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Antibody-Based Modulation of Humoral Immune Responses in Adults and Infants

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Antibody-Based Modulation of Humoral Immune Responses in Adults and Infants

By

Chelsey L. Goins

B.S., Saint Louis University, 2005

Advisor: Joshy Jacob, Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT: Antibody-Based Modulation of Humoral Immune Responses in Adults and Infants Chelsey L. Goins

Antibodies have powerful stimulatory abilities, particularly when combined with specific antigen in the form of immune complexes (ICs). These complexes can activate immune responses in such a way as to enhance both the speed and the magnitude of the response. Here we examine two instances in which ICs enhance humoral immune responses.

The reigning paradigm of immunologic memory has long been that secondary antibody responses are derived from memory B cells. However, this model lacks the flexibility necessary to survive in an ever-evolving world. We examined the origins of the secondary response, and in particular the role of ICs. ICs are formed naturally upon secondary antigen encounter, making them of particular interest in examining B cell activation at this time. We found that naïve B cells participate heavily in the secondary antibody response. This participation is mediated by ICs acting through Fc receptors.

While antibodies can be stimulatory, there are also instances of antibodies suppressing immune responses. This is particularly true in the case of maternally derived antibodies in newborns, which protect infants from infection during the early months of life but inhibit their ability to form antibody responses of their own. Since maternal antibodies are primarily IgG, which is known to suppress responses to particulate antigen while enhancing responses to soluble antigen, we investigated the effect of different immunogens on the infant immune response in the presence and absence of maternal antibodies. We found that maternal antibodies were able to enhance antibody responses to soluble antigens only. This enhanced response is short-lived, as maternal antibodies appear to block the formation of B cell memory, regardless of antigen type.

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Immunologic memory and vaccination

Human beings have evolved in a world filled with disease-causing microbes, and so have evolved a complex and sophisticated defense mechanism to protect themselves. The importance of this mechanism – the immune system – cannot be overstated. It allows for survival in a world where survival should be impossible. Indeed, when the immune system becomes non-functional, through disease or genetics, death from infection always follows (1). We simply cannot live in this world without the protection the immune system provides.

The main way the immune system effects this protection is through a phenomenon known as immunological memory. In this, cells of the immune system "remember" the pathogens they have seen, staying active but dormant and waiting to respond should these pathogens return. This is how immunity is developed – an infection once had will likely not be had again because the immune system will remember and be ready.

When a pathogen is first encountered it is the cells of the innate immune system that respond. These cells include macrophages, dendritic cells (DCs), neutrophils, and natural killer cells, among others (2). While these cells are able to respond quickly, they are not specific and therefore cannot recognize individual pathogens. Because of this they respond to every pathogen in a similar way – find it, eat it, kill it. It is a simple system, and one that is easy to evade. At the same time these cells also function as messengers, calling in the cells of the adaptive immune system.

The adaptive immune system is made up of T and B lymphocytes. Unlike cells of the innate immune system, these lymphocytes are specific, meaning each individual cell

recognizes only one pathogen. They also have the capacity for memory. Naïve B and T cells, which have not yet encountered their specific pathogen, must be activated before they can respond and form memory. This is a long and slow process, which is why initial pathogen encounter usually results in infection. The cells simply cannot respond quickly enough. Once activated, however, the adaptive cells are able to rapidly clear away infection. After the pathogen has been cleared, memory T and B cells remain. These cells wait, ready to strike immediately should the pathogen return. Because of this, secondary pathogenic encounters rarely result in infection, as the adaptive immune system is able to respond immediately to clear the pathogen. This is the very definition of immunological memory, a rapid and specific immune response to a pathogen that has been previously encountered (3).

Humans have manipulated the adaptive immune system's ability to form memory to enhance their own survival through vaccination. With vaccination, the initial encounter with a pathogen is not through infection, which results in illness and potential death, but through controlled administration of a non-harmful version of that pathogen (4). In this way, memory develops without the unpleasant effects of infection. Vaccination first became common through Edward Jenner's use of cowpox, or vaccinia, to protect against smallpox. Today vaccination is a common and necessary part of life; children receive vaccines against upwards of 15 pathogens (5), and continue receiving boosters throughout life. This has resulted in vastly improved quality of life and increased life expectancy. It is estimated that each year upwards of two million deaths are prevented through vaccination (6).

While vaccination mimics pathogen exposure through infection, its immune stimulating effects differ. Infection results in the activation of both T and B cells and the ultimate formation of T and B cell memory. T cells play an important role in immune defense. Vaccines, however, rely almost entirely on B cell responses (1). T cells may be activated, but it is the effector action of the B cells that provides vaccine-induced protection. All vaccines in use today function to stimulate B cells. Thus, to understand how vaccines work and how to develop better vaccines, it is important to understand how B cells are activated and how B cell memory forms.

Formation of B cell memory

B cells originate and develop in the bone marrow (1). From here, naïve B cells are released into the blood stream, where they pass through the circulatory and lymphatic systems, waiting to encounter antigen. Until this antigen encounter occurs, these cells are dormant, unable to produce antibody or respond to pathogens in any way. If antigen encounter never occurs, naïve B cells will eventually undergo programmed cell death (7). However, if antigen encounter occurs, the B cell rapidly undergoes selection and differentiation to become a powerful effector cell.

Primary antigen encounter

The primary foci of initial antigen encounters are the secondary lymphoid tissues, the spleen and the lymph nodes (LNs). While naïve B cells do pass through the bloodstream, they are unlikely to encounter the stimulation necessary for activation in so

vast an area. The secondary lymphoid organs act to concentrate the players in B cell activation, allowing very rare cells and antigens to congregate in a single location (8).

Activation of B lymphocytes requires at least two signals (9). The first is antigen binding by the B cell receptor. Binding of more than one B cell receptor on the same cell by a single antigen results in a much stronger signal, and therefore more rapid activation, than binding of one receptor alone. The second signal required for B cell activation is costimulation from $CD4^+$ helper T cells or complement. If the B cell receives only the first signal, antigen binding, it will enter into a state of anergy until it receives the necessary costimulation (10). Without costimulation, the cell will eventually die. This is a protective mechanism of peripheral tolerance, which prevents immune responses against self-antigen.

Costimulatory help from $CD4^+$ T helper (Th) cells is important in activating naïve B cells and determining their fate. After encountering an antigen, a B cell will internalize, process, and present the antigen on its MHC Class II molecules, which are upregulated upon antigen encounter (11). The B cell can then present this antigen to Th cells. Recognition of antigen presented by the naïve B cell stimulates the Th cell to deliver activating signals to the B cell. These signals are generally mediated by a variety of costimulatory molecules, including CD40-CD40L, ICOS-ICOSL, and OX40-OX40L (11, 12). Th cells also secrete cytokines such as IL-4, IL-6, and IFN-γ, which help direct B cell fate (13). There are also T cell independent mechanisms of B cell activation. These generally occur in response to large polysaccharide antigens, which crosslink B cell receptors so extensively that they do not require costimulation to become activated.

Following interactions with Th cells, a small subset of newly activated B cells migrates to the marginal zones of lymphoid organs and undergoes differentiation into short-lived plasma cells, beginning immediate production of antigen-specific antibody (14). These cells generally arise 7-10 days after initial antigen exposure and are responsible for the first wave of antibodies seen in response to immunization or infection. They produce primarily low-affinity IgM antibodies, as the plasma cells have not participated in the reactions necessary for isotype switching or affinity maturation. As their name implies, short-lived plasma cells live only a short time, having a half-life of approximately 5 days *in vivo* (15). They function solely to produce protective antibody as early as possible during an immune response, filling the gap while the majority of newly activated B cells participate in germinal center reactions.

Germinal center reactions

Germinal centers (GCs) are follicles within secondary lymphoid organs where naïve B cells are converted into mature, memory B cells. They arise 7-10 days following exposure to T-dependent antigens and the reactions that take place within last anywhere from one to several weeks (11). GCs form when newly activated B cells and a small number of Th cells travel along a gradient of CXCL12 from the T-B cell interface of the medulla to take residence in secondary lymphoid follicles within the cortex (16). While B cells form the majority of the cell population within the GC, 5-10% of cells are Th cells and approximately 1% are a special subgroup of dendritic cells known as follicular dendritic cells (FDCs), which capture and present the antigen necessary to drive the GC reaction (17).

GC morphology is related directly to the B cells it contains. When newly activated B cells enter GCs they undergo several changes to become centroblasts. Chief among these is downregulation of surface immunoglobulin (Ig). Centroblasts are also characterized by large size and expanded cytoplasm. The area of the GC in which they congregate is known as the 'dark zone,' as the morphology of the centroblasts creates an area of darkness in tissue sections of GCs (18). While in the dark zone, centroblasts undergo several rounds of rapid proliferation, with the ultimate goal of increasing antibody affinity through somatic hypermutation.

Somatic hypermutation is a process in which mutations are made in antibody heavy and light variable regions. These mutations are random and uncontrolled, meaning sometimes they will be advantageous, increasing the relative affinity of the antibody for its cognate antigen, and sometimes they will be deleterious, resulting in antibody that no longer recognizes antigen. The process begins when centroblasts proliferate, driven by T cell derived cytokines IL-2, IL-4, and IL-5 (19). Through multiple rounds of division, point mutations, substitutions, and deletions occur at random throughout the antibody variable regions. This process of mutation is unique because it occurs at a rate $10^5 – 10^6$ times higher than the mutation rate within other cells (20). The exact mechanism of this mutation, how it is driven and how it is targeted, remains poorly understood. This centroblast division cycle results in a number of newly created centrocytes, which express mutated Ig on their surface and require selection.

Selection of centrocytes occurs in the 'light zone' of the GC and is regulated by FDCs. These are specialized dendritic cells that capture antigen, usually in the form of antigen-antibody complexes, on their surface through Fc receptors and complement

receptors (21). FDCs create a pocket of concentrated antigen on which the newly mutated antigen receptors of centrocytes can be tested. Centrocytes require survival signals from FDCs, both in the form of antigen receptor stimulation and through various cell-cell interactions, including signals from the anti-apoptotic BAFF and the pro-proliferative IL-15 (17). Without these signals, the centrocyte will undergo apoptosis. Although FDCs create a pocket of antigen, antigen concentration is still limited in comparison to the number of centrocytes. Because of this, there is intense competition between centrocytes for stimulation from FDCs. Centrocytes whose somatic mutation has created higher affinity antibodies will have greater success in binding to FDC-presented antigen and receiving stimulatory signals, while centrocytes with lower affinity receptors will not receive necessary stimulation and will die (19). In this way, only antibody receptors with the highest affinity, those best able to bind antigen, will survive the GC selection process.

The final function of the GC is the creation of memory B cells. Following interactions with FDCs, centrocytes once again encounter Th cells. These cells also provide signals necessary for the centrocytes' survival, including antigen binding through the B cell's MHC Class II molecule and CD40 engagement (19). Signal from CD40L on the Th cell not only promotes centrocyte survival, but is also responsible for the induction of isotype switching. Until this point, all B cells express antibodies of IgM or IgD isotype (11). CD40L stimulation signals to the cell that it is time to change its heavy chain gene expression. Following class switch, centrocytes differentiate into mature B cells and migrate out of the GC, signaling the completion of the GC reaction.

B cell memory

Upon exiting the GC, centrocytes differentiate into two populations of mature B cells, some becoming memory B cells and some undergoing transformation into the terminally differentiated state of a plasma cell. Unlike the PCs formed prior to the GC reaction, GC-derived PCs are long-lived. After exiting the GC they migrate to the bone marrow, where they take up residency and receive survival signals from the surrounding microenvironment (22). These cells essentially function as antibody factories, secreting large amounts of antibody for the entirety of their lives. This antibody circulates through the body and provides immediate protection from any invading pathogens.

Long-lived PCs can produce up to 10,000 antibody molecules per second (23). In order to maintain such a high production rate, differentiation into PCs involves several physiological changes. PCs have large, irregularly shaped nuclei and greatly enlarged cytoplasms. The PC cytoplasm is devoted almost entirely to protein processing and packaging, including a large Golgi apparatus and endoplasmic reticulum and numerous mitochondria. These changes allow the PC to maintain a high level of antibody production. Transcription of antibody heavy and light chain genes is increased in PCs, and RNA processing is altered to produce secreted rather than membrane bound forms of antibody (24). Because PCs function only to secrete antibody, they have very low levels of Ig on their surface. Indeed PCs produce very few proteins other than antibodies. They are most easily differentiated from other cell types by their unusual morphology and high expression of CD138 (syndecan-1).

The other cells produced through the GC reaction are memory B cells. These cells comprise the majority of GC-derived B cells. Following GC reactions, memory B

cells migrate to the spleen where they enter a state of dormancy. During this time they neither produce antibody nor replicate to any great extent (11). They remain here and in this state until they reencounter their cognate antigen, at which time they will activate and differentiate into PCs.

Unlike PCs, resting memory B cells are capable of proliferation and further differentiation. Memory B cells also retain the general morphology of a naïve B cell, though they are slightly larger. Besides class switched antibody, there are few membrane molecules that distinguish memory from naïve B cells. Memory B cells are generally considered to be $CD19^+$ $CD27^+$ (25). They also express higher levels of CD80, CD86, and MHC molecules (1). Classically, memory B cells have been defined as being IgM^-, having undergone class switching as part of their GC reaction. However, this is an error: nearly 40% of memory B cells express IgM (25-27). Some have cast aspersions on the identification of these IgM^+ $CD27^+$ B cells as true memory (28, 29), suggesting they are derived independently of GCs and only respond to T independent antigens. However, these studies have been refuted (30) and IgM^+ memory B cells have become accepted as a part of the immune repertoire.

While PCs receive their survival signals from the bone marrow microenvironment, memory B cells appear to need no such stimulus. Early experiments suggested that antigen persistence was essential for memory B cell survival (31), but it is now known that memory B cells do not require antigenic stimulus and persist for upwards of 70 years in the absence of specific antigen (32). It is still possible that memory B cells receive survival signals from elsewhere, particularly those that reside in the bone marrow (33).

It is not well understood how the fate of a mature B cell is determined. Some believe the relative affinity of the antibody expressed by the B cell is the primary determinant, as PCs in the bone marrow have very high affinity but fewer overall mutations than memory B cells (34). Using transgenic mice in which cellular Igs recognized the same antigen but at varying affinities, Loren Erickson and his group showed that the affinity of the naïve B cell receptor affects ultimate B cell fate (35). Naïve B cells with the highest affinity antigen receptors are recruited into the pool of short-lived PCs, whereas those with the lowest affinity undergo GC reactions such as affinity maturation to become memory B cells. It is the group between these, naïve B cells with moderate natural affinity for antigen, that seed the bone marrow population of long-lived PCs.

Secondary antibody responses

Unlike long-lived PCs, which continuously produce a steady-state amount of antibody, memory B cells remain dormant until they reencounter their specific antigen. When antigen from a primary immune response is reintroduced, usually through repeat pathogen exposure, memory B cells are activated and begin producing antibody. The subsequent antibody response occurs rapidly, producing an excess of antibodies to neutralize the invading pathogen. This response is the ultimate goal of humoral immunological memory and provides the protection necessary to prevent repeat infections.

Memory B cell activation occurs rapidly upon pathogen encounter. Because memory B cells received activating signals during the primary antigen encounter and GC

reaction, they can activate rapidly, with minimal or no help (11, 36). Upon antigen encounter memory B cells awaken from a resting state and immediately begin to proliferate, creating large numbers of daughter cells with identical antigen receptors, all capable of producing antibody against the invading pathogen. During expansion the memory B cells differentiate into plasma cells, downregulating surface molecules such as antigen receptors, MHC, and costimulatory molecules while upregulating production of secreted antibody molecules. The result is a massive number of antigen-specific plasma cells producing large amounts of antibody.

As discussed above, primary antibody responses are characterized by a lengthy lag followed by very low antibody production, meaning the response is very rarely protective. In contrast, secondary antibody responses occur very rapidly, reaching antibody titers several logs higher than primary responses within 3-4 days after antigen encounter (37). This antibody varies from that found in the primary response in that much of it has higher affinity for antigen, coming from cells that have undergone GC reactions. These antibodies also carry different heavy chain genes, and these different isotypes allow them to perform a number of effector functions that aid in the removal of infectious agents.

Unlike other cells of the immune system, B cells mediate their effector functions almost exclusively through the production of antibodies. Depending on isotype, secreted antibody can perform a variety of functions. These include activating cells through Fc receptor binding, activating complement components, neutralizing invading pathogens by blocking cell and receptor interactions, and opsonizing pathogens to aid in removal through phagocytosis (38). Though B cells themselves can act as antigen presenting cells

to activate T cells, this function is secondary to their antibody production. In particular, the speed and magnitude of the secondary antibody response is key in ensuring repeat pathogen encounters do not result in repeat infection.

Current dogma suggests that the secondary antibody response is entirely the result of the activation of memory cells (1, 11, 39). What cell other than a memory cell could act so quickly and produce antibodies at such high levels? While this may seem logical initially, there are a number of discrepancies between what is thought to be true and what has been observed to be true. Additionally, a major flaw of this theory is simply its lack of variability. Pathogens change and adapt, and so the immune response should be allowed to change and adapt accordingly. Experimental evidence has shown a number of instances where memory cells alone cannot possibly explain the secondary antibody response.

One such instance is a phenomenon known as repertoire shift. This refers to the often-observed change in antibody repertoires following secondary antigen encounter. Repertoire shift has been observed against numerous antigens, including influenza hemagglutanin (40), rheumatoid factor (41), and respiratory syncticial virus (42). The best characterized occurrence of repertoire shift is in the immune response to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) (43). The majority of antibodies produced upon immunization of Igh^b (C57BL/6) mice with NP are encoded by V_H186.2-DFL6.1-J_H2 Ig heavy chain rearrangements and utilize the λ_1 light chain (44). However, only 20% of antibodies produced during the secondary response contain this specific rearrangement (45). In addition, many of the novel antibodies found during the secondary response use the κ light chain, which is virtually undetectable during the primary response. Other

hapten immunization models, including 2-phenyl oxazolone and p-azophenylarsonate (46-48), demonstrate the same Ig repertoire shift following secondary immunization. The current model for B cell memory and secondary antibody responses does not account for this, as all antibodies produced during secondary responses should come from cells activated during primary responses, and therefore be identical to those antibodies.

One theory to explain repertoire shift is that different B cell subsets are responsible for the primary antibody response and B cell memory, and so different antibodies are produced by these subsets (49). Since the primary response comes from short-lived PCs and the secondary response from B cells that have undergone a GC reaction, it seems obvious that the antibodies produced would differ between primary and secondary responses. However, the change seen in repertoire shift is between antibodies present at memory time points - those being produced by long-lived PCs that have undergone GC reactions - and antibodies from the secondary response. This means that any change in antibody production or any new subset that is producing antibodies must arise after secondary antigen encounter. Additionally, many of the new antibodies seen during secondary responses have not undergone somatic hypermutation (45), meaning they cannot be explained by GC-altered memory B cells.

A possible explanation for repertoire-shifted antibodies may lay in observations made in our laboratory regarding the cellular composition of secondary responses. While investigating a mouse model in which all GC-derived B cells are permanently marked with yellow fluorescent protein (YFP), we observed that almost 75% of PCs participating in secondary responses did not originate in the GC (50). While some cells can develop in an extra-GC environment (51), these make up no more than 20% of the memory B cell

pool, and thus do not explain the large number of extra-GC-derived PCs observed. These cells had not undergone hypermutation and produced antibody of lower affinity than GC-derived PCs. In many ways, these cells resembled naïve B cells.

As discussed earlier, naïve B cells are characterized primarily by their extremely slow responses to antigen. Were naïve B cells to be activated following secondary antigen encounter, they would be burdened by this response time and would not yet have begun producing antibody by day 4, when these extra-GC-derived PCs have been observed. However, if naïve B cells were activated in an alternate way, one which would speed their response time, they might explain both this population of cells and the phenomenon of repertoire shift. Naïve B cells raised under such conditions could also add diversity to an otherwise static memory pool.

Immune complex mediated B cell activation

One powerful method for rapid activation of naïve B cells involves using complexes of antigen and specific antibody, immune complexes (ICs), instead of antigen alone. ICs have powerful stimulating effects on the immune system, including activation of T and B lymphocytes and enhancement of innate immune responses (52). For years researchers have been experimenting with the idea of using ICs as therapies or vaccinations. As early as 1913 Nobel Prize winner Emil von Behring had begun experimenting with diphtheria vaccines made of toxin and antitoxin conjugates (53). Though the beginning of World War I led these experiments to an abrupt end, the idea of using antibodies to stimulate immune responses remained.

It was not until G. G. Klaus's seminal studies in the late 1970's and early 1980's that the powerful stimulating capabilities of antibodies were truly understood. In a series of elegant experiments (54-58), Klaus not only showed that were ICs stimulatory, but he also defined the parameters under which this stimulation occurred – antibody isotype requirements, cellular requirements, and speed of B cell priming. According to Klaus, IgG complexes, particularly those containing IgG_2, could stimulate B cell responses up to 100x greater than antigen. These IC-primed B cells reacted much more rapidly than those primed by antigen alone or in alum. With IC priming, antibody-producing B cells reached substantial numbers by 5-7 days, as compared to 2-3 weeks, and high affinity memory cells began appearing as early as 6 days post-priming.

