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Quantitative Analysis of Influenza Virus-Specific B Cell Responses Generated by Infection and Vaccination

Hye Mee Joo
University of Tennessee - Chattanooga

To the Graduate Council:

I am submitting herewith a dissertation written by Hye Mee Joo entitled "Quantitative Analysis of Influenza Virus-Specific B Cell Responses Generated by Infection and Vaccination." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Mark Y. Sangster, Major Professor

We have read this dissertation and recommend its acceptance:

Barry T. Rouse, Tim Sparer, Stephen J. Kennel

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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Quantitative analysis of influenza virus-specific B cell responses generated by infection and vaccination

A Dissertation Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Hye Mee Joo

August 2009

DEDICATION

This dissertation is simply impossible without my parents, Dong-Choon Joo and Soon-Seok Hong. They bore me, raised me, loved me, and supported me spiritually throughout my life. To them I dedicate this thesis.

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It is a pleasure to thank the many people who made this thesis possible. Foremost, I would like to express my sincere gratitude to my advisor Dr. Mark Sangster for the continuous support of my Ph. D study and research, for his patience, motivation, enthusiasm and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. In addition, he was always accessible and willing to help his students. As a result, research life became smooth and rewarding for me. I could not have imagined having a better advisor and mentor for my Ph. D study.

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My deepest gratitude goes to my parents who always besides me and encouraged me to come this far. I am eternally grateful for their inseparable support and prayers. My brother, Sehwan Joo, thanks for being supportive and caring siblings.

Last but not the least, I greatly express my appreciation to my husband Jae Hoon Bahn for his dedication, love and persistent confidence in me. Thanks for putting up with me through this process. I could not have done this without you.

ABSTRACT

The two cellular components of B cell memory are antibody (Ab)-secreting cells (ASCs) and memory B cells (B_{Mem}). B_{Mem} are quiescent cells that respond to "recall" antigen and contribute substantially to vaccine effectiveness. The rational evaluation of vaccination strategies is dependent on quantitative information on the state of B cell memory. Despite the importance of B_{Mem} in resistance to infection, there is little information on the nature of B_{Mem} populations generated by influenza infection or vaccination. The major goal of this dissertation was a comprehensive quantitative analysis of the frequency and distribution of influenza-specific B_{Mem} generated by influenza infection of the respiratory tract, or by vaccination with inactivated virus.

To achieve this, a prerequisite was the development of a sensitive and reproducible limiting dilution assay (LDA) for determining influenza-specific B_{Mem} frequencies. The results in Chapter 2 demonstrated that a strategy utilizing BPL-inactivated virus particles was effective for the in vitro activation of virus-specific B_{Mem}. This strategy selectively activates virus-specific B_{Mem} and, importantly, achieves this without a requirement for CD4+ T cell associated factors.

Using this strategy, the frequency of Influenza specific B_{Mem} in different anatomical sites was measured and we also tested three different forms of influenza virus immunization strategy; (i) intranasal (i.n.) infection, (ii) i.n. vaccination, (iii) intramuscular (i.m.) vaccination. The results in Chapter III, IV showed that following different forms of immunization, B_{Mem} dispersed broadly to organized lymphoid tissues throughout the body, and a population of ASCs was established in the bone marrow. ASC and B_{Mem} frequencies in these locations reflected the magnitude of the primary B cell response to the form of immunization. Thus, the frequencies were higher following i.n. infection. Interestingly, the state of B cell memory in the lung was

profoundly influenced by the form of immunization. The lung was a preferential site of B_{Mem} localization after i.n. infection. However, this was not the case after i.m. immunization when B_{Mem} frequencies in the lung were considerably lower than in other sites. Our findings point to an effect of influenza infection on the lung environment that profoundly influences ASC and B_{Mem} trafficking to this site.

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ABBREVIATIONS

ASC……..... Antibody-secreting cell

BALT…… Bronchus-associated lymphoid tissue

BCM…….. B cell medium

BM……. Bone marrow

B_{Mem}……... Memory B cell

BPL…… β-propiolactone

BPL-flu…….. BPL-inactivated influenza virus

BPL-HSV……... BPL-inactivated HSV

CLN……... Cervical lymph node

d-NALT……. Diffuse nasal-associated lymphoid tissue

ELT……...……. Effector lymphoid tissue

FBS……...……. Fetal bovine serum

HEV……...…… High endothelial venule

HSV……...…… Herpes simplex virus

IliLN……...…… Iliac lymph node

ILN……...…….. Inguinal lymph node

i.n. ……. Intranasal

i.m. …… Intramuscular

LDA……..…. Limiting dilution assay

LCMV……...……. Lymphocytic choriomeningitis virus

LLPC…….. Long-lived plasma cell

MedLN…….. Mediastinal lyph node

MesLN……. Mesenteric lymph node

MLN……..……..…….. Mediastinal lymph node

MOI…….……..…… Multiplicity of infection

NALT…….…….. Nasal-associated lymphoid tissue

o-NALT…….…….…….. Organized nasal-associated lymphoid tissue

PLN…….…….. Popliteal lymph node

PP……...…….…….. Peyer's patches

S1P…….……...…… Sphingosin-1-phosphate

Chapter 1

Background and Overview

Introduction

Influenza is a contagious respiratory viral illness of global importance and is a significant cause of morbidity and mortality annually. Four or five pandemics of influenza occurred during the last century with intervals of 9-39 years. By far the worst was the pandemic of 1918 – 1920. Over a 10-month period, this "Spanish flu" killed an estimated 40-50 million people worldwide with many of the deaths occurring in young, previously healthy adults.

When symptomatic, influenza typically produces an acute febrile illness characterized by cough, headache and myalgia. The majority of morbidity and mortality occurs in the elderly with underlying chronic cardiorespiratory disease or diabetes mellitus, particularly those in residential care. Complications of acute influenza include viral and secondary bacterial pneumonias, exacerbations of pre-existing cardiopulmonary disease and, in children and infants, croup, otitis media, bronchiolitis and febrile convulsions (Glezen 1982; Nicholson 1992).

Each year, circulating influenza viruses are responsible for approximately 40,000 deaths and up to 150,000 hospitalizations in the United States (Thompson, Shay et al. 2003; Harper, Fukuda et al. 2005). On top of this are growing concerns of a developing influenza pandemic. Avian influenza viruses of the H5N1 subtype with potential for high virulence in humans continue to expand their distribution from initial sites of emergence in Southeast Asia. Mutations that enable efficient spread of H5N1 viruses within an immunologically naïve human population may occur and serve as a pandemic trigger. Clearly, there is a strong need to continue to develop and improve strategies to combat influenza.

The challenge of antigenic change in influenza viruses

Influenza viruses have segmented genomes and show great antigenic diversity. Of the three types of influenza viruses-A, B, and C-only types A and B cause widespread outbreaks. Influenza A viruses are classified into subtypes based on antigenic differences between their two surface glycoproteins, haemagglutinin and neuraminidase. 15 haemagglutinin subtypes (H1–H15) and nine neuraminidase subtypes (N1–N9) have been identified for influenza A viruses. Viruses of all haemagglutinin and neuraminidase subtypes have been recovered from aquatic birds, but only three haemagglutinin subtypes (H1, H2, and H3) and two neuraminidase subtypes (N1 and N2) have established stable lineages in the human population since 1918. Only one subtype of haemagglutinin and one of neuraminidase are recognised for influenza B viruses. Haemagglutinin facilitates entry of the virus into host cells through its attachment to sialic-acid receptors. It is the major antigenic determinant of type A and B viruses to which neutralizing antibodies (Abs) are directed and the crucial component of current influenza vaccines. Five Ab-combining sites have been mapped on the HA of H3 subtype human influenza A viruses (Wilson and Cox 1990); however, less is known about HAs of avian influenza A subtypes.

An important function of neuraminidase, the second major antigenic determinant, is to catalyze the cleavage of glycosidic linkages to sialic acid, thereby assisting in the release of progeny virions from infected cells. Accordingly, neuraminidase has become an important target for antiviral activity. The M2 ion channel of influenza A, which is blocked by the antiviral drug amantadine, regulates the internal pH of the virus, which is crucial during early viral replication.

Influenza vaccination is the primary method for preventing influenza and its severe complications. However, the biology of influenza virus presents a major challenge to effective

vaccination. Influenza viruses utilize two mechanisms, referred to as antigenic drift and antigenic shift, to evade the human immune response. Point mutations that occur during viral replications continuously generate antigenic change (antigenic drift), especially in the surface hemagglutinin (HA) and neuraminidase (NA) glycoproteins of influenza A viruses. These surface molecules are the basis for categorizing influenza A viruses into subtypes (e.g. H3N2, H1N1). Abs induced by vaccination with one antigenic form may confer profound protection against infection with the homologous virus. However, the protection may be substantially less against a "drifted" variant, even though the viral subtype remains the same. As a general rule, Abs raised against one subtype provide little, if any, protection against a completely novel subtype. This feature enables the virus to evade immune recognition, leading to repeated outbreaks during interpandemic years.

Antigenic shift is a rare but occurs with the emergence of a "new", potentially pandemic, influenza A virus that possesses a novel HA alone or with a novel NA. The novel HA and NA genes in pandemic influenza viruses are derived from the reservoir of avian influenza viruses in nature (Reid, Fanning et al. 2004; Reid, Taubenberger et al. 2004). The new virus is antigenically distinct from circulating human influenza viruses and Abs towards the previously circulation subtype do not cross-react with the new subtype.

A major goal of influenza vaccination is to generate broad protection against variants within a subtype (heterovariant protection) and, optimally, against a range of different subtypes (heterosubtypic protection).

Human influenza vaccines

Since the 1940s, inactivated influenza vaccines have been developed for the control of annual influenza epidemics (Hilleman 2000). Two forms of influenza vaccine are currently licensed for use in humans in the United States: (i) an inactivated vaccine administered intramuscularly (i.m.), and (ii) a live attenuated intranasal (i.n.) vaccine. Both vaccine forms are prepared from the same set of three viruses, two influenza A viruses and an influenza B virus, that are predicted to match the predominant circulating viruses in the next flu season.

Three types of inactivated influenza vaccine are currently recommended by WHO; whole virus, detergent-treated split product and purified surface antigen. All commercially produced influenza vaccines are grown in eggs and inactivated by either formaldehyde or β-propiolactone (Davenport, Hennessy et al. 1964; Bachmayer 1975; Brady and Furminger 1976; Brady and Furminger 1976). Early whole virion inactivated vaccines were associated with frequent local and systemic adverse effects in young children(Davenport, Hennessy et al. 1964). Accordingly, whole virion influenza vaccines are little used and are unlicensed in many countries. At the present time, inactivated split-virus and subunit influenza vaccines receive more widespread use, and it is the only vaccine form approved for administration to children aged <5 years and adults aged ≥50 years.

Intranasal delivery of live influenza vaccines offers the advantage of mimicking natural infection, thereby providing a broader immunological response and more durable protection than with inactivated vaccines (Murphy and Clements 1989; Murphy and Coelingh 2002). However, in the United States, live attenuated virus vaccine is only approved for the age group of 5-49,

thus excluding two major high-risk groups, infants and the elderly, in addition to immunocompromised patients and pregnant woman.

. A key determinant of the efficacy of both vaccine forms is the ability to induce strong B cell responses against the component viruses. The inactivated vaccine is essentially a "B cell vaccine". Protective efficacy of the live vaccine is also ascribed primarily to the induction of virus-specific Abs (Murphy and Coelingh 2002), although replicating virus also facilitates priming of virus-specific cytotoxic T cells (Gorse, Campbell et al. 1995). The key point is that B cell responses are of central importance in vaccine-induced protection against influenza. More precisely, it is the vaccine-induced state of B cell memory that is all important.

Immune responses and memory in a mouse model of influenza infection

Influenza virus infection of the respiratory tract in mice is a well-characterized model that has many features in common with human influenza infection. This model has been used extensively to study the immune response to infection and vaccination, and to identify the components of the adaptive immune response that are most effective in mediating virus clearance and providing protection against re-infection (Doherty, Topham et al. 1997; Gerhard, Mozdzanowska et al. 1997).

Influenza viruses infect epithelial cells of the upper respiratory tract and bronchi, as well as mononuclear cells (Ronni, Sareneva et al. 1995). Intranasal administration of the A/HKx31 (H3N2) influenza A virus to C57BI/6J (B6) mice leads to the development of primary viral pneumonia. In the B6 model, virus is cleared 10 days after infection, with no indication of

persistent antigen or viral RNA (Allan, Tabi et al. 1990). Recovery or prevention of influenza relies on both innate and adaptive response in the immunologically intact mouse. This has been evidenced by increased mortality or delayed recovery of mice that have a defective type I interferon response system (Lindenmann, Lane et al. 1963; Haller, Arnheiter et al. 1979; Garcia-Sastre, Durbin et al. 1998) or complement system (Hicks, Ennis et al. 1978; Kopf, Abel et al. 2002) or are deficient in major histocompatibility complex class I (MHC-I)-restricted CD8+ T (Bender, Croghan et al. 1992) or B cells (Gerhard, Mozdzanowska et al. 1997; Graham and Braciale 1997; Topham and Doherty 1998; Baumgarth, Herman et al. 2000; Kopf, Brombacher et al. 2002; Webby, Andreansky et al. 2003).

Innate response to influenza virus infection

Respiratory epithelial cells are the primary targets for influenza A virus infection. This localized pathology reflects that the production of new virus requires cleavage of the surface HA molecule by an enzyme restricted in distribution to the superficial epithelial layer of the respiratory tract (Walker, Sakaguchi et al. 1992; Rott, Klenk et al. 1995). Following influenza infection, respiratory epithelial cells produce large amounts of virus which can then infect alveolar macrophages and immature respiratory dentritic cells (RDC). In addition, cell necrosis/apoptosis triggers innate immune responses and local production of cytokines such as type I interferon, TNF-α, IL-12, IL-1 and chemokines such as monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted (RANTES), MIP-1α/β, interferon induced protein (IP-10) (Matsukura, Kokubu et al. 1996; Sprenger, Meyer

et al. 1996; Adachi, Matsukura et al. 1997; Bussfeld, Kaufmann et al. 1998; Dawson, Beck et al. 2000). These proinflammatory cytokines and chemokines produced by infected cells cause the extravasation of small numbers of blood-derived neutrophils initially, followed by larger numbers of blood monocytes/macrophages from the peripheral blood across the endo-epithelial barrier into infected lung tissue within the first 3 days after influenza A virus infection (Table 1.) (Ada and Jones 1986; La Gruta, Kedzierska et al. 2007). During this period, viral replication continues in the epithelial cells and infection spreads to the recruited macrophages (Fujisawa, Tsuru et al. 1987; Hofmann, Sprenger et al. 1997). These processes alone are not able to clear the virus from the lung. By day 7, CD8+ cytotoxic T cells (CTLs) from mediastinal lymph nodes begin to accumulate in the infected lung and the efficient process of T cell mediated viral clearance begins (Bender and Small 1992; Shaw, Arden et al. 1992).

Adaptive response to influenza virus infection

The adaptive response to influenza virus is mediated by neutralizing antibody producing B cells, and CD4+ helper T cells, and CD8+ cytotoxic T lymphocytes (CTL) that kill infected cells (Doherty, Topham et al. 1997; Gerhard, Mozdzanowska et al. 1997; Topham, Tripp et al. 1997; Doherty and Christensen 2000; Wong and Pamer 2003; Brown, Roman et al. 2004). To initiate anti-influenza adaptive immune response, Antigen presenting cells (macrophages and DCs) are essential. Influenza infection via TLR3, TLR7/8 results in DC activation, maturation and migration of the DC from the respiratory tract through the afferent lymphatics to draining lymph nodes (Figure 1). DC then present viral antigen to virus-specific naïve T cells which

triggers T cell activation/differentiation. influenza-specific naïve T cells can differentiate into Th1 and Th2. Th1 cells secrete IFN-γ, IL-2 and help IgG2a/c Ab production, while Th2 cell secrete IL-4 and IL-5 and help IgA, IgG1 Ab production (Ada and Jones 1986) in the germinal center of the lymph node. Each antibody isotype produced during the immune response is known to have specific functions (Table 2.). Typically, influenza infection leads to a strong Th1 type of response followed by dominating IgG2a/c production (Sangster, Riberdy et al. 2003). Th1 cells are also essential in the generation and maintenance of CD8+ T cell memory by secreting IL-2, though in the primary response, CD4+ T cells are not required for expansion or development of functional CD8+ CTL (Riberdy, Christensen et al. 2000; Thomas, Keating et al. 2006).

The consequences of the immune response in the lymph node, production of influenza-specific Abs contributing to viral neutralization due to binding with viral antigen. In addition, differentiated effector T cells can leave the draining lymph nodes through the efferent lymphatics and circulatory system and then localize to infected lung (Legge and Braciale 2003; Langlois and Legge 2007).

Characteristics of B cell memory

Studies of the mouse model have established that influenza infection elicits a vigorous primary B cell response and extensive anti-viral Ab production that contributes substantially to virus clearance and recovery (Gerhard, Mozdzanowska et al. 1997). A consequence of this B cell response is a state of B cell memory that provides profound long-term protection against re-infection with the same virus or an antigenically related virus. Two components of B cell

memory can be identified: (i) plasma cells, and (ii) B_{Mem}. Long-lived plasma cells (Slifka, Antia et al. 1998) secreting high affinity anti-viral Abs are found predominantly in the bone marrow (Hyland, Sangster et al. 1994; Sangster, Hyland et al. 1995; Slifka, Matloubian et al. 1995) and at mucosal surfaces (Liang, Hyland et al. 2001). IgA Abs produced by sub-mucosal plasma cells, and serum IgG derived from the bone marrow plasma cell population, provide anti-viral activity in the upper and lower respiratory tract, respectively. B_{Mem} are also long-lived (Schittek and Rajewsky 1990) but, unlike plasma cells, they are quiescent, non-Ab-secreting cells that distribute to lymphoid tissues throughout the body (Bachmann, Kundig et al. 1994; Slifka and Ahmed 1996). If a previously encountered virus manages to escape the barrier of pre-existing Abs and re-establishes an infection, B_{Mem} are stimulated to produce a secondary anti-viral Ab response that is even more rapid and effective than the primary response (Sangster, Smith et al. 1995). Thus, a B_{Mem} response functions to greatly curtail the duration and severity of infection.

Characteristics of memory B cells

The B_{Mem} generated in germinal centers as part of T cell-dependent B cell responses (McHeyzer-Williams, Driver et al. 2001) typically express an isotype-switched surface Ig receptor (IgG, IgA, or IgE) with high Ag-binding affinity, and also the mature B cell markers CD19 and B220 (Ridderstad and Tarlinton 1998). Recent evidence for a $CD19^-$ $B220^-$ B_{Mem} population (McHeyzer-Williams, Driver et al. 2001) is controversial (Bell and Gray 2003). It is generally thought that B_{Mem} recirculate and establish similar frequencies in lymphoid tissues throughout the body (Bachmann, Kundig et al. 1994). Although there is some evidence that B_{Mem}

may preferentially localize at mucosal surfaces (Liu, Barthelemy et al. 1995), this has not been fully investigated and may relate to the Ab isotype expressed and the route of immunization (Moser and Offit 2001).

B_{Mem} are quiescent, non-Ab-producing cells. It is only upon activation that B_{Mem} divide and differentiate into plasma cells producing high affinity Abs. Typically, this process is initiated in vivo when B_{Mem} are exposed to specific Ag in the presence of $CD4^+$ T cell help (Vieira and Rajewsky 1990). B_{Mem} are strong Ag-presenting cells. High affinity surface Ig receptors on B_{Mem} facilitate Ag capture for presentation to specific $CD4^+$ T cells, and the constitutive expression of the costimulatory molecules CD80 and CD86 by B_{Mem} promotes cognate T-B interaction (Liu, Barthelemy et al. 1995). In vitro studies have demonstrated that B_{Mem}, but not naïve B cells, generate substantial numbers of plasma cells when stimulated with T cell-derived signals such as CD40 ligand, IL-2, and IL-10. The presence of specific Ag was not a requirement (Bernasconi, Traggiai et al. 2002; Tangye, Avery et al. 2003). Indeed, non-specific methods of B_{Mem} activation have been used as a basis for assays to determine Ag-specific B_{Mem} frequencies (Nanan, Heinrich et al. 2001), but the sensitivity of such an approach remains unclear. It has recently been suggested that "bystander" B_{Mem} may differentiate into plasma cells in response to non-specific signals from $CD4^+$ T cells activated in the course of normal in vivo immune responses (Bernasconi, Traggiai et al. 2002).

