ABSTRACT

Title of Dissertation: The M167 mu+kappa Immunoglobulin Transgene Increases Susceptibility to Pristane-Induced Plasmacytomas In BALB/C mice

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The goal of this research was to investigate the role of Ig transgenes (Tgs) in plasma cell tumor development as a useful means of examining potential antigen-specific B cell precursors. The Ig Tg M167mk (V1 mu plus Vk24) on a genetically PCT-susceptible BALB/c background was used to determine if B cells expressing anti-phosphorylcholine (PC) B cell receptors are more susceptible than non-Tg littermates to pristane induction of plasma cell tumors. The M167mk Tg mice, which express the anti-PC M167 BCR on >97% of B cells, developed a higher percentage of plasma cell tumors (63%) compared to non-Tg BALB/cAnPt littermates (35%), $p<0.01$, and had a reduced latent period from 240 to 200 days. Serologic and nucleic acid analysis of Igs expressed by Tg tumors revealed co-expression of the M167-id V1-mu/Vk24 transgenes and a rearranged endogenous VH-alpha/Vk Ig. In an attempt to learn something of the natural

history of the PCT precursor B cell, the M167mk transgene and endogenous VH/VL Ig mRNA from Tg PCT cells were sequenced for evidence of somatic hypermutation, a hallmark of T-cell dependent germinal center activity. M167mu and k Tg mRNA transcripts revealed no evidence of mutation of the transgenes; however, sequencing of endogenous Ig mRNA from M167mk Tg PCTs showed that 80% contained somatic hypermutation (SHM) and 20% had germline Ig genes. PCTs from control groups, BALB/c non-Tg littermates and C.B20 mice, also contained 80% SHM and 20% germline Ig genes.

M167mk Tg mice have an increased (MZ) B cell population (15-25% $B220^{+}$), a hyper-activated TI responsive B cell phenotype, compared to non-Tg WT mice (5-10% $B220^{+}$). These data suggest that PC-responsive M167mk B cells are a PCT-susceptible subset, as the M167mk Tg BCR generates B cell clones that are responsive to bacterial PC antigen that likely enhances overall proliferation of B cells during PCT development, thereby increasing the chances of a neoplastic B cell clone to expand and increasing the susceptibility of M167mk Tg mice to the induction of PCTS.

M167 mu+kappa Immunoglobulin Transgene Increases Susceptibility to Pristane-Induced Plasmacytomas in BALB/c Mice

by

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LIST OF ABBREVIATIONS

APC	allophycocyanin (red fluorescent label)
B	biotinylated
BCR	B cell receptor
BM	bone marrow
CDR	complementary determining region
CFSE	5,6-carboxyfluorescein succinimidyl ester
CRP	C-reactive protein
DC	dendritic cells
ELISA	enzyme linked immunosorbent assay
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate (green fluorescent label)
FO	follicular
FSC	forward scatter
g0	generation 0 primary tumor
g1	generation 1 transplanted tumor
GC	germinal center
Hy	hemocyanin
Id	idiotope
Ig	Immunoglobulin
IgA	Immunoglobulin heavy chain alpha
IgG	Immunoglobulin heavy chain gamma

IgH	Immunoglobulin Heavy chain
IgM	Immunoglobulin heavy chain mu
i.p.	intra-peritoneal
Kc	Kappa chain constant variable region
mIg	membrane Ig
MOPC	Mineral Oil-induced PlasmaCytomas
MZ	marginal zone
NCBI	National Center for Biotechnology Information
NF	newly formed
OxLDLs	oxidatively modified low density lipoproteins
PC	phosphorylcholine
PC-Hy	phosphorylcholine conjugated to hemocyanin
PCT	Plasma Cell Tumor or plasmacytoma
PE	phycoerytherin (yellow fluorescent label)
PerCP	peridinin chlorophyll protein (red fluorescent label)
PnC	pneumococcal C polysaccharide
PtC	phosphatidylcholine
PC	phosphorylcholine
RT-PCR	reverse transcriptase-polymerase chain reaction
SAV	streptavidin
SSC	Side Scatter
SHM	somatic hypermutation
sIg	surface Ig

SPE	serum protein electrophoresis
t(12:15)	Chromosomal translocation involving murine chromosomes 12 and 15
T15	TEPC15
Tg	transgenic
TI	T-cell independent
TD	T-cell dependent
VH	Heavy chain variant region
VL	Light chain variant region
Vκ	Kappa light chain variant region
WT	wild type

CHAPTER 1

Introduction

Plasmacytomas

A plasma cell tumor (PCT), such as multiple myeloma in humans or a plasmacytoma in mice, is an immunoglobulin-secreting, neoplastic plasma cell that represents a megaclonal expansion of one plasma cell, and ultimately, of one selected B cell clone. B lymphocytes each express a unique antibody molecule on the plasma membrane that acts as a receptor for antigen. Interaction with antigen triggers the B-lymphocyte to differentiate into a plasma cell that secretes soluble antibody molecules (Error! Reference source not found.). Like normal plasma cells, PCTs secrete large amounts of monoclonal immunoglobulin (Ig), the same antibody that their precursor B cell clone rearranged and expressed on its cell surface. Unlike normal plasma cells, the majority of which are short-lived and die within several days of activation, PCT cells continue to divide indefinitely in an unregulated fashion. They expand to fill the various microenvironments where they are supported, e.g., the bone marrow in humans and the peritoneal cavity in mice.

Plasmacytomas can be induced in genetically susceptible mice by intraperitoneal injection of non-metabolizable light white mineral oils, of which the chemically defined alkane, pristane (2, 6, 10, 14-tetramethylpentadecane), is a frequent component (1). While pristane is not thought to be a DNA damaging carcinogen that directly causes

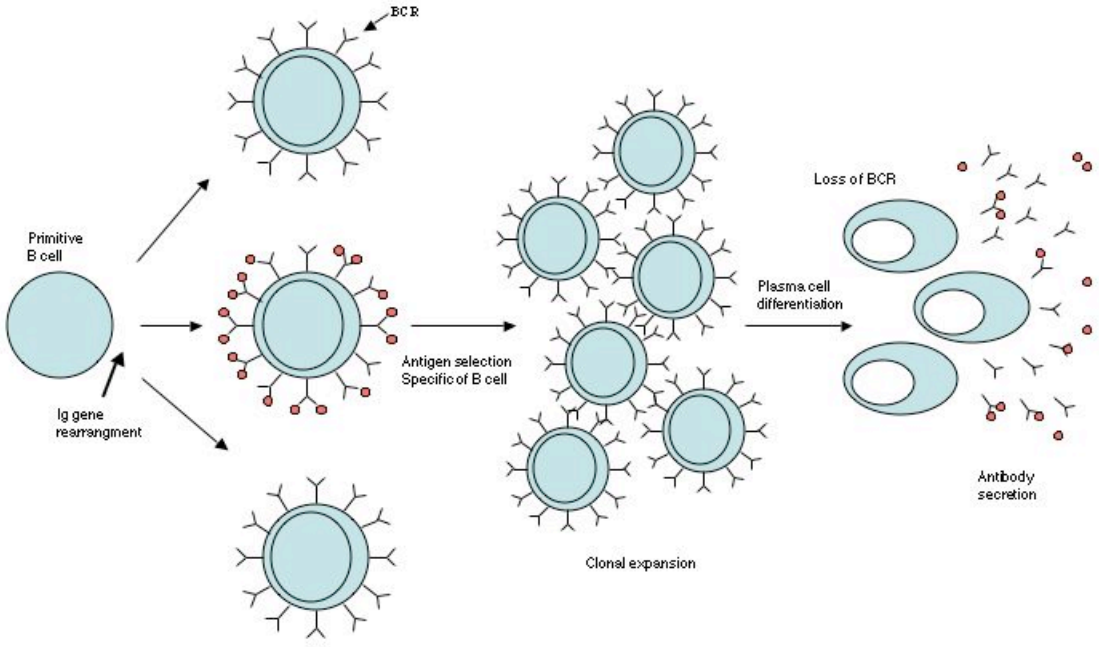


Figure 1**. Clonal selection of B lymphocyte.** B-lymphocytes are antigen selected to form monoclonal Ig secreting plasma cells. Once a primitive B cell has rearranged its Ig genes and expresses a complete H+L chain antigen-binding B cell receptor (BCR) on its cell surface (explained in following section), it circulates throughout the body, "looking" for antigen. Within the total pool of heterogeneous B cells, only a small number of B cells will have a BCR that recognizes a specific antigen. Therefore, clonal expansion of an antigen-selected B cell becomes important during an immune response. Ag selection stimulates cell proliferation and subsequent differentiation into a plasma cell that secretes antibody that was originally expressed as the antigen receptor on the B cell. During the clonal expansion stage, numerous molecular changes to the Ig genes occur to make the antibody have a higher affinity for antigen, and adopt different effector functions by switchin its constant region from IgM to IgA, IgG, or IgE.

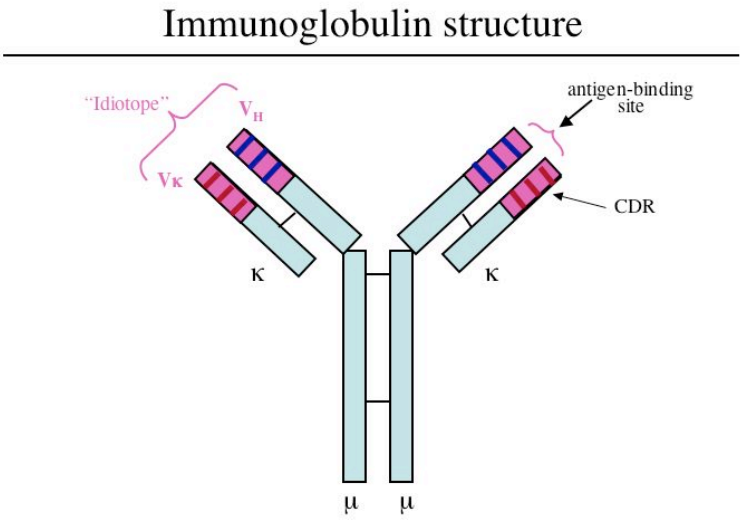
oncogenic mutations in maturing plasma cells, the current hypothesis is that the phagocytic neutrophils of the oil granuloma produce large amounts of reactive oxygen radicals that can potentially cause chromosomal damage (2, 3). In response to pristane,

large numbers of macrophages and neutrophils are recruited to the peritoneal space where they surround the oil droplets of various sizes in an attempt to clear this foreign substance from the body. The complexes of oil, macrophages, and neutrophils adhere to peritoneal surfaces and become an oil granuloma (OG), a chronic inflammatory tissue that persists in the peritoneum and is vascularized by out growth of mesenteric blood vessels. Plasmacytomas arise within the peritoneal oil granuloma tissue, which provides a source of essential factors, such as IL-6, that are important in supporting tumor growth and survival (4-6). IL-6 is a macrophage-derived soluble factor required for survival and proliferation of PCTs *in vivo* (7).

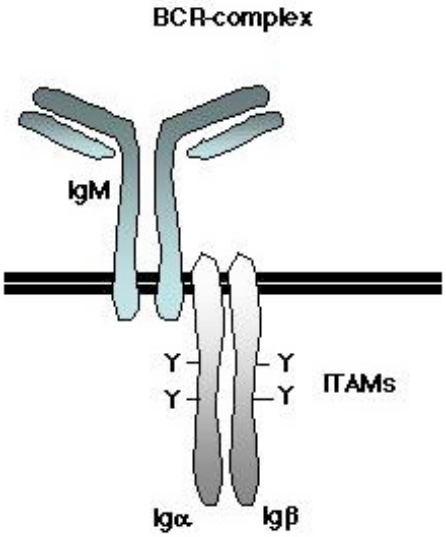
Consistently occurring oncogenic mutations in peritoneal plasmacytomas are the *Myc*-activating chromosomal translocations t(12;15) and t(6;15) that are found in over 95% of pristane–induced PCTs.

B Cell Receptor

Development and maintenance of B cells depend upon surface expression and constitutive signaling through the IgM B cell receptor (BCR) (**Figure2a**) expressed on the B cell's surface (8). B cells are generated in the BM and progress through several stages of development, from pro-B to pre-B to the immature B cell stage, depending on the status of the IgM receptor expressed on the surface of the cell. The IgM BCR is made from pairing two heavy and two light chain Ig polypeptides, joined together with disulfide bonds. Each chain has a constant region and a variable region. The heavy



A.



B.

Figure 2. B Cell Receptor. The B cell receptor is a rearranged immunoglobulin (or antibody) molecule displayed on the surface of the cell, where it can to bind antigen. A) An example of an IgM molecule. An Ig molecule is made from pairing 2 heavy (H) and 2 light (L) chains, joined by disulfide bonds. Antibody molecules are made up of various gene segments - each chain has a constant region (blue) and a variable region (purple). The H and L chain variable regions make up the antigen-binding site. Each B cell produced in the bone marrow generates a unique Ig molecule from a different combination of V region genes The idiotype of an antibody is the unique structure made by the pairing of the H+L chain variable region. Each V region has 3 hypervariable sites

(called complementary determining regions, or CDRs). These regions have the greatest impact on antigen binding ability of the antibody. During affinity maturation of the Ig molecules, amino acid changes resulting from somatic hypermutation have the greatest affect in these regions. Newly formed B cells express IgM (mu) constant region with a transmembrane region allowing it to be inserted on the cell surface. B) The B cell receptor is comprised of the surface IgM molecule and the Igα/β heterodimer. Igα/β is responsible for cytoplasmic signal transduction through its ITAM sites, which are phosphorylated by tyrosine kinases (PTKs). Figure of BCR complex is a modified version based on diagram from Immunology, Kuby.

Once a complete surface IgM receptor is displayed, immature B cells leave the bone marrow and enter the peripheral lymphoid system. The immature B cells travel through the blood/lymph to the spleen, where they receive further positive selection to become mature B cell lymphocytes. Basal levels of signaling through the BCR is necessary to for the survival and maintenance of mature B cells in the periphery.

Further, activation and differentiation of a B cell begins with the binding of antigen to the BCR. Physiological and transgenic BCRs are both made up of membrane IgM paired with the Igα/Igβ heterodimeric signal transducing molecule (9) (**Figure 2b**). Transmission of signals from the BCR to the nucleus is mediated through the long cytoplasmic tails of the Igα/β heterodimer that contain immunoreceptor tyrosine-based activation motifs (ITAM) and depends on activation of cytoplasmic protein tyrosine kinases (PTKs), their adaptor molecules and various secondary messengers that activate intermediary signaling pathways (**Figure 3**).

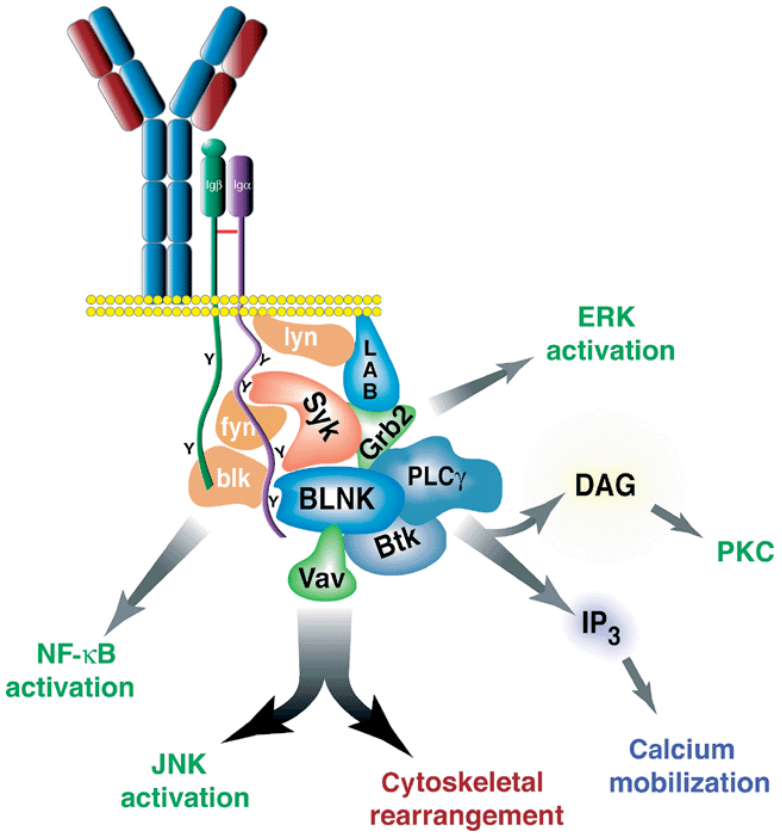


Figure 3. BCR Signaling. The Src family tyrosine kinases (Blk, Lyn, Fyn) and Syk tyrosine kinase are all associated with the resting BCR and become activated upon ligation of the receptor. These kinases phosphorylate the tyrosines in the ITAM motifs and the non-ITAM tyrosine 204 on Igα, which in turn recruits adaptor molecules to the cytoplasmic tails of the receptor. Syk kinase and BLNK (B-cell linker protein), an important B-cell adaptor protein, bind the phosphorylated Igα tail. Multiple pathways are activated: the Src kinases activate the nuclear factor NF-κB; Btk activates phospholipase C(PLC)γ2, leading to calcium flux and PKC activation; the GTPase, VAV, is important in JNK activation and cytoskeleton remodeling, and the linker molecule, Grb2, activates the extracellular signal-regulated kinase (ERK) pathway. Figure and text are reprinted with permission from Wang, 2003 (10).

Antigen stimulation through the BCR also results in the formation of a membrane signaling complex that includes the BCR (mIgM/Igα/αIgβ) and a co-receptor complex (CD19, CD21/CR2, CD81/TAPA-1). The B cell co-receptor complex modulates B cell

receptor signaling. CD21 recognizes complement fragment C3d bound to antigen and the antigen activates the BCR. Antigen that binds the IgM receptor is internalized and processed in specialized cytoplasmic compartments for presentation as peptides to helper T cells by an MHC class II molecule located on the surface of a B cell.

B cell development, survival, migration and differentiation require constant signaling through the B cell antigen receptor. Through activation of its BCR, a B cell is signaled to perform one or more of its effector functions: (1) Ag processing and presentation, (2) antibody production, (3) cytokine secretion, (4) formation of a memory B cell and/or (5) differentiation to an Ig secreting plasma cell.

Mature B cell compartments (B-1 and B-2: FO, MZ)

There are two major B cell compartments, B-1 and B-2 cells, based on phenotypic and functional characteristics, as well as different developmental and maintenance requirements. B-2 cells make up the majority of the B cell population in the mouse and can be found in all lymphoid compartments: spleen, lymph nodes, peyers patches in the gut, peritoneal cavity, and peripheral blood. B-2 B cells can be further divided into follicular (FO) and marginal zone (MZ) B cell phenotypes. FO B cells are resting, naïve B cells that recirculate throughout the body and are found in B cell follicles. MZ B cells are non-recirculating B cells found only in the marginal zone of the spleen. The marginal zone is a distinct compartment found only in the spleen around the marginal sinus, surrounding the B cell follicles and the periarteriolar T cell area. MZ B cells typically make up about 5% of splenic B cells, but are of great importance in early immune responses to blood-borne pathogens. The other major B cell compartment, B-1 cells, are

found predominantly in the peritoneal and pleural cavities of the mouse and make up only a very small percentage (1-5%) of splenic B cells. B-1 cells are fetally-derived, self-renewing B cells that produce low affinity, autoreactive natural IgM antibodies.

B cells are selected through ligation of their BCRs into the B-1 and B-2 subsets. It had been unclear whether the antigen specificity of the BCR may play an role in selecting B cell subsets (11) or whether the signaling strength through the BCR ultimately influenced the B cell subset selection (12). However, a recent publication by S. Casola *et al*. (13) supports the conclusion that B subset selection depends primarily on the strength of signaling through the BCR. The major characteristics of each of these B cell phenotypes are listed in the appendix (see 'Summary of B Cell Subtypes' on page 178, as reviewed by Vinuesa and Cook (14), and also by Martin and Kearney (12, 15)).

Functionally, B1 and MZ B cells appear to be close relatives. It has been suggested that B1 and MZ B cells have evolved to provide first line responses for gut/peritoneum (B1) and blood-borne antigens (MZ). Restriction of their BCR repertoire helps to ensure rapid development of short-term antibody responses to a limited number of conserved antigens. MZ and B1 B cells are a part of the host's natural immune memory and are considered to be part of a bridge between the rapid responses of innate immunity and the slower responses provided by adaptive immunity.

Relationship of Ig Producing PCTs to Ab Formation (Response to Ag)

The induction of paraffin oil-induced plasmacytomas in mice provided the first source of large quantities of monoclonal Igs. Laboratories with monoclonal Ig libraries questioned what relationship these proteins had to "antibody", and this led to a search for

antigen binding activities of these homogeneous Igs. Antibody was defined at the time as a molecularly heterogenous collection of Ig molecules that bound to the same antigenic macromolecules. Binding activity for chemically defined antigens and haptens were identified in only about 5% of these paraffin oil induced antibody proteins (16). Many of the haptens identified were oligosaccharides, α1-3 dextrans, β2-1 and β2-6 fructofurans, β1-6 D galactan, phosphorylcholine and nitrophenyl (**Table 1**). These antigenic 'determinants' are widely available on macromolecules in plants and animals. Finding several PCT proteins, generated independently in different mice, that bind the same haptenic determinant (e.g. phosphorylcholine) suggests that antigens carrying these haptens may be biologically active in the pathogenesis of the tumor.

Table 1. Hapten Binding of PCTs (16).

Associated hapten binding	Plasma Cell Tumor
β1-6 D galactan	J539, XRPC24, XRPC44, TEPC601
PC Choline	TEPC15, HOPC8, MOPC167, MOPC511, McPC603, CBBPC3, W3207
β2-1 and β2-6 fructofurans	UPC61, ABPC64, ABPC47N, EPC109
α1-3 dextrans	MOPC104E, J558, HDEX1-10;
DNP	MOPC315
Arsonate	ARS1, H9367, H124E1
NP (4-hydroxy-3-nitrophenylacetyl)	B1-8, S43

Natural Antigens are an Important Factor in PCT Development

There are several lines of evidence that suggest that PCTs are derived from Ag-selected B cells. First, previous PCT induction studies in several types of defined antigenic environments suggest the need for antigenic stimulation for development of plasmacytomas. Animal colonies are classified based on the status of the

microorganisms present within the colony. The animals in a conventional mouse colony are raised and maintained under standard conditions, with an undetermined microbiological status. Animals in a specific pathogen free (SPF) colony are free of disease-causing microbes or pathogens and are maintained in a special environment that prevents exposure to or transmission of pathogens. Germ-free animals are completely free of all microorganisms, including natural gut flora, and are maintained under sterile conditions. In 1969, K.R. McIntire *et al*. showed that the incidence of plasmacytomas was drastically reduced in mineral oil- treated germ-free mice (17). Byrd *et al.* later compared the incidence of PCTs induced in genetically susceptible BALB/c mice in both conventional and SPF mouse colonies (18) and found a very low incidence of PCTs in SPF mice. The low incidence of PCTs (5%) that occurred in SPF mice compared to 58-60% of mice in conventional colonies suggested that natural antigens provided by microorganisms from natural gut flora and food contaminants are important sources of B cell clone stimulation (18).

Second, a large percent (60 to 70%) of the Ig protein produced by pristane-induced tumors are IgA class immunoglobulins (19-21). IgA-secreting plasma cells are infrequently found in spleen or lymph nodes, but abound in the lamina propria and respiratory tract mucosal membranes, where they make up ~80% of the plasma cell population (22, 23). When the primary antigenic stimulation occurs across mucosal surfaces of the gastrointestinal or respiratory tracts, B cells are not only activated and selected by the antigen, but differentiate selectively to produce IgA (through a process called isotype switching). In the lamina propria of the gut, the primary function of these IgA antibodies is to be secreted across the intestinal epithelium into the lumen of the gut

where the IgA antibodies bind to potential invasive microorganisms and impedes their entry into the internal environment of the host. Although close in proximity, PCTs develop in the peritoneal oil granuloma, a tissue that is not vascularly connected with the lamina propria. IgA committed B cells presumably respond to antigens of normal gut flora that migrate or circulate and are diverted to the peritoneal cavity, where they become precursors to plasmacytomas (24).

And lastly, in order to establish a link between the plasma cell tumors, their secreted immunoglobulins, and microbial natural antigens, the homogenous Igs produced by PCTs were screened against various antigens. Antigen-binding specificities for about 5% of the tumors were determined. A 1970 paper published by Potter (25) reported that precursors of the neoplastic plasma cells were actively forming antibodies that bound polysaccharide antigens originating from gastrointestinal microorganisms. One recurring Ag that was detected by Igs from several PCTs was phosphorylcholine (PC). In early studies, eight independently induced PCTs produced structurally and genetically related Igs that bound PC: H8, S63, S107, T15, M167, M299, M511, and M603. Three subclasses of PC binding antibodies were devised based on the unique antigenic specificities of the murine PC-reactive myeloma proteins studied: class I M167, class II M603, and class III T15 (**Table 2**) (26, 27). All three families share the common germline V_H1 heavy chain (28, 29). In each of these tumors, the V_H1 heavy chain combines with a different light chain, Vk24 (M167), Vk8 (M603), Vk22 (T15), to form distinct anti-PC binding antibodies with different antigenic specificities.

Table 2. Three classes of PC binding antibodies.

PC antibody		H chain $(VDJ)_H$	L chain $(VJ)\kappa$	Unique PC binding activities
I.	M167	V_H1	Vk24	PC on lipoproteins
II.	M603	V_H1	Vk8	PC on morganii LPS
III.	T15, H8, M299 S63, S107	V_H1	Vk22	PC on bacterial Ags

MOPC 167

The PC antibody of particular interest in this research project is M167. M167 is an IgA/kappa myeloma protein from a BALB/c MOPC tumor, originally described by Potter and Leon (30). The authors showed the M167 IgA myeloma protein was one of several related monoclonal Igs that could specifically bind to and precipitate the pneumococcal C polysaccharide (PnC). Leon and Young (27) later reported that the relevant hapten for M167 and its related myeloma proteins was phosphorylcholine found within the structure of PnC.

Phosphorylcholine

Phosphorylcholine (PC) is a naturally occurring haptenic antigen found in many potentially disease-causing pathogenic organisms, including bacteria such as *Streptococcus pneumoniae, Lactobacillus,* and *Haemophilus influenzae*, as well as in fungi, protozoa, tapeworms, and in the cuticle of certain nematodes (31, 32). PC is associated with teichoic acid (33), a polysaccharide constituent of bacterial cell walls, and

lipoteichoic acids (34), a polysaccharide in cell membranes. In both of these polysaccharides, PC is attached directly to the sugar residues (**Figure 4**), usually *N*-acetylgalactosamine (GalNAc). PC is involved in the maintenance of normal shape, size and physiology of a cell and plays an important role in normal cell growth and cell division in *Streptococcus pneumoniae* (35). Studies have also shown that PC mediates persistence and invasiveness of certain pathogens within host organisms. One such study demonstrated that PC on *S. pneumoniae* is important for allowing the bacteria to invade human endothelial cells through a receptor for platelet-activating factor (PAF) (36). In normal unimmunized mice, IgM and IgG3 antibodies directed against PC are found in naturally high levels (that vary depending on mouse strain) in the serum (31). The presence of these natural PC antibodies demonstrate the important role these natural antibodies play in targeting PC antigen in host immune responses against bacterial infection. Immunization with *Streptococcus pneumoniae* is a well-studied mouse model demonstrating the importance of natural PC antibodies in host protection against bacterial infection (32).

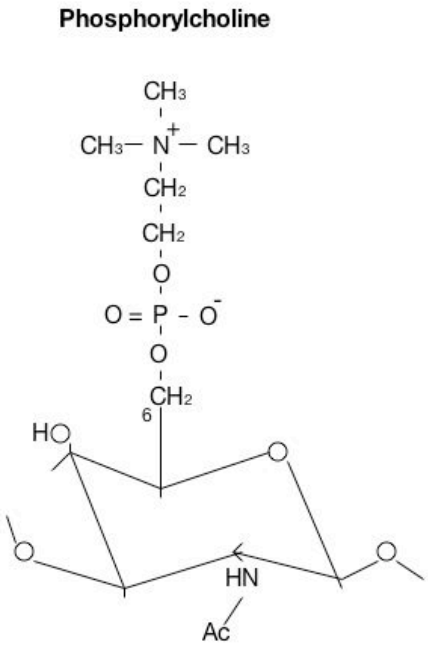


Figure 4. Phosphorylcholine hapten attached to a sugar ring.

Autoantigen Origin of PC: oxidized lipoproteins and apoptotic membranes

It has recently been shown that apoptotic cells are a source of PC-antigen. Shaw, et al (37) demonstrated that PC-antibodies, and specifically the M167 antibody, bind both microbes as well as "neo-self" antigens linked to apoptotic cells, atherosclerosis and autoimmune disease. These studies have demonstrated an enrichment of PC-containing phospholipids in apoptotic blebs (38). Further, oxidatively modified low density lipoproteins (oxLDLs) also display PC hapten that can be recognized by anti-PC antibodies, whereas normal LDLs do not (38). The OG tissue is made up of

inflammatory macrophages and neutrophils, many of which are undergoing apoptosis, thus creating a potential and persistent source of autogenous antigen to promote proliferation of anti-PC specific B cells present in the peritoneum. Further studies to show that the oil granuloma tissue is in fact a rich source of apoptotic cellular PC antigen.

Immune Responses to PC Antigen

The innate immune system produces natural IgM antibodies that recognize PC determinants primarily associated with the early stage of apoptosis, while the higher affinity, hypermutated PC antibodies that are generated during T cell dependent immune responses recognize determinants found at the late stages of apoptosis (37). In addition to the PC-binding natural antibodies, C-reactive protein (CRP) is another part of the innate tier of the immune system that recognizes PC determinants. C-reactive protein has been shown to bind PC found on microbrial pathogens, apoptotic cells (39), and oxidatively modified low density lipoproteins (OxLDLs) (40). PC-antibodies and CRP are both thought to be important in protecting against microbial pathogens and clearance of apoptotic cells and oxidatively modified moieties found in inflammatory lesions.

Ig Transgenes in Pristane-Induced Plasmacytoma Studies

To our knowledge, no one has studied pristane induction of plasmacytomas in immunoglobulin transgenic (Ig Tg) mice. Further, no one has studied the Ig production in plasmacytomas generated in Ig Tg mice. The antigen specificity of the majority of B cells in these Tg mice is dictated by the specific Ig transgenic BCR, which can be used to

address the role of antigen as a critical factor in plasmacytoma development. Specifically, are there certain antigens that are more influential in promoting tumor development in BALB/c mice?

As previously discussed, a small percentage of PCT myeloma proteins have been shown to bind bacterial antigens. In particular, a well-studied group of PCT tumors bind PC in bacterial polysaccharides and apoptotic cells. In this study we specifically ask the question: are phosphorylcholine (PC)-specific Tg B cells more likely to cause an increased incidence of plasmacytomas when stimulated by antigen? For example, if the Ig transgene forms a functional BCR, then the presence of specific antigen can stimulate preferential survival and proliferation of the B cells that contribute to generation of oncogenic mutations. The net effect of pristane may be to create an inflammatory environment that provides factors, such as IL-6, that aid in the maintenance and survival of PCT cells.

Transgenes and Immunoglobulin Transgenes

Transgenes are genes that are introduced into an organism by microinjection of DNA into newly fertilized eggs. Transgenic positive animals that develop from the injected eggs carry the foreign gene(s), which have been randomly incorporated in the genome, and are then passed on to their progeny. A single copy or multiple copies of the transgene(s) are usually integrated in tandem into a single insertion site (41). Expression levels of the transgene are dependent upon the number of copies integrated into the genome, as well as the site of integration within the chromosome and are heavily dependent upon the regulatory sequences (promoters and/or enhancers) that are included

with the transgene. The regulatory sequences control tissue specificities of transgene expression.

Immunoglobulin transgenes (Ig Tgs) are heavy (H) or light (L) chain Ig genes that have had their variable regions (VDJ_HC_H or VJ_LC_L) previously rearranged so that they form a productive H or L chain. Some Ig transgenic mice carry both H and L chain transgenes (and are referred to as double Ig Tgs). Functional transgenic Ig molecules can be generated using a combination of both endogenous Igs and transgenic Ig products. When only one Tg Ig chain is introduced (i.e. IgH-only or IgL-only), the Tg can bind to endogenous H or L chains to generate a limited antibody repertoire. The microinjected Ig transgenic cDNA constructs can be expressed specifically on the surface of B cells as B cell receptors (BCR) and/or as secreted Ig molecules. In order to be expressed on the cell surface of developing and mature B cells, the antibody molecule must have a constant region containing a transmembrane domain so that it can be inserted into the membrane and act as an antigen receptor.

Ig Transgenic BCR Influences B Cell Phenotype

Ig Tgs have not only been shown to be influential in designating the specificity of the expressed BCR on the surface of B cells, but also in determining the phenotype of the B cell on which they are expressed. An accumulating body of evidence shows that certain B cell clones appear to be uniquely selected into a particular mature B cell compartment, such as B1, marginal zone (MZ) or follicular (FO) B cell populations based upon its BCR. For example, it has been demonstrated that M167-id and VH81X-id B cells localize to the marginal zone of the spleen (42). The current dogma states that the

specificity of the BCR as well as the strength of selective positive signaling through the receptor determine what mature B cell subset the precursor cell enters (11). **Table 3** highlights a few of the known VH/VL clones that influence a particular B cell phenotype.

Table 3. BCR influences B cell phenotype. The table lists specific examples of B cell receptors that have been shown to determine B cell phenotype. NP = anti-4-hydroxy-3-nitrophenyl acetyl, HEL = hen egg lysozyme, PC = phosphocholine, PtC = phosphatidylcholine.

B cell clone	B cell phenotype	Antigen Specificity	Ig Genes	Ref
T15	B-1	PC	V_HS107 (V_H1)-Dfl16.1-J_H1 Vk22	(43)
V_H12/Vk24	B-1	PtC	V_H12 Vk24	(44)
M167	MZ	PC	V_HS107 (V_H1)-Dfl16.1-J_H1 Vk24	(42)
81x (35-1$^+$ clone)	MZ		V_H7183 (V_H81x)-Dfl16.1-J_H1 Vk1C	(42)
MD2	FO/B2			(42)
HEL	FO/B2	HEL		(42)
V_HB1-8f	FO/B2	NP		(8, 45)
$V_H$glD42i	FO/B2	DNA	unmutated germline V_H11-Dsp2.3-J_H1	(46)

Project

I used the BALB/c M167μk transgenic mouse (C.M167μk Tg) to determine if B cells expressing the M167 anti-PC natural antibody as a B cell receptor are more susceptible to pristane induction of plasma cell tumors. The M167μk transgenic mouse, originally made in Ursula Storb's lab (47) and well-studied as reported in the literature, expresses the M167μk (heavy and light chain) receptor on >97% of B cells in these mice, making it a good model in which to attempt the induction of plasmacytomas from phosphorylcholine-specific B cells. I will further investigate the effects of the transgene on peripheral B cell populations in the spleen and peritoneal cavity using flow cytometry to test for transgene expression and for B cell characterization.

Since one of the main characteristics shared by a PCT and its precursor B cell is the Ig it produces, I will characterize the Ig genes produced by the M167μk Tg PCTs. Will the Tg PCTs secrete a monoclonal M167 Ig or will other Igs be expressed? What will the mutational status of the expressed Igs be? How will the M167μk Tg affect expression of endogenous Ig genes? Tracing the natural history of in a PCT using a known sequence marker such as the M167μk Ig transgene would allow us to determine whether antigen selection is indeed important in plasmacytoma development. Somatic hypermutation (SHM) of the Ig variable (V) region genes expressed by a B cell is a T cell dependent process induced upon antigen stimulation of the B cell receptor and CD40/CD40L interactions in germinal centers (GCs) and is necessary in generating high-affinity antibodies during an immune response. Therefore, seeing an accumulation of somatic hypermutations in the hyper-variable regions (or CDRs) of Igs is considered a classic hallmark of GC activity and indicating that the B cell underwent antigen selection.

And finally, because the M167μk Tg BCR has to be able to respond to PC antigens in order to affect the incidence of tumors, I want to investigate the functional ability of the Tg BCR to respond to physiological, PC antigens, such as the PC-containing bacteria *S. Pneumoniae*. Does immunization with this bacterial antigen stimulate an anti-PC antibody response and are Tg B cells stimulated to proliferate upon activation of the M167 BCR?

Specific Aims

To study the effects of immunoglobulin (Ig) transgenes (Tgs) on the induction of plasma cell tumors (PCTs) by pristane in BALB/c mice and the characterization of Igs produced by PCTs that have been induced in these M167μk Tg mice

1. Compare the incidence of C.M167μk Tg mice and C.VH12μ Tg mice to wild-type BALB/c littermates in a pristane plasmacytoma study.

2. Use flow cytometry to evaluate the B cell subpopulations in the spleen and pertioneal cavity of C.M167μk Tg mice.

3. Sequence the immunoglobulin genes produced by PCTs derived from C.M167μk Tg mice.

4. Determine if the M167μk Tgs or other expressed immunoglobulin genes have undergone SHM, as an indication of antigen selection of the precursor B cell clone.

5. Test the functionality of the M167μk Tg BCR and show that it can respond to PC antigen.

CHAPTER 2

Plasmacytoma Studies in C.M167μk Tg mice

The original experiment using Ig transgenics was to determine if mice that produce an expanded population of B-1 cells were more prone (susceptible) to PCT induction by pristane than parental BALB/c mice. This was instigated by the finding that BALB/c xid/xid mice, which lack B-1 cells, were refractory to PCT induction by pristane. Also certain monoclonal Igs produced by pristane-induced plasmacytomas, e.g. TEPC 15, come from B-1 cells (43), and therefore led us to believe that B-1 cells could be the primary B cell precursors of pristane-induced PCTs. Accordingly, transgenic mice with the V_H12 gene were imported for this purpose. V_H12 is a VH gene that is almost exclusively expressed on B-1 cells and the $V_H12\mu$ transgene generates a dominant B-1 phenotype in V_H12 Tg mice. Due to technical difficulties in backcrossing the V_H12 transgene onto BALB/c, results were delayed. BALB/c M167μk Tg mice were also imported and used as a control for Ig Tg mouse. A pristane induction experiment was underway when a substantial difference in PCT incidence between the M167μk Tg and non-Tg littermates was noted. I decided to further investigate this intriguing model.

Several questions emerged: 1) would M167μk Tg mice be susceptible to PCT-induction, 2) would plasma cells derived from these mice secrete large quantities of monoclonal M167μk antibody, and 3) would the M167 Ig transgenes carry any marks of somatic hypermutation? Since >95% of M167μk Tg B cells carry the M167-idiotope B

cell receptor and can bind PC (48, 49), the targeted B cell population of interest includes almost every B cell in the Tg mouse.

M167μk Tg BCR Increases Incidence of Pristane Induced PCTs

Figure 5 describes the protocol used in pristane induction of plasmacytomas in M167μk Tg mice. Induction studies in M167μk Ig Tg mice were carried out to determine whether B cells with an anti-PC specific B cell receptor would be more susceptible to developing plasma cell tumors. Results of the PCT induction study surprisingly showed that M167μk Tg mice have a consistently increased susceptibility to developing pristane-induced peritoneal PCTs (**Figure 6**), with PCTs developing more rapidly than in non-Tg (WT) littermates given pristane. Results were confirmed in two independent PCT induction studies, with a total of 80 Tg+ mice and 74 non-Tg littermates. M167μk Tg mice had a 63% PCT incidence while non-Tg littermate mice only had a 35% incidence. Further, this was associated with a decrease in the mean latent period from 240 days in WT to 200 days in the Tg mice. Chi squared (χ^2) analyses of the combined results showed a significant difference ($p < 0.01$) in PCT development between Tg+ and non-Tg mice. For all PCTs from both M167μk Tg and non-Tg mice, either ascites cells or diced tissue from solid primary (g0) tumors were transplanted into pristane primed BALB/c mice for further analysis.

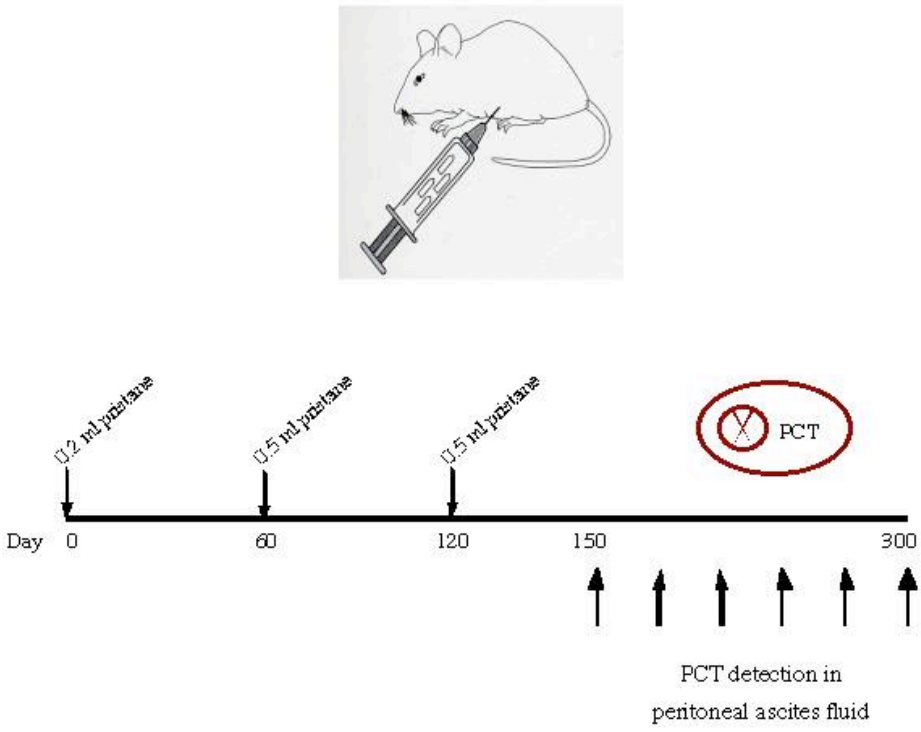


Figure 5. Schedule of pristane (i.p.) injection for PCT induction. M167μk Tg+ and non-Tg littermate mice (2-3 months) were intra-peritoneally (i.p.) injected with 0.2ml, 0.5 ml, and 0.5 ml pristane on day 0, 60, and 120. Plasmacytoma positive mice were diagnosed by screening peritoneal ascites fluid. Beginning at around day 125, cytospin slides were made every 21 days in order to detect the presence of abnormal PCT cells in the ascites. Mice with >10 large atypical plasma cells on a slide were considered to have a PCT. Subsequent cytospins revealed the mice had progressively expanding numbers of PCT cells. Autopsy results indicated widespread PCT in the OG.

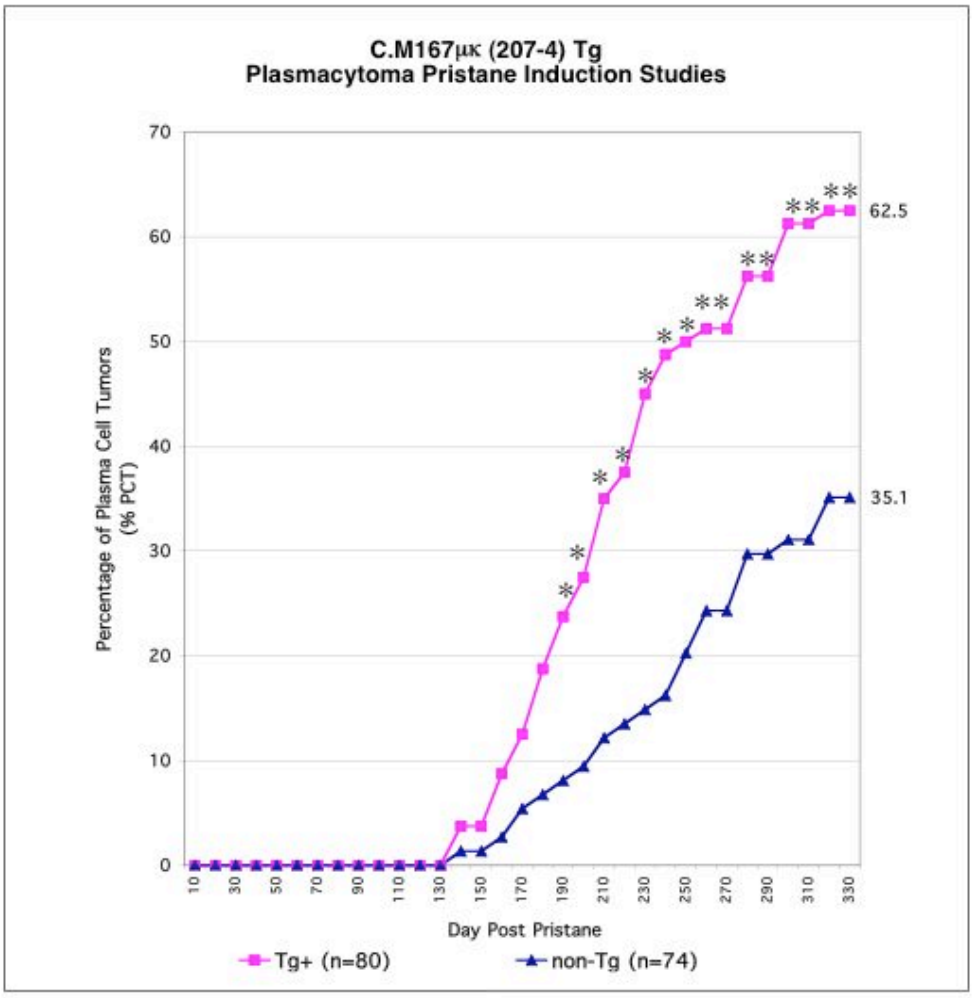


Figure 6. Pristane Plasmacytoma Study in C.M167μk Tg Mice. C.M167μk Tg+ (207-4) mice have increased incidence of PCTs. The results of the plasmacytoma pristane induction showed that the C.M167μk Tg mice develop a significantly ($p < 0.01$*) higher percentage of plasma cell tumors (63%) as compared to non-Tg (Tg-) littermates (35%). There was an associated decrease in the mean latent period from 240 days in non-Tg mice to 200 days in the Tg mice. The latent period is defined as the number of days at which 50% of the total tumors within a group had developed. Two independent study results are combined, totaling 80 Tg and 74 non-Tg C.M167μk mice.

