

Tracking germinal center B cells expressing germ-line immunoglobulin γ 1 transcripts by conditional gene targeting

Stefano Casola^{*†‡}, Giorgio Cattoretti[§], Nathalie Uyttersprot^{*¶}, Sergei B. Koralov^{*}, Jane Seagal^{*}, Zhenyue Hao^{*||}, Ari Waisman^{***}, Angela Egert^{*‡}, Dvora Ghitza^{*}, and Klaus Rajewsky^{*†‡}

^{*}CBR Institute for Biomedical Research, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115; [†]Institute for Genetics, University of Cologne, Weyertal 121, D-50931 Cologne, Germany; and [§]Institute for Cancer Genetics and the Herbert Irving Comprehensive Cancer Center, Columbia University, 1150 Saint Nicholas Avenue, New York, NY 10032

Contributed by Klaus Rajewsky, March 23, 2006

Germinal centers (GCs) represent the main sites for the generation of high-affinity, class-switched antibodies during T cell-dependent antibody responses. To study gene function specifically in GC B cells, we generated $C\gamma$ 1-cre mice in which the expression of Cre recombinase is induced by transcription of the Ig γ 1 constant region gene segment ($C\gamma$ 1). In these mice, Cre-mediated recombination at the *fas*, *Ig β* , *IgH*, and *Rosa26* loci occurred in GC B cells as early as 4 days after immunization with T cell-dependent antigens and involved >85% of GC B cells at the peak of the GC reaction. Less than 2% of IgM⁺ B cells showed Cre-mediated recombination. These cells carried few Ig somatic mutations, expressed germ-line $C\gamma$ 1- and activation-induced cytidine deaminase-specific transcripts and likely include GC B cell founders and/or plasma cell precursors. Cre-mediated recombination involved most IgG1, but also a fraction of IgG3-, IgG2a-, IgG2b-, and IgA-expressing GC and post-GC B cells. This result indicates that a GC B cell can transcribe more than one downstream C_H gene before undergoing class switch recombination. The efficient induction of Cre expression in GC B cells makes the $C\gamma$ 1-cre allele a powerful tool for the genetic analysis of these cells, as well as, in combination with a suitable marker for Cre-mediated recombination, the tracking of class-switched memory B and plasma cells *in vivo*. To expedite the genetic analysis of GC B cells, we have established $C\gamma$ 1-cre F₁ embryonic stem cells, allowing further rounds of gene targeting and the cloning of compound mutants by tetraploid embryo complementation.

class switch recombination | Cre recombinase | memory B | plasma cells

During T cell-dependent (TD) antibody responses, germinal centers (GCs) are formed in secondary lymphoid organs (1). GCs result from an orchestrated series of cellular interactions between antigen-specific B and CD4 T helper (Th) cells starting at the border between B and T cell areas and continuing at later stages in close proximity to the follicular dendritic cell (FDC) network within established GCs (2–5). During the GC reaction, rapidly proliferating B cells accumulate nontemplated point mutations (and less commonly deletions or duplications) within their rearranged Ig heavy (H) and light (L) chain variable region genes at a high rate (6). This process, which is initiated by activation-induced cytidine deaminase [AID; (7)], leads to the establishment of a pool of mutated B cells, expressing a repertoire of B cell receptor (BCR) specificities. From this pool, a subset of cells, namely those recognizing antigen on FDCs through their high-affinity BCRs, is selected into the compartment of long-lived resting memory-B and antibody-secreting plasma cells, whereas the majority of the remaining cells with low-affinity BCRs or lacking BCR expression because of crippling mutations in rearranged Ig genes die by apoptosis (8).

GCs also represent sites where B cells undergo Ig class switch recombination (CSR), leading to the replacement of the $C\mu$ and $C\delta$ constant regions with those of other isotypes. Initiated by AID,

CSR is based on intrachromosomal recombination between DNA “switch” regions, one positioned upstream of $C\mu$ and the other preceding one of the downstream C_H isotypes (9). This deletional event often occurs in both IgH loci and involves, in most cells, the same downstream C_H isotype (10, 11). CSR is triggered in antigen-engaged B cells in response to cytokine stimulation and crosslinking of coreceptors, such as CD40, resulting from the interaction with antigen-specific Th cells. Targeting of CSR to a specific C_H locus is preceded by the transcription from that locus of “preswitch,” spliced, noncoding polyadenylated RNAs (also called “germ-line” transcripts), in response to Th1- or Th2-type cytokines (9).

Despite extensive experimentation in the past, the nature of the signals that influence proliferation, differentiation, and selection of B cells within the GC are still largely unknown. Cre/loxP-mediated conditional gene targeting provides a unique experimental approach to tackle these biological questions in the context of the whole animal. The present study characterizes the $C\gamma$ 1-cre knockin strain, a mouse mutant in which expression of Cre recombinase, induced upon onset of germ-line $C\gamma$ 1 transcription, promotes conditional gene targeting in the majority of GC B cells generated in response to immunization with TD antigens. Driving Cre expression from the $C\gamma$ 1 locus allowed us to track the fate of B cells undergoing sterile $C\gamma$ 1 transcription and thus to investigate to what extent this process controls the targeting of CSR *in vivo*.

Results

Generation of $C\gamma$ 1-cre Knockin Mice. $C\gamma$ 1-cre mice were generated from 129-derived ES cells in which an internal ribosome entry site (IRES) followed by the Cre-coding sequence was inserted into the 3' region of the $C\gamma$ 1 locus between the last membrane-coding exon and its polyadenylation sites (Fig. 1a). This approach allows for the expression from the $C\gamma$ 1 locus of a bicistronic mRNA consisting of the $C\gamma$ 1 and the Cre transcript, respectively. Results presented in this study were obtained from the analysis of $C\gamma$ 1-cre animals on the C57BL/6J genetic background.

Conflict of interest statement: No conflicts declared.

