

Construction of an sIgE:FLAG-mIgE:GFP Reporter Mouse Strain

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Key Words

Antigen receptor · IgE · Gene targeting · Reporter mouse strain

Abstract

Like all other immunoglobulins, IgE can be secreted into the blood or expressed as a membrane receptor on the surface of B lymphocytes. Secreted immunoglobulins trace the antigen and contribute to its destruction. Membrane immunoglobulins accompany the B cell along its differentiation pathway, regulating processes like the induction and maintenance of immunological memory and differentiation of plasma cells. The regulation of the expression of IgE is very complex. A lot of positive and negative regulators influence the synthesis of IgE. In previous publications, we were able to show that the membrane IgE (mIgE) antigen receptor itself controls the quantity and quality of serum IgE produced. However, the knowledge about the regulatory function of the antigen receptor on these processes is at best limited. In the present paper, we present the construction of a reporter mouse strain, which will help us to follow an mIgE-bearing B cell during the immune response more precisely.

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Introduction

Depending on their differentiation stage or activation state, B cells can express different immunoglobulin heavy chain isotypes. However, under all conditions, expression of the membrane immunoglobulin (mIg) is fundamental for all further differentiation steps. Immature B cells in the bone marrow express only mIgM [1]. After a specificity check [2, 3] of the receptor and the removal of autospecificities, B cells exit from the bone marrow into the periphery and undergo further maturation steps towards becoming fully immuno-competent, mature B cells. The expression of a second membrane-bound immunoglobulin, mIgD, seems to signal the successful completion of this process. In answer to antigen contact and a specific cytokine milieu, mature B cells undergo an isotype switch to IgGs, IgE or IgA [4, 5].

Among all isotypes, IgE has a unique position. IgE was the last of the immunoglobulins to be detected [6, 7]. It is present in very low amounts (in the range of nano- to micrograms per millilitre) in the serum of healthy individuals and normal laboratory mouse strains and has a very short half-life [8]. This contrasts with the other immunoglobulin classes, which are present in much higher concentrations (in the range of micro- to milligrams per mil-

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lilitre) and form a substantial component of serum proteins. However, like all other immunoglobulin isotypes, IgE may exist in two forms. In the first form, it is integrated into the plasma membrane and expressed as a B cell receptor on the cell surface, while in the other, it is secreted as a soluble antibody in tissue fluids and blood. At the molecular level, little is known about regulatory factors determining the expression of mIgE and the resulting magnitude and quality of the IgE-mediated immune response. Previous data from our laboratory [9–11] suggest a different regulation of mIgE expression in comparison to all other immunoglobulin isotypes. In contrast to mIgG1, which can easily be detected on B cells shortly after the induction of a class switch, it is very difficult to detect mIgE under these conditions. Even in ϵ -transgenic mice, hardly any mIgE was found [12]. However, detection of mIgE-bearing B cells is not only difficult because of the very low number of these cells. Antibodies specifically recognizing mIgE are not yet available, which means that in FACS experiments, one must be very careful to discriminate mIgE-carrying B cells from B cells presenting serum IgE bound via CD23, the low-affinity IgE receptor [13]. To bypass these problems, we developed a reporter mouse strain (sIgE:FLAG-mIgE:GFP), in which we primarily fused the green fluorescence protein (GFP) reporter protein to the cytoplasmic domain of the mIgE antigen receptor. Because of GFP's fluorescence, mIgE-bearing B cells could now doubtlessly be detected in FACS experiments. A second reporter element, the FLAG tag, was fused to the last constant exon of the secreted form. The advantage of this marker is that after adoptive transfer of mIgE-GFP cells and subsequent re-stimulation, secreted IgE expressed by these transferred cells is distinguishable from endogenous secreted IgE via the FLAG tag. This will be a useful tool to perform experiments investigating the kinetics of re-stimulation and to analyse the IgE memory response more precisely. In summary, the employment of the reporter mouse strain will help to understand the role of mIgE B cells during an immune response more precisely.

Materials and Methods

Construction of the Target Vector

The target vector consists of 5,830 base pairs (bp) of genomic DNA, spanning a region from 2,830 bp upstream of the M2 to 3 kb downstream of the M2 exon (fig. 1, 2). Polymerase chain reaction (PCR) was used to fuse constant exon 4 with the FLAG tag. To introduce the GFP reporter, an *Nco/Not* site was introduced by PCR after the M2 exon. Between M2 and GFP, 10 leucines and 1 proline were

introduced by PCR. An *Sma* I site, 3' of exon M2, was used to introduce a selection cassette consisting of the neomycin resistance gene and the herpes simplex virus thymidine kinase gene, flanked by Cre-recombinase recognition (loxP) sequences. Targeting was performed as described before [9].

