dependent on Th2 associated cytokines (48), and elevated levels of IgG1 to allergens often associate with IgE production including patients 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. 5, F). 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 antibody 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. 5, G and H). 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 to the isotype-treated controls (Fig. 5, I–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 antibody production.

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 antigens (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 antibody response characterized by a predominance of IgG1 and IgE, and measured GC responses and NP-specific antibody production (Fig. 6, A). Results demonstrated that total IgE levels were similarly increased in mice that received NP Ag in tick extract compared to those that were immunized subcutaneously with NP Ag in alum (Fig. 6, B). Mice immunized with NP-KLH Ag in tick extract or in alum also exhibited significantly increased NP-specific IgE (Fig. 6, C), which included high affinity antibodies (Fig. 6, D). 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. 6, E–G). The frequency of NP-specific GC B cells was greater in mice immunized with NP Ag in tick extract compared to 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 antibodies were produced only in animals that were immunized with tick extract (Fig. 6, H and I). 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 toll-like receptors (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 (Fig. S4). 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. 7, A–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 immunoglobulin heavy constant gamma 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 antibodies were found when we performed the same experiment using the Ighγ1^{Cre}MyD88^{fl/fl} mouse strain as with germline MyD88^{-/-} mice (Fig. 7, D–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.

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 antibodies associated with tick-borne allergic diseases. Subcutaneous immunization with proteins isolated from lone star ticks induced IgE antibody production, including tick-specific IgE, as well as tick-specific IgG1 antibodies. Our model is unique in that wild type mice and alpha-gal-deficient mice were utilized, which were both sensitized to tick extract and produced robust total and tick-specific IgE. Moreover, only alpha-gal-deficient mice produced alpha-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 due to 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 since there does not appear to be a correlation between anaphylaxis and alpha-gal-specific IgE titers. Our study also supported a subcutaneous route was needed for an IgE antibody response to tick immunization compared to an intraperitoneal 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 CD40 ligand-CD40 interactions during either the sensitization or challenge phase prevented IgE production in immunized mice, while 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 antibody 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 naïve 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 germinal center (GC)-derived