Abbreviations: GC, germinal center; CSR, class switch recombination; AID, activation-induced cytidine deaminase; R26, Rosa 26; Th, T helper; IRES, internal ribosome entry site; SPL, spleen; LN, lymph node; SRBC, sheep red blood cell; PNA, peanut agglutinin; STAT, signal transducer and activator of transcription; NP-CG, (4-hydroxy-3-nitrophenyl) acetyl coupled to chicken gamma globulin; BCR, B cell receptor; TD, T cell-dependent.

[†]To whom correspondence may be addressed. E-mail: stefano.casola@ifom-ieo-campus.it or rajewsky@cbr.med.harvard.edu.

[¶]Present address: Artemis Pharmaceuticals GmbH, Neurather Ring 1, 51063 Cologne, Germany.

^{||}Present address: The Campbell Family Institute for Breast Cancer Research, University Health Network, University of Toronto, Toronto, ON, Canada M5G 2C1.

^{***}Present address: I Medical Department, Johannes Gutenberg–University Mainz, Verfügungsgebäude, Obere Zahlbacher Strasse 63, 55131 Mainz, Germany.

© 2006 by The National Academy of Sciences of the USA

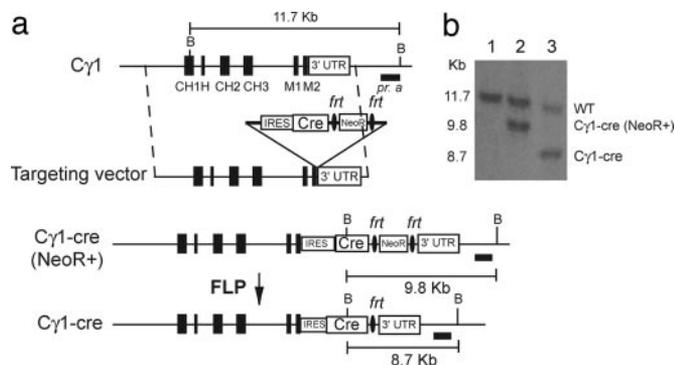


Fig. 1. Knockin of the Cre transgene into the $C\gamma 1$ locus. (a) Strategy to insert the IRES-Cre cassette into the mouse $C\gamma 1$ locus. The targeted locus is depicted before and after removal by FLP recombinase of the frt-flanked selection marker. BamHI (B) sites within the targeted genomic region are indicated. (b) Southern blot analysis, using probe a, of BamHI-digested tail DNA from WT (lane 1) and $C\gamma 1$ -cre heterozygous mice, before (lane 2) and after (lane 3) removal of the selection marker.

Induction of Cre-Mediated Recombination in $IgG1^+$ $C\gamma 1$ -cre B Cells *in Vitro*. Purified splenic B cells from C57BL/6J controls and $C\gamma 1$ -cre mice carrying a conditional *rag-2* allele (12) as a reporter gene were stimulated *in vitro* with LPS and IL-4 to promote IgG1 CSR. B cells treated with LPS alone served as negative controls. The fraction and absolute number of $IgG1^+$ B cells measured by flow cytometric analysis 4 days later was comparable between mutant and control cell cultures (data not shown). B cells from 4-day LPS and LPS plus IL-4 cultures were sorted based on surface IgM and IgG1 expression, respectively, and subjected to Southern blotting analysis. Southern blot quantification showed that 95% of $IgG1$ -switched B cells had undergone Cre-mediated recombination in $C\gamma 1$ -cre-bearing cells. In contrast, IgM^+ B cells retained the loxP-flanked ("floxed") *rag-2* gene in >90% of the cells (Fig. 2a).

Cell-Type and Stage-Specific Expression of the $C\gamma 1$ -cre Transgene. To study the efficiency of Cre-mediated recombination *in vivo* at the single-cell level, $C\gamma 1$ -cre mice were bred to the Rosa26 (R26)-EYFP reporter (R26-EYFP) strain (13). Flow cytometric analysis was performed to measure the fraction of EYFP⁺ cells within different B cell subsets isolated from spleen (SPL), lymph nodes (LNs), bone marrow (BM), and peritoneal cavity (PC) of the compound mutants. In BM, pro-B ($IgM^- B220^{lo} CD43^+$) and pre-B ($IgM^- B220^{lo} CD43^-$) cells lacked expression of the re-

porter gene. The same result was obtained when we analyzed transitional ($IgM^+ B220^{lo} AA41^+$) cells isolated from both BM and SPL (Fig. 2b and data not shown). Less than 2% of IgM^+ follicular ($CD23^+ CD21^+ CD38^+$) and marginal zone ($CD23^{lo} CD21^{hi} CD38^{hi}$) B cells expressed the reporter gene (Fig. 2b) in unimmunized compound mutants. Similar fractions of EYFP-expressing cells were observed among non-GC B-2 cells ($CD23^+ CD21^+ CD38^+$) in peripheral LNs (data not shown). Less than 1% of PC B-1 cells ($B220^{lo} CD19^{hi} CD43^+ IgM^{hi}$) showed EYFP expression (data not shown). The largest fraction of EYFP-expressing B cells was within the population of PNA^{hi}Fas^{hi}CD38^{lo} GC B cells in SPL and LN after i.p. immunization with TD antigens such as alum-precipitated (4-hydroxy-3-nitrophenyl) acetyl coupled to chicken gamma globulin (NP-CG) or sheep red blood cells (SRBC). Within the GC B cell fraction, 85–95% of these cells expressed the reporter gene 10–14 days after immunization (Fig. 2b). Furthermore, within the fraction of splenic $IgG1^+$ B cells, 85–95% of the cells with a GC B cell phenotype ($CD38^{lo} IgG1^{lo}$) and 70–80% of those identified as memory B cells ($IgG1^+ CD38^+$) had undergone Cre-mediated recombination, respectively (Fig. 2c). Immunofluorescence analysis of splenic histological sections prepared from $C\gamma 1$ -cre;R26-EYFP mice 10 days after SRBC immunization showed distinct areas expressing EYFP within $CD21^+$ B cell follicles that were intensively stained with the GC marker peanut agglutinin (PNA; Fig. 2d). EYFP⁺ GC B cells expressed the proliferation marker Ki-67 and were in intimate contact with the follicular dendritic cell network expressing high levels of the complement receptor CD21 (Fig. 2d).

