Immunologic Research 2000;22/2–3:223–236

Antigen-Specific Immunity

Th Cell-Dependent B Cell Responses

M.G. McHeyzer-Williams
L.J. McHeyzer-Williams
J. Fanelli Panus
G. Bikah
R. R. Pogue-Caley
D.J. Driver
M.D. Eisenbraun

Duke University Medical Center,
Department of Immunology,
Durham, NC.

Abstract

Helper T cell-regulated B cell responses constitute a major component of the primary immune response to many pathogens. The subsequent development of antigen-specific immune memory is one critical outcome of this primary adaptive immune response. Antigen-specific immunity develops through a series of intercellular information exchanges organized around cognate T cell receptor-peptide/MHC interactions. Here, we discuss these complex molecular events and their cellular consequences in a serial synapsis model of adaptive immunity. Our laboratory has developed strategies to isolate antigen-specific Th cells and B cells to analyze gene expression and cellular function in single responding lymphocytes directly ex vivo. These studies provide insight into the regulation and cellular organization of antigen-specific immune responses in vivo.

Key Words

B lymphocytes
Helper T lymphocytes
Immune memory
Murine models
Germinal centers
Immune synapse

Introduction

Th-cell-regulated B cell responses develop within specialized microenvironments of secondary lymphoid organs. Antigen-presenting cells (APC), antigen-specific Th cells, and antigen-specific B cells exchange molecular information in an orchestrated progression of cognate cellular interactions. These interactions are organized in different microenvironmental locations within lymphoid organs and occur separated over time after exposure to antigen. Here, we present an ordered model of adaptive immunity that consists of overlapping stages of immune synapse-dependent and synapse-independent lymphocyte differentiation (a serial synapsis model). Inherent to this model is the notion of antigen-specific

Michael G. McHeyzer-Williams
Box 3010; Department of Immunology
Duke University Medical Center
Durham, NC 27705
E-mail: mchey002@duke.edu

© 2000
Humana Press Inc.
0257–277X/00/
22/2–3:223–236/$13.50

lymphocyte subsets, each with cell surface complementarity that helps to promote successful synapse formation. To evaluate the extent of cellular heterogeneity within an adaptive immune response, it is imperative to analyze the attributes of lymphocyte populations one cell at a time. Our laboratory has focused its efforts on flow cytometric strategies to isolate antigen-specific Th and B lymphocytes from intentionally immunized mice. These cells remain accessible to the analysis of gene expression directly ex vivo and measurements of physiologic responsiveness following short-term stimuli in vitro. In this review, we will outline these general strategies and highlight the types of assays that provide single-cell resolution to the assessment of lymphocyte physiology in vivo.

Antigen-Specific Immunity

The Immune Synapse

Recognition of peptide-MHC molecules by the T cell receptor (TCR) of specific Th cells is central to the development of adaptive immunity. Successful cognate interactions occur through organized rearrangement of cell surface molecules at the cellular interface now commonly referred to as the immunological synapse *(1–3)*. Specific TCR-peptide/MHC interactions cluster at the center of the immune synapse surrounded by complementary adhesion molecule interactions. Within the first 5 min of intercellular junction formation, peptide/MHC is transported centrally and stabilized *(3,4)*. Actin cytoskeleton changes enable the translocation of major costimulatory molecules to the cellular interface *(5,6)* and may also help to preferentially recruit membrane microdomains rich in glycolipids and signaling intermediates *(5)*. Thus, immune synapse formation can encourage efficient signal transduction by promoting localized high concentrations of positive regulators and potentially diluting the local influence of negative regulators. Furthermore, immune deficits associated with aging have been associated with suboptimal recruitment of positive signaling intermediates into specific immune synapses *(7,8)*. Overall, immune synapse formation appears to be the critical organizing principle underlying cognate cellular interactions in vivo.

The Serial Synapsis Model

Over the course of a primary immune response, synapse formation must occur between antigen-specific Th cells and a variety of different cell types. These cognate cellular interactions occur at different times after antigen exposure and in distinct microenvironments within secondary lymphoid organs. To help unravel these complex events, we consider a Th-cell dependent primary B cell response as a series of six overlapping phases of synapse-dependent and synapse-independent cellular development. The Serial Synapsis Model depicted in Figs. 1 and 2 attempts to integrate the need for cognate information exchange with timing, cellular development, and tissue localization in vivo.

Phase 1 signifies the initial activation of resident APC at the site of antigen entry through nonspecific means of antigen uptake. Dendritic cells (DC), as the most efficient APC, activate in response to antigen and local inflammatory signals and migrate to the T cell zones of draining lymph nodes. In the T cell zones, primed DC recruit naive antigen-specific Th and initiate the first cognate cellular interactions (Phase 2: Synapse I). It is generally thought that all naive Th cells are equally able to differentiate into a spectrum of effector Th cells as pluripotent antigen-specific precursors. However, the quality of the TCR-peptide/MHC interaction can also clearly influence functional commitment of Th cells *(9)*. Hence, the available TCR repertoire can influence the outcome