IgE⁺ B cells and IgE⁺ memory B cells that are directly derived from IgM⁺ naïve 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 antigen 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 non-cognate 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 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 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 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 antibodies 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 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 interleukin 1 receptor family (including IL-1R, IL-18R, and IL-33R) also signals through MyD88 (76). IL-1 β 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-1 β 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 C γ 1^{Cre}MyD88^{f/f} mice had significantly defective antibody responses to tick extract immunization, antibody titers were higher than naïve animals. The Toll-interleukin receptor domain containing adaptor-inducing interferon- β (TRIF) molecule can promote MyD88-independent NF- κ B activation in TLR4 signaling pathways and is essential for LPS driven activation of the C ϵ 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-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 antigen exposure through the skin leads to a loss of tolerance to food antigens in the gut. Sensitization of alpha-gal deficient mice by subcutaneous injections of saliva obtained from the *Amblyomma sculptum* tick species has been shown to produce IgE antibodies against alpha-gal (80). In contrast, no detectable levels of IgE against alpha-gal were observed in alpha-gal deficient mice when sensitized to virus-like particles containing alpha-gal epitopes. This suggests that *A. sculptum* saliva might have an adjuvant effect that is needed to induce IgE against alpha-gal despite alpha-gal not present in these mice, similar to humans. Our findings that alpha-gal-deficient mice sensitized to lone star tick extract and alpha-gal drives the production of alpha-gal-specific IgE and a greater hypersensitivity response following meat consumption compared to wild type mice suggest a role for alpha-gal-specific B cells in the gut. Further work will be required to assess how gut-associated lymphoid tissue responds to food antigens and its relationship with cutaneous immune responses such as the potential trafficking of antigen-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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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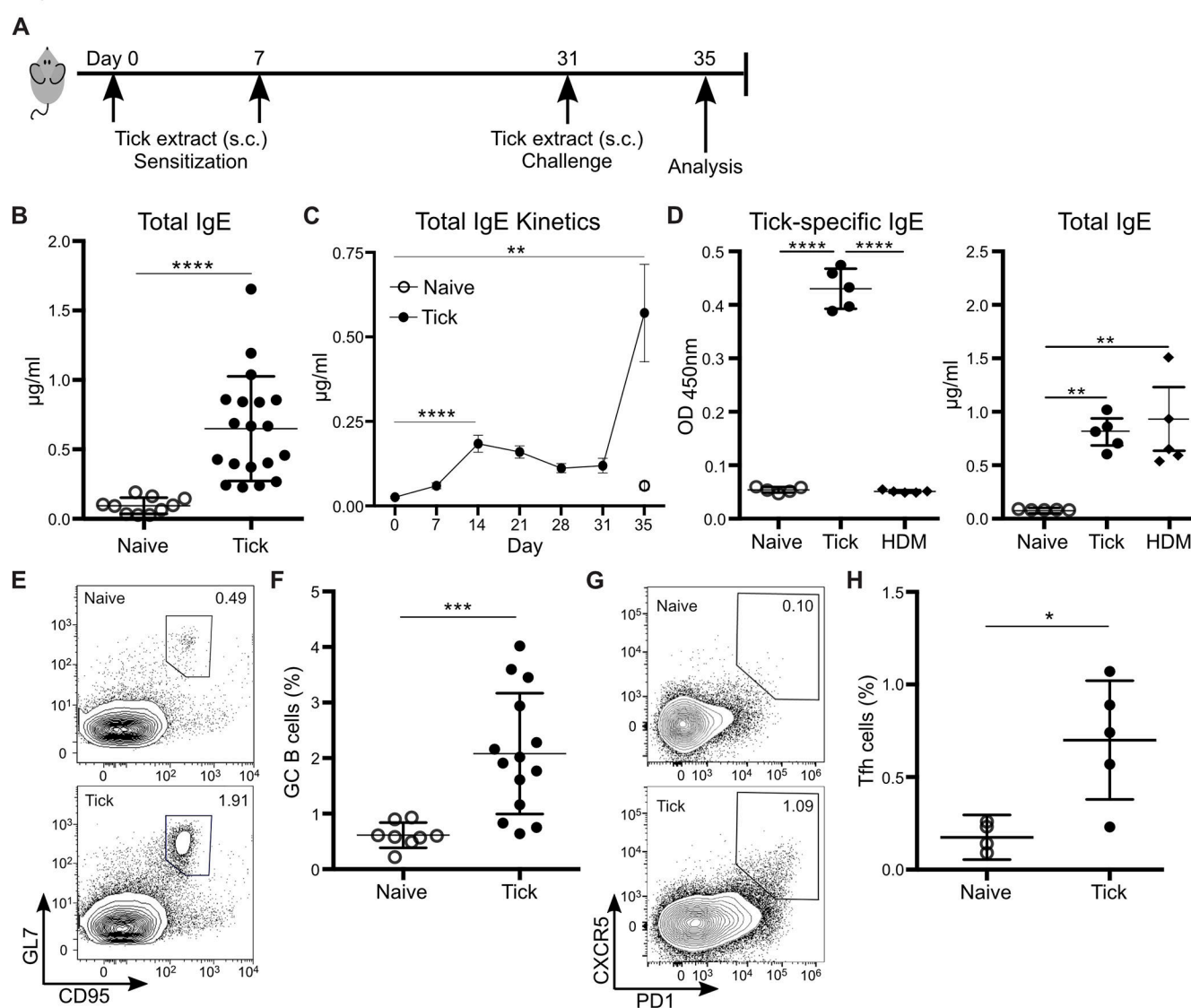
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Key Points

- Lone star tick proteins induce IgE production and hypersensitivity reactions in mice.
- CD4⁺ T cell help is required for both sensitization and recall IgE production.
- MyD88 deficiency in B cells disrupts IgE production.

**FIGURE 1.**

Cutaneous tick exposure induces an IgE response. *A*, Mice were injected subcutaneously 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 house dust mite (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-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 $\pm$ SEM. One of two experiments with similar results is shown. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