Efficiency of Cre-mediated recombination in GC B cells was further tested by breeding $C\gamma 1$ -cre knockin animals to mice carrying conditional alleles for the death receptor fas (*fas^{fl}*) or the BCR signaling subunit $Ig\beta$ (*Ig $\beta^{fl-EGFP}$*). In the latter case, Cre-mediated recombination is monitored by induction of EGFP expression from the *Ig β* locus (Fig. 6, which is published as supporting information on the PNAS web site). After immunization with NP-CG or SRBC, >85% of GC-B and $IgG1^+$ memory B cells showed Cre-mediated recombination in both compound mutant strains (Fig. 7, which is published as supporting information on the PNAS web site, and data not shown).

Efficiency of Cre-mediated recombination population was only marginally improved in mice carrying two copies of the $C\gamma 1$ -cre transgene (Fig. 8, which is published as supporting information on the PNAS web site). However, in these animals, the fraction of $IgG1^+$ memory B cells as well as the $IgG1$ serum antibody titers were significantly reduced (Fig. 8 and data not shown), suggesting that the IRES-Cre cassette may interfere with expression of proper

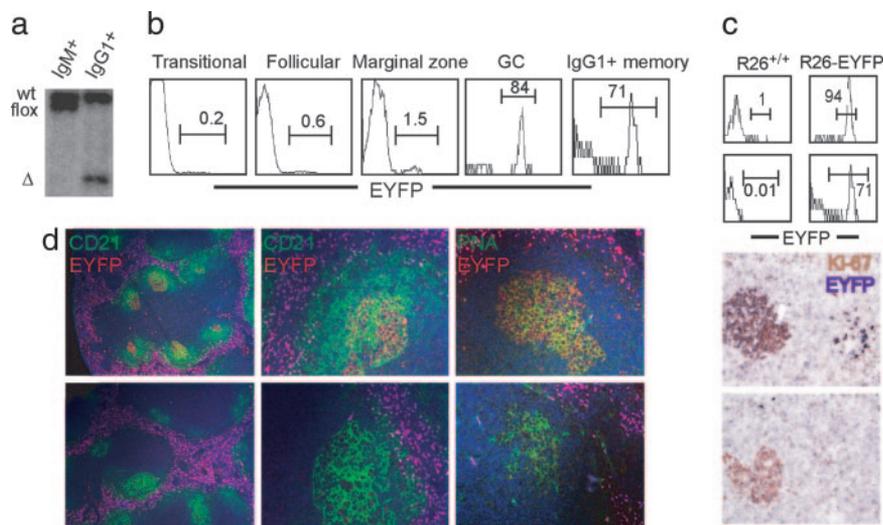


Fig. 2. Cre-mediated recombination in B cell subsets of $C\gamma 1$ -cre mice. (a) Status of the *rag-2* allele. wt, wild type; fl, floxed; Δ , deleted) in IgM^+ and $IgG1^+$ B cells sorted from cultures of $C\gamma 1$ -cre; *Rag-2^{fl/+}* B cells. (b) Percentage of EYFP⁺ cells within gated splenic B cell subsets of $C\gamma 1$ -cre/+; R26-EYFP mice. (c) Percentage of gated $IgG1^+ EYFP^+$ within the GC B ($IgG1^{lo} CD38^{lo}$; Upper) and memory B ($IgG1^+ CD38^+$; Lower) cell subsets of $C\gamma 1$ -cre/+; R26-EYFP and $C\gamma 1$ -cre/+ control mice. EYFP gates were set based on controls. (d) Immunofluorescence analysis of SPL sections from day-10 SRBC-immunized $C\gamma 1$ -cre/+; R26-EYFP (Upper) and R26-EYFP control animals (Lower) stained as indicated. Results shown in b–d are representative of at least four independent experiments. Experiments shown in a were repeated twice.

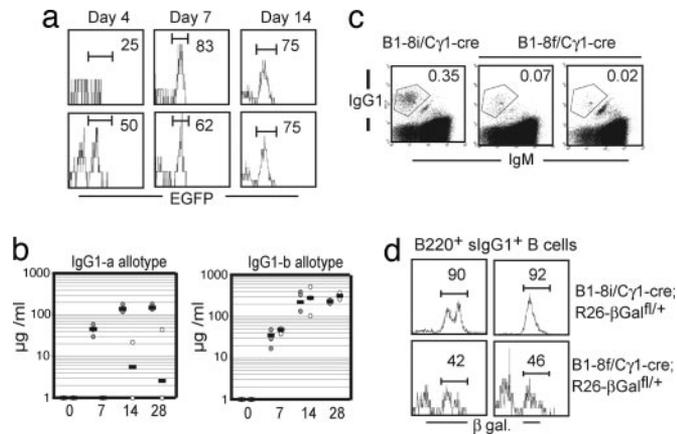


Fig. 3. Early induction of Cre-mediated recombination in GC responses of $C\gamma 1$ -cre mice. (a) Percentage of $EGFP^+$ cells among gated splenic $PNA^{hi}Fas^{hi}$ GC B cells at different time points after NP-CG immunization. Two representative $C\gamma 1$ -cre; $Ig\beta^{fl-EGFP}$ mice are shown. (b) NP- and allotype-specific serum IgG1 titers measured >28 days after NP-CG immunization of $C\gamma 1$ -cre heterozygous (\circ) and CB6F1 (\bullet) control mice; bars indicate geometric mean of IgG1 levels. (c) Percentage of boxed $IgG1^+$ B cells in $C\gamma 1$ -cre mice carrying either the B1-8i or B1-8f alleles. (d) Percentage of β -gal $^+$ cells among gated $B220^+IgG1^+$ B cells of $C\gamma 1$ -cre mice carrying the indicated B1-8 alleles. Two mice per group are shown. β -gal gates were defined based on control mice. Results shown are representative of at least three independent experiments

levels of IgG1 from the adjacent $\gamma 1$ locus and thus the selection of GC B cells into the compartments of memory B and antibody-secreting cells.