Examination of Correct Splicing

For verification of correct splicing, the 5'-*Xma*-*Hind*III fragment containing VDJ-CH1-CH2-CH3 was fused with the genomic sequence CH4-Flag-M1-M2-Leu spacer-GFP (fig. 1, 2). K46 cells were transfected, RNA was isolated and cDNA was constructed using oligo(dT) priming. RT-PCR was performed with the following primers: 201: 5'-GAGGCACTTCAGAAACCCAGGAAACT-3'; 206: 5'-TGGACCTGGAAAGCGAGAAGAATG-3'; 207: 5'-ACGCCGT-AGGTCAGGGTGGTCA-3'; 209: 5'-CCCTCGCCGGACACGCTGA-3'. PCR products were re-cloned and sequenced.

ELISA Assays and Spleen B Cell Induction with Lipopolysaccharide and Interleukin-4

Splenocyte induction and ELISA experiments, as well as antibody usage for IgE detection, were performed as described in Achatz et al. [9].

FACS Analysis

Lipopolysaccharide (LPS)- and interleukin-4 (IL-4)-treated splenocytes were harvested after 6 days. 1×10^6 cells were stained with PE (fluorescence channel [FL]-2)-labelled anti-CD19 and FITC (FL-1)-labelled rat anti-mouse IgG1 or FITC-labelled rat anti-mouse IgE. Because of GFP's fluorescence (FL-1), splenocytes of mIgE-GFP reporter mice were only stained with anti-CD19-PE. Viability of the cells was checked by adding propidium iodide (FL-3) directly before FACS analysis. For detection of mIgE-GFP+ cells in mesenteric lymph nodes, double-boosted Schistosoma egg-immunized mice (day 99) were dissected. After homogenization of the lymph nodes, cells were stained with antibodies as described before. To get rid of cells presenting IgE via CD23, cells were stripped by acid hydrolysis as described before [13].

Schistosoma Egg Immunization

Mice were immunized intraperitoneally on day 0, 14 and 74 with 5,000 inactivated eggs of *Schistosoma mansoni*. Schisto-specific immunoglobulin serum concentrations were measured by coating ELISA plates with Schisto antigen.

Results

Functional Splicing of the 'Reporter Elements' in vitro

For the construction of the reporter mouse strain, we decided to use two 'reporter elements'. First, we fused the green fluorescence protein (EGFP) from the jellyfish *Aequorea victoria* to the cytoplasmic tail of mIgE (fig. 1a). To guarantee full accessibility of the cytoplasmic tail, the M2 exon and GFP were separated by a spacer sequence, coded by 10 leucine amino acids followed by 1 proline. This configuration was calculated (Lasergene software package) as the best conformational secondary structure.