Interestingly, Klaus found that IgM containing complexes did not prime B cell responses and concluded that only IgG complexes could stimulate immune responses (55). Soon after it would be shown that the stimulatory or suppressive effect of an antibody depended not only on the isotype of the antibody, but also on the antigen to which it was bound (59). Klaus used a soluble antigen, DNP-KLH, in his IgG IC studies. If he had chosen a different antigen he might have seen a different response. IgG is stimulatory when complexed with soluble antigens, but suppressive when complexed to particulate antigens. IgM, on the other hand, can stimulate as well as IgG, but only when complexed with particulate antigen (60). Klaus used a soluble antigen in his studies, and so did not observe the powerful stimulating abilities inherent in IgM ICs.

After ICs were shown to be stimulatory, a plethora of work was done elucidating their effects on primary B cell responses. Researchers specifically wanted to know how this stimulation occurred. Klaus had theorized that the ICs worked by concentrating

antigen in lymphoid follicles, inducing the rapid formation of germinal centers (61). This theory was later demonstrated to be true by evidence showing IC immunization led to accelerated germinal center formation in the spleen (62). It was also shown that IgG-mediated enhancement was dependent upon presence of Fcγ receptors but independent of complement (63, 64). The mechanism of IgM-mediated enhancement is not yet, known, but it has been shown to depend on the presence of complement and complement receptors (65, 66).

Since the discovery of the enhancing effect of ICs, several scientists have returned to von Behring's idea of complexed vaccines (52). In 1977 William Houston and Richard Spertzel showed that a vaccine against Venezuelan equine encephalitis given in a complex resulted in enhanced primary responses and more rapid secondary responses (67). More recently, a variety of studies in animals using immune complex-based vaccines against Newcastle disease virus, simian virus 5 paramyxovirus, and SIV, among others, have shown that antigen given in complex results in a faster, stronger immune response against a greater number of epitopes (68-70). In all cases, these vaccines were IgG-based, as in Klaus's original studies. Recent work on the fungal pathogen *Cryptococcus neoformans*, however, has shown that IgM-based vaccines also have enhanced stimulatory effects (71). These preliminary studies, coupled with what is known about the immune-enhancing effects of ICs, suggest a potential future for IC-based vaccines.

While most studies examining the effects of ICs have looked at primary antibody responses, there is reason to suspect a similar role for ICs during the secondary response. ICs form naturally following secondary antigen encounter between the newly introduced

antigen and the specific antibody produced by long-lived plasma cells. While it seems unlikely that ICs have a great effect on memory B cells, naïve B cells are still present and could still be activated by the newly formed ICs. Indeed, the magnitude and kinetics of naïve B cell responses to ICs is remarkably similar to the antibody responses seen upon secondary antigen encounter. If naïve B cells were activated by ICs early in the secondary response, this could explain several inconsistencies between what is thought to be true about secondary responses and what is actually known to occur. Specifically, IC-activated naïve B cells could account for the antigen diversity of the secondary response, as well as the large population of non-GC derived plasma cells that appears at this time. With this issue in mind, we sought to examine the role of ICs in the secondary response, and specifically to look at the potential activation of naïve B cells at this time. Results from this investigation can be found in Chapter 2.

Maternally derived antibodies

One of the greatest challenges in the life of any mammal is surviving the initial months after birth. Mortality is great at this time because the infant must rely on others for protection, both physically and immunologically. The intricate immune network that allows adults to survive has not yet fully developed, and the pathogens and vaccines that will provide immunological memory have not yet been encountered. Because of this, infants must receive their immunologic protection in another form – namely antibodies from their mother.

The infant immune system

At birth humans have immune systems that are underdeveloped and poorly capable of handling the burden of infection. At least part of this is due to the strange immunologic environment of gestation (72). In order to prevent the mother's immune system from attacking and rejecting the fetus due to its foreign antigens, the immunologic profile of the uterus differs from that of other tissues in a normal, healthy adult woman. The most substantial change is the creation of a Th2-biased environment within the uterus (73). This change is driven primarily by the placental tissue, which increases production of TGFβ, progesterone, and prostoglandin, and fails to ligate Toll-like receptors (74, 75). This helps protect the fetus from rejection by abortifacient Th1 cells, and causes a decreased sensitivity to Fas-promoting cytokines such as IFN-γ, which would increase sensitivity to apoptosis (76). There is also an absence of human leukocyte antigens, which would promote fetal rejection, in areas of placental contact (72).

The result of the fetus developing in this protective environment during pregnancy is that it cannot protect itself from pathogens after birth. The newborn immune system is skewed towards a Th2 response (73, 77). These responses are directed at large, extracellular antigens, such as parasites and allergens. They are specifically not responsive to viruses and bacteria, which make up the majority of pathogens an infant will encounter. While Th2 cells are responsible for stimulating humoral responses, the antibodies produced under these conditions are primarily IgE. Additionally, the Th2 produced cytokine IL-10 acts to depress immune responses, particularly the development of macrophage phagocytic functions (78). Over the course of the first year of life, numerous viral and bacterial encounters will skew the balance of the immune system to a

Th1 response. Some have suggested that if this does not occur, perhaps due to increased hygiene leading to decreased pathogen encounters, that the immune response will stay skewed towards Th2 responses and result in a greater incidence of allergies and decreased overall health (79).

Vaccination does little in the early months of life to enhance immunologic protection. Because of the Th2 bias, typical immune stimulatory adjuvants such as alum cannot be used, as they enhance Th2 skewing (80). The only adjuvant that has had success in infant vaccines are those containing CpG motifs (81). With adjuvants or without, vaccines very rarely stimulate strong humoral immune responses in these early months. Newborns are capable of raising weak IgG and IgM responses to pathogens. This is true even in the prenatal period, as well as for babies born prematurely (82). However, vaccines given in newborns generally raise very few antibodies. For example, more than 50% of babies receiving an oral polio vaccine (OPV) at birth have no response at all (83). With the exception of the tuberculosis vaccine BCG, all infant vaccines require several doses to elicit protection when administered before six months of age (84).

At least part of the response – or lack of response – to vaccines in newborns can be attributed to the underdeveloped nature of the immune system at birth. This includes downregulated levels of CD21/CR2 on B cells, impaired ligation of CD40, poor responsiveness to stimulatory factors such as IFN-γ, and difficulty forming GC reactions, leading to a predominance of low affinity IgM when antibody is produced (84). These factors combine to create an immune system that is only weakly able to respond to any form of stimulation. This state continues for most of the first year of life.

As much of the research in infant immune responses is performed in mice, it is important to consider the similarities and differences between mouse and human immune development. In mice the immaturity of the neonatal immune system is even more pronounced than in humans. Several developments that occur during the last few months of human gestation do not occur in mice until after birth. These include the development of secondary lymphoid organs, the ability to form T cell independent immune responses, and the upregulation of terminal deoxynucleotidyl transferase, which is necessary for the development of B and T cell antigen receptors (8, 85, 86). Because of this delay in immune maturation, the murine equivalent of the human neonatal period does not begin until more than one week after birth. After birth, however, mice develop much more rapidly than humans, reaching a state of immune competence by five weeks of age (77). Thus the window for investigating infant immune responses in mice is limited. For both humans and mice, however, the period after birth is a time when they must rely on passively acquired immunologic protection in order to survive.

Transfer of maternal antibodies

Because the infant immune system is underdeveloped at birth, infants must have another source of immunologic protection to survive their earliest months. This protection comes in the form of antibodies passed from mother to child during pregnancy and breastfeeding. These antibodies reflect the mother's repertoire and offer protection from pathogens the mother has encountered previously, either through infection or vaccination.

Depending on the species, maternal antibody (MatAb) transfer can happen during pregnancy, after birth during breastfeeding, or both. In humans IgG is passed transplacentally, while only IgA can be absorbed from the breast milk (87). In mice some antibodies are transferred during pregnancy, but the majority of MatAbs come after birth, through colostrums and then breastmilk (88). These antibodies include both IgG and IgA. For horses, cows, and other ruminants, no antibody is transferred during pregnancy – all MatAbs come after birth through colostrum. A delay in breastfeeding for even a few hours in livestock can lead to a severely deadly agammaglobulinemia (89).

The restricted passage of antibodies in humans is due to the mechanism of transfer. IgG crosses the placenta through neonatal Fc receptors (FcRn) found on placental syncytiotrophoblasts. Starting at approximately 17 weeks gestation FcRns appear and begin transferring antibody, though substantial transfer does not occur until week 30 (90). These receptors preferentially transfer IgG1 and IgG3 at a somewhat variable rate of 0.41% per hour (fetal/maternal ratio) for the remaining prenatal period (91). At birth, infant IgG concentrations are often higher than those found in the mother. Infants born prematurely are particularly immunocompromised, as they have low or no levels of MatAbs in addition to underdeveloped immune systems. The antibodies transferred transplacentally are entirely IgG, as this is the only isotype that can bind the FcRn (92). Transfer in this manner allows the infant to receive a representative sampling of its mother's immune repertoire. The limited number of FcRns, however, causes some level of competition for binding sites, meaning antibody doses are skewed towards the most commonly found antibodies (90). If the mother experiences an infection or receives

a vaccination during pregnancy, a larger number of antibodies specific for these antigens will be transferred.

Antibodies transferred after birth arrive through breastfeeding. Due to the lack of Fc receptors in the breast tissue in humans, antibodies transferred through breast milk must be secreted (89). This limits MatAb transfer to IgA. Dimeric IgA is able to bind to poly-Ig receptors on the baso-lateral side of breast epithelial cells, which causes the IgA to be endocytosed and transferred to the lumen where it is secreted into breast milk (93). Here it is ingested by the infant and taken to the gut musoca, where it remains and aids in protecting the infant from orally transmitted pathogens, as well as helping in the establishment of a normal intestinal flora. No antibody transferred via breast milk is taken into the bloodstream in humans. In other mammals, this is not the case, as IgG is transferred through breast milk and can be absorbed by Fc receptors found in the small intestines. Though small amounts of IgG are transferred in human breast milk, human infants lack the receptors necessary for absorption (92).

Once MatAbs reach the infant, they offer passive immunity to any pathogens the mother has encountered. Historically this phenomenon was first noticed in 1846 during an outbreak of measles in the Faroe Islands, when babies born to mothers who had survived infection did not become infected themselves. It gained additional attention in 1879, when babies born to women who had received Edward Jenner's vaccinia vaccine were immune, although temporarily, to smallpox (90). Finally, in 1892, Paul Ehrlich demonstrated conclusively the transmission of maternal antibodies through infant breast milk. Further studies showed this antibody to be protective, and that breastfed infants almost always fared better than artificially fed infants during infections (94).

Widespread acceptance of the phenomenon of maternally derived protection from infection has led to many campaigns for maternal vaccination. In areas where a specific disease is endemic, or at times when outbreaks are occurring, many believe it is prudent to immunize the mother for the future protection of the child. This has been occurring since 1938, when mothers were immunized with a whole cell pertussis vaccine to protect their babies from whooping cough (90). It is now quite common for women to receive vaccinations during pregnancy. This is particularly true for influenza (95). The most common infecting strains of influenza change each year, so a mother's previous influenza immunity might not provide sufficient protection for her infant. The disease is particularly dangerous in the very young. In order to ensure the newborn is protected against the newest influenza strains, updated vaccines are often given during pregnancy.

Women now routinely receive a number of immunizations during pregnancy, both to protect themselves from infection during pregnancy and to increase the transfer of these antibodies to their fetus. The diseases for which women are most often immunized include tetanus, meningitis, poliovirus, and hepatitis A and B (90, 96). The specific vaccines each woman receives depends on her individual risk level. Because of competition for FcRn, maternal vaccination during pregnancy must be utilized judiciously. If too many antibody responses are raised in the mother, useful antibodies from older vaccinations or infections may not be transferred due to competition for receptor binding and therefore not represented in the infant's repertoire. This results in decreased protection for the fetus.

Maternal suppression of infant immune responses

There is a downside to the protection offered by maternal antibodies – suppression of the infant immune response. As discussed earlier, antibodies can have extraordinary power to stimulate immune responses, but they can also prevent these responses from developing. In the case of MatAbs, very often the antibodies meant to protect and aid the infant will prevent its immune system from developing its own responses. This is particularly true in the case of infant vaccination, when immune responses to the vaccine can be suppressed or even completely inhibited by MatAbs.

Maternal suppression of infant vaccine responses is very poorly understood. While it has been observed on several occasions, much of the data is conflicting, with studies of the same vaccine offering differing results (97). Beyond titer of MatAbs at the time of immunization, there seems to be no theme to when a response might be inhibited and when it might not. Despite the conflicting nature of some MatAb inhibition data, the phenomenon is widely accepted. MatAb blocking of vaccine responses was first characterized, and is most often studied, in live virus vaccinations. This includes oral polio, oral rotavirus, and influenza (98-100). However, MatAb blocking also occurs in cases of vaccination with killed virus and subunit vaccines, including tetanus and diptheria (101, 102), hepatitis A (103), *Haemophilus influenzae* type b (HiB) (104), and pertussis (105), among others.

Perhaps the best-studied case of MatAb inhibition of immune responses to vaccine involves the response to measles vaccine. Measles is the leading cause of vaccine-preventable death in infants (106). Despite the pressing need for increased

measles immunity in infants, vaccination is delayed until 12 months of age because of MatAb inhibition (107). MatAbs provide protection from infection for some of this time, but begin to wane around 6 months after birth. There is a period of time after MatAbs titers have decreased to a non-protective level but before measles vaccine immunity can be induced in which the infant is highly susceptible to measles infection. The vast majority of deaths due to measles and measles-related complications occurs at this time (108). The timing of this window is not precise, so vaccine attempts cannot be made earlier than recommended. Additionally, using higher doses of vaccine antigen results in high levels of mortality, meaning measles immunity cannot be induced simply by overwhelming the immune system with antigen (109). This time period is one of the many reasons why the need to understand and overcome vaccine response inhibition by MatAbs is so high.

There are a number of different theories to explain how and why maternal suppression of vaccine responses occurs. The most common of these is that MatAbs simply bind and remove all vaccine antigen so none remains to stimulate the infant immune system (110). This is supported by several cases in which lower levels of maternal antibody or higher vaccine results in overcoming suppression (97, 111, 112). However, increasing vaccine doses leads to unpredictable and unnecessary death, so this strategy is not often employed.

While removal of vaccine antigen certainly plays a role in maternal inhibition, the cause is likely much more complicated than this. Importantly, T cell responses are not affected by MatAbs, suggesting sufficient antigen must remain to support their activation (113, 114). This also points to a more B cell-centric suppressive mechanism. As

discussed previously, antibodies are powerful regulators of B cell responses, particularly when complexed with specific antigen. While these complexes are often stimulatory, under the right conditions, complexed with the right antigens, they are excellent suppressors of response. In studies of IgG complexed with sheep red blood cells, it was shown that these ICs greatly downregulate antibody responses, often to less than 1% of the control response (59). However, much like the suppression mediated by MatAbs, these ICs have little to no effect on T cell responses (115).

The precise mechanism of IgG-mediated suppression of immune responses is unknown. The effect is known to be dose-dependent, though even very small doses of IgG will result in some level of suppression (116). Much work investigating mechanisms of IgG-mediated suppression has focused on the inhibitory Fc receptor found on B cells, FcγRIIB. It is known that IgG antibodies lacking the Fc region are poor suppressors, and the same is true for deglycosylated antibodies, which cannot bind FcRs (117). It has also been shown that blocking FcγRIIB results in reversal of suppression (118). These points seem to strongly suggest crosslinking of the inhibitory receptor as the mechanism for IgG-mediated suppression. However, studies in FcγRIIB have shown that effective IgG-mediated suppression occurs in the receptor's absence (115).

The most likely mechanism for IgG-mediated suppression, including MatAb suppression, is epitope masking. Several pieces of evidence support this, including the need for antibodies to be high affinity in order to suppress. Additionally, non-IgG antibodies, which cannot bind FcγRIIB, are able to suppress responses (119). Epitope masking would not interfere with processing and presentation of antigen, meaning T cell responses would be unaffected. This would also explain why IgG-suppression,

particularly as seen in studies of infant vaccination, would be dose dependent – as antigen levels rise or antibody levels fall, more epitopes would be left unmasked and available to stimulate B cell responses.

Regardless of the mechanism of suppression, IgG is known to only suppress immune responses to particulate antigens, while enhancing responses to soluble antigens. This is explored further in Chapter 2. If MatAb suppression acts in a manner similar to other IgG-mediated suppression, it will only occur in the presence of particulate antigens. Indeed most, if not all, vaccines given to infants are particulate. Those in which MatAb suppression have been noted, including influenza, polio, measles, hepatitis A, and HiB, all contain particulate antigen. It seems, then, that if infant vaccination were done using soluble antigens, which form stimulatory complexes in the presence of specific IgG, the result would be enhancement, rather than suppression of responses. In Chapter 3 we explore this theory further, investigating the effects of using different types of both soluble and particulate antigens in immunizations in young mice containing antigen specific MatAbs.

Chapter 2

Immune complex-mediated enhancement of secondary antibody responses

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Abstract

Immunological memory is a hallmark of the vertebrate immune system. The first antigenic exposure leads to a slow and modest immune response while repeat exposure, even many years later, leads to a rapid and exaggerated response that is 2-3 orders of magnitude greater than the primary. In the case of humoral immunity, the increased efficacy of recall responses is due to the production of amplified levels of antigen-specific antibody, as well as the accelerated kinetics of their production. Current dogma suggests that this is due to selective activation of long-lived antigen-specific memory B cells. A downside of restricting secondary responses solely to memory cells is that the repertoire of the memory B cell pool remains static while pathogens continue to evolve. Here we propose that during secondary responses naïve, antigen-specific B cells participate alongside memory cells. We show that immune complexes formed in vivo between the antigen and pre-existing antibodies from the primary response, activate these naive B cells, inducing them to respond with accelerated kinetics and increased magnitude. Thus the continued recruitment of new B cell clones following each antigenic exposure enables the immune system to stay abreast of rapidly changing pathogens.

Introduction

Unlike primary antibody responses, which occur after an extended lag, too late to prevent infection, secondary responses occur rapidly following antigen encounter. Current understanding of antibody responses is that antibodies formed during the secondary response come from memory B cells that differentiate into secondary plasma cells (1, 39). These are cells that are activated during the primary response and continue to persist in the host for a lifetime. Their expressed Ig have been altered through somatic hypermutation and isotype switching but still bear the antigen specificity of their progenitor (11, 120). If this were the case, the repertoire of antibodies responding to a particular antigen would remain static. The cells activated upon primary exposure would be the source of all subsequent antibody, meaning the antibodies seen years later would be virtually identical to those seen early in memory. This would be disadvantageous to the host especially since the pathogens continue to evolve. Interestingly, antibody repertoires of responding B cells change with time and repeated antigen exposure (48). The antibodies responding following a third or fourth antigen exposure are not identical to those responding to a primary or secondary exposure. This has been shown to be true in a number of systems, including the haptens (4-hydroxy-3-nitrophenyl)acetyl (NP) and 2-phenyl oxazolone (46, 47), as well as for respiratory syncytial virus (42), influenza hemagglutanin (40), and rheumatoid factor (41).

Recently our laboratory has shown that a large portion of the secondary antibody response comes from non-germinal center derived memory B cells (50). These cells were antigen-specific, and their expressed IgV regions lacked somatic hypermutation. While a small component of B cell memory comes from extra-germinal center B cells (10), these

cells are not numerous enough to account for the observed population. One possible explanation for this population is that some or all of these non-germinal center-derived cells are naïve B cells that have only been activated upon secondary antigen exposure. If this were true, that a large portion of secondary antibody responses come from naïve cells, it would explain how and why antibody repertoires change upon each repeat antigen exposure.

During the primary response, naïve B cell differentiation and antibody production occur several days after antigen encounter. In contrast, following secondary antigenic exposure B cells expand with a shortened lag phase and produce larger quantities of antibodies. The difference between the primary and secondary exposures is the presence of memory B cells and pre-existing antigen-specific antibodies. These antibodies can form immune complexes (ICs) with the incoming antigen and it is known that ICs can induce the production of higher antibody titers than antigen alone (56). One possible mechanism of IC-mediated enhancement is the activation of complement cascade. ICs, particularly those containing the antibody isotypes IgG2a and IgG3, are able to activate the classical complement pathway (121). As the complement receptor CD21 is part of the B cell co-receptor complex, this could lead to enhanced B cell activation. Additionally, ICs are able to bind to a variety of cell types, particularly dendritic cells (DCs), through IgG Fc receptors (FcγR). For DCs, engagement of FcγRs leads to cell activation, which results in enhanced antigen presentation and increased expression of costimulatory molecules (122). These effects lead to more efficient B and T cell activation.

Here we propose that the shortened lag phase and exaggerated antibody production characteristic of secondary responses is due to IC-mediated enhancement of naïve and memory antigen-specific B cells. We show that ICs are able to activate naive B cells with accelerated kinetics, that naive B cells are activated early during secondary responses, and that this activation is dependent on activating FcγR engagement.