Flow cytometric analyses failed to detect Cre-mediated recombination in peripheral $CD4^+$ or $CD8^+$ T cells, $Gr1^+$ granulocytes, $CD19^-Mac1^+$ macrophages, $CD11c^+$ dendritic cells, and $NK1.1^+$ NK cells of $C\gamma 1$ -cre mice (Fig. 9a, which is published as supporting information on the PNAS web site). Similar results were obtained when genomic DNA from kidney, brain, liver, and heart of $C\gamma 1$ -cre mice were tested for Cre-mediated recombination by Southern blot or genomic PCR analyses (Fig. 9b). However, surprisingly, Cre-mediated recombination was observed in germ cells of 10–30% of $C\gamma 1$ -cre mice older than 4 months (oocytes being more often subject to Cre-mediated recombination than spermatocytes), thus resulting in target gene deletion in the germ line of the corresponding offspring.

Early Onset of $C\gamma 1$ -Cre Expression During the GC Reaction. To determine the onset of Cre-mediated recombination during the GC reaction, $C\gamma 1$ -cre; $Ig\beta^{fl-EGFP/+}$ mice were immunized with NP-CG and euthanized at different time points after immunization. Flow cytometric analysis revealed that, by 4 days after immunization, a significant fraction of GC B cells (25–50%) had already undergone Cre-mediated recombination. This fraction increased over time, reaching a maximum of 75–85% 12–14 days after immunization, after which it remained stable (Fig. 3a and data not shown).

IgG1 Expression in $C\gamma 1$ -cre B Cells Occurs Mainly from the Nontargeted IgH Allele. To test whether the insertion of IRES-Cre cassette was detrimental for IgG1 expression from the same allele *in vivo*, we measured allele-specific IgG1 serum titers in heterozygous $C\gamma 1$ -cre mice after immunization with NP-CG. This assay was possible because the $C\gamma 1$ -cre transgene was originally inserted into an IgH locus of an allotype. The measurement of IgG1 serum titers >28 days after immunization revealed equivalent contribution of NP-specific IgG1a and -b antibody titers in $C57BL/6J \times BALB/c$ F_1 control mice. In contrast, heterozygous $C\gamma 1$ -cre mice showed a significant and selective impairment in antigen-specific IgG1a titers deriving from the targeted $C\gamma 1$ -locus (Fig. 3b). Thus, the impairment in the

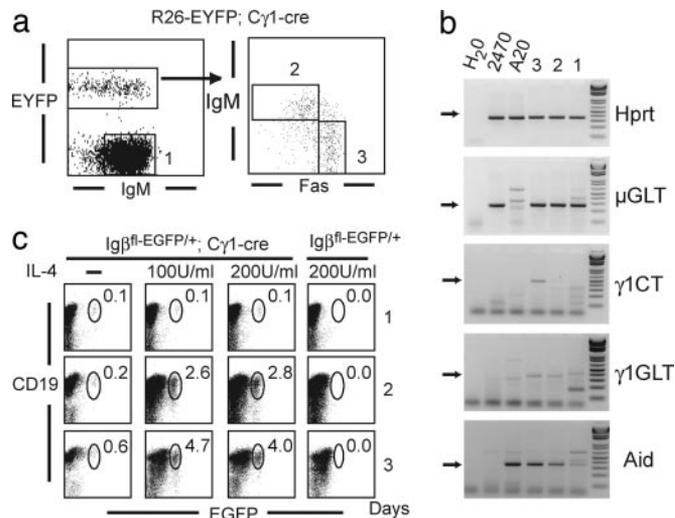


Fig. 4. Germ-line $C\gamma 1$ transcription induces Cre-mediated recombination in resting $C\gamma 1$ -cre B cells. (a) $EYFP^+IgM^+$ B cells in $C\gamma 1$ -cre; R26-EYFP mice are divided into two subsets according to Fas expression. (b) RT-PCR analysis to detect IgH germ-line (GLT), circle (CT)-switched and *AID*-specific transcripts in IgM^+EYFP^- (lane 1), $IgM^+EYFP^+Fas^{lo}$ (lane 2), and $IgM^{lo}EYFP^+Fas^{hi}$ (lane 3) sorted B cells. cDNA from $IgG1^+$ A20 B lymphoma cells (lane 4) and IgM^+ c-MYC transformed pre-GC mouse B lymphoma cells were included as controls; arrows indicate specific PCR fragments. (c) Percentage of $EGFP^+$ $C\gamma 1$ -cre; $Ig\beta^{fl-EGFP/+}$ and $Ig\beta^{fl-EGFP/+}$ control B cells throughout a 3-day culture period in the presence of the indicated amounts of IL-4. Results shown are representative of at least three independent experiments

generation of IgG1-switched memory B and plasma cells in $C\gamma 1$ -cre mice seems to be limited to those cells that express a functional IgH chain from the chromosome carrying the $C\gamma 1$ -cre transgene.

