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

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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 μ MT 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](#).

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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](#).

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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^{hi} 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^{hi} 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^{hi} 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).