THE ABCs OF ANTIGEN PRESENTATION: DEFINING ANTIGEN PROCESSING PATHWAYS

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF ZOOLOGY AND BIOTECHNOLOGY LABORATORY

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

OCTOBER 2004

ABSTRACT

The ATP-binding cassette (ABC) transporter TAP (ABCB2/3) is an integral component of the endogenous antigen processing pathway. Without TAP, antigenic peptides cannot enter the ER to bind to class I MHC molecules *en route* to the cell surface, and as a result, no cell-mediated immune response can occur. All normal cells can present endogenous antigens, but exogenous antigen presentation is conducted solely by professional antigen presenting cells (APCs). The precise mechanism of intracellular transport of these exogenous antigens remains unknown. As TAP plays an integral role in endogenous peptide transport, it is conceivable that other ABC transporters play a similar role in exogenous antigen processing. Using RT-PCR, gene expression of all 46 known murine ABC transporters was assayed in APCs and non-APCs in response to various stimulatory cytokines and exogenous antigens. A pattern emerged, whereby APCs induced many specific ABC genes compared to non-APCs, and activated dendritic cells (DCs) expressed the highest number. Incorporating information based on the evolutionary phylogeny of sequence similarity to known antigen transporters, 18 candidate antigen presenting genes were identified.

The ABCB9 transporter gene shared the highest sequence homology to the TAP genes, was differentially expressed in activated DCs, and localized to endosome and lysosomal compartments. An ABCB9$^{-/-}$ mouse was made and characterized for an immunological phenotype. While no defect in live viral immune response was observed, a defective T-cell response to cross-presented exogenous antigens such as ovalbumin and heat-inactivated Sendai virus was discovered, but not to MHC class-II restricted antigens. This defect was traced back to an inability of ABCB9$^{-/-}$ DCs to present these antigens on the cell surface, and was consistent

with a lack of antigen transport between the endo/lysosome and the cytoplasm, as introduction of endogenous antigen corrected the defect. The contribution of the ABCB9 gene to cancer immunology was then evaluated, and it was found that upon challenge with live tumour cells, pre-immunized ABCB9$^{-/-}$ mice were no more susceptible than wild-type mice. Thus, exogenous antigen presentation is not the primary method by which a cellular anti-tumour response is primed *in-vivo*.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER II

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

LIST OF ABBREVIATIONS

(RT)-PCR	(Reverse transcriptase)-polymerase chain reaction
Ab	Antibody
ABC	ATP-binding cassette
APC	Antigen presenting cell
ATP	Adenosine triphosphate
B04	Helper T-cell clone recognizing HEL peptide on I-A^b
B16	Murine melanoma cell line (B6 background)
B220	B-cell specific marker
B3Z	CTL clone recognizing OVA peptide on H2-K^b
B-cell	Bone-marrow derived lymphocyte
C57BL/6	B6 background inbred mouse
CD	Cellular differentiation marker
CD11b	MØ marker (also found on some DCs)
CD11c	DC specific marker
CD19	B-cell specific marker
CD3	T-cell specific marker
CD4	Helper T-cell marker
CD40	DC maturation marker
CD8	Cytotoxic T-cell marker
CD80	B-7.1 'danger' signal, DC maturation marker
CD86	B-7.2 'danger' signal, DC maturation marker

cDNA	Complementary DNA
CIIV	Class II vesicle
CMT.1-4	CMT.64 transfected with TAP1
CMT.64	Murine SCLC cell line (B6 background)
CNS	Central nervous system
CTL	Cytotoxic T-lymphocytes
DC	Dendritic cell
DEC-205	DC surface receptor and maturation marker
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ER	Endoplasmic reticulum
ERAAP	ER-associated amino peptidase
ES cell	Embryonic stem cell
FITC	Fluorescein isothiocyanate fluorescent marker
H-2K^b (K^b)	Class I MHC molecule in C57BL/6 mice
HEL	Hen egg lysozyme
HI-Sendai	Heat inactivated Sendai (parainfluenza) virus
HLA	Human leukocyte antigen
i.p.	Intraperitoneal
I-A^b	Class II MHC molecule in C57BL/6 mice
IB3B	Anti-human ABCB9 specific polyclonal Ab
IFNγ	Interferon gamma
IL-2	Interleukin 2

IR	Immune response
IRE	Interferon response element
LMP	Low molecular-weight protein (part of the proteasome)
LPS	Lipopolysaccharide
Ltk	Murine fibroblast cell line
mAb	Monoclonal antibody
MDR	Multiple drug resistance
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
MØ	Macrophage
NK	Natural killer cell
NOD	Non-obese diabetic mouse model
OVA	Chicken egg ovalbumin
PE	Phycoerythrin fluorescent marker
PGP	P-glycoprotein
RNA	Ribonucleic acid
s.c.	Subcutaneous
SCLC	Small-cell lung carcinoma
Sv/Ev	B6 background inbred mouse
TAA	Tumour associated antigen
TAP	Transporter associated with antigen presentation
T-cell	Thymically educated lymphocyte
TCR	T-cell receptor

TD_{50}	Timepoint at which 50% mortality is reached
TIL	Tumour infiltrating lymphocytes
TNFα	Tumour necrosis factor alpha
VSV	Vesicular stomatitus virus
VV	Vaccinia virus
β2-m	Beta-2-microglobulin

ACKNOWLEDGEMENTS

The process of completing this dissertation has been a long and not always easy one. Many challenges had to be overcome, which has resulted in much growth, both personal and professional. Having completed this thesis feels as thought I've walked through a doorway emerging as a different person.

There are a number of people who have been very important to me during this time, and I would like to acknowledge their support. For the generation and sharing of the ABCB9$^{-/-}$ mouse, reagents and discussion, I would like to thank following individuals: V. Ling, F. Zhang, R. Wang, A. Chan, and D. Leveson-Gower. Thanks to T. Mak for his generous gift of ES cells, R. Grewal and C.D.Helgason for blastocyst injection in the generation of chimeric mice, and L. Munroe for embryo re-derivation of the ABCB9$^{-/-}$ mouse. I thank A. Jeffries and R. Gopaul for mouse breeding and technical assistance. I would like to acknowledge J. Yewdell for the kind gift of ovalbumin vaccinia-virus constructs, J. Blum for protocols, N. Shastri for making the B3Z and B04 T-cell clones available, and R. Germain for providing the 25-D1.16 antibody. Thanks to Q-J. Zhang, G. Basha and G. Lizée for discussions and procedural advice. For endless repetitions of cell purification and RT-PCR for the ABC transporter screen, I thank my project student N. Tracey. Thanks also to technicians L. Li and S. Lok, and to R. Seipp for proofreading and editing my thesis without complaint. I also want to thank my supervisor Wilf Jefferies, who has been pivotal in my growth and maturation as a scientist, and has helped guide my decisions with his advice.

On a personal level, I want to thank my husband, Ken Johnson, who has always supported my decisions, and encouraged my career without concern for himself, all the while providing a

warm and welcoming home environment. Last but not least, I would like to thank my parents, Eric and Sandra Merz who got me interested in science at an early age when they gave me my first microscope and chemistry set. Since my childhood, they have always created an environment where my goals were encouraged, but I never felt pressured. To this day, my father remains the one of the most knowledgeable people I have ever met.

CHAPTER I

WHAT DO ABC TRANSPORTERS HAVE TO DO WITH ANTIGEN PRESENTATION?

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1.1 Introduction

The immune system normally destroys diseased cells in the body by sampling antigens presented in the context of major histocompatibility molecules (MHC) on cell surfaces and generating a T-cell response against those antigens determined to be dangerous (i.e. of viral, bacterial or cancerous origin). These primed T-cells then circulate throughout the body, conducting immuno-surveillance by sampling cell surface antigens to identify and destroy diseased cells. Problems arise when components of this immuno-surveillance mechanism break down. If for some reason diseased cells are unable to present antigens, no antigen-specific immune response will take place, and the disease can progress. Indeed, many cancers and pathogenic infections occur in which known components of the antigen presentation pathway are defective (Ehrlich 1997; Gabathuler, Alimonti et al. 1998; Alimonti, Zhang et al. 2000; Tortorella, Gewurz et al. 2000). Although there are many different pathogens and genetic defects that can cause disease, there appears to be a uniform mechanism of endogenous antigen processing and presentation (Heath and Carbone 2001). Recent work in a tumour model has established that *in-vivo* correction of a known antigen presenting defect was sufficient to resurrect antigen presentation and result in elimination of the tumour (Alimonti, Zhang et al. 2000). Characterization of all the genes involved in the antigen presentation process would present an enormous potential to cure a broad spectrum of diseases through gene therapy.

1.2 Antigen presentation

Most cells throughout the body present endogenous antigens for immuno-surveillance, while specialized antigen presenting cells (APC) (macrophages (MØ), B lymphocytes (B-cells) and dendritic cells (DC)) also process and present exogeous antigens. This exogenous antigen presentation utilizes a unique mechanism, and it results in the antigen-specific priming of naïve T-cells (Heath and Carbone 2001). Currently, the full process involved in APC antigen processing and T-cell priming remains unknown.

Endogenous antigen presentation

Endogenous antigen presentation involves the processing of cytosolic antigens, such as viral antigens in infected cells or self antigens, into peptides to mount on class I MHC molecules. Cytosolic antigen is degraded by the proteasome (Hirsch and Ploegh 2000; Thery and Amigorena 2001; Yewdell and Bennink 2001) and is transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP), formed by the TAP1 (ABCB2) and TAP2 (ABCB3) subunits (Kelly, Powis et al. 1992), there to be further processed by the ER-associated aminopeptidase (ERAP) (Serwold, Gonzalez et al. 2002). The resulting peptides can then be mounted on nascent class I MHC molecules with the help of accessory molecules tapasin (Ortmann, Copeman et al. 1997), calnexin (Saito, Ihara et al. 1999), calreticulin (Sadasivan, Lehner et al. 1996) and ERp57 (Hughes and Cresswell 1998) as shown in Figure 1.1. The complete peptide-MHC complex is then moved to the cell surface to be presented to CD8+ cytolytic T-cells (CTL) for immuno-surveillance. This endogenous pathway occurs in

essentially all nucleated cells in the body, and its primary function is to present cellular antigens for immuno-surveillance by T-cells.

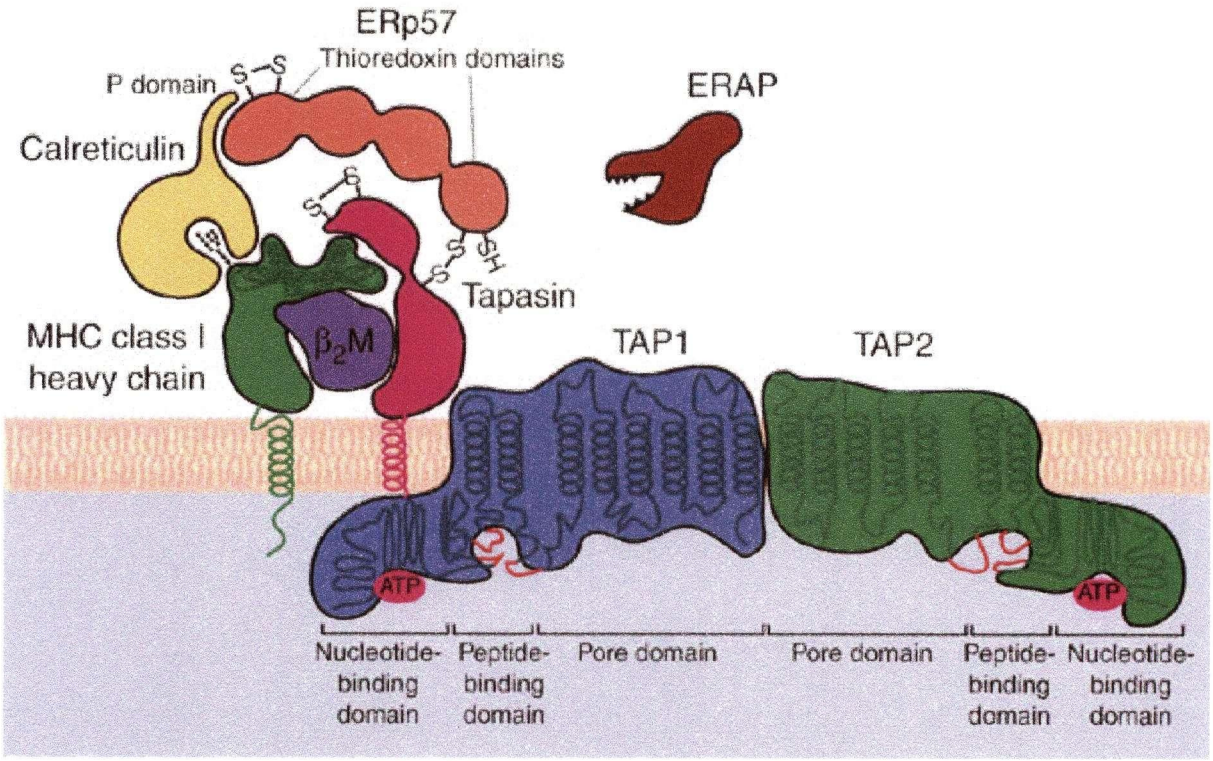


Figure 1.1. The MHC class I peptide-loading complex. ERp57, calreticulin and tapasin are all involved in facilitating loading of TAP-transported, class I restricted, ERAP-trimmed peptides onto the MHC heavy chain / β2-m complex. Possible modes of interaction are indicated (Ackerman and Cresswell 2004). Reproduced from *Nature Immunology*.

Exogenous antigen presentation

In contrast, exogenous antigens are endocytosed by APCs, where they are processed in one of several ways (Moore, Carbone et al. 1988; Trowsdale, Hanson et al. 1990; Kovacsovics-Bankowski and Rock 1995). Exogenous antigen presentation requires extracellular antigens and peptides to pass from phagosomes or endosomes into endolysosomes or MHC class II compartments (MIIC) (Peters, Neefjes et al. 1991; Amigorena, Drake et al. 1994) for exogenous presentation on class II MHC or cross-presentation on class I MHC (Bevan 1987; Bachmann, Lutz et al. 1996; den Haan, Lehar et al. 2000). In the MIIC, class II MHC restricted antigens undergo cathepsin cleavage to form peptides (Kleijmeer, Ossevoort et al. 1995; Pierre and Mellman 1998; Trombetta, Ebersold et al. 2003; Watts 2004). These peptides can then be loaded onto post-golgi class II MHC molecules for transport to the surface for presentation to CD4+ helper T-cells.

Cross-presentation

Cross-presentation is the process of presenting exogenous antigens on class I MHC molecules, previously thought to bind only endogenous antigens. Antigen presentation by dendritic cells (DCs) in the form of cross-presentation is pivotal to the initiation of T-cell mediated immunity and in particular, the priming of naïve T-cells (Banchereau and Steinman 1998; Mellman and Steinman 2001). Uptake and presentation of exogenous antigens by DCs is integral to the production of an effective adaptive immune response against viruses, bacteria, tumour antigens, as well as for the generation of tolerance to self proteins (von Boehmer and

Hafen 1986; Matzinger and Guerder 1989; Germain 1994; Kurts, Heath et al. 1996; Kurts, Carbone et al. 1997; Steinman, Turley et al. 2000; Steinman and Nussenzweig 2002).

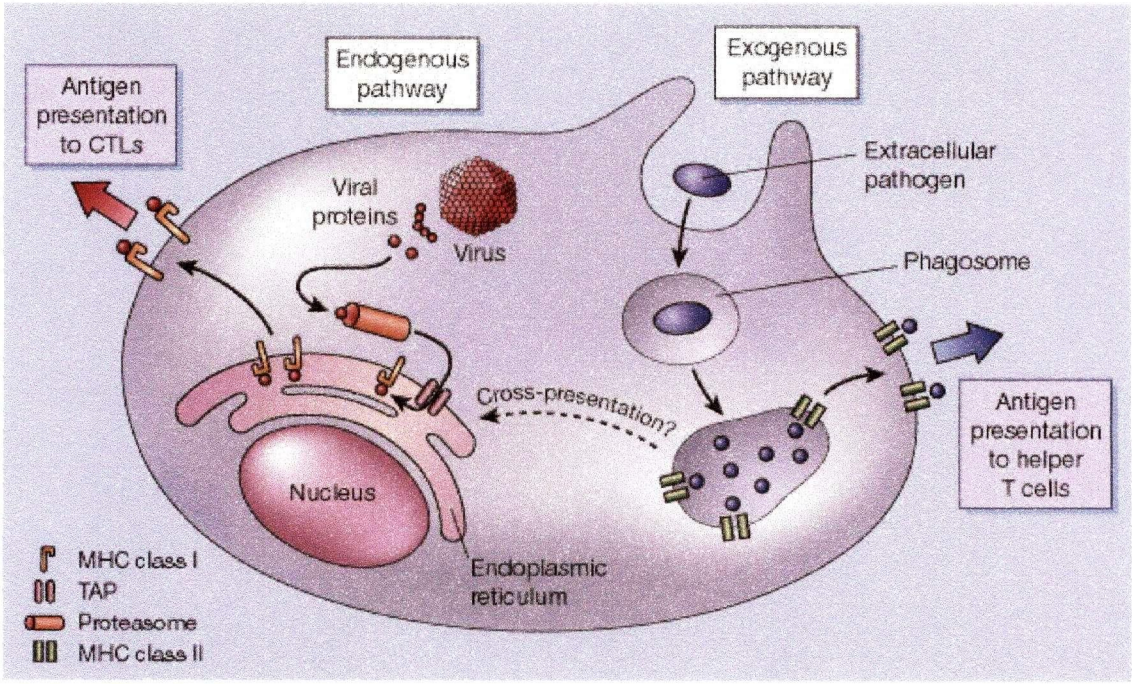
Evidence has supported three principal, non-mutually exclusive models explaining how DCs cross-present exogenous antigens on class I MHC molecules (Heath and Carbone 2001)a,b: (1) TAP-dependent ER-loaded peptides (conventional), (2) phagosome-ER fusion loading (involving cathepsins, TAP, Sec61, and proteosomes), and (3) TAP-independent endolysosome or MIIC loaded peptides (via cathepsin degradation). Specific antigens are known to undergo processing via each of these pathways as shown in Figure 1.2a:

1. The TAP-dependent pathway (Moore, Carbone et al. 1988; Kovacsovics-Bankowski and Rock 1995) processes endocytosed antigens and exogenous proteins in the cytosol (Brossart and Bevan 1997). TAP-dependent cross-presentation involves exogenous antigen uptake whereby fluid antigens or antigens taken up by receptor-mediated endocytosis are endocytosed. These antigens are then transferred into the cytosol to be degraded into peptides by the proteasome. The peptides are then transported by TAP into the ER, similarly to the well-defined endogenous antigen presentation pathway. Alternatively, at this point the peptides may enter specialized antigen presenting compartments, such as the endolysosome or MIIC, where they can access post-golgi class I MHC molecules and then proceed to the cell surface.

2. Cross-presentation via the ER-phagolysosome has recently been demonstrated in DCs (Ackerman, Kyritsis et al. 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003). The phagolysosome is a compartment capable of phagocytosing particulate antigens or dying cells, translocating those to the cytoplasm via Sec61 for degradation by the proteasome, and passing the generated peptides back into the phagosome through localized TAP transporters for access to class I MHC (Figure 1.2b).

3. A third pathway has been described as the TAP-independent pathway (Bachmann, Oxenius et al. 1995; Liu, Zhou et al. 1995; Gromme and Neefjes 2002). In this pathway, endocytosed exogenous proteins move into MIIC vesicles or endolysosomal compartments, where antigens are degraded by proteolytic cathepsins, similarly to the class II MHC restricted antigen presentation pathway (Amigorena, Drake et al. 1994; Mellman, Pierre et al. 1995). Peptides generated in this way are then able to access class I MHC molecules in these secretory vesicles, which have presumably been recycled from the cell surface or post-golgi membrane. Additional support for this pathway comes from recent work, showing that MHC I molecules lacking a sorting signal within their cytoplasmic domain renders them unable to access this endolysosomal compartment and are thus incapable of cross-presenting antigens (Lizee, Basha et al. 2003).

a


Antigen presentation to CTLs
Endogenous pathway
Exogenous pathway
Extracellular pathogen
Viral proteins
Virus
Phagosome
Cross-presentation?
Antigen presentation to helper T cells
Nucleus
Endoplasmic reticulum
MHC class I
TAP
Proteasome
MHC class II

b

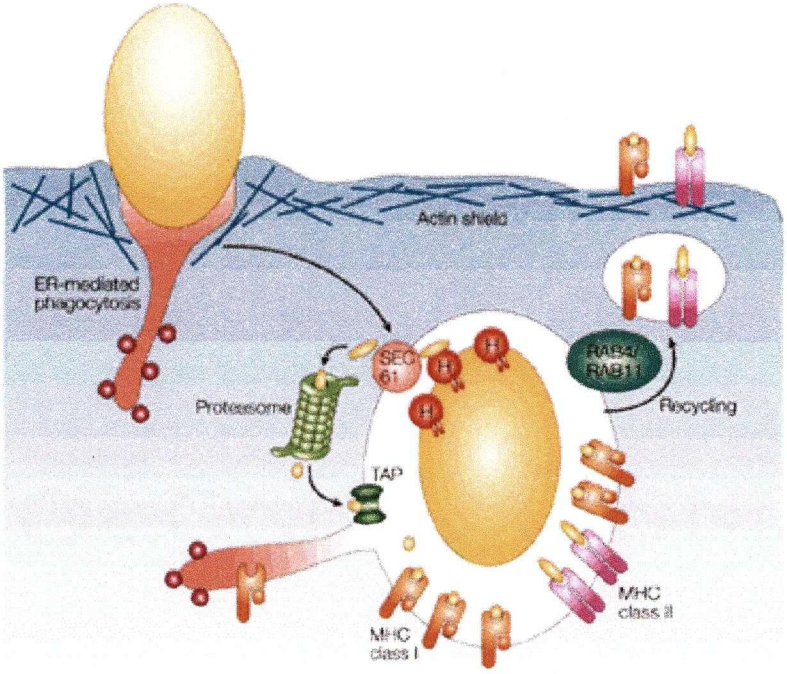

Actin shield
ER-mediated phagocytosis
SEC 61
Proteasome
TAP
RAB4/ RAB11
Recycling
MHC class II
MHC class I

Figure 1.2. ER-phagosome cross-presentation. Professional antigen presenting cells process intracellular and extracellular pathogens differently. a) In the endogenous pathway, proteins from intracellular pathogens, such as viruses, are degraded by the proteasome and the resulting peptides are shuttled into the endoplasmic reticulum by TAP proteins. These peptides are loaded onto MHC class I molecules and the complex is delivered to the cell surface, where it stimulates cytotoxic T-lymphocytes (CTLs) that kill the infected cells. In contrast, extracellular pathogens are engulfed by phagosomes (exogenous pathway) (Roy 2003). Reproduced from *Nature*. b) Inside the phagosome, the pathogen-derived peptides are either loaded directly onto MHC class II molecules, which activate helper T-cells that stimulate the production of antibodies, or degraded via the proteasome and cross-presented on MHC class I molecules (Desjardins 2003). Reproduced from *Nature Immunology Reviews*.

Antigen presentation pathways for common antigens

Different pathogens or antigens are processed and presented in different ways. Viral infections, such as vesicular stomatitis virus (VSV), are injected directly into the cell cytoplasm and are processed through the endogenous pathway (endogenous Class I). DC cross-presentation of these endogenous viral antigens from phagocytosed infected cells is required to prime the anti-viral immune response (Sigal, Crotty et al. 1999; Sigal and Rock 2000). Chicken egg ovalbumin (OVA) is taken up exogenously by endosomes and cross-presented by DCs in a

TAP-dependent manner. The immunodominant H-2K^b restricted peptide, OVA$_{(257-264)}$ is generated in the cytoplasm via proteasome-dependent processing (exogenous class I) (Moore, Carbone et al. 1988; Kovacsovics-Bankowski and Rock 1995). Heat-inactivated Sendai virus (HI-Sendai) is an endocytosed antigen cross-presented in a TAP-independent manner. This antigen produces an H-2K^b restricted immunodominant peptide, Sendai$_{(324-332)}$, which is processed through the endosome to endolysosome pathway (exogenous class I) (Bachmann, Oxenius et al. 1995; Liu, Zhou et al. 1995; Gromme and Neefjes 2002). Hen egg lysozyme (HEL) is another exogenous protein, endocytosed and processed in the endosome to lysosome or MIIC pathway, producing an immunodominant class II MHC restricted peptide, HEL$_{(74-88)}$ on the I-A^b class II molecule (exogenous class II). These pathways are represented graphically in Figure 1.3 below, reproduced from *Nature Immunology Reviews*.

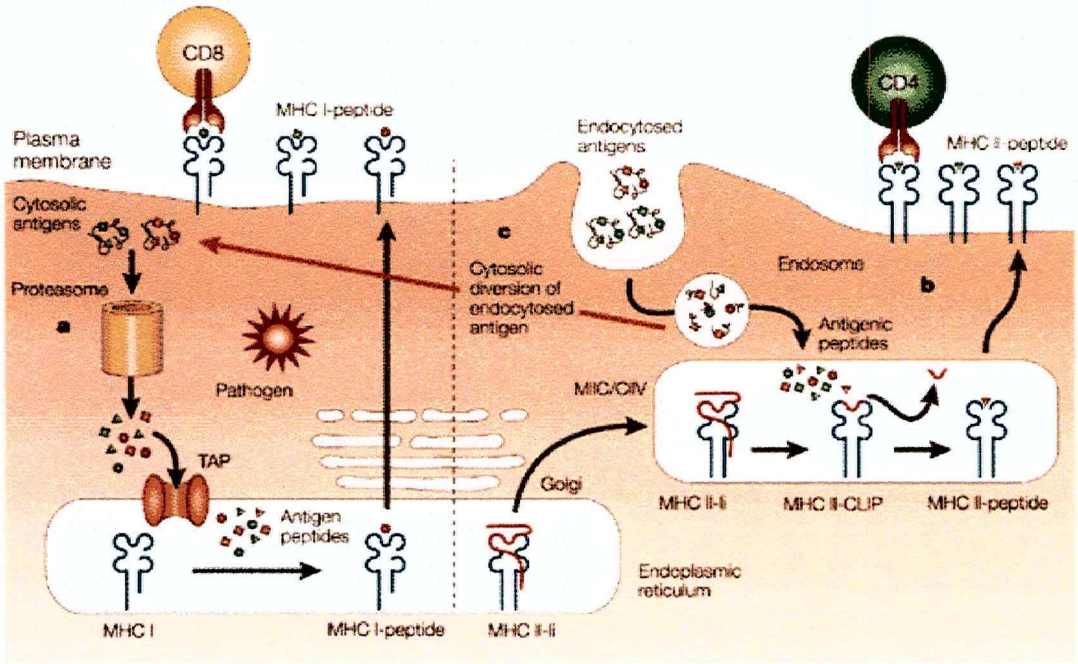


Figure 1.3. Antigen processing and presentation pathways. (a) Endogenous antigens, such as viral antigens in infected cells, are presented on ER-localized class I MHC via the TAP transporter. (b) Exogenous 'TAP-independent' antigens are presented on class II or cross-presented on class I MHC molecules. (c) Exogenous 'TAP-dependent' antigens are cross-presented on class I MHC (Heath and Carbone 2001)a. Reproduced from *Nature Immunology Reviews*.

Cytokine production and activation of APC

The cellular immune system, and in particular the APCs, act in response to stimulatory cytokines. Infection by different pathogens generates an immune response, which results in production of different cytokines within the body. Interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) are generally produced in response to various DNA and RNA-based viral infections (Wong and Goeddel 1986). Both of these cytokines have been shown capable of activating antigen presenting cells, and DCs in particular (Brossart and Bevan 1997). Upon activation, the B7 family (CD80, CD86) of co-stimulatory 'danger' signals are upregulated, along with CD40 and DEC-205 in DCs, and antigen presentation is maximized through a marked increase in both class I and class II MHC expression at the cell surface, as shown in Figure 1.4. It is in this state, through the combined expression of MHC-bound antigen and co-stimulatory signals, that APCs are able to prime an antigen-specific T-cell immune response.

