Mechanisms of affinity maturation and selection of the antibody produced by plasma cells

(プラズマ細胞の解析による抗体の親和性成熟と選択機構の解明)

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Abbreviations

Ab: antibody

APC: allophycocyanin

ASC: antibody secreting cell

Ag: antigen

BCR: B cell receptor

CDR: complementarity-determining region

CFP: cyan fluorescence protein

CGG: chicken gammaglobulin

GC: germinal center

GL: germline

mIg: membrane-form immunoglobulin

NP: (4-hydroxy-3-nitrophenyl) acetyl

SHM: somatic hypermutation

sIg: secreted-form immunoglobulin

TD: T-cell dependent
Abstract

Studies on the structural basis of antibody (Ab) affinity maturation have been carried out by measuring the affinity of secreted Abs, and information on the structure has often been obtained from nucleotide sequences of memory B cell receptors (BCRs). I considered that it is important to establish whether the repertoire of secreted Abs from plasma cells is really in accord with that of BCRs on memory B cells at the same time points postimmunization. To this end, the novel assay system for analyzing Abs secreted by plasma cells was established; Plasma cells secreting Abs specific to (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten were isolated by the affinity matrix technology. They were labelled with a complex of biotin-anti-CD138 and streptavidin-NP-allophycocyanin, to which anti-NP Abs secreted by autologous plasma cells were captured preferentially. This technology enabled us to detect live plasma cells as secreted Ig⁺APC⁺ using flow cytometry. By using this plasma-cell based Ab repertoire analysis, I demonstrated that the affinity maturation of anti-NP Abs proceeds in two steps. The first step begins with a germinal center (GC) reaction through which memory B cells as well as plasmablasts are generated from GC B cells, which is accompanied by a gradual increase in Ab affinity. Plasmablasts occupied more than 90 % of the Ab secreting cell (ASC) compartment and secret V186.2⁺Tyr95⁺Leu33⁺
Abs having an NP₂/NP₂₆ ratio of ~0.7 during primary immunization. The second step begins with secondary immunization, and the appearance of a large number of plasma cells secreting V₁₈₆₂⁺Gly₉₅⁺ Abs with multiple somatic hypermutations (SHMs; SHM⁺), which was thought to bring about an increase in the NP₂/NP₂₆ ratio up to ~1.0. Surprisingly, V₁₈₆₂⁺G₉₅⁺SHM⁺ plasmablasts were absent even in the secondary response, suggesting that the repertoire of plasma cells is different from that of plasmablasts, i.e., their respective repertoires are asymmetric. On the basis of these findings, the relationship between plasmablasts and plasma cells as pertaining to their ontogeny is discussed.
**Introduction**

*Antibody evolution: two types of affinity maturation pathway*

Studies on antibody (Ab) affinity maturation have often been carried out using hapten-protein conjugates as antigens (Ags) due to their convenience in measuring Ab affinities. The immune response of C57BL/6 mice to a hapten, (4-hydroxy-3-nitrophenyl)acetic acid (NP), has been widely employed in the analysis of affinity maturation since anti-NP Abs are known for their unique structural properties; they are encoded by the canonical gene segments, $V_{186.2}$, $DFL16.2$, and $JH2$ for the heavy chain and $V_{\lambda1}$ and $J_{\lambda1}$ for the light chain (Bothwell et al., 1981) although $V_H$ genes analogous to $V_{186.2}$ have also been employed (Gu et al., 1991). Therefore, anti-NP Abs are rather homogeneous in terms of usage of their gene segments. However, even though they are encoded by canonical genes, these Abs are heterogeneous in sequences of complementarity-determining regions (CDRs), especially in the third CDR of heavy chains (CDRH3), due to the addition of an N-region by terminal deoxyribonucleotidyl transferase, which adds non-germline encoded nucleotides during Ig gene rearrangement (Benedict et al., 2000). By preparing hybridoms, Furukawa et al. (1999) showed previously that there are two distinct anti-NP Ab populations that were characterized by the amino acid residue at position 95 (Kabat numbering, (Kabat et al.,
gene and harbored Tyr95 (V186.2^Tyr95^). This Ab appeared at the early stage of immunization, and its affinity increased ~10-fold with the introduction of an amino acid replacement of Trp33 with Leu (Leu33^) by means of somatic hypermutation (SHM) (Fig. 3) (Cumano et al., 1986; Torigoe et al., 1995). The other was also encoded by V186.2 but harbored Gly95 (V186.2^Gly95^) instead of Tyr95. Since Gly95 appeared on pairing with His100, this Ab having both Gly95 and His100 was referred to as Gly95^+. This type of maturation pathway required a long time to gain higher affinity up to approximately 100-fold increase than that of V186.2^Tyr95^Leu33^ Abs due to the induction of multiple SHMs in both heavy and light chains (Fig. 3). Although the affinities of germline Abs were similar, the maximum affinity attainable by SHM (referred to as the ceiling affinity) was different between V186.2^Tyr95^ and V186.2^Gly95^ Abs, reflecting the difference in their ability to raise affinity by SHM (referred to as evolvability) (Furukawa et al., 1999). V186.2^Gly95^ Abs generated through the late maturation pathway was potentiated to have higher evolvability than V186.2^Tyr95^ that had completed affinity maturation at an earlier time of immune courses.
The relationship between affinity maturation and B-cell differentiation

Naïve B cells are activated with Ags and undergo one of three fates with the help of CD4+ T cells: they become short-lived plasma cells that secrete primary Abs (Ho et al., 1986), primary memory B cells (Dogan et al., 2009; Pape et al., 2011; Taylor et al., 2012), or germinal center (GC) B cells that can differentiate into memory B cells and/or long-lived Ab-secreting cells (ASCs) (Manz et al., 1998; Blink et al., 2005; Paus et al., 2006; Radbruch et al., 2006; Fig. 2). The third B cell fate via the GC is referred to as the GC-dependent pathway through which affinity maturation of Abs was induced (Jacob and Kelsoe, 1992). Previously, Nishimura et al. (2011) followed changes in the affinity of BCRs on memory B cells using a chromophore protein, allophycocyanin (APC), which was conjugated with varying numbers of NP, and showed that V186.2^-Tyr95^-Leu33^+ memory B cells were dominant at the early phase of the immune response, while V186.2^-Gly95^+ cells required a longer period to attain a high affinity (Sagawa et al., 2002). This result clearly demonstrated that affinity matured B cells were selected into memory B-cell compartment preferentially, not randomly. However, this late appearance of V186.2^-Gly95^+ cells seemed not to be in accord with a rapid increase in the affinities of secreted Abs in the blood in response to antigen rechallenge. Moreover, the fact that V186.2^-Gly95^+ hybridomas started being recovered frequently
after boost immunization could not be explained on the memory B-cell level.