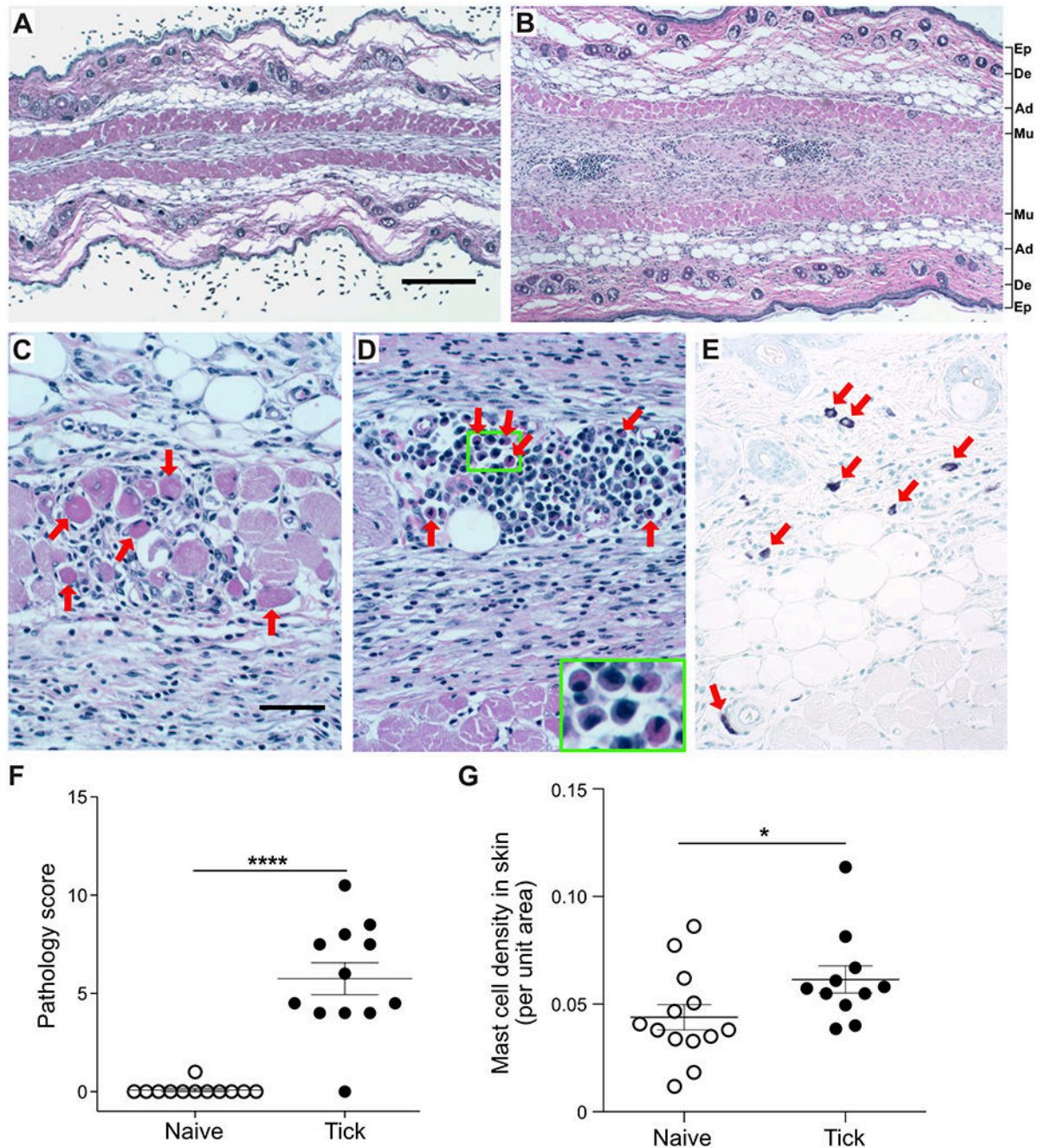