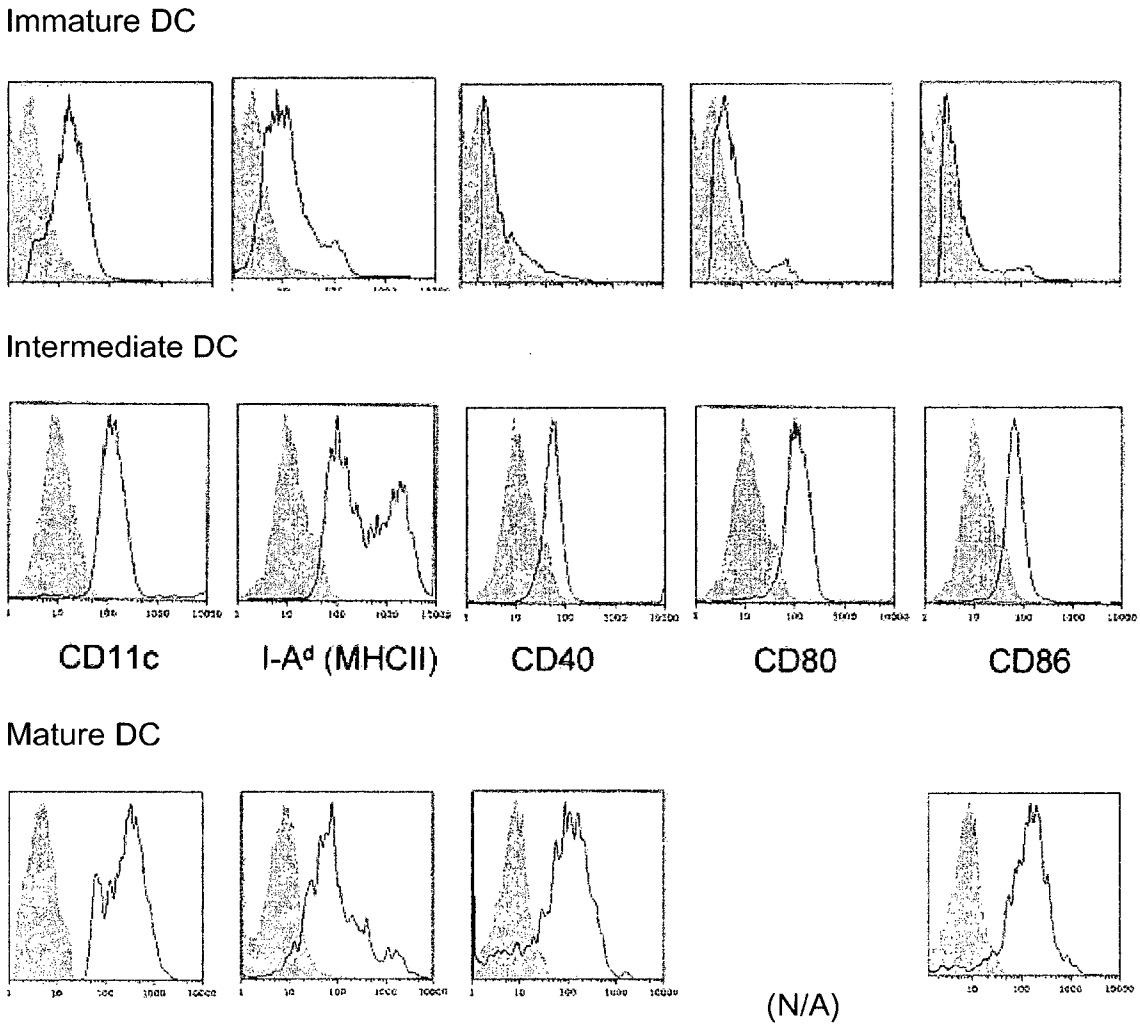


Figure 1.4. Dendritic cell activation markers. Fluorescent activated cell sorting (FACS) analysis of DC (CD11c) cells, demonstrating the upregulation of DC cell surface markers as the cells progress from immature DCs (CD40-, CD80-, CD86-, MHC-II lo), through intermediate DCs (CD40 lo, CD80 lo, CD86 lo, MHC-II mid), and into mature 'activated' DCs (CD40 hi, CD80 hi, CD86 hi, MHC-II hi). Shaded histograms show negative staining controls, thin black outlines show stained cells (Calder, Liversidge et al. 2004). Reproduced from *Journal of Immunological Methods*.

1.3. ATP-binding cassette transporters

The ATP-binding cassette (ABC) transporter family of proteins is the largest known group of transmembrane (TM) proteins. Well represented in organisms ranging from bacteria to humans, the predicted numbers of ABC transporters range from 29 in yeast (Michaelis and Berkower 1995; Decottignies and Goffeau 1997) to as many as 114 in the plant *Arabidopsis* (Dean, 2002), with humans and mice numbering in the middle, with an estimated 48-51 each (Dean, Rzhetsky et al. 2001; Dean 2002). Structurally, functional ABC proteins are comprised of 2 TM regions, each several (6-7) membrane-spanning alpha-helices long, which determine the substrate specificity; TM domains are paired together with 2 cytosolic nucleotide binding domains (NBD), which bind ATP and power the transport (Higgins 1992; Childs and Ling 1994; Dean and Allikmets 1995; Dean 2002). ABC transporters generally take 1 of 2 forms: a 'full' transporter, in which 1 gene encodes both TM and NBD paired regions, or 'half' transporters, coding for 1 NBD and 1 TM.

Half-transporters must pair up to form homo- or hetero-dimers to function. In prokaryotes, ABCs are generally embedded in the cellular membrane and function to transport metabolic substrates into the cell from the surrounding environment. Most of the known functions of ABC transporters in eukaryotes are to shuttle compounds throughout the cell or from inside the cell out to the extracelluar environment for metabolic, protective or immunological purposes (Dean 2002).

ABC transporter proteins have been localized to many different subcellular compartments within cells from phylogenetically diverse groups. The physiological functions for most of the

48-51 transporters found in mammals remain unknown, with the exception of a few, including the peptide transporters TAP1 and TAP2. Recent research has shown a peptide-transport role for an ABC transporter in an organelle other than the ER. In yeast, the ABC transporter Mdl1 is located in mitochondria and has been shown to transport self peptides from the mitochondria into the cytosol (Young, Leonhard et al. 2001).

ABC transporters are involved in processes as diverse as lipid and sterol transport, drug efflux, eye colour pigment movement, and peptide or chemical transport (Dean 2002). The downstream results of these processes become very apparent once ABC function becomes disrupted. Such human diseases as cystic fibrosis, cholesterol defects, anemia, and multidrug resistance in cancerous tumours are all attributable to abnormal ABC transporter expression.

1.4 Rationale and hypotheses

Rationale

The goals of this dissertation were to further clarify the cell biological mechanisms by which exogenous antigen presentation occurs, by identifying candidate antigen presenting genes and characterizing those for their role in this process. I have selected one particular family of genes to evaluate, the ABC transporters. There is precedence for the involvement of ABC transporters in antigen processing, in the well-characterized movement of cytosolic peptides through the TAP transporters. TAP has been shown to be crucial for endogenous antigen

movement from the cytosol into the ER, and its presence is required for peptides to access MHC class I molecules prior to surface expression (Moore, Carbone et al. 1988; Trowsdale, Hanson et al. 1990; Kovacsovics-Bankowski and Rock 1995). The existence of the Mdl1 mitochondrial peptide transporter in eukaryotic cells (Young, Leonhard et al. 2001) provides precedent for the existence of additional peptide transporters in organelles other than the ER.

The different pathways of exogenous antigen presentation require antigenic movement through subcellular compartments (Heath and Carbone 2001). This suggests the need for additional transporter involvement. Since antigens and peptides must be transported between intracellular vesicles, and TAP and Mdl1 are known to transport endogenous peptides, the family of ABC transporters may contain other such peptide-transport proteins.

Of the 48 putative murine ABC transporter genes, on a phylogenetic basis, ABCB9/TAPL (TAP-like) shows exceptional similarity to the two known TAP antigen-presenting transporters (Zhang, Zhang et al. 2000). Since conserved structure is often indicative of conserved function, this gene holds the most interest for a potential role in antigen presentation. There are however several notable differences between ABCB9 and the TAP genes. ABCB9 is known to be expressed in only a few tissues, and it localizes to the endosome and lysosome membranes (Zhang, Zhang et al. 2000), while TAP expression is ubiquitous. TAP1 and 2 are located genomically within the class II MHC region on chromosome 6p21 (human) and 17 (mouse), while ABCB9 is located on 12q24, and 5F, respectively. While many immune response genes involved in antigen presentation are located within the MHC locus, there are exceptions. For example, the β2-microglobulin (β2-m) subunit of the class I MHC complex is located on 15q21 (human) and 2 (mouse), which is outside of the MHC region.

Endogenous antigen presentation for immuno-surveillance does not utilize endosomal or lysosomal compartments, but exogenous antigen presentation for T-cell priming does. Combined with its similarity to TAP, the endosomal and lysosomal localization of ABCB9 suggests a possible role in exogenous antigen presentation. The restriction of exogenous antigen presentation to APCs may also account for the reduced array of tissue expression of ABCB9 compared to the known TAP genes.

A knock-out mouse model of the ABCB9 gene was made available by the laboratory of Dr. Victor Ling at the BC Cancer Research Centre (Canada).

Hypotheses

Hypotheses and predictions were as follows:

1. Since APCs process exogenous antigens through several intracellular compartments, and there is precedent for peptide transport by ABC transporters (TAP), this intracellular antigen movement will be facilitated via localized ABC transporters.
2. Since resting APCs take up antigen from their surroundings, but do not process nor present it on the cell surface, and activated APCs primarily process and present antigens, activated APCs should express higher numbers of ABC transporters than non-activated APCs, and these should be involved specifically in antigen transport.
3. Since ABCB9 bears significant similarity to the TAP transporter, and is located in those compartments in which exogenous antigens are processed (i.e. endosomes and

lysosomes) of activated dendritic cells, it may function in the transport and processing of exogenous antigens.

4. It is expected that DCs from an animal lacking ABCB9 may be unable to process and present exogenous antigens, and therefore these animals may be unable to prime a cell-mediated immune response upon challenge with either MHC class I or class II restricted exogenous antigens.

1.5 Goals

Expression of all mouse ABC transporters will be evaluated in primary APCs derived from C57BL/6 wild-type mice. This expression profile will be repeated following exposure to the APC activating cytokines TNFα and IFNγ, and to the exogenous antigens ovalbumin and HI Sendai virus. The profile of transporter expression in untreated APCs will be compared with that of treated APCs, as well as to a non-antigen presenting cell fibroblast control. We expect the results to reveal a series of ABC transporters that are inducible in activated APCs. These genes will be subjected to phylogenetic analysis, and the results will be used to predict ABC transporters as potential candidates for a role in antigen presentation.

The ABCB9$^{-/-}$ mouse representing the knockout of the TAP-like ABC transporter gene will be evaluated for an immunological phenotype. Lymphoid organs will be analyzed for a differential cellular distribution and compared to wild-type mice. Immune function will be evaluated *in-vivo* by challenge with both live and heat-killed virus, as well as for the ability to respond to exogenous antigens on class I and class II MHC. Finally, the ability to generate an

anti-tumour response will be evaluated by challenge with MHC expressing and non-expressing tumour cells.

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CHAPTER II

EXPRESSION PROFILING OF ABC TRANSPORTERS IN APCs

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2.1 Overview

Ubiquitious endogenous antigen presentation on class I major histocompatibility (MHC) molecules drives immuno-surveillance by CD8+ T-cells. The transporter associated with antigen presentation (TAP), comprised of 2 half ABC transporters, ABCB2 (TAP1) and ABCB3 (TAP2), transports peptides for endogenous antigen presentation. Specialized antigen presenting cells (APCs) conduct both endogenous and exogenous antigen presentation, which is crucial for priming the immune response. Exogenous antigen presentation requires uptake and processing of extracellular antigens through subcellular compartments for presentation on MHC molecules. In this study, we examined gene expression of 46 murine ABCs in resting and stimulated APCs, compared with non-APC controls. Genes that were inducible in APCs by immune cytokines were also found to be induced by exposure to exogenous antigen and were identified as potential antigen transporters. These results were combined with a phylogenetic analysis of these 46 ABCs, and they revealed clustering of many of our candidate antigen transporters including TAP1 and TAP2. In this manner, 18 ABC genes were identified for their potential involvement in antigen presentation.

2.2 Introduction

The ability of the adaptive immune system to mount a response to antigens requires peptide processing and presentation by antigen presenting cells (APCs) (Unanue 2002). Dendritic cells (DC), macrophages (MØ), and B-cells are all known to act as specialized APCs. APCs are known for their ability to process and present exogenous antigens, both on class I and on class II MHC, as required for priming a cellular immune response to viral, bacterial, or tumour antigens, as well as for generation of tolerance to self proteins. Dendritic cells in particular have been shown to function exceptionally in this role (Mellman and Steinman 2001). Endogenous antigen presentation is constitutive in most cells, and the mechanisms of this pathway are well known (Monaco, Cho et al. 1990; Trowsdale, Hanson et al. 1990; Powis, Townsend et al. 1991; Sadasivan, Cariappa et al. 1995; Hughes and Cresswell 1998). Endogenous antigens are cleaved into peptides by the proteasome before being transported through the TAP transporter into the ER to bind to nascent class I MHC en route to the cell surface. In less well-defined pathways, exogenous antigens are taken up by APCs and processed through various intracellular compartments to be presented on MHC-II or cross-presented on MHC-I (Moore, Carbone et al. 1988; Trowsdale, Hanson et al. 1990; Kovacsovics-Bankowski and Rock 1995).

In the cellular immune system, APCs are activated by exposure to cytokines, or specific receptor-binding by pathogens. IFNγ is produced in response to a variety of viral infections, while tumour necrosis factor alpha (TNFα) is commonly released in response to viral or bacterial infection and/or inflammation (Holler 2002). Both of these cytokines have been shown to be

capable of activating APCs, and dendritic cells in particular (Brossart and Bevan 1997; Gallucci and Matzinger 2001).

The purpose of this investigation was to determine whether additional ABC transporters other than TAPs might be candidates for involvement in antigen processing and presentation. To address this question, differential gene expression of 46 identified ABC transporters was evaluated in DCs, MØs and B-cells, and compared to non-APC controls. Each subset of APCs was exposed to stimulatory immune cytokines, or to various antigens, and the differential ABC gene expression was evaluated. The results were used to determine that specialized APCs express differential ABC transporters compared to non-APCs, that these transporters are cytokine-inducible, and that specific antigens induce these same ABC transporters, suggesting that exposure to antigen alone is capable of inducing ABC transporter activity. Several of those transporters induced in APCs share sequence similarity, and are phylogenetically clustered into groups with the known TAP antigen presenting genes. It was also revealed that DCs express specific ABC transporters that differ from those expressed in other APCs, suggesting genes that may be specific to cross-presentation.

2.3 Materials and Methods

Media

All assays were conducted in CTL media: RPMI 1640 + 1% antibiotics (penicillin/streptomycin) + 1% NEAA + 1% L-glutamine + 1% HEPES + 0.1% 2-Mercaptoethanol + 10% Hyclone (UT) fetal bovine serum (FBS).

Cell purification

All cells were obtained from C57BL/6 mice. Dendritic cells, macrophages and B-cells were purified from mouse spleen using positive selection for CD11c+, CD11b+ (following CD11c+ depletion), and CD19+ antibody-bound microbeads (Miltenyi Biotec, Canada) respectively, as per manufacturer's directions on an AutoMACS (Miltenyi Biotec, Canada) cell separator. Briefly, spleens were injected with 1mg/mL Collagenase D (Boehringer-Mannheim, NJ) in PBS, disaggregated, and incubated for 1 hr at 37 °C. Splenocytes were then passed through 70 μm nylon mesh and washed with PBS supplemented with 0.5% FBS (buffer). The cells were resuspended in 400 μL buffer per two spleens, and 100 μL microbeads was added. After 15 minutes incubation at 4 °C, cells were washed, resuspended in 500μL buffer and separated on AutoMACS by double positive selection (POSSELD).

Antigenic induction

Cells were cultured overnight in 6-well plates in 5 mL of CTL media. Cells were stimulated with 10 ng/mL TNFα (R&D Systems, MN) to induce general inflammatory immune response genes. To activate the endosome to lysosome exogenous MHC Class I pathway of antigen presentation, heat-inactivated Sendai (HI-Sendai) virus (65 degrees C., 2 hours) was added to the media prior to overnight incubation at 37 °C. Chicken egg ovalbumin (Worthington, NJ) was added (2 mg/mL) to the media to stimulate the endosome to cytosol

exogenous class I pathway. Hen egg lysosome (HEL) (Sigma-aldrich, Canada) was added (2 mg/mL) to stimulate the exogenous class II antigen presentation pathway. To simulate viral infection initiation of endogenous antigen presentation, 50 ng/mL IFNγ (R&D Systems, MN) was added to the cells prior to incubation. An untreated control was included for all cell types.

Bioinformatics

Phylogenetic analysis was conducted using ClustalX software, version 1.81 (National Center for Biotechnology Information). Sequences for alignment were obtained based upon the GenBank accession numbers published on http://www.humanabc.org, hosted by the Nutrition, Metabolism and Genomics Group, Wageningen University, the Netherlands. Phylograms were obtained by ClustalX multiple amino acid sequence alignment of all available sequences for human and mouse, followed by creation of a tree using neighbour-joining (N-J) and bootstrap algorithms.

Sequence specific RT-PCR primers (shown in Table 2.1) were selected corresponding to each of the murine ABC transporter expressed sequences recorded in NCBI GenBank database (accession numbers published at http://www.humanabc.org). Primer selection was conducted using the web-based Primer3 software and checked against a rodent mispriming library (Rozen 1996, 1997).

RT-PCR

RNA was extracted using the Qiagen (Canada) RNEasy kit. Concentration of RNA was determined by spectrophotometric quantification, and equal amounts (1.0 μg) of RNA from each cell type treatment were used for each reverse-transcription (RT) reaction. Complementary DNA

(cDNA) was produced from the RNA using random hexanucleotide primers and SuperscriptII RT enzyme (Gibco-BRL, Canada) as per manufacturers' directions. Equal amounts of cDNA were then used as template for each PCR reaction. Expression of ABC genes was evaluated by RT-PCR with Taq polymerase (Gibco-BRL, Canada) using the primers indicated in Table 2.1 for 35 PCR cycles, annealing temperature of 60° C with 1 minute each denaturing, annealing and elongation. Equal volumes of the PCR product were run on a 1% agarose gel alongside a 100 bp DNA ladder (Gibco-BRL, Canada).

2.4. Results

Interferon-γ (IFNγ) is produced in response to immune stimulation by several stimuli, including viral infections and results in a primarily Th-1 type T-cell response. Many genes involved in the immune response are regulated by this cytokine, and are considered to be interferon responsive (IRE) (York, Goldberg et al. 1999). TAP1 and TAP2 have been shown to be upregulated in response to interferon, and other immune stimulatory cytokines such as TNFα (Figure 2.1).

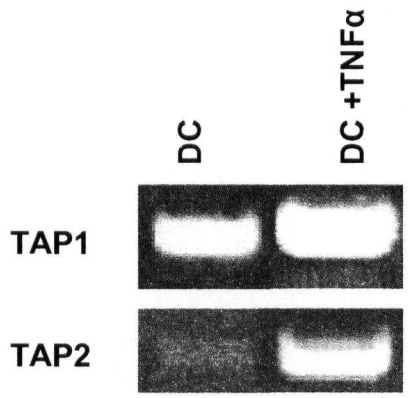

Figure 2.1. Cytokine treatment resulted in increased expression of the transporters involved in antigen presentation, TAP1 (ABCB2) and TAP2 (ABCB3). CD11c+ dendritic cells were purified from C57BL/6 mouse spleens (>98% pure) and incubated at 37° overnight in the absence or presence of the maturation stimulus TNFα to induce DC maturation. Total RNA was extracted and gene expression was evaluated using RT-PCR.

Many other genes located in the MHC-locus (chromosome 6p21 in humans and 17,4 in mice) are involved in antigen presentation and are IFN-responsive, such as the immunoproteasome subunits LMP2 and 7, and tapasin (York, Goldberg et al. 1999). Other genes outside of this MHC locus are involved in the immune response and also show this IFN-responsiveness, such as β2-m or ER-proteases. Initially, we wanted to see whether ABC

transporters were induced in APCs by pulsing with IFNγ or TNFα. Having chosen gene-specific primers for all 46 available mouse ABC transporter sequences (shown in Table 2.1), we evaluated gene expression in APCs both untreated and treated with cytokine. We observed several genes to be upregulated, and these provided a starting point from which to examine potential antigen presentation function (see RT-PCR example in Figure 2.2 and full results in Table 2.2).

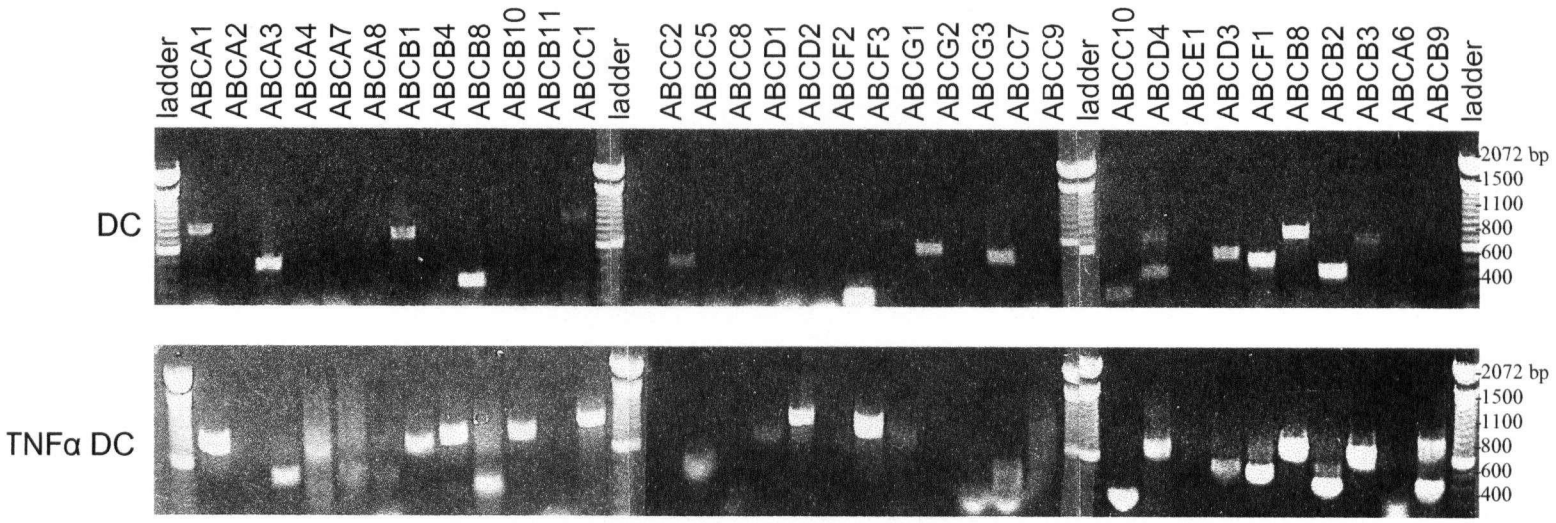

Figure 2.2. Sample subset RT-PCR of untreated (NT) and 10 ng/mL TNFα treated DCs. Each lane represents 1 of the 46 total ABC transporter gene-specific primer sets. Note additional bands present in TNFα treated DCs compared to untreated DCs. PCR amplification was controlled using the same inital amounts of RNA from each cell type and treatment to provide the same amount of cDNA template.

Transporter	fwd primer (5'-3')	rvs primer (5'-3')	product size (bp)
1) ABCA1	ACCGCCTATGTGGTTCTCAC	GAGAGCTTTCGTTTGTTGCC	866
2) ABCA2	GACACAGCCAAGATTGCTCA	GTTGTTGCCACACAAGATGG	390
3) ABCA3	TGGGTGGCTTCCTGTACTTC	CTACCAAGTCCTCTGGCTCG	453
4) ABCA4	GTCTGACCTCCTGTGTGGGT	AAGTCTTTGACGGTTGGGTG	432
5) ABCA5	TTACAGTTCTGCAGGCATCG	CTCAAAGCCTCCACGTTCTC	976
6) ABCA6	GCACAATATACCGGACGCTT	TCTGCAACAGAACAGCCATC	617
7) ABCA7	ACGCAGACGTTAGCTCTGGT	GGTGGCAGTTGGAATCTCAT	380
8) ABCA8	AATATGGCAAGCAATCAGGG	TCCAGGTCAAAGGTCTGCTT	369
9) ABCA9	ACTCAGTGGACTGTCACCCC	AACCCAGCAGTTGGTTCATC	357
10) ABCA12	CTGAGCATGCTGGAGTACCA	GTACCCTTGCCGTGCAGTAT	343
11) ABCA13	AAAGGGCTCTCACAGCAAAA	CACCTCTTCCAAGGTGGTGT	993
12) ABCB1	TTGGTGGCACAACAACTCAT	CTGATGTTGCTTCGTCCAGA	727
13) ABCB2	GACTTGCCTTGTTCCGAGAG	CTGTGCTGGCTATGGTGAGA	393
14) ABCB3	GCTGTGGGGACTGCTAAAAG	TATTGGCATTGAAAGGGAGC	665
15) ABCB4	AAGAAATGACCACGTACGCC	GTGCCCACTCTTTGAGAAGC	824
16) ABCB6	GGTCCTGCGTTATGTGACCT	CGAAACTTGGCTCTCCACTC	750
17) ABCB7	ATACAGTTGCAACCATGGCA	TAGCAGCATTACCTGCATCG	453
18) ABCB8	AAGATTGTGGCTCTTGTGGG	ACAGTGCTGTAGCCATCGTG	311
19) ABCB9	TCGGGAGCCACACTACAGAA	AAGCCCATGAAGGTGACCAG	480
20) ABCB10	ATGTACGCTTTCTGGGTTGG	TGTGTCCCGTGTTCACAGAT	839
21) ABCB11	GAACAGCTGTGGAAAGAGGC	GGAAGCATCTGTAGCAAGCC	704
22) ABCC1	TTCTCAGACTGCTCACCCCT	TCCCATTGAGGATCTCGTTC	967
23) ABCC2	ACACACTCCCCCAGACTTTG	GGCCACCTGCAGACTCTAAG	792
24) ABCC3	ACAACAGGGTCCTGGTCTTG	TGTAGAGGCAGGAGCAAGGT	344
25) ABCC4	TGGTCTCAGCAGTCATCCAG	TGCATCAAACAGCTCCTGAC	810
26) ABCC5	GAAGCATCAGTGGCTGTTGA	TGGCTGAAACGAGAGAGGTT	448
27) ABCC6	CTACTCAGGGCTATCTGGCG	AAAGGACACTCGAGCCTGAA	903
28) ABCC7	TGCTAGTGCTGATTTGGTGC	GCTCGAAGTGTCCAGAGTCC	622
29) ABCC8	ACTGGATGGTGAGGAACCTG	CTGGTCTTTCTCACGAAGGC	616
30) ABCC9	GAAACAGCTGAAGGGCTCAC	AAAGCCAGGGATAAGGAGGA	548
31) ABCC10	GGTTTTCATGAGGAAGCCAG	CAGCAAGTGTGGACCAGAAG	395
32) ABCC12	CCGTGTTTCCTGTTGTCCTT	AAGGGGTGAGTGTGTTCAGG	508
33) ABCD1	GCCGTCTAGTAGCTCATGCC	AACATCTCGTACACCCTGGC	778
34) ABCD2	AGCAGAAGCTTACTCGCCTG	TCGCTAACCATAGCCTGCTT	999
35) ABCD3	CGAAAACTGGTGGAACACCT	GGTCAGAGATCCCCCTCTTC	730
36) ABCD4	AGAGCTTAGCACCCTGGTCA	CTGGAGGTAGAAGAGACGGG	712
37) ABCE1	AATGCTTGTTGGAAGGCTTA	CCAGCCAAAAGAGTCTGAGG	492
38) ABCF1	GCAAGCTGACACCGACCAAC	CTTTCTGCCCACCGGAGAGTT	376
39) ABCF2	ACCTCAGGTGGCAGAAGAGA	GCCAAACCTGGCAATGTAGT	946
40) ABCF3	GCCAGGCTCTCAACAACTTC	GAGCGGAACTGTTCTTGGAG	166
41) ABCG1	CTGCCTCACCTCACTGTTCA	TCTTGGCTTCATTCCCAATC	808
42) ABCG2	CGCAGAAGGAGATGTGTTGA	CATCCAGGAAGAGGATGGAA	536
43) ABCG3	GCTCTTCGTCTCCCAGTGAC	GACACATGTTGTCTCCACGG	622
44) ABCG4	GTACTTGGGCATTGGTTGCT	AAGGGATGGTGCCTGTGTAG	803
45) ABCG5	GTCCTGCTGAGGCGAGTAAC	ACAAGGGTCAGACACAAGGG	963
46) ABCG8	CCAGGAATCCTCATTCTGGA	AGGAGGACATGTGGAAGGTG	988

Table 2.1. RT-PCR primer sequences for mouse ABC transporter genes

It has been well documented that APCs *in-vitro* are able to take up extracellular antigens from the surrounding media, process, and present the antigenic peptides on the cell surface in the context of MHC molecules (Banchereau and Steinman 1998; Mellman and Steinman 2001; Belz, Carbone et al. 2002). In order to further refine our search for ABC transporters that may specifically transport antigens, we examined ABC induction following exposure to antigen. We found that most of the ABC transporters that were inducible by the immune cytokines were also induced by at least one, and often several, of the antigens as shown in Table 2.2. We assayed the DCs for maturation levels both prior to and following overnight incubation and found that splenic DCs *in-vivo* are initially in an intermediate stage (Wu, Vremec et al. 1995; Kamath, Pooley et al. 2000) in naïve mice (Figure 2.3). The process of purifying them from spleen and culturing them overnight *in-vitro* was sufficient to drive them into a mature state, upregulating class II MHC ($I\text{-}A^b$), CD40, CD80 and CD86 without additional maturation stimulus, as shown in Figure 2.3. As these mature cells were used as untreated controls, this demonstrates that maturation alone was not sufficient to induce expression of these ABCs, and an additional cytokine or antigenic treatment was required to upregulate their expression.