*Antibody secreting cells*

ASCs are cells that have differentiated from such B cells as GC B cells and memory B cells, and consist of plasmablasts and plasma cells; the former were considered to be a precursor of the latter that occupy the final differentiation stage of B lineage cells (Oracki et al., 2010). However, it is not yet focused on the functional difference between them, such as physiological role and Ab repertoire. Furthermore, although memory B cells were also found to form ASCs in response to antigen restimulation, the relationship between these three subsets remains unknown due to a lack of the method to recover live plasma cells. In addition to the unavailability of any crucial surface markers to separate plasmablasts and plasma cells, loss of BCR expression on the latter compartment (Oracki et al., 2010) makes it difficult to isolate Ag-specific plasma cells based on NP-binding ability. The ELISPOT assay that has been used for the detection of cells secreting Ag-specific Abs is not suitable for the study aiming to analyze the phenotype and genotype of viable cells (Crotty et al., 2004; Tarlinton 2006). Although intracellular staining to detect Ag-specific Abs is available, this method cannot be employed for the isolation of viable plasma cells since cells must
be fixed and permeabilized (Manz et al., 1998).

**Purpose of this study**

Increase in the affinities of secreted Abs in the blood after secondary immunization appear to be attributable to the robust expansion of V186.2^Gly95^ cells that were selected based on affinity (Fig. 3) (Furukawa et al., 1999). However, the previous study focused on the memory B cells revealed that V186.2^Gly95^ memory B cells were not dominant at the same time point where V186.2^Gly95^ sequences were frequently observed in hybridomas (Fig. 3). Therefore, it remains difficult to explain the hypothesis that the hybridoma study raised on memory B-cell level (Fig. 3). It is thus necessary to examine which Abs are involved in anti-NP Ab affinity maturation through the analysis of ASCs. In the present experiments, the process of Ab affinity maturation was tracked by measuring the binding ratio of NP_2-BSA to NP_26-BSA (NP_2/NP_26 ratio) of secreted Abs by ELISA, by measuring the number of memory B cells, plasmablasts, and plasma cells at the same immunization time points by flow cytometry, and by analysis of nucleotide sequences of V186.2 genes of isolated cells. On the basis of the findings obtained in this study together with those reported by Murakami et al. (Murakami et al., 2010), I discuss here the relationship between plasmablasts and
plasma cells that appeared after secondary immunization with respect to plasma cell ontogeny.
**Materials and methods**

*NP-protein conjugates and anti-NP Abs*

NP-protein conjugates were prepared as described (Azuma et al., 1987). Briefly, the hydroxysuccinimide ester of NP (NP-OSu) was coupled to carrier proteins such as chicken $\gamma$-globulin (CGG), BSA, APC, and streptavidin-APC (APC$_{avi}$) by incubation in 0.1 M sodium bicarbonate buffer (pH 8.5) containing 0.15 M NaCl for 1 h at room temperature. Any excess reagents or byproducts of the reaction were removed by passing the mixture through a Sephadex G-25 column (Amersham Biosciences) or performing dialysis against PBS. The average molar ratio of NP per carrier protein was calculated by measuring OD at 280 nm and 430 nm except in the case of NP-APC or NP-APC$_{avi}$. The number of NPs per APC or APC$_{avi}$ was approximated based on the molar ratio of NP-OSu and APC or APC$_{avi}$ in the reaction mixture and expressed as NP$_{lo}$, NP$_{med}$, and NP$_{hi}$, respectively. Hybridomas producing the anti-NP Abs, B2 and E11, were prepared as described previously (Azuma et al., 1987). N1G9 was provided by Prof. K. Rajewsky. The equilibrium association constants ($K_a$) of N1G9, B2, and E11 to $\varepsilon$-NP-aminocaproic acid, as determined by isothermal titration calorimetry, are $2.9 \times 10^5$ M$^{-1}$, $3.4 \times 10^6$ M$^{-1}$, and $3.3 \times 10^8$ M$^{-1}$, respectively (Torigoe et al., 1995; Furukawa et al., 1999).
**Immunization**

The experiments using mice were carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of Tokyo University of Science. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tokyo University of Science (Permit Number: S13017). All efforts were made to minimize suffering. Female C57BL/6 mice (Crea) at 6-10 weeks of age were immunized i.p. with 100 μg NP40-CGG adsorbed on 100 μl of alum gel. For analysis of the memory response, mice received a secondary i.p. immunization with 100 μg NP40-CGG in PBS at 7 weeks after the primary immunization. Spleens were excised at various time points and single-cell suspensions were prepared. In some experiments, bone marrow was aspirated from both femurs.

**ELISA and ELISPOT assay**

For measuring the binding of antibodies to NP, plates (Nunc) were coated with 50 μl per well of either 1 μg ml⁻¹ NP₂-BSA or NP₂₆-BSA in PBS and left overnight, followed by blocking with 1% BSA. After washing with PBS containing 0.05% Tween 20, PBST, antibodies of known concentrations, purified Abs or antisera at various dilutions were added and the plates were then incubated for 2 h at room temperature. The bound
antibodies were detected using HRP-conjugated goat antibody specific to mouse Igλ or IgG1 (SouthernBiotech).

NP-specific Ab-secreting cells (ASCs) were detected using ELISPOT assay with a MultiScreen 96-well filtration plate (Millipore) coated with 50 μl per well of either 10 μg ml⁻¹ NP₁-HEL, NP₂-BSA or NP₂₆-BSA, respectively. Serially diluted cells were added to individual well in triplicate, then incubated for 12 h in a humidified atmosphere at 37°C under 5% CO₂. Anti-NP IgG1 spots were visualized with HRP-conjugated goat anti-mouse IgG1 antibodies (SouthernBiotech) in conjugation with TMB substrate (Sigma-Aldrich).

Transfectants

A mouse B-cell lymphoma, K46, and a plasmacytoma, SP2/0, were used for preparing B cell transfectants expressing NP-specific BCRs and secreting anti-NP Abs, respectively. The membrane-form immunoglobulin (mlg) μ chain gene was amplified by PCR from cDNA of BALB/c mouse spleen cells using the primers: CH-XhoI-F1, 5’-agtctcgagtgagatcgtcctc-3’; and IgMm-R1, 5’ taatggcccagcccccttcattcactgtgacag gg-3’. V(D)J genes of N1G9, B2, and E11 were obtained by PCR using cDNA from the respective hybridomas and primers: Bud Hs, 5’-agctcgagtcgtctcagctc-3’; and
VH-XhoI, 5’-ttactcgagctgagagagtggtgcctt-3’. The \( \mu \) gene was cloned into pCR2.1-TOPO vector, followed by ligation with \( V(D)J \) genes. The complete anti-NP membrane form \( Ig \mu \) gene was cloned into pBud CE4.1 vector (Invitrogen). The \( \lambda_1 \) chain gene was amplified from cDNA of N1G9, B2, and E11 using primers: BudEF-NotI-F, 5’-aggcggccgcttagacctggtttgtgaattatg-3’; and EFLa, 5’-aggcggccgcaggtt agatgacctaggaac-3’, and cloned into pBudCE 4.1 vector. These were digested with FspI for linearization and transfected into K46 cells using an electroporater Gene Pulser Cuvettes (BIO-RAD). The transfectants were selected in the presence of 0.4 mg/ml Zeocin. The SP2/0 plasmacytoma secreting anti-NP Abs, N1G9, B2, and E11, were prepared by transfecting the respective H-chain genes of secreted-form immunoglobulin (slg) and L-chain genes. In some experiments, SP2/0 cells transfected with cyan fluorescence protein (CFP) were used.

