

IgE⁺ memory B cells and plasma cells generated through a germinal-center pathway

Oezcan Talay¹, Donghong Yan², Hans D Brightbill¹, Elizabeth E M Straney³, Meijuan Zhou², Ena Ladi¹, Wyne P Lee², Jackson G Egen¹, Cary D Austin³, Min Xu² & Lawren C Wu¹

Immunoglobulin E (IgE) antibodies are pathogenic in asthma and allergic diseases, but the *in vivo* biology of IgE-producing (IgE⁺) cells is poorly understood. A model of the differentiation of IgE⁺ B cells proposes that IgE⁺ cells develop through a germinal-center IgG1⁺ intermediate and that IgE memory resides in the compartment of IgG1⁺ memory B cells. Here we have used a reporter mouse expressing green fluorescent protein associated with membrane IgE transcripts (IgE-GFP) to assess *in vivo* IgE responses. In contrast to the IgG1-centered model of IgE switching and memory, we found that IgE⁺ cells developed through a germinal-center IgE⁺ intermediate to form IgE⁺ memory B cells and plasma cells. Our studies delineate a new model for the *in vivo* biology of IgE switching and memory.

Despite the importance of immunoglobulin E (IgE) in hypersensitivity reactions and the pathogenesis of asthma and allergic diseases^{1–4}, the *in vivo* biology of IgE-producing (IgE⁺) B cells and plasma cells, including the pathway by which IgE⁺ B cells differentiate from naive B cells and the nature of IgE memory, remains poorly understood. Two challenges for the study of IgE⁺ cells *in vivo* are a low cellular frequency and a lack of reagents with which to directly detect IgE-switched B cells. The present method for the detection of IgE-switched cells, which may not be completely specific for IgE⁺ cells, uses an indirect two-step process consisting of acid treatment of cells to remove serum IgE bound to CD23 (the low-affinity receptor for IgE) on all B cells, followed by detection of the membrane B cell antigen receptor form of IgE with antibody to IgE (anti-IgE)^{5,6}.

The generation of isotype-switched B cells and plasma cells has been well studied for IgG1 (refs 7–12); IgG1 and IgE constitute the main immunoglobulin isotypes formed during T helper type 2 responses. T cell-dependent IgG1 B cell responses develop in germinal centers, which are follicular structures in secondary lymphoid organs that are sites of B cell isotype-switch recombination, somatic hypermutation and affinity maturation. The products of germinal centers include isotype-switched IgG1⁺ memory B cells and long-lived IgG1⁺ plasma cells that migrate to the bone marrow and contribute to cellular IgG1 memory and serological IgG1 memory, respectively.

In contrast to studies of IgG1, few studies have assessed the location of IgE switching, the location and kinetics of IgE plasma-cell responses and the existence of IgE⁺ memory B cells^{5,8,13,14}. IgE-switched cells have been studied *in vivo* and *ex vivo* either by immunization of mice bearing monoclonal T cells and B cells or by infection of wild-type mice with the helminth *Nippostrongylus brasiliensis* to generate a high frequency of IgE-switched cells¹⁴. By acid treatment

and staining with anti-IgE, that study found IgE⁺ cells located largely outside of germinal centers, with the phenotypic characteristics of plasma cells¹⁴. It was therefore proposed that IgE⁺ cells have a unique differentiation program distinct from that of IgG1⁺ cells that consists of isotype switching to a germinal-center IgG1⁺ B cell intermediate, followed by rapid switching and differentiation to IgE⁺ plasma cells outside germinal centers. In addition, it was proposed that cellular IgE memory resides mainly in IgG1⁺ memory B cells that undergo a sequential switch to IgE after reexposure to antigen.

Here we use a reporter mouse in which a bicistronic reporter gene encoding enhanced green fluorescent protein (called 'GFP' here) is inserted into the mouse locus encoding IgE just downstream of the M2 exon that encodes the cytoplasmic domain of membrane IgE (IgE-GFP reporter mice)¹⁵. Like other immunoglobulin isotypes, IgE is present in two forms: a secreted serum immunoglobulin form, and a membrane B cell antigen receptor form encoded by distinct mRNA splice variants. The GFP reporter is associated only with membrane IgE. In addition to the reporter gene encoding GFP, these mice also have sequence encoding an extracellular 52-amino acid region (M1') inserted at the 5' end of the M1 exon that encodes the transmembrane domain of mouse membrane IgE. M1' is found in human membrane IgE but not mouse membrane IgE^{16–19} and is inserted in the reporter mice at a position in mouse membrane IgE similar to that in human IgE, which thereby enables specific detection and targeting of IgE-switched cells with anti-M1'. As reported before, the reporter mice are normal, healthy and fertile, with no alterations in hematopoietic compartments, and have basal and immunization-induced immunoglobulin concentrations for all isotypes, including IgE, similar to those of wild-type mice¹⁵. Here we investigate IgE switching and memory in these IgE-GFP reporter mice.

¹Department of Immunology, Genentech, South San Francisco, California, USA. ²Department of Translational Immunology, Genentech, South San Francisco, California, USA. ³Department of Pathology, Genentech, South San Francisco, California, USA. Correspondence should be addressed to L.C.W. (lawren@gene.com).

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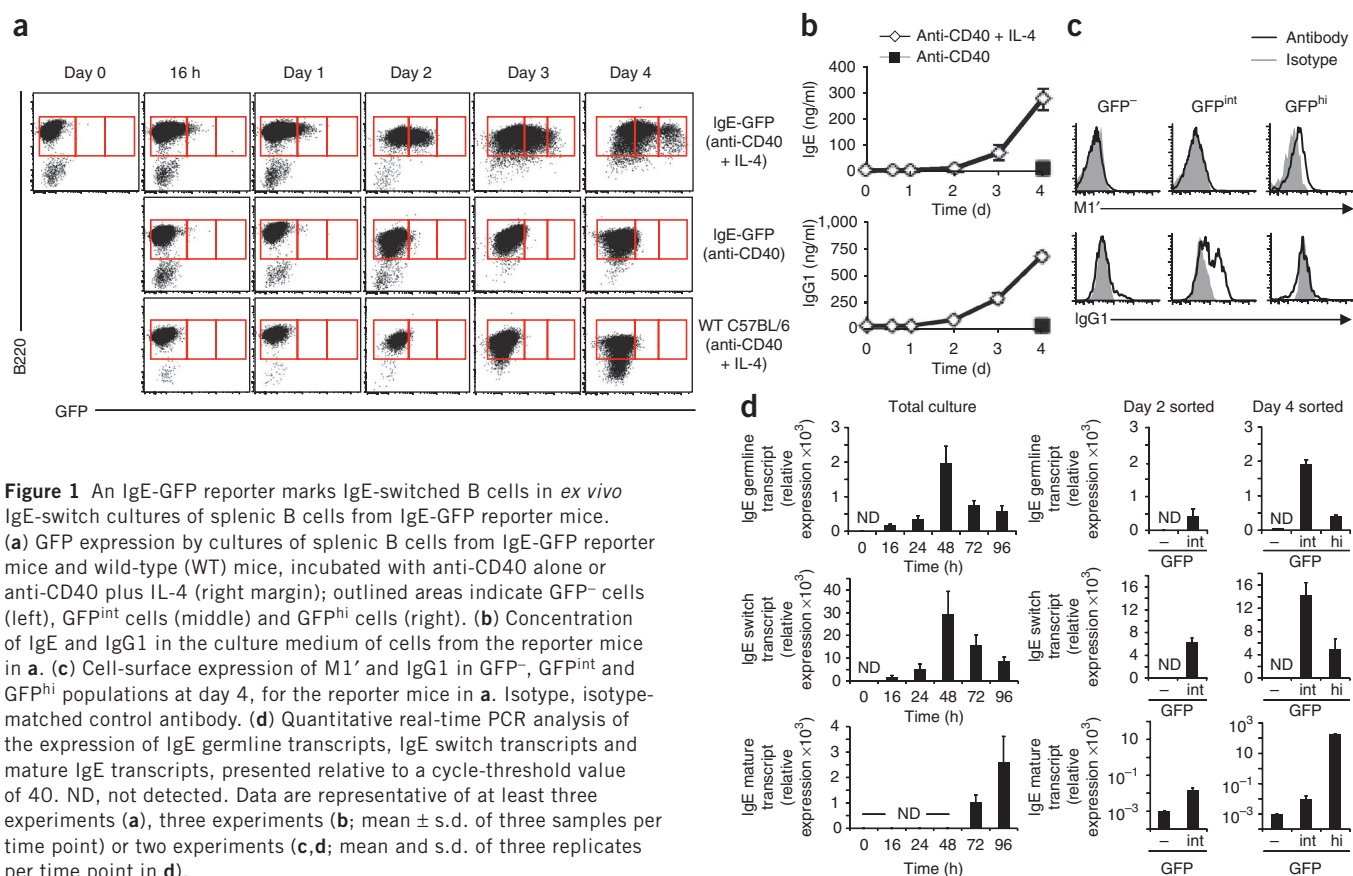


Figure 1 An IgE-GFP reporter marks IgE-switched B cells in *ex vivo* IgE-switch cultures of splenic B cells from IgE-GFP reporter mice. (a) GFP expression by cultures of splenic B cells from IgE-GFP reporter mice and wild-type (WT) mice, incubated with anti-CD40 alone or anti-CD40 plus IL-4 (right margin); outlined areas indicate GFP⁻ cells (left), GFP^{int} cells (middle) and GFP^{hi} cells (right). (b) Concentration of IgE and IgG1 in the culture medium of cells from the reporter mice in a. (c) Cell-surface expression of M1' and IgG1 in GFP⁻, GFP^{int} and GFP^{hi} populations at day 4, for the reporter mice in a. Isotype, isotype-matched control antibody. (d) Quantitative real-time PCR analysis of the expression of IgE germline transcripts, IgE switch transcripts and mature IgE transcripts, presented relative to a cycle-threshold value of 40. ND, not detected. Data are representative of at least three experiments (a), three experiments (b); mean ± s.d. of three samples per time point) or two experiments (c,d; mean and s.d. of three replicates per time point in d).

RESULTS

IgE-switched B cells are GFP^{hi}

We used *in vitro* IgE-switch cultures to assess the ability of the IgE-GFP reporter to mark IgE-switched B cells. We purified splenic B cells from the IgE-GFP reporter mice and stimulated the cells with anti-CD40 and interleukin 4 (IL-4) to promote switching to IgE, then analyzed the cells by flow cytometry. Control cultures consisted of IgE-GFP splenic B cells stimulated with anti-CD40 alone (without IL-4) and wild-type mouse splenic B cells stimulated with anti-CD40 plus IL-4. Stimulation of IgE-GFP B cells with anti-CD40 plus IL-4 induced a GFP^{int} population as early as 16 h and a GFP^{hi} population starting at day 3 (Fig. 1a). We did not detect GFP^{int} or GFP^{hi} B cells in either of the control cultures. The appearance of GFP^{hi} B cells coincided with the appearance of IgE in the cell culture medium, and GFP^{hi} cells from 4-day switch cultures were positive for intracellular IgE but not intracellular IgG1 (Fig. 1b and Supplementary Fig. 1a), which suggested that the GFP^{hi} B cells were IgE-switched B cells. Consistent with that, in 4-day switch cultures GFP^{hi} B cells expressed membrane IgE, but GFP^{int} B cells did not, as detected by staining with anti-M1' (Fig. 1c), and staining of M1' on GFP^{hi} cells with anti-M1' was blocked by preincubation of anti-M1' with a soluble M1'-containing protein (Supplementary Fig. 1b).

The kinetics of the appearance of GFP^{int} cells suggested that they may have been cells that expressed the GFP reporter from IgE germline and switch transcripts. We confirmed this by RT-PCR analysis of IgE germline transcripts, IgE switch transcripts and an arbitrary mature IgE transcript at various times during the switch culture. We detected IgE germline and switch transcripts as early as 16 h and did not detect the mature IgE transcripts until day 3 (Fig. 1d and Supplementary Fig. 2).