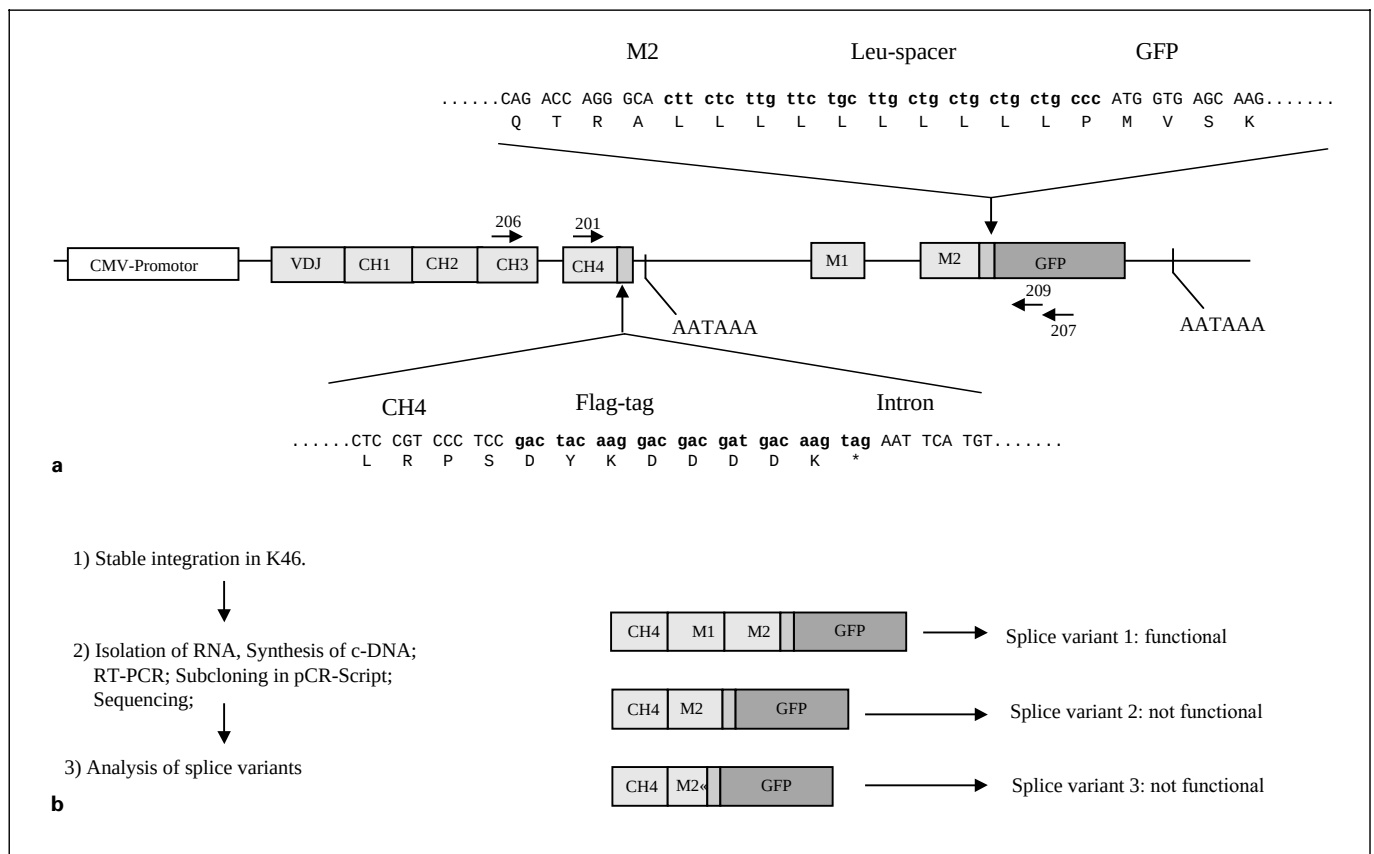


Fig. 1. a For determination of functional transcriptional expression, spliced NP-specific VDJ, CH1, CH2 and CH3 exons were cloned together with genomic DNA harbouring CH4-FLAG, followed by membrane exon 1 and membrane exon 2-LEU-GFP reporter gene. **b** The construct was re-cloned in vector pCMV-Script (Stratagene) and stably transfected in K46 lymphoma cells. Re-cloning of transcripts by RT-PCR (primer locations 206–209 are indicated) and sequencing led to the isolation of three splice variants. Variant 1 expressed the correct transcriptional product, whereas variants 2 and 3 lacked exon M1. The direct splicing of CH4 to M2 (or M2') resulted in a frame shift.

Second, we fused the FLAG tag to the 3' end of the secretory part of constant exon 4.

To test correct splicing and functionality of the reporter elements, we constructed an expression vector (pGA171) consisting of a recombined NP-specific VDJ antigen recognition part fused to spliced constant exons CH1, CH2 and CH3, followed by genomic DNA consisting of the CH4 fused to the FLAG tag, membrane exon 1 (M1) and membrane exon 2 (M2) with the leucine spacer and the GFP reporter gene (fig. 1a). This construct was cloned in vector pCMV-script (Stratagene) and stably transfected into the B cell lymphoma line K46. RNA was isolated and reverse transcribed into cDNA using an oligo(dT) primer. To test correct splicing, the first-strand cDNA was amplified by nested PCR. PCR products were

subcloned in pCR-Script cloning vector (Stratagene) and sequenced. Three splice variants could be detected (fig. 1b). Splice variant one represents a fully functional mIgE cDNA with the Leu spacer and the GFP reporter fused in frame to the cytoplasmic tail of IgE. Thus, the FLAG and the GFP fusion did not influence the accessibility of the splice donor and splice acceptor sites in the targeted gene. Splice variant two lacks the complete transmembrane domain, thus showing the M2-Leu-GFP cassette directly spliced to constant exon 4. Splice variant three lacks the transmembrane domain too, but additionally uses a splice acceptor site located inside exon M2, leading to a truncated M2-Leu-GFP product. However, direct splicing of constant exon 4 to membrane exon 2 in variants 2 and 3 results in a frame shift. The reporter cas-