**Flow cytometry**

Cells were stained on ice for 15 min in PBS containing 1% BSA at 1 x 10^7 /ml with Biotin- or fluorophore-conjugated Abs using a FACSCantoII (BD Biosciences). The mean fluorescence intensity of the cells was recorded. Abs used for flow cytometry were anti-mouse IgM (PerCP-Cy5.5-II/41), anti-mouse IgG1 (PE-A85-1),
FITC/PE-labeled rat anti-mouse λ chain (Ig λ), anti-CD45R/B220 (PE-Cy7-RA3-6B2), Biotin-anti-CD138/syndecan (281.2), and PE-streptavidin. The data were analyzed using FlowJo software (Tree Star).

*cDNA synthesis and sequencing*

Cells were sorted using a FACSariaII (BD Biosciences). Individual cells were sorted into 10 μl of an oligo(dT)-primed cDNA reaction mixture containing PrimeScript™ Reverse transcriptase (Takara) with the recommended reverse transcriptase buffer, 10 mM each dNTP, and 20U RNase inhibitor. Fifty μl reaction mixtures containing 1ul of cDNA from individual cDNA preparations were set up using the following reagents: 1.25U PrimeSTAR™ HS DNA polymerase (Takara) with the recommended reaction buffer, 2.5 mM each dNTP, and varying concentrations of primers. The first PCR was performed for 30 cycles.

*Adoptively transfer of isolated cells*

Total spleen cells collected from NP-CGG/alum-immunized C57BL/6 mice were stained with either Biotin-anti-CD138/syndecan (281.2), or Bitoin-anti-CD45R/B220 (RA3-6B2) and Bitoin-anti-CD19 (1D3), followed by sequential negative isolation.
using MACS system (Miltenyi Biotec) and iMAG system (BD Biosciences). Memory B cells and plasmablasts were enriched as CD138- and B220/CD19-negative fractions, respectively. Purified cells were transferred i.v. into Ly-congeneric mice (CD45.1), which were then boosted with soluble NP-CGG on the next day.

**Statistical analysis**

Statistical analysis were analyzed by the Mann-Whitney test or the Tukey test. GraphPad Prism software was used for analysis, and the data were considered significant at * $p \leq 0.05$, **$p \leq 0.01$, and $p \leq 0.001$. 

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Results

*Affinity maturation of anti-NP IgG Abs*

Ags harboring a different number of hapten groups per carrier protein, such as NP-BSA, are widely employed in estimating the relative affinity. Figure 4A shows the binding of control Abs to NP2- and NP26-BSA. These Abs bound to NP26-BSA irrespective of affinity, while the binding to NP2-BSA was affinity-dependent. Therefore, the ratio of binding to NP2-BSA vs NP26-BSA (NP2/NP26) provided a useful measure of Ab affinity; N1G9 (V186.2’Tyr95’Trp33’, $K_a=2.9 \times 10^5 \text{ M}^{-1}$) had an NP2/NP26 ratio close to 0, and B2 (V186.2’Tyr95’Leu33’, $K_a=3.4 \times 10^6 \text{ M}^{-1}$) had a ratio of ~0.7, and E11 (V186.2’Gly95’SHM’, $K_a=9 \times 10^8 \text{ M}^{-1}$) exhibited the highest ratio, ~1.2 (Fig. 4A).

The changes in the amounts of IgG1 Abs bound to NP2- and NP26-BSA as well as the NP2/NP26 ratios are shown in Figure 4B. Day 7 Abs were characterized by little binding to NP2-BSA and provided an NP2/NP26 ratio < ~0.1. Abs capable of binding to NP2 appeared on day 14 and their amounts increased with time. The NP2/NP26 ratio reached a plateau value of ~0.7 on day 42. After secondary immunization, Abs with higher affinities were produced, having NP2/NP26 ratios of ~1. I divided the process of Ab production into 3 periods based on the magnitude of the NP2/NP26 ratio of the secreted Abs as indicated in Fig. 4B; Phase I corresponds to early production of primary
Ab with low affinity on day 7, Phase II, to the subsequent period of Ab production accompanied by a gradual increase in affinity between day 14 and 49, and Phase III, to the period during which high-affinity Ab production is maintained after secondary immunization (Fig. 4C).

Establishment of a method for isolating plasma cells secreting anti-NP Abs

With the cellular affinity matrix approach, cells are first coated with capture Abs that recognize molecules secreted from autologous cells, which are captured by second Ab reagents so that the secreting cells can be detected by flow cytometry (Brosterhus et al., 1999; Campbell 2003). I modified this technology for isolation of plasma cells secreting NP-specific Abs by labeling cells using a complex of biotin-anti-CD138 and streptavidin-NP-allophycocyanin (NP-APCavi) to which anti-NP Abs secreted from parent plasma cells were trapped (Manz et al., 1995; Fig. 5).

CD138 molecules on SP2/0 cells were reacted with biotin-anti-CD138, allowing for NP-streptavidin-APC (NP-APCavi) with different NP valences (NP_{lo-}, NP_{med-} and NP_{hi-APCavi}) to be conjugated through the biotin-avidin reaction. Biotin-anti-CD138/NP-APCavi labels CD138^+ cells with fluorescent chromophore and, simultaneously, provides NP groups to which anti-NP Abs secreted from autologous
CD138+ cells can bind. Since the bound Abs were detected by FITC-anti-Igλ, anti-NP Ab-secreting cells (ASCs) were detected by flow cytometry as APC+FITC+ double-positive cells. Firstly, I examined whether the anti-NP Abs added to the medium could bind to NP-APCavi via CD138 on SP2/0 cells. All anti-NP Abs bound to NPh-i-APCavi irrespective of their affinities (Fig. 6A), while Ab binding to NPmed-APCavi and NPlo-APCavi was affinity-dependent, N1G9 failed to bind to both, while B2 bound to NPmed-APCavi but not to NPlo-APCavi. Only E11 bound to NPlo-APCavi (Fig. 6A).

Next, I examined the binding of Abs secreted from autologous plasma cells. In order to distinguish between BCRs (mIg) expressed on the surface and Abs (sIg) bound to the anti-CD138/ NP-APCavi complex, anti-Igλ Abs conjugated with different chromophores, FITC or PE, were used. PE-anti-Igλ was added before cultivation to insure that BCR+ cells were labelled with PE. The following cells were used; K46 cells (IgG2a, κ) as B cells of unknown specificity, K46 cells transfected with the membrane form anti-NP IgG or IgM as memory B cells with anti-NP specificity, hybridomas secreting anti-NP Abs as plasmablasts, SP2/0-CFP as plasma cells secreting Abs with unknown specificity (CFP genes were transfected for labeling SP2/0), and SP2/0 transfectants secreting B2 (SP2/0-B2γ) as plasma cells secreting anti-NP Abs. After independent cultivation of these cells, Abs secreted from SP2/0-B2γ or B2 hybridoma,
which bound to biotin-anti-CD138/NP<sub>hi</sub>-APC<sub>avi</sub>, were detected by FITC-anti-Igλ. (Fig. 6B). The Abs secreted were trapped by the biotin-anti-CD138/NP<sub>hi</sub>-APC<sub>avi</sub> complex but not by biotin-anti-CD138/NP<sub>0</sub>-APC<sub>avi</sub> that lacked NP.