We also sorted GFP⁻, GFP^{int} and GFP^{hi} cells from 2-day and 4-day switch cultures and assessed the various IgE transcripts in these sorted cells. Only GFP^{hi} cells had substantial expression of the mature IgE transcript, whereas GFP^{int} cells expressed IgE germline and switch transcripts (Fig. 1d). Some GFP^{int} cells from 4-day switch cultures expressed membrane IgG1 (Fig. 1c), which suggested that these were IgG1⁺ B cells with low expression of GFP from IgE germline and/or switch transcripts from the nonproductive heavy-chain allele. Consistent with that, some GFP^{int-neg} cells from 4-day switch cultures were positive for intracellular IgG1 but not intracellular IgE (Supplementary Fig. 1a). Thus, the IgE-switched cells in the IgE-GFP reporter mice were GFP^{hi} cells, whereas GFP^{int-neg} cells were not IgE-switched cells and included other immunoglobulin isotypes such as IgG1.

In vivo IgE⁺ germinal-center and memory B cells

Infection of mice with the helminth *N. brasiliensis* results in a strong T helper type 2 response associated with the robust generation of serum IgE and IgG1 (refs. 5,20,21). In wild-type C57BL/6 mice, the peak IgE response occurred around 15 d after infection, with a subsequent decrease in IgE to approximately 30–50% of the peak by 25 d after infection, which was sustained for at least 50 d (Supplementary Fig. 3a). A second infection of mice with *N. brasiliensis* at day 28, after steady-state concentrations of serum IgE had been achieved, resulted in a more rapid and slightly larger IgE response than that of the primary infection, with IgE peaking around 10 d after the secondary infection before decreasing to approximately 30–50% of the peak by 40 d after secondary infection (Supplementary Fig. 3b). These characteristics of the secondary IgE response (faster and stronger than the primary response) are indicative of a memory B cell response.

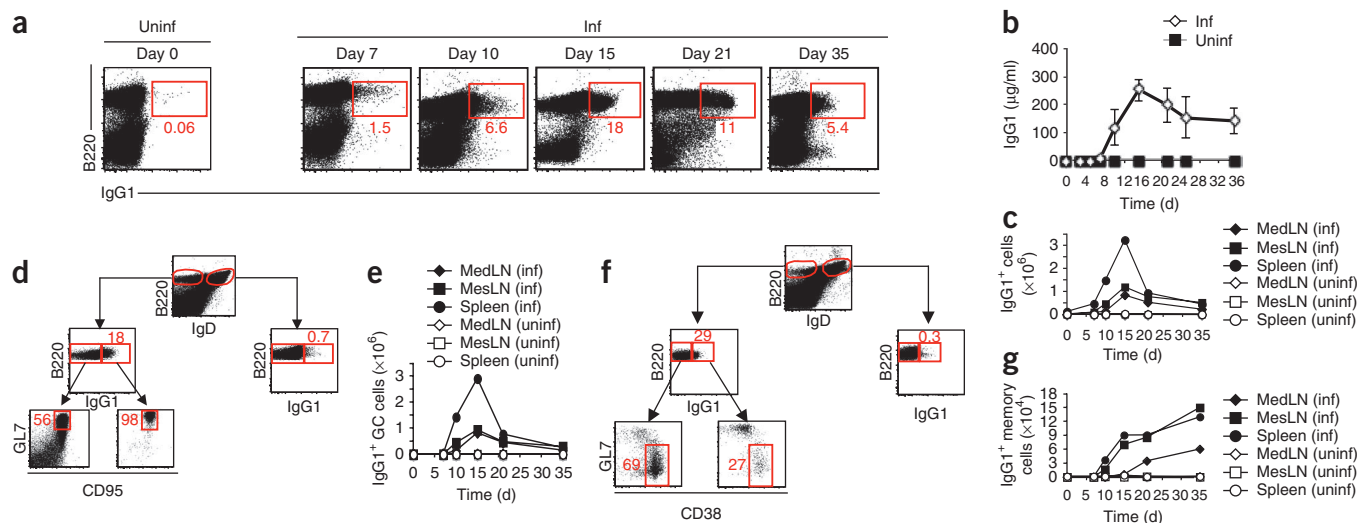


Figure 2 *In vivo* generation of IgG1-switched B cells after infection of IgE-GFP reporter mice with *N. brasiliensis*. (a) Flow cytometry profiles of IgG1⁺ B cells in the mediastinal lymph nodes of mice left uninfected (Uninf) or infected (Inf) with *N. brasiliensis*. Numbers adjacent to outlined areas indicate percent cells in each throughout. (b) Kinetics of serum IgG1 concentrations in mice left uninfected or infected with *N. brasiliensis*. (c) Number and kinetics of IgG1⁺ B cells in the mediastinal lymph nodes (MedLN), mesenteric lymph nodes (MesLN) and spleens of mice left uninfected or infected with *N. brasiliensis*. (d) Flow cytometry of germinal-center IgG1⁺ (B220⁺IgD⁻GL7⁺CD95⁺IgG1⁺) B cells in the mediastinal lymph nodes of mice at day 15 after infection with *N. brasiliensis*. (e) Number and kinetics of germinal-center (GC) IgG1⁺ B cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. (f) Flow cytometry of IgG1⁺ (B220⁺IgD⁻GL7⁻CD38^{hi}IgG1⁺) memory B cells in the mediastinal lymph nodes at day 35 after infection with *N. brasiliensis*. (g) Number and kinetics of IgG1⁺ memory B cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. Data are representative of three experiments (mean of three mice per time point in a,c-g; mean ± s.d. of three mice per group in b).

We first assessed the generation of IgG1-switched B cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of IgE-GFP mice during primary infection with *N. brasiliensis*. We detected IgG1⁺ B cells by flow cytometry in all tissues examined, starting at

days 7–10 after infection and peaking around day 15 (Fig. 2a). The IgG1⁺ B cells had intermediate to low GFP expression similar to that of cells generated by *in vitro* switch cultures (Supplementary Fig. 4a) and did not express membrane IgE, as detected by M1' staining

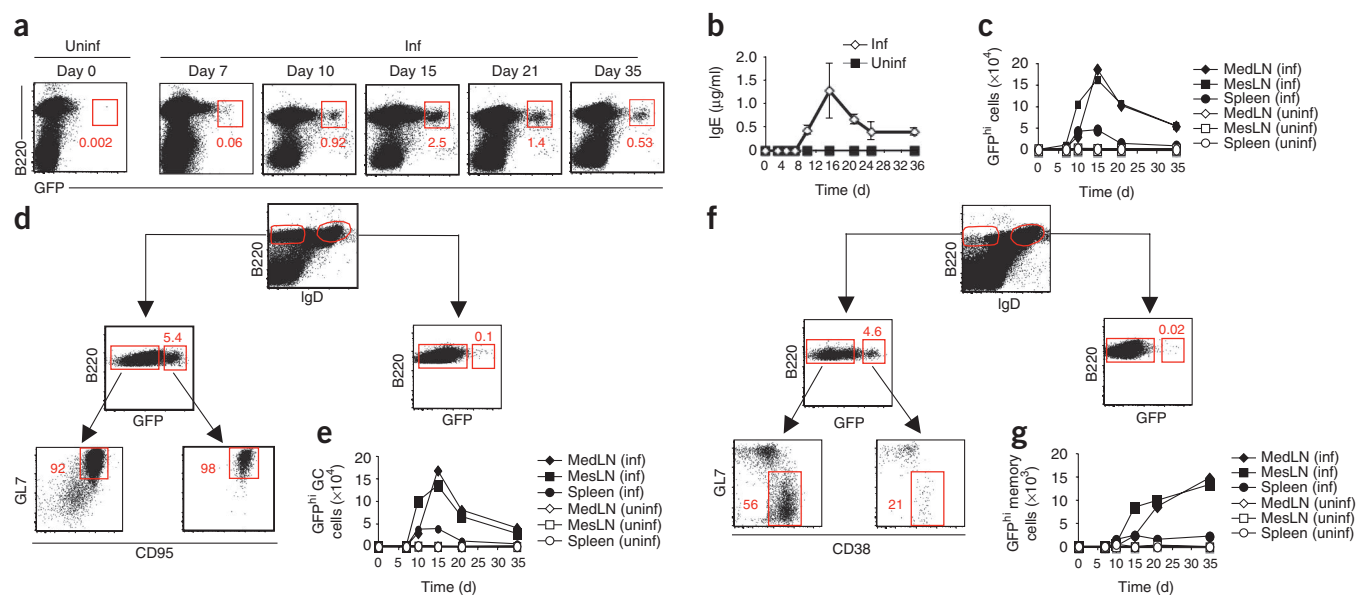


Figure 3 *In vivo* generation of IgE-switched B cells after infection of IgE-GFP reporter mice with *N. brasiliensis*. (a) Flow cytometry of GFP^{hi} B cells in the mediastinal lymph nodes of mice left uninfected or infected with *N. brasiliensis*. (b) Kinetics of serum IgE protein concentrations in mice left uninfected or infected with *N. brasiliensis*. (c) Number and kinetics of GFP^{hi} B cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. (d) Flow cytometry of germinal-center IgE⁺ (B220⁺IgD⁻GL7⁺CD95⁺GFP^{hi}) B cells in the mediastinal lymph nodes at day 15 after infection with *N. brasiliensis*. (e) Number and kinetics of germinal-center IgE⁺ B cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. (f) Flow cytometry of IgE⁺ (B220⁺IgD⁻GL7⁻CD38^{hi}GFP^{hi}) memory B cells in the mediastinal lymph nodes at day 35 after infection with *N. brasiliensis*. (g) Number and kinetics of IgE⁺ memory B cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. Data are representative of three experiments (mean of three mice per time point in a,c-g; mean ± s.d. of three mice per group in b).

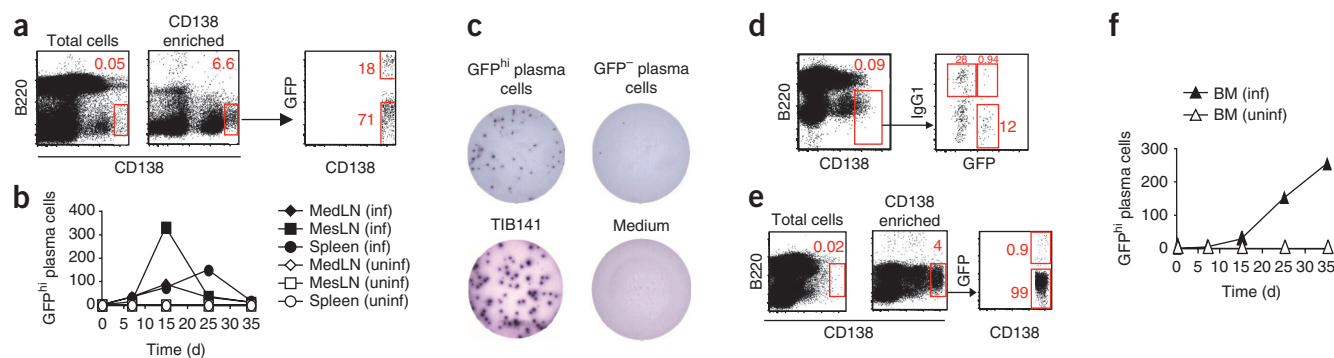


Figure 4 *In vivo* generation of IgE⁺ plasma cells after infection of IgE-GFP reporter mice with *N. brasiliensis*. (a) Flow cytometry of IgE⁺ (B220-CD138^{hi}GFP^{hi}) plasma cells in the mediastinal lymph nodes at day 15 after infection with *N. brasiliensis*. (b) Number and kinetics of IgE⁺ plasma cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. (c) Enzyme-linked immunospot analysis of sorted GFP^{hi} and GFP⁻ plasma cells from mediastinal lymph nodes of mice at day 16 after infection with *N. brasiliensis* (top row), TIB141 mouse IgE hybridoma cells (bottom left) and cell culture medium (bottom right). (d) Flow cytometry of the expression of GFP and intracellular expression of IgG1 by plasma cells. (e) Flow cytometry profile of IgE⁺ (B220-CD138^{hi}GFP^{hi}) plasma cells in the bone marrow (BM) of mice at day 35 after infection with *N. brasiliensis*. (f) Number and kinetics of IgE⁺ plasma cells in the bone marrow of mice left uninfected or infected with *N. brasiliensis*. Data are representative of two experiments (mean of five mice per time point in a,b,d-f).