Materials and Methods

Mice and immunizations

Animals were housed in an American Association of Laboratory Animal Care-accredited facility under specific pathogen-free conditions at the Emory University Vaccine Research Center. We purchased FcR$\gamma^{-/-}$ mice from Taconic Farms (Albany, NY) and C3$^{-/-}$ mice from The Jackson Laboratory (Bar Harbor, Maine). All other studies were performed using C57BL/6 mice (Charles River Laboratories, Wilmington, MA). The hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) coupled to chicken gamma globulin (CGG) (Biosearch Technologies, Navato, CA) (NPCG) was used for these studies. Primary immune responses were induced by i.p. injections of 50μg of alum-precipitated NPCG, CGG, or OVA along with 250ng Pertussis Toxin (List Biological Laboratories, Campbell, CA). Secondary responses were induced by tail-vein injection of 20μg soluble NPCG. In experiments where immune complexes were used for immunization, these complexes were made *in vitro* by incubating 25μg NPCG with 50μg NP-specific monoclonal antibody (pEVCHγ1) at 37°C for two hours and were administered i.p. For immune complex blockade experiments, 10μg each of plasmids encoding the human CD32A-Ig R and H isoform DNA was mixed and given i.v. in 1.6ml sterile PBS 2 days prior to secondary immunization (123). Human CD32A-Ig is capable of binding mouse IgG subtypes. For depletion of complement, mice received 5μg Cobra Venom Factor (Sigma-Aldrich) i.p. at 28, 24, and 4 hours prior to secondary immunization as described (124). Emory University's Institutional Animal Care and Use Committee approved all studies.

Adoptive transfers

Single cell spleen suspensions of TcR transgenic OT-II mice (on C57BL6 background) were RBC-lysed, washed, and resuspended in PBS. 1-2x10^6 purified TCRβ5.1/5.2$^+$ CD4 T cells were administered to Ly5.1 congenic mice in the tail vein one day prior to immunization.

ELISA

Serum NP or CGG Ab titers were measured by modification of an ELISA assay. Briefly, 96-well microplates were coated with a solution of 20μg/mL NP-BSA (Biosearch Technologies) or 20μg/mL CGG (Sigma-Alderich) in PBS overnight at 4°C. Plates were blocked for 1h at 37°C using a solution of PBS containing 4% non-fat dry milk (Bio-Rad, Hercules, CA). Serum samples were applied and allowed to react at room temperature for 1.5h. Anti-mouse antibodies with specificity for IgG or IgM isotypes coupled to horseradish peroxidase (HRP) (Southern Biotech, Birmingham) were applied and allowed to incubate 1.5h at room temperature. Plates were developed with TMB substrate and read at 450nm absorbance. Values were compared to known dilutions of IgG or IgM to calculate Ab concentrations. An ELISA for complement component C3 was performed as directed using a mouse C3 ELISA kit (Immunology Consultants Laboratory, Newberg, OR).

ELISPOT assay

ELISPOT assays were performed as described (125) with the exception that 96-well nitrocellulose plates (Millipore) were coated overnight with 20μg/ml NP-BSA in

100µl PBS. Spots were visualized using an ELISPOT reader (Cellulara Technologies, Ltd., Cleveland, OH) and counted manually.

Statistics

Unpaired, two-tailed Student's *t* test was used to generate all statistical values except where otherwise indicated. For statistical designations, * denotes $p<0.05$; ** denotes $p<0.01$; *** denotes $p<0.001$.

Results

ICs enhance immune responses in naïve animals

Previous reports have identified enhanced immune responses following immunization either with ICs or with specific antibody administered passively prior to immunization (62, 126). To revisit these results we immunized naive mice with ICs formed from NPCG and a monoclonal anti-NP antibody. Four days following immunization we examined their sera for anti-CGG antibody levels (Figure 1a) and splenocytes for the development of NP-specific antibody-secreting cells by ELISPOT (Figure 1b). Mice receiving ICs had anti-CGG levels of 5416±1484 ng/ml; this was significantly greater ($p=0.0043$) than that observed in mice immunized with NPCG/alum (226±52 ng/ml). Similarly, IC immunized naïve mice exhibited significantly greater ($p=0.0006$) numbers of NP-specific antibody forming cells that those immunized with NPCG/alum. From these data we conclude that ICs are able to stimulate antigen-inexperienced cells in such a way as to produce a more rapid and efficient immune response than antigen alone.

ICs stimulate secondary responses through FcγR binding, not complement activation

Having confirmed the potent stimulating capabilities of ICs, we next sought to determine the extent to which ICs participate in the generation of secondary antibody responses, as ICs form naturally *in vivo* following secondary immunization. To determine the role of ICs in the secondary response we targeted two of the main activating pathways used by ICs, FcγR binding and complement activation.

ICs exert their potent stimulating effect on a variety of cell types by binding to FcγRs. To determine if this plays a role in the generation of secondary antibody responses, we blocked ICs from binding to FcγRs by using CD32-Ig, a recombinant soluble FcγR dimer that binds the Fc regions of ICs with higher avidity than cell-surface FcγRs (127, 128). We immunized cohorts of B6 mice i.p. with 50μg NPCG/alum, allowed them develop to memory phase (>day 30) and administered CD32-Ig two days prior to a secondary immunization with 20μg NPCG i.v. Four days following immunization we harvested their spleens and quantitated them for NP-specific plasma cells using ELISPOT assay. Mice that received CD32-Ig prior to immunization had significantly reduced IgG ($p=0.035$) and IgM ($p=0.034$) B cell responses compared to mice that received secondary NPCG immunization without the CD32-Ig treatment (Figure 2a). Thus blocking the FcγR binding of ICs *in vivo* resulted in severely diminished secondary immune responses, suggesting that ICs play an essential role in the development of secondary antibody responses through their interactions with FcγRs.

Next we examined the role of the complement pathway during secondary antibody responses by depleting complement using cobra venom factor (CVF). CVF is a C3b analogue that induces uncontrolled activation of the complement cascade, resulting in temporary depletion of all complement proteins (129). Briefly, we gave NPCG-immune C57B/6 mice three injections of 5μg CVF prior to administering a secondary immunization of 20μg NPCG i.v. We bled mice prior to CVF treatment and one day following treatment and examined the level of complement depletion by ELISA for C3. Mice that received CVF treatments had significantly lower levels of the complement protein C3 (one order of magnitude lower; $p<0.0001$) as compared to both their pre-CVF

levels and to mice that had not received CVF, confirming successful complement depletion (Figure 2b). Four days after secondary immunization we harvested and examined splenocytes for NP-specific antibody-secreting cells by ELISPOT (Figure 2c). Mice that received CVF prior to secondary immunization had statistically insignificant reductions of both IgG ($p=0.59$) and IgM ($p=0.70$) producing cells.

The CVF treatment decreased C3 levels but did not completely eliminate it (Figure 2b). Therefore to confirm these results in the complete absence of C3, we examined secondary responses in C3 deficient mice ($C3^{-/-}$). Briefly we immunized $C3^{-/-}$ and control wild type mice with 50μg NPCG/alum and >30 days later gave them a secondary challenge of 20μg NPCG i.v. Four days later we harvested splenocytes and tested for NP-specific antibody secreting cells by ELISPOT (Figure 2d). $C3^{-/-}$ mice exhibited no deficiency in mounting a secondary antibody response; they mounted comparable IgG ($p=0.48$) and IgM ($p=0.208$) antigen-specific plasma cell responses as compared to wild type control animals, confirming our previous results with CVF-mediated depletion of C3. Taken together, these data suggest that complement may play a minimal role in the generation of secondary antibody responses.

Mice that lack the signaling chain of activating FcγRs have diminished secondary antibody responses

FcγRs bind IgG in many forms, including as ICs. Once IgG has bound to these receptors, a signal is sent to the cell through an attached signaling chain. Some FcγRs cause cell activation when IgG ICs are bound. These receptors contain the γ signaling chain (FcRγ) and are found primarily on APCs (130). To determine if the secondary

antibody response was a product of signaling through an activating FcγR, we examined secondary responses in mice lacking the activating FcR signaling chain ($FcR\gamma^{-/-}$). The prediction is that these mice would fail to mount a secondary response. Briefly, we immunized cohorts of $FcR\gamma^{-/-}$ and wild type $FcR\gamma^{+/+}$ mice with 50μg NPCG in alum and allowed them to develop immunity (>30 days). We bled these mice and examined their sera for antibodies by ELISA to determine whether γ-chain deficient animals could mount a successful primary response. We found that $FcR\gamma^{-/-}$ mice mount similar IgG ($p=0.10$) responses as compared to $FcR\gamma^{+/+}$ animals (Figure 3a). We next immunized these mice with 20ug soluble NPCG. Four days later, mice were sacrificed and examined for secondary B cell responses by measuring NP-specific antibody-secreting splenocytes by ELISPOT (Figure 3b). As anticipated, $FcR\gamma^{-/-}$ had a significantly diminished secondary B cell response ($p=0.01$) compared to control wildtype $FcR\gamma^{+/+}$mice, producing fewer antigen-specific IgG secreting cells in response to repeat antigenic exposure.

In order to better understand the kinetics of secondary responses in $FcR\gamma^{-/-}$ mice, we collected serum at various time-points following secondary immunization and measured antigen-specific IgG antibodies by ELISA (Figure 3c). Control $FcR\gamma^{+/+}$ wild type mice developed normal IgG secondary responses, with a rapid increase in antibody levels peaking 8 days following secondary immunization. However, IgG responses in $FcR\gamma^{-/-}$ mice developed much more slowly, with modest increase in antibody levels not appearing until day 15 post-secondary immunization. Overall $FcR\gamma^{-/-}$ mice achieved significantly lower IgG titers. Taken together these data show that signaling through the FcR γ-chain, initiated by IC binding, is critical for the rapidity and enhanced magnitude of secondary antibody responses.

ICs stimulate naive CD4 T cell activation

One possible way that ICs could enhance B cell responses is by augmenting CD4 T cell help via increased APC-mediated pMHCII presentation to CD4 T cells. To determine whether ICs could generate rapid activation and expansion of naive CD4 T cells, we transferred CFSE-labeled Ly5.2^{+} OVA-specific TCR transgenic OT-II CD4 T cells into congenic Ly5.1 C57BL/6 mice. We then immunized these recipients intravenously with either immune-complexed OVA, OVA alone, or PBS. Four days later we sacrificed the recipient mice and examined the donor Ly5.2^{+} CD4 T cells for activation and proliferation (Fig. 4a). Activation, as measured by upregulation, of CD44 was highest in the immune complex immunized group. Similarly, cell division as measured by dilution of CFSE was highest in mice that received ICs as compared to OVA or PBS controls. Not surprisingly, the overall numbers of OVA-specific CD4 T cells from IC-immunized mice was significantly greater ($p=0.01$) than controls (Fig. 4b). Taken together, these data suggest that ICs can enhance activation and proliferation of naïve antigen-specific CD4 T cell *in vivo.*

Naïve B cells participate in secondary antibody responses

In a previous report we have shown that naïve B cells play a major role in secondary antibody responses (50). Having shown that ICs have powerful activating effects on both naïve B cells and the secondary antibody response, we wanted to determine to what extent ICs stimulated naïve cell participation during the secondary response. To demonstrate the ability of naïve cells to respond in the environment of a

secondary response, we immunized mice with CGG, allowed immunity to develop, and then immunized with NPCG. Prior to secondary immunization these mice contained CGG-specific memory B cells and plasma cells producing anti-CGG antibodies, but were completely naïve to NP (Figure 5a). However, following immunization with NPCG, the mice had a rapid anti-NP response, with large numbers of NP-specific antibody-secreting cells responding, as detected by ELISPOT 4 days post-immunization. The resulting response resulted in a greater number of cells responding much more rapidly, and it more closely resembled a secondary NP response than a primary response (Figure 5b). To show that the enhancement is dependent upon antigen specificity of the pre-existing antibodies, we immunized OVA-immune mice with NPCG. As anticipated no more NP-specific B cells than a normal primary immunization were generated in this cohort. This shows that the presence of immune complexes, generated with CGG-specific antibodies, is essential for the recruitment of naïve B cells in the secondary response. Unlike a normal secondary response, more of the responding NP-specific cells expressed IgM than IgG. As all NP-specific cells must be naïve and will have not yet undergone germinal center reactions, this is unsurprising. To confirm that this NP-specific naïve B cell activation requires ICs, we blocked FcγR binding with CD32-Ig. As expected, blocking FcγR binding prevented naïve NP-specific B cell participation in the response (Figure 5b). From this we conclude that naïve B cells can be enticed in to secondary responses via IC-mediated activation.

Memory CD4 T cells alone are not sufficient to enhance the antibody response through naïve cell activation.

In the above experiment (Figure 5b), we showed that CGG-immune mice upon immunization with NPCG, exhibited enhanced primary responses to NP. We attribute this enhancement to the presence of CGG-specific antibodies. However, CGG-primed T cells are also present and capable of lending help to newly activated B cells. In order to observe the effect of CGG-primed T cells alone on the antibody response we isolated $5x10^6$ $CD4^+$ T cells from CGG-immune B6 mice and adoptively transferred them into a naïve B6 mouse. One day following transfer, we immunized these mice with 20μg NPCG i.v., and four days following immunization we examined splenocytes by ELISPOT for NP-specific antibody producing cells (Figure 6). Compared to mice receiving naïve T cells prior to immunization, the mice receiving CGG-primed T cells had no increase in IgM responses ($p=0.8101$). Interestingly IgG responses were increased ($p=0.0338$). This is most likely due to enhanced class-switching in the presence of memory CD4 T cells. From these data we conclude that, while the presence of primed $CD4^+$ T cells can enhance antibody responses modestly, they alone are not sufficient to induce an intense secondary-like antibody response.

Discussion

Here we provide evidence that the long-accepted model for secondary antibody responses lacks a key component, the participation of naïve B cells. These cells are activated by ICs that are formed upon secondary antigen exposure, causing rapid and robust antibody production. This activation is dependent on the ability of ICs to bind to FcγRs, though the role of complement appears to be minimal. Additionally we have shown that mice lacking the γ signaling chain of FcRs are unable to mount a secondary response to immunization, despite mounting a successful primary response.

The ability of ICs to induce potent humoral immune responses has long been known. A series of early experiments (54, 56, 57, 131) demonstrated the activating capacity of these molecules, finding them able to enhance antibody production, generate germinal centers, and stimulate memory formation with increased kinetics. Furthermore, it has been shown that passively administered antibody can provide the same effect as ICs, enhancing antibody production and cellular activity (132). Our results confirm these reports, and extend the findings to include stimulating secondary antibody responses as a function of ICs.

Because ICs are able to activate naïve cells with enhanced kinetics, and because ICs are present following secondary antigen encounter, it seems likely that ICs activate naïve cells to participate in secondary responses. Previous studies, from our laboratory and others, have suggested that the secondary antibody response is not an entirely memory-based phenomenon. Using a mouse model in which germinal center-derived B cells are permanently marked with YFP, we found that unmarked naïve B cells account for close to 2/3 of the secondary antibody response (50). Experiments utilizing adoptive

transfer have also shown that naïve B cells are recruited during memory responses (133, 134). Here we have shown that naïve cells can and do participate in secondary responses, and that antigen-specific serum antibody generated following primary antigen exposure is required for this participation. In our experiments, CGG-immune mice have the necessary cellular and humoral environment to stimulate a secondary response to CGG, but they lack memory B cells and antibodies specific for NP. However, when given a secondary immunization of NP coupled to CGG, these mice develop a strong NP-specific response. Since these mice have no previous immunity to NP, all responding cells must be naïve and all NP-specific antibodies must be derived from naïve cells. In other words, in the environment of a secondary response naïve B cells are activated and respond as quickly and in as great a number as memory cells.

Current understanding of naïve B cell activation does not account for the rapidity of a secondary response. However, activation by membrane-bound ICs incorporates the BCR into a synapse of integrins and co-receptors that lower the signaling threshold required for B cell activation and result in more rapid antibody responses (135). While either FcγRs or complement could anchor these ICs to the cell surface, and while complement can also be involved in B cell co-stimulation through CD21, our results suggest that it is FcγRs rather than complement that give ICs their functionality in secondary antibody responses. This is supported by work showing that the stimulating effect of ICs is dependent on activating FcγRs (63), but does not require complement (64). Additionally, it has been shown that FcγR-bound ICs can remain intact in their native form on the cell surface and, in that manner, be presented to B cells (136). That the secondary response is entirely dependent on FcγRs seems curious, as even in the

absence of naïve cell activation memory and long-lived plasma cells are still present to respond to antigenic challenge. This suggests either that memory B cell activation also depends on IC interaction or that perhaps memory cells may not be a major component of protective immunity, as has been previously suggested (137).

While FcγRs are present on a number of cell types, our results suggest that FcγRs utilizing the γ-signaling chain are necessary for IC enhancement of secondary responses. As these activating FcγRs are not found on B cells (130), an intermediary cell must be involved in their activation. The most likely candidates are antigen-presenting cells (APCs), as these cells possess the necessary FcγRs as well as the ability to activate T cells. The identity of the APCs involved in this process warrants further investigation.

The reigning paradigm in humoral immunity is that immunological memory is responsible for protective immunity, as demonstrated by robust antibody response upon repeat antigen exposure. However, we show that naïve B cells are also active participants in the secondary response, and that their recruitment relies upon activating FcRs and pre-existing serum antibodies raised during primary antigen exposure. These data reveal a previously underappreciated positive feedback mechanism, mediated by antigen-specific serum antibody, which operates between the primary and secondary immune responses. This mechanism both accelerates secondary antibody responses via rapid CD4 T cell activation and diversifies the antibody repertoire through the recruitment of novel B cell clonotypes. Thus, the immune system retains high-affinity memory B cell responses while actively recruiting new B cell clones, thereby continuously diversifying the antibody response yet ensuring high-affinity antibodies are produced upon each antigen exposure. This enables the immune system to stay abreast of rapidly changing pathogens.

Figure Legends

FIGURE 2.1. Immune complexes enhance immune responses in naïve animals. Naïve C57B/6 mice were immunized with either complexes of NPCG and anti-NP antibody formed *in vitro* (IC) or NPCG precipitated in alum (NPCG/alum). Four days following immunization sera and spleens were harvested for ELISA and ELISPOT assays, respectively. **(a)** The levels of serum anti-CGG antibody in the two groups are shown. Error bars denote mean +/- SEM. **(b)** The number of NP-specific antibody-secreting plasma cells per 10^6 splenocytes is plotted. Error bars denote mean +/- SEM. Data shown are representative results from 2 independent experiments (n=5-6). (IC = IC immunized mice; Alum = NPCG/alum immunized mice)

FIGURE 2.2. Immune complexes stimulate secondary responses through FcγR binding, not complement activation. (a) Cohorts of C57BL/6 mice were immunized with 50μg of NPCG/alum and > 30 days later immunized again with 20μg NPCG i.v. Mice received either PBS or 20μg CD32-Ig i.v two days prior to the secondary immunization. Four days following immunization spleens were harvested and examined *ex vivo* by ELISPOT. Numbers of NP-specific antibody-producing cells per 10^6 splenocytes are plotted, with the antibody isotype indicated on the x-axis **(b)** NPCG-immune C57B/6 mice were immunized i.v. with 20μg NPCG. Mice received injections of either PBS or 5μg CVF at 28, 24, and 4 hrs prior to immunization. Serum was collected prior to and 1 day following CVF treatment and analyzed by ELISA for C3 levels. C3 levels at both time points are plotted in μg/ml. Here $p<0.0001$ by two-way ANOVA. **(c)** Four days following immunization splenocytes were harvested and NP-

specific plasma cells were enumerated *ex vivo* by ELISPOT. The number of NP-specific antibody-secreting cells per 10^6 splenocytes is plotted, with antibody isotype indicated on the x-axis. Data shown are representative results of three independent experiments (n=5-8). **(d)** We immunized $C3^{-/-}$ and control wild type mice first with 50μg NPCg/alum and >30days later with 20μg NPCG i.v. Four days later, we quantitated NP-specific plasma cells by ELISPOT. The number of NP-specific IgG and IgM antibody-secreting cells per 10^6 splenocytes is plotted, with antibody isotype indicated on the x-axis. Data shown are representative results of two independent experiments (n=4 per experiment). For all experiments error bars denote mean +/- SEM.

FIGURE 2.3. Mice that lack the signaling chain of activating FcγRs have diminished secondary antibody responses. **(a)** $FcR\gamma^{-/-}$ mice were immunized with 50μg NPCG in alum. 30 days following immunization sera were collected and analyzed for antibody levels by ELISA. Results were compared with control wild type $FcR\gamma^{+/+}$ mice receiving identical treatment. Levels of NP-specific IgG are plotted in ng/ml. **(b)** NPCG-immune mice were given a secondary immunization with 20μg NPCG i.v. Four days following immunization, splenocytes were harvested and examined *ex vivo* by ELISPOT. Number of NP-specific IgG-secreting cells per 10^6 splenocytes is plotted, with antibody. **(c)** Serum was collected at various time points before and after secondary immunization and analyzed for NP-specific antibody levels by ELISA. Levels of NP-specific IgG are plotted in ng/ml with time relative to secondary immunization denoted on the x-axis. For all experiments error bars denote mean +/- SEM. Data are representative of two independent experiments (n=4-6).

FIGURE 2.4. ICs stimulate naïve CD4 T cell activation. CFSE-labeled, naive $Ly5.2^+$ OT-II TcR transgenic CD4 T cells were adoptively transferred into naïve $Ly5.1^+$ mice. These mice were then immunized with either immune complexed OVA, OVA only, or PBS as a negative control. Four days following immunization $Ly5.2^+$ donor CD4 T cells were examined by flow cytometry. **(a)** Cell division as indicated by CFSE dilution and the expression of activation marker, CD44 are shown. Columns represent different antigen treatments. **(b)** The total number of donor $CD4^+$ $Ly5.2^+$ T cells per spleen is plotted. Error bars denote mean +/- SEM. Data shown are representative results from 2 independent experiments (n=3-5).