Characteristics of Plasma cells

Long-lived antibody-secreting B cells can also be considered part of the memory B cell compartment. The long-lived plasma cells (LLPC) are terminally differentiated cells that continually produce high-affinity antibody and survive for the life of the mouse in the absence of antigen and cell division (Manz, Thiel et al. 1997; Manz, Lohning et al. 1998; Slifka, Antia et al. 1998). These nondividing cells differ from B_{Mem} in many respects. For instance, plasma cells down-regulate surface expression of many typical B cell markers, including MHC class II and surface Ig (Abney, Cooper et al. 1978; Halper, Fu et al. 1978). These changes indicate that unlike memory B cells, mature plasma cells are unlikely to function as antigen presenting cells. LLPC preferentially homes to the bone marrow for their survival. The survival factors could include CXCL12, IL-6, B cell-activation factor (BAFF), a proliferation inducing ligand (APRIL), and CD44. Recently, the plasma-cell marker CD138 (also known as syndecan-1) was identified as a receptor for APRIL that is involved in the homing of plasma cells to the bone marrow. Inhibition of BAFF and APRIL, both of which are lignads for the B cell maturation antigen (BCMA) receptor, which is expressed by plasma cells (O'Connor, Raman et al. 2004), but not BAFF alone, was found to prevent the survival and/or the migration of newly formed plasma cells to the bone marrow (Ingold, Zumsteg et al. 2005). In addition to the bone marrow, survival factors for LLPC are also expressed in inflamed tissues (Baggiolini 1998; Cassese, Lindenau et al. 2001; Muehlinghaus, Cigliano et al. 2005). The presence of interferon-γ (IFN-γ), a hallmark of inflamed tissue, increase both the expression of CXCR3 on B cells differentiating into plasmablasts and the expression of CXCR3 ligands by cells in the inflamed tissue, indicating that IFN-γ might be particularly important for the attraction of plasmablasts to inflamed tissues.

When migratory plasmablasts reach the inflamed tissue, they become resident long-lived plasma cells, which do not have migratory capacity. It is not clear, however, whether resident plasma cell population in inflamed tissues contributes to serum antibody concentration or provides local protection as the humoral memory. It also remains to be determined whether the maintenance of antibody concentrations that are observed following a primary immune response is due to long lived plasma cells generated from activated naïve B cells or whether it results from reactivation of specific B_{Mem} that were generated in the primary immune response (Blink, Light et al. 2005; Dorner and Radbruch 2005).

Quantitation of memory B cells

Despite the important contribution that a B_{Mem} response makes to resistance to infection, there have been few quantitative studies of the B_{Mem} pool induced by virus infection. Many studies of B cell memory have focused on responses to simple haptens or fluorochromes, since these molecules can be utilized in cell staining strategies to identify B_{Mem} expressing specific, high affinity receptors (Schittek and Rajewsky 1990; Ridderstad and Tarlinton 1998; Shimoda, Nakamura et al. 2001; Bell and Gray 2003). However, such an approach cannot be applied to virus-specific B cells which generally bind poorly defined conformational epitopes. Virus-specific B_{Mem} can be identified by the transfer of immune cells to a recipient animal followed by virus challenge (Bachmann, Kundig et al. 1994), but this approach is time consuming, increases animal usage, and is only semi-quantitative. Alternatively, virus-specific B_{Mem} frequencies can be determined by limiting dilution analysis after an in vitro stimulation step to initiate the

proliferation and differentiation of B_{Mem} into Ab-secreting plasma cells (Nanan, Heinrich et al. 2001). Slifka and Ahmed (Slifka and Ahmed 1996) optimized this approach by applying the ELISPOT assay for the rapid and sensitive detection of plasma cell formation, and used the assay to determine the frequencies of B_{Mem} generated by lymphocytic choriomeningitis virus infection. To use this assay as a reliable and broad method for measuring B_{Mem} frequencies, this method has to be tested on other virus system.

CONCLUSION

The purpose of vaccination is to provide protection from disease via induction and recall of immunological memory responses. Despite the important contribution that a B_{Mem} response makes to resistance to infection, there have been few quantitative studies of the B_{Mem} pool induced by virus infection or vaccination. One commonly held premise is that a live virus infection of the respiratory tract is likely to induce the "optimal" B_{Mem} pool in terms of providing resistance to re-infection. However, no studies have been conducted to provide a quantitative basis for this premise. Comparative studies of the nature of the MBC pool induced by live virus infection and by different approaches to vaccination are clearly required. Fundamental questions to be addressed include: "How many virus-specific B_{Mem} are there?", "What is their anatomical distribution?", and "What Ab isotypes do they express?" The answers to such questions are essential if rational decisions are to be made about the development and optimization of vaccination regimens.

The work described in this dissertation has been aimed at (i) developing a sensitive and reproducible limiting dilution assay (LDA) for measuring influenza-specific B_{Mem} frequencies and (ii) applying the influenza-specific B_{Mem} assay to determine the state of influenza-specific B cell memory generated by respiratory tract infection, and also by different forms of vaccination with inactivated virus in a mouse model. We found ASCs and B_{Mem} preferentially localize in the lung after i.n. infection whereas, there is minimal localization of the components of B cell memory in the lung after i.m. vaccination. Thus, we concluded that the lung is a repository of B cell memory after influenza infection and must be taken into account in the development of effective local vaccines to protect specifically respiratory tract. However, there are many remaining questions concerning the functional significance and anatomical location of B cell memory in the lung. It is also need to be determined how homing mechanisms and local retention of B cell memory is regulated.

Although these studies have focused on influenza infection and vaccination, it is anticipated that the findings will be generally applicable to other viruses that target the respiratory tract.

APPENDIX

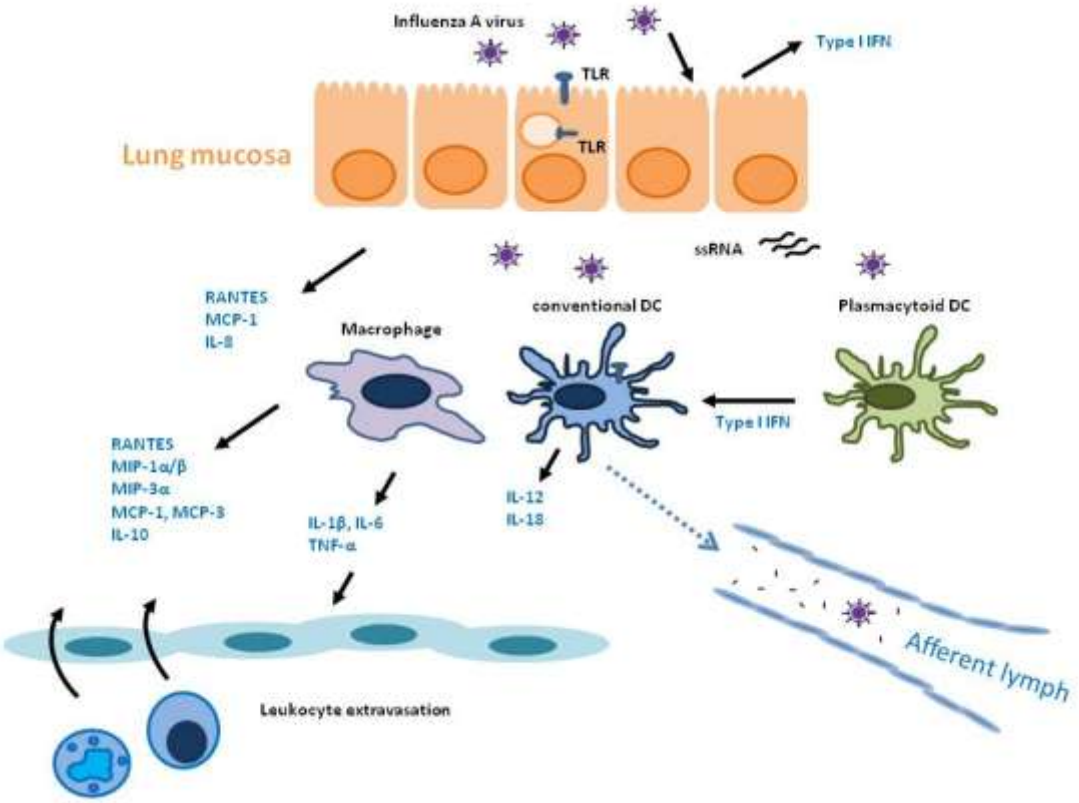


Figure 1. Cytokine interplay in innate and adaptive immunity. Influenza A virus is capable of infecting both epithelial cells in respiratory tract and tissue macrophages. Infected epithelial cells produce RANTES, MCP-1, IL-8 and type I IFN, whereas macrophages produce various chemokines that attract monocytes/macrophages and proinflamatory cytokines such as IL-1, TNF-α that are involved in functional maturation of tissue macrophages and dendritic cells. Cytokines and chemokins produced following influenza infection lead to massive infiltration of leukocytes from the circulation to the lung which is hallmark of the host defense mechanism against viral infection. In addition, Matured macrophages and dendritic cells exit the lung through lymphatic system and reach regional lymph nodes to initiate adaptive immune response.

Table 1. Links between influenza infection and chemokine/cytokine-mediated immunopathogenesis (adapted from La Gruta, Kedzierska et al. 2007)

Chemokine/cytokine	Function	Produced by
IFN-γ	Inhibits viral replication Stimulates CTL mediated killing Increases MHC I expression Activates macrophages and neutrophils Promotes T-cell proliferation	T cells, NK cells
TNF-α	Direct antiviral effects Neutrophil chemoattractant Stimulates macrophage phagocytosis and production of IL-1 Increases vascular permeability	T cells Monocytes/macrophages Dendritic cells Neutrophils
IL-1	Increases expression of adhesion factors on endothelium Increases vascular permeability Stimulates IL-6 production	Monocytes/macrophages Dendritic cells
IL-6	Pro-inflammatory cytokine Activates T cells	Respiratory epithelium T cells Monocytes/macrophages Dendritic cells
MIP-1b (CCL4)	Monocyte, T cell chemoattractant Activates neutrophils	Monocytes/macrophages Neutrophils T cells Dendritic cells
MIG (CXCL9)	Monocyte and T-cell chemoattractant	Respiratory epithelium Monocytes/macrophages
IP-10 (CXCL10)	Monocyte and T-cell chemoatrractant	Monocytes/macrophages T cells Respiratory epithelium
RANTES (CCL5)	Monocyte, T cell, DC chemoattractant Activates T cells	T cells Respiratory epithelium
IL-8 (CXCL8)	Neutrophil and T-cell chemoatrractant Activates neutrophils	Respiratory epithelium Monocytes/macrophages Neutrophils

Table 2. Mouse immunoglobulin isotypes

Isotype	Switch regulation	Function	
IgG1	+: IL-4, IL-6 -: IFN-α/β, TLR ligands	Viral neutralization	
IgG2a/c	+: IFN-γ, IL-21, IFN-α/β, TLR ligands -: IL-4		High affinity for activating FcR Complement activation
IgG2b	+: TGF-β, IFN-γ -: IL-4		
IgG3	+: IFN-γ -: IL-4		T-independent responses to carbohydrates
IgA	+: IL-4, IL-5, IL-6, IL-10, TGF-β, APRIL		Mucocal protection (poly-IgR transport)
IgE	+: IL-4, IL-13 -: IFN-γ, IFN-α/β, TLR ligands	Parasite responses Allergy	

Chapter 2

A strategy for selective, CD4+ T cell-independent activation of virus-specific memory B cells for limiting dilution analysis

Research described in this chapter is a modified version of an aticle published in 2006 in *Journal of immunological methods* by Xiaofeng Li, Daisy J. Vanitha, Hye Mee Joo, Yuxia He, Barry T. Rouse and Mark Y. Sangster.

Li X, Vanitha DJ, Joo HM, He Y, Rouse BT, Sangster MY. A strategy for selective, $CD4^+$ T cell-independent activation of virus-specific memory B cells for limiting dilution analysis. *J Immunol Methods.* 2006 Jun 30;313(1-2):110-8.

In this chapter "our" and "we" refers to me and co-authors. My contribution in the paper includes (1) planning experiments and data analysis (2) interpretation of the results (3) Compiling and interpretation of the literature (4) understanding how results fit with the literature (5) Preparation of graphs, figures and tables (6) editing.

Abstract

Complete characterization of the B cell response to infection or vaccination is dependent on accurate quantitation of the memory B cell (B_{Mem}) pool. An established method for measuring B_{Mem} frequencies is limiting dilution analysis based on in vitro stimulation of B_{Mem} to divide and differentiate into antibody-secreting cells (ASCs). The presence of specific antibody then serves to identify cultures positive for precursor B_{Mem}. The sensitivity of this approach is critically dependent on optimal in vitro B_{Mem} activation. To develop a limiting dilution assay (LDA) for measuring influenza-specific B_{Mem} frequencies, we evaluated strategies for the in vitro

stimulation of influenza-specific MBCs. An ELISPOT assay to enumerate influenza-specific IgG B_{Mem} was used as the readout for B_{Mem} activation. Culture of influenza-specific B_{Mem} with influenza-infected splenocytes was effective for B_{Mem} activation, but T cell-associated factors were required for optimal LDA sensitivity and clonal expansion of activated B_{Mem}. However, optimal influenza-specific B_{Mem} activation was T cell-independent when B_{Mem} were simply cultured with β-propiolactone (BPL)-inactivated influenza virus particles (BPL-flu). BPL-flu did not stimulate naïve B cells to produce influenza-specific IgG, demonstrating that only B_{Mem} were activated. In addition, BPL-flu acted selectively and only activated influenza-specific B_{Mem}, not B_{Mem} of other specificities. Analysis of influenza-specific B_{Mem} frequencies in different anatomical locations in influenza-immune mice established that in vitro stimulation with BPL-flu provided the basis for a sensitive and reproducible LDA. Extending our studies to the herpes simplex virus (HSV) system, we demonstrated that HSV-specific B_{Mem} cultured with BPL-inactivated HSV were selectively activated to IgG secretion in the absence of T cells. Our studies identify BPL-inactivated viral particles as a valuable tool for selective, T cell-independent activation of virus-specific B_{Mem} in vitro. This strategy eliminates the influence of poorly-defined T cell-associated factors on B_{Mem} frequency determinations.

Introduction

The B cell response to infection or vaccination generates antibody-secreting plasma cells and a population of antigen-specific memory B cells (B_{Mem}). Typically, B_{Mem} are long-lived products of the germinal center reaction that express high affinity, isotype-switched

immunoglobulin receptors and distribute to lymphoid tissues throughout the body (McHeyzer-Williams and McHeyzer-Williams 2005). Unlike plasma cells, B_{Mem} are quiescent, non-antibody-secreting cells. It is only upon activation that B_{Mem} divide and differentiate into antibody-secreting cells (ASCs), a process that generally occurs in vivo when B_{Mem} encounter recall antigen. The activation requirements of B_{Mem} are less stringent than those of naïve B cells (Yefenof, Sanders et al. 1986), but still remain incompletely understood. In at least some experimental systems, B_{Mem} activation is dependent on $CD4^+$ T cell help (Ochsenbein, Pinschewer et al. 2000).

Quantitative studies of antigen-specific B_{Mem} have frequently focused on responses to simple haptens, since these molecules can be tagged with fluorochromes for flow cytometric identification of B_{Mem} expressing specific receptors (Shimoda, Nakamura et al. 2001; Blink, Light et al. 2005). Recently, biotinylated influenza virus hemagglutinin was used to enumerate hemagglutinin-specific B cells by flow cytometry (Doucett, Gerhard et al. 2005). The analysis of antigen-specific B cells by flow cytometry has obvious appeal, but currently this approach provides an incomplete picture of the B_{Mem} pool generated by many pathogens. The B cell response to pathogens is generally complex, being directed against multiple, often poorly-defined epitopes on multiple molecules.

An alternative approach to B_{Mem} quantitation that may better accommodate the diverse B cell response to pathogens is the limiting dilution assay (LDA). This assay is based on the in vitro stimulation of B_{Mem} to divide and differentiate into ASCs, with the presence of specific antibody serving to identify cultures positive for precursor B_{Mem}. The period required for B_{Mem} stimulation can be minimized by applying an ELISPOT assay to directly detect antibody-

secreting daughter cells (Slifka and Ahmed 1996). A key requirement for accurate MBC quantitation by LDA is effective in vitro B_{Mem} activation. One approach is to non-specifically activate B cells with polyclonal stimulators (Nanan, Heinrich et al. 2001; Crotty, Aubert et al. 2004), but massive cell proliferation and antibody production are likely to have detrimental effects on culture conditions and assays for specific antibody. Alternatively, a more selective activation of B_{Mem} may be achieved by incubation with appropriate antigenic forms (Hebeis, Klenovsek et al. 2004) . Virus-specific MBCs have been activated by culture with virus-infected cells or purified viral particles (Jones and Ada 1987; Slifka and Ahmed 1996; Moser and Offit 2001), but the in vitro culture conditions required for optimal B_{Mem} activation and clonal expansion are not well defined.

The B_{Mem} response to a previously encountered virus contributes substantially to immune protection, and is likely to be a key determinant of the effectiveness of inactivated viral vaccines that target the humoral arm of the immune system. It is therefore surprising that little quantitative information on the B_{Mem} pool induced by viral infection or vaccination has been collected. Notably, Slifka and Ahmed used a limiting dilution approach to determine lymphocytic choriomeningitis virus (LCMV)-specific B_{Mem} frequencies. In vitro B_{Mem} activation was achieved by incubation with splenocytes from LCMV carrier mice, a cell population heavily infected with the virus. Supplementation of stimulation cultures with ConA-conditioned medium increased LCMV-specific MBC frequencies, indicating a requirement for T cell-derived factors (Slifka and Ahmed 1996).

The current report describes the development of a limiting dilution assay for determining influenza virus-specific B_{Mem} frequencies in a mouse model. Our initial strategy of using

influenza virus-infected splenocytes to activate specific B_{Mem} required T cell-associated factors for optimal effectiveness. However, the use of β-propiolactone (BPL)-inactivated influenza virus particles achieved T cell-independent B_{Mem} activation. Extension of our studies to a mouse model of herpes simplex virus (HSV) infection demonstrated that BPL-inactivated HSV particles activated HSV-specific B_{Mem} in the absence of T cells. Our findings identify a simple, broadly applicable strategy for in vitro activation of virus-specific B_{Mem} for limiting dilution analysis. Using this strategy, the proportion of $CD4^+$ T cells present in culture is unlikely to be a factor in B_{Mem} activation, and any detrimental effects of viral replication on leukocyte function are eliminated.

Materials and Methods

Mice

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions until infection, and thereafter in BSL2 containment. Female mice were used in all studies and were infected at 8-10 wk of age. The Animal Care and Use Committee of the University of Tennessee approved all animal procedures.

Viruses

Influenza virus A/HKx31 (H3N2) grown and titrated in the allantoic cavity of embryonated hen's eggs was obtained from Dr. P. C. Doherty (St. Jude Children's Research Hospital, Memphis, TN). The KOS strain of HSV-1 was grown and plaqued in Vero cells. HSV was concentrated from clarified tissue culture grown virus by centrifugation at 75,000*g* for 1h. For HSV inactivation by BPL treatment, concentrated virus in 0.1% BPL (Acros Organics), 0.1 M Tris-HCl (pH 7.5) was incubated for 2 h at 37°C with frequent mixing, and then held overnight at 4°C. Complete inactivation was confirmed by the absence of cytopathic effect in Vero cell monolayers inoculated with the treated virus. Preparations of sucrose gradient-purified and BPL-inactivated influenza A/HKx31 were purchased from Charles River (Wilmington, MA). Viral protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). All virus preparations were stored at -80°C.

Infection and sampling

Mice were anesthetized with Avertin (2,2,2-tribromoethanol) given intraperitoneally, and then infected intranasally with $10^{6.8}$ 50% egg infectious doses of influenza virus (30 μl in PBS). Influenza-immune mice were sampled 60-90 days after infection. Mice were infected intraperitoneally with 10^6 PFU of HSV, and sampled 30-45 days after infection. The right posterior mediastinal lymph node (MLN), inguinal lymph nodes (ILN), and spleen were collected and gently disrupted to generate single cell suspensions. Splenocytes were cleared of erythrocytes by ammonium chloride lysis. Nasal-associated lymphoid tissue (NALT) was

collected attached to the palate (Asanuma, Thompson et al. 1997) and cells were released by teasing.