(data not shown). Serum IgG1 responses in infected IgE-GFP mice corresponded to the appearance of IgG1⁺ B cells (Fig. 2b,c). As expected on the basis of published studies of the switching and development of IgG1⁺ B cells^{7–12}, more detailed analysis of the IgG1⁺ B cells indicated that germinal-center IgG1⁺ B cells, defined as B220⁺IgD⁻GL7⁺CD95⁺IgG1⁺ cells, constituted almost 100% of the IgG1⁺ B cells at days 10–15 and decreased to approximately 60% of IgG1⁺ B cells at day 35 (Fig. 2d,e). In addition, we detected IgG1⁺ memory B cells, defined as B220⁺IgD⁻GL7⁻CD38^{hi}IgG1⁺ cells, starting at days 10–15 after infection, and they increased to approximately 30% of IgG1⁺ B cells at day 35 (Fig. 2f,g).

We next assessed the generation of IgE-switched B cells, identified as GFP^{hi} B cells, in *N. brasiliensis*-infected IgE-GFP mice. We detected GFP^{hi} B cells by flow cytometry in mediastinal lymph nodes, mesenteric lymph nodes and spleen starting at days 7–10 after infection, and they peaked around days 10–15 (Fig. 3a). Similar to results obtained with the *in vitro* switch cultures, GFP^{hi} B cells expressed membrane IgE, as detected by M1' staining that was blocked by preincubation of anti-M1' with M1'-containing protein (Supplementary Fig. 4b). In addition, GFP^{hi} B cells were not generated during a general non-IgE-inducing inflammatory response triggered by challenge of IgE-GFP mice with lipopolysaccharide (Supplementary Fig. 5). Serum IgE responses in infected IgE-GFP mice corresponded with the appearance of GFP^{hi} B cells (Fig. 3b,c).

More detailed analysis of GFP^{hi} B cells demonstrated both germinal-center and memory IgE⁺ B cell populations that were distinct from germinal-center and memory IgG1⁺ B cell populations but that appeared with kinetics and frequency similar to that of the IgG1⁺ B cells. We detected IgE⁺ germinal-center B cells, defined as B220⁺IgD⁻GL7⁺CD95⁺GFP^{hi} cells, in IgE-GFP mice starting at day 10 after infection. These germinal-center cells constituted almost 100% of the GFP^{hi} B cells at day 10 and decreased to approximately 60–80% of GFP^{hi} B cells at day 35 (Fig. 3d,e). We detected a small population of IgE⁺ memory B cells, defined as B220⁺IgD⁻GL7⁻CD38^{hi}GFP^{hi} cells, in mediastinal lymph nodes, mesenteric lymph nodes and spleen starting at day 15 after infection (Fig. 3f,g). This IgE⁺ memory B cell population increased through day 35, at which time it constituted approximately 25% of GFP^{hi} B cells. Our observations of a substantial IgE⁺ B cell population in the germinal center and an IgE⁺ memory B cell population *in vivo* were in contrast to a

published study in which IgE⁺ cells were rarely found in germinal centers and mainly had a plasma cell-like phenotype¹⁴.

In vivo IgE⁺ plasma cells

We assessed the generation of IgE⁺ plasma cells, defined as B220⁻CD138^{hi}GFP^{hi} cells, in mediastinal lymph nodes, mesenteric lymph nodes, spleens and bone marrow of IgE-GFP mice during the course of infection with *N. brasiliensis*. Given the expected low frequency of IgE⁺ plasma cells, we used enrichment for CD138⁺ cells by column purification to greatly enhance our ability to detect these cells. We detected IgE⁺ plasma cells in the mediastinal lymph nodes, mesenteric lymph nodes and spleen at day 15 after infection, and they constituted approximately 15–20% of total CD138^{hi} plasma cells in the lymph nodes and approximately 3% of total CD138^{hi} plasma cells in the spleen (Fig. 4a,b). We confirmed that these were IgE-secreting plasma cells by enzyme-linked immunospot analysis of sorted GFP⁺ and GFP⁻ plasma cells from mediastinal lymph nodes at 16 d after infection (Fig. 4c). In addition, only GFP⁻ plasma cells, but not GFP⁺ plasma cells, from mediastinal lymph nodes at 14 d after infection expressed intracellular IgG1, as assessed by flow cytometry (Fig. 4d). The kinetics and frequency of IgE⁺ plasma cells in the secondary lymphoid organs closely mirrored the kinetics and overall concentration of serum IgE during the course of infection, which suggested that the plasma cells in the secondary lymphoid organs were the main sources of serum IgE. We detected a very low frequency of IgE⁺ plasma cells in the bone marrow of *N. brasiliensis*-infected mice. These cells were first detectable around day 15 after infection and continued to increase during the course of infection, constituting approximately 1% of total CD138^{hi} plasma cells in the bone marrow at day 35 after infection (Fig. 4e,f).

Spatial localization of IgE⁺ cells in lymph nodes

To determine the location of IgE⁺ cells in secondary lymphoid tissues, we used immunofluorescence confocal microscopy to examine tissue sections from mediastinal and mesenteric lymph nodes of IgE-GFP mice infected with *N. brasiliensis*. To correlate the GFP⁺ cells visualized by microscopy with our previous flow cytometry results, we imaged sorted GFP^{hi}, GFP^{int} and GFP⁻ cells in cell pellets that were processed similarly to tissue sections. We detected only GFP^{hi} cells under these imaging conditions (Supplementary Fig. 6a). In agreement with our

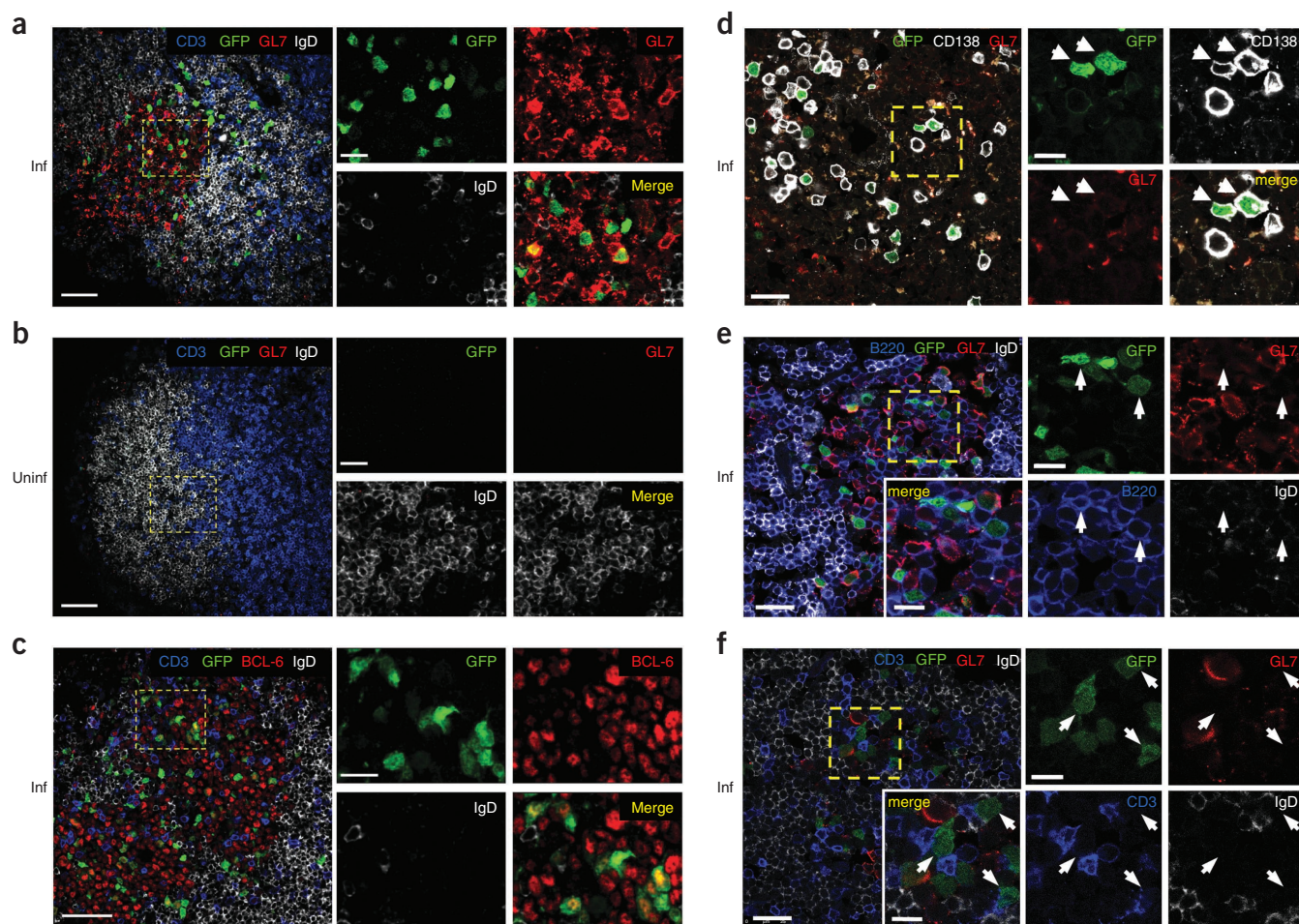


Figure 5 Immunofluorescence confocal microscopy of IgE⁺ germinal-center cells, plasma cells and memory B cells in the lymph nodes of IgE-GFP reporter mice left uninfected or infected with *N. brasiliensis*. (**a–c**) Germinal-center IgE⁺ (GFP⁺) cells (green) in sections of mediastinal lymph nodes from mice 14–15 d after infection with *N. brasiliensis* (**a,c**) or from an uninfected mouse (**b**), counterstained with anti-CD3 (blue) and anti-IgD (white) and antibodies to the germinal center markers GL7 (red; **a,b**) or Bcl-6 (red; **c**). Scale bars, 50 μ m (left) or 15 μ m (right). (**d**) IgE⁺ (GFP⁺) plasma cells (green) of the medullary zone of a mesenteric lymph node from a mouse 14 d after infection with *N. brasiliensis*, counterstained with anti-CD138 (white) and anti-GL7 (red). Scale bars, 25 μ m (left) or 10 μ m (right). (**e,f**) IgE⁺ (GFP⁺) memory B cells (green) in sections of mediastinal lymph nodes from mice 35 d after infection with *N. brasiliensis*, counterstained with anti-GL7 (red) and anti-IgD (white), plus either anti-B220 (blue; **e**) or anti-CD3 (blue; **f**). Scale bars, 25 μ m (left) or 10 μ m (right and insets). Right and insets, enlargement of dashed outlined regions in main images at left. Data are representative of two experiments.

flow cytometry results, we identified GFP⁺ cells in IgD⁺GL7⁺ germinal centers in lymph nodes of infected IgE-GFP mice 14–15 d after infection (**Fig. 5a**) but not in lymph nodes of uninfected control mice (**Fig. 5b**). Germinal-center GFP⁺ cells expressed the transcription factor Bcl-6 (**Fig. 5c**) and stained negatively for the plasma-cell marker CD138 (**Supplementary Fig. 6b**). IgE⁺ plasma cells, identified as GFP⁺CD138⁺ cells, were commonly located distant from germinal centers; for example, in the lymph node medullary regions (**Fig. 5d**). We identified IgE⁺ memory B cells in mediastinal lymph nodes 35 d after infection as GFP⁺B220⁺GL7⁺CD138⁺ cells; they were located in loosely arranged GFP⁺ cell clusters in association with GL7⁺ germinal centers and their collapsed remnants (**Fig. 5e**). In addition, the GFP⁺ cells in these follicular clusters frequently abutted CD3⁺ T cells (**Fig. 5f**). We were also able to visualize, by two-photon microscopy, a dynamic population of IgE-switched B cells in intact mesenteric lymph nodes of mice at 13 d after infection (**Supplementary Video 1**), which demonstrated the utility of these mice for analysis of the migration and motility of IgE⁺ B cells during T helper type 2 responses.