FIGURE 2.5. Naïve B cells participate in secondary antibody responses. (a) Naïve C57B/6 mice were immunized with 50μg CGG in alum. Thirty days following immunization serum was collected and analyzed for antibody levels by ELISA. IgG levels in ng/ml are plotted, with antibody specificity, CGG or NP, indicated on the x-axis. **(b)** CGG-immune mice were immunized i.v. with 20μg NPCG. Four days following immunization splenocytes were harvested and examined *ex vivo* by ELISPOT (CGG/NPCG; black bars). Number of NP-specific antibody-secreting cells per 10^6 splenocytes is plotted, with antibody isotype indicated on the x-axis. Data are compared to a primary response 4 days following immunization with 50μg NPCG in alum (NPCG primary; striped bars), to a secondary response to NPCG in mice given a primary immunization of 50μg OVA in alum (OVA/NPCG; white bars), and to a secondary response to NPCG in CGG immune mice which received 20μg CD32-Ig 2 days prior to

secondary immunization (CGG/NPCG + CD32-Ig; gray bars). For both IgG and IgM $p<0.0001$ by one-way ANOVA. For all experiments error bars denote mean +/- SEM. Data are representative of three independent experiments (n=3-7).

FIGURE 2.6. T cells alone are not sufficient to induce secondary antibody response. We immunized cohorts of mice with CGG and >30days later, we purified their splenic $CD4^+$ T cells and adoptively transferred them into age-matched sex-matched naïve recipients. Twenty-four hours later these mice received 20μg NPCG i.v. Four days later splenocytes were harvested and examined *ex vivo*. Number of NP-specific antibody-secreting cells per 10^6 splenocytes is plotted, with antibody isotype indicated on the x-axis. Error bars denote mean +/- SEM. Data are representative samples from two independent experiments (n=5-6).

Figure 2.1

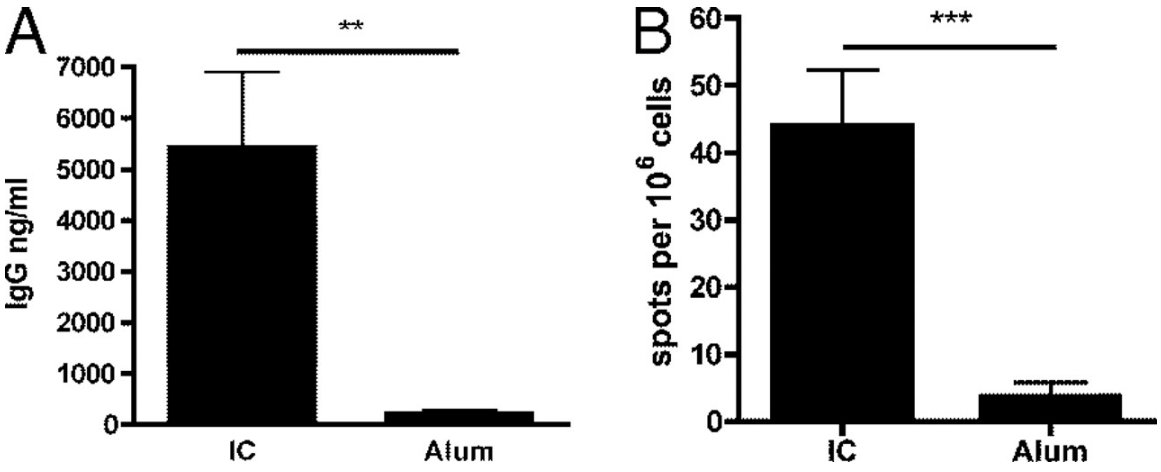
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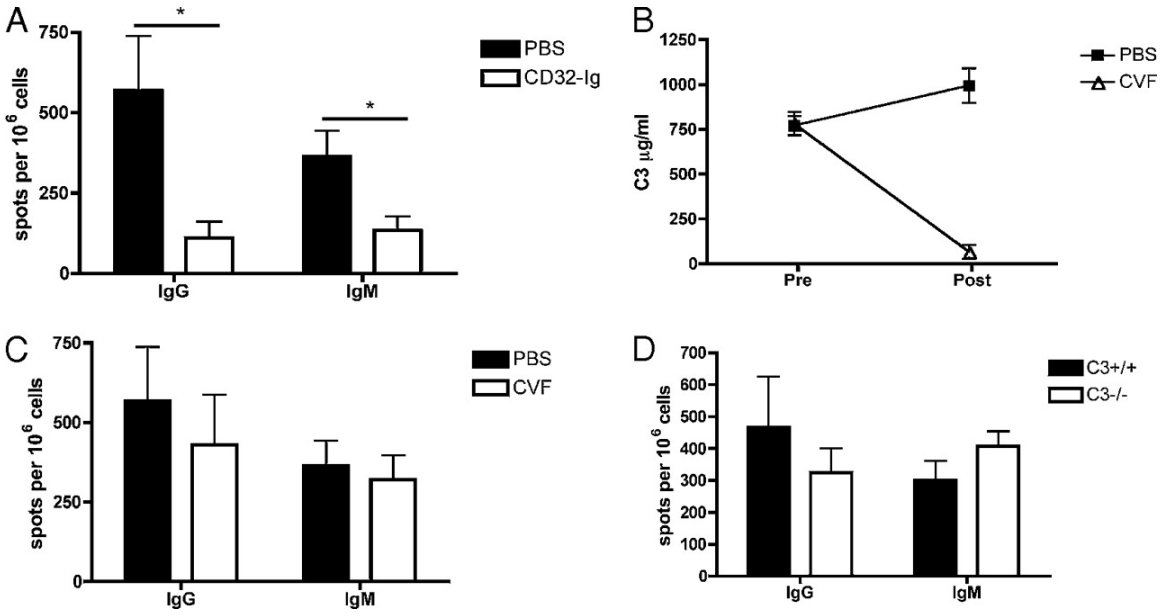
Figure 2.2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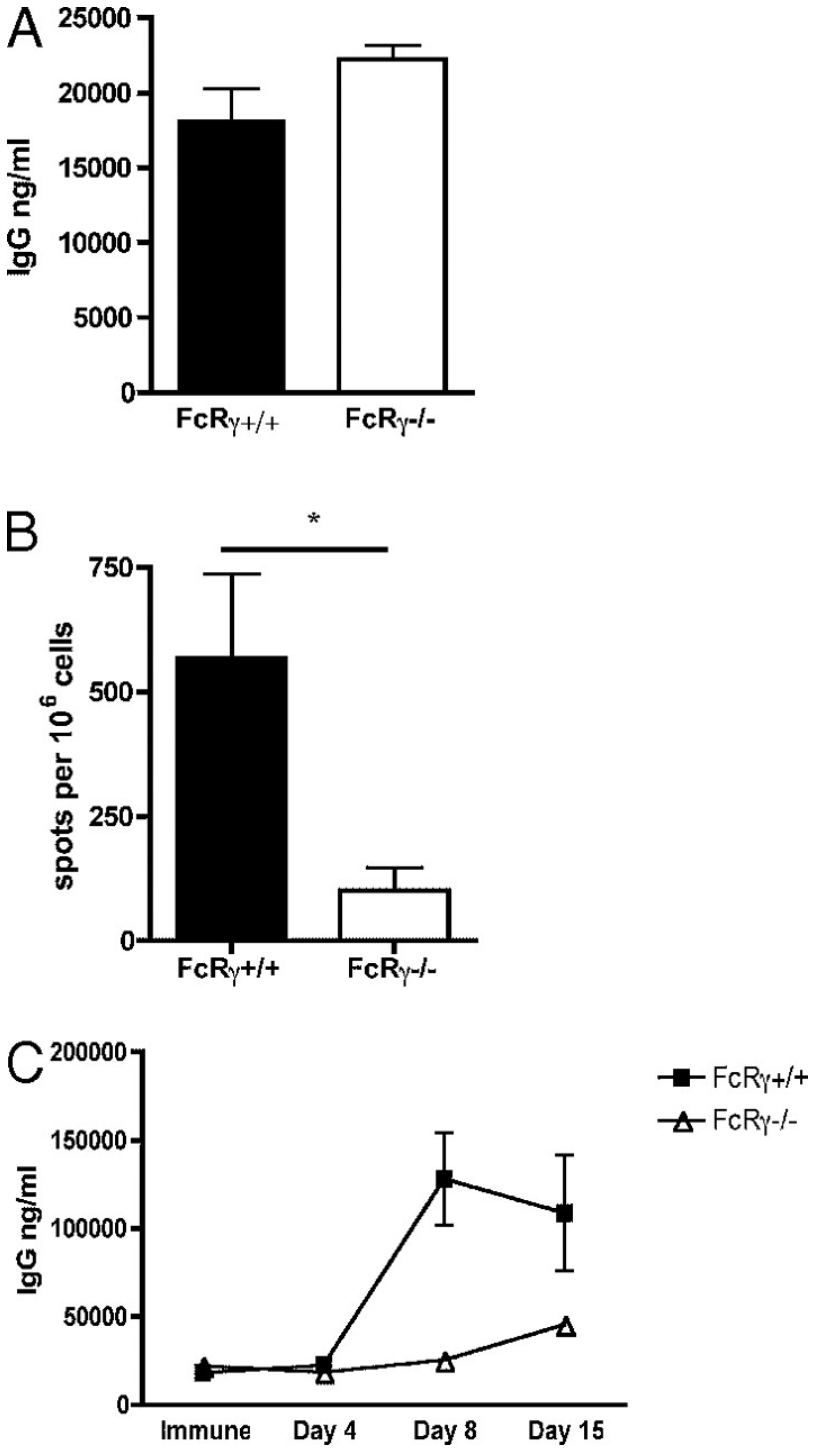
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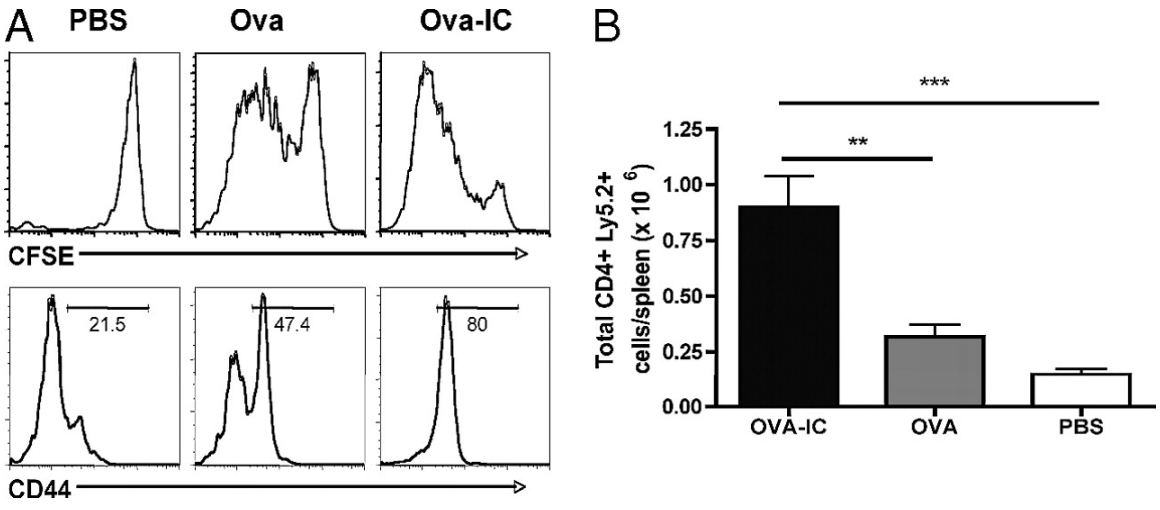

Experiment performed by Craig P. Chappell

Figure 2.5

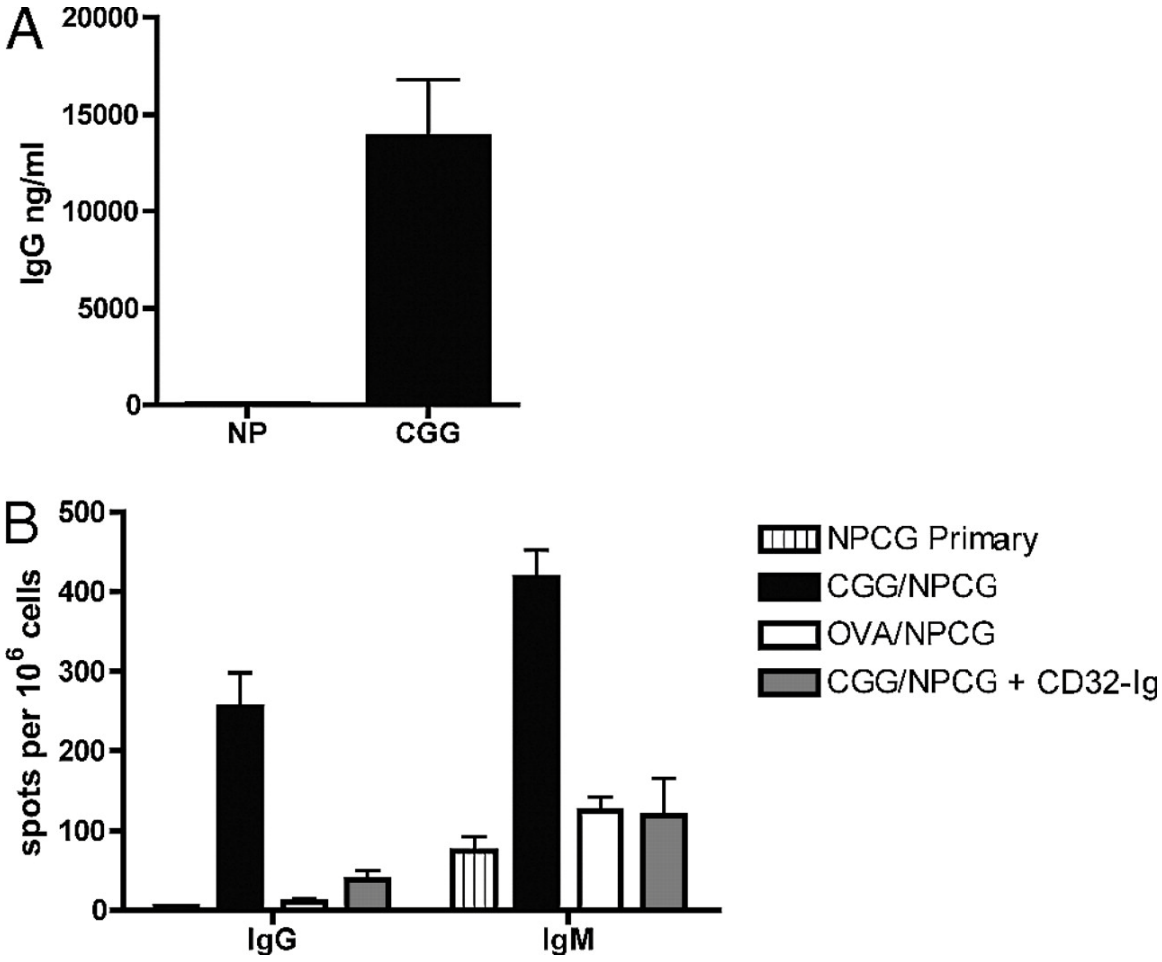
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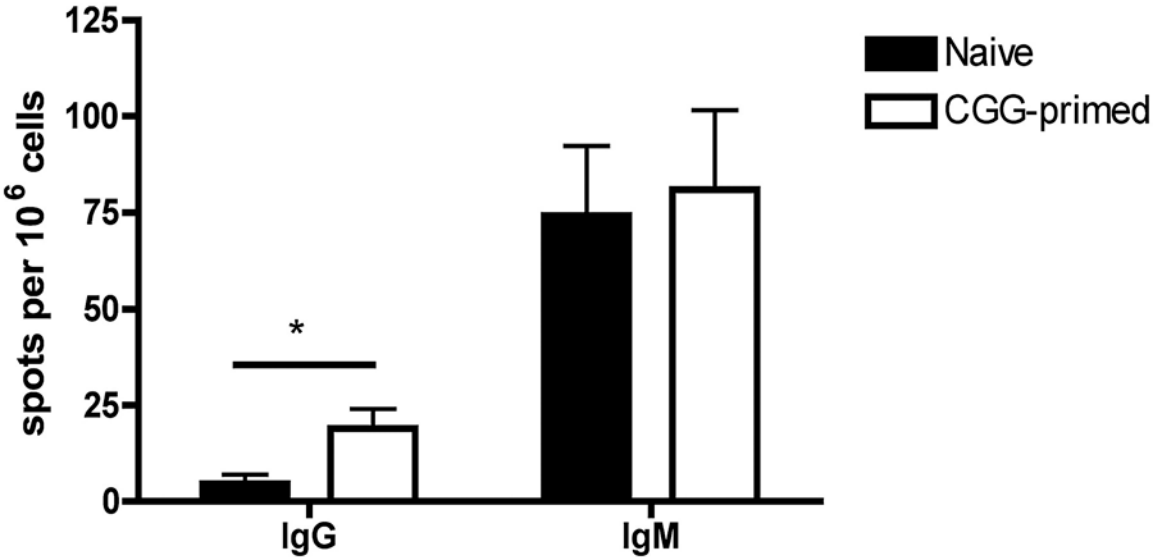
Figure 2.6



Chapter 3

Maternal Antibodies Enhance Infant B cell Reponses to Soluble Antigens but Block the Formation of Memory

A manuscript submitted to *The Journal of Immunology*.

By: **Chelsey L. Goins**, Ryan P. Powers, William C. Weldon, Richard W. Compans, and Joshy Jacob.

Abstract

Maternally derived antibodies are essential in protecting infants during their first months of life. However, they are also a burden to vaccination because they impair the ability of the infant immune system to form immune memory. Because of this, many necessary and life-saving vaccines such as measles are given at delayed times, up to a year after birth. This leaves a wide window of time in which the infant is vulnerable to infection but unable to develop the antibody response necessary for its own protection. Better understanding of maternal antibody could lead to the development of more efficacious infant vaccines. We reasoned that this suppression could in part be due to the form of the antigen. Here we show that maternal suppression of infant immune responses occurs because most vaccine antigens are particulate, and that this suppression of initial immune responses can be overcome partially by the use of soluble antigens. However, despite this initial enhancement the presence of maternal antibodies led to impaired immune memory generation and diminished recall responses. Interestingly, newborn pups that lacked antigen-specific maternal antibodies could be successfully vaccinated; they mounted robust protective immune responses when challenged later in life. Thus maternal antibodies are critical for protection early in life but they hamper the development of vaccine-induced immune memory generation.

Introduction

Maternal antibodies, passed from mother to child during pregnancy and breastfeeding, are essential for protection during the first months of life. The newborn immune system is underdeveloped and completely naïve to all pathogens, and so lacks the ability to ward off infection (84). Instead, maternal antibodies provide this protection while the immune system develops.

The downside of this protection is that maternal antibodies often inhibit the activation of the infant humoral immune response. This is particularly true in regards to vaccination (98, 101, 102, 110). This is presumably because maternal antibodies will clear away antigens and block epitopes before a response can develop. Hence infants must often undergo multiple rounds of immunization in order to induce protective responses to vaccines. Some vaccines, such as measles, essential for the good health of the population, are delayed until later in life simply because they cannot overcome maternal blocking (107). If it were possible to overcome maternal blocking, vaccinations could be given earlier, resulting in accelerated development of immune memory.

Maternal blocking is the result of IgG-mediated immune suppression, which occurs only with particulate antigens and is believed to be a result of epitope masking (115). In contrast, several studies have documented the immune-stimulating effects of IgG in response to soluble antigen (54, 56, 62, 64, 138). Here, the immune-stimulating effects of IgG occur only in response to soluble antigens, as IgG inhibits responses to particulate antigens (119). Since most, if not all, infant vaccines contain particulate antigens, maternal blocking could likely be due to the inhibitory effect of IgG in response

to these antigens. Thus, the use of soluble, rather than particulate, vaccine antigens could overcome maternal blocking.

Here we examine the infant immune response to both soluble and particulate antigens in the form of influenza hemagglutanin (HA) and inactivated influenza virus. We show enhanced immune activation in response to soluble antigens in pups containing antigen-specific maternal antibodies. We find that immune responses in the presence of maternal antibodies are short-lived, and that presence of maternal antibodies during immunization results in lack of protective memory formation, regardless of the nature of the immunogen.

Materials and Methods

Mice and immunizations

Animals were housed in an American Association of Laboratory Animal Care-accredited facility under specific pathogen-free conditions at the Emory University Vaccine Research Center. Parent BALB/c and C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA). All pups were bred on site. Immunizations with the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) coupled to chicken gamma globulin (CGG) (Biosearch Technologies, Navato, CA) (NPCG) were given intraperitoneally as 50μg NPCG in PBS. Immunizations with BPL-inactivated virus were given as 10μg intramuscularly for mothers or 1μg i.p. for pups. We administered soluble HA trimers in a dose of 1μg given i.p. For live virus infection, we infected mice intranasally with 25μl of a 0.1 or 20 × 50% lethal dose (LD_{50}) dose of mouse-adapted live virus under anesthesia. Emory University's Institutional Animal Care and Use Committee approved all studies.

Viruses

Madin-Darby canine kidney (MDCK) cells were grown in DMEM containing antibiotics, glutamine, and 10% FBS and serially passed before the cells reaching 90% confluence. All virus studies were done using mouse adapted A/PR/8/34 (PR8) influenza virus. For the purpose of immunization with inactivated viruses, we amplified viruses in 11-day-old chicken embryonic eggs for 48 h and harvested the allantoic fluid by centrifugation. We further pelleted the virus from the allantoic fluid supernatants by ultracentrifugation and purified them by sucrose density gradient ultracentrifugation.

