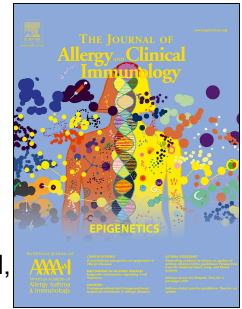


Journal Pre-proof

The B cell antigen receptor of IgE-switched plasma cells regulates memory IgE responses

Michaela E.R. Schmitt, PhD, Johannes Lutz, PhD, Paul Haase, MSc, Michael R. Bösl, PhD, Jürgen Wienands, PhD, Niklas Engels, PhD, David Voehringer, PhD



PII: S0091-6749(20)30268-2

DOI: <https://doi.org/10.1016/j.jaci.2020.02.015>

Reference: YMAI 14432

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 20 September 2019

Revised Date: 12 February 2020

Accepted Date: 14 February 2020

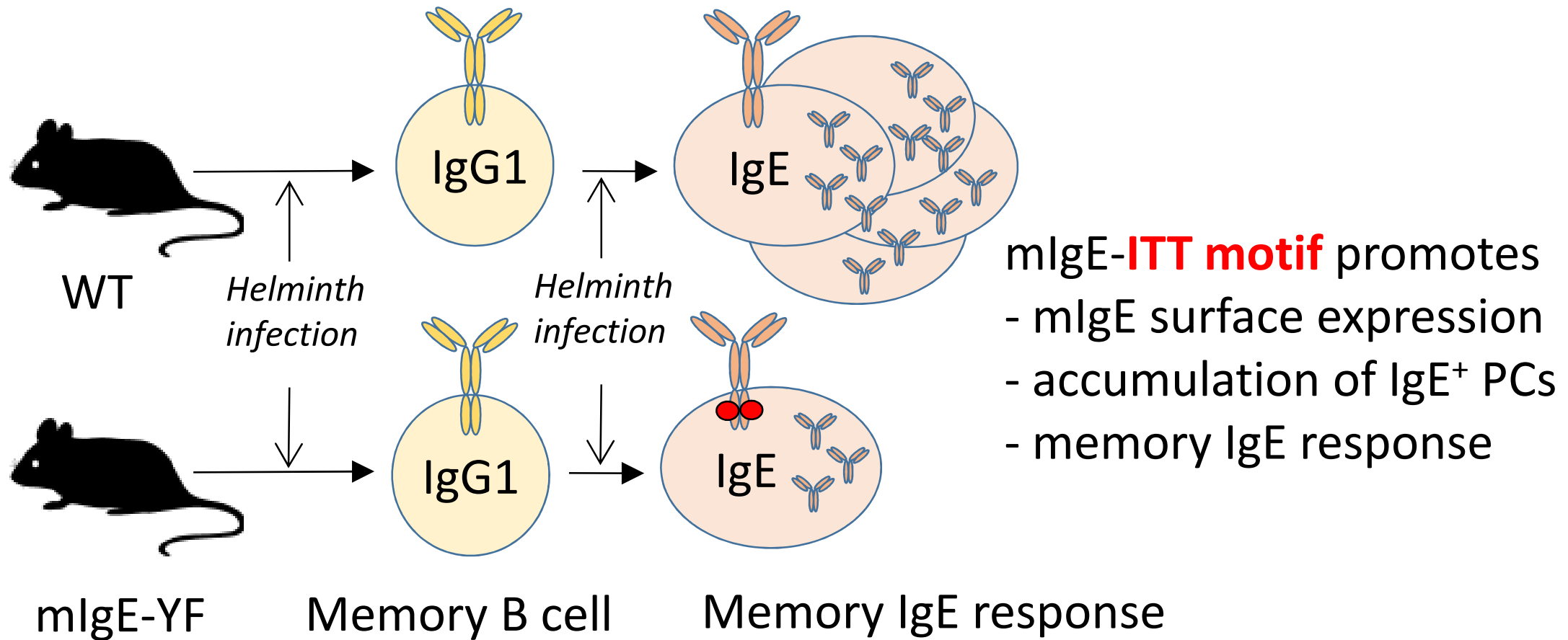
Please cite this article as: Schmitt MER, Lutz J, Haase P, Bösl MR, Wienands J, Engels N, Voehringer D, The B cell antigen receptor of IgE-switched plasma cells regulates memory IgE responses *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.02.015>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology.



The mIgE-ITT motif promotes the memory IgE response



ITT: immunoglobulin tail tyrosine, mIgE-YF: mice with mutant ITT motif in the IgE B cell receptor, PC: plasma cells, WT: wild-type mice

The B cell antigen receptor of IgE-switched plasma cells regulates memory IgE responses

Michaela E. R. Schmitt, PhD¹, Johannes Lutz, PhD², Paul Haase, MSc¹,
Michael R. Bösl, PhD³, Jürgen Wienands, PhD², Niklas Engels, PhD^{2*},
and David Voehringer, PhD^{1*}

¹Department of Infection Biology, University Hospital Erlangen, 91054 Erlangen,
Germany.

²Institute for Cellular and Molecular Immunology, University Medical Center
Göttingen, 37073 Göttingen, Germany.

³Department of Experimental Biomedicine I, University Hospital and Rudolf Virchow
Center, University of Würzburg, Würzburg, Germany.

Funding: This work was supported by the Deutsche Forschungsgemeinschaft (DFG)
grant no. TRR130_TP08 to N.E. and J.W. and TRR130_TP20 to D.V..

Conflict of interests statement: All authors declare to have no conflict of interests.

Correspondence: David Voehringer, PhD
Department of Infection Biology
University Hospital Erlangen
Wasserturmstrasse 3-5
91054 Erlangen
Phone +49-9131-8532735
Email: david.voehringer@uk-erlangen.de

Abstract

Background: Allergic inflammation is driven by IgE-producing plasma cells (PCs) which are required for IgE-mediated activation of mast cells and basophils. Repeated antigen encounter elicits a memory IgE response with elevated serum IgE titers and accumulation of IgE-producing PCs. However, the cellular compartment and molecular signals that underlie the immunological memory of IgE responses remain unclear.

Objective: With this study we aimed at clarifying whether inactivation of the cytoplasmic immunoglobulin tail tyrosine (ITT) motif in transmembrane IgE (mIgE) impairs the memory IgE response in mice.

Methods: We generated mice with an inactivated mIgE-ITT motif and analyzed serum IgE levels as well as the generation of IgE-producing germinal center B cells and PCs upon primary and secondary infection with helminths. In vitro cultures were used to study the mIgE-ITT-controlled expression of mIgE on the surface of PCs. Systemic mast cell activation was determined by serum Mcpt1 ELISA in response to ovalbumin challenge.

Results: mIgE-ITT mutant mice showed an impaired memory IgE response upon helminth infection. Furthermore, sensitization and challenge of mIgE-ITT-mutant mice with ovalbumin resulted in diminished serum IgE titers and reduced mast cell activation. The mIgE-ITT motif was required for optimal cell surface expression of mIgE B cell antigen receptors (BCRs) but not for intracellular IgE expression in PCs.

Conclusion: These results indicate that the mIgE-BCR plays a critical role in establishing or maintaining the population of IgE-producing PCs during memory IgE responses.

Key messages:

- The immunoglobulin tail tyrosine (ITT) motif of the mIgE-BCR promotes the memory IgE response.
- The ITT motif is required for optimal surface expression of mIgE on plasma cells and their accumulation upon secondary helminth infection.

Capsule summary:

B cell-intrinsic mechanisms that regulate memory IgE responses are poorly understood. Here we show evidence that accumulation of IgE⁺ plasma cells upon secondary antigen challenge is regulated by the mIgE-ITT motif.

Key words:

Allergy, helminths, IgE, plasma cells, B cell receptor

Abbreviations:

ITT, immunoglobulin tail tyrosine; mIgE, transmembrane IgE; mIgG1, transmembrane IgG1; CSR, class switch recombination; GC, germinal center; PC, plasma cell; NES, *N. brasiliensis* excretory/secretory products

Introduction

The immunoglobulin (Ig) isotype IgE is associated with protective immunity against parasitic worms and insect venoms, yet also with type I hypersensitivity reactions and allergic inflammation¹⁻³. Secreted IgE antibodies sensitize mast cells and basophils by binding to the high-affinity IgE receptor (FcεRI) on their cell surface⁴. Antigen-mediated cross-linking of FcεRI-bound IgE results in the rapid release of a variety of effector molecules including histamine, proteases, cytokines, chemokines and lipid mediators, which facilitate antigen clearance but may also result in life threatening anaphylaxis^{5, 6}. Like other Ig isotypes, IgE can be expressed either as secreted antibody molecule or as plasma membrane-bound isoform (mIgE) that associates with the Igα/β heterodimer (CD79) to form a functional B cell antigen receptor (BCR)⁷⁻⁹. The half-life of free serum IgE is only about 12 hours and therefore is the shortest of all Ig isotypes^{10, 11}. Accordingly, serum IgE concentrations are about 10,000-fold lower compared to other isotypes¹². Despite the importance of IgE for allergic reactions, the mechanisms regulating primary and memory IgE responses *in vivo* are still insufficiently understood, which is in part due to the fact that IgE-expressing B cells and plasma cells (PCs) are very rare cells with a rather short lifespan. Class switch recombination (CSR) to IgE can occur by either a direct (IgM → IgE) or a sequential (IgM → IgG1 → IgE) pathway in mice¹³ and (IgM → IgG1/IgG2/IgG3/IgG4 → IgE) in humans¹⁴. Although the mechanisms that regulate direct versus sequential IgE CSR remain unclear, increasing evidence suggests that directly switched IgE⁺ germinal center (GC) B cells acquire less somatic mutations, rapidly undergo apoptosis and/or give rise to short-lived IgE-producing PCs^{8, 9, 15-17}. These properties may be caused by low mIgE-BCR expression, which may result in an impaired competitive fitness in the GC reaction¹⁵. In addition, other studies

reported antigen-independent, i.e. mIgE-autonomous apoptosis induction as well as enhanced PC differentiation in certain B cell cultures *in vitro*¹⁸. These effects may involve an association of the extracellular membrane-proximal domain (EMPD) of mIgE with the B cell co-receptor CD19^{19, 20}. However, spontaneous apoptosis induction by mIgE could not be confirmed by another study, in which mIgE was rather found to dampen cell cycle progression in GC B cells and to promote antigen-independent PC differentiation²⁰.