Detection of *in vivo* IgE⁺ cells in a second model

We sought to confirm our observations of *in vivo* germinal-center IgE⁺ B cells, IgE⁺ memory B cells and IgE⁺ plasma cells in a second mouse model that consisted of the immunization of IgE-GFP reporter mice with the model antigen 2,4,6-trinitrophenyl-conjugated ovalbumin (TNP-ovalbumin). Published studies have demonstrated a low but substantial antigen-specific IgE response after primary immunization of IgE-GFP mice with TNP-ovalbumin mixed with aluminum hydroxide (alum), as well as a secondary antigen-specific IgE response with the characteristics of a memory response after rechallenge of IgE-GFP mice with TNP-ovalbumin at day 28 (ref. 15). Similar to results obtained in our *N. brasiliensis*-infection studies, we identified by flow cytometry a major IgE⁺ B cell population in the germinal center in the mesenteric lymph nodes and spleens of IgE-GFP mice immunized with TNP-ovalbumin, which constituted >90% of GFP^{hi} B cells at the peak of the antigen-specific IgE response at day 9 after immunization (**Fig. 6a**). We detected a small but distinct IgE⁺ memory B cell population at day 28 after immunization (**Fig. 6b**), but not at day 9 (data not shown), and we also detected IgE⁺ plasma

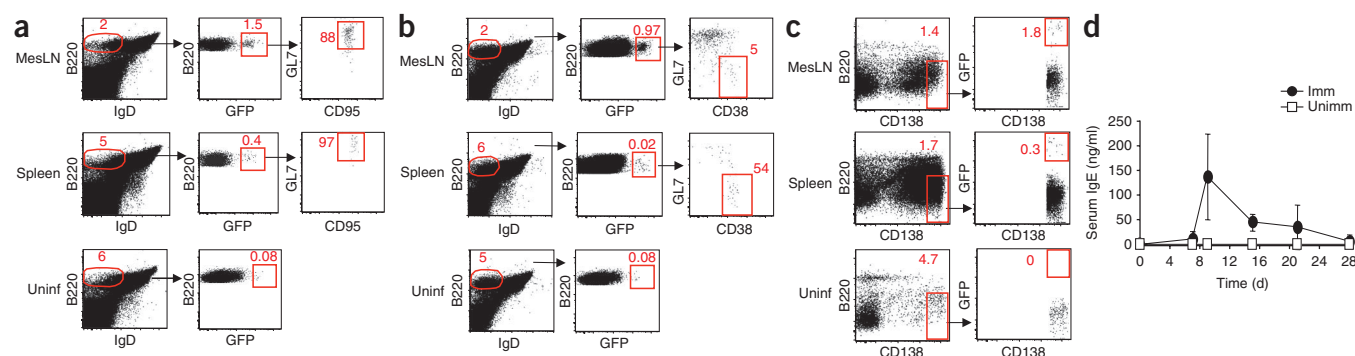


Figure 6 *In vivo* generation of IgE-switched B cells after immunization of IgE-GFP reporter mice with TNP-ovalbumin. (**a–c**) Flow cytometry of IgE⁺ (B220⁺IgD⁺GL7⁺CD95⁺GFP^{hi}) germinal-center B cells (**a**), IgE⁺ (B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{hi}) memory B cells (**b**) and IgE⁺ (B220⁺CD138^{hi}GFP^{hi}) plasma cells (**c**) in the mesenteric lymph nodes and spleens of mice at day 9 (**a,c**) or day 28 (**b**) after immunization with TNP-ovalbumin (top two rows) and in the mesenteric lymph nodes of mice left unimmunized (bottom row); $n = 4$ mice per group (**a,b**), or $n = 3$ mice per group (**c**, immunized) and $n = 2$ mice (**c**, unimmunized). (**d**) Kinetics of TNP-specific serum IgE concentrations in mice left unimmunized or immunized with TNP-ovalbumin. Data are representative of two experiments (mean $\pm$ s.d. of six mice per group in **d**). Imm, immunized; unimm, unimmunized.

cells at day 9 (Fig. 6c). The TNP-specific serum IgE concentrations were similar to those observed in published studies¹⁵ (Fig. 6d).

Memory IgE responses to *N. brasiliensis* infection

A key feature of memory B cell responses is a faster response rate than that of primary B cell responses. We assessed the development of IgE responses in IgE-GFP mice after a second challenge with *N. brasiliensis* 35 d after the first infection and observed a serum IgE response that was faster in kinetics and slightly greater in magnitude than that of the primary infection (Fig. 7a). Whereas serum IgE did not increase until 10 d after a primary infection, it increased as early as 5 d after a second infection. Consistent with the faster serum IgE response, GFP^{hi} cells increased in the mediastinal lymph nodes, mesenteric lymph nodes and spleen 3–5 d after the second infection (Fig. 7b). These GFP^{hi} cells were mainly of a germinal-center phenotype (Fig. 7c). IgE⁺ memory B cells (Fig. 7d) and IgE⁺ plasma cells (Fig. 7e,f) also increased in the same time frame.

IgE⁺ memory B cells encompass cellular IgE memory

The role of IgE⁺ memory B cells in the IgE response to a second *N. brasiliensis* infection was difficult to discern in intact IgE-GFP mice, given that there were still ongoing B cell responses at the

time of the second infection. To better understand the basis of IgE memory, we assessed whether B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{hi} cells, which had the phenotypic characteristics of IgE⁺ memory B cells, also had the functional characteristics of IgE memory B cells. We sorted B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{hi} cells from *N. brasiliensis*-infected IgE-GFP mice at day 35 after infection (Supplementary Fig. 7) and transferred them, along with memory T cells and carrier spleen cells from recombinase activating gene 2-deficient (*Rag2*^{−/−}) mice, into B cell-deficient μ MT recipient mice²², which we then challenged with *N. brasiliensis*. Control groups for this study included mice given B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{int-neg} cells (non-IgE memory B cells; Supplementary Fig. 7) along with memory T cells; naive B cells plus memory T cells; or naive B cells plus naive T cells. The number of B cells transferred in the control groups was the same as the number of B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{hi} cells transferred.

Mice that received B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{hi} IgE⁺ memory B cells generated a significantly higher serum IgE response than that of control mice, starting at 5 d after infection (Fig. 8a). The kinetics of the IgE response in the recipient mice were faster than those associated with a primary *N. brasiliensis* infection and the same as those associated with a secondary *N. brasiliensis* infection in intact IgE-GFP mice. The IgE⁺ memory B cell compartment in mice that received

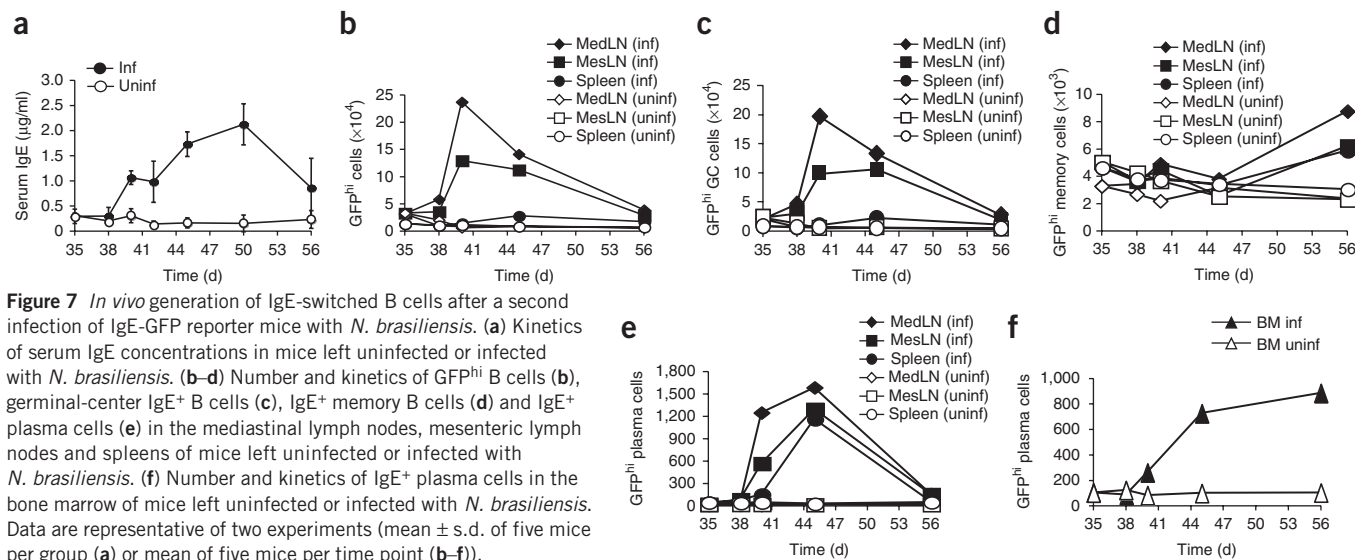


Figure 7 *In vivo* generation of IgE-switched B cells after a second infection of IgE-GFP reporter mice with *N. brasiliensis*. (**a**) Kinetics of serum IgE concentrations in mice left uninfected or infected with *N. brasiliensis*. (**b–d**) Number and kinetics of GFP^{hi} B cells (**b**), germinal-center IgE⁺ B cells (**c**), IgE⁺ memory B cells (**d**) and IgE⁺ plasma cells (**e**) in the mediastinal lymph nodes, mesenteric lymph nodes and spleens of mice left uninfected or infected with *N. brasiliensis*. (**f**) Number and kinetics of IgE⁺ plasma cells in the bone marrow of mice left uninfected or infected with *N. brasiliensis*. Data are representative of two experiments (mean $\pm$ s.d. of five mice per group (**a**) or mean of five mice per time point (**b–f**)).