C.VH12μ Tg Pristane Plasmacytoma Induction Study

The mice used in this study were received from Steve Clarke and backcrossed from C57/B6 to BALB/c. Accelerated backcrossing was done using MIT chromosomal

markers to selectively eliminate C57/B6 genes and the PCT pristane study was done on generation (n5) BALB/c mice. The V_H12 Cμ founder line 6-1 used contains 15-20 copies of the IgM Tg. The construction of the V_H12 Cμ transgene is specified in **Table 4**.

Table 4. VH12μ Tg cDNA construct. B cells expressing the transgene have VH12 heavy chain that selectively pairs with the Vk4 light chain. Mice with the VH12 transgene make primarily PtC binding B cells that express the CD5+ B1 phenotype (50)**.**

GENE	ORIGIN
V_H12-DSP2.9-J_H1 segment 3.3-kb EcoR1 fragment	CH27.LX (CD5+ B1 cell lymphoma) of B10.H-2^aH-4^bp/Wts origin
Cμ-μM segment 12.8-kb EcoR1 fragment	HyHEL10-μ-δ (C. Goodnow)

The V_H12 Cμ Tg produces high numbers of PtC-specific peritoneal and spleen B cells, due to V_H12μ Tg heavy chains selectively pairing with endogenous Vk4 light chains. Exclusive segregation of the PtC specificity to the B-1 phenotype in all peripheral B cells. The peripheral PtC B1 cells have the following cell surface characteristics: most are CD5+, all are IgM^{high}, $B220^{low}$, $CD23^{neg}$ (and peritoneal B cells are also MAC-1/CD11b+).

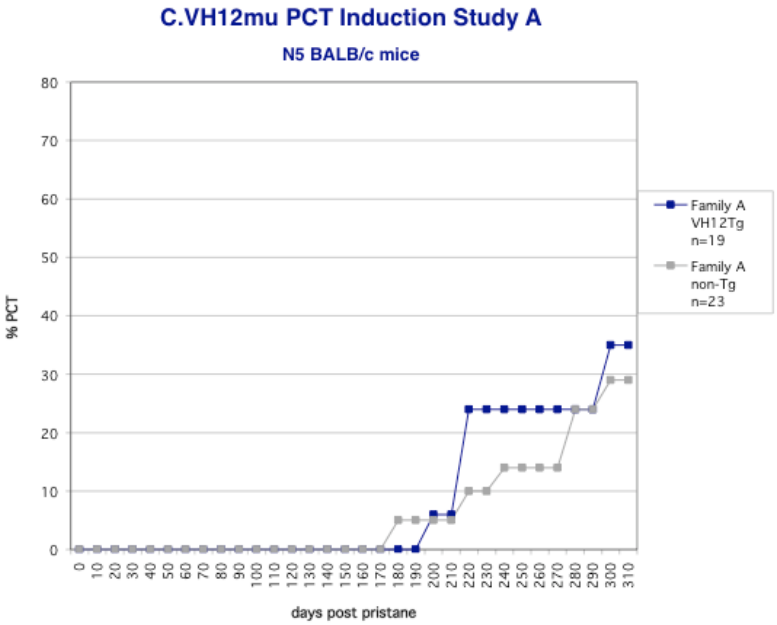
Several breeder lines of N5 C.VH12μ Tg mice were tested in pristane induction of PCTs in case genetic background effects from C57/B6 genes still remained. **Figure 7** demonstrates that the VH12μ Tg did not cause any change in PCT incidence. Tg and non-Tg (WT) littermates developed the same number of plasma cell tumors in both

studies shown. Other studies not reported here on further generations of C.VH12μ Tg mice also support these findings.

Discussion

Finding an increased incidence of PCTs with shorter latent period in C.M167μk Tg mice was a novel result. Why are the PC-specific C.M167μkTg mice developing more plasmacytomas than BALB/c non-Tg littermates?

The chromosomal integration site of the transgenes is an important factor to consider when discussing the influence of the M167μk transgenes upon increased plasmacytoma development. The transgenes may have inserted into an oncogenic locus and are deregulating a gene important in plasma cell tumor development. Susceptibility of mice to plasmacytomas is a complex genetic trait involving multiple loci. Resistance/susceptibility genes have been identified on chromosomes 1, 4, and 11 (51, 52). Previous reports of M167μk (207-4) mice in the literature have not identified the chromosomal insertion point of the transgenes. However, I have preliminary studies that show the Tg M167k light chain is located on either chromosome 2 or 3 (see **Figure 39**



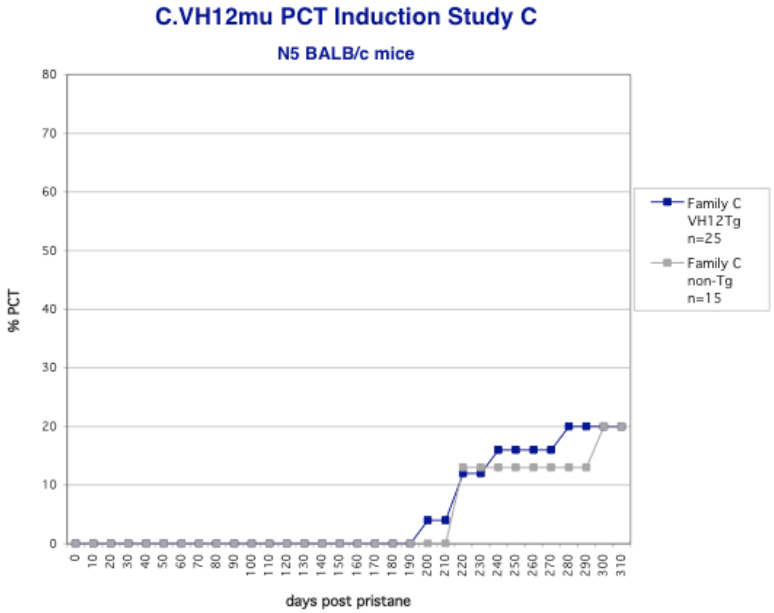


Figure 7. Pristane Plasmacytoma Study in C.VH12µ Tg Mice. Induction curves for 2 different breeder lines or "families" of VH12 BALB/c mice are shown. Although PCT incidences in non-Tg control mice were generally lower than seen in pure BALB/c mice, the VH12µ Tg did not significantly affect the number of PCTs that developed.

in Appendix), and I think disruption of an important gene is an unlikely probability. Further studies need to be done to isolate the exact location of the M167μk transgenes.

Alternatively, the M167μk Tg could be causing changes in the B cell repertoire that effect immune responsiveness and influence the ability of the B cells to become plasmacytomas. The abundant presence of environmental PC (or other related antigen) found in the diet or on commensal bacteria is a likely source of antigen that is stimulating many of the C.M167μk Tg PC-specific B cells to divide and differentiate into plasma cells. The inflammatory pristane OG generates a rapid turnover of cells and there are many apoptotic cells that potentially become a source of phosphorylcholine-containing autoantigens.

Another possible factor affecting the enhanced PCT development in these M167μk mice if they have an increased MZ B cell population because of the M167 BCR expression on their cell surface (see **Table 3**). A high surface density of M167-id B cell receptor confers the MZ B cell phenotype. The MZ B cells, strategically located at the marginal sinus of the spleen where they can filter blood born antigens, are more rapidly activated by T-cell independent antigens and differentiate into plasma cells at a faster rate than the follicular splenic cells (see 'Summary of B Cell Subtypes' in appendix on page 178). The hyper-activated marginal zone B cells may be more susceptible to obtaining oncogenic mutations under the pressure of inflammatory conditions peritoneal OG tissue.

My hypothesis is that the M167μk Tg changes the immune response of the B cell repertoire, based on the antigen-specificity of the Tg B cell receptor, allowing the B cell population as a whole to respond to the widely available environmental PC antigen.

Chapter 2 conclusions:

- The M167μk Tg increases the incidence of plasmacytomas (PCTs) and shortens the latent period in conventional BALB/c An mice induced with pristane.
- Two independent studies confirmed that there is a significant increase ($p<0.01$) in PCT incidence from 35% PCTs in WT controls to 65% PCTs in C.M167μk Tg mice (**Figure 6**).
- The mean latent period of PCT development decreased from 240 days in WT controls to 200 days in Tg mice
- Pristane induction in anti-PtC C.VH12μ only Tg mice did not induce the dramatic increase of PCTs seen in C.M167μk Tg mice

These findings led to studies designed to explain why the M167μk Tg mouse is more prone to developing PCTs. This was approached by studying the immunology of the M167μk Tg mouse.

CHAPTER 3

Characterization of B cell Subpopulations in C.M167μk Tg Mice

Background on M167μk (207-4) Tg Mice

The M167 μ and k transgenes utilize the V_H and V_k genes from a well-studied mineral oil-induced PCT antibody (MOPC 167 or M167). The monoclonal immunoglobulin produced by this PCT is an IgA molecule that binds with low affinity phosphorylcholine groups found in various antigens.

The original M167μk transgenic mouse (line 207-4) was produced in the lab of Ursula Storb (47). Storb *et al*. produced two cDNA transgenes: one of the M167 Vk L chain and the other of a modified M167 H chain of IgM isotype. The kappa light chain contains the functional variable (V_κ24-J_k2) region of the M167 gene (obtained from P. Gearhart, Johns Hopkins University, Baltimore) ligated to the BALB/c MOPC21 kappa constant gene (Ck^a) created by Brinster *et al.* (53). The heavy chain contains the VDJ (V_H1-DFL16.1-J_H1) region of the rearranged plasmacytoma M167-IgA gene recombined with a BALB/c germline IgM^a (μ^a) constant region (**Figure 8**). The M167 kappa and M167 IgM transgenes were co-injected into $(C57BL/6 \times SJL)_{F2}$ ova and both genes inserted themselves into the chromosome at the same integration site. Integration of the transgene was not targeted to the IgH locus but occurred at a random (and undetermined) site

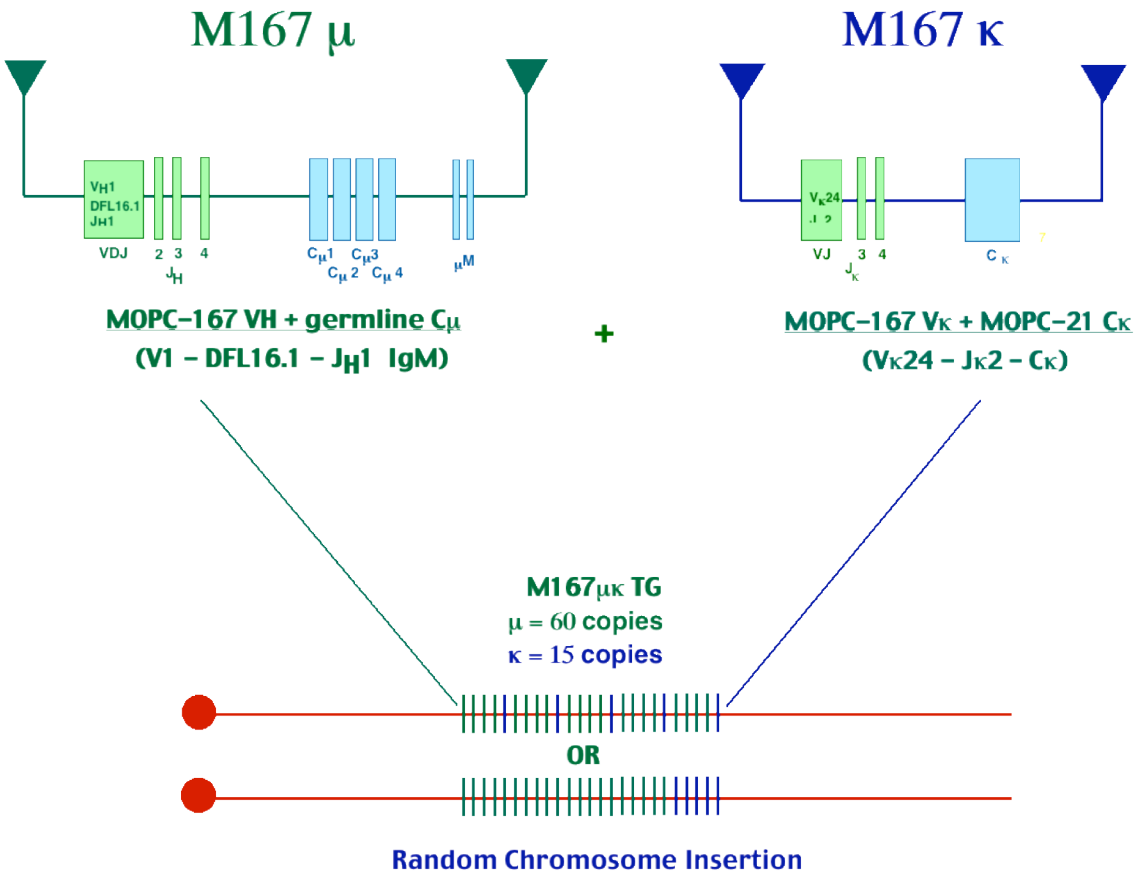


Figure 8. M167μ and M167k transgene constructs (line 207-4). Multiple copies of the M167 μ heavy chain and M167 kappa light chain transgenes were tandemly integrated into a single site in the chromosome of M167μk Tg mice. The chromosomal integration site is unknown. Original estimates of copy numbers were 60 copies of the M167μ gene and 15 copies of the M167k transgene. Figure of M167μk transgenes are as described by Storb *et al.* (47).

within the genome. The original ratio of μ:k was 4:1 (60 copies μ and 15 copies κ), tandemly inserted in the chromosome as determined by quantitative slot hybridization using tail DNA of offspring (47). The transgene-encoded M167-idiotype (V_H1 plus V_k24) IgM B cell receptor is expressed on >97% of B cells (48) and high levels of

background anti-PC antibodies have been reported in the serum of unimmunized transgenic mice (54 321). Expression of the M167μk transgene was reported to be tissue specific as high levels of μ and k RNA were detected in spleen, but not in heart, kidney or liver tissues (47). B cell specificity is due to the presence of Ig specific promoters and enhancers present in the Ig gene segments used in the transgenic construct.

The BALB/c IgM constant region gene contains the transmembrane domain sequence that allows the heavy chain to be inserted into the membrane. Alternate mRNA processing of the M167μk Tg heavy chain transcript allows the production of both the membrane and secreted forms of the Tg-IgM antibody, as both RNAs are detected in spleen (47). In a B lymphocyte, the M167 μ heavy chain transcript is inserted into the cell surface membrane. Further tests have shown that RNA processing favors the secreted form of M167μ RNA in plasma cells as demonstrated when the M167 μ gene was transfected into the J558L myeloma (47).

No IgD constant region gene included in the rearranged M167 IgM Tg heavy chain construct and, therefore, transgenic M167 $V_HD_HJ_H$-IgD expression on B cells is not possible. Furthermore, there is no IgM switch region (Sμ) included in the M167μ cDNA, so isotype switching of the heavy chain transgene with the endogenous IgH constant region on chromosome 12 would be an unlikely event.

Kenny *et al.* (48, 55) previously reported expression patterns of transgenic and endogenous Ig antibodies on C57BL/6.M167μk Tg (207-4) B cells. In the M167μk Tg mice studied by these researchers, the transgenic BALB/C IgM^a could be distinguished

serologically from endogenous C57BL/6 IgM^b using allotypic[1] determinants found on the C region. Allotypic anti-IgM antibodies (anti-IgM^a mAb and anti-IgM^b mAb) were used to determine transgenic IgM (μ^a) versus endogenous IgM (μ^b) expression in splenic B cells. Most B cells, 97-99% from Storb's M167μκ Tg mice (Ig μ^a/μ^b), expressed high surface densities of transgenic IgM^a on their cell surface (48). Only 2-11% of total spleen cells in Tg mice stained positive for endogenous IgM^b and 2-4% stained for IgD isotype expression. These results indicated that the expression of endogenous μ^b Ig heavy chain had been suppressed, although not completely eliminated, by the presence of the productively rearranged M167μ Ig transgene. Co-expression of endogenous and transgene receptors occurred on 4 to 30% of the splenic Tg B cells. Kenny reported that Tg+ mice typically had half the number of B cells of non-Tg littermates. Tg spleens contain ~25% B cells, whereas non-Tg spleens are ~50% B cells. There were also fewer total splenic cells in Tg mice compared to non-Tg mice (3 to 4 x 10^7 Tg+ vs. 1x 10^8 non-Tg spleen cells). My research with BALB/c.M167μk Tg mice supports these data.

Naïve M167μk Tg mice produce 10-100x more PC antibodies than normal wild-type mice (47, 54). Pinkert *et al.* conducted immunization studies in both germ-free (described as caesarian-derived pups maintained under sterile conditions in a gnotobiotic environment, i.e. with a carefully defined natural gut flora to aid in digestion) and conventional mice (exposed to many environmental antigens) to investigate whether

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[1] Allotypic determinants (or allotopes) are created by polymorphic differences in the amino acid sequence of a conserved Ig constant region between strains of mice and can be detected by anti-allotypic antibodies.

environmental antigen was important to the spontaneous production of anti-PC serum antibodies in Tg mice. Sources of PC contaminants due to dead microorganisms in diet and bedding were significantly limited in the gnotobiotic environment in order to maintain a sub-threshold level of PC contamination. Because PC is an abundant environmental contaminant found in many antigens both dietary and microbial, conventional wild type (WT) BALB/c mice have naturally high levels of anti-PC serum antibodies. However, germ-free BALB/c WT mice normally express very little to no anti-PC serum antibodies (54) in the absence of immunization. Results showed that M167μk Tg germ-free, as well as conventional, mice spontaneously secreted elevated levels of anti-PC antibodies (54). Elevated levels of anti-PC antibodies were detected in sera of these Tg mice as early as 16 days of age, at a time when normal non-Tg mice are not fully immunocompetent (summarized in **Table 5**). In vitro studies also showed that Tg B cells, in contrast to non-Tg B cells, spontaneously secreted PC-antibodies without the presence of antigen. Therefore, the authors concluded that a triggering antigenic stimulus was not necessary to evoke production of the anti-PC antibodies in M167μk Tg mice (54).

Table 5. Reported levels of PC Ab in M167μk Tg mice. Levels of PC Ab in germ-free gnotobiotes and conventionally housed mice at 16 days of age (54).

	Germ-free (gnotobiotes)	**Conventional**
M167μk Tg	very high	very high
Non-Tg littermates	low to none	high
BALB/c WT	low to none	high

Several reports indicate that M167μk Tg mice can respond to T cell dependent PC antigens. Pinkert *et al.* (54) reported that M167μk Tg B cells of mice pre-immunized with T-cell dependent PC-hemocyanin (Hy) antigen had an increased frequency of PC-responsiveness by using *in vitro* splenic fragment cultures. In this experiment, recipient F1 mice were injected i.p. with 100 μg of T-cell dependent PC-Hy antigen in IFA, boosted i.p. with 100 μg Hy in saline at 4 weeks. Another 4-8 weeks after the boost, these recipient mice were whole body irradiated 1-4 hours before receiving M167μk Tg B cell transfers. Fragment cultures were prepared from the spleen of recipient mice and stimulated with PC-Hy for 3 days. Culture medium tested for PC antibody production over a period of 28 days determined that the Tg B cells were responsive to PC antigen.

Kenny (48) reported that M167μk Tg mice can respond to PC-KLH *in vivo*. In addition to PC, M167μk Tg mice can respond normally to other antigens and are not immunocompromised by conventional conditions. The M167μk Tg mice can respond to endemic pathogens when reared in a conventional colony (54). These mice also have the ability to produce endogenous antibodies to such antigens as the KLH protein carrier and FITC molecules (48), as well as to DNP, or dinitrophenol (54).

BALB/c.M167μk Tg Mice

The original M167μk Tg (207-4) line was maintained and studied on a C57BL/6 genetic background, a strain resistant to PCT induction by pristane, where the transgene could be detected using specific antibodies against the allotypic μ^a marker on the IgM heavy chain (BALB/c μ^a vs. C57BL/6 μ^b). The M167μk transgene needed to be expressed in conventionally raised PCT-susceptible BALB/c mice in order to conduct

plasmacytoma induction studies. BALB/c.M167μk (207-4) Tg mice were a gift of Dr. J. Kenny (National Institute on Aging, GRC, NIH, Baltimore) and had been previously backcrossed to BALB/c at least 20 generations. The mice used in the studies reported here are maintained in a conventional mouse facility at the NIH, Bethesda. C.M167μk Tg+ male mice were backcrossed to female BALB/cAnPi mice at least 4 more generations (N24) before beginning PCT induction studies.

Peripheral blood lymphocytes (PBL) from progeny were typed for presence of the transgene by flow cytometry using anti-B220 and an anti-V_H1-specific antibody. Non-Tg mice have <1% V_H1 expression on PBLs, whereas almost all B220+ B cells are V_H1+ in Tg mice (**Figure 9**). The rat anti -V_H1 antibody (56) from hybridoma T68.3 specifically recognizes the transgenic V_H1 heavy chain, in combination with various light chains, was a gift of Dr. J. Kenny, originally obtained from Dr M. Scharff, Albert Einstein College of Medicine, Bronx, NY.

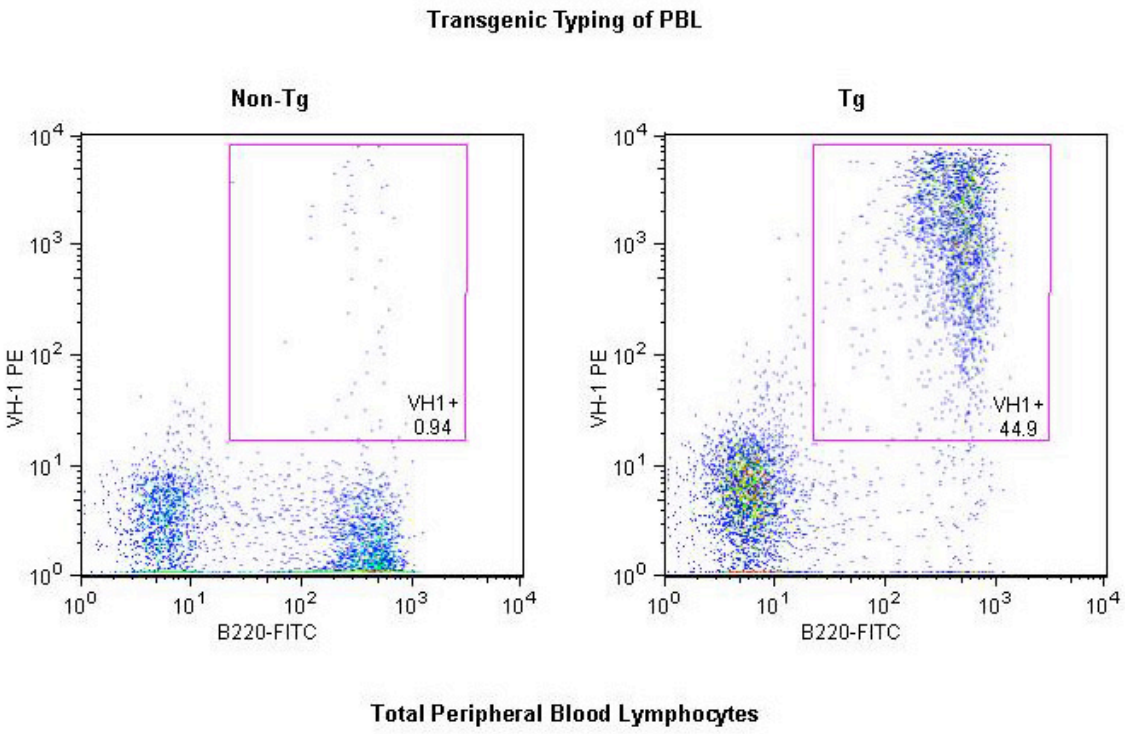

Figure 9. Transgenic Typing of PBL. Flow cytometry is used to type C.M167μk mice for presence or absence of the transgene using peripheral blood cells. Cell staining with antibodies against anti-B220 (FITC) and anti-V_H1 (PE) heavy chain.

The T15 subfamily (currently classified as S107 VH family) contains four gene segments, designated V1, V3 (defective), V11, and V13 (57). The four genes have a 75% nucleotide homology with each other and all are structurally very similar. V1 (V_H1) is the VH gene family found in most PC antibodies. V_H1 can pair with 3 different L chains to form 3 different families of PC-binding antibodies: T15, M167, and M603 (**Table 2**). The anti-V_H1 antibody used for typing the Tg mice can recognize the V_H1 gene product used in any of the PC-antibodies. An antibody's idiotype refers to the collection of idiotopes (or determinants) created by a particular H+L chain combination. Three

idiotypes found in PC antibodies are T15-id (VH1/Vk22), M167-id (VH1/Vk24), and M603-id (VH1/Vk8). Anti-idiotypic antibodies can recognize idiotypes.

VH1 and M167 BCR expression levels

Splenic B cells were screened from our C.M167μk Tg mice to determine whether the expression levels of the Tg V_H1 heavy chain were comparable with previous reports. Flow cytometry analysis shown in **Figure 10** confirms that the transgene is expressed on most splenic B cells in Tg mice. The anti-V_H1 antibody (56) specifically recognizes the transgenic V_H1 heavy chain (clone T68.3) used in the M167μk Tg. As seen in **Figure 10**a, the V_H1 heavy chain Tg is highly expressed on most B cells (> 95%) in the spleen, whereas non-Tg (WT) littermates have only a small population of V_H1 B cells (< 3%). These expression levels agree with previously reported levels (48). The V_H1 histogram in **Figure 10**a, however, shows two distinct levels of heavy chain surface expression on Tg B cells. An anti-M167 antibody (rat, clone 28-6-20) that recognizes the Vk24 light chain was utilized to determine levels of M167 idiotype expression (49, 58). **Figure 10**b show that the Tg-encoded M167 B cell receptor is expressed at low to high surface densities on splenic B cells in Tg mice.

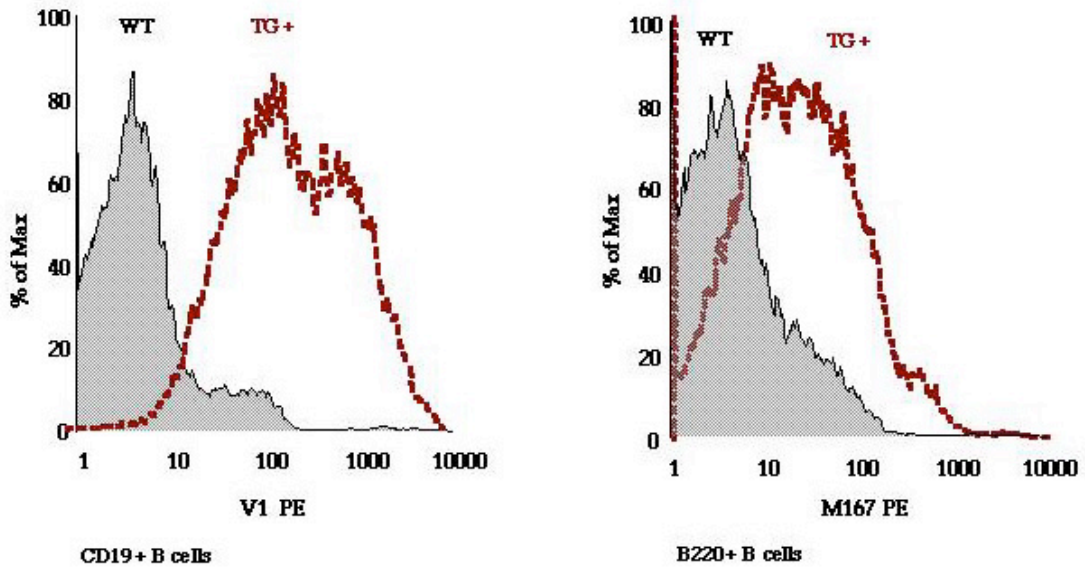

Figure 10. Transgene expression in C.M167μk Tg mice. Splenic B cells were gated on using either CD19 (a B cells surface marker) or B220. (A) The rat anti-V_H1-id hybridoma, T68.3, recognizes all antibodies utilizing a V_H1 heavy chain. V_H1 is highly expressed on B cells in Tg+ mice. (B) The rat anti-M167-id hybridoma, 28-6-20, recognizes only the M167 V_k24 light chain, which can be paired with various heavy chains. The Tg-encoded M167 V_k24 light chain is expressed at low to high surface densities on B cells in M167μk Tg mice.

Anti-PC antibody Sera Titers in M167μk Mice

To determine whether anti-PC antibodies sera levels of C.M167μk Tg study mice used in the plasmacytoma studies were elevated, comparable to previously published levels, their sera were compared to BALB/c non-Tg littermates as well as from conventional C57BL/6 M167μk Tg mice from J. Kenny's colony. BALB/c WT mice are known to naturally produce PC antibody in serum (59). We show here that conventional BALB/c (non-Tg littermates) mice have high levels of natural PC antibodies, as can be seen in **Figure 11**. Because PC is an abundant environmental antigen, conventional

BALB/c titers are equivalent to the M167 protein (ascites) positive control. However, both C57BL/6 and BALB/c M167μk mice have naturally higher levels of anti-PC antibodies in the serum of unimmunized mice than their WT controls as they are elevated ~10 fold over non-Tg mice (**Figure 11**). The conventional C.M167μk Tg mice that were rederived by caesarean section into an SPF colony also have elevated sera titers comparable to their conventional counterparts (data not shown). I conclude that anti-PC sera titers in our conventional and SPF Tg mice are spontaneously elevated due to the presence of the PC-specific M167μk Tg and are comparable to previously published reports.

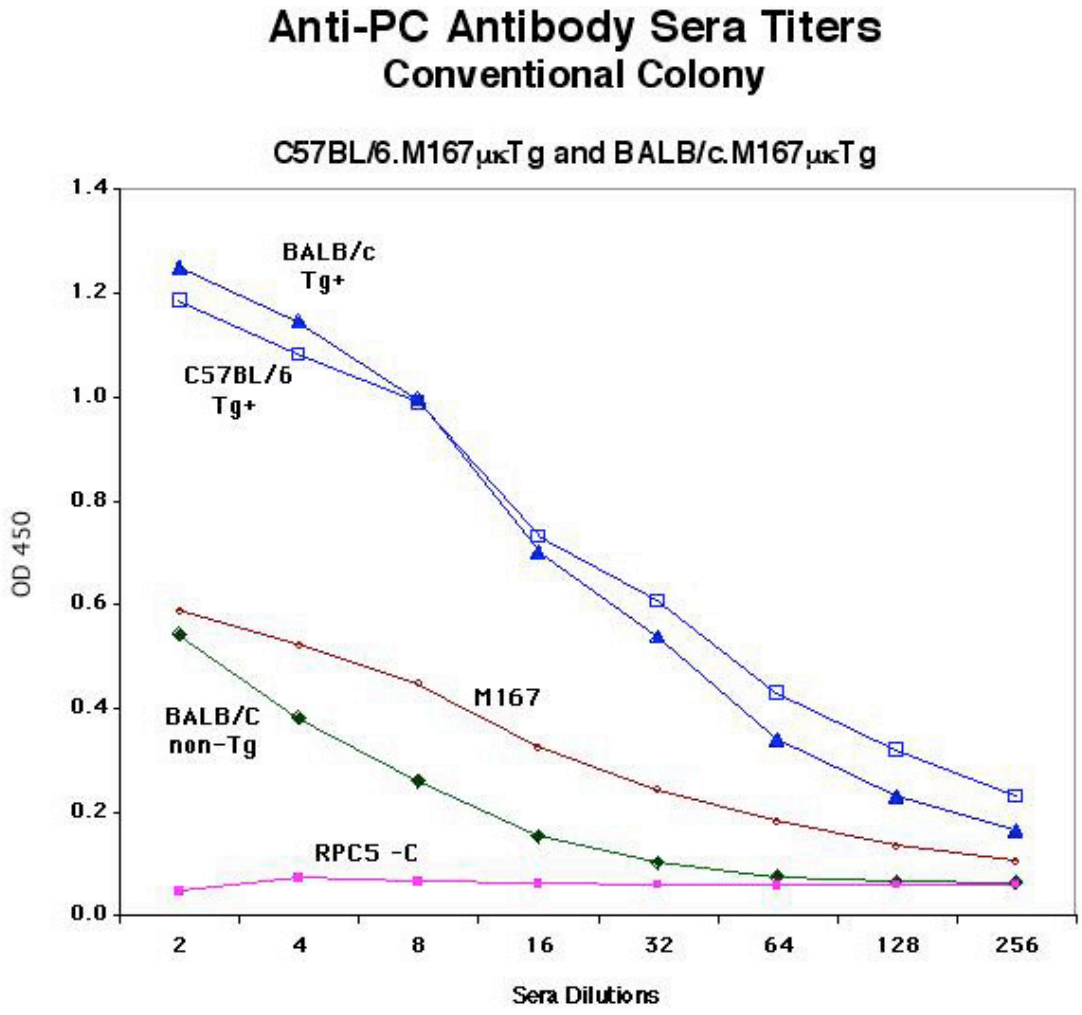

Figure 11. Serum titers of anti-PC antibodies in conventional M167μk Tg mice. High levels of background anti-PC antibodies are detected in the serum of unimmunized transgenic mice compared to WT BALB/c mice in a conventional colony. C57BL/6 sera (n=3) from another facility and BALB/c sera (n=3) from our conventional colony were tested and have comparable titers. RPC5 (a non-PC binding myeloma protein) was used as a negative control. M167 ascites (1:100) was our positive control. (Titers are from n=3 mice in each group, sera dilution factor x 100.)

Expression of Endogenous sIgD on M167μk Tg B cells

Expression of the M167μk transgenes does not prevent VDJ rearrangement of endogenous H chain genes and their subsequent expression on the surface of Tg B cells in our conventional BALB/c M167μk mice. As described earlier, the M167μ transgenic construct does not include the IgD gene (47) and therefore cannot produce an IgD heavy chain protein. Kenny *et al* (48) originally reported that the majority of B cells in M167μk Tg mice do not express IgD on their cell surface, and co-expression of endogenous IgM occurs on only 4 - 30% of the Tg B cells (48, 55). Our BALB/c conventionally housed M167μk Tg mice clearly express cell surface IgD on all B cells, shown in the flow cytometry profile of B220+ spleen cells in **Figure 12**, indicating that endogenous Ig is being expressed on the cell surface of Tg B cells. The two IgD+ groups gated are the mature B cells (IgM^{low} IgD^{high}) and the immature and marginal zone B cells (IgM^{high} IgD^{low}). Unfortunately, after backcrossing the M167μκ Tg from C57BL/6 to BALB/c, easily distinguishing surface expression of transgene from endogenous IgM on B cells is no longer possible using IgM allotypic markers as Kenny's laboratory had done. The transgenic IgM^a is now allotypically indistinguishable from the endogenous BALB/c IgM^a allele. However, knowing that IgD is not included in the transgene, this observation leads me to conclude that most of the IgM+ B cells in these Tg mice are co-

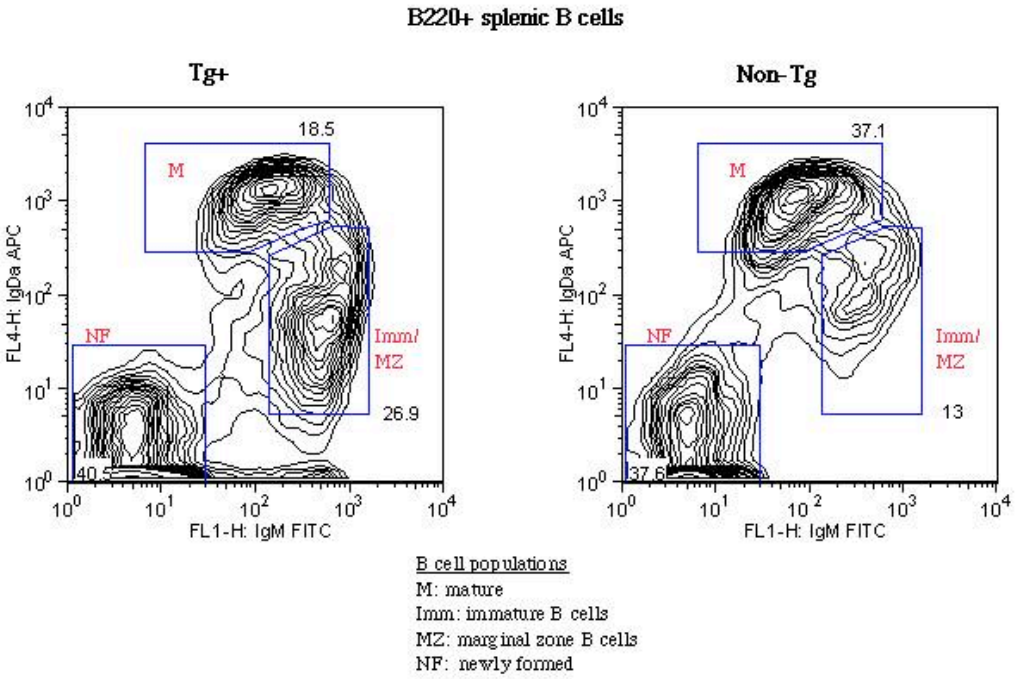

Figure 12. Expression of IgD on mature and immature/marginal zone B cells in C.M167μk Tg mice. Splenic cells were stained with B220 PerCp, IgM-FITC and IgD-biotin/SAV-APC. Unlike previously published reports, our conventionally housed BALB/c M167μk Tg mice express IgD on the surface of splenic B cells, indicating heavy chain VDJ rearrangement and expression of endogenous IgM/IgD genes. There are two IgD+ populations of splenic B220+ B cells shown above. IgD^{high}/IgM^{low} B cells are mature (M) follicular B cells, whereas IgD^{low}/IgM^{high} B cells are immature (Imm) follicular B cells or marginal zone (MZ) B cells. Note the increased percentage of $IgM^{high}IgD^{low}$ B cells seen in the Tg+ mice (26.9%) compared to non-Tg littermate mice (13%). This difference is examined in the following section.

expressing endogenous IgM and IgD genes in addition to the M167μ and k Tgs[2]. These results are one of the large differences between my study mice and their C57BL/6 M167μk Tg counterparts, which have little to no IgD expression on their B cells.

Increased Marginal Zone B cell population in M167μk Tg Mice

Upon examining IgM/IgD B220+ populations in Tg and non-Tg mice in Figure 12, we noticed a significantly enlarged $IgM^{high}IgD^{low}$ population in the Tg mice. In order to further define which type of B cells were increased in the M167μk Tg mice, Tg and non-Tg B cells were stained with CD21-FITC, B220-PerCP, and CD23-biotin/SAV-APC and analyzed by flow cytometry. As seen in **Figure 13**, the phenotypic $CD21^{high}CD23^{low}$ MZ B cells are significantly expanded in Tg spleens. MZ B cells make up 15-25% of the $B220^+$ cells as compared to 5-10% of $B220^+$ cells in non-Tg littermates.

The Ig BCR displayed on B cells plays an important role in determining the phenotype a mature B cell will eventually adopt (such as a B1, MZ or FO phenotype). It has been demonstrated in some Ig transgenic mice that the specified VH/VL BCR generated by the Tg dictates such a B cell phenotype (as discussed in

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[2] If IgD is expressed on the B cell's surface, then it is due to an endogenous IgM/IgD allele being rearranged and expressed. IgD is formed from alternate splicing of a primary RNA transcript that includes the rearranged variable region $V_HD_HJ_H$ and both the IgM and IgD gene segments.

in the introduction). B cells utilizing the M167-idiotype B cell receptor in normal mice and in M167μ only transgenic models are selected into the MZ B cell compartment where they are more adept at responding rapidly to blood-borne T cell independent particulate antigens (42). I have shown here that the double transgenic C.M167μk Tg (C.207-4) mice also have an increased IgM^{high}, IgD^{low}, $CD21^{high}$, $CD23^{low}$ MZ B cell population compared to their non-Tg littermates, providing more confirmatory evidence that M167-id drives development of the MZ phenotype.

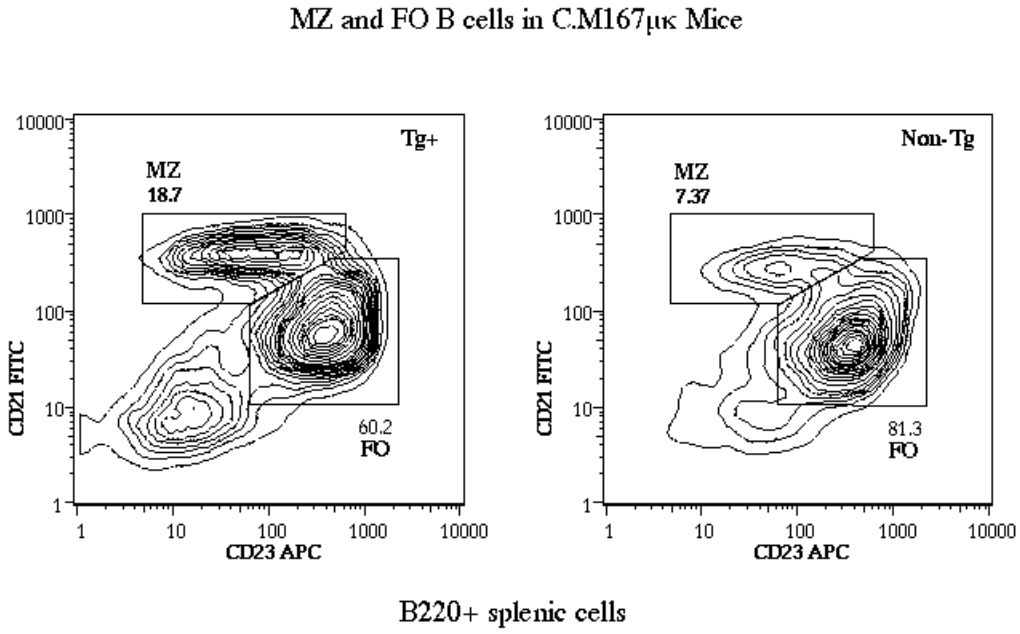

Figure 13. Increased MZ B cell Population in C.M167μk Tg mice. Flow cytometry analysis of B220+ splenic cells show an increase in the $CD21^{high}$ $CD23^{low}$ MZ population of M167μk Tg+ mice compared to non-Tg littermates. Data are representative of five mice in each group.

High Surface Density of M167-id BCR Produces MZ B cell phenotype

Differences in surface density of Tg receptor on the surface of B cells were first observed when examining V_H1 heavy chain expression in splenic B cells (Figure 10). Two distinct peaks were clearly seen in the M167μk Tg spleen cells that indicated the presence of two populations of B cells with different levels of Tg V_H1 expression: low/intermediate $V_H1$and high V_H1. To determine the phenotype of the V_H1^{high} and V_H1^{low} B cells, spleen cells were stained with anti- CD21-FITC, VH1-PE, B220-PerCP, and CD23-biotin/SAV-APC. The FO ($CD21^{low}$, $CD23^{high}$), MZ ($CD21^{high}$, $CD23^{low}$), and NF ($CD21^{low}$, $CD23^{low}$, newly formed) B cells compartments were compared between the V_H1 low and high populations. As shown in **Figure 14**, B cells with lower surface levels of V_H1 contain mostly FO and NF B cells, but lack the MZ cells. Tg B cells with a high density of V_H1 surface expression contained the MZ B cells (14-17% V_H1^{high} B cells are MZ) and distinctly lack the NF population. This evidence led me to conclude that a high receptor density of V_H1 Tg is driving B cells to develop into MZ B cells.

As demonstrated, the V_H1^{high} population contains the $CD21^{high}$, $CD23^{low}$ MZ B cells. The M167 V_H1 is only one of the two Ig transgenes expressed in Tg B cells; the M167 V_k24 Tg is also expressed. The V_H1 heavy chain pairs with V_k24 to make the M167-id antibody. However, endogenous heavy chains and possibly endogenous light chains, are also present on the surface of B cells. Also, the V_H1 may be pairing with any endogenously expressed light chains

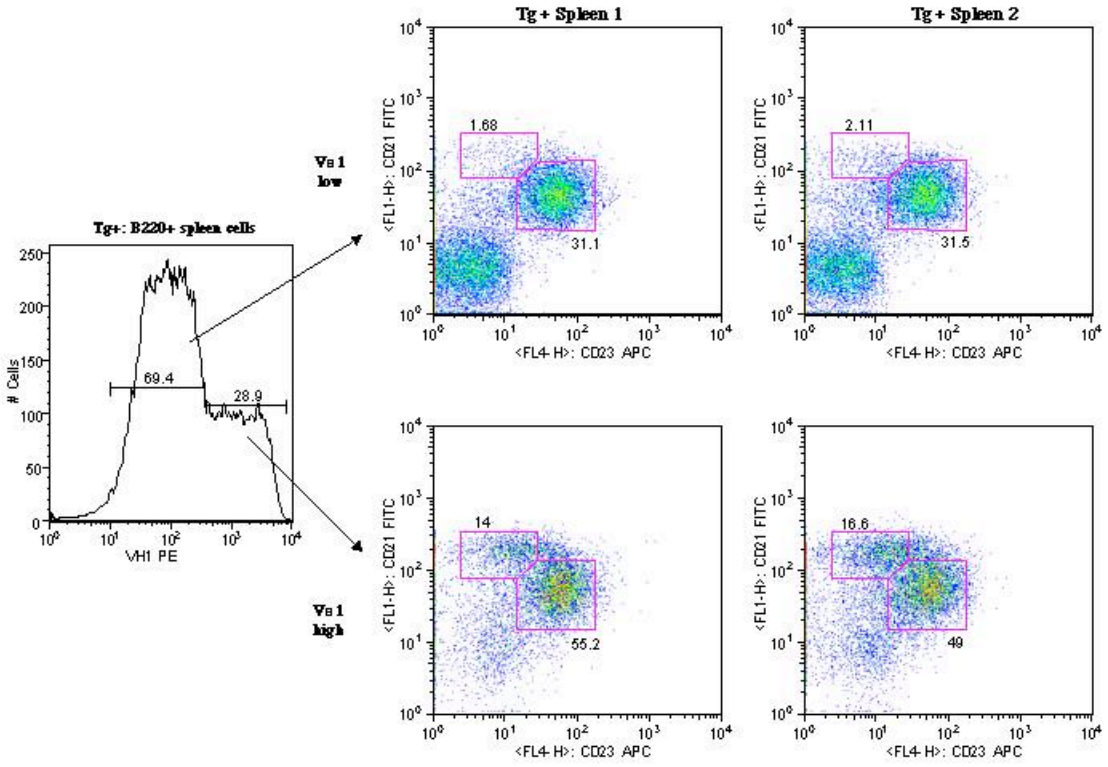

Figure 14. V_H1 expression on M167μk Tg splenic B cells. Tg+ spleen cells have two distinct densities of V_H1 Tg (low/intermediate vs. high) surface expression on their cell surface. Spleen cells were stained with CD21-FITC, VH1-PE, B220-PerCP, and CD23-biotin/SAV-APC and their FO ($CD21^{low}$, $CD23^{high}$), MZ ($CD21^{high}$, $CD23^{low}$), NF ($CD21^{low}$, $CD23^{low}$) B cell compartments are shown. The $VH1^{high}$ B cells contain the MZ B cell population (shown in the 14% and 16.6% gates in the two bottom dot-plots.

present, and therefore, not creating as high a density of M167-id BCR on the cell surface. Because the density of M167-id receptors could be diluted by the presence endogenous Ig, the levels of M167 idiotype expression on FO and MZ (CD21/CD23) B220+ splenic B cells, as well as on the mature and immature/MZ (IgM/IgD), were examined. In both instances, B cells with M167-id^{high} were found in the MZ compartments (**Figure 15** and **Figure 16**), supporting the hypothesis that M167^{high} receptor density plays role in selecting a MZ phenotype. B cells with M167^{low} receptor density were found in FO compartment.