Although only cells secreting anti-NP Abs, B2 hybridoma and SP2/0-B2γ, were detected by FITC-anti-Igλ when experiments were carried out using independent cultures (Fig. 6B), the Ab binding to all CD138<sup>+</sup> cells was observed when a mixture of cells was cultured, suggesting that Abs secreted from plasma cells diffused during cultivation and bound to the biotin-anti-CD138/NP<sub>hi</sub>-APC<sub>avi</sub> complexes on neighboring plasma cells (data not shown). To prevent this diffusion, we cultured cells in a medium that contained both 1% agarose and 50 ng/ml NP<sub>13</sub>-BSA. The former was expected to decrease the diffusion rate of the secreted Abs (25) and the latter, to absorb the diffused Abs. When the same numbers of SP2/0-CFP and SP2/0-B2γ were cultured with K46, K46B2μ, and B2 hybridomas, ~50% of CD138<sup>+</sup> cells were detected as an sIgλ<sup>-</sup> population, indicating that agarose and NP<sub>13</sub>-BSA synergistically prevented Abs from binding to neighboring cells (Fig. 6C). In addition, secreted Abs were detected only on CFP<sup-</sub> cells (SP2/0-B2γ) but not on CFP<sup+</sup> cells (Fig. 6D).

*Isolation of ASCs from mice immunized with NP<sub>40</sub>-CGG*
Plasma cells were isolated from day 7 mice immunized with NP_{40}-CGG, using biotin-anti-CD138/NP-APC_{av}, followed by detection of the bound Abs with FITC-anti-Ig\(\lambda\), as described above. B cells and plasmablasts were sorted into NP_{bright}B_{220}^{+}CD138^{+}Ig\(\lambda\) (PE)^{+} cells and NP_{dull}B_{220}^{+}CD138^{+}Ig\(\lambda\) (PE)^{-} cells, respectively (Fig. 7A). Plasmablasts were also characterized as NP_{hi}-APC-binding (NP^+) CD138^{+} cells (Fig. 7B). On the other hand, plasma cells were separated as CD138^{+}Ig\(\lambda\) (FITC)^{+} cells and were characterized by their large size, dull expression of B220, and the absence of BCR expression (Fig. 7B), phenotypes which were consistent with those reported previously (Smith et al., 1997; Manz et al., 1997; Takahashi et al., 1998; Kallies et al., 2004). RT-PCR analysis of cDNA revealed that mIg mRNA was not expressed in plasma cells, in contrast to the significant expression of sIg (Fig. 7C). Although both plasmablasts and plasma cells contained sIg mRNA, mIg mRNA was detected only in the former. On the other hand, B cells (represented by NP_{bright}B_{220}^{+}CD138^{-} cells) synthesized only a small amount of sIg mRNA (Sidman 1981).

*Variation in the numbers of ASCs and B cells during immunization*

Figure 8 shows the variation in the numbers of plasmablasts, plasma cells, and B cells
with time after immunization with NP_{40}-CGG. A large number of NP_{hi}-APC_{avi}-binding IgG1\(^+\) plasmablasts were present on day 7 in spleen (~5 x 10^5, Fig. 8A) and bone marrow (1 x 10^3, Fig. 8C). Plasma cells were observed in spleen on day 7 (~1 x 10^4, Fig. 8B) but not in bone marrow (Fig. 8D). Therefore, the ratio of plasma cells to total ASCs at this time points was less than 0.02. Judging from the small number of plasma cells compared to plasmablasts, the former seemed to provide a rather small contribution to primary IgG1 Ab production. The absence of plasma cells in bone marrow on day 7 suggested that they were generated in spleen but did not migrate into bone marrow immediately.

The number of plasmablasts and plasma cells in spleen had decreased to ~1/10 by day 14, suggesting that these cells were short-lived (Fig. 8A and B). They were further decreased by day 28 and their numbers remained the same thereafter. This decrease in these cells on day 14 was not seen in bone marrow (Fig. 8C and D) (Manz et al., 1998). I also examined the number of plasmablasts capable of binding to NP_{med}-APC_{avi} (Fig. 8A and C). A certain fraction of NP_{med}-APC_{avi}-binding cells were present in spleen and bone marrow. The ratio of NP_{med}-APC_{avi}-binding cells relative to NP_{hi}-APC_{avi}-binding cells (NP_{med}/NP_{hi} ratio) also increased with time, indicating that affinity maturation had proceeded in plasmablasts (Fig. 8E), although the number of
plasma cells was too small to allow for an accurate estimation of the $NP_{med}/NP_{hi}$ ratio (data not shown). On the other hand, memory B cells exhibited a rather small change in their number compared with ASCs (Fig. 8F). On day 42, at a time point when primary immunization had reached a steady-state, there were $4.5 \times 10^3$ memory B cells but only $1.2 \times 10^3$ plasmablasts and $0.1 \times 10^3$ plasma cells, suggesting that more memory B cells were generated compared with ASCs during Phase II. Plasma cells occupied only 6-7% of the ASC compartment, and it is not yet clear whether the Abs have a similar repertoire to that of plasmablasts. It is also not clear whether such small numbers of plasma cells are derived from plasmablasts.

Secondary immunization on day 49 caused a remarkable increase in the cell number on day 56, especially in the number of plasma cells (Table 1). The ratio of cell numbers on day 56 vs day 42 was 20 in spleen and 19 in bone marrow for plasmablasts, while that of plasma cells was 91 in spleen and 19 in bone marrow. Since plasma cells were unable to proliferate in response to Ag stimulation, they may have differentiated either from memory B cells or plasmablasts, although no information was available regarding whether plasmablasts are capable of responding to Ag stimulation (Radbruch et al., 2006; Yoshida et al., 2010). Regardless of their origin, this selective expansion of plasma cells brought their percentage in total ASCs to 26%, much higher than the 7%
seen with primary immunization on day 42, suggesting that plasma cells contribute significantly to Ab production after secondary immunization. In contrast to the rather large increase in the number of ASCs, B cells showed a small change in their number following secondary immunization (Fig. 8F).