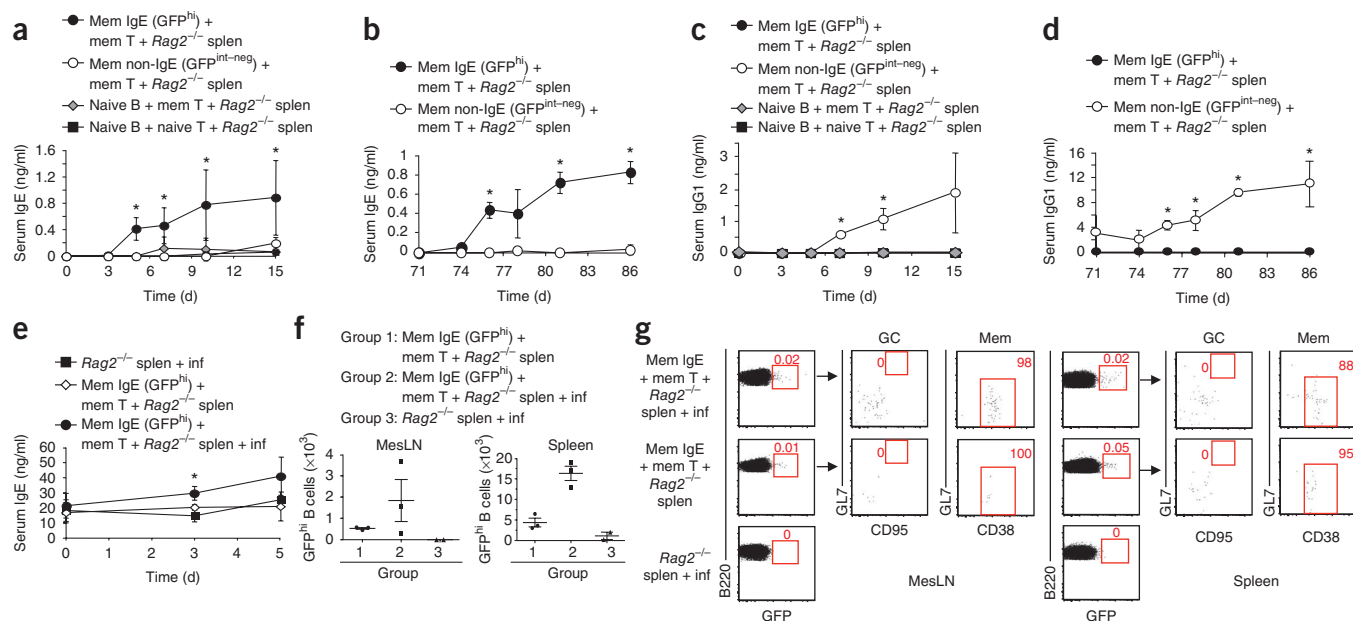


Figure 8 Purified IgE⁺ memory B cells produce IgE after adoptive transfer and infection with *N. brasiliensis*. **(a)** Serum IgE concentrations in B cell-deficient μ MT recipient mice infected with *N. brasiliensis* 1 d after adoptive transfer of purified B cells and T cells and splenocytes from Rag2^{-/-} mice (Rag2^{-/-} splen). Mem, memory. **P* < 0.05, compared with control (naive T + naive B; Bonferroni correction for multiple comparisons). **(b)** Serum IgE concentrations after a second infection of μ MT recipient mice with *N. brasiliensis* 71 d after adoptive transfer of cells and primary infection as in **a**. **P* < 0.05 (Student's *t*-test). **(c)** Serum IgG1 concentrations after treatment of mice as in **a**. **P* < 0.05 (as in **a**). **(d)** Serum IgG1 concentrations after treatment of mice as in **b**. **P* < 0.05 (Student's *t*-test). **(e)** Serum IgE concentrations in wild-type C57BL/6 recipient mice infected with *N. brasiliensis* (+ inf) 1 d after adoptive transfer of purified B cells and T cells and splenocytes from Rag2^{-/-} mice. **P* < 0.05, compared with control (uninfected memory IgE⁺ B cell; Bonferroni correction for multiple comparisons). **(f)** GFP^{hi} cells in mesenteric lymph nodes and spleens of wild-type C57BL/6 recipient mice 6 d after adoptive transfer of cells with or without infection (key). Each symbol represents an individual mouse; horizontal lines indicate the mean ($\pm$ s.d.). **(g)** Flow cytometry of IgE⁺ germinal-center and memory B cells in the mesenteric lymph nodes and spleens of wild-type C57BL/6 recipient mice 6 d after adoptive transfer of cells and infection (left margin). Data are representative of two experiments with two recipient mice (purified IgE⁺ memory B cells; **a–d**) or three recipient mice (all other groups; **e–g**; mean $\pm$ s.d. in **a–e**).

B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{hi} cells persisted beyond the first infection with *N. brasiliensis*, as a second infection of the recipient mice with *N. brasiliensis* at day 71 generated a second serum IgE response (Fig. 8b). Neither group of mice that received naive B cells mounted an IgE response to infection with *N. brasiliensis*, probably because of a low frequency of *N. brasiliensis*-specific responder B cells in the naive B cell pool.

Mice that received B220⁺IgD⁺GL7⁺CD38^{hi}GFP^{int-neg} non-IgE⁺ memory B cells, which included IgG1⁺ memory B cells, did not mount an IgE response after infection with *N. brasiliensis* (Fig. 8a). Rechallenge of the mice with a second infection at day 71 also failed to induce a serum IgE response (Fig. 8b). In contrast, we observed serum IgG1 responses only in the mice that received IgG1⁺ memory B cells (Fig. 8c), and the serum IgG1 concentrations in these mice remained higher through day 70. Rechallenge of mice with a second infection of *N. brasiliensis* at day 71 further increased serum IgG1 concentrations in the mice that had received IgG1⁺ memory B cells but not in the mice that had received IgE⁺ memory B cells (Fig. 8d). These results indicated that cellular IgE memory resided with IgE⁺ memory B cells and that IgG1⁺ memory B cells were not a substantial source of IgE memory.

To further evaluate the activation of IgE⁺ memory B cells *in vivo*, we sorted IgE⁺ memory B cells from *N. brasiliensis*-infected IgE-GFP mice at day 40 after infection, transferred them along with memory T cells and carrier spleen cells from Rag2^{-/-} mice into wild-type C57BL/6 mice, and assessed by flow cytometry germinal-center and memory GFP^{hi} IgE⁺ cells from the mesenteric lymph

nodes and spleens of recipient mice with or without *N. brasiliensis* infection. In this scheme, only the transferred cells from IgE-GFP mice were able to express the GFP reporter. We restricted our analyses to no more than 6 d after infection to avoid serum IgE responses that arose from B cells of the host mice, which we monitored by including a control group of wild-type C57BL/6 mice that received only carrier spleen cells from Rag2^{-/-} mice followed by infection with *N. brasiliensis*. Similar to the results obtained by transfer of IgE⁺ memory B cells into B cell-deficient μ MT mice, higher serum IgE concentrations were detectable as early as 3 d after infection in wild-type C57BL/6 mice that received IgE⁺ memory B cells (Fig. 8e). Neither the uninfected mice that had received IgE⁺ memory B cells nor the infected mice that had received only carrier Rag2^{-/-} spleen cells produced more serum IgE during this time frame. We observed more GFP^{hi} B cells at day 6 after infection in the mesenteric lymph nodes and spleens of infected mice that had received IgE⁺ memory B cells (Fig. 8f). None of these GFP^{hi} B cells had a germinal-center phenotype (Fig. 8g). These results suggest that IgE⁺ memory B cells differentiate directly into IgE⁺ plasma cells without progressing through a germinal-center intermediate.

Together our studies support a model of IgE biology in which the main pathway for the generation of IgE-switched cells is through an IgE⁺ intermediate cell in the germinal center (Supplementary Fig. 8). We propose that these germinal-center IgE⁺ B cells give rise to IgE⁺ memory B cells and IgE⁺ plasma cells. Cellular IgE memory is encompassed by IgE⁺ memory B cells, which respond rapidly after rechallenge to produce memory IgE responses.

DISCUSSION

Here we have used IgE-GFP reporter mice to study the *in vivo* biology of IgE switching and IgE memory during infection with the helminth *N. brasiliensis* and after immunization with the model antigen TNP-ovalbumin. These IgE-GFP reporter mice enabled the direct detection of IgE-switched cells by GFP fluorescence, which we confirmed by antibody staining of the human M1' segment inserted into membrane IgE in these mice. The sensitivity and specificity of the GFP reporter facilitated identification of IgE-switched cells *in vivo* and *ex vivo* and enabled the study of *in vivo* IgE biology in the context of a normal polyclonal T cell and B cell repertoire.

Our model of IgE biology is analogous to the model established for the differentiation of IgG1⁺ cells and contrasts with the model of IgE biology proposed before, in which IgG1⁺ cells are the main germinal-center intermediate in the generation of IgE responses and IgG1⁺ memory B cells are the main source of cellular IgE memory¹⁴. In our studies, we identified a major population of IgE-switched B cells with the phenotypic characteristics of germinal-center cells, as assessed by flow cytometry, and that localized to germinal-center structures, as visualized by fluorescence microscopy. We also identified IgE⁺ memory B cells *in vivo*, and our studies indicated that these IgE⁺ memory B cells, and not IgG1⁺ memory B cells, were the source of cellular IgE memory.

Although our studies and other published studies¹⁴ have both used infection of wild-type mice with *N. brasiliensis*, our results differ in that we were able to identify a substantial population of germinal-center IgE⁺ cells and IgE⁺ memory B cells. These differences may be due to the methods used for detecting IgE-switched cells (direct detection by the GFP reporter and M1' staining versus indirect detection by acid treatment followed by staining with anti-IgE), as well as differences in the mouse model systems (all our studies used mice with wild-type polyclonal T cell and B cell repertoires, whereas many of the experiments in the other published study, including the assessment of memory IgE responses, used only mice with monoclonal T cells and B cells¹⁴). In addition, immunohistochemistry with anti-IgE to detect IgE⁺ cells may be biased toward the detection of IgE⁺ plasma cells, which have much higher expression of IgE than do IgE-switched B cells (that is, secreted IgE in plasma cells versus membrane IgE on IgE-switched B cells). Our IgE-GFP reporter was linked specifically to membrane IgE and therefore may have allowed us to better detect both IgE-switched B cells and IgE⁺ plasma cells.

Although we identified IgE⁺ plasma cells in both secondary lymphoid organs and bone marrow after infection with *N. brasiliensis*, we did not assess the lifespan of these cells and therefore do not know whether they were short-lived or long-lived plasma cells. However, some indirect evidence suggests that most serum IgE generated after infection with *N. brasiliensis* is derived from short-lived plasma cells. First, serum IgE peaks and decreases with the same kinetics and in approximately the same proportion as those of the population of IgE⁺ plasma cells in the secondary lymphoid organs, which are thought to be short-lived plasma cells^{23,24}. The serum half-life of IgE in mice is less than a day^{25,26}, so the rates of decrease in serum IgE concentrations are a good indicator of the turnover of IgE⁺ plasma cells and the half-life of IgE⁺ plasma cells. Second, published studies have demonstrated that the treatment of IgE-GFP reporter mice at the peak of the *N. brasiliensis*-induced IgE response with anti-M1' results in the depletion of IgE-switched cells and in a rapid decrease in serum IgE of over 90% (ref. 15), which suggests that most serum IgE is generated by short-lived plasma cells. We also observed a rapid decrease in serum IgE of over 90% after treatment of IgE-GFP mice with anti-M1' at 40 d after *N. brasiliensis* infection, when serum IgE had reached

steady-state concentrations (data not shown). The residual IgE that remains after treatment of *N. brasiliensis* infection with anti-M1' may be due to the presence of long-lived IgE⁺ plasma cells, which are thought to reside in the bone marrow^{7,13,23,24}. Consistent with that, we detected a low frequency of IgE⁺ plasma cells in the bone marrow of *N. brasiliensis*-infected mice as early as 15 d after infection, which seemed to reach a plateau by approximately 35 d after infection.

Our study is the first to our knowledge to directly identify IgE-switched memory B cells, and it indicates these cells are the main source of cellular IgE memory. Our finding that cellular IgE memory resided in an IgE-switched memory B cell compartment, with minimal contributions from secondary switching of IgG1⁺ memory B cells, was consistent with a published study indicating that there are no differences in primary or memory IgE responses in mice that are unable to switch to IgG1 (ref. 27) and also with observations that IgG1⁺ memory B cells rapidly differentiate into IgG1-producing plasma cells after secondary challenge and do not form new germinal centers, which would be required for a secondary switch to IgE^{28,29}. Restricting the IgE memory compartment to a small population of IgE-switched memory B cells, rather than secondary switching of a much larger population of IgG1⁺ memory B cells, may enable better regulation of memory IgE responses to minimize undesirable anaphylactic reactions.

Our studies suggest three mechanisms by which memory IgE responses can be generated more rapidly than primary IgE responses, which have also been proposed for memory IgG1 responses^{28–30}. First, memory IgE⁺ B cells can be found in lymph nodes in association with GL7⁺ germinal centers and their collapsed remnants. The retention of IgE⁺ memory B cells in the lymph nodes may expedite reencounter with antigen. Second, memory IgE⁺ B cells clustered in these lymph nodal regions are in close proximity to T cells. This close proximity may expedite cellular activation, differentiation or survival after a secondary antigen challenge. Finally, our analysis of the *in vivo* response of transferred IgE⁺ memory B cells to *N. brasiliensis* infection suggested that IgE⁺ memory B cells may differentiate directly into IgE⁺ plasma cells without reinitiating a germinal-center reaction, thereby facilitating a faster IgE response. Additional studies with the IgE-GFP reporter mouse may further improve the understanding of the biology of IgE⁺ cells and IgE memory.

Note added in proof: Another group, using a different IgE reporter mouse, has found evidence of IgE⁺ germinal-center B cells and 'preferential' differentiation of IgE⁺ B cells into short-lived plasma cells (Yang, Z. and Allen, C.D.C., personal communication).