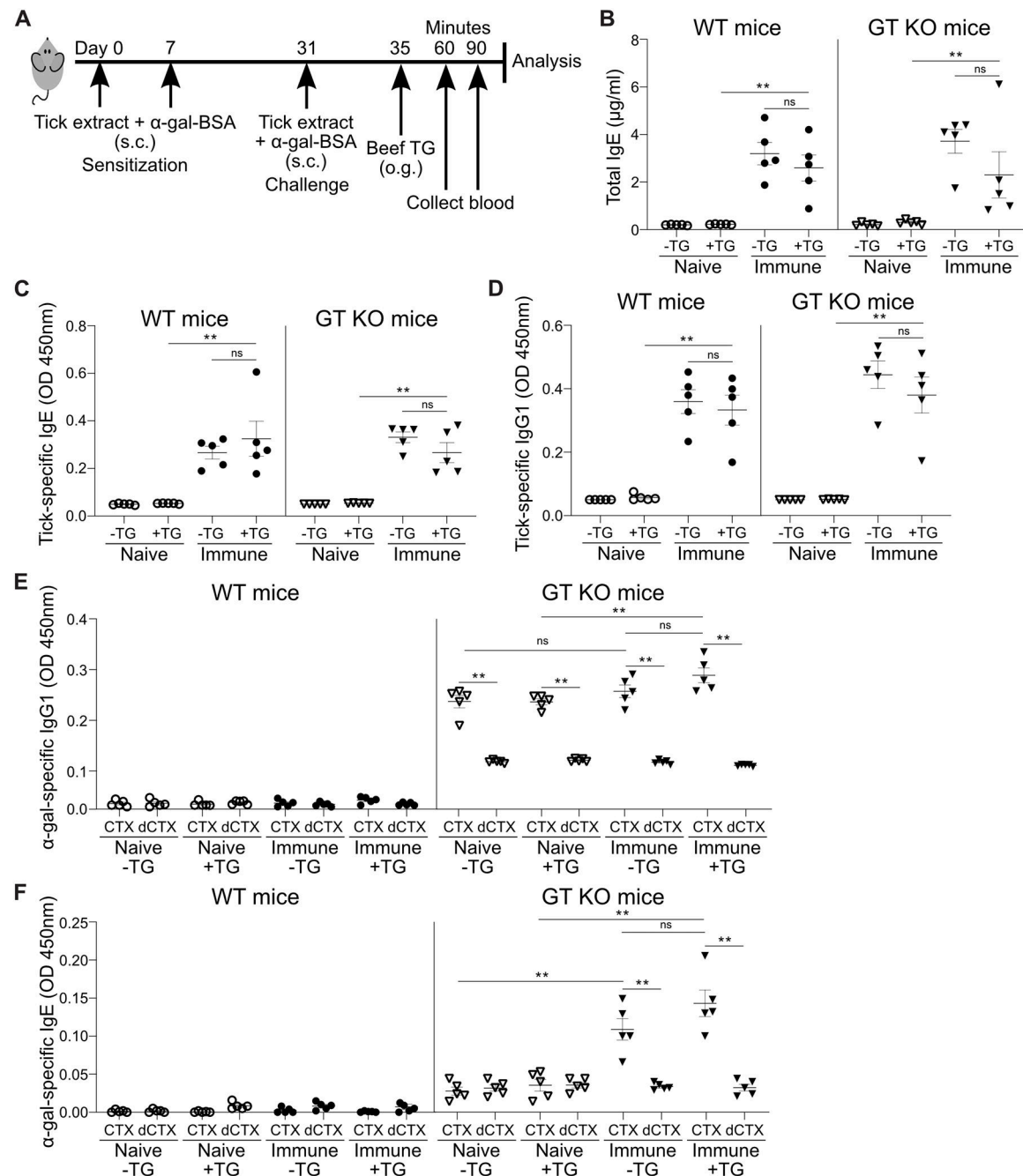