The $C\gamma 1$ -cre Transgene Promotes Efficient Cre-Mediated Recombination When Expressed from an IgH Locus Carrying $D_H\mu_H$ Rearrangements. Because up to 90% of $IgG1^+$ memory B cells undergo Cre-mediated recombination in heterozygous $C\gamma 1$ -cre mice, we suspected that the majority of these cells expressed Cre from the nonfunctional IgH chromosome. To test this hypothesis, we bred $C\gamma 1$ -cre transgenics to mice carrying a prearranged V_H gene (B1-8), either flanked by loxP sites [B1-8f; (14)] or, as a control, lacking such sites [B1-8i; (15)]. A subset of the compound mutant mice also carried the R26- β -gal $^{fl-STOP}$ reporter allele to further assess efficiency of Cre-mediated recombination. The majority of B cells in B1-8/ $C\gamma 1$ -cre double knockin mice express the prearranged B1-8 IgH chain, and thus carry the second IgH chromosome in germ-line configuration or bearing a D_HJ_H joint, as a result of IgH allelic exclusion. After immunization with NP-CG, a normal sized compartment of $IgG1^+$ memory B cells was found in B1-8i/ $C\gamma 1$ -cre; R26- β -gal $^{fl-STOP}$ mice (Fig. 3c). Over 90% of these cells expressed β -gal, confirming efficient Cre-mediated recombination in memory B cells (Fig. 3d). In contrast, a significant reduction ($>80\%$) in the fraction of surface $IgG1^+$ B cells was observed in B1-8f/ $C\gamma 1$ -cre; R26- β -gal $^{fl-STOP}$ mice as a consequence of efficient Cre-mediated deletion of the B1-8f allele (Fig. 3c). Furthermore, the remaining $sIgG1^+$ B cells in these mice largely lacked expression of β -gal, possibly representing a selected pool of cells that had escaped Cre-mediated recombination to preserve BCR expression (Fig. 3d). Thus, $C\gamma 1$ and $D_H/I\mu$ promoters, respectively, before and after completion of CSR to IgG1, can drive expression of sufficient Cre protein to induce efficient Cre-mediated recombination in $C\gamma 1$ -cre B cells.

Identity of IgM^+ B Cells Undergoing Cre-Mediated Recombination in $C\gamma 1$ -cre Mice. Flow cytometric analysis of SPL and LN cells from unimmunized $C\gamma 1$ -cre; R26-EYFP mice identified $<2\%$ of

form of the signal transducer and activator of transcription (STAT)-3 protein (STAT-3c) (21) followed by an IRES-EGFP cassette (Fig. 10, which is published as supporting information on the PNAS web site). Correctly targeted C γ 1-cre; STAT-3c^{fl-STOP} ES cells were used to generate 18 cloned mice from two sessions of ES microinjection into tetraploid blastocysts. Upon SRBC immunization, induction of STAT-3c expression was readily detected in the majority of GC and IgG1 memory B cells of cloned mice by means of EGFP expression (Fig. 9 and data not shown).

Discussion

Germ-Line C γ 1 Transcription Induces Efficient Cre-Mediated Recombination in GC B Cells of C γ 1-cre Mice. In C γ 1-cre mice, the insertion of the Cre coding sequence into the C γ 1 locus, leading to its expression from a bicistronic mRNA together with the C γ 1 transcript, induced efficient Cre-mediated recombination in GC B cells, in accordance with previous reports identifying the GC as the major site of IgG1 CSR (22). Within the GC B cell population, the fraction of cells undergoing Cre-mediated recombination increased over time, reaching an average of >85% at the peak of the GC reaction, when most cells carried high loads of Ig somatic mutations.

Approximately 25–50% of GC B cells in these animals were subject to Cre-mediated recombination as early as 4 days after immunization, suggesting that transcription from the C γ 1 locus (and thus C γ 1-cre expression) represents an early event in activated B cells driven into a TD antibody response, in accordance with previous reports (4, 23). Several pieces of evidence suggest that germ-line C γ 1 transcription was sufficient to promote efficient Cre-mediated recombination. First, recombination mediated by the C γ 1-cre transgene, using the Ig β ^{fl-EGFP} reporter allele, was induced *in vitro* in resting, IgM⁺ B cells upon stimulation with IL-4, a specific inducer of sterile C γ 1 transcripts. Second, B1-8i/C γ 1-cre mice showed Cre-mediated recombination in >90% of GC B cells, although most of these cells expressed the B1-8H chain and thus had the second IgH allele (carrying the C γ 1-cre transgene) in germ-line configuration or at the most bearing D_HJ_H rearrangements. This result indicates that, in GC B cells of these mice, Cre is expressed from germ-line C γ 1 transcripts derived from the nonfunctional IgH allele (24). Third, in unimmunized C γ 1-cre mice carrying the EYFP reporter allele, we observed \approx 2% of IgM⁺ EYFP⁺ B cells, lacking GC B cell markers. RT-PCR analysis showed expression in these cells of germ-line C γ 1 transcripts, whereas circular I γ 1-C μ switch transcripts were not detected, indicating that transcription at the C γ 1 locus had just been initiated.

As descendants of GC B cells, up to 90% of IgG1⁺ memory B cells in C γ 1-cre mice had also undergone Cre-mediated recombination. These cells expressed IgG1 preferentially from the nontargeted locus. By using the C γ 1-cre system in combination with a reporter gene, IgG1⁺ memory B cells can be easily tracked *in vivo*, allowing one to monitor the homing and activation properties of these cells in steady-state conditions as well as during primary and secondary TD antibody responses.

To accelerate the analysis of gene function in GC B cells, we established C γ 1-cre F₁ ES cells allowing further rounds of genetic manipulations and ultimately the cloning of compound mutants by tetraploid blastocyst complementation. Through this approach, a large cohort of cloned C γ 1-cre; STAT-3c^{fl-STOP} compound mutants was readily obtained, allowing the rapid analysis of GC responses in these mice.

Transcription at Multiple C_H Loci Precedes CSR in Individual GC B Cells.

