chemicals [8]. DNA microarray assays have also been applied to the analysis of the side effects of medicines [9]. Recently, the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have, either individually or together, started to review submissions for the qualification of biomarkers for medical products for specific purposes proposed by industry [10]. The introduction of pharmacogenomics, or pharmacogenetics, to the evaluation of medicines is a global trend.

For a better understanding of the molecular toxicology regarding vaccines, DNA microarray analysis promises to be an ideal method, as has been the case for pharmaceuticals. The FDA now encourages the voluntary submission of genomic data to the FDA outside of the regular review process [11]. However, no studies similar to those described above for pharmaceuticals have yet been conducted in the field of vaccines. At the beginning of this review, we summarized the current efforts used for the control of vaccine safety using conventional animal tests. We then referred to our recent efforts using DNA microarray analysis to identify "genetic signatures" for the toxicants remaining in vaccines. Since pertussis and influenza vaccines are among the most commonly used vaccines, we tried to develop a system to evaluate the "genetic signatures" of the toxicity of these vaccines.

2. Current Vaccine Safety Test

2.1. Body Weight Change in Vaccinated Animals. To screen for general toxicity of vaccines, the body weight of vaccine-treated animals can be analyzed as the general safety test [12]. Five mL of the vaccine are injected into the peritoneum of guinea pigs weighing 300–400 g, and the weight loss experienced by the animals is analyzed at days 1, 2, 3, 4, and 7 after administration. None of the animals should show any abnormal signs; no statistically significant ($P = .01$) difference in weight loss should be observed between the treated animals and the control group on any observation day. This test has been applied to a wide variety of vaccines in a unified way, and plays an important role in ensuring the safety and consistency of vaccine batches [12]. For pertussis vaccine (inactivated whole cell formulation), the effects of vaccine treatment were also measured using test for toxicity to mouse weight gain, in addition to the general safety test. All mice were weighed on days 0, 1, 2, 3, 4, and 7 after vaccine administration. The criterion for judgment is that mean body weight 3 days after injection should be no less than that at the time of injection upon statistical analysis, and no mice showed any abnormal sign during the observation periods [12]. When the reference vaccine (RE: the inactivated whole cell pertussis vaccine) was administrated, weight loss was observed on day 1 after administration (Figure 1(a)).

2.2. Leukocytosis-Promoting Toxicity in Vaccinated Animals. To detect the toxin present in pertussis vaccines, the number of peripheral leukocytes can also be analyzed. Pertussis vaccine is injected into the peritoneum of mice at a dose of 0.5 mL. Leukocytes present in peripheral blood are then counted 3 days after injection [12]. The white blood cell (WBC) counts in peripheral blood of reference vaccine-treated mice reach approximately 2,500 cells/μL (Figure 1(b)). The standard criterion of safety for pertussis vaccine (inactivated whole cell formulation) is that the mean count of leukocytes in peripheral blood, 3 days after injection, should not exceed 10 times that before injection [12].

2.3. Leukopenic Toxicity Test in Vaccinated Animals. Quality control of influenza vaccines is performed using the general safety test and the leukopenic toxicity test (LTT), which is based on peripheral WBC counts in mice 12–18 hours after intraperitoneal injection of a vaccine. The criterion for judgment is that the leukopenic toxicity of the test sample relative to that of the toxicity reference sample should be no higher than the value corresponding to 80% of the leukocyte count of the control relative to that of the toxicity reference sample [12–14].

3. DNA Microarray-Based Safety Test

The currently used quality control and safety tests, such as the LTT and the general safety test, have been used to evaluate vaccine safety for over 50 years [3]. We are now developing a new quality control method for vaccines using DNA microarray analysis as a substitute for the conventional animal tests [15–17]. The principle of this method is to translate vaccine quality, immunogenicity, and reactogenicity, into gene expression profile data. This method is expected to be informative, rapid, and highly sensitive.

For DNA microarray analysis using vaccines, 8 week-old male rats, weighing 180–220 g, were intraperitoneally administered with 5 mL of vaccine or physiological saline (SA). Three to 6 rats were used for each group. Vaccinated rats were sacrificed to obtain whole lung, kidney, brain, and the lateral left lobe of the liver on day 1, 2, 3, and 4 postadministration (Figure 2). Tissues were immediately frozen in liquid nitrogen for storage. Thawed tissue was homogenized and poly(A)$^+$ RNA was purified from the lysate. Cyanine 5-labeled poly(A)$^+$ RNA was subjected to DNA microarray analysis. Blood was also collected, however, this could not be analyzed due to the low quality of purified RNA.

For DNA microarray analysis, a set of synthetic polynucleotides (80-mers) representing 11,468 rat transcripts and including most of the RefSeq genes deposited in the NCBI database (MicroDiagnostic, Tokyo, Japan) was arrayed on aminosilane-coated glass slides [18, 19]. Cyanine 5-labeled poly(A)$^+$ RNA was competitively hybridized on the slide with cyanine 3-labeled common reference RNA. Hybridization signals were measured, processed into primary expression ratios ([Cyanine 5-intensity obtained from each sample]/[Cyanine 3-intensity obtained from common reference RNA]), and then normalized by multiplying normalization factors calculated for each microarray feature.

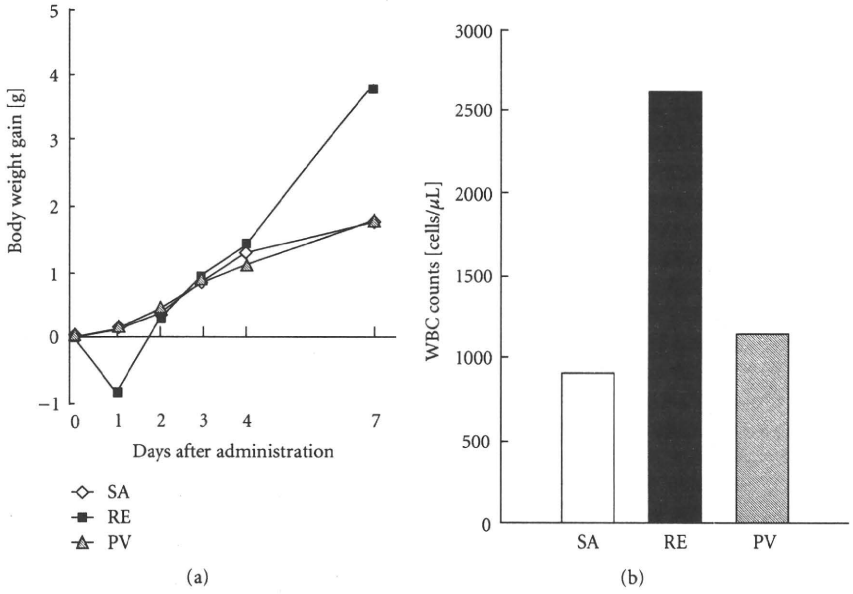

FIGURE 1: Safety control tests for pertussis vaccines. (a) Test for toxicity to mouse weight gain. Physiological saline (SA), an inactivated whole-cell pertussis vaccine (RE), or an acellular pertussis vaccine (PV)-administered mice were weighed on 0, 1, 2, 3, 4, and 7 days postadministration. Ten mice in each group were used, and the mean changes in body weight are indicated. (b) Leukocytosis promoting activity of various pertussis vaccines. White blood cell (WBC) counts in peripheral blood were measured 3 days after vaccine administration. Ten mice in each group were used and the mean WBC counts are indicated.

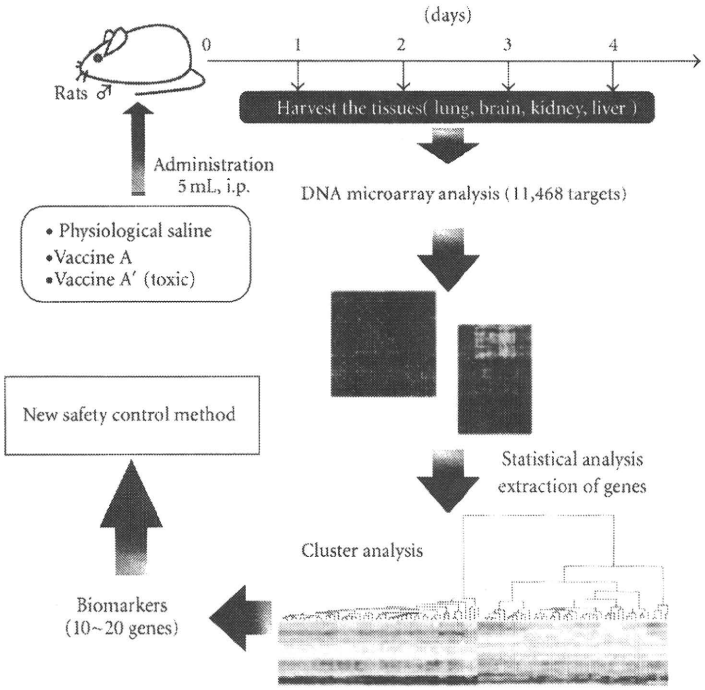

FIGURE 2: The gene expression analysis procedure. The detail of the procedure is described in the text.