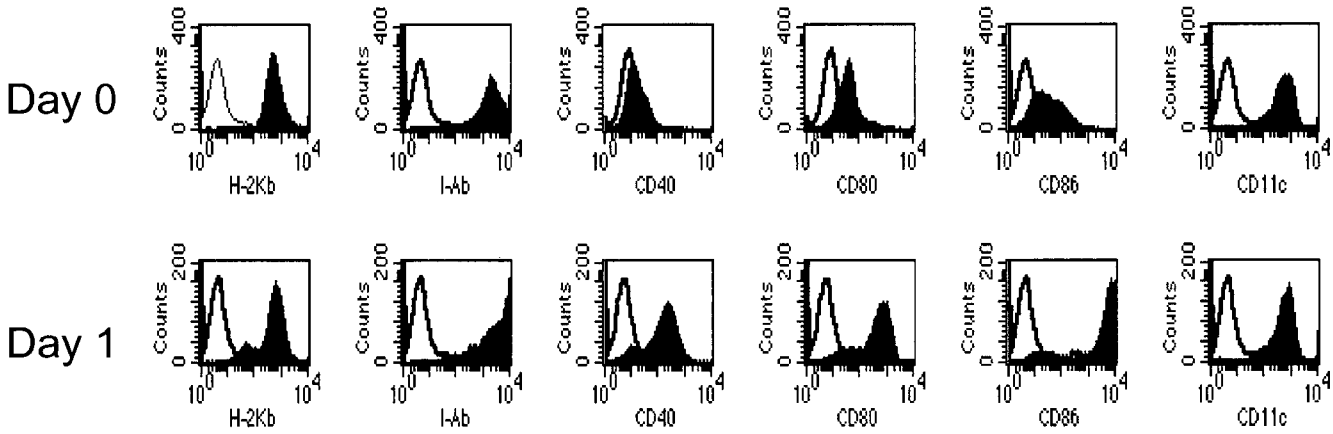

Figure 2.3. Splenic DC activation markers. Splenic DCs stained immediately following purification (Day 0) show 99% CD11c purity and are of an intermediate phenotype as determined by MHC class I (H-2K^b) and II (I-A^b) levels, CD40, CD80 and CD86. Following overnight incubation *in-vitro* (Day 1) in the absence of any other stimulus, DCs advance to a mature phenotype.

RT-PCR results by cell type

As shown in Table 2.2, untreated DCs expressed 8 ABC transporters, while DCs stimulated by cytokines expressed 36. Untreated MØ constitutively expressed 13 transporters, whereas cytokine activation resulted in expression of 23 ABC transporter genes. B-cells constitutively expressed 10 transporter genes without cytokine treatment, and expressed 31 following treatment. In the Ltk fibroblast non-professional antigen presenting cell control, there

were 14 ABCs constitutively expressed, and 15 expressed after treatment with cytokine (Table 2.2). This ABC induction observed in APCs was often the same, regardless of whether the cytokine was IFNγ or TNFα, and was also observed upon addition of different antigens.

	ABCA1	ABCA2	ABCA3	ABCA4	ABCA5	ABCA6	ABCA7	ABCA8	ABCA9	ABCA12	ABCA13	ABCB1	ABCB2	ABCB3	ABCB4	ABCB6	ABCB8	ABCB9	ABCB10	ABCB11	ABCC1	ABCC2	ABCC3	ABCC4	ABCC5	ABCC6	ABCC7	ABCC8	ABCC9	ABCC10	ABCC12	ABCD1	ABCD2	ABCD3	ABCD4	ABCE1	ABCF1	ABCF2	ABCF3	ABCG1	ABCG2	ABCG3	ABCG4	ABCG5	ABCG8
DCs																																													
NT	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
TNF	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1	1	0	1
IFN	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0	0	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1	0	1
OVA	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	1	1	1	1	0	1	1	0	0
HI Sen	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	1	1	1	0	0
HEL	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	1	0	0
MØ																																													
NT	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	1	0	0
TNF	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1	1	0	0
IFN	1	1	1	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0
OVA	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0
HI Sen	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0
HEL	1	1	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
B-cells																																													
NT	0	1	1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
TNF	1	0	1	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
IFN	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	0	1	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	1	1	1	1	1	1	0	0	0
OVA	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	0	0	0	0	1	0	1	0	1	1	0	1	1	1	1	1	1	1	0	0
HI Sen	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0
HEL	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0
LTK																																													
NT	0	1	0	0	0	0	0	0	1	0	0	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	1	0	0	0	1	0	0
IFN	1	1	0	0	0	0	0	0	1	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0	1	0	0	0	1	0	0	0

Table 2.2. Differential ABC expression in APCs under various stimuli. 0 = gene not expressed, 1 = gene expressed. Highlighted genes are of particular interest as potential candidate antigen presenting transporters due to their cytokine inducibility in APCs and phylogenetic clustering with known peptide transporters. Cell treatments: NT = no treatment, TNF = TNFα, IFN = IFNγ, OVA = ovalbumin, HI Sen = Heat inactivated Sendai virus, HEL = hen egg lysozyme.

Cytokine and antigen inducible transporters that were highly expressed and shared between the different APCs include (ABC) A1, A2, A3, B1, B2, B3, B4, B6, B8, B10, F2, F3, G1, G2, G3, and G4. Of these ABC genes, many are clustered together in groups based upon their sequence similarity. As shown in the dendrogram in Figure 2.4, the ABCB subfamily, especially (ABC) B2, B3, B9 and B10 are very closely related, with B1 and B6 only slightly more divergent, while G1, G2, G3 and G4 are also very closely grouped together.

Ltk murine fibroblast cells were evaluated as a non-professional APC control. Although Ltk cells do constitutively present endogenous antigen, they are not considered a specialized APC as they do not present exogenous antigens. ABC genes that were induced in Ltk cells by cytokine stimulation include (ABC) A1, B2, C3, C10, D3, E1 and G4. These represent candidate genes for involvement in endogenous antigen presentation. Interestingly, these genes were generally not upregulated by cytokines or antigen in APC.

ABC genes that were constitutively expressed in specialized APCs but not in Ltk cells include (ABC) A1, A3, A4, A8, A13, B3, B4, C1, C2, C5, and G2. Resting APCs function primarily to sample and uptake antigens prior to their processing and MHC-restriceted surface presentation. In this 'inactive' state, these constitutive transporters are worth considering for their APC specific expression, in particular for potential function in the initial uptake of exogenous antigens.

All of the APCs showed induction of transporter genes following treatment with cytokines or antigens, while the total number of ABC transporters expressed by the non-APC fibroblasts remained constant. This result is supportive of our hypothesis that professional APCs utilize additional ABC transporters for exogenous antigen processing and presentation.

Phylogenetic analysis

Phylogenetic analysis of the ABC transporter superfamily (Figure 2.4) indicates the relative evolutionary divergence of the various ABC proteins. Groups of proteins clustered together show evolutionary conservation and similarity of these genes, suggestive of sharing a common function. Comparison of orthologous sequences between human and mouse is useful, in that both species are dependent upon cellular immunology for protection against pathogenic infections. Since this process is integral to survival, natural selection predicts that the genes involved will be extremely conserved, in that mutations are selected against by reduced survival. Thus, genes with conserved sequence between human and mouse potentially share an important function. This information is useful in that once an ABC transporter is known to be involved in antigen presentation, such as ABCB2 (TAP1) or ABCB3 (TAP2), other genes similar to these can be targeted for further analysis of a potential antigen transport role. Comparison of conserved clustering between the orthologous mouse and human genes lends further support to shared function in these related species. ABCB2 and ABCB3, known to form the functional TAP heterodimer, are shown as closely related on the dendrogram. For half-transporters with unknown partners, this analysis can readily identify those that share the most similarity, therefore suggesting a possible partner for heterodimer formation. Based upon our analysis, ABCB9 is the most likely candidate for an antigen presentation function. Bearing significant sequence homology to both TAP genes, ABCB9 is located in the same cluster, and this gene was differentially expressed in activated DCs.

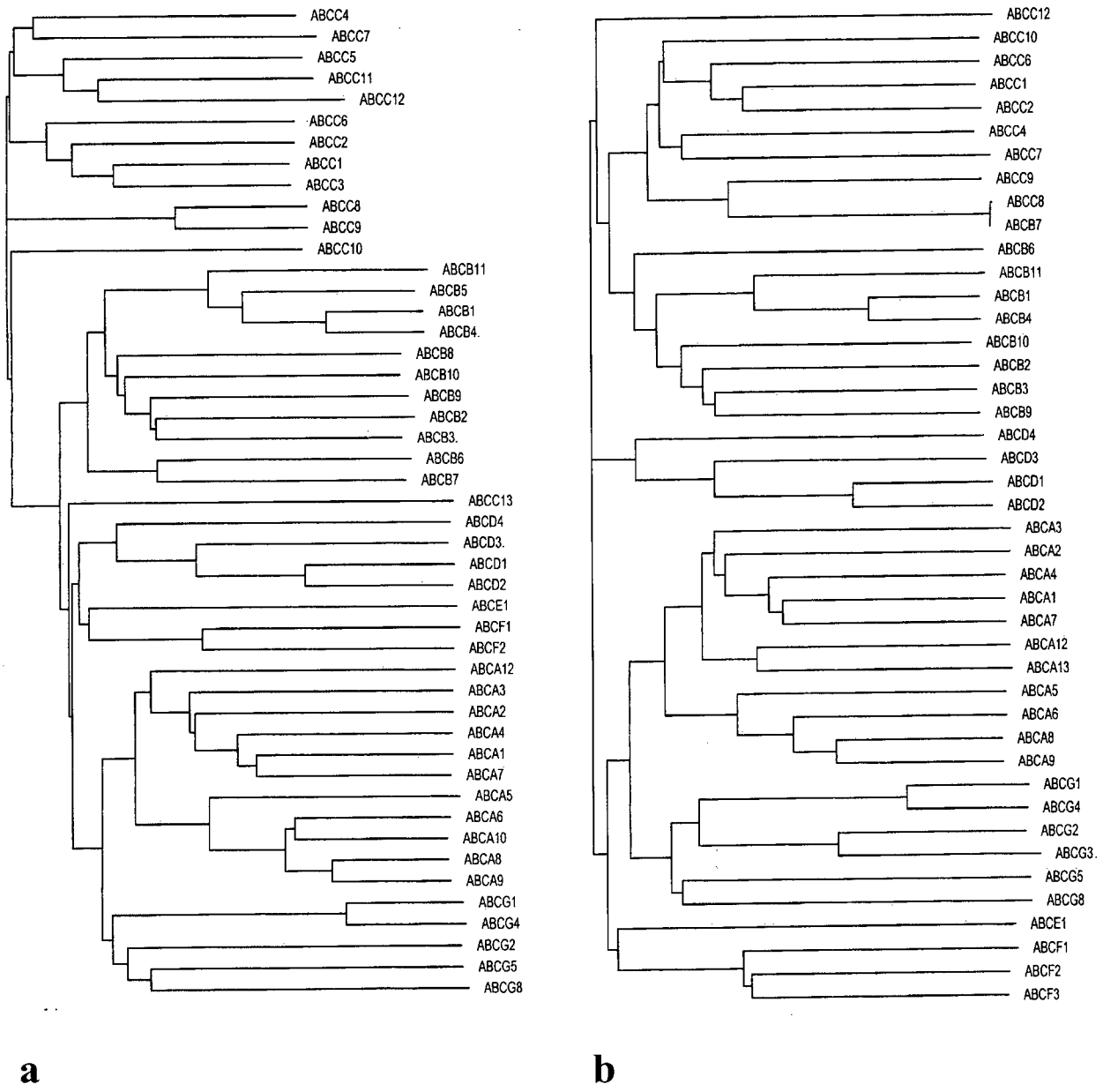

Figure 2.4. Phylogenetic analysis of all known members of the ABC transporter protein superfamily in (a) humans and (b) mice. Genes such as those belonging to the ABCB and ABCG family show distinct clustering, and these clusters are similar between human and mouse. Distance from the node indicates relative time of evolutionary divergence.

2.5 Discussion

We have shown that APCs constitutively express 8 ABC transporters, and these were generally also expressed constitutively in the Ltk fibroblast cell line (Table 2.2). We have also shown that in all APCs, the addition of immune cytokines induces expression of a number of transporters, while the addition of antigen was also found to upregulate several ABCs, and that this upregulation was independent of maturation state (Figure 2.3). One caveat to analyzing the antigenic stimulus data that should be considered is the possibility that it may not be antigen alone stimulating the APCs, but perhaps endotoxin contamination inherent in these commercial preparations (Gao and Tsan 2003). We have attempted to minimize this possibility by using reagents previously shown to have minimal endotoxin content (Rafiq, Bergtold et al. 2002). Regardless, since endotoxins are known to upregulate antigen presentation in APCs, possible endotoxin contamination should not adversely affect our analysis of transporter expression, although it may limit our ability to determine transporter substrate specificity. Additionally, the limitations of RT-PCR should be considered; while RT-PCR is a useful tool to determine presence or absence of a gene transcript, it is of limited use in quantifying gene expression. To properly quantify expression, not only are matching amounts of RNA and cDNA required, but internal controls, such as primers to identify structural genes (i.e. β-actin) and metabolic genes (i.e. S15) must be incorporated and used in a real-time RT-PCR experiment.

The consistent recruitment of certain ABC transporters, in particular by more than one type of APC, should be followed up to determine whether they play a mechanistic role in antigen presentation. Combined with other information such as interferon inducibility, homology to known antigen transporters (ABCB9), and chromosomal location (ABCF1 and ABCC10 are

located within the MHC class II region), certain transporters can be singled out for further study. In order to identify candidate antigen transporting genes, we have incorporated a combinatorial approach, utilizing evolutionary conservation of protein similarity both within and between species, combined with a screen of inducible APC gene expression. This combination of indicators of potential antigen presentation involvement allows us to identify specific proteins upon which to focus our efforts to find new antigen or peptide transporters.

ABC transporters inducible in APCs

Several of the ABC transporters revealed here as candidates for antigen presentation have been characterized, though the evidence for substrate specificity of many is indirect and non-physiological. Transporters that we found to be induced in APCs will be discussed further here.

The ABCA subfamily of transporters comprises full-length transporters (2 TM regions and 2 NBD regions), of which we found 3 to be activated in DCs. Expressed in all 3 APCs and upregulated by cytokine and antigen, ABCA1 maps to 9q31 human, 4,23.1cM mouse (Luciani, Denizot et al. 1994). This gene has been shown to be involved in cholesterol transport, with $ABCA1^{-/-}$ mice resulting in low levels of high density lipoproteins and accumulation of cholesterol in tissues (McNeish, Aiello et al. 2000; Orso, Broccardo et al. 2000; van Eck, Bos et al. 2002) and is the causative gene in Tangier disease, a disorder of cholesterol transport between tissues and the liver (Bodzioch, Orso et al. 1999; Brooks-Wilson, Marcil et al. 1999; Marcil, Brooks-Wilson et al. 1999; Remaley, Rust et al. 1999; Rust, Rosier et al. 1999; Schmitz, Kaminski et al. 1999; Young and Fielding 1999). Of particular interest to our study, ABCA1 has

also been identified as playing a role in the phagocytosis of apoptotic cells (Moynault, Luciani et al. 1998), thus lending support to the hypothesis that it may be involved in some form of antigen presentation process.

ABCA2, expressed in APCs and Ltk cells, maps to 9q34.3 (human), 2,12.6 cM (mouse) (Luciani, Denizot et al. 1994; Allikmets, Gerrard et al. 1995; Dean 2002), is closely related to ABCA1 (see Figure 2.4) and as such has been proposed to have a similar function, with evidence of sterol-dependent gene regulation in macrophages (Kaminski, Piehler et al. 2001) and possible drug-efflux properties (Laing, Belinsky et al. 1998). Intriguingly, ABCA2 has been localized by immunofluorescence to the endosomal / lysosomal intracellular vesicles (Vulevic, Chen et al. 2001), known to be involved in exogenous antigen presentation.

ABCA3 maps to 16p13.3 (human) (Klugbauer and Hofmann 1996), and its location in mouse is currently unknown. With high expression in lung, this protein has been characterized for its apparent involvement in the production of surfactant over the alveolar surface of the lung (Zen, Notarfrancesco et al. 1998; Mulugeta, Gray et al. 2002). In our study, ABCA3 was inducible in all stimulated APCs, but not expressed in Ltk cells.

The ABCB subfamily of ABC transporters is comprised of a combination of full and half transporters (1 TM region and 1 NBD region). This subfamily includes the well-characterized MDR (Juliano and Ling 1976; Kartner, Riordan et al. 1983) and TAP (Trowsdale, Hanson et al. 1990; van Kaer, Ashton-Rickardt et al. 1992) genes. ABCB1, located on 7q21.1 (human), 5,1 (mouse), is better known as the multiple drug resistance (MDR) protein, due to its ability to confer a multidrug resistance phenotype to cancer cells (Juliano and Ling 1976) (Kartner, Riordan et al. 1983; Kartner, Evernden-Porelle et al. 1985; Riordan, Deuchars et al. 1985; Roninson, Chin et al. 1986). ABCB1 was expressed strongly in all APCs and Ltk cells. ABCB1

has been shown to transport several different drug compounds, as well as lipids and, interestingly, peptides (Gottesman, Fojo et al. 2002). This gene has also been shown to be specifically involved in transport at the blood-brain barrier (Schinkel, Smit et al. 1994; Schinkel, Mayer et al. 1997), although its precise physiological function *in-vivo* remains unknown.

ABCB2 (TAP1) and ABCB3 (TAP2) both map to 6p21.3 (human) and chromosome 17 in mouse (Dean 2002), where they are located within the MHC gene complex. Many of the genes located in this region, including TAP1 and 2, are involved in antigen presentation and are stimulated by immune regulatory cytokines. Each a half transporter, they come together to form a functional heterodimer involved in the transport of cytosolic antigenic peptides. These proteins are located in the ER membrane of most nucleated cells, where they translocate peptides into the ER lumen to complex with nascent class I MHC molecules (Monaco, Cho et al. 1990; Spies, Bresnahan et al. 1990; Trowsdale, Hanson et al. 1990). TAP proteins are required for class I MHC restricted antigen presentation, and $TAP1^{-/-}$ mice are unable to mount a cytotoxic T-cell response (van Kaer, Ashton-Rickardt et al. 1992). TAP1 and 2 have also recently been found to be present in ER-phagosome membranes in antigen presenting cells, where they appear to function in antigen cross-presentation (Ackerman, Kyritsis et al. 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003). In our screen, both were expressed strongly in all APCs, particularly following stimulation.

ABCB4, which maps to 7q21.1 (human) (Allikmets, Gerrard et al. 1996) and 12,60 (mouse) (Dean 2002), was expressed in activated and antigen-pulsed APCs. This is a full transporter most closely related to ABCB1 (Figure 2.3). This protein, highly expressed in bile cannilicular membrane of the liver, has been shown to be capable of transporting phosphatidylcholine *in-vitro* (Ruetz and Gros 1994; van Helvoort, Smith et al. 1996), and mice

with an ABCB4 deficiency exhibit liver pathology due to a defect in fatty acid secretion in bile (Smit, Schinkel et al. 1993).

ABCB6 maps to chromosome 2q35 (human), and 1,C3 (mouse), and subcellularly localizes to the mitochondria (Allikmets, Gerrard et al. 1996; Mitsuhashi, Miki et al. 2000). Expressed in activated DCs and B-cells, this half transporter has been shown capable of complementing iron transport in deficient yeast cells, and as such has been suggested to be involved in iron homeostasis (Mitsuhashi, Miki et al. 2000).

ABCB8 was upregulated in APCs by maturation stimulus and also constitutively in non-APC Ltk cells, and it maps to 7q36.1 (human). It also encodes a half transporter that is located in the mitochondria, although its function is unknown (Allikmets, Raskind et al. 1999; Hogue, Liu et al. 1999). Based upon localization, this is less likely to be involved in exogenous antigen transport.

ABCB9 (also known as TAPL), is located on 12q24.31 (human), 5,F (mouse) (Allikmets, Gerrard et al. 1996; Zhang, Zhang et al. 2000) was expressed specifically in stimulated DCs. With extremely high sequence identity to TAP1 and TAP2 (Figure 2.4), and localization to endosome / lysosomes (Zhang, Zhang et al. 2000), this half transporter is of great interest to us for its potential role in exogenous peptide transport. It is also intriguing that this gene is highly expressed in the sertoli cells of testis, as these are phagocytic cells involved in antigen uptake and processing (Zhang, Zhang et al. 2000).

ABCB10 maps to 1q42.13 (human) and 8,67 (mouse) (Allikmets, Gerrard et al. 1996; Dean 2002), and was expressed in all stimulated APCs. This half transporter localizes to the mitochondria (Zhang, Hogue et al. 2000), and although its function is currently unknown, it is the mammalian transporter most closely related to Mdl1 in yeast (Zhang, Hogue et al. 2000).

Mdl1 has been shown to transport peptides out of the mitochondria in *Saccharomyces cerevisiae* (Young, Leonhard et al. 2001), and as such, we suggest that ABCB10 may function as a mammalian ortholog to Mdl1.

The ABCC transporter subfamily is comprised of full transporters that transport a variety of compounds. ABCC1 (also known as MRP1, a multiple drug resistance protein), is located on chromosome 16p13.1 (human) and 16 (mouse) (Cole and Deeley 1998; Dean 2002). Expressed in all of the stimulated APCs, it is best known for its high incidence in tumour cells (Cole and Deeley 1998). Although this protein has been shown to confer a multiple drug resistant phenotype to tumour cells against several chemotherapeutic agents, the physiological phenotype of an ABCC1 null mouse was quite a surprise. These animals were found to mount an impaired immune respone, due to a defect in dendritic cell migration into the lymphatic vessels. ABCC1 was found to be able to transport the immune regulator leukotriene C4, an important signaling molecule for DC migration (Robbiani, Finch et al. 2000). As such, it is not surprising that immune cytokines induced this gene, however it was interesting that antigen pulsing was also sufficient to do so. Based upon the previous characterization of this protein, it appears unlikely to function directly in antigen transport, however combined with the high expression in DCs and MØ observed here, its involvement in DC priming of the immune response would be worth investigating.

ABCC3, located on 17q21.3 (human) (Allikmets, Gerrard et al. 1996), unknown for mouse, is capable of effluxing organic ions, and can confer a resistant state to certain compounds (Zeng, Bain et al. 1999). With high expression in activated DCs and B-cells, the physiological function is unknown, but it has been suggested to play a role in liver for bile acid transport (Zelcer, Saeki et al. 2003).

ABCC4 on 13q32 (human), 13,E4 (mouse), was expressed in stimulated DCs, and ABCC5 on 3q27 (human) and 16,14 (mouse), was expressed in all APCs. These are two related transporters for which very little physiological data is known, besides the ability to confer resistance to nucleoside analogues in transfected cells *in-vitro* (Schuetz, Connelly et al. 1999; Wijnholds, Mol et al. 2000; Reid, Wielinga et al. 2003). With generally unknown function, both ABCC4 and ABCC5 are of interest as candidate antigen presenting genes.

ABCC10, located on 6p21.1 (human) (Allikmets, Gerrard et al. 1996), its location in mouse unknown, is induced by IFNγ in DCs and Ltk cells, but otherwise not highly expressed in APCs. This transporter is of interest to us due to its chromosomal location within the MHC gene complex. Genomically located proximally to the two TAP transporters within the MHC locus, we suspect it to have a potential immune response function. The physiological transport function remains unknown.

The ABCD transporter subfamily is comprised of half transporters, all of which are located in the peroxisome. ABCD1 is located on Xq28 (human) and X,29.5 (mouse) and mutations in this gene appear to result in adrenoleukodystrophy (ALD), an X-linked recessive disorder characterized by neurodegenerative phenotype, with typical onset in late childhood (Mosser, Douar et al. 1993). ALD patients have an accumulation of saturated long-chain fatty acids in the cholesterol esters of the brain and in the adrenal cortex. ABCD1$^{-/-}$ mice also show an accumulation of long-chain fatty acids in the brain and kidneys (Forss-Petter, Werner et al. 1997; Yamada, Shinnoh et al. 2000). Expressed in actived DCs and B-cells, this gene shows potential for involvement in antigen presentation, in particular for the processing of lipids for antigen presentation on CD1, a non-classical MHC molecule (Pamer and Cresswell 1998; Kang and Cresswell 2002; Park, Kang et al. 2004).

ABCD3 maps to 1p21-22 (human), 3,56.6 (mouse) (Dean 2002). Expressed in cytokine stimulated DCs and B-cells, this is another peroxisomal protein, the function of this gene has yet to be determined and as such is of interest as a potential antigen-presenting gene.

The ABCF transporter gene subfamily is unusual in that members of this group encode a pair of NBDs but no TM domains, and as such have not been shown to directly transport substrates (Dean 2002). ABCF1 is located on 6p21.33 (human) (Allikmets, Gerrard et al. 1996), 17, 20.5 (mouse) (Dean 2002) in the MHC region, marking it another gene of interest for potential antigen presentation regulation. Expressed in all APCs and Ltk cells, the protein product of this gene has been shown to interact with the eukaryotic initiation factor 2 (eIF2) and associate with ribosomes (Tyzack, Wang et al. 2000). It is thought to play a role in the initiation of protein translation.

ABCF2 maps to 7q36 (human) (Allikmets, Gerrard et al. 1996), 13,40 (mouse) (Dean 2002) and is expressed by all stimulated APC subsets, as well as untreated Ltk cells. This gene has no known function and should be examined for an antigen presentation function.

The ABCG subfamily is comprised of 'reverse' half transporters, with the NBD at the N-terminus, and TM domain C-terminal, opposite to the rest of the family members. ABCG1, 2, 3, and 4 were all expressed in all of our stimulated APC subsets. ABCG1 is located on 21q22.3 (human) and 17, A2-B (mouse) (Dean 2002), and is expressed by all APC subsets upon activation. Although the physiological function remains unknown, it has been suggested to be involved in macrophage cholesterol efflux (Klucken et al, 2000). This suggests a potential function in lipid antigen presentation.

