[Frontiers in Bioscience 12, 1594-1609, January 1, 2007]

Immune response to MMTV infection

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1. ABSTRACT

Mouse mammary tumor virus (MMTV) has developed a strategy of exploitation of the immune response. It infects dendritic cells and B cells and requires this infection to establish an efficient chronic infection. This allows transmission of infection to the mammary gland, production in milk and infection of the next generation via lactation. The elaborate strategy developed by MMTV utilizes several key elements of the normal immune response. Starting with the infection and activation of dendritic cells and B cells leading to the expression of a viral superantigen followed by professional superantigen-mediated priming of naive polyclonal T cells by dendritic cells and induction of superantigen-mediated T cell B cell collaboration results in long-lasting germinal center formation and production of long-lived B cells that can later carry the virus to the mammary gland epithelium. Later in life it can induce transformation of mammary gland epithelium by integrating close to proto-oncogenes leading to their overexpression. Genes encoding proteins of the Wnt-pathway are preferential targets. This review will put these effects in the context of a normal immune response and summarize important facts on MMTV biology.

2. INTRODUCTION

Pathogens have developed strategies of sabotage, stealth and exploitation to better survive in the hostile environment (1). In recent years, much has been learnt about the interaction of mouse mammary tumor virus (MMTV) with the immune system. In fact, the virus induces an extremely strong T cell-dependent B cell response due to expression of a gene in its long terminal repeat that encodes a superantigen produced in infected cells. The effect of the superantigen is to induce a strong T cell-mediated immune response. This exaggerated response, however, does not lead to the elimination of the virus in most muse strains but in the fixation of integrated viruses in long-lived memory and effector B cells (plasmocytes) and later also in T cells. Surprisingly, this response is required for efficient long-term infection of the host and therefore, MMTV exploits the immune response. In addition, the virus also uses the strategies of sabotage and stealth the latter being a result of the superantigen-mediated immune response. It uses the classical consequences of an immune response such as induction of anergy in responsive T cells (stealth) infection of dendritic cells and long-term survival in effector and memory B cells (sabotage). The virus serves as an excellent model to study

T cell-dependent immune responses as well as the innate immune response. In addition, as it can induce mammary carcinomas after integration in the proximity of proto-oncogenes, thereby inducing their overexpression it represents as a good model for cancer. MMTV biology has led to the description of a variety of host proto-oncogenes highly relevant for basic research and cancer biology.

From the discovery of spontaneous mammary tumors in mice to the description of an extrachromosomal transmission of the susceptibility it took nearly 80 years (2, 3). After the description of milk-transmission from mothers to babies it was called Bittner agent (4). To convince the research community of the retroviral origin of this agent required extensive studies as non-tumor-bearing mice also showed expression of these retroviruses as a consequence of integration of proviruses into the germline and the presence of MMTV strains with low oncogenicity (5). But finally the retroviral induction of mammary carcinomas was accepted. It again took many years to discover that the immune system plays a role in this transmission (6), and only in the 90'ies the role of the immune system was understood (For reviews see (7-9). The current knowledge on how MMTV exploits the immune response and how it is stabilized in long-lived B cells and T cells before infecting the final target, the mammary gland, is described in this review. As citing all the relevant articles would overcharge this review, the author is referred to the following reviews for older citations (9-11).

3. STRUCTURE, EXPRESSION AND TRANSMISSION OF THE VIRUS

Morphologically, due to its acentric nucleocapsid, MMTV is classified as a B-type retrovirus with immature A type particles with a diameter of 85 nm and mature particles of 105 nm are generated. These particles contain a pair of polyadenylated capped positive strand 8.5 kb long RNA molecules as well as the reverse transcriptase and integrase proteins to produce viral cDNA and to integrate it into the host DNA. Once integrated, three major mRNA molecules are produced (9kb encoding gag-protease-pol, 3.6 kb encoding env, 1.7 kb encoding the superantigen ORF (open reading frame). The longest mRNA molecules encodes the classical retroviral genes gag (p10, p21, p27, p14), protease, cleaved from the gag-protease precursor p110, p13), pol and integrase (cleaved from the gag-protease-pol precursor p160). As for other retroviruses, the pol and protease genes can only lead to functional proteins by changing the reading frame via mechanisms of slipping at stem loop structures or pseudoknots leading to 4 fold lower expression of protease and 20 fold lower expression of pol proteins than gag proteins. The 3.6 kb mRNA encodes env (cleaved from the p73 precursor into the membrane bound gp36 and gp52) (12). The gp52 determines host specificity for infection of epithelia, dendritic cells and B cells (13, 14). In addition, the nonclassical 1.4 kb mRNA encodes the superantigen (Orf) but a role for the env mRNA molecule has recently been found (15). The open reading frame protein is encoded in the 3' long terminal repeat. Several other sometimes minor mRNA molecules were found with known or unknown function. A doubly spliced mRNA has been characterized encoding a 301 amino acid protein with rev-like functions (16). It increases gag protein production. In the LTR a hormone-responsive element is found allowing increased virus production during lactation in response to glucocorticoids.

MMTV preferentially infects mouse epithelial (mammary gland, salivary gland, sebaceous gland, male reproductive organs) and lymphoid tissues (dendritic cells, B cells, later in infection also T cells, (14, 17-20). Most efficient infection occurs via milk but sporadic cases of transmission via biting have been observed. Transmission via sperm is not clearly shown (21, 22). Recently, infection of other species such as monkey and man has been described (23).

Different viruses share about 97-98% amino acid homology whereas homology for the o*rf, superantigen* proteins is lower (82.5%) with a highly variable stretch at the 3'end of the coding region (last 63-114 nucleotides, see below) (24).

There exist many integrated MMTVs (*mtv*-loci) in the genome of laboratory and wild mice, which are mostly not able to produce infectious viral particles. An example of endogenous copies after digestion of DNA with PvuII is shown. This enzyme cuts once inside most of the *mtv* loci and therefore generates 2 bands per locus in Southern blots probed with full-length MMTV probes. Frequent mutations in *env* genes rendering the loci nonproductive are found, whereas nearly all *orf* genes remained functional. Endogenous copies are, as expected, transmitted following Mendelian transmission routes. Several fully functional *Mtv*-loci have been described: *mtv-1*, *mtv-2*, *mtv-3* and *mtv-4, mtv-48*) and several of these loci can form infectious virus particles. When outbred Swiss mice, which transferred infectious MMTV(SW) virus into a BALB/c mouse colony were analyzed a new *mtv*-locus, *mtv-53*, was found that deleted the same T cell populations as the infectious copy and had the same predicted amino acid sequence (Figure 1). Most likely this endogenous copy can in some cases form infectious virus particles as not all the mice carrying the *mtv*-locus show virus expression in milk. Care has to be taken with these loci as even embryo-derived mice raised in a germ-free environment can transmit infectious viruses. Special care has to be taken in addition, with the use of foster mothers, which can carry such viruses. At least two recent isolates (MMTV(SW) and MMTV(SIM)) have been introduced into the SPF BALB/c mouse strain using outbred Swiss foster mothers (25-27). In the case of MMTV(SW) the endogenous copy *mtv-53* is most likely responsible for this transmission (Figure 1).