For data processing and hierarchical cluster analysis, the primary expression ratios were converted into $\log_2$ ratios ($\log_2$ Cyanine-5 intensity/Cyanine-3 intensity). The genes with $\log_2$ ratios over 1 or under -1 in at least one sample were extracted from the primary data matrix, then subjected to two-dimensional hierarchical cluster analysis for samples and genes.

For the identification of biomarker genes for pertussis vaccines, we extracted differentially expressed genes from physiological saline and pertussis toxin-treated lung samples using the t-test ($P < .01$). Among the extracted genes, we further selected genes that exhibited mean average $\log_2$ ratio differences greater than 0.75 between the two sample groups [17]. For influenza vaccines, we extracted differentially expressed genes from physiological saline and inactivated whole-virion vaccine-treated lung samples using the t-test ($P < .005$) [16].

4. Pertussis Vaccines

Pertussis, or whooping cough, is an infectious respiratory disease caused by a Gram-negative bacillus, *Bordetella pertussis*. *Bordetella pertussis* possesses several pathogenic components, including pertussis toxin (PT) [20]. PT is known as a leukocytosis promoting factor, a major contributor to the pathogenesis of pertussis, and an antigen in immunity to pertussis [21]. At present, whole-cell pertussis vaccines and acellular pertussis vaccines containing inactivated PT are in commercial use [20].

Although pertussis vaccines are effective in the prevention of whooping cough, they have occasionally caused local reactions such as redness, swelling, and pain at the injection site. However, little is known about the overall responses to these vaccines. To address this problem, we applied DNA microarray analysis and quantification of specific genes to analyze the toxicants in pertussis vaccines [15, 17]. Three preparations, an acellular vaccine containing inactivated pertussis toxin (PV), an inactivated whole-cell vaccine (RE), and a purified pertussis toxin (PT) were prepared. RE is a reference vaccine for National Quality Control Tests of pertussis vaccines in Japan and is made from formaldehyde-inactivated *Bordetella pertussis* preparations. Physiological saline (SA) was used as a control. For comprehensive gene expression analysis, 5 mL of SA, PV, PT, and RE were each injected into 3 rats and the vaccinated tissues, lung, brain, kidney, and liver, were harvested at 1, 2, 3, and 4 days after vaccine administration. The experiments were performed twice and purified poly(A)$^+$ RNA from a total of 384 samples was subjected to DNA microarray analysis.

Of the 4 organs tested, the lung expressed genes that were extracted by DNA microarray analysis were classified sharply into clusters depending on sample treatment. From the DNA microarray analysis of vaccinated rat lungs at day 1, 13 genes for which expression levels were dynamically changed in response to PT treatment were [17] (accession numbers were updated in Table 1). Interestingly, the DNA microarray-based gene expression data correlated well with the body weight change of vaccine-treated mice (Figure 1(a)) and rats [17]. The real-time PCR quantification results of the expression levels of the 13 genes were comparable to the relative expression ratios from the DNA microarray analysis. Furthermore, cluster analysis using the 13 genes could distinguish SA- and PV-treated groups from PT- and RE-treated groups. These 13 genes are likely to be closely involved in the toxicity of pertussis vaccines. To quantify these genes in a convenient way, the QuantiGene Plex assay was applied. The QuantiGene Plex assay enabled the simultaneous analysis of the 13 genes. We evaluated the expression levels of the 13 genes in the lungs of rats vaccinated with various doses of RE. Nine genes, *S100A9*, *S100A8*, *IRF7*, *MX2*, *IFI27L*, *BEST5*, *MMP9*, *MMP8*, and *CYP2E1* (indicated in bold letters in Table 1) showed dose-dependent up-or down-regulation in response to the various doses of RE treatment. RE vaccine toxicity could be measured by the expression level in lung lysate of these 9 genes. The quantification of these 9 genes using the QuantiGene Plex assay is, we believe, a promising candidate for a new control test for pertussis vaccines.

5. Influenza Vaccines

Influenza virus triggers a highly contagious acute respiratory disease and has caused epidemics and global pandemics, partly because it possesses the capacity for gradual antigenic change in two surface antigens, hemagglutinin (HA) and neuraminidase (NA) [22]. To combat influenza, split vaccines consisting of subvirion preparations and whole-virus vaccines are manufactured using strains recommended annually by the WHO, based on the antigenic characteristics of HAs and NAs. Furthermore, the recent circulation of the highly pathogenic avian influenza A (H5N1) virus has raised concerns about the preparations for a coming influenza pandemic [23]. Many efforts are underway to develop vaccines against influenza A (H5N1).

To identify biomarkers for influenza vaccine toxicity, 3 vaccines were used: trivalent influenza HA vaccine (HAv, a split vaccine), trivalent influenza vaccine (WPv, an inactivated whole-virion vaccine), and prepandemic influenza vaccine (PDv, inactivated whole-virion (A/H5N1) absorbed onto an aluminum salt). All were produced by Kaketsuken, The Chemo-Sero-Therapeutic Research Institute, Japan. Physiological saline (SA) was used as a control. For comprehensive gene expression analysis, SA, HAv, WPv, and PDv were each injected into 5 rats, and the vaccinated tissues, lung, liver, brain, and peripheral blood, were harvested at 1, 2, 3, and 4 days after vaccine administration. Purified poly(A)$^+$ RNA from a total of 320 samples was subjected to DNA microarray analysis [16]. Based on the analysis of pertussis vaccines, described above, the gene expression profiles from lung samples were subjected to two-dimensional hierarchical cluster analysis. PDv- and WPv-treated samples at day 1 formed an independent cluster from other samples, indicating distinct profiles in gene expression of these groups. As was the case with pertussis vaccines, we tried to identify several biomarkers from the analysis of lung gene expression. The analysis of lungs from vaccinated rats at day 1 resulted in the extraction of 76 genes, whose expression levels were statistically different between SA- and

Table 1: Biomarkers for pertussis vaccine toxicity.

Category	Accession no.	Symbol	Brief description
Inflammation	NM_053587	***S100A9***	A calcium binding protein that may be associated with acute inflammatory processes, coupled with S100a8
	NM_053822	***S100A8***	May play a role in inflammatory responses such as cell motility, coupled with S100a9
	NM_019323	*MCPT9*	A serine protease expressed in mast cells, but the precise function has not yet been determined
	NM_031530	*CCL2*	A ligand for CCR2 that acts as a chemoattractant of monocytes
IFN inducible, immune response	NM_001033691	***IRF7***	Unknown
	NM_134350	***MX2***	Involved in inhibiting vesicular stomatitis virus
	NM_203410	***IFI27***	Induced by steroid hormone, IFN, and LPS in endometrium at implantation, dendric cells, and macrophases
	NM_001007694	*IFIT3*	May induced by IFN or virus infection
	Y07704	***BEST5***	Induced by IFN and involved in bone formation
Peptidoglycan metabolism	NM_031055	***MMP9***	Metalloproteinase involved in extracellular matrix remodeling, bone resorption, and immune responses
	NM_022221	***MMP8***	May play a role in appositional bone formation and regulation of the extracellular matrix
Xenobiotic metabolism	J02627	***CYP2E1***	Protects hepatocytes from stress-induced cell death
Others	NM_001106862	*NGP*	Unknown

Table 2: Biomarkers for influenza vaccine toxicity.