Expression of germ-line transcripts is an essential step preceding Ig CSR (16, 25). These transcripts, expressed from C_H promoters, are induced in response to stimulation by Th1- or Th2-type cytokines (9). *In vitro* induction of CSR in C γ 1-cre B cells followed the rules outlined above, as efficient Cre-mediated recombination was observed in cultures stimulated with CD40 (or LPS) plus IL-4, predominantly switching to IgG1. Over 85% of the B cells under-

went Cre-mediated recombination under these conditions regardless of whether they became IgG1-expressing cells, further confirming the role of sterile C γ 1 transcription in driving Cre-mediated recombination. A subset of CD40 plus IL-4-stimulated B cells switched to IgE expression, most of which had undergone Cre-mediated recombination. This result suggests that induction of Cre expression occurred either in activated IgM⁺ B cells expressing both C γ 1 and C ϵ germ-line transcripts or in IgG1⁺ B cells, which subsequently switched to IgE (26, 27). Conversely, culture conditions promoting switching to IgG3, IgG2a, IgG2b, and IgA failed to induce Cre-mediated recombination by the C γ 1-cre transgene. However, germ-line transcription of C γ 1 did not always result in IgG1 or IgE CSR *in vivo*. Molecular, immunofluorescence, and flow cytometric analyses identified a subset of IgG3-, IgG2a-, IgG2b-, or IgA-switched GC B or extrafollicular plasma cells in C γ 1-cre mice that had undergone Cre-mediated recombination. This result suggests a scenario where at early stages of the GC reaction, possibly during the initial T cell–B cell interactions (28), both Th1- and Th2-type cytokines are secreted by antigen-specific and/or by-stander Th cells. This process then activates the simultaneous or sequential expression of germ-line transcripts from multiple C_H loci in individual GC B cells or their precursors. Ultimately, the concentration of individual cytokines determines the extent of germ-line transcription from the individual C_H loci and thus the frequency of switching to a particular isotype. In accordance with this scenario, <30% of C γ 1-cre B cells underwent Cre-mediated recombination in spontaneous GCs of gut-associated lymphoid tissues where TGF- β represents the predominant cytokine, promoting switching to IgA (29). Cre-mediated recombination in C γ 1-cre B cells switching to isotypes other than IgG1 may also result from CSR involving different C_H genes on the two IgH chromosomes (30) or sequential switching from C γ 1 to distal C_H isotypes (31), except in the case of cells expressing IgG3. The latter mechanism may contribute to the generation of IgA⁺ B cells because the predominant IgH allotype expressed by IgG1⁺ B cells in C γ 1-cre mice was specifically overrepresented within the fraction of IgA⁺ cells that had undergone Cre-mediated recombination (data not shown).

Methods

Generation of C γ 1-cre Mice. The C γ 1-cre vector is a derivative of a plasmid previously used to target the mouse C γ 1 locus (32). The encephalomyocarditis (EMCV)-derived IRES DNA segment (gift from E. Wimmer) followed by the NLS-Cre coding sequence was inserted downstream of the termination codon in the last membrane-coding exon of IgG1. The targeting vector was electroporated into 129Sv-derived IB10 ES cells (gift from A. Berns). ES clones were screened by Southern blot analysis by using an external probe as described (32). Two independent targeted ES clones were injected into blastocysts to generate chimeric mice from which germ-line-transmitted animals were obtained. Removal of the frt-flanked selection marker was achieved breeding C γ 1-cre (NeoR⁺) mice to FLPe deleter mice.

Other Mouse Reagents. Rag-2^{fl}, fas^{fl}, R26- β -gal^{fl-STOP}, R26-EYFP, and FLPe mice have been described (12, 13, 33–35). Ig β ^{fl-EGFP} and R26-STAT3c^{fl-STOP} conditional mice are described in *Supporting Text*, which is published as supporting information on the PNAS web site. Mice were bred and maintained under specific pathogen-free conditions; mouse protocols were approved by the University of Cologne and the Harvard University Institutional Animal Care and Use Committee and by the CBR Institute for Biomedical Research.

Mouse Immunizations. Eight- to 12-week-old mice were immunized i.p. with 100 μ g of alum-precipitated (4-hydroxy-3-nitrophenyl) acetyl coupled to chicken gamma globulin (Biosearch) or with 1 \times 10⁸ SRBC (Cedarlane Laboratories) in PBS.

Flow Cytometry and Cell Sorting. Cell suspensions were stained with biotin or fluorescent-labeled mAbs as described (36). Additional antibodies included anti-IgG1 (X56), -IgG3 (R40-82), -IgE (R35-118), -IgG2a (R19-15) (BD Pharmingen), -IgA (Southern Biotechnology Associates), -AA4.1, and -CD38 (90) (eBioscience, San Diego). Biotin conjugates were visualized with PerCP or APC-streptavidin (BD Pharmingen). Flow cytometric determination of β -gal enzymatic activity was performed as described (37). All analyses were performed on a FACSCalibur (BD Biosciences), and results were analyzed with CELLQUEST or FLOWJOW software. Cell sorting was performed on a FACS Vantage or FACS Aria (BD Biosciences).

Immunohistochemistry and Immunofluorescence. Paraffin-embedded sections were stained for hematoxylin/eosin (H&E) or immunostained as described (38). Briefly, antigen-retrieved, peroxidase-inhibited sections were blocked in 5% defatted milk powder, incubated overnight with a rabbit anti-EGFP/EYFP peptide Ab (BD Pharmingen) or rabbit Ig (Sigma), washed, counterstained with peroxidase-conjugated anti-rabbit polymer (DakoCytomation), washed, and tyramide amplified in Cy3 (PerkinElmer). Subsequently, peroxidase was quenched again, and biotin-conjugated anti-Ig isotype-specific antibodies (goat anti-IgG1, -IgG2a, -IgG2b, -IgM; Southern Biotechnology Associates) or biotin-PNA (Vector Laboratories) were added, washed, incubated with HRP-conjugated Avidin (DakoCytomation), washed, and tyramide-amplified in FITC. An FITC-conjugated goat anti-mouse CD21 (sc-7028; Santa Cruz Biotechnology) was counterstained with a peroxidase-conjugated goat anti-FITC (Roche Diagnostics), washed, and amplified with tyramide FITC. Slides, mounted with DAPI-containing mounting medium (Molecular Probes), were viewed on an E600-Nikon Microscope (Nikon). Images were edited for optimal color contrast with Adobe PHOTOSHOP 7 and Adobe ILLUSTRATOR 10 (Adobe Systems, San Jose, CA).