**Frequency of SHM in Ab \(V_H\) regions secreted from plasmablasts and BCRs of memory B cells with time postimmunization**

I examined the frequency of SHM in Abs secreted from plasmablasts and in BCRs of memory B cells. SHM was not observed in Abs on day 7 but became apparent on day 14, and the average frequency increased with time (Fig. 9). These profiles of SHM induction were similar to those of BCRs of memory B cells and GC B cells (Nishimura et al., 2011). In fact, SHMs had accumulated to a similar extent in the plasmablast and memory B cell compartment on day 42 (Fig. 9D). \(V_H\) gene sequence analysis showed that \(V_H\) regions of both memory B cells and plasmablasts were predominantly encoded by \(V_{186.2}^{+}Tyr95^{+}Leu33^{+}\) genes and those encoded by \(V_{186.2}^{+}Gly95^{+}\) genes were observed only in memory B cells and not in plasmablasts. Although I attempted to analyze \(V_H\) gene sequences of the plasma cells, only preliminary sequence data were obtained and were insufficient for analysis due to the low numbers of cells recovered.
Characterization of ASCs responsible for recall Ab production

VH gene sequence analysis revealed that there was no significant difference in the frequency of SHM between ASCs on day 42 and 56, or among memory B cells, plasmablasts, and plasma cells (Fig. 10A). These results suggested that additional SHMs would not be induced by secondary immunization. Analysis focusing on the amino acid residue at position 95 also showed that little change in the repertoire of memory B cells occurred with secondary immunization, which consisted largely of components encoded by $V186.2^{+}Tyr95^{+}Leu33^{+}$ genes in addition to those encoded by $V186.2^{+}Gly95^{+}$, suggesting that the immunological memory was maintained without drastic changes in the repertoire brought on by boost immunization (Fig. 10B). Alteration in the repertoire was also not observed in Abs secreted from plasmablasts; these cells were encoded by $V186.2^{+}Tyr95^{+}Leu33^{+}$ genes and the components encoded by $V186.2^{+}Gly95^{+}SHM^{+}$ genes were absent (Fig. 10B). On the other hand, the repertoire of plasma cells was different, i.e., components secreting $V186.2^{+}Gly95^{+}SHM^{+}$Abs became predominant (Fig. 10B). Moreover, NP1-binding Abs with extreme high affinity were secreted only from plasma cells (Fig. 11). These results
suggested that V186.2\(^{\circ}\)Gly95\(^{\circ}\)SHM\(^{\circ}\) memory B cells that had gained high affinity
differentiated into plasma cells that were responsible for affinity maturation at the late
stage of immunization.

Identification of the precursor of plasma cells during secondary response

Plasma cells have been considered to be a maturated form of ASCs derived
from plasmablasts (Oracki et al., 2010). \(\text{V}_{H}\) sequencing analysis revealed that
V186.2\(^{\circ}\)Gly95\(^{\circ}\)SHM\(^{\circ}\) type of cells were only found within the memory B-cell
compartment, but not plasmablast one, at the late time point of primary response.
Together with the finding that the outbreak of V186.2\(^{\circ}\)Gly95\(^{\circ}\)SHM\(^{\circ}\) plasma cells was
brought about after boost immunization, it seems reasonable to hypothesize that
memory B cells might be a major source of plasma cells during secondary response. To
test this hypothesis, I compared plasma-cell differentiation potency between memory B
cells and plasmablasts by boosting mice adoptively transferred with each subset
separately (Fig. 12A). Plasma-cell formation was observed only in mice containing
memory B cells, but not plasmablasts, indicating that the former were a main source of
plasma cells during secondary responses (Fig. 12B).
Discussion

Comparison of the Ab repertoires of plasma cells and plasmablasts or memory B cells at the same time points of immunization deepened the understanding of the relationships between these cells in the developmental pathway as well as the mechanism of Ab affinity maturation at the cellular level. To achieve this understanding, I firstly established experimental conditions for isolating plasma cells using a method based on affinity matrix technology (Manz et al., 1995). I employed CD138 as an absolute marker of ASCs and labelled these cells using biotin-anti-CD138 and NP-APC<sub>avi</sub>. With 30 min cultivation in the presence of 50 ng/ml NP<sub>13</sub>-BSA and 1 % agarose, anti-NP Abs secreted from autologous plasma cells bound preferentially to the biotin-anti-CD138/NP-APC<sub>avi</sub> complex. Plasma cells were discriminated from plasmablasts based on the expression of BCRs on the cell surface. The number of ASCs thus obtained in the present experiments was in good agreement with those reported previously (Yoshida et al., 2010).

I tracked Ab affinity changes using NP<sub>2</sub>/NP<sub>26</sub> ratio as a measure of affinity, which was found to increase in a stepwise manner (Fig. 4B), namely, the time course of Ab production was divided into 3 periods, Phases I, II, and III. Day 7 IgG Abs had an NP<sub>2</sub>/NP<sub>26</sub> ratio of <0.1 (Fig. 4B) and were known to be encoded largely by the germline
29

*V186.2^+Tyr95^+* gene (Smith et al., 1997; Takahashi et al., 1998). Since Abs having an NP2/NP26 ratio < 0.1 appeared only on day 7 but not later, the ASCs responsible for their production would be short-lived. In fact, a large number of plasmablasts appeared on day 7 in spleen but their incidence declined to 1/20-1/50 by day 14. A similar decline in the number of ASCs in bone marrow was not evident, suggesting that they had achieved longevity (Manz et al., 1998). The ratio of plasma cells vs plasmablasts was ~0.07, which is in agreement with results reported by Smith et al. (Smith et al., 1997) who showed that 90% of ASCs were capable of binding to Ags. Therefore, at Phase I, only a small fraction of the ASC compartment was occupied by plasma cells. If I assume that plasmablasts are intermediates of plasma cell ontogeny, most of them seem to remain undifferentiated into the final stage of development.

The Abs secreted from short-lived plasmablasts were substituted by those capable of binding to NP2-BSA after day 14. Phase II corresponds to the duration of GC activity and induction of SHM in GC B cells which then differentiate into either memory B cells or plasmablasts. Since GC B cells at this time point consisted largely of V186.2^+Tyr95^+ cells, they were able to raise affinity promptly by a single amino acid substitution of Trp33 (Trp33^+) to Leu (Leu33^+) (Cumano et al., 1986; Torigoe et al., 1995). Simultaneous to the increase in the NP2/NP26 ratio, SHMs began to accumulate in
plasmablasts (Fig. 9A and B). The NP2/NP26 ratio reached a plateau value of ~0.7 on day 42, which was similar to the value of V186.2+Tyr95+Leu33+ Abs such as B2 (Fig. 4A). On day 42 when the primary immune response had reached a steady state, plasmablasts occupied 93 % of the ASC compartment (Table 1) and 65 % of them secreted V186.2+Tyr95+Leu33+ Abs (Fig. 9E). From these results, I concluded that the gradual increase in the NP2/NP26 ratio up to ~0.7 at Phase II reflected the increase in the fraction of V186.2+Tyr95+Leu33+ plasmablasts. In addition, the fraction of plasma cells on days 7 and 42 in the ASC compartment was as low as ~8 %, suggesting these cells are not generated in the primary response.

Ab affinity maturation at Phase II was demonstrated by the appearance of V186.2+Tyr95+Leu33+ plasmablasts and the essential lack of contribution from V186.2+Gly95+ cells, although V186.2+Gly95+ memory B cells were observed at this time point. Nishimura et al. (2011) showed that the affinity of V186.2+Gly95+ memory B cells before secondary immunization was lower than those of V186.2+Tyr95+Leu33+ cells, therefore it is probable that the former (as well as GC B cells) did not reach the threshold value necessary for plasmablast differentiation. It was also speculated that V186.2+Gly95+ GC B cells differentiated into plasma cells but were too rare to be detected. V_H sequencing of plasma cells would clarify this point. It should be noted that
the GC reaction generated 3-fold more memory B cells, consisting of both V186.2^Tyr95^ and V186.2^Gly95^ cells, than ASCs (Table 1), suggesting that Phase II represents the period in which immunological memory is established.