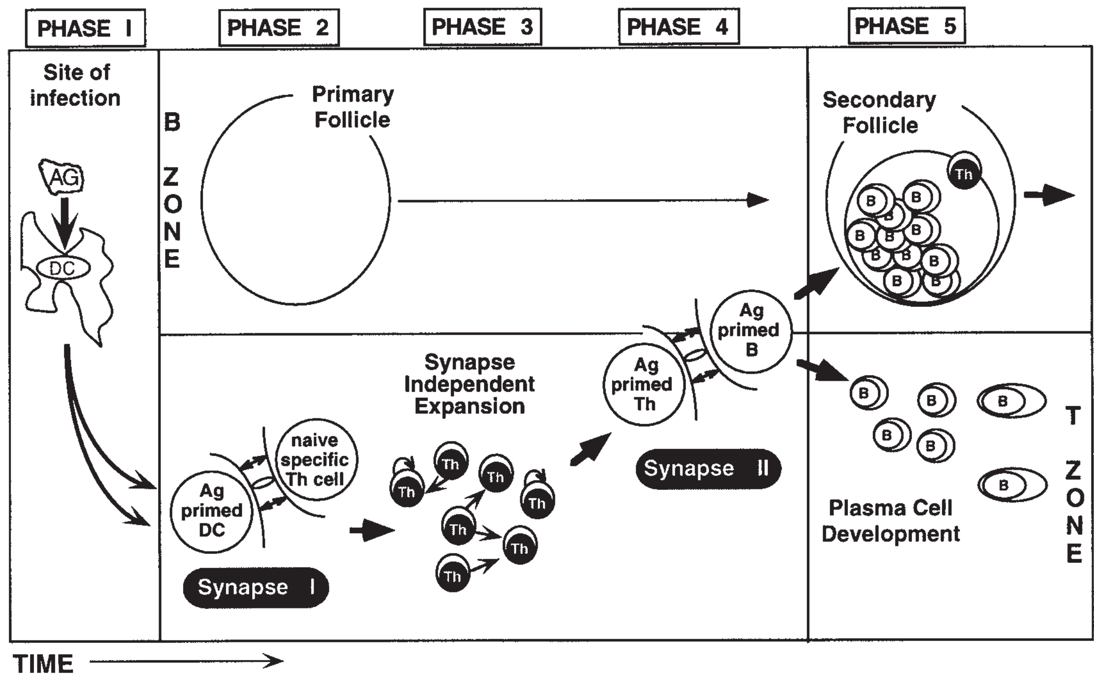

Fig. 1. The Serial Synapsis Model, Phase 1-5. The five phases depicted in this figure are used to broadly outline the synapse-dependent and synapse-independent phases of a developing primary immune response. Phase 1 depicts the initiation of DC activation at the site of antigen entry. The antigen-primed DC migrates to the T cell zones of secondary lymphoid organs to recruit naive but antigen-specific Th cells to form the first immune synapse (Phase 2: Synapse I). Clonal expansion of the antigen-specific Th cells ensues in the T cell zone in a synapse independent manner (Phase 3). There is significant migration of these antigen-primed Th cells to the T/B follicular borders of these organs. The next stage involves cognate delivery of T cell help to antigen-primed B cells and the less well characterized signaling to the Th cells by antigen-primed B cells (Phase 4: Synapse II). The outcome of this information exchange is clonal expansion of antigen-specific B cells and their subsequent differentiation into short-lived plasma cells in the T cell zones or alternatively, secondary follicle formation in the B cell zones (Phase 5: Synapse Independent). Antigen-specific Th cells also migrate to the follicular regions during this phase of the response.

of an immune response *(10,11)*. This initial stage of antigen-specific Th cell development is heavily influenced by the DC expression pattern of cytokines (such as IL-12 and IL-6) and costimulatory molecules (such as CD80 and CD86). The antigen-primed Th cells can then deliver signals to the DC by way of cell contact (such as CD40L) and perhaps also immediate early cytokine production (such as TNF-α). Thus, intercellular synapsis encourages efficient local exchange of molecular information that remains antigen-specific.

The next phase involves synapse-independent clonal expansion of antigen-specific Th cells (Phase 3: Synapse Independent). At this stage, developmental programs initiated during the first synapse can be consolidated and propagated through selective cellular expansion. Selection for Th cells with preferred TCR occurs very rapidly (d 3–5 after priming) and is associated with extensive clonal expansion in the T cell zones *(12)*. Subdominant clonotypes selected in this phase of the response express distinct cell fates (such as GC vs non-

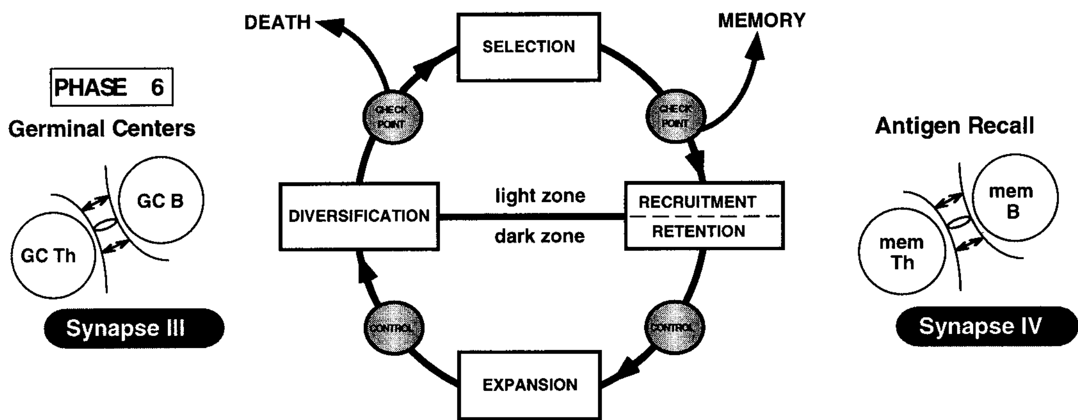

Fig. 2. The germinal center cycle and antigen recall. The last stage of the primary response involves the polarization of secondary follicles to initiate the GC reaction (Phase 6: Synapse III). The activities associated with the GC reaction are depicted in the center of the figure as the GC cycle identifying its general points of control and checkpoints for developmental progression. Antigen-specific B cells are recruited into the follicular regions within the B cell zones of secondary LNs. Rapid clonal expansion describes the formation of the secondary follicle. The polarization of expanding centroblasts to the T proximal regions of the secondary follicle and the appearance of resting centrocytes apically defines the emergence of the germinal center reaction. Antigen-specific B cells diversify their BCR by somatic hypermutation and then "test" their variant BCR for antigen binding in the light zone. Antigen is present in the GC as immune complexes deposited on the FDC networks of the light zone. Diminished binding for antigen leads to programmed cell death and rapid clearance of apoptotic B cells locally by tingle body macrophage. Improved binding to antigen results in positive selection of the variant. There are two possible outcomes. The first option is to return to the dark zone and resume the cycle of expansion, diversification, and selection. The second option is to exit the GC cycle and thereby enter the memory B cell compartment. Th cell regulation of these events is depicted as Synapse III in an antigen-specific interaction between germinal center Th cells and germinal center B cells (most likely centrocytes). Accelerated memory B cell responses to antigen recall are also regulated by antigen-specific memory Th cells depicted as Synapse IV in this general schematic. These cognate interactions are proposed to occur between memory lymphocyte subsets with cell surface complementary.