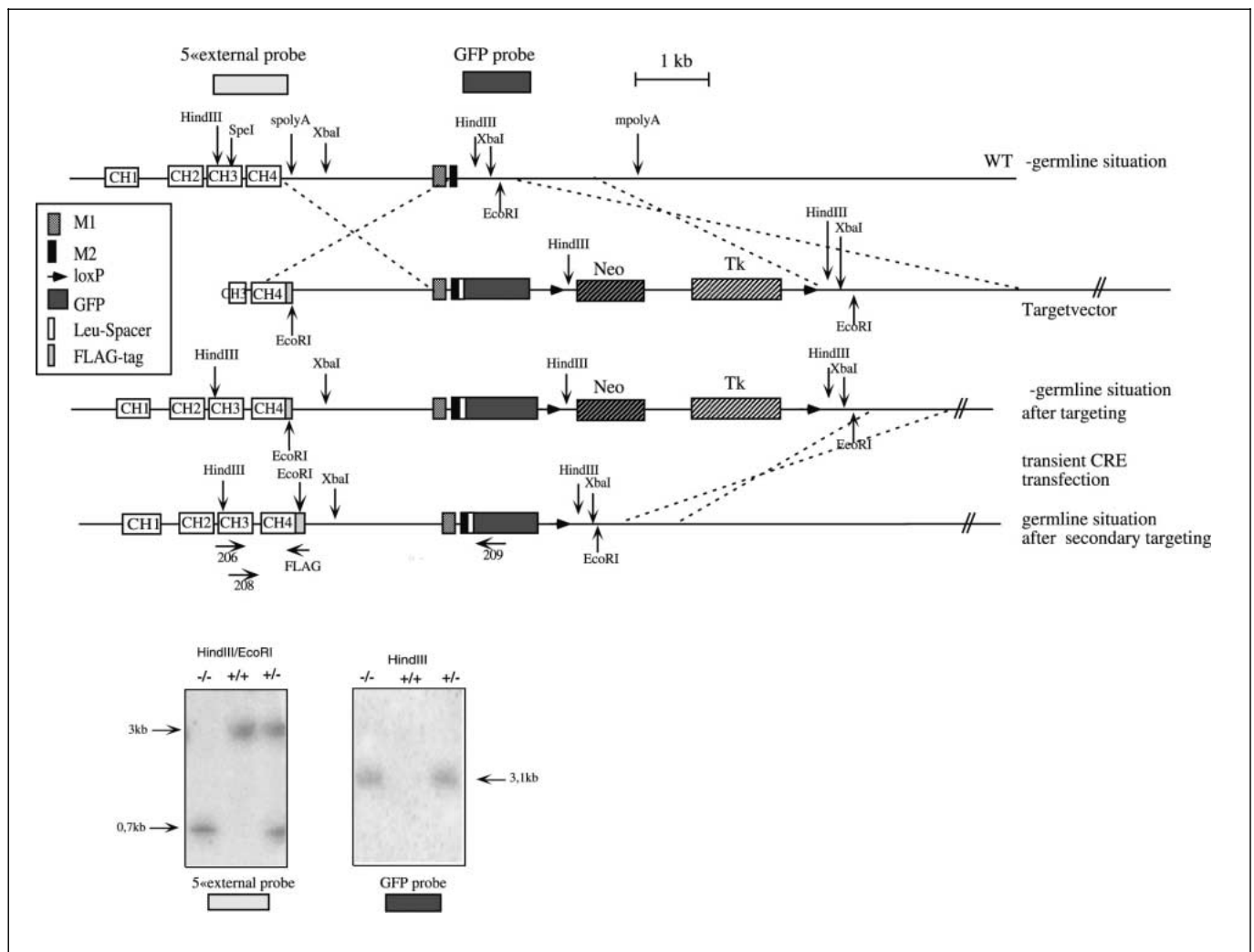


Fig. 2. Construction of the target vector. Reporter elements were introduced as described in figure 1. The 3' end of M2-LEU-GFP, a selection cassette consisting of the neomycin resistance gene for positive selection and the thymidine kinase gene for negative selection, flanked by two loxP sites, were introduced. Balb/C ES cells were transfected with the target vector. Heterozygous targeting events were checked by nested PCR. After Cre transfection, the selection cassette was lost. Positive ES cells were injected into C57/Bl6 blasto-

cysts, followed by re-implantation into foster mothers. To check germ line transmission, chimaeric offsprings were crossed with WT Balb/C mice. Heterozygous mice of the F1 generation were crossed for homozygosity. Southern analysis was performed by digesting genomic DNA, isolated from mouse tails, and probing with a 5' external and a GFP DNA probe. Southern analysis showed clean homozygous targeting events.

sette will not be expressed correctly. All splice variants were in full agreement with variants published by Anand et al. [14].