4. SUPERANTIGENS

In classical T cell immune responses, there is only about 1 T cell in a million that can recognize a particular MHC/peptide complex. For superantigens, the frequencies can be as high as 30%. Superantigens are microbial proteins that bind to major histocompatibility complex (MHC) class II molecules and stimulate T cells

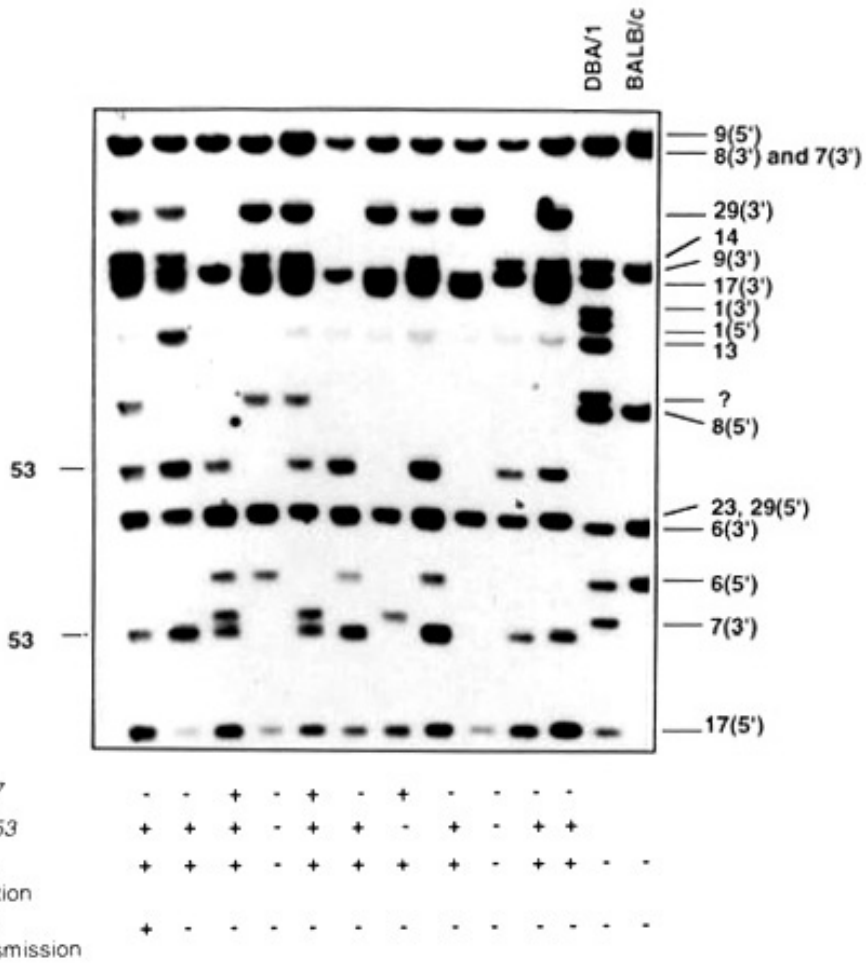
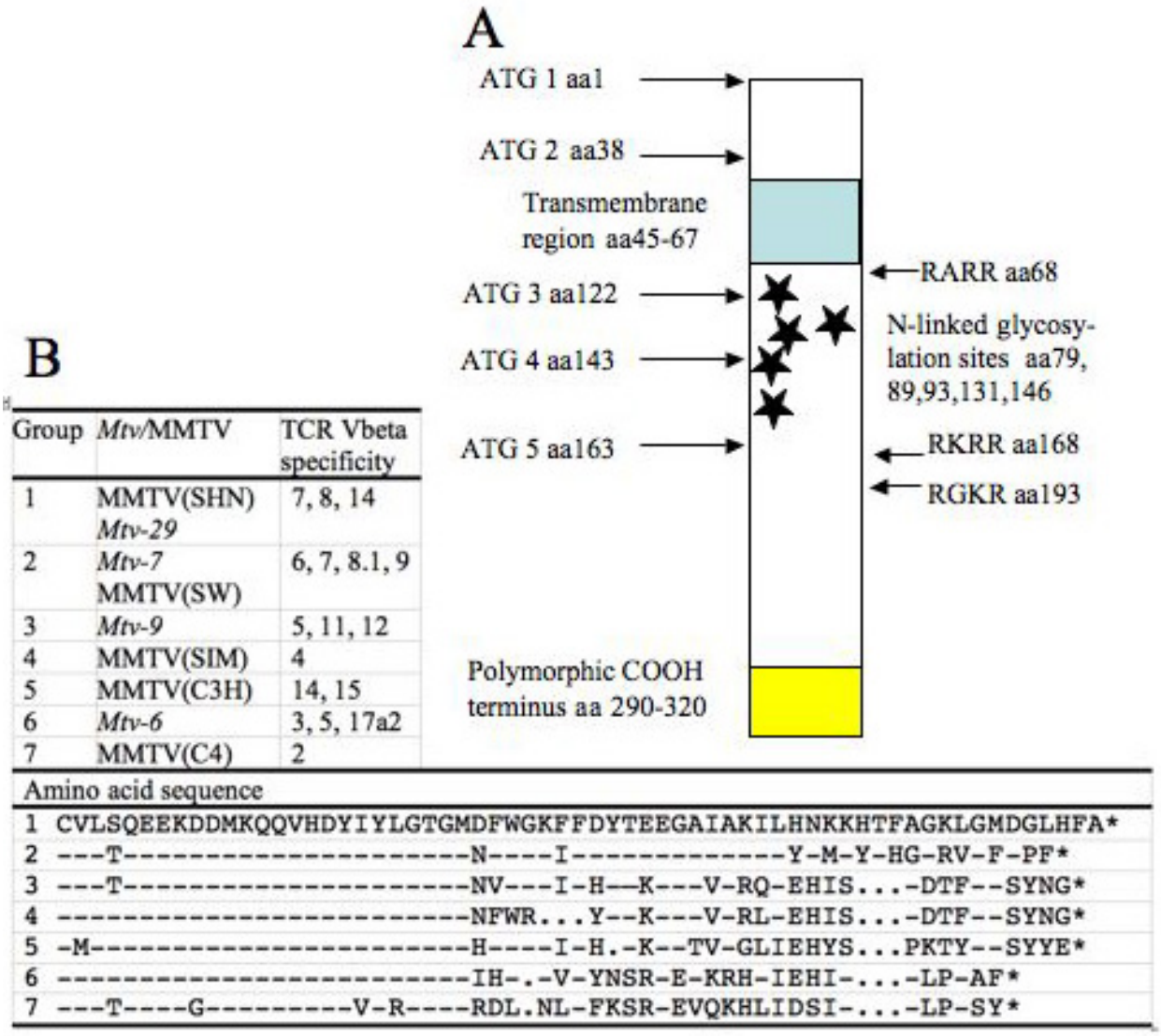


Figure 1. Comparison of endogenous mtv-loci from BALB/c, DBA/1 and outbred Swiss mice. This Swiss mouse colony was used to derive SPF mouse colonies by embryo transfer and forster nursing. It introduced MMTV(SW) into the SPF-derived BALB/c mice. A Southern blot after PvuII digestion of genomic DNA is shown. It cuts once inside the mtv-loci and generates 2 bands depending on the integration site per mtv-locus. The new mtv-53 locus is indicated to the left and the known mtv-loci to the right and the perfect correlation with deletion of T cells expressing TCR Vbeta 6, which is also deleted by the infectious MMTV(SW). Sequences of mtv-53 and MMTV(SW) were identical indicating a high likelihood that this new endogenous mtv locus can form sporadically infectious particles.

via interaction with the lateral side of the Vbeta domain of the T cell receptor (TCR). They have been characterized from many different bacterial strains (staphylococci, streptococci, mycoplasma, mycobacteria, yersinia etc.), rhabdoviruses (rabies) as well as retroviruses (MMTV, MuLV, HERV-K). Also a plant lectin with TCR Vbeta specificity has been found. Analysis by mutagenesis and co-crystallization showed that they all bind to the lateral side of MHC class II molecules and the TCR complex with high affinity. In some cases the peptide bound to the MHC can influence the TCR reactivity and only a proportion of the TCR Vbeta expressing T cells responds. For most cases, the peptide does not seem to play a major role. Therefore superantigens often bypass antigen-specificity and imitate a cognate antigen for a superantigen. They can either be secreted (bacterial exotoxins), part of the nuclear capsid (rabies) or be produced after infection and integration (MMTV).

