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Both mutated and unmutated memory B cells accumulate mutations in the course of the secondary response and develop a new antibody repertoire optimally adapted to the secondary stimulus

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Abstract

High-affinity memory B cells are preferentially selected during secondary responses and rapidly differentiate into antibody-producing cells. However, it remains unknown whether only high-affinity, mutated memory B cells simply expand to dominate the secondary response or if in fact memory B cells with a diverse V_H repertoire, including those with no mutations, accumulate somatic mutations to create a new repertoire through the process of affinity maturation. In this report, we took a new approach to address this question by analyzing the V_H gene repertoire of IgG1$^+$ memory B cells before and after antigen re-exposure in a host unable to generate IgG$^+$ B cells. We show here that both mutated and unmutated IgG1$^+$ memory B cells respond to secondary challenge and expand while accumulating somatic mutations in their V_H genes in a stepwise manner. Both types of memory cells subsequently established a V_H gene repertoire dominated by two major clonotypes, which are distinct from the original repertoire before antigen re-exposure. In addition, heavily mutated memory B cells were excluded from the secondary repertoire. Thus, both mutated and unmutated IgG1$^+$ memory cells equally contribute to establish a new antibody repertoire through a dynamic process of mutation and selection, becoming optimally adapted to the recall challenge.

Keywords: Antibody repertoire, memory B cells, secondary response, somatic hypermutation

Introduction

During primary immune responses to T-cell-dependent antigens, B cells interact with T cells at the border of B- and T-cell areas, migrate to the B-cell follicle, proliferate and undergo class switching (1, 2). The activated B cells can then follow either of two distinct pathways of differentiation, becoming memory B cells or plasma cells prior to germinal center (GC) formation (3, 4) or forming GCs upon expression of the transcriptional repressor Bcl6 (5–7). Within GCs, proliferating B cells accumulate somatic mutations in their rearranged V genes and their progeny are selected by antigen to generate high-affinity memory B cells and plasma cells (reviewed in references 8, 9). GC-dependent memory B cells are joined by GC-independent memory cells in roughly equal proportions, and both home to B-cell follicles in the spleen for a long period of time. Accordingly, IgG memory B cells consist of two types of cells, expressing either an unmutated or a mutated BCR (4, 10).

A defining feature of memory B cells is their ability to undergo terminal plasma cell differentiation rapidly and efficiently upon re-exposure to the same antigen (secondary

response), as a hallmark of adaptive immunity. However, the nature and mechanism of this dynamic response by memory B cells, which have a diverse and heterogeneous antibody repertoire, remain largely unknown. In this context, earlier hybridoma studies of secondary and tertiary responses elicited in primed and intact animals provided evidence of selection of certain somatic mutations (11–14). However, those studies using hybridoma technology may have led to misleading conclusions, as B-cell blasts rather than terminally differentiated antibody-secreting cells (ASCs) were predominantly immortalized by fusion with the plasmacytoma (15). To overcome this problem, the antibody repertoire in a secondary response was analyzed in antigen-binding cells by direct V_H gene sequencing, instead of by making hybridomas (15). Based on this approach, it has been proposed that the memory B-cell pool can acquire additional mutations (15). However, this experimental system was unable to distinguish memory B-cell progeny from other types of cells, including primary and secondary GC B cells (16) and long-term plasma cells arising during the primary response. Furthermore, it has recently been observed that naive B cells also actively participate in the secondary response (17). Therefore, a method for tracking memory B cells *in situ* is essential to clarify whether or not they are involved in the hypermutation and selection pathway during the secondary response.

In the present study, we introduce a new approach to address the V_H gene repertoire of memory B cells before and after antigen re-exposure. We purified (4-hydroxy-3-nitrophenyl)acetyl (NP)-specific/IgG1$^+$ memory B cells from immunized mice (4, 18), transferred the cells intravenously into chicken γ-globulin (CG)-primed activation-induced cytidine deaminase (AID)$^{-/-}$ mice (19), which are unable to generate their own IgG1$^+$ B cells and monitored NP-specific IgG1 responses in the recipients after challenge with soluble NP-CG. This experimental system provides a unique opportunity to selectively characterize the IgG1 memory B-cell response independently of preexisting long-term plasma cells (20) or GC B cells derived from naive B cells during the secondary response (16, 17). In addition, this approach has an advantage of allowing analysis of memory responses in non-irradiated hosts that retain intact secondary lymphoid structures (21).

The present study demonstrates that IgG1$^+$ memory B cells elicited a long-term and high-affinity antibody response in AID$^{-/-}$ mice upon secondary challenge. Analysis of antigen-specific V_H gene sequences from the IgG1$^+$ cells revealed that both mutated and non-mutated memory B cells responded to the secondary challenge and accumulated somatic mutations during proliferation, indicative of GC-mediated selection. As the immune response progressed, a limited number of clones became enriched among the V_H gene repertoire derived from the memory B cells. Finally, two major clonotypes became predominant in the repertoire, and these were not found in the original repertoire before antigen re-encounter. These results suggest that upon antigen re-exposure, memory B cells undergo a dynamic process of hypermutation and selection to establish a new antibody repertoire adapted to the secondary antigen challenge.

Methods

Mice

Eight- to ten-week-old C57BL/6 mice were purchased from Clea Inc. (Tokyo, Japan). AID-deficient mice (19) were kindly provided by Dr Honjo (Kyoto University, Japan). All experiments were performed in accordance with guidelines established by the RIKEN Research Center for Allergy and Immunology.

Memory B-cell purification

Memory B-cell purification was performed as described previously (4). Briefly, C57BL/6 mice were intra-peritoneally immunized with 100 μg NP_{15}-CG precipitated in alum. Single-cell suspensions prepared from pooled spleens of 20 mice 40 days after immunization were blocked with anti-FcγRII/III mAb (2.4G2; ATCC), followed by incubation with a mixture of biotinylated mAbs against IgM, IgD, CD3, CD5, CD90, TER119, Gr-1, F4/80, DX5, AA4.1 and NK1.1 (eBioscience, San Diego, CA, USA). After incubation with streptavidin microbeads (Miltenyi Biotec, Gladbach, Germany), cells were enriched by using a magnetic-activated cell sorting (MACS) system (Miltenyi Biotec). Cells were then stained with anti-CD38 [CS2 (4)] conjugated with AlexaFluor647 (anti-CD38$^{\text{AlexaFluor647}}$), anti-B220 conjugated with PE-Cy7 (anti-B220$^{\text{PE-Cy7}}$; eBioscience), anti-IgG1 (BD Pharmingen, San Diego, CA, USA) conjugated with Pacific Blue (anti-IgG1$^{\text{Pacific Blue}}$), anti-Igλ coupled with FITC (anti-Igλ$^{\text{FITC}}$; BD Pharmingen), PE-conjugated (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP)-BSA (NIP-BSA$^{\text{PE}}$) and streptavidin conjugated with PE-TexasRed (streptavidin$^{\text{PE-TexasRed}}$; BD Pharmingen). Cells were analyzed using a FACS Aria (BD Biosciences, San Jose, CA, USA) and viable NIP-binding memory B cells were sorted as B220$^+$IgG1$^+$CD38$^+$Igλ$^+$ cells.

ELISA and ELISPOTs

ELISA and ELISPOT assays were performed with NP_2-BSA and NP_{18}-BSA as described previously (10).