Tg MZ B cells have higher expression of M167 receptor than the Tg FO B cells (**Figure 15**). Although almost all of the B cells in the M167μκ Tg mice express the M167 receptor, those with the highest densities of M167 adopt a marginal zone phenotype rather than a follicular phenotype. In non-Tg WT mice, the M167 positive B cells are found strictly in the marginal zone B cell compartment. These results agree with the data published by Martin *et al.* (42) where M167 idiotype positive (Id+) B cells are shown to localize in the MZ compartment of normal mice as well as in M167μ only Tg mice.

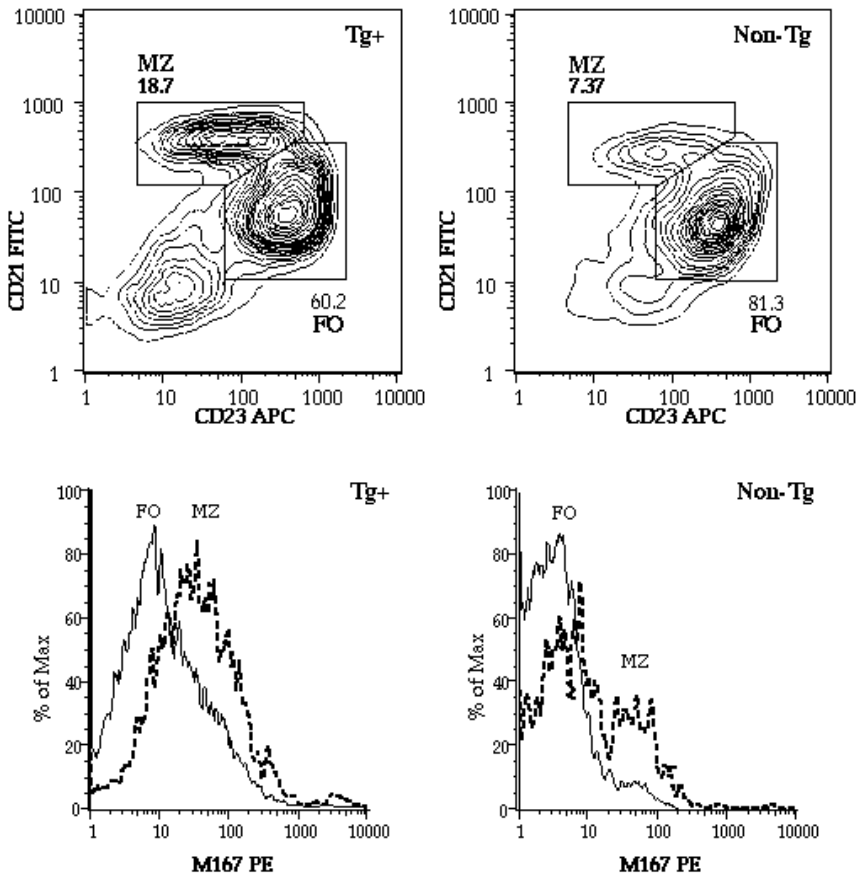

Figure 15. High surface expression of M167 BCR in Tg MZ B cells. Marginal zone B cells express higher levels of cell surface M167 BCR than follicular (FO) B cells in both Tg and non-Tg mice. M167-id positive B cells in non-Tg WT mice are strictly found in the MZ compartment, not in the FO B cell compartment. Data are representative of five mice in each group.

In **Figure 16**, the enlarged $IgM^{high}IgD^{low}$ fraction, which includes both the immature as well as MZ B cells, also contains the $M167^{high}$ B cells. Taking the evidence as a whole, I believe that Tg B cells with the higher cell surface densities of V_H1 were able to pair with Vk24 light chain to make higher levels of M167-idiotypic receptor, and are directed by a receptor-mediated event to become marginal zone (MZ) B cells.

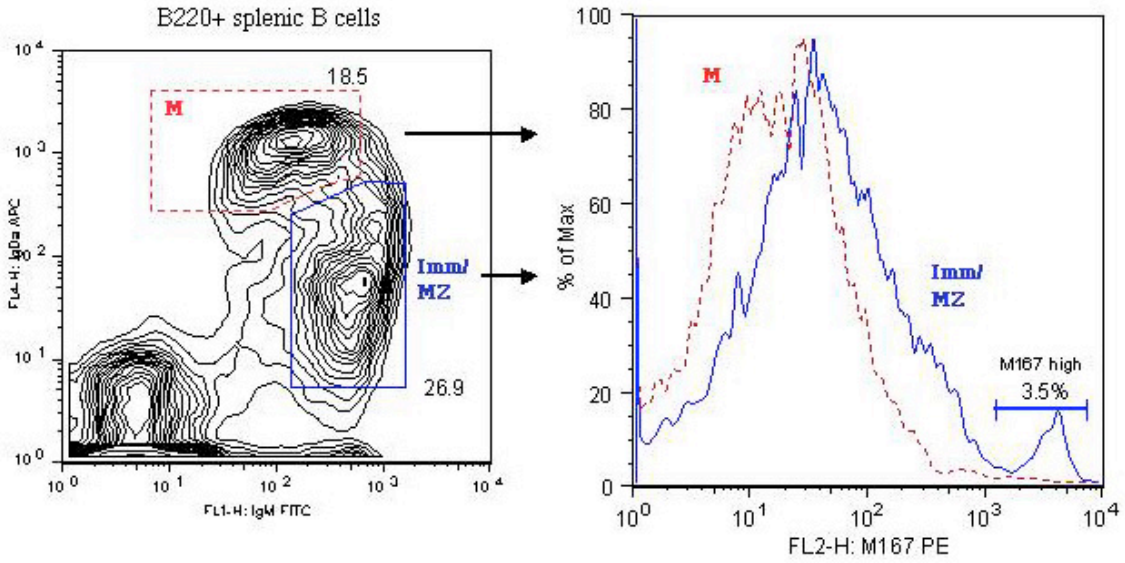

Figure 16. B cells with high expression of M167 BCR are found in the IgM^{high}/IgD^{low} compartment of spleen in M167μk Tg mice. A subgroup of IgM^{high}/IgD^{low} B cells has higher expression of M167-idiotype on their cell surface (dotted line, Mature B cells; solid line, immature and marginal zone B cells). This population includes the MZ B cells and supports the previous observation that the high V_H1 receptor density on Tg+ B cells results in the MZ phenotype.

Peritoneal B Cell Populations in C.M167μk Tg Mice

Peritoneal cells were analyzed to determine if the M167μk Tg caused any changes to occur in the B cell repertoire, particularly in the B1 compartment. **Figure 17** shows that Tg and non-Tg (WT) littermates did not have any significant differences in the size of the various peritoneal B cell populations, except where the presence of the V_H1 Tg was concerned. V_H1 was expressed on 88% of Tg peritoneal B cells, whereas only 2% of the non-Tg peritoneal B cells carried the V_H1 heavy chain. CD5+ IgM- T cells were similar (13% and 12.2% of PEC lymphocytes in Tg and non-Tg, respectively.)

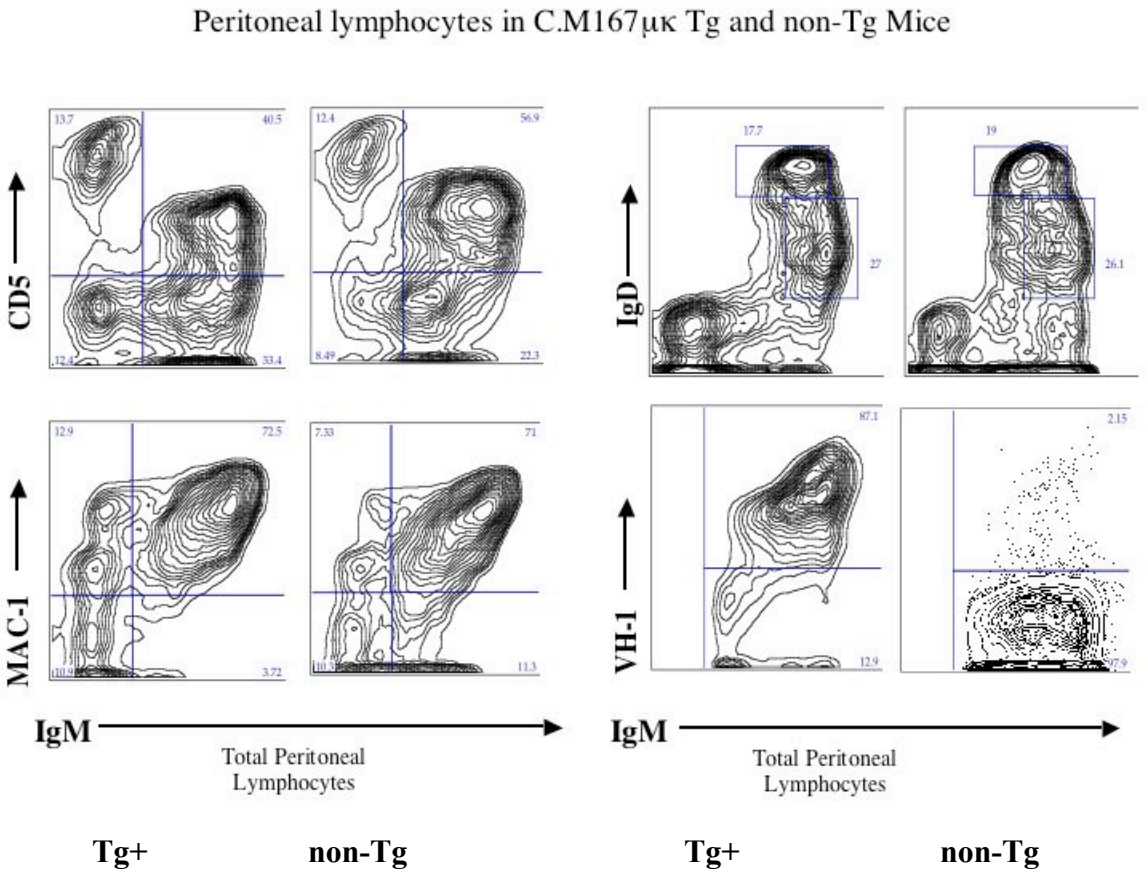

Figure 17. Peritoneal B cells from C.M167μk Tg and non-Tg mice. In our conventional colony mice, Tg and non-Tg mice have the same size populations of B1 and B2 cells in their peritoneal cavities. B1 cell surface markers, CD5 and MAC-1, have similar expression profiles on peritoneal B1 cells in both Tg+ and non-Tg littermates. The two IgM/IgD fractions, B1 and B2 cells, are the same size in both Tg and non-Tg mice. The main difference between the peritoneal B cells is the expression of VH1. (SPF mice, not shown, give similar peritoneal B cell profiles as the conventional mice shown above.)

Chapter 3 Conclusions:

- The BALB/c M167μk Tg mouse expresses the transgenic M167-id B cell receptor on the cell surface of 97-99% of splenic, peritoneal and MLN B cells.
- Confirming the observations of Jim Kenny in C57BL/6 M167μk Tg mice, I found a striking reduction in the peripheral B cell population in the BALB/c M167μk Tg mice.
- M167μk Tg induces a shift in B cell population - the marginal zone (MZ) B cell population in spleen is expanded in M167μk Tg mice (**Figure 13**).
- There is variation in surface density of Tg V_H1 heavy chain on B cells
    - **Figure 10** shows two distinct population of VH1+ B cells
    - V_H1 low/intermediate and V_H1 high (**Figure 14**) and M167-id (μ+k) B cell receptor (**Figure 15**).
- Contrary to previous reports in the literature, IgD was found to be expressed on >90% of peripheral IgM+ B cells in M167μk Tg mice (**Figure 12**).

CHAPTER 4

Analysis of the Immunoglobulins Produced by PCTs from C.M167μk Tg mice

One of the main characteristics shared by a PCT and its precursor B cell is the Ig they produced. This chapter examines the immunoglobulins produced in PCTs derived from M167μk Tg mice in order to learn which type of B cells give rise to PCTs. Analysis of the PCT immunoglobulins is divided into two sections: (I) secreted Ig proteins and (II) expressed Ig gene sequences.

I. Analysis of the Secreted Immunoglobulin Proteins

Electrophoretic Analysis of Secreted Ig Proteins from C.M167μk Tg PCTs

Do PCTs generated in M167μk Tg mice secrete large quantities of monoclonal M167-IgM transgenic protein? Plasma cell tumors, such as the original MOPC167 (IgA), typically secrete a monoclonal "myeloma" protein. One of the side effects of pristane injection in mice is the generation of ascites fluid in the peritoneal cavity. This fluid contains the immunoglobulins secreted from the plasma cell tumors. Electrophoresis of ascites on polyacrylamide gels was done to determine whether a monoclonal M167 immunoglobulin protein was being secreted by plasma cell tumors generated in M167μk Tg mice.

The Potter lab has observed for many years that the ascites from a primary PCT may not have an unequivocal Ig band, but ascites from the transplanted tumor usually

does. Therefore, PCTs from M167μk Tg mice were transplanted into pristane-primed WT BALB/c mice, although not all transplanted tumors successfully grew. Ascites from mice carrying first generation (g1) of transplanted Tg PCTs were compared to normal ascites on Paragon serum protein electrophoresis (SPE) gels (top panel, Figure 18A.) Tg PCTs that did not grow in transplant are presented in the second panel of **Figure 18**B as primary ascites (g0). Ascites from WT PCTs (either primary and transplanted) are shown in **Figure 18**C.

It appeared that the Tg PCTs were not secreting large quantities of M167μk Ig, as an IgM monoclonal (M) component could not be identified in the SPE profiles of Tg ascites. If the Tg-derived PCTs were secreting a large amount of monoclonal transgenic product, we would expect to see a large Ig band of M167 IgM transgenic product running at or near the origin (center). However, each of the Tg tumors had a unique Ig banding profile. The presence of the monoclonal (M) components of different mobilities indicates that many PCTs were secreting Ig other than the one controlled by the transgene. Many of the M components had gamma (γ) mobility that suggested that the tumors were producing M proteins from endogenous Ig genes. It was not possible to distinguish M167μk protein product from endogenously produced Ig protein on the SPE gels.

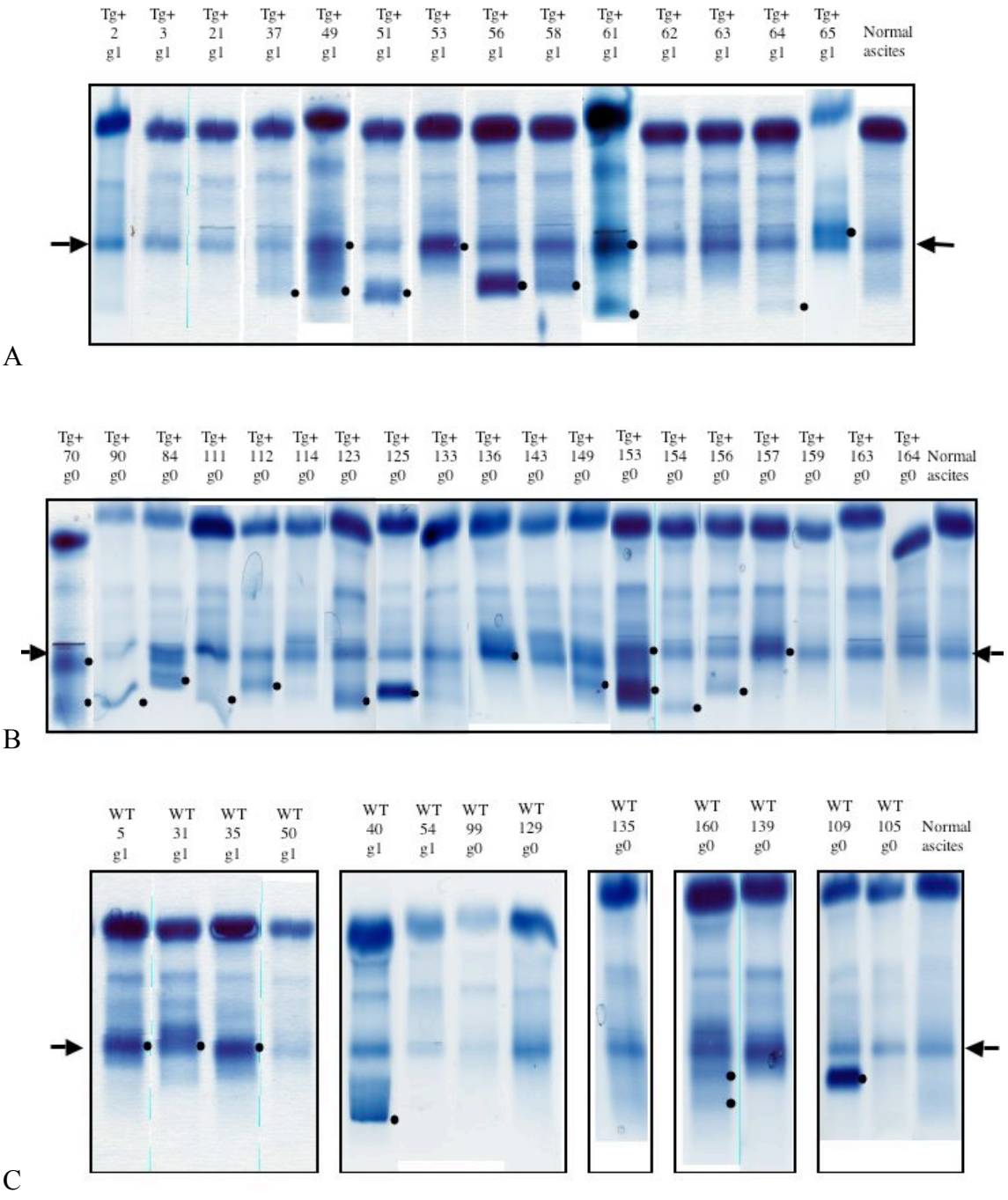

Figure 18. SPE gels of PCT ascites. Serum protein electophoresis in a non-reducing polyacrylamide gel. (A) Tg g1, (B) Tg g0, and (C) WT g1 and g0 ascites. Ascites (1 μl) from M167μκ Tg and non-Tg WT primary (g0) and transplanted (g1) PCTs were run beside normal ascites from a pristane mouse with no PCT. Black dots (•) indicate Ig proteins bands detected in some of the PCTs. The anode (+) is at the top and the cathode (–) is at the bottom of the gel. Ascites was loaded at the center (origin, indicated by arrows).

Of those PCTs analyzed by SPE, 18 Tg and 3 WT PCTs had detectable monoclonal Ig bands located below the origin, as indicated by black dots in Figure 18. Eight Tg PCTs appeared to have monoclonal bands at or near the origin that could be the IgM transgenic product (49, 53, 61, 65, 70, 136, 153 and 157), also notated by black dots. Only 6 of the PCTs induced in Tg mice (51, 56, 61, 70, 125, 153) and 2 of the PCTs from the WT mice (40 and 109) produced very large amounts of monoclonal Ig protein**.** Even though the IgM, IgA, and IgG often have characteristic electrophoretic patterns, it was not possible to determine isotype with the banding patterns produced here.[3]

Are the Ig proteins being secreted by M167μk PCTs from the M167 transgene or from endogenous Ig? The next step was to determine by an ELISA whether the tumors were secreting PC-binding antibodies (reflecting antigen-specific stimulation of the Tg anti-PC receptor on precursor B cells) and if those PC antibodies were using the transgenic M167 idiotope. Further, the Ig isotypes produced by the PCTs were examined by an ELISA to determine whether the transgenic IgM isotype was present or if other isotypes were being expressed.

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[3] In the SPE gel shown in Figure 18, there is a strong albumin protein band at the top (anode) and another distinct band at the origin, both of which can be seen in the normal ascites control lanes. IgM is known to run around the origin (at the center of gel where ascites is loaded). IgG runs toward the negative electrode (below the origin) and IgA towards the positive (above the origin).

Tg+ PCTs Secrete anti-PC, M167-id Antibodies

The primary question regarding the M167 Tg plasma cell tumors was whether they secrete monoclonal anti-PC antibodies, and specifically, whether the PC-binding antibodies have the M167 (μ+k) idiotype. If precursor B cells were initially activated through the transgenic B cell receptor (M167μk) by PC antigen, it could be expected that the plasma cell tumors would produce monoclonal Ig of transgenic origin.

The Tg and non-Tg WT tumors were tested for PC-specific antibodies using ELISA to screen ascites fluid. PC antigen (PC-BSA) was used to capture anti-PC antibodies from ascites on a 96-well plate, and an HRP-labeled secondary antibody detected any mouse (H+L) immunoglobulin that could bind PC. The ELISA results show that every PCT derived from M167μk Tg mice secreted anti-PC antibodies (Figure **19**). Only one of the 12 non-Tg PCTs (#40WT) produced antibodies with specificity for PC antigen.

Because the Tg-derived PCTs were secreting PC-specific antibodies, ascites from these tumors were further screened for PC antibody containing the unique (idiotypic) specificity of the M167 protein. J. Kenny prepared such a M167 specific antiserum (clone 28-6-20), which he kindly donated. Once again using the PC-ELISA assay to capture secreted PC-binding antibodies, the ascites was screened for M167-idiotopes using the anti-M167-id antibody. **Figure 20** shows that the Tg tumors did indeed secrete PC-binding M167-id antibodies, while the non-Tg (WT) tumors were negative for M167 idiotopes. Although the Ig protein from #40WT PCT binds PC (**Figure 19**), this WT tumor does not secrete an M167-id antibody. (This tumor will be analyzed further in later sections).

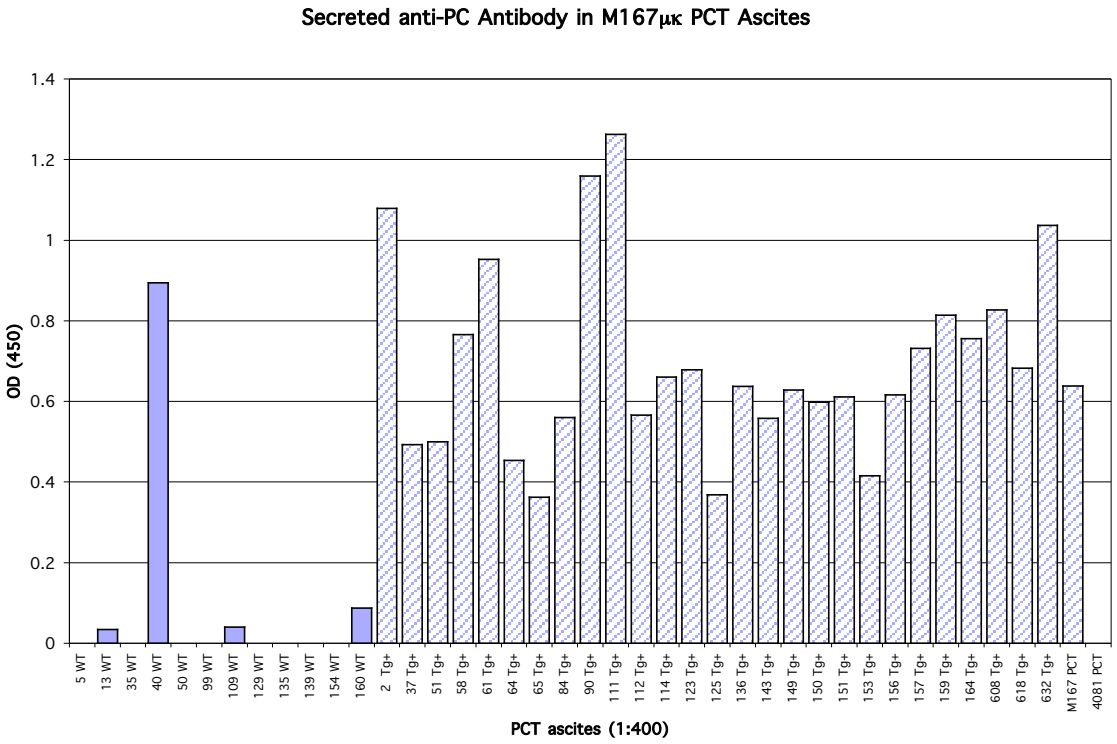


Figure 19. M167μk Tg PCTs are secreting anti-PC antibodies. Peritoneal ascites fluid was analyzed by ELISA for presence of secreted anti-phosphorylcholine (PC) antibodies. ELISA plates were coated with PC-BSA antigen. Anti-PC antibodies are detected in ascites from all Tg+ PCTs (n=27), and in only one of the non-Tg (WT) PCTs (n=12). Positive and negative controls were ascites from M167 (IgA, anti-PC) and 4081 (IgG2b, unknown specificity), respectively. OD reading reported at 1/400 dilution of ascites.

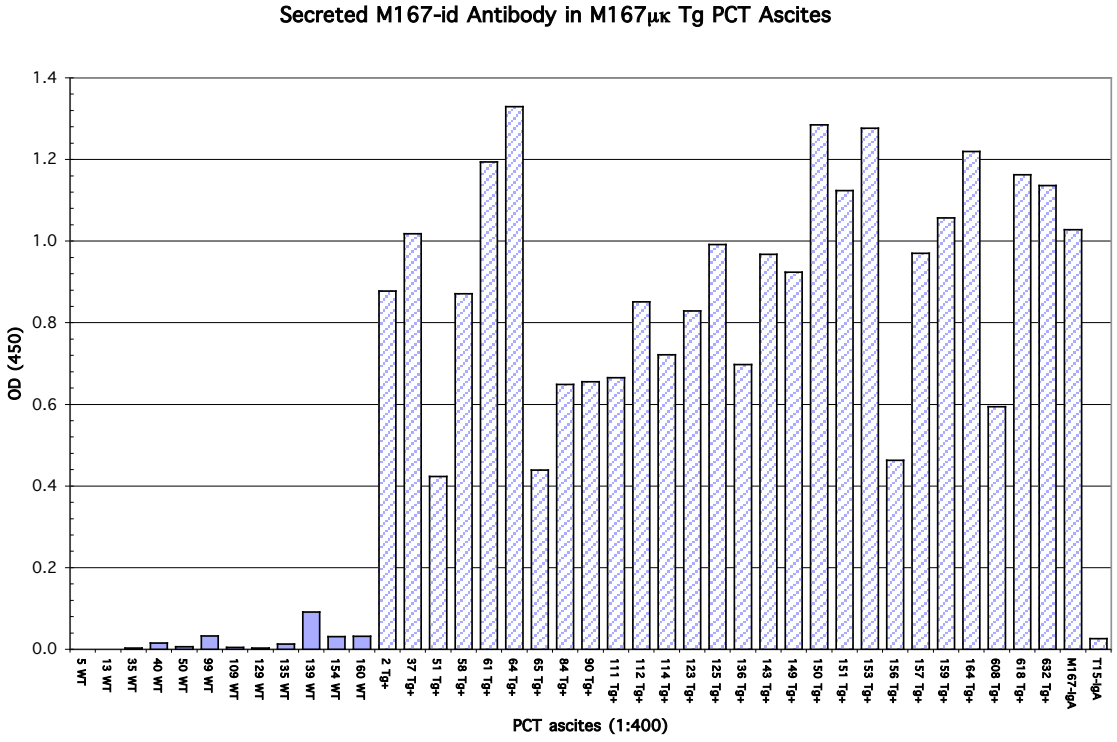


Figure 20. M167μk Tg PCTs are secreting M167-id Antibody. Peritoneal ascites fluid was analyzed by PC-ELISA for presence of secreted Tg M167-id antibody. ELISA plates were coated with PC-BSA antigen to capture PC-specific antibodies from ascites. M167-idiotypic antibody is detected in ascites from Tg PCTs (n=27), but not in non-Tg (WT) PCTs (n=12). Positive and negative ELISA controls were the anti-PC binding idiotypic antibodies, M167 and T15, respectively. OD reading reported at 1/400 dilution of ascites.

The 28-6-20 Ab was provided and used as an anti-idiotypic Ab for M167. However, it is not truly an anti-idiotypic antibody as its specificity is for determinants found on the Vk24 L chain. This antibody can detect Vκ24 L chains in combination with any type of heavy chain, including V_H1 and non-V_H1 heavy chains. Evidence thus far indicates that Vk24 is rarely found paired with other heavy chain genes and appears to be

the unique light chain partner for V_H1 in anti-PC antibodies. Therefore, for it serves our purpose as a detector of M167 antibody.

Isotyping of Igs from M167μk PCTs: IgM, IgA, IgG ELISA

The isotypes produced by the Tg PCTs were initially examined to determine whether the transgenic IgM isotype was being utilized. The Tg PCTs were shown to secrete M167-id PC-specific Ig, which should have an IgM constant region as dictated by the transgenic construct. Additionally, the presence of monoclonal bands with different mobilities seen on the SPE gels indicated that the Tg PCTs were also secreting Igs other than the one controlled by the transgene.

Using an ELISA, I screened ascites for the presence of IgM, IgA, and IgG isotypes secreted from Tg PCTs. Murine Ig proteins were captured on a 96-well plate using a goat anti-mouse (H+L) antibody. The individual isotypes present in ascites fluid were identified with HRP-labeled anti-IgM, anti-IgA, and anti-IgG (a mix of anti-G1, -G2a, -G2b, and -G3) antibodies. All of the PCTs generated from Tg mice had IgM antibodies present in their ascites (Table 6). Secreted IgM antibodies were expected in Tg PCTs because the transgene has an IgM isotype. Significantly, most of the Tg positive PCTs (90%) also secreted IgA antibodies in addition to the IgM antibodies. The remaining 10% secreted IgG in addition to the Tg IgM, but these two tumors were not able to be further analyzed. Unlike the IgM antibodies, the presence of secreted IgA (or IgG) from the Tg PCTs was a surprise. The presence of possible IgA Igs being secreted

Isotyping of PCT Ascites (ELISA)

WT PCT	IgM	IgA	IgG
5	–	+	–
13	–	+	–
35	–	–	–
40	+	–	–
50	–	+	–
99*	–	+	–
109*	–	+	+
129	–	+	+
139	–	+	–
152*	–	+	+
160	–	–	+
TG PCT	IgM	IgA	IgG
2	+	+	–
37	+	+	–
51	+	+	–
58	+	+	–
61	+	–	+
64	+	+	–
65	+	+	–
84*	+	+	–

TG PCT	IgM	IgA	IgG
90*	+	-	+
111*	+	-	+
112*	+	+	–
114*	+	+	–
123*	+	+	–
125	+	+	–
136*	+	+	–
143*	+	+	–
149	+	+	–
150	+	+	–
151	+	+	–
153*	+	+	–
156	+	+	–
157*	+	+	–
159*	+	+	–
164*	+	+	–
608*	+	+	–
618*	+	+	–
632*	+	+	–
T15-IgA	-	+	-
4081-IgG2b	-	-	+

Table 6. Isotyping (IgM, IgA, IgG) peritoneal ascites from PCTS of C.M167μk Tg and non-Tg (WT) mice. The isotypes were determined using ELISA. A positive (+) isotype was designated if the OD was >0.2 at a 1:12,800 dilution. A * denotes ascites from the primary (g0) PCT; otherwise, the ascites tested was from a transplanted g1 PCT.

by the Tg tumors might mean either (1) the M167 IgM heavy chain transgene had switched to IgA in these tumors or (2) that the tumors have rearranged an endogenous heavy chain locus and are secreting separate species of isotype switched antibody. However, the M167μ Tg does not contain switch regions, so isotype switching of the transgene is unlikely to explain the presence of IgA protein.

Of the 11 non-Tg (WT) PCTs tested, one secreted an IgM protein (#40WT), eight secreted IgA, and one secreted IgG (#160WT). Three WT PCTs had both IgA and IgG isotypes detectable in the ascites. Tumor #35WT did not have a detectable Ig isotype of any kind and, therefore, is probably a non-secreting PCT. Further, all of Tg and WT tumors secrete kappa light chains, and not lambda light chains (data not shown). These isotyping data were used for further sequencing analysis of Ig from the PCTs.

Immunohistochemistry: Secreted M167, IgM, and IgA Proteins in Tg PCTs

Immunochemical staining of tissue sections was used to confirm that individual tumor cells from Tg PCTs were secreting IgA as well as the Tg IgM isotype. Serially cut sections of mesentery oil granuloma from 5 of the transplanted g1 Tg PCTs were fixed and stained with HRP-labeled anti-M167-id, anti-IgM, or anti-IgA antibodies. Immunohistochemical staining of individual plasma cells in the Tg tumors confirmed the presence of both IgA and IgM, as well as Tg M167-id antibodies. **Figure 21** shows staining of a representative tumor, PCT #58Tg, where all tumor cells in the mesentery oil granuloma tissue stain positive for IgM and IgA, as well as for the transgenic M167-idiotype (IgM+, IgA+, and M167+).

Because it appears that Tg PCTs secrete both IgM and IgA antibodies, we wanted to know if these antibodies were either (1) isotype-switched transgenic M167-id IgA or (2) endogenous Ig genes being rearranged and secreted as IgA antibodies. If the latter is true, then are the endogenous Ig proteins contributing to the production of PC-specific antibodies?

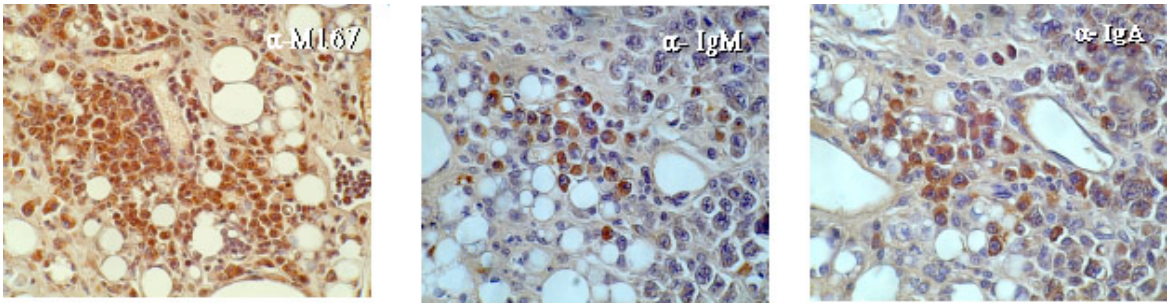


Figure 21. Immunohistochemical staining of PCT #58Tg in mesentery oil granuloma tissue. Plasmacytoma cells from PCT #58Tg have stained positive for the transgenic M167-id and IgM proteins. However, the same monoclonal tumor also stains positive for IgA antibody, which indicates another species of antibody utilizing endogenous IgA. Staining for IgG1, IgG2a, and IgG2b was negative on PCT #58Tg cells (not shown).

IgM from Tg PCTs Binds PC Antigen, but IgA Does Not

A simple immunoprecipitation assay using g1 ascites from #37 Tg PCT (37g1) and PC antigen (from LB4 bacterial cell wall) mixed in a capillary tube provided a visual confirmation that #37 Tg PCT produces antibody proteins with a specificity for PC antigen by the formation of an Ab/Ag precipitate. The PC-reactive #37 Tg PCT has been previously shown to secrete IgM and IgA isotypes (ELISA isotyping, **Table 6**) as well as the anti-PC M167-id (ELISA, **Figure 20**). However, we still didn't know which of the antibody isotypes (IgM, IgA, or both) produces the anti-PC reactivity.

An immunoelectrophoresis (IEP Paragon) gel was used to separate the Ig isotypes in ascites and test the individual Ig proteins with an antigen of interest to see which of the isotypes will react with the PC antigen. The anti-PC monoclonal antibody, T15, has an IgA isotype (anti-PC/IgA) that serves as a positive control for PC-specific IgA. The IEP assay

shown in **Figure 22** demonstrates that the IgM antibody from #37 Tg PCT reacts to PC antigens, but the IgA component does not.

The T15-IgA/PC precipitation arc shows where the IgA reaction would have taken place had the IgA antibody in #37 Tg PCT contributed to PC specificity. There is no precipitation reaction between IgA and PC in the #37 Tg PCT ascites, as seen in theT15-IgA control lane. This provides evidence that the IgA does not contribute to the anti-PC binding

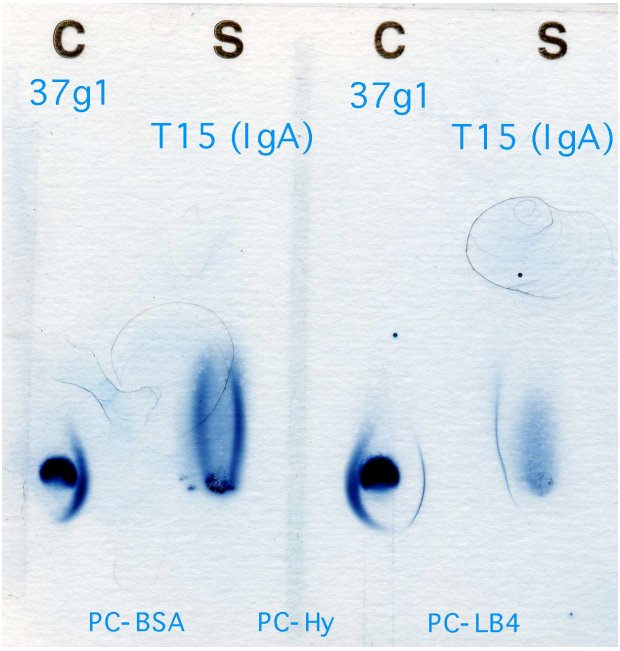


Figure 22. Immunoelectrophoresis (IEP) of 37Tg167PCT precipitated with PC antigens. Ascites from a M167μk Tg PCT (37g1) and PC-specific T15 (IgA) control both react to various PC antigens: PC-BSA, PC-Hy (PC attached to hemocyanin protein), and PC-LB4 (PC antigen in bacterial cell wall of Lactobacillus). IgM protein runs at the origin, as seen in 37g1. IgA runs toward the anode (above the origin), as seen in the IgA protein, T15. The IgM antibody from #37 Tg PCT binds PC, but there is no IgA in this tumor that reacts to PC. If PC-reacting IgA antibodies were present, they would be detected at the same position as the control T15-IgA.

antibody repertoire produced in Tg PCTs. Therefore, the IgA is probably not isotyped-switched PC-binding M167 H chain. (As demonstrated in a later section, RT-PCR testing of Tg tumors for the presence of M167-IgA transcripts corroborates this result.)

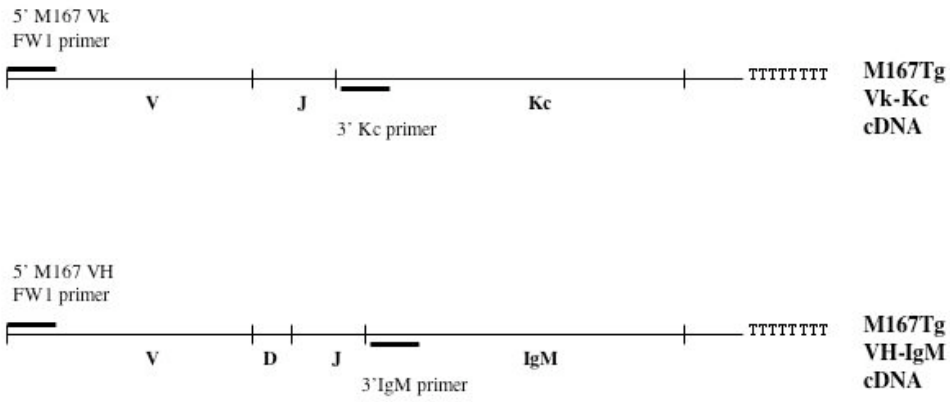
Thus far, we can conclude that PCTs arising in M167μk Tg mice are secreting the transgenic M167-IgM antibody, as shown by ELISA and immunohistochemical staining. Further, the electrophoretic analysis showed that the tumors from Tg mice collectively produced a variety of different M components (monoclonal Ig proteins). These Igs were not the products of the M167μκ transgene. IEP analysis provided evidence that the secreted Tg IgM is the only species of Ig present that binds PC antigen (**Figure 22**).

II. Analysis of Expressed Immunoglobulin Gene Sequences

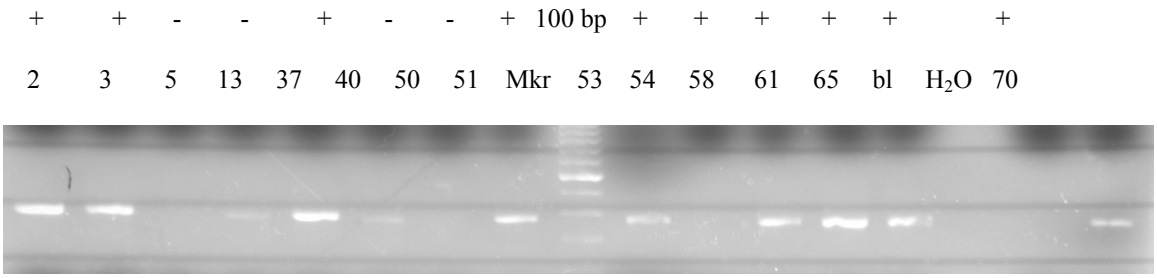
M167-IgM and M167-kappa RNA Expression in PCTs

As shown in the previous section, the PC-binding M167-id antibody protein is secreted into ascites by Tg tumors, but not by the non-Tg PCTs. To confirm that the plasmacytoma cells from Tg mice are expressing mRNA for both the transgenes, total RNA from Tg and non-Tg WT PCT cells were screened for transcripts of transgenic M167 IgM heavy and M167 kappa light chains. Total RNA was extracted from tumor tissue and first strand cDNA was generated from mRNA using oligo d(T) primers. Tg-specific primers at the 5' end of the variable framework 1 region (V_H or Vκ FW1) and a 3' constant region primer (positioned at the most 5' end of the IgM or kappa Kc region) were used to amplify the variable regions of the M167 Tg heavy and light chains by PCR (shown in **Figure 23**A). For primer sequences, see pg165 in M&M. PCR products were visualized on an agarose gel.

A.



B. M167Tg Vk



C. M167Tg V_H - IgM

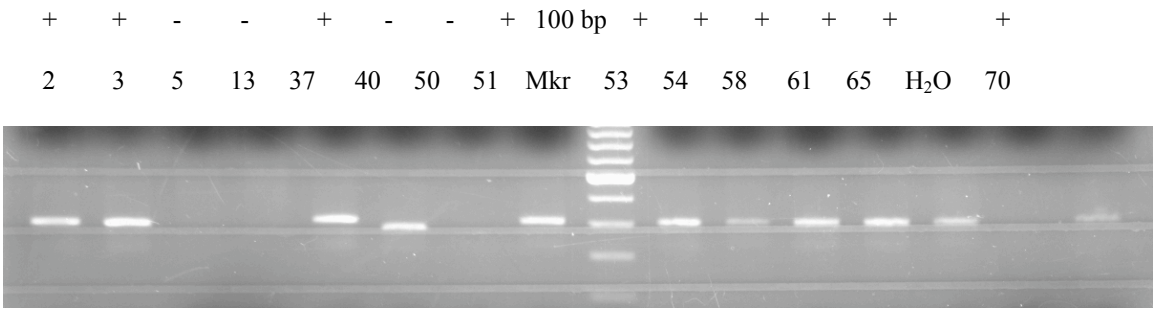


Figure 23. Tg mRNA transcripts from PCTs. (A) Tg-specific primers in FW1 of the variable region were paired with at the corresponding 3' constant region primer to amplify the VκJ and V_HDJ variable regions of the M167 transgenes. (B-C) RT-PCR products show expression of Tg M167 light (B) and heavy (C) chain genes in a panel of Tg (+) and non-Tg WT (-) PCTs. Variable region RT-PCR products were sequenced to confirm expression of

the transgenes. A 100 bp ladder was used as a size marker, the brightest band being 600 bp, and the V region bands were ~400bp. Sequencing of the smaller, non-specific IgM heavy chain band in 40WT (C: lane 6) shows that it is not M167-IgM, but VH6 (VH22.1)-dsp2.8-Jk3 IgM. Weak Vκ bands in #13WT and #40WT PCTs (B: lanes 4 and 6) were non-specific products: Vκ19-15/Jκ2 (#40WT) and Vκ19-15/Jκ1 (#13WT). One of the Tg PCTs (#54Tg, lane 11) does not express the Tg M167 Vκ24 light chain, but does express the M167 V1-IgM heavy chain.

Figure 23(B-C) shows a panel of PCT tumors that were screened by RT-PCR for the presence of Tg M167 kappa and M167 IgM transcripts. PCTs generated from Tg mice all contained RNA transcripts of both the M167 light and heavy chain transgenes. The one exception was #54Tg PCT, which only contained the mRNA transcript for M167 IgM heavy chain, but was not positive for the M167 Vκ24 light chain transgene mRNA. (**Figure 23**, B and C, lane 11). Supporting these data, ascites from #54Tg PCT tested by ELISA does not contain the M167-id antibody (data not shown), which is consistent with the lack of RNA expression of Vκ24 to pair with the expressed M167μ V_H1 transgene. PCTs from non-Tg WT littermates did not contain M167 heavy or light chain mRNA. All of the variable region RT-PCR products were sequenced to confirm they were actually the M167μκ transgene sequences. Strict precautions were taken to control for PCR cross-contamination between samples. Samples were independently prepared and separate benchtop areas, pipettes, and disposables were used for each of the nucleic acid isolation, amplification, and sequencing steps. Importantly, nucleic acid was prepared and sequenced from multiple samples of frozen tumor to confirm accuracy of the Ig sequences.

Two non-Tg PCTs (#13WT and #40WT) had very weak, non-Vκ24 products that when sequenced were actually unique gene products from the Vκ19 family (closest

homology to gene Vκ19-15), not the M167 Vκ24 transgene. There is an IgM RT-PCR product in #40WT PCT that is smaller than the transgene VH-IgM product, and sequencing showed that it is not the M167μ transgene. The IgM in #40WT PCT uses the VH6(VH22.1) gene combined with DJ genes dsp2.8 and JH3.