5. THE STRUCTURE OF THE MMTV SUPERANTIGEN (ORF)

The superantigen is a type II glycoprotein, which is anchored in the membrane and therefore has its polymorphic COOH-terminus at the outside of the cell (28-32). The *orf* gene has 5 in frame start codons but superantigen mRNA starting at the second ATG has reduced functions and the shorter forms do not act as superantigens (28, 33, 34). A potential function of these shorter forms has not yet been described. Several furin-sensitive sites have been detected just outside of the transmembrane region and in the middle of the molecule but it is not clear whether these cleavage products have biological function. The presence of different cleavage products was detected in cells and association with MHC class II molecules was detected (35-37). Evidence for a role of cleavage for superantigen function was found (38).



Group	*Mtv*/MMTV	TCR Vbeta specificity
1	MMTV(SHN) *Mtv-29*	7, 8, 14
2	*Mtv-7* MMTV(SW)	6, 7, 8.1, 9
3	*Mtv-9*	5, 11, 12
4	MMTV(SIM)	4
5	MMTV(C3H)	14, 15
6	*Mtv-6*	3, 5, 17a2
7	MMTV(C4)	2

Amino acid sequence	
1	CVLSQEEKDDMKQQVHDYIYLGTGMDFWGKFFDYTEEGAIAKILHNKKHTFAGKLGMDGLHFA*
2	---T----------N----I----------Y-M-Y-HG-RV-F-PF*
3	---T----------NV---I-H--K---V-RQ-EHIS...-DTF--SYNG*
4	----------NFWR...Y--K---V-RL-EHIS...-DTF--SYNG*
5	-M----------H----I-H.-K--TV-GLIEHYS...PKTY--SYYE*
6	----------IH-.-V-YNSR-E-KRH-IEHI-...-LP-AF*
7	---T----G---------V-R----RDL.NL-FKSR-EVQKHLIDSI-...-LP-SY*

Figure 2 A) Predicted superantigen structure. The transmembrane region is indicated in light blue. The nucleotide sequence shows 5 conserved initiation codons allowing the formation of superantigen proteins with shorter NH2 protein sequences (arrows left with amino acid position (aa)). Five potential N-linked glycosylation sites are indicated with black stars. The three furin cleavage sites (RARR, RGKR) are marked with arrows from the right with the indicated amino acid positions. At the COOH-terminal end the highly polymorphic is indicated (yellow). B) The MMTVs and mtv's, TCR Vbeta specificities and amino acid sequences of the polymorphic COOH terminal sequences of representative members of the 7 described families are indicated. Dashes represent sequences identical to the MMTV(SHN) sequence, dots stand for single amino acids lacking in comparison to this sequence introduced to achieve better alignment. Always the sequence of the upper member is indicated when two members are listed. The second member differs from the first in 6-9 amino acids in this COOH-terminal sequence whereas the rest of the molecule shows high sequence homology. Sequences were from (24-26, 32, 63, 64, 108, 109).

Indirect evidence for a superantigen function of cleaved molecules came from the ability of transferring superantigens in the absence of cell contact from cell to cell (28, 39, 40).

So far 7 families of ORF/superantigen proteins have been described varying as described above strikingly in the sequence at the COOH terminus. 21-38 highly polymorphic amino acids have been found correlating well with the TCR Vbeta specificity. The predicted structure of the superantigen is shown in Figure 2 A, the COOH-terminal predicted amino acid sequence of selected members of the 7 superantigen families in Figure 2 B. Chimeric superantigens carry the TCR specificity in this polymorphic region and antibodies specific for COOH-terminal peptides block superantigen function (28, 39, 40).

6. NORMAL T-DEPENDENT IMMUNE RESPONSES

After infection with classical pathogens or injection of antigens in adjuvant subcutaneously, several waves of T cell activation have been observed in the

draining lymph node. Especially the introduction of intravital microscopy and the use of adoptively transferred naive TCR transgenic T cells have provided the tools to study the different phases of an immune response *in situ* (41, 42). Similar processes occur in Peyer's patches but this review will only focus on the best-characterized lymph node reaction. The different steps are illustrated in Figure 3. Within 30 minutes of injection, T cells in the paracortex of the lymph node interact shortly with dendritic cells and receive partial activation signals as detected by upregulation of the early activation marker CD69. Approximately 18 hours later, tissue-resident dendritic cells carry the antigens to the lymphoid tissues. This leads to prolonged T cell-dendritic cell interactions with a duration of several hours. During this process activated dendritic cells present pathogen/immunogen-derived peptides in the context of their MHC class II molecules Figure 3A,1. This interaction has been called synapse 1. T cells enter into cell cycle and differentiate into effector T cells. Depending on the signals they receive from the dendritic cells (cytokines, co-stimulation, antigen) they differentiate into cytokine-secreting effector cells with TH1 or TH2 bias. They also upregulate expression of co-stimulation molecules such as CD40, Ox40L, etc.

During this phase of the immune response, B cells become activated T-cell independently in the cortex as well as the paracortex of the lymph node by the pathogen/immunogen (Figure 3A1, (41, 43, 44). It has recently been shown that B cells require interaction with pathogens/immunogens through surface immunoglobulins as well as toll-like receptors (TLR3) to induce an efficient immune response (45). B cells internalize immunoglobulin-bound antigens and process them in early endosomes to peptides to be presented in the context of MHC class II Figure 3B. The efficiency of presenting proteins for which their immunoglobulin is specific is about 10.000 times higher than presentation of peptides of other proteins for which their immunoglobulin is not specific (46). This guarantees that the ensuing T cell-B cell interaction only helps B cells that are specific for the immunogen. Activated B cells migrate to the cortical-paracortical boundary. About three days after antigen encounter, the differentiated CD4 T cells change their migration pattern and localize to the boundary of the cortex/paracortex where antigen-primed B cells are found. This is achieved by upregulation of the chemokine receptor CXCR5 that directs them to the paracortical-cortical junction as well as to the later forming germinal centers where the chemokine CXCL13, the ligand of CXVR5, is produced (47). In the border between cortex and paracortex, T cells can interact with B cells and will provide cognate help if the peptide presented originally by dendritic cells and now by B cells is the same. This prolonged interaction is an important checkpoint to assure that only B cells recognizing the immunogen are induced to switch isotypes, somatically hypermutate to increase the affinity of their immunoglobulin and initiate germinal center formation. This interaction is called synapse 2.

At this stage B cells have to decide between differentiation into either plasmablasts, plasma cells or memory B cells. B cells can directly differentiate into plasmablasts, switching their isotype, migrating to the medulla and secreting large amounts of mostly low affinity IgM or switched isotypes (43). Alternatively, 1-3 activated B cells can induce the formation of germinal centers in the cortex Figure 3B2. There, B cells quickly divide as apoptosis-sensitive centroblasts in the dark zone of the germinal center Figure 3C. Their susceptibility for apoptosis induction is partially explained by the downregulation of the survival molecule Bcl-2. They induce isotype switching and affinity maturation, stop dividing and become centrocytes. At this stage there is another checkpoint. The centrocytes compete for recognition and endocytosis of their antigen from the local follicular dendritic cells in the light zone of the germinal center and receive for a second time cognate help from the antigen-specific follicular T cells. This step is called synapse 3 and assures that affinity maturation does not change antigen-specificity. This step is thought to be of importance to avoid autoimmunity. After receiving the required signals from the follicular helper T cells, Bcl-2 is re-induced allowing survival. Therefore, in the absence of help developing B cells die if they do not receive the appropriate signals within time.