GC entry) *(13)*. Autocrine and paracrine influences of cytokines may also play a major role in shaping the mix of Th cell functions within the responsive population during this phase of the response. Our recent studies on cytokine production in vivo clearly demonstrate a broad spectrum of functional outcomes associated with the Th cell response to one dominant peptide epitope *(14)*. This phase of synapse-independent development is also associated with the migration of antigen-specific Th cells toward the T/B follicular borders in vivo *(15,16)* to initiate the second critical synaptic interaction between antigen-primed Th cells and antigen-primed B cells.

Antigen-specific B cells must have bound, processed, and presented antigenic peptide-MHC class II complexes to initiate the formation of Synapse II with antigen-primed Th cells (Phase 4: Synapse II). At this phase in the primary response, there may be multiple antigen-primed Th cell subsets based on cell surface phenotype and potential for cytokine production *(14,17)*. We have also recently demonstrated substantial changes in Th cell physiology, antigen responsiveness, and costim-

ulatory requirements that would impact qualitatively and quantitatively on the outcome of Synapse II in vivo *(17)*. The predominant cellular outcome of Synapse II is extensive clonal expansion of antigen-specific B cells (Phase 5: Synapse Independent). Some of this B cell expansion proceeds in the T cell zones and gives rise to short-lived antibody-secreting plasma cells. The remainder of the B cell expansion proceeds in the B cell zones within primary follicles. Initial expansion occurs with a 6–8 h doubling time and creates large IgD negative regions within primary follicles, now referred to as the secondary follicle. Around d 7–10 after initial priming, the secondary follicle polarizes into a T cell zone-proximal region of rapidly dividing B cells (centroblasts) and a region of quiescent noncycling B cells (centrocytes) at the opposite pole. Once polarity is established, this dynamic microenvironment is called the germinal center reaction.

The Germinal Center Cycle

Figure 2 depicts the progress of B cell development within the GC reaction and graphically outlines the accompanying molecular and cellular process (Phase 5: Synapse Dependent) *(18,19)*. Antigen-specific B cells recruited into the GC reaction rapidly expand (centroblasts) and then diversify their antigen receptors through somatic hypermutation. The vast majority of these essentially random changes are deleterious to antigen binding and lead to death of the resulting centrocyte within the GC light zone. Some rare mutations increase affinity for antigen binding with the resulting centrocyte being positively selected either to remain within the GC and reenter the cycle or to exit into the long-lived memory B cell pool. There is clear evidence for antigen-specific Th cells within this dynamic microenvironment *(12,13,20,21)*, still little is known of their precise role within this GC cycle of activity. Nevertheless, Th cell involvement is most likely antigen-specific involving synapsis between GC Th cells and GC B cells. GC Th cell are phenotypically distinct from their T zone counterparts (*22*; MMW unpublished) suggesting qualitatively different cell surface complementarity in Synapse III interactions with GC B cells.

Immune Memory

It is useful to consider the development of antigen-specific memory in four broad phases: induction, maintenance, expression, and replenishment *(18,19)*. Immune memory is *induced* as one outcome of the emergent cellular response to primary antigenic exposure. Although it is yet unclear when and where memory Th cells are selected, the main cellular outcomes of the GC cycle are long-lived memory B cells. In either case, a cohort of the primary response lymphocytes is selected to enter the long-lived memory compartment based on the "quality" of the antigen receptor they express. In the Th cell compartment, selection appears based on slower dissociation kinetics of TCR and peptide/MHC interactions *(23)*, whereas in the B cell compartment selection is based on high affinity for antigen after somatic diversification of the BCR. Memory cells are then *maintained* for long periods after the initial clearance of antigen and recirculate throughout the body in surveillance of antigen reexposure. Upon antigen rechallenge, memory is *expressed* through massive antigen-specific cellular expansion and rapid development of effector cell function. Memory B cell expansion and plasma cell differentiation are dependent on the presence of antigen-specific memory Th cells. Although these sets of interactions can be broadly classified as Synapse IV, they must involve multiple sets of complementary interactions depending on the nature of the recall response and the

memory lymphocyte subsets. At some point after rechallenge, the long-lived memory compartment must be *replenished* to protect against recurrent antigen incursion.

Antigen-specific Th Cell Responses

Isolation and Quantification of Antigen-Specific Th Cells

Access to antigen-specific Th cells was the first major obstacle to detailed analysis of immune responses in vivo. Initially, antigen specificity could only be revealed through antigen-dependent selection in vitro and the subsequent formation of short-term cell lines or T cell hybridomas. These types of studies provided critical information on TCR structure and offered access to analyzing the fine specificity of the TCR regarding peptide recognition. Transgenic animals expressing TCR with known peptide specificity and MHC restriction pattern represented the next substantial breakthrough to analysis of T cell fate in vivo. These TCR transgenic animals were best used for unraveling the molecular and cellular events associated with T cell development in the thymus. Unfortunately, immunizing animals with monoclonal peripheral TCR repertoires was inappropriate and distorted the dynamics of normal T cell responsiveness in vivo. Jenkins and colleagues championed the use of adoptively transferred TCR transgenic T cells into normal syngeneic recipients rendering them immunocompetent with elevated precursors to a known peptide/MHC *(24)*. These animal models have been used extensively to analyze many aspects of the Th cell response in vivo *(15,24–27)*.