Viruses were inactivated by incubation with a 1:2000 (v/v) dilution with beta-propiolactone (BPL) overnight at 37°C.

ELISAs

Serum NP Ab titers were measured by modification of an ELISA assay. 96-well microplates were coated with a solution of 20µg/mL NP-BSA (Biosearch Technologies) in PBS overnight at 4°C. Plates were blocked for 1h at 37°C using a solution of PBS containing 4% non-fat dry milk (Bio-Rad, Hercules, CA). Serum samples were applied and allowed to react at room temperature for 1.5h. Anti-mouse antibodies with specificity for IgG isotypes coupled to horseradish peroxidase (HRP) (Southern Biotech, Birmingham) were applied and allowed to incubate 1.5h at room temperature. Plates were developed with TMB substrate and read at 450nm absorbance. Values were compared to known dilutions of IgG to calculate Ab concentrations.

ELISPOTs

ELISPOT assays were performed as described (125) with the exception that 96-well nitrocellulose plates (Millipore) were coated overnight with either 10µg/ml BPL-inactivated PR8 in 100µl PBS for flu-specific ELISPOTS or 20µg/ml NP-BSA in 100µl PBS for NP-specific ELISPOTs. Spots were visualized using an ELISPOT reader (Cellulara Technologies, Ltd., Cleveland, OH) and counted manually.

Plaque assay

Viral titers in mouse lungs were assessed using plaque assays. MDCK cells were grown in six-well plates to >99% confluence before serial dilutions of lung lysates were added and allowed to absorb at 37°C for 1 hour. The lysates were aspirated and cells were covered in agar containing DMEM, glutamine, antibiotics, DEAE-dextran, nonessential amino acids, TPCK-trypsin, and HEPES buffer. The plates were incubated for 5 days. The cells were then fixed with 0.25% glutaraldhyde. Following fixation, the agar plug was removed, the adherent cell layers were stained with 1% crystal violet, and the plaques were counted.

Serum HAI assay

Mouse sera were treated with receptor destroying enzyme II (RDE II; Denka Seiken) overnight at 37°C and then diluted 1:10 in PBS. Dilutions of sera were mixed with influenza viruses freshly grown in MDCK cells at a dose of 8 HA/50µl. Mixtures of virus and serum dilutions were incubated for 15 min, followed by addition of 50µl 0.5% chicken RBC (Innovative Reasearch). The highest serum dilution inhibiting hemagglutination was taken as the HAI titer.

Statistics

Student's *t* test was used to generate all statistical values stated. For statistical designations, * denotes $p < 005$; ** denotes $p < 0.01$; *** denotes $p < 0.001$.

Results

Maternal antibodies enhance immune responses in pups from NPCG immune mothers

In previous studies (139) we have shown that antigen-specific antibody can enhance naïve B cell responses to immunization. To determine if this held true in the case of maternally derived antibodies, we examined immune responses in young mice (18-21 days post-natal) possessing antigen-specific maternal antibodies. We immunized age-matched female C57Bl/6 mice with 50μg NPCG in alum. One month following immunization immune females were mated with naïve C57BL/6 males. Ensuing progeny were examined for NP-specific antibodies at 21 days of age. Pups from immune mothers had significantly higher levels of NP specific IgG (1899.7±102.7 ng/ml) than pups from naïve mothers (Fig 1a). We then immunized these pups from naïve and immune mothers with 50μg NPCG and examined splenocytes for NP-specific B cell activation and differentiation to plasma cells by ELISPOT 4 days post-immunization. Pups from immune mothers had nearly four times higher levels of antigen-specific plasma cells ($p=0.0009$) as compared to pups from naïve mothers (Fig 1b). All cells examined produced antibodies of the IgM isotype; no IgG isotype antibodies were observed. These data suggest that presence of antigen-specific maternal antibodies enhances the infant immune response by increasing the number of B cells activated and the speed with which they respond.

Maternal antibodies provide protection from influenza infection but do not enhance infant immune responses

We next wanted to determine whether the enhancement of infant immune responses to hapten immunization could be observed in a real-life scenario of infection. In order to do this we immunized aged-matched female Balb/c mice with 10μg BPL-inactivated A/PR/8/34 (PR8) influenza virus. One month following immunization these females were mated with naïve male Balb/c mice and ensuing progeny were weaned and taken for experimentation after 2.5 weeks of age. We examined levels of maternal antibodies in pups from PR8 immune mothers over a period of three weeks to track the decline of maternal antibody levels. We collected serum from pups at half-week intervals beginning at 2.5 weeks post-natal and examined it for PR8-specific IgG by ELISA (Fig 2). We found that at the time of weaning (2.5 weeks of age) pups had PR8-specific antibody titers of 2269±195 ng/ml. These titers steadily declined over time and all traces of maternal antibody were gone by 5 weeks of age.

Next, we determined whether maternal antibodies protect by neutralizing the pathogens, enhancing immune responses, or both. Briefly, we lethally challenged pups from PR8-naïve or -immune mothers with 20 x LD_{50} live mouse-adapted PR8 virus. None of the mice from immune mothers died following lethal infection while 100% of the mice from naïve mothers succumbed to infection and died by day 5 (Fig 3a). All infected mice displayed evidence of illness as defined by decrease in body weight. Mice from immune mothers lost on average 7.1±4.5% of their initial body weight before recovering, while all mice from naïve mothers lost >20% body weight and either died or were euthanized as per IACUC guidelines (Fig 3b). To further analyze the extent to which maternal antibodies neutralize and lower the virus loads, we isolated lung samples from infected mice 4 days post-infection and quantitated the lung viral titers by plaque

assay. Mice from naïve mothers had significantly higher viral titers ($p<0.0001$) than mice from immune mothers (Fig 3c) demonstrating that maternal antibodies are efficient at lowering viral loads.

While maternal antibodies protected pups from influenza infection, they did not enhance *de novo* B cell responses to infection (Fig 3d). Four days following challenge with 20 x LD_{50} live PR8 virus, mice from immune mothers had similar numbers of influenza virus-specific plasma cells as compared to mice from naïve mothers ($p=0.236$). To rule out the possibility that this was due to the high viral load challenge, we infected separate cohorts of pups with lower viral loads ($0.1xLD_{50}$). Even at this low dose there was no significant difference in the extent of B cell activation and plasma cell differentiation ($p=0.565$) between the two groups of pups. From these data we conclude that maternal antibodies play a vital role in protecting the infant from infection early in life, but they do not enhance the infant's immune response to the infecting pathogen.

Maternal antibodies enhance infant immune responses to soluble but not particulate antigen

Most infant vaccinations are done using particulate antigens. Hence we ascertained whether maternal antibodies enhance or inhibit immune responses to particulate antigens. Briefly, we immunized pups from naïve and PR8 immune mothers with 1µg inactivated PR8, a particulate antigen. Four days following immunization we sacrificed pups and quantitated their splenocytes *ex vivo* by ELISPOT for PR8-specific plasma cells (Fig 4a). Mice from immune mothers, containing antigen-specific maternal antibody, did not exhibit an enhancement in immune activation over mice from naïve

mothers (p=0.369). This is not unexpected, as IgG has been shown to suppress immune responses to particulate antigen (59, 115).

Since IgG enhances responses to soluble antigens, we next determined if soluble antigens could result in the enhancement of immune responses in the presence of maternal antibodies. We immunized pups from naïve or PR8-immune mothers with 1μg of a soluble PR8 hemagglutinin (HA) trimer. Four days following immunization we sacrificed these pups and examined their splenocytes *ex vivo* for PR8-specific plasma cells (Fig 4b). Unlike immunization with a particulate antigen, immunization with the soluble PR8 HA resulted in a significant increase of more than 30% (p=0.0088) in the immune response in pups from immune mothers. From these data we conclude that immunization with soluble antigen rather than the standard particulate antigen can overcome maternal antibody inhibition of infant immune responses and lead to enhanced activation of the infant humoral immune system.

Presence of maternal antibodies impairs the formation of immune memory and recall responses

The objective of vaccination is to provide long-lasting immune protection from infection. Having shown that maternal antibodies enhance the activation of infant immune responses to soluble but not particulate antigen, we followed the immune responses of pups from naïve and PR8 immune mothers immunized with either soluble (1μg PR8 HA) or particulate (1μg inactivated PR8) at 18-21 days of age. We collected serum samples every two weeks post-immunization and examined by hemagglutination inhibition assay (HAI) the development of PR8-specific antibody responses. In pups

receiving particulate antigen immunization, titers in mice from naïve mothers reached as high as 2240 (±320) HAI at 8 weeks post-immunization, whereas pups from immune mothers had titers of only 280 (±40) HAI at the same time (p=0.0009) (Fig 5a). Likewise, at 8 weeks post-immunization with soluble antigen, pups from naïve mothers had HAI titers of 1120±160), while pups from immune mothers had titers of only 400±80 (p=0.0069) (Fig 5b). Taken together, these data show that maternal antibodies prevent the establishment of memory.

Next we determined if this decrease in antibody levels corresponded to a decrease in immune effectiveness. We challenged these pups i.n. with 20 x LD_{50} live PR8 virus at two months post-immunization and tracked their mortality and morbidity for 14 days. As expected, particulate Ag-immunized pups from naïve mothers, which had higher antibody titers prior to infection, showed 80% survival rate (Fig 5c). These mice also had decreased severity of infection, as measured by weight loss over the course of the infection (Fig 5d). In contrast, 100% of the particulate antigen-immunized mice from immune mothers lost weight rapidly and succumbed to infection and died. Similarly, in pups that were immunized with soluble antigen, 100% of mice from immune mothers lost >20% of their initial body weight and perished, despite the early boost in immune response (Fig 5e). Mice from naïve mothers fared better; though all mice experienced a severe infection as measured by weight loss, 50% of the mice were able to survive (Fig 5f). From these results we conclude that, regardless of success or failure of initial immune response, maternal antibodies inhibit B cell memory from developing. Our data also suggest that immunization of pups from naïve mothers can lead to robust protective immunity.

Discussion

Maternal antibodies, particularly IgG, are transmitted from mother to child during pregnancy and after birth, via breastfeeding. Because of the ability of IgG to interact with and modulate immune responses, it is important to understand how maternal antibodies affect infant immune responses. Here we show that maternal antibodies are necessary for the protection of the infant but that the infant is unable to mount strong de novo immune responses to pathogens. We also show that pups bearing maternal antibodies upon immunization with a soluble antigen results in the strongest overall immune response, far greater than immunization in the absence of maternal antibodies but this response was short-lived. Finally, we show that maternal antibodies block the development of immune memory, as the initially strong immune responses do not convert to long-term protection in the presence of maternal antibodies.

Understanding the mechanism of maternal antibody suppression, and particularly how to overcome it, has long been the focus of childhood vaccine research (97, 140-142). Overcoming this suppression would lead to enhanced quality of life for infants, perhaps extending into healthier immune systems as adults. In adults IgG-mediated suppression of immune responses occurs only in response to particulate antigens. Immunization with soluble antigens leads to enhanced immune responses (119). It seems likely, then, that the immune modulation seen with maternal antibodies, which are primarily IgG, might occur in a similar manner.

In a previous experiment (139) we showed that naïve B cells respond rapidly and produce high titers of antibody in response to the soluble antigen NPCG when in the

presence of antigen-specific IgG. Replicating this experiment in pups containing NPCG-specific maternal antibodies, we saw similar results. B cell responses were enhanced in the presence of the maternal antibodies, while they were low in their absence. However, the opposite was seen in response to influenza infection. When pups containing influenza-specific maternal IgG were infected with the same strain of influenza, the maternal antibodies did not lead to enhancement of the immune response. In many similar cases, the result of maternal antibody presence is inhibition of immune response.

Maternal antibody suppression has been demonstrated against a number of vaccines. Those most often studied include measles, oral poliovirus, hepatitis, pertussis, and *Hemophilus influenzae B* (98, 105, 107, 143, 144). In all of these examples maternal antibodies have been shown to suppress infant immune responses, and in all of these examples the vaccines use particulate antigen. Indeed, in the example of pertussis, maternal antibodies inhibit vaccine responses when a whole-cell, or particulate, vaccine is used, but not when an acellular, soluble vaccine is used (105). The same is true of vaccine studies against herpes simplex virus, which showed that maternal antibodies suppressed responses to whole-virus vaccines but did not affect responses to DNA vaccines (145).

We examined the infant immune response to soluble and particulate influenza vaccines to determine if the nature of the vaccine antigen was the deciding factor influencing maternal antibody-based inhibition. We found that this was indeed the case: maternal antibodies greatly enhanced responses to soluble vaccines, but not to particulate vaccines. That we saw no inhibition of response to particulate vaccines, which might be expected, is likely due either to low maternal antibody titers or high doses of vaccine

antigen. Both of these factors have been shown to influence the degree of maternal antibody inhibition, or whether it occurs at all (113).

The rapid enhancement of immune responses seen following soluble antigen immunization in the presence of maternal antibody could be a great boon to the immune system early in life. This accelerated response will provide additional protection against pathogens, and may happen to be the deciding factor in battling an infection if maternal antibody levels were low. Additionally it has been shown that early immune responses in the presence of maternal antibody have the potential to shape the adult immune repertoire (146). Unfortunately, this early enhancement does not seem to result in protective memory later in life. Indeed, it seems presence of maternal antibody blocks the formation of immunological memory independent of the antigen type.

The lack of a memory response in infants containing maternal antibodies raises a number of questions. Since this memory blockade occurs regardless of the nature of the antigen, it seems likely that it occurs through a different mechanism than IgG-mediated suppression of responses to particulate antigen. What this mechanism is remains unknown. Depending on the mechanism of action, there is the possibility that this blockade might be overcome, possibly through the use of adjuvants, or by stimulating the immune system in another manner. Additionally, there is the possibility that the enhanced humoral immune response to soluble antigen in the presence of maternal antibody, while short-lived, may prime the infant immune system for greater responses later in life. Whether this might be in the form of T cell activation, low levels of remaining antibody, or some other mechanism remains an interesting question.

Based on our studies of both short and long term immune responses, the ideal vaccination scenario would be a particulate antigen immunization in an infant lacking maternal antibodies. As we have demonstrated, however, maternal antibodies are necessary for the survival of the infant in the early months of life. In our experiments, infants without virus-specific maternal antibodies succumbed rapidly to influenza infection and perished, while those with maternal antibodies were protected, experiencing only mild infections and ultimately surviving. This is supported by data from other studies as well (90). Since maternal antibodies are both necessary and inevitable, it seems prudent to find an immunization strategy that will work with the maternal antibodies, rather than fighting against them. While immunization with soluble antigens in the presence of maternal antibodies does not provide a memory benefit, it does seem to enhance the protective level of antibodies early in life, when protection is most needed. The additional effects this immunization scheme has on immune responses remain to be investigated.

Figure Legends

FIGURE LEGENDS

Figure 1. Maternal antibodies enhance immune responses in pups from NPCG immune mothers. Age-matched female C57Bl/6 mice were immunized with 50μg NPCG i.p. and rested for four weeks to allow the immune response to mature to memory phase. Control female mice were immunized with PBS. We then mated these mice with male mice. Pups from these matings were weaned at 21 days of age. **(a)** Sera were collected from pups from naïve and immune mothers and assayed for NP-specific IgG antibodies by ELISA. Antibody levels are plotted in ng/ml. **(b)** Pups were immunized i.p. with 50μg NPCG. Four days following immunizations splenocytes were harvested and examined *ex vivo* by ELISPOT. The numbers of NP-specific IgM-producing cells per 10^6 splenocytes are shown. Data shown are representative of two separate experiments (n=5). For all experiments error bars denote mean +/- SEM. *denotes $p <$ 005; **denotes $p < 0.01$; ***denotes $p < 0.001$.

Figure 2. Maternal antibody levels decline in pups after weaning. Age-matched female Balb/c mice were immunized with 10μg inactivated PR8. Four weeks later they mated with male mice. Pups from these matings were examined at half-week intervals, starting upon weaning at 2.5 weeks, for PR8-specific IgG by ELISA. The levels of maternal antibody over time is plotted, with titers of PR8-specific IgG indicated on the y-axis and age of mice (time post-natal) indicated on the x-axis. Results are representative data from two independent experiments (n=5). Error bars denote mean +/- SEM.

Figure 3. Maternal antibodies provide protection from influenza infection but do not enhance infant immune responses. Age-matched female Balb/c mice were immunized with 10μg inactivated PR8 influenza virus. Four weeks later they mated with male mice. Pups from these matings (△) or from naïve Balb/c mothers (■) were weaned and taken for experiments at 18-21 days of age. **(a)** Pups were infected i.n. with 20 x LD_{50} live PR8 virus and the morbidity and mortality were assessed for 14 days. Percent survival over the course of a 14-day period post-challenge is plotted. **(b)** Weight loss as a percentage of starting weight is shown for the course of the experiment. In these figures, the x-axis denotes time post lethal challenge. **(c)** In separate cohorts, we sacrificed the pups four days following lethal challenge, collected their lung samples and quantitated the viral titers by plaque assay. Viral lung titers four days post-infection from pups born from naïve or immune mothers are shown in pfu/ml. **(d)** Pups were infected i.n. with either 0.1 x LD_{50} or 20 x LD_{50} live PR8 virus. Four days following infection splenocytes were harvested and examined *ex vivo* by ELISPOT. Number of PR8-specific IgM-secreting cells per 10^6 splenocytes is plotted, with dosage indicated on the x-axis. Data shown are representative samples from two independent experiments (n=5-6). For all experiments error bars represent mean +/- SEM. *denotes $p < 005$; **denotes $p < 0.01$; ***denotes $p < 0.001$.

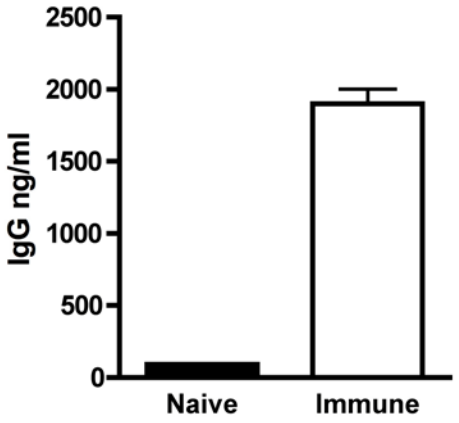
Figure 4. Maternal antibodies enhance infant immune responses to soluble but not particulate antigen. Pups from PR8 immune and naïve mothers were immunized with either 1μg inactivated PR8 (particulate) or 1μg PR8 HA trimer (soluble) at 18-21 days of age. Four days following immunization, splenocytes were harvested and examined *ex*

vivo by ELISPOT. Responses to particulate antigen **(a)** or soluble antigen **(b)** are shown as number of PR8-specific IgM-producing cells per 10^6 splenocytes. Data shown are representative data from two independent experiments (n=5-6). For all experiments error bars denote mean +/- SEM. *denotes $p < 005$; **denotes $p < 0.01$; ***denotes $p < 0.001$.

Figure 5. Maternal antibodies inhibit the formation of memory. Pups from PR8 immune (△) and naïve (■) mothers received immunizations of either 1μg inactivated PR8 (particulate) or 1μg PR8 HA trimer (soluble) at 18-21 days of age. Serum samples were taken every two weeks and the PR8-specific humoral immune responses were examined by HAI. HAI titers on a $\log_2$ scale are shown for pups receiving particulate antigen immunizations **(a)** or soluble antigen immunizations **(b)**, with time post-immunization denoted on the x-axis. Error bars represent mean +/- SEM. **(c)** Two months following immunization with particulate antigen (inactivated PR8) pups were lethally challenged i.n. with 20 x LD_{50} live PR8 virus and survival and weight loss were monitored for two weeks post-challenge. Percent survival over a course of 14-days post-challenge is shown, with days post-infection indicated on the x-axis. **(d)** Weight loss as a percentage of starting weight is shown. **(e)** Two months following immunization with soluble antigen (HA trimer) pups were infected i.n. with 20 LD_{50} live PR8 virus and survival and weight loss were followed for two weeks post-infection. Percent survival is shown, with days post-infection indicated on the x-axis. **(f)** Weight loss as a percentage of starting weight is shown. Data shown are representative results from two independent experiments (n=4-5).