With respect to specific therapeutic intervention of allergic diseases, it is important to understand how memory IgE responses are regulated. Long-term persistence of IgE memory is evident, since helminth-specific IgE can be observed in humans years after de-worming²¹. Furthermore, immunoglobulin repertoire analyses revealed that the same IgE-expressing B cell or PC clones can be detected in peripheral blood of birch pollen allergy patients in two consecutive pollen seasons²². Mice that lack the cytoplasmic tail of mIgE have been shown to develop impaired primary and memory serum IgE responses upon helminth infection²³. Although the potential existence of rare long-lived mIgE⁺ memory B cells in mouse or human cannot be ruled out completely, numerous studies found no evidence for the existence of *bona fide* mIgE-expressing memory B cells and thus rather support the model according to which the memory IgE response originates from mIgG1-expressing memory B cells that undergo secondary CSR to IgE followed by rapid differentiation into IgE-secreting PCs^{8, 15, 16, 24}.

The memory response of mIgG1-expressing B cells is greatly enhanced by the cytoplasmic tail domains of the mIgG1 molecule²⁵. These tail domains contain a tyrosine-based signaling motif termed immunoglobulin tail tyrosine (ITT) motif²⁶. The ITT motif is, however, not restricted to mIgG1 cytoplasmic tails but is found in all mIgG isotypes and in mIgE of mouse, human and other mammals as well as in their

common ancestor isotype, mIgY, from reptiles and birds ²⁷ (Figure E1e in the Online Repository). On phosphorylation of the central tyrosine residue, ITT motifs provide docking sites for the universal SH2 domain-containing adaptor protein Grb2, which in turn recruits the protein tyrosine kinase Btk to the activated BCR and thereby amplifies its signaling activity ²⁶⁻²⁸. Whether the ITT motif of mIgE is involved in the regulation of IgE antibody production *in vivo*, or whether IgE production relies on the performance of mIgG1-expressing cells remains unknown. To address these questions, we have generated mutant mice, in which the ITT motif of mIgE was inactivated by a tyrosine to phenylalanine substitution.

We used the *Nippostrongylus brasiliensis* helminth infection model and systemic ovalbumin-induced mast cell activation to investigate the role of the ITT motifs in mIgG1 and mIgE for generation of IgE⁺ and IgG1⁺ GC B cells, PCs as well as the production of serum IgG1 and IgE antibodies. The mIgG1-ITT motif was essential for the IgG1 response but not for that of IgE. By contrast, mIgE-ITT mutant mice revealed a role of this motif for the memory IgE response and for the accumulation of IgE⁺ PCs. Mechanistically, the mIgE-ITT motif promoted surface expression of mIgE on PCs, indicating a so far unknown role for the mIgE-BCR in this cell type. Our data demonstrate, that the IgE memory response not only originates from mIgG1-expressing memory B cells, but also relies on the proper function of the mIgE-switched BCR on plasma cells.

Methods

Mice

mlgE-YF knock-in mice were generated by homologous recombination in ES cells. In brief, the 19th codon of the transmembrane exon M2 of the ϵ immunoglobulin locus was mutated from TAT to TTT (causing an amino acid exchange from tyrosine to phenylalanine). Simultaneously, a loxP-neo-loxP cassette was inserted into the SmaI site 3' of exon M2. The linearized targeting vector was electroporated into BALB/c-I ES cell (kindly provided by Lars Nitschke) and correct integration was determined by Southern blot. The confirmed clone I4B was expanded, transiently transfected with a Cre recombinase expression plasmid to delete the neo resistance cassette and subsequently was injected into blastocysts to generate chimeric offspring mice, which were bred to BALB/c mice for germline transmission and then inter-crossed to obtain the final homozygous mlgE-YF mutant line.

mlgE-KVK Δ tail, mlgG1-YF and IgE-deficient mice have been described before. mlgG1-YF mice²⁵ were generated and used on pure C57BL/6 background whereas mlgE-KVK Δ tail²³, mlgE-YF and IgE-deficient²⁹ mice were on BALB/c background. BALB/c mice were originally obtained from the Jackson Laboratory, C57BL/6J mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were maintained under specific pathogen-free conditions and mice of both sexes were used at 6-12 weeks of age. All animal experiments were approved by the Federal Government of Lower Franconia and performed in accordance with German animal protection law.

Helminth infection

Third stage larvae (L3) of *N. brasiliensis* were washed in sterile 0.9% saline (37°C). Mice were infected with 500 larvae subcutaneously in the base of the tail. For the first five days after infection mice were treated with antibiotics in drinking water (2 g/l neomycin sulfate, 100 mg/l polymyxin B sulfate). Mice were analyzed at indicated time points.

Systemic mast cell activation

To induce systemic mast cell activation, mice were sensitized with 50 µg OVA (Sigma-Aldrich) in 200 µl imject alum (Thermo Scientific) i.p. and re-challenged 14 days later with 200 µg OVA in 200 µl PBS i.p.. Blood was taken 2-3 h after re-challenge from the tail vein.

Cell preparation and flow cytometry

Single cell suspensions from mesenteric lymph nodes (MLNs) were prepared by passing MLNs through a 70 µm cell strainer. Cell suspensions were treated with 1 ml ice-cold acetic acid buffer to remove unspecific binding of IgE antibodies from the cell surface⁸. After neutralization with 500 µl fetal calf serum (FCS) (Gibco at Thermo Fisher Scientific, Waltham, MA) cells were washed twice with FACS buffer (PBS, 2% FCS, 1 mg/ml NaN₃). All samples were incubated with anti-CD16/CD32 blocking antibody (clone 2.4G2; BioXCell) and afterwards were stained in FACS buffer at 4°C for 25 min. Following antibodies were purchased from BioLegend (San Diego, CA): PerCP-Cy5.5-labeled anti-CD45R (clone RA3-6B2), PE-Cy7-labeled anti-CD38 (clone 90) and PE-labeled anti-CD79b (clone HM79-12). PE-labeled anti-CD267 (clone eBio8F10-3), eFluor 660-labeled anti-GL-7 (clone GL-7), PE-Cy7-labeled anti-CD45R (clone RA3-6B2), PE-Cy7-labeled anti-IgM (clone eB121-15F9) were

purchased from eBioscience. FITC-, BB700- and BV421-labeled anti-IgE (clone R35-72), APC-labeled anti-CD138 (clone 281-2), V450- and PE CF594-labeled anti-IgG1 (clone A85-1) and PE-labeled anti-CD49b (clone HM ALPHA2) were purchased from Pharmingen at BD Biosciences (San Diego, CA). Dead cells were excluded by using fixable ViaDye eFluor 506 or eFluor 780 (Invitrogen). For intracellular staining, cells were fixed with 4% PFA and permeabilized with Perm/Wash Buffer (BioLegend). For combined surface and intracellular isotype stainings BD Cytofix/Cytoperm and BD Phosflow Perm/Wash buffers were used. Samples were acquired on either a FACSCanto II or a LSR Fortessa cytometer (BD Biosciences). Data were analyzed with FlowJo software (FlowJo LLC, Ashland, OR).

In vitro culture

Single cell suspensions from spleens of naïve mice were generated by mechanical disruption. Primary B cells were purified with the EasySep mouse B cell isolation kit (Stemcell technologies, Vancouver, BC) or Mojosort mouse Pan B cell isolation Kit II (BioLegend). 2×10^6 B cells in 24 well plates were cultured in RPMI 1640 containing 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamin and 50 µM β-mercaptoethanol. Cells were stimulated with 10 µg/ml anti-CD40 (BioXCell) and 25 ng/ml IL-4 (R&D Systems, Minneapolis, MN) to induce PC differentiation and class switching to IgG1 and IgE. Cells were cultured for four days at 37°C in 5% CO₂.

Serum ELISA

To measure IgG1 and IgE serum levels, ELISA plates were coated with goat anti-mouse Ig (SouthernBiotech, Birmingham, AL), purified rat anti-mouse IgE (clone R35-72, Pharmingen) or excretory/secretory products of *N. brasiliensis* L3 larvae and blocked with PBS + 3% BSA. Sera were incubated overnight at 4°C. Alkaline

phosphatase-coupled anti-mouse IgG1 (SouthernBiotech) and goat-anti-mouse IgE (SouthernBiotech) were used for detection. mMcpt1 ELISA was performed with a commercial Kit (Invitrogen) according to the manufacturer's protocol. ELISAs were measured at 405 nm with Multiskan FC multiplate photometer (Thermo Fisher Scientific).

Statistics

Two-tailed Student's t-test was used for normally distributed (Shapiro-Wilk test) data and Mann-Whitney U-test was used for non-normally distributed data. Analysis was performed with SigmaPlot 12.3 software. P-values < 0.05 were considered statistically significant.

Online Repository

The Online Repository contains Figures E1-E5.

Results

The mIgE-ITT motif promotes the IgE memory response upon helminth infection.