Category	Accession No.	Symbol	Brief description
IFN inducible gene	NM_172019	*IFI47*	Mouse homolog may be a guanine nucleotide-binding protein induced by IFN-gamma
	AF329825	*TRAFD1*	Putative TRAF-interacting zinc finger protein
	NM_019242	*IFRD1*	May be involved in proliferation of neuronal and glial precursors
IFN inducible, immune response	NM_001033691	*IRF7*	Unknown
	NM_134350	*MX2*	Involved in inhibiting vesicular stomatitis virus
Immune response	NM_172222	*C2*	Likely component of the classical pathway of the complement cascade
	NM_012708	*PSMB9*	Subunit of the proteasome complex, which may play a role in protein catabolism
	NM_032056	*TAP2*	Transports peptides into the ER lumen for binding with MHC class I molecules; plays a role in antigen processing and presentation
	NM_033098	*TAPBP*	Facilitates the binding of MHC class I molecules to the transporter associated with antigen processing (TAP) in MHC class I assembly
	NM_017264	*PSME1*	May play a role in proteasome activation
Chemokine and Cytokine function	AF065438	*LGALS3BP*	Displays differential expression in a fibroblast cell line transformed by human T-cell leukemia virus type 1 Tax protein
	NM_012977	*LGALS9*	A highly selective urate transporter/channel
	NM_053819	*TIMP1*	Acts as an inhibitor of metalloprotease activity; may play a role in vascular tissue remodeling
	NM_023981	*CSF1*	Plays a role in macrophage formation
	NM_145672	*CXCL9*	Chemokine which plays a role in the recruitment of mononuclear cells and in allograft rejection
	XM_223236	*CXCL11*	Mouse homolog is a chemokine and is involved in the immune response
Transcription activity	AJ302054	*ZBP1*	DNA binding protein; thought to bind Z-DNA, which is largely controlled by the amount of supercoiling

WPv-treated samples ($P < .005$) [16]. The cluster analysis using these 76 genes successfully distinguished WPv- and PDv-treated groups at day 1 from other groups, indicating the suitability of the 76 genes as biomarkers for influenza vaccines.

The extracted 76 genes were categorized according to function, such as interferon-inducible, chemokine and cytokine function, immune response, transcriptional activity, and so on. Among the 76 genes, 17 genes met the requirement for high expression levels and were chosen as representatives for each functional category (Table 2). Among the 17 genes, *IRF7* and *MX2* were also nominated for biomarkers of pertussis vaccine toxicity. Real-time PCR quantification results of the expression levels of the 17 genes were comparable to the relative expression ratios determined by DNA microarray analysis. We are now working to establish a rapid quantification system for these 17 biomarkers using the QuantiGene Plex assay.

6. Japanese Encephalitis Vaccines

Japanese encephalitis (JE) is a seasonal and sporadic encephalitis in East Asia caused by the JE virus. Vaccination is very important to prevent JE infection, because palliative care is the only treatment available for JE patients. Recently, a Vero cell-derived JE vaccine had been licensed in Japan as an alternative to the long-used mouse brain-derived JE vaccines. The newly developed Vero cell-derived vaccine should be at least equivalent to the mouse brain-derived vaccines, because the mouse brain-derived vaccines were considered generally safe and succeeded in the near elimination of JE in certain endemic regions. In this context, we performed DNA microarray analysis of tissues from rats administered with mouse brain-derived or Vero cell-derived JE vaccine and compared the gene expression profiles. As expected, the gene expression patterns in brain and liver were comparable between mouse brain-derived and Vero cell-derived vaccines, indicating that both vaccines possessed equivalent reactivity characteristics in rats [24].

7. Conclusions

Over recent decades, the safety control of vaccines has been assessed using several animal tests, including the body weight change test and white blood cell counts. However, conventional animal safety tests need to be improved in many aspects. For example, the number of test animals used needs to be reduced and the test period needs to be shortened. This requires the development of a new vaccine evaluation system. In this review, we showed that gene expression patterns were well correlated to the biological responsiveness of vaccinated animals. From the DNA microarray analysis of lungs from vaccinated rats, we identified 13 and 17 biomarkers to detect the toxicity of pertussis and influenza vaccines, respectively.

Furthermore, the QuantiGene Plex assay for gene expression analysis is being introduced. The QuantiGene Plex assay was revealed to be as accurate as real-time PCR and has the great benefit of being able to evaluate all biomarkers simultaneously. Using the QuantiGene Plex assay, we could rapidly and sensitively detect the gene expression changes that accompany biological reactivity in vaccinated rats.

Thus, it may be concluded that DNA microarray technology is an informative, rapid, and highly sensitive method with which to evaluate vaccine quality. Our data suggest that this new method has the potential to shorten the time for safety tests and can reduce the number of animals used. In addition, our test may contribute to the development of urgently required vaccines. Further analyses are required to confirm that gene expression changes correlate with vaccine quality.

In this review, we referred to our recent efforts of exploring new safety control methods using gene expression pattern indexes, focusing on pertussis and influenza vaccines. In the future, for the evaluation of all kinds of vaccines, microarray analysis is expected to play an important role in the new safety control test, especially for checking toxin-reactive transcripts.

Acknowledgment

This work was supported by Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

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Immunological Reviews

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Unique properties of memory B cells of different isotypes

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Acknowledgements
We thank Dr. Peter Burrows for critical reading of the manuscript. This work was supported by grants to Y. A., Y. T. and T. K. from Ministry of Education, Culture, Sports, Science and Technology in Japan and by a grant to T. K. from JST, CREST.

Immunological Reviews 2010
Vol. 237: 104–116
Printed in Singapore.
Immunological Reviews
0105-2896

Summary: Memory antibody responses are typically seen to T-cell-dependent antigens and are characterized by the rapid production of high titers of high-affinity antigen-specific antibody. The hallmark of T-cell-dependent memory B cells is their expression of a somatically mutated, isotype-switched B-cell antigen receptor, features that are mainly generated in germinal centers. Classical studies have focused on isotype-switched memory B cells (mainly IgG isotype) and demonstrated their unique intrinsic properties in terms of localization and responsiveness to antigen re-exposure. However, recent advances in monitoring antigen-experienced B cells have revealed the considerable heterogeneity of memory B cells, which include unswitched IgM^+ and/or unmutated memory B cells. The IgM and IgG type memory B cells reside in distinct locations and appear to possess distinct origins and effector functions, together orchestrating humoral memory responses.

Keywords: *memory B cell, class-switch, somatic hypermutation, follicular helper T cell, PLC-γ2*

Introduction

One striking feature of the adaptive immune response is the generation of immunological memory. When the primary immunological challenge is resolved, several features of the immune response, such as the increased cellularity of secondary lymphoid tissues, subside. For many antigens, however, resolution of the immune response does not completely reset the immune system to the state prior to antigen exposure; the system retains a memory of the response, and it is long lasting, sometimes persisting for a lifetime in both rodents and humans (1, 2).

Immunological memory is characterized by qualitative and quantitative differences between the response that occurs after the primary exposure and the subsequent response that occurs after re-exposure to the same antigen. T-cell-dependent memory humoral responses are typically more rapid, have a greater magnitude, and contain antibodies of higher affinity (1). This improved responsiveness is thought to derive from a relatively small number of memory B cells and T cells that participated in the primary immune response and then survived in a quantitatively and qualitatively altered state. Thus, understanding

the mechanism by which memory B cells are generated and maintained, as well as defining the basis for their heightened responsiveness, is of fundamental interest to reveal the basis of immunological memory.

It is generally thought that memory B cells develop under T-cell help within the germinal center (GC), where somatic hypermutation and isotype switching of the immunoglobulin (Ig) heavy chain take place (3–5). The isotype-switched B-cell antigen receptor (BCR), for instance of IgG type, has been considered the 'gold standard' for identifying memory B cells. However, accumulating evidence has shown that unswitched and unmutated memory B cells exist in both rodents and humans, raising the question of whether these memory B cells have distinct or overlapping functions. The purpose of this review is to summarize the major recent advances in our understanding of how heterogeneous memory B-cell populations are generated in response to initial antigen exposure, mainly focusing on T-cell-dependent antigens, where these cells are located, and how they respond to antigen re-exposure, with specific emphasis on their intrinsic properties.

How to identify memory B cells

In this review, memory B cells are defined as quiescent antigen-experienced lymphocytes that promptly differentiate into effector cells after antigen re-exposure. Studies of memory B cells have been done in rodent and human systems, and both models have contributed to our understanding of the unique properties of memory B cells. Here, we first describe approaches to identify and isolate memory B cells from humans and mice, the most widely studied of the rodents, and then point out several advantages and disadvantages of each model.

Human system

Due to the inherent obstacles to experimentation in humans, isolation of antigen-specific memory B cells, either before or after their activation *in vivo*, is not as easy as in mice. Therefore, human memory B cells of unknown antigen-specificity have been isolated solely by cell surface markers. Due to the lack of any positive markers, human memory B cells were first isolated by negative selection (IgD^-CD38^-) from total tonsillar B cells (6, 7). However, later studies suggested that CD27, a tumor necrosis factor receptor (TNF-R) family member of unknown function, could be used as a marker of human memory B cells (8, 9). The idea that $CD27^+$ B cells represent memory B cells was supported by several observations. $CD27^+$ B cells responded to several stimuli more robustly than naive B cells *in vitro*, their numbers were very low in the antigen inexperienced newborn but increased with age, and more importantly, most of the mutated and class-switched B cells were $CD27^+$ (8–12). It is impractical to study the *in vivo* behavior of human $CD27^+$ B cells during the course of primary and secondary responses, however recent adoptive transfer studies using immune-compromised host mice showed that human memory B cells could at least differentiate into plasma cells after antigen re-challenge (13).