Figure 3.1

A.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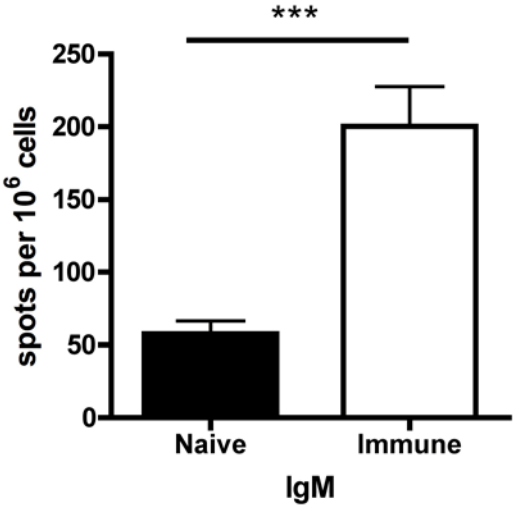
IgG ng/ml
Naive
Immune

B.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200
150
100
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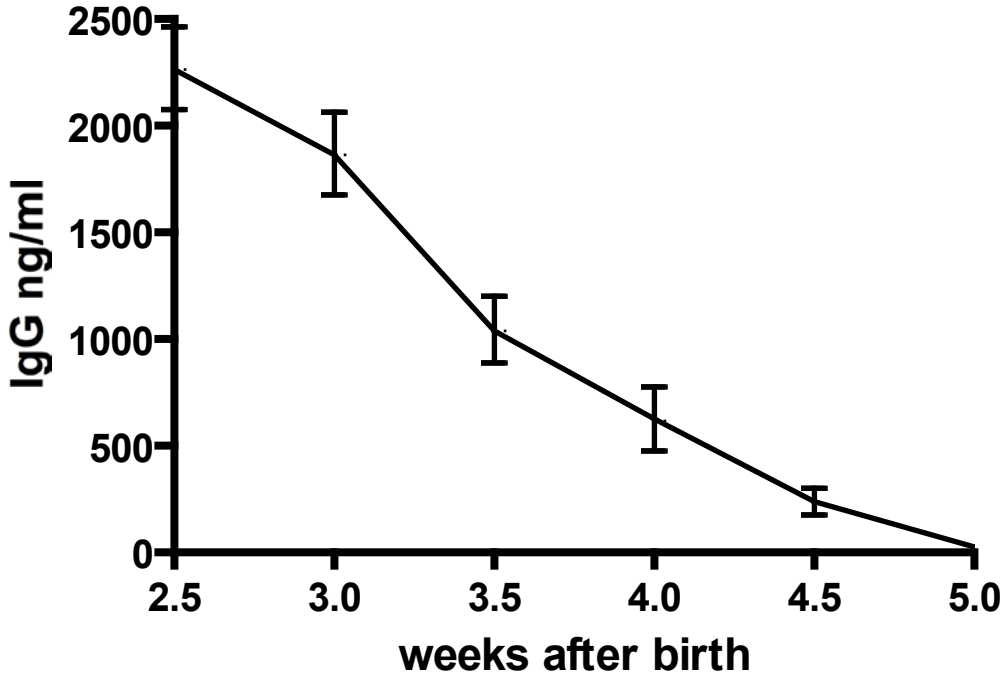
spots per 10^6 cells
Naive
Immune
IgM

Figure 3.2


2500
2000
1500
1000
500
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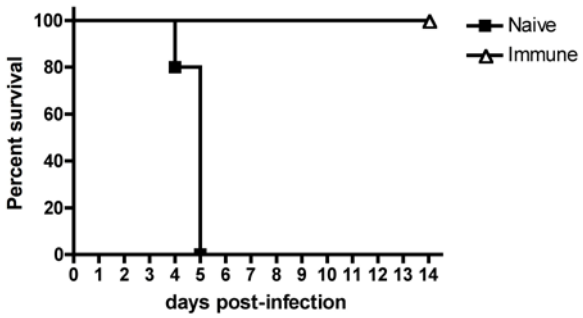
IgG ng/ml
2.5
3.0
3.5
4.0
4.5
5.0
weeks after birth

Figure 3.3

A.



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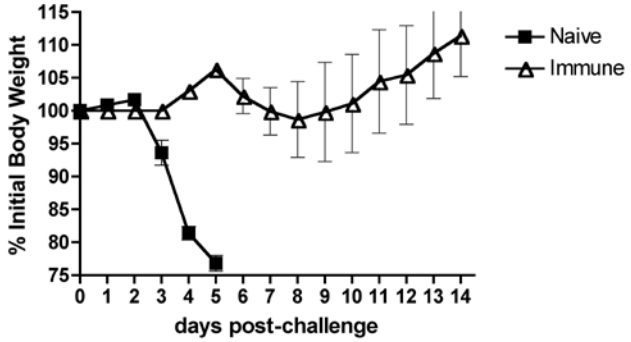

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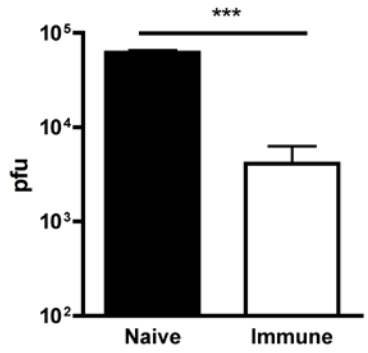

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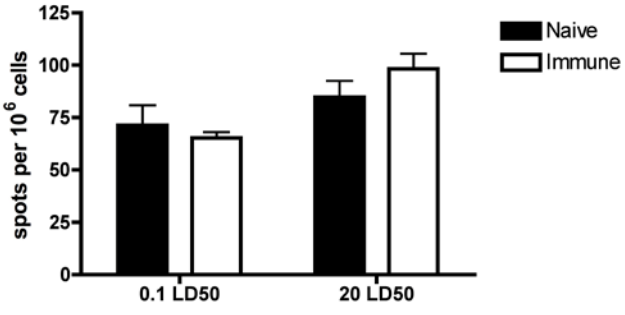

Figure 3.4

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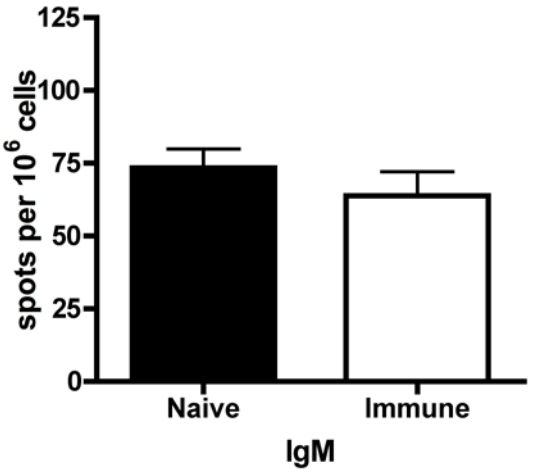
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100
75
50
25
0
spots per 10^6 cells
Naive
Immune
IgM

B.

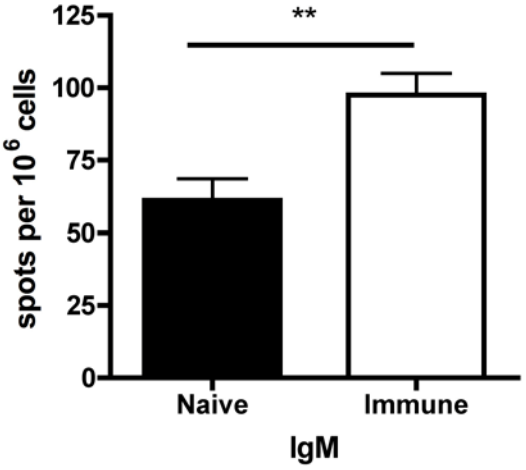
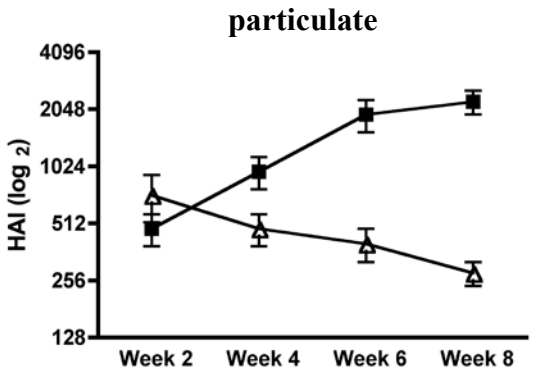

**
125
100
75
50
25
0
spots per 10^6 cells
Naive
Immune
IgM

Figure 3.5

A.



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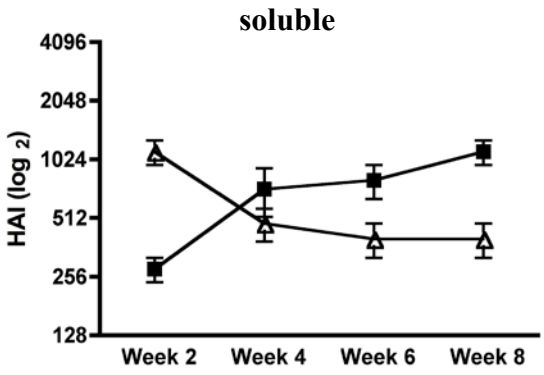

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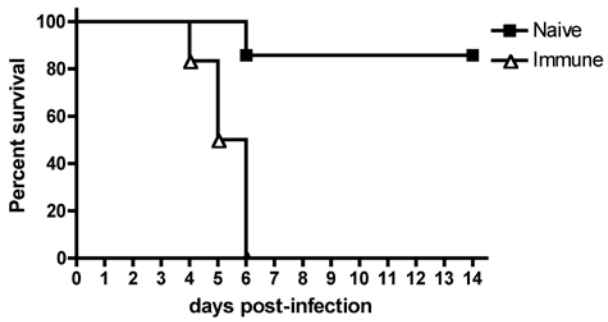

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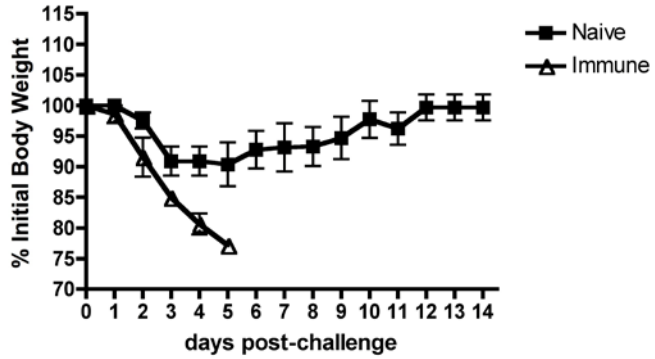
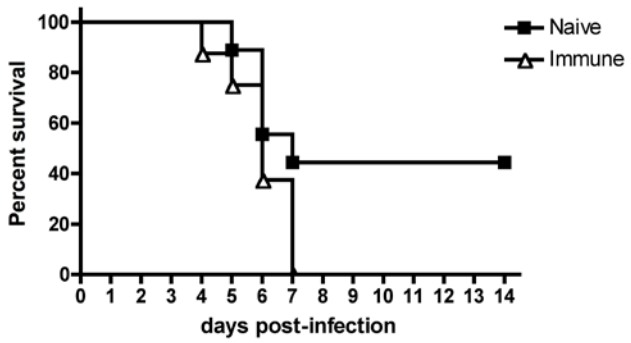


Figure 3.5 cont.

E.



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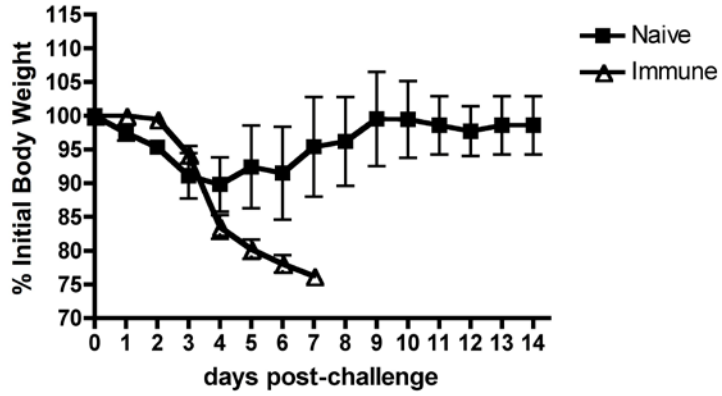

Chapter 4

Discussion

By: Chelsey L. Goins

Introduction

The powerful ability of antibody to modulate humoral immune responses is well documented. Here we add to that knowledge base with two studies examining the effects of stimulation or suppression of immune responses by antibodies. In the first we examined the role of immune complexes (ICs) in creating the secondary antibody response. We found that ICs, acting through Fc receptors, were responsible for the majority of the cellular activation seen during secondary antibody responses. Additionally, we showed that many of the cells participating in the secondary antibody response are naïve B cells, newly activated by ICs. In the second study we looked in depth at the ability of maternally-derived antibodies (MatAbs) to influence the development of the infant immune system. We found that we could overcome the often-cited suppression of vaccine responses in the presence of MatAbs by using a soluble, rather than a particulate, vaccine antigen. We also found that MatAbs inhibit the formation of B cell memory in infants in a process that is independent of antigen type.

The new information presented here opens a variety of avenues for future study. In particular, this knowledge can be used to enhance public health. Understanding of how antibodies affect immune responses may lead to more sophisticated vaccine design. An example might be utilizing the stimulating effects of immune complexes to build more effective vaccines. In Chapter 2 we showed that pre-existing immunity to the carrier chicken gamma globulin (CGG) could enhance immune responses to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) when NP was coupled to CGG. A way to utilize this in a real-life vaccination scenario might be to create fusion vaccines, where the desired vaccine antigen is coupled to a different antigen to which most people are

immune. For example, the HIV envelope glycoprotein coupled to tetanus toxin. As with the CGG/NPCG studies, the tetanus-specific antibodies would form ICs with the tetanus/envelope fusion, causing enhanced B cell activation directed towards the HIV envelope.

In the case of infant vaccines, better understanding how MatAbs interact with vaccine antigens will lead to more precise and useful infant vaccination schedules. In Chapter 3 we showed that using soluble vaccines in the presence of MatAbs led to significantly higher B cell responses. Though these responses were short-lived, they would provide much needed immune protection early in life. It is feasible that using soluble vaccines on newborns and young infants might lead to a decrease in morbidity during the first year of life. We also showed that MatAbs block the development of B cell memory in infants. While this may cause infant vaccination to seem futile, the early protection provided by soluble vaccination along with the potential for a priming effect later argues for in-depth study of infant vaccination. Understanding how maternal antibodies block memory cell formation may lead to a method for overcoming this block, possibly through use of adjuvants or some concurrent form of immune stimulation.

Our studies raise a number of interesting questions regarding how the humoral arm of the immune system functions and, in particular, how antibodies modulate these responses. The potential applications in the field of vaccine design are numerous, but it is clear that before we can move forward in that regard we must first answer a number of questions. Here we will now address three important questions raised by this research.

Fate of antibody-activated B cells

The first, and likely most important, question we must ask concerns the fate of B cells activated by antibody in the form of ICs. Based on the data presented in Chapter 2, we know that ICs activate naïve B cells rapidly, but we do not know what happens after the initial B cell response. Do these IC activated B cells differentiate into memory, adding to the diversity of the immune pool, or do they simply fulfill their function and die? While the fate of IC-activated cells in infants has been clearly demonstrated – short-lived cell activation followed by cell death and a lack of functional memory – the immune repertoire of adult mice differs substantially, suggesting a different outcome for these cells.

Early work by Klaus on IC-mediated activation of B cells showed clear memory formation after the primary response (56). In fact, immunization with ICs resulted in more rapid formation of germinal centers, accelerated affinity maturation, and enhanced numbers of memory cells as compared to immunization with antigen in alum. In Klaus's work, however, the effect of ICs was examined during the primary response, which has the formation of memory as its duty.

The secondary response exists to provide immunity, not to form memory, so the fate of cells activated at this time is less clear. However, germinal centers form after secondary immunization (147, 148). This has long been a puzzling feature of the secondary response, as memory cells do not reenter germinal centers (149). Were they to undergo further hypermutation, the effect on their already highly mutated antigen receptors would only be deleterious. Since memory B cells do not enter germinal centers, naïve B cells must feed the follicles that develop after secondary immunization. These

cells will undergo hypermutation and ultimately join the memory pool. While future memory cells undergo maturation within the germinal center, their short-lived, non-mutated counterparts are able to participate in secondary responses (50).

Examining the likely players in B cell activation during the secondary antibody response may go a long way towards understanding the fate of these activated cells. Because of the size and the nature of ICs, the type and location of cells that may present them as an antigen is different from those that present non-complexed antigens. In Chapter 2 we showed that the intermediary cell involved in IC presentation must express an activating Fcγ receptor, containing the γ signaling chain. This limits the potential cells involved to macrophages and dendritic cells (130).

One cell type that is certainly involved in IC-mediated cell activation is the follicular dendritic cell (FDC). These cells are involved in all B cell activation that results in memory formation, as they drive the germinal center response. IC bound to their surfaces through complement and Fc receptors serves as antigen for B cell stimulation (21). Another cell type potentially involved in IC-mediated activation of B cells is the large population of antigen-presenting cells found just below the subcapsular sinus in secondary lymphoid organs. These cells transport large antigens, such as ICs, to B cells within secondary follicles, making them available for B cell stimulation as well as transfer to FDCs (150). While called 'macrophages', these cells are functionally and phenotypically distinct from other macrophages. In particular, they have limited phagocytic function, and so are able to present intact antigen on their surface. In terms of B cell activation by ICs, this is an extremely important ability.

Immunological synapses have been most thoroughly described for T cell-APC interactions (151). There is a growing body of research, however, that suggests that these synapses also form between B cells and APCs (152-154). B cells can respond to antigen through many different mechanisms, but membrane associated antigens are the most powerful B cell activators (155). This is because membrane-bound antigen has at least two abilities that soluble antigen does not. Membrane-bound antigen can crosslink multiple B cell receptors, as well as B cell coreceptor molecules such as CD19, enhancing the activating signal received by the cell. Membrane-bound antigen can also work in synergy with cell-surface integrins to form a synapse, causing colocalization of a multitude of B cell signaling molecules, and increasing the amount of time the B cell is in contact with the APC (154).

All of these activating properties of membrane-bound antigen occur because the antigen can be held in its native, unprocessed form on the APC surface. This happens most often with ICs, which can bind to Fc receptors. Many cells will internalize and degrade ICs bound to Fc receptors. Cells with limited phagocytic ability, such as subcapsular macrophages, do not do this. Cell-surface presentation of intact antigen also occurs through a process of endocytic vesicle recycling, where cells internalize Fc-bound ICs, hold them intact in vesicles, and then redisplay the Fc-bound IC on its surface at the appropriate time. This process is mediated by the FcγRIIB (156). Since IC-mediated activation of B cells occurs independently of FcγRIIB, and is even enhanced in its absence, this method is likely not responsible for B cell activation by ICs (63).

Because of their ability to hold antigen intact on their surface, and because they transfer large antigens that could otherwise not diffuse into the lymph node, subcapsular

macrophages certainly play an important role in IC-mediated B cell activation. In fact, it has been shown that these macrophages are the main players in the presentation of large antigens to B cells (157, 158). Their depletion results in an inability to retain antigen within the lymph node, dissemination of pathogen, and decreased B cell activation (159). Because subcapsular macrophages shuttle antigen to the lymph nodes, they would be responsible for the development of memory cells. The cells that respond early after secondary immunization are not memory cells, nor are they naïve cells undergoing memory development. They are short-lived plasma cells, which are not activated in lymph nodes. This suggests there may be an alternate activation method for these early-responder cells.

The population of DCs resident in the T cell area, or paracortex, of the lymph node is a likely candidate for activators of IC-induced short-lived plasma cells. These DCs congregate around high endothelial venules and take up antigen as soon as it enters the lymph node (160). Because they are located in the paracortex, these DCs are able to interact with both T and B cells. This would allow concurrant activation of T and B cells, and provide T cell help for newly activated B cells. It has been suggested that these DCs are responsible for the development of short-lived plasma cells (21).

Based on these pieces of information, the model for B cell fate following IC-induced activation, including following secondary immunization, is as follows: Paracortical DCs take up ICs in the T cell zone. Some of this antigen is held in its native form on the cell surface, while some is processed and presented on MHC molecules. Antigen is presented to B cells and T cells, causing their activation. T cells aid in the activation of B cells, which differentiate into short-lived plasma cells and begin

producing antibody immediately. This is the source of a large amount of the antibody seen during the secondary antibody response. At the same time, subcapsular macrophages are acquiring ICs and transporting them to lymphoid follicles. Here they aid in the activation of B cells and the beginning of a GC reaction. The cells that participate in the reaction eventually form memory B cells and long-lived plasma cells. These cells incorporate into the B cell memory pool, adding to its diversity. This model is summarized in Figure 4.1.