To investigate the role of the signaling function of the mIgE-BCR for the generation of IgE antibodies in an *in vivo* model system, we specifically compromised its signaling function by introducing an ITT-inactivating tyrosine to phenylalanine-substituting mutation into the M2 exon of the Ig ϵ heavy chain locus of BALB/c mice (mIgE-YF mice, Supplementary Figure S1). To analyze the humoral immune response in these animals to a complex physiological antigen, we infected the mice once or twice with the nematode *Nippostrongylus brasiliensis* to study the primary and memory IgE response. As controls, we infected wild-type BALB/c mice and mice in which the cytoplasmic tail domain of mIgE is shortened from 28 to three amino acids (mIgE-KVK Δ tail mice²³). Ten days after primary or secondary infection, cells were isolated from mesenteric lymph nodes (MLN) and treated with acidic buffer to remove cytophilic IgE from the cell surfaces, followed by flow cytometric analysis of germinal center (GC) cells and plasma cells (PC).

Following primary infection, the frequencies of total and IgE⁺ GC B cells as well as the total number of IgE⁺ GC B cells increased in all three groups of mice (Fig. 1a-b, Fig. E1 and E2 in the Online Repository). Following secondary infection four weeks after the primary infection, we did not observe a further increase in the frequency or number of IgE⁺ GC B cells irrespective of the genotype (Fig. 1a-b, Fig. E3 in the Online Repository). We also analyzed the formation of IgG1- and IgE-producing plasma cells (PCs) in all animals 10 days after primary or secondary infection, respectively. Whereas the frequencies of PCs were similar in all groups, only wild-type mice showed an increase in frequency of IgE⁺ PCs between primary and

secondary infection with *N. brasiliensis* (Fig. 1c). The total number of IgE⁺ PCs was also higher in wild-type mice as compared to both mIgE mutant mice, although a slight increase in IgE⁺ PC numbers was detected in mIgE-YF but not in mIgE-KVKΔtail mice (Fig. E3 in the Online Repository). This further translated into severely reduced concentrations of serum IgE antibodies in mIgE mutant mice, whereas IgG1 concentrations were similar in all mouse lines (Fig. 1d). Notably, the reduction of IgE⁺ PCs frequency, number and soluble IgE antibodies was more severe in mIgE-KVKΔtail mice than in mIgE-YF mice, indicating a role of the mIgE cytoplasmic tail that goes beyond the signaling function of the ITT motif. To further analyze the *N. brasiliensis*-specific IgE response we performed ELISA with plates coated with excretory/secretory products of *N. brasiliensis* L3 larvae. The pronounced *N. brasiliensis*-specific memory IgE response in wild-type mice was basically abrogated in both mutant mice (Fig. 1e) In conclusion, the data show that the mIgE-ITT motif plays an important role in promoting the primary and particularly the secondary IgE antibody responses.

The mIgG1-ITT motif is dispensable for the IgE memory response.

Several previous reports support the concept that IgG1⁺ memory B cells serve as precursors for the memory IgE response^{8, 15, 16, 24}. We have previously shown that the memory IgE response to *N. brasiliensis* requires the extracellular part of IgG1 as IgE^{ki/ki} mice in which the extracellular domain of IgG1 is replaced by the extracellular part of IgE are not able to mount a normal memory IgE response after secondary infection⁸. To further investigate whether the cytoplasmic mIgG1-ITT motif is required for the memory IgE response, we compared normal C57BL/6J mice to mice in which the mIgG1-ITT motif has been inactivated by an amino acid exchange from tyrosine to phenylalanine (mIgG1-YF mice)²⁵. The different mice were analyzed

under steady-state conditions (naïve) or 10 days after primary or secondary infection with *N. brasiliensis* (Fig. 2).

The frequencies of mIgG1⁺ GC B cells doubled in both strains of mice from about 15% in naïve mice to roughly 30% of all GC B cells after primary infection (Fig. 2b, middle left). However, only wild-type mice showed a further increase of mIgG1⁺ GC B cells to approximately 50% after secondary infection (Fig. 2b, middle left). The total numbers of mIgG1⁺ GC B cells were also higher after secondary infection of wild-type as compared to mIgG1-YF mice (Fig. E4 in the Online Repository). The frequencies of mIgE⁺ GC B cells were generally below 2% and only increased after primary infection in both wild-type and mIgG1-YF mice (Fig. 2b, lower left).

Similar to the GC response, we observed an increase of mIgG1⁺ PCs after secondary infection in wild-type mice, which was not the case in mIgG1-YF animals (Fig. 2b, middle right). By contrast, the frequencies of mIgE⁺ PCs increased in both wild-type and mIgG1-YF-mutant mice after primary infection and again after secondary infection (Fig. 2b, lower right). The frequencies of total PCs were not changed in mIgG1-YF mice, which may be due to compensatory formation of PCs expressing other IgG isotypes as observed before^{25, 30} (Fig. 2b, upper right).

As before, we also investigated the serum concentrations of IgG1 and IgE antibodies in the *N. brasiliensis*-infected mice. The IgG1 titers in mIgG1-YF mice were strongly reduced as compared to wild-type mice at all three time points tested (Fig. 2c, left diagram). However, in stark contrast to what we observed for IgG1 antibodies, the concentrations of serum IgE antibodies increased equally in both strains of mice, especially after secondary infection (Fig. 2c, right). These findings demonstrate that the ITT motif in mIgG1 is essential for the IgG1 memory response to *N. brasiliensis*, which is similar to what we previously observed for non-infectious antigens²⁵.

However, the mIgG1-ITT motif is dispensable for a *bona fide* memory response of the IgE antibody isotype.

The mIgE-ITT motif enhances IgE⁺ PC accumulation in an antigen-independent *in vitro* system.

The reduced IgE response in mIgE-YF and mIgE-KVK Δ tail mice after *N. brasiliensis* infection could be due to impaired antigen-dependent signaling from the mIgE-BCR. Alternatively, it may indicate a requirement for the mIgE cytoplasmic tail to establish the mIgE⁺ PC population in an antigen-independent manner as suggested by previous reports^{19, 20}. To address this point, we adapted a published *in vitro* culture system to generate mIgE⁺ PCs from B cells without engaging the BCR²⁰. To this end, we isolated B cells from the spleens of naïve mice and stimulated them with IL-4 and anti-CD40 antibodies to induce proliferation and differentiation to IgE⁺ and IgG1⁺ PCs. After an incubation period of four days, we observed a reduced frequency of IgE⁺ PCs in cultures derived from mIgE-YF and mIgE-KVK Δ tail mice, whereas the IgG1⁺ PC populations were not diminished (Fig. 3). Together, our data indicate that the truncation of the entire mIgE cytoplasmic tail or the selective inactivation of the mIgE-ITT motif both lead to an impaired formation or survival of IgE⁺ PCs *in vivo* and *in vitro*. The latter observation indicates that this effect may occur in an antigen-independent, i.e. in a mIgE-BCR-autonomous manner.

The mIgE-ITT motif is required for BCR surface expression in PCs and for accumulation of IgE⁺ PCs upon helminth infection *in vivo*.

To further address the potential mechanism by which the mIgE tail regulates the IgE⁺ PC compartment, we investigated whether the mIgE-ITT motif is required for efficient surface expression of mIgE on *in vivo*-derived PCs. Although PCs generally down-

regulate their BCR expression, it has been described that some isotypes including mIgE can be expressed on the surface of PCs^{9, 31}. We infected wild-type, mIgE-YF and mIgE-KVK Δ tail mice with *N. brasiliensis* as before and determined the frequency of mIgE⁺ PCs isolated from MLN on day 10 after primary infection. In addition, we determined the expression level of mIgE on the cell surface of PCs.

Wild-type mice contained about 24% mIgE⁺ PCs whereas this population was reduced to about 9% and 3% in mIgE-YF and mIgE-KVK Δ tail mice, respectively (Fig. 4a and b). Lack of mIgG1/mIgE double-positive cells and detection of Ig β (CD79b) on the cell surface confirmed that the mIgE⁺ cells indeed expressed mIgE-BCRs rather than having bound secreted IgE (Fig. 4a). Interestingly, the mean fluorescence intensity (MFI) for mIgE was reduced by roughly 50% in IgE⁺ PCs from mIgE-YF mice while the MFI for intracellular IgE was comparable to PCs from wild-type animals (Fig. 4c and d). PCs from mIgE-KVK Δ tail mice also showed lower cell surface mIgE expression but also reduced intracellular IgE levels (Fig. 4c and d).

These data indicate that the mIgE-ITT motif promotes cell surface expression of mIgE-BCRs on PCs, which may be required for optimal formation and/or survival of these cells, potentially in an antigen-independent manner. To further determine whether all surface IgE⁺ or IgG1⁺ PCs generally express the corresponding isotypes intracellularly we analyzed in vitro differentiated PCs. Here we observed a tight correlation between surface and intracellular staining for both isotypes (Fig. E5 in the Online Repository).

Mast cell activation and active systemic anaphylaxis are reduced in mIgE-BCR-mutant mice.

The reduced IgE responses in mIgE-YF and mIgE-KVK Δ tail mice after helminth infection suggested that these mice may also develop an attenuated mast cell response during a systemic anaphylaxis model. To test this hypothesis, we primed mice by intraperitoneal (i.p.) injection of 50 μ g ovalbumin (OVA) in alum and re-challenged them two weeks later by another i.p. injection with 200 μ g OVA. We determined serum IgE titers, the frequencies of IgE-sensitized basophils in the blood and the concentration of mouse mast cell protease 1 (mMcp1) in the sera as readout for mucosal mast cell activation³². All three parameters were reduced in mIgE-YF and mIgE-KVK Δ tail mice when compared to wild-type controls (Fig. 5). As additional control we used IgE-deficient mice (IgE-ko)²⁹, which showed basically no response. These data demonstrate that the mIgE-BCR controls the production of anaphylactic IgE antibodies and thus is a critical determinant of systemic allergic immune reactions that are carried out by mast cells and basophils.