Memory B cell assay

Single-cell suspensions of immune cells were prepared in IMDM (Invitrogen, Carlsbad, CA) containing L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), gentamicin (10 μg/ml), and 5 x 10^{-5} M β-mercaptoethanol (designated B cell medium, BCM), and supplemented with 10% fetal bovine serum (FBS). Initially, immune cells were stimulated in vitro by culture with virus-infected splenocytes. Two-fold dilutions of immune cells (12 wells per dilution) were incubated in 96-well flat-bottom tissue culture plates (Corning) containing 5 x 10^5 irradiated (3,000 rad) syngeneic naïve spleen cell feeders and 5 x 10^5 irradiated (3,000 rad) syngeneic virus-infected splenocytes. The total volume per well was 200 μl. Plates were incubated for 4 days (routinely) at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, cells in each well were washed 3 times as described previously (Slifka and Ahmed 1996). Briefly, this involved removing a volume of supernatant, adding fresh BCM containing 2% FBS, and pelleting the cells by centrifugation. After the final wash, cells in each well were resuspended in the remaining volume of medium and transferred to antigen-coated plates for enumeration of virus-specific ASCs by ELISPOT assay (described below). Pre-existing virus-specific ASC numbers in immune cell populations at the time of sampling were determined by direct ex vivo ELISPOT assay. After in vitro B_{Mem} activation and ELISPOT analysis, individual wells were scored positive for virus-specific B_{Mem} if progeny ASC

numbers were greater than the mean pre-existing ASC number plus 3 standard deviations. A minimum of 6 progeny ASCs were also required for a well to be scored positive. In an alternative strategy, graded doses of immune cells were stimulated in vitro by culture with 10^6 irradiated (3,000 rad) syngeneic naïve spleen cell feeders plus BPL-inactivated viral particles (generally. 0.05-0.1 μg/well). On occasion, mitomycin C treatment (Slifka and Ahmed 1996) was used instead of irradiation to block proliferation of naïve spleen cell feeders. Otherwise, the B_{Mem} assay was performed as described above. The virus-specific B_{Mem} frequency was calculated from the number of negative wells per cell dilution by extrapolation to the dilution that gave 37% negative wells (Topham and Doherty 1998). In some experiments, in vitro stimulation cultures were supplemented with 10^5 $CD4^+$ T cells enriched from the spleens of immune mice by negative selection to >85% purity (Topham and Doherty 1998). Briefly, immune splenocytes treated with anti-MHC class II (TIB-120) and anti-CD8 (53-6.72) were incubated with sheep anti-mouse and sheep anti-rat Ig-coupled Dynabeads (Invitrogen), and a magnet was applied according to the manufacturer's instructions to remove labeled cells. Input cell volumes in the B_{Mem} assay were adjusted to maintain the total volume per well at 200 μl. T cell-depleted immune splenocytes (<1% $CD3^+$ T cells) were prepared by complement lysis of cells coated with anti-Thy-1.2 mAb (AT83). Splenocytes were depleted of $CD19^+$ B cells (<0.6%) by treatment with biotinylated anti-CD19 mAb (1D3; BD Biosciences, San Diego, CA), 1 μg/10^6 cells, followed by incubation with streptavidin-coupled Dynabeads (Invitrogen), and magnetic removal of labeled cells according to the manufacturer's instructions. Cell populations were characterized by flow cytometry using FITC-conjugated mAbs to CD3ε (145-2C11) and CD4 (RM4-5); PE-

conjugated mAbs to CD8α (53-6.7) and CD19 (1D3); and APC-conjugated anti-CD3ε mAb (145-2C11; BD Biosciences) as staining reagents.

Influenza-infected splenocytes

Up to 10^8 erythrocyte-free naïve splenocytes in HBSS were pelleted and resuspended in 1 ml HBSS. Influenza virus stock was added to give the required multiplicity of infection, and the preparation was incubated for a total of 4 h with occasional mixing in a 37°C waterbath. After 1 h of the incubation period, 10 ml BCM containing 10% FBS was added per ml of cell suspension. The infected cells were then irradiated (3,000 rad), washed once, and resuspended in complete BCM.

ELISPOT assay

The ELISPOT assay was adapted to enumerate influenza-specific or HSV-specific IgG ASCs. A preparation of concentrated viral particles was disrupted for 10 min at room temperature in a 1 in 10 dilution of disruption buffer (0.5% Triton X-100, 0.6 M KCl, and 0.05 M Tris-HCl, pH 7.5) in PBS, further diluted in PBS, and plated at 1 μg/well in nitrocellulose-bottomed 96-well Multiscreen HA filtration plates (Millipore, Bedford, MA). After overnight incubation at 4°C, plates were washed with PBS, and blocked with BCM containing 10% FBS. Plates were emptied by flicking, and cell suspensions (including resuspended cells from 96-well MBC assay plates) were added in volumes of approximately 100 μl/well. After incubation for 3-

4 h at 37°C in a humidified atmosphere containing 5% CO_2, plates were thoroughly washed with PBS alone and PBS containing 0.1% Tween 20. Alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) diluted to 2 μg/ml in PBS containing 5% bovine serum albumin was added (100 μl/well), and the plates were incubated overnight at 4°C. The plates were then washed extensively with PBS alone and PBS containing 0.1% Tween 20, including washing the underside of the nitrocellulose filters. Spots were developed at room temperature by the addition of 1 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO) in diethanolamine buffer (10% diethanolamine, 0.1 M NaCl, 5 mM $MgCl_2$, and 0.1 M Tris-HCl, pH 9.5), 100 μl/well. After optimal spot development, plates were washed with PBS and dried, and spots representing individual ASCs were counted using an Olympus SZX9 stereozoom microscope.

Statistics

Statistical comparisons of mean values were performed using the nonparametric Mann-Whitney *U* test for unpaired samples.

Results

In vitro activation of influenza virus-specific B_{Mem} using virus-infected splenocytes

Our primary goal of determining influenza-specific B_{Mem} frequencies by limiting dilution assay required an effective method for the in vitro activation of influenza-specific B_{Mem}. Initial experiments evaluated the use of irradiated, influenza-infected syngeneic splenocytes as "stimulators" for this purpose, since a similar approach activates B_{Mem} in the LCMV system (Slifka and Ahmed 1996). In addition, virus-infected splenocytes stimulate the activation of primed, influenza-specific $CD4^+$ T cells, and these would be expected to contribute cytokines to the cultures (Sarawar and Doherty 1994). Limiting, two-fold dilutions of spleen cells from influenza immune mice (the source of B_{Mem}) were cultured in 96-well plates with virus-infected stimulator cells. The starting input dose of immune spleen cells was 10^5. After 4 days incubation, cells were resuspended and transferred to influenza-coated, 96-well ELISPOT plates for the enumeration of influenza-specific ASCs. For each input dose of immune cells, post-stimulation ASC numbers were compared to the number of pre-existing influenza-specific ASCs in the immune spleen, determined by ELISPOT assay at the time of sampling. Wells were scored as positive for influenza-specific B_{Mem} if the post-stimulation ASC count was greater than the mean background count plus 3 SD. All post-stimulation ASC counts of ≤ 5 were considered negative. The influenza-specific B_{Mem} frequency was calculated from the number of negative wells per cell dilution by extrapolation to the dilution that gave 37% negative wells (Topham and Doherty 1998). Studies of the LCMV system by Slifka and Ahmed validated the ELISPOT-based limiting dilution assay for quantitation of virus-specific B_{Mem} (Slifka and Ahmed 1996).

Influenza virus is completely cleared from sublethally infected mice by 10-12 days after infection, corresponding to a decrease in the number of influenza-specific ASCs in responding lymphoid tissue (Liang, Hyland et al. 2001). By 8 weeks or more after infection, the mean number of influenza-specific IgG ASCs in the spleen at an input dose of 10^5 immune cells was <10. In contrast, stimulation of the same input dose of cells with virus-infected splenocytes typically generated uncountable (>100) influenza-specific IgG ASCs. No influenza-specific IgG ASCs were detected after in vitro stimulation of splenocytes from naïve mice, demonstrating that the ASCs generated by stimulation of immune splenocytes reflected activation of virus-specific B_{Mem}.

Influenza-specific B_{Mem} frequencies were determined after in vitro stimulation with virus-infected splenocytes prepared using a range of multiplicities of infection (MOIs). Highest frequencies were obtained at MOIs ranging from 0.01–1.0 (Table I), and an MOI of 0.1 was selected for subsequent experiments using virus-infected splenocytes.

An important question concerned the requirement for $CD4^+$ T cell help for optimal in vitro B_{Mem} activation. To evaluate the contribution of T cells, we determined influenza-specific B_{Mem} frequencies using total or T cell-depleted immune splenocytes. Results were standardized by expressing the B_{Mem} frequency for $CD19^+$ cells. Depletion of $CD19^+$ cells to <0.6% eliminated influenza-specific B_{Mem} from immune splenocytes (data not shown), indicating that B_{Mem} reside in the $CD19^+$ population (Bell and Gray 2003; Blink, Light et al. 2005). The use of total immune splenocytes gave up to three-fold higher frequencies than did the T cell-depleted populations (Table II), indicating an important role for T cell-associated factors during in vitro stimulation. Although the key T cell-associated factors in this system have not been defined, it is

likely that they are derived primarily from $CD4^+$ T cells (Sarawar and Doherty 1994). To determine whether $CD4^+$ T cell help was limiting even during stimulation of total immune splenocytes, a cell population that contained approximately 20% $CD4^+$ T cells, we evaluated the effect of supplementing the stimulation cultures with enriched $CD4^+$ T cells from influenza-immune mice. Addition of enriched, immune $CD4^+$ T cells (10^5 cells/well; >85% $CD4^+$ T cells) to total immune splenocytes cultured with virus-infected stimulator splenocytes had little effect on the number of wells scored positive for B_{Mem} (and hence the B_{Mem} frequency), but increased the number of ASCs generated in positive wells (data not shown). Taken together, our findings indicate that $CD4^+$ T cell-derived factors promote the activation and clonal expansion of influenza-specific B_{Mem} during in vitro culture with virus-infected splenocytes.

In vitro activation of influenza virus-specific B_{Mem} using BPL-inactivated virus particles

Recently, Hebeis and colleagues demonstrated that the form of challenge antigen determines the $CD4^+$ T cell dependence of B_{Mem} responses. The in vivo B_{Mem} response elicited by antigen in the form of non-replicating, virus-like particles was $CD4^+$ T cell-independent, whereas that elicited by soluble monomeric antigen was not (Hebeis, Klenovsek et al. 2004). Apparently, the repetitive arrangement of antigens on the virus-like particles provided a potent activating signal for B_{Mem} that circumvented the requirement for $CD4^+$ T cell help. We therefore tested whether BPL-inactivated influenza virus particles (BPL-flu) would effectively activate virus-specific B_{Mem} in vitro for limiting dilution analysis. Graded doses of immune splenocytes were simply cultured with a feeder population of irradiated naïve syngeneic splenocytes to which

BPL-flu had been added. Titration of the input amount of BPL-flu established that effective influenza-specific B_{Mem} activation was achieved with as little as 0.01 μg/well (Fig. 1A). BPL-flu was used at 0.1 μg/well in subsequent experiments. B_{Mem} frequencies were similar after in vitro stimulation of immune cells with BPL-flu for periods of 3, 4, or 5 days (data not shown), and an incubation period of 4 days was used routinely. No influenza-specific IgG ASCs were generated during the incubation of splenocytes from naïve mice with BPL-flu, demonstrating that only B_{Mem} are activated. B_{Mem} frequencies were comparable when total immune splenocytes were stimulated in vitro with BPL-flu or with virus-infected splenocytes (Table III). However, a consistent trend was for higher ASC counts in positive wells after BPL-flu stimulation, indicating that this approach generated greater clonal expansion of B_{Mem}.

We next evaluated the requirement for $CD4^+$ T cells during in vitro B_{Mem} activation with BPL-flu. T cell-depleted splenocytes were stimulated with BPL-flu, or with virus-infected splenocytes supplemented with enriched, immune $CD4^+$ T cells to provide optimal B_{Mem} activation. In two separate experiments, stimulation with BPL-flu produced B_{Mem} frequencies similar to those obtained using virus-infected stimulators plus immune $CD4^+$ T cells, and approximately 2.4-fold greater than those obtained by stimulation with virus-infected splenocytes alone (Table IV). In addition, stimulation with BPL-flu or with virus-infected splenocytes plus immune $CD4^+$ T cells resulted in similar clonal expansion of B_{Mem}, as indicated by the number of ASCs generated in positive wells (Fig. 2). These experiments emphasize the contribution of $CD4^+$ T cell-associated factors to in vitro B_{Mem} activation. Importantly, our findings indicate that BPL-flu effectively activates influenza-specific B_{Mem} in vitro in the absence of $CD4^+$ T cells.

To assess the specificity of BPL-flu in the activation of B_{Mem}, we cultured splenocytes from HSV-immune mice with BPL-flu. No HSV-specific ASCs were generated in these cultures, demonstrating that BPL-flu activates only influenza-specific B_{Mem} (Fig. 1B). LPS was below the level of detection at the BPL-flu concentrations used for activating influenza-specific B_{Mem}.

Measurement of influenza-specific B_{Mem} frequencies in different anatomical locations

We next determined whether in vitro stimulation with BPL-flu provided the basis for a sensitive limiting dilution assay to measure influenza-specific B_{Mem} frequencies in different anatomical locations. Cell populations for analysis were prepared from the NALT, MLN, spleen, and ILN of mice 8-12 weeks after i.n. influenza infection. The tissues sampled included sites (NALT, MLN) that generate strong B cell responses to influenza infection of the respiratory tract (Liang, Hyland et al. 2001; Sangster, Riberdy et al. 2003), and a site (ILN) that does not. Our analysis demonstrated influenza-specific B_{Mem} in all sites examined (Fig. 3), supporting the notion that B_{Mem} distribute to lymphoid tissues throughout the body (Bachmann, Kundig et al. 1994). However, this distribution was not equal. Significantly higher B_{Mem} frequencies were present in the NALT and MLN compared with the spleen and ILN, indicating a concentration of B_{Mem} in lymphoid tissue associated with the site of infection even long after the infectious agent has been cleared. This preliminary analysis establishes that our approach is sufficiently sensitive and reproducible to demonstrate differences between anatomical locations in influenza-specific B_{Mem} frequencies.

BPL-inactivated HSV activates HSV-specific B_{Mem} for limiting dilution analysis

Our studies of the influenza system raised the possibility that the use of BPL-inactivated virus particles may have broad applicability for in vitro B_{Mem} activation. We therefore turned to a mouse model of HSV infection and analyzed approaches to the in vitro activation of HSV-specific B_{Mem} for limiting dilution analysis. An ELISPOT assay for HSV-specific IgG ASCs was used to detect B_{Mem} activation and plasma cell formation. Splenocytes from HSV-immune mice (the source of B_{Mem}) were cultured in limiting dilution format with feeder splenocytes to which BPL-inactivated HSV (BPL-HSV) had been added. This strategy effectively activated HSV-specific B_{Mem} at BPL-HSV doses as low as 0.0025 μg/well (Fig. 1B). No HSV-specific IgG ASCs were generated from naïve splenocytes stimulated with BPL-HSV, demonstrating that only B_{Mem} were activated. The specificity of BPL-HSV stimulation was tested by incubating splenocytes from influenza-immune mice with BPL-HSV. No influenza-specific ASCs were generated in these cultures, demonstrating that B_{Mem} activation by BPL-HSV is antigen specific (Fig. 1A). LPS was undetectable in the BPL-HSV preparation.

To evaluate the requirement for T cells during in vitro B_{Mem} activation by BPL-HSV, intact or T cell-depleted immune splenocytes were incubated in limiting dilution format with irradiated feeder splenocytes plus BPL-HSV. HSV-specific MBC frequencies (adjusted to accommodate differences in the proportion of $CD19^+$ input cells) were similar for the intact and T cell-depleted immune populations (Table V). Our findings indicate that BPL-HSV (like BPL-flu in the influenza system) effectively activates virus-specific B_{Mem} in vitro in the absence of T cell-associated factors.

Discussion

The measurement of virus-specific B_{Mem} frequencies by limiting dilution analysis typically relies on B_{Mem} activation and differentiation during a period of in vitro stimulation. Cultures are then scored as positive or negative for virus-specific B_{Mem} on the basis of specific antibody secretion. In this report, we describe a strategy utilizing BPL-inactivated virus particles for the in vitro activation of virus-specific B_{Mem}. This strategy selectively activates virus-specific B_{Mem} and, importantly, achieves this without a requirement for $CD4^+$ T cell-associated factors.

It is well-established that B_{Mem} have less stringent activation requirements than do naïve B cells. In fact, this is reflected in the limiting dilution assay described in this report, since only B cells from immune mice, but not from naïve mice, were stimulated in vitro to produce virus-specific antibodies. However, the requirements for optimal B_{Mem} activation in vitro are not known. Initially, we used virus-infected splenocytes as "stimulators" for the activation of influenza-specific B_{Mem}. This approach produced consistently higher influenza-specific B_{Mem} frequencies when total, compared with T cell-depleted, immune splenocytes were tested. Supplementation of total immune splenocytes with enriched immune $CD4^+$ T cells had no effect on the influenza-specific B_{Mem} frequency, but did increase the number of ASCs generated in positive wells. Thus, when influenza-infected splenocytes were used as stimulators, there was a minimum requirement for T cell-associated factors to promote B_{Mem} activation and plasma cell formation. Exceeding this minimum requirement by adding immune $CD4^+$ T cells simply increased the number of plasma cells formed, presumably by driving the clonal expansion of activated B_{Mem}. Although the precise nature of the T cell "help" remains to be determined, T

cell-derived cytokines are likely to be important factors. In studies of the LCMV system, B_{Mem} activation during culture with virus-infected splenocytes was improved by supplementary cytokines (Slifka and Ahmed 1996).

Our studies demonstrate that in vitro B_{Mem} activation with BPL-inactivated virus particles, at least in the influenza and HSV systems, circumvents the requirement for T cell-associated factors. This strategy provides the basis for a limiting dilution assay that can be applied with confidence to determine B_{Mem} frequencies in lymphoid populations that differ substantially in the proportion of $CD4^+$ T cells. For instance, in our analysis of influenza-specific B_{Mem} frequencies in different anatomical locations, the percentage (mean±SD) of $CD4^+$ T cells in the tissues sampled were 5.6±0.3 (NALT), 20.1±1.6 (spleen), 23.6±0.9 (MLN), and 31.7±2.6 (ILN). The use of BPL-flu for in vitro B_{Mem} activation minimized the possibility that results would be influenced by the proportion of $CD4^+$ T cells present in the cultures.

Recently, Hebeis and colleagues emphasized the importance of antigen form in the induction of $CD4^+$ T cell-independent B_{Mem} responses in vivo (Hebeis, Klenovsek et al. 2004). The effectiveness of BPL-inactivated particles in our studies may be due to repetitively arranged epitopes that cross-link B cell receptors and generate potent activating signals. If this is the case, then the B_{Mem} activated will only be those specific for epitopes displayed by molecules on the surface of virions. Generally, however, the most reactive B cell determinants are those present on the abundant viral surface glycoproteins, and it is these determinants that are the targets of neutralizing antibody responses. The majority of influenza-specific antibodies generated during infection, including the primary protective antibodies, target the hemagglutinin and neuraminidase envelope glycoproteins (Marshall, Sealy et al. 1999). It remains to be determined

whether culture with BPL-flu activates the same or a more restricted set of B_{Mem} specificities than does culture with influenza-infected splenocytes. The effectiveness of BPL-inactivated viral particles as B_{Mem} activators raises the possibility that antigens appropriately arranged on an artificial lipid vesicle (Huckriede, Bungener et al. 2003) may also be used for in vitro B_{Mem} activation. This strategy may permit the activation of B_{Mem} specific for selected molecules.

Numerous strategies for viral inactivation may generate particles that are suitable for in vitro B_{Mem} activation. An important consideration is the need to achieve viral inactivation with minimal modification of B cell epitopes. In the current study, viral inactivation was achieved by treatment with BPL, a potent alkylating agent that acts primarily through modification of the viral genome (Perrin and Morgeaux 1995). Compared with other methods of viral inactivation, BPL treatment completely inhibits replicative capacity with much less severe effects on protein structure (Sangster, Mo et al. 1997).

In summary, this report describes a simple, inexpensive strategy for selective, virus-specific B_{Mem} activation in vitro. Most importantly, B_{Mem} activation and clonal expansion is achieved independently of poorly-defined $CD4^+$ T cell-associated factors, thus eliminating a variable that adds uncertainty to estimates of B_{Mem} frequencies. In addition, the use of inactivated virus particles for B_{Mem} activation avoids problems that may be caused by live virus. Although this may not be a significant concern with influenza virus, many viruses including HSV induce the functional impairment or death of leukocytes infected in vitro (Brucher, Domke et al. 1984; Jones, Fernandez et al. 2003). The fact that only antigen-specific B_{Mem} are activated avoids the extensive cell proliferation and detrimental effects on cell culture conditions associated with the use of polyclonal B cell activators. This makes the strategy particularly suitable for limiting

dilution assays performed in 96-well plates and requiring incubation for three or more days. Finally, our demonstration that the strategy is effective with both influenza virus and HSV suggests that it is likely to have broad applicability in a wide range of viral systems.