The biggest advantage of using human rather than murine memory B cells is the greater abundance of the cells. Around 0.01% of total B cells in the spleen of an immunized mouse are antigen-specific memory B cells. However, based on CD27 expression, close to 40% of the B cells in adult human venous blood are considered to be memory cells, although they are polyclonal (8). Many of the phenotypic features and several intrinsic properties of memory B cells in terms of their responsiveness to various *in vitro* stimuli were in fact first described in studies of humans. Many of these findings have proven to be true for both humans and mice, but there are notable exceptions, for example, dependency on Toll-like receptors, as described in detail below. A drawback to studies of the phenotypes and intrinsic properties of human memory B cells is the number of variables that cannot be controlled or even determined, for example the form of initial antigen exposure, its delivery route, and the age of the memory cells. Therefore, human $CD27^+$ memory B cells are clearly composed of several subsets that likely have different features and possibly functions, as described in the next section.

Murine system

There are two major advantages for studying the memory B-cell responses in mice rather than humans. First, by using model antigens and BCR-transgenic mice in the study of immune responses, identifiable precursor cells can be tracked over the course of the response. Second, because synchronous activation of the antigen-specific cells is feasible *in vivo* and because antigen-experienced cells can be permanently labeled by using methods such as insertion of tamoxifen-inducible Cre into an *Aicda* locus [encoding activation-induced cytidine deaminase (AID)] (14, see below), the age, behavior, and function of memory B cells can be tracked *in vivo* (Fig. 1).

Classically, many groups have isolated murine memory B cells as antigen-binding, isotype-switched B cells that also express or not a combination of other markers that are presumed to discriminate memory B cells from other antigen-experienced B-cell populations, such as GC B cells and plasma

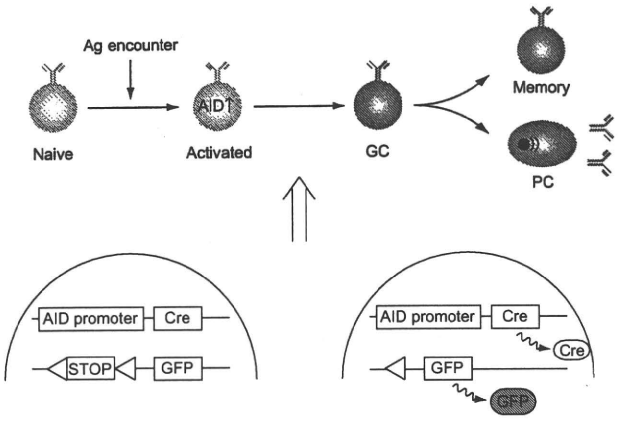

Fig. 1. Fate mapping for memory B cells. In the mice expressing Cre recombinase under the control of activation-induced cytidine deaminase (AID) promoter, antigen-experienced B cells upregulate Cre recombinase which can mediate excision of the loxP-flanked stop cassette to allow the permanent expression of green fluorescence protein (GFP). Germinal center B cells, memory B cells, and isotype-switched plasma cells are irreversibly labeled by GFP even after Cre expression is ceased. (triangles: loxP sites)

cells (15, 16). Plasma cells are easily marked by several surface receptors (e.g. CD138) (17) or transcription factors (e.g. Blimp-1) (18), and GC B cells can be separated from memory B cells by virtue of their binding to the GL-7 mAb (19) or peanut agglutinin (PNA) (20) and because they have down-regulated CD38 (15).

While the above approach has enabled us to isolate isotype-switched memory B cell, unswitched memory B cells are not captured by this type of analysis. Recently, antigen-experienced cells including both switched and unswitched, have been successfully labeled using a Cre/loxP system. AID was discovered as an indispensable enzyme for isotype-switching and somatic hypermutations of the BCR (21). As it turns out, B cells are activated by antigen exposure *in vivo*, almost all of them upregulate AID at the transcriptional level, thereby making AID expression a signature of antigen encounter. Indeed, mice bearing an *Aicda* locus with a knocked-in tamoxifen-inducible Cre recombinase effectively turned on enhanced green fluorescence protein in the cells that had expressed AID during the course of an immune response (14). Importantly, this strategy does not depend on the phenotype of the cells at the moment of analysis, but rather on their previous antigen-experience or that of their precursors. Because a memory B cell is by definition antigen-experienced, these mice provide a novel tool to identify memory B cells and to trace their functions *in vivo*, regardless of whether they have isotype-switched or not.

Although an excellent model system, the above fate mapping strategy using AID has a potential weak point, because not only memory B cells but also GC B cells are labeled. In this regard, it would definitely be desirable to find specific markers, analogous to FoxP3 for regulatory T cells (22–24), that would allow the specific isolation of the memory population. Now that global gene expression data from antigen-binding murine memory B cells is available and several genes that are upregulated on memory B cells have been found (25, 26), this approach should become a feasible one for the identification of novel memory B-cell markers.

Heterogeneity of memory B cells

There is now accumulating evidence of heterogeneity of memory B cells. This heterogeneity is particularly evident in humans because, by using $CD27^+$ as an isolation marker for memory B cells, a broad range of B cells in terms of antigen-specificity, isotype expression, and ages can be identified. Indeed, in addition to isotype-switched $CD27^+$ memory cells, about half of $CD27^+$ B cells in blood expressed IgM, and these cells can be further subdivided into IgM^+IgD^+ and IgM-only compartments (8, 12). Both of these populations primarily contain somatically mutated V regions, although unmutated cells also exist (8, 27). The origin of somatically mutated $CD27^+IgM^+$ memory B cells has recently become a topic of debate (28, 29). Three major mechanisms have been postulated. First, the cells are generated independently of GC processes and in fact undergo somatic hypermutation during diversification of the pre-immune repertoire (30, 31). However, in terms of the accepted definition that memory B cells are antigen experienced, such cells cannot be considered as *bona fide* memory B cells. Second, the cells are generated by exposure to T-independent antigens, such as bacterial polysaccharides, probably through a GC-independent manner (12, 31). Indeed, $CD27^+IgM^+$ human B cells have been reported to play a role in immunity against encapsulated bacteria by producing anti-polysaccharide IgM (12). Finally, the cells are generated in the GC by canonical T-dependent antigens. Supporting this third mechanism, $CD27^+IgM^+$ memory B cells, like class-switched memory cells, have somatic mutations in the Bcl-6 gene, a genetic footprint of GC experience, demonstrating that many, if not all of the $CD27^+IgM^+$ cells, derive from a GC reaction during a T-cell-dependent response (32).

Due to the low precursor frequency of murine antigen-specific memory B cells, their phenotypic characterization has lagged behind that possible with the human polyclonal memory cells. The usage of BCR-transgenic and knock-in mice has somewhat circumvented this problem. Through such analysis, Shlomchik and his colleagues (33) have recently found that (4-hydroxy-3-nitrophenyl)acetyl (NP)-binding memory B

cells could be separated into subpopulations based on CD80 and CD35 expression. Intriguingly, 70% of the $CD35^+$ fraction of $CD80^+$ memory B cells had unmutated V genes, whereas almost all the $CD35^-CD80^+$ memory B cells were mutated. These data clearly indicate that unmutated memory B cells are generated and maintained for a long period as distinct subsets expressing unique surface markers. These unmutated memory B cells may originate from a pathway that is independent of GCs as discussed in detail later.

Memory B-cell development

Where are memory B cells generated?

By using rodent systems, information about the behavior of naive B cells during immune responses has been accumulated, thereby providing insights into where memory B cells are generated. The following general scenario has emerged from these types of studies. After initial exposure to antigen, antigen-binding IgM^+ B cells migrate from random locations within the B cell follicles to the border between the follicles and the T cell-rich area, where cognate interactions with antigen-specific CD4 T cells take place (34, 35). Next, B cells proliferate and foci of antibody (Ab)-secreting plasmablasts, some of which contain isotype-switched antibodies, appear at the border between the T cell area and the red pulp of the spleen, 6–8 days after primary immunization. Shortly after these foci appear, clusters of isotype-switched cells such as $IgG1^+$ B cells that are stained with PNA and GL-7 mAb appear in GCs within the areas of the follicles occupied by follicular dendritic cells (FDCs) (36–38).