Discussion

In this study we investigated the influence of the mIgE-BCR on primary and memory IgE responses after infection of mice with the gastrointestinal helminth *N. brasiliensis* and in a model of active systemic anaphylaxis. Our previous work demonstrated that the extracellular constant Ig domains of mIgG1 are important for the memory IgE response⁸. This finding supported the concept that the immunological memory of IgE is preserved at the cellular level in mIgG1-expressing long-lived memory B cells^{16, 24}. However, even if long-term IgE memory resides in the cellular compartment of mIgG1⁺ memory B cells, this does not rule out a function of the mIgE-BCR in the memory IgE response. Indeed, a previous study reporting impaired IgE antibody production in mIgE-KVK Δ tail mice provided evidence in favor of a role of the mIgE-BCR in that process²³. Yet, the mechanistic details as well as the cellular stage at which the mIgE-BCR exerts its function remained unclear. We previously identified the ITT motif as a key molecular determinant of humoral immunological memory for IgG1^{25-27, 33}. Even though the mIgE-ITT motif, like that of mIgG1, functions to amplify BCR signaling and employs a similar set of cytoplasmic effector molecules^{26, 28}, its physiological role for IgE antibody production remained unclear. By inactivating the mIgE-ITT motif in the mouse, we showed here that it indeed promotes the production of IgE antibodies *in vivo*. By contrast, inactivation of the ITT in mIgG1 had no effect on IgE serum titers, while it was absolutely essential for production of IgG1 antibodies to *N. brasiliensis*. This shows that even though IgE memory may persist at the cellular level in mIgG1⁺ memory B cells, optimal signaling of the mIgG1-BCR is not required for IgE antibody production. An explanation for this seeming discrepancy may be that the mIgG1-ITT is particularly important for the generation of IgG1-producing PCs, yet not for the generation of IgG1 memory cells²⁵. Since mIgG1⁺

memory B cells need to undergo secondary CSR in a GC reaction in order to give rise to IgE-producing PCs, this process is most likely not promoted by the mIgG1-ITT motif. Hence, mIgG1-ITT-mutant memory B cells are not impaired in their capacity to generate IgE-producing plasma cells, at least not within the four weeks interval between primary and secondary infection with *N. brasiliensis* that we used in our experiments. Why exactly the extracellular part of mIgG1 is required for the memory IgE response remains to be clarified. One possible scenario is that the extracellular domains of mIgE⁺ B cells very potently induce autonomous, antigen-independent cellular differentiation into IgE⁺ plasma cells, thus preventing the establishment of (long-lived) mIgE⁺ memory B cells^{19, 20}. This would explain why IgE^{ki/ki} mice which express the extracellular domains of IgE instead of IgG1 lack an IgE memory response⁸.

However, our analyses of mIgE-mutant mice clearly revealed that a second level of regulation of the memory IgE response is provided by the mIgE cytoplasmic tail and its ITT motif which promote the accumulation of mIgE⁺ PCs and thus the production of IgE antibodies after secondary *N. brasiliensis* infection. *In vitro* cultures with anti-CD40 and IL-4 furthermore demonstrated that the diminished mIgE⁺ PC accumulation of mIgE-mutant cells occurred in the absence of a germinal center. Moreover, reduced PC accumulation was not dependent on antigen recognition by the BCR. These findings are consistent with two recent reports, showing that formation of IgE⁺ PCs can occur in an antigen-independent, mIgE-BCR-autonomous manner in *in vitro* cultures^{19, 20}. Even though the mechanistic details of such a mIgE-BCR autonomous differentiation signal are insufficiently understood, cell surface expression of mIgE is obviously required.

Unlike most other Ig isotypes, IgE-switched PCs show a very strong cell surface expression of mIgE⁹. Although it is generally believed that PCs downregulate

expression of the BCR during their differentiation from B cells, also mIgM⁺ and mIgA⁺ PCs have been identified in mice and humans³⁴⁻³⁶. Moreover, mIgM⁺ PCs have been shown to sense antigen and to secrete cytokines upon antigen recognition³¹. Therefore, it will be important to address the function of mIgE on PCs using defined model antigens and *in vivo* experiments in the future. However, here we showed that cell surface mIgE on PCs was indeed associated with CD79 and thus could serve as a functional BCR. Notably, PCs in mIgE-ITT-mutant mice expressed lower levels of the mIgE-BCR on their surface, while they produced similar amounts of secreted IgE. This finding suggests that the mIgE-ITT motif plays a role either in transporting the BCR to the cell surface or in reducing its internalization. The Hax-1 protein was initially identified as a protein involved in BCR internalization that could be immunoprecipitated together with the cytoplasmic tail of mIgE³⁷. However, Hax-1 does not bind the ITT motif but rather the membrane-proximal KVKWI(V)F motif of the mIgE tail³⁸. By removing the hydroxyl group necessary for phosphorylation of the ITT motif in mIgE-YF mice, we may have created an improved binding site for Hax-1, which may result in enhanced internalization of YF-mutant mIgE.

The phenotype of mIgE-KVKΔtail mice was consistently more pronounced than that of mIgE-YF mice and this correlated with a more severe reduction in mIgE-BCR surface expression in PCs, supporting the notion that cell surface expression of mIgE-BCRs on PCs is a key mechanism to control IgE antibody production. This idea is furthermore supported by additional recent reports. In a mouse model, in which all B cells were forced to express mIgE-BCRs during their development, inefficient surface expression of mIgE interfered with B cell maturation, a defect that could be rescued by ectopic expression of mIgE³⁹. In contrast to mouse mIgE-BCRs, human mIgE-BCRs can be expressed as splice variant that contains an additional 52 amino acid extracellular membrane proximal domain (EMPD) between the Cε4 and the M1

domains^{40, 41}. This extended EMPD causes retention of human mIgE BCRs in the endoplasmatic reticulum and limits their surface expression and signaling capacity⁴². When introduced into murine mIgE, the extended human EMPD caused a severe reduction of serum IgE antibodies in two independent mouse models^{43, 44}. Taken together, the available data from several independent studies indicate that surface expression of mIgE-BCRs is a mechanism by which the production of the potentially dangerous IgE antibody isotype can be restricted. Our data furthermore indicate that the cell type that is subject to regulation by the mIgE-BCR is the IgE-switched PC. Therefore, therapeutic strategies aiming at targeting mIgE⁺ PCs could prove useful for improving the conditions of patients suffering from severe allergic inflammation.

Acknowledgments

We thank G. Achatz-Straussberger for permission to work with IgE-KVK Δ tail mice and H. Oettgen for the IgE-deficient mice. We furthermore thank D. Döhler and K. Castiglione for technical assistance and members of the Voehringer lab and colleagues from the collaborative research center TRR130 for helpful comments.

References

1. Ogilvie BM, Smithers SR, Terry RJ. Reagin-like antibodies in experimental infections of *Schistosoma mansoni* and the passive transfer of resistance. *Nature* 1966; 209:1221-3.
2. Marichal T, Starkl P, Reber LL, Kalesnikoff J, Oettgen HC, Tsai M, et al. A beneficial role for immunoglobulin E in host defense against honeybee venom. *Immunity* 2013; 39:963-75.
3. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* 2008; 8:205-17.
4. Kraft S, Kinet JP. New developments in FcεRI regulation, function and inhibition. *Nat Rev Immunol* 2007; 7:365-78.
5. Gilfillan AM, Beaven MA. Regulation of mast cell responses in health and disease. *Crit Rev Immunol* 2011; 31:475-529.
6. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 2010; 125:S73-80.
7. Venkitaraman AR, Williams GT, Dariavach P, Neuberger MS. The B-cell antigen receptor of the five immunoglobulin classes. *Nature* 1991; 352:777-81.
8. Turqueti-Neves A, Otte M, Schwartz C, Schmitt ME, Lindner C, Pabst O, et al. The Extracellular Domains of IgG1 and T Cell-Derived IL-4/IL-13 Are Critical for the Polyclonal Memory IgE Response In Vivo. *PLoS Biol* 2015; 13:e1002290.
9. Yang Z, Sullivan BM, Allen CD. Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. *Immunity* 2012; 36:857-72.
10. Hirano T, Hom C, Ovary Z. Half-life of murine IgE antibodies in the mouse. *Int Arch Allergy Appl Immunol* 1983; 71:182-4.

11. Vieira P, Rajewsky KJEjoi. The half-lives of serum immunoglobulins in adult mice. 1988; 18:313-6.
12. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. J Allergy Clin Immunol 2012; 129:635-45.
13. Mandler R, Finkelman FD, Levine AD, Snapper CM. IL-4 induction of IgE class switching by lipopolysaccharide-activated murine B cells occurs predominantly through sequential switching. J Immunol 1993; 150:407-18.
14. Mills FC, Mitchell MP, Harindranath N, Max EE. Human Ig S gamma regions and their participation in sequential switching to IgE. J Immunol 1995; 155:3021-36.
15. He JS, Meyer-Hermann M, Xiangying D, Zuan LY, Jones LA, Ramakrishna L, et al. The distinctive germinal center phase of IgE+ B lymphocytes limits their contribution to the classical memory response. J Exp Med 2013; 210:2755-71.
16. Erazo A, Kutchukhidze N, Leung M, Christ AP, Urban JF, Jr., Curotto de Lafaille MA, et al. Unique maturation program of the IgE response in vivo. Immunity 2007; 26:191-203.
17. Xiong H, Dolpady J, Wabl M, de Lafaille MAC, Lafaille JJJJoeM. Sequential class switching is required for the generation of high affinity IgE antibodies. 2012; 209:353-64.
18. Laffleur B, Duchez S, Tarte K, Denis-Lagache N, Peron S, Carrion C, et al. Self-Restrained B Cells Arise following Membrane IgE Expression. Cell Rep 2015; 10:900-9.
19. Haniuda K, Fukao S, Kodama T, Hasegawa H, Kitamura D. Autonomous membrane IgE signaling prevents IgE-memory formation. Nat Immunol 2016; 17:1109-17.