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

O.T., H.D.B., E.L. and J.G.E. designed and did experiments, and wrote the manuscript; D.Y., E.E.M.S. and M.Z. did experiments; C.D.A. designed experiments and wrote the manuscript; W.P.L. and M.X. designed experiments; and L.C.W. designed experiments, wrote the manuscript, and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/natureimmunology/>.

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ONLINE METHODS

Mice. C57BL/6 and B6.129S2-*Igh-6^{tm1Cgn}/J* (μ MT) mice were from The Jackson Laboratory. B6.129S6-*Rag2^{tm1Fwa}* N12 (*Rag2*^{-/-}) mice were from Taconic. Human M1'-eGFP knock-in (IgE-GFP) mice were generated as described¹⁵. All mice were housed under specific pathogen-free conditions, and all experiments were conducted in accordance with guidelines from the US National Institutes of Health for the care and use of mice in research with protocols approved by the Genentech Institutional Animal Care and Use Committee.

Antibodies and flow cytometry. The following monoclonal antibodies were used for flow cytometry: anti-mouse B220 (RA6B2; BD Bioscience), antibody to mouse and human GL7 (GL7; eBioscience), anti-mouse CD38 (90; eBioscience), anti-mouse CD95 (Jo2; BD Bioscience), anti-mouse CD138 (281-2; BD Bioscience), anti-mouse IgG1 (A85-1; BD Bioscience), anti-mouse CD4 (RM4-5; BD Bioscience), anti-mouse CD44 (IM7; BD Bioscience), anti-mouse CD45RB (16A; BD Bioscience), anti-mouse IgD (11-26c.2a; BioLegend), biotin-conjugated anti-mouse IgG1 (RMG-1; BioLegend) and anti-human M1' (47H4; Genentech). Samples were enriched for plasma cells (CD138⁺ cells) with magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). Cells were analyzed on an LSR II, FACSCalibur or LSRFortessa (Becton Dickinson) and were sorted on a FACS Vantage or FACS Aria (Becton Dickinson). Data were processed with FlowJo software (TreeStar).

In vitro IgE-switch cultures. Naive B cells were purified from C57BL/6 and IgE-GFP mouse splenocytes by magnetic bead separation according to the manufacturer's instructions (Miltenyi Biotec) and were cultured with anti-mouse CD40 (100 ng/ml; BD Biosciences) with or without recombinant mouse IL-4 (100 ng/ml; R&D Systems).

Analysis of ϵ -transcripts. Total RNA from *in vitro* IgE-switch cultures or sorted B cell populations was prepared by RNeasy protocol (Qiagen), with DNase treatment to remove genomic DNA contamination. Quantitative RT-PCR used 50 ng total RNA per sample and a Taqman One-step RT-PCR kit (Applied Biosystems) with a 7500 Realtime PCR system (Applied Biosystems). Sequences for analysis of ϵ -germline transcripts³¹ were as follows: forward primer, 5'-GCAGAAGATGGCTTCGAATAAGACAGT-3'; reverse primer, 5'-TCGTTGATGATGGAGGATGTGTACAGT-3'; Probe, 5'-CAGCCACTCACTTATCAGAG-3'. Sequences for analysis of ϵ -switch transcripts¹⁴ were as follows: forward primer, 5'-CTCTGGCCCTGCTTATGTGTG-3'; reverse primer, 5'-AGTTCACAGTGCTCATGTTACAG-3'; probe, 5'-TCTGAATACCAGGTCACAGT-3'. Sequences for analysis of the V-H7183 mature transcript¹⁴ were as follows: forward primer, 5'-TACGACGAGAACGGGTTTGCTTAC-3'; reverse primer, 5'-AGTTCACAGTGCTCATGTTACAG-3'; probe, 5'-TCTGAATACCAGGTCACAGT-3'. Sequences for analysis of the control transcript encoding ribosomal protein L19 were as follows: forward primer 5'-GCGCATCCTCATGGAGCACA-3'; reverse primer 5'-GGTCAGCCAGGAGCTTCTTG-3'; Probe 5'-CACAAGCTGAAGGCAGACAAGGCC-3'. Results for ϵ -transcription were normalized to those of control transcription of the gene encoding ribosomal protein L19, and relative transcript expression was determined by the calculation $2^{-\Delta CT}$ with reference to an absolute baseline cycling threshold (C_T) of 40 cycles.

***N. brasiliensis* infection.** Larvae of *N. brasiliensis* (R. Locksley) were isolated from the feces of infected Lewis rats (Charles River). Mice were infected subcutaneously with 500 L3 larvae in 200 μ L PBS.

TNP-ovalbumin immunization. Mice were immunized intraperitoneally with 100 μ g TNP-ovalbumin (Biosearch Technologies) in 2 mg aluminum hydroxide in 100 μ L PBS.

Adoptive-transfer experiments. Memory B cells were sorted from the pooled lymph nodes and spleens of IgE-GFP mice ($n = 20$ –23) at 35 or

42 d after infection with *N. brasiliensis*. Memory T cells were sorted from the pooled lymph nodes and spleens of C57BL/6 mice ($n = 20$) at 35 d after infection with *N. brasiliensis*. Naive B cells and naive T cells were purified from the spleens of GFP-IgE and C57BL/6 mice, respectively, with magnetic beads (Miltenyi Biotec). B cells (1.2×10^4 to 1.4×10^4), T cells (1×10^5) and/or splenocytes from *Rag2*^{-/-} mice (as carrier cells; approximately 1×10^6) were combined and injected intravenously, in 100 μ L sterile PBS, into each recipient mouse.

IgE and IgG1 enzyme-linked immunosorbent assay. Cell culture supernatant and mouse serum IgE was measured with a mouse IgE ELISA set according to the manufacturer's instructions and with the manufacturer's IgE standard (BD Biosciences Pharmingen). Culture supernatants and mouse serum IgG1 was measured with purified anti-mouse IgG1 (A85-3; BD Bioscience), biotin-conjugated anti-mouse IgG1 (A85-1; BD Bioscience) and streptavidin-horseradish peroxidase (BD Bioscience Pharmingen), and with a purified mouse IgG1 standard (BD Bioscience). TNP-ovalbumin-specific mouse serum IgE was measured with TNP-ovalbumin-coated plates, biotinylated rat anti-mouse IgE (R35-118; BD Bioscience), and streptavidin-horseradish peroxidase (BD Bioscience Pharmingen), and with purified anti-TNP mouse IgE standard (TIB-141; American Type Culture Collection).

IgE enzyme-linked immunospot assay. Anti-mouse IgE and biotinylated anti-mouse IgE capture and detection antibodies (OptEIA mouse IgE; BD), respectively, and streptavidin-alkaline phosphatase were used for enzyme-linked immunospot assay of mouse IgE. TIB-141 cells expressing mouse IgE antibody to TNP (American Type Culture Collection) were used as a positive control cell line. Immunospots were counted manually.

Fluorescence confocal microscopy. Mediastinal and mesenteric lymph nodes were collected and then immersed for 12 h in PLP buffer (0.05 M phosphate buffer containing 0.1 M L-lysine, pH 7.4, NaIO₄ (2 mg/ml) and paraformaldehyde (10 mg/ml)), dehydrated for 8 h in 30% sucrose, and embedded in optimum cutting temperature freezing medium. Sections 10 μ m in thickness were cut on a CM3050s cryostat (Leica) and made to adhere to Superfrost Plus slides (VWR). Sections were made permeable, blocked and stained with the appropriate combinations of the following antibodies: Alexa Fluor 488-conjugated rabbit anti-GFP (A-21311; Invitrogen), biotinylated rat anti-IgD (11-26; eBioscience), V450-conjugated rat anti-CD3 (17A2; BD Biosciences), Alexa Fluor 647-conjugated rat anti-GL7 (GL7; BD Biosciences), Alexa Fluor 647-conjugated mouse anti-BCL6 (K112-91; BD Biosciences), V450-conjugated rat anti-B220 (RA3-6B2; BD Biosciences) and/or rat anti-CD138 (281-2; BD Biosciences), followed by staining with or without biotinylated anti-rat IgG (619540; Invitrogen), then finally Alexa Fluor 546-streptavidin (S-11225; Invitrogen). Stained slides were mounted with Prolong Gold (Invitrogen) and images were acquired on a Leica TCS-SPE confocal microscope (Leica Microsystems). Images were visualized and exported with Imaris (Bitplane) or Photoshop CS3 (Adobe). For comparison of GFP expression in infected and uninfected lymph nodes, image-capture parameters and minimum and maximum pixel intensity values were held constant.

Statistical analysis. *P* values were calculated with JMP statistics software (SAS). For experiments with more than two groups, Dunnett's test was used for comparison of group means, in which all groups were tested against a control group as a reference (μ MT recipients of naive B cells plus naive T cells, or uninfected wild-type C57BL/6 recipients of memory IgE B cells). For experiments with only two groups, Student's *t*-test was used for comparison of group means. *P* values of less than 0.05 were considered significant.

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Addendum: IgE⁺ memory B cells and plasma cells generated through a germinal-center pathway

Oezcan Talay, Donghong Yan, Hans D Brightbill, Elizabeth E M Straney, Meijuan Zhou, Ena Ladi, Wyne P Lee, Jackson G Egen, Cary D Austin, Min Xu & Lawren C Wu

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It has come to our attention that in our studies of the differentiation and memory of IgE⁺ B cells in mice, we did not appropriately distinguish IgG1⁺ and IgE⁺ B cells because of technical flaws in our original flow cytometry^{1,2}. After changing the fluorochrome of the antibody to IgG1 from phycoerythrin to allophycocyanin to overcome problems in the compensation between the green fluorescent protein (GFP) and IgG1 channels of our original nine-color analysis of *in vivo* IgG1⁺ and IgE⁺ B cell populations, we identified the following three distinct IgG1⁺ and IgE⁺ B cell populations in our IgE-GFP reporter mice infected with *Nippostrongylus brasiliensis*: IgG1⁺GFP[−] IgG1-expressing cells, IgG1⁺GFP^{hi} IgG1-expressing cells and IgG1[−]GFP^{hi} IgE-expressing cells (**Fig. 1a**). The mean fluorescence intensity for GFP in the population of IgG1⁺GFP^{hi} cells was slightly lower than that of the IgG1[−]GFP^{hi} IgE⁺ cells, but the range of GFP fluorescence of these two cell populations overlapped significantly, in contrast to the results we obtained before (Supplementary Fig. 3 in ref. 2) and also in contrast the results we obtained for *in vitro*-derived IgG1⁺ and IgE⁺ cells, which exhibited a more distinct separation in GFP fluorescence (Fig. 1 in ref. 1). The IgG1⁺GFP^{hi} cell population was present in both germinal center (GC) B cell populations and memory B cell populations and, although it constituted only ~10% of all IgG1⁺ B cells, it ranged from approximately equal to the number of IgG1[−]GFP^{hi} IgE⁺ B cells (for memory B cells) to approximately threefold that number (for GC B cells) (**Fig. 1b**). We further confirmed that for cells in GCs, both IgG1⁺GFP[−] and IgG1⁺GFP^{hi} cells were IgG1-expressing B cells and IgG1[−]GFP^{hi} cells were IgE-expressing B cells, as assessed by quantitative real-time PCR analysis of transcripts encoding membrane IgG1 and membrane IgE (**Supplementary Fig. 1**), as well as by flow cytometry staining of each cell population for membrane IgE with antibody to IgE after treatment of the cells with acid to remove serum IgE bound to the low-affinity CD23 receptor for IgE (**Supplementary Fig. 2**). Thus, our original studies, which defined *in vivo* IgE⁺ B cells as B220⁺IgD[−]GFP^{hi} cells¹, probably included contributions from both IgG1⁺GFP^{hi} B cells and IgG1[−]GFP^{hi} IgE⁺ B cells, since the fluorescence intensity of GFP in the IgG1⁺GFP^{hi} cells was similar to that of the IgG1[−]GFP^{hi} IgE⁺ cells.