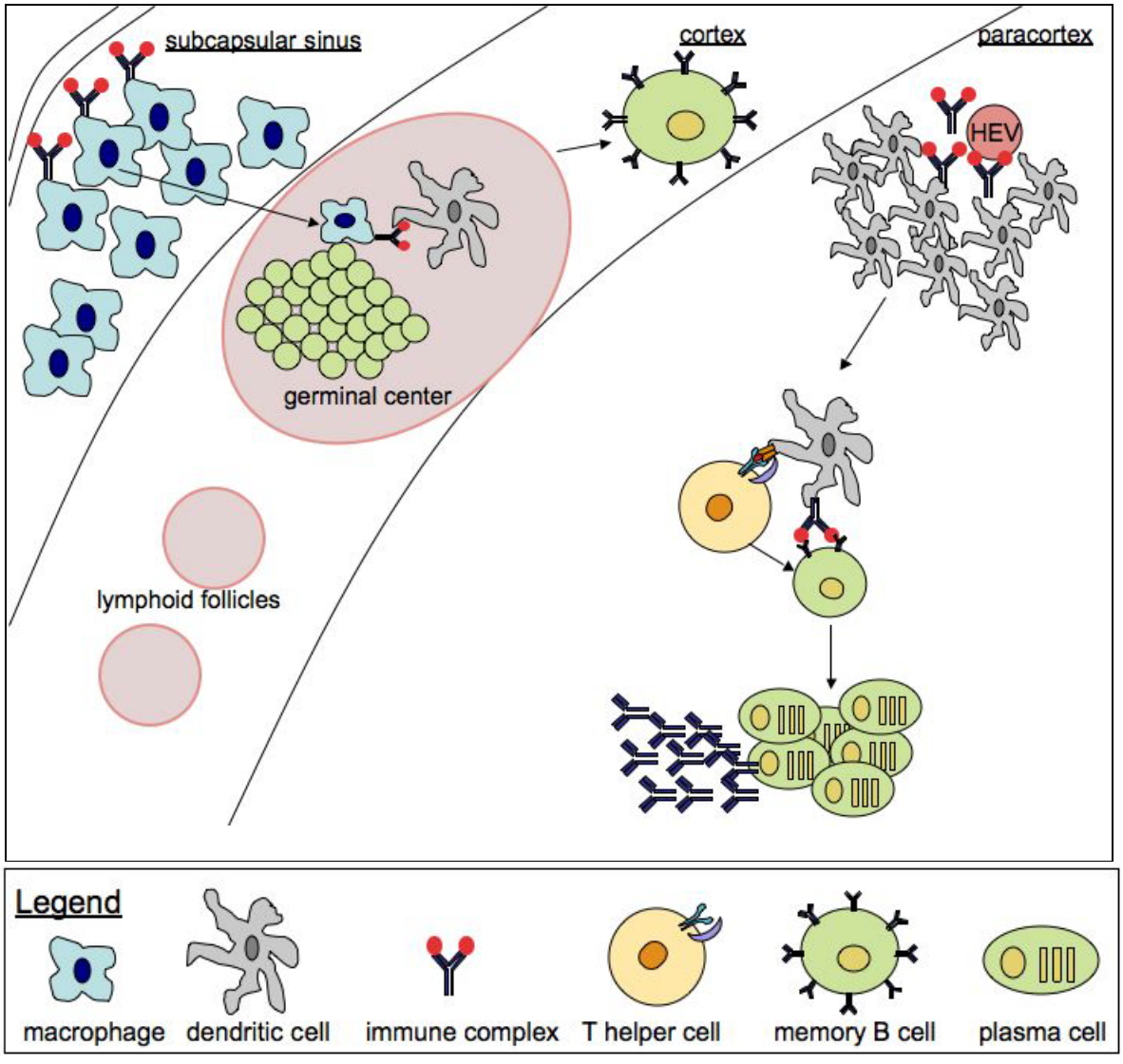

Figure 4.1: A model for IC-mediated activation of naïve B cells in the lymph node.

IgM immune complexes

As discussed in Chapter 1, IgG ICs are only stimulatory when coupled with soluble antigen. Indeed, as we saw in Chapter 3, IgG complexed with particulate antigen results in suppression of immune responses. Our studies of the secondary antibody response in Chapter 2 focused on immunizations with a soluble antigen, namely NPCG. We must then wonder if our results would have been similar using a particulate antigen. Most antigens the immune system will encounter, including pathogens and vaccines, will be particulate. It is necessary to understand how antibodies modulate responses to particulate antigen in order to better understand how immune responses form.

Unlike IgG, IgM antibodies enhance humoral immune responses when complexed with particulate antigen. This is the most commonly observed immunoregulatory function of IgM (119). In studies of IgM-mediated responses, IgM given with RBCs resulted in enhanced IgM, IgG, and IgE titers, as well as increased Th and memory B cell formation (59, 60, 161-163). The enhancing effect of IgM can be seen as early as three days following immunization (60) and the resulting enhancement lasts for more than three months (59). Enhancement of immune responses via IgM have also been observed in pathogenic infections and vaccines.

The mechanism of this enhancement obviously differs from that of IgG-mediated enhancement, as IgM cannot bind Fcγ receptors. It is T cell dependent, as it does not occur in nude mice which lack T cells (164). Unlike IgG, IgM-mediated enhancement is complement dependent. It does not occur when complement has been depleted, or in the

absence of the complement receptor Cr1/2 (65, 66). In Chapter 2 we showed that IgG enhancement is unaffected by the absence of complement or its associated receptors.

The question we must then ask is whether IgM ICs play a role similar to that of IgG ICs in creating the secondary antibody response. In Chapter 2 we showed that blocking IgG ICs resulted in almost complete ablation of the secondary response, suggesting that ICs did not just enhance the response, but were entirely responsible for its creation. What remains unknown is whether blocking IgM ICs would have a similar effect in a secondary response induced by particulate antigen. Since we do not know the precise mechanism of IgM-mediated enhancement, this is a difficult question to resolve. Complement has been shown to be necessary for IgM-mediated enhancement, likely due to its role in the formation of IgM ICs. Therefore depletion of complement prior to secondary immunization may be one way to study this question.

IgG ICs exert their function through Fcγ receptors. While IgM complexes will not bind to Fcγ receptors, they may function through the Fcμ receptor. Tools for studying this receptor are few, as it has been difficult to find and characterize. Recently the receptor was identified in humans and found to be present on $CD19^+$ memory and naïve B cells, as well as $CD4^+$ and $CD8^+$ T cells and some natural killer cells (165). Early studies of this receptor have found that it plays a role in B cell activation, survival, and proliferation. This suggests a mechanism for IgM action similar to that for IgG. Hopefully this new information about the IgM receptor will lead to a greater understanding of IgM IC function, particularly through the development of new reagents to study the Fcμ receptor and its interactions with ICs.

Effects of maternal antibody on memory formation

Perhaps the most puzzling piece of new data presented here is the discovery that MatAbs somehow inhibit the development of memory B cell responses. In Chapter 3 we examined the effects of maternally-derived IgG on infant immune responses to soluble and particulate antigen. While short-term responses to soluble antigen were enhanced in the presence of maternal antibody, protective memory failed to develop. This was also the case when particulate antigen was used as the immunogen. When MatAbs were not present, B cell memory developed unimpeded. Since MatAbs are a necessary and unavoidable obstacle in infant vaccine development, it is important to understand how and why this memory blockage occurs.

Certainly the immaturity of the infant immune system plays a role in this blockade. In particular, secondary lymphoid organs are poorly defined. This suggests the possibility that not all cells involved in the formation of memory have developed. Specifically, T cells and DCs are slow to populate secondary lymphoid organs, and marginal zones have not yet formed, suggesting a deficiency in marginal zone B cells (84). However, infants that lack MatAbs are able to develop memory responses that, while weak, are functional. Clearly the underdeveloped nature of the infant immune system is not the only factor involved in this memory blockade.

Another related possibility is that MatAbs do not block the development of memory cells themselves, but instead exert a suppressive effect on another cell involved in memory B cell development. We know that MatAbs do not inhibit T cell development (97, 166), and so it is likely any suppressive effect would not impact Th cells. The effect of MatAbs on DCs and other antigen-presenting cells is not known. A MatAb-mediated

suppression of response at this level would certainly affect the development of B cell memory, and so is an area worthy of further investigation.

While the lack of memory following immunization in the presence of MatAbs is discouraging, it does not have to be the death knell for infant vaccination. Certainly the immune boost seen shortly following soluble antigen immunization is encouraging, particularly as these additional antibodies will provide much needed protection from infection early in life. When MatAb levels are too low to be protective on their own, this may serve as a way to boost protection early in life. Additionally, despite not converting to memory, this early antibody response may serve to prime the infant immune system for later boosting. Several studies have shown that priming of immune responses occurs following immunizations early in life, even if no immune response is detected at the time (83, 167). In the case where an immune response is readily observed, such as with soluble antigen immunization, this priming almost certainly occurs. We know that T cells are activated, and the additional antibody produced will provide an IC-mediated boost to immune responses later when the immune system is more mature. Additionally, certain non-alum adjuvant formulations, such as CpG or Titermax, can enhance infant vaccine responses where others cannot (168, 169). All of our infant vaccination studies were done using unadjuvanted antigen. It will be interesting to see what immunological differences appear with the additional immune stimulation of an adjuvant.

The discovery of an immune enhancing benefit to using soluble antigens in infant vaccination is an important step in improving infant vaccine regimens and improving overall health early in life. Before soluble vaccines can be considered, though, we must better understand how MatAbs interact with the infant immune system. The first step of

this process will be discerning the mechanism through which MatAbs inhibit memory B cell formation.

References

1. Tangye, S. G., and Tarlinton, D.M. 2009. Memory B cells: Effectors of long-lived immune responses. *Eur J Immunol* 39:2065-2075.
2. Janeway, C. A., Jr., and Medzhitov, R. 2002. Innate immune recognition. *Ann Rev Immunol* 20.
3. Beverley, P. C. L. 2002. Immunology of vaccination. *British Medical Bulletin* 62:15-28.
4. Siegrist, C.-A. 2008. Vaccine immunology. In *Vaccines, 5th edition*. S. A. Plotkin, Orenstein, W.A., and Offit, P.A., ed. Elsevier. 17-36.
5. CDC. 2010. Recommended immunization schedule for persons aged 0 through 6 years.
6. WHO. 2005. Immunization against diseases of public health importance [Fact Sheet].
7. Cambier, J. C., S. B. Gauld, K. T. Merrell, and B. J. Vilen. 2007. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol* 7:633-643.
8. Friedberg, S. H., and Weissman, I.L. 1974. Lymphoid tissue architecture. II. Ontogeny of peripheral T and B cells in mice: evidence against Peyer's patches as the site of generation of B cells. *J Immunol* 113:1477-1492.
9. Lanzavecchia, A. 1985. Antigen-specific interaction between T and B cells. *Nature* 314:537-539.
10. Takahashi, Y., D. M. Cerasoli, J. M. Dal Porto, M. Shimoda, R. Freund, W. Fang, D. G. Telander, E-N Malvey, D. L. Mueller, T. W. Behrens, and G. Kelsoe. 1999. Relaxed negative selection in germinal centers and impaired affinity maturation in bcl-xL transgenic mice. *J Exp Med* 190:399-410.
11. McHeyzer-Williams, L. J. and M. G. McHeyzer-Williams. 2005. Antigen-specific memory B cell development. *Ann Rev Immunol* 23:487-513.
12. Armitage, R. J., Fanslow, W. C., L. Strockbine, T. A. Sato, K. N. Clifford, B. M. Macduff, D. M. Anderson, S. D. Gimpel, T. Davis-Smith, C. R. Maliszewski, E. A. Clark, C. A. Smith, K. H. Grabstein, D. Cosman, and M. K. Spriggs. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature* 357:80-82.
13. Snapper, C. M., and W. E. Paul. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236:944-947.
14. Jacob, J., Kassir, R., and Kelsoe, G. 1991. 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* 173:1165-1175.
15. Ho F., J. E. L., I. C. M. MacLennan, and M. Khan. 1986. Distinct short-lived and long-lived antibody-producing cell populations. *Eur J Immunol* 16:1297-1301.
16. Allen, C. D. C., K. M. Ansel, C. Low, R. Lesley, H. Tamamura, N. Fujii, and J. G. Cyster. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 5:943-952.

17. Park, C.-S. a. C., Yong Sung. 2005. How do follicular dendritic cells interact intimately with B cells in the germinal centre? *Immunology* 114:2-10.
18. Cozine, C. L., K. L. Wolniak, and T. J. Waldschmidt. 2005. The primary germinal center response in mice. *Current Opinion in Immunology* 17:298-302.
19. MacLennan, I. C. M. 1994. Germinal Centers. *Annual Review of Immunology* 12:117-139.
20. French, D., R. Laskov, and M. Scharff. 1989. The role of somatic hypermutation in the generation of antibody diversity. *Science* 244:1152-1157.
21. Batista, F. D., and Harwood, N.E. 2009. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* 9:15-27.
22. Slifka, M. K., Antia, R., Whitmire, J.K., and Ahmed, R. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* 8:363-372.
23. Hibi, T., and Dosch, H.M. 1986. Limiting dilution analysis of the B cell compartment in human bone marrow. *Eur J Immunol* 16:139-145.
24. Tarlinton, D., Radbruch, A., Hiepe, F., and Doner, T. 2008. Plasma cell differentiation and survival. *Curr Opin Immunol* 20:162-169.
25. Klein, U., Rajewskey, K, and R Küppers. 1998. Human Immunoglobulin (Ig)M+IgD+ Peripheral Blood B Cells Expressing the CD27 Cell Surface Antigen Carry Somatically Mutated Variable Region Genes: CD27 as a General Marker for Somatically Mutated (Memory) B Cells. *J Exp Med* 188:1679.
26. Tangye, S. G., Liu, Y.J., Aversa, G., Phillips, J.H., and de Vries, J.E. 1998. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J Exp Med* 188:1691-1703.
27. Klein, U., Kuppers, R., and Rajewsky, K. 1997. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood* 89:1288-1298.
28. Weller, S., M. C. Braun, B. K. Tan, A. Rosenwald, C. Cordier, M. E. Conley, A. Plebani, D. S. Kumararatne, D. Bonnet, O. Tournilhac, G. Tchernia, B. Steiniger, L. M. Staudt, J.-L. Casanova, C.-A. Reynaud, and J.-C. Weill. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 104:3647-3654.
29. Weller, S., A. Faili, C. Garcia, M. C. Braun, F. L. Deist, G. d. S. Basile, O. Hermine, A. Fischer, C.-A. Reynaud, and J.-C. Weill. 2001. CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc Natl Acad Sci U S A* 98:1166-1170.
30. Tangye, S. G., and K. L. Good. 2007. Human IgM+CD27+ B Cells: Memory B Cells or "Memory" B Cells? *J Immunol* 179:13-19.
31. Gray, D., and Skarvall, H. 1988. B-cell memory is short-lived in the absence of antigen. *Nature* 336:70-73.
32. Maruyama, M., Lam, K-P, and Rajewsky, K. 2000. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature* 407:636-642.
33. Paramithiotis, E., and Cooper, M.D. 1997. Memory B lymphocytes migrate to the bone marrow in humans. *Proc Natl Acad Sci U S A* 94:208-212.
34. Smith, K. G., A. Light, G. J. Nossal, and D. M. Tarlinton. 1997. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO J* 16:2996-3006.

35. O'Connor BP, V. L., Zhang W. Loo W, Shnider D, Lind EF, Ratliff M, Noelle RJ, Erickson LD. 2006. Imprinting the fate of antigen-reactive B cells through the affinity of the B cell receptor. *J Immunol* 177:7723-7732.
36. Good, K. L., and Tangye, S.G. 2007. Decreased expression of Kruppel-like factors in memory B cells induces the rapid response typical of secondary antibody responses. *Proc Natl Acad Sci U S A* 104:13420-13425.
37. Chappell, C. P., J. Dauner, and J. Jacob. 2009. Ontogeny of the secondary antibody response: origins and clonal diversity. In *Crossroads between Innate and Adaptive Immunity II*. N. Back, I. R. Cohen, D. Kritchevsky, A. Lajtha, and R. Paoletti, eds. Springer New York. 27-41.
38. Bruggemann, M., Williams, G.T., Bindon, C.I., Clark, M.R., Walker, M.R., Jefferis, R., Waldmann, H., and Neuberger, M.S. 1987. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med* 166:1351-1361.
39. Schmidlin H, D. S., Blom B. 2009. New insights into the regulation of human B-cell differentiation. *Trends Immunol* 30:277-285.
40. Kavaler, J., Caton, A.J., Staudt, L.M., and W.U. Gerhard. 1991. A B cell population that dominates the primary response to influenza virus hemagglutinin does not participate in the memory response. *Eur J Immunol* 21.
41. Randen, I., Pascual, V., Victor, K., Thompson, K.M., Forre, O., Capra, J.D., and J.B. Natvig. 1993. Synovial IgG rheumatoid factors show evidence of an antigen-driven immune response and a shift in the V gene repertoire compared to IgM rheumatoid factors. *Eur J Immunol* 23:1220-1225.
42. Scott, P. D., Ochola, R., Sande, C., Ngama, M., Okiro, E.A., Medley, G.F., Nokes, D.J., and P.A. Cane. 2007. Comparison of Strain-Specific Antibody Responses During Primary and Secondary Infections with Respiratory Syncytial Virus. *J Med Virol* 79:1943-1950.
43. Blier, P. R., and A. L. Bothwell. 1988. The immune response to the hapten NP in C57BL/6 mice: insights into the structure of the B-cell repertoire. *Immunol Rev* 105:27-43.
44. Weiss, U., and Rajewsky, K. 1990. The repertoire of somatic antibody mutants accumulating in the memory compartment after primary immunization is restricted through affinity maturation and mirrors that expressed in the secondary response. *J Exp Med* 172:1681-1689.
45. Lu, Y.-F., M. Singh, and J. Cerny. 2001. Canonical germinal center B cells may not dominate the memory response to antigenic challenge. *Internat Immunol* 13:643-655.
46. Berek, C., G. M. Griffiths, and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. *Nature* 316:412-418.
47. Griffiths, G. M., C. Berek, M. Kaartinen, and C. Milstein. 1984. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature* 312:271-275.
48. Berek, C., and C. Milstein. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol Rev* 96:23-41.

49. Linton, P.-J., Decker, D.J., and N.R. Klinman. 1989. Primary Antibody-Forming Cells and Secondary B Cells are Generated from Separate Precursor Cell Subpopulations. *Cell* 59:1049-1059.
50. Chappell, C. P., and J. Jacob. 2006. Identification of Memory B Cells Using a Novel Transgenic Mouse Model. *J Immunol* 176:4706-4715.
51. Matsumoto, M., S. F. Lo, C. J. L. Carruthers, J. Min, S. Mariathasan, G. Huang, D. R. Plas, S. M. Martin, R. S. Geha, M. H. Nahm, and D. D. Chaplin. 1996. Affinity maturation without germinal centres in lymphotoxin-[alpha]-deficient mice. *Nature* 382:462-466.
52. Brady, L. J. 2005. Antibody-mediated immunomodulation: a strategy to improve host responses against microbial antigens. *Infection and Immunity* 73:671-678.
53. Park, W. H., Schroder, M.C., and Zingher, A. 1922. The Control of Diphtheria. *Am J Pub Health*:23-32.
54. Klaus, G. G. 1978. The generation of memory cells. II. Generation of B memory cells with preofrmed antigen-antibody complexes. *Immunology* 34:643-652.
55. Klaus, G. G. B. 1979. The generation of memory cells. III. Antibody class requirements for the generation of B-memory cells by antigen-antibody complexes. *Immunology* 37:345-351.
56. Kunkl, A., and G. G. Klaus. 1981. The generation of memory cells. IV. Immunization with antigen-antibody complexes accelerates the development of B-memory cells, the formation of germinal centres, and the maturation of antibody affinity in the secondary response. *Immunology* 43:371-378.
57. Kunkl, A., and G. G. Klaus. 1981. The generation of memory cells. V. Preferential priming of IgG1 B memory cells by immunization with antigen IgG2 antibody complexes. *Immunology* 44:163-168.
58. Klaus, G. G. 1979. The role of antigen-antibody complexes in generating immunological memory and auto-antiidiotypic immunity. *Adv Exp Med Biol* 114:289-294.
59. Heyman, B., and Wigzell, H. 1985. IgM enhances and IgG suppresses immunological memory in mice. *Scand J Immunol* 21:255-266.
60. Heyman, B., Andrighetto, S., and Wigzell, H. 1982. Antigen dependent IgM-mediated enhancement of the sheep erythrocyte response in mice: evidence for induction of B cells with specificities other than that of the injected antibodies. *J Exp Med* 155:994-1009.
61. Klaus, G. G. 1982. Immune complexes and the problem of immunological memory. *Ann Immunol (Inst Pasteur)* 133:221-227.
62. Wiersma, E. J. 1992. Enhancement of the antibody response to protein antigens by specific IgG under different experimental conditions. *Scand J Immunol* 36:193-200.
63. Wernersson, S., M. C. Karlsson, J. Dahlstrom, R. Mattsson, J. S. Verbeek, and B. Heyman. 1999. IgG-mediated enhancement of antibody responses is low in Fc receptor gamma chain-deficient mice and increased in Fc gamma RII-deficient mice. *J Immunol* 163:618-622.
64. Wiersma, E. J., M. Nose, and B. Heyman. 1990. Evidence of IgG-mediated enhancement of the antibody response in vivo without complement activation via the classical pathway. *Eur J Immunol* 20:2585-2589.