We have developed an alternate strategy to isolate antigen-specific Th cells from nontransgenic animals. Our studies focus on dominant antigen-specific Th cells in the murine B10.BR response (I-E^k restricted) to pigeon cytochrome c (PCC) *(28)*. This dominant clonotype expresses restricted V region genes (Vα11Vβ3) with distinguishable third hypervariable (CDR3) sequences that confer peptide specificity *(29,30)*. It is possible to purify PCC-specific Th cells using expression of Vα11Vβ3 and antigen-dependent comodulation of cell surface molecules (up-regulation of CD44 and down-regulation of CD62L) (Fig. 3A) *(12,31)*. Owing to the very low frequencies of responders in nontransgenic animals (approx 0.1% of total lymph node cells even at the peak of a developing immune response), high resolution flow cytometry was required to ensure accurate cellular quantification in vivo. Using this strategy, we quantify the emergence of PCC-specific Th cells from as early as 3 d after primary immunization, through the peak of clonal expansion (d 7–9). There is a steady local decline in specific Th cells over the next 2 wk (d 11–21) that stabilizes around d 28 after initial priming *(12,31)*. The accelerated memory response to this same antigen (peaks at d 3–4 and decline begins by d 6) typifies the expectations of the cellular response to antigen recall.

Antigen-Specific TCR Repertoire

Single-cell repertoire analysis provided confirmation of peptide specificity and the first direct evidence for clonal maturation in the Th cell compartment *(12,31)*. Between d 3 and 5 of the primary response, PCC-specific Th cells with preferred CDR3 motifs are rapidly selected in vivo *(12)*. Clonal dominance is further propagated through selective expansion of these TCR with the "best fit." Thus, much of this clonal maturation occurs during Phases 2–3 of the schematic outlined above and is primarily a consequence of Synapse I formation in vivo. PCC-specific Th cells expressing the preferred TCR accumulate to large numbers in the T cell zones by d 7 after priming and subsequently migrate into germinal centers by d 9 *(12)*. These trends are consistent with a

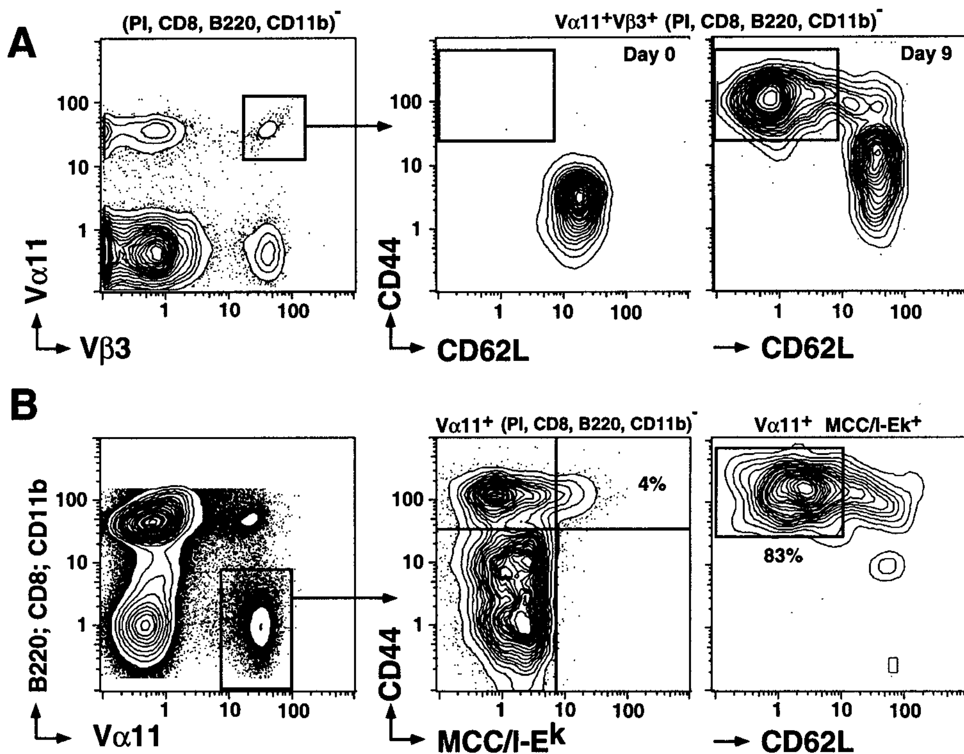

Fig. 3. Isolating PCC-specific Th cells directly ex vivo **(A)** B10.BR mice were immunized with 400 μg PCC in RIBI adjuvant. Cells from the draining lymph nodes were stained with Cy5PE-53-6.7 (anti-CD8), Cy5PE-M1/70.15 (anti-CD11b), Cy5PE-6B2 (anti-B220), FITC-RR8.1 (anti-Vα11), APC-KJ25 (anti-Vβ3), PE-Mel14 (anti-CD62L), and biotin-IM7 (anti-CD44). Forward and obtuse light scatter gates were used to exclude neutrophils and macrophages but include T cell blasts. Cells stained with PI are excluded in the Cy5PE channel at acquisition. A representative probability contour of Vα11-Vβ3 expressing T cells from an unimmunized animal (d 0) and an animal immunized 7 d earlier (d 7) are shown. Vα11/Vβ3 expressing T cells that have up-regulated CD44 and down-regulated CD62L at each time point are presented in the small insert box. **(B)** LN cells from B10.BR mice labeled with MCC/I-Ek tetramers 8 d post priming with PCC as described above. KJ25 (anti-Vβ3) blocks MCC/I-Ek labeling so cannot be used together with the tetramer. Five percent probability contours display cells binding MCC/I-Ek tetramers within the $V\alpha11^+$ LN cells. >80% of $V\alpha11^+$MCC/I-Ek^+ cells are $CD44^{hi}$ $CD62L^{lo}$ (right panel) and express CDR3 regions typical of antigen binding (L. McHeyzer-Williams, unpublished). This figure is reproduced from ref. *15*.

role for the dominant clonotype in Phases 4–6 of the primary immune response, delivering T cell help to B cells in the T zones and assisting memory B cell development in the germinal center. Finally, the dominant clonotype rapidly reemerges upon antigen recall with little evidence for further antigen-driven selection *(12,31)*.

