The Role of Cav1.4 Calcium Channel in T Cell Activation, Proliferation, Effector Functions and Death

by

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ABSTRACT

T lymphocytes are an important part of the immune system that identify and destroy foreign antigens in the body as well as activate and deactivate other immune cells. In T lymphocytes, calcium is a secondary messenger that regulates activation and proliferation, effector function, survival and death. Although calcium release from the intracellular stores within T lymphocytes is well characterized, the calcium entry pathway from extracellular sources into T lymphocytes is unclear, despite contributing to the majority of elevated intracellular calcium ions during T lymphocyte activation. Preliminary studies have shown that L-type calcium channels play significant roles in the calcium influx pathways, mediating T lymphocyte activation and proliferation in vitro. Cav1.4 L-type calcium channel has been found to be expressed in both mouse and human T lymphocytes. To date, three Cav1.4 calcium channel splice variants have been identified with differential expression throughout the T lymphocyte proliferation process.

I hypothesized that pore forming subunit of a L-type calcium channel, Cav1.4, regulates T lymphocyte activation and proliferation in vivo. To test this hypothesis, I used loss of function L-type calcium channel knock-out (KO) mice lacking the entire gene coding for this calcium channel and its splice variants to study its effects on T cell activation, proliferation, death, calcium uptake, and effector responses. From these studies, I predict that the lack of L-type calcium channels will cause T lymphocyte activation, proliferation and development to be impaired. These studies shed light on the mechanism of T lymphocyte activation and also enabled us to better understand how antagonistic drugs such as nifedipine may cause immunosuppressive effects.
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<tr>
<td>2-APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>ADPR</td>
<td>ADP-ribose</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>APCs</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAK</td>
<td>bcl-2 homologous antagonist/killer protein</td>
</tr>
<tr>
<td>BAX</td>
<td>bcl-2-associated x protein</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell antigen receptor</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>cADPR</td>
<td>cyclic ADPR</td>
</tr>
<tr>
<td>CaMK</td>
<td>calmodulin-dependent kinases</td>
</tr>
<tr>
<td>CCE</td>
<td>capacitative calcium entry</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>ConA</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>COS1</td>
<td>African green monkey kidney cell line 1</td>
</tr>
<tr>
<td>CRAC</td>
<td>calcium release activated calcium</td>
</tr>
<tr>
<td>CREB</td>
<td>target cyclic AMP-responsive element-binding protein</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DHPs</td>
<td>1, 4-dihydropyridines</td>
</tr>
<tr>
<td>DN</td>
<td>double negative</td>
</tr>
<tr>
<td>DP</td>
<td>double positive</td>
</tr>
<tr>
<td>EDTA</td>
<td>diaminoethane-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptors</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FHLH</td>
<td>familial hemophagocytic lymphohistiocytosis</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>ICM</td>
<td>immunofluorescence confocal microscopy</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>IL-7</td>
<td>interleukin 7</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP₃R</td>
<td>IP₃ receptor</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor 2</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NAADP</td>
<td>nicotinic acid adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK cells</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>P2X receptors</td>
<td>purinoreceptors</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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PBTs - peripheral blood T lymphocytes
PCR - polymerase chain reaction
PHA - phytohemagglutinin
PIP$_2$ - phosphatidylinositol 3,4-bisphosphate
PLC-$\gamma$ - phospholipase C gamma
PMA - 12-O-tetradecanoylphorbol-13-acetate
RBL - rat basophilic leukemia
PBMCs - human peripheral blood mononuclear cells
RPMI - Roswell Park Memorial Institute
RT-PCR - reverse transcription PCR
Ry - ryanodine
SOC - store-operated calcium channels
SP - single positive
SCID - severe combined immunodeficiency
STIM/ORAI - stromal interaction molecules
Tc cells - cytotoxic T cells
TCR - T cell antigen receptor
TRP - mammalian homologues of transient receptor potential
Th cell - T helper cell
Treg cells - regulatory T cells
VDCC - L-type voltage dependent calcium channel
VSV - vesicular stomatitis virus
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Special thanks are owed to my parents, who have supported me throughout my years of education, both morally and financially.
DEDICATION

This work is dedicated to my parents, who have been there to support me through everything in life.

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CHAPTER ONE: INTRODUCTION

1.1 Calcium ion channels functions in physiological events

Calcium ion acts as a secondary messenger to regulate many physiological events such as motility (1), photoreceptor function (2), skeletal muscle functions (3), lymphocyte activation (4), proliferation (5), differentiation (4), effector functions (6, 7), transcription (8-10), survival (11), anergy and death in many cell types (12, 13).

1.2 Calcium ion channel functions in the immune system

Calcium signaling is important in immune responses, including those associated with autoimmunity. Calcium signals act as an important secondary messenger for many immune cells such as T cells, B cells, mast cells and natural killer (NK) cells (4). In immune cells, calcium signals are tightly regulated by membrane receptors, signaling molecules, and ion channels.

Immune cells use calcium signals as a secondary messenger to induce signaling transduction pathways (Diagram 1), leading to such diverse functions as activation, proliferation, effector function, anergy, and death. Lymphocytes in their resting state have a calcium concentration of approximately 100-200nM. During activation, these intracellular stores of calcium are mobilized through a series of coordinated events. Antigen presenting cells (APCs) present antigen to the immunoreceptors: T cell antigen receptor (TCR) in T cells, B cell antigen receptor (BCR) in B cells, and Fc receptors (FcR) on mast cells and NK cells. Once the corresponding immunoreceptor recognizes the specific antigen, immunoreceptor ligation takes place, causing non-receptor tyrosine kinases to phosphorylate and activate phospholipase C gamma (PLC-γ) (PLC-γ1 in T lymphocytes, and PLC-γ2 in B cells) (14-17-19). PLC-γ cleaves phosphatidylinositol, 3,4-bisphosphate (PIP₂) from plasma membrane phospholipids to generate...
diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$) (4). The released IP$_3$ binds to IP$_3$
receptor in the membrane of endoplasmic reticulum (ER), which leads to the release of
intracellular calcium ions from the ER through store-related Ins(1, 4, 5)P$_3$ receptor calcium
channels, ryanodine (Ry) receptors and nicotinic acid adenine dinucleotide phosphate (NAADP)
receptors (20, 21). The entire process from immunoreceptor ligation to intracellular calcium
release takes place in tens of seconds. The decrease in the ER calcium level causes an opening of
the store-operated calcium channels (SOCs) on the plasma membrane, which causes a sustained
calcium influx from the extracellular space (22, 23). Calcium influx through the SOCs activates
calmodulin dependent serine/threonine phosphatase calcineurin, calmodulin-dependent kinases
(CaMK) and downstream transcription factors such as nuclear factor of activated T cells
(NFAT), CaMK and its target cyclic AMP-responsive element-binding protein (CREB), myocyte
enhancer factor 2 (MEF2). Sustained calcium signal ranging from a concentration of
approximately 20 nM to 1μM for up to 48 hours is required for the phosphorylation and
translocation of NFAT into the nucleus. Elevated levels of NFAT in the nucleus causes various
downstream signals to be transcribed, such as interleukin-2 (IL-2) (24). Similar calcium
signaling pathway is observed for cytotoxic T (Tc) cells and T helper (Th)-cell activation and
proliferation (5).
Diagram 1. T lymphocyte activation signaling cascade

Immediate effects of calcium signaling, independent of gene transcription, are within minutes. Examples include regulation of lymphocyte motility (4), and granule exocytosis in allergen-sensitized mast cells (4). Long term effects of calcium signaling may take several hours to days. Utilizing downstream gene transcription, a plethora of immunological functions take place, including T and B lymphocyte activation, cytokine and chemokine production, lymphocyte proliferation, lymphocyte development, differentiation, effector function, anergy establishment and death (4).

Calcium signaling is also pivotal for downstream effector functions, such as perforin-dependent cytotoxic T lymphocyte (CTL) killing (6) and helper T cell (Th cell) cytokine production. Perforin dependent killing is utilized by CD3⁺ CD8⁺ CTLs, NK cells (25, 26), some subsets of CD3⁺ CD4⁺ effector cells (7), including some regulatory T (Treg) cells (27).
CD3⁺ CD8⁺ CTL perforin dependent killing is a crucial effector function used to kill virus infected cells, transformed cancerous cells, non-self cells in allografted tissues and organs (30-34), and also cells infected by intracellular bacteria such as *Salmonella typhimurium* (35) and *Mycobacterium tuberculosis* (36).

In Th cell cytokine production, calcium influx in Th cells occurs primarily via capacitative calcium entry (CCE), where calcium channel at the plasmic membrane is triggered by depletion of intracellular calcium stores (37). The specific channel responsible has been coined as the calcium release activated calcium (CRAC) channel (14, 22, 38-40), similar to the channel used for T cell activation.