Although the NP_2/NP_26 ratio reached a plateau on day 42, secondary immunization induced a further increase in the ratio to ~1.0 (Fig. 4B). Since a mutation that brings a further increase in the affinity of V186.2^Tyr95^Leu33^ Abs has not yet been found (26), V186.2^Gly95^SHM^ Abs were considered to be candidates that brought about this increase in the NP_2/NP_26 ratio. In fact, secondary immunization induced a large number of plasma cells, of which 65 % secreted V186.2^Gly95^SHM^ Abs (Fig. 10B). These plasma cells are expected to have differentiated from memory B cells or plasmablasts. However, Murakami et al. (2010) showed that hardly any V186.2^Tyr95^Leu33^ Abs were converted to V186.2^Gly95^SHM^ Abs by SHM. They showed that not only replacement of Tyr95 to Gly but also shortening of the CDR3H length were necessary for conversion of Tyr95 Ab to Gly95 Ab. They also showed that V186.2^Tyr95^ and V186.2^Gly95^ antibodies employed different strategies for affinity maturation; the mutation of Trp33Leu contributed to an increase in the affinity of the former while it had a negative effect on that of the latter. Moreover, the stepwise increase in affinity was yielded by amino acid replacement of Gly66 with Asp in V_L
region of Gly95+ Abs, while these mutations had not been detected in Tyr95+ ones (Fig. 1). In addition to the evidence obtained from these sequence analysis, adoptive transfer experiment revealed that only memory B cells, but not plasmablasts, were potentiated to form plasma cells in response to antigen rechallenge (Fig. 12B). These results thus strongly support the notion that the V186.2+Gly95+SHM+ plasma cells that appeared after secondary immunization were not derived from V186.2+Tyr95+Leu33+ plasmablasts but from V186.2+Gly95+SHM+ memory B cells via V186.2+Gly95+SHM+ plasmablasts, although the latter plasmablasts were rarely observed.

Plasma cell ontogeny is considered to consist of the processes of plasmablast and plasma cell differentiation. We suppose that each process depends on the BCR affinity of memory B cells. The plasma cell compartment generated after secondary immunization consisted of ~30% V186.2+Tyr95+Leu33+ and ~65% V186.2+Gly95+SHM+ cells (Fig. 10B), and therefore contained only affinity-maturated cells (Fig.11). This is contrast to the plasmablast compartment that contained V186.2+Tyr95+Trp33+ cells that had immature affinity (Fig. 10B). These results suggest that the affinity threshold required for differentiation of plasmablasts into plasma cells is higher than that of memory B-cell differentiation into plasmablasts. After secondary immunization, memory B cells differentiate into plasmablasts and only a portion of
V186.2^Tyr95^Leu33^ plasmablasts would be converted into plasma cells because their affinities would hardly reach the threshold and the majority would continue to reside in the plasmablast compartment. On the other hand, V186.2^Gly95^SHM^ plasmablasts would differentiate promptly into plasma cells because of their high affinities and did not remain in the plasmablast stage. Therefore, the absence of V186.2^Gly95^SHM^ cells in the plasmablast compartment and the abundance of V186.2^Gly95^SHM^ cells in the plasma cell compartment can be explained in terms of the high affinity of V186.2^Gly95^SHM^ memory B cells that tend to differentiate into plasma cells (Phan et al., 2006; Fig.13). Plasmablasts and plasma cells appear to have different roles in Ab production; the former are responsible for the prompt secretion of Abs at primary responses, while the latter act to secrete Abs with maturated affinity after secondary immunization (Fig. 13).

Memory B cells that have completed affinity maturation during primary immunization either remain memory B cells, or become plasmablasts, or plasma cells after secondary immunization. Since no significant difference was observed between the memory B cell repertoire before and after secondary immunization (Fig. 9E and 10B), selective expansion of particular components did not occur on Ag stimulation, and “memory” was preserved after proliferation. In view of the longevity of memory B cells
and their limited variation in number during immunization, the role of these cells would appear to be to preserve and transmit their complete “memory” to ASCs and daughter cells. On the other hand, the quantity and quality of Abs in blood were regulated by a change in the numbers of plasmablasts and plasma cells which were promptly generated from the appropriate memory B cells when necessary; V186.2^+Tyr95^+Leu33^+ memory B cells differentiated into plasmablasts and V186.2^+Gly95^+SHM^+ cells, into plasma cells.

In conclusion, I demonstrated here the functional roles of memory B cells, plasmablasts, and plasma cells in the immune response to NP-CGG, with a particular notion that secondary immunization is essential for memory B cells that require a long period for affinity maturation in order to differentiate into plasma cells. The data present in this thesis strongly support the hypothesis that antigen restimulation is necessary for inducing differentiation of silent memory B cells that had gained higher affinity as plasma cells over the course of the immunization schedule. Together with dynamic changes in Ab affinities reported in other immune systems (Griffiths et al., 1984; Berek et al., 1985; Guo et al., 1996; Wiens et al., 2003), the observations obtained from NP hapten system used here are expected to help in the understanding of the immune response and to contribute to the establishment of the effective vaccination schedules.
Acknowledgements

I would thank all people who supported and contributed to the achievement of this study. I could not have completed this study without their help and encouragement.

I would like to first thank my supervisor, Professor Takachika Azuma, for showing me the pleasure of science, for discussions and his broad-minded treatment for my work, and for the greatest wines.

Equally, I deeply appreciate Dr. Akikazu Murakami for continuous supports and precious advices for this work and my life.

And, I would like to give my special thank to Professor Ryo Abe for giving me a great opportunity to start my research life; to learn Immunology; to meet my ideal persons; to stay motivated.

I also appreciate Professor Yoichiro Iwakura for giving me a lot of opportunity to learn more about science and supporting my student life.

And, I would like to thank collaborators throughout this study and my student life, particularly Professor Ryo Goitsuka, Professor Daisuke Kitamura, Yasushi Hara, Dr. Miyuki Nishimura, Dr. Toshihiro Suzuki, and Dr. Kei Haniuda for their great supports, discussions and encouragement. I would also thank all members of Research Institute for Biomedical Sciences, all people who have been there to support me, and “science”.

Finally, for their supporting and understanding my work and dream, I can never thank my parents and family enough.
References


and germinal center B cell differentiation. J. Exp. Med. 203, 1081-1091.