APPENDIX

Table I

Influenza-specific B_{Mem} frequencies determined after in vitro stimulation with virus-infected splenocytes prepared using different multiplicities of infection[a]

MOI	B_{Mem} Frequency[b]
5	1.4×10^4
1	8.8×10^3
0.1	8.2×10^3
0.01	7.7×10^3
0.001	2.3×10^4

[a] Total splenocytes from influenza-immune mice were incubated in limiting dilution format with influenza-infected syngeneic splenocytes.

[b] Numbers represent the reciprocal of calculated influenza-specific B_{Mem} frequencies per total splenocytes. Results are representative of two separate experiments.

Table II

Comparison of influenza-specific B_{Mem} frequencies determined using total or T cell-depleted immune splenocytes[a]

Immune splenocytes	B_{Mem} Frequency[b]		
	Spleen 1	Spleen 2	Spleen 3
Total	1.3×10^4	1.6×10^4	5.8×10^3
T cell-depleted	4.0×10^4	2.8×10^4	8.3×10^3

[a] Total or T cell-depleted splenocytes from influenza-immune mice were incubated in limiting dilution format with influenza-infected syngeneic splenocytes.

[b] Numbers represent the reciprocal of calculated influenza-specific B_{Mem} frequencies per $CD19^+$ splenocytes.

Table III

Influenza-specific B_{Mem} frequencies determined after in vitro stimulation with virus-infected splenocytes or BPL-flu

In vitro stimulation[a]	B_{Mem} Frequency[b]		
	Experiment 1	Experiment 2	Experiment 3
Virus-infected splenocytes	7.1×10^3	7.3×10^3	8.2×10^3
BPL-flu	8.3×10^3	7.1×10^3	5.6×10^3

[a] Total splenocytes from influenza-immune mice were cultured in limiting dilution format, together with either influenza-infected syngeneic splenocytes or BPL-flu.

[b] Numbers represent the reciprocal of calculated influenza-specific B_{Mem} frequencies per total splenocytes.

Table IV

In vitro stimulation with BPL-flu activates influenza-specific B_{Mem} in the absence of $CD4^+$ T cells

In vitro stimulation[a]	$CD4^+$ T cells[b]	B_{Mem} Frequency[c]	
		Experiment 1	Experiment 2
Virus-infected splenocytes	-	8.3×10^3	1.1×10^4
Virus-infected splenocytes	+	4.3×10^3	4.5×10^3
BPL-flu	-	3.4×10^3	4.1×10^3

[a] T cell-depleted splenocytes from influenza-immune mice were cultured in limiting dilution format, together with either influenza-infected syngeneic splenocytes or BPL-flu.

[b] Cultures were supplemented as indicated with enriched $CD4^+$ T cells from the spleens of influenza-immune mice (10^5 cells/well; >85% $CD4^+$ T cells; <0.1% $CD8^+$ T cells). Control cultures established that this population was free of influenza-specific B_{Mem}.

[c] Numbers represent the reciprocal of calculated influenza-specific MBC frequencies per $CD19^+$ splenocytes.

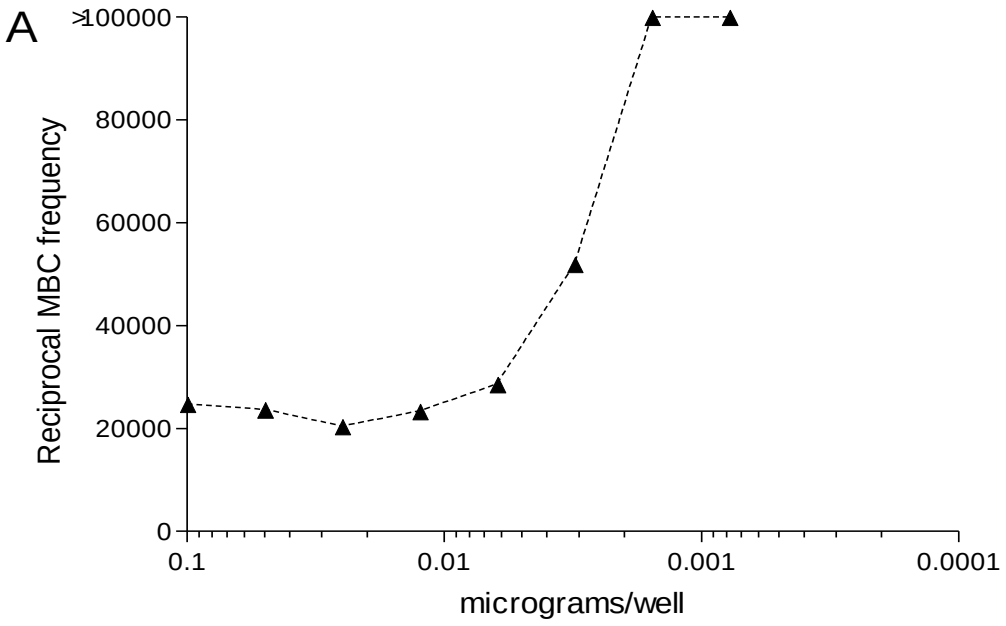
Table V

Comparison of HSV-specific B_{Mem} frequencies determined using total or T cell-depleted immune splenocytes[a]

Immune splenocytes	B_{Mem} Frequency[b]	
	Spleen 1	Spleen 2
Total	4.1×10^3	8.7×10^3
T cell-depleted	4.0×10^3	1.0×10^4

[a] Total or T cell-depleted splenocytes from HSV-immune mice were incubated in limiting dilution format with BPL-HSV (0.01 μg/well).

[b] Numbers represent the reciprocal of calculated HSV-specific B_{Mem} frequencies per $CD19^+$ splenocytes.


A
>100000
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60000
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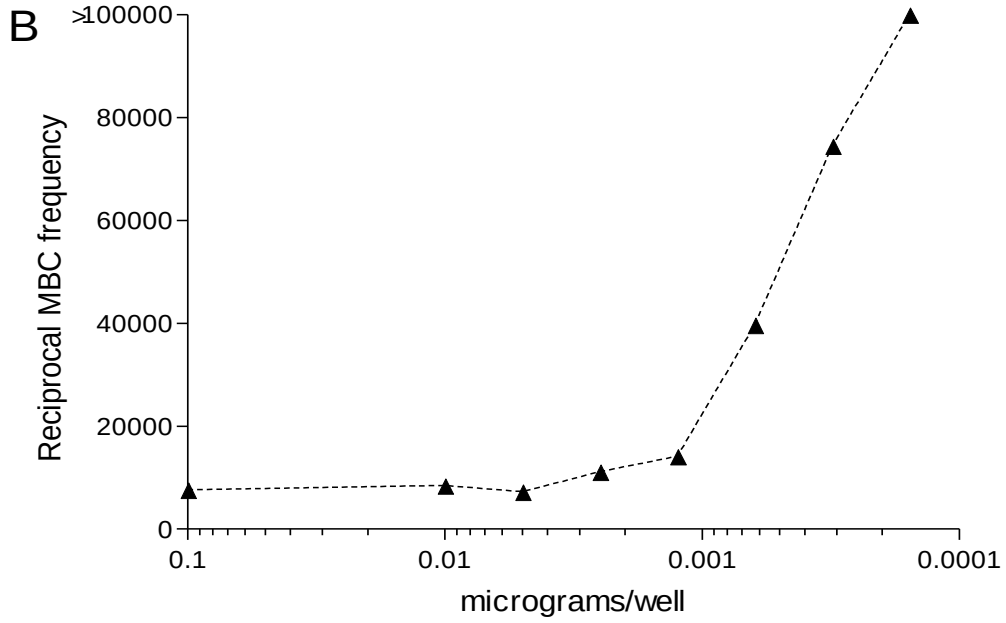
20000
0
Reciprocal MBC frequency
0.1
0.01
0.001
0.0001
micrograms/well


B
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Reciprocal MBC frequency
0.1
0.01
0.001
0.0001
micrograms/well

Figure 1. Titration of BPL-inactivated viral particles for in vitro B_{Mem} activation. (A) Influenza-specific B_{Mem} frequencies determined after in vitro stimulation with different doses of BPL-flu. Total splenocytes from influenza-immune mice were incubated in limiting dilution format with irradiated syngeneic splenocytes and different doses of BPL-flu. Parallel sets of control wells received BPL-HSV instead of BPL-flu. After 4 days incubation, influenza-specific ASC numbers were determined by ELISPOT assay, and wells were scored as positive or negative for precursor B_{Mem}. Numbers represent the reciprocal of calculated influenza-specific B_{Mem} frequencies per total splenocytes. No influenza-specific ASCs were generated in wells that contained 0.1, 0.01, or 0.005 μg/well of BPL-HSV. Results are representative of two separate experiments. (B) HSV-specific B_{Mem} frequencies determined after in vitro stimulation with different doses of BPL-HSV. HSV-specific B_{Mem} frequencies were determined as described above using total splenocytes from HSV-immune mice and a range of doses of BPL-HSV. Control wells received BPL-flu instead of BPL-HSV. An ELISPOT assay for HSV-specific ASCs was used to score wells as positive or negative for precursor B_{Mem}. Numbers represent the reciprocal of calculated HSV-specific B_{Mem} frequencies per total splenocytes. No HSV-specific ASCs were generated in wells that contained 0.1, 0.01, or 0.005 μg/well of BPL-flu. Results are representative of two separate experiments.

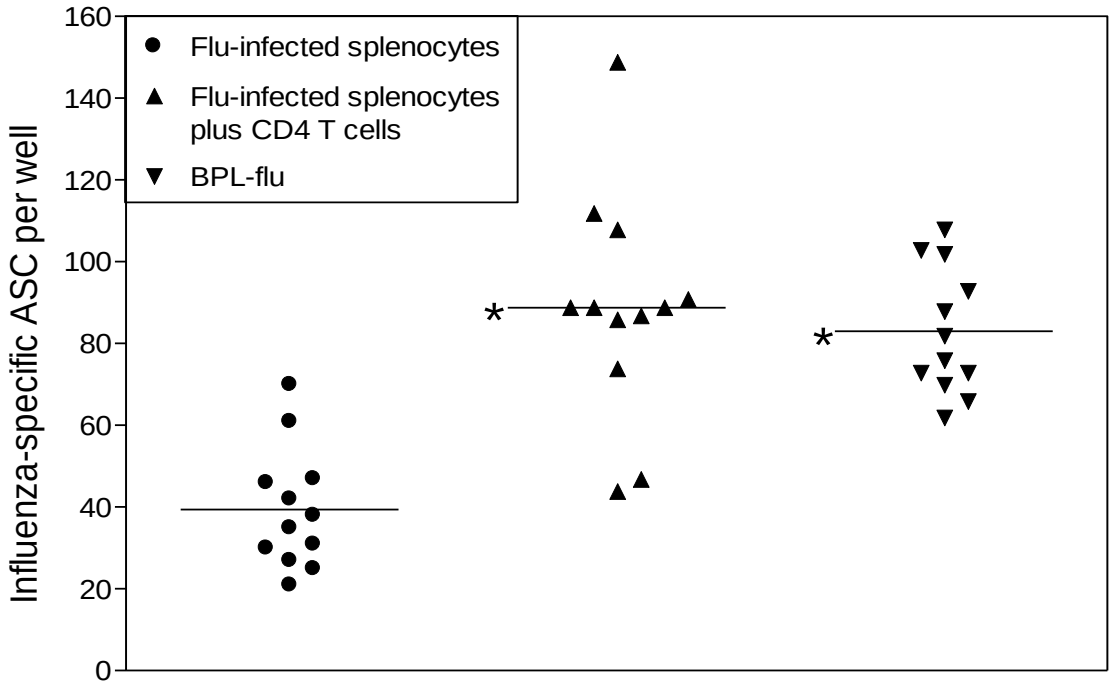

Figure 2. Clonal expansion of influenza-specific B_{Mem} after in vitro stimulation. Two-fold dilutions of T cell-depleted splenocytes from influenza-immune mice (12 wells per dilution) were stimulated in vitro by culture with (i) influenza-infected splenocytes, (ii) influenza-infected splenocytes plus enriched $CD4^+$ T cells from the spleens of influenza immune mice, or (iii) BPL-flu. After 4 days incubation, influenza-specific ASC numbers in each well were determined by ELISPOT assay. ASC numbers are shown for wells receiving 5×10^4 T cell-depleted splenocytes. A horizontal bar identifies the mean. Results are representative of two separate experiments. *, $P < 0.0005$ compared with cultures stimulated with influenza-infected splenocytes alone.

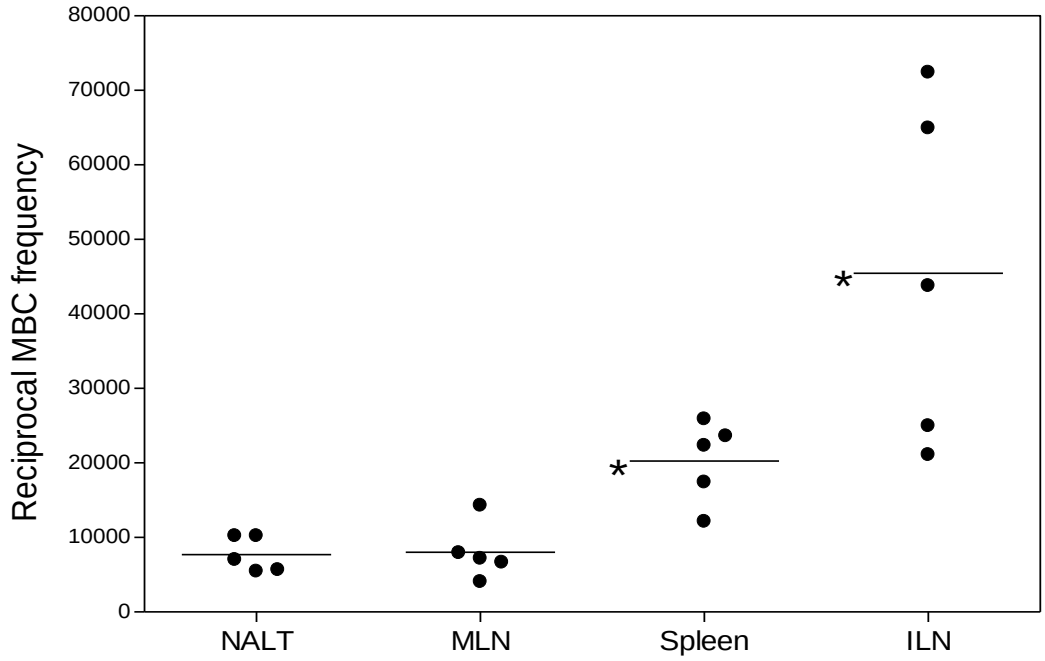

Figure 3. Influenza-specific B_{Mem} frequencies in different anatomical locations. Cell populations from influenza-immune mice were cultured for 4 days in limiting dilution format, together with BPL-flu for in vitro stimulation. An ELISPOT assay for influenza-specific ASCs was used to identify wells positive for precursor B_{Mem}, and B_{Mem} frequencies were calculated. Numbers represent the reciprocal of influenza-specific B_{Mem} frequencies per total input cells. A horizontal bar identifies the mean. *, $P < 0.02$ compared with NALT and MLN.

Chapter 3

Broad dispersion and lung localization of virus-specific memory B cells induced by influenza pneumonia

Research described in this chapter is a modified version of an aticle published in 2008 in *the National Academy of Sciences of the USA* by Hye Mee Joo, Yuxia He and Mark Y. Sangster.

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In this chapter "our" and "we" refers to me and co-authors. My contribution in the paper includes (1) the majority of the lab work and (2) most of planning experiments and the data analysis and (3) Preparation of graphs, figures and tables and (4) editing and discussions.

ABSTRACT

Although memory B cells (B_{Mem}) contribute significantly to resistance to infection, B_{Mem} population characteristics that may relate to protective efficacy have received little attention. Here we report a comprehensive quantitative analysis of virus-specific IgG and IgA B_{Mem} dispersion following transient influenza pneumonia in mice. From early in the response, B_{Mem} circulated continuously and dispersed widely to secondary lymphoid tissues. However, a complicated picture emerged with B_{Mem} frequency differences between secondary lymphoid tissues indicating an influence of local tissue factors on trafficking. B_{Mem} numbers increased and stabilized at tissue-specific frequencies without contraction of the B_{Mem} pool during the period of

analysis. The lung was notable as a non-secondary lymphoid tissue where a rapid influx of IgG and IgA B_{Mem} established relatively high frequencies that were maintained long-term. Our findings provide insights into the pattern of B_{Mem} dispersion, and emphasize the lung as a complex repository of immune memory following local infection.

INTRODUCTION

Memory B and T cell subsets generated during adaptive immune responses play a key role in long-term resistance to infectious agents expressing previously encountered antigens. After formation in organized secondary lymphoid tissues, the cellular elements of immunological memory migrate via function-related trafficking pathways and populate lymphoid and non-lymphoid tissues throughout the body. This process establishes a dispersed state of cellular memory that optimizes protective efficacy. The dispersed CD4 and CD8 memory T cell populations have been well-characterized (Marshall, Turner et al. 2001; Masopust, Vezys et al. 2001; Reinhardt, Khoruts et al. 2001), but similar comprehensive studies of B cell memory have not been performed (Anderson, Tomayko et al. 2006).

The two cellular components of B cell memory, long-lived plasma cells (LLPCs) and memory B cells (B_{Mem}), are the products of germinal center reactions in secondary lymphoid tissue (McHeyzer-Williams and McHeyzer-Williams 2005). LLPCs are classically recognized as Ab-secreting cells (ASCs) that localize in the bone marrow (BM) and maintain circulating Ab levels (Slifka and Ahmed 1996). However, long-lasting populations of IgA-secreting cells (in

particular) are also established at mucosal surfaces after mucosal immunization where they provide local protection (Jones and Ada 1986; Liang, Hyland et al. 2001). In contrast to plasma cells, B_{Mem} are quiescent cells that require stimulation to divide and differentiate into ASCs. Upon encounter with recall antigen, B_{Mem} mediate the rapid, vigorous, and high affinity secondary Ab response that plays a key role in immune protection. The impression from studies to date is that B_{Mem} spread widely to organized lymphoid tissues (Bachmann, Kundig et al. 1994; Slifka, Antia et al. 1998; Vanitha, Joo et al. 2007), but the full extent of B_{Mem} dispersion has not been determined. It is also unclear whether B_{Mem} trafficking patterns relate to the Ab isotype expressed.

The present study provides a comprehensive quantitative analysis of the dispersed cellular components of B cell memory induced by influenza A virus infection. Intranasal (i.n.) influenza virus administration results in a highly localized pulmonary infection because of the dependence of viral replication on a trypsin-like enzyme that is largely restricted to respiratory epithelial cells (Steinhauer 1999). Although infected pulmonary dendritic cells are thought to travel to regional lymphoid tissues and spleen and drive influenza-specific T and B cell responses, little if any infectious virus is detected outside the lung (Eichelberger, Wang et al. 1991; Hamilton-Easton and Eichelberger 1995; Legge and Braciale 2003). The development of adaptive immune responses correlates with the clearance of infectious virus from the lung, which is complete approximately 10 days after infection (Gerhard, Mozdzanowska et al. 1997; Woodland 2003). The strong influenza-specific B cell response to infection is dominated by the production of IgG isotypes, but there is also a substantial IgA response. Thus, the model provides an opportunity to compare the state of both IgG and IgA memory. Our findings provide insights

into the anatomically dispersed state of B cell memory established during the return to homeostasis following a transient, localized viral infection of the lung.

Materials and METHODS

Mice and infection

C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were housed under specific pathogen-free conditions until infection, and thereafter in BSL2 containment. Female mice were used in all experiments and were infected at 8-10 weeks of age. Influenza virus A/HKx31 (H3N2) was grown in the allantoic cavity of embryonated hen's eggs. Mice were anesthetized with Avertin (2,2,2-tribromoethanol) given intraperitoneally, and then infected i.n. with $10^{6.8}$ 50% egg infectious doses of influenza virus (30μl in Dulbecco's PBS). Animal procedures were approved by the Animal Care and Use Committee of the University of Tennessee.