In this third synapse, B cells differentiate into memory B cells as well as short and long-lived plasma cells depending on co-stimulation signals. Plasma cells are often returning to the bone marrow after leaving the draining lymph node (Figure 3B) to continue antibody secretion life-long whereas memory B cells recirculate or remain in the draining lymph node prepared to differentiate efficiently into centroblasts after second antigen encounter.

7. LIVE CYCLE OF MMTV, EXPLOITING THE T CELL-DEPENDENT B CELL IMMUNE RESPONSE

7.1. Transmission of infectious MMTV

MMTV is transmitted from mother to babies via milk during the first two weeks after birth before the stomach acidifies (Figure 4A) (3, 48, 49). In milk large amounts of MMTV can be found (about 10^{10} - 10^{12} particles/ml). Due to the neutral pH viruses pass the stomach unaffected and can enter via the dome region into the Peyer's patches (50). Less efficiently, they can also enter in other sites along the small intestine. Although it has never been formally shown, it is likely that they enter through M cells, which are specialized in sampling luminal components. These cells are not sheltered from the gut lumen by mucus and have hence easier access to commensals and pathogens. It has been speculated that they enter through occasional breaches in the epithelial layer. The structure of the follicle-associated epithelium is shown in (Figure 4B,C). Note the distribution of the CD11c positive dendritic cells in the FAE: In this region B cells and dendritic cells are accumulated (Figure 4B,C).

7.2. Infection of dendritic cells and B cells

Underneath the M cells Peyer's patch B cells and dendritic cells are found, which are readily infected by MMTV (Figure 5). As retroviruses require cell division for integration into the host DNA, it was a puzzle for a long

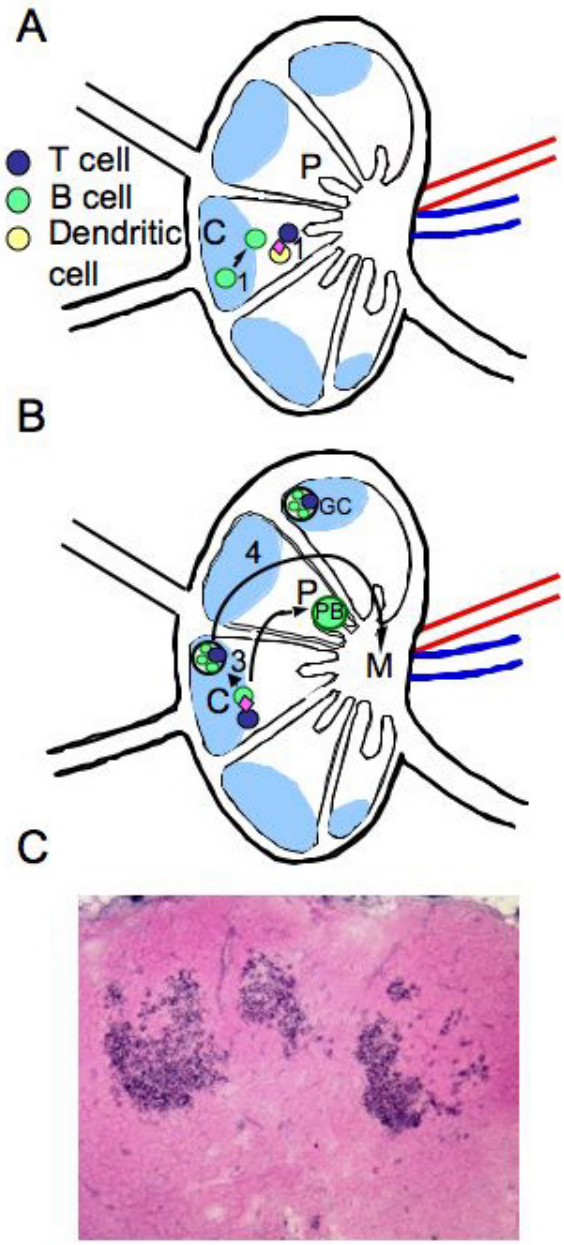


Figure 3. Stages of a normal T-cell-dependent B cell response. A) Antigen-presenting dendritic cells (DC) (yellow) enter the paracortex (P) of the draining lymph node from the periphery via lymphatic vessels and can transfer their antigen also to local DC. If they are issued from an infection they show an activated phenotype allowing priming of an effector T cell response. T and B cells are activated independently (1). T cells in the paracortex, B cells in the B cell follicles in the cortex (C). T cells make prolonged interaction with the dendritic cells to become effectors. This interaction has been termed synapse 1. B cells migrate to the cortical-paracortical junction after activation. B) Around day 3 after encountering DC, primed T cells migrate to the cortical-paracortical boundary to interact with B cells presenting the same peptide as the originally priming DC. This interaction is called synapse 2 and leads to two waves of B cell differentiation. They can differentiate directly into isotype switched plasmablasts migrating to the medulla (M) secreting IgM or switched isotypes of low affinity and being exported into the periphery or dying locally (2). They have a short lifespan. Alternatively, 1-3 B cells can initiate the formation of germinal centers (GC) in B cell follicles (3). There, they rapidly proliferate as centroblasts in the dark zone of the GC to reach 10.000 cells, switch their isotype and mutating their variable Ig regions. To survive they have to stop proliferating, take up their antigen from local stromal cells (follicular dendritic cells) in the light zone of the GC and receive a second time cognate help from T cells to rescue them from cell death and differentiate into long-lived memory or plasma cells. This interaction is called synapse 3. Long-lived plasma cells home to spleen or bone marrow (4) where they can survive life-long in niches. C) Germinal center formation. Lymph node sections were labeled with a follicular-dendritic cell-specific marker M2.

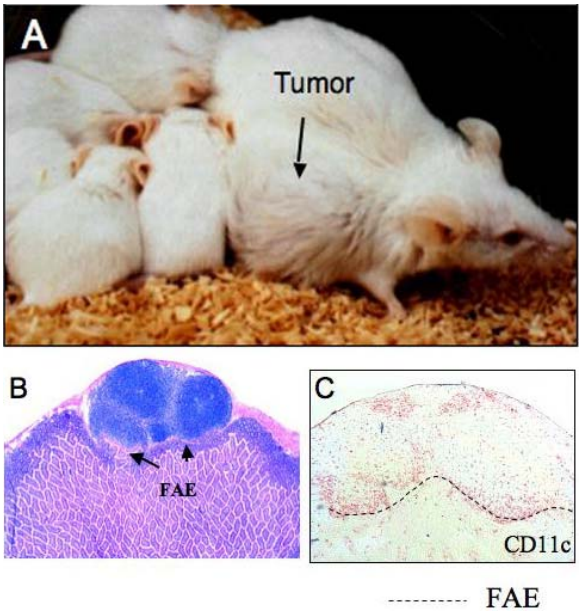


Figure 4. A. Natural transmission of MMTV from mother to babies. Infected mothers transmit MMTV to their babies during the first two weeks after birth. During lactation large amounts of viruses are produced through the action of steroid hormones. The mouse shown has a large mammary tumor but transmission occurs long before tumors appear. B). Histological sections of small intestine showing a Peyer's patches, the entry site for naturally transmitted MMTV. The follicle-associated epithelium (FAE) is indicated by arrows C. Immunohistological staining of dendritic cells in Peyer's patch and FAE (CD11c-positive dendritic cells are red). The dotted line indicates the FAE.