Tg PCTs do not have Isotype-Switched M167 VH-IgA mRNA Transcripts

Serum analysis showed that the Tg tumors were also secreting IgA antibodies in addition to IgM. To find out if the M167μ heavy chain transgene was able to switch to IgA, I screened mRNA from Tg PCTs for M167V_H-IgA transcription products. Tg mRNA was tested by RT-PCR using the 5' Tg-specific M167 VH FW1 primer paired with a 3' IgA constant region primer. No transgenic M167 heavy chain IgA transcripts were detected by RT-PCR (Figure 24) in any of the Tg PCTs, but the control cDNA from the original M167(IgA) tumor was successfully amplified. Further, ELISA could not detect M167-id IgA antibodies in the ascites of these Tg PCTs (data not shown). Therefore, the IgA antibodies detected by ELISA isotyping are not a result of M167Tg-switched antibodies. The lack of M167-IgA transcripts is further evidence of the inability of M167-IgM transgene to isotype switch from its location on chromosome 2/3 with endogenous IgA locus on chromosome 12.

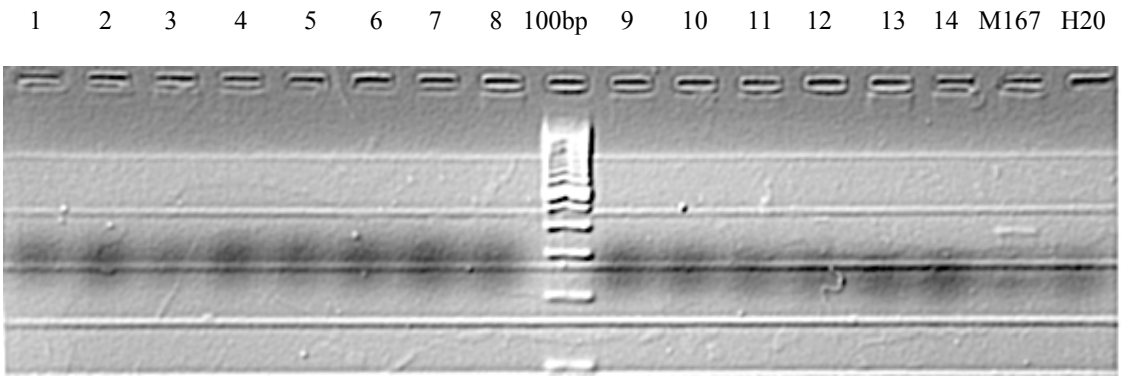


Figure 24. PCR of M167-IgA transcripts. RT-PCR using 3' M167-specific framework (FWR1) and 5' IgA constant primers were run on an agarose gel to see if any isotype switched M167-IgA transcripts were being produced by the Tg PCTs. Lanes 1-14 are Tg PCT cDNA (37,2,61,112,123,154,162,27,64,65,153,62,160, 5), 15 is M167-IgA cDNA, and 16 is H_20. The M167(IgA) positive control cDNA (lane 16) has a band at the appropriate size (V_HDJ ~400bp), but no M167-IgA transcripts were detected in the Tg PCTs tested. A100 bp ladder size marker was used, the brightest band being 600 bp, and the M167VH-IgA product size is ~400bp.

Sequencing of Coexpessed Endogenous lg Genes

If the IgA antibodies are not from isotype-switched M167Tg, their source must be from endogenously rearranged IgH genes. To determine whether there are species of endogenous Ig heavy and light chain genes being rearranged and expressed in Tg PCTs, RT-PCR was used to amplify the unknown IgA transcripts and any possible unknown IgM, IgG or kappa transcripts. In four independent reactions, four different degenerate VH primers (named SG1, 2, 3, and 4; Huppi and Smith-Gill (52), see pg166 in M&M) were paired with the 3' primer of the heavy chain constant region (IgA, IgG, or IgM) to amplify unknown VH regions from PCT mRNA. Each of these degenerate (or mixed base) primers includes multiple species of oligonucleotides that are homologous to the framework 1 (FW1) region of VH genes within related families. In this manner, one or more of the four 5'VH primers

would amplify the unknown VH region expressed by a tumor. Similarly, one 5' Vκ degenerate FW1 primer (5' Mκ) paired with the 3' kappa constant region primer (3' Kc) was comprehensive enough to amplify the Vκ region of all PCTs screened.

RT-PCR routinely produced a single distinct DNA band for Vκ products, and sequencing of these bands always gave specific unique kappa sequences. For the VH products, occasionally non-specific banding products were present. For each tumor, the correct size VH band (~400bp) was excised from an agarose gel and purified before sequencing. Independent preparations of RNA were made from multiple tissue samples of the same tumor to confirm Ig sequencing results and to prevent PCR cross-contamination of samples. Several independent RT-PCR reactions were performed at different times on each RNA prep. Sequencing was routinely repeated at least twice on each RT-PCR reaction with forward and reverse primers in order to ensure accuracy of base calls during sequencing. Identification of VH and Vκ family genes was done using a blast search of the NCBI database of germline Ig mouse genes. The D and J genes were identified by greatest homology to germline genes reported in the NCBI IgBLAST database.

All the transgenic PCTs co-expressed endogenous heavy and light chain mRNA (**Table 7**) in addition to the transgenic M167 IgM and M167 Vκ mRNAs. PCTs from non-Tg littermates were also sequenced and are reported in **Table 8**.

Table 7. M167μk Tg PCT: endogenous Ig sequences (n=24). Endogenously expressed Vk and VH Ig genes from PCTs generated in BALB/cAnPt M167μk Tg. VH families are classified on the basis of homology to reported germline genes as reported in the NCBI IgBLAST. Vk families classified on basis of homologous germline gene segments as reported by Thiebe *et al*. (60). ND= not determined.

M167μk Tg PCT: endogenous Ig sequences (n=24).

M67μk Tg PCT	Vk family	Jk	VH family	D	JH	Ig
2 TG	Vk8 (8-21)	Jk1	VH1 J558	dsp2.x	JH2	A
37 TG	Vk1 (bb1)	Jk1	VH3 36-60	dsp2.x	JH2	A
44 TG	Vk9/10 (bv9)	Jk2	VH1 J558		JH4	A
51 TG	Vk4/5 (kj4)	Jk1	VH1 J558	dfl16.2	JH2	A
53 TG	Vk12 (12-46)	Jk2	VH6 VH22.1	dsp2.2	JH4	A
54 TG	Vk19/28 (19-32)	Jk2	VH1 J558	dq52	JH3	A
58 TG	Vk19/28 (19-32)	Jk2	VH1 J558	dsp2.2	JH4	A
61 TG	Vk9 (bv9)	Jk1	VH10	none	JH4	A
62 TG	Vk1 (bb1)	Jk1	VH1 J558	dsp2.x	JH2	A
64 TG	Vk4/5 (aq4)	Jk5	VH1 J558	dsp2.7	JH4	A
70 TG	Vk19/28 (19-32)	Jk1	VH3 36-60		JH3	A
114 TG	Vk21 (21-7)	Jk1	VH7 V1	dfl16.2	JH4	A
123 TG	Vk4/5 (ac4)	Jk4	VH5 VH61-1p		JH4	A
135 TG	Vk24/25 (hg24)	Jk5	VH1 J558	dsp2.2	JH2	A
149 TG	Vk23 (23-39)	Jk2	VH4 Gal55.1	dfl16.1	JH4	A
150 TG	ND		VH5 VH61-1p		JH3	A
151 TG	Vk9/10 (cb9)	Jk2	VH1 J558		JH2	A
153 TG	Vk9/10 (ba9)	Jk5	VH1 J558	dfl16.1	JH4	A
157 TG	Vk9/10 (cp9)	Jk1	VH1 J558	dsp2.2	JH3	A
162 TG	Vk23 (23-37)	Jk2	VH1 J558	dsp2.10	JH3	A
608 TG	Vk9/10 (ce9)	Jk2	VH3 36-60	dsp2.9	JH4	A
618 TG	Vk9/10 (ce9)	Jk1	VH1 J558	dsp2.8	JH4	A
26697 TG	ND		VH1 J558	dfl16.1	JH4	A
27534 TG	Vκ9/10 (bv9)	Jκ1	VH1 J558		JH2	A

Table 8. M167μk non-Tg PCT: endogenous Ig sequences (n=10). Endogenously expressed Vk and VH Ig genes from PCTs generated in BALB/c M167μk non-Tg (WT). VH families are classified on the basis of homology to reported germline genes as reported in the NCBI IgBLAST. Vk families are classified on basis of homologous germline gene segments as reported by Thiebe et al. (60). ND= not determined.

WT PCT (non-Tg)	Vk family	Jk	V_H family	D_H	J_H	Ig
40 WT	Vk19/28 (19-15)	Jk2	VH6 VH22.1	dsp2.8	JH3	M
50 WT	Vk11 (if11)	Jk2	VH6 VH22.1		JH3	A
154 WT	Vk12/13 (12-44)	Jk2	VH3 36-60		JH1	A
13 WT	Vk19/28 (19-15)	Jk1	VH2 Q52		JH4	A
35 WT	Vk9/10 (ba9)	Jk2	VH1 J558	dfl16.1,2	JH1	A
5 WT	Vk9/10 (ce9)	Jk1	VH9 VGK9	dsp2.8	JH2	A
109 WT	Vk4/5 (aq4)	Jk5	VH6 VH22.1		JH3	A
129 WT	ND		VH1 V130	dfl16.1	JH2	A
152 WT	Vk9/10 (ba9)	Jk5	VH Gal55.1	dfl16.1	JH4	A
160 WT	Vk4/5 (at4)	Jk5	VH1 J558.47	dfl16.1	JH1	A

Analysis of the endogenous VH sequences shows that the endogenous antibodies found in Tg PCTs do not use the V1 (S107/VH7) family genes known to form PC-binding antibodies (61). The exception is #114Tg PCT, which expresses an IgA heavy chain using

an endogenous V1 allele recombined with D_H and J_H genes (V1/DFL16.2/JH4-IgA). This VDJ gene rearrangement is different from the M167μ transgene (V1/DFL16.1/JH1).

Further, only three kappa light chains that pair with the V1 gene to form PC-binding antibodies: Vκ8, Vκ22, Vκ24. Further, Segal, et al. studied the antigenic binding site of the PC-binding myeloma M603, and reported that residue 96 (leucine) in the kappa light chain makes contact with the PC hapten (62). Jk5 is the only J region coding for a leucine at this position (49). All the Vk genes expressed in PC-binding myelomas and hybridomas have included the Jk5 gene segment (63). Only two of the Tg PCTs, #2Tg and #135Tg, utilize one of these three kappa genes and neither of these PCTs rearranged an endogenous V1 heavy chain gene. PCT #2Tg expresses a Vk8/Jk1 gene and #135Tg expresses a germline (unmutated) Vk24/Jk5 that was similar, but not identical, to the M167 Vk transgene. The endogenous kappa genes may be able to bind to the Tg V1 heavy chain to form a PC antibody. Finally, the PCT with an endogenous V1 heavy chain gene, #114Tg PCT, expresses an endogenous Vk21/Jk1 gene, which is not one of the three kappa genes involved in PC-binding antibodies. However, it is possible that the endogenous V1 gene expressed in the #114Tg PCT could pair with the Tg Vk24 gene to form a PC-binding antibody.

Because the majority of Tg PCTs are expressing endogenous VH genes that are not from the V1 family, they are unlikely to be contributing to the Tg tumors' specificity for PC antigen. This supports the evidence that the endogenous IgA antibodies are not contributing to the PC reactive antibodies secreted by Tg PCTs (as shown by IEP analysis, **Figure 22**). In all probability, the endogenously rearranged VH and Vk genes form antibodies that are specific for an antigen other than PC. In conclusion, the Tg PCTs are co-expressing

endogenous H and L chain genes, however, the PC-specificity of the Tg PCTs appear to be due solely to the M167μk transgenes.

Analysis of VH Family Usage in Endogenous Igs of M167μκ Tg PCTs

The endogenous heavy chains expressed in Tg and non-Tg primarily used variable region genes from the most commonly utilized V_H families, V_HJ558 (63%) and V_H36-60 (13%) as shown in **Table 9**. The presence of the M167μk Tg does not appear to dramatically skew the choice of VH genes found in Tg PCTs, as the non-Tg WT PCTs utilize a similar pattern of VH family

Table 9. VH Family Usage in C.M167μk Tg and non-Tg PCTs. VH families are classified on the basis of homology to germline genes as reported in the NCBI IgBLAST. C.B20 tumors analyzed were included as a control, and are reported later in the chapter. The most frequently utilized VH family from each of the three groups of PCTs in VH1/J558. The J558 VH family is the dominant group of V genes used in the Igs of the normal B cell repertoire in mice.

VH family	Tg			Non-Tg			C.B20	
VH1/J558	15/24	63%		3/10	30%		6/9	67%
VH2/Q52				1/10	10%		1/9	11%
VH3/36-60	3/24	13%		1/10	10%		2/9	22%
VH5/7183	2/24	8%						
VH6/J606	1/24	4%		3/10	30%			
VH7/S107	1/24	4%						
VH4/Gal55.1	1/24	4%		1/10	10%			
VH9/VGAM3.8				1/10	10%			
VH10	1/24	4%						

genes. Although the M167μk Tg PCTs show a heavier usage of J558 family VH genes (63%) than PCTs from non-Tg littermates (30%), a separate analysis of VH genes used in PCTs from C.B20 mice show an identical usage (67%) of J558VH as the Tg mice. A more accurate analysis of VH gene usage would include higher numbers of sequenced immunoglobulins from PCTs in each of the BALB/c WT and C.B20 control groups.

Tg M167μk RNA Remains Unmutated

In an attempt to learn something of the natural history of the tumors and their precursor B cells, we asked whether the M167-IgM transgene had undergone somatic hypermutation, a hallmark of germinal center activity within lymphoid tissues, in any of the tumors. As previously described, M167μ and k mRNA transcripts were detected by RT-PCR from total RNA preps of PCT tumor cells. Sequence analysis of the M167 mRNA transcripts revealed no evidence of somatic hypermutation in μ or k copies of the transgene in any of the PCTs from Tg mice (data not shown).

Published data suggest that potential SHM mechanism is somehow confined to the λ, H, and k chain gene complexes (chromosomes 6, 12, and 16) and is not global in the nucleus. Transcription of the M167μk Tg from chromosome 2/3 may be excluded from the SHM mechanism. Examples of PCTs with endogenous H and L chain genes that are highly mutated will be discussed later, whereas no mutations were found when sequencing Tg mRNA transcripts from any of the tumors. However, if somatic hypermutation did occur in

one or more copies of the M167 transgenes, it is likely not detectable among the mRNA transcripts produced from the multiple copies of heavy and light chain Tgs inserted in the genome. Other possibilities will be addressed in the discussion section. Therefore, to examine the mutational status of the PCT immunoglobulins generated in this study, the endogenous Ig genes were sequenced and studied rather than the M167μk transgenic Ig genes.

Analysis of SHM in Endogenous Ig Genes

Accumulation of mutations in Ig genes is a generally accepted indication that activated B cells have been exposed to somatic hypermutation (SHM) processes in germinal centers of lymphoid tissues. Pre- and post-germinal center B cells can be distinguished through lack of or presence of mutations in the V genes. A clustering of mutations in and around an Ig's hypervariable CDR regions suggests that antigen played a role in B cell selection. The mutational status of the Ig V gene in numerous B cell cancers, such as multiple myeloma (MM), follicular lymphoma (FL), and B cell chronic lymphocytic leukemia (B-CLL), is being recognized as an important factor in the clinical severity of the disease and has a significant impact on patient survival (64). In sequence studies of VH genes in human multiple myeloma, a pattern of extensive hypermutation is seen in neoplastic plasma cells (65). These findings suggest that the malignant plasma cell in humans arises from an antigen-selected B cell at a mature, post-germinal stage of B-cell development.

One factor considered critical in plasmacytoma development is the role of antigen in selecting precursor B cells, and therefore, I was interested in studying the Ig genes produced in the PCTs to see if there were variations from germline genes, particularly mutations

concentrated in the CDR hypervariable regions. Is there a difference in Ig mutational status between M167μk Tg and non-Tg PCT groups? With more complete databases of the murine genome now available from the NCBI GenBank and Celera, it becomes easier to compare Ig sequences from murine PCTs to germline genes.

NCBI has a collection of murine germline V genes that includes functional V genes and V genes with Open Reading Frame (ORF) for the heavy chain and the kappa and lambda light chains. Only one gene sequence is included for each V, D or J gene and, therefore, does not include polymorphism information. IgBLAST uses the collected germline genes to report the top three V, two D and J genes for a query sequence. Ig sequences from PCTs were searched against the Genbank Ig sequences database for information regarding germline gene polymorphisms and to other rearranged Ig sequences seeking to find the best homology.

Gene searches resulted in homologous sequence information, but Celera's murine genomic database has not been fully annotated. Polymorphism data was also not available. The kappa V genes reported by Thiebe *et al*. (60) in the NCBI IgBLAST germline database found perfect matches in the Celera genomic database. Germline information on the VH genes was not as consistent between the two databases as it was with the Vκ genes. This may have to do with the lack of a fully annotated VH locus. Information on germline VH genes was collectively considered from both the Celera and the NCBI databases for each Ig heavy chain sequence to determine the mutational status of a PCT Ig gene.

I sequenced endogenously rearranged heavy (VDJ) and light (VJ) chain variable regions from Tg M167μk plasma cell tumors. In order to compare V regions used in PCTs from M167μk Tg mice to those from normal BALB/c mice, I also sequenced and analyzed variable regions from PCTs generated in the non-Tg BALB/c littermates and C.B20 mice.

Vκ and VH Ig genes from nine PCTs generated in C.B20 mice were sequenced (**Table 10**) in order to utilize the C57BL/6 IgH locus that is found in these genetically susceptible BALB/c congenics for germline gene comparisons in the available databases. Concern about polymorphic differences between mouse strains that would hinder analysis of somatic mutation levels in the Ig genes prompted the inclusion of C.B20 tumors in SHM analysis. Most of the IgH sequences reported in the NCBI database were from other strains of mice than BALB/c, and were predominantly of C57BL/6 origin. A fairly comprehensive (but not

Table 10. CB20 PCT Ig sequences. Sequencing of endogenously expressed Vk and VH Ig genes from nine tumors induced in C.B20 mice were done in order to utilize the C57BL/6 IgH locus in a genetically susceptible BALB/c congenics. VH families are classified on the basis of homology to germline genes as reported in the NCBI IgBLAST. Vk families are classified on the basis of homologous germline gene segments as reported by Thiebe et al (60).

C.B20	Vk family	Jk	V_H family	D_H	J_H	Ig
5 CB20	Vk12/13 12-41	Jk4	VH1 J558	–	–	A
19 CB20	Vk33/34 gn33	Jk2	VH1 J558	dq52	2	A
23 CB20	Vk9/10 ce9	Jk1	VH1 J558		4	A
24 CB20	Vk1 bb1	Jk5	VH2 Q52		4	A
35 CB20	Vk1 bb1	Jk5	VH1 J558	dq52	2	A
6 CB20	Vk23 23-39	Jk1	VH5 7183.32b	dsp2.6	4	A
26 CB20	Vk4/5 af4	Jk2	VH5 7183.3b	dsp2.2	1	A
27 CB20	Vk23 23-48	Jk5	VH1 J558	dfl16.1	2	A
2 CB20	Vk19/28 19-15	Jk5	VH1 J558	dfl16.1	2	G2b

quite complete) set of germline genes from the largest VH family, J558 VH1, were sequenced from BALB/c mice by Brodeur (66) and added to NCBI Ig Sequence database in 2001.

The C.M167μk Tg and non-Tg (WT) tumors all utilized endogenously rearranged kappa light chains. In the Tg PCTs, the endogenous kappa genes were different from the M167 Vk24 transgene. Both Tg and WT PCTs primarily had isotype-switched IgA heavy chains, as shown previously by ELISA isotyping in **Table 6**. As described earlier, 5' degenerate V_H region primers for the FRW1 region were paired with primers in the IgA or 5' degenerate V_k region primers with a kappa 3' constant region primer to amplify V_H and V_k from tumor cDNA.

I observed that a small percentage of tumors in all three groups, C.M167μκ Tg, non-Tg (WT), and C.B20 PCTs, contained germline V_k and V_H genes, while the majority had mutated Vk and VH genes (**Table 11**). Vk germline genes were observed in 46% (11/24) of M167μk Tg PCTs, 30% (3/10) of WT littermates PCTs, and 33% (3/9) of C.B20 PCTs. Heavy chain V genes in the PCTs were compared to both Genbank and then Celera databases to identify germline genes with the best homology. In most cases, the best V_H gene homology could be found in the Celera database, although many of the sequences were located on unannotated scaffolds.

Table 11. Germline vs. Somatic Hypermutated Ig genes in PCTs. Germline (GL) or somatically hypermutated (SHM) immunoglobulin genes are identified in pristane-induced plasmacytomas from 3 groups of BALB/c mice. PCTs are considered germline tumors if both (V_H+V_L) Ig genes are rated GL. The numbers/ percentages of tumors that contained GL V_L genes and those that contained GL V_H are also notated.

Germline vs. Somatic Hypermutated Ig genes in PCTs

TG PCT #	GL or SHM		WT PCT# controls	GL or SHM		CB20 PCT# controls	GL or SHM	
	VJ_k	VDJ_H		VJ_k	VDJ_H		VJ_k	VDJ_H
2	SHM	SHM	5	SHM	SHM	2	SHM	SHM
37	SHM	SHM	13	GL	GL	5	SHM	SHM
44	GL	SHM	35	SHM	SHM	6	GL	GL
51	SHM	SHM	40	GL	GL	19	GL	SHM
53	GL	SHM	50	SHM	SHM	23	GL	GL
54	GL	SHM	109	GL	SHM	24	SHM	SHM
58	GL	GL	129	nd	SHM	26	SHM	SHM
61	GL	GL	152	SHM	SHM	27	SHM	SHM
62	SHM	SHM	154	SHM	SHM	35	SHM	SHM
64	GL	GL	160	SHM	SHM			
70	SHM	SHM						
114	GL	SHM						
123	SHM	SHM						
135	SHM	SHM						
149	GL	SHM						
150	nd	SHM						
151	SHM	SHM						
153	SHM	GL						
157	GL	GL						
162	GL	GL						
608	SHM	SHM						
618	GL	SHM						
26697	nd	SHM						
27534	SHM	SHM						
V_H+V_L: SHM	19/24	79%		SHM	8/10	80%	SHM	7/9	78%
V_H+V_L: GL	5/24	21%		GL	2/10	20%	GL	2/9	22%
GL V_H:	6/24	25%		GL	2/10	20%	GL	2/9	22%
GL V_L:	11/24	46%		GL	3/10	30%	GL	3/9	33%

One important consideration is that the Celera murine database is not the BALB/c genome, and therefore, some nucleotide differences are likely to be due to polymorphisms. V_H germline genes were observed in 25% (6/24) of M167μk Tg PCTs, 20% (2/10) of WT littermates PCTs, and 22% (2/9) of C.B20 PCTs.

Ig genes in PCTs were considered to be germline (GL) if both of the V_k and V_H genes had no mutations. In each of the three PCT groups (Tg, non-Tg WT, and C.B20), ~20% of the tumors turned out to be germline (**Table 11**). Five clear cases of germline sequences were seen: M167μκ Tg PCTs: 58Tg, 61Tg, 64Tg, 157Tg and 162Tg. Two of the non-Tg WT PCTs had germline Igs: 13WT and 40 WT. C.B20 PCTs also had two examples of germline tumors: 6CB20 and 23CB20. The remaining 80% of PCTs had somatically hypermutated Ig genes and, according to the commonly accepted concepts about SHM of B cell Igs, must have undergone TD processing in germinal centers. Mutated V_H and V_k genes of Ig proteins were observed in all 3 groups: 19/24 (79%) M167μkTg, 8/10 (80%) non-Tg, and 7/9 (78%) CB20. Interestingly, SHM of Ig genes in PCTs was frequently found in V_H chains only, and not the V_L chain. However, there was only one case where SHM was found solely in the V_L chain and not the V_H chain. Why the Ig heavy chain would be the primary target for SHM, and not the light chain, is not known.

The #114Tg PCT expresses an endogenous Ig-A gene from the anti-PC forming VH7/S107 family and is 98% homologous to the V1 germline gene. Three nucleotide mutations are found within CDR1 and CDR2 of the VH region of #114Tg, each causing an amino acid change. There are no mutations in the D_HFL16.1 or J_H4 regions of #114Tg. Numerous SHM antibodies are reported in the NCBI GenBank that also have a 98% homology to the VH region of the #114Tg IgA heavy chain, all of which are anti-PC and/or

anti-DNA antibodies shown to be produced from as little as a single mutation to change the anti-PC V1 heavy chain into a DNA binding antibody (67, 68 170:5095). The endogenous Vk gene is Vk21 (21-7)/Jk2, which has no known usage in anti-PC antibodies.

SHM Analysis of FRW & CDR From Endogenous IgH Chain Genes

SHM introduces point mutations, and more rarely deletions and insertions, into the V region of the Ig gene. In GCs or in extrafollicular sites, antigen-driven mutation occurs during the rapid division in the centroblast stage of plasma cell differentiation at particularly high rates, $\sim 1 \times 10^{-3}$ bp per cell division (69), the rate of mutation being a function of transcription efficiency. Mutations occur at random throughout the V region, but when amino acid substitutions occur, antigen specificity can be increased or decreased depending on how the antigen-binding site of the Ig is affected. The hypervariable complimentary determining regions (CDRs) of the Ig gene have the greatest affect on an Ig's ability to bind antigen, and therefore, amino acid substitutions tend to cluster in and around the CDRs if antigen selection is occurring through the affinity maturation process. The heavy chain and light chain each have 3 such regions (CDR1, CDR2, and CDR3). CDR3 includes the joining (J) gene in light chains and the diversity and joining (DJ) genes in heavy chains.

Figure 25 provides a closer look at the locations of SHM point mutations in the Ig VH regions and whether amino acid changes occurred in PCTs from M167μk Tg and non-Tg WT mice. Each red "**i**" along a gene indicates a mutation resulting in an amino acid substitution, whereas the blue "**I**" indicates a silent mutation. Most of the mutations occurring throughout the VH sequences resulted in an amino acid change, and many of the Igs show a clear clustering of mutations around the CDRs. Some clear examples of this

include 2, 44, 62, 149, 114, and 26697 in M167μk Tg PCTs and 5, 35, 152, and 160 in non-Tg (WT) PCTs. Because clustering of mutations is seen around the CDRs, antigen-driven selection does appear to play a role in the mutation of the Ig genes in plasma cell tumors.

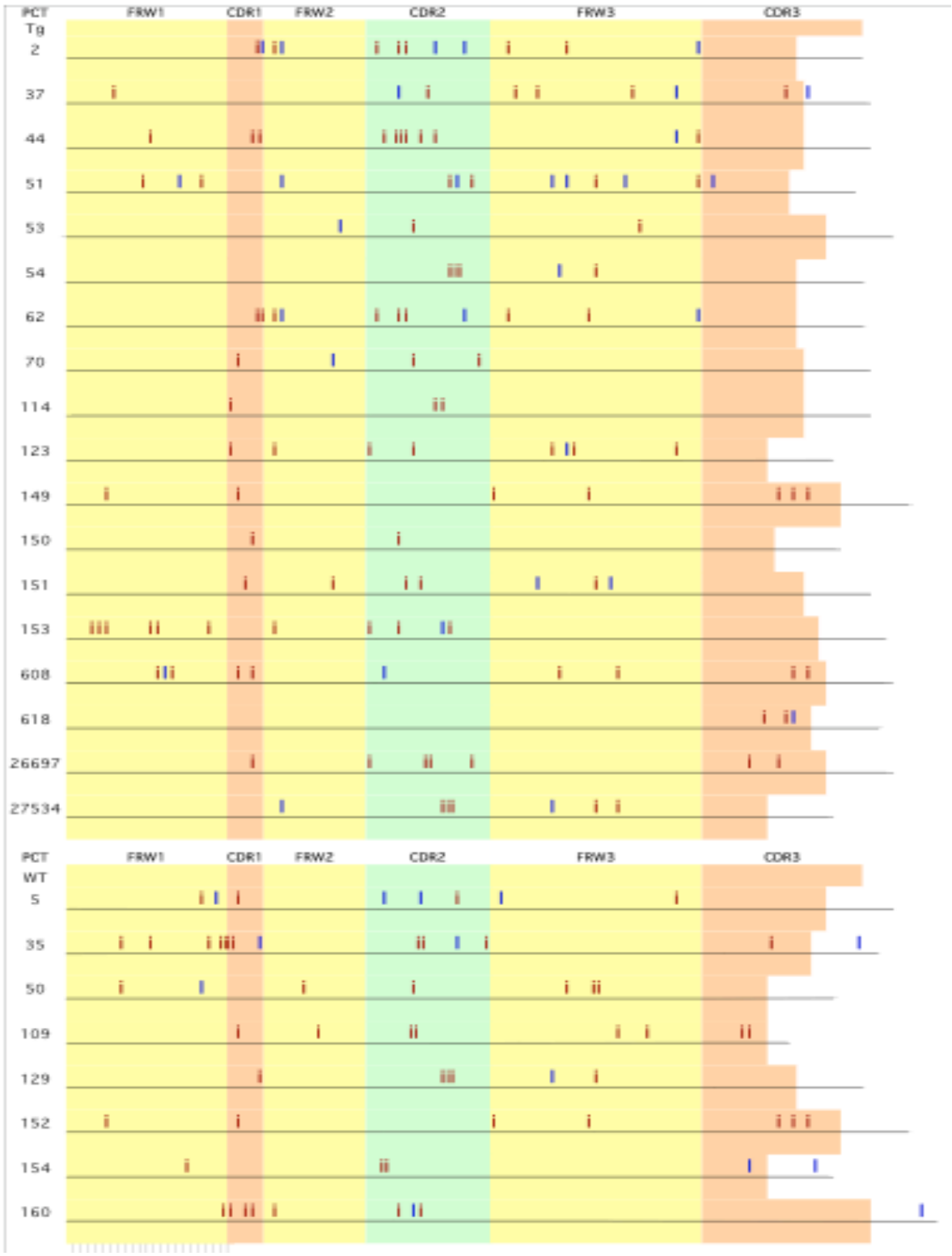


Figure 25. Somatic hypermutation in endogenous Ig heavy chain genes rearranged in PCTs from BALB/c M167μκ Tg and non-Tg (WT) mice. The 18 M167μk Tg PCTs and 8 non-Tg PCTs with somatic hypermutation are marked showing their mutation sites. The

framework regions (FWR 1, 2, 3) are shown in yellow, while the three hypervariable complimentary determining regions (CDR 1, 2, 3) are shown in color and the location of each nucleotide mutation is marked. Grouping of nucleic/amino acid changes that occur in the hypervariable CDRs are a sign of antigen-driven mutation. The red (i) denotes a nucleotide mutation that resulted in an amino acid change; the blue (I) marks a silent nucleotide mutation.

Analysis of CDR3 Regions

The CDR3 region of the VH gene plays a large role in the antigen binding region of the Ig protein and can reflect large differences in size as well as the amino acid composition among antibodies. **Table 12** shows the VH CDR3 regions of the Igs from PCTs of all three groups of BALB/c mice: M167μk Tg, non-Tg WT littermates, and C.B20 mice. In order to prevent bias in length measurements, CDR3 lengths started at the first residue following the invariant cysteine (C), which is coded by the VH gene, to the last residue before the invariant tryptophan (W) that is common to all 4 JH segments. CDR3 nucleotides are grouped into their gene segments (VH FRW3, DH, and JH), allowing identification of the intervening N nucleotides that could not be attributed to the V, D, or J gene segments.

Table 12. CDR3 regions of Igs from M167μk Tg, non-Tg and C.B20 PCTs. To prevent bias in length measurements, CDR3 lengths started at the first residue following the invariant cysteine (underlined, black) coded by the VH gene to the last residue before the invariant tryptophan that is common to all 4 JH segments. This means of calculation is based on that reported by Klonowski (70). A minimum of 4 homologous and contiguous nucleotides was required to assign a germline D segment.

M167μκ Tg PCT

		FW3		DH		JH3	Average: 11.6 ± 0.7	AA
61 TG	IgA	TGTGC		TAGTAGG	GGGGGGTT	CTGGGGT	CASRGGFWG	6
123 TG	IgA	TGTGCAAGA	GGGGG			GGACTACTGGGGT	CARGGDYWG	6
27534 TG	IgA	TGTGCGTCA	CGGGG	CGGCTAC		TGGGGC	CASRGGYWG	6
150 TG	IgA	TGTGCGAG	G			GCCTGGTTTGCTTACTGGGGC	CARAWFAYWG	7
51 TG	IgA	TGTGCCAGA	G	ATTACTACGGCTAC		GACTACTGGGGC	CARDYYGYDYWG	9
2 TG	IgA	TGTGCAAGA	G	ATAGTAACTAC	GT	CTTTGACTACTGGGGC	CARDSNYVFDYWG	10
54 TG	IgA	TGTGCTAGA	TTCCC	CAACTGGG	CC	TTTGCTTACTGGGGC	CARFPNWAFAYWG	10
135 TG	IgA	TGTGCTA	GATC	CTATGATTACGACC		TTGACTACTGGGGC	CARSYDYDLDYWG	10
37 TG	IgA	TGTGCAAGA	GA	TGGGGGTAAGAGA	G	ACTTTGACTCCTGGGGC	CARDGGKRDFDSWG	11
44 TG	IgA	TGTGCAAGA		TATGG	GTAC	TACTATGCTATGGACTACTGGGGT	CARYGYYYAMDYWG	11
70 TG	IgA	TGTGCAAGA	AGGG	GATTA	ACTGGC	TGGTTTGCTTACTGGGGC	CARRGLTGWFAYWG	11
114 TG	IgA	TGTGCAAGA	GATG	ACTACGGC	C	ATGCTATGGACTACTGGGGT	CARDDYGHAMDYWG	11
153 TG	IgA	TGTGCTAGA	G	ACTACGGTAG	AC	ATTACTATGCTATGGACTACTGGGGT	CARDYGRHYYAMDYWG	13
157 TG	IgA	TGTGCAAGA	AGGGGAGGGG	ATGATTACGAC	G	GGTTTGCTTACTGGGGC	CARRGGDDYDGFAYWG	13
53 TG	IgA	TGTACCAGG	A	ACTATGATTACG	TGGGAG	ACTATGCTATGGACTACTGGG	CTRNYDYVGDYAMDVWG	14
608 TG	IgA	TGTGCAAGA	ACGAG	GAATGGTTACTAC	CGG	TATGCTTTGGACCACTGGGGT	CARTRNGYYRYALDHWG	14
618 TG	IgA	TGTGCAAGA	GG	GTATGGTAACTAC	G	ATGCTATGGAATATTGGGGT	CARGYGNYDAMEYWGWG	14
26697 TG	IgA	TGTGCAAGA	TCTGGA	TACTTCGGC	CTT	TACTTTGCTATGGACTACTGGG	CARSGYFGLYFAMDYWG	14
58 TG	IgA	TGTGCAAGA	TCGAGA	ATGATTAC	AGGTTTT	TACTATGCTATGGACTACTGGGGT	CARSRMITGFYYAMDYWG	15
149 TG	IgA	TGTGCAAGA	CCGGGGGT	TTACTACGGTAGTCGC	C	ACTCTGCTCTGGACTACTGGGGT	CARPGVYYGSRHSALDYWG	16
64 TG	IgA	TGTGCAAGA	GAGGGGTA	CTACTATGGTAACTAC	G	ATTACTATGCTATGGACTACTGGGGT	CAREGYYYGNYDYYAMDYWG	17
162 TG	IgA	TGTGCAAGA	AGGGG	GGGTACGAC	GGACTTC	TTGCTTACTGGGGCCAAGGGACTCTGGTCA	CARRGGTTDFLLTGAKGLWS	17

Non-Tg WT PCT

		FW3		DH		JH3	Average: 10.4 ± 1.4	AA
40 WT	IgM	TGTACCAGGCCT		TATGGTAAC		TAC	CTRPYGNYWG	7
50 WT	IgA	TGTACCA	CTG			GGTTTGCTTACTGGGGC	CTTGFAYWG	6
109 WT	IgA	TGTAC	CACGG			GGTTTCCTCACTGGGGC	CTTGFPHWG	6
154 WT	IgA	TGTGCAA	AGG			ACTTCGATGTTTGGGGC	CAKDFDVWG	6
130 WT	IgA	TGTGCAAG	G			GCCTGGTTTGCTTACTGGGGC	CARAWFAYWG	7
13 WT	IgA	TGTGTAA	TCGACC			ATTACTATGCTATGGACTACTGGGGT	CVIDHYYAMDYWG	10
129 WT	IgA	TGTGCTAGA	GGGG	ACTACGGTAGT	GGGGG	CTACTGGGGC	CARGDYGSGGYWG	10
35 WT	IgA	TGTGCAAGA	TTCGA	CTACGG	AA	ACTGGTGCTTCGATGTCTGGGGC	CARFDYGNWCFDVWG	12
5 WT	IgA	TGTACAAGACA	GAATAA	TAACTAC		TTTGGTTTGACTACTGGGGC	CTRQNNNYGGVWFDYWG	14
152 WT	IgA	TGTGCAAGA	CCGGGGGT	TTACTACGGTAGTCGC	C	ACTCTGCTCTGGACTACTGGGGT	CARPGVYYGSRHSALDYWG	16
160WT	IgA	TGTGCAAGA	AGAACGGCGTCCGTTTGGGT	TACGGTAGTAG	ACAGGA	ACTGGTACTTCGATGTCTGGGGC	CARRTASVWVTVVDRNWYFDVWG	20

C.B20 PCT

		FW3		DH		JH3	Average : 12.4 ± 0.9	AA
5 C.B20	IgA	TGTGCAAGAT						
19 C.B20	IgA	TGTGCAAA		AACTGGGAC	GTCC	TATTTTGACTACTGGGGC	CAKTGTSYFDYWG	10
23 C.B20	IgA	TGTACTA	CAGAATACCG			CTATGCTATGGACTACTGGGGT	CTTEYRYAMDYWG	10
24 C.B20	IgA	TGTGCCAA	ACGGGGAGGTGGCTCTGTTC			TGGACTACTGGGGT	CAKRGGGSVLDYWG	10
35 C.B20	IgA	TGTGCAAA		AACTGGGAC	GTCC	TATTTTGACTACTGGGGC	CAKTGTSYFDYWG	10
6 C.B20	IgA	TGTGCAAGA	GAGG	ATGGTTACGAC	GGA	TATGCTATGGACTACTGGGGT	CAREDGYDGYAMDYWG	13
26 C.B20	IgA	TGTGCAAGG	CT	CTATGATTACGAC	GGT	TGGTACTTCGATGTCTGGGGC	CARLYDYDGWYFDVWG	13
2 C.B20	IgG2b	TGTGCAAGA	CGGGAACACAACGATGATA	ACTACGGT		TACTTCGACTACTGGGGC	CARREHNDDNYGYFDYWG	15
27 C.B20	IgA	TGTACAAG	GAGTTCGCT	TTACTACGATAGTAGC	CCCGTGAT	CTTTGACCACTGGGGC	CTRSSLYYDSSPVIFKHWG	16

The amino acid lengths of the CDR3 regions vary greatly in the Igs secreted by PCTs from M167μk Tg and non-Tg mice, ranging from 6 –20 amino acids (aa) (**Table 12**). Interestingly, the C.B20 PCTs had a smaller range of from 10 – 16 aa. However, the overall mean length does not differ between groups derived from the M167μκ transgenic mice and the WT control groups. Also of note, N additions are found between the junctions of the VDJ genes in most of the CDR3 regions in all three groups of PCTs. During neonatal development, N-region diversification of the H chain (71) appears to play little role in the generation of antibodies and are rarely found in natural IgM antibodies. Later in B cell development, the presence of terminal deoxynuceotidyl (Tdt) helps to create antibody diversity by the addition of N nucleotides in the CDR3 during Ig rearrangements (72).

New studies in mice show that in addition to recruitment from a more restricted repertoire of naïve B cells, a portion of MZ B cells are selected from the pool of recirculating and memory B cells that have undergone antigen affinity maturation in GCs (14). The hypermutated MZ B cells that are responsive to TI-2 antigens adopt the MZ B cell phenotype. Recruitment of memory B cells into the MZ would have the benefit of expanding the B cell repertoire against a wider range of TI bacterial polysaccharide antigens, as these cells would have developed in the presence of Tdt that generates Ig heavy chain diversity in the V-region during rearrangement. Interestingly, the only tumor that did not include N additions was #40 from a non-Tg WT PCT and was the only IgM-secreting tumor.

The fact that most of the Ig genes from the three groups of BALB/c PCTs, including the Tg tumors, have N additions and are somatically hypermutated seem to point away from the natural antibody-producing B cells produced early in ontogeny as precursors of pristane-induced PCTs in mice, and toward an Ag-selected B cell population. This Ag-driven B cell

precursors of PCTs may potentially be from the MZ (or B1) B cell subset. It is still unclear how to differentiate from which B cell subset a PCT originated.

I conclude that murine plasmacytoma development involves post-antigen selected B cells, where 80% of Igs are somatically hypermutated in both M167μk Tg and non-Tg mice. In most cases, the point mutations cause amino acid changes around the hypervariable CDR regions, a further indication of antigen selection. The 20% of PCTs from M167μk Tg and non-Tg mice with germline VH/Vk genes might be generated either from early GC reactions, where little to no SHM is seen, or from the natural antibody producing B cells from the B1 or MZ subsets. Clear examples of both endogenously rearranged germline and somatically hypermutated Ig proteins are seen in all three BALB/c congenic groups of PCTs (M167μkTg BALB/C, non-Tg WT BALB/c, and C.B20 mice). The M167μkTg does not appear to influence the occurrence of hypermutation occurring in these PCTs, as the distribution of germline and mutated Ig genes PCTs (20% GL and 80% SHM) is the same as in non-Tg BALB/c and C.B20 derived-PCTs.

Chapter 4 Conclusions:

- Both M167μk Tg and endogenous Ig proteins are expressed in the PCTs generated from C.M167μk Tg mice.
- Surprisingly, a large monoclonal (M) component of the M167μk protein was not found in Tg PCTs.
- The M167μk protein was not clearly demonstrable by electrophoresis (Figure 18), but was specifically detected by ELISA (Figure **20**).
- The presence of the monoclonal (M) components of varying mobilities on the SPE gels indicated that many PCTs were secreting Igs electrophoretically different than the ones controlled by the transgenes.
- The predominant electrophoretic M components in sera from M167μk Tg PCTs were in fact Ig derived from endogenously rearranged genes.
- The majority of M component generated by endogenously rearranged immunoglobulin genes (demonstrated by ELISA) was IgA (**Table 6**).
- Immunohistochemical staining of tumor cells from #58 Tg PCT gave a clear example of a Tg PCT that produces intracellular Ig not only with the Tg M167-id, but expresses both IgM (Tg) and IgA (endogenous) antibodies (**Figure 21**).
- Immunoelectrophoresis (IEP) on tumor sera (**Figure 22**) provided evidence that the endogenous IgA antibodies are not specific for PC antigen.

Nucleic acid studies

- Confirmed mRNA expression of both the M167μ and M167k transgenes in PCTs derived from M167μk Tg mice
- M167μ and k Tg transcripts showed no evidence of somatic hypermutation.
- Confirmed mRNA expression of endogenous Ig genes in PCTs derived from M167μk Tg mice
- VH family usage in endogenous Igs expressed in Tg PCTs is not related to VH genes used in PC-binding antibodies.
- Endogenously rearranged Ig genes in PCTs from C.M167μk Tg mice are isotype switched to IgA.
- Of the PCTs from M167μk Tg mice, 80% have somatically hypermutated endogenous Ig, while the other 20% express unmutated germline gene VH/Vk regions.
- The IgH CDR3 lengths varied greatly among the M167μκ Tg PCTs, from 6 – 17 aa, with a mean of 11.6 ± 0.7 aa. All contained N additions of varying lengths.
- The control group of PCTs derived from non-Tg littermates also showed an 80% to 20% ratio of somatic hypermutation to germline gene usage in the VH/Vk regions of pristane induced tumors.
- Another control study on PCTs derived from C.B20 mice, BALB/c mice that have a C57BL/6 Ig heavy chain locus, also had 80% hypermutated to 20% germline VH/Vk regions.

- Therefore, I conclude that the presence of M167μk Tg does not influence the somatic hypermutation status of the endogenously rearranged Ig genes, but is refractory to SHM itself.

CHAPTER 5

Functional Properties of The M167μk Tg BCR

Increased plasma cell tumor incidence in M167μk Tg mice suggested that the Tg PC-specific B cell receptor (and other such BCRs with critical Ag specificities in normal mice) plays an important role in inducing and accelerating plasma cell tumor development in the pristane plasmacytoma model. Accumulation of oncogenic mutations is potentially greater in B cells that (1) are able to survive longer and (2) are actively dividing. Naïve B cell populations are kept alive through ligand-independent activation (tonic signaling) of their BCRs (73, 74). The increased number of B cells capable of responding to a plentiful supply of environmentally available PC antigens could be an important factor in the increased incidence of plasma cell tumors in C.M167μk Tg mice. If PC antigen is a major source of B cell activation in the M167μk Tg pristane-induction model of plasma cell tumor development, the M167μk BCR must be functionally able to respond to PC stimuli. A functional PC-specific BCR would bind PC antigen on a macromolecule and activate a signal transduction pathway that maintains B cell viability, triggers proliferation, stimulates anti-PC antibody secretion in response to autologous and exogenous PC antigens, and triggers differentiation of B-lymphocytes into Ig secreting plasma cells.

What are the sources of PC antigen in the mouse? Storb *et al.* (47) proposed that environmental antigen is responsible for M167μk Tg B cell activation and the secretion of naturally high levels of Tg antibody in the serum. In support of this hypothesis, Kenny

et al. used M167μ-only and M167k-only Tg mice to provide evidence that M167 (H+L) Tg B cells are selected and expanded *in vivo* through encounter with antigen – either autologous or environmental PC – through the Tg antigen-specific receptor (49).

The PC haptenic group is found on commensal bacteria that colonize the mouse intestinal tract. PC is also found on apoptotic host cells and on oxidized lipoproteins (37, 75, 76) produced under conditions of oxidative stress (such as the chronic inflammation existing in the pristane treated mouse.) These sources of PC antigen have recently been shown to bind certain anti-PC antibodies (T15, M167, M603, and others) *in vivo* (37). Therefore, PC is an important environmental- and self-antigen. Two sources of PC antigen that could stimulate B cells and drive PCT development in the pristane induction model are 1) the pristane produced oil granuloma inflammatory tissue, which is known to contain apoptotic cells and to increase oxidation through phagocytosis (77) and 2) normal gut flora.