In situ detection of isotype-switched B cells at early times after the switch had been difficult, because of the rarity of these antigen-specific B cells. However, now this problem has been circumvented by employing B cells harboring antigen-specific BCR knocked-in the endogenous heavy chain locus and therefore capable of class switching, thereby allowing us to track the genesis and fate of antigen-experienced IgM as well as class-switched B cells. By using this approach, Jenkins and his colleagues traced the behavior of IgM and IgG type B cells after primary immunization (39). They observed that antigen-specific isotype-switched B cells (in their experimental setting, IgG2a type) appeared on day 2 in the follicles rather than in foci of plasmablasts in the red pulp and medullary cords, as expected from previous work (40, 41). Thus, it now appears more likely that, after receiving signals from helper T cells at the T–B border, IgM B cells migrate into follicles where they proliferate and initiate class switching. On day 4, some of the antigen-experienced IgM and IgG2a B cells appeared in the pre-GC region, where antigen was concentrated, presumably on FDCs. On day 10, many of the surviving antigen-experienced B cells in these regions expressed IgG2a, whereas IgM^+ cells were located outside the GCs. At least two explanations are possible for the failure of antigen-experienced IgM B cells to persist in GCs on day 10. First, antigen-specific IgM B cells might promptly undergo isotype-switching after they enter this location due to abundance of antigen-specific follicular type T cells. Thus, by day 10, no more precursor IgM type B cells would remain in the GC region. Alternatively, although some IgM type progeny are generated in the GC region, IgG2a type B cells might have a competitive survival advantage. As suggested in this paper, the antigen-experienced IgG2a B cells located in the GC region on day 10, some of which expressed CD38, can be considered as memory or memory-precursor B cells. As mentioned above, CD38 is a good marker for distinguishing memory versus GC B cells among class-switched B cells. The antigen-specific IgM type B cells scattered in the follicles on day 10 were likely memory or memory-precursor cells, because they had undergone several rounds of proliferation. Thus, this study not only identifies the initial site of class switching, but also provides significant insight into where IgM and IgG2a type memory B cells are initially generated.

Potential mechanisms for development of memory B cells

In terms of the developmental pathways of memory B cells, published studies have only focused on the IgG type cells. Most models of post-GC B cell development posit that cells destined to become plasma cells and memory B cells develop separately (bifurcative differentiation model) (*Fig.* 2). However, an alternative possibility is that there is a single developmental path, beginning with memory cells and progressing to plasmablast and plasma cells (linear differentiation model). Although there is little data at present to validate either model, the early appearance of $Blimp^+$ plasmablasts in the GC somewhat favors the bifurcative model (42). Assuming that the bifurcative model is correct, does this plasma versus memory decision come about as a result of different progeny receiving different cytokine, chemokine, and/or niche cues from their environment? Or is the decision the result of differences in the duration or strength of antigen stimulation? Or is it instead a stochastic event that occurs owing to cell-intrinsic unequal segregation of lineage-determining factors to daughter cells, as has been proposed for T-cell fate determination? These important questions have not been adequately addressed so far, but some fragmentary evidence has accumulated.

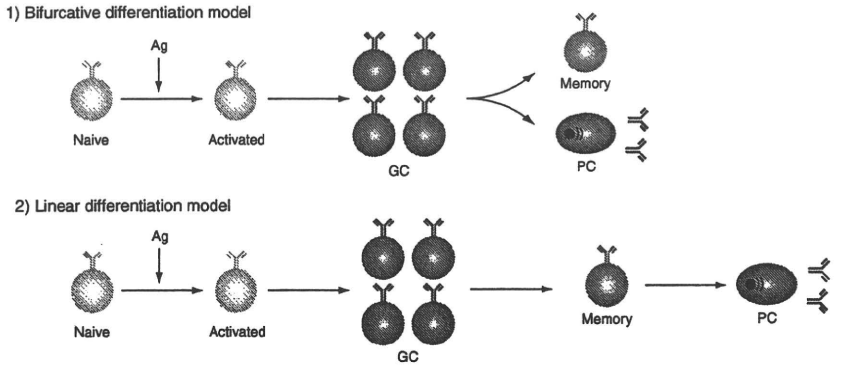

Fig. 2. Two possible models for the generation of memory B cells and plasma cells from germinal center (GC) B cells. Model-1 'Bifurcative differentiation model': Naive B cells are activated and differentiated into GC B cells. GC B cells develop both memory B cells and plasma cells. Model-2: 'Linear differentiation model': GC B cells first differentiate into memory B cells, and subsequently plasma cells are generated from memory B cells.

BCR affinity

As for the BCR-antigen affinity issue, by using B cells harboring a knockin anti-hen egg lysozyme (HEL) BCR, Brink and his colleagues (43) have tried to determine whether *in vivo* plasma cell differentiation of IgG1 GC B cells occurs in an affinity-based selection manner. Indeed, the IgG1 GC B cells that had acquired high affinity for the immunizing antigen were apt to differentiate into plasma cells, suggesting that stronger BCR signaling favors plasma cell differentiation. Therefore, it is possible that the BCR signal could play a direct role in facilitating plasma cell differentiation, for example by inducing Bcl6 degradation and thereby releasing Blimp-1 from its repressed state (44, 45). Alternatively, responding GC B cells may stochastically commence plasma cell differentiation but require the strong BCR signal to survive beyond the very earliest stages of this process. Although the study of Brink and his colleagues did not examine the affinity of anti-HEL IgG1 memory B cells, given the evidence that IgG1 memory B cells in the NP-hapten system do not necessarily have higher affinity for antigen (46), it seems reasonable to anticipate that IgG GC B cells require a relatively permissive BCR signal strength to enter the memory pool, in contrast to the high stringency needed for entering the plasma cell pool.

Cytokine requirements

While analysis of mice with germline deletion of cytokine and cytokine receptor genes provides some insights into memory B-cell biology, interpretation of these data needs to be done with caution because the phenotype is obviously the sum of effects on both B and T cells, as well as other cell types. In this regard, recent studies of B-cell-specific deletion of Stat3 and Stat5 genes are informative. B-cell-specific deletion of Stat3 resulted in profound defects in T-dependent IgG responses after primary immunization (47). Because GC formation, isotype switching, and generation of antigen-specific IgG B cell appeared to be intact in these mice, Stat3 was proposed to play a major role in differentiation of IgG GC B cells to plasma cells. Given that IL-6, IL-2, IL-10, and IL-21 activate Stat3 (48–51), these cytokines, singly or in combination, are likely to utilize Stat3 for plasma cell differentiation. The function of Stat3 in memory B cell formation and maintenance remains to be determined. By *in vitro* gain-of-function experiments, Stat5 has been shown to control the differentiation and self-renewal potential of human memory B cells (52). By contrast, when the Stat5 gene was deleted in murine B cells *in vivo*, memory B cell formation as well as plasma cell differentiation occurred normally (53).

In the case of human GC B cells, the requirement for different cytokines in memory versus plasma cell formation has been defined by using *in vitro* experiments. In response to IL-4, human tonsillar GC B cells (roughly 50% are IgG positive) acquired a memory B cell phenotype (54). These cells express high levels of Bcl-2, CD80, and CD86 mRNA, a hallmark of *in vivo* memory B cells (7), and, with appropriate stimulation by IL-10, develop rapidly into plasma cells, also consistent with the memory features mentioned above. When GC B cells are grown with IL-10, they develop into cells with a plasma cell phenotype (54). Thus, exposure to IL-4 or IL-10 appears to bias GC B cells toward memory or plasma development, respectively. Despite the importance of IL-4 for *in vitro* human memory differentiation, its *in vivo* role in the mouse is unclear. The numbers of $IgG1^+$ GC B cells and memory B cells were equally reduced in IL-4-deficient mice, suggesting that IL-4 is required for GC formation but not for development of

memory B cell from GC B cells (55). However, IL-13, a closely related cytokine that shares similar biological activities and signaling pathways, could compensate for of IL-4 (56).