Tissue sampling and treatment

Tissues collected from exsanguinated mice were processed to generate single-cell suspensions in IMDM (Invitrogen, Carlsbad, CA) containing L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), gentamicin (10 μg/ml), and 5×10^{-5} M β-mercaptoethanol (designated B cell medium, BCM), and supplemented with 10%

FBS. Lymph nodes (CLN, MedLN, ILN, and MesLN) and spleen were disrupted between the frosted ends of microscope slides. BM cell suspensions were obtained by flushing the femurs and tibiae. Red blood cells were removed from the spleen and BM preparations by ammonium chloride lysis. Cell populations from the o-NALT and d-NALT were collected as previously described (Asanuma, Thompson et al. 1997). Lungs were finely minced and incubated for 1 h at 37°C in BCM containing 10% FBS and 4 mg/ml collagenase type II (Worthington, Lakewood, NJ). Cells pelleted from the lung digest were resuspended in 40% isotonic Percoll and layered over 75% isotonic Percoll. After centrifugation at 600*g* for 20 min at 25°C, cells at the interface were collected and washed. PP were dissected from the small intestine and washed, and cells were released by digestion with 2 mg/ml collagenase type I (Worthington) for 30 min at 37°C. Cells from heparinized blood were collected over Lympholyte-Mammal (Cedarlane, Burlington, NC) according to the manufacturer's instructions. Spleens were processed individually; otherwise tissues were pooled from 2-5 mice to generate sufficient cells for analysis. Cell populations were characterized by flow cytometry using FITC-conjugated mAb to CD3ε (145-2C11) and PE-conjugated mAb CD19 (1D3; BD Biosciences) as staining reagents.

Cell enrichment

$CD19^+$ B cells were enriched (purity >95%) from single cell suspensions by MACS using murine CD19 MicroBeads and LS columns according to the manufacturer's instructions (Miltenyi Biotec).

ELISPOT assay

Influenza-specific ASCs were enumerated by ELISPOT assay as previously described (Li, Vanitha et al. 2006). Plate-bound secreted Abs were detected using alkaline phosphatase-conjugated goat anti-mouse Abs with specificity for IgG or IgA (Southern Biotechnology, Birmingham, AL).

Memory B cell assay

Influenza-specific B_{Mem} frequencies were determined by a previously described LDA based on in vitro stimulation of B_{Mem} to differentiate into ASCs (Li, Vanitha et al. 2006). Briefly, two-fold dilutions of cells were incubated in 96-well tissue culture plates (routinely 12 wells per dilution), together with 10^6 irradiated (3000 rad) syngeneic naïve spleen cell feeders plus β-propiolactone-inactivated HKx31 (Charles River, Wilmington, MA). After incubation, cells in each well were transferred to ELISPOT plates for the enumeration of influenza-specific IgG or IgA ASCs. Pre-existing virus-specific ASC numbers at the time of sampling were determined by direct ex vivo ELISPOT assay. After in vitro B_{Mem} activation and ELISPOT analysis, individual wells were scored positive for virus-specific B_{Mem} if progeny ASC numbers were greater than twice the mean pre-existing ASC. The approach was modified for tissues that had high numbers of virus-specific ASCs at the time of sampling. For analysis of BM and d-NALT, wells were scored positive for B_{Mem} relative to unstimulated cultures (without inactivated-HKx31). Lung cell suspensions prepared 3 wk or more after infection were enriched for $CD19^+$ cells to reduce ASC numbers before in vitro stimulation, and were compared to unstimulated cultures for identification of positive wells. The virus-specific B_{Mem} frequency was calculated from the

number of negative wells per cell dilution by extrapolation to the dilution that gave 37% negative wells (Topham and Doherty 1998). Linearity between the proportion of negative cultures and the input cell dose indicated direct measurement of B_{Mem}. Virus-specific IgG and IgA B_{Mem} were defined as cells that generated IgG and IgA ASCs, respectively, after in vitro stimulation (Okumura, Julius et al. 1976; Coffman and Cohn 1977). No influenza-specific IgG or IgA ASCs were detected after in vitro stimulation of spleen, lung, or PP lymphocytes from naïve mice or from mice infected i.n 8 wk previously with an unrelated virus (murine gammaherpesvirus 68). IgG and IgA B_{Mem} frequencies (per $CD19^+$ cells) in the lung were similar regardless of whether total or CD19-enriched immune cell populations were analyzed, indicating that results were independent of tissue-specific non-B cell factors (Mora, Iwata et al. 2006). This was supported by a B_{Mem} analysis of CD19-enriched immune spleen cells stimulated in the presence of CD19-depleted immune PP cells, or CD19-enriched immune PP cells stimulated in the presence of CD19-depleted immune spleen cells (data not shown).

Statistical analysis

Mean values were analyzed using one-way ANOVA and the Tukey post test for multiple comparisons. The nonparametric Kruskal-Wallis test and Dunn's post test were used as required to accommodate values below the limit of assay sensitivity. Tests were performed using GraphPad software (SanDiego, CA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

The primary influenza-specific ASC response to i.n. administered virus has been well-characterized. Initially, responses develop in the organized nasal-associated lymphoid tissues (o-NALT), which samples antigens in the upper respiratory tract, and in the cervical (CLN) and mediastinal (MedLN) lymph nodes, which drain the upper and lower respiratory tract, respectively(Liang, Hyland et al. 2001; Sangster, Riberdy et al. 2003). A delayed response develops in the spleen, presumably reflecting the transit time of antigen-laden dendritic cells migrating from the lung. Responses in these sites wane rapidly after the elimination of infectious virus. The response kinetics are notably different in the BM and in the submucosa of the upper and lower respiratory tract, where long-maintained populations of influenza-specific ASCs are established (Jones and Ada 1986; Hyland, Sangster et al. 1994; Liang, Hyland et al. 2001). To expand our understanding of B cell memory generated by primary influenza pneumonia, we determined the frequencies of virus-specific ASCs and B_{Mem} in a broad range of anatomical locations 8-12 weeks after infection. At this time, a fully dispersed and stabilized state of B cell memory would be expected (Slifka, Antia et al. 1998).

Tissue distribution of influenza-specific IgG B_{Mem}

Influenza-specific IgG B_{Mem} were present in all of the organized lymphoid tissues examined (Fig. 1*A*). B_{Mem} frequencies expressed as a proportion of total cells were significantly higher in the o-NALT, MedLN, and Peyer's patches (PP) than in multiple other sites (Fig. 1*A*, legend). Interestingly, IgG B_{Mem} preferentially localized in the PP, even though influenza-

specific ASCs were not generated at this site during the primary response to infection (data not shown). Notably, a relatively high B_{Mem} frequency was also present in the lung, a site of plasma cell concentration (Fig. 1*B*). The BM contained a substantial plasma cell population, but B_{Mem} were infrequent (<1 in 10^5) and may have been contaminating cells in the vasculature. B_{Mem} were consistently present in the blood, indicating maintenance of B_{Mem} circulation long after viral clearance.

There was a considerable range in the proportions of $CD19^+$ B cells in the different anatomical sites examined, with high percentages in the o-NALT and PP and low percentages in the diffuse(d)-NALT and lung (Fig 1*C*). Enrichment and depletion experiments established that the $CD19^+$ population contained all of the influenza-specific B_{Mem} detected in our analysis (data not shown). Therefore, to standardize site-to-site comparisons, influenza-specific IgG B_{Mem} frequencies were calculated as a proportion of $CD19^+$ cells (Fig. 1*D*). This analysis clearly established anatomical differences in IgG B_{Mem} localization ($P < 0.0001$) and identified the MedLN, lung, and d-NALT as sites of preferential concentration. The proportion of IgG B_{Mem} in the $CD19^+$ cell population was significantly higher in the MedLN than in all other sites except the lung and d-NALT, and was significantly higher in the lung and d-NALT compared with the spleen, inguinal lymph node (ILN), and blood (Fig. 1*D*, legend). The prominence of the o-NALT and PP as sites of B_{Mem} localization (Fig. 1*A*) was less marked after standardizing the expression of B_{Mem} frequencies. With this adjustment, frequencies in the o-NALT and PP were still consistently higher than in the spleen and some lymph nodes, but differences were not statistically significant. B_{Mem} frequencies did not correlate with participation of the tissue in the immune response to influenza, as evidenced by significantly higher frequencies in the MedLN

compared with the CLN (both responding sites), and similar frequencies in the CLN and non-responding sites such as the PP and mesenteric lymph node (MesLN). The overall impression is one of widespread IgG B_{Mem} dispersion modulated by local tissue factors.

The total number of IgG B_{Mem} in each anatomical location was calculated from the frequency and cell yield (Fig. 1*E*). Because of its cellularity, the spleen was by far the major repository with approximately 5,000 IgG B_{Mem}, representing close to two-thirds of the total IgG B_{Mem} measured in the analysis.

Tissue distribution of influenza-specific IgA B_{Mem}

Influenza-specific IgA B_{Mem} frequencies differed depending on anatomical location (P = 0.0004) (Fig. 1*F*). Measurable IgA B_{Mem} frequencies were present on the majority of samplings in the o-NALT, MedLN, lung, and blood. In all other locations examined, frequencies were always below the level of sensitivity of the assay (frequency $<1/10^5$ cells). IgA B_{Mem} frequencies in the o-NALT, MedLN, and lung were approximately one-tenth of the IgG B_{Mem} frequencies, perhaps reflecting the overall IgA:IgG composition of the primary response to infection (Fig. 2*A*). The presence of IgA B_{Mem} in the blood suggests continuous re-circulation, as was the case for IgG B_{Mem}.

Kinetics of influenza-specific AFC and B_{Mem} generation and dispersion

To gain insights into the establishment of the dispersed state of B cell memory, we determined influenza-specific ASC and B_{Mem} frequencies in selected sites at intervals after infection (Fig. 2). A vigorous IgG ASC response accompanied by B_{Mem} production developed first in the MedLN and later in the spleen (Fig. 2*A*). In contrast to the peak and decline in IgG ASC frequencies in the MedLN and spleen, the pattern for IgG B_{Mem} was the quick establishment and maintenance of the frequencies that were characteristic of these tissues in the long-term (Fig. 2*B*). As early as day 7 after infection, the presence of circulating IgG B_{Mem} indicated that B_{Mem} dispersion from sites of generation had commenced. Notably, IgG B_{Mem} were detected in the lung as early as day 7 ($<1/10^5$ total cells) and had increased substantially in numbers by day 9, raising the possibility of a functional significance of these cells during the acute response. A limited analysis of IgA B_{Mem} on days 9 and 14 demonstrated circulating cells and relatively high frequencies in the lung, emphasizing the lung as a site of rapid and preferential B_{Mem} localization (Fig. 2*C*). ASC frequencies in the lung progressively increased from 1-3 wk after infection and then plateaued, a kinetic pattern consistent with the immigration of ASCs (and presumably also B_{Mem}) from other sites, rather than local synthesis. Although IgA ASCs were consistently present in the blood from 1-3 wk after infection (5-10 IgA ASC/10^5 cells), circulating IgG ASCs were never detected, perhaps reflecting an influence of isotype expression on the differentiation rate of activated B cells. IgG B_{Mem} were detected earlier and established higher frequencies in the PP compared with the ILN, even though immune responses to influenza do not occur in either site. The general pattern was for IgG B_{Mem} frequencies in all tissues to increase and stabilize, with no evidence of contraction of the B_{Mem} pool during the period of analysis.

DISCUSSION

The state of B cell memory generated by influenza infection of the respiratory tract provides profound and long-lasting resistance to reinfection with the homologous virus, as well as varying levels of protection against non-homologous but serologically related strains. The protection provided by pre-existing virus-specific Abs produced by ASCs in the upper and lower respiratory tract and in the BM is well-recognized. However, much less attention has been given to the virus-specific B_{Mem} pool, even though this is likely to be of considerable importance in conferring long-term protection, especially if pre-existing Ab levels begin to fall. The present analysis focuses on the virus-specific B_{Mem} population generated by primary influenza infection.

Our findings are consistent with the concept of widespread B_{Mem} dispersion to secondary lymphoid tissues throughout the body. However, what also emerge are significant tissue-specific influences on B_{Mem} localization. After adjusting for the proportion of $CD19^{+}$ cells, the MedLN, d-NALT, and lung stood out as sites of preferential IgG B_{Mem} localization. The high B_{Mem} frequency in the MedLN is not simply a consequence of a vigorous B cell response and B_{Mem} formation in this site, since the CLN also responds strongly to infection. Indeed, multiple site-to-site comparisons indicate that participation in the response to infection is not a determinant of the IgG B_{Mem} frequency that is established. IgG B_{Mem} frequencies in the o-NALT and PP, although significantly lower than in the MedLN, were still consistently higher than in the spleen and some lymph nodes, suggesting additional complexity in the regulation of B_{Mem} localization. The set of expressed homing molecules that direct B_{Mem} migration is not well-established and may vary within the B_{Mem} population (Roy, Kim et al. 2002). Since naïve and B_{Mem} traffic through many of

the same lymphoid tissues, there is likely to be considerable overlap in the homing molecules that regulate the process (Rodrigo Mora and Von Andrian 2006). However, even subtle differences between naïve and memory B cells in the expression levels of homing molecules may be sufficient to bias B_{Mem} localization. For example, up-regulation of $\alpha4\beta7$ on B_{Mem} (Roy, Kim et al. 2002) may favor entry into the PP, o-NALT, and mucosa-associated lymph nodes, where high endothelial venules (HEV) display relatively high levels of MadCAM-1, the $\alpha4\beta7$ ligand (Csencsits, Jutila et al. 1999). Our findings may also reflect tissue differences in the rate of egress of lymphocytes. A key component of this process is receptor-mediated cell migration along an increasing sphingosine-1-phosphate (S1P) concentration gradient between the lymphoid tissue interior and adjacent blood or lymph (Cyster 2005). Inflammatory mediators released early in immune responses stimulate local overproduction of S1P in lymphoid tissues, leading to disruption of the S1P concentration gradient or down-regulation of cell receptors for S1P. The result is a transient block in lymphocyte egress from lymphoid tissues. Thus, high B_{Mem} frequencies in constitutively active lymphoid tissues like the PP and o-NALT may be a consequence of both preferential entry and delayed egress.

Importantly, our studies demonstrate that a relatively high frequency of virus-specific IgG B_{Mem} is established and maintained in the lung following influenza infection. This finding emphasizes the complexity of the lung as a site for the localization of the cellular elements of adaptive immunity. Previous studies have established that virus-specific ASCs and memory $CD4^+$ and $CD8^+$ T cells persist in the lung long after influenza and other viruses that target the respiratory tract have been cleared (Jones and Ada 1986; Hogan, Usherwood et al. 2001; Hogan, Zhong et al. 2001; Ostler, Hussell et al. 2001). The kinetics of B_{Mem} (and ASC) movement into

the lung resembled that previously reported for virus-specific $CD4^+$ and $CD8^+$ T cells (Cauley, Cookenham et al. 2002; Roman, Miller et al. 2002; Lawrence, Ream et al. 2005). There is little information on molecules specifically expressed by B_{Mem} that direct entry into the lung, but at least initially the process is likely to resemble that for activated lymphocytes in general as they exit the vasculature in response to local inflammation-associated changes (Kim 2004; Luster, Alon et al. 2005). Up-regulation of $\alpha4\beta1$ on B_{Mem} may be expected, since the $\alpha4\beta1$/VCAM-1 adhesion pathway is a key element in lymphocyte trafficking into the inflamed lung (Feng, Britton et al. 2000). The microanatomical location of B_{Mem} in the lung has not been identified, but they may come to reside primarily in the relatively organized regions of lymphoid tissue that form in lung within 10 days of infection (Moyron-Quiroz, Rangel-Moreno et al. 2004). This lymphoid tissue, collectively referred to as induced bronchus-associated lymphoid tissue (BALT), includes areas that resemble secondary lymphoid tissue with distinct B and T cell zones and HEV. Lymphocyte homing to BALT, as well as to PP and peripheral lymph nodes, is highly dependent on L-selectin and LFA-1 expression (Xu, Wagner et al. 2003). However, the BALT homing pathway may rely particularly on $\alpha4\beta1$/VCAM-1 interactions, since VCAM-1 is expressed at much higher levels on BALT HEV than on HEV in other secondary lymphoid tissues (Xu, Wagner et al. 2003). T cell studies indicate that lung lymphocytes are part of a re-circulating pool (Moyron-Quiroz, Rangel-Moreno et al. 2006), moving via lymphatics through draining lymph nodes to eventually reach the venous blood. A preferential lymphocyte trafficking circuit from lung → draining lymph nodes → blood → lung has been proposed (Zammit, Turner et al. 2006), and may underlay the high B_{Mem} frequencies that we consistently observed in the MedLN. The IgG B_{Mem} frequency (as a proportion of $CD19^+$ cells) was also

relatively high in the d-NALT sample, which consisted of cells isolated from the thin tissue lining the nasal cavity (o-NALT excluded). This result reflects the presence of a small number of B_{Mem} in a tissue that contained few lymphocytes.

Influenza-specific IgG B_{Mem} frequencies in the BM were always below the level of sensitivity of the assay ($<1/10^5$ cells), in contrast to the concentration of LLPCs in this site. Our findings are consistent with other studies that reported the scarcity of B_{Mem} in the BM (Bachmann, Kundig et al. 1994; Slifka, Antia et al. 1998). Interestingly, this contrasts with the role of the BM as a significant reservoir of memory T cells (Marshall, Turner et al. 2001; Di Rosa and Pabst 2005). Although distinct trafficking characteristics may contribute to the difference between memory T and B cell frequencies in the BM (Mazo, Honczarenko et al. 2005), another factor may be B_{Mem} differentiation in the BM microenvironment to replenish the LLPC population.

Only the o-NALT, MedLN, lung, and blood had measurable influenza-specific IgA B_{Mem} frequencies when analyzed 8 wk or more after infection. This finding may simply reflect a consistent ratio of IgG:IgA B_{Mem} (approximately 10:1) in all of the solid tissues sampled, since this could result in IgA B_{Mem} frequencies below the level of assay sensitivity in all sites except the o-NALT, MedLN, and lung. Alternatively, our data can be taken to reflect preferential IgA B_{Mem} trafficking to the respiratory tract and associated lymphoid tissues. It is well established that the expression of different sets of adhesion molecules and homing receptors by IgG- and IgA-producing ASCs results in preferential trafficking to the BM and mucosal sites, respectively (Cyster 2003). There is evidence for further compartmentalization of IgA ASC trafficking, with preferential migration to either the small intestine or to other mucosal sites like the lung

(McDermott and Bienenstock 1979). Further studies are required to firmly establish whether an analogous situation applies to IgA B_{Mem}. Our analysis demonstrated a rapid increase in IgA B_{Mem} frequency in the lung during the acute response, with maintenance of a lower frequency in the long-term. In contrast, IgA B_{Mem} frequencies in the MedLN (presumably a key site of IgA B_{Mem} formation) stabilized at higher frequencies after clearance of the virus. This may reflect the lymphocyte trafficking pathway from lung to draining lymph nodes discussed above.

In our analysis of the MedLN and spleen, long-term B_{Mem} frequencies (per total cells) were comparable to previous limiting dilution assay (LDA) determinations of memory $CD4^+$ and $CD8^+$ T cell frequencies (Tripp, Sarawar et al. 1995; Topham, Tripp et al. 1996), although more recent studies (for example, using tetramers) indicate that the $CD8^+$ T cell frequencies may be somewhat higher (Flynn, Belz et al. 1998; Hogan, Usherwood et al. 2001; Hogan, Zhong et al. 2001). A more dramatic difference exists in the lung; at least when comparing memory B and $CD8^+$ T cell frequencies (and numbers). During the memory phase after a primary infection, influenza-specific $CD8^+$ T cell numbers in the lung may exceed our estimate of B_{Mem} numbers by up to 100-fold (Hogan, Usherwood et al. 2001; Lawrence, Ream et al. 2005). This difference may be much less marked for memory $CD4^+$ T cells, which decrease in frequency in the lung much more rapidly than do $CD8^+$ T cells after viral clearance (Cauley, Cookenham et al. 2002). A stable population of approximately 30,000 influenza-specific ASCs was maintained in the lung following infection. These cells, which can be considered the effector form of B_{Mem}, are numerically more comparable to the memory $CD8^+$ T cell population in the lung. It is not known whether our LDA strategy underestimates influenza-specific B_{Mem} frequencies, as was shown to be the case for LDA analysis of $CD8^+$ T cell frequencies prior to the introduction of tetramers.

However, at present, our approach may be the best option for enumerating a B_{Mem} population representing specificities for multiple, often poorly defined epitopes on different molecules.

In summary, the current report provides the most comprehensive picture currently available of the dispersed virus-specific B_{Mem} pool generated by primary influenza infection. Perhaps primarily, our findings focus attention on the lung as a complex repository of B and T cell memory that may contribute substantially to resistance to respiratory infections. The concept of effector lymphoid tissue (ELT) has been introduced to describe the stable accumulations of functionally significant memory T cells that are established in non-lymphoid tissues as a consequence of infection (van Panhuys, Perret et al. 2005). Our studies demonstrate that B_{Mem} (as well as ASCs) should be recognized as components of the ELT that develops in the lung following influenza infection. Future studies are required to relate B_{Mem} numbers, location, and isotype expression to protective immunity and may have important implications for vaccine development.