Figures and table
Figure 1

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Figure 1. Molecular characteristics of anti-NP Ab $V_H$ and $V_L$ regions. Anti-NP Abs are encoded by canonical gene segments, $V186.2$, $DFL16.1$ and $J_{\mu}2$ for the heavy chain and $V\lambda 1$ and $J\lambda 1$ for the light chain. An increase in affinity of Tyr95 type Abs is induced by a mutation of W33L in $V_H$ region. Gly95 type Abs having His at position 100j which corresponds to the junction of D-J segments is raised by mutations such as K59R in $V_H$ region, A57V and G66D mutations in $V_L$ region. Appropriate length of CDR3H (5 amino acid residues) may be necessary for the evolvability of Gly95 type Abs.
Figure 2

- TD Ag
- IgM+ naïve B cell
- TH help
- class switch
- IgG-
- short-lived ASC

Phase I
(0-7 days post-immunization)

SHM
affinity maturation
GC B cell
IgG-

Phase II
(14-49 days post-immunization)

memory B cell
IgG+

maturation and survival
long-lived ASC
IgG+

Phase III
(after boost-immunization)

TD Ag
IgG+
TH help
IgG+
memory B cell
IgG+
IgG+
short-lived ASC
Figure 2. B-cell response against TD-Ags. Ag-engaged naive B cells that receive help signals from helper T (Th) cells either differentiate into short-lived ASCs or form germinal center (GC). GC B cells that increase their affinity by accumulating SHM undergo affinity maturation. Affinity-matured GC B cells are selected to develop into memory B cells and long-lived ASCs. Memory B cells provide a long-term defense by differentiating ASCs secreting affinity-matured Abs at the time of Ag reexposure. The time course of Ab production was divided into three periods; Phase I, Phase II, and Phase III. Phase I: primary production of germline Abs (day 0-7); Phase II: Ab production accompanied by a gradual increase in affinity through GC reaction (day 14-49); Phase III: affinity-matured Ab production after secondary immunization (day 56-).
Figure 3

Hybridoma
(Furukawa et al., Immunity 1999)

Memory B cell
(Nishimura et al., Int Immunol 2011)
Figure 3. Discrepancy in the time point when Gly95 type becomes dominant between hybridomas and memory B cells. Tyr95\(^+\) type with W33L mutation became dominant at the same time point postimmunization in hybridomas and memory B cells. Gly95\(^+\) type hybridomas were frequently observed at earlier stage of secondary response when Gly95\(^+\) type memory B cells were rare. Each circle represents an individual clone obtained at the indicated time points and colors represent the \(V_H\) repertoires as follows: green, Gly95\(^+\) type; orange, Tyr95\(^+\)W33L\(^+\) type; black, Tyr95\(^+\) germline (GL) type (top). Pie charts represent the \(V_H\) repertoire of memory B cells at 7 and 28 days after secondary immunization, corresponding to 56 and 77 days after primary immunization (middle). The time course of Ab production was divided into three periods; Phase I, Phase II, and Phase III. Phase I: primary production of germline Abs (day 0-7); Phase II: Ab production accompanied by a gradual increase in affinity through GC reaction (day 14-49); Phase III: affinity-matured Ab production after secondary immunization (day56-).
Figure 4. Changes in the amount of Abs bound to NP$_2$-BSA or NP$_{26}$-BSA and in the NP$_2$/NP$_{26}$ ratio during the immune response following immunization with NP$_{40}$-CGG. All mice primed with NP$_{40}$-CGG/alum were received a secondary immunization with NP$_{40}$-CGG in PBS. (A) Binding of control IgG Abs having different $K_a$ values to NP, N1G9 ($K_a = 2.9 \times 10^5$ M$^{-1}$), B2 ($K_a = 3.4 \times 10^6$ M$^{-1}$), and E11 ($K_a = 9 \times 10^8$ M$^{-1}$) (NP$_2$-BSA, open bars; and NP$_{26}$-BSA, closed bars). NP$_2$/NP$_{26}$ ratios are represented by hatched circles. (B) Changes in the binding of IgG1 Abs in the immune sera. Serum from an individual immunized mouse (n = 5) was diluted (20,000-fold) and its binding measured binding by ELISA. Open circles (individual mice) and open bars (averaged values) represent the binding to NP$_2$-BSA. Closed circles (individual mice) and closed bars (averaged values) represent binding to NP$_{26}$-BSA. The time course of Ab production was divided into three periods; Phase I, Phase II, and Phase III. Phase I: primary production of germline Abs (day 0-7); Phase II: Ab production accompanied by a gradual increase in affinity through GC reaction (day 14-49); Phase III: affinity-matured Ab production after secondary immunization (day 56-). (C) NP$_2$/NP$_{26}$ ratios of each serum sampled at indicated time point post-immunization represented by black circles. NP$_2$/NP$_{26}$ ratios of control IgG Abs are shown as colored circles (N1G9: gray; B2: orange; E11: green).
Figure 5

1st

Biotin-anti-CD138
streptavidin-NP-APC
PE-anti-Ig λ

2nd
culture

3rd
FITC-anti-Ig λ

Plasma Cells

→

FITC + PE + APC +

Plasmablasts

→

FITC + PE + APC +

CD138
Biotin-anti-CD138
streptavidin
NP-APC
anti-NP BCR/Abs
PE-conjugated anti-Ig λ
FITC-conjugated anti-Ig λ
Figure 5. Schematic representation of a modified cellular affinity matrix system for isolation of plasma cells. Cells are stained with and Biotin-anti-CD138, followed by NP-conjugated streptavidin-APC, together with PE-anti-Igλ in order to detect BCRs (1st). After culturing cells for 30 min, NP-specific Abs secreted from autologous CD138^+ cells are captured on the surfaces of themselves (2nd). The bounded Abs are detected by FITC-anti-Igλ (3rd).
Figure 6

A

![Graph showing IgG-positive cells (%) for N1G9, B2, and E11.]](image)

Legend:
- □ NP0
- ■ NPh
- ▲ NPmed
- ▼ NPl0

B

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<td>sIg λ</td>
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Legend:
- □ NP0
- ■ NPh

C

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<td>mIg λ</td>
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NP0-APCavi: Nonculture | Medium | Agarose NP-BSA
0.45 | 3.85 | 84 |
| mIg λ    | 49.6      |

D

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Legend:
- □ NP0
- ■ CFP−
- ▼ CFP+

E

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Legend:
- □ NP0
- ■ CFP−
- ▼ CFP+
Figure 6. Development of a modified cellular affinity matrix system for isolation of plasma cells. (A) Binding of control Abs to biotin-anti-CD138/NP-APC_{avi} complex on an SP2/0 surface. The complex was pre-formed by adding biotin-anti-CD138 to an SP2/0 cell suspension followed by a biotin-streptavidin reaction with NP-APC_{avi} having different NP valences, NP_{lo-}, NP_{med-}, and NP_{hi-}APC_{avi}. The control Abs were added to the SP2/0 cell suspension and bound Abs were titrated by flow cytometry using FITC-anti-Ig\(\lambda\). (B) Binding of secreted Abs from SP2/0 transfectants to biotin-anti-CD138/NP-APC_{avi} complex on an autologous cell surface. Cells were cultured for 30 min and bound Abs were measured using FITC-anti-Ig\(\lambda\). (C) Specificity of detection of ASCs, B2 and SP2/0-B2\(\gamma\), by NP_{hi-}APC_{avi}. Secreted Abs were detected on the cell surface of ASCs using NP_{hi-}APC_{avi} but not NP_{lo-}APC_{avi} which lacked NP. No difference in binding between NP_{hi-}APC_{avi} and NP_{lo-}APC_{avi} was observed in the B cell lymphoma, K46, and its transfectant, K46B2\(\mu\). Discrimination between mIg and sIg was carried out using PE-anti-Ig\(\lambda\) and FITC-anti-Ig\(\lambda\), respectively. (D) The sIg\(\lambda^+\) fraction showed no CFP fluorescence, and conversely, the CFP\(^+\) fraction showed no sIg\(\lambda^+\) cells (E), indicating that there was little contamination by non-anti-NP Ab secreting plasma cells in those of the secreting (sIg\(\lambda^+\)) fraction.
Figure 7