In a more extensive analysis of the PCC-specific TCR repertoire, additional Vb3-Va11+ Th cells could be found to emerge in the primary response and reemerge on antigen recall *(13)*. Unlike the dominant clonotype, these additional PCC-specific clonotypes were rarely found in GCs but were still retained into the memory compartment. These data pro-

vide evidence for alternate cellular fates based on expressed TCR structures and highlights one division of function that must exist in the antigen-responsive Th cell compartment. There also appears to be a preimmune bias in B10.BR animals. One of the eight distinguishing CDR3 sequence features preexisted antigenic challenge in these animals *(18)*. In a collaborative study with Ashwell and colleagues *(11)*, reducing the level of glucocorticoid receptors in the thymus of B10.BR animals altered this preimmune bias and created a "hole" in the T cell repertoire. These results lend support to a role for thymic selection in subsequent clonal dominance, however, the connection between the self-peptide in the former and foreign peptide in the latter is yet to be resolved.

Tetramers of Peptide/MHC Class II

An isolation strategy based on V region gene expression has its strengths and does not require direct antigen binding. More recently, John Altman, Mark Davis, and I developed a new approach for identifying antigen-specific Th cells using fluorophore-labeled tetramers of peptide MHC complexes. The first tetramers produced and tested were I-Ek class II molecules bearing the dominant peptide epitope of moth cytochrome c (MCC), broadly analogous to PCC *(32)*. This new class of reagent was rapidly and extensively adapted to labeling CD8 T cells using peptide/MHC class I complexes in both humans *(33,34)* and mouse *(35–37)*. The class II versions of this reagent were more difficult to produce and the target populations in normal animals were orders of magnitude lower than their CD8 counterparts. These issues have been overcome in a variety of ways with class II versions of these reagents providing new tools for the analysis of TCR affinities *(38)* and the kinetics of binding *(23)*. We have used these reagents to confirm the specificity of our original isolation strategy *(14,17)* and can reliably isolate PCC-specific Th cells from all stages of the immune response outlined above. One example of this type of labeling is presented in Fig. 3B. These data indicate the level of tetramer binding in Vα11-expressing Th cells from d 8 after initial priming when the vast majority of tetramer-binding cells also express high levels of CD44 and low levels of CD62L as predicted from the previous studies. Binding of these reagents is affinity dependent and known to efficiently transduce signals through the TCR *(39)* so are best avoided for analysis of low affinity TCR or direct ex vivo analysis of T cell function.

Changes in Th Cell Physiology

A great deal is known about naive Th cell activation; however, it is not clear how Th cell physiology changes during the course of a primary immune response. Our recent study focuses on the peak of clonal expansion in the T cell zones, 7 d after initial priming *(17)*. We evaluated changes to intrinsic signaling potentials and proliferative capacity of single PCC-specific Th cells at this phase of development. To our surprise, all d 7 PCC-specific Th cells exhibited a profound block in CD3- and CD4-mediated mobilization of intracellular calcium stores. The levels of intracellular calcium stores were equivalent to naive Th cells, but when artificially emptied by ionomycin or thapsigargin, there was no capacitative calcium entry (CCE). This more global calcium blockade would seriously impair numerous calcium-dependent activities fundamental to developmental progression in vivo (such as chemotaxis that drives migration to GCs). The proliferative response of these refractory cells to conventional polyclonal stimuli in vitro (anti-CD3, anti-CD28, and IL-2) was also severely blunted. Thus, known costimulatory factors were not sufficient to drive cell cycle entry at this stage of the immune response. Overall, these data imply that the Th cell phys-

iology governing Phase 4: Synapse II formation and its outcome in vivo are very different to those at play during first contact (Phase 2: Synapse I).

In subsequent studies, we noted the expression of CD69 on a substantial fraction (25–30%) of the d 7 PCC-specific Th cells. As CD69 had been previously associated with promoting sustained high intracellular calcium levels, we assayed this activity directly ex vivo *(17)*. Whereas CD69 crosslinking had no effect on calcium flux by itself, there was a notable rescue of TCR-independent CCE when the d 7 Th cells were treated with ionomycin or thapsigargin. Crosslinking CD69 with plate-bound antibodies also restored proliferative capacity in vitro. This CCE rescue operated through a CD69-coupled G protein and required calcium-bound calmodulin and calcineurin. Thus, CD69 ligation may operate as an efficient costimulatory signal at this phase of the primary immune response in vivo. This refractory phenotype and the role of CD69 in its regulation also provides a new means for analyzing the molecular mechanisms that underpin the fundamental process of CCE. These previous two sets of data highlight how little we still know of Th cell regulation during late phase events in adaptive immunity.

Cytokine Production In Vivo

The production of cytokines by antigen-specific Th cells and their cognate delivery to B cells is a major regulating principle of humoral immunity. In a recent study, we isolated PCC-specific Th cells from the primary and memory response to evaluate their change in cytokine-producing potential due to antigen experience *(14)*. Using short-term mitogen restimulation in vitro, there was surprisingly little evidence for the selective preservation of IL-2, TNF-α, IL-4, and IFN-γ producing potentials into the memory response. IL-10 provided one exception to this pattern with significant increases in the frequency of PCC-specific memory responders able to produce this cytokine in vitro. Thus, the development of functional potential appeared to be the consequence of initial antigen experience with a proportional preservation of specialized function into the memory compartment. We also developed an RT-PCR assay with single-cell sensitivity to estimate more directly the frequency of cytokine-producing cells in vivo. Frequencies of PCC-specific Th cells expressing IL-2, TNF-α, IFN-γ, and IL-10 increased in the memory response compared to their primary response counterparts. However, these frequencies directly ex vivo were markedly lower for most cytokines (IL-2, TNF-α, IFN-γ, and IL-10) than revealed with mitogen restimulation in vitro.