In perforin-dependent CTL target exocytosis, the process is initiated when CTL adheres to a target. When its TCR recognizes the specific antigen, an immunological synapse is formed (41-43). This triggers a downstream signaling transduction pathway which results in the polarization of the microtubule organizing center, Golgi apparatus and lytic granules towards the target. Exocytosis then occurs at the site of contact. (44-46). The entire perforin-mediated target killing process takes minutes to tens of minutes (30, 47-50) and does not require new gene transcription (31, 32, 51-52).

Calcium signaling is also utilized in controlling whether T cells undergo pathways leading to survival, anergy or cell death. Extremely high levels of calcium influx from the extracellular matrix disrupt homeostasis and activate calcium-sensitive proteases, leading to cell death (49).
The fate of the T lymphocyte depends significantly on calcium signaling. The survival of naive T cells is dependent on both low-grade TCR signaling upon encounter with self-peptides/self-MHC molecules and exposure to the cytokine interleukin 7 (IL-7) (54). Together, TCR and IL-7 receptor signaling promote T cell survival by influencing the balance and function of pro-survival and pro-apoptotic proteins. Moreover, a critical function of IL-7 receptor signaling has been postulated to be the up-regulation of the anti-apoptotic protein Bcl-2 since enforced Bcl-2 expression can rescue T cell development in IL-7 receptor-deficient mice (55-56). Important pro-survival transduction mechanisms involve promoting the expression and function of members of the Bcl-2 family or activating the phosphoinositide kinase-AKT pathway. Either of these singlet pathways represses the function of Bcl-2-associated x protein (BAX) and Bcl-2 homologous antagonist/killer protein (BAK) that are required for release of lymphocyte mitochondrial cytochrome c (57). Bcl-2 is a potent inhibitor of apoptosis and is an integral membrane protein located on the ER and mitochondria. Bcl-2 inhibits calcium release from the ER (58-62) while another member of the family Bcl-2 family, Bcl, inhibits mitochondrial calcium waves initiated by inositol-P3-mediated calcium signals (61, 63). Furthermore, Bcl-2 binds calcineurin and thereby inhibits NF-AT activation by calcium (64).
Diagram 2. T lymphocyte survival signaling cascade

Calcium signaling is also important in anergy. When self reactive lymphocytes escape the thymus during negative selection and becomes distributed in the periphery, several mechanisms are used to inactivate or eliminate the presence of these self reactive lymphocytes. The mechanisms employed include induction of anergy, dominant suppression by regulatory T cells, and peripheral deletion of self-reactive T cells. Anergy is used to cause self reactive lymphocytes to be functionally inactivated. It has been found that calcium signaling pathways such as calcium/calcineurin pathways and calcium/NFAT pathways directly control anergy induction (64).

Calcium signaling is also significant for programmed cell death. Also termed apoptosis, it is a process where cells shrink and dissociate from their surrounding neighbours, their organelles retain in size, and in the nucleus chromatin forms dense aggregates on the nuclear membrane and eventually undergoes fragmentation (65). The lack of apoptosis leads to autoimmune diseases or...
cancer (65); whereas an overreactive amount of apoptosis leads to chronic pathologies such as neurodegenerative disease like Alzheimer type dementia or immune deficiencies such as acquired immune deficiency syndrome (AIDS) (66). The induction of apoptotic pathway by different stimuli such as caspases and B-cell lymphoma (Bcl) family of proteins eventually converge to lead to an increase in calcium signaling. Therefore, calcium signal is a general mediator of apoptotic events (65). Whether T lymphocytes undergo activation, proliferation and sustainance or follow the pathway of anergy or cell death depends significantly on calcium signaling.

1.3 Candidates of SOCs

Although the mechanisms of calcium release from the intracellular stores is well characterized, the calcium signaling pathway from extracellular space into T lymphocytes through SOC is unclear, despite contributing to the majority of the calcium signaling in the T lymphocyte signaling pathway (67). Downstream effects of SOC channel may provide vital information on how T lymphocyte activation, proliferation, death is controlled as well as providing potential insights and therapeutic functions to disease phenotypes associated with T lymphocyte responses. One form of severe combined immunodeficiency (SCID) is a result of severe defects in SOC channel function, which leads to severe defects in cytokine impression and lymphocyte functions (41, 68, 69). Various SOC channel candidates have been put forward including IP$_3$R calcium channels (70), STIM/ORAI (71-73), TRP calcium channels (74-76), P2X receptors (77-79), and L-type voltage dependent calcium channels (80-87).

The availability of electrophysiology enabled whole-cell patch clamping profile of the calcium channel for a cell type to be elucidated. Electrophysiological profile of the calcium
channel responsible for calcium influx from the extracellular matrix is therefore defined. Originally identified in hematopoietic cells in a basophil-mast-cell line, the as yet unindentified calcium channel responsible for the observed calcium influx in T lymphocytes is termed calcium release activated calcium (CRAC) channels (88-89 into 84-85). CRAC calcium current is found to be inwardly rectifying, highly selective for calcium ions over other cations (except barium ions) and with an extremely low calcium ion conductance.

Identification of CRAC candidate has been difficult as it is likely multimeric and no candidate has expressed the exact electrophysiological profiles of CRAC without lacking certain properties associated with CRAC or containing additional properties separate from what is observed in CRAC. Potentially, several calcium channels may be taken together to be responsible for the observed CRAC current (86).

1.3.1 IP₃ receptor calcium channels

IP₃ receptor (IP₃R) calcium channels (70) were initially found as intracellular calcium channels on the ER (91). Studies have shown that T lymphocytes express three isoforms of the IP₃R channels as integral plasma membrane proteins (92). However, functional redundancies of the three isoforms have rendered it difficult to define the contribution of these channels to calcium signaling in T lymphocyte (93).

1.3.2 Stromal interaction molecules

One candidate of CRAC channel is stromal interaction molecules (STIM1). Found with the use of RNA interference (RNAi) screening in Drosophila and human, STIM1 has been
identified as the calcium sensor within stores. (71-73). Using the same approach, a pore forming subunit of CRAC has been identified as ORAI1 (also named CRACM1 or TMEM142A) (94-96).

Upon MHC engagement, downstream tyrosine phosphorylation of kinases and adaptor molecules take place. This facilitates STIM1 and ORAI1 to colocalize to the site of MHC engagement (97, 98), forming a cap-like structure at the distal pole of the cell. This cap moves from the distal pole to an existing or a newly formed immunological synapse. It is postulated that the cap may contain preassembled calcium channel components that can be delivered to newly formed immunological synapses (98).

Evidence indicates that both STIM1 and ORAI1 knockout mice exhibit T lymphocyte abnormalities. ORAI1 knockout mice exhibits partial decrease of calcium influx (99, 100); STIM1 -/- mice were found to exhibit abolishment of peripheral T lymphocytes and double positive (DP) thymocytes (4, 101).

However, it remains elusive whether STIM1/ORAI1 composes the sole calcium channel responsible for directing calcium influx through the extracellular matrix. STIM1 -/- mice demonstrated no developmental defect in αβ+ TCR T cells (4, 101). This may be due to the rescue effect from STIM2; however, CD4Cre knockout of both STIM1 and STIM2 loci did not show a phenotype that affects thymocyte development (102). In addition, in ORAI1 -/- mice, calcium influx was not completely abolished, 10-15% of calcium influx remained to be observed in ORAI1 deficient DP thymocytes as compared to wildtype (99, 100). Currently, no ORAI2 mouse model is available to demonstrate whether ORAI2 alone or the combinations of the two are used as the CRAC channel. But ORAI2 is found to be more highly expressed in the thymus as compared to ORAI1, and it has been shown to conduct CRAC currents in in vitro over-
expression systems (103, 104). Furthermore, CRAC channel inhibitors such as SKF96365, 2-aminoethoxydiphenyl borate (2-APB) and lanthanum ion do not affect thymocyte motility (105). Most importantly, it has been found that human patients with mutations in STIM1 or ORAI1 are found to develop mature T cells in the periphery (68, 69, 106). This evidence taken together, indicate that although further work is required, it is plausible that other calcium channels may be present in addition to STIM1/ORAI1.

1.3.3 Mammalian homologues of transient receptor potential (TRP) calcium channels

There has been accumulating evidence indicating that store-independent calcium channels may contribute to calcium signaling in T lymphocyte (74-76). One candidate for the CRAC channel is the transient receptor potential (TRP) calcium channel. It was originally found in *Drosophila melanogaster* (107). TRP calcium channel is a six-transmembrane cation-permeable channel. It regulates intracellular concentrations of sodium, calcium and magnesium ions. Twenty-eight TRP channel proteins are grouped into six sub-families based on sequence homology: TRPC1-6, TRPV1-6, TRPM1-8, TRPA, TRPML1-3, and TRPP2,3,5. They are found to be widely expressed in mammalian tissues (108). In particular, in Jurkat and human T lymphocytes, TRPC1, TRPC3, TRPC6, TRPM2 and TRPV6 are found to be expressed and functional (74, 109). TRPM2 has been extensively studied in T lymphocyte cell lines.