APPENDIX

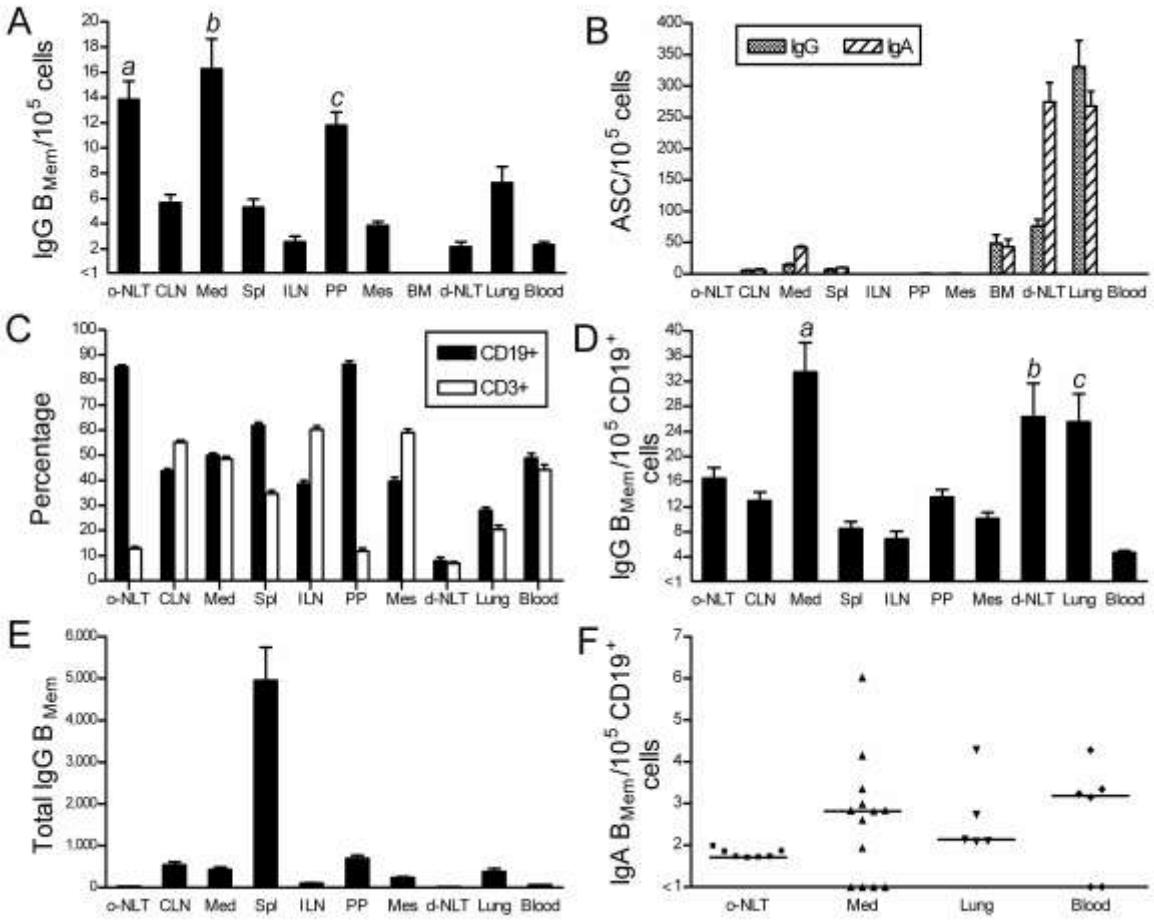

A
IgG B Mem/10^5 cells
a
b
c
o-NLT CLN Med Spl ILN PP Mes BM d-NLT Lung Blood
B
IgG
IgA
ASC/10^5 cells
o-NLT CLN Med Spl ILN PP Mes BM d-NLT Lung Blood
C
Percentage
CD19+
CD3+
o-NLT CLN Med Spl ILN PP Mes d-NLT Lung Blood
D
IgG B Mem/10^5 CD19+ cells
a
b
c
o-NLT CLN Med Spl ILN PP Mes d-NLT Lung Blood
E
Total IgG B Mem
o-NLT CLN Med Spl ILN PP Mes d-NLT Lung Blood
F
IgA B Mem/10^5 CD19+ cells
o-NLT Med Lung Blood

Figure 1. Quantitative analysis of anatomically dispersed influenza-specific B_{Mem} and ASCs. Mice were sampled 8-12 wk after i.n. influenza infection. Abbreviations: o-NLT, organized nasal-associated lymphoid tissue; CLN, cervical lymph node; Med, mediastinal lymph node; Spl, spleen; ILN, inguinal lymph node; PP, Peyer's patches; Mes, mesenteric lymph node; BM, bone marrow; d-NLT, diffuse nasal-associated lymphoid tissue. (*A*) IgG B_{Mem} frequencies expressed as a proportion of total cells. B_{Mem} frequencies (*A*, *F*) were determined by LDA based on in vitro stimulation of B_{Mem} to generate ASCs. Formation of IgG and IgA ASCs were taken to reflect precursor IgG and IgA B_{Mem} respectively. $P < 0.0001$ for frequency differences among anatomical locations. [a]o-NLT vs ILN, d-NLT, and blood ($P < 0.001$), and vs CLN, spleen, and Mes ($P < 0.01$). [b]Med vs CLN, Spl, ILN, Mes, d-NLT, lung, and blood ($P < 0.001$). [c]PP vs ILN, d-NLT, and blood ($P < 0.05$). Data are mean + s.e.m. (n = 4-6 for PP, Mes, BM, d-NLT, and blood; otherwise n = 8-12). (*B*) IgG and IgA ASCs were enumerated by direct ex vivo ELISPOT assay and are expressed as a proportion of total cells. Data are mean + s.e.m. (n = 5-12). (*C*) Percentages of B cells ($CD19^+$) and T cells ($CD3^+$) were determined by flow cytometry. Data are mean + s.e.m. (n = 5-12). (*D*) IgG B_{Mem} frequencies shown in *A* expressed as a proportion of $CD19^+$ cells. Data are mean + s.e.m. $P < 0.0001$ for frequency differences among anatomical locations. [a]Med vs CLN, Spl, ILN, and blood ($P < 0.001$), vs o-NLT and Mes ($P < 0.01$), and vs PP ($P < 0.05$). [b]d-NLT vs ILN and blood ($P < 0.01$), and vs Spl ($P < 0.05$). [c]Lung vs vs ILN and blood ($P < 0.01$), and vs Spl ($P < 0.05$). (*E*) Total IgG B_{Mem} were calculated from the frequency (shown in *A*) and cell yield. Data are mean + s.e.m. (*F*) IgA B_{Mem} frequencies expressed as a proportion of $CD19^+$ cells. Only tissues that had measurable IgA B_{Mem} frequencies on at least one occasion are shown. The median of each group is indicated by a line. Frequencies were

below the level of assay sensitivity in the following tissues (n = 3-5): CLN, Spl, ILN, PP, Mes, and BM. P = 0.0004 for frequency differences among all anatomical locations analyzed. IgA B_{Mem} frequencies were not significantly different in pairwise comparisons of anatomical locations.

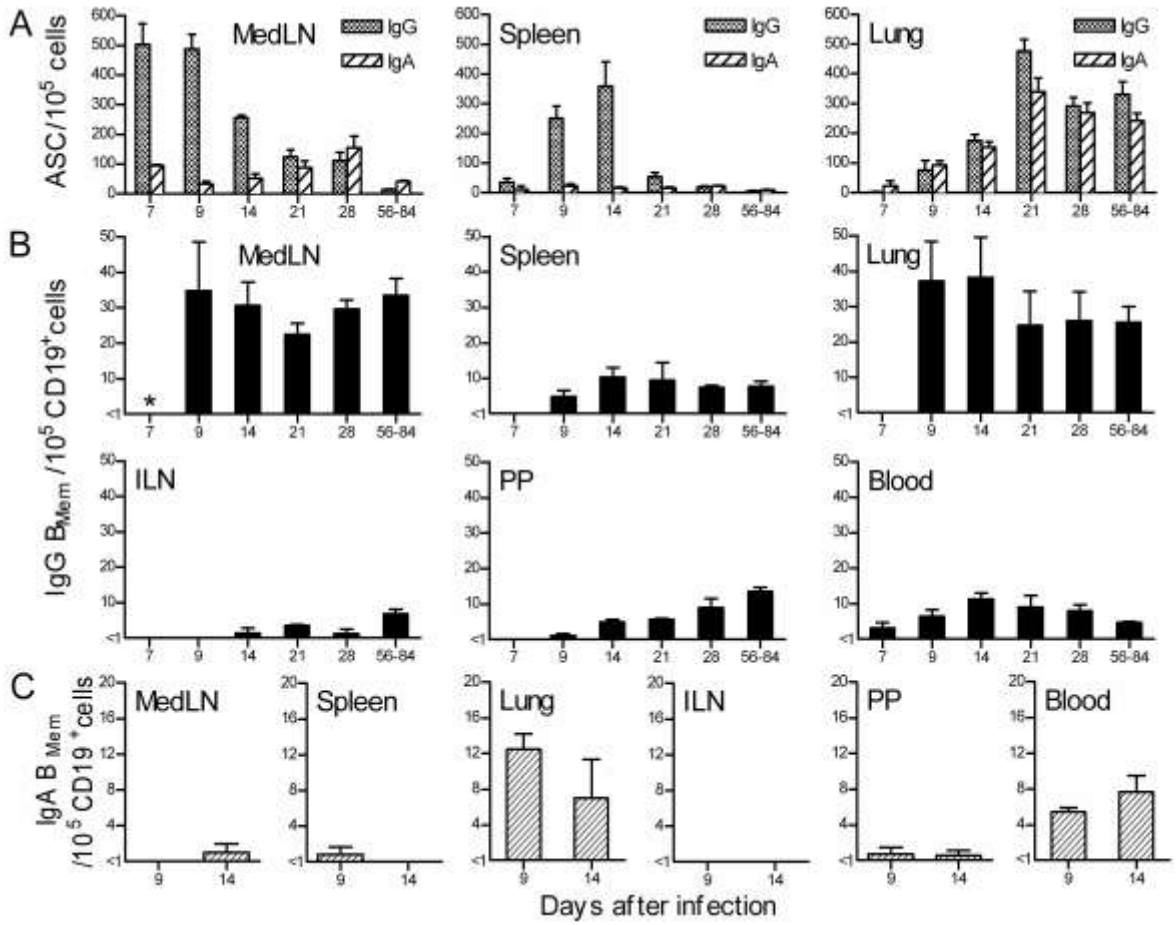

A
ASC/10^5 cells
MedLN
Spleen
Lung
IgG
IgA
B
IgG B_Mem /10^5 CD19+cells
MedLN
Spleen
Lung
ILN
PP
Blood
*
C
IgA B Mem /10^5 CD19 + cells
MedLN
Spleen
Lung
ILN
PP
Blood
7
9
14
21
28
56-84
Days after infection

Figure 2. Kinetic analysis of influenza-specific ASC and B_{Mem} generation and dispersion. Mice were sampled at intervals after i.n. influenza infection. Data for days 56-84 from the analysis shown in Fig. 1 are included for comparison. Abbreviations: MedLN, mediastinal lymph node; ILN, inguinal lymph node; PP, Peyer's patches. (*A*) IgG and IgA ASC frequencies in the MedLN, spleen, and lung were enumerated by direct ex vivo ELISPOT assay and are expressed as a proportion of total cells. Data are mean + s.e.m. (n = 3-7 for days 7-28). (*B*) IgG B_{Mem} frequencies in the MedLN, spleen, lung, ILN, PP, and blood expressed as a proportion of $CD19^+$ cells. Frequencies were determined by LDA based on in vitro stimulation of B_{Mem} to generate ASCs. Formation of IgG ASCs was taken to reflect precursor IgG B_{Mem}. Data are mean + s.e.m. (n = 3 for days 7-28). *The IgG B_{Mem} frequency in the responding MedLN on day 7 was $>1/10^5$ cells, but could not be accurately measured by LDA. (*C*) IgA B_{Mem} frequencies in the MedLN, spleen, lung, ILN, PP, and blood expressed as a proportion of $CD19^+$ cells. Frequencies were determined as for *B*, with formation of IgA ASCs taken to reflect precursor IgA B_{Mem}. Data are mean + range (n = 2).

Chapter 4

Quantitative analysis of influenza virus-specific B cell memory generated by different routes of inactivated virus vaccination

Abstract

The humoral response to inactivated viral vaccines generates a state of B cell memory in the form of Ab-secreting cells (ASCs) and memory B cells (B_{Mem}) that determines protective efficacy. Although the activation of B_{Mem} by recall Ag contributes substantially to protection, the population characteristics of B_{Mem} generated by vaccination has received little attention. Here, we consider both ASC and B_{Mem} populations in a quantitative analysis of virus-specific B cell memory generated by intramuscular or intranasal vaccination of mice with inactivated influenza virus. The memory phase after both forms of vaccination was characterized by localization of ASCs in the bone marrow and dispersion of B_{Mem} to organized lymphoid tissues. Other features of B cell memory reflected the form of vaccination: the stronger IgG response to intramuscular vaccination correlated with larger numbers of IgG ASCs in the bone marrow and IgG B_{Mem}; IgA production was only prominent in the response to intranasal vaccination and was associated with IgA ASC localization in the lung and IgA B_{Mem} formation. Nevertheless, the B_{Mem} pools generated by both forms of vaccination were comparably effective in responding to viral challenge. Notably, few IgG ASCs or B_{Mem} localized in the lung after intramuscular vaccination, a marked contrast with the preferential localization of ASCs and B_{Mem} in the lung following influenza pneumonia. Our analysis links the route of Ag administration to characteristics of B cell memory that may relate to protective immunity, and emphasizes the potentially suboptimal state of B cell memory in the lung after intramuscular vaccination.

Introduction

Characteristics of the B cell response, and particularly the nature of long-term B cell memory, are key determinants of the protective capacity of many vaccines (Subbarao and Joseph 2007). Ab-secreting cells (ASCs)[3] generated during B cell responses and persisting into the memory phase provide one component of B cell memory (McHeyzer-Williams and McHeyzer-Williams 2005). Long-lived ASCs are primarily recognized as a bone marrow (BM)-resident population that maintains circulating Ab levels (Hyland, Sangster et al. 1994; Slifka, Antia et al. 1998). ASC populations may also be established and maintained at mucosal surfaces, typically following mucosal immunization (Jones and Ada 1986; Liang, Hyland et al. 2001). A second cellular component of B cell memory is formed by memory B cells (B_{Mem}) which are generated in parallel with ASCs during B cell responses (Anderson, Tomayko et al. 2006). B_{Mem} are long-lived, non-ASCs that, upon activation, divide and differentiate into ASCs and mediate the rapid, vigorous, and high affinity secondary Ab response (Ahmed and Gray 1996). The contribution of the B_{Mem} response to protection is particularly important when pre-existing ASC numbers (and thus Ab levels) begin to wane.

As a result of recent studies, a more complete picture of the B_{Mem} pool generated by viral infection is emerging. It is now well-established that B_{Mem} generated during the acute response circulate and disperse to secondary lymphoid tissues throughout the body (Bachmann, Kundig et al. 1994; Vanitha, Joo et al. 2007; Joo, He et al. 2008). In addition, some non-lymphoid organs that are a target of infection may become significant repositories of B_{Mem}, perhaps reflecting de novo lymphoid tissue formation in these locations (Carragher, Rangel-Moreno et al. 2008). An

analysis of B cell memory induced by influenza infection identified the lung as a site where B_{Mem} (as well as ASCs) preferentially localize (Joo, He et al. 2008).

In contrast to the situation following infection, little information is available on the nature of B_{Mem} populations generated by vaccination. These cells are likely to be especially important when the protective efficacy of a vaccine is largely determined by its ability to elicit a humoral response. Such vaccines include the most widely used form of influenza vaccine, an inactivated split virus preparation that is administered i.m (Gerdil 2003). Generally, influenza-specific circulating Ab levels induced by this vaccine have declined by 6 mo after immunization (Powers, Smith et al. 1995; Kunzel, Glathe et al. 1996), but a longer-lasting B_{Mem} pool remains to respond to recall Ag and enhance resistance to infection (Wrammert, Smith et al. 2008). Here we report a comprehensive quantitative analysis of the state of virus-specific B cell memory generated by vaccination with inactivated influenza virus. Our analysis considers the B_{Mem} and ASC populations resulting from i.m. or intranasal (i.n.) Ag administration. We show that parameters of B cell memory that may relate to the level of protective immunity are influenced by the route of immunization. In particular, our analysis emphasizes the marked difference between B cell memory in the lung generated by i.m. vaccination and the situation following influenza pneumonia.

Materials and methods

Mice and immunizations

C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were housed under specific pathogen-free conditions. Female mice were used in all experiments and were immunized at 8-12 weeks of age. The Animal Care and Use Committee of the University of Tennessee approved all animal procedures.

Preparations of sucrose gradient-purified influenza virus A/HKx31 (H3N2) inactivated by treatment with formaldehyde or β-propiolactone were purchased from Charles River (Wilmington, MA). Results were not affected by the method of inactivation. A stock of infectious influenza virus HKx31 was grown and titrated in the allantoic cavity of embryonated hen's eggs. Mice were anesthetized with Avertin (2,2,2-tribromoethanol) given i.p. before all immunizations. For i.m. vaccination, a total dose of 20 μg of inactivated virus was given in two injections, each of 10 μg (50 μl in PBS), into the tibialis anterior muscle of each leg. A plastic sleeve over the needle controlled the depth of injection. The dose of inactivated virus for i.n. vaccination was 30 μg (30 μl in PBS). Vaccinated mice were challenged i.n. with $10^{6.8}$ 50% egg infectious doses of influenza virus (30 μl in PBS).

Tissue sampling and treatment

Anesthetized mice were exsanguinated via the retro-orbital plexus before tissue sampling. Tissues were processed to generate single-cell suspensions in IMDM (Invitrogen, Carlsbad, CA)

containing L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), gentamicin (10 μg/ml), and 5 x 10^{-5} M β-mercaptoethanol (designated B cell medium), and supplemented with 10% FBS. Lymph nodes and spleen were collected and gently disrupted between the frosted ends of microscope slides. BM cell suspensions were obtained by flushing the femurs and tibiae. RBCs were removed from the spleen and BM preparations by ammonium chloride lysis. The organized nasal-associated lymphoid tissue was collected attached to the palate (Asanuma, Thompson et al. 1997) and cells were released by teasing. Lungs were finely minced and incubated for 1 h at 37°C in B cell medium containing 10% FBS and 4 mg/ml collagenase type II (Worthington, Lakewood, NJ). Cells pelleted from the lung digest were resuspended in 40% isotonic Percoll and layered over 75% isotonic Percoll. After centrifugation at 600*g* for 20 min at 25°C, cells at the interface were collected and washed. PP were dissected from the small intestine and washed, and cells were released by digestion with 2 mg/ml collagenase type I (Worthington) for 30 min at 37°C. Blood was collected into heparin sodium (1,000 USP Units/ml, Abraxis, Schaumburg, IL), diluted in an equal volume of HBSS containing 0.1% BSA, layered over Lympholyte-Mammal (Cedarlane, Burlington, NC) and centrifuged at 800*g* for 20 min at 25°C. Cells at the interface were then collected and washed.

Lungs to be titrated for infectious virus were homogenized in 1 ml HBSS containing 0.1% BSA. Homogenates were clarified by centrifugation, and supernatants were stored at -80°C.

Virus titration

Viral titers in lung homogenates were determined by 50% tissue culture infective dose assay using Madin Darby canine kidney cells grown in MEM containing L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5% FBS. Confluent cell monolayers in 96-well tissue culture plates were washed once with serum-free MEM immediately before the addition of viral inocula. Serial 10-fold dilutions of lung homogenates were prepared in MEM containing 0.3% BSA and 1 μg/ml L-(toslyamido 2-phenyl) ethyl chloromethyl ketone-treated trypsin (Worthington), and 0.2 ml volumes were added to the appropriate wells. After 2 days incubation at 37°C with 5% CO_2, wells positive for virus growth were identified by the presence of hemagglutinating activity.

ELISPOT assay

Influenza-specific ASCs were enumerated by ELISPOT assay as previously described (Li, Vanitha et al. 2006). Briefly, plates were coated with purified influenza HKx31 (Charles River) and single cell suspensions were plated and incubated. Alkaline phosphatase-conjugated goat anti-mouse Abs with specificity for Ig isotypes or IgG (Southern Biotechnology, Birmingham, AL) were used in combination with the substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma, St. Louis, MO) to generate spots.