ABCG2 maps to 4q22 (human) (Allikmets, Gerrard et al. 1996), 6, 28.5 (mouse) (Dean 2002). This gene has been shown to be capable of transporting several chemotherapeutic drugs

and dyes *in-vitro* (Brangi, Litman et al. 1999; Ross, Yang et al. 1999; Litman, Brangi et al. 2000). With high expression in trophoblast cells of the placenta, it has been suggested to function transporting compounds in or out of the fetal blood supply (Jonker, Smit et al. 2000). Its expression in all three APC types suggests that his gene should also be evaluated for a potential antigen presentation function.

ABCG3 is found in mouse, on chromosome 5, 59 (Dean 2002), and has no human ortholog. Closely related to ABCG2 (Figure 2.3), the physiological function of this gene is not known. It is expressed in all three types of APC upon activation. With no human ortholog, this may well be a duplicated gene in the mouse genome.

ABCG4 is located on 11q23 (human), 9 (mouse) (Dean 2002). Expressed by all stimulated APCs and the Ltk cells, the function of this gene remains unknown and should be evaluated for antigen presentation ability.

ABCG8 maps to 2p21 (human) (Berge, Tian et al. 2000) and 17 (mouse) (Dean 2002) and is expressed differentially in stimulated DCs. This gene is thought to be involved in sterol transport (Berge, von Bergmann et al. 2002), and when overexpressed in combination with ABCG5 increases biliary sterol secretion (Yu, Li-Hawkins et al. 2002). This is another transporter potentially involved in lipid antigen presentation.

There are other potential functions for antigen presenting cell ABC transporters worth considering, such as antigen uptake or transport of intermediates involved in peptide processing, like cathepsins or other proteases. Another potential function is the secretion of immunomodulatory cytokines. APCs and T-cells are known to produce cytokines, as well as to respond to them in a positive feedback loop. The physiological purpose of anti-toxin

transporters, such as the MDR types of transporters, is readily apparent in such organs as liver, where detoxification of compounds is a primary function. The function of these transporters in antigen presenting cells is not clear, particularly when these genes are induced in response to immune cytokine exposure. A possible explanation could be that certain of these transporters function in the secretion or endocytic processing of cytokines.

2.6 Conclusions

Based upon a combination of assessment of differential antigenic or cytokine induction in APCs and phylogenetic analysis, the genes that show the most potential as candidate antigen transporters are (ABC) A1, A2, A3, B1, B2, B3, B4, B6, B8, B9(DC), B10, F1, F2, F3, G1, G2, G3 and G4. In particular, the inducible members of the ABCB subfamily are of interest as they are closely related and have been shown to have 2 members (ABCB2 and B3) involved in peptide transport, while the yeast ortholog to ABCB10, Mdl-1, has also been shown to transport peptides (Young, Leonhard et al. 2001). Since these results were based upon RT-PCR analysis utilizing the same concentrations of starting RNA and PCR template, it would be useful to evaluate the candidate genes identified here in a real-time RT-PCR screen, incorporating a house-keeping gene for the purposes of quantification of gene expression. The information provided by such quantitation would determine the relative contribution of each gene under a given stimulus, as well as determining a precise measure of individual gene induction under the various stimuli applied here. This would indicate those genes whose expression are most

increased upon the initiation of antigenic presence, and as such, the most likely to be involved in antigen presentation.

2.7 Future directions

Different subsets of CD11c+ DCs have been recognized to perform specific antigen-capturing tasks, generally defined by their differential expression of cellular differentiation markers and toll-like receptors. In particular, the DC plasmacytoid subset, expressing DEC-205, have been indicated as the group specifically responsible for cross-presentation of bacterial and viral antigens (Mahnke, Guo et al. 2000; Liu 2001; Steinman 2001; Bonifaz, Bonnyay et al. 2002). It would certainly be worth investigating whether these discrete subsets of DCs express different ABC transporters. This information could be of particular relevance in addressing questions of substrate specificity, as certain DC subsets have been shown to take up and present specific pathogens through receptor-mediated intake. Potential antigen-presenting ABC transporters identified in each of these subsets may indicate specificity for these pathogenic antigen substrates.

Having identified several ABC transporter genes with potential antigen presenting functions, the next step will be to characterize each one independently with regards to their effects on antigen presentation. To do this, each one should be evaluated with loss-of function and gain-of function experiments. Since antigen presentation occurs in primary antigen presenting cells, the best model would be to work *in-vivo* in mouse models that could be

compared to previous work in the antigen presentation field, such as the TAP1 (Van Kaer 2001), and ABCB9 (Chapter III) knockout mice. For each of these genes, subcellular location will be determined, and an array of tissues will be evaluated for their levels of expression. We are currently in the process of generating knockout mice to several of these candidate genes, and we will evaluate their *in-vivo* response to endogenous VSV viral antigens, exogenous heat-killed viral antigens (HI Sendai), and the well-defined cross-presented exogenous antigen ovalbumin. Should any exhibit an abnormal phenotype, primary APCs will then be evaluated for their ability to process and present the respective antigens. Any ABC transporters that are found to be involved in antigen presentation may also have the potential to be eventually used for gene therapy to treat a variety of diseases that are combated by cell-mediated immunity. This potential has been demonstrated for cancer treatment using the ABC transporter ABCB2 (TAP1) in a mouse model, resulting in tumour reduction (Alimonti, Zhang et al. 2000).

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CHAPTER III

IMMUNOLOGICAL CHARACTERIZATION OF THE ABCB9 KNOCKOUT MOUSE

CHAPTER III. IMMUNOLOGICAL CHARACTERIZATION OF THE ABCB9 KNOCKOUT MOUSE

3.1 Overview

The uptake of exogenous antigen by dendritic cells (DCs), and the processing of these antigens into peptides for loading onto major histocompatibility complex (MHC) proteins, termed cross-presentation, is a key event in mounting an immune response (Banchereau and Steinman 1998; Heath and Carbone 2001; Mellman and Steinman 2001). Although the transporter associated with antigen processing (TAP), composed of an ABCB2/ABCB3 heterodimer, is required to transport endogenous peptides through the endoplasmic reticulum (ER) (Kelly, Powis et al. 1992), to date, ATP-binding cassette (ABC) genes that play a role in exogenous antigen presentation have not been identified (Moore, Carbone et al. 1988; Kovacsovics-Bankowski and Rock 1995; Schmitt and Tampe 2002). We have characterized the role of a novel ABC protein, ABCB9 (also known as TAPL), whose expression is induced in mature dendritic cells and is localized to endosomes (Zhang, Zhang et al. 2000). Here we show that ABCB9$^{-/-}$ mice generate deficient T-cell responses to exogenous MHC class I (MHC-I) antigens, but not to exogenous MHC class II (MHC-II) or endogenous MHC-I antigens. This deficiency reduces the ability of ABCB9$^{-/-}$ DCs to cross-present exogenous antigens on MHC-I molecules, thus identifying ABCB9 as the first ABC family member to specifically function in cross-presentation.

3.2 Introduction

Uptake and presentation of exogenous antigens by DCs is key to the production of an effective immune response against viruses, bacteria, tumor antigens, as well as for the generation of tolerance to self (Bachmann, Lutz et al. 1996; Steinman, Turley et al. 2000; Steinman and Nussenzweig 2002; Valdez, Mah et al. 2002). Exogenous antigen presentation requires extracellular antigens to pass from phagosomes or endosomes through the cytosol into endolysosomes (Lizee, Basha et al. 2003) or MHC-II compartments (Amigorena, Drake et al. 1994) for presentation on MHC-II or cross-presentation on MHC-I (Bevan 1987; Mellman, Pierre et al. 1995). DCs cross-present exogenous antigens on MHC-I in three ways: TAP-dependent ER-loaded peptide (Moore, Carbone et al. 1988; Kovacsovics-Bankowski and Rock 1995; Brossart and Bevan 1997; Yewdell and Bennink 2001) (conventional), phagolysosome peptide loading (Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003) and TAP-independent endolysosome-loaded peptides (Bachmann, Oxenius et al. 1995; Liu, Zhou et al. 1995; Gromme and Neefjes 2002).

Well-characterized for its role in endogenous antigen presentation, the TAP protein complex is located within the ER membrane (Kelly, Powis et al. 1992). TAP functions to transport endogenously-synthesized peptides from the cytosol into the ER to access nascent MHC-I. ABCB9 is a member of the same ABC family as TAP but localizes to the endo/lysosomal membrane (Zhang, Zhang et al. 2000). Based on homology to TAP, and endo/lysosomal location, we hypothesized that this protein may act as a transporter involved in exogenous antigen presentation. To test this hypothesis, we characterized an ABCB9 knockout

mouse, the phenotype of which demonstrates that ABCB9 is integral to cross-presentation of exogenous antigens.

3.3 Materials and methods

Generation of ABCB9$^{-/-}$ mutant mice

The ABCB9$^{-/-}$ mice were obtained from the lab of Dr. Victor Ling at the BC Cancer Research Centre, Vancouver, Canada. Briefly, the mice were made in 129Sv/Ev embryonic stem cells by germline homologous recombination of two fragments of cloned DNA surrounding a neomycin (NEO) cassette. 6.3 Kb of genomic DNA including 215 bp of coding sequence from exons 2-4 were replaced with the NEO cassette, followed by introduction of an in-frame stop codon. The GenBank (NCBI) genome database was used to identify the exon-intron boundaries of ABCB9 mRNA sequences; mABCB9 Genbank (NCBI) genomic sequence accession number NT_039312; mRNA accession number AF216495.

Selected ES cell lines were subsequently used to produce chimeric mice. Of these chimeras, one with germline transmission was selected. The ABCB9$^{-/-}$ mice used in this manuscript were all backcrossed to C57BL/6 mice a minimum of 7 generations. C57BL/6 were used as wild-type contols.

ABCB9$^{-/-}$ genotyping

ABCB9$^{-/-}$ offspring were genotyped using PCR primers 5' GGA AAA GCG CCT CCC CTA CCC GGT AG 3' (neo fwd), 5' GGA ACT GTC TCT TCC GCT CAC TGG 3' (WT fwd) and 5' GGG AAT AAT GGG GAG CAC TTC TGG 3' (uniform rvs).

Antibodies

Anti-CD4:PE (L3T4, RM4-5), anti-CD8a:FITC (Ly-2, 53-6.7), anti-H2-Kb:FITC (AF6-88.5), anti-I-Ab:biotin (KH74) with streptavidin:PE secondary antibody (Jackson, ME), anti-I-Ab:PE, anti-CD3:biotin, streptavidin:PE, anti-B220:PE, anti-Mac1:FITC or anti-CD11c (HL3):biotin, anti-mIL-2 (BD Biosciences, California). Unless otherwise noted, all antibodies were obtained from Pharmingen, Canada.

Media

Unless otherwise specified, all *in-vitro* assays were conducted in CTL media: RPMI 1640 + 1% antibiotics (penicillin/streptomycin) + 1% NEAA + 1% L-glutamine + 1% HEPES + 0.1% 2-Mercaptoethanol + 10% Hyclone (UT) fetal bovine serum (FBS).

Cell lines

RMA-S (Ljunggren, Paabo et al. 1989) is an H-2K^{b}-haplotype T-cell line deficient in TAP2. B04 (Shastri, Gammon et al. 1986) is a helper T-cell clone recognizing $HEL_{(74-88)}$ peptide on I-A^{b}. B3Z (Shastri and Gonzalez 1993) is a CTL clone specific for H-2K^{b}/$OVA_{(257-264)}$.

Peptides

H-2K^b-restricted VSV-N$_{(52-59)}$ peptide (RGYVYQGL); H-2K^b-restricted Sendai$_{(324-332)}$ peptide (FAPGNYPAL); H-2K^b-restricted OVA$_{(257-264)}$ peptide (SIINFEKL); I-A^b-restricted HEL$_{(74-88)}$ peptide (NLANIPASALLSSDI).

CTL Assays

All CTL assays were conducted by stimulating mice with i.p. injection of antigen or antigen-pulsed DCs, as described (Brossart and Bevan 1997). Target cells were ^{51}Cr-pulsed RMA-S cells, pulsed with or without relevant peptide.

Tetramer staining

CTLs bearing the T-cell receptor specific for H-2K^b/OVA$_{(257-265)}$ or H-2K^b/VSV-N$_{(52-59)}$ were identified by staining on day 0 and 4 *ex-vivo* with the relevant tetramer (iTAg from Beckman Coulter, CA) as per manufacturer's directions and analyzed on a FACScalibur™ flow cytometer.

Tissue expression of ABCB9

RNA was extracted using a Qiagen (Canada) RNEasy kit and cDNA produced using SuperscriptII RT enzyme (Gibco-BRL, Canada) as per manufacturers' directions. Expression of ABCB9 was evaluated by RT-PCR using the primers 5' TCG GGA GCC ACA CTA CAG AA 3' (fwd) and 5' AAG CCC ATG AAG GTG ACC AG 3' (rvs). Bone marrow CD11c+ dendritic cells from C57BL/6 mice were cultured for 24-48 hours in the presence or absence of 10 ng/mL TNFα to evaluate ABCB9 inducibility.

Antigen presentation assays

MHC-I. DCs were purified using CD11c microbeads (Miltenyi Biotec, Canada) as per manufacturer's directions. Cells were cultured overnight with 0-10 mg/mL ovalbumin (Worthington, NJ), or 1μM $OVA_{(257-265)}$ peptide. Antigen presentation of H-$2K^b$/OVA was measured directly by staining with 25-D1.16 antibody (Porgador, Yewdell et al. 1997). Endogenous ovalbumin processing was evaluated using a vaccinia virus (VV) construct containing either vector alone (pjs-5), whole ovalbumin (VV-OVA), or the $OVA_{(257-265)}$ peptide (vv-SIINFEKL). DCs were infected at a multiplicity of infection (MOI) of 10:1, washed and co-incubated with B3Z. B3Z activation was determined by IL-2 ELISA (BD Pharmingen, CA). *MHC-II.* B04 T-cells were co-incubated with splenic B-cells or DCs and pulsed overnight with 0, 2 mg/mL HEL protein, or 1 μM $HEL_{(74-88)}$ peptide. B04 activation was determined by IL-2 ELISA as above.

Cellular profiles

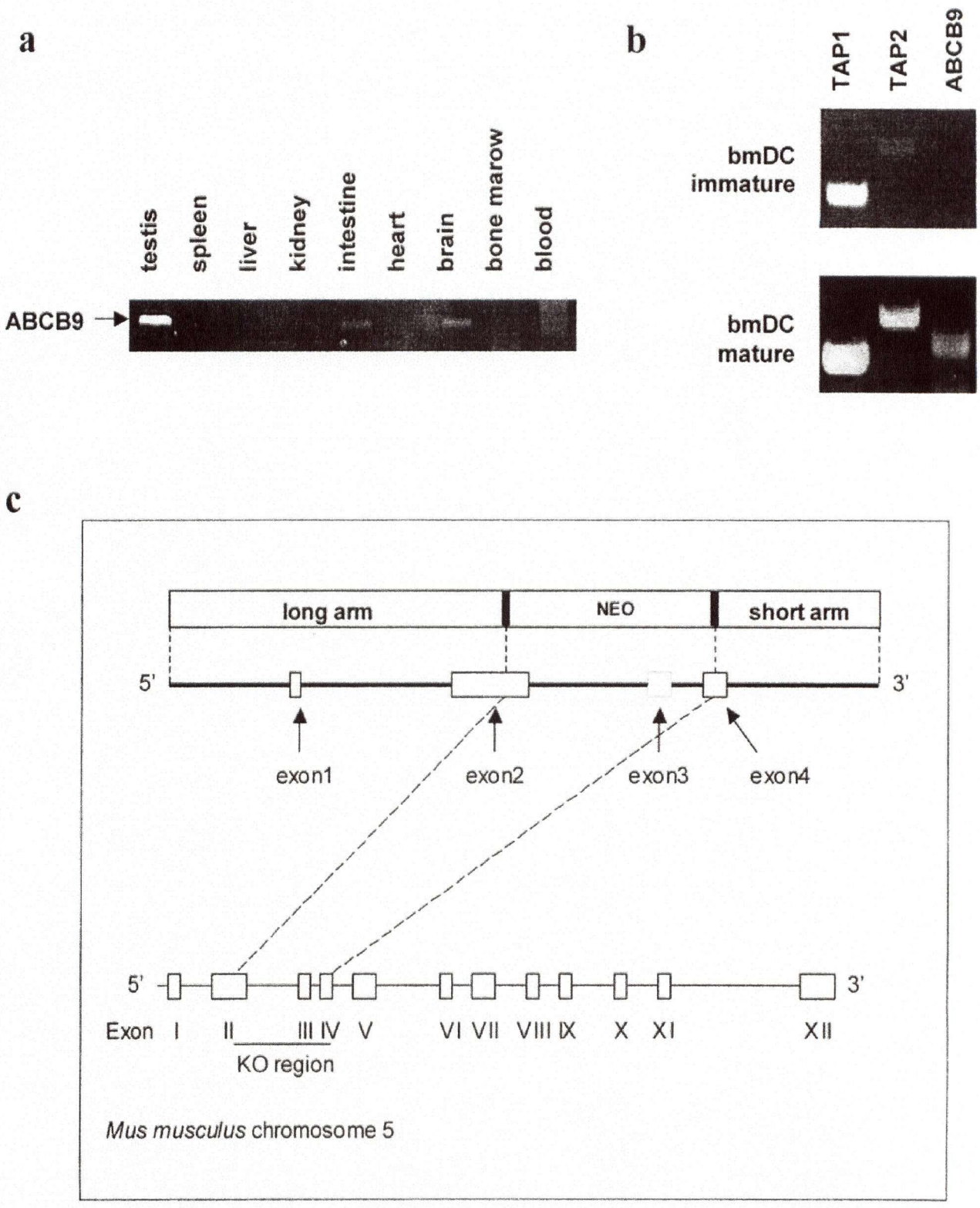
Cell profiles were evaluated in the peripheral lymphoid organs (lymph nodes, thymus and spleen) by flow cytometry of antibody staining using a FACScan™ (Becton-Dickinson, CA) and CellQuest™ software. CD3, CD11c, CD11b (CD11c depleted), and B220 were used to differentiate T-cells, DCs, MØs, and B-cells respectively.

Statistics

Statistical analyses of data were conducted using the paired T-test.

3.4 Results

Based on RNA analysis, the expression of ABCB9 has been observed at very low levels in most tissues, with the exception of brain and testis (Zhang, Zhang et al. 2000), where expression is higher (Figure 3.1a). We find negligible expression in B-cells, macrophages and immature dendritic cells. ABCB9 induction was compared to TAP1 and TAP2 (known antigen-presenting ABCs) in immature and mature (TNFα stimulated) DCs. Transcripts of all 3 transporters were seen to be upregulated (Figure 3.1b) in response to cytokine stimulation.


a
testis
spleen
liver
kidney
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brain
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ABCB9
b
TAP1
TAP2
ABCB9
bmDC immature
bmDC mature
c
long arm
NEO
short arm
5'
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exon1
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exon3
exon4
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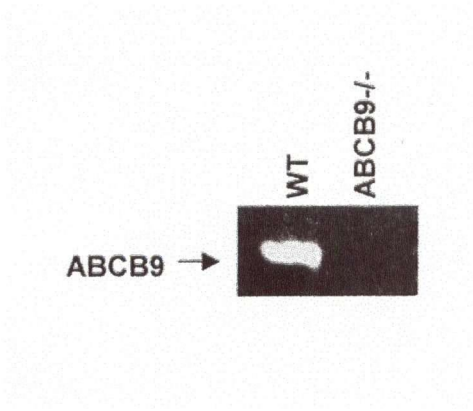
Exon I II III IV V VI VII VIII IX X XI XII
KO region
Mus musculus chromosome 5

Figure 3.1. ABCB9 gene knockout construct and expression profile. RT-PCR of ABCB9 expression in C57BL/6 mouse (a) tissues or (b) purified bone marrow derived (bm)DCs in an immature state, and matured with TNFα, including TAP1 and TAP2 expression levels. (c) Diagram of the ABCB9 genomic DNA knockout region.

To assess the role of ABCB9 in immune function, we derived the ABCB9$^{-/-}$ mouse (summarized in Figure 3.1c) and observed that this mouse does not express ABCB9 mRNA (Figure 3.2a). The ABCB9$^{-/-}$ mice are normal in appearance, fertility, and growth rate compared to wild-type mice. To determine whether ABCB9 affected leukocyte development, we examined leukocyte sub-populations in lymphoid tissues using flow cytometry. The thymus and lymph nodes of the ABCB9$^{-/-}$ mice show a sharp reduction in total cellularity (-50%). Examination of the total numbers of different cell types in these organs revealed half as many B- and T-cells as wild-type (Figure 3.2b,c). The total numbers of DCs and MØ in these organs were normal, however, as were the splenocytes, with the exception of slightly elevated numbers of B-cells (Figure 3.2b,c). This decrease in LN B- and T-cells, accompanied by a decrease in numbers of thymic MHC–I and MHC–II expressing cells, clearly suggests a role for ABCB9 in T-cell development.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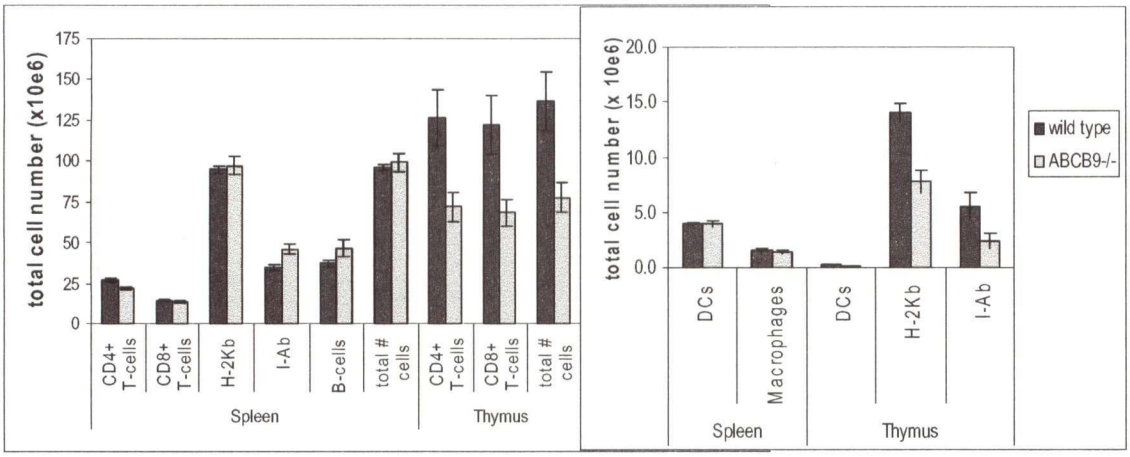
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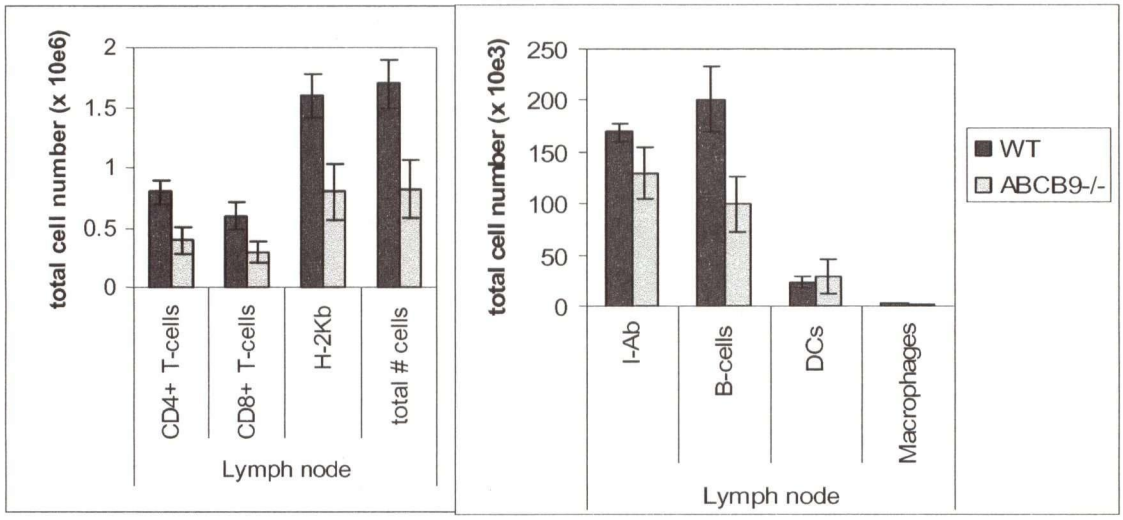
150
125
100
75
50
25
0
CD4+ T-cells
CD8+ T-cells
H-2Kb
I-Ab
B-cells
total # cells
CD4+ T-cells
CD8+ T-cells
total # cells
Spleen
Thymus
total cell number (x 10e6)
20.0
15.0
10.0
5.0
0.0
DCs
Macrophages
DCs
H-2Kb
I-Ab
Spleen
Thymus
wild type
ABCB9-/-

b


total cell number (x 10e6)
2
1.5
1
0.5
0
CD4+ T-cells
CD8+ T-cells
H-2Kb
total # cells
Lymph node
total cell number (x 10e3)
250
200
150
100
50
0
I-Ab
B-cells
DCs
Macrophages
Lymph node
WT
ABCB9-/-

c

Figure 3.2. Cellular profiles of ABCB9$^{-/-}$ mice show decreased B-cells and T-cells in lymph node and thymus, and a fewer MHC class I+ or II+ thymocytes. (a) RT-PCR of ABCB9 expression in wild-type and ABCB9$^{-/-}$ mouse brain. (b-c) Total organ cellularity of various cellular subtypes in (b) spleen and thymus and (c) lymph nodes as measured by flow cytometry, multiplied by total cell counts. The profiles depicted represent mean +/- SE of 3 mice.

We investigated whether the loss of ABCB9 resulted in a phenotype similar to the TAP1$^{-/-}$ mouse (van Kaer, Ashton-Rickardt et al. 1992), with no CD8+ T-cells nor K^b expression, and defective endogenous antigen presentation. In response to infection with vesicular stomatitus virus (VSV), ABCB9$^{-/-}$ mice produced normal numbers of total K^b/VSV-$N_{(52-59)}$ (K^b/VSV) specific CTLs, as determined directly by staining with K^b/VSV tetramers (Figure 3.3a,b). ABCB9$^{-/-}$ mice were also able to generate a functional VSV CTL response, measured by ^{51}Cr-release assay (Figure 3.3c). In contrast to the TAP1$^{-/-}$ mouse, ABCB9$^{-/-}$ mice were fully capable of priming a T-cell response by presenting peptides from phagoctyosed infected cells. ABCB9 therefore does not appear to function analogously to the TAP molecule because a live virus infection results in a robust CTL response.