Adoptive transfer experiment

Naive B cells from unimmunized AID$^{-/-}$ mice were prepared by using anti-B220 microbeads (Miltenyi Biotec) according to the manufacturer's instruction. Purified NIP-binding IgG1$^+$Igλ$^+$ memory B cells (1×10^3 per mouse) together with naive B cells (5×10^4 per mouse) were injected intravenously into AID$^{-/-}$ mice immunized with 100 μg CG precipitated in alum 1 month previously. The recipient mice were challenged with 50 μg of soluble NP-CG in PBS 12 h later. NP-specific IgG1 antibodies in sera and NP-specific IgG1 ASCs were determined by ELISA and ELISPOT, respectively, at the indicated time points. In some experiments, splenocytes and bone marrow (BM) cells were labeled with biotinylated anti-CD43 mAb and anti-CD138 mAb (BD Pharmingen), followed by incubation with streptavidin microbeads. Positive and negative fractions were separated by using a MACS system, followed by an ELISPOT assay.

Sequence analysis of the V_H186.2 gene

Sequence analysis of the *V_H186.2* gene was carried out as described previously (4) with minor modifications. Total RNA was prepared from 1×10^7 splenocytes and 5×10^6 BM cells of the recipient AID$^{-/-}$ mice at the indicated time points using the RNeasy mini kit (Qiagen, Hilden, Germany). Two micrograms of total RNA was subjected to RT–PCR reaction using the SuperScript One-Step High Fidelity kit (Invitrogen, Carlsbad, CA, USA) as follows: 50°C for 30 min, 94°C for 2 min, 30 cycles of 94°C for 15 s, 58°C for 20 s, 68°C for 1 min and a final extension at 68°C for 5 min. Primer pairs used were as follows: V_H186.2-sense 5′-TTCTTGGCAGCAACAGCTACA-3′ and Cγ1-external-anti-sense 5′-GGATCCAGAGTTCCAGGTCACT-3′. One micro liter of first RT–PCR product was used as a second semi-nested PCR template. The second round of PCR was performed using Platinum Pfx DNA polymerase (Invitrogen) and the following conditions: 94°C for 2 min, 30 cycles of 94°C for 15 s, 58°C for 20 s, 68°C for 1 min and a final extension at 68°C for 5 min. Primer pairs used were as follows: V_H186.2-sense and Cγ1-internal-anti-sense 5′-GGAGTTAGTTTGGGCAGCAG-3′. A 3′ adenine overhang was added to the second-round PCR products by treatment with ExTaq polymerase (Takara, Shiga, Japan) at 72°C for 10 min, followed by cloning into the pCR2.1 TOPO vector (Invitrogen). Inserted products were sequenced using a Cγ1-internal-anti-sense primer. The nucleotide sequences of the cloned V_H genes were determined as described previously (10).

Results

IgG1 memory B cells differentiate into high-affinity ASCs in AID$^{-/-}$ adoptive hosts upon secondary challenge

To examine the antibody repertoire formed by memory B cells during the secondary response, NP-specific/IgG1$^+$ memory B cells were purified from the pooled spleens of immunized mice at day 40 after immunization. One thousand IgG1$^+$ memory B cells were transferred into CG-primed AID$^{-/-}$ mice, and the IgG1 response in the recipients was monitored after challenge with soluble NP coupled to CG.

As shown in Fig. 1A, the recipient AID$^{-/-}$ mice transplanted with IgG1 memory B cells produced a significant amount of anti-NP IgG1 antibodies after challenge with NP-CG, which consisted predominantly, but not exclusively, (i.e. 60–80%) of high-affinity antibodies during 1–8 weeks after challenge with substantial variations among recipients. The antibodies did not contain any other isotypes besides IgG1 (data not shown), suggesting that IgG1$^+$ memory B cells did not undergo further sequential class switching upon antigen re-encounter, as previously suggested (22). Consistent with the previous observations indicating that ASCs in the spleen home to the BM via blood circulation (20, 23), the number of ASCs in the spleen of AID$^{-/-}$ mice was reduced as the secondary response progressed (Fig. 1B). By contrast, BM became the major reservoir for ASCs at the late stage of the secondary response (Fig. 1B), where they may retain their numbers for a long period of time. As shown in Fig. 1C, depletion of CD43$^+$CD138$^+$ cells from the IgG1$^+$ cells recovered from the spleens of AID$^{-/-}$ recipient mice significantly reduced the number of NP-specific ASCs detected by ELISPOT, suggesting that ASCs produced in the secondary response have the same surface phenotype as the plasma cells in the primary response (24–26). Together, these results suggest that NP-specific/IgG1$^+$ memory B cells in AID$^{-/-}$ recipient mice differentiate into plasma cells upon secondary challenge, and subsequently home to the BM, where they are maintained for long-term antibody production.

A limited number of clones are selected into the secondary response

In C57BL/6 mice, the anti-NP response is dominated by antibodies composed of λ light chains and heavy chains in which the *V_H186.2* gene is used to encode the V_H region (27). A previous study that analyzed a large number of V_H186.2 gene transcripts from a single mouse revealed clonal dominance with a close genealogical relationship in the anti-NP response, as indicated by the numbers and sequences of clonally associated V_H gene transcripts that shared the same complementarity determining region (CDR)3 sequence (14, 28, 29). Using this experimental strategy, we examined the genealogical relationship among the clones in the anti-NP secondary response as indicated by over 100 V_H186.2-Cγ1 transcripts amplified from RNA extracted from the spleen and BM of a single AID$^{-/-}$ mouse that received day 40 IgG1$^+$ memory B cells followed by immunization with NP-CG.

As shown in Table 1, sequence analysis revealed that 110 out of 119 $V_HD_HJ_H$ transcripts from day 40 IgG1$^+$ memory B cells had distinct junctional nucleotides, suggesting that the memory B-cell population was derived from distinct progenitors in the primary response. On the other hand, such transcripts amplified from the progeny of day 40 memory B cells from immunized AID$^{-/-}$ mice grouped into a limited number of clonally related families based on their identical CDR3 and J_H segments and shared mutations (Table 1 and data not shown). The frequency of somatic mutations in the *V_H186.2* genes in IgG1$^+$ B cells was increased after secondary challenge ($P < 0.05$), but the average number of mutations per V_H gene was comparable before and after the secondary antigen challenge. The majority of $V_HD_HJ_H$ transcripts contained tyrosine in the V_H–D_H boundary at amino acid position 99 (Tyr99), which is the predominant amino acid found at this position in the antibodies selected by the NP hapten in the primary response to T-cell-dependent antigens(30).

The secondary memory B-cell response is dominated by two major clonotypes

In the NP response, the Trp to Leu exchange at amino acid position 33 of V_H186.2 (W33L) confers a 10-fold increase in affinity for the hapten NP (31), and this substitution is dominant in GC B cells (Fig. 2A; 10). Approximately 20% of rearranged *V_H186.2* genes from day 40 IgG1$^+$ memory B cells (before transfer to AID$^{-/-}$ recipients) had this W33L substitution (Fig. 2A), and this frequency was significantly increased among V_H186.2-Cγ1 transcripts recovered from the spleen and BM of some but not all immunized AID$^{-/-}$ recipients