$CD4^+$ T cells and NKT cells produce IL-21 (57, 58). Among the $CD4^+$ T cells, IL-21 is expressed at the highest levels by T follicular helper (Tfh) cells (59). Its receptor is expressed on T cells, B cells, NK cells, macrophages, and dendritic cells (DCs) (60). Therefore, in the context of TD humoral responses, the *in vivo* function of IL-21 is thought to be mediated by its dual effects on, at least, two types of cells, Tfh and B cells. In terms of GC B cell maintenance, initial reports proposed a dominant role for IL-21 on Tfh cell formation, which in turn affects B-cell biology, rather than any direct action on B cells (61, 62). Indeed, in naive T cells, IL-21 leads to upregulation of Bcl6, the transcriptional regulator of Tfh cells (63–65). However, more recent reports by using mixed bone marrow chimeras have shown that IL-21 acts directly on B cells, thereby manifesting the defects of B-cell responses in IL-21-deficient mice (55, 66). In contrast to its effect on GC B-cell maintenance, IL-21 is dispensable for the generation of memory B cells (55). In addition, extrafollicular antibody responses, as seen with Salmonella species, are not affected in the absence of IL-21 (66).

Non-classical pathways for memory B-cell development

Immunoglobulin V genes of T-cell-dependent memory B cells carry somatic hypermutations which are introduced by GC reaction. However, unmutated V genes have been repeatedly observed in IgG type memory B cells by several groups (16, 33, 55), raising the idea that memory B cell development does not necessarily require GCs. Supporting this, immunization of mice having Bcl-6-deficient B cells with NP-hapten coupled to a carrier protein failed to generate GCs and somatic hypermutations, however it elicited IgG type memory B cells lacking somatic hypermutations (67). They persisted for a long period and promptly differentiated into plasma cells after secondary challenge, indicating that they represent functional memory B cells. The idea that unmutated memory B cells could develop via a GC-independent pathway was further supported by a different experimental setup in which memory B-cell development within GCs was blocked by treatment with an anti-inducible co-stimulator (ICOS) mAb (68). Consistent with the results from Bcl-6-deficient mice, the frequency of unmutated memory B cells was highly elevated in the treated mice. Thus, these data clearly indicate that even in T-cell-dependent responses, unmutated memory B cells can be generated independently of GCs (*Fig.* 3).

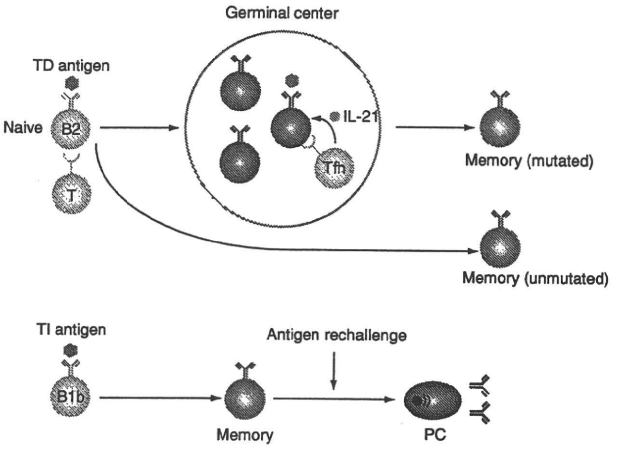

***Fig.* 3. Multiple pathways for memory B-cell development.** In response to T-dependent (TD) antigen, naive B cells differentiate into memory B cells through germinal centers (GCs) or independently of GCs. Within GCs, the precursors for high-affinity memory B cell are generated by somatic hypermutations, while GC-independent pathway generates only low-affinity memory B cells. The maintenance of GC B cells partly depends on IL-21 from Tfh cells. In response to T-independent (TI) antigens, B1b cells differentiate into low-affinity memory B cells, which are maintained at resting state for a long period and promptly differentiate into plasma cells without T cell help after antigen re-exposure.

One of the classical features of memory B cells is that they require T-cell help both for their development and re-activation. T-cell-dependent memory B cells have long been the major targets of memory B-cell studies, especially in the mouse. However, there are several reports describing the appearance of memory B cells during T-cell independent responses in rodents. Antigen-specific B cells persisted for a long period in rats that had been immunized with trinitrophenyl-lipopolysaccharide (TNP-LPS), a T-cell independent type 1 antigen and these cells showed enhanced responsiveness following antigen re-exposure (69). More recently, two papers have described the development and persistence of memory B cells in response to T-cell independent type II antigens (70, 71). In one report, memory B cells specific for a T-cell independent antigen on the surface of *Borrelia hermsii* were shown to originate from B1b cells ($CD11b^+CD21^-CD23^-$), which had been thought to behave as 'innate' rather than inducible memory cells (70). These B1b type memory B cells persisted as quiescent cells and promptly differentiated into plasma cells following bacterial re-challenge. Additionally, T-cell independent memory B cells generated in response to NP-Ficoll expressed unswitched IgM BCR with no somatic hypermutations (71). Thus, T-cell independent memory B cells indeed exist and differ from canonical T-cell-dependent memory B cells in their origins and phenotypes (surface markers and immunoglobulin genes).

Localization of memory B cells

Since memory B cells are believed to be long-lived irrespective of whether they express IgM or a switched isotype, the question arises of whether the initial lodging sites for IgG2a and IgM type memory B cells, identified by Jenkins and his colleagues, can function as niches for longer time periods, or do these sites function simply as transit stations for memory B cells prior to their joining the recirculating pool. These questions have been answered, in part, by tracing their lodging sites over longer periods.

By using a transgenic mouse line harboring a rearranged V_H186.2 IgM type heavy chain gene, Shlomchik and his colleagues (33) demonstrated that long-lived IgM type memory B cells reside not just in the marginal zone (MZ), as had been observed in the rat system, but also scattered in splenic follicles on 12 weeks after immunization with NP-CGG. By using non-transgenic mice and similar immunization protocols, we have recapitulated their results; IgM type memory cells were seen scattered in splenic follicles on day 60 after immunization. Moreover, we observed that IgG1 type memory B cells preferentially localized adjacent to contracted GCs (Aiba *et al.*, manuscript in preparation). Together, these data favor the idea that the initial lodging sites for IgG and IgM type memory B cells can function like niches for their long-term survival, rather than as a transit station. Persistence of the GC-like structures on day 60 after injection of NP-CGG is consistent with the recent report demonstrating that GC-like structures can be detected for up to 8 months after challenge with sheep red blood cells (14). Thus, it is reasonable to anticipate that the GC-like structures persist longer than previously thought, and that the duration of such GC-like structures is dependent, at least partly, on the nature of the immunogen and adjuvants.

Although many experimental systems have shown that memory T cells reside in peripheral non-lymphoid organs, which are likely to be the first line of defense (73), there is limited data so far regarding the presence of memory B cells in these sites. Virus infection in the lower respiratory tract generates lymphoid tissue in the lungs named induced bronchus-associated lymphoid tissue (iBALT), which resembles secondary lymphoid organs with distinct B and T-cell areas and HEV (74). iBALT developed by day 7 after infection, concomitantly with the formation of GL-7^+ GC structures. Moreover, virus-specific plasma cells persist in the lungs for a long period even after viral clearance, although the origin and lifespan of these plasma cells remain elusive (75, 76). Sangster's group (76) has recently enumerated memory B cells in infected lungs by an approach similar to that utilized for counting human memory B cells with a particular antigen-specificity. After stimulation of lung B cells under limiting dilution conditions, these investigators detected considerable numbers of virus-specific plasma cells of IgG and IgA isotypes, and concluded that virus-specific class-switched memory B cells reside in the infected lungs. Notably, IgA^+ B cells preferentially localized to the lungs, whereas IgG^+ B cells were equivalently dispersed in the draining LN and lungs. Although we have to await the isolation and further characterization of those antigen-experienced B cells in the lungs, these data suggest that, in analogy with memory T cells, memory B cells with distinct functions may persist in non-lymphoid organs and confer protection against recurrent infections.

Maintenance of memory B cells

Humans with loss-of-function mutation in genes encoding CD40/CD40L, NEMO (NFκB essential connector), ICOS, signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), and CD19 have dramatic reductions in the frequencies and absolute numbers of memory B cells (30, 77–80). These patients are essentially devoid of isotype-switched memory B cells and lack the majority (more than 60%) of IgM type memory B cells. However, these reports have struggled to distinguish whether a gene is required for GC formation, memory differentiation, or memory maintenance. Such problems are now being resolved by the generation of mouse strains containing inducible forms of Cre recombinase, such as ERT2-Cre, or Cre driven by promoters that either become active after B cell activation or are expressed late in development (81). These include Cγ1-Cre, in which deletion of floxed genes is restricted to B cells initiating CSR to IgG1, and an AID-Cre strain in which Cre is expressed from a bacterial artificial chromosome (BAC) transgene under the control of the AID promoter and thus restricted to B cells after *in vivo* activation (82, 83).