20. Yang Z, Robinson MJ, Chen X, Smith GA, Taunton J, Liu W, et al. Regulation of B cell fate by chronic activity of the IgE B cell receptor. *Elife* 2016; 5.
21. Mitre E, Nutman TB. IgE memory: persistence of antigen-specific IgE responses years after treatment of human filarial infections. *J Allergy Clin Immunol* 2006; 117:939-45.
22. Otte M, Mahler V, Kerppe A, Pabst O, Voehringer D. Persistence of the IgE repertoire in birch pollen allergy. *J Allergy Clin Immunol* 2016; 137:1884-7 e8.
23. Achatz G, Nitschke L, Lamers MC. Effect of transmembrane and cytoplasmic domains of IgE on the IgE response. *Science* 1997; 276:409-11.
24. He JS, Subramaniam S, Narang V, Srinivasan K, Saunders SP, Carbajo D, et al. IgG1 memory B cells keep the memory of IgE responses. *Nat Commun* 2017; 8:641.
25. Lutz J, Dittmann K, Bosl MR, Winkler TH, Wienands J, Engels N. Reactivation of IgG-switched memory B cells by BCR-intrinsic signal amplification promotes IgG antibody production. *Nat Commun* 2015; 6:8575.
26. Engels N, König LM, Heemann C, Lutz J, Tsubata T, Griep S, et al. Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched B cells. *Nat Immunol* 2009; 10:1018-25.
27. Engels N, König LM, Schulze W, Radtke D, Vanshylla K, Lutz J, et al. The immunoglobulin tail tyrosine motif upgrades memory-type BCRs by incorporating a Grb2-Btk signalling module. *Nat Commun* 2014; 5:5456.
28. Vanshylla K, Bartsch C, Hitzing C, Krumpelmann L, Wienands J, Engels N. Grb2 and GRAP connect the B cell antigen receptor to Erk MAP kinase activation in human B cells. *Sci Rep* 2018; 8:4244.

- 551 29. Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P.
552 Active anaphylaxis in IgE-deficient mice. *Nature* 1994; 370:367-70.
- 553 30. Kaisho T, Schwenk F, Rajewsky K. The roles of gamma 1 heavy chain
554 membrane expression and cytoplasmic tail in IgG1 responses. *Science* 1997;
555 276:412-5.
- 556 31. Blanc P, Moro-Sibilot L, Barthly L, Jagot F, This S, de Bernard S, et al. Mature
557 IgM-expressing plasma cells sense antigen and develop competence for
558 cytokine production upon antigenic challenge. *Nat Commun* 2016; 7:13600.
- 559 32. Khodoun MV, Strait R, Armstrong L, Yanase N, Finkelman FD. Identification of
560 markers that distinguish IgE- from IgG-mediated anaphylaxis. *Proc Natl Acad*
561 *Sci U S A* 2011; 108:12413-8.
- 562 33. Wienands J, Engels N. The Memory Function of the B Cell Antigen Receptor.
563 *Curr Top Microbiol Immunol* 2016; 393:107-21.
- 564 34. Racine R, McLaughlin M, Jones DD, Wittmer ST, MacNamara KC, Woodland
565 DL, et al. IgM production by bone marrow plasmablasts contributes to long-
566 term protection against intracellular bacterial infection. *J Immunol* 2011;
567 186:1011-21.
- 568 35. Reynolds AE, Kuraoka M, Kelsoe G. Natural IgM is produced by CD5- plasma
569 cells that occupy a distinct survival niche in bone marrow. *J Immunol* 2015;
570 194:231-42.
- 571 36. Pinto D, Montani E, Bolli M, Garavaglia G, Sallusto F, Lanzavecchia A, et al. A
572 functional BCR in human IgA and IgM plasma cells. *Blood* 2013; 121:4110-4.
- 573 37. Oberndorfer I, Schmid D, Geisberger R, Achatz-Straussberger G, Cramer R,
574 Lamers M, et al. HS1-associated protein X-1 interacts with membrane-bound
575 IgE: impact on receptor-mediated internalization. *J Immunol* 2006; 177:1139-
576 45.

38. Wolkerstorfer S, Schwaiger E, Rinnerthaler M, Karina Gratz I, Zoegg T, Brandstetter H, et al. HAX1 deletion impairs BCR internalization and leads to delayed BCR-mediated apoptosis. *Cell Mol Immunol* 2016; 13:451-61.
39. Tong P, Granato A, Zuo T, Chaudhary N, Zuiani A, Han SS, et al. IgH isotype-specific B cell receptor expression influences B cell fate. *Proc Natl Acad Sci U S A* 2017; 114:E8411-E20.
40. Peng C, Davis FM, Sun LK, Liou RS, Kim YW, Chang TW. A new isoform of human membrane-bound IgE. *J Immunol* 1992; 148:129-36.
41. Zhang K, Saxon A, Max EE. Two unusual forms of human immunoglobulin E encoded by alternative RNA splicing of epsilon heavy chain membrane exons. *J Exp Med* 1992; 176:233-43.
42. Vanshylla K, Opazo F, Gronke K, Wienands J, Engels N. The extracellular membrane-proximal domain of membrane-bound IgE restricts B cell activation by limiting B cell antigen receptor surface expression. *Eur J Immunol* 2018; 48:441-53.
43. Talay O, Yan D, Brightbill HD, Straney EE, Zhou M, Ladi E, et al. IgE(+) memory B cells and plasma cells generated through a germinal-center pathway. *Nat Immunol* 2012; 13:396-404.
44. Vigl B, Salhat N, Parth M, Pankevych H, Mairhofer A, Bartl S, et al. Quantitative in vitro and in vivo models to assess human IgE B cell receptor crosslinking by IgE and EMPD IgE targeting antibodies. *J Immunol Methods* 2017; 449:28-36.

Author contributions

M.E.R.S. and D.V. designed experiments; M.E.R.S. and P.H. performed experiments and generated figures; J.L., M.R.B., J.W. and N.E. constructed and provided mIgG1-YF and mIgE-YF mice; M.E.R.S., P.H., D.V. and N.E. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Figure legends

Fig. 1. The mIgE-ITT motif promotes the memory IgE response upon helminth infection.

(a) Frequency of GC B cells (B220⁺CD49b⁻CD38^{lo}GL7⁺), PCs (B220⁻CD138⁺TACI⁺) and IgG1 and IgE from GC B cells and PCs on day 10 after 1st *N. brasiliensis* infection gated as indicated in Fig. E2 in the Online Repository. **(b and c)** Mean+SEM percentage of GC B cells **(b, upper)**, IgE⁺ GC cells **(b, lower)**, PCs **(c, upper)** and IgE⁺ PCs **(c, lower)** before and after 1st or 2nd infection with 4-26 mice per group from 1-6 pooled experiments. Diamonds indicate individual data points. * $P < 0.05$ by Student's t-test, # $P < 0.05$ by Mann-Whitney U-test. **(d)** Mean+SEM IgG1 and IgE serum levels from WT (black circle), mIgE-YF (light grey triangle), and mIgE-KVKΔtail (dark grey square) mice with 4-28 mice per group from 1-4 pooled experiments. **(e)** Mean+SEM serum level of *N. brasiliensis*-specific IgE with 4-6 mice per group. # $P < 0.05$ between WT and mIgE-KVKΔtail by Mann-Whitney U-test, § $P < 0.05$ between WT and mIgE-YF by Mann-Whitney U-test.

Fig. 2. The mIgG1-ITT motif promotes the IgG1 but not the IgE immune response.

(a) Frequency of GC B cells (B220⁺CD49b⁻CD38^{lo}GL7⁺), PCs (B220⁻CD138⁺TACI⁺) and IgG1 and IgE from GC B cells and PCs on day 10 after 2nd infection gated as indicated in Fig E2 in the Online Repository. (b) Mean+SEM percentages of indicated populations before and after 1st or 2nd infection with 3-16 mice per group from 1-4 pooled experiments. (c) Mean+SEM IgG1 and IgE serum levels with 8-26 mice per group from 2 (IgG1) or 4 (IgE) pooled experiments. (d) Mean+SEM serum level of *N. brasiliensis*-specific IgE with 3-6 mice per group. Diamonds indicate individual data points. * P < 0.05 by Student's t-test, # P < 0.05 by Mann-Whitney U-test.

Fig. 3. The mIgE-ITT motif enhances IgE⁺ PC accumulation in an antigen-independent *in vitro* system.

In vitro differentiation of PCs from isolated primary B cells on day 4 after stimulation with anti-CD40 and IL-4. (a) Frequency of PCs (TACI⁺CD138⁺ of B220⁻ gated cells) and intracellular staining for IgG1 and IgE in gated PCs. (b and c) Mean+SEM percentages of IgE⁺ PCs (b) and IgG1⁺ PCs (c) with 3 mice per group from 3 pooled experiments. Diamonds indicate individual data points. Indicated P values were calculated by Student's t-test.

Fig. 4. IgE⁺ PCs require the mIgE-ITT motif for mIgE expression and accumulation upon helminth infection *in vivo*.