FIGURE 2.

Subcutaneous 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 3-5 sections per animal from (*A*) naive and (*B*) tick immunized mice showed increased cellular infiltration and enlarged dermis from immune mice. Two butterflyed layers of skin per mouse are shown. Ep, epidermis; De, dermis; Ad, adipose; Mu, muscle. Original magnification $\times 40$. Bar scale = 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 x200. Bar scale = 20 μ m. *F*, Skin pathology was scored based on dermal thickening, vasculitis, 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 $\pm$ SEM. Data are representative of two independent experiments. * $p < 0.05$, **** $p < 0.0001$.

**FIGURE 3.**

Mice deficient in alpha-gal expression produce alpha-gal-specific antibodies following cutaneous exposure to tick extract. *A*, Wild type (WT) and alpha-gal deficient (GT KO) mice were injected subcutaneously with tick extract and alpha-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 naïve 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 alpha-gal-specific IgG1 and IgE from mice at 90 min

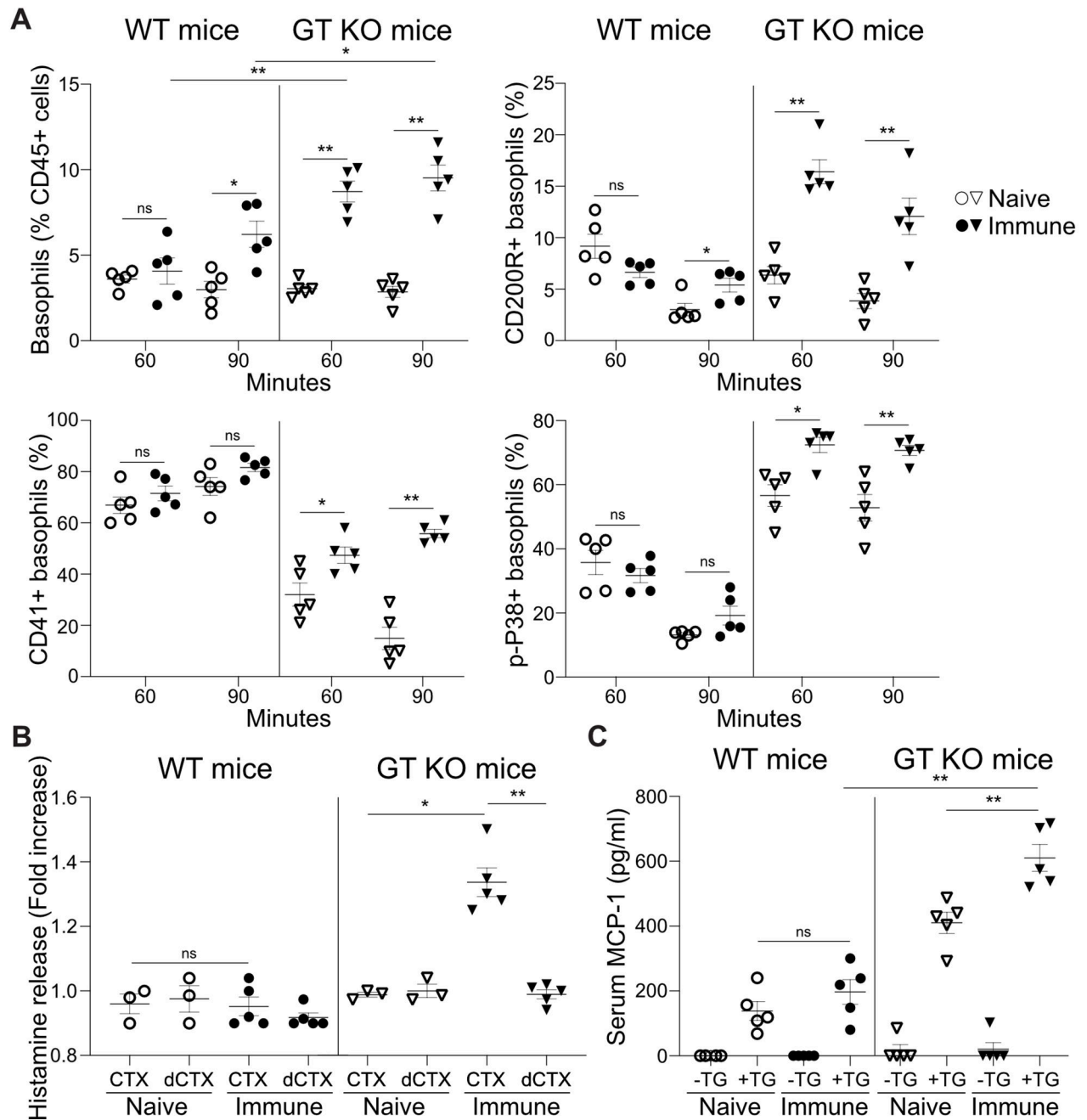
after gavage were measured by ELISA using cetuximab (CTX) or deglycosylated cetuximab (dCTX) for the presence and absence of alpha-gal epitopes, respectively. Each symbol represents an individual mouse and error bars show the mean $\pm$ SEM. ** $p < 0.01$. Data are representative of two independent experiments, each with 5 mice per group.