ELISA

Influenza-specific Ab levels in sera were determined by ELISA (Sangster, Topham et al. 2000) using plates coated with purified, detergent-disrupted influenza HKx31 (0.5 μg/well). Briefly, serial 3-fold serum dilutions were added to the plates, and bound Ab was detected with ALP-conjugated goat anti-mouse IgG (Southern Biotechnology) and *p*-nitrophenyl phosphate substrate. Ab concentrations in arbitrary units were calculated from standard curves constructed using goat anti-mouse IgG capture Ab and a purified mouse Ig standard (Southern Biotechnology).

Memory B cell assay

Influenza-specific B_{Mem} frequencies were determined by a previously described limiting dilution assay based on in vitro stimulation of B_{Mem} to differentiate into ASCs (Li, Vanitha et al. 2006). Briefly, two-fold dilutions of cells from immune mice were incubated in 96-well tissue culture plates (routinely 12 wells per dilution starting with 10^5 cells per well), together with 10^6 irradiated (3000 rad) syngeneic naïve spleen cell feeders plus β-propiolactone-inactivated HKx31. Plates were incubated for 4 days at 37°C in a humidified atmosphere containing 5% CO_2. After incubation, cells in each well were washed thoroughly and transferred to ELISPOT plates for the enumeration of influenza-specific IgG or IgA ASCs. Pre-existing virus-specific ASC numbers in immune cell populations at the time of sampling were determined by direct ex vivo ELISPOT assay. After in vitro B_{Mem} activation and ELISPOT analysis, individual wells were scored positive for virus-specific B_{Mem} if progeny ASC numbers were greater than twice the

mean pre-existing ASC. The virus-specific B_{Mem} frequency was calculated from the number of negative wells per cell dilution by extrapolation to the dilution that gave 37% negative wells (Topham and Doherty 1998). B_{Mem} frequencies of less than one per 10^5 input cells could not be accurately determined. Virus-specific IgG and IgA B_{Mem} were defined as cells that generated IgG and IgA ASCs, respectively, after in vitro stimulation (Okumura, Julius et al. 1976; Coffman and Cohn 1977). At the time of sampling, cell populations were characterized by flow cytometry using FITC-conjugated anti-CD3ε (145-2C11) and PE-conjugated anti-CD19 (1D3) mAbs (BD Biosciences, San Diego, CA) as staining reagents. Spleens were processed individually; otherwise tissues were generally pooled from 2-5 mice to generate sufficient cells for analysis. To accomodate tissue differences in the proportion of $CD19^+$ B cells, the population that includes the B_{Mem} (Bell and Gray 2003; Blink, Light et al. 2005; Li, Vanitha et al. 2006), B_{Mem} frequencies are expressed for $CD19^+$ cells.

Statistical analysis

Mean B_{Mem} values were analyzed using one-way ANOVA and the Tukey post test for multiple comparisons. The nonparametric Kruskal-Wallis test and Dunn's post test were used as required to accommodate B_{Mem} values below the limit of assay sensitivity. Other comparisons of group means were performed using the nonparametric Mann-Whitney *U* test for unpaired samples. Tests were performed using GraphPad software (SanDiego, CA). Values of $P < 0.05$ were considered statistically significant.

Results

Influenza-specific Ab responses to intramuscular and intranasal administration of inactivated virus

Initial experiments evaluated the magnitude and quality of the influenza-specific ASC response following two routes of administration of inactivated virus. The i.m. route was used to model human vaccination with inactivated influenza virus, and the i.n. route was included to determine whether mucosal Ag administration influenced parameters of B cell memory. Influenza-specific ASCs in different tissues were enumerated by ELISPOT assay. Responses in the draining lymph nodes and the spleen after i.m. vaccination were characterized by early IgM production, followed by increased numbers of cells producing IgG2c and, to a lesser degree, IgG2b (Fig 1). IgG production in responding lymphoid tissues was weaker following i.n. compared with i.m. vaccination (Fig 2), likely reflecting some loss of effective Ag following mucosal administration. However, the response to i.n. vaccination included a substantial IgA component (see CLN and spleen), which was essentially absent in the response to i.m. vaccination.

Responses in draining lymph nodes and spleen had substantially waned by 3 wk after vaccination by both routes, but ASC populations were established in the BM and maintained into the memory phase (Fig 3A and D). BM ASC populations reflected the isotype composition of the acute response, with IgG or IgA ASCs predominating after i.m. or i.n. vaccination, respectively. Higher IgG ASC numbers in the BM and serum IgG levels following i.m. compared with i.n. vaccination were consistent with a generally stronger acute IgG response (Fig 3C and F).

IgA ASCs generated by i.n. vaccination localized in the lung as well as in the BM (Fig 3D and E). In contrast, very few IgG ASCs were detected in the lung after either route of vaccination (Fig 3B and E), consistent with the notion of differential ASC trafficking patterns related to isotype expression.

Influenza-specific B_{Mem} generated by intramuscular and intranasal vaccination with inactivated virus

Influenza-specific B_{Mem} frequencies in a range of anatomical locations were measured by limiting dilution analysis 8-12 wk after vaccination, a time when fully dispersed and stable B_{Mem} populations would be expected. To standardize site-to-site comparisons, B_{Mem} frequencies were calculated as a proportion of $CD19^+$ cells, the cell population that contained the influenza-specific B_{Mem} in this analysis (Joo, He et al. 2008). IgG B_{Mem} generated by i.m. vaccination dispersed widely to all secondary lymphoid tissues sampled (Fig 4A). Significant variation among mean IgG B_{Mem} frequencies in these tissues ($p < 0.02$) indicated local influences on trafficking. Participation of the tissue in the immune response to vaccination did not correlate with higher B_{Mem} frequencies. IgG B_{Mem} were consistently detected in the blood, indicating a process of continuous recirculation. Lymphocyte populations isolated from the lung after i.m. vaccination occasionally contained influenza-specific IgG B_{Mem}, but frequencies were generally below the limit of sensitivity of the assay, a situation that contrasted with preferential localization of B_{Mem} in the lung after influenza pneumonia (Joo, He et al. 2008).

IgG B_{Mem} frequencies were markedly lower after i.n. compared with i.m. vaccination (Fig 4B), reflecting the relatively weaker acute IgG response. The presence of IgG B_{Mem} in all of the sampled sites was evident by the presence of influenza-specific IgG ASC after in vitro stimulation, emphasizing broad dispersion of these cells and continuous recirculation. However, only the o-NALT, MLN, and PP consistently had B_{Mem} frequencies above the limit of assay sensitivity after i.n. vaccination. Significant variation among mean IgG B_{Mem} frequencies ($p < 0.001$) indicated a non-uniform pattern of dispersion to organized lymphoid tissues. IgG B_{Mem} were infrequent in the lung, indicating that preferential B_{Mem} localization in this tissue (Joo, He et al. 2008) is not simply a consequence of i.n. Ag administration.

A limited analysis of the CLN, MLN, o-NALT, and lung indicated a higher frequency of IgA B_{Mem} after i.n. compared with i.m. vaccination, consistent with the isotype composition of the primary response. Only cell populations (particularly o-NALT cells) from i.n. vaccinated mice gave rise to influenza-specific IgA ASCs during in vitro stimulation of limiting dilution cultures, indicating the presence of precursor IgA B_{Mem} (data not shown). However, IgA B_{Mem} frequencies were less than 1 in 10^5 $CD19^+$ cells and could not be accurately determined.

To further evaluate B_{Mem} populations generated by inactivated virus vaccination, mice primed by the i.m. or i.n. routes were challenged i.n. with live homologous virus. The Ag load following challenge may have differed in the two groups of primed mice because of differences in the levels of pre-existing local and systemic Abs (Fig 3). Nevertheless, the route of vaccination markedly influenced the isotype composition of the secondary response. The secondary response in the CLN of i.n. primed mice had a strong IgA component (Fig 5), consistent with a higher proportion of IgA B_{Mem}. There was a small IgA component in the

secondary response of i.m. primed mice, even though IgA B_{Mem} in these mice were not detected by limiting dilution analysis. Both i.m. and i.n. priming provided a high level of protection. At the time of sampling (day 5), infectious virus titers in the lungs of both groups of primed mice were not significantly different, and were more than 100-fold lower than in the lungs of unprimed mice (data not shown).

Lung localization of B cell memory is poorly induced by intramuscular vaccination

A recent study of virus-specific B cell memory generated by influenza pneumonia identified the lung as a site of preferential localization of ASCs and B_{Mem} (Joo, He et al. 2008). In contrast, the current analysis of the response to a single i.m. vaccination demonstrated systemic B cell memory, but very few ASCs or B_{Mem} in the lung (Figs 3 and 4). To test for an effect of response magnitude, the state of B cell memory in the lung was evaluated after priming and boosting with inactivated virus given i.m. Effective expansion of influenza-specific B cell memory by boosting was indicated by a vigorous secondary B cell response in draining lymph nodes and a significant increase in IgG ASC numbers in the BM and serum IgG levels (Fig 6A-D). However, there was no significant change in the minimal numbers of ASCs or B_{Mem} present in the lung (Fig 6E and F). Surprisingly, IgG B_{Mem} frequencies in the spleen were not obviously increased by boosting, raising the possibility that B_{Mem} expansion does not parallel ASC formation. Overall, the impression is that factors other than response magnitude regulate localization of B cell memory in the lung.

Discussion

The state of B cell memory in the form of ASCs and B_{Mem} is the critical determinant of the protective efficacy of inactivated viral vaccines. Our analysis provides a comprehensive quantitative picture of the state of virus-specific B cell memory generated by i.m. and i.n. vaccination with inactivated influenza virus. The localization and maintenance of ASC populations in the BM (Hyland, Sangster et al. 1994; Slifka, Antia et al. 1998) and the continuous circulation and broad dispersion of B_{Mem} to organized lymphoid tissues (Bachmann, Kundig et al. 1994; Vanitha, Joo et al. 2007; Joo, He et al. 2008) are established features of B cell memory that were common to both forms of vaccination. However, other features that may impact protective efficacy depended on the route of Ag administration.

The i.m. administration of inactivated virus is widely used for human influenza vaccination (Nichol and Treanor 2006). The levels of circulating IgG induced by this strategy correlate with resistance to influenza in humans (Cox, Brokstad et al. 2004), but Ab levels typically begin to fall after vaccination (Powers, Smith et al. 1995; Kunzel, Glathe et al. 1996). As this happens, B_{Mem} and the rapid recall response they mediate are likely to become increasingly important determinants of protection (Wrammert, Smith et al. 2008). In our analysis, acute IgG production was appreciably stronger following i.m. compared with i.n. vaccination, probably simply reflecting differences in the efficiency of Ag delivery to the immune system. The stronger IgG response correlated with a larger IgG ASC population in the BM, higher circulating IgG levels, and a quantitatively greater IgG B_{Mem} pool. Our analysis establishes that IgG B_{Mem} generated by i.m. vaccination would have rapid access to Ag entering the body via any

route, as reflected in the vigorous secondary Ab response following i.n. influenza challenge. In particular, localization of B_{Mem} in organized lymphoid tissues associated with the respiratory tract would ensure prompt activation in the event of influenza infection. Furthermore, the presence of B_{Mem} in the circulation suggests a mobile population that continuously traffics through lymphoid tissues surveying for cognate Ag.

As would be expected in a comparison of mucosal and non-mucosal Ag administration, IgA production was prominent in the response to i.n. but not to i.m. vaccination (Neutra and Kozlowski 2006), and this was reflected in differences in the state of B cell memory. Only i.n. vaccination resulted in a durable IgA ASC population in the lung, thus providing a potentially valuable, broadly protective barrier to infection at the site of virus encounter. In addition, the B_{Mem} population generated by i.n. vaccination included a greater number of IgA B_{Mem}. A practical benefit of this is suggested by the substantial IgA component of the secondary B cell response to i.n. challenge of i.n. vaccinated compared with i.m. vaccinated mice (Fig. 5). Rapid B_{Mem} activation is undoubtedly a valuable protective response, regardless of the Ab isotype expression profile. However, the presence of IgA B_{Mem} may confer an additional advantage, since IgA ASCs generated by IgA B_{Mem} activation would rapidly localize to sites of viral replication in the upper respiratory tract (Liang, Hyland et al. 2001; Sangster, Riberdy et al. 2003). Interestingly, the secondary response to i.n. challenge of i.m. vaccinated mice included an IgA component (Fig. 5), perhaps reflecting the activation of small numbers of IgA B_{Mem}. Analysis of lymphoid tissue populations after i.m. vaccination did not identify IgA B_{Mem}, but this may reflect numbers below the limit of assay sensitivity. It may be noteworthy that IgA ASCs were not completely absent from the response to i.m. vaccination. The presence of IgA ASCs in

the BM after i.m. vaccination (Fig. 3A) suggests a germinal center origin (Smith, Light et al. 1997) and it is therefore likely that some IgA B_{Mem} were generated in parallel. Alternatively, IgG B_{Mem} may retain the potential to switch to IgA expression when activated in an IgA-inductive environment.

The state of B cell memory in the lung following influenza infection is profoundly different from that following i.m. vaccination. Recent analysis of a mouse model of influenza pneumonia demonstrated preferential localization of IgG- and IgA-expressing ASCs and B_{Mem} in the lung (Joo, He et al. 2008). In contrast, the current analysis detected few virus-specific ASCs or B_{Mem} in the lung after i.m. vaccination, and the situation was unchanged after a second i.m. immunization to expand B cell memory (Fig. 6). Data presented in Fig. 7 emphasize the contrast in ASC and B_{Mem} localization after influenza infection and i.m. vaccination: infection resulted in approximately 10-fold higher ASC frequencies in the lung than in the BM, and approximately 3-fold higher IgG B_{Mem} frequencies in the lung than in the spleen; i.m. vaccination resulted in essentially the opposite ratios with preferential localization of ASCs in the BM and B_{Mem} in the spleen. Apparently, ASC and B_{Mem} trafficking to the lung is modulated by local environmental factors associated with influenza infection. Factors associated with inflammation are likely to play a role, but mechanisms may differ depending on cell type and Ab isotype expression.

The stable population of approximately 30,000 virus-specific ASCs established in a mouse lung after influenza infection consists of similar numbers of IgA and IgG ASCs (Joo, He et al. 2008). Molecular interactions that regulate ASC homing to the lung are not fully understood. IgA ASCs are programmed to migrate to mucosal sites by the expression of chemokine receptors responsive to ligands constitutively produced at mucosal locations. The

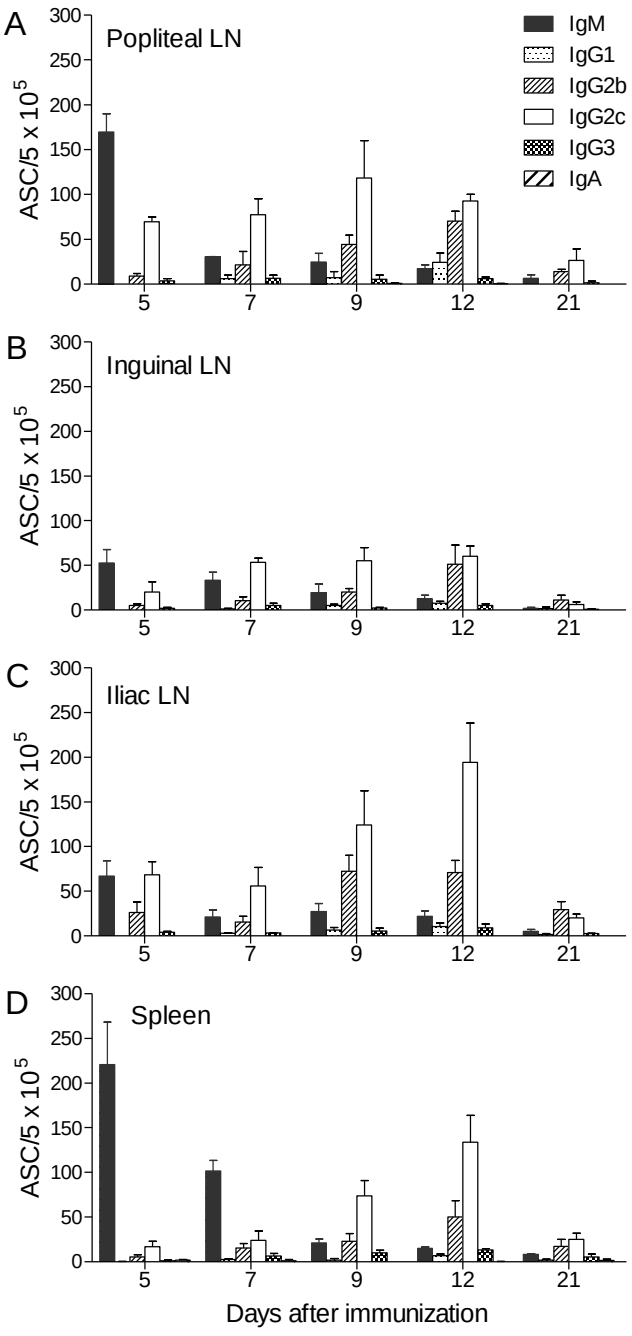
expression of different chemokine receptors may direct IgA ASC trafficking to specific mucosal compartments, such as the lung or the small intestine (Mora and von Andrian 2009). Interestingly, chemokine receptor expression may be influenced by the site of cell activation, such that IgA ASCs formed in lymph nodes draining the respiratory tract are programmed to home to the lung (McDermott and Bienenstock 1979). The regulation of IgG ASC trafficking is somewhat different (Cyster 2003). IgG ASCs express CXCR4 and generally localize in the BM in response to the production of CXCL12, the CXCR4 ligand. However, at least a proportion of IgG ASCs also express CXCR3 and are responsive to chemokines produced at sites of inflammation (Hauser, Debes et al. 2002). CXCL12 production may be upregulated at inflammatory sites (Nanki, Hayashida et al. 2000), and circulating inflammation-associated molecules decrease CXCL12 levels in the BM (Ueda, Yang et al. 2004). The potential for IgG ASCs to bind to activated endothelium and enter sites of inflammation is enhanced by expression of P-selectin glycoprotein ligand-1 and $\alpha 4\beta 1$ (Underhill, Minges Wols et al. 2002). Overall, the impression is that IgA ASCs, and perhaps specifically those generated in lymph nodes draining the respiratory tract, are pre-programmed to home to the lung, whereas IgG ASC trafficking is modulated by ongoing inflammation, as would occur during influenza infection. Our observations are consistent with this scenario. Lung inflammation is unlikely to be a consequence of the i.m. (in particular) or i.n. administration of inactivated virus. Thus, IgG ASCs generated by i.m. vaccination localized in the BM but not in the lung, and lung localization of ASCs after i.n. vaccination was essentially limited to IgA ASCs. Little information is available on molecules expressed by B_{Mem} that direct entry into the lung. IgG B_{Mem} were readily detectable in diverse lymphoid tissues after i.m. vaccination but, in contrast to the situation after influenza

infection, few localized in the lung. Inflammation in the lung may also be a prerequisite for at least IgG B_{Mem} localization. A consequence of influenza infection is formation in the lung of regions of relatively organized lymphoid tissue referred to as inducible bronchus-associated lymphoid tissue (iBALT) (Moyron-Quiroz, Rangel-Moreno et al. 2004). The iBALT has characteristics of secondary lymphoid tissue and may provide a microenvironment for the concentration of B_{Mem} (and perhaps ASCs), but this has not been established.

Our analysis of the state of B cell memory generated by i.m. vaccination emphasizes a number of features that provide valuable protection against influenza. It is well-recognized that this vaccination strategy effectively generates circulating virus-specific IgG that is likely maintained by BM ASCs. Ready access of circulating IgG to sites of influenza replication, especially in the lower respiratory tract, is likely to result from transudation, and this would be enhanced by local inflammation (Persson, Erjefalt et al. 1998). Importantly, our analysis establishes that the B_{Mem} pool generated by i.m. vaccination has characteristics that ensure a potent secondary B cell response to influenza infection. However, our analysis also raises the possibility that the state of B cell memory established in the lung after i.m. vaccination is suboptimal. In particular, this is suggested by a consideration of the situation following influenza infection. What remains to be determined is the protective advantage of lung localization of the cellular elements of B cell memory. Certainly, a case can be made for the value of virus-specific ASCs in the lung. It is well-established that IgA produced by submucosal ASCs is transcytosed across epithelial cells to provide a barrier to infection at the luminal surface. IgG produced by ASCs in the lung parenchyma may supplement this barrier, perhaps after transcytosis across respiratory tract epithelial cells by a process involving FcRn (Spiekermann, Finn et al. 2002).

The advantage of a lung-localized B_{Mem} population is less clear, although these cells are well-positioned to respond rapidly to influenza infection. Future studies aimed at understanding the relationship between B cell memory in its various forms and protective immunity will be important for the optimization of vaccination strategies.