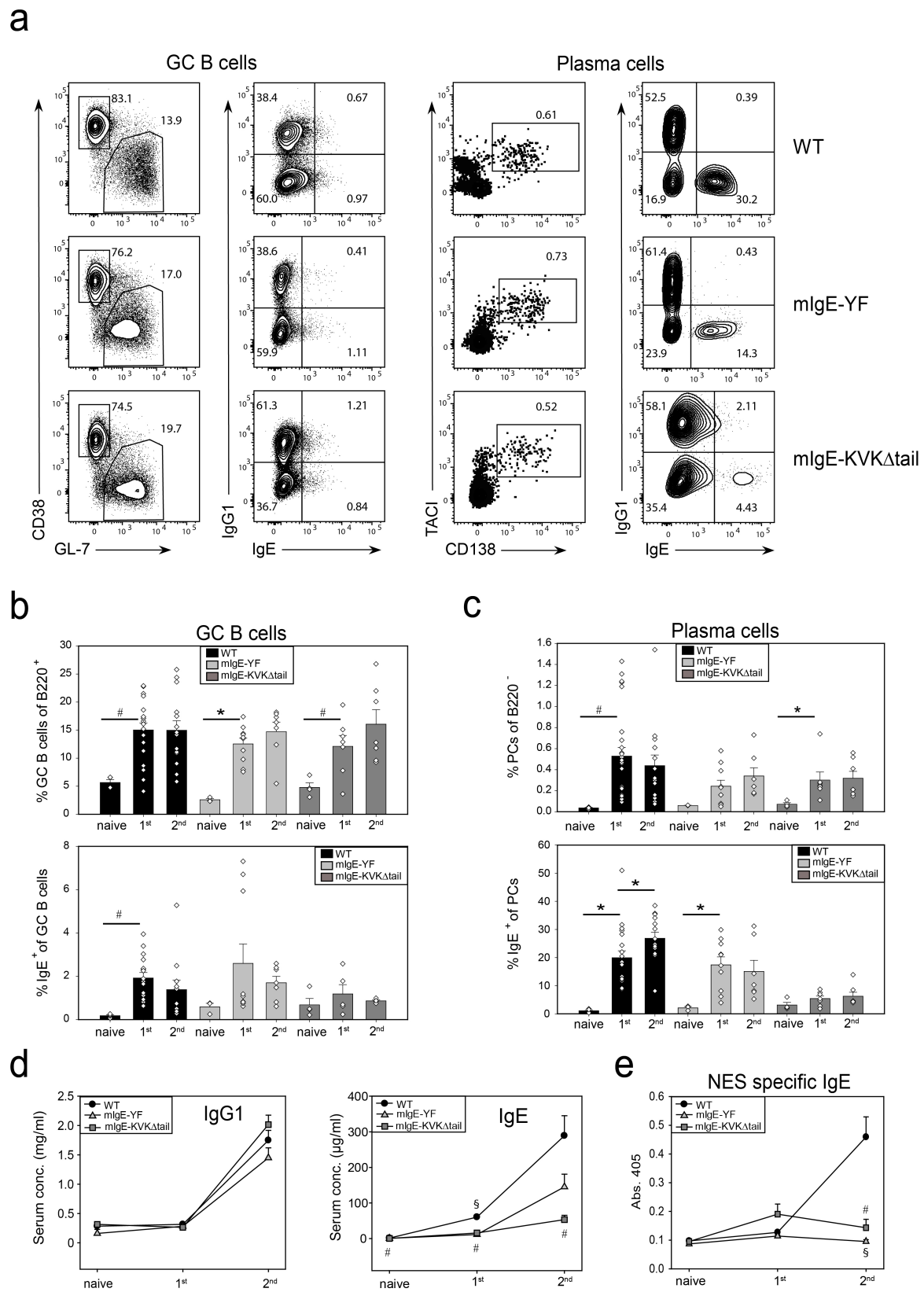
(a) Expression of surface IgG1 (mIgG1) and IgE (mIgE) on PCs from mesenteric lymph nodes of indicated mice on day 10 after 1st *N. brasiliensis* infection. Histograms show the expression of CD79b on gated mIgE⁺ PCs. (b) Mean+SEM

percentage of mIgE⁺ PCs from 7-9 mice per group from 2 pooled experiments. (c and d) Mean+SEM MFI for mIgE (c) and total IgE (surface and intracellular IgE, d) on gated PCs after 1st infection with 5-14 mice per group of 2-4 pooled experiments. *P < 0.05 by Student's t-test, #P < 0.05 by Mann-Whitney U-test. Diamonds indicate individual data points.

Fig. 5. Reduced mast cell activation in mIgE-mutant mice in an active systemic anaphylaxis model.

Active systemic anaphylaxis was induced in BALB/c (WT), mIgE-YF, mIgE-KVKΔtail, and IgE-deficient mice as described in Materials and Methods. (a) Total serum IgE levels. (b) Mean+SD frequency of IgE-sensitized basophils in the blood from 4-5 mice per group of one experiment. *P < 0.05 by Student's t-test. (c) Mean+SEM mMcp1 serum levels of indicated mice from 8-9 mice per group of 2 pooled experiments. *P < 0.05 by Student's t-test, #P < 0.05 by Mann-Whitney U-test. Diamonds indicate individual data points.