We continued the project with the construction of a gene targeting vector expressing the reporter cassettes as described before (fig. 2).

Construction of the sIgE:FLAG-mIgE:GFP Reporter Mouse

Targeting was performed using a Cre-loxP-mediated gene targeting technique [15, 16]. To check homologous recombination after the primary targeting event, 2,500 ES cell clones were analysed by nested PCR. Eight positive PCR clones were further tested by Southern analyses (fig. 2). Six of the 8 clones showed a correct homologous recombination. Southern-positive clones were re-trans-

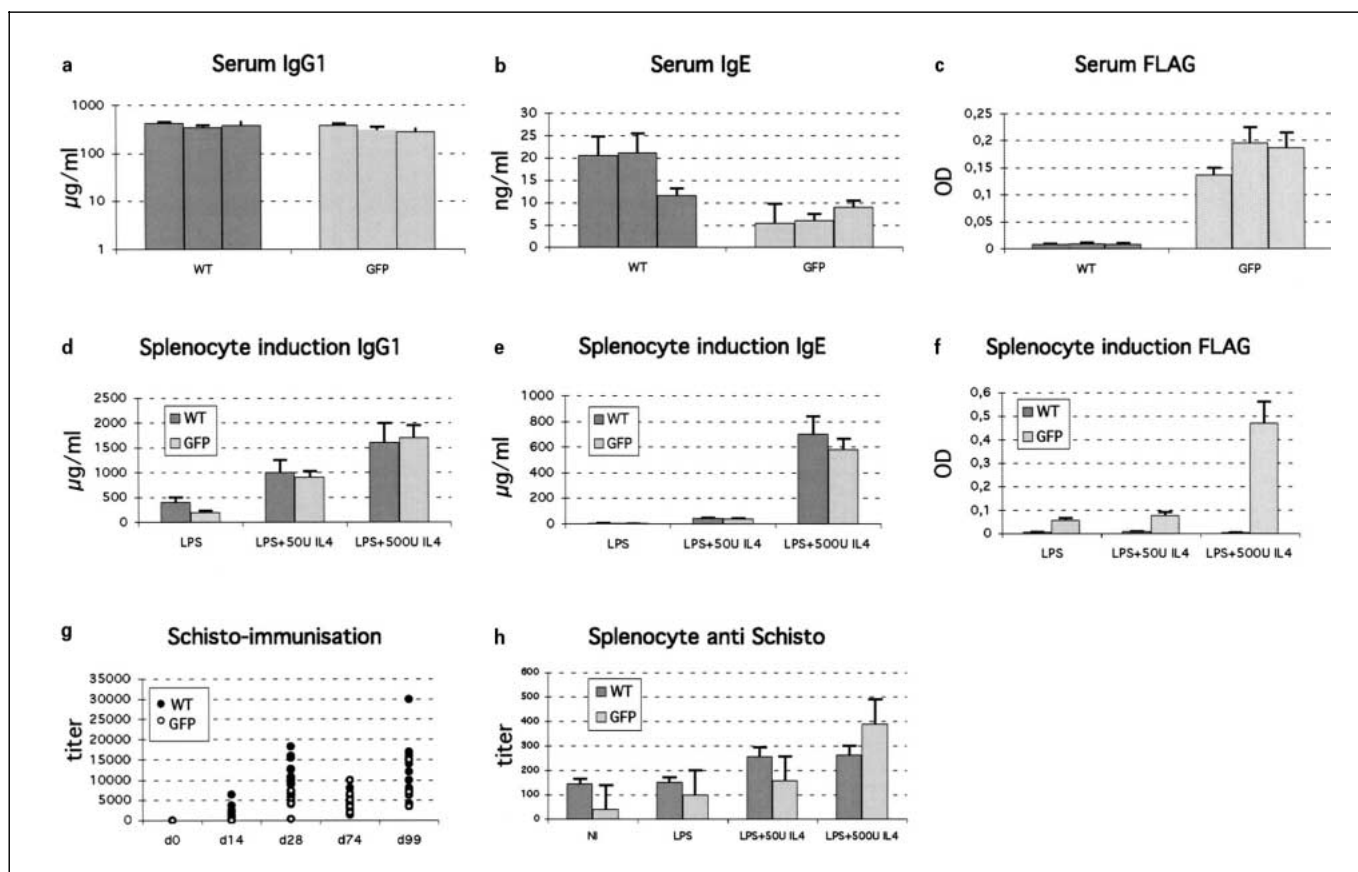


Fig. 3. Determination of the IgG1 and IgE levels of WT and reporter mice. While serum IgG1 (**a**) was comparable in WT and reporter mice, serum IgE was reduced by 60% in reporter GFP mice (**b**). However, reporter mice show a clear expression of 'flagged' IgE antibodies (**c**). Splenocytes of WT and reporter mice were isolated and stimulated with LPS and IL-4. Secreted IgG1 (**d**), IgE (**e**) and 'flagged' IgE (**f**) was measured in the cell culture supernatant. Under these in vitro

conditions, the IgE expression in reporter mice was comparable with that in WT mice. Only reporter mice showed expression of 'flagged' IgE (**c**, **f**). WT and reporter mice were infected with inactivated eggs of the parasite *S. mansoni* and the immune response was measured over a period of 99 days. The block of IgE production could be partially bypassed by the strong Th2 response (**g**). After splenocyte induction, WT and reporter mice showed comparable titres (**h**).

infected with a Cre-recombinase-expressing plasmid and negatively selected for the loss of the selection cassette. Positive ES clones were injected into C57/Bl6 blastocysts as described before [9]. Chimaeric mice were bred for hetero- and homozygosity and finally tested by Southern blot analysis (fig. 2).

Serum Immunoglobulin Level of Wild-Type and Reporter Mice

In the following experiments, we measured the serum levels of IgG1 and IgE in 8-week-old unprimed wild-type (WT) and sIgE:FLAG-mIgE:GFP reporter mice. As expected, we measured no difference in the IgG1 level between WT and reporter mice (fig. 3a). However, the

serum IgE level in reporter mice was reduced by 60%, indicating an influence of the GFP fusion on the signalling capacity (fig. 3b). Interestingly, this difference could be bypassed in vitro, when we incubated splenocytes with LPS and 50 or 500 units of recombinant IL-4 (fig. 3e). In the supernatant of the induced splenocyte culture, equal amounts of secreted IgG1 (fig. 3d) and IgE (fig. 3e) could be detected. The functionality of the FLAG tag fused to the secretory part of IgE was proven by ELISA experiments, in which we identified 'flagged' IgE antibodies in the serum as well as in the supernatant of stimulated splenocytes (fig. 3c, f).

We were also interested in the IgE immune response of WT and reporter mice to immunizations with inactivated