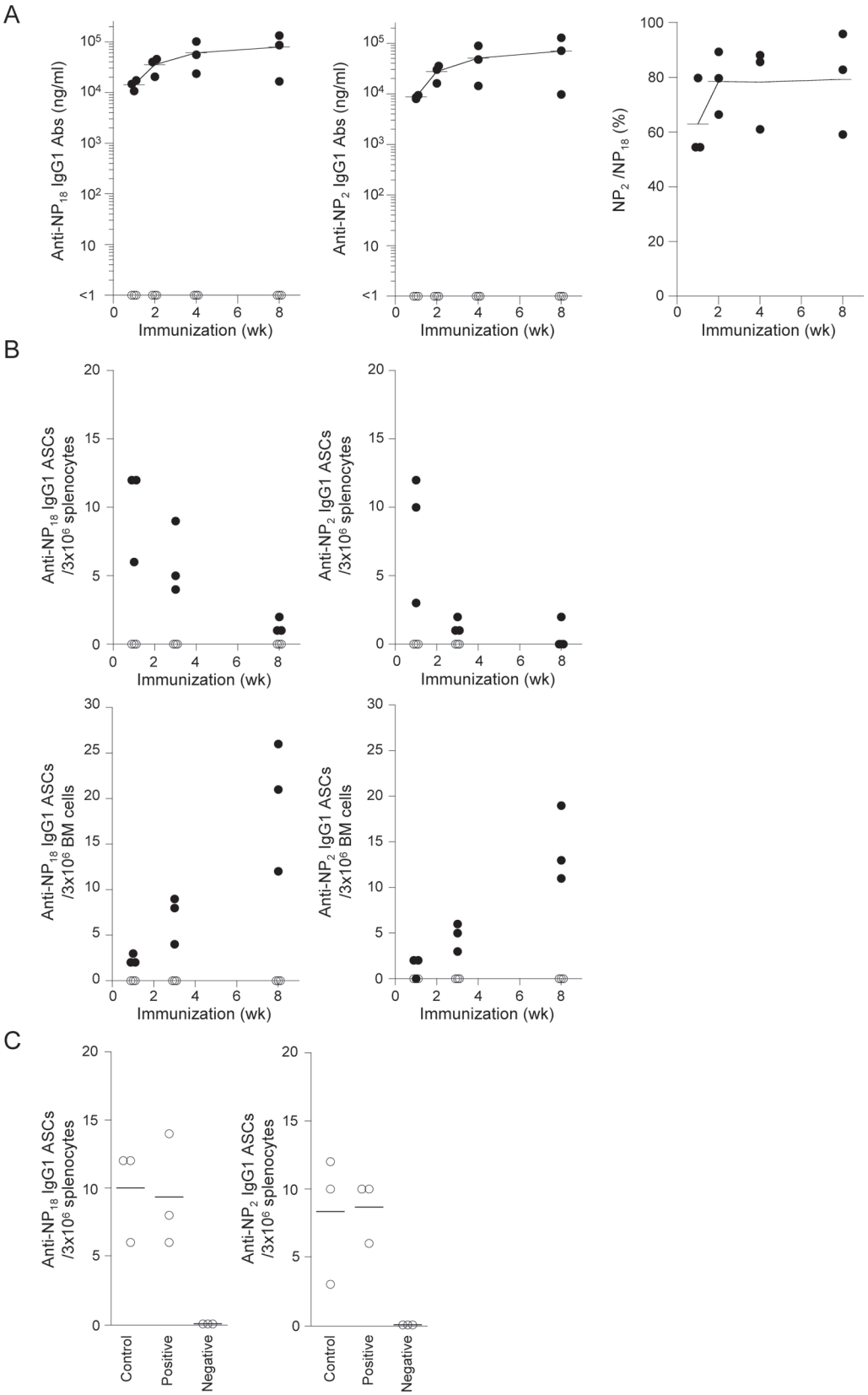

Fig. 1. Secondary responses of IgG1 memory B cells. NP-specific IgG1$^+$ memory B cells were purified from 20 C57BL/6 mice immunized with NP-CG in alum 40 days previously and 1000 of the cells were transferred into CG-primed AID$^{-/-}$ mice. (A) Serum anti-NP_{18} (left panel) and NP_2 (middle panel) IgG1 titers, and the percentage of anti-NP_2 in total anti-NP_{18} IgG1 titers (right panel) were determined by ELISA at the indicated

Table 1. Summary of V_H gene sequence analysis of memory B cells in primary and secondary responses

Mouse[a]		Group[b]	DFL16.1 family[c]	JH2[d]	Mutations/ VH[e]	Mutated clones[f]	Tyr[g]	W33L (%)[h]	K58R (%)[i]	W33L/ K58R[j]	K58R/ Gly[k]
C57BL/6 D40 memory		114/119	34/114 (29.8)	53/114 (46.5)	3.25	78/119 65.5% (4.59)	78/114 (68.4)	21/119 (17.6)	14/119 (11.8)	0/14	2/5
AID$^{-/-}$ mouse											
1 week											
1	Spl	9/23 (5)	4/9	5/9	5.17	23/23 100% (5.17)	9/9	16/23 (69.6)	1/23 (4.3)	0/1	0
	BM	11/27 (5)	5/11	7/11	6.41	27/27 100% (6.41)	7/11	19/27 (70.4)	5/27 (18.5)	1/5	1/1
2	Spl	12/29 (1)	6/12	8/12	4.41	27/29 93% (4.74)	6/12	1/29 (3.45)	14/29 (48.3)	0/14	2/6
	BM	2/7 (1)	4/7	4/7	5.0	6/7 85.7% (5.83)	2/2	0/7 (<14.3)	3/7 (42.8)	0/3	0
3	Spl	13/24 (4)	3/13	7/13	7.46	23/24 95.8% (7.78)	12/13	12/24 (50.0)	6/24 (25)	0/6	0
	BM	11/18 (4)	5/11	9/11	3.67	15/18 83% (4.40)	8/11	6/18 (33.3)	3/18 (16.7)	1/3	0
Total			27/66 (40.9)	40/63 (63.3)							
3 weeks											
1	Spl	5/25 (3)	2/5	4/5	5.8	25/25 100% (5.80)	4/5	23/25(92)	2/25(8.0)	0/2	0
	BM	4/28 (3)	3/4	3/4	9.1	28/28 100% (9.14)	4/4	28/28(100)	0/28	–	0
2	Spl	7/30 (6)	3/7	3/7	7.33	30/30 100% (7.33)	6/7	5/30 (16.7)	24/30 (80.0)	1/24	0
	BM	10/21 (6)	4/10	7/10	5.5	19/21 90% (6.10)	9/10	9/21 (42.8)	11/21 (52.3)	0/11	0
3	Spl	11/27 (6)	4/11	8/11	4.04	26/27 96.3% (4.19)	8/11	7/27 (25.9)	8/27 (29.6)	0/8	2/2
	BM	12/37 (6)	7/12	11/12	4.3	34/37 91.9% (4.68)	8/12	18/37 (48.6)	7/37 (18.9)	2/7	1/3
Total			23/49 (46.9)	36/49 (73.5)							
8 weeks											
1	Spl	3/29	1/3	2/3	7.79	29/29 100% (7.79)	2/3	0/29 (<3.4)	23/29 (79.3)	0/23	0
	BM	1/16	0/1	0/1	7.93	16/16 100% (7.94)	1/1	0/16 (<6.2)	16/16 (100)	0/16	0
2	Spl	7/21 (4)	5/7	7/7	4.96	20/21 95.2% (4.7)	6/7	6/21 (28.6)	7/21 (33.3)	0/7	3/5
	BM	5/26 (4)	3/5	4/5	7.88	26/26 100% (7.88)	6/5	2/26 (7.7)	16/26 (61.5)	0/16	15/17
3	Spl	7/35 (4)	1/7	6/7	6.34	34/35 97.1%(6.91)	4/7	12/35 (34.3)	22/35 (62.9)	1/22	9/11
	BM	9/40 (4)	3/9	7/9	5.58	38/40 95%(5.87)	7/9	27/40 (67.5)	6/40 (15.0)	0/6	3/4
Total			13/40 (32.5)	26/32 (81.2)							

BM, bone marrow; Spl, spleen.
[a]NP-specific IgG1 memory B cells in the primary response (day 40) and IgG1 memory cells in the secondary response at 1, 3 and 8 weeks after challenge in AID$^{-/-}$ mice as depicted in Figs 2–6.
[b]Number of clones/number of *V186.2* genes sequenced (number of clones in both spleen and bone marrow).
[c]Number of *V186.2* genes joined to *DFL16.1*/number of clones sequenced (% positive clones).
[d]Number of *V186.2* genes joined to *JH2*/number of clones sequenced (% positive clones).
[e]Frequency of somatic mutations in *V186.2* genes sequenced.
[f]Number of mutated clones/number of *V186.2* genes sequenced and % positive clones (frequency of somatic mutations in mutated V186.2 genes sequenced).
[g]Number of clones carrying tyrosine at position 99 of the CDR3 region/number of clones sequenced (% positive clones).
[h]W33L: number of positive clones carrying the affinity-enhancing amino acid exchange from tyrosine to leucine at position 33 of the CDR1 region/number of *V186.2* clones (% positive clones).
[i]K58R: number of positive clones carrying the substitution from lysine to arginine at position 58 of the CDR2 region/number of *V186.2* clones (% positive clones).
[j]Number of W33L clones/number of K58R clones.
[k]Number of K58R clones/number of clones carrying glycine at position 99 of the CDR3 region.