TRPM2 has been found to be activated by intracellular second messengers such as ADP-ribose (ADPR), nicotinamide adenine dinucleotide (NAD\(^+\)), H\(_2\)O\(_2\), and cyclic ADPR (cADPR) (110-112). When TCR engagement takes place, it activates soluble ADP-ribosyl cyclase which increases intracellular cADPR. In T lymphocyte lines, when ADPR and cADPR are directly
applied to cells, calcium signaling was induced through TRPM2 channels; therefore implying that TRP channels may contribute to calcium signaling in T lymphocytes (4).

Another channel, TRPV6, also termed CaT1 and ECaC2, exhibits many electrophysiological properties associated with CRAC when over-expressed in Jurkat T lymphocytes.

However, there are arguments against TRP channel being the CRAC channel. First, CaT1 does not exhibit all the electrophysiological properties associated with CRAC current (75, 77). It has also been found to exhibit additional electrophysiological properties not found in CRAC (113). Second, TRP channels are only partially regulated by store-depletion (75), contrary to what is observed for CRAC. Furthermore, major abnormalities in T cell development and function have not been reported in TRP mouse models (114).

1.3.4 P2X receptors

Another candidate for the CRAC channel is the P2X receptor. P2X receptors are adenosine triphosphate (ATP)-gated channels with high calcium permeability, which are activated by the increase in extracellular ATP, causing an influx of calcium signal upon activation (115). As it has been found that TCR engagement triggers a rapid release of ATP, it's plausible that the release of ATP may activate P2X receptors (78). P2X receptors form homo or heteromeric P2X receptors. There are 7 P2X receptors (P2X1-P2X7). Evidence indicate that P2X1, P2X2, P2X6, P2X7 subunits are found to be expressed in lymphocytes. However, major abnormalities in T cell development and function have not been reported in P2X mouse model (79).
1.3.5 L-type voltage dependent calcium channels (VDCCs)

There has been accumulating evidence that a store independent L-type voltage dependent calcium channel (VDCC) may participate in T lymphocyte functions as the CRAC channel candidate. L-type VDCCs are heteromultimeric proteins containing a channel forming α subunit, a β subunit, and a γ subunit. It is voltage gated as it responds to changes to the electrical potential across the plasma membrane and thereby change its conformation to become activated (82).

Four subtypes of L-type voltage dependent calcium channels are found in T and B lymphocytes: Cav1.1, Cav1.2, Cav1.3, Cav1.4 (83, 84, 87). Studies done on Jurkat T lymphocytes by Densmore et al. (83, 84) identified an electrically responsive current in the plasma membrane of T lymphocytes which had different electrophysiological properties from CRAC but was activated through TCR/CD3 complex and calcium store depletion (83, 84). Reverse transcription (RT)-PCR showed Cav1.1 (α1s) and Cav1.2 (α1c) pore forming subunits of L-type calcium channels are expressed in Jurkat T lymphocytes (85). Additionally, Savignac et al. demonstrated that 2G12.1 murine T cell hybridoma line expresses L-type calcium channel mRNA and protein, and modulate calcium-dependent IL-4 gene transcription (86). Cav1.2 mRNA and protein as well as the auxiliary β-subunit were also found to be expressed in various human B and T cell lines (87). In human L3055 B cell line, antibodies against extracellular region of Cav1.2 L-type calcium channel caused sustained calcium influx (87).
There are also pharmacological evidences supporting the function of VDCCs in T lymphocytes as calcium signal modulators. 1, 4-dihydropyridines (DHPs), a class of synthetic derivatives that selectively binds to L-type voltage-dependent calcium channels, are found to modulate calcium signaling in lymphocytes (80, 86, 87), whereas synthetic 1, 4-dihydropyridine (DHP) L-type calcium channel antagonist, nifedipine, is a potent suppressor of T lymphocyte proliferation. Birx et al. demonstrated that using an in vitro [3H]thymidine uptake assay, that 0.001-100μM nifedipine prevents the proliferation of human T lymphocytes in response to the mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) (116). Another study shows that human peripheral blood mononuclear cells (PBMCs), stimulated with PHA cannot proliferate in the presence of 10-200μM nifedipine, whereas IL-2 addition restores the proliferative response in the nifedipine-treated cells (117). Nifedipine is found to be a dose-dependent inhibitor for T lymphocyte proliferation when added in combination with immunosuppressive agent cyclosporine A, as demonstrated in in vitro proliferation assays (118, 119).

Previous work in our laboratory has shown that a subtype of VDCCs, Cav1.4 (α1F), may be responsible for the calcium influx through the plasma membranes in T lymphocytes (80, 81, 120). Cav1.4 L-type calcium channel is coded by the CACNA1F gene. It was initially cloned from human retina (121). In photoreceptors, it modulates calcium signal (122). A knockout of Cav1.4 calcium channel causes night blindness (123). Our laboratory has used PCR to prove that the mRNA of pore forming α1F-subunit L-type calcium channel transcript is expressed in human T lymphocytes (80). This was confirmed by McRory et al., that Cav1.4 is found to be expressed in human spleen, thymus and bone marrow as well as in the retina (124). In addition, (+/-) Bay K 8644 (agonist) and nifedipine (antagonist) were found to modulate early T lymphocyte activation.
through calcium signaling in a dosage dependent manner in both Jurkat T cell leukemia line and in human peripheral blood T lymphocytes (PBTs) (80). Furthermore, (+/-) Bay K 8644 and nifedipine were additionally found to modulate late T lymphocyte activation signals such as phospho-p44/42 mitogen activated protein (MAP) kinase activation, NFAT transcription, IL-2 secretion, and splenocyte proliferation during T lymphocyte activation and proliferation in Jurkat T cells and in human PBTs (80).

Although VDCCs are typically observed in excitable cell types such as photoreceptors and skeletal muscle, they may still function in non-excitable cell types such as T lymphocytes. It’s possible that a mechanism such as alternative splicing is used to generate splice variants expressing calcium channels. These alternatively spliced calcium channels may share common structural similarities to VDCCs, but are not gated by changes in membrane potential. This has been previously demonstrated in our laboratory by Koturri et al (81). Using nested RT-PCR, we identified two novel alternative splice variants of the Cav1.4 in human spleens with alternative splicing in their carboxy termini. Cav1.4a variant had a deletion in the IVS4 voltage sensor domain; Cav1.4b variant had a deletion of the IVS3-S4 interlinker domain. This could explain the feature of T lymphocyte insensitivity to membrane depolarization as well as its low affinity for DHP as compared to electrically excitable cell types. In Jurkat cells, Cav1.4a and Cav1.4b mRNA expression following TCR engagement are found to be differentially expressed in different splenocytes and temporally regulated (81). This has also been confirmed by Badou et al. (125). Our laboratory also found that Cav1.4a expression is limited to lymphocytes; whereas Cav1.4b expression was also found in monocytes. To establish Cav1.4 protein expression in T lymphocytes, Cav1.4 protein was found in both Jurkat T lymphocytes as well as human PBTs. In Jurkat T lymphocytes, Cav1.4 protein expression is increased following TCR engagement,
suggesting alternative functional expression patterns (81). However, all work on VDCCs have been done on cell lines, no work has been done to characterize the expression and function of voltage dependent L-type calcium channels in mouse models.

We propose that an L-type voltage dependent calcium channel may be at least partially responsible for the extracellular influx of calcium signal associated with downstream T lymphocyte functions. Previously, mRNA from two splice variants of L type calcium channel Cav1.4 has been identified (81). The original Cav1.4 gene, which was cloned from retina, is 5.8 kb in length, with 48 exons. The Cav1.4 protein has 55-62% amino acid sequence identity to other Cav1 L-type calcium channels. The retinal Cav1.4 channel mediates calcium signal entry into the photoreceptors to promote tonic neurotransmitter release. Mutations in the CACNA1F gene causes night blindness.

Since very little is understood about the molecular structure of the Cav1.4 channel in lymphocytes, we demonstrated that the full length Cav1.4 mRNA is present in T cells and gave rise to a functional protein. The two splice isoforms were found to be expressed in T lymphocytes, but not in the human retina. cDNA sequencing showed that these two splice variants (Cav1.4a and Cav1.4b) had alternatively spliced out exons 31, 32, 33, 34 and 37. This caused deletion in the transmembrane segments S3, S4, S5 and half of S6 in motif IV of the spleen Cav1.4a channel. Transmembrane segment IVS4 (exon33) deletion caused the removal of a voltage sensor domain; whereas IVS6 (exon 37) deletion caused the removal of the DHP binding site and an EF-hand Ca^{2+}-binding motif. Cav1.4b splice variant alternatively spliced out only exon 32 and 37, and retained the voltage sensor domain in IVS4. Exon 32 deletion removed an extracellular loop between segments IVS3-S4. The removal of either the IVS4 voltage sensor
domain or the IVS3-S4 interlinker may prevent Cav1.4 splice isoforms from being gated by membrane depolarization. At the 3 terminus region of both splice isoforms, deletion of exon 37 caused a frameshift and led to a premature termination of channel protein translation at the carboxy-terminus and caused the amino acid sequence downstream of the frameshift to lose 55% of the amino acid sequence identity compared to the human Cav1.1 L-type Ca\textsuperscript{2+} channel wild type found in skeletal muscle (GenBank accession number XP001910). The frameshift is not a PCR artifact or a sequencing error since this unique sequence has been repeatedly isolated and sequenced not only from human spleen, but also from many different human, rat and mouse T cell lines, mouse splenocytes and thymocytes, and naïve human PBTs. Both splice isoforms had 99% nucleotide and 95% amino acid sequence identity to the Cav1.4 channel from human retina. There were no additional differential splice sites found upstream of exon 31 in Cav1.4a and Cav1.4b. In summary, the Cav1.4 splice variants expressed in T lymphocytes contain novel structural features at the carboxy-terminus which may have a unique impact on the Ca\textsuperscript{2+} kinetics gated by these channels in T lymphocytes.