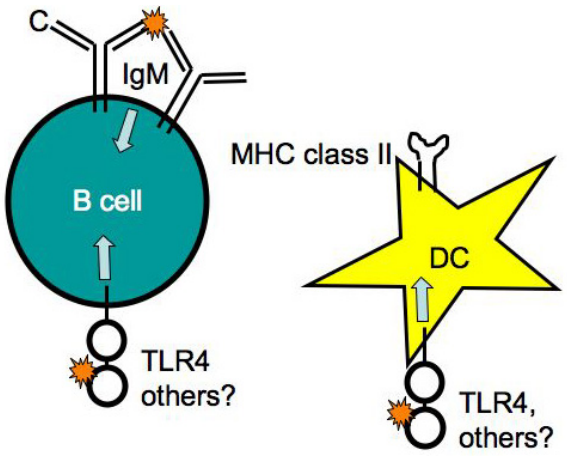


Figure 5. Activation and infection of dendritic cells and B cells. MMTV binds TLR 4 amongst other receptors. This interaction leads to activation of B cells and dendritic cells. A contribution of Ig crosslinking by MMTV has been observed (not shown). These interactions most likely allow entry into cell cycle and infection of the host cells.

time how naive B cells and dendritic cells can become infected. There are two scenarios that could explain this problem: 1) the positive strand RNA molecules introduced by the virus could be transcribed and form small amounts of superantigen and 2) MMTV induces B and dendritic cell activation with induction of cell cycle in a proportion of them. Experimental evidence for the second scenario has been obtained (51-53). Naive B cells become activated upon incubation with MMTV (51). A large proportion of B cells can be activated by MMTV and show expression of co-stimulatory molecules such as CD86. It was then shown that one of the MMTV receptors allowing B and dendritic cell infection is the toll-like receptor TLR4 also known as LPS receptor (52),(54). Other MMTV receptors have been characterized and little is known on their role in B cell activation (55, 56). Similarly, dendritic cells are activated by TLR4-MMTV interaction (53). Recent evidence also showed that dendritic cells can enter cell cycle upon activation explaining how B and T cells can become infected by MMTV. As described above, full B cell activation requires three signals: 1) immunoglobulin crosslinking, 2) TLR signals, and 3) later on CD40L signals from T cells to fully differentiate into antibody-secreting cells. MMTV may have found a strategy to induce classical B cell activation in the early phases of the immune response. Only the Ig crosslinking was lacking so far as classically only a very small proportion of B cells has a sufficiently high affinity IgM on the cell surface to recognize a single protein. This last point was recently addressed, when we could show that MMTV can interact with intermediate affinity with about 10% of peripheral B cells via their immunoglobulin (Finke and Acha-Orbea, unpublished observations) which closes the circle of the three activation steps required for optimal B cell activation provided by MMTV (for CD40L see below).

MMTV infection leads to the accelerated maturation of B cell follicles. In normal mice, B cell follicles have an immature appearance on day 11 after birth but after MMTV infection they mature much quicker and allow the required superantigen response already shortly after birth (Figure 6).

7.3. Superantigen-mediated induction of helper T cells

Evidence for endogenous loci encoding superantigens came from the observation that MHC class II I-E expressing mouse strains clonally delete whole families of specific TCR expressing T cells in the thymus during negative selection of autoreactive T cells. The major reason for this MHC class II I-E dependency turned later out to be a better efficiency of I-E to present most superantigens than I-A. Among the less efficient presenters a clear hierarchy for presentation via MHC class II I-A isotypes has been observed: I-Ak>I-Ab>I-Ad>>I-As>>>I-Aq. For the so far described MMTV superantigens I-E was always a more efficient presenter that I-A. Some superantigens are more efficient than others to associate with I-A and also I-A^q-binders have been described (Figure 7) (57, 58). Several MMTV superantigens do not even lead to thymic deletion of the superantigen-responsive T cells in the absence of I-E.

Soon thereafter, it was shown that the classical *mls* (minor lymphocyte stimulating) loci also show such a superantigen activity. *Mls* loci are sequences in the genome encoding proteins, which induce very strong mixed

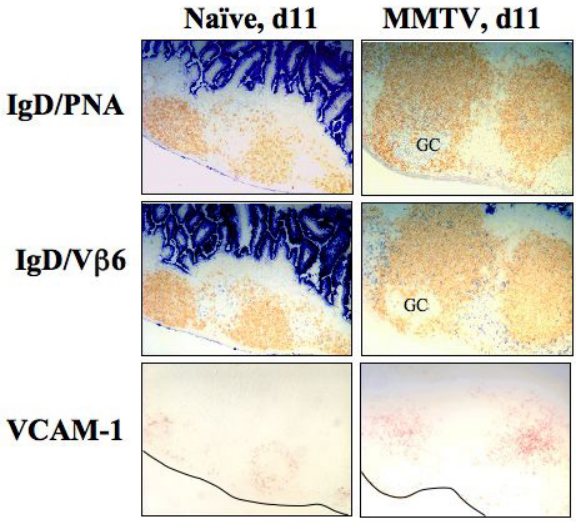


Figure 6. Accelerated maturation of Peyer's patches after MMTV infection. Peyer's patches of BALB/c mice fed on wild type or MMTV(SW) infected mothers from birth were stained with IgD. Germinal center B cells in the germinal centers (GC) are IgD-, B cells outside GC IgD+. Note the better developed B cell follicles and early formation of GC in infected mice. In the second part of the Figure the superantigen-reactive Vbeta6+ T cells are labeled in blue. They accumulate after MMTV infection. In the lowest part the faster appearance of ICAM-1, a marker for mature B cell follicles, shows accelerated maturation of B cell follicles in infected mice.

Mouse strain	*Mtv*													
	-1	-3	-6	-7	-8	-9	-11	-13	-14	-17	-23	-29	-30	-31
A/J			+		+			+			+			
AKR				+	+	+				+	+		+	+
BALB/c			+		+	+								
BALB.D2			+	+	+	+								
C3H/HeJ	+		+		+		+		+					
C57BL/6					+	+				+			+	
CBA/J			+	+	+	+			+	+				
DBA/1	+		+	+	+		+	+	+	+				
DBA/2	+		+	+	+		+	+	+	+				
MRL/lpr					+	+	+		+	+	+			
NOD		+								+				+
NZB		+		+		+			+	+			+	
NZW		+	+		+					+	+	+		+
SJL					+							+		+
129		+			+	+	+	+		+				

Figure 7. Distribution of *mtv*-loci in commonly used mouse strains. In yellow the *mtv*-loci that express superantigens that can be presented by I-E and to a lesser extent by I-A, in light blue the strictly MHC class II I-E-dependen*t* *mtv*-loci are shown. Black indicates mouse strains lacking MHC class II I-E. Expression of the indicated loci is indicated in dark blue with a + sign. Adapted with permission from (9).