These studies indicate that the expression of preferred memory function is not simply a reflection of the increased functional capacity of the memory Th cells; rather, their accelerated expression and increased prevalence appear differentially regulated by the microenvironment of the memory response. These data also clearly highlight the functional heterogeneity among antigen-specific Th cells with respect to cytokine-producing potentials and their expression patterns in vivo. These patterns indicate extensive subset assortment that most likely occurs as a consequence of Phase 2: Synapse I interactions and must be reconciled in any comprehensive model of adaptive immunity.

Antigen-Specific B Cell Responses

Cognate cellular interactions promote the exchange of information between both cell types involved. Although the outcome of Phase 2: Synapse I is a critical initiating event, the continued maturation of antigen-specific Th cells during Phases 4–6 most likely depends on receiving the appropriate signals from

antigen-primed B cells. To more completely understand these activities, it is important to dissect the cellular and molecular development of antigen-specific B cells directly ex vivo. In the last section of this review, we will summarize the parallel experimental model we have chosen for these studies and present the most recent results that define a new cellular pathway for antigen-specific memory B cells and their development during the primary response.

Isolation and Quantification of Antigen-Specific B Cells

An ideal system for direct ex vivo analysis of antigen-specific B cells is the immune response in C57BL/6 mice to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) *(40, 41)*. NP-specific B cells can be visualized directly *in situ (42,43)* or through NP binding by flow cytometry *(44–46)*. The vast majority of NP-specific B cell clones from a primary response express B cell receptors (BCR) with the V_H186.2 heavy chain coupled to $V\lambda_1$ light chain *(47,48)*. Somatic mutation proceeds in the GC microenvironment *(43)* and is seen in NP-specific B cells as early as d 6 after initial priming *(49)*. Most NP-binding cells express mutated heavy and light chain genes by the end of the second week after priming and can be visualized upon antigen rechallenge in vivo *(44)*. Direct access to NP-specific B cells through flow cytometry provides a reliable means to quantify NP-specific B cell responses directly ex vivo.

Our recent application of this high-resolution flow cytometric strategy *(46)* is broadly outlined in Fig. 4. Light scatter parameters and propidium iodide labeling are used in conjunction with an "exclusion channel" to clarify the starting population for analysis. This first step in analysis excludes dead and dying cells, T cells, macrophage, and cells that nonspecifically bind reagents for a variety of reasons (such as Fc receptor binding). We then identify cells able to bind the NP using a fluorophore (allophycocyanin in this case) with a low conjugation ratio of the hapten. The figure displays profiles from a memory response to NP in which d 0 represents d 56 after initial priming before antigen rechallenge. In response to NP, most memory response precursors are expected to have switched to downstream immunoglobulin isotypes such as IgG, during the primary immune response. The starting population of putative memory-response precursors can be seen at low frequencies as NP-binding cells that are negative for sIgD (Fig. 4 center panels). By d 3 after antigen recall, there has been significant expansion in the NP+IgD– cell compartment that persists at high numbers in the spleen (Fig. 4: d 9 displayed) *(46)*. These memory responders emerge rapidly in the spleen and can also be seen at high numbers in the bone marrow (Fig. 4 bottom panels). The dynamics of cellular expansion in the bone marrow are more consistent with migration from the spleen rather than *de novo* local development.

Memory B Cell Subsets

The strategy outlined above leaves two fluorescence channels available for further phenotypic analysis. In these initial studies, we used antibodies against CD138 (281.2: anti-syndecan) and the B cell isoform of CD45R (6B2: anti-B220) to subtype the NP+IgD– memory responders. CD138 is an excellent marker of antibody-secreting cells in this response and can be seen to label a large fraction of NP+IgD– cells at d 4 (Fig. 4 last panel top row) *(46)*. As expected, these antibody-secreting cells express reduced levels of B220. The second subset clearly visible in this panel is negative for CD138 and expresses high levels of B220. This subset is consistent with a GC phenotype as well the expected phenotype of postgerminal center memory B cells. Appearance of CD138-B220– cells as a third