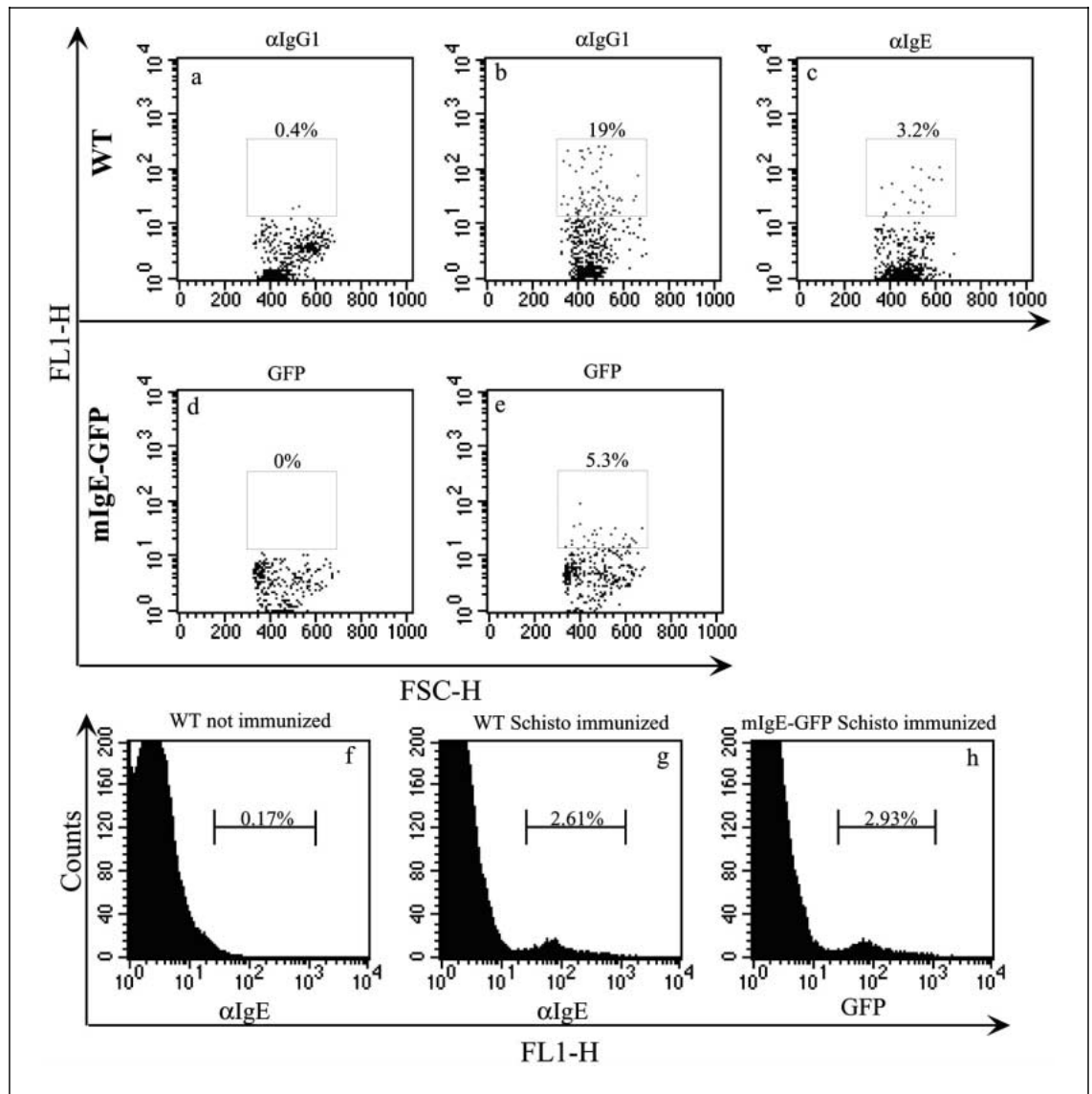


Fig. 4. Splenocytes were incubated with LPS and 500 U of IL-4 for 6 days. IgG1 and IgE populations were gated for living (propidium iodide-negative) CD19⁺ cells. Uninduced WT splenocytes expressed 0.4% mIgG1⁺ cells (**a**). Induced splenocytes expressed 19% mIgG1⁺ (**b**) and 3.2% mIgE⁺ (**c**) cells. Uninduced splenocytes of reporter mice expressed no mIgE-GFP⁺ cells, whereas induced splenocytes expressed 5.3% mIgE-GFP⁺ cells. Mesenteric lymph nodes of unprimed WT mice showed a minor population of mIgE⁺ lymphocytes (**f**). Schisto egg-immunized WT and reporter mice presented a population of 2.61% mIgE⁺ cells (**g**) and 2.93% mIgE-GFP⁺ lymphocytes.

eggs of the parasite *S. mansoni*. *S. mansoni* induces a strong T helper 2 (Th2) response, resulting in a class switch to IgG1 and IgE. We measured the Schisto-specific serum IgE level over a period of 99 days (fig. 3g). A group of seven WT and seven reporter mice were immunized with 5,000 inactivated Schisto eggs and boosted on day 14 and day 74. WT mice showed a clear secondary response

to schistosome eggs. However, the Schisto-specific IgE serum level decreased within a short period. On re-infection, a strong increase in specific IgE antibodies could be detected during the memory response. The reduced IgE level in the reporter mice was not as significant as in unprimed mice (fig. 3a). We concluded that the strong Th2 response partially bypassed the block of IgE synthe-