lymphocyte reactions. They induced even stronger responses than MHC differences but were called minor as the unique place "major" was already taken by the MHC (59). It only later turned out that the 4 described endogenous loci (*mlsa,b,c,d*) actually were encoded by endogenous copies of MMTV (Figure 8). In this nomenclature small letters indicate differences and in the figure, the tip of the arrow means that T cells of this Mls-type are stimulated by antigen-presenting cells of the other Mls type. The nomenclature was complicated by the fact that the different *mls* genes were often present as independently segregating loci in different chromosomal localizations (60). Later, when it became clear that these were independently segregating loci and not alleles and they were subsequently named *mls-1a or b*, *mls-2a or b* etc with *a* being the stimulatory and *b* the non-stimulatory allele (60). Initial mapping studies and the observation of multiple loci with stimulatory and non-stimulatory functions in different mouse strains led to the idea that *mls* loci were retroviral insertions (61). Unfortunately from the start, *mtv*-loci were excluded despite the observed linkage correlation until mistakes in the mapping were corrected in the early 90ies. Then it was finally shown that *mls* loci were endogenous copies of MMTV (62-67). Their integrated copies were named *mtv's* followed by a dash and a number (*mtv-1* to *mtv-54* with several gaps). In laboratory mouse strains there are 2-9 endogenous copies found, in wild mice 0-20. T cells of strains not having deleted the responsive T cells will mount a very strong mixed lymphocyte reaction towards Mls-different antigen-presenting cells as most of the T cells expressing one of several Vbeta family members will recognize the superantigen. Mouse strains expressing *mtv*-loci delete superantigen-reactive T cells in the thymus (57, 62-64, 67-72). A strong expansion of superantigen-responsive CD4 and to a lesser extent CD8 T cells is observed. As many as 30% of peripheral T cells can respond to a single *mtv* difference, compared to 1-5% for allo-responses differing at the whole MHC. Therefore mouse strains expressing several *mtv* genes can have large holes in their T cell repertoire due to thymic deletion of T cells expressing superantigen-specific TCR Vbeta regions..

As described above, many different MMTV strains and *mtv*-loci have been characterized and many of the infectious and integrated forms have been sequenced, especially the open reading frame sequences. The sequences revealed large homology between the different strains (<97%) except for the 3' region of the long terminal repeat which was very heterogeneous (24). It turned out that it is this polymorphism that explains the superantigen activity and TCR specificity of the different endogenous and exogenous MMTVs and *mtv's*. In Figure 2 the strain distribution of these endogenous *mtv*-loci, their I-E dependence and their TCR specificities for commonly used mouse strains is given. The characteristics of some selected MMTV strains is illustrated in Table 1.

After infection of dendritic cells, superantigen is expressed at their cell surface bound to MHC class II and T cells expressing the corresponding Vbeta chain are activated as in classical immune responses by the professional antigen presenting cells leading to perfect synchronized activation of superantigen-reactive T cells. (Figure 9) (73). As dendritic cells become activated by MMTV they will upregulate co-stimulation molecules, may enter into cell cycle, integrate virus and produce more of the superantigen at the cell surface. Then they can prime a polyclonal, superantigen-driven CD4 T cell response characterized by the TCR Vbeta of the amplifying T cell

Table 1. Characteristics of the best described infectious MMTV strain

MMTV strain	Vbeta-specificity	I-E dependence	Extent of stimulation after injection	CD4 T cell deletion in blood, % of reactive T cells	Half-maximal deletion in blood or lymph node in weeks	Tumori-genicity, months
MMTV(C3H)	14, 15	+	+/-	75	8	High, 6-12
MMTV(SW) MMTV(JYG)	6, 7, 8,1, 9	-	++	95	5-6	Low, > 1 year
MMTV(SHN)	7, 8, 14	-	++	75	12	High, 6-9
MMTV(C4)	2	-	++	95	6	Intermediate, 10-12
MMTV(FM)	2, 8, 14	-	++	90	<8	ND
MMTV(SIM)	4	+	++	95	5-6	High, 6-12

Characteristics of the best described infectious MMTV strains after infection of BALB/c mice (neonatal or after injection into adult mice). -: I-E-independent; +: I-E-dependent; +/-: weak stimulation; ++: strong stimulation of superantigen-reactive T cells after subcutaneous injection (day 4-6) (25-27, 63, 64, 108, 109, 111-114).

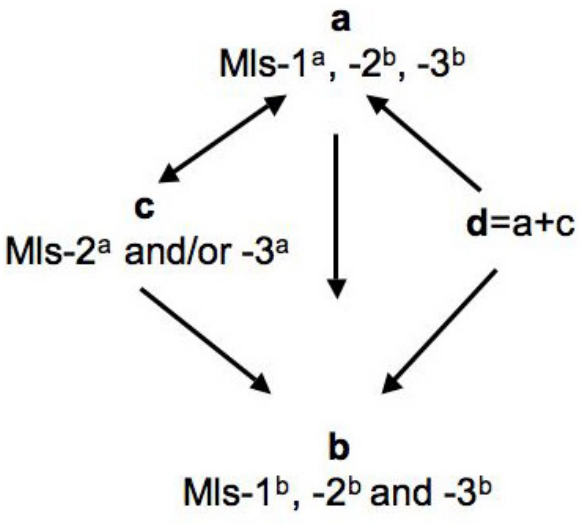


Figure 8. Classical summary of mls interaction (60). The four classical Mls-phenotypes mls a,b,c,d are shown. Arrows indicate in which direction strong mixed lymphocyte reactions are induced. The tip of the arrow points to the strain that vigorously responds to antigen presenting cells from the other strain.

repertoire. The responding T cells will mostly differentiate into TH1 cells secreting interferon-gamma. As a consequence infected B cells will preferentially switch their switch towards IgG2a in the first phase of the immune response. A proportion of T cells differentiate into TH2 cells, which is important for the germinal center reaction (74). Chronic responses are predominated by IgG1 isotypes.

Several genetic experiments have shown the key role of T cell-dependent B cell activation for establishing chronic infection: Mice lacking the following genes or cells show an absence of a superantigen response and hence only inefficiently allow chronic infection: 1) Absence of superantigen-presentation or lack of T or B cells: MHC class II or invariant chain-deficient mice or mice with MHC haplotypes not presenting superantigens. T and B cell-deficient mice (75); IgM-deficient mice, rag-deficient mice, nude mice, CD4-deficient mice (6, 75, 76). 2) mice lacking the TCR Vbeta-expressing T cells required for superantigen recognition (TCR transgenic mice, mice carrying endogenous copies of the infectious MMTV with the same TCR specificity) (13, 77). 3) Mice lacking key co-stimulation signals: CD40 or CD40L deficient mice (78).

7.4. Formation of germinal centers and long-lived memory cells

After superantigen priming by infected dendritic cells, T and B cells form the synapse 2 in the cortical-paracortical border. This interaction again is formed between superantigen presenting infected B cells and superantigen-specific T cells (Figure 10A). Except for the high frequency of reactive cells this interaction is fully equivalent to the events in classical immune responses described above. Between day 5 and 7 after virus injection large numbers of extrafollicular plasmablasts are observed in the medulla representing the extrafollicular B cell response not having passed through germinal centers. These cells have switched isotypes (IgG2a) and secrete large quantities of polyclonal immunoglobulins since all infected B cells receive superantigen-mediated help (17, 79) (80). These cells home to a variety of peripheral organs and carry integrated copies of MMTV (81). A few days later, germinal centers are formed. The majority of follicular T cells express the superantigen-specific Vbeta chain and give superantigen-mediated help to infected B cells independently on their antigen-specificity. Immunohistological stainings illustrating this point are shown in Figure 10 B. During this interaction, weak neutralizing antibody responses are generated suggesting preferential infection of virus-specific B cells (74, 82). This may rely on the fact that B cells with low affinity for viral envelope may receive a stronger activation signal leading to entry into cell cycle. At this stage the preferential help to infected B cells leads to a striking increase of infected B cells in the lymph node. Most but not all activated B cells now carry integrated proviral copies. Very few T cells are infected at this stage. Germinal centers last for several months (79). Follicular helper T cells select plasma cells and memory B cells, which now are long-lived virus carriers. As in classical responses, the response contracts but for the life-span of the mice a few percent of infected long-lived B cells are found in the B cell repertoire. In a later phase of the MMTV-induced immune response, infected T cells are also found. It has been shown that infected B or T cells can lead to infection of other subsets showing their capacity of forming infectious virus live-long (19, 83).