(Table 1 and Fig. 2B). That is, some recipients had V_H186.2-Cγ1 transcripts with a replacement of lysine (K) to arginine (R) at amino acid position 58 (K58R) at a higher frequency than the W33L substitution (Table 1 and Fig. 2B). Thus, the V_H186.2-Cγ1 transcripts in the progeny derived from the day 40 IgG1$^+$ memory B cells upon secondary antigen challenge were predominantly from clones carrying either the W33L or K58R substitution. The dominance of either the W33L or K58R clonotype in each individual recipient mouse could be a result of competition between these two clonotypes (Fig. 2B).

Unmutated memory B cells develop mutated progeny during the secondary response

We searched among the V_H186.2 transcripts for all possible sets of clonally related genes. Complete sequencing of the V_H-Cγ1 transcripts revealed many shared somatic mutations

time points after challenge with NP-CG in PBS. Each point indicates an individual recipient. Closed and open circles represent mice reconstituted with or without IgG1$^+$ memory B cells, respectively. (B) In a similar experiment as in (A), spleen (upper panels) and BM (lower panels) cells were isolated from AID$^{-/-}$ mice at the indicated time points after challenge with NP-CG. Frequencies of NP-specific/IgG1$^+$ ASC were measured by ELISPOT assay. Symbols are as in (A), NP_{18} (left panel) and NP_2 (right panel). (C) Splenocytes were isolated from AID$^{-/-}$ mice at 1 week after challenge and CD43 and CD138 positive and negative cells were separated by the MACS system. Unfractionated cells are also shown as controls. Frequencies of NP-specific/IgG1$^+$ ASC were measured by ELISPOT assay and each point indicates an individual recipient. The data are representative of two experiments with three mice per group. NP_{18} (left panel) and NP_2 (right panel).

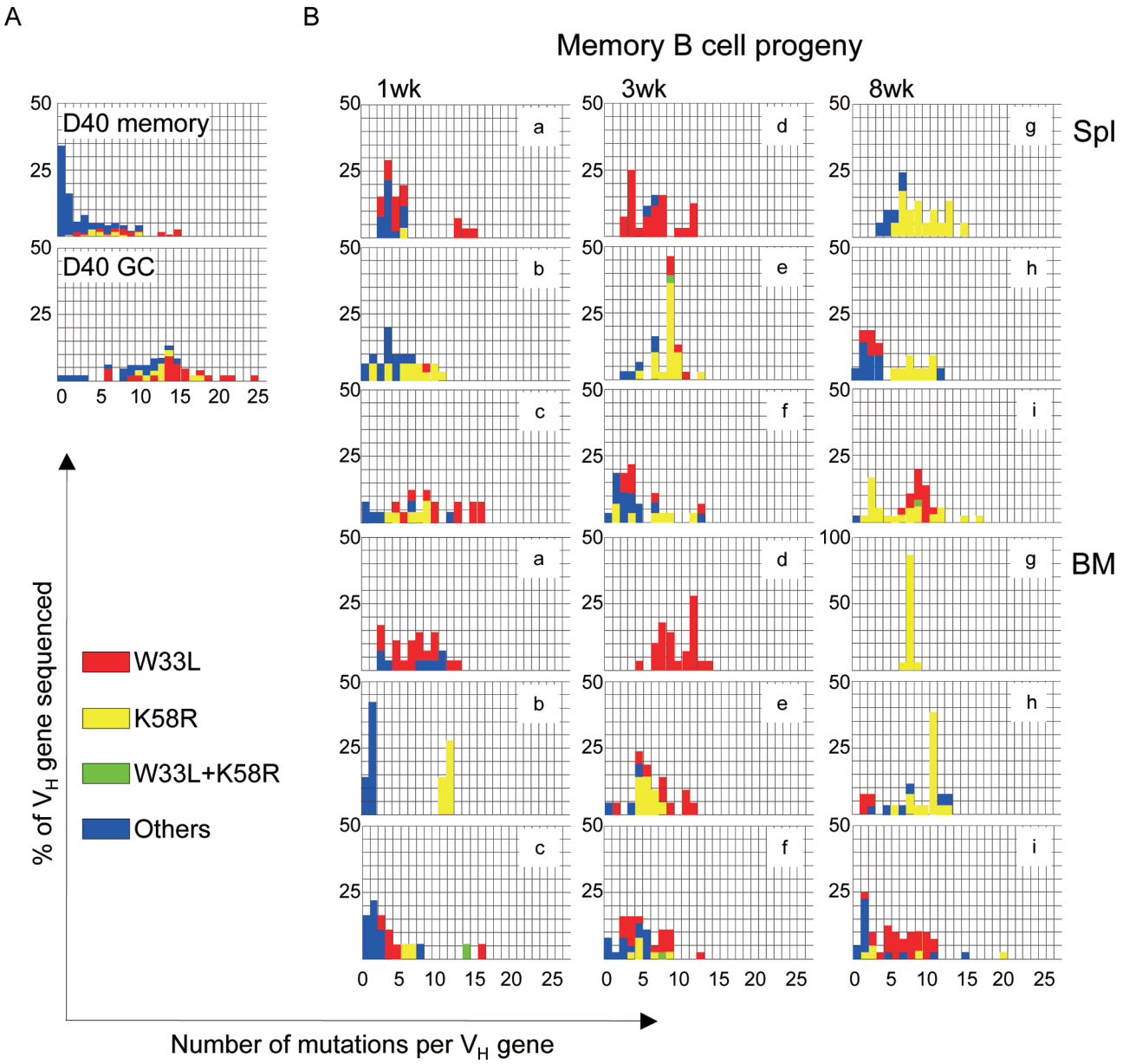

Fig. 2. Somatic mutations in V_H genes during the NP response. Frequency of rearranged V_H genes accumulating W33L (red columns) and K58R (yellow columns) substitutions in (A) IgG1 memory and GC B cells in the primary response (day 40) and (B) IgG1 memory B-cell progeny in the secondary response at 1, 3 and 8 weeks after challenge. The ordinate represents the percentage of clones in the total number of V_H genes analyzed and the horizontal axis represents the number of mutations accumulated in the V_H genes. The blue columns represent the V_H genes without accumulation of W33L or K58R and the green columns represent the V_H genes carrying both substitutions. The data are illustrated from the analysis of 16–40 V_H genes sequenced from spleen (Spl) and bone marrow (BM) of three individual recipients at 1 week (a–c), 3 weeks (d–f) and 8 weeks (g–i) of the response. The data for BM in No. 2 (middle panel) recipient at 1 week of the response are based on analysis of seven V_H genes sequenced.

among the clones that had the same D_HJ_H regions and junctional diversity. Given that clones carrying V_H genes with these features originated from the same precursor (14, 28, 29), accumulation of mutations within memory B-cell progeny with the same CDR3 region allows the construction of clonal genealogies based on shared and distinct mutations.