Figure 1



Journal Pre-proof

Figure 2

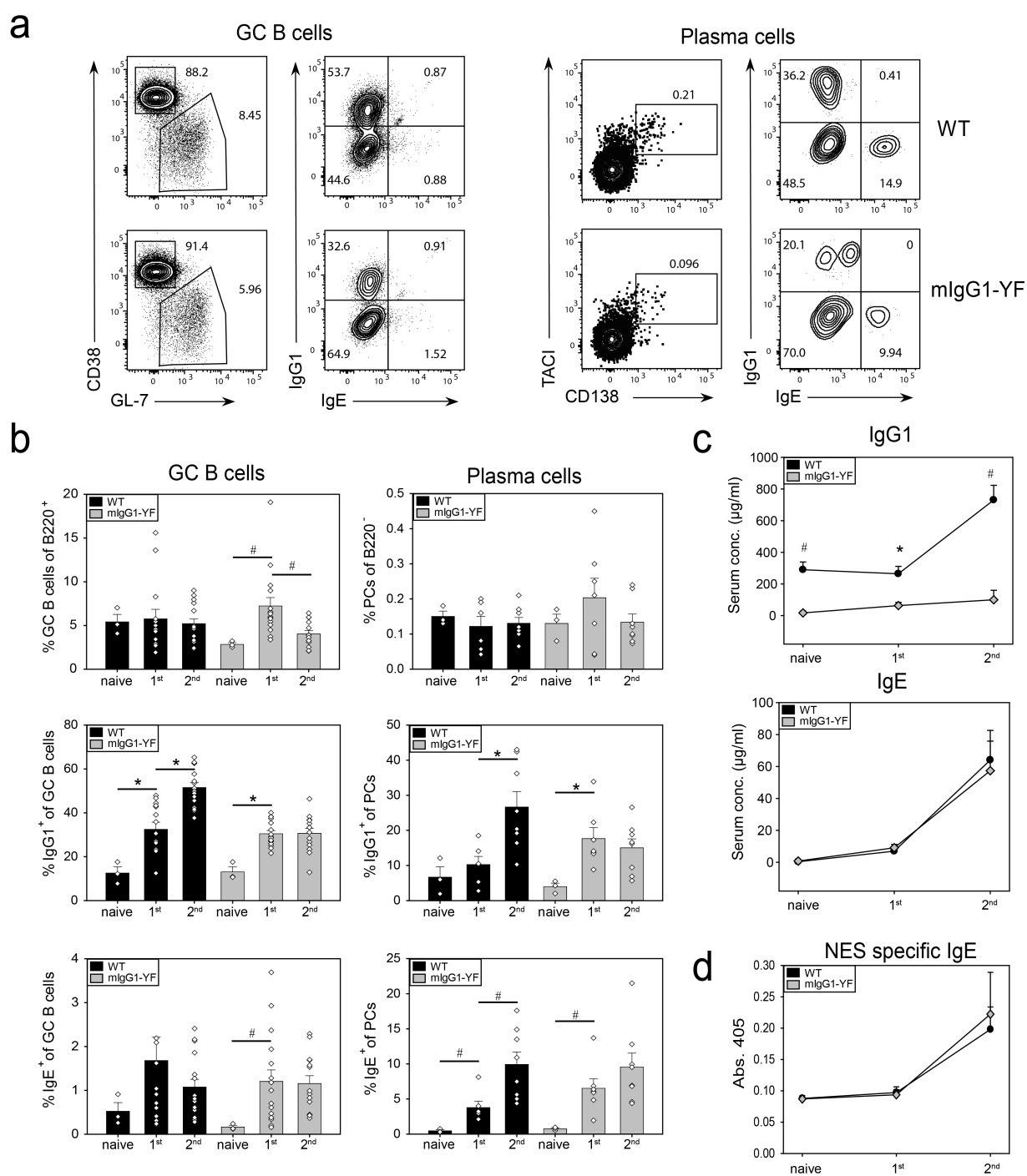


Figure 3

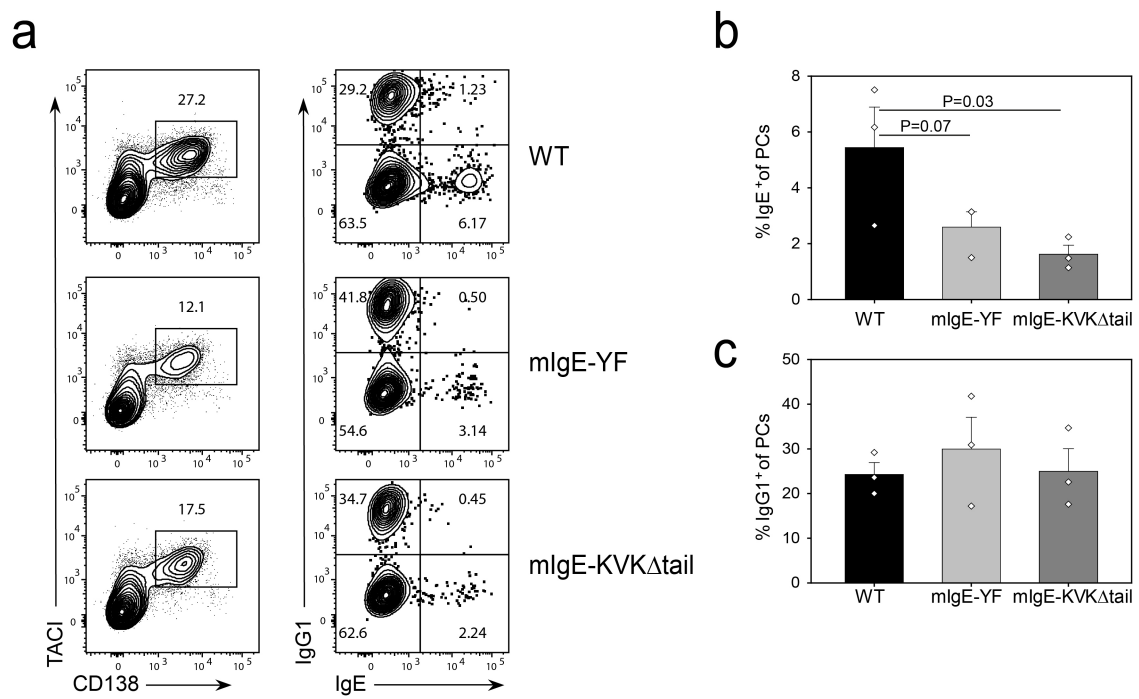


Figure 4

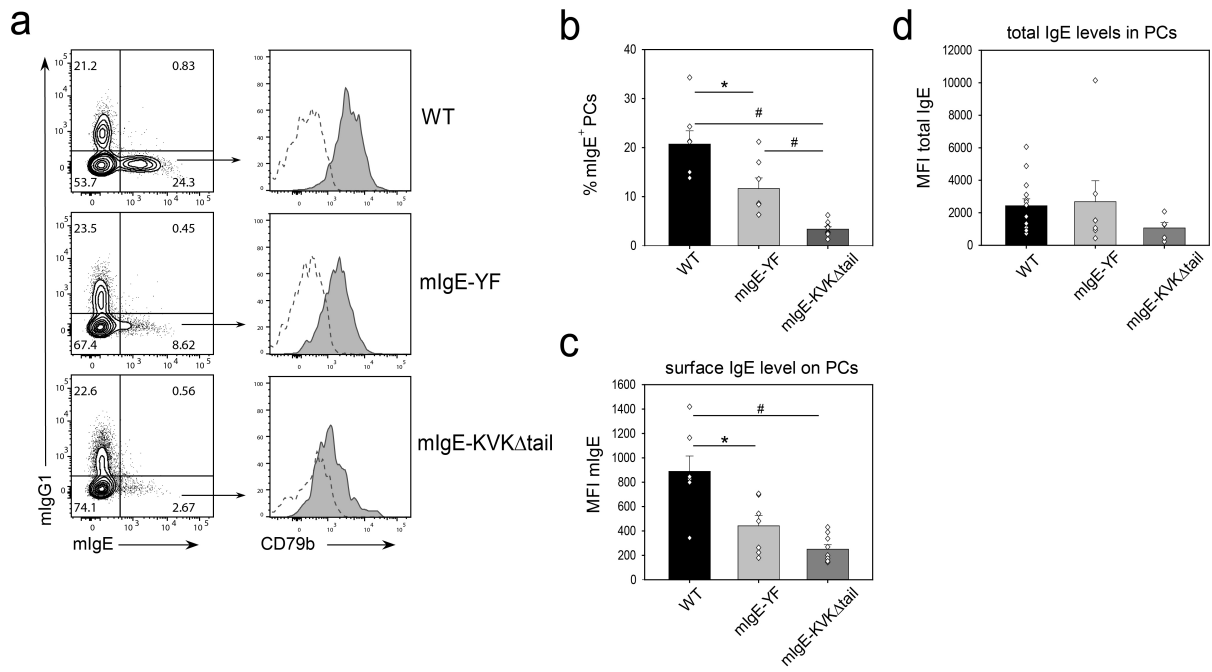
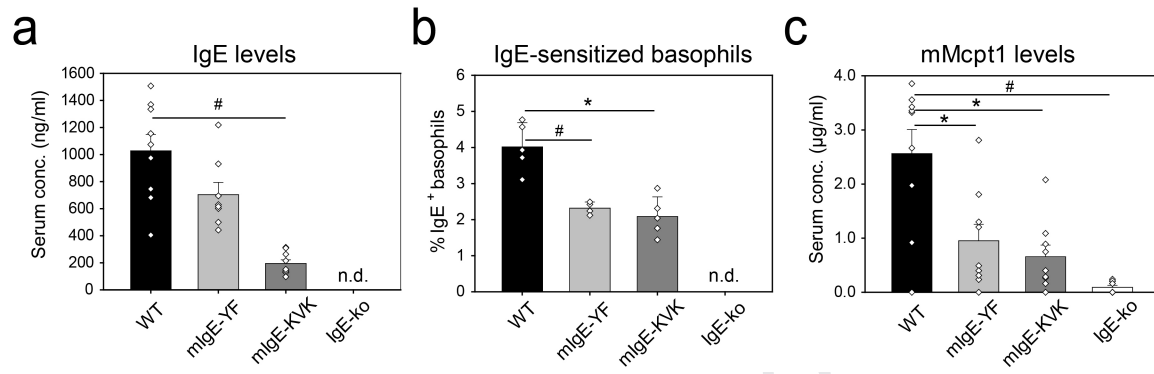


Figure 5



Online repository Figure legends

Figure E1. Generation of mIgE-YF mice. (a) Targeting strategy. Upper panel: Schematic organization of the murine ϵ immunoglobulin locus. A point mutation was introduced in the ϵ M2 exon leading to a tyrosin-to-phenylalanine (YF) substitution in the cytoplasmic domain of membrane-bound ϵ . The targeting vector was integrated into BALB/c I ES cells (middle panel) and the neomycin resistance cassette was deleted by transfection of a Cre expression plasmid before injection into blastocysts (lower panel). (b) Genomic DNA sequence of targeted region. Nucleotides that were introduced into the mouse genome by gene targeting are indicated in upper case italics. The remaining loxP site is indicated in green. The coding region of the manipulated M2 exon is depicted in blue, the introduced point mutation in red, and the two polyadenylation sites of the membrane form in purple. (c) Sequencing chromatogram showing the ITT-inactivating mutation in the mouse genome. (d) Amino acid sequences of wild type and ITT YF-mutant cytoplasmic domains of mouse ϵ m. (e) Amino acid sequences of cytoplasmic tails from mouse and human mIgE. The yellow box indicates the ITT motif.

Figure E2. Gating strategy to detect germinal center (GC) B cells and plasma cells (PC). (a) Single cell suspensions from mesenteric lymph nodes of wild-type (WT) and IgE-deficient (IgE ko) mice on day 10 after 2nd *N. brasiliensis* infection were first gated on SSC^{lo} cells, then doublets and dead cells were excluded, before gating on B cells (B220⁺CD49b⁻) with a GC phenotype (CD38⁻GL-7⁺) and intracellular detection of IgG1 and IgE. (b) PCs were gated from a large FSC/SSC gate, followed by doublet, dead cell and B cell (B220⁺) exclusion. From there, the PCs were gated

as CD138⁺TACI⁺ cells and intracellular staining was performed to detect IgE and IgG1.

Figure E3. Total number of IgE⁺ GC B cells and plasma cells.

BALB/c (WT), mIgE-YF and mIgE-KVK Δ tail mice were infected with *N. brasiliensis* and mesenteric lymph nodes and serum were analyzed on day 10 after 1st or 2nd infection. Bars show the mean+SEM total number of IgE⁺ GC B cells (**a**) and IgE⁺ PCs (**b**) from WT (black), mIgE-YF (light grey), and mIgE-KVK Δ tail (dark grey) mice before and after 1st or 2nd infection with 2-14 mice per group from 1-6 pooled experiments. Diamonds represent individual data points. * P < 0.05 by Student's t-test, [#] P < 0.05 by Mann-Whitney U-test.

Figure E4. Total number of IgG1⁺ and IgE⁺ GC B cells and plasma cells.

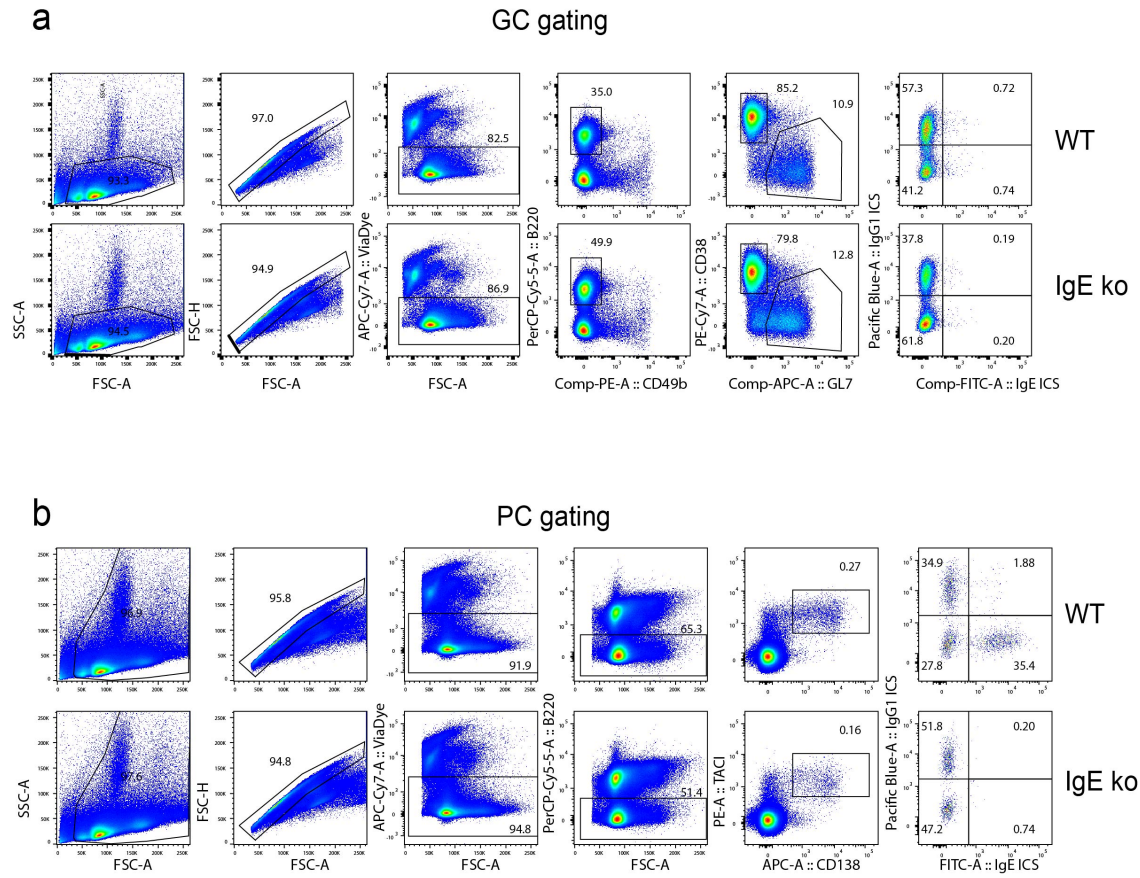
C57BL/6 (WT) and mIgG1-YF mice were infected with *N. brasiliensis* and mesenteric lymph nodes and serum were analyzed on day 10 after 1st or 2nd infection. Bars show mean+SEM total number of indicated populations from WT (black) and mIgG1-YF (grey) mice before and after 1st or 2nd infection with 3-16 mice per group from 1-4 pooled experiments. Diamonds represent individual data points. * P < 0.05 by Student's t-test, [#] P < 0.05 by Mann-Whitney U-test.