sis. Interestingly, the rapid decrease in Schisto-specific IgE antibodies could not be observed in the period after day 28, nor could we find a remarkable increase in specific IgEs during the memory response. The reduction of the Schisto-specific IgE level could again be bypassed in vitro when we stimulated splenocytes with LPS and IL-4 (fig. 3h).

FACS Analysis of Cells from WT and Reporter Mice

To detect mIgG1+, mIgE+ and mIgE-GFP+ cells, FACS analysis was performed under the same experimental conditions as those used for the ELISA experiments. First, we were interested in the frequency of mIgG1+ and mIgE+ cells in uninduced and induced splenocytes of naïve WT and reporter mice. Presented FACS data were gated for living, CD19-positive cells. Additionally, cells were stripped by acid hydrolysis. In unstimulated splenocytes of WT mice, a very small population of mIgG1+ cells (fig. 4a) and no mIgE+ cells (data not shown) could be detected. Six days after incubation with LPS and 500 U of IL-4, a population of 19% mIgG1+ (fig. 4b) and 3.2% mIgE+ (fig. 4c) could be observed. In reporter mice, no mIgE-GFP+ cells were detected in unstimulated (fig. 4d) splenocytes. However, under stimulated conditions, 5.3% mIgE-GFP+ cells were observed (fig. 4e).