As shown in Fig. 3, among 20–40 independent V_H gene transcripts from either spleen or BM of a single recipient, 60% fell into families of 2–11 members (3.8 on average) at 1 week of the response and ~80% of clones were derived from families of 2–23 members at 3 and 8 weeks of the response (6 and 8.4 on average, respectively). The distribution of family size may imply that each clone underwent proliferation to a different extent.

Figures 4, 5 and 6 illustrate genealogical relationships among V_H186.2 sequences derived from a common

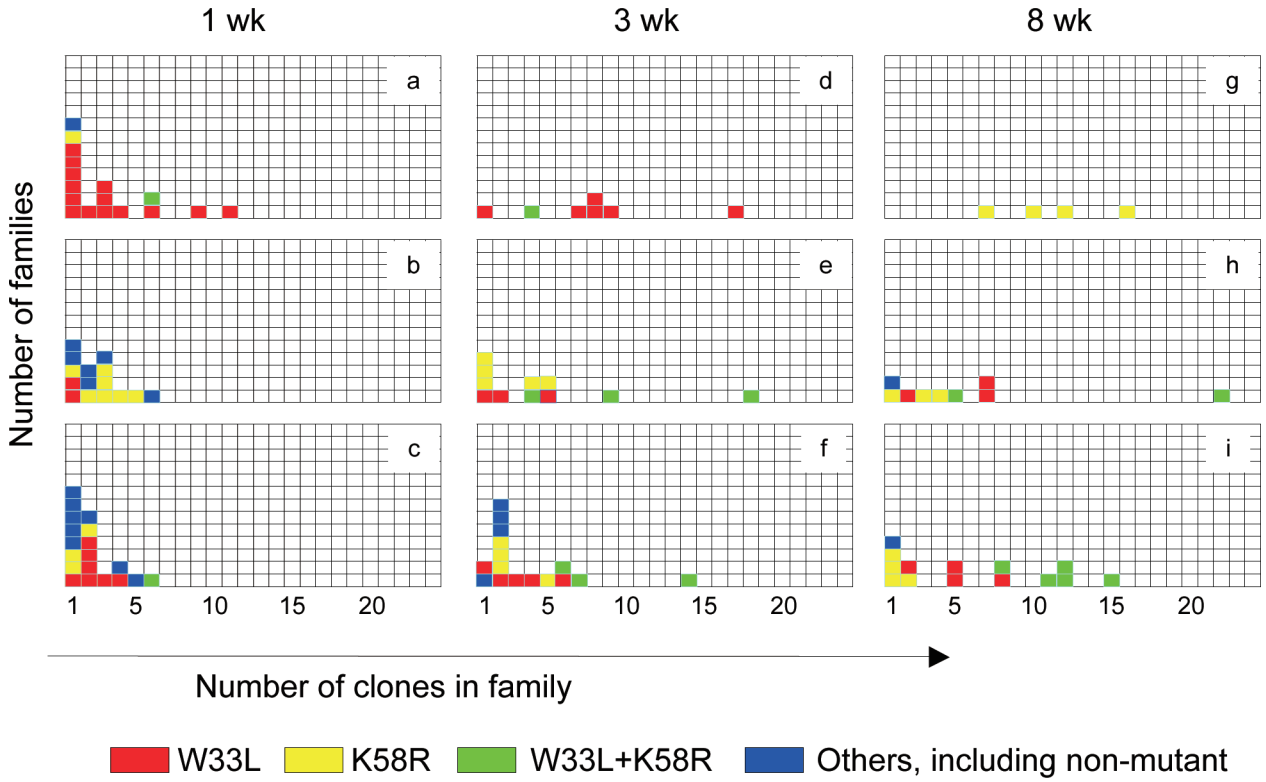

Fig. 3. Size of the oligoclonal families in the secondary response. The clonal diversity for three individual recipients at 1 week (a–c), 3 weeks (d–f) and 8 weeks (g–i) of the secondary response was analyzed. The ordinate represents the number of each single family that has the same D_HJ_H regions and junctional diversity and the horizontal axis represents the number of clones involved in the family. Columns are as in Fig. 2.

progenitor at 1, 3 and 8 weeks after secondary challenge. IgG1$^+$ memory B-cell progeny were recovered from both spleen (blue circles) and BM (red circles) of each individual recipient. In some cases, V_H gene transcripts with the same sequence were detected in both tissues (purple circles), suggesting that memory B cells expanded in the absence of further somatic mutation and homed to different lymphoid organs. Sequence analysis also identified clones with a nucleotide substitution creating a stop codon (*stop*, see clones 1B7S3(1)-Tyr-JH4 and 8B1(1)-DFL16.1-Tyr-JH1), indicating that the progeny of memory B cells must have undergone somatic mutations *in situ* during their expansion, since the cells were originally isolated based on IgG1 expression. Although the A to G substitution is reported to be the most common artifact introduced by PCR (32, 33), this substitution was not found in the base changes in seven clones that led to new stop codons. Also, the generation of non-productive immunoglobulin genes by introduction of stop codons during the GC reaction has been identified by PCR analysis in other studies with different experimental systems (15, 16, 34–36).

The IgG1$^+$ memory B cells that had acquired either the W33L (Fig. 4A and B) or K58R substitution (Fig. 4D) before or immediately after the secondary challenge seem to have undergone significant expansion by 1 week of the response as indicated by the stepwise accumulation of mutations in their V_H gene sequences. On the other hand, IgG1$^+$ memory B cells carrying a V_H gene with the germline configuration also seemed to be actively involved in the secondary response to generate a small number of progeny with an accumulation of mutations (Fig. 4E and F), during which some clones acquired either the W33L or K58R substitution.

Sequence analysis at 3 weeks of the response suggested that memory B cells with either the W33L or K58R substitution produced a large number of progeny with a further accumulation of mutations in their rearranged V_H genes (Figs 2B and 5). Importantly, some unmutated IgG1$^+$ memory B cells generated progeny with either the single W33L or K58R substitution or with double substitutions (Fig. 5B–E). This observation indicates that unmutated IgG1$^+$ memory B cells are capable of giving rise to diverse antibodies in response to secondary antigen challenge. By 8 weeks of the response, each family dramatically expanded its clonal size in both spleen and BM (Fig. 6), in which a single clone with either the W33L or K58R substitution produced 10–20 daughter cells with the accumulation of fewer than 10 mutations per V_H gene. Although heavily mutated memory B cells (>10 mutations per V_H genes) were identified at 1 week of the response (Fig. 2B), such clones and their putative progeny were barely detected at the late stage of the secondary response.

Together, the results indicate that both mutated and unmutated memory B cells responded to the recall antigen, proliferated and accumulated mutations during the secondary response. Both mutated and unmutated memory cells gave rise to a number of progeny and formed a new secondary