The work presented in this thesis characterizes the function of Cav1.4 in T lymphocyte activation, proliferation and death by using a Cav1.4 knockout mouse model, lacking the entire gene sequence coding for the Cav1.4 L-type voltage dependent calcium channel. These studies will shed light on the mechanism of Cav1.4 calcium channel in T lymphocyte activation, proliferation, effector function, anergy, survival, and death.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Mice - Cav1.4 knockout heterozygous female and homozygous knockout male were provided by Dr. Bech-Hansen at the University of Calgary. As described in Mansergh et al. (126), Cav1.4 calcium channel is coded by the Cacna1f gene. Cacna1f knockout mice were derived by a targeted disruption strategy with a 70bp insertion in exon 7 of the Cacna1f gene which created an in-frame (TAA) stop codon to be placed in position 305, which resulted in the premature termination of Cacna1f translation. The 70bp insertional mutation transmission is X-linked. PCR, RT-PCR and RNA isolation conducted by Bech-Hansen’s laboratory confirmed the existence of the 70bp mutation. In our laboratory, Cacna1f knockout mice and C57B1/6 wildtype mice were housed at the animal facilities at the University of British Columbia. Mice were kept in specific pathogen-free conditions, and all animal experiments were conducted according to institutional guidelines and animal care regulations. All mice were used between 6 to 12 weeks of age. All mouse studies were approved by the Committee on Animal Care at the University of British Columbia using the guidelines set out by the Canadian Council on Animal Care.

2.2 Cell line and culture conditions - The human T cell leukemia line Jurkat clone E6-l was obtained from American Type Culture Collection (ATCC, Manassas, VA) maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1mM sodium pyruvate. African green monkey kidney cell line 1 (COS1) was also cultured under the same medium condition and used as a control.

2.3 PCR – PCR reactions were performed using the following primers: exon 7F: ATA TGG AAG CAG AGG AGG ACC and exon 7R: CCA GTA GAG GAC GTC TGT CCA. PCR Taq
polymerase (Invitrogen) was used and the PCR reaction was conducted in a Whatman Biometra UnoII Thermocycler at 94°C for 30 sec, followed by a 10-min extension at 72°C. PCR fragments were resolved on a 1.4% agarose gel and visualized by staining with CyberSafe.

2.4 Isolation of splenocytes, thymocytes and peripheral blood for T cell subset distribution analysis - Whole blood (10-50ml), thymus and spleen were collected from three C57Bl/6 and Cacn1f males and females. Thymus and spleen were homogenized. Thymocytes, splenocytes and peripheral blood were separated by centrifugation at 900 × g for 30 min at 4°C over a ficoll-paque PLUS (Amersham Biosciences) gradient. The resulting mono-nuclear cell layer was washed and resuspended in FACS buffer (5% FBS in Phosphate buffered saline (PBS)), then stimulated for 20 min with anti-CD3, CD4, and CD8 monoclonal antibodies, and resuspended in FACS buffer. Thymocytes, splenocytes and PBLs were analyzed on a FACSCalibur cytometer (BD Biosciences) with fluorescein isothiocyanate (FITC)-conjugated CD3(BD Pharmingen), PE-conjugated CD4(BD Pharmingen) and PE-conjugated CD8(BD Pharmingen).

2.5 Isolation of splenocytes, thymocytes and peripheral blood for T cell surface marker analysis - Monoclonal antibodies (Abs) against CD4 (GK1.5), CD8a (53-6.7), CD8b (53.38) TCRb (H57-597), CD25 (PC61.5) CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), Cd127 (A7R34), Thy1.1 (HIS51), CD45.2 (104) were purchased from eBioscience. Data were acquired using either a FACSCalibur/Cell Quest software or LSRII/FACSDiva software (BD Biosciences). Data were analyzed with FlowJo software.

2.6 Calcium phenotype cell sorting – Splenocytes were extracted from 7 or 12 week old C57B1/6 and Cacna1f male and females. Spleens were homogenized, splenocytes were separated by centrifugation at 900 × g for 20 min at 4°C over a ficoll-paque PLUS gradient. The resulting
ficoll layer was washed and resuspended in optiMEM buffer, then stained for 20 min with anti-CD3 FITC and Endo-1 dye for intracellular calcium measurement. Splenocytes were analyzed on a FACS cell sorter. Intracellular calcium level was measured upon T cell specific mitogen stimulation for 7 minutes.

2.7 VSV Infection for tetramer staining and CTL Assays — C57B1/6 or CACNA1F knockout mice were infected by intraperitoneal injection of VSV virus at $2 \times 10^5$ TCID50 (50% tissue culture/infectious dose). At 7 days post viral infection, mice were sacrificed and spleens were collected. Splenocytes were cultured for 5 days in RPMI 1640 complete medium (RPMI 1640 supplemented with 10% FBS, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 50 uM 2-mercaptoethanol, 2 mM L-glutamine) and 1 uM of the H-2Kb-restricted peptide, VSV N(52-59), RGYVYQGL. To identify the CD8+ cells specific for the VSV-N(52-59) peptide complexed with the H-2Kb allele, splenocytes were double stained with anti-CD8 specific monoclonal antibody (BD Pharmingen) conjugated to FITC and anti-H-2K-VSV-NP52-59 iTAgTM Tetramer Streptavidin-Phycoerythrin (SA-PE, immunomics-Beckman CoulterTM) for 30 min at 4°C. The cells were examined using a FACSCalibur flow cytometer (Beckton Dickinson) and analyzed using FlowJo software to assess the percentage of VSV N(52-59) specific CD8+ cells. Cytotoxicity was assessed with a standard Chromium 51 release assay. Target cells (H-2Kb-transfected L cell fibroblasts were incubated with 1 uM VSV N(52-59) peptide, labeled for 1.5 hours with sodium chromate (100 μCi; Amersham), then washed and resuspended in RPMI 1640 complete medium. CTLs were incubated for 4 h at 37°C with target cells (1×10^4 cells per well in 96-well plates) at various effector/target ratios. Spontaneous Chromium release by labeled cells was measured in the absence CTL and maximum release was quantified by lysing target cells in...
2.5% Triton X-100 detergent. All experiments were performed in triplicates and virus-specific Chromium release was calculated using the below formula.

\[
\text{% Specific } ^{51}\text{Cr release} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100\%
\]

2.8 Bone marrow transfer experiments - Bone marrow (BM) cells were prepared from thigh bone extracts of Thy1.1 wild type (Thy1.1⁺ CD45.2⁺ or Cav1.4⁻/- (Thy1.2⁺ CD45.2⁺)). Wild type and mutant BM cells were then mixed 50:50 based on number and confirmed by flow cytometry (Thy1.1⁺:Thy1.2⁺=1:1) before being transferred intravenously into sub-lethally irradiated (1000 rads) CD45.1⁺ hosts (Thy1.2⁺ CD45.1⁺). Splenocytes were extracted 30 days after adoptive transfer; Thy1.1 and CD45.2 were used as markers to discriminate wildtype and mutant donors.

CD4⁺ and CD8⁺ T cell purification and in vitro proliferation assay: Single cell suspensions from lymph nodes and spleens of C57Bl/6 wildtype or Cav1.4 knockout mice were prepared and then incubated with biotinylated anti-CD4 (GK1.5) or anti-CD8 (53-6.7) mAb on ice for 30 min, followed by positive selection using the MiniMACs system (Miltenyi Biotec), according to the manufacturer’s specifications.

2.9 NF-κB mobilization is modulated in the Cacna1f knockout mice - Single cell suspensions from spleens of C57 wildtype or cacna1f knockout mice were prepared and T lymphocytes were positively selected using CD90 MACS beads (Miltenyi Biotec), according to the manufacturer’s specifications. Following selection, 1x10⁶ cells were activated with ionomycin and ConA for 120 minutes. The cells were fixed in 4% formaldehyde for 30 min then permeabilized with 0.2% Triton X-100 detergent for 2 min. Non-specific binding was blocked by 30 min incubation in 3% BSA and 5% normal goat serum. The cells were stained with a goat anti-NF-κB (nuclear factor
kappa-light-chain-enhancer of activated B cells) antibody (1:200 dilution) followed by a rabbit anti-goat antibody conjugated to Alexa fluor 555 (1:600 dilution). The cells were counterstained with Hoechst stain solution for 5 min, and fluorescence was visualized by immunofluorescence confocal microscopy (ICM). Data were analyzed using Image J.1 to select single slices and Adobe Photoshop 7.0 to merge images.