To address the importance of BCR signaling in formation and/or maintenance of memory B cells, we had chosen the PLC-γ2 gene, because PLC-γ2 is well known to function downstream of the BCR and to be required for calcium/PKCβ activation following antigen stimulation (84, 85). By crossing PLC-$\gamma2^{f/f}$ mice with Cγ1-Cre or ERT2-Cre mice, we demonstrated that PLC-γ2 is critical for GC and memory B-cell formation and, more importantly, that it is also required for memory B-cell maintenance (86). Antigen-dependency of memory B-cell survival is still debated (87, 88). For instance, it had been shown that antigen could be retained on FDCs for many months, and possibly years after primary immunization

(89). It was therefore hypothesized that memory B cells needed to interact with this retained antigen in the form of immune complexes possibly trapped by Fc and/or complement receptors expressed on FDCs for long-term survival. In contrast, using an elegant *in vivo* system in which BCR specificity could be changed after memory B cells had been generated, Maruyama *et al.* (90) found that memory B cells that could no longer bind the immunizing antigen had a lifespan comparable to that of memory B cells that had not switched receptor specificity. Although Maruyama's data suggest that antigen occupancy is dispensable for the maintenance of memory B cells, they do not necessarily mean that BCR signaling is not required. Thus, based on our observations using the PLC-$\gamma 2^{f/f}$ mice, we proposed that survival receptors, probably the BCR, use PLC-γ2 for the maintenance of memory B cells.

In addition to BCR, the involvement of TLR receptors, TNF-receptor family, and cytokine receptors in maintenance of memory B cells has been postulated. The idea that memory B cells can be polyclonally activated by TLR ligands was proposed based on *in vitro* responsiveness of human memory B cells and the observed linear correlation of persistent serum Ab titers and memory B-cell frequencies in humans (91). However, in mice, deletion of key signaling molecules for TLR signaling [MyD88 and TIR domain containing adaptor-inducing interferon-beta (TRIF), together] did not significantly affect the secondary antibody responses (92). Although the number of antigen-specific memory B cells during extended time periods such as 3 months was not analyzed in these studies, they do raise the question of whether the TLR-dependent maintenance mechanism operates *in vivo* during typical TD-antigen-induced immune responses. As for the TNF-receptor and cytokine receptor families, so far there is no convincing genetic evidence demonstrating their involvement in maintenance of memory B cells at least in the mouse model systems.

Reactivation of memory B cells

The cellular and molecular basis for the faster and more robust humoral responses after antigen re-exposure still remains a mystery, although some suggestive data has been gleaned as described below. In terms of this important issue, at least, three layers of regulation are worth considering. First, not only the B cells, but also other types of cells, such as T cells, might be changed during primary immune responses. For instance, memory type Tfh cells may be formed during primary immune responses, and they may be specialized for helping memory B cells upon antigen re-exposure. Second, assuming that such other cell types are required for activating memory B cells, spatial reorganization of these cells might be occurring in the host, which could then contribute to the faster and more robust humoral memory responses. Finally, in regard to B cells, the increased frequency of antigen-specific memory B cells and the intrinsic properties that memory B cells acquire after primary antigen experience could contribute to the features of memory responses.

Contribution of other cell types and their spatial reorganization to rapid memory responses

It is now clear that antigen-specific T-helper cell development can proceed along multiple paths depending upon the nature of the antigen assault. In this context, Tfh cells have been considered a separable T-helper cell subset specialized for regulating the evolution of primary and memory B-cell responses (93). McHeyzer-Williams and his colleagues (94) demonstrated the existence of a memory compartment of antigen-specific effector Tfh cells that persists locally to regulate accelerated memory B-cell responses. Importantly, the local retention of Tfh memory cells in draining lymphoid tissues appears to require cell-associated cognate peptide-MHCII, suggesting that the primary antigen is not completely eliminated in the periphery, but still remains, at least to some extent, for maintenance of the memory Tfh cells. Assuming that memory Tfh cells are a major player in T-dependent memory humoral responses, clarifying the exact location of the memory Tfh cells in the secondary lymphoid tissues and their activation mechanisms is very important to understand why humoral memory responses are rapid and robust. In regard to localization of memory Tfh cells, we have recently shown that memory Tfh cells reside close to IgG type memory B cells surrounding the contracted GCs in the spleen (72), allowing us to propose that such close proximity of memory B and Tfh cells can explain, at least partly, the more rapid kinetics of humoral memory responses (Fig. 4).

What is the intrinsic difference between naive and antigen-experienced memory B cells?

Because of the relative ease of obtaining large numbers of memory B cells, the intrinsic properties in the *in vitro* reactivation of these cells have been extensively analyzed in humans. Most studies have utilized purified $CD27^+$ cells and then compared their responsiveness to $CD27^-$ naive B cells. Activation of splenic $CD27^+$ memory B cells with CD40L, IL-2, and IL-10 induced terminal differentiation into plasma cells, more efficiently than with naïve cells (95). Additionally, after

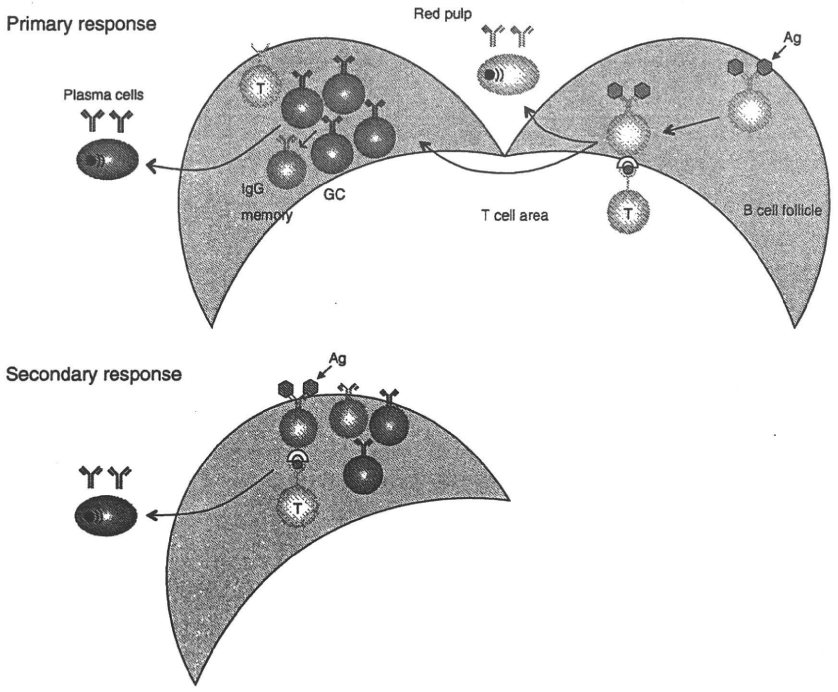

Fig. 4. Unique localization of IgG type memory B cell allows the prompt memory response. After primary antigen exposure, antigen-reactive B cells move to T–B boundary and interact with helper T cells. B cells activated by helper T cells differentiate into plasma cells, germinal center (GC) B cells or memory B cells. IgG type memory B cells preferentially localize adjacent to contracted GCs and promptly interact with Tfh cells proximal to them and rapidly differentiate into plasma cells, which appear to explain, at least partly, the rapid kinetics of secondary IgG responses.

treatment with T-cell-dependent (CD40L+anti-Ig) and T-cell independent (CpG+anti-Ig) stimuli, $CD27^+$ memory B cells entered the cell cycle earlier and underwent greater numbers of division than naive B cells (96). Some of these studies carefully examined the *in vitro* properties of non-class-switched and class-switched $CD27^+$ memory B cells, showing that both possess similar characteristics. These *in vitro* properties support the idea that there are intrinsic differences between naïve and memory B cells, irrespective of which isotype is expressed, and explain partly why memory B cells are able to respond faster upon secondary exposure to antigen. Global gene expression analysis of human memory B cells identified several candidate genes and signaling pathways that may underlie the rapid secondary response (97). Compared to naive B cells, both IgM^+ and isotype-switched memory B cells expressed reduced levels of negative cell cycle regulators, but increased levels of positive regulators. To address the role of these molecules in the rapid entry of memory B cells into cell cycle, Good and Tangye (96) have focused on the KLF4, KLF9, and promyelocytic leukemia zinc finger (PLZF) transcription factors, which are negative regulators of the cell cycle that are down-regulated in memory B cells. They observed that enforced expression of these genes in memory B cells delayed their entry into cell cycle, concluding that the reduced expression of these molecules in memory B cells allows their rapid response to external stimuli. In contrast to these recent clues about potential mechanisms for rapid entry into cell cycle, the molecular basis for why human memory B cells are prone to differentiate into plasma cells remains elusive (98).