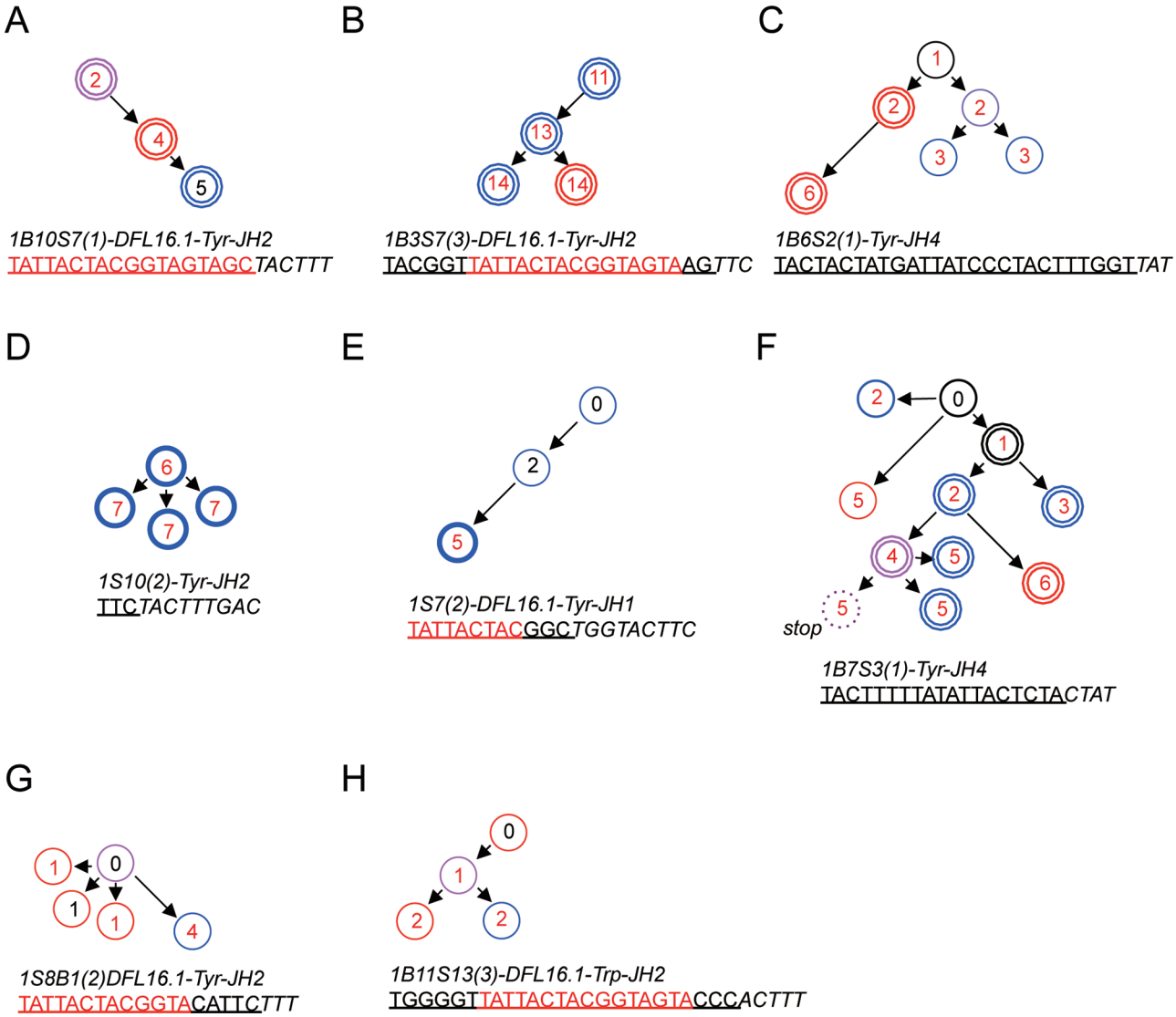

Fig. 4. Genealogical trees generated from sequences of IgG1$^+$ memory B cells transferred into three AID$^{-/-}$ mice. Genealogical relatedness of the $V_HD_HJ_H$ sequences of typical secondary memory B cells specified by clone numbers in spleen (blue circles) and BM (red circles) recovered from individual recipient (A, C and F from a; D, E and G from b and B and H from c) at 1 week after challenge are illustrated. RNA was purified from spleen and BM from individual mice, and rearranged V_H gene transcripts were amplified using V_H186.2-Cγ1 primer pairs and sequenced as described in Methods. Individual clones were assigned to a particular genealogical tree on the basis of having identical $V_HD_HJ_H$ sequence and junctional diversity, together with shared and unique mutations. The numbers of mutated codons are indicated within circles. Clones carrying amino acid changes and those carrying silence mutations compared with their hypothetical parent clone are indicated by red and black characters, respectively. The length of each branch represents the number of mutations. Hypothetical clone members (black circles) are deduced from the sequences of internal clones. The clone with a dotted line represents the one carrying a V_H gene with a stop codon generated by somatic mutations. Purple circles represent clones identified in both spleen and BM. Circles with double and bold lines represent clones carrying an amino acid substitution from Trp to Leu at amino acid 33 of CDR1 and Lys to Arg at amino acid position 58 of CDR2, respectively. The clone name is shown below the genealogical tree and the sequence represents D_H and J_H regions that are underlined and in italics, respectively. The red sequence represents DFL16.1.

antibody repertoire adapted to the secondary antigen challenge that was dominated by V_H genes either with the W33L or K58R mutation or with the Trp33Gly99 clonotype (Fig. 6C and E; Table 1).

Discussion

The immune system has evolved two distinct differentiation pathways to form two types of memory B-cell compartments, one that has been subject to evolutionary selection and the other that is somatically selected for high-affinity antigen binding in the GC reaction after a process of hypermutation (4). The present results reveal that both mutated and unmutated NP-specific/IgG1$^+$ memory B-cell populations are involved in the secondary NP response in AID$^{-/-}$ recipient mice. The response analyzed in this study is composed of memory B cells and ASCs that have undergone a process

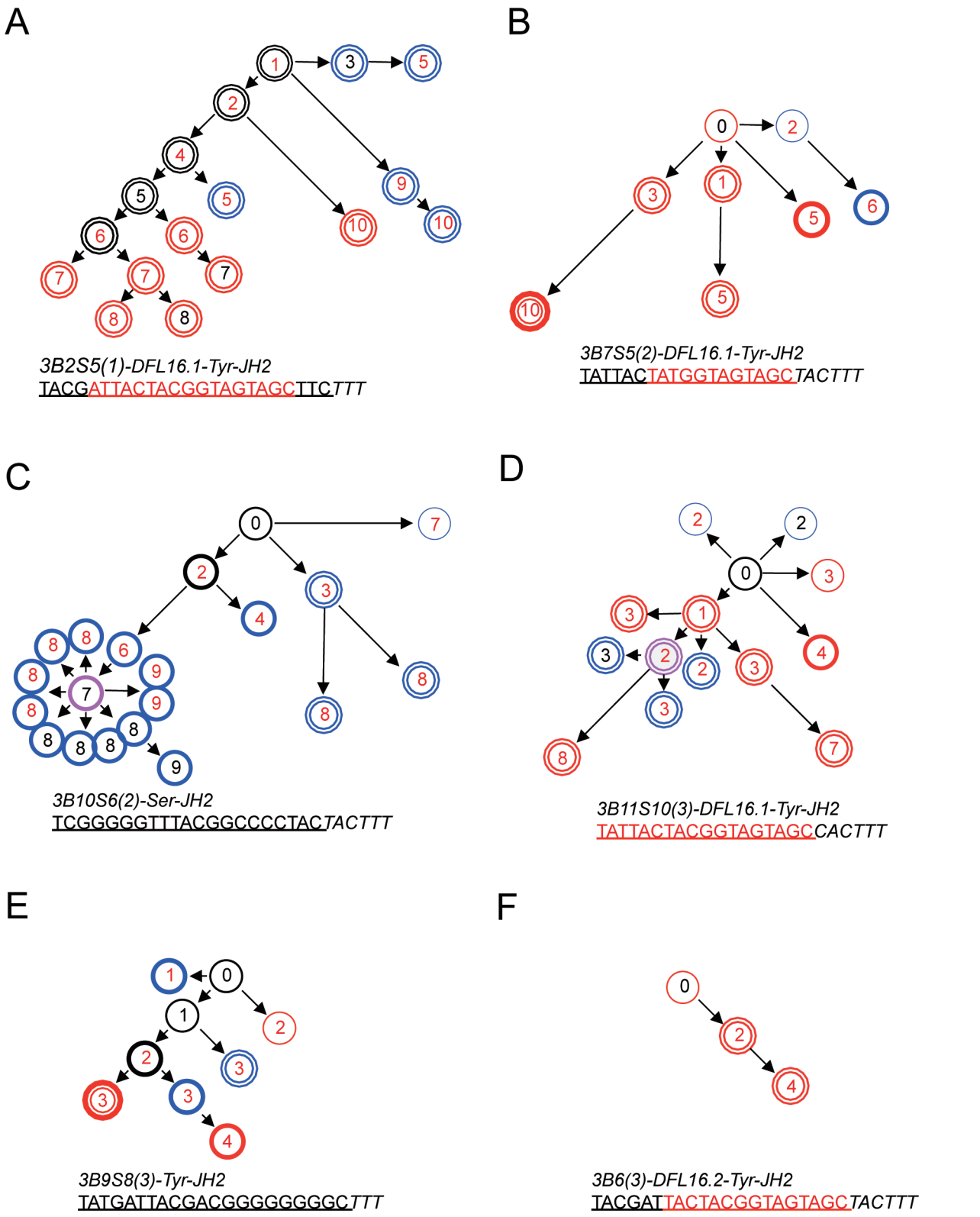

Fig 5. Genealogical relatedness of the $V_HD_HJ_H$ sequences at 3 weeks post challenge. Symbols and methods are as in Fig. 4, but the cells were isolated at 3 weeks post challenge from three individual mice (A from d, B and C from e and D–F from f).