65. Heyman, B., Pilstrom, L., Shulman, M.J. 1988. Complement activation is required for IgM-mediated enhancement of the antibody response. *J Exp Med* 167:1999-2004.
66. Applequist, S. E., Dahlstrom, J., Jiang, N., Molina, H., and Heyman, B. 2000. Antibody production in mice deficient for complement receptors 1 and 2 can be induced by IgG/Ag and IgE/Ag, but not IgM/Ag complexes. *J Immunol* 165:2398-2403.
67. Houston, W. E., Kremer, R.J., Crabbs, C.L., and Spertzel, R.O. 1977. Inactivated Venezuelan equine encephalomyelitis virus vaccine complexed with specific antibody: enhanced primary immune response and altered patter of antibody class elicited. *J Infect Dis* 135:600-610.
68. Porkric, B., Sladic, D., Juros, S., and Cajavec, S. 1993. Application of the immune complex for immune protection against viral disease. *Vaccine* 11:655-659.
69. Randall, R. E., Young, D.F., and Southern, J.A. 1988. Immunization with solid matrix-antibody-antigen complexes containing surface or internal virus structural proteins protects mice from infection with the paramyxovirus, simian virus 5. *J Gen Virol* 69:2517-2526.
70. Hanke, T., Botting, C., Green, E.A., Szawlowski, P.W., Rud, E., and Randall, R.E. 1994. Expression and purification of nonglycosylated SIV proteins and their use in induction and detection of SIV-specific immune responses. *AIDS Res Hum Retroviruses* 10:665-674.
71. Taborda, C. P., and Casadevall, A. 2001. Immunoglobulin M efficacy against *Cryptococcus neoformans*: mechanism, dose dependence, and prozone-like effects in passive protection experiments. *J Immunol* 166:2100-2107.
72. Barker C.F., B., R.E. 1977. Immunologically priviledged sites. *Adv Immunol* 25:1-54.
73. Wegman, T. G., Lin, H., Guildbert, L., and Mosman, T.R. 1993. Bi-directional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? *Immunology Today* 14:353-356.
74. Lin, H., Mosman, T.R., Guilbert, L., Tuntipopipat, S., and Wegman, T.G. 1993. Synthesis of T-helper 3 type cytokines at the maternal-fetal interface. *J Immunol* 151:4562-4573.
75. Sachs, G., Sargent, I., and Redman, C. 1999. An innate view of human pregnancy. *Immunology Today* 20:114-118.
76. Aschkenazi, S., Straszewski, S., Verwer, K.M., Foellme, H., Rutherford, T., and Mor, G. 2002. Differential regulation and function of the FAS/FAS ligand system in human trophoblast cells. *Biology of Reproduction* 66:1853-1861.
77. Barrios, C., Brawand, P., Berney, M., Brandt, C., Lambert, P.H., and Siegrist, C.A. 1996. Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. *Eur J Immunol* 26:1489-1496.
78. Romagnani, S. 2001. Th1/Th2 cells. *Inflamm Bowel Dis* 5:285-294.
79. Zinkernagel, R. M. 2001. Maternal antibodies, childhood infections and autoimmune diseases. *N Engl J Med* 345:1331-1335.

80. Morein, B., Blomqvist, G., and Hu, K. 2007. Immune Responsiveness in the Neonatal Period. *J Comp Path* 137:S27-S31.
81. Brazolog Millan, C. L., Weeratna, R., Krieg, A.M., Siegrist, C.A., and Davis, H.L. 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatits B surface antigen in young mice. *Proc Natl Acad Sci U S A* 95:15553-15558.
82. Stoll, B. J., Lee, F.K., Hale, E., Schwartz, D., Holmes, R., Ashby, R., Czerkinsky, C., and Nahmias, A.J. 1993. Immunoglobulin secretion by the normal and the infected newborn infant. *J Pediatr* 122:780-786.
83. Dong, D. X., Hu, X.M., Liu, W.J., Li, J.S., Jin, Y.C., Tan, S.G., Chen, T.Q., Fu, J.Z., Niu, B.Y., Yu, H.M., and Yun, Z. 1986. Immunization of neonates with trivalent oral poliomyelitis vaccines (Sabin). *Bull WHO* 64:853-860.
84. Siegrist, C.-A. 2001. Neonatal and early life vaccinology. *Vaccine* 19:3331-3346.
85. Rothenberg, E., and Triglia, D. 1983. Clonal proliferation unlinked to terminal deoxynucleotidyl transferase synthesis in thymocytes of young mice. *J Immunol* 130:1627-1633.
86. Wells, W. J., Parkman, R., Smogorzewska, E., and Barr, M. 1998. Neonatal thymectomy: does it affect immune function? *J Thorac Cardiovasc Surg* 115:1041-1046.
87. Hanson, L. A., Korotkova, M., Lundin, S., Haversen, L., Silfverdal, S.A., Mattsby-Baltzer, I., Strandvik, B., and Telemo, E. 2003. The transfer of immunity from mother to child. *Ann NY Acad Sci* 987:199-206.
88. Reuman, P. D., Paganini, C.M.A., Ayoub, E.M., and Small, P.A. 1983. Maternal-infant transfer of influenza-specific immunity in the mouse. *J Immunol* 130:932-936.
89. Van de Perre, P. 2003. Transfer of antibody via mother's milk. *Vaccine* 21:3374-3376.
90. Englund, J. A. 2007. The influence of maternal immunization on infant immune responses. *J Comp Path* 137:S16-S19.
91. Firan, M., R. Bawdon, C. Radu, R. J. Ober, D. Eaken, F. Antohe, V. Ghetie, and E. S. Ward. 2001. The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of {gamma}-globulin in humans. *Int. Immunol.* 13:993-1002.
92. Roopenian, D. C., and Akilesh, S. 2007. FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol* 7:715-725.
93. Corte, E. D., and Parkhouse, R.M.E. 1973. Biosynthesis of immunoglobulin A (IgA). Secretion and addition of carboyhydrate to monomer and polymer forms of a mouse myeloma protein. *Biochem J* 136:589-596.
94. Kaul, A., and Smith, G.F. 1980. Immunobiology of the Fetus and Newborn -- Historical Perspectives and Recent Advances. In *Historical Review and Recent Advances in Neonatal and Perinatal Medicine*. G. F. Smith, and Vidyasagar, D., ed. Mead Johnson Nutritional Division.
95. Zaman, K., Roy, E., Arifeen, S.E., Rahman, M., Rqib, R., Wilson, E., Omer, S.B., Shahid, N.S., Breiman, R.F., and Steinhoff, M.C. 2008. Effectiveness of maternal influenza immunization in mothers and infants. *N Engl J Med* 359:1555-1564.

96. Gall, S. A. 2005. Maternal immunization to protect the mother and neonate. *Expert Review of Vaccines* 4:813-818.
97. Siegrist, C.-A. 2003. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. *Vaccine* 21:3406-3412.
98. Perkins, F. T., Yetts, R., and Gaisford, W. 1959. A comparison of the responses of 100 infants to primary poliomyelitis immunization with two and with three doses of vaccine. *Br Med J* 1:1083-1086.
99. Ruiz-Palacios, G. M. 2002. Impact of maternal antibodies on the immune response to an oral human rotavirus vaccine in Mexican infants. In *In: Proceedings of the 3rd World Congress of Pediatric Infectious Dseases*, Chile: Santiago.
100. Karron, R. A., Steinhoff, M.C., Sbbarao, E.K., et al. 1995. Safety and immunogenicity of a cold-adapted influenza A (H1N1) reassortant virus vaccine administered to infants less than 6 months of age. *Pediatr Infect Dis J* 14:10-16.
101. Sarvas, H., Kurikka, S., Seppala, I.U., Makela, P.H., and Makela, O. 1992. Maternal antibodies partly inhibit an active antibody response to routine tetanus toxoid immunization in infants. *J Infect Dis* 165:977-979.
102. Bjorkholm, B., Granstrom, M., Taranger, J., Wahl, M., Hagberg, L. 1995. Influence of high titers of maternal antibody on the serologic response of infants to diphtheria vaccination at three, five and twelve months of age. *Pediatr Infect Dis J* 14:846-850.
103. Troisi, C. L., Hollinger, F.B., Krause, D.S., Pickering, L.K. 1997. Immunization of seronegative infants with hepatitis A vaccine (HAVRIX SKB): a comparative study of two dosing schedules. *Vaccine* 15:1613-1617.
104. Daum, R. S., Siber, G.R., Ballanco, G.A., Sood, S.K. 1991. Serum anticapsular antibody response in the first week after immunization of adults and infants with the *Haemophilus influenzae* type b-*Neisseria meningitidis* outer membrane protein complex conjugate vaccine. *J Infect Dis* 164:1154-1159.
105. Englund, J. A., Anderson, E.L., Reed, G.F., et al. 1995. The effect of maternal antibody on the serologic response and the incidence of adverse reactions after primary immunization with acellular and whole-cell pertussis vaccines combined with diphtheria and tetanus toxoids. *Pediatrics* 96:580-584.
106. Nair, N., Gans, H., Lew-Yasukawa, L, Long-Wagar, A.C., Arvin, A., and Griffin, D.E. 2007. Age-depdendent differences in IgG isotype and avidity induced by measles vaccine received during the first year of life. *J Infect Dis* 196:1339-1345.
107. Gans, H., De Hovitz, R., Forghani, B., Deeler, J., Maldonado, Y. and Arvin, A.M. 2003. Measles and mumps vaccination as a model to investigate the developing immune system: passive and active immunity during the first year of life. *Vaccine* 21:3398-3405.
108. Maldonado, Y. A., Lawrence, E.C., DeHovitz, R., Hartzell, H., and Albrecht, P. 1995. Early loss of passive measles antibody in infants of mothers with vaccine-induced immunity. *Pediatrics* 96:447-450.
109. Aaby, P., Ibrahim, S.A., Libman, M.D., and Jensen, H. 2006. The sequence of vaccinations and increased female mortality after high-titre measles vaccine: trials from rural Sudan and Kinshasa. *Vaccine* 24:2764-2771.

110. Albrecht, P., Ennis, F.A., Saltzman, E.J., Krugman, S. 1977. Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. *J Pediatr* 91:715-718.
111. Dagan R., A., J., Mijalovsky, A., Kalmanovitch, I., Bar-Yochai, A., Thoelen, S., Safary, A., and Ashkenazi, S. 2000. Immunization against hepatitis A in the first year of life: priming despite the presence of maternal antibody. *Pediatr Infect Dis J* 11:1045-1052.
112. Cutts, F. T., Nyandu, B., Markowitz, L.E., Forsey, T., Zell, E.R., Othepa, O., and Wilkins, K. 1994. Immunogenicity of high-titre AIK-C or Edmonston-Zagreb vaccines in 3.5-month-old infants, and of medium or high-titre Edmonston-Zagreb vaccine in 6-month-old infants, in Kinshasa, Zaire. *Vaccine* 12:1311-1316.
113. Siegrist, C.-A., Cordova, M., Brandt, C., Barrios, C., Berney, M., Tougne, C., Kovarik, J., and Lambert, P.H. 1998. Determinants of infant responses to vaccines in presence of maternal antibodies. *Vaccine* 16:1409-1414.
114. Siegrist, C.-A., Barrios, C., Martinez, X., Brandt, C., Berney, M., Cordova, M., Kovarik, J., and Lambert, P.H. 1998. Influence of maternal antibodies on vaccine responses: inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice. *Eur J Immunol* 28:4138-4148.
115. Karlsson, M. C. I., Wernersson, S., Diaz de Stahl, T., Gustavsson, S., and Heyman, B. 1999. Efficient IgG-mediated suppression of primary antibody responses in Fc-gamma receptor-deficient mice. *Proc Natl Acad Sci U S A* 96:2244-2249.
116. Heyman, B., and Wigzell, H. 1984. Immunoregulation by monoclonal sheep erythrocyte specific IgG antibodies: Suppression is correlated to level of antigen binding and not to isotype. *J Immunol* 132:1136-1143.
117. Heyman, B., Nose, M., Weigle, W.O. 1985. Carbohydrate chains on IgG2b: a requirement for efficient feedback immunosuppression. *J Immunol* 134:4018-4023.
118. Heyman, B. 1989. Inhibition of IgG-mediated immunosuppression by a monoclonal anti-Fc-receptor antibody. *Scand J Immunol* 29:121-126.
119. Heyman, B. 2000. Regulation of antibody responses via antibodies, complement, and Fc receptors. *Ann Rev Immunol* 18:709-737.
120. Liu, Y.-J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C.M. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature* 342:929-931.
121. Carroll, M. C. 2004. The complement system in B cell regulation. *Mol Immunol* 41:141-146.
122. Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., Amigorena, Sebastian. 1999. Fcγ Receptor-mediated Induction of Dendritic Cell Maturation and Major Histocompatibility Complex Class I-restricted Antigen Presentation after Immune Complex Internalization. *J Exp Med* 189:371-380.
123. Liu, F., Song, YK, and Liu, D. 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* 6:1258-1266.

124. Test, S. T., J. K. Mitsuyoshi, and Y. Hu. 2005. Depletion of complement has distinct effects on the primary and secondary antibody response to a conjugate of pneumococcal serotype 14 capsular polysaccharide and a t-cell-dependent protein carrier. *Infection and Immunity* 73:277-286.
125. Slifka, M., and Ahmed, R. 1996. Limiting dilution analysis of virus-specific memory B cells by an ELISPOT assay. *J Imm Met* 199:37-46.
126. Enriques-Rincon, F. a. G. G. B. K. 1984. Differing effects of monoclonal anti-hapten antibodies on humoral responses to soluble or particulate antigens. *Immunology* 52:129-136.
127. Shashidharamurthy, R., R.A. Hennigar, S. Fuchs, P. Palaniswami, M. Sherman, and P. Selvaraj. 2008. Extravasations and emigration of neutrophils to the inflammatory site depend on the the interaction of immune-complex with Fcgamma receptors and can be effectively blocked by decoy Fcgamma receptors. *Blood* 111:894-904.
128. Shashidharamurthy R, Z. F., Amano A, Kamat A, Panchanathan R, Ezekwudo D, Zhu C, and Selvaraj P. 2009. Dynamics of the interaction of human IgG subtype immune complexes with cells expressing R and H allelic forms of a low-affinity Fcgamma CD32A. *J Immunol* 183:8216-8224.
129. Vogel, C.-W., R. Bredehorst, D. C. Fritzinger, T. Grunwalkd, P. Ziegelmüller, and M. A. Kock. 1996. Structure and function of cobra venom factor, the complement-activating protein in cobra venom. *Adv Exp Med Biol* 391:97-114.
130. Nimmerjahn, F. a. J. V. R. 2007. Fc-Receptors as Regulators of Immunity. *Adv Immunol* 96:179-204.
131. Laissue, J., H. Cottier, M. W. Hess, and R. D. Stoner. 1971. Early and enhanced germinal center formation and antibody responses in mice after primary stimulation with antigen-isologous antibody complexes as compared with antigen alone. *J Immunol* 107:822-831.
132. Coulie, P. G., and J. Van Snick. 1985. Enhancement of IgG andi-carrier responses by IgG2 anti-hapten antibodies in mice. *Eur J Immunol* 15:793-798.
133. Gray, D., I. C. MacLennan, and P. J. Lane. 1986. Virgin B cell recruitment and the lifespan of memory clones during antibody responses to 2,4-dinitrophenyl-hemocyanin. *Eur J Immunol* 16:641-648.
134. Lane, P. J., D. Gray, S. Oldfield, and I.C. MacLennan. 1986. Differences in the recruitment of virgin B cells into antibody responses to thymus-dependent and thymus-independent type-2 antigens. *Eur J Immunol* 16.
135. Batista, F. D., E. Arana, P. Barral, Y.R. Carrasco, D. Depoil, J. Eckl-Dorna, S. Fleire, K. Howe, A. Vehlow, M. Weber, and B. Treanor. 2007. The role of integrins and coreceptors in refining thresholds for B-cell responses. *Immunol Rev* 218:197-213.
136. Berftold, A., D. D. Desai, A. Gavhane, and R. Clynes. 2005. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity* 23:503-514.
137. Zinkernagel, R. M. a. H. H. 2006. Protective 'immunity' by pre-existent neutralizing antibody titers and preactivated T cells but not by so-called 'immunological memory'. *Immunol Rev* 211.

138. Hamano, Y., Arase, H., Saisho, H., and Takashi Saito. 2000. Immune Complex and Fc receptor-Mediated Augmentation of Antigen Presentation for in vivo Th Cell Responses. *J Imm* 164:6113-6119.
139. Goins, C. L., C. P. Chappell, R. Shashidharamurthy, P. Selvaraj, and J. Jacob. 2010. Immune Complex-Mediated Enhancement of Secondary Antibody Responses. *J Immunol* 184:6293-6298.
140. Obeid, O. E., and Steward, M.W. 1994. The potential of immunization with synthetic peptides to overcome the immunosuppressive effect of maternal anti-measles virus antibodies in young mice. *Immunology* 82:16-21.
141. Hagglund, S., Hu, K.-F., Larsen, L.-E., Hakhverdyan, M., Valarcher, J.-F., Taylor, G., Morein, B., Belak, S., and Alenius, S. 2004. Bovine respiratory syncytial virus ISCOM-protection in the presence of maternal antibodies. *Vaccine* 23:646-655.
142. Wesley, R. D., and Lager, K.M. 2006. Overcoming maternal antibody interference by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of swine influenza virus. *Veterinary Microbiology* 118:67-75.
143. Hu, Y., Q. Wu, B. Xu, Z. Zhou, Z. Wang, and Y.-H. Zhou. 2008. Influence of maternal antibody against hepatitis B surface antigen on active immune response to hepatitis B vaccine in infants. *Vaccine* 26:6064-6067.
144. Nahm, M. H., Glezen, P., Englund, J. 2003. The influence of maternal immunization on light chain response to *Haemophilus influenzae* type b vaccine. *Vaccine* 21:3393-3397.
145. Manickan, E., Yu, Z, and Rouse, B.T. 1997. DNA immunization of neonates induces immunity despite presence of maternal antibody. *J Clin Invest* 100:2371-2375.
146. Fink, K., Zellweger, R., Weber, J., Manjarrez-orduno, N., Holdener, M., Senn, B.M., Hengartner, H., Zinkernagel, R.M., and Macpherson, A.J. 2007. Long-term maternal imprinting of the specific B cell repertoire by maternal antibodies. *Eur J Immunol* 38:90-101.
147. Nossal, G. J. V. 1994. Differentiation of the Secondary B-Lymphocyte Repertoire: The Germinal Center Reaction. *Immunological Reviews* 137:173-183.
148. Saitoh, H., Maeda, K, and Yamakawa, M. 2006. In situ observation of germinal center cell apoptosis during a secondary immune response. *J Clin Exp Hematopathol* 46:73-82.
149. Siekevitz, M., Kocks, C., Rajewsky, K, and Dildrop, R. 1987. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. *Cell* 48:757.
150. Szakal, A., Kolmes, K., and Tew, J. 1983. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells. *J Immunol* 131:1714-1727.
151. Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., and Dustin, M.L. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 9:221-227.
152. Depoil, D., Fleire, S., Treanor, B.L., Weber, M., Harwood, N.E., Marchbank, K.L., Tybulewicz, V.L.J., and Batista, F.D. 2008. CD19 is essential for B cell

activation by promoting B cell recetor-antigen microcluster formation in response to membrane-bound ligand. *Nat Immunol* 9:63-72.

153. Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature* 411:489-494.
154. Harwood, N. E., and Batista, F.D. 2008. New insights into the early molecular evens underlying B cell activation. *Immunity* 28:609-619.
155. Carrasco, Y. R., and Batista, F.D. 2006. B cell recognition of membrane-bound antigen: an exquisite way of sensing ligands. *Curr Opin Immunol* 18:286-291.
156. Bergtold, A., D. D. Desai, A. Gavhane, and R. Clynes. 2005. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity* 23:503-514.
157. Phan, R. G., I. Grigorova, T. Okada, and J. G. Cyster. 2007. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol*.
158. Carrasco, Y. R., and Batista, F.D. 2007. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity* 27:160-171.
159. Junt, T., Moseman, E.A., Iannacone, M., Massberg, S., Lang, P.A., Boes, M., Fink, K., Henrickson, S.E., Shayakhmetov, D.M., Di Paolo, N.C., van Rooijen, N., Mempel, T.R., Whelan, S.P., and von Andrian, U.H. 2007. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* 450:110-114.
160. Qi, H., Egen, J.J., Huang, A.Y., and Germain, R.N. 2006. Extrafollicular activation of lymph node B cells by antigen-bearing dendritic cells. *Science* 312:1672-1676.
161. Heyman, B., Hobbs, M., and Weigle, W.O. 1985. IgM-mediated enhancement of in vivo anti-sheep erythrocyte antibody responses: isotype analysis of the enhanced responses. *Cell Immunol* 92:134-141.
162. Harte, P. G., Cooke, A., Playfair, J.H.L. 1983. Specific monocolonal IgM is a potent adjuvant in murine malaria vaccination. *Nature* 302:256-258.
163. Lehner, P., Hutchings, P., Lydyard, P.M., and Cooke, A. 1983. Regulation of the immune response by antibody. II. IgM-mediated enhancement: dependency on antigen does, T-cell requirement, and lack of evidence for an idiotype-related mechanism. *Immunology* 50:503-509.
164. Coutinho, A., and Forni, L. 1981. The enhancement of antibody response by IgM antibodies is dependent on antigen-specific T helper cells. *Immunobiology* 158:182-190.
165. Kubagawa, H., Oka, S., Kubagawa, Y., Torii, I., Takayama, E., Kang, D-W., Gartland, G.L., Bertoli, L.F., Mori, H., Takatsu, H., Kitamura, T., Ohno, H., and Wang, J-Y. 2009. Identity of the elusive IgM Fc receptor in humans. *J Exp Med* 206:2779-2793.
166. Gans, H., Maldonado, Y., Yasukawa, L.L., et al. 1999. IL12 IFN-gamma and T cell proliferation to measles in immunized infants. *J Immunol* 162:5569-5575.
167. Kurikka, S., Kayhty, H. Peltola, H., Saarinen, L., Eskola, J., and Makela, P.H. 1995. Neonatal immunization: response to *Haemophilus influenzae* type b-tetanus toxoid conjugate vaccine. *Pediatrics* 95:815-822.

168. Barrios, C., Brandt, C., Berney, M., Lambert, P.H., and Siegrist, C.A. 1996. Partial correction of the TH2/TH1 imbalance in neonatal murine responses to vaccine antigens through selective adjuvant effects. *Eur J Immunol* 26:2666-2670.
169. Brazolot Millan, C. L., Weeratna, R., Krieg, A.M., Siegrist, C.A., and Davis, H.L. 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatits B surface antigen in young mice. *Proc Natl Acad Sci U S A* 95:15553-15558.