We were concerned that in our previous study we may not have clearly separated IgG1⁺ and IgE⁺ B cells in our studies of IgE memory, which used the fluorescence intensity of GFP to separate IgG1⁺ and IgE⁺ memory B cell populations for adoptive transfer¹. If the GFP^{hi} B cells used in those studies included both IgG1⁺GFP^{hi} and IgG1[−]GFP^{hi} cells, then both IgG1⁺ memory B cells and IgE⁺ memory B cells could have contributed to IgE memory responses. To address this, we repeated the cell-transfer studies as follows: we sorted equal numbers of memory B cells from each of the three different populations (IgG1⁺GFP[−], IgG1⁺GFP^{hi} and IgG1[−]GFP^{hi}) obtained from mice 33–35 d after infection with *N. brasiliensis* and transferred those cells into B cell-deficient μMT mice along with memory T cells and carrier splenocytes from mice deficient in recombination-activating gene 2. Subsequently, we challenged the recipient mice with *N. brasiliensis* and measured the concentration of IgE and IgG1 in serum. Similar to results obtained in our earlier study (Fig. 8 in ref. 1), IgG1[−]GFP^{hi} IgE⁺ memory B cells gave rise to serum IgE responses but not serum IgG1 responses, while IgG1⁺GFP[−] cells memory B cells gave rise to serum IgG1 responses but not serum IgE responses (**Fig. 2**). IgG1⁺GFP^{hi} memory B cells produced both serum IgE responses and serum IgG1 responses after transfer and challenge with *N. brasiliensis*. Given the approximately equal frequency of IgG1⁺GFP^{hi} and IgG1[−]GFP^{hi} memory B cells in *N. brasiliensis*-infected mice (**Fig. 1b**), we calculate that the IgG1⁺GFP^{hi} memory B cells contributed approximately 25% of the total serum IgE response in one study (**Fig. 2a**) and significantly less in a second study (**Fig. 2b**). Thus, we have identified a small population of IgG1⁺ memory B cells that contributed to IgE memory responses in *N. brasiliensis*-infected IgE-GFP reporter mice. However, IgE⁺ memory B cells (i.e., IgG1[−]GFP^{hi} memory B cells) were still the main contributors to IgE memory responses in our new studies, consistent with our earlier work in which we concluded that IgE-switched memory B cells were the main source of cellular IgE memory¹.

In summary, after changing the conditions of our flow cytometry to rectify the analysis of IgG1⁺ and GFP⁺ B cells, we have resolved three distinct GC and memory B cell populations in our IgE-GFP reporter mice: a large IgG1⁺GFP[−] IgG1-expressing B cell population, a small IgG1⁺GFP^{hi} IgG1-expressing B cell population, and a small IgG1[−]GFP^{hi} IgE-expressing B cell population. Since the GFP fluorescence of the IgG1⁺GFP^{hi} B cells and the IgG1[−]GFP^{hi} IgE⁺ B cells was similar, our analyses of GFP^{hi} B cells in earlier publications included contributions from both IgG1⁺GFP^{hi} B cells and IgG1[−]GFP^{hi} IgE⁺ B cells. Notably, in our new studies, we still observed IgE⁺ GC B cells (i.e., IgG1[−]GFP^{hi} GC B cells), and the *in vivo* kinetics of those cells were still consistent with our original observations^{1,2} and other published studies³. In addition, we still found that IgE⁺ memory B cells were the main source of IgE memory responses and that the majority of IgG1⁺ memory B cells did not contribute significantly to IgE memory. However, we have now identified a small subset of IgG1⁺ memory B cells that made minor contributions to cellular IgE memory. With this Addendum, we seek to correct the mistake in our earlier flow cytometry and to clarify the different populations of IgG1⁺ and IgE⁺ B cells that we found in our IgE-GFP reporter mice. We apologize for any confusion that this error may have caused.

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

O.T. designed and did the experiments; L.C.W. wrote the manuscript and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the [online version of the paper](#).

1. Talay, O. *et al.* IgE⁺ memory B cells and plasma cells generated through a germinal-center pathway. *Nat. Immunol.* 13, 396–404 (2012).
2. Talay, O. *et al.* On the differentiation of mouse IgE⁺ cells. *Nat. Immunol.* 13, 623–624 (2012).
3. Yang, Z., Sullivan, B.M. & Allen, C.D. Fluorescent *in vivo* detection reveals that IgE⁺ B cells are restrained by an intrinsic cell fate predisposition. *Immunity* 36, 857–872 (2012).

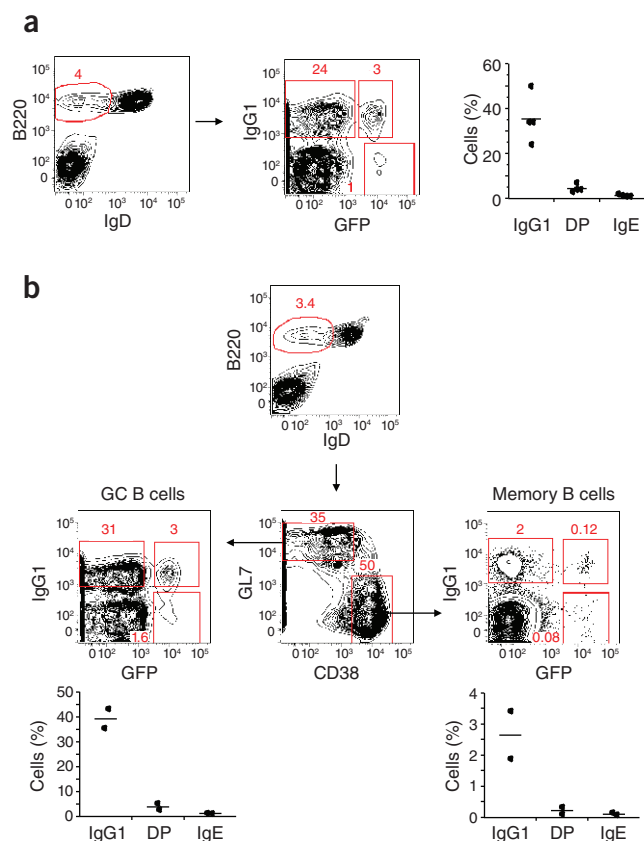


Figure 1 IgG1⁺ and GFP⁺ cell populations in IgE-GFP reporter mice infected with *N. brasiliensis*. (a) Flow cytometry (left and middle) of mesenteric lymph node cells from IgE-GFP reporter mice at day 14 after infection with *N. brasiliensis*, among cells in the B220⁺IgD⁺ gate (outlined area at left). Right, quantification of IgG1⁺GFP⁺ cells (IgG1), IgG1⁺GFP^{hi} cells (DP) and IgG1⁺GFP^{lo} cells (IgE) among B220⁺IgD⁺ B cells. Each symbol represents an individual measurement ($n = 4$); small horizontal lines indicate the mean. (b) Flow cytometry (top and middle) of mesenteric lymph node cells from IgE-GFP reporter mice at day 34 after infection with *N. brasiliensis*, in the GC or memory B cell gate (outlined areas, middle). Bottom, quantification of IgG1⁺GFP⁺, IgG1⁺GFP^{hi} and IgG1⁺GFP^{lo} cells (as in a) in the gates above. Each symbol represents an individual measurement ($n = 2$); small horizontal lines indicate the mean. Numbers adjacent to outlined areas indicate percent cells in each throughout. Data are representative of four experiments (a) or two experiments with cells pooled from three mice (b).

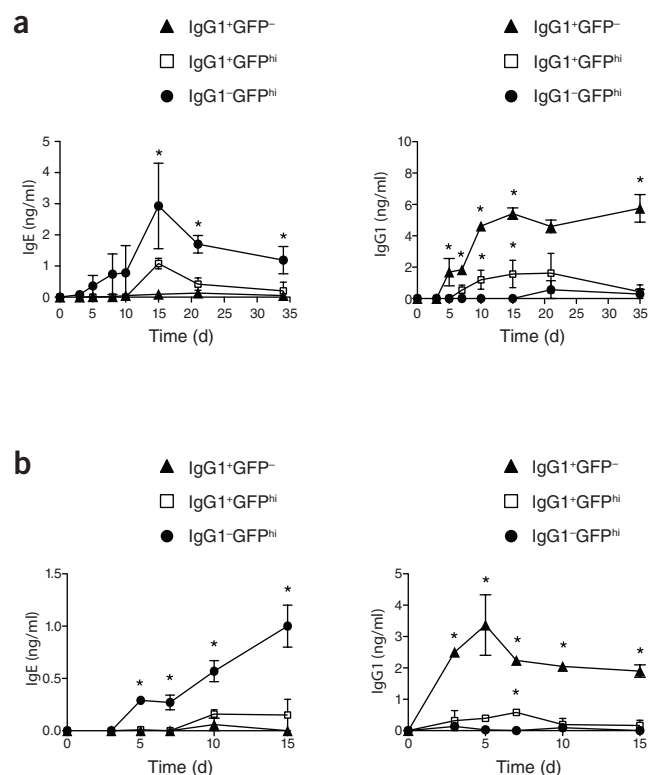


Figure 2 Concentration of IgE and IgG1 in serum from B cell-deficient μ MT recipient mice infected with *N. brasiliensis* after adoptive transfer of purified memory B cells (1.4×10^4 per mouse; gated as B220⁺GL7⁺CD38⁺ memory B cells and the IgG1 and GFP combinations in Fig. 1b) and T cells from *N. brasiliensis*-infected mice, plus carrier splenocytes from mice deficient in recombination-activating gene 2 (a and b are two different experiments). * $P < 0.01$, versus IgG1⁺GFP⁺ cells (for IgE) or IgG1⁺GFP^{hi} cells (for IgG1) (Dunnett's test). Data are representative of two independent experiments with two mice per group (IgG1⁺GFP^{hi} and IgG1⁺GFP^{lo} memory B cells) or two to three mice per group (IgG1⁺GFP⁺ memory B cells) (mean $\pm$ s.e.m.).

METHODS

***N. brasiliensis* infection.** Larvae of *N. brasiliensis* (R. Locksley) were isolated from the feces of infected Lewis rats (Charles River). Mice were infected subcutaneously with 500 L3 larvae in 200 mL PBS.

Antibodies and flow cytometry. The following monoclonal antibodies and reagents were used for flow cytometry: Pacific Blue–conjugated antibody to anti-mouse B220 (RA3-6B2; BD Bioscience), allophycocyanin–indotricarbocyanine–conjugated antibody to mouse IgD (11-26c.2a; BioLegend), biotin–conjugated antibody to mouse GL7 (GL7; eBioscience), phycoerythrin–indotricarbocyanine–conjugated anti-mouse CD95 (Jo2; BD Bioscience), Alexa Fluor 700–conjugated antibody to mouse CD38 (90; eBioscience), allophycocyanin–conjugated antibody to mouse IgG1 (A85-1; BD Bioscience), phycoerythrin–conjugated antibody to mouse IgE (R35-72; BD Bioscience), streptavidin–V500 (BD Bioscience) and 7-amino-actinomycin D (eBioscience). Cells were analyzed on an LSR II (Becton Dickinson) or were sorted on a FACS Aria (Becton Dickinson). Data were processed with FlowJo software (TreeStar).