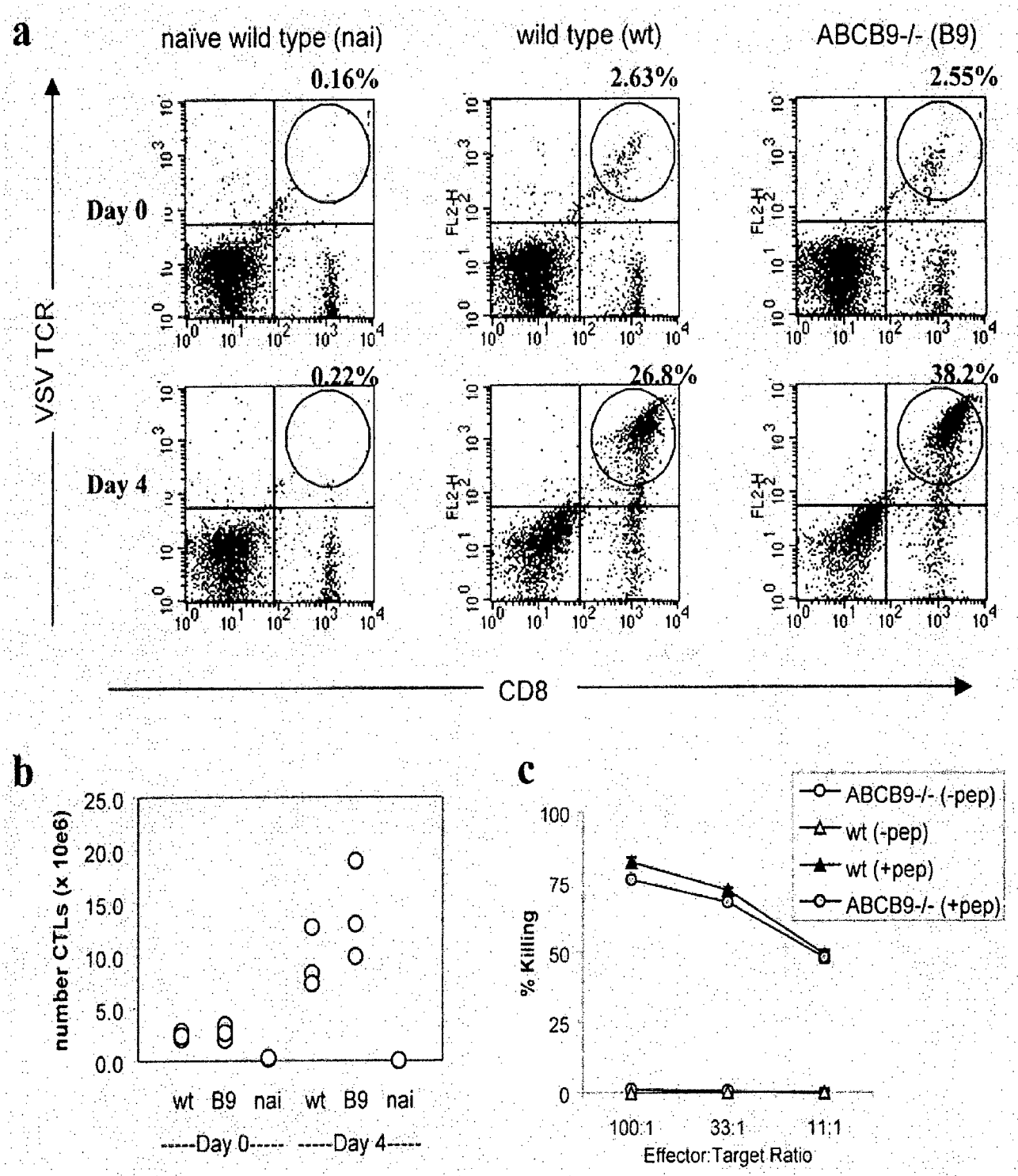

a
naïve wild type (nai)
wild type (wt)
ABCB9-/- (B9)
0.16%
2.63%
2.55%
Day 0
0.22%
26.8%
38.2%
Day 4
VSV TCR
CD8
b
number CTLs (x 10e6)
25.0
20.0
15.0
10.0
5.0
0.0
wt B9 nai wt B9 nai
-----Day 0----- -----Day 4-----
c
% Killing
100
75
50
25
0
100:1
33:1
11:1
Effector:Target Ratio
ABCB9-/- (-pep)
wt (-pep)
wt (+pep)
ABCB9-/- (+pep)

Figure 3.3. ABCB9$^{-/-}$ mice generate a normal CTL response to live virus. (a) Flow cytometric tetramer analysis of splenocytes from mice infected 7 days with VSV. Percent K^b/VSV-$N_{(52-59)}$ specific CTLs from total splenocytes on (top) day 0 and (bottom) day 4 *ex-vivo*. (b) Quantity of K^b/VSV-$N_{(52-59)}$ specific CTLs from total wild-type (wt), ABCB9$^{-/-}$ (B9), or naïve (naï) splenocytes, N=3 mice. (c) ^{51}Cr-release CTL assay of VSV primed effector splenocytes against targets pulsed + or – VSV peptide.

To further evaluate the role of ABCB9 in viral cross-presentation, we injected mice with heat-inactivated (HI) Sendai virus, an exogenous antigen cross-presented on K^b molecules in a 'TAP-independent' manner. The immunodominant peptide $NP_{(324-332)}$ is processed via the endocytic pathway and loaded onto K^b molecules in endolysosomal compartments. We found that ABCB9$^{-/-}$ mice were unable to mount an effective CTL response as measured by ^{51}Cr-release assay. CTL function appears to be reduced approximately 10-fold, requiring 10x more CTL to achieve the same amount of killing as wild-type cells (Figure 3.4). As DCs are the most relevant APC involved in cross-presentation (Banchereau and Steinman 1998; Mellman and Steinman 2001), we asked whether the ABCB9$^{-/-}$ mouse CTL deficiency to HI-Sendai was due to a specific defect in DC cross-presentation and priming of T-cells. We pulsed DCs with HI-Sendai and injected these into syngeneic mice to prime a DC-specific CTL response (Inaba, Metlay et al. 1990; Brossart and Bevan 1997). Our results show that although antigen-pulsed DCs were able to more efficiently prime a CTL response than DCs stimulated by intra-peritoneal (i.p.) injection of antigen alone, the overall CTL response in the ABCB9$^{-/-}$ mice was still reduced 10-fold

compared to their wild-type counterparts (Figure 3.4b). This supports the hypothesis that loss of ABCB9 results in a defect in cross-presentation of HI-Sendai antigen.

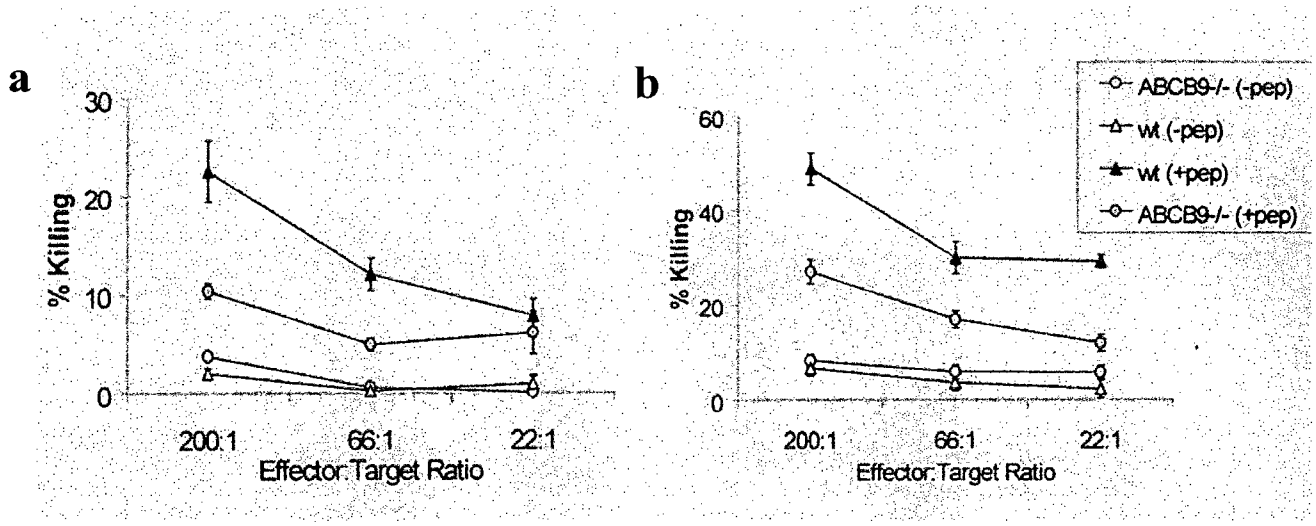


Figure 3.4. ABCB9$^{-/-}$ mice generate a deficient CTL response to killed virus. ^{51}Cr-release CTL assay against HI-Sendai, primed by (a) direct i.p. HI-Sendai injection, or (b) i.p. injection of syngeneic DCs pulsed with HI-Sendai. Mean+/-SE, triplicate samples. Similar results were obtained in three different experiments.

This TAP-independent pathway of antigen presentation, typified by HI-Sendai antigen, is known to be chloroquine sensitive, brefeldin-A resistant, and TAP independent (Liu, Zhou et al.

1995; Liu, Chambers et al. 1997). It is generally assumed that peptides from these endocytosed antigens are generated within the endolysosome by acidification and cathepsin activity. We show however that these TAP-independent antigens access the cytosol to undergo proteasome degradation into peptides. Treating DCs with lactacystin, a proteasome inhibitor, while pulsing with HI-Sendai, abrogated CTL priming ability (Figure 3.5).

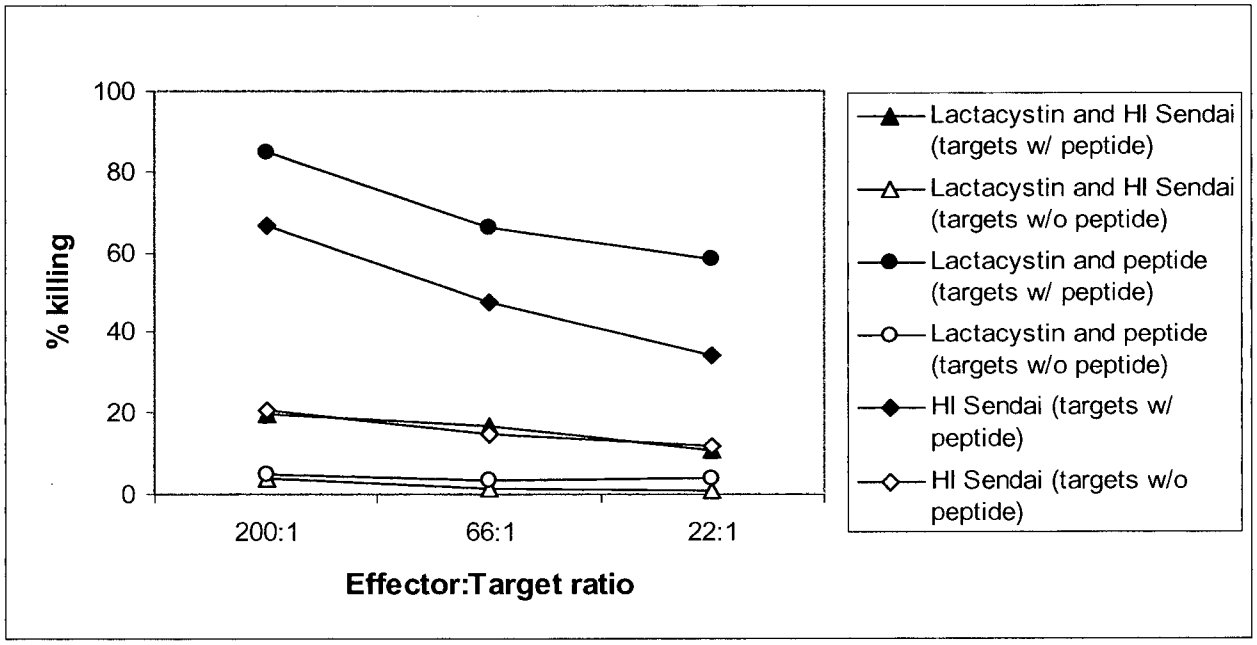


Figure 3.5. HI Sendai antigen processing is proteasome-dependent. ^{51}Cr-release CTL assay against HI Sendai in wild-type mice. DCs were purified from C57BL/6 mouse spleens and pulsed +/- the proteasome inhibitor lactacystin prior to incubation with HI Sendai antigen, or Sendai$_{(324-332)}$ peptide. Effector CTLs were primed *in-vivo* by i.p. injection of these +/- lactacystin, +/- HI Sendai/peptide pulsed DCs into syngeneic

animals on day 0 and 7. On day 14, spleens were collected from primed animals and restimulated in-vitro with 1 μM Sendai$_{(324-332)}$ peptide. Splenocytes were used as effectors in a standard ^{51}Cr-release assay against chromium-labelled RMA-S target cells. Filled shapes indicate target cells pulsed with Sendai$_{(324-332)}$ peptide, open shapes indicate target cells with irrelevant peptide bound (negative controls). Results represent mean of triplicate samples.

In order to assess whether loss of ABCB9 also affects presentation of exogenous non-viral antigens, we examined the CTL response to ovalbumin (OVA). DCs were pulsed with soluble OVA and injected into syngeneic mice. To determine whether cross-presentation of OVA protein in ABCB9$^{-/-}$ mice affected K^b/OVA$_{(257-264)}$ (K^b/OVA) peptide-specific CTL production *in-vivo*, we measured the generation of splenic CTLs directly with K^b/OVA tetramer (Figure 3.6a). ABCB9$^{-/-}$ mice on average produced 10-fold fewer K^b/OVA CTLs than wild-type animals (Figure 3.6b). K^b/OVA CTL function was further examined by ^{51}Cr-release assay. Similar to the response to HI-Sendai, ABCB9$^{-/-}$ splenocytes were 10x less effective at killing K^b/OVA target cells (Figure 3.6c).

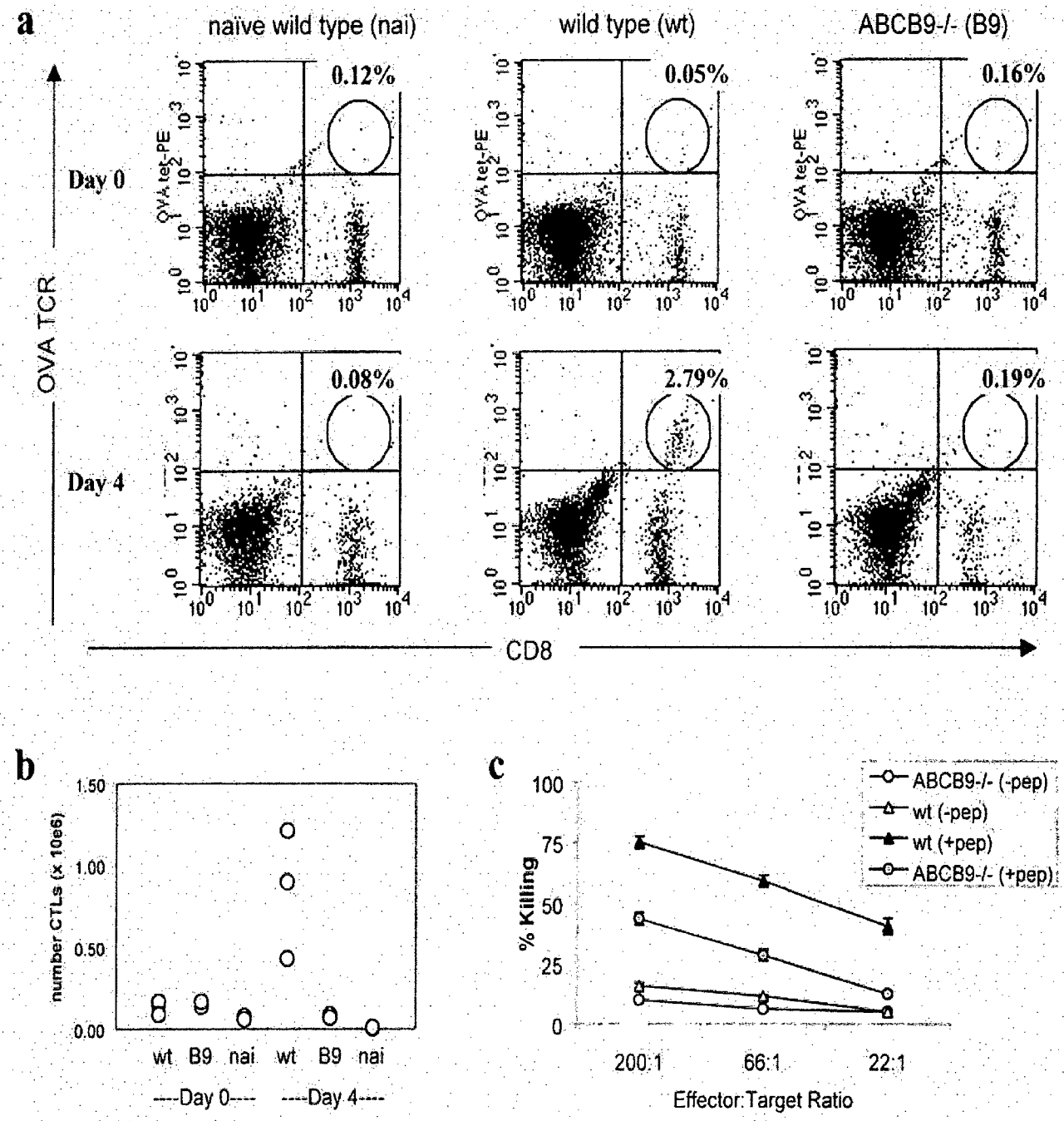


Figure 3.6. ABCB9$^{-/-}$ mice have defective DC cross-presentation and CTL response to exogenous antigen. (a) Flow cytometric tetramer analysis of splenocytes from OVA primed mice. Percent $K^b/OVA_{(257-264)}$ specific CTLs from total splenocytes on (top) day 0 and (bottom) day 4 *ex-vivo*. (b) Quantity of $K^b/OVA_{(257-264)}$ specific CTLs from total

splenocytes (each circle represents one mouse, N=3). (c) ^{51}Cr-release CTL assay for $K^b/OVA_{(257-264)}$ specific CTLs primed by i.p. injection of syngeneic DCs pulsed with OVA.

We sought to directly confirm whether this defective CTL response was due to an antigen presentation defect in DCs by pulsing DCs with OVA, then measuring surface K^b/OVA with either the 25-D1.16 monoclonal antibody (Porgador, Yewdell et al. 1997) (Figure 3.7) or the K^b/OVA specific T-cell, B3Z (Shastri and Gonzalez 1993) (Figure 3.8). ABCB9$^{-/-}$ mouse DCs were found to be significantly deficient ($p<0.05$) in their ability to process and present K^b/OVA on the cell surface. Peptide-pulsing restored K^b/OVA surface expression, indicating conformationally intact K^b molecules at the cell surface. As both HI-Sendai and ovalbumin are egressed from endosomes into the cytosol following their endocytosis, the defective presentation of both antigens in ABCB9$^{-/-}$ mice is consistent with a defect in antigen transport out of the endosome.

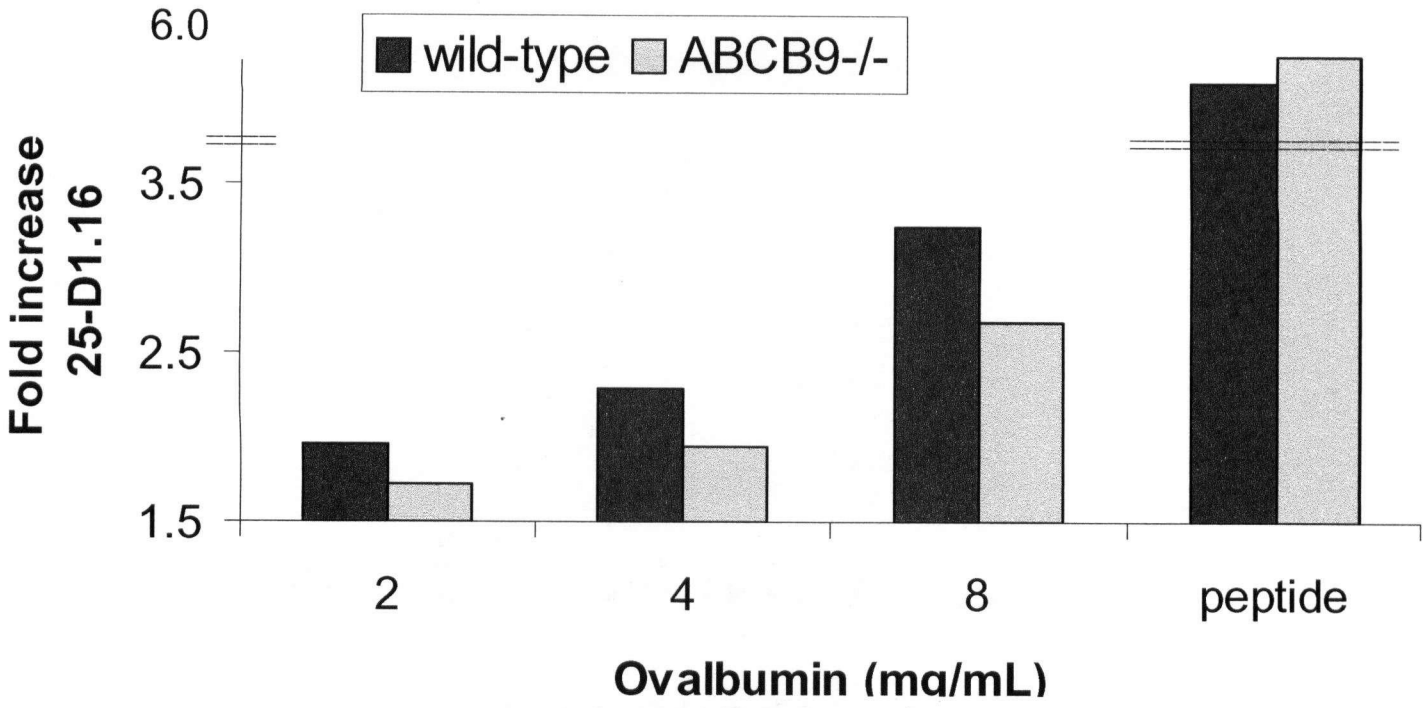


Figure 3.7. ABCB9$^{-/-}$ DCs are defective in cross-presentation of ovalbumin antigen on H-2K^{b}. DCs were pulsed *in-vitro* with various concentrations of ovalbumin antigen overnight or peptide prior to staining for K^{b}/OVA surface presentation with 25-D1.16 antibody.

Based upon localization of ABCB9 to endo/lysosomes (Zhang, Zhang et al. 2000), we examined whether the inability to present exogenous OVA antigen was due to a block of antigen transport from endosomes into the cytosol, or a block of cytosolic antigen movement into the ER and endolysosomes. Using vaccinia virus constructs encoding OVA (VV-OVA), or OVA$_{(257-264)}$ peptide (VV-SIINFEKL), we introduced these antigens directly into the cytosol of DCs, bypassing the need for endosomal uptake and transport. Co-incubation with B3Z demonstrated

that ABCB9$^{-/-}$ DCs infected with VV-OVA or VV-SIINFEKL presented K^b/OVA at levels comparable to wild-type DCs (Figure 3.8). This indicates a defect in exogenous antigen transport from endocytic vesicles into the cytosol in ABCB9$^{-/-}$ mice.

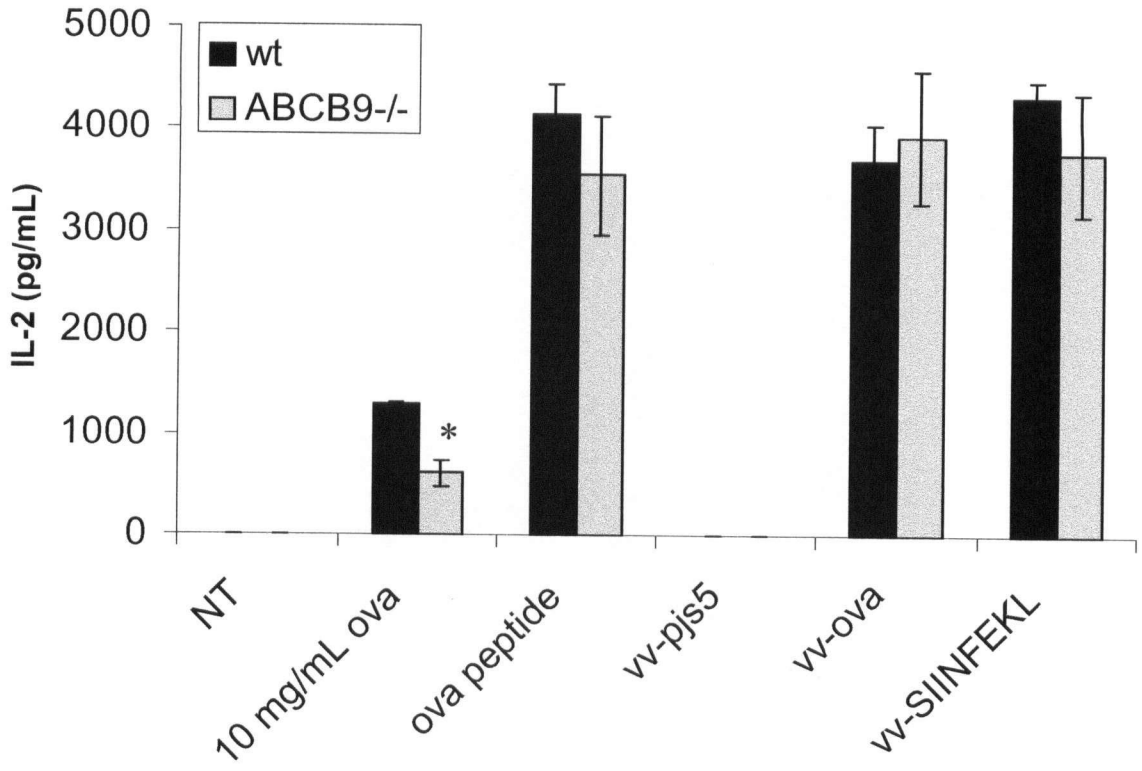


Figure 3.8. Endogenously supplied OVA antigen rescues surface K^b/OVA presentation in ABCB9$^{-/-}$ mouse DCs. DCs were pulsed *in-vitro* with whole ovalbumin or OVA peptide, or infected with vector alone (vv-pjs5), vector carrying full-length ovalbumin (vv-ova), or carrying ova peptide (vv-SIINFEKL). DCs were then co-incubated with B3Z CD8+ T-cells and IL-2 production was measured by ELISA assay. Values are

mean+/-SE, triplicate samples. Similar results were obtained in three different experiments. *$p<0.05$, T-test.

We next examined whether the defect in exogenous antigen processing in ABCB9$^{-/-}$ mice extended to presentation of the MHC-II (I-A^b)-restricted antigen, hen egg lysozyme (HEL). The presentation of I-A^b/HEL$_{(74-88)}$ peptide (I-A^b/HEL) was examined using a B04 T-cell clone (Shastri, Gammon et al. 1986) functional assay for T-cell activation. DCs and B-cells were separately pulsed with HEL and assayed for I-A^b/HEL presentation. The APCs from both ABCB9$^{-/-}$ and wild-type mice presented I-A^b/HEL, resulting in comparable IL-2 production by B04 cells (Figure 3.9). Disruption of the ABCB9 gene does not appear to impact exogenous MHC-II antigen presentation, thus endocytosis is not affected and function of the ABCB9 gene appears to be specific to exogenous MHC-I antigens.

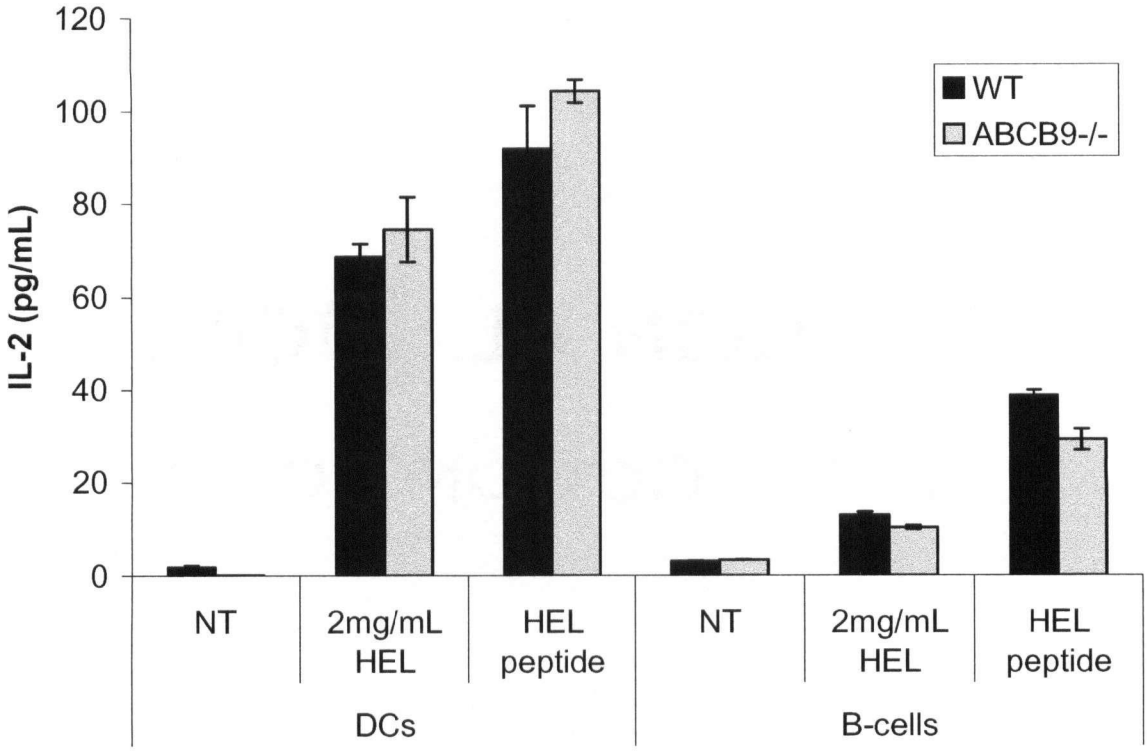


Figure 3.9. ABCB9$^{-/-}$ mice have normal exogenous antigen presentation of MHC-II restricted HEL antigen. DCs or B-cells were pulsed *in-vitro* with whole hen egg lysozyme or $HEL_{(74-88)}$ peptide prior to incubation with B04 CD4+ T-cells. IL-2 production was measured by ELISA assay. Mean+/-SE, triplicate samples. Similar results were obtained in three different experiments.