2.10 Statistical analysis - statistical significance was determined by the student’s t test, using two factorial design without replication. For all tests, p ≤ 0.01 was considered to indicate statistical significance.
CHAPTER THREE: RESULTS

3.1 Genotyping

In order to study the effect of Cav1.4 calcium channel on T lymphocyte activation, proliferation, effector functions, survival and death, we have obtained a Cav1.4 knockout mouse model from the Bech-Hansen laboratory at the University of Alberta. One heterozygous female and one homozygous male were provided. Mice were backcrossed 7-10 times with C57Bl/6 wildtype mice prior to arriving to our laboratory. The Cav1.4 knockout mouse model was derived by a targeting disruption strategy where a 7Obp insertion in exon 7 of the Cav1.4 gene (CACNA1F) was used to create a in frame TAA stop codon at position 305. This stop codon results in the premature termination of CACNA1F translation. The 7Obp insertional mutation is X-linked. The Cav1.4 knockout mice were further backcrossed with C57Bl/6 for seven generations in our laboratory prior to this study. Age and sex matched homozygous mutants were used in our study.

In order to confirm the insertional mutation in the Cav1.4 knockout mouse model, PCR with primers flanking the region of insertion and 200bp upstream of the insertion were used. Lane 1 represents the 1kb ladder. Lane 2 represent homozygous wildtype C67Bl/6 mice with the 200bp PCR product from primers flanking the region of insertion; lane 3 is the negative control; lane 4-6 represent homozygous Cav1.4 mutant with the 70bp insertion, making the sequenced region 270bp in length. The results were identical for all mice used in the studies of more than 100 mice. Therefore, we conclude the existence of the 70bp insertion in the Cav1.4 knockout mouse model.
Fig. 1. PCR indicating Cav1.4 identity of individual mice. 1. 1kb ladder. 2. C57B1/6 WT. 3. Negative control. 4-6. Cacna1f homozygous KO progeny. Figure representative of over 20 individual experiments. All mice used in these studies were genotyped to confirm for Cav1.4 identity.

3.2 Isolation and flow cytometry of T cell subset analysis of T lymphocytes from spleen, thymus, lymph nodes and peripheral blood

In order to assess whether Cav1.4 calcium channel is important in regulating T lymphocyte development and function, spleens, thymuses, lymph nodes and peripheral blood were extracted from C57B1/6 wildtype and Cav1.4 knockout mice (three replicates, experiment repeated three times) in order to analyze whether Cav1.4 calcium channel has functional significance in T lymphocyte development and maturation. Splenocytes, thymocytes, lymph node cells and peripheral blood cells were gated for analysis based on granularity and size. T cell subsets from splenocytes, thymocytes, lymph nodes and peripheral blood were stained with CD3, CD4 and CD8 antibodies to analyze the population of T cell subsets. T lymphocytes were recognized by the CD3 antibodies. DP T lymphocytes were recognized by the presence of CD3,
CD4, and CD8 antibodies; double negative (DN) T lymphocytes were recognized by the presence of CD3 alone, and the absence of CD4 and CD8 antibody staining. Single positive (SP) cytotoxic T (Tc) cells were recognized by both CD3 and CD8 antibodies; whereas SP helper T (Th) cells were recognized by CD3 and CD4 antibodies. All the results shown are representative FACS plots and were found to be significant with \( p < 0.01 \).

Results indicated that in the thymus, there is an overall decrease in the number of T lymphocytes in the thymus (Fig. 2a); there is also a reduction in the overall number of each of DN, CD4 SP and CD8 SP cells in the Cav1.4 knockout as compared to the wildtype; but an increase in the total number of DP cells were observed (Fig. 3a). The ratio of thymic CD4 SP cells in the Cav1.4 knockout is approximately half of that in the C57B1/6 wildtype (Fig. 2a); the ratio of thymic DN cells decreased by approximately 2.5 times in the Cav1.4 knockout as compared to the wildtype; whereas the ratio of thymic CD8 SP cells is decreased by approximately 1.4 times in the Cav1.4 knockout as compared to the wildtype. The ratio of thymic CD4 SP to CD8 SP cells changed in the Cav1.4 mutants as compared to the wildtype (Fig. 2a). In the Cav1.4 wildtype thymocytes, CD4 SP to CD8 SP cell ratio is approximately 8 to 1; whereas in the Cav1.4 mutant, the ratio becomes 6 to 1 (Fig. 2a). This suggests a strong up-regulation of CD8 SP T cell development in the Cav1.4 knockout mice. In the Cav1.4 knockout mice, there is a decrease in the frequency of DN thymocytes as compared to the wildtype. 1.79% of C57B1/6 wildtype thymocytes were DN; whereas 0.77% of Cav1.4 thymocytes were DN. There is an increase in the number of DP thymocytes as compared to the wildtype. Indicating that the lack of Cav1.4 calcium channel blocks the transition of DP cells into CD4 SP and CD8 SP cells.
In the spleen, the physical sizes of spleens in the Cav1.4 knockout were smaller than in the C57B1/6 wildtype. There is a decrease in the number of CD4 SP in the Cav1.4 knockout as compared to the wildtype (Fig. 2b). There is also a marked decrease in the number of DN in the Cav1.4 knockout as compared to the wildtype. The total number of splenocytes in the Cav1.4 knockout was also found to be less than in the C57B1/6 wildtype (Fig. 3b). There is an overall decrease in the total number of CD4 SP cells in the Cav1.4 knockout as compared to the wildtype. The total number of CD8 SP cells in the Cav1.4 knockout is also decreased as compared to the wildtype (Fig. 3b). The frequency of CD4 SP cells decreased in the Cav1.4 knockout as compared to the wildtype, however, the ratio of CD8 SP cells is similar to the ratio seen in the wildtype. The CD4 SP to CD8 SP ratio in the wildtype is approximately 2 to 1; however, in the Cav1.4 knockout, the ratio becomes 1.2 to 1.

In the Cav1.4 knockouts, lymph nodes were found to be enlarged as compared to the wildtype. Interestingly, there is a large decrease in the CD4 SP population in the Cav1.4 knockouts as compared to the wildtype; however, there is a marked increase in the CD8 SP population in the Cav1.4 knockouts (Fig. 2c). There is a decrease in the total T lymphocyte number in the Cav1.4 knockout as compared to the wildtype (Fig. 3c). This indicates a chronically activated and exhausted phenotype as apoptosis takes place. The frequency of CD4 SP cells are found to be decreased in the Cav1.4 knockout as compared to the wildtype. The frequency of CD4 SP cells in the Cav1.4 knockout is approximately half that in the C57B1/6 wildtype. However, the frequency of CD8 SP cells is increased in the Cav1.4 knockout as compared to the wildtype. The ratio of CD4 SP to CD8 SP cells changed from 1.3 to 1 as seen in the wildtype to 0.56 to 1 as seen in the Cav1.4 knockout. Indicating that in the periphery, the lack of Cav1.4 calcium channel prevents the establishment of CD4 SP cells.
Peripheral blood lymphocyte (PBL) T cell subset analyses showed a decrease in the total number of CD4 SP and CD8 SP cells in the periphery in the Cav1.4 knockout as compared to the wildtype. As well, CD8 SP Tc cells (Fig. 2d) were slightly down-regulated in peripheral blood in the knockout compared to the wildtype; the CD4 SP T helper cell populations were also down regulated in the Cav1.4 as compared to the wildtype (Fig. 2d). This result indicates that although there was an up regulation of CD8 SP cells in the lymph nodes, the number of CD8 SP cells was reduced in the periphery. As well, there was a general decrease in the total number of T lymphocytes in the periphery. This indicates that Cav1.4 is important in establishing T lymphocyte survival and regulating homeostasis. Therefore, the lack of Cav1.4 calcium channel causes T cell lymphopenia in the periphery despite the high frequency of CD8 SP cells in the thymus.
Fig. 2. T cell subset profiles. C57BL/6 wildtype and Cav1.4 knockout thymocyte (a), splenocyte (b), lymph node (c), and peripheral blood cell (d) CD4, CD8 profiles. Results shown are representative FACS plots. Experiment was conducted using 3 mice in 3 replicates. Results were found to be significant with $p \leq 0.01$. 

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Fig. 2. T cell subset profiles. C57BL/6 wildtype and Cav1.4 knockout thymocyte (a), splenocyte (b), lymph node (c), and peripheral blood cell (d) CD4, CD8 profiles. Results shown are representative FACS plots. Experiment was conducted using 3 mice in 3 replicates. Results were found to be significant with $p \leq 0.01$. 
Fig. 3

Fig. 3. T cell subset cell number. C57B1/6 wildtype (WT) in blue; Cav1.4 knockout (KO) in red. a. Thymocyte, b. splenocyte, and c. lymph node total cell number profiles. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. *Results were found to be significant with p ≤ 0.01. Results without * were found to have a statistical significance of p ≤ 0.5.