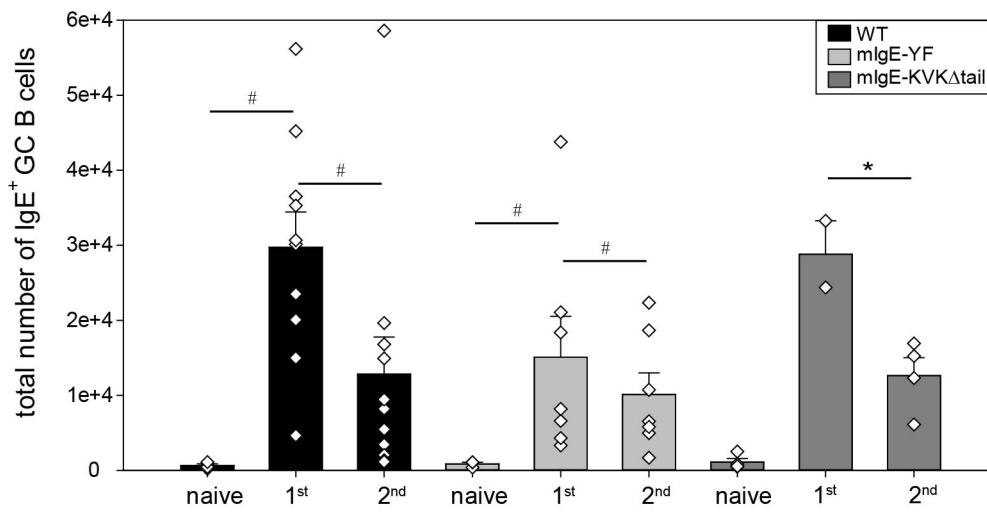
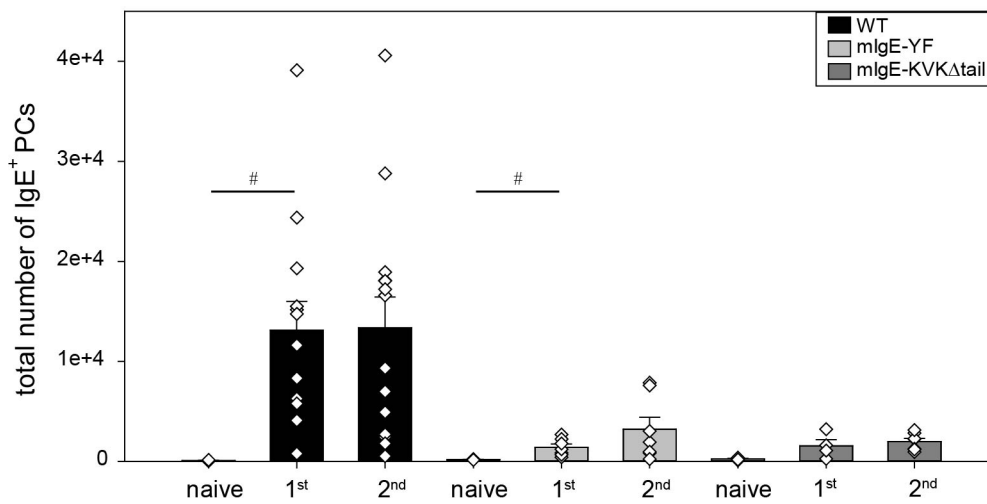
Figure E5. Double staining for surface and intracellular IgG1 and IgE on plasma cells.

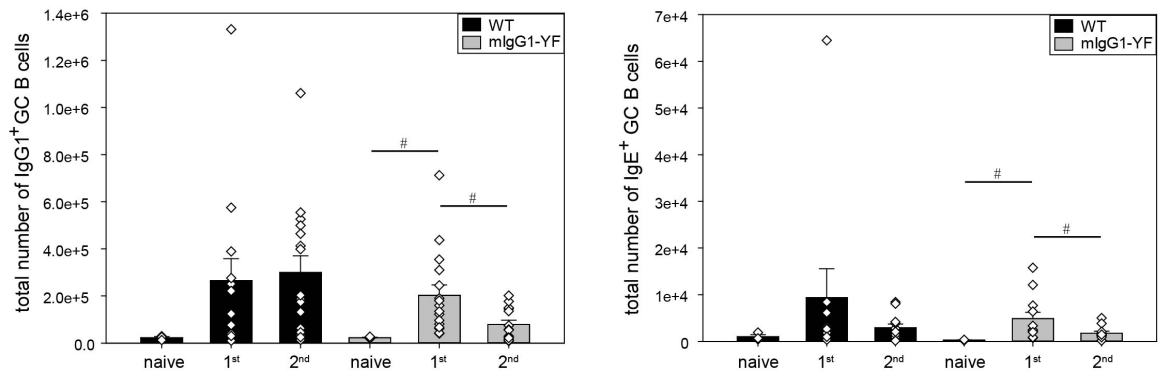
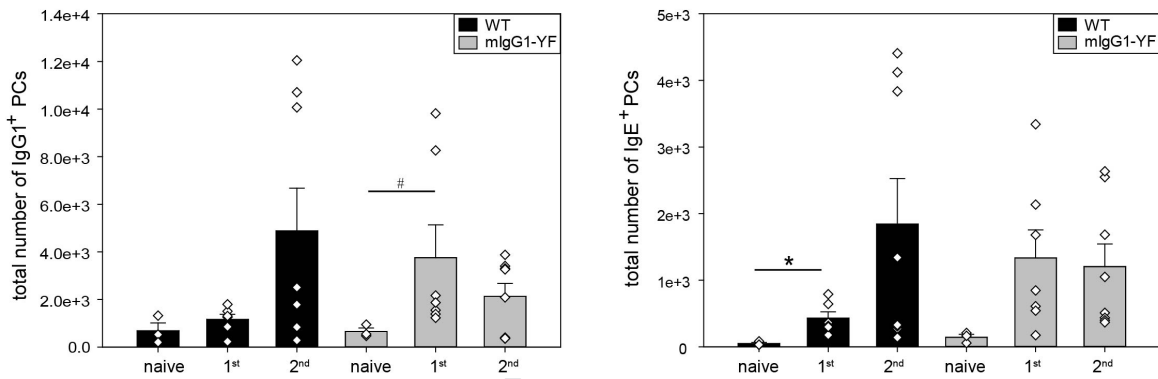
BALB/c mice were infected with *N. brasiliensis* and the mesenteric lymph nodes analyzed on day 10 after infection. Cells were stained for surface and intracellular IgG1 (**a**) and IgE (**b**) with same respective antibody clones but different fluorescent

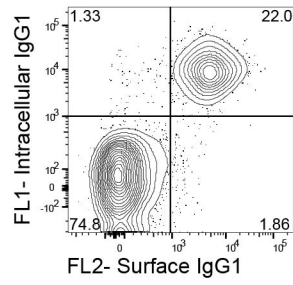
markers. For staining 2 the fluorescent markers for surface and intracellular were switched. Contour plots show plasma cells (B220⁻CD138⁺TACI⁺) were gated as indicated in Fig. E2 in the Online Repository. **(a)** FL1: V450; FL2: PE CF594. **(b)** FL1: FITC; FL2: BB700.

Mouse mIgE-tail: ...KVKWVFSTPMQDTPQTFQDYANILQTRA
Human mIgE-tail: ...MVORFLSATROGRBPOTSLDYTNVLOPHA

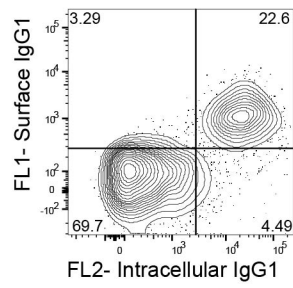


a**b**

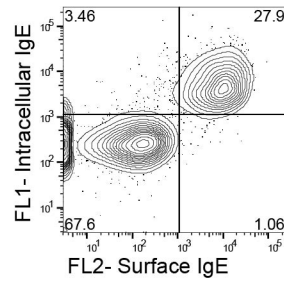
a**b**

a

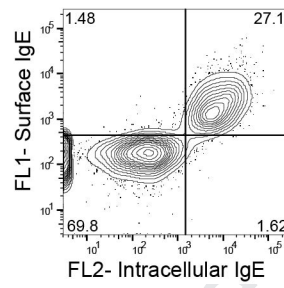
Staining 1



Staining 2

b

Staining 1



Staining 2