3.5 Discussion

We show here that ABCB9 is involved in cross-presentation of exogenous antigen on MHC-I, but not on MHC-II. Moreover, it is not involved in presentation of endogenous antigens in the MHC-I pathway. ABCB9$^{-/-}$ mice make fewer T-cells and B-cells than wild-type mice, a phenotype partially shared by TAP1$^{-/-}$ mice, in which endogenous antigen presentation is disrupted. This indicates a function in lymphocyte development, potentially during thymic selection. The observed reduction in DC surface MHC-I restricted exogenous antigen presentation may affect the T-cell repertoire selected in the thymus, additionally contributing to the observed CTL deficiency. ABCB9 has been shown to be localized to the endo/lysosomes (Zhang, Zhang et al. 2000), and recent evidence delineating the mechanisms of cross-presentation in APCs has proposed that MHC-I peptide loading takes place in specialized late endolysosomal compartments and the ER-phagosome, which are likely one and the same (Ackerman, Kyritsis et al. 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003; Lizee, Basha et al. 2003).

Here we demonstrate that ABCB9 appears to be involved in regulating the movement of exogenous MHC-I-restricted antigens out of the endosome into the cytosol, or as a substrate-specific transporter for moving exogenous antigens from the phagosome-associated proteasome back into the endolysosome / ER-phagosome. This effect on exogenous antigen presenatation may be the result of direct antigenic substrate transport, or it may function in the endosome by transporting in proteases to allow the denaturing or partial digestion of the antigen. We conclude that the ability of DCs to process and cross-present exogenous antigens is modulated by the endo/lysosomal ABCB9 protein. The ability of ABCB9$^{-/-}$ mice to generate a strong CTL

response to VSV suggests that cross-presentation of live virus accesses this compartment in a different manner than do exogenous antigens. Alternate expanations for this observation include the existence of alternate pathways, or differential transporter substrate specificities; less likely is the possibility of direct infection of DCs (Sigal, Crotty et al. 1999). Viral antigens, such as VSV, are endogenously expressed within infected cells. These infected cells are phagocytosed by DCs, where the viral antigens pass, presumably via Sec61, from the ER-derived phagosome into the cytosol for proteasome degradation (Ackerman, Kyritsis et al. 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003). Exogenous antigens, such as ovalbumin and HI-Sendai, are endocytosed into endosomes. Lacking Sec61 transporter, endosomes must move antigens into the cytosol by another method. Our results suggest that ABCB9 may be involved in antigen transport from endosomes. Alternatively, ABCB9 may allow substrate specific transport of exogenous antigens from the cytosol into the ER-phago/endolysosome compartment. Presence in this latter compartment could also explain the residual levels of exogenous antigens on MHC-I, as having been diverted from the cytosol through the endogenous ER-restricted pathway. As a result, we observe some antigen presentation on the DC cell surface, but this does not appear to be sufficient to prime a CTL response. This model is depicted in Figure 3.10.

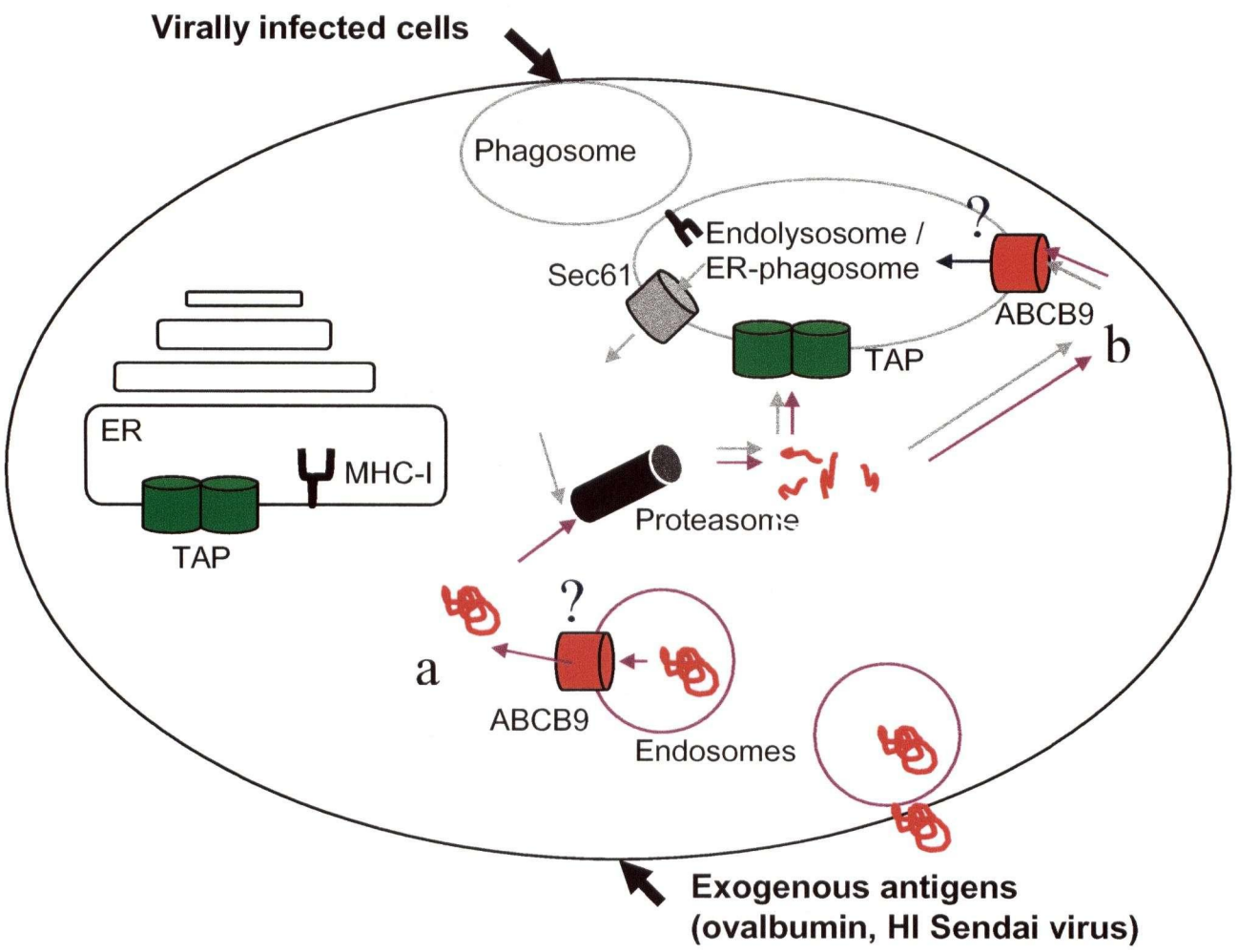


Figure 3.10. Proposed models of ABCB9 as an endosomal or endolysosomal antigen transporter in cross-presentation. (a) Exogenous antigens are taken up through the endosomal pathway, processed through ABCB9 into the cytosol to be degraded by the proteasome. Alternatively, (b) following proteasome degradation, antigens from both the phagocytic and endocytic pathways pass through the substrate specific ABCB9 or TAP into the ER or ER-phagosome / endolysosome to access MHC-I in this antigen presenting compartment.

Barrier-protected organs such as brain and testis are considered to be immune-privileged sites. The cross-presentation of self antigens by DCs has been shown to be the main mechanism of induction of tolerance to self (Bachmann, Lutz et al. 1996; Steinman, Turley et al. 2000; Heath and Carbone 2001; Steinman and Nussenzweig 2002). The high levels of ABCB9 in brain microglia (unpublished observation) and testis Sertoli cells (Zhang, Zhang et al. 2000) may provide a high constitutive level of exogenous self-antigens in order to promote tolerance in these immunologically sensitive sites. Our data suggests a possible role for ABCB9 in thymic T-cell selection and peripheral self-tolerance, in addition to immune priming. A role for ABCB9 in exogenous antigen movement through the endolysosome may begin to reveal the molecular processes involved in this crucial immunological event.

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CHAPTER IV

EXOGENOUS CROSS-PRIMING AND TUMOUR IMMUNOLOGY

CHAPTER IV. EXOGENOUS CROSS-PRIMING AND TUMOUR IMMUNOLOGY

4.1 Overview

Cancer arises in the body when genetic mutations accumulate and allow cells to proliferate in an unchecked manner. The cellular immune response regularly surveys for such cells by testing for tumour associated antigens (TAA) bound to class I MHC molecules on the cell surface. When such a cancerous cell is found by a dendritic cell (DC), it will be processed by the DC in order to cross-prime a T-cell response. Primed cytotoxic T-lymphocytes (CTLs) will then proliferate and circulate through the body sampling for these same MHC-I bound TAAs. Upon encountering the tumour, if they display their TAAs on the surface, CTLs can recognize and destroy the cancerous cells. Normally this process is very efficient, but tumours may arise whereby their antigen presentation is abrogated. The transporter associated with antigen presentation (TAP) has been shown to be integral to this process. Here we investigate the role of another TAP-like protein (ABCB9) in tumour immunology. ABCB9$^{-/-}$ mice are unable to mount an effective immune response to exogenous cross-presented antigens. We wanted to evaluate whether exogenous cross-presentation plays a major role in the anti-tumour immune response. We injected ABCB9$^{-/-}$ mice with the B16 melanoma, CMT.64 or CMT.1-4 small cell lung carcinoma and followed tumour progression and animal survival. Further, we immunized mice with CMT.64 or CMT.1-4 prior to injecting them with live tumour cells. Our results show no significant effect of ABCB9 on survivorship of mice injected with antigen-expressing tumours, whether immunized or not. ABCB9$^{-/-}$ mice do show a slight increase in survival and reduction of tumour growth when injected with non-antigen presenting tumour cells,

and this trend is reversed if they are pre-immunized. This suggests a possible supportive, although not primary role for ABCB9 and exogenous antigen cross-presentation in tumour immunology and a possible function in induction of tolerance.

4.2 Introduction

Cancerous cells arise spontaneously in the body in response to any number of external or internal stimuli. Cancer-inducing agents are often mutagenic agents and include such omnipresent environmental factors as ultraviolet radiation from the sun's rays, cigarette smoke breathed in the air, and naturally occurring or man-made radioactive materials that emit alpha, beta or gamma irradiation. A cancer is essentially a cell that proliferates unchecked and does not die, or one in which the rate of proliferation is greater than that of elimination (Laiho and Latonen 2003). Any number of genetic mutations can contribute to this, and indeed it generally requires several such mutations so that the cell's natural cycle checkpoints can be overridden (Wright, Egan et al. 1990; Laiho and Latonen 2003; Sarasin 2003; Malaguarnera 2004). This requirement for an accumulation of mutations explains why cancers tend to be more prevalent as people age, after increased years of exposure to environmental factors (Sarasin 2003). Cancers also appear in clusters of individuals who are exposed to large amounts of mutagens, such as the incidence of mouth cancer in early watch dial painters, who used radium-infused paint to illuminate the numbers on watch faces, and habitually licked the brush tip to a point (McGrayne 1996).

Cancerous cells arise frequently, but are eliminated by the cellular immune system, which has evolved a very intricate recognition mechanism to eliminate diseased cells. Expression of tumour-associated antigens (TAA) on cellular class I MHC molecules allows the body to recognize them as dangerous and destroy them with cytotoxic T-lymphoctyes (CTLs). Generating an immune response to tumours requires two steps of antigen presentation. First, the antigen presenting cells (APCs), particularly dendritic cells (DCs), must encounter the tumour cell and determine that it is abnormal. Once that occurs the cell itself is phagocytosed and the TAAs are processed and cross-presented on the DC class I MHC. The DCs must then migrate to the lymphoid organs (spleen, lymph nodes) to encounter naïve T-cells and prime them to activate, proliferate and destroy the tumour target cells. Secondly, for the purposes of immunosurveillance, the tumour cells themselves must express the TAAs on their own surface MHC-I molecules, so that activated T-cells can identify and destroy them.

A major method of tumour immune evasion involves the elimination of TAAs on MHC-I of tumour cells. This can be accomplished by mutation or down-regulation of any number of genes and proteins involved in the antigen presentation pathway. Many tumours that do not express TAAs have been examined and found to be defective in their expression of antigen processing transporter TAP1 and/or TAP2, or the proteasome subunits LMP2 and/or LMP7 (Tanaka, Isselbacher et al. 1985; Singal, Ye et al. 1996; Garrido, Ruiz-Cabello et al. 1997). It has been shown that re-introducing these genes by gene therapy is sufficient to partially restore TAA and MHC-I surface expression and allows the cells to be recognized and killed as CTL targets (Tanaka, Isselbacher et al. 1985; Wallich, Bulbuc et al. 1985; Garrido, Ruiz-Cabello et al. 1997; Seliger, Maeurer et al. 1997; Alimonti, Zhang et al. 2000).

Small-cell lung carcinomas (SCLC) are highly malignant in both humans and mice and are generally fatal. The CMT.64 cell line is a murine SCLC that arose spontaneously in a C57BL/6 mouse (Franks, Carbonell et al. 1976) and shows extremely reduced endogenous MHC-I restricted TAA expression at the cell surface (Jefferies, Kolaitis et al. 1993; Mandelboim, Bar-Haim et al. 1997). CMT.64 cells are defective in their expression of proteasome subunits LMP2 and LMP7, and transporter associated with antigen presentation (TAP) subunits TAP1 and TAP2 (Gabathuler, Reid et al. 1994). However, antigen presentation can be rescued in these cells by the addition of TAP1 alone (Gabathuler, Reid et al. 1994; Alimonti, Zhang et al. 2000). This demonstrates that even in tumours where many components are disrupted, it is possible to reimplement immunosurveillance by introducing only one integral gene.

Having established that ABCB9 plays a role in cross-priming, and that ABCB9$^{-/-}$ mice cannot cross-prime exogenous antigens effectively, we wanted to investigate whether exogenous cross-priming has any effect on cancer progression *in-vivo*. This was done using the ABCB9$^{-/-}$ mouse model to evaluate tumour growth and disease progression. As ABCB9 is normally expressed in activated DCs, expression of ABCB9 in the tumours themselves would not be expected to affect immune response. However, a lack of ABCB9 in APCs may create a more permissive environment for tumour growth *in-vivo* by decreasing or eliminating the activation of anti-tumour T-cells by DCs.

Several H-2K^{b} restricted murine tumour cell lines are available for this purpose, such as the melanoma B16/F10 (B16) (Fidler, Gersten et al. 1976) and small-cell lung carcinoma CMT.64 and CMT.1-4 (CMT.64 transfected with TAP1, expressing surface H-2K^{b} molecules) (Alimonti, Zhang et al. 2000). It has been established (Alimonti, Zhang et al. 2000; Thompson,

Timme et al. 2000) that certain H-2K^b expressing tumour cell lines can be destroyed by an anti-tumour cellular immune response, if animals are immunized first with irradiated tumour cells. Our laboratory has previously established that expressing TAP in tumour cells that have downregulated antigen presentation machinery is sufficient to re-implement partial H-2K^b surface expression and incite an anti-tumour immune response (Gabathuler, Reid et al. 1994; Alimonti, Zhang et al. 2000).

To test this hypothesis, we separately injected each of the non-H-2K^b expressing tumour cell lines above (B16/F10, CMT.64) and the CMT tumour cell line stably transfected with TAP1 (CMT.1-4) into wild-type (C57BL/6) and ABCB9$^{-/-}$ mice deficient in exogenous antigen cross-priming. For both the H-2K^b expressing (CMT.1-4) and non-H-2K^b tumour cells, we injected live cells or injected irradiated tumour cells followed by injection of live tumour cells, to determine if there was any differential anti-tumour response between our ABCB9$^{-/-}$ mice and wild-type controls, and whether this was influenced by prior immunization with the tumour antigen.

4.3 Materials & Methods

Models

Mouse models C57Bl/6 and ABCB9$^{-/-}$ (on C57BL/6 background) mice (both H-2K^b haplotype).

Tumour cell lines: B16, a non-K^b expressing H-$2K^b$ haplotype murine melanoma; CMT.64, a non-K^b expressing, H-$2K^b$ haplotype murine small-cell lung carcinoma; CMT.1-4 (CMT.64 stably transfected with TAP1 to reconstitute partial K^b expression).

Tumour injections

For B16 cells, $1.5x10^5$ were injected sub-cutaneously (s.c.) in 100 μL of PBS with a 25G needle on the right hind leg. B16 were separately injected s.c. on matched cohorts of mice from the wild-type and ABCB9$^{-/-}$ groups. Tumour growth was monitored until any single tumour was estimated at ~10% body weight, at which point all animals were sacrificed, the tumours excised and weighed.

For CMT cells, $3.5x10^5$ were injected intra-peritoneally (i.p.) in 500 μL of PBS with a 25G needle. CMT.64 or CMT.1-4 (an adenocarcinoma) was separately injected intraperitoneally (i.p.) into wild-type and ABCB9$^{-/-}$ groups of mice. To immunize animals, CMT cells were irradiated with 10^4 rads before injecting $3.5x10^5$ cells per mouse i.p.. 14 days later the animals were challenged with $3.5x10^5$ live cells per mouse i.p.. The animals were monitored for signs of morbidity, at which time each individual morbid animal was euthanized, and a survivorship record was kept in graphical form.

Statistics

All experimental results were evaluated for statistical significance by the paired, 2-tailed T-test.

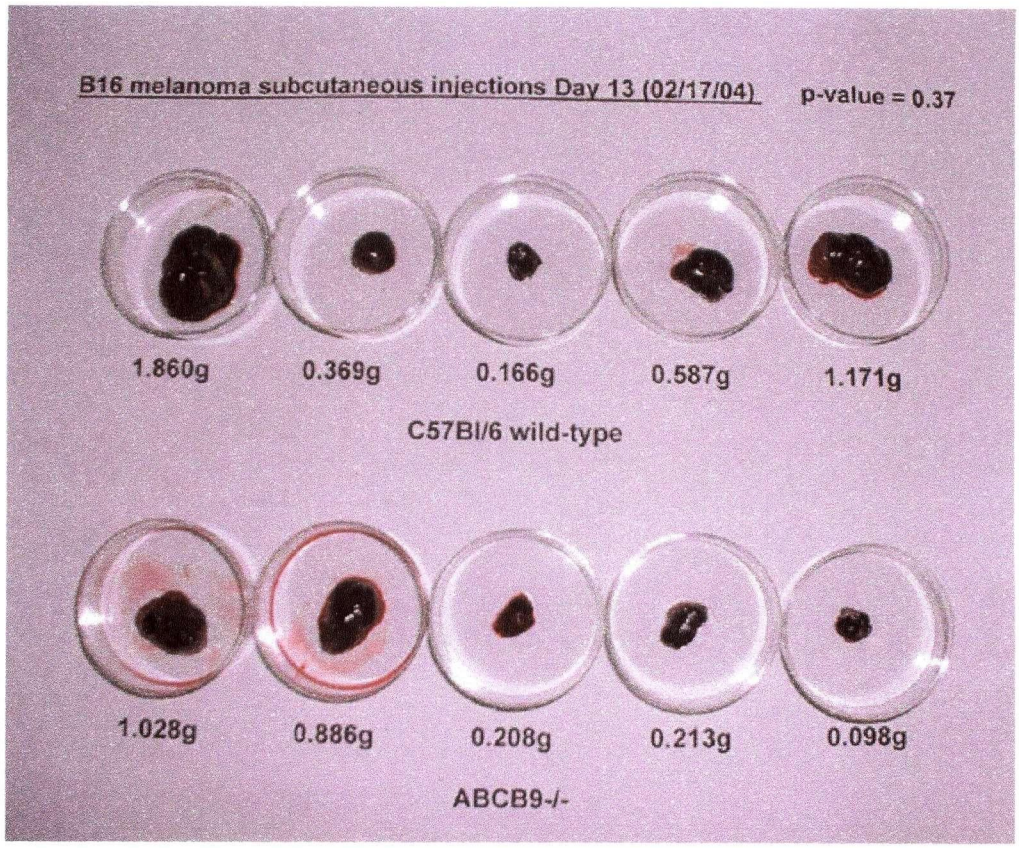
4.4 Results

Very little is known about the specific details of antigen processing pathways for initiation of an anti-tumour response, other than that DC cross-priming appears to be involved. We were interested to evaluate whether exogenous cross-presentation was the primary mechanism of anti-tumour T-cell cross-priming. Since the ABCB9$^{-/-}$ mouse model is deficient in exogenous cross-presentation (Chapter III), it provides a method to evaluate the involvement of exogenous cross-priming in tumour responses *in-vivo*. We challenged these animals by injecting them with tumour cells of a shared haplotype background (B6). Normally tumours such as B16 and CMT.64 that do not present MHC antigens succeed in proliferating and metastasizing and result in mortality of the mice by evading CTL-mediated immunosurveillance and killing (Franks, Carbonell et al. 1976; Gabathuler, Alimonti et al. 1998; Alimonti, Zhang et al. 2000). These non-MHC expressing tumour cells can be targeted for destruction by natural killer (NK) cells (Trinchieri 1989; Papamichail, Perez et al. 2004). As attested to by the prevalence of aggressive cancers however, the NK response alone is often not sufficient to eliminate the disease. Those tumours that do express MHC antigens however, are capable of being destroyed in an immuno-competent mouse. If exogenous cross-presentation is involved in anti-tumour cross-priming, it would be expected that those tumours that express little or no MHC (and therefore do not present antigens at the cell surface) will grow similarly in both mouse genotypes. It would also be expected that those tumours presenting antigens on the cell surface, such as CMT.1-4 should show a differential growth rate between the two animal groups, with the ABCB9$^{-/-}$ mice being more susceptible to tumour progression due to a lack of immune response to TAAs. As the type of tumour and route of injection can result in a differential induction of the

immune response, we chose two tumour types to evaluate initially: an adenocarcinoma small-cell lung cancer, CMT.64 to be injected intraperitoneally (i.p.) and B16, a melanoma to be injected sub-cutaneously (s.c.).

Our experimental results from the B16 tumour cell injections show that wild-type and ABCB9$^{-/-}$ mice do not show statistically significant differences in growth of these non-H-2K^{b} expressing tumours, as shown in Figure 4.1 below. While there was a trend towards smaller tumours in the ABCB9$^{-/-}$ mice, the size differences were not statistically significant ($p>0.25$).

a


B16 melanoma subcutaneous injections Day 13 (02/17/04)
p-value = 0.37
1.860g
0.369g
0.166g
0.587g
1.171g
C57Bl/6 wild-type
1.028g
0.886g
0.208g
0.213g
0.098g
ABCB9-/-

b

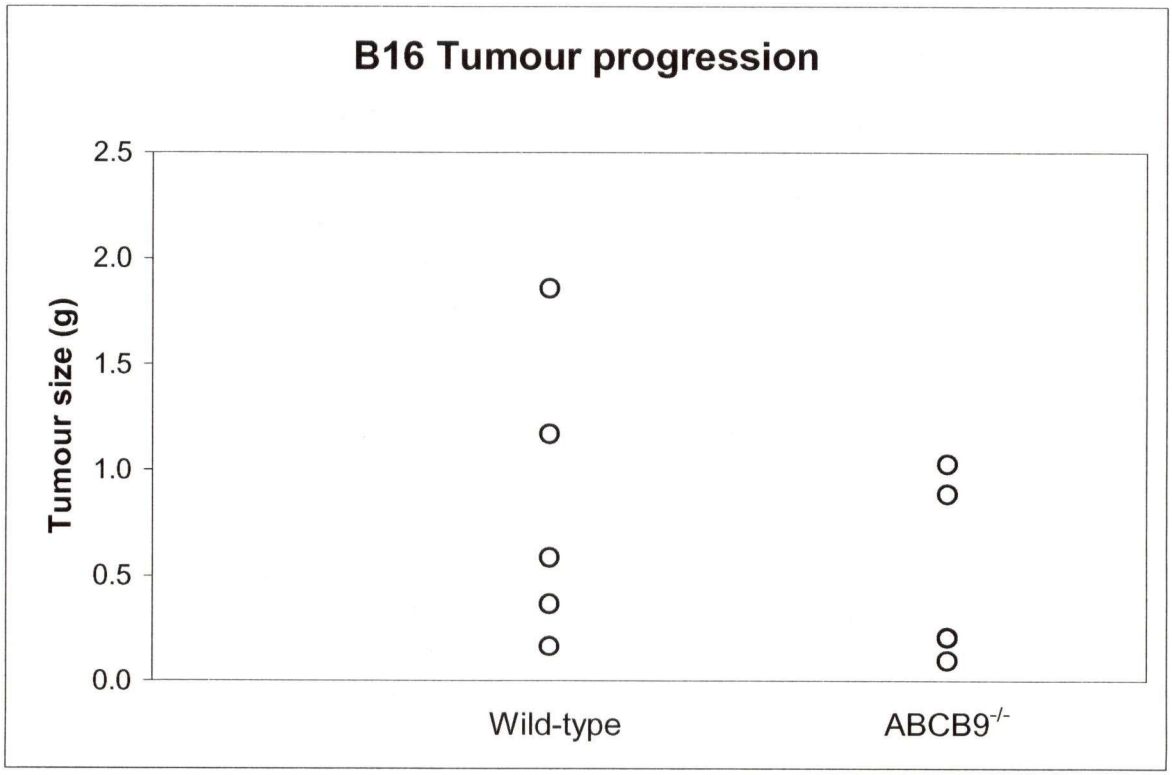
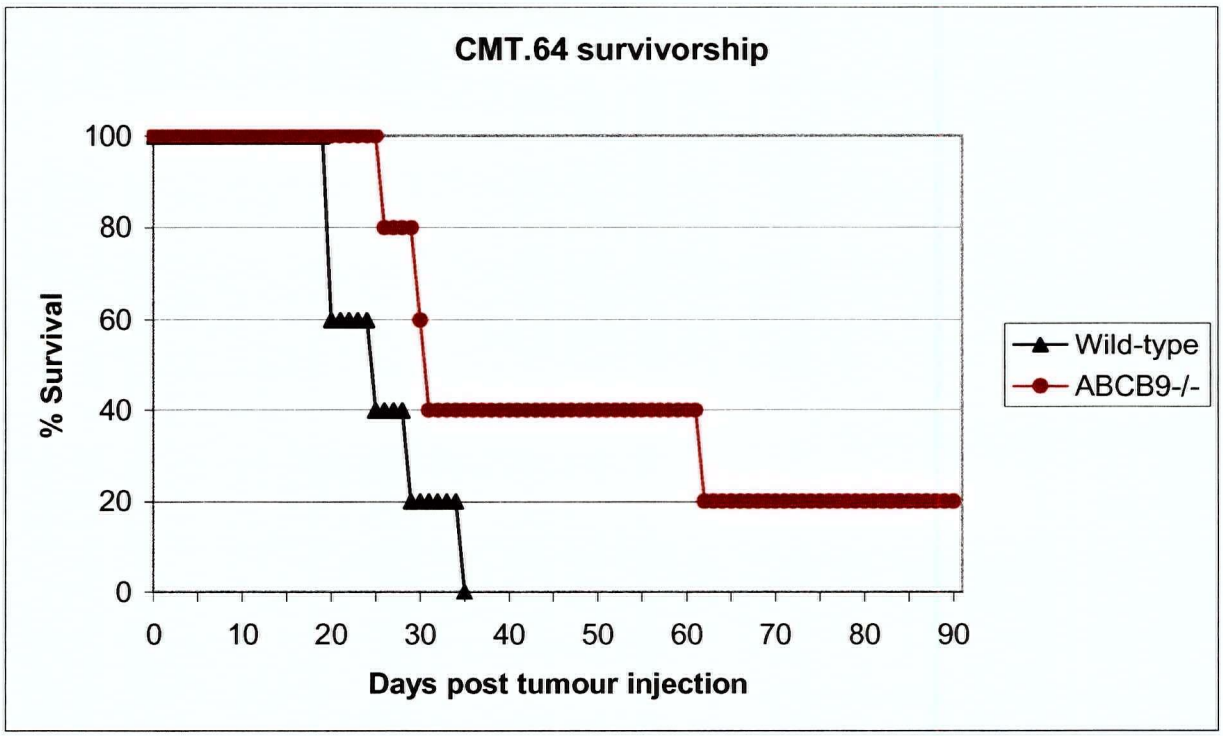
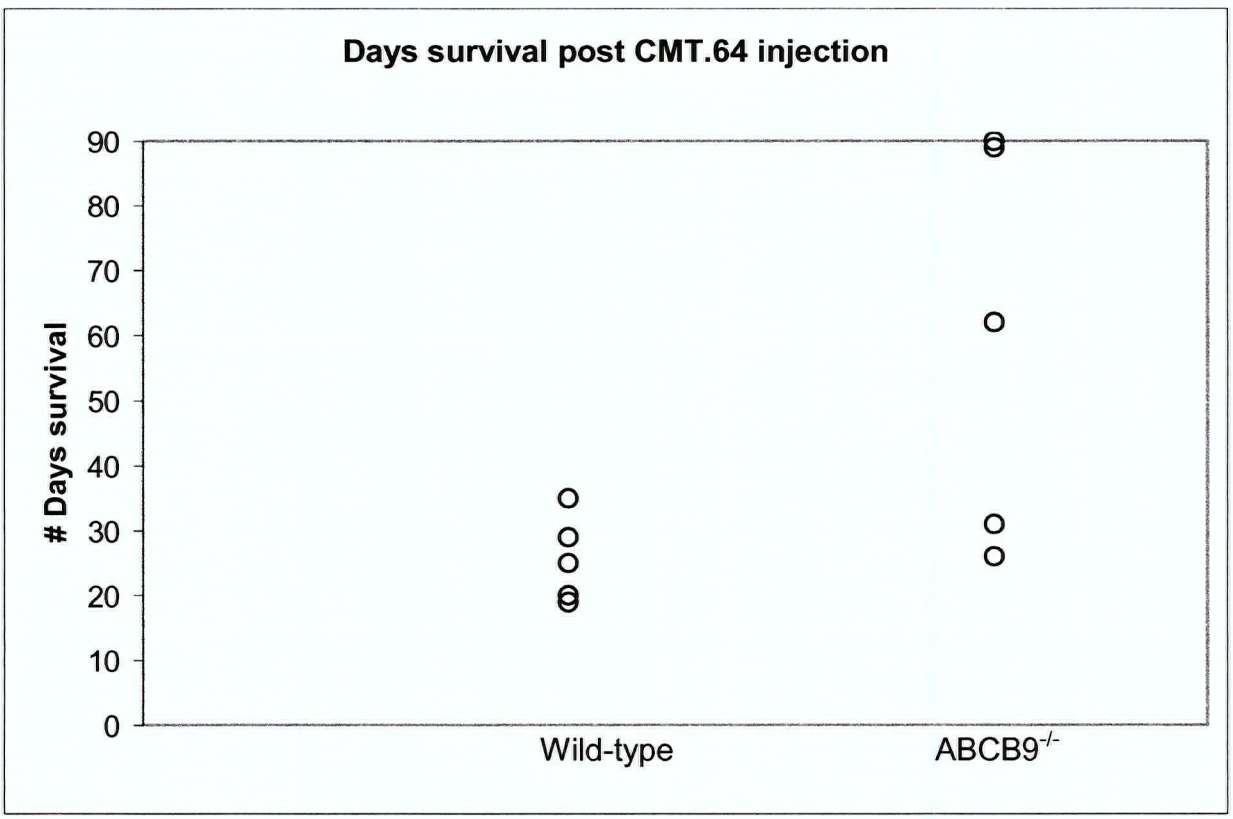