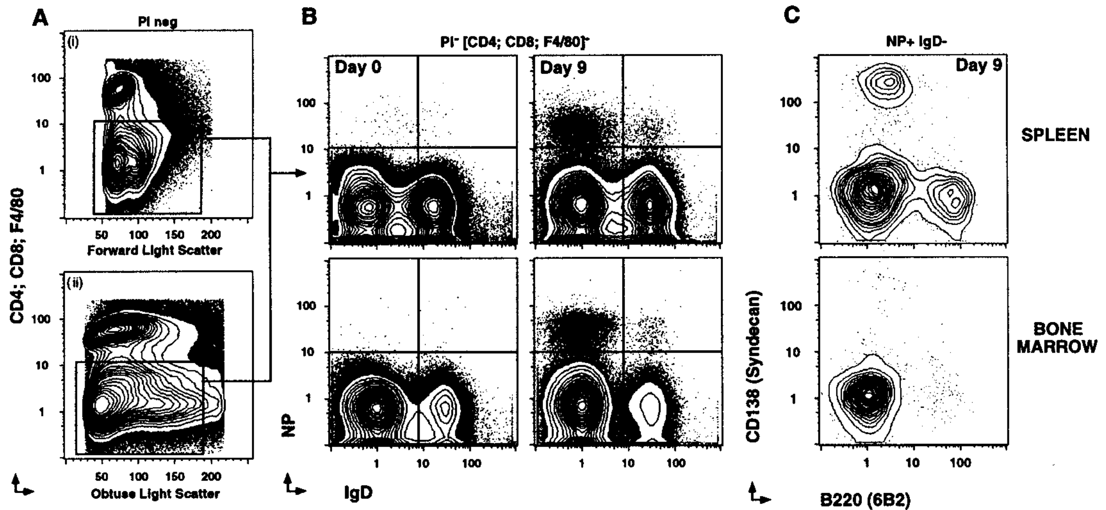

Fig. 4. Three major subsets of NP-specific memory B cells. Previously primed C57BL/6 mice, rechallenged with 400 µg NP-KLH in Ribi adjuvant, were sacrificed at various times post-recall to label spleen and bone marrow cells for "five-color" flow cytometry. **(A)** Propidium iodide labeling is excluded at time of acquisition. One example of light scatter parameters and Cy5PE labeling (anti-CD4, CD8, and F4/80) used to exclude T cells, macrophage and cells that nonspecifically label with antibodies, light scatter gates are broad to include B cell blasts. **(B)** Examples of IgD (texas red-11.26) and NP (NP-allophycocyanin) labeling on the Cy5PE- cells for spleen (top) and bone marrow (bottom) d 0 and d 9 after antigen rechallenge. **(C)** Examples of CD138 (PE-281.2) and B220 (FITC-RA3-6B2) levels on NP-specific B cells ($CD4^- CD8^- F4/80^- IgD^- NP^+$) of the spleen (upper) and bone marrow (lower) for cells 4 d after antigen recall. This figure is largely reproduced from ref. *48*.

major subset of NP+IgD– cells was a complete surprise. On closer examination, these cells expressed the lambda light chain typical of the NP response (on only 5% of background B cells) with mRNA from single-cell analysis displaying evidence of somatic hypermutation *(46)*. Although not antibody-secreting cells themselves, they rapidly gave rise to NP-specific antibody-secreting B cells upon adoptive transfer. Interestingly, these novel memory B cells were the majority population of NP-binding cells found in the bone marrow (Fig. 4 last panel bottom row) *(46)*.

The extended cell surface phenotype of this novel B220– memory B cell compartment identifies at least two major subtypes *(46)*. One predominantly expresses IgG and the other IgE. These B220– B cells have different proliferative capacity from their B220+ counterparts. Atypical coreceptor expression indicates unique means for responding to antigen (lack of CD19) and recruiting T cell help (expressing CD43) *(46)*. Integrin expression patterns also suggest unique recirculating behavior that may explain the preponderance for bone marrow homing. Overall, these B220– memory B cells comprise a major cellular subset of the memory response and the long-term memory B cell compartment (at least d 42 post-recall) *(46)*. These cells act most like memory response precursors, primed for rapid clonal expansion and ready to differentiate into plasma cells on antigen recall. Although it may be expected that these cells have their origin in the primary response to antigen, this cannot be taken for granted and must be demonstrated directly. The presence of B220– antigen-specific B cells during the

emergent primary response may impact the appropriate and ongoing development antigen-specific Th cell as discussed above.

Summary

In this review, we have presented a model for Th cell dependent B cell development that proceeds as overlapping phases of change across time after antigen exposure and in distinct microenvironments within secondary lymphoid organs. The central organizing principle of this model is the formation of a series of immune synapses. Synapsis occurs between different sets of cells with an exchange of molecular information that impacts continued developmental progression. We refer to this model as the Serial Synapsis Model of adaptive immunity. Our laboratory is committed to identifying the cellular and molecular events that regulate this procession of behavior in vivo. Our first step has been to develop strategies to reliably isolate the antigen-specific lymphocytes directly ex vivo. Using flow cytometry, these cells remain physiologically responsive and amenable to functional analysis. Our initial studies have helped to identify the complex cellular organization of primary and memory immune responses in vivo and begin to unravel the nature of the changes in lymphocyte physiology that accompany antigen-specific development. It is clear that antigen-specific lymphocyte subsets with specialized functions develop in both Th cell and B cell compartments. The basis of this functional specialization and its utility in vivo remains the major focus of our studies.

Acknowledgments

This work has been supported by a Biomedical Sciences Grant from the Arthritis Foundation and NIH grants no. AI40215 and AI47231.

References

1. Monks CR, Kupfer H, Tamir I, Barlow A, Kupfer A: Selective modulation of protein kinase C-theta during T-cell activation. Nature 1997;385:83–86.
2. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A: Three-dimensional segregation of supramolecular activation clusters in T cells. Nature 1998;395:82–86.
3. Grakoui A, Bromley SK, Sumen C, et al.: The immunological synapse: a molecular machine controlling T cell activation. Science 1999;285:221–227.
4. Dustin ML, Cooper JA: The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. Nature Immunol 2000;1:23–29.
5. Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A: T lymphocyte costimulation mediated by reorganization of membrane microdomains. Science 1999;283: 680–682.
6. Villalba M, Coudronniere N, Deckert M, Teixeiro E, Mas P, Altman A: A novel functional interaction between Vav and PKCtheta is required for TCR-induced T cell activation. Immunity 2000;12: 151–160.
7. Eisenbraun MD, Tamir A, Miller RA: Altered composition of the immunological synapse in an anergic, age-dependent memory T cell subset. J Immunol 2000;164: 6105–6112.
8. Tamir A, Eisenbraun MD, Garcia GG, Miller RA: Age-dependent alterations in the assembly of signal transduction complexes at the site of T cell/APC interaction. J Immunol 2000;165:1243–1251.
9. Blander JM, Sant'Angelo DB, Bottomly K, Janeway CA, Jr: Alteration at a single amino acid residue in the T cell receptor alpha chain complementarity determining region 2 changes the differentiation of naive CD4 T cells in response to antigen from T helper cell type 1 (Th1) to Th2. J Exp Med 2000;191:2065–2074.
10. Tolosa E, King LB, Ashwell JD: Thymocyte glucocorticoid resistance alters positive selection and inhibits autoimmunity and lymphoproliferative disease in MRL-lpr/lpr mice. Immunity 1998;8: 67–76.
11. Lu FW, Yasutomo K, Goodman GB, et al.: Thymocyte resistance to glucocorticoids leads to antigen-specific unresponsiveness due to "holes" in the T cell repertoire. Immunity 2000;12:183–192.
12. McHeyzer-Williams LJ, Panus JF, Mikszta JA, McHeyzer-Williams MG: Evolution of antigen-specific T cell receptors in vivo: preimmune