3.3 Calcium Influx

Splenocytes were utilized to measure calcium influx in order to assess the function of Cav1.4 calcium channel in T lymphocyte. Activated and resting splenocytes exhibit different granularity and size. As shown in Fig. 4, the gate on the left (4a) captured the resting splenocytes, whereas the right hand gate (4b) captured the activated splenocytes. Splenocytes were labeled with endo-1 dye to measure intracellular calcium levels. Fig. 5 shows splenocyte calcium influx profile
shown by intensity of Indo-1 dye release over time. T lymphocytes were specifically labeled with anti-CD3 FITC to distinguish T lymphocytes amongst various splenocyte subpopulations (Fig. 6). Ionomycin/PMA (12-O-Tetradecanoylphorbol-13-acetate) in different titrations were used as an activator to trigger lymphocyte activation (data not shown). 2C11, a hamster anti-mouse CD3ε antibody was used to trigger T lymphocyte specific activation. EDTA (Diaminoethane-tetraacetic acid) is a calcium chelator used to sequester free calcium in the media. This was used as a control to show that the calcium influx observed was due to an extracellular calcium influx instead of intracellular calcium release from the ER and the sarcoplasmic reticulum. Fig. 7 shows the T lymphocyte calcium influx profile. Fig. 8 shows calcium influx levels between splenocytes and the T lymphocyte subset. T lymphocyte subset can account for most of the calcium influx observed from splenocytes. Results were preliminary, later data done in our laboratory by others have confirmed these data.

Fig. 4. Resting (4a.) and Activated (4b.) splenocyte.
Fig. 5. splenocyte calcium influx profile shown by intensity of Indo-1 dye release over time.

Fig. 6 CD3+ T lymphocyte gate.
EDTA, a calcium chelator, sequesters free calcium in the media, thereby eradicating calcium influx from the extracellular matrix. Wild type mice splenocytes (Fig. 9), in the presence of EDTA and mitogen (Ionomycin/PMA) showed no extracellular calcium influx, but show a small calcium influx peak which quickly disintegrates, characteristic of intracellular calcium fluxes. In normal conditions, wildtype mice splenocytes generated normal extracellular calcium influx profile as expected. While knockout mice splenocytes exhibit marked reduction in the
ability to induce extracellular calcium influx in the presence of mitogen alone. They still
maintained the low level intracellular calcium influx in the presence of both mitogen and EDTA.

T lymphocyte results, however, indicate that while wildtype mice T lymphocytes (Fig. 10)
exhibited both intracellular and extracellular calcium influx as expected. Cav1.4 knockout mice
T lymphocytes do not have either intracellular or extracellular calcium influx. This finding
suggests that Cav1.4 calcium channel is involved in intracellular and extracellular calcium influx
in T lymphocytes, and is involved in only extracellular calcium influx in other splenocyte subsets
such as B cells, NK cells & mast cells.

Fig. 9. 7 week old Cav1.4 KO splenocytes shows severe reduction in extracellular and intracellular calcium influx. Red: 7 week WT splenocytes, calcium influx profile upon activation with 50μM Ionomycin/PMA. Blue: 7 week KO splenocytes, 50μM Ionomycin/PMA. Green: 7 week WT splenocytes, 50μM Ionomycin/PMA, 10μM EDTA. Yellow: 7 week KO splenocytes, 50μM Ionomycin/PMA, 10μM EDTA. Results shown are representative FACS plots. Five mice were used in this experiment in 8 replicates. Results between WT and KO were found with a statistical significance of p ≤ 0.5.
Fig. 10. 7 week old Cav1.4 KO T lymphocytes shows more reduction in extracellular and intracellular calcium influx. Red: 7 week WT T lymphocytes, calcium influx profile upon activation with 50µM Ionomycin/PMA. Blue: 7 week KO T lymphocytes, 50uM Ionomycin/PMA. Green: 7 week WT T lymphocytes, 50µM Ionomycin/PMA, 10µM EDTA. Yellow: 7 week KO T lymphocytes, 50µM Ionomycin/PMA, 10µM EDTA. Results shown are representative FACS plots. Five mice were used in this experiment in 8 replicates. Results between WT and KO were found with a statistical significance of p ≤ 0.5.

Twelve week old knockout mice showed a 20% reduction in the amount of calcium influx observed in T lymphocytes, as well as a faster calcium influx response (Fig. 11); whereas seven week old mice showed an almost complete annihilation of calcium influx in T lymphocytes (Fig. 10).

Fig. 11. 12 week old Cav1.4 KO mice T lymphocytes show faster and reduced calcium influx. Red: Splenocytes were extracted from 12 week old wildtype mice. Calcium influx profile is induced by activation with 20µM Ionomycin/PMA. Blue: Splenocytes were extracted from 12 week old knockout mice. Calcium influx is measured by activation with 20µM Ionomycin/PMA. Results shown are representative FACS plots. Three mice were used in this experiment in three replicates. Results were found with a statistical significance of p ≤ 0.5.
Fig. 12. Higher concentration of Ionomycin/PMA causes moderate amount of increase in calcium influx in splenocytes. Blue: 7 week WT splenocyte calcium influx profile activated with 50μM Ionomycin/PMA. Red: 7 week WT splenocyte calcium influx profile activated with 20μM Ionomycin/PMA. Results shown are representative FACS plots. Three mice were used in this experiment in three replicates. Results were found with a statistical significance of p ≤ 0.5.

Fig. 13. Higher concentration of Ionomycin/PMA causes moderate amount of increase in calcium influx in T lymphocytes. Blue: 7 week WT T lymphocyte calcium influx profile activated with 50μM Ionomycin/PMA. Red: 7 week WT T lymphocyte calcium influx profile activated with 20μM Ionomycin/PMA. Results shown are representative FACS plots. Three mice were used in this experiment in three replicates. Results were found with a statistical significance of p ≤ 0.5.

In both splenocytes (Fig. 12) and T lymphocytes (Fig. 13) 50μM of Ionomycin/PMA caused a higher calcium influx than 20μM of Ionomycin/PMA. However, 50μM of Ionomycin/PMA also caused more apoptosis in both WT and KO (data not shown).
3.4 Activation and maturation markers

To further investigate the decrease of peripheral T cells in the Cav1.4 knockout mice, the activation and maturation markers on developing thymocytes, splenocytes, and lymph node T cells were examined (Fig. 14-16). CD4 and CD8 markers were used to distinguish cytotoxic T cells and helper T cells. TCRβ and CD69 were development markers. CD127 is an IL7 receptor, signaling for activation, memory and survival in T cells. CD62L is an activation and memory marker. CD44 and CD25 are T cell activation and development markers. Thymocytes, splenocytes and lymph node cells were activated and then stained with activation and maturation markers. Splenocytes, thymocytes and lymph node cells were stained against CD4, CD8, CD44, CD69, TCRβ, CD25, CD62L and CD127 markers. In thymocytes, there is a decrease in TCRβ in CD8 SP populations, as well as a decrease in CD69 in CD4 and CD8 SP cells; whereas there is no difference in CD44, CD25 and CD62L markers between C57B1/6 wildtypes and Cav1.4 knockouts (Fig. 4a, 4b). TCRβ and CD69 are development markers, appearing during CD4 CD8 DP stage, before T lymphocytes transition into CD4 and CD8 SPs. This suggests that the lack of Cav1.4 calcium channel interferes with development. This aligns with our T lymphocyte subset analysis, where development seems to be blocked from the double positive stage to CD4 and CD8 SP stage. The decrease in TCRβ and CD69 signaling is particularly obvious with CD8 SP cells. According to the strength of signaling hypothesis (120), weak TCR/MHC engagement leads to CD8 SP T cell development; whereas moderate/strong TCR/MHC engagement leads to CD4 SP T cell development. Therefore, the decreased frequency of CD4 SPs as observed in T cell subset analysis could be due to the lack of calcium signaling in the Cav1.4 calcium channel knockout mice. This led to weak downstream signaling, which favors the development of CD8 SP T cells in the thymus of Cav1.4 knockout mice.
Furthermore, there is decrease in CD127 (also termed IL7Rα) signaling in both CD4 SP, CD8 SP and double negative cells in the thymus. CD127 is a pro-survival IL7 receptor. As IL7 signaling is important for positive selection of thymocytes, this suggests that the developmental defects of T cells in the thymus of Cav1.4 knockout mice is due to the lack of IL7 receptor signaling resulting from dysregulated IL7 receptor. Therefore, it is postulated that Cav1.4 calcium channel provides calcium signals which either directly or indirectly regulate IL7 receptor expression.
Fig. 14a. Lack of Cav1.4 calcium channel favours CD8 SP T lymphocyte development. CD4, CD8, DN and DP profiles of thymocytes stained with TCRβ, CD69 and CD25. Wildtype in red; Cav1.4 knockout in blue. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. TCRβ, CD69 results were found to be significant with p < 0.01. CD25 results were found to be significant with p ≤ 0.5.
Fig. 14b. Lack of Cav1.4 calcium channel favors CD8 SP T lymphocyte development. CD4, CD8, DN and DP profiles of thymocytes stained with CD44, CD62L and CD127. Wildtype in red; Cav1.4 knockout in blue. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. CD127 results were found to be significant with p < 0.01. CD44, CD62L results were found to be significant with p ≤ 0.5.
To investigate the effects of the knockout of Cav1.4 calcium channel on T lymphocytes in the periphery, splenocytes and lymph node cells were extracted and activation and maturation markers were analyzed post activation. In splenocytes, TCRβ, CD69, CD62L and CD127 expression showed no statistically significant difference between C57B1/6 wildtype and Cav1.4 knockout (Fig. 15a, 15b). However, there is down regulation of CD25 in the Cav1.4 knockout compared to the wildtype; there is also a distinct population of up regulated CD44 signaling in the Cav1.4 knockout as compared to the wildtype, exhibiting a more activated phenotype in the Cav1.4 knockout. Since acute activation markers such as CD44 is heightened in the Cav1.4 knockout as compared to the wildtype, this indicates the chronically activated phenotype.