We next tried to stain mIgE+ and mIgE-GFP+ B cells directly in lymphatic tissues. We dissected mesenteric lymph nodes of three double-boosted Schisto egg-immunized mice and compared the number of mIgE+ cells with those in unprimed mice. In mesenteric lymph nodes of unprimed WT mice (fig. 4f), only 0.17% of the living CD19-positive population was positive for mIgE. In immunized WT mice, a population of 2.61% mIgE+ cells (fig. 4g) could be observed. This population correlated with the mIgE-GFP+ population (fig. 4h) of Schisto egg-immunized reporter mice.

Discussion

We have chosen a genetic approach to evaluate the role of mIgE immunoglobulins. We constructed a mouse line where we fused the FLAG tag to the secretory part of IgE and the GFP reporter protein to the cytoplasmic tail of mIgE. This mouse strain will be a useful tool to follow mIgE-expressing B cells during an IgE immune response in vivo. In independent experiments, we were able to clearly show that the reporter elements are functionally expressed in vitro and in vivo. As previously published by our group [9], the IgE antigen receptor is the prerequisite

for the expression of secreted IgE. We point to this conclusion because without expression of mIgE-GFP+ cells in homozygous reporter mice, it would never have been possible to detect 'flagged' IgE antibodies. Thus, it becomes clear that membrane expression of mIgE-GFP is not impaired in the reporter mouse strain. However, the reduction of the serum IgE level in the reporter mice, in our view, reflects a problem in the signalling capacity of the whole mIgE-GFP antigen receptor complex. Interestingly, the induction of a strong Th2 response partially bypassed the block of serum IgE synthesis. With this experimental background, we are convinced that we will be able to study the induction and maintenance of the IgE memory response in the future.

In general, immunological memory is defined as the ability to generate a more effective immune response after a second encounter with antigen [17]. However, little is known about the generation and maintenance of immunological memory in general and the development of memory IgE cells in particular. Immunological memory is characterized by an antibody response, which occurs more rapidly, is of greater magnitude, is long-lived and is characterized by the secretion of antibody of higher than average binding affinity and of classes of immunoglobulin other than IgM [18]. The mechanisms underlying the generation of memory B and also memory T cells have long been controversial. Two theories have been suggested. One theory is that precursors of antibody-forming plasma cells and memory cells are derived from a common B cell. Implicit in this theory is that during antigen-driven cell division, some daughter cells of a given B cell clone differentiate into plasma cells and others do not. When the supply of antigen is exhausted, generation of more plasma cells ceases and the dividing B cells return to their G0 resting state and become memory cells. An opposing theory states that different precursor B cells are programmed to give rise to memory cells or plasma cells within a clone prior to antigen exposure. In both cases, it remains to be determined how commitment to each pathway is regulated. Our reporter system could help to follow an mIgE B cell during an immunization reaction, thus supporting one or the other theory.

Another important question that needs to be answered is what determines the life span of a memory cell. In the case of some vaccines and infections, subsequent immunity can last for the lifetime of the host. In other cases, it lasts a few months to a few years. B and T memory cells were originally thought to remain in a resting state indefinitely. There is evidence that in humans, some memory T cells can remain in the resting state without cell division

for at least 30 years [19]. Other studies indicate that antigen, perhaps in the form of immune complexes, remains on follicular dendritic cells for long periods of time, leading to continued rounds of low-level activation of B cells [20]. A third possibility is that long-term memory is maintained by periodic exposure of memory T and/or B cells to cross-reactive antigens [21], super antigens or mitogens, leading to limited rounds of clonal expansion. Our reporter system will also be able to follow the life span and apoptosis of cells because protein degradation could easily be detected by this system. This would also enable us to investigate the long-standing question of the role of the mIgE-carrying B cell during antigen presentation. It is generally agreed that memory B cells are generated in the germinal centres of the lymph nodes and spleen. They then enter marginal zones and re-circulate in the lymph. Finally, long-term memory cells appear to return to the

bone marrow, which is a highly immunosuppressive environment such that antigens do not readily activate these cells [22–24]. This might provide a mechanism for preserving memory cells, i.e. preventing them from being used up by modest antigenic exposure.

In summary, the employment of an mIgE-GFP mouse line will help us to understand the role of mIgE cells during an immune response and will perhaps also help us to understand the mechanism of allergic reactions more precisely.

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