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**FIGURE 4.**

Mice deficient in alpha-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 naïve and immune mice at 60 and 90 minutes after oral gavage were evaluated by flow cytometry. **B**, Heparinized blood from naïve and immune mice before oral gavage was incubated with either cetuximab (CTX) or deglycosylated cetuximab (dCTX), and basophil release of

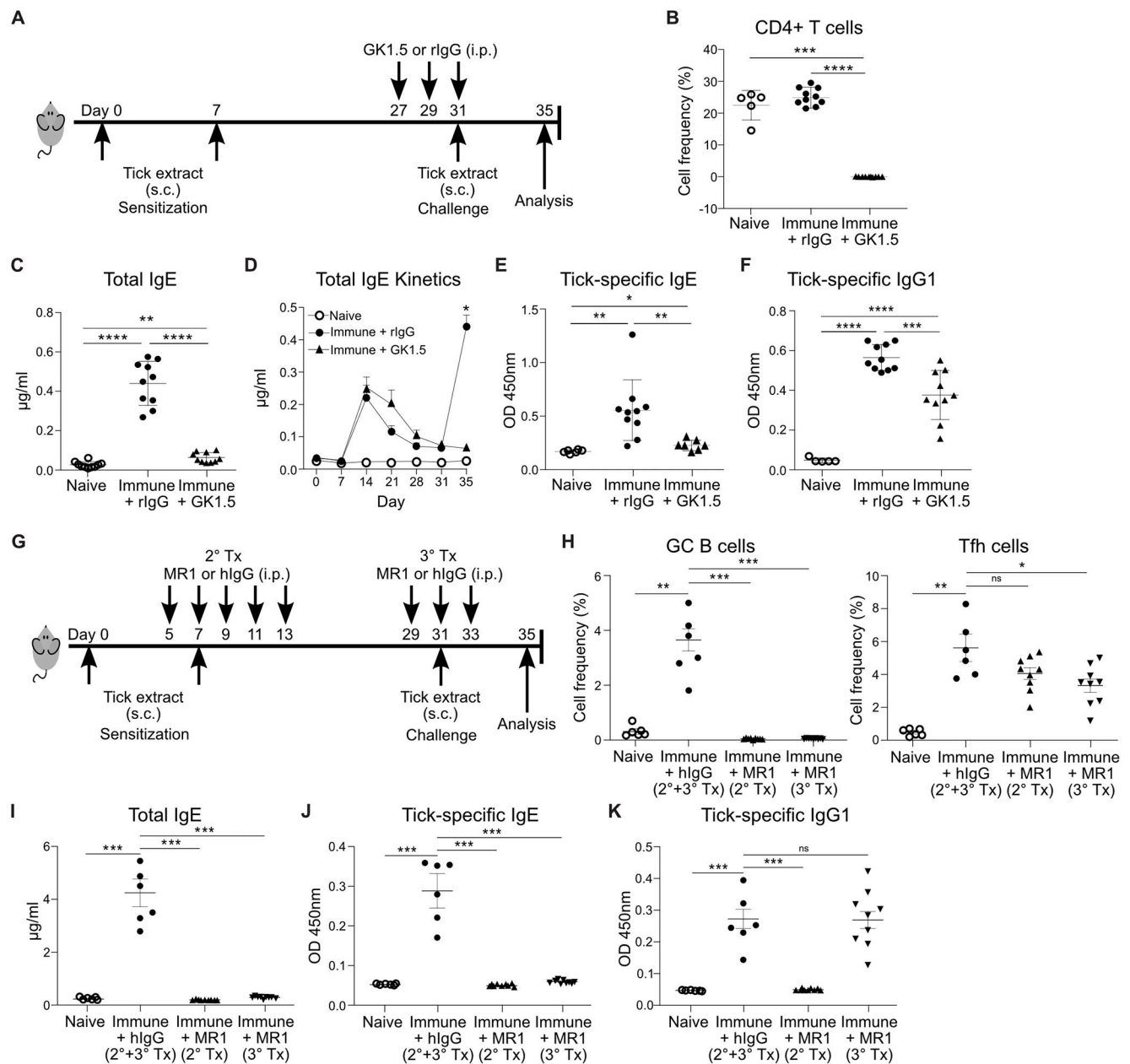
histamine was measured by ELISA. *C*, Serum levels of MCP-1 were measured by ELISA. Each symbol represents an individual mouse and error bars show the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$. ns, not significant. Data are representative of two independent experiments.