APPENDIX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IgM
IgG1
IgG2b
IgG2c
IgG3
IgA
B
Inguinal LN
C
Iliac LN
D
Spleen
ASC/5 x 10^5
300
250
200
150
100
50
0
5
7
9
12
21
Days after immunization

Figure 1. The acute influenza-specific ASC response to i.m. vaccination. The kinetics of virus-specific ASC responses in the popliteal lymph node (*A*), inguinal lymph node (*B*), iliac lymph node (*C*), and spleen (*D*) were determined for mice vaccinated i.m. with inactivated influenza virus. The ELISPOT assay was used to enumerate influenza-specific ASCs in cell suspensions from individual mice. Results are expressed as the number of ASC/5 x 10^5 nucleated cells. Data are mean + SE (n = 3-5).

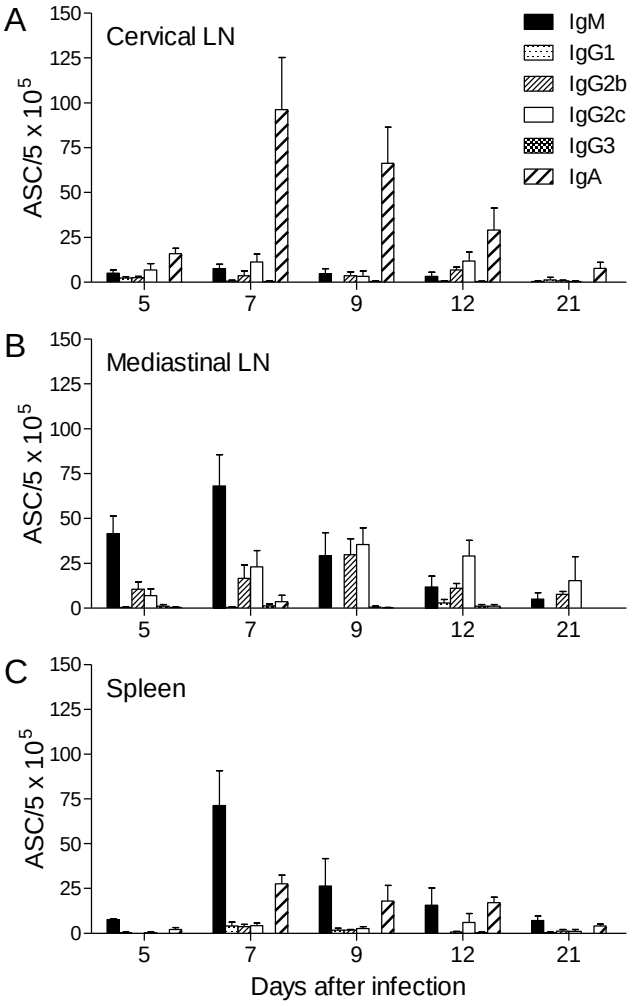


Figure 2. The acute influenza-specific ASC response to i.n. vaccination. The kinetics of virus-specific ASC responses in the cervical lymph node (*A*), mediastinal lymph node (*B*), and spleen (*C*) were determined for mice vaccinated i.n. with inactivated influenza virus. The ELISPOT assay was used to enumerate influenza-specific ASCs in cell suspensions from individual mice. Results are expressed as the number of ASC/5 x 10^5 nucleated cells. Data are mean + SE (n = 3-7).

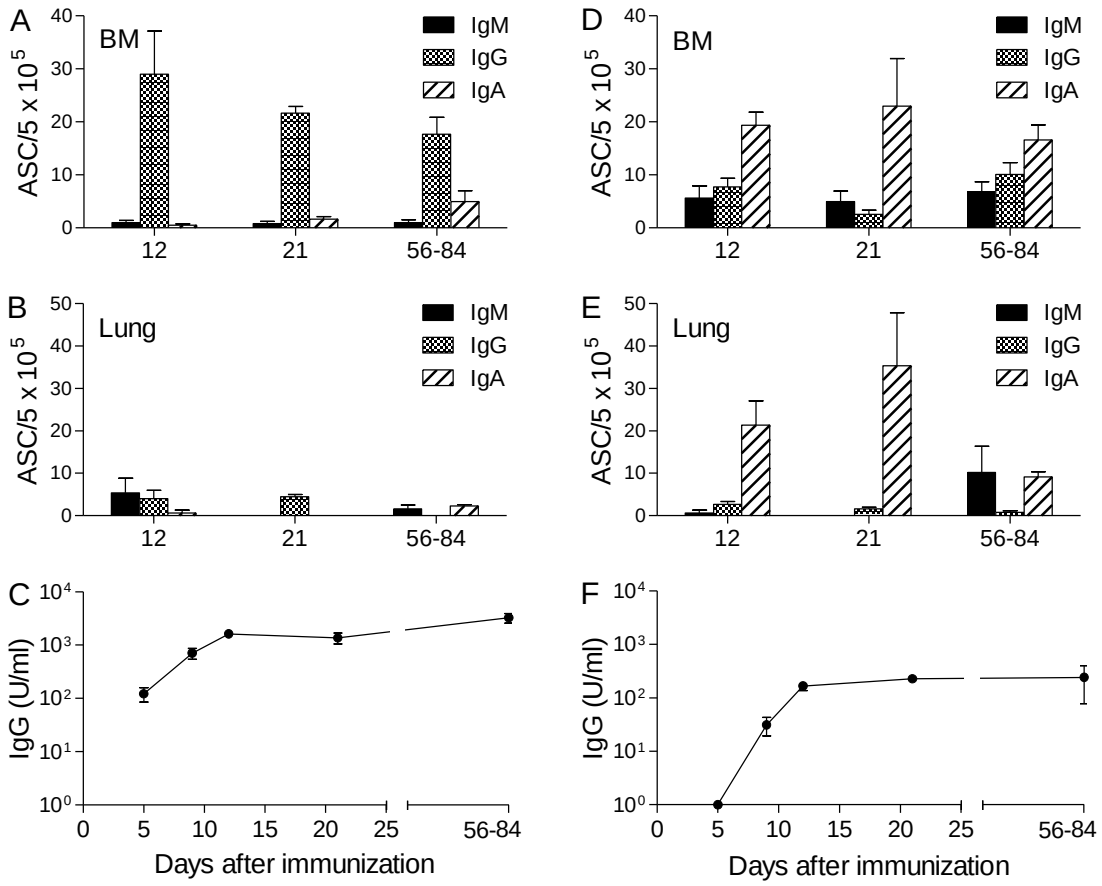


Figure 3. ASC populations in BM and lung and circulating IgG levels after vaccination. Responses are shown after i.m. (*A*, *B* and *C*) and i.n. (*D*, *E* and *F*) vaccination with inactivated influenza virus. Virus-specific ASC numbers in the BM (*A* and *D*) and lung (*B* and *E*) were determined by ELISPOT assay and are expressed per 5 x 10^5 nucleated cells. Serum concentrations of virus-specific IgG (*C* and *F*) were determined by ELISA and are expressed in arbitrary units. Data are mean + SE (n = 3-7).

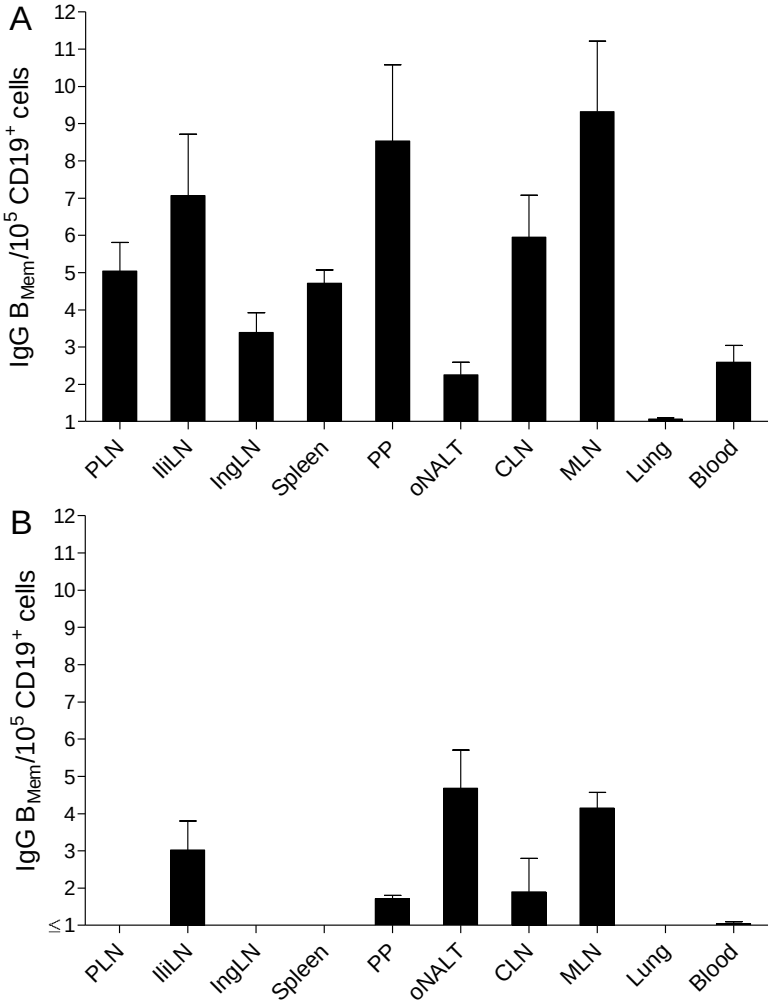

A
IgG $B_{Mem}/10^5$ $CD19^+$ cells
12
11
10
9
8
7
6
5
4
3
2
1
PLN
IliLN
IngLN
Spleen
PP
oNALT
CLN
MLN
Lung
Blood
B
IgG $B_{Mem}/10^5$ $CD19^+$ cells
12
11
10
9
8
7
6
5
4
3
2
1
PLN
IliLN
IngLN
Spleen
PP
oNALT
CLN
MLN
Lung
Blood

Figure 4. Distribution of influenza-specific IgG B_{Mem} after vaccination. IgG B_{Mem} frequencies in different anatomical locations are shown 8-12 wk after i.m. (*A*) and i.n. (*B*) vaccination. PLN, popliteal lymph node; IliLN, iliac lymph node; IngLN, inguinal lymph node; PP, Peyer's patches; oNALT, organized nasal-associated lymphoid tissues; CLN, cervical lymph node; MLN, mediastinal lymph node. Individual spleens were analyzed; otherwise cells were pooled from two or more mice. B_{Mem} frequencies were determined by limiting dilution assay based on in vitro stimulation of IgG B_{Mem} to generate IgG ASCs. Frequencies are expressed as a proportion of $CD19^+$ cells to accommodate differences in the proportion of B cells in different locations. Data are mean + SE (n = 3-10). Mean IgG B_{Mem} frequencies varied significantly among organized lymphoid tissues (i.e. exclusive of lung and blood) after i.m. ($p < 0.02$) and i.n. ($p < 0.001$) vaccination.

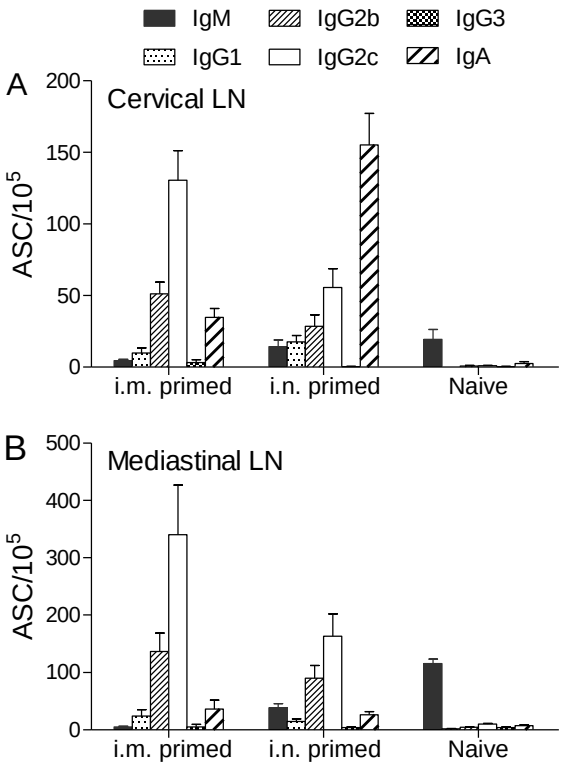
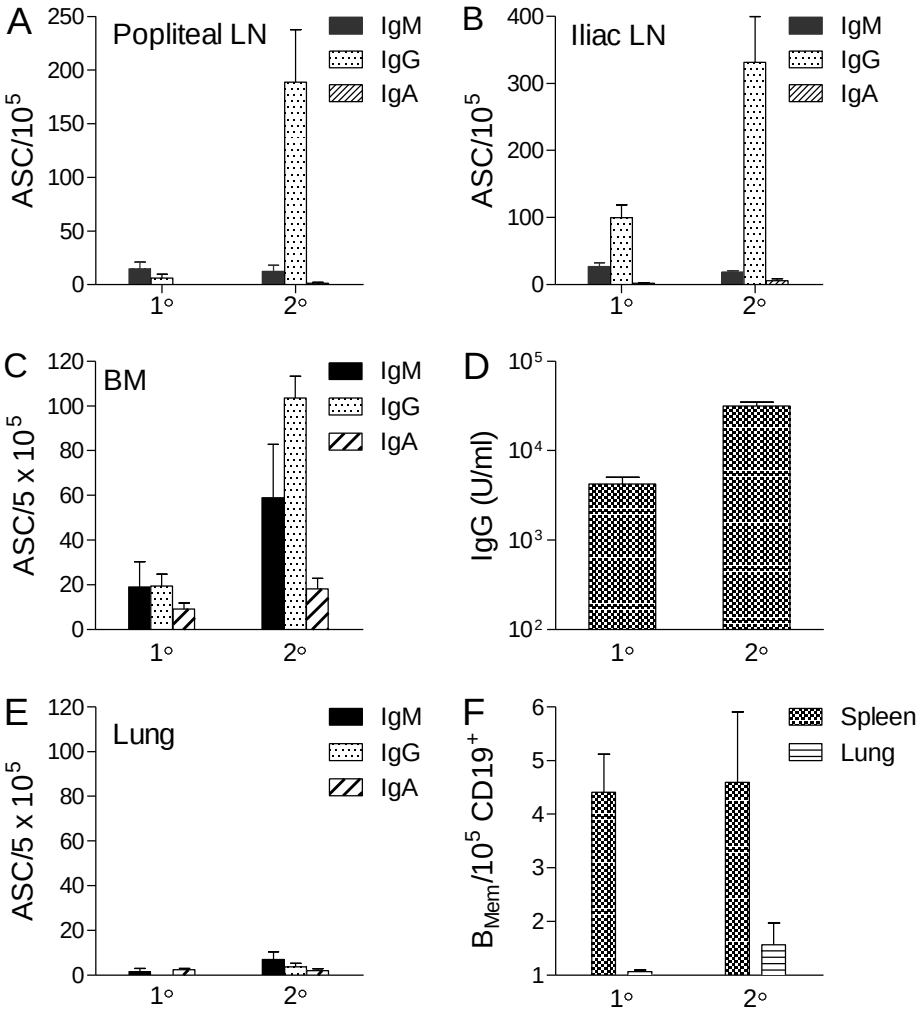


Figure 5. The influenza-specific secondary ASC response after viral challenge of vaccinated mice. Mice were vaccinated i.m. or i.n. with inactivated virus, and challenged with infectious influenza virus given i.n. 12 wk after priming. Virus-specific ASC frequencies in the cervical (*A*) and mediastinal (*B*) lymph nodes of individual mice were determined by ELISPOT assay 5 d after challenge of vaccinated control mice. The response in naïve mice is shown for comparison. Results are expressed as the number of ASC/10^5 nucleated cells. Data are mean + SE (n = 5-9).


A
Popliteal LN
IgM
IgG
IgA
ASC/10^5
250
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0
1°
2°
B
Iliac LN
IgM
IgG
IgA
ASC/10^5
400
300
200
100
0
1°
2°
C
BM
IgM
IgG
IgA
ASC/5 x 10^5
120
100
80
60
40
20
0
1°
2°
D
IgG (U/ml)
10^5
10^4
10^3
10^2
1°
2°
E
Lung
IgM
IgG
IgA
ASC/5 x 10^5
120
100
80
60
40
20
0
1°
2°
F
B_Mem/10^5 CD19^+
Spleen
Lung
6
5
4
3
2
1
1°
2°

Figure 6. Influenza-specific B cell memory after i.m. priming and boosting with inactivated virus. Primed mice were boosted with an identical i.m. vaccination after 8 wk. Virus-specific ASC frequencies were determined by ELISPOT assay and are expressed as a proportion of nucleated cells. Virus-specific IgG B_{Mem} frequencies were determined by limiting dilution analysis based on in vitro stimulation of B_{Mem} to generate ASCs and are expressed as a proportion of $CD19^+$ cells. Lungs were pooled from 2-3 mice for B_{Mem} analysis; otherwise data points represent individual mice. *A* and *B*, Boosting generated a vigorous secondary ASC response. ASC frequencies in draining popliteal (*A*) and iliac (*B*) lymph nodes were measured 5 d after boosting (2º). The response in naïve mice (1º) is shown for comparison. Data are mean + SE ($n = 3$). *C*, Boosting significantly expanded the virus-specific ASC population in the BM. ASC frequencies are shown 8 wk after priming (1º) and 6 wk after boosting (2º). IgG ASCs 1º vs. 2º, $p < 0.005$. Data are mean + SE ($n = 4$ for 1º and 9 for 2º). *D*, Boosting significantly increased circulating virus-specific IgG levels. Serum IgG levels determined by ELISA are shown 8 wk after priming (1º) and 6 wk after boosting (2º). IgG 1º vs. 2º, $p < 0.0001$. Data are mean + SE ($n = 10$-12). *E*, Boosting had no significant effect on virus-specific ASC numbers in the lung. ASC frequencies are shown 8 wk after priming (1º) and 6 wk after boosting (2º). Data are mean + SE ($n = 3$ for 1º and 8 for 2º). *F*, Boosting had no significant effect on virus-specific IgG B_{Mem} numbers in the lung. B_{Mem} frequencies are shown 8 wk after priming (1º) and 6 wk after boosting (2º). Data are mean + SE ($n = 3$-4).

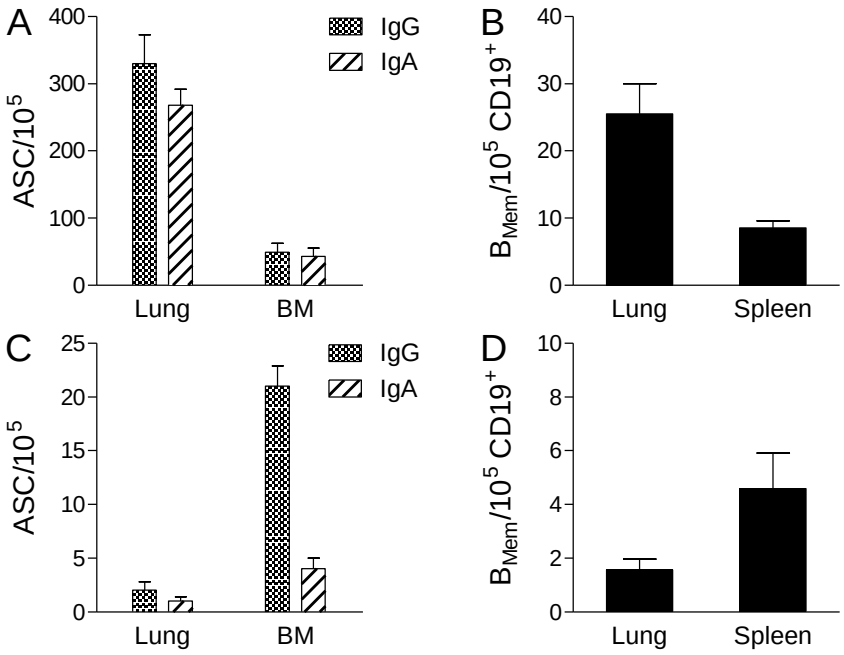

Figure 7. Contrasting patterns of influenza-specific B memory localization in the lung after i.n. infection and i.m. vaccination. Data are presented from (Joo, He et al. 2008) showing virus-specific ASC and B_{Mem} frequencies 8-12 wk after i.n. influenza infection (*A* and *B*). These data are compared with data selected from Fig. 6 of the current study showing virus-specific ASC and B_{Mem} frequencies after i.m. priming and boosting with inactivated influenza virus (*C* and *D*). Mice vaccinated i.m. were boosted at 8 wk and sampled 6 wk later. *A* and *C*, IgG and IgA ASCs are compared in the lung and BM (n = 5-12). *B* and *D*, IgG B_{Mem} frequencies are compared in the lung and spleen (n = 3-12). Data are mean + SE.

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