Fig. 15a

Fig. 15a. Lack of Cav1.4 calcium channel causes a chronic activated phenotype. CD4, CD8, DN and DP profiles of splenocytes stained with TCRβ, CD69 and CD25. Wildtype in red; Cav1.4 knockout in blue. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. CD25 results were found to be significant with p < 0.01. TCRβ, CD69 results were found to be significant with p ≤ 0.5.
Fig. 15b. Mice lacking Cav1.4 calcium channel exhibit a chronic activated phenotype. CD4, CD8, DN and DP profiles of splenocytes stained with CD44, CD62L and CD127. Wildtype in red; Cav1.4 knockout in blue. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. CD44 results were found to be significant with p < 0.01. CD62L and CD127 results were found to be significant with p ≤ 0.5.

In lymph node cells (Fig. 16a, 16b), TCRβ, CD25 and CD69 expression showed no statistically significant difference between C57B/6 wildtype and Cav1.4 knockout. However, there is a distinct population of up regulated CD44 signaling in the Cav1.4 knockout as compared to the wildtype, exhibiting a more activated phenotype in the Cav1.4 knockout. There is also a down regulation of CD62L marker in the Cav1.4 knockout. There is a down regulation of CD127, which is an important signal for survival and the generation of memory T cells. Since
acute activation markers such as CD25 and CD69 expression were comparable to the wildtype, and CD127 is a memory marker; taken together, the CD44 high, CD62L low, CD127 low phenotype is indicative of the chronic activation effects of the knockout of Cav1.4 calcium channel. This effect is even more prominent for CD4 SP cells. This suggests that Cav1.4 is important for T cell development and lineage commitment. This explains why the lack of Cav.4 calcium channel causes T cell lymphopenia, chronic activation of CD4 SP cells, and the decrease in CD8 SP cells that exhibit memory T cell phenotypes.

**Fig. 16a**

Fig. 16a. Cav1.4 is important for T cell development and lineage commitment. CD4, CD8, DN and DP profiles of lymph node cells stained with TCRβ, CD69 and CD25. Wildtype in red; Cav1.4 knockout in blue. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. TCRβ, CD69 and CD25 results were found to be significant with p ≤ 0.5.
Fig. 16b. Cav1.4 is important for T cell development and lineage commitment. CD4, CD8, DN and DP profiles of lymph node cells stained with CD44, CD62L and CD127. Wildtype in red; Cav1.4 knockout in blue. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. CD44, CD62L and CD127 results were found to be significant with p < 0.01.

3.5 Bone Marrow Transfer experiment

To further investigate whether Cav1.4 knockout mice exhibit defects in development and peripheral T cell expansion, bone marrow transfer experiment was performed (Fig. 17). Thigh bone marrow was extracted from Thy1.1 positive, CD45.2 positive wildtype mice, and Thy1.2 positive, CD45.2 positive Cav1.4 knockout mice. Mixed in 50:50 portions, they were injected into Thy1.2 positive, CD45.1 positive irradiated congenic hosts. The spleens of these recipient mice were analyzed one month post injection. Results indicate that the ratio of T lymphocytes in Thy1.1 positive, CD45.2 positive C57B1/6 wildtype mice to Thy1.2 positive, CD45.2 positive Cav1.4 knockout mice were 44 to 1 (Fig. 17b), suggesting that Cav1.4 calcium channel is important for peripheral T cell expansion and survival of T lymphocytes.
Furthermore, in the congenic host, while 68% of the total wildtype donor T lymphocytes were expressing CD4 marker; only 22.4% of the Cav1.4 knockout T lymphocytes were CD4 positive. Taken together, the bone marrow transfer experiment indicates that Cav1.4 calcium channel is important for T cell development and peripheral T cell expansion. In addition, in the congenic host, 23.6% of wildtype donor T lymphocytes were expressing CD8 marker; and only 14.1% of the Cav1.4 knockout T lymphocytes were expressing CD8 marker, showing a slight reduction in the number of CD8 cells in its ability to develop and expand in the periphery.

Fig. 17

Fig. 17. Cav1.4 calcium channel is important for peripheral T cell expansion and survival of T lymphocytes. Bone marrow transfer experiment. a. showing granularity and size of recipient splenocytes, b. showing gating of the C57B1/6 wildtype and Cav1.4 knockout T lymphocytes. C. showing the CD4 and CD8 distribution of C57B1/6
wildtype T lymphocytes d. showing the CD4 and CD8 distribution of Cav1.4 knockout T lymphocytes. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. Results were found to be significant with \( p < 0.01 \).

3.6 Tetramer analysis

To further investigate on the effects of the Cav1.4 calcium channel knockout on cell mediated immune responses, we analyzed the ability of Cav1.4 knockout mice to generate vesicular stomatitis virus (VSV) specific cytotoxic T lymphocytes (CTLs) by \textit{in vivo} priming followed by \textit{in vitro} re-stimulation (Fig. 18). The results indicate that the number of VSV specific CTLs generated by Cav1.4 knockout mice is 4 times less than that generated by C57B1/6 wildtype mice. This demonstrates a severe impairment in the ability of Cav1.4 knockout mice to generate antigen specific CTLs, illustrating the severe immunodeficiency that the Cav1.4 knockout mice demonstrates.
Fig. 18. Cav1.4 calcium channel is important in generating antigen specific CTLs. Tetramer analysis. a. showing CD4, CD8 profile of C57Bl/6 wildtype mice. b. showing CD4, CD8 profile of Cav1.4 knockout mice. c. showing VSV-tetramer specific cytotoxic T cells of wildtype mice. d. showing VSV-tetramer specific cytotoxic T cells of knockout mice. e. control with no tetramer. Results shown are representative FACS plots. Three mice were used in this experiment in 2 replicates. Results were found to be significant with p < 0.01.

3.7 Chromium release assay

In order to establish whether the limited repertoire of antigen specific cytotoxic T lymphocytes are able to mount appropriate cytolytic effector functions, a standard chromium release assay was conducted. Chromium release assay uses the amount of radioactive Chromium that the target cells release when killed, to measure the ability of the CTLs to perform target killing response. Results indicate that while C57Bl/6 wildtype mice mounted appropriate CTL target killing response as expected, Cav1.4 knockout mice wasn’t able to effectively mount effector functions to effectively kill target cells on a cell per cell basis in comparison to the
wildtype (Fig. 19). These results indicate that Cav1.4 knockout mouse model generates reduced number of CD8 SP and CD4 SP cells, which are chronically exhausted and have reduced effector function.

![Fig. 19](image)

**Fig. 19** CTL

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Fig. 19. Cav1.4 calcium channel is important in CTL killing. CTL assay. Yellow and dark blue line representing wildtype and Cav1.4 knockout control, respectively, without peptide. Pink line representing wildtype incubated with peptide, showing percentage killing rate, and knockout, in light blue, showing percentage killing rate. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. Results were found to be significant with p < 0.01 between WT and KO samples under the same conditions.

### 3.8 NF-κB nuclear mobilization expression

In order to assess whether downstream T cell activation and proliferation is affected by the lack of Cav1.4 calcium channel, NF-κB nuclear mobilization was analyzed using confocal microscopy. C57B1/6 wildtype and Cav1.4 knockout mice T lymphocytes were purified with magnetic beads and stained with anti-NF-κB monoclonal antibody and counterstained with nuclear stain at 0 minute and 120 minutes post activation with Ionomycin and ConA Blue color represents the nucleus, red color represents NF-κB dye. Pink colour indicates co-localization.