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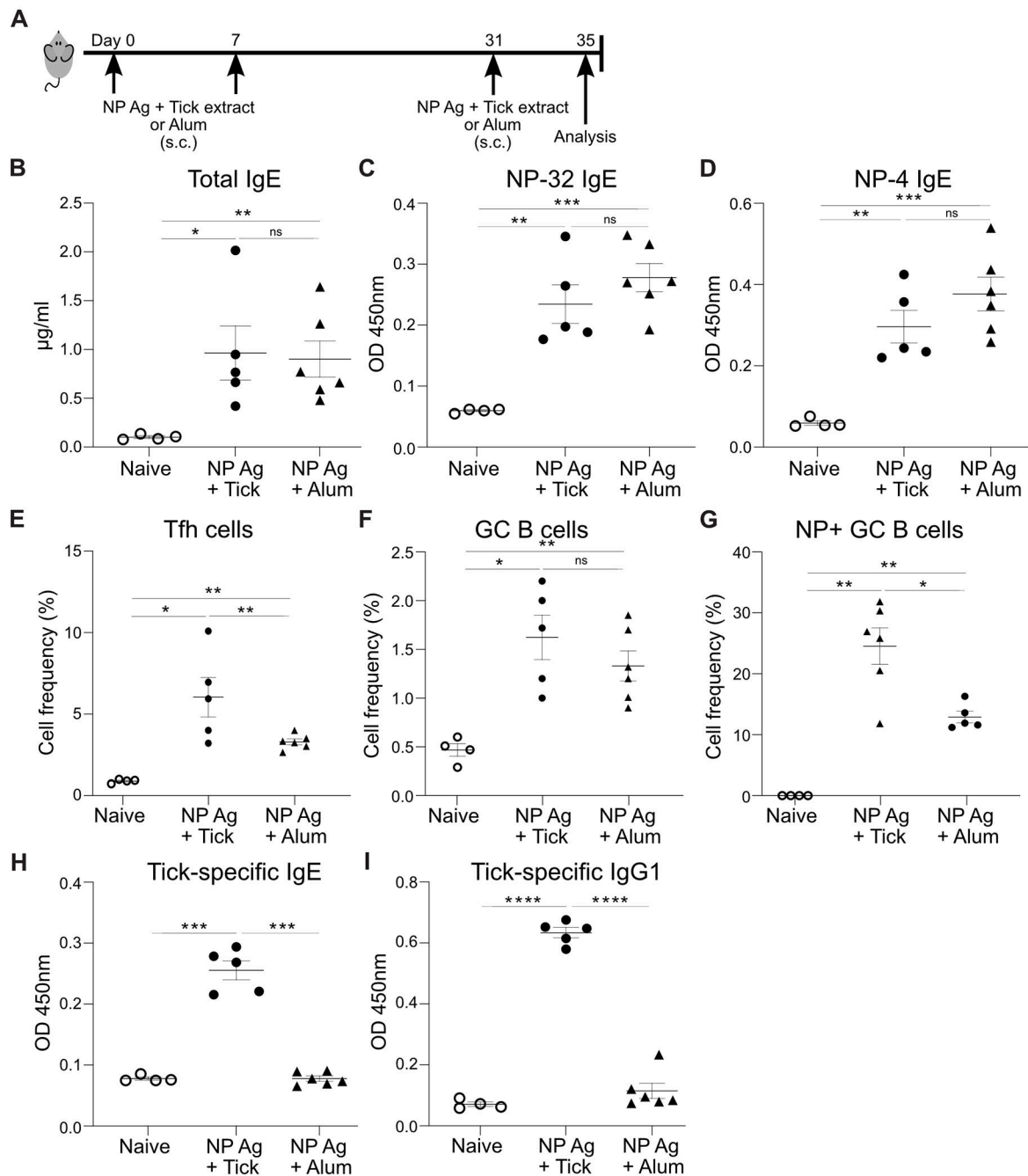
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**FIGURE 5.**

T cell help is required for the induction of IgE response to tick extract. *A*, Mice were injected subcutaneously 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 subcutaneously with tick extract on days 0, 7 and 31, and administered MR1 or isotype control mAb intraperitoneally 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 was 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 $\pm$ 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.