As described above, it is now possible to label not only IgG type but also IgM type memory B cells by utilizing tamoxifen-inducible Cre inserted into *Aicda* locus. This method, combined with adoptive transfer of these memory subsets into mice, has revealed unappreciated differences between IgM and IgG type memory B cells (14) (Fig. 5). After antigen re-exposure, most transferred IgG^+ memory B cells differentiated into IgG-secreting plasma cells, similar to the biased terminal differentiation of human tonsillar memory B cells into plasma cells *in vitro* (98). Furthermore, this behavior is very consistent with a recent report showing that IgG type memory B cells have little capacity to reinitiate a GC response (99). Intriguingly, however, IgM type memory B cells predominantly gave rise to IgM and IgG GC B cells rather than differentiating into plasma cells, disclosing their very different effector properties in comparison to IgG type memory B cells. This *in vivo* property of IgM type memory B cells differs markedly from their *in vitro* characteristics mentioned above. One interpretation that reconciles these data is that these IgM type

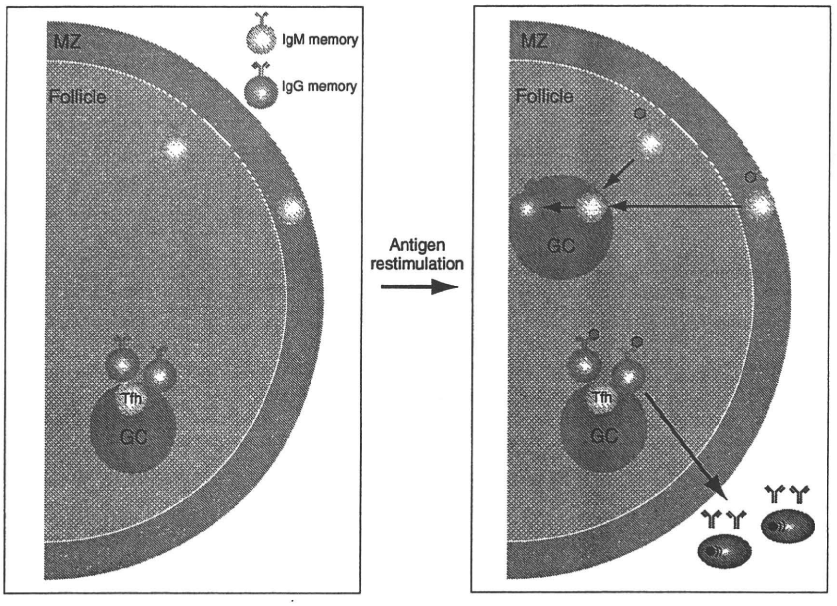

Fig. 5. Different effector functions of IgM and IgG type memory B cells. After priming with T-cell-dependent antigens, IgM type memory B cells emerge not only in marginal zone (MZ), but also scatter in splenic follicles. IgG type memory B cells, however, preferentially localize adjacent to contracted germinal center (GC) together with Tfh cells. After antigen re-exposure, IgM type memory B cells preferentially differentiate into GC B cells, while IgG type memory B cells terminally differentiate into plasma cells, exerting different effector functions.

memory B cells, despite having a greater potential to differentiate into plasma cells than naive B cells, are in an appropriate microenvironment where sufficient cytokines for GC B-cell differentiation are provided *in vivo*.

The molecular mechanisms underlying these differential effector properties of IgM and IgG type memory B cells are currently unknown, but their differential properties are likely to have a significant biological impact; IgM type memory B cells contribute to replenishment of the memory pool by their rapid mobilization into GC and switching to IgG, while IgG type memory B cells promptly differentiate into isotype-switched plasma cells to immediately confer protective functions. More detailed analysis of IgM and IgG type memory B cells in both mice and humans is definitively required for dissecting the molecular mechanisms underlying the differential effector properties of these B cells.

Future perspectives

Many intrinsic properties of memory B cells have been elucidated by using human memory B cells, however, recent advances that have allowed visualization of murine memory B cells, defined as antigen-experienced, long-lived B cells, after synchronous activation with antigen have provided new insights into the overall picture of memory B-cell responses. Consistent with the human studies, murine memory B cells are heterogeneous in their phenotypes, isotypes, and mutational status of their immunoglobulin V genes. Moreover, through the analysis of *in vivo* dynamics of murine memory B cells, it has been elucidated that considerable diversification exists in their localization and effector functions, issues that were not possible to directly address in the human studies. At present, we do not know where and how this heterogeneity of memory B cells originates, but it appears to be generated by the complexity of precursors and pathways for memory B-cell development. Because the choice for each developmental pathway is determined in part by immunization methods, we may need to use several types of antigens and delivery routes to visualize and dissect as many memory components as possible. In particular, many antigens that have great biological impacts are not pure protein antigens, but also include sugars and complex carbohydrates with unique structures. Dissection of memory B-cell response against these non-conventional antigens will be necessary for the translation of basic information into vaccine design, or treatment of allergy and autoimmune diseases.

In addition to these newly arising questions, two long-standing fundamental questions about the intrinsic properties of memory B cells still remain unanswered. How is the survival of memory B cells achieved? What determines the robust and prompt responsiveness of memory B cells after antigen re-exposure? Because PLC-γ2 has been identified as a key

molecule for memory B-cell survival, and the responsiveness of re-stimulated memory B cells can now be monitored *in vivo*, it will be possible in the near future to finally answer these questions.

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International Immunology, Vol. 22, No. 3, pp. 179–189
doi:10.1093/intimm/dxp126
Advance Access publication 5 February 2010

Transient role of $CD4^+CD25^+$ regulatory T cells in mycobacterial infection in mice

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Transmitting editor: S. Koyasu

Received 22 April 2009, *accepted* 22 December 2009

Abstract

$CD4^+CD25^+$ regulatory T (Treg) cells cause immune suppression by inhibiting T cell effector functions and play pivotal roles not only in self-tolerance but also in immune response to parasitic microbial pathogens. Mycobacteria are major parasitic bacterial pathogens, but the role of $CD4^+CD25^+$ Treg cells in mycobacterial infection is not yet defined. In this study we found that, at the early stage of infection, depletion of $CD25^+$ cells reduced both bacterial load and granuloma formation in mice infected with *Mycobacterium tuberculosis* strains, such as *M. tuberculosis* Erdman or *M. tuberculosis* Kurono. However, at a later stage of infection, bacterial burden and histopathology were similar regardless of depletion of $CD25^+$ cells. Severe combined immunodeficient (SCID) mice reconstituted with $CD4^+CD25^-$ T cells alone or a combination of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells showed similar bacterial loads and survival kinetics after infection with *M. tuberculosis* Erdman. Consistent with *in vivo* data, *in vitro* studies revealed that mycobacterial antigens, purified protein derivative of tuberculin (PPD), failed to induce the suppressive function of $CD4^+CD25^+$ Treg cells to $CD4^+CD25^-$ effector T cells, as demonstrated by the lack of response of $CD4^+CD25^+$ T cells to PPD, in mice chronically infected with *Mycobacterium bovis* bacillus Calmette–Guérin and *M. tuberculosis*. Our data show that $CD4^+CD25^+$ Treg cells have a transient effect at the early stage of mycobacterial infection but, contrary to the expectation, have little impact on the overall course of infection.

Keywords: bacterial, T cells, rodent, inflammation, lung

Introduction

Mycobacteria are intracellular bacterial pathogens, which persistently infect eukaryotes, including mammals, and cause diseases not only following primary infection but also by reactivation from latent state. Several species of mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium bovis*, are known to cause human tuberculosis. The World Health Organization estimates that *M. tuberculosis* infects one-third of the world's population and is responsible for 2 million deaths each year (1). While the infection remains latent in 95% of the infected cases of *M. tuberculosis*, 5–10% of those who initially controlled the infection later develop active disease at some stage during their lifetime. To suppress intracellular growth of mycobacteria, macrophage activation by IFN-gamma is critical in both mice (2, 3) and humans (2, 3).

The important role of the $CD4^+CD25^+$ regulatory T (Treg) cells in immune response has recently been recognized. This T cell subset maintains immunologic self-tolerance and suppresses the onset of autoimmune diseases (4). The vast majority of Treg cells constitutively express CD25/IL-2 receptor alpha chain in the physiological state (5, 6). $CD4^+CD25^+$ Treg cells also express cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; 7, 8), glucocorticoid-induced tumor necrosis factor receptor (GITR; 9, 10) and the transcription factor,