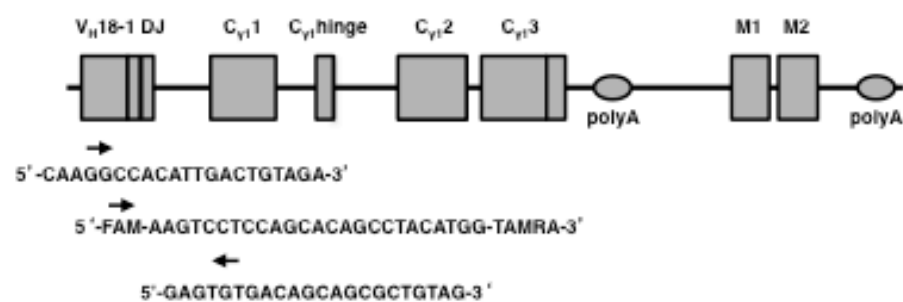
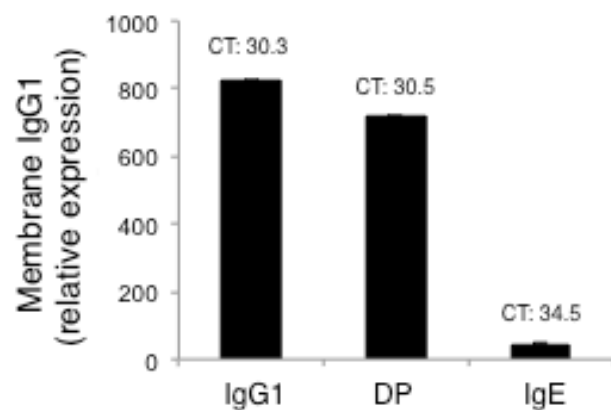
Quantitative real-time PCR analysis of transcripts encoding membrane IgG1 and membrane IgE. Total RNA was prepared by an RNeasy protocol (Qiagen) with DNase treatment to remove genomic DNA contamination from cells sorted from lymph nodes of IgE-GFP reporter mice ($n = 39$) at 15 d after infection with *N. brasiliensis*. Cells were first gated as B220⁺IgD⁺GL7⁺CD95⁺CD38[−] GC B cells and were then sorted into IgG1⁺GFP[−], IgG1⁺GFP^{hi} and IgG1[−]GFP^{hi} cell populations. Sequences for real-time PCR analysis of membrane IgG1 were as follows: forward primer, 5′-CAAGGCCACATTGACTGTAGA-3′; probe, 5′-FAM-AAGTCCTCCAGCACAGCCTACATGG-TAMRA-3′; and reverse primer, 5′-GAGTGTGACAGCAGCGCTGTAG-3′. Sequences for real-time PCR analysis of membrane IgE were as follows: forward primer, 5′-CAAGGCCACATTGACTGGTAGA-3′; probe, 5′-FAM-TCTGAATACCAGGTCACAGT-TAMRA-3′; and reverse primer, 5′-AGTTCACAGTGCTCATGTTTCAG-3′. The approximate cell input for each assay was as follows: 4×10^4 IgG1⁺GFP[−] cells, 1×10^4 IgG1⁺GFP^{hi} cells, and 1×10^4 IgG1[−]GFP^{hi} cells. For each assay, results were normalized to those of control transcription of the gene encoding ribosomal protein L19, and relative transcription was determined by calculation of the change in cycling threshold (ΔC_t) as $2^{-\Delta C_t}$, with reference to an absolute baseline cycling threshold of 40 cycles.

Adoptive-transfer experiments. B220⁺IgD⁺GL7⁺CD38⁺ memory B cells were sorted from the pooled lymph nodes and spleens of IgE-GFP mice ($n = 30$ – 40) at day 35 after infection with *N. brasiliensis*. Memory T cells were sorted from the pooled lymph nodes and spleens of C57BL/6 mice ($n = 20$) at day 35 after infection with *N. brasiliensis*. Approximately 1.4×10^4 memory B cells, along with 1×10^5 memory T cells and 1×10^6 splenocytes from mice deficient in recombination-activating gene 2 (as carrier cells), were injected intravenously (in 100 μ L sterile PBS) into μ MT recipient mice ($n = 2$ mice per group for IgG1⁺GFP^{hi} and IgG1[−]GFP^{hi} memory B cells; $n = 2$ – 3 mice per group for IgG1⁺GFP[−] memory B cells). Recipient μ MT mice were infected with *N. brasiliensis* 1 d after cell transfer.

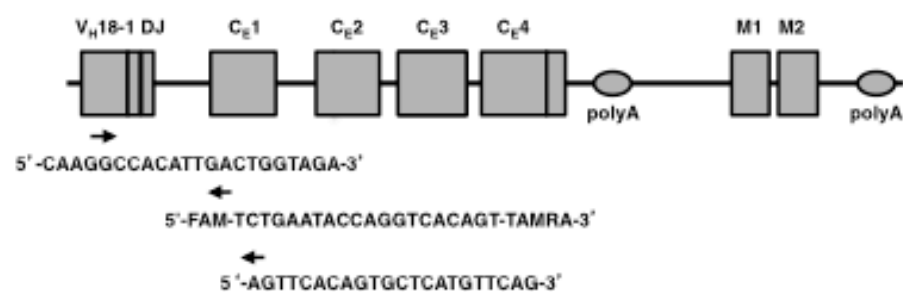
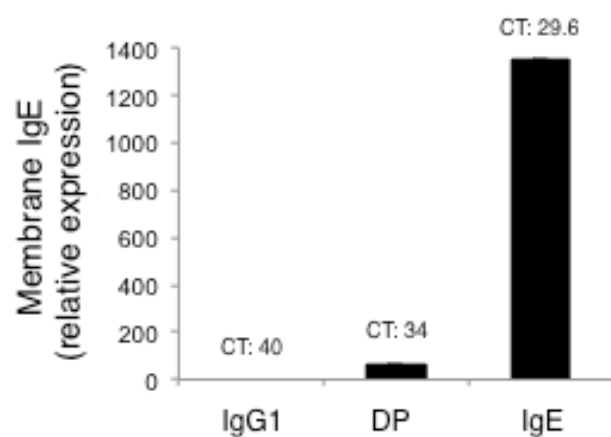
Enzyme-linked immunosorbent assay of IgE and IgG1. Mouse serum IgE was measured with a mouse IgE ELISA set according to the manufacturer's instructions and the manufacturer's IgE standard (BD Biosciences Pharmingen). Mouse serum IgG1 was measured with purified antibody to mouse IgG1 (A85-3; BD Bioscience), biotin–conjugated antibody to mouse IgG1 (A85-1; BD Bioscience) and streptavidin–horseradish peroxidase (BD Bioscience Pharmingen) with a purified mouse IgG1 standard (BD Bioscience).

Statistical analysis. *P* values were calculated with JMP statistical software (SAS).

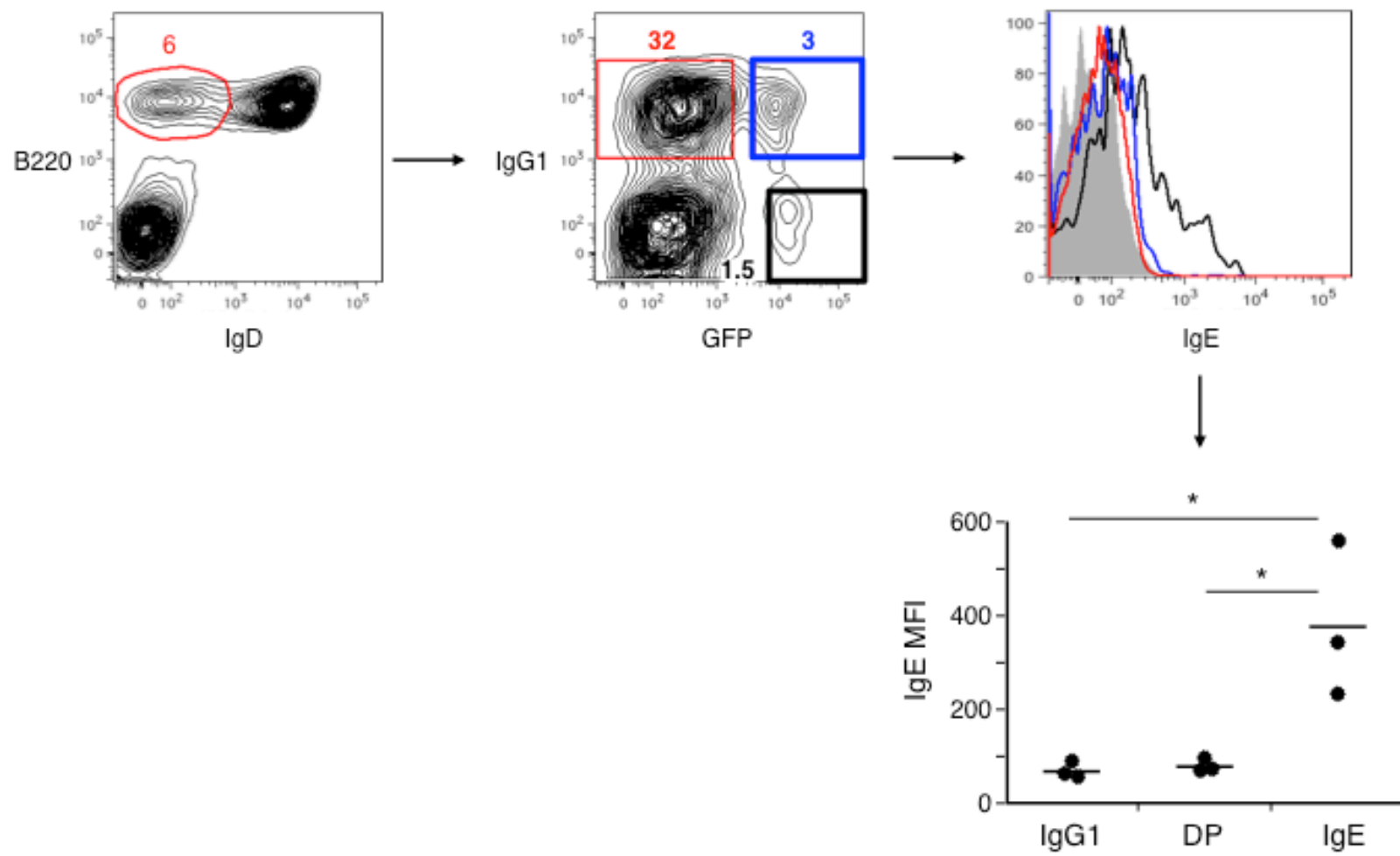
a



b



Supplementary Figure 1. Quantitative real-time PCR analysis of membrane IgG1 and membrane IgE transcripts in IgG1⁺ GFP⁻ (IgG1), IgG1⁺ GFP^{hi} (DP), and IgG1⁻ GFP^{hi} (IgE) cells sorted from pooled mesenteric and mediastinal lymph nodes of IgE-GFP reporter mice 15 days after infection with *N. brasiliensis*. **(a)** Membrane IgG1 transcript. **(b)** Membrane IgE transcript. Cells were first gated as B220⁺ IgD⁻ B cells, then as GL7⁺ CD95⁺ CD38⁻ germinal center B cells, and then separated based on IgG1 and GFP expression. The location and sequences of primers and probes for the membrane IgG1 and membrane IgE assays are as shown in the figure. Approximate cell input for each assay was as follows: 4x10⁴ IgG1 cells, 1x10⁴ DP cells, and 1x10⁴ IgE cells. Data are plotted as relative expression, with the normalized cycling threshold (CT) value denoted above each bar graph. For each assay, results were normalized to those of control transcription of the gene encoding ribosomal protein L19, and relative transcription was determined by the calculation $2^{-\Delta CT}$ with reference to an absolute baseline CT of 40 cycles. The graph depicts the mean $\pm$ s.e.m. of two technical replicates.



Supplementary Figure 2. Membrane IgE staining of IgG1⁺ GFP⁻ (red, IgG1), IgG1⁺ GFP^{hi} (blue, DP), and IgG1⁻ GFP^{hi} (black, IgE) cells from mesenteric lymph nodes of IgE-GFP reporter mice at 12 days after infection with *N. brasiliensis*. At this time point, almost all B220⁺ IgD⁻ cells are germinal center B cells. Membrane IgE staining with an anti-IgE antibody was performed after treatment of cells with an acid buffer to first remove serum IgE bound to the B cells through the low affinity IgE receptor CD23. The graph shows the results of three measurements of the mean fluorescence intensity (MFI) of each cell population, with a line drawn at the mean value of the three measurements. * p=0.01 using a Student's t-test for each pair-wise comparison.

1.txt

Figure 3
IgE MFI

IgG1	DP	IgE
56,1	70,4	233
62,9	72,4	342
89,2	96,7	561