and antigen-driven selection of preferred complementarity-determining region 3 (CDR3) motifs. J Exp Med 1999;189: 1823–1838.
13. Mikszta JA, McHeyzer-Williams LJ, McHeyzer-Williams MG: Antigen-driven selection of TCR In vivo: related TCR alpha-chains pair with diverse TCR beta-chains. J Immunol 1999;163: 5978–5988.
14. Panus JF, McHeyzer-Williams LJ, McHeyzer-Williams MG: Antigen-specific T helper cell function: Differential cytokine expression in primary and memory responses. J Exp Med 2000;192:1301–1316.
15. Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK: Visualization of specific B and T lymphocyte interactions in the lymph node. Science 1998; 281:96–99.
16. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG: In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. J Exp Med 1999;190: 1123–1134.
17. Bikah G, Pogue-Caley RR, McHeyzer-Williams LJ, McHeyzer-Williams MG: Regulating T helper cell immunity through antigen responsiveness and calcium entry. Nature Immunol 2000;1:402.
18. McHeyzer-Williams MG, Ahmed R: B cell memory and the long-lived plasma cell. Curr Opin Immunol 1999;11:172–179.
19. McHeyzer-Williams LJ, Driver DJ, McHeyzer-Williams MG: The germinal center reaction. Curr Opin Hematol 2001;8:52–59.
20. Zheng B, Han S, Zhu Q, Goldsby R, Kelsoe G: Alternative pathways for the selection of antigen-specific peripheral T cells. Nature 1996;384:263–266.
21. Gulbranson-Judge A, MacLennan I: Sequential antigen-specific growth of T cells in the T zones and follicles in response to pigeon cytochrome c. Eur J Immunol 1996;26:1830–1837.
22. Zheng B, Han S, Kelsoe G: T helper cells in murine germinal centers are antigen-specific emigrants that downregulate Thy-1. J Exp Med 1996;184:1083–1091.
23. Savage PA, Boniface JJ, Davis MM: A kinetic basis for T cell receptor repertoire selection during an immune response. Immunity 1999;10:485–492.
24. Kearney ER, Pape KA, Loh DY, Jenkins MK: Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity 1994;1: 327–339.
25. Ingulli E, Mondino A, Khoruts A, Jenkins MK: In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. J Exp Med 1997; 185:2133–2141.
26. Swain SL, Hu H, Huston G: Class II-independent generation of CD4 memory T cells from effectors. Science 1999;286:1381–1383.
27. Dutton RW, Bradley LM, Swain SL: T cell memory. Annu Rev Immunol 1998;16:201–223.
28. Schwartz RH: T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. Annu Rev Immunol 1985;3:237–261.
29. Winoto A, Urban JL, Lan NC, Goverman J, Hood L, Hansburg D: Predominant use of a Vα gene segment in mouse T-cell receptors for cytochrome c. Nature 1986;324: 679–682.
30. Hedrick SM, Engel I, McElligott DL, et al.: Selection of amino acid sequences in the beta chain of the T cell receptor. Science 1988;239: 1541–1544.
31. McHeyzer-Williams MG, Davis MM: Antigen-specific development of primary and memory T cells in vivo. Science 1995;268: 106–111.
32. McHeyzer-Williams MG, Altman JD, Davis MM: Tracking antigen-specific helper T cell responses. Curr Opin Immunol 1996;8: 278–284.
33. Altman JD, Moss PAH, Goulder PJR, et al.: Phenotypic analysis of antigen-specific T lymphocytes. Science 1996;274:94–96.
34. Ogg GS, Jin X, Bonhoeffer S, et al.: Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998;279:2103–2106.
35. Murali-Krishna K, Altman JD, Suresh M, et al.: Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity 1998;8: 177–187.
36. Busch DH, Pilip IM, Vijh S, Pamer EG: Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity 1998;8:353–362.
37. Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL, Doherty PC: Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity 1998;8: 683–691.
38. Crawford F, Kozono H, White J, Marrack P, Kappler J: Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. Immunity 1998;8:675–682.
39. Boniface JJ, Rabinowitz JD, Wulfing C, et al.: Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. Immunity 1998;9:459–466.
40. Makela O, Karjalainen K: Inherited immunoglobulin idiotypes of the mouse. Immunol Rev 1977;34: 119–138.
41. Reth M, Hammerling GJ, Rajewsky K: Analysis of the repertoire of anti-NP antibodies in C57BL/6 mice by cell fusion. I. Characterization of antibody families in the primary and hyperimmune response. Eur J Immunol 1978;8: 393–400.
42. Jacob J, Kassir R, Kelsoe G: In situ studies of the primary immune response to (4-hydroxy-3- nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. J Exp Med 1991;173: 1165–1175.
43. Jacob J, Kelsoe G, Rajewsky K, Weiss U: Intraclonal generation of antibody mutants in germinal centres. Nature 1991;354: 389–392.
44. McHeyzer-Williams MG, Nossal GJ, Lalor PA: Molecular characterization of single memory B cells. Nature 1991;350:502–505.

45. McHeyzer-Williams MG, McLean MJ, Lalor PA, Nossal GJ: Antigen-driven B cell differentiation in vivo. J Exp Med 1993;178:295–307.
46. McHeyzer-Williams LJ, Cool M, McHeyzer-Williams MG: Antigen-specific B cell memory: expression and replenishment of a novel B220– memory B cell compartment. J Exp Med 2000;191: 1149–1166.
47. Allen D, Cumano A, Dildrop R, et al.: Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. Immunol Rev 1987;96:5–22.
48. Bothwell AL, Paskind M, Reth M, Imanishi-Kari T, Rajewsky K, Baltimore D: Heavy chain variable region contribution to the NPb family of antibodies: somatic mutation evident in a gamma 2a variable region. Cell 1981;24: 625–637.
49. Weiss U, Zoebelein R, Rajewsky K: Accumulation of somatic mutants in the B cell compartment after primary immunization with a T cell-dependent antigen. Eur J Immunol 1992;22:511–517.