**FIGURE 6.**

Tick extract functions as an adjuvant. *A*, Mice were injected subcutaneously with NP Ag in the presence of tick extract or in the presence of alum on days 0, 7 and 31, and antibody 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 was 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

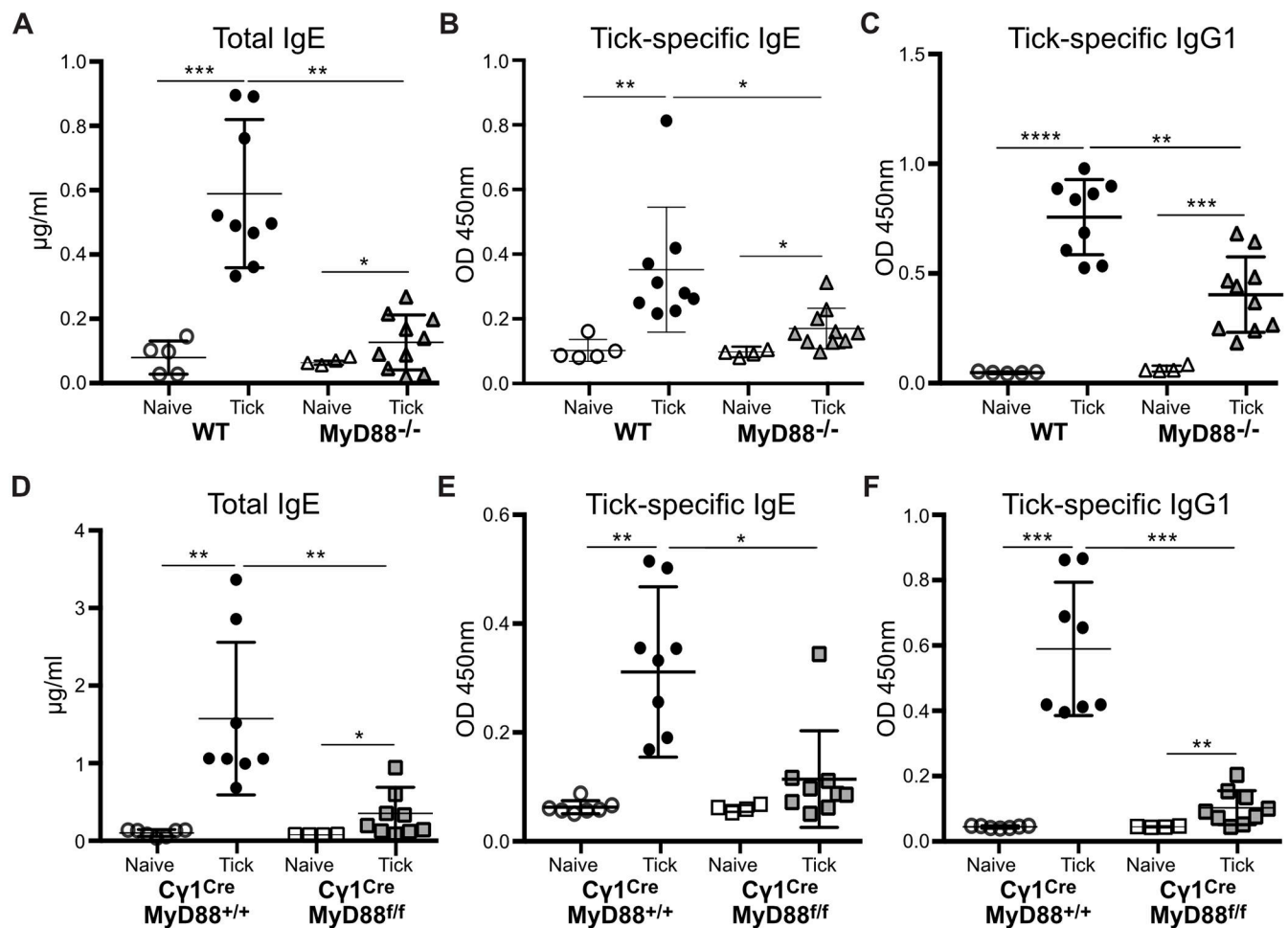
bars show the mean $\pm$ SEM. One of three experiments with similar results is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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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*, WT and MyD88^{-/-} mice were injected subcutaneously with tick extract or PBS (naïve) on days 0, 7 and 31, and then antibody 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 subcutaneously 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.