MMTV strain	Vbeta-specifi-city	I-E depend-ence	Extent of stimulation after injection	CD4 T cell deletion in blood, % of reactive T cells	Half-maximal deletion in blood or lymph node in weeks	Tumori-genicity, months
MMTV(C3H)	14, 15	+	+/-	75	8	High, 6-12
MMTV(SW) MMTV(JYG)	6, 7, 8,1, 9	-	++	95	5-6	Low, > 1 year
MMTV(SHN)	7, 8, 14	-	++	75	12	High, 6-9
MMTV(C4)	2	-	++	95	6	Intermediate, 10-12
MMTV(FM)	2, 8, 14	-	++	90	<8	ND
MMTV(SIM)	4	+	++	95	5-6	High, 6-12

Figure 9. T cell priming by MMTV superantigens. A) After infection of DC, superantigens and co-stimulation molecules are expressed by DC (CD80, CD86, CD40 as well as others). The superantigen associates with MHC class II and interacts with constitutively CD28 expressing CD4+ T cells expressing the corresponding TCR Vbeta. This interaction corresponds to synapse 1 in Figure 2A. (B). Activated T cells induce the expression of other co-stimulation molecules such as CD40L, which interact with CD40 expressed by DC.

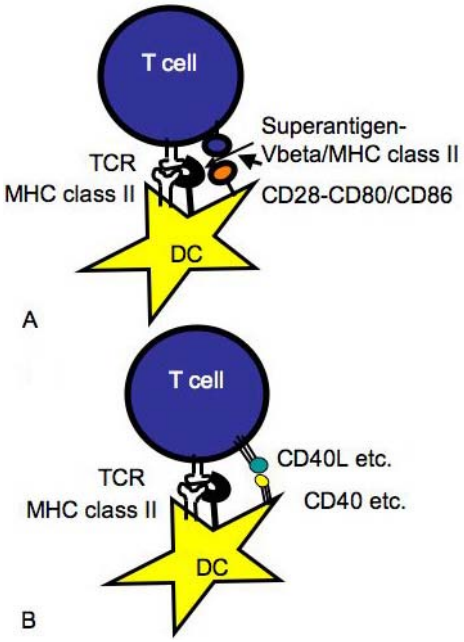


Figure 10. Around day 3 T cells form the synapse 2 with activated B cells in the paracortical-cortical region of the lymph node. This again is induced by superantigen interactions. T cells secrete cytokines such as interferon g to induce isotype switching in B cells to IgG2a. Co-stimulation via CD40-CD40L is crucial at this step. As in classical response, direct differentiation into IgG2a secreting plasmablasts around day 5-7 after virus encounter is induced as well as formation of germinal centers. B) B cells from germinal centers as described in Figure 2 but again superantigen not antigen is driving rescue of plasma cells or memory cells independently on Ig specificity of the B cells. Germinal centers appear only a week after termination of the extrafollicular response. a NP-specific immune response. CD3+ T cells in blue, proliferating centroblasts in light red. IgD in brown) b) PNA staining of MMTV-induced germinal centers 22 days after virus injection. IgD (brown), proliferation (red), germinal center (PNA, blue) c) same as b) but blue indicates staining for CD3+ T cells. d) same as b) but blue indicates staining for superantigen-reactive TCR Vbeta 6+ T cells. Note that most T follicular T cells express the superantigen-reactive TCR. Reproduced with permission from (79)).

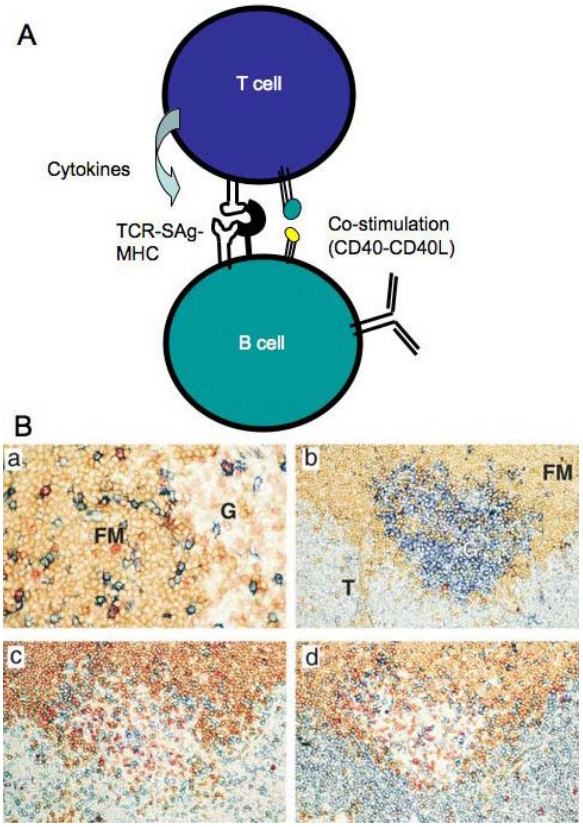


Figure 11. Deletion of superantigen-reactive T cells after MMTV injection in adult BALB/c mice. In the draining popliteal lymph node the strong expansion of superantigen-reactive T cells is observed starting around day 5, peaking on day 6-7 and slowly decreasing thereafter. It takes around 60 days to return to pre-injection levels and slowly decreases thereafter. In non-draining lymph nodes a reduction is seen as soon as 4 days and continues thereafter steadily until 90-95% of superantigen reactive T cells are deleted. Note that even after 220 days a clear difference between draining and non-draining lymph nodes is observed.

7.5. Fate of superantigen-reactive T cells

At the peak of the response, superantigen-responsive, activated T cells have expanded dramatically. They represent 10-50% of the T cells in the lymph node (25). As in classical responses, these numbers contract after the peak of the response (Figure 11). In nondraining lymph nodes and spleen the percentages slowly decrease leading to peripheral deletion of T cells carrying the superantigen-responsive TCR Vbeta. The remaining T cells became anergic and therefore cannot be restimulated to secrete cytokines or to proliferate after restimulation. It was shown that the surviving T cells express CD25, a marker for regulatory T cells (84). The deletion in the draining lymph node, however, is much slower indicating an ongoing immune response. It takes many months until their percentages reach levels observed in nondraining lymphoid organs, and there are indications that these calls can be restimulated *in vivo*.

7.6. Transmission to the mammary gland

The final target of infection is the mammary gland epithelium. For efficient infection of these cells a functional immune system is required. Mice lacking an efficient superantigen-mediated T-cell B cell interaction or crucial co-stimulation molecules cannot efficiently transfer the infection to the mammary gland. T cells and CD4 or CD8+ T cells can transfer infection to exocrine organs such as the mammary gland and to the other lymphocyte subsets. It is not clear which cells transfer the virus to the mammary gland (19).