In Vitro B Cell Cultures. Splenic B cells purified by immunomagnetic depletion of CD43⁺ cells (Miltenyi Biotec, Auburn, CA) were cultured in B cell medium in the presence of stimuli including LPS (20 μ g/ml, *Escherichia coli*, 055:B5; Sigma), anti-CD40 (3 μ g/ml; IC10; eBioscience), IL-4 (100–200 units/ml; Preprotech), IFN- γ (100 ng/ml), and TGF- β (1 ng/ml; R & D Systems).

RT-PCR Analysis. Total RNA was extracted from sorted cells by using the TRIzol reagent (Invitrogen). RNA from 1×10^5 cell equivalents was subject to first strand cDNA synthesis by using random hexamers and Superscript RT II (Invitrogen). cDNA from 10^4 cell equivalents was amplified by PCR. Intron-spanning primers and PCR conditions to amplify respectively Hprt, AID, circle, and isotype-switched IgH transcripts have been described (17, 36). PCR products were cloned into pGEM-T Easy (Promega) and sequenced.

IgH Somatic Mutation Analysis. IgH V gene rearrangements from sorted B cells were PCR-amplified by using the Expand High fidelity PCR system (Roche Molecular Diagnostics) and primers as described (39). PCR-amplified IgH rearrangements carrying a J_H4 segment were cloned and sequenced.

Establishment of C γ 1-cre F₁ ES Cell Lines and Generation of Cloned Mice by Tetraploid Embryo Complementation. Day 3.5 C γ 1-cre/+ (C57BL/6J \times BALB/c) F₁ embryos were isolated and plated onto mitomycin-treated mouse embryonic fibroblasts, in ES cell medium (40) containing 1,000 units/ml leukocyte inhibiting factor, and 50 μ M MEK1 inhibitor PD98059 (Cell Signaling Technology, Beverly, MA). After two successive rounds of trypsinization, cells were transferred onto a six-well plate, at which time point individual ES colonies were identified, isolated, and further expanded. ES clones displaying <30% of differentiated cells were subjected to a karyotype analysis and genotyped by PCR for the presence of the Y chromosome (41). Generation of cloned mice from one of the established C γ 1-cre/+ XY ES cell lines was performed as described (42).

We thank T. Takemori, U. Klein, K. L. Otipoby, and D. Schenten for helpful discussions; U. Klein and Riccardo Dalla-Favera for sharing unpublished results and for critical reading of the manuscript; E. Wimmer (Stony Brook University, Stony Brook, NY) for providing the pMPS1-ECAT plasmid containing the ECMV IRES DNA segment; A. Berns (Netherlands Cancer Institute, Amsterdam) for IB10 ES cells; and S. Willms, A. Roth, C. Goettlinger, N. Barteneva, K. Ketman, V. Dreier, and M. Curnutte for technical assistance. This work was supported by National Institutes of Health Grants P01 CA92625 and R01 CA098285 (to K.R.) and fellowships from the Alexander von Humboldt Foundation, the Human Frontier Science Program, and the Cancer Research Institute (to S.C.).