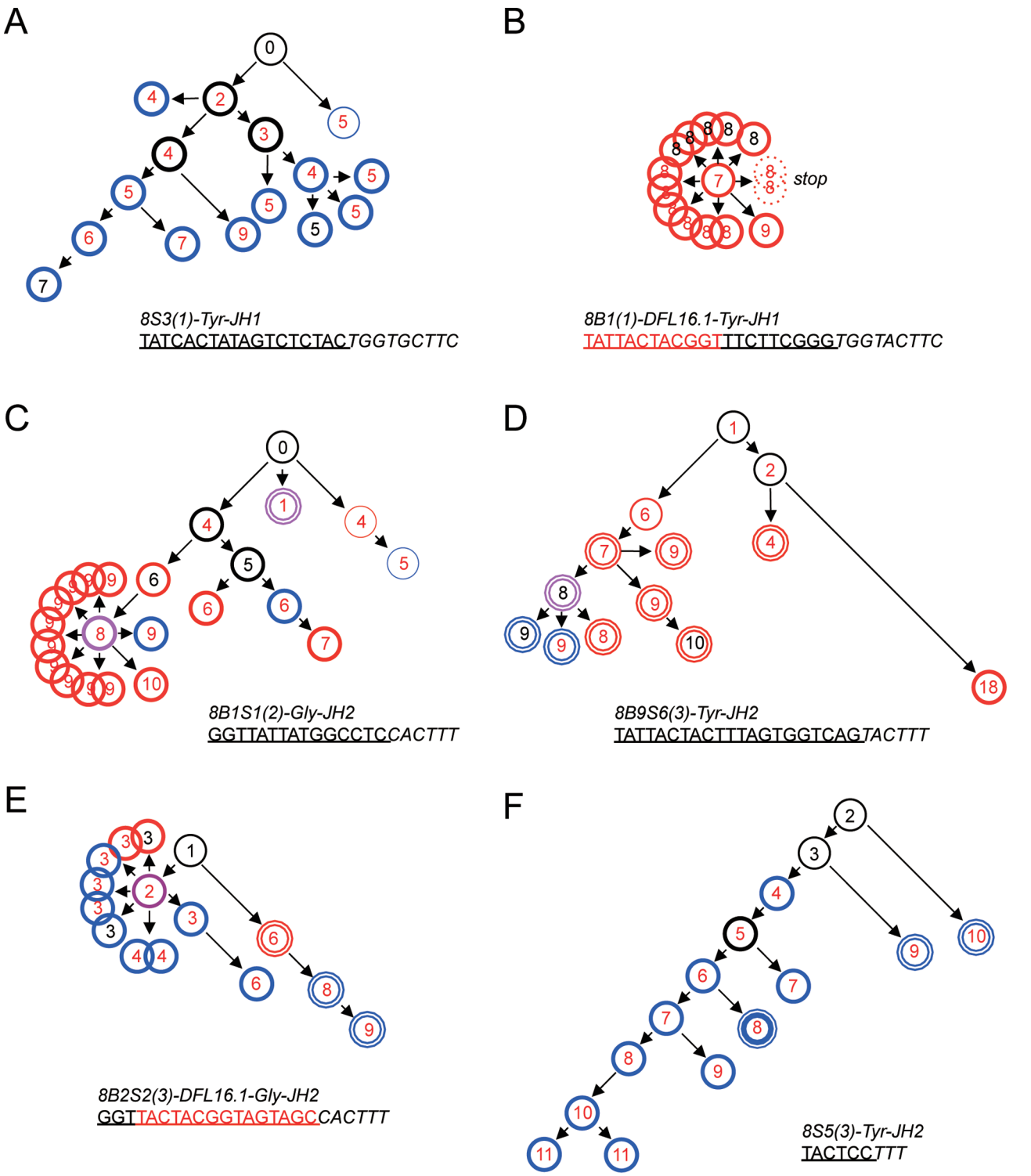

Fig. 6. Genealogical relatedness of the $V_HD_HJ_H$ sequences of typical secondary memory B cells. Symbols and methods are as in Fig. 4, but the cells were isolated at 8 weeks post challenge from three individual mice (A and B from g; C from h and D, E and F from i).

of somatic hypermutation and affinity-based selection presumably through GC reactions. Considering that ASCs are likely to have higher overall immunoglobulin levels compared with memory B cells and GC B cells, we could not exclude the possibility that the data would show bias toward the ASC compartments. Nonetheless, the results demonstrate that unmutated memory B cells generated a large number of progeny that accumulated stepwise mutations in their V_H

genes. Furthermore, even in the presence of mutated memory B cells, unmutated memory B cells generated progeny both with and without the high-affinity W33L V_H region substitution, which confers a 10-fold increase in affinity for the hapten NP (31). In addition, as shown in Fig. 2A, the percentage of high-affinity antibodies in the secondary response by memory B cells was predominantly, but not exclusively, high affinity (i.e. 60–80%) with variations among recipients. These results favor the view that IgG1$^+$ memory B cells responded to the secondary antigen challenge irrespective of their initial BCR properties, either mutated or unmutated and either low or high affinity for the initiating antigen. Such non-discriminating responses could be possible in part because of the activation signal threshold for memory B cells, which is thought to be lower than that for naive B cells in the primary response (37). An alternative but not mutually exclusive possibility would be that antigen–antibody complexes formed upon secondary antigen exposure may enhance the B-cell response (17), thus allowing even low-affinity B cells to enter the secondary reaction.

How could memory B cells rapidly expand and accumulate mutations in the secondary response? Because GCs are dynamic anatomical structures that can be reutilized by newly activated B cells during immune responses (38), it is conceivable that, after challenge with NP-CG, NP-specific memory B cells entered into pre-existing GCs that had formed in the CG-primed AID$^{-/-}$ recipients, where they could expand and accumulate mutations. Supporting this notion, we observed that the secondary response was not efficiently elicited in naive AID$^{-/-}$ recipients that had been transferred with IgG1$^+$ memory B cells and carrier-primed T cells, and subsequently challenged with NP-CG (Supplementary Figure S1, available at *International Immunology* Online). In addition, after secondary challenge we recovered V_H186.2-Cγ1 transcripts from the PNA$^+$ B-cell population of the carrier-primed AID$^{-/-}$ recipients that had been transplanted with IgG1$^+$ memory B cells (data not shown). Heavily mutated memory B cells also expanded and accumulated mutations in their V_H genes upon secondary antigen challenge in AID$^{-/-}$ recipients although such progeny were excluded from the response, probably due to a loss of integrity of the antibody structure or grossly impaired antibody-binding activity as a consequence of further accumulation of mutations, as discussed previously (22, 27, 36). Accordingly, the frequency of mutated V_H genes was increased in the memory B-cell pool in AID$^{-/-}$ recipients after the secondary challenge, whereas the average number of mutations per V_H gene was comparable between the memory B-cell pools before and after the secondary challenge. The latter outcome could be a combined result of ongoing accumulation of mutations in both mutated and unmutated clones as well as deletion of heavily mutated clones.