B16 Tumour progression
Tumour size (g)
2.5
2.0
1.5
1.0
0.5
0.0
Wild-type
ABCB9-/-

Figure 4.1. B16 tumour progression. Wild-type and ABCB9$^{-/-}$ mice do not show differential growth of non-H-2K^{b} expressing B16 tumours. 4x10^{5} B16 tumour cells were injected sub-cutaneously on day 0 and excised on day 13. (a) B16 tumours excised from mice on day 13 post-injection, with corresponding tumour weights. (b) Graphical plot of tumour sizes for each group of mice, each circle represents one mouse (N = 5). Statistically not significant (T-test, $p>0.25$).

With no apparent effect of the loss of ABCB9$^{-/-}$ on B16 tumour growth, mice were injected i.p. with CMT.64 SCLC and monitored for survivorship for 90 days (Figure 4.2). Initially, the ABCB9$^{-/-}$ mice were seen to survive on average approximately 5 days longer than the wild-type, with all wild-type mice dying by day 35 post-injection. 40% (2 mice) from the ABCB9$^{-/-}$ group survived until day 60, and 20% (1 mouse) of wild-type survived throughout the 90-day study period. Although this difference was considered to be statistically significant ($p<0.05$) based upon a paired T-test, due to the small sample size, no conclusion can be drawn until larger populations are evaluated. The time until 50% mortality (TD_{50}) was reached for wild-type and ABCB9$^{-/-}$ mice were 25 and 31 days respectively. Because the sample size was small (N=5), the differences observed may not be truly representative of an ABCB9$^{-/-}$ population differential survival rate.


CMT.64 survivorship
% Survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Days post tumour injection
Wild-type
ABCB9-/-

a

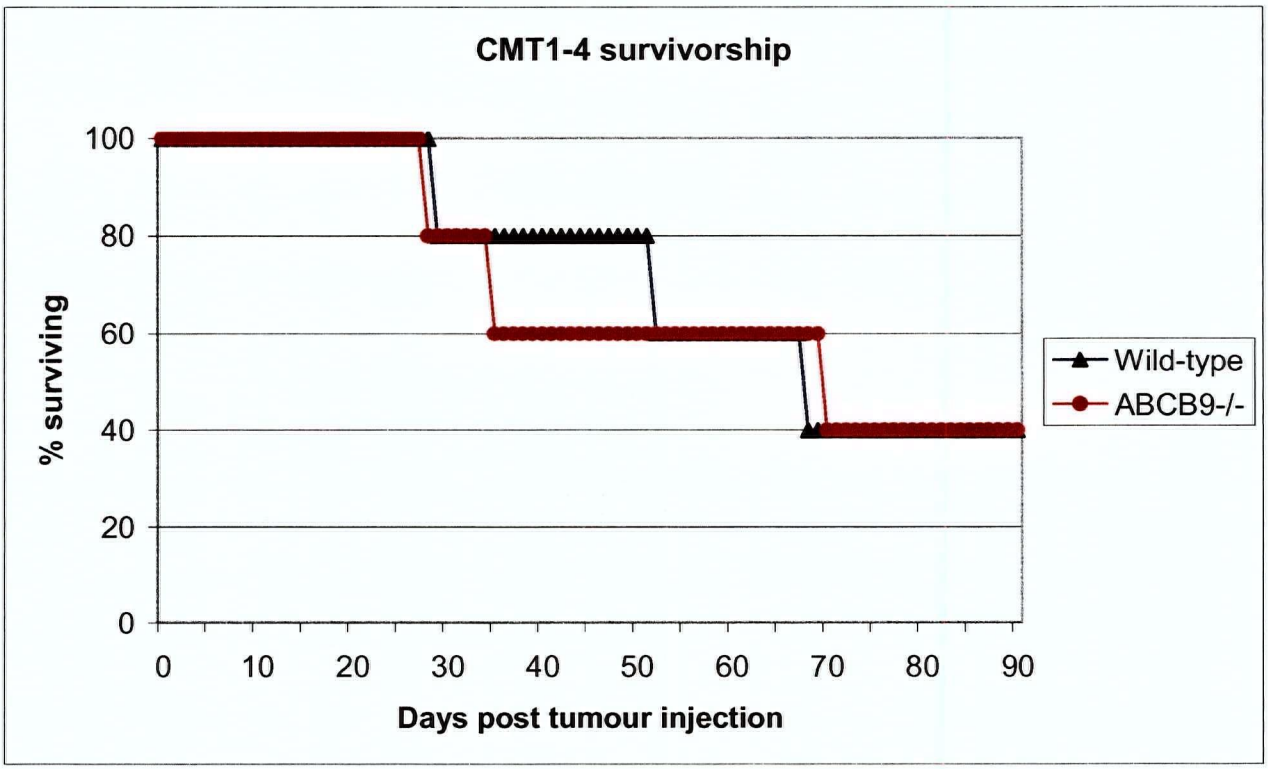

Days survival post CMT.64 injection
Days survival
90
80
70
60
50
40
30
20
10
0
Wild-type
ABCB9-/-

b

Figure 4.2. Survivorship of CMT.64 injected mice. $3.5x10^5$ CMT.64 cells (non- H-$2K^b$ expressing, transfected with empty vector) were injected i.p. for each mouse. (a) Survivorship curve. (b) Chart marking days survival for each mouse per group, each circle represents one mouse (N = 5). Statistically significant (T-test, $p<0.05$).

Mice were injected i.p. with the K^b expressing CMT.1-4 (CMT.64+TAP) and monitored for survivorship for 90 days (Figure 4.3). Both wild-type and $ABCB9^{-/-}$ mice showed a similar survival rate, with TD_{50} being 68 and 70 days respectively. This was significantly longer than for mice injected with CMT.64 tumours, with TD_{50} at 25 and 31 days respectively for wild-type and $ABCB9^{-/-}$ mice (Figure 4.2).

a


CMT1-4 survivorship
100
80
60
40
20
0
% surviving
0
10
20
30
40
50
60
70
80
90
Days post tumour injection
Wild-type
ABCB9-/-

b

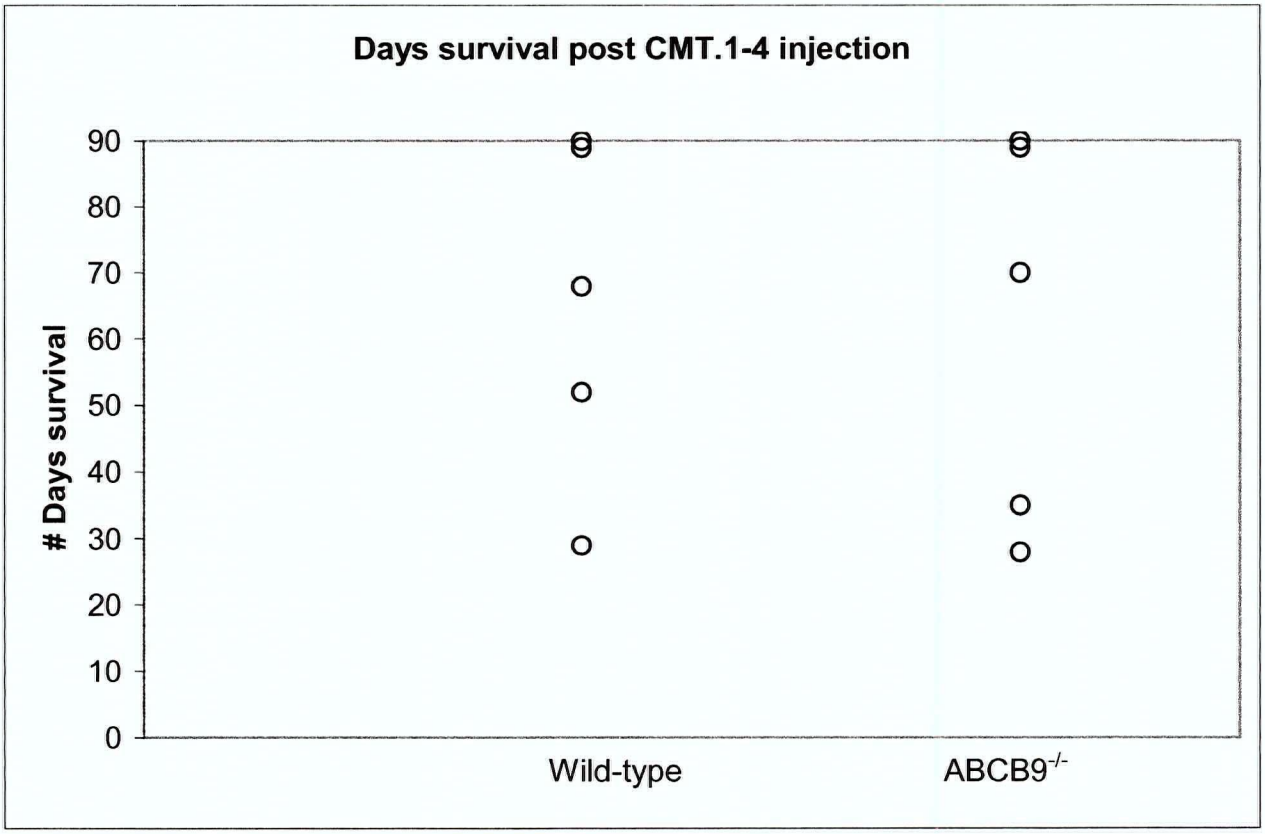
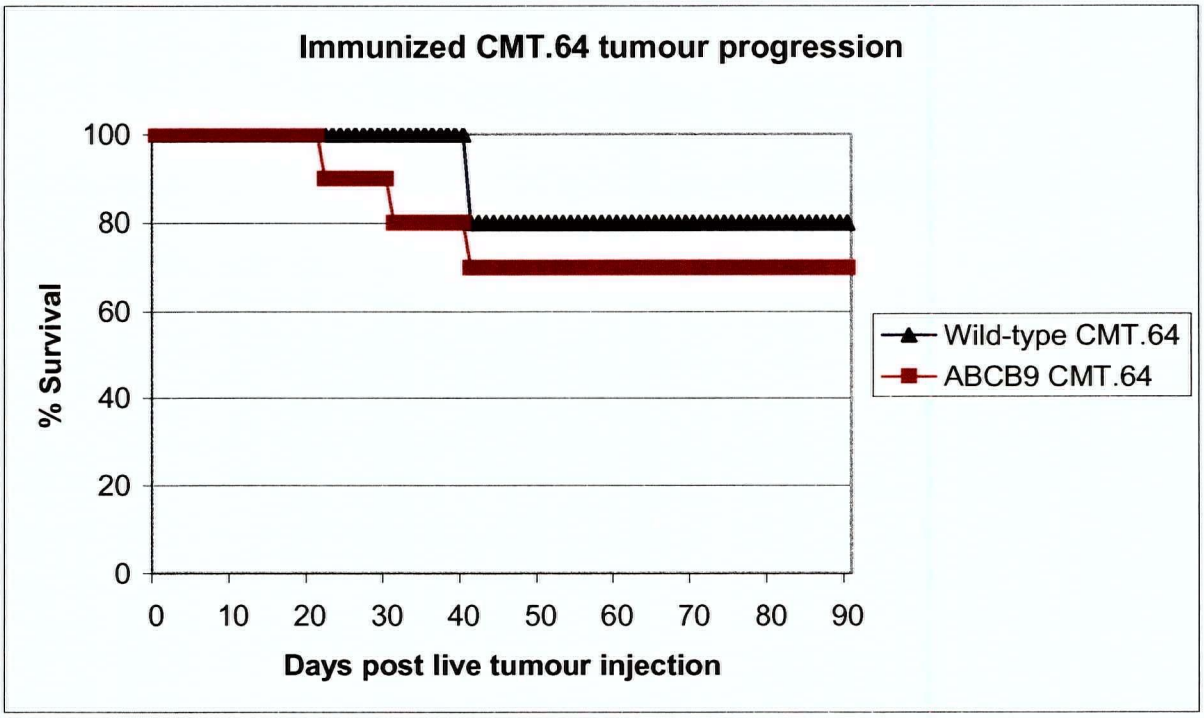

Days survival post CMT.1-4 injection
90
80
70
60
50
40
30
20
10
0
Days survival
Wild-type
ABCB9-/-

Figure 4.3. Survivorship of CMT.1-4 injected mice. $3.5x10^5$ CMT.1-4 cells (transfected with TAP1 to restore $H\text{-}2K^b$ expression) were injected i.p. for each mouse. (a) Survivorship curve. (b) Chart marking days survival for each mouse per group, each circle represents one mouse (N = 5). Statistically not significant (T-test, $p>0.05$).

Mice were then injected with irradiated CMT cells (either CMT.64 or CMT.1-4) and given 14 days to allow them to generate a robust cellular immune response. On day 14 following immunization, the animals were injected with live CMT cells corresponding to those with which they had been originally immunized. In the CMT.64 immunized mice, both $ABCB9^{-/-}$ and wild-type mice showed an increased survival compared to non-immunized CMT.64 challenge (Figure 4.4). This indicates that even the reduced MHC on the cell surface was sufficient to induce a cellular immune response. In the first 40 days, there was an increase in the morbidity of the $ABCB9^{-/-}$ mice compared with wild-type, however beyond 40 days, both groups showed a similar response with 70-80% long-term survival.

a


Immunized CMT.64 tumour progression
% Survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Days post live tumour injection
Wild-type CMT.64
ABCB9 CMT.64

b

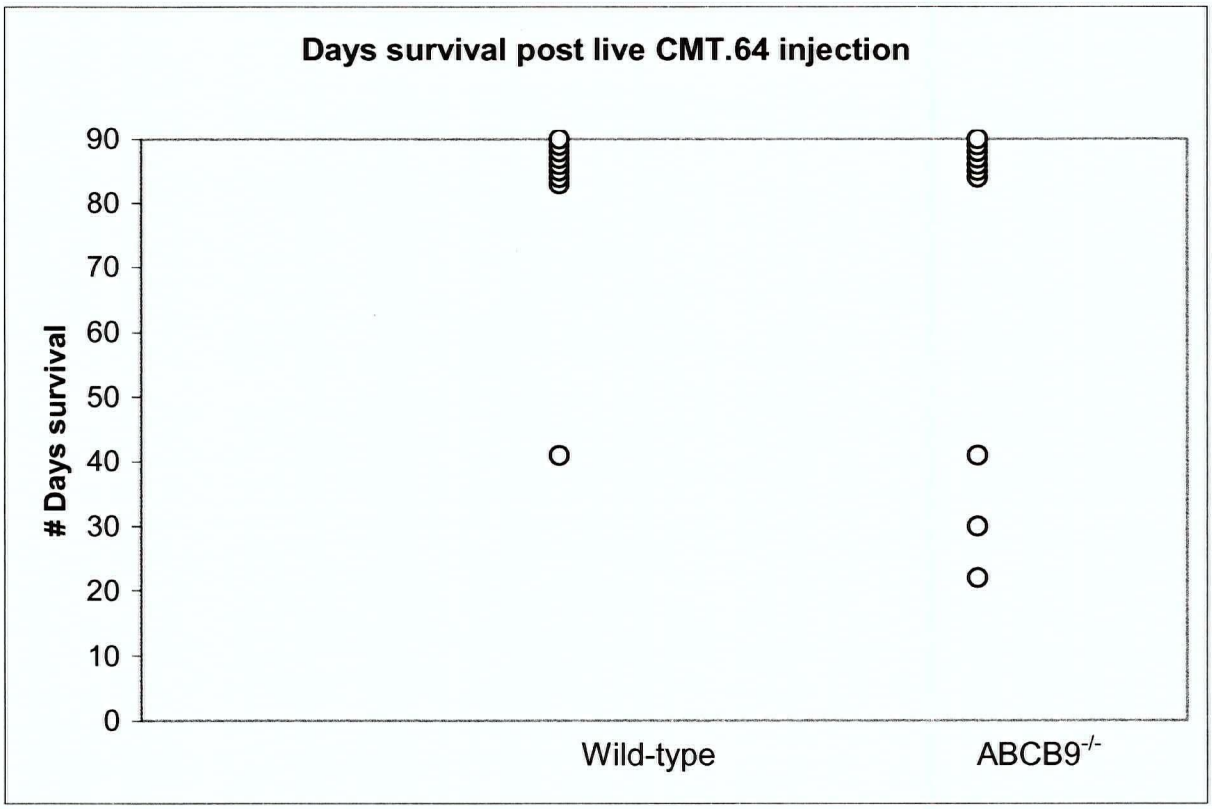
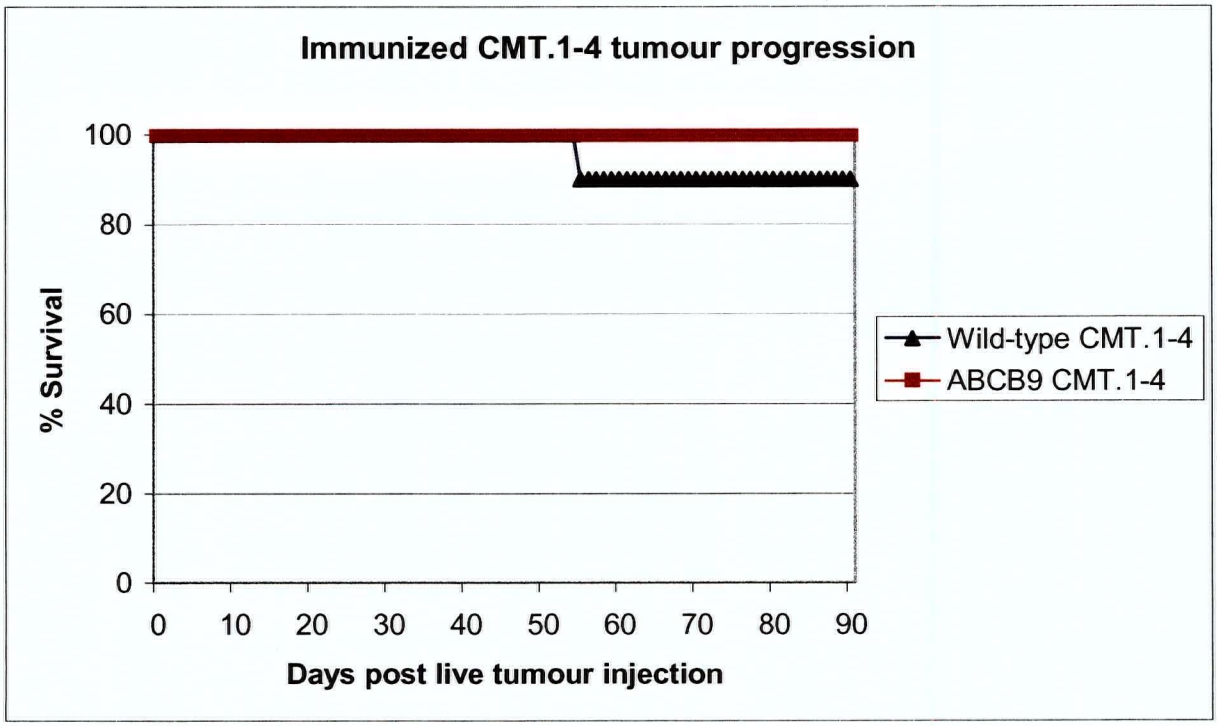

Days survival post live CMT.64 injection
Days survival
90
80
70
60
50
40
30
20
10
0
Wild-type
ABCB9-/-

Figure 4.4. Survivorship of pre-immunized CMT.64 injected mice. Mice were injected i.p. with $3.5x10^5$ irradiated CMT.64 cells 14 days prior to challenge by i.p. injection of $3.5x10^5$ live CMT.64 cells. (a) Survivorship curve. (b) Chart marking days survival for each mouse per group, each circle represents one mouse (N = 10). Statistically not significant (T-test, $p>0.05$).

For both ABCB9$^{-/-}$ and wild-type mice, when animals were pre-immunized, survival was also increased remarkably when challenged with the CMT.1-4 cells, as 90-100% of both groups survived the entire study period (Figure 4.5), compared to only 40% survival in non-immunized CMT.1-4 injected mice from both groups (Figure 4.3a). This demonstrates the potential of the cellular immune response to eliminate MHC expressing tumour cells. Since the ABCB9$^{-/-}$ mice generated a similar anti-tumour response compared to wild-type mice, this demonstrates that exogenous antigen presentation does not make a major contribution to this type of anti-tumour immunity.

a


Immunized CMT.1-4 tumour progression
% Survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
Days post live tumour injection
Wild-type CMT.1-4
ABCB9 CMT.1-4

b

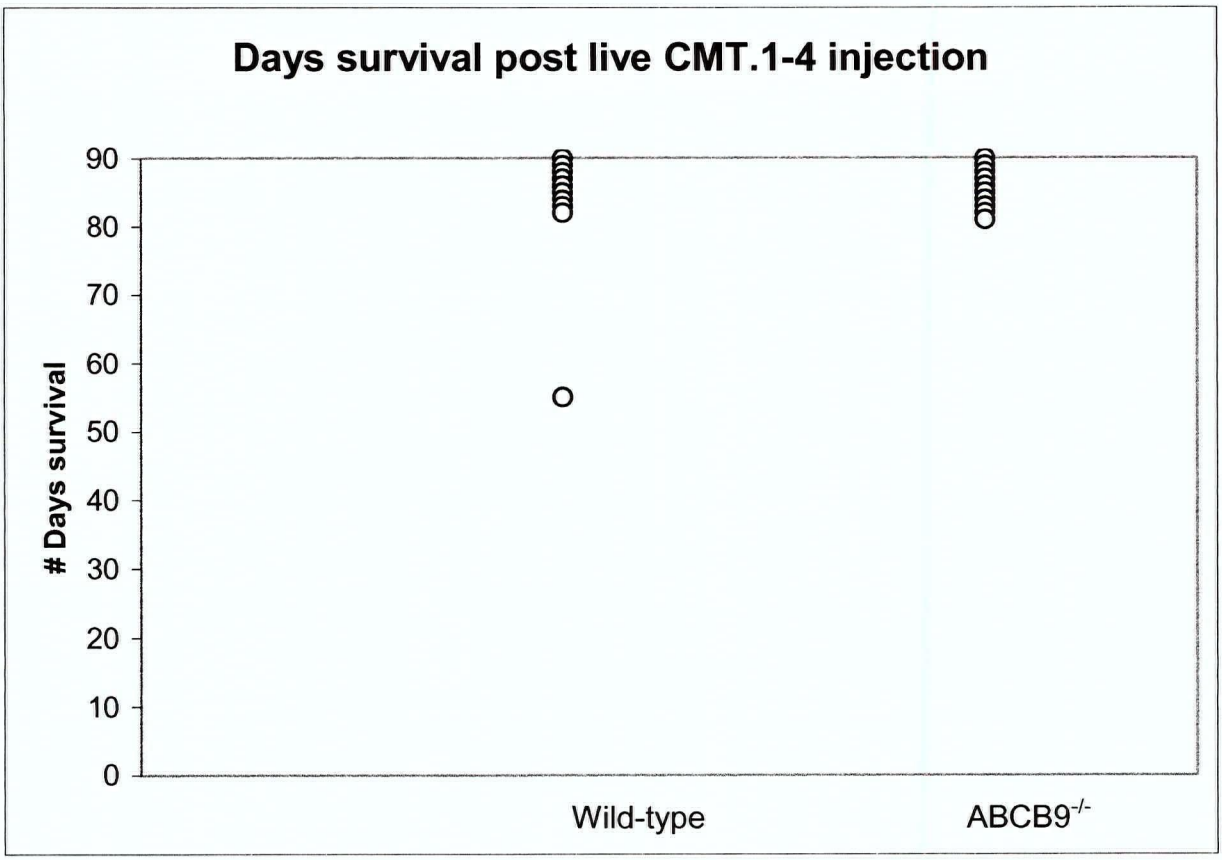

Days survival post live CMT.1-4 injection
Days survival
90
80
70
60
50
40
30
20
10
0
Wild-type
ABCB9-/-

Figure 4.5. Survivorship of pre-immunized CMT.1-4 injected mice. Mice were injected i.p. with $3.5x10^5$ irradiated CMT.1-4 cells 14 days prior to challenge by i.p. injection of $3.5x10^5$ live CMT.1-4 cells. (a) Survivorship curve. (b) Chart marking days survival for each mouse per group, each circle represents one mouse (N = 10). Statistically not significant (T-test, $p>0.05$).