I. Immunization Studies

One test for functionality of the M167μk Tg BCR on B cells of C.M167μk Tg mice would be PC-specific antibody secretion in response to PC antigens. *In vivo* immunization with different kinds of PC antigenic macromolecules (such as break-down products of cell membranes, PC-conjugated foreign proteins and polysaccharides, or PC-containing whole bacteria) and monitoring primary and secondary antibody levels would show whether M167μk Tg B cells are capable of responding to PC antigen.

Previous immunization studies with T-cell dependent PC-KLH (48) provided evidence that the M167μk Tg B cells are able to produce a strong immune response to PC

antigen after a secondary antigen boost, as determined by detecting secreted Tg+ IgM PC-antibodies. These immunization studies indicate that the Tg M167μk BCR is functional in C57/B6 mice. I have extended these PC immunization studies to include the conventionally raised BALB/c M167μk Tg mice used in the pristane model system in order to show that M167μk Tg B cells can be stimulated by PC-specific antigen to 1) secrete PC-specific antibodies and 2) to proliferate. *In vivo* immunizations were carried out using various PC antigens and serum anti-PC antibody was measured to monitor immune responses in these mice. Pristane, which induces chronic inflammation and increased production of oxidants during phagocytosis of oil droplets, was also studied as a potential immunogen that might generate a specific PC immune response by increasing formation of auto-immunological materials such as oxidized lipids and apoptotic cells.

Further experiments measured M167μk Tg BCR activation through *in vitro* cell proliferation in response to various PC antigens and to antibodies that specifically cross-linked M167-id Tg BCR.

Anti-PC Immune Response to Pristane

Recent studies have demonstrated the presence of PC-containing phospholipids in apoptotic cells (38) and anti-PC antibodies can bind to determinants on thymoctyes undergoing dexamethasone-induced apoptosis (37). Different subgroups of PC antibodies bind various stages of these apoptosing cells. Group I (natural PC Abs) bind PC-determinants on cells in the early stages of apoptosis, whereas group II (mutated PC-Abs) recognize late stage apoptotic cells. Shaw *et al.* (37) also demonstrated the

autoreactivity of anti-PC antibodies for PC determinants found on atherosclerotic lesions, which involve chronic inflammation and lipid abnormalities. The T15 set of natural Abs, which are derived solely from B1 cells (43) and are the dominant anti-PC clone (78-84) in BALB/c mice as well as in M167μΔmem Tg mice unable to insert the M167μ chain in the B cell membrane (49), recognizes not only microbial antigens, but also oxidatively altered lipoproteins (produced in inflammatory environments under oxidative stress) and apoptotic cells (37, 75, 76). Of specific interest among the group I antibodies was the M167-IgA monoclonal antibody shown to bind to atherosclerotic inflammatory lesions and apoptotic thymoctyes (37).

Injection of pristane leads to the development of oil granuloma, a chronic inflammatory tissue in the peritoneal cavity. The OG contains reactive, cytokine-secreting macrophages and neutrophils that generate tissue-damaging oxygen radical molecules. Similar to the inflammatory atherosclerotic lesions mentioned above, PC antigen may also be present in OG tissue created by the presence of pristane in the peritoneal cavity, and thus may be a source of PC antigen for the transgenic M167 B cell receptor.

Because pristane may be creating a source of PC antigen in oil granuloma that stimulates B cell precursors of plasmacytomas, anti-PC sera titers were monitored in pristane "immunized" mice in order to determine if an anti-PC immune response is generated as a result of i.p. pristane injection. Anti-PC sera titers were monitored in C.M167μk Tg and non-Tg littermate mice injected with 0.2 ml pristane, the initial dose administered in plasmacytoma studies. A prebleed (PB) for each mouse was taken on day 0, before i.p. injection of 0.2 ml pristane. Sera were collected for time points

spanning the primary (day 0) and secondary (day 60) doses of pristane given in a plasmacytoma study. PC-binding antibody titers were analyzed by PC-ELISA for days 10, 20, 35, and 50 and were reported as the OD (450 nm) reading for 1/1600 sera dilution (**Figure 26**). No changes in anti-PC titers in either group were seen within the first 60 days after the injection of pristane to indicate a chronic stimulation of anti-PC B cells. Several injections of the non-metabolizable pristane may be necessary, providing a cumulative affect of PC-antigen generated by the inflammatory tissue, before a PC immune response in detectable. However, sera tested at day 150, after all three injections of pristane were administered, still did not generate a change in anti-PC sera titers over prebleed (day 0) controls in M167 Tg or non-Tg mice (data not shown). However, plasmacytomas can be drastically accelerated by use of the Abelson murine leukemia virus (85) or the $Bcl_{X\,L}$ anti-apoptotic transgene in BALB/c pristane-treated mice ($Bcl_{X\,L}$ x C.M167μk Tg F1 mice, **Figure 40** in appendix). This evidence suggests that the influence of antigen stimulation on B cells driving proliferation and differentiation would probably occur in the early stages of pristane induction.

PC antigen may still be provided by apoptotic cells and/or oxidatively modified LDLs in the OG, but it clearly does not generate a systemic anti-PC immune response. Rather, the main function of the OG in plasmacytoma-genesis is probably to provide a persistent inflammatory environment rich in oxidative stress and growth factors that nurtures PCT development, instead of generating a primary source of antigen. Thus, exogenous sources of PC - the environmental antigens from microorganisms of the natural gut flora, diet, and bedding - is probably the principal stimulus of B-cells in C.M167μk Tg mice.

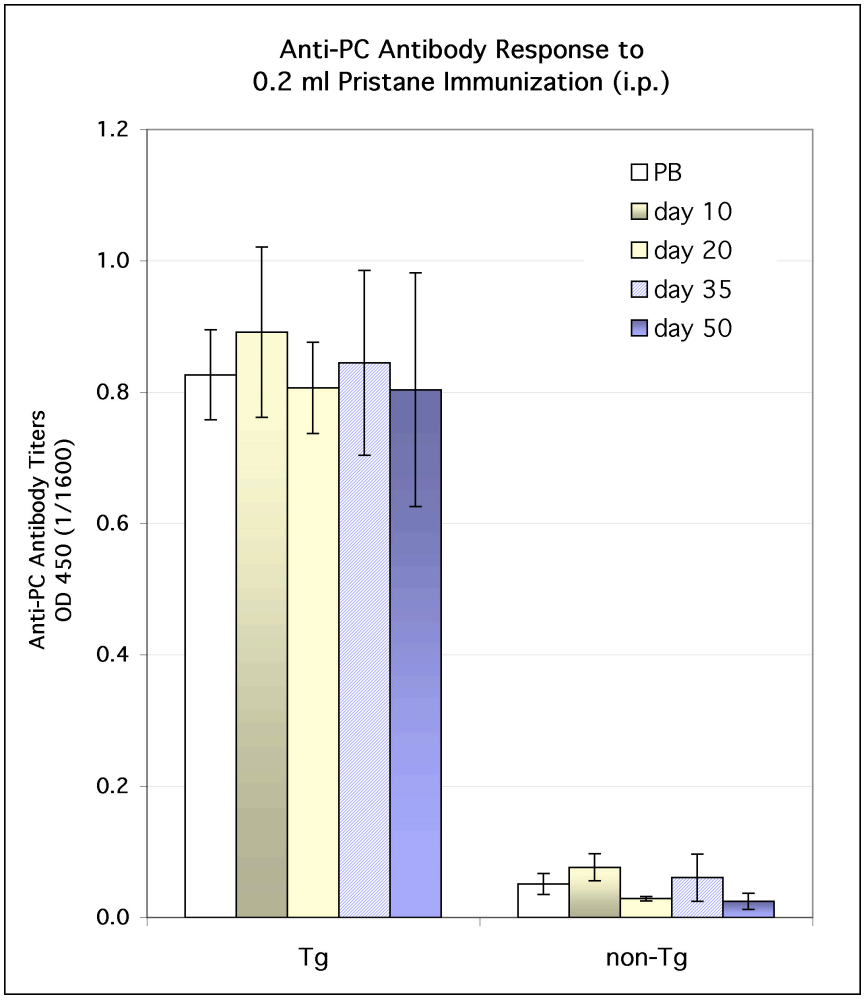


Figure 26. Anti-PC antibodies in response to pristane. One injection of pristane (0.2 ml, injected i.p. on day 0) produced no increase in the anti-PC immune response within the first 50 days of a pristane induction in either Tg+ or non-Tg BALB/c M167μk mice. Each mouse was bled on day 0 (PB), 10, 20, 35 and 50. Sera titers are reported at 1/1600 dilution. Values represent the arithmetic mean ± SEM of n=4-5 mice.

Immune Responses to PC-KLH (TD Ag) and PnC (TI Ag) Were Poor

Initial *in vivo* immunization experiments with PC antigen (described below) included soluble 1) a PC-conjugated hemocyanin protein carrier (PC-Hy) that induces a T-cell dependent (TD) immune response, and 2) PnC, a PC-containing bacterial cell wall polysaccharide that is a T-cell independent (TI) antigen. For an explanation of T-cell dependent and independent antigens and immune responses, see Appendix (page 180). However, results from intraperitoneal (i.p.) immunization in M167μk Tg mice were surprising in that neither of these PC antigens produced the expected high levels of anti-PC antibody immune response in Tg mice, although large anti-PC responses were seen in non-Tg control mice.

PC-Hemocyanin (Protein) in CFA

As previously mentioned, Kenny, et al (48) showed that intraperitoneal (i.p.) immunization of M167μκ Tg mice with 200 μg PC-KLH in CFA on day 0 and 14 produced a T-cell dependent immune response (at day 21) as indicated by increased levels of anti-PC IgM antibodies in the serum. Kenny measured serum antibody by capturing PC antibodies from serum on PC-BSA coated plates and using secondary antibodies to detect IgM, IgG, V_H1-id, or T15-id. His results showed an increase in PC-antibodies using IgM (1586 μg/μl) and V_H1-id (>1000 μg/μl), but very little IgG (19 μg/μl) and no T15-id (<1 μg/μl).

Similar immunization experiments were performed in our conventionally raised C.M167μk Tg mice using PC-conjugated hemocyanin (PC-Hy) in order to show the PC-specific M167 Tg BCR is functional in mice used for plasmacytoma studies. The

hemocyanin protein carrier used in the following experiments is slightly different, as Kenny used keyhole limpet hemocyanin (KLH) and in the studies described here, horseshoe crab hemocyanin (Hy) was used. C.M167μκ Tg and non-Tg littermate mice were i.p. immunized at 12 - 20 weeks of age with PC-Hy/CFA (100μg or 200μg) or PBS/CFA on day 0 and again on day 14, or were left completely untreated (naïve, unimmunized mice). Day 7 (primary response) and day 21 (secondary response) sera were collected and analyzed by ELISA for total anti-PC specific antibodies. Total anti-PC antibodies (instead of specific IgM or IgG antibodies) were detected using a goat anti-mouse Ig(H+L) antibody.

In both groups of Tg and non-Tg mice, the sera from injected PBS/CFA control mice had identical amounts of anti-PC antibody as sera from unimmunized, naïve mice (**Figure 27**). The naïve and PBS-treated Tg mice have significantly higher amounts of PC-specific antibodies (3-4x more) than their non-Tg littermates. However, an attempt to induce a robust T cell dependent immune response in M167μκ Tg mice with PC-Hy antigen was only moderately successful, as shown in **Figure 27**. OD readings for anti-PC antibodies are reported at 1/160 sera dilution. Immunization of C.M167μκ Tg mice with 100 μg PC-Hy/CFA generated no anti-PC Ab response at all. However, a moderate increase in anti-PC Abs is seen with 200 μg PC-Hy/CFA in Tg mice, and the secondary immune response is no higher than the original primary response. In comparison, the non-Tg littermates responded with large increases of anti-PC Abs, and had even higher levels during the secondary response.

PC-specific Antibody Titers after PC-Hy/CFA Immunization

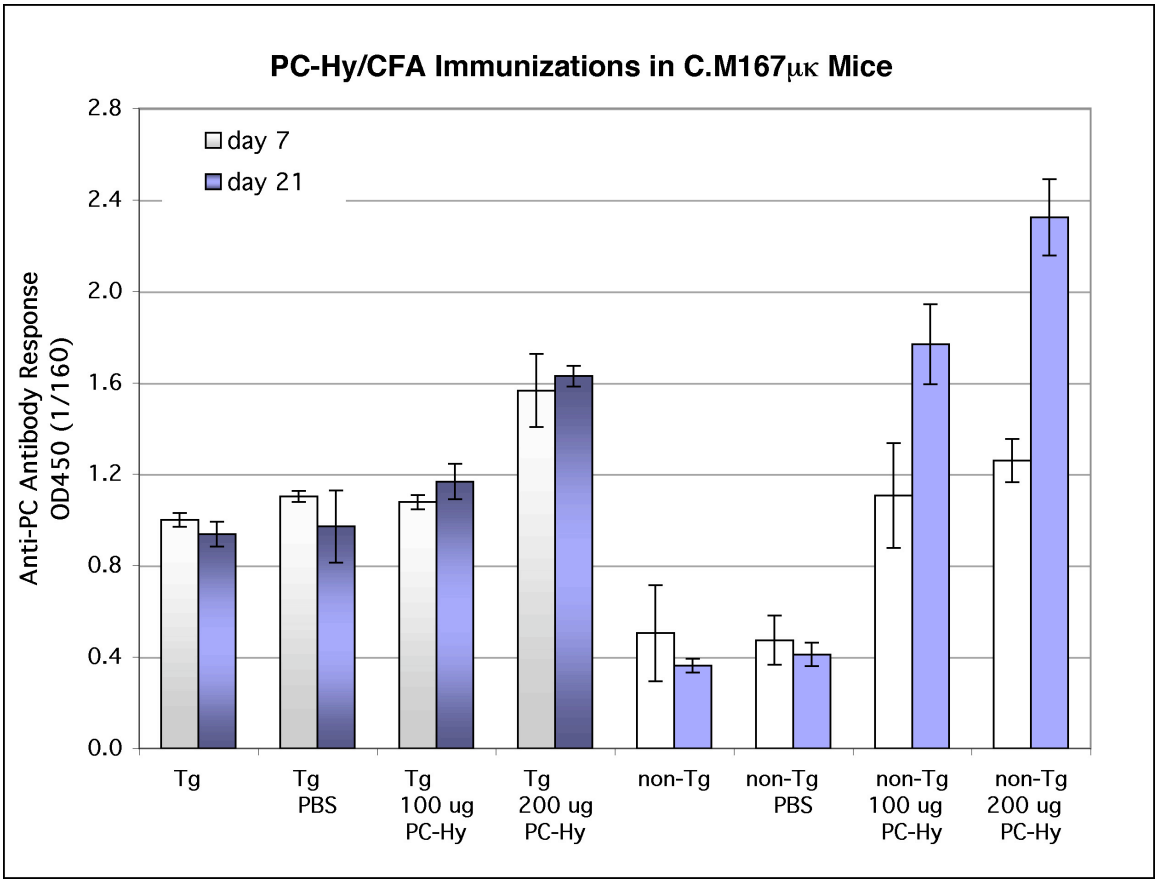


Figure 27. Anti-PC Antibody Responses to PC-Hy/CFA. C.M167μk Tg and non-Tg mice were injected i.p. with either, PBS/CFA, 100 μg, or 200 μg PC-Hy emulsified in CFA adjuvant, or were left immunized (naive). On day 14 after initial immunization, mice were given a second injection of PC-Hy or PBS in CFA. Sera were collected on both day 7 and day 21 and anti-PC Ab titers were assayed by PC-ELISA. Transgenic naive (Tg) and PBS control (Tg PBS) mice have a significantly higher amount of anti-PC antibody compared with naive (non-Tg) and PBS-treated (non-Tg PBS) littermates. In C.M167μk Tg mice, anti-PC Ab titers were not increased with immunization using 100 μg PC-Hy. Tg mice responded to 200 μg PC-Hy/CFA at day 7 and 21, but no significant differences were seen between the primary (day 7) and secondary responses (day 21). Non-Tg littermate mice showed a much greater anti-PC immune response to both concentrations of PC-Hy antigen at both 7 and 21 days. The non-Tg mice had an initial primary anti-PC Ab response and anti-PC Ab titers were further boosted in the secondary immune response, as expected in a T cell dependent response. Values represent the arithmetic mean ± SEM of n= 3-5 mice/group.

The same data is presented in an alternate form in Table 13 as anti-PC Abs titers for the PC-Hy/CFA immunization experiment, emphasizing a moderate response to TD PC-antigen in Tg mice compared to the prolific anti-PC immune response in the non-Tg littermates. There was a small, 2 fold immune response in Tg mice with 200 μg PC-Hy/CFA, although no further increase was seen between day 7 and day 21, after mice were boosted a second time with PC-Hy antigen. Non-Tg littermate mice responded to PC-Hy antigen with an 8-16 fold (day 7) and >64 fold (day 21) increase in anti-PC Ab sera titers for both concentrations of PC-Hy.

Table 13. Serum titers for the T-cell dependent PC-Hy/CFA immunization study. Reported is the lowest titer that had an OD > 0.25 (at 450nm). The fold differences are noted in parenthesis. The anti-PC antibody day 0 (prebleed) titers of Tg mice are 4x higher than non-Tg day 0 titers. A 2x difference is seen in Tg mice between day 0 and day7 or 21 given 200 mg of PC-Hy/IFA. Non-Tg mice responded with 8x to >64x increases in PC Ab titers given PC-Hy/IFA.

Serum Titers for PC-Hy/CFA Immunization Study

Mice	Day 0	Day 7	Day 21
Tg+ (100μg/PC-Hy)	1/320	1/320	1/320
Tg+ (200μg/PC-Hy)	1/320	1/640 (2x)	1/640 (2x)
Non-Tg (100μg/PC-Hy)	1/80	1/640 (8x)	1/5120 (64x)
Non-Tg (200μg/PC-Hy)	1/80	1/280 (16x)	> 1/5120 (>64x)

Kenny (48) reported large increases in total anti-PC antibody in Tg mice [B6.CBA/N x M167Tg F1 females] from day 0 (46 μg/ml) to day 21 (1605 μg/ml) after immunization with 200 μg PC-KLH in CFA, whereas the non-Tg littermates had increases in anti-PC titers from day 0 (<1 μg/ml) to day 21 (3961 μg/ml), both significant increases in PC-antibody titers. Compared to these previously reported immune responses, the anti-PC antibody responses seen in BALB/c.M167μk Tg study mice were lower than expected. Therefore, these low responses are a cause of concern in my study that the mice are not responding to PC-antigen as expected.

PnC (Polysaccharide) Produces no Anti-PC Ab Response in Tg mice

A second immunization with T-cell independent type 2 antigen pneumococcal C polysaccharide (PnC) was also performed. A soluble extract made from S. pneumoniae R36A was tested in M167μk Tg mice. Emulsified PnC/IFA (100 μg) or PBS/IFA was injected i.p. into C.M167μk Tg and non-Tg littermate mice at day 0 and again on day 14. Day 7 and day 21 sera were collected and analyzed by ELISA for anti-PC specific antibodies. Intraperitoneal immunization with PnC elicited no anti-PC immune response in M167μk Tg mice (**Figure 28**); there were no increases in anti-PC antibody titers after injections with PnC antigen in IFA. PnC immunized M167μk Tg mice had titers at day 7 and 21 that were similar to non-immunized Tg control mice. Immunization of WT controls, non-Tg littermates, was successful as there was a 4-fold anti-PC antibody titer increase at day 21 after two injections with PnC in IFA.

PC-specific Antibody Titers after PnC/IFA Immunization

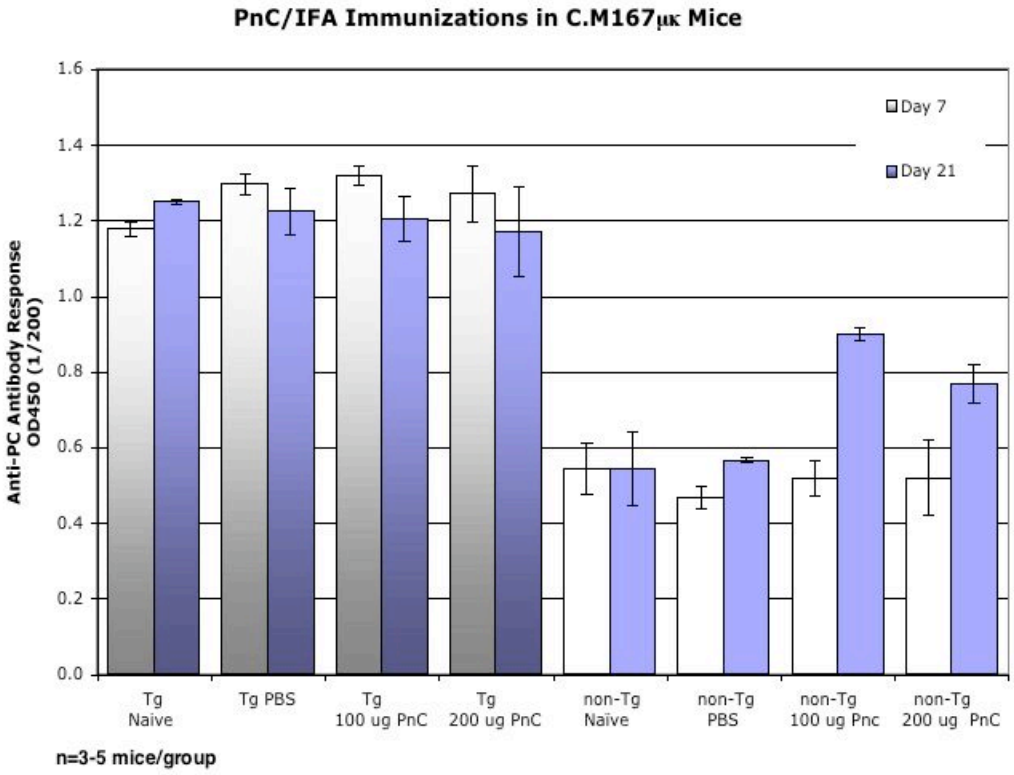


Figure 28. PC-specific Antibody Titers after PnC/IFA Immunization. C.M167μk Tg and non-Tg mice were injected i.p. with 100 μg of the T-cell independent antigen pneumococcal C polysaccharide (PnC/IFA), or with PBS (PBS/IFA). Mice were boosted again on day 14. Day 7 and 21 sera were analyzed for PC-specific Ab titers. There is no increase in the anti-PC Ab sera titers in Tg mice at either day 7 or 21 after 100 μg PnC immunization compared to unimmunized Tg control sera. However, non-Tg littermates showed a definitive spike in anti-PC antibodies with both 100μg and 200 μg PnC polysaccharide antigen at day 21 (n= 3-5 mice/group ± SEM).

The lack of responsiveness to PnC in M167μk Tg mice has been previously reported by Caulfield and colleagues (86). Using C57BL/6 M167μk Tg mice, Caulfield *et al*. found that Tg B cells were unresponsive to R36A-derived pneumococcal cell wall

polysaccharide (PnC) antigen, delivered without the use of adjuvants. Even repeated injection of PnC in their experiments did not significantly increase the basal levels of anti-PC antibodies in Tg mice. However, C57BL/6 M167μk Tg mice could respond to PnC antigen if it was given as a preformed antigen/antibody complex with the BALB/c anti-PC IgA myeloma antibodies, T15 or M603 (these complexes were also injected i.p. without adjuvant) (86). The authors had previously reported a strain-specific genetic restriction to PnC and PnC/T15 immune complex responsiveness: C57BL/6 non-Tg littermates were low responders to both PnC and PnC/T15 complexes, whereas the control BALB/c mice were high responders (84) and are able to produce large amounts of anti-PC antibody. Their data also showed that these strain-specific PnC responses correlated with expression levels of the dominant anti-PC T15 idiotypic antibodies produced in response to PC antigen. In the early 1970's, certain genetic strains were shown to have naturally high levels of T15 antibodies in their serum, while other strains had little to no T15 serum antibodies (81). Strains such as BALB/c and A/J that express high levels of T15 idiotype are high responders to PnC antigen and T15/PnC immune complexes, whereas strains that genetically express low levels of T15 antibody (C3H, AKR, C58 and CE/J) are poor responders to PnC and T15/PnC immune complex (84). The carrier antibody's idiotype, rather than the strain specific allotype of the IgC_H region, is an important factor in determining responsiveness to PnC or PnC/Ab complexes (84).

The immune response to PnC/T15 antigen/antibody complex is dependent on CD4 T cells, as shown by the abrogation of immune response when anti-CD4 was used *in vivo* to remove T cells (86). The PnC-antigen/antibody complex acts as a special case of the classical hapten-carrier conjugate, allowing the immune system to respond in a T cell

dependent manner to PC groups on the polysaccharide component. Polysaccharide antigen (PnC) cannot be presented by the MHC II molecules that are necessary to achieve T cell help (and is therefore classified as a T-cell independent antigen). Instead, B cells that recognize PC antigen will process 'carrier' epitopes (or the idiotypic determinants on the T15 Ab) from the immune complexes and present them to helper T cells. The stimulated T helper cells secrete cytokines that stimulate B cell antibody production in the PC-reactive B cells. The provided T cell help is therefore a non-cognate response. These data presented by Caulfield suggest the M167μk Tg B cells that are unresponsive to soluble T cell independent PnC-antigen are not autoreactively tolerized cells, but rather lack the T cell help necessary to stimulate an immune response.

M167μk Tg mice on BALB/c background had not been tested for their immune responses to PnC or T15/PnC complexes. The studies reported here show that BALB/c.M167μk Tg mice do not respond to soluble PnC free antigen (**Figure 28**), despite being on a high responder genetic background. PnC antigen (from *S.pneumococcus* or *LB4* bacteria) mixed with T15 Ab to form immune complexes were injected i.p. (without adjuvant) into C.M167μk Tg mice. PnC/T15 immune complexes (100μg and 200 μg, based on the concentration of protein present in the complex) were able to elicit an anti-PC immune response in BALB/c M167μk Tg and non-Tg study mice and corroborated Caulfield's results. These data are not shown.

In Vivo Immunization with R36A Pneumococcal Bacteria

Martin *et al*. showed that M167-id B cells respond to the particulate bacterial antigen, R36A *Streptococcus pneumoniae*, injected i.v. or i.p. without the use of adjuvants (42). As shown earlier, BALB/c.M167μk Tg mice have an increased population of marginal zone B cells, dependent on the higher surface density of the M167-id receptor on many of its B cells. Therefore, the M167 receptor in these BALB/c.M167μk Tg mice is probably highly susceptible to stimulation by particulate bacterial or immune-complexed PC-antigens. Taking into consideration Caulfield's evidence that PnC antigen had to be presented as an immune complex and Martin's report that M167-id MZ B cells responded to particulate bacterial antigens, C.M167μκ Tg mice were immunized with heat-killed particulate R36A S. pneumoniae bacteria.

Figure 29 shows results from i.p. immunization with R36A in C.M167μk Tg and non-Tg mice. Intraperitoneal injection of R36A bacteria (without adjuvant) induced a significant PC-antibody response. There was a 2-3 fold increase in PC Abs detected in sera of Tg mice at day 7. PC-Abs remained elevated and were even higher after secondary boost with R36A on day 14. Non-Tg WT mice had a 4-6 fold increase in anti-PC Abs at day 7 and on day 21. Overall, the anti-PC Ab titers in immune sera of Tg mice were much higher than those reached in the non-Tg mice (5000 μg/ml in Tgs vs. 1500 μg/ml in non-Tgs) in response to PC+ bacteria. M167μk Tg mice can respond to particulate whole PC+ bacteria by secreting anti-PC Abs despite not being able to respond to soluble forms of the T-cell independent PnC antigen.

Immunization with *S. pneumoniae* R36A Bacteria

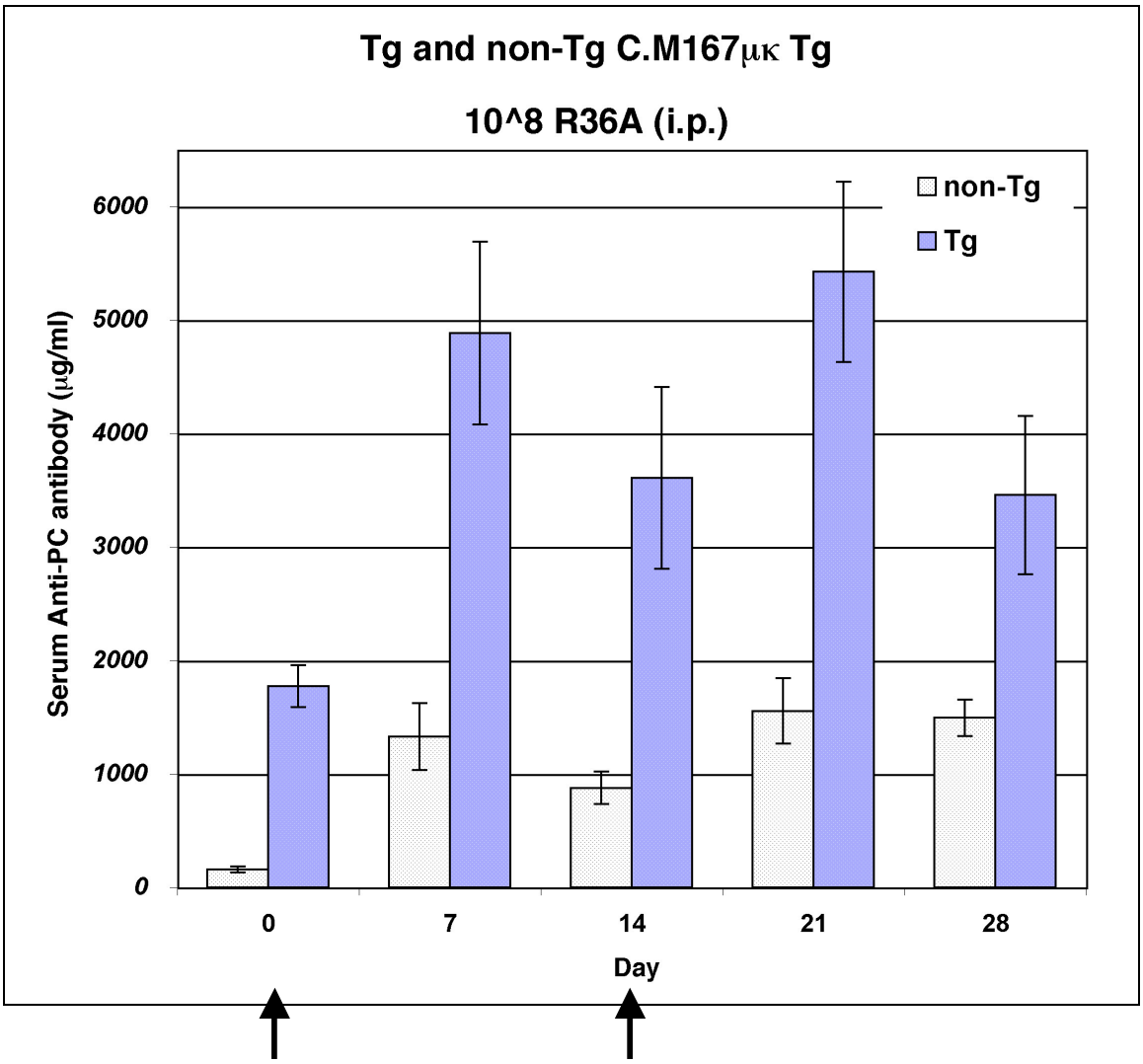


Figure 29. Anti-PC immune response to PC-containing bacteria, R36A. M167μk Tg (n=8) and non-Tg (n=7) mice were immunized by i.p. injection with R36A heat-killed *Streptococcus pneumoniae* ($1x10^8$ CFU/mouse) on day 0 and again on day 14. Sera were collected on day 0, 7,14, 21 and 28 for analysis of anti-PC antibody titers. A T15 Ab standard was used to quantitate anti-PC Ab levels in sera. M167 Tg mice immunized with PC+ R36A bacteria had a significant increase in anti-PC Ab titers, indicating that Tg B cells have functional PC receptors that respond to PC antigen on bacteria. Values represent the arithmetic mean of anti-PC Abs ± SEM (*$p < 0.05$).

In vivo i.p. immunization with heat-killed, particulate R36A S. pneumoniae was successful in stimulating increased anti-PC antibody production in both Tg and non-Tg C.M167μκ mice (**Figure 29**) indicating that the PC antigen from R36A specifically stimulated PC-specific receptors. However, with the co-expression of endogenous heavy and light chains on M167μκ Tg B cells (as demonstrated in **Figure 12**), it is possible that other "mixed molecule" antibody combinations could be contributing to the PC-specificity of the surface receptors. In order to prove that the B cells in transgenic mice can be activated specifically through the Tg M167μκ BCR, not the endogenously expressed receptors, *in vitro* proliferation assays were done to target the M167-id BCR.

II. Induction of In Vitro Cell Proliferation In M167μk Tg B cells

Stimulation of M167μk Tg BCR: CFSE *In vitro* Proliferation Studies

To directly show that activation of the M167 receptor on Tg+ B cells could initiate cellular proliferation, M167-specific Ig was used to stimulate proliferation *in vitro* by cross-linking the M167μk receptors on the cell surface with anti-M167 idiotypic antibodies. This mechanism has been shown in many other studies to be a way to trigger cell proliferation in B cells (58). I used CFSE (5,6-carboxyfluorescein succinimidyl ester) labeling of B cells (see below) to determine the number of proliferative cycles M167μk Tg+ B cells underwent when stimulated by various crosslinkers. I confirmed that the M167μk Tg B cell receptor in B cells of conventional C.M167μk Tg mice would

proliferate when specifically activated by anti-idiotypic antibodies to M167 idiotypic receptors.

CFSE is a membrane-permeable dye can be cleaved by intracellular esterases, enabling it to be excited by a 480 nm argon laser and detected in the fluorescein (or green) channel of a flow cytometer. The cells are stably labeled because the cleaved molecules are highly reactive with the amine groups on cytosolic proteins, which they bind, and therefore, cannot easily escape from the cell. Proliferation of CFSE-labeled cells can be tracked by flow cytometry because the high fluorescent signal of stained undivided cells is reduced by half with each cell division. A limited number of cell divisions that can be detected before the dividing cells look like unlabeled control cells (background), perhaps 8-10 divisions.

Although M167μk Tg B cells have been shown to respond normally to both *in vivo* (48) and *in vitro* (87) immunization with T-cell dependent PC-KLH, they fail to proliferate when treated with soluble anti-M167 idiotype, anti-IgM, or other anti-IgG antibodies (55, 88). However, anti-IgM and anti-M167 idiotypic antibodies (28-5-15, anti-V_H1/Vk24; 28-6-20, anti-Vk24; and 28-4-3, anti-Vk24) conjugated to sepharose beads were able to induce proliferation of PC-antigen specific M167μk Tg B cells *in vitro* (58). This is not surprising because the M167Tg BCR appears to respond best to large particulate antigens, such as whole bacteria or immune complexes that are more efficient in crosslinking the BCRs, rather than soluble ones.

Therefore, the anti-M167 idiotypic antibody, 28-5-15, which recognizes antigenic determinants formed by elements of both the M167V_k and M167V_H polypeptide chains, was used to stimulate Tg B cells in cell culture. Anti-IgM sepharose, PC-sepharose, and

unconjugated sepharose beads were included as controls. Splenic B220+ B cells were purified, stained with CFSE, and stimulated for 3 days *in vitro*, then harvested and analyzed on the flow cytometer. Proliferation was induced by the anti-M167-sepharose complexes in 25% of the B cell population (**Figure 30**), but not in non-Tg B cell controls. One possible explanation for this is that only 25% of splenic B cells have a surface density of Tg M167-id BCR high enough to cross-link with the anti-idiotypic antibody *in vitro*. The M167-id Tg receptor being diluted by endogenously expressed receptors. PC-sepharose could promote proliferation in over 50% of the cells, although non-Tg B cells also were able to divide in response to the PC antigen. The proliferation seen in the non-Tg B cells is more excessive than expected for a heterogeneous population of antigen-specific cells, and the proliferation may be due to a mitogenic antigen contamination (such as LPS) of PC-sepharose reagent. The PC-sepharose was obtained from Jim Kenny's lab, but should have been screened for the presence of LPS contamination before use in culture. Proliferation in Tg B cells was not induced with soluble anti-IgM or soluble anti-M167 (data not shown), confirming previously reported data.

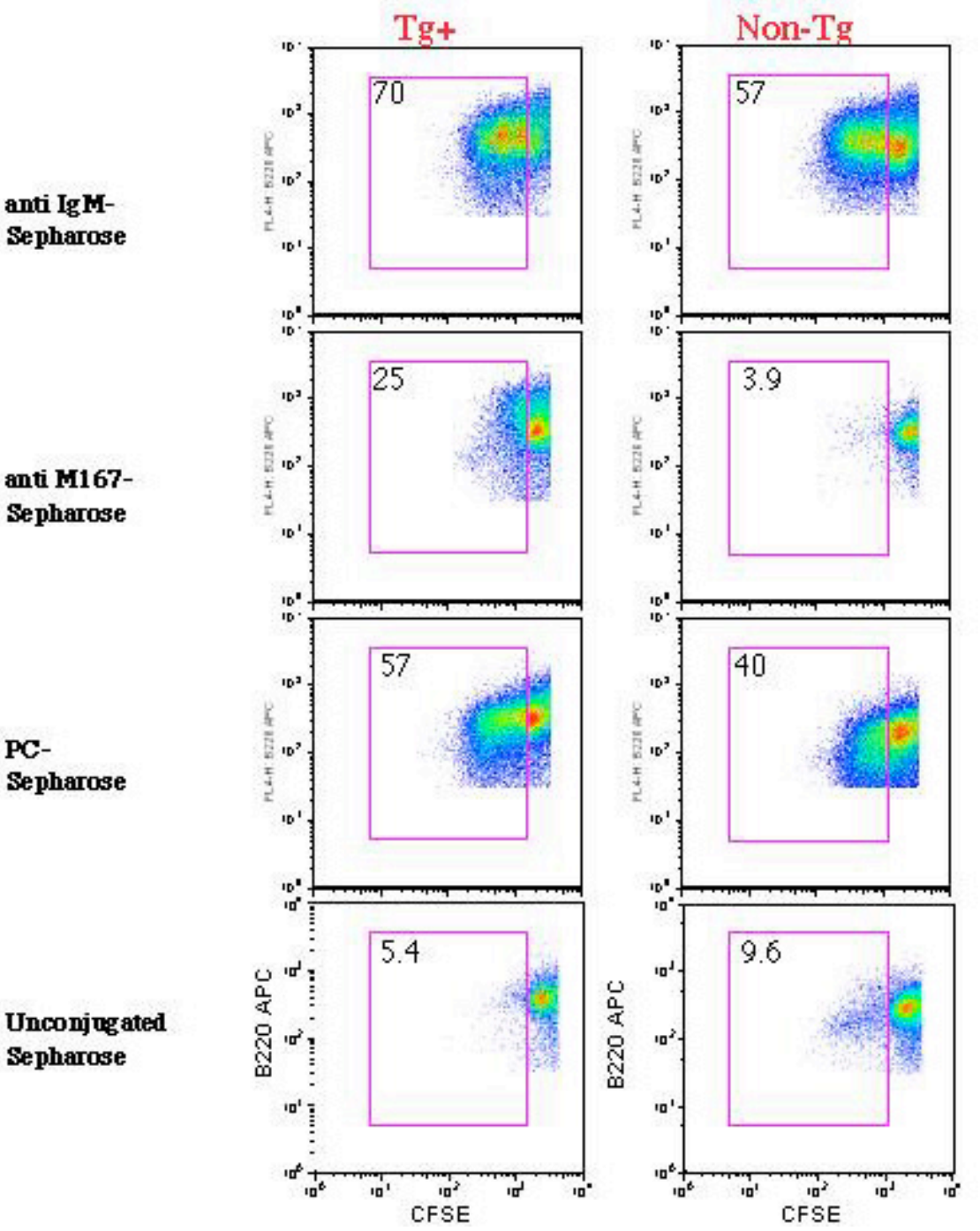


Figure 30. Proliferation of M167μk Tg B cells *in vitro*. Purified B220+ B cells stained with CFSE+ from Tg and non-Tg M167μk mice were stimulated with anti-IgM-, anti-M167- (28-5-15, V_H1/Vk24), PC-conjugated or unconjugated sepharose beads. Numbers inside gates indicate the percent of B cells that divided in response to stimulant. Anti-M167 sepharose caused 25% of B220+ B cells to divide in Tg cultures, whereas non-Tg B cells were not stimulated to divide with anti-M167Ab.

Figure 31 shows forward (FSC/size) and side (SSC/complexity) scatter graphs of stimulated B cells. FSC/SSC data show that the naïve B cells become larger and more complex blast cells when stimulated to divide. Unconjugated sepharose controls show background levels of stimulation, where most of the cells are still small, non-complex B cells (long, thin arrow indicates naïve B cells, whereas short, thick arrows indicate blast cells in Tg cultures). Tg B cells produced blasts in all three treatments, whereas non-Tg B cells blasted in response to anti-IgM and PC, but not anti-M167. The FSC/SSC plots support CFSE proliferation data shown in **Figure 30**.

Figure 32 shows the number of divisions cells have gone through in response to each of the sepharose-conjugated stimulants. IgM and PC produced higher number of cell divisions in both Tg and non-Tg cells (4 to 7 cell divisions), while the M167-responders in Tg B cell cultures had only undergone one to two cell divisions by day 3. **Figure 30** shows that 25% of these Tg splenic B cells were stimulated into cell division by anti-M167-sepharose.

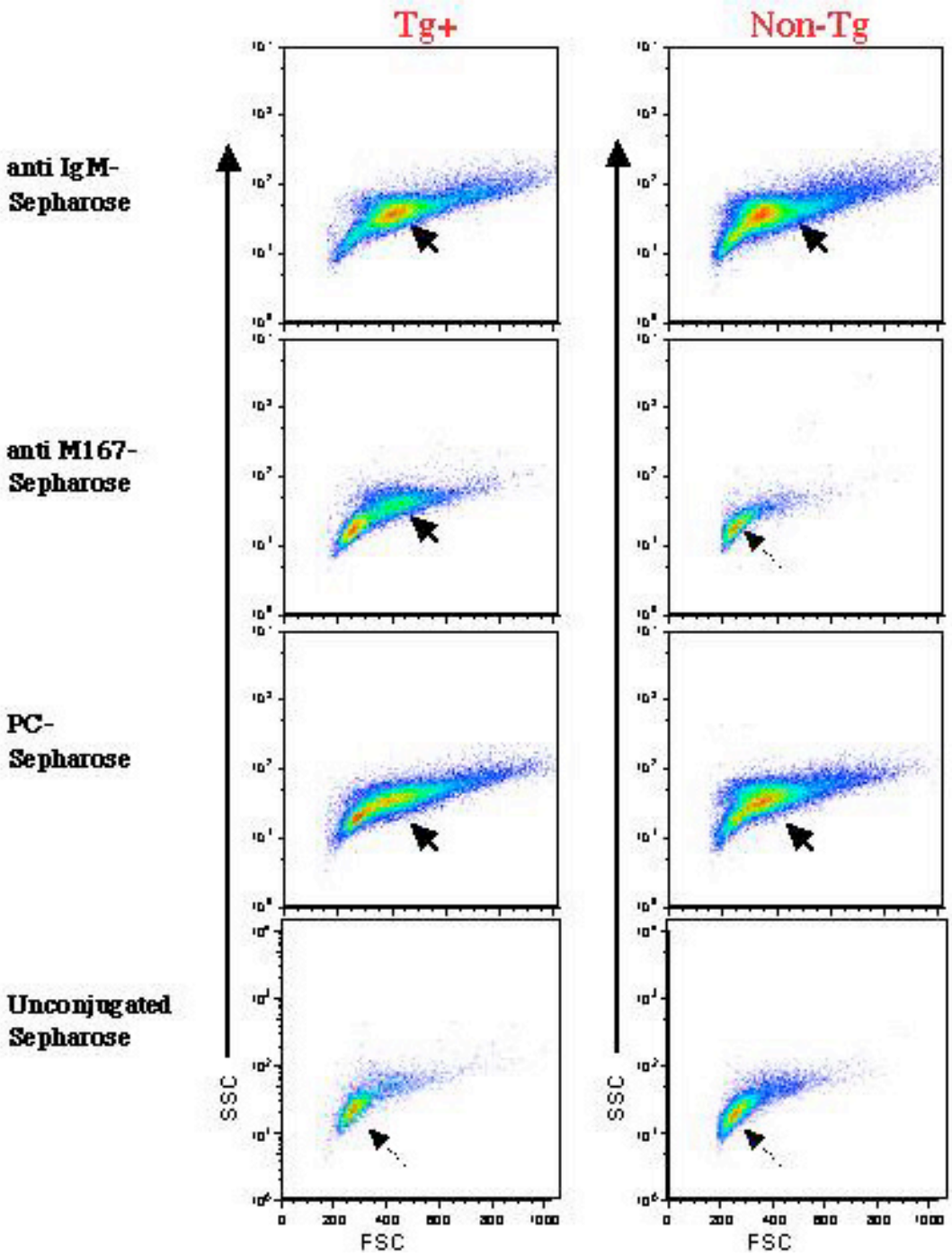


Figure 31. FSC/SSC plots of stimulated B cells. Purified B220+ B cells from Tg and non-Tg M167μk mice were stimulated *in vitro* with IgM-, M167-(28-5-15, V_H1/Vk24), PC- or unconjugated sepharose beads. Long, thin arrow indicates population of naïve, unstimulated B cells; short, thick arrow indicates the larger, more complex B220+ plasmablasts. Tg+ B cells can be stimulated by anti-IgM-, anti-M167- as well as PC-sepharose to differentiate into plasmablasts. Anti-M167does not promote plasmablasts in non-Tg B cells.