7.7. Mammary tumors and B cell lymphomas

MMTV does not carry an oncogene. It acts as an insertional mutagen and due to the hormone-responsive element in the LTR, steroid hormones induces higher expression of neighboring genes. MMTV induces tumors by integrating in the vicinity of proto-oncogenes inducing their overexpression through their 3'LTR. In the early 80ies genetic mapping approaches allowed the characterization of regions where MMTV preferentially had inserted in developing mammary carcinomas (85, 86). The findings enforced the multi-stage cancer model. As most tumors are mono- or oligoclonal these mapping approaches were successful. At the time little was known about the discovered genes. Nowadays, several of these are at the center stage of cancer and developmental biology research. The first integration site was called int-1 and it later turned out that the gene was a close homolog of the drosophila wingless gene, which coordinates wing development in drosophila (87, 88). The gene was responsible for producing a secreted gycoprotein acting on neighboring cells. These interspecies comparisons showed that *wnt* genes are important regulators of cancer and development. In an effort to standardize the nomenclature the int-1 and *wnt* gene names were fused together to yield the *Wnt-1* gene. The Wnt pathway is involved in developmental decision and is overexpressed in a variety of cancers. Amongst the 7 *int* genes 3 are key elements of the Wnt pathways highlighting the central role that this pathway plays in cancer development (89). The int-2 gene turned out to be a member of the fibroblast growth factor familyFGF3 and other members of this family were found close to integration sites: int-8 (FGF8) and FGF2 (90). These two pathways act in synergy for mammary cancer development as elegantly shown that often integration in int-1 and in-2 regions leading to overexpression of the two genes are found in MMTV-induced mammary tumors (91). The characteristics of the known MMTV *int* sites are summarized in Table 2. Many of these proto-oncogenes have been shown to be important for human cancer development.

Integration of MMTV was found up-or downstream, sometimes even within these genes. Due to the activities of enhancers and promoters in the LTR, overexpression of these genes was routinely found. A role for an env-encoded ITAM motif in mammary carcinoma development was recently suggested (92).

MMTV rarely causes B cell lymphomas despite the vigorous proliferation induced after integration in these

Table 2. Proto-oncogenes activated by nearby insertion of MMTV in the mammary gland epithelium

Gene	Chromosomal location	Gene function	Expression pattern	Frequency of integration
Int-1/*wnt-1*	15	Wnt pathway	Adult testis, developing CNS	30–80%
Int-2/*fgf-3*	7	Growth factor	Embryonic	5–65%
Int-3/*Notch 4*	17	Notch related protein	All tissues	20%
Int-4/*wnt-3*	11	Wnt pathway	Embryonic, developing CNS	5%
Int-5/Cyp19	9	Aromatize	Breast, Ovaries, Testis	rare
Int-6/EIF3	11	Translation	All tissues	10%
Fgf-4/Hst	7	Growth factor	Embryonic	Less than10%
Wgf-8/AIGF	19	Growth factor	Ovaries, testis, embryo	10%
wnt-10b	15	Wnt pathway	Mammary gland, lung, uterus	Unknown
Fgf-10		Growth factor	Embryonic	10%

Based on (89, 115)

cells. Results indicate that transformation is actively inhibited by lymphocyte infection. Negative regulatory elements in the LTR were found, which can in part explain this cell-specific protective effect of reduced susceptibility of lymphocytes to be transformed. In thymomas and B lymphocyte tumors, repeatedly small deletions in the LTR were observed (93-96). An exceptional situation has been found in SJL mice, which develop B cell lymphomas late in life (97, 98). In these mice overexpression of *mtv-29* has been associated with lymphoma development.

7.8. Role of the superantigen response for infection

Having described the exaggerated immune response to MMTV, an obvious question arose, whether this response was helping to protect from infection or whether the virus exploited the immune response to obtain stable fixation in the host. The second possibility seemed more likely as MMTV encodes a superantigen in the LTR. To address this question either MMTV transgenic mice or congenic mice harboring a natural endogenous copy of an infectious virus were used which clonally delete the superantigen-reactive T cells in the thymus and hence are not able to mount a superantigen response (99, 100). Alternatively, mice expressing an MHC haplotype not able to present MMTV superantigens were infected (I-E-dependent superantigen in I-E-non-expressing mice) or virus transmission was studied in B, CD4 or T cell-deficient mice (6, 58, 75, 101, 102). In all these models the superantigen was, as expected, absent and the amplification of infected B cells did not take place. As a consequence virus transmission to the next generation was severely reduced despite the fact that infection still occurred. In some mice transmission to the mammary gland still occurs but less efficiently and at later time points. In the majority of mice the virus is cleared although this has not yet been convincingly shown. As mentioned above, MMTV still can reach the mammary gland in a minority of mice even in the absence of an immune response (103). Taken together, MMTV exploits the immune response and the immune response helps to fix the infection in long-lived memory B cells and plasma cells. As infected lymphocytes can transmit the virus to other lymphocytes and to epithelial cells, the maintenance of an infected pool of lymphocytes is guaranteed even after induction of anergy in the superantigen-responsive T cell population.

7.9. Role of virus neutralization and mechanisms of resistance to MMTV infection

MMTV infection induces a weak neutralizing antibody response, which is not sufficient for blocking the mammary gland infection to virus strains expressing other TCR specificities (74). Also even overexpressed endogenous MMTV does not induce complete tolerance for induction of a neutralizing immune response and does not lead to receptor interference (74, 104). As the superantigen response is not favoring antigen-specific help and affinity maturation for envelope-reactive antibodies is not selected for, this response is not optimal. Two experimental attempts have addressed the question whether an efficient anti-MMTV neutralizing antibody response can prevent transfer to the mammary gland. The first was based on the observation that injection of higher virus doses induces a stronger neutralizing antibody response (74, 82). Mice injected with high MMTV doses showed the classical even enhanced lymph node response but transmission to the mammary gland was severely impaired. This originally difficult to interpret result made sense taking into consideration the above described observation that strong neutralizing responses would block mammary gland infection. An alternative approach consisted in removing the draining popliteal lymph node once virus infected cells had populated the periphery. After removal of draining lymph nodes, the original neutralizing response was lost over time resulting in accelerating mammary gland infection (82). These results also show that the continuous presence of the draining lymph node is required to maintain a neutralizing antibody response arguing against the formation of sufficient long-lived plasma cells in the limited bone marrow niches. This is most likely another consequence of the superantigen response generating plasma cells of many specificities.

Other mechanisms of resistance have been described, few of which have been further analyzed. I/LNJ mice have a single recessive gene that prevents from MMTV susceptibility. It was shown that an increased interferon production in these mice correlates with a strong neutralizing antibody response preventing mammary gland infection (105, 106). YBR/Ei mice can be normally infected with MMTV, but they clear MMTV infection more efficiently infection than other mouse strains. In these mice virus clearance is dependent on T cells leading to reduced transmission (107).

8. CONCLUSIONS AND PERSPECTIVES

With the current knowledge on MMTV biology, it has teached us a great deal on how a retrovirus can manipulate the immune response to achieve chronic

infection in its host. Understanding its strategies it can serve as an excellent tool for a better understanding of key aspects of T cell dependent B cell activation, germinal center development, development of extrafollicular B cell responses and memory/plasma cell generation. On top of serving as a tool for inducing B cell responses, the future discoveries on the genetic factors protecting mice from chronic infections may lead to discovery of genes as important as the ones discovered through MMTV biology such as the notch/wnt-related molecules associated with cancer development.

9. ACKNOWLEDGMENTS

We would like to thank the Swiss National Science Foundation for financing our research and IFFA CREDO, L'Arlabesque, France for providing outbred Swiss mice used for foster nursing.

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Key Words: Provide a number of key words for indexing purposes.MMTV, superantigen, cancer, B cell, T cell, infection, germinal center, integration, transmission, mouse strain, T cell receptor, neutralization, Review

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