Results are quantified by counting the number of cells showing pink representing colocalization.
As shown in figure 20, following activation, while the localization of NF-κB in the nucleus was seen in the wild type T lymphocytes; the localization of NF-κB in the nucleus was largely absent in Cav1.4 knockout mice T lymphocytes. In addition, co-localization at time zero in Cav1.4 knockout mice seem higher than the corresponding wildtype mice, which suggests that T lymphocytes of the Cav1.4 knockout mice are in a state of exhaustion prior to encountering stimulation.

Fig. 20. Cav1.4 calcium channel is important for NF-κB localization. NF-κB confocal staining. Blue color representing nuclear dye, red color representing NF-κB dye. Pink colour indicating co-localization. Two graphs on top showing wildtype expression, two panels on the bottom showing Cav1.4 knockout expression. Two panels on the left indicating co-localization at time 0 minutes, and two panels on the right indicating co-localization at time 120 minutes post activation. Results shown are representative FACS plots. Three mice were used in this experiment in 3 replicates. Results were found to be significant with p ≤ 0.01.
CHAPTER FOUR: DISCUSSION

In this study, a knockout mouse model lacking Cav1.4 calcium channel was used to elucidate the effects and biological functions of Cav1.4 calcium channel in a physiological setting.

The identity of the Cal.4 knockout mice was confirmed using PCR. Results showed that the DNA of Cav1.4 knockout mice contained the 70 bp insertion as compared to the C57B1/6 wildtype.

Cav1.4 calcium channel is found to be important for both T lymphocyte extracellular and intracellular calcium influx; whereas in total splenocyte population, Cav1.4 calcium channel is only important for extracellular calcium influx but not intracellular calcium influx. We have also found older Cav1.4 KO mice showed faster but reduced calcium influx profile.

We have found that Cav1.4 calcium channel is important for T cell development. In the thymus, CD8 SP cells are preferentially selected. In the wildtype, the ratio of CD4 SPs to CD8 SP cells are 3 to 1; however, in the knockout, the ratio becomes 1.3 to 1.

Furthermore, in the thymus, the number of CD8 SP cells in Cav1.4 knockout are approximately 1.5 times that of the wildtype. In the thymus, there is an increase in the number of double positive T lymphocytes in the Cav1.4 knockout as compared to the wildtype, suggesting that Cav1.4 deficiency prevents double positive cells to progress into single positive cells.

To further investigate on the effect of Cav1.4 calcium channel knockout on development, thymocytes were stained with surface markers. In the thymus, a general trend of decrease in TCRβ and CD69 were seen in the Cav1.4 knockout. This corresponds to the previous T lymphocyte subset analysis, where thymocyte development was blocked from the double
positive stage to CD4 or CD8 SP stage. The decrease in TCRβ and CD69 is especially severe in CD8 positive cells, confirming that development is hindered.

As well, CD127, which is an IL7 marker that regulates survival, has shown marked decrease in the Cav1.4 knockout compared to the wildtype. One hypothesis to explain the preferential selection of CD8 SP cell development in thymus is that weak TCR/MHC engagement signals for CD8 SP cell development; whereas strong TCR/MHC engagement signals for CD4 SP cell development, the lack of Cav1.4 calcium channel causes the calcium signaling downstream of TCR/HMC engagement to be hindered, resulting in weak downstream signaling, which eventually leads to the preferential development of CD8 SP cells instead of CD4 SP cells. In conclusion, Cav1.4 calcium channel is important for the development, lineage commitment and survival of T lymphocytes.

To elucidate whether the developmental defect observed in Cav1.4 calcium channel knockout mouse model stems from defective developmental conditions or whether they stem from the lack of Cav1.4 calcium channel in these specific T cells, a bone marrow transfer experiment was conducted. Thy1.1 positive, CD45.2 positive wildtype mice bone marrow were extracted and mixed 50:50 with the bone marrow of Thy1.2 positive, CD45.2 positive Cav1.4 knockout mice bone marrow. This mixture was injected into previously eradicated Thy1.2 positive, CD45.1 positive recipient mice. One month post injection, the spleen of the recipient mice was analyzed. It was found that the ratio of surviving wildtype to Cav1.4 knockout T lymphocytes in the recipient mice were 44 to 1, indicating that despite normal developmental environment, T lymphocytes from Cav1.4 knockout mice were unable to survive and proliferate. Alternatively, this could indicate that the Cav1.4 knockout T lymphocytes were outcompeted by the wildtype T lymphocytes. Furthermore, the percentage of wildtype CD4 SP cells were
approximately three times that of the Cav1.4 knockout, reconfirming our previous observations on T cell lineage commitment.

The effect of Cav1.4 calcium channel on T lymphocytes in the periphery was also analyzed. The total number of T lymphocytes in the periphery was decreased in the Cav1.4 knockout as compared to the wildtype. It is also found that periphery T cells in the Cav1.4 knockout exhibits a CD44 high, CD62L low, CD127 low phenotype, which is typically associated with chronic activation. This profile is more prominent in CD4 SP cells, which is in line with our developmental data. Whereas CD44 high, CD62L high, CD127 high phenotype is associated with what is observed in memory T cells, we can conclude that the lack of Cav1.4 calcium channel causes lymphopenia, chronic activation of CD4 SP T cells, and a decrease in CD8 SP memory T cells.

In order to elucidate the importance of Cav1.4 calcium channel in T cell effector functions, we conducted a tetramer analysis to elucidate whether Cav1.4 knockout mice were able to generate antigen specific cytotoxic T cells. Our results indicate that the ability of Cav1.4 knockout mice to generate VSV specific cytotoxic T cells were 3.4 times less than that of the C57B1/6 wildtype, demonstrating that Cav1.4 calcium channel is important in regulating T lymphocyte effector functions.

In order to further investigate whether the limited antigen specific cytotoxic T cells were also functionally defective, we conducted a standard chromium release assay to test the ability of these cytotoxic T lymphocytes to kill target cells. Using equivalent numbers of wildtype and Cav1.4 knockout antigen specific cytotoxic T cells, it was found that while C57B1/6 wildtype antigen specific cytotoxic T cells were able to kill target cells rigorously as expected, the ability of Cav1.4 knockout antigen specific cytotoxic T cells to kill target cells is reduced. This could be
due to the chronically activated phenotype observed in the Cav1.4 knockout, which causes the Cav1.4-/- cytotoxic T cells to be chronically exhausted, thereby, hamper its effector functions. Alternatively, as calcium signaling is a critical secondary messenger in the signaling transduction pathways used to initiate effector functions, it is possible that Cav1.4 calcium channel plays crucial roles in signaling for cytotoxic killing, causing the Cav1.4-/- cytotoxic T cells unable to effectively perform cytotoxic effector functions.

In order to demonstrate that the knockout of Cav1.4 calcium channel causes downstream signaling transduction pathways to be affected, we tracked the nuclear mobilization of NF-kB into the nucleus. NF-κB translocation is one of the major initial steps in T cell activation, proliferation, effector function and death. At time zero, it was observed that the Cav1.4 knockouts exhibited slightly more NF-κB translocation as compared to the wildtype. This could be due to the observed chronically activated/exhausted phenotype we have found to be associated with Cav1.4 knockout mice. At time 120 minutes, we can see a clear contrast in the number of wildtype T lymphocytes exhibiting NF-κB translocation as compared to the Cav1.4 knockout. Indicating that the lack of Cav1.4 calcium channel induced calcium signaling causes downstream signaling transduction essential for T cell functioning to be reduced.

In conclusion, Cav1.4 is important for T cell development, lineage commitment, survival, establishing homeostasis, generating functional effector and memory T cells. It would be important to determine the function of Cav1.4 calcium channel in cell death. As well, electrophysiological profiles of the Cav1.4 knockout mice would provide important insight in whether Cav1.4 contributes at least in part to the proposed CRAC channel electrophysiology.
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Appendix I: Ethics Approval

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A07-0373

Investigator or Course Director: Wilfred A. Jefferies

Department: Michael Smith Laboratories

Animals:

Mice Cacna1F 100
Mice Cacna1c 100

Approval Date: April 9, 2009

Funding Sources:

Unfunded
title: Breeding - L-Type Calcium Channels and T-Lymphocytes

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.
A copy of this certificate must be displayed in your animal facility.

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102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

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This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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**BREEDING PROGRAMS**

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The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
The University of British Columbia

Biohazard Approval Certificate

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PROJECT OR COURSE TITLE: Aspects of Antigen Presentation by Breast and Lung Carcinomas and Dendritic Cells

APPROVAL DATE: December 11, 2009  START DATE: January 27, 2006

APPROVED CONTAINMENT LEVEL: 2

Genus Species and Strain
Genus Species
Strain
There are no items to display

FUNDING TITLE: Epigenetic regulation of antigen processing machinery in prostate carcinomas
FUNDING AGENCY: Prostate Cancer Canada

FUNDING TITLE: Studies on antigen presentation by dendritic cells in immune responses
FUNDING AGENCY: Canadian Cancer Society Research Institute

FUNDING TITLE: Regulation of antigen processing machinery in prostate carcinomas
FUNDING AGENCY: Prostate Cancer Canada
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The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093