4.5 Discussion

Wild-type and ABCB9$^{-/-}$ mice that were injected with the live B16 tumour cells showed statistically similar tumour growth, albeit with ABCB9$^{-/-}$ showing a tendency toward smaller tumours. Wild-type mice injected with CMT tumour cells showed the expected survival curve, with CMT.1-4 (+TAP) resulting in a much longer survival (Figure 4.2, 4.3), as shown previously (Alimonti, Zhang et al. 2000). The ABCB9$^{-/-}$ mice did not show an increased susceptibility to tumour proliferation after injection with CMT.64 cells, and surprisingly a sub-group of ABCB9$^{-/-}$ mice even showed increased longevity compared to wild-type mice (Figure 4.2). In the initial stages of the CMT.64 tumours, the survival of the knockout mice and wild-type animals differed slightly, with ABCB9$^{-/-}$ mice surviving 5 days longer to the TD_{50} point. In the period following 35 days post tumour injection, there were two ABCB9$^{-/-}$ mice that survived to day 60, and one to the end of the study at 90 days, while none of the wild-type mice survived past 35 days. While this may be a true representation of the population, the sensitivity of the assay must be

considered. For this size of group (N=5), having one or two mice survive could merely represent difference in the original injection, or simply animal to animal variation.

In the immunized mice injected with the H-2K^b expressing CMT.1-4 (CMT.64+TAP), the survivorship of both wild-type and ABCB9$^{-/-}$ mice was the same (Figure 4.5). This indicates that exogenous antigen cross-priming has little or no effect on the progression of MHC-I antigen expressing tumour cells in the body. Those immunized mice injected with CMT.64 however, showed an initial difference between the survivorship of ABCB9$^{-/-}$ animals and wild-type, although later in the study, the susceptibility became equal between the two groups (Figure 4.4). In the early stages, more knockout mice succumbed to the tumours than wild-type. By day 30 post tumour challenge, two out of ten (20%) of the ABCB9$^{-/-}$ mice had succumbed, while all wild-type mice survived until day 40, at which point one animal of each type succumbed.

When comparing non-immunized to immunized mice, immunized mice show increased survival in all cases, indicating the initiation of a cellular immune response (Figures 4.2-4.5). The immunization response to CMT.1-4, which expresses MHC plus bound antigens at the cell surface, provided almost complete protection, with 90% and 100% of wild-type and ABCB9$^{-/-}$ mice, respectively, surviving beyond the study period (Figure 4.5). In comparison, the immunization response against CMT.64 was also protective, although to a lesser degree (Figure 4.4), as would be expected based upon the significantly reduced MHC surface expression compared to CMT.1-4 (Jefferies, Kolaitis et al. 1993; Alimonti, Zhang et al. 2000). This shows that the cellular immune response is important even in those cancers with reduced MHC surface expression.

4.6 Conclusions

Results from these experiments should be interpreted with caution, since the numbers of animals tested in each case was relatively small (N=5 or 10). Should these results reflect the typical response to tumour challenge in wild-type and ABCB9$^{-/-}$ mice, it appears that exogenous antigen presentation is not the primary method by which a cellular anti-tumour response is primed *in-vivo*, as assessed by the ABCB9$^{-/-}$ mouse model. Animals deficient in exogenous antigen presenation, but with intact phagocytic and endogenous antigen presentation can mount a normal anti-tumour response to s.c. B16 melanoma, or to i.p. injected CMT-64 or CMT.1-4 (CMT.64 + TAP) SCLC. Pre-immunization with irradiated tumour cells resulted in a similarly increased survival in ABCB9$^{-/-}$, as compared to wild-type mice. One notable difference was observed in non-immunized mice challenged with CMT.64. In this group, two of five ABCB9$^{-/-}$ mice survived longer than their wild-type counterparts. This result is the opposite of what would be expected if ABCB9 played a role in priming the immuological T-cell response against tumours, suggesting a possible role for ABCB9 in the generation of immune tolerance.

4.7 Future directions

Further experiments should be conducted to determine whether this data is significant. If significant, then a potential explanation for the results observed here would be if ABCB9 also plays a role in immune tolerance. T-cell priming by DCs is a double-edged sword. On one hand, MHC-restricted antigen presentation is required to prime a CTL response to dangerous antigens.

On the other hand, the same MHC-restricted antigen presentation of self proteins is required to initiate tolerogenic T-cells into the anergic T-cell response. The mechanism by which the DC 'decides' to prime a CTL or tolerogenic response is still very much a mystery, although clues are beginning to emerge (Liu, Iyoda et al. 2002). It has recently been shown that DCs purified from inflamed brain and central nervous system were incapable of priming a CTL response, and were only able to promote tolerance (Suter, Biollaz et al. 2003). As ABCB9$^{-/-}$ is highly expressed in the brain, a specific role in tolerance to self tissues should be considered. As DCs encounter tumour cells that do not present surface antigens, they may not be recognized as dangerous. Instead, a tolerogenic response to these cells could be produced, whereby CTLs specific for these antigens are destroyed. Such a role in tolerance would account for an increase in survival of non-immunized ABCB9$^{-/-}$ mice compared with wild-type when challenged with CMT.64 tumour cells. It is also predictable that this response is not observed in immunized animals as the irradiated CMT.64 cells should provide sufficient danger signals through their apoptosis to override tolerance and promote a phagocytic-primed CTL response.

Future investigation of TIL cytokines pruoduced in ABCB9$^{-/-}$ mice should focus upon whether these animals do indeed produce fewer tolerogenic signals, such as IL-10 and tumour growth factor-β (TGFβ), as well as evaluation of their levels of the Th-1 stimulating cytokines IL-2, IL-12 and IFNγ, or Th-2 stimulating cytokine IL-4.

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CHAPTER V

A VIEW TO THE FUTURE

CHAPTER V. A VIEW TO THE FUTURE

5.1 In review

With the initial aims of this thesis completed, it is time to evaluate what has been done, how this may impact the field, and where these discoveries may lead. The identification of several novel candidate antigen presenting genes should provide a starting place from which to further evaluate the mechanisms of antigen transport. The characterization of ABCB9 as a transporter involved in dendritic cell cross-presentation should lead to further evaluation of its ability to modulate T-cell priming. Of particular interest, having defined a new step in the exogenous antigen cross-presentation pathway, this information has clearly delineated a difference between viral antigen cross-priming through the phagosome, and endocytic exogenous antigen cross-priming. The model shown in Figure 3.10 of Chapter III adds to the mechanistic model of antigen cross-presentation built over recent years (Brossart and Bevan 1997; Yewdell, Norbury et al. 1999; Sigal and Rock 2000; Heath and Carbone 2001; Yewdell and Bennink 2001), and adds an additional level of refinement to the most recent models put forth elucidating the ER-phagolysosome (Ackerman, Kyritsis et al. 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003; Lizee, Basha et al. 2003). In addition, the unexpected finding that HI Sendai antigen accesses the cytosolic antigen processing pathway redefines a previously poorly understood mechanism and provides biologically relevant information that can be used to illuminate the details of TAP-independent antigen cross-presentation (Liu, Chambers et al. 1997). Finally, having shown a limited effect of exogenous antigen cross-priming on cancer immunology, future immunotherapy can focus on cross-presentation of endogenous

phagocytosed antigens instead. However, the potential for ABCB9 to act in tolerance as well as CTL priming should be evaluated for possible treatment of autoimmune disease.

5.2 ABCB9

Having established ABCB9 as a transporter involved in dendritic cell cross-presentation, there are several directions from which to pursue future experiments. With the demonstration that ABCB9$^{-/-}$ mice cannot cross-present the exogenous antigen ovalbumin, the next step will be to determine why ovalbumin processing is deficient. It is possible that ABCB9 transports this protein directly, or indirectly by transporting some intermediate substance that is involved in antigen processing. It will be very important to determine the precise substrate specificity of ABCB9, in particular, whether it transports ovalbumin protein as intact protein, partially degraded as large antigenic epitopes, or as discrete specific peptides, similarly to TAP. A cross-presentation peptide transport assay would be a useful method by which to determine this (Heemels, Schumacher et al. 1993; Schumacher, Kantesaria et al. 1994). Such an assay could also provide information as to whether loss of ABCB9 alters the peptide repertoire. This type of assay would have to be conducted in dendritic cells, purified from wild-type and ABCB9$^{-/-}$ mice.

We have identified that ABCB9$^{-/-}$ mice can respond normally to endogenous viral antigens cross-presented by DCs, presumably through phagocytosis of infected cells. ABCB9$^{-/-}$ mice do not respond normally to exogenous antigens endocytosed and cross-presented by DCs. An assay should be conducted to determine if ABCB9$^{-/-}$ DCs are able to present ovalbumin that has been phagocytosed as a coating on latex beads (Ackerman, Kyritsis et al. 2003;

Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003). This can be done by pulsing DCs *in-vitro* with latex beads coated in ovalbumin, and evaluating H2-K^b/OVA peptide at the cell surface with B3Z T-cells (Shastri and Gonzalez 1993), measuring IL-2 production, similarly to Figure 3.8 in Chapter III. Alternatively, DCs can be 'fed' cells known to be phagocytosed (i.e. *Listeria monocytogenes*) (Paschen, Dittmar et al. 2000), or other irradiated non-APCs that have been transfected with ovalbumin. If H2-K^b/OVA surface presentation is restored in ABCB9$^{-/-}$ DCs following co-incubation with these OVA-transfected cells, it would show that phagocytic uptake of ovalbumin was sufficient to restore cross-presentation, contrasted with the endocytic antigen processing pathway. This will allow for the differentiation between ABCB9 as a transporter in the endosomal compartment (H2-K^b/OVA presentation will be increased), or as a substrate-specific transporter in the phagolysosome (H2-K^b/OVA presentation will not be increased).

5.3 Cross-presentation and cancer immunology

For the cancer immunology studies, the differential tumour response based upon ABCB9$^{-/-}$ animal model indicates that further experiments should be conducted. These animals should be evaluated for presence of tumour infiltrating lymphocytes (TIL). These lymphocytes can be monitored for subtype (CD4+ versus CD8+) and production of immunological cytokines to determine whether loss of ABCB9$^{-/-}$ results in release of regulatory or immunogenic cytokines. IL-2 and a high proportion of CD8+ T-cells would indicate a primarily Th-1 type CTL response. Conversely, predominantly IL-4 and CD4+ cells would indicate a Th-2 type helper T-cell

response. Production of cytokines such as IL-10 would indicate an immunosuppressive reaction, promoting immune tolerance (Alimonti, Zhang et al. 2000; Steinman, Hawiger et al. 2003).

5.4 New avenues for ABCB9

Last but not least, the avenue of autoimmunity is well worth considering. Dendritic cells have lately been revealed as integral to generating tolerance by the mechanism of cross-presentation (Steinman and Nussenzweig 2002; Steinman, Hawiger et al. 2003), and as such, the possible effects of ABCB9 on autoimmune disease should be fully evaluated. The role of ABCB9 in antigen cross-presentation in DCs, and particularly the results of the tumour challenge experiments, suggests a possible role in creating tolerance and protecting against autoimmunity. There are readily available mouse models of autoimmune disease such as the non-obese diabetic (NOD) mouse, with an immune response against the pancreatic insulin-secreting islet cells (Gallegos and Bevan 2004). The ABCB9$^{-/-}$ mouse should be crossed onto the NOD background mouse to determine whether the autoimmune diabetic pathology is accelerated or increased. Alternatively, young NOD mice could be irradiated and injected with syngeneic *ex-vivo* bone marrow that had been transduced to highly express ABCB9, to observe whether there was relief of the diabetic phenotype later in life.

5.5 Multiple RT-PCR screen for ABC transporter gene expression

The ABC transporter family-wide screen utilized in this thesis is a novel, although straightforward approach. Since the publication of the human (and mouse) genome, large-scale screening on microarrays has gained in popularity. These gene 'chips' generally compare cDNA from several samples, and give a readout of increased or decreased gene expression for thousands of different genes (most still uncharacterized) (Goldsmith and Dhanasekaran 2004). While the semi-quantitative gene screen used here is based on a similar concept, it focuses on one specific gene family, and is limited to a manageable number of genes (N=46). Having identified several ABC transporter genes with potential antigen presenting functions, the next step will be to characterize each one independently with regards to their effects on antigen presentation. Each one should be evaluated using loss-of function and gain-of function experiments. Since antigen presentation occurs in primary antigen presenting cells, the best model would be to work *in-vivo*, in mouse models which could be compared to previous work in this field, similarly to the TAP1 (van Kaer, Ashton-Rickardt et al. 1992) and ABCB9 knockout mice (Chapter III).

Any ABCs found to be involved in antigen presentation should also be examined for the potential for use in gene therapy for any variety of diseases that utilize cell-mediated immunity.

This particular screening method of evaluating ABC expression could also be used as a tool to evaluate patterns of transporter expression in several other systems. In particular, it could be applied to evaluate ABC genes active in barrier-protected immune-privileged organs, such as the brain, testis, eye and at the fetal / maternal placental barrier. This should be useful in providing candidate transport proteins used to facilitate the movement of substrates across these otherwise impermeable barriers. There is precedent for the hypothesis that ABC transporters are

involved in these functions, as ABCB1 (PGP/MDR1) was coincidentally found to play a role in blood-brain barrier biology in ABCB1$^{-/-}$ mice (Schinkel, Smit et al. 1994; Schinkel, Mayer et al. 1997). The phenotype was discovered only when the mouse colony received an otherwise harmless spray of the insecticide vermectin, used to remove external parasites living on the mice. While normally harmless to vertebrates, the ABCB1$^{-/-}$ mice died when subjected to this treatment, and the underlying cause was found to be passage through the otherwise impermeable blood-brain barrier, indicating an important role for ABCB1$^{-/-}$ in regulation of components crossing into the brain.

5.6 Conclusion

In conclusion, I feel that the goals of this thesis, namely identifying ABC transporters involved in exogenous antigen presentation, have been attained. Having characterized the first ABC protein (ABCB9) specifically involved in cross-presentation has been a major accomplishment in both the cell biology of antigen presentation as well as illumination of the mechanism of cross-priming in the immune response. Evaluation of the ABCB9$^{-/-}$ mouse when challenged with various antigens has revealed differential phagocytic processing of cells infected with endogenous antigens versus direct processing of endocytosed exogenous antigens, which are specifically regulated by the ABCB9 transporter protein. A tumour model has revealed that exogenous antigen presentation may play a supportive, although not principal role in tumour immunology. Having identified several additional (N=17) ABC transporters with potential functions in antigen presentation has opened the door to elucidating the mechanisms of

exogenous antigen presentation, through the participation of antigen transporters. This should contribute to both the fields of cellular biology and immunology.

5.7 Bibliography

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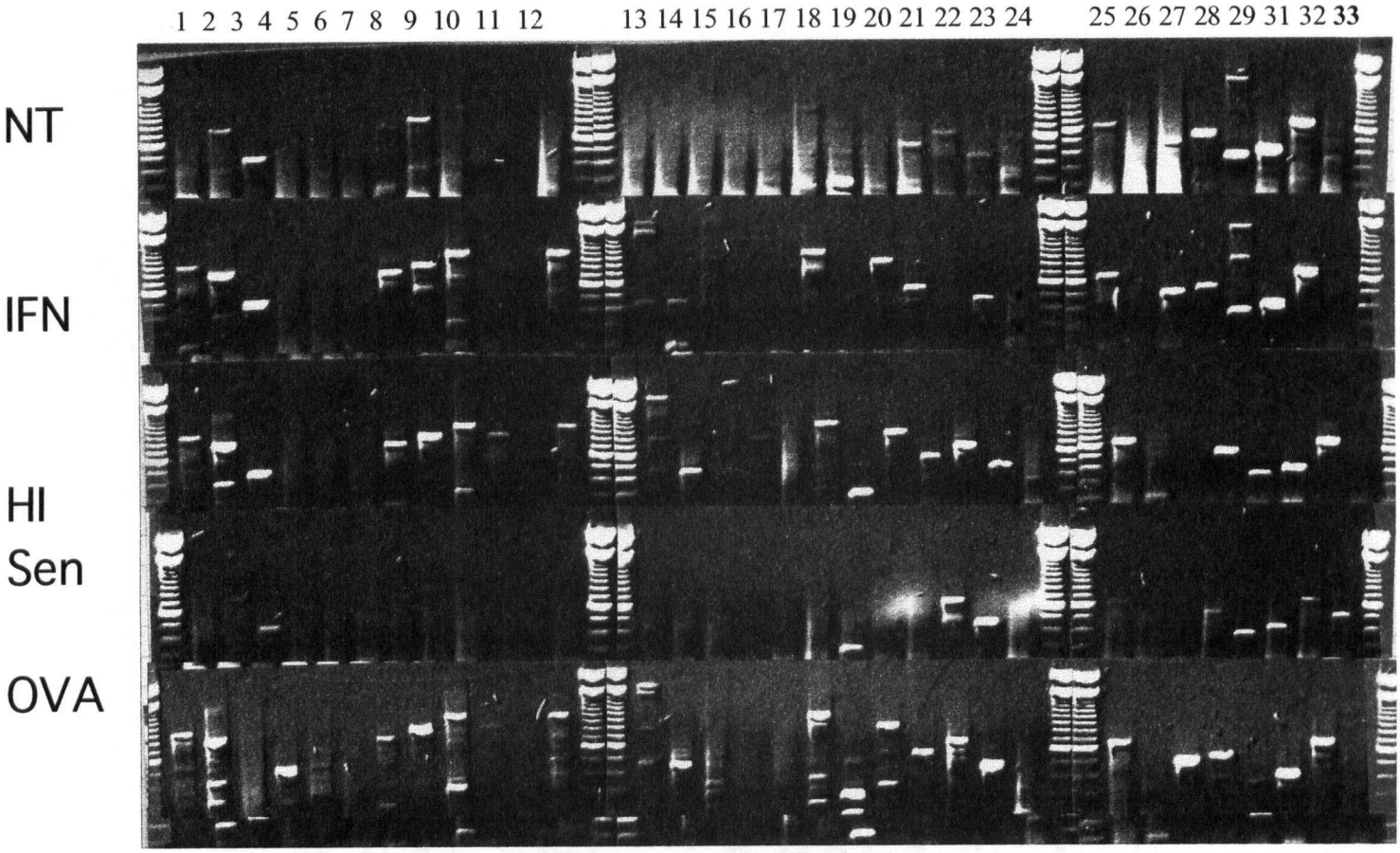
APPENDIX I

MULTIPLE ABC TRANSPORTER RT-PCR DATA

Appendix I. MULTIPLE ABC TRANSPORTER RT-PCR DATA

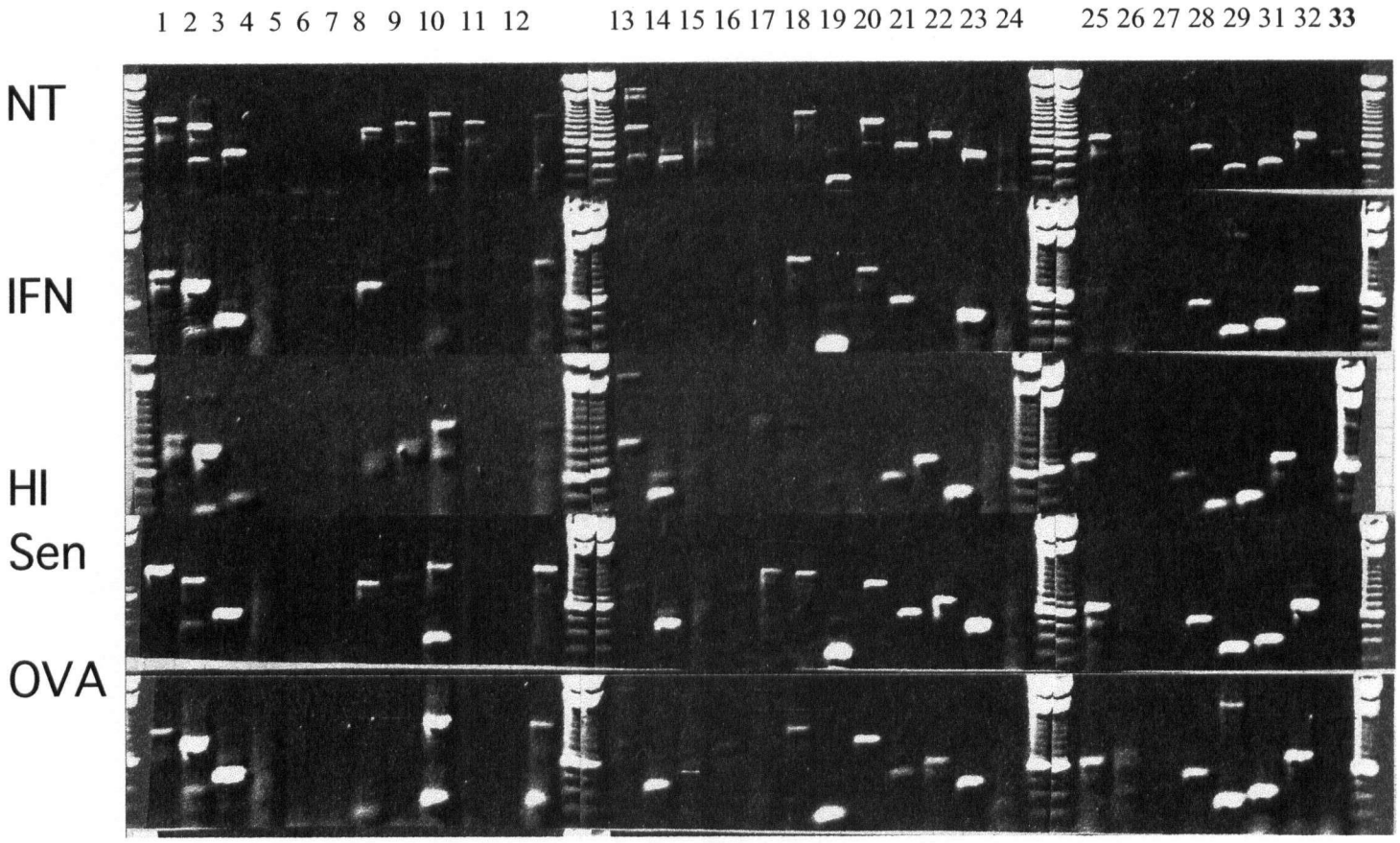
RT-PCR data that comprised Table 2.1 is included below: NT = no treatment, IFN = interferon-γ, HI Sen = heat inactivated Sendai virus, OVA = ovalbumin, HEL = hen egg lysozyme

Splenic DCs



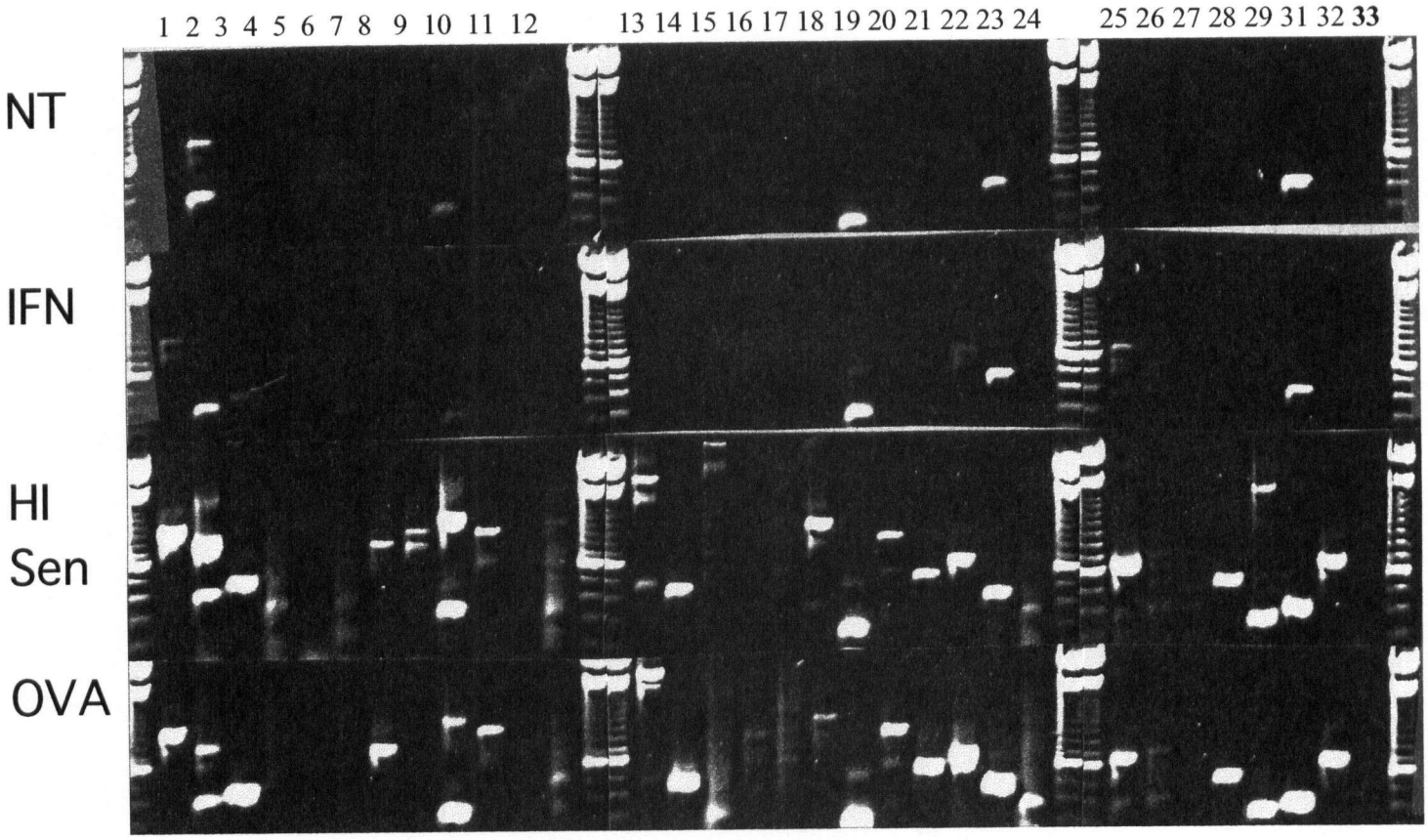
1=ABCA1, 2=A2, 3=A3, 4=A4, 5=A7, 6=A8, 7=B1, 8=B4, 9=B8, 10=B10, 11=B11, 12=C1, 13=C2, 14=C5, 15=C8, 16=D1, 17=D2, 18=F2, 19=F3, 20=G1, 21=G2, 22=G3, 23=ABC7, 24=C9, 25=C10, 26=D4, 27=E1, 28=D3, 29=F1, 31=B2(TAP1), 32=B3(TAP2), **33=B9**(TAPL).

Splenic macrophages



1=ABCA1, 2=A2, 3=A3, 4=A4, 5=A7, 6=A8, 7=B1, 8=B4, 9=B8, 10=B10, 11=B11, 12=C1, 13=C2, 14=C5, 15=C8, 16=D1, 17=D2, 18=F2, 19=F3, 20=G1, 21=G2, 22=G3, 23=ABC7, 24=C9, 25=C10, 26=D4, 27=E1, 28=D3, 29=F1, 31=B2(TAP1), 32=B3(TAP2), **33=B9**(TAPL).

Splenic B-lymphocytes



1=ABCA1, 2=A2, 3=A3, 4=A4, 5=A7, 6=A8, 7=B1, 8=B4, 9=B8, 10=B10, 11=B11, 12=C1, 13=C2, 14=C5, 15=C8, 16=D1, 17=D2, 18=F2, 19=F3, 20=G1, 21=G2, 22=G3, 23=ABC7, 24=C9, 25=C10, 26=D4, 27=E1, 28=D3, 29=F1, 31=B2(TAP1), 32=B3(TAP2), **33=B9**(TAPL).