- MacLennan, I. C. (1994) *Annu. Rev. Immunol.* **12**, 117–139.
- Garside, P., Ingulli, E., Merica, R. R., Johnson, J. G., Noelle, R. J. & Jenkins, M. K. (1998) *Science* **281**, 96–99.
- Liu, Y. J., Zhang, J., Lane, P. J., Chan, E. Y. & MacLennan, I. C. (1991) *Eur. J. Immunol.* **21**, 2951–2962.
- Pape, K. A., Kouskoff, V., Nemazee, D., Tang, H. L., Cyster, J. G., Tze, L. E., Hippen, K. L., Behrens, T. W. & Jenkins, M. K. (2003) *J. Exp. Med.* **197**, 1677–1687.
- Allen, C. D., Ansel, K. M., Low, C., Lesley, R., Tamamura, H., Fujii, N. & Cyster, J. G. (2004) *Nat. Immunol.* **5**, 943–952.
- Jacob, J., Kelsoe, G., Rajewsky, K. & Weiss, U. (1991) *Nature* **354**, 389–392.
- Muramatsu, M., Kinoshita, K., Fagarasan, S., Yamada, S., Shinkai, Y. & Honjo, T. (2000) *Cell* **102**, 553–563.
- Rajewsky, K. (1996) *Nature* **381**, 751–758.
- Manis, J. P., Tian, M. & Alt, F. W. (2002) *Trends Immunol.* **23**, 31–39.
- Radbruch, A., Muller, W. & Rajewsky, K. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3954–3957.
- Hummel, M., Berry, J. K. & Dunnick, W. (1987) *J. Immunol.* **138**, 3539–3548.
- Hao, Z. & Rajewsky, K. (2001) *J. Exp. Med.* **194**, 1151–1164.
- Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. & Costantini, F. (2001) *BMC Dev. Biol.* **1**, 4.
- Lam, K. P., Kuhn, R. & Rajewsky, K. (1997) *Cell* **90**, 1073–1083.
- Sonoda, E., Pewzner-Jung, Y., Schwers, S., Taki, S., Jung, S., Eilat, D. & Rajewsky, K. (1997) *Immunity* **6**, 225–233.
- Esser, C. & Radbruch, A. (1989) *EMBO J.* **8**, 483–488.
- Kinoshita, K., Harigai, M., Fagarasan, S., Muramatsu, M. & Honjo, T. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 12620–12623.
- Klein, U., Kuppers, R. & Rajewsky, K. (1997) *Blood* **89**, 1288–1298.
- Rothman, P., Lutzker, S., Cook, W., Coffman, R. & Alt, F. W. (1988) *J. Exp. Med.* **168**, 2385–2389.
- Shinall, S. M., Gonzalez-Fernandez, M., Noelle, R. J. & Waldschmidt, T. J. (2000) *J. Immunol.* **164**, 5729–5738.
- Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C. & Darnell, J. E., Jr. (1999) *Cell* **98**, 295–303.
- Liu, Y. J., Malisan, F., de Bouteiller, O., Guret, C., Lebecque, S., Banchereau, J., Mills, F. C., Max, E. E. & Martinez-Valdez, H. (1996) *Immunity* **4**, 241–250.
- Toellner, K. M., Gulbranson-Judge, A., Taylor, D. R., Sze, D. M. & MacLennan, I. C. (1996) *J. Exp. Med.* **183**, 2303–2312.
- Delpy, L., Le Bert, M., Cogne, M. & Khamlichi, A. A. (2003) *Eur. J. Immunol.* **33**, 2108–2113.
- Stavnezer-Nordgren, J. & Sirlin, S. (1986) *EMBO J.* **5**, 95–102.
- Jung, S., Siebenkotten, G. & Radbruch, A. (1994) *J. Exp. Med.* **179**, 2023–2026.
- Mandler, R., Finkelman, F. D., Levine, A. D. & Snapper, C. M. (1993) *J. Immunol.* **150**, 407–418.
- Toellner, K. M., Luther, S. A., Sze, D. M., Choy, R. K., Taylor, D. R., MacLennan, I. C. & Acha-Orbea, H. (1998) *J. Exp. Med.* **187**, 1193–1204.
- Letterio, J. J. & Roberts, A. B. (1998) *Annu. Rev. Immunol.* **16**, 137–161.
- Schultz, C., Petrini, J., Collins, J., Clafflin, J. L., Denis, K. A., Gearhart, P., Gritzmacher, C., Manser, T., Shulman, M. & Dunnick, W. (1990) *J. Immunol.* **144**, 363–370.
- Radbruch, A., Liesegang, B. & Rajewsky, K. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2909–2913.
- Kaisho, T., Schwenk, F. & Rajewsky, K. (1997) *Science* **276**, 412–415.
- Hao, Z., Hampel, B., Yagita, H. & Rajewsky, K. (2004) *J. Exp. Med.* **199**, 1355–1365.
- Mao, X., Fujiwara, Y. & Orkin, S. H. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 5037–5042.
- Rodriguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A. F. & Dymecki, S. M. (2000) *Nat. Genet.* **25**, 139–140.
- Casola, S., Otipoby, K. L., Alimzhanov, M., Humme, S., Uyttersprot, N., Kutok, J. L., Carroll, M. C. & Rajewsky, K. (2004) *Nat. Immunol.* **5**, 317–327.
- Nolan, G. P., Fiering, S., Nicolas, J. F. & Herzenberg, L. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2603–2607.
- Cattoretti, G., Pasqualucci, L., Ballon, G., Tam, W., Nandula, S. V., Shen, Q., Mo, T., Murty, V. V. & Dalla-Favera, R. (2005) *Cancer Cell* **7**, 445–455.
- Jolly, C. J., Klux, N. & Neuberger, M. S. (1997) *Nucleic Acids Res.* **25**, 1913–1919.
- Casola, S. (2004) *Methods Mol. Biol.* **271**, 91–109.
- Kunieda, T., Xian, M., Kobayashi, E., Imamichi, T., Moriwaki, K. & Toyoda, Y. (1992) *Biol. Reprod.* **46**, 692–697.
- Eggen, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W. M., 3rd, Yanagimachi, R. & Jaenisch, R. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 6209–6214.

IMMUNOLOGY. For the article “Tracking germinal center B cells expressing germ-line immunoglobulin γ 1 transcripts by conditional gene targeting,” by Stefano Casola, Giorgio Cattoretti, Nathalie Uyttersprot, Sergei B. Koralov, Jane Segal, Zhenyue Hao, Ari Waisman, Angela Egert, Dvora Ghitza, and Klaus Rajewsky, which appeared in issue 19, May 9, 2006, of *Proc Natl Acad Sci USA* (103:7396–7401; first published May 1, 2006; 10.1073/pnas.0602353103), the authors note that the author name Jane Segal should have appeared as Jane Seagal. The corrected author line appears below. The online version has been corrected.

Stefano Casola, Giorgio Cattoretti, Nathalie Uyttersprot, Sergei B. Koralov, Jane Seagal, Zhenyue Hao, Ari Waisman, Angela Egert, Dvora Ghitza, and Klaus Rajewsky

www.pnas.org/cgi/doi/10.1073/pnas.0611564104

MEDICAL SCIENCES. For the article “Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK,” by Anna V. Galkin, Jonathan S. Melnick, Sungjoon Kim, Tami L. Hood, Nanxin Li, Lintong Li, Gang Xia, Ruo Steensma, Greg Chopiuk, Jiqing Jiang, Yongqin Wan, Peter Ding, Yi Liu, Fangxian Sun, Peter G. Schultz, Nathanael S. Gray, and Markus Warmuth, which appeared in issue 1, January 2, 2007, of *Proc Natl Acad Sci USA* (104:270–275; first published December 21, 2006; 10.1073/pnas.0609412103), the authors note that the first two authors, Anna V. Galkin and Jonathan S. Melnick, contributed equally to this work.

www.pnas.org/cgi/doi/10.1073/pnas.0611563104