**A**

NP → CD138

Memory B cells

B220 → NP

Plasmablasts

**B**

CD138-NP → mlg λ

Plasma Cells

**C**

<table>
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Figure 7. Isolation of NP specific B cells as well as plasmablasts or plasma cells secreting anti-NP Abs from mice immunized with NP40-CGG. (A) NP-specific B cells and plasmablasts were isolated using NPhi-APC, which bound to their BCRs on the cell surface. B cells were then separated as B220+CD138- cells and plasmablasts, as B220+CD138+ cells. (B) Plasma cells secreting anti-NP Abs were isolated as CD138+mIgλ- cells, while plasmablastss were distinguished as NP-binding mIgα+ cells. (C) RT-PCR analysis of mRNAs encoding the membrane- (mIgγ) or secreted-form (sIgγ) of γ-chains obtained from memory B cells, plasmablasts, and plasma cells, respectively. β-Actin was used as a control.
Figure 8

A  Spleen Plasmablasts

B  Spleen Plasma Cells

C  BM Plasmablasts

D  BM Plasma Cells

E  Memory B cells

F  Memory B cells
Figure 8. Appearance of plasmablasts, plasma cells, and memory B cells with time postimmunization with NP_{40}-CGG in spleen and bone marrow. (A) Changes in the numbers of plasmablasts secreting anti-NP IgG Abs at the early stage of immunization. (B) Variation in the number of IgG^+ plasmablasts in spleen. (C) Changes in the number of IgG^+ plasma cells in spleen. (D) Changes in the number of IgG^+ plasmablasts in bone marrow. (E) Changes in the number of IgG^+ plasma cells in bone marrow. (F) Changes in the number of IgG^+ memory B cells in spleen.
Figure 9

A  Spleen Plasmablasts

B  BM Plasmablasts

C  Memory B cells

E  Spleen Plasmablasts  BM Plasmablasts  Memory B cells

- V186.2  Tyr95  Trp33
- V186.2  Tyr95  Leu33
- V186.2  Gly95
- Others

D  Spleen Plasmablasts  BM Plasmablasts  Memory B cells

Number of mutations per V186.2 gene

Weeks after immunization

N=18

N=26

N=26
Figure 9. Frequency of SHM induced in the $V_H$ region of IgG Abs secreted from plasmablasts with time postimmunization in spleen (A) and bone marrow (B). The distribution of SHM in memory B cells also shown in (C). Comparison of the frequency of SHM (D) and the $V_H$ repertoire (E) of memory B cells and plasmablasts on day 42 after primary immunization. Diversity in the amino acid residues at positions 33 and 95 is shown by pie charts. V186.2²Tyr95²Trp33²; V186.2²Tyr95²Leu33²; V186.2²Gly95²; others.
Figure 10

A

Number of mutations per V186.2 gene

Plasmablasts  Plasma Cells  Memory B cells

0  5  10  15  20

B

<table>
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<tr>
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<th>B Plasmablasts</th>
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<tr>
<td>N</td>
<td>20</td>
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<td>16</td>
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<tr>
<td>%</td>
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<td>12.5%</td>
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- V186.2 Tyr95 Trp33
- V186.2 Tyr95 Leu33
- V186.2 Gly95
- Others
Figure 10. Comparison of the frequency of SHM (A) and the \( V_{11} \) repertoire (B) among memory B cells, plasmablasts, and plasma cells 7 days after secondary immunization, corresponding to 56 days after primary immunization. Data are taken from 3 independent experiments using 2 mice per experiment.
Figure 11

The figure shows a bar graph comparing the spot number of Plasmablasts and Plasma cells for two different samples, NP₁ and NP₂. The y-axis represents the spot number ranging from 0 to 100, while the x-axis categorizes the samples as Plasmablasts and Plasma cells. The graph indicates a higher spot number for NP₂ in the Plasma cells category compared to NP₁.
Figure 11. ELISPOT assay of plasmablasts and plasma cells 7 days after secondary immunization using NP₁ and NP₂ plates. The numbers of spots per well using 100 each cell obtained from spleen and bone marrow on the respective plates are shown.
Figure 12

A

Memory B cells

Plasmablasts

B

![Image of Figure 12 with text and graphs]

**Number of donor cells**

- Memory B cells
- Plasmablasts
Figure 12. Comparison of plasma-cell differentiation potential between memory B cells and plasmablasts. CD45.1 mice were adoptively transferred with either memory B cells (CD138-negative fraction) or plasmablasts (B220/CD19-negative fraction) isolated from NP40-CGG primed C57B/6 mice. On the next day posttransfer, the mice were challenged with NP40-CGG in PBS. (A) Donor-derived (CD45.2+) plasma cells were isolated from each mice 7 days after boostimmunization. (B) The actual number of donor-derived CD45.2+ plasma cells was shown as in Fig. 7A. Data are taken from 3 independent experiments.
Figure 13

Affinity

Phase I
(0-7 days post-immunization)

Phase II
(14-49 days post-immunization)

Phase III
(after boost-immunization)
Figure 13. A working model of affinity maturation on cellular level. During Phase 1, plasmablasts secreting germline(GL) type Abs with low-affinity are predominantly generated to provide a prompt protection against exposed Ags. During Phase II, Tyr95$^+$ B cells that increase affinity by introducing W33L mutation develop into memory B cells and plasmablasts, while the majority of them are selected into the former compartment. Following the early expansion of Tyr95$^+$Leu33$^+$ type, Gly95$^+$ SHM$^+$ type appears in memory B-cell fraction. During Phase III, the secretion of affinity-matured Abs is accomplished by a burst of Gly95$^+$SHM$^+$ plasma cells originating from memory B cells. NP$_2$/NP$_{26}$ ratio of IgG1 binding shown in Fig. 4B is represented by faint red line.
Table 1

**Table 1.** Cell number of each subsets at indicated time points

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<td>Spleen Plasma cells</td>
<td>8.92(±4.2)</td>
<td>0.69(±4.2)</td>
<td>0.07(±0.06)</td>
</tr>
<tr>
<td>Spleen Memory B cells</td>
<td>23.3(±10.8)</td>
<td>5.91(±3.02)</td>
<td>5.01(±4.37)</td>
</tr>
<tr>
<td>BM Plasmablasts</td>
<td>1.7(±0.46)</td>
<td>1.13(±0.52)</td>
<td>0.8(±0.3)</td>
</tr>
<tr>
<td>BM Plasma cells</td>
<td>n/d</td>
<td>0.13(±0.11)</td>
<td>0.11(±0.1)</td>
</tr>
</tbody>
</table>

Cell number (x10^3) (±standard deviation)