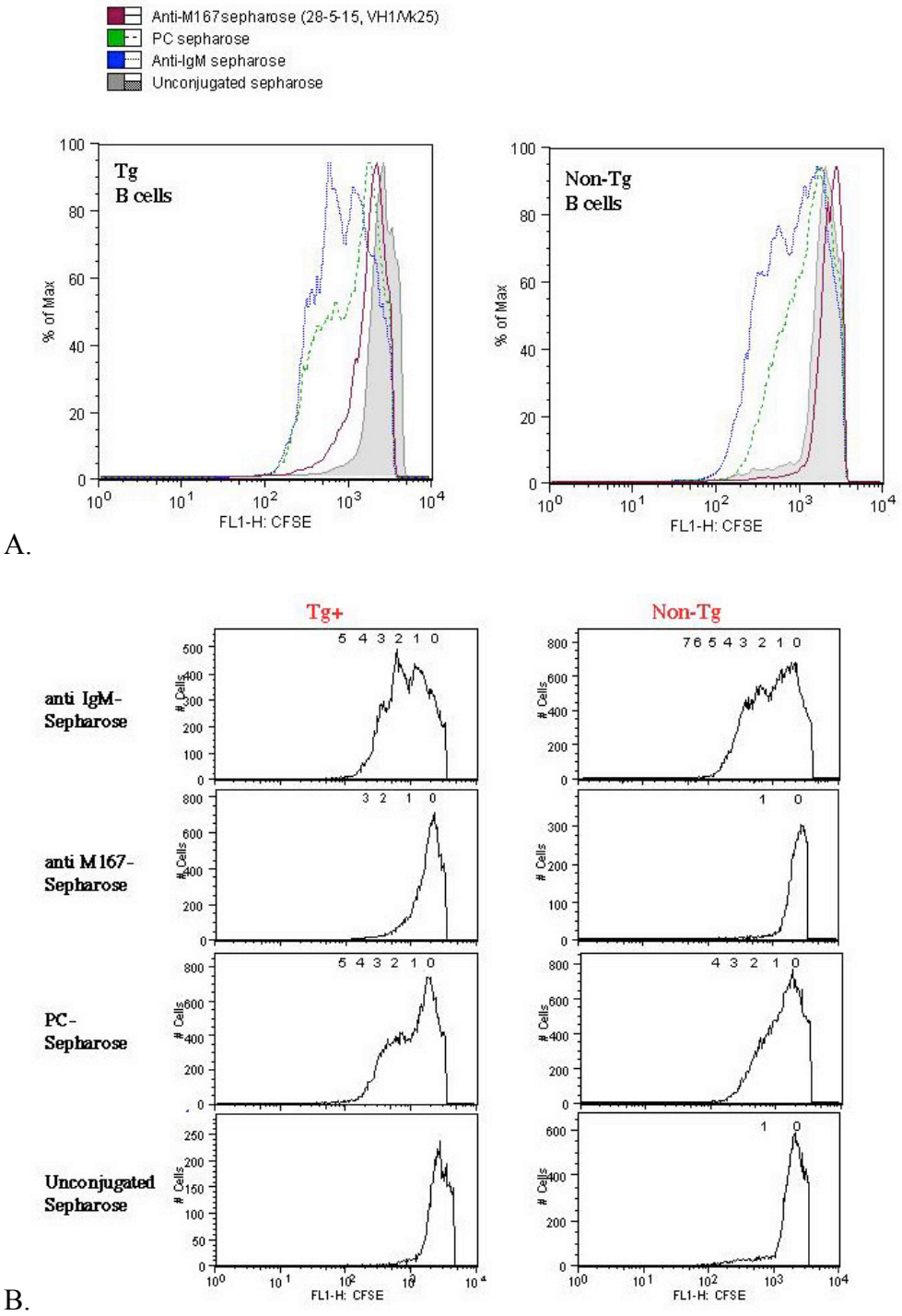


Figure 32. CFSE: *In vitro* cell division in Ag stimulated Tg+ and non-Tg C.M167μk B cells. (A) Shows overlays of stimulated B220+ cells from Tg+ and non-Tg C.M167μk mice. Anti-M167 sepharose stimulates cell division only in M167μk Tg B cells. (B) Shows the number of cell divisions in each of the stimulated B cell cultures as measured by a decrease in CFSE signal.

Figure 33 shows stimulation through other mitogenic receptors using LPS is normal in M167μk mice - most of the M167 Tg B cells were stimulated to divide (non-Tg WT B cells responded with identical vigor, not shown).

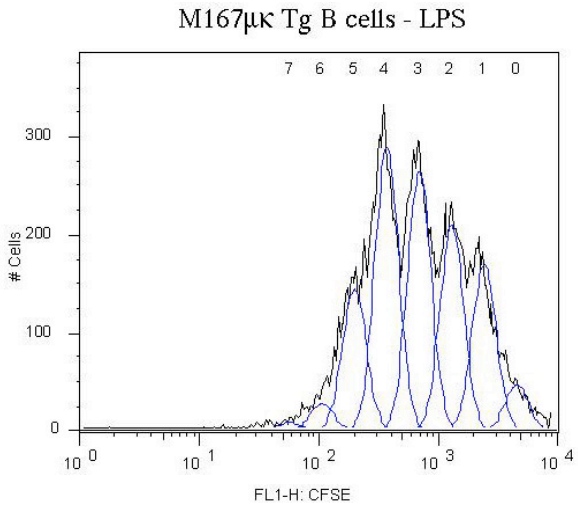


Figure 33. LPS stimulation of C.M167μk Tg B cells. Stimulation through other mitogenic receptors using LPS is normal in M167μk Tg mice. Proliferation algorithms in the FlowJo analysis program performed cell division calculations.

R36A Heat-Killed Bacteria Promotes Proliferation of Tg B Cells *In Vitro*

As previously demonstrated in Figure 29, R36A heat killed bacteria stimulated M167 Tg B cells to secrete the M167 IgM PC-binding antibody *in vivo*. Here, I show that heat-killed R36A whole bacteria also stimulate proliferation of PC-specific Tg B cells *in vitro* (**Figure 34**). R36A bacteria was used to stimulate CFSE-labeled C.M167μk Tg and non-Tg B cells in cultures that were >85% B220+ purified B cells. As reported in

M167μk Tg spleen cells – Day 3 *in vitro* stimluation

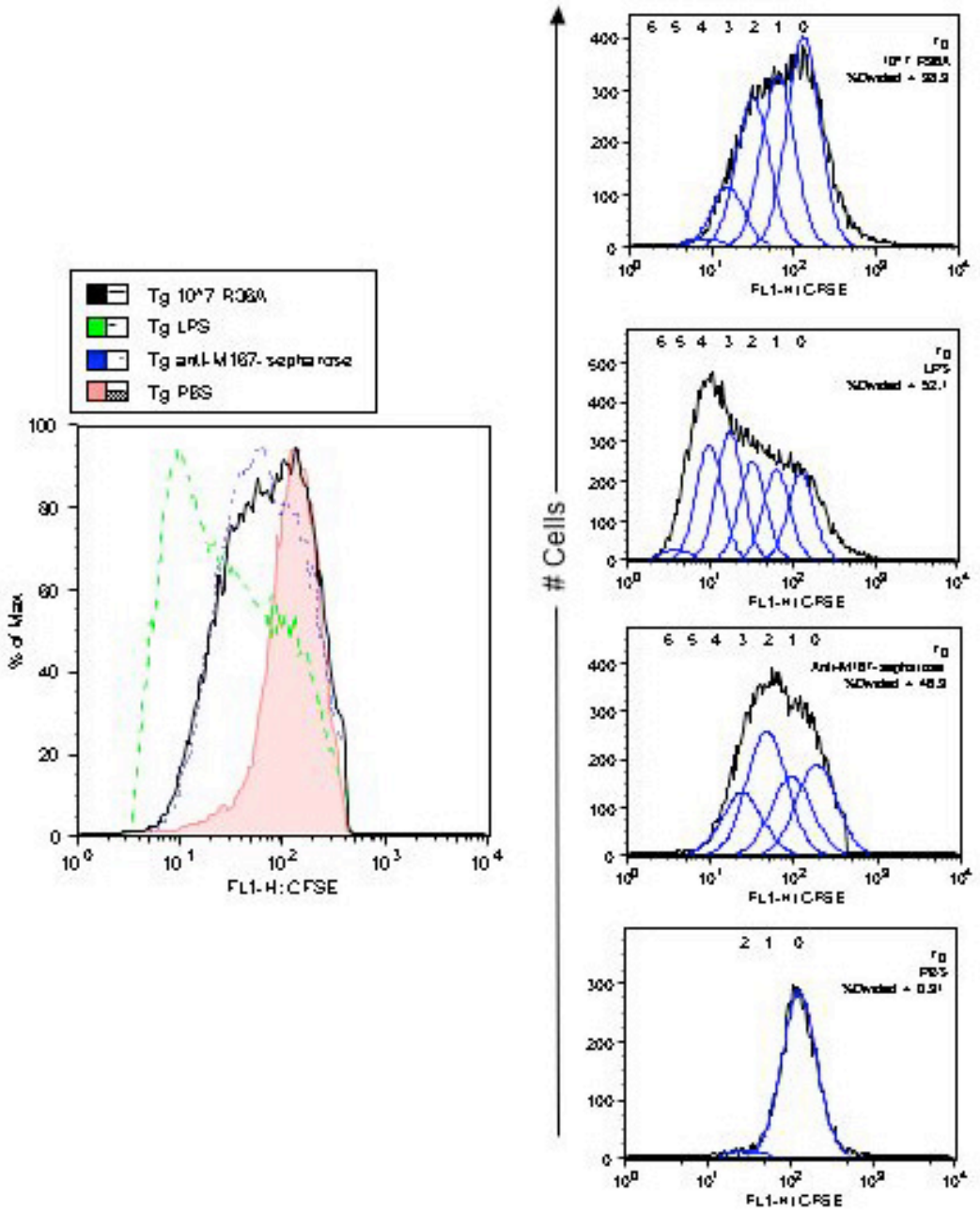


Figure 34. *In vitro* stimulation R36A heat-killed bacteria, LPS, or anti-M167-sepharose beads. Day 3, M167 Tg+ splenic B cells: overlay histograms (left) and individual histograms (right) showing number of cell divisions. By day 3, 38.3% of the Tg+ B cells had responded to 10^7 R36A bacteria, dividing up to 3 times. Non-Tg B cells had little to no division with 10^7 R36A (0% divided) compared to PC-specific Tg B cells. Background proliferation in PBS cultures was typically 0-4%. Cell division histograms were reported for proliferation controls using anti-M167 Ab (VH1/Vk24) and LPS also reported. See Table 17 for summary of cell division in M167μκ Tg and to non-Tg B cell controls.

Figure 34 and **Table 14**, 10^7 R36A stimulated proliferation of 38% of the Tg B cells in culture, but not in non-Tg WT B cells. M167-id sepharose beads were able to stimulate 42% of Tg B cells to divide up to 3 times, compared to 0 % of the non-Tg B cells.

Table 14. In vitro proliferation of C.M167μk purified splenic B cells. Percentage of purified B cells that divided after 3 days of *in vitro* stimulation, as calculated by the FlowJo proliferation algorithm. *1:100 dilution of anti-M167 (28-5-15, V_H1/Vk24) sepharose beads.

***In Vitro* Proliferation of Purified Splenic B cells**

	% Cell Division (Max # cell divisions)	
	M167μk Tg	**Non-Tg littermates**
10^7 R36A bacteria	38.3% (3 divisions)	0% (0 divisions)
LPS (10μg/ml)	52.1% (5 divisions)	67% (3 divisions)
Anti-M167-sepharose*	46.3% (3 divisions)	0% (0 divisions)
PBS	0.9% (0 divisions)	0% (0 divisions)

III. Effect of Immunization on PCT Induction

Role of PC-Hy Immunization on PCT Development

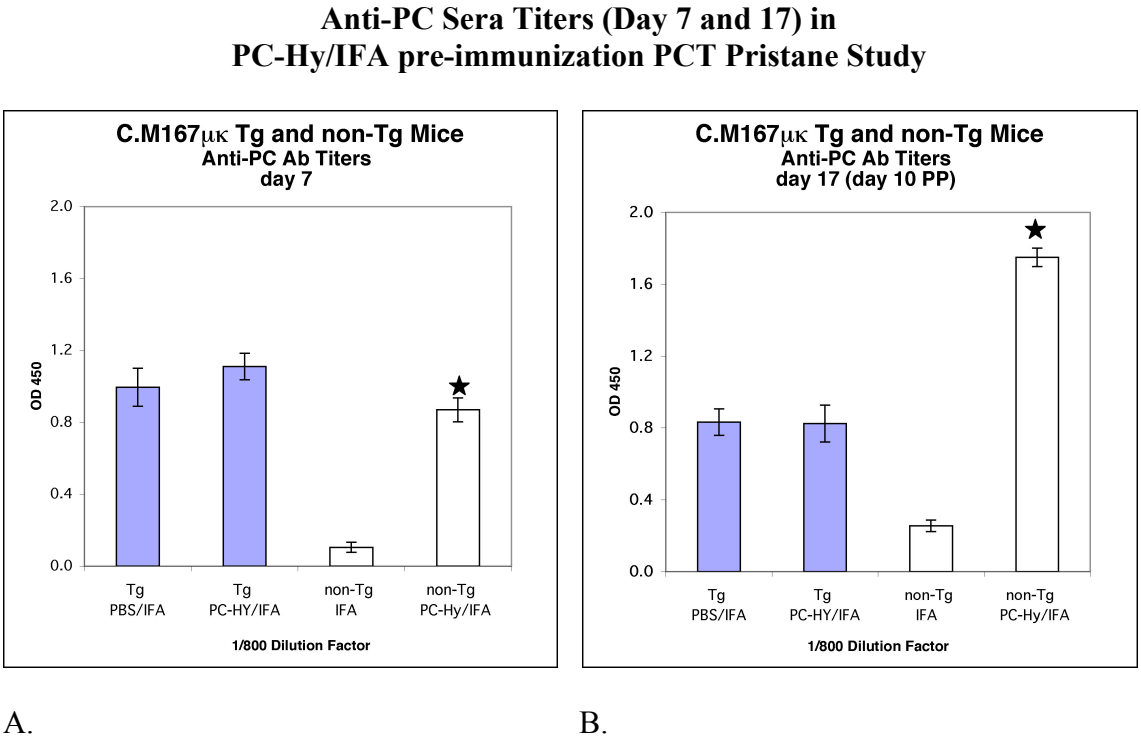
Pristane induction of plasmacytomas in C.M167μk Tg mice produced twice as many PCTs in Tg mice that had anti-PC BCRs as in non-Tg littermates. Despite the lack of systemic anti-PC immune response detected in M167 Tg and non-Tg BALB/c mice in response to pristane, stimulation of Tg B cells with PC antigen is predicted to be necessary to drive proliferation of B cells and, ultimately, plasma cell tumor

development. To this end, we sought to test the hypothesis that pre-stimulation of B cells with low doses of PC antigen in a pristane induction study would enhance incidence of PCTs, particularly in M167Tg mice.

In order to determine if chronic stimulation of B cells with PC antigen would increase or decrease PCT incidence in C.M167μk Tg and non-Tg littermate mice, a pristane induction study using PC-Hy antigen was designed to pre-immunize mice with PC antigen before each pristane injection. In previous experiments (48), Kenny *et al.* had shown that M167μk Tg mice respond very well to the T-cell dependent antigen, PC-keyhole limpet hemocyanin (KLH). Therefore, the PC antigen used for immunization of study mice was PC conjugated to horseshoe crab hemocyanin (PC-Hy). Taking into account that under physiological conditions, environmental PC would be present at chronic, yet low levels, the preparation of PC-Hy was ultracentrifuged to remove highly immunogenic particulates and i.p. injected at a concentration of 100μg/mouse (based on protein concentration in conjugate). The immunization schedule was administered as follows: 3 doses of pristane were injected i.p. at day 0, 60 and 120 (.2 ml, 0.5 ml, and 0.5 ml) as usual; 100μg of PC-Hy emulsified in IFA was injected i.p. 7 days before each injection of pristane (at -7, 53 and 113 of the pristane schedule). IFA (Incomplete Freund's Adjuvant) is a mix of 0.85 ml mineral oil and 0.15 ml mannide monooleate emulsifier that was used to help slow release of the antigen and stimulate long-lasting antibody responses

To determine if 100 μg PC-Hy/IFA produced an anti-PC immune response in pre-immunized pristane study mice, sera was collected after the initial i.p. immunization at day 7 and again at day 17 (10 days after the first pristane injection). The anti-PC

antibody titers for C.M167μk Tg and non-Tg mice at day 7 (A) and day 17 (B) post-PC immunization are reported in Figure 35.

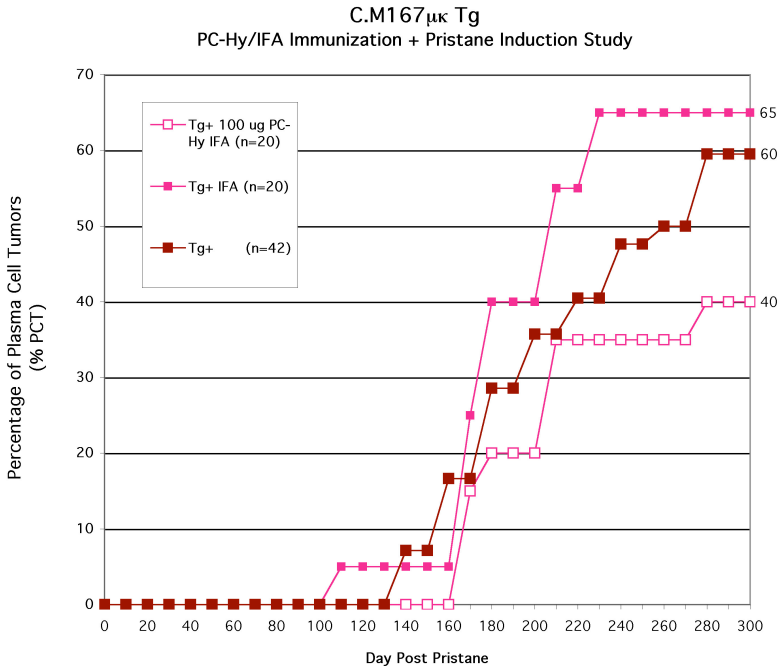


A. B.

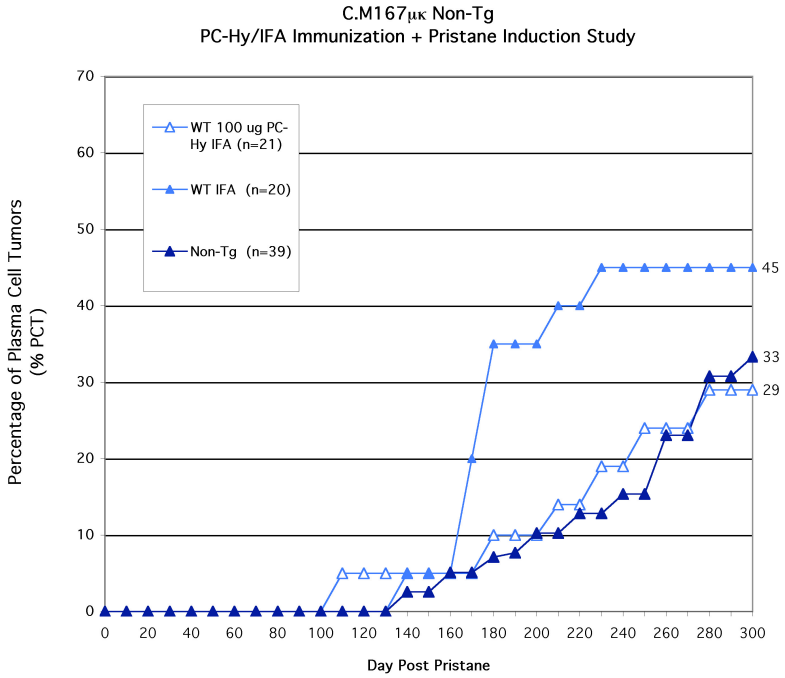
Figure 35. C.M167μk Anti-PC sera titers from PC-Hy/IFA pre-immunized mice. Tg and non-Tg mice were injected i.p. with the T-cell dependent antigen, PC-Hemocyanin (100μg PC-Hy in IFA) or with PBS (in IFA) as a negative control. (A) Day 7 (no pristane) and (B) day 17 (day 10 post pristane, PP) sera were analyzed for PC-specific Ab titers. Normal sera titers in Tg mice are naturally elevated and were similar to IFA controls. No change of anti-PC Ab levels was seen at day 7 or day 17 post-immunization from normal, PBS/IFA treated mice. Non-Tg control mice, however, did have a significant anti-PC immune response to PC-Hy antigen at day 7 and 17. (n= 5 mice/group ± SEM, statistics analysis using the Student's T test, * $p < 0.05$).

In non-Tg littermates, PC-Hy/IFA caused increased anti-PC Ab titers, indicating a normal secondary T cell dependent immune response to PC-Hy antigen, and providing a positive control for the integrity of the PC-Hy antigen injected. Surprisingly, in the M167μk Tg mice there were no detectable increases in anti-PC Ab sera titers between PC-Hy/IFA and PBS/IFA injected mice on either day 7 or day 17 of immunization, indicating a lack of immune response to PC. These results were of concern because the M167μk Tg BCR did not appear to be responding to this PC antigen, and may not be functioning as expected.

Results of PC-Hy immunization/PCT pristane induction study are reported in **Figure 36**. Pre-immunization with 100μg PC-Hy had no affect on PCT incidence in BALB/c WT mice as compared to untreated mice (29% in PC-treated mice vs. 33% in untreated mice). In C.M167μk Tg mice, pre-immunized PC treated mice had a decreased incidence of PCTs compared to the pristane only or IFA/pristane treated mice (40% vs. 60-65% PCTs). Because there was no detectable anti-PC immune response in Tg mice to PC-Hy antigen, it is not clear what the reason for the decrease in PCTs might be. The 20% decrease seen may be due to Tg B cells either being deleted or "tolerized" by too much PC antigen, and therefore the anti-PC B cell population is not able to respond as vigorously with subsequent proliferation and anti-PC antibody secretion.



A.



B.

Figure 36. PCT pristane induction results in mice pre-immunized with untreated, PBS/IFA or PC-Hy/IFA. M167μk Tg+ mice (A) and non-Tg WT mice (B). In C.M167μk Tg mice, the pristane and IFA+pristane control groups had similar induction curves and the same numbers of PCTs generated in this study. However, a decrease in the numbers of PCTs generated when PC-Hy+IFA was administered. The injection of PC-Hy + IFA didn't change the number of PCTs that developed in non-Tg WT BALB/c

mice. However, IFA only did cause a small 12% increase in the number of PCTs. IFA has been noted to cause an increase in pristane PCTs in BALB/c mice (data not published, Mike Potter, personal communication), but the the mechanism of the increase is not understood.

Interestingly, PBS/IFA causes acceleration of and increased incidence of PCTs in WT mice (**Figure 36**), although the explanation for this result remains unclear. This intriguing result has also been seen in other IFA-treated PCT pristane induction study groups (Potter, personal communication). As shown in **Figure 35**, the initial pre-immunization of non-Tg mice did generate higher titers of anti-PC antibody responses to PC-Hy antigen at day 7 and day 17. The initial anti-PC immune response seen at day 7 and 17 (**Figure 35**) indicates stimulation of PC-specific B cells in non-Tg BALB/c mice, although it did not result in an increase incidence of PCTs. Serological analysis was not documented for 2^{nd} and 3^{rd} injection of PC-Hy + pristane in this study. The type of PC antigen and the immunization schedule used in this study may be not have been suitable and further investigation is warranted.

As demonstrated earlier in **Figure 27**, immunization of C.M167μk Tg mice with 100μg PC-Hy (in CFA) did induce an anti-PC Ab immune response, seen after the secondary boost with antigen. In the pristane induction study reported here, an attempt was made to mimic low levels of endogenous PC antigen suspected to be present in a pristane treated mouse. The preparation of PC-Hy antigen had been ultra-centrifuged to remove all particulate complexes from solution, leaving only soluble protein. The M167μk Tg B cells do not appear to respond as well to soluble antigen, but rather need a particulate antigen presented in an immune complex or as part of a whole bacterium

suggested by our successful R36A bacterial immunization studies. Repeating the study using either particulate PC-Hy or R36A bacteria to ensure a detectable anti-PC immune response is advisable.

Thus far, I have three independent examples where the M167mk Tg has caused increased PCT incidences in BALB/c mice in pristane induction studies (**Table 15**).

Table 15. Three examples where the M167μk Tg increases PCT incidence. M167μk Tg mice consistently generate more pristane induced PCTs than non-Tg WT littermates.

	Non-Tg % PCT	M167μk Tg %PCT
Pristane only	33	60
Pristane + IFA	45	65
Pristane + PC-Hy/IFA	29	40

Chapter 5 Conclusions:

- C.M167μk Tg B cells can respond to PC antigens administered in the form of immune complexes.
- C.M167μk Tg B cells can respond to PC on whole bacteria, as demonstrated by the generation of secreted PC-specific antibody *in vivo* and by undergoing several rounds of cell division *in vitro*.
- The M167μk BCR can be specifically cross-linked using anti-idiotypic antibody that also stimulates proliferation *in vitro*. Together, these results provide proof that the Tg receptor is indeed functional in the M167μk Tg mice used in plasmacytoma induction studies.
- Pristane does not generate a systemic anti-PC immune response. PC antigen, however, may still be provided by apoptotic cells and/or oxidatively modified LDLs in the OG tissue (not directly demonstrated). The alternative source of PC - the environmental antigens from microorganisms of the gut flora - is probably the primary stimulus of B-cells in C.M167μk Tg mice.

CHAPTER 6

Discussion

This project was conceived to study plasma cell tumor development in immunoglobulin (Ig) transgenic conventional BALB/c An mice as there were no reports in the literature on this topic and to ask if an Ig transgene could be expressed in plasma cell tumors. I focused on Ig transgenes whose products were associated with binding to phosphorylcholine and phosphatidylcholine antigens, as much was known about the antibodies against these antigenic haptens. Several of these Ig transgenes had previously been constructed by other researchers (47, 50) and the mice were available. Further, plasmacytomas that secreted immunoglobulins with anti-phosphorylcholine activity had been described, the best known of these were T15, M603, and M167 monoclonal Igs. Most Ig transgenes are either rearranged L or H chains, but the M167μκ transgenic mouse had a tandem cluster of both M167μ (H) and M167k (L) transgenes. Two reproducible studies on M167μk Tg mice showed that when these mice were treated with pristane, there was a statistically significant increase in PCT incidence from 35% in WT controls to 63% in the Tg mice. Further this occurred with a decrease in the mean latent period from 240 days in WT to 200 days in the Tg mice. These intriguing results suggested that the M167μk Tg had changed the immune responsiveness of mice carrying the Tg that predisposes the mouse to an increased risk of developing a PCT. This thesis explores some of the changes in the M167μk Tg mouse that contribute to this predisposition.

Changes in the B cell populations of M167μκ Tg mice

1) Reduction in total B-cells

2) Increase in MZ population

Previous studies in the laboratory of James Kenny demonstrated that a high percentage of B cells (>97%) in M167μk Tg mice express M167 H + L chain B cell receptor on the plasma membrane where it has the potential to interact with PC containing antigens and transmit signals to the B cell to proliferate and differentiate to the plasma cell stage. The transgenic M167μk BCR expressed in conventional BALB/c M167μk Tg mice causes changes to the overall B cell population that are likely to have impacted their susceptibility to develop increased numbers of PCTs. Two such interesting changes are a reduction in the total number of splenic B cells and an enlargement of the splenic MZ B cell subset in C.M167μk Tg mice.

Kenny reported that the number of IgM+ B cells in C57BL/6 M167μk Tg mice can range from a low of 18% to a close to normal 45% of the total splenic cells (48). On average, IgM+ B cells make up about 25% of spleen cells in Tg mice, whereas normal mice have about 50% IgM+ B cells in the spleen. Further, Kenny reported that Tg mice contain fewer total spleen cells (3-4 x 10^7 spleen cells) than their non-Tg littermates (1 x 10^9 spleen cells) (48), which means that there is a 5x reduction in the number of B cells in Tg mice compared to non-Tg mice (see **Table 16**). I confirmed this observation in the BALB/c M167μk Tg mice, which also have a reduced number of total spleen cells and fewer total B cells in the spleen. Therefore, the effect of the M167μk transgenes on B cell number does not appear to be regulated by the genetic background of the mice. This

reduction in B cell numbers was of great interest because, despite having only 1/5 the B cells of non-Tg littermates, M167μk Tg mice are more than twice as likely to develop plasmacytomas due to the presence of the Tg PC-specific receptor.

Table 16. Splenic B cell numbers in M167μk Tg mice. These numbers were confirmed in C.M167μk mice used in the current plasmacytoma studies. Table adapted from Kenny, 1989 (48).

	Total Spleen cells	% B cells in Spleen	Total Splenic B cells
Tg	3-4 x 10^7	25%	0.75-1.0 x 10^7
Non-Tg	1.0 x 10^8	50%	5.0 x 10^7

Kenny does not offer an explanation as to why the M167μk Tg mice might have reduced numbers of total spleen and B cells. The presence of the rearranged M167μk Ig transgene has apparently affected the immune system's ability to regulate B cell development and maintain a normal number of B cells. With a significantly reduced B cell population that is also skewed toward PC-binding antigens, the antigen-binding B cell repertoire in M167μk Tg mice must be more limited than normal WT mice.

The other major change in the B cell population is an increased percentage of MZ B cells found in the spleen compared to non-Tg mice. For the first time in the M167 double transgenic mice, I have presented data that shows that the $CD21^{high}/CD23^{low}$ marginal zone B cell population is expanded in the C.M167μk Tg mice (15-25% of

B220+ cells) compared to non-Tg mice (5-10% of B220+ cells) in **Figure 13**. It is the M167^{high} B cells in M167μk Tg mice that are found in the MZ compartment, as are the M167+ B cells in non-Tg BALB/c mice. My observations in M167μk Tg mice agree with previously published data by Martin and Kearney that show certain B cell clones like M167 are favored to become MZ B cells (42) and that heavy chain only M167μ Tg mice, which have an enriched number of M167-Id+ B cells, also have an enlarged MZ B cell subset (42, 89).

Further calculation reveals that the absolute numbers of MZ B cells are generally much lower in M167μk Tg mice than in non-Tg mice (**Table 17**). Tg mice have 1.5 – 2.5 million MZ B cells compared to the 2.5 - 5 million MZ B cells in non-Tg littermates. Although Tg mice do not have greater numbers of MZ B cells, a much greater proportion of the splenic B cells are MZ B cells, which may be potential B cell precursor to pristane induced PCTs.

Table 17. Absolute number of splenic MZ B in M167μk Tg mice.

[a]total splenic B cells calculated in Table 16; [b]percentage data from Figure 11

	Total number Splenic B cells[a]	% MZ B cells in Spleen[b]	Absolute number MZ B cells in spleen
Tg	1.0 x 10^7	15-25%	1.5 – 2.5 x 10^6
Non-Tg	5.0 x 10^7	5-10%	2.5 – 5.0 x 10^6

Changes and characterizations of the B cells themselves in M167μk

Variations in M167 BCR density
IgD co-expressed with Tg Ig
Proliferation activity of B cells to aggregated, but not soluble, PC Ag
Significance of proliferation and generation t12;15 *myc* activating CT

Characterization of the M167μk Tg B cells in our conventional BALB/c mice turned up some interesting observations and highlighted certain differences from those in published reports of C57BL/6 M167μk Tg mice. First, there were variations in the density of M167 receptor on the surface of Tg B cells, first noted when looking at the histogram of V_H1 expression, which showed two distinct subsets of Tg B cells: V_H1^{low} and V_H1^{high} (**Figure 10**). This variation in the surface density of antigen receptors is particularly important to plasmacytoma studies in the M167μk Tg mice, as these mice have a larger proportion of splenic B cells with the MZ phenotype.

Figure 14 showed that MZ B cells in Tg mice are among B cells with higher surface densities of Tg V_H1. This discovery was further extended to include B cells with a high receptor density of the paired M167 V_H1 + Vk24 BCR, specifying the $M167^{high}$ B cells such as those that develop the MZ B cell phenotype (as shown in **Figure 15**).

Secondly, the M167μk Tg mice express surface IgD on most of the peripheral Tg B cells in the BALB/c study mice, where the original characterization of peripheral B cells in C57BL/6 M167μk Tg mice distinctly showed a lack of IgD expression (48). The M167μk Tg mice used in PCT induction studies co-express the Tg M167μ heavy and M167k light chains as well as endogenously rearranged IgA heavy and κ light chains. The presence of sIgD on B220+ cells in M167μk Tg mice (**Figure 10**) indicated expression of endogenous IgH. We know that this IgD must have originated from an endogenous non-Tg heavy chain, as the M167-IgM transgene does not contain an IgD

segment. Endogenous Ig light chains are also likely to be present (as sequencing of tumor Igs showed both H and L chains were rearranged from endogenous Ig genes). If endogenous heavy and light chains on the cell surface pair with the transgenic M167 heavy and light chains, there would be multiple antigen binding specificities present on each peripheral Tg B cell. Kenny *et al*., using B cells from F1 hybrid mice that expressed two IgM allotypes (IgM^a and IgM^b), provided evidence that the M167μk Tg B cells were capable of this mixed pairing of Ig chains on the cell surface using anti-IgM^a and IgM^b fluorescent antibodies in flow cytometry. His lab initially reported that ~10% of the B cells in C57BL/6 M167μk Tg mice produce mixed molecules ($\mu^a + \mu^b$) antibody molecules on their surface (48), coupling one Tg heavy chain and one endogenously-derived heavy chain to make a single antibody molecule.

One could argue that the C.M167μk Tg mice are expressing endogenously rearranged Ig genes on most of the B cells because the antigenic stimulation provided in a conventional colony makes it necessary for adequate immune function. Expression of multiple antigen binding specificities would expand the B cell repertoire and guard against immune incompetence. However, C.M167μk Tg mice that have been rederived into an SPF colony have the same percentage of IgD on their IgM+ B cells as the conventional mice (data not shown), so this may not be a primary reason for co-expression of the endogenous Ig chains along with the Tg receptor.

Another possibility might be that the endogenous Ig chains are rescuing the PC-specific M167+ Tg B cells from an autoreactive fate. Autoreactive B cells are regulated in one of three ways, depending on what developmental stage the B cell encounters

antigen and/or the affinity of the autoreactive BCR for self-Ag: 1) receptor editing, 2) tolerance induction, or 3) deletion (90).

Sieckmann and Kenny had suggested that the transgenic sIgM^{high} sIgD^{neg} B cell phenotype they reported in B cells from the M167μk Tg C57BL/6 mice was similar to that of recently emigrated immature B cells from the bone marrow and thus might be more susceptible to tolerance induction (55, 88). Tolerance results from an autoreactive BCR previously encountering antigen during its early developmental stages, and thus the B cell is rendered non-functional (i.e. cannot proliferate or secrete Ab in response to antigens). The increase of PCTs in our conventional BALB/c M167μk Tg mice may be due to the "rescue" of the autoreactive M167 PC receptor with the additional rearrangements of endogenous Ig, where otherwise the Tg B cell might exist in a tolerized state, as suggested by Kenny, and not able to proliferate in the presence of antigen. If Tg B cells expressed only the M167μk receptor, there might not have been an increase in plasma cell tumors due to tolerized autoreactive PC-binding B cells, and there may have even been a decrease in PCTs.

Further studies by Kenny's lab suggested this mechanism of receptor co-expression contributed to the rescue of self-reactive cells from deletion, as autoreactive anti-PC B cells created in Rag2 -/- M167μk Tg mice are rescued from deletion by expressing multiple antigen receptors on their cells (91). In these experiments, because Rag2 deficient mice are not able to rearrange and express endogenous Ig genes, the Tg B cells can only express the already rearranged M167μk chains, thus allowing autoreactive anti-PC B cells to persist. The autoreactive anti-PC Tg B cells in Rag2-/- mice were developmentally arrested in the bone marrow, could not secrete antibody, and were

functionally anergic (or non-functional). Introducing a second light chain resulted in mixed molecule BCR expression, where both PC and non-PC specific antibodies were expressed on the B cell's surface (91). The dilution of the autoreactive BCR specificity with endogenous BCR rescued these cells from a tolerized B cell fate, and allowed them to be responsive to antigen. Based on these experiments, it may be that the C.M167μk Tg mice in my study are re-arranging endogenous Ig genes in order to compensate for the autoreactive M167-Id PC receptor, as well as to expand their Ig repertoire to deal with antigens present in a typical conventional environment.

There are further implications involved in the expression of multiple B cell receptors in regards to mature B cell phenotype. Two factors have been shown to play significant roles in determining whether a B cell becomes a mature B-1, MZ, or FO B cell: (1) the antigen specificity of the BCR and (2) the receptor density of the BCR on the cell surface (8, 91, 92). Key experiments using Ig Tg mice with B cells that could dilute their surface B1 specificity (VH12f, anti-PC) with a B2 receptor (either glD42 anti-DNA or B1-8 anti-NP) showed that B1 segregation depended on a threshold level of the B1 receptor expression (8). If the B1 receptor density on the cell surface was diluted below this threshold density, then the B cell developed a B2 phenotype instead of a B1 phenotype. Further, comparisons between the receptor density of hemizygous and homozygous anti-RBCμk Tg mice (with an autoreactive B1 phenotype) showed that threshold levels of BCR expression are required to regulate not only the number of peritoneal B1 cells, but also the efficiency of endogenous H+L allelic exclusion (92). Higher levels of the B1 receptor in the homozygous mice more efficiently inhibited

endogenous H + L gene expression and also created a larger peritoneal B1 compartment (thought to be a reflection of enhanced signals through sIg by the RBC self-antigen (92)). These two examples of receptor dilution in Ig Tg mice reflect what I believe is occurring in the C.M167μk Tg mice, as it is the B cells with a high surface density of PC-specific M167-Id (i.e. the ones without multiple antigen receptors from endogenous Ig) that become MZ B cells, resulting in a population of B cells more quickly activated by PC antigen due to their hyper-activated phenotype.

Antigen-driven proliferation of B cells is a key factor in generating t(12;15) chromosomal translocations during plasma cell tumor development. It was necessary to investigate the proliferative capability of the M167μk Tg B cells, as Kenny's group suggested that tolerance induction was occurring in the IgM^{high}, IgD^{low} Tg B cells.

Previous studies done in C57BL/6 mice had shown that M167μk Tg B cells respond normally to both *in vivo* (48) and *in vitro* (87) immunization with T-cell dependent PC-KLH. However, the M167μk Tg B cells failed to proliferate when treated with soluble anti-IgM, anti-M167 idiotype, or other anti-IgG antibodies (55, 88). *In vitro* assays that I performed with the conventional BALB/c M167μk Tg B cells using soluble anti-IgM and anti-M167 antibodies to activate B cells through BCR cross-linking experiments also failed to stimulate proliferation. Further, my initial immunization studies in C.M167μk Tg mice with PnC and low amounts of ultra-centrifuged, soluble PC-KLH failed to produce stimulation of an anti-PC antibody immune response. Caulfield and Stanko had previously demonstrated this lack of B cell responsiveness to PnC antigen, a T cell independent type 2 antigen, in C57BL/6 M167μk Tg mice.

However, the B cells were able to respond if the PnC was presented as an antibody/antigen complex (PnC/T15 immune complex) (86) and further evidence suggested that this lack of responsiveness to PnC was not a result of tolerance in Tg B cells, but rather was due to a lack of T cell help (86). I was able to initiate Ab response to PC using PnC/T15 complexes in the BALB/c M167μk Tg mice, but not using soluble PnC.

The *in vivo* immunization experiments I carried out in conventional BALB/c M167μk Tg mice and the *in vitro* proliferation assays on M167μk Tg B cells using various PC antigen to stimulate the M167μk BCR are summarized in **Table 18**. The results of these experiments showed that the M167 BCR in C.M167μk Tg mice was indeed functionally able to respond to various T-dependent particulate PC antigens and PC+ bacteria by initiating both antibody secretion and B cell proliferation. The anti-M167 sepharose conjugated antibodies that crosslink the Tg BCR and particulate PC+ bacteria stimulated splenic B cells to proliferate an average of 3 cell cycles after three days *in vitro*, most of the proliferation likely due to the MZ B cells, as there were no T cells present in the cultures.

This proliferative capability of C.M167μk Tg B cells to respond to PC antigen was an important point to demonstrate as repeated and vigorous proliferation is thought to be necessary for the generation of t(12:15) chromosomal translocations found in PCTs. I am suggesting that environmental/autogenous PC antigen increases the number of activated, proliferating B cells from the pool of anti-PC M167μk Tg B cells, leading to increased plasma cell tumor development. Dendritic cells may also play an important role by providing a stable source of antigen that stimulates the further rounds of B cell

proliferation (93) necessary to generate the t(12:15) chromosomal translocation that deregulate the *myc* gene.

Table 18. Functional Studies of M167μk Tg BCR. *In vivo* anti-PC antibody production. Antigens used to test for production of anti-PC antibody responses: PC-conjugated hemocyanin (PC-Hy); pneumococcal C polysaccharide (PnC); LB4 is a PC-containing antigen from French-pressed extracts of Lactobacillus sp.4. *In vitro* proliferation studies. PC-antigens and antibodies to cell surface receptors found on M167μk Tg B cells used to stimulate proliferation of splenic cells *in vitro*.

Functional Studies of M167μk Tg BCR

***In Vivo* Immunization Studies**	**Anti-PC Ab Response**	
	M167μk Tg	**Non-Tg**
PC-Hy (PC-hemocyanin, a TD Ag)	–/+	+
PnC (pneumococcal C polysaccharide, a TI Ag)	–	+
R36A *S.pneumoniae* heat killed bacteria	+	+
PnC/T15 or LB4/T15 Immune complexes	+	+
Pristane	–	–
Pristane with IFA	–	–
Pristane with PC-Hy/IFA (TD Ag)	–	+
***In Vitro* Proliferation Assay**	**Cell Proliferation**	
	M167μk Tg	**Non-Tg**
Rat anti-M167-id sepharose (28-5-15)	+	–
Goat anti-IgM sepharose	+	+
PC-sepharose	+	+
R36A heat-killed bacteria	+	–

PC Antigens likely to bind to and stimulate M167μk BCRs and trigger proliferation (autogenous vs. exogenous)

Antigen has been implicated as having an important role in plasma cell tumor development, as it initiates an immune response that ultimately leads to B cell proliferation that is essential for the chance generation of the oncogenic t(12:15) chromosomal translocation. The link between PC antigen and plasmacytoma development was established early on, when immunoglobulins from a number of individually derived PCTs were identified as having a common specificity for PC (the best known are T15, M603, and M167). Gut flora provides the largest source of PC as well as the other bacterially derived antigens identified as targets for PCT immunoglobulins. The PC hapten plays an important role in the immune system's attempts to protect itself against microbial infections, and normal BALB/c mice have a plentiful supply of natural antibody-producing B cells, particularly of T15-Id+ B1 cells in the peritoneal cavity, that are specific for PC antigen.

Autogenous PC antigen may be an important source of immune stimulation during plasma cell tumor development. Apoptotic cells are one such source, recently demonstrated by researchers to present PC on the surface of apoptotic blebs (94) and available for presentation to immune cells and anti-PC antibodies. Apoptosis occurs throughout the body during the normal processes of immune regulation and inflammation. The oil granuloma, as an inflammatory tissue, produces an abundance of apoptotic cells. Oxidatively modified low density lipoproteins (LDLs), also found in inflammatory lesions, express PC groups (38) and are another possible autogenous source

of PC antigen that could stimulate an immune responses during plasma cell tumor development.

A recent and important finding that various forms of PC-specific antibodies, including M167 and T15, recognize the PC hapten on apoptotic blebs and modified LDL (37) provides another connection between these autogenous antigens and pristane-induction of plasmacytomas. One of my original premises was that pristane might, at least indirectly, be an initiator of B cell immune responses in PCT development by creating antigen in the form of apoptotic cells and/or oxidatively modified LDLs during the formation of OG tissue. With the large population of the dominant anti-PC clones present in the M167μk Tg mice, I thought an antibody response to PC might be detectable. Pristane was not an effective stimulator of the anti-PC immune response in either M167μk Tg or non-Tg BALB/c mice, as there were no detectable changes in anti-PC antibody response in either group. However, since my studies were limited, this needs to be more fully investigated. Therefore, the primary role of the oil granuloma in plasma cell tumor development may not lie in generating PC antigen that triggers B cell activation, but in providing a cytokine-rich environment for the circulating plasmablasts to survive, to proliferate, and to be exposed to oxidative stress that is potentially important in creating t(12;15) chromosomal translocations. Apoptotic cells and/or oxidatively modified LDLs generated in the inflammatory tissue of the OG may be a secondary source of PC antigen initiating B cell proliferation in plasma cell tumor development, but more likely, the primary source of PC is from environmental bacterial antigens.

For a number of reasons bacterial antigens (particularly the PC-containing bacterial antigens in C.M167μk Tg mice) are thought to be the most likely principal stimulus of B cells that become PCTs after induction with pristane. There is the dramatic decrease in the incidence of PCTs that occurs in BALB/c mice housed in SPF colonies, where sources of bacterial antigen are stringently restricted. Many PCTs have been shown to make monoclonal Igs with specificity for a variety of TI type 2 bacterial antigens found in the gut (95). And importantly, one such Ig against the bacterial PC antigen, M167, has now been shown to increase the number of PCTs induced by pristane when it is displayed as the dominant BCR on B cells in C.M167μk Tg mice.

The predominant usage of IgA by the M167μk Tg and non-Tg PCTs is another indication that gut microflora are involved in the initial immune activation of B cell precursors. Hypermutated secretory IgA plays an important role in defense against microbial invaders and in maintaining the commensal bacteria in the gut (23, 96). Normal IgA secreting cells are found at mucosal linings in the respiratory and intestinal tracts where they provide a protective barrier of secreted IgA molecules. And interestingly, 50% of the IgA-secreting plasma cells in the mucosal lining of the intestine are B1 cell derived (97, 98).

Clues from the Igs produced by PCTs in M167μk mice

Plasma cell tumors are a reflection of the susceptible B cell population. By taking a careful look at the Ig proteins produced in PCTs derived from M167μk Tg and non-Tg control mice, we can get some idea about the B cells that originated the tumors. Sera and cells were studied from primary (g0) PCTs from Tg mice, as well as from PCTs from Tg

mice that had been transplanted (g1) into non-Tg BALB/c mice. Analysis included determining the amino acid sequence of secreted Ig proteins found in ascites, as well as sequence analysis of both transgenic and endogenously rearranged Ig genes from tumor mRNA in Tg PCT cells.

One expectation from study of tumor serology was that the tumors produced in Tg mice would secrete a large amount of monoclonal M167 protein. But this was not the case. Instead, I found that the panel of Tg PCTs produced a variety of monoclonal (M) protein components with different electrophoretic mobilities, instead of each tumor producing the same large M167-IgM band. This indicated that the Tg PCTs were secreting Ig molecules other than the one controlled by the M167μk transgene. There are no switch sequences in the M167μ construct to allow for isotype switching of the transgene. Therefore, since many of the M components seen on the SPE gels had what appeared to be gamma (γ) mobility (Figure 18), this suggested the Tg tumors were producing Ig proteins using endogenous Ig genes.