Our results suggest that IgG1$^+$ memory B cells expanded and accumulated mutations in GCs after re-stimulation. However, Siekevitz *et al.* (22) did not find any evidence for renewed mutations in memory cells in the secondary response when utilizing an adoptive cell transfer system in which NP–keyhole limpet hemocyanin (KLH)-primed spleen cells were transferred into irradiated recipient mice followed by stimulation with KLH-conjugated anti-idiotope antibody. We think that the discrepancy between the present and previous results may be attributed to the differences in experimental systems employed. In our study, we used primed mice, which had established GCs already, as an adoptive recipient without irradiation, whereas Siekevitz *et al.* used unprimed, irradiated mice as adoptive recipients. Thus, the latter did not have preformed GCs, and GCs were not well-developed after immunization with KLH-conjugated anti-idiotope. Under such conditions, memory B cells could not have renewed somatic mutations through the GC response.

In response to sheep red blood cells (SRBC), IgG memory B cells mostly differentiated into plasma cells in SRBC-primed recipients after re-stimulation (39), which is distinct from our observation of the IgG memory response upon re-stimulation with soluble antigen without adjuvant. The discrepancy observed here may reflect the form of antigen used in each study. SRBC-specific IgG production depends on complement receptor (CR)1/2 on B cells. SRBC, which are essentially a dense polyvalent antibody epitope surface become opsonized with complement factors and are captured by follicular dendritic cells (FDCs) via CR1/2, resulting in extensive synergist BCR- and CR2-mediated signaling in antigen-specific B cells (40). Considering the fact that B cells undergoing strong initial interaction with antigen can efficiently differentiate into extrafollicular plasma cells (41), and that IgG1 BCRs dramatically enhance the earliest BCR-intrinsic events (42), it is conceivable that SRBC do indeed optimize the differentiation pathway of memory B cells to extrafollicular plasma cells, as previously observed (39).

The high-affinity W33L substitution was detected in ~20% of day 40 mutated memory B cells. The frequency of V_H genes carrying this substitution was significantly increased among the progeny of memory B cells in the secondary response because of the expansion of W33L$^+$ memory B cells. Furthermore, W33L$^+$ clones were newly generated in the secondary response from unmutated memory B cells, as a consequence of somatic mutations and selection during the secondary GC response. In addition to W33L$^+$ clones, the frequency of clones with the K58R V_H gene substitution was significantly increased in the progeny of memory B cells generated in AID$^{-/-}$ recipients. This may reflect the expansion of the K58R$^+$ progeny generated from unmutated memory B cells as a result of accumulation of mutations and/or the expansion of pre-existing K58R$^+$ clones that were underrepresented among the day 40 memory B cells. Transfer of a limited number of day 40 memory B cells resulted in the dominance of either the W33L$^+$ or K58R$^+$ clonotype in the lymphoid tissues of individual AID$^{-/-}$ recipient mice after the secondary challenge, suggesting that there was interclonal competition between W33L$^+$ and K58R$^+$ memory B cells during their expansion in the immunized recipients. Thus, it is likely that B cells with the K58R substitution and those with the W33L substitution have a similar competitive advantage for expansion over the other clones.

It has previously been suggested that a single key mutation such as the W33L substitution in the anti-NP response (31) alone sufficiently increases the affinity of antibodies (8). In addition, the junctional amino acid at the border of the V_H–D_H segments (Gly at amino acid position 99) also determines the pathway of affinity maturation in the late stage of the anti-NP response. Thus, by accumulating mutations, the Gly99

clonotype could increase the antibody affinity to NP by 10- to 100-fold compared with that in cells with the W33L clonotype (43). Notably, the Gly99 clonotype examined in the previous study exclusively had the K58R substitution (43). Therefore, it is reasonable to speculate that IgG memory B cells may increase the affinity of antibodies by using the K58R in addition to the W33L substitution, indicating that several pathways may provide affinity maturation in the anti-NP response. Whereas the W33L substitution constitutes an important single mutation for the anti-NP memory B-cell response, the introduction of the K58R substitution in non-W33L$^+$ BCRs may increase the probability of their selection to produce high-affinity antibodies optimally adapted to the antigen upon secondary challenge. Such a mechanism could be beneficial for efficiently eliminating pathogens.

We intended to measure the relative affinity of these two major clonotypes of memory B-cell progenies in the secondary response by either expressing several V_H gene clones with W33L or K58R clonotypes in myeloma cell lines expressing lambda chain (31) or expressing Fab fragment in bacteria using a phagemid (44). However, neither approach could provide definitive information on affinities of these two clonotypes due to lack of V_L sequence information. Therefore, we should address the point in future studies such as by single cell sorting of plasma cells and cloning both V_H and V_L genes in order to reconstitute complete antibodies for reconstitution and affinity measurement.

AID$^{-/-}$ recipients transplanted with IgG memory B cells and subsequently immunized showed significantly increased levels of high-affinity serum antibodies from 1 to 3 weeks, compared with naive AID$^{-/-}$ recipients, and maintained these high levels up to 8 weeks after antigen exposure. During this time frame, we found that the clonal complexity among the progeny of the transplanted memory B cells gradually decreased, accompanied by the expansion of a limited number of clones carrying either the W33L or K58R substitution in their rearranged V_H genes. Whether or not such quantitative and kinetic differences in serum antibodies also reflect dynamic changes in the repertoire of the ASC compartment during a secondary response needs further investigation.

Our genealogical analysis suggests that both mutated and unmutated IgG1$^+$ memory B cells respond to a secondary challenge and expand while accumulating somatic mutations in their V_H genes in a stepwise manner. How do we know that the genealogies seen in the present study reflect the state of memory cells after transfer, but not before transfer? In the current study, we sampled 114 cell groups (i.e. a single group carrying the identical CDR3 sequence) in 119 memory B-cell clones at the primary response and 9 groups in 23 memory B-cell clones at 1 week after the secondary response. With the assumption that the numbers of cells among the 114 cell groups were uniform, we carried out a Monte-Carlo probabilistic simulation to address whether or not random sampling of 23 memory B-cell clones from the discrete uniform distribution could belong to only nine cell groups. Within 1 million Monte-Carlo iterations, we observed that this never happened (data not shown). Performing additional Monte-Carlo simulations, we found that this could happen with probability 0.02 when the number of cell groups was reduced to 15 from 114 (data not shown). These calculations support our suggestion that particular groups were selected and driven in the secondary response. Furthermore, the clonotype distributions in each adoptive recipient after secondary challenge shown in Fig. 2 differed from the pattern detected in memory B cells at the primary response before transfer and changed according to the period of time after transfer and subsequent challenge, supporting our conclusion that our genealogy data reflect the dynamics of memory B cells after the secondary response. Finally, sequence analysis identified several clones wherein nucleotide substitution created a stop codon, supporting the notion that the memory B-cell progeny underwent somatic mutations *in situ* during their expansion in adoptive hosts.

In summary, we propose that the antibody repertoire of IgG1$^+$ memory B cells adapts to a recall antigen via accumulation of mutations in the rearranged V genes followed by selection. The unmutated IgG1$^+$ memory B-cell population is particularly flexible in nature to create a new diverse antibody repertoire, probably via the GC reaction, by ensuring that high-affinity antibodies are produced upon antigen re-exposure. This property may guarantee rapid generation of antibodies optimally adapted to the antigenic variants that can arise during the course of an infection.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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