It was not possible to detect a separation of M167μk product from endogenous Ig products using the serum protein electrophoresis, but further testing of ascites for the M167 idiotype by ELISA showed that M167 protein is, in fact, being secreted by the Tg PCTs, but not as the predominant antibody species. PCTs from Tg mice, but not the non-Tg controls, secrete a PC-binding M167 antibody protein (**Figure 20**). An unexpected result from isotyping the PCT ascites was that the majority of Tg PCTs also secreted IgA M-component antibodies, in addition to IgM from the M167 Tg. The isotyping data from ELISA supported the electrophoretic evidence that other Ig isotypes were being expressed, as most of the Tg PCTs had high levels of IgA protein present in the ascites.

Immunohistochemistry on cells from Tg PCT#58 provided a clear example of an individual PCT cell that secreted the M167-id antibody and both IgM and IgA antibody species (**Figure 21**). The M protein from endogenously rearranged genes appears not to have PC specificity, as demonstrated in the example of PC-immunoprecipation of Tg PCT #37 ascites. Only IgM antibodies, and not the IgA, precipitated with PC antigen (**Figure 22**). Even if PC antigen were the original activator of the Tg M167 BCR in these Tg PCTs, as supposed, the endogenous Ig expressed by the B cell precursor is likely passively produced by the plasma cell tumor as the 'natural' Ig of the B cell, even though it's antigen specificity is not related to PC antigen.

To conclusively prove that the IgA proteins being produced were from endogenous Ig genes, I sequenced the rearranged IgA sequences from tumor mRNA. Endogenously rearranged Ig transcripts in M167μk Tg PCTs were present and, in almost every case, were IgA isotype. **Table 7** reported the variety of VH and Vk genes utilized by the endogenous Igs in M167μk Tg PCT, but their antigen specificities is not known. Based on gene analysis alone, none appear to be PC-binders, with the possible exception of #114 Tg. Further analysis of M167μk Tg PCTs, revealed that 80% of the endogenous Ig genes were somatically hypermutated, indicating that a large number of the B cell precursors probably went through mutational processes in germinal centers of follicles, while the remaining 20% of PCTs had both VH/Vk germline Ig genes. This same 80% to 20% ratio of mutated to germline Igs also occurred in PCTs examined from the non-Tg (WT) BALB/c control group, as well as from C.B20 mice, which carry the C57BL/6 IgH loci.

Somatic hypermutation (SHM) and class switch recombination (CSR) of Ig genes are considered hallmarks of GC reactions and both are dependent upon the expression of activation-induced cytidine deaminase (AID) (99, 100). AID initiates the mutation of Ig genes by deaminating DNA (converting cytosine to uracil nucleotides within the V region), which is then "repaired" using error-prone DNA polymerases and/or mismatch repair enzymes. AID is specifically found in germinal centers where both SHM and CSR occur simultaneously in B cells with the assistance of T cells. Antigen activation of B cells through the BCR and subsequent CD40 interactions with CD40L on T cell initiate both processes. Transcription of DNA and DNA cleavage are also essential elements involved in both CSR and SHM, but the DNA repair mechanisms differ (100). Interestingly, even though both SHM and CSR occur in the same time and place, they can occur independently from one another. Isotype switched Igs do not have to have SHM (and vice versa) (100), as is reflected in the IgA derived from PCTs, as 20% had germline genes in both the VH and Vk chains.

Curiously, the M167μk Ig Tgs are not mutated. There are several possible explanations as to why the M167μk Tg complex does not undergo SHM. One reason may be a low transcription rate associated with the transgenes. The first and essential step in SHM is transcription of the targeted Ig DNA, where the Ig enhancers are generally thought to be necessary for targeting SHM to the Ig V gene (101). Genes with high transcription rates, such as those of Ig genes in activated B cells, may be more likely to become mutated by AID, as suggested by Martin and Scharff (69). The M167μk transgenes may have a low transcription rate, and therefore have little to no mutation, because the DNA is not accessible to AID. I am uncertain of the status of promoters and

enhancers included with the M167μk transgenes. However, Storb *et, al.* reports high levels of splenic RNA expression in early generations of the founder M167μk Tg mice (102). Further, although there may be B cell specific factors involved in targeting AID to the V region of Ig genes, they are as yet unknown. Therefore, another possibility for the lack of somatic hypermutation may be because the transgenes do not provide a target sequence for AID.

Finally, the transgenes may actually be mutated, but the mutant sequences cannot be detected from mRNA analysis. The reason for this may be due to the multiple copies of the M167 H + L chain genes that were integrated into the chromosome. If any of the numerous transgene copies were mutated, transcripts from the mutated copy could be obscured by transcription from multiple copies of unmutated transgenes.

Therefore, despite its direct effect of creating an enlarged splenic MZ B cell population that is associated with non-germinal center TI immune responses, the M167μk Tg BCR does not appear to influence somatic hypermutation events that occur in the Ig of PCT precursor B cells.

Why and how M167μk Tg did not decrease, but increases incidence of PCT

The M167μk Tg creates an increase in the incidence of PCTs by putting proliferative stress on the available B cell population, forcing them all to react with the same environmental antigen, PC, which is very abundant in a conventional mouse colony. PC-containing particulate bacteria and immune complexed bacterial antigens can induce proliferation of a greater number of functional B cells in M167μk Tg mice than in non-Tg mice. This large-scale B cell proliferation occurs within a stressful inflammatory

environment induced by pristane that likely causes systemic effects on regulation of lymphoid proliferation, SHM, and isotype switching, such as changes in cytokine levels throughout the animal. MacLennan, et al. (93) has shown that activated B cells that turn into plasmablasts need dendritic cells to differentiate into the end stage plasma cells. This requirement may effectively be a bottleneck to the response in Ig Tg mice where a large number of Tg B cells are activated, as speculated by Qian *et al*. (103). Most antigen specific B cells die as plasmablasts because they are unable to associate with dendritic cells.

An autoreactive Ig Tg, one not rescued by co-expression of endogenous Ig, would likely have reverse affect on the incidence of PCTs, decreasing or suppressing their development altogether. B cells that were tolerized due to their autoreactive nature would not be able to react to antigen and undergo proliferation necessary for progression to PCTs. The M167μk Tg also creates an excess of splenic MZ B cells, as well as a homogenous PC-specific B1 population, that exist in a pre-activated state and are ready to collectively and rapidly respond to bacterial (PC) antigens.

Can the greater percentage of M167-id+ MZ B cells seen in the spleen of M167μk Tg mice explain the increases in pristane induced PCTs? It is possible that both PC-specific MZ and B1 B cells are particularly susceptible precursors of PCTs due to their hyper-activated phenotype that responds particularly well to bacterial antigens. Splenic MZ B cells are strategically placed at the blood-lymphocyte border in the spleen where exposure to endothelial cell and professional antigen presenting cells, such as macrophages and dendritic cells, assist their function to filter blood-born antigens (15). The MZ B cells exist in a "pre-activated" state that allows them to respond with rapid and

intense plasmablast production to viral and bacterial blood-borne antigens (15, 89, 104). MZ B cells, in contrast to recirculating FO B cells, are able to generate plasma cells faster in response to T-cell independent polyclonal activators, such as LPS (104), and respond more rapidly to bacterial T-cell independent antigens (89). The MZ B cells differentiate into plasma cells within 3 days of stimulation, whereas it take at almost 2 weeks for FO B cells to generate peak T cell dependent immune responses in GCs.

Splenic MZ B cells have similar functional abilities as B1 cells, found predominantly in the peritoneal cavity and also in the spleen (see appendix for a comparison of B cell types). Both MZ and B1 B cells normally have low affinity, polyreactive B cell receptors with a broad range of antigen specificities, particularly against TI type 2 bacterial polysaccharide antigens and self-antigens. Specific B cell clones are selected into the MZ or B1 subsets based on antigen interaction with the BCR and the strength of their BCR signaling. Each of these subsets shows signs of this previous interaction with antigen, which is reflected in upregulation of various activation markers. B1 cells, in particular, have been shown to be selected through interaction with self-antigens (105), but at least one MZ clone (M167) has been identified that can bind autoantigens on oxidatively modified low density lipoprotein (LDLs) and apoptotic cells. Instead of being deleted, receptor edited, or tolerized because of their autoreactive natures, B1 and MZ B cells are sequestered to special microenvironments where local regulatory factors keep these cells in check, so that the B cell clones are available to help protect against bacterial and viral infections.

Current dogma suggests that B1 and MZ B cell subsets are a part of the first line of defense by providing early immune responses against bacterial antigens in the

peritoneum/gut (B-1 B cells) and blood-born antigens (MZ B cells) (106). Located at sites such as these, where they can encounter bacterial antigen early and help prevent systemic infection. Because of their anatomical locations and their ability to respond early and rapidly to pathogens, MZ and B-1 B cells are considered to be a bridge between the immediate reactions of the natural innate immunity and the slower, adaptive immune responses of follicular B cells that generate high affinity antibodies and establish immune memory.

Monoclonal immunoglobulins from a number of PCTs have been shown to have T-cell independent antigen specificity for numerous microorganisms found in gut flora, food, and bedding (95). New studies suggest the MZ and B1 cells that are optimized to respond to bacterial TI type 2 antigens are linked together during immune responses, as the presence of both cell subsets seem to be required for the production of specific IgM in response to different repetitive polysaccharide antigens (107). These experiments using splenectomized mice, show that the presence of the spleen, including the MZ B cells, is not sufficient for an adequate response to TI-2 antigens in mice where B1 cells are absent. Conversely, mice possessing B1 cells but lacking a spleen and MZ B cells also show an impaired response to the TI-2 antigen TNP-Ficoll after intraperitoneal administration (108). These data imply a close relationship between MZ and B1 cells and include them both as connected precursor populations for PCTs in BALB/c mice.

Peritoneal B1 cells are ideal PCT precursor candidates due to their location in the peritoneal cavity, their enhanced ability to respond to bacterial antigens, and their involvement in providing 50% of the IgA-secreting plasma cells and secreted IgA in the gut (44, 45). In addition, although most of the B1 cells are located in the peritoneal

cavity and to a lesser extent in the spleen, it has been suggested that B1 cells are able to migrate to sites of infection (107). Wardemann, et al. detected B1 cells in the peripheral blood (107) and B1 cells have also been found in the lungs of mice after infection with influenza (109). The ability of B1 cells to circulate via the blood to sites of infection could be an important mechanism to prevent systemic infection of invading pathogens. The TEPC15 plasmacytoma would be a perfect example of having originated from a B1 cell, in as much as Masmoudi *et al.* demonstrated that Igs with the T15-id are produced by the B-1 cell subset (43).

It is my belief that the original MOPC167 PCT (with the M167-idiotypic BCR) was derived from a splenic MZ B cell, as this receptor idiotype characteristically develops a $CD21^{high}$, $CD23^{low}$, IgM^{high}, IgD^{low} phenotype and is enriched in the MZ subset of the spleen. Despite their remote location from the site of plasma cell tumor development, MZ B cells are potential precursors of pristane-induced PCTs. MZ B cells would rapidly be activated during the later stages of infection, once bacteria had entered the bloodstream trying to spread throughout the body. Local effects of pristane in the peritoneum may contribute to the release of gut bacteria into the circulatory system of blood and lymph. Blood-derived dendritic cells (DCs) can also carry bacterially-derived antigen from sites of infection to the marginal zone of the spleen where the presented antigens can activate MZ B cells, T cells and macrophages (110).

My results from the pristane induction study in C.M167μk Tg mice provide new evidence to support the hypothesis that MZ B cells are highly susceptible precursors. Having an enlarged PC-binding M167-Id+ MZ B cell population and greater than 97% of the B cells expressing varying levels of the anti-PC M167 BCR, the C.M167μk Tg mice

are twice as susceptible to PCT induction as non-Tg BALB/c mice. It would be of great interest to test Ig Tg mice with an anti-PC T15-id transgene in a plasmacytoma pristane induction study to see if the B1 derived T15 B cell receptor would increase the incidence of plasmacytomas, as does the MZ-derived M167 BCR. I predict that it would. Therefore, I suspect that the best candidates for PCT precursor B cells are hyper-activated B1 cells of the peritoneal cavity and spleen and splenic MZ B cells.

What might happen during plasma cell tumor development to a MZ B cell once it is activated by (PC) antigen? Within hours of antigen stimulation, activated MZ B cells move out of the marginal zone and within 3 days either become plasma cells in the red pulp or enter the B-T cell border of follicles and become rapidly dividing plasmablasts (111). MZ B cells can also enter the B-T follicles, but not much is known about the MZ B cell immune responses in germinal centers. MZ-derived plasmablasts from the spleen might then travel via the lymphatic system into the peritoneal oil granuloma tissue, where they could continue proliferating and complete their oncogenic transformation into plasma cell tumors under the pressure of oxidative stress and increased availability of cytokines such as IL6.

SHM in potential MZ precursors

Most of the Igs from C.M167μk Tg PCTs (80%) were hypermutated, indicating antigen selected B cell precursors. Antibody mutations normally occur in germinal centers (GC), and SHM, affinity maturation, and isotype switching are generally accepted hallmarks of T-cell dependent GC processing (112, 113). The TD immune responses generate GCs over a period of at least 2 weeks. In newly formed germinal centers, VH

genes in B cells have few to no mutations (112, 113). Cells generated in the GCs during this initial period of B cell growth could explain the lack of SHM in the remaining 20% PCTs if they arose from GC B cells.

I suspect that the hyper-activated B1 and MZ B cells are particularly susceptible precursors to PCTs, both being optimally responsive to T-cell independent (TI) bacterial polysaccharide antigens. But how does one explain that 80% of Igs produced in PCTs are mutated when it is generally thought that TI immune responses do not generate SHM since they do not receive T cell help in GCs? TI immune responses provide short-term antibody production, fail to induce GC formation (with a few exceptions), do not generate memory or affinity maturation, and have a more restricted Ig isotype profile (which is mostly IgM and IgG3 in mice) (114). There is some evidence that certain types of T-cell independent (TI) antigens, such as bacterially derived $\alpha(1\rightarrow6)$dextran, can initiate the formation of GCs (115, 116).

Studies in rats have shown that the MZ compartment in spleen contains memory B cells generated from immune response to both TD and TI type 1 antigens (117-120). The memory B cells in the MZ have been derived from B cells that have undergone antigen-driven proliferation. They are no longer in cell cycle but can be induced to re-enter cell cycle by subsequent exposure to antigen. These B cells have a phenotype characteristic of other marginal zone B cells. In addition, there is another subset of MZ B cells that respond to the repetitive polysaccharide TI type 2 antigens, but these do not generate B cell memory (117). In rat MZ B cells, most VH genes are germline (naive MZ-B cells) but a minor fraction (about 20%) of the MZ-B cells carry somatic mutations

(memory MZ-B cells). Human splenic MZ B cells include highly mutated post-switched memory MZ B cells (121-123). In mice, MZ B cells may be recruited directly from immature B cells (42), but memory B cells from germinal centers can also colonize the MZ (14, 119). The memory B cells that populate the MZ and adopt a MZ B cell phenotype (IgM^{high}, IgD^{low}, $CD21^{high}$) are associated with a responsiveness to TI type 2 polysaccharide antigens (14). This study showed that IgM+ memory B cells in mice are recruited into TI-2 antibody responses (14).

Marginal zone B cells are not restricted to TI Ab responses. Song (124) provide evidence that MZ B cells also respond to TD antigens with 1) early antibody forming cell responses and 2) GC/memory responses. Each of these MZ B cell responses originates from different MZ precursors in what appears to be a heterogeneous population. Interestingly, the GCs derived from MZ B cells were similar to those derived from FO B cells, with respect to the usage of VH genes, level of SHM, and production of secreted antibody. Further, recent research on SHM and isotype switching in extrafollicular immune responses suggests that it is possible for TI immune responsive MZ B cells to generate hypermutated Igs.

These studies support the idea that MZ B cells can generate the types of hypermutated Igs that have been found in plasma cell tumors from in pristane-induced BALB/c mice. **Figure 37** depicts the specialized B cell subsets found in the spleen and peritoneal cavity, and the highlights the MZ B cells' potential as precursors of plasmacytomas.

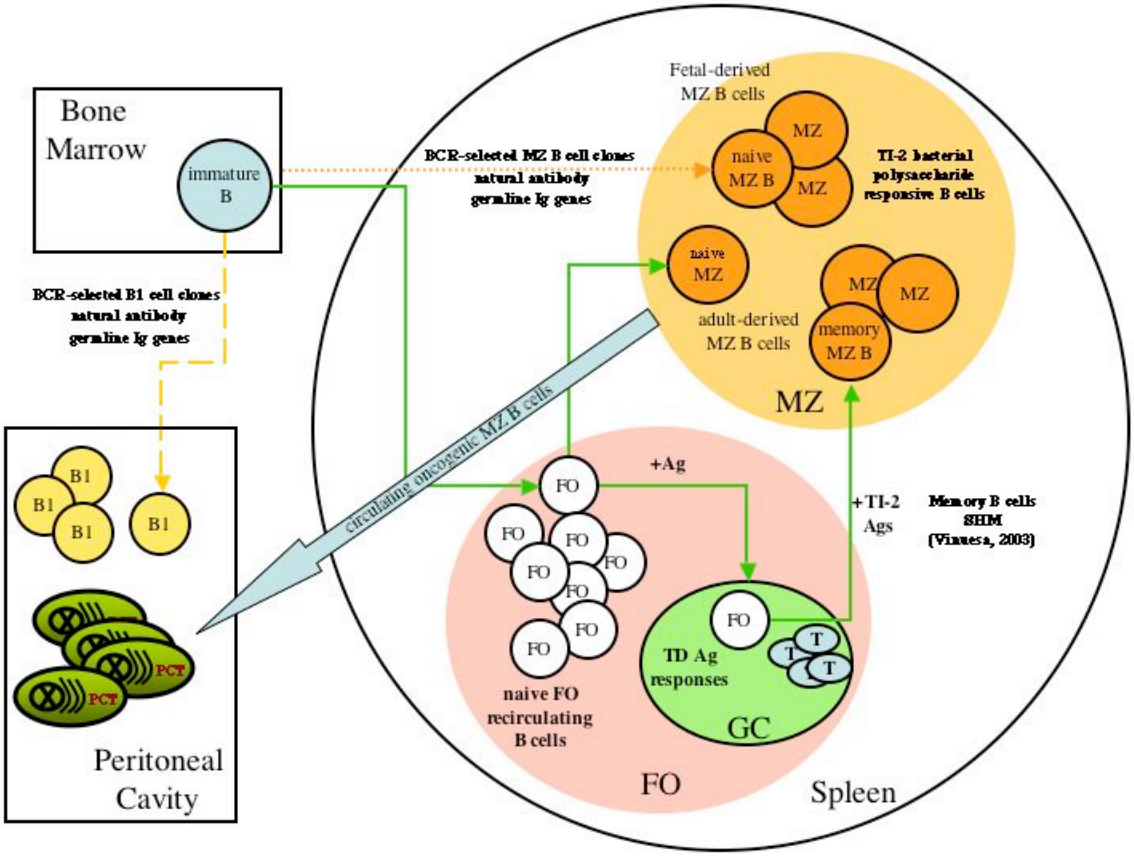


Figure 37. Marginal zone B cells are potential precursors of peritoneal plasmacytomas. Marginal zone (MZ) B cells have a preactivated phenotype that enables them to quickly respond to blood-born bacterial antigens, particularly the TI-2 type polysaccharide antigens that the Igs from PCTs are known to bind. In large part, the splenic MZ B cells in mice are BCR-selected with polyreactive, germline Igs that are typically derived from naïve B cells. However, a small subset of these MZ B cells are now shown to be hypermutated memory B cells, selected from the follicular B cells (FO) that were previously antigen selected and hypermutated during T cell dependent (TD) processing in germinal centers (GC). These mutated memory B cells that are responsive to TI-2 bacterial antigens can take up residence in the MZ and adopt a MZ B cell phenotype. Upon activation by antigen, the MZ B cells rapidly divide and become plasmablasts within 1 – 3 days. This rapid division and differentiation process could be a potential opportunity for developing oncogenic mutations. B1 cells are also a polyreactive, BCR-selected subset that responds to TI-2 bacterial antigens that end up sequestered in the peritoneal cavity instead of the marginal zone. The B1 cells are of considerable interest as PCT precursors due to their location, as plasmacytomas develop within the pristane-induced oil granuloma tissue in the peritoneal cavity. It is not inconceivable, however, that once MZ B cells have been activated by blood-born antigen in the spleen, they leave the MZ, enter the circulation and end up in becoming trapped in the peritoneal cavity milieu of the oil granuloma.

IgA in PCTs

In addition to most of the Igs being hypermutated, nearly all of the plasmacytomas from both Tg and non-Tg C.M167μk mice secreted a rearranged immunoglobulin expressing the IgA isotype. Conventional thinking about Ig hypermutation and class switching is that these processes must occur in a T-cell dependent manner in the germinal centers of lymphoid tissue.

Generation of IgA+ B cells occurs in germinal centers of intestinal Peyer's patches (PP) and are the precursors of the IgA secreting plasma cells in the lamina propria of the gut (96, 125). Gardby *et al*. found that somatically hypermutated mucosal gut IgA against T-cell dependent antigens can occur without GC formation in PPs, as demonstrated in studies with mice that cannot form GCs (126). In addition to the T-cell dependent induction of IgA in PPs, new studies report a primitive mechanism of IgA class switching against commensal bacteria that is independent of T cell help and occurs outside of germinal centers in gut tissues (127). In this manner, a large part of the intestinal IgA against cell wall antigens and proteins of commensal bacteria is derived mostly from B1 cells without germinal center formation or the help of T cells. This GC- and T-cell independent generation of IgA is probably assisted by mucosal dendritic cells and stromal cells (96).

T-cell independent CSR can occur in human B cells though the interaction of BlyS (B-cell-activating factor) on cytokine-activated DCs and its receptor BR3 on B cells. The B cells of the MZ and intestinal lamina propria are able to class switch in this manner (100, 128). IgA production has been documented in patients with CD40L-

defiencies, which suggests that the B-T cell CD40/CD40L interactions are not absolutely necessary in CSR (100, 129, 130).

The recent data that CSR and SHM is possible without T cognate interactions supports the theory that Ag-activated B1 and MZ B cells stimulated by TI polysaccharide antigens could be a source of these plasmacytomas with somatically mutated IgA antibodies without requiring the B cell precursors having to migrate to germinal centers. The B-1 cells, which provide 50% of the IgA secreting plasma cells in the gut (98) appear able to migrate to sites of infection in other mucosal tissues, and the MZ B cells of the spleen are both cell subsets that are hyper-activated to quickly respond to TI bacterial antigens and able to differentiate into plasmablasts faster than the normal recirculating FO B cell.

Preferential switching and differentiation to IgA secreting plasma cells is assisted by stromal cells in the lamina propria that secrete cytokines involved in IgA+ development, such as IL6, TGF-β, and IL10 (131). Pristane oil granuloma provides a rich source of these kinds of cytokines, especially IL6 and TGF-β. Pristane may have an effect on the overall immune system responses, such as deregulating expression patterns of cytokines, causing IgA switching to occur in areas other than the normal GCs of Peyer's patches in the intestine. Antigen stimulated MZ B cells may undergo IgA isotype switching due to these influences.

In order for IgA secreting plasmacytomas to develop in the OG, the IgA-secreting B cells/plasmablasts must leave the site of original antigenic stimulation, circulate, and end up trapped in the rich environment of the oil granuloma tissue. The oil granuloma may preferentially sequester IgA+ B cells if they express other IgA homing chemokines,

such as TECK/CCL25 or MEC/CCL28 or CXCL12. TECK (thymus-expressed chemokine) is normally expressed within the epithelial crypts of the small intestine, and preferentially attracts IgA-, but not IgG- or IgM-expressin B cells from spleen, PPs, and mesentery lymph nodes (MLN) (132). MEC (mucosal-associated epithelial chemokine) is expressed on other types of mucosal cells and also preferentially attracts IgA^+, but not IgM^+ or IgG^+, B cells. IgA+ B cells express $\alpha 4\beta 7$ integrin and home to the cells that express MADCAM-1 (mucosal addressin cell adhesion molecule 1), such as high endothelial cells in the lamina propria of the gut.

Uniqueness of PCT induction in M167 compared to VH12 and VH2-12 Ig Tgs

In addition to the C.M167μk Tg mouse, I have conducted pristane-induced plasmacytoma studies on several other BALB/c Ig transgenic mouse lines, including C.VH12μ Tg and C.VH2-12μ Tg mice. The majority of B cells in VH12μ Tg mice display the VH12/Vk4 anti-phosphatidylcholine B1 cell receptor (50) and VH2-12μTg mice display an autoreactive anti-Sm B1 cell receptor . What I have discovered thus far is that Ig transgenes do not universally affect the susceptibility of BALB/c mice to plasmacytomas. Only the anti-PC M167 receptor caused an increase in PCTs, while anti-PtC and anti-Sm receptors have not effected any changes in PCT incidence.

What is the key factor regarding a specific BCR that increases or decreases a B cell's susceptibility to PCTs? It may be due to the particular type of antigen specificity conferred by the B cell receptor, such as autoreactive BCR that can react to an auto-antigen. Alternatively, it might be a function of the mature B cell phenotype (B1, MZ or

FO) that was positively selected due to strength of antigen signaling though the BCR during the maturation process.

Because its VH12 heavy chain preferentially pairs with endogenous Vk4 light chain, VH12μ Tg mice express a PtC-specific BCR on most of the B cells (50). In addition to having a single antigen specificity for PtC, all the peripheral B cells with anti-PtC activity display a CD5+ B1 cell phenotype, making the these B cells interesting precursor candidates for PCT induction. And much like anti-PC B cells, normal mice have a naturally abundant number of peritoneal B cells that are specific for PtC (5-15% of the B cells). Unlike the M167μk Tg, repeated pristane plasmacytoma studies in anti-PC BALB/c VH12Tg mice failed to produce a significant increase in development of PCTs.

Interestingly, unlike the various PC-specific antibodies studied, the VH12/Vk4 anti-PtC antibodies do not stain apoptotic cells nor are the anti-PtC B cells activated by apoptotic cells (103), even though selection into the B1 compartment indicates positive selection from some source of auto-antigen (105). Perhaps the source of exogenous/autogenous PtC antigen is not as an abundant or available as PC and, therefore, less likely to stimulate a generalized proliferation of the Tg anti-PtC B cells. Alternatively, CD5+ cells are proposed to have previously encountered antigen, and are being negatively regulated by its expression in order to control polyreactive or autoreactive B cell receptors. The expression of CD5 on Tg+ B cells throughout the VH12μ Tg mouse may keep an overall proliferation of the anti-PtC B cells in check in the case of pristane-induced plasmacytomas.

The VH2-12 Tg mouse produces an autoreactive B1 phenotype with specificity against the ribonucleoprotein Smith antigen (Sm) that is associated with apoptotic blebs from the late stages of apoptosis (103). Positively selected anti-Sm B1 cells in the peritoneum are functional and can respond to antigen. In this case, Qian *et al*. (103) have demonstrated that apoptotic cells specifically activate Sm-specific MZ B cells in the spleen and the B-1 cells in the peritoneum. However, many of the splenic anti-Sm B cells exist in a transitional state and do not fully mature. Those B cells that do mature have low affinity for Sm and are tolerized to antigen. However, the Sm antigenic specificity conferred by this Ig transgene also did not alter the incidence of plasmacytomas that develop in BALB/c mice.

Using these three examples of receptor antigen specificity (PC, PtC, and Sm), it seems probable that particular kinds of antigens are more likely to stimulate a B cell immune response during pristane induction that result in PCTs. Antigen accessibility may play an important role in determining which varieties of B cell receptors are more likely to increase PCT development. The type of antigen specificity of the BCR displayed on the B lymphocyte cell surface may play the primary role in determining whether a B cell is more or less susceptible to becoming a plasma cell tumor under pristane conditions. This scenario would require that sufficient antigen was available to signal through the BCR and stimulate the number of rounds of proliferation necessary to give rise to the illegitimate recombination process involved in t(12:15) chromosomal translocation formation. However, if the antigen specificity of the BCR were highly autoreactive, as in the case of the anti-Sm mouse, peripheral B cells would be in a tolerized state having encountered antigen during development. The functional

autoreactive B cells are found in privileged compartments such the peritoneum or marginal zone of the spleen where they can be regulated by local factors. The impact of the BCR on what developmental stage or functional state a B cell is able to reach is a confounding factor.

T15-idiotype in normal mice vs. M167μk Tg mice

The expression of the M167μk Tg product alters the normal immune response to PC antigen in Tg mice because their PC-binding B cell repertoire is different than BALB/c non-Tg mice. In BALB/c wild-type mice, the primary anti-PC antibodies utilized during an immune response to PC are T15 idiotype antibodies, which use the unmutated, germline VH1 heavy chain and the Vk22-33 light chain (61, 78, 80-83). Other strains, such as C.B20, do not have this inherent ability to mount a T15 antibody response. The T15 idiotype is not utilized by endogenous Ig during immune responses in C57B/6.M167μk Tg mice after immunization with PC antigen (48, 54). In normal BALB/c mice, the T15 idiotype may be the more expanded PC antibody because it has a higher affinity for PC than M167-id antibodies (133, 134). M167-id+ PC-antibodies are selectively amplified in M167μ Tg mice in an Ag-driven, receptor-mediated process, resulting in a 100-500x higher number than expected from random pairing with other endogenous L chains that could also result in PC-binding antibodies (49, 91). In M167μ Tg and M167μk Tg mice, Kenny has shown that the Tg M167μ heavy chain can associate with Vk22 light chain to form a T15-id, but this clone has little to no affinity for PC. According to Kenny's hypothesis that PC-binding B cells need to encounter environmental or autologous PC antigen in the presence of low levels of cytokines, these

non-PC reactive T15 B cells cannot be clonally expanded. This explanation makes sense as to why there are no T15 antibodies seen in immune responses in M167μ or μk Tg mice.

It would be interesting to test anti-PC T15 Tg mice in a pristane induction study to see if the T15 antigen receptor would also increase the incidence of plasmacytomas. There is a heavy chain T15 knock-in (T15i) mouse, but T15-id+ B cells are not the dominant PC-binding B cells in the unimmunized mouse. Only 5-10% of Tg VH1+ B cells can bind PC, and only 20% of these PC-binders are T15-id+ (135). However, upon immunization with PC antigen, the T15-Id+ B cells are expanded 6x and differentiate to antibody-secreting cells. Therefore, the ability of the T15+ PC-specific subset to expand in response to PC may still be enough to generate a larger source of PCT-susceptible precursors and result in increased PCT development in a pristane study.

CONCLUSION

In summary, the acceleration of plasma cell tumor development in C.M167μk mice is a novel result. The PC-specific receptor, found on the majority (97-99%) of B cells in C.M167μk Tg mice, increases the incidence and decreases the latency period of plasma cell tumors over non-Tg littermates. This result suggests that the antigen-specific receptor is playing a role in the increased development of these tumors and that PC antigens are playing a key role in stimulating these PC-specific B cells. Phosphorylcholine, abundantly found on apoptotic cells (present in inflammatory lesions) and in commensal bacteria inhabiting the gut, appears to be one important antigen that can stimulate B cell proliferation necessary for the generation of oncogenic mutations needed in development of plasmacytomas. The simple fact that there is now a vast pool of PC-specific B cells, of which an enlarged MZ subset is hyperactivated to antigen, able to be activated by the abundant bacterially-derived PC antigen is the most plausible explanation for the increased incidence of PCTs in C.M167μk Tg mice. The most likely source of relevant antigens involved in stimulation of the M167μk PC-specific receptors would be from a systemic immune response generated locally in the mucosal lining of the intestinal tract. Evidence indicating that the immune response probably originated in the intestinal mucosa in response to commensal or pathogenic bacteria is the predominance of the IgA isotype in the endogenously rearranged Ig genes expressed in the M167Tg tumors. Hypermutated IgA B cells are meant to protect the integrity of the mucosal barrier throughout the host against invading microbes.

I suggest that the best candidates for PCT precursors are B cells that are hyper-activated against polysaccharide bacterial TI antigens, which includes both the MZ B cells in the spleen and the B1 cells of the peritoneal cavity and spleen. A growing number of PCT-derived immunoglobulin idiotypes have been identified in B cells that are consistently selected and enriched into the hyper-activated subsets of marginal zone B cells (M167-id) and B-1 cells (T15-id). Adding to this growing body of evidence is the fact the M167 BCR increases the incidence of PCTs in BALB/c mice.

MATERIALS AND METHODS

Animal studies

All studies were carried out according to protocols and methods approved by the University of Maryland ACUC as well as the National Institutes of Health ACUC.

Transgene typing of M167μk mice

Peripheral blood (>100 μl) was collected by retro-orbital bleeds of anesthetized mice. Blood was collected in heparin (Sigma, 200 μl at 1000 units/ml) to prevent clotting. Red blood cells were removed using (2x) 3 ml AKC lysis buffer, pH 7.5 (Biofluids) and washed in FACS staining buffer (1xPBS/0.1% BSA/0.1% NaN_3 (sodium azide)). Cells were blocked with anti-FcR antibody (5% 2.4G2/i10% rat serum/FACS staining buffer) on ice for 10 minutes to block any non-specific binding to Fc receptors and then labeled for 20 minutes on ice with anti-B220-FITC and the rat anti-mouse V_H-1-PE (hybridoma T68.3) (56) antibody provided by J. Kenny, GRC, NIH, Baltimore. Cells were washed with 2 ml FACS wash buffer (1xPBS/0.1% NaN_3), resuspended in 500 ml wash buffer and analyzed by flow cytometry (FACS). Tg+ mice had double positive B220/V_H1 B cells, whereas non-Tg mice were B220+ only.

Induction Studies

Three pristane injections of 0.2 ml, 0.5 ml, and 0.5 ml were administered to eight- to twelve-week-old N_{24} M167μk Tg+ and non-Tg littermate mice on day 0, 60, and 120,

respectively. Plasmacytomas were detected by finding >10 atypical plasma cells in the peritoneal fluid using Wright-Giemsa stained cytofuge preparations of peritoneal ascites fluid; mice were sacrificed when 100+ PCTs developed. The study was carried to 300+ days, at which time mice in the study were euthanized by CO_2 inhalation and necropsied. Kidneys, liver, gut sections, and oil granuloma tissues were fixed in Telly's fixative and embedded in paraffin for immunohistochemistry for all study mice. Sections of tumor tissue from PCT positive mice were either flash frozen in LN_2 for nucleic acid recovery or viably frozen in freeze media [100 ml EMEM with Earle's Salts, no Glutamine (Gibco), 15 ml Tryptose Phosphate Broth (Gibco), 15 ml heat inactivated FCS (Gibco), 24 ml DMSO (Sigma), 2 ml 200 nM L-Glutamine (Gibco), and 2 ml 100x Penicillin (Gibco)]. Either finely minced peritoneal PCT tumor tissue or cells recovered from ascites were washed and resuspended in 1x PBS, then injected i.p into pristane-primed BALB/c mice for transplantation of tumors. All mice were bred and maintained in our conventional facility on the National Institutes of Health Bethesda Maryland campus.

Flow cytometry analysis of B cell populations

Peritoneal washes: The peritoneal wall was carefully exposed by retracting the skin, 10 ml of cold 1x PBS w/ 10 mM EDTA was injected into peritoneal cavity and removed using a syringe with a 19g needle. Spleen and mesentery lymph node cells were obtained by either teasing the tissue with cold 1x PBS or using a MediMachine, a device from Becton Dickinson Company which gently disaggregates tissue into single cell suspension suitable for flow cytometric analysis of surface antigens. Red blood cells were

lysed with ACK buffer. Splenic cells were washed and resuspended in staining buffer (1x PBS/0.1% BSA/0.1% NaN_3).

The following monoclonal antibodies against the following molecules were used in these studies: M167 and V_H-1 antibodies (biotin- or PE-tagged, provided by J. Kenny), CD45R/B220 (RA3-6B2), CD11b (Mac-1; M1/70), CD5 (53-7.3), CD19 (ID3), CD21/CD35 (CR2/CR1; 7G6), CD23 (IgE Fc receptor; B3B4), CD43 (Leukosialin; S7), CD138 (Syndecan-1; 281-2), IgM (R6-60.2), and IgD (11-26c.2a). Antibodies obtained from PharMingen (San Diego, CA), were either fluoresceinated, biotinylated, or conjugated to PE, PerCP, or APC. In four-color experiments, directly fluoresceinated and PE-, and APC-conjugated antibodies were combined with biotinylated antibody revealed with streptavidin-PerCP.

Electrophoresis of Myeloma Proteins from PCTs

One microliter of PCT+ ascites diluted 1:5 in barbital buffer was run on a polyacrylamide gel using the Beckman Paragon Serum Protein Electrophoresis (SPE) system to determine whether the plasma cell tumor was secreting a myeloma protein.

ELISA Isotyping of C.M167μk Tg Myeloma Proteins

Secreted myeloma proteins from the C.M167μk Tg PCTs were isotyped by ELISA using HRP-labeled anti-isotype monoclonal antibodies: goat anti-mouse IgM, IgG (IgG1, IgG2a, IgG2b, IgG3), and IgA (Southern Biotechnology, Birmingham, Ala). Goat anti-mouse kappa and lambda HRP antibodies were used to determine light chain usage.

Immulon 2 HB plates were coated with 50 μl 1:200 goat anti-mouse (H+L) antibody-unlabelled in carbonate buffer and incubated o/n at 4°C. Plates were blocked with 0.5% gelatin/PBS for 1 hour at 37°C. Twofold serial dilutions of ascites fluid in 0.1% gelatin/PBS are added to plates and incubated for 1 hour at 37°C. Plates were washed three times with PBS/0.05% Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma). Secondary isotype-specific antibody goat anti-mouse Ig(H+L)-, IgM-, IgA-, or IgG-HRP (Southern Biotechnologies) was added at predetermined dilution and incubated for 1 hour at 37°C.

Conjugation of Phosphorylcholine to BSA and Hemocyanin (Hy)

Phosphorylcholine (PC) hapten was conjugated to the BSA or Hemocyanin protein carrier as previously described by Wu *et al*. (136).

Collection of Sera

Peripheral blood was collected by retro-orbital bleeds and serum was harvested by allowing blood to clot at RT° for 2 hours or overnight at 4°C, then centrifuging at 14,000 rpm for 10 minutes and collecting sera (supernate).

Sera were normalized before analysis in ELISA by diluting sera 1:100 in 1x PBS and measuring protein content in a spectrophotometer. Total protein concentration was determined using following formula: μg/μl = 1.55 x 100df x A280 – 0.67 x 100df x A260. The IgG concentration, which comprises the largest portion of antibody in mouse

serum, was then determined using the average IgG fraction (0.685). IgG protein was normalized to 30 μg/μl, and serially diluted starting at 75 μg/μl in ELISA.

PC-ELISA: Detecting anti-PC Abs in Sera/Ascites

ELISA was used to detect isotype- or idiotype-specific phosphorylcholine serum antibodies: PC hapten was conjugated to BSA protein carrier as previously described (136) and used to capture PC-specific serum or PCT ascites antibodies. Immulon 2 HB plates were coated with PC-BSA at 1 μg/well and stored o/n at 4°C. Plates were blocked with 0.5% gelatin/PBS for 1 hour at 37°C. Twofold serial dilutions of ascites fluid in 0.1% gelatin/PBS are added to plates and incubated for 1 hour at 37°C. Plates were washed three times with PBS/0.05% Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma). Secondary isotype-specific antibody goat anti-mouse Ig(H+L)-, IgM-, IgA-, IgG-HRP (Southern Biotechnologies), or M167-id (clone 28-5-15 FITC, later revealed by a third step using anti-FITC HRP), was added at predetermined dilution and incubated for 1 hour at 37°C. Plates washed twice with PBS/0.05% Tween 20. Color revealed with TMB substrate (Sigma) at 100 μl/well. Color reaction stopped with 1M H_2SO_4. Absorbance was measured at 450 nm using a VersAmax spectraphotometer.

Sequencing of Ig variable regions

RNA

RNA was obtained from tumor tissue in the following manner. Tumor was ground into a fine paste in liquid nitrogen using a mortar and pestle. Ground tissue was

lysed in TriReagent (Sigma, MO), a mixture of guanidine thiocyanate and phenol in a mono-phase solution, according to manufacturer's instructions, followed by isopropanol precipitation of RNA. RNA was washed with 75% ETOH, resuspended in diethyl pyrocarbonate (depc)-treated H_20 and quantitated by OD_{260}.

First strand cDNA

The cDNA was synthesized from purified tumor RNA using reverse transcriptase (Moloney murine leukemia virus, Gibco BRL) in a mixture of: 5 μl RNA (~1 μg RNA), 2 μl 5x first strand buffer (Promega, Madison, WI), l μl 10 mM dNTPs, (Promega, Madison, WI), 1 μl oligo$(dT)_{12\text{-}18}$ (0.5 μg, Boehringer Mannheim), 0.1 μl RNAse Inhibitor (Promega, Madison, WI) and dH2O to the final volume of 20 μl. After a 10-minute incubation at 70˚C, the reaction was placed on ice for 1-2 minutes and 5 U of M-MLV RT was added. The reaction was then incubated at 37˚C for 1 hour and heated at 99˚C for 10 minutes, and cooled to 4˚C.

Primers

The following 5' FRW1 degenerate primers for amplifying the variable heavy chains were obtained from K. Huppi (52). Four individual PCR reactions, using each of the four mouse 5' heavy chain degenerate primers paired with the appropriate isotype constant region primer, were run for each myeloma RNA. One or all primer combinations may produce successful variable heavy (V_H) region band between 400 and 500 bp in length. The mouse kappa light chain degenerate FRW1 primer (103) was paired with the kappa constant-region primer produces a band between 350 and 400 bp.

Mouse 5’ heavy chain degenerate primers:

VH-SG1	5’ GTGCAGCTKMAGSAGTCRGG 3’
VH-SG2	5’ CARCTGCARCARYCTGG 3
VH-SG3	5’ GTGAAGCTKSWSGARTCTGG 3’
VH-SG4	5’ GTYCARCTKCARCAGTCTGG 3’

Mouse 5’ Kappa chain FR1 region universal degenerate primer:

5’ Mk:	5’ GAYATTGTGMTSACMCARWCTMCA 3’

Mixed bases:

r = a, g; y = c, t; m = a, c; k = g, t; s = c, g; w = a, t; v = a, c, g; n = a, c, g t

Isotype-specific 3’ primers were designed from the first domain of the constant region of each heavy isotype gene (51). All 3’ heavy chain constant region primers include an EcoR1 cloning site (5’ gga agatct 3’) at the 5’ end. The primer used in PCR amplification depended on the previous isotyping of myelomas done using ELISA.

Mouse 3’ heavy chain constant region primers:

IgA:	5’ GATGGTGGGATTTCTCGCAGACTC 3’
IgG1:	5’ ATAGACAGATGGGGGTGTCGTTTTGGC 3’
IgG2A:	5’ CTTGACCAGGCATCCTAGAGTCA 3’

IgG2B: 5’ AGGGGCCAGTGGATAGACTGATGG 3’

IgM: 5’ GACATTTGGGAAGGACTGACTCTC 3’

Mouse 3’ Kappa chain constant region primer:

3’ Kc: 5’ GGATACAGTTGGTGCAGCATC 3’

The kappa 3’ primer (51) is from the constant region of the kappa light chain gene and also contains a SphI (5’ ggt gcatgc 3’) cloning site at its 5’ end.

PCR

Variable regions of Ig heavy and light chain genes were amplified by PCR using the cDNA and Taq polymerase (Perkin Elmer/Roche). Each PCR reaction contained: 2 μl cDNA, 20 pmol/μl each of 5’ and 3’ primers, 1 μl 10 mM dNTPs, 10 μl 10 X Taq buffer (15 mM $MgCl_2$), and an additional 2 μl of 25 mM $MgCl_2$ for a final concentration of 2mM $MgCl_2$, and 2.5 U Taq polymerase. The final volume was brought to 100 μl with depc-H_2O. The cycling program used had an initial melt at 94˚C for 3 minutes, followed by 30 cycles of 1 minute at 94˚C, l minute at 45˚C, and 2 minutes at 72˚C. Finally, the reaction was held at 72˚C for 10 minutes and cooled to 10˚C.

VH and VL PCR products were either excised from a low-melt 2% NuSieve agarose gel or directly purified using the silica based GeneClean Spin kit (BIO 101). Purified DNA was then eluted from silica in 50 μl TE buffer at 50˚C. Amersham’s Big Dye Terminating Kit was used for sequencing of purified PCR products. Variable light chain products are sequenced using the kappa 3’ constant region primer, while the cloned

variable heavy chain fragments are sequenced using M13 Forward or Reverse primers. The sequencing reaction mix was 4 μl Big Dye mix, 2 μl 3.2 pmol/μl primer, and 4 μl DNA. The cycling program for Big Dye is as follows: 25 cycles of 96˚C for 30 seconds, 50˚C for 15 seconds, and 60˚C for 4 minutes. Reaction products are filtered in Centriflex Gel Filtration Cartridges (Edge BioSystems) and lyophilized in a speed vac before being sent to the NCI Sequencing Core Facility for processing. Sequence data is analyzed and aligned using Sequencher software.

Sequence Databases

NCBI IgBLAST: http://www.ncbi.nlm.nih.gov/igblast/ is the link for the National Center for Biotechnology Information's 'Immunoglobulin Blast' website, as special search database designed to facilitate analysis of immunoglobulin sequences in GenBank. Only human and mouse germline genes are included in thee current Ig germline gene collections. As described on the NCBI website:

"IgBLAST has three functions:

1. Reports the three germline V genes, three D* and two J* genes that show the closest match to the query sequence.
2. Annotates the immunoglobulin domains (FWR1 through FWR3) according to Kabat *et al*.
3. Matches the returned hits from the nr database to the closest germline V genes, thus making it easier to identify related sequences.

* D and J genes will be reported only for nucleotide sequence query and a stretch of seven or more nucleotide identity is required for them to be reported."

Celera: http://www.celeradiscoverysystem.com/index.cfm

As stipulated in the Celera agreement: "This data was generated through the use of the Celera Discovery System and Celera's associated database.

Ig variable region sequences are searched using IgBLAST in the NCBI GenBank database to determine which variable region families the myeloma proteins are derived and against the Celera mouse database to further search for GL matches to myeloma genes.

PC-Hy and PnC *in vivo* immunizations

For immunization experiments using PC-antigens, 200 μl of either 200 μg PC-Hy/IFA, 100 μg PnC/IFA or PBS/IFA (1:2 Ag:IFA emulsion) were injected i.p into C.M167μκ Tg and non-Tg littermate mice. Mice were given a secondary boost at day 14. Day 7 and day 21 sera were collected by retro-orbital eye bleeds and analyzed by ELISA for anti-PC specific antibodies. Control sera were unimmunized mice and PBS/IFA mice, which were not significantly different. Sera were normalized before analysis in PC-specific ELISA.

PnC, or polysaccharide C component (teichoic acid) from Streptococcus pneumoniae type III bacterial cell wall, contains the PC hapten. PC-Hy is PC conjugated to the protein carrier, horseshoe crab Hemocyanin (Hy), as previously described.

R36A *in vivo* immunizations

Eight- to twelve-week-old N_{24} M167μκ Tg+ and non-Tg littermate mice were immunized with heat-killed *Streptococcus pneumonia*, R36A (kindly provided by Dr. Cliff Snapper, USUHS and John Kearney, University of Alabama at Birmingham). Aliquots of 10^9 cfu/ml R36A were thawed and 100 ml of bacteria was injected i.p. so that each mouse received a dose of 10^8 R36A at day 0 and day 7. Blood was collected by retro-orbital puncture according the NIH Animal Care guidelines on day 0, 7, 14, 21, and 28, and the serum titered onto PC-BSA-coated ELISA plates. PC-ELISA was done as described above.

Cell Culture

RPMI/10%FCS complete culture media: RPMI 1640 without Glutamine, 10% FCS, 4 mM L-Glutamine, 50 μg/ml Pen-Strep (all from Biofluids, Rockville, MD) and 0.05 mM 2-ME (Sigma, St. Louis, MO) were used for culturing cells.

Conjugation of Antibody to Sepharose Beads

Conjugation of M167 (28-6-20, anti-Vk4) and polyclonal goat anti-IgM (Southern Biotech) to sepharose beads was 2carried out according to the previously published protocol (55). Briefly, one gram of cyanogen bromide activated Sepharose 4B (Sigma) was hydrated in 50 ml of 0.001 M HCl at RT° for 15 minutes. One gram of hydrated beads is equal to 3.5 ml wet packed volume. Hydrated beads were washed thoroughly using a scintered glass filter funnel attached to a vacuum with 3 liters of 0.001 M HCl,

which removes the dextrose inhibitor found in the bead preparation. Immediately following washing, 1.34 ml of activated beads were scraped into 15 ml screw cap tubes containing 1.34 ml of the antibody to be conjugated and well mixed overnight on a rotating wheel at 4°C. The antibodies had been previously diluted to 1 mg/ml in 0.1 M $NaHCO_3$-NaCl buffer, pH 8-9. The pH of the bead/antibody slurry was checked by placing a small drop on pH paper and adjusted to pH 8-9 with 0.1 N NaOH, if necessary. The following day, beads were centrifuged at 1000 rpm for 5 minutes; the supernatant removed and read in a spectrophotometer to determine how much protein did not conjugate to the beads. Beads were then washed 2x with 0.1 M $NaHCO_3$-NaCl buffer, pH 8-9 by centrifugation and decanting of wash fluid. To block remaining conjugation sites on the beads, 1 ml of 0.5 M ethanolamine was added to beads and rotated at 4°C for 2 hours. Beads were then washed 2x with 5 ml sterile PBS and finally resuspended at 1 part beads to 1 part PBS/0.02% NaN_3. Bead preparations were sterilized for 1 hour in a 60°C waterbath.

In Vitro Stimulation of B cells with anti-M167 mAb-sepharose

The following rat anti-idiotypic antibodies conjugated to sepharose were used to stimulate Tg+ B cells: anti-M167 (28-5-15) and anti-M167 (28-6-20). Clone 28-5-15 (1:50) is M167 heavy-light chain specific, its binding to M167 can be inhibited with PC-antigen (58). Clone 28-6-20 (1:100) is specific for the Vk24 light chain in combination with any heavy chain. PC-Sepharose (1:50), IgM-sepharose (1:100), a goat anti-IgM polyclonal, and unconjugated sepharose were kindly provided by Ana Lustig, GRC, NIH. LPS (Sigma) was used as a proliferation control.

Table 19. Rat anti-idiotypic M167 monoclonal antibodies. Adapted from Sieckmann, 1997 (58).

Clone	Specificity	Isotype	
28-5-15	anti-V_H1/Vk24	IgG2b	lambda
28-6-20	anti-Vk24	IgG2a	kappa

Enriched B cells were obtained from spleens from eight- to twelve-week-old N_{26} M167μk Tg+ and non-Tg littermate mice. Lymphocytes were recovered from spleen suspensions using Ficoll (LSM, ICN Biomedicals, Aurora, OH). T cells were cytotoxically lysed using a cocktail of monoclonal rat IgM anti–thy 1.2 (H013-4, Pharmingen), rat IgG2a anti-CD4 (CT-CD4, Caltag), and rat IgG2a anti-CD8 (CT-CD8a; Caltag) with rabbit complement (Sigma, St. Louis, MO) (137). Macrophages were depleted by "panning" the spleen cells in a petri dish at 37°C for 1-2 hours. B cells from Tg+ and non-Tg mice were enriched to between 85% and 95%.

B cells were labeled with the cytoplasmic proliferation dye, CFSE (carboxyfluoresein succinimidyl ester), as follows. Cells were aliquoted at 1 x 10^7/1ml in PBS/2% FCS and incubated with 20 μl of 500 mM CFSE [10 mM final CFSE] for 10 minutes at 37°C in dark. Reactions were stopped by addition of 100ul FCS. Cells were washed 2 times with PBS/2% FCS and once in RPMI/10%FCS culture media.

CFSE labeled B cells were cultured at 2 x 10^6 cell/ml with 1000units/ml IL-4 and stimulated with 10 μg/ml LPS, PC-antigen, dilutions of R36A bacteria/ml, soluble or sepharose-conjugated anti-BCR antibodies to stimulate proliferation. Three days after *in*

vitro stimulation, cells were harvested, labeled with anti-B220-APC (BD Pharmingen) and proliferation of B cells was measured using a BD FACSCalibur. PI was used to gate out dead cells.

Statistical Analysis

Statistical analysis was performed using the one-tailed Student's t test. A value of $p < 0.01$ was considered to be significant.

APPENDIX

Chromosomal location of VH12μ Tg heavy chain

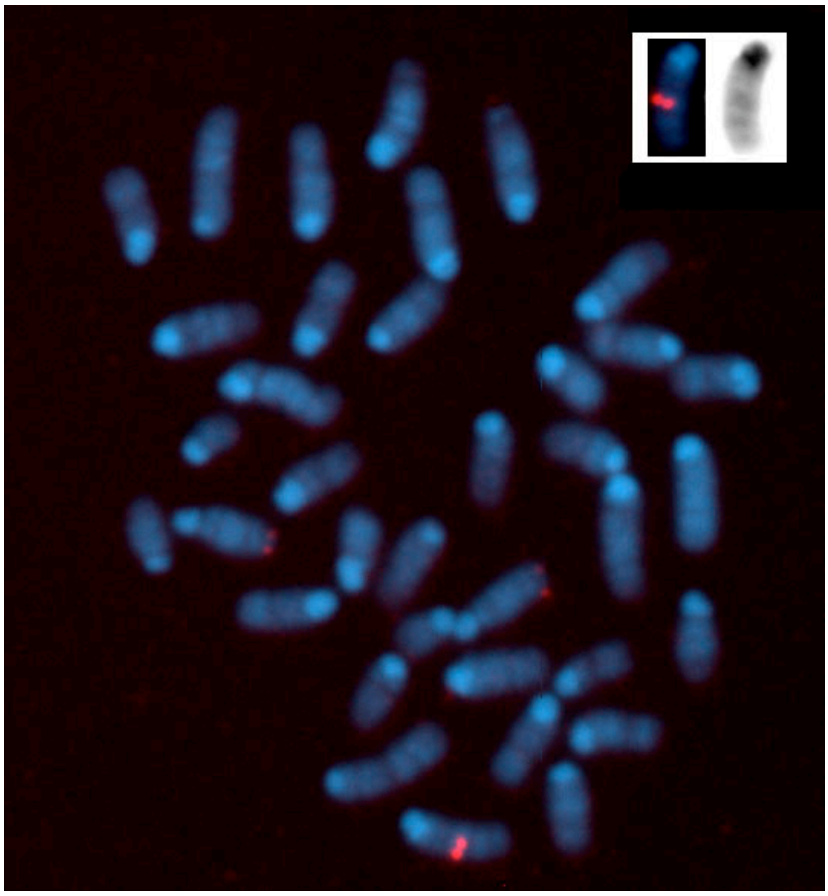


Figure 38. VH12μ Tg on murine chromosome 6. The transgene is inserted in chromosome 6 of the mouse. The VH12μ plasmid was used as a probe. FISH analysis was performed by Allen Coleman, Laboratory of Genetics, NIH.

Chromosomal location of M167μk Tg heavy and light chains

Using the original heavy chain pV167m(13) and light chain pJRD/vk 167 plasmids as probes (47), we used fluorescent *in situ* hybridization (FISH) to identify which chromosome the transgenes were inserted onto. Preliminary reports showed that the M167k Tg was inserted into either chromosome 2 or 3 (**Figure 39**, Santiago Silva, personal communication).

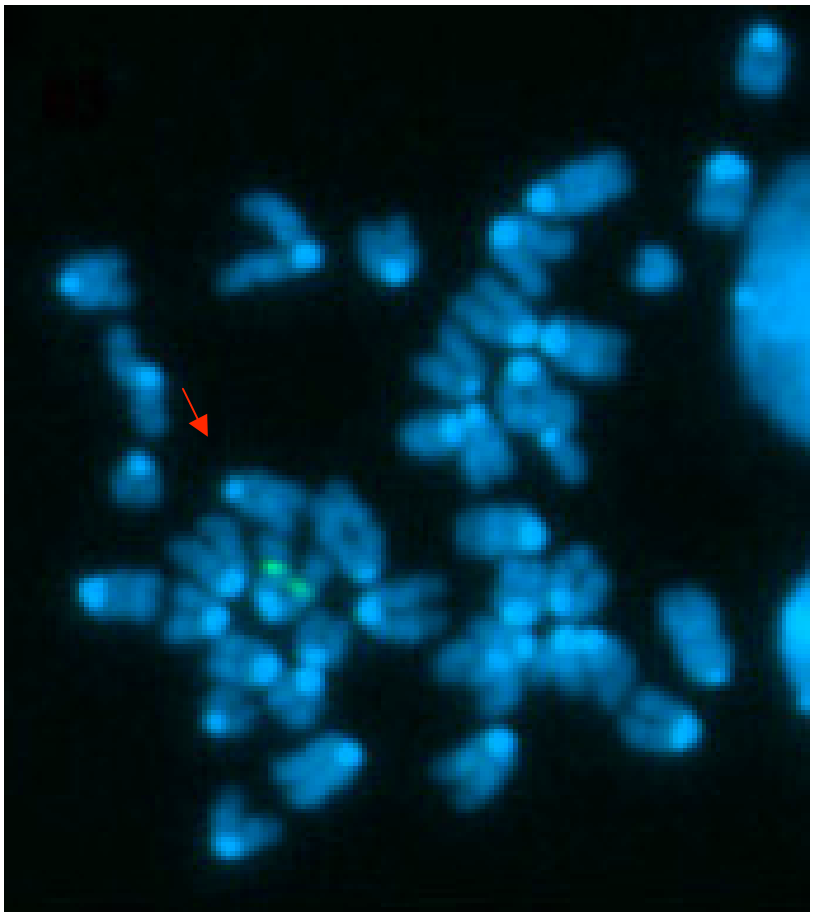


Figure 39. FISH on metaphase chromosomes from M167μk Tg B cells. The light chain pJRD/vk167 plasmid was used as a probe to locate the position of the tandemly inserted M167μk transgenes. The location of the transgene insertion has been narrowed to either chromosome 2 or 3 (Dr. Santiago Silva). The M167 heavy chain (V_H1-μ) and light chain (Vk24) have the same integration site (47).

The heavy chain gene is reported to be co-inserted with the light chain gene (47). Work on this project is still in progress. Susceptibility of mice to plasmacytomas is a complex genetic trait involving multiple loci. Resistance/susceptibility genes have been identified on chromosomes 1, 4, and 11 (51, 52, 138). The Ig loci are found on chromosomes 12 (IgH), 6 (Igk), and 16 (Igλ). The data presented here confirm that the M167 kappa transgene was not inserted on any of the Ig chromosomes or one of the chromosomes linked to PCT resistance/susceptibility genes. However, a more precise survey of the transgenes' location is necessary to rule out the possibility of an important oncogene being deregulated by their insertion.

(M167μk Tg x Bcl-xL Tg) F1 PCT pristane induction study

The Bcl-xL Tg was introduced onto the C.M167μk Tg background to test how this anti-apoptotic gene would affect pristane induction of plasmacytomas. PCTs studies in C. Bcl-xL Tg mice appeared with reduced latency periods of approximately 40 days (Potter, personal communication) and increased PCT incidence to 100% of study mice. **Figure 40** shows that the Bcl-xL Tg accelerated the development of PCTs over controls, while the double transgene (M167μk/Bcl-xL Tg) caused tumor development even earlier than the Bcl-xL Tg alone. By day 110 of this study, the M167μk/Bcl-xL double Tg mice had produced more tumors (100%) than the Bcl-xL Tg mice (95%), the M167μk Tg mice (45%), or the non-Tg BALB/c WT mice (20%).

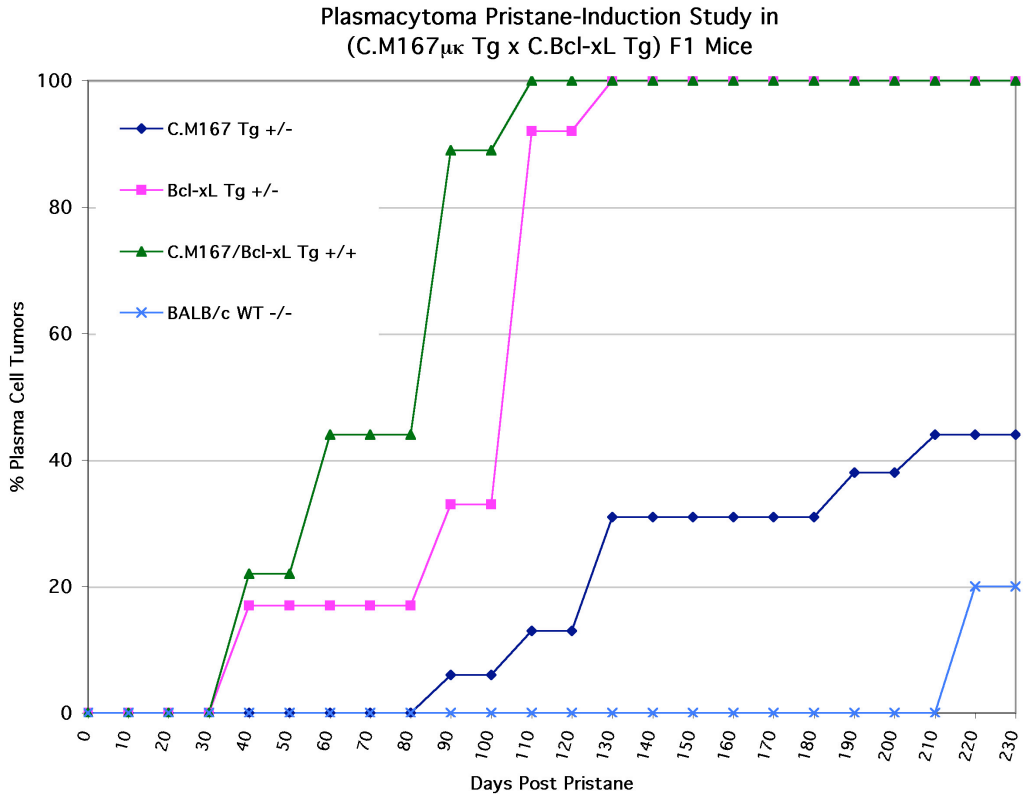


Figure 40. (C.M167μk Tg x C.Bcl-xL) F1 PCT pristane induction study results. C.M167μk Tg x C.Bcl-xL (n=9), C.M167μk Tg (n=16), C.Bcl-xL (n=12), and BALB/c non-Tg (n=5) littermates. Acceleration of PCT development occurs in the double Tg M167μk/Bcl-xL mice over Bcl-xL Tg, M167μk Tg, and BALB/c WT mice. Study also reconfirms the increased incidence and acceleration of PCTs in C.M167μk Tg over BALB/c non-Tg WT mice.

Summary of B cell Subtypes

Follicular (FO) B cells (subset of B-2 cells):

- Found in lymphoid follicles of secondary lymphoid organs: spleen, LN, PP
- Represent most of the recirculating cells in adult animals
- Naïve, resting B cell state
- sIgM ^{low} sIgD ^{high} and CD23 ^{high} CD21/CR2 ^{low}
- Develop continuously from the progenitor cells in the BM
- Contain a large repertoire of B cells that respond to BCR-dependent antigen stimulation and participate in T-cell dependent immune responses within germinal centers

- In germinal centers, intense proliferation of FO B cells results in production of high affinity antibodies through somatic hypermutation, isotype switching, and formation of memory B cells

Marginal zone (MZ) B cells (subset of B-2 cells):

- Found in the marginal zone of spleen- area surrounding the marginal sinus, bordering the white and the red pulp
- Typically 5% of splenic B220+ cells
- Do not circulate, but remain in MZ until activated
- Persist in a pre-activated state (are larger size, have less condensed chromatin, and express higher levels of B7-1 and B7-2 co-stimulatory molecules than FO B cells)
- sIgM ^{high} sIgD ^{low} and CD23 ^{low} CD21/CR2 ^{high}
- Can be induced to rapidly differentiate into plasma cells within 1-3 days upon antigen stimulation of BCR
- Have a far greater ability to proliferate and differentiate into plasma cells in response to T-independent antigens than FO B cells
- Selection of MZ B cells may require a strong BCR signaling threshold
- Positive selection of MZ B cells also appears to be based on preferential selection of certain antigen specific BCR clones
- Multi-reactive cells with "sticky" antigen receptors appear to be efficiently recruited into MZ

B-1 cells (B-1a and B-1b):

- Localized in the peritoneal and pleural cavities
- Make up only 1-5% of splenic B cells
- Develop from the neonatal repertoire, are long-lived and self-renewing
- Responsible for production of natural IgM antibodies (low antigen affinity)
- Also responsible for 50% of the intestinal IgA-secreting plasma cells
- Have little or no N sequence additions on their VDJ borders
- Express an activated phenotype
- $sIgM^{+}$, $sIgD^{+}$, $CD23^{low}$, $CD43^{+}$
- Peritoneal B-1 cells express Mac 1^{+} and $CD5^{+}$ (B-1a)
- Splenic B-1 cells are Mac 1 and CD5 negative
- Have a restricted antibody repertoire skewed toward reactivity to self antigens
- B-1 selection depends on strong self-antigen binding and low threshold signaling through the IgM receptors
- Decreased BCR activation thresholds lead to expanded B-1 populations, whereas increased signaling capabilities through BCR decrease B-1 cells

Table 20. T cell independent and dependent antigen classification.

T-cell independent		T-cell dependent
TI type 1	**TI type 2**	**TD**
B cell mitogens LPS Hsp Bacterial cell wall components	Highly repetitious molecules Polymeric proteins Bacterial cell wall polysaccharides with repeating units	Proteins
Activates both immature and mature B cells	Activates mature B cells and inactivates immature B cells	Antibody response includes isotype switching, affinity maturation and memory B cells
Requires no direct T helper cell contact or cytokines for B cell activation	No direct T helper cell contact needed, but does need T_H cytokines for efficient B cell proliferation and class switching to other isotypes than IgM	Requires T cell recognition of MHC II presented antigens on B cells to stimulate immune response
Polyclonal activators that stimulate B cells irrespective of antigenic specificity	TI-2 antigens extensively crosslink BCRs, triggering a strong signal transduction pathway to activate B cells	BCR recognizes TD antigens and are internalized for processing and presentation to T cells

T cell independent type 2 responses:

- Do not show affinity maturation
- Do not show memory B cell development
- Characterized by early B cell proliferation in all splenic compartments
- Induces differentiation of B cells into antibody-production plasma cells starting at day 3 after immunization
- Number of plasma cells falls markedly during 2nd week after immunization
- Lack of affinity maturation and memory in T1-2 responses increases the importance of initial selection of B cells producing high-affinity antibodies

REFERENCES

1. Anderson, P. N., and M. Potter. 1969. Induction of plasma cell tumours in BALB-c mice with 2,6,10,14-tetramethylpentadecane (pristane). *Nature 222:994*.
2. Potter, M., J. S. Wax, A. O. Anderson, and R. P. Nordan. 1985. Inhibition of plasmacytoma development in BALB/c mice by indomethacin. *J Exp Med 161:996*.
3. Shacter, E., E. J. Beecham, J. M. Covey, K. W. Kohn, and M. Potter. 1988. Activated neutrophils induce prolonged DNA damage in neighboring cells. *Carcinogenesis 9:2297*.
4. Potter, M., and R. C. Maccardle. 1964. Histology of Developing Plasma Cell Neoplasia Induced by Mineral Oil in Balb/C Mice. *J Natl Cancer Inst 33:497*.
5. Cancro, M., and M. Potter. 1976. The requirement of an adherent cell substratum for the growth of developing plasmacytoma cells in vivo. *J Exp Med 144:1554*.
6. Potter, M., and F. Wiener. 1992. Plasmacytomagenesis in mice: model of neoplastic development dependent upon chromosomal translocations. *Carcinogenesis 13:1681*.
7. Nordan, R. P., and M. Potter. 1986. A macrophage-derived factor required by plasmacytomas for survival and proliferation in vitro. *Science 233:566*.
8. Lam, K. P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell 90:1073*.
9. Reth, M. 1992. Antigen receptors on B lymphocytes. *Annu Rev Immunol 10:97*.
10. Wang, L. D., and M. R. Clark. 2003. B-cell antigen-receptor signalling in lymphocyte development. *Immunology 110:411*.
11. Lam, K. P., and K. Rajewsky. 1999. B cell antigen receptor specificity and surface density together determine B-1 versus B-2 cell development. *J Exp Med 190:471*.
12. Vinuesa, C. G., and M. C. Cook. 2001. The molecular basis of lymphoid architecture and B cell responses: implications for immunodeficiency and immunopathology. *Curr Mol Med 1:689*.
13. Casola, S., K. L. Otipoby, M. Alimzhanov, S. Humme, N. Uyttersprot, J. L. Kutok, M. C. Carroll, and K. Rajewsky. 2004. B cell receptor signal strength determines B cell fate. *Nat Immunol 5:317*.
14. Vinuesa, C. G., D. M. Sze, M. C. Cook, K. M. Toellner, G. G. Klaus, J. Ball, and I. C. MacLennan. 2003. Recirculating and germinal center B cells differentiate into cells responsive to polysaccharide antigens. *Eur J Immunol 33:297*.
15. Martin, F., and J. F. Kearney. 2002. Marginal-zone B cells. *Nat Rev Immunol 2:323*.
16. Potter, M. 1983. Ch 15: Immunoglobulins and Immunoglobulin Genes. In *The Mouse in Biomedical Research*, Vol. Vol. 3. H. L. Foster, Small, J.D. and Fox, J.G., ed. Academic Press, New York, p. 347.

17. McIntire, K. R., and G. L. Princler. 1969. Prolonged adjuvant stimulation in germ-free BALB-c mice: development of plasma cell neoplasia. *Immunology 17:481*.
18. Byrd, L. G., A. H. McDonald, L. G. Gold, and M. Potter. 1991. Specific pathogen-free BALB/cAn mice are refractory to plasmacytoma induction by pristane. *J Immunol 147:3632*.
19. Potter, M. 1986. *J. National Cancer Institute 39:1197*.
20. Potter, M. 1963. *J. National Cancer Institute 31:997*.
21. Potter, M. 1986. *Cancer Research 28:1891*.
22. Cebra, J. J. 1969. Immunoglobulins and immunocytes. *Bacteriol Rev 33:159*.
23. Craig, S. W., and J. J. Cebra. 1971. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. *J Exp Med 134:188*.
24. Potter, M. 1974. The Molecular Biology of Cancer, Vol. Chapter XIV, p. 535.
25. Potter, M. 1970. Experimental approaches to homogenous antibody populations. Mouse IgA myeloma proteins that bind polysaccharide antigens of enterobacterial origin. *Fed Proc 29:85*.
26. Potter, M., and R. Lieberman. 1970. Common individual antigenic determinants in five of eight BALB-c IgA myeloma proteins that bind phosphoryl choline. *J Exp Med 132:737*.
27. Leon, M. A., and N. M. Young. 1971. Specificity for phosphorylcholine of six murine myeloma proteins reactive with Pneumococcus C polysaccharide and beta-lipoprotein. *Biochemistry 10:1424*.
28. Rudikoff, S., and M. Potter. 1974. Variable region sequence of the heavy chain from a phosphorylcholine binding myeloma protein. *Biochemistry 13:4033*.
29. Rudikoff, S., and M. Potter. 1976. Size differences among immunoglobulin heavy chains from phosphorylcholine-binding proteins. *Proc Natl Acad Sci U S A 73:2109*.
30. Potter, M., and M. A. Leon. 1968. Three IgA myeloma immunoglobulins from the BALB/ mouse: precipitation with pneumococcal C polysaccharide. *Science 162:369*.
31. Harnett, W., and M. M. Harnett. 1999. Phosphorylcholine: friend or foe of the immune system? *Immunol Today 20:125*.
32. Briles, D. E., M. Nahm, K. Schroer, J. Davie, P. Baker, J. Kearney, and R. Barletta. 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 streptococcus pneumoniae. *J Exp Med 153:694*.
33. Brundish, D. E., and J. Baddiley. 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. *Biochem J 110:573*.
34. Briles, E. B., and A. Tomasz. 1973. Pneumococcal Forssman antigen. A choline-containing lipoteichoic acid. *J Biol Chem 248:6394*.
35. Horne, D. S., and A. Tomasz. 1993. Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in Streptococcus oralis. *J Bacteriol 175:1717*.
36. Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen. 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature 377:435*.

37. Shaw, P. X., C. S. Goodyear, M. K. Chang, J. L. Witztum, and G. J. Silverman. 2003. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cells. *J Immunol 170:6151*.
38. Huber, J., A. Vales, G. Mitulovic, M. Blumer, R. Schmid, J. L. Witztum, B. R. Binder, and N. Leitinger. 2002. Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. *Arterioscler Thromb Vasc Biol 22:101*.
39. Gershov, D., S. Kim, N. Brot, and K. B. Elkon. 2000. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med 192:1353*.
40. Chang, M. K., C. J. Binder, M. Torzewski, and J. L. Witztum. 2002. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc Natl Acad Sci U S A 99:13043*.
41. Lacy, E., S. Roberts, E. P. Evans, M. D. Burtenshaw, and F. D. Costantini. 1983. A foreign beta-globin gene in transgenic mice: integration at abnormal chromosomal positions and expression in inappropriate tissues. *Cell 34:343*.
42. Martin, F., and J. F. Kearney. 2000. Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and btk. *Immunity 12:39*.
43. Masmoudi, H., T. Mota-Santos, F. Huetz, A. Coutinho, and P. A. Cazenave. 1990. All T15 Id-positive antibodies (but not the majority of VHT15+ antibodies) are produced by peritoneal CD5+ B lymphocytes. *Int Immunol 2:515*.
44. Mercolino, T. J., A. L. Locke, A. Afshari, D. Sasser, W. W. Travis, L. W. Arnold, and G. Haughton. 1989. Restricted immunoglobulin variable region gene usage by normal Ly-1 (CD5+) B cells that recognize phosphatidyl choline. *J Exp Med 169:1869*.
45. Reth, M., G. J. Hammerling, and K. Rajewsky. 1978. Analysis of the repertoire of anti-NP antibodies in C57BL/6 mice by cell fusion. I. Characterization of antibody families in the primary and hyperimmune response. *Eur J Immunol 8:393*.
46. Pewzner-Jung, Y., D. Friedmann, E. Sonoda, S. Jung, K. Rajewsky, and D. Eilat. 1998. B cell deletion, anergy, and receptor editing in "knock in" mice targeted with a germline-encoded or somatically mutated anti-DNA heavy chain. *J Immunol 161:4634*.
47. Storb, U., C. Pinkert, B. Arp, P. Engler, K. Gollahon, J. Manz, W. Brady, and R. L. Brinster. 1986. Transgenic mice with mu and kappa genes encoding antiphosphorylcholine antibodies. *J Exp Med 164:627*.
48. Kenny, J. J., F. Finkelman, F. Macchiarini, W. C. Kopp, U. Storb, and D. L. Longo. 1989. Alteration of the B cell surface phenotype, immune response to phosphocholine and the B cell repertoire in M167 mu plus kappa transgenic mice. *J Immunol 142:4466*.
49. Kenny, J. J., C. O'Connell, D. G. Sieckmann, R. T. Fischer, and D. L. Longo. 1991. Selection of antigen-specific, idiotype-positive B cells in transgenic mice expressing a rearranged M167-mu heavy chain gene. *J Exp Med 174:1189*.

50. Arnold, L. W., C. A. Pennell, S. K. McCray, and S. H. Clarke. 1994. Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. *J Exp Med 179:1585*.
51. Mock, B. A., J. Hartley, P. Le Tissier, J. S. Wax, and M. Potter. 1997. The plasmacytoma resistance gene, Pctr2, delays the onset of tumorigenesis and resides in the telomeric region of chromosome 4. *Blood 90:4092*.
52. Mock, B. A., M. M. Krall, and J. K. Dosik. 1993. Genetic mapping of tumor susceptibility genes involved in mouse plasmacytomagenesis. *Proc Natl Acad Sci U S A 90:9499*.
53. Brinster, R. L., K. A. Ritchie, R. E. Hammer, R. L. O'Brien, B. Arp, and U. Storb. 1983. Expression of a microinjected immunoglobulin gene in the spleen of transgenic mice. *Nature 306:332*.
54. Pinkert, C. A., J. Manz, P. J. Linton, N. R. Klinman, and U. Storb. 1989. Elevated PC responsive B cells and anti-PC antibody production in transgenic mice harboring anti-PC immunoglobulin genes. *Vet Immunol Immunopathol 23:321*.
55. Sieckmann, D. G., K. Holmes, P. Hornbeck, E. Martin, G. Guelde, S. Bondada, D. L. Longo, and J. J. Kenny. 1994. B cells from M167 mu kappa transgenic mice fail to proliferate after stimulation with soluble anti-Ig antibodies. A model for antigen-induced B cell anergy. *J Immunol 152:4873*.
56. Desaymard, C., A. M. Giusti, and M. D. Scharff. 1984. Rat anti-T15 monoclonal antibodies with specificity for VH- and VH-VL epitopes. *Mol Immunol 21:961*.
57. Siu, G., E. A. Springer, H. V. Huang, L. E. Hood, and S. T. Crews. 1987. Structure of the T15 VH gene subfamily: identification of immunoglobulin gene promotor homologies. *J Immunol 138:4466*.
58. Sieckmann, D. G., E. Martin, G. Guelde, D. L. Longo, and J. J. Kenny. 1997. Anti-idiotype monoclonal antibodies specific for the MOPC167 anti-phosphocholine transgene-encoded antibody. *Hybridoma 16:503*.
59. Sigal, N. H., P. J. Gearhart, and N. R. Klinman. 1975. The frequency of phosphorylcholine-specific B cells in conventional and germfree BALB/C mice. *J Immunol 114:1354*.
60. Thiebe, R., K. F. Schable, A. Bensch, J. Brensing-Kuppers, V. Heim, T. Kirschbaum, H. Mitlohner, M. Ohnrich, S. Pourrajabi, F. Roschenthaler, J. Schwendinger, D. Wichelhaus, I. Zocher, and H. G. Zachau. 1999. The variable genes and gene families of the mouse immunoglobulin kappa locus. *Eur J Immunol 29:2072*.
61. Crews, S., J. Griffin, H. Huang, K. Calame, and L. Hood. 1981. A single VH gene segment encodes the immune response to phosphorylcholine: somatic mutation is correlated with the class of the antibody. *Cell 25:59*.
62. Segal, D. M., E. A. Padlan, G. H. Cohen, S. Rudikoff, M. Potter, and D. R. Davies. 1974. The three-dimensional structure of a phosphorylcholine-binding mouse immunoglobulin Fab and the nature of the antigen binding site. *Proc Natl Acad Sci U S A 71:4298*.
63. Rudikoff, S. 1983. Immunoglobulin structure--function correlates: antigen binding and idiotypes. *Contemp Top Mol Immunol 9:169*.

64. Schriever, F., and D. Huhn. 2003. New directions in the diagnosis and treatment of chronic lymphocytic leukaemia. *Drugs 63:953*.
65. Kosmas, C., K. Stamatopoulos, and D. Loukopoulos. 1997. Antigen selection of multiple myeloma clonogenic B cells as evidenced by V(H) and V(L) gene mutations. *Blood 90:1334*.
66. Haines, B. B., C. V. Angeles, A. P. Parmelee, P. A. McLean, and P. H. Brodeur. 2001. Germline diversity of the expressed BALB/c VhJ558 gene family. *Mol Immunol 38:9*.
67. Giusti, A. M., N. C. Chien, D. J. Zack, S. U. Shin, and M. D. Scharff. 1987. Somatic diversification of S107 from an antiphosphocholine to an anti-DNA autoantibody is due to a single base change in its heavy chain variable region. *Proc Natl Acad Sci U S A 84:2926*.
68. Wiens, G. D., M. Brown, and M. B. Rittenberg. 2003. Repertoire shift in the humoral response to phosphocholine-keyhole limpet hemocyanin: VH somatic mutation in germinal center B cells impairs T15 Ig function. *J Immunol 170:5095*.
69. Martin, A., P. D. Bardwell, C. J. Woo, M. Fan, M. J. Shulman, and M. D. Scharff. 2002. Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature 415:802*.
70. Klonowski, K. D., L. L. Primiano, and M. Monestier. 1999. Atypical VH-D-JH rearrangements in newborn autoimmune MRL mice. *J Immunol 162:1566*.
71. Feeney, A. J. 1990. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. *J Exp Med 172:1377*.
72. Benedict, C. L., S. Gilfillan, T. H. Thai, and J. F. Kearney. 2000. Terminal deoxynucleotidyl transferase and repertoire development. *Immunol Rev 175:150*.
73. Monroe, J. G. 2004. Ligand-independent tonic signaling in B-cell receptor function. *Curr Opin Immunol 16:288*.
74. Cabatingan, M. S., M. R. Schmidt, R. Sen, and R. T. Woodland. 2002. Naive B lymphocytes undergo homeostatic proliferation in response to B cell deficit. *J Immunol 169:6795*.
75. Binder, C. J., S. Horkko, A. Dewan, M. K. Chang, E. P. Kieu, C. S. Goodyear, P. X. Shaw, W. Palinski, J. L. Witztum, and G. J. Silverman. 2003. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. *Nat Med 9:736*.
76. Chang, M. K., C. Bergmark, A. Laurila, S. Horkko, K. H. Han, P. Friedman, E. A. Dennis, and J. L. Witztum. 1999. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A 96:6353*.
77. Shacter, E., G. K. Arzadon, and J. Williams. 1992. Elevation of interleukin-6 in response to a chronic inflammatory stimulus in mice: inhibition by indomethacin. *Blood 80:194*.
78. Cosenza, H., and H. Kohler. 1972. Specific inhibition of plaque formation to phosphorylcholine by antibody against antibody. *Science 176:1027*.
79. Lee, W., H. Cosenza, and H. Kohler. 1974. Clonal restriction of the immune response to phosphorylcholine. *Nature 247:55*.

80. Claflin, J. L., R. Lieberman, and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. I. Specificity, class, and idiotype of phosphorylcholine-binding receptors on lymphoid cells. *J Exp Med 139:58*.
81. Lieberman, R., M. Potter, E. B. Mushinski, W. Humphrey, Jr., and S. Rudikoff. 1974. Genetics of a new IgVH (T15 idiotype) marker in the mouse regulating natural antibody to phosphorylcholine. *J Exp Med 139:983*.
82. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1975. Heterogeneity of the BALB/c antiphosphorylcholine antibody response at the precursor cell level. *J Exp Med 141:56*.
83. Sher, A., and M. Cohn. 1972. Inheritance of an idiotype associated with the immune response of inbred mice to phosphorylcholine. *Eur J Immunol 2:319*.
84. Caulfield, M. J., K. J. Luce, D. Shaffer, and J. P. Lake. 1986. The antibody response to specific immune complexes is under genetic control and correlates with the expression of a recurrent idiotype. *J Exp Med 163:75*.
85. Potter, M., M. D. Sklar, and W. P. Rowe. 1973. Rapid viral induction of plasmacytomas in pristane-primed BALB-c mice. *Science 182:592*.
86. Caulfield, M. J., and D. Stanko. 1995. T-cell dependent response to immune complexes abrogates B-cell unresponsiveness to pneumococcal cell wall polysaccharide. *Immunology 86:331*.
87. O'Brien, R. L., P. Marrack, U. Storb, and J. W. Kappler. 1988. B cells expressing Ig transgenes respond to a T-dependent antigen only in the presence of Ia-compatible T cells. *J Immunol 141:3335*.
88. Kenny, J. J., R. T. Fischer, A. Lustig, H. Dintzis, M. Katsumata, J. C. Reed, and D. L. Longo. 1996. bcl-2 alters the antigen-driven selection of B cells in mukappa but not in mu-only Xid transgenic mice. *J Immunol 157:1054*.
89. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity 14:617*.
90. Goodnow, C. C., R. Glynne, S. Akkaraju, J. Rayner, D. Mack, J. I. Healy, S. Chaudhry, L. Miosge, L. Wilson, P. Papathanasiou, and A. Loy. 2001. Autoimmunity, self-tolerance and immune homeostasis: from whole animal phenotypes to molecular pathways. *Adv Exp Med Biol 490:33*.
91. Kenny, J. J., L. J. Rezanka, A. Lustig, R. T. Fischer, J. Yoder, S. Marshall, and D. L. Longo. 2000. Autoreactive B cells escape clonal deletion by expressing multiple antigen receptors. *J Immunol 164:4111*.
92. Watanabe, N., S. Nisitani, K. Ikuta, M. Suzuki, T. Chiba, and T. Honjo. 1999. Expression levels of B cell surface immunoglobulin regulate efficiency of allelic exclusion and size of autoreactive B-1 cell compartment. *J Exp Med 190:461*.
93. MacLennan, I., and C. Vinuesa. 2002. Dendritic cells, BAFF, and APRIL: innate players in adaptive antibody responses. *Immunity 17:235*.
94. Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman, and J. L. Witztum. 2000. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest 105:1731*.
95. Potter, M. 1970. Mouse IgA myeloma proteins that bind polysaccharide antigens of enterobacterial origin. *Fed Proc 29:85*

96. Fagarasan, S., and T. Honjo. 2004. Regulation of IgA synthesis at mucosal surfaces. *Curr Opin Immunol 16:277*.
97. Bos, N. A., J. J. Cebra, and F. G. Kroese. 2000. B-1 cells and the intestinal microflora. *Curr Top Microbiol Immunol 252:211*.
98. Kroese, F. G., W. A. Ammerlaan, and A. B. Kantor. 1993. Evidence that intestinal IgA plasma cells in mu, kappa transgenic mice are derived from B-1 (Ly-1 B) cells. *Int Immunol 5:1317*.
99. Martin, A., and M. D. Scharff. 2002. Somatic hypermutation of the AID transgene in B and non-B cells. *Proc Natl Acad Sci U S A 99:12304*.
100. Durandy, A., P. Revy, K. Imai, and A. Fischer. 2005. Hyper-immunoglobulin M syndromes caused by intrinsic B-lymphocyte defects. *Immunol Rev 203:67*.
101. Betz, A. G., C. Milstein, A. Gonzalez-Fernandez, R. Pannell, T. Larson, and M. S. Neuberger. 1994. Elements regulating somatic hypermutation of an immunoglobulin kappa gene: critical role for the intron enhancer/matrix attachment region. *Cell 77:239*.
102. Storb, U., K. A. Ritchie, R. O'Brien, B. Arp, and R. Brinster. 1986. Expression, allelic exclusion and somatic mutation of mouse immunoglobulin kappa genes. *Immunol Rev 89:85*.
103. Qian, Y., H. Wang, and S. H. Clarke. 2004. Impaired clearance of apoptotic cells induces the activation of autoreactive anti-Sm marginal zone and B-1 B cells. *J Immunol 172:625*.
104. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur J Immunol 27:2366*.
105. Hayakawa, K., M. Asano, S. A. Shinton, M. Gui, D. Allman, C. L. Stewart, J. Silver, and R. R. Hardy. 1999. Positive selection of natural autoreactive B cells. *Science 285:113*.
106. Martin, F., and J. F. Kearney. 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunol Rev 175:70*.
107. Wardemann, H., T. Boehm, N. Dear, and R. Carsetti. 2002. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J Exp Med 195:771*.
108. Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat Immunol 1:31*.
109. Baumgarth, N., O. C. Herman, G. C. Jager, L. Brown, and L. A. Herzenberg. 1999. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc Natl Acad Sci U S A 96:2250*.
110. Balazs, M., F. Martin, T. Zhou, and J. Kearney. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity 17:341*.
111. Lopes-Carvalho, T., and J. F. Kearney. 2004. Development and selection of marginal zone B cells. *Immunol Rev 197:192*.
112. Berek, C., and M. Ziegner. 1993. The maturation of the immune response. *Immunol Today 14:400*.

113. Kelsoe, G. 1996. The germinal center: a crucible for lymphocyte selection. *Semin Immunol 8:179*.
114. Snapper, C. M., Colino, Jesus, Khan,Abdul Q. and Wu, Zheng Qi. 2004. The Humoral Immune Response To Streptococcus Pneumoniae. In *The Pneumococcus*. E. I. Toumanen, ed. ASM Press, Washington, D.C., p. 367.
115. Goodlad, J. R., and J. C. Macartney. 1995. Germinal-center cell proliferation in response to T-independent antigens: a stathmokinetic, morphometric and immunohistochemical study in vivo. *Eur J Immunol 25:1918*.
116. Wang, D., S. M. Wells, A. M. Stall, and E. A. Kabat. 1994. Reaction of germinal centers in the T-cell-independent response to the bacterial polysaccharide alpha(1->6)dextran. *Proc Natl Acad Sci U S A 91:2502*.
117. Zhang, J., Y. J. Liu, I. C. MacLennan, D. Gray, and P. J. Lane. 1988. B cell memory to thymus-independent antigens type 1 and type 2: the role of lipopolysaccharide in B memory induction. *Eur J Immunol 18:1417*.
118. MacLennan, I. C., and Y. J. Liu. 1991. Marginal zone B cells respond both to polysaccharide antigens and protein antigens. *Res Immunol 142:346*.
119. Liu, Y. J., S. Oldfield, and I. C. MacLennan. 1988. Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones. *Eur J Immunol 18:355*.
120. Liu, Y. J., J. Zhang, P. J. Lane, E. Y. Chan, and I. C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. *Eur J Immunol 21:2951*.
121. Tangye, S. G., Y. J. Liu, G. Aversa, J. H. Phillips, and J. E. de Vries. 1998. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J Exp Med 188:1691*.
122. Klein, U., K. Rajewsky, and R. Kuppers. 1998. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med 188:1679*.
123. Dunn-Walters, D. K., P. G. Isaacson, and J. Spencer. 1995. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. *J Exp Med 182:559*.
124. Song, H., and J. Cerny. 2003. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *J Exp Med 198:1923*.
125. Dunn-Walters, D. K., L. Boursier, and J. Spencer. 1997. Hypermutation, diversity and dissemination of human intestinal lamina propria plasma cells. *Eur J Immunol 27:2959*.
126. Gardby, E., J. Wrammert, K. Schon, L. Ekman, T. Leanderson, and N. Lycke. 2003. Strong differential regulation of serum and mucosal IgA responses as revealed in CD28-deficient mice using cholera toxin adjuvant. *J Immunol 170:55*.
127. Macpherson, A. J., D. Gatto, E. Sainsbury, G. R. Harriman, H. Hengartner, and R. M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science 288:2222*.

128. Litinskiy, M. B., B. Nardelli, D. M. Hilbert, B. He, A. Schaffer, P. Casali, and A. Cerutti. 2002. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol 3:822*.
129. Weller, S., A. Faili, C. Garcia, M. C. Braun, F. F. Le Deist, G. G. de Saint Basile, O. Hermine, A. Fischer, C. A. Reynaud, and J. C. Weill. 2001. CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc Natl Acad Sci U S A 98:1166*.
130. Kruetzmann, S., M. M. Rosado, H. Weber, U. Germing, O. Tournilhac, H. H. Peter, R. Berner, A. Peters, T. Boehm, A. Plebani, I. Quinti, and R. Carsetti. 2003. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med 197:939*.
131. Fagarasan, S., K. Kinoshita, M. Muramatsu, K. Ikuta, and T. Honjo. 2001. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature 413:639*.
132. Bowman, E. P., N. A. Kuklin, K. R. Youngman, N. H. Lazarus, E. J. Kunkel, J. Pan, H. B. Greenberg, and E. C. Butcher. 2002. The intestinal chemokine thymus-expressed chemokine (CCL25) attracts IgA antibody-secreting cells. *J Exp Med 195:269*.
133. Feeney, A. J., S. H. Clarke, and D. E. Mosier. 1988. Specific H chain junctional diversity may be required for non-T15 antibodies to bind phosphorylcholine. *J Immunol 141:1267*.
134. Feeney, A. J., and D. J. Thuerauf. 1989. Sequence and fine specificity analysis of primary 511 anti-phosphorylcholine antibodies. *J Immunol 143:4061*.
135. Hu, L., L. J. Rezanka, Q. S. Mi, A. Lustig, D. D. Taub, D. L. Longo, and J. J. Kenny. 2002. T15-idiotype-negative B cells dominate the phosphocholine binding cells in the preimmune repertoire of T15i knockin mice. *J Immunol 168:1273*.
136. Wu, P., and R. E. Ward. 1993. Ig repertoire expression of BALB/c primary and secondary B cell precursors specific for phosphorylcholine. *J Immunol 150:3862*.
137. 1991. In *Current Protocols in Immunology*, p. 3.4.1.
138. Potter, M., K. K. Sanford, R. Parshad, K. Huppi, and B. Mock. 1988. Susceptibility and resistance to plasmacytomagenesis: possible role of genes that modify efficiency of chromatin repair. *Curr Top